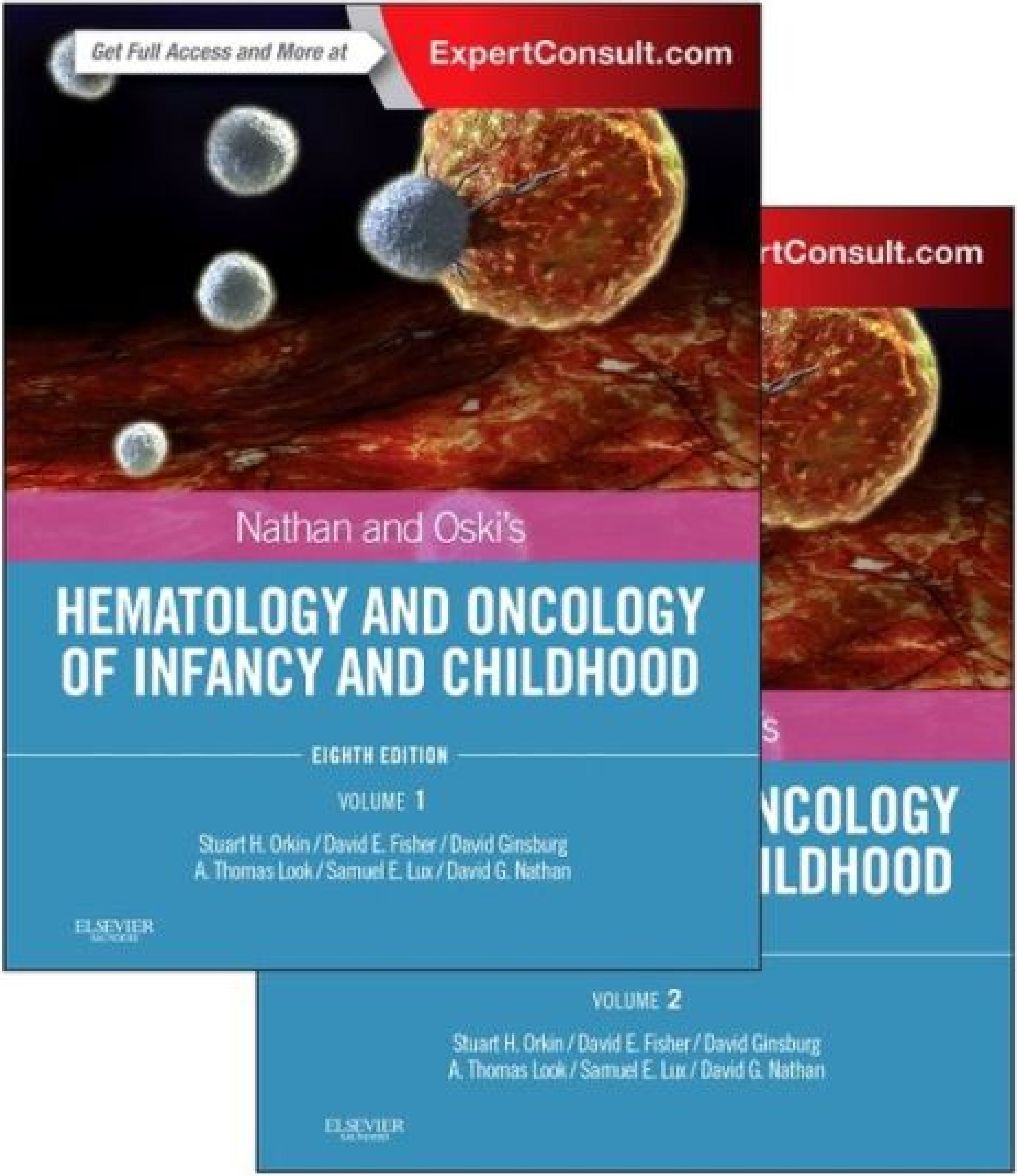

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PREFACE

On behalf of the editors, I welcome you to the eighth edition of *Nathan and Oski's Hematology of Infancy and Childhood*, which has been renamed *Nathan and Oski's Hematology and Oncology of Infancy and Childhood* to reflect the new format begun with the last edition. The classic *Nathan and Oski* text continues to evolve in concert with development in the field of pediatric hematology and oncology. Four decades ago David Nathan and Frank Oski had the vision of creating a resource for students, practitioners, and clinical and basic investigators in which pathophysiology relating to diseases of children would inform clinical care and research in the present and for the future. They hoped to stimulate bench-to-bedside and bedside-to-bench activities in order to understand and treat pediatric patients with hematologic disorders and cancer. The first six editions were decidedly hematology-centric. They concentrated on normal blood cells and their abnormalities, hemostasis, and immune disorders. Except for relatively brief discussion of childhood leukemias, the emphasis was on what might be classified as non-malignant hematology. In recognition of the enormous growth of interest, new knowledge, and treatment possibilities in pediatric oncology, we expanded the scope of the seventh edition with extensive coverage of cancer therapeutics, solid tumors, and oncologic supportive care. In some respects the seventh edition was an experiment. We intentionally separated hematology and oncology into two volumes, thereby allowing readers to consider and purchase either *Hematology of Infancy and Childhood* or *Oncology of Infancy and Childhood*. To accomplish the task of creating an oncology volume de novo, we invited our colleagues Tom Look and David Fisher to join as editors.

By branding the eighth edition with a unified title, the editors have declared the experiment of the seventh edition a success. We believe the expanded scope of the textbook has been well received by readers and more accurately reflects the state of the entire field of pediatric hematology and oncology. The rather artificial distinction between hematology and oncology pained us as editors. Where would we now place leukemia if restricted to one or the other volume? Don't the bone marrow failure syndromes that often evolve to frank malignancy constitute an important bridge between the two areas? As many trainees and practitioners in pediatric hematology and oncology devote a majority of their time to oncology, the need to bring a unified package forward was clear.

We hope that the current form of *Hematology and Oncology of Infancy and Childhood* captures the enormous progress in our understanding and management of non-malignant and malignant conditions in pediatrics. The last two decades have been revolutionary in terms of discovery. The impact of molecular biology, human genetics, immunology, and chemistry on pediatric hematology and oncology, as on all medicine, has been extraordinary. It is now commonplace to anticipate that the entire genome of every child with cancer or a blood disorder will be determined going forward based on the expectation that a full genetic profile will lead to personalized and more effective treatment. To encompass areas of broad relevance that were not covered previously, we have added new chapters. Mel Greaves, a distinguished leader in childhood leukemia, has graced our text with his views on the origin and evolution of cancer, indeed retracing some of Darwin's logic for us. Because informatics has become a critical component of all genomic efforts, we solicited a new chapter on informatics that has been written by Sam Volchenboum. Given the increasing excitement and relevance of epigenetics to childhood cancer, we asked two experts in this area, Charles Roberts and Alex Kentsis, to provide a new contribution on this topic. As always, we hope that readers will provide frank and constructive feedback on this edition. To do justice to the entire field of pediatric hematology and oncology may be a near impossible task. The editors have given their best efforts on behalf of the cause. The readers are our judges.

The eighth edition has truly been a team effort. I cannot thank the other editors enough for their dedication to this project. We are most grateful to the authors of each chapter for their contributions. The editorial staff at Elsevier kept us on course, even when it seemed that we would not make our deadlines. We appreciate everything they have done and more.

It is an honor to be in the field of pediatric hematology and oncology.

Stuart H. Orkin, MD

ACRONYMS

A

A2: annexin 2
AA: arachidonic acid
AABB: American Association of Blood Banks
AAE: acquired angioedema
AAIR: age-adjusted incidence rate
AAP: American Academy of Pediatrics
AAV: adeno-associated virus
ABC: ATP-binding cassette transporter superfamily
ABCB7: ATP-binding cassette subfamily B member 7, mitochondrial
ABL: Abelson kinase, Abelson gene product
ABO: ABO(H) blood group system
ABP: actin-binding protein
ABP-1: actin-binding protein-1
ACO1: aconitase 1
ACS: acute chest syndrome
ACE: angiotensin-converting enzyme
ACIP: Advisory Committee on Immunization Practices
ACTH: adrenocorticotropic hormone, adrenocorticotropin
AD: anauxetic dysplasia; autosomal dominant
ADA: adenosine deaminase
ADAD: ADA deficiency
ADC: apparent diffusion coefficient
ADCC: antibody-dependent cellular cytotoxicity; antibody-dependent cell-mediated cytotoxicity
AdoCbl: adenosylcobalamin, 5′-deoxyadenosylcobalamin
AdoHcy: *S*-adenosylhomocysteine
AdoMet: *S*-adenosylmethionine
ADP: adenosine diphosphate; ALA-D deficiency porphyria; antibody-dependent phagocytosis
ADPase: adenosine diphosphatase
AE1: anion exchange protein 1 (band 3 protein of the red cell membrane)
AFP: α-fetoprotein
AGLT: acidified glycerol lysis test
AGM: aorta-gonad-mesonephros
aGVHD: acute graft-versus-host disease
AH50: alternative pathway complement hemolysis 50%
AHA: acute hemolytic anemia
AHG: antihuman globulin
AHSP: α-hemoglobin–stabilizing protein
aHUS: atypical hemolytic uremic syndrome
AICAR: amino-4-imidazolecarboxamide ribonucleotide
AICD: activation-induced cell death
AICDA: activation-induced cytidine deaminase (gene)
AID: activation-induced cytidine deaminase, activation-induced deaminase
AIDS: acquired immunodeficiency virus
AIHA: autoimmune hemolytic anemia
AIN: autoimmune neutropenia
AIP: acute intermittent porphyria
AIRE: autoimmune regulator
AIS: arterial ischemic stroke
AK: adenylate kinase
AK2: adenylate kinase 2
ALA: δ-aminolevulinic acid
ALA-D: δ-aminolevulinic acid dehydratase
ALA-S: δ-aminolevulinic acid synthase
ALCL: anaplastic large-cell lymphoma
ALD: adrenoleukodystrophy
ALDH: aldehyde dehydrogenase
ALG: antilymphocyte globulin
ALK: anaplastic lymphoma kinase
ALL: acute lymphoblastic leukemia; acute lymphocytic leukemia; acute myeloblastic leukemia; acute myeloid leukemia
ALM: activation loop mutations
ALPS: autoimmune lymphoproliferative syndrome
AMKL: acute megakaryoblastic leukemia
AML: acute myelogenous leukemia; acute myeloid leukemia
AMT: amegakaryocytic thrombocytopenia
ANC: absolute neutrophil count
ANCA: antineutrophil cytoplasmic antibody
α_2-AP: α_2-antiplasmin
AP3B1: adaptor protein 3 complex β3A subunit
APA: antiphospholipid antibody
APC: activated protein C, adenomatous polyposis coli, antigen-presenting cell
APCC: activated prothrombin complex concentrate
APECED: autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy
APHON: Association of Pediatric Hematology/Oncology Nurses
APL: acute promyelocytic leukemia
APLA: antiphospholipid antibody
APOL1: apolipoprotein L1
APRCA: acquired pure red cell aplasia
APRIL: α-proliferation–inducing ligand
APS-1: autoimmune polyglandular syndrome type 1
APTT: activated partial thromboplastin time
AQP1: aquaporin-1
AR: autosomal recessive; androgen receptor
Ara-CMP: arabinosylcytosine 5′ monophosphate
ARC: Australia Research Council
ARF: alternate reading frame
ARSB: arylsulfatase B
ARTUS: amegakaryocytic thrombocytopenia with radio-ulnar synostosis
ASA: arylsulfatase A
ASBMT: American Society for Blood and Marrow Transplantation
ASCO: American Society of Clinical Oncology
ASCT: autologous stem cell transplant
ASH: American Society of Hematology

ASL: arterial spin labeling
ASM: acid sphingomyelinase; aggressive systemic mastocytosis
AST: aspartate aminotransferase
AT: antithrombin; ataxia-telangiectasia
ATG: antithymocyte globulin
ATLS: acute tumor lysis syndrome
ATM: ataxia-telangiectasia mutated
ATOX1: antioxidant 1 copper chaperone
ATP: adenosine triphosphate
ATPase: adenosine triphosphatase
ATP7A: ATPase, Cu^{2+} transporting, alpha polypeptide
ATRA: all-*trans*-retinoic-acid
ATRUS: amegakaryocytic thrombocytopenia with radioulnar synostosis
AUC: area under the plasma concentration vs. time curve
AVN: avascular necrosis
AVWS: acquired von Willebrand syndrome
AZT: zidovudine

B

β-LCR: β-locus control region
BACT: BCNU (carmustine)/Ara-C (cytarabine)/cyclophosphamide/6-thioguanine
BACH1: BTB and CNC homology 1
BAEP: brainstem auditory evoked potential
BAER: brainstem auditory evoked responses
BAF: BRG1-associated factor
BAFF: B-cell–activating factor
BAFFR: BAFF receptor
BAL: bronchoalveolar lavage
BAL: British anti-Lewisite
B-ALL: B-cell acute lymphoblastic leukemia; B-precursor ALL
BCAM: basal cell adhesion molecule
BCG: bacille Calmette-Guérin
BCL10: B-cell lymphoma 10
BCNU: carmustine
BCR: B-cell receptor; breakpoint cluster region
BDNF: brain-derived neutrotrophic factor
BEAM: BCNU (carmustine)/etoposide/Ara-C (cytarabine)/melphalan
BET: bromodomain and extraterminal
BFU-E: burst-forming unit–erythrocyte
BFU-Meg: burst-forming unit–megakaryocyte
bHLH: basic helix-loop-helix
BiPAP: bilevel positive airway pressure
BIRC4: baculoviral IAP repeat-containing 4
BiTEs: bispecific T-cell engagers
BLM: Bloom syndrome, RecQ helicase-like
BLNK: B-cell linker protein
BM: bone marrow
BMD: bone mineral density
BMF: bone marrow failure
BMI: body mass index
BMP: bone morphogenetic protein
BMT: bone marrow transplantation
BMT-MA: bone marrow transplantation–associated microangiopathy
BPGM: 2,3-bisphosphoglycerate mutase
BPI: bactericidal/permeability-increasing protein
BRB: blood-retinal barrier; blood-eye barrier
BRCA1: breast cancer 1, early onset
BrdU: bromodeoxyuridine
BrOb: bronchiolitis obliterans
BRM: biologic response modifier
BSAP: B-cell–specific activator protein
BSP: bromsulfophthalein
BSS: Bernard-Soulier syndrome
BT: bleeding time
Btk: Bruton tyrosine kinase
BU: Bethesda units, busulfan
BUGT: bilirubin UDP-glucuronyltransferase
BWS: Beckwith-Wiedemann syndrome
bZIP: basic region–leucine zipper domain

C

C282Y: cysteine 282 to tyrosine
C: constant
CAD: cold agglutinin disease
CADP: collagen/adenosine diphosphate
CAFC: cobblestone area–forming cell
CAIV: carbonic anhydrase IV
CALGB: Cancer and Leukemia Group B
CaM: calmodulin
CAM: complementary and alternative medicine
cAMP: cyclic adenosine monophosphate
CAMT: congenital amegakaryocytic thrombocytopenia
CaNaEDTA: calcium disodium-ethylenediaminetetraacetic acid
CAR: Central African Republic; chimeric antigen receptor
CARD: caspase recruitment domain family member
CASP: caspase
CAT: calibrated automated thrombography
CB: cord blood
CBB: cord blood bank
C4bBP: C4b-binding protein
CBF: core-binding factor
CBP: cyclophosphamide/BCNU (carmustine)/cisplatin
CBV: cyclophosphamide/BCNU (carmustine)/VP-16 (etoposide)
CCG: Children's Cancer Study Group
CCI: corrected count increment
CCSK: clear cell sarcoma of the kidney
CCyR: complete cytogenetic response
CD: cluster of differentiation
CD3D, CD3E, CD3G, CD3Z, CD3: δ, ε, γ, chains, respectively
CD40L: CD40 ligand
CDA: congenital dyserythropoietic anemia
CDA: cytidine deaminase
CDC: complement-dependent cytotoxicity
Cdc42: cell division control protein 42
CDK: cyclin-dependent kinase
CDP: CCAAT displacement protein; cytidine diphosphate
CDR3: complementarity-determining region 3
C/EBP: CCAAT/enhancer-binding protein α
CEP: congenital erythropoietic porphyria

CEPI: collagen/epinephrine
CESD: cholesteryl ester storage disease
CFC: colony-forming cell
CFC-Eo: eosinophil colony-forming cell
CFU-C: colony-forming unit in culture
CFU-E: colony-forming unit–erythrocyte
CFU-Eo: colony-forming unit–eosinophil
CFU-GEMM: colony-forming unit–multipotent (granulocyte, erythrocyte, macrophage, megakaryocyte)
CFU-GM: colony-forming unit–granulocyte-macrophage
CFU-Meg: colony-forming unit–megakaryocyte
CFU-S: colony-forming unit–spleen
CGD: chronic granulomatous disease
CGH: comparative genomic hybridization
cGMP: current good manufacturing practice; cyclic guanosine monophosphate
CGP: circulatory granulocyte pool
cGVHD: chronic graft-versus-disease
CH: calponin homology domain
CHBA: congenital Heinz body hemolytic anemia
CHF: congestive heart failure
CH50: classical pathway complement hemolysis 50%
CHH: cartilage-hair hypoplasia
CHO: Chinese hamster ovary
CHL: classic Hodgkin lymphoma
CHOP: **c**yclophosphamide, **h**ydroxydaunorubicin, **o**ncovin, and **p**redsinone or **p**rednisolone
CHR: complete hematologic response
CHr: cellular hemoglobin of the reticulocyte
CHS: Chédiak-Higashi syndrome
CIBMTR: Center for International Blood and Marrow Transplant Research
CIK: cytokine-induced killer cell
CIITA: class II *trans*-activator
C1-INH: C1 esterase inhibitor
CI: confidence interval
CIS: cytokine-inducible SH2-containing protein
Cl^-: chloride anion
CLABSI: central line–associated bloodstream infection
CLC: Charcot-Leyden crystal
ClC: chloride channel (may be followed by -7 or other number)
CLIA: Clinical Laboratory Improvement Amendment
CLL: chronic lymphocytic leukemia
CLN-1: neuronal ceroid lipofuscinosis type 1
CLP: common lymphoid progenitor
CMC: chronic mucocutaneous candidiasis
CML: chronic myelocytic leukemia; chronic myelogenous leukemia
CMN: cellular congenital mesoblastic nephroma
cMOAT: canalicular multispecific organic anion transporter
CMP: common myeloid progenitor; cytidine monophosphate
CMV: cytomegalovirus
CN: Crigler-Najjar syndrome, type I (CNI) or type II (CNII); cyanide; cystic nephroma
CNB: core needle biopsy
CNCbl: cyanocobalamin
CNS: central nervous system
CNSHA: congenital nonspherocytic hemolytic anemia
CNTF: ciliary neurotrophic factor
CNV: copy number variation
CO: carbon monoxide
CO_2: carbon dioxide
CoA: coenzyme A
COG: Children's Oncology Group
COJEC: cisplatin, vincristine, carboplatin, etoposide, and cyclophosphamide
COL1A1: collagen, type I, α1
COPA: cancer outlier profile analysis
COX-1: cyclooxygenase-1
COX-2: cyclooxygenase-2
CP: connecting peptides
CPAP: continuous positive airway pressure
CPD: citrate-phosphate-dextrose
CPDA-1: citrate-phosphate-dextrose-adenosine
CPDN: cystic, partially differentiated nephroblastoma
CPO: coproporphyrinogen oxidase
CPP: choroid plexus papillomas
CR: complete remission
CR1: complement receptor 1
CRAC: calcium release–activated calcium
CREG: cross-reactive group
CRF: chronic renal failure
CRHR1: corticotropin-releasing hormone receptor 1
CRIg: complement receptor of the immunoglobulin superfamily
CRISPR: clustered regularly interspaced short palindromic repeats
CRLF2: cytokine receptor-like factor 2
CRM: cross-reacting material
CRP: cross-reactive protein; C-reactive protein
CRT: catheter-related thrombosis
CRX: cone-rod homeobox
CSA: congenital sideroblastic anemia
CSF: cerebrospinal fluid; colony-stimulating factor
c-SMAC: central supramolecular activation cluster
CSR: class switch recombination
CSC: cancer stem cells
CSSCD: Cooperative Study of Sickle Cell Disease
CSVT: cerebral sinovenous thrombosis
CT: closure time; computed tomography
CTCL: cutaneous T-cell leukemia
CIITA: MHC class II transactivator
CT-1: cardiotropin-1
CTD: C-terminal domain
CTL: cytotoxic T lymphocyte
CTLA-4: cytotoxic T-lymphocyte antigen 4
CTP: cytidine triphosphate
CTR1: copper transporter 1
Cu-ATPase: adenosine triphosphatase transmembrane copper transporter
CVID: common variable immunodeficiency
CVL: central venous line
CVS: chorionic villus sampling
CXCR4: CXC chemokine receptor 4
CXR: chest radiography
CY: cyclophosphamide
CYP: cytochrome P

D

D: diversity
DA: diffuse anaplasia
DAF: decay accelerating factor
DAG: diacylglycerol
DAMP: damage-associated molecular pattern
DAP: DNAx activating protein (au query; set OK?)
DAT: direct antibody test, direct antiglobulin test
dATP: deoxyadenosine triphosphatase
DBA: Diamond-Blackfan anemia
DC: dyskeratosis congenita
DCC: delayed cord clamping
DCE: downstream core element
DCK: deoxycytidine kinase
DCLRE1C: DNA cross-link repair enzyme 1C
DCRLRE1C: DNA cross-link repair enzyme 1C
DCT1: divalent cation transporter 1
DDAVP: 1-deamino-8-D-arginine vasopressin
DEB: diepoxybutane
deoxy-Hb: deoxygenated hemoglobin
DFMO: a-difluoromethylornithine
DFS: dermatofibrosarcoma; disease-free survival
DGS: DiGeorge syndrome
dGTP: deoxyguanosine triphosphatase
DHAP: dihydroxyacetone phosphate
DHF: dengue hemorrhagic fever; 7,8-dihydrofolate
DHFR: dihydrofolate reductase
DHR 123: dihydroxyrhodamine 123
DIC: disseminated intravascular coagulation
DIDMOAD: diabetes insipidus, diabetes mellitus, optic atrophy, and deafness
DIDS: 4,4′-diisothiocyanostilbene-2,2′-disulfonate (anion channel inhibitor)
DIIHA: drug-induced immune hemolytic anemia
DJS: Dubin-Johnson syndrome
DLI: donor lymphocyte infusion
DLBCL: diffuse large B-cell lymphoma
DLT: dose-limiting toxicity
DMS: demarcation membrane system
DMSO: dimethyl sulfoxide
DMT1: divalent metal transporter 1
DNA: deoxyribonucleic acid
DNA-PKcs: DNA-dependent protein kinase catalytic subunit
DNET: dysembryoplastic neuroepithelial tumors
DNM3TA: DNA methyltransferase 3A
DNM3TB: DNA methyl transferase 3B
DNTs: double-negative T cells
DOCK8: dedicator of cytokinesis 8
DLI: donor lymphocyte infusion
DOPA: 3,4-dihydroxyphenylalanine
DOT1L: Histone-*lysine*-N-methyltransferase, H3 *lysine*-79–specific
DP: double-positive
2,3-DPG: 2,3-diphosphoglycerate
DPT: diphtheria and tetanus toxoids and pertussis
DRESS: drug rash with eosinophilia and systemic symptoms (syndrome)
dRTA: distal renal tubular acidosis
DRVVT: dilute Russell viper venom time
DS: Down syndrome
DS-AMKL: Down syndrome–acute megakaryoblastic leukemia
DSRCT: desmoplastic small round blue cell tumors
DSS: dengue shock syndrome
DS-TMD: Down syndrome–transient myeloproliferative disorder
DTB: ditaurobilirubin
DTI: Diffusion tensor imaging
dTMP: thymidylate (deoxythymidine monophosphate)
DTS: dense tubular system
DVI: partial D category VI phenotype
DVT: deep venous thrombosis
DWI: diffusion-weighted imaging

E

EA: early antigen
EBA140: *Plasmodium falciparum* erythrocyte binding antigen 140
EBMT: European Group for Blood and Marrow Transplantation
EBP: elastin-binding protein
EBRT: external beam radiation therapy
EBV: Epstein-Barr virus
EC: Enzyme Commission
ECLT: euglobulin clot lysis time
ECMO: extracorporeal membrane oxygenation
EDA-ID: anhidrotic ectodermal dysplasia with immunodeficiency
EDAS: encephaloduroarteriosynangiosis
EDAMS: encephaloduroarteriomyosynangiosis
EDS: Ehlers-Danlos syndrome
EDTA: ethylenediaminetetraacetic acid
EFS: event-free survival
EGA: estimated gestational age
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
EHEC: enterohemorrhagic *Escherichia coli*
EKLF: erythroid Krüppel-like factor
ELISA: enzyme-linked immunoabsorbent assay
EM: extramedullary
eNOS: endothelial nitric oxide synthase
ENU: *N*-ethyl-*N*-nitrosourea
EPCR: endothelial cell protein C receptor
EPEC: enteropathogenic *Escherichia coli*
EPO: erythropoietin
EpoR: erythropoietin receptor
EPP: erythropoietic protoporphyria
EPR: electron paramagnetic resonance
ER: endoplasmic reticulum; estrogen receptor
ERGIC-53: endoplasmic reticulum–Golgi intermediate compartment, 53 kilodalton protein
ERT: enzyme replacement therapy
ES: embryonic stem
ESC: embryonic stem cell
ESL-1: E-selectin ligand-1
ESRD: end-stage renal disease
ET: essential thrombocythemia
ETANTRs: embryonal tumors with abundant neuropile and true rosettes
EV: epidermodysplasia verruciformis

EVER: epidermodysplasia verruciformis
EZH2: enhancer of zeste 2

F

FA: Fanconi anemia; focal anaplasia
FAAP: Fanconi anemia–associated protein
FAB: French-American-British (staging)
Fab: antigen-binding fragment
FACS: fluorescence-activated cell sorting
FACT: Foundation for the Accreditation of Cell Therapy
FAD: flavin adenine dinucleotide
FADD: Fas-associated via death domain
FAH: fumarylacetoacetate hydrolase
FAICAR: formamido-4-imidazolecarboxamide ribonucleotide
FAK: focal adhesion kinase
FASLG: FAS ligand
FBN: fibronectin
FBXL5: F-box and leucine-rich repeat protein 5
FCP: F-cell production (locus)
FcR: Fc receptor
FcγR: IgG Fc receptor
FDA: Food and Drug Administration
FDC: follicular dendritic cell
FDG-PET: fluorine 18–labeled deoxyglucose positron emission tomography
FDP: familial platelet deficiency; fibrin(ogen) degradation product; fructose 6-diphosphate
FECH: ferrochelatase
FEIBA: factor eight inhibitor–bypassing activity
FEP: free erythrocyte protoporphyrin
Fe-SIH: ferric salicylaldehyde-isonicotinyl-hydrazone
FFP: fresh-frozen plasma
FGAR: formylglycinamide ribonucleotide
FGF: fibroblast growth factor
fH: factor H
FHL: familial hemophagocytic lymphohistiocytosis
fI: factor I
FIGlu: formiminoglutamic acid
FISH: fluorescence in situ hybridization
FKBP: FK-binding proteins
FL: ligand for the Flks/Flt3 receptor
FLAER: fluorescent aerolysin
FLAIR: fluid-attenuated inversion recovery
FLIP: FLICE-like inhibitory protein
FLT3: fms-related tyrosine kinase 3
FMH: fetomaternal hemorrhage
FN: fever and neutropenia
FNA: fine needle aspiration
FOG-1: friend of GATA-1
FOX: forkhead box transcription factor
FPA: fibrinopeptide A
FPB: fibrinopeptide A
F-PCT: familial porphyria cutanea tarda
FPN1: ferroportin 1
FRAP: fluorescence recovery after photobleaching
FSGS: focal sclerosing glomerulosclerosis
FTCD: formiminotransferase-cyclodeaminase
5-FU: 5-fluorouracil
FUO: fever of unknown origin

G

γc: cytokine receptor common gamma chain
G6PD: glucose-6-phosphate dehydrogenase
GABA: γ-aminobutyric acid
GAG: glycosaminoglycan
β-gal: β-d-galactosidase
GalNAc-T: *N*-acetylgalactosaminyltransferase
GALNS: *N*-acetylgalactosamine-6-sulfatase
GAP: GTPase-activating protein
GAR: glycinamide ribonucleotide
GAS: IFN-γ–activated sequence
GAT: granulocyte agglutination test
GBS: Guillain-Barré syndrome
GC: germinal center; glucocorticoids
GCase: acid β-glucosidase; glucocerebrosidase (i.e, the Gaucher disease enzyme)
G-CSF: granulocyte colony-stimulating factor
G-CSFR: granulocyte colony-stimulating factor receptor
GDF: growth and differentiation factor
GDF15: growth differentiation factor 15
GDP: guanosine diphosphate
GEMMs: genetically engineered mouse models
GFR: glomerular filtration rate
GH: growth hormone
GI: gastrointestinal
GIF: gastric intrinsic factor
GIFT: granulocyte immunofluorescence test
GIST: gastrointestinal stromal tumors
GITMO: Gruppo Italiano Trapianti di Midollo Osseo
GlcNAc: *N*-acetylglucosamine
GLRX5: glutaredoxin 5
Glu-Plg: glutamic acid plasminogen
GluR1: glutamate receptor type 1
GM2A: GM2 activator protein
GM-CSF: granulocyte-macrophage colony-stimulating factor
GMP: granulocyte-monocyte precursor, guanosine monophosphate; granulocytic-monocytic–restricted progenitors
GMTZ: gemtuzumab ozogamicin
GNPT: UDP-*N*-acetylglucosamine:lysosomal-enzyme *N*-acetylglucosamine-1-phosphotransferase
GnRH: gonadotropin-releasing hormone
GP: glycoprotein
GPA: glycophorin A
GPB: glycophorin B
GPC: glycophorin C
GPCR: G protein–coupled receptor
GPD: glycophorin D
G3PD: glyceraldehyde-3-phosphate dehydrogenase
G6PD: glucose-6-phosphate dehydrogenase
GPE: glycophorin E
GPI: glucose phosphate isomerase, glycosylphosphatidylinositol
GPIb: glycoprotein Ib
GPS: gray platelet syndrome
GRF: guanine nucleotide–releasing factor
GRO: growth-regulated oncogene
GS: Gilbert syndrome; Griscelli syndrome
GS2: Griscelli syndrome type 2

GSCbl: glutathionylcobalamin
GSH: reduced glutathione, γ-secretase inhibitor
GSK3: glycogen synthase kinase 3
GSL: glycosphingolipid
GSSG: oxidized glutathione
GSHPX: glutathione peroxidase
GST: glutathione-*S*-transferase
GT: Glanzmann thrombasthenia
GTP: guanosine triphosphate; guanosine nucleotide phosphate
GTPase: guanosine triphosphatase
GV: gemcitabine and vinorelbine
GVHD: graft-versus-host disease
GVL: graft versus leukemia

H

H3K27: lysine 27 of histone 3
H3K79: lysine 79 of histone 3
H63D: histidine 63 to aspartic acid
H^+: hydrogen cation
HAART: highly active antiretroviral therapy
HABA: 2(4′-hydroxyazobenzene) benzoic acid
HAE: hereditary angioedema
HAMP: hepcidin antimicrobial peptide
Hb: hemoglobin
HB: Heinz body
HbA: adult hemoglobin
HbF: fetal hemoglobin
HBV: hepatitis B virus
HC: homocysteine; haptocorrin (also called transcobalamin I or R binder)
4-HC: 4-hydroperoxycyclophosphamide
HCP: hereditary coproporphyria, hematopoietic cell phosphatase
HCV: hepatitis C virus
HD: Hodgkin disease; homodimerization
HDAC: Histone deacetylase
HDL: high-density lipoprotein
HDN: hemolytic disease of the fetus and newborn; hemorrhagic disease of the newborn
HDW: hemoglobin distribution width
HE: hereditary elliptocytosis
HELLP: hemolytic anemia, elevated liver enzymes, low platelet count
HEMPAS: hereditary erythroblastic multinuclearity with a positive acidified serum lysis test
HEP: hepatoerythropoietic porphyria
HES: Hairy and Enhancer of Split, hypereosinophilic syndrome
HETE: 15(*S*)-hydroxyeicosatetraenoic acid
Hex-A: hexosaminidase A
Hex-B: hexosaminidase B
HFE: HLA-linked human hemochromatosis protein
HGF: hematopoietic growth factor; hepatocyte growth factor
HGFR: hematopoietic growth factor receptor
HHS: Hoyeraal-Hreidarsson syndrome
HHV-6: human herpesvirus 6
HHV-8: human herpesvirus 8
HiB: *Haemophilus influenzae* type B
HIDA: hepatobiliary iminodiacetic acid
HIES: hyperimmunoglobulin E syndrome (hyper-IgE syndrome)
HIF: hypoxia-inducible factor
HIF-1: hypoxia-inducible factor 1
HIM: hyper-IgM syndrome
HIT: heparin-induced thrombocytopenia
HITTS: HIT with thrombosis syndrome
HIV: human immunodeficiency virus
HJV: hemojuvelin
HK: hexokinase
HLA: human leukocyte antigen
HLH: hemophagocytic lymphohistiocytosis
HMB: hydroxymethylbilane
HMGB1: high–molecular group box 1 protein
HMP: hexose monophosphate
HMPV: human metapneumovirus
HMS: hyperreactive malarial splenomegaly
HMWK: high-molecular-weight kininogen
HNE: hydroxynonenal
hnRNA: heterogeneous nuclear RNA
HO: heme oxygenase
HOCl: hypchlorous acid
H_2O_2: hydrogen peroxide
H_2OCbl: aquocobalamin
holo-HC: holohaptocorrin
holo-TC: holotranscobalamin
HOX: homeobox transcription factor
HPA-1a: human platelet antigen 1a
HPC: hematopoietic progenitor cell
HPCHA: high-phosphatidylcholine hemolytic anemia
HPFH: hereditary persistence of fetal hemoglobin
HPLC: high-performance liquid chromatography; high-pressure liquid chromatography
HPP: hereditary pyropoikilocytosis
HPP-CFCs: high–proliferative potential colony-forming cells
HPRT: hypoxanthine phosphoribosyltransferase
HPS: Hermansky-Pudlak syndrome
HPV: human papillomavirus
HR: homologous recombination
HRE: hypoxia response element
HRI: heme-regulated inhibitor kinase
HRM: heme regulatory motif
HRQoL: Health-related quality of life
HS: hereditary spherocytosis; hypersensitivity site
HSC: hematopoietic stem cell
HSCT: hematopoietic stem cell transplantation
HSP: heat shock protein; Henoch-Schönlein purpura
HSt: hereditary stomatocytosis
HSV: herpes simplex virus
5-HT: *5′*-hydroxytryptamine (serotonin)
HTLV: human T-cell leukemia/lymphoma virus; human T-cell lymphotropic virus
HUS: hemolytic uremic syndrome
HUSOFT: Hydroxyurea Safety and Organ Toxicity
HUVEC: human umbilical vein endothelial cell

I

IAP: integrin-associated protein or CD47
IAS: ischemic arterial stroke
IBMFS: inherited bone marrow failure syndrome

IBMTR: International Bone Marrow Transplant Registry
ICAM: intercellular adhesion molecule
ICE: ifosfamide, carboplatin, and etoposide
ICD-9: International Classification of Diseases, 9th revision
ICF: immunodeficiency-centromeric instability facial anomalies
ICG: indocyanine green
ICH: intracranial hemorrhage
ICN: intracellular domain of Notch
ICOS: inducible costimulator; inducible T-cell costimulator
ICOSL: inducible costimulator ligand
IDO: indolamine 2,3-dioxygenase
IDS: iduronate 2-sulfatase
IDUA: α-l-iduronidase
IE: ineffective erythropoiesis
IEF: isoelectric focusing
IF: intrinsic factor
IFAR: International Fanconi Anemia Registry
IFI: invasive fungal infection
IFN: interferon
IFN-α: interferon-α
IFN-γ: interferon-γ
IFNGR: interferon γ receptor
IFRT: involved-field radiotherapy
IFS: infantile sarcoma
IgA: immunoglobulin A
IGAD: IgA deficiency
IGF-1: insulin-like growth factor 1
IGFBP: insulin-like growth factor binding protein
IgE: immunoglobulin E
IgG: immunoglobulin G
IgM: immunoglobulin M
IGGSD: IgG subclass deficiency
IHC: immunohistochemistry
IκB: inhibitor of NF-κB
IKBA: inhibitor of NF x-B α chain
IKK: IκB kinase
IL: interleukin
IL-1RA: IL-1 receptor antagonist
IL-2: interleukin-2 (also IL-1, IL-3, IL-4, etc.)
IL2RG: interleukin-2 receptor gamma (IL-2R γ)
IL-6R: interleukin-6 receptor
IL-“X”R: IL-“X” receptor
ILNR: intralobar nephrogenic rests
IMP: inosine 5′-monophosphate; intramembrane particle
IMRT: intensity-modulated radiation therapy
IMT: inflammatory myofibroblastic tumor
iNKT: invariant natural killer T cell
iNOS: inducible nitric oxide synthase
INPC: International Neuroblastoma Pathology Classification
INR: international normalized ratio
INRG: International Neuroblastoma Risk Group
INSS: International Neuroblastoma Staging System
IRS: Intergroup Rhabdomyosarcoma Study
IP: isoprostenoid
IP_3: inositol 1,4,5-triphosphate
IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked (syndrome)
IPH: idiopathic pulmonary hemosiderosis
IPT: intraperitoneal fetal transfusion
IRAG: IP_3 receptor–associated cGMP
IRAK-4: interleukin-1 receptor–associated kinase 4
IRE: iron response element, iron responsive element
IREG1: iron-regulated transporter
IRF: interferon regulatory factor
IRIDA: iron-refractory iron deficiency anemia
IRP: IRE-binding protein, iron regulatory protein
IRSS: International Retinoblastoma Staging System
ISBT: International Society for Blood Transfusion
ISC: irreversibly sickled cell
ISH: in situ hybridization
ISHAGE: International Society for Hematotherapy and Graft Engineering
ISI: International Sensitivity Index
ISM: indolent systemic mastocystosis
ISP: intermediate single positive
ISRE: interferon-stimulated response element
IST: immunosuppressive therapy
ISTH: International Society on Thrombosis and Hemostasis
ITAM: immunoreceptor tyrosine–based activation motif, immunoreceptor tyrosine activation motif
ITCH: itchy E3 ubiquitin protein ligase
ITD: internal tandem duplication
ITIM: immunoreceptor tyrosine–based inhibitory motif, immunoreceptor tyrosine activation motif
ITP: immune thrombocytopenia purpura
ITK: IL-2 inducible tyrosine kinase
iTreg: inducible Treg
IVIG: intravenous immune serum globulin, intravenous immunoglobulin
IVS: intervening sequence (intron)
IVT: intravascular transfusion

J

J: joining
JAK: Janus kinase
JAK-STAT: Janus kinase–signal transducer and activator of transcription
JAM-1: junctional adhesion molecule 1
JCML: juvenile chronic myelomonocytic leukemia
JIA: juvenile idiopathic arthritis
JME: juxtamembrane expansion
JMML: juvenile myelomonocytic leukemia

K

kb: kilobase
KCC: K^+-Cl^- cotransporter
K_d: dissociation constant
KD: Kostmann’s disease, kyphomelic dysplasia
KEYY: lysine–glutamic acid–tyrosine-tyrosine
KIF1B: kinesin family member 1B
KIR: killer cell immunoglobulin-like receptor
KL: Klotho (gene)
K_m: Michaelis constant
KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
KTLS: c-Kit$^+$, Thy-1^{lo}, Lin$^-$, Sca-1$^+$ cell

KLF4: Krüppel-like factor 4
KLH: keyhole limpet hemocyanic
KSS: Kearns-Sayre syndrome

L

LA: lupus anticoagulant
LAD I: leukocyte adhesion deficiency type I
LAD II: leukocyte adhesion deficiency type II
LAD III: leukocyte adhesion deficiency type III
LAL: lysosomal acid lipase
LAMP-3: lysosome-associated membrane protein 3
LASD: lysosomal autophagy system disease
LAP: localized aggressive periodontitis; leukocyte alkaline phosphatase
LAT: linker for activation of T cells
LCAT: lecithin-cholesterol acyltransferase
Lck: lymphocyte-specific protein tyrosine kinase
LCL: large cell lymphoma
LCR: locus control region
LDH: lactate dehydrogenase
LDHL: lymphocyte-depleted Hodgkin lymphoma
LD-RIPA: low-dose ristocetin-induced platelet aggregation
LDL: low-density lipoprotein
LEF-1: lymphoid enhancer–binding factor 1
LELY: low-expression allele Lyon
LEPRA: low-expression allele Prague
LFA-3: lymphocyte function antigen 3
LFS: Li-Fraumeni syndrome
LGL: large granular lymphocyte leukemia
LHRF: luteinizing hormone–releasing factor
LIC: leukemia-initiating cells
LIF: leukemia inhibitory factor
LIFR: leukemia inhibitory factor receptor
LIG4: DNA ligase IV
LIR: leukocyte immunoglobulin-like receptor
LJP: localized juvenile periodontitis
LL: lymphoblastic lymphoma
LMIC: low- and middle-income countries
LMO2: LIM domain only 2 (rhombotin-like 1)
LMWH: low-molecular-weight heparin
LMWK: low-molecular-weight kininogen
LNR: Lin12-NOTCH repeats
LOH: loss of heterozygosity
LPS: lipopolysaccharide
LRHL: lymphocyte-rich Hodgkin lymphoma
LRP: lipoprotein receptor–related protein
LRRC8: leucine-rich repeat–containing 8
LSD: lysosomal storage disease
LSP1: lymphocyte-specific protein 1
LT: leukotriene
LTB$_4$: leukotriene B$_4$
LTC-IC: long-term culture–initiating cell
LTR: long terminal repeat
LTR-HSCs: long-term–reconstituting hematopoietic stem cells
LW: Landsteiner-Weiner glycoprotein
Lyso-PC: lysophosphatidylcholine
Lys-Plg: lysine plasminogen
LYST: lysosomal trafficking regulator

M

mAbs: monoclonal antibodies
MAGE: melanoma antigen-encoding gene
MAGT1: magnesium transporter protein 1
MAGUK: membrane-associated guanylate kinase
MAIT: mucosa-associated invariant T
MALT1: mucosa-associated lymphoid tissue lymphoma translocation gene 1
MAP: mitogen-activated protein
MAPK: mitogen-activated protein kinase
MARCKS: myristoylated alanine-rich C kinase substrate
MAS: macrophage activation syndrome
MASP: MBL-associated serine protease
MASCC: Multinational Association of Supportive Care in Cancer
Mb: myoglobin
MBD: membrane-binding domain
MBL: mannose-binding lectin
MBP: major basic protein
MCH: mean corpuscular hemoglobin
MCHL: mixed-cellularity Hodgkin lymphoma
MCHC: mean corpuscular hemoglobin concentration
MCL: mast cell leukemia
MCM4: mini-chromosome maintenance-deficient 4
MCP: membrane cofactor protein
MCP-1: monocyte chemotactic protein 1
M-CSF: macrophage colony-stimulating factor
MCV: mean cell volume; mean corpuscular volume
MCV4: meningococcal conjugate vaccine
MCyR: major cytogenetic response
MDCK: Madin-Darby canine kidney (cells)
MDM2: murine double minute 2
MDS: myelodysplastic syndrome
MDWH: metaphyseal dysplasia without hypotrichosis
MeCbl: methylcobalamin
MEK: MAPK kinase
MELAS: mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes
MEN1: multiple endocrine neoplasia type I
MEP: megakaryocyte, erythroid, and basophil (lineage); megakaryocyte/erythroid precursor
met-Hb: methemoglobin
5-methyl-THF: 5-methyltetrahydrofolate
α_2-MG: α_2-macroglobulin
MGF: myelomonocytic growth factor
MGP: marginating granulocyte pool
MGSA: melanoma growth stimulatory activity
mHA: minor histocompatibility antigen
MHA: May-Hegglin anomaly
MHC: major histocompatibility complex
MIBG: metaiodobenzylguanidine
micro-PET: micro–positron emission tomography
MIDAS: metal ion–dependent adhesion site
MIM: Mendelian Inheritance in Man
MIP-1α: macrophage inflammatory protein 1α
miR: micro–ribonucleic acid
MIS: minimally invasive surgery
MKI: mitosis-karyorrhexis index
MLASA: mitochondrial myopathy with lactic acidosis and sideroblastic anemia

MLD: metachromatic leukodystrophy
MLL: mixed-lineage leukemia
mM: millimolar
MMA: methylmalonic acid
MMC: mitomycin C
MMF: mycophenolate mofetil
MMP: matrix metalloproteinase
MMP-25: membrane type matrix metalloproteinase 25; the membrane-associated metalloproteinase leukolysin
MoAb: monoclonal antibody
M6P: mannose 6-phosphate
MPD: myeloproliferative disease
MPCF: multiparameter flow cytometry
MPGM: monophosphoglycerate mutase
Mpl: ligand for the myeloproliferative ligand receptor
MPO: myeloperoxidase
MMR: major molecular response
MRP: multidrug resistance-associated protein
MPS: Montreal platelet syndrome, mucopolysaccharidosis
MPS I: mucopolysaccharidosis I
MPS I H: Hurler disease
MPS III: Sanfilippo disease
MPS III A: Sanfilippo disease III A
MPS III B: Sanfilippo disease III B
MPS VI: Maroteaux-Lamy disease
MPS VII: Sly syndrome
MPSV4: meningococcal polysaccharide vaccine
MPV: mean platelet volume
M_r: molecular weight
MRA: magnetic resonance angiography
MRI: magnetic resonance imaging
MRC: Medical Research Council
MRE11A: meiotic recombination 11 homolog A
mRNA: messenger RNA
mRNP: messenger ribonucleoprotein
MRP2: multidrug resistance–associated protein 2 (a.k.a. ABCC2, cMOAT)
MRT: malignant rhabdoid tumor
ms: millisecond
MSC: multipotent mesenchymal stem cells
MSCV: murine stem cell virus
MSH: Multicenter Study of Hydroxyurea
MSH6: MutS homologue 6
MSMD: mendelian susceptibility to mycobacterial diseases
MSP1: *Plasmodium falciparum* merozoite surface protein 1
MST: metanephric stromal tumor
MTD: maximum tolerated dose
mtDNA: mitochondrial DNA
mTEC: medullary thymic epithelial cell
MTHFD1: methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1
MTHFR: methylenetetrahydrofolate reductase (methylene-THF reductase)
mTOR: mammalian target of rapamycin
MTTP: microsomal triglyceride transfer protein
MTP1: metal tolerance protein 1
Munc: mammalian homolog of *Caenorhabditis elegans* unc
MVB: multivesicular body
MWC: Monod, Wyman, and Changeaux model
MYD88: myeloid differentiation primary response gene (88)
MYHIIA: myosin heavy chain IIA
MYO5A: myosin 5 Va

N

NAA: *N*-acetylaspartate
NACHRI: National Association of Children's Hospitals and Related Institutions
NAD: nicotinamide adenine dinucleotide
NAD 47/89: neutrophil actin dysfunction with 47- and 89-kD protein abnormalities
NADH: reduced nicotinamide adenine dinucleotide
NADP: nicotinamide adenine dinucleotide phosphate
NADPH: reduced nicotinamide adenine dinucleotide phosphate
NAFLD: nonalcoholic fatty liver disease
NAIT: neonatal alloimmune thrombocytopenia
NANT: the New Approaches to Neuroblastoma Therapy consortium
NAP: neutrophil-activating protein
NAP-2: neutrophil-activating protein 2
NAT: nucleic acid testing
NATP: neonatal alloimmune thrombocytopenic purpura
NB-DGJ: *N*-butyldeoxygalactonojirimycin
NBS: Nijmegen breakage syndrome
NBT: nitroblue tetrazolium
NCI: National Cancer Institute
NCV: nerve conduction velocity
NDPase: nucleoside diphosphatase
NE: neutrophil elastase
NEMO: NF-κB essential modulator
NET: neutrophil extracellular trap
NF-1: neurofibromatosis type 1
NF-AT: nuclear factor of activated T cells
NF-κB: nuclear factor κB
NGAL: neutrophil gelatinase–associated lipocalin
NGF: nerve growth factor
NH: neonatal hemochromatosis
NHANES: National Health and Nutrition Examination Survey
NHEJ: nonhomologous end joining
NHL: non-Hodgkin's lymphoma
NHLBI: National Heart, Lung, and Blood Institute
NICU: neonatal intensive care unit
NIH: National Institutes of Health
NIK: NF-κB–inducing kinase
NK: natural killer (cell)
NKT: natural killer T cell
NLR: NOD-like receptor
NLPHL: nodular lymphocyte–predominant Hodgkin lymphoma
nm: nanometer
NMDP: National Marrow Donor Program
NMR: nuclear magnetic resonance
NNJ: neonatal jaundice
NO: nitric oxide
NOD: nonobese diabetic

NOS: nitric oxide synthase
NOX: NAD(P)H oxidase
NPA: Niemann-Pick disease type A
NPB: Niemann-Pick disease type B
NPC: Niemann-Pick disease type C
NR: nephrogenic rest
Nramp2: natural resistance-associated macrophage protein 2
NRAS: neuroblastoma RAS viral (v-ras) oncogene homolog
Nrf-1: nuclear regulatory factor 1
NRTI: nucleoside reverse transcriptase inhibitors
ns: nanosecond
NS: Noonan syndrome
NSAID: nonsteroidal anti-inflammatory drug
NSE: nonspecific esterases
NSCLC: non–small cell lung cancer
NSHL: nodular sclerosis Hodgkin lymphoma
NTBI: non–transferrin-bound iron
NTD: neural tube defect
nTreg: natural Treg
NuMA: nuclear mitotic apparatus (protein)
NWTSG: National Wilms Tumor Study Group

O

O_2: oxygen
OATP: organic acid–transporting polypeptide
OCA: oral contraceptive agent
OCS: open canalicular system
OCT1: organic cation transporter-1
ODC1: ornithine decarboxylase–1
OF: osmotic fragility
OGT: orogastric tube
OHCbl: hydroxycobalamin
OLTX: orthotopic liver transplant
OMIM: Online Mendelian Inheritance in Man
OMS: opsoclonus-myoclonus syndrome
OPG: Optic pathway gliomas
OPSI: overwhelming postsplenectomy infection
OR: odds ratio
OS: Omenn syndrome; overall survival
OSA: osteosarcoma
OSM: oncostatin M
OSMR: oncostatin M receptor
oxy-Hb: oxygenated hemoglobin

P

PA: phosphatidic acid
PAF: platelet-activating factor
PAFc: polymerase-associated factor complex
PAGE: polyacrylamide gel electrophoresis
PAI-1: plasminogen activator inhibitor 1
PAIgG: platelet-associated IgG
PALS: periarterial (or periarteriolar) lymphoid sheath
PAMP: pathogen-associated molecular pattern
PANDAS: pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections
PAR: protease-activated receptor
PAS: periodic acid–Schiff
PBG: porphobilinogen
PBG-D: porphobilinogen deaminase
PBREM: phenobarbital-responsive enhancer module
PBSC: peripheral blood stem cell
PC: phosphatidylcholine
PCBP1: poly C–binding protein 1
PCC: prothrombin complex concentrate
PCFT: proton-coupled folate transporter
PCH: paroxysmal cold hemoglobinuria
PCP: *Pneumocystis carinii* pneumonia
PCR: polymerase chain reaction
PCT: porphyria cutanea tarda
PCV: polycythemia vera
PCV7: 7-valent pneumococcal polysaccharide vaccine
PCV23: 23-valent pneumococcal polysaccharide vaccine
PD-1: programmed death 1
PDB: Protein Data Bank
PDGF: platelet-derived growth factor
PDGFB: platelet-derived owth factor β
PE: phosphatidylethanolamine; pulmonary embolism
PEBP2: polyomavirus enhancer–binding protein 2
PECAM-1: platelet–endothelial cell adhesion molecule 1
PEG: polyethylene glycol
PEG-ADA: polyethylene glycol conjugated ADA
PEG-MGDF: pegylated megakaryocyte growth and development factor
PEMR: progressive epilepsy with mental retardation
PET: partial exchange transfusion
PF4: platelet factor 4
PFA-100: Platelet Functional Analyzer 100
PFK: phosphofructokinase
PFS: progression-free survival
PFT: pulmonary function test
PGC-1a: peroxisomal proliferator–activated cofactor 1α
PGD: preimplantation genetic diagnosis
6-PGD: 6-phosphogluconate dehydrogenase
PGI_2: prostaglandin I_2 (prostacyclin)
PGK: phosphoglycerate kinase
PH: Pleckstrin homology
PHA: phytohemagglutinin
PHS: polycythemia hyperviscosity syndrome
PHT: primary hypertension; pulmonary hypertension
P_i: inorganic phosphate
PI: phosphatidylinositol
PIDD: primary immunodeficiency disease
PIG-A: phosphatidylinositol glycan class A (gene: *PIGA*)
PI3: phosphoinositide 3′
PI3K: phosphatidylinositol 3′-kinase
PIK3R2: phosphoinositide-3-kinase, regulatory subunit 2 (beta)
PIP: phosphatidylinositol 4-phosphate
PIP_2: phosphatidylinositol 4,5-bisphosphate
PIP_3: phosphatidylinositol 3,4,5-triphosphate
PIT: plasma iron turnover
PIVKA: protein induced by vitamin K antagonists
PK: pyruvate kinase
PKA: protein kinase A
PKB: protein kinase B
PKC: protein kinase C
PKG: protein kinase G
PKR: protein kinase regulated by RNA

PLC: phospholipase C
PLCγ: phospholipase Cγ
PLCγ2: phospholipase Cγ2
PLDN: pallidin
PLK1: polo-like kinase–1
PLNR: perilobar nephrogenic rests
PLP: pyridoxal 5-phosphate
PLTP: phospholipid transfer protein
PM: primary myelofibrosis
PMA: phorbol myristate acetate
PMBCL: Primary mediastinal (thymic) large B-cell lymphoma
PMD: AU QUERIED
PML: progressive multifocal leukoencephalopathy
PMN: polymorphonuclear neutrophil
PMPS: Pearson marrow-pancreas syndrome
PMS2: postmeiotic segregation 2
PN: parenteral nutrition
P-5′-N: pyrimidine-5′-nucleotidase
PN2/APP: protease nexin-2/amyloid β-protein precursor
PNET: primary neuroectodermal tumor
PNH: paroxysmal nocturnal hemoglobinuria
POD: PML oncogenic domains
POG: Pediatric Oncology Group
PNP: purine nucleoside phosphorylase
PNPD: purine nucleoside phosphorylase deficiency
Po_2: partial pressure of oxygen
PPCA: protective protein cathepsin A
PPO: protoporphyrinogen oxidase
PPP: platelet-poor plasma
PPTID: pineal parenchymal tumor of intermediate differentiation
PPTP: Pediatric Preclinical Testing Program
PR: partial remission
pRBCs: packed red blood cells
PRC2: Polycomb repressive complex 2
PRCA: pure red cell aplasia
PRCC: papillary renal cell carcinoma
PRES: Posterior reversible leukoencephalopathy
PRETEXT: pretreatment imaging-defined staging protocol
PRF1: perforin
PRKDC: protein kinase, DNA-activated, catalytic polypeptide
PROWESS: Protein C Worldwide Evaluation in Severe Sepsis (trial)
PRP: platelet-rich plasma
PRPP: 5-phosphoribosylpyrophosphate
PRR: pattern recognition receptor
PS: Pearson's marrow-pancreas syndrome; phosphatidylserine
PSA: prostate-specific antigen
PSD95: postsynaptic density protein of 95 kD
PSGL-1: P-selectin glycoprotein ligand-1
p-SMAC: peripheral central supramolecular activation cluster
PT: prothrombin time
PTB: phosphotyrosine binding
PTD: partial tandem duplication
P-TEFB: positive transcription elongation factor b
PTH: parathyroid hormone
PTK: phosphotyrosine kinase
PTLD: posttransplant lymphoproliferative disease
PTP: posttransfusion purpura
PTS: postthrombotic syndrome
PTT: partial thromboplastin time
PUS1: pseudouridylate synthase 1
PV: polycythemia vera
PXA: Pleomorphic xanthroastrocytomas

Q

QoL: quality of life

R

RA: refractory anemia
RAB27A: member *RAS* oncogene family 27A
***RAD*:** recombinase-activating gene
RAEB: refractory anemia with an excess of blasts
RAEBIT (RAEB-T): RAEB in transformation
***RAG*:** recombination-activating gene
***RAG1* and *RAG2*:** recombinase activating gene 1 and 2
RANK: receptor activator of nuclear factor κB
RANTES: regulated on activation, normal T-cell expressed and secreted
RAR: retinoic acid receptor
RARS: refractory anemia with ringed sideroblasts
RBC: red blood cell
RCC: renal cell carcinoma
RCT: randomized control trial
RDA: recommended dietary allowance
REAL: Revised European-American Classification of Lymphoid Neoplasms
RECIST: Response Evaluation Criteria in Solid Tumors
REM: rapid eye movement
RES: reticuloendothelial system
RDS: respiratory distress syndrome
RDW: red cell distribution width, red cell volume distribution width
RFC: reduced folate carrier
RFLP: restriction fragment length polymorphism
rFVIIa: recombinant activated factor VII
RFX5: regulatory factor X, 5
RFXANK: regulatory factor X-associated ankyrin-containing protein
RFXAP: regulatory factor X-associated protein
RGD: arginine, glycine, aspartic acid
Rh: rhesus
RhAG: Rh-associated glycoprotein
rhG-CSF: recombinant human granulocyte colony-stimulating factor
RhoH: Ras homology family member H
r-HuEPO: rhEPO, rHuEPO, recombinant human erythropoietin
RIG: retinoic acid–inducible gene
RIAM: $Rap1_{GTP}$-interacting adaptor molecule
RIS: reduction of immunosuppression
RLR: RIG-1–like receptor
RMC: renal medullary carcinoma
RMRP: RNase mitochondrial RNA processing

RNA: ribonucleic acid
rNAPc2: recombinant nematode anticoagulant protein c2
RNP: ribonucleoprotein
ROHHAD: rapid-onset obesity with hypothalamic dysfunction, hypoventilation, and autonomic dysregulation
ROS: reactive oxygen species
RPA: replication protein A
RPE: retinal pigment epithelium
RPL: ribosomal protein of the large ribosomal subunit
RPS: ribosomal protein of the small ribosomal subunit
RPS19: ribosomal protein S19
RR: rate ratio; response rate
rRNA: ribosomal RNA
RS: Rotor syndrome
RSC: reversibly sickled cell
RSP-2: ring surface protein 2
RSV: respiratory syncytial virus
RT: reverse transcriptase
RTA: renal tubular acidosis
RTK: receptor tyrosine kinase; rhabdoid tumor of the kidney
rVIIa: recombinant factor VIIa
RVT: renal vein thrombosis
RVV: Russell viper venom

S

SAA: severe aplastic anemia
SABD: spectrin-actin binding domain
SADNI: specific antibody deficiency with normal immunoglobulins
SAHA: suberoylanilide hydroxamic acid
SAGE: serial analysis of gene expression
SAO: Southeast Asian ovalocytosis
SAP: SLAM-associated protein
SARS: severe acute respiratory syndrome
SBB: Sudan black B
SBDS: Shwachman-Bodian-Diamond syndrome
SCA: sickle cell anemia
Sca-1: stem cell antigen 1
SCI: silent cerebral infarct
SCID: severe combined immunodeficiency disease
SCD: sickle cell disease
SCDSC: subacute combined degeneration of the spinal cord
SCF: stem cell factor
SCFR: stem cell factor receptor
scFv: single-chain variable fragments
SCID: severe combined immunodeficiency
SCN: severe congenital neutropenia
SCNIR: Severe Chronic Neutropenia International Registry
SCT: sacrococcygeal teratomas; stem cell transplantation
SD: standard deviation
SDF-1: stromal cell–derived factor 1
SDS: Shwachman-Diamond syndrome; sodium dodecyl sulfate
SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEER: Surveillance Epidemiology and End Results
SEGA: subendymal giant cell astrocytoma
SERCA: sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SF3B1: splicing factor 3B subunit 1
SGD: specific (secondary) granule deficiency
SH2: src homology domain 2
SHD: sex hormone–binding domain
SH2D1A: SH2-containing protein 1A, Src homology 2 domain–containing protein
Shh: Sonic hedgehog
SHIP1: SH2 domain–containing inositol 5′-phosphatase 1
sHLH: secondary hemophagocytic lymphohistiocytosis
SHML: sinus histiocytosis with massive lymphadenopathy
SHP-2: SH2 domain–containing protein tyrosine phosphatase-2
SIFD: sideroblastic anemia, immunodeficiency, fever, and developmental delay
SIGAD: selective IgA deficiency
sIgM: surface IgM
SIOP: International Society of Paediatric Oncology
SIR: standardized incidence ratio
SIV: simian immunodeficiency virus
S-JIA: systemic onset juvenile idiopathic arthritis
SLAM: signaling lymphocyte activation molecule
SLC4A: solute carrier family 4A
SLC11A2: solute carrier family 11, member 2
SLC19A2: solute carrier family 19 (thiamine transporter), member 2
SLC25A37: solute carrier family 25 (mitochondrial iron transporter), member 37
SLC40A1: solute carrier family 40 member 1
SLE: systemic lupus erythematosus
SL-IC: severe combined immunodeficiency (SC)-ID mouse leukemia–initiating cell
SLP3: stomatin-like protein 3
SLP76: SH2 domain–containing leukocyte protein of 76 kD
SM: sphingomyelin; systemic mastocytosis
SMAC: supramolecular activation cluster
SMARCAL1: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a-like 1
SMC3 and SMC1A: structural maintenance of chromosomes 3 and 1A
SMD: spondylometaphyseal dysplasia
SMMHC: smooth muscle myosin heavy chain
SMN: second malignant neoplasms
Smo: Smoothened receptor
SMRT: silencing mediator of retinoid and thyroid receptors
SNARE: soluble *N*-ethylmaleimide–sensitive attachment protein receptor
snoRNA: small nucleolar RNA
SNP: single nucleotide polymorphism
SOCbl: sulfitocobalamin
SOCS: suppressor of cytokine signaling
SOD2: superoxide dismutase 2
SOS: sinusoidal obstruction syndrome; son of sevenless (protein)
SOT: solid organ transplantation
S-PCT: sporadic porphyria cutanea tarda
SP: single-positive

SP110: SP110 nuclear body protein
SPD: storage pool deficiency
sPLA2: secretory phospholipase A_2
SQUID: superconducting quantum interference device
SRC: SCID-reconstituting cell
SREBP: sterol regulatory element-binding proteins
SRT: substrate reduction therapy
SSCP: single-strand conformation polymorphism
SSP: stage-selector protein
STAG: stromal antigen 2
STAT: signal transducer and activator of transcription
STEAP3: six-transmembrane epithelial antigen of prostate 3
sTfR: serum transferrin receptor
STIM1: stromal interacting molecule 1
STOP: Stroke Prevention Trial in Sickle Cell Anemia
STR-HSC: short-term–reconstituting hematopoietic stem cell
Stx: Shiga-like toxin
STX11: syntaxin 11
STXBP2: syntaxin binding protein 2
SUMF1: sulfatase-modifying factor
SVC: superior vena cava
SWI/SNF: switch/sucrose nonfermentable
SWiTCH: Stroke With Transfusions Changing to Hydroxyurea study

T

TACI: transmembrane activator and calcium modulator and cyclophilin ligand interactor
TACL: Therapeutic Advances in Childhood Leukemia and Lymphoma consortium
TAFI: thrombin-activatable fibrinolysis inhibitor
TALEN: transcription activator-like effector nucleases
T-ALL: T-cell acute lymphoblastic leukemia
T antigen: Thomsen-Friedenreich cryptoantigen
TAM: transient abnormal myelopoiesis
TAMs: tumor-associated macrophages
TAMV: time-averaged maximum velocity
TAP1 or TAP2: transporter associated with antigen processing 1 or 2
TAPS: Alternatives Preoperatively in SCD
TAPA1: target of antiproliferative antibody 1
TAR: thrombocytopenia–absent radius (syndrome)
TAT: thrombin-antithrombin complex
TBI: total-body irradiation
TBK1: TANK-binding kinase 1
TBP: TATA-binding protein
TBX1: T-box 1
TcB: transcutaneous bilirubin
TCD: transcranial Doppler
TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCN2: transcobalamin II
TCR: T-cell antigen receptor, T-cell receptor
TCT: thrombin clotting time
TD: thymus dependent (antigen)
TdT: terminal deoxyribonucleotidyl transferase
TE: thromboembolism
TEC: transient erythroblastopenia of childhood
TEG: thromboelastography
TEMRA: T effector memory cell CD45RA+
TERT: telomerase reverse transcriptase
TF: tissue factor
TFEB: transcription factor EB
TFH: follicular helper T cell
TFPI: tissue factor pathway inhibitor
TFR2: transferrin receptor 2
TGA: thrombin generation assay
TGN: thioguanine nucleotides
TGF-β: transforming growth factor β
TGTP: 6-thioguanosine 5-triphosphate
TH: tyrosine hydroxylase
tHcy: plasma total homocysteine
THF: 5,6,7,8-tetrahydrofolate
THI: transient hypogammaglobulinemia of infancy
TI: thymus independent (antigen)
TIA: transient ischemic attack
TIBC: total iron-binding capacity
TIC: tumor-initiating cell
TICAM1: TIR domain-containing adapter molecule 1
TIMP: tissue inhibitor of matrix metalloproteinase–2
TIPS: intrahepatic portosystemic shunting
TKI: tyrosine kinase inhibitor
TL: transient leukemia
TLR: Toll-like receptor
TLR2: Toll-like receptor 2
TLS: translesion synthesis
TLT-1: TREM cells–like transcript-1
TM: tropomyosin
TMC: transmembrane channel-like
TMD: transient myeloproliferative disorder
TM8: transmembrane domain 8
TMD: transient myeloproliferative disorder
TMod: tropomodulin
TMP-SMX: trimethoprim-sulfamethoxazole
TMPRSS6: transmembrane protease, serine 6
7-TMR: seven-transmembrane–spanning receptor
7-TMS: seven-transmembrane–spanning domain
TNF: tumor necrosis factor
TNF-α: tumor necrosis factor-α
TNFRSF: TNF receptor superfamily
TNFSF: TNF superfamily
TNM: tumor-nodes-metastasis
TOF: time-of-flight (imaging)
TORCH: toxoplasmosis, rubella, cytomegalovirus, and herpesvirus infections
tPA: tissue plasminogen activator
TPE: therapeutic plasma exchange
TPH: transplacental hemorrhage
TPI: triose phosphate isomerase
TPMT: thiopurine methyltransferase
TPN: total parenteral nutrition
TPO: thrombopoietin
TPOR: thrombopoietin receptor
TPR: tetratricopeptide repeat
TQM: total quality management
TRAF: TNF receptor–associated factor
TRAF3: TNF receptor-associated factor 3
TRAIL: tumor necrosis factor–related apoptosis-inducing ligand
TRALI: transfusion-related acute lung injury

TRAP: Trial to Reduce Alloimmunization to Platelets
TREC: T-cell receptor excision circle, T-cell receptor gene excision circle
Treg: T-regulatory
TREM, TREM-1, TREM-2: triggering receptor expressed on myeloid cells (1, 2, etc)
TfR: transferrin receptor
TRIF: Toll-interleukin-1 receptor domain-containing adapter protein inducing interferon β
TRJV: tricuspid regurgitant jet velocity
TRK: tropomyosin receptor kinase
TRM: treatment-related mortality
TRMA: thiamine-responsive megaloblastic anemia
tRNA: transfer RNA
TSB: total serum bilirubin
TSH: thyroid-stimulating hormone
TSLC: tumor suppressor in lung cancer–1
TSLP: thymic stromal thymopoietin
TT: thrombin time
TTP: thrombotic thrombocytopenic purpura
TTT: transpupillary thermotherapy
TTTS: twin-twin transfusion syndrome
TVTG: topotecan, vinorelbine, thiotepa, dexamethasone, and gemcitabine
TWSG1: twisted gastrulation 1
TXA_2: thromboxane A_2
TYK2: tyrosine kinase 2
TYMS: thymidylate synthetase

U

UBIL: unconjugated bilirubin
UCB: umbilical cord blood
UDP: uridine diphosphate
UDPG: uridine diphosphate glucose
UDPGA: uridine diphosphate glucuronic acid
UDPGT1: Uridine diphosphogluconosyl transferase 1
UDPNAG: uridine diphosphate *N*-acetylglucosamine
UGT1A1: UDP glucuronosyltransferase 1A1 gene
UKCCG: United Kingdom Children's Cancer Group
ULVWF: *unusually large multimers of VWF*
UMP: uridine monophosphate
UNC: unc homolog *(C. elegans)*
UNG: uracil nucleoside glycosylase
UNOS: United Network of Organ Sharing
uPA: urokinase plasminogen activator
uPAR: uPA receptor
UPR: unfolded protein response
URD: unrelated donor
URO-D: uroporphyrinogen decarboxylase
URO3-S: uroporphyrinogen III synthase
UTI: urinary tract infection
UTP: uridine triphosphate
UTR: untranslated regions
UV: ultraviolet

V

V: variable
VACTERL: vertebral abnormalities, anal atresia, cardiac abnormalities, tracheoesophageal fistula, and/or esophageal atresia, renal agenesis and dysplasia, and limb defects
VAMP-1, -2: vesicle-associate membrane protein 1, 2, etc
VASP: vasodilator-stimulated phosphoprotein
VATER: vertebral defects, imperforate anus, tracheoesophageal fistula, renal defects and radial dysplasia
VCA: viral capsid antigen
VCAM-1: vascular cell adhesion molecule 1
Vd: volume of distribution
VDJ: variable, diversity, joining
VDRL: Venereal Disease Research Laboratory
VEGF: vascular endothelial growth factor
VHL: von Hippel-Lindau (protein)
VK: vitamin K
VK1: phylloquinone
VK2: menaquinone
VKDB: vitamin K–dependent bleeding
VKDP: vitamin K–dependent protein
VKOR: vitamin K 2,3-epoxide reductase
VLA-4: very late antigen 4
VLBW: very low birth weight
VLDL: very-low-density lipoproteins
VOD: veno-occlusive disease
VODI: veno-occlusive disease with immunodeficiency
VP: variegate porphyria
VP-16: etoposide
VTE: venous thromboembolism
V/Q: ventilation-perfusion
VWD: von Willebrand disease
VWF: von Willebrand factor
VWF:Ag: von Willebrand factor antigen
VWF:CB: collagen-binding assay for von Willebrand factor
VWFpp: von Willebrand factor propeptide
VWF:RCo: VWF activity by ristocetin cofactor assay
VZV: varicella-zoster virus

W

WAGR: **W**ilms tumor, **A**niridia, **G**enitourinary abnormalities, and **R**etardation
WAS: Wiskott-Aldrich syndrome
WASP: Wiskott-Aldrich syndrome protein
WBC: white blood cell
WBRT: whole-brain radiotherapy
WHIM: warts, hypogammaglobulinemia, infections, myelokathexis (syndrome)
WIP: WASP-interacting protein
WIPF1: WAS/WASL interacting protein family, member 1
WNDP: Wilson disease protein
WNV: West Nile virus
WRK: Woodward's reagent K
WSXWS: Trp-Ser-Xaa-Trp-Ser

X

X-CGD: X-linked chronic granulomatous disease
XHIM: X-linked immunodeficiency with normal or elevated IgM
XIAP: X-linked inhibitor of apoptosis

XLA: X-linked agammaglobulinemia
XLF: XRCC4-like factor
XLP1, XLP2: X-linked lymphoproliferative syndrome type 1 and 2
XLPD: X-linked lymphoproliferative disease
XLSA: X-linked sideroblastic anemia
XLSA/A: X-linked sideroblastic anemia with ataxia
XLT: X-linked thrombocytopenia
XPD: xeroderma pigmentosum disease
XRT: x-ray therapy; pulmonary radiation therapy
XSCID: X-linked severe combined immunodeficiency

Y

YARS2: tyrosyl-tRNA synthetase 2, mitochondrial
YTRF: tyrosine-threonine-arginine-phenylalanine (sequence)

Z

ZAP-70: TCRζ–associated protein of 70 kD
ZBTB24: zinc-finger and bric-a-brac, tramtrack, broad complex-domain-containing 24
ZIP: zinc transporter
ZFN: zinc-finger nucleases
ZPI: protein Z–dependent protease inhibitor
ZPP: zinc protoporphyrin

CHAPTER 1

Anatomy and Physiology of Hematopoiesis

Colin A. Sieff, George Q. Daley, and Leonard I. Zon

CHAPTER OUTLINE

HISTORY
PHYLOGENY
MARROW ANATOMY
HEMATOPOIETIC CELLS
Stem Cells
Progenitor Cells
Precursors and Mature Cells
THE HEMATOPOIETIC GROWTH FACTORS
The Lymphohematopoietic Cytokines
The Receptor Tyrosine Kinase Ligands
The Interleukin 1 Gene Family
The Chemokines
The Tumor Necrosis Factors
The Interferons
The Transforming Growth Factor Beta Gene Family
ACTIONS OF HEMATOPOIETIC GROWTH FACTORS
Predominantly Lineage Specific HGFs: G-CSF, M-CSF, Il-5, TPO, and EPO
Multilineage Hematopoietic Growth Factors: Il-3 and GM-CSF
Early-Acting Hematopoietic Growth Factors: SCF and FLT3 Ligand
Other Stem Cell Factors: Wnt, Jagged, BMP, and Angiopoietin-Like Growth Factors
Synergistic Factors: Il-1, Il-6, and Il-11
Negative Regulator of Hematopoiesis: Transforming Growth Factor Beta
MOLECULAR BIOLOGY OF HEMATOPOIETIC GROWTH FACTORS
Genomic Analysis of Cytokine and Cytokine Receptor Genes
Structures of Hematopoietic Growth Factor Proteins
Hematopoietic Growth Factor Gene Disruptions
Hematopoietic Growth Factor Genes: Regulation of Expression
HEMATOPOIETIC GROWTH FACTOR RECEPTORS
Types of Hematopoietic Growth Factor Receptors
Structure and Binding Properties
Receptor Function
BIOLOGY OF HEMATOPOIESIS
Stem Cells
Erythropoiesis
Myelopoiesis
Megakaryocytopoiesis
CLINICAL USE OF HEMATOPOIETIC GROWTH FACTORS
Human Clinical Studies
Toxicity of Treatment with Colony-Stimulating Factors
Antibodies to Recombinant Factors
CONCLUSION

This chapter offers a review of the anatomy and physiology of normal hematopoiesis that is intended to provide a basis for understanding the marrow failure syndromes described at length in the following chapter. In this chapter we briefly discuss the phylogeny of hematopoiesis and describe marrow anatomy and the egress of recognizable hematopoietic cells from the marrow into the peripheral blood. A more detailed analysis then follows of the cellular bases of erythrocyte, granulocyte-macrophage, and megakaryocyte development, including discussions of the pluripotent stem cell and its niche, the more committed but still undifferentiated progenitor cells, and the differentiated precursors of the mature formed elements of the blood. Much of this chapter is devoted to the interactions of growth factors and the cells that produce them in the upregulation of hematopoiesis. The mechanisms of downregulation of hematopoiesis by cell interactions and cytokines are touched upon here, but they are less well understood despite the fact that they are likely to influence the pathophysiology of aplastic anemia and other marrow failure syndromes.

HISTORY*

That "blood is life" was appreciated by Empedocles in the fifth century BC. The theory that the vasculature contains blood, phlegm, black bile, and yellow bile, all revealed when freshly let blood is permitted to separate, is attributed to Polibus, the son-in-law of Hippocrates.

Servetus recognized the systemic and lesser circulations in the sixteenth century. He was burned at the stake, in part because he did not accept the dogma that blood must pass through the intraventricular cardiac septum. (Grant disapproval and, more recently, approval without funding have been substituted for immolation. The effects are not entirely dissimilar.)

In view of the present growth of knowledge of hematology, it is remarkable to realize that the concept of the circulation of the blood was finally established by Harvey in 1628, not even 400 years ago. This began the clinical application of blood transfusion, of which Pepys wrote "it gave rise to many pretty wishes as of the blood of a Quaker to be let into an Archbishop and such like."

In the mid–seventeenth century, Swammerdam observed red blood corpuscles in the microscope and Malpighi discovered the capillary circulation in the lung and in the omentum. But it was not until the nineteenth century that the source of blood cell production began to be successfully explored. Houston suggested that red cells were derived from leukocytes in the lymphoid system. Zimmerman believed that erythrocytes were derived from platelets, an opinion shared by Hayem. Addison, perhaps not surprisingly, attributed red cell production to the adrenals, and Reikert finally suggested that red cells might be produced in the embryonic liver. In fact, it was not until 1868 that Neumann demonstrated that red cells arise from precursors in the marrow. The modern understanding of the physiology of hematopoiesis then began.

PHYLOGENY

Much can be learned about the physiology of hematopoiesis from study of the evolution of oxygen transport, a subject reviewed by Lehman and Huntsman.[1]

One of the major advantages of mammalian life over that of invertebrates is the capacity to package large amounts of hemoglobin within cells. This permits the delivery of oxygen to tissues without the enormous increase in oncotic pressure that would be induced by a similar concentration of high–molecular-weight hemoglobin free in the plasma. The renewal rate of red cells is a function of metabolic rate or basal heat production. This is illustrated dramatically in studies of the animal kingdom, ranging from the turtle to the pygmy shrew, and by comparisons of red cell renewal in marmots during periods at ambient and cold temperatures,[2] in rats,[3] and in frogs.[4]

The production of blood cells in bone marrow is a late development in phylogeny. Red cells are found in the coelomic cavity of the worm, and are produced in the kidneys of the goldfish. The influence of oxygen demand on the production of red cells[5] is illustrated by the effects of hyperoxia on bled rats[6] and the behavior of the European eel, one of the few vertebrate forms that ordinarily lacks erythrocytes in its juvenile state. When the adult eel struggles against the current up the rivers of Europe, nucleated cells containing hemoglobin appear in its plasma. This influence of oxygen demand on respiratory pigment production is also illustrated in organisms that do not produce red cells, such as *Daphnia*, the English water flea, a creature that produces high–molecular-weight hemoglobin in its ovaries when it is exposed to low oxygen tension in stagnant ponds. The discovery of transcription factors that function as oxygen sensors provides a potential molecular explanation for these regulatory mechanisms.[7-10]

MARROW ANATOMY

The active red marrow space of a child is much greater than that of an adult, presumably because the high requirements for red cell production during neonatal life demand the resources of the entire production potential of the marrow. During postnatal life the demands for red cell production ebb, and much of the marrow space is slowly and progressively filled with fat (Fig. 1-1). In certain disease states that are usually associated with anemia, such as myeloid metaplasia, hematopoiesis may return to its former sites in the liver, spleen, and lymph nodes and may also be found in the adrenals, cartilage, adipose tissue, thoracic paravertebral gutters, and even in the kidneys.

The microenvironment of the marrow cavity is a vast network of vascular channels or sinusoids in which float fronds of hematopoietic cells. This complex area of cell biology and anatomy has been the subject of several reviews.[11-15] The vascular and hematopoietic compartments are joined by reticular fibroblastoid cells that form the adventitial surfaces of the vascular sinuses and extend cytoplasmic processes to create a lattice on which blood cells are found. The lattice itself is illustrated by reticulin stains of marrow sections (Fig. 1-2). The conformation of the meshwork of reticulin and the location of hematopoietic cells in the network of vascular sinuses are best illustrated by scanning electron microscopy (Fig. 1-3). The fibroblastoid network has two major functions: (1) it provides an adhesive framework onto which the developing cells are bound, and (2) it participates in the production by these cells of essential hematopoietic colony-stimulating factors,[16] to be discussed below. Cell-cell adhesion may be mediated by binding of the hematopoietic very late antigen 4 (VLA-4) integrin to stromal fibronectin or vascular cell adhesion molecule 1 (VCAM-1).[17-20] In addition, cytokine receptors such as KIT can bind to the membrane-bound form of stem cell factor (SCF),[21] and the extracellular matrix proteins secreted by

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*For an entertaining review from which this precis was in part drawn, see Robb-Smith AHT: The growth of knowledge of functions of the blood, in MacFarlane RG, Robb-Smith AHT, editors: *Functions of the blood*, Oxford, 1961, Blackwell Scientific Publications. For more details, see also *Blood pure and simple* by the late Maxwell M. Wintrobe, New York, 1980, McGraw-Hill.

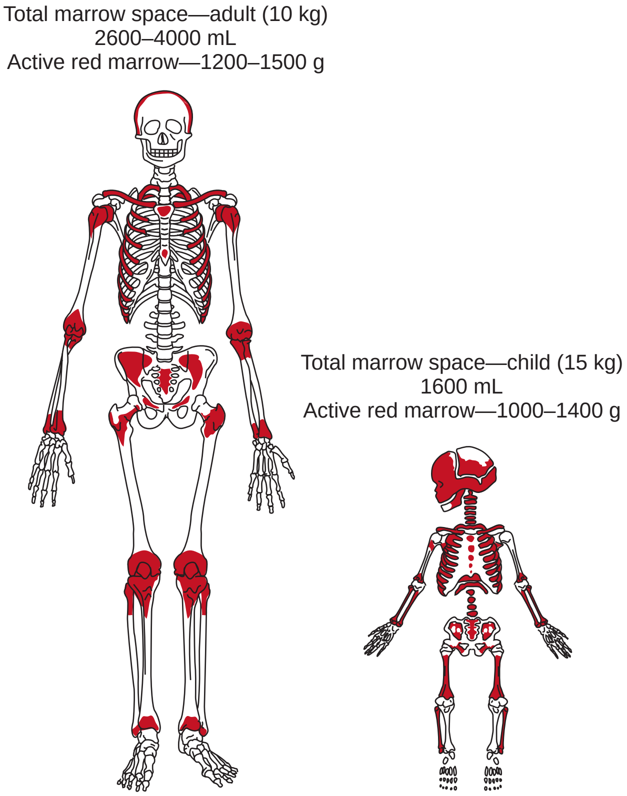

Total marrow space—adult (10 kg)
2600–4000 mL
Active red marrow—1200–1500 g
Total marrow space—child (15 kg)
1600 mL
Active red marrow—1000–1400 g

Figure 1-1 The comparison of active red marrow–bearing areas in the child and the adult. Note the almost identical amount of active red marrow in the child and adult despite a fivefold discrepancy in body weight. *(From MacFarlane RC, Robb-Smith AHT, editors:* Functions of the blood, *Oxford, 1961, Blackwell Scientific, p. 357.)*

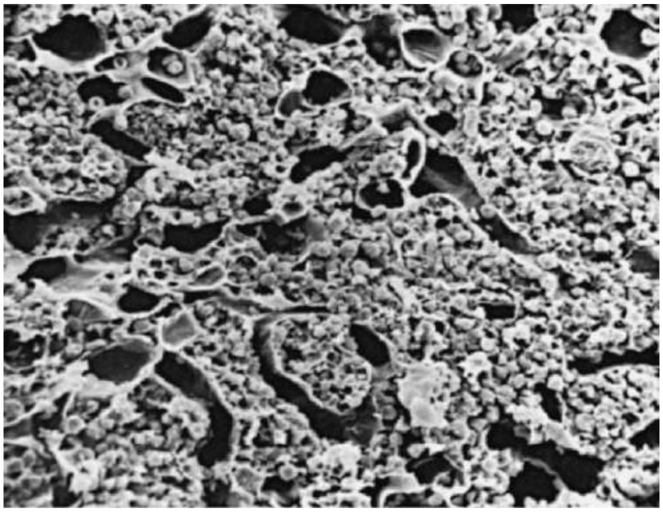


Figure 1-3 Scanning electron micrograph of rat femoral marrow. The hematopoietic cells are grouped between the interlacing network of vascular sinuses. Many cells are dislodged when the marrow is transected, and separate spaces are present where cells had been. *(From Lichtman MA, Chamberlain JK, Santillo PA: Factors thought to contribute to the regulation of egress of cells from marrow. In Gordon AS, Silber K, LoBue L, editors:* The year in hematology, 1978, *New York, 1978, Plenum Medical, p. 243–279.)*

stromal cells may actually provide a binding site for some growth factors or for hematopoietic cells.[22-24] In addition, chemokines, a family of small molecules, play major roles in stromal function. Specifically, stromal-derived factor 1 (SDF-1) is a potent attractant for hematopoietic cells (both mature leukocytes and progenitor cells that express its CXC chemokine receptor 4 [CXCR4]).[25] Gene disruption studies in mice show that both ligand and receptor are lethal in the embryonic stage, with defects in B lymphopoiesis and myelopoiesis.[26,27] These relate to a critical role for CXCR4 in bone marrow engraftment in immunodeficient nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice[28]; SDF-1 has been shown to activate the integrins VLA-4, VLA-5, and lymphocyte function–associated antigen 1 (LFA-1) on CD34+ cells.[29] Last, SDF-1 plays a role in the egress of progenitor cells from bone marrow to blood during stem cell mobilization.[30,31] Indeed, pharmacologic inhibition of the SDF-1 receptor CXCR4 with the drug plerixafor is employed clinically to enhance mobilization.[32]

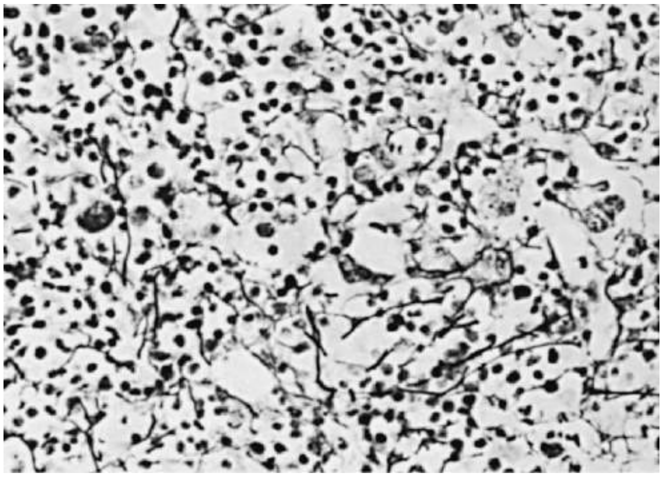


Figure 1-2 Bone marrow biopsy of a patient with mild myelofibrosis showing a slight increase in the number of reticulin fibers in a delicate discontinuous fiber network. (Gomori stain ×350.) *(From Lennert K, Nagai K, Schwarze EW: Patho-anatomical features of the bone marrow.* Clin Hematol *4:335, 1975.)*

A schema of the marrow circulation is shown in Figure 1-4. The central and radial arteries ramify in the cortical capillaries, which in turn join the marrow sinusoids and drain into the central sinus. Cells that egress from the marrow sinusoids then join the venous circulation through comitant veins. The inner, or luminal, surface of the vascular sinusoids is lined with endothelial cells, the cytoplasmic extensions of which overlap, or interdigitate, with one another. The escape of developing hematopoietic cells into the sinus for transport to the general circulation occurs through gaps that develop in this endothelial lining and even through endothelial cell cytoplasmic pores.

The location of the different hematopoietic cells is not random. Clumps of megakaryocytes are found adjacent to marrow sinuses. They shed platelets, the fragments of their cytoplasm, directly into the lumen. This reduces the requirement for movement of bulky mature megakaryocytes, a mobility characteristic of the granuloid- and erythroid-differentiated precursors as they approach the

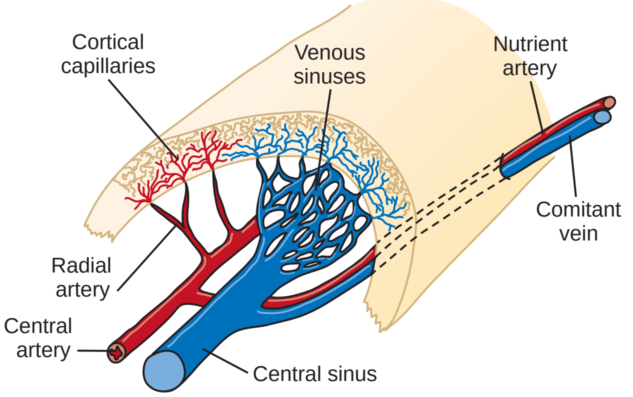


Figure 1-4 A schematic representation of the circulation of the marrow. The nutrient artery, central arteries, and radial arteries feed the cortical capillaries. The cortical capillaries anastomose with the marrow sinuses, which drain into the large central sinus. The central sinus enters the comitant vein by which the marrow effluent enters the systemic venous circulation. An interesting feature of the circulation of marrow is the transit of nearly all arterial blood through cortical capillaries before it enters the marrow sinuses. Not shown are the arterial communications from muscular arteries that feed the periosteum and penetrate the cortex to anastomose with intracortical vessels. *(From Lichtman MA, Chamberlain JK, et al: Factors thought to contribute to the regulation of egress of cells from marrow. In Silber K, LoBue J, et al, editors:* The year in hematology, 1978. *New York, 1978, Plenum Medical, p. 243–279.)*

point at which they egress from the marrow. A schema that illustrates the transfer of hematopoietic cells into the sinus is shown in Figure 1-5. Disruption of the function of microenvironmental cells inhibits long-term murine marrow cultures.[33] Such disruptions may be responsible for certain cases of aplastic anemia. In recent years significant attention has been paid to the cellular components of the hematopoietic stem cell (HSC) niche, with several reports of the role of osteoblasts as supportive microenvironmental cells that liberate critical cytokines such as SDF-1.[34-36] However, alternative views argue for a major role of vascular endothelium of the marrow sinusoids in sustaining HSCs (see Fig. 1-5).[37,38] See the review by Krause and Scadden[39] for further details.

HEMATOPOIETIC CELLS

Stem Cells

The concept that sustained hematopoiesis derives from pluripotent stem cells was first suggested in 1949 by Jacobson,[40] who showed that mice can be protected from the lethal affects of whole-body irradiation by exteriorization and shielding of the spleen. This protective effect was shown to be cell mediated by the observation that the injection of spleen cells could initiate recovery and reestablish hematopoiesis in irradiated animals.[41] The clonal nature of hematopoiesis and the concept that a single pluripotent stem cell exhibits the capacity to repopulate the entire hematopoietic system was first demonstrated experimentally by Till and McCulloch,[42] who also used the mouse as an experimental system. They found colonies of hematopoietic cells containing erythrocytes, granulocytes, macrophages, and megakaryocytes in the spleens of irradiated recipients within 10 days after transplant. Subsequent experiments using karyotypically marked donor cells confirmed the clonal origin of the differentiated cells in the colony, proving that a single multipotent stem cell had given rise to these differentiated cells.[43] It was also shown that each colony contained a number of stem cells that could again form a colony of differentiated progeny in a second irradiated recipient, demonstrating their self-renewal capacity. This is true only of spleen colonies that are present on days 12 to 14 (colony-forming unit–spleen,[CFU-S_{12}]). Colonies observed on days 7 to 8 after marrow infusion are transient, disappear by day 12, and are neither multipotential nor self-maintaining.[44] Under steady-state conditions no more than 10% of the CFU-S become committed to differentiation during any given 3-hour period. The demonstration of a stem cell that can differentiate to form progenitor cells for erythropoiesis, granulopoiesis, and megakaryopoiesis is consistent with subsequent observations in disease states such as chronic myelogenous leukemia[45,46] and polycythemia vera,[47,48] in which a clonal origin of abnormal erythroid, granulocytic, and megakaryocytic precursor cells and lymphocytes can be demonstrated.

The demonstration of a multipotent stem cell in adult bone marrow led to a systematic search for the ontogenic origins of HSCs. Experiments performed by Moore and Metcalf[49] demonstrated the presence of cells capable of repopulating the adult marrow in the yolk sac and the murine fetal liver.[42] Subsequent work has corroborated these observations.[50] It is widely accepted that the first blood cells that circulate in the mammalian embryo arise from the yolk sac. This so-called "primitive" blood lineage consists primarily of nucleated erythrocytes that express embryonic forms of hemoglobin.[51] The classic view of the developmental origins of HSCs holds that the first progenitors of the yolk sac later populate the fetal liver,[52,53] where they mature to become the "definitive" HSCs of the adult bone marrow, whose erythroid progeny express mature adult forms of beta hemoglobin. However, painstaking dissections of midgestation mouse embryos prior to the onset of blood circulation suggest that the HSCs that can engraft the bone marrow of an irradiated adult mouse first arise within the embryonic trunk.[54,55-57] Refinements of the anatomic location of these nascent definitive HSCs show their emergence from a specialized hemogenic endothelium along the base of the developing aorta,[58-62] where the first pulsations of the heartbeat serve as a trigger[63,64] to activate an endothelial-to-hematopoietic transition.[65,66] Debate persists, and some argue that HSCs may arise simultaneously in the yolk sac and intraembryonically from the hemogenic endothelium of the aorta.[67] Complicating the true origins of the first definitive HSCs further, the placenta also appears to be a source of HSCs during development,[68,69] although it remains possible that the stem cells arrive through the circulation from intraembryonic sources such as the hemogenic endothelium of the aorta. Alternatively, given its rich network of arteries, the placenta may indeed be a de novo producer of blood stem cells. The placenta is quantitatively a large source of HSCs during development.

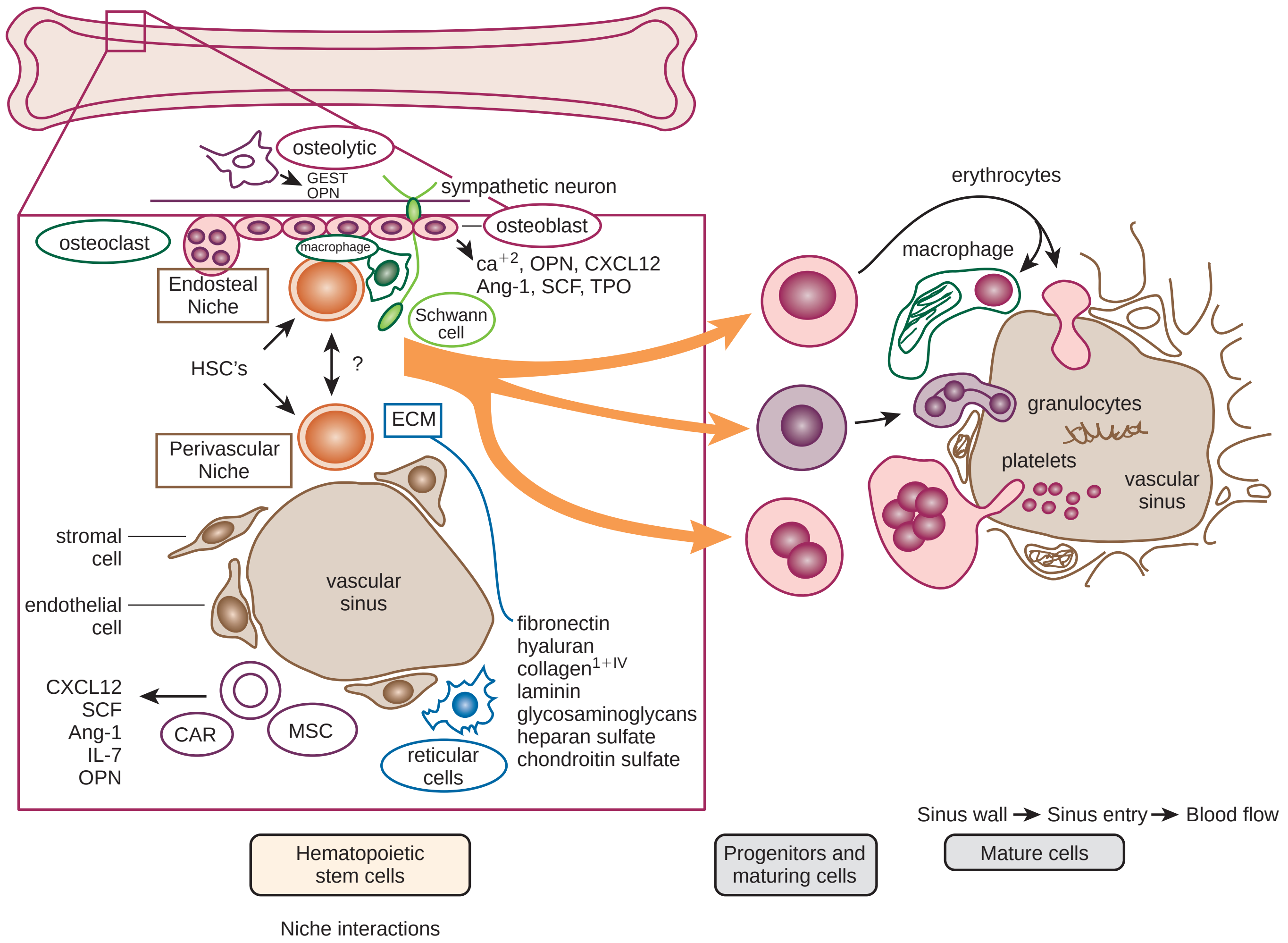

osteolytic
GEST
OPN
sympathetic neuron
osteoclast
macrophage
osteoblast
ca^{+2}, OPN, CXCL12
Ang-1, SCF, TPO
Endosteal Niche
Schwann cell
HSC's
?
ECM
Perivascular Niche
stromal cell
endothelial cell
vascular sinus
fibronectin
hyaluran
collagen^{1+IV}
laminin
glycosaminoglycans
heparan sulfate
chondroitin sulfate
CXCL12
SCF
Ang-1
IL-7
OPN
CAR
MSC
reticular cells
erythrocytes
macrophage
granulocytes
platelets
vascular sinus
Sinus wall → Sinus entry → Blood flow
Hematopoietic stem cells
Progenitors and maturing cells
Mature cells
Niche interactions

Figure 1-5 A schematic diagram of the factors that may be involved in controlling stem cell regulation in the perivascular and endosteal niches as well as the release of marrow cells. The central relationship between the hematopoietic compartment and the marrow sinus is depicted. The stem cell niche is involved in the regulation of hematopoietic stem cell quiescence, self-renewal, mobilization, and differentiation; niches involve cell contact and secretory functions of osteolineage cells, osteoclasts, sinusoidal endothelial cells, macrophages, and mesenchymal stromal and stem cells *(MSCs)*, including CXCL12 abundant reticular cells *(CARs)*, sympathetic neurons, and extracellular matrix. There are nestin-positive stromal cells that are thought to be derived from neural crest, which support hematopoiesis. The drawing highlights the similarity of the egress process for the three major hematopoietic cells: reticulocytes in the top pathway, granulocytes and monocytes in the center pathway, and platelets in the lower pathway. Immature cells undergo biophysical changes under the influence of cytopoietins that favor egress. In the case of the reticulocyte, enucleation precedes egress. This is shown by the solid red inclusion in the perisinal macrophage representing nucleophagocytosis antecedent to digestion of the erythroblast nucleus. The cytoplasmic protrusion of the megakaryocyte presumably detaches itself from the cell and will further fragment into platelets in the circulation. *OPN,* Osteopontin. *(Modified from Lichtman MA, Chamberlain JK, et al: Factors thought to contribute to the regulation of egress of cell from marrow. In Silber K, LoBue J, et al, editors:* The year in hematology, 1978. *New York, 1978, Plenum Medical, p. 243–279.)*

The most primitive cells with the greatest self-renewal capacity reconstitute long-term hematopoiesis after transfer into irradiated recipient mice. These cells, termed long-term reconstituting hematopoietic stem cells (LTR-HSCs), were shown to be separable from CFU-S$_{12}$ in a limiting-dilution assay designed to detect and enumerate "cobblestone areas."[70] This assay is derived from the original "Dexter" technique for long-term culture of murine marrow in which CFU-S, colony-forming unit–granulocyte-macrophage (CFU-GM), and burst-forming unit–erythroid (BFU-E) flourish for many months on and within an adherent stromal monolayer.[71] The areas of active hematopoiesis have a "cobblestone" appearance. In the limiting-dilution assay, different concentrations of bone marrow cells are plated onto a series of microwells that contain a preestablished stromal monolayer, and at 5 weeks the cobblestone areas that comprise proliferating blast cells within the stromal cell layer are counted.[70] In cell separation experiments, their numbers correlate with a cell fraction that is characterized by low mitochondrial mass per cell (minimal retention of the supravital fluorochrome rhodamine-123); this Rho– cell fraction is enriched for marrow repopulating cells but depleted of CFU-S$_{12}$.[70,72] Intermediate and rapidly sedimenting cells contained more than 99% CFU-S$_{12}$ as well as the cells responsible for short-term reconstitution. In contrast, long-term reconstituting cells (>60 days) came from a slowly sedimenting fraction that contained only 0.25% CFU-S$_{12}$.

The stem cell model of hemopoiesis has parallels in other organ systems. That rapidly self-renewing epithelial tissues such as skin and intestine have stem cells that continually replenish the cells lost by differentiation is well described. However, the demonstration of the existence of neural stem cells[73,74] has raised the possibility that many organ systems might retain a population of self-renewing stem cells. Muscle satellite cells also appear to fulfill this role. These organ- or tissue-specific stem cells arise during early fetal development from embryonic stem cells of the inner cell mass of the blastocyst that are pluripotent and give rise to all cell types in the body.

Many assays have been proposed as "surrogate" stem cell assays, but until homogeneous populations can be evaluated in both in vitro and in vivo assays it will be impossible to determine the precise cell type measured by these methods. Fauser and Messner and others[46,75-77] demonstrated colonies in semisolid media that contain granulocytes, erythrocytes, monocytes, and megakaryocytes (CFU-GEMM) in methylcellulose cultures of human bone marrow. A unique type of in vitro blast cell colony that comprises small numbers of blast cells with higher self-renewal capacity (secondary colonies on replating) than CFU-GEMM has been described.[78] Evidence for the presence of pluripotent HSCs is also derived from the human "Dexter" technique for liquid culture of marrow, in which myeloid progenitors (mostly CFUs-GM) are sustained for about 2 months on and within an adherent stromal monolayer.[79,80] The progenitors can be detected by replating into methylcellulose with several growth factors at 5 to 8 weeks, thereby demonstrating that unipotent and multipotent cells are generated in this culture system. Eaves and colleagues[81] have adapted this long-term culture technique to a limiting=dilution assay in which long-term culture-initiating cells (LTC-ICs) can be quantitated after culture at different concentrations on a stromal layer for 5 weeks, followed by replating in methylcellulose to score for the number of wells that do not contain colonies. The analogous cobblestone area–forming cell (CAFC) assay has also been adapted to human cells.[82] The high proliferative potential colony-forming cell (HPP-CFC) assay that gives rise to macroscopically visible (>5 mm) in vitro colonies[83,84] is another method that measures the enormous proliferative capacity of primitive progenitor cells. In an effort to establish a more direct measure of the ability of human stem cells to reconstitute hematopoiesis, Dick and colleagues[85] developed an in vivo assay. In this method human cells are injected into immunodeficient nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, and 5 to 8 weeks later the animals are sacrificed and tested for the presence of human cells in blood and progenitors in bone marrow. By limiting-dilution assay these SCID reconstituting cells (SRCs) can be quantitated. Because NOD/SCID mice develop thymic lymphomas that prevent long-term studies and have natural killer (NK) cell activity that confers graft resistance, improvements in the model have been achieved by using mice with additional truncated (NOG) or deleted (NSG) interleukin 2 receptor (IL-2R) common gamma chains[86,87]; these animals support a -fivefold higher level of CD34+ engraftment. Cell fractionation and gene marking studies provide some evidence that SRCs are more primitive than LTC-ICs.[88,89] Although SRCs may still be a heterogeneous population of cells that includes long-term repopulating HSCs, this assay provides a better measure of stem cell properties than the LTC-IC assay, especially with regard to the multipotent (i.e., myeloid and lymphoid) and self-renewal properties of HSCs. A tentative relationship of the cells measured in these different assays to the stem cell is shown in Figure 1-6.

Application of these assays and analysis of HSCs, in general, has been hindered by the low frequency of the cell in the hematopoietic population and the lack of reagents to identify stem cells. However, it is now possible to purify murine stem cells by several methods. Murine HSCs can be highly purified by density gradient centrifugation combined with labeling with antibodies, lectins, or

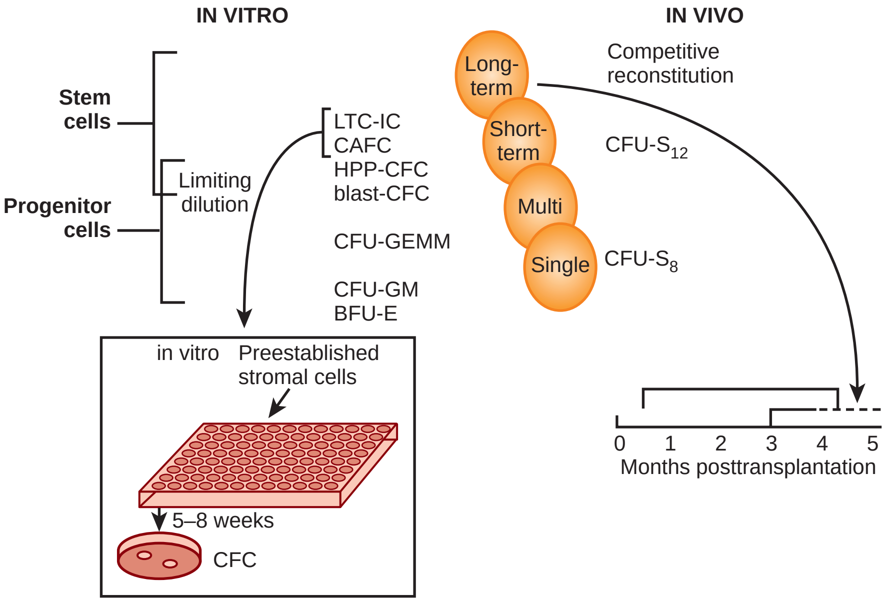


Figure 1-6 Schematic view of some general properties and assays for the heterogeneous cells that comprise the stem cell and progenitor cell compartments. As indicated on the diagram, cells capable of permanently reconstituting in vivo hematopoiesis are separable from cells that give rise to day 12 CFU-S, but the precise developmental stage of in vitro long-term culture initiating cells *(LTC-ICs)*, cobblestone area–forming cells *(CAFCs)*, high proliferative potential colony-forming cells *(HPP-CFCs)*, and blast CFCs is not established. In the progenitor compartment, mixed colonies of almost all lineage combinations have been described.

intracellular dyes (alone or in combination) followed by separation using fluorescence-activated cell-sorting (FACS), immunopanning, or immunomagnetic beads. Immunologic reagents that define murine stem cell populations include antibodies to Thy-1 (CD90), Kit, and stem cell antigen (Sca-1). Weissman and coworkers[90] used the FACS in combination with negative expression of T-cell, B-cell, granulocyte, and monocyte lineage-specific markers; low expression of Thy-1; and expression of Sca-1 to isolate HSCs. Transplantation of single Kit$^+$, Thy-1^{lo}, Lin–, Sca-1$^+$ (KTLS) cells can ensure long-term survival of lethally irradiated syngeneic recipients,[91] and approximately 20% of such cells are LTR-HSCs. Murine HSCs can be even further enriched using cell surface receptors of the signaling lymphocyte activation molecule (SLAM) family. HSCs are CD150+, CD244–, and CD48–, whereas multipotent progenitors lose CD150 expression and begin to express the other family members CD244 and CD48. Purification of CD150+, CD48–, and CD41– cells (CD41 is also expressed on megakaryocyte lineage cells) provides a population of cells 45% of which are LTR-HSCs.[92]

Expression of the CD34 antigen has been used to enrich for human stem cells. Although most colony-forming cells (CFCs) express both the CD34 and CD33 antigens, cells that give rise to CFC in long-term bone marrow cultures (i.e., pre-CFCs) can be separated by their expression of CD34, lack of expression of CD33, and intermediate forward light-scattering properties.[93] A G_0 CD34+ cell population has been isolated by exploiting the resistance of these cells to 5-fluorouracil (5-FU) in the presence of stem cell factor (SCF) and interleukin-3 (IL-3).[94] The G_0 cells are also positive for KIT, interleukin-6 receptor (IL-6R), and IL-1R, and they do not form progenitor-derived colonies upon direct culture in methylcellulose; after 5 weeks in culture on stromal cells, however, they do form primary colonies in methylcellulose 40% of which are replatable. The importance of CD34-positive marrow cells is emphasized by in vivo simian studies. Similar to human bone marrow, the CD34 antigen is expressed by a minority of baboon cells, and infusion of these cells isolated by immunoabsorption chromatography and FACS can reconstitute lymphohematopoiesis in lethally irradiated baboons.[95] The cloning of the murine CD34 complementary DNA (cDNA) has cast some doubt on expression of CD34 by LTR-HSCs, at least in the mouse. A monoclonal antibody raised to murine CD34–glutathione-S-transferase (GST) fusion protein was used to separate purified Sca-1+, Kit+, lin– bone marrow cells into CD34^{lo}/–, CD34^{lo}, and CD34+ fractions. Interestingly, long-term multilineage reconstitution was observed after transplantation of the CD34^{lo}/– cells, whereas the CD34+ fraction gave early but unsustained multilineage reconstitution.[91] These data are supported by experiments demonstrating that a tiny subset of murine bone marrow cells that exclude the Hoechst 33342 dye (called the *side population*) contains all the LTR-HSC activity but is CD34–.[96] It is possible that murine and primate LTR-HSCs differ in their expression of CD34; however, the human and primate transplants have not used very highly purified cells, and so it is also possible that CD34^{lo}/– cells could account for the long-term engraftment. Primate and human studies have also raised the possibility that HSCs do not express CD34.[97] When primitive human lin– cells are separated into CD34+ and CD34– fractions, the capacity to reconstitute hemopoiesis in immunodeficient mice (SRC) is found in both cell fractions.[98] A resolution to this controversy may come from the demonstration that resting murine HSCs are CD34–, whereas HSCs activated with 5-FU or cytokines such as G-CSF express the CD34 antigen.[99,100] Most interestingly, transplantation of activated CD34+ HSCs showed that these HSCs can lose CD34 expression after return of the recipients to the resting steady state and still retain the capacity to reconstitute secondary recipients, demonstrating that CD34 expression is reversible.[100] The current state of the art for HSC purification in the mouse includes the following marker profile: CD34–, CD150+, CD48–, CD41–, flt3–, and CD49b lo[101,102]; whereas in the human system, the current purification would exploit a different marker profile: CD34+, CD38–, CD45RA–, CD90+, and CD49f+, with further enrichment (capacity for single-cell engraftment) in a Rho$^-$ fraction of these cells.[103,104]

Progenitor Cells

The long-term repopulating HSCs of the marrow (LTR-HSCs) slowly self-replicate while occasionally (and stochastically) differentiating into cells that are multipotent but have reduced self-renewal capacity (called *myeloid lymphoid progenitors* in humans) and then into either common lymphoid progenitors or common myeloid progenitors. These cells can be prospectively isolated according to their expression of unique combinations of cell surface markers (Fig. 1-7).[105-108] Lymphoid differentiation will not be further considered here. The common myeloid progenitor differentiates into all of the progenitors of the blood cells other than lymphoid cells. These include more committed progenitors of the granulocyte, monocyte (GMPs), and eosinophil lineages or progenitors of the megakaryocyte, erythroid, and basophil lineages (MEPs). These cells can be isolated prospectively, and transplantation into lethally irradiated mice can confer transient but not long-term hematopoiesis.[109]

The current view of the mouse hierarchy derived from transplant studies has defined a linear track from LTR-HSCs to short-term reconstituting hematopoietic stem cells (STR-HSCs) (the latter most likely equivalent to the human myeloid lymphoid progenitor), and finally a multipotential progenitor, and then several branch points. There is a lymphocyte-myeloid multipotent progenitor (LMPP) that gives rise to common lymphoid progenitors and GMPs, whereas GMPs can also arise from common myeloid progenitors. There is certainly heterogeneity in these populations, and future fate mapping and sorting techniques may further define the populations.[108,110]

Erythroid Colony-Forming Cells

The erythroid progenitor compartment is invisible under the light microscope. Erythroid progenitors are committed progenitors of a single lineage derived from the stochastic differentiation of bipotential or multipotential

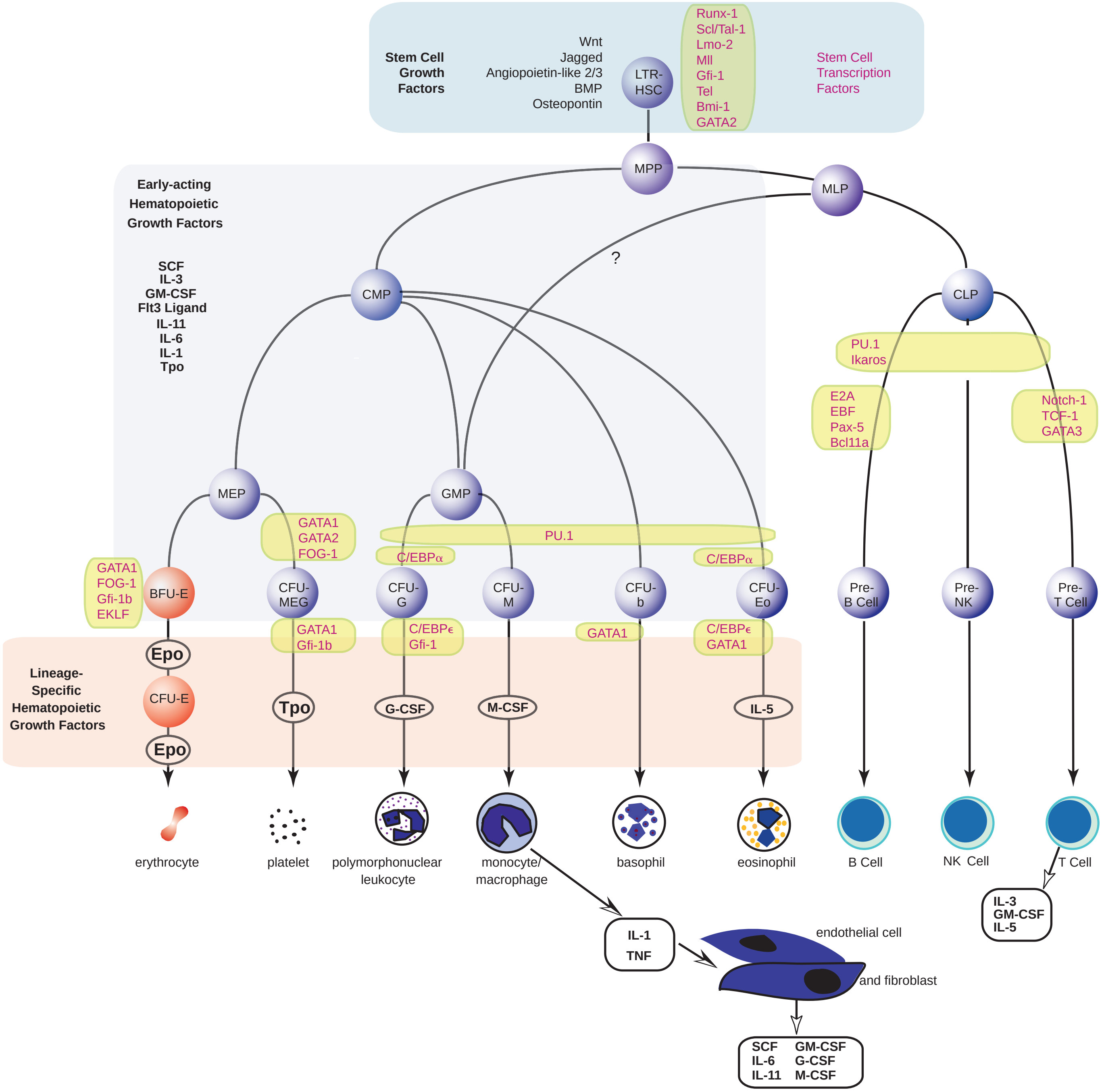

Stem Cell Growth Factors
Wnt
Jagged
Angiopoietin-like 2/3
BMP
Osteopontin
LTR-HSC
Runx-1
Scl/Tal-1
Lmo-2
Mll
Gfi-1
Tel
Bmi-1
GATA2
Stem Cell Transcription Factors
MPP
MLP
Early-acting Hematopoietic Growth Factors
SCF
IL-3
GM-CSF
Flt3 Ligand
IL-11
IL-6
IL-1
Tpo
?
CMP
CLP
PU.1
Ikaros
E2A
EBF
Pax-5
Bcl11a
Notch-1
TCF-1
GATA3
MEP
GMP
GATA1
GATA2
FOG-1
PU.1
C/EBPα
C/EBPα
GATA1
FOG-1
Gfi-1b
EKLF
BFU-E
CFU-MEG
CFU-G
CFU-M
CFU-b
CFU-Eo
Pre-B Cell
Pre-NK
Pre-T Cell
GATA1
Gfi-1b
C/EBPε
Gfi-1
GATA1
C/EBPε
GATA1
Epo
Lineage-Specific Hematopoietic Growth Factors
CFU-E
Tpo
G-CSF
M-CSF
IL-5
Epo
erythrocyte
platelet
polymorphonuclear leukocyte
monocyte/macrophage
basophil
eosinophil
B Cell
NK Cell
T Cell
IL-3
GM-CSF
IL-5
IL-1
TNF
endothelial cell
and fibroblast
SCF
IL-6
IL-11
GM-CSF
G-CSF
M-CSF

Figure 1-7 Major cytokine sources and actions and transcription factor requirements for hematopoietic cells. Cells of the bone marrow microenvironment, such as macrophages, endothelial cells, and reticular fibroblastoid cells, produce M-CSF, GM-CSF, G-CSF, IL-6, and, probably, SCF (cellular sources not precisely determined) after induction with endotoxin (macrophage) or IL-1/TNF (endothelial cells and fibroblasts). T cells produce IL-3, GM-CSF, and IL-5 in response to antigenic and IL-1 stimulation. These cytokines have overlapping actions during hematopoietic differentiation, as indicated, and for all lineages optimal development requires a combination of early-acting and late-acting factors. Transcription factors important for survival or self-renewal of stem cells are shown in *red* at the top, whereas stages of hematopoiesis blocked after the depletion of indicated transcription factors are shown in *red* for multipotent and committed progenitors.

progenitors[111] that are, in turn, derived from a tiny population of multipotential stem cells (see Fig. 1-7). In humans, the most primitive single-lineage committed erythroid progenitor is the erythroid burst-forming unit (BFU-E). In response to the combination of erythropoietin and either SCF, IL-3, or GM-CSF in vitro in semisolid (methylcellulose) cultures, BFU-E divide several times while still motile, thereby forming subpopulations of erythroid colony-forming units (CFU-E).[112] Then, each of the latter forms a large colony of proerythroblasts that go on to form more mature erythroblasts and even reticulocytes. The burstlike morphology of the colony is responsible for the name of the progenitor. The entire process requires about 2 weeks in vitro. Bone marrow also contains the more mature CFU-E that, under the influence of erythropoietin, form small colonies of erythroblasts in 7 days.

The murine erythroid progenitor phenotype (pre–CFU-E) can be defined as Sca-1–, Kit+, CD41–, CD150+, and endoglin+.[113] The human BFU-E counterpart is CD34+, CD38+, IL-3Rα–, and CD45RA–.

Granulocyte and Macrophage Colony-Forming Cells

The first colony assays relevant to the study of the production of granulocytes and monocytes in the mouse were described in 1965 by Pluznik and Sachs[115] and in 1966 by Bradley and Metcalf.[116] Analogous assays have been developed in the human system.[117] These groups demonstrated that individual cells derived from mouse spleen or bone marrow could give rise to colonies of up to several thousand differentiated granulocytes and/or macrophages in a soft agar medium. A period of 7 to 8 days was required for full maturation of these colonies (12 to 14 days is required in humans). Appropriate studies were performed to demonstrate the single-cell origin of the colonies. These studies also demonstrated that a single progenitor cell, which was termed the colony-forming unit in culture, or CFU-C, was capable of differentiation into both granulocytes and macrophages, giving rise to the designation CFU-GM. Unit gravity sedimentation and other separation methods have been used to demonstrate that CFU-GM represent a cell population distinguishable from the pluripotent stem cell.[118] Long-term liquid bone marrow cultures have been particularly helpful in defining humoral and cell-cell interactions that induce myeloid differentiation.[119] CFU-GM give rise to the more mature granulocyte and macrophage colony-forming units, CFU-G and CFU-M, respectively.[116,117] In addition, CFU-GM can be distinguished from the eosinophil progenitor, CFU-Eo, each arising independently from the common myeloid progenitor (CMP). Human CFU-GM are CD34+, CD38+, IL-3Rα^{lo}, and CD45RA–.[114]

Megakaryocyte Colony-Forming Cells

Figure 1-7 provides an accepted if idealized schema of lineage-restricted megakaryocyte progenitor development. Evidence strongly suggests that the initial phase of differentiation into erythrocyte and megakaryocyte commitment involves a single progenitor capable of giving rise to colonies of differentiated cells, all of which express a nuclear transcription factor known as GATA1.[120,121] Of great interest is the fact that another transcription factor, nuclear factor, erythroid 2 (NF-E2), has been shown to regulate platelet production.[122] Mice rendered NF-E2 deficient have increased numbers of immature megakaryocytes in the marrow but die from bleeding resulting from absence of platelets. The thrombocytopenia is due to a block late in megakaryocyte maturation. Interestingly, these animals do not show an increase in thrombopoietin levels, suggesting that the megakaryocyte mass rather than the platelet count regulates thrombopoietin production.

Precursors and Mature Cells

The erythroid precursor or erythroblast pool represents about one third of the marrow cell population in the normal child above the age of 3 years or in the adult. Proerythroblasts are the earliest recognizable forms. These divide and mature through various stages that involve nuclear condensation and extrusion and hemoglobin accumulation. On average, each erythroblast can form about eight reticulocytes. Measurement of the total marrow proerythroblast content[123] and daily reticulocyte production shows that under normal conditions, replicating proerythroblasts largely maintain the reticulocyte pool being renewed from the progenitor compartment at a rate of about 10% per day.[124]

Erythroid Development

Up to this point we have discussed the nondescript progenitors of erythropoiesis without reference to their physical appearance or to the appearance of their differentiated daughter cells. The best evidence to date suggests that hematopoietic progenitors or stem cells look like lymphoblasts,[125-127] and studies of peripheral blood have shown that BFU-E reside in the nonadherent “null” lymphocyte population.[128]

The pathway of erythroid *precursor* differentiation between the development of proerythroblasts and the mature red cell is known as the *erythron* and includes the functioning differentiated precursor cells observed in bone marrow aspirates and biopsies. The morphology of erythroid precursor maturation is well described in several texts and will not be repeated here. The salient features of the morphologic changes during cell development are related to biochemical and kinetic alterations that were reviewed by Granick and Levere[129] and are shown in Figure 1-8. The residence times spent in each morphologic compartment are shown at the bottom of the figure, but the average transit time from proerythroblast to emergence of the reticulocyte into the circulation is approximately 5 days.[130] In acute anemia, the transit time may decrease to as little as 1 or 2 days by means of skipped divisions.[131] The red cells that emerge are macrocytic and may bear surface i antigen and other fetal characteristics because the abbreviated time in the marrow compartment does not permit complete conversion of i antigen to I antigen or acquisition of certain other adult characteristics.[132] The cells also contain excessive burdens of the rubbish that normally accumulates during cell assembly,[133] because less time is available for the cleansing action of cell proteases and nucleases.[134] Thus stress erythropoiesis is associated with circulating Pappenheimer bodies (iron granules), basophilic stippling (ribosomes), Heinz bodies (hemoglobin inclusions), and Howell-Jolly bodies (nuclear remnants).

The kinetics of erythropoiesis can be monitored by the use of radioactive iron and surface scanning. The various ferrokinetic patterns in human diseases are shown in Figure 1-9. The total distribution of erythroid marrow can be determined by scintigraphy using ^{111}InCl bound to transferrin, as shown in Figure 1-10. Both ^{59}Fe kinetics and ^{111}InCl scintigraphy can be useful in the diagnosis of

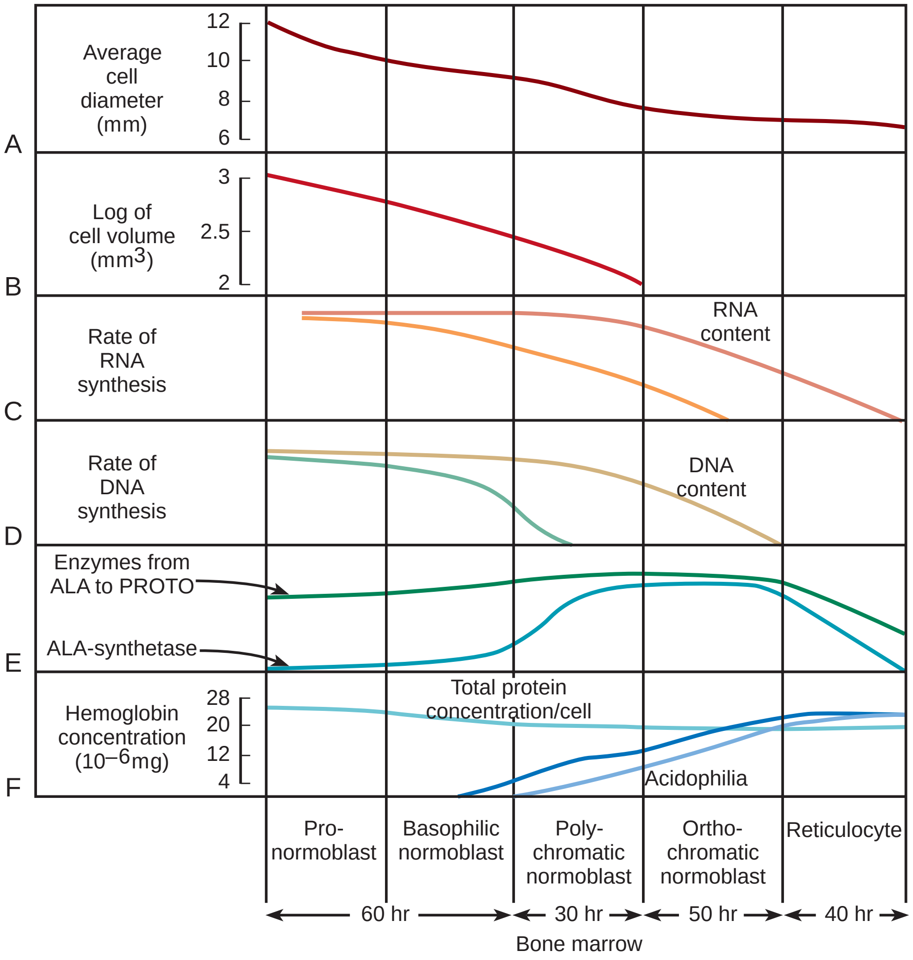


Figure 1-8 Erythroid maturation: alterations in cell size, rates of DNA and RNA synthesis, enzymes involved in heme synthesis, and hemoglobin concentration. Substances listed in the left-hand column are represented by corresponding solid black lines. Unless specified, graphs represent relative values. It will be noted that considerable protein synthesis takes place during the earliest phase. Following this, the nucleolus disappears but mitochondria remain. As the concentration of DNA decreases and the concentration of RNA starts to fall, hemoglobin begins to appear, increasing rapidly in amount. *ALA*, δ-Aminolevulinic acid; *PROTO*, protoporphyrin. *(From Granick S, Levere RD: Heme synthesis in erythroid cells. In Moore CV, Brown EB, editors:* Progress in hematology, *vol IV. New York, 1964, Grune & Stratton, p. 1.)*

marrow failure, but these techniques are rarely necessary. The initial uptake of ^{59}Fe in marrow is found primarily in proerythroblasts and early basophilic erythroblasts.[135]

Mouse proerythroblasts; basophilic, polychromatic, and orthochromatic normoblasts; reticulocytes; and mature erythrocytes can be separated on the basis of increasing expression of glycophorin A (Ter119) and loss of expression of CD71.[136] Highly purified populations can be obtained by substituting CD44 for CD71, since there is a thirtyfold loss of expression of CD44 during erythroid maturation compared with a fourfold decrease in CD71 expression.[137]

Neutrophil Production

A model that describes the production and kinetics of neutrophils in humans is shown in Figure 1-11. It is highly compartmentalized. The relatively tiny peripheral blood pool is divided into two components in equilibrium; the circulatory pool (CGP) and the marginating pool (MGP). These pools provide entrance into the tissues. The level of circulating cells is buffered by an immense marrow reserve of identifiable precursors, some of which are in the mitotic compartment and others in a maturing storage compartment. The transit times within each compartment are relatively long, so that a huge reserve remains available. The responses of these compartments to various diseases are detailed in Chapter 22. The kinetics of proliferation of recognizable cell precursors have been studied using labeled precursors of DNA. The so-called labeling indices from which measurements of cell cycle times can be made have served as important approaches to the study of the pharmacology and toxicity of chemotherapy (see Chapter 45).

The final stages of granulocyte production and their release from the marrow are also multifaceted.[138] At least four factors may influence granulocyte egress: the organization and localization of the cells in relation to vascular channels; the development of nuclear and cytoplasmic changes that increase cell deformability; factors that cause cell release; and, finally, the regulation of blood flow through vascular channels in the marrow.

Bone Marrow Examination and Megakaryocytopoiesis

Despite the fact that the morphology of megakaryocyte development is fairly well established (Table 1-1), bone marrow examinations can be of limited value in the various platelet disorders. Bone marrow smears and biopsies provide insufficient data about thrombopoiesis because the final stage of platelet production, extrusion of cytoplasm into the sinusoid and shedding of platelets (to be described later), is not appreciated by these techniques; only the relative numbers of megakaryocytes and

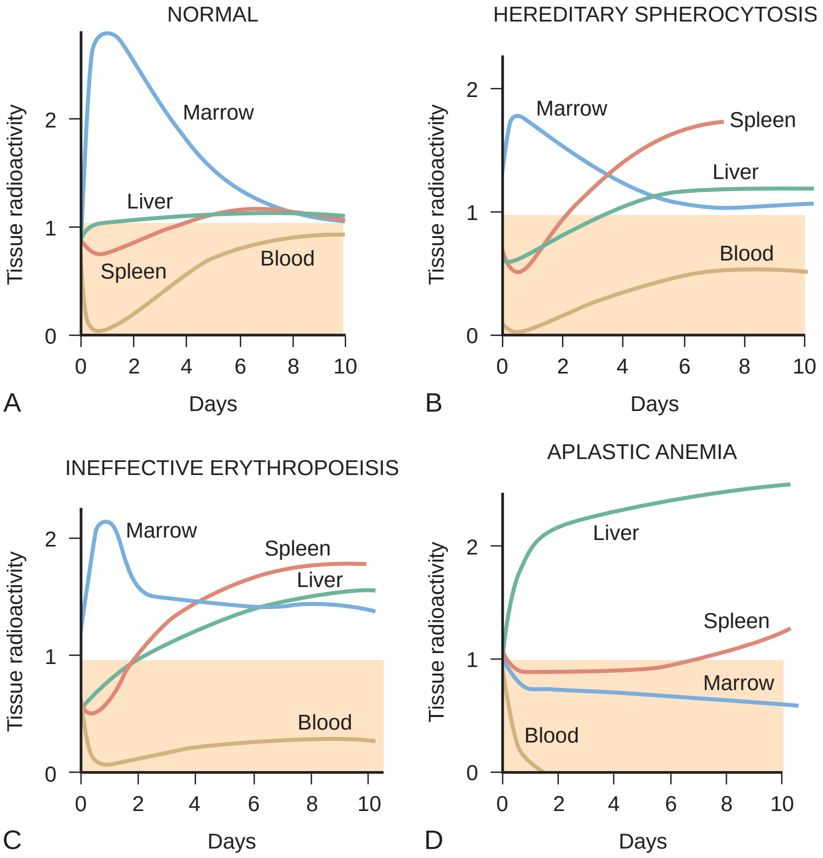


Figure 1-9 Pattern of radioactivity in blood and over sacrum (marrow), liver, and spleen during a ferrokinetic study. Representative data from patients with three different disorders and one normal individual are presented. *(From Finch CA, Dubelbeiss K, Cook JD, et al: Ferrokinetics in man.* Medicine [Baltimore] *49:17–53, 1970.)*

their size and ploidy can be appreciated by routine morphologic methods. It is not surprising that such information is only loosely correlated with platelet production (Fig. 1-12). Furthermore, sampling errors can be responsible for serious misinterpretations. This is a particular hazard in aspirates of neonatal marrow, in which megakaryocytes may be hard to detect whether platelet production is normal or not. Furthermore, megakaryocytes are not evenly distributed in marrow smears. They are more readily found around the edges of the particles, and they may be mistaken for broken cells by the untrained observer. Megakaryocyte nuclei are often found lying free in marrow smears, where they may be erroneously scored as tumor cells. Biopsy sections provide more accurate assessments of megakaryocyte number and distribution than smears, though the latter are usually sufficient (except in neonates) if examined carefully. Biopsies should not be attempted in neonates merely to define megakaryocytes; clinical judgment is a safer tool in these patients.

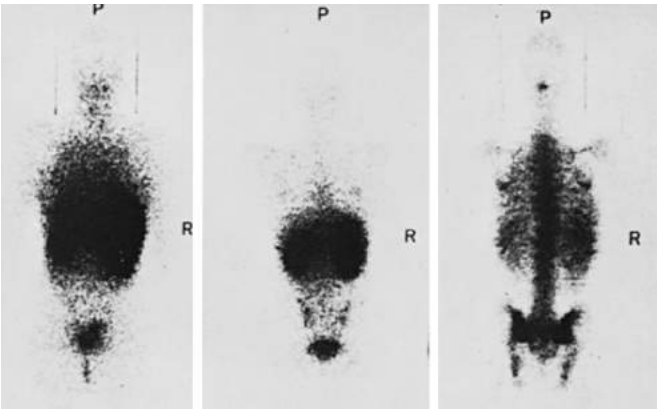


Figure 1-10 Spectrum of scintigraphic patterns in patients with idopathic aplastic anemia. On the left is a scintigram of a patient prior to transplantation with renal activity but no marrow activity. In the middle is a scintigram of a patient with activity in the kidneys and borderline uptake within the marrow. On the right is a normal bone marrow scan in a patient after transplantation. Liver activity is seen in all three patterns, and splenic activity is seen in none. *(From McNeil BJ, Rappeport JM, Nathan DG: Indium chloride scintigraphy: an index of severity in patients with aplastic anaemia.* Br J Haematol *34:599–604, 1976.)*

Examinations of routine marrow smears and biopsies, though instructive, are limited by their two-dimensional views and the thickness of the sections. Megakaryocytes have a peculiar predilection to lie next to the endothelial cell lining of the fronds of developing marrow cells, perhaps because thrombopoietin is produced by these cells. In general, megakaryocytes are too large to squeeze through the sinusoidal meshwork, so they merely push their cytoplasms through the fenestrations. These cytoplasmic structures, called *proplatelets,* serve as microtubule conduits for platelet packaging and release at sinusoids.[139] The protruding cytoplasms form demarcation lines and then shatter into platelets, which are swept into the blood, regulated in part by fibrinogen interacting with platelet integrin αIIbβ3.[140] The megakaryocyte

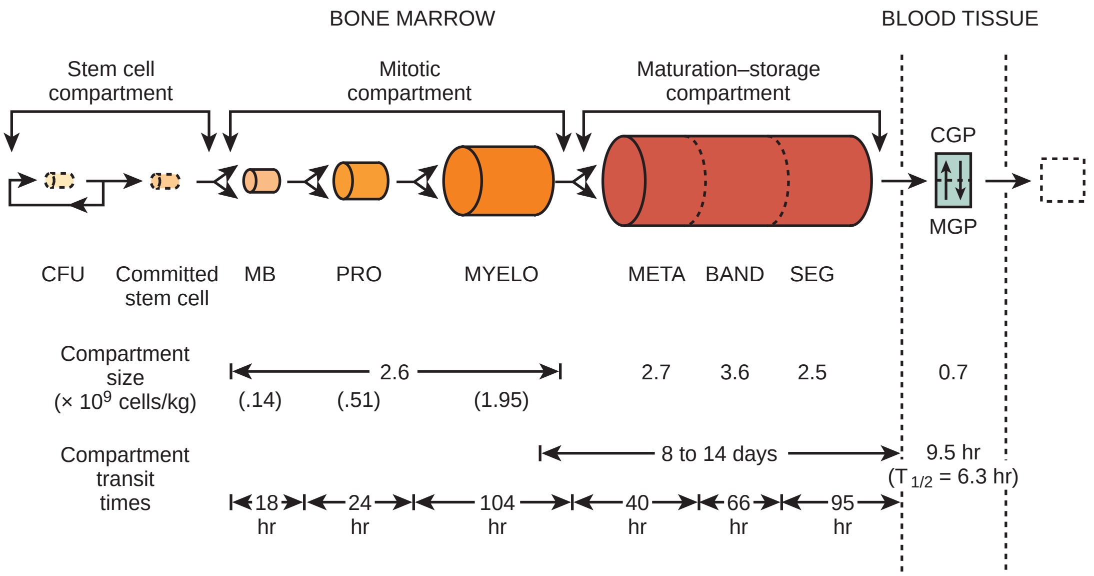


Figure 1-11 Model of the production and kinetics of neutrophils in humans. The marrow and blood compartments have been drawn to show their relative sizes. The compartment transit times, as derived from di-isopropylfluorophosphate ^{32}P studies, are shown on the next to the last line. Values derived from titrated thymidine studies are shown on the last line. *(From Skubitz KM: Neutrophil leukocytes. In: Wintrobe MM, Lee RG, et al, editors:* Clinical hematology, *Philadelphia, 1975, Lea & Febiger, p. 244.)*

nuclei rarely make the transfenestration journey into the sinusoids and thence the blood. If they do, they may be mistakenly interpreted by the unwary microscopist as tumor cells in the blood. Intact or partial megakaryocytes are regularly observed in the blood of patients with marrow-invasive diseases such as certain leukemias, metastatic cancers, granulomatous disorders, and fibrosis.

THE HEMATOPOIETIC GROWTH FACTORS

Since the pioneering work in the early 1960s by Metcalf[116] and Sachs[115] and their coworkers, it has been recognized that normal and leukemic blood progenitor cells can be propagated in culture in the presence of soluble protein growth factors. These factors were originally termed colony-stimulating factors, or CSFs, based on their ability to support the formation of colonies of blood cells by bone marrow cells plated in semisolid medium.[141] During the 1970s and 1980s, it was recognized that there exist multiple types of CSFs based on the different types of blood cells found in the colonies that grew in the presence of the different factors, leading to the hypothesis that the growth and differentiation of different lineages of blood cells are controlled, at least in part, by exposure of progenitor cells to CSFs having different lineage specificities.[141,142] With the molecular cloning of the genes for many of these factors and their receptors during the 1980s and 1990s it became possible to study in detail the structure, function, and biology of the recombinant CSFs as well as the molecular biology of their respective genes.[141-146] This analysis, along with similar work on the regulation of cells in the immune system, led to the realization that there exist a large number of interacting regulatory molecules now generally known as *cytokines* or *lymphohematopoietic cytokines* that together serve to control the hematopoietic and immune systems and to integrate the responses of these systems with those of many other physiologic systems.[144,147-150] These control

TABLE 1-1 Cytologic Characteristics of Megakaryocyte Maturation Stages

Stage	Nuclear Morphology	Cytoplasmic Staining (Wright-Giemsa)	Approximate Size Range	Demarcation Membranes	Granules	Suggested Name
I	Compact (lobed)	Basophilic	6-24 µm	Present by electron microscopy	Few present by electron microscopy	Megakaryoblast
II	Horseshoe	Pink center	14-30 µm	Proliferating to center of cell	Starting to increase	Promegakaryocyte
III	Multilobed	Increasingly more pink than blue	15-56 µm	Extensive but asymmetric	Great numbers	Granular megakaryocyte
IV	Compact but highly lobulated	Wholly eosinophilic	20-50 µm	Evenly distributed	Organized into “platelet field”	Mature megakaryocyte

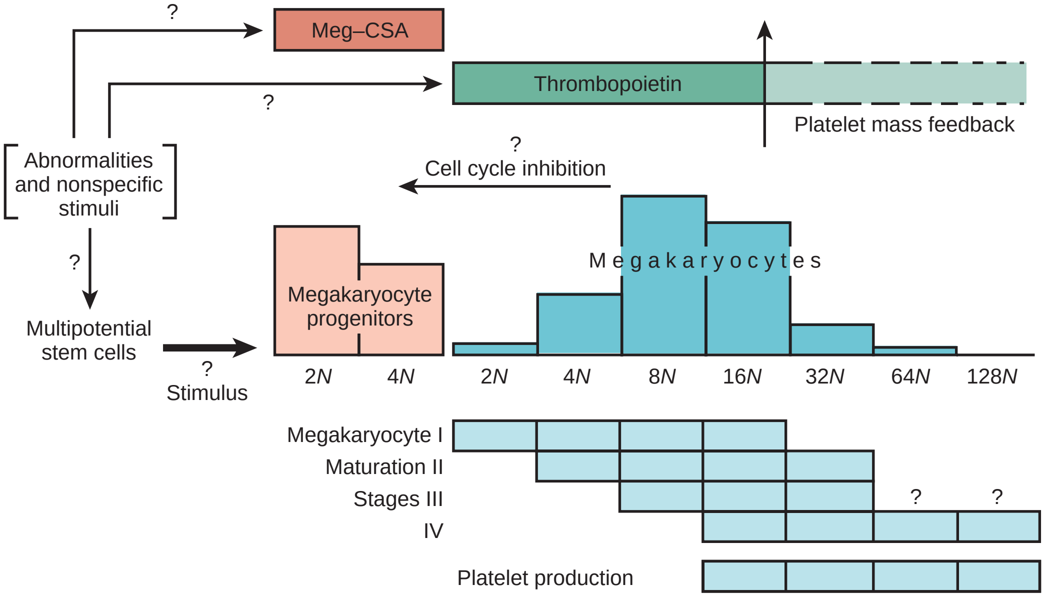


Figure 1-12 Thrombopoiesis. Shown horizontally in the center is the progression from uncommitted multipotential stem cells, through the proliferating progenitors detected in the in vitro clonogenic assays, to the spectrum of maturing megakaryocytes. The relative DNA levels of megakaryocytes and their precursors are given as ploidy values *(N)*, where 2N is a diploid cell. The megakaryocytes are also presented vertically in terms of their maturation stages, ending in platelet shedding; a detailed classification of these stages in given in Table 1-1. The columns show the maturation stages at particular ploidy ranges. Thus 4N megakaryocytes are found at maturation stages I and II. The top row shows a postulated two-level regulatory process with megakaryocyte colony-stimulating activity *(Mega-CSA)* primarily influencing proliferation of progenitors and thrombopoietin required for megakaryocyte ploidy amplification and possibly for maturation. It is not certain that these regulators are completely exclusive in their target cell specificities as shown. Furthermore, no clear distinction is currently possible between the specific or nonspecific control of the two factors. Thrombopoietin production is sensitive to variations in platelet mass, as indicated in the figure; it is assumed that this feedback mechanism operates on the source of thrombopoietin. The progenitors might be controlled by cell cycle inhibition, perhaps as a consequence of normal numbers of megakaryocytes. *(From Williams N, Levine RF: The origin, development, and regulation of megakaryocytes.* Br J Haematol *52:173–180, 1982.)*

molecules serve to regulate the growth, development, differentiation, activation, and trafficking of cells within the hematopoietic and immune systems. With the elucidation of the sequence of most of the human genome in the final years of the 1990s, even more cytokine and growth factor genes have been discovered, providing new insight into how all these systems have evolved with common themes but also providing further challenges to cell biologists in their attempts to understand the functions and interactions of all of the different molecules.

The cytokine gene families that contain at least one member that functions within the hematopoietic or immune system include the lymphohematopoietic cytokines (including many interleukins and CSFs[141,146]); the receptor tyrosine kinase ligand CSF-1,[151] the stem cell factor (SCF) (also known as *steel factor*),[152] and the ligand for the Flk2/Flt3 receptor (FLT-3)[153]; the IL-1 gene family[154]; the chemokines[155]; the tumor necrosis factors[156]; the interferons[157]; the IL-10 gene family[158]; the transforming growth factor β (TGF-β)[159]; and the IL-17 gene family.[160] We will briefly describe these families and the molecules among the family members selected as hematopoietic growth factors (HGFs) for further discussion.

The Lymphohematopoietic Cytokines

For our purposes, the lymphohematopoietic cytokines are defined as the protein ligands for the members of the hematopoietin receptor gene family, also known as the *cytokine receptor class I gene family*.[161] This gene family is characterized by an extracellular domain with pairs of conserved cysteine residues and the distinctive amino acid sequence element Trp-Ser-Xaa-Trp-Ser (WSXWS).[162] These receptors all signal at least partly through the JAK-STAT pathway.[163,164] This rather large family of hematopoietins now includes many interleukins[165] (IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, IL-21, and IL-23),[146,166,167] granulocye-macrophage colony-stimulating factor (GM-CSF),[141,168] granulocyte colony-stimulating factor (G-CSF),[169] erythropoietin (EPO),[170] thrombopoietin (Tpo),[171] thymic stromal thymopoietin (TSLP),[172-174] leukemia inhibitory factor (LIF),[175] oncostatin M (OSM), cardiotropin 1 (CT-1), and ciliary neurotrophic factor (CNTF).[176] The cytokine receptors on the cell surface signal as multimers, either homomeric or heteromeric, and the family can be further divided into subfamilies based on the molecular composition of their receptor complexes. We will focus on cytokines that have major actions on hematopoietic cells. Those with homodimeric receptors include EPO, Tpo, and G-CSF.[146] A second subgroup comprising GM-CSF, IL-3, and IL-5 in the human system have receptor complexes that share a common subunit known as the *beta common chain* that forms heterodimers with unique cytokine alpha subunits to give selectivity for the respective cytokines.[146] A third group includes IL-6 and IL-11 (as well as LIF, OSM,[177] CNTF, and CT-1, not considered further); all share a common receptor-signaling chain known as *gp130*, with unique chains that provide ligand specificity.[176]

The Receptor Tyrosine Kinase Ligands

Three HGFs, macrophage CSF (M-CSF, also known as *CSF-1*[151]), stem cell factor (SCF),[153,178] and the ligand for FLT3 (FLT3L)[179] have been identified that signal through related receptors, which themselves have tyrosine kinase activity.[153] The receptors for these HGFs are all evolutionarily related, as are the ligands themselves, and each plays an important role in controlling blood cell production.[180,181]

The Interleukin 1 Gene Family

The IL-1[182,183] gene family has grown substantially and now includes at least many IL-1s (IL-1α through IL-1ξ), the IL-1 receptor antagonist (IL-1RA), and IL-18.[154,184] IL-1α, IL-1β, and IL-18 all have potent ability to induce cytokine production (IL-18 particularly interferon gamma (IFN-γ) in combination with IL-12[185]), but the two IL-1s have also been shown to synergize with other HGFs in support of early blood cell proliferation and, therefore, will be considered further here.[186] The functions of the other novel members of the IL-1 gene family are still unknown.

The Chemokines

The chemokines represent a large family of generally small proteins (usually 8 to 12 kDa) readily identifiable by sequence homology, including twin characteristic cysteine residues near the amino termini that separate the family into CC chemokines if the cysteines are adjacent or CX chemokines if they are separated by one residue.[155] Currently, there are more than 50 members of the gene family that have demonstrated important roles in cell trafficking in many systems. The receptor gene family for this cytokine family comprises about 20 members of the G-protein–coupled receptor (GPCR) family. However, their biologic function is not limited to chemotaxis, because several have been shown to effect gene transcription, apoptosis, and cell proliferation. IL-8 is a prominent CX chemokine that regulates neutrophil trafficking and therefore plays a role in inflammatory responses.[155] However, two chemokines, SDF-1[25-28] and macrophage inflammatory protein 1α (MIP-1α)[187] have been reported to inhibit the cycling of very early stem cells and therefore may be important molecules in halting cycling stem cells and returning them to their normal, quiescent state. SDF-1 is clearly very interesting and important in the hematopoietic system as a chemotactic factor for very primitive stem cells, providing possible mechanisms for the homing of cells both as hematopoiesis moves from the fetal liver to the bone marrow and during transplantation of cells in adults.[188]

The Tumor Necrosis Factors

The TNF gene family[161] now includes TNFα, lymphotoxin α and β, CD27Ligand, CD30Ligand, CD40Ligand, FasLigand, TRAIL, and Trance.[150] Many of these molecules are involved in regulating activation and death of various cells, including T lymphocytes and erythroid progenitors. TNFα itself has many biologic activities, particularly in inflammation. However, it is mentioned here because of its potent and important role in promotion of dendritic cell differentiation and activation[189,190] and its capacity to depress hematopoiesis.

The Interferons

The type I interferons (13 species of IFN-α, IFN-β, and IFN-ω) are closely linked on human chromosome 9. The type II interferon gamma (IFN-γ) demonstrates little if any sequence homology with the type I interferons, but the receptors for all of these cytokines are members of cytokine receptor class 2, which is related to but distinct from cytokine receptor class I (HGF receptors) because class 2 receptors lack the distinctive WSXWS sequence.[191] In addition to their antiviral activities, both type I and type II interferons have multiple other regulatory activities with many cell types, including T cells. In the human system, IFN-γ plays a prominent role in polarizing T helper cells toward the type 1 (Th1) phenotype.[150] However, none of these molecules appear to have prominent roles in directly controlling blood cell production and will not be considered further here.[157]

The Transforming Growth Factor Beta Gene Family

The large TGF-β gene family includes TGF-β1, TGF-β2, and TGF-β3; the bone morphogenetic proteins (BMPs) 2 through 16; the growth and differentiation factor (GDF) genes 1 to 10; activin; and inhibin. Like those for many of the other cytokines, the receptors for the TGF-β family members are multimers comprised of two different members of the serine/threonine kinase receptor gene family.[159] Receptors of different ligand specificities are generated by forming these gene products in different combinations. TGF-β1 itself is a very important regulator of cell cycling in many cell systems, including the hematopoietic system, and will be considered here further as a negative regulator of hematopoiesis.[192,193] Mutations in its receptor cause Loeys-Dietz syndrome.[194] The BMPs and GDFs have been implicated in many developmental systems. Interestingly, BMP-4 has been shown to be essential in *Xenopus* species for the embryonic development of HSCs, and it seems at least possible that this molecule might also play a role in mammals in blood cell development during embryogenesis if not in adult hematopoiesis, an area worth following.[194a] However, for our purposes and the sake of simplicity, we will only include TGF-β1 as an HGF.

ACTIONS OF HEMATOPOIETIC GROWTH FACTORS

Predominantly Lineage Specific HGFs: G-CSF, M-CSF, IL-5, TPO, and EPO

Early on, it was recognized that different colony-stimulating factors could selectively support the growth of specific types of hematopoietic colonies.[157] Thus when human bone marrow cells are cultured in semisolid medium in the presence of G-CSF, colonies emerge 7 to 8 days later that consist largely of mature neutrophilic granulocytes and their precursors.[157-169] This finding led to the model that G-CSF, to a large degree, interacts with relatively late hematopoietic progenitors that have already committed to the neutrophil lineage (CFU-G) and thus G-CSF supports their growth and final maturation into functional neutrophils.[169] Similar analysis has revealed that the other major hematopoietic cell lineages have analogous, lineage-specific, late-acting factors and that these molecules frequently serve as important if not primary regulators of the respective pathways. Thus M-CSF supports monocyte/macrophage colony growth and is important in supporting the growth and maturation of monocyte progenitors (CFUs-M)[151]; IL-5 supports eosinophilic granulocyte colony formation and therefore supports the growth and maturation of eosinophil progenitors (CFUs-Eo) as well as activating eosinophils[195,196]; EPO is necessary for the growth and maturation of both earlier (BFU-E) and later (CFU-E) progenitors of the erythroid lineage[170]; and TPO directly supports the growth and maturation of megakaryocyte progenitors (CFUs-meg) and the subsequent production of functional platelets.[171,197]

Although the regulation of the respective blood cell pathways by the lineage-specific HGFs is likely to be their major function, in no case is this lineage specificity absolutely maintained. G-CSF has been found to influence the migration and proliferation of endothelial cells, cells that express high-affinity receptors for this cytokine.[198] IL-5 serves as a growth factor for activated B cells, particularly in the mouse, and affects the type of immunoglobulin secreted by mature B cells.[199] Epo[200] and TPO have been noted to interact with megakaryocyte and erythroid progenitors,[201] respectively. M-CSF appears to be important in trophoblast development.[151] Finally, populations of early HSCs have been found to express receptors for many cytokines; typically, these cells do not respond to single factors but require combinations of factors to trigger them into cycle.[202,203] “Lineage-specific” factors that have been reported to act in various combinations to trigger cycling of early “stem” cells include G-CSF,[204] M-CSF,[205] and TPO,[206] demonstrating that the molecules are not strictly “lineage specific” even within the hematopoietic system. Mice deficient in either the Tpo receptor (*Mpl*) or G-CSF are deficient in levels of all progenitor cells, consistent with the idea that these factors indeed are involved in expansion of early lineage cells.[145] However, when administered in vivo each of these molecules largely influences the growth and development of the expected lineage, so that the designation of lineage specificity seems warranted.

Multilineage Hematopoietic Growth Factors: IL-3 and GM-CSF

Initial analysis of human bone marrow cell cultures grown in the presence of GM-CSF revealed that a variety of different colony types develop over a period of 10 to 14 days.[207] Mature blood cells that could be readily identified included neutrophils, monocytes/macrophages, and eosinophils; this finding led to the designation of the molecule as a “granulocyte-macrophage” colony-stimulating factor, or GM-CSF. In comparison with G-CSF, it was found that it took longer to produce colonies with identifiable neutrophils but the ultimate variety of cell types was greater. This led to a model in which GM-CSF acts on progenitors committed to produce either neutrophils or monocytes (CFU-GM), a precursor to the G-CSF–responsive CFU-G and the M-CSF–responsive CFU-M.[157,207] These later progenitors apparently retain responsiveness to GM-CSF as well because mature monocytes and neutrophils can be observed in cultures supported by GM-CSF alone. That this model is not strictly correct was shown when recombinant GM-CSF was introduced into human bone marrow cultures in the presence of EPO and it was found that this combination of factors was very effective in supporting the development of erythroid colonies (murine GM-CSF is somewhat less effective in this regard).[157,208,209] Thus despite its name, GM-CSF generally interacts with intermediate multilineage progenitors that yield neutrophils, eosinophils, monocytes, erythroid cells, and megakaryocytes (CFU-GEMM). At the time, these activities were similar to those ascribed to IL-3 in the murine system.[210] When human IL-3 was identified, it proved to have abilities to support multilineage colony formation similar to those of human GM-CSF, indicating that it interacts with slightly different but strongly overlapping subsets of progenitors.[211,212] In comparison with GM-CSF, IL-3 is somewhat more effective in supporting multilineage, erythroid, and megakaryocyte colony formation and GM-CSF is slightly more effective with granulocyte and monocyte/macrophage colony formation.[211,212] In serum-free conditions, the ability of IL-3 to support final neutrophil and monocyte maturation is significantly depressed, indicating that the later-acting factors, G-CSF or GM-CSF in the case of neutrophils or M-CSF or GM-CSF in the case of monocytes, are necessary for final end cell production.[213]

In addition to acting slightly earlier than GM-CSF, IL-3 is clearly distinguished in its activity by its ability to support the growth and maturation of mast cells and basophils.[214,215] In the mouse, this was one of the first recognized activities of IL-3,[210] and when IL-3 was first administered to primates, basophilia was one of the most prominent findings.[215,216] Thus IL-3 appears to be capable of supporting the growth and development of basophil and mast cell progenitors. In the human system, both IL-3 and GM-CSF can support the development and differentiation of dendritic cells of either myeloid or lymphoid origin, especially in combination with SCF, FLT3L, and TNF.[217-219]

Other Interleukins

IL-4 in mice and humans has been reported to support multilineage colony formation, including colonies that contain cells from the erythroid, megakaryocytic, neutrophilic, and monocytic lineages.[220-222] IL-4 in the mouse supports mast cell growth and therefore shares many activities with IL-3.[199] However, IL-4 plays very important roles in the development and maturation of T cells and B cells,[199,222,223] particularly in the polarization of cytokine production by helper T cells toward the type 2 (Th2) response.[224] Therefore, on balance IL-4 is likely to be more important in controlling immune cell development and function and will not be discussed further here. IL-9 in both the murine and human systems has been shown to enhance erythroid colony formation in the presence of Epo[225] and appears to play a role in T-cell and B-cell development as well.[226] More recently, IL-9 has been shown to have some effects on the growth and activation of mast cells and may play some role in the pathology of asthma.[227]

Early-Acting Hematopoietic Growth Factors: Stem Cell Factor and Flt3 Ligand

Stem cell factor (SCF)[152,153,178] and FLT3 ligand (FLT3L),[179,228,229] both receptor tyrosine kinase ligands, interact with a variety of hematopoietic progenitor cells, perhaps most importantly with very early stem cell populations. SCF also plays an important role in melanocyte growth and development, which is reflected in the coat color effects of mutations in SCF or its receptor, KIT.[152] Genetic analysis of mice clearly showed that mice defective in either SCF *(Sl* mice*)* or in *Kit (W* mice*)* have serious hematopoietic (and many other) defects, including macrocytic anemia, mast cell deficiencies, and deficiencies in the stem cell compartment (reviewed in reference 152). These early studies had already indicated the critical importance of SCF in the survival and development of stem cells. Mutations in the human *KIT* gene lead to a similar phenotype in melanocyte development in humans known as the *piebald mutation;* however, these individuals do not have any hematologic problems, probably because severe mutations in this locus are likely to be lethal.[230] In vitro, the activities of SCF are generally most evident when combined with other HGFs; the proliferative activity of SCF with hematopoietic cells in culture as a single factor is minimal.[152] In fact, culture of murine bone marrow cells in SCF alone ultimately yields largely mast cells.[231] However, SCF acts synergistically to enhance the activities of most of the other HGFs in culture and is particularly effective when combined with HGFs such as IL-3, IL-1, or IL-11 at promoting the expansion of "blastlike cells" that retain considerable potential for yielding multilineage colonies in secondary culture.[232-234] These colonies, when replated under conditions that support B-lymphocyte development or when transplanted into animals also yield B and T lymphocytes, indicating that SCF-responsive cells include primitive stem cells with both lymphoid and myeloid potential.[235,236] SCF has also been implicated in combination with IL-2 or IL-7 in early stages of T-cell development in the thymus,[237] with IL-7 in pre–B-cell growth,[238] and with IL-7 in enhancing NK cell responsiveness to IL-2.[152] However, the fact that none of these lineages are dramatically affected in *W* or *Sl* mice indicates that SCF-independent mechanisms can compensate in these systems.

SCF,[239] M-CSF,[240] and FLT3LG[181] are all expressed as both membrane-bound and soluble forms. In the case of SCF, expression of membrane-bound forms of the molecule in the marrow microenvironment provides a nice model for how this growth factor might act locally. Indeed, cell lines that express membrane-associated SCF exclusively are much more effective in supporting long-term hematopoiesis in vitro than cell lines that produce soluble forms of the molecule exclusively.[239] This interaction of membrane-associated SCF with KIT provides one mechanism for the adherence of hematopoietic cells to stroma; binding of human megakaryocytes to fibroblasts can be blocked by antibodies to KIT.[241] Finally, if membrane-associated forms of SCF are present in which the cytoplasmic domain is essentially missing, the result is male but not female sterility, suggesting that the cytoplasmic domain may have an as yet undetermined important biologic function.[152,242]

As shown by the early genetic studies and confirmed through analysis of the recombinant protein, SCF is not specific for the hematopoietic system. It is an important growth factor for melanocytes and primordial germ cells, and it appears to play a role in development of the nervous system, perhaps as a neuronal guidance factor, although it has been difficult to demonstrate neurologic defects in *W* or *Sl* mice[152] other than the fact that the mice are severely constipated due to SCF dependence of intestinal Cajal cells.[243]

The FLT3 receptor tyrosine kinase was originally identified as a novel receptor present in HSCs; with human marrow, the expression is largely limited to the CD34+ cell population.[244-246] FLT3L alone yields low numbers of CFU-GM colonies from human bone marrow but acts synergistically with other cytokines, including IL-3, GM-CSF, EPO, and SCF, to yield enhanced colony formation, both in terms of size and numbers of colonies.[228,247,248] The synergy observed between FLT3L and the other HGFs is comparable to that observed with SCF in similar systems, with the exception that FLT3L has little effect on BFU-E.[229] Multifactor combinations with SCF have been used for expansion of colony-forming cells in long-term cultures; FLT3L has effects comparable to those of SCF when combined with IL-1, IL-3, IL-6, and EPO in 4-week cultures.[249,250] Like SCF, FLT3L in combination with other cytokines such as GM-CSF supports dendritic cell development from CD34+ bone marrow cells.[217] In contrast to SCF, FLT3L does not support the growth and development of mast cells.[228,247] Despite this overlap in bioactivities, mice in which the Flt3 receptor tyrosine kinase has been disrupted appear to have normal hematopoiesis, with the only detectable defects observed within the B-lymphocyte lineage.[251] However, mice in which both the *Kit* and *Flt3* receptor tyrosine kinase genes have been disrupted display more severe hematologic complications than mice with mutation of only one of these genes, suggesting that the two pathways can to some degree compensate for one another.[251] The importance of FLT3L in B-cell growth has also been shown with cultures of primitive B-cell progenitors (CD43+, B220^{low}) in combination with either IL-7 or SCF.[252] Altogether, these findings argue for an important role for FLT3L in hematopoiesis.

Other Stem Cell Factors: Wnt, Jagged, BMP, and Angiopoietin-like Growth Factors

Several new growth factors have been shown to regulate HSC production during embryogenesis as well as adulthood. The wnt family of factors regulates stem cell homeostasis.[253-259] Overexpression of wnt 3A led to a threefold expansion of HSCs. This is the first time that stem cell number was actually increased in a competitive repopulation assay. In separate experiments, overexpression of β-catenin led to an expansion of HSCs, and sorting by activated β-catenin in HSCs based on a wnt reporter line that turned cells green when wnt was activated demonstrated HSC activity. Further studies in chronic myeloid leukemia (CML) demonstrated that wnt activation was present in leukemia.[260] These studies demonstrated that wnt activity could drive HSC production; however, other experiments have demonstrated that the knockout of β-catenin within the HSCs is not required for stem cell homeostasis.[261] Two papers demonstrated that a constitutively activated β-catenin in HSCs leads to aplastic anemia.[262,263] The activation of the wnt pathway may not be necessary for stem cell homeostasis, but it may be sufficient to drive the HSCs to produce more self-renewal cell divisions.

The wnt pathway clearly interfaces with HSC production. It is possible that pulses of wnt will have different activity than a constitutively active wnt signal. In addition, redundancy within the marrow space among other signaling pathways, including the notch and BMP pathways, has left the stem cell compartment with significant chances of survival. Wnt acts through canonical and noncanonical pathways. In the former case this leads to activation of β-catenin to the nucleus through the complex involving adenomatous polyposis coli (APC) and β-catenin. In the latter, there is stem cell activation by the Rho GTPases. Wnt activation acts at multiple places during hematopoiesis, at the stem cell level, at the pre-B-cell level, and at multiple places in T-cell development. Wnts bind to canonical frizzled receptors and activate a signaling pathway. An antagonist wnt signal, wnt 5a, suppresses B-cell proliferation and may act to suppress stem cell homeostasis. Other wnts have been found to regulate hematopoiesis, including wnt 2b and wnt 10b for human bone marrow CD34+ cells.

The notch pathway has been shown to regulate HSC production based on retroviral transduction of hematopoietic cells.[264,265] This expands the population of HSCs significantly. Notch works through a ligand receptor interaction in which deltas and jaggeds are presented on the stem cell niche. In general, jagged2 is thought to be the predominant notch ligand in the marrow and is presented by the osteoblast. Modulation of jagged2 leads to an expansion of HSC production. The notch pathway has been put into a genetic pathway with runx1, based on the zebrafish mutant mindbomb.[266] Mindbomb mutants have no notch signaling due to a defect in an E3 ubiquitin ligase that promotes ligand recycling. This mutant lacks HSCs during development, and overexpression of runx1 rescues hematopoiesis. A pulse of notch signaling has been shown to expand HSCs in the zebrafish system. Furthermore, the runx1 rescue of notch 1 mutant was demonstrated in the mouse system and it was also shown that GATA2 was a target of the notch pathway.[267,268] Modulation of wnt and notch demonstrates synergy of wnt 3a and activation of the notch pathway. It is possible to activate wnt pathways using the chemical called *BIO drug*, which is an inhibitor of glycogen synthase kinase 3beta GSK-3β. This modulation of the wnt and notch pathways represents a significant role in stem cell homeostasis.

The bone morphogenetic protein family has been demonstrated to induce HSC production in the *Xenopus* and zebrafish systems.[194a,270-276] This leads to an expansion of the erythroid blood islands during early development. Interestingly, a dominant-negative version of BMP activated later in development leads to enhanced erythropoiesis, demonstrating two independent effects of BMP regulation of HSC differentiation and proliferation. Furthermore, BMP has recently been shown to expand HSC production in the aorta-gonads-mesonephros region. BMP was shown to expand or maintain HSC production in human embryonic stem cells and functions as a key regulator of stress-induced erythropoiesis.[277] BMP also regulates the stem cell niche. It is unclear how BMP and the notch and wnt pathways interact.

Angiopoietin-related proteins are potent activators of HSC production.[278] Thus angiopoietin-like 2 and angiopoietin-like 3 were shown to lead to a twentyfold or thirtyfold expansion of long-term HSCs when they were cultured for 10 days in the presence of the ligands. This was demonstrated by a limiting-dilution competitive repopulation assay. Thus angiopoietin-like genes must also participate in the stem cell niche. Another growth factor glycoprotein called *osteopontin* was also involved in stem cell homeostasis; it negatively regulates the stem cell pools.[279,280] A number of factors, including TGF-β1, have a negative regulation as well. Ligands produced by the stem cells themselves may also regulate stem cell homeostasis.

Synergistic Factors: IL-1, IL-6, and IL-11

Early in the 1980s, activities were identified that had the ability to enhance hematopoietic colony formation supported by other HGFs, particularly with early progenitor cells. One activity, designated hematopoietin 1, was subsequently purified and discovered to be IL-1.[205] In this fashion, IL-1 was recognized as having little ability on its own to stimulate hematopoietic colony formation but was able to act in synergy with other HGFs, notably IL-3, in increasing both the frequency of colony formation and the numbers of cells per colony.[186] Subsequent to the discovery of the synergistic activity of IL-1, numerous other cytokines have emerged with similar activity, including IL-6,[281] IL-11,[282] leukemia inhibitory factor (LIF),[283] and IL-12.[284] Of these, IL-6, IL-11, and LIF all signal through a common signal-transducing molecule, the gp130 component of the IL-6 receptor.[285] Because these molecules are likely to behave similarly in most systems and because IL-6 and IL-11 have been the most thoroughly studied, we will limit our discussion to these two members of this family. IL-12, which signals through a distinct but perhaps similar pathway to gp130,[286] has interesting effects in combinations with other cytokines[284] but appears to be more important in regulating the development and activities of T and NK cells[287,288] and will therefore not be discussed further here.

The effects of IL-1 on hematopoiesis have been highly complicated by the fact that this cytokine is a potent inducer of secondary cytokine production, frequently by accessory cells in the culture.[289] The induction of other growth factors, notably IL-6, G-CSF, GM-CSF, and IL-11, is likely to contribute to the activity of IL-1 as a synergistic factor in hematopoietic colony formation. Nevertheless, combinations of IL-1 with other factors in cultures of highly purified hematopoietic cells typically yield synergistic effects, suggesting that at least some of the effects are direct.[290,291] However, even with highly purified hematopoietic progenitor cell populations, the effects of IL-1 can be indirect. For example, Rodrigues and colleagues[292] have reported that IL-1 can prevent apoptotic death of CD34+ lin– human bone marrow cells but the effect is largely abrogated by antibodies to GM-CSF, suggesting that some progenitor cells in the population can produce their own GM-CSF. Thus the survival effect of IL-1 in some systems may also be indirectly mediated by induction of GM-CSF expression in CD34+ lin– bone marrow cells themselves.

IL-6 is an extremely pleiotropic cytokine with important effects on the growth and differentiation of T and B cells, on the induction of the hepatic acute-phase response, and on the enhancement of the proliferation of hematopoietic progenitor cells.[244] IL-6 signaling, mediated by two members of the hematopoietin receptor gene family, IL-6R[293] and gp130,[294] was the first example of signaling through a commonly shared receptor subunit, gp130, which now is known to be involved in the signaling of LIF, IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1).[285,295] Because most cells express gp130, the expression of other receptor components such as IL-6R or IL-11R generally determines whether or not a cell will respond to one of these family members; cells that express receptors for multiple members of this cytokine subgroup generally exhibit identical or nearly identical responses to each member whose receptor component is expressed by the cell.[176,285,296]

IL-6 has little if any ability to support colony formation on its own but can enhance colony formation supported by other HGFs, particularly IL-3 and SCF.[232,281] This effect was most prominent using bone marrow cells from mice isolated for 2 days following treatment with 5-FU, a drug that enriches for primitive progenitors by selectively killing later, actively cycling cells in the bone marrow.[202] Bone marrow cells treated in this fashion generally yield significant numbers of colonies only when plated in the presence of multiple HGFs. In this system, IL-6 was found to enhance colony formation supported by IL-3 or SCF. These early cells are typically quiescent in the G_0 phase of the growth cycle, and combined effects of cytokines, such as IL-3 and IL-6, are required to push them into active cycling.[202] Similar effects have been observed with cultures of purified early human hematopoietic progenitor cells.[297]

As noted above, the IL-11 receptor complex employs the IL-6 gp130 signal-transducing system. Thus IL-11 and IL-6 appear to have somewhat overlapping biologic activities.[298] In general, IL-6 has proved to have more effects on T and B cells than IL-11[244,299] and similar effects in the hepatic acute-phase response[300] and on osteoclast formation,[301] but IL-11 is somewhat more potent in megakaryocytopoiesis.[302] Like IL-6, the effects of IL-11 in hematopoietic cultures are largely observed only in combination with other factors. IL-11 in combination with SCF or FLT3L has proved to be very effective in supporting the growth of primitive hematopoietic progenitors.[248] Soon after its discovery, IL-11 was shown to have important effects on the growth and development of megakaryocyte progenitors.[299,302,303] Again, in this system, IL-11 had little effect on colony formation on its own but was found to act in synergy with IL-3, SCF, or, more recently, TPO in supporting CFU-meg colony formation.[304] Its clinical use in thrombocytopenic states has been disappointing.

Negative Regulator of Hematopoiesis: Transforming Growth Factor Beta

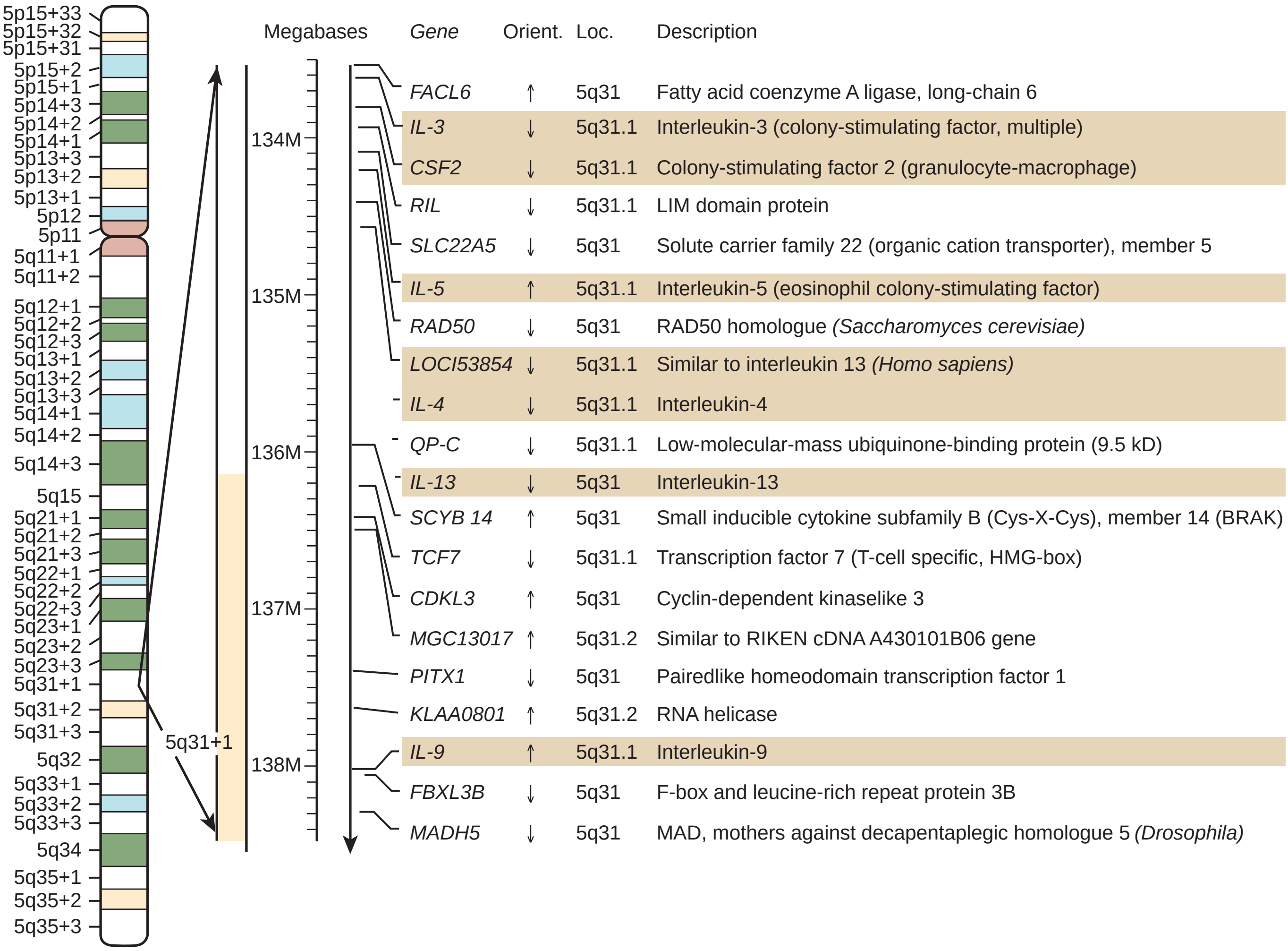
A variety of cytokines have been reported to reversibly inhibit cycling of early hematopoietic cells, including the chemokines SDF-1,[208] MIP-1α,[187] and TGF-β, perhaps the most potent of all.[305-307] Very interestingly, when individual early cells were plated in wells, either antibody to TGF-β1 or an antisense oligonucleotide to the mRNA for this factor could trigger the release of the cells from quiescence in the presence of appropriate combinations of early-acting cytokines, which by themselves alone failed to trigger the cycling of the cells.[306,307]

MOLECULAR BIOLOGY OF HEMATOPOIETIC GROWTH FACTORS

Genomic Analysis of the Cytokine and Cytokine Receptor Genes

With analysis of the human genome, several common themes and relationships have emerged from the different types of cytokine/growth factor and receptor genes. First, as we have already noted, most of the cytokines and their receptors exist as members of distinct, evolutionarily related gene families; examples of a single cytokine or growth factor gene with no relatives are rare. Similarly, the receptors also fall into gene families. Thus a relatively small number of primordial growth factor/cytokine genes were duplicated many times, forming families of genes that evolved into serving regulatory functions for many different systems. Interestingly, in most cases, each of the individual cytokines of a particular gene family use members of a single receptor gene family. For example, all of the chemokines use G-coupled protein receptors as their signaling/recognition molecules,[155] and all of the members of the TGF-β gene family use different combinations of the serine/threonine kinase receptors as their recognition and signaling molecules.[159] All of these receptor-ligand pairs must have evolved in concert as they were selected to control different cellular processes.

Despite the obvious gene duplications that have occurred in all of these gene families, there is no absolute rule as to how the genes are arranged today. In some cases, the related genes are still closely linked in tandem in the genome, as is the case for the IL-3 and GM-CSF genes on chromosome 5 at 5q31.1 (Fig. 1-13). In this case, another duo of related genes, IL-4 and IL-13, are very closely linked to one another but are slightly more separated from the IL-3/GM-CSF cluster.



Gene	Orient.	Loc.	Description
FACL6	↑	5q31	Fatty acid coenzyme A ligase, long-chain 6
IL-3	↓	5q31.1	Interleukin-3 (colony-stimulating factor, multiple)
CSF2	↓	5q31.1	Colony-stimulating factor 2 (granulocyte-macrophage)
RIL	↓	5q31.1	LIM domain protein
SLC22A5	↓	5q31	Solute carrier family 22 (organic cation transporter), member 5
IL-5	↑	5q31.1	Interleukin-5 (eosinophil colony-stimulating factor)
RAD50	↓	5q31	RAD50 homologue *(Saccharomyces cerevisiae)*
LOCI53854	↓	5q31.1	Similar to interleukin 13 *(Homo sapiens)*
IL-4	↓	5q31.1	Interleukin-4
QP-C	↓	5q31.1	Low-molecular-mass ubiquinone-binding protein (9.5 kD)
IL-13	↓	5q31	Interleukin-13
SCYB 14	↑	5q31	Small inducible cytokine subfamily B (Cys-X-Cys), member 14 (BRAK)
TCF7	↓	5q31.1	Transcription factor 7 (T-cell specific, HMG-box)
CDKL3	↑	5q31	Cyclin-dependent kinaselike 3
MGC13017	↑	5q31.2	Similar to RIKEN cDNA A430101B06 gene
PITX1	↓	5q31	Pairedlike homeodomain transcription factor 1
KLAA0801	↑	5q31.2	RNA helicase
IL-9	↑	5q31.1	Interleukin-9
FBXL3B	↓	5q31	F-box and leucine-rich repeat protein 3B
MADH5	↓	5q31	MAD, mothers against decapentaplegic homologue 5 *(Drosophila)*

Figure 1-13 Cytokine gene cluster at 5q31.1. The region from chromosome 5, comprising five megabases of DNA sequence, is expanded as shown with the structures of the individual genes with GenBank notations, descriptions, and gene orientations as indicated. The region contains approximately 115 genes, 21 of which are illustrated. The data were collected and assembled from the National Center for Biotechnology Information database.

Structures of the Hematopoietic Growth Factor Proteins

The availability of the cDNAs for the individual cytokines allowed for the rapid determination of their primary amino acid sequence, for their expression in quantities large enough for direct x-ray determination of their crystal structures, and, more broadly, for predicting structures based on those of proteins related even distantly by sequence. One of the clear results of this analysis of the structures of the different cytokines was the realization that, despite generally low conservation of amino acid sequence, many of the lymphohematopoietic cytokines are distantly related in evolution. Most of the lymphohematopoietic cytokines are believed to assume a tightly packed, antiparallel, four—α-helix–bundled structure with either short or long helical bundles.[146,150] Lymphohematopoietins having the short helical bundle structure include IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-13, IL-15, and GM-CSF; cytokines having the long helical structure include the gp130 signaling proteins IL-6, IL-11, LIF, and OSM; IL-12p35; and the homodimeric receptor signaling proteins EPO, TPO, and G-CSF.[146,197,308]

Sequence and structural information suggest that the cytokines with the long helical bundle structure are all distantly related in evolution. Members of the IL-6 subfamily, including IL-6, IL-11, and G-CSF, show rather modest sequence similarity but display common gene organization and certain structural features that suggest evolutionary relatedness.[149,308,309] Sequence alignments of IL-6 and G-CSF show conservation of cysteine residues.[310] Interestingly, a cytokine identified in the chicken, myelomonocytic growth factor (MGF),[311] displays sequence similarities with G-CSF and IL-6, which suggests that all three are derived from a common ancestral gene. Erythropoietin shows some structural features similar to those of growth hormone and to G-CSF but at a very low level.[308] In contrast, the amino-terminal half of TPO shows strong sequence similarity with EPO, whereas the carboxy-terminal half is unrelated to any known protein.[312-314] The four-helical-bundle structure of EPO is clearly related to the amino-terminal, helically structured portion of TPO, whereas the additional domain of TPO, which is heavily glycosylated, may serve to increase the

TABLE 1-2 Genetic Defects in Receptors or Signaling Proteins

A. Human Mutations

Gene	Mutation	Phenotype	Reference
IL2Rγc	Deletion	X-linked severe combined immunodeficiency (SCID)	909
JAK3	Deletion	SCID	910
GCSFR	C-terminal deletion	Kostmann disease developing leukemia	419
EPOR	C-terminal deletion	benign erythrocytosis	911

B. Nonhuman Mutations

Species	Gene	Name	Phenotype	Reference
Mouse	*Kit*	White-spotting	Macrocytic anemia, mast cell deficiency, lack of pigmentation, sterile	520, 519, 564
	Sl	Steel	Macrocytic anemia, mast cell deficiency, lack of pigmentation, sterile	520, 519, 564
	Csf1	Microphthalmia	Osteopetrosis due to decreased osteoclast function	342
	Hcp	Motheaten	Immunodeficiency, autoimmune disease, increased sensitivity to EPO	487
Drosophila	JAK homologue	Hopscotch	TumL allele, gain-of-function mutation that causes leukemia	912, 913

C. Targeted Disruption in Murine Embryonic Stem Cells

Gene	Phenotype	Reference
Il-6	Decreased CFU-S and stem cell function	329
Gmcsf	Hematopoiesis normal; progressive accumulation of pulmonary surfactant	340, 341
Gcsf	Neutrophils 25% normal; impaired response to *Listeria monocytogenes* infection	317
Epo	Embryonic lethal; failure of definitive erythropoiesis	327
Epor	Embryonic lethal; failure of definitive erythropoiesis	327
Tpor (Mpl)	Platelets 15% to 20% normal	322
IL-3/-5/GMRβc	Similar to *Gmcsf*$^{-/-}$ mice; also low eosinophils and impaired eosinophil response to *N. brasiliensis*	712
Vav	Not required for hematopoiesis	914

serum half-life of the molecule. Both of these family members are believed to act as monomers.

The ligands for the receptor tyrosine kinase gene family members, SCF, FLT3L, and M-CSF, share many structural similarities.[152,181,315] All three contain transmembrane domains and are initially synthesized as homodimeric integral membrane proteins. Although the membrane-bound forms are functional, dimeric soluble forms that also display biologic activity are released from the cell by proteolysis. The extracellular domains display low but significant sequence similarity, including conservation of the positions of the cysteine residues involved in disulfide bridge formation.[181] Modeling of these extracellular domains indicates that they, too, are likely to form four α-helix–bundled structures tethered to the membrane through variable spacer domains, with dimerization through a cysteine located in the spacer domain.[146,180,181]

Hematopoietic Growth Factor Gene Disruptions

Hematopoietin Receptor Ligands

G-CSF Gene. The combination of technology for disrupting genes in cultured mammalian cells has led to methodology for studying gene function in vivo (mutants related to HGFs are summarized in Table 1-2).[316] Using this approach, Lieschke and associates[317] were able to generate mice with both copies of the *G-CSF* gene disrupted. These animals develop normally but as adults display a 70% to 80% reduction in levels of circulating neutrophils, a 50% reduction in the numbers of progenitors in all lineages, and impaired resistance to challenge to infections with *Listeria monocytogenes*. These findings, which are in agreement with earlier observations by Hammond and colleagues[318] in dogs that had developed cross-reacting antibodies to canine G-CSF, demonstrate the central role for G-CSF in controlling neutrophil levels. However, the decline in all populations of progenitors is consistent with a role for G-CSF prior to commitment to the neutrophil lineage, perhaps at the early stem cell stage.[319,320]

Erythropoietin and Thrombopoietin. The *TPO* and *EPO* genes have clearly evolved from a common ancestor; at some point the extra protein-coding region found in the final coding exon of the *TPO* gene was either removed (in the case of the *EPO* gene) or added (in the case of the *TPO* gene) following the early duplication event.

Mice with deletion of either the *Tpo* or *Mpl* gene have been described.[321,322] Although these animals develop normally and do not display signficant abnormalities in bleeding times, they do have only 15% to 20% of the normal levels of circulating platelets. This result implies that TPO is very important for maintaining circulating levels of platelets but that other cytokines might have some capacity to generate platelets at low levels. TPO has also been shown to have effects on stem cell homeostasis.[323-326] In the case of *Epo*, gene disruption results in embryonic lethality due to failure of fetal liver erythropoiesis.[327]

IL-6–gp130 Complex Ligands: IL-6 and IL-11. Animals that have been engineered to lack the *IL-6* gene develop normally but display significant deficiencies in various immune and inflammatory responses.[328] The hepatic acute-phase response in these animals is severely compromised after tissue damage but only moderately in response to lipopolysaccharide (LPS) The effects of disruption of the *IL-6* gene on normal hematopoiesis are relatively minor; a slight decrease in peripheral blood leukocyte counts, a 10% reduction in bone marrow cellularity, a 50% reduction in CFU-S_{d12}, and a fourfold to fivefold reduction of pre–CFU-S.[329]

GM-CSFR Complex Ligands: IL-3, GM-CSF, and IL-5. The genes for each of these proteins comprise approximately 3000 base pairs divided among four exons (GM-CSF[330,331] and IL-5[199,332]) or five exons (IL-3[331,333]) with somewhat similar structures. All of these genes have been localized to the long arm of chromosome 5 at 5q31.1,[334] a region commonly disrupted or deleted in patients with various malignant myeloid neoplasms.[335] This region also contains the genes for other important cytokines, including IL-4, IL-9, IL-12p40, and IL-13.[334-336] Detailed mapping of the region has demonstrated that several of these genes are very closely linked: the *GM-CSF* and *IL-3* genes are tandemly arrayed within 9 kb of one another;[337] the *IL-4* and *IL-13* genes are separated by only 12.5 kb.[334] This clustering of molecules with similar structures and functions, including the sharing of receptor components in the case of the *GM-CSF*, *IL-3*, and *IL-5* genes,[338,339] provides further strong support for the evolutionary relatedness of their respective genes.

Mice with homozygous disruption of the *GM-CSF* gene develop normally and display neither numerical defects in the levels of circulating granulocytes or monocytes nor defects in the levels of CFU-GM progenitors in marrow or spleen.[340,341] These animals exhibit alveolar proteinosis with surfactant accumulation in the lungs based on defective aveolar macrophage function. GM-CSF plays an irreplaceable role in the function and development of certain macrophage populations, but any function played by this cytokine in controlling basal or stimulated production of granulocytes or monocytes can be replaced by other factors.

To evaluate the role of the entire IL-3/GM-CSF/IL-5 complex, Nishinakamura and coworkers[341a] engineered a mouse lacking the beta-c receptor component shared by all three cytokines as well as a disruption in the *IL-3* gene. Mice lacking beta-c show a pulmonary alveolar proteinosis-like disease and reduced numbers of peripheral eosinophils, which are explained by the lack of GM-CSF and IL-5 functions, respectively. Combination with the *IL-3* gene disruption showed no further abnormalities in hematopoiesis, demonstrating that the entire IL-3/GM-CSF/IL-5 axis is dispensable for hematopoiesis both in normal and stressed mice.

Receptor Tyrosine Kinase Ligands: M-CSF, Stem Cell Factor, and FLT3 Ligand. The *M-CSF*, *SCF*, and *FLT3* genes are evolutionarily related.[180,181] The gene for M-CSF proved to be defective in the spontaneous mutant osteopetrosis (*op/op*).[342] Female *op/op* mice are infertile, confirming the role of M-CSF in the biology of the pregnant uterus.[343] They also have a severe deficiency in the ability to develop osteoclasts derived from the monocytic lineage, resulting in the development of osteopetrosis and the failure to develop teeth.[344] The animals do develop some macrophage populations, indicating that M-CSF is important in many but not all macrophage-related lineages. The observation that some of the defects in the *op/op* mouse are corrected as the animals mature has led to speculation that alternative splicing of the mutant M-CSF transcript can lead to production of some functional M-CSF.[345] The OP9 stromal cells are often used in the expansion of stem cells, showing that M-CSF suppresses stem cell growth.[346]

Naturally occurring mutations in the SCF locus (*Sl*) or in the receptor for SCF, Kit (*W*), result in profound effects on many stages of hematopoiesis, most notably in the stem cell, erythroid, and mast cell compartments.[152] These studies have clearly demonstrated the central role of SCF in controlling hematopoietic cell function. Interestingly, disruption of the *Flt3* receptor gene has relatively minor effects on hematopoiesis, the most notable being somewhat depressed levels of B-lymphocyte precursors.[251] However, mice with disruption of both the *Kit* and *Flt3* receptor tyrosine kinase genes display more severe hematologic complications than mice with a mutation of only one of these genes,[251] suggesting that the two factors do, at some level, complement one another in vivo.

Hematopoietic Growth Factor Genes: Regulation of Expression

Expression of Hematopoietic Growth Factors by Monocytes/Macrophages and Dendritic Cells: G-CSF, M-CSF, GM-CSF, IL-6, and SCF

Monocytes/macrophages and related cells, including dendritic cells, Kupfer cells in the liver, and Langerhans cells in the skin, are key cells that serve many important functions in regulating the immune system.[347] Among the functions performed by these cells is the production of many cytokines, including TNF,[348] IL-1,[185] G-CSF,[169] M-CSF,[348] GM-CSF,[142] IL-6,[244] and IL-12,[347] in response to various stimuli, including LPS,[347,348] IL-1, IL-3,[349] GM-CSF,[350] and M-CSF.[351] Other cytokines, including IL-4,[352] IL-10,[353] and IL-11,[354] downregulate monocyte production of many of these cytokines. Several of the cytokines, including IL-18,[185] various members of the *IL-17* gene family,[355] and IL-23,[167] are playing important roles in controlling cytokine production by monocytes and macrophages.

All of these interactions are key components of the interactions between T cells and monocytes in determining the nature and direction of the immune response. Different regulators have been found to induce different subsets of these cytokines. For example, LPS has been shown to induce expression of IL-1, IL-6, TNF, IL-1 receptor antagonist, GM-CSF, and G-CSF.[349] In contrast, IL-3 and GM-CSF failed to induce the expression of either GM-CSF or G-CSF but were found to induce M-CSF expression. M-CSF has been shown to induce peritoneal macrophages to activate expression of GM-CSF and IL-6 at the transcriptional level, but additional signals are necessary to induce the release of the functional cytokine proteins from the cells.[356] Murine bone marrow–derived macrophages have been reported to constitutively express the messenger RNA (mRNA) for M-CSF and SCF, and the level of expression can be enhanced by treating the cultures with pokeweed mitogen.[357]

The different HGF genes in monocytes can be upregulated by a variety of different mechanisms. In the case of IL-6, LPS and *Mycobacterium tuberculosis* activate gene expression at the transcriptional level through activation of the transcription factors NF–IL-6 and NF-κB.[358] In contrast, activation of IL-1 and IL-6 expression by *Salmonella typhimurium* porins is largely mediated by mRNA stabilization.[359] Cytokine activation of monocyte IL-6 expression also occurs by various mechanisms, often involving interactions between NF-κB and NF–IL-6.[360] IL-2, IL-3, and GM-CSF have all been shown to induce monocyte expression of IL-6, and this expression is inhibited posttranscriptionally by treatment with IL-4.[349,352] In the case of G-CSF, expression can be activated by treatment of monocytes with either IL-1 or LPS (IFN-γ potentiates the LPS response[361]); IL-4 given simultaneously can block this induction, an effect that is not mediated by mRNA stability but rather is mediated at the transcriptional level.[362] These interactions between monocytes and T cells and the cytokines they produce, although complicated, are clearly important in determining the direction and extent of the resulting immune responses and are likely to play a key role in controlling the cytokine network.

Dendritic cells, which have been characterized as potent antigen-presenting cells, are also an important source of HGFs and other cytokines.[363] With the availability of cytokines such as FLT3L, GM-CSF, IL-3, and IL-4, it has become much easier to generate dendritic cells from both myeloid and lymphoid sources in significant numbers for further studies.[218,364] Originally believed to be largely of myeloid cell origin, it is now recognized that some are likely derived from myeloid cells and others have their origins in the lymphoid lineages.[365] Interestingly, different types of dendritic cells, matured under different conditions, can serve to polarize cytokine production by maturing T-helper cells[366] and therefore are likely to play key roles in determining the outcomes of immune responses.

Different populations of fibroblasts, endothelial cells, and epithelial cells can be stimulated to produce cytokines by treatment with LPS,[367] phorbol esters,[331] or other cytokines, including IL-1,[182] TNF,[368] and platelet-derived growth factor (PDGF).[369] In many tissues, this is likely to be an integral part of the host response to infection: Cytokine production at the site of infection should result in local activation of host defense effector cells and the systemic recruitment of more effector cells until the infection is cleared.[370] In bone marrow, thymus, and spleen, these cell populations are likely to be critical components of the local microenvironment, which is involved in the normal proliferation, development, and differentiation of cells of the hematopoietic and lymphopoietic systems. The control of production of the various HGFs in the steady state is still poorly understood.[370]

Fibroblasts and endothelial cells are important sources of many of the HGFs, including G-CSF,[169] M-CSF,[151] GM-CSF,[331] IL-6,[371] IL-11,[299] SCF,[152] and FLT3L.[228,247] This expression is regulated both transcriptionally and posttranscriptionally by exposure to LPS, phorbol esters, or cytokines such as IL-1, TNF, and IFN-γ.[169,372,373] SCF expression has been reported to be constitutive in human stromal bone marrow cultures, in endothelial cells and in bone marrow– derived fibroblasts, and is not responsive to IL-1.[374]

Although it is not clear that stromal fibroblasts from bone marrow are any more or less capable of cytokine production than fibroblasts from other tissues, because of their proximity to stem and progenitor cells they have long been studied for their ability to express HGFs. Many of these cells constitutively express the tyrosine kinase receptor ligands M-CSF and SCF.[374,375]

Following myeloablation, circulating levels of many HGFs increase dramatically. In some cases this might result from mechanisms that sense low levels of the various blood cells. However, Hachiya and colleagues[376] have found that in culture, TNF and IL-1 synergize with irradiation to upregulate the expression of GM-CSF both through mRNA stabilization and activation of gene transcription. These observations raise the possibility that the myeloablative agents themselves may directly induce or enhance HGF production in vivo following various cancer therapy regimens.

Endothelial cells are another important source of HGFs. Human endothelial cells produce G-CSF, GM-CSF, and M-CSF in response to inflammatory cytokines such as IL-1 and TNF.[377,378] In contrast to G-CSF and GM-CSF, SCF mRNA is expressed constitutively by human umbilical vein endothelial cells (HUVECs).[379] The levels of SCF mRNA are further increased in response to inflammatory mediators, including IL-1 and LPS. This induction results predominantly from mRNA stabilization by approximately threefold. Increased expression of GM-CSF mRNA in HUVECs is also at least in part mediated by mRNA stabilization.[380] Possibly the induction of SCF mRNA in endothelial cells contributes to the elevated plasma levels of this factor in patients with sepsis or inflammatory diseases.[379]

Regulation of EPO Expression by Hypoxia

Erythropoietin has long been recognized as the physiologic regulator of red cell production.[381-383] It is produced in the kidney and in the fetal liver in response to hypoxia or exposure to cobalt (II) chloride; the mechanism of the

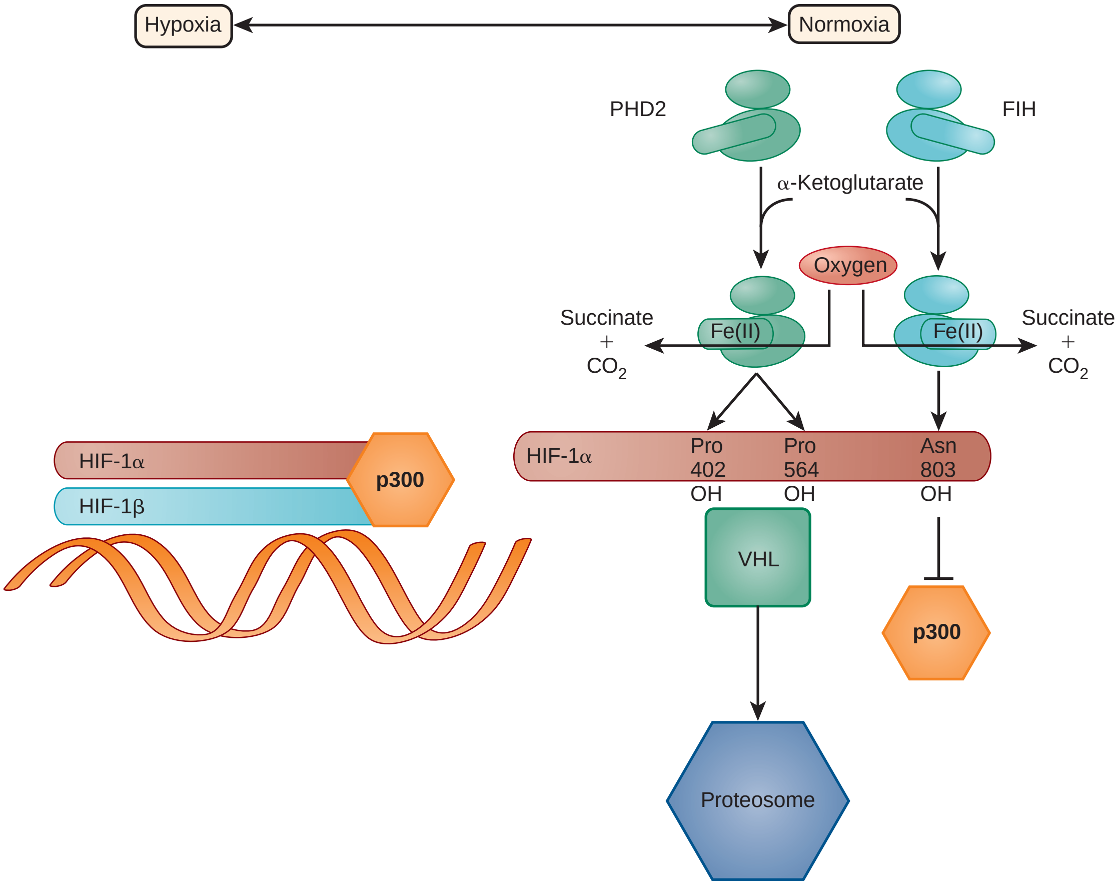


Figure 1-14 Hypoxia-inducible regulation of Epo production. Under normoxic conditions hypoxia-inducible factor 1α *(HIF-1α)* undergoes prolyl hydroxylation at Pro402 and Pro564 that leads to binding of HIF-1α to the von Hippel–Lindau *(VHL)* protein and subsequent ubiquitin-mediated destruction. This dioxygenase enzymatic reaction is catalyzed by a prolyl hydroxylase termed prolyl hydroxylase domain 2 *(PHD2)*. A second hydroxylation at asparagine 803 prevents binding of HIF-1α to its transcriptional coactivator p300, and is catalyzed by a hypoxia-inducible factor asparaginyl hydroxylase called *factor-inhibiting hypoxia-inducible factor (FIH)*. Both these reactions are dependent on oxygen, Fe(II), and α-ketoglutarate, which is oxidized to succinate with the release of CO_2. Under hypoxic conditions both hydroxylases are inhibited, leading to an increase in HIF-1α and recruitment of the p300 coactivator, which together with the ubiquitously expressed HIF-1β, activates transcription.

switch of production from predominantly fetal liver to predominantly kidney in adults is largely unknown.[384] Beck[385] and Semenza[386] showed that the *Epo* gene contains a hypoxia response element in the 3′ flanking sequence.

Nuclear factors present in hypoxic but not normoxic cells were identified as a heterodimeric basic helix-loop-helix transcription factor complex designated hypoxia-inducible factor–1 (HIF-1), comprising inducible HIF-1α and constitutively expressed HIF-1β.[8,9,387] HIF-1 is induced in a variety of cell types in response to hypoxia or cobalt, indicating that while it is important in the activation of EPO expression, it is also important in the activation of many other hypoxia-inducible genes.[8,388] HIF-1 has been shown to bind to an enhancer sequence located approximately 130 bp downstream from the poly-A addition signal of the *Epo* gene. This enhancer segment has been shown to render other promoter-reporter gene constructs responsive to hypoxia, with typical inductions in the range of 4-fold to 15-fold, significantly less than the 50-fold to 100-fold induction observed with the chromosomal *Epo* gene in Hep3B cells.[386] In addition to HIF-1 and its role on the 3′ *Epo* gene enhancer, other studies have identified a 53-bp sequence from the *Epo* promoter that confers oxygen sensitivity (sixfold to tenfold inducibility) to a luciferase reporter gene.[389] Combining the enhancer and promoter sequences results in cooperative (fiftyfold) inducibility of transcription in response to hypoxia, approaching that observed in vivo.

HIF-1α protein levels are regulated by binding to the von Hippel–Lindau (VHL) tumor suppressor followed by ubiquitin-mediated proteosomal degradation (Fig. 1-14).[390,391] Under normoxic conditions, a prolyl hydroxylase uses molecular O_2, iron (Fe II), and α-ketoglutarate to hydroxylate Pro402 and Pro564 in human HIF-1α, which leads to VHL binding and proteosome-mediated proteolysis. A second hydroxylation of Asn 803 by an asparaginyl hydroxylase (similarly dependent on O_2, Fe II, and α-ketoglutarate) blocks the C-terminal interaction of HIF-1α with the transcriptional coactivators p300/CBP.[10] Under hypoxic conditions both hydroxylases are inhibited, leading to escape from ubiquitin-mediated proteolysis and an increase in HIF-1α protein, as well as recruitment of the p300/CBP and transcriptional activation.[392-395]

Regulation of Thrombopoietin Gene Expression

The cloning and expression of the gene for thrombopoietin[396] has provided new insight into the regulation of levels of platelets.[153] Gene disruption studies of *Mpl*, the receptor for TPO in mice, have shown that in the absence of the function of this pathway, mice have only 15% of the normal levels of circulating platelets.[322] Thus while redundancy among the growth factors, perhaps including SCF, IL-3, IL-6, and IL-11, can partially compensate for dysfunctional Mpl signaling, TPO, like EPO in the erythrocyte lineage, appears to be the major regulator of circulating platelet levels.[197] However, in contrast to the control of EPO production, *Tpo* gene expression does not seem to be transcriptionally regulated or significantly influenced by platelet levels.[397] It may be regulated by the megakaryocyte/platelet mass.[398] In adult mice, the major sources of Tpo mRNA are the liver and kidney; in both organs, the gene is constitutively expressed and is not significantly upregulated during thrombocytopenia. However, circulating levels of TPO increase rapidly during thrombocytopenia and decline as platelet counts return to normal.[399,400] The observation that platelets themselves can remove TPO from thrombocytopenic plasma in vitro has led to the model that TPO is constantly produced and released into circulation but, in normal circumstances, is rapidly removed by the circulating platelets and possibly megakaryocytes as well.[398-400] During thrombocytopenia, the platelet shortage fails to remove TPO as fast as it is made, resulting in elevated levels and stimulation of platelet production. This mechanism is similar to one proposed some years ago by Stanley and associates[401] for the regulation of circulating M-CSF levels directly through consumption by the monocytes themselves.

HEMATOPOIETIC GROWTH FACTOR RECEPTORS

Types of Hematopoietic Growth Factor Receptors

Analysis of the actions of the HGFs on purified murine or human stem and progenitor cells (see below) has shown that there is considerable overlap in the action of the HGFs. Some insight into the apparent overlap in biologic activities of many cytokines has come from analysis of their structural homologies and from the cloning of many of their receptors. These receptors are all type 1 membrane glycoproteins with extracellular N-termini and single transmembrane domains, and they fall into two major classes. Receptors (Rs) such as KIT, FMS (the M-CSFR), and FLT3 are characterized by a cytoplasmic tyrosine kinase domain (RTKs), whereas most of the other receptors lack cytoplasmic tyrosine kinase activity and can be divided into four subclasses (Table 1-3). Most of the HGFRs (class 1) fall into a superfamily with structural features based on two linked fibronectin (FBN) type III domains (Fig. 1-15). Analogous FBN domains are found in the IFN (class 2) receptors, and the class 1 and 2 structures probably evolved from a common primitive adhesion molecule.[402] Although the TNF family of receptors (class 3) are characterized by an extracellular fourfold repeat of approximately 40 amino acids that contains 6 conserved cysteine residues (Cys repeat),[149] the IL-1Rs (class 4) feature extracellular immunoglobulin-like repeats. Although the discussion below is focused mainly on the HGFRs, the RTKs activate many of the same signaling cascades.[403,404]

TABLE 1-3 Hematopoietic Growth Factor Receptor Classes

Type	Receptor	Cytokine
Tyrosine Kinase (Ig repeats)	KIT, M-CSFR (FMS)	Four-α-helix bundle
Non–Tyrosine Kinase		
Class 1: HGFRs (FBN III domains)		Four-α-helix bundle
Shared βc	IL-3R, GM-CSFR, IL-5R	
Shared gp130	IL-6R, LIFR, IL-11R, IL-12R, OSMR	
Shared γc	IL-2R, IL-4R, IL-7R, IL-9R, IL-15R	
Single chain	G-CSFR, EpoR, TpoR (MPL)	
Class 2 (FBN III domains)	IFNα/βR, IFNγR	
Class 3 (Cys repeats)	TNFR I, TNFR II, FAS, CD40	β-jellyroll wedge
Class 4 (Ig repeats)	IL-1R I and II	β-trefoil fold

Structure and Binding Properties of Receptors

Prototypes for the class 1 structure are the (nonhematopoietic) prolactin and growth hormone receptors.[405-407] These polypeptides share a number of features (Fig. 1-16). The major homology lies in the extracellular domain, which is characterized by four conserved cysteine residues in the N-terminal FBN III repeat and a Trp-Ser-X-Trp-Ser (WSXWS) motif in the linked C-terminal FBN III repeat that forms a hydrophobic hinge between the two domains. In addition, the IL-6 family of receptors has members that each have an N-terminal immunoglobulin-like domain as well as extra FBN III repeats. The cytoplasmic regions of the receptor family show much less homology, although membrane proximal domains rich in prolines (box 1, within 20 amino acids of the membrane) and acidic residues (box 2), separated by a positionally conserved tryptophan, have been defined. Mutations within the box1/2 domains inactivate the mitogenic function of the receptors that have been examined,[408] and mutations of the conserved tryptophan inactivate some receptors but not others.[409] An additional interesting feature of many of the HGFRs is that receptor subunits are shared. GM-CSF, IL-3, and IL-5 all have receptors with unique alpha chains that bind their respective ligands with low affinity. They share a common beta chain (βc) that converts ligand binding from low to high affinity in each case and is thought to be critical for signal transduction.[410] A similar arrangement is evident with the IL-6, LIF, OSM, IL-11, and CNTF receptors, all of which share a common beta chain, namely gp130. The finding

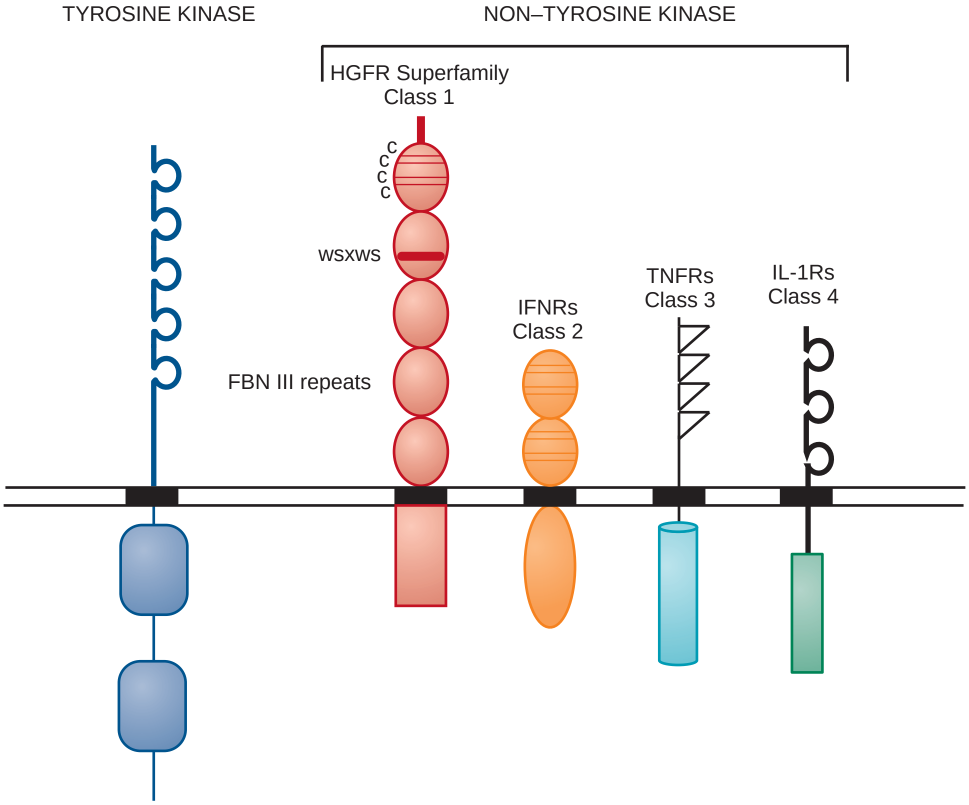


Figure 1-15 Receptors such as KIT, FMS (the macrophage colony-stimulating factor receptor), and FLT are characterized by a cytoplasmic tyrosine kinase domain, whereas most of the other receptors lack cytoplasmic tyrosine kinase activity and can be divided into four subclasses. Most of the hematopoietic growth factor receptors *(HGFRs)* (class 1) fall into a superfamily with structural features based on two linked fibronectin *(FBN)* type III domains; analogous FBN domains are found in the interferon receptors *(IFNRs)* (class 2); the tumor necrosis factor family of receptors *(TNFRs)* (class 3) are characterized by an extracellular fourfold repeat of approximately 40 amino acids that contains 6 conserved cysteine residues (Cys repeat); and the interleukin-1 receptors *(IL-1Rs)* (class 4) feature extracellular immunoglobulin-like repeats.

of shared subunits explains to a certain extent why the actions of GM-CSF and IL-3 overlap on many cells, and why IL-6, LIF, and IL-11 all share pleiotropic actions on HSCs and hepatic cells. The gamma chain of IL-2R is now known to be shared by the IL-4,[411,412] IL-7,[413] IL-9,[414] IL-15,[415] and IL-21[416] receptors. Other members of the HGFR superfamily that act on lineage-restricted cells, such as EPOR, G-CSFR, and thrombopoietin (TPO)R, appear not to require an additional subunit for ligand binding or signal transduction.

The number of IL-3, GM-CSF, G-CSF, and EPO receptors per cell is strikingly low (≈1000 sites per cell), whereas those for M-CSF are about one log higher. In all cases affinity of a receptor for its ligand is high, with dissociation constants usually in the picomolar range. Stimulation of target cells can occur at concentrations of factor that are orders of magnitude lower than the equilibrium constant at which 50% of receptors are occupied, and therefore it is apparent that low receptor occupancy is sufficient to produce biologic effects.

Receptor Function

Lineage-Specific Factors and Induction of Differentiation

Do lineage-specific receptors direct differentiation, or do intracellular proteins specific for lineage-restricted cells have that function? With respect to receptor-driven events, a proximal cytoplasmic domain of the G-CSFR is essential for proliferation, whereas a more distal domain is important for induction of acute-phase plasma protein expression when the receptor is transfected into hepatoma cell lines,[417] or granulocyte-specific proteins when it is introduced into murine IL-3–dependent factor-dependent cell (FDC-P1) cells.[418] Support for a role for the G-CSFR in granulocyte differentiation comes from sequence analysis of the receptor in two patients with severe congenital neutropenia (Kostmann disease) who went on to develop AML.[419] Two different point mutations in the G-CSFR gene resulted in truncations of the C-terminal cytoplasmic region of the receptor and coexpression of both mutant and wild-type genes. The mutation was present in the neutropenic phase in one of the patients, suggesting that the mutation was not acquired along with the leukemia. Functional analysis by transfection of mutant or wild-type genes into murine 32D.C10 cells showed that the mutation acted as a “dominant negative” and prevented differentiation in response to G-CSF. Other evidence for an inductive role of receptors comes from murine long-term bone marrow cultures infected with a retroviral *Fms* vector that yielded a pre–B line with an immunoglobulin heavy-chain gene rearrangement. This line grew in IL-7 or M-CSF;

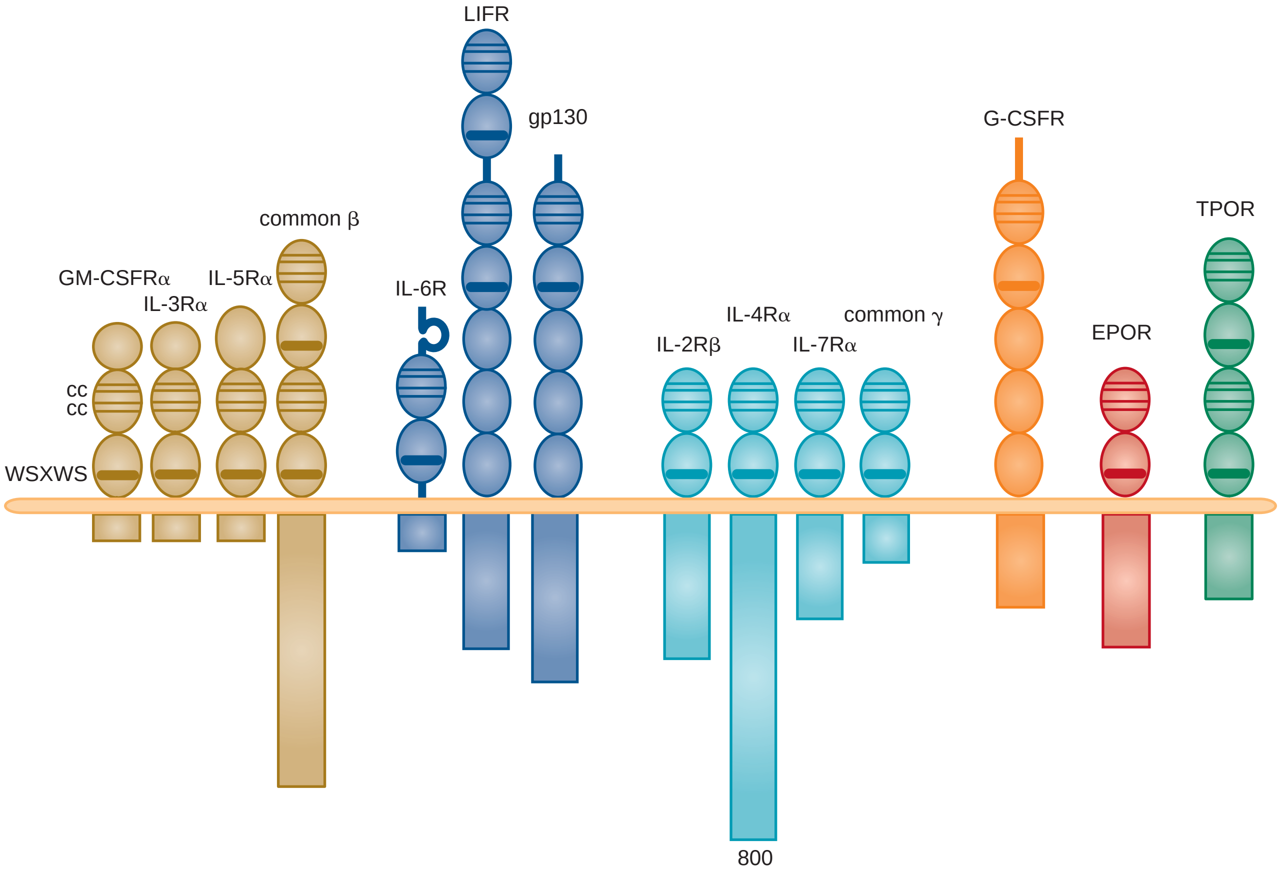


Figure 1-16 Hematopoietic growth factor receptor family. Diagram of some of the hematopoietic growth factor receptors that have been cloned. The extracellular domains are all characterized by one or two regions that contain four conserved cysteines and a tryptophan-serine-x-tryptophan-serine *(WSXWS)* motif, like the prolactin receptor. Unique features: IL-6 contains an immunoglobulin-like domain *(open circle)*; and G-CSF contains a fibronectin type III–like region *(cross-hatching)*. The intracellular domains show less homology. An additional interesting feature of many of the hematopoietic growth factor receptors is that receptor subunits are shared (in particular, the IL-3R, IL-6R, and IL-2R groups. In contrast, lineage-restricted receptors such as the EPOR, G-CSFR, and thrombopoietin (Tpo)R appear not to require an additional subunit for ligand binding or signal transduction.

interestingly, the switch to M-CSF led to macrophage maturation, suggesting that signals from this receptor can determine differentiation in these bipotent cells.[420] Last, transduction and stable expression of the EPOR in IL-3–dependent Ba/F3 cells results in cells that produce globin mRNA upon EPO stimulation but not IL-3 stimulation[421]; and a chimeric receptor that comprises the extracellular domain of GM-Rα and the cytoplasmic domain of the EPOR can induce increased glycophorin A expression in Ba/F3 cells, in contrast to the GM-Rα/βc control.[422] Although these experiments suggest that elements of the cytoplasmic domains of these receptors can direct both proliferation and differentiation, experiments with cell lines must be interpreted with caution. Definitive proof that the EPOR is not specifically required for erythroid differentiation comes from retroviral expression of the prolactin receptor in wild-type and *Epor*$^{-/-}$ progenitors; these cells differentiate efficiently in prolactin, indicating that there is no unique instructive role for the EPO in erythropoiesis.[423] However, the prolactin receptor activates similar signaling pathways to the EPOR such as STAT5, and therefore could perhaps readily substitute for EPOR function. In contrast, GM-CSF cannot rescue lymphopoiesis in GM-CSFR transgenic mice crossed with IL-7–deficient animals; furthermore, common lymphoid progenitors that express ectopic GM-CSFR differentiate into myelomonocytic cells, indicating plasticity in common lymphoid progenitors and an instructive role for GM-CSFR in this context.[424]

Taken together, it is reasonable to conclude that the major role of HGFRs is the survival, the amplification, and, particularly in the case of the lineage-specific receptors, the completion of an intrinsic differentiation program of committed progenitor cells. There is, however, overlap of expression, as with EPOR expression on megakaryocyte progenitors.[113]

Signal Transduction

Hematopoietic Growth Factor–Induced Tyrosine Phosphorylation. Several signaling proteins and pathways are common to many different receptor types. A paradigm common to receptors both with and without endogenous tyrosine kinase activity is that ligand binding induces homodimerization or heterodimerization of receptor subunits, and this is followed rapidly by transient tyrosine phosphorylation of the cytoplasmic domain of the

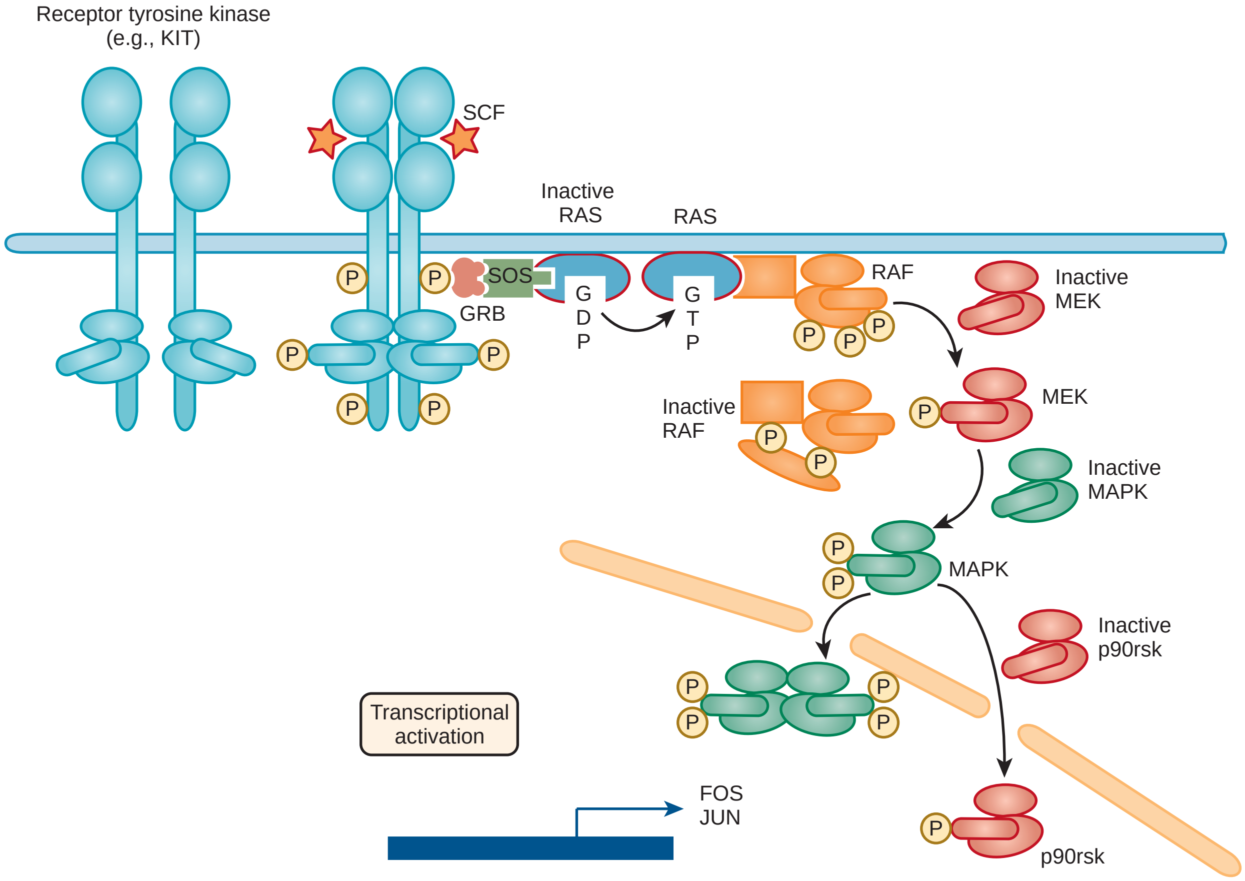


Figure 1-17 Signal transduction through a receptor tyrosine kinase such as KIT. The figure illustrates that the cytosolic domains contain a protein tyrosine kinase catalytic site that forms a functional dimer after ligand binding. The kinases phosphorylate each other, which causes the activation lip to move out of the catalytic site, allowing the binding of adenosine triphosphate or a protein substrate. The kinase phosphorylates other tyrosines in the receptor cytosolic domain that function as docking sites for other signaling proteins. One of the major pathways is RAS activation and phosphorylation of c-raf, MEK (MAPKK), MAPK, and p90S6k (or p90RSK) and induction of FOS/JUN and probably bcl-2, important for cell proliferation and survival.

receptor itself, of cytoplasmic tyrosine kinases, and of other cytoplasmic proteins involved in generating different signaling cascades (Fig. 1-17). In the case of the receptors with intrinsic tyrosine kinases, ligand-induced activation of their catalytic function leads to transphosphorylation or autophosphorylation of dimerized receptor subunits and activation of three major signaling cascades: RAS/MAP kinase; phosphoinositol (PI)-3′ kinase/protein kinase B (PKB, also known as *AKT*); and phospholipase C (PLC)/inositol trisphosphate (IP_3)/diacylglycerol (DAG)/protein kinase C (PKC). Receptors that lack a tyrosine kinase domain must recruit cytoplasmic tyrosine kinases. For the class 1 cytokine receptors the family of Janus kinases (JAKs) fulfills this role, but other nonreceptor kinases have been identified and may also be important. Tyrosine phosphorylation within the receptor cytoplasmic domain creates docking sites for substrates characterized by the presence of *src* homology 2 (SH2) domains. These SH2 domains recognize phosphotyrosine in the context of specific short sequences of amino acids.[425] This leads to activation of the three signaling cascades mentioned above (RAS, PI -3′ kinase, and PLC) as well as the activation by JAK of a critical family of *s*ignal *t*ransducers and *a*ctivators of *t*ranscription, the STAT proteins.

There are four known members of the JAK family, JAK1, JAK2, JAK3, and TYK2.[426-428] All are 130- to 134-kD related proteins that have an amino-terminal receptor-binding domain, a carboxy-terminal kinase domain, and a middle domain that regulates kinase activity (Fig. 1-18). Although it first appeared that HGFs activated JAK proteins in a rather promiscuous manner, some patterns have emerged (Table 1-4). Thus receptors that are comprised of dimers such as the EPOR, G-CSFR, and TPOR associate with JAK2 (or to a lesser extent JAK1) in either a constitutive or ligand-dependent manner. Ligand binding and consequent clustering of receptor molecules leads to JAK2 aggregation and transphosphorylation at the lysine–glutamic acid–tyrosine–tyrosine (KEYY) site in the kinase activation loop. For example, the inactive EPOR exists as a homodimer each cytoplasmic domain of which is irreversibly bound to a molecule of JAK2. EPO binding to the extracellular domain of the receptor induces a conformational change that brings the JAK kinase domains together, resulting in rapid tyrosine transphosphorylation.[429] In vitro experiments show that

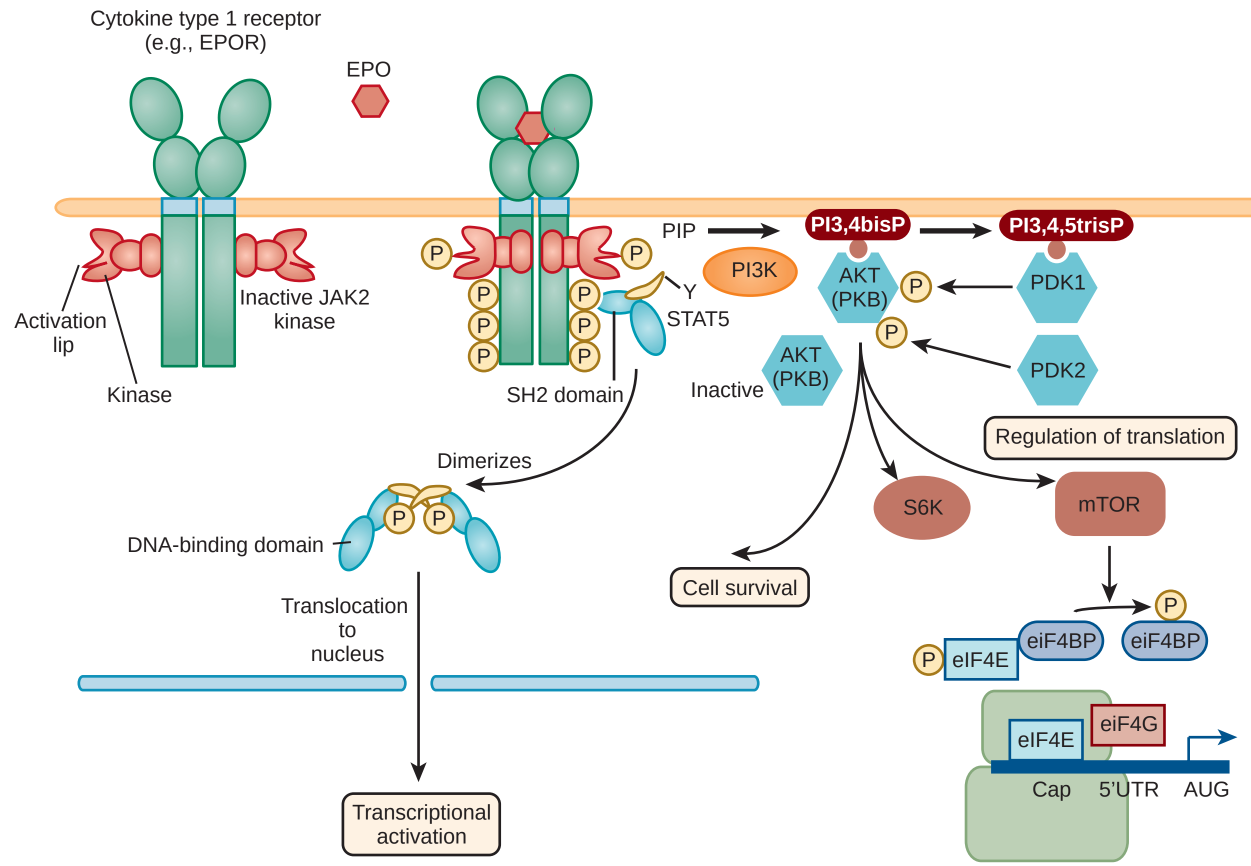

Cytokine type 1 receptor
(e.g., EPOR)
EPO
Activation lip
Kinase
Inactive JAK2 kinase
P
Y
STAT5
SH2 domain
PIP
PI3K
PI3,4bisP
PI3,4,5trisP
AKT (PKB)
PDK1
PDK2
Inactive
AKT (PKB)
Regulation of translation
Dimerizes
DNA-binding domain
S6K
mTOR
Cell survival
Translocation to nucleus
eIF4E
eiF4BP
eiF4G
Cap
5'UTR
AUG
Transcriptional activation

Figure 1-18 Signal transduction through a cytokine receptor such as the EPOR: JAK2 is tightly bound to the receptor. The JAKs contain a C-terminal tyrosine kinase domain and an N-terminal receptor-binding domain separated by a regulatory domain. The homodimeric receptor undergoes a conformational change after ligand binding that brings together associated JAK2 molecules, which then phosphorylate each other on a tyrosine in the activation lip, leading to tyrosine phosphorylation of the receptor. These tyrosines act as docking sites for STAT5 and other signal transduction proteins containing *src* homology 2 *(SH2)* and phosphotyrosine-binding (PTB) domains. The STAT proteins contain an SH2 domain that contains the completely conserved GTFLLRFSS sequence; mutation of the arginine residue eliminates function. The SH2 domain is critical for recruitment to the receptor and for STAT dimerization. The N-terminal DNA-binding domain is highly conserved, and after STAT dimerization binds similar symmetric dyad sequences (e.g., STAT5: TTCC[A→T]GGAA). DNA binding is totally dependent on phosphorylation of a critical tyrosine at the C-terminus. *PI3K,* Phosphatidylinositol 3′-kinase; *PI3,4bisP,* phosphatidylinositol 3,4-bisphosphate; *PI3,4,5trisP,* phosphatidylinositol 3,4,5-trisphosphate; *PIP,* phosphatidylinositol 4-phosphate; *PKB,* protein kinase B; *UTR,* untranslated region. *(For an excellent review see Cell signaling II: signaling pathways that control gene activity. In Lodish H, Berk A, Kaiser CA, et al, editors:* Molecular Cell Biology, *6th ed. WH Freeman and Company, 2007.)*

TABLE 1-4 JAK/STAT and Nonreceptor Protein Tyrosine Kinases Activated by Hematopoietic Growth Factor Receptors*

Receptor	JAK Family	JAK-Activated STATs	Other Nonreceptor Protein Tyrosine Kinases
KIT	JAK2[915]		PI3K[916-917]
IFNRα/β	JAK1,[918,452,919] Tyk2[920]	STAT1,[921] STAT2,[921] STAT3[450]	
IFNRγ	JAK1,[918,452,919] JAK2[918,452,919]	STAT1[922]	
IL-3Rα (→βc)	**JAK1,**[438] **JAK2**[438]	STAT5,[457,458] STAT6[923]	LYN,[440,441] FYN,[441] FES,[442] TEC[924]
GM-CSFRα (→βc)	JAK1,[439,443] **JAK2**[439,443]	STAT5[457,458]	**FES**[442]
IL-6Rα (→gp130)	**JAK1,**[453] **JAK2,**[453] **Tyk2**[453]	STAT1,[454,454,925] STAT3[453]	**HCK,**[926] **BTK,**[927] **TEC**[927]
IL-2Rα (→β, γc) IL-4, IL-7, IL-9, IL-15Rαs (→γc)	**JAK1,**[928,929] **JAK3**[928,929]	STAT3,[930] STAT5[930,931] STAT6 (IL-4[923])	**LCK,**[932] **FYN,**[441] LYN[441,933] **SYK**[934]
EPOR	**JAK2**[429]	STAT5[458,935,936]	**FES**[435]
GCSFR	**JAK1,**[937] **JAK2**[938-939]	STAT3[938]	**LYN,**[940] **SYK,**[940] REL[941]

From Taniguchi T: Cytokine signaling through nonreceptor protein tyrosine kinases. Science *268:251–255, 1995.*
*Nonreceptor tyrosine kinases that associate with receptors are indicated in bold, and references are in superscript.

JAK2 phosphorylation leads to activation of its kinase function, which, taken together with evidence of association of JAK2 with the EPOR, suggests that JAK2 may act as the "master" protein tyrosine kinase that mediates the biologic response to EPO. Support comes from observations that a kinase-negative JAK2 acts as a dominant-negative suppressor of the proliferative response to EPO,[430] and that the box1/box2 proximal cytoplasmic domain of the EPOR is the region that both binds JAK2 and is essential for receptor function.[429] Furthermore, an acquired JAK2V617F mutation, found in almost all patients with polycythemia vera and about half of those with essential thrombocytosis and myelofibrosis, confers constitutive tyrosine kinase activity upon JAK2; it transforms hematopoietic cells when it is coexpressed with homodimeric cytokine type 1 receptors, and is sufficient for the development of polycythemia vera in murine recipients of transplanted JAK2V617F stem cells.[431] A number of other substrates are phosphorylated in response to EPO,[432-434] including nonreceptor kinases such as the p85 subunit of PI-3 K, substrates such as PLC-γ1 and the adaptor molecule Shc, and the tyrosine phosphatases hematopoietic cell phosphatase (HCP or PTP-1C) and Syp (also known as *PTP-1D* or *SH-PTP2*) that down-regulate signal transduction; other kinases such as FES[435] and Vav are also activated.

The other heterodimeric receptors appear to associate with and activate several JAKs (see Table 1-4).[426] The beta common component of the IL-3/GM-CSF receptor is tyrosine phosphorylated after IL-3 or GM-CSF stimulation.[436,437] The tyrosine kinase that is responsible for ligand-induced receptor phosphorylation could be JAK2[438] or JAK1,[439] but the role of other recruited nonreceptor kinases such as LYN,[440] FYN,[441] and FES[442] has not been clearly defined. Truncation mutants of βc show that the proximal cytoplasmic domain (amino acids 449 to 517) can induce JAK2 tyrosine phosphorylation and kinase activation,[443] and this truncated receptor also retains the capacity to induce myc and pim-1 and to induce proliferation in serum-replete conditions but not serum-free conditions.[436,444-446] Both gp130 and leukemia inhibitory factor receptor (LIFR) can associate with and activate JAK1, JAK2, and TYK2, but the pattern of such JAK family activation is distinct in different cell types.[447]

Hematopoietic Growth Factor–Induced Activation of STAT Proteins. The paradigm of the response to IFN has provided another major insight into a subsequent step in JAK family signal transduction. The kinase activities of TYK2 and JAK1 that are activated after IFN-α binding lead to phosphorylation of STAT1α/STAT1β, and STAT2. STAT proteins are characterized by an amino-terminal DNA-binding domain, a conserved SH2 domain, and a critical tyrosine at the carboxyl-terminus. Tyrosine phosphorylated STAT1α/β and STAT2 form heterodimers through their SH2 domains, bind to a 48-kD protein, translocate to the nucleus, and activate gene expression by binding to an interferon-stimulated response element (ISRE).[448-450] STAT1α is also tyrosine phosphorylated (at Y^{701}) after IFN-γ binds to the IFN-γR, and this event is associated with tyrosine phosphorylation of JAK1 and JAK2.[451,452] STAT1α homodimerizes, translocates to the nucleus, and binds to the IFN-γ activated sequence (GAS), thereby transcriptionally activating genes that contain this sequence in their promoters.

The JAK proteins activated by HGFRs also lead to phosphorylation and DNA binding of STAT proteins; four additional members of the STAT family have been cloned: STAT3, STAT4, STAT5, and STAT6 (see Table 1-4). Thus ligand-induced activation of gp130, the signal-transducing component of the IL-6, LIF, IL-11, OSM, and CNTF family of receptors, leads to tyrosine phosphorylation of the JAK-Tyk family and activation of STAT3.[447,453,454] STAT4 expression is restricted to spermatogonia as well as thymic and myeloid cells. STAT4 mRNA levels appear to decline when 32Dcl1 cells differentiate in either G-CSF or EPO.[455] An ovine DNA-binding activity induced by prolactin called *mammary gland factor* was cloned and is named STAT5.[456] STAT5 is activated by EPO and growth hormone[457] as well as IL-3, GM-CSF, and IL-5.[458] This 92-kD protein exists as two highly related proteins, STAT5A and STAT5B (96% identical), which are encoded by different genes. STAT6 is most closely related to STAT5 and is induced by IL-4.[459]

Hematopoietic Growth Factor Signaling Through the RAS Pathway. RAS is a "turnstile" through which signals from many receptors are routed.[460] RAS guanosine triphosphate (GTP) associates with and activates RAF-1 and mitogen-activated protein kinase (MAPK).[461] RAS is a 21-kD membrane-associated protein that cycles between the active GTP-bound forms and the inactive guanosine diphosphate (GDP)-bound forms in response to extracellular signals from various HGFRs, including KIT, IL-3, and GM-CSF receptors, T-cell receptor, IL-2R, and EPO R.[462-465] Activation of RAS is mediated by guanine-nucleotide–releasing factors (GRFs) that catalyze the release of bound GDP and its exchange for GTP, while deactivation occurs by GTPase-activating proteins (GAPs) such as $p120^{GAP}$ and neurofibromin that accelerate the intrinsic activity of RAS, leading to hydrolysis of GTP to GDP. The mammalian prototype for GRF-mediated RAS activation is the epidermal growth factor receptor (EGFR) the cytoplasmic domain of which bears intrinsic tyrosine kinase activity. After ligand-mediated dimerization, the EGFR is activated through autophosphorylation or transphosphorylation of tyrosine 1068. An adaptor protein, Grb2, binds to this phosphotyrosine through its SH2 domain. This in turn leads to the formation of a ternary complex of EGFR, Grb2, and a GRF called *SOS,* which is analogous to the *Drosophila* son of sevenless (SOS) protein.[466] SOS contains a proline-rich region that recognizes two SH3 domains of Grb2, and the complex then activates membrane-bound RAS GDP to RAS GTP (Fig. 1-17). The protooncogene vav also has RAS GDP/GTP nucleotide exchange activity[467] and is activated through tyrosine phosphorylation after cross-linking of the T-cell antigen receptor–CD3 complex. With respect to the HGFRs, IL-2–mediated activation of the IL-2R leads to IL-2Rβ–chain tyrosine phosphorylation and association of the receptor with Shc, another adaptor protein that is characterized by both an SH2 domain and a second phosphotyrosine-binding (PTB) domain.[468,469] Shc itself becomes phosphorylated on tyrosine, and this in turn leads to recruitment of Grb2 and SOS.[470] The IL-3/GM-CSF–mediated signaling cascade involves phosphorylation of the beta subunit itself as well as Shc, with subsequent increased levels of RAS GTP and activation of RAF-1, MEK (also called *MAPK kinase*), and MAPK, followed by induction of FOS and JUN (see Fig. 1-17). This activity was mapped to the cytoplasmic domain of βc between amino acids 626 and 763, whereas a more proximal domain (amino acids 449 to 517) retains the capacity to induce myc and pim-1.[445] The PTB domain of Shc binds to tyrosine 577 of βc after JAK2-mediated phosphorylation of βc.[471] RAS activation is important for both the proliferative and survival (prevention of apoptosis) functions.[446,472] In the case of the EPOR, ligand-induced phosphorylation of Shc and the association of Shc with the EPOR has also been demonstrated; phosphorylated Shc associates with Grb2, and RAS and the MAP kinase pathway is activated.[473-477] In summary, these three examples show that RAS is activated by a number of HGFRs, and that the RAS-RAF-MEK-MAP kinase pathway is important for proliferation and survival of hematopoietic cells.

The RAS pathway may also be important in certain leukemias. Neurofibromin, encoded by the *NF-1* gene, is mutated in patients with autosomal dominant neurofibromatosis type 1 (NF-1). NF-1 shows sequence homology with yeast and mammalian GAP genes. Children with neurofibromatosis have an increased risk of juvenile myelomonocytic leukemia (JMML), monosomy 7 syndrome, and acute myeloblastic leukemia.[478,479] Importantly, leukemic cells from children with NF-1 and myelodysplastic syndrome show loss of the normal NF-1 allele, thus implicating NF-1 as a tumor suppressor gene.[479] NF-1^{GAP} activity is significantly lower in cell lysates prepared from NF-1–associated leukemias than in normal bone marrow or non–NF-1 leukemic lysates.[480] Bone marrow mononuclear cells from patients with JMML show an increase in CFU-GM in response to GM-CSF but not in response to IL-3. Interestingly, fetal liver cells from mice that are rendered null for the *NF-1* gene show similar hypersensitivity to GM-CSF, indicating that NF-1^{GAP} may play a crucial and specific role in the response of myeloid cells to GM-CSF.[480,481] NF-$1^{-/-}$ mice die in utero around day 13.5 to 14.5 from complex cardiac defects, but transplantation of fetal liver cells on day 11.5 to 13.5 into lethally irradiated recipients produces a myeloproliferative syndrome similar to that of the human disease.[481] BCR-ABL is a chimeric oncogenic protein that shows dysregulated tyrosine kinase activity and is implicated in the pathogenesis of Philadelphia chromosome–positive chronic myeloid leukemia. A phosphorylated tyrosine (Y177) in the BCR first exon binds to the SH2 domain of Grb2 and activates RAS.[482] Mutation of the Y177 BCR-ABL to phenylalanine abolishes Grb2 binding and abrogates both BCR-ABL–induced RAS activation and transformation of primary bone marrow cultures.[482] In summary, RAS dysregulation may contribute to the increased proliferation that characterizes these two chronic leukemias.

Phosphatases and Receptor Signaling. The C-terminal domain of the EPOR has a negative regulatory role.[483] It is likely that this effect is mediated by a phosphatase that dephosphorylates kinase-associated tyrosine(s) in this region, as has been shown for the IL-3R.[484] There is a fascinating report of a Finnish family with dominantly inherited benign erythrocytosis.[485] The proband, an Olympic cross-country skier, has a mutation in the EPOR that introduces a premature stop codon and generates a receptor lacking the C-terminal 70 amino acids. This mutation cosegregates with the disease phenotype in this large family. Recent data show that the nontransmembrane protein tyrosine phosphatase SH-PTP1 (also called SHP-1, HCP, and PTP1C) associates via its SH2 domain with tyrosine-phosphorylated EPOR.[486] Mutational analysis mapped the binding site most probably to Y429, and this tyrosine is deleted with the C-terminal truncation of the EPOR in the Finnish family with benign erythrocytosis. Factor-dependent cells that express a Y429,431F mutant EPOR show increased sensitivity to EPO, as do cultured erythroid progenitors from patients with benign erythrocytosis.[486] CFU-E from mice that lack or have impaired SH-PTP1 activity (motheaten or motheaten viable) show a similar increased sensitivity to EPO.[487,488] Therefore, EPO-induced activation and subsequent tyrosine phosphorylation of the EPOR leads to recruitment of SH-PTP1, which then plays a major role in terminating the EPO signal, possibly through dephosphorylation of JAK2[486] or other tyrosine kinases.

Another nonreceptor protein tyrosine phosphatase called SH-PTP2 or SHP2, encoded by the *PTPN11* gene, has 50% to 60% identity with SH-PTP1 in both SH2 and catalytic domains. Despite this similarity, SH-PTP2 appears to be a positive regulator of some growth factor pathways,[489] in particular RAS. Approximately 50% of patients with Noonan syndrome (NS), a developmental disorder characterized by short stature, dysmorphic features, and cardiac defects, have germline mutations in SH-PTP2, and 35% of patients with JMML but not NS have somatic mutations in this gene. Some patients with NS develop JMML, and it appears that the mutations in NS/JMML differ from those found in de novo nonsyndromic JMML,[490] and differ in the degree to which they activate the phosphatase.[491]

Other Inhibitors of Hematopoietic Growth Factor Signaling. Another family of negative regulators is made up of the suppressors of cytokine-signaling (SOCS) proteins. SOCS-1 was discovered by three groups, contains an SH2 domain, and has sequence and structural similarities to an immediate early gene called *CIS* (cytokine-inducible SH2–containing protein).[404,492-494] It is one of an eight-member gene family (SOCS-1 to SOCS-7 and CIS) that is characterized by a central SH2 domain and a C-terminal conserved motif called a *SOCS box*. Unlike the constitutively expressed phosphatases, SOCS expression is induced by many cytokines, including IL-3, EPO, TPO, IL-6, and LIF, and the proteins are expressed in hematopoietic and other tissues.[495] SOCS-1, expressed predominantly in the thymus, interacts directly with the phosphorylated activation loop of (activated) JAKs, and its N-terminus is thought to inhibit adenosine triphophate

(ATP) binding to the activation cleft.[404,492,496] Subsequently, the SOCS box is hypothesized to target the SOCS/signaling complex to the proteosome for degradation by recruiting components of the proteasome machinery, including elongins B and C and Cullin 2.[497,498] SOCS-3 has been shown to inhibit signaling by a different mechanism, which is binding directly to activated receptors and to JAKs without inhibiting JAK activity,[499] whereas CIS can bind to phosphorylated tyrosines on the EPO receptor and thereby inhibit STAT5 signaling.[500]

SOCS knockouts have provided insight into the important physiologic roles of some of these proteins. SOCS-$1^{-/-}$ mice die before weaning with fatty degeneration of the liver, reduced thymic size, lack of T and B cells, and monocytic infiltration of organs.[501,502] This phenotype is similar to mice with elevated IFN-γ, and indeed SOCS-$1^{-/-}$ mice have increased IFN-γ levels; rescue of the animals can be achieved with antibodies to IFN-γ or by crossing mice with IFN-$\gamma^{-/-}$ mice.[503,504] SOCS-$2^{-/-}$ mice are normal at birth but then become significantly larger than wild-type mice, with thickening of the dermis due to excess collagen deposition.[505] Therefore SOCS-2 appears to play an important role in postnatal growth, possibly through the growth hormone/insulin-like growth factor-1 axis. SOCS-$3^{-/-}$ mice are hematopoietically most interesting in that they die at embryonic day 12 to 14 because of massive erythrocytosis.[506] Progenitor cells appear to be hyperresponsive to IL-3 and EPO. Thus SOCS-3 plays a crucial role in inhibiting fetal erythropoiesis. Although mice in which *CIS* has been deleted show no abnormalities, overexpression of CIS induces a phenotype similar to that of *Stat5a*$^{-/-}$ or *Stat5b*$^{-/-}$ animals.[507-510]

BIOLOGY OF HEMATOPOIESIS

Stem Cells

The formed elements of the blood in vertebrates, including humans, continuously undergo replacement to maintain a constant number of red cells, white cells, and platelets. The number of cells of each type is maintained in a very narrow range in the physiologically normal adult, approximately 5,000 granulocytes, 5×10^6 red blood cells, and 150,000 to 300,000 platelets/μL of whole blood (see Appendix for normal values in infancy and childhood). In this section we examine the normal regulatory mechanisms that maintain a balanced production of new blood cells. These regulatory systems are far from completely understood, but present evidence strongly supports the following basic principles, schematically depicted in Figure 1-7:

1. A single pluripotent stem cell is capable of giving rise to many committed progenitor cells. These committed progenitors are destined to form differentiated recognizable precursors of the specific types of blood cells.
2. The pluripotent stem cell is capable of self-renewal. The committed progenitor cells are limited in proliferative potential and are not capable of indefinite self-renewal. In addition to their limited proliferative potential, committed progenitors also "die by differentiation"[511] and their numbers depend on influx from the pluripotent stem cell pool.
3. The proliferative potential and differentiation of stem cells and committed progenitors may be influenced by niche cells or factors derived from them. Thymus-derived lymphocytes appear to contribute to the induction of spleen colony formation in irradiated recipients.[512-515] The hematopoietic role of thymus-derived cells is further supported by experiments demonstrating the defective restorative capacity of bone marrow from congenitally athymic[516] or neonatally thymectomized[517] mice, or rigorously T-depleted human bone marrow.[518]
4. Committed progenitor cells are capable of response to humoral- or marrow stroma–derived regulators, some produced in reaction to the circulating levels of a particular differentiated cell type. In this response, they proliferate and differentiate to form the recognizable blood cells. Under this type of control, amplification of production occurs at the committed progenitor cell level. Most of the regulatory molecules are produced by hematopoietic accessory cells in close proximity to progenitor cells. As differentiation proceeds, sensitivity to the late-acting factor increases. This late-acting regulator may serve to protect the highly proliferative but numerically limited immature progenitor compartment from exhaustion or death by differentiation under these stress conditions.
5. Hematopoietic differentiation requires an appropriate microenvironment. In normal humans, this environment is confined to the bone marrow, whereas in the mouse the microenvironment includes both the spleen and the bone marrow. The existence of the Steel (Sl) strains of mutant mice[519,520] that exhibit a deficiency in the hematopoietic microenvironment (see below) suggests that the interactions between hematopoietic progenitors and the bone marrow microenvironment involve very specific molecular mechanisms.[521] A number of early hematopoietic cells, including the pluripotent stem cells and certain committed progenitor cells, have been demonstrated in the circulation of normal individuals and/or experimental animals.[75,522-524] The capacity of HSCs to negotiate nonhematopoietic tissues via the circulation is especially significant in relation to bone marrow transplantation, which is carried out by infusion of bone marrow or blood cells from the donor into the circulation of the recipient.[525] For example, blockade of hepatic asialoglycoprotein receptors enhances stem cell engraftment in murine spleen and marrow.[526] There is also convincing evidence that murine stem cells (CFU-S) and progenitors (CFU-GM) express "homing" or adhesive lectin receptors that bind to the mannose and/or galactose presented on stromal cells. For example, homing of transplanted mouse progenitors can be blocked by synthetic neoglycoproteins with these specificities, suggesting that stromal cells are likely to express these sugars.[527] SDF-1/chemokine (CXCR4) signaling, adhesion molecules, including VLA-4/VCAM-1 and CD44/hyaluronic acid interactions, are also important in the regulation of homing.[528]

If only 10% to 20% of stem cells are in cycle in the resting state, what is their role with regard to self-renewal versus differentiation, and what is the role of the noncycling stem cells? The hypothesis that a series of stem cells may contribute clones successively to maintain hematopoiesis throughout the life span of an individual was first advanced by Kay.[529] Support for this hypothesis comes from transplant studies of recipients of small numbers of bone marrow cells[530,531] and from experiments in which mixtures of fetal liver cells from different inbred mouse strains were introduced into W-mutant fetuses. W mutants exhibit a genetic deficiency of HSCs (as well as germ and follicle cells) (see below). Long-term monitoring showed clonal dominance followed by a decline of cells of particular genotypes.[531]

The use of retroviral-mediated gene transfer to mark HSCs at first lent support to this hypothesis. Analysis of the viral integration patterns at intervals after transplantation documented concurrent contributions of small numbers of stem cells to hematopoiesis, with changing patterns over time.[532,533] These conclusions are not supported by the work of Harrison,[534] who measured variances at successive intervals in recipients of marrow mixtures from two congenic donors. The high correlations observed in this study suggest that the transplanted stem cells continuously produce descendants. However, if 10 to 20 (or more) clones are contributing to hematopoiesis simultaneously, then even with clonal succession one might expect variances between two alloenzymes to be minimized at sequential samplings. Furthermore, a longer-term analysis of viral integration patterns in multiple-lineage progeny of stem cells transplanted into mice shows that integration patterns are unstable initially but then stabilize and maintain consistency over many months.[535] Although this consistency does not appear to support the clonal succession hypothesis, the contributory role of unmarked stem cells in these experiments cannot be assessed. Furthermore, it is not possible to determine whether the immediate progeny of the transplanted (and retrovirally marked) stem cells might not all still have self-renewal capacity and subsequently contribute to hematopoiesis successively. Such clones would carry the same integration marker. A problem with the transplant approach discussed above is that the preparation required for successful transplantation may perturb hematopoiesis and complicate interpretation of the results. A resolution of the problem came from studies in which a nontoxic dose of bromodeoxyuridine in drinking water was fed to mice.[536,537] Bromodeoxyuridine is incorporated into DNA by proliferating cells, and measurement of incorporation rates into primitive stem cell populations over time can be used as a monitor of proliferative history. Under steady-state conditions, approximately 5% of the LTR-HSCs were in cycle and another 20% in the G_1 phase, with approximately 8% entering the cell cycle per day. By 6 days, 1 month, and 6 months, 50%, 90%, and 99%, respectively, of the stem cell pool had incorporated the label.[537] Because the size of the stem cell pool remains constant, these data indicate that 50% of dividing LTR-HSCs must self-renew; this could be accomplished by asymmetric division or by symmetric division in which half the cells give rise to LTR-HSCs and the other half to differentiating cells. More recently, fluorescent marking studies and bar-coding strategies have suggested that significant numbers of clones participate in hematopoiesis.[538,539]

Transcription Factors and Stem Cells

Although the factors that control the "decisions" of stem cells to undergo either self-renewal or commitment to differentiate down one of the alternate lineage pathways are poorly understood, in recent years nuclear transcription factors have been characterized that play a role in stem or early progenitor cell proliferation and lineage commitment.[540,541] The Tal1, LMO2, and GATA family of transcription factors is important in this regard. In particular, tal-1/SCL, a basic helix-loop-helix (bHLH) transcription factor, is expressed in biphenotypic (lymphoid/myeloid) and T-cell leukemias[542,543] and in both early hematopoietic progenitors and more mature erythroid, mast, megakaryocyte, and endothelial cells.[544,545] Targeted disruption of the *Tal-1/Scl* gene leads to death in utero from absence of blood formation, and lack of in vitro myeloid colony formation suggests a role for this factor very early during hematopoiesis at the pluripotent or myeloid-erythroid stem cell level.[546,547] Another transcription factor implicated in T-cell ALL is the LIM domain–only nuclear protein (Lmo2).[548,549] Mice that lack this factor die in utero and have the same bloodless phenotype as the *Tal1*$^{-/-}$ animals.[550] Interestingly, although Lmo2 does not show sequence-specific DNA binding, immunoprecipitates in erythroid cells show that Lmo2 exists in a complex with Tal1,[551,552] suggesting a physiologic interaction in vivo. GATA2 is expressed in the regions of the *Xenopus* and zebrafish embryos that are fated to become hematopoietic, and is highly expressed in progenitor cells.[545,553-555] Overexpression of GATA2 in chicken erythroid progenitors leads to proliferation at the expense of differentiation.[556] Targeted disruption of the *Gata2* gene by homologous recombination in embryonic stem cells leads to reduced primitive hematopoiesis in the yolk sac and embryonic death by day 10 to 11.[557] Definitive hematopoiesis in liver and bone marrow is profoundly reduced, with loss of virtually all lineages, and in vitro differentiation data show a marked deficiency of SF-responsive definitive erythroid and mast cell colonies and reduced macrophage colonies, suggesting that GATA2 serves as a regulator of genes that control HGF responsiveness or proliferation of stem and/or early progenitor cells. Dominant mutations in *GATA2* have recently been described in the monoMAC syndrome, characterized by nontuberculous mycobacterial infections (frequently *Mycobacterium avium* complex [MAC] infections), fungal infections, disseminated human papillomavirus infection, and pulmonary alveolar proteinosis. Patients have a marked reduction of monocytes, NK cells, and B cells, and can develop myelodysplasia and leukemia.[558,559]

These data contrast with the later time of embryonic death (day 15) from anemia during the midfetal liver stage in mice with targeted disruption of *Myb*[560] and *Rb*[561-563] and severe forms of *W* and *Sl* mutations (see below).[564] Myb has been shown to be absolutely required for normal hematopoietic production during embryogenesis.[565] Embryonic erythropoiesis in *Myb* knockout mice is normal, but there is complete failure of erythropoiesis in fetal liver. Progenitors of other lineages, but not megakaryocytes, were also decreased, indicating that Myb is required for early definitive cellular expansion.[560] Furthermore, a knockdown allele of *Myb* shows that suboptimal levels of Myb favor macrophage and megakaryocyte differentiation whereas higher levels are particularly important for erythropoiesis and lymphopoiesis.[566] An ethylnitrosourea (ENU)-induced allele of *Myb* (M303V) affects the transactivation domain of the protein that interacts with the transcriptional coactivator p300; recessive mice ($Myb^{\mathrm{M303V/M303V}}$) demonstrate increased platelet production, anemia, absence of eosinophils, and defects in B-cell and T-cell lymphopoiesis.[567] These animals were found to have an increase in transplantable HSCs, indicating that Myb has a negative role in HSC self-renewal. Thus Myb appears to have a negative role in HSC and megakaryocyte progenitor proliferation and positive effects on erythropoiesis, eosinophil production, and lymphopoiesis.

Loss of function of the *runx1* (or *AML1*) gene, which encodes the alpha subunit of the heterodimeric core-binding factor (CBF), results in fetal death by day 12.5 because of failure of production of all definitive hematopoietic lineages.[568] CBF recognition sequences are present in the IL-3, GM-CSF, M-CSFR, and T-cell antigen receptor promoters. The *runx1* gene is frequently rearranged in acute myelogenous leukemia (AML) and childhood acute lymphoblastic leukemia (ALL), and is expressed in myeloid and lymphoid cells.[549] Runx1 has been shown to interact with a number of repressors, including GROUCHO domain factors. Runx1 is also a regulator of HSC production in the aorta-gonads-mesonephros region, as well as in adult stem cell homeostasis. A rescue of runx1 by a Tek (Tie2) promoter rescues hematopoiesis during development.

There are GATA1 PU.1 interactions regulating an erythroid/myeloid decision, and there are many other transcription factor interactions, such as CEBPa induction of PU.1 in the myeloid system. Such competition for transcription factors and the regulation of stem cell differentiation appears to be a recurring mechanism in the hematopoietic system. Manipulation of the process actually leads to altered hematopoiesis that can favor a single lineage. This involves a cell fate change rather than a proliferative event.

Homeobox (*HOX*) genes encode transcription factors involved in the establishment of body pattern and tissue identity during development.[569] The human genes occur in four clusters, HOX A to D, on chromosomes 7, 17, 12, and 2, respectively. *HOXA* and *HOXB* genes are expressed in CD34+ cells and downregulated as these cells mature. Intriguingly, murine bone marrow cells engineered to overexpress Hoxb4 by retrovirus-mediated gene transfer show a dramatic upregulation in stem cell activity both in vitro and in vivo, although it may not be required for normal hematopoiesis.[570,571] Serial transplantation studies showed a greatly enhanced ability of *Hoxb4*-transduced bone marrow cells to regenerate the most primitive HSC compartment, resulting in fiftyfold-higher numbers of transplantable totipotent HSCs in primary and secondary recipients, compared with serially passaged control cells. This in vivo expansion of *Hoxb4*-transduced HSCs was not accompanied by identifiable anomalies in the blood of these mice, suggesting that Hoxb4 is an important regulator of very early but not late hematopoietic cell proliferation. Recent data show that ectopic expression of Hoxb4 in yolk sac and embryonic stem cells confers long-term engraftment capacity in primary and secondary recipients.[572] Daley and colleagues[573] reported that nuclei from *Rag2* knockout ($\mathrm{Rag2}^{-/-}$) tail cells could be transferred into enucleated oocytes, and embryonic stem (ntES) cell lines could be derived from the resulting blastocysts. The Rag2 mutation was then corrected by homologous recombination. Insertion of Hoxb4 into the corrected embryonic stem cells allowed their expansion into transplantable HSCs. These cells were able to partially correct the defect in immunoglobulin production in $\mathrm{Rag2}^{-/-}$ recipients after bone marrow transplantation.[573] The HOX pathway has been expanded to include HOX A7 and A9 as major regulators of hematopoiesis and leukemia formation.[574] The posterior *Hox* genes therefore drive HSC production; this was highlighted by the finding that mutants of Cdx4, a controller of *HOX* gene expression, lack HSCs in the zebrafish and Cdx controls *Hox* gene expression for the formation of HSCs.[570]

The *Gfi1* locus was originally identified as an oncogene causing lymphoma based on retroviral insertion.[575] Gfi1 contains an internal SNAG domain and a six–zinc-finger DNA-binding domain. There are two nearly identical genes called *Gfi1* and *Gfi1b*. Gfi1 is restricted to the lymphoid system and neutrophils, whereas Gfi1b seems to be distributed throughout the entire hematopoietic system. *Gfi1* knockout animals have a severe reduction in lymphoid progenitors. This leads to an early lack in both CD4+ and CD8+ cells. In addition, IL-7 receptor signaling is suppressed. Gfi1 is a major regulator of neutrophil maturation. Normal neutrophils are absent in the knockout animals but eosinophils are present in normal numbers. The response of neutrophils to G-CSF is preserved, but differentiation does not occur. There is a significant interaction of Gfi1 with other transcription factors, including PU.1, Cebpβ, and Cebpε, which regulate neutrophil maturation.

Gfi1 also has been shown to be involved in HSC self-renewal. *Gfi1* knockout animals actually increase and outcompete other cells. This leads to stem cell exhaustion. Gfi1b may also regulate stem cell homeostasis. The HSCs appear to be more active in terms of their cell cycle and have increased proliferation rates. It is clear that p21 is likely one of the mediators of Gfi1 in HSC production.[576] There is also modulation of the STAT3 activity as a consequence of the interaction of Gfi1 with PIAS-3, a factor that simulates transcription factors. This is interesting because other genes such as *Tal1*, *Runx1*, *Notch 1*, *Notch 2*, *RBPJ*, integrin-1, *Hoxb4*, and β-catenin (*CTNNB*) are dispensable for most HSC activities, whereas p21 (*CDKN1A*), Bmi 1, tel1, Cebp, PU.1, and Gfi are actually required.

Growth Factors and Stem Cells

HGFs also appear to influence at least some classes of stem cells. Analysis of the actions of HGFs on "purified" murine or human stem and progenitor cells has led to several major conclusions. These conclusions should be interpreted in light of the fact that, to date, even the most highly purified "stem cell" fractions are still heterogeneous with respect to content of LTR-HSC, HSC with short-term reconstituting potential, and lineage-committed progenitors. HGFs such as SCF and perhaps IL-3, GM-CSF, and G-CSF can independently support the survival of murine and/or human stem cell populations.[297,577-579] Of all of these HGFs, however, only SCF has been shown to enhance the survival of murine LTR-HSCs, and no single HGF or combination of HGFs has unequivocally shown the capacity to induce significant self-renewal of LTR-HSCs.[580,581] Mice deficient in IL-6 show a reduction in CFU-S and IL-$6^{-/-}$ bone marrow cells fail to contribute to long-term hematopoiesis after serial transfer.[329] In addition to its effect on megakaryocyte and platelet formation, TPO is also vital for stem cell production. Mice that lack the receptor for TPO, Mpl, show reduced numbers of blast colonies and CFU-S, and fail to compete effectively with normal stem cells for reconstitution of hematopoiesis in irradiated recipients, even when transplanted in tenfold excess.[582] Furthermore, both murine and human repopulating activity segregates with primitive cells that express MPL.[583] In the human system, information on HSC self-renewal is not available, because no human assay measures this property.

Analysis of mutations that affect the function of stem cells and the hematopoietic microenvironment through HGF interactions has provided important insights. Two murine mutations have been characterized in some detail, the Steel (Sl) and dominant white spotting (W) mutations.[591,520,564] Genetically affected mice that are defective at these loci have a severe macrocytic anemia associated with increased radiation sensitivity, lack of pigmentation, poor gastrointestinal motility, and sterility. Although phenotypically similar, these mutations map to different chromosomes (W to chromosome 5, Sl to chromosome 10) and have distinct hematologic characteristics. The W mutation, which is best characterized at the molecular level, results in a functional deficiency of HSCs and, particularly, erythroid progenitors. Transplantation of normal bone marrow into homozygous W recipients completely corrects the hematopoietic abnormalities, whereas transplantation of W bone marrow into normal mice fails to reconstitute hematopoiesis.[591,584] The W gene is allelic with the Kit protooncogene,[585,586] which, as discussed above, is known to be a member of a tyrosine kinase receptor family.[587] Several variants of the W mutation have been shown to be phenotypic expressions of specific mutations of the *Kit* gene, and the severity of the phenotypic changes associated with specific alleles correlates with the degree of functional impairment of kinase activity.[588]

In contrast, the Steel mutation affects the function of the cells of the microenvironment. Sl/Sl^d bone marrow cells are capable of reconstituting hematopoiesis in irradiated normal mice, whereas transplantation of normal bone marrow into Sl/Sl^d animals fails to cure the defect.[589] A deeper insight into the molecular nature of this defect was provided by the purification of a factor that is active on mast cells and on mouse bone marrow that has been exposed to 5-FU (enriched for primitive stem cells but depleted of mature progenitors).[590,591] Labeling studies with the purified recombinant protein demonstrate that it is the ligand for Kit.[592-595] Furthermore, administration of the factor to Sl/Sl^d mice corrects their macrocytic anemia and repairs their mast cell deficiency. Less severe mutations at the Sl locus, such as Sl^d, are associated with smaller deletions at the Sl locus. The gene encodes a protein that exists in both transmembrane and secreted forms.[592,596,597] Recent studies have shown that SCF is expressed by HSCs and perivascular stromal cells in the marrow niche, but expression by endothelial cells and leptin receptor perivascular cells is critical for HSC survival.[37]

Recently, other growth factor pathways that are important during embryogenesis and have been subverted in certain cancers have also been implicated in stem cell self-renewal. Notch receptors (four members, Notch 1 to 4) are expressed on the surface of a variety of hematopoietic cells as proteolytically cleaved dimers comprising an EGF-repeat–rich extracellular domain and a transmembrane/intracellular domain that contains two motifs (RAM and ANK) that can bind to a transcriptional repressor CBF1/RBPJκ (see Kojika and Griffin[598] for a detailed review). After ligand binding (three ligands, Delta, Jagged1, and Jagged2), the transmembrane/intracellular domain is cleaved to release the active intracellular domain of Notch (ICN), which translocates to the nucleus, binds to CBF1/RPBJκ, and transactivates genes such as Hairy and Enhancer of Split (HES)-1, a bHLH protein that suppresses transcription of N-box (CACNAG) target genes. Human Notch 1 was first identified as a gene called *TAN-1* that is involved in some T-cell leukemias, in which it generates an intracellular activated form of Notch 1 (ICN-1).[599] Remarkably, overexpression of ICN-1 in murine stem cells leads to immortalization of cytokine-dependent cell lines that can generate lymphoid or myeloid progeny in vitro and in vivo, suggesting a role for Notch 1 in stem cell self-renewal.[600] Activating mutations in Notch have been found in many T-cell leukemias.[601] Addition of soluble Jagged1 to cultures of human purified stem and progenitor cells also increased NOD/SCID repopulating activity.[602] A clinical trial was recently completed using soluble Delta as a means of expanding hematopoiesis in vitro. Cord blood was treated with the soluble Delta, and the expanded progenitors were then infused into the patient. There was no effect on stem cells. This treatment enhanced neutrophil recovery following preparative chemotherapy and irradiation.[603]

A second signaling pathway that may be involved in stem cell self-renewal involves the Sonic hedgehog[604] protein. Shh activates the Smoothened receptor (Smo) by binding to Patched, its inhibitor. Smo initiates signaling that regulates transcription factors of the Gli family, which modify expression of genes that encode BMP-4 and its inhibitor Noggin. Shh has recently been shown to induce expansion of human NOD/SCID repopulating cells, possibly through regulation of BMP-4. Last, Wnt signaling may also be involved in stem cell self-renewal. Wnt signals through the receptors Frizzled and lipoprotein receptor–related protein (LRP) to activate a cytoplasmic protein called *Dishevilled,* which in turn stabilizes β-catenin, a transcriptional coactivator that associates with the Tcf/LEF family of transcription factors.[605] Without Wnt signaling, β-catenin is normally destabilized by a cytoplasmic complex that comprises Axin, adenomatous polyposis coli, and glycogen synthase kinase 3β (GSK-3β). Overexpression of activated β-catenin in highly purified murine HSCs appears able to expand the pool of transplantable cells.[606] Controversy still exists about the role of wnt signaling during homeostasis, but it seems to have a role during stress. Mammalian target of rapamycin (mTOR) may cooperate with wnt at homeostasis.[607] The wnt pathway established different responses based on level of activation.[608]

Two models have been proposed to explain the mechanisms that influence the choice of stem cells between self-renewal and commitment. A *stochastic model* was proposed by Till, McCullough, and Siminovitch.[511] In this model, self-renewal or differentiation is considered to occur in a random or stochastic manner, dictated only by a certain probability. Because no extrinsic source of stem cells exists, under steady-state conditions the overall probability of stem cell division resulting in self-renewal must be 0.5; probabilities less or greater than this value would lead to progressive stem cell depletion (aplasia) or expansion (leukemia), respectively. The commitment of stem cells could occur symmetrically, where overall, half the stem cells divide to produce progeny both of which enter a differentiation pathway, and the other half of the stem cells generate progeny that are both stem cells. Alternatively, asymmetric division would give rise to one stem cell and one cell committed to differentiate. Analysis of single cells from blast cell colonies has shown that hematopoietic cells can divide asymmetrically; these micromanipulated single cells give rise to colonies that comprise almost all possible lineage combinations.[111] Single-cell analysis of purified human CD34+CD45RA^{lo}CD71^{lo} provides evidence for quiescent survival, self-renewal, and asymmetric division.[609] These data provide support for a stochastic mechanism to explain both commitment and restriction of differentiation potential. The best purification strategies still allow only one of two cells to be an HSC, and paired daughter analysis has some caveats with regard to lineage tracing. During the regeneration of hematopoietic tissues after injury by irradiation or cytotoxic drugs, the probability of generating stem cells must increase. How this probability is altered is one of the outstanding unanswered questions in hematopoiesis.

The osteoblastic niche in bone marrow plays a role in stem cell self-renewal, whereas the sinusoidal endothelial niche regulates proliferation, differentiation, and mobilization. These functional differences suggest a mechanism for the external control of stem cell expression. This observation and others related to the morphology of the focal development of hematopoietic islands attached to adherent cells have led to postulation that HSCs reside within stromal cell "niches" that play a vital role in supporting stem cell survival, perhaps by intimate cell contact (see Fig. 1-5).[15] Interestingly, a recent study showed that deletion of Dicer1, an RNAseIII endonuclease important for microRNA biogenesis, in osteoprogenitors led to myelodysplasia (MDS) and acute myeloid leukemia (AML). Most intriguing, the dysplastic or leukemic hematopoietic cells retained Dicer1 expression. Subsequent gene expression studies showed reduced expression of the *SBDS* gene, which is mutated in 90% of patients with Shwachman-Diamond syndrome, who also have an increased risk of development of MDS and AML. Thus this study suggests, surprisingly, that stromal cell dysfunction may have an important role in secondary oncogenesis.[610]

Whether a stochastic mechanism or one influenced by environmental cues dictates the outcome of the stem cell decision to commit to one or the other hematopoietic lineage is also uncertain. Recent experiments suggest that cross-antagonism among lineage-specific transcription factors may provide a mechanistic explanation for lineage specification. Forced expression of the erythroid-specific factor GATA1 in myelomonocytic cells perturbs their ability to differentiate,[611] and evidence is accumulating that this is due to direct physical interaction and cross-antagonism of PU.1, the myeloid transcription factor, and GATA1; GATA1 inhibits PU.1 by preventing it from interacting with its cofactor JUN, whereas PU.1 prevents GATA1 from binding to DNA.[612-615] In Down syndrome patients, there are mutations in GATA1, which lead to a truncated protein missing its N-terminus.[616] This appears to give competitive advantage to a yolk sac/fetal liver progenitor in the genesis of leukemia and transient myeloproliferative disorder. Another example is the antagonism between FOG1 and C/EBP-β in an eosinophilic cell line, in that enforced expression of FOG1 blocks C/EBP-β mediated eosinophil differentiation.[617] It is possible that lineage-specific transcription factors not only have potent inductive effects but also exert blocking effects on alternate lineage choices. The observation that forced expression of C/EBP-β in pancreatic cells promoted their differentiation into a hepatic phenotype raises the intriguing possibility that this mechanism underlies stem cell plasticity as well[618] (reviewed by Cantor and Orkin[541]).

Erythropoiesis

It is well established that the level of oxyhemoglobin and the rate of delivery of oxygen to the tissues is the fundamental stimulus of erythropoiesis.[5] In species that package hemoglobin in erythrocytes, erythropoietin mediates the response to oxygen demand and does so by interacting with specific receptors[619] that are found on the surface of committed erythroid progenitor cells[620-622] and erythroblasts.[623] Insight into the mechanism by which hypoxia induces a transcriptional increase in the expression of the *Epo* gene has come from the identification of the hypoxia-inducible transcription factor HIF-1α.[387,624]

It is believed that BFU-E do not directly give rise to erythroblasts in vivo except under conditions of extreme anemic stress. Instead, they mature to single erythroid colony-forming units, or CFU-E, which divide in vivo. Under the influence of lower concentrations of erythropoietin these CFU-E form single, relatively small colonies of proerythroblasts at about 1 week in vitro. Many (at least half) of CFU-E will form erythroid colonies in vitro in response to erythropoietin alone. They do not require the additional presence of either GM-CSF or IL-3, having differentiated beyond this requirement.[625] All proerythroblasts can differentiate in the presence of erythropoietin alone. In the steady state, the number of human CFU-E exceeds the number of BFU-E by a factor of 10. CFU-E are larger than BFU-E, and because a higher fraction of CFU-E are in the S phase of DNA synthesis than are BFU-E, the former exhibit a higher rate of "suicide" in response to exposure to tritiated thymidine. The membranes of mature BFU-E and CFU-E are CD34+ and HLA-DR+, and various negative and positive selection methods may be used to purify them from sources such as bone marrow and human fetal liver.[125,626] They have the appearance of lymphoblasts.

The bone marrow of an adult mouse contains about 500 CFU-E per 10^5 nucleated cells. In response to anemic stress, as in hemorrhage or hemolysis, nearly the entire burden of accelerated reticulocyte production is borne by the rapid erythropoietin-dependent influx into the proerythroblast pool from the progenitor compartment.[124,135] This produces an expanded proerythroblast pool. Little or no increase in the mitotic rate of recognizable erythroid precursors occurs.[124] Instead, the late BFU-E and CFU-E proliferate in response to the engagement of their erythropoietin receptors by the hormone and, as well, differentiate to proerythroblasts and beyond. In normal murine marrow, following experimental hemorrhage or hemolysis, the CFU-E frequency of about 500 per 10^5 nucleated cells increases to 1000 to 2000 CFU-E per 10^5 nucleated cells. In contrast, hypertransfused mice exhibit a reduced number of CFU-E per 10^5 nucleated marrow cells (values between 10% and 20% of normal have been reported).

During stress erythropoiesis induced by anemia, the orderly progression from immature BFU-E through CFU-E to proerythroblast is interrupted as high erythropoietin levels appear to permit or induce differentiation of immature progenitors to proerythroblasts. In the rhesus monkey this premature terminal differentiation may account for the marked increase in fetal hemoglobin content and F cells seen in simian stress erythropoiesis.[627] The ability of human progenitors to generate erythroblasts capable of synthesizing large quantities of fetal hemoglobin is less pronounced.[628] Thus the accumulation of F cells in peripheral blood in response to anemia is relatively small.[629] Such red cells are usually macrocytic and carry the i antigen as well, but these two characteristics may relate to a short transit time through the marrow in response to erythropoietin rather than to an intrinsic characteristic of fetal hemoglobin-containing cells themselves (see below). F cells are also present in very small numbers in the blood of normal individuals, but these cells are not macrocytic and do not bear i antigen. F cell progenitors can be detected easily in the bone marrow of patients with various hemoglobinopathies[630,631] and even in normal marrow.[632]

Transcription Factors and Erythropoiesis

As discussed above, targeted disruption of the *Tal1*, *Lmo2*, and *Gata2* genes leads to embryonic lethality because of failure of blood production, probably at the level of the HSC. GATA1 expression is limited to multipotent progenitors and erythroid, megakaryocyte, mast, and eosinophil lineages.[120,633,634] Analysis of chimeric mice injected at the blastocyst stage with $Gata1^{-/-}$ embryonic stem cells shows a failure of a contribution of the $Gata1^{-/-}$ cells to erythrocytes but development into other hematopoietic lineages, as well as other tissues.[635,636] Differentiation assays in vitro show proerythroblast arrest and apoptosis of these cells.[637-639] A transcriptional cofactor of GATA1, Friend of GATA1 (FOG1), was discovered and found to be essential for erythropoiesis and megakaryopoiesis.[640,641] Evidence that this interaction is essential in vivo comes from studies of a family with severe dyserythropoietic anemia and thrombocytopenia. Affected family members have a mutation in GATA1 that disrupts its interaction with FOG1 without affecting DNA-binding affinity.[642] The EKLF transcription factor was found as an activity bound to the CACCC site of the β-globin promoter.[643] It controls globin expression and some aspects of erythroid development. The NF-E2 protein complex binds to the locus control region (LCR) to regulate globin gene transcription; it is a dimer of a tissue-restricted (erythroid/megakaryocyte/mast cell) cnc domain subunit of 45 kD and a smaller, widely expressed subunit of 18 kD, and is essential for platelet, but not erythrocyte, production.[644,645]

Growth Factors and Erythropoiesis

The regulation of the proliferation and maturation of erythroid progenitors depends on interaction with a number of growth factors. The availability of pure recombinant growth factors, the enrichment of target progenitor cells, and the use of defined "serum-free" culture conditions have provided insights into the role of these factors during hematopoiesis.

Erythropoietin (EPO) is essential for the terminal maturation of erythroid cells. Its major effect appears to be at the level of the CFU-E during adult erythropoiesis, and recombinant preparations[625] are as effective as the natural hormone.[646,647] These progenitors do not require "burst-promoting activity"[648,649] in the form of IL-3 or GM-CSF, and their dependence on EPO is emphasized by the observation that they will not survive in vitro in the absence of EPO. Because most CFU-E are in cycle, their survival in the presence of EPO is probably tightly linked to their proliferation and differentiation to mature erythrocytes. EPO also acts on a subset of presumptive mature BFU-E, which also require EPO for survival and terminal maturation. A second subset of BFU-E, presumably less mature, survive EPO deprivation if IL-3 or GM-CSF (IL-3 more than GM-CSF) is present. When EPO is added to these cultures on day 3 these BFU-E form typical colonies. Under serum-deprived culture conditions the combination of IL-3 and GM-CSF results in more BFU-E than either factor alone when EPO is added on day 3.[650] Although EPO is crucial for the terminal differentiation of erythroid progenitors, mice with homozygous null mutations of the *Epo* or *Epor* genes form BFU-E and CFU-E normally, but they fail to differentiate into mature erythrocytes.[327] Both the $Epo^{-/-}$ and $Epor^{-/-}$ mutations are embryonically lethal due to failure of definitive (fetal liver) erythropoiesis. However, yolk sac erythropoiesis is only partially impaired, indicating that a population of EPO-independent primary erythropoietic precursors exists. SCF has marked synergistic effects on BFU-E cultured in the presence of EPO,[152] although alone it has no colony-forming ability. SCF is crucial for the normal development of CFU-E, because mice that lack SCF (Steel mutants) or its receptor Kit (W mutants) are severely anemic and show a reduction in fetal liver CFU-E.[651] Further studies showed that SCF induces tyrosine phosphorylation of the EPOR and that Kit associates with the cytoplasmic domain of the tyrosine phosphorylated EPOR. Further insight into the molecular mechanism by which Epo prevents apoptosis in erythroid cells comes from studies of $Stat5a^{-/-}5b^{-/-}$ mice. During fetal development the embryos are severely anemic; the erythroid progenitors in fetal liver are reduced in number and show increased apoptosis.[652] This result was explained by the finding that STAT5 mediates the early induction of the antiapoptotic gene $Bcl\text{-}X_L$ through direct binding to its promoter. Interestingly, although adult animals were thought to be hematologically normal, about half have chronic anemia with splenomegaly, due to an increase in early erythroid precursors; erythropoiesis is ineffective because of an increase in apoptosis in these early normoblasts that show reduced levels of Bcl-X_L.[653]

Factors distinct from the classic CSFs may positively regulate erythropoiesis, either directly or indirectly. Limiting-dilution studies of highly purified CFU-E in serum-free culture show that insulin and insulin-like growth factor 1 (IGF-1) act directly on these cells.[654] The presence of Epo is essential in these studies, which contrast with earlier murine studies of unfractionated cells in which CFU-E respond to IGF-1 or insulin in the absence of Epo.[655] Activin is another factor that enhances both BFU-E and CFU-E colony formation. This protein dimer, also known as *follicle-stimulating hormone–releasing protein,* appears to have a lineage-specific effect on erythropoiesis that is indirect because removal of monocytes and/or T lymphocytes abrogates its effect.[656] It is interesting that activin has been identified as the factor produced by vegetal cells during blastogenesis that induces animal ectodermal cells to form primary mesoderm.[657] Hepatocyte growth factor has also been shown to have synergistic effects on CFUs-GEMM and BFU-E in cultures containing EPO.[658]

Negative Regulation of Erythropoiesis

Observations that subsets of lymphocytes with an immunologic suppressor phenotype isolated from normal subjects can inhibit erythroid activity in vitro[659-661] correlate with reports of patients with a variety of disorders in whom anemia or granulocytopenia is associated with an expansion of certain T-lymphocyte populations (see also Chapter 7).[662-664] In the rare disorder T lymphocytosis with cytopenia,[665] in vitro suppression of erythropoiesis has been correlated with the expansion of a T-lymphocyte population that may be the counterpart of the hematopoietic suppressor cells isolated from normal blood. The phenotype of these cells has been described in detail.[665,666] The cell is a large granular lymphocyte that is positive for both E rosettes and CD8 (classic suppressor phenotype). Suppressor T cells may also be involved in some cases of aplastic anemia[667,668] or neutropenia[669] without an underlying immunologic disorder or an overt T-cell proliferation. Exactly how such suppressor T cells interact with hematopoietic progenitors and what surface antigens are "seen" by the suppressors is uncertain. There is evidence to support the concept that suppression of erythroid colony expression in vitro is regulated by T cells and may be genetically restricted.[666,670] Cell-cell interactions in immunologic systems have been well characterized with regard to surface determinants that allow for cellular recognition. That certain phenotypes of T cells "recognize" distinct classes of histocompatibility antigens on immunologic cell surfaces has been well described.[671] Thus the observation that hematopoietic progenitors have a unique distribution of class II hiscompatibility antigens on their cell surfaces[672-675] suggests a role for these antigens in the cell-cell interactions that regulate hematopoietic differentiation.

TNF also suppresses erythropoiesis in vitro.[676,677] The injection of peritoneal macrophages into Friend murine leukemia virus–infected animals results in rapid but transient resolution of the massive erythroid hyperplasia associated with this disease. This may be due to elaboration by macrophages of IL-1α, which does not suppress erythropoiesis itself but acts by the induction of TNF. This effect is reversed by Epo.[678]

Myelopoiesis

Phagocyte Development

Although a clonogenic assay of granulocyte progenitors was developed almost a decade before the erythroid clonogenic assay, the various factors responsible for granulocyte-macrophage development remain incompletely understood. Excellent reviews of early work in this field have been provided by Metcalf[679] and Moore.[117]

It is now accepted that the granulocyte-macrophage colony-forming unit, or CFU-GM, is derived from the pluripotent progenitor and, under the influence of SCF, IL-3, GM-CSF, G-CSF, and/or M-CSF, ultimately gives rise to mature granulocytes and monocytes. Monocytes irreversibly leave the circulation[680,681] and differentiate further into fixed-tissue macrophages, a category comprising the alveolar macrophages,[682] hepatic Kupffer cells,[683] dermal Langerhans cells,[684] osteoclasts,[685] peritoneal and pleural macrophages, and, perhaps, brain microglial cells.[686] The enormous diversity of this system and the high turnover rate of granulocytes, as well as the necessity of maintaining splenic, marginated, and bone marrow granulocyte pools in order to meet sudden demands caused by infection, have led to the evolution of an extremely complex regulatory network.

Granulopoiesis associated with myelosuppression, cyclic neutropenia, and infection depends on IL-23 production by macrophages and dendritic cells that in turn stimulates IL-17 and G-CSF upregulation, a pathway revealed by study of leukocyte adhesion molecule–deficient mice.[687] Recent data suggest that the innate immune mechanisms that involve Toll-like receptor 4 (TLR4) and its adaptor protein TRIF may be important in steady-state and emergency granulopoiesis.[688]

Transcription Factors and Myelopoiesis

A family of transcription factors important for commitment to myeloid and lymphoid lineages as well as regulation of myeloid-specific promoters such as those of the myeloperoxidase (MPO) and neutrophil elastase (NE) genes are factors that were first identified on the basis of their ability to bind to the polyomavirus enhancer–binding protein 2/core binding factor (PEBP2/CBF). Two alpha subunits (CBFα1 and CBFα2 [the human homologue is known as *AML-1* or *RUNX1*]) bind DNA with low affinity, and affinity is strengthened in the presence of the non–DNA-binding beta subunit (CBFβ). As noted above, runx1$^{-/-}$ mice die in utero by day 12.5 because of failure of production of all definitive hematopoietic lineages.[568] The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors bind to DNA through a basic region–leucine zipper domain (bZIP). There are several family members (C/EBPα, C/EBPβ (NF–IL-6), C/EBPγ, C/EBPδ, C/EBPζ, and C/EBPε) that are differentially expressed during myelopoiesis, with increasing (C/EBPβ), or an increase followed by either a partial (C/EBPδ) or a marked (C/EBPα) reduction in level of, expression during maturation of 32D cl3 cells to terminally differentiated granulocytes.[689] C/EBPα has been implicated in the regulation of hepatocyte and adipocyte differentiation (levels are low in undifferentiated dividing cells but high in quiescent terminally differentiated cells), and also in myeloid CSF receptor promoter function; RUNX1 can act synergistically with C/EBPα to activate the M-CSFR promoter.[690] *C/ebpα*$^{-/-}$ mice show arrested myelopoiesis at the myeloblast stage resulting from failure of expression of G-CSF and IL-6 receptors.[691,692] Mice that lack C/EBPβ produce monocytes that are defective in bactericidal and tumoricidal function; their macrophages and fibroblasts, but interestingly not their endothelial cells, fail to produce G-CSF in response to LPS.[693] Thus C/EBPβ is important for "emergency" granulopoiesis.[694] C/EBPε is produced primarily in myeloid lineages. Knockout mice are viable and fertile but die at 50 to 75 days from opportunistic infections with *Pseudomonas aeruginosa*.[695] Nullizygous animals fail to produce normal neutrophils and eosinophils. Functional abnormalities include diminished release of hydrogen peroxide in response to phorbol myristate acetate (PMA) stimulation, because of failure to produce secondary and tertiary granules, which are important reservoirs of membrane-bound components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase apparatus. Additional components of secondary and tertiary granules, lactoferrin and gelatinase B, are absent because of failure of transcriptional activation of C/EBPε.[695,696] Interestingly, similar abnormalities characterize the rare neutrophil-specific granule deficiency,[697] and indeed mutations in *C/EBPε* have recently been found in these patients.[698-700] The PU.1(Spi-1) transcription factor is a member of the Ets family and is expressed principally in monocytes/macrophages and B lymphocytes, and also in erythroid cells and granulocytes.[701,702] Potential target genes include the integrin CD11b, M-CSFR, GM-CSFRα, G-CSFR, and the Igλ light chain.[703-707] Mice that lack PU.1 die in utero with absence of monocytes, granulocytes, and T and B lymphocytes; anemia is variable and thus does not explain the prenatal mortality.[708]

Growth Factors and Myelopoiesis

Murine IL-3 stimulates a broad spectrum of myeloid progenitor cells, including pluripotent stem cells, granulocyte and/or macrophage colony-forming cells or units (CFU-GM, CFU-G, and CFU-M), erythroid burst-forming units (BFU-E), eosinophil colony-forming units (CFU-Eo), megakaryocyte colony-forming units (CFUs-Meg), and mast cells. As its name implies, GM-CSF was initially shown to be more restricted as a stimulus of the proliferation and development of CFU-GM. However, murine studies with purified or recombinant factor have shown that it stimulates the initial proliferation of other progenitors such as BFU-E as well (see above).[209,709] The other murine factors, G-CSF and M-CSF, are more restricted and predominantly stimulate granulocyte and monocyte colony-forming units (CFU-G and CFU-M), respectively.[710,711]

With the possible exception of GM-CSF, the activities of the human CSFs are similar to those of the corresponding murine factors. Both IL-3 and GM-CSF affect a similar broad spectrum of human progenitor cells. This includes CFU-GEMM, CFU-GM, CFU-G, CFU-M, CFU-Eo, and CFU-Meg. In full-serum cultures, IL-3 and GM-CSF alone stimulate the formation of colonies derived from CFU-GM, CFU-G, CFU-M, CFU-Eo, and CFU-Meg. Data from serum-free cultures suggest that in the presence of IL-3 or GM-CSF alone, myeloid colony formation is much reduced, and that optimal CFU-G or CFU-M proliferation requires the addition of G-CSF or M-CSF, respectively, to the cultures.[213,650] Even in serum-replete conditions, IL-3 acts additively or synergistically with G-CSF to induce more granulocyte colony formation than is observed with either factor alone.[212]

Insight into the in vivo role of GM-CSF comes from studies in which the GM-CSF gene was disrupted by homologous recombination in embryonic stem cells.[341] GM-CSF knockout mice showed normal basal hematopoiesis but developed progressive accumulation of surfactant lipids and proteins in the alveolar space, the defining characteristic of idiopathic human pulmonary alveolar proteinosis. Extensive lymphoid hyperplasia associated with lung airways and blood vessels was also found. Surfactant proteins and lipids are synthesized by type II pneumocytes and cleared from the alveolar space by type II cells and by alveolar macrophages. The lungs from the null animals showed normal surfactant synthetic capacity and no accumulation in type II pneumocytes. In contrast, the alveolar macrophages showed a marked increase of surfactant protein and lipid, suggesting strongly that these cells cannot process surfactant as a result of the lack of GM-CSF. Mice that were generated with null mutations of the common chain of the GM-CSF/IL-3/IL-5 receptor (βc) show similar pulmonary pathology, and also show low basal numbers of eosinophils and absence of blood and lung eosinophilia in response to infection with the parasite *Nippostrongylus brasiliensis*.[341a] The G-CSF gene has also been disrupted by homologous recombination in embryonic stem cells.[317] G-CSF$^{-/-}$ mice have a chronic neutropenia (20% to 30% of normal levels) and reduced bone marrow myeloid precursors and progenitors. The animals also had a markedly impaired capacity to increase neutrophil and monocyte counts after infection with *Listeria monocytogenes*.

In addition to their effects on progenitor differentiation, the CSFs also induce a variety of functional changes in mature cells. GM-CSF inhibits polymorphonuclear neutrophil migration under agarose,[713] induces antibody-dependent cytotoxicity (ADCC) for human target cells,[714] and increases neutrophil phagocytic activity.[207] Some of these functional changes may be related to GM-CSF–induced increase in the cell surface expression of a family of antigens that function as cell adhesion molecules.[715] The increase in antigen expression is rapid and is associated with increased aggregation of neutrophils; both are maximal at the migration inhibitory concentration of 500 pM, and granulocyte-granulocyte adhesion can be inhibited by an antigen-specific monoclonal antibody. GM-CSF also acts as a potent stimulus of eosinophil ADCC, superoxide production, and phagocytosis.[716] G-CSF acts as a potent stimulus of neutrophil superoxide production, ADCC, and phagocytosis,[717] whereas M-CSF activates mature macrophages[718] and enhances macrophage cytotoxicity.

Megakaryocytopoiesis

The cloning of thrombopoietin (TPO) has greatly clarified our understanding of the regulation of megakaryocytopoiesis.[197] Prior to the discovery of TPO, several factors,[719] including IL-3,[720] IL-6,[721] IL-11,[722] SCF,[723,724] and even erythropoietin[725,726] were shown to stimulate megakaryocytopoiesis and thrombopoiesis in vitro and in vivo. IL-3, IL-6, and IL-11 engage heterodimeric receptors of the beta common family (IL-3R) and gp130 family (IL-6R and IL-11R). SCF engages a receptor the intracellular domain of which expresses tyrosine kinase activity upon ligand binding. As already emphasized in this chapter, ligand engagements of these receptor families are known to induce early multipotent progenitors to proliferate and even differentiate toward lineage-specific progenitors, and SCF, IL-3, and IL-11 participate in the induction of the proliferation and differentiation of lineage-specific progenitors. Single-cell analysis of prospectively isolated murine megakaryocyte progenitors shows that a large proportion (≈60%) express EPOR,[113] which is consistent with the potent effect of EPO on megakaryocyte colony formation.[727]

The discovery of TPO provides a major step in understanding megakaryocytopoiesis because this factor induces lineage-restricted megakaryocyte progenitor proliferation, differentiation of those committed progenitors to megakaryoblasts, and, finally, differentiation of megakaryoblasts to the megakaryocytes that in turn produce platelets.

Thrombopoietin

TPO is produced at the site of hematopoiesis. Therefore, though its activity is increased in the blood during episodes of thrombopenia, it does not necessarily function as a hormone, because it is produced directly at the site of thrombopoiesis. In this sense, it differs from erythropoietin, which is not produced at all in marrow stroma. Blood levels may increase in thrombopenic states merely because circulating platelets and/or tissue megakaryocytes sop up the growth factor and carry it out of the circulation.[399,728] This theory has received support from observations in mice with disruption of the murine transcription factor gene called *NF-E2*;[122] although these animals are thrombocytopenic they have an increase in megakaryocyte mass and no increase in serum TPO levels.[729]

As reviewed by Kaushansky,[197] the TPO molecule is considerably longer than the other HGF polypeptides. Its 5′ half bears 23% sequence homology to erythropoietin, whereas the 3′ half bears no structural homology to any cytokine and may be removed by a proteolytic mechanism. Indeed, removal of this half does not ablate physiologic function. The resemblance of the 5′ domain of the molecule to erythropoietin may explain the synergy of thrombopoietin and erythropoietin in megakaryocyte colony formation and platelet production.[197,719] It is well-recognized that splenectomized individuals with persistent anemia usually have significant thrombocytosis and many individuals with red cell aplasia and high EPO levels also have thrombocytosis and megakaryocytosis.

The function of TPO has been studied carefully in vivo and in vitro. Lineage-specific CD34+ meg progenitors bear receptors for SF, IL-3, IL-11, and TPO, the four major classes of hematopoietic cytokine receptors. Maximal megakaryocyte colony formation probably requires signaling by all four receptors, but TPO is absolutely required for the final stages of CFU-MEG maturation, including maximal ploidy and cytoplasmic volume, and, therefore, platelet production. Therapeutic trials of TPO in mice have shown that TPO is species specific. Treatment of mice with murine TPO induces massive thrombocytosis, whereas human TPO is much less active in these animals. More importantly, TPO is active in reducing the platelet nadir in mice and primates rendered thrombopenic by chemotherapy or irradiation.

Circulating Platelets

The differential diagnosis of thrombocytopenia rests first on evaluation of platelet morphology. In conditions in which megakaryocytopoiesis is accelerated, circulating platelet volume (and usually diameter) is increased. The reasons for this shift in volume are disputed. Some claim that young platelets are larger than old platelets.[730] Others suggest that large megakaryocytes give rise to large platelets.[731] Neither explanation satisfies all experimental and clinical conditions, but in general thrombocytopenia secondary to increased destruction of platelets is associated with platelets of large volume, and thrombocytopenia related to decreased production of platelets is associated with platelets of normal size. There are major exceptions to this rule. Patients with hyposplenism tend to have large platelets in their blood, whether thrombopoiesis is increased or not, and patients with primary abnormalities of platelet function, such as Wiskott-Aldrich syndrome or Bernard-Soulier syndrome, have platelet sizes that bear no relationship to platelet production. Thrombopoietin increases platelet production by increasing both the number and size of individual megakaryocytes. Though TPO is probably solely responsible for the later stages of recognizable megakaryocyte differentiation and proliferation of megakaryocyte progenitors, its function depends, at least in part, on the additional stimulation of megakaryocyte progenitors (and probably early megakaryocytes as well) with other growth factors, including IL-3, IL-11, SF, and EPO.

CLINICAL USE OF HEMATOPOIETIC GROWTH FACTORS

Correction or amelioration of marrow failure syndromes by administration of HGFs has been and continues to be a major practical goal of research in hematopoiesis. The goal could not be achieved, however, until recombinant DNA technology provided sufficient amounts of the hormones to permit interpretable investigations.

Human Clinical Studies

Several recombinant HGFs are currently under evaluation or used in a variety of clinical settings. G-CSF and erythropoietin are widely used clinically. G-CSF shortens the hypoplasia that follows chemotherapy and makes more intensive myeloablative chemotherapy regimes possible.

Malignant Disease

G-CSF. In the first phase I/II clinical studies in patients with malignant disease, administration of G-CSF by bolus or continuous intravenous (IV) infusion for 5 to 6 days before chemotherapy led to a dose-related increase in polymorphonuclear neutrophils.[732,733] Rapid increases in neutrophil counts were observed, with maximal counts of 80 to 100 × 10^9/L at doses of 10 to 30 mg/kg/day. A transient depression in neutrophil counts was noted to precede this increase in one study.[734] A double-blind, randomized, placebo-controlled multicenter trial was conducted in the United States; patients with lung carcinoma received up to six cycles of cyclophosphamide, doxorubicin, and etoposide and were given G-CSF or placebo from day 4 to 17 of each cycle. The results showed a reduction in the median duration of severe neutropenia ($<0.5 \times 10^9$/L) from 6 to 3 days in the G-CSF arm and a 50% reduction of febrile neutropenia, hospitalizations, confirmed infections, and antibiotic use with G-CSF.[735] This study and other reports[736,737] have led the American Society of Clinical Oncology (ASCO) and the European Organization for Research and Treatment of Cancer (EORTC) to recommend the primary use of G-CSF in patients receiving chemotherapy protocols with an expected rate of neutropenia that exceeds 20%.[738,739] The use of G-CSF is recommended to support dose-dense or dose-intense chemotherapy regimes in adult carcinoma patients, but most studies do not show a significant effect on progression-free survival rates.[739] Patients in the placebo arm of the U.S. multicenter trial[735] who developed fever in the first treatment cycle were subsequently treated with G-CSF, and this reduced the duration of severe neutropenia from 6 to 3 days and neutropenic fever from 100% in cycle 1 to 23% in cycle 2. Thus the prophylactic use of G-CSF in chemotherapy-induced neutropenia is supported. However, the literature does not support the use of G-CSF or GM-CSF once afebrile[740] or febrile neutropenia has developed,[741,742] with the exception of one study.[743] Although days of neutropenia may be shortened, it has been difficult to show an effect on clinically significant end points such as shortened duration of hospitalization, which was seen in only one of the studies.[742]

GM-CSF. Recombinant hGM-CSF has been administered in phase I/II studies to several adult groups of patients with advanced malignant disease, both before and after chemotherapy.[744-746] Glycosylated GM-CSF produced in either mammalian (Chinese hamster ovary) cells or yeast, and *Escherichia coli*–derived nonglycosylated GM-CSF, have been evaluated with comparable results. A rapid, dose-related increase in polymorphonuclear neutrophils, monocytes, and eosinophils is observed in patients treated before chemotherapy. Neutrophils peak at around 20 to 30 × 10^9/L at doses of 4 to 32 mg/kg/day, and GM-CSF is well tolerated at doses up to this level. A capillary leak syndrome and venous thrombi are observed at higher doses (64 mg/kg/day).[744] Phase III randomized trials in patients with lymphoma or breast cancer have confirmed that GM-CSF given after chemotherapy is associated with shorter periods of neutropenia and higher leukocyte nadirs.[747-749] However, one of these studies included only the patients able to tolerate treatment and was not significant on an intention-to-treat basis. Furthermore, another study of GM-CSF after chemotherapy showed decreased neutropenia after the first treatment cycle only,[750] whereas two other studies showed no improvement in neutrophil counts.[751,752] In one of these studies[751] patients with small cell lung cancer were given chemotherapy and irradiation; compared with the placebo control, patients in the GM-CSF arm suffered more infections, toxic deaths, and longer hospital stays, and therefore the ASCO panel has cautioned against the use of CSFs in combined chemotherapy/irradiation regimens.[753,754]

Similar encouraging results have been obtained in children with solid tumors undergoing intensive chemotherapy.[755-757] Significantly shorter durations of severe neutropenia and thrombocytopenia were observed in a study of 25 children in whom yeast-derived GM-CSF was given at 60 to 1500 μg/m^2/day for 14 days after chemotherapy.[755]

Like G-CSF, GM-CSF also produced an increase in blood progenitor cells of both erythroid and myeloid lineages. Before chemotherapy, an eighteenfold increase in blood CFU-GM and an eightfold increase in BFU-E was noted; after chemotherapy, GM-CSF produced a much greater increase of progenitors (sixtyfold) when given during the recovery period.[755]

Erythropoietin. Anemia develops frequently in cancer patients, and correlates with poor performance status and decreased quality of life.[758] There is good evidence that rhEPO can increase the hemoglobin level in adults[759,760] and children[761] with anemia associated with cancer or its treatment. Three different EPO preparations are available: epoetin alfa (Epogen [Amgen], Procrit [Ortho Biotech], and Eprex [Ortho Biologicals, not sold in the United States]), epoetin beta (NeoRecormon [Hoffmann-LaRoche], sold in Europe), and darbopoetin alfa (Aranesp [Amgen], sold in the United States, Europe, Canada, and Australia). Differences in sialic acid carbohydrate composition of epoetin alfa and beta account for an increase in half-life from 8.5 hours (natural EPO) to 20 to 24 hours, whereas hyperglycosylation of darbopoetin results in a half-life of 49 hours.

Two 2003 randomized studies of patients with anemia and either head and neck cancer or breast cancer showed shorter survival times in the groups receiving EPO, raising concern that EPO can lead to disease progression.[762,763] Another study of advanced non–small cell lung cancer designed to compare EPO with placebo was stopped early when a safety analysis of 70 patients showed a significant survival advantage in the placebo group. Whether carcinoma cells express EPO receptors or not and whether they respond to EPO is controversial. Antibodies to EPO receptor lack specificity and do not distinguish cell surface from intracellular expression, and functional in vitro effects are frequently slight and found at supraphysiologic doses. However, EPO could still have adverse effects through indirect effects on endothelial cells or on angiogenesis.[764]

TPO. Because of TPO's potent in vitro activity and its role as the factor essential for terminal megakaryocytic differentiation, analogous to EPO for the erythroid lineage, clinical studies designed to assess its effect on platelet production have been reported. Both rhTPO and pegylated megakaryocyte growth and development factor (PEG-MGDF) are safe and show no organ toxicity, and in normal volunteers a single bolus of 3 μg/kg/day of PEG-MGDF doubles the blood platelet concentration by day 12, with a return to baseline by day 28.[765] A stimulatory effect on platelet production was observed when TPO or PEG-MGDF was administered after chemotherapy to more than 100 cancer patients, with a decrease in the time for platelet counts to return to normal and elevated platelet nadirs.[766-768] However, PEG-MGDF has caused the development of antibodies to TPO that resulted in severe thrombocytopenia (see below), and these cytokines have been withdrawn from clinical use. Small molecules, peptides, and a peptide-Fc antibody (AMG 531 "peptibody") that have no sequence homology with TPO but activate the receptor are under investigation.[769]

Bone Marrow Transplantation

GM-CSF and G-CSF have been evaluated in clinical autotransplantation trials.

GM-CSF. Patients with nonhematopoietic malignant diseases were treated with high-dose combination chemotherapy, autologous bone marrow transplantation, and rhGM-CSF given by continuous IV infusion for 14 days beginning 3 hours after bone marrow infusion. There was a dose-related increase in the neutrophil count at day 14.[770] Although not statistically significant, there was an improved neutrophil response in patients who had not received previous chemotherapy compared with those who had. Lower rates of morbidity and mortality were also noted among the patients who received the GM-CSF; bacteremia occurred in 16% of treated patients compared with 35% of evaluable controls. Comparable results were reported in a study of patients with lymphoid malignant diseases who received rhGM-CSF as a 2-hour infusion daily for 14 days following chemotherapy, radiotherapy, and autologous bone marrow transplantation.[771] Neutrophil and platelet counts recovered more rapidly; there were fewer days with fever; and the extent of hospitalization was reduced in comparison with an historical control group. In a pediatric study, nine patients received 5 to 10 μg/kg/day of GM-CSF after bone marrow transplantation. Neutrophil recovery was accelerated, although there was no difference in fever, infection, or length of hospitalization when compared with historical controls.[772] Similar results were obtained in a larger prospective double-blind randomized study of 40 patients.[773]

The response to GM-CSF after myelosuppression may be dependent on the infusion of sufficient progenitor cells. In an autotransplantation study of patients with acute lymphoblastic leukemia, bone marrows were purged with 4-hydroperoxycyclophosphamide and anti-T–cell or anti-B–cell lineage-specific antibodies before transplantation.[774] Thirty percent of the patients who received more than 64 μg/m^2/day of GM-CSF achieved an absolute neutrophil count of more than 1000/μL by day 21, whereas none of the nonresponders reached this level by day 27 following transplant. The responders required only one third as many platelet transfusions, and there was a trend toward fewer red cell transfusions, a higher myeloid : erythroid (M : E) ratio, and an earlier day of discharge in the responder group as well. Although bone marrow cell dose did not differ among the two groups, the number of CFU-GM progenitors infused per kilogram was significantly higher in the responders as compared with the nonresponders (17.5 [12 to 27] × 10^3/kg vs. 2 [0 to 7.2] × 10^3/kg). Although it is possible that the CFU-GM infusion, rather than the GM-CSF treatment, accounted for the more rapid recovery, all patients in the responder group showed a rapid decrease in ANC within 48 to 72 hours of discontinuing GM-CSF; this is consistent with a stimulatory effect on bone marrow. One can conclude that GM-CSF is effective in this context provided that sufficient progenitor cells are present.

G-CSF. Recombinant human G-CSF (rhG-CSF) was evaluated in patients with hematopoietic and nonhematopoietic malignant diseases after intensive chemotherapy and reinfusion of cryopreserved autologous bone marrow.[775] The rhG-CSF was given by continuous IV infusion from 24 hours after marrow infusion for a maximum of 28 days, beginning at 20 μg/kg/day and with the dose reduced after the neutrophil count persistently exceeded 1×10^9/L. The mean time to neutrophil recovery (>0.5×10^9/L) occurred by day 11, 9 days earlier than in historical controls. This led to significantly fewer days of parenteral antibiotic therapy, but there was no effect on red cell or platelet recovery. Although the rate of recovery from neutropenia was faster than that reported for rhGM-CSF, the latter studies were phase I dose escalation evaluations, and many patients did not receive an optimal dose of GM-CSF. In a prospective randomized study of 221 children receiving allogeneic or autologous bone marrow or peripheral blood stem cells, the patients treated with G-CSF showed more rapid neutrophil recovery, and this translated to clinical benefit in the bone marrow group (platelet transfusion independence and time to discharge).[776] A study compared G-CSF with GM-CSF in exactly the same bone marrow transplant protocol. Overall, total leukocyte recovery was slightly more rapid in the G-CSF group in comparison with the GM-CSF group. G-CSF administration resulted in two leukocyte peaks. The first at day 10 consisted of lymphocytes, while the second at day 14 consisted mostly of granulocytes; in contrast, GM-CSF produced a single peak at the end of the period of infusion. A major difference was observed with respect to neutrophil migration to an inflammatory site during CSF infusion after hematopoietic reconstitution.[777] Neutrophils did not migrate to skin chambers filled with autologous serum during GM-CSF treatment, a defect not encountered during the administration of G-CSF. There was, however, a similar reduction in the incidence of bacteremia with both GM-CSF and G-CSF (18% and 19%, respectively) in comparison with the historical controls (35%).

In conclusion, if neutrophil recovery is the goal following bone marrow transplantation, then G-CSF appears to be the factor of choice. It preserves neutrophil function and is well tolerated. The inability to hasten platelet recovery is still the major challenge.

Myelodysplasia

GM-CSF. Despite the theoretic risks of treating patients with myeloid stem cell clonal diseases with the CSFs, the factors have been evaluated in patients with refractory anemia (RA), RA with an excess of blasts (RAEB), RAEB in transformation (RAEBIT), and chronic myelomonocytic leukemia.[778-780] In an early study, GM-CSF was given by continuous infusion for 14 days, repeated after a 2-week rest period.[778] Five of the eight patients had received chemotherapy up to 4 weeks before the study. Doses of 30 to 500 μg/m^2 were used. Blood leukocytes rose 5-fold to 70-fold and neutrophils 5-fold to 373-fold, and an absolute increase in monocytes, eosinophils, and lymphocytes was also observed. Three of the eight patients also had a twofold to tenfold increase in platelet count and improvement in erythropoiesis, and two of the three became transfusion independent. Marrow cellularity increased, and there was a reduction in the proportion of blasts, although there was a transient dose-related increase in the absolute number of circulating blasts. No patient developed overt leukemia during the period of follow-up (≤32 weeks). There was no cytogenetic evidence for a reduction in abnormal clones; it is likely that stimulation of hematopoiesis affects both normal and abnormal cells. A dose-related increase in neutrophils, monocytes, eosinophils, and lymphocytes was also noted in a study of 11 patients with myelodysplasia. However, four patients who presented with more than 14% blasts in the bone marrow showed an increase in blasts after therapy, and an additional three patients showed an increase in blasts in the blood; five patients progressed to acute leukemia either during or within 4 weeks after treatment.[780] Unlike in the earlier report, none of these patients had received previous chemotherapy.

G-CSF. A number of patients have been treated with G-CSF.[781,782] Neutrophil responses were seen in five patients with myelodysplasia who received 50 to 1600 μg/m^2/day by intravenous infusion daily for 6 days. At the higher doses (400 μg/m^2/day) the increase was sustained and associated with an increase in bone marrow cellularity. No reticulocyte or platelet increases were observed, and no patient progressed to an acute phase. Similar results were reported in a study of 12 patients given G-CSF by daily subcutaneous injection, with dose escalation from 0.1 to 3 μg/kg/day over an 8-week period.[782] Although 10 of the 12 patients showed elevations in neutrophils (fivefold to fortyfold), an increase in reticulocytes occurred in five patients, with reduction in transfusion requirement in two patients. There was no response in other cell lineages and no conversion to acute leukemia.

Other Hematopoietic Growth Factors and Hematopoietic Growth Factor Combinations. Approximately 20% of patients show an increase in platelet counts during treatment with IL-3,[783,784] and a similar proportion show an increase in hematocrit or reduced transfusion requirement with EPO.[785] This erythroid response can be increased to about 40% if EPO is combined with either G-CSF or GM-CSF.[786,787]

In a review of this subject, Marsh and colleagues[788] concluded that although EPO can increase hemoglobin levels and reduce the need for transfusion, the cost-effectiveness of the approach is uncertain, as is safety with regard to the risk of progression. There is no compelling evidence for a survival benefit with the use of EPO or G-CSF in myelodysplastic syndrome (MDS).[788]

Aplastic Anemia

GM-CSF. Establishing a role for the CSFs in aplastic anemia has challenged investigators. Severe aplastic anemia is a heterogeneous disease that may result from either absent or defective stem cells, from microenvironmental defects, or from immunologically mediated suppression. The mortality rate is high, and therapeutic options are limited to bone marrow transplantation if an appropriate donor is available, or to immunosuppression (see Chapters 7 and 8). Although bone marrow transplantation can be curative, treatment with antithymocyte globulin (ATG) and cyclosporine is frequently effective but generally not curative. For these reasons rhGM-CSF has been evaluated in a number of phase I/II studies.

Administration of rhGM-CSF by bolus or continuous IV infusion for 7 or 14 days resulted in increased granulocytes, monocytes, and reticulocytes in six of eight cases.[779] In another small study, rhGM-CSF was given to cohorts of patients in escalating doses from 4 to 64 μg/kg/day by continuous IV infusion for 14 days. Although a dose-related effect was not observed, 10 of 11 evaluable patients had partial or complete responses in neutrophils, monocytes, and eosinophils, with increases in bone marrow cellularity. Importantly, the greatest increments occurred in patients with higher pretreatment neutrophil counts and more cellular marrows. Only 10% to 20% of patients show an increase in hemoglobin concentration and platelet count, and in all cases counts return to baseline after cessation of treatment.[778,789] In the first report of rhGM-CSF treatment in childhood, three quarters of the evaluable patients responded with a significant rise in neutrophil count during the 28-day induction period.[790] Although neutrophil counts returned to baseline after cessation of treatment in all the responders with severe aplastic anemia, one patient with moderate aplasia maintained a trilineage response when not receiving therapy for more than 1 year. One cannot extrapolate from such a small experience, but the data underscore the point that responses are more likely in less severely affected cases.

Thus in summary, it appears as though rhGM-CSF is palliative in aplastic anemia, with greater neutrophil responses evident in less severely affected patients. The most severely affected patients respond poorly.[791] No infections were observed during the study period in several reports, whereas infections were observed in others. Longer-term prospective comparative studies will be necessary to investigate morbidity rates.

G-CSF. Neutrophil responses have also been reported in a study of 20 children given 400 μg/m^2/day of G-CSF for 14 days.[792] Twelve patients responded by doubling their neutrophil counts, but other lineages were unaffected. In another pediatric study, high doses of G-CSF (400 to 2000 μg/m^2/day) induced neutrophil responses in 6 of 10 patients with very severe aplastic anemia.[793] Long-term treatment may be associated with multilineage responses when given alone[794] or in combination with cyclosporine.[795]

IL-3, IL-6, and EPO. Hematopoietic responses have been reported in a small phase I study of nine aplastic anemia patients given IL-3 in a dosage of 250 to 500 μg/m^2 (five of the nine doubled their neutrophil counts; four of the nine showed an increase in reticulocyte counts but no reduction in transfusion requirement; and one increased platelet counts from 1 to 31 × 10^9/L).[796] An increase in platelet count was reported in one of six patients entered in a phase I study of IL-6, although a dose-related decrease in neutrophils, monocytes, and lymphocytes was observed; and one of three patients given EPO showed a reduced transfusion requirement and/or an increase in hemoglobin levels (reviewed by Kojima[797]). Trilineage responses have been reported in a small number of refractory aplastic anemia patients treated with low doses of GM-CSF plus EPO[798] or G-CSF plus EPO.[799] The effects of EPO are unexpected because endrogenous levels are very high in aplastic anemia.

Human Immunodeficiency Virus Infection

GM-CSF. Human immunodeficiency virus (HIV) infection is associated with several hematologic abnormalities, including neutropenia, anemia, and thrombocytopenia. Anemia and neutropenia can be exacerbated by treatment with azidothymidine, and rhGM-CSF has been evaluated in an effort to enhance immunologic and hematopoietic function and improve tolerance to therapeutic agents. In the first report of the use of rhGM-CSF in humans, cohorts of acquired immunodeficiency syndrome (AIDS) patients were treated with increasing doses of factor given by 14-day continuous IV infusion.[800] This resulted in a rapid, dose-related increase in neutrophils, bands, and eosinophils, with a slight increase in monocytes. A follow-up study with subcutaneous administration showed that these effects could be sustained for up to 6 months without evidence of tachyphylaxis.[800]

A concern with the use of GM-CSF in AIDS is possible enhancement of HIV replication. In one study of azidothymidine given on an alternate-week schedule with GM-CSF, some patients showed increased viral p24 levels during therapy with GM-CSF. There is evidence, however, that GM-CSF may in fact augment azidothymidine levels in monocytes, which suggests that the combination of azidothymidine and GM-CSF might be advantageous.[801] This is not the case with newer nucleoside analogues such as dideoxycytidine and dideoxyinosine (reviewed by Mueller[802]). Two clinical studies have documented an increase in HIV p24 antigen while patients were receiving GM-CSF,[803,747] and therefore GM-CSF has been replaced by G-CSF for the treatment of neutropenia.

G-CSF. In a pilot study at the National Institutes of Health (NIH), G-CSF was evaluated in 19 pediatric AIDS patients who developed neutropenia while receiving azidothymidine. The neutrophil count increased from a median of 1 × 109/L to 2.9 × 109/L at doses of 1 to 20 μg/kg/day, and in 17 of the 19 patients, continued azidothymidine was well tolerated.[804] Of note was the development of thrombocytopenia in some patients; two patients developed G-CSF–dependent disseminated intravascular coagulation, and one patient developed an increase in myeloblasts that disappeared after the G-CSF was stopped.[802]

Erythropoietin. EPO has been used in 12 anemic pediatric patients to determine if tolerance to azidothymidine could be improved.[802] EPO was well tolerated, and at doses from 150 to 400 U/kg subcutaneously or intravenously 3 times a week all patients could be maintained on azidothymidine, with marked reduction (four patients) or moderate reduction (four patients) in transfusion requirement.

Inherited Bone Marrow Failure Syndromes

The use of HGFs in the treatment of inherited bone marrow failure syndromes has been reviewed in detail.[805,806]

Fanconi Anemia. GM-CSF has been studied in seven patients with Fanconi anemia (FA),[807] and trials of IL-3 and G-CSF[805] show that all three HGFs can improve the neutrophil count in most pancytopenic patients but that platelet and hemoglobin levels are unaffected.

Diamond-Blackfan Anemia. Although there is no evidence that Diamond-Blackfan anemia (DBA) is due to deficiency of EPO,[808] IL-3, or GM-CSF,[809] or to an abnormality of KIT or its ligand SCF,[810,811] it is possible that pharmacologic doses of these factors might stimulate erythropoiesis. Niemeyer and colleagues[812] observed no reticulocyte or hemoglobin responses in nine patients treated with rhEpo doses as high as 2000 U/kg/day. In contrast, three of six patients treated with IL-3 (60 to 125 μg/m^2/day subcutaneously for 4 to 6 weeks) had reticulocyte increases, and two of the responders remained transfusion independent for 1.5 to 2 years when not receiving therapy.[813] Another IL-3 study[814] showed 4 responders out of 18 patients treated with 0.5 to 10 μg/kg subcutaneously. Two responders developed deep vein thromboses, necessitating discontinuation of treatment, and the other two patients sustained their responses, one on maintenance IL-3 for 31 months and one without treatment for 12 months after 30 months of therapy. In another study of 13 patients, no responses were observed.[815] Thus 6 patients have had significant erythroid responses to IL-3 out of a total of 37 patients (14%), a rate similar to that reported by the European working group for DBA (3 of 25 patients).[816]

Amegakaryocytic Thrombocytopenia. Amegakaryocytic thrombocytopenia (AMT) is a rare disease that presents in infancy or early childhood with thrombocytopenia, frequent anemia, and progression to pancytopenia. Bone marrow megakaryocytes are absent or extremely scarce, and the disease results from mutations in the TPO receptor MPL. A phase I/II IL-3 dose escalation study without or with sequential GM-CSF in five children with AMT showed that IL-3 (but not IL-3/GM-CSF) induced platelet responses in two patients and improvement in bruising, bleeding, and transfusion requirement in the other three patients.[720] The two platelet responders became unresponsive after several months of IL-3 maintenance (125 to 250 μg/m^2/day), and another patient became platelet transfusion independent after 4 months of IL-3 and had a trilineage response sustained for almost 2 years.

Severe Congenital Neutropenia. Severe congenital neutropenia (SCN) (see also Chapter 8) is a disorder of myelopoiesis characterized by impaired neutrophil differentiation and absolute neutrophil counts less than 200/μL. In contrast, monocytes and eosinophils are normal or increased. The bone marrow shows maturation arrest at the promyelocyte stage, and these cells are often atypical, with abnormal nuclei and vacuolated cytoplasm. The pathophysiology is uncertain, despite the identification of mutations in neutrophil elastase (ELA2) and, more recently, HAX1 in recessively inherited disease.[817] Serum from these patients contains normal or elevated levels of G-CSF, as determined by Western blot testing[818] and bioassay.[819] Other investigations showed mutations in the C-terminal “differentiation” domain of the G-CSFR in two patients.[419]

The responses of these patients to exogenous G-CSF and GM-CSF are therefore of great interest. One study has compared the effects of GM-CSF and G-CSF in a small number of patients, and G-CSF alone was evaluated in other studies.[820-822] In these investigations, *G-CSF produced a remarkable increase in neutrophils in all patients*. The dose necessary to maintain a neutrophil count of more than 1000/μL varied, and ranged from 3 to 15 μg/kg/day. The monocyte count was also increased. In contrast, rhGM-CSF produced an increase in neutrophils in only one patient, and the others showed an increase in eosinophils and monocytes. Although the period of the study was short, no new episodes of severe bacterial infection occurred during either GM-CSF or G-CSF treatment; this contrasts with the recurrent bacterial and fungal infections that occurred before treatment. A multicenter phase III study of G-CSF in 120 patients with severe chronic neutropenic disorders, including severe congenital neutropenia, Shwachman-Diamond syndrome, and myelokathexis showed complete responses in 108 patients (ANC >1.5 × 10^9/L), partial responses in 4 patients, and failure of response in 8 patients.[823] Although the majority of patients responded with an ANC equal to or more than 1 × 10^9/L at doses in the range of 3 to 20 μg/kg/day, about 25% of patients needed 20 to 100 μg/kg/day or more.[806]

Somatic mutations of a single allele of the G-CSFR that result in loss of the C-terminal “differentiation” domain of the receptor were described in several SCN patients, and shown to act as a dominant negative in transduced cells.[824,825] These patients may go on to develop MDS and or acute myeloblastic leukemia (AML).[419] Data from the Severe Chronic Neutropenia International Registry[826] regarding 506 patients with severe congenital neutropenia, cyclic neutropenia, and idiopathic neutropenia who were treated during phase I to III clinical G-CSF trials showed that 23 of 249 patients with severe chronic neutropenia (SCN)/Schwachman-Diamond syndrome (neutropenia and pancreatic insufficiency) developed MDS/AML, in comparison with none of the 97 patients with cyclic neutropenia or the 160 patients with idiopathic neutropenia. The observation that SCN patients receiving G-CSF develop MDS/AML but cyclic or idiopathic neutropenia patients do not is consistent with reports of AML in SCN before the introduction of G-CSF treatment,[827,828] and show that SCN is a preleukemic disease. Additional cytogenetic changes (translocations and monosomy 7) and oncogene (RAS) mutations are common in the patients that go on to develop MDS/AML.[829] The G-CSFR mutation is present in myeloid cells only and was not present in first-degree relatives of affected patients, including a sibling with SCN,[830] demonstrating that the mutation is acquired. Interestingly, detailed study to identify the risks for leukemic transformation show increased risk over time (2.9% per year after 6 years; 8% per year after 12 years; cumulative incidence, 21% after 10 years). Importantly, the risk of MDS/AML correlates with the dose of G-CSF; 40% of the poor responders developed MDS/AML (G-CSF dose higher than the median of 8 μg/kg/day and ANC less than the median of 2188 cells/μL at 6 to 8 months), compared with only 11% of the good responders.[831] Similar results were reported by the French Severe Chronic Neutropenia study group.[832]

In summary, treatment with recombinant G-CSF has had a major positive impact on the lives of the majority of these patients. This continued treatment has increased the risk of emergence of leukemic clones in this preleukemic disease, and patients need to be monitored regularly for their acquisition. Patients who respond poorly to G-CSF should be considered earlier for stem cell transplantation.

Cyclic Neutropenia. Cyclic neutropenia (see also Chapter 8) is a rare HSC disease due to mutations in neutrophil elastase (ELANE). It is characterized by regular 21-day cyclic fluctuations in numbers of neutrophils, monocytes, eosinophils, lymphocytes, platelets, and reticulocytes. Patients typically have recurrent episodes of fever, malaise, mucosal ulceration, and, occasionally, life-threatening infection during periods of neutropenia. Six patients were treated with IV or subcutaneous rhG-CSF for 3 to 15 months at doses ranging from 3 to 10 μg/kg/day.[833] The median neutrophil count increased from 0.7 × 10^9/L to 9.8 × 10^9/L. In five of the patients, cycling of blood counts continued, but the length of cycles decreased from 21 to 14 days. The number of days of severe neutropenia (<200/μL) was reduced from a mean of 12.7 per month to less than 1 per month, and, importantly, the nadir counts increased; neutrophil turnover increased almost fourfold; and migration to a skin window was normal. Average counts of other cells did not increase. One patient with disease of adult onset had a qualitatively different response, with an increase in neutrophils and disappearance of the cyclic fluctuations in count. Therapy reduced the frequency of oropharyngeal inflammation, fever, and infection, demonstrating that treatment with rhG-CSF is effective management for such patients. These results have been confirmed in other studies, and treatment is well tolerated.[834-836] In one study, GM-CSF was not effective in two patients but eliminated severe neutropenia when given in a low dose (0.3 μg/kg/day).[837-839]

Anemia of Chronic Renal Failure

Erythropoietin. Anemia is a major complication of end-stage renal failure and is due primarily to a reduction in erythropoietin production. Other mechanisms that may be involved are shortened red cell survival, iron deficiency, hypersplenism, possible circulating inhibitors of erythropoiesis, and aluminum-induced microcytosis. Several phase I, II, and III studies have documented that rhEpo can induce a dose-dependent increase in effective erythropoiesis.[840-842]

In a phase III study, patients received 150 or 300 U of rhEpo 3 times per week following hemodialysis, and reached a target hematocrit of 35% by 8 to 12 weeks.[841,842] Most patients required 50 to 125 U/kg intravenously 3 times per week following dialysis to maintain a hematocrit of approximately 35%. The increase in erythropoiesis needed to normalize the hemoglobin level requires mobilization of a considerable amount of iron; patients who improve, particularly those who improve rapidly, can develop absolute or relative iron deficiency. If iron is not given, the response to rhEpo becomes blunted; the standard corrective measure is IV administration of iron dextran or oral iron supplementation. Regular measurements of ferritin and transferrin saturation are necessary to ensure that iron stores are adequate.

Erythropoietin has been well tolerated and has resulted in elimination of transfusion dependency. Hypertension, occasionally with encephalopathy, has been observed, particularly with rapid rises in hematocrit that lead to an increase in peripheral vascular resistance; induction with doses of not more than 150 U/kg is recommended to produce a gradual increase in hemoglobin levels. Some patients require either the initiation of antihypertensive medication or an adjustment of dosage. A marked increase in pure red cell aplasia occurred from 1998 to 2004 because of the development of anti-EPO antibodies,[843-845] with reports of approximately 250 suspected or proven cases. The complication, characterized by reticulocytopenia, absence of erythroid precursors, and severe anemia, has occurred mostly in adults (a few children are included) with renal disease who were treated with Eprex outside the United States, but cases have been reported in the United States as well. Differences in EPO glycosylation status and drug formulation, processing, storage, and route of administration appear to affect the immunogenicity of EPO. The estimated exposure-adjusted incidence per 100,000 patient-years was 18 cases for Eprex without serum albumin (epoetin alfa, Ortho Biotech), 6 for Eprex with serum albumin, 1 for Neorecormon (epoetin beta), and 0.2 for Epogen (epoetin alfa).[845] Changes in drug formulation and drug monitoring programs have led to an 80% reduction in the incidence of pure red cell aplasia, and EPO should not be withheld for this reason.

The effect of rhEpo in most studies appears to be restricted to the erythroid lineage. However, data from a phase III study of 303 patients show a significant increase in mean platelet count (224 × 10^9/L to 241 × 10^9/L at 6 months), which is not biologically meaningful. There is also a slight increase in blood urea nitrogen, creatinine, and serum potassium levels. The bone marrow progenitors from patients with end-stage renal failure were studied before and 2 weeks after rhEpo treatment. The concentration of BFU-E, CFU-E, and CFU-Meg increased after therapy. Surprisingly, an increment in CFU-GM also occurred, and the number of progenitors of all classes in cell cycle almost doubled.[846]

Subjective improvement in appetite, energy, sleep pattern, and libido are also noted. A Canadian double-blind placebo-controlled study of rhEpo treatment has provided objective evidence of benefit.

Extending this treatment to patients who do not yet require dialysis has met with similar success.[847] An issue that has yet to be resolved is the financial cost; rhEpo is not inexpensive, and the National Kidney Foundation in the United States has issued guidelines for the use of rhEpo in which all patients with a hematocrit of less than 30% will be eligible. rhEpo is more effective when administered by the subcutaneous route; this route is more convenient for patients and also allows for a dose reduction.[848,849] In predialysis patients, 100 U/kg given subcutaneously provided a response similar to that when 150 U/kg was given intravenously.[848] There is evidence that the bioavailability of subcutaneous EPO is seven times greater than that of the intravenously administered drug, and a large controlled study showed that the mean EPO dose given to maintain a stable hematocrit was 32% lower when EPO was given by the subcutaneous route rather than the IV route.[850]

Similar results have been obtained in several rhEpo studies in children (reviewed by Müller-Wiefel[851]). Anemia was corrected by 3 to 4 months in 24 children with preterminal chronic renal failure treated with rhEpo and iron, which was adjusted by careful monitoring of iron status. Hypertension is the most common side effect.[852-855] Interestingly, although the growth failure of children with terminal chronic renal failure is unaffected by rhEpo treatment, the mean growth velocity in 22 children with preterminal chronic renal failure increased from −2.29 to −0.56 within the first 6 months of treatment.[851] Other possible benefits include reports of delay in progression of renal dysfunction in children with preterminal chronic renal failure and of improvement in cognitive function.

Other Indications for Recombinant Erythropoietin Therapy

Several small clinical trials have evaluated the use of rhEpo in the anemia of prematurity.[856-862] These studies have been reviewed by Mentzer and Shannon,[863] and although study differences in patient population, transfusion criteria, rhEpo dose, and iron therapy make comparisons difficult, rhEpo treatment has been shown to be safe and to stimulate a reticulocyte response. An effect on hematocrit was observed only in the studies in which doses of more than 500 U/kg/week were used,[856,858,860,861] and there appears to be a modest effect on transfusion requirement. Increasing the dose from 750 U/kg/week to 1500 U/kg/week was not supported by a large European study of 184 very–low-birth-weight infants.[864]

Preliminary data suggest that rhEpo may be useful in the treatment of patients with the anemia of chronic disease associated with rheumatoid arthritis[865] and the anemia that complicates azidothymidine treatment in patients with AIDS (see discussion above).

In simian studies, hemoglobin F levels can be increased by administration of erythropoietin.[866] If similar changes occur in humans, erythropoietin may have a role in the management of sickle cell disease and thalassemia. Several small studies in sickle cell disease have shown that an F reticulocytosis can occur in some patients, whereas other patients show an increase in hemoglobin without a sustained F-reticulocyte response, a finding that is of some concern because blood viscosity might be increased.[867] There are also data to suggest that hydroxyurea in combination with EPO can augment the F-reticulocyte response, although the contribution of EPO is still uncertain.[868,869] In patients with transfusion-dependent or untransfused thalassemia, variable responses to EPO alone have also been reported,[870-873] with some patients showing an increase in hemoglobin and F cells. The only report to show a consistent increase in fetal hemoglobin was a study of 10 untransfused thalassemic patients who received rhEpo (400 to 800 U/kg 3 times weekly), as well as iron and hydroxyurea 4 days per week. Hemoglobin counts increased significantly in 8 of the 10 patients, with concomitant increases in fetal hemoglobin 5% to 20% above baseline.[873]

Finally, erythropoietin can be used to increase the number of units of blood that can be obtained preoperatively in the context of autologous blood donation.[874]

Immune Thrombocytopenic Purpura. TPO Mimetics. Two small molecules that can activate the TPO receptor (MPL) are under intense study and have been approved by the Food and Drug Administration (FDA) for immune thrombocytopenic purpura (ITP). Romiplostim has four peptides linked to an IgG Fc fragment that binds to the TPO receptor in the same way as TPO; it is administered by weekly subcutaneous injection. Eltrombopag is an orally administered nonpeptide drug that activates the TPO receptor by binding to its transmembrane domain. In normal individuals both agents produce a dose-dependent rise in platelet count beginning on day 5 and peaking at around 2 weeks. There is evidence that ITP is not only due to increased platelet destruction but that immune-mediated megakaryocyte damage and decreased platelet production are also important,[875] providing a compelling rationale for the use of TPO mimetics in ITP.[876-878] In an open-label study of nonsplenectomized ITP patients who had failed standard therapy, 234 adult patients were randomized to receive romiplostim or standard care. Romiplostim was associated with better outcomes with respect to sustained platelet response, less bleeding, fewer transfusions, a decreased requirement for other treatments, and improved quality of life.[879] Two smaller placebo-controlled pediatric studies showed similar results.[880,881] In the EXTEND (Eltrombopag eXTENed Dosing Study), safety, efficacy, and tolerability were studied in 299 patients with ITP of more than 6 months' duration for up to 3 years.[882] Bleeding symptoms affected 56% of patients at baseline and decreased to 20% and 11% at 2 and 3 years, respectively. Platelet counts were maintained at more than 50,000/μL and 2 times baseline in at least two thirds of the cumulative study weeks for both splenectomized and nonsplenectomized subjects. Furthermore, 65% of the 69 patients who attempted to decrease concomitant treatment were able to sustain a reduction or permanently stop one or more of these therapies.

Stem Cell and Progenitor Cell Mobilization

The effect of G-CSF on progenitor cells is interesting. After melphalan and G-CSF, the absolute number of *circulating* progenitor cells of the granulocyte-macrophage, erythroid, mixed, and megakaryocyte lineages show a dose-related increase up to 100-fold after 4 days of treatment with rhG-CSF,[883] confirming earlier animal studies.[884,885] The relative proportion of the early and late granulocyte progenitors and of early progenitors of different lineages remains unchanged; however, CFU-E, normally undetectable in the blood, are markedly increased. The mechanism for the increase in all progenitors in the blood is unclear; G-CSF has been shown to affect immature blast colony-forming cells, and it is possible that stem cells are stimulated in vivo to produce increased progenitors of all classes; alternatively, G-CSF may indirectly stimulate progenitors by induction of HGF production or by release into the circulation of bone marrow progenitor cells.

These early studies set the stage for attempts at high-dose chemotherapy followed by mobilized blood progenitor cell rescue in a number of chemosensitive tumors such as lymphoma, breast cancer, and multiple myeloma. Time to engraftment is critically related to the number of CD34+ cells infused, and a number of studies indicate that a dose of 5 × 10^6 CD34+ cells per kilogram or higher results in prompt neutrophil and platelet engraftment.[886-889] G-CSF is the usual HGF used to mobilize progenitors, and multiple apheresis procedures (four or more) are commonly required to achieve this dose of CD34+ cells. Strategies to increase the yield and thereby reduce the number of apheresis procedures include the combination of G-CSF with chemotherapy or SCF.[889-892] Two studies suggest that SCF in combination with G-CSF can decrease the number of required apheresis procedures[889,893]; this might be expected to have several advantages, including reduction of apheresis-associated morbidity, less delay in administration of chemotherapy, and decreased cost. Another strategy is to combine G-CSF with plerixafor,[894] an inhibitor of the interaction between stem cell CXCR4 and stromal-derived SDF-1, or to use plerixafor alone.[895]

Toxicity of Treatment with Colony-Stimulating Factors

The CSFs tested in all these clinical trials have in general been well tolerated. Both GM-CSF and G-CSF induce a transient leukopenia in the first 30 minutes after administration by IV bolus. GM-CSF rapidly induces surface expression of the leukocyte adhesion protein CD11b (MO1) in vitro, and this is accompanied by an increase in neutrophil aggregation.[715] CD11a (LFA-1) and CD11c (gp150/95), two other members of this family of cell surface adhesion glycoproteins that have distinct alpha chains but share a common beta chain (CD18) with CD11b, are unaffected by GM-CSF. These results have been corroborated by in vivo studies of sarcoma patients who received 32 or 64 µg/kg/day of GM-CSF. A marked increase of CD11b was noted that was evident by 30 minutes and persisted for 12 to 24 hours after treatment.[896] Radionuclide-labeled leukocytes are sequestered in the lungs after GM-CSF treatment,[734] probably because of the aggregability and adhesiveness induced by increased CD11b expression. Breathlessness and hypoxia have been observed in some patients, particularly after short-duration IV administration. CD11b is not modulated by G-CSF, and the reason for the transient leukopenia following treatment with G-CSF is at present unclear.

Both GM-CSF and G-CSF have been associated with bone pain coincident with or shortly after administration. Occasional increases in leukocyte alkaline phosphatase and/or lactate dehydrogenase have been noted. In contrast, GM-CSF has induced flulike symptoms, including fever, flushing, malaise, myalgia, arthralgia, anorexia, and headache. Mild elevations of transaminase levels and rash are also reported. These effects are usually mild, are alleviated by antipyretics, and disappear with continued administration. More serious GM-CSF toxicity has been observed at higher dose levels (>32 µg/kg/day given intravenously or >15 µg/kg/day given subcutaneously). These include a capillary leak syndrome, with weight gain due to fluid retention, manifested as pericardial or pleural effusions, ascites, and/or edema.[744,770] Phlebitis was noted in initial studies when the GM-CSF was infused into small veins; large-vessel thrombosis has occurred with infusion of high doses into central veins.[744]

Antibodies to Recombinant Factors

rhGM-CSF that is produced in mammalian cells (Chinese hamster ovary [CHO] cells) is variably glycosylated on both O-linked and N-linked sites. Production in *E. coli* results in nonglycosylated GM-CSF, and the yeast product is glycosylated at N-linked sites. All three products appear to be equally efficacious, but antibodies have been reported in 4 of 13 patients given the product derived from yeast in phase I/II studies.[897] The IgG antibodies developed by 7 days after the start of the infusion in all four patients, three of whom had received a bolus test dose; the antibodies were nonneutralizing as judged by bone marrow colony-forming assay, and they were directed at sites on the protein backbone of the GM-CSF molecule that are normally protected by O-linked glycosylation but that are exposed in the products derived from yeast or *E. coli*. Transient antibodies to TPO have been reported in one cancer patient.[898] However, the development of antibodies in both patients and volunteers given PEG-MGDF led to termination of further clinical development of this TPO formulation, because transient decreases in platelet count were noted.[899,900,765,901] Antibodies to erythropoietin can also develop in patients with the anemia of chronic renal failure who are receiving treatment with erythropoietin.[902] Some formulations of EPO were associated with an antibody to EPO that resulted in pure red cell aplasia; this complication is discussed more fully above in the section on the use of EPO in renal failure.

No dose-limiting toxicity has been observed with G-CSF. However, one case of pathogenic neutrophil infiltration (acute febrile neutrophilic dermatosis, or Sweet syndrome) has been reported.[903] One patient with severe congenital neutropenia developed cutaneous necrotizing vasculitis (leukoclastic vasculitis) while receiving G-CSF treatment.[819,821] As noted above, there is also concern that G-CSF will induce or accelerate the development of acute myeloblastic leukemia or myelodysplasia with monosomy 7 in aplastic anemia and SCN; such a development is perhaps more likely in patients with G-CSF receptor mutations that can transduce a proliferative signal but not a differentiation signal.

As noted above, erythropoietin treatment has been associated with an increased risk of thromboembolic complications in adults, particularly when target hemoglobin concentrations were in the normal range, with a possible adverse effect on breast cancer progression.[763,904] Two randomized studies in which the targeted hemoglobin level was either in the normal range (>13.5 g/dL) or subnormal range (10.5 to 11.5 g/dL) showed an increased risk of adverse cardiovascular effects for the normal hemoglobin groups.[905,906] A 2006 update of a Cochrane review of 57 trials including more than 9000 patients showed strong evidence that epoetin and darbepoetin increased the risk of thromboembolic complications compared with controls (relative response [RR] 1.67; 95% confidence interval 1.35 to 2.06).[907] There are no comparable large studies in children. As a result of these and other studies, the FDA has recommended the lowest dose possible to increase the hemoglobin to a level that avoids transfusion, not to exceed 12 g/dL or a rise of more than 1g in any 2-week period (see).

TPO mimetics have been associated most frequently with nausea and vomiting (5% to 7% of patients), and serious adverse events include thromboembolism (2%) and elevations in alanine transaminase and bilirubin (4% to 7%).[876,907a] Studies in rats showed an increase in bone marrow reticulin fibrosis with high doses of romiplostim, and this has raised concern in clinical studies. In general, fibrosis appears to be mild and reversible with cessation of the drug.[908]

CONCLUSION

Advances in molecular and cell biology have led to an unraveling of some of the most challenging and confusing aspects of the study of hematopoiesis. Therapeutic benefits have emerged from these discoveries. The field of hematopoiesis, once a jumble of unknown factors and activities, has come of age and is providing direct benefits to countless patients.

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CHAPTER 2

The Neonatal Erythrocyte and Its Disorders

Patrick G. Gallagher

CHAPTER OUTLINE

INTRODUCTION
THE NEONATAL ERYTHROCYTE
NORMAL HEMATOLOGIC VALUES
NEONATAL ANEMIA
HEMORRHAGE IN THE FETUS AND NEWBORN
- Prenatal Hemorrhage
- Intrapartum Hemorrhage
- Postpartum Hemorrhage
- Clinical Manifestations of Fetal and Neonatal Hemorrhage

HEMOLYSIS IN THE FETUS AND NEWBORN
- Extrinsic Hemolysis
- Intrinsic Hemolysis

DECREASED ERYTHROCYTE PRODUCTION
PHYSIOLOGIC ANEMIA AND ANEMIA OF PREMATURITY
APPROACH TO THE ANEMIC INFANT
POLYCYTHEMIA HYPERVISCOSITY SYNDROME

INTRODUCTION

At no other time in the life of a patient is the physician confronted with as many diagnostic considerations in the interpretation of apparent disturbances of the erythrocyte as during the neonatal period. Neonatal erythrocytes are fundamentally different from those of older children and adults, varying in size, shape, globin composition, oxygen transport, membrane characteristics, cellular metabolism, and life span.[1] Because of these differences, evaluation of a neonate with a suspected erythrocyte disorder mandates a thorough understanding of developmental erythropoiesis (reviewed in Chapter 1), appreciation of the differences between neonatal and adult red blood cells (RBCs), understanding of perinatal conditions influencing the fetus and newborn, and knowledge of inherited and acquired disorders of the erythrocyte that manifest in the neonate.

THE NEONATAL ERYTHROCYTE

Erythrocyte Size

Early in embryogenesis, the size and volume of erythrocytes are significantly greater than that of neonatal and adult erythrocytes, with the mean corpuscular volume (MCV) ranging from 150 to 180 fl and cellular diameter ranging from 20 to 25 μ. Erythrocyte size and volume decrease throughout gestation to values of MCV between 108 to 118 fl and diameter between 8 to 10 μ at term (Table 2-1). After birth,[2] erythrocytes continue to decrease in size and volume, with values similar to those of adults by 1 year of age.

Erythrocyte Shape and Deformability

Marked variability exists in the shape of fetal and neonatal erythrocytes. Irregularly shaped erythrocytes are found in larger numbers on the peripheral blood smears of hematologically normal term neonates than on those of adults (Fig. 2-1). Acanthocytes, target cells, and immature erythroid cells with various membrane projections are frequently seen.[3] These variations have been attributed to poor or absent splenic function in neonates. Decreased splenic function is also associated with membrane surface "pits," the site of formation of endocytic vacuoles, attributed to immaturity of the neonatal macrophage pitting process.[4] Nearly half of erythrocytes from preterm infants and a quarter of erythrocytes from term infants have surface pits compared with approximately 2% of normal adult erythrocytes.[5] Erythrocyte deformability is determined primarily by three factors: the surface/volume relationship of the cell, cytoplasmic viscosity, and intrinsic membrane rigidity. Cellular deformability influences several processes, including whole blood viscosity, which in turn affects peripheral vascular resistance and cardiac workload, flow in the peripheral circulation, and the life span of the erythrocyte. As erythrocytes age, neonatal cells lose more volume, have a higher mean corpuscular hemoglobin concentration, and become less

TABLE 2-1 The Effect of Gestational Age of Fetuses in Utero and Newborn Infants On Their Hematocrit, Hemoglobin, Mean Corpuscular Volume, and Reticulocytes

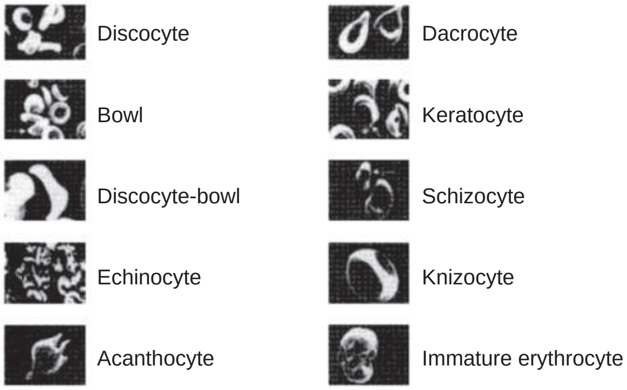
Gestational Age (wk)	Hematocrit (%)[*]	Hemoglobin (g/dL)	MCV (fl)	Reticulocytes (%)
18-20[†]	36 ± 3	11.5 ± 0.8	134 ± 9	NR
21-22[†]	38 ± 3	12.3 ± 0.9	130 ± 6	NR
22-23[†]	38 ± 1	12.4 ± 0.9	125 ± 1	NR
24-25	63 ± 4	19.4 ± 1.5	135 ± 0	6.0 ± 0.5
26-27	62 ± 8	19.0 ± 2.5	132 ± 14	9.6 ± 3.2
28-29	60 ± 7	19.3 ± 1.8	131 ± 14	7.5 ± 2.5
30-31	60 ± 8	19.1 ± 2.2	127 ± 13	5.8 ± 2.0
32-33	60 ± 8	18.5 ± 2.0	123 ± 16	5.0 ± 1.9
34-35	61 ± 7	19.6 ± 2.1	122 ± 10	3.9 ± 1.6
36-37	64 ± 7	19.2 ± 1.7	121 ± 12	4.2 ± 1.8
Term	61 ± 7	19.3 ± 2.2	119 ± 9	3.2 ± 1.4

From Christensen RD: Expected hematologic values for term and preterm neonates. In Christensen RD, editor: Hematologic problems of the neonate, *Philadelphia, 2000, Saunders, p. 120.*

MCV, Mean corpuscular volume; *NR,* not reported.

[*] Values reported as the mean ± standard deviation.

[†] Fetuses in utero.



The Erythrocyte Differential Count
Median (5% to 95% range)

Cell type	Premature infant	Term infant	Normal adult
Discocyte	39.5 (18–57)	43 (18–62)	78 (42–94)
Bowl	29.0 (13–53)	40 (14–58)	18 (4–50)
Discocyte-bowl	3.0 (0–10)	2 (0–5)	2 (0–4)
Spherocyte	0.0 (0–3)	0 (0–1)	0 (0–0)
Echinocyte	5.5 (1–23)	1 (0–4)	0 (0–3)
Acanthocyte	0.0 (0–2)	1 (0–2)	0 (0–1)
Dacrocyte	1.0 (0–5)	1 (0–3)	0 (0–1)
Keratocyte	3.0 (0–7)	2 (0–5)	0 (0–1)
Schizocyte	2.0 (0–5)	0 (0–2)	0 (0–1)
Knizocyte	3.0 (0–11)	3 (0–8)	1 (0–5)
Immature erythrocyte	1.0 (0–6)	0 (0–2)	0 (0–0)

Figure 2-1 Varied morphology associated with neonatal smears. *(From Zipursky A, Brown E, Palko J, et al: The erythrocyte differential count in newborn infants.* Am J Pediatr Hematol Oncol *5:45, 1983.)*

deformable than adult RBCs, with decreased deformability leading to increased splenic sequestration. Together these properties lead to accelerated membrane loss and decreased erythrocyte life span.[6,7]

Membrane Differences

Fetal and neonatal erythrocyte membranes demonstrate additional characteristics that differ from those of adult cells (Box 2-1). Fetal and neonatal erythrocytes contain more phospholipid and cholesterol per cell,[8] resulting in a larger surface to volume ratio and rendering them slightly more osmotically resistant.[9] This observation has practical clinical importance, because when osmotic fragility testing is used in a neonate to diagnose hereditary spherocytosis (HS), a normative neonatal osmotic fragility curve should be used rather than the standard adult curve. The surface charge of neonatal erythrocytes is

Box 2-1 Some Structural Characteristics of Fetal Erythrocytes Compared with Adult Erythrocytes

Membrane Structure and Function

- Less osmotically fragile
- Differences in surface antigen expression
  - A and B antigens diminished
  - Lewis system diminished
  - I antigen very diminished (or absent): i antigen present
- Possible decreased structural stability as a result of increased levels of unbound 2,3-diphosphoglycerate
- Membranes less deformable
- Red cells of different shapes with a greater predominance of stomatocytes than adult cells
- Increased variability of the smear with multiple morphologic changes
- Red cell membrane pits (as a result of decreased splenic function)

From Wolfe LC: Neonatal anemia. In Handin RI, Lux SE, Stossel TP, editors. Blood: principles and practice of hematology, *Philadelphia, 1995, JB Lippincott, p. 2107.*

more negative than that of adult cells because of a higher sialic acid content.[10] This increased negative charge contributes to the decreased erythrocyte sedimentation rate observed in newborns.[11] Neonatal erythrocytes contain increased amounts of total and membrane-associated myosin compared with adult cells.[12] Membrane acetylcholinesterase is decreased.[13] Permeability to monovalent cations is increased, and less sodium-potassium adenosine triphosphatase activity is required for monovalent cation removal.

The expression of various antigens and surface receptors vary between neonatal and adult erythrocytes. The major RBC antigens—Rh, MN, Kell, and Duffy—are well developed in early intrauterine life. Other membrane-associated antigens—Lutheran, ABO, I, and XgA—are incompletely developed at birth. Other antigens, such as the Lewis antigen, are absent at birth. Neonatal erythrocytes express higher levels of several membrane-associated proteins, such as the insulin and insulinlike growth factor receptors, while expressing fewer digoxin receptors than adult erythrocytes.[14]

The i antigen is a commonly used marker of fetal erythropoiesis. Differences in the glycans attached to the external surface of band 3, the anion exchanger, account for this developmental marker.[15] In fetal cells the chain is arranged in an unbranched, linear fashion (i antigen with i reactivity), whereas the chain is arranged in a branched manner in adult cells (I antigen with I reactivity).[16] The i antigen predominates in fetal and neonatal cells, gradually decreasing in a reciprocal relationship with the I antigen, which gradually increases, until approximately 18 months of age. Adult erythrocytes express minimal to no i antigen.

Globin Composition and Oxygen Transport

The synthesis and composition of embryonic, fetal, and neonatal globins are described in Chapter 19. Depending on gestational age, approximately 70% to 90% of the hemoglobin in the erythrocyte of the fetus and neonate is fetal hemoglobin (HbF). HbF is more soluble in strong phosphate buffers and is resistant to acid denaturation.[17] This latter characteristic is the biochemical basis of the Kleihauer-Betke test (discussed later).

An important functional difference between HbF and adult hemoglobin (HbA) results from the ability of HbA, but not HbF, to interact with phosphorylated compounds, particularly adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG). The interaction of HbA with 2,3-DPG leads to a marked decrease in oxygen affinity.[18] In contrast, HbF has no or minimal interaction with these organic phosphates, leading to a relatively higher oxygen affinity of fetal blood, with a leftward shift of the oxygen dissociation curve (Table 2-2; Fig. 2-2) compared with adult blood. In utero, this characteristic is of significant benefit to the fetus because it facilitates placental oxygen exchange from maternal blood to fetal erythrocytes.

Cellular Metabolism

Fetal and neonatal erythrocytes have a number of metabolic differences compared with adult cells (Box 2-2).[19]

TABLE 2-2 Theoretical Hemoglobin Levels (g/dL) Required in Low Birth Weight Infants to Maintain a Central Venous Oxygen Tension of 30 mm Hg*

	ARTERIAL OXYGEN SATURATION (%)†			
P_{50} (mm Hg)	95	90	85	80
20‡	10.0	13.0	20.0	>25.0
23	7.3	9.0	11.0	15.5
25	6.2	7.3	8.8	11.0
27§	5.3	6.3	7.3	8.9

Modified from Oski FA: Hematology: the erythrocyte and its disorders. In Nathan DA, Oski FA, editors: Hematology of infancy and childhood, *ed 4, Philadelphia, 1993, WB Saunders, p. 40.*

*Assumes a cardiac output of 250 mL/kg/min and an oxygen consumption of 6.5 mL/kg/min.

†The lower the arterial oxygen saturation, the less resting pulmonary function (e.g., bronchopulmonary dysplasia).

‡P_{50} of fetal hemoglobin.

§P_{50} of adult hemoglobin.

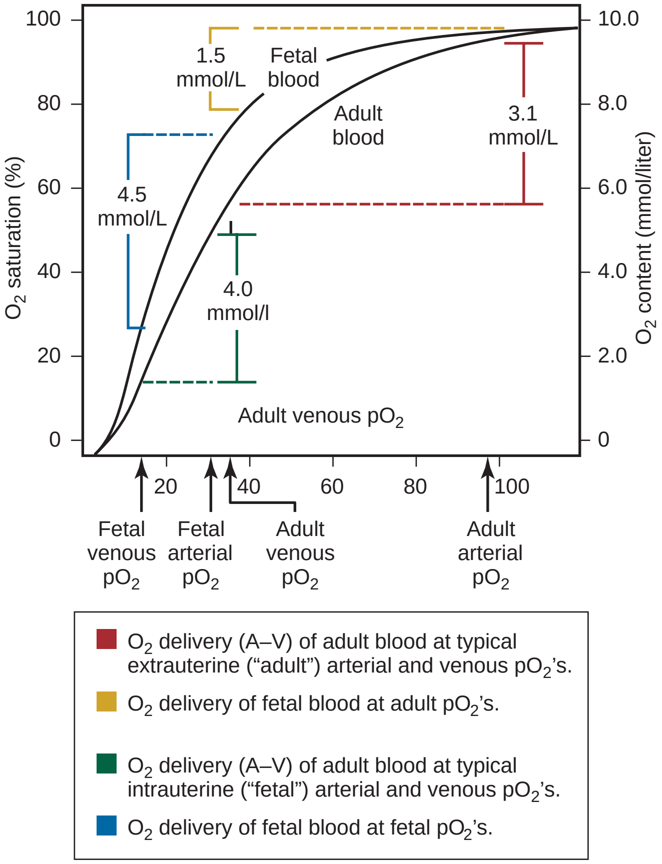


Figure 2-2 Difference in the hemoglobin–oxygen (O_2) dissociation curve between newborns and adults. The left shift of the newborn curve enhances the amount of O_2 that can be delivered at lower O_2 tensions. For example, comparing the difference in O_2 content of blood at fetal arterial and venous (A-V) partial pressure of oxygen (pO_2) values, fetal blood delivers 4.9 mmol/L, whereas adult blood delivers only 4 mmol/L. In contrast, at typical adult arterial and venous pO_2 values, adult blood delivers more O_2 (3.1 mmol/L) compared with fetal blood (1.5 mmol/L). *(From Stockman JA: Fetal hematology. In Eden RD, Boehm FH, editors:* Assessment and care of the fetus, *Norwalk, CT, 1989, Appleton & Lange, p. 124.)*

Box 2-2 Metabolic Characteristics of Neonatal Erythrocytes

CARBOHYDRATE METABOLISM

Glucose consumption increased
Galactose more completely used as substrate under normal circumstances and for methemoglobin reduction*
Decreased activity of sorbitol pathway*
Decreased triokinase activity*

GLYCOLYTIC ENZYMES

Increased activity of hexokinase, phosphoglucose isomerase,* aldolase, glyceraldehyde-3-phosphate dehydrogenase,* phosphoglycerate kinase,* phosphoglycerate mutase, enolase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, galactokinase, and galactose-1-phosphate uridyl-transferase
Decreased activity of phosphofructokinase*
Distribution of hexokinase isoenzymes differs from that of adults*

NONGLYCOLYTIC ENZYMES

Increased activity of glutamic oxaloacetic transaminase and glutathione reductase
Decreased activity of NADP-dependent methemoglobin reductase catalase,* glutathione peroxidase, carbonic anhydrase,* adenylate kinase,* and glutathione synthetase*
Presence of alpha-glycerol-3-phosphate dehydrogenase

ATP AND PHOSPHATE METABOLISM

Decreased phosphate uptake,* slower incorporation into ATP and 2,3-diphosphoglycerate*
Accelerated decline of 2,3-diphosphoglycerate during red blood cell incubation*
Increased ATP levels
Accelerated decline of ATP during storage or incubation

STORAGE CHARACTERISTICS

Increased potassium efflux and greater degrees of hemolysis during short periods of storage
More rapid assumption of altered morphologic forms during brief incubation*

MEMBRANE CHARACTERISTICS

Decreased ouabain-sensitive ATPase*
Decreased potassium influx*
Decreased permeability to glycerol and thiourea*
Decreased membrane filterability*
Increased sphingomyelin, decreased lecithin content of stromal phospholipids
Decreased content of linoleic acid*
Increase in lipid phosphorus and cholesterol per cell
Greater affinity for glucose*

OTHER CHARACTERISTICS

Increased methemoglobin content*
Increased affinity of hemoglobin for oxygen*
Glutathione instability*
Increased tendency for Heinz body formation in the presence of oxidant compounds*

From Gallagher PG: Disorders of erythrocyte metabolism and shape. In Christensen RD, editor: Hematologic problems of the neonate, *Philadelphia, 2000, Saunders, p. 212.*
ATP, Adenosine triphosphate; *ATPase,* adenosine triphosphatase; *NADP,* nicotinamide adenine dinucleotide phosphate.
*Appears to be a unique characteristic of the newborn's erythrocytes and not merely a function of the presence of young red blood cells.

Glucose and ATP production are increased, probably representing compensatory activity to maintain normal intracellular homeostasis by energy-dependent ion transport.[20] Several glycolytic enzymes demonstrate increased activity,[21] but this increase may reflect a larger percentage of younger RBCs in the neonatal circulation compared with the adult circulation rather than a characteristic specific to neonatal erythrocytes. Activities of other enzymes, including carbonic anhydrase and methemoglobin reductase, are reduced.

One important characteristic of the neonatal erythrocyte is increased susceptibility to oxidant-induced damage,[22-24] leading to glutathione instability, Heinz body formation, and methemoglobinemia. Alterations in the pentose phosphate pathway, decreased glutathione peroxidase,[25] diminished oxidant capacity of neonatal plasma, variations in superoxide dismutase levels, and decreased membrane sulfhydryl groups have all been implicated in the susceptibility of the neonatal erythrocyte to oxidative stress.[26] This increased susceptibility to oxidative injury may lead to hemolysis when severe hypoxia and acidosis is present in the fetus and newborn.[24]

Life Span

The life span of the erythrocyte in a term neonate is between 60 and 90 days, with preterm infants demonstrating even shorter life spans, from 35 to 50 days.[27] This shortened life span has been attributed to several properties specific to neonatal erythrocytes, including increased membrane deformability, more rapid loss of membrane surface area, increased susceptibility of membrane proteins and lipids to peroxidation, and a rapid decrease in intracellular enzyme activity and ATP content. When neonatal erythrocytes are transfused into adults, they exhibit a shortened life span that is attributed to the intrinsic differences between neonatal and adult erythrocytes. When adult erythrocytes are transfused into a neonate, they exhibit a normal life span.[28,29]

NORMAL HEMATOLOGIC VALUES

A number of factors influence hematologic values in the neonate, including many of the developmental changes previously listed. Interpretation of laboratory data in the neonate requires consideration of a number of factors, because a value that is normal for one infant may not be normal for another infant. The gestational and chronologic age of the infant, the infant's sex, timing of cord clamping, and the site of blood sampling are important factors to be considered.

Hemoglobin and Hematocrit

Hemoglobin concentration rises gradually throughout gestation and peaks shortly after birth.[30,31] The mean hemoglobin at 10 weeks' gestation is 9 g/dL, increasing to 11 to 12 g/dL by approximately 23 weeks and to 13 to 14 g/dL by 30 weeks' gestation (see Table 2-1).[2] Hemoglobin concentration is relatively stable the last 6 to 8 weeks of gestation, with a mean concentration 16 to 17 g/dL at term. Hemoglobin may increase by 1 to 2 g/dL at birth as a result of placental transfusion. Decreased plasma volume leads to a peak in hemoglobin between 2 to 6 hours of life, with levels stabilizing by 8 to 12 hours of age.

Erythropoiesis decreases at birth, leading to a gradual decrease in hemoglobin concentration during the next

TABLE 2-3 Normal Hematologic Values during the First 2 Weeks of Life in the Term Infant*

Value	Cord Blood	Day 1	Day 3	Day 7	Day 14
Hemoglobin (g/dL)	16.8	18.4	17.8	17.0	16.8
Hematocrit (%)	53.0	58.0	55.	54.0	52.0
Red cells (mm^3)	5.25	5.8	5.6	5.2	5.1
MCV (fl)	107	108	99	98.0	96.0
MCH (pg)	34	35	33	32.5	31.5
MCHC (g/dL)	31.7	32.5	33	33	33
Reticulocytes	3-7	3-7	1-3	0-1	0-1
Nucleated RBCs (/mm^3)	500	200	0-5	0	0
Platelets (1000's mm^3)	290	192	213	248	252

From Oski FA, Naiman JL: Hematologic problems in the newborn, *ed 2, Philadelphia, 1972, WB Saunders, p. 13.*

MCH, Mean corpuscular hemoglobin; *MCHC,* mean corpuscular hemoglobin concentration; *MCV,* mean corpuscular volume; *RBC,* red blood cell.

*During the first 2 weeks of life, a venous hemoglobin value below 13.0 g/dL or a capillary hemoglobin value below 14.5 g/dL should be regarded as anemic.

several weeks of life (Table 2-3). The nadir of hemoglobin concentration is reached at 8 weeks of life, with levels of approximately 11 g/dL in healthy term infants before erythropoiesis increases and the hemoglobin rises. This process is called *physiologic anemia* (discussed later).

Cord Clamping and Placental Transfusion

The timing of cord clamping significantly influences hemoglobin values in the newborn (discussed later).[30,32,33] Around the time of birth, placental blood is rapidly transferred to the infant. Approximately a quarter of this transfusion occurs within 15 seconds of birth and half occurs by the end of the first minute after birth. Delayed cord clamping (DCC) typically increases the infant's blood volume by 30%, because the placental vessels contain between 75 to 125 mL of blood at birth. Holding the infant above the level of the placenta prevents placental transfusion and may even lead to neonatal transfusion into the placenta, resulting in neonatal anemia.

Site of Blood Sampling

Significant variation in hemoglobin levels may exist, depending on the site of blood sampling.[30] Hemoglobin levels from capillary blood samples typically are higher than those obtained from indwelling venous or arterial catheters. Capillary samples may lead to overestimation of the hemoglobin, particularly when the samples are obtained from a poorly perfused extremity. The largest differences between capillary and venous hematocrits exist in very preterm infants,[34] particularly those with acidosis, hypotension, and anemia. In infants younger than 30 weeks' gestation, capillary hematocrit values are 20% higher than venous hematocrit values, compared with capillary values that are 12% higher than venous hematocrit levels at term. "Arterializing" a capillary sample by prewarming the extremity may improve the correlation between capillary and venous hematocrit levels. Finally, values of samples from similar sources, such as arterial, venous, and capillary samples, correlate independent of the site of sampling. For example, umbilical, radial, and femoral arterial hemoglobin levels show little variation from sample to sample.

Reticulocytes

Reticulocyte counts and the absolute reticulocyte count are elevated at birth, with values from 4% to 7% and 200,000 to 400,000/μL in term infants and 6% to 12% and 400,000 to 550,000/mL in preterm infants, respectively.[30] In healthy infants, the reticulocyte count falls over the first few days of life to levels of 0 to 1% by day 4 of life (see Table 2-3). A reticulocyte count of 0 on day 1 of life indicates that there is erythrocyte under production (discussed later).

Erythrocyte Indices

Mean Corpuscular Volume

The average volume of circulating erythrocytes is estimated by the MCV. During pregnancy an inverse relationship exists between gestational age and MCV, with MCV values decreasing throughout gestation to 108 to 118 fl at term (see Table 2-1).[2] When an MCV less than 95 fl is observed in a term neonate, α-thalassemia trait or iron deficiency should be considered.

Mean Corpuscular Hemoglobin

Similar to the MCV, the mean corpuscular hemoglobin is higher in preterm and term neonates, ranging from 33 to 41 pg/cell compared with 27 to 31 pg/cell in adults.

Mean Corpuscular Hemoglobin Concentration

The mean corpuscular hemoglobin concentration does not vary between neonates and adults. Although neonatal erythrocytes are larger and contain more hemoglobin, the hemoglobin in the erythrocyte is not more concentrated.

Erythrocyte Morphology

As previously noted, significant variations in neonatal erythrocyte morphology are commonly seen on peripheral blood smears, particularly in preterm infants, without intrinsic abnormalities of the erythrocyte. These variations include discocytes, bowls, echinocytes, and keratocytes (see Fig. 2-1).[3]

NEONATAL ANEMIA

Determining the cause of neonatal anemia can present a significant challenge. The differential diagnosis is extensive and includes not only many of the causes of anemia seen in older patients but also many causes unique to the fetus and newborn that are associated with pregnancy, labor, and delivery (Fig. 2-3). Knowledge of these disorders and an understanding of the associated

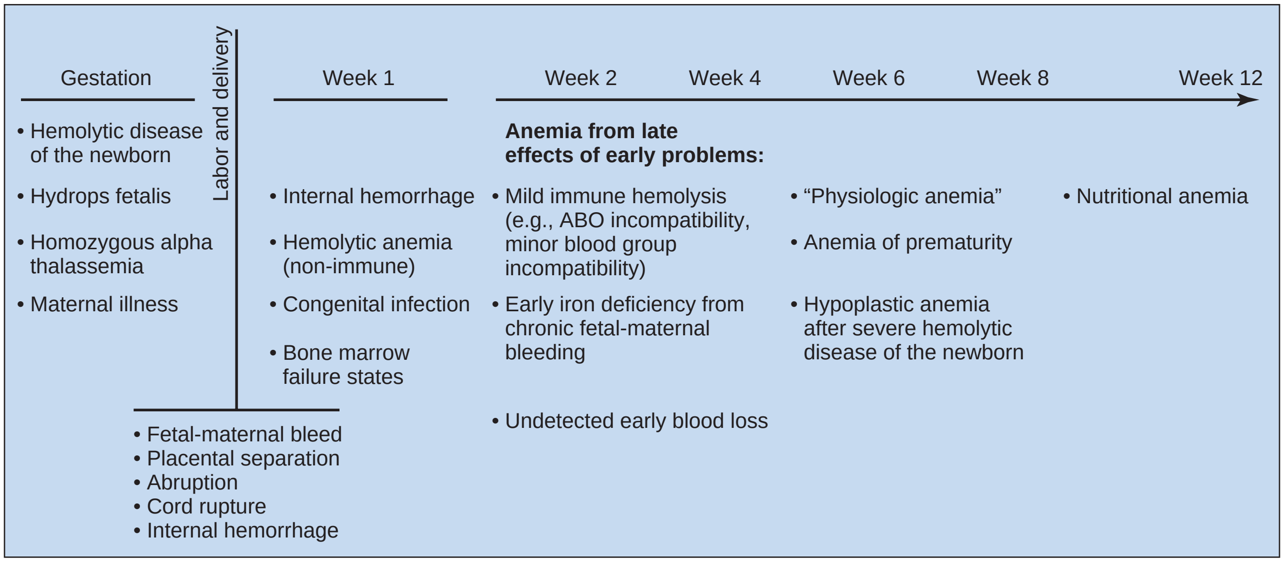

Gestation
Labor and delivery
Week 1
Week 2
Week 4
Week 6
Week 8
Week 12
• Hemolytic disease of the newborn
• Hydrops fetalis
• Homozygous alpha thalassemia
• Maternal illness
• Internal hemorrhage
• Hemolytic anemia (non-immune)
• Congenital infection
• Bone marrow failure states
Anemia from late effects of early problems:
• Mild immune hemolysis (e.g., ABO incompatibility, minor blood group incompatibility)
• Early iron deficiency from chronic fetal-maternal bleeding
• Undetected early blood loss
• "Physiologic anemia"
• Anemia of prematurity
• Hypoplastic anemia after severe hemolytic disease of the newborn
• Nutritional anemia
• Fetal-maternal bleed
• Placental separation
• Abruption
• Cord rupture
• Internal hemorrhage

Figure 2-3 Developmental differential diagnosis of neonatal anemia.

pathophysiology provide the appropriate context for the evaluation, diagnosis, and treatment of neonatal anemia. This chapter reviews anemia by etiology using the broad classifications of hemorrhage, hemolysis, and inadequate erythrocyte production.

HEMORRHAGE IN THE FETUS AND NEWBORN

Prenatal Hemorrhage

Fetal blood loss prior to birth is usually via the transplacental route, including fetomaternal hemorrhage (FMH), placental abruption, placenta previa, vasa previa, and twin-twin transfusion (Box 2-3).[35,36] Fetal blood loss, for instance due to intracranial hemorrhage in a thrombocytopenic infant with platelet isoimmunization or in a fetus after traumatic amniocentesis or cordocentesis, is less common.

Fetomaternal Hemorrhage (FMH)

FMH is the passage of fetal blood into the maternal circulation before or during delivery. Antenatal FMH is associated with variable effects on the fetus and neonate, ranging from none to neurologic injury, hydrops fetalis, and in utero demise of the fetus or, in the neonate, congestive heart failure (CHF), hypovolemic shock, persistent pulmonary hypertension, neurologic injury, and death.[36-38] The structure of the fetal-placental unit sets up a large pressure gradient from the umbilical artery to the intervillous space. The maternal intervillous space is separated from the pulsatile fetal circulation by a thin, two-cell membrane, with the constant possibility for rupture of the trophoblastic-endothelial juncture and contamination of the maternal intervillous space with fetal blood (Fig. 2-4, *A*). Given this very fragile mechanical relationship, it is surprising that significant FMH does not occur more frequently. Most spontaneous FMH occurs during the third trimester (Fig. 2-4, *B*), most commonly during labor and delivery,[39] although hemorrhage has been reported as early as 3 to 4 months' gestation (Fig. 2-4, C). Approximately 99% of deliveries are associated with FMH of less than 15 mL.[36] However, 3 in 1000 deliveries are associated with FMH greater than or equal to 30 mL, and 1 in 2000 pregnancies is associated with an FMH greater than or equal to 100 mL.[36,40]

Fetal RBCs can be detected in the maternal circulation in a number of ways. The most frequently used test is microscopic demonstration of fetal RBCs in the maternal circulation with use of the Kleihauer-Betke staining procedure.[17] With this method, HbA is denatured and eluted from smears of adult RBCs with an acid solution, leaving behind largely empty RBC membranes. Because HbF resists denaturation and elution, fetal cells stain darkly. Simple observation of the acid-treated maternal blood smear can provide a qualitative picture. The actual amount of fetal blood lost is estimated using the following formula: 2400 × the ratio of fetal to maternal cells = 1 mL of fetal blood.[41,42] Using this equation, it has been shown that most deliveries result in less than 3 mL of fetal blood in the maternal circulation, and only 0.3% have a fetomaternal transfusion of 10 mL or more. However, the volume of fetal blood in the maternal circulation does not reflect the timing or acuity of the hemorrhage.[36] Anti-HbF–based flow cytometry is an alternative method for detecting and quantifying cells that contain HbF in the maternal circulation.[36,43] Conditions that elevate maternal HbF production (e.g., thalassemia minor, sickle cell disease, hereditary persistence of HbF, and pregnancy-induced maternal HbF production) confound these analyses.[44] ABO blood group incompatibility can complicate interpretation of testing for FMH because removal of fetal cells from the maternal circulation by maternal anti-A or anti-B antibodies may lead to a false-negative result.

Abruptio Placentae

Placental abruption—that is, separation of the placenta from the uterine wall—occurs in approximately 1% of pregnancies. Abruption is more common in pregnancies

Box 2-3 Excessive Erythrocyte Loss Due to Hemorrhage

PRENATAL HEMORRHAGE

- Transplacental Hemorrhage
  - Fetomaternal hemorrhage
  - Abruptio placentae
  - Placenta previa
  - Vasa previa
  - Velamentous insertion of umbilical cord
  - Twin-twin transfusion
- Traumatic Fetal Hemorrhage
  - Maternal trauma
  - Amniocentesis
  - Cordocentesis
- Placental Lesions
  - Chorioangioma
  - Hematoma
  - Hemangioma
  - Choriocarcinoma
- Other Causes of Fetal Hemorrhage
  - Intracranial bleeding
  - Gastrointestinal bleeding

INTRAPARTUM HEMORRHAGE

- Placenta
  - Abnormalities of placentation previously listed
  - Surgical laceration, such as incision of the anterior placenta or cord at delivery
- Umbilical Vessels
  - Rupture of normal cord
  - Rupture of abnormal cord
    - Aneurysm
    - Varix
    - Cyst
    - Funisitis with weakened vessels
    - Short cord
  - Cord entrapment, for example, by forceps
  - Tight nuchal cord
  - Occult cord prolapse
- Intrapartum Neonatal Trauma
  - Cranial hemorrhage including subgaleal, subarachnoid, and intraventricular hemorrhage
  - Cephalohematoma
  - Splenic rupture
  - Adrenal hemorrhage
  - Liver laceration
  - Retroperitoneal hemorrhage

POSTPARTUM HEMORRHAGE

- Intracranial bleeding in the VLBW infant
- Gastric or intestinal ulceration
- Hemorrhage from vascular malformation
- Coagulopathy
- Iatrogenic causes
  - Phlebotomy
  - Tracheal mucosal tear during endotracheal intubation
  - Posterior pharyngeal tears during laryngoscopy or OGT placement
  - Vessel perforation during umbilical catheterization
  - Intercostal vessel laceration during thoracostomy tube placement
  - Gastric rupture from overdistention or OGT placement
  - Pulmonary hemorrhage during ventilation
  - Bladder mucosal tear during catheterization
  - Surgical wounds

OGT, Orogastric tube; *VLBW,* very low birth weight.

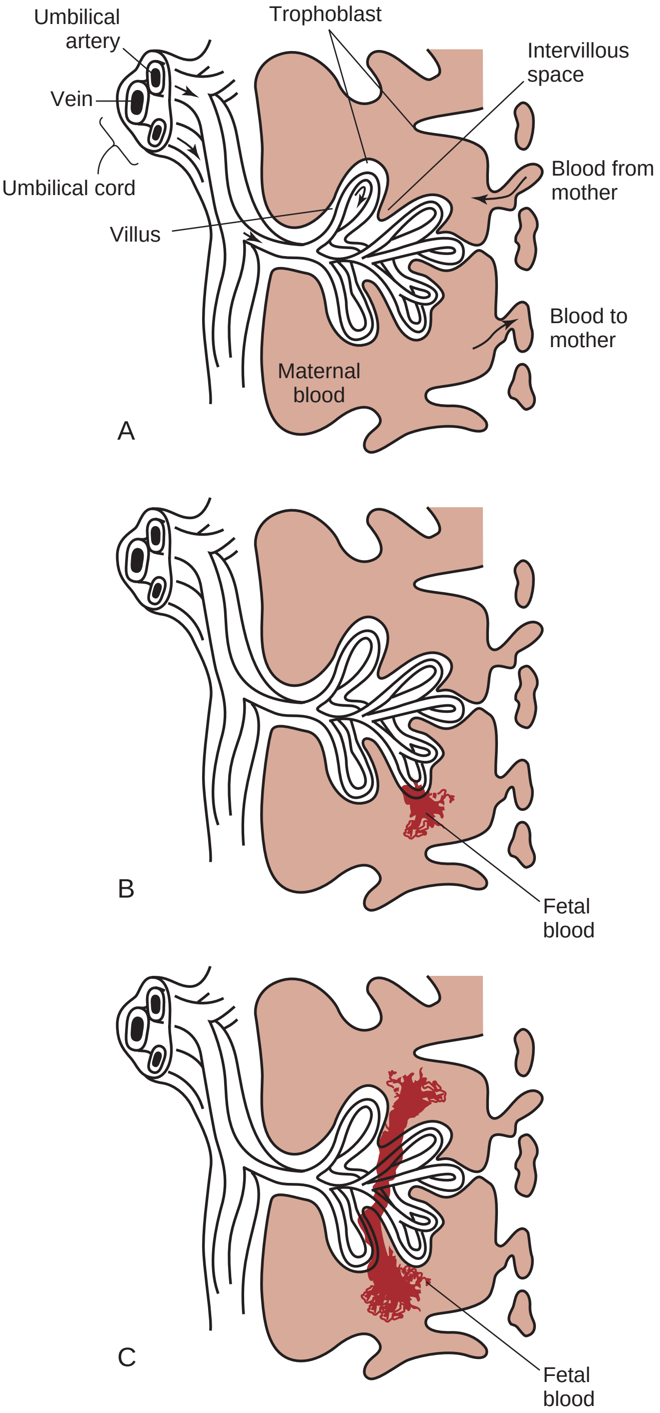


Figure 2-4 Anatomic issues in fetomaternal hemorrhage. **A,** Resting state: fragile capillary networks are present. **B,** A minor small bleed, known to occur in almost all pregnancies. **C,** A major possibility of significant exchange of fetal and maternal blood as a result of abruption (more severe) or normal childbirth.

complicated by chronic or pregnancy-induced hypertension.[31,45] The classic triad of placental abruption is vaginal bleeding, uterine contractions, and an irritable, tender uterus. If bleeding occurs in a retroplacental location, recognition and treatment may be delayed. Fetal hypoxemia may develop as a result of decreased placenta surface area and may be worsened by fetal hemorrhage accompanying the maternal bleeding. If fetal hemorrhage is significant, severe anemia, hypovolemia, and heart failure may rapidly develop, leading to in utero fetal demise.[46,47] Placental abruption is typically an acute event. However, fetal blood loss may occur gradually when the area of placental separation is small with a low-grade, chronic hemorrhage. At birth, these neonates exhibit anemia with compensation of varying degree, marked reticulocytosis, and prominent normoblastosis. In severe cases, iron deficiency may occur.

Placenta Previa

Placenta previa—that is, implantation of the placenta in a low-lying position in advance of the fetal presenting part, usually overlying part or all of the cervical os—occurs in approximately 1 in 250 births.[31,48] The classic presentation is acute onset of painless vaginal bleeding, with a peak incidence around 34 weeks' gestation. When placenta previa persists to the third trimester,[49,50] a cesarean section is indicated.[51] Fetal blood loss of varying amounts occurs in approximately 50% of cases. Signs of fetal anemia include intrauterine growth retardation, abnormal biophysical testing, and tachycardia. Vaginal bleeding may be intermittent and recurrent or massive and acute. In the former situation, the neonate presents with a partially or completely compensated anemia and marked reticulocytosis. In the latter situation, the neonate may present with acute hypovolemic shock with risk of death.[52] Up to 10% of neonates born after the occurrence of placenta previa exhibit significant anemia, making it the most common placental anomaly that causes neonatal anemia.[53]

Vasa Previa

Vasa previa occurs when fetal vessels exiting the placental end of the umbilical cord travel across the fetal membranes and then return to the fetal placental surface. If the fetal vessels are near the cervical os, they may tear when membranes rupture or during labor, when progressive cervical dilatation occurs. In both situations, laceration of the fetal vessels may lead to rapid exsanguination and death of the fetus.[54] Acute onset of vaginal bleeding (usually at the initiation of labor or during the progression of labor) with acute fetal tachycardia is a classic presentation of vasa previa. Prompt recognition of this condition, delivery, and resuscitation of the anemic infant are indicated to prevent peripartum death.[50]

Velamentous Insertion of the Cord

In cases of velamentous insertion of the cord, which occurs in approximately 1% of pregnancies, the umbilical vessels are unprotected and may tear spontaneously or during labor.[55] Rarely the unprotected umbilical vessel crosses the cervical os, combining a velamentous insertion with vasa previa, with a high potential for tearing when the membranes rupture.[56] Velamentous insertion is more common in twins and with low-lying placentas. The presentation of painless vaginal bleeding followed by signs of fetal distress is similar to vasa previa. The incidence of fetal loss is 1% to 2%.

Twin-Twin Transfusion and Related Disorders

Monochorionic twin pregnancies are at high risk for an adverse outcome because of vascular anastomoses that create shared fetal circulations. This situation may lead to complications, including twin-twin transfusion syndrome (TTTS), the twin anemia polycythemia sequence, the twin reversed arterial perfusion sequence, and monoamniotic twinning.[57-59] TTTS complicates 8% to 15% of twin gestations with monochorionic diamniotic placentation,[60,61] with the unbalanced transfer of blood from one fetus to the other via vascular anastomoses in the placenta.[62] Classically, the donor twin is small and anemic and the recipient is large and plethoric. Fetal demise may occur from exsanguination of the donor or circulatory overload in the recipient.[63] This condition may be dynamic with varying consequences to the twin fetuses, because the vascular anastomoses may vary in both size and number, as well as proceed in opposite directions.[64] Antenatal factors that may predict outcome include the presence of hydrops fetalis and gestational age.[65] Perinatal therapies have included serial amnioreduction, fetoscopic laser occlusion of communicating placental anastomoses,[66,67] maternal digoxin therapy, and selective feticide.[68,69]

At birth, both infants may be critically ill. The anemic donor twin may experience CHF and respiratory failure, whereas the plethoric recipient twin may require treatment for polycythemia hyperviscosity syndrome (PHS) and its complications, including thrombosis, hyperbilirubinemia, and, rarely, disseminated intravascular coagulation (DIC). In the classic presentation of TTTS in which the donor twin has been chronically hemorrhaging into the recipient, the donor anemic twin has marked reticulocytosis and normoblastosis, whereas the plethoric, polycythemic infant weighs greater than or equal to 20% more with a hemoglobin greater than or equal to 5 g/dL higher than its sibling. Acute transfusions, which typically occur closer to the time of delivery, do not lead to significant differences in neonatal weights, and there is an equal chance of either twin being the donor. Despite advances in pregnancy management, mortality in fetuses with TTTS remains high. The risk of long-term neurodevelopmental morbidity in survivors is significant.[70]

Traumatic Fetal Hemorrhage

Fetal bleeding has been described after multiple trauma and after blunt abdominal trauma to the pregnant mother.[71-73] It has also been described after traumatic amniocentesis or cordocentesis and after external cephalic version.[71-75]

Placental Lesions

The marked vascularity and unique double circulation of the placenta predisposes it to numerous vascular abnormalities, some of which may lead to fetoplacental hemorrhage and/or FMH.[76,77] Chorioangioma is the most common vascular anomaly of the placenta associated with neonatal anemia from fetoplacental hemorrhage and/or FMH. Chorioangiomas may number from one to many. In severe cases, they may lead to severe in utero hemorrhage and fetal demise.[78] Other placental lesions associated with neonatal anemia are hemangioma, hematoma, and intraplacental choriocarcinoma.[79-81]

Other Causes of Fetal Hemorrhage

In utero hemorrhage into fetal organs is uncommon,[82,83] with intracranial and intraabdominal hemorrhage predominating. Intracranial hemorrhage typically occurs when the pregnancy is complicated by thrombocytopenia (autoimmune or alloimmune), severe fetal hypoxemia, or maternal coumadin use.[84-87] Intraabdominal bleeding may occur after in utero volvulus, leading to ischemic bowel necrosis with venous hemorrhage.[88]

Intrapartum Hemorrhage

Hemorrhage due to events around the time of delivery is classified into cases involving the placenta and umbilical cord and cases related to neonatal trauma (see Box 2-3).

Placenta

Labor may exaggerate or worsen abnormalities of placentation (such as vasa previa, abruptio placentae, and velamentous insertion of the cord during labor) and lead to significant hemorrhage.

Laceration of the Placenta. An inadvertent placental incision during a cesarean section can lead to severe hemorrhage.[89,90] The risk of this complication is highest when the placenta is anteriorly placed and is lacerated during an emergency cesarean section or in cases in which placentation is unknown. Lower uterine incisions have also been associated with increased risk of placental injury.

Umbilical Vessels

One in 10,000 deliveries is associated with umbilical cord bleeding or rupture, which is associated with the risk of anemia, hypovolemic shock, and death. Cord rupture is most common in precipitous deliveries. Typically, traction on a shortened or abnormal umbilical cord leads to cord rupture, usually on the fetal side. Abnormalities such as cysts, vascular malformations (including aneurysms, hemangiomas, or varices), funisitis, or a velamentous insertion can weaken the cord and predispose it to rupture.[91] Entrapment of the cord accompanied by direct pressure, for example, by forceps, can also lead to traumatic cord laceration.

Neonates born with a tight nuchal cord can lose up to 20% of their blood volume into the placenta, leading to anemia and hypovolemic shock.[92] In cases of a tight nuchal cord, placental blood is blocked from flowing into the fetus by constriction of the thin-walled umbilical vein, while blood flow out of the fetus into the placenta via the muscular umbilical arteries continues.[93]

Intrapartum Trauma

Neonates may experience varying degrees of anemia as a result of internal bleeding associated with intrapartum trauma.[89] These internal hemorrhages may or may not be obvious at the time of delivery. In severe cases, signs of hypovolemic shock are seen shortly after birth. In other cases the infant may present with sudden pallor, tachycardia, and hypotension later on the first or second day of life. The site of internal bleeding may be discerned by the intrapartum history and other individual characteristics. Marked hyperbilirubinemia may occur.

Cranial Hemorrhage. Cranial hemorrhage is common in neonates because the head is typically the presenting part. Trauma to the scalp, the skull, or intracranial structures may result from traction applied manually—for example, by forceps or vacuum-assist devices. Cranial hemorrhage is associated with varying degrees of blood loss.

Subgaleal Hemorrhage. Bleeding into the subgaleal space usually occurs after a difficult, instrument-assisted delivery.[94,95] Trauma and/or traction tears of the emissary or "bridging" veins, which are connections between the dural sinuses and the scalp veins, leads to accumulation of blood in the potential space between the galea aponeurotica and the periosteum of the skull. The subgaleal space extends from the base of the skull forward to the orbital ridge and laterally to the temporal fascia and is thus able to accommodate significant amounts of blood.[96] Prompt recognition of subgaleal hemorrhage, with the finding of ballotable fluid in dependent regions of the head, is critical, and close monitoring should be provided. The degree of bleeding may lead to hypovolemic shock,[97] and exsanguination into a subgaleal hemorrhage with neonatal death has been reported. The occurrence of an extensive subgaleal hemorrhage should prompt evaluation for intracranial hemorrhage and neonatal coagulopathy.

Cephalohematoma. In cases of cephalohematoma, the hemorrhage is confined by the periosteum and limited to the area overlying a single bone of the skull. Giant cephalohematomas may result in neonatal anemia and hyperbilirubinemia.[98]

Intracranial Hemorrhage. Intracranial bleeding is most frequently found after a difficult delivery, in preterm infants, and in infants with disorders of coagulation, such as platelet isoimmunization or hemophilia.[99] Traumatic delivery leading to subdural, subarachnoid, and/or epidural hemorrhage may lead to anemia, even in term infants without coagulopathy. Preterm infants are at high risk for intraventricular hemorrhage,[100] which may approach 10% to 15% of the total blood volume. Although signs of acute blood loss may be present, anemia is rarely the only associated clinical finding.

Abdominal Hemorrhage. Traumatic intraabdominal hemorrhage is typically observed after a difficult delivery with prolonged traction and/or compression of the abdomen, particularly during breech extraction.[101] Patients may present with shock, abdominal distention, and discoloration of the abdominal wall or scrotum anytime from shortly after birth to up to 10 to 14 days of life. The diagnosis is suspected on the basis of the intrapartum history and clinical course, with confirmation by abdominal imaging—usually ultrasound. Some persons advocate repeat imaging of the abdomen after 12 to 24 hours to examine whether equilibration has occurred.

Adrenal Hemorrhage. Hemorrhage of the adrenal gland is uncommon in the neonatal period.[89,90] The adrenal gland is normally enlarged in the neonate, which, combined with its vascularity, predisposes it to birth trauma and hemorrhage. Adrenal hemorrhage may also occur in neonates with coagulopathy or infection or after acute hypoxia or asphyxia.[102] Adrenal hemorrhage has been associated with renal dysfunction, intestinal obstruction, and hyperbilirubinemia.[103] Bilateral adrenal hemorrhage, independent of the cause, can predispose the patient to adrenal collapse and an Addisonian crisis.

Hepatic Hemorrhage. Liver laceration, which occurs more frequently than recognized, is present in 1.2% to 5.6% of fetal and neonatal autopsies. Liver laceration may present immediately after birth or the infant may appear well for the first 12 to 24 hours of life because the hemorrhage is initially confined within the surrounding capsule.[104] When the hepatic capsule ruptures, hemoperitoneum, abdominal distention and discoloration, and hypovolemic shock rapidly ensue.[105] In some cases, a palpable mass in the right upper quadrant is present. Aggressive resuscitation and prompt surgical intervention have been lifesaving in affected neonates, but mortality is high.[106]

Splenic Hemorrhage. Splenic rupture may occur after traumatic delivery of a normal infant or after delivery of an infant with marked splenomegaly.[107] Splenic enlargement may be due to a congenital infection or extramedullary hematopoiesis—for example, as a result of erythroblastosis fetalis. Rupture may occur at delivery or later.[108] It has been reported to occur during exchange transfusion.[109] Symptoms of splenic rupture are similar to those of hepatic laceration.

Postpartum Hemorrhage

Neonatal Coagulopathy

Neonatal anemia may result from marked bleeding due to abnormalities in hemostasis, particularly at intracranial and gastrointestinal sites, or after trauma, such as circumcision. Disorders most commonly associated with significant hemorrhage in the neonate include congenital factor deficiencies, particularly hemophilia, and acquired and inherited platelet abnormalities.[110] Neonatal hemorrhage with secondary anemia has occurred in infants with vitamin K deficiency as a result of maternal anticonvulsant therapy or in exclusively breast-fed infants who were not treated with vitamin K at birth.[111] Neonatal anemia has also been reported in infants with Wiskott-Aldrich syndrome and thrombocytopenia–absent radius syndrome.

Iatrogenic Causes

Phlebotomy-associated blood loss leading to anemia may occur in hospitalized neonates, particularly premature infants. It is not surprising that these critically ill premature infants require the most phlebotomy procedures for diagnostic testing[112] or that they experience the most additional blood wastage (phlebotomy overdraw) during this testing.[113] Numerous noninvasive monitoring techniques and microscale laboratory analyses have been developed to avoid these phlebotomy-associated losses. Other iatrogenic causes of hemorrhage and anemia include vascular or tissue damage during diagnostic or therapeutic procedures, accidental disconnection of vascular catheters, and postoperatively associated with operative site or wound.

Other Causes of Hemorrhage

Gastric and intestinal ulceration with hematemesis or hematochezia may cause significant anemia in the neonate. The Apt test permits differentiation of neonatal blood from swallowed maternal blood. Hemorrhage into large tumors such as sacrococcygeal teratomas and bleeding from vascular malformations such as hemangiomas, hemangioendotheliomas, and arteriovenous malformations as causes of neonatal anemia have been described.[114-117]

Clinical Manifestations of Fetal and Neonatal Hemorrhage

Neonates are less tolerant of acute hypovolemia than are adults. Whereas an adult can tolerate a 10% loss of blood volume, a loss of this degree in the neonate results in significant peripheral and circulatory effects. Loss of greater than 20% of blood volume in the neonate leads to circulatory collapse, hypovolemic shock, and a high mortality rate. Symptomatology in neonatal hemorrhage is variable and is affected by the timing and amount of blood loss and the degree of resultant hypovolemia, if any (Table 2-4).

Hemorrhage prior to delivery may present as anemia without signs of hypovolemia. If adequate time has elapsed from the time of hemorrhage until delivery, reticulocytosis may be present, which may be confused with a hemolytic process. However, jaundice is not prominent, blood smear morphology is not suggestive of a hemolytic process, and the Coombs test is negative, all providing additional evidence for prenatal hemorrhage rather than hemolysis.

If the in utero blood loss is chronic, the neonate may be asymptomatic or present at birth with findings of CHF, including pulmonary edema, hepatomegaly, and peripheral edema, because over time, the fetus compensates for blood loss by increasing total blood volume. If the amount of in utero blood loss has been significant but has occurred over a protracted period, the neonate may be normovolemic but pale at birth, with hypochromic, microcytic erythrocytes on a blood smear indicating iron deficiency.

Neonates with significant acute blood loss at the time of delivery are pale, tachypneic, tachycardic, and have decreased pulses and hypotension. Pallor may be striking. Differentiation from other causes of pallor in the neonate is critical because immediate resuscitation with volume replacement is indicated to prevent circulatory collapse and death (Table 2-5).[90] When the neonate is in shock, a blood transfusion is indicated. In cases of acute hemorrhage, hemoglobin levels are a poor guide to

TABLE 2-4 Characteristics of Acute and Chronic Blood Loss in the Newborn

Findings	Acute Blood Loss	Chronic Blood Loss
Clinical	Acute distress; pallor; shallow, rapid, and often irregular respirations; weak or absent peripheral pulses; low or absent blood pressure; no hepatosplenomegaly	Marked pallor disproportionate to distress; occasionally signs of CHF are present; pulses are usually normal; blood pressure is usually normal; hepatomegaly
Venous pressure	Low	Normal or elevated
Hemoglobin	May be normal initially, then drops quickly in first 24 hours	Low at birth
RBC morphology	Normochromic and macrocytic	Hypochromic and microcytic, anisocytosis
Serum iron	Normal at birth	Low at birth
Course	Prompt treatment of anemia and shock required to prevent death	Generally uneventful
Treatment	IV fluids, blood, and iron therapy later	Iron therapy, occasionally pRBCs

CHF, Congestive heart failure; *IV,* intravenous; *pRBCs,* packed red blood cells; *RBC,* red blood cell.

diagnosis and therapy because hemodilution has not yet occurred and hemoglobin levels may not have fallen dramatically enough to immediately indicate that crystalloid volume replacement without transfusion is adequate.[89,90]

Supplemental iron should be provided to all infants with significant blood loss, acute or chronic, to replace lost stores.

HEMOLYSIS IN THE FETUS AND NEWBORN

Hemolytic processes are common causes of anemia in the neonate (Box 2-4). As with adults, it is useful to classify hemolytic disorders as extrinsic or intrinsic. Specific hemolytic disorders are discussed in detail in other chapters. Elements specific to the fetus and newborn are discussed in this section.

Extrinsic Hemolysis

Blood Group Incompatibility

Immune-mediated hemolytic anemia due to blood group incompatibility is the most common cause of hemolytic anemia in the newborn period. Details of fetal and neonatal immune hemolytic disease are provided in Chapter 3.

Other Causes of Extrinsic Hemolysis

Hemolytic anemia is present in some neonates born to mothers with autoimmune disease, particularly systemic lupus erythematosus and rheumatoid arthritis.[118] Neonatal lupus syndrome is associated with the transplacental passage of maternal autoantibodies, including Ro/SS-A, Ro/SS-B, and La, as well as other poorly characterized

TABLE 2-5 Differential Diagnosis of Pallor at Birth

Feature	Asphyxia	Acute Blood Loss	Chronic Blood Loss	Hemolytic Disease
Respirations	Retractions, cyanosis	Rapid without effort	Little distress unless congestive heart failure	Rapid if congestive heart failure
Arterial blood pressure	May be elevated or decreased	May be elevated or decreased	Usually normal	Usually normal
Central venous pressure	Normal or elevated	Low	Normal or elevated	Usually normal
Hepatosplenomegaly	Organs may be pushed down by lungs	None	Hepatomegaly if congestive heart failure	Hepatosplenomegaly
Jaundice	Perhaps	If loss of blood into tissues (e.g., scalp)	Usually not	Yes
Hemoglobin	Normal	Normal initially, drops after 24 h	Low at birth	Low at birth
Red blood cell morphology	Normal	Normal/macrocytic	May be hypochromic microcytic	May have hemolytic morphology
Other	Responds to O_2 intrauterine	Weak pulses	—	May have positive direct Coombs test or positive family history

Modified from Oski F: Hematology: the erythrocyte and its disorders. In Nathan DA, Oski FA, editors: Hematology of infancy and childhood, *ed 4, Philadelphia, 1993, WB Saunders, p. 34.*

Box 2-4 **Etiology of Hemolysis in the Neonate**

EXTRINSIC HEMOLYSIS

- Isoimmunization
  - Rh sensitization
  - ABO
  - Others, such as Duffy, Kell, and Lewis
  - Maternal autoimmune disorders
  - Maternal medication use
- Microangiopathic Anemias
  - DIC
    - Sepsis
    - Congenital infection—TORCH, malaria
- Vascular—Related Causes
  - Kasabach-Merritt syndrome
  - Renal artery
  - Large vessel thrombosis
  - Severe aortic coarctation
  - Arteriovenous malformation
- Oxidant Exposure
- Other
  - Galactosemia
  - Prolonged or recurrent acidosis

INTRINSIC HEMOLYSIS

- Enzymopathies
  - Hexose monophosphate shunt abnormality, such as G6PD
  - Embden-Meyerhof defect (glycolysis), such as pyruvate kinase
  - Other
- Red Blood Cell Membrane Defects
  - Hereditary spherocytosis
  - Hereditary elliptocytosis and related disorders
  - Hereditary stomatocytosis
- Hemoglobinopathies
  - α-Thalassemia syndromes
  - β-Globin cluster deletions
  - Unstable hemoglobins

DIC, Disseminated intravascular coagulation; *G6PD,* glucose-6-phosphate dehydrogenase; *TORCH,* toxoplasmosis, rubella, cytomegalovirus, and herpes virus infections.

contributing factors.[119] Clinical findings may include cutaneous lesions, congenital heart block, thrombocytopenia, anemia, neutropenia, hepatobiliary disease, and pericarditis.[120,121] With the exception of heart block, which is permanent, clinical manifestations, including anemia, are self-limited.[122]

Maternal autoimmune hemolytic anemia has led to neonatal anemia of varying degree with a positive direct antiglobulin test.[123-125]

Cases of maternal medication administration (including penicillin) reported to cause hemolytic anemia in the newborn have been described.

Angiopathic Anemias

Disseminated Intravascular Coagulation. Disseminated intravascular coagulation (DIC), which is commonly associated with anemia in neonates, occurs most frequently in association with systemic infection. DIC may complicate neonatal sepsis, particularly sepsis due to group B streptococcus, *Escherichia coli*, or *Pseudomonas aeruginosa*. Congenital infections due to cytomegalovirus, toxoplasmosis, syphilis, rubella, herpes simplex, and human immunodeficiency virus (HIV) have been associated with neonatal hemolytic anemia, with or without DIC.[31,121,126-128] Such cases may present a diagnostic dilemma when other causes of hemolysis are not found. Differentiating these common disorders clinically can be an aid to diagnosis (Table 2-6). Neonatal enteroviral infection has been complicated by DIC and anemia.[129] Although typically associated with hypoplastic anemia, parvovirus B19 infection may present with hemolysis as well (discussed later).

Neonates with necrotizing enterocolitis and infection with *Clostridium welchii* or *Clostridium perfringens* have experienced the acute development of severe, rapidly progressive intravascular hemolysis. This condition may lead to hemolysis of the entire RBC mass with acute renal failure, hypovolemic shock, and death.[130,131] These clostridial organisms produce several hemolytic toxins, one of which cleaves sialic acids from erythrocyte membrane glycoproteins, thus forming and exposing the Thomsen-Friedenreich cryptoantigen (or T antigen).[132] Anti-T antibodies are present in almost all adult plasma, and thus transfusion of adult blood to a neonate lacking T antibodies can lead to life-threatening hemolysis.

Vascular-Related Causes, Including Kasabach-Merritt Syndrome. Neonatal hemolytic anemia with microangiopathic changes has been associated with arteriovenous malformations, cavernous hemangiomas, renal artery stenosis, severe coarctation of the aorta, and large vessel thrombi. Kasabach-Merritt syndrome, a local consumptive coagulopathy with microangiopathic anemia, thrombocytopenia, and hypofibrinogenemia in association with significant vascular malformations, typically presents in the neonatal period.[133,134] The coagulopathy and anemia, which may be persistent and severe, improve with regression or removal of the malformation.

Oxidant Exposure

Oxidant hemolysis in the newborn is not rare, and it is not always associated with an inherited metabolic abnormality of the erythrocyte (see Chapters 17 and 18) or with congenital Heinz body anemia (see Chapter 19). An acute hemolytic event occurs, presumably related to the oxidant sensitivity of neonatal red cells. Infants present with acute onset anemia and hyperbilirubinemia with “bite” and “blister” cells, spherocytes, and irregularly shaped RBCs on a peripheral blood smear. Typically no obvious source for the presumed oxidant stress is identified and the episode resolves in a few days. Transfusion is rarely required. Infants with ongoing hemolysis warrant further diagnostic evaluation (see Chapters 18 and 19).

Other Causes of Hemolysis

Other causes of neonatal hemolysis include galactosemia, prolonged or recurrent metabolic acidosis, lysosomal storage disease, and some amino acidopathies. Although typically associated with a hypoplastic anemia and thrombocytopenia, hemolysis may be found in neonates with osteopetrosis. Maternal ingestion of valproic acid has caused hemolysis and thrombocytopenia in a breastfed infant.[111]

TABLE 2-6 Diagnostic Features of Some Transplacentally Acquired Infections Compared with Erythroblastosis Fetalis

Finding	Congenital Syphilis	Toxoplasmosis (Generalized Form)	Cytomegalic Inclusion Disease	Rubella Syndrome	Erythroblastosis Fetalis	Nonimmune Hemolytic Anemia
Anemia	++++	+++	++	++	+++	++
Jaundice	+++	+++	+++	+	+++	++
Thrombocytopenia	++	+	+++	+++	+	0
Hepatomegaly	++++	+++	++++	++	++	+
Splenomegaly	++++	++++	++++	++	++	+
Purpura	++	+	+++	+++	+	0
Skin rash	+	+	0	+	0	0
Chorioretinitis	+	+++	+	+	0	0
Intracranial calcifications	0	+	+++	7	0	0
Generalized edema	++	+	+	7	+	0
Additional findings	Mucocutaneous lesions; periostitis; snuffles; positive serology	Convulsions; microcephaly; hydrocephaly; positive dye test; lymphadenopathy	Pneumonia; cytomegalic inclusion cells in urine	Cataract; glaucoma; heart defects; deafness; microcephaly; hydrocephaly; bone lesion; rubella virus recoverable	Positive Coombs test; evidence of blood group incompatibility between mother and child	Morphology usually helpful

Modified from Oski F, Naiman J: The hematologic aspects of the maternal-fetal relationship. In Oski F, Naiman J, editors: Hematologic problems of the newborn, *ed 3, Philadelphia, 1982, WB Saunders, p. 36.*

0, Not described; *+,* present in about 1% to 25% of cases; *++,* present in about 26% to 50% of cases; *+++,* present in about 51% to 75% of cases; *++++,* present in about 76% to 100% of cases.

Intrinsic Hemolysis

Disorders of Erythrocyte Metabolism

Congenital nonspherocytic hemolytic anemia includes disorders not attributed to immune-mediated disturbances, defects of hemoglobin or the erythrocyte membrane, or other disorders. The disorders in this heterogeneous group are caused by a variety of metabolic abnormalities of the erythrocyte, including enzymopathies of glucose, glutathione, and nucleotide metabolism (see Chapters 17 and 18). Significant clinical, laboratory, and genetic heterogeneity exists within individual disorders. Hemolysis develops as a result of enzyme or antioxidant deficiency or impaired enzyme function.

Disorders of the Hexose Monophosphate Shunt and Associated Pathways

Disorders of the hexose monophosphate shunt or the glutathione metabolic pathways impair the ability of the erythrocyte to adequately respond to oxidant stress. In the normal erythrocyte, reduced glutathione detoxifies intracellular oxidants. In glucose-6-phosphate dehydrogenase (G6PD) deficiency, the inability to generate reduced nicotinamide adenine dinucleotide phosphate leads to inadequate levels of reduced glutathione, leaving oxidants free to damage critical erythrocyte proteins. Oxidation of the sulfhydryl groups of hemoglobin produces functionless methemoglobin and intracellular precipitates of hemoglobin called Heinz bodies.[135]

Three clinical syndromes have been described in patients who are deficient in G6PD: neonatal jaundice (NNJ), congenital nonspherocytic hemolytic anemia, and acute hemolysis after an oxidative stress.[136] All three have been observed in the neonatal period.[136] NNJ, which is rarely present at birth, has a peak onset between the second and third day of life. The severity of hyperbilirubinemia is variable, but it may be severe, resulting in kernicterus or even death.[137] Hyperbilirubinemia is usually adequately treated with phototherapy,[138] but occasionally exchange transfusion is necessary. Anemia is rarely severe in neonates with NNJ, and when it is observed, it should prompt investigation for additional abnormalities.[139] The cause of NNJ in neonates with G6PD deficiency is unknown. Contributions from decreased hepatic excretion of bilirubin, low-grade hemolysis, or manifestation of an additional genetic disorder have been suggested.[140] The incidence of NNJ is increased in infants who are deficient in G6PD, with a polymorphism of the uridine diphosphoglucuronosyl transferase gene (*UGT1A*) associated with Gilbert syndrome (see Chapter 4).[141]

Hemolytic anemia, even with nonimmune hydrops fetalis, has occurred in the G6PD-deficient fetus or neonate after maternal ingestion of an oxidant agent such as a sulfa drug, ascorbic acid, or fava beans.[142-144] Hemolysis has occurred in breastfed, G6PD-deficient infants after maternal ingestion of fava beans.[145-147] These cases have led to the term *favism by proxy*.

Significant hemolysis after transfusion or exchange transfusion with G6PD-deficient erythrocytes in neonates has been described.[148,149] In areas endemic for G6PD deficiency, it has been recommended that blood donors be screened for G6PD deficiency prior to neonatal transfusion.

The diagnosis and treatment of G6PD deficiency is discussed in Chapter 18.

Disorders of the Embden-Meyerhof Pathway

Defects of the Embden-Meyerhof pathway are inherited in an autosomal-recessive fashion, and typically hemolysis is only seen in homozygotes or compound heterozygotes. An exception is phosphoglycerate kinase, an X-linked disorder in which hemolysis is only seen in affected males.

Pyruvate kinase deficiency, which is the most common abnormality of the Embden-Meyerhof pathway, usually presents in the neonatal period or early childhood with anemia and jaundice (see Chapter 17). Severe cases have presented with anemia in utero or in the first days of life.[150,151] Nonimmune hydrops fetalis has been described.[150] Neonatal hyperbilirubinemia is common in persons deficient in pyruvate kinase, and phototherapy or exchange transfusion may be required.[152] Kernicterus has been reported.

Other abnormalities of the Embden-Meyerhof pathway may present in the neonatal period.[19] Glucose phosphate isomerase deficiency is the third most common hemolytic enzymopathy and is frequently manifested in infancy. Marked fetal and neonatal anemia, nonimmune hydrops, hyperbilirubinemia, and perinatal death have been described.[153] Patients with hexokinase deficiency have experienced neonatal anemia and hyperbilirubinemia. Similarly, NNJ and anemia have been associated with 2,3-biphosphoglycerate mutase deficiency and phosphoglycerate kinase deficiency.

Disorders of Glutathione Metabolism

Deficiency of γ-glutamylcysteine synthetase and glutathione synthetase, enzymes involved in erythrocyte glutathione synthesis, has been associated with anemia in the neonatal period.[154,155]

Disorders of the Erythrocyte Membrane

Disorders of the erythrocyte membrane (see Chapter 16) make up an important group of hemolytic anemias that may present in the fetal or neonatal period. HS, hemolytic hereditary elliptocytosis (HE), and hereditary pyropoikilocytosis are the membrane disorders most likely to present in the fetus and newborn.

Hereditary Spherocytosis. HS frequently presents in the neonatal period with anemia and jaundice.[156,157] Other findings include pallor, jaundice, tachypnea, and poor feeding. Anemia is present in more than half of patients and may require transfusion. Neonates with HS typically experience more significant hemolysis than do adults. Alterations in the neonatal intraerythrocytic environment, microcirculatory effects, and decreased membrane stability due to destabilization of the membrane junctional complex by elevated levels of 2,3-DPG all may worsen hemolysis.[158] In addition, the shorter life span of the neonatal erythrocyte and the sluggish response of the marrow to erythropoietic stress in the first year of life contribute to the anemia. A longitudinal study of 23 infants with HS from birth to 1 year demonstrated an inadequate reticulocyte response and the need for transfusion in the first few months of life that gradually improved.[159] Severe HS cases with in utero anemia and nonimmune hydrops fetalis have been described.[160,161]

Hyperbilirubinemia, which is present in more than half of neonates with HS, occurs in the first few days of life. Levels of bilirubin may rise rapidly. Kernicterus has been described, and exchange transfusion may be required.[156] Similar to G6PD deficiency and other inherited anemias, co-inheritance of the Gilbert syndrome *UGT1A* gene polymorphism may accentuate hyperbilirubinemia in neonates with HS.[162]

Hereditary Elliptocytosis, Hereditary Pyropoikilocytosis, and Related Disorders. HE rarely presents in the neonatal period. The exceptions are hemolytic HE and hereditary pyropoikilocytosis[163] in which hemolysis, anemia, and jaundice may be severe, requiring treatment with phototherapy and blood transfusion.[164-166] Elliptocytosis, pyknocytosis, and fragmented cells may be prominent on a peripheral blood smear.[167,168] In most infants with hemolytic HE and hereditary pyropoikilocytosis, hemolysis wanes during the first year of life and typical HE proceeds to develop in the child with minimal to no anemia. Many of these patients have parents with typical HE and thus are homozygotes or compound heterozygotes for defects inherited from each of the parents. Southeast Asian ovalocytosis, a common HE syndrome in Southeast Asia and the South Pacific, usually presents with neonatal anemia and hyperbilirubinemia.[169]

Hereditary Stomatocytosis Syndromes

The hereditary stomatocytosis (HSt) syndromes are a heterogeneous group of disorders characterized by variable numbers of stomatocytes on a peripheral blood smear and abnormal permeability of erythrocytes to sodium and potassium, with resultant modification of intracellular water content.[170] HSt has presented in the neonatal period with severe hemolysis, anemia, hyperbilirubinemia, and hydrops fetalis. The dehydrated variant of HSt, hereditary xerocytosis, may present in utero with nonimmune hydrops fetalis unrelated to the degree of fetal anemia.[171,172] Hereditary xerocytosis has been linked to mutations in the mammalian mechanosensory transduction protein PIEZO1.[173,174]

Hemoglobin Disorders

Significant variation exists in the fetal and neonatal manifestations of α- and β-thalassemia because of developmental differences in globin chain synthesis. α-Globin synthesis is found in the yolk sac by 3 weeks' gestation. By 9 weeks' gestation, α-globin is the main α-like globin in the fetus, with only low levels of the major embryonic α-like embryonic hemoglobin, hemoglobin Portland ($\zeta_2\gamma_2$). In contrast, the switch from fetal to adult β-like globin chains, γ to β-globin, is not complete until 6 to 12 months of life. The primary hemoglobin in fetal and neonatal life is fetal hemoglobin, HbF ($\alpha_2\gamma_2$). Thus abnormalities of α-globin, such as homozygous α-thalassemia, lead to severe anemia in utero, whereas abnormalities of β-globin, such as homozygous β-thalassemia, are typically ameliorated in the first few months of life by sustained production of HbF. (See Chapter 21 for a detailed discussion of the thalassemias.)

α-Globin Defects

Clinical severity of the α-thalassemia syndromes is related to the numbers of functional α-globin genes present.[175,176] Deletion of a single α-globin gene results in a clinically silent asymptomatic carrier state. Deletion of two α-globin genes results in α-thalassemia trait with marked microcytosis, hypochromia, and mild anemia. Deletion of three α-globin genes results in hemoglobin H (Hb H) disease (see the section on Hb H disease in this chapter) with varying degrees of anemia, ranging from mild to severe.[177] Deletion of all four α-globin genes results in homozygous α-thalassemia, which usually leads to death of the affected fetus in utero from severe hemolytic anemia, CHF, and nonimmune hydrops fetalis.

Homozygous α-Thalassemia. In homozygous α-thalassemia, the major hemoglobin present is a tetramer of unpaired non-α chains, γ_4, or hemoglobin Bart's. Survival of the homozygous fetus to late gestation depends on the presence and amount of embryonic hemoglobin Portland. In the rare case where the child is born alive, death from cardiopulmonary collapse usually occurs shortly after birth. Advances in prenatal genetic diagnosis, interventional obstetric practice, and neonatal critical care have altered the clinical course of patients with homozygous α-thalassemia.[178,179] Genetic screening of at-risk populations, particularly Southeast Asians, has allowed identification of couples at risk for conceiving an affected fetus. In more than a dozen cases of homozygous α-thalassemia, careful pregnancy surveillance, in utero transfusion therapy, and postnatal transfusion therapy with iron chelation have led to prolonged survival.[179-181] Concerns about congenital malformations and potential long-term neurologic effects of prolonged intrauterine hypoxia exist.[178] Similar to some patients with β-thalassemia, bone marrow transplantation may provide a potential cure for these patients.

Hb H Disease. Infants with Hb H disease present with hypochromic, hemolytic anemia with microcytosis and RBC fragmentation.[175,178] Neonatal hyperbilirubinemia may be severe.[182] Hydrops fetalis has been described in cases of nondeletional Hb H disease.[183,184] Hemoglobin electrophoresis demonstrates hemoglobin H (β_4) and hemoglobin Bart's ($^{\gamma}4$). The differential diagnosis of neonatal microcytosis, with or without anemia, includes Hb H disease, acute-on-chronic fetomaternal hemorrhage with iron deficiency, or early presentation of α-thalassemia trait. Family history and ethnic origin (such as Southeast Asian), pregnancy history, maternal Kleihauer-Betke testing, and neonatal hemoglobin electrophoresis will clarify the diagnosis.

β-Globin Defects

As previously noted, structural variants of β-globin, including β-thalassemia and sickle cell disease, typically do not present with neonatal anemia. However, the incidence of neonatal hyperbilirubinemia in sickle cell disease is increased. In one rare case of sudden infant death in an infant with sickle cell disease, widespread neonatal vasoocclusion was observed.[185]

γδβ and εγδβ Thalassemia. Large deletions of the β-globin gene cluster may lead to the phenotype of γδβ and εγδβt halassemia.[186-188] These patients frequently present as neonates with a hypochromic hemolytic anemia with prominent normoblastosis. Over time, the anemia improves and the erythrocyte morphology evolves to that similar to β-thalassemia trait.

Unstable Hemoglobins

Unstable hemoglobins (see Chapter 19) due to abnormalities in the α-globin or the β-globin chain have been reported to cause NNJ and hemolytic anemia. Precipitated globin chain fragments form Heinz bodies, thus the name *congenital Heinz body hemolytic anemia*. Two interesting representative hemoglobin variants that present with hemolytic anemia in the neonate due to congenital Heinz body hemolytic anemia are hemoglobin Hasharon and hemoglobin F Poole. Hemoglobin Hasharon is due to a mutation in the α-globin chain such that, when mutant α-globin chains are paired with γ-globin, an unstable hemoglobin is produced.[189] When mutant α-globin chains are paired with β-globin, it is no longer unstable, presumably because of the tenfold higher affinity between α and β chains than between α and γ chains. Hemoglobin F Poole is an unstable hemoglobin due to mutation in the γ-globin chain.[190] As might be expected, Hemoglobin Hasharon, hemoglobin F Poole, and other hemoglobin variants influencing HbF structure and function produce hemolytic anemia in the neonatal period that resolves as the fetal-to-adult hemoglobin switch occurs.

DECREASED ERYTHROCYTE PRODUCTION

Decreased erythrocyte production is an uncommon cause of neonatal anemia. The etiologies of this state can be classified as genetic syndromes,[191] bone marrow replacement syndromes, or infectious suppression, or they may be due to other rare causes (see Chapters 6 and 7; Box 2-5).

Genetic Syndromes

Diamond-Blackfan Anemia

Diamond-Blackfan anemia (DBA) is a congenital, pure RBC aplasia often diagnosed before the age of 6 months (see Chapter 7).[191-193] Thirteen percent to 16% of children

Box 2-5 **Etiology of Erythrocyte Underproduction in the Neonate**

GENETIC SYNDROMES

Diamond-Blackfan syndrome
Congenital dyserythropoietic anemia
X-linked familial dyserythropoietic anemia
Fanconi anemia
Shwachman-Diamond syndrome
Pearson syndrome
Aase syndrome

BONE MARROW REPLACEMENT SYNDROME

Congenital leukemia
Transient myeloproliferative disorder—Down syndrome
Osteopetrosis

INFECTIOUS SUPPRESSION

Viral—parvovirus B_{19}, cytomegalovirus, rubella, herpes simplex, and Coxsackie B
Protozoal—toxoplasmosis
Overwhelming bacterial infection

OTHER

Maternal deficiencies (usually nutritional or genetic) leading to neonatal anemia—iron, folate, and B_{12}
Medications to mother or neonate
"Late" hyporegenerative anemia of Rh disease or other blood group antigens

affected with DBA are diagnosed at birth. Prenatal manifestations of severe anemia such as hydrops fetalis have been described, but they are rare in fetuses with DBA.[194-196,197,198] It has been suggested that fetal erythropoiesis is spared in utero or that the erythropoietic defect is rescued by a putative placental or maternal factor.[192] Growth retardation occurs in approximately one quarter of patients. A wide variety of nonhematologic abnormalities may be observed that bring the affected neonate to medical attention, including physical anomalies, particularly unusual thumb changes (bifid, triphalangeal, hypoplastic, or absent) or radial abnormalities (hypoplasia or absent pulse).[199,200] Less specific anomalies include microcephaly, a low hairline, hypertelorism, a flat nose, a cleft palate, a high-arched palate, retrognathia, low-set ears, and a webbed neck. Rarer abnormalities include genitourinary, cardiac (septal defects), and bony involvement.

Fanconi Anemia

Fanconi anemia,[201] an autosomal-recessive disorder involving a number of organ systems, is characterized by a steroid-responsive, hypoplastic macrocytic anemia with reticulocytopenia (see Chapter 7).[191,202,203] Diagnosis in the neonatal period rarely occurs as a result of anemia but instead is suggested by associated physical abnormalities,[204] including hypoplasia and/or aplasia of the thumbs and/or radii.[205] Other findings apparent in the neonatal period may include intrauterine growth retardation, genitourinary or cranial defects, and hyperpigmentation of the skin. It has been observed that patients with Fanconi anemia who are diagnosed in the neonatal period have a much higher incidence of multisystem involvement than do patients diagnosed later in life.[206]

Shwachman-Diamond Syndrome

Shwachman-Diamond syndrome is a rare autosomal-recessive disorder associated with exocrine pancreatic insufficiency, growth failure in the newborn period or early infancy, and bone marrow failure (see Chapter 7).[207,208] Affected patients have presented in the neonatal period with neutropenia, thrombocytopenia, and/or anemia,[209] as well as thoracic abnormalities.[210] Aplastic anemia may develop in up to 25% of patients.

Pearson Syndrome

Pearson syndrome, a rare mitochondrial deoxyribonucleic acid deletion disorder, is associated with anemia, metabolic acidosis, and exocrine pancreatic insufficiency as a result of the deficiency of several enzymes involved in oxidative phosphorylation (see Chapter 7).[211-214] Affected patients occasionally lack the metabolic derangements and present only with sideroblastic anemia. Macrocytic anemia is present in the neonatal period in a quarter of patients and by 6 months of age in about three quarters of patients.[215] Nonimmune hydrops fetalis has been described. Neutropenia and thrombocytopenia, usually in a mild form, may also be present.[216]

Aase Syndrome

Aase syndrome is a very rare congenital hypoplastic anemia with abnormally digitized thumbs that may present in the neonatal period.[217] Growth failure and other bony abnormalities may occur.[218]

Congenital Dyserythropoietic Anemia

Congenital dyserythropoietic anemia (CDA) is the name given to a group of rare disorders characterized by ineffective erythropoiesis and anemia with distinct abnormalities of cellular architecture as seen on electron microscopy (see Chapter 7). Although not technically a bone marrow failure syndrome, CDA results in anemia with ineffective reticulocytosis. Three primary subtypes and numerous variants of CDA have been described.[219]

Type I CDA (CDA-I), characterized by megaloblastoid erythroid hyperplasia and distinct chromatin bridges between cells, is due to mutations in codanin-1.[220] Approximately half of infants with CDA-I present in the neonatal period with anemia, and occasionally they present with nonimmune hydrops fetalis and hyperbilirubinemia.[219,221-223] Nonhematologic manifestations of CDA-I identified in the neonatal period include intrauterine growth retardation, syndactyly, and other bony abnormalities.

Type II CDA (CDA-II) is the most common variant of CDA. It is characterized by multinuclear late erythroblasts and one positive acidified serum test and is due to mutations in *SEC23B*.[221,224] One study of patients with CDA-II revealed that approximately one quarter of patients had severe anemia in the neonatal period.[225] Hyperbilirubinemia was common, and exchange transfusions were required in a few cases. Nonimmune hydrops fetalis has been observed in patients with CDA-II.[226]

Type III CDA, which is characterized by multinuclear erythroblasts and macrocytosis and associated with mutations in *KIF23*, has presented in the neonatal period with anemia and hyperbilirubinemia.[227]

Patients with variant CDA due to mutations in erythroid transcription factors have presented in the perinatal period. Infants with X-linked anemia and thrombocytopenia due to a mutation in the erythroid and megakaryocyte transcription factor GATA1 have been described.[228-230] Affected male fetuses experience severe fetal anemia with nonimmune hydrops fetalis and require in utero transfusion. Neonatal thrombocytopenia and anemia are present, and marrow examination shows dyserythropoiesis. Infants who have severe fetal anemia with nonimmune hydrops fetalis and neonatal anemia associated with a mutation in the second zinc finger of the erythroid transcription factor KLF1 have been described. Affected patients experience hemolysis and ineffective erythropoiesis with elevated HbF and changes in numerous membrane proteins, including several blood group antigens.[231-233]

Variant CDA with extramedullary erythropoiesis presenting in the neonatal period has been described in patients with mevalonate kinase deficiency.[234,235] Other symptoms include intrauterine growth retardation, dysmorphic features, skeletal anomalies, cholestatic liver disease, diarrhea, and failure to thrive.[236]

Dyserythropoietic anemia presenting in infancy, along with exocrine pancreatic insufficiency and calvarial hyperostosis, has been reported due to mutations in COX412, a structural component of the cytochrome c oxidase complex.[237]

Bone Marrow Replacement Syndromes

Anemia can develop as a secondary effect of bone marrow replacement. This symptom has been observed in cases of transient myeloproliferative disorder (TMD) and is frequently associated with trisomy 21 (see Chapter 50), congenital leukemia (see Chapter 51), and osteopetrosis.

Transient Myeloproliferative Disorder

TMD is characterized by the proliferation of myeloblasts in the marrow and blood of affected newborns, mimicking congenital leukemia and leukemoid reaction.[238] It is typically observed in patients with trisomy 21. In contrast to congenital leukemia, spontaneous remission occurs early in infancy. Anemia and thrombocytopenia are commonly observed. Cases of TMD with nonimmune hydrops fetalis have been described.

Congenital Leukemia

True malignant congenital leukemias have been associated with in utero fetal demise, nonimmune hydrops fetalis, fetal or neonatal anemia, and thrombocytopenia.[239-242] Widespread infiltration of leukemic cells with visceral fibrosis due to megaloblastic proliferation in the liver has been described in cases of both TMD and congenital leukemia.[239,240,243]

Osteopetrosis

Osteopetrosis is a heterogeneous group of disorders.[244] Recessive malignant forms of osteopetrosis may present in the neonatal period with hepatosplenomegaly, craniomegaly, bony sclerosis, and disordered hematopoiesis, including progressive anemia.[244,245,246,247] The failure of osteoclasts to resorb and remodel bone leads to progressive ablation of the bone marrow cavity and extramedullary hematopoiesis in the spleen and liver and a progressive macrocytic anemia with reticulocytosis. Early bone marrow transplantation has been successfully used to treat these patients.[246,248]

Other Replacement Syndromes

Fetal and neonatal anemia has been described in cases of mucopolysaccharidosis type VII and neonatal hemochromatosis.[194]

Infectious Suppression

Congenital infection, in addition to hemolysis, may also lead to bone marrow failure in the neonatal period (see Box 2-5). A detailed discussion of associated infectious agents is beyond the scope of this chapter; however, infection with parvovirus B_{19} warrants discussion.

Parvovirus B_{19} Infection

Parvovirus B_{19} is the etiologic agent of erythema infectiosum (fifth disease),[249] a benign illness of children and adults that presents with fever, chills, vomiting, and diarrhea,[250] with the appearance of a maculopapular exanthem on the face (slapped-cheek syndrome) temporally associated with the abatement of the fever. Parvovirus B_{19} selectively infects erythroid precursors and inhibits their growth. Erythropoietic arrest leads to an acute decrease in hemoglobin with reticulocytopenia. Although this drop is tolerated by healthy children and adults, infection in susceptible pregnant women may bring about fetal infection,[251] leading to profound fetal anemia, nonimmune hydrops fetalis, and fetal demise.[252,253] Hydrops fetalis will develop in approximately 10% of fetuses whose mothers acquire a primary parvovirus B_{19} infection. If fetal demise does not occur, fetal anemia and the hydrops fetalis may gradually resolve. In utero transfusion has been successfully used to treat anemic, infected fetuses.[254,255]

When parvovirus B_{19} infection is suspected in an anemic, hydropic newborn, detection of parvovirus immunoglobulin M antibody in the infant, analysis of serial, paired fetal/maternal immunoglobulin G antibody, or detection of parvovirus protein or deoxyribonucleic acid in the neonate or placenta can confirm the diagnosis.[256] Typically the anemia associated with parvoviral infection resolves in 10 to 14 days, during which time supportive care is required. The long-term outcome of infants receiving in utero transfusion to treat parvoviral-induced anemia has been good.[257-259]

Other Causes of Erythrocyte Underproduction

Nutritional Anemia

Nutritional deficiencies are the most common cause of anemia in the first year of life, but they are an infrequent cause of anemia in the neonatal period.

Iron deficiency anemia is uncommon at birth. When present, it is most common in infants who have sustained significant chronic or acute prenatal blood loss, because approximately three quarters of the neonate's total body iron is stored as hemoglobin. Several factors contribute to the increased risk of preterm infants for iron deficiency, including rapid growth without adequate supplemental iron, iron losses due to phlebotomy, and their inability to regulate their iron status on the basis of physiologic needs with poor regulation of iron absorption and failure to increase gastrointestinal iron absorption despite decreased serum ferritin levels.[260]

Vitamin B_{12} deficiency has occurred in the breastfed infants of strict vegetarian mothers, in the infants of mothers with pernicious anemia, and in breastfed infants of mothers with chronic tropical sprue.[261,262] Vitamin B_{12} deficiency can also occur in infants with malabsorptive abnormalities or with inborn errors of vitamin B_{12} or related proteins, including deficiency of intrinsic factor, haptocorrin, or transcobalamin II.[263] Other related disorders that may be associated with vitamin B_{12} deficiency include the methylmalonic acidurias and deficiency of adenosylcobalamin or methylcobalamin (or both).

Folate deficiency is more likely to develop in preterm infants, in infants who are fed a folate-poor diet (including goat's milk or milk that has been boiled), and in infants with malabsorption. Other disorders associated with folate deficiency in infancy include congenital folate malabsorption, defective cellular folate uptake, and inborn errors of folate metabolism. Folate deficiency has also been reported in infants after exchange transfusion because the high levels of folate in the infant's blood have been replaced by normal adult values, which are relatively lower. The megaloblastic anemia of vitamin B_{12} or folate deficiency in infancy is similar to that observed in adults.

The sideroblastic, hypochromic anemia of copper deficiency, which is often associated with neutropenia, is rare in the neonatal period. Infants at risk for copper deficiency include preterm infants fed only milk, infants receiving prolonged parenteral nutrition without trace elements, and infants with chronic diarrhea and malnutrition.

Medications

Hypoplastic anemia has been observed in neonates born to mothers taking various medications. Administration of zidovudine (AZT) alone[264,265] and in combination with other antiretroviral agents[266-268] for the treatment of maternal HIV infection may result in a hyporegenerative neonatal anemia.

The Late Anemia of Rh Disease

In infants with Rh or Kell alloimmunization, a "late" anemia characterized by significant anemia with reticulocytopenia may develop at 1 to 3 months of age as a result of decreased erythrocyte production and/or continued destruction of reticulocytes from the persistence of maternal antibody.[269-271] This late anemia is found in infants who have received a transfusion and in infants who have not received a transfusion. Similar to anemia of prematurity (see related section in this chapter), relative reticulocytopenia is present despite elevated erythropoietin levels.[272] Transfusion may be required, particularly if the infant feeds poorly, has inadequate weight gain, and has persistent tachypnea or tachycardia. Although erythroid progenitors from these patients are responsive to erythropoietin in vitro, response to treatment with erythropoietin has been disappointing because its administration has failed to have an impact on the need for transfusion.

PHYSIOLOGIC ANEMIA AND ANEMIA OF PREMATURITY

Physiologic Anemia

Immediately after birth, a sudden increase in hemoglobin saturation occurs as the infant moves from maternal oxygenation to extrauterine circulation and pulmonary respiration. The degree of relative tissue hypoxia is dramatically altered, erythropoietin levels fall, and RBC production begins to decrease. Eventually, decreased oxygen-carrying capacity caused by decreasing hemoglobin concentration stimulates erythropoietin production, leading to increased RBC production and a higher hemoglobin concentration. Term infants experience this gradual decrease in hemoglobin concentration during the first 8 to 12 weeks of life (Table 2-7). The hemoglobin concentration then remains stable for several weeks before gradually increasing. In term infants, this decline and rise in hemoglobin concentration has been termed *physiologic anemia*. These infants are asymptomatic, and this process appears to be a normal adaptation of the switch from intrauterine to extrauterine life.

Anemia of Prematurity

Preterm infants also experience this decline and rise in hemoglobin concentration. Although mean cord hemoglobin levels do not vary significantly between preterm and term infants, the decline in hemoglobin concentration occurs earlier in preterm infants, around 4 to 8 weeks of age, and to values significantly lower than those of term infants, with the nadir varying inversely with gestational age (Fig. 2-5; Table 2-8).[273] This dramatic decrease in hemoglobin may lead to symptomatic anemia requiring transfusion. This condition, characterized by normocytic, normochromic anemia; reticulocytopenia; bone marrow hypoplasia; and erythropoietin levels inappropriately low for the level of anemia, is nonphysiologic[274-276] and is termed the *anemia of prematurity*. The degree of anemia varies with the degree of prematurity, nutritional status and intake, chronologic age, and the type and severity of the underlying illness.

Pathogenesis

The pathogenesis of anemia of prematurity is multifactorial.[277,278] Preterm infants have a blunted response to erythropoietin, the primary growth factor for erythropoiesis. Hypoxia is the stimulus for erythropoietin production and secretion. After secretion erythropoietin travels via the blood to hematopoietic tissues where it stimulates

TABLE 2-7 Normal Hematologic Values during the First Year of Life in Healthy Term Infants[*]

	AGE (mo)[†]						
n	0.5 (N = 232)	1 (N = 240)	2 (N = 241)	4 (N = 52)	6 (N = 52)	9 (N = 56)	12 (N = 56)
Hemoglobin (mean ± SE)	16.6 ± 0.11	13.9 ± 0.10	11.2 ± 0.06	12.2 ± 0.14	12.6 ± 0.10	12.7 ± 0.09	12.7 ± 0.09
–2 SD	13.4	10.7	9.4	10.3	11.1	11.4	11.3
Hematocrit (mean ± SE)	53 ± 0.4	44 ± 0.3	35 ± 0.2	38 ± 0.4	36 ± 0.3	36 ± 0.3	37 ± 0.3
–2 SD	41	33	28	32	31	32	33
RBC count (mean ± SE)	4.9 ± 0.03	4.3 ± 0.03	3.7 ± 0.02	4.3 ± 0.06	4.7 ± 0.05	4.7 ± 0.04	4.7 ± 0.04
–2 SD + 2 SD	3.9-5.9	3.3-5.3	3.1-4.3	3.5-5.1	3.9-5.5	4.0-5.3	4.1-5.3
MCH (mean ± SE)	33.6 ± 0.1	32.5 ± 0.1	30.4 ± 0.1	28.6 ± 0.2	26.8 ± 0.2	27.3 ± 0.2	26.8 ± 0.2
–2 SD	30	29	27	25	24	25	24
MCV (mean ± SE)	105.3 ± 0.6	101.3 ± 0.3	94.8 ± 0.3	86.7 ± 0.8	76.3 ± 0.6	77.7 ± 0.5	77.7 ± 0.5
–2 SD	88	91	84	76	68	70	71
MCHC (mean ± SE)	314 ± 1.1	318 ± 1.2	318 ± 1.1	327 ± 2.7	350 ± 1.7	349 ± 1.6	343 ± 1.5
–2 SD	281	281	283	288	327	324	321

From Saarinen UM, Slimes MA: Developmental changes in red blood cell counts, and indices of infants after exclusion of iron deficiency by laboratory criteria and continuous iron supplementation. J Pediatr *92:414, 1978.*

MCH, Mean corpuscular hemoglobin; *MCHC,* mean corpuscular hemoglobin concentration; *MCV,* mean corpuscular volume; *RBC,* red blood cell; *SD,* standard deviation; *SE,* standard error of the mean.

[*] These values were obtained from a selected group of 256 healthy term infants followed up at the Helsinki University Central Hospital who were receiving continuous iron supplementation and who had normal values for transferrin saturation and serum ferritin.

[†] Values at the ages of 0.5, 1, and 2 months were obtained from the entire group, and those at the later ages were obtained from the iron-supplemented infant group after exclusion of iron deficiency.

the proliferation and differentiation of erythroid cells. In the fetus, erythropoietin is produced primarily in the liver, with production switching to the kidney near term. The sensitivity of the mechanisms that sense hypoxia in the adult kidney are decreased tenfold in the fetal liver, and thus preterm infants have a decreased response to comparable levels of hypoxia and anemia compared with adults. Erythropoietin has greater clearance and a shorter half-life in preterm infants.[279,280] Other factors influencing hemoglobin concentration in the preterm infant include a shorter erythrocyte life span and dilution of hemoglobin by rapid growth. Continued phlebotomy and transfusion of adult blood with hemoglobin A further blunts the hypoxic stimulus for erythropoietin production and worsens anemia.

Diagnosis, Treatment, and Prevention

Infants thought to be symptomatic as a result of anemia of prematurity are reported to exhibit apnea, bradycardia, tachycardia, tachypnea, lethargy, poor feeding, increased oxygen requirement, and inadequate weight gain despite adequate caloric intake.[281,282] It has never been convincingly shown that symptoms of anemia are reduced after transfusion in a randomized, controlled prospective trial.[283-285] In fact, several studies have documented a lack of improvement in clinical status in transfused versus untransfused infants with anemia of prematurity,[286-289] leading to controversy regarding when—and at what levels—hemoglobin transfusion is indicated in preterm infants. Concerns about potential adverse effects of transfusion in preterm neonates, including transfusion-associated gut injury,[290-293] have led to further reexamination of the risks and benefits of transfusion therapy in this population.[294,295]

The success of erythropoietin therapy in decreasing transfusion in adult patients with end-stage renal disease offered a potentially novel treatment modality for anemia of prematurity. Pilot studies were encouraging, with erythropoietin enhancing erythropoiesis in anemic, preterm infants. At present, with more than 20 studies reported,[296] a consistent increase in erythropoiesis has been observed in erythropoietin-treated preterm neonates but without a consistent reduction in transfusions. Reviews of controlled clinical trials analyzing the efficacy of erythropoietin in reducing blood transfusions in infants with anemia of prematurity detected benefit across high-quality studies using conservative transfusion criteria, but the authors concluded that insufficient data were available to recommend erythropoietin therapy as standard treatment for anemia of prematurity.[297,298,299,300]

Retrospective analyses have raised concerns about the use of erythropoietin and the occurrence of severe retinopathy of prematurity in preterm infants.[301-303] Because previous studies were not designed to characterize the relationship between erythropoietin administration and retinopathy of prematurity, further studies have been proposed to address this issue.[304]

A more important goal than decreasing the number of blood transfusions in the treatment of infants with anemia of prematurity has been reducing the number of blood donors to which an infant is exposed.[305] This goal has led to the development of conservative transfusion criteria, decreases in phlebotomy-related losses, adoption of noninvasive monitoring technology, changes in

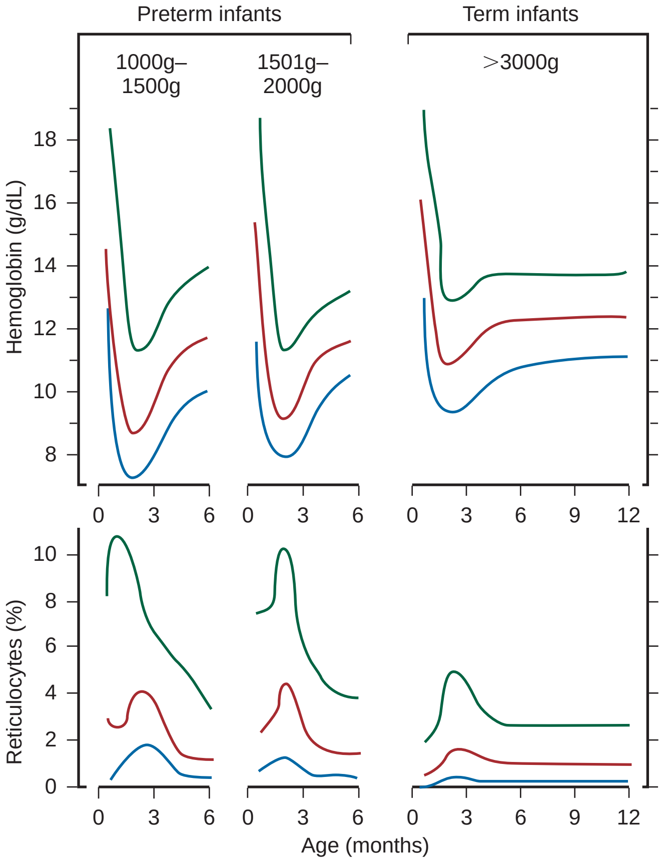


Figure 2-5 The mean upper *(green)* and lower *(blue)* ranges of normal hemoglobin and reticulocyte values for healthy and preterm infants. Premature infants become more anemic sooner after birth and their hemoglobin returns toward normal later. *(From Dallman PR: Anemia of prematurity.* Annu Rev Med *32:143, 1981.)*

clinical practice, provision of adequate supplemental iron and early protein supplementation, and the use of dedicated units of erythrocytes in neonatal transfusion therapy.[278,306,307,308-310] A single-donor unit that can be used multiple times over a period of 42 days is dedicated to an individual infant to avoid multiple donor exposure.[311]

Resurging interest has recently been expressed in delayed umbilical cord clamping at the time of birth.[312] Delayed cord clamping (DCC) leads to an increase in neonatal blood volume of up to 30%.[313] In the term infant, DCC increases iron stores, thereby decreasing the risk of anemia.[314] In the preterm infant, DCC improves transitional circulation, decreases the need for and the number of blood transfusions to treat anemia, decreases the risk of intraventricular hemorrhage, and may decrease the risk of late onset sepsis and necrotizing enterocolitis.[315-317] The American College of Obstetricians and Gynecologists supports DCC in preterm but not term infants.[318]

APPROACH TO THE ANEMIC INFANT

Detailed evaluation of the anemic neonate combines history, physical examination, and laboratory investigation. Use of a diagnostic algorithm may facilitate evaluation (Fig. 2-6).

A detailed maternal history should be obtained that includes medical history with particular attention to anemia, jaundice, transfusions, gallstones, or splenectomy, as well as ethnicity. The obstetric history also should be obtained, including complications of labor and delivery and a history of anemia or jaundice in previously delivered infants. Details of the pregnancy should be

TABLE 2-8 Red Blood Cell Parameters of Very Low Birth Weight Infants during the First 6 Weeks of Life

	Day of Life*	Valid *n*	PERCENTILES								
			3	5	10	25	Median	75	90	95	97
Hemoglobin (g/dL)	2	559	11.0	11.6	12.5	14.0	15.6	17.1	18.5	19.3	19.8
	12-14	203	10.1	10.8	11.1	12.5	14.4	15.7	17.4	18.4	18.9
	24-26	192	8.5	8.9	9.7	10.9	12.4	14.2	15.6	16.5	16.8
	40-42	150	7.8	7.9	8.4	9.3	10.6	12.4	13.8	14.9	15.4
Hematocrit (%)	3	561	35	36	39	43	47	52	56	59	60
	12-14	205	30	32	34	39	44	48	53	55	56
	24-26	196	25	27	29	32	39	44	48	50	52
	40-42	152	24	24	26	28	33	38	44	47	48
Red blood cells (1012/L)	3	364	3.2	3.3	3.5	3.8	4.2	4.6	4.9	5.1	5.3
	12-14	196	2.9	3.0	3.2	3.5	4.1	4.6	5.2	5.5	5.6
	24-26	188	2.6	2.6	2.8	3.2	3.8	4.4	4.8	5.2	5.3
	40-42	148	2.5	2.5	2.6	3.0	3.4	4.1	4.6	4.8	4.9
Corrected reticulocytes (%)	3	283	.6	.7	1.9	4.2	7.1	12.0	20.0	24.1	27.8
	12-14	139	.3	.3	.5	.8	1.7	2.7	5.7	7.3	9.6
	24-26	140	.2	.3	.5	.8	1.5	2.6	4.7	6.4	8.6
	40-42	114	.3	.4	.6	1.0	1.8	3.4	5.6	8.3	9.5

From Obladen M, Diepold K, Maier RF, et al: Venous and arterial hematologic profiles of very low birth weight infants. Pediatrics *106:708, 2000.*

*On day 3, all infants are included irrespective of antenatal steroids and transfusions up to day 3. Thereafter, infants who did not receive recombinant human erythropoietin were studied, irrespective of the use of antibiotics and steroids.

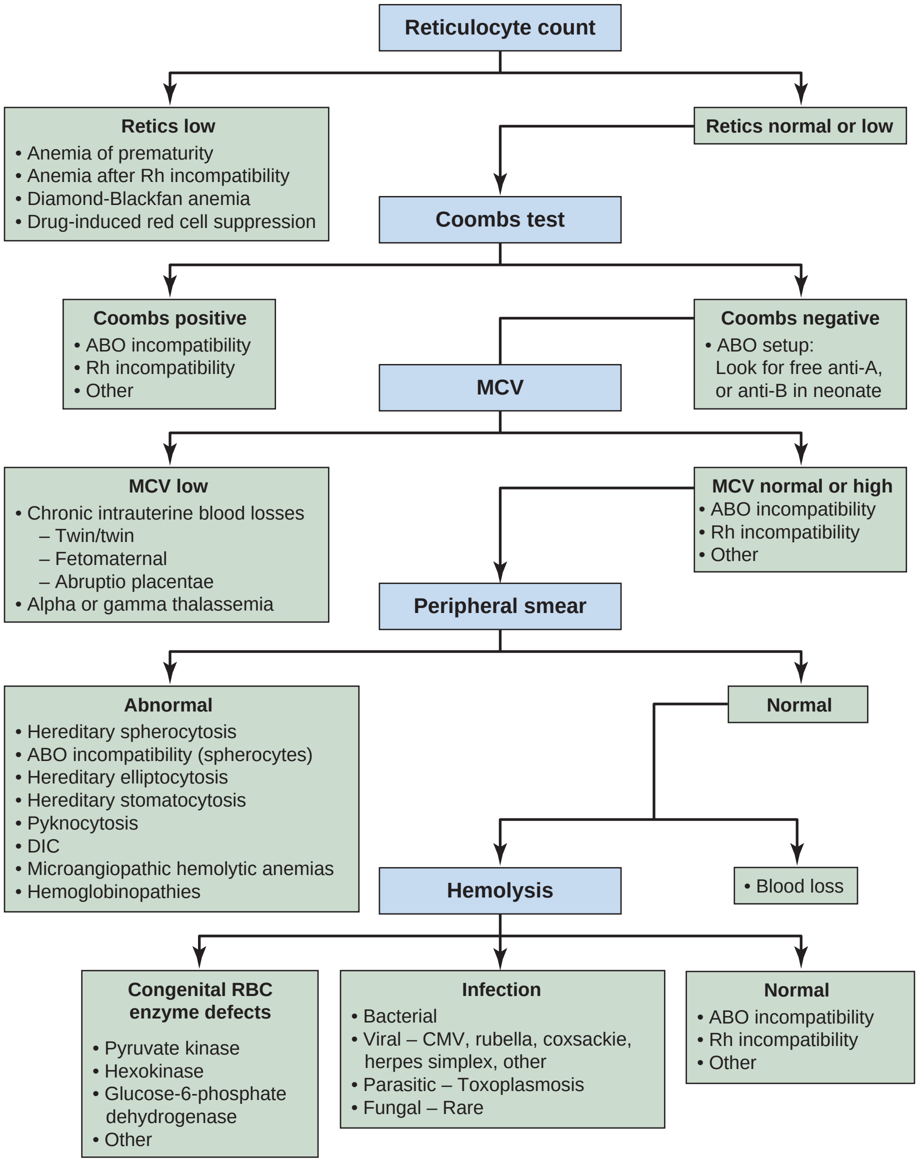

Reticulocyte count
Retics low
• Anemia of prematurity
• Anemia after Rh incompatibility
• Diamond-Blackfan anemia
• Drug-induced red cell suppression
Retics normal or low
Coombs test
Coombs positive
• ABO incompatibility
• Rh incompatibility
• Other
Coombs negative
• ABO setup: Look for free anti-A, or anti-B in neonate
MCV
MCV low
• Chronic intrauterine blood losses
– Twin/twin
– Fetomaternal
– Abruptio placentae
• Alpha or gamma thalassemia
MCV normal or high
• ABO incompatibility
• Rh incompatibility
• Other
Peripheral smear
Abnormal
• Hereditary spherocytosis
• ABO incompatibility (spherocytes)
• Hereditary elliptocytosis
• Hereditary stomatocytosis
• Pyknocytosis
• DIC
• Microangiopathic hemolytic anemias
• Hemoglobinopathies
Normal
Hemolysis
• Blood loss
Congenital RBC enzyme defects
• Pyruvate kinase
• Hexokinase
• Glucose-6-phosphate dehydrogenase
• Other
Infection
• Bacterial
• Viral – CMV, rubella, coxsackie, herpes simplex, other
• Parasitic – Toxoplasmosis
• Fungal – Rare
Normal
• ABO incompatibility
• Rh incompatibility
• Other

Figure 2-6 An approach to neonatal anemia. The asterisk indicates a smear with no predominant morphology. *CMV,* Cytomegalovirus; *DIC,* disseminated intravascular coagulation; *MCV,* mean corpuscular volume; *RBC,* red blood cell; *Retics,* reticulocyte count. *(From Oski F: Hematology: the erythrocyte and its disorders. In Nathan DA, Oski FA, editors:* Hematology of infancy and childhood, *ed 4, Philadelphia, 1993, WB Saunders, p. 38.)*

reviewed, including zygosity, medication use, history of vaginal bleeding, infections and exposure to infectious persons, exposure to oxidant compounds such as fava bean ingestion, and history of amniocentesis or trauma. Maternal blood type, antibody testing, and available hematologic data should be collected. Documentation of fetal status throughout the pregnancy should be obtained. The presence of congenital malformations, abnormal ultrasound findings, and amniocentesis and cordocentesis results, if performed, should be obtained.

Details of the labor and delivery including placentation, fetal distress, and history of external cephalic version should be sought. The route of delivery, use of instrumentation to assist extraction (such as forceps or vacuum-assist devices), breech delivery or difficult extraction, and the presence of a tight nuchal cord should all be noted. Placental or vaginal bleeding should be recorded. Placental findings should be collected.

A family history of anemia, jaundice, transfusions, gallstones, or splenectomy and paternal ethnicity should be sought.

The neonate's hospital course and available laboratory data should be reviewed. Particular attention should be directed to amounts obtained at phlebotomy, performance of invasive procedures, surgery, and the need for transfusion or phototherapy.

Physical examination begins with inspection of the placenta, umbilical cord, and vessels, with special attention to the integrity and insertion of the umbilical vessels, the presence of lesions of the cord or placenta, and the presence of placental abruption. Careful physical examination of the neonate should be undertaken with evaluation for pallor, jaundice, hepatosplenomegaly, and congenital anomalies.

The initial basic laboratory evaluation includes a blood cell count with erythrocyte indices, examination of a peripheral blood smear, a reticulocyte count, blood type, a direct antiglobulin test, and serum bilirubin determination.

A positive direct antiglobulin test indicates immune hemolysis. Neonatal and maternal blood types should be compared, and maternal serum should be evaluated for the presence of antibodies. A low reticulocyte count indicates inadequate RBC production. A maternal history of possible parvoviral infection should be sought and additional diagnostic testing should be pursued, if indicated. Microcytosis in an anemic infant suggests the presence of a hemoglobinopathy, Hb H disease or γδβ thalassemia, or recovery from chronic in utero hemorrhage. Hypochromia due to iron deficiency is common with chronic hemorrhage. Neonatal normoblastosis is prominent in infants who have experienced chronic hemorrhage and γδβ thalassemia. Hemoglobin electrophoresis and maternal Kleihauer-Betke testing can help differentiate these disorders.

The presence of hyperbilirubinemia and hemolysis on a peripheral blood smear in an infant with a negative antiglobulin test suggests an intrinsic erythrocyte defect. In some cases, such as HS, erythrocyte morphology on a blood smear may be suggestive of the diagnosis. Additional diagnostic evaluation is indicated. Serum haptoglobin is frequently low or undetectable in healthy infants without hemolysis and is not a useful adjunct in the investigation of neonatal hemolytic anemia.[319]

The infant with a normocytic, normochromic nonhemolytic anemia and a negative direct antiglobulin test should be suspected of having had acute blood loss. Maternal Kleihauer-Betke testing and placental examination should be performed. If the cause remains cryptic, ultrasonography of the head and abdomen to detect occult hemorrhage should be considered.

POLYCYTHEMIA HYPERVISCOSITY SYNDROME

Neonatal polycythemia is common; it occurs in 1% to 2% of live births at sea level and up to 5% of births at high altitude.[320-325] Numerous risk factors have been associated with the development of polycythemia, primarily associated with increased erythropoiesis due to fetal hypoxia or fetal erythrocyte transfusion. Reported risk factors include maternal diabetes, smoking, alcohol or substance use, hyperthyroidism, hypertension, or preeclampsia, intrauterine growth restriction, fetal trisomy or other genetic syndromes including Beckwith-Wiedemann syndrome, congenital adrenal hyperplasia and hypothyroidism,[326-337] maternal-fetal transfusion, twin-twin transfusion, delivery outside of labor and delivery, perinatal asphyxia, nuchal cord, cord stripping, and DCC.[338,339] Increased erythrocyte mass may lead to increased blood viscosity (Fig. 2-7), alterations in blood flow, and end organ damage, which results in a constellation of clinical and laboratory findings called PHS (Box 2-6).[326,340] In some (but not all) reports, infants with PHS have been reported to have worse developmental and neurologic outcomes than their nonpolycythemic counterparts.

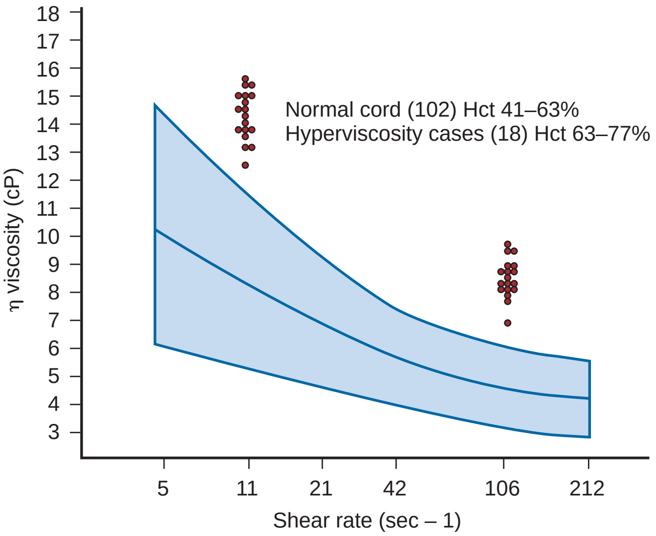


Figure 2-7 Blood viscosity versus hematocrit (Hct) in normal and polycythemic infants. The shaded area represents the viscosity of cord blood at shear rates from 2 to 212/s for 102 healthy term appropriate-for-gestational-age infants (mean ± 2 standard deviation). Viscosity for 18 "symptomatic" infants is plotted at shear rates of 11/s and 106/s. Hct values for each group are indicated. *(From Gross GP, Hathaway WE, McGaughey HR: Hyperviscosity in the neonate.* J Pediatr *83:1004–1012, 1973.)*

Partial exchange transfusion (PET) has been used in infants with PHS to relieve clinical symptomatology and potentially improve neurologic outcome by reducing the hematocrit. The rationale for PET is that decreasing presumed hyperviscosity decreases vascular resistance, improves tissue perfusion, limits end organ damage, and thereby prevents or ameliorates complications of PHS.[341-344] PET improves symptomatology, such as hypoglycemia, poor perfusion, cyanosis, and cardiac function.[345-348] However, it has not been convincingly shown to improve long-term developmental outcomes,[345,346,349] and it may be associated with complications of its own,[350] leading many persons to question the utility of PET.[351] The American Academy of Pediatrics

Box 2-6 **Findings Attributed to Neonatal Polycythemia Hyperviscosity Syndrome**

- Plethora
- Tachypnea
- Jitteriness
- Cyanosis
- Thrombocytopenia
- Hypotonia
- Apnea
- Seizures
- Feeding intolerance
- Hyperbilirubinemia
- Hypoglycemia
- Thrombocytopenia
- Pulmonary hypertension
- Necrotizing enterocolitis
- Thrombotic/ischemic event

guidelines for PET in the treatment of PHS are: “The accepted treatment of polycythemia is PET. However, there is no evidence that exchange transfusion affects long-term outcome.”[352]

Studies providing guidance about when to perform PET for PHS are lacking, making it difficult to determine when and at what threshold of hematocrit a PET for PHS should be performed.[353] The standard in many hospital nurseries is to perform PET when the venous hematocrit is greater than or equal to 65% in an infant with symptoms of PHS or when the venous hematocrit is greater than or equal to 70% in an asymptomatic infant, although few data support this approach.[354] In some hospitals, PET is not performed in asymptomatic neonates until the venous hematocrit is greater than or equal to 75%. Adding to the controversy regarding the appropriate threshold of hematocrit is the observation that hematocrit is an imprecise surrogate of blood viscosity, making it a poor indicator of who needs PET. Polycythemia and hyperviscosity are not synonymous. In addition to hematocrit, many other factors such as plasma proteins, leukocytes, fibrinogen, platelets, blood pH, and erythrocyte deformability may influence blood viscosity.[326,328,355-358] The inability to directly measure blood viscosity in most hospitals has led to the adoption of hematocrit as a surrogate marker of blood viscosity. However, hematocrit is an imperfect measure of viscosity, because not all infants with an elevated hematocrit level have increased blood viscosity.[359] Given the risk of complications from both PHS and PET, a more precise test of blood viscosity is needed to better determine which patients might benefit from PET.

Several studies have demonstrated that symptomatic patients with PHS are at risk of adverse neurologic outcomes,[349,360,361] highlighting the need for careful developmental follow-up of these infants, even if they do not undergo PET. These data are consistent with the suggestion that in some patients, an adverse intrauterine environment or a significant perinatal event leads to tissue hypoxia, polycythemia, hyperviscosity, with irreversible tissue damage that would not be ameliorated by PET.[340,345,349] Given the potential risk of complications from both PHS and PET, a more precise test of blood viscosity is needed to better determine which patients might benefit from PET.

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CHAPTER 3

Immune Hemolytic Disease

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CHAPTER OUTLINE

HISTORICAL ASPECTS
THE Rh BLOOD GROUP SYSTEM
Biochemistry and Molecular Genetics
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HISTORICAL ASPECTS

Whereas many serious complications of pregnancy were well known in antiquity, maternofetal blood group incompatibility was recognized more recently. Against the high perinatal mortality prevailing until the mid-twentieth century, deaths from hemolytic disease were of little statistical significance, even though about half of the affected babies died.

In 1932 Diamond and coworkers deduced the connection between “congenital anemia,” icterus gravis, and hydrops fetalis, and coined the term *erythroblastosis fetalis* for the disease characterized by hemolytic anemia, intramedullary and extramedullary erythropoiesis, and hepatosplenomegaly.[1] Darrow correctly speculated in 1938 that the disease was caused by maternal antibodies to fetal antigens developed as a result of transplacental fetomaternal hemorrhage (FMH).[2]

In 1939 Levine and Stetson linked a woman’s severe transfusion reaction to her husband’s blood with her recent delivery of a hydropic stillborn infant.[3] She was found to have an antibody that agglutinated her husband’s red blood cells (RBCs). Levine postulated that she had become sensitized to an antigen that the fetus had inherited from its father.

In 1940 Landsteiner and Wiener[4] proposed the identity of the antigen by generating antibodies to rhesus monkey RBCs in guinea pigs and rabbits. The antisera agglutinated RBCs from 85% of white people. These people were designated rhesus (Rh) positive. The 15% whose RBCs were not agglutinated were rhesus (Rh) negative.

Subsequent years saw rapid and dramatic progress, including the development of the Coombs test.[5] This test uses anti–human immunoglobulin G (IgG) antibodies to agglutinate IgG-labeled RBCs and remains important in the detection and management of hemolytic disease of the fetus and newborn (HDFN). Chown reported in 1954[6] that mothers could be sensitized by a transplacental hemorrhage of fetal blood, although until crossmatching for Rh groups became routine, transfusion for postpartum hemorrhage and other indications also contributed to the incidence of the disease. Chown’s observation and the subsequent recognition that most sensitizing FMHs occurred at delivery, explaining why Rh HDFN was

TABLE 3-1 Example of the Variation in Incidence of Common Rh Haplotypes

			Allele Frequency in United States (%)			
Gene	**Allele**	**Translated Product**	Whites	Blacks	Native Americans	Asians
RHD	*RHD*	RhD polypeptide	61	97	99	99
	RHD deletion or silent	None	39	3	1	1
RHCE	*RHCe*	RhCe polypeptide	44	19	46	72
	RHcE	RhcE polypeptide	15	11	40	21
	RHce	Rhce polypeptide	41	70	8	6
	RHCE	RhCE polypeptide	0	0	6	1

From Huang CH: Blood Group Antigen Gene Mutation Database: Rh Blood Group System. HUGO Mutation Database Initiative, *2001. Accessed 2007 at* http://www.bioc.aecom.yu.edu/bgmut/index.htm. *Note that distribution differs in other regions worldwide.*

mainly a disease of multipara, paved the way for development of immunoprophylaxis. Since the late 1960s RhD immunoglobulin has been available to treat RhD-negative mothers of RhD-positive infants at delivery to prevent Rh HDFN.

For many problems of pregnancy, obscurity of the pathophysiology often led to management that, until recently, was symptomatic at best. In contrast, with hemolytic disease the genetic predilection, molecular mechanism, and rationale for treatment were rapidly deduced, which led to remarkable success in preventing the disease and mitigating its effects. Indeed, the history of the prevention and management of RhD HDFN includes a number of pivotal discoveries that have influenced the management of other diseases. These breakthroughs include exchange transfusion for neonatal treatment, development of screening programs for maternal blood group antibodies, strategies for managing early delivery to optimize neonatal outcomes, the use of amniocentesis for fetal diagnosis, the first example of intrauterine fetal treatment, the development of one of the earliest and most successful forms of immunoprophylaxis using human antibodies, and the noninvasive prediction of fetal RHD type using DNA from maternal blood. Subsequently, many of the successes of fetal and neonatal treatment of Rh HDFN have been extrapolated to HDFN caused by other blood group antibodies and other problems of pregnancy.

Advances in molecular biology and biochemistry allowed the identification of the Rh polypeptides in 1982 and the *RH* genes in 1991. These discoveries amended previous suggested models to explain inheritance, although like many disorders that were once thought to have been genetically simple and phenotypically diverse, the diversity of Rh genotype/phenotype relationships is much greater than once imagined.

THE Rh BLOOD GROUP SYSTEM

Biochemistry and Molecular Genetics

Although the incidence of Rh HDFN has declined and the incidence of HDFN caused by other alloantibodies has increased, the Rh blood group system remains the most common cause of HDFN (particularly severe HDFN), especially in white persons, for whom approximately one in seven conceptions involves an Rh-negative woman and an Rh-positive man. Individuals are classed as Rh positive or negative based on expression of the major D antigen on their RBCs; however, more than 50 other Rh antigens have been identified, of which the most commonly recognized in clinical practice are those designated D, C, c, E, and e. The Rh blood group system is the most complex blood group system in humans.[7,8]

After the landmark descriptions of the RhD protein in 1982,[9,10] the non-D[11,12] and D complementary DNAs (cDNAs) and genes were cloned in the early 1990s.[13,14] In most individuals the Rh blood group loci are the products of two very similar genes, one of which encodes the polypeptide that carries the Rh CcEe epitopes in combinations (Ce, ce, cE, or CE); the other encodes the RhD antigen polypeptide (Table 3-1).[8,15-17] The two genes are separated by only 30 kb (including the small *SMP1* gene) and are organized in opposite orientation on chromosome bands 1p36.1-p34.3.[18] They share a very similar 10-exon structure, and their intronic sequences are also highly conserved. Their sequence identity strongly suggests that they evolved by duplication of an ancestral gene approximately 8 million years ago.[19] Worldwide, the Rh blood group system displays considerable polymorphism. A wide variety of both small and large mutations (including gene deletion and recombination to form hybrid *RHD-RHCE* products) and the effects of other modifying genes account for the diverse common and rare Rh phenotypes.[18,20] Although some genotypic and phenotypic variants are confined to a single family, for others the incidence in various gene pools is much higher or unknown. This diversity mandates caution in the use of DNA-based clinical diagnostic approaches, especially when these approaches are applied to populations other than the ones in which they were piloted, but these techniques may open the door for future individualization of prevention and therapy.[21]

It has been well established for decades that no d antigen exists. RhD negativity is defined as the absence of RhD antigen, and its incidence is highest in the Basque population.[22] It has been suggested that peripatetic Basque traders and fishermen (or their ancestors) may have spread this genotype to other populations, which explains its higher prevalence in those of European ancestry (Table 3-2). In European-descended populations in which RhD negativity is relatively common, most RhD-negative individuals are homozygous for an *RHD* gene deletion.[20,21] This mutation appears to have arisen because two highly

TABLE 3-2 Incidence of Rh Allelic Combinations in a North American Sample Expressed by Fisher-Race and Wiener Nomenclature

	Nomenclature			Frequency[243]		
	Fisher-Race	Wiener	Expressed Alleles	Whites	Blacks	Asians*
RhD positive	CDe	R_1	*RHD RHCe*	0.42	0.17	0.70
	cDE	R_2	*RHD RHcE*	0.14	0.11	0.21
	cDe	R_0	*RHD RHce*	0.04	0.44	0.03
	CDE	R_z	*RHD RHCE*	0.00	0.00	0.01
TOTAL				0.6	0.72	0.95
RhD negative†	cde	r	*RHce*	0.37	0.26	0.03
	Cde	r’	*RHCe*	0.02	0.02	0.02
	cdE	r”	*RHcE*	0.01	<0.01	<0.01
	CdE	r^y	*RHCE*	<0.01	<0.01	<0.01
TOTAL				0.4	0.28	0.05
Ratio of RhD-positive to RhD-negative phenotypes				0.84:0.16	0.92:0.08	0.99:<0.01

*The predominant reasons for failure to express RHD vary by racial group. RHD deletions predominate in whites, but other variants, such as partial deletions and RHD-RHCE recombinations, are more common in other populations.

†Rh negativity is more common in Indo-Asians.

homologous sequences (1463 bp “rhesus boxes”) flank the *RHD* gene and provide the opportunity for unequal crossing over.[23] However, although RhD negativity is less common among them, other mechanisms for D negativity appear more commonly in other populations, such as those of African, Chinese, or Japanese ancestry.[24-26] These alternative mechanisms include partial deletion, recombination between *RHD* and *RHCE* genes, and point mutation. An Rh allele that comprises a pseudogene (*RHDψ*) is prevalent among RhD-negative people of African ancestry, including approximately 66% in South Africa and about one in five RhD-negative African Americans.[26,27] *RHDψ* is not expressed because of two inactivating mutations. Singleton found that 15% of RhD-negative black South Africans carry a hybrid *RHD-CE-D* that also encodes an altered C antigen.[26]

The molecular basis of the RhD-positive phenotype is also intricate. Studies using site-directed mutagenesis, naturally occurring variants of *RHD*, and monoclonal antibodies or molecular modelling indicate that the RhD antigen is composed of at least 40 different but overlapping epitopes,[18,28-31] some of which are missing in individuals who express partial D antigenicity. The *RHD* and *RHCE* gene products are unique among blood group antigens in being nonglycosylated, palmitoylated integral membrane proteins (Fig. 3-1). Their expression is confined to erythroid cells, whereas many other blood group antigens are present in many tissues.

The RhD and RhCE antigens are found in association with a glycoprotein, formerly known as RH50 glycoprotein and now designated Rh-associated glycoprotein (RhAG). *RHAG* shares about one third homology with *RHD* and *CE*; however, it maps to 6p21.1-p11. The Rh complex comprises multimers of RhD or CE subunits with RhAg.[32]

Cartron[7] and Cartron and Colin[16] have reviewed the Rh deficiency states. Rare individuals with the Rh deficiency syndrome (Rh$_{\text{null}}$) lack expression of all or virtually all RhD, C/c, and E/e antigens. This recessive condition can result (in RhD-negative individuals) from homozygous silent mutations in the *RHCE* gene that appear to reduce cell surface expression of RhAG (“amorph type”). Homozygotes for mutations that prevent expression of RhAG have the more common “regulator type” or the Rh$_{\text{mod}}$ phenotype. Despite normal transcription of RH genes, the proteins are not found at the cell surface.[33]

The functional significance of the Rh complex is indicated by the fact that Rh-deficient individuals have spherocytosis, stomatocytosis, and chronic hemolysis of varying severity. Their RBCs have altered ion transport and membrane composition and are partly or completely deficient in certain other membrane proteins including RhAG, CD47, LW (Landsteiner-Wiener glycoprotein, or intracellular adhesion molecule 4 [ICAM-4]), GPB (glycophorin B, which confers the Ss blood group), and Duffy Fy5 (Duffy antigen receptor for chemokines). These deficiencies suggest that Rh proteins form complexes with RhAG and other proteins and that the complexes fail to form if components are missing (see Chapter 15).[7,33] There is also strong evidence for an interaction with the anion-exchange protein (band 3) and linkage of the Rh complex to the spectrin-based RBC membrane skeleton, thus indicating a role in maintaining the biconcave disc shape of RBCs (see Fig. 15-6 in Chapter 15).[34] Failure of this association may explain some of the dysfunction of Rh-deficient cells. However, many details of when, how, and why the Rh complexes form remain to be explained.

In contrast to the common Rh-negative and very rare Rh deficiency states, weak and partial D phenotypes are fairly common (e.g., 0.2% to 1% of populations of European ancestry) and are caused by *RHD* coding region alterations that affect the number and antibody binding characteristics of RhD sites per cell. The term “partial D” has been used to describe RBCs that react with some monoclonal anti-D antibodies and not others, and their RhD proteins typically have one or more amino acid

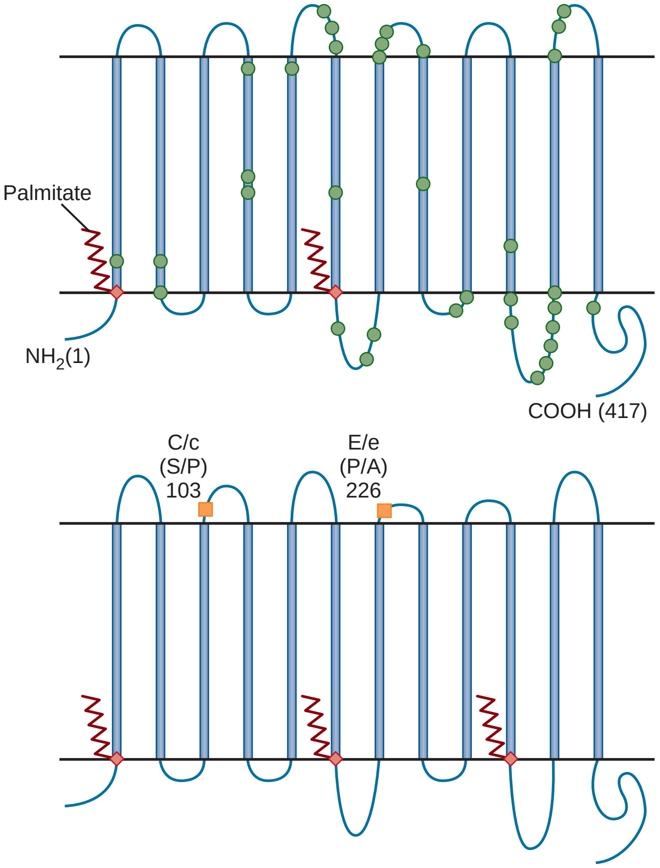

Figure 3-1 Diagram of the Rh peptides: RhD *(top)* and RhCE *(bottom)*, inserted in the cell membrane *(parallel horizontal lines)*. The amino acid sequence of both proteins predicts 12 transmembrane domains. Neither is glycosylated, but RhD has two sites of palmitoylation *(zigzag lines)*, and RhCE has three. Approximate positions of D-specific amino acids are marked with *open circles*. The predicted position of most of them is in transmembrane and cytoplasmic domains. E/e specificity appears to be conferred by a single amino acid substitution (P226A) encoded by exon 5 of the *RHCE* gene and C/c specificity by the S103P substitution encoded by exon 2. Four other amino acid differences are also associated with C/c polymorphism but do not appear to affect antigen characteristics: C16W encoded by exon 1 and I60L, S68N, and S103P all encoded by exon 2 of the RhCE gene.[8]

substitutions on or near the RBC surface, or consist of RhD-RhCE hybrids. In contrast, "weak D" has been used for RBCs with markedly reduced numbers of antigenic sites and in which one or more amino acid substitutions are found in regions that are presumed to be in or below the membrane and may interfere with assembly of Rh complexes.[35] Most individuals with weak D, especially those with types 1, 2, and 3, do not make anti-D. However, it has been recognized that there is overlap in, and some imprecision in allocation of, these phenotypes, and the terms "aberrant D" or "D variant" have been proposed as collective terms for these plus the (very weak) DEL phenotype (Table 3-3).[35]

Because of variable antigenic potential and the potential to form antibodies to RhD, certain weak and partial D phenotypes are significant in transfusion therapy and pregnancy. Some individuals are considered RhD-negative in pregnancy and as transfusion recipients (eligible for anti-RhD immunoprophylaxis and Rh-negative blood) but Rh-positive as blood donors.[35] For example, if some blood donors with aberrant, or variant, D are regarded as D negative, their blood can cause alloimmunization of D-negative recipients. On some occasions, women with D variants (especially partial D VI) develop anti-D antibodies capable of causing severe HDFN if they carry a D-positive child, or D-negative women can develop antibodies if their babies have some types of aberrant, or variant, D.[35] Some partial D phenotypes are also associated with distinct antigens.

The C antigen is most common in southeast Asia, parts of South America, and the southern tip of Greenland, whereas the E antigen occurs most often in populations indigenous to parts of South America and Alaska.[22] The genetics underlying RhCc/Ee phenotypes also display more subtle variation than was once envisaged, and weak, partial, and variant antigen phenotypes have also been recognized. Internet databases are a good source of up-to-date information about the phenotype/genotype relationships for RhD and RhCE.[36,37]

RhD ALLOIMMUNIZATION

HDFN is a disease of the fetus caused by a maternal response to pregnancy. As such, the pathogenesis can be

TABLE 3-3 Examples of Variant Rh Phenotypes for Which a Corresponding Genotype Has Been Described, Illustrating the Diversity of the RHD Genotype

Category	Phenotype	Typical Mutations
Partial D	D^{II} to D^{VII} and others	Variety of point mutations and RHD-RHCE hybrids
Weak D	Weak D (D^u) types 1-16	Variety of point mutations leading to amino acid substitutions in transmembrane or cytoplasmic domains
D negative (silent D or d)	D negative	Variety of mechanisms: RHD complete or partial deletion, CE hybrids, point mutations; some prevent transcription, but all prevent expression at the cell surface
CE variants	RN, E^{I-III}, VN, CX, C^W, etc.	Variety of hybrids and point mutations
CE silent	D.., D-, and various others	Mostly RHD-RHCE hybrids
Rh deficiency states (no D or CE expression)	D_{null} amorph $D_{null\ reg}$, $D_{null\ mod}$	RHCE silent genes plus RHD deletion RHAG mutations that prevent expression

considered in three stages: maternal alloimmunization, antibody transfer to the fetus, and fetal response.

Maternal Rh Sensitization

Maternal sensitization to Rh antigens can occur as a result of exposure to antigenically dissimilar fetal RBCs during pregnancy, as a result of therapeutic transfusion, or occasionally as a result of needle sharing.[38,39] In high-resource countries, transfusion rarely causes RhD sensititization because compatibility testing for RhD antigen has been a routine practice for several decades. However, it accounts for a high proportion of sensitization to other blood group antigens, including RhCE, and among those with RhD and RhCE variants. Furthermore, the need for emergency transfusion of Rh-positive blood to Rh-negative women is likely to remain a cause of sensitization to RhD in locations where the Rh-negative phenotype is rare and Rh-negative blood is scarce.

Exposure to fetal antigens remains the most common cause of maternal antibodies to RhD. Although Chown first described maternal Rh immunization that occurred as a result of FMH, the test of Kleihauer and associates in 1957[40] was needed to show the incidence, size, and timing of these events and to show that most maternal Rh sensitization occurs this way.[41] The test, which depends on the resistance of fetal hemoglobin (HbF) to acid elution (Fig. 3-2), can detect 1 fetal RBC in 200,000 adult RBCs, corresponding to a fraction of a milliliter in the circulating maternal blood volume. However, acid elution studies must be interpreted with some caution because of rapid destruction of fetal cells when ABO incompatibility exists and natural variation and pregnancy-induced increases in expression of HbF among adults. Using flow cytometric techniques and DNA amplification of fetal sequences, researchers have subsequently confirmed that some fetal cells can be detected in maternal blood during all trimesters of most pregnancies.[42,43] Because the strategies for detection vary among studies, there are inaccuracies of the Kleihauer-Betke test and there are uncertainties about the persistence of fetal cells in the maternal circulation, the volume and timing of this transplacental passage of fetal cells remain unclear.[44] However, most of these "hemorrhages" are likely to be small in volume. Delivery is the time of greatest risk for FMH and larger hemorrhages, especially when complications of delivery of the fetus or placenta occur.[42]

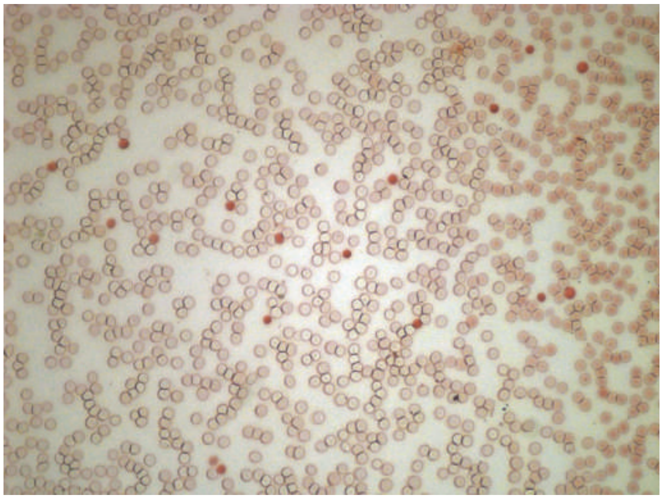

Figure 3-2 Acid elution technique of Kleihauer. Fetal red blood cells stain with eosin and appear dark. Adult red blood cells do not stain and appear as ghosts.

Larger FMHs are uncommon; the amount of fetal blood has been estimated to exceed 2.5 mL in fewer than 1% of gestations and is greater than 30 mL in fewer than 0.25%.[42] However, certain obstetric situations increase the risk of significant FMH; these include antepartum hemorrhage, placental abruption, preeclampsia, external cephalic version, cesarean section, and manual removal of the placenta.[44] Abdominal trauma and placental vascular malformations are additional but uncommon causes. FMH occurs after early-gestation chorionic villus sampling (CVS)[45] and after amniocentesis,[46,47] although the risk of maternal sensitization after amniocentesis is typically higher, perhaps because CVS is usually performed in earlier gestation when there is lower expression of fetal blood group antigens. The level of risk of sensitization as a result of first trimester pregnancy loss is also uncertain and in the case of spontaneous miscarriage will depend on whether an embryo with circulating blood ever formed, but surgical evacuation of the uterus is thought to present a greater risk of sensitization than medically induced or spontaneous evacuation.[48] The Rh antigen may be expressed by approximately 38 days after conception,[49] early enough in gestation for early CVS or pregnancy loss to present a potential risk.

All studies have focused on fetal cells in the maternal bloodstream, but the responses to submucosal, subcutaneous, or intramuscular antigen presentation differ from the responses to intravascular presentation. Therefore deposition of fetal RBCs in these sites could be more immunogenic than with the intravascular route and could account for some of the propensity for Rh sensitization after invasive procedures. If this is the case, techniques such as those recommended to minimize exposure of the fetus to maternal blood[50] might be effective in reducing risk of blood group sensitization.

The Nature of Rh Sensitization

Primary and Secondary Immune Responses

In most RhD-negative individuals, the primary immune response develops slowly. In experimental Rh immunization of male volunteers, antibody responses are typically detected at 8 to 9 weeks, although they can occur at any time from 4 weeks to 6 months. Hemolytic anemia caused by anti-RhD has been detected as early as 10 days after a massive Rh-incompatible transfusion in a previously unsensitized patient.[51] A primary, often weak immunoglobulin M (IgM) response will not affect the fetus even if it occurs before birth in a sensitizing pregnancy, because IgM anti-RhD does not cross the placenta. However, immunoglobulin G (IgG) anti-RhD production usually ensues, and if sensitization occurs in early pregnancy there is the potential for continued antigenic challenge, accounting for the rare occurrence of HDFN in the progeny of women during their first Rh-positive pregnancy.

After a primary response has been invoked, subsequent exposure to Rh-positive RBCs induces a rapid increase in anti-RhD IgG, which can cross the placenta and affect the fetus. Repeated exposures can progressively increase the antibody titer and change the characteristics (including IgG subclass distribution) of the RhD antibody, thus increasing the severity of Rh erythroblastosis in successive pregnancies.

Dose of Antigen Necessary to Produce RhD Sensitization

Experiments using injection of RhD-positive blood into RhD-negative male subjects show, not unexpectedly, that the likelihood of Rh sensitization depends on both dose and repetition of exposure to the antigen.[52] A proportion of individuals are anergic to RhD regardless of dose and repetition of exposure ("nonresponders").[52]

Studies during and immediately after pregnancy using the Kleihauer technique indicated that if the volume of FMH is always less than 0.1 mL of RBCs, the prevalence of RhD sensitization detectable up to 6 months after delivery was 3%, whereas when volumes exceed 0.4 mL, the prevalence was 22%.[52,53] Nevertheless, because in 75% to 80% of pregnancies the amount of the FMH is always less than 0.1 mL, it is likely that either small or undetectable FMHs or fetal cells deposited in other sites account for the majority of sensitizations. Secondary immune responses may occur after exposure to very small amounts of RhD-positive RBCs (as little as 0.03 mL).

Incidence of RhD Sensitization

RhD sensitization has decreased markedly with the introduction of RhD immunoprophylaxis. In its absence, about 16% of RhD-negative women become sensitized in their first ABO-compatible RhD-positive pregnancy. Of these, approximately one half have detectable anti-D 6 months after delivery, and in one half a rapid secondary response is seen in the next susceptible pregnancy, indicating that previous primary sensitization had occurred.[53,54] If sensitization does not occur, the risk in a second RhD-positive, ABO-compatible pregnancy is similar. By the time an RhD-negative woman has completed her fifth ABO-compatible, RhD-positive pregnancy, the probability that she will be RhD sensitized is approximately 50%.

As parity increases and the number of women capable of an RhD immune response diminishes because they have already become immunized, the proportion of the remainder whose systems mount a primary immune response decreases because of a greater residual number of nonresponders. About 25% to 30% of RhD-negative women are nonresponders, in that they do not become RhD sensitized despite having many RhD-positive pregnancies; however, some may yet become RhD sensitized if they are exposed to a very large amount of RhD-positive blood. Immunologic tolerance can be induced by several different mechanisms and can depend on the context of antigen presentation to the immune system. It is not known which mechanisms of tolerance predominate in nonresponding RhD-negative women.

ABO incompatibility provides partial protection against RhD sensitization. Without anti-RhD immunoprophylaxis, it has been estimated that in white persons blood group A incompatibility between mother and fetus reduces the risk of RhD sensitization by 90% and group B by 55%.[55] Bowman[54] suggested that this partial protection is due to rapid intravascular hemolysis of the ABO-incompatible, RhD-positive RBCs, with sequestration of RhD-positive cells in the stroma in the liver (an organ with poor antibody-forming potential) rather than in the spleen (the site of RBC sequestration when extravascular RBC destruction occurs). However, it is not clear whether the immune system remains naïve with respect to the RhD antigens or tolerance is induced in these instances. Although ABO incompatibility provides substantial protection against the primary RhD immune response, it provides no protection against the secondary RhD immune response[56] and should not influence management of an already affected pregnancy. Maternal HLA type does not have any consistent effect on risk of sensitization, although it may affect antibody titre and severity of HDFN.[57,58]

Other factors that influence the likelihood and severity of sensitization include fetal gender (male to female, 1.44-1.74 to 1) and fetal red cell phenotype, which affects the number and antigenicity of RhD antigen sites on fetal RBCs. For example, R1r (CDe/cde) cells, with only 9900 to 14,400 D antigen sites per cell, are less immunogenic than R2r (cDE/cde) cells, which have 14,000 to 16,000 sites.[55,59] The possibility that some RhD-negative women are exposed to RhD during their own fetal life (the so-called grandmother theory) has been considered, but the evidence from a case-control study is against it.[60] Nevertheless, convincing evidence of maternal-to-fetal hemorrhage has been found in other circumstances, and these events could explain the occasional presence of RhD antibodies in RhD-negative men who have never had a transfusion.

RhD sensitization during pregnancy occurs in about 1.5% of susceptible pregnancies.[61] The proportion of all occurrences of RhD sensitization that are attributable to antenatal sensitization now varies among studies, depending on the application of antenatal immunoprophylaxis.

In summary, some general rules apply to RhD HDFN. The probability of incompatible pregnancies is predictable from Hardy-Weinberg genetic principles, but the impact of HDFN on a population will also depend on typical family size and on careful management of obstetric complications.[62] The risk should be low when good blood bank procedures, parsimonious approaches to blood transfusion, effective screening of pregnant women, and evidence-based administration of RhD immunoprophylaxis are applied. The disease should be uncommon or mild in a woman's first incompatible pregnancy, and 50% of the infants of heterozygous fathers and sensitized mothers should be safe. However, the striking feature of pregnancies in severely isoimmunized women is their clinical variability. Because these women have often become sensitized despite the predictions of conventional wisdom, their care requires astute and conscientious management to ensure satisfactory outcomes.[56]

Prevention of Rh Sensitization

The background to the prevention of RhD sensitization lies in the experiments of Emil von Dungern at the beginning of the twentieth century.[63] He showed that serum from rabbits that had previously been injected with ox cells prevented the development of antibodies to ox cells in a second group of rabbits. The partial protective effect of ABO incompatibility also suggested that antibody-mediated destruction of fetal RBCs could prevent maternal RhD sensitization. Because anti-RhD immunoglobulin was strikingly successful in preventing sensitization in males,[41,64] clinical trials were then undertaken in which RhD-negative, unsensitized women were given anti-D intramuscularly after delivery of an RhD-positive infant.[65-67] The licensing of RhD immunoglobulin in 1968 profoundly influenced the prevalence of Rh sensitization. Despite its success, the exact mechanism of action of prophylactic RhD immunoglobulin is not yet fully elucidated.[68]

The level of evidence for the use of postpartum RhD immunoglobulin is high. A systematic review collated data from the six eligible trials involving more than 10,000 women that compared postpartum RhD immunoglobulin with no treatment or placebo.[69] The conclusion was that RhD immunoglobulin strikingly lowered the incidence of RhD alloimmunization 6 months after birth (relative risk 0.04, 95% confidence interval 0.02 to 0.06) and in a subsequent pregnancy (relative risk 0.12, 95% confidence interval 0.07 to 0.23). These benefits were seen regardless of the ABO status of the mother and baby when RhD immnoglobulin was given within 72 hours of birth. Higher doses (up to 200 μg) were more effective than lower doses (up to 50 μg) in preventing RhD alloimmunization in a subsequent pregnancy. The usual dose after full-term delivery in the United States is 300 μg (1500 International Units),[70,71] which will effectively suppress the immunizing potential of approximately 17 mL of RhD RBCs (approximately 30 mL of fetal blood),[72] although other guidelines recommend lower standard doses.[73,74,75,76] Routine assessment of the size of FMH and subsequent titration of RhD immunoglobulin dose is recommended[44,70,73,75,77] because although larger volume FMH is uncommon, it is not reliably predicted by clinical history. Postpartum prophylaxis is recommended whenever an RhD-positive infant is born to an unsensitized RhD-negative mother. RhD immunoglobulin is also recommended if the infant is positive for DVI or weak D.[70,77-79] After postnatal immunoprophylaxis, the risk of RhD alloimmunization is between 1% and 2%. Administration of 100 μg (500 IU) of RhD immunoglobulin at 28 and 34 weeks' gestation to women in their first pregnancy can reduce this risk to less than approximately 0.2%.[80] Most current guidelines therefore recommend routine administration of RhD immunoglobulin at doses of 250 to 300 μg (1250-1500 IU) at about 28 weeks or 100 to 125 μg (500-750 IU) at 28 and 34 weeks' gestation to all RhD-negative pregnant women.[81]

Some transfusion services also recommend prophylaxis to weak D-positive women,[82] although it is not recommended in most guidelines.[78] Additional or earlier doses (depending on the timing and circumstances) for RhD-negative women who are not already sensitized and who experience trauma or who are undergoing amniocentesis, CVS, or other relevant procedures may further reduce the risk of sensitization.[71,73,74-76] There is no high-quality evidence for immunoprophylaxis after early pregnancy loss or termination,[83] but RhD immunoglobulin (typically in smaller doses) is widely recommended.[71,73,74-76]

Small amounts of the RhD immunoglobulin cross the placenta and cause a weakly positive antiglobulin test, but there are no apparent adverse effects on the infant.[84] It is important to note that after antenatal RhD immunoglobulin, results of maternal antibody screening tests done near term may be positive (albeit at low titer), but under the current guidelines these results should not preclude routine estimation of peripartum FMH and administration of postnatal immunoprophylaxis.

Approximately 1 in 400 women will have a FMH of greater than 30 mL of fetal blood at the time of delivery. Such hemorrhages are often clinically silent but can be detected by routine performance of a Kleihauer-Betke test on RhD-negative mothers of RhD-positive neonates. Prevention of RhD sensitization in the presence of such a large dose of antigen requires prompt assessment (by the Kleihauer-Betke test, preferrably confirmed by flow cytometry, which is more accurate but less widely available[85,86]) and administration of a titrated dose of RhD immunoglobulin. Pediatricians must be aware of this possibility and promptly notify their obstetric colleagues of any suspected FMH in the newborn infant of an RhD-negative mother. Assessment of the magnitude of FMH is also recommended after pregnancy complications such as antepartum hemorrhage and abdominal trauma.

Certain knowledge of RhD negativity in the biologic father of the fetus can obviate the need for antenatal immunoprophylaxis. However, this information should be established with a very high degree of certainty because of the potential for severe consequences in a future pregnancy in case of error.

Reports from numerous countries document the reduction in RhD HDFN as a result of RhD immunoglobulin. Nevertheless, there is no room for complacency because 30% to 40% of the reduction in incidence of HDFN over recent decades has been attributed to decreased family size rather than to immunoprophylaxis.[87] Chavez and colleagues[88] noted that the incidence of RhD sensitization in the United States is three times the rate predicted if RhD immunoglobulin was always applied according to guidelines issued by the American College of Obstetricians and Gynecologists. The failure of the immunoprophylaxis program to completely abolish RhD HDFN can be partly attributed to noncompliance with guidelines. In addition, recommendations for additional doses during pregnancy have led to scarcity of RhD immunoglobulin in some locations. These problems are even greater in low-resource countries.[89] Giving booster doses to donors, using carefully screened RhD-positive RBCs, has been a necessity as the number of suitable sensitized donors decreases, but this practice requires extreme care to protect both the donor and eventual recipients. The distribution of the RhD-negative phenotype may increase as a result of migration. As with use of any blood product, issues of safety cannot be disregarded.[90] All these forces threaten the supply of RhD immunoglobulin. Recombinant monoclonal or polyclonal preparations—which could, in principle, replace the current polyclonal RhD immunoglobulin, thereby resolving problems of availability and safety—have been under development in recent years. However, although monoclonal antibodies have significant diagnostic and research applications, they are not yet available for prophylaxis against sensitization.[91]

Pathogenesis of Rh Hemolytic Disease

Erythropoiesis begins in the yolk sac by 14 days' gestation, and Rh antigen is expressed by the sixth week. By 6 to 8 weeks' gestation, the liver replaces the yolk sac as the main site of RBC production. Normally, erythropoiesis then diminishes in the liver and takes place nearly entirely in the bone marrow by the late third trimester of pregnancy.[92]

Erythroblastosis is caused by maternal IgG coating of fetal RBCs, leading to their destruction. The subsequent fetal anemia stimulates erythropoiesis via the production of erythropoietin and other erythroid growth factors. When fetal marrow RBC production cannot keep up with RBC destruction, marked extramedullary erythropoiesis can be found in other organs, including the liver and in more severe cases also in the spleen, kidneys, skin, intestine, and adrenal glands. Hepatosplenomegaly is a hallmark of erythroblastosis fetalis. In the presence of erythroblastosis, immature nucleated RBCs, from normoblasts to early erythroblasts, are poured into the circulation.

SEVERITY OF Rh HEMOLYTIC DISEASE

Degrees of Severity

For purposes of audit, research, and planning of treatment, degrees of severity of HDFN have been used since the 1950s.

In general, fetuses and newborns with mild HDFN have had antibody-coated RBCs, yielding positive results on a direct antiglobulin (Coombs) test, but they have no anemia or anemia that is well-compensated for in utero and after birth and do not require exchange transfusion to prevent bilirubin toxicity. This group historically accounted for approximately one half of affected fetuses. Nearly all survive and do well without invasive treatment.

Moderately affected fetuses are at risk of neural toxicity from bilirubin if they do not receive treatment (see Chapter 4). Historically, they are a group who routinely underwent exchange transfusion after birth. They are likely to have some signs of anemia in utero and ex utero but are not significantly acidotic or hydropic. Peripheral blood typically shows polychromasia, anisocytosis, and reticulocytosis, although these can be suppressed by intrauterine treatment (Fig. 3-3). Maternal clearance copes with the products of hemolysis, and the fetus is usually born in good condition at or near term. This category historically represented approximately one third of fetuses. Moderately affected neonates do require vigilant neonatal management, including intensive phototherapy or exchange transfusion to manage jaundice (Fig. 3-4).

Severely affected fetuses have hydrops or hydrops is impending, and they would die before, during, or after birth unless managed intensively. Untreated, hydrops develops in half between 18 and 34 weeks' gestation and the other half between 34 and 40 weeks' gestation.

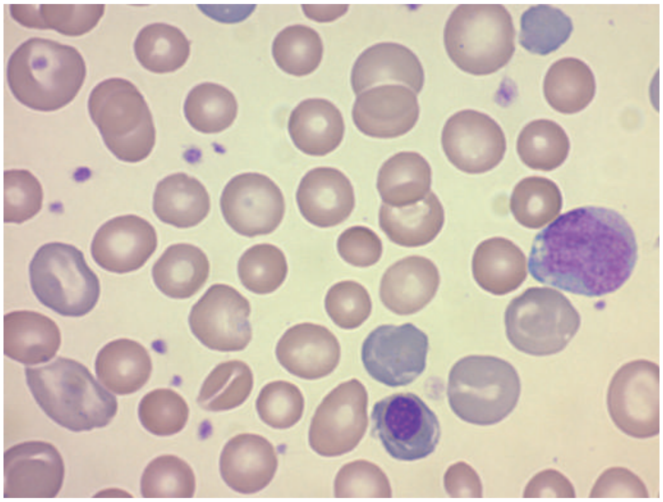

Figure 3-3 Neonatal blood sample of baby with severe Rh erythroblastosis fetalis who required an exchange transfusion at about 16 hours of age for progressive anemia with acidosis and rapidly rising bilirubin. Note the spherocytes, a promyelocyte, a nucleated red blood cell, and polychromasia.

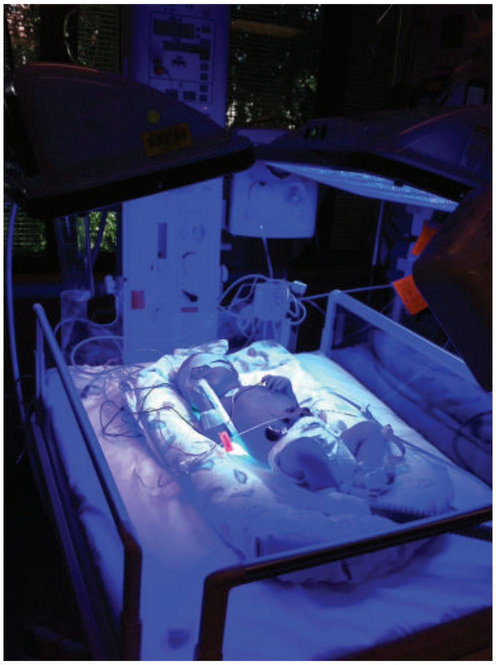

Figure 3-4 A baby receiving intensive phototherapy for anti-D hemolytic disease of the fetus and newborn. Umbilical catheters are in place for bilirubin and blood gas sampling and to undertake exchange transfusion if needed.

Polyhydramnios usually occurs early in the course of hydrops and results from decreased swallowing of amniotic fluid. Ascites is usually the first feature to develop, with severe anemia followed by skin edema and pleural and pericardial effusions. In infants with the most extreme conditions, compression hypoplasia of the lungs makes gas exchange after birth precarious.

When these categories of disease were first devised, severe fetal disease predicted severe neonatal disease. However, as fetal and neonatal management has been refined, the predictions must be updated, and the proportion of mildy and moderately affected neonates has risen. For example, some mildly anemic but not hydropic neonates who met previous criteria for exchange transfusion are now managed effectively with intensive phototherapy and would therefore be regarded as having mild rather than moderate disease. Similarly, a fetus with early development of hydrops (severe HDFN) may respond well to one or more intrauterine transfusions and therefore be born with mild neonatal disease and yet still have severe late anemia that is as much a consequence of hematopoietic suppression caused by effective intrauterine treatment as of hemolysis caused by the underlying disease. A suggested update to the working definitions that separates the criteria into epochs and includes a summary of implications for practice is given in Table 3-4.

Pathogenesis of Hydrops

Hydrops in HDFN was originally attributed to fetal heart failure, but other factors probably play a role.[93] With severe hemolysis and progressively greater extramedullary erythropoiesis, the hepatic architecture and circulation are distorted by the islets of erythropoiesis. It has been suggested that this results in portal and umbilical venous obstruction and portal hypertension. Impairment of umbilical venous return would exacerbate the fetal tissue hypoxia caused by anemia and explain the placental edema that is usually seen in severe hydrops. The placental edema, by increasing the placental barrier to gas exchange, could then impair oxygen delivery even more. The theory that liver distortion and dysfunction are at least as important as heart failure in the pathogenesis of hydrops fetalis could explain why hydrops is not always accompanied by hypervolemia or even severe anemia.[93] Support for the suggestion that portal hypertension is present is provided by ultrasound assessment of waveforms in the portal venous system.[94]

Hypoalbuminemia is a common feature, but the mechanism has not been explained. It could be caused by a greater volume of distribution for albumin or reversion to α-fetoprotein expression rather than failure of hepatic synthesis.

The mechanisms that lead to hydrops, both in anemic fetuses and in nonanemic fetuses with "nonimmune" hydrops, remain poorly understood. Various inflammatory mediators could play a role but have not been examined. Genes that are regulated by hypoxia include those for vascular endothelial growth factor and other molecules involved in capillary morphogenesis. Depending on the pattern of expression, these molecules can markedly affect capillary permeability and could also play a role.

Factors Affecting the Severity of Hemolytic Disease of the Fetus and Newborn

If a sensitized mother is pregnant with an Rh-incompatible fetus, a number of factors influence the severity of disease in the fetus. These factors include placental transfer of antibody, antibody characteristics, antigen characteristics, state of maturation of the fetal spleen, and resilience of fetal erythropoiesis.

Transfer of Antibody to the Fetus

All four IgG subclasses are transported across the placenta, via FcRn receptor–mediated endocytosis and then exocytosis.[95-97] The fetal-to-maternal ratio for total IgG is approximately 0.4 at 28 weeks' gestation and increases to 1.4 at term,[98] but fetal levels of IgG1 are close to maternal levels by 17 to 20 weeks' gestation.[99] The efficiency of placental transport of antibody may be an important factor in the severity of Rh HDFN, and reduced IgG transport can result in unexpectedly mild disease.[100]

Mechanism of Red Blood Cell Hemolysis

When anti-RBC antibodies bind complement and are present in high concentrations (as with transfusion reactions to ABO-incompatible blood in adults), RBC intravascular destruction occurs and may cause a clinically severe reaction including hemoglobinemia, shock, and disseminated intravascular coagulopathy. Products of RBC destruction are cleared predominantly by the liver.

When antibodies do not fix complement, as is typical with anti-RhD in the fetus and neonate,[101,102] the mechanisms of hemolysis are more subtle but, in the end, equally destructive. Three Fc receptor–mediated pathways have been implicated in the destruction of opsonized RBCs: direct phagocytosis, phagocytic damage leading to later lysis, and killer cell–mediated damage. When anti-RhD becomes concentrated on the membrane of RhD-positive RBCs, the RBCs adhere to macrophages and form rosettes. This occurs particularly in the red pulp of the spleen, where RBCs and macrophages come into close apposition. The phagocytic cell can then destroy the RBC or damage and deplete the membrane, causing loss of RBC deformability. If the RBC escapes being consumed by the macrophage, its membrane is damaged, and its osmotic fragility and likelihood of lysis are greater.

Antibody-dependent cellular cytotoxicity (ADCC) probably also accounts for the destruction of some RBCs.[103] Large granular "K cells" (natural killer lymphocytes) or myeloid cells (polymorphonuclear leukocytes and monocytes) bind antibody-labeled cells and release lysosomal enzymes and perforin, causing lysis.

Hemolysis also requires a competent system of phagocytic cells in the fetus. The clinical manifestations of the disease suggest that their maturation is sufficient as early as the middle of the second trimester.

Antibody Specificity and Severity of Hemolytic Disease

Antigen characteristics, including details of antibody distribution, density, and structure, all affect risk for HDFN; these have been reviewed by Hadley.[104] Antibodies with

TABLE 3-4 Degrees of Severity of Rh Hemolytic Disease of the Fetus and Newborn

Severity	Mild	Moderate	Severe
In Fetus			
Historical proportion of Rh HDFN before intrauterine therapy was available	~50%	25%-30%	20%-25%
Likely history in previous siblings	No exchange transfusion	Exchange transfusion unless in utero treatment	Death unless in utero treatment
Likely maternal anti-D titer	<1:64	>1:64	>1:64
Ultrasound abnormalities	None	Altered patterns of flow in fetal vessels	Signs of severe anemia (see Table 3-5), which may lead to hydrops, portal hypertension, ascites and other effusions, polyhydramnios
In Neonate			
Clinical condition at birth	Normal	Mildly or moderately ill	Hydrops
Hemoglobin at birth	No anemia	Mild, or moderate anemia; Hg >0.6 MoM for gestation	Severe, symptomatic anemia Hg <0.6 MoM
Hemoglobin nadir	Transfusion not needed	At risk for symptomic anemia	Risk of death unless transfusion
Bilirubin cord	<3.5 mg/dL (≈70 μmol/L)	>3.5 mg/dL (≈70 μmol/L)	>3.5 mg/dL (≈70 μmol/L)
Bilirubin peak	Term: <20 mg/dL (≈380 μmol/L) Premature: lower levels depending on prematurity	Term: >20 mg/dL (≈380 μmol/L) Premature: lower levels depending on prematurity	Rate of RBC destruction depends on (low) hemoglobin B; peak bilirubin is variable, toxicity increased
Phototherapy	If bilirubin increases faster than 0.4-0.5 mg/dL/hr; lower threshold for premature infants	If bilirubin increases faster than 0.4-0.5 mg/dL/hr; lower threshold for premature infants	Immediate, intensive, pending measurement of bilirubin increase
"Early" exchange	No	If rate of increase of bilirubin >0.8-1 mg/dL/hr despite several hours of intensive phototherapy; lower threshold for premature infants	If rate of increase of bilirubin >0.8-1 mg/dL/hr despite several hours of intensive phototherapy; lower threshold for premature infants
"Late" exchange	If bilirubin > exchange level	If bilirubin ≥ exchange level	If bilirubin ≥ exchange level
Heme oxygenase inhibition	Unnecessary; number needed to treat to avoid exchange will be high	In context of well-designed clinical trials	In clinical trial to avert late exchange; unlikely to allow avoidance of early exchange
Intravenous immunoglobulin	Unnecessary; number needed to treat to avoid exchange will be high	Evidence from high-quality trials to date does not support; therefore only in context of further well-designed randomized trials	Evidence from high-quality trials to date does not support; therefore only in context of further well-designed randomized trials
Phenobarbital	No	Neonatal—no clear benefit if optimal phototherapy; may be role for antenatal treatment in context of well-designed clinical trials	Neonatal—no clear benefit if optimal phototherapy; may be role for antenatal treatment in context of well-designed clinical trials
Erythropoietin (or analogue) for late anemia	No	In context of well-designed clinical trials	In context of well-designed clinical trials

Modified from Peterec SM: Management of neonatal Rh disease. Clin Perinatol *22:561–592, 1995.*
Hg, Hemoglobin; *MoM,* multiples of mean; *RBC,* red blood cell.

the potential to cause HDFN generally recognize antigens restricted to erythroid cells (e.g., Rh antigens) rather than those with a wider tissue distribution (e.g., A and B antigens, found on many cell types). The antigens must be expressed in the fetus and neonate. Thus antibodies to Kell and Rh antigens, which are expressed early in fetal life, cause severe HDFN, whereas antibodies to Lewis antigens, which are not synthesized in erythrocyte progenitors, never cause HDFN. The circulating glycosphingolipids carrying the antigenic Le^a or Le^b epitopes generally only adsorb to the erythrocyte membrane during postnatal life. Lewis antibodies are also usually

IgM. Lewis is the only human blood group system that has never been reported to cause HDFN of any severity.

Antigen density is also an important factor. Within the Rh system, even in the presence of high maternal anti-RhD titers, fetuses with weak RhD phenotypes are unlikely to be severely affected.[79] As might be expected, homozygotes for *RHD* appear to have higher RhD antigen density than heterozygotes,[59] although this is only potentially relevant in unusual situations such as ovum donation, because otherwise RhD positive infants of negative mothers are obligate heterozygotes. *RHCE* genotype also affects RhD antigen density, and in particular there seems to be higher expression of D where CE is deleted or underexpressed.[105,106] The density of several other antigens, including A and B and the Lutheran antigens,[107] is developmentally regulated, and antigen density on immature RBCs limits the severity of hemolysis. Antigen structure may also play a role, with some antigens being more capable of promoting recognition of RBCs by phagocytic cells.

Antibody characteristics also play a role in determining the severity of HDFN. IgG subclasses bind different classes of Fc receptors, although it has not yet been established conclusively which receptors are involved in RBC destruction in vivo. Fc receptor polymorphisms that bind IgG more avidly (and may play a role in ABO HDFN[108]) may yet be found to account for some of the heterogeneity of Rh HDFN.[104]

Whereas anti-A and anti-B antibodies are predominantly IgG2, IgG1 and IgG3 anti-D antibodies tend to predominate in Rh-sensitized women.[109-112] The different IgG subclasses are often present in combination.

The capacity of RBC-bound IgG3 antibodies to bind to Fc receptors of monocytes is greater than that of IgG1 antibodies. Threshold levels of antibody binding for rosette formation in vitro are approximately 1000 IgG3 or 2000 IgG1 molecules per RBC.[113] Both can promote phagocytosis, but their relative potencies depend on circumstances. IgG3 usually causes more efficient lysis by monocytes than does IgG1.[113-115] Clearance of Rh-positive RBCs is caused by fewer molecules of IgG3 anti-D than of IgG1 anti-D.[116] However, there is some evidence that IgG1 is more important in the pathogenesis of severe HDFN.[117]

Human leukocyte antigen (HLA) antibodies have been found in the sera of some women who have infants with mild HDN despite high antibody levels. The HLA antibodies may block antibody-antigen interactions on the macrophage membrane.[104]

ANTIBODY DETECTION

ABO and RhD typing plus antibody screening is recommended at the first prenatal visit in all pregnancies, including visits for elective abortion. A history of prior pregnancies and blood transfusions should also be taken. If the blood group is D-negative or weak D, another antibody screening is recommended at 24 to 28 weeks' gestation, before administration of anti-D immunoprophylaxis. If an alloantibody is detected at a low titer, screening is usually repeated at regular intervals to detect any increase. There are a variety of methods for assessing maternal antibodies, some that are better suited for screening methods and some that are more useful for predicting severity of disease.[104]

Manual Methods

Coombs serum is an anti–human globulin produced by immunizing another species.[5] Rh-positive RBCs are incubated with the serum sample that is being tested for the presence of anti-RhD. If anti-RhD is present in the sample, it adheres to the RhD-positive RBC membrane. The RBCs are then washed to remove nonadherent human protein and suspended in Coombs serum. If the RBCs are coated with anti-RhD, they are agglutinated by the antibodies to human immunoglobulin (positive test result). The reciprocal of the highest dilution of maternal serum that produces agglutination is the indirect antiglobulin titer.

Incubation of RBCs with enzymes such as papain, trypsin, and bromelin reduces the electrical potential of the RBC membranes, allowing agglutination of Rh-positive cells by IgG anti-D even in saline. Enzyme screening methods are the most sensitive manual techniques available for detecting Rh sensitization, but they detect many antibodies that have no clinical significance.[118]

These manual methods have the advantages of sensitivity and flexibility, but the correlation between antibody titer and disease severity is poor.

Automated Analysis

Autoanalyzer methods have been developed and refined for more efficient and reproducible detection and quantitation of Rh and other antibodies.[119] Autoanalyzer techniques are very sensitive, and the finding of an Rh antibody detected only by an autoanalyzer method does not necessarily indicate the presence of HDFN or even true sensitization, especially if the titer is very low and stable. However, rising titers indicate the need for urgent assessment of the fetus.

PREDICTION OF FETAL HEMOLYTIC DISEASE

Once sensitization is detected, subsequent investigative measures are required. To refine the estimation of risk, one or more of the following may need to be considered.

Fetal blood type is determined by the following:

- Paternal blood typing
- Fetal blood typing

Severity of disease is predicted by the following:

- History indicating the severity of HDFN in previous infants
- Maternal antibody quantitation
- Fetal ultrasound
- Amniotic fluid spectrophotometry
- Percutaneous fetal blood sampling

Determination of Fetal Blood Type

Paternal Blood Typing

In some instances paternal blood typing can indicate that the baby has no risk for HDFN. This situation occurs if the father is Rh negative and the mother was sensitized not by his offspring but by those of a previous partner or by transfusion. If the father is predicted to be Rh negative or heterozygous, the fetus may be completely unaffected regardless of the maternal antibody titer. In predominantly white populations, approximately 56% of Rh-positive individuals are heterozygous. Because the *RHD* and *RHCE* genes are closely linked, paternal RHD zygosity can be estimated from his Rh c/C and e/E type and his race, or more accurately using molecular techniques.[20,120]

Fetal Blood Typing

Fetal Rh typing by genotyping, using DNA amplification or microarray techniques, is becoming increasingly feasible and widespread and is supplanting the need for antigen typing of fetal blood. Although the need for it can be obviated if paternal homozygosity is certain, it can be an especially important technique when paternity is uncertain or the father is heterozygous.

Molecular methods are now used routinely and with high accuracy in many locations to detect fetal *RHD*, *RHCE*, *KEL*, *DARC* (Duffy), and *SLC14A1* (Kidd) genes in free fetal DNA in maternal blood, obviating the need for chorionic villus biopsy or amniocentesis to obtain fetal DNA.[121,122] Cell-free fetal DNA, presumed to be from apoptotic syncytiotrophoblast, is now most commonly used for fetal genotyping because unlike some fetal cells found in the maternal circulation, it is cleared rapidly after delivery and therefore reflects only the current pregnancy.[123] Fetal genotyping has the potential to reduce or predict the need for assessment throughout the pregnancy, including the need for invasive tests, and to avert the need for RhD immunoglobulinduring some pregnancies. Single-cell preimplantation diagnosis has also been described.[124]

The genetic diversity of the Rh blood group system outlined in an earlier section of this chapter provides an important reminder to use caution in choosing primers that minimize false-positive and false-negative test results and to keep in mind the fact that genotype does not always obviously predict phenotype. The prevalence of *RHD-RHCE* hybrids and partially intact but functionally inert RH genes in certain populations can markedly reduce the validity of tests designed for application in predominantly white populations. The use of appropriate controls; amplification of at least two diagnostic sites, preferably checking the correlation between parents' genotype and phenotype in parallel; and performance of the tests with a good understanding of the typical genotypes in the populations for which they were developed and to which they are being applied should minimize errors.[78,122,125,126,127] The need to have a good positive control for fetal DNA to identify false-negative results is critical. The *SRY* sequence can be used if the fetus is male, but more complicated comparison of multiple fetal and parental polymorphisms in other genes may be necessary if the fetus is female. Differential methylation of the maspin gene in fetal and maternal tissues recommends it as a universal positive control.[128,129]

RHD genotyping of amniotic and chorionic cells agrees with serologic and tissue typing,[130] allowing CVS or amniocentesis to be used for typing of the fetus. However, the need to use these procedures should diminish as procedures using fetal DNA from maternal blood become more widely available. Some advantages of diagnosis using maternal blood samples are the ability to type early in pregnancy and the fact that sample acquisition neither risks harming the fetus nor increases maternal exposure to fetal antigens.

Prediction of Severity of Disease

The relative roles of methods for assessing the severity of hemolytic disease in the fetus have changed in recent years.

Pregnancy History

Although it is usually true that the severity of HDFN remains the same or increases during subsequent affected pregnancies, the disease sometimes becomes less severe. If a previous baby was hydropic, a subsequent affected fetus has a 90%, but not a 100%, chance of developing hydrops. If hydrops is going to develop, usually it does so at the same gestation or earlier, but occasionally it develops later. In a first RhD-sensitized pregnancy, with no prior history of HDFN, there is an 8% to 10% probability that hydrops will develop. If a mother has had a previous fetus with hydrops and the father is heterozygous for the offending antigen, the fetus may be negative and completely unaffected or positive and very severely affected, a dilemma that requires resolution, usually by fetal typing. Thus there are numerous situations in which the history of previous pregnancies is unreliable, although it is nevertheless worth noting.

Maternal Antibody Titers

Although antibody titrations carried out in the same laboratory by the same experienced personnel using the same methods and test cells are reproducible and do give the physician some indication of risk, their predictive value is inadequate to guide invasive fetal treatment. Use of automated analyzers provides somewhat better prediction of the severity of HDFN than manual methods do, but the two approaches have not been compared rigorously. Hadley[104] has noted that whereas many laboratories can and should undertake screening for alloantibodies, antibody quantitation is best limited to regional centers with proven reliability. Ideally, these laboratories will also have strategies for auditing the outcomes of pregnancies in which antibodies have been detected. This can lead over time to the recognition of a critical titer, below which no cases of severe disease have occurred; this allows targeting of more extensive and invasive tests to those with titers above the threshold. However, the follow-up needed is laborious, prone to ascertainment bias, and possibly a violation of patient privacy. Generally, if the obstetric history is good, low titers (≤1:16 by manual methods or <5 IU/mL [1 μg/mL] by autoanalyzer) that increase slowly or not at all are reassuring and indicate continued surveillance using antibody titers and ultrasound.

Although rising titers suggest increasing risk to the fetus, it has not been possible, even with autoanalyzer methods, to establish a titer above which the fetus must be sensitized,[131,132] and sometimes antibody levels increase significantly (but presumably nonspecifically) despite the presence of a compatible and therefore unaffected fetus. Nevertheless, in the presence of high or rising titers, fetal assessment becomes urgent.

Amniotic Fluid Spectrophotometry

Bevis[133] showed that bilirubin could be found in the amniotic fluid of infants with HDFN, and in 1961 Liley[134] plotted fetal outcomes by gestation and results of amniotic fluid spectrophotometry, thus devising a prenatal test of severity of HDFN that has only recently been surpassed as the standard method of assessing fetuses with hemolytic disease. Liley used the ΔOD_{450} reading (the deviation from linearity at 450 nm caused by the absorption spectrum of bilirubin) as a measurement of the amniotic fluid bilirubin level. Three zones of predicted severity of disease were defined (Fig. 3-5). Readings in zone I indicated either no disease or no anemia at the time of testing but reflected a 10% chance that exchange transfusion would be needed. Readings in zone II indicated moderate disease that becomes more severe as readings approach the zone III boundary. Readings falling into very high zone II or zone III indicated severe disease: hydrops was present or would develop within 7 to 10 days. When serial ΔOD_{450} measurements are taken, the overall accuracy of prediction of hemolytic disease with the amniotic fluid technique is 95%. Accuracy is higher in the third trimester than in the second trimester. Because the graphs were developed before intervention at earlier than 27 weeks' gestation was contemplated, zone boundaries in the second trimester were not defined. The Liley zone boundaries were subsequently modified by inclining them downward before 24 weeks' gestation because of the observation that ΔOD_{450} readings peak at 23 to 24 weeks' gestation in pregnancies unaffected by HDFN.[135] The division lines for Queenan's chart are lower at all gestations than those of Liley's system. Although it was suggested that Queenan's modification could overestimate severity, leading to too many interventions,[136] others concluded that a more conservative approach was appropriate as safer and more effective intervention became available.[137] Monitoring by ultrasound and selective direct fetal blood sampling has markedly reduced the need for assessment by amniocentesis. Amniocentesis may still sometimes be useful as an adjunct to ultrasound to assess severity. Increases in antibody titer occur after some amniocenteses, although the risk is reported to be lower than that after umbilical blood sampling.[46,138]

Ultrasound Assessment

The development of obstetric ultrasonography in the late 1970s was a major advance in the management of maternal blood group alloimmunization. Ultrasound allows estimation of placental and hepatic size and determination of the presence or absence of polyhydramnios, edema, ascites, and other effusions. It increased the safety

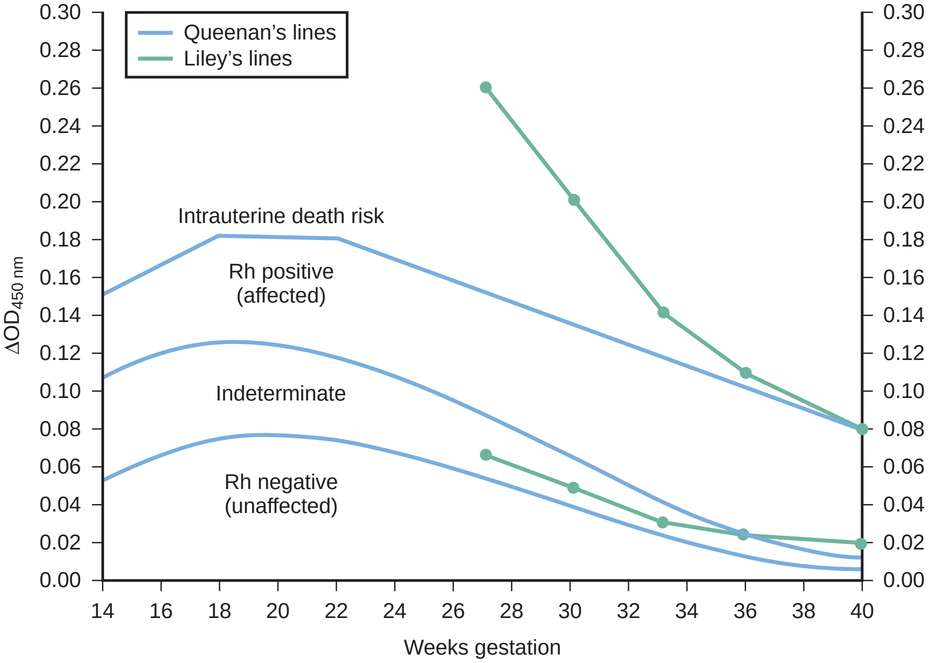

Figure 3-5 Superimposed Queenan and Liley charts of the deviation of absorbance at a wavelength of 450 nm (ΔOD_{450}) versus gestation. Liley's lines began at 27 weeks, and they were rectilinear when shown on a log-linear plot.[134] They were subsequently extrapolated back to 20 weeks.[135] Queenan's chart demonstrates that the lines should deflect downward in the early second trimester.[136] However, the division lines for Queenan's chart are lower at all gestations and may overestimate risk in some fetuses. Current practice is to use fetal cerebral velicometry instead of, or sometimes in addition to, amniotic fluid results in determining the need and timing for intrauterine treatment. *(Redrawn from Scott F, Chan FY: Assessment of the clinical usefulness of the "Queenan" chart versus the "Liley" chart in predicting severity of rhesus iso-immunization.* Prenat Diagn *18:1143-1148, 1998.)*

of amniocentesis and intraperitoneal transfusion and is essential for umbilical blood sampling and intravascular fetal transfusions.

Although ultrasound was previously said to be of little use in assessing the severity of disease until hydrops had developed, assessment of flow velocities in major fetal vessels has now been shown by several groups to be reliable for detecting significant fetal anemia. Middle cerebral artery (MCA) velocimetry is the most extensively used assessment.[139] Published reports comparing MCA peak systolic velocity (MCA-PSV) to threshold levels of fetal or neonatal hemoglobin, show some variation in prediction, but MCA-PSV above 1.5 multiples of the median (MoM) is strongly correlated with severe fetal anemia.[139] As the test becomes more widespread and reduces the need for fetal sampling, it may become more difficult for practitioners to audit outcomes and refine their judgments accordingly. Nevertheless, when skills to perform it are available, MCA velocimetry has become the method of choice for monitoring women with blood group–sensitized pregnancies and in the planning and timing of invasive fetal sampling, fetal treatment, and delivery (Table 3-5, Fig. 3-6).[139,140,141]

Percutaneous Fetal Blood Sampling

With the development of sophisticated ultrasound equipment and the availability of perinatologists skilled in its use, percutaneous fetal umbilical blood sampling became feasible in the mid-1980s.[142] This procedure allows direct diagnosis of fetal blood type, hemoglobin, and acidosis and therefore provided the "gold standard" for diagnosis of fetal anemia against which the accuracy of other assessment techniques has been assessed. The procedure does carry with it a likelihood of fetal-to-maternal hemorrhage and a small risk of fetal mortality.[138,143] Generally, it should be reserved for use when ultrasound (or amniocentesis) indicates moderately severe or severe disease with a probable need for intrauterine treatment. It is generally not indicated unless fetal treatment of severe anemia appears to be justified for continuation of the pregnancy and safe delivery. Fetal blood sampling and intrauterine blood transfusion may be possible as early as 13 weeks' gestation[144]; it usually is feasible by 16 weeks' gestation.[145] The preferred sampling site is the umbilical vein at its insertion into the placenta. For this reason the procedure is technically easier to perform if the placenta is implanted on the anterior uterine wall.

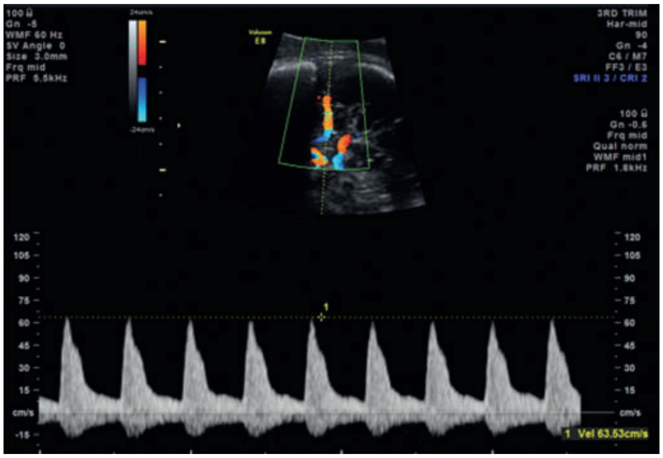

Figure 3-6 Middle cerebral artery Doppler study showing measurement of the elevated peak systolic velocity that is now the method of choice for detecting fetal anemia. Middle cerebral artery peak systolic velocity (MCA-PSV) can predict fetal anemia with sufficient accuracy to determine management, including the need for intrauterine transfusion or in the mid to late third trimester, early delivery. Fetal hemoglobin is typically measured at the start and end of intravascular transfusion to validate the prediction from MCA-PSV results. The reliability of MCA-PSV can decrease after intrauterine transfusion because of the altered rheostatic characteristics of transfused adult blood.

TABLE 3-5 Comparison of Test Characteristics for Peak Systolic Velocity in the Middle Cerebral Artery Measured via Doppler Ultrasonography and Amniotic Fluid ΔOD_{450nm} in Predicting Severe Fetal Anemia*[140]

	Sensitivity	Specificity	Accuracy
Test	Percent (95% Confidence Interval)		
MCA blood flow >1.5 MoM[245]	88 (78-94)	82 (73-89)	85 (79-90)
Amniotic fluid ΔOD_{450nm}			
Modified Liley chart—upper third of zone II or zone III[136]	76 (65-84)	77 (67-84)	76 (69-82)
Queenan chart—zone IV[135]	81 (71-88)	81 (72-88)	81 (75-86)

MoM, Multiples of the median.
*Defined as a hemoglobin level 5 SD or more below the mean for gestation.

MANAGEMENT OF MATERNAL ALLOIMMUNIZATION

Suppression of Alloimmunization

Since the mid-1940s, efforts have been made to suppress the strength of already developed maternal immune responses against fetal RBC. Administration of Rh immune globulin, of great value in preventing Rh sensitization, has been shown to be ineffective in suppressing an established antibody response, no matter how weak, once it has begun.[146]

Two measures that have potential to reduce maternal antibody levels and ameliorate hemolytic disease are (1) intensive plasma exchange[147,148] and (2) the administration of nonspecific intravenous immune serum globulin (IVIG).[149-151] With intensive plasma exchange, alloantibody levels can be lowered by as much as 75%, but they tend to rebound, at times to levels higher than they were before.[152] Vascular access becomes difficult, and IgG and albumin administration is needed. Plasma exchange may have a role in a mother with a fetus known to be susceptible to the antigen to which she is immunized and who has a prior history of severe, early HDFN, especially before 20 weeks' gestation, when the risks and technical difficulty of intrauterine transfusion are higher.[145,153,154] However, the risk-to-benefit ratio remains insufficiently high to recommend it for routine management.

High-dose IVIG administration can also be effective in the severely alloimmunized pregnant woman. The mechanism of action could include inhibition of antibody synthesis or competitive blockade of receptor-mediated transfer across the placenta or binding sites on fetal immune cells. If IVIG therapy is considered, it should be used for indications similar to those for intensive plasma exchange, beginning at 10 to 12 weeks' gestation. Ongoing assessment of fetal disease remains essential because not all fetuses respond adequately. Because of its cost, inconvenience, and side effects, its use, like that of plasmapheresis, is reserved for fetal disease that is expected to be refractory to other therapy.[154]

Fetal Treatment

Induced Early Delivery

Premature delivery of fetuses with Rh HDFN has been important in prevention of mortality since the late 1940s. With the introduction of amniotic fluid assessment in 1961, it became possible for the first time to predict which fetuses should be considered candidates for early delivery. Subsequent advances in perinatal and neonatal management of premature infants have had a major impact on survival. However, HDFN is different from many other pregnancy complications in that intrauterine treatment has been very effective in prolonging many gestations. As the effectiveness of fetal treatment has improved, particularly after the introduction of intravascular intrauterine transfusions, the need for premature delivery of infants with HDFN has diminished. Nevertheless, delivery at 36 to 37 weeks is frequently advisable, particularly in moderate and severe HDFN, to prevent the progression of anemia at late gestation, when fetal treatment often becomes impracticable and neonatal treatment presents fewer risks.[154,155] If premature delivery is required before 34 weeks' gestation, antenatal betamethasone therapy should be given to the mother according to consensus guidelines. It is probable that infants with HDFN have as much or more to gain from use of this important preparation for extrauterine life than their gestation-matched peers with other fetal conditions. Glucocorticoids are often given if intrauterine transfusion is needed (provided the fetus is of sufficient gestation, size, and condition) in case complications of the procedure lead to premature birth. The blood bank should be forewarned so that appropriate blood for exchange transfusion can be obtained if needed. A possible role for antenatal phenobarbital treatment to reduce the need for exchange transfusion of the neonate warrants investigation in randomized controlled trials.[156,157]

Intrauterine Transfusions for Fetal Hemolytic Disease

Until 1961, induced early delivery and neonatal treatment offered the only hope for infants with severe Rh HDFN, but at that time it could not be undertaken before 31 to 32 weeks' gestation without the risk of prohibitive mortality from prematurity. Hydrops develops in 8% of fetuses before 32 weeks' gestation. In 1963 the introduction of intraperitoneal fetal transfusion (IPT) by Liley[158] completely altered the prognosis for these most severely affected of all fetuses

Since the beginning of the twentieth century, it was known that RBCs placed in the peritoneal cavity are absorbed and function normally. At one time, IPT was a preferred method for giving transfusions to children with thalassemia. It was abandoned in favor of vascular transfusions because of the severe discomfort that it caused. Absorption is by way of the subdiaphragmatic lymphatic lacunae, up the right lymphatic duct, and into the venous circulation. In the fetus, breathing movements are necessary for absorption to occur.[159] In the absence of hydrops, 10% to 12% of infused RBCs are absorbed daily. Ascites does not prevent absorption per se, although the rate of absorption in its presence is less predictable and the procedure is more risky. Rupture of membranes and spontaneous labor after IPT are also risks.

Although IPT was a major advance in the management of severe erythroblastosis fetalis, it has been largely superseded by intravascular transfusion (IVT). The earliest attempts at direct IVTs, either into a fetal or placental blood vessel approached through a hysterotomy incision, were made in the mid-1960s, but outcomes were poor because complications such as premature labor almost invariably ensued.[160,161] In 1981 Rodeck and coworkers[162] reported direct fetal transfusions through a fetoscope. This method was soon superseded by percutaneous transfusion into umbilical vessels.[163-167] Under ultrasound guidance the tip of a needle is introduced into an umbilical vein, usually either at its insertion into the placenta (Fig. 3-7) or the umbilical or hepatic vein within the fetus (Fig. 3-8). Fetal blood sampling usually precedes and follows the transfusion, and confirmation that the needle tip remains in the correct position can be obtained throughout the procedure by observing altered echoes in the vessel on ultrasonography.

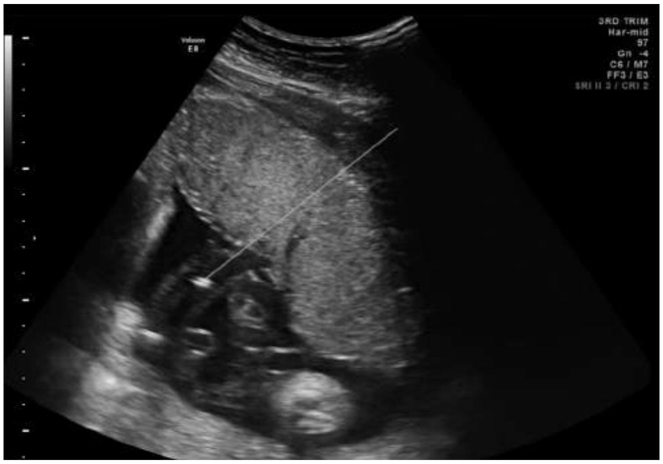

Figure 3-7 Real-time two-dimensional ultrasound view of intravascular transfusion near the insertion of the umbilicus into an anterior placenta. The path of the needle past the placenta is illustrated by the *superimposed line*.

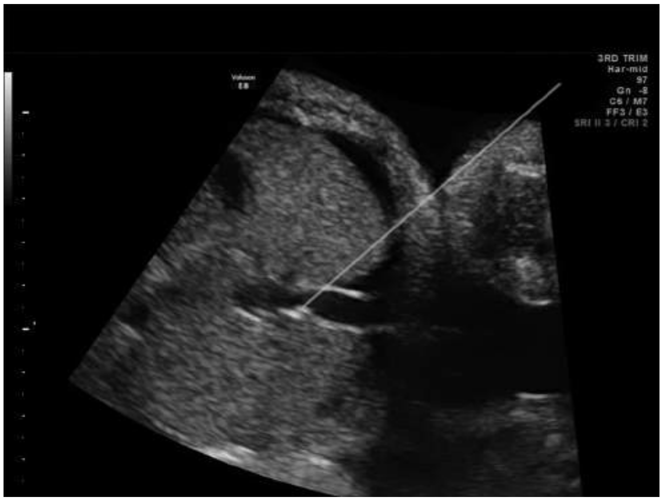

Figure 3-8 Real-time two-dimensional ultrasound view of intravascular transfusion into the intraabdominal portion of the umbilical vein, near the porta hepatis. The path of the needle past the placenta is illustrated by the *superimposed line*. This technique is preferred by some and used selectively by others when (owing to placental and fetal position) the insertion of the umbilical vein to the placenta is inaccessible. Fetal movement can result in dislodgment of the needle, but if that occurs, in some cases, the intrauterine transfusion can be completed as an intraperitoneal transfusion.

IVT offers a number of advantages compared with IPT. IVT allows direct diagnostic sampling at the onset of the procedure, to determine fetal well-being and determine volume to transfuse, and at completion to confirm success and to help predict need for subsequent intrauterine treatments.[154] For skilled practitioners the failure rate, procedure-related complication rate, and mortality rate are low, even in hydropic fetuses.[154,168,169] It allows larger transfusions than IPT and administration of platelets if needed. It is effective in correcting anemia and its immediate physiologic consequences and in reversing hydrops.[166] Survival and neonatal condition were improved so rapidly by the introduction of IVT that, just as with IPT before it, its merits have been determined by comparison with historical control data rather than with prospective randomized studies. Comparison with historical control data may fail to take into account numerous other improvements in perinatal and neonatal care since the 1970s. However, babies with more severe disease respond to IVT, and they need less invasive and intensive neonatal care afterward. Undoubtedly, if an intrauterine procedure is necessary and IVT is feasible, it is the procedure of choice. The overall survival rate of severely anemic fetuses with HDFN who are treated with IVT averages 90% to 95%.[170] Follow-up studies indicate that most survivors of both IPT and IVT have good prognoses for neurologic development and general health.[171] Umbilical and inguinal hernias are reported to occur often after IPT.[172]

Despite the great advantages of IVT, in some situations IPT remains necessary. These include the fetus that is severely afflicted early in pregnancy, at a time when the cord vein is too small for a successful venipuncture. In late pregnancy or in twin gestation, fetal size and position may preclude access to the umbilical vessels. IPT may also be used to “top-up” an incomplete IVT if needle dislodgment or other immediate complications occur.

Both IVT and IPT can expose the mother to fetal cells, raising her antibody titer and potentially worsening disease. As stated earlier, there is no benefit to giving anti-D antibody to these already sensitized mothers. In the future other methods of immunomodulation might prove beneficial to improve the safety of this aspect of treatment.

It has been recommended that both IPT and IVT be performed in centers that perform at least 20 to 30 cordocenteses and 10 to 15 transfusions annually to maintain competence and to allow adequate surveillance of outcomes.[173,174] In experienced hands the risk of procedure-related perinatal loss after IVT is 1.6% per procedure.[168] Some perinatologists routinely administer a muscle relaxant drug before IVT to prevent fetal movement, but others use it selectively or rarely. Transfusion volume should be optimized.[175,176] Complications include fetal bradycardia, cord hematomas, thrombosis or bleeding, rupture of membranes, infection, and premature labor.

Blood for Intrauterine Transfusion

No recent general guidelines have been published for many aspects of intrauterine transfusions. Although advances in equipment design and blood banking techniques have undoubtedly been made, reports of their application to intrauterine transfusion have not been published in peer-reviewed literature. By extrapolation from principles of neonatal transfusion therapy, it seems reasonable to propose that cytomegalovirus-negative packed RBCs should be used for intrauterine transfusion. Cells should be as fresh as is compatible with careful screening and crossmatch against maternal blood and should be leukocyte depleted and irradiated. The risk of graft-versus-host disease is very low but not zero.[177,178] The use of Kell antigen–negative blood is optimal, if it is available.

Treatment of the Neonate with Hemolytic Disease

General Measures

As a result of advances in antenatal assessment and treatment, the pediatrician often has the benefit of treating an infant with HDFN whose condition is better and whose disease course is more predictable than those of infants with HDFN in previous decades. The management of the infant with severe hydrops fetalis remains difficult, and because the disease is now uncommon in high-resource countries, there is reduced opportunity for well-designed trials to test various approaches to management. Uncertainties include the role of antenatal drainage of pleural effusions and ascites, which resuscitation fluids and drugs to use, and what criteria should be used for exchange transfusion. Hydropic fetuses often require extensive resuscitation at birth, including intubation (which can be complicated because of general edema), mechanical ventilation using high pressures, and prompt drainage of pleural effusions or ascites to facilitate lung expansion. Surfactant therapy may be necessary because of prematurity or surfactant inactivation.[179,180] Top-up

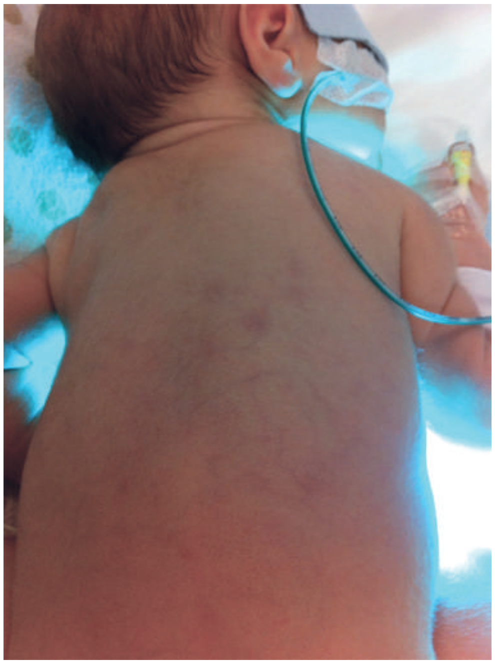

Figure 3-9 Photograph of palpable purpuric lesions that likely represent intradermal erythropoiesis on the back of an infant receiving phototherapy for severe RhD hemolytic disease.

transfusion may be useful for stabilization in hydropic infants who are severely anemic and may have low central venous pressures.[181]

Even in the mildly affected infant, careful assessment and anticipatory management are required. The fetus who has coped well in the intrauterine environment and has not needed or has responded well to intrauterine treatment can still have significant problems with the transition to independent extrauterine life. Problems attributable to HDFN such as hyperinsulinemic hypoglycemia[182,183] may be compounded by the problems of prematurity. The baby should be assessed promptly at birth and resuscitated if necessary. Physical examination may reveal hepatic and splenic enlargement and signs of anemia, including intradermal erythropoiesis (Fig. 3-9). Occasionally, stigmata resulting from accidents of intrauterine therapy are found.

Laboratory assessment should include a complete blood count, albumin level, assessment of blood group and relevant antibodies, and baseline measurement of (conjugated and unconjugated) bilirubin level so that the rate of increase of bilirubin can be calculated. In severely affected infants, liver function, acid-base balance, and coagulation profile should also be assessed. Cord blood can be useful for these tests but may provide misleading results unless the sample is promptly obtained and processed. Many blood banks will not accept cord blood for crossmatch. In infants who have received multiple transfusions, assessment of iron status should be considered,[184] especially if there is evidence of liver dysfunction.[185-188]

In regard to general care, breastfeeding should commence early in infants who are not deemed likely to need exchange transfusion. Infants who are mature and in good condition can be breastfed while receiving phototherapy by way of a suitable fiberoptic blanket or spotlight. Although Rh antibodies are present in breastmilk, particularly in colostrum, very little antibody is absorbed.[189] There is no evidence that forced fluid administration (beyond requirements for nutrition and compensation for any increased insensible losses) improves the outcome of the disease, and it is hazardous in premature infants and those in heart failure.

Folate is normally stored in the liver in late gestation, but reserves may be deficient in premature infants and depleted by ineffective erythropoiesis or by exchange transfusion, which replaces the infant's blood with adult blood that has a lower folate level. Supplemental folic acid at a dose of 50 to 200 μg/day is recommended to approximately 3 months of age.[190] Vitamin B_{12} is usually supplied in adequate amounts in breastmilk (unless the mother has a deficiency). Supplemental iron is not only unnecessary (because of increased iron storage at birth) but potentially hazardous.[184,186,190] Drugs that interfere with bilirubin binding to albumin or with bilirubin metabolism should be avoided.

Intensive phototherapy implies the use of high levels of irradiance distributed to as much of the infant's surface as possible. In intensive phototherapy the diaper should be removed to achieve optimal surface area exposure. Intensive phototherapy consists of at least two phototherapy lamps above and a fiberoptic pad under the infant. The use of phototherapy and other measures for hyperbilirubinemia is discussed in Chapter 4. The principles we now apply in deciding when to begin and end phototherapy in infants with HDFN have not, for the most part, been validated in well-designed trials. However, it seems likely that phototherapy is effective in preventing many exchange transfusions in infants with hemolysis.[191] Only limited evidence supports lower thresholds for using phototherapy and exchange transfusion for hemolytic than for nonhemolytic jaundice with respect to the risk of neurologic sequelae,[192] but it is common to use lower thresholds for phototherapy in HDFN to minimize the need for exchange transfusion. Most neonatal units experienced in care of infants with HDFN use intensive phototherapy prophylactically for infants at high risk and in others whenever there are high or rapidly rising bilirubin levels. The risks and resource implications of phototherapy are low compared with those of exchange transfusion.

Neonates with HDFN require, on average, 5 days of phototherapy, although there is a wide range.[193] It is standard practice to discontinue phototherapy lights one by one when serum bilirubin levels decrease and need for an exchange transfusion becomes less likely. It is also standard to reduce or discontinue phototherapy if the conjugated fraction of bilirubin increases substantially (e.g., to a third to half the total bilirubin level), although there is no good evidence as to what threshold should be used. Commonly, well near-term infants who have received intrauterine transfusions can be discharged by about 1 week of age.[180] Conversely, many weeks of tertiary-level neonatal intensive care may be needed for premature infants and very anemic prehydropic or hydropic infants.

The administration of IVIG to prevent the need for exchange transfusion in HDFN has been the subject of investigation.[194] These studies used different IVIG preparations and for different thresholds. Meta-analysis of all trials suggests a benefit, but the only two high-quality trials found no difference in need for exchange transfusion, peak serum bilirubin, or duration of phototherapy.[195,196] IVIG therapy may merit further investigation, but there is insufficient published evidence to recommend it outside of further carefully conducted clinical trials, and intensive phototherapy and preparation for exchange transfusion should not be delayed in the expectation that IVIG will be effective.

Exchange Transfusion

Exchange transfusion originated in the 1920s in a procedure done by way of the anterior fontanelle. Refinements to the technique of "exsanguination transfusion" were introduced by Wallerstein and Wiener in 1946.[197,198] Generations of infants and pediatric residents should be grateful that Diamond pioneered the use of umbilical catheters[199] because Wiener's technique involved infusion of blood through a saphenous vein cutdown and drainage (after heparinization of the baby) through an incision in the radial artery at the wrist. A randomized controlled trial in 1952 confirmed that the technique could save lives and prevent kernicterus.[200]

During exchange transfusion RBCs coated with antibody are replaced with RBCs negative for the antigen to which the mother is alloimmunized. Exchange transfusion corrects the anemia of the severely affected newborn, mitigates hyperbilirubinemia by removing the infant's hemolyzing erythrocytes and suppressing erythropoiesis, removes some of the already formed bilirubin, and depletes unbound anti-D antibody. Only modest amounts of the offending antibody are removed by exchange transfusion because unbound antibody is widely distributed in extracellular fluid, both within and outside the vascular compartment.

Indications for Exchange Transfusion. Exchange transfusion is generally performed when phototherapy and other conservative measures fail to control the bilirubin level, according to American Academy of Pediatrics (AAP) guidelines.[201] Although these guidelines provide thresholds for exchange transfusion that are lowest in the first hours after birth, neonatal intensive care units that have prompt blood bank support and the ability to perform exchange transfusion at short notice will often make preliminary preparations but delay commencing exchange transfusion in babies who do not have symptomatic anemia for at least several hours after birth in order to assess the effect of intensive phototherapy and to allow the procedure to be performed with optimal safety (e.g., in a neonatal intensive care unit, in daylight hours, with optimal staffing). The decision is complicated by imprecise knowledge regarding the risk boundaries for serious procedure-related morbidity on the one hand and bilirubin-induced brain injury on the other.

Free Bilirubin and Reserve Albumin Binding Studies. It appears that the level of free unconjugated bilirubin is the determining factor in the pathogenesis of bilirubin encephalopathy (kernicterus; see Chapter 4). Measurements of free bilirubin are not routinely available to guide clinical practice, but the AAP guidelines suggest taking the bilirubin-to-albumin ratio (which correlates with free bilirubin) into account in determining the threshold for phototherapy.[201]

Technique of Exchange Transfusion. Because the specific technical details of exchange transfusion are clearly described elsewhere,[202] they are discussed only briefly here. The safest location for catheter tips and whether it is better to use a single catheter "push-pull" technique or a double-catheter continuous technique have not been conclusively determined. In practice, these decisions are often based on local tradition and on the number and type of catheters that can be easily inserted. The procedure most often used in the authors' institutions consists of withdrawing blood in aliquots through an arterial catheter, with timing of each aliquot carefully matched to a continuous infusion (using a peristaltic pump) of donor blood into an umbilical or other venous catheter. Compared with techniques using both bolus withdrawals and injections, this method seems likely to reduce the risk of human error and avoids the need for rapid injection of boluses of donor blood. Regardless of technique, the procedure should be conducted precisely and with meticulous attention to precautions against infection. A second operator records the infusion and each withdrawal, plus the baby's vital signs and results of blood gases, blood sugar, and ionized calcium.

The blood should be ABO compatible and, for anti-D HDFN, Rh negative. If the mother is alloimmunized to an antigen other than D, the blood should be missing that antigen. It should be crossmatch compatible with the mother's serum. Ideally, the blood should also be negative for Kell antigen (to keep from sensitizing the infant) and hemoglobin S (to prevent problems with filtration). If the initial exchange transfusion is carried out using group O blood, any further exchange transfusions should use group O blood. Otherwise, brisk hemolysis and jaundice resulting from ABO incompatibility may become a further complication. Graft-versus-host disease occurs rarely after exchange transfusion, but blood should be irradiated if possible, especially for premature infants. The question of whether to use whole blood or packed RBCs partially reconstituted with plasma or saline (plus or minus platelets) has not been subjected to a rigorous study, but component therapy is increasingly widely used. Component therapy potentially exposes the infant to more donors and greater risk of handling errors, but less risk of white blood cell–related complications. For very unusual antibodies or combinations of antibodies, it may be necessary to transport RBCs over long distances. Occasionally, stored washed maternal RBCs must be used. In these circumstances, whole blood is unlikely to be available.

Depending on the local strategy for neonatal screening for metabolic and other diseases, it is strongly recommended that some of the blood from the first drawback be applied to a neonatal screening card. Otherwise, the diagnosis of some disorders, such as galactosemia, may be masked for some time to come. If any blood for serologic analysis or a DNA sample is needed for other diagnostic purposes, such as congenital infection, it should also be collected at this time.

Aliquots of the infant's blood are removed and replaced with antigen-negative donor blood. In choosing a technique for transfusion, the practitioner should take into account the following considerations: the infant's tolerance of the metabolic, hemodynamic, and hematologic demands; the effectiveness of the procedure in treating anemia and preventing an increase in bilirubin level; and safe handling of the blood for transfusion. The size of aliquot (generally 3 to 5 mL/kg) should be chosen to take into account the tolerance of the infant, and if a single catheter is used, its dead space. Principles of calculus make it clear that the largest aliquots the infant can tolerate will correct anemia most rapidly, but very rapid correction of anemia is not usually the prime objective. Allowing sufficient time for equilibration of bilirubin and antibody between vascular and extravascular compartments is another concern, but with very long procedures there is the risk of clotted catheters and degradation of the donor blood. Generally, between 1.5 and 2 infant blood volumes are exchanged (130 to 170 mL/kg of body weight) over about 2 to 3 hours.[202,203] A two–blood volume exchange transfusion removes approximately 90% of the affected RBCs of the infant, but the response is asymptotic rather than linear and 70% removal occurs after the first blood volume has been exchanged. Only about 25% to 30% of total bilirubin is removed because it is sequestered in extravascular and cellular compartments. A decrease of about 50% in the serum bilirubin level can be expected, but a rapid rebound to about 75% of the pre-exchange level typically is seen. The results of a recent randomized controlled trial suggest that two sequential single-volume exchange transfusions (with about a 3-hour interval between transfusions) may reduce the rate of rebound serum bilirubin level and decrease the need for repeated exchange transfusions, when compared with a single-stage, double volume exchange transfusion.[204]

Many earlier guidelines suggested routine administration of calcium gluconate. However, contemporary anticoagulants affect the ionized calcium much less than the citrate-phosphate-dextrose used in previous years. Furthermore, most neonatal units now have immediate access to blood gas analyzers that also measure ionized calcium, electrolytes, and glucose in small samples. Periodic measurement of ionized calcium, electrolytes, glucose, and blood gas is therefore a good alternative (e.g., every 30 minutes or earlier if the infant manifests any instability). Continuous electrocardiographic monitoring and oximetry should be performed.

Complications of Exchange Transfusion. Complications of exchange transfusion include all those of simple transfusion plus a variety of others related to the large size of the transfusion, to the catheters, and to the technique.[205,206,180]

Mortality rates have been estimated at 0.5% to 2%, but most studies are too small to estimate the risk precisely. Furthermore, the risk for each infant may be influenced by many factors, particularly prematurity and prior severity of illness.

Exchange transfusion produces a significant drop in leukocyte levels, and this is associated with increased risk for culture-proven sepsis, necessitating a high index of suspicion.[180] However, leukopenia and subsequent "left shift" during the rebound from it can also complicate the diagnosis of sepsis in the hours and days after exchange. A similar decrease in platelets is reported after exchange transfusion, with an associated increased risk of hemorrhage. Because of the risk for intraventricular hemorrhage,[180,207] platelet therapy should be considered in sick and premature infants who manifest thrombocytopenia. Platelet count should be measured before, half-way through, and after an exchange transfusion to detect and treat severe thrombocytopenia. Hypocalcemia is also common (because of the use of citrate in donor blood) and could contribute to the risk of hemorrhage in the presence of other coagulation defects.

Necrotizing enterocolitis is a potentially life-threatening hazard that may be related to the technique. In the absence of evidence-based guidelines, effort should be made to prevent wide pressure and volume variations during exchange transfusion. Blood injection and withdrawal should be carried out smoothly without undue haste. Prolonging the interval until oral feeding is restarted and administering prophylactic antibiotics or probiotics might also influence the risk of necrotizing enterocolitis, but there is currently no clear evidence for any of these practices in relation to exchange transfusion.

Declining experience with exchange transfusion among neonatal and pediatric medical and nursing staff as fewer procedures are performed may be counterbalanced by the use of detailed, standardized protocols and improvements in other aspects of intensive care. However, the performance of exchange transfusions in neonatal intensive care units with experienced staff and with blood banks familiar with supplying neonates is likely to yield the best results for infants and the best hope of accumulating evidence to guide future practice.

MANAGEMENT OF SPECIAL PROBLEMS

Syndrome of Hepatocellular Damage

Obstructive jaundice is frequent in infants with HDFN, occuring in 13% in one study, of whom a quarter had conjugated bilirubin levels exceeding half the total bilirubin level.[188] RhD HDFN and intrauterine treatment increased the risk. In a few infants there is extreme hepatomegaly and other clinical evidence of biliary canalicular obstruction. Some infants may develop extreme or prolonged cholestasis, necessitating fat soluble vitamin supplements or other management. The need for chelation for iron overload has also been reported.[187,188]

Infants Who Have Undergone Fetal Transfusions

Assessment of infants who have undergone IVT or IPT does not differ from that of other babies with HDFN.

After multiple IVTs these infants may be delivered with few residual Rh-positive hemolyzing RBCs; their early neonatal management may be simple. However, a high proportion still require one or more exchange transfusions and later top-up transfusions.[208] Multiple transfusions often suppress erythropoiesis (reduced reticulocyte count at birth), leading to an increased need for top-up transfusions to treat neonatal anemia. In addition, thrombocytopenia is often detected in HDFN and is increased in infants treated with intrauterine transfusions.[209]

Follow-Up Care of the Infant with Hemolytic Disease

Whether or not the affected infant has required fetal transfusions or exchange transfusions, anemia in the first few weeks of life is common.[190] Hemoglobin levels should be checked at 7- to 14-day intervals until the hemoglobin has stabilized (usually at approximately 8 to 12 weeks of age). The anemia is due to the gradual loss of transfused Rh-negative RBCs and to the failure of the infant's Rh-positive RBCs to replace them. This failure is initially caused by hemolysis of any RBCs produced and by transient erythroblastopenia, which usually resolves spontaneously at about 6 to 8 weeks of age. The risk is increased by prior intrauterine treatment and decreased by exchange transfusion.[190,208,210]

On average, neonates with HDFN require one top-up transfusion within the first 3 months after delivery.[190] In rare cases, however, some neonates may require up to six top-up transfusions. If symptomatic anemia develops or if hemoglobin levels are falling rapidly toward symptomatic levels, a simple transfusion of 15 to 20 mL/kg of body weight of crossmatched, packed RBCs compatible with the infant's serum is administered. In the authors' institutions, usual hemoglobin thresholds for top-up transfusion are approximately 7.2 to 8 g/dL for asymptomatic babies and approximately 9.6 g/dL for symptomatic babies. Use of these restrictive thresholds for asymptomatic babies after discharge from hospital depends on very good follow-up and rapid access to transfusion if symptoms develop. It is important to remember that international consensus regarding the optimal hemoglobin level threshold for transfusion is lacking.

Recombinant erythropoietin has been used with some success in the treatment of the anemia of prematurity. Its use for prevention of "late" anemia in infants with HDFN has been tested in small trials.[211-213] A larger trial is needed to evaluate the role of erythropoietic stimulating agents in mitigating late anemia of HDFN.

Long-Term Neurodevelopment Outcome After Severe Fetal Anemia

In a recent long-term follow-up study on the neurodevelopment outcome after severe fetal anemia in HDFN treated with intrauterine transfusions (LOTUS study), the risk of neurodevelopmental impairment was reported to be low (4.8%). Severe fetal hydrops was independently associated with neurodevelopmental impairment (odds ratio 11.2; 95% confidence interval, 1.7-92.7), suggesting that prevention of fetal hydrops by timely detection, referral, and treatment may improve long-term outcome.[171]

OTHER MATERNAL ALLOANTIBODIES CAUSING FETAL AND NEONATAL HEMOLYTIC DISEASE

Alloantibodies Other than A and B

Although reports from numerous geographic areas demonstrate the effectiveness of administration of anti-D IgG to prevent RhD alloimmunization, most find that Rh is still the blood group system causing most cases of HDFN and anti-D is still the most common single antibody causing severe HDFN. Of non-D Rh antigens, anti-c is the next most important cause of clinically significant HDFN (although it is typically surpassed in severity, and in many countries in frequency, by anti-Kell HDFN—discussed later). About half of anti-c antibodies are caused by transfusion, and they are often present at low titer or the fetus is c-negative. However, anti-c can cause severe HDFN or fetal death, and careful assessement is warranted.[214] Anti-E is relatively common but most commonly causes only mild HDFN.[215] A wide variety of other non-D Rh antigens, such as anti-C, anti C^w, and anti-e, have also been reported to very occasionally cause moderate or severe HDFN.[216,217] Rh antigens are not infrequently found in combination, such as anti-D plus –C, –D plus –E, or –c plus –ce. Often, one of the antibodies (e.g., anti-D) is present in higher titer and has the predominant influence on disease severity, but the combination adds to the complexity of finding suitable donors for intrauterine, exchange, or top-up transfusion.

There are at least 26 blood group systems, with more than 900 alleles of 39 genes, and the molecules carrying these antigens are very diverse in structure, function, and tendency to cause HDFN. The molecules include transporters and channels (e.g., Diego, Kidd, Colton, and Rh), receptors for pathogens (Duffy, P, and others) and for adhesion to other cells (e.g., Landsteiner-Wiener), enzymes (ABO, H, Lewis, P, and Kell), structural proteins (Ge), and complement receptors (Ch/Rg)[16,218] (see Chapters 15 and 36).

As the incidence of RhD HDFN has been decreasing, other blood group antibodies have assumed a greater importance. Through the interval when anti-D immunoprophylaxis became available, Bowman[219] found that in Manitoba, Canada (population 1 million), the mean annual occurrence of D alloimmunization in pregnant women dropped from 194 in 1962 through 1967 to 23 in 1989 through 1994. In the same two periods, the mean annual occurrence of detected non-D alloimmunization in pregnant women, excluding ABO alloimmunization, increased from 14 to 108. This increase was partially the result of the increased screening of pregnant D-positive women. It also reflected a real increase in the occurrence of non-D alloimmunization because of the increased incidence of blood transfusion (transfused blood is generally only crossmatched for ABO and D). A summary of results from eight studies from the 1960s to the 2000s is shown in Table 3-6.

There is marked international variation in frequency and distribution of alloantibodies. For example, in Asian countries where RhD negativity is rare, so is anti-D. Lee and colleagues identified one case in 28,303 women in

TABLE 3-6 Antibody Occurrence in Eight Series

Place	Minnesota		New York		Australia		Sweden		New York		Liverpool UK		Croatia		Netherlands[†]	
Time period	**1960-1966**		**1960-1967**		**1965-1975**		**1980-1991**		**1993-1995**		**1993-1994**		**1995-2000**		**2003-2005**	
Antibody*	**Polesky et al[246]**		**Queenan et al[247]**		**Pepperell et al[248]**		**Filbey et al[249]**		**Geifman-Holtzman et al[250]**		**Howard et al[251]**		**Jovanovic-Szentic et al[252]**		**Koelewijn et al[253]**	
D	1864	63.1%	304	48.3%	958	65.3%	159	19.0%	101	18.4%	100[‡]	40.9%	200[‡]	35%	335	20%
E	80	2.7%	34	3.5%	69	4.7%	51	6.1%	77	14.0%	29	11.9%	67	11.6%	289	17.9%
C	448	15.2%	34	5.3%	9	0.6%	36	4.3%	26	4.7%	15	6.1%	145	25.2%	21	1.3%
c	68	2.3%	12	1.9%	59	4.0%	38	4.5%	32	5.8%	28	8.6%	17	3.0%	152	9.4%
C^w	4	0.14%	—		—		10	1.2%	1	0.2%			2	0.3%	82	5%
e	2	0.07%	3	0.4%	6	0.04%	1	0.1%	—						9	0.5%
Kell	93	3.1%	30	4.7%	34	2.3%	48	5.7%	121	22%	42	17.2%			242	15.0%
Duffy	17	0.6%	12	1.9%	8	0.5%	26	3.1%	31	5.6%	9	3.6%	6	0.9%	69	4.2%
MNS	45	1.5%	20	3.1%	18	1.2%	35	4.2%	26	4.7%	10	4.1%	27	4.7%	53	3.2%
Kidd	7	0.2%	7	1.1%	2	0.14%	10	1.2%	8	1.5%	10	4.1%	4	0.6%	43	2.6%
Lutheran	—		3	0.4%	—		13	1.6%	7	1.3%						
P_1	27	0.9%	15	2.3%	129	8.8%	48	5.7%	1	0.2%			9	1.6%	NR	
Le^a, Le^b	94	3.2%	51	8.1%	174	11.9%	241	28.8%	113	20.5%	NR		50	8.7%	NR	
I	13	0.4%	15	2.3%	—		—		5	0.9%	NR				NR	
Others	194	6.6%	90	14.3%	1	0.07%	120	14.4%	1	0.2%	NR		49	8.5%	20	1.2%
Total antibodies	2956		630		1467		836		550		244[‡]		576[‡]		1614	
Blood samples	43,000		18,378		72,138		110,765		37,506		22,264		21,730		43,500	
D[§]	43.3		16.5		1.3		1.4		2.6		4.5		9.2		0.83	
E[§]	1.9		1.9		1.0		0.5		2.0		1.3		3.1		0.71	
C[§]	11.3		1.9		0.1		0.3		0.7		0.7		6.7		0.05	
c[§]	1.6		0.7		0.8		0.3		0.8		1.3		0.8		0.37	
Kell[§]	2.2		1.6		0.5		0.4		3.2		1.9		0		0.60	
Lea, Leb[§]	2.2		2.8		2.4		2.2		3.2		NR		2.3		NR	

NR, Not reported.
*Values are given as number of antibodies and % of positive samples.
[†] Denominator is antibodies with risk of causing hemolytic disease of the fetus and newborn.
[‡] Excludes women given anti-D during pregnancy.
[§] Values are given as number of antibodies per 1000 samples.

Hong Kong over a 5-year period, and Rh antibodies accounted for only 27.3% overall. Anti-Mi, recognizing an antigen in the MNS blood group system, was about twice as common, and although it caused no cases of severe HDFN in this series, its potential to do so has been reported.[220]

The alloantibodies listed by various authors as having been reported to cause HDFN are listed in Table 3-7.[42,216,221] Of the multitude of antibodies implicated as causes of HDFN, fortunately most are rare or rarely cause severe disease (Table 3-8). Some are the subject of single case reports.

There are various reasons that the incidence and severity of HDFN differ from one blood group antigen system to another. In some cases it is because of the rarity of the offending antigen or the lack of it and the likelihood of incompatibility between parents being low. An example is the Colton blood group system, which comprises the high-incidence antigens Co$_{a}$ and Co$_{3}$ found on aquaporin-1. Rare individuals lacking this aquaporin can produce antibodies that lead to severe HDFN.

TABLE 3-7 Non-ABO Alloantibodies Reported to Cause Hemolytic Disease[42,219,221]

	Any HDFN	Can Cause Moderate or Severe HDFN
Within the Rh system	Anti-D, -c, -C, -C^{w}, -C^{x}, -e, -E, -E^{w}, -ce, -ceBI -Ce^{s}, -Rh32, -Go^{a}, -Be^{a}, -Evans, -Rh17	Anti-D (commonly) Anti-c, -C, -C^{w}, -C^{x}, -e, -E, -E^{w}, -ce, -Ce^{s}, -Rh32, -Go^{a}, -Be^{a}, -Evans, -Rh17
Outside the Rh system		
Kell	Anti-K, -k, -Ku, -Kp^{a}, -Kp^{b} , -Jk^{a}, -Jk^{b} -Js^{a}, -Js^{b},	Anti-K (commonly) Anti-k, -Kp^{a} -Jk^{a}, -Js^{a}, -Js^{b}, -Ku
Duffy	Anti-Fy^{a}, -By3	Anti-Fy^{a}
Kidd	Anti-Jk^{a}, -Jk^{b}, -Jk^{3}	Anti-Jk^{a}
MNSs	Anti-M, -N, -S, -s, -U, -Mi^{a}, -M^{v}, -Mit	Anti-M, -S, -s, -U, -Mi^{a}
MSSs	Anti-Mt^{a}, -Mur, -Hil, -Hut	Anti-Mt^{a}
Lutheran and LW	Anti-Lu^{a}, -Lu^{b}, -Lu9, -LW	
Diego	Anti-Di^{a}, -Di^{b}	Anti-Di^{a}, -Di^{b}
Xg	Anti-Xg^{a}	Anti-LW
P	Anti-PP$_{1pk}$	Anti-PP$_{1pk}$
Examples of antibodies to other low-frequency antigens	Anti -By, -Fr^{a}, -Sc2, -Rd, -Re^{a}, -Zd, -Vw, -Batty, -Becker, -Berrens, -Biles, -Gonzales, -Good, -Heibel, -Hunt, -Jobbins, -Radin, -Rm, -Ven, -Wr^{a}, -Wr^{b}, -Zd	Anti-Biles, -Good, -Heibel, -Wr^{a} -Zd
Examples of antibodies to high-incidence antigens	Anti-At^{a}, -Co^{a}, -En^{a}, -Do^{a}, -Ge, -Jr^{a}, -Lan, -Yt^{a}, -Yt^{b}	Anti-Co^{a}, -En^{a}, -Lan, -Yt^{a}

HDFN, Hemolytic disease of the fetus and newborn.

*Anti-Lewis, anti-I and -Fy^{b} antibodies do not cause HDFN of any severity. For antibodies that can cause moderate or severe HDFN, assessment of risk, including paternal typing and fetal surveillance, should be considered if the antibodies are present at or above the critical titer for the laboratory or are rising. Note that Kell antibodies can cause severe HDFN at relatively low titers. More than one antibody can coexist, and prognosis may depend most on the antibody with the highest predilection for causing HDFN, even if it is not present at the highest titer.

In others it is because of low levels of expression on the RBC surface. For this reason maternal anti-I and anti-Lutheran antibodies, even when present at high titer, usually do not cause HDFN because of low cell surface expression of these antigens on fetal RBCs. Lutheran antigens are also widely distributed in endothelial cells.

Disease caused by anti-Kell antibodies can be very severe. Maternal antibodies to KEL1 causing neonatal anemia were first described in 1945,[5] and the blood group system was named for the woman in whom the antibodies were found. Kell glycoprotein is an endopeptidase involved in endothelin activation,[218,221] and it is more properly regarded as a subunit of a larger protein because it is linked by disulfide bond to the XK protein. Absence of XK can cause weak Kell expression and dysfunction of other organs (McLeod syndrome). Null Kell alleles also occur. Redman and associates[222] reviewed the Kell blood group and summarized the low- and high-incidence antigens, of which approximately 25 have been described. They occur as variants of several epitopes. The most immunogenic antigen, KEL1, is second to RhD in its immunizing potential and occurs in about 9% of white subjects and 2% percent of black subjects. It results from a point mutation causing a Thr193 (KEL2) to Met193 (KEL1) substitution, which in turn leads to loss of a glycosylation site. The other Kell phenotypes are also generally caused by single base substitutions, so genotyping is more straightforward than that in the Rh system.

Sensitization occurs most commonly through unmatched blood transfusion but can arise during pregnancy. Although the number of incompatible pregnancies is lower for Kell than for RhD, the proportion of anti-KEL1–affected fetuses that are severely affected is thought to be 20% or higher.[223-227]

Kell antibodies, which recognize antigens expressed in early erythroid progenitors and at an early stage of erythropoiesis in fetal liver, appear to be capable of suppressing erythropoiesis.[228] This is the putative reason for the tendency for hydrops early in pregnancy seen in anti-Kell HDFN. It has also been proposed as the reason that amniotic fluid spectrophotometry is not as reliable as in Rh hemolytic disease, although not all studies agree on this point.[224,229,230] Paternal typing followed, where necessary, by fetal genotyping and early, close monitoring by ultrasound is recommended in pregnancies complicated by anti-Kell antibodies. Because of the suppression of fetal erythropoiesis, the immediate neonatal management in Kell HDFN is different from RhD HDFN. As shown in a recent study, neonates with Kell hemolytic disease require less phototherapy and fewer exchange transfusions compared with neonates with RhD hemolytic disease, but an equal number of top-up transfusions.[227]

There are rare instances of a wide variety of other alloantibodies, usually benign ones, causing hemolytic

TABLE 3-8 Severity of Hemolytic Disease in Manitoba over 32 Years*[219]

Alloantibody Specificity	No. of Patients	Affected Patients (%)	No Treatment Required (%)	Phototherapy and/or Exchange Transfusion Required (%)	Hydropic or Hb <60 g/L (%)
D (19 yr)	566	257 (47)	51	30	19
E	633	162 (26)	89	11	—
c, cE	302	164 (54)	70	23	7
C, Ce, C^w, e	193	50 (36)	86	14	—
Kell	478	16 (3.3)	50	37	13
Kp^a	7	3 (43)	67	33	—
k	1	1 (100)	—	100	—
Fy^a	35	6 (17)	67	16	16
S	20	11 (55)	64	36	—

Hb, Hemoglobin.
*From November 1, 1962, to October 31, 1994, except for anti-D (November 1, 1975, to October 31, 1994).

disease sufficiently severe to require intrauterine or postnatal treatment (e.g., anti-Coa, anti-Fya, anti-Di, and anti-M).

ABO Hemolytic Disease

ABO HDFN behaves differently from HDFN because of either anti-D or other blood group antibodies. Anti-A and anti-B, which bind complement in adults, cause violent, life-threatening intravascular hemolysis after transfusion of ABO-incompatible blood. However, although kernicterus may develop if the baby with ABO HDFN is left untreated, symptomatic fetal, neonatal, or late anemia is very rare. There are occasional reports of hydrops fetalis attributed to ABO erythroblastosis.[231] It is possible that some of these involve other causes of increased ineffective erythropoiesis (such as RBC enzyme deficiency or α-thalassemia trait) or nonimmune hydrops superimposed on ABO HDFN. If clinically significant anemia or hydrops is found in a fetus or newborn with a positive direct antiglobulin test (DAT) owing to anti-A or anti-B, other potential etiologies should still be investigated. Some but not all series have found that ABO antibodies are a more common cause of HDFN (and of severe HDFN) in Southeast Asia, Africa, and Latin America, for reasons that have yet to be determined.[42]

Several reasons have been proposed for the mildness of ABO HDFN compared with HDFN caused by other antibodies and with adult ABO hemolysis.[232] First, there are fewer A and B antigenic sites on the fetal RBC membrane,[233] and anti-A and anti-B do not bind complement on the fetal RBC membrane.[234] Second, anti-A and anti-B are often IgM, which does not cross the placenta, or IgG2, which plays a minor role in HDFN.[235] Third, the small amounts of IgG anti-A and anti-B that do traverse the placenta have myriad antigenic sites both on tissues other than on RBCs and on secretions to which they may bind. Because there is very little antibody on the RBC, the cord blood direct antiglobulin test result in ABO hemolytic disease is usually only weakly positive and may be negative unless a sensitive test is used. Capillary blood taken at 2 or 3 days of age often yields a negative direct antiglobulin test result regardless of test sensitivity. In at least 25% of ABO-incompatible babies, cord blood RBCs give weakly positive results on the direct antiglobulin test at delivery, and an excess of babies with blood types A and B of mothers with blood type O become severely jaundiced.[236,237] The A- or B-incompatible infants of mothers with blood type A or B do not appear to be at increased risk for hemolysis or jaundice.[238]

However, only a small fraction of A- or B-incompatible infants of O mothers develop clinical evidence of HDFN (early and severe jaundice), even when the direct antiglobulin titer is positive.[238-240] One study found that only 0.07% of more than 88,000 infants required exchange transfusion for anti-A or -B HDFN.[239] Nevertheless, ABO HDFN continues to account for a significant proportion of infants with troublesome jaundice, necessitating prolongation of hospital stay, readmission to the hospital, or home therapy. The same large study found that ABO incompatibility accounted for 5.5% of infants with jaundice.

Although anemia is rarely severe, mild hemolysis appears to be common. In a series of 1704 infants of blood group O mothers, the infants with blood group A or B had significantly higher cord bilirubin and lower cord hemoglobin concentrations than the babies with blood group O.[241] Blood films may show polychromasia, reticulocytosis, and prominent spherocytosis, which can sometimes be difficult to differentiate from hereditary spherocytosis.

Control of hyperbilirubinemia is usually the major issue in infants with ABO HDFN, and thresholds for phototherapy and exchange transfusion are generally regarded as similar to those for Rh HDFN. The risk of symptomatic late anemia in ABO HDFN is very low. The risk of recurrence of jaundice caused by anti-A or -B in future incompatible siblings is high, and mothers should be counseled accordingly.[242]

CONCLUSIONS

Advances in prevention of Rh sensitization and the management of immune HDFN in the past 30 years have been dramatic. Statistics from high-resource countries have indicated that the prevalence of Rh sensitization in pregnant women has been reduced by more than 90%, although worldwide the disease burden remains large.[89] The number of exchange transfusions has also decreased by more than a hundredfold. In addition, the number of perinatal deaths caused by HDFN has plummeted. However, for those still affected by anti-D or other significant antibodies, the disease burden remains significant.

Despite a recent explosion in knowledge about antigen presentation and provocation of the immune response in general, little detail at the molecular and cellular level is known about maternal sensitization to RhD. A better understanding of these events, taken together with the recent elucidation of the molecular genetics of RhD and other blood groups, is likely to provide new tools for prevention, diagnosis, and treatment.

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CHAPTER 4

Neonatal Jaundice and Disorders of Bilirubin Metabolism*

Elissa Downs and **Glenn R. Gourley**

CHAPTER OUTLINE

INTRODUCTION
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Bilirubin Production and Transport
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Bilirubin Conjugation
Bilirubin Excretion
Enterohepatic Circulation of Bilirubin
DIAGNOSIS OF HYPERBILIRUBINEMIA
Total Serum Bilirubin Measurements
Transcutaneous Bilirubinometry
TOXICITY OF UNCONJUGATED HYPERBILIRUBINEMIA
Kernicterus
NONPATHOLOGIC UNCONJUGATED HYPERBILIRUBINEMIA
Physiologic Jaundice
Breastfeeding and Jaundice
DISORDERS OF PATHOLOGIC UNCONJUGATED HYPERBILIRUBINEMIA
Disorders of Production
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Disorders of Enterohepatic Circulation
Other Causes of Unconjugated Hyperbilirubinemia
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Rotor Syndrome
Dubin-Johnson Syndrome

INTRODUCTION

Elevation of the serum bilirubin level is a common, if not universal, finding during the first week of life and has been reviewed elsewhere.[1-4] This can be a transient phenomenon that will resolve spontaneously or can signify a serious or even potentially life-threatening condition. There are many causes of hyperbilirubinemia and related therapeutic and prognostic implications. Independent of the cause, elevated serum bilirubin levels can be potentially toxic to the newborn infant. This chapter will review perinatal bilirubin metabolism and address assessment, etiology, toxicity, and therapy for neonatal jaundice. The diseases in which there is a primary disorder in the metabolism of bilirubin will be reviewed regarding their clinical presentation, pathophysiology, diagnosis, and treatment.

BILIRUBIN METABOLISM

Bilirubin Production and Transport

In 1864 Städeler used the term *bilirubin,* derived from Latin (*bilis,* bile; *ruber,* red), for the red-colored bile pigment.[5] Bilirubin is formed from the degradation of heme-containing compounds (Fig. 4-1), the largest source of which is hemoglobin, and other sources are the cytochromes, catalases, tryptophan pyrrolase, and muscle myoglobin.[6]

The formation of bilirubin is initiated by cleaving the tetrapyrrole ring of protoheme (protoporphyrin IX), which results in a linear tetrapyrrole (biliverdin). The first enzyme system and rate limiting step in bilirubin synthesis is microsomal heme oxygenase (HO).[7] Two major forms of HO have been identified. HO1, the inducible form, is located in the spleen, liver, and bone marrow. HO2, the constitutive form, is located in testes, central nervous system (CNS), vasculature, liver, kidney, and gut.

HO results in reduction of the porphyrin iron (Fe^{III} to Fe^{II}) and hydroxylation of the α methine (=C-) carbon.

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*URL referenced in this chapter includes: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/.

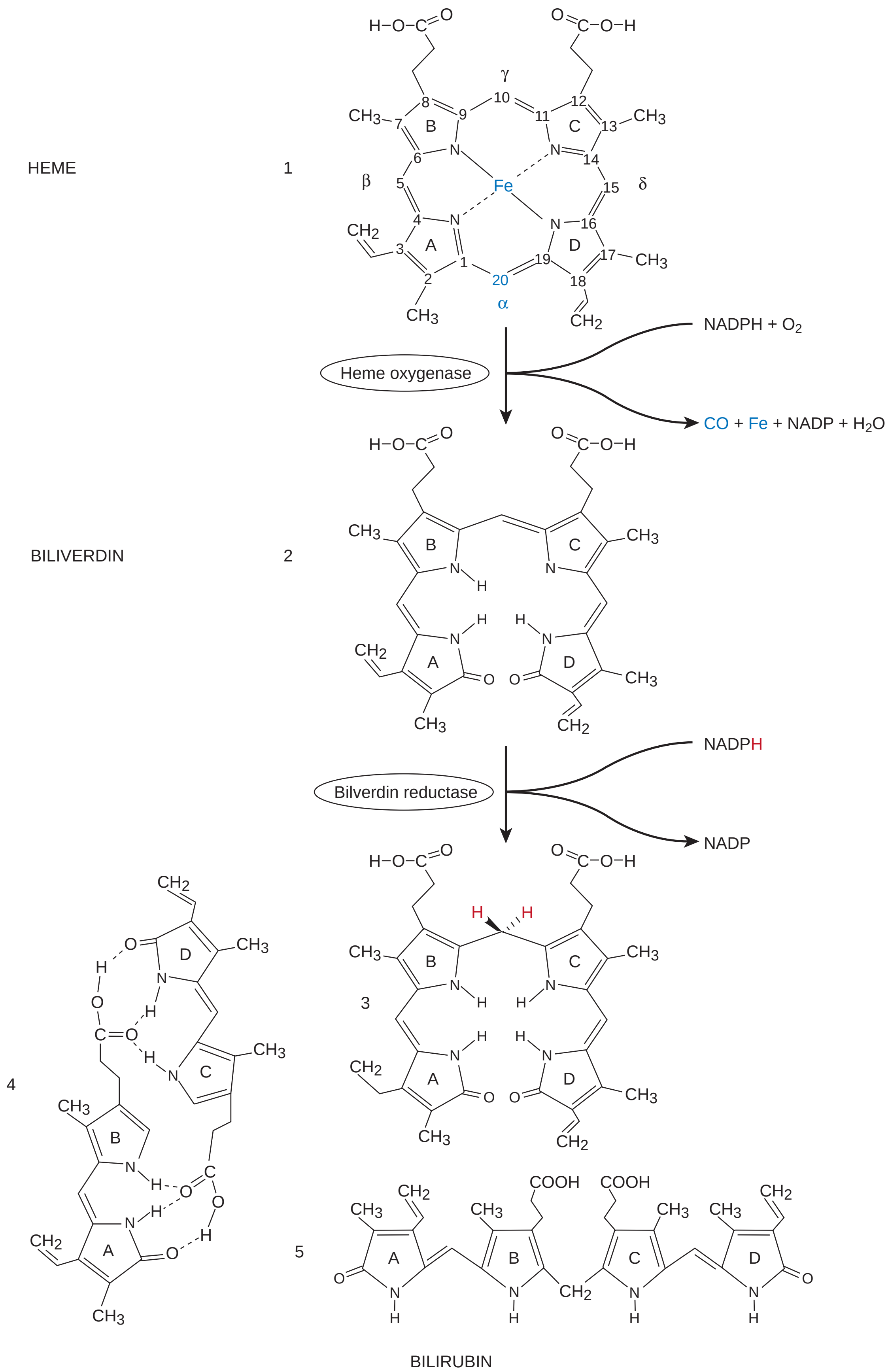
HEME
1
BILIVERDIN
2
3
4
5
BILIRUBIN
Heme oxygenase
NADPH + O2
CO + Fe + NADP + H2O
Bilverdin reductase
NADPH
NADP
Fe
α
β
γ
δ
A
B
C
D
COOH
CH3
CH2

Figure 4-1 Chemical structures depicting the conversion of heme to bilirubin. Bilirubin is frequently represented by any of the three structures (3-5) shown at the bottom. *(From Gourley GR: Neonatal jaundice and disorders of bilirubin metabolism. In Suchy FJ, Sokol RJ, Balistreri WF, editors:* Liver disease in children, *ed 3, New York, 2007, Cambridge University Press.)*

This α carbon is then oxidatively excised from the tetrapyrrole ring, yielding carbon monoxide (CO); this is the only physiologic source of CO. This excision opens the ring structure and is associated with oxygenation of the two carbons adjacent to the site of cleavage. The cleaved α carbon is excreted as CO, which has numerous biological effects, including neurotransmission, vasodilation as well as mediation of apoptosis and anti-inflammatory processes.[7] The iron released by HO can be reutilized by the body. HO produces a linear tetrapyrrole, biliverdin IXα. The IX designation is a result of Fischer's grouping of the protoporphyrin isomers, group IX being the physiological source of bilirubin.[8] In utero, bilirubin IXβ is the first bile pigment seen and can be found in bile or meconium by 15 weeks' gestation.[9] Small amounts of bilirubin IXβ are also found in adult human bile.[10] The central (C-10) carbon on biliverdin IXα is then reduced from a methine to a methylene group (-CH_2-), thus forming bilirubin IXα. This is accomplished by the cytosolic enzyme biliverdin reductase.[11] The proximity of this enzyme results in very little biliverdin ever being present in the circulation.

Bilirubin formation can be assessed by measurement of CO production.[12] Such assessments indicate that the daily production rate of bilirubin is 6 to 8 mg/kg/24 hr in healthy term infants and 3 to 4 mg/kg/24 hr in healthy adults.[13,14] In mammals, 80% to 85% of bilirubin produced daily originates from hemoglobin.[15] Degradation of hepatic and renal heme appears to account for most of the remainder, reflecting the very rapid turnover of certain of these heme proteins. Although the precise fate of myoglobin heme is unknown, its turnover appears to be so slow as to be relatively insignificant.[16]

Catabolism of hemoglobin occurs very largely from the sequestration of erythrocytes at the end of their life span (120 days is adult humans, 90 days in newborns). A small fraction of newly synthesized hemoglobin is degraded in the bone marrow. This process, termed *ineffective erythropoiesis,* normally represents less than 3% of daily bilirubin production but may be substantially increased in people with hemoglobinopathies, vitamin deficiencies, and heavy metal intoxication.[17-19] Infants produce more bilirubin per unit of body weight because of their greater red blood cell mass and shorter red blood cell life span. Additionally, hepatic heme proteins represent a larger fraction of total body weight in infants.

Although bilirubin has long been thought of solely as a waste product, data suggest that some mild degree of hyperbilirubinemia may be helpful because of the antioxidant capacity of bilirubin and its potential role as a free-radical scavenger and cytoprotectant.[20]

Bilirubin is poorly soluble in aqueous solvents and thus requires biotransformation to more water-soluble derivatives for excretion from the body. This poor solubility is related to the structure of bilirubin.[21] Rather than being linear (structure 5, Fig. 4-1), bilirubin undergoes extensive internal hydrogen bonding (structure 4, Fig. 4-1). This shields the polar propionic acid side chains and makes bilirubin very nonpolar and lipophilic. The carbon–carbon double bonds at positions 4-5 and 15-16 can assume two different configurations (similar to *cis* and *trans*) depending on whether the higher priority atoms or groups (based on atomic number) are on the same (*Z, zusammen,* German: *together*) or opposite (*E, entgegen, opposite*) sides of the double bond. The naturally occurring form of bilirubin, 4Z,15Z-bilirubin IXα, can be represented by any of the three structures (3-5) depicted at the bottom of Figure 4-1. Knowledge of this stereochemistry is important in understanding phototherapy, which will be discussed later.

Bilirubin's poor aqueous solubility necessitates a carrier molecule, albumin, for transport from sites of production in the reticuloendothelial system to the liver for excretion (Fig. 4-2).[22] Each albumin molecule possesses a single high-affinity ($K_a = 7 \times 10^7\ M^{-1}$) binding site for one molecule of bilirubin.[23] A binding affinity of this magnitude implies that, at normal serum bilirubin levels, all bilirubin will be transported to the liver bound to albumin, with negligible amounts free to diffuse into other tissues. Secondary binding sites of lesser affinity also exist on albumin. Albumin also serves as a carrier for other compounds such as xenobiotics and fatty acids.

Hepatic Uptake of Bilirubin

The structure of the liver is well suited for the uptake of bilirubin by individual hepatocytes. Cords of hepatocytes are arranged radially so that adjacent sinusoids border all hepatocytes. The flow of blood through the sinusoids is slower than that of other capillary beds because it is generated by portal venous pressure rather than arterial pressure. There is easy passage of albumin-bound bilirubin from the plasma into the tissue fluid space (space of Disse) between the endothelium and the hepatocyte because the sinusoidal endothelium of the liver lacks the basal laminae, which are found in other organ capillary systems.[24] The pores of the endothelium allow direct contact with the plasma membrane of the hepatocyte.

A hepatocyte with a schematic illustration of bilirubin metabolism is shown in Figure 4-2. In the first step, bilirubin dissociates from its albumin carrier[25] and enters the hepatocyte via a membrane-receptor carrier, which facilitates entry into the hepatocyte. Carrier-mediated transport into the hepatocyte has been demonstrated for several organic anions including bilirubin, bromsulfophthalein (BSP), and indocyanine green (ICG),[26] though bilirubin has also been shown to be able to pass through membranes by simple passive diffusion.[27] Evidence suggests that bilirubin, BSP, and ICG share the same hepatocyte-receptor carrier because they exhibit competitive inhibition when injected simultaneously. This finding cannot be explained by subsequent intrahepatic metabolism, because these anions are handled differently by the hepatocyte: Bilirubin is conjugated with glucuronic acid in the endoplasmic reticulum (ER), BSP is conjugated with glutathione in the cytosol, and ICG is excreted directly without biotransformation. Data from rat hepatocytes suggest that the anion binding–receptor carrier is a dimeric protein with subunit molecular weight of 55,000.[28-30] Antibody studies confirm the expected location in the plasma membrane[29] and demonstrate blocking of uptake.[30] Organic anion transporting polypeptide 2 (OATP2, recently named OATP1B1 under

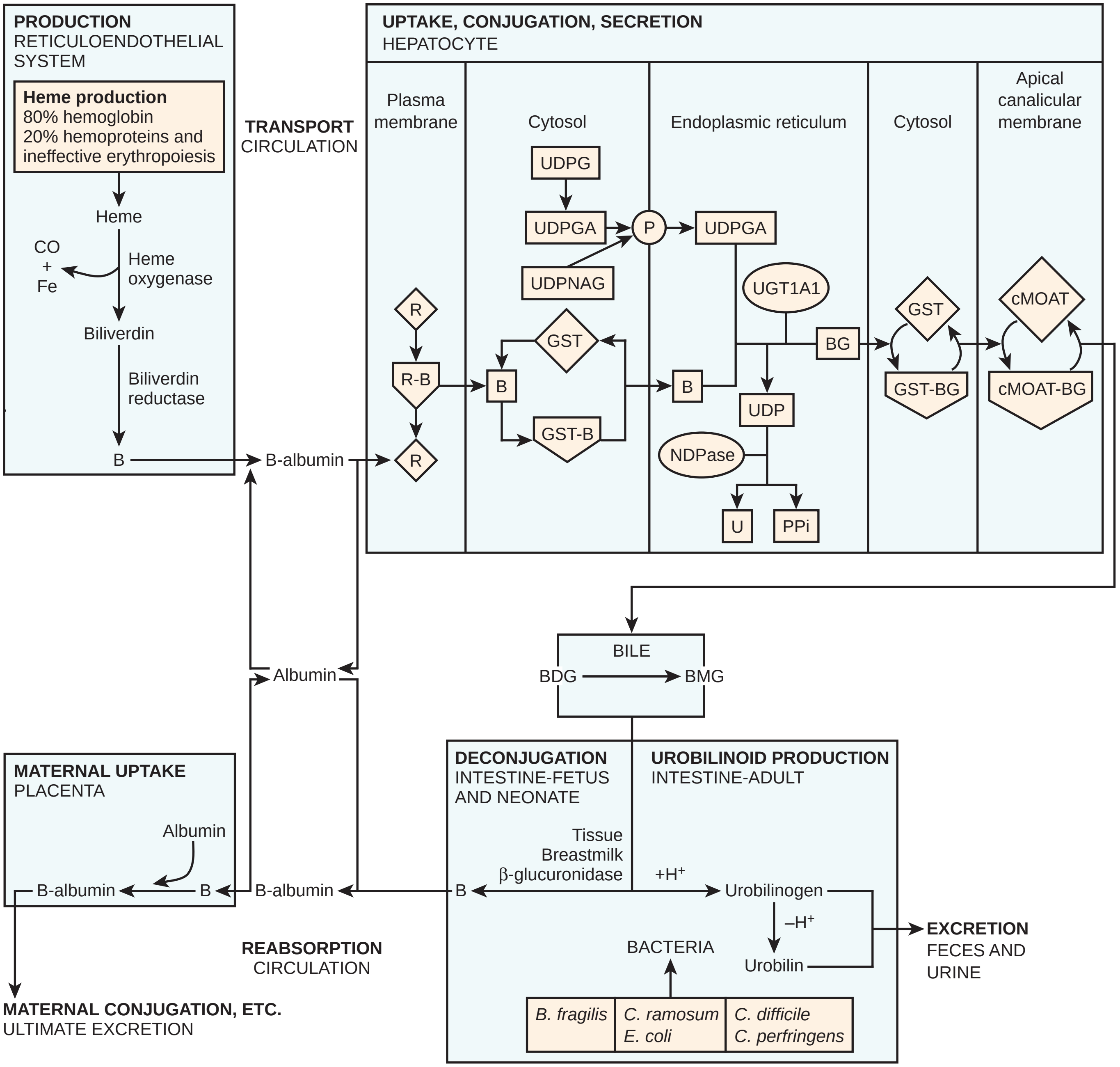
PRODUCTION
RETICULOENDOTHELIAL SYSTEM
Heme production
80% hemoglobin
20% hemoproteins and ineffective erythropoiesis
Heme
CO + Fe
Heme oxygenase
Biliverdin
Biliverdin reductase
B
TRANSPORT
CIRCULATION
B-albumin
UPTAKE, CONJUGATION, SECRETION
HEPATOCYTE
Plasma membrane
Cytosol
Endoplasmic reticulum
Cytosol
Apical canalicular membrane
UDPG
UDPGA
P
UDPGA
UDPNAG
UGT1A1
R
R-B
B
GST
GST-B
R
B
BG
UDP
NDPase
U
PPi
GST
GST-BG
cMOAT
cMOAT-BG
BILE
BDG
BMG
Albumin
MATERNAL UPTAKE
PLACENTA
Albumin
B-albumin
B
B-albumin
DECONJUGATION
INTESTINE-FETUS AND NEONATE
Tissue
Breastmilk
β-glucuronidase
B
UROBILINOID PRODUCTION
INTESTINE-ADULT
+H+
Urobilinogen
–H+
Urobilin
BACTERIA
B. fragilis
C. ramosum
E. coli
C. difficile
C. perfringens
EXCRETION
FECES AND URINE
REABSORPTION
CIRCULATION
MATERNAL CONJUGATION, ETC.
ULTIMATE EXCRETION

Figure 4-2 A schematic overview of bilirubin *(B)* metabolism in the fetus, neonate, and adult. *BDG/BMG,* Bilirubin di- or monoglucuronide; *BG,* bilirubin glucuronide; *cMOAT,* canalicular multispecific organic anion transporter (also known as MRP2 or ABCC2); *GST,* glutathione S-transferase (ligandin); *NDPase,* nucleoside diphosphatase; *R,* membrane carrier; *P,* permease; *PPi,* inorganic pyrophosphate; *UDPG,* uridine diphosphate glucose; *UDPGA,* uridine diphosphate glucuronic acid; *UDPNAG,* uridine diphosphate N-acetyl glucosamine; *UGT1A1,* bilirubin glucuronosyltransferase. *(From Gourley GR: Neonatal jaundice and disorders of bilirubin metabolism. In Suchy FJ, Sokol RJ, Balistreri WF, editors:* Liver disease in children, *ed 3, New York, 2007, Cambridge University Press.)*

new nomenclature[31]) has shown high-affinity uptake of bilirubin in the presence of albumin and is a member of the OATP family, transporter symbol SLC21A.

Carrier-mediated transport of bilirubin into the hepatocyte is necessary because of differences in protein-binding inside and outside the hepatocyte. Outside the hepatocyte, bilirubin is bound to albumin (affinity constant ~10^8, concentration: 0.6 mM).[23] Inside the hepatocyte, bilirubin is bound to glutathione S-transferase B (GST), historically known as ligandin or the Y protein (affinity constant ~10^6, concentration 0.04 mM).[32,33] GST constitutes a family of proteins that exhibit important functions both as enzymes and as intracellular binding proteins for nonsubstrate ligands such as bilirubin.[34] Carrier-mediated uptake helps generate a concentration gradient for bilirubin uptake despite the difference in affinity between albumin and GST. GST is important in the intracellular storage of bilirubin and bilirubin

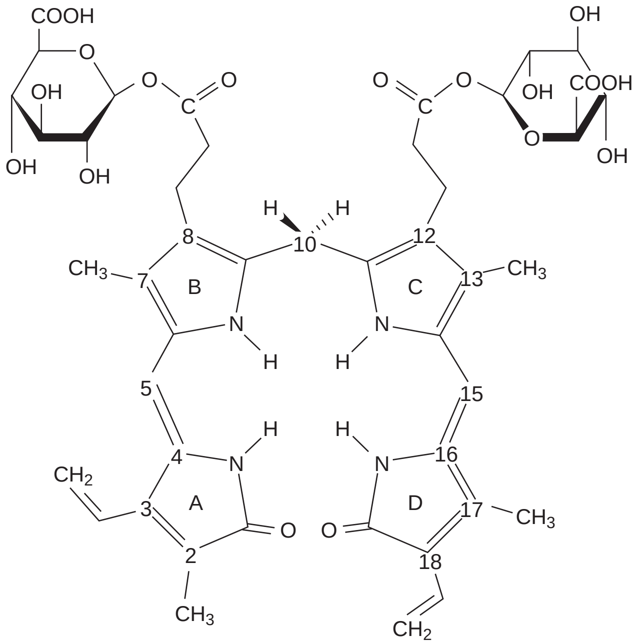

Figure 4-3 Bilirubin diglucuronide. In bilirubin monoglucuronide, only one propionic acid side chain (C-8 or C-12) is glucuronidated. *(From Gourley GR: Neonatal jaundice and disorders of bilirubin metabolism. In Suchy FJ, Sokol RJ, Balistreri WF, editors:* Liver disease in children, *ed 3, New York, 2007, Cambridge University Press.)*

conjugates and reduces efflux from the hepatocyte back into plasma.[33]

Bilirubin Conjugation

Inside the hepatocyte, bilirubin is conjugated with glucuronic acid[35] within the ER (microsomes). The glucuronic acid donor is uridine diphosphate glucuronic acid (UDPGA). The conjugation results in an ester linkage formed with either or both of the propionic acid side chains on the B and C pyrrole rings of bilirubin (Fig. 4-3). The enzyme responsible for this esterification is bilirubin UDP-glucuronosyltransferase (BUGT; Online Mendelian Inheritance in Man [OMIM] *191740). BUGT is distinct from the other glucuronosyltransferase isoforms which catalyze the conjugation of thyroxine, steroids, bile acids, and xenobiotics.[36] BUGT (which is the same as UGT1A1, see below) is embedded in the lipid environment of the microsomal membrane, and perturbations of this environment greatly affect in vitro measurements of BUGT activity. Since BUGT is located on the interior of the ER, the existence of a permease has been hypothesized to facilitate the transport of UDPGA from the cytosol across the lipid layers of the ER. The permease has been proposed because uridine diphosphate glucose (UDPG) is present in the cytosol in higher concentrations, yet UDPGA serves as the preferred donor for bilirubin conjugation.[37] Uridine diphosphate N-acetyl glucosamine (UDPNAG) is considered to be a natural regulator of BUGT[38] because UDPNAG increases in vitro BUGT activity threefold. The mechanism for this is unknown and could possibly involve facilitation of the permease UDPGA transporter.[39] After providing glucuronic acid for conjugation, UDP is converted to uridine and inorganic pyrophosphate by a nucleoside diphosphatase (NDPase) that is also located on the interior of the ER[40] and prevents the reverse reaction.

The specific isoform responsible for bilirubin conjugation is UGT1A1 (trivial name HUG-Br1 or BUGT, EC 2.4.1.17).[36] This is part of the UDP glycosyltransferase superfamily of enzymes encoded by the UGT gene complex on chromosome 2 that is involved with metabolism of many xenobiotic and endogenous substances. The UGT1 gene encodes several isoforms and has a complex structure consisting of four common exons (2-5) and 13 variable exons encoding different isoforms (Fig. 4-4).[41,42] At least 30 different *UGT1* mutant alleles have been described that cause Gilbert's syndrome and Crigler-Najjar (CN) syndromes I and II.[43] UGT1A1 catalyzes the formation of both bilirubin mono- and diglucuronides[44] but also metabolizes other hormones and drugs, so mutations could be involved in carcinogenesis and adverse drug reactions.[45] In normal adult humans the majority of bilirubin conjugates are excreted in the bile as bilirubin diglucuronides (approximately 80%)[46-48,49,50,51] (Fig. 4-5, middle panel). Lesser amounts of bilirubin monoglucuronides (approximately 15%) are also excreted along with very small amounts of unconjugated bilirubin (UBIL) and other bilirubin conjugates (e.g., glucose, xylose, and mixed diesters). In infants, because there is lower UGT1A1 activity, bile contains less bilirubin diglucuronide and more bilirubin monoglucuronide than the adult (Fig. 4-6, middle panel).

Bilirubin Excretion

Following conjugation, bilirubin conjugates are excreted against a concentration gradient from the hepatocyte through the canalicular membrane into the bile. The ATP-dependent transporter responsible for bilirubin glucuronide passage from the hepatocyte through the canalicular

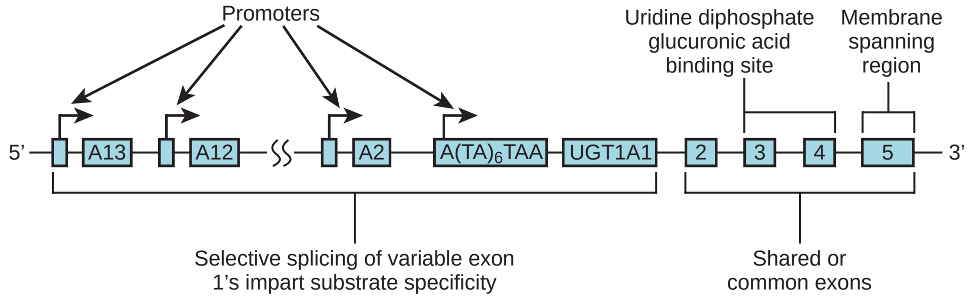
Promoters
Uridine diphosphate glucuronic acid binding site
Membrane spanning region
5'
A13
A12
A2
A(TA)6TAA
UGT1A1
2
3
4
5
3'
Selective splicing of variable exon 1's impart substrate specificity
Shared or common exons

Figure 4-4 The human uridine diphosphate glucuronosyltransferase-1 gene. *(From Gourley GR: Neonatal jaundice and disorders of bilirubin metabolism. In Suchy FJ, Sokol RJ, Balistreri WF, editors:* Liver disease in children, *ed 3, New York, 2007, Cambridge University Press.)*

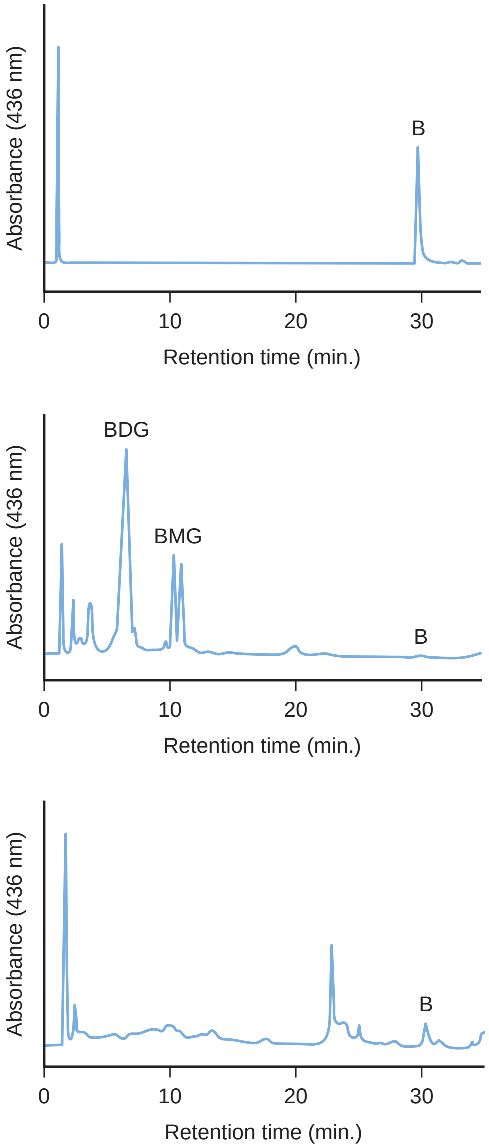

Figure 4-5 Bile pigment excretion in the adult human as assessed by high-performance liquid chromatography. Chromatograms represent analysis of serum (20 µl; *top*), duodenal bile (20 µl; *middle*) and stool extract (equivalent to 50 mg of wet stool; *bottom*) from a normal man. Scale of y axes varies. Serum bile pigments are almost all bilirubin. The bilirubin di- and monoglucuronides *(BDG, BMG)* that predominate in adult bile are not present in adult feces because of metabolism by intestinal bacteria. *(From Gourley GR: Neonatal jaundice and disorders of bilirubin metabolism. In Suchy FJ, Sokol RJ, Balistreri WF, editors:* Liver disease in children, *ed 3, New York, 2007, Cambridge University Press.)*

membrane is canalicular multispecific organic anion transporter (cMOAT). cMOAT is a member of the ATP-binding cassette (ABC) transporter superfamily and is homologous to the multidrug resistance–associated protein (MRP2).[52,53] It is also known as ABCC2 because it is encoded by the ABCC2 gene.[54] cMOAT/MRP2/ABCC2 is involved with ATP-dependent transport across the apical canalicular membrane of a variety of endogenous compounds and xenobiotics[55] including both bilirubin mono- and diglucuronide.[56] cMOAT has previously been described as the nonbile acid organic anion transporter, the glutathione S-conjugate export pump, or the leukotriene export pump.[57] Genetic mutations which alter these ABC transporters cause diseases which include cystic fibrosis, hyperinsulinemia, adrenoleukodystrophy, multidrug resistance,[58] and, as will be discussed later in this chapter, Dubin-Johnson syndrome. This mechanism can be saturated with increasing amounts of bilirubin or bilirubin conjugates.[59-61] Many other organic anions (e.g., BSP, ICG), share this same canalicular membrane

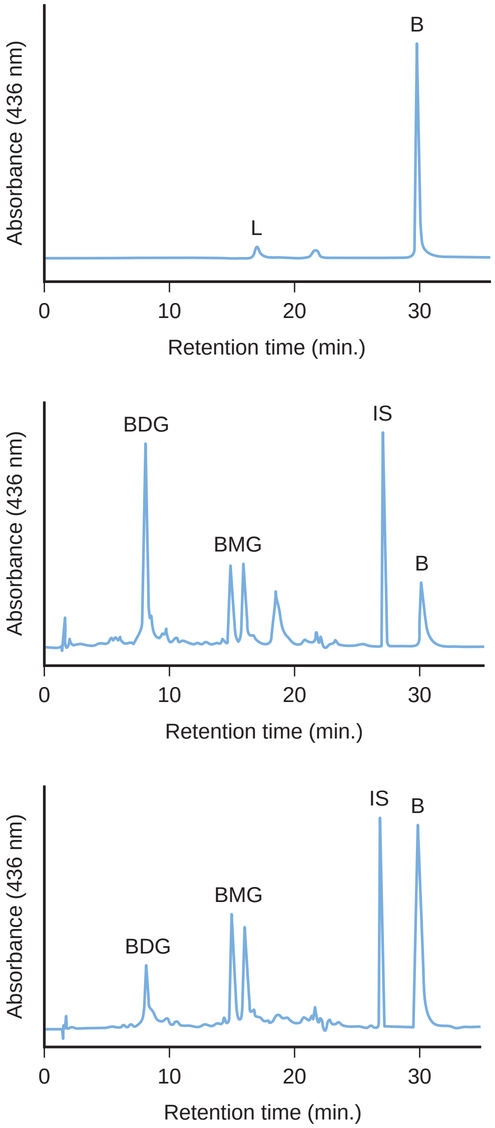

Figure 4-6 Bile pigment excretion in the newborn human as assessed by high-performance liquid chromatography. Chromatograms represent analysis of serum (20 µl; *top*) of an infant receiving phototherapy in the first week of life, and duodenal bile (20 µl; *middle*) and stool extract (equivalent to 50 mg of wet stool; *bottom*) from a normal full-term, formula-fed female infant on day 3 of life. Serum bile pigments include lumirubin *(L)*. Scale of y axes varies. Neonates lack an intestinal bacterial flora and, hence, large quantities of bilirubin di- and monoglucuronides *(BDG, BMG)* and bilirubin *(B)* are present in feces. *(From Gourley GR: Neonatal jaundice and disorders of bilirubin metabolism. In Suchy FJ, Sokol RJ, Balistreri WF, editors:* Liver disease in children, *ed 3, New York, 2007, Cambridge University Press.)*

excretion mechanism.[62] Simultaneous infusions of BSP and ICG will decrease the maximal canalicular excretion of bilirubin and vice versa.[63,64] The canalicular excretion mechanism for bilirubin and BSP are different from that of bile salts. Biliary excretion of conjugated bilirubin and BSP is decreased in individuals with the Dubin-Johnson syndrome,[65] though bile salt excretion in not impaired. However, bile salt and bilirubin conjugate excretion by the canalicular membrane are not completely independent since infusion of bile salts does increase the maximal excretion of bilirubin conjugates.[66] A similar effect in seen with phenobarbital.[67] Conversely the maximal excretion of bilirubin conjugates can be decreased by cholestatic agents like estrogens and anabolic steroids.[68,69]

Under normal conditions there is evidence that bilirubin conjugates equilibrate across the sinusoidal membrane of hepatocytes resulting in small amounts of bilirubin conjugates being present in the systemic circulation.[70,71,72] If there is diminished hepatic glucuronidation of bilirubin (e.g., in the neonate), there will be a decreased amount of bilirubin conjugates present in the serum.[70,71,73] Data show that in full-term newborns there is an increase in the serum level of bilirubin diconjugates (0.55 ± 0.25% on days 2 to 4 to 1.62 ± 0.99% on days 9 to 13) which are consistent with the maturation of bilirubin glucuronidation.[74] In contrast, in premature infants less than 33 weeks' gestation, bilirubin diconjugates were very low and remained so, suggesting a more severe immaturity of the glucuronidation process.

In many pathologic circumstances, bilirubin mono- and diglucuronides are not excreted from the hepatocyte fast enough to prevent significant reflux back into the circulation. The resulting elevation of serum bilirubin conjugate levels results in the transesterification of bilirubin glucuronide with an amino group on albumin, producing a covalent bond between albumin and bilirubin.[75] This product is formed spontaneously and is known as *delta bilirubin* or *bilirubin-albumin*.[76] Similar nonenzymatic reactions have been demonstrated between albumin and various drugs.[75,77-79] Delta bilirubin is not formed in hyperbilirubinemic conditions unless there is elevation of the conjugated bilirubin fraction. Both delta bilirubin and bilirubin conjugates are direct reacting, which explains a situation that has long confounded clinicians. The direct bilirubin may continue to be elevated in patients who otherwise are recovering from a hepatic insult because the delta bilirubin formed lingers due to the long (~20 day[80]) half-life of albumin.

Enterohepatic Circulation of Bilirubin

When bilirubin conjugates enter the intestinal lumen (see Fig. 4-2), several possibilities for further metabolism arise. In adults, the normal bacterial flora hydrogenate various carbon double bonds in bilirubin to produce assorted urobilinogens (Fig. 4-7). Subsequent oxidation of the middle (C-10) carbon produces the related urobilins. Because there are a large number of unsaturated bonds in bilirubin, there are many compounds formed by reduction and oxidation of these bonds. This large family of related reduction–oxidation products of bilirubin is known as *urobilinoids*[81] and excreted in the feces and urine. Bacteria capable of producing urobilinoids include *Clostridium ramosum*,[82] *Escherichia coli, Bacteroides fragilis*,[83] *Clostridium perfringens*, and *Clostridium difficile*.[84] The conversion of bilirubin conjugates to urobilinoids is important because it blocks the intestinal absorption of bilirubin known as the *enterohepatic circulation*.[85,86] Neonates lack an intestinal bacterial flora and are more likely to absorb bilirubin from the intestine. This difference in bile pigment excretion between adults and neonates is demonstrated by comparing Figures 4-5 and 4-6 (lower panels).

Bilirubin conjugates in the intestine can also act as substrate for either bacterial[87-89] or endogenous tissue[90] β-glucuronidase. This enzyme hydrolyses glucuronic acid from bilirubin glucuronides. The UBIL produced is more rapidly absorbed from the intestine.[91] In the fetus, tissue β-glucuronidase is detectable by 12 weeks' gestation and is believed to play an important role in facilitating intestinal bilirubin absorption that enables bilirubin to be cleared via the placenta. Following birth, increased intestinal β-glucuronidase can increase the neonate's likelihood of experiencing higher serum bilirubin levels.[92] The ability of endogenous tissue β-glucuronidase to deconjugate bilirubin glucuronides has been demonstrated in germ-free animals.[93] Breastmilk can contain high levels of β-glucuronidase, and it has been suggested that this is one factor related to the higher jaundice levels seen in breastfed infants.[94] Feeding specific nutritional ingredients that inhibit β-glucuronidase, such as hydrolyzed casein or L-aspartic acid[95] have been shown to result in increased fecal bilirubin excretion and lower levels of jaundice.[96]

DIAGNOSIS OF HYPERBILIRUBINEMIA

Jaundice and icterus both refer to the yellow discoloration of the tissues (skin, sclerae, etc.) caused by deposition of bilirubin. *Jaundice*, from the French *jaune* means yellow. *Icterus* is derived from the Greek word for jaundice *(ikteros)*. Jaundice is a sign that hyperbilirubinemia exists (i.e., total serum bilirubin greater than approximately 1.4 mg/dL after 6 months of age, 1 mg/dL = 17 μmol/L). The degree of jaundice is directly related to the level of serum bilirubin and the amount of bilirubin deposited in the extravascular tissues. Hypercarotenemia, occasionally mistaken for jaundice, can impart a yellow hue to the skin, but the sclerae remain white. There are many conditions associated with neonatal jaundice. Some of these states are so commonly recognized as to be termed *physiologic*. Alternatively jaundice can be a sign of severe disease.

Total Serum Bilirubin Measurements

Measurement of the total serum bilirubin concentration allows quantitation of jaundice. Such measurements are very common in the newborn nursery and in one study were made at least once in 61% of term newborn infants.[97] Two components of total serum bilirubin can be routinely measured in the clinical laboratory: conjugated bilirubin ("direct" reacting, because in Van den Bergh's test, color development takes place directly without adding methanol), and UBIL ("indirect" fraction). Although the terms

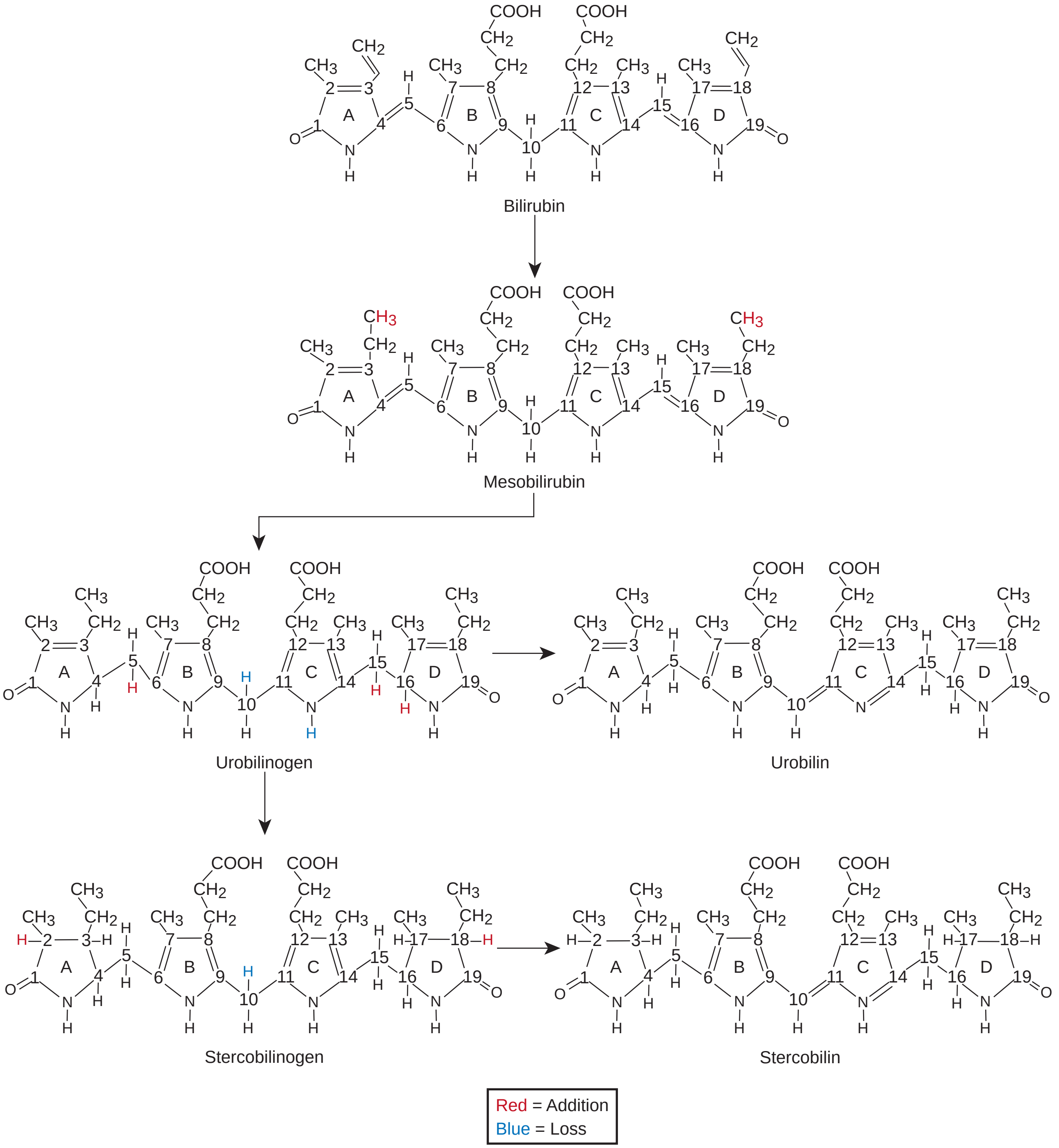

Figure 4-7 The reduction and oxidation of bilirubin to a family of related compounds known collectively as *urobilinoids*. Only several examples are given of this much larger family. *(From Gourley GR: Neonatal jaundice and disorders of bilirubin metabolism. In Suchy FJ, Sokol RJ, Balistreri WF, editors:* Liver disease in children, *ed 3, New York, 2007, Cambridge University Press.)*

direct and *indirect* are often used equivalently with conjugated and UBIL, this is not quantitatively correct because the direct fraction includes both conjugated bilirubin and albumin-bound delta bilirubin.[98] Elevation of either of these fractions can result in jaundice. There is a long history of undesirable variability in the measurement of serum bilirubin fractions.[99,100] One problem is the ditaurobilirubin (DTB) content of standards set by the College of American Pathologists (CAP).[100] The DTB influences the result variably because of protein matrix differences related to the specific bilirubin measurement used. This has prompted the suggestion that standards consist of human serum enriched solely with UBIL rather than bovine serum containing a mixture of UBIL and DTB.[101] The automated laboratory methods now used to measure serum bilirubin have been reviewed elsewhere.[98,102-104] The Jendrassik-Grof procedure is the method of choice for total bilirubin measurement, though this method also has problems.[105] When the total serum bilirubin level is high, factitious elevation of the direct fraction has been reported.[106] Three newer methods have been developed which can more accurately determine the various bilirubin fractions (unconjugated, monoconjugated, diconjugated, and albumin-bound or delta): high-performance liquid chromatography (HPLC),[107] multilayered slides,[108,109] and use of bilirubin oxidase.[110] HPLC analysis is superior and used as the gold standard,[111,112] but it is too expensive and time consuming for the clinical laboratory.[98] HPLC analysis of serum from normal human neonates in the first 4 days of life[113] showed unconjugated and conjugated bilirubin levels rose in parallel with the conjugated fraction, making up only 1.2% to 1.6% of total pigment (3.6% in adults). Although the absolute concentration of conjugates was 2 to 6 times higher in neonates, only 20% were diconjugates (54% in adults). These sensitive HPLC data are consistent with the increased bilirubin production and relatively deficient glucuronidation seen in the neonate. Analysis with automated multilayered slide technology (VITROS, Johnson & Johnson) used in many clinical laboratories allows measurement of specific conjugated and UBIL fractions without inclusion of delta bilirubin. The conjugated bilirubin measurement is an earlier indicator of relief from biliary cholestasis than is direct bilirubin because of the long half-life of delta bilirubin.[114] A comparison of the bilirubin oxidase method concluded that determinations of total bilirubin in neonatal serum were not advanced by this method.[115]

Newer methods of total bilirubin measurement (Twin Beam, Ginevri, Rome, Italy; ABL 735, Radiometrer, Copenhagen, Denmark; Roche OMNI S, Roche Diagnostics, Graz, Austria) using nonenzymatic photochemical analysis offer the convenience of bilirubin quantitation outside of a core lab setting (i.e., blood gas analyzer in the nursery) using a smaller blood sample than traditional serum analysis. However, these measurements must be interpreted with caution because the instruments tend to underestimate the total bilirubin concentration at greater than 15 mg/dL.[116]

There are conflicting data regarding the accuracy of capillary versus venous serum bilirubin levels.[117,118] However, as Maisels has pointed out, the literature regarding kernicterus, phototherapy, and exchange transfusion is based on bilirubin measurements in capillary samples.[119]

Transcutaneous Bilirubinometry

Noninvasive methods to measure jaundice levels have been shown to be useful in neonates. Current commercially available methods include the BiliChek (Respironics, Pittsburgh, Pennsylvania)[111,112] and the Minolta/Hill-Rom Air-Shields Transcutaneous Jaundice Meter 103 (Air-Shields, Hatboro, Pennsylvania).[120] The technique utilizes principles of reflectance spectrophotometry, has been validated against both HPLC and clinical laboratory measures,[111,112] and is advocated by the American Academy of Pediatrics (AAP).[121] The device is touched to the skin in a painless manner with the immediate resulting point-of-care measurement of transcutaneous bilirubin (TcB). Significant TcB levels (e.g., greater than 13 mg/dL) should prompt measurement of a serum or plasma bilirubin level. Some suggest that the yellow color of the skin is a better risk indicator of bilirubin-dependent brain damage than is the serum bilirubin level.[122] A less expensive[123] method useful in assessing jaundice[124,125] utilizes a plexiglass color chart pressed against the baby's nose (Ingram icterometer, Thos. A. Ingram and Co. Ltd, Birmingham, England).

TOXICITY OF UNCONJUGATED HYPERBILIRUBINEMIA

Kernicterus

Reviews of neonatal bilirubin toxicity have been published elsewhere.[1,126,127] Kernicterus (German: *kern*: nucleus; Latin: *icterus*: yellow) is the neuropathologic finding associated with severe unconjugated hyperbilirubinemia and is named for the yellow staining of certain regions of the brain, particularly the basal ganglia, hippocampus, cerebellum, and nuclei of the floor of the fourth ventricle.[126,128] Acute clinical findings associated with kernicterus, termed *acute bilirubin encephalopathy,* include sluggish Moro reflex, opisthotonos, retrocollis, hypotonia, vomiting, high-pitched cry, hyperpyrexia, seizures, paresis of gaze ("setting sun sign"), oculogyric crisis, and death. Long-term findings (chronic bilirubin encephalopathy) include spasticity, choreoathetosis, dental enamel dysplasia of the desiduous teeth, and sensorineural hearing loss. Milder forms of bilirubin encephalopathy include cognitive dysfunction and learning disabilities.[129,130] In 17-year-old males, the risk of having an IQ score less than 85 was found to be significantly higher among full-term subjects with neonatal serum bilirubin levels above 20 mg/dL.[131] The mechanisms of bilirubin cytotoxicity are complex and have been reviewed elsewhere.[126,127,132-134] Though the neonatal period is the most common time for bilirubin-related brain damage, the neurotoxicity of bilirubin has also been documented in older children and adults with CN syndrome, type I.[135,136]

The absolute level of serum bilirubin has not been a good predictor of the risk of bilirubin encephalopathy. However, it has long been known that kernicterus is likely with serum UBIL levels greater than 30 mg/dL and unlikely with levels less than 20 mg/dL.[137-139] In one study, 90% of the patients who had a bilirubin level greater than 35 mg/dL either died or had cerebral palsy or physical retardation.[139] Alternatively, no developmental retardation was found in 129 infants with bilirubin levels less than 20 mg/dL. Albumin concentration is an important variable because of the high affinity binding with bilirubin, and the ratio of bilirubin to albumin is a risk factor that has been included in the most recent AAP guidelines.[121] Drugs and organic anions also bind to albumin and can displace bilirubin, thereby increasing the free bilirubin which can diffuse into cells and cause toxicity,[140,141] such as when sulfisoxazole was given to premature infants.[142] Walker has reviewed neonatal bilirubin toxicity and drug-induced bilirubin displacement.[132] In recent years there has been a reemergence of kernicterus.[127] While it has been acknowledged that total serum bilirubin may not be the most important factor related to risk, there are, at present, no other generally accepted tests (e.g., albumin saturation, reserve bilirubin binding capacity, or free bilirubin concentration) which are more helpful in identifying infants at risk for bilirubin encephalopathy. Recently the basics of bilirubin–albumin binding and neonatal jaundice have been reviewed by Ahlfors and Wennberg.[143] Additionally, Ahlfors and colleagues have recently described how to use zone fluidics (the precisely controlled physical, chemical, and fluid-dynamic manipulation of zones of miscible and immiscible fluids and suspended solids in narrow bore conduits to accomplish sample conditioning and chemical analysis) to measure free bilirubin and overcome some of the existing problems with unbound (free) bilirubin measurement.[144] Wennberg and colleagues speculate that enhanced sensitivity and specificity of newer methods of free bilirubin measurement will provide for the establishing of improved risk threshold for kernicterus and therefore reduce unnecessary aggressive intervention (i.e., exchange transfusion) and its associated cost and morbidity.[145]

Another approach aimed at measuring early changes in the CNS caused by bilirubin has assessed brainstem auditory evoked potentials (BAEP).[146,147] Abnormalities in the BAEP have been demonstrated in jaundiced infants and shown to improve after exchange transfusion.[148,149] Data have shown that even moderate hyperbilirubinemia (mean ± SD: 14.3 ± 2.8 mg/dL) affects the BAEP, specific components of the Brazelton Neonatal Behavioral Assessment Scale,[150] and cry characteristics.[151]

Another reported toxicity of neonatal jaundice relates not to the CNS of the jaundiced infant, but rather to the attitude of that infant's parents. Mothers of jaundiced infants more frequently exhibit behavior suggesting the "vulnerable-child syndrome,"[152,153] including inappropriate visits to the physician because of unrealistic perceptions of illness, separation difficulties, and early termination of breastfeeding. Another study showed, however, that hyperbilirubinemia and/or phototherapy during the neonatal period are not associated with impaired mother–child attachment after the first year of life.[154]

NONPATHOLOGIC UNCONJUGATED HYPERBILIRUBINEMIA

Physiologic Jaundice

The term *physiologic jaundice* has been used to describe the frequently observed jaundice in otherwise completely normal neonates. However, physiologic jaundice is merely the result of a number of factors involving increased bilirubin production and decreased excretion.

Jaundice should always be considered to be a sign of a possible disease and not routinely explained as physiologic. Specific characteristics of neonatal jaundice to be considered abnormal until proven otherwise include: (1) development before 36 hours of age; (2) persistence beyond 10 days of age; (3) serum bilirubin greater than 12 mg/dL at any time; (4) elevation of the direct bilirubin (direct bilirubin greater than 1 mg/dL if the total bilirubin is less than 5 mg/dL, or direct bilirubin greater than 20% of the total bilirubin if the total bilirubin is greater than 5 mg/dL[155]). The physiologic immaturity of premature infants is associated with both higher peak serum bilirubin levels and pathologic conditions.[156]

In general, infants are not jaundiced at the moment of birth, due to the impressive ability of the placenta to clear bilirubin from the fetal circulation. However, within the next few days most if not all infants will develop elevated serum bilirubin levels (greater than 1.4 mg/dL), with clinical jaundice evident when the total bilirubin level exceeds 2 to 5 mg/dL. As the serum bilirubin rises, the skin becomes more jaundiced in a cephalocaudal manner.[157] Kramer found the following serum indirect bilirubin levels as jaundice progressed: head and neck: 4 to 8 mg/dL; upper trunk: 5 to 12 mg/dL; lower trunk and thighs: 8 to 16 mg/dL; arms and lower legs: 11 to 18 mg/dL; palms and soles: greater than 15 mg/dL.[158] Hence when the bilirubin was greater than 15 mg/dL, the entire body was icteric. However, darker skin tones can make jaundice difficult to estimate visually.[121] Jaundice is best observed by blanching the skin with gentle digital pressure under well-illuminated (white light) conditions. At least one third of infants develop visible jaundice. A combined analysis of several large studies involving thousands of infants during the first week of life showed that moderate jaundice (bilirubin greater than 12 mg/dL) occurs in at least 12% of breastfed infants and 4% of formula-fed infants, while severe jaundice (greater than 15 mg/dL) occurs in 2% and 0.3% of these respective feeding groups.[159]

There are a number of epidemiologic risk factors related to neonatal jaundice that have been reviewed elsewhere.[121,160-162] Risk factors include male gender, low birth weight, prematurity, polycythemia, certain ethnicities (Asian, Native American, Greek), maternal medications (e.g., oxytocin, promethazine hydrochloride), premature rupture of the membranes, increased weight loss after birth, delayed meconium passage, breastfeeding, and neonatal infection. Delivery with the vacuum extractor increases the risk of cephalohematoma and neonatal jaundice.[163] Data suggest pancuronium is associated with an increased risk of hyperbilirubinemia.[164] There is a significant correlation between umbilical cord serum bilirubin level and subsequent hyperbilirubinemia.[165,166] Maternal serum bilirubin level at the time of delivery and transplacental bilirubin gradient also correlate positively with neonatal serum bilirubin concentrations.[166] Factors associated with decreased neonatal bilirubin levels[121,167] include African race, exclusive formula feeding, gestational age 41 weeks, maternal smoking, and certain drugs given to the mother (e.g., phenobarbital).

Breastfeeding and Jaundice

Breastfeeding has been clearly identified as a factor related to neonatal jaundice,[159] and this subject has been reviewed elsewhere.[168,169] Breastfed infants have been shown to have significantly higher serum bilirubin levels than formula-fed infants on each of the first 5 days of life,[170] and this unconjugated hyperbilirubinemia can persist for weeks[171] to months.[172,173] Jaundice during the first week of life is sometimes described as *breastfeeding jaundice* in order to differentiate it from breastmilk jaundice syndrome, which occurs after the first week of life.[174-176] The former is frequently associated with inadequate breastmilk intake, whereas the latter generally occurs in otherwise thriving infants. There is probably overlap between these conditions and physiologic jaundice. Early reports linking breastmilk and neonatal jaundice with a steroid (pregnane-3(α),20(β)-diol) in some milk samples[177] have not been confirmed by more recent larger studies utilizing more sensitive methods.[178] There are also conflicting data regarding efforts to attribute this jaundice to increased lipase activity in the breastmilk, resulting in elevated levels of free fatty acids, which could inhibit hepatic glucuronosyltransferase.[179] It has been suggested that the enterohepatic circulation of bilirubin can be facilitated by the presence of β-glucuronidase[92,180] or some other substance in human milk.[181] Other factors possibly related to jaundice in breastfed infants include caloric intake, fluid intake, weight loss, delayed meconium passage, intestinal bacterial flora, and inhibition of bilirubin glucuronosyltransferase by an unidentified factor in the milk.[94] Lascari suggests that a healthy breastfed infant with unconjugated hyperbilirubinemia, normal hemoglobin concentration, reticulocyte count, blood smear, no blood group incompatibility, and no other abnormalities on physical examination may be presumed to have early breastfeeding jaundice.[175] Because there is no specific laboratory test to confirm a diagnosis of breastmilk jaundice, it is important to rule out treatable causes of jaundice before ascribing the hyperbilirubinemia to breastmilk. Some infants with presumed breastmilk jaundice exhibit elevated serum bile acid levels suggesting mild hepatic dysfunction or cholestasis,[182] though in general this is not the case. Breastfed infants who are fed specific nutritional ingredients, such as L-aspartic acid, that inhibit β-glucuronidase[95] excrete more fecal bilirubin and have lower levels of jaundice[96] than breastfed infants who receive no supplements. No commercial preparations of these specific ingredients are presently available.

DISORDERS OF PATHOLOGIC UNCONJUGATED HYPERBILIRUBINEMIA

Disorders of Production

Isoimmunization

The most common cause of severe early jaundice is fetal–maternal blood group incompatibility with resulting isoimmunization (see Chapter 3). Maternal immunization develops when erythrocytes leak from fetal to maternal circulation. Fetal erythrocytes carrying different antigens are recognized as foreign by the maternal immune system that forms antibodies against them (maternal sensitization). These antibodies (IgG immunoglobulins) cross the placental barrier into the fetal circulation and bind to fetal erythrocytes. In Rh incompatibility, sequestration and destruction of the antibody-coated erythrocytes takes place in the reticuloendothelial system of the fetus. In ABO incompatibility, hemolysis is intravascular, complement mediated, and usually not as severe as in Rh disease.[183] Significant hemolysis can also result from incompatibilities between minor blood group antigens (e.g., Kell).[184] Although hemolysis is predominantly associated with elevation of UBIL, the conjugated fraction can also be elevated.[185] Any type of isoimmune hemolytic disease is a risk factor for kernicterus.[121]

Rh incompatibility problems do not usually develop until the second pregnancy, thus prenatal blood typing and serial testing of Rh-negative mothers for the development of Rh antibodies provide important information to guide possible intrauterine care. If maternal Rh antibodies develop during pregnancy, potentially helpful measures include serial amniocentesis (with bilirubin measurement),[186,187] ultrasound assessment of the fetus,[188,189] intrauterine transfusion,[190,191] and premature delivery. The prophylactic administration of anti-D gamma globulin[192] has been most helpful in preventing Rh sensitization. The newborn infant with Rh incompatibility presents with pallor, hepatosplenomegaly, and a rapidly developing jaundice in the first hours of life. In severe cases the infant may be born with generalized edema (fetal hydrops). Laboratory findings in the neonate's blood include reticulocytosis, anemia, a positive direct Coombs' test, and a rapidly rising serum bilirubin level. Conjugated hyperbilirubinemia is occasionally observed in Rh erythroblastosis, particularly when intrauterine transfusions have been used.[113] This may be the result of exceeding the hepatic excretory capacity. Alternatively, cholestasis may also result from hepatic congestion from extramedullary erythropoiesis and heart failure, or tissue hypoxia resulting from reduced hepatic blood flow and anemia.[193] Cholestasis in infants with erythroblastosis fetalis may also be exacerbated by secondary complications, including biliary obstruction, sepsis, or hepatic failure.[194]

Therapeutic intervention with phototherapy and consideration of exchange transfusion should be initiated at lower serum bilirubin concentrations in infants with isoimmune hemolysis, and intravenous gamma-globulin has been shown to reduce the need for exchange transfusions in Rh and ABO hemolytic disease.[121] Serum bilirubin measurements should be obtained as frequently as every 4 hours to establish the rate of increase and to assess the effect of therapy. Exchange transfusions may be performed as emergency procedures in severe cases almost immediately after delivery in order to treat anemia and heart failure.

In contrast to Rh incompatibility, ABO incompatibility usually presents clinically with the first pregnancy. ABO hemolytic disease is largely limited to blood group A or B infants born to group O mothers.[195] ABO hemolytic disease is relatively rare in type A or B mothers. Development of jaundice is not as rapid as with Rh disease, and a serum bilirubin greater than 12 mg/dL on day 3 of life would be typical. Laboratory abnormalities include reticulocytosis (greater than 10%) and a weakly positive direct Coombs' test, though this is sometimes negative. Anti-A or anti-B antibodies may be seen in the serum of the newborn if examined within the first few days of life, before they rapidly disappear. Spherocytes are the most prominent feature seen in the peripheral blood smear with ABO incompatibility.

Erythrocyte Enzymatic and Structural Defects

A number of specific abnormalities of the red blood cell can result in neonatal jaundice, including hemoglobinopathies and red blood cell membrane and enzyme defects. Hereditary spherocytosis (see Chapter 16) can be a neonatal problem with significant hemolysis and present with a rising bilirubin level and a falling hematocrit.[196] A family history of spherocytosis, anemia or early gallstone disease (before age 40) is helpful in suggesting this diagnosis. The characteristic spherocytes seen in the peripheral blood smear may be impossible to distinguish from those seen with ABO hemolytic disease. Other erythrocyte conditions associated with neonatal jaundice include drug-induced hemolysis, deficiencies of the erythrocyte enzymes (glucose 6-phosphate dehydrogenase [G6PD] deficiency[197,198] [see Chapter 17], pyruvate kinase deficiency, and others) (see Chapter 18), and hemolysis induced by vitamin K or bacteria. Recent studies of G6PD activity in African-American neonates suggest that hemolysis is neither the predominate factor in the pathogenesis of hyperbilirubinemia, nor is it alone predictive of hyperbilirubinemia,[199] though African-American male neonates may be at higher risk for hyperbilirubinemia, particularly if breastfed and premature.[200] This emphasizes the important relationship between bilirubin excretion and serum bilirubin concentration.[201] α-thalassemia (see Chapter 21) can result in severe hemolysis and lethal hydrops fetalis.[202] β-thalassemia (see Chapter 21) may also present with hemolysis and severe neonatal hyperbilirubinemia.[203] There are a wide variety of clinical findings associated with the thalassemias, extending from profound intrauterine hydrops and death, to mild neonatal jaundice and anemia, to no jaundice or anemia. Southeast Asian ovalocytosis (see Chapter 16) has been associated with severe hyperbilirubinemia.[204] These red blood cell abnormalities are more likely to result in hyperbilirubinemia in the presence of Gilbert's syndrome as described later in this chapter.

Drugs or other substances responsible for hemolysis can be passed to the fetus across the placenta or to the neonate via breastmilk. Induction of labor with oxytocin has been shown to be associated with neonatal jaundice. There is a significant association between hyponatremia and jaundice in infants of mothers who received oxytocin to induce labor.[205,206] The vasopressin-like action of oxytocin prompts electrolyte and water transport, and it is suggested that the erythrocyte swells, and increased osmotic fragility and hyperbilirubinemia can result. Steroid administration at the initiation of oxytocin and 4 hours later may be helpful in preventing this hyperbilirubinemia.[207]

Infection

Bacterial sepsis increases bilirubin production through initiation of erythrocyte hemolysis or via endotoxin-mediated reduction of canalicular bile secretion, resulting in a combination of conjugated and unconjugated hyperbilirubinemia. However, hyperbilirubinemia is rarely the only manifestation of bacteremia or sepsis,[208] and some data suggest sepsis may result in lower peak levels of UBIL through consumption of bilirubin as an antioxidant in response to oxidant production by phagocytic cells.[209]

Sequestration

Extravascular blood within the body can be rapidly metabolized to bilirubin by tissue macrophages. Examples of this increased bilirubin production include cephalohematoma, ecchymoses, petechiae, and hemorrhage. Although the diagnosis can often be made on physical examination, occult intracranial, intestinal, adrenal, or pulmonary hemorrhage can also produce hyperbilirubinemia. Similarly, swallowed blood can be converted to bilirubin by the HO of intestinal epithelium. From a red sample of bloody gastric contents, meconium, or stool, the Apt test can be used to distinguish blood of maternal or infant origin because of differences in alkali-resistance between fetal and adult hemoglobin;[210,211] however, this test has low sensitivity and poor reproducibility, and an HPLC method has been shown to be superior.[212]

Polycythemia

Polycythemia can cause hyperbilirubinemia because the absolute increase in red cell mass results in elevated bilirubin production through normal rates of erythrocyte breakdown. A number of mechanisms may result in neonatal polycythemia (usually defined by venipuncture hematocrits greater than 65%) as reviewed by Werner.[213] Maternal–fetal transfusion during peripartum placental separation, a delay in umbilical cord clamping at birth, and twin–twin transfusion syndrome are all commonly encountered causes of polycythemia. Similarly, intrauterine hypoxia and maternal diseases such as diabetes mellitus can result in neonatal polycythemia. Therapy for symptomatic polycythemia is partial exchange transfusion, though therapy for asymptomatic polycythemia remains controversial.[214]

Disorders of Conjugation

The disorders described in this section, summarized in Tables 4-1 and 4-2, are those in which there is a primary abnormality in bilirubin metabolism without other liver disease.[215] The disorders can best be understood in the context of the normal pathway in which bilirubin is cleared from the circulation (see Fig. 4-2). Defects in these metabolic steps are responsible for the disorders to be described in the final section of this chapter.

Gilbert's Syndrome

Clinical Presentation. Gilbert's syndrome (GS) (OMIM #143500) was first described in 1901 by Gilbert and Lereboullet.[216] It is characterized by a hereditary chronic or recurrent, mild unconjugated hyperbilirubinemia with otherwise normal liver function tests (for reviews, see[217-219]). The serum UBIL elevation is variable and usually ranges from 1 to 4 mg/dL (17 µmol/L = 1 mg/dL). Frequently patients are first identified when an elevated serum bilirubin is found on screening blood chemistry or

TABLE 4-1 Comparison of Disorders of Unconjugated Hyperbilirubinemia

		Gilbert's Syndrome	**Crigler-Najjar Type I**	**Crigler-Najjar Type II**
Prevalence		3%	Rare	Rare
Inheritance		Autosomal dominant or recessive	Autosomal recessive	Autosomal recessive, rarely dominant
Genetic Defect		UGT1A1 gene	UGT1A1 gene	UGT1A1 gene
Hepatocyte Defect Site		Microsomes +/– plasma membrane	Microsomes	Microsomes
Deficient Hepatocyte Function		Glucuronidation +/– uptake	Glucuronidation	Glucuronidation
BUGT Activity		5%-53% of controls	Severely decreased	2%-23% of controls
Hepatocyte Uptake		Decreased in 20%-30%	Normal	Normal
Serum Total Bilirubin Level (mg/dL)		0.8-4.3	15-45	8-25
Serum Bilirubin Decrease with Phenobarbital (%)		70	0	77
HPLC Serum Bilirubin Composition				
	(Normal %)			
Unconjugated	(92.6)	98.8	~100	99.1
Diglucuronide	(6.2)	1.1	0	0.6
Monoglucuronide	(0.5)	0	0	0
Bile Bilirubin Conjugates				
	(Normal %)			
Diglucuronide	(~80)	60	0 to trace	5-10
Monoglucuronide	(~15)	30	Predominant if measurable	90-95
Other Routine Liver Function Tests		Normal	Normal	Normal
Prognosis		Benign	Kernicterus common	Occasional kernicterus

From Suchy FJ, Sokol RJ, Balistreri WF, editors: Liver disease in children, *ed 3, New York, 2007, Cambridge University Press.*

mild jaundice (perhaps only scleral icterus) is noted during a period of fasting associated with a nonspecific viral illness or religious activities.[220] Icteric plasma from a blood donor may suggest GS.[221] Alternatively, hyperbilirubinemia post liver transplant may be a sign that the donor had GS.[222-225] GS in normal adults is generally associated with no negative implications for health or longevity; however, a large variety of common symptoms have been reported by patients with GS.[226,227] These symptoms include vertigo headache, fatigue, abdominal pain, nausea, diarrhea, constipation, and loss of appetite. The possible relationship of these symptoms to GS was evaluated in a group of 2395 Swedish subjects.[228] The only symptom that was more common in GS was diarrhea in males aged 57 to 67 years. The authors suggested that this was most likely type I error because of the large number of comparisons made and concluded that there was no higher prevalence of symptoms associated with GS. There are limited reports suggesting that GS is a risk factor for chronic fatigue syndrome.[229,230]

In two large surveys of normal individuals, approximately 3% of the population had serum bilirubin levels greater than 1 mg/dL.[231,232] If the normal upper limit of serum bilirubin is defined as 1.4 mg/dL, then there is a strong male predominance (approximately 4:1). This finding might be related to the observation that females clear bilirubin better than males.[233] GS may be inherited in either an autosomal dominant[234-237] or recessive[238] fashion.

Although GS is a congenital disorder, it rarely becomes clinically apparent until after puberty. The reasons for this are unknown but have been suggested to be related to the hormonal changes of puberty. Steroid hormones can suppress hepatic bilirubin clearance.[69] During pregnancy, increased estrogen levels are associated with impaired clearance of exogenous bilirubin.[239] Gonadectomy has been shown to alter BUGT activity.[240] Odell speculated that some infants with nonhemolytic neonatal jaundice are manifesting GS.[241] Use of genetic markers (see below) has allowed investigation of the role GS plays in neonatal jaundice. Individuals carrying such markers have been shown to have a more rapid rise in their jaundice levels during the first 2 days of life,[242] a predisposition to prolonged or severe neonatal hyperbilirubinemia,[243-245] variably increased jaundice when the GS polymorphism occurs with pyloric stenosis[246,247] or is coinherited with hematologic abnormalities such as G6PD deficiency,[201,248] β-thalassemia,[248-251] or hereditary spherocytosis.[252] Thus studies from several different parts of the world indicate that GS, as detected by

TABLE 4-2 Comparison of Disorders of Conjugated Hyperbilirubinemia

	Rotor's Syndrome	Dubin-Johnson Syndrome
Prevalence	Rare	Rare
Inheritance	Autosomal recessive	Autosomal recessive
Genetic Defect	SLCO1B1/ SLCO1B3 gene	cMOAT/MRP2/ ABCC2 gene
Hepatocyte Defect Site	GST	Apical canalicular membrane
Deficient Hepatocyte Function	Intracellular binding of bilirubin and conjugates	Cannalicular secretion of bilirubin conjugates
Brown-Black Liver	No	Yes
Serum Total Bilirubin Level (mg/dL)	2-7	1.5-6
Serum Conjugated Bilirubin (%)	>50	>50
Other Routine Liver Function Tests	Normal	Normal
Oral Cholecystogram	Usually visualizes	Usually does not visualize
^{99m}Tc- HIDA Cholescintigraphy: Liver Gallbladder	 Poor to no visualization Poor to no visualization	 Intense, prolonged visualization Delayed or no visualization
BSP Clearance Test	Serum BSP levels elevated (delayed clearance)	Serum BSP levels normal at 45 min, but elevated at 90-120 min
ICG Clearance Test	Delayed clearance	Normal
Response to Estrogens or Pregnancy	No change	Increased jaundice
Total Urinary Coproporphyrin Excretion (Isomers I + III)	2.5-5 times increased	Normal
Urinary Coproporphyrin Isomer I Composition (%) (Normal = 25%)	Usually <80% of total	>80% of total
Prognosis	Benign (asymptomatic)	Benign (occasional abdominal complaints; probably incidental)

From Suchy FJ, Sokol RJ, Balistreri WF, editors: Liver disease in children, *ed 3, New York, 2007, Cambridge University Press.*

UGT1A1 analysis, does play some role in neonatal jaundice. Kaplan *et al* noted that, in their study,[201] neither G6PD deficiency nor the GS type UDPGT1 promoter polymorphism (also known as *UGT1A1*28*) alone increased the incidence of hyperbilirubinemia, but both in combination did (Fig. 4-8). They speculated that this gene interaction may serve as a paradigm of the interaction of benign genetic polymorphisms in the causation of disease, in other words, it may take two genetic abnormalities to produce disease symptoms. Numerous studies indicate the increased risk of cholelithisis in patients with GS.

Pathophysiology. GS is a heterogeneous group of disorders all of which share at least a 50% decrease in hepatic BUGT activity.[253-256] Based on plasma clearance of other organic anions (BSP and ICG) that share the same hepatocyte uptake receptor carrier, there appear to be at least four subtypes of GS.[257,258] In GS type I, clearance of BSP and ICG are normal. In GS type II there is delayed BSP clearance, but ICG clearance is normal. Because BSP uptake is normal in type II, delayed clearance must be related to subsequent intrahepatic metabolism or canalicular excretion.[259] In GS type III, clearance of both BSP and ICG are delayed. The delay in the initial rate of disappearance from the plasma suggests a defect in uptake at the hepatocyte plasma membrane.[259] Those with GS type IV have delayed uptake of ICG but not BSP. Thus some individuals with GS have delayed uptake of bilirubin into the hepatocyte, others have delayed biotransformation, and others demonstrate both abnormalities.[51,260-262] Immunohistochemical staining for UGT shows a clear reduction throughout the hepatic lobule in specimens from individuals with GS, when compared to normals.[263]

The elucidation of the structure of the *UGT1* gene, which encodes human bilirubin, phenol and other UDP-glucuronosyltransferase isozymes[264,265] led to the discovery of *UGT1A1* mutations or polymorphisms associated with GS. In Caucasian populations, the homozygous

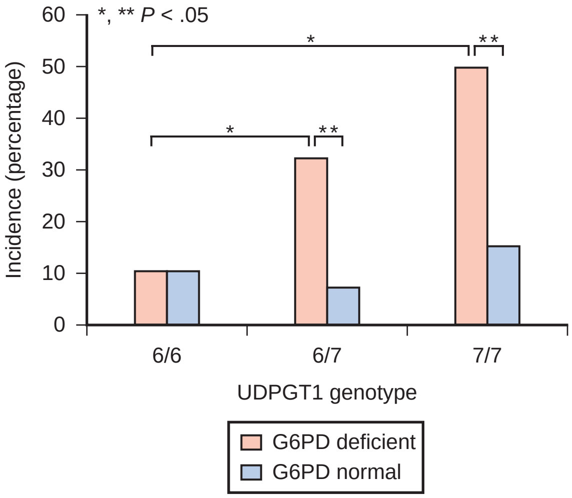

Figure 4-8 Incidence (percentage) of hyperbilirubinemia (serum total bilirubin ≥257 μmol/liter) in G6PD-deficient neonates and normal controls, stratified for the three genotypes of the UGT1A1 promoter. *(From Kaplan M, Renbaum P, Levy-Lahad E, et al: Gilbert syndrome and glucose-6-phosphate dehydrogenase deficiency: a dose-dependent genetic interaction crucial to neonatal hyperbilirubinemia.* Proc Natl Acad Sci U S A *94:12128–32, 1997.)*

finding of an additional TA repeat in the promoter region, or so called TATA box (i.e., $[TA]_7TAA$, rather than $[TA]_6TAA$) of the *UGT1A1* gene has been shown to be a necessary, though not sufficient, condition for GS.[266-268] Individuals who are heterozygous for seven TA repeats have significantly higher serum bilirubin levels than the homozygous wild type six repeats.[266] In Asian populations, the $(TA)_7TAA$ mutation is relatively rare,[269] but several different *UGT1A1* mutations have been associated with GS.[237,270,271] These Asian mutations involve exon one of the *UGT1A1* gene, rather than the TATA promoter region. One of the most common mutations in Asians, a Gly71Arg mutation in exon 1, has also linked GS and severe neonatal hyperbilirubinemia.[244] It has been reported that although within Caucasians promoter TA repeat number and bilirubin level are strongly positively correlated, in other ethnic groups (e.g., Africans, where two other variants, $[TA]_5$ and $[TA]_8$, have been identified) there is a negative correlation.[272] Rarely the $(TA)_8$ variant has been reported in Caucasians,[273] thus the ethnic implications of these genetic polymorphisms of the *UGT1A1* gene require further analysis.

Although decreased hepatic BUGT activity is universal in GS, there is poor correlation between measured enzyme activity in liver and serum bilirubin level.[274] This may be explained by the increased bilirubin production associated with the decreased red cell half-life seen in as many as 40% of patients with GS.[236,262] While early authors[254,256] concluded that there was little or no induction of BUGT following administration of phenobarbital, more recent work has revealed that, in fact, phenobarbital does increase UGT1A1 activity via a phenobarbital-responsive enhancer module (PBREM) of the *UGT1A1* promoter region[275] and that such polymorphisms could represent an additional risk factor for GS.[276]

Related to the decreased BUGT activity is the observation that duodenal bile from individuals with GS contains a decreased amount of bilirubin diglucuronides and an increased amount of bilirubin monoglucuronides compared to normals[48,51,256,277] (Fig. 4-9). This distribution is similar to that seen in infants.[278] Administration of phenobarbital has been shown to normalize the bile pigment profile in duodenal fluid,[51] lower plasma bilirubin levels, and increase hepatic clearance of bilirubin.[279,280] Clofibrate and glutethimide also normalize serum bilirubin concentrations but do not normalize the duodenal bile pigment profile.[281] In animals clofibrate is an inducer of BUGT but does not effect BSP uptake or GST.[282] An additional unexplained phenomenon in GS is the exaggerated rise in serum bilirubin associated with fasting.[283-286] Although normal individuals can double their serum bilirubin level in response to fasting, in GS a more pronounced rise occurs. This is not related to decreased hepatic blood flow because ICG clearance is not affected.[284] Although fasting can reduce BUGT activity[286] and increase HO (the enzyme responsible for bilirubin production) activity,[287] neither of these effects are believed to explain the fasting hyperbilirubinemia of GS.[279,288] Enhanced enterohepatic circulation of bilirubin is suggested to be a major factor in the pathogenesis of fasting-induced hyperbilirubinemia.[289] Intraluminal noncalorie food-bulk can blunt the bilirubin rise.[290]

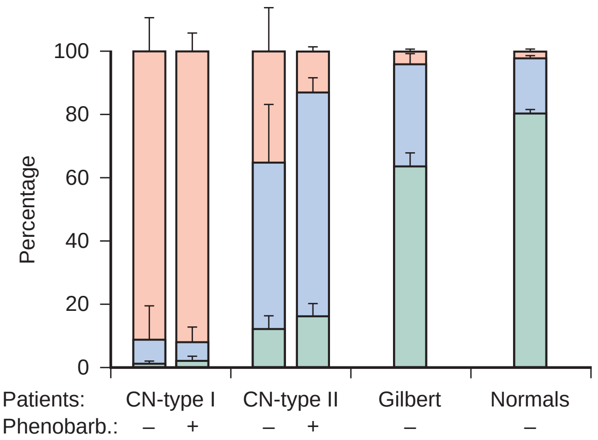

Figure 4-9 Bile pigment composition in bile from normal (n = 8), Crigler-Najjar (CN) type I (n = 3), CN type II (n = 3), and Gilbert's syndrome (n = 16) patients, receiving (+) or not receiving (-) phenobarbital. Relative bile pigment composition is indicated in the vertical columns as percentage of total ± SD. *Blue,* Bilirubin monoconjugate; *green,* bilirubin diconjugate; *red,* unconjugated bilirubin. *(From Sinaasappel M, Jansen PL: The differential diagnosis of Crigler-Najjar disease, types 1 and 2, by bile pigment analysis.* Gastroenterology *100:783–9, 1991.)*

Diagnosis and Treatment. Generally, a diagnosis of GS can be made when there is a mild, fluctuating unconjugated hyperbilirubinemia, the rest of the liver function tests are normal, and there is no hemolysis. Hemolysis can add confusion because it can result in similar findings and it is not unusual in GS. Hence other tests are sometimes used to aid in diagnosis, including the intravenous administration of nicotinic acid (niacin)[291] or one oral dose or rifampin[292] with assessment of the subsequent rise in serum bilirubin concentration.

Another test suggested to aid in the diagnosis of GS involves fractionation of the total serum bilirubin using alkaline methanolysis and thin-layer chromatography.[73,185] This allows precise measurement of the conjugated and UBIL levels. This approach has shown that in GS ~6% of the total serum bilirubin was conjugated compared to approximately 17% in normals and those with chronic hemolysis. Individuals with chronic persistent hepatitis had 28% of their total bilirubin present as conjugates. Fasting did not change the percentage of conjugates in GS, despite the rise in total serum bilirubin concentration. An overlap of only three individuals was seen among the 77 with GS and 60 normal subjects.[185] Other studies support these findings.[293] In patients with GS, fractionation of the total serum bilirubin by HPLC showed significantly decreased bilirubin monoglucuronides (1.1% vs. 6.2% in normals) and increased UBIL (98.8 vs. 92.6 in normals).[294]

Currently genetic testing is easily available to diagnose GS.[295] Monaghan et al.[243] have suggested GS genetic screening for the *UGT1A1* TA repeat as a simple, useful additional test in the investigation of very prolonged neonatal jaundice in North American, African, and

European populations; and for the Gly71Arg mutation in Asians. However, the value of such a genetic test cannot be fully determined until accurate data regarding the prevalence and penetrance of the GS genotype are known.[296] Thus genetic testing for GS cannot be routinely recommended.[296]

GS has no significant negative implications regarding morbidity or mortality. In general, drug metabolism studies have revealed no major dangers,[293,297] although there appears to be an increased incidence of slow acetylators,[298,299] and lorazepam clearance is 20% to 40% decreased.[300] Concurrent genetic deficiencies in other xenobiotic pathways may put individuals with GS at increased risk of drug toxicity to such compounds as acetaminophen,[301,302] cancer chemotherapeutic agents CPT-11 (irinotecan)[303] or TAS-103,[304] or the viral protease inhibitor, indinavir.[215,305] For this reason, Bosma suggests that screening for GS is of clinical importance.[215] No specific treatment is necessary for GS, though phenobarbital has been shown to lower serum bilirubin levels in these patients.[306] If the well-documented antioxidant effect of bilirubin[307] provides a biologic advantage,[308] then the mild hyperbilirubinemia of GS might actually be a significant benefit against such things as vascular disease,[309,310] in which free radicals are involved in pathogenesis. However, Bosma concludes that a selective advantage in GS because of the antioxidant capacity of bilirubin seems unlikely.[215]

Crigler-Najjar Syndrome Types I and II

In 1952, Crigler and Najjar described seven infants with congenital familial nonhemolytic jaundice who developed severe unconjugated hyperbilirubinemia shortly after birth and died from kernicterus within months.[311] These infants were from three related families. The serum bilirubin concentration reached 25 to 35 mg/dL despite a lack of hemolytic disease. Other liver function tests were normal. Liver histology was normal except for the deposition of bile pigments. Subsequent reports document that the main risk for patients with the CN is kernicterus.[1] An excellent review of the neurologic perspectives of CN has been published.[312] Although some patients survive into the second decade with normal development,[313] the possibility of developing late kernicterus is always a concern, even in adulthood.[314-317] Serum bilirubin levels vary from approximately 15 to 45 mg/dL.

In 1969, Arias and colleagues described a second, more frequent type of severe nonhemolytic hyperbilirubinemia.[318] The previous syndrome was termed CN type I (CNI, OMIM #218800), while the new findings were termed CN type II or Arias' syndrome (CNII, OMIM #606785).[318] Hyperbilirubinemia is less severe in type II patients and varies from approximately 8 to 25 mg/dL. Hence these individuals have a much lower incidence of kernicterus, though such damage occurs.[319,320]

Pathophysiology. Both CNI and CNII are generally inherited in an autosomal recessive manner, though one case of autosomal dominant inheritance of CNII has been reported.[321] CNI and CNII result from mutations to the *UGT* gene complex.[41] Patients with one normal allele demonstrate normal metabolism of bilirubin.[322] The genetic details determine the severity of clinical disease. In CNI there is a complete absence of functional UGT1A1, while in CNII, UGT1A1 activity is markedly reduced.[323] In CNI, 18 of 23 described mutations of the *UGT1* gene are found in the common exons 2 to 5 (see Fig. 4-4) and thus affect many UGT1 enzymes.[41,324] Intronic mutations causing CNI have also been reported.[325] However, in CNII four out of nine known mutations are found in exon 1A1. There is some overlap in classification of mild CNII and GS (e.g., Gly71Arg) which relate to differences in definitions based on serum bilirubin levels.[41] The TATA box TA_7 repeat mutation seen in GS can be seen along with other mutations resulting in either CNI or CNII.[326] In both CNI and CNII, assays of liver tissue from affected patients demonstrate negligible or very low BUGT activity.[48,49,256,315,318,327,328] Hence patients with these disorders experience a profound block in bilirubin excretion, because they lack the ability to conjugate bilirubin with UDPGA. Thus liver biopsy is not helpful in differentiating CNI and CNII. Study of the resected livers from four patients with CNI undergoing liver transplantation showed that there was heterogeneous glucuronidation of various substrates other than bilirubin.[329] Hence several in the family of glucuronosyltransferase isoenzymes can be affected in the same patient. There is considerable overlap of hepatic BUGT activity between CNII and GS[256] (see Table 4-2).

In family studies of CNI patients,[330,331] partial deficiencies have been found in the glucuronidation of salicylate and menthol among siblings, parents, and grandparents. Hence it has generally been accepted that this represents an autosomal recessive inheritance.[330] In family studies of CNII patients, the original report by Arias and colleagues found abnormalities of glucuronidation (menthol) in only one parent, suggesting autosomal dominant inheritance. Subsequent studies of siblings or parents often found elevated serum bilirubin levels (1.2 to 4 mg/dL), delayed bilirubin clearance tests, and decreased hepatic BUGT activity, as would be seen in GS.[280,315,318,320,332,333] These findings in both parents[280,332] suggested that CNII may represent homozygous GS. However, if CNII were truly the homozygous form of GS, one would expect many more affected individuals because GS occurs in at least 3% of the population. Pertinent genetic data have been reviewed[326] supporting the inheritance mode of CNII as autosomal recessive.

The major differentiating characteristic between CNI and CNII is the response to drugs that stimulate hyperplasia of the ER. When CNII patients received phenobarbital or diphenylhydantoin there was a significant decline in the serum bilirubin level, increased hepatic clearance of radiolabeled bilirubin,[280,318,319,334-337] and increased biliary levels of bilirubin diglucuronides[48,320] (see Fig. 4-9). In a study of five CNII patients, the magnitude of the phenobarbital induced decrease in serum bilirubin ranged from 2.1 to 12.1 mg/dL (27% to 72%) with pre- and postphenobarbital serum bilirubin levels ranging from 7.8 to 16.9 and 4.7 to 10.1 mg/dL, respectively.[48] Summarizing data from seven earlier studies[280,318,319,334-337] regarding the response of CNII patients to oral phenobarbital treatment revealed the following: 11 females and 13 males had a total serum bilirubin of 15.7 ± 13.8 (mean ± SD) prior to phenobarbital. After doses ranging from 90 to 390 mg/day, or alternatively 4 mg/kg/day, the serum bilirubin decreased 12 ± 4 mg/dL (77% ± 13%). The lowest total serum bilirubin following phenobarbital therapy was 5.9 mg/dL. In contrast, CNI patients show neither decrease in serum bilirubin nor significantly increased biliary bilirubin conjugates in response to drugs[48,314] (see Fig. 4-9). The response to phenobarbital is the criterion used to differentiate between these two disorders.[338] Bile analysis has also been suggested as another method to differentiate CNI and CNII.[48] In CNI, bile contains insignificant bilirubin conjugates (less than 10%), and UBIL predominates. In CNII, bile contains small amounts of bilirubin conjugates, and those present are predominantly bilirubin monoglucuronides (greater than 60%).[256]

Two cousins with CN have been described with unique features that raise the possibility of a new variant of this syndrome (type III).[339] This new variant resembled CNI in that there was no biliary excretion of bilirubin di- or monoglucuronide. However, the type III patients did excrete mono- and diglucoside conjugates of bilirubin. It has been speculated that type III patients lack the long proposed permease[39] that has been hypothesized to transport UDP-glucuronic acid to the luminal side of the ER, where glucuronosyltransferase is located. This absence is suggested to force utilization of a very inefficient substrate for conjugation to bilirubin, UDP-glucose.

Diagnosis and Treatment. Although CN can be diagnosed during the prenatal period,[340,341] evaluation of infants with CN more typically begins during the first days of life when serum bilirubin levels exceed 20 mg/dL. The conjugated fraction will not be elevated except possibly for the factitious elevation sometimes seen when the total serum bilirubin level is very high.[106] Evaluation of such infants will eliminate hemolysis, hypothyroidism, infection, and other more common causes of jaundice. Formula feedings will help identify those infants with jaundice related to human milk. During this period of testing, the magnitude of the serum bilirubin elevation will prompt use of phototherapy to avoid kernicterus.[126] Exchange transfusion may be necessary. Yet despite these efforts, CN patients will have persistent jaundice. There is currently no widely available simple clinical test to confirm a diagnosis of CN. CN can be excluded by finding significant amounts of bilirubin conjugates in neonatal stools, if collected prior to establishment of sufficient intestinal bacteria that convert bilirubin conjugates to urobilinoids.[1] High pressure liquid chromatographic (HPLC) analysis of duodenal bile will show that in CNI, there are negligible bilirubin di- or monoglucuronides, while in CNII these conjugates are present but in low concentration.[41,48] An easy method to collect such fluid for analysis utilizes the pediatric Entero-Test capsule.[277] This approach to diagnosis is much less invasive than performing a liver biopsy to confirm negligible BUGT activity with in vitro assay. The ratio of serum bilirubin conjugates (as determined by alkaline methanolysis with thin-layer chromatography) to total bilirubin, although abnormally low, does not allow differentiation of CN patients from those with GS.[185] Similar overlap occurs with HPLC fractionation of serum bilirubin conjugates.[294] DNA analysis can be very helpful in establishing the correct diagnosis,[342] and in the future it is expected that DNA array technology will allow rapid screening for known mutation.

A world registry of patients with CNI aimed at developing management guidelines has been published.[343] Phenobarbital (4 mg/kg/day in infants) should be used when there is concern about deficiency of BUGT. Within 48 hours CNII patients can demonstrate a significant decrease in serum bilirubin levels (as detailed above) and an increased biliary excretion of bilirubin di- and monoglucuronide,[49,320] while the CNI patients will show no significant response. Occasionally CNII patients do not respond to the first trial of phenobarbital therapy, but subsequent trials months later will demonstrate the significant decrease in serum bilirubin level.[338] However, despite the decrease in serum bilirubin in response to phenobarbital, CNII patients will usually continue to manifest a significant hyperbilirubinemia (approximately 5 to 15 mg/dL). Phototherapy for 6 to 12 hours daily has been the primary modality to keep serum bilirubin levels below 20 mg/dL during the first several months of life[344] because CNI patients can excrete all bilirubin photoisomers.[345] CNI patients will require lifelong treatment with phototherapy until more definitive therapy such as liver transplant. Phototherapy has been found to be least intrusive when given at night, and improvements have been made in effectiveness and comfort.[346,347] Although phototherapy is very helpful in infancy, in adolescence social inconvenience and compliance problems can bring increased risk of kernicterus.[343]

Other therapeutic considerations involve the oral administration of binding agents such as agar or cholestyramine or calcium phosphate.[348-351] These agents bind to bilirubin in the intestinal lumen due to phototherapy or through direct intestinal permeation.[352] They prevent the enterohepatic circulation of bilirubin. Problems associated with the use of cholestyramine include cost, taste, and concern about bile salt depletion and fat malabsorption. Problems regarding agar include significant variation in bilirubin binding affinity among various preparations and batches.[315,348,353] During acute episodes of severe hyperbilirubinemia after the first year of life, plasmapheresis has been shown to rapidly decrease serum bilirubin levels.[315,354] Peritoneal dialysis and exchange transfusion have not been helpful in this setting.[314] Repeated intramuscular injections of tin protoporphyrin, an HO inhibitor that blocks bilirubin formation, have been used in one CNI patient with data suggesting a decreased need for phototherapy.[355] Two patients with CNI were treated with tin mesoporphyrin to block bilirubin formation and also received daily phototherapy and intermittent plasmapheresis over a 400-day period.[356] They developed an iron deficiency anemia believed to be due to the porphyrin therapy,[357] but tin mesoporphyrin (stannsoporfin) (2 to 4 μmol/kg)[358] is suggested to offer a promising, though still experimental, additional therapy for controlling episodes of acute, severe jaundice.[359] Drugs which bind to albumin and can potentially displace bilirubin should be avoided at all times.[360]

Because patients with CN syndrome have good hepatic function other than conjugating bilirubin, they are ideal candidates for auxiliary liver transplantation, wherein the patient's own liver remains within the body while another whole or partial liver is transplanted just beneath or adjacent to the recipient's. This option has recently become clinically available.[361-363] More commonly, orthotopic liver transplantation has been performed,[364-371] and this represents the only true cure for the hyperbilirubinemia of CNI. Ideally the timing of transplantation would precede irreversible neurologic injury.[372,373] Transplantation of other BUGT-containing tissues (e.g., segments of small intestine,[374,375] kidneys,[376] or hepatocytes[377]) remain experimental. Successful cloning of the gene responsible for bilirubin glucuronosyltransferase activity offers the hope of future gene therapy to correct this deficiency based on studies in Gunn rats, the congenitally jaundiced model for CNI.[372,378-380]

Disorders of Enterohepatic Circulation

Increased enterohepatic circulation of bilirubin is believed to be an important factor in neonatal jaundice. As previously reviewed, neonates are at risk for the intestinal absorption of bilirubin because (1) their bile contains increased levels of bilirubin monoglucuronide, which allows easier conversion to bilirubin; (2) they have significant amounts of β-glucuronidase within the intestinal lumen, which hydrolyses bilirubin conjugates to bilirubin, which is more easily absorbed from the intestine; (3) they lack an intestinal flora to convert bilirubin conjugates to urobilinoids; and (4) meconium, the intestinal contents accumulated during gestation, contains significant amounts of bilirubin.[381] Conditions that prolong meconium passage (e.g., Hirschsprung's disease, meconium ileus, meconium plug syndrome) are associated with hyperbilirubinemia.[382,383] Earlier passage of meconium has been shown to be associated with lower serum bilirubin levels.[384,385] This may be facilitated by rectal temperature measurement during the neonatal period.[386] The enterohepatic circulation of bilirubin can be blocked by the enteral administration of compounds which bind bilirubin, such as agar,[85,348,353,387] charcoal,[388] and cholestyramine.[349]

Other Causes of Unconjugated Hyperbilirubinemia

Various hormones may cause development of neonatal unconjugated hyperbilirubinemia. Congenital hypothyroidism can present with serum bilirubin greater than 12 mg/dL prior to the development of other clinical findings.[389] Prolonged jaundice is seen in one third of infants with congenital hypothyroidism.[390] Similarly hypopituitarism and anencephaly may be associated with jaundice due to inadequate thyroxine, which is necessary for hepatic clearance of bilirubin.

Certain drugs may affect the metabolism of bilirubin and result in hyperbilirubinemia or displacement of bilirubin from albumin. Such displacement increases the risk of kernicterus and can be caused by sulfonamides,[391] moxalactam,[392] and ceftriaxone[393] (independent of its sludge-producing effect[394]). The popular Chinese herb, Chuen-Lin, given to 28% to 51% of Chinese newborn infants, has been shown to have a significant effect in displacing bilirubin from albumin.[395] Pancuronium bromide[164] and chloral hydrate[396] have been suggested as causes of neonatal hyperbilirubinemia.

Infants of diabetic mothers have higher peak bilirubin levels and a greater frequency of hyperbilirubinemia compared with normal neonates.[397] These patients have shown a positive correlation between total bilirubin and hematocrit, thus implicating polycythemia as one possible mechanism.[397] Other potential reasons for this hyperbilirubinemia include prematurity, substrate deficiency for glucuronidation (secondary to hypoglycemia), and poor hepatic perfusion (secondary to either respiratory distress, persistent fetal circulation, or cardiomyopathy).

The Lucey-Driscoll syndrome[398] consists of neonatal hyperbilirubinemia within families in whom there is in vitro inhibition of glucuronosyltransferase by both maternal and infant serum. It is presumed that this is caused by gestational hormones.

Prematurity is frequently associated with unconjugated hyperbilirubinemia in the neonatal period. Hepatic UDP-glucuronosyltransferase activity is markedly decreased in premature infants and rises steadily from 30 weeks' gestation until reaching adult levels 14 weeks after birth.[399] In addition there may be deficiencies for both uptake[400] and secretion.[401] Bilirubin clearance improves rapidly following birth.[402]

Hepatic hypoperfusion can result in neonatal jaundice. Inadequate perfusion of the hepatic sinusoids may not allow sufficient hepatocyte uptake and metabolism of bilirubin. Causes might include patent ductus venosus,[403] congestive heart failure, and portal vein thrombosis. Other specific liver diseases, as listed in Box 4-1 and described elsewhere in this text, can result in neonatal jaundice.

MANAGEMENT AND TREATMENT OF UNCONJUGATED HYPERBILIRUBINEMIA

In recent years changes in perinatal care have made severe neonatal jaundice a larger problem and there has been a reemergence of kernicterus.[404-406] Possible reasons for this include[406,407] (1) early hospital discharge (before extent of jaundice is known and signs of impending brain damage have appeared);[408] (2) lack of adequate concern for the risks of severe jaundice in healthy term and near newborns;[405] (3) an increase in breastfeeding incidence;[169] (4) medical care cost constraints; (5) paucity of educational materials to enable parents to participate in safeguarding their newborns; (6) limitations within in health care systems to monitor the outpatient progression of jaundice;[409] (7) difficulty in estimating the degree of jaundice, particularly in dark-skinned infants;[410] and (8) demonstration of bilirubin being an antioxidant.[20] Newborn infants are now being described who are

Box 4-1 **Causes of Neonatal Hyperbilirubinemia**

INCREASED PRODUCTION OF BILIRUBIN

- Fetal–maternal blood group incompatibilities
    - Extravascular blood in body tissues
    - Polycythemia
    - Red blood cell abnormalities (hemoglobinopathies, membrane, and enzyme defects)
    - Induction of labor

DECREASED EXCRETION OF BILIRUBIN

- Increased enterohepatic circulation of bilirubin
    - Breastfeeding
    - Inborn errors of metabolism
    - Hormones and drugs
    - Prematurity
    - Hepatic hypoperfusion
    - Cholestatic syndromes
    - Obstruction of the biliary tree

COMBINED INCREASED PRODUCTION AND DECREASED EXCRETION OF BILIRUBIN

- Sepsis
    - Intrauterine infection
    - Congenital cirrhosis

From Suchy FJ, Sokol RJ, Balistreri WF, editors: Liver disease in children, *ed 3, New York, 2007, Cambridge University Press.*

discharged early, develop severe hyperbilirubinemia (30 to 40 mg/dL) at home, and go on to develop classic signs of kernicterus.[1,121,405,411-414] This has been reported in otherwise healthy breastfed infants with no other identified etiology for their jaundice.[415-417] Although early postpartum discharge has advantages, one disadvantage is the risk associated with delayed diagnosis of severe hyperbilirubinemia. The AAP has recommended that infants discharged at less than 48 hours of age be seen in follow-up within 48 hours of discharge.[418] Many physicians do not follow these recommendations,[121,419] despite the serious impact that short hospital stay has on the jaundiced newborn.[420] The AAP updated their 1994 guidelines[421] for the management of hyperbilirubinemia in newborn infants in 2004.[121]

Jaundice can be caused by increased bilirubin production, decreased bilirubin excretion, or combinations of these mechanisms (see Box 1 for specific examples). An approach to management of jaundice in the newborn nursery has been published elsewhere.[121] Although Newman and colleagues found that obtaining a direct bilirubin measurement was seldom helpful because of low yield and poor specificity,[422] others advocate measurement of an early conjugated bilirubin as a population screening test, which could lead to the earlier diagnosis of neonatal liver disease.[423] Bhutani and colleagues advocated universal bilirubin measurement prior to hospital discharge in order to identify infants at risk for severe neonatal hyperbilirubinemia on the basis of a predischarge hour specific total serum bilirubin measurement[424,425] (Fig. 4-10). Such universal predischarge screening is now recommended by the AAP[121] and has been shown in one recent study to reduce the incidence of significant hyperbilirubinemia and the rate of hospital readmission of infants for the treatment of jaundice.[426] Despite the large number of etiologies for neonatal jaundice, no cause for the jaundice could be identified in nearly half of the infants evaluated during one study of 447 infants[97] and in one third of the infants in a kernicterus registry.[417]

Hyperbilirubinemia is the most frequent reason that infants are readmitted to the hospital in the first weeks of life.[408,427,428] The management of neonatal jaundice has been reviewed elsewhere[121] (Figs. 4-11 and 4-12). The most important step in treatment of jaundice is determination of the primary etiology. However, independent of the etiology of the jaundice, elevation of the serum UBIL fraction prompts concern about possible kernicterus. As previously reviewed,[132,141] when the UBIL fraction is elevated, care must be given to avoid administration of agents which bind to albumin and displace bilirubin, thus promoting kernicterus. Although

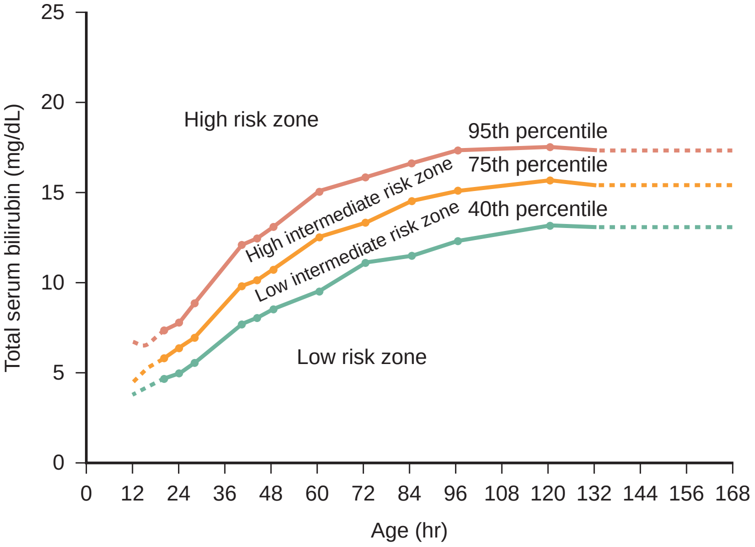

Figure 4-10 Risk designation of term and near term–well newborns based on their hour-specific serum bilirubin values. *(From Bhutani VK, Johnson L, Sivieri EM: Predictive ability of a predischarge hour-specific serum bilirubin for subsequent significant hyperbilirubinemia in healthy term and near-term newborns.* Pediatrics *103:6–14, 1999. American Academy of Pediatrics Subcommittee on Hyperbilirubinemia: Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation.* Pediatrics *114:297–316, 2004.)*

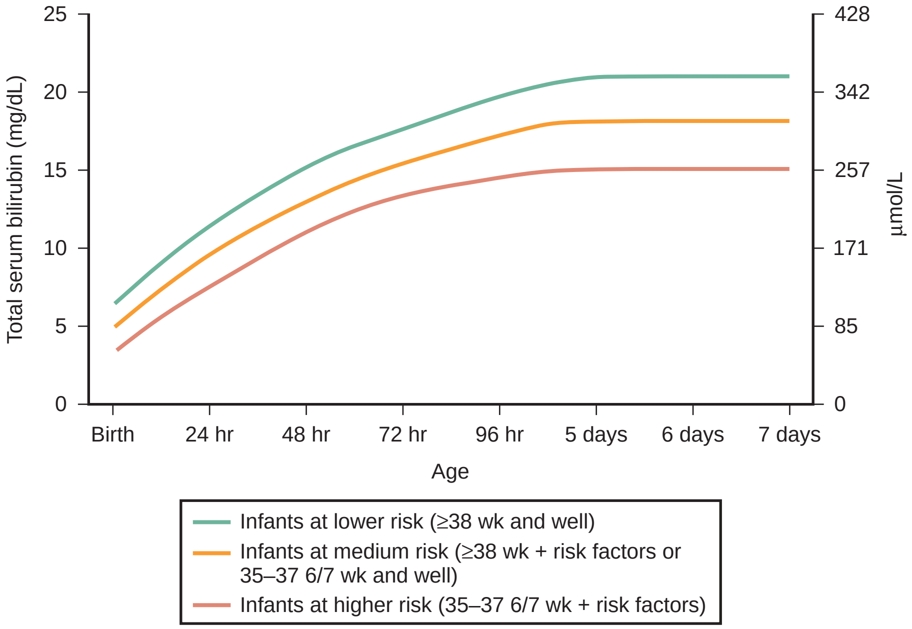

Figure 4-11 Guidelines for phototherapy in hospitalized infants of 35 or more weeks' gestation. These guidelines are based on limited evidence, and the levels shown are approximations. The guidelines refer to the use of intensive phototherapy, which should be used when the TSB exceeds the line indicated for each category. *Intensive phototherapy* implies irradiance in the blue-green spectrum (wavelengths of approximately 430 to 490 nm) of at least 30 µW/cm^2 per nm (measured at the infant's skin directly below the center of the phototherapy unit) and delivered to as much of the infant's surface area as possible. If the total serum bilirubin does not decrease or continues to rise in an infant who is receiving intensive phototherapy, this strongly suggests the presence of hemolysis. Infants who receive phototherapy and have an elevated direct-reacting or conjugated bilirubin level (cholestatic jaundice) may develop the bronze-baby syndrome. Use total bilirubin; do not subtract direct reacting or conjugated bilirubin. Risk factors: isoimmune hemolytic disease, G6PD deficiency, asphyxia, significant lethargy, temperature instability, sepsis, acidosis or albumin less than 3 g/dL. For well infants 35 to 37 6/7 wk can adjust TSB levels for intervention around the medium risk line. It is an option to intervene at lower TSB levels for infants closer to 35 wks and at higher TSB levels for those closer to 37 6/7 wk. It is an option to provide conventional phototherapy in hospital or at home at TSB levels 2 to 3 mg/dL (35 to 50 µmol/L) below those shown, but home phototherapy should not be used in any infant with risk factors. *(From American Academy of Pediatrics Subcommittee on Hyperbilirubinemia: Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation.* Pediatrics *114:297–316, 2004.)*

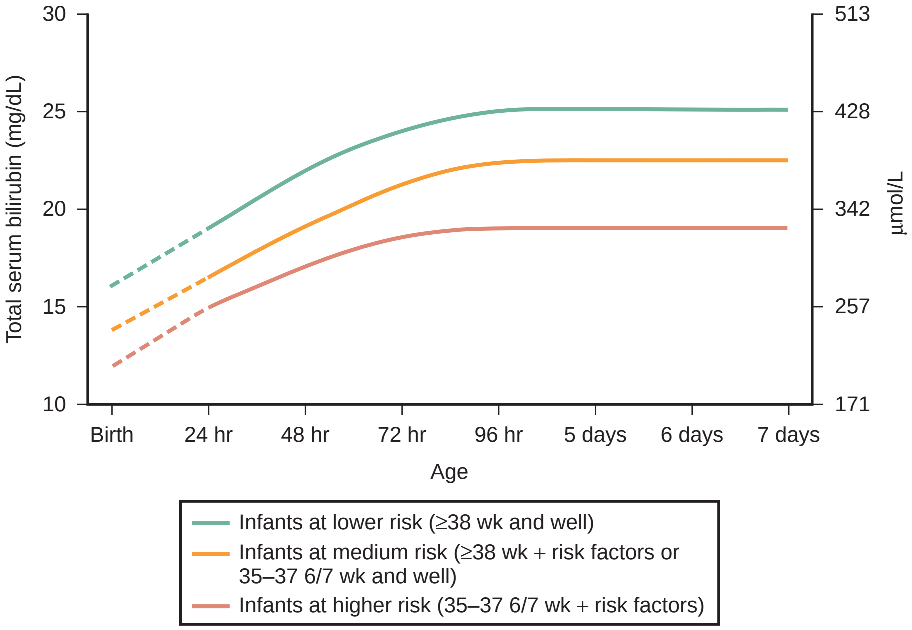

Figure 4-12 Guidelines for exchange transfusion in infants 35 or more weeks' gestation. These suggested levels are based on limited evidence, and the levels shown are approximations. During birth hospitalization, exchange transfusion is recommended if the TSB rises to these levels despite intensive phototherapy. For readmitted infants, if the TSB level is above the exchange level, repeat TSB measurement every 2 to 3 hours and consider exchange if the TSB remains above the levels indicated after intensive phototherapy for 6 hours. The dashed lines for the first 24 hours indicate uncertainty due to a wide range of clinical circumstances and a range of responses to phototherapy. Immediate exchange transfusion is recommended if an infant shows signs of acute bilirubin encephalopathy (hypertonia, arching, retrocollis, opisthotonos, fever, high-pitched cry) or if TSB is greater than or equal to 5 mg/dL (85 µmol/L) above these lines. Risk factors: isoimmune hemolytic disease, G6PD deficiency, asphyxia, significant lethargy, temperature instability, sepsis, acidosis. Use total bilirubin; do not subtract direct reacting of conjugated bilirubin. If infant is well and 35 to 37 6/7 wk (median risk), one can individualize TSB levels for exchange based on actual gestational age. *(From American Academy of Pediatrics Subcommittee on Hyperbilirubinemia: Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation.* Pediatrics *114:297–316, 2004.)*

historically, sulphonamides are the most well known bilirubin displacing agents, drugs such as ceftriaxone and ibuprofen are also strong bilirubin displacers with a potential for inducing bilirubin encephalopathy.[393,429] Therapeutic options to lower UBIL levels include phototherapy, exchange transfusion, interruption of the enterohepatic circulation, enzyme induction, and alteration of breastfeeding. Research into these and other modalities continues actively. The outcome of rational therapeutic guidelines for unconjugated hyperbilirubinemia during the first 7 days of life have been published elsewhere;[430] phototherapy can reduce the incidence of exchange transfusion and the risk of bilirubin-induced neurologic disease.

Phototherapy

Phototherapy consists of irradiation of the jaundiced infant with light.[431,432] Photon energy derived from light changes the structure of the bilirubin molecule in two ways (Fig. 4-13), allowing bilirubin to be excreted into bile or urine without the usual requirement for hepatic glucuronidation. One change involves a 180-degree rotation around the double bonds between either the A and B or C and D rings,[433] converting the normal Z configuration to the E configuration. 4Z,15E bilirubin is preferentially formed and can spontaneously reisomerize to native bilirubin. More importantly a new seven-membered ring structure can be formed between rings A and B, resulting in *lumirubin*[434] or *cyclobilirubin*[435] via the 4E,15Z-isomer intermediate.[436] The isomerization interferes with the internal hydrogen bonding of native bilirubin and by exposing the propionic acid groups result in a more polar compound. Thus the lumirubin and E isomers can be excreted directly into bile. Lumirubin appears to be the major route by which bilirubin is eliminated with phototherapy.[437,438] The choice of light for phototherapy is a subject about which there is much conflicting data.[431] Special blue light (or Super Blue, but not regular blue) appears to be better than white or green light, though white is less disturbing to nursery personnel.[439] Phototherapy devices utilizing woven fiberoptic pads are currently available,[440] effective (comparable to conventional phototherapy), safe, eliminate the need for eye patches, and permit greater time for maternal–infant bonding.[441-443]

Proposed guidelines for phototherapy have been published elsewhere.[121] In general, phototherapy is used to prevent serum bilirubin concentrations from reaching levels necessitating exchange transfusion (see Fig. 4-11). Phototherapy is now frequently done at home,[444,445] a practice accepted and recommended by the AAP.[121] Despite documented complications, such as skin rash, loose bowel movements, overheating, and dehydration,[446-448] phototherapy is widely used and generally safe.[449] Although phototherapy does effect cardiac output, blood flow to other organs (e.g., increased cerebral blood flow), and may be associated with opening of the ductus arteriosus, these effects are generally not problematic.[450,451] In extremely premature infants (less than 800 g birthweight) prolonged phototherapy and low peak serum bilirubin levels (less than 9.4 mg/dL) were shown in one study to be independently associated with blindness.[452] This could possibly be related to direct effects of light on the unshielded immature eye or decreased antioxidant protection by low serum bilirubin levels. Phototherapy should not be employed without prior diagnostic evaluation of the cause of the jaundice. The shielding of phototherapy units should never be removed because of the ultraviolet radiation hazards that can result.[453] Although it has been recommended that the infants position be changed every 6 hours during phototherapy,[454] data from one study showed position change made no difference in serum bilirubin levels.[455] One study of 264 neonates (some with hemolytic jaundice) who completed an average of 121 hours of phototherapy indicated that significant rebound hyperbilirubinemia did not occur.[456] Similarly no significant rebound hyperbilirubinemia occurred in 163 healthy, full-term infants with nonhemolytic jaundice (including breast-, formula- and mixed-feedings) receiving an average of 54 to 65 hours.[457] In full-term, healthy newborns, phototherapy may be stopped when the serum bilirubin level falls below 14 to 15 mg/dL, and hospital discharge need not be delayed to observe for rebound.[421]

Exchange Transfusion

Exchange transfusion is the most rapid method to acutely lower the serum bilirubin concentration. Indications for exchange transfusion vary, have been published elsewhere,[121,446,458-460] and are detailed in Chapter 2. They can be related to either anemia or elevated serum bilirubin level. The AAP has published guidelines which represent the most widely accepted indications for initiation of exchange transfusion based on risk factors, including hour-specific total bilirubin level; response to intensive phototherapy; serum bilirubin-to-albumin ratio; and the presence of hemolytic disease, sepsis, acidosis, or other acute conditions (see Fig. 4-12). Additionally exchange transfusion should be initiated if the infant displays signs of acute bilirubin encephalopathy (see section on bilirubin encephalopathy). In neonatal hemolytic disease, suggested indications for transfusion include (1) anemia (hematocrit less than 45%), positive direct Coombs test, and bilirubin greater than 4 mg/dL in the cord blood; (2) postnatal rise of the serum bilirubin concentration that exceeds 1 mg/dL/hr for more than 6 hours; (3) progressive anemia and rate of increase in serum bilirubin of greater than 0.5 mg/dL/hr; and (4) continuing progression of anemia despite control of hyperbilirubinemia. Sometimes exchange transfusion for hemolytic disease can be avoided through the use of high-dose intravenous immunoglobulin therapy.[461] Suggested indications for exchange transfusion because of hyperbilirubinemia alone include (1) bilirubin concentration greater than 15 mg/dL for more than 48 hours; (2) salicylate saturation index greater than 8 or HABA binding less than 50% on two successive determinations 4 hours apart; (3) ratio of total serum bilirubin (mg/dL) divided by total serum protein (g/dL) greater than 3.7; and (4) molar concentration ratio of serum bilirubin to serum albumin of greater than 0.7.[446] Although there are many well described risks with exchange transfusion, mortality should be low (less than 0.6%) if it is performed properly.[402,462,463]

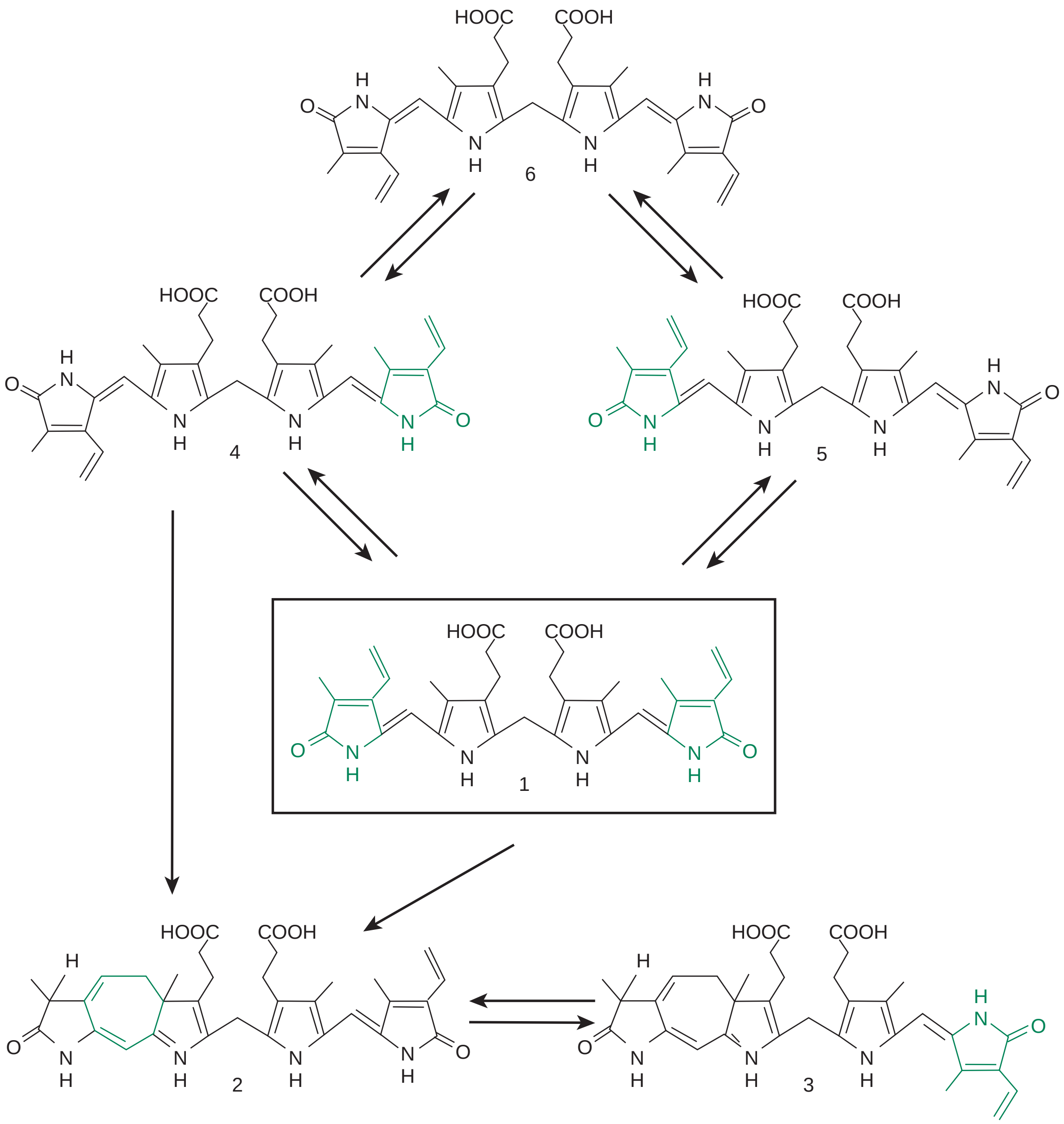

Figure 4-13 The products of phototherapy. Light induces an isomerization of 4*Z*,15*Z*-bilirubin (1) to produce the configurational isomers 4*E*,15*Z*-bilirubin (4), 4*Z*,15*E*-bilirubin (5), 4*E*,15*E*-bilirubin (6) and the structural isomers, *Z*-lumirubin (2) and *E*-Lumirubin (3) as shown. Isomerization exposes one or both of the propionic acid side chains, increasing the polarities of the isomers with respect to biosynthetic bilirubin (1) and allowing excretion in bile without hepatic glucuronidation. Formation of 4*E*,15*Z*-bilirubin, 4*Z*,15*E*-bilirubin, 4*E*,15*E*-bilirubin is reversible, and these isomers spontaneously revert to the parent 4*Z*,15*Z*-bilirubin (1) isomer after excretion in bile. The predominant isomer present in the circulation of infants exposed to light or phototherapy is 4*Z*,15*E*-bilirubin (5). Although the structural isomers (2 and 3) are formed more slowly than the configurational isomers, they may contribute more to the overall effect of the treatment because of their relatively faster excretion. *(From Antony McDonagh: Phototherapy: From ancient Egypt to the new millenium.* J Pernatol *21:S7–12, 2001.)*

Pharmacotherapy

There are a number of pharmacologic approaches to the prevention and treatment of neonatal hyperbilirubinemia that have been recently reviewed.[464] The enterohepatic circulation can be interrupted by enteral administration of agents that bind bilirubin in the intestine and prevent reabsorption. Such agents include agar,[85,348,353] cholestyramine,[349] activated charcoal,[388] and calcium phosphate.[351,465,466] Increased intestinal peristalsis would be expected to allow less time for bilirubin absorption. Frequent feedings[467] and rectal stimulation[386] are associated with lower serum bilirubin levels. Enteral feedings of bilirubin oxidase, an enzyme that degrades bilirubin to biliverdin, dipyrrols, and other unidentified products,[468] are an additional method to block the enterohepatic circulation that remain experimental at present.[469] Another experimental approach utilizes intravenous bilirubin oxidase.[470,471]

Because neonatal hepatic BUGT activity has been shown to be low in neonates,[399] it is not surprising that induction of hepatic BUGT results in lower serum bilirubin levels. Such induction in the neonate can be accomplished with prenatal maternal use of phenobarbital or diphenylhydantoin.[472,473] Even very low birth weight infants (less than 2000 g) have been shown to respond to in utero phenobarbital therapy, with significantly increased serum levels of conjugated bilirubin and decreased need for phototherapy.[474] In the postnatal period, use of phenobarbital by the neonate has the same bilirubin-lowering effect.[475] Clofibrate is advocated by French workers as a simple, nontoxic pharmacologic treatment which induces BUGT and is used increasingly in France to prevent and treat neonatal jaundice.[476] An alternative approach to treat neonatal hyperbilirubinemia is to block the first enzyme responsible for the production of bilirubin, HO. This can be accomplished by several different metalloporphyrins.[477,478] Sn-protoporphyrin has been used successfully in the experimental management of jaundice in neonates with ABO incompatibility.[479] Treatment of neonatal jaundice with HO inhibition remains experimental at this point in time, though clinical trials appear promising. Two studies with Sn-mesoporphyrin (Sn-MP) showed that phototherapy could be abolished by using Sn-MP.[480,481] In addition to inhibiting bilirubin production, metalloporphyrins are photosensitizers, which can accelerate the destruction of bilirubin by light but also cause other unwanted side effects.[482] Because CO is now suggested to be a significant neurotransmitter,[483] blocking HO activity early in life may not be a completely benign process. Thus a major concern about metalloporphyrins is the lack of detailed long term follow-up studies addressing safety issues.[484] At the present time, phototherapy and exchange transfusion remain the primary modes to control neonatal unconjugated hyperbilirubinemia, and pharmacologic interventions remain largely adjunctive or experimental.

Feeding Strategies

Optimization of breastfeeding in the perinatal period is important.[485] If the bilirubin level is rising, published recommendations support encouraging mothers to breastfeed more frequently,[421,486,487] with an average suggested interval between feeds of 2 hours and no feeding supplements[488] or at least 8 to 10 feedings per 24 hours.[421] A strong dose-response relationship has been shown between feeding frequency and a decreased incidence of hyperbilirubinemia.[489] More frequent nursing may not increase intake, but it has been suggested to increase peristalsis and stool frequency, thus promoting bilirubin excretion.[458] Frequency of breastfeeding during the first 24 hours of life has been shown to be correlated significantly with the frequency of meconium passage.[489] The serum bilirubin level at which breastfeeding should be discontinued is controversial, and recommendations include 14,[490] 15,[175] 16 to 17,[487] and 18 to 20 mg/dL.[488] When breastfeeding is interrupted, formula feeding may be initiated for 24 to 48 hours, or breastfeeding and formula-feeding can be alternated with each feeding.[421] No studies have addressed the cost effectiveness of various formulas in their jaundice-lowering effects, though two independent studies have shown that infants exclusively fed Nutramigen™ (a casein hydrolysate formula, Mead Johnson) have lower jaundice levels than infants fed routine formula.[491,492] Supplementing breastfed infants with small volumes of specific nutritional ingredients has been shown to result in increased fecal bilirubin excretion and lower TcB levels,[96] though no commercial preparations of these ingredients are currently available. Feeding heated, expressed breastmilk is also said to reduce serum bilirubin levels.[488] A fall in the serum bilirubin level of 2 to 5 mg/dL[458] is consistent with a diagnosis of breastmilk jaundice. Breastfeeding may then be resumed with the acknowledgment that serum bilirubin levels may rise for several days but will gradually level off and decline.[175,486] If breastfeeding is to be resumed following the interruption, it is important to preserve lactation with the use of a breast pump. In one study, interruption of breastfeeding for approximately 50 hours (during which time a formula was given) was shown to have the same bilirubin-lowering effect as a similar duration of phototherapy.[493] Interruptions of breastfeeding for 24 to 48 hours have been shown to be successful at lowering serum bilirubin levels and avoiding the need for phototherapy in 81 of 87 jaundiced infants.[490] Formula feedings to Asian neonates receiving phototherapy resulted in greater decrease of serum bilirubin levels than infants who were exclusively breastfed.[457] Careful counseling and support can prevent the interruption of breastfeeding from becoming a permanent discontinuance of nursing.[490]

DISORDERS OF CONJUGATED HYPERBILIRUBINEMIA

Rotor Syndrome

Rotor syndrome, (RS) (OMIM #237450) first described in 1948, is a familial disorder which involves chronic elevation of both the conjugated and unconjugated serum bilirubin fractions.[494,495] Half or more of the total serum bilirubin is conjugated, and total bilirubin levels usually range from 2 to 7 mg/dL but occasionally may reach 20 mg/dL.[496] Liver functions tests are otherwise normal, and there is no evidence of hemolysis. Liver histology is normal when examined with both light and electron microscopy. Oral cholecystograms reveal normal gallbladder opacification. This disorder can present in early childhood[497] or possibly the first months of life, if coinherited with G6PD deficiency or heterozygous β-thalassemia,[498] and manifests no gender predisposition. Family studies suggest an autosomal recessive mode of inheritance.[494,495,499]

Pathophysiology

OMIM now indicates that the primary abnormality in RS is simultaneous mutation in the SLCO1B1 and SLCO1B3 genes, which are neighboring genes, leading to nonfunctional organic anion transporter peptides OATP1B1 and OATP1B3. Recent data in eight RS families found these mutations and suggest that interference with the hepatic uptake of bilirubin glucuronide by the nonfunctional OATP1B1/1B3 transporters results in elevated conjugated and unconjugated serum bilirubin levels.[500] Previously there was thought to be a deficiency in the intracellular storage capacity of the liver for binding anions,[501,502] which can be demonstrated by constant infusions of BSP and ICG.[503] Patients with RS demonstrate a delayed plasma clearance of both BSP and ICG, and heterozygotes show delayed BSP clearance with values intermediate between normals and those with homozygous RS.[501] GST serves as an intracellular carrier protein for certain organic molecules, acting as an intracellular equivalent to albumin in blood plasma.[504] A patient with RS has been shown to have a deficiency of hepatic GST,[505] and this would be consistent with observations regarding the pathogenesis of this disorder. Deficiency of GST would result in impaired uptake of bilirubin within the cytosol. In addition, because bilirubin conjugates are bound to GST while awaiting excretion from the hepatocyte via the canalicular membrane,[506] deficient intracellular storage would result in leakage of bilirubin conjugates back into the circulation. Serum elevations of both conjugated and nonconjugated bilirubin result.

Another important observation in RS relates to the urinary excretion of coproporphyrin. In normal, healthy individuals only the I and III isomers of coproporphyrin are excreted in the urine. In RS there is a marked increase in urinary coproporphyrin excretion, and, usually, less than 80% of the total (I + III) is isomer I (normal is 25%). Heterozygotes demonstrate urinary coproporphyrin values that are intermediate between normals and homozygotes.[507] Urinary excretion of coproporphyrin is believed to be increased because biliary excretion is impaired, similar to findings in other liver diseases.[508]

Diagnosis and Treatment

A diagnosis of RS should be considered in all individuals having elevation of both conjugated and nonconjugated serum bilirubin fractions along with otherwise normal liver function tests. The diagnosis can be confirmed by measuring urinary coproporphyrin levels, which are 2.5 to 5 times higher than normal levels.[507] Of the total urinary coproporphyrin isomers I plus III, isomer I constitutes less than 80% of the total in RS.[509] ^{99m}Tc-HIDA cholescintigraphy has also been shown to be useful to diagnose RS and demonstrates poor to no visualization of the liver.[510,511] Patients with RS require no specific therapy and are asymptomatic. Although jaundice is a lifelong finding, it is associated with no morbidity or mortality unless the cosmetics are a concern.

Dubin-Johnson Syndrome

Dubin-Johnson syndrome (DJS) (OMIM #237500), first described in 1954,[512] involves elevation of both the conjugated and unconjugated serum bilirubin fractions.[513] More than half of the total serum bilirubin is conjugated, and total bilirubin levels usually range from 1.5 to 6 mg/dL, though values have been reported as high as 25 mg/dL during intercurrent illness.[514] It is not unusual for patients with DJS to report vague abdominal complaints, though this is not believed to reflect serious pathology. Although hepatomegaly is sometime seen, liver functions tests are otherwise normal, including bile acids,[515] and there is no evidence of hemolysis.[512,513,516] Although this syndrome occurs in both sexes, males predominate and present at an earlier age. It occurs in all races; however, Iranian Jews have an increased incidence.[517,518] It is usually diagnosed after puberty, though cases have also been reported in neonates,[519] at which time cholestasis can be significant.[520-524] DJS is inherited as an autosomal recessive trait with heterozygotes manifesting normal serum bilirubin levels.[517,525,526] This syndrome is far more common than RS, and jaundice can be worsened by pregnancy and oral contraceptives.[527] Often the gallbladder is not visualized when patients with DJS undergo an oral cholecystogram.[513]

A striking characteristic of DJS is the brown to black discoloration of the liver. There is still debate about the identity of this pigment, which is located in the lysosomes.[528] Although originally thought to be lipofuscin, more recent data provide conflicting evidence for a relationship to melanin[529-531] or polymerized epinephrine or other metabolites[532,533] that accumulate in the lysosomes. It is hypothesized that these pigments accumulate in the liver because of impaired secretion of various metabolites from the hepatocyte into the bile.[533] This pigment disappears from the liver during acute viral hepatitis, with subsequent reappearance.[534] Other than this striking pigmentation, the liver is histologically normal. Recently, a black liver was reported in an individual who did not have DJS.[535]

Pathophysiology

The primary defect in the DJS is deficient hepatic excretion of non–bile-salt organic anions at the apical canalicular membrane, by the ATP-binding cassette (ABC) transport system known variously as cMOAT, MRP2, or ABCC2 (OMIM 601107).[536,537] cMOAT is encoded by a single copy gene located on chromosome 10q24.[538] Mutations of this gene have been shown to produce a highly defective cMOAT that is associated with DJS.[58,536,537,539-542] Similar findings made in the homologous cMOAT gene of two rat models of hyperbilirubinemia (TR[-] and Eisai) have been very helpful in understanding DJS in humans.[58]

Although hepatic BSP clearance tests are no longer performed, they nicely demonstrate the effect of deficient transport via the canalicular membrane, characteristic of DJS.[543] Initially the clearance rate of intravenously administered BSP from the circulation is rapid and results in a BSP retention that is often normal at 45 minutes. However, a subsequent rise in serum BSP concentration occurs at 90 and 120 minutes, because the conjugated BSP cannot be excreted and thus refluxes out of the hepatocyte back into the circulation.[543-546] Data suggest that BSP hepatic storage is normal, but there is a 90% decrease in the BSP excretory transport maximum.[527,545] Other substances (e.g., ICG, rose bengal, and dibromosulfophthalein) have also been shown to have a decreased excretory transport maximum, though these substances do not require hepatic biotransformation and do not show the late rise in plasma levels during clearance tests.[547] Hence in DJS, deficient excretion of bilirubin glucuronides at the canalicular membrane, in the presence of otherwise normal intrahepatic metabolism, results in reflux of conjugated bilirubin back into the circulation.

An important observation in DJS patients relates to the urinary excretion of coproporphyrins.[548,549] Patients with this disorder have an increase in the urinary excretion of coproporphyrin I with a concomitant decrease in the excretion of coproporphyrin III. This results in a total coproporphyrin excretion (I + III) that is normal or only slightly increased, but which consists of greater than 80% coproporphyrin I (normal 25%).[549-552] In heterozygotes the coproporphyrin I:III ratios are intermediate between normals and homozygotes,[549,551,552] though there is some overlap between heterozygotes and normals. The explanation for these findings regarding urinary coproporphyrin excretion is unclear. Several pathogenetic mechanisms have been suggested.[549] Fecal coproporphyrin levels are normal.[549] Healthy neonates have been shown to have impressive elevations of urinary coproporphyrin levels with more than 80% isomer I on the first 2 days of life.[553] By day 10, levels fell to overlap normal adult values.

Diagnosis and Treatment

A diagnosis of DJS should be considered in all individuals having elevation of conjugated bilirubin in the serum, along with otherwise normal liver function tests. The diagnosis can be confirmed by measuring urinary coproporphyrin levels of isomers I and III. While the total coproporphyrin level will be approximately normal, more than 80% will be isomer I. This finding is pathognomonic for DJS when congenital erythropoietic porphyria[554] or arsenic poisoning[555] have been excluded. Although oral cholecystogram may fail to visualize the gallbladder, ultrasound examination will show a normal biliary tree. Cholescintigraphy demonstrates prolonged intense visualization of the liver with delayed appearance of the gallbladder and only faint or nonvisualization of the biliary ducts.[511,556,557] Computed tomography of the liver has shown increased attenuation in one report.[558] Because cMOAT transport of leukotrienes into bile is defective in DJS, there is increased excretion of leukotriene metabolites into urine, and this has been suggested to be a new approach to the noninvasive diagnosis of this disease.[559] Another proposed diagnostic tool for DJS involves micro–positron emission tomography (microPET) imaging of a copper complex studied in normal and cMOAT-deficient (TR[-]) rats.[560] In normal rats, the radioactive copper complex was cleared quickly from the liver into the intestine, while radioactivity accumulated continuously in the liver of TR(-) rats and was not excreted into the small intestine.

Patients with DJS require no specific therapy. Although jaundice is a lifelong finding, it is associated with no morbidity or mortality, as demonstrated in a 30-year follow-up of 10 Japanese individuals.[541] Avoidance of the oral contraceptive has been recommended[561] because it can increase jaundice. Anticipatory guidance regarding pregnancy[527] is appropriate. Increased fetal wastage has been reported in one study.[562] In one case report of neonatal DJS with severe cholestasis, phenobarbital significantly decreased serum levels of bilirubin and bile acids,[520] though chronic phenobarbital therapy is not recommended.[563] Drugs such as rifampin and ursodeoxycholic acid should be used with caution if MRP2 expression is decreased, as in DJS.[564]

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   In nine children with Crigler-Najjar, the bile pigment composition and fractional biliary excretion ratio was studied, as was their response to phenobarbital administration. Serum bilirubin levels either decreased in response to phenobarbital administration with an increase in bilirubin monoconjugates and diconjugates, leading to a classification of CN type II, or did not significantly change with largely unconjugated bilirubin, leading to a diagnosis of CN type I. Bile pigment analysis fractional bilirubin excretion could differentiate between Crigler-Najjar and Gilbert's syndromes.
49. Fevery J, Blanckaert N, Heirwegh KPM, et al: Unconjugated bilirubin and an increased proportion of bilirubin monoconjugates in the bile of patients with Gilbert's syndrome and Crigler-Najjar disease. *J Clin Invest* 60:970–979, 1977.
   Bilirubin pigment analysis demonstrated that in those with Gilbert's syndrome there was an increase in bilirubin monoconjugates. This was further increased in those with Crigler-Najjar, suggesting that Crigler-Najjar has a more pronounced expression of hepatic bilirubin UDP-glucuronosyltransferase.
51. Goresky CA, Gordon ER, Shaffer EA, et al: Definition of a conjugation dysfunction in Gilbert's syndrome: studies of the handling of bilirubin loads and of the pattern of bilirubin conjugates secreted in bile. *Clin Sci* 55:63–71, 1978.
   Intravenous bilirubin given to patients with Gilbert's syndrome led to sustained elevation of the plasma bilirubin and reduced clearance that was not observed in normal subjects. Bile analysis of Gilbert's patients demonstrated a smaller average proportion of bilirubin diglucuronide and increased average proportion of bilirubin monoglucuronide. Caloric restriction raised bilirubin levels, and phenobarbital administration decreased bilirubin in patients with Gilbert's and reversed the biliary pattern towards normal.
69. Zimmerman HJ: Hormonal derivatives and other drugs used to treat endocrine disease. In *Hepatotoxicity: the adverse effects of drug and other chemicals on the liver*, New York, 1978, Appleton, Crofts, pp 436–467.
   A book chapter detailing effects of synthetic anabolic steroids and contraceptive steroids causing hepatic dysfunction and disease; natural androgens and estrogens and their antagonists in liver lesions; glucocorticoids and hepatic steatosis; and oral hypoglycemic and antithyroid medications and injury.
71. Muraca M, Fevery J, Blanckaert N: Relationships between serum bilirubins and production and conjugation of bilirubin—studies in Gilbert's syndrome, Crigler-Najjar disease, hemolytic disorders, and rat models. *Gastroenterology* 92:309–317, 1987.
   Serum bilirubin patterns in humans and rats were studied. In Gilbert's syndrome, the concentration of unconjugated pigment was increased. Those with Crigler-Najjar had higher unconjugated bilirubin levels and undetectable diconjugated bilirubin. With hemolytic disease, the fraction of conjugated to total bilirubin remained within the normal range as both increased proportionally.
85. Poland RL, Odell GB: Physiologic jaundice: the enterohepatic circulation of bilirubin. *N Engl J Med* 284:1–6, 1971.

Bilirubin levels were evaluated after full-term infants were fed either a formula containing agar supplementation, a bilirubin stabilizer, or nonsupplemented formula for the first 5 days of life. The infants fed the agar-supplemented formula had no rise in serum bilirubin, excreted more bilirubin in their feces, and lost less weight than the nonsupplemented group. By day 6 of life, fecal bilirubin excretion was similar in each group. The authors concluded that the agar-stabilized bilirubin decreased enterohepatic circulation of bilirubin and subsequent elevations in serum bilirubin.

92. Gourley GR, Arend RA: β-glucuronidase and hyperbilirubinemia in breast-fed and formula-fed babies. *Lancet* i:644–646, 1986.

Bilirubin levels were studied in breastfed and formula-fed infants, with total serum levels significantly higher at both timepoints in the breastfed infants. Fecal studies at day 21 of life demonstrated higher β-glucuronidase levels in breastfed infants. β-glucuronidase activity was considerable in human milk and undetectable in formula and must be considered in the etiology of hyperbilirubinemia in breastfed infants.

95. Kreamer BL, Siegel FL, Gourley GR: A novel inhibitor of β-glucuronidase: L-aspartic acid. *Pediat Res* 50:460–466, 2001.

Infants who consume formulas with casein-hydrolysate, which contains a β-glucuronidase inhibitor, have lower bilirubin levels than their counterparts who are breastfed or regular formula-fed. The use of inhibition assays and kinetic studies demonstrated that the L-aspartic acid component of casein is responsible for β-glucuronidase inhibition.

96. Gourley GR, Li Z, Kreamer BL, et al: A controlled, randomized, double-blind trial of prophylaxis against jaundice among breastfed newborns. *Pediatrics* 116:385–391, 2005.

A group of 64 breastfed infants were randomized to receive supplements (6 mL 5 times daily) of either L-aspartic acid, enzymatically hydrolyzed casein (EHC), whey/casein (w/c), or a control group for the first week of life. Lower transcutaneous bilirubin levels were noted in the L-aspartic acid, EHC, and w/c groups versus the control group on days 3 to 7 of life and did not disrupt the breastfeeding experience. Fecal bile excretion was significantly greater in the L-aspartic acid group.

105. Schlebusch H, Axer K, Schneider C, et al: Comparison of five routine methods with the candidate reference method for the determination of bilirubin in neonatal serum. *J Clin Chem Clin Biochem* 28:203–210, 1990.

Five routine methods to determine serum bilirubin levels were compared to Doumas's reference method. Four of the methods (Jendrassik and Grof's method, Hertz's spectrographic, bilirubinometer procedure, and 2,5-dichlorophenyldiazonium method) produced higher values than the reference. A study of Vink's spectrographic method differed negligibly from the reference. Accuracy of some of the other methods could be improved with calibration.

111. Bhutani VK, Gourley GR, Adler S, et al: Noninvasive measurement of total serum bilirubin in a multiracial predischarge newborn population to assess the risk of severe hyperbilirubinemia. *Pediatrics* 106:e17, 2000.

In a group of 490 ethnically diverse, full-term and near-term infants, transcutaneous bilirubin levels were measured with a portable BiliChek device and found to correlate with total serum bilirubin values. In those infants with total serum bilirubin greater than the 95th percentile for age and at high risk, the transcutaneous bilirubin values were all greater than the 75th percentile for age.

120. Maisels MJ, Ostrea EM, Jr, Touch S, et al: Evaluation of a new transcutaneous bilirubinometer. *Pediatrics* 113:1628–1635, 2004.

In a group of 849 ethnically diverse, full-term and near-term infants, a close correlation was demonstrated between total serum bilirubin and transcutaneous bilirubin values as obtained by the Minolta-Hill-Rom Air-Shields Transcutaneous Jaundice Meter model. The correlation in black infants was less close (r = 0.822), with differences increasing with rising total serum bilirubin values, but the transcutaneous bilirubin value always overestimated the bilirubin levels.

121. American Academy of Pediatrics Subcommittee on Hyperbilirubinemia: Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. *Pediatrics* 114:297–316, 2004.

In these guidelines by the AAP, it is proposed that successful breastfeeding be promoted and supported, infants should be systematically assessed for hyperbilirubinemia prior to discharge, follow-up should be dictated based on this assessment, and infants should be treated with phototherapy or exchange transfusion if dictated by levels of hyperbilirubinemia to prevent the development of adverse effects.

126. Odell GB, Schutta HS: Bilirubin encephalopathy. In McCandless DW, editor: *Cerebral energy metabolism and metabolic encephalopathy*, New York, 1985, Plenum Publishing Corp, pp 229–261.

This chapter discusses the typical clinical manifestations of kernicterus, histopathologic changes due to bilirubin injury, and treatment options.

129. Odell GB, Storey GNB, Rosenberg LA: Studies in kernicterus. III. The saturation of serum proteins with bilirubin during neonatal life and its relationship to brain damage at five years. *J Pediatr* 76:12–21, 1970.

In a group of 5-year-old children that were jaundiced during the neonatal period, psychometric studies were performed with 14 children classified as normal and 18 children classified as brain damaged. No correlations were found between brain damage and max serum bilirubin; however, a significant correlation was identified between brain damage and the saturation of serum proteins with bilirubin during the neonatal period.

132. Walker PC: Neonatal bilirubin toxicity-a review of kernicterus and the implications of drug-induced bilirubin displacement. *Clin Pharmacokinet* 13:26–50, 1987.

Interactions between bilirubin and common drugs given to newborn infants are reviewed in this article, including the methodology, mechanisms, and pharmacokinetics of various classes such as antibiotics, diuretics, and other commonly administered drugs.

140. Odell GB: The dissociation of bilirubin from albumin and its clinical implications. *J Pediatr* 55:268–279, 1959.

Bilirubin can be increased in the extracellular fluid by reducing the binding capacity of plasma proteins, such as albumin, for bilirubin. This is seen in premature infants due to hypoalbuminemia, and in full-term infants if decreased pH, or if sulfonamides, salicylates, or caffeine is present that reduces albumin's binding capacity, leading to hyperbilirubinemia.

155. Moyer V, Freese DK, Whitington PF, et al: Guideline for the evaluation of cholestatic jaundice in infants: recommendations of the north american society for pediatric gastroenterology, hepatology and nutrition. *J Pediatr Gastroenterol Nutr* 39:115–128, 2004.

A consensus statement for evaluation of cholestatic jaundice in infants was crafted after a comprehensive and systematic literature review, with recommendations for detection, pediatric gastroenterology referral, and management of jaundice. Infants who remain jaundiced at 2 weeks of age need evaluation of total and direct serum bilirubin. Breastfed infants with normal history and exam may defer serum determination of total and direct serum bilirubin until 3 weeks if jaundice persists.

159. Schneider AP: Breast milk jaundice in the newborn—a real entity. *JAMA* 255:3270–3274, 1986.

This pooled data analysis of 12 studies demonstrated that moderate and severe jaundice was significantly higher in breastfed versus formula-fed infants, breastfed infants have higher mean serum bilirubin levels, and that a significant relationship existed between jaundice and breastfeeding.

169. Gourley GR: Breast-feeding, neonatal jaundice and kernicterus. *Semin Neonato* l7:135–141, 2002.

In this review article, the author discusses mechanisms for the development of higher bilirubin levels in breastfed infants versus formula-fed infants, including poor fluid and caloric intake, inhibition of hepatic excretion of bilirubin, and enterohepatic circulation. With almost all of the cases of kernicterus in the last 15 years in full or partially breastfed newborns, this population should be followed closely.

201. Kaplan M, Renbaum P, Levy-Lahad E, et al: Gilbert syndrome and glucose-6-phosphate dehydrogenase deficiency: a dose-

dependent genetic interaction crucial to neonatal hyperbilirubinemia. *Proc Natl Acad Sci U S A* 94:12128–12132, 1997.

DNA from term neonates was analyzed for UDP-glucuronyltransferase-1 polymorphism and G6PD deficiency. Either one alone did not increase the incidence of hyperbilirubinemia, but in combination they did.

242. Bancroft JD, Kreamer B, Gourley GR: Gilbert syndrome accelerates development of neonatal jaundice. *J Pediatr* 132:656–660, 1998.

Gilbert's syndrome is usually diagnosed after puberty and has an unknown impact on neonatal jaundice. In 151 infants transcutaneous bilirubin measurements were taken, and genomic analysis of the UGT1A promoter of the gene for bilirubin UDP-glucuronosyltransferase (BUGT) was performed to evaluate for polymorphisms associated with Gilbert's syndrome (A$(TA)_7$*TAA polymorphism). The 13% of subjects homozygous for the Gilbert-associated polymorphism had a greater increase in jaundice versus heterozygotes or those without the polymorphism in the first 2 days of life.*

266. Bosma PJ, Chowdhury JR, Bakker C, et al: The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N Engl J Med* 333:1171–1175, 1995.

The coding and promoter regions of the gene for bilirubin UDP-glucuronosyltransferase 1 were sequenced in patients with Gilbert's syndrome, Crigler-Najjar type II, and normal subjects. Abnormalities were noted in the promoter region of the gene in those with Gilbert's, encoding two extra bases in the TATAA element. This promoter region abnormality appeared to be necessary but not sufficient for complete manifestation of the Gilbert's syndrome.

295. Bartlett MG, Gourley GR: Assessment of UGT polymorphisms and neonatal jaundice. *Semin Perinatol* 35:127–133, 2011.

This is a review of the current understanding of genetic polymorphisms of bilirubin conjugation that lead to Gilbert's syndrome and Crigler-Najjar syndrome. In white populations, a $(TA)_7$*TAA repeat in the promoter region of the UGT1A1 is a necessary although not sufficient condition for GS. In Asian populations, mutations have been identified in exon 1 of the UGT1A1 gene rather than the promoter region. In Crigler-Najjar, functional UGT1A1 is absent in type I, or markedly reduced in type II.*

399. Kawade N, Onishi S: The prenatal and postnatal development of UDP-glucuronyltransferase activity towards bilirubin and the effect of premature birth on this activity in the human liver. *Biochem J* 196:257–260, 1981.

Liver UDP-glucuronyltransferase activity was studied in a number of human subjects and divided into four developmental phases of middle fetal, late fetal, neonatal, and mature. Activity was significantly lower in the early phases compared to the mature phase (0.1%, 0.1% to 1%, and 1% to 100% respectively).

404. Johnson L, Brown AK: A pilot registry for acute and chronic kernicterus in term and near-term infants. *Pediatrics* 104:736, 1999.

A pilot kernicterus registry from 21 states was analyzed, and characteristics that put infants at risk for kernicterus were identified. It identifies the reemergence of kernicterus in a small subset of otherwise healthy-appearing newborns due to early disharge, and lack of mandated early follow-up, as well as lactation counseling.

415. Maisels MJ, Newman TB: Kernicterus in otherwise healthy, breast-fed term newborns. *Pediatrics* 96:730–733, 1995.

Cases of neonatal hyperbilirubinemia alleged to be responsible for brain damage were reviewed in apparently healthy, full-term newborns. Six infants were inluded in analysis and had no evidence of hemolysis, and no other cause identified, other than breastfeeding.

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CHAPTER 5

Hemostasis in the Newborn and Infant

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CHAPTER OUTLINE

ONTOGENY OF THE HUMAN HEMOSTATIC SYSTEM
Coagulation Factors
Natural Inhibitors of Coagulation
Vitamin K–Dependent Factors
The Fetal and Neonatal Fibrinolytic System
D-Dimers
von Willebrand Factor
Platelets
Blood Vessel Wall: Age and Anticoagulant Properties
Maternal Preeclampsia and Fetal Hemostasis
Summary of Hemostatic Differences between Neonates and Older Children and Adults
CLINICAL ASPECTS OF DEVELOPMENTAL HEMOSTASIS
Hemorrhagic Disorders in the Neonate
Thrombotic Disorders in the Neonate
CONCLUSION

Development of the human hemostatic system begins in utero and continues until well after birth. As a result, functional levels of many of the procoagulants, coagulation inhibitors, fibrinolytic components, and platelet-associated factors differ from those of older children and adults, with "adult" values often not reached until about 6 months of age. These unique aspects of fetal and neonatal coagulation were first fully documented in the pioneering work of Dr. Maureen Andrew in the 1980s and have been termed *developmental hemostasis*. A firm understanding of these differences, as well as access to the "normal" ranges of various hemostatic factors in infants at different gestational and postnatal ages, is critical to proper interpretation of laboratory tests performed on newborns and infants. Despite broad differences from adults in functional levels of hemostatic factors, healthy newborns rarely have difficulties with hemorrhage or thrombosis. Thus these differences in prothrombotic and antithrombotic factors are uniquely balanced in the neonate and fetus and represent a normal physiologic state. However, this system provides little reserve, which often contributes to significant morbidity in sick and preterm infants. In addition to these developmental aspects of hemostasis, many inherited bleeding and clotting disorders can present in the newborn period. This chapter describes the normal development of the hemostatic system and reviews disorders of hemostasis and thrombosis that can occur in the newborn period. Data tables listing normal ranges of hemostasis laboratory tests for term and preterm infants are included in the Appendix. For additional discussion of the investigation and management of neonatal hemostasis and thrombosis, see several recent evidence-based guidelines.[1,2]

ONTOGENY OF THE HUMAN HEMOSTATIC SYSTEM

Coagulation Factors

The biochemistry and function of the individual coagulation factors are reviewed in detail in Chapter 27. Coagulation proteins do not effectively cross the placental barrier and are independently synthesized by the fetus.[3-15] Messenger RNA (mRNA) transcripts for coagulation factors VII, VIII, IX, and X and fibrinogen are detectable at about 5 weeks of gestation in hepatocytes of the embryo (Fig. 5-1).[16] By 10 weeks of gestation, plasma protein levels of most factors are measurable but range from 10% of the normal adult level for factor IX to about 30% for most of the other coagulation factors.[17] In general, these levels continue to increase gradually in parallel with gestational age (Appendix Table A5-1). However, with the exception of fibrinogen and factors V, VIII, and XIII, these levels remain considerably below the normal adult range, even at the time of birth for full-term infants.[17,18-23,24,25] They then continue to gradually increase and reach the normal adult range by about 6 months of age (Appendix Tables A5-2 and A5-3). Premature infants have an accelerated increase in levels after birth and, in

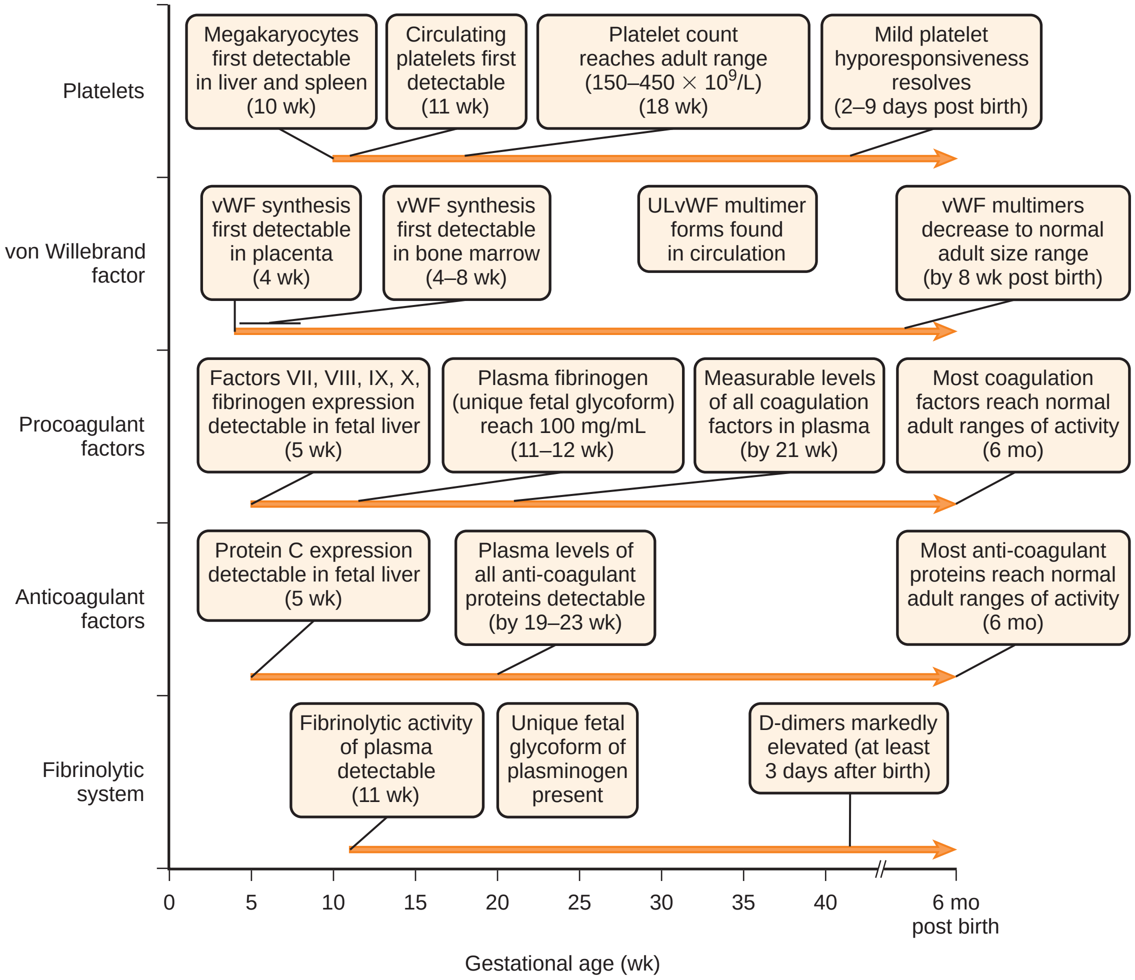
Platelets
Megakaryocytes first detectable in liver and spleen (10 wk)
Circulating platelets first detectable (11 wk)
Platelet count reaches adult range (150–450 × 10⁹/L) (18 wk)
Mild platelet hyporesponsiveness resolves (2–9 days post birth)
von Willebrand factor
vWF synthesis first detectable in placenta (4 wk)
vWF synthesis first detectable in bone marrow (4–8 wk)
ULvWF multimer forms found in circulation
vWF multimers decrease to normal adult size range (by 8 wk post birth)
Procoagulant factors
Factors VII, VIII, IX, X, fibrinogen expression detectable in fetal liver (5 wk)
Plasma fibrinogen (unique fetal glycoform) reach 100 mg/mL (11–12 wk)
Measurable levels of all coagulation factors in plasma (by 21 wk)
Most coagulation factors reach normal adult ranges of activity (6 mo)
Anticoagulant factors
Protein C expression detectable in fetal liver (5 wk)
Plasma levels of all anti-coagulant proteins detectable (by 19–23 wk)
Most anti-coagulant proteins reach normal adult ranges of activity (6 mo)
Fibrinolytic system
Fibrinolytic activity of plasma detectable (11 wk)
Unique fetal glycoform of plasminogen present
D-dimers markedly elevated (at least 3 days after birth)
0
5
10
15
20
25
30
35
40
6 mo post birth
Gestational age (wk)

Figure 5-1 Development of the hemostasis system in human embryos and newborns. Reported detection or other key developmental changes that occur in the different components of the hemostatic system during gestation and the first 6 months of postnatal life are presented in this schematic timeline. *ULVWF*, ultralarge von Willebrand factor multimers. *(Modified from Manco-Johnson MJ: Development of hemostasis in the fetus.* Thromb Res *115[Suppl 1]:55–63, 2005.)*

general, "catch up" to the full-term infant range by 3 months' postnatal age (Appendix Table A5-4).[26] True reference ranges for extremely premature infants (<30 weeks' gestation) are not available, because the majority of these infants have postnatal complications. Appendix Table A5-5 provides reference ranges for older children.[22] The nearly normal "adult" levels of fibrinogen and factors V and VIII in neonates make them useful markers for the investigation of possible consumptive coagulopathy or hemophilia A.

Regulation of fetal coagulation protein levels occurs by both transcriptional and posttranscriptional mechanisms.[16,27] In addition, ratios of hepatocyte levels to plasma levels of many factors are relatively high in comparison with adults, suggesting that delayed hepatocyte release may also contribute to the relatively low coagulation factor levels in fetuses and newborns.[28]

Qualitative differences in procoagulant proteins also exist between neonates and adults. Fibrinogen from fetuses has increased sialic acid content, increased phosphorus content, and different chromatographic profiles compared with fibrinogen from adults.[29-32]

Natural Inhibitors of Coagulation

Like many of the procoagulant factors, functional levels of most coagulation inhibitors in fetuses and neonates are significantly lower than those in adults (Appendix Tables A5-6 and A5-7; see also Appendix Table A5-3).[17,19-21] Awareness of these differences is critical to proper interpretation of laboratory studies obtained during evaluation of neonatal thrombosis. Most coagulation inhibitors reach the adult range of activity by 6 months of age.

As reviewed in Chapter 27, at least three mechanisms/pathways exist for inhibition of procoagulant activity: (1) cleavage of factors V and VIII by the protein C/S system; (2) direct inhibition of thrombin by antithrombin (AT), heparin cofactor, and α_2-macroglobulin; and (3) inhibition of factor VIIa by the tissue factor pathway inhibitor (TFPI)/factor Xa complex. When thrombin binds to thrombomodulin on endothelial surfaces, it no longer cleaves fibrinogen but it activates protein C. Activated protein C, in complex with protein S, proteolytically inactivates factors V and VIII. At birth, plasma concentrations of protein C are quite low and remain decreased during the first 6 months of life.[18,20,21,26] Neonates also have about a twofold higher amount of the single-chain form of protein C than adults do, and they may exhibit increased protein C glycosylation. Despite these alterations, there is no evidence that protein C from neonates differs functionally from that of adults when compared at equivalent concentrations.[33,34]

In adults, protein S circulates in either a free form or in a form that is bound to the carrier protein C4b binding protein. In fetuses and neonates, circulating protein S is almost entirely of the free type because levels of C4b binding protein are extremely low.[35,36] Thus the protein S activity-to-antigen ratio is greater for fetuses and neonates than for adults. Nonetheless, both total and free protein S levels are lower in fetuses and neonates. They rise gradually after birth, with the adult range of free protein S reached by 4 months and total protein S by about 10 months.[1] The interaction of protein S with activated protein C in the plasma of newborns may be modulated by increased levels of α_2-macroglobulin.[37]

Healthy full-term newborns have lower functional AT levels than adults do, with an average of 0.55 U/mL based on several studies.[13,14,19,20,38-41] This is most likely due to a reduced amount of absolute AT protein inasmuch as most studies have found no significant difference in the antigen-to–functional activity ratio or the immunoelectrophoretic or chromatographic properties of purified AT from neonates and adults.[13,38,41,42] The reduced AT levels in neonates probably account, at least in part, for the relative heparin resistance seen in this age group. AT levels typically reach the adult range by 3 months of age in full-term healthy infants. Preterm infants with respiratory distress syndrome (RDS) have significantly lower levels of AT, typically less than 0.2 U/mL, and the AT present often has additional dysfunctional properties.[38,43-46] Lower AT levels correlate with increased catheter-related thromboses, intracranial hemorrhages (ICH), and mortality rates.

Like AT, levels of heparin cofactor II are also substantially lower in infants than adults.[47] A circulating fetal dermatan sulfate proteoglycan has been described that has natural anticoagulant properties mediated through heparin cofactor II.[48] This fetal anticoagulant is also present in the plasma of pregnant women and is produced by the placenta. The length of time that it circulates in newborns is not known; however, it is still present during the first week of life in sick premature infants with RDS.

α_2-Macroglobulin is a more important inhibitor of thrombin in plasma from newborns than in adults.[49] It compensates, in part, for the low levels of AT and heparin cofactor in newborns, even in the presence of endothelial cell surfaces.[49,50] However, the overall rate of thrombin inhibition is still lower in newborns than adults.[49]

TFPI binds to factor VIIa/tissue factor complexes in a factor Xa calcium-dependent reaction that results in the inhibition of factor VIIa. After generation of small amounts of thrombin, TFPI prevents further generation of thrombin via tissue factor/factor VIIa. TFPI levels in cord plasma have been reported to be approximately 50% of adult values.[51]

Vitamin K–Dependent Factors

Factors II, VII, IX, and X and proteins C, S, and Z undergo posttranslational γ-carboxylation dependent on vitamin K (VK) of certain glutamic acid residues (see Chapter 27). Typically, 9 to 12 glutamic acid residues, often clustered within a 45–amino acid region referred to as the "Gla" domain, are modified.[28,52] γ-Carboxylation of glutamic acids enhances calcium-dependent phospholipid interactions of the coagulation factors and helps regulate the coagulation cascade. Because of the association of relative VK deficiency and hemorrhagic disease of the newborn (HDN; see detailed description later), development of these VK-dependent factors in utero has been the focus of considerable research. During gestation, a steep gradient of VK concentration is maintained across the placenta, with fetal levels being 10% or less of maternal levels.[28,53-58] This gradient is even further enhanced during the third trimester.[54] Consequently, VK stores in newborns are low, as shown by low levels of VK in the cord blood and liver of aborted fetuses and increases in VK-dependent coagulation factor activity following VK administration to neonates.[53,56,59,60] The teleologic explanation for maintenance of low fetal VK levels is not known. However, VK has been shown to promote DNA mutagenesis in vivo. For this reason, several investigators have suggested that maintenance of low fetal VK levels might be a mechanism to prevent chromosomal damage during the rapid cellular proliferation of embryogenesis.[61-63] In addition to these low VK levels, deficiency of VK reductase activity (a rate-limiting step in the reaction) as a result of immaturity of the hepatocyte may contribute to inefficient γ-carboxylation of coagulation factors.[28] In fact, undercarboxylated prothrombin and protein C have been detected in the cord and peripheral blood of up to 7% and 27% of healthy term infants, respectively, and these levels correlate with gestational age.[64,65] Even when corrected for this relative VK deficiency, levels of VK-dependent factors are still considerably lower than the normal adult range because the values presented in Appendix Tables A5-1 to A5-4, A5-6, and A5-7 were measured in infants who received VK prophylaxis at birth.

VK-dependent glutamic acid carboxylation also occurs on proteins outside the coagulation system, many of which play roles in development. Such proteins include Gas-6, the ligand for the receptor kinases Sky and Axl; the bone growth–related and calcification-related factors osteocalcin and matrix Gla protein; the urine calcium binding protein nephrocalcin; and PRPG1 and PRPG2, two proline-rich predicted signaling proteins expressed in the central nervous system and endocrine tissues.[52,65-67] Thus VK is predicted to have pleiotropic effects on development.

Although VK is maintained at low levels in the fetus, further deficiency of VK during gestation can reduce functional levels of the VK-dependent coagulation factors to an even greater extent. Maternal use of certain anticonvulsant medications, including phenytoin, phenobarbital, valproic acid, and carbamazepine, is associated with an increased risk of VK deficiency in the fetus and neonate.[28,68] Fetal or neonatal bleeding has been linked to maternal use of these medications on rare occasions. Fetal VK deficiency can be prevented in such cases by antenatal oral administration of 10 mg of VK_1 daily to the mother beginning at 36 weeks' gestation.[69]

Warfarin is a potent VK antagonist commonly used for long-term anticoagulation. It crosses the placenta and inhibits VK-dependent carboxylation in the fetus. Its use during pregnancy at 6 to 12 weeks of gestation is associated with an embryopathy syndrome consisting of limb deformities, nasal hypoplasia, ophthalmologic malformations, mental retardation, hypotonia, and ear abnormalities.[70] Warfarin exposure during the second or third trimester is associated with fetal wastage and central nervous system anomalies. The developmental defects related to warfarin use during pregnancy are thought to be due to bleeding into the developing limb buds or effects on bone and cartilage growth from inhibition of bone-associated Gla proteins, or both.

The Fetal and Neonatal Fibrinolytic System

The enzyme plasmin degrades polymerized fibrin and is responsible for clot dissolution. It is generated from its precursor plasminogen by two known activators, tissue plasminogen activator (tPA) and urokinase plasminogen activator. These activators, in turn, are inhibited by the plasminogen activator inhibitors PAI-1 and PAI-2. In addition, plasmin itself is inhibited by the circulating blood protein α_2-antiplasmin. See Chapter 28 for a more detailed description of the fibrinolytic system. In newborns, plasminogen levels are only 50% of adult values, α_2-antiplasmin levels are 80% of adult values, and plasma concentrations of PAI-1 and tPA are significantly greater than adult values (Appendix Table A5-8).[18,20,21,26,71,72,73,74] The increased levels of tPA and PAI-1 found on day 1 of life contrast markedly with values obtained from cord blood, which are significantly lower than adult levels. The discrepancy between newborn and cord plasma concentrations of tPA and PAI-1 may be explained by the enhanced release of both proteins from the endothelium shortly after birth. PAI-2 levels are detectable in cord blood but are significantly lower than they are in pregnant women.[75] Unique glycoforms of plasminogen with an increased content of mannose and sialic acid exist in the fetus.[76-78] This fetal form of plasminogen is less efficiently converted to plasmin by tPA compared with adult plasminogen.[76] No significant differences exist in activation kinetics by urokinase plasminogen activator.[76]

D-Dimers

D-dimers are the breakdown product of fibrin mesh cross-linked by factor XIII. Neonates have markedly elevated D-dimer levels in comparison with older children and adults that persist for at least 3 days after birth (Appendix Table A5-9).[19] These high levels suggest activation of the coagulation system at birth or decreased clearance of D-dimers in neonates, or both.

von Willebrand Factor

von Willebrand factor (VWF) is a large protein that mediates adhesion of platelets to exposed subendothelium and is involved in platelet aggregation (see Chapter 26). Its synthesis can first be detected as early as 4 weeks of gestation in placental endothelial cells and by 4 to 8 weeks in bone marrow.[28,79,80] In contrast to other coagulation factors, VWF functional levels are considerably elevated in neonates in comparison with the normal adult range (see Appendix Tables A5-2 and A5-5). They gradually decline to the normal adult range by about 3 to 6 months. It is important to keep these physiologically elevated levels in mind when using VWF as a marker of inflammation in infants.

VWF is released from endothelial cells and megakaryocytes as large multimers with molecular weights in the millions-of-dalton range. They are then cleaved into smaller multimers by the metalloproteinase ADAMTS-13. Unusually large VWF (ULVWF) multimers are VWF multimer forms larger than those found in normal plasma. These ULVWF multimers are 10 to 20 times more active in shear stress–induced platelet aggregation and bind more avidly to the extracellular matrix of fibroblasts than do the VWF multimer forms found in normal plasma.[81,82] In older children and adults, the presence of ULVWF plasma forms is the hallmark of thrombotic thrombocytopenic purpura, a microangiopathic disorder. It is due to deficiency, acquired or congenital, of ADAMTS-13. ULVWF forms are consistently found in platelet-poor plasma (PPP) from fetuses less than 35 weeks' estimated gestational age (EGA) and most fetuses older than 35 weeks' EGA, as well as in umbilical cord blood.[83,84] These forms are not seen in simultaneously sampled maternal PPP, indicating that the ULVWF forms are fetal specific. The ULVWF in fetal PPP is similar to the VWF directly released from endothelial cells. This similarity may be explained by the finding of low VWF cleaving protease (ADAMTS-13) activity in many neonates.[85,86] Serial monitoring of PPP from neonates shows a gradual reduction in ULVWF to the normal size range within 8 weeks after birth.[84] The presence of ULVWF during fetal development is probably physiologically relevant because plasma derived from umbilical cord blood shows increased shear stress–induced and ristocetin-induced VWF platelet-binding activity in comparison with plasma derived from adults, even when adult platelets are used in the assay.[87] The enhanced hemostatic efficacy of ULVWF may therefore partially balance the low functional levels of other coagulation factors to achieve reliable hemostasis in the fetus and newborn.

Platelets

Megakaryocytes During Fetal Development

In humans, megakaryocytes are first detectable in the liver and spleen by 10 weeks of gestation.[88] By 30 weeks, megakaryocytes are present in bone marrow and actively contribute to thrombopoiesis. Circulating megakaryocyte progenitors are detectable in the blood of neonates, and their number decreases as a function of postconceptional age (gestational age plus days of life).[89] Neonatal megakaryocytes have distinct properties compared with adult megakaryocytes, including increased thrombopoietin sensitivity, increased proliferation, lower ploidy, and increased cytoplasmic maturation for the degree of ploidy.[90] The differences in megakaryocyte size resolve by about 4 years of life.[91]

Platelet Number, Size, and Survival During Development

In humans, circulating platelets are first detected by 11 weeks' gestational age.[88] Thereafter, the plasma platelet concentration rises rapidly and reaches the adult range of 150 to 450 × 10^9/L by about 18 weeks of gestation.[21,28,72,88,92-98] During the earliest phase of platelet production, mean platelet volume is larger than the adult range.[28] However, platelet volume "normalizes" soon thereafter and reaches the adult range of 7 to 9 fL.[99,100] It is possible that the larger platelets arise from a unique wave of "primitive" megakaryopoiesis that occurs early in gestation, as demonstrated in several mouse studies,[101,102] but this mechanism remains to be determined in humans. Platelet survival has not been measured in healthy infants. In rabbits, survival of ^{111}In-oxine–labeled platelets is similar in adult and newborn rabbits.[103] However, in humans, levels of reticulated platelets, which represent those recently released from megakaryocytes, are elevated about twentyfold in comparison with adults.[104] Concentrations of thrombopoietin (TPO), the major cytokine for megakaryocyte progenitor proliferation and development, are increased about twofold in fetal versus adult plasma.[105] These findings suggest possible increased platelet turnover in human fetuses versus adults.

Platelet Structure

Peripheral blood platelets from newborns contain similar numbers of platelet-specific granules as platelets from older children and adults; however, serotonin and adenosine diphosphate (ADP), which are stored in dense granules, are present at concentrations of less than 50% of adult values.[106] Newborn platelets contain normal levels of the receptors glycoprotein Ib (GPIb) (part of the VWF binding complex), GPIa/IIa, P-selectin, and HPA-1a. Variable levels of GPIIb/IIIa have been reported, with some studies indicating significantly reduced expression.[107,108] Functional epinephrine receptors are significantly diminished in newborn platelets.[109]

Platelet Function

Platelet aggregometry and other functional assays of neonatal and cord blood platelets have shown variable results, but most demonstrate a modest hyporesponsiveness to ADP, epinephrine, collagen, and thrombin when compared with platelets from older children and adults (Fig. 5-2).[107,110-118] The hyporesponsiveness spontaneously resolves by 2 to 9 days after birth in full-term infants.[119] Extremely low birth weight neonates (<1 kg, gestation < 30 weeks) have markedly hyporesponsive platelets.[114,120] This impaired platelet response may contribute to the propensity of extremely low birth weight neonates for ICH. The platelet hyporesponsiveness improves over the

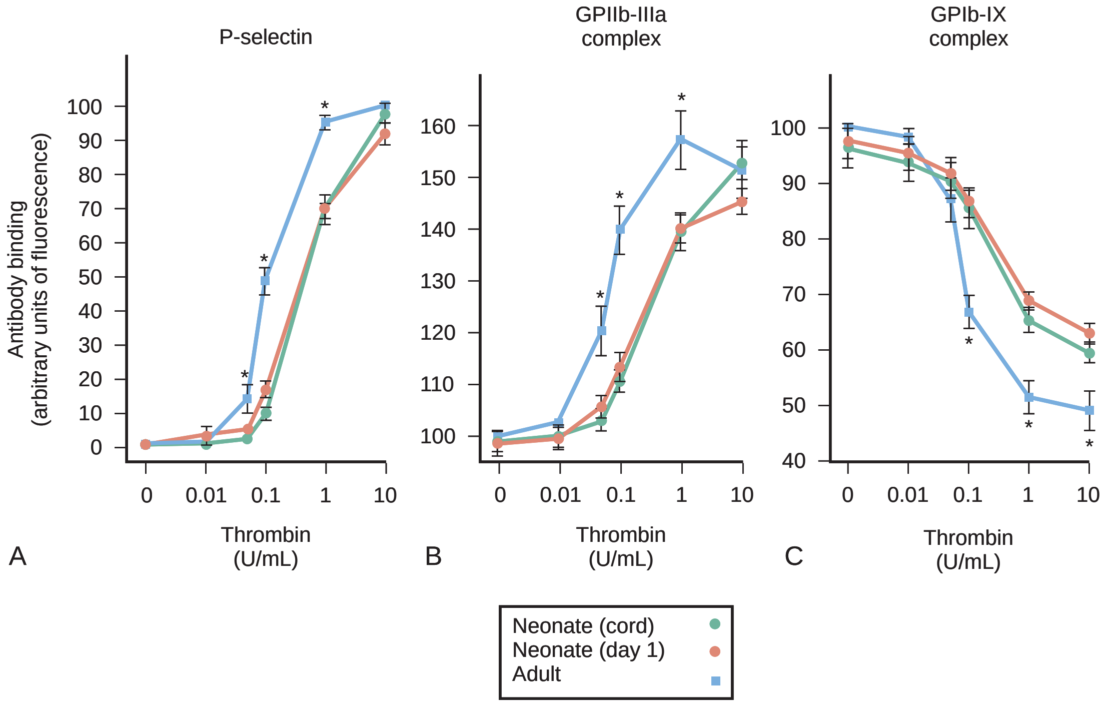

Figure 5-2 Effect of thrombin on the surface expression of P-selectin, the glycoprotein (GP) IIb/IIIa complex, and the GPIb/IX complex on neonatal and adult platelets in whole blood. Results were similar for cord blood and neonatal day 1 platelet values. Expression of P-selectin and GPIIb/IIIa complexes was decreased in newborns, whereas GPIb/IX expression was relatively well preserved in newborns in comparison with adults after stimulation with thrombin. Data are expressed as means ± SEM; $N = 20$. *Asterisks* indicate $P > .05$ for both cord blood and day 1 neonatal platelets in comparison with adult platelets. *(Redrawn from Rajasekhar D, Kestin AS, Bednarek FJ, et al: Neonatal platelets are less reactive than adult platelets to physiological agonists in whole blood.* Thromb Haemost *72:957–963, 1994.)*

first 10 to 14 days after birth, but platelet reactivity does not reach adult levels even by day 14.[114] Several explanations have been proposed to account for this hyporesponsiveness, including impaired calcium channel transport and signal transduction,[121-124] decreased availability of α-adrenergic receptors because of either delayed receptor maturation or occupation by catecholamines released during birth,[106,110,111,113,121,125-128] and the presence of inhibitory placenta-derived prostaglandins.[129]

In contrast to the agonists just described, agglutination in response to low concentrations of ristocetin is significantly enhanced in fetal and newborn platelet-rich plasma in comparison with that of adults,[106] probably partly because of high VWF levels and the presence of ULVWF forms in newborn plasma.[83,84,121,130-132] In the end, the hyporesponsiveness to certain platelet agonists and the hyperresponsiveness to VWF appear to balance each other out inasmuch as most healthy term infants do not experience bleeding or thrombotic complications in the newborn period.

Platelet Activation During the Birth Process

There is strong evidence that platelets are activated during the birth process. Cord plasma levels of thromboxane B_2, β-thromboglobulin, and platelet factor 4 are increased; the granular content of cord platelets is decreased; and epinephrine receptor availability is reduced, perhaps as a result of occupation.[133-136] The mechanisms of activation are probably multifactorial and include thermal changes, hypoxia, acidosis, adrenergic stimulation, and the thrombogenic effects of amniotic fluid.

Blood Vessel Wall: Age and Anticoagulant Properties

The endothelium fulfills a complex role in hemostasis in that it prevents thrombotic complications under physiologic conditions and promotes fibrin formation when injured. One of the anticoagulant properties of endothelial cell surfaces is mediated by lipoxygenase and cyclooxygenase metabolites of unsaturated fatty acids. Prostacyclin (prostaglandin I_2) production by cord vessels exceeds that by blood vessels in adults.[137] A second endothelial cell–mediated antithrombotic property is promotion of AT neutralization of thrombin by cell surface proteoglycans. Structurally, there is evidence that the vessel wall glycosaminoglycans of the young differ from those of adults.[138,139] In a rabbit venous model, increased amounts of glycosaminoglycans are seen in the inferior venae cavae of rabbit pups as compared with adults. Similarly, in the rabbit arterial model, greater glycosaminoglycan-mediated AT activity is seen in rabbit pups than in adult rabbits.[140,141]

Maternal Preeclampsia and Fetal Hemostasis

Preeclampsia is a pregnancy-associated syndrome consisting of maternal hypertension and proteinuria. Alterations in maternal coagulation and fibrinolysis, including reduced AT and PAI-2 levels and increased tPA antigen, PAI-1 antigen, and fibrin degradation product levels, have been described in women with preterm preeclampsia.[142-146] This suggests a state of enhanced thrombin generation and fibrinolysis in the maternal circulation. Analysis of cord blood from infants born to mothers with preeclampsia shows no statistical difference in the levels of these factors versus those of age-matched controls born to mothers without preeclampsia, thus indicating a probable protective effect of the placenta on the fetal circulation.[142,147]

Summary of Hemostatic Differences Between Neonates and Older Children and Adults

In summary, the salient differences in the hemostatic system of neonates and older children or adults are (1) decreased plasma concentrations of many of the procoagulant proteins, including factors II, VII, IX, X, XI, and XII, prekallikrein, and high–molecular-weight kininogens; (2) a unique fetal glycoform of fibrinogen; (3) decreased plasma concentrations of the coagulation inhibitors AT, heparin cofactor II, TFPI, protein C, and protein S, with a concomitantly slower rate of thrombin inhibition; (4) a unique glycoform of plasminogen that is less efficiently converted to plasmin by tPA; (5) markedly elevated D-dimer levels until at least 3 days after birth; (6) increased plasma VWF concentrations and elevated levels of circulating ULVWF multimers; (7) smaller and more proliferative megakaryocytes; and (8) modest, transient hyporesponsiveness of platelets to certain agonists such as collagen and epinephrine, but increased agglutination with low-dose ristocetin. In general, most of these differences resolve within the first 6 months of life.

CLINICAL ASPECTS OF DEVELOPMENTAL HEMOSTASIS

Hemorrhagic Disorders in Neonates

Although acquired disorders are more commonly seen, severe forms of congenital factor deficiencies or platelet disorders can present in early infancy and should be seriously considered in otherwise healthy infants who are bleeding.[28,43,148-151] About 15% to 30% of children with inherited bleeding disorders have hemorrhage in the neonatal period.[152,153] In addition, a third of new cases of severe hemophilia represent new mutations and are therefore not accompanied by any antecedent family history.[153]

Clinical Findings

The clinical manifestations of bleeding disorders are different in newborns than in older children and adults. Bleeding may appear as oozing from the umbilicus, bleeding into the scalp, large cephalhematomas, bleeding after circumcision, bleeding from peripheral phlebotomy sites, and bleeding into the skin. A small but important proportion of infants are seen with ICH as the first manifestation of their bleeding tendency.[151,154-160] Sick infants can bleed from mucous membranes, the bladder, and sites of invasive procedures. Joint bleeding is rare. The most common causes of bleeding in healthy infants are thrombocytopenia secondary to transplacental passage of a maternal antiplatelet antibody, sepsis, VK deficiency, and congenital coagulation factor deficiencies.

Laboratory Evaluation

In addition to a workup for sepsis, laboratory evaluation of infants with bleeding complications should include determination of the prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin clotting time (TCT), fibrinogen level, and platelet count. As discussed earlier, results need to be compared with the expected range of values for neonates because some may not reflect the normal adult range. It is also critical to apply age-specific reference ranges generated using the same analyzer and reagents because these can influence the measured absolute levels.[161] Tests that provide exogenous AT should be avoided because they do not reflect the normal physiologic state of low AT levels in neonates. Values obtained from cord blood may be different from those obtained from neonatal peripheral blood samples.[32] Abnormalities in test results usually prompt additional tests, such as specific factor assays. For a male child in whom hemophilia A or B is suspected, specific factor assays should be performed regardless of the APTT value. The PT, APTT, and TCT are not prolonged in those with factor XIII and α_2-antiplasmin deficiency. Therefore levels of these factors must be measured directly if deficiencies are suspected.

Sample Collection. Collection of samples for laboratory investigation of hemostatic defects in neonates, especially premature infants, presents particular challenges because of the patient's small size and increased hematocrit. Specialized microcollection tubes (1 mL) should be available in the neonatal unit. Blood sampling techniques should avoid contamination with intravenous fluids and heparin and should also avoid activating the coagulation system. Slow transit time and contact with plastic tubing are most likely to cause activation. All neonatal samples should be inspected for the presence of fibrin clumps before processing, and those containing clumps should not be used for analysis. Ideally, the volume of anticoagulant in the sample should be based on the volume of plasma, not the total volume of blood, because the increased hematocrit of neonates causes accentuated dilution of the coagulation factors if not taken into account.[1] This is particularly important for neonates with very high hematocrit values, such as those with cyanotic congenital cardiac disease. Overfilled or underfilled samples should not be analyzed.

Screening Coagulation Tests in Neonates. Screening tests of plasma clotting activity, including the PT, APTT, and TCT, are all prolonged during fetal development and in neonates when compared with the normal adult range (see Appendix Tables A5-1 to A5-3), probably because of physiologic "deficiencies" of the VK-dependent factors and contact factors and the presence of fetal glycoforms of fibrinogen.[1] The absolute values for these tests, as well as specific coagulation factor tests, depend on the reagents and analyzer used. Appendix Table A5-3 provides reference ranges using the STA Compact analyzer and Diagnostica Stago reagents, a system currently used in many clinical laboratories.[19] In contrast to some earlier systems, such as the ACL system, the STA Compact analyzer is not affected by plasma bilirubin levels, which are frequently elevated in neonates. Reference ranges using the Behring Coagulation System (BCS) and the Sysmex CA-1500 System with various reagents have also recently been published.[20] The differences observed underscore the need for each clinical laboratory to establish its own normal age-related reference ranges.[19]

Bleeding Time. The bleeding time has traditionally been used as an in vivo test of platelet function, particularly for testing adhesion to injured vasculature.[130] However, it is subject to considerable operator-dependent variability and is not routinely used in newborns. Automated devices modified for newborns and children are available and have been standardized.[162] In contrast to the prolonged coagulation screening tests, multiple studies have shown that bleeding times in infants during the first week of life are significantly shorter than those in adults.[121,130-132,162,163] This somewhat paradoxic finding can be explained by several mechanisms, including higher plasma concentrations of VWF,[18,21,26] enhanced function of VWF because of a disproportional increase in ULVWF multimers,[83,84] large red cells,[164] and high hematocrits.[165]

Platelet Function Analyzer. The Platelet Function Analyzer 100 (PFA-100) system provides an in vitro method of assessing primary platelet-related hemostasis by measuring the time (closure time) required for a platelet plug to occlude a microscopic aperture cut into a membrane coated with collagen and either epinephrine or ADP. It uses the patient's own platelets and plasma. The PFA-100 system is attractive for use in neonates because of the small volume required, rapidity of testing, and standardization of testing cartridges. Like the bleeding time, most studies of neonates' peripheral or cord blood report PFA-100 closure times, for both epinephrine and ADP, that are shorter than those for older children and adults (Appendix Table A5-10).[1,166-170] This is probably due to similar mechanisms as described earlier for the shortened bleeding time.[167] Ranges for premature infants have been reported and show an inverse relationship between ADP closure time and gestational age.[170] Although the PFA-100 system may eventually become a standard screening test for platelet function in neonates, older children, and adults, its clinical utility has not yet been clearly established.[171] Thus further studies will be required to determine the optimal method of assessing primary hemostasis in newborns and children, particularly premature infants.

Hereditary Coagulation Factor Deficiencies

For most hemostatic components, both severe and mild forms of deficiency can occur, with severe deficiencies often characterized by significant bleeding in newborns. Chapters 31 and 32 discuss hereditary factor deficiencies in detail.

Inheritance. Deficiencies of factors II, V, VII, XI, and XII, prekallikrein, and high–molecular-weight kininogen are rare autosomally inherited disorders, with consanguinity present in many families. Deficiencies of factor XII, prekallikrein, and high–molecular-weight kininogen do not result in hemorrhagic complications and thus are not considered further here. Deficiencies of factors VIII and

IX are sex linked and the most common congenital bleeding disorders to present in newborns. Rarely, combined deficiencies of factors II, VII, and IX and/or factors V and VIII may also present in the neonatal period.[172-174] Tests are available to diagnose most congenital factor deficiencies prenatally so that either termination of pregnancy or management of affected infants can be planned (see Chapters 31 and 32).

Clinical Findings. In the majority of newborns with congenital coagulation factor deficiencies, bleeding does not occur in the perinatal period unless a hemostatic challenge is present. On the other hand, unexplained bleeding in an otherwise healthy newborn should be carefully investigated because it may be due to a congenital coagulation factor deficiency.

ICH is rare in full-term newborns and usually occurs either spontaneously or after insults such as birth asphyxia or trauma, VK deficiency, or severe congenital factor deficiencies. Other risk factors include low birth weight, young gestational age, and ethnic background.[175-183] The risk for ICH in children with severe hemophilia A or B ranges from 2% to 8%. The location of the ICH is most commonly the subarachnoid area, but subdural and parenchymal bleeding also occurs. Head ultrasound may not detect small parafalcine or subdural bleeding, so computed tomography or magnetic resonance imaging is the preferred modality when deficiency of a coagulation factor is suspected.[184] Some infants require surgical intervention, and many have long-term neurologic deficits.

Full-term infants with unexplained ICH should be carefully evaluated for congenital or acquired hemostatic defects. Unfortunately, the diagnosis of ICH may be delayed because of the nonspecific nature of the early clinical findings, including poor feeding, lethargy, apnea, vomiting, and irritability. Further delays can result when secondary coagulopathies such as disseminated intravascular coagulation (DIC) occur or because of confusion related to the physiologically low levels of some coagulation factors in newborns.[185,186] The more extreme clinical manifestations of ICH are usually recognized early and include seizures, meningismus, and a tense fontanelle.

Although severe deficiency of factor VIII is the most common cause of ICH from a coagulation factor deficiency, severe congenital deficiencies of fibrinogen and factors II, V, VII, VIII, IX, X, XI, and XIII can also cause ICH at birth.[187] The incidence of ICH in newborns is unknown and is probably changing because of improvements in perinatal care. The widespread use of ultrasound, a safe modality for monitoring fetuses at risk, has resulted in the detection of ICH in utero. In utero factor replacement has been accomplished in several infants. Though less common than ICH, subgaleal bleeding with concurrent shock and DIC may be the initial manifestation of a congenital factor deficiency.[188]

Diagnosis. In newborns, the diagnosis of many congenital factor deficiencies based on plasma concentrations can be difficult because of neonates' physiologically low levels at birth (see Appendix Tables 5-2 and 5-3). Mild to moderate hereditary deficiencies of factors II, VII, IX, X, and XI result in plasma concentrations that may overlap with the normal neonatal range. In contrast, plasma concentrations resulting from either mild to moderate factor VIII deficiency or severe deficiency of factors V, VII, VIII, IX, X, and XIII can easily be distinguished from physiologic values. Prenatal diagnosis of most hereditary factor deficiencies is performed by amniocentesis or chorionic villus biopsy and is largely confined to severe hemophilia A and B, although deficiencies of factors V, VII, and XIII and VWF have also been diagnosed prenatally.[10,11,189-191]

Treatment. In the presence of active bleeding or a planned hemostatic challenge, the fundamental principle of management is to increase the plasma concentration of the deficient coagulation protein to a minimal hemostatic level. This target level varies, depending on the protein and nature of the hemostatic challenge (see Chapters 31 and 32). When possible, recombinant protein products or detergent-treated pooled donor preparations should be used to reduce the risk of infectious complications. Most products available for the treatment of older children and adults can be used in neonates. Although it has not been well studied, the half-lives and volumes of distribution of recombinant factor VIII (rFVIII) and recombinant factor IX (rFIX) appear to be similar in full-term newborns and older children, so standard initial dosing regimens can be used. However, it is critical to monitor trough levels closely and make appropriate dose modifications to ensure achievement of the proper target level. There are a few case reports of extremely premature infants (<1500 g or <30 weeks' EGA) having a slightly shortened half-life of factor VIII (about 6 hours vs. 8 to 12 hours in full-term infants and older children).[192,193]

Fresh frozen plasma (FFP) should be given for bleeding emergencies in neonates in whom a coagulation defect is suspected but a specific factor deficiency has not yet been documented. There are case reports of a small number of infants (<4 months) treated with recombinant factor VIIa (rFVIIa) for catastrophic bleeding of varying causes.[194-196] However, rFVIIa treatment of patients without congenital factor VII deficiency or hemophilia with high-titer inhibitors should be considered investigational at this time. Infants with known or suspected deficiencies in coagulation factors should receive vaccinations by the subcutaneous rather than the intramuscular route, and arterial puncture should be avoided when possible.

Mode of Delivery. The optimal mode of delivery for infants of hemophilia carrier mothers has yet to be determined.[153,184] However, normal vaginal delivery appears to be generally safe for many babies with hemophilia.[184,197] Use of vacuum assistance is associated with an increased risk for significant cranial hematomas in newborns with hemophilia and should be avoided if possible.[153,184,197]

Specific Coagulation Factor Deficiencies

Fibrinogen Deficiency. Deficiency of fibrinogen is rare. Bleeding secondary to afibrinogenemia has been reported in newborns after circumcision or as umbilical stump bleeding and soft tissue hemorrhage, with some cases being fatal. Reported replacement therapies have included whole blood, cryoprecipitate, FFP, and fibrinogen concentrates.[198-201] One fibrinogen concentrate (Haemocomplettan HS, Centeon/Aventis Behring) is available in Europe and North America.

Factor II Deficiency. Deficiency of prothrombin is very rare. Reported bleeding complications in newborns include gastrointestinal bleeding and ICH.[202,203] Reviews of adult patients have reported bleeding after invasive events such as circumcision and venipuncture or as soft tissue hematomas.[199] Although FFP can be used as initial therapy, factor II concentrate or prothrombin complex concentrate (PCC) is the preferred replacement product.

Factor V Deficiency. Bleeding as a result of severe factor V deficiency has been reported in newborns. Clinical manifestations include ICH, subdural hematoma, bleeding from the umbilical stump, gastric hemorrhage, and soft tissue hemorrhage.[204-207] Replacement therapy includes whole blood and FFP. Although thrombotic complications occur in some patients with factor V deficiency, they have not been reported in newborns.[204-208]

Factor VII Deficiency. Severe factor VII deficiency (factor VII level <1%) usually causes significant bleeding equivalent to that seen in patients with severe hemophilia. Patients with factor VII levels greater than 5% generally have mild hemorrhagic episodes. The most commonly reported bleeding complication in newborns with congenital factor VII deficiency is ICH.[209,210] In a review of 75 patients with factor VII deficiency, ICH was observed in 12 (16%). In 5 (42%) of these 12 patients, ICH occurred in the first week of life, with a fatal outcome. Congenital factor VII deficiency may occur in infants with Dubin-Johnson syndrome[210,211] or Gilbert syndrome[212] in certain populations. Bleeding episodes secondary to factor VII deficiency can be treated with FFP, PCC, purified factor VIIa concentrates, or, preferably, rFVIIa.[194,209,210,213,214] Lower doses of rFVIIa (15 to 20 μg/kg) than those used for patients with severe hemophilia and high-titer inhibitors (90 μg/kg) are often sufficient to control bleeding in patients with factor VII deficiency.[215,216] However, this has not been carefully defined in prospective clinical trials. Moreover, dosing has not been optimized for neonates and infants. Caution should be used in combining treatment with rFVIIa and PPC because thrombotic complications have been reported.

Factor VIII Deficiency. The severity of factor VIII deficiency is determined by the plasma concentration of factor VIII, with a level of less than 1% being severe, 1% to 5% being moderate, and 5% to 50% being mild. Severe factor VIII deficiency is the most common inherited bleeding disorder of the neonatal period. In addition, a small number of neonates with moderate and mild hemophilia are seen after a hemostatic challenge.[156,158,217-219] Large cohort studies have revealed that approximately 10% of children with hemophilia have clinical symptoms in the neonatal period.[187] In this group of factor VIII–deficient children experiencing bleeding in the neonatal period, approximately 50% of bleeding episodes occur after circumcision, with almost 20% of patients suffering intracranial bleeding; some deaths also occur. In contrast to older infants and children, bleeding into joints is extremely rare in neonates.[220] Severe factor VIII deficiency can, on rare occasion, occur in female infants as a result of homozygosity or compound heterozygosity for an FVIII gene mutation[221] or skewed lyonization.[222,223] Management of bleeding secondary to factor VIII deficiency is discussed in detail in Chapter 31.

Factor IX Deficiency. Classification of the severity of factor IX deficiency is identical to that used for factor VIII deficiency. Diagnosis of milder forms of factor IX deficiency is complicated by physiologic levels of factor IX that can be as low as 0.15 U/mL and, in rare infants, by the potential for concurrent VK deficiency. Bleeding after circumcision and ICH can occur in neonates with factor IX deficiency.[156,158,217,224,225] Management of bleeding secondary to factor IX deficiency is discussed in detail in Chapter 31.

Factor X Deficiency. Severe factor X deficiency can be manifested as bleeding in the newborn period.[226-230] ICH was present in a high proportion of reported cases, several of which were fatal. Additional sites of bleeding that have been noted include umbilical, gastrointestinal, and intraabdominal sites. Whole blood, FFP, factor X concentrate, and PCC have been used as replacement therapy.

Factor XI Deficiency. Severe deficiency of factor XI is rare. It is different from other coagulation protein deficiencies in that bleeding symptoms are not necessarily tightly correlated with factor plasma concentrations. Bleeding complications as a result of severe factor XI deficiency were reported in two newborns. One bled after circumcision at the age of 3 days and had a factor XI level of 0.07 U/mL. In another newborn, factor XI deficiency with bilateral subdural hemorrhage was diagnosed prenatally.[231-233] Either FFP or cryoprecipitate can be used for the treatment of factor XI–deficient patients if factor XI concentrate is not available.

Factor XIII Deficiency. Severe factor XIII deficiency is typically manifested at birth as bleeding from the umbilical stump or ICH.[157,234-243] Other clinical manifestations of homozygous factor XIII deficiency include delayed wound healing, abnormal scar formation, and recurrent soft tissue hemorrhage with a tendency to form hemorrhagic cysts. ICH occurs even in the absence of trauma in approximately a third of all affected patients.[153] Newborns with heterozygous factor XIII deficiency are not clinically affected.

FFP, cryoprecipitate, or factor XIII concentrates can be used for the treatment of factor XIII–deficient patients. Newborns with known factor XIII deficiency should receive a prophylactic regimen of factor XIII because of the high incidence of ICH. Plasma concentrations of factor XIII greater than 1% are effective, and the very long half-life of factor XIII permits once-per-month therapy. Therefore prophylactic replacement therapy consists of either small doses of FFP (2 to 3 mL/kg) administered every 4 to 6 weeks, cryoprecipitate at a dose of 1 bag/10 to 20 kg of weight every 3 to 6 weeks, or, preferably, factor XIII concentrate at a dose of 10 to 20 U/kg every 4 to 6 weeks, depending on the clinical situation and the preinfusion plasma concentration of factor XIII.[157,234-245]

Hereditary Deficiencies of Multiple Coagulation Factors. Hereditary deficiencies of two or more coagulation proteins have been reported for 16 different combinations of coagulation factors.[172,246,247] Combined deficiency of factors V and VIII has been described in patients with mutations in the genes encoding LMAN1 (also called ERGIC-53) and MCFD2.[174,248] Administration of FFP is usually the initial therapy. Subsequent treatment varies, depending on the specific factors affected.

Hemorrhagic Disease of the Newborn

Historical Background. HDN, as first described by Townsend in 1894, consists of hemorrhage on days 1 through 5 of life from multiple sites in otherwise healthy infants.[249] Subsequently, a causal link between HDN and abnormal blood coagulation was established.[72,250,251] Initial treatment of infants with HDN consisted of intravenous, intramuscular, or subcutaneous injection of blood or serum. Even at this very early time, the difficulty of separating treatment response from spontaneous improvement was recognized. Later, a randomized, controlled trial showed that intramuscular injection of blood was not helpful in preventing the abnormalities in blood coagulation that occurred during the first week of life.[250] The link between VK deficiency and spontaneous hemorrhage was first recognized in chicks in 1929.[252] The association between VK deficiency and HDN quickly followed, as subsequently did treatment of infants with HDN.[72,253-255]

The next historical step was recognition of the link between decreased prothrombin activity and increased PT on days 2 to 4 of life in the absence of prophylactic VK. Prothrombin activity was observed to return to normal by days 5 to 7 of life.[72,256-259] These observations led to the hypothesis that VK administered prophylactically could prevent HDN.[256,257,260-262] There was uniform agreement that the prophylactic administration of VK to mothers or infants prevented the decrease in prothrombin activity during the first 3 to 4 days of life.[72,255,256] On the basis of these studies, VK prophylaxis was widely recommended. Subsequently, the scientific basis for this policy became less clear for several reasons. First, plasma concentrations of other coagulation proteins in addition to prothrombin were shown to be low in newborns. Second, there was increasing recognition that bleeding in neonates was often not due to VK deficiency.[72,251,263-265] Third, administration of high amounts (50 to 70 mg) of a water-soluble form of VK resulted in hemolytic anemia with kernicterus in some infants.[72,266,267] Fourth, many clinicians suggested that VK prophylaxis was not needed for all healthy full-term infants.[265,268-270] Consequently, VK prophylaxis was suspended in some countries, and recurrence of HDN ensued.[72,257,271-282] Most of the controversy concerning prophylactic use of VK can be explained by the design of the trials and subsequent interpretation of their results. The best evidence comes from large randomized, placebo-controlled trials. Such trials have consistently shown a statistically significant benefit from VK prophylaxis in terms of clinical bleeding.[283-285] No randomized, controlled trials with large enough sample size have shown that prophylactic VK does not prevent bleeding.

Prophylactic Vitamin K Administration. VK exists in three forms: VK_1 (phytonadione), which is present in leafy green vegetables; VK_2 (menaquinone), which is synthesized by intestinal bacterial flora; and VK_3 (menadione), which is a synthetic, water-soluble form. VK_3 is rarely used in newborns because at high doses it causes hemolytic anemia, which results in jaundice and potential morbidity.[72,266,267] The recommendations for VK prophylaxis in many countries are similar.[55] Daily requirements of VK are approximately 1 to 5 μg/kg of body weight for newborns.[55] Most groups recommend a single dose of 0.5 to 1 mg intramuscularly or an oral dose of 2 to 4 mg at birth, with subsequent dosing for breastfed infants. Oral VK prophylaxis is preferable to parenteral prophylaxis; studies have shown that oral administration of VK is as effective, less expensive, and less traumatic than intramuscular administration in preventing the classic signs and symptoms of VK deficiency.[55] However, orally administered VK_1 or VK_3 may not be as effective as intramuscularly injected VK in the prevention of late VK deficiency.[286] Strategies for preventing late VK deficiency include repeat administration of oral VK[281,287] or continuous low-dose VK supplementation.[281] A mixed micelle oral VK_1 preparation that is readily absorbed has been tested in children.[55] However, optimal dosing of this preparation to prevent late VK deficiency bleeding remains to be determined.[288]

In addition to general prophylaxis at birth, patients in certain risk groups require additional VK prophylaxis (e.g., infants with α_1-antitrypsin deficiency, chronic diarrhea, cystic fibrosis, or celiac disease). Pregnant women receiving oral anticonvulsant therapy should take 10 mg of oral VK_1 daily beginning at 36 weeks' gestation for the prevention of overt VK deficiency in their infants at birth.[69]

In 1990, a cohort study aimed at determining perinatal risk factors for childhood cancer reported an association between drugs administered in the peripartum period (maternal pethidine or neonatal VK) and childhood cancer (odds ratio, 2.6).[289] A subsequent case-control study by the same group found no association between maternal pethidine and childhood cancer; however, they again reported an increased risk of childhood cancer after neonatal intramuscular VK administration (odds ratio, 1.97).[290] In contrast, a later large case-control study of 2530 children with cancer, one ecologic study, and one metaanalysis have shown no association between VK administration and childhood cancer.[291,292] The current consensus of the American Academy of Pediatrics Committee on Fetus and Newborn is that the risk for increased childhood cancer is probably minimal, if any, and the benefits of VK prophylaxis in terms of reduced bleeding are substantial.[293]

Bleeding Caused by Vitamin K Deficiency in the Newborn. In the absence of prophylactic VK, the incidence of vitamin K–dependent bleeding (VKDB) ranges from 0.25% to 1.7%.[72,283] Infants are at greater risk for hemorrhagic complications from VK deficiency than similarly affected adults are because their plasma concentrations of VK-dependent factors are physiologically

TABLE 5-1 Forms of Bleeding Resulting from Vitamin K Deficiency in Infancy

Parameter	Early Form	Classic Form	Late Form
Age	<24 hours	2-7 days	0.5-6 months
Causes and risk factors	Medications during pregnancy Anticonvulsants Oral anticoagulants (rifampin, isoniazid) Antibiotics (rarely, idiopathic or hereditary)	Breastfeeding Inadequate VK intake	Marginal VK content in breastmilk resulting from low VK intake and absorption Cystic fibrosis Diarrhea α_1-Antitrypsin deficiency Hepatitis Celiac disease
Localization in order of occurrence	ICH Gastrointestinal region Umbilicus Intraabdominal region Cephalhematoma	ICH Gastrointestinal region Umbilicus ENT region Injection sites Circumcision	ICH (>50%) Gastrointestinal region Skin ENT region Injection sites Urogenital tract Intrathoracic region
Occurrence without VK prophylaxis	Very rare	1.5% (1/10,000 births)	4-10/10,000 births*
Prophylaxis	Discontinue or replace offending medications Maternal VK prophylaxis	Adequate VK supply Early and adequate breastfeeding Formula VK prophylaxis	Adequate VK supply Adequate breastfeeding Formula VK prophylaxis†

ENT, Ear, nose, and throat region; *VK*, vitamin K.
*More common in Southeast Asia.
†A single intramuscular injection is better than a single oral dose; repeated small doses are closer to physiologic conditions.

decreased.[18,21,26,72,282,294] The clinical manifestation of VKDB can be classified as classic, early, or late on the basis of the timing and type of complications (Table 5-1). Classic VKDB initially occurs on days 2 to 7 of life in breastfed, healthy full-term infants.[64,72,277,294-297] Causes include poor placental transfer of VK,[54,56-58] marginal VK content in breastmilk (<20 mg/L), inadequate milk intake, and a sterile gut.[72] VKDB rarely occurs in formula-fed infants because commercially available formulas are supplemented with VK.[298,299] How often classic VKDB occurs in the absence of VK prophylaxis depends on the population studied, the supplemental formula, and the number of mothers breastfeeding. Early VKDB develops in the first 24 hours of life and is linked to maternal use of specific medications that interfere with VK stores or function, such as some anticonvulsants.[300-302] Late VKDB occurs between weeks 2 and 8 of life and is linked to disorders that compromise ongoing VK supply.[303,304]

Laboratory Diagnosis of Vitamin K Deficiency in the Newborn. Laboratory tests used to detect VK deficiency include screening coagulation tests, specific factor assays, measurement of decarboxylated forms of VK-dependent factors, PIVKA (protein induced by VK antagonists) assays, and direct measurements of VK levels. The results of these tests must always be compared with values for age-matched, healthy, non–VK-deficient infants to distinguish physiologic and pathologic deficiencies.[65,305]

Treatment. An infant suspected of having VK deficiency should be treated immediately with VK while laboratory confirmation is awaited. All infants with VKDB should be given VK either subcutaneously or intravenously, depending on the clinical problem. VK should not be given intramuscularly to infants with VKDB, because large hematomas may form at the site of the injection. Absorption of subcutaneously administered VK is rapid, and its effect occurs only slightly slower than that of systemically administered VK. Intravenous VK should be given slowly and with a test dose because it may induce an anaphylactoid reaction. Infants with major bleeding because of VK deficiency should also be treated with plasma products to rapidly increase levels of VK-dependent proteins. Plasma is the product of choice for treatment of a non–life-threatening hemorrhagic event, whereas PCCs should be considered for the management of life-threatening bleeding.

Liver Disease

The coagulopathies associated with liver disease in newborns are similar to those of adults and reflect the failure of hepatic synthetic function. However, in newborns this problem may be superimposed on the already “physiologic” immaturity of the coagulation system, activation of the coagulation and fibrinolytic systems, poor clearance of activated coagulation factors, and loss of hemostatic proteins into ascitic fluid.[306] Secondary effects of liver disease on platelet number and function also occur in newborns.[307,308] Common causes of hepatic dysfunction in newborns include viral hepatitis, hypoxia, total parenteral nutrition, shock, and fetal hydrops. Rare causes include genetic diseases such as α_1-antitrypsin deficiency, galactosemia, and tyrosinemia.

Laboratory abnormalities induced by acute liver disease include prolongation of the PT and low plasma concentrations of several coagulation proteins, including fibrinogen.[309,310] However, it is important to compare these levels with those of normal age-matched controls (see Appendix Tables A5-1 to A5-3). Fibrinogen is present at adult levels in newborns and may be a useful marker. Chronic liver failure with cirrhosis is also characterized by a coagulopathy and mild thrombocytopenia.[311-314] Secondary VK deficiency may occur as a result of impaired absorption from the small intestine, particularly in those with intrahepatic and extrahepatic biliary atresia.[315] Patients with clinical bleeding may benefit temporarily from replacement of coagulation proteins with FFP, cryoprecipitate, or exchange transfusion. However, without recovery of hepatic function, replacement therapy is futile. VK should be administered to infants in whom cholestatic liver disease is suspected.

von Willebrand Disease

Although von Willebrand disease (VWD) is the most common inherited bleeding disorder, patients with the disease rarely present in the neonatal period. Plasma concentrations of VWF are increased in neonates, as is the proportion of ULVWF multimers.[83,84] Nonetheless, severe deficiency (type 3) and some qualitative VWF disorders (type 2) have been reported to present in the newborn period.[316-319] Management of VWD is discussed in detail in Chapter 31. Use of arginine vasopressin in the newborn period is contraindicated because of associated excess free water retention.

Platelet Disorders

Quantitative Platelet Disorders. The definition of thrombocytopenia in newborns is the same as that in adults: a platelet count less than $150 \times 10^9/L$. Consequently, platelet counts less than $150 \times 10^9/L$ are abnormal and indicate the need for investigation and, sometimes, treatment. Mean platelet volumes in newborns are similar to those of adults (range, 7 to 9 fL).[96-98] Postnatally, the mean platelet volume increases slightly over the first 2 weeks of life, concomitantly with an increase in platelet count.[99,100]

Epidemiology. Thrombocytopenia is the most common hemostatic abnormality in newborns admitted to neonatal intensive care units (NICUs).[93,95,97,98,320] A single prospective cohort study[97] and six retrospective reviews[98,321-325] have provided the most reliable information on the occurrence, natural history, mechanisms, and clinical impact of thrombocytopenia in newborns. There is general agreement that thrombocytopenia indicates the presence of an underlying pathologic process; however, the clinical relevance of mild thrombocytopenia is unknown. Thrombocytopenia develops in approximately 22% to 35% of infants and approximately 75% of extremely low birth weight neonates admitted to the NICUs of tertiary hospitals.[97,326,327] In some infants the thrombocytopenia is trivial, with platelet counts between 100 and 150 $\times 10^9/L$. However, in 50% of affected infants, platelet counts decrease to less than $100 \times 10^9/L$, and in 20% of infants, platelet counts are lower than 50 $\times 10^9/L$. What constitutes a safe platelet count in a neonate remains to be rigorously determined,[327-329] but many neonatal units attempt to keep the platelet count higher than $50 \times 10^9/L$, although this figure may vary, depending on the postconceptional age of the infant and comorbid conditions.

Pathogenesis. Causes of thrombocytopenia in neonates, like those in older children and adults, can be divided into disorders involving increased platelet destruction, decreased platelet production, or sequestration (Box 5-1). Although many of these causes overlap with those seen in older children and adults, several entities are unique to neonates or may occur in the newborn period and are considered in more detail here. Increased platelet destruction is the mechanism responsible for thrombocytopenia in most infants.[97,330] Characterization of mechanisms responsible for thrombocytopenia is important because they have practical implications in assessing the risk for bleeding and in guiding management.

Increased Platelet Destruction. Thrombocytopenia secondary to increased platelet destruction can be divided into immune and nonimmune causes.

Immune Thrombocytopenia. Immune thrombocytopenia is defined as an increased rate of platelet clearance caused by platelet-associated immunoglobulin G (IgG) or complement. It is the most common form of increased platelet destruction in newborns and should always be suspected in otherwise healthy infants with isolated severe thrombocytopenia. Neonatal immune thrombocytopenia can be further broken down into one of three processes: transplacental passage of a maternal antibody directed against a nonshared platelet antigen (termed alloimmune thrombocytopenia or, sometimes, isoimmune thrombocytopenia), transplacental passage of a cross-reactive maternal autoimmune-derived antiplatelet antibody, or generation of an autoreactive antiplatelet antibody by the newborn itself (termed autoimmune thrombocytopenia). The second can be distinguished from the other two by examining the mother for a low platelet count. Differentiation of autoimmune thrombocytopenia from alloimmune thrombocytopenia in neonates is critical because the management and severity of these disorders are quite different. Chapter 34 discusses these two forms of immune thrombocytopenia and their management in detail.

Neonatal Alloimmune Thrombocytopenia. Neonatal alloimmune thrombocytopenia (NAIT) is the most common cause of immune-mediated thrombocytopenia in the newborn period, with an estimated incidence of about 1 in 1000 to 5000 live births in white populations.[331-335] It arises when the mother, who lacks a common platelet antigen, is exposed to the antigen (inherited from the father) on neonatal platelets and generates a neutralizing IgG. This crosses the placenta and mediates premature clearance of fetal and newborn platelets. First-born infants can be affected. Frequently, the thrombocytopenia is severe. It is critical to make the diagnosis because the risk of life-threatening bleeding and central nervous system morbidity is high, estimated at up to 10% to 15% of cases. A useful diagnostic test is to assay the reactivity of the mother's serum with the father's platelets. If such samples are not available, it is possible to look for reaction of the mother's serum with the newborn's platelets or the presence of antiplatelet antibodies in the newborn's serum. The most common antigen is HPA-1a (formerly called Pl^{A1}), although other antigens, such as HPA-3a

Box 5-1 Causes of Thrombocytopenia in Newborns

- INCREASED DESTRUCTION OF PLATELETS
  - Immune-mediated destruction
    - Neonatal alloimmune thrombocytopenia
    - Maternal idiopathic thrombocytopenic purpura
    - Drug-dependent antibody (could be from maternal medication use)
    - Autoimmune thrombocytopenic purpura
  - Non–immune-mediated destruction
    - Pseudothrombocytopenia (platelet clumping) in vitro
    - Disseminated intravascular coagulation
    - Asphyxia and placental insufficiency
    - Perinatal aspiration
    - Necrotizing enterocolitis
    - Hemangiomas (Kasabach-Merritt syndrome)
    - Thrombosis (may be occult)
    - Respiratory distress syndrome
    - Maternal preeclampsia
    - Cardiopulmonary bypass (including extracorporeal membrane oxygenation [ECMO])
    - Familial thrombotic thrombocytopenic purpura
  - Other syndromes of destruction
    - Hyperbilirubinemia
    - Phototherapy
    - Polycythemia
    - Rh hemolytic disease
    - Total parenteral nutrition
- DECREASED PRODUCTION OF PLATELETS
  - Acquired disorders
    - Viral infection (e.g., human immunodeficiency virus, cytomegalovirus, rubella virus, human herpesvirus 6)
    - Congenital leukemia
    - Down syndrome–transient myeloproliferative disorder (DS-TMD)
    - Neuroblastoma
    - Histiocytosis
    - Osteopetrosis
  - Inherited disorders
    - *Disorders associated with small platelets*
      - Wiskott-Aldrich syndrome (WAS)
      - X-linked thrombocytopenia (associated with Wiskott-Aldrich syndrome protein [WASP])
    - *Disorders associated with normal-size platelets*
      - Thrombocyopenia with absent radii (TAR)
      - Amegakaryocytic thrombocytopenia with radioulnar synostosis (ALTRUS)
      - Congenital amegakaryocytic thrombocytopenia (CAMT)
      - Familial platelet disorder with propensity for development of acute myelogenous leukemia (FPD/AML)
      - ANKRD26-related thromobocytopenia (THC2)
      - Cytochrome C–related thrombocytopenia (CYCS)
    - *Disorders associated with large platelets*
      - X-linked macrothrombocytopenia with or without anemia (*GATA-1* mutations)
      - MYH9-related disorders (May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, Epstein syndrome)
      - Bernard-Soulier syndrome
      - Paris-Trousseau/Jacobsen syndrome
      - Velocardiofacial syndrome
      - Montreal platelet syndrome
      - Gray platelet syndrome
      - Platelet-type von Willebrand diseaes (pseudo–von Willebrand disease)
      - Filamin-related thrombocytopenia
      - Tubulin-related thrombocytopenia
      - Thrombocytopenia associated with sitosterolemia
    - *Other disorders*
      - Fanconi anemia
      - Metabolic disorders (e.g., methylmalonic acidemia, ketotic glycinemia, isovaleric acidemia)
      - Drug-mediated suppression
- SEQUESTRATION
  - Hypersplenism

(formerly called Bak^{a}), HPA-4a (formerly called Pen^{a} [more common in Asian populations]), HPA-5a (formerly called Br^{b}), and HPA-5b (formerly called Br^{a}), have been described.[336] Not all cases in which the pregnant mother generates allosensitive antiplatelet antibodies result in neonatal thrombocytopenia.

Treatment of NAIT includes transfusion with washed platelets donated by the mother. Because the mother's platelets lack the offending antigen, they should have a normal life span. Many blood banks also maintain registries of HPA-1a–negative donors who can be contacted for platelet donation in cases involving antibodies against HPA-1a. If antigen-negative platelets are not available, treatment with intravenous immunoglobulin, 1 g/kg as a single daily bolus for 1 or 2 consecutive days, along with random donor platelets if the patient is bleeding, is effective in reducing the rate of platelet destruction.[337-340] Endogenous platelet counts typically rise by 36 to 48 hours. The role of corticosteroids in the treatment of infants with NAIT is unclear, and they are typically not used as initial therapy. All infants with NAIT should undergo head ultrasonography or computed tomography to rule out ICH. The natural history of NAIT is gradual resolution of the thrombocytopenia over the first few months of life as the offending antibody is cleared. Close monitoring of the platelet count is indicated for at least the first month of life until the platelet count normalizes. The safety of breastfeeding in the setting of NAIT has not been rigorously studied. One case report suggested that breastfeeding did not significantly worsen the clinical course, even though the breastmilk contained antiplatelet IgG.[341]

Immune Thrombocytopenia Caused by Maternal Antiplatelet Autoantibodies. Maternal autoimmune antiplatelet IgG can cross the placenta and result in increased platelet destruction in the fetus. A common cause is maternal systemic lupus erythematosus. Like NAIT, the thrombocytopenia gradually resolves over the first few months of life as the maternal antibodies are cleared. In severe cases, use of intravenous immunoglobulin in the neonate may help reduce the rate of platelet destruction. Corticosteroids are of no clear utility. Drug-dependent antibodies can result in both maternal and neonatal thrombocytopenia.[125]

Autoimmune Thrombocytopenia. True autoimmune thrombocytopenia, in which neonates generate an antibody against their own platelets, is very rare in the newborn period. If present, it typically signals an underlying immune dysregulatory disorder. If heparin-induced thrombocytopenia is suspected, heparin therapy should be discontinued immediately and alternative forms of anticoagulation considered if necessary (see Chapter 34).

Nonimmune Thrombocytopenia

Sepsis and Disseminated Intravascular Coagulation. A common nonimmune cause of thrombocytopenia in the newborn period is sepsis, with or without DIC. Viral infections (rubella virus, herpesvirus, echovirus, cytomegalovirus, human herpesvirus 6, and human immunodeficiency virus), protozoal infections (Toxoplasma), and bacterial infections cause severe thrombocytopenia.[342] Mechanisms responsible for bacterial sepsis–induced thrombocytopenia are multifactorial and include consumption resulting from DIC, endothelial damage, platelet aggregation caused by binding of bacterial products to platelet membranes, and decreased production as a result of marrow infection.[343] Mechanisms responsible for virus-induced thrombocytopenia include loss of sialic acid from platelet membranes because of viral neuraminidase, intravascular platelet aggregation, and impaired megakaryopoiesis (see later). Congenital rubella causes thrombocytopenia in three quarters of infants, with platelet counts ranging from 20 to 60 × 10^9/L for the first 4 to 8 weeks of life.

In premature infants, thrombocytopenia often complicates other disorders such as RDS, persistent pulmonary hypertension, necrotizing enterocolitis, and hyperbilirubinemia treated by phototherapy. Activation of coagulation with platelet consumption occurs in RDS, and mechanical ventilation may be an independent factor contributing to thrombocytopenia.[344] It has been suggested that persistent pulmonary hypertension in newborns may be due in part to intrapulmonary platelet aggregation and the release of platelet-derived vasoactive substances such as thromboxane A_2.[345] Approximately half of infants with necrotizing enterocolitis are thrombocytopenic, with about 20% having laboratory evidence of DIC.

Exchange Transfusion, Hyperbilirubinemia, and Phototherapy. Intrauterine and exchange transfusions cause thrombocytopenia by a dilutional effect that depends on the amount of blood transfused.[322] After exchange transfusion, platelet counts increase within 3 days and reach preexchange levels by about 7 days.[43] Both hyperbilirubinemia and phototherapy are associated with mild thrombocytopenia in newborn humans and shortened platelet survival in rabbits.[330]

Hypoxia and Placental Insufficiency. Acute asphyxia is a consistent cause of DIC and thrombocytopenia.[103] Chronic hypoxia is associated with placental dysfunction, intrauterine growth retardation, and significant thrombocytopenia.

Vascular Malformations. Certain types of large vascular malformations are associated with Kasabach-Merritt syndrome, in which a local consumptive coagulopathy occurs as a result of abnormal endothelium. This causes hypofibrinogenemia, elevated levels of fibrinogen-fibrin degradation products, microangiopathic fragmentation of red cells, and thrombocytopenia (see Chapter 34).[346-350] The thrombocytopenia can be severe, with platelet counts lower than 50 × 10^9/L. Approximately 50% of affected infants experience systemic bleeding during the first month of life. Sometimes the vascular malformations are not apparent on physical examination and require imaging studies to detect.

Thrombosis and Familial Thrombotic Thrombocytopenic Purpura. Thrombocytopenia is loosely associated with thromboembolic complications and polycythemia in infants with hematocrit values greater than 70%. However, thrombocytopenia may also indicate the presence of other concurrent disease processes. Infants with a familial form of hemolytic-uremic syndrome or thrombotic thrombocytopenic purpura have a microangiopathic hemolytic anemia in association with transient neurologic or renal abnormalities (see Chapter 34).

Decreased Platelet Production. Thrombocytopenia secondary to decreased platelet production is rare and accounts for less than 5% of thrombocytopenic infants.[43,93-95] Causes include viral infections, drug-induced thrombocytopenia, congenital leukemia, Down syndrome transient myeloproliferative disorder (DS-TMD), neuroblastoma, histiocytosis, osteopetrosis, congenital amegakaryocytic thrombocytopenia (CAMT), thrombocytopenia with absent radius syndrome (TAR), other inherited genetic platelet disorders, and bone marrow failure syndromes.

CONGENITAL VIRAL INFECTION. Congenital viral infections with rubella virus, herpesvirus, echovirus, cytomegalovirus, human herpesvirus 6, and human immunodeficiency virus have been associated with thrombocytopenia in the newborn period, in part through deleterious effects on megakaryopoiesis and thrombopoiesis.

DRUG-INDUCED THROMBOCYTOPENIA. Transplacental passage of drugs or the use of drugs in neonates can cause thrombocytopenia via bone marrow suppression or the development of drug-dependent antibodies, or both.[125] However, these causes are rare, and evidence for them is weak.[351-354] Agents implicated are salicylates, quinine, hydralazine, tolbutamide, and thiazide diuretics.

CONGENITAL LEUKEMIA AND OTHER MALIGNANT DISEASES. Infantile leukemia, as well as other neonatal malignant diseases characterized by bone marrow infiltration, such as neuroblastoma, can be accompanied by thrombocytopenia in the newborn period. Typically, other hematopoietic lineages are affected and other clinical signs are apparent. An infantile form of myelofibrosis has been described in several families in Saudi Arabia.[355,356]

DOWN SYNDROME–TRANSIENT MYELOPROLIFERATIVE DISORDER. Some infants with Down syndrome (DS) (trisomy 21) are born with a transient myeloproliferative disorder (DS-TMD) characterized by leukocytosis, thrombocytopenia, predominance of circulating early erythromegakaryocytic precursor cells, pancytopenia, and, in some cases, severe liver fibrosis.[357] Remarkably, this myeloproliferation resolves spontaneously over the first few months of life. In about 20 % of symptomatic cases, acute megakaryocytic leukemia (DS-AMKL) develops within a few years.[358] The DS-TMD and DS-AMKL cells harbor acquired mutations in the gene encoding the erythromegakaryocytic transcription factor GATA-1.

Inherited Platelet Disorders. Though considerably less common than consumptive processes or viral suppression, inherited platelet disorders can occur in the newborn period and should be considered in the differential diagnosis of thrombocytopenia in a neonate. They are discussed in more detail in Chapter 30 (also see Box 5-1 for a complete listing). Several classifications, diagnostic algorithms, and management recommendations have been published.[359,360] Infants with aplastic disorders have the greatest risk of serious bleeding in the form of ICH in the first months of life. Brief descriptions of selected syndromic disorders with specific neonatal manifestations are given here.

Disorders with Small Platelets

WISKOTT-ALDRICH SYNDROME. Wiskott-Aldrich syndrome (WAS; OMIM 301000) is an X-linked disorder caused by deficiency of the Wiskott-Aldrich syndrome protein, which functions in actin dynamics in megakaryocytes and T lymphocytes. It is one of the few disorders characterized by small platelets. The small size of the platelets is not always apparent by standard automated measurements of blood cell mean platelet volume. Therefore if the diagnosis is being considered, it is imperative to evaluate platelet size by examination of the peripheral smear and to confirm small platelet volume with cytometer devices calibrated for platelets. The other clinical hallmarks of WAS are immunodeficiency (usually severe) and eczema. Bloody diarrhea is often seen and can be an initial sign. The immunodeficiency is frequently the most significant clinical problem associated with this disease. It is important to recognize the syndrome early so that appropriate interventions can be instituted, including prophylactic treatment against opportunistic infections such as Pneumocystis carinii infection. A destructive component of the thrombocytopenia in WAS is also likely because splenectomy increases platelet counts in a large number of patients. In addition, the thrombocytopenia can be exacerbated by development of immune thrombocytopenic purpura secondary to the immune dysregulation. Bone marrow transplantation is curative for WAS, and early diagnosis is helpful in initiating listing for transplantation before significant complications arise.

X-LINKED THROMBOCYTOPENIA. X-linked thrombocytopenia (OMIM 313900) is a less severe form of WAS that is manifested predominantly as thrombocytopenia. Typically, there is no or minimal immunologic dysfunction. However, more severe immunologic disturbances may develop in some patients over time. This clinical entity is distinct from X-linked macrothrombocytopenia, which is associated with germline mutations in GATA-1.

Disorders with Normal-Size Platelets

CONGENITAL AMEGAKARYOCYTIC THROMBOCYTOPENIA. Congenital amegakaryocytic thrombocytopenia (CAMT; OMIM 604498) is characterized by severe thrombocytopenia with a markedly reduced number or absence of megakaryocytes. Patients are typically seen shortly after birth with petechiae, bruising, or bleeding. Biallelic inactivating mutations in the gene for c-mpl, the receptor for the major megakaryocyte cytokine TPO, have been identified in a large number of patients. Interestingly, no patients have been reported to date with mutations in the TPO gene itself. Patients with severe CAMT are at high risk for the development of complete bone marrow failure, usually within the first few years of life.[361] Such failure is most likely due to a role of TPO signaling in hematopoietic stem cell function.[362,363] Bone marrow transplantation has been used successfully to treat patients with severe CAMT.

THROMBOCYTOPENIA WITH ABSENT RADII. Thrombocytopenia with absent radii syndrome (TAR; OMIM 274000) is characterized by hypomegakaryocytic thrombocytopenia and bilateral radial hypoplasia with the presence of thumbs.[364] Other hematopoietic lineages are not affected. Additional skeletal features may include shortening or, rarely, aplasia of the humerus and ulna. The lower limbs are frequently affected but to a lesser extent. Congenital cardiac disease and cow's milk intolerance have also been associated in some cases. The genetic basis of TAR has recently been clarified.[365-367] Common interstitial microdeletions of 200 kb on chromosome 1q21.1 have been detected in several families with TAR, and the key gene within this region is thought to be RBM8A, which encodes a subunit of the exon junction complex involved in RNA processing. Microdeletion or monoallelic null mutations in RBM8A are typically insufficient to cause disease. However, coinheritance of a low-frequency single-nucleotide polymorphism in an RBM8A gene regulatory sequence on the opposite allele is associated with disease penetrance.

Neither splenectomy nor steroids are beneficial for infants with TAR.[368,369] Platelet transfusions are highly effective but should be reserved for symptomatic infants because prophylactic platelet transfusions could result in refractoriness as a result of allosensitization.[369] By several months of age, increased numbers of megakaryocytes usually appear in the bone marrow, and platelet counts increase.[368,369] A functional platelet defect may be present in some children with TAR.[370]

AMEGAKARYOCYTIC THROMBOCYTOPENIA WITH RADIOULNAR SYNOSTOSIS. Amegakaryocytic thrombocytopenia with radioulnar synostosis (ATRUS; OMIM 605432) is a rare disorder characterized by reduced or absent megakaryocytes, severe thrombocytopenia, possible aplastic anemia, and proximal radioulnar synostosis leading to difficulty with forearm pronation. Sensorineural hearing loss may also be present. Mutations in the gene encoding the homeobox containing transcription factor HOXA11 have been found in some familial cases of ATRUS.[371,372]

Disorders with Large Platelets

PARIS-TROUSSEAU SYNDROME OR JACOBSEN SYNDROME. Paris-Trousseau syndrome (OMIM 188925) (also called Jacobsen syndrome; OMIM 147791) is a constitutional contiguous gene deletion disorder involving the long arm of chromosome 11 (11q23).[373,374] The constellation of findings in this syndrome includes severe congenital cardiac abnormalities, trigonocephaly, mental retardation, dysmorphogenesis of the hands and face, and macrothrombocytopenia.[375] The cause of the thrombocytopenia appears to be related to impaired platelet production because platelet survival time is normal.[376] These patients can also exhibit a predisposition to thrombosis, thus suggesting possible qualitative defects in platelet production. Examination of bone marrow reveals significant dysmegakaryopoiesis with an abundance of micromegakaryocytes and death of large numbers of megakaryocytes during the late stages of maturation.[377] Peripheral blood platelets contain giant alpha granules, which are thought to arise from aberrant alpha-granule fusion during prolonged residence in bone marrow.[378] The minimal chromosome regions deleted in Paris-Trousseau or Jacobsen syndrome associated with thrombocytopenia include genes for the Ets family transcription factors FLI-1 and ETS-1, both of which play roles in megakaryocyte development.[379] Lentiviral expression of FLI-1 in CD34+ cells from patients with Paris-Trousseau thrombocytopenia rescues megakaryocyte differentiation in vitro, thereby providing evidence that FLI-1 deficiency is the cause of impaired thrombopoiesis in these patients.[380]

VELOCARDIOFACIAL SYNDROME. Velocardiofacial syndrome (OMIM 192430) is a contiguous gene deletion disorder involving chromosome 22q11.2. Clinical manifestations include cleft palate, cardiac anomalies, typical facies, learning disabilities, and defective GPIb/IX/V function. The platelet effects are due to heterozygous loss of the GPIbβ gene, which resides in this region, and the condition should be considered a mild form of Bernard-Soulier disorder.[381-384]

HYPERSPLENISM. As in adults, thrombocytopenia resulting from hypersplenism is usually mild to moderate, with platelet counts ranging from 50 to 100 × 10^9/L in newborns.

Clinical Impact of Neonatal Thrombocytopenia. Clinically important bleeding is less likely to occur in patients with consumptive platelet disorders than in those with production defects. The bleeding risk is increased in patients who have both thrombocytopenia and a defect in platelet function. Selection of a platelet count at which one should intervene, though a simplistic response, provides a guideline for therapy. A platelet count less than 50 × 10^9/L places some otherwise healthy full-term newborns at risk for serious ICH.[385] The importance of "moderate" thrombocytopenia (platelet counts between 50 and 100 × 10^9/L) in sick premature infants has been a subject of controversy. A randomized, controlled trial assessed the potential benefits of platelet concentrate transfusions in 154 premature thrombocytopenic infants during the first 72 hours of life.[386] Treated infants received platelet concentrates to maintain platelet counts higher than 150 × 10^9/L. No beneficial effect on the incidence or extension of ICH was shown in this study, which was designed to detect an effect of 25% or greater. However, infants who received transfusions had shortened bleeding times and required significantly less blood product support. A retrospective study of 53 neonates with severe thrombocytopenia treated at a single NICU showed no significant difference in major hemorrhage in those transfused to maintain a platelet count greater than 30 × 10^9/L versus greater than 50 × 10^9/L.[325]

Treatment. Management of thrombocytopenic infants depends in part on the underlying disorder. If an infant is bleeding, a trial of platelet concentrates (10 to 20 mL/kg) is indicated. The increased platelet count usually shortens the bleeding time and is frequently clinically effective.[132] Autoimmune and alloimmune thrombocytopenia typically does not respond to random donor platelet concentrates and requires additional specific forms of therapy.

Thrombocytosis in the Newborn. Elevated platelet counts are frequently observed in premature infants at approximately 4 to 6 weeks after birth.[387] There are no clinical manifestations of neonatal thrombocytosis, and therapeutic intervention is not indicated.

Qualitative Platelet Disorders. Despite the physiologic hyporeactivity of neonatal platelets in response to exposure to some agents, healthy infants do not have an increased risk of bleeding. Pathologic impairment of platelet function may occur, to a variable extent, as a result of the use of certain drugs or the presence of pathologic states in either mothers or infants. In mothers, causative factors include the use of some drugs, diabetes,[388-391] dietary abnormalities,[392-397] smoking,[398-400] and ethanol abuse[401]; in infants, they include the use of some drugs, perinatal aspiration syndrome,[322,402-405] hyperbilirubinemia,[406,407] phototherapy,[407,408] renal failure,[409] and hepatic failure.

Phototherapy. When platelets are exposed to a broad-spectrum blue fluorescent light in vitro, aggregation is decreased and microscopic alterations in granules and external membranes occur.[410]

Medications

ASPIRIN. Salicylate crosses the placenta and can be detected in fetuses after maternal ingestion.[351-354,411-415] Clearance of salicylate is slower in newborns than in adults, and thus infants have a potential risk of bleeding for a prolonged time.[352] However, in vitro studies have not demonstrated increased sensitivity of newborn platelets to salicylates as compared with adult platelets,[134,413] and the evidence linking maternal aspirin ingestion to clinically important bleeding in newborns is weak.[354] There is little reason to have serious concerns about maternal ingestion of aspirin, but it is reasonable to advise mothers to not ingest aspirin unless specifically instructed by their physician.

INDOMETHACIN. Indomethacin is used for nonsurgical closure of a patent ductus arteriosus in premature infants, but it also has antiplatelet activities. Indomethacin, like salicylate, has a longer half-life in newborns than in adults (21 to 24 hours in newborns vs. 2 to 3 hours in adults).[416] This extended half-life is probably due to underdevelopment of hepatic drug metabolism or renal excretory function or to altered protein binding.[417,418] Indomethacin inhibits platelet function in newborns, as shown by prolongation of bleeding times.[419,420] Randomized, controlled trials have provided conflicting conclusions on the effect of indomethacin on intraventricular hemorrhage in premature infants.[421]

Maternal Diabetes. The reactivity of platelets from diabetic mothers and their infants is increased, as demonstrated by enhanced thromboxane B_2 production, enhanced platelet aggregation,[390,422] and a lower threshold to many aggregating agents.[422] The enhanced platelet function in diabetes is associated with increased synthesis of a prostaglandin E–like substance that crosses the placenta and affects the fetus.[423] Despite these in vitro findings, the evidence linking enhanced platelet reactivity to clinically significant thromboembolism in newborns of diabetic mothers is weak.[424,425]

Diet. Alterations in the diet of mothers or infants during the postnatal period can affect newborn platelet function. Increases in the ratio of polyunsaturated fatty acids to saturated fatty acids in the diet of mothers who breastfeed their infants result in increases in the concentration of linoleic acid and enhanced thromboxane B_2 production.[392] Infants receiving a diet deficient in essential fatty acids may exhibit arachidonic acid depletion and platelet dysfunction.[393,395] Vitamin E functions as an antioxidant and as an inhibitor of platelet aggregation/release in humans.[396,397,426,427] Instances of vitamin E–deficient infants with increased platelet aggregation that reversed after vitamin E supplementation have been reported.[397,428]

Amniotic Fluid. Amniotic fluid contains procoagulant activity that enhances the generation of thromboxane A_2 by platelets.[402-405] Infants in whom perinatal aspiration syndrome develops have pulmonary hypertension with platelet thrombi in the pulmonary microcirculation. The exact mechanism or mechanisms leading to persistent pulmonary hypertension in these infants is unknown.

Nitric Oxide. Nitric oxide prevents adhesion of platelets to endothelial cells and inhibits ADP-induced aggregation of cord platelets in a manner similar to that in adults.[429-432]

Extracorporeal Membrane Oxygenation. Extracorporeal membrane oxygenation (ECMO) permits transfer of oxygen into blood across a semipermeable membrane and is currently used for infants with life-threatening severe respiratory insufficiency. Underlying respiratory disorders include meconium aspiration syndrome, severe RDS, congenital diaphragmatic hernia, persistent pulmonary hypertension, and sepsis. Hemorrhage, particularly ICH, is one of the most serious complications of this technique. Hardart and Fackler[433] reported an overall incidence of new ICH of about 10% in infants included in the Extracorporeal Life Support Organization Registry (N = 4550) from 1992 through 1995. Of 1398 evaluable premature infants (born at <37 weeks' EGA) reported to the registry between 1992 and 2000, ICH developed in 13%.[434] Similar to that found with cardiopulmonary bypass, the increased risk of bleeding during ECMO is due mostly to the use of heparin in combination with other hemostatic defects, including significantly decreased plasma concentrations of coagulation factors and platelet dysfunction secondary to chronic activation.[435-437] Other recognized contributing factors include prolonged hypoxia, ischemia, general anesthesia, acidosis, sepsis, and treatment with epinephrine.[433,435,438-443] In premature infants, the risk for ICH with ECMO correlates inversely with postconceptional age.[434] Although anticoagulation is required for ECMO, the optimal use of heparin has never been tested in clinical trials.

Thrombotic Disorders in Neonates

Congenital Prothrombotic Disorders

Patients with heterozygous defects for recognized inherited prothrombotic disorders are rarely seen with their first thromboembolic complication during childhood unless another pathologic event unmasks the problem. In contrast, patients who are homozygotes or double heterozygotes for a congenital prothrombotic disorder are often initially encountered in the newborn period or during early childhood. The following discussion is limited to the unique aspects of these inherited deficiencies in newborns. Chapter 33 discusses congenital prothrombotic disorders in detail.

Homozygous Prothrombotic Disorders. Though rare, the most commonly reported homozygous prothrombotic disorder encountered during the newborn period is protein C deficiency.[444-466] Homozygous protein S deficiency is even less common. All patients seen in the newborn period had undetectable levels of protein C (or protein S), whereas children with a delayed manifestation had detectable levels ranging between 0.05 and 0.20 U/mL. Rare cases of homozygous AT deficiency have been reported in newborns.[467]

Clinical Findings. The classic clinical manifestation of homozygous protein C/protein S deficiency consists of cerebral or ophthalmic damage (or both) in utero, purpura fulminans within hours or days of birth, and, on rare occasion, large-vessel thrombosis. Purpura fulminans is an acute, lethal syndrome of DIC characterized by rapidly progressive hemorrhagic necrosis of the skin secondary to dermal vascular thrombosis.[468-471] The skin lesions start as small, ecchymotic sites that increase in radial fashion, become purplish black with bullae, and then turn necrotic and gangrenous.[468,470,471] The lesions develop mainly on the extremities but can occur on the buttocks, abdomen, scrotum, and scalp. They also occur at pressure points, at sites of previous puncture, and at previously affected sites. Moreover, affected infants have severe DIC with hemorrhagic complications. Large-vessel arterial thromboses have been described in a neonate with homozygous AT deficiency.[467]

Diagnosis. The diagnosis of homozygous protein C/protein S deficiency in infants is based on the appropriate clinical picture, a protein C/protein S level that is usually undetectable, a heterozygous state in the parents, and, ideally, identification of the molecular defect. The presence of very low levels of protein C/protein S in the absence of clinical manifestations and a family history cannot be considered diagnostic, because physiologic plasma levels can be as low as 0.12 U/mL. Molecular diagnosis is available for identified families (see Chapter 33).

Initial Treatment. The diagnosis of homozygous protein C/protein S deficiency is usually unanticipated and made at the time of clinical evaluation. Although numerous forms of initial therapy have been used, 10 to

20 mL/kg of FFP every 6 to 12 hours is usually the form of therapy that is most readily available.[471,472] Plasma levels of protein C achieved with these doses of FFP vary from 15% to 32% at 30 minutes after the infusion and from 4% to 10% at 12 hours.[455] Plasma levels of protein S (which was entirely bound to C4b) were 23% at 2 hours and 14% at 24 hours, with an approximate half-life of 36 hours.[473]

A protein C concentrate (Ceprotin) received approval by the Food and Drug Administration in 2007 for use in treating congenital protein C deficiency. A second plasma-derived concentrate (Protexel) is also available in Europe. Recommended initial doses for acute thrombotic episodes and short-term prophylaxis in patients with severe protein C deficiency are 100 to 120 IU/kg in neonates, with subsequent doses of 60 to 80 IU/kg every 6 hours and maintenance doses of 45 to 60 IU/kg thereafter every 6 to 12 hours. However, dosing should be adjusted to maintain trough protein C activity of 50%. After resolution of the acute event, the patient should continue on a dose to maintain the trough protein C activity level above 25% for the duration of treatment. Replacement therapy should be continued until all the clinical lesions resolve, which usually takes place at 6 to 8 weeks. In addition to the clinical course, plasma D-dimer concentrations may be useful for monitoring the effectiveness of protein C replacement.[35,471]

Long-Term Therapy. Modalities used for the long-term management of infants with homozygous protein C/protein S deficiency include oral anticoagulation therapy, replacement therapy with either FFP or protein C concentrate, or liver transplantation.[463] When oral anticoagulation therapy is initiated, replacement therapy should be continued until the international normalized ratio (INR) is at a therapeutic value so that skin necrosis can be avoided. The therapeutic range for the INR can be individualized to some extent but is usually between 2.5 and 3.5. Risks associated with oral anticoagulation therapy include bleeding with high INR values and recurrent purpuric lesions with low INR values. Frequent monitoring of INR values is required if these complications are to be avoided. Bone development should also be monitored because the long-term effect of warfarin use on the bones of young infants is unknown. Long-term use of low–molecular-weight heparin (LMWH) could be considered, but it also has the potential for osteopenia. Moreover, it is expensive and requires twice-daily subcutaneous injections.

Heterozygous Prothrombotic Disorders. Thromboembolic events related to heterozygous genetic prothrombotic disorders rarely occur in infants. When they do, a secondary, acquired insult is usually present. The few case reports in the literature describe a diversity of clinical manifestations that usually reflect the site of the thrombus. Purpura fulminans did not occur in any case.[474-480]

Treatment. Treatment consists of supportive therapy alone, anticoagulation with heparin, thrombolytic therapy, and replacement with specific factor concentrates.[481,482] Removal or treatment of the secondary acquired insult is important. For AT deficiency, AT concentrates were administered to four infants as either boluses or continuous infusions. Boluses of 52 and 104 U/kg of AT concentrate increased AT levels from 0.10 U/mL to 0.75 and 1.48 U/mL, respectively, at 1 hour.[482] At 24 hours, levels of AT had decreased to approximately 0.20 U/mL. A continuous infusion of AT concentrate at a rate of 2.1 U/kg/hr maintained a plasma level of 0.40 to 0.50 U/mL.

Acquired Prothrombotic Disorders

Symptomatic secondary thromboembolic complications occur more frequently in sick newborns than in children of any other age, with an incidence of approximately 2.4 per 1000 hospital admissions to the NICU.[483] Intravascular catheters are responsible for more than 80% of venous and 90% of arterial thrombotic complications.[483] Their contribution to thrombosis is probably multifactorial, including the presence of a foreign surface, endothelial cell damage, impairment of flow, and infusion of noxious substances. Renal vein thrombosis (RVT) is the most common form of non–catheter-related thrombosis. Other risk factors include increased blood viscosity because of a high hematocrit, poor deformability of physiologically large red cells, dehydration, and activation of the coagulation and fibrinolytic systems secondary to a variety of medical problems. Very low AT levels have been observed in preterm infants with RDS, which probably contributed to their increased risk for thromboembolic complications.[38]

Venous Catheter–Related Thrombosis. The use of umbilical venous catheters and other forms of central venous catheters is associated with a significant risk for thrombosis.[483-485] According to autopsy studies, 20% to 65% of infants who die with an umbilical venous catheter in place have an associated thrombus. Appropriate placement of umbilical venous catheters is critical to prevention of serious organ impairment, such as portal vein thrombosis and hepatic necrosis. Long-term sequelae of umbilical venous catheters have not been rigorously studied but include portal vein thrombosis with portal hypertension, splenomegaly, gastric and esophageal varices, and hypertension. Until recently, pulmonary embolism was rarely diagnosed in sick newborns because its clinical signs were easily confused with those of RDS. Ventilation lung scintigraphy and computed tomography pulmonary angiography have been used in newborns to facilitate the diagnosis of pulmonary embolism, but there are no studies to date that have determined their sensitivity and specificity in this setting.[486,487]

Arterial Catheter–Related Thrombosis. Seriously ill infants require indwelling arterial catheters, which are associated with a risk of thrombosis regardless of the vessel or catheter type chosen.[488-492] Catheter-related thrombosis not only occludes catheters but may also obstruct major arterial vessels. In a retrospective examination of approximately 4000 infants who underwent umbilical artery catheterization, 1% had severe symptomatic vessel obstruction. Asymptomatic catheter-related thrombi occur more frequently, as evidenced by postmortem (3% to 59% of cases) and angiographic (10% to 90% of cases) studies.

Diagnosis. Contrast-enhanced angiography is considered the reference test for the diagnosis of arterial thrombosis. Noninvasive techniques such as Doppler ultrasound offer advantages, but their sensitivity and specificity are unknown. A review of 20 neonates with aortic thrombosis treated in one institution revealed that ultrasound failed to identify thrombi in four patients, three of whom had complete aortic obstruction.[491]

Sequelae. The sequelae of catheter-related thrombosis can be immediate or long term. Acute symptoms reflect the location of the catheter and include renal hypertension, intestinal necrosis, and peripheral gangrene.[493] The long-term side effects of symptomatic and asymptomatic thrombosis of major vessels have not been studied but are probably significant.

Prophylaxis with Heparin. A low-dose continuous heparin infusion (3 to 5 U/hr) is commonly used to maintain catheter patency. The effectiveness of heparin was assessed in seven studies focusing on three outcomes: patency, local thrombus, and ICH.[489,490,492,494-496] Patency, which is probably linked to the presence of local thrombus, is prolonged with the use of low-dose heparin. Local thrombosis was assessed by ultrasound in two randomized studies. The evidence linking low-dose heparin prophylaxis to ICH in newborns is weak.[492,497,498] One study had a sample size of only 15 per arm[492]; another case-control study had a broad odds ratio that ranged from 1.4 to 11[497]; and a third study was a retrospective association analysis that may be confounded by other variables.[498] A randomized study involving 113 premature infants using a 1-U/hr continuous heparin infusion showed no difference in ICH.[499] Thus the magnitude of risk for ICH is uncertain. Heparin is used in at least three quarters of American nurseries.[493]

Renal Vein Thrombosis. RVT occurs primarily in newborns and young infants. Approximately 80% of cases occur within the first month and usually within the first week of life. In some infants RVT develops in utero. The incidence in male and female infants is similar, and the left and right sides are affected equally. Bilateral thrombosis occurs in 24% of pediatric RVT patients.

Clinical Findings and Etiology. The initial symptoms and clinical findings are different in neonates and older patients and are influenced by the extent and rapidity of thrombus formation. Neonates generally have a flank mass, hematuria, proteinuria, thrombocytopenia, and impaired function of the involved kidney. Clinical findings suggesting acute inferior vena cava thrombosis include cold, cyanotic, and edematous lower extremities. RVT results from pathologic states characterized by reduced renal blood flow, increased blood viscosity, hyperosmolality, or hypercoagulability.

Coagulation Abnormalities. The most common coagulation abnormality in RVT is thrombocytopenia, which is usually mild, with average values of 100×10^9/L. Thus RVT should be considered in the differential diagnosis of thrombocytopenia in neonates. Coagulation may be prolonged and levels of fibrinogen-fibrin degradation products increased. Children with RVT are often evaluated for a congenital prothrombotic disorder, although the significance of thrombophilic markers in this disease is unknown.[476]

Diagnosis and Treatment. Ultrasound is the radiographic test of choice for the diagnosis of RVT because of ease of testing and sensitivity to an enlarged kidney. Treatment options include supportive care, anticoagulation, and thrombolytic therapy. The use of anticoagulants and thrombolytic agents is controversial. Evidence-based guidelines have recently been published.[2] Either supportive care with serial radiographic monitoring or heparin therapy with therapeutic doses for 6 weeks to 3 months should be considered for unilateral RVT in the absence of uremia and extension into the inferior vena cava. Heparin therapy with therapeutic doses for 6 weeks to 3 months is recommended for unilateral RVT that extends into the inferior vena cava or for bilateral RVT because of the risk of pulmonary embolism and complete renal failure. Thrombolytic therapy should be considered in the presence of bilateral RVT and renal failure. Thrombectomy, though a common therapeutic choice in the past, is rarely indicated.

Outcome. RVT has changed from a frequently lethal complication to one that is now associated with nearly 100% survival.[2,500,501,502-504] However, there are considerable long-term renal sequelae,[500,502-505] with approximately 70% of affected kidneys having irreversible atrophy, approximately 20% of children having hypertension, and approximately 3% of children having chronic renal failure.[501]

Spontaneous Venous and Arterial Thrombosis. Spontaneous venous thrombosis occurs in the adrenal veins, inferior vena cava, portal vein, hepatic veins, and venous system of the brain.[493,506] Spontaneous occlusion of arterial vessels in the absence of a catheter is unusual, but it can occur in ill infants. As in catheter-related thrombosis, the clinical findings reflect the vessel that is occluded. Complete occlusion of a vessel can lead to gangrene and loss of the affected limb or to ischemic organ damage. The presence of systemic hypertension in newborns is frequently related to renal artery thrombosis, even in the absence of a catheter.

Anticoagulation Therapy in Newborns

Heparin Therapy in Newborns. Guidelines for antithrombotic therapy in neonates have been published.[2,507] However, the limited strength of the recommendations reflects the lack of controlled trials in this area. Recommendations for adult patients provide useful guidelines but probably do not reflect the optimal therapy for newborns. Current therapeutic options include supportive care alone, anticoagulant therapy, thrombolytic therapy, and thrombectomy. For most infants in whom thrombotic complications develop, the cause of thrombosis is a catheter-related thrombus that does not produce clinical symptoms. In most nurseries, catheters are not routinely checked for associated thrombosis; thus, by exclusion, most infants with clinically silent thrombi receive supportive care alone.

Age-Dependent Features. Although the benefits of heparin therapy in newborns are probably similar to those in adults, the relative risk that major bleeding will occur with its use may be increased. Infants with thromboses that are extending or infants whose organ or limb viability is threatened by thrombosis may benefit from heparin therapy.

Heparin's anticoagulant activities are mediated primarily through acceleration of inhibition of thrombin and factor Xa by AT. Although dosing for heparin therapy in newborns differs from that in adults, optimal dosing cannot be predicted. Several observations suggest that the heparin requirements of neonates are decreased in comparison to those of adults. First, the capacity of plasma from healthy newborns to generate thrombin is both delayed and decreased in comparison to that of adult plasma, but similar to that of plasma from adults receiving therapeutic amounts of heparin.[508,509] Second, at heparin concentrations in the therapeutic range, the capacity of plasma from healthy newborns to generate thrombin is barely measurable.[510] Third, the amount of clot-bound thrombin is decreased in newborns because low plasma concentrations of prothrombin probably reduce heparin requirements.[511] Observations suggesting higher heparin requirements include the following: (1) clearance of heparin is accelerated in newborns,[38,512,513] and (2) plasma concentrations of AT are decreased to levels frequently less than 0.40 in premature infants, which may limit heparin's antithrombotic activities.[18,21,26,482,514]

Therapeutic Range and Dose. Therapeutic ranges reflect the optimal risk-benefit ratio of anticoagulant therapy with regard to recurrent thrombotic events and bleeding complications. In the absence of clinical trials in newborns, one approach is to use heparin in doses that achieve the lower therapeutic range for adults (see Chapter 33).

General guidelines for initial dosing and subsequent dose adjustment of unfractionated heparin in full-term and premature neonates have been published.[2,507,515] Average doses of heparin required in newborns are bolus doses of 25 to 100 U/kg, depending on gestational age, and average maintenance doses of 15 to 28 U/kg/hr, again depending on gestational age. Adjustments are made most reliably with heparin assays, with a target-level anti-Xa range of 0.35 to 0.70 U/mL. Because of the short half-life of unfractionated heparin, simple discontinuation of the infusion is typically sufficient to reverse anticoagulation in the event of hemorrhage. For more rapid reversal, protamine sulfate can be used to neutralize heparin in vivo. However, anaphylactoid reactions with hypotension and bradycardia have been associated with protamine use in children and adults, and protamine should therefore be used with caution.[516]

The use of LMWH offers significant therapeutic advantages over unfractionated heparin, and there is now considerable experience using these preparations in newborns.[2,493,515,517,518] Potential advantages include more predictable pharmacokinetics, the need for less frequent monitoring than with unfractionated heparin, ease of administration, decreased bleeding, and possibly a lower incidence of heparin-induced thrombocytopenia. Full-term and moderately preterm (28 to 36 weeks EGA) infants metabolize heparin at considerably faster rates than older children and adults do and typically require higher doses to maintain the same levels. Very premature infants (<28 weeks) have similar pharmacokinetic parameters for heparin as do older children and adults. General guidelines for initial therapeutic dosing and subsequent sliding-scale dose adjustments of LMWH in neonates of different gestational ages have been published.[2,507,515] Typically, higher doses of LMWH are required in neonates compared with adults to achieve the same anti-Xa levels. Traditional doses of enoxaparin in full-term neonates for full anticoagulation are 1.5 mg/kg given subcutaneously every 12 hours,[2,507] but they can be as high as 2 mg/kg given subcutaneously every 12 hours in preterm neonates.[507] Levels should be monitored with anti-Xa heparin assays on samples drawn via fresh venipuncture 4 to 6 hours after the subcutaneous dose is given to achieve levels of 0.5 to 1 U/mL for full therapeutic dosing. Levels should be checked every two to four doses for the first week of therapy, then once a week for a month, and then monthly if the infant is stable.[515] Infants who do not respond appropriately to heparin therapy, despite adequate levels, may benefit from infusions of AT concentrate, even if the levels are close to the normal physiologic range for their age.[515] Intramuscular and arterial puncture and the use of antiplatelet medications should be avoided in newborns receiving heparin therapy, and platelet counts should be monitored periodically.

Like unfractionated heparin, LMWH can be reversed with the use of protamine, with dosing dependent on the last dose of LMWH given.[2,519,520] As discussed earlier, protamine must be given slowly, and caution must be exercised to avoid potential hypersensitivity reactions. Repeat doses of protamine may be required to reverse the effects of LMWH because the half-life of protamine is relatively shorter than that of LMWH.

Close monitoring of the thrombus with objective means such as ultrasound is recommended. The duration of heparin therapy required for the treatment of thromboembolic complications is uncertain. One approach is to treat the infant for 10 to 14 days with heparin alone. If there is subsequent extension of the thrombus in the absence of anticoagulation therapy, treatment with oral anticoagulants may be considered.

Adverse Effects. There are two clinically important adverse effects of heparin therapy: hemorrhage, including ICH, and heparin-induced thrombocytopenia.[521,522] In the absence of an alternative cause, thrombocytopenic patients should be evaluated for heparin-induced thrombocytopenia and treated with alternative therapies.

Oral Anticoagulant Therapy in Newborns

Age-Dependent Features. Oral anticoagulation therapy in children is discussed in Chapter 33, and only specific issues related to newborns are discussed in this section. The oral anticoagulant warfarin works by reducing functional plasma levels of the VK-dependent proteins. At birth, levels of the VK-dependent proteins are similar to those found in adults receiving therapeutic amounts of warfarin for deep venous thrombosis/pulmonary embolism.[18,21,26,92,510,523,524] In addition, stores of VK are low, and a small number of newborns have evidence of functional VK deficiency.[65] These features significantly increase the sensitivity of newborns to warfarin and potentially their risk for bleeding. Oral anticoagulant therapy should be avoided when possible during the first month of life.[65,525] Unfortunately, a small number of infants require extended anticoagulation therapy, and heparin cannot be used for extended periods because of the risk of osteopenia.

Indications, Therapeutic Range, and Dose. The optimal therapeutic INR range is unknown for newborns and almost certainly differs from that for adults.[2] Recommendations for oral anticoagulation therapy in adults can be used as a guideline for determining the lowest effective dose, which to some extent can be individualized. Maintenance doses for warfarin are age dependent, with infants requiring the highest doses (0.32 mg/kg).[526]

Adverse Effects. Close monitoring of oral anticoagulation in newborns is required if both hemorrhagic and recurrent thrombotic complications are to be prevented. Unfortunately, these infants often have poor venous access, as well as complicated medical problems. Weekly or biweekly INR measurements and frequent dose adjustments are required.[527] Doses are affected by diet, medications, and intercurrent illnesses. Breastfed infants are very sensitive to oral anticoagulants because of low concentrations of VK in breastmilk.[53,59,72,298,528] Daily supplementation of breastfed infants with small amounts of commercial formulas reduces their sensitivity to oral anticoagulants and the risk of sudden increases in INR values. In contrast to breastfed infants, infants receiving commercial formulas or total parenteral nutrition are resistant to oral anticoagulants because of VK supplementation.[298,529] Reducing or removing VK supplementation in infants receiving total parenteral nutrition significantly decreases the dose requirements. Most infants requiring oral anticoagulants also require other medications on an intermittent and long-term basis. The effects of dosage changes and the introduction of new medications must be closely supervised.

Antiplatelet Agents in Newborns. Antiplatelet agents are rarely used in newborns for antithrombotic therapy. The hyporeactivity of neonatal platelets and the paradoxically short bleeding time suggest that optimal use of antiplatelet agents differs in newborns and adults. Aspirin is the most commonly used antiplatelet agent. The empiric use of low doses of 1 to 5 mg/kg/day has been proposed as adjuvant therapy for patients with Blalock-Taussig shunts, some endovascular stents, and some thrombotic cerebrovascular events.[530] Use of the antiplatelet agent clopidogrel (Plavix) in infants as young as 6 weeks of age has been reported in a small retrospective series.[531] A prospective, multicenter, placebo-controlled pharmacodynamic trial of clopidogrel in children (from birth to 24 months) with a cardiac condition demonstrated that a dose of 0.20 mg/kg/day achieved a platelet inhibition level similar to that in adults receiving a standard dose of 75 mg/day, although there was significant interpatient variability in platelet inhibition levels.[532,533] Clopidogrel was well tolerated by the infants.

Thrombolytic Therapy in Newborns

Age-Dependent Features. The activity of thrombolytic agents depends on endogenous concentrations of plasminogen, which are physiologically decreased at birth.[534] Low plasminogen levels result in impairment of the capacity to generate plasmin[73] and a decrease in the capacity to thrombolyse fibrin clots.[534] In addition, fetal glycoforms of plasminogen are less efficiently converted to plasmin by tPA than is adult plasminogen.[76] Thus neonates have an overall reduced capacity to respond to tPA in comparison with adults. If an infant's condition does not respond to thrombolytic therapy, replacement of plasminogen should be considered.

Indications, Therapeutic Range, and Dose. Infants in whom serious thrombotic complications develop, as defined by organ or limb impairment, may benefit from thrombolytic therapy. The clinical objective is removal of the clot as quickly and safely as possible. Surgical removal of a clot in a major vessel can be curative; however, it is technically difficult and poses a considerable life-threatening risk to infants, who are often premature. In the absence of contraindications, the use of thrombolytic agents in these infants is a preferred approach (see Chapter 33).

CONCLUSION

Over the past several decades, the developmental timing and functional maturation of the human hemostatic system have been examined in great detail. These studies show critically important differences in many components of the hemostatic system in premature and full-term newborns versus older children and adults. The reason for this unique physiologic state in the fetus and neonate is not clear but appears to be appropriately balanced for healthy full-term infants. Of clinical importance has been the determination of gestational and postnatal age-specific activity ranges of factors involved in hemostasis, which has allowed appropriate interpretation of clinical data. As newer diagnostic tests and treatments are introduced for older children and adults, it will be critical to carefully evaluate them separately in the context of neonates, both premature and full-term, given their unique physiologic state.

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APPENDIX

Normal Ranges of Hemostasis Laboratory Tests for Term and Preterm Infants

TABLE A5-1 Coagulation Screening Tests and Coagulation Factor Levels in Fetuses, Full-Term Infants, and Adults[*]

	FETUSES (WEEKS' GESTATION)				
Parameter	19-23 (*n* = 20)	24-29 (*n* = 22)	30-38 (*n* = 22)	Newborn (*n* = 60)	Adult (*n* = 40)
PT (seconds)	32.5 (19-45)	32.2 (19-44)[†]	22.6 (16-30)[†]	16.7 (12-23.5)[‡]	13.5 (11.4-14)
PT (INR)	6.4 (1.7-11.1)	6.2 (2.1-10.6)[†]	3 (1.5-5)[‡]	1.7 (0.9-2.7)[‡]	1.1 (0.8-1.2)
APTT (seconds)	168.8 (83-250)	154 (87-210)[†]	104.8 (76-128)[†]	44.3 (35-52)[‡]	33 (25-39)
TCT (seconds)	34.2 (24-44)[‡]	26.2 (24-28)	21.4 (17-23.3)	20.4 (15.2-25)[†]	14 (12-16)
Factor I von Clauss (g/L)	0.85 (0.57-1.50)	1.12 (0.65-1.65)	1.35 (1.25-1.65)	1.68 (0.95-2.45)[†]	3 (1.78-4.50)
Factor I antigen (g/L)	1.08 (0.75-1.50)	1.93 (1.56-2.40)	1.94 (1.30-2.40)	2.65 (1.68-3.60)[†]	3.5 (2.50-5.20)
Factor IIc (%)	16.9 (10-24)	19.9 (11-30)[‡]	27.9 (15-50)[†]	43.5 (27-64)[†]	98.7 (70-125)
Factor VIIc (%)	27.4 (17-37)	33.8 (18-48)[‡]	45.9 (31-62)	52.5 (28-78)[†]	101.3 (68-130)
Factor IXc (%)	10.1 (6-14)	9.9 (5-15)	12.3 (5-24)[†]	31.8 (15-50)[†]	104.8 (70-142)
Factor Xc (%)	20.5 (14-29)	24.9 (16-35)	28 (16-36)[†]	39.6 (21-65)[†]	99.2 (75-125)
Factor Vc (%)	32.1 (21-44)	36.8 (25-50)	48.9 (23-70)[†]	89.9 (50-140)	99.8 (65-140)
Factor VIIIc (%)	34.5 (18-50)	35.5 (20-52)	50.1 (27-78)[†]	94.3 (38-150)	101.8 (55-170)
Factor XIc (%)	13.2 (8-19)	12.1 (6-22)	14.8 (6-26)[†]	37.2 (13-62)[†]	100.2 (70-135)
Factor XIIc (%)	14.9 (6-25)	22.7 (6-40)	25.8 (11-50)[†]	69.8 (25-105)[†]	101.4 (65-144)
PK (%)	12.8 (8-19)	15.4 (8-26)	18.1 (8-28)[†]	35.4 (21-53)[†]	99.8 (65-135)
HMWK (%)	15.4 (10-22)	19.3 (10-26)	23.6 (12-34)[†]	38.9 (28-53)[†]	98.8 (68-135)

From Reverdiau-Moalic P, Delahousse B, Body G, et al: Evolution of blood coagulation activators and inhibitors in the healthy human fetus. Blood *88:900–906, 1996.*

APTT, Activated partial thromboplastin time; *HMWK*, high–molecular-weight kininogen; *INR*, international normalized ratio; *n*, number of subjects; *PK*, prekallikrein; *PT*, prothrombin time; *TCT*, thrombin clotting time.

[*] All values are given as means, followed in parentheses by the lower and upper boundaries including 95% of the population.

[†] $P < .01$.

[‡] $P < .05$.

TABLE A5-2 Reference Values for Coagulation Tests in Healthy Full-Term Infants During First 6 Months of Life with ACL Analyzer[*]

Coagulation Tests	Day 1	Day 5	Day 30	Day 90	Day 180	Adult
PT (seconds)	13 (10.1-15.9)[†]	12.4 (10-15.3)[†]	11.8 (10-14.3)[†]	11.9 (10-14.2)[†]	12.3 (10.7-13.9)[†]	12.4 (10.8-13.9)
INR	1 (0.53-1.62)	0.89 (0.53-1.48)	0.79 (0.53-1.26)	0.81 (0.53-1.26)	0.88 (0.61-1.17)	0.89 (0.64-1.17)
APTT (seconds)	42.9 (31.3-54.5)	42.6 (25.4-59.8)	40.4 (32-55.2)	37.1 (29-50.1)[†]	35.5 (28.1-42.9)	33.5 (26.6-40.3)[†]
TCT (seconds)	23.5 (19-28.3)[†]	23.1 (18-29.2)	24.3 (19.4-29.2)[†]	25.1 (20.5-29.7)[†]	25.5 (19.8-31.2)	25 (19.7-30.3)[†]
Fibrinogen (g/L)	2.83 (1.67-3.99)[†]	3.12 (1.62-4.62)[†]	2.70 (1.62-3.78)[†]	2.43 (1.50-3.79)[†]	2.51 (1.50-3.87)	2.78 (1.56-4)[†]
Factor II (U/mL)	0.48 (0.26-0.70)	0.63 (0.33-0.93)	0.68 (0.34-1.02)	0.75 (0.45-1.05)	0.88 (0.60-1.16)	1.08 (0.70-1.46)
Factor V (U/mL)	0.72 (0.34-1.08)	0.95 (0.45-1.45)	0.98 (0.62-1.34)	0.90 (0.48-1.32)	0.91 (0.55-1.27)	1.06 (0.62-1.50)
Factor VII (U/mL)	0.66 (0.28-1.04)	0.89 (0.35-1.43)	0.90 (0.42-1.38)	0.91 (0.39-1.43)	0.87 (0.47-1.27)	1.05 (0.67-1.43)
Factor VIII (U/mL)	1 (0.50-1.78)[†]	0.88 (0.50-1.54)[†]	0.91 (0.50-1.57)[†]	0.79 (0.50-1.25)[†]	0.73 (0.50-1.09)	0.99 (0.50-1.49)
VWF (U/mL)	1.53 (0.50-2.87)	1.40 (0.50-2.54)	1.28 (0.50-2.46)	1.18 (0.50-2.06)	1.07 (0.50-1.97)	0.92 (0.50-1.58)
Factor IX (U/mL)	0.53 (0.15-0.91)	0.53 (0.15-0.91)	0.51 (0.21-0.81)	0.67 (0.21-1.13)	0.86 (0.36-1.36)	1.09 (0.55-1.63)
Factor X (U/mL)	0.40 (0.12-0.68)	0.49 (0.19-0.79)	0.59 (0.31-0.87)	0.71 (0.35-1.07)	0.78 (0.38-1.18)	1.06 (0.70-1.52)
Factor XI (U/mL)	0.38 (0.10-0.66)	0.55 (0.23-0.87)	0.53 (0.27-0.79)	0.69 (0.41-0.97)	0.86 (0.49-1.34)	0.97 (0.67-1.27)
Factor XII (U/mL)	0.53 (0.13-0.93)	0.47 (0.11-0.83)	0.49 (0.17-0.81)	0.67 (0.25-1.09)	0.77 (0.39-1.15)	1.08 (0.52-1.64)
PK (U/mL)	0.37 (0.18-0.69)	0.48 (0.20-0.76)	0.57 (0.23-0.91)	0.73 (0.41-1.05)	0.86 (0.56-1.16)	1.12 (0.62-1.62)
HMWK (U/mL)	0.54 (0.06-1.02)	0.74 (0.16-1.32)	0.77 (0.33-1.21)	0.82 (0.30-1.46)[†]	0.82 (0.36-1.28)[†]	0.92 (0.50-1.36)
Factor XIIIa (U/mL)	0.79 (0.27-1.31)	0.94 (0.44-1.44)[†]	0.93 (0.39-1.47)[†]	1.04 (0.36-1.72)[†]	1.04 (0.46-1.62)[†]	1.05 (0.55-1.55)
Factor XIIIb (U/mL)	0.76 (0.30-1.22)	1.06 (0.32-1.80)	1.11 (0.39-1.73)[†]	1.16 (0.48-1.84)[†]	1.10 (0.50-1.70)[†]	0.97 (0.57-1.37)

From Andrew M, Paes B, Milner R, et al: Development of the human coagulation system in the full-term infant. Blood *70:165–172, 1987.*

APTT, Activated partial thromboplastin time; *HMWK*, high–molecular-weight kininogen; *INR*, international normalized ratio; *PK*, prekallikrein; *PT*, prothrombin time; *TCT*, thrombin clotting time; *VWF*, von Willebrand factor.

[*] All factors except fibrinogen are expressed as units per milliliter, with pooled plasma containing 1 U/mL. All values are expressed as means, followed by the lower and upper boundary encompassing 95% of the population. Between 40 and 77 samples were assayed for each value in newborns. Some measurements were skewed because of a disproportionate number of high values. All infants received 1 mg of vitamin K intramuscularly at birth. The following reagents were used for coagulation screening tests: prothrombin time (Dade C rabbit thromboplastin), activated partial thromboplastin time (Dade FS). Measurements were made with an ACL analyzer.

[†] Values indistinguishable from those of adults.

TABLE A5-3 Reference Values for Coagulation Tests in Healthy Full-Term Infants and Children with the STA Analyzer[*]

Coagulation Tests	Age 1 Day	Age 3 Days	Age 1 Month to 1 Year	Age 1 to 5 Years	Age 6 to 10 Years	Age 11 to 16 Years	Adults
APTT (seconds)[†]	38.7 (34.3-44.8)[‡]	36.3 (29.5-42.2)[‡]	39.3 (35.1-46.3)[‡]	37.7 (33.6-43.8)[‡]	37.3 (31.8-43.7)[‡]	39.5 (33.9-46.1)[‡]	33.2 (28.6-38.2)
PT (seconds)[§]	15.6 (14.4-16.4)[‡]	14.9 (13.5-16.4)[‡]	13.1 (11.5-15.3)	13.3 (12.1-14.5)[‡]	13.4 (11.7-15.1)[‡]	13.8 (12.7-16.1)[‡]	13 (11.5-14.5)
INR	1.26 (1.15-1.35)[‡]	1.20 (1.05-1.35)[‡]	1 (0.86-1.22)	1.03 (0.92-1.14)[‡]	1.04 (0.87-1.20)[‡]	1.08 (0.97-1.30)[‡]	1 (0.80-1.20)
Fibrinogen (g/L)	2.80 (1.92-3.74)	3.30 (2.83-4.01)	2.42 (0.82-3.83)[‡]	2.82 (1.62-4.01)[‡]	3.04 (1.99-4.09)	3.15 (2.12-4.33)	3.10 (1.9-4.3)
COAGULATION FACTORS							
Factor II (%)	54 (41-69)[‡]	62 (50-73)[‡]	90 (62-103)[‡]	89 (70-109)[‡]	89 (67-110)[‡]	90 (61-107)[‡]	110 (78-138)
Factor V (%)	81 (64-103)[‡]	122 (92-154)	113 (94-141)	97 (67-127)[‡]	99 (56-141)[‡]	89 (67-141)[‡]	118 (78-152)
Factor VII (%)	70 (52-88)[‡]	86 (67-107)[‡]	128 (83-160)	111 (72-150)[‡]	113 (70-156)[‡]	118 (69-200)	129 (61-199)
Factor VIII (%)	182 (105-329)	159 (83-274)	94 (54-145)[‡]	110 (36-185)[‡]	117 (52-182)[‡]	120 (59-200)[‡]	160 (52-290)
Factor IX (%)	48 (35-56)[‡]	72 (44-97)[‡]	71 (43-121)[‡]	85 (44-127)[‡]	96 (48-145)[‡]	111 (64-216)[‡]	130 (59-254)
Factor X (%)	55 (46-67)[‡]	60 (46-75)[‡]	95 (77-122)[‡]	98 (72-125)[‡]	97 (68-125)[‡]	91 (53-122)[‡]	124 (96-171)
Factor XI (%)	30 (7-41)[‡]	57 (24-79)[‡]	89 (62-125)[‡]	113 (65-162)	113 (65-162)	111 (65-139)	112 (67-196)
Factor XII (%)	58 (43-80)[‡]	53 (14-80)[‡]	79 (20-135)[‡]	85 (36-135)[‡]	81 (26-137)[‡]	75 (14-117)[‡]	115 (35-207)
COAGULATION INHIBITORS							
AT (%)	76 (58-90)[‡]	74 (60-89)[‡]	109 (72-134)[‡]	116 (101-131)[‡]	114 (95-134)[‡]	111 (96-126)[‡]	96 (66-124)
Protein C, chromogenic (%)36 (24-44)[‡]	44 (28-54)[‡]	71 (31-112)[‡]	96 (65-127)[‡]	100 (71-129)	94 (66-118)[‡]	104 (74-164)	
Protein C, clotting (%)	32 (24-40)[‡]	33 (24-51)[‡]	77 (28-124)[‡]	94 (50-134)[‡]	94 (64-125)[‡]	88 (59-112)[‡]	103 (54-166)
Protein S, clotting (%)	36 (28-47)[‡]	49 (33-67)[‡]	102 (29-162)[‡]	101 (67-136)[‡]	109 (64-154)[‡]	103 (65-140)[‡]	75 (54-103)

Reproduced with permission from Monagle P, Barnes C, Ignjatovic V, et al: Developmental haemostasis: impact for clinical haemostasis laboratories. Thromb Haemost *95:362–372, 2006.*

APTT, Activated partial thromboplastin time; *AT*, antithrombin; *INR*, international normalized ratio; *PT*, prothrombin time.

[*] Values represent means. Boundaries including 95% of the population are shown in parentheses. All infants received 1 mg of vitamin K intramuscularly at the time of birth.

[†] STA-PTT-A reagent used.

[‡] Denotes values that are significantly different from adult values ($P < .05$).

[§] Neoplastine Cl reagent used.

TABLE A5-4 Reference Values for Coagulation Tests in Healthy Premature Infants (30 to 36 Weeks' Gestation) During First 6 Months of Life[*]

Coagulation Tests	Day 1	Day 5	Day 30	Day 90	Day 180	Adult
PT (seconds)	13 (10.6-16.2)[†]	12.5 (10-15.3)[†]	11.8 (10-13.6)[†]	12.3 (10-14.6)	12.5 (10-15)[†]	12.4 (10.8-13.9)
INR	1 (0.61-1.70)	0.91 (0.53-1.48)	0.79 (0.53-1.11)	0.88 (0.53-1.32)	0.91 (0.53-1.48)	0.89 (0.64-1.17)
APTT (seconds)	53.6 (27.5-79.4)[‡]	50.5 (26.9-74.1)	44.7 (26.9-62.5)	39.5 (28.3-50.7)	37.5 (27.2-53.3)	33.5 (26.6-40.3)
TCT (seconds)	24.8 (19.2-30.4)	24.1 (18.8-29.4)[†]	24.4 (18.8-29.9)	25.1 (19.4-30.8)	25.2 (18.9-31.5)	25 (19.7-30.3)
Fibrinogen (g/L)	2.43 (1.50-3.73)[†‡]	2.80 (1.60-4.18)[†‡]	2.54 (1.50-4.14)	2.46 (1.50-3.52)	2.28 (1.50-3.60)	2.78 (1.56-4)
Factor II (U/mL)	0.45 (0.20-0.77)	0.57 (0.29-0.85)[‡]	0.57 (0.36-0.95)	0.68 (0.30-1.06)	0.87 (0.51-1.23)	1.08 (0.70-1.46)
Factor V (U/mL)	0.88 (0.41-1.44)[†‡]	1 (0.46-1.54)[†]	1.02 (0.48-1.56)[†]	0.99 (0.59-1.39)	1.02 (0.58-1.46)[†]	1.06 (0.62-1.50)
Factor VII (U/mL)	0.67 (0.21-1.13)	0.84 (0.30-1.38)	0.83 (0.21-1.45)	0.87 (0.31-1.43)	0.99 (0.47-1.51)[†]	1.05 (0.67-1.43)
Factor VIII (U/mL)	1.11 (0.50-2.13)	1.15 (0.53-2.05)[†‡]	1.11 (0.50-1.99)	1.06 (0.58-1.88)[†‡]	0.99 (0.50-1.87)[†‡]	0.99 (0.50-1.49)
VWF (U/mL)	1.36 (0.78-2.10)	1.33 (0.72-2.19)	1.36 (0.66-2.16)	1.12 (0.75-1.84)[†‡]	0.98 (0.54-1.58)[†]	0.92 (0.50-1.58)
Factor IX (U/mL)	0.35 (0.19-0.65)[‡]	0.42 (0.14-0.74)[‡]	0.44 (0.13-0.80)	0.59 (0.25-0.93)	0.81 (0.50-1.20)	1.09 (0.55-1.63)
Factor X (U/mL)	0.41 (0.11-0.71)	0.51 (0.19-0.83)	0.56 (0.20-0.92)	0.67 (0.35-0.99)	0.77 (0.35-1.19)	1.06 (0.70-1.52)
Factor XI (U/mL)	0.30 (0.08-0.52)[‡]	0.41 (0.13-0.69)[‡]	0.43 (0.15-0.71)[‡]	0.59 (0.25-0.93)[†]	0.78 (0.46-1.10)	0.97 (0.67-1.27)
Factor XII (U/mL)	0.38 (0.10-0.66)[‡]	0.39 (0.09-0.69)[‡]	0.43 (0.11-0.75)	0.61 (0.15-1.07)	0.82 (0.22-1.42)	1.08 (0.52-1.64)
PK (U/mL)	0.33 (0.09-0.57)	0.45 (0.25-0.75)	0.59 (0.31-0.87)	0.79 (0.37-1.21)	0.78 (0.40-1.16)	1.12 (0.62-1.62)
HMWK (U/mL)	0.49 (0.09-0.89)	0.62 (0.24-1)[‡]	0.64 (0.16-1.12)[‡]	0.78 (0.32-1.24)	0.83 (0.41-1.25)[†]	0.92 (0.50-1.36)
Factor XIIIa (U/mL)	0.70 (0.32-1.08)	1.01 (0.57-1.45)[†]	0.99 (0.51-1.47)[†]	1.13 (0.71-1.55)[†]	1.13 (0.65-1.61)[†]	1.05 (0.55-1.55)
Factor XIIIb (U/mL)	0.81 (0.35-1.27)	1.10 (0.68-1.58)[†]	1.07 (0.57-1.57)[†]	1.21 (0.75-1.67)	1.15 (0.67-1.63)	0.97 (0.57-1.37)

From Andrew M, Paes B, Milner R, et al: Development of the human coagulation system in the healthy premature infant. Blood *72:1651–1657, 1988.*

APTT, Activated partial thromboplastin time; *Factor VIII*, factor VIII procoagulant; *HMWK*, high–molecular-weight kininogen; *INR*, international normalized ratio; *PK*, prekallikrein; *PT*, prothrombin time; *TCT*, thrombin clotting time; *VWF*, von Willebrand factor.

[*] All factors except fibrinogen are expressed as units per milliliter, with pooled plasma containing 1 U/mL. All values are given as means, followed by the lower and upper boundary encompassing 95% of the population. Between 40 and 96 samples were assayed for each value in newborns. Some measurements were skewed because of a disproportionate number of high values. All infants received 1 mg of vitamin K intramuscularly at birth. The following reagents were used for coagulation screening tests: prothrombin time (Dade C rabbit thromboplastin), activated partial thromboplastin time (Dade FS). Measurements were made with an ACL analyzer.

[†] Values indistinguishable from those of adults.

[‡] Measurements are skewed because of a disproportionate number of high values.

TABLE A5-5 Reference Values for Coagulation Tests in Healthy Children (Ages 1 to 16 Years) and Adults[*]

Coagulation Tests	Age 1 to 5 Years	Age 6 to 10 Years	Age 11 to 16 Years	Adult
PT (seconds)	11 (10.6-11.4)	11.1 (10.1-12.1)	11.2 (10.2-12)	12 (11-14)
PT (INR)	1 (0.96-1.04)	1.01 (0.91-1.11)	1.02 (0.93-1.10)	1.10 (1-1.3)
APTT (seconds)	30 (24-36)	31 (26-36)	32 (26-37)	33 (27-40)
Fibrinogen (g/L)	2.76 (1.70-4.05)	2.79 (1.57-4)	3.0 (1.54-4.48)	2.78 (1.56-4)
Bleeding time (minutes)	6 (2.5-10)[†]	7 (2.5-13)[†]	5 (3-8)[†]	4 (1-7)
Factor II (U/mL)	0.94 (0.71-1.16)[†]	0.88 (0.67-1.07)	0.83 (0.61-1.04)[†]	1.08 (0.70-1.46)
Factor V (U/mL)	1.03 (0.79-1.27)	0.90 (0.63-1.16)[†]	0.77 (0.55-0.99)[†]	1.06 (0.62-1.50)
Factor VII (U/mL)	0.82 (0.55-1.16)	0.85 (0.52-1.20)	0.83 (0.58-1.15)[†]	1.05 (0.67-1.43)
Factor VIII (U/mL)	0.90 (0.59-1.42)	0.95 (0.58-1.32)	0.92 (0.53-1.31)	0.99 (0.50-1.49)
VWF (U/mL)	0.82 (0.60-1.20)	0.95 (0.44-1.44)	1 (0.46-1.53)	0.92 (0.50-1.58)
Factor IX (U/mL)	0.73 (0.47-1.04)	0.75 (0.63-0.89)[†]	0.82 (0.59-1.22)[†]	1.09 (0.55-1.63)
Factor X (U/mL)	0.88 (0.58-1.16)	0.75 (0.55-1.01)[†]	0.79 (0.50-1.17)[†]	1.06 (0.70-1.52)
Factor XI (U/mL)	0.97 (0.56-1.50)	0.86 (0.52-1.20)	0.74 (0.50-0.97)	0.97 (0.67-1.27)
Factor XII (U/mL)	0.93 (0.64-1.29)	0.92 (0.60-1.40)	0.81 (0.34-1.37)[†]	1.08 (0.52-1.64)
PK (U/mL)	0.95 (0.65-1.30)	0.99 (0.66-1.31)	0.99 (0.53-1.45)	1.12 (0.62-1.62)
HMWK (U/mL)	0.98 (0.64-1.32)	0.93 (0.60-1.30)	0.91 (0.63-1.19)	0.92 (0.50-1.36)
Factor XIIIa (U/mL)	1.08 (0.72-1.43)	1.09 (0.65-1.51)[†]	0.99 (0.57-1.40)	1.05 (0.55-1.55)
Factor XIIIb (U/mL)	1.13 (0.69-1.56)	1.16 (0.77-1.54)[†]	1.02 (0.60-1.43)	0.97 (0.57-1.37)

From Andrew M, Vegh P, Johnston M, et al: Maturation of the hemostatic system during childhood. Blood *80:1998–2005, 1992.*

APTT, Activated partial thromboplastin time; *HMWK*, high–molecular-weight kininogen; *INR*, international normalized ratio; *PK*, prekallikrein; *PT*, prothrombin time; *TCT*, thrombin clotting time; *VWF*, von Willebrand factor.

[*] All factors except fibrinogen are expressed as units per milliliter, with pooled plasma containing 1 U/mL. All data are expressed as means, followed by the upper and lower boundary encompassing 95% of the population. Between 20 and 50 samples were assayed for each value in each age group. Some measurements were skewed because of a disproportionate number of high values. The lower limit, which excludes the lower 2.5% of the population, has been given. The following reagents were used for coagulation screening tests: prothrombin time (Dade C rabbit thromboplastin), activated partial thromboplastin time (Dade FS). Measurements were made with an ACL analyzer.

[†] Values significantly different from those of adults.

TABLE A5-6 Blood Coagulation Inhibitor Levels in Fetuses, Full-Term Infants, and Adults[*]

Parameter	Fetuses (Weeks' Gestation)		Newborns	Adults	
19-23 (*n* = 20)	24-29 (*n* = 22)	30-38 (*n* = 22)	(*n* = 60)	(*n* = 40)	
AT (%)	20.2 (12-31)[†]	30 (20-39)	37.1 (24-55)[‡]	59.4 (42-80)[‡]	99.8 (65-130)
HCII (%)	10.3 (6-16)	12.9 (5.5-20)	21.1 (11-33)[‡]	52.1 (19-99)[‡]	101.4 (70-128)
TFPI (%)[§]	21 (16-29.2)	20.6 (13.4-33.2)	20.7 (10.4-31.5)[‡]	38.1 (22.7-55.8)[‡]	73 (50.9-90.1)
PC antigen (%)	9.5 (6-14)	12.1 (8-16)	15.9 (8-30)[‡]	32.5 (21-47)[‡]	100.8 (68-125)
PC activity (%)	9.6 (7-13)	10.4 (8-13)	14.1 (8-18)[†]	28.2 (14-42)[‡]	98.8 (68-125)
Total PS (%)	15.1 (11-21)	17.4 (14-25)	21 (15-30)[‡]	38.5 (22-55)[‡]	99.6 (72-118)
Free PS (%)	21.7 (13-32)	27.9 (19-40)	27 (18-40)[‡]	49.3 (33-67)[‡]	98.7 (72-128)
Free PS/total PS	0.82 (0.75-0.92)	0.83 (0.76-0.95)	0.79 (0.70-0.89)[‡]	0.64 (0.59-0.98)[‡]	0.41 (0.38-0.43)
C4b-BP (%)	1.8 (0-6)	6.1 (0-12.5)	9.3 (5-14)	18.6 (3-40)[‡]	100.3 (70-124)

From Reverdiau-Moalic P, Delahousse B, Body G, et al: Evolution of blood coagulation activators and inhibitors in the healthy human fetus. Blood *88:900–906, 1996.*

AT, Antithrombin; *BP*, binding protein; *HCII*, heparin cofactor II; *n*, number of subjects; *PC*, protein C; *PS*, protein S; *TFPI*, tissue factor pathway inhibitor.

[*] All values are given as means, followed in parentheses by the lower and upper boundaries including 95% of the population.

[†] *P* <.05.

[‡] *P* <.01.

[§] Twenty samples were assayed for each group, but only 10 for 19- to 23-week-old fetuses.

TABLE A5-7 Reference Values for Inhibitors of Coagulation in Infants During the First 6 Months of Life[*]

Inhibitor Levels	Day 1	Day 5	Day 30	Day 90	Day 180	Adult
HEALTHY FULL-TERM INFANTS						
AT (U/mL)	0.63 (0.39-0.87)	0.67 (0.41-0.93)	0.78 (0.48-1.08)	0.97 (0.73-1.21)[†]	1.04 (0.84-1.24)[†]	1.05 (0.79-1.31)
α_2-Macroglobulin (U/mL)	1.39 (0.95-1.83)	1.48 (0.98-1.98)	1.50 (1.06-1.94)	1.76 (1.26-2.26)	1.91 (1.49-2.33)	0.86 (0.52-1.20)
C1E-INH (U/mL)	0.72 (0.36-1.08)	0.90 (0.60-1.20)[†]	0.89 (0.47-1.31)	1.15 (0.71-1.59)	1.41 (0.89-1.93)	1.01 (0.71-1.31)
α_1-Antitrypsin (U/mL)	0.93 (0.49-1.37)[†]	0.89 (0.49-1.29)[†]	0.62 (0.36-0.88)	0.72 (0.42-1.02)	0.77 (0.47-1.07)	0.93 (0.55-1.31)
HCII (U/mL)	0.43 (0.10-0.93)	0.48 (0.00-0.96)	0.47 (0.10-0.87)	0.72 (0.10-1.46)	1.20 (0.50-1.90)	0.96 (0.66-1.26)
Protein C (U/mL)	0.35 (0.17-0.53)	0.42 (0.20-0.64)	0.43 (0.21-0.65)	0.54 (0.28-0.80)	0.59 (0.37-0.81)	0.96 (0.64-1.28)
Protein S (U/mL)	0.36 (0.12-0.60)	0.50 (0.22-0.78)	0.63 (0.33-0.93)	0.86 (0.54-1.18)[†]	0.87 (0.55-1.19)[†]	0.92 (0.60-1.24)
HEALTHY PREMATURE INFANTS (30-36 WEEKS' GESTATION)						
AT (U/mL)	0.38 (0.14-0.62)[‡]	0.56 (0.30-0.82)	0.59 (0.37-0.81)[‡]	0.83 (0.45-1.21)[‡]	0.90 (0.52-1.28)[‡]	1.05 (0.79-1.31)
α2-Macroglobulin (U/mL)	1.10 (0.56-1.82)[‡]	1.25 (0.71-1.77)	1.38 (0.72-2.04)	1.80 (1.20-2.66)	2.09 (1.10-3.21)	0.86 (0.52-1.20)
C1E-NH (U/mL)	0.65 (0.31-0.99)	0.83 (0.45-1.21)	0.74 (0.40-1.24)[‡]	1.14 (0.60-1.68)[†]	1.40 (0.96-2.04)	1.01 (0.71-1.31)
α_1-Antitrypsin (U/mL)	0.90 (0.36-1.44)[†]	0.94 (0.42-1.46)[†]	0.76 (0.38-1.12)[‡]	0.81 (0.49-1.13)[†‡]	0.82 (0.48-1.16)[†]	0.93 (0.55-1.31)
HCII (U/mL)	0.32 (0.10-0.60)[‡]	0.34 (0.10-0.69)	0.43 (0.15-0.71)	0.61 (0.20-1.11)	0.89 (0.45-1.40)[†‡]	0.96 (0.66-1.26)
Protein C (U/mL)	0.28 (0.12-0.44)[‡]	0.31 (0.11-0.51)	0.37 (0.15-0.59)[‡]	0.45 (0.23-0.67)[‡]	0.57 (0.31-0.83)	0.96 (0.64-1.28)
Protein S (U/mL)	0.26 (0.14-0.38)[‡]	0.37 (0.13-0.61)	0.56 (0.22-0.90)	0.76 (0.40-1.12)[‡]	0.82 (0.44-1.20)	0.92 (0.60-1.24)

From Andrew M, Paes B, Johnston M: Development of the hemostatic system in the neonate and young infant. Am J Pediatr Hematol Oncol *12:95–104, 1990.*

AT, Antithrombin; *C1E-INH*, C1 esterase inhibitor; *HCII*, heparin cofactor II.

[*] All values are expressed in units per milliliter, with pooled plasma containing 1 U/mL. All values are given as means, followed by the lower and upper boundary encompassing 95% of the population. Between 40 and 75 samples were assayed for each value in newborns. Some measurements were skewed because of a disproportionate number of high values. The lower limits exclude the lower 2.5% of the population.

[†] Values indistinguishable from those of adults.

[‡] Values different from those of full-term infants.

TABLE A5-8 Reference Values for Components of the Fibrinolytic System During the First 6 Months of Life[*]

Fibrinolytic System	Day 1	Day 5	Day 30	Day 90	Day 180	Adult
HEALTHY FULL-TERM INFANTS						
Plasminogen (U/mL)	1.95 (1.25-2.65)	2.17 (1.41-2.93)	1.98 (1.26-2.70)	2.48 (1.74-3.22)	3.01 (2.21-3.81)	3.36 (2.48-4.24)
tPA (ng/mL)	9.6 (5-18.9)	5.6 (4-10.0)[†]	4.1 (1-6.0)[†]	2.1 (1.0-5)[†]	2.8 (1.0-6)[†]	4.9 (1.4-8.4)
α2-Antiplasmin (U/mL)	0.85 (0.55-1.15)	1 (0.70-1.30)[†]	1 (0.76-1.24)[†]	1.08 (0.76-1.40)[†]	1.11 (0.83-1.39)[†]	1.02 (0.68-1.36)
PAI-1 (U/mL)	6.4 (2-15.1)	2.3 (0.0-8.1)[†]	3.4 (0.0-8.8)[†]	7.2 (1-15.3)	8.1 (6-13)	3.6 (0-11)
HEALTHY PREMATURE INFANTS (30-36 WEEKS' GESTATION)						
Plasminogen (U/mL)	1.70 (1.12-2.48)[‡]	1.91 (1.21-2.61)[‡]	1.81 (1.09-2.53)	2.38 (1.58-3.18)	2.75 (1.91-3.59)[‡]	3.36 (2.48-4.24)
tPA (ng/mL)	8.48 (3-16.70)	3.97 (2-6.93)[†]	4.13 (2-7.79)[†]	3.31 (2-5.07)[†]	3.48 (2-5.85)[†]	4.96 (1.46-8.46)
α2-Antiplasmin (U/mL)	0.78 (0.40-1.16)	0.81 (0.49-1.13)[‡]	0.89 (0.55-1.23)[‡]	1.06 (0.64-1.48)[†]	1.15 (0.77-1.53)	1.02 (0.68-1.36)
PAI-1 (U/mL)	5.4 (0.0-12.2)[†‡]	2.5 (0.0-7.1)[†]	4.3 (0.0-10.9)[†]	4.8 (1-11.8)[†‡]	4.9 (1-10.2)[†‡]	3.6 (0.0-11)

From Andrew M, Paes B, Johnston M: Development of the hemostatic system in the neonate and young infant. Am J Pediatr Hematol Oncol *12:95–104, 1990.*

PAI-1, Plasminogen activator inhibitor 1; *tPA*, tissue plasminogen activator.

[*] Plasminogen units are those recommended by the Committee on Thrombolytic Agents. Values for tPA are given as nanograms per milliliter. For α_2-antiplasmin, values are expressed as units per milliliter, with pooled plasma containing 1 U/mL. Values for PAI-1 are given as units per milliliter, with 1 unit of PAI-1 activity being defined as the amount of PAI-1 that inhibits the 1 IU of human single-chain tPA. All values are given as means, followed by the lower and upper boundaries encompassing 95% of the population.

[†] Values indistinguishable from those of adults.

[‡] Values different from those of full-term infants.

TABLE A5-9 D-Dimer Reference Values for Neonates and Children*

	Age 1 Day	Age 3 Days	Age 1 Month to 1 Year	Age 1 to 5 Years	Age 6 to 10 Years	Age 11 to 16 Years	Adults
D-dimers (µg/mL)	1.47 (0.41-2.47)† n = 20 (10F/10M)	1.34 (0.58-2.74)† n = 23 (12F/11M)	0.22 (0.11-0.42) n = 20 (7F/13M)	0.25 (0.09-0.53)† n = 40 (19F/21M)	0.26 (0.10-0.56)† n = 39 (12F/27M)	0.27 (0.16-0.39)† n = 21 (6F/15M)	0.18 (0.05-0.42) n = 32 (19F/13M)

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n, number of subjects.

*Mean and boundaries including 95% of the population of D-dimer (µg/mL) concentrations are shown. Measurements were made with the Liatest D-Di test kit (Diagnostica Stago, France).

†Denotes values that are significantly different from adult values (*P* <.05).

TABLE A5-10 PFA-100 Closure Time Values from Healthy Cord Blood Samples and Adults

Study	CT-Epi (seconds): Neonate	CT-Epi (seconds): Adult	CT-ADP (seconds): Neonate	CT-ADP (seconds): Adult
Boudewijns et al[149] (n = 80)	108 ± 30 (49-168) (n = 20)	85-65 (n = 80)	66 ± 13 (40-92) (n = 20)	72-120
Roschitz et al[150] (n = 70)	75 (50-112) (n = 25)	106 (84-150) (n = 70)	58 (43-98) (n = 25)	83 (64-98)
Carcao et al[151]	81 ± 17 (61-108) (n = 17)	106 ± 21 (82-142) (n = 31)	56 ± 6 (48-65) (n = 17)	85 ± 16 (67-111) (n = 31)
Israels et al[152]	82 ± 27 (n = 31)	111 ± 22 (n = 21)	60 ± 16 (n = 31)	91 ± 17 (n = 21)

Modified from Saxonhouse MA, Sola MC: Platelet function in term and preterm neonates. Clin Perinatol *31:15–28, 2004.*

ADP, Adenosine diphosphate; *CT*, closure time; *Epi*, epinephrine; *n*, number of subjects.

CHAPTER 6

Acquired Aplastic Anemia and Pure Red Cell Aplasia

Akiko Shimamura and **David A. Williams**

CHAPTER OUTLINE

APLASTIC ANEMIA
Epidemiology
Causal Factors
Pathophysiology
Clinical Evaluation
Management and Outcome
PURE RED CELL APLASIA
Primary Acquired Pure Red Cell Aplasia
Secondary Acquired Pure Red Cell Aplasia

Acquired and congenital bone marrow failure syndromes are characterized by a reduction in the effective production of mature erythrocytes, granulocytes, and platelets by the bone marrow. Bone marrow failure leads to various peripheral blood cytopenias. In some conditions, only one or two cell lines may be affected. In others such as aplastic anemia the result is pancytopenia. In this chapter, we deal only with the acquired bone marrow failure syndromes, though we use the term rather loosely because the acquired marrow failure syndromes may have a genetic basis. The known *inherited/congenital* bone marrow failure syndromes are described in detail in Chapter 7. The acquired marrow failure syndromes that affect only platelets or granulocytes are described in Chapters 22 and 34 respectively. The acquired single lineage deficiency syndromes that involve red cell production (pure red cell aplasia) are also described in this chapter, except that the 5Q-syndrome is discussed in Chapter 7 and in more detail Chapter 51.

APLASTIC ANEMIA

Decreased production of *mature blood cells* may result from a reduction in the number or function of their *progenitors*. *Aplastic anemia* is a descriptive term referring to a clinical state in which peripheral blood pancytopenia results from reduced or absent production of blood cells in the bone marrow. Aplastic anemia may arise in the setting of inherited/congenital syndromes associated with a predisposition to marrow failure (discussed in Chapter 7), may develop secondary to marrow-toxic stressors in an otherwise seemingly normal host, or may lack any apparent underlying cause. Classifications of the aplastic anemias are presented in Box 6-1. A careful medical history, physical examination, and laboratory evaluation are critical to discern inherited versus acquired causes of aplastic anemia. The distinction between inherited and acquired causes of aplastic anemia carries profound implications for medical management and treatment, so a careful search for possible underlying inherited syndromes should be undertaken prior to initiation of therapy.

Camitta and co-workers classified the severity of aplastic anemia in an effort to make possible the comparison of diverse groups of patients and different therapeutic approaches.[1] Diagnosis of *severe aplastic anemia* requires that the patient have at least two of the following anomalies: a granulocyte count below 500/μL, a platelet count below 20,000/μL, and an absolute reticulocyte count less than or equal to 40×10^9/L. In addition the bone marrow biopsy must contain less than 25% of the normal cellularity or less than 30% hematopoietic elements (Fig. 6-1). Very severe aplastic anemia is further defined by a granulocyte count of less than 200/μL.[2] *Mild* or *moderate aplastic anemia*, sometimes called *hypoplastic anemia*, is distinguished from the severe form by the presence of mild or moderate cytopenias and more variable, but still deficient, bone marrow cellularity. These distinctions are more than semantic; they are critical for the prediction of outcome and the choice of therapy.

Epidemiology

Epidemiologic studies performed in Europe estimate that the annual incidence of aplastic anemia is 2 per million per year.[3] By comparison, the incidence of acute leukemia is about 50 per million per year. Higher figures for the

Box 6-1 Classification of the Aplastic Anemias[339]

ACQUIRED

- Secondary
  - Radiation
  - Drugs and chemicals
    - Direct toxicity: chemotherapy; benzene
    - Idiosyncratic: chloramphenicol; antiinflammatory drugs; antiepileptics; carbonic anhydrase inhibitors
  - Viruses
    - Epstein-Barr virus
    - Hepatitis (non-A, B, C, E, or G)
    - Human immunodeficiency virus
  - Immune diseases
    - Eosinophilic fasciitis
    - Hypoimmunoglobulinemia
    - Systemic lupus erythematosis (uncommon)
    - Thymoma
    - Graft-versus-host disease in immunodeficiency
  - Pregnancy
  - Paroxysmal nocturnal hemoglobinuria
  - Myelodysplasia
- Idiopathic

INHERITED

- Fanconi anemia
- Dyskeratosis congenita
- Shwachman-Diamond syndrome
- Amegakaryocytic thrombocytopenia
- Diamond-Blackfan anemia
- Reticular dysgenesis
- GATA-2 syndromes
- Familial aplastic anemias
- Nonhematologic syndromes (e.g., Down, Dubowitz, and Seckel syndromes)

incidence of aplastic anemia have been obtained from smaller studies in the United States and earlier surveys in Europe, but these figures may have been inflated by the inclusion of cases of myelodysplasia, a much more common syndrome. Aplastic anemia is more common in Asia (4 to 7 per million per year)[4] than in the West. Chloramphenicol, a known cause of aplastic anemia, has been widely used in much of Asia because of its efficacy and low cost, but reductions in its use have not been accompanied by reductions in aplastic anemia incidence in Japan[5,6] or elsewhere,[7,8] and no association was observed in the case control studies in Thailand.[9] A recent case control study[10] in Thailand spanning 1989-2002 described an association between aplastic anemia and exposure to benzene or pesticides. Although an increased risk of aplastic anemia was also associated with animal exposures and ingestion of nonbottled or nondistilled water, no significant associations with known infections or hepatitis were observed.

The peak age of presentation of aplastic anemia is at 15 to 25 years or over 60 years. The male-to-female ratio in acquired aplastic anemia is approximately 1:1.

Causal Factors

When no causative factors are ascertained, the cases are classified as idiopathic. A search for possible causative agents is warranted because some patients may improve following the removal of the offending agent. However, most cases of aplastic anemia remain idiopathic. The distinction between acquired versus inherited causes of aplastic anemia is crucial to guide clinical management and treatment (see Chapter 8).

Drugs

The incidence of drug- and chemical-related aplastic anemia[11] varies over time and from place to place. Many drugs and toxins have been implicated by inferential and circumstantial evidence; the magnitude of the risk is usually unknown (Box 6-2). Presence of an agent on this list suggests caution regarding its use, but no drug on this list should be proscribed if there are strong clinical indications for its use. From a public health perspective, even drugs associated with an increased risk of marrow failure do not cause large numbers of cases of aplastic anemia.[3]

Note that even confirmed associations do not substantiate causality. Antibiotics felt to be causative may have been administered for the viral infection that led to the aplastic anemia or for symptoms from already established neutropenia in an undiagnosed case of aplastic anemia. Bleeding may be precipitated in undiagnosed thrombocytopenic patients who receive nonsteroidal antiinflammatory drugs. As an example of known errors in associations, among six patients reported to have sniffed glue and

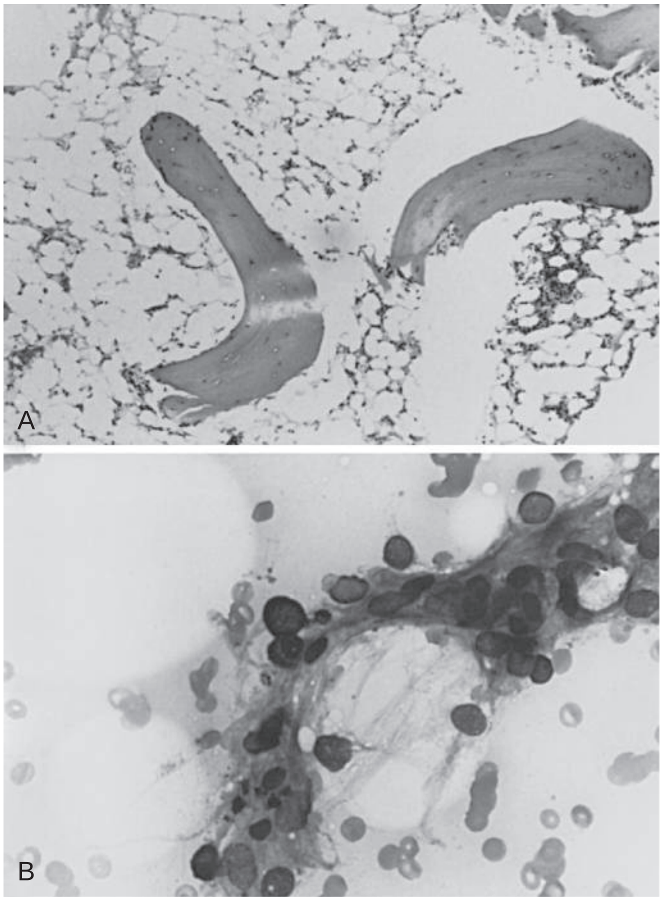

Figure 6-1 Bone marrow examination in severe aplastic anemia. **A**, Note hypocellularity in biopsy. **B**, Residual cells seen in aspirate are lymphocytes, stromal cells, plasma cells, and mast cells. *(Courtesy of Dr. Gail Wolfe, Boston, Mass.)*

Box 6-2 **Classification of Drugs and Chemicals Associated with Aplastic Anemia***

AGENTS THAT REGULARLY PRODUCE MARROW DEPRESSION

Antibiotics: daunorubicin, doxorubicin hydrochloride (Adriamycin), chloramphenicol
Antimetabolites: antifolic compounds, nucleotide analogues
Antimitotics: vinblastine, colchicine
Benzene and chemicals containing benzene: carbon tetrachloride, chlorophenols, kerosene, Stoddard solvent
Cytotoxic cancer chemotherapy with alkylating drugs: busulfan, melphalan, cyclophosphamide

AGENTS POSSIBLY ASSOCIATED BUT WITH A LOW PROBABILITY RELATIVE TO USE

Chloramphenicol
Insecticides: chlordane, chlorophenothane (DDT), γ-benzene hexachloride (lindane), parathion
Anticonvulsants: carbamazepine, hydantoins, phenacemide
Nonsteroidal antiinflammatory agents: indomethacin, ibuprofen, oxyphenylbutazone, phenylbutazone, sulindac
Antihistamines: cimetidine, chlorpheniramine, ranitidine
Antiprotozoal drugs: quinacrine, chloroquine
Sulfonamides: some antibiotics, antidiabetics (chlorpropamide, tolbutamide), antithyroid drugs (methimazole, methylthiouracil, propylthiouracil), carbonic anhydrase inhibitors (acetazolamide, methazolamide)
Penicillamine
Metals: gold, arsenic, bismuth, mercury

AGENTS MORE RARELY ASSOCIATED

Allopurinol (may potentiate marrow suppression by cytotoxic drugs)
Antibiotics: flucytosine, mebendazole, methicillin, sulfonamides, streptomycin, tetracycline, trimethoprim/sulfamethoxazole
Carbimazole
Guanidine
Lithium
Methyldopa
Potassium perchlorate
Quinidine
Sedatives and tranquilizers: chlordiazepoxide, chlorpromazine, meprobamate, methyprylon, piperacetazine, prochlorperazine
Thiocyanate

Modified from Young NS, Alter BP: Aplastic anemia: acquired and inherited*, Philadelphia, 1994, WB Saunders, p 104.*

*Agents are listed because they have been cited in the literature; inclusion in this list does not imply acceptance by the author of a causal relationship.

become aplastic, five had sickle cell anemia and aplastic crises now known to be due to parvovirus infection.[12]

The incidence of drug-related aplasia in pediatric cases is low, mainly because many of the drugs felt to be related to aplasia are not used in childhood, with the exception of antiepileptic drugs, carbonic anhydrase inhibitors, nonsteroidal antiinflammatory medications, and some antibiotics.

Drug-related aplasia may occur in several ways. Drugs may exert direct cytotoxic or suppressive effects on the bone marrow. Myelosuppressive drugs, such as those used in cancer chemotherapy, lead to predictable and dose-related marrow suppression. Benzene, too, can be demonstrated regularly to suppress the bone marrow in animals in a dose-linked manner, and most individuals exposed to sufficient amounts of benzene would probably suffer some type of marrow damage. In practice, most drug-related aplastic anemia is idiosyncratic and occurs unpredictably in only rare individuals who are prescribed the medication, sometimes weeks to months after its administration is discontinued. This last category of patients may possess a genetic propensity for this phenomenon or be due to a direct effect of the drug on antigen presentation of abnormal peptides (see below).

Chloramphenicol

Chloramphenicol was considered to be the commonest cause of aplastic anemia at the peak of its use, which began in 1949.[13] A genetic predisposition may exist. The mechanism of the idiosyncratic aplasia remains unknown despite extensive investigation.

Chloramphenicol contains a nitrobenzene ring and thus resembles amidopyrine, a drug known to cause agranulocytosis.[14,15] Chloramphenicol is the prime example of a drug that causes both dose-related marrow suppression through mechanisms that include mitochondrial inhibition,[16] and idiosyncratic aplastic anemia.[17]

The signs of dose-related toxicity appear more rapidly in patients with hepatic or renal disease because the drug must be inactivated by conjugation with glucuronide in the liver and excreted in the urine. High doses and high plasma levels correlate with the characteristic reversible erythroid depression. In vitro, chloramphenicol inhibits the growth of both colony-forming units granulocyte/macrophage (CFU-GMs) and colony-forming units erythroid (CFU-Es)[18,19,20,21,22] and also may inhibit the hematopoietic microenvironment (HM).[20,23]

Other Drugs

Nonsteroidal antiinflammatory drugs, which are used more extensively in adults than in children, are associated with aplasia.[3] Nonsteroidal antiinflammatory drugs associated by occasional case reports with aplastic anemia include aspirin,[24] indomethacin,[25,26,27,28] and ibuprofen.[29] Several large studies reported an increased risk of aplastic anemia with phenylbutazone[30,31] and identified even higher probabilities with some of the other nonsteroidal antiinflammatory drugs.[3] Cimetidine, another commonly used drug, is associated with a 2 per 100,000 user risk of cytopenias.[32,33] Sulfa-containing compounds, which appear as risk factors in most case-control studies of drugs and aplastic anemia, are used in a wide variety of clinical circumstances.[34,35,36,37,38,39,40,41,42,43] Other drugs implicated in aplastic anemia that are commonly used in the pediatric population include anticonvulsants (hydantoins,[44,45,46] carbamazepine[47,48,49,50,51]) and carbonic anhydrase inhibitors[52] (acetazolamide[53,54] and methazolamide[55,56]). Many of the drugs listed in Figure 6-1 also have been associated with agranulocytosis. In general only a minority of cases of aplastic anemia can be assigned a drug association. The distinction between aplasia secondary to the medication versus aplasia arising from the underlying disorder (or occult viral infection) requiring treatment can be difficult.

Chemicals and Toxins

Benzene

Benzene is a particularly dangerous environmental contaminant.[57,58] It is found in organic solvents, coal tar derivatives, and petroleum products.[59,60,61,62] Fatal aplastic anemia, leukemia, or both, have been reported years later in factory workers who had benzene exposure. Benzene is concentrated in bone marrow fat,[63] forms water-soluble intermediates,[64,65,66] and damages DNA.[67,68,69] It decreases the numbers of progenitors and damages stroma. The risk of cytopenias is likely related to cumulative exposure.

Other Aromatic Hydrocarbons

Toxicity thought to be due to other organic solvents may in some instances be caused by benzene contaminants. Neither pure toluene nor xylene is a marrow toxin. Aplastic anemia has been linked by many case reports to insecticides, particularly γ-hexachlorobenzene (lindane) in children.[70] Aromatic hydrocarbons are present in insecticides and herbicides and may comprise the solvents for these agents. Some organophosphate insecticides have been shown to inhibit in vitro hematopoietic colony formation, as has lindane.[71]

Ionizing Radiation

Marrow aplasia may occur as an acute toxic sequela of irradiation due to nuclear bomb explosion, radioactive fallout, reactor accidents, and accidental exposure in medicine and industry. Bone marrow cells may be affected by high-energy γ-rays as well as by ingested or absorbed lower-energy α particles. The radiation injury is to the actively replicating pool of precursor and progenitor cells and also to stem cells, in which DNA damage may have a more severe effect. Nonetheless, radiation-related marrow aplasia is infrequent. Even in a restricted episode, such as the Chernobyl reactor accident in 1986, most immediate deaths were due to skin burns and damage to gastrointestinal and pulmonary systems.

Chronic radiation-induced aplasia is dose related. Patients who were irradiated for ankylosing spondylitis had an increased risk of aplastic anemia, and American radiologists have been reported to have an increased death rate from aplasia (for both groups, the pathologic distinction of aplasia and myelodysplasia was not made). The incidence of late aplasia in atomic bomb victims was not increased, nor was it increased in patients receiving radiation therapy for malignancies. Knospe and co-workers suggested that irradiation with an exposure greater than 4.4 Gy was required for the development of aplasia; they also postulated that low doses might damage only stem cells, whereas high doses would also damage the supportive hematopoietic stromal microenvironment.[72]

Infectious Agents

Patients with bacterial or viral illnesses frequently develop mild pancytopenia during or following the infection (see Chapter 37). Patients with bacterial or viral infections often receive antibiotics and other medications, and it is frequently not clear whether an ensuing aplastic anemia was caused by the infection, by the drug, or by the combination of the two, or even whether the infectious illness was the result and not the cause of the pancytopenia.

Hepatitis

Although hepatitis is frequently associated with mild depression of blood cell counts, aplasia is a rare sequela, estimated to occur in fewer than 0.07% of the total number of pediatric hepatitis cases[73] and in fewer than 2% of those with non-A, non-B hepatitis.[74] Nonetheless as an identifiable clinical event, a prior episode of hepatitis is recognized in 2% to 5% of aplastic anemia patients in a Western series.[75] The prevalence of prior hepatitis is about twofold this proportion in the Far East.[76] Among children with aplastic anemia in Taiwan, 24% had a history of recent acute hepatitis.[77] Antecedent hepatitis may be subclinical, as about 50% of patients with aplastic anemia may have elevated hepatic transaminases before their first transfusion. In a report of 32 patients with liver transplantation for hepatic failure following non-A, non-B hepatitis, 28% developed aplastic anemia.[78] While aplasia has been reported following both hepatitis A and B virus infections,[79, 80] the majority of cases of the hepatitis/aplasia syndrome are not associated with serotypable hepatitis virus.[81, 82]

A study of aplastic anemia patients reported to the European Registry from 1990-2007 found that 5% of patients with aplastic anemia had an antecedent seronegative hepatitis. Hepatitis-associated aplastic anemia was slightly more common in males and tended to present at a younger age. Actuarial survival after treatment with either immunosuppression or hematopoietic stem cell transplant was similar between patients with hepatitis-associated aplastic anemia versus other acquired aplastic anemia.[83]

Flaviviruses

Flaviviruses cause arbovirus hemorrhagic fevers, dengue, and other hematodepressive syndromes. Dengue can propagate in bone marrow cultures without direct cytotoxicity, and dengue antigens induce lymphocyte activation and the release of marrow suppressive cytokines.[84]

Epstein-Barr Virus

Herpesviruses such as Epstein-Barr virus (EBV) are large, complex DNA viruses.[85] EBV causes infectious mononucleosis, which has pancytopenic complications in less than 1% of cases. More than 12 such cases have been reported, and one half of these cases had a fatal outcome. In one study, EBV was demonstrated by immunologic and molecular methods in the bone marrow of six patients with aplastic anemia.[86] Only two had a history of typical mononucleosis, although all six had serologic evidence, suggesting that EBV may be an unrecognized cause of aplastic anemia. The EBV's target is B cells, although T cells also may be infected. Because EBV is a common infection, issues of ascertainment can render the causative determination of aplasia difficult.

Cytomegalovirus and Human Herpesvirus 6

Infection with cytomegalovirus (CMV) may lead to graft failure in immunosuppressed bone marrow transplant (BMT) recipients.[87] CMV can infect marrow stromal cells in vitro and can inhibit their ability to produce growth factors;[88] direct progenitor cell infection by some CMV strains also has been documented.[88,89] Herpesvirus 6 is the cause of exanthem subitum[90] and, like CMV, may be found in the marrow of patients with graft failure after transplantation,[91] as well as in hematopoietic progenitors infected in vitro.[92] As with other viruses, both of these are ubiquitous infections making causality difficult to ascertain.

Human Immunodeficiency Virus

Patients with acquired immunodeficiency syndrome (AIDS) often have cytopenias,[93] but their marrow is much more commonly cellular and dysplastic than empty. Colony formation by marrow from patients may be diminished.[94,95,96,97] The action of human immunodeficiency virus-1 (HIV-1), a lentivirus, on hematopoietic cells remains a subject of controversy. HIV-1 infection of CD34+ cells has been difficult to detect in vivo from patient material[96,98] or after tissue culture infection of normal cells.[96,99] The virus apparently directly infects megakaryocytes, which bear the CD4 receptor present on T cells.[100] The virus also may affect stroma functions, at least in vitro.[101,102] HIV-1 can act indirectly on hematopoiesis through inhibitory lymphokine production: The envelope glycoprotein can stimulate macrophages to produce tumor necrosis factor (TNF), which in turn inhibits hematopoietic colony formation.[103] Hematologic suppression can also be due to opportunistic infections, neoplasms, or marrow suppression from the drugs used to treat AIDS and its complications.

Other Viruses

Blood count abnormalities, which are rarely severe, may be observed in the course of rubella, measles, mumps, varicella, and influenza A.[104]

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is a disease characterized by variable combinations of mild to severe intravascular hemolysis, large venous thromboses, and aplastic anemia.[105] It is uncommon in adults and even rarer in children (see Chapter 13). There is a clear association of PNH with aplastic anemia: Many patients with PNH develop pancytopenia and marrow hypoplasia, and PNH clones are detectable in up to 50% to 70% of patients with acquired aplastic anemia.[106,107,108,109]

PNH is characterized by an inability to inactivate complement on the erythrocyte cell surface, resulting in increased sensitivity to complement. Deficits were subsequently identified in a family of membrane proteins, all of which were anchored to the cell membrane via glycosylphosphatidylinositol (GPI). GPI binds covalently to specific carboxyl terminal protein sequences and attaches them to cell membrane phosphatidylinositol residues. The genetic defect in PNH was localized to the X-linked phosphatidylinositol glycan class A (PIG-A) gene, whose product functions in the transfer of N-acetylglucosamine to phosphatidylinositol as an early step in GPI anchor formation. Early tests for PNH, such as the Ham test or sucrose hemolysis test, relied on the demonstration of increased sensitivity to complement-mediated red cell lysis. Increased sensitivity and specificity are achieved using flow cytometry assays for the absence of GPI-anchored proteins such as CD59 and CD55.[110] More sensitive tests utilize aerolysin, a channel-forming toxin that binds GPI-anchored proteins to result in cell lysis but leaves the GPI-deficient PNH cells intact.[111] The use of a fluorescently labeled aerolysin variant that binds to GPI but fails to lyse the cells offers increased sensitivity and specificity for the detection of the PNH clone.[112]

The clinical significance of small populations of PNH clones is unclear, and most of the published data are derived from adult patients. Small numbers of PNH cells are detectable in healthy controls,[113] although the PNH cells in this context are typically polyclonal. Despite the frequent finding of PNH clones in the bone marrows of aplastic anemia patients, only 10% to 15% of patients subsequently develop the clinical syndrome of PNH.[114] In the majority of patients, such clones may persist or disappear; however, some patients may develop large symptomatic PNH clones requiring therapeutic intervention.[115,116] Patients with small asymptomatic PNH clones may respond to the same treatments as other patients with aplastic anemia. Patients who develop large PNH clones are at risk for developing symptoms of hemolysis or thrombosis.[115] One recent retrospective study included 47 pediatric patients with aplastic anemia of whom 14 had a PNH clone greater than 1% at baseline (median clone size 16%). The median clone size remained small for all patients, and none of these patients developed clinical symptoms of PNH.[115]

The etiology of marrow aplasia in PNH remains an area of active investigation. Although severe combined immunodeficiency (SCID) mice infused with bone marrow from PNH patients show preferential engraftment with the PNH clones,[117] studies of hematopoiesis in PNH patients have not detected any selective proliferative advantage of the *PIG-A*$^-$ hematopoietic clones.[118] A subsequent study comparing in vitro proliferation of *PIG-A*$^-$ and *PIG-A*$^+$ CD34 cells from PNH patients found a selective growth deficiency in the *PIG-A*$^+$ cell population rather than an advantage for the *PIG-A* mutant cells: Fas expression was elevated on the wild type compared with the GPI-deficient cells, suggesting increased resistance to apoptosis as one potential mechanism to explain their findings.[119] No proliferative advantage of PNH hematopoietic clones was observed in mice mosaic for the *PIG-A* gene.[120,121] To evaluate the hypothesis that autoreactive T cells might preferentially eliminate *PIG-A*$^+$ hematopoietic stem cells while sparing the PNH clones, the sensitivity of normal versus PNH EBV-transformed B-cell lines to autologous EBV-specific T-cell lines was examined.[122] The PNH cells were no less sensitive to T-cell-mediated cytotoxicity than the non-PNH cells; thus the GPI-linked cell surface molecules are not required for killing by T cells. An abnormal distribution of expanded T-cell clones detected by size analysis of the complementarity-determining region 3 (CDR3) in the β-variable region (BV) mRNA of the T-cell receptor (TCR) has been noted in PNH patients,[123] although the targets of such T-cell populations remain to be ascertained. The mechanisms underlying the clonal expansion of the GPI-negative cells in PNH or aplastic anemia is currently unclear.[124] Despite the presence of clonal populations of PNH cells, patients with PNH do not exhibit an increased incidence of leukemias, and the GPI-negative clones do not behave in a malignant fashion.

Immunologic Diseases

Ten percent of patients with the rare collagen vascular syndrome, *eosinophilic fasciitis,* have associated aplastic anemia.[125] This condition is usually limited to adults. Thymoma associated with hematopoietic failure generally but not exclusively presents as pure red cell aplasia.[126] Some of the adult cases had also received drugs such as chloramphenicol or sulfonamides, and the aplasia could have been due to the drugs alone or to the combination. An iatrogenic aplasia can be induced by the transfusion of competent lymphocytes into immunodeficient hosts. In these cases, marrow failure arises from the transfusional graft-versus-host disease (GVHD).[127]

Pathophysiology

The purported mechanisms for failure of hematopoiesis are predicated on our current knowledge of normal hematopoiesis (see Chapter 7). Disease could result from decreased numbers or defective function of the cellular or soluble components required for blood cell production or from exogenous factors that result in damage or elimination of hematopoietic cells. Figure 6-2 depicts different potential mechanisms for the loss of hematopoietic cells in aplastic anemia. In model I, marrow toxins such as irradiation, drugs, or chemicals or direct invasion by viruses might cause hematopoietic stem and/or progenitor cell death. In model II, an underlying abnormality of the hematopoietic cells may result in a predisposition to hematopoietic stem cell damage, premature stem cell loss, or insufficient stem cell production (see Chapter 1). In model III, viruses, drugs, toxins, or immune dysregulation may incite a cellular or humoral immunologic reaction against a normal hematopoietic compartment. In model IV, abnormalities of the marrow stromal environment may actively inhibit hematopoiesis. These possibilities are not mutually exclusive, and supportive data for each model have been described. Current data suggest that aplastic anemia may represent the final seemingly common outcome of different pathogenic mechanisms, with different underlying causes entailing different medical management considerations.

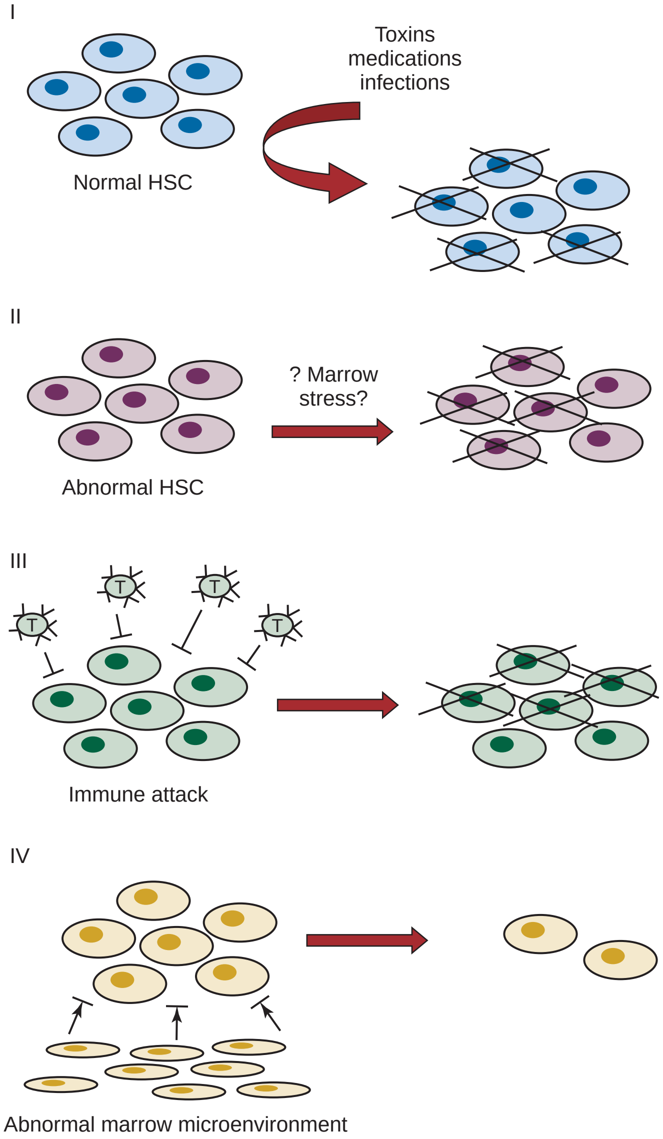

Figure 6-2 Models for the pathogenesis of aplastic anemia. I. Healthy hematopoietic stem cells and progenitors are damaged by exogenous agents such as toxins, medications, or infectious pathogens resulting in marrow aplasia. II. Abnormal marrow cells (e.g., inherited marrow failure syndromes) undergo premature attrition. Marrow aplasia might be exacerbated by external factors. III. Immune-mediated attack (cellular or humeral) eliminates hematopoietic stem cells and progenitors. IV. Abnormal marrow microenvironment impairs hematopoiesis. *HSC,* Hematopoietic stem cell.

Hematopoietic Stem Cells and Clonality

Patients with aplastic anemia have decreased numbers of blood and marrow committed progenitor cells, assayed as myeloid colony-forming cells (CFU-GMs); erythroid colony-forming cells (CFU-Es and burst-forming units erthyroid [BFU-Es]); megakaryocytic progenitors (CFU-megas); multipotent colony-forming cells (CFU granulocyte, erythrocyte, monocyte, megakaryocyte [CFU-GEMMs]); and *long-term culture-initiating cells* (LTC-ICs).[128,129] Hematopoietic cells can also be assessed phenotypically for the presence of the CD34 antigen, which defines a compartment of about 1% of marrow cells that includes progenitors and possibly activated stem cells.[130,131,132,133] CD34+ cells are reduced in aplastic anemia, and almost all patients show not only severe reduction in the numbers of these cells but also poor plating efficiency for colony formation from purified CD34+ cells.[134,135]

Several lines of evidence point to primary abnormalities in the hematopoietic stem cell as a potential etiology for aplastic anemia. Multiple studies have noted that simple infusion of stem cells from an identical twin donor without prior conditioning was sufficient to treat the aplasia in many cases.[136,137,138,139] These results strongly support a pathogenic stem cell defect and argue against permanent disorders of the bone marrow environment, including immunologic mechanisms, as the cause of stem cell destruction. Low stem cell numbers persist even with hematologic recovery after medical therapy with antithymocyte globulin (ATG) and cyclosporine (see following discussion), and blood cell parameters, such as macrocytosis, do not return to normal in many cases, again suggestive of an underlying stem cell abnormality. A primary stem cell defect likely also contributes to the observation of clonal hematopoiesis in aplastic anemia[140] as well as late development of other hematologic diseases such as myelodysplasia and acute leukemia (see following discussion).

A subset of patients with seemingly idiopathic aplastic anemia may actually carry an inherited/congenital predisposition to marrow failure (see Chapter 7). Although the inherited/congenital marrow failure syndromes are often associated with characteristic physical findings, patients may present with isolated aplastic anemia as the sole manifestation of their underlying inherited syndrome.[141] In some patients lacking significant physical findings or cytopenias at the time of evaluation, an elevated mean corpuscular volume (MCV) may be the only clue to an underlying inherited marrow failure syndrome. A careful family history for hematologic abnormalities, malignancy predisposition, or other characteristic clinical stigmata may be helpful in distinguishing an inherited marrow failure syndrome.[142] For example a subset of patients presenting with aplastic anemia without other clinical stigmata of an inherited marrow failure syndrome were found to harbor mutations in the dyskeratosis congenita genes *hTERC,*[143,144] which encodes the RNA component of telomerase, or hTERT,[145,146,147] which encodes the catalytic subunit of the telomerase complex. Telomeres are specialized structures that stabilize the ends of each chromosome to prevent excessive shortening with replication, to distinguish chromosome ends from internal DNA breaks, and to prevent end-to-end fusions. Telomeres consist of six–base pair repeated sequences (TTAGGG) associated with a specific protein complex. Telomerase is a ribonucleoprotein enzyme consisting of an H/ACA RNA component (TERC) complexed with a reverse transcriptase (TERT) and additional H/ACA binding proteins including dyskerin, GAR1, NHP2, and NOP10. When telomerase levels are diminished or absent, telomeres progressively shorten with each cell division. At a critically short telomere length, a checkpoint is triggered for cells to stop dividing and undergo senescence to prevent chromosomal rearrangements.

Shortened telomeres have been observed in the leukocytes of aplastic anemia patients.[148] Of five patients noted to have mean telomere lengths less than 5 kb, three had acquired cytogenetic abnormalities, suggesting that compromise in the telomere's probable role in stabilizing chromosome ends may contribute to the increased incidence of myelodysplasias and acute leukemias. A study by Brummendorf and colleagues reported relatively normal telomere lengths in patients who responded to immunosuppression, while untreated or unresponsive patients showed significant telomere shortening.[149] Telomere shortening is a common feature associated with marrow failure, though telomere lengths are particularly short in patients with dyskeratosis congenita, a disorder of telomerase function, even in comparison to other marrow failure patients.[150] Whether telomere shortening in marrow failure patients lacking telomerase mutations represents a primary defect in telomere maintenance or a secondary effect of increased cycling of the few remaining hematopoietic stem cells remains to be clarified. Shorter telomere lengths have been associated with a higher risk of relapse and clonal progression with decreased survival in patients with aplastic anemia.[151]

Immune Destruction

Several lines of data suggest that immune phenomena may result in aplastic anemia.[152] In some syngeneic (identical twin) BMTs, simple infusion of stem cells is not sufficient to reconstitute hematopoiesis. The requirement for prior conditioning of the host may reflect some operational immunologic process, though it is also consistent with the presence of an underlying clonal abnormality that requires ablation.[153] Histocompatibility studies conducted in Europe, Asia, and America have indicated an increased representation of HLA-DR2 among aplastic anemia patients, consistent with a genetically determined immune susceptibility to disease.[153,154,155] Evidence for specific immune mechanisms is reviewed below.

Lymphocytes

Lymphocyte infusions in mouse models result in marrow destruction and aplastic anemia.[156] Autologous recovery has been reported after either mismatched or matched transplants in patients prepared with antilymphocyte sera or cyclophosphamide.[157,158,159] While the etiology of aplastic anemia in children remains unclear, evidence supports an immunologic mechanism of disease in the absence of known environmental toxins, drugs, and constitutional syndromes. Overriding these considerations are the large proportion of responses in acquired aplastic anemia to immunosuppressive therapies, although the antilymphocyte preparations contain heterogeneous mixtures of antibodies to many cells, including lymphocytes, and are clearly immunosuppressive as well as generally cytotoxic. Cyclosporine exerts inhibitory T cells effects and inhibits transcription of genes for cytokines including IL-2 and interferon-gamma (IFN-γ).[160] It has many other toxic effects on several tissues, including brain and kidney. The diverse antibodies in ATG react with a wide range of antigens, including signal transduction and adhesion molecules.[161] Thus the attribution of immune dysregulation as causal in aplastic anemia based on therapy must be tempered with caution because ATG and cyclosporine exert multiple effects in addition to immunosuppression. Of note, therapies with mixtures of monoclonal antibodies specific for human T cells have not been effective in clinical trials to date[162] (see following discussion). Furthermore ATG can increase colony growth in normal, myelodysplastic syndrome (MDS), and aplastic anemia CD34+ bone marrow cells in culture.[163,164] ATG treatment also reduces the expression of Fas-Ag on aplastic anemia bone marrow CD34+ cells.[165] Effective treatment of myelodysplasia with immunosuppressive medications has been reported[166] and may suggest that such agents might exert additional effects on the bone marrow.[167]

Nonetheless multiple reports have demonstrated an association of aplastic anemia with alterations in lymphocyte numbers or specific immunologic changes at presentation and in some cases further changes with hematologic responses. Hoffman and colleagues showed inhibition of erythroid growth in vitro utilizing coculture of peripheral blood lymphocytes from five out of seven aplastic anemia patients studied. [168] Zoumbos and colleagues noted increased circulating and bone marrow levels of IFN-γ in aplastic anemia patients and a response of increased colony growth in vitro in response to inhibition with neutralizing antibody to this proinflammatory cytokine.[169] Hinterberger and colleagues failed to confirm the reported increased circulating levels of proinflammatory cytokines in aplastic anemia patients, but did demonstrate increased TNF and IFN-γ production by peripheral blood mononuclear cells (PBMCs) from aplastic anemia patients at baseline and after stimulation in vitro.[170] Risitano and colleagues reported a dominant clonal T-cell immune response characterized by flow cytometry using antibodies against different TCR-β subfamilies and by sequencing the CDR3 region of the TCR-β chain in 54 patients studied with aplastic anemia.[171] The size of these clones and the total number of T cells decreased following successful treatment with ATG and increased with relapse. This phenomenon was associated with HLA-DR1501 genotype and activated cytotoxic T cells assayed in vitro. The association with HLA type was not confirmed by a subsequent study of Japanese patients.[172] Solomou and colleagues reported reduced T regulatory cells (TRegs) (CD4+, CD25+, FoxP3$^+$) in 20 patients with aplastic anemia that was consistent with increased IFN-γ and a TH1 polarization phenotype of T cells.[173] Six of seven patents showed slightly increased numbers of TRegs after immunosuppression therapy (IST) during hematologic responses. In a case report, Ikeda and colleagues noted marked alteration of the CD4/CD8 T-cell ratio after hepatitis that was subsequently followed by onset of aplastic anemia.[174] IST was followed by normalization of lymphocyte subsets with hematologic recovery.

These studies imply concomitant immune dysregulation with aplastic anemia and suggest a mechanism by which IST could be working therapeutically. However, no specific antigen has previously been implicated mechanistically in the evolution of aplasia. In a recent report, it was shown that a prodrug of the anti-HIV drug, abacavir, which is associated with a hypersensitivity reaction in treated patients, can bind to the HLA-B*57:01 molecule, resulting in altered peptide presentation.[175] The altered peptides appear as foreign and lead to activation of CD8+ T cells. One can imagine such a scenario leading to acquired aplastic anemia if the abnormal peptide presentation of "self-antigens" leads to CD8 activation directed against hematopoietic stem cells in the bone marrow. While such a finding is not reported yet in aplastic anemia patients, this could underlie the immunologic mechanism long hypothesized as the etiology of this disease. Interestingly Weinstock and colleagues also recently demonstrated somatic alterations in 20% of adult patients with acquired aplastic anemia utilizing next generation sequencing technology.[176] Alterations included mutations of *DNMT3A* and *ASCL1,* both associated with MDS in adults, and mutations of β_2-microglobulin, which is required for class I expression and CD8 T-cell function. Thus previously unrecognized mutations may predispose to acquired aplastic anemia via either mechanisms leading to a prodrome of myelodysplasia or by subtle alternations in immune functions. Limitations of these studies include the paucity of purified cell populations required for sequencing and the lack of functional data that addresses the biologic relevance of these alterations with respect to stem cell function and hematopoiesis. Additional studies will undoubtedly follow.

Lymphokines

Whether overproduction or dysregulated production of inhibitory cytokines represents a primary etiology versus a secondary effect of an underlying bone marrow abnormality remains unclear. IFN-γ, a soluble inhibitor produced by T cells, was found to be produced at high levels in cultures from aplastic anemia patients.[177,178,169] IFN-γ is expressed in the marrow of most patients with aplastic anemia but not in normal people or in patients with other hematologic diseases who have undergone frequent transfusions.[179,180] High pretreatment intracellular γ-interferon levels correlated with response to immunosuppressive therapy.[181] Other inhibitory cytokines, such as *TNF*[182] and *macrophage inflammatory protein-1,*[183,184] also are overexpressed in aplastic marrow. Both interferon and TNF directly and synergistically inhibit hematopoiesis in vitro.[185] In long-term bone marrow culture, constitutive low-level expression of IFN-γ is sufficient to markedly reduce the output of committed progenitor cells and LTC-ICs, the stem cell surrogate.[186] Both interferon and TNF increase the potential for programmed cell death within the CD34+ compartment by increasing Fas antigen expression on target cells.[187] Fas antigen is a cell surface molecule in the TNF receptor family; its activation signals apoptosis.

Microenvironment

In theory aplastic anemia could be due to a microenvironment that fails to support hematopoiesis: a lesion of "soil" rather than "seed."[72] The role of the HM in etiology of aplastic anemia or the response to treatment remains controversial. Ershler and colleagues showed decreased erythroid growth in whole bone explants from a single patient with constitutional red cell aplasia but no effect from the patient's plasma, mononuclear cells, or T cells, implying an HM defect.[188] This observation is reminiscent of the HM defect seen in *Steel* mice,[189] which lack production of normal stem cell factor (SCF) in nonhematopoietic cells, but it seems more likely that this was simply a demonstration of a cell-intrinsic erythroid cell defect. Dexter and colleagues described an in vitro long-term marrow culture (LTMC) that consisted of an adherent cell compartment made up of a number of different cell types that supported hematopoiesis for weeks to months ex vivo.[190] Subsequently Marsh and colleagues examined LTMC from 32 adult aplastic anemia patients and found 31 of the 32 had abnormal hematopoiesis in these cultures.[191] However, using cross-over cultures, only 1 of 23 patients had defective adherent components. Juneja and colleagues demonstrated reduced cobblestone areas (containing active hematopoiesis) but normal adherent cell fractions in eight aplastic anemia patients compared with normal controls.[192] Marsh and colleagues utilized human LTMC and assessed the HM in cross-over experiments.[193] They demonstrated that stroma generated from aplastic anemia patients performed normally when inoculated with CD34+ cells from normal donors. Nissen and colleagues compared short-term stroma and progenitor colony growth in aplastic anemia patients treated with or without IST and found a modest increase in both of these cell fractions in treated versus untreated patients.[194] Chatterejee and colleagues used a cytotoxic drug animal model of aplasia to demonstrate failure of recovery of marrow stromal cells with time.[195] While this study may be relevant to some isolated instances of acquired aplastic anemia, it seems more relevant to marrow damage in cancer or leukemia patients undergoing therapy. Shipounova and colleagues showed impaired adipogenesis and osteogenic potential with increased size and number of fibroblast colonies from aplastic anemia patients' bone marrow.[196] These authors also demonstrated increased stromal-derived cytokines, including ANG1, VEGF, and VCAM-1. Overall these studies suggest some alterations in the HM of aplastic anemia patients, but there is currently no definitive evidence in humans of a mechanistic link between defective marrow microenvironment and development of acquired aplastic anemia.

More recently interest has focused on defining specific anatomic and/or biochemical spaces (niches) in the bone marrow occupied by hematopoietic stem cells. Schofield first termed the phrase *stem cell niche* to define a supporting cellular environment in which hematopoietic stem cells reside.[197] Currently controversy exists about the overall relevance of the two best-studied stem cell niches.[198] Hematopoietic stem cells appear to reside in close proximity to osteoblasts that line the cortical bone of the marrow space, and osteoblasts may be important in maintaining quiescence of the primitive hematopoietic stem cells. This area of the marrow has thus been termed the *osteoblastic niche*. A subset of these cells, spindle-shaped N-cadherin$^+$ CD45$^-$ osteoblasts (SNO cells) residing in trabecular bone, may be derived from mesenchymal stem cells, and their number correlate with the content of long-term hematopoietic stem cells.[199] Ablation of osteoblasts leads to loss of HSC[200] and failure of hematopoiesis, while manipulations that increase osteoblasts have the opposite effect.[201]

An anatomically distinct putative hematopoietic stem cell niche is made up in part by sinusoidal endothelial cells and has been termed the *perivascular niche*.[202] Some data suggest this niche may be critical for mobilization and homing of hematopoietic stem cells via the Rac GTPase pathway[203,204] and/or for progenitor cell proliferation and differentiation,[205,206] although these studies have not examined long-term hematopoietic stem cells in functional assays. Alterations of the sinusoidal endothelium with antibodies to VE-cadherin leads to failure of megakaryocytopoiesis in murine models.[205] Some models suggest that quiescent hematopoietic stem cells may migrate from the osteoblastic niche to the perivascular niche via β1 integrin function and chemokine/growth factor stimulation. Overall, whether there are truly distinct functional differences between these putative hematopoietic stem cell niches remains to be determined, and the role of specific growth factors, adhesion molecules, and chemokines are incompletely understood. A long-term goal of investigators in this field is to recapitulate the HM in vitro such that true expansion of hematopoietic stem cells may occur in the laboratory setting. Success in this area would have a major impact in BMT and regenerative medicine.

Hematopoietic growth factor production and plasma levels are usually increased rather than decreased in patients with aplastic anemia.[207] Circulating levels of erythropoietin, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF), thrombopoietin, and flt-3 ligand are elevated in patients with aplastic anemia. SCF levels have been reported to be low to normal. Levels of interleukin 1 (IL-1), produced by monocytes, may be low in aplastic anemia. Therapeutic trials with these factors have yielded divergent and incomplete responses,[208] casting doubt on the pathophysiologic significance of deficiency of these factors. The report of inhibitory effects of marrow adipocytes on hematopoiesis in mouse models is intriguing given the typical fatty replacement observed in aplastic bone marrows.[209]

Clinical Evaluation

The differential diagnosis of pancytopenia is broad (Box 6-3). The evaluation and diagnostic work up of the patient with presumed acquired aplastic anemia should be directed at eliminating alternative diagnoses for which curative therapies are available or for which the therapies for aplastic anemia would be inappropriate (Table 6-1). Because the inherited marrow failure syndromes entail different medical management issues, a careful medical history, family history, physical examination, and appropriate laboratory evaluation to assess for underlying inherited bone marrow failure syndromes should be pursued. The diagnosis of an inherited marrow failure syndrome also carries profound implications for hematopoietic stem cell donor selection from family members and may be important for family planning. Given the wide clinical variability of the inherited marrow failure syndromes even amongst members of a given family, all siblings of a patient with an inherited marrow failure disorder should be tested whether or not they appear to carry clinical stigmata of the disease.

History

The chief presenting symptoms relate to low blood counts. Bleeding, such as gum oozing, nosebleeds, easy bruising with minimal trauma, or heavy menses may be seen. Chronic anemia may present with fatigue, decreased

Box 6-3 Causes of Pancytopenia

HYPOCELLULAR BONE MARROW

Acquired aplastic anemia
Inherited bone marrow failure syndrome
(Fanconi anemia, amegakaryocytic thrombocytopenia, dyskeratosis congenita, Shwachman–Diamond syndrome, GATA-2 syndromes)
Paroxysmal nocturnal hemoglobinuria*
Hypoplastic myelodysplastic syndrome
Virus-associated aplastic anemia

CELLULAR BONE MARROW

PRIMARY BONE MARROW DISEASE

Myelodysplasia
Paroxysmal nocturnal hemoglobinuria*

SECONDARY TO SYSTEMIC DISEASE

Systemic lupus erythematosis, Sjögren syndrome
Hypersplenism
Vitamin B_{12}, folate deficiency
Infection
Storage disease (Gaucher, Niemann-Pick)
Alcoholism
Sarcoidosis
Infiltrative bone marrow disorders
Acute myelogenous leukemia
Acute lymphoblastic leukemia
Hemophagocytic lymphohistiocytosis
Metastatic solid tumors
Osteopetrosis
Myelofibrosis

activity, or exercise intolerance, though the gradual onset of severe anemia can be surprisingly well compensated in young children. Serious infection is not a frequent presenting symptom early in the course of aplastic anemia. Many patients feel well with few symptoms on initial presentation. A history of steatorrhea or fatty food intolerance may be a manifestation of Shwachman–Diamond syndrome, though the absence of these symptoms does not rule out this diagnosis.[210] Gastrointestinal disorders may also be associated with other inherited marrow failures syndromes such as dyskeratosis congenita or Fanconi anemia. A careful family history for blood disorders, malignancies (particularly ovarian and breast cancer or hepatocellular carcinoma), premature graying, hepatitis, pulmonary fibrosis, or congenital anomalies that might suggest an inherited bone marrow syndrome should be sought. Patients with a history suggestive of a familial marrow failure syndrome or familial MDS should be evaluated further with genetic and laboratory studies. A developmental history can provide a helpful screen for potential congenital, metabolic, or storage diseases. A detailed medication, environmental, and infectious history, particularly for the period 1 to 12 months prior to presentation, should be obtained.

Physical Examination

Clinical appearance is related to the severity and duration of the underlying pancytopenia. Hemorrhagic manifestations from thrombocytopenia occur early and include petechiae (particularly on the border of the hard and soft palate), ecchymoses, epistaxis, and bleeding from mucous membranes. Neutropenia may be associated with oral ulcerations, bacterial infections, and fever; these signs are rarely present early. Evidence of erythropoietic failure characterized by pallor, fatigue, and tachycardia is often late because the life span of the erythrocyte (120 days) far exceeds that of platelets (10 days) or white cells (variable, but measured in hours for granulocytes). Mucous membranes and nail beds may be pale. Lymphadenopathy, splenomegaly, and severe weight loss are uncommon and may suggest other underlying disorders. Short stature, congenital anomalies (particularly of the thumbs and forearms), areas of hyper- or hypopigmentation, dystrophic nails, immunologic abnormalities, or pulmonary disease should alert the examiner to possible inherited bone marrow failure disorders. Immunologic abnormalities or lymphedema raises consideration of inherited GATA-2 syndromes (see Chapter 24).

Laboratory Studies

Evaluation should include a complete blood count and reticulocyte count. A careful evaluation of the peripheral blood smear may suggest infection or dietary deficiency. Blood counts are most often uniformly depressed. The blood smear shows a paucity of platelets and leukocytes. The anemia is often macrocytic and particularly useful in pointing to a possible constitutional syndrome, though if erythropoiesis is entirely arrested, it may be normocytic. The red cell distribution width (RDW), a numeric measure of anisocytosis, is most often normal. Platelet size is not increased, as it is in most cases of immune thrombocytopenias. The absolute reticulocyte count is decreased. Granulocyte numbers are clearly diminished, as may be those of monocytes and lymphocytes.[211,212] Increases in fetal hemoglobin (Hb F) and red cell *i* antigen are manifestations of the fetal-like erythropoiesis seen in stress hematopoiesis[213] but occur irregularly in aplastic anemia patients, have no prognostic value, and can persist in recovered patients.

Laboratory evaluation for possible infectious etiologies should be obtained as clinically indicated. Additional testing for possible immunologic or autoimmune diseases may be considered. Blood tests to rule out Fanconi anemia and PNH are important, as these diagnoses would entail alternative treatments. Telomere length measurements may be useful as a screen for possible underlying telomere disorders. Very short telomeres in long-lived lymphocyte populations should prompt consideration of additional genetic workup for dyskeratosis congenita. Data on the use of telomere measurements in isolation to guide clinical treatment decisions in this setting are sparse. A growing array of genetic testing is available to assist in the diagnosis of many of the inherited marrow failure syndromes (see Chapter 7). Patients with stigmata of collagen vascular disease should be appropriately evaluated. Early HLA typing of the patient and family members is helpful in guiding future therapies. Baseline renal and hepatic laboratory evaluations are also indicated prior to the initiation of potentially nephrotoxic or hepatotoxic therapies.

Bone marrow examination must be done by both aspiration and biopsy so that cellularity can be evaluated

TABLE 6-1 Clinical Evaluation[340]

Clinical History	**Rationale**
Recent illnesses	Infectious etiology
Medications	Marrow suppression
Toxic exposures	Marrow suppression
Family history of blood disorders, malignancy, congenital anomalies	Assess for inherited marrow failure
Physical Examination	
Vital signs	Clinical sequelae of cytopenias
Height and weight	Assess for inherited marrow failure
Congenital anomalies, stigmata	Assess for inherited marrow failure Assess for other systemic diseases
Laboratory Tests	
Complete blood count and differential	Defines severity
Morphology	Malignant versus benign Vitamin B_{12} deficiency Storage disease
Reticulocyte count	Defines severity Differentiate production versus destruction
Bone marrow biopsy	Assess cellularity Assess architecture (granuloma, fibrosis, hemophagocytosis, infiltrative or metastatic disease)
Bone marrow aspirate	
Morphology	Malignant versus benign Storage disease Hemophagocytosis Congenital disorder
Cytogenetics	Myelodysplasia
Culture	Infectious agent (e.g., tuberculosis, virus)
Other	DNA/antigen-based viral tests
Peripheral blood	
Chemistry	
AST, ALT, GGT, Bilirubin, LDH	Hepatitis
Coombs test	Autoimmune cytopenias
BUN, creatinine, electrolytes	Chronic renal failure
Serologic testing/PCR	Hepatitis, EBV, HIV, other virus
Flow cytometry for CD55/CD59	Paroxysmal nocturnal hemoglobinura
Diepoxybutane or mitomycin C chromosomal breakage	Fanconi anemia
Telomere length, dyskeratosis congenita	
Autoimmune disease evaluation	Evidence of collagen vascular disease
Histocompatibility testing of patient and family	Establish potential donor pool

ALT, Alanine aminotransferase; *AST,* aspartate aminotransferase; *BUN*, blood urea nitrogen; *GGT*, γ-glutamyl transferase; *LDH*, lactate dehydrogenase.

both qualitatively and quantitatively. The bone marrow in aplastic anemia is hypocellular, with empty spicules, and increased fat, reticulum cells, plasma cells, and mast cells. In young children there may be residual lymphocytes that appear prominent due to the lack of myeloid elements in the marrow cavity. Some dyserythropoiesis, with megaloblastosis and nuclear-cytoplasmic asynchrony, may be seen. Aspirates alone may appear hypocellular owing to dilution with peripheral blood, or they may look hypercellular because of areas of focal residual hematopoiesis. Biopsies provide more reliable specimens for assessment of cellularity. Biopsies also provide critical information regarding bone marrow architecture such as fibrosis or granulomas. Bone marrow aspirates should also be sent for karyotype analysis and fluorescence in situ hybridization (FISH) to assess for clonal cytogenetic abnormalities associated with myelodysplasia or malignancy. While FISH studies are more sensitive, they can only detect the specific chromosomal abnormality tested. Bone marrow may also be sent for culture or DNA-based antigen detection of infectious agents such as viruses, though interpretation is tempered by the frequent persistence of viral DNA long after the active infection has passed.

Management and Outcome

Prognosis

Outcome depends on the types of treatments offered, the causes of the aplasias, and the eras and countries being analyzed. In a large series conducted before 1957, Wolff found a survival rate of only 3%.[214] In other series ending no later than 1965, complete recoveries were reported in 10% to 35% of patients. In a series of 40 pediatric patients seen before 1958, Shahidi and Diamond found that only two patients recovered, one of whom later had recurrence,[215] though these studies may have included patients with moderate disease for whom spontaneous recovery is more common.[216,217] Long-term prognosis is adversely affected by delayed complications such as clonal disease. The clinical course of 24 pediatric patients with moderate aplastic anemia was followed for a median of 66 months (range 10 to 293 months).[218] Moderate aplastic anemia was defined as marrow hypoplasia of less than 50%, at least two cytopenias such as an absolute neutrophil count (ANC) less than 1500/mm^3, platelet count less than 100,000/mm^3, or reticulocytopenic anemia lasting at least 6 weeks. Sixteen patients (67%) progressed to severe aplastic anemia, five patients (21%) remained in the moderate range, and three patients (12%) resolved. It is currently unclear whether early institution of therapy for moderate aplastic anemia confers improved outcome. Because current therapies are associated with significant side effects, their institution for children with moderate aplastic anemia, for whom the marrow aplasia may spontaneously resolve, warrants careful consideration.

Spontaneous Recovery

Spontaneous recovery has been reported anecdotally, although most cases were probably of moderate rather than of severe aplastic anemia. Good supportive care (see following discussion) may contribute to this; in one series, 14 of 33 children recovered,[219] and children with moderate aplastic anemia have been found to have an excellent prognosis even without specific treatment.[220] A study of immunosuppressive therapy in which 21 patients were randomized (some to receive only supportive care) found no improvement in the control group after 3 months.[221] Spontaneous recovery is sufficiently rare in severe aplastic anemia that all modalities of current therapy should be initiated.

Supportive Care

Blood product support should be used judiciously to avoid sensitization, and blood product donors should never be from the patient's family for the same reason. Complete red cell phenotyping should be considered where feasible to minimize the potential risk of erythrocyte antibodies in patients who may receive long-term blood product support. All blood products should be irradiated because standard treatments are associated with profound immunosuppression. Leukoreduction appears prudent to reduce the potential risk of infection transmission and allosensitization because aplastic anemia patients may require a hematopoietic stem cell transplant. Exposure to potentially hazardous drugs or toxins should be eliminated. Treatment with single hematopoietic growth factors, such as granulocyte-colony stimulating factor (G-CSF) or erythropoietin should be avoided in part due to the resulting delay in definitive therapy.[222]

Bleeding

Platelet (and red cell) support has probably had the greatest impact on survival in aplastic anemia and has changed the cause of death from bleeding to infection. Platelets can be provided from several individual units of blood or, preferably, by platelet pheresis from a single donor to reduce antigenic exposure (see Chapter 36).

The main role of prophylactic platelet support is reduction of the risk of intracranial hemorrhage, which is a rare but potentially devastating event. The general threshold platelet count has traditionally been 20,000 cells/μL; however, this figure and the entire practice of prophylactic administration of platelet transfusions have recently been questioned.[223,224,225] Platelet transfusion at platelet counts below 10,000/μL in stable outpatients with chronic severe aplastic anemia was feasible and safe.[226] Chronic platelet transfusions for aplastic anemia are generally given when there are symptoms of bleeding or if the patient is at increased bleeding risk (e.g., toddlers learning to walk, patients with hypertension while on cyclosporine, patients with fever and infection). In some institutions a higher platelet transfusion trigger point of 20,000/μL is used during initiation of IST.

Other measures to reduce bleeding include maintenance of good dental hygiene, use of a soft toothbrush, stool softeners, and avoidance of trauma. Drugs that may interfere with platelet function, such as aspirin or nonsteroidal antiinflammatory drugs, should be avoided in thrombocytopenia. Antifibrinolytic agents such as aminocaproic acid and tranexamic acid may decrease bleeding, particularly from the gums and nasal mucosa.

Infections. There are few reported studies of infection in aplastic anemia.[227,228] Severe granulocytopenia may last for years, and infection remains the leading cause of death in aplastic anemia patients, particularly bacterial and fungal infections.[229,230] However, the immune systems of granulocytopenic patients remain intact; this is in contrast to the situation of patients with malignancies who receive chemotherapy and who experience simultaneous neutropenia and immunosuppression (as during IST for aplastic anemia). Neutropenia (and perhaps monocytopenia) increases the risk of bacterial infection in aplastic anemia.[211] Because neutropenia precludes the development of an inflammatory response, identification of an infected locus is often difficult. The bacterial organisms may be gram-negative bacilli such as *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa,* as well as gram-positive cocci, including *Staphylococcus aureus* and *S. epidermidis* and streptococci. Use of G-CSF has been studied in a few prospective analysis and other randomized trials, and these studies have been examined by meta-analysis. Gluckman and colleagues randomized aplastic patients receiving IST to prophylactic G-CSF treatment or not.[231] These investigators reported enhanced recovery of neutrophils in the G-CSF group but no effect on overall survival. More recently, Tichelli and colleagues randomized 192 aplastic patients to ATG/cyclosporine +/– G-CSF and reported significantly fewer infectious episodes and hospitalization days in the G-CSF group but no differences in overall survival or event-free survival between groups.[230] Interestingly they found that the lack of neutrophil response at day 30 post initiation of treatment was significantly associated with a lower response rate overall and a lower survival rate. They postulated that if confirmed by additional studies, the lack of neutrophil recovery at 30 days posttreatment could identify a group of patients in which alternative treatment might begin earlier than in the past. This study did not confirm a prior study by Teramura and colleagues that reported a lower incidence of relapse in patients treated with G-CSF.[232] Meta-analysis of a large number of studies has also reported that in aggregrate these studies showed no effect of G-CSF on overall survival, hematologic responses, or specific infections and no association of the use of G-CSF with MDS, AML or PNH.[233]

Immunosuppression due to preparation for BMT or as primary therapy for the aplastic anemia may lead to unusual bacterial, fungal, viral, and protozoan infections. Recommendations for specific antibiotics and other anti-infection agents are beyond the scope of this chapter, and regimens are changing rapidly as new generations of treatments are developed.

The use of routine prophylactic antibiotics has no demonstrated role in the “well” patient with aplastic anemia. In many institutions, guidelines are derived from experience in neutropenic cancer and post–BMT patients because prospective studies in pediatric aplastic anemia patients are rare.[229] In the opinion of many, even without supporting outcomes data, aplastic anemia patients with ANC less than 500 should receive antibacterial and antifungal prophylaxis. Patients undergoing IST are typically given prophylaxis for *Pneumocystis carinii*, although data supporting the need for this treatment are sparse or nonexistent. In the context of fever and neutropenia, complete evaluation and cultures of all possible sites should be followed by the administration of broad-spectrum parenteral antibiotics. Fungal infections occur frequently in neutropenic patients who have received repeated or extended courses of antibiotics; candidiasis and especially aspergillosis, which lead to sinusitis, lung disease, or disseminated infection, are distressingly frequent causes of death in aplastic anemia.[228] Antecedent or concurrent administration of steroids increases this risk. Aggressive introduction of antifungal treatment is indicated in the persistently febrile patient who is unresponsive to antibiotics or in the appropriate clinical setting.

Anemia. Red cells should be provided as clinically indicated. The hemoglobin should be maintained at a level consistent with normal activities if routine transfusions are to be used. Chronic anemia can be well tolerated once adaptation has occurred, and some children who can sustain a hemoglobin level greater than 6 to 7 g/dL without transfusions (i.e., a child who is not bleeding) may be carefully observed. Long-term transfusions lead to iron overload, with accumulation in critical organs. Permanent damage to heart, liver, and endocrine glands from iron overload may be prevented by iron chelation therapy.[234]

Treatment

Hematopoietic Stem Cell Transplantation. Hematopoietic stem-cell transplant offers curative therapy for aplastic anemia. The general topic of BMT is described in Chapter 8. The role of BMT in aplastic anemia treatment is discussed in Chapter 8.

Immunosuppression

Antithymocyte Globulin. *Antilymphocyte globulin* (ALG) and ATG (the sera produced by immunization of horses or rabbits with human thoracic duct lymphocytes or thymocytes) are highly cytotoxic reagents with activity against all blood and marrow cells, including progenitors. These reagents were initially used to decrease the rate of rejection of HLA-matched bone marrow. ALG and/or cyclophosphamide were also employed to permit at least temporary engraftment with mismatched marrow in aplastic patients without a matched donor.[157,158,159,235,236] Autologous recoveries occurred in some of these patients; they encouraged several groups to examine the use of ATG or ALG with and without haploidentical marrow. Treatment with ALG was superior to controls in both response rate and 3-year survival.[221] The addition of additional agents to ALG therapy can further enhance therapeutic effect.

ATG or ALG/ALS is often given to a total cumulative dose of 100 to 160 mg/kg, typically divided over 4 days. Immediate allergic reactions to ALG are rare and can be predicted by skin testing followed by desensitization in those who are allergic.[237] The patient begins to make antibodies to horse protein about 1 week after first exposure; at this point, equine antibodies are rapidly cleared from the circulation. This reduces the effective dose and enhances immune complex formation. *Serum sickness* due to immune complex deposition may manifest around 11 days after initiation of treatment as fever, a serpiginous rash at the volar–dorsal border of the hands and feet, arthralgia, myalgia, lassitude, and changes in urine sediment. Bronchospasm or liver chemistry abnormalities may also occur. A short course of corticosteroids is usually given (prednisone, 1 mg/kg) beginning at the start of ATG treatment and for 10 to 14 days to reduce ATG-related side effects. ATG binds to determinants on all cell types in blood and marrow and reduces platelet and granulocyte counts in addition to the lymphocyte counts, and it may lead to a positive Coombs test. Thrombocytopenia may require intensive blood product support during this time.[238]

Responses to ATG given as a single agent usually occur within the first 3-months, if at all, and blood count improvement by 3 months correlates with survival.[239] The earliest response to therapy may be heralded by the appearance of a few granulocytes and nucleated red cells in the circulation. Red-cell size distribution histograms typically show a macrocytic shoulder.[240] Reticulocytes appear, transfusions decline, and the hemoglobin level increases slowly. The white cell count rises next. The last cell line to return is often the platelets, whose numbers may remain low for months or even years. The recovering red cells are not normal; in addition to macrocytosis, Hb F level is increased, and fetal membrane antigens may remain present.[241] Long-term survivors (except those with successful transplants) often have residual abnormalities, such as thrombocytopenia, red cell macrocytosis, and elevated Hb F levels.[242] Clearly we do not understand either the mechanism of action of this poorly characterized drug, the ultimate course of patients with ATG-induced remissions, or the basis of their partial recovery.

Cyclosporine. Cyclosporine (formerly cyclosporin A) is a fungal cyclic undecapeptide and an effective immunosuppressive agent.[243] Among its many effects, cyclosporine can inhibit T cells, preventing production of IL-2 and IFN-γ.[160] About one half of patients who had failed ATG or ALG treatment subsequently experienced remission with cyclosporine.[244,245] Treatment with cyclosporine alone yielded significantly lower response rates and higher rates of disease progression as compared with combined therapy.[246,247]

Oral cyclosporine should be administered twice daily to maintain blood trough levels between 100 and 250 ng/mL as measured by radioimmunoassay, though the target levels may vary depending on laboratory methods used to determine cyclosporine levels. Hematologic responses can take weeks to months, and an initial trial period of 3 to 6 months is generally recommended.

Toxic effects from cyclosporine use are not insignificant and include hypertension, hypertensive encephalopathy, azotemia, hirsutism, gingival hypertrophy, coarsening of facial features, tremor, immunodeficiency, increase in serum creatinine levels, transient or irreversible nephrotoxicity, hepatotoxicity, seizures, and *P. carinii* pneumonia.[243] As with ATG, in vitro tests of the effect of cyclosporine on progenitor cells do not predict responses.[248]

Combination Immunosuppressive Therapy. The combination of ATG with cyclosporine has resulted in higher rates of hematologic recovery than either drug alone.[246,247,249] Importantly, intensive IST has greatly improved the results of therapy in two groups commonly refractory to ALG or ATG alone: children and patients with very severe disease.[250,251] In the National Institutes of Health (NIH) Clinical Center trial,[252] survival curves of those older and younger than 35 years of age were similar. A large study including both children and adults at the NIH reported a response rate of 67% at 43 months and a 5-year actuarial survival rate of 70% (86% for responders and 16% for nonresponders).[253] The Gruppo Italiano Trapianti di Midolio Osseo (GITMO)/European Group for Blood and Marrow Transplantation (EBMT) study of 100 patients with severe aplastic anemia including children (median age 16 years) treated with ALG, cyclosporine, prednisolone, and G-CSF found trilineage hematopoietic response in 77% after one or more courses of ALG.[254] Several trials in children have reported the efficacy of combined ATG and cyclosporine in the pediatric population with an overall response rate of close to 80%[255,256,250,251,257] (Table 6-2). Although greater lymphocyte depletion is achieved with rabbit ATG as compared to that with horse ATG, a randomized trial comparing horse ATG versus rabbit ATG in the upfront treatment of aplastic anemia demonstrated that superior hematologic response (68% versus 37%) and survival (96% versus 76%) was achieved with horse ATG.[258]

ATG is typically given over a 4-day course in combination with a 6 to 12 month course of cyclosporine. The lack of a unified definition for response complicates comparison of different studies, but generally a *complete response* (CR) refers to normal or functionally normal blood counts, a *partial response* (PR) denotes hemoglobin and platelet counts adequate to avoid the need for transfusion, and neutrophil counts typically greater than 500/μL, and *no response* commonly refers to continued severe aplastic anemia. Improvements in blood counts are delayed and may not be evident for many weeks. In a study including both children and adults, response rates, defined as at least a PR in two blood cell lineages, were 67% at 3 months and 71% at 6 months following initiation of combined ATG and cyclosporine therapy.[252] Cyclosporine should be tapered gradually with close monitoring of the blood counts prior to each decrease in dosage. A persistent drop in the blood counts can often be treated with either an increase in cyclosporine dosage or reinitiating the full dose. Some patients require continuous cyclosporine, and these patients should be maintained on the lowest effective dose. Relapse risk was reduced when cyclosporine was tapered slowly.[259] In a review of ongoing trials by the German multicenter group and the NIH, 29% and 14% of initial responders, respectively, were found to require continuous treatment.[253] The reoccurrence of frank pancytopenia generally requires a second course of ATG followed by full-dose cyclosporine. The relapse rate after combined IST varies widely from study to study. Most studies do not include only children. Kamio and colleagues recently reported a relapse rate of 16% in 264 responding aplastic patients[260] (which compared to 33% reported in an NIH study).[261] Relapse rates were even lower in a Japanese study.[232] Patients treated with IST often continue to have subnormal counts and may remain macrocytic. While the majority of patients respond within 6 months, delayed responses beyond that time may be seen for some patients.

Around 20% of patients in pediatric studies fail to respond to ATG and cyclosporine therapy (Table 6-2), while a recent study of mostly adult patients reported failure of response in around 40% of patients.[262] In

TABLE 6-2 Pediatric Studies of Immunosuppressive Therapy for Aplastic Anemia

Study	Years	Patient Number	Treatment	Follow-Up (years)	Response	Survival	Relapse	Clonal Abnormality
Fuher et al[255]	1993-1997	86 (49vSAA, 30SAA, 7MAA)	ALG, CSA, G-CSF*	4	80% (OR)	87%	15%	3% clonal abnormalities 4.7% AML
Kojima et al[250]	1992-1997	119 (50 vSAA, 36 SAA, 33 MAA)	ATG, CSA, DAN, +/-G-CSF	4	71% (OR) vSAA 61%(OR) SAA+G-CSF, 83% (OR) SAA-G-CSF	83% vSAA 91-93% MAA+SAA	13% vSAA 29% MAA/SAA +G-CSF 64% MAA/SAA -G-CSF	3%
Kojima et al[278]	1992-1997	113 (39vSAA, 47SAA, 27MAA)	ATG, CSA, DAN, +/- G-CSF	Median 5.3 Range 3.75-8.9	30% CR 31% PR 42% NR at 6 months	87%	NR	13.7% cumulative incidence MDS at 8 years, 21.9% cumulative incidence clonal cytogenetic abnormalities at 8 years
Goldenberg et al[257]	1990-2003	14	ATG, CSA +/-G-CSF	Median 4.4 Range 0.8-13.3	93% (OR)	93%	0	0
Fuher et al[251]	1993-2001	97 vSAA 49 SAA	ATG CSA G-CSF	Median 4.1	69% (CR) vSAA 44% (CR) SAA	93% vSAA 81% SAA	13% vSAA 14% SAA	NR NR

CR, Complete response; *CSA*, cyclosporine A; *DAN*, danazol; *MAA*, moderate aplastic anemia; *NR*, not reported; *OR*, overall response; *PR*, partial response; *SAA*, severe aplastic anemia; *vSAA*, very severe aplastic anemia.
*G-CSF added for SAA and vSAA.

most studies, failure of response is typically defined as persistence of severe aplastic anemia. In adult patients failing the first treatment with horse ATG, 63% of patients receiving a second course of horse ATG responded,[263] while 77% (23 out of 30) of patients initially treated with horse ATG and cyclosporine responded to a second course with rabbit ATG and cyclosporine.[264] In some small studies in children, the response rate to second immunosuppressoin has been less,[265] and treatment with rabbit ATG as a second therapy was associated with only 27% response rate.[266] In a large NIH study that included 77 children less than 18 years of age, of 14 relapsed patients, 6 were retreated with ATG and were alive at report, but the hematologic response of these patients was not reported.[261] Less acute reactions were noted when the source of ATG was varied between the first and second treatments. The time frame to response following a second course of immunosuppression was similar to that observed following initial treatment. Patients failing to respond to two courses of immunosuppressive therapy are unlikely to respond to a third treatment course.[267]

Because androgens are effective in treating some patients with aplastic anemia (see following discussion), the effect of combining androgen treatment with ATG was examined in several trials. Champlin and colleagues reported no difference in response rate or survival in patients with moderate to severe aplastic anemia.[268] Studies by Kaltwasser and colleagues[269] and the EBMT[270] found significantly higher response rates after ALG plus androgens (73% and 56%) than in patients receiving ALG alone (31% and 40%). A recent retrospective study in adults reported an overall response rate of 77% and overall survival rate of 78% at 5 years following treatment with ATG plus androgens.[271] At least one report showed increased relapse after ATG and cyclosporine when danazol was included in the treatment.[260] Because some patients with inherited causes of aplastic anemia may experience hematologic improvement following androgen therapy,[272] it is important to rule out an underlying inherited marrow failure syndrome. Androgen side effects may be unacceptable to some patients, and their use in acquired aplastic anemia in children is generally limited.

Relapse. Relapse after immunosuppressive therapy has been reported in around one third of patients in studies including both children and adults,[262,273] although lower rates of relapse may be attained with slow tapering of cyclosporine and continued maintenance doses at the lowest required cyclosporine dose for some patients. Lack of standardized definitions of *relapse* hamper comparison between studies. An EBMT study including both children and adults reported an actuarial relapse risk of 35% at 14 years.[274] Relapses were observed out to 10 years after treatment. Disease severity, age, sex, and etiology were not predictive of relapse. Large studies of relapse risk following IST therapy in the pediatric population are scarce. Results of these studies are summarized in Table 6-2.

Tichelli and colleagues studied the efficacy of repeated treatment with ALG for patients with severe aplastic anemia who either failed to respond to an initial course of ALG or who experienced hematologic relapse.[263] Transfusion independence was achieved in 63%, and survival probability was 52%± 8% at 10 years. No differences were noted between patients retreated for nonresponse versus relapse. No increases in acute toxicity were noted with additional courses of ALG, though the timing of serum sickness tended to occur earlier following repeat ALG administration. Relapse had little effect on survival for those patients responding to reinstitution of immunosuppression. A study of 22 patients refractory to combined ATG/cyclosporine reported an overall response rate of only 30% at 6 months. This study included two refractory patients under the age of 18, neither of whom responded to a second course of immunosuppression. Of 21 patients relapsing after ATG/cyclosporine treatment, an overall response rate of 65% was observed.[266] A retrospective study of 18 relapsed or refractory patients reported responses to a third course of an ATG-containing regimen only for those patients who had previously responded to a prior course of treatment.[267]

Complications of Immunosuppressive Therapy. Patients treated for aplastic anemia with immunosuppressive therapy have an increased risk of developing clonal hematopoietic disorders.[275,276] Because ATG and cyclosporine are not associated with an increased risk of MDS or leukemia in patients treated for other disorders, these complications are believed to result from the underlying dyspoiesis of aplastic anemia. In an analysis of 860 aplastic anemia patients treated with immunosuppression, 42 malignant conditions were reported, including 19 cases of PNH, 11 cases of myelodysplasia, 15 cases of acute leukemia, 7 cases of solid tumors, and 1 case of non-Hodgkin's lymphoma.[277] Of 748 aplastic anemia patients treated with transplantation, 9 malignancies were reported: 2 acute leukemias and 7 solid tumors. The cumulative incidence of developing a malignant tumor at 10 years was 18.8% following immunosuppression and 3.1% following transplantation. A recent NIH study of 122 patients treated with immunosuppression showed a 33% actuarial risk of developing a clonal hematopoietic disorder at 10 years.[4] An increased risk of clonal abnormalities as high as 21.9%[278] has been reported in some studies of pediatric patients with aplastic anemia. An increased risk of MDS or AML has also been observed in pediatric patients (Table 6-2). Certain clonal abnormalities, such as monosomy 7, were associated with worse clinical outcomes.[279] While an association between G-CSF use and risk of clonal disease has been observed,[278] in the absence of randomized studies, the question of causality remains unanswered. These data suggest that a clonal transformation of stem cells may underlie aplastic anemia, at least in a subset of patients. Prolonged survival caused by ATG/ALG/CYA may permit the expected clonal evolution.

Transplantation versus Immunosuppression. The decision to treat with immunosuppression versus an HLA-identical sibling stem-cell transplant for the 25% of patients for whom a suitable donor is available is based on a consideration of risks and benefits that vary depending on patient age and neutrophil count. Upfront toxicities associated with the conditioning regimens, GVHD and graft rejection are the major problems associated with stem-cell transplantation, but this treatment offers the potential for long-term cure. Cytotoxic and immunosuppressive therapy is associated with lower acute toxicities but often yields incomplete hematologic recovery and is associated with late mortality from long-term complications such as relapse and clonal hematologic disorders. Comparison of outcomes with BMT and immunosuppression in children support the benefit of HLA-matched sibling stem-cell transplant. Kojima and colleagues[280] reported a study of 100 children under the age of 17 years with aplastic anemia between 1984 and 1998. Thirty-seven patients received allogeneic BMT and 63 patients received immunosuppression with either ATG plus danazol or ATG and cyclosporine with or without danazol. Some patients also received G-CSF. Eleven patients initially treated with IST received subsequent salvage treatment with unrelated donor transplant. The projected 10-year survival was 55% ± 8% with immunosuppression and 97% ± 3% with transplant. Failure-free survival at 10 years was 40% ± 8% for the immunosuppression group and 97% ± 3% for the transplant group. Seven patients in the immunosuppression group developed MDS with monosomy 7, and the estimated 10-year cumulative incidence of MDS was 20% ± 7%.

A recent report from the EBMT analyzed 1765 patients with aplastic anemia receiving initial treatment with either an HLA-identical sibling transplant or immunosuppressive therapy.[281] Analysis was performed on an "intent-to-treat" basis. Patients were aged 1 to 50 years and treated between 1974 and 1996. Five hundred eighty-three patients received HLA-identical sibling transplant, and 1182 patients received immunosuppression. A comparison of immunosuppression versus BMT in patients stratified by age and neutrophil count showed superior failure-free survival with transplant for children regardless of their neutrophil count. The advantage of transplant increased with increasing follow-up time, most likely due to the ongoing risks of late relapse and evolution to myelodysplasia and leukemia in the group receiving immunosuppression. Children have a lower mortality with stem-cell transplant and a longer posttreatment life expectancy, placing them at higher risk for late complications than older adults. Thus the current recommendation is to treat young patients (10 years or younger) with HLA-identical sibling stem-cell transplant when a suitable donor is available. Patients with severe disease also do better with BMT. It is important to note that many patients on these studies of long-term outcomes were treated prior to the development of current combined immunosuppressive regimens containing both ATG and cyclosporine.

For those patients lacking a suitable HLA-matched sibling donor, ATG and cyclosporine treatment is generally the preferred line of therapy given the higher risk of toxicities and morbidity associated with current alternative donor transplant regimens. For patients who fail to respond to an initial course of ATG and cyclosporine, either a second course of ATG/cyclosporine or an alternative donor hematopoietic stem-cell transplant may be considered. The risks and benefits of each approach will vary for each patient, but the risks associated with prolonged delay to potential transplant[282] should be weighed against the most current data on alternative donor regimens.

Hematopoietic Growth Factors. Although patients with aplastic anemia do not have a deficiency of most hematopoietic growth factors (see previous discussion), it was hypothesized that pharmacologic levels might stimulate hematopoiesis of one or more cell lines.[208] Small trials of GM-CSF, G-CSF, IL-3, IL-1, IL-6, and SCF in mostly adult patients with aplastic anemia do not support the use of growth factors as primary treatment for aplastic anemia, and serious side effects were noted with some agents. To date, no consistent benefit in terms of response rate or survival following the adjunctive use of growth factors such as G-CSF in immunosuppressive regimens has been observed (see previous discussion).[250,283] No improvement in overall response rates were noted in a retrospective study of 18 pediatric patients receiving GM-CSF in addition to ATG and cyclosporine.[284] Responses to single or combined growth factors have been reported in a few refractory patients.

G-CSF is associated with flulike symptoms, bone pain, and splenomegaly. Symptoms of “cytokine flu”—fever, rash, hives, headache, and myalgia—are common but do respond to acetaminophen or resolve with the passage of time. Bone pain may be a symptom of increased marrow activity, though this is more commonly seen with G-CSF. At higher doses, the severity of symptoms increases, and there may be evidence of visceral engorgement and fluid retention.

The potential risk of promoting or stimulating the development of abnormal hematopoietic clones leading to a further increased risk of myelodysplasia or leukemia following prolonged treatment with growth factors remains a concern in aplastic anemia patients. Although associations between G-CSF treatment and the subsequent development of MDS and leukemia have been reported,[285] in the absence of randomized prospective trials, it is not clear whether this represents an effect of growth factor treatment or merely an association reflecting the natural history of aplastic anemia of sufficient severity to warrant the addition of G-CSF.

Eltrombopag. A recent report suggests that the thrombopoietin-mimetic eltrombopag may have activity in aplastic anemia.[286] Thrombopoietin is a major regulator of platelet production and is the ligand for c-mpl on megakaryocytes. C-mpl is also expressed on hematopoietic stem cells, and mice deficient in either thrombopoietin or c-mpl show deficiencies in hematopoietic stem and progenitor cells.[287,288] In addition a significant number of children with amegakaryocytic thrombocytopenia develop pancytopenia (discussed in Chapters 30 and 34). In a phase 2 study, investigators at the NIH demonstrated a 44% hematologic response rate of aplastic anemia patients refractory to IST. In this nonrandomized study, 9 out of 25 patients became platelet transfusion–independent, 6 out of 25 demonstrated increased hemoglobin levels, and 9 out of 25 showed increased leukocyte counts within 3 months of treatment with minimal side effects. Studies in children have not been reported, and this study has not been reproduced to date, so caution is warranted in utilizing this drug long-term outside of a clinical trial.

Androgens. Androgens no longer have a primary role in the management of aplastic anemia, unless the first-line therapies discussed above are unavailable or unsuccessful. As described earlier, androgens have been used as adjuncts to immunosuppression

The mechanism of action has been reviewed extensively by Gardner and Besa.[289] Androgens increase erythropoietin production,[290] and stimulate erythroid stem cells.[291] Hemoglobin levels increase in normal males at puberty and in patients treated with androgens for arthritis, breast cancer, or in old age. Shahidi and Diamond were the first to report success with testosterone[292] and large confirmatory series were published from France[293] and Mexico.[294] However, later critical evaluations revealed no evidence supporting the use of androgens. Despite the use of androgens, greater than 75% mortality has been reported in children[216] and adults.[295] Androgen-treated patients often did not do as well as those who were not treated with these hormones.[217,296] The older publications often failed to distinguish moderate from severe patients. Camitta and colleagues performed a prospective, multicenter analysis beginning in 1974 and concluded that androgens (whether oral or intramuscular) were no more effective than supportive care alone for severe aplastic anemia.[297,298]

Improvement, if it occurs, is usually greatest in the hematocrit, though increases in the granulocyte and platelet counts may also be seen. Response may take weeks to months, and trials of at least 3 months are recommended before declaring a patient as unresponsive. The major side effect of androgens is liver toxicity. Cholestatic jaundice and hepatomegaly are usually reversible. Peliosis hepatis (blood lakes) have been reported.[299] Hepatic tumors are a serious risk, although the pathologic picture is usually that of a benign adenoma; such tumors may be reversible when use of the androgen is discontinued. Other side effects include masculinization with hirsutism, baldness, deepening of the voice, and enlargement of the genitalia. Acne, flushing of the skin, nausea, and sodium and fluid retention also occur. The appetite is stimulated, and there is weight gain with increased muscular development. There may be an increase in the rate of skeletal maturation, with eventual premature fusion and ultimate short stature.[300] All patients who receive androgens should be monitored with frequent liver function testing, annual liver ultrasound examinations, and annual bone age determinations.

Corticosteroids. Modest doses of methylprednisolone are generally given during ATG or ALG treatment to lessen the side effects of serum sickness. As a single agent, very high doses of corticosteroids have been reported to lead to responses in a few patients.[301,302] Treatment with high-dose steroids is associated with substantial toxicities, including hypertension, hyperglycemia, fluid retention, potassium wasting, psychosis, aseptic necroses of femoral and humeral heads, and increased susceptibility to fungal infections. Given the higher response rate and lower toxicity profile of ATG, high-dose steroids are not recommended as first-line therapy for aplastic anemia.

Splenectomy. Splenectomies were performed until recently, perhaps for want of something better to offer to patients. A review in 1957 indicated improvements in 20 of 35 patients.[303] More recently, the operation has been performed to facilitate supportive care.[304] Unless there are clear indications of hypersplenism significantly impeding platelet and red cell transfusion support, the operation does not seem warranted.

Other Therapies. New therapeutic regimens are aimed at improving rates of hematologic response and minimizing acute and long-term complications of therapy.[162] The current status of a few investigational regimens is summarized below.

Cyclophosphamide. Administration of high doses of cyclophosphamide is an effective IST and is not myeloablative in normal hosts. Historically a few patients who received high-dose cyclophosphamide as preparation for marrow transplant rejected their grafts and had autologous recovery. An early study of treatment with this drug without transplant indicated a CR in 7 of 10 patients treated with 45 mg/kg per day for 4 days.[305] A study of 19 previously untreated patients with aplastic anemia reported a 73% overall response rate at 24 months with a 65% probability of achieving CR at 50 months and an 85% survival rate at 24 months.[306] No relapses or clonal disorders were observed in the responding patients. A phase III prospective randomized trial compared high-dose cyclophosphamide plus cyclosporine to ATG plus cyclosporine as first-line therapy.[307] The trial was terminated prematurely due to invasive fungal infections and three early deaths in the group treated with cyclophosphamide plus cyclosporine (none were noted in the ATG/cyclosporine group). Responses at 6 months' follow-up were seen in 6 of 13 (46%) patients treated with cyclophosphamide plus cyclosporine versus 9 of 12 (75%) patients treated with ATG plus cyclosporine. Trials of high-dose cyclophosphamide as a single agent in patients refractory to prior treatment with IST reported a 53% response rate and 59% survival rate at a median follow-up of 29 months.[308] The generally prolonged time to response and associated toxicities are potentially limiting factors, and additional studies are required to determine whether long-term complications such as relapse or clonal disease will be superior to that associated with ATG and cyclosporine.

Alemtuzumab. Several recent adult studies have examined the use of alemtuzumab, a humanized anti-CD52 monoclonal antibody, in both naïve and relapsed/refractory aplastic anemia. Alemtuzumab eliminates CD52+ lymphocytes by both direct cytotoxicity and by complement-mediated cellular lysis. The studies are small and included naïve as well as relapsed and refractory patients.[309,310-312] Dosage ranged from 70 to 100 mg (total) delivered over 5 to 10 days either intravenously or subcutaneously with or without low dose Cytoxan as a chronic maintenance. Study results were variable, with 30% to 50% response rates (combined CR and PR) and approximately 70% to 80% overall survival. Side effects also varied with multiple infectious complications, clonal evolution (MDS with monosomy 7) and PNH and in one case progressive multifocal leukoencephalopathy. Studies are ongoing, including a consensus regimen by the Severe Aplastic Anemia Working Group in Europe.[312]

Recombinant Humanized Anti-IL-2 Receptor Antibody. The IL-2 receptor is found on activated T cells and is required for T-cell expansion.[313] A pilot study of 17 patients with moderate aplastic anemia treated for 3 months with a humanized monoclonal antibody against the IL-2 receptor (daclizumab) reported improved blood counts in 38% of patients.[314] Daclizumab was well tolerated with minimal side effects. Further studies on the use of daclizumab in aplastic anemia are warranted.

Mycophenolate Mofetil. Mycophenolate mofetil (MMF; Cell-Cept) is the prodrug of mycophenolic acid, which inhibits B- and T-cell proliferation through inhibition of inosine monophosphate dehydrogenase, which is involved in guanine nucleotide synthesis.[315] MMF has been in clinical use for prevention of solid organ transplant rejection and to treat autoimmune diseases. Although generally well tolerated, MMF side effects include leukopenia and marrow suppression. A study of 104 patients with severe aplastic anemia treated with ATG, cyclosporine, and MMF reported an overall response rate of 62% at 6 months.[316] Relapses were seen in 37% of responders with over half of relapses occurring while the patients were still receiving MMF. Clonal abnormalities were noted in 8.7% of patients after a median follow-up of 42 months. No difference in overall survival was noted between children and adults. The addition of MMF did not result in improved rates of response, relapse, or clonal abnormalities in comparison to those reported following treatment with ATG and cyclosporine.

ACQUIRED PURE RED CELL APLASIA

Acquired pure red cell aplasia (APRCA) is a term that embraces a range of incompletely understood aregenerative anemias (sometimes associated with neutropenia and thrombocytopenia).[317] Unifying characteristics of this broad range of disorders are anemia and a paucity of recognizable erythroid precursors in the bone marrow. The anemia may be macrocytic if some residual "stress" erythropoiesis is extant. If there is complete arrest of erythropoiesis, the anemia will be normocytic or even slightly microcytic and hyperchromic if the arrest of erythropoiesis has existed for 2 months or more because red cell volume shrinks as red cells age in the circulation. In some cases, particularly those associated

Box 6-4 Classification of Acquired Pure Red Cell Aplasia

PRIMARY

Autoimmune (antibodies against erythropoietin, erythroid progenitors, and precursors and T-cell suppression of erythropoiesis)
Idiopathic (transient erythroblastopenia of childhood)
Preleukemic (the 5Q-syndrome)

SECONDARY

Thymoma
Nonthymic solid tumors (gastric, breast, lung carcinomas, Kaposi sarcoma)
Hematologic malignancies (lymphoma, CLL, CML)
Infections (particularly parvovirus)
Chronic hemolytic anemia (aplastic crisis)
Collagen vascular disease
Drugs and chemicals (chemotherapy particularly azathioprine in renal transplant)
Pregnancy
Severe renal failure
Severe vitamin and calorie malnutrition

with parvovirus infection, mild neutropenia, and even thrombocytopenia may coexist with the anemia. Thus the term *pure red cell aplasia* may be honored in the breech.

Much of our understanding of APRCA seen in adults has been derived from the clinical research of Sanford Krantz and Emmanuel Dessypris and their colleagues at Vanderbilt University.[318] The reader is referred to Dr. Dessypris's excellent monograph for a history and reasonable classification and description of these arcane disorders. A more recent set of reviews has been edited by Stephen A Feig and Melvin H Freedman.[319]

APRCA may be classified as outlined in Box 6-4.

Primary Acquired Pure Red Cell Aplasia

Circulating antibodies and suppressing T cells: Dessyrpis and Krantz have carefully described the course of patients who suddenly develop antibodies reactive with erythroid precursors that presumably suppress either erythroid progenitors (BFU-E and CFU-E) or recognizable precursors or both. Dessyypris's monograph[318] and Krantz's more recent review in the volume edited by Feig and Freedman[319] exhaustively document the evidence for such antibodies and their suppressing activities. In brief the antibodies may arise de novo and hence are considered primary or in the setting of immune deficiency, neoplastic disease, collagen vascular disease, or infection in which they are considered secondary. In either case, the antibodies cause severe aregenerative anemia and, though they are often responsive to immunosuppressive therapy, commonly return to the circulation and cause relapse unless therapy is continued for protracted periods of time. Fortunately antibody mediated APRCA is quite rare in children. The pathophysiology, course, and treatment of the disease are better understood in adults.

Autoantibodies that bind and inhibit erythropoietin and are responsible for erythroid aplasia were first inferred from the work of Jepson and Lowenstein.[320] Though similar reports later emerged, three of which were associated with recombinant erythropoietin therapy, the pathophysiologic evidence for such antibodies was marginal until 2002, when French investigators reported a high incidence of antierythropoietin antibodies in patients (largely with renal failure) who had been treated with a particular brand of recombinant erythropoietin that had been altered by a variation in glycosylation and by improper storage and thereby rendered antigenic.[321] The findings have been confirmed worldwide. The offending brand of erythropoietin has since been modified, and the incidence of such autoantibodies has been markedly reduced. IST induces a disappearance of these antibodies in most cases. Successful treatment with a peptide-based erythropoietin-receptor agonist has been reported.[322] In truth it might be reasonable to classify the PRCA that results from antierythropoietin antibodies that arise in the face of recombinant erythropoietin treatment as an example of secondary PRCA.

A case of primary APRCA associated with T-cell suppression of erythropoiesis by large granular lymphocytes that carried both T-cell and monocyte surface markers was described in 1983 by Lipton and his associates.[323] In this case the T cells recognized erythroid but not myeloid progenitors in the context of HLA and suppressed in vitro erythropoiesis in the patient and in HLA-identical normal individuals. Prior to and since that report there have been several papers that describe such T cells in cases of APRCA,[317] but the Lipton study provides the best evidence for an HLA-specific erythroid inhibition by an offending clone of T cells. Interestingly this patient was rapidly cured by treatment with cyclophosphamide.

Transient Erythroblastopenia of Childhood

Though probably described in the 1960s by Schmid and by DiGiacomo and their colleagues,[324,325] who each reported a potpourri of cases of APRCA, transient erythroblastopenia of childhood (TEC) was first clearly enunciated by Wranne in 1970.[326] This frequent disease is well described by Glader.[319] TEC is certainly the most common aregenerative anemia in childhood and is likely to be a very frequent complication of a host of relatively trivial viral infections. It is well established that erythropoiesis is briefly interrupted during such infections in most individuals. But anemia is not documented because the red cell life span is 120 days, and the suppression usually lasts for only a week. In many patients the suppression is protracted, and anemia ensues. Spontaneous recovery without any treatment is virtually the rule, but confusion may result if the pediatrician sees the patient at the time of recovery when reticulocytosis is in full spate.[327] This may lead to a false diagnosis of hemolytic anemia and an ensuing workup that simply produces more anemia in the blood as well as in the pocketbook. It is important to suspect TEC in an otherwise healthy child whose blood smear does not suggest a red cell disorder or leukemia. A bone marrow examination is not indicated in the vast majority of cases.[328] Because neutropenia and mild thrombocytopenia are common concomitants of many viral infections, TEC is often not a "pure" red cell disease; however, the anemia is usually the more striking anomaly.

Many efforts have been made to implicate parvovirus serotype 19 as an offender in TEC. Though rare cases have been noted, parvovirus is usually not detected.[329-331]

It is, however, a serious risk in patients with immune deficiency, such as those undergoing organ or marrow transplantation, and in patients with foreshortened red cell lifespans, such as sickle cell anemia or hemolytic anemias (see Chapter 20).

Preleukemia (the 5Q-syndrome)

The 5Q-syndrome is an acquired clonal defect in which a segment of chromosome 5Q bearing at least 40 genes is deleted. Severe aregenerative anemia, absent marrow erythroblasts, abnormal megakaryocytes, and a high incidence of acute myelogenous leukemia are the results. The recent discovery by Ebert and his colleagues that the responsible deletion is the gene encoding a ribosomal protein[332] classifies the 5Q- syndrome as an acquired and clonal form of the Diamond–Blackfan anemia.[333] It is discussed in extenso in Chapters 7 and 51.

Secondary Acquired Pure Red Cell Aplasia

The secondary causes of APRCA are very rare in childhood, and thymoma is even rarer. Freedman and Feig reviewed the pediatric literature in 1993,[319] and little has emerged since. Thymoma, when associated with PRCA may be successfully treated with surgery coupled with immunosuppression or intravenous immunoglobulin.[334-337]

Very rarely, children with B-cell proliferative diseases may have concomitant PRCA, but almost all of the therapeutic experience is recorded in adults. Of the few cases of PRCA reported in children, most are in the setting of immunodeficiency.

Drug-induced PRCA is by far the most common cause of secondary PRCA in childhood, and azathioprine treatment of renal transplant rejection is by far the commonest offender.[338] The latter drug usually causes a megaloblastic anemia, but it may result in near total aplasia. Severe renal failure may also cause PRCA in childhood.

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CHAPTER 7

Inherited Bone Marrow Failure Syndromes

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CHAPTER OUTLINE

INHERITED BONE MARROW FAILURE SYNDROMES ASSOCIATED WITH PANCYTOPENIA	INHERITED BONE MARROW FAILURE SYNDROMES ASSOCIATED WITH ISOLATED CYTOPENIAS
Fanconi Anemia	Diamond-Blackfan Anemia
Dyskeratosis Congenita	Congenital Dyserythropoietic Anemias
Shwachman-Diamond Syndrome	Severe Congenital Neutropenia
Cartilage-Hair Hypoplasia	Inherited Thrombocytopenias
Pearson Syndrome	Amegakaryocytic Thrombocytopenia
Familial Marrow Dysfunction	Thrombocytopenia with Absent Radii

Inherited bone marrow failure syndromes (IBMFSs) are a diverse collection of genetic disorders associated with inadequate blood cell production. Bone marrow failure (BMF) may manifest as an isolated cytopenia (pure red cell aplasia, neutropenia, or thrombocytopenia) or as pancytopenia with the clinical picture of aplastic anemia. Table 7-1 summarizes the more common IBMFSs, the blood cell lineages affected, the genes mutated and their modes of inheritance, and the pathways involved. Other organ systems are often affected by these genetic abnormalities, resulting in birth defects or clinical disease in nonhematopoietic organs. Birth defects and extra hematopoietic manifestations are often characteristic and may be noticed before the onset of BMF or may be the only manifestations of disease. BMF may be present at birth (congenital BMF) or develop later in life. IBMFSs are not always inherited; the genetic mutations responsible may occur de novo during early embryonic development. Several, but not all, IBMFSs are characterized by a predisposition for malignant transformation, in particular of the hematopoietic system, including the development of myelodysplastic syndrome (MDS) and acute leukemia, but also for a variety of other forms of cancer (see Table 7-1).

The prevalence of IBMFSs is difficult to ascertain because BMF registries are biased by referral, founder effect, and the enrollment of mainly pediatric patients. The incidence of IBMFSs has been estimated to be 40 to 65 per 10^6 live births.[1,2] Originally, IBMFSs were considered to be mainly diseases of childhood. In children 25% to 30% of BMF cases were thought to have a genetic cause, whereas among adults an inherited genetic cause was considered in only a minority of patients. However, more recent data suggest that the prevalence of IBMFS patients has probably been underestimated, particularly in adults. In addition, improved medical care after bone marrow transplantation (BMT) has extended life expectancy and shifted the management of IBMFS patients to adult hematologists.

Timely and correct diagnosis of an IBMFS is paramount because management of an IBMFS differs from that of acquired BMF. Treatment of IBMFSs often requires a complex interdisciplinary approach that includes hematologists, geneticists, hematopathologists, BMT specialists, oncologists, endocrinologists, orthopedists, neurologists, gastroenterologists, nephrologists, dentists, and other specialists. Differentiation between acquired and inherited BMF is of importance not only for affected individuals but also for their family members. Early and correct diagnosis of an IBMFS allows intensified and focused disease surveillance, anticipatory guidance, and avoidance of treatment-related toxicities. Identification of a gene mutation known to be associated with BMF can definitively establish the diagnosis before clinical symptoms advance and help identify an unaffected bone marrow donor in the family. It is therefore imperative that at the time of initial evaluation maximal effort be made to distinguish acquired from inherited forms of BMF.

Considerable advances have been made in the last 15 years in elucidating the genetic causes of IBMFSs

Text continues on p. 187

TABLE 7-1 Inherited Bone Marrow Failure Syndromes

Syndrome	**Inheritance Pattern**	**Peripheral Blood Manifestations**	**Associated Malignant Diseases**	**Gene**	**Chromosomal Location**	**Affected Pathway**
Fanconi anemia (FA, MIM #227650)	AR	Pancytopenia	MDS/AML Squamous cell carcinoma Other tumors	*FANCA*	16q24.3	DNA repair, homologous recombination
	XLR			*FANCB*	Xp22.31	
	AR			*FANCC*	9q22.3	
	AR			*FANCD1/BRCA2*	13q12-13	
	AR		AML Wilms tumor Medulloblastoma	*FANCD2*	3p25.3	
	AR		MDS/AML Squamous cell carcinoma Other tumors	*FANCE*	6p21.3	
	AR			*FANCF*	11p15	
	AR			*FANCG/XRCC*	9p13	
	AR			*FANCI*	15q26.1	
	AR			*FANCJ/BACH1/BRIP1*	17q22-q24	
	AR			*FANCL*	2p16.1	
	AR			*FANCM*	14q21.3	
	AR		Wilms tumor Medulloblastoma	*FANCN/PALB2*	16p12.2	
	AR		?	*FANCO/RAD51C*	17q22	
	AR		?	*FANCP*/SLX4	16p13.3	
Dyskeratosis congenita (DC)	XLR (MIM, #305000)	Pancytopenia	MDS/AML Squamous cell carcinoma Other tumors	*DKC1*	Xq28	Telomere maintenance, ribosome biogenesis
	AD (MIM #127550)			*TERC*	3q26.2	Telomere maintenance
	AD (MIM #613989)			*TERT*	5p15.33	Telomere maintenance
	AD (MIM #613990)		Unknown	*TINF2*	14q11.2	Telomere maintenance
	AR (MIM #224230)		Unknown	*NOP10/NOLA3*	15q14-q15	Telomere maintenance, ribosome biogenesis
	AR (MIM # 613987)		Unknown	*NHP2/NOLA2*	5q35.5	Telomere maintenance, ribosome biogenesis
	AR (MIM # 613988)		Unknown	*WDR79/TCAB1/WRAP53*	17p13.1	Telomere maintenance
	AR (MIM # 612199)		Unknown	*CTC1*	17p13.1	Telomere maintenance
	AR (MIM #615190)		Unknown	*RTEL1*	20q13.3	DNA repair, homologous recombination, telomere maintenance
Shwachman-Diamond syndrome (SDS, MIM #260400)	AR	Neutropenia with progression to pancytopenia	MDS/AML	*SBDS*	7q11.21	Ribosome biogenesis, ribosome assembly
Cartilage-hair hypoplasia (CHH, MIM #250250)	AR	Neutropenia Lymphopenia Anemia	Lymphoma Basal cell carcinoma	*RMRP*	9p13.3	Mitochondrial DNA replication, ribosome biogenesis, degradation of *CLB2* mRNA

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TABLE 7-1 Inherited Bone Marrow Failure Syndromes (Continued)

Syndrome	Inheritance Pattern	Peripheral Blood Manifestations	Associated Malignant Diseases	Gene	Chromosomal Location	Affected Pathway
Pearson marrow-pancreas syndrome (PS, MIM #557000)	Mitochondrial	Neutropenia with progression to pancytopenia	None	mt DNA deletion		Mitochondrial function
Diamond-Blackfan anemia (DBA, MIM #105650)	AR	Anemia with rare progression to pancytopenia	MDS/AML[*] Osteosarcoma	*RPS19* *RPS24* *RPS17* *RPS10* *RPS26* *RPS7* *RPS29* *RPL35a* *RPL5* *PRL11* *RPL26* *RPL15*	19q13.2 10q22-23 15q25.2 6p21.31 12q13.2 2p25.3 14q21.3 3q29-qter 1p22.1 1p36.1-35 17p13.1 3p24.2	Ribosome biogenesis
	XLR	Anemia and neutropenia		*GATA1*	Xp11.23	
Congenital dyserythropoietic anemia (CDA) type I (MIM #224120)	AR	Anemia	None	*CDAN1*[†]	15q15.1-15.3	Unknown
CDA type II (MIM #224100)	AR		None	*SEC23B (CDAN2)*	20q11.23	Unfolded protein response
CDA type III (MIM #105600)	AD		Myeloma	*KIF23MKLP1*	15q21-25	Cytokinesis
CDA type IV (MIM #613673)	AD			*KLF1 (CDAN4)*	19p13.2	Erythroid transcription factor
Severe congenital neutropenia due to *ELANE* mutation (SCN, MIM #202700)	AD	Neutropenia	MDS/AML	*ELANE*	19p13.3	Unfolded protein response (?)
Cyclic neutropenia (CN, MIM #162800)	AD	Neutropenia	None	*ELANE*	19p13.3	Unfolded protein response (?)
Kostmann syndrome (MIM #610738)	AR	Neutropenia	MDS/AML	*HAX1*	1q21.3	Regulation of apoptosis (?)
Severe congenital neutropenia due to *G6PC3* mutation (MIM #612541)	AD	Neutropenia	MDS/AML	*G6PC3*	17q21.31	Glucose metabolism, glycosylation (?), unfolded protein response
Severe congenital neutropenia due to *GIF1* mutation (MIM #613107)	AD	Neutropenia Lymphopenia	MDS/AML	*GIF1*	1p22.1	Transcription repressor of ELANE
Severe congenital neutropenia due to *CSF3R* mutation	AD Sporadic	Neutropenia	MDS/AML	*CSF3R*	1p34.3	GCSF receptor signaling
Barth syndrome (BTHS, MIM #302060)	AR	Neutropenia	None reported	*TAZ1*	Xq28	Changes in mitochondrial architecture and function

TABLE 7-1 Inherited Bone Marrow Failure Syndromes (Continued)

Syndrome	Inheritance Pattern	Peripheral Blood Manifestations	Associated Malignant Diseases	Gene	Chromosomal Location	Affected Pathway
Cohen syndrome (COH1, MIM #216550)	AR	Neutropenia	None reported	*COH1*	8q22-q23	Lysosomal protein trafficking
Chédiak-Higashi syndrome (CHS1, MIM #214500)	AR	Neutropenia	None reported	*CHS1/LYST*	1q42.3	Lysosomal protein trafficking
Griselli syndrome (GS2, MIM # 607624)	AR	Neutropenia Thrombocytoepnia	None reported	*RAB27A*	15q21.3	Lysosomal function (?)
Hermansky-Pudlak syndrome 2 (HPS2, MIM #608233)	AR	Neutropenia	None reported	*AP3B1*	5q14.1	Lysosomal protein trafficking, unfolded protein response (?)
Immunodeficiency due to defect in MAPBP-interacting protein P14 (MIM #610798)	AR	Neutropenia	None reported	*MAPBPIP*	1q22	Lysosome biogenesis
Glycogen storage disease 1b (GSD1B, MIM #23220)	AR	Neutropenia	Renal carcinoma	*G6PT1*	11q23.3	Glycogen storage
Hyper-IgM immunodeficiency syndrome (XHIM, MIM #308230)	XLR	Neutropenia Pancytopenia	None reported	*CD40LG (HIGM1)*	Xq26.3	B-cell activation
WHIM syndrome (MIM #19370)	AD	Neutropenia Lymphopenia	None reported	*CXCR4*[†]	2q22.1	Neutrophil mobilization
Poikiloderma with neutropenia (MIM #604173)	AD	Neutropenia	MDS/AML Skin cancer	*USB1*	16q21	Processing of U6 spliceosomal RNA
Congenital amegakaryocytic thrombocytopenia (CAMT, MIM #60448)	AR	Thrombocytopenia with progression to pancytopenia	AML	*c-MPL*	1q35	Megakaryopoiesis
Thrombocytopenia with absent radii (TAR, MIM # 274000)	AR	Thrombocytopenia	AML	*RBM8A*	1q21.1	Part of exon junction complex
Amegakaryocytic thrombocytopenia with radioulnar synostosis (ARTUS, MIM #605432)	AD	Thrombocytopenia with progression to pancytopenia	AML	*HOXA11*	7p15-p14.2	Morphogenesis
Familial platelet disorder with associated myeloid malignancy (FPD/AML, MIM #601399)	AD	Thrombocytopenia	MDS/AML	*AML1*	21q22.3	Hematopoietic cell differentiation

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TABLE 7-1 Inherited Bone Marrow Failure Syndromes (Continued)

Syndrome	Inheritance Pattern	Peripheral Blood Manifestations	Associated Malignant Diseases	Gene	Chromosomal Location	Affected Pathway
Wiskott-Aldrich syndrome (WAS, MIM #301000)	XLR	Thrombocytopenia	Lymphoma	*WAS*†	Xp11.23	Loss of function, signal transduction from cell surface to actin cytoskeleton
X-linked severe congenital neutropenia (SCNX, MIM #300299)		Neutropenia Monocytopenia	MDS/AML	*WAS*†		Gain of function; disrupt autoinhibiton
X-linked thrombocytopenia (XLT, MIM #313900)		Thrombocytopenia	Lymphoma	*WAS*		Loss of function, signal transduction from cell surface to actin cytoskeleton
Congenital dyserythropoiesis associated with thrombocytopenia (MIM #300367)	XLR	Thrombocytopenia	None	*GATA1*	Xp11.23	Erythroid and megakaryocytic development
Rare Forms of Inherited Bone Marrow Failure Syndromes						
Nijmegen breakage syndrome (NBS, MIM #251260)	AR	Pancytopenia	AML Lymphoma	*NSB1*†	8q21	DNA repair
DNA ligase IV syndrome (LIG4, MIM #606593)	AR	Pancytopenia	Leukemia	*LIG4*	13q22-q34	DNA repair
Seckel syndrome (SCKL1, MIM #210600)	AR	Pancytopenia (not genetically subtyped)	Leukemia Lymphoma Oropharyngeal cancer (not genetically subtyped)	*ATR* *SKC1*	3q22-q24	DNA repair
				PCNT *SCKL4*	21q22.3	Centrosomal function, ATR signaling pathway
	AR			*CENPJ SCKL4*	13q12.12	Centrosomal function, ATR signaling pathway
	AR			*CEP152* *SCKL5*	15q21.1	Centrosomal function, ATR signaling pathway
	AR			*CtIP/RBBP8* *SCKL2*	18q11.2	Centrosomal function, ATR signaling pathway
	AR			*ATRIP*	3p21.31	Interacts with ATR, ATR signaling pathway
Dubowitz syndrome (MIM %223370)	AR	Pancytopenia	Leukemia Lymphoma Neuroblastoma Cancer	Unknown	Unknown	DNA repair (?)
Schimke syndrome (SIOD, MIM #242900)	AR	Pancytopenia	Lymphoma	*SMARCAL1*	2q34-q36	Chromatin remodeling
Duncan/Purtilo syndrome (XPL, MIM #308240)	XLR	Pancytopenia	EBV lymphoma	*SH2D1A/SAP*	Xq25	B-cell and T-cell function

AML, Acute myeloid leukemia; *AR*, autosomal recessive; *AD*, autosomal dominant; *ATR*, ataxia-telangiectasia and Rad3 related; *GCSF*, granulocyte colony-stimulating factor; *MDS*, myelodysplastic syndrome; *MIM #*, Mendelian Inheritance in Man with responsible gene identified; *MIM %*, Mendelian Inheritance in Man with responsible gene not identified; *XLR*, X-linked recessive.

*No specfic ribosomal gene mutation has been assocated with AML/MDS.

†Specific mutations only.

(see Table 7-1). In the past investigations of IBMFSs were largely restricted to careful observations and descriptions of clinical manifestations in the patient and family, as well as meticulous study of the clinical course of the disease. However, innovations in genomic sequencing technology have led to the identification of a rapidly increasing number of genes that when mutated are responsible for certain IBMFSs. Characterization of these genes and investigation of their role in hematopoiesis are powerful new tools to explore the pathways responsible for BMF.

Traditionally, IBMFSs have been classified according to their clinical features. Increasingly this classification scheme is being supplanted by one based on the affected biochemical pathway (see Table 7-1). Surprisingly, the affected pathways often entail "housekeeping" functions important for most cell types (DNA repair, telomere maintenance, or ribosome biosynthesis) rather than functions unique to hematopoietic cells. Why hematopoiesis is preferentially and sometimes even exclusively affected by a derangement in these pathways is intriguing and the focus of current investigations in the field of BMF research. One feature these pathways share is that their derangement usually causes the activation of p53, suggesting that this molecule plays a central role in the pathogenesis of IBMFSs (Fig. 7-1).[3,4]

With the increased knowledge of how the genetic framework influences the development of BMF, distinguishing between inherited and acquired BMF has become more difficult. Previously, IBMFSs were restricted mainly to patients with BMF caused by gene mutations with high disease penetrance. Today, it is increasingly being recognized that, in addition, a growing number of patients have BMF caused by gene mutations that have low or very low penetrance, with disease developing only occasionally or just under certain precipitating circumstances. Thus the majority of patients in whom acquired idiopathic aplastic anemia was previously diagnosed may have an underlying genetic component. Figure 7-1 schematically illustrates the possible interrelationship between genetic mutation, environment, and gene modifiers in the pathogenesis of BMF. In the coming years, advances in DNA sequence technology are likely to bring a wealth of new information to the field of BMF research that in combination with the power of bioinformatics is likely to shift some of the current disease paradigms. The increased understanding of the genetic components and their role and importance in the pathogenesis of disease will allow us to improve the diagnosis, management, and treatment of patients with BMF.

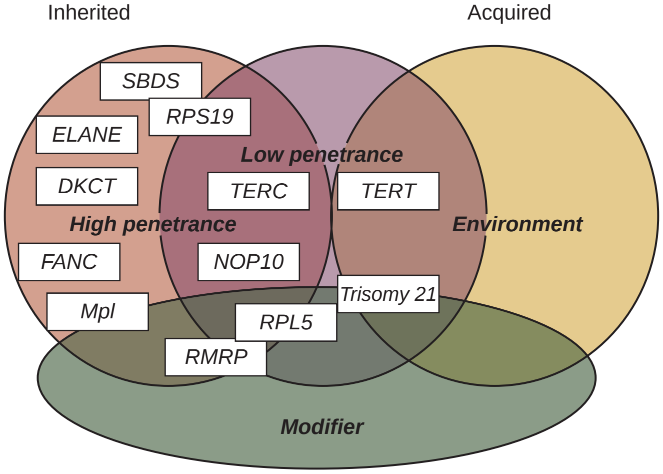


Figure 7-1 Interrelationships of disease penetrance of a genetic mutation, the environment, and modifiers in the pathogenesis of bone marrow failure.

Box 7-1 Fanconi Anemia

Fanconi anemia (FA, MIM #227650) is an autosomal recessive and X-linked disorder characterized by progressive bone marrow failure, congenital abnormalities, and a predisposition for malignant disease. Cells from patients with FA exhibit spontaneous chromosomal instability and a characteristic hypersensitivity to DNA interstrand cross-linking agents such as diepoxybutane (DEB) and mitomycin C (MMC).

INHERITED BONE MARROW FAILURE SYNDROMES ASSOCIATED WITH PANCYTOPENIA

Fanconi Anemia

Fanconi anemia (FA) is a genetically and phenotypically heterogeneous disorder. The disease is named after the Swiss pediatrician Guido Fanconi (1892-1979), who first described the syndrome in 1927.[5] Clinically, FA is characterized by a variety of congenital abnormalities, progressive BMF, and a propensity for the development of leukemia and other forms of cancer. Cells from FA patients have a striking hypersensitivity to DNA interstrand cross-links[6]; this trait has become the basis of a clinical diagnostic test for FA (Box 7-1).[7]

Epidemiology

The incidence of FA is difficult to ascertain. The Fanconi Anemia Research Fund, Inc., estimates that about 30 persons with FA are born in the United States each year; this would indicate that the prevalence of FA is about 2.5 per million and the carrier frequency is approximately 1 in 180.[8] The male-to-female ratio is 1.2 : 1.[9] FA has been reported in all races and ethnic groups. The world's highest prevalence of FA, caused by a founder effect, is seen in Spanish gypsies, who have a carrier frequency of 1 in 70.[10] A high prevalence of FA is also found in individuals of Ashkenazi Jewish decent, who have an approximate heterozygous frequency of 1 in 77,[11] and in South African Afrikaans, in whom carrier frequency is estimated to be 1 in 83.[12]

Clinical Manifestations

The clinical manifestations of FA are heterogeneous (variable penetrance and expressivity; for definitions, see Box 7-2). Affected family members can have a wide variety of abnormalities (variable expressivity).[13-15] Even monozygotic twins have been described to differ in their physical and hematologic manifestations.[15] However, the occurrence of malformations is nonrandom. Siblings are usually

Box 7-2 **Heritability, Penetrance, and Expressivity**

Heritability, penetrance, and expressivity are distinct but related concepts. *Heritability* is the number of family members who have inherited a genotype versus the number of family members who have not. *Penetrance* is defined by the number of individuals who show a phenotype versus the number of individuals who carry the genotype. *Expressivity* measures the variability and severity of an expressed phenotype.

concordant for the presence or absence of multiple congenital abnormalities; likewise, the age at onset of hematologic manifestations is similar within an individual family.[16] Thus the variability in clinical findings appears to be to some extent linked to the different genotypes. The emergence of revertant cells leading to hematopoietic mosaicism further contributes to disease variability (see the section on Somatic Mosaicism, Functional Reversion, and Attenuation of Fanconi Anemia Cells).[15,17-19,20-22] Originally, the diagnosis of FA was based on the presence of both aplastic anemia and a specific, though broad, range of physical abnormalities.[23] With the availability of more sensitive and specific tests for the diagnosis of FA, including the availability of genetic testing, it has become evident that a significant proportion of patients lack congenital abnormalities (25% to 40%) or fail to develop aplastic anemia.[24-28] Nevertheless, these individuals are still at risk for late complications such as leukemia and cancer.

Congenital Abnormalities

The frequency and nature of congenital abnormalities within the FA population is summarized in Box 7-3. Figure 7-2, *A* shows a 1-year-old girl with FA, and Figure 7-2, *C* shows a 12-year-old boy with several classic phenotypic features, including short stature, microencephaly, broad nasal bridge, epicanthal folds, micrognathia, café-au-lait spots and hypopigmentation, and one absent thumb and one triphalangeal thumb. FA patients may have a combination of the congenital anomalies listed or none of them.

Box 7-3 **Specific Types of Anomalies in Fanconi Anemia**[*26-28]

SKIN (40%)

Generalized hyperpigmentation on the trunk, neck, and intertriginous areas; café-au-lait spots; hypopigmented areas

BODY (40%)

Short stature, delicate features, small size, underweight

UPPER LIMBS (35%)

Thumbs (35%): absent or hypoplastic; supernumerary, bifid, or duplicated; rudimentary; short, low-set, attached by a thread; triphalangeal, tubular, stiff, hyperextensible
Radii (7%): absent or hypoplastic (only with abnormal thumbs); absent or weak pulse
Hands (5%): clinodactyly; hypoplastic thenar eminence; six fingers; absent first metacarpal; enlarged, abnormal fingers; short fingers; transverse crease
Ulnae (1%): dysplastic or absent

LOWER LIMBS (5%)

Feet: toe syndactyly, abnormal toes, flatfeet, short toes, clubfeet, six toes, supernumerary toe
Legs: congenital hip dislocation, Perthes disease, coxa vara, abnormal femur, thigh osteoma, abnormal legs

GONADS

Males (25%): hypogenitalia, undescended testes, hypospadias, abnormal genitalia, absent testis, atrophic testes, azoospermia, phimosis, abnormal urethra, micropenis, delayed development
Females (2%): hypogenitalia; bicornuate uterus; abnormal genitalia; aplasia of uterus and vagina; atresia of uterus, vagina, and ovary

OTHER SKELETAL ANOMALIES

Head (20%) and face (2%): microcephaly, hydrocephalus, micrognathia, peculiar face, bird-like face, flat head, frontal bossing, scaphocephaly, sloped forehead, choanal atresia, dental abnormalities
Neck (1%): Sprengel deformity; short, low hairline; webbed
Spine (2%): spina bifida (thoracic, lumbar, cervical, occult sacral), scoliosis, abnormal ribs, sacral agenesis, sacrococcygeal sinus, Klippel-Feil syndrome, vertebral anomalies, extra vertebrae

EYES (20%)

Small eyes, strabismus, epicanthal folds, short or almond-shaped palpebral fissures, hypertelorism, ptosis, slanting, cataracts, astigmatism, blindness, epiphora, nystagmus, proptosis, small iris

EARS (10%)

Deafness (usually conductive); abnormal shape; atresia; dysplasia; low-set, large, or small; infections; abnormal middle ear; absent eardrum; dimples; rotated; canal stenosis

KIDNEY (20%)

Ectopic or pelvic; abnormal, horseshoe, hypoplastic, or dysplastic; absent; hydronephrosis or hydroureter; infections; duplicated; rotated; reflux; hyperplasia; no function; abnormal artery

GASTROINTESTINAL SYSTEM (5%)

High-arched palate, atresia (esophagus, duodenum, jejunum), imperforate anus, tracheoesophageal fistula, Meckel diverticulum, umbilical hernia, hypoplastic uvula, abnormal biliary ducts, megacolon, abdominal diastasis, Budd-Chiari syndrome

UROGENITAL

Males (25%): Micropenis, penile-scrotal fusion, undescended or atrophic or absent testes, hypospadias, chordee, phimosis, azoospermia
Females (2%): bicornate uterus, aplasia or hypoplasia of vagina and uterus, atresia of vagina, hypoplastic uterus, hypoplastic or absent ovary, hypoplastic fused labia

CARDIOPULMONARY SYSTEM (6%)

Patent ductus arteriosus, ventricular septal defect, abnormal heart, peripheral pulmonic stenosis, aortic stenosis, coarctation, absent lung lobes, vascular malformation, aortic atheromas, atrial septal defect, tetralogy of Fallot, pseudotruncus, hypoplastic aorta, abnormal pulmonary drainage, double aortic arch, cardiac myopathy

CENTRAL NERVOUS SYSTEM (3%)

Hyperreflexia, Bell palsy, central nervous system arterial malformation, Moyamoya syndrome, Arnold-Chiari malformation, stenosis of internal carotid artery, small pituitary gland, absent corpus callosum
Slow development (10%)

From Auerbach A, Buchwald M, Joenje H: Fanconi anaemia. In Vogelstein B KK, editor: The genetic basis of human cancer. *New York, 1999, McGraw Hill, p. 317–332; Shimamura A, Alter BP: Pathophysiology and management of inherited bone marrow failure syndromes.* Blood Rev *24:101–122, 2010.*
*Abnormalities are listed in the approximate order of frequency within each category.

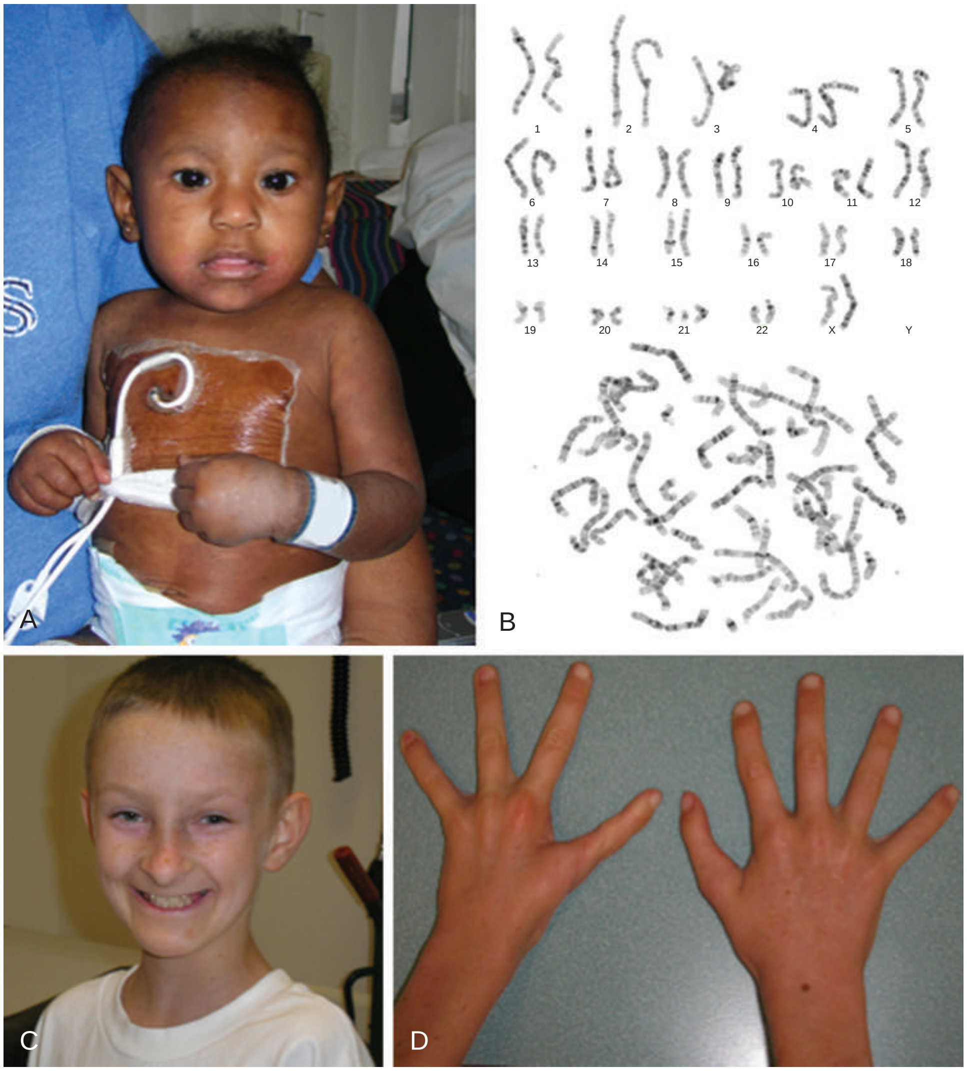


Figure 7-2 **A**, Infant female with Fanconi anemia, who exhibited several classic phenotypic features, including short stature, microcephaly, broad nasal base, epicanthal folds, micrognathia, and café-au-lait spot (left forehead). **B**, Cytogenetic findings in peripheral blood lymphocytes in Fanconi anemia. **C** and **D**, A 12-year-old boy with Fanconi anemia. Classic phenotypic features are microcephaly, broad nasal base, epicanthal folds, micrognathia, café-au-lait spot (chin and left temple), hyperpigmentation, and an absent thumb on one hand and a triphalangeal thumb on the other hand. *(B, Courtesy of Shashikanat Kulkarni, Washington University.)*

The phenotypic variability makes a diagnosis of FA difficult when it is based only on clinical features.

Endocrinopathies

Data from the International Fanconi Anemia Registry and from the Cincinnati FA cohort indicate that endocrine abnormalities are present in about 80% of children and adults with FA, primarily glucose/insulin abnormalities with a blunted first-phase insulin release, mild hypothyroidism, and male hypogonadism.[15,29-31]

Short Stature. Although short stature is an integral feature of FA,[31,32] a superimposed endocrinopathy may have an additional impact on growth and is responsive to replacement therapy.[31] Detection of an anatomic abnormality of the hypothalamic-pituitary region by magnetic resonance imaging is a diagnostic marker of permanent growth hormone deficiency.[33] In contrast to previous reports, osteopenia or osteoporosis, when adjusted for body size and height, is not frequent.[31]

Glucose Intolerance. Impaired glucose tolerance, elevated insulin levels, and overt diabetes mellitus are common in FA. Importantly, HgbA1c or elevated fructosamine does not identify glucose intolerance in FA, possibly because of the high fetal hemoglobin or abnormalities in glycosylation.[31]

Fertility. Female patients with FA usually have a shortened reproductive life because of secondary amenorrhea, irregular menses, anovulatory menstrual cycles, and premature menopause. Congenital abnormalities in the female genitourinary tract further limit fecundity. Though rare, pregnancies have been reported in women with FA. The babies born were normal, and the pregnancies seemed to have had a transient but not lasting impact on the mother's hematologic manifestations.[26,34] Pregnancy in women with FA has also been reported after BMT.[35,36] Cryptorchidism, hypogenitalism, hypergonadotropic hypogonadism, and reduced sperm counts are frequent in male patients with FA. Reports of male FA patients fathering children are rare.[26]

Hematologic Abnormalities

Progressive BMF is one of the hallmarks of FA, although the hematologic findings vary. Thrombocytopenia and macrocytosis often precede anemia and neutropenia. Elevated hemoglobin F levels[37] and elevated expression of "i" antigen on red cells usually coincide with macrocytosis and are suggestive of stress hematopoiesis.[38] Serum alpha-fetoprotein levels are consistently elevated in FA patients irrespective of the presence of liver abnormalities.[39,40] Erythropoietin levels are usually elevated in anemic FA patients. The first hematologic abnormalities in individuals with FA are detected at a median age of 7 years. The majority of FA patients already have pancytopenia at the time of diagnosis (53%). By the age of 40, the cumulative incidence of hematologic abnormalities is 90% to 98%.[19,41]

In rare cases, thrombocytopenia may be present at birth and progress to pancytopenia in the neonatal period or infancy.[16,42] Thus FA also has to be considered in cases of aplastic anemia in neonates and infants. In other patients, hematologic abnormalities never become clinically evident, and the diagnosis of FA is made later in life as a result of late FA-associated complications such as cancer or leukemia. The emergence of revertant cells leading to hematopoietic mosaicism is often associated with a mild or absent hematologic phenotype (see later). Finally, in a small proportion (2% to 5%) of FA patients, MDS or leukemia is diagnosed but the underlying BMF never becomes clinically apparent.[16] Bone marrow examination generally shows reduced cellularity; however, it may also be normocellular or hypercellular, particularly in disease that evolves into MDS or MDS/acute myeloid leukemia (AML). The cumulative incidence of BMF is approximately 50% by 40 to 50 years of age.[19,41] BMF is usually progressive. Fifty percent of individuals who are initially found to have thrombocytopenia progress to pancytopenia within 3 to 4 years.[16]

Myelodysplastic Syndrome and Acute Leukemia in Patients with Fanconi Anemia

The cumulative incidence of MDS in FA patients is approximately 30%, and of acute leukemia about 10% by the age of 40 to 50 years.[19,41] In the literature the definition of MDS and acute leukemia in FA patients varies, which makes a strict comparison difficult. Clonal hematopoiesis, as suggested by nonrandom X-inactivation, may be present even before the onset of MDS.[43,44] Clonal cytogenetic abnormalities are found in 34% to 48% of FA patients.[16] Fluctuations are frequent, including the disappearance of clones, the appearance of new clones, and clonal evolution.[45] Chromosomal abnormalities in MDS most frequently involve chromosomes 1q+, 3q+, −7/7q, 11q− 20q−, 6, 13, and 21q− (Fig. 7-3).[44,46]

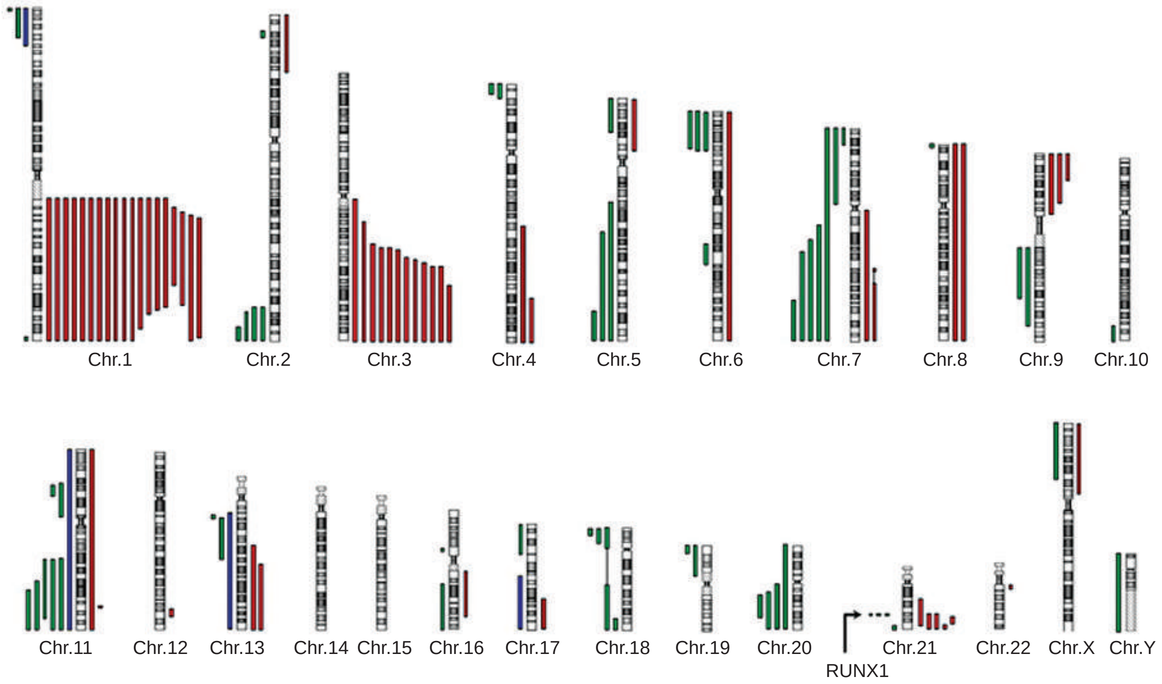


Figure 7-3 A recurrent pattern of somatic copy number abnormalities and uniparental disomy (UPD) in Fanconi anemia bone marrow cells. All acquired copy number and UPD regions found in the bone marrow cells of 57 individuals with Fanconi anemia are shown. Green lines represent deletions; red lines, gains; and blue lines, regions of UPD (somatic copy-neutral loss of heterozygosity). Constitutional copy-neutral homozygosity regions are not shown. The RUNX1 gene location at 21q22 is shown. *(From Quentin S, Cuccuini W, Ceccaldi R, et al: Myelodysplasia and leukemia of Fanconi anemia are associated with a specific pattern of genomic abnormalities that includes cryptic RUNX1/AML1 lesions.* Blood *117:e161–170, 2011.)*

Individuals in whom clonal cytogenetic abnormalities develop have a reduced survival rate of 35% to 40% as opposed to 92% in FA patients without such abnormities. An exception is patients with an isolated 1q+ that may be present without morphologic MDS and is not associated with progression to MDS/AML.[44,46] MDS morphology, as defined by dysplasia in multiple blood cell lineages, portends a poor outcome, with a 5-year survival rate of 9%. Gain of the distal portion of chromosome 3q(26-29) is frequently present in FA patients and is associated with a poor prognosis, decreased survival times, and an increased frequency of MDS/AML.[44,46-48]

Leukemia is the most common malignant disease in patients with FA.[19,41] AML is the most frequent leukemic phenotype, although patients with acute lymphocytic leukemia and chronic myelomonocytic leukemia have been described. Leukemia in patients with FA shows many molecular and clinical similarities to leukemia occurring in other IBMFSs; cases of FA-associated leukemia are notoriously difficult to treat, and most patients die within 6 months of diagnosis. Leukemia in patients with FA may arise de novo or develop from MDS. In some FA patients, AML may be the first hematologic manifestation of disease. Leukemic cells often show complex cytogenetic abnormalities, including abnormalities similar to those seen in MDS. Chromosomal translocations and mutations in oncogenes and tumor-suppressor genes frequently found in de novo AML are rare in FA MDS/AML with the exception of *RUNX1* mutations, which are seen in approximately 20% of FA patients with MDS/AML.[44] Abnormalities of chromosome 7 (monosomy, isochromosome, or other structural rearrangement), followed by abnormalities of 1q (usually duplications), are frequent in FA-associated leukemia.[44,46-48]

Predisposition to Malignancy

The risk of cancer developing is about 500 to 700 times higher than in the normal population (Table 7-2). By the age of 40 years, the cumulative incidence of solid tumors is estimated to be 26% to 30%.[41] The median age at development of cancer is 16 years, versus 68 years for the same types of cancer in the general population.[41] The risk of solid tumors and leukemia developing increases with age. Thus patients with mild hematologic and phenotypic abnormalities and the longest survival times are at the highest risk for the development of tumors or leukemia. However, with increased survival after hematopoietic stem cell transplantation (HSCT), the frequency of cancer might also increase in patients with more severe disease. The most frequent cancers occur in the neck, head, and upper esophagus, followed by the vulva, anus, and lower esophagus. Human papillomavirus (HPV), particularly HPV16, is frequently isolated from patients with FA.[49] Although the FA DNA repair pathway is involved in limiting HPV replication[49] and patients with FA are frequent carriers of HPV, the role of HPV in the pathogenesis of squamous cell cancer in patients with FA remains controversial. HPV vaccination at an early age should be considered for all patients with FA. The risk for development of squamous cell cancer of the head and neck and particularly the tongue is higher in FA patients after HSCT than in those without HSCT and correlates with the presence and severity of graft-versus-host disease.[50] Adenoma of the liver and hepatocarcinoma were found mainly in patients undergoing androgen therapy.[40] Hepatoadenomas usually regress after cessation of androgen therapy.[40]

The risk and type of cancer vary for different FA subtypes (also see the section on Risk of Malignant Disease). FA patients belonging to complementation group D1 (FA-D1) with biallelic mutations in *FANCD1/BRCA2* have a high risk for the development of malignant disease at a very young age, frequently before manifestations of BMF appear, and the cumulative risk for AML or other cancers is 97% by the age of 5.2 years. In addition, the spectrum of cancer is distinct, with medulloblastoma being the predominant malignant disease,[41,51,52] possibly because BRCA2 participates in DNA repair outside the FA pathway. An increased risk for childhood cancer with a very similar spectrum has also been found in families belonging to the FA-N subtype.[53] FANCN/PALB2 binds to BRCA2 and facilitates its binding to chromatin.[54] The interaction between these two proteins explains the similar cancer predisposition in FA individuals belonging to the FA-D1 and FA-N subgroups.

All FA cancer patients commonly have a low tolerance for DNA-damaging chemotherapeutic agents. Accordingly, chemotherapeutic regimens often need to be modified, given at low dosage, or avoided in favor of alternative or surgical approaches.

Diagnosis

The most widely used diagnostic test for FA is hypersensitivity to the clastogenic (chromosome-breaking) effect of diepoxybutane (DEB) or mitomycin C (MMC).[7,55,56] The increased spontaneous chromosomal instability of FA cells leads to distinctive chromosomal breaks and gaps and various chromatid interchanges, which were previously used as a cellular marker for FA. The frequency of spontaneous chromosomal instability in FA cells is highly variable, and it is thought to be responsible for the increased susceptibility of FA patients to cancer. The sensitivity of FA cells to the clastogenic effect has been studied for numerous cross-linking agents, but DEB and MMC are the agents most widely used for the diagnosis of FA.[7,55] Figure 7-2, *B* shows the increased spontaneous chromosome fragility in peripheral lymphocytes in a patient with FA. The diagnosis of FA is made when DEB-treated cultured lymphocytes demonstrate a 3- to 10-fold increase in chromosomal breakage over normal controls. The tests are highly sensitive and fairly specific for FA and are used for the diagnosis of FA before the development of clinical disease and for prenatal diagnosis.[7,55] Possible rare exceptions that can result in a pattern of DEB- or MMC-induced chromosomal breakage similar to that found in FA patients are individuals with specific mutations in the *NBS1* gene in Nijmegen breakage syndrome and patients with cohesinopathies, that is, those with Roberts syndrome and Warsaw breakage syndrome.[57-59] However, more frequently, the presence of revertant cells in the specimen may complicate the interpretation of DEB or MMC test results (see the section on

TABLE 7-2 Cancer in Fanconi Anemia*

	No. of Patients (%)			
Type	**Alter et al[672]**	**Alter et al[41]**	**Kutler 2003[19]**	**Rosenberg[673]**
Reporting period	1927-2001	2002-2008	1982-2001	2001
Patients evaluated	1301	66	754	145
Hematologic malignant diseases	214 (16)	12 (18)	128 (17)	32 (22)
Cumulative incidence	—	30	33 (by age 40)	10 (by age 10)
Acute myeloid leukemia	116 (9)	4 (6)	60 (8)	9 (6)
Myelodysplastic syndrome	89 (7)	8 (12)	53 (7)	23 (16)
Acute lymphocytic leukemia	7	—	5	—
Chronic myelomonocytic leukemia	—	1	1	—
Lymphoma	2	—	2	—
NONHEMATOLOGIC MALIGNANT DISEASES	89 (7)	12 (18)	73 (10)	18 (12)
Cumulative incidence	—	—	28 (by age 40)	29 (by age 49)
Squamous cell carcinoma	66 (5)	5 (8)	39 (5)	16 (11)
Oropharyngeal cancer	26 (2)	—	19 (3)	6 (4)
Esophageal cancer	9	—	1	2
Vulva and anus cancer	10	3	—	—
Vulva cancer	3	—	8	3
Anus cancer	6	—	2	2
Cervical cancer	6	—	6	3
Cutaneous cancer (nonmelanoma)	6	2	3	
Hepatic malignant diseases	37 (3)	—	18 (2)	2 (1)
Adenoma	6	—	11	11
Hepatocellular carcinoma	14	—	6	22
Renal cancer	0	—	6 (1)	—
Brain cancer	1	—	5 (1)	1
Miscellaneous tumors			11 (1)	
Breast cancer	4	1	3	4
Lung cancer	3	—	—	2
Gastric cancer	2	—	—	2
Colon cancer	0	—	—	1
Osteogenic sarcoma	1	—	1	1
Retinoblastoma	1	—	—	1
Other tumors	—	—	7	1

From Alter BP: Cancer in Fanconi anemia, 1927-2001. Cancer 97:425–440, 2003; *Alter BP, Giri N, Savage SA, et al: Malignancies and survival patterns in the National Cancer Institute inherited bone marrow failure syndromes cohort study.* Br J Haematol 150:179–188, 2010; *Kutler DI, Singh B, Satagopan J, et al: A 20-year perspective on the International Fanconi Anemia Registry (IFAR).* Blood 101:1249–1256, 2003; *Rosenberg PS, Greene MH, Alter BP: Cancer incidence in persons with Fanconi anemia.* Blood 101:822–826, 2003.

*The four cohorts are not mutually exclusive.

Somatic Mosaicism, Functional Reversion, and Attenuation of Fanconi Anemia Cells). It is estimated that 10% to 25% of FA patients exhibit two populations of phytohemagglutinin-stimulated lymphocytes, one that is sensitive to the clastogenic effects of DNA cross-linking agents and one that is resistant to them.[17,60] Thus the presence of only a few metaphases with multiple structural chromosomal changes might indicate FA with somatic mosaicism. In these cases, demonstration of increased chromosomal breakage in DEB- or

MMC-treated fibroblasts might help confirm the diagnosis of FA.

Immunoblotting for FANCD2 Monoubiquitination

Biallelic mutations in 10 of the 15 FA genes (*FANC* genes) lead to failure of FANCD2 monoubiquitination, and failure of FANCD2 monoubiquitination is specific for a defect in the FA pathway (see the section on Function of FANC Proteins). Testing for FANCD2 monoubiquitination by immunoblotting of peripheral blood lymphocytes and fibroblasts is therefore used in some laboratories as an alternative first-line tool for the diagnosis of FA.[61,62] Biallelic mutations in *FANCD1/BRCA2*, *FANCJ*, *FANCN/PALB2*, *FANCO/RAD51C*, and *FANCP/SLX4* that have normal FANCD2 monoubiquitination but an abnormal DEB or MMC test (or both) are missed with this assay and, therefore, additional testing is required.

Genetic Testing for *FANC* Gene Mutations

Only the identification of biallelic mutations in a *FANC* gene definitively confirms the diagnosis of FA. Currently genetic testing for *FANC* gene mutations is not usually recommended as a first-line tool for the diagnosis of FA. Possible exceptions are families with a known *FANC* gene mutation and FA in a population with a high frequency of a founder mutation, for example, a case of FA in Spanish gypsies. The indications, advantages, and difficulties of genetic testing for biallelic mutations in *FANC* genes are discussed later. As costs decrease, targeted or whole-genome next-generation sequencing is likely to become the diagnostic test of choice for patients clinically suspected to have FA (see the section on Molecular Diagnosis of Fanconi Anemia).

Cellular Phenotype of Fanconi Anemia Cells

In addition to increased spontaneous and induced chromosomal instability, FA cells have several other phenotypic characteristics (Table 7-3). FA cells have increased sensitivity to other DNA-damaging agents, such as ionizing radiation and oxygen radicals.[6,7,63,64] FA cells have an abnormality in cell cycle distribution, with an increased number of cells with 4N DNA content, suggesting a delay in the G_2/M or late S phase (or both) of the cell cycle.[65] FA cells have a failure in cytokinesis forming an increased number of ultrafine DNA bridges (UFB) during the M phase of the cell cycle, leading to increased chromosome instability and binucleated cells that undergo apoptosis.[66] The increase in cells with 4N DNA content is accentuated after treatment with interstrand DNA cross-linking agents. Flow cytometric measurements of the increase in the proportion of cells with 4N DNA content after such treatment may be used as an additional criterion for the diagnosis of FA.[67] However, similar results have been obtained in patients with ataxia-telangiectasia. FA cells show accelerated telomere shortening in vitro and an increase in telomere free ends and chromosome end fusions, suggesting a defect in maintenance of telomere integrity and increased breakage at telomeric sequences.[68,69] Cells deficient in the FA repair pathway are hypersensitive to both formaldehyde and acetaldehyde.[70,71,72]

TABLE 7-3 Cellular Abnormalities in Fanconi Anemia

Feature	References
Spontaneous chromosome breaks	60
Sensitivity to cross-linking agents	7, 55, 64
Sensitivity to formaldehyde	70-72, 89
Prolongation of G_2/M phase and S phase of cell cycle	67, 674
Failure in cytokinesis, with increased number of ultrafine DNA bridges (UFB) during M phase, binucleated cells	66
Accelerated telomere shortening	10, 69, 675, 676[*]
Hypersensitivity to ionizing radiation[†]	677
SENSITIVITY TO OXYGEN	Reviewed in 139
Poor growth at ambient O_2	63
Overproduction of O_2 radicals	678
Deficient O_2 radical defense	679
Deficiency in superoxide dismutase	680
Overproduction of TNF-α	681
Increased TNF-α and IFN-γ sensitivity	682, 683
DIRECT DEFECTS IN DNA REPAIR	
Defective repair of DNA cross-links	684
Hypermutability (by deletion)	685, 686
Increased apoptosis	687, 688
Increased induction of p53	4, 687, 689
Defective end-joining fidelity	690-692
Increased homologous recombination	693, 694
INTRINSIC STEM CELL DEFECT	
Decreased colony growth in vitro	695
Decreased gonadal stem cell survival	696

Modified from Kaddar T, Carreau M: Fanconi anemia proteins and their interacting partners: a molecular puzzle. Anemia 2012:425814, 2012.

[*] Experiments in mice suggest that accelerated telomere shorenting is not a direct effect of FA gene products on telomeres but rather an indirect effect, such as the increased sensitivity to oxygen radicals.

[†] Hypersensitivity to ionizing irradiation is controversial. More recent results suggest an increased sensitivity in the majority of fa complementation groups.

Differential Diagnosis

Clinically, FA has to be differentiated from other IBMFSs, particularly dyskeratosis congenita (DC), Diamond-Blackfan anemia (DBA), Shwachman-Diamond syndrome (SDS), Wiskott-Aldrich syndrome (WAS), thrombocytopenia with absent radii (TAR), and other nonclassified forms of BMF. Clinical manifestations, associated physical abnormalities, family history (pattern of inheritance), and increased chromosomal fragility in the DEB or MMC test performed on cultured peripheral lymphocytes or fibroblasts, or both, are helpful in strengthening the diagnosis of FA. However, only identification of pathogenic mutations on both copies of an FA gene (biallelic mutation) may definitely consolidate the diagnosis of FA. Congenital abnormities in FA may have an overlap with malformations seen in other disorders with genetic and nongenetic causes. Thrombocytopenia and radial ray abnormalities are also characteristic of TAR. However, in FA, if the radii are affected, the thumbs are always abnormal; in TAR, in which the radii are absent, thumbs are always present. Other syndromes that have phenotypic overlap with FA include Holt-Oram syndrome (MIM 142900), which is characterized by thumb anomalies and atrial septal defects and caused by mutations in the *TBX5* gene, and Baller-Gerold syndrome (MIM 218600) and Rothmund-Thomson syndrome (MIM 278400), which are caused by mutations in the *RECQL4* gene and characterized by radial defects, craniosynostosis, and cancer predisposition but not BMF. FA patients may present with VATER (vertebral defects, imperforate anus, tracheoesophageal fistula, and radial and renal dysplasia) or VACTERL (vertebral abnormalities, anal atresia, cardiac abnormalities, tracheoesophageal fistula and/or esophageal atresia, renal agenesis and dysplasia, and limb defects) (MIM 192350) or the IVIC syndrome (MIM 147750), which is an acronym for Instituto Venezolano de Investigaciones Cientificas, the institution at which a family with autosomal dominant radial ray defects, hearing impairment, internal ophthalmoplegia, and thrombocytopenia was first described. In patients presenting with these associated anomalies, FA should be excluded.

The increased chromosomal breakage in FA has to be distinguished from that seen in Bloom syndrome (MIM 210900), another single-gene disorder with a predisposition for leukemia as a result of mutation in the helicase RecQ protein-like-3 gene (*RECQL3*). Chromosomal aberrations are in general more numerous in FA cells than in Bloom cells. Furthermore, in Bloom syndrome, most interchanges occur between homologous chromosomes, whereas in FA they are more frequently seen between nonhomologous chromosomes.

Nijmegen breakage syndrome (NBS; MIM 251260), caused by mutations in the *NBS1* gene and characterized by immunodeficiency and a predisposition to lymphoma, is another autosomal recessive chromosomal breakage disease. A small number of patients with NBS may have FA-like features, including BMF and the development of myeloid leukemia,[73,74] and specific mutations in the *NSB1* gene cause an increased clastogenic response to MMC and DEB testing.

A third disease associated with spontaneous chromosomal breakage and hematopoietic malignant disease is ataxia-telangiectasia (MIM 208900), which is inherited in an autosomal recessive manner. Cells from patients with ataxia-telangiectasia are hypersensitive to ionizing radiation but not to cross-linking agents. Furthermore, the disease is associated with immunodeficiency and progressive cerebellar neuronal degeneration, which clearly distinguish this condition from FA.

Seckel syndrome (*SCKL1*, MIM 210600), caused by a defect in the signaling pathway dependent on ataxia-telangiectasia and Rad3-related (ATR), may have some clinical similarities to FA, including anemia and an increased risk for AML.

Patients deficient in DNA ligase IV (*LIG4,* MIM 606593) are characterized by microcephaly, growth retardation starting in utero, distinctive facial appearance (birdlike face), developmental delay, immunodeficiency, pancytopenia, and pronounced clinical and cellular radiosensitivity. LIG4 deficiency is another rare IBMFS caused by defective DNA repair.[75,76]

Two of the cohesinopathies, Roberts syndrome (MIM 268300) (caused by mutations in *ESCO2,* encoding an acetyltransferase involved in sister chromatid cohesion) and Warsaw breakage syndrome (MIM 613398) (caused by mutations in *DDX11/ChlR1,* encoding an XPD-like DNA helicase), show excessive MMC-induced chromosomal breakage in primary lymphocyte cultures and an increased arrest of primary skin fibroblasts in the G_2/M phase of the cell cycle that need to be distinguished from FA.[59]

Genetic Characteristics

Inheritance. FA is a genetically heterogeneous recessive disorder. Mutations in at least 15 different genes, all of which have been identified, may be responsible for FA. Inheritance of FA is autosomal or, in rare cases, X-linked.

Complementation Groups. At least 16 genetic subtypes or complementation groups have been identified (FA-A, FA-B, etc.), each with a distinctive disease gene (Fanconi anemia gene A [*FANCA*], *FANCB*, etc.). The majority of patients belong to complementation group A (≈ 60% to 70%), followed by complementation groups C and G. Table 7-4 shows the complementation groups and the genes responsible for FA and their frequency. Complementation groups were originally defined by somatic cell fusion and the ability of the heterokaryon to correct the MMC hypersensitivity as measured in a growth inhibition assay.[77,78] Correction of the MMC hypersensitivity indicates that the two fusion partners belong to different complementation groups, whereas no correction indicates that they belong to the same complementation group. More recently and as more *FANC* genes are identified, complementation groups are assigned through the correction of MMC hypersensitivity after introduction of the respective wild-type cDNA (complementation by cDNA expression). Retroviruses expressing *FANC* cDNA may be used to correct the phenotype of T cells from FA patients, which determines the complementation group in a rapid, accurate manner.[79]

Fanconi Genes (*FANC* Genes). *FANC* genes have been identified by expression cloning, positional cloning, protein complex purification, or a combination thereof and are summarized in Table 7-4. Figure 7-4 schematically represents the 15 *FANC* genes and their functional domains.

Function of FANC Proteins. The FANC proteins have been shown to act mainly during the S phase of the cell cycle. Their main role is in the repair of DNA interstrand cross-links that impede replication and transcription by inhibiting DNA strand separation.[80-82] It is generally thought that FANC proteins sense, promote, and coordinate three DNA repair pathways: Repair is initiated by recognition of a lesion by nucleotide excision repair components, followed by incisions made flanking the interstrand cross-links; the gapped intermediate that is produced is filled by translesion synthesis or by repair of broken replication forks by homologous recombination.[83]

TABLE 7-4 Complementation Groups and Genes Responsible for Fanconi Anemia

Complementation Group	Responsible Gene	Percentage of FA Patients	Locus	Protein (kDa)	FANCD2 Monoubiquitination	Main Function of Protein	Comments	References
FA-A	*FANCA*	≈66	16q24.3	163	+	FA core complex	Phosphorylated following DNA damage	697
FA-B	*FANCB*	≈2	Xp22.31	95	+	FA core complex		121
FA-C	*FANCC*	≈10-15	9q22.3	63	+	FA core complex		698
FA-D1	*FANCD1/BRCA2*	3-4	13q12-13	380	–	Recruits RAD51 and promotes HR repair		699
FA-D2	*FANCD2*	≈3	3p25.3	155, 162	+	Monoubiquitinates and recruits FANCP to chromatin	Phosphorylated and monoubiquitinated following DNA damage	700
FA-E	*FANCE*	2-3	6p21.3	60	+	FA core complex, probable adaptor for FANCD2	Direct binding to FANCD2, phosphorylated following DNA damage	701
FA-F	*FANCF*	≈2	11p15	42	+	FA core complex		702
FA-G	*FANCG/XRCC9*	8-10	9p13	70	+	FA core complex		703
FA-I	*FANCI*	<2	15q25-26	150	+	Monoubiquitinated protein (part of FANCI-FAND2 subcomplex)	Phosphorylated and monoubiquitinated following DNA damage, binds to FACND2	448, 704-706
FA-J	*FANCJ/BACH1/Brip1*	<2	17q22.3	150	–	5′→3′ DNA helicase/ATPase	Binding to BRCA1	707
FA-L	*FANCL/PHF9/POG/FAAP43*	Rare	2p16.1	43	+	FA core complex, ubiquitin ligase	Recruits UBE2T, monoubiquitinates FANCD2 and FANCI	708
FA-M	*FANCM/ Hef/ FAAP250*	Rare	14q21.3	250	+	FA core complex, branch migration/ATPase activity, sensor for DNA cross-link damage	Helicase/nuclease motif, phosphorylated following DNA damage, interacts with BLM	709
FA-N	*FANCN/PALB2*	Rare	16p12.1	130	–	Mediates interaction between BRCA1 and BRCA2 during HR	Binding to BRCA2	54, 108, 710
FA-O	*FANCO (RAD51C)*	Rare	17q22	43	–	Promotes HR, interacts with RAD51 and RAD51 homologues		711
FA-P	*FANCP (SLX4)*	Rare	16p13.3	200	–	Functions as holiday junction resolvase with SLX1	Interacts with XPF-ERCC1 and EME1-MUS81 nucleases	712

ATPase, Adenosine triphosphatase; *BLM*, product of the gene mutated in Bloom syndrome; *HR*, homologous recombination; +, gene is required for FANCD2 monoubiquitination; –, gene is not required for FANCD2 monoubiquitination.

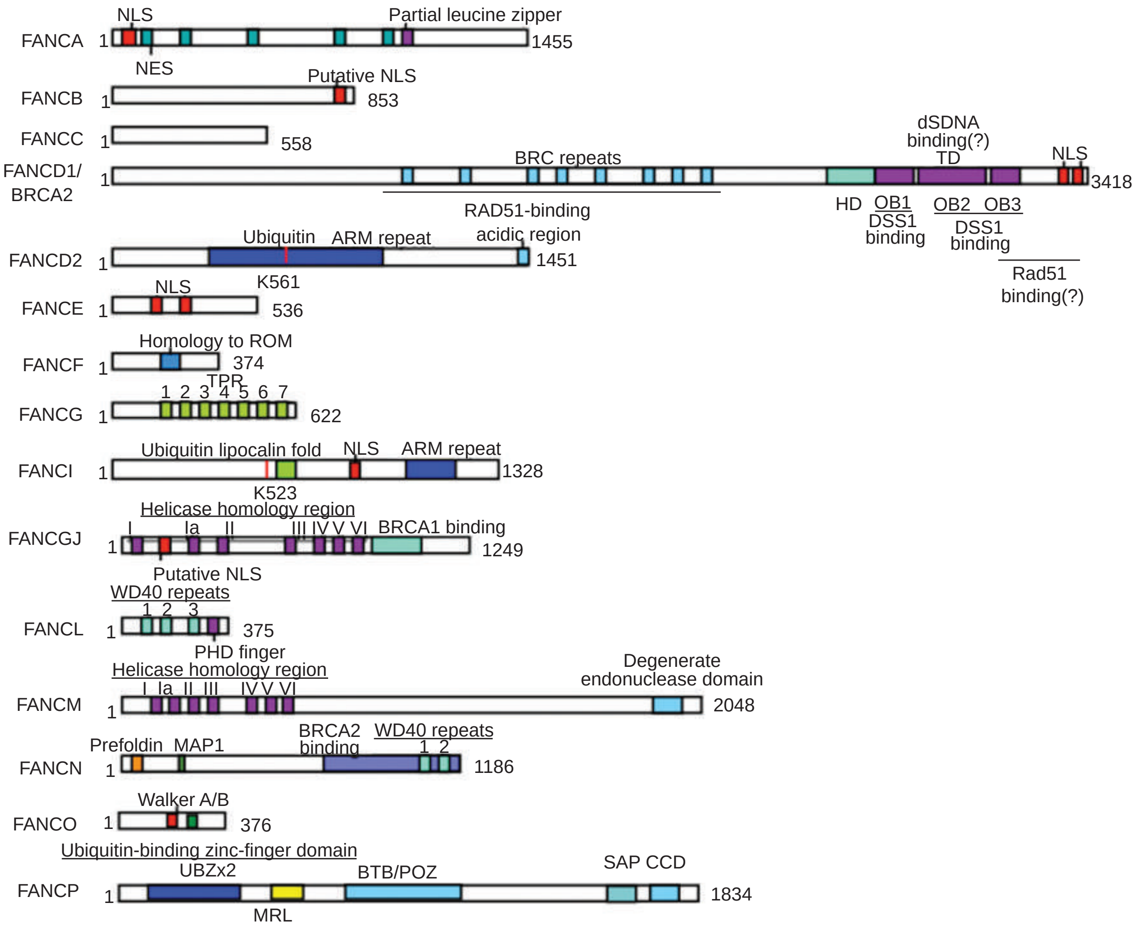

FANCA
NLS
Partial leucine zipper
1
1455
NES
FANCB
Putative NLS
1
853
FANCC
1
558
FANCD1/
BRCA2
BRC repeats
dSDNA
binding(?)
TD
NLS
1
3418
RAD51-binding
acidic region
HD
OB1
DSS1
binding
OB2
OB3
DSS1
binding
Rad51
binding(?)
FANCD2
Ubiquitin
ARM repeat
1
1451
K561
FANCE
NLS
1
536
FANCF
Homology to ROM
1
374
FANCG
TPR
1 2 3 4 5 6 7
1
622
FANCI
Ubiquitin lipocalin fold
NLS
ARM repeat
1
1328
K523
FANCGJ
Helicase homology region
I Ia II III IV V VI
BRCA1 binding
1
1249
Putative NLS
FANCL
WD40 repeats
1 2 3
1
375
PHD finger
FANCM
Helicase homology region
I Ia II III IV V VI
Degenerate
endonuclease domain
1
2048
FANCN
Prefoldin
MAP1
BRCA2
binding
WD40 repeats
1 2
1
1186
FANCO
Walker A/B
1
376
FANCP
Ubiquitin-binding zinc-finger domain
UBZx2
BTB/POZ
SAP CCD
1
1834
MRL

Figure 7-4 Schematic representation of the Fanconi anemia genes and their functional domains. *BTB/POZ*, Broad-complex, tramtrack, and bric-a-brac/poxvirus and zinc finger; *CCD*, conserved C-terminal domain; *dsDNA*, double-stranded DNA; *MLR*, MEI9^{XPF}-interaction–like region; *NES*, nuclear export sequence; *NLS*, nuclear localization signal; *OB*, oligonucleotide/oliosaccharide-binding fold; *ssDNA*, single-stranded DNA; *TD*, tower domain; *TRP*, tetratricopeptide repeat; *UBZ*, ubiquitin-binding zinc-finger domain. *(Modified from Taniguchi T, D'Andrea AD: Molecular pathogenesis of Fanconi anemia: recent progress.* Blood *107:4223–4233, 2006.)*

Thus it is thought, and schematically summarized in Figure 7-5, that in normal cells the FA pathway serves to minimize the severity of mutational outcomes by favoring error-free DNA repair and error-prone DNA repair that results in base pair substitutions or small deletions over DNA repair that results in larger deletions and chromosomal rearrangements (Box 7-4 summarizes the main DNA repair pathways).[84,85]

The current model suggests that FANCM recognizes the DNA interstrand cross-links and initiates DNA repair by recruiting eight FA core complex proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) to the DNA damage site, stabilizing the stalled replication fork, and initiating ATR mediated checkpoint signaling. The FA core complex functions as an ubiquitin ligase, of which FANCL is the catalytic subunit. FANCE recruits FANCD2 to the FA core complex, whereas disease-associated *FANCE* mutations disrupt the FANCE-FANCD2 interaction.[86] Figure 7-6 schematically summarizes the current view of the FANC protein interconnections in the FA DNA repair pathway.[80-82,87] During activation, multiple FA proteins undergo phosphorylation by ATR-CHK1 checkpoint kinases, highlighting the close interconnection of the FA pathway with DNA damage response signaling. The FA core complex catalyzes monoubiquitination of the FANCI-FANCD2 complex, which is a key regulator of the FA pathway. A defect in any of these core proteins results in failure to monoubiquitinate the FANCI-FANCD2 complex, underscoring the importance of this modification in the FA pathway. FANCI and FANCD2 are dependent on each other for their respective monoubiquitination. The FANCL subunit is a ubiquitin E3 ligase, and in concert with the UBE2T E2 enzyme, it conjugates a single ubiquitin to FANCD2 and to FANCI. The monoubiquitinated FANCI-FANCD2 complex is relocalized to the DNA lesion, where it coordinates cross-link repair together with downstream FA proteins. Monoubiquitinated FANCD2 acts as a platform to recruit multiple nucleases, including FANCP, to coordinate nucleolytic incisions flanking the interstrand cross-links. The incision creates a DSB, which is repaired by homologous recombination. Downstream FA proteins, including FANCD1, FANCJ, FANCN, and FANCO, promote

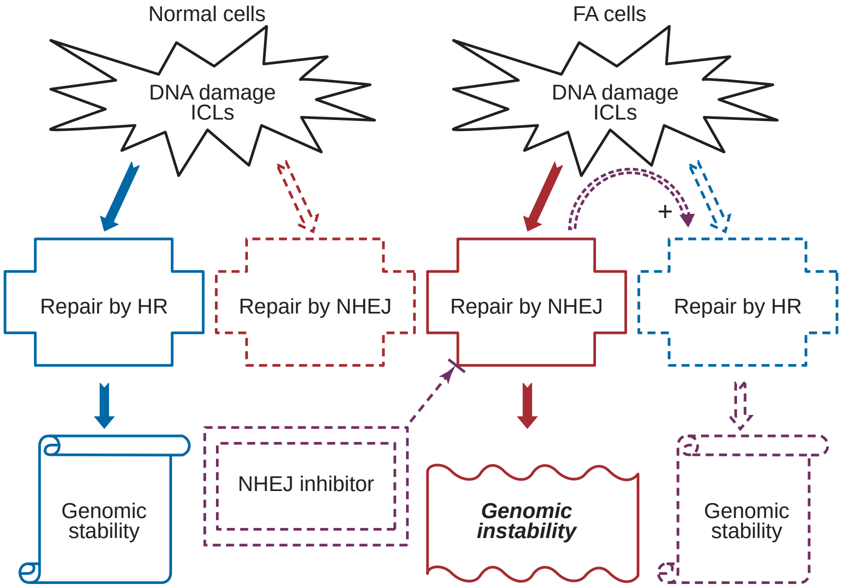


Figure 7-5 Presumed function of the Fanconi anemia DNA repair pathway. In normal cells the Fanconi anemia pathway ensures correct DNA repair by homologous recombination, whereas error-prone alternative pathways using nonhomologous end joining are rarely used. In Fanconi anemia cells the error-free DNA repair by homologous recombination is impaired, so alternative DNA repair pathways are used more frequently, leading to genomic instability and cell death. Suppression of nonhomologous end joining by deleting a component of this pathway or by using DNA-dependent protein kinase catalytic subunit inhibitors promotes the repair of the double-strand breaks by FA-independent pathways of homologous recombination and improves in some experimental systems the sensitivity to DNA cross-linking agents *(dashed arrow). HR,* Homologous recombination; *ICLs,* intrastrand cross-links; *NHEJ,* non-homologous end joining. *(Modified from Deans AJ, West SC: DNA interstrand crosslink repair and cancer.* Nat Rev Cancer *11:467–480, 2011.)*

RAD51-dependent strand invasion and the resolution of homologous recombination intermediates.

The FA core complex is assembled sequentially from subcomplexes, which may have additional functions aside from DNA repair. Several FA proteins, including FANCA, FANCE, FANCG, FANCD2, FANCI, and FANCM, are posttranslationally modified by phosphorylation, and two FA proteins, FANCC and FANCD2, are regulated through a caspase-mediated proteolytic process. FANC proteins have been reported to interact with a number of other proteins, including proteins involved in transcription, the cell cycle, oxidative metabolism, cell signaling, and cytokine response, suggesting that FANC proteins/subcomplexes might have a separate role in cell cycle control, apoptosis, hematopoiesis, and tumorigenesis that is independent of DNA damage recognition/repair.[88]

Bone Marrow Failure in Fanconi Anemia

Although the impairment of DNA repair seems to explain the genomic instability and possibly the increased risk for malignant disease in patients with FA, its link to the pathogenesis of BMF is less obvious, because mutations associated with genomic instability outside the FA DNA repair pathway are not usually associated with BMF. There is an ongoing debate on whether BMF is a direct consequence of the FA DNA repair defect or is caused by impairment of functions apart from DNA repair. Naturally, FA DNA repair and non–DNA repair pathways in the pathogenesis of BMF may not be mutually exclusive but, rather, may overlap. However, two recent findings point strongly towards defective DNA repair being at least the initiating cause of BMF in FA. In a recent study Ceccaldi and colleagues[4] demonstrated that the basal and MMC-induced levels of p53 and its downstream target p21 are elevated in the blood, bone marrow, and liver of fetuses with FA, with a simultaneous accumulation of cells in phase G_0/G_1. These findings suggest that in FA patients the pool of available hematopoietic stem cells and progenitor cells is compromised, with fewer cells and less proliferation of cells than in normal individuals. The impairment is intrinsic, occurs even in the fetus, and leads to overt BMF later in life.[4] Although cross-linking agents have been used experimentally to

Box 7-4 **DNA Repair Pathways**

DNA repair pathways are highly regulated pathways that involve detection of DNA lesions, signaling to recruit DNA repair factors, activation of DNA repair enzymes, and disassembly or degradation of DNA repair factors after repair.

- EXCISION OF DAMAGED AND MISMATCHED BASES
  - Nucleotide excision repair, including global genome repair and transcription-coupled repair
  - Base pair excision repair
  - Mismatch repair
- REPAIR OF DOUBLE-STRANDED BREAKS
  - Homologous recombination (generally error free)
  - Nonhomologous end joining (generally error prone)
- DNA STRESS REPLICATION PATHWAY, REPLICATION PATHWAY OF DAMAGED TEMPLATES DURING DNA SYNTHESIS
  - Translesion DNA synthesis (error prone)

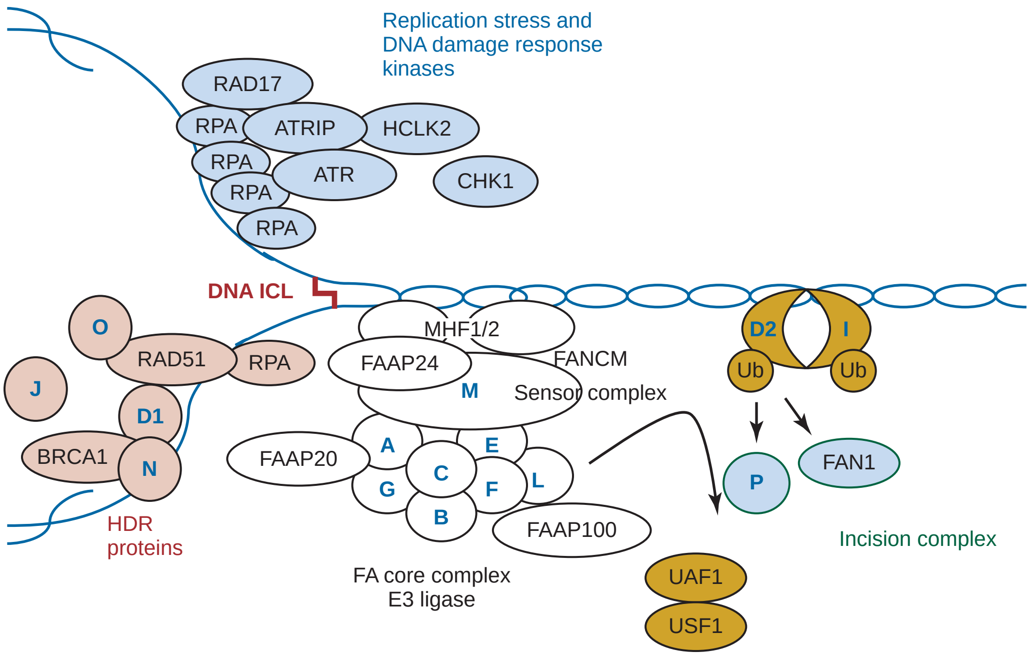


Figure 7-6 Schematic overview of protein interconnections in the Fanconi anemia DNA repair pathway (see also text). The Fanconi anemia pathway maintains genomic stability in response to stalled replication forks, particularly in the context of interstrand DNA cross-links (DNA ICL). Fork stalling is detected by the FANCM sensor complex and triggers ATR (ataxia-telangiectasia and rad3-related)–mediated checkpoint signaling, resulting in the phosphorylation of several Fanconi anemia proteins. FANCM becomes a platform for the other Fanconi anemia core complex proteins, including the FANCL E3 ubiquitin ligase. The core complex monoubiquinates FANCD2 and FANCI (Ub) to promote interstrand DNA cross-link repair. The incision complex, including FANCP/SLX, is essential for resolving the cross-link. Repair and replication fork restart is further accomplished using translesion synthesis and homology-directed repair, which involves BRCA2/FANCD1, PALB2/FANCN, and RAD51C/FANCO. The USP1-UAF1 DUB complex removes monoubiquitin from FANCD2-I and completes the repair. *(Modified from Kee Y, D'Andrea AD: Molecular pathogenesis and clinical management of Fanconi anemia.* J Clin Invest *122:3799–3806, 2012; and Hays LE, Meyer S, van de Vrugt HJ: A molecular, genetic, and diagnostic spotlight on Fanconi anemia.* Anemia *2012:650730, 2012. For further review see also Crossan GP, Patel KJ: The Fanconi anaemia pathway orchestrates incisions at sites of crosslinked DNA.* J Pathol *226:326–337, 2012; Deans AJ, West SC: DNA interstrand crosslink repair and cancer.* Nat Rev Cancer *11:467–480, 2011; Kim H, D'Andrea AD: Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway.* Genes Dev *26:1393–1408, 2012; Kottemann MC, Smogorzewska A: Fanconi anaemia and the repair of Watson and Crick DNA crosslinks.* Nature *493:356–363, 2013.)*

demonstrate the FA defect in DNA repair, experiments by Ridpath and colleagues[70] and Langevin and colleagues[71] suggest that naturally occurring aldehydes might be a major source of endogenous cross-linking and FA pathway activation under steady-state conditions in vivo. Interestingly, HSCs express aldehyde hydrogenase type 2 (*Aldh2*), a condition that is essential for the protection of HSCs against acetaldehyde toxicity.[89] The experiments were performed in mice and need to be confirmed in patients with FA, but the findings suggest that the FA pathway is needed to protect HSCs from aldehyde-mediated genotoxicity (see the section on Mouse Models for Fanconi Anemia). Moreover, these observations explain the tissue specificity of HSC and solve the conundrum of why HSC is affected in FA but is not affected, or not to the same extent, in other non-FA DNA repair disorders.

FANC Gene Mutations in Fanconi Anemia

Detailed information on mutations identified in the *FANC* genes in patients with FA is maintained in the FA Mutation Database at Rockefeller University through the International Fanconi Anemia Registry (available at rockefeller.edu/fanconi/[19,90]) and in the Leiden Open Variation Database; chromium.liacs.nl/lovd/[91]). Frequent FA mutations and mutations with founder effects are presented in Table 7-5.

FANCA is the gene most frequently mutated in FA patients. The mutations are spread throughout the gene and include missense and nonsense mutations, small deletions, insertions, and duplications, as well as splicing mutations.[92] Of note is the substantial number of large genomic, usually intragenic DNA deletions, which account for about 40% of all pathogenic mutations within the *FANCA* gene and are thought to be due to the frequent occurrence of Alu repeats in this genomic region. Large gene deletions are not generally detected by routine nucleotide sequence–based mutation detection methods but require more specialized analysis (haplotype analysis) or molecular methods specifically designed for the detection of large gene deletions. Two small deletions (deletion of three nucleotides between positions 3788 and 3790, with c.3788_3790delTCT leading to deletion of phenylalanine at position 1263, 1263delF, and a 4–base pair (bp) deletion at position 1115, c.1115-1118delTTGG, leading to a frameshift and premature stop codon) are the most common pathogenic mutations in the *FANCA* gene, with a frequency of 8.8% and 5.5% in the

TABLE 7-5 Frequent *FANC* Gene Variants and Mutations with Founder Effect Identified in Fanconi Anemia Patients

Gene	Founder Effect	Variation at DNALevel (Old Nomenclature)[*]	Variation at Protein Level[*]	Frequency within Mutated FA Allele	Population with Founder Effect (% of All FA Patients)	References
FANCA	Yes	c.3788_3790delTCT[†]	p.1263delF	8.8%	51% Brazilians, relatively mild	90, 713
	No	c.1115-1118delTTGG[†]	p.V372fs	5.5%	2.2% Brazilians	90
	Yes	Intragenic deletion of exons 12-31, intragenic deletion of exons 17-31	del		60% Afrikaner in South Africa	714
	Yes	c.295C→T	p.Q55X		Spanish Gypsies (highest prevalence of mutant *FANCA* allele)	10
	Yes	c.65G→A	W22X		Unique to Ashkenazi Jews	715
FANCC	Yes	c.711 +4A→T (IVS4+4A→T)	Splicing		Homozygous in 80% of Ashkenazi-Jewish FA (carrier frequency 1/100), relatively common in Japan; severe phenotype in Jews, milder in Japanese	715-718
	Yes	c.67delG (322delG)	fs		Homozygous in 50% of Dutch FA patients, relatively mild phenotype	18, 716, 719
FANCG	Yes	c.1077-2A→G	Splicing		Portuguese Brazilians	94, 720, 721
	Yes	c.1480+1G→C	Splicing		French Acadians	721
	Yes	c.307+1G→C	Splicing		Koreans and Japanese	721
	Yes	c.1794_1803del10	p.W599fs		Europeans	721
	Yes	313G→T	E105X		44% of mutated FANG alleles in Germans	720
	Yes	c.637 643del	p.Y213fs		Sub-Saharan Africa, 82% of all black FA patients	722
FANCD1/ BRCA2	Yes	c.6174delT	fs		Unique to Ashkenazi-Jewish persons	715
	No	c.859+1G→A (IVS7+1G→A)[‡]	Splicing			93, 120, 723
	No	c.859+1G→A (IVS7+1G→A)[‡]	Splicing			51, 93, 119
	No	c.886delGT[§]	fs			
	No	c.6174delT[§]	fs			
FANCD2	Yes	c.1948-16T→G	Splicing	Turkish	Founder mutation	94
FANCJ		c.2392C→T	p.R798X	50%	Found in ≈50% of FA-J patients of diverse ancestry; ancient mutation or hot spot	707

From Levran O, Diotti R, Pujara K, et al: *Spectrum of sequence variations in the FANCA gene: an International Fanconi Anemia Registry (IFAR) study.* Hum Mutat 25:142–149, 2005; Fokkema IF, den Dunnen JT, Taschner PE: *LOVD: easy creation of a locus-specific sequence variation database using an "LSDB-in-a-box" approach.* Hum Mutat 26:63–68, 2005; Gille JJ, Floor K, Kerkhoven L, et al: *Diagnosis of Fanconi anemia: mutation analysis by multiplex ligation-dependent probe amplification and PCR-based Sanger sequencing.* Anemia 2012:603253, 2012.

Del, Deletion; *fs*, frameshift, premature stop codon; *splicing*, splice-site mutations with unknown consequences at the protein level.

[*] For coding DNA reference sequence see rockefeller.edu/fanconi/, with the first base of the Met-codon counted as position 1. Single-letter abbreviation is used for amino acids: *E*, glutamic acid; *F*, phenylalanine; *Q*, glutamine; *R*, arginine; *W*, tryptophan; *X*, stop codon; *Y*, tyrosine.

[†] Most frequent *FANCA* gene mutations.

[‡] Associated with high frequency of leukemia at very young age (acute myeloid leukemia and acute lymphocytic leukemia).

[§] Associated with brain tumors.

non-Brazilian and 51.1% and 2.2% in the Brazilian mutated *FANCA* alleles (see Table 7-5).

Mutations in *FANCC* genes account for 10% to 15% of all FA cases. The splicing mutation c.711+4A→T (old nomenclature, IVS4+4A→T) in the *FANCC* gene accounts for the majority (80%) of FA cases in Ashkenazi Jewish families. Mutations in *FANCG* are responsible for about 8% to 9% of patients with FA. Several mutations with founder effects have been identified in specific populations (see Table 7-5).

Biallelic mutations in *BRCA2* are found in individuals with FA-D1. Biallelic *FANCD1/BRCA2* mutations account for about 3% to 4% of all FA patients. The majority of *FANCD1/BRCA2* mutations in FA lead to frameshifts or truncations (see Table 7-5). Interestingly, however, FA-D1 cells are not biallelic for *FANCD1/BRCA2* null alleles but have at least one hypomorphic *FANCD1/BRCA2* allele expressing a FANCD1/BRCA2 protein with some residual activity. Biallelic null *FANCD1/BRCA2* mutations have never been identified and in mice are embryonically lethal. Biallelic mutations in *FANCD1/BRCA2* have a very high risk of early malignant disease. The splice-site mutations c.859+1G→A (IVS7+1G→A) and c.859+1G→A (IVS7+1G→A) are associated with a high frequency of leukemia at a very young age,[93] whereas the frameshift mutations c.886delGT and c.6174delT are associated with brain tumors.[52]

Mutation in *FANCD2* accounts for 3% to 6% of FA-affected patients. Malformations are frequent in these patients, and hematologic manifestations appear early and progress rapidly. Although hypomorphic mutations exist, patients with *FANCD2* mutations have a relatively severe form of FA. Mutation analysis revealed 66 mutated alleles, 34 causing aberrant splicing. Many mutations are recurrent and have ethnic associations and shared allelic haplotypes. There were no biallelic null mutations, suggesting that in contrast to the *Fancd2* knockout mouse, complete absence of FANCD2 does not exist in patients with *FANCD2* mutations.[52,94]

Mutations in *FANCN/PALB2* were identified in families with the new subtype FA-N and cancer in early childhood. Similar to biallelic *BRCA2* mutations, biallelic *FANCN/PALB2* mutations confer a high risk for childhood cancer.[53,54]

Molecular Diagnosis of Fanconi Anemia

Molecular diagnosis is not routinely performed for FA, because it is expensive, labor intensive, and, in some cases, possible only in specialized laboratories using research tests that are currently not approved by Clinical Laboratory Improvement Amendments. However, only identification of biallelic mutations in a *FANC* gene (or hemizygosity for a mutation in the *FANCB* gene) definitively establishes the diagnosis of FA. Furthermore, molecular diagnostic testing enables the identification of mutation carriers, facilitates prenatal diagnosis for an existing pregnancy, and allows preimplantation genetic diagnosis for partners identified as mutation carriers.[95] For a definition of preimplantation genetic diagnosis see Box 7-5. In addition, because the clinical manifestations and severity of disease may vary depending on the affected *FANC* gene and the nature of individual mutations, molecular diagnostic testing may influence medical management not only for FA individuals carrying biallelic *FANC* gene mutations but possibly also for heterozygous *FANC* gene mutation carriers (see the section on Heterozygous Carriers of *FANC* Gene Mutations). Currently, an educated candidate gene approach is used to identify the affected *FANC* gene by taking into account the origin and ethnicity of the affected FA individual.[92,96] After exclusion of the most frequent mutations, it might be necessary to determine the complementation group and perform mutational screening of the entire coding sequence, including methods that will detect large deletions in compound heterozygotes. Protein expression analysis and functional assays are used to test for the pathogenicity of unclassified variants.[97] Multiplexed next-generation sequencing with massively parallel sequencing is becoming increasingly available for clinical genetic testing and has been shown to be an effective molecular diagnostics approach for FA.[27,98,99,100-103,104] With the decreasing costs of next-generation sequencing, customized targeted, whole-exome, or whole-genome sequencing is likely to become the future diagnostic standard for patients with FA, enabling genetic molecular diagnosis to be performed at a reasonable price in a short turnaround time (Box 7-6). Although genetic mutation analysis is likely to be successful in the majority of FA patients, in some, the responsible gene mutations might remain unclear. In addition to the large number of mutations in *FANC* genes associated with FA, a number of nonpathogenic mutations and mutations of unknown

Box 7-5 Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis (PGD) is the genetic diagnosis of a preimplantion embryo (typically on day 3 of embryo development at the 8-cell stage or on day 5 as a blastocyst) obtained by in vitro fertilization. It is usually performed in families with a severe genetic disorder or for selection of a human leukocyte antigen–compatible donor for a sibling in need of a hematopoietic stem cell transplant. The procedure is often successful but is ethically controversial.[95,100,103]

Box 7-6 Next-Generation Sequencing

DNA sequencing determines the order of the nucleotide bases (adenine, guanine, cytosine, thymine) in a molecule of DNA. Next-generation sequencing is a high-throughput technology that uses massively parallel sequencing, producing thousands or millions of sequences at once. Next-generation sequencing reduces the turnaround time and number of assays and significantly reduces the cost of analysis. In the future, next-generation sequencing will allow simultaneous screening for mutations in most or all of the loci associated with inherited bone marrow failure syndromes at once as well as screen the whole genome for the detection of novel mutations. With decreasing costs, next-generation sequencing is likely to become the diagnostic test of choice for all patients clinically suspected to have an inherited bone marrow failure syndrome or presenting with bone marrow failure in general.[104]

pathogenic significance are identified and require further functional investigations. The multitude of FA complementation groups, the presence of pseudogenes, the heterogeneity of the mutation spectrum, and the frequency of large intragenic deletions present a considerable challenge for the current and future molecular diagnosis of FA.

Heterozygous Carriers of *FANC* Gene Mutations

Heterozygous carriers of *FANC* gene mutations are not at risk for the development of BMF. Similarly, cell lines heterozygous for *FANC* gene mutations do not show the chromosomal instability (either spontaneously or in response to cross-linking agents) characteristic of FA cells with biallelic *FANC* gene mutations. However, an increased frequency of skeletal and genitourinary abnormalities[105] and an increased risk of malignant disease have been reported in heterozygous *FANC D1/J/N/O* and possibly *FANCP* and female *FANCB* gene mutation carriers.[105]

Risk of Malignant Disease in Heterozygous Carriers. A strong association exists between heterozygous mutation of *FANC* genes and susceptibility to breast or ovarian cancer,[106] and other forms of malignant disease have been identified in heterozygous mutation carriers. Carriers of mutations in *BRCA1* or *BRCA2/FANCD1* have a 65% and 35% risk, respectively, of developing breast cancer and a 39% (*BRCA1*) and 6% (*BRCA2/FANCD1*) risk for ovarian cancer.[107] Heterozygous *BRCA2/FANCD1* mutations additionally predispose to pancreatic, prostate, and gastric cancers, as well as melanoma. Monoallelic truncating *FANCN/PALB2* mutations are associated with familial breast cancer, although the risk of breast cancer is lower (2.3-fold) than that in *BRCA2* mutation carriers.[108] Heterozygosity for *FANCJ* mutations has been identified in patients with early-onset breast cancer.[109] Monoallelic pathogenic mutations in *RAD51C/FANCO* confer an increased risk for breast and ovarian cancers.[110] Heterozygous germline mutations in *FANCC* and *FANCG* and loss of heterozygosity or functional biallelic loss in the tumor sample have been identified in rare patients with pancreatic cancer.[111] Furthermore, heterozygous germline mutations of *FANCA* have been identified in a small percentage of patients with AML.[112]

Somatic *FANC* Gene Mutations in Cancer

Cancer cells often accumulate a large number of mutations within an individual cancer cell, many of which promote cell growth and survival. It is therefore thought that a cancer cell with an increased mutation rate, a mutator phenotype, will have a selective advantage in tumor growth and development. Inactivation or mutation of genes involved in maintaining genetic stability is thought to be an early event in cancer development. The FA pathway has been observed to be one of the pathways that maintains genomic stability and is targeted in several forms of sporadic cancer.[113,114]

Identification of acquired abnormalities in the FA pathway might become clinically important because these tumors are expected to have increased sensitivity to cross-linking agents such as cisplatin, cyclophosphamide, MMC, and other novel agents.[115] Thus identification of abnormalities in the FA pathway may serve as a biomarker that identifies a subgroup of patients with cancers selectively sensitive to specific chemotherapeutic agents. Similarly, inhibition of the FA pathway in cancer may render cancer cells sensitive to chemotherapy.[113,116,117]

Genotype-Phenotype Correlation

Clinical findings in FA are highly variable, although within an individual family the presence or absence of multiple congenital abnormalities or the age at onset of other disease manifestations is usually concordant.[21] More recent studies have demonstrated that disease severity and manifestations are determined, at least in part, by the *FANC* gene affected (complementation group) and by the nature of the mutations.[14,19,118] For example, patients with mutations in the *FANCA* gene usually have a milder form of disease and BMF develops at a later age than it does in FA individuals with mutations in the *FANCC* or *FANCG* gene,[18] whereas in FA patients with biallelic *FANCD1* or *FANCN* mutations, cancer develops even before the manifestation of BMF.[51,53,119,120] Furthermore, splice-site mutations in *FANCD1* are more frequently associated with the development of leukemia,[52,120] whereas frameshift mutations within the same gene more frequently lead to brain tumors (see the section on *FANC* Gene Mutations in Fanconi Anemia).[51,52,119] Malformations are frequent in *FANCD2* patients, and hematologic manifestations appear earlier and progress more rapidly than they do in non-*FANCD2* patients.[94]

FA-B is X-linked, in contrast to all other forms of FA, which are autosomal. Thus a single mutation is sufficient to cause a phenotype and disease in males. In females, because of inactivation of one of the two X chromosomes, a phenotype is expressed only in cells with the *FANCB* mutation on the active X chromosome. Because X-chromosome inactivation occurs at random in early embryogenesis and, once determined, persists clonally, one might expect female *FANCB* mutation carriers to have a mild form of FA-B. Interestingly, however, to date no female FA-B patient with clinical manifestations of FA has been reported, and careful clinical examination of females heterozygous for *FANCB* gene mutations showed no manifestations of disease. Molecular studies investigating X-inactivation revealed that only the X chromosome with the normal *FANCB* gene is expressed in blood and bone marrow and also in fibroblasts.[121] This finding indicates a proliferative disadvantage of cells expressing the mutant *FANCB* allele and complete outgrowth by phenotypically normal cells, not only in bone marrow but possibly also in all other tissues. This situation differs from other X-linked IBMFSs such as X-linked DC (see the section on Dyskeratosis Congenita). For individuals carrying a *FANCB* mutation, however, longer follow-up and examination of additional mutation carriers will be necessary to determine whether some FA-B cells persist in female carriers and whether

Box 7-7 Genetic Mosaicism

Genetic mosaicism is defined as the presence of two or more genetically distinct populations of cells in a single individual that differ from each other at the DNA sequence level but are derived from a single zygote. In Fanconi anemia, genetic mosaicism results from reversion, which is the genetic correction of the *FANC* mutation.[126]

these persisting FA-B cells are at risk for malignant transformation. Whether somatic *FANCB* inactivation can be found in malignant disease (only a single mutation on the active X chromosome is required) remains to be determined.

Somatic Mosaicism, Functional Reversion, and Attenuation of Fanconi Anemia Cells

About 10% to 25% of FA patients have been found to have mosaicism in the peripheral blood inasmuch as two populations of lymphocytes can be detected, one with the classic increased sensitivity to cross-linking agents characteristic of FA cells and the other usually a slowly increasing second population that is resistant to treatment with these agents.[17] For a definition of genetic mosaicism see Box 7-7. The presence of mosaicism is usually considered if the clinical course of the disease is milder than expected in comparison with individuals with the same genetic abnormality, if the course of the disease improves spontaneously rather than worsens, or in the presence of classic phenotypic abnormalities but rather mild or absent hematologic disease. The molecular mechanisms of genetic reversion include intragenic recombination (in compound heterozygotes), mitotic gene conversion (nonreciprocal exchange of genetic information as a result of heteroduplex formation between nonsister chromatids),[22] DNA slippage, second-site compensating mutations,[122,123] and site-specific correction by an as yet unknown mechanism.[22] Gene reversion may occur in vivo or in vitro. In FA patients the reverting mutation is thought to occur in progenitor cells with self-renewing capability,[124] possibly stem cells, thereby conferring a proliferative advantage to the revertant cells; this may lead to expansion of the revertant clone and progressive replacement of the FA cells in bone marrow.[17,125,126] Genetic reversion in FA has been documented to date only in hematopoietic cells, not in fibroblasts.[122,123] However, the fact that females heterozygous for *FANCB* mutations have biased X-inactivation in fibroblasts suggests that an intact FA pathway confers a proliferative advantage to nonhematopoietic cells. Thus the frequency of genetic reversion is largely unknown and so are its clinical consequences. In many patients genetic reversion is associated with hematologic improvement.[17,123] However, genetic reversion has also been found in leukemic cells from FA patients,[127] indicating that genetic reversion may confer a selective growth advantage and resistance of leukemic or preleukemic cells to specific chemotherapy drugs. Furthermore, genetic reversion in hematopoietic cells has no effect on cancer frequency in nonhematopoietic tissues. The strong selection for a functional FANC pathway may be used to advantage in gene therapy approaches. Genetic mosaicism is responsible at least in part for phenotypic variability, particularly within an individual family, and may create difficulty in interpretation of the chromosomal breakage testing with DEB and MMC (see the section on Diagnosis) or even lead to false-negative testing results.[128] DEB or MMC testing of fibroblasts should therefore be considered in patients with clinical or genetic suspicion of FA but a negative DEB or MMC test result for peripheral blood lymphocytes.

The term *attenuation* has been introduced by Ceccaldi and associates[4] for FA patients whose peripheral blood cells lack monoubiquitination of FANCD2 and have the characteristic increased chromosomal breaks after MMC treatment but whose cells, unlike those of most patients, do not show the characteristic G_2 arrest after MMC treatment. Most of these patients developed MDS or leukemia later in life. Attenuation was accompanied by clonal hematopoiesis, implying that all of the peripheral blood cells were derived from a single progenitor cell in which a molecular event took place that gave rise to attenuated G_2 arrest. The molecular mechanism underlying attenuation remains to be determined. Attenuation in patients with FA contributes to the variability in disease expression and identifies patients with a milder presentation of BMF but who might need continuous, intensified tumor surveillance.

Mouse Models for Fanconi Anemia

Orthologues of FANCD2, FANCM, and FANCL are found in nonvertebrates, whereas the other members of the FA core complex have evolved in vertebrates. To study clinical phenotypes and develop possible new therapeutic strategies, several mouse models for FA have been generated by introducing mutations into murine *Fanc* genes, including *Fanca*, *Fancc*, *Fancg*, *Fancd2*, *Fancd1*, *Fancf*, *Fancl*, *Fancm*, *Fancn*, *Fanco, and Fancp*.[129] Homozygous null mutations were lethal for *Fancd1*, *Fancn, Fanco,* and some *Fancl* embryonic mice, depending on the genetic background; this finding confirms what had already been suspected from the mutation analysis in patients with FA, namely that some residual function in these *FANC* genes is required for a living organism. Some models recapitulate the clinical features seen in patients with FA, specifically microphthalmos, germ cell defects, chromosomal instability, and increased sensitivity to cross-linking agents. In some models tumors develop with increased frequency, and tumor formation is more pronounced in the absence of p53. These models underline the importance of the FA pathway in tumorigenesis. Hematopoietic progenitor cells from mice homozygous for a null mutation in the *Fancc* gene demonstrate a distinct hypersensitivity to IFN-gamma that leads to cell death independent of the DNA repair pathway mediated by FAS-induced apoptosis, suggesting that IFN-gamma hypersensitivity may play a major role in the pathogenesis of BMF in patients with FA. Interestingly, however, although *Fancd1*- and *Fancp*-deficient mice have a reduced number of HSCs and decreased white blood cell and platelet counts, spontaneous development of aplastic anemia was not observed in these mice. Mice deficient in *Fancd1* are exquisitely sensitive to excess acetaldehydes from endogenous and exogenous sources.[71] Aldehydes are produced endogenously during normal metabolism or as a by-product of ethanol metabolism and can induce DNA-DNA and DNA-protein cross-links. Aldehydes are detoxified by aldehyde dehydrogenases. Interestingly, hematopoietic stem cells in mice express the aldehyde dehydrogenase *Aldh2*, which is essential for protection against acetaldehyde toxicity, suggesting that the FA pathway is needed to protect from aldehyde-mediated genotoxicity.[89] Although these findings need to be confirmed in humans, they would explain why hematopoiesis is preferentially affected in FA but is not affected, or not to the same extent, in other non-FA DNA repair disorders.

Induced Pluripotent Stem Cells Derived from Patients with Fanconi Anemia

Induced pluripotent stem cells (iPSCs) are derived from human somatic cells through ectopic expression of transcription factors.[130] Since their discovery these cells have become a widely used tool for modeling and studying IBMFSs.[131] Their unlimited self-renewal capacity, pluripotency, and ease of accessibility are very promising for disease modeling, cell-based therapy, and drug discovery. Interestingly, the generation of iPSCs from FA patients is challenging due to a low reprogramming efficiency in the presence of a defective FA pathway,[132] suggesting that cellular reprogramming or the methodology (integrating viruses) of reprogramming requires an intact FA pathway. FA iPSC lines are capable of undergoing hematopoietic differentiation, but the hematopoietic progenitors undergo increased apoptosis and have a reduced clonogenic potential.[133,134] Further investigation using the iPSC FA disease model is likely to provide exciting insights into the disease pathology and future treatment options.

Clinical Management

Once the diagnosis is confirmed, the family should be referred to a clinical geneticist for counseling and careful examination of family members. Genetic testing or chromosome breakage analysis should be offered to all family members. Early diagnosis is important for correct management of hematologic complications, diagnosis and appropriate treatment of coexisting congenital abnormalities and associated endocrinopathies, and identification of affected but asymptomatic family members and unaffected family members or pregnancies that may serve as potential hematopoietic stem cell donors. In addition, early diagnosis allows targeted cancer surveillance, not only in patients with FA but also in some cases in heterozygous mutation carriers (also see earlier). Management of patients in whom FA is diagnosed depends on the age at diagnosis and the presence or absence of congenital or hematologic abnormalities (see also Fanconi Anemia: Guidelines for Diagnosis and Management, Third Edition, 2008; available from the Fanconi Anemia Research Fund at fanconi.org/). All diagnosed FA patients should have a full hematologic assessment, including examination of bone marrow, and HLA typing should be performed in anticipation of possible HSCT. Other investigations at diagnosis should include audiometry, ophthalmic examination, renal ultrasound studies, and endocrine assessment. Referral to specialized surgeons might be necessary for correction of radial ray defects.

Management of Hematologic Disease

Initially, in the absence of hematologic abnormalities or clinical and laboratory signs of mild BMF, monitoring of peripheral blood values and yearly examination of bone marrow might be sufficient. Bone marrow examination should include a trephine biopsy and marrow aspirate for examination of marrow cellularity and morphologic changes characteristic of MDS, as well as for cytogenetic examination by G banding and fluorescent in situ hybridization (FISH) analysis, including probes that identify the most frequent cytogenetic abnormalities seen in patients with FA and progression to MDS or AML (see the section on Myelodysplastic Syndrome and Acute Leukemia in Patients with Fanconi Anemia).[44] Plans for possible HSCT should be in place because hematologic complications may develop rapidly. With progression of BMF it will become necessary to increase the frequency of hematologic monitoring. HSCT is usually considered in patients with moderate BMF and an available HLA-matched unaffected sibling donor or when BMF becomes severe and an unrelated donor is available. Preventive HSCT in the absence of hematologic disease is highly controversial and currently considered investigational because the risk and time course of disease progression are highly variable and have to be weighed against the morbidity and mortality associated with HSCT. However preventive HSCT might be considered in patients with biallelic *BRCA2* mutations associated with a higher risk of leukemic transformation.

Management of FA patients with MDS or AML, or both, is less standardized. The significance of transient clonal cytogenetic abnormalities limited to a single chromosome is unclear, but if they are persistent or complex, such abnormalities may predict a poor prognosis; the exception is an isolated trisomy 1q that might be present years before the development of MDS (see the section on Myelodysplastic Syndrome and Acute Leukemia in Patients with Fanconi Anemia).[44] HSCT with or without induction chemotherapy or enrollment in clinical trials for MDS or MDS/AML is usually considered.[135] Androgens such as oxymetholone, methyl testosterone, or danazol and hematopoietic growth factors transiently improve BMF in 50% to 60% of FA patients.[136,137] Side effects of androgen therapy include masculinization, acne, hyperactivity, growth spurt followed by premature closure of the epiphyseal growth plates, high serum lipid levels, liver enzyme abnormalities, hepatic adenomas (with danazol), and a risk for hepatic adenocarcinomas (with androgens) that requires frequent monitoring of liver function and liver ultrasound scanning.[40,138] The effect of pretransplantation androgen use is controversial. Hematopoietic growth factors [granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF)] may transiently improve neutrophil counts and, in rare FA patients, hemoglobin levels and platelet numbers. The use of hematopoietic growth factors in patients with clonal cytogenetic abnormalities is controversial because of the potential risk of inducing or promoting leukemia. Anti-inflammatory agents such as anti-TNF-alpha and antioxidants have been shown to improve hematopoiesis in vitro and in mouse models and are currently being tested in clinical trials for efficacy and safety in patients with FA.[139,140] Red cell transfusion therapy and iron chelation (after multiple red cell transfusions) are frequently used in symptomatic FA patients. Platelet transfusions may be indicated in thrombocytopenic patients with bleeding or before surgical procedures.

HSCT from an HLA-matched sibling donor is accepted as the best treatment to cure BMF in FA patients and to prevent progression to MDS and AML. Early experiences with HSCT for the treatment of BMF were disastrous because of excessive regimen-related toxicity. Early conditioning regimens consisted of cyclophosphamide alone or in combination with total-body irradiation (TBI). Unfortunately, severe toxicities (e.g., gastrointestinal hemorrhage, hemorrhagic cystitis, cardiomyopathy, and skin burns) were common. Gluckman and colleagues[141] proposed the use of a less intensive conditioning regimen consisting of low-dose cyclophosphamide and thoracoabdominal irradiation for patients with FA. This approach proved to be a major advance in the treatment of patients who had HLA-matched sibling donors.[118,142] However, it did not afford consistent engraftment in recipients of unrelated donor transplants, with graft rejection rates exceeding 20%,[143] and head and neck cancer was frequent after HSCT.[144] Consequently, HSCT without irradiation is increasingly used for patients with FA and a matched sibling donor using fludarabine and cyclophosphamide and with T cell depletion either of the graft or in vivo with rabbit ATG or alemtuzumab to prevent graft-versus-host disease (GVHD).[145] Recent studies suggest that fludarabine-based conditioning regimens are associated with improved survival rates in patients undergoing related or unrelated donor HSCT and have significantly improved survival rates for alternative donor transplant recipients.[146-148] The use of irradiation for alternative donor transplant varies among countries and transplant centers.[149-151] Transplant-related mortality and GVHD remain a problem. Furthermore chronic GVHD significantly increases the risk of squamous cell carcinoma in FA patients who undergo HSCT.

PGD may be used in parents with a previously affected child to select embryos that are both unaffected by FA (if a *FANC* gene mutation is known) and have HLA matched to the affected sibling. HSCT is performed with the HLA-matched umbilical cord blood of the unaffected newborn.[152,153]

Unrelated cord blood as an alternative HSC source showed an overall survival rate of 53% with fludarabine in the conditioning regimen (vs. a rate of 19% without fludarabine). There was a relatively high incidence of GVHD and transplant-related mortality rates of 32% and 59%, respectively, suggesting that unrelated cord blood transplant should be considered only when other alternative donor stem cell sources cannot be found. Alternatively a mismatched or haploidentical family donor may be a valid option if no matched unrelated donor can be found.[154]

Cancer Surveillance and Treatment

For FA patients who survive the hematologic complications, follow-up surveillance for solid malignant tumors becomes increasingly important. There are no clear guidelines on the treatment of cancer in FA patients, which is complicated because the increased sensitivity to irradiation and certain chemotherapeutic agents may result in enhanced toxicity and an increased risk of therapy-induced secondary malignant diseases.

Gene Therapy

HSCT may cure hematologic disease in FA patients; however, HLA-matched sibling donor cells are available only for a minority of patients. Gene therapy to correct HSCs, followed by autologous transplantation, offers an attractive alternative for the treatment of hematologic disease. Indeed, gene transfer corrects the hypersensitivity of most FA cell lines to DNA cross-linking agents in vitro (with the exception of FANCM, most likely because of the insolubility of the protein) and the hypersensitivity of FA knockout mice in vivo. The high incidence of somatic mosaicism and the often ameliorated course of hematologic disease in these patients (frequently referred to as the *gene therapy of nature*) suggest that FA cells corrected by gene therapy are likely to have a selective growth and survival advantage. However, gene therapy attempts in FA patients have thus far been rather disappointing,[155,156] mainly because of the lack of permanent gene correction and the difficulty in culturing FA bone marrow cells in vitro. Multiple investigations are currently addressing the major difficulties of gene therapy for FA, including the scarcity of hematopoietic stem cells in patients with BMF, the extraordinary fragility of FA hematopoietic progenitor cells, and the increased sensitivity of FA hematopoietic progenitor cells to oxidative stress, which makes it difficult to culture and transduce FA hematopoietic progenitor cells in vitro. There is also a risk that the transduced hematopoietic cells might already have acquired secondary somatic, and possibly oncogenic, mutations and that correction by gene therapy would promote rather than prevent leukemogenesis. The International FA Gene Therapy Working Group was formed to accelerate the transition from research to clinical gene therapy trials and coordinating activities on an international scale.[157]

Dyskeratosis Congenita

Dyskeratosis congenita (DC), also known as *Zinsser-Cole-Engman syndrome*, is genetically and phenotypically heterogeneous (Box 7-8). It was first described by Zinsser and recognized as a clinical entity by Cole and Engman.[158-160] DC was originally perceived to be a dermatologic disorder affecting the skin, nails, and mouth. Its association with BMF and squamous cell cancer arising from the skin or from leukoplakia in the mouth or anus was recognized later.[161]

Box 7-8 Dyskeratosis Congenita[162,163]

Dyskeratosis congenita is a rare inherited bone marrow failure syndrome with an X-linked, autosomal dominant, and autosomal recessive inheritance. However, more than half of patients present with sporadic disease. Classically, bone marrow failure in these dyskeratosis congenita patients is associated with the mucocutaneous triad including abnormal pigmentation, dystrophic nails, and mucosal leukoplakia. Patients with dyskeratosis congenita have a defect in telomere maintenance, and at the time of bone marrow failure peripheral leukocytes have very short telomeres (see Fig. 7-9).[162,163]

Clinical Manifestations

Originally, DC was clinically recognized by the classic diagnostic triad of reticular pigmentation of the skin, nail dystrophy, and mucosal leukoplakia (Figure 7-7).[158-160] Other signs and symptoms found frequently in classic DC are summarized in Table 7-6.[164,165] The classic clinical course is mucocutaneous features first appearing in childhood, BMF developing in adolescence, and death occurring in young adulthood. Cytopenias due to BMF, immune deficiency, pulmonary fibrosis, and malignant disease are the major causes of death.

The identification of nine of the genes responsible for DC and the realization that these genes converge into a common pathway have changed the perception of this disease. It is now thought that individuals in whom DC is diagnosed on the basis of BMF and the classic cutaneous manifestations represent only a fraction of those who suffer from this condition.[166,167] Whether affected individuals in the absence of cutaneous disease should be labeled with the diagnosis of "dyskeratosis congenita" or whether the disease should be renamed with a term better reflecting the pathogenetic pathway is still under debate. Here we will use the conventional nomenclature and clinically classify patients as having classic, atypical, or silent DC. Patients who have two or more of the three diagnostic mucocutaneous features or who have one of the mucocutaneous features and hypocellular bone marrow with all three blood cell lineages affected are those with classic DC. Patients with BMF but lacking mucocutaneous features or patients with clinical manifestations of DC other than hematologic disease or

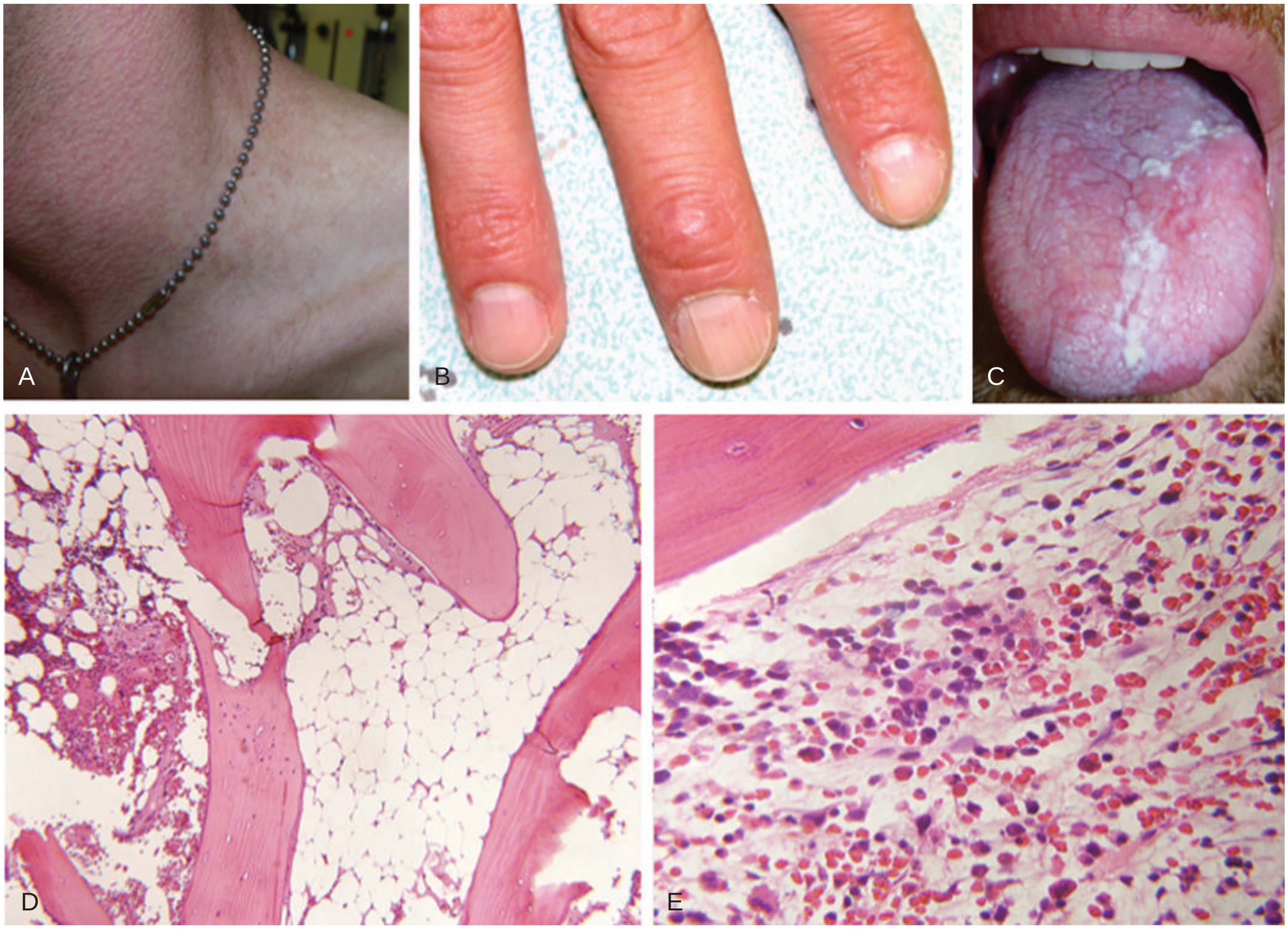


Figure 7-7 Physical findings in a 31-year-old patient with classic dyskeratosis congenita and homozygosity for a *TERT* gene mutation. **A**, Reticular skin pigmentation. **B**, Dystrophic fingernails, rather mild in this case. **C**, Leukoplakia of the tongue. **D**, Hypocellular myelodysplastic syndrome in a 41-year-old patient with atypical dyskeratosis congenita due to a *TERC* gene mutation. **E**, Hypocellular bone marrow. *(Photographs courtesy of Susan Bayliss, Washington University.)*

TABLE 7-6 Prevalence of Clinical Features in Patients with Classic Dyskeratosis Congenita

Characteristic	Alter 2003[27] (%)	Vullamy & Dokal 2006[724] (%)
Diagnostic mucocutaneous features		
Skin pigmentation	88	89
Nail dystrophy	73	88
Leukoplakia	64	78
Eye abnormalities/epiphora	38	31
Teeth abnormalities/caries/loss	19	17
Learning difficulties/ developmental delay/cognitive disability	14	25
Pulmonary diease/fibrosis	20	
Skeletal anomalies	14	
osteoporosis/avascular necrosis/scoliosis		5
Short stature	14	20
Hyperhidrosis	10	15
Hair loss, early graying	18	16
Urinary tract abnormalities	7	
Urethral stricture/phimosis		5
Gastrointestinal abnormalities	14	
Liver fibrosis/ulcer/enteropathy	7	
Esophageal stricture		17
Other conditions	11	
Gonadal anomalies/ hypogonadism/undescended testes	5	6
Intrauterine growth retardation		
Ataxia/cerebellar hypoplasia	7	
Microencephaly	8	
Malignant disease	12	10

mucocutaneous features, such as pulmonary fibrosis, are those with atypical DC. Individuals with no clinical manifestations but carrying a pathogenic mutation are classified as having silent DC. Our knowledge about the natural history of DC is biased because early studies focused on patients with the classic clinical manifestations. Our experience is still limited about the course of disease, response to treatment, and late complications in individuals with atypical or silent DC.

Classic Dyskeratosis Congenita

Mucocutaneous Features. Mucocutaneous abnormalities are the hallmark of classic DC (see Fig. 7-7).[168] The most commonly observed mucocutaneous abnormality (>90%) is reticulated skin hyperpigmentation on the neck, face, chest, and arms. It may be extensive or localized and tends to increase with age. Other cutaneous manifestations include alopecia, sparse hair, premature hair graying, hyperhidrosis, hyperkeratosis of the palms and soles, and loss of dermal ridges on the fingers and toes.

Nail dystrophy is another frequent finding (>80%). Progressive nail dystrophy starts with ridging and longitudinal splitting and may proceed via atrophy and thinning to complete nail loss. The severity of nail dystrophy may vary among the digits; fingernails are usually more severely affected than toenails.[169]

Mucosal leukoplakia occurs in the majority of patients (>70%) and typically involves the tongue, buccal mucosa, and oropharynx, with the tongue being the most frequently affected site.[170] Mucosal leukoplakia may be the initial clinical manifestation of disease. Consequently, dentists or oral surgeons may be the first health professionals consulted. Histologic examination reveals hyperkeratosis or a lichenoid reaction. Other sites of leukoplakia include the urethra, glans penis, vagina, and anorectal region.[168] DC patients have an increased risk of malignant disease arising from the preexisting leukoplakia; the incidence of malignant transformation has been estimated to be 35%.[171]

Hematologic Abnormalities. About 85% of patients with classic DC are initially found to have cytopenia of one or more lineages, and pancytopenia develops in more than 95% by 40 years of age.[164] The overall cumulative incidence of BMF in all patients with DC (typical and atypical) is approximately 50%.[41] Often the degree of aplasia in the bone marrow is striking despite only a moderate degree of peripheral blood cytopenia. Complications of BMF, such as hemorrhage or opportunistic infection, represent the major causes of death in patients with DC. In patients with atypical DC, BMF may appear before the classic mucocutaneous features and lead to an initial diagnosis of idiopathic aplastic anemia. Hypoplastic MDS and MDS/AML are increasingly diagnosed in patients with DC, especially in those with a milder course or an atypical form of the disease. Indeed, MDS or MDS/AML may be the initial manifestation of patients with atypical DC.

Combined immune deficiency is frequently associated with DC, particularly in children with severe and early onset of disease. Reduced or absent B cells and reduced NK cells are frequent; other findings in young patients include decreased T-cell numbers, low immunoglobulin levels, and a lack of lymphocyte proliferation in response to antigens.[172,173]

Other Clinical Features. Pulmonary complications are common (>20%) and include pulmonary fibrosis and vascular abnormalities.[174,175] Pulmonary fibrosis is usually a more prominent finding in older patients or after BMT.[176] In some patients with atypical DC, pulmonary fibrosis may be the only clinical manifestation. Figure 7-8 shows a family with autosomal dominant DC and pulmonary fibrosis. Families with hereditary pulmonary fibrosis of adult onset should be investigated for DC, particularly when pulmonary fibrosis is associated with macrocytic anemia or other cytopenias.[177,178] In families with pulmonary fibrosis and BMF, a pathogenic DC gene mutation is almost always responsible for both conditions; in such kindreds pulmonary disease typically manifests in the sixth decade of life,

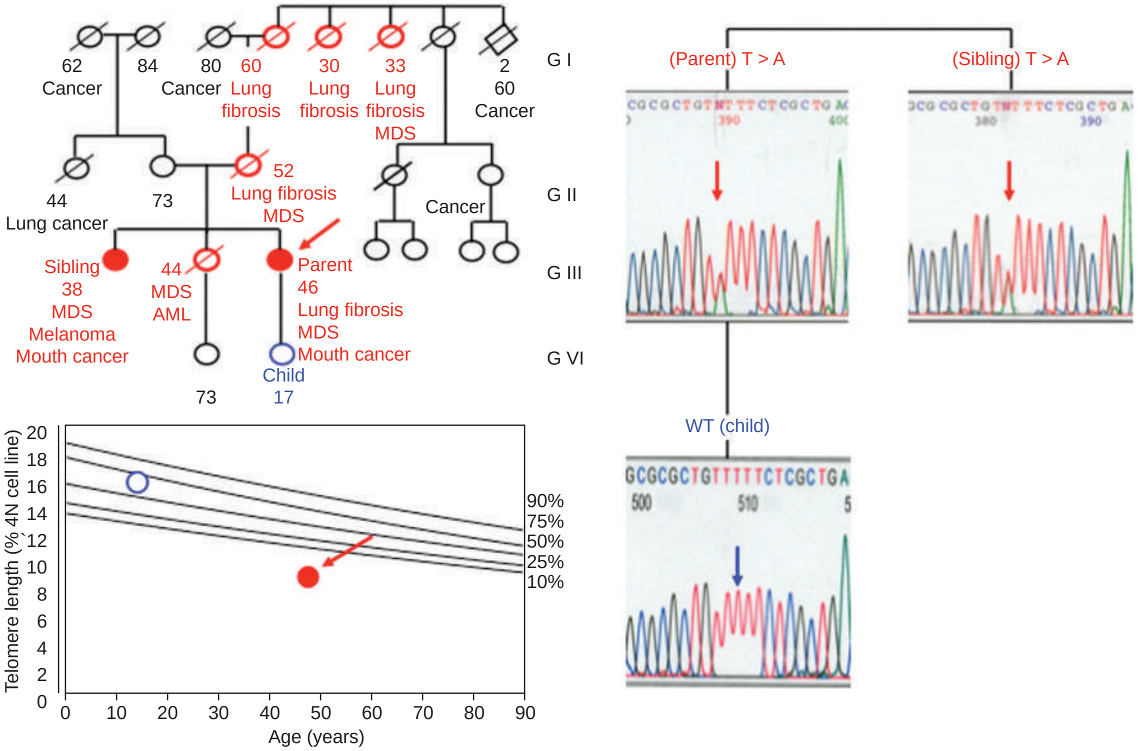


Figure 7-8 **A**, Pedigree of a family with dyskeratosis congenita due to a *TERC* gene mutation. Note, in addition to myelodysplastic syndrome, pulmonary fibrosis is a prominent feature of dyskeratosis in this family (to protect privacy, gender identification has been removed). **B**, Chromatogram demonstrating that the parent and the sibling are heterozygous for the *TERC* gene mutation, whereas the child has not inherited the mutation. **C**, Telomere length of the affected parent (below and outside of telomere length distribution of normal controls) and the nonaffected child (normal telomere length). Lines indicate the 10th, 25th, 50th 75th, and 90th percentile of telomere length determined in 105 healthy controls between the ages of 1 and 95 years.

whereas BMF manifests in the first or second decade (see Fig. 7-8).[179]

Liver fibrosis may be a late manifestation of DC. As with pulmonary fibrosis, liver fibrosis tends to occur in older patients or after BMT. In some patients liver fibrosis might be the only or first manifestation of disease. Excessive alcohol consumption or acute hepatitis is thought to accelerate liver fibrosis and severity in patients with a pathogenic DC mutation.[180-182] The presence of a DC mutation does not preclude the development of hepatocellular carcinoma.[182]

Ocular findings include epiphora (excessive tearing caused by blocked lacrimal ducts), blepharitis, cataracts, loss of eyelashes, conjunctivitis, ectropion, glaucoma, strabismus, ulcers, ocular albinism, and exudative retinopathy (*Coats retinopathy*).[183-186] Interestingly, Coats retinopathy is specific for *Revesz syndrome* (caused by mutations in *TINF2*) and *Coats- plus syndrome* (caused by mutations in *CTC1*); exudative retinopathy is not typical of other forms of DC. Corneal limbal insufficiency owing to impaired corneal stem cell function has also been described in patients with DC.[187]

Sparse hair and early graying are common. Dilated cardiomyopathy due to cardiac fibrosis has been associated with DC.[188]

Multiple dental caries early loss of teeth, and a bifid uvula are common oral manifestations of DC.[189,190] Gastrointestinal abnormalities include web formation in the upper esophagus, esophageal stricture, severe necrotizing ulcerative pancolitis in infants, and chronic diarrhea with malabsorption due to mucosal villous blunting in young adults.[191-194] Patients have an increased risk of esophageal, rectal, and possibly gastric cancers.[41]

Genitourinary abnormalities include hypospadias, phimosis, and urethral strictures. Underdeveloped testes and azoospermia have been reported in more severe and advanced forms of DC.

Cognitive deficiencies such as learning disorders are common in classic DC. In rare cases an association with schizophrenia has been described. Other neurologic manifestations may include cerebellar ataxia due to cerebellar hypoplasia, intracranial calcification, and hypoplasia of the corpus callosum, pituitary gland, or brainstem.

Osteoporosis and avascular necrosis of the bones, in particular the hips, is frequent and probably underappreciated and may complicate the course of the disease. In some forms of DC avascular necrosis of the bones occurs at a very young age and may be the dominating clinical feature. Short stature and an elf-like appearance have been associated with classic DC.[195]

Predisposition to Malignant Disease

DC is a syndrome that predisposes to cancer.[161,162,164] After BMF and pulmonary complications, malignant disease is the third leading cause of death. The cumulative incidence has been estimated to be 20% to 30% by the age of 50 years.[41] The most frequent malignant tumors in patients with DC are squamous cell carcinoma of the head and neck (especially the tongue but also the lip, mouth, palate, cheek, nasopharynx, and larynx),[170,171,190] the upper and lower gastrointestinal tract (esophagus and anus), and the female genital tract. Other reported malignant tumors include skin cancer and adenocarcinoma of the stomach, colon, lung, and pancreas.[41] Whether all forms of DC have equal risks of predisposition to cancer remains to be determined. Malignant tumors usually develop in the third decade and are therefore more frequently found in individuals with milder and late-onset forms of the disease (see Table 7-1).

MDS and MDS/AML are common in patients with atypical DC and in patients with late-onset of the disease (see Fig. 7-7, *E*). The cumulative incidence of MDS has been estimated to be about 30% and that of MDS/AML approximately 10% by the age of 50 years.[41] MDS or MDS/AML may be the first hematologic manifestation of disease. Although DC is rarely a cause of MDS or MDS/AML in children,[196] it should be considered in the differential diagnosis in young adults with MDS or MDS/AML. Abnormalities of chromosome 7 (monosomy, isochromosome, or other structural rearrangements) are the most common cytogenetic abnormalities in patients with DC-associated MDS or MDS/AML.

Little is known about the biology of leukemia or other cancers in patients with DC and to what extent these malignant diseases differ from those seen in patients without DC. The cancer recurrence rate is high in patients with DC.

Laboratory Findings

Common laboratory findings are summarized in Box 7-9. In most patients, the initial hematologic abnormalities are thrombocytopenia or macrocytic anemia followed by pancytopenia. Bone marrow examination often reveals increased cellularity at the outset, suggesting an element of hypersplenism. However, hypocellularity then ensues, consistent with the diagnosis of aplastic anemia. Macrocytosis and elevated hemoglobin F levels *(stress erythropoiesis)* are common.[197] The number of circulating hematopoietic progenitor cells is decreased, indicative of the impairment in hematopoiesis at the stem cell level.

Bone marrow examination usually shows hypocellular marrow affecting all three blood cell lineages. The hypocellularity of the bone marrow is often striking in comparison with rather moderate cytopenias in the peripheral blood. Hypocellular MDS or MDS/AML is increasingly being found in patients with atypical DC or a late onset of disease. Monosomy 7 is the most frequent clonal cytogenetic abnormality in patients with MDS or MDS/AML.

Long-term culture assays of bone marrow cells show a decreased number of progenitor cells and an impaired capability for colony formation, indicative of a quantitative and qualitative defect.[198]

Immunologic abnormalities may include humoral and cellular defects (summarized in Box 7-9).[172,173,199] Severe combined immunodeficiency is characteristic of severe forms of DC.

Box 7-9 Laboratory Findings in Patients with Dyskeratosis Congenita*

1. **Peripheral blood findings**
   - Cytopenia of one or more lineages (80%):
   - Initial presentation highly variable:
     - macrocytosis +/– anemia
   - Thrombocytopenia
   - Neutropenia
   - Pancytopenia
   - Low number of circulating progenitor cells
   - Elevated hemoglobin F
   - Elevated von Willebrand factor
2. **Bone marrow examination**
   - Hypocellular bone marrow affecting all three lineages
     - Increased number of mast cells
     - Dyserythropoiesis
   - Hypocellular myelodysplastic syndrome
   - Myelodysplastic syndrome/acute myeloid leukemia
3. **Immunologic findings**[172,173]
   - Humoral abnormalities:
     - Hypogammaglobulinemia or hypergammaglobulinemia
   - Cellular abnormalities:
     - Lymphopenia: B cells, T cells, NK cells
   - Functional abnormalities:
     - Proliferation upon stimulation is reduced
     - Increased apoptosis upon stimulation
     - Anergy
     - Difficulty in immortalization by Epstein-Barr virus transformation
4. **Cellular abnormalities**
   - Premature replicative senescence[201,208]
   - No increased chromosomal breakage after cross-linking agents
   - Hypersensitivity to ionizing irradiation (controversial)
   - Increased sensitivity to certain cytotoxic agents
   - Difficulty in immortalization by TERT[208]
   - Difficulty in reprogramming to induce pluripotent stem cells[210]
5. **Cytogenetic abnormalities**[200-202]
   - Chromosomal instability: Nonclonal complex anomalies in cultured cells:
     - Chromosomal breaks, hypodiploidy, micronuclei,
     - anaphase and telophase bridges,
     - short telomeres/signal free ends[725]
     - end-to-end fusion with no telomeric signal
   - No increase in chromsomal instability after clastogenic effects of
     - DNA
     - cross-linking agents
   - Biased X-inactivation (female carrier of X-linked dyskeratosis congenita)[204,205]
   - Clonal abnormalities in bone marrow cells : monosomy 7 (myelodysplastic syndrome, myelodysplastic syndrome/acute lymphocytic leukemia)

*Laboratory findings in bold are characteristic for dyskeratosis congenita.

DC cells have increased spontaneous chromosomal instability that leads to distinctive chromosomal breaks, rearrangements,[200,201] and sister chromatid exchanges.[202,203] Chromosomal instability increases as DC cells are passaged in vitro. Chromosome breakage does not increase after treatment with alkylating agents such as DEB, MMC, or nitrogen mustard,[202] a finding that distinguishes DC from FA. Chromosomal abnormalities characteristic of DC include end-to-end fusions, dicentric or tricentric chromosomes, anaphase or telophase bridges, short telomeres, and telomere free ends.

Paternally or maternally biased X-inactivation in blood cells, as well as in fibroblasts from buccal mucosa

Box 7-10 **Measurement of Telomeres, Telomerase, and Telomere Length**

Telomeres are complex DNA/protein structures at the ends of chromosomes that are needed to protect chromosome ends from degradation and distinguish them from double-stranded breaks.[233] Human telomere DNA consists of thousands of repeats of TTAGGG (see Fig. 7-10, *A* and *B*). Most somatic cells lack telomerase, so telomeres shorten with each cell division[234] owing to the inability of DNA polymerase I to copy the extreme end of a DNA strand.[235] In most tissues, therefore, telomere length decreases with age.[236] When telomeres become critically short, cell cycle arrest or cell death occurs (see Fig. 7-11).[237] In germ cells,[238] some stem cells,[239,240] and most cancer cells,[237,241] telomeres are maintained by the action of the enzyme *telomerase*,[242] which contains two core components, a reverse transcriptase TERT (telomerase reverse transcriptase) and an RNA molecule, *TERC* (telomerase RNA component), which acts as a template for the synthesis of telomere repeats (see Fig. 7-10, *C* and *D*).

and skin biopsy samples in female mutation carriers, is characteristic of X-linked DC.[204,205]

The presence of short telomeres in circulating peripheral blood cells is a hallmark of patients with BMF and DC.[162,163] Telomere length in individuals with BMF secondary to DC is far below the normal distribution of telomere length in age-matched healthy control individuals (Box 7-10 and Fig. 7-9).[162,163] In contrast to other IBMFSs, which may have short telomeres in circulating myeloid cells, telomeres are exquisitely short in the myeloid and lymphoid cells of patients with DC-associated BMF.[163] Telomere length measurements of circulating peripheral blood lymphocytes (or total blood cells, although less sensitive) are therefore used to screen patients with BMF for DC; normal telomere length makes DC an unlikely cause of BMF.[163] Similarly, very short telomeres in circulating lymphocytes are highly suggestive of DC, but the diagnosis should be reserved for patients with classic clinical DC features or an identified pathogenic DC mutation.[163]

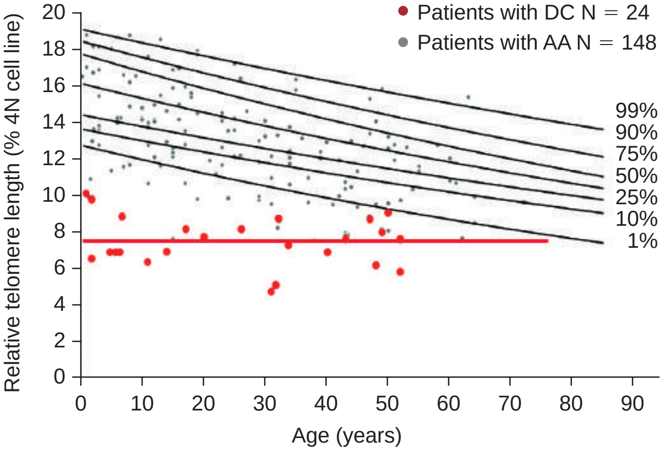


Figure 7-9 Telomere length in peripheral blood mononuclear cells in individuals with dyskeratosis congenita and bone marrow failure. In all individuals with dyskeratosis congenita and bone marrow failure, telomere lengths are below and outside of telomere length distribution of normal controls. The black lines indicate the 1st, 10th, 25th, 50th 75th, 90th, and 99th% percentile of telomere length determined in 148 healthy controls between the ages of 1 and 95 years. The gray dots indicate telomere length in 148 patients with aplastic anemia due to other causes than dyskeratosis congenita. *(Modified from Du HY, Pumbo E, Ivanovich J, et al: TERC and TERT gene mutations in patients with bone marrow failure and the significance of telomere length measurements.* Blood *113:309–316, 2009.)*

Cellular Phenotype of Dyskeratosis Congenita Cells

In addition to increased spontaneous chromosomal instability, DC cells exhibit several other phenotypic characteristics, as summarized in Box 7-9. DC cells have been reported to have increased sensitivity to ionizing radiation and certain chemotherapeutic agents (e.g., bleomycin), although these findings were not consistent among all investigators.[202,206,207] Decreased proliferation capacity in vitro and premature replicative senescence are also characteristic of DC cells (see Box 7-9).[201,208] Cells from classic and more severe forms of DC (X-linked DC) fail to immortalize after Epstein-Barr virus–induced transformation or after transfection of a plasmid expressing the telomerase catalytic subunit TERT.[208] Similarly the reprogramming of DC skin cells into iPS cells is difficult,[209,210] and reprogrammed iPS cells are not immortal.[210]

Differential Diagnosis

Clinically, BMF in patients with DC has to be distinguished from other forms of BMF, including acquired aplastic anemia, familial aplastic anemia, and IBMFSs such as FA (MIM 227650), Seckel syndrome (MIM 606593), DNA ligase IV deficiency (MIM 606593), and Shwachman-Diamond syndrome (SDS, MIM 260400). Reticulate hyperpigmented disorders including Naegeli-Franceschetti-Jadassohn syndrome (MIM 161000), dermatopathia pigmentosa reticularis (MIM 125595), X-linked reticulate pigmentary disorder, incontinentia pigmenti (MIM 308300), and poikiloderma with neutropenia (MIM 604173) need to be distinguished from DC. Clinical manifestations, associated physical abnormalities, the family history (pattern of inheritance), and the lack of increased chromosomal fragility in the DEB or MMC test performed on cultured peripheral lymphocytes or fibroblasts (or both) are helpful in strengthening the diagnosis of DC. As noted earlier, telomere length below the normal distribution strongly suggests DC, whereas normal telomere length makes DC an unlikely cause for BMF in these patients.[163] X-inactivation studies may be helpful in identifying female carriers of the X-linked form of DC.[205]

Genetic Characteristics

Inheritance. There are four distinct forms of inheritance of DC. Historically, before molecular diagnostic testing was available, inheritance was determined by examining family members for the presence of clinical manifestations. Today, we know that such classification was often mistaken because of the variability of penetrance of gene mutations.

X-linked Dyskeratosis Congenita. The most common form of classic DC is the X-linked form (MIM 30500), and many families with classic DC and X-linked inheritance have been described. The X-linked form is almost always due to single point mutations in the *DKC1* gene encoding dyskerin.

Autosomal Dominant Dyskeratosis Congenita. Several families have been described with an autosomal dominant (AD) form of inheritance of DC (AD DC, MIM 127550). This form exhibits genetic anticipation (Box 7-11). Several pedigrees in which AD DC is caused by mutations in the *TERC* gene encoding telomerase RNA have been described. In addition, families in whom AD DC appears to be due to mutations in *TERT*, which encodes the telomerase reverse transcriptase, have been reported. Mutations in the shelterin component TIN2, encoded by *TINF2*, are dominant in that a severe form of DC is caused by heterozygous mutations. However, these patients seldom have children, and the disease is sporadic, caused by de novo mutations.[211,212]

Autosomal Recessive Dyskeratosis Congenita. A number of families have been described with autosomal recessive (AR) DC (MIM 224230). AR DC is usually inferred when neither parent has any signs of DC but two or more children have the disease or when the parents of an affected child are consanguineous. Some families originally thought to have AR DC were found to have AD DC with variable penetrance and expressivity, mimicking sporadic or AR disease.[213-215] Rare cases of AR DC are due to biallelic mutations in *TERT, TERC, NOP10, NHP2*, and *WRAP53*. More commonly, recessive forms of DC are due to mutations in *CTC1* and *RTEL1*.

Sporadic Dyskeratosis Congenita. A large proportion of DC cases arise sporadically (i.e., neither parent of an affected child has the disease). Of 123 such male cases from the DC registry in the United Kingdom, 40 were found to have a de novo *DKC1* mutation and 3 were found to have an inherited *TERC* mutation with variable penetrance and expressivity in the affected family. Eighty had neither *TERC* nor *DKC1* mutations. Similarly, all 35 female cases had neither *TERC* nor *DKC1* mutations. These sporadic cases account for more than half of DC patients.[215,216] Most cases due to *TINF2* mutations are sporadic, due to the fact that most patients with this severe form of DC do not reproduce.[211]

Dyskeratosis Congenita Genes and Their Functions

DKC1 and Dyskerin. X-linked DC is caused by mutations in the *DKC1* gene at Xq28. *DKC1* was identified by positional cloning in families with affected males and biased X-inactivation in female carriers.[217] The *DKC1*-encoded protein dyskerin is a 58-kD nucleolar protein orthologous to proteins in yeast, flies, and rats (Cbf5p,[218] Nop60B/minifly,[219] and NAP57,[220] respectively).

Dyskerin is associated with box H/ACA RNA molecules in ribonucleoprotein (RNP) complexes. Box H/ACA RNP complexes consist of four different proteins (dyskerin, GAR1, NOP10, and NHP2) and an integral RNA species.[221] The box H/ACA RNA determines the function of the RNP complex. The majority of box H/ACA RNAs are small nucleolar RNAs (snoRNAs) found in the nucleolus. These snoRNAs guide the protein complex containing dyskerin by complementary base pairing to specific uridine residues of newly synthesized ribosomal RNA (rRNA) molecules, which are then modified by pseudouridylation. In snoRNP dyskerin is the enzyme, a pseudouridine synthase, that actually catalyzes the pseudouridylation reaction.[222,223] The role of pseudouridylation is not known, but pseudouridines tend to be found in the conserved sequences of rRNA and transfer RNA (tRNA) in regions of high secondary structure, and it is likely that these modifications stabilize the secondary and tertiary structures of RNA molecules. Other box H/ACA RNAs that associate with dyskerin and the three other proteins of the complex participate in ribosome biogenesis downstream of rRNA modification or messenger RNA (mRNA) splicing or are orphan RNAs, the function of which remains to be determined.[221]

Telomerase RNA (*TERC*, telomerase RNA component), which along with TERT (telomerase reverse transcriptase) forms the core of the telomerase enzyme, also associates with dyskerin and the other three proteins present in H/ACA-class snoRNP (Fig. 7-10, *C*).[224,225] The conserved secondary structure of the 3′ end of telomerase RNA is very similar to that of H/ACA RNAs. This part of the telomerase RNA and its association with H/ACA RNP components are found only in vertebrates, whereas the H/ACA RNP components themselves are highly conserved among all eukaryotes. The four proteins may be involved in transport, stability, and assembly of the telomerase complex in the nucleolus. There is no evidence that the H/ACA part of telomerase RNA functions as a guide RNA or that dyskerin exhibits any pseudouridylation activity as part of the telomerase complex.

The diverse function of dyskerin in humans raises the interesting question of whether and to what extent the pathogenesis of X-linked DC is caused by defects in telomerase activity or in other functions of RNP, such as rRNA metabolism.

NOP10. Autozygous mutations (homozygous mutations in a consanguineous family) in the *NOP10* gene on chromosome 15q14 have been found to be responsible for disease in a single family.[226] NOP10, like dyskerin, is an essential component of H/ACA RNP complexes and thus participates in the same pathways as dyskerin (see Fig. 7-10, *C*).[225,227]

NHP2. Two families have been described with biallelic mutations in *NHP2*. NHP2, like dyskerin and NOP10, is an essential component of H/ACA snoRNP complexes (see Fig. 7-10, *C*).[225,227]

TERC, TERT, and Telomerase. AD DC is caused by mutations in the *TERC* gene, also abbreviated as *hTR or TER*, which is found on chromosome 3q26.[228] *TERC* was identified as the gene responsible for AD DC through positional cloning. It encodes the RNA component of the telomerase complex. Telomerase, the enzyme responsible for elongating and maintaining telomeres, synthesizes telomeric DNA repeats by using *TERC* RNA as a template (see Box 7-10 and Fig. 7-10, *C and D*). Nonsynonymous mutations have also been identified in the *TERT* gene localized on 5p15.33.[229-231] TERT is the catalytic part of the telomerase complex (see Box 7-10 and Fig. 7-10, *C*). *TERT* was identified as a gene responsible for

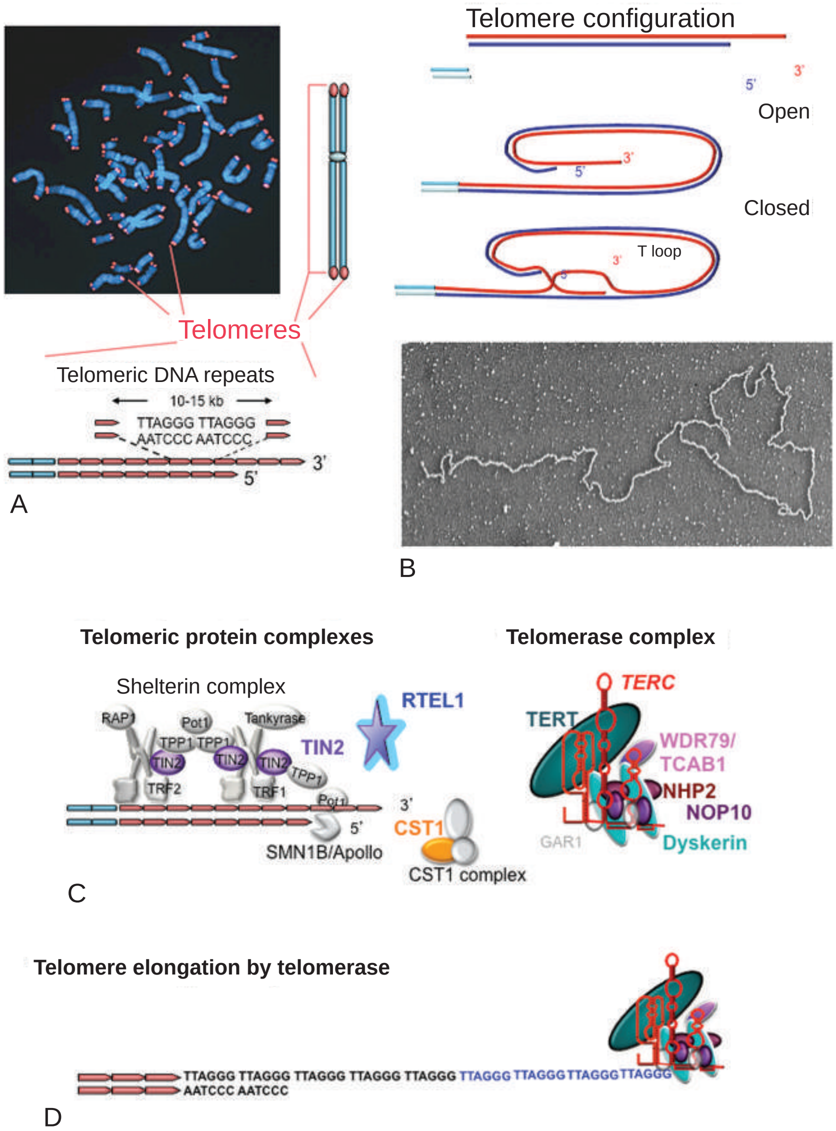

Telomere configuration
5'
3'
Open
5'
3'
Closed
3'
T loop
Telomeres
Telomeric DNA repeats
10-15 kb
TTAGGG TTAGGG
AATCCC AATCCC
3'
5'
A
B
Telomeric protein complexes
Telomerase complex
Shelterin complex
RAP1
Pot1
Tankyrase
TPP1 TPP1
TIN2
TIN2
TIN2
TPP1
TRF2
TRF1
Pot1
RTEL1
TIN2
3'
5'
SMN1B/Apollo
CST1
CST1 complex
TERC
TERT
WDR79/
TCAB1
NHP2
NOP10
GAR1
Dyskerin
C
Telomere elongation by telomerase
TTAGGG TTAGGG TTAGGG TTAGGG TTAGGG TTAGGG TTAGGG TTAGGG TTAGGG
AATCCC AATCCC
D

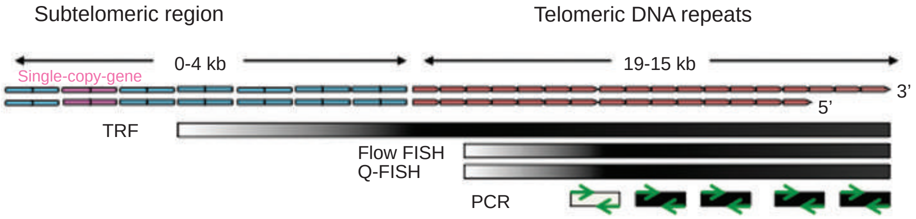
Figure 7-10 **A**, Telomeres and telomere structure. Telomeres are complex DNA protein structures that protect the ends of chromosomes in every cell. The organization of DNA ends at telomeres is highly conserved. Telomeric DNA is composed of short, direct repeats. **B**, In quiescent cells the telomeres adopt a looplike structure, the T loop, which hides the end of chromosomes from degradation and end-to-end fusion (see also Box 7-10).[101] **C**, A number of additional telomeric proteins and protein complexes are responsible for maintaining this unique telomeric DNA structure.[748-750] Highlighted here are the shelterin complex, telomerase, and CST1 and RTEL1 enzymes involved in telomere elongation and replication. Patients with dyskeratosis congenita have mutations in one of eight gene products participating in telomere maintenance (highlighted in color; mutations in the telomerase complex are TERT, TERC, DKC1, NHP2, and NOP10; mutations in the shelterin complex are TIN2; mutations in enzymes involved in the replication of telomeres are CTC1 and RTEL1. **D**, Telomerase is a ribonucleoprotein complex. It uses its RNA component, TERC, as a template to synthesize telomeric repeats on the 3′ end of telomeres, thereby elongating the ends of chromosomes (see also Box 7-10).

Continued on following page

AD DC through a candidate gene approach. Telomerase RNA, or the *TERC* component, which along with TERT forms the core of the telomerase enzyme, also associates with the four proteins present in H/ACA-class RNPs, including dyskerin, NOP10, and NHP2 (telomerase RNP complex; see Fig. 7-10, C). Dyskerin, TERT, and *TERC* were the three components identified in purified active telomerase.[232]

The measurement of telomere length has become a widely used screening test to identify patients who have BMF due to DC. Several methods are available that provide this type of information (for review, see Aubert et al, 2012,[754] and Vera and Blasco, 2012[755]). Although the methods in general produce similar results, individual measurements are difficult to compare. Methods most frequently used clinically include the measurement of telomere restriction fragment (TRF) lengths; PCR amplification of telomere repeats; and FISH, quantitative FISH (Q-FISH), and flow FISH, which are based on digital microscopy and flow cytometry, respectively (see Fig. 7-10, *E*).

TINF2. Heterozygous mutations in *TINF2* represent the second most frequent cause of DC, after *DKC1* mutations.[211,212] *TINF2* encodes TIN2, one of the six



Clinically Most Frequently Used Methods for Telomere Measurements

Method	Approach	Advantages	Drawbacks
Telomere restriction fragment (TRF)	Southern blot hybridization using genomic DNA and probes against telomeric repeats or single-copy gene probe • Quantifies average telomere length per sample (i.e., total peripheral white blood cells)	• Well-known and widely used technique • Simple and relatively cheap	• Difficult to quantify • Requires many cells (~1 million) • Subtelomeric polymorphisms influence results
Telomere measurement by PCR	• Measures ratio of telomere repeat copy number to single-copy gene number • Quantifies average telomere length per sample	• Simple • Fast • Only little DNA required • A high throughput analysis possible • Can use archived DNA	• Very sensitive to quality of DNA
Flow FISH	• Telomere fluorescence in individual interphase cells using flow cytometry (FACS) • Quantifies average telomere length per cell in interphase nuclei	• Simple • Quantitative • Reproducible • Accurate • Can determine lineage-specific telomere lengths	• Restricted to single-cell solution • Requires live cells • Requires expensive and technically demanding equipment
Quantitative FISH (Q-FISH)	• Fluorescence microscopy to determine individual telomere lengths • Fluorescence after hybridization of metaphase spreads with a fluorescent PNA telomeric probe	• Measurement of telomere length of each individual chromosome • Allows quantification of "signal-free ends" • Qualitative analysis of telomeres	• Labor intensive and time consuming • Requires expensive and technically demanding equipment • External calibration is difficult • Requires dividing cells

E

Figure 7-10, cont'd E, Graphic illustration of the telomeric regions detected using the most frequently used telomere measurement methods. Telomere length measured by terminal restriction fragment includes subtelomeric DNA and telomeric repeats of the telomere. Both the quantitative fluorescent in situ hybridization and flow fluorescent in situ hybridization techniques use probes that are specific for pure telomeric repeats. Quantitative polymerase chain reaction compares amplification of pure telomere reads using telomeric primers (green arrows). The table below summarizes the different methods and their advantages and disadvantages. *(B, Tungsten shadow-cast visualization of a mammalian telomeric T loop using transmission electron microscopy courtesy of Jack Griffith, Department of Biochemistry, University of North Carolina, Chapel Hill, N.C.; E, Figure modified from Aubert G, Hills M, Lansdorp PM: Telomere length measurement: caveats and a critical assessment of the available technologies and tools.* Mutat Res *730, 59–67, 2012; Table modified from Vera E, Blasco MA: Beyond average: potential for measurement of short telomeres.* Aging [Albany, N.Y.] *4:379–392, 2012.)*

components of the shelterin complex, which interacts with telomere DNA repeats and protects the ends of chromosomes from degradation and from being recognized by the cell's DNA repair machinery (see Fig. 7-10, C).[233] TIN2, along with another shelterin component, TPP1, is important in recruiting telomerase to its site of action at the telomere.[243] TIN2 also associates with a protein called HP1-gamma, which binds to telomeres in S phase and is important in cohesion, the physical pairing of sister chromatids by association with proteins called cohesions.[244] The HP1-gamma binding site in TIN2 is specifically required for cohesion of sister telomeres, and mutations in this site can affect telomere length maintenance.

TCAB1. Two unrelated cases have been reported where DC was caused by compound heterozygous mutations in *TCAB1*.[245] TCAB1, also known as WDR79 and WRAP53, contains WD40 repeats and binds to the CAB box, a motif found in scaRNAs and TERC (see Figs. 7-10, C and 7-12, C). Binding of TCAB1 is necessary for localization in Cajal bodies, the nuclear sites of assembly of telomerase and scaRNAs.[246] Telomerase assembly in Cajal bodies is important in the translocation of telomerase to its site of action at the telomere, and TCAB1 inhibition leads to telomere shortening.[210]

CTC1. Patients with biallelic mutations in *CTC1* (conserved telomere component 1) present with a variable set of abnormalities, including some characteristic features of DC.[247,248] CTC1 forms a complex with two other proteins, Suppressor of cdc ThirteeN 1 (STN1) and TElomeric pathways with stN1 (TEN1). This complex is known as the CST telomere-capping complex (see Fig. 7-10, C). CTC1 and STN1 stimulate DNA polymerase primase by binding single-stranded DNA, suggesting that the complex may be involved in priming telomeric C-strand synthesis.[249] The corresponding complex in yeast is involved in maintaining telomeric DNA integrity. CTC1 may have a general role in DNA metabolism rather than a specifically telomeric one.

RTEL1. Both homozygous and heterozygous mutations in *RTEL1* have been described in DC patients.[250,251] RTEL1 is a helicase originally identified in a screen of genes that affect telomere length.[252] RTEL1 plays an essential role in maintaining telomere length and genome stability and is an antagonist of homologous recombination. It has recently been shown that RTEL1 is essential for the disassembly of telomeric T loops; without proper disassembly telomere loss occurs because of the action of the SLX4 nuclease complex, which resolves the T loop as a circle.[253] G-quadruplex structures at the telomere are a major source of telomere fragility in *RTEL1*-deficient cells.

Other Genes. For about 50% of patients in whom DC and BMF are diagnosed, the mutations or the genes mutated have not been identified. Like patients with DC and BMF caused by mutations in known genes, most of these patients, but not all, also have very short telomeres, suggesting that the affected gene or genes participate in the same pathway.

Unraveling of the molecular pathology of DC has greatly advanced our understanding of the disease. The finding that all forms of DC in which the pathogenesis is understood are caused by disruption of telomere maintenance highlights the importance of telomeres in the disease (see Fig. 7-9). Figure 7-11 summarizes our current model of the pathogenesis of DC.

Dyskeratosis Congenita Gene Mutations

Mutations in telomere maintenance genes that have been found in patients with DC are listed on the comprehensive Telomerase Database, which is updated regularly.[254]

***DKC1* Mutations.** The mutations in the *DKC1* gene that cause DC are mainly single–amino acid missense mutations[255] (Fig. 7-12, *A*) and can be inherited or occur de novo. The *A353V* mutation is the most frequent de novo *DKC1* mutation and is found in approximately 40% of patients with DC and *DKC1* mutations.[215] *DKC1* mutations are not randomly distributed but are concentrated in the N-terminal region of dyskerin. The crystal structure of the archaeal homologue of the H/ACA RNP complex, the Cbf5-Nop10 complex,[256,257] has enabled modeling of human dyskerin. In this model most of the residues mutated in cases of DC are tightly clustered in or near the PUA domain, which is predicted to bind H/ACA RNA, including *TERC* RNA (see Fig. 7-12, *B*). The mutations would thus be expected to decrease binding of the cognate RNA and lead to instability. Only two mutations have been found in the TruB catalytic domain, one of which, S121G, is located three residues from the essential aspartate required for pseudouridylation and, interestingly, is associated with a severe form of DC known as Hoyeraal-Hreidarsson syndrome (HHS, MIM 300240). The absence of mutations that would predict a "null" allele, such as frameshifts and nonsense mutations, suggests that a dyskerin null mutation is incompatible with life. In support of this notion, a dyskerin null mutation is embryonically lethal in mice.[258]

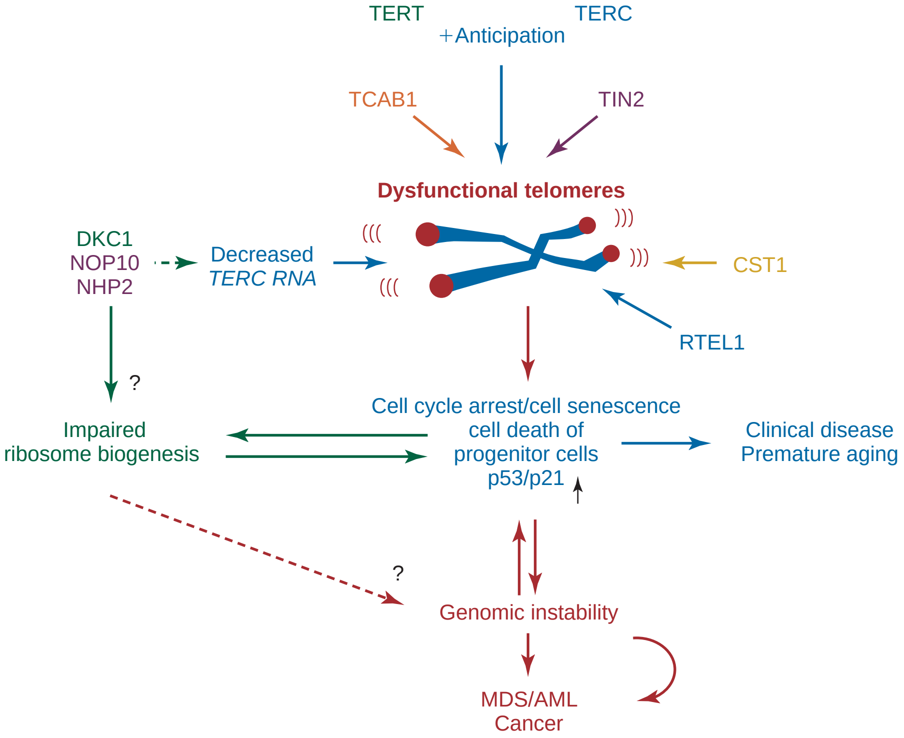


Figure 7-11 Model of the pathogenesis of disease in patients with dyskeratosis congenita. Our hypothesis is that excessive telomere shortening and dysfunctional telomeres play a central role in the pathogenesis of dyskeratosis congenita. Dysfunctional telomeres lead to cell cycle arrest, cell senescence, and cell death. In patients with *DKC1, NHP2,* or *NOP10* mutations, *TERC* RNA stability and accumulation are decreased, which leads to decreased telomerase activity and telomere shortening. Impaired ribosome biogenesis may also limit cell survival and proliferation. *TERC* and *TERT* mutations cause telomere shortening through haploinsufficiency. The inheritance of preshortened telomeres is the molecular basis of anticipation in autosomal dominant dyskeratosis congenita. *TCAB1* mutations prevent the recruitment of telomerase to the telomeres. RTEL1 and CTC1 are important for the proper replication of telomeres. Dysfunctional telomeres lead to genomic instability that may contribute to the cancer predisposition in patients with dyskeratosis congenita.

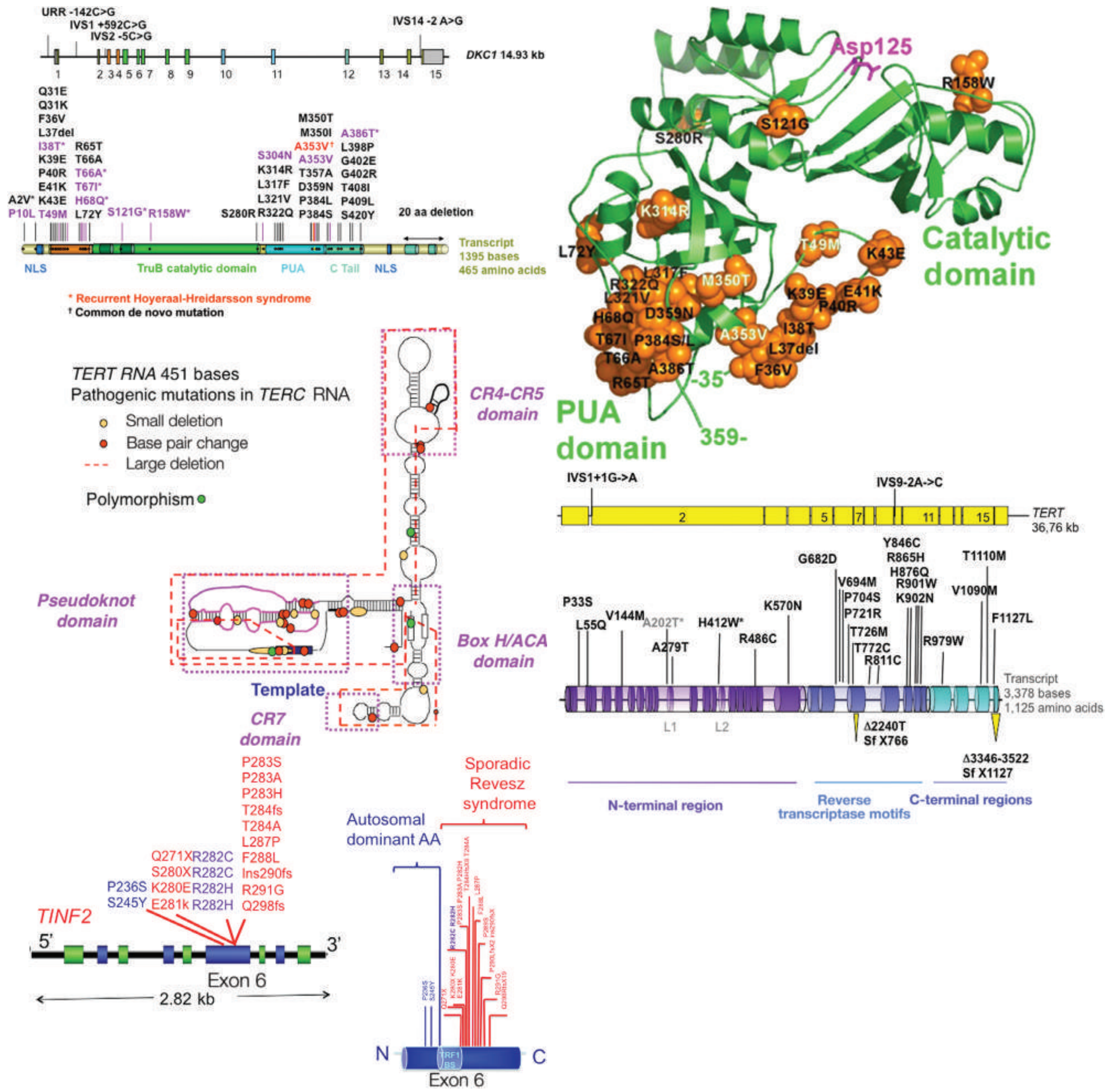

URR -142C>G
IVS1 +592C>G
IVS2 -5C>G
IVS14 -2 A>G
DKC1 14.93 kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Q31E
Q31K
F36V
L37del
I38T* R65T
K39E T66A
P40R T66A*
E41K T67I*
A2V* K43E H68Q*
P10L T49M L72Y S121G* R158W*
M350T
M350I A386T*
A353V† L398P
S304N A353V G402E
K314R T357A G402R
L317F D359N T408I
L321V P384L P409L
S280R R322Q P384S S420Y
20 aa deletion
Transcript
1395 bases
465 amino acids
NLS
TruB catalytic domain
PUA
C Tail
NLS
* Recurrent Hoyeraal-Hreidarsson syndrome
† Common de novo mutation
Asp125
R158W
S121G
S280R
K314R
L72Y
T49M
K43E
Catalytic domain
R322Q
L321V
L317F
M350T
K39E
E41K
P40R
H68Q
D359N
I38T
T67I
P384S/L
A353V
L37del
T66A
A386T
F36V
R65T
-35
PUA domain
359-
TERT RNA 451 bases
Pathogenic mutations in TERC RNA
Small deletion
Base pair change
Large deletion
Polymorphism
CR4-CR5 domain
Pseudoknot domain
Box H/ACA domain
Template
CR7 domain
IVS1+1G->A
IVS9-2A->C
2 5 7 11 15
TERT 36,76 kb
Y846C
R865H
H876Q
R901W
K902N
G682D
T1110M
V694M
P704S
P721R
T726M
T772C
R811C
V1090M
P33S
L55Q
V144M
A202T*
H412W*
K570N
R486C
A279T
F1127L
R979W
Transcript
3,378 bases
1,125 amino acids
L1
L2
Δ2240T
Sf X766
Δ3346-3522
Sf X1127
N-terminal region
Reverse transcriptase motifs
C-terminal regions
P283S
P283A
P283H
T284fs
T284A
L287P
Q271X R282C F288L
S280X R282C Ins290fs
P236S K280E R282H R291G
S245Y E281k R282H Q298fs
Sporadic Revesz syndrome
Autosomal dominant AA
TINF2
5′
3′
Exon 6
2.82 kb
N
C
TRF1 BS
Exon 6

Figure 7-12 **A**, Mutations in the DKC1 gene. Schematic representation of the *DKC1* gene, below the schematic representation of dyskerin protein and its functional domains. A series of patient-derived mutations are shown in bold. An asterisk indicates recurrent mutations. The A353V mutation is shown in red; this mutation is a frequent de novo mutation and accounts for about one third of *DKC1* mutations. Mutations found in patients with the severe form of dyskeratosis congenita known as Hoyeraal-Hreidarsson syndrome are shown in purple. **B**, Clustering of pathogenic mutations in dyskerin. Crystal structure of the archaeal homologue of dyskerin. In this model the majority of residues mutated in cases of dyskeratosis congenita are tightly clustered in or near the pseudouridine synthase and archaeosine transglycosylase (PUA) domain, which is predicted to bind box H/ACA RNAs, including the TERC RNA. **C**, Schematic representation of the tertiary structure of the telomerase RNA (TERC). The pseudoknot and the CR4-CR5 domain are important for the association of TERC with the catalytic subunit TERT and for telomerase activity. Mutations found in families with autosomal dominant dyskeratosis congenita are shown, including two large deletions, one affecting the 5′ end and the other the 3′ end of TERC. **D**, Mutations in the *TERT* gene. Schematic representation of the 16 exons of the TERT gene, below the schematic representation of the TERT protein with its functional domains. Patient-derived mutations are shown in bold. Sequence variants of uncertain pathogenicity are shown in gray. Small deletions are indicated below the diagram. L1 and L2 are linker regions; Sf indicates a frameshift, which results in a premature stop codon X. The asterisk indicates recurrent mutations. **E**, *TINF2* mutations are tightly clustered in exon 6. Mutations at 5′ of the TRF1 binding site have been found in families with familial aplastic anemia, whereas mutations at 3′ of the TRF1 binding site are sporadic and cause a severe phenotype of disease with early onset, intrauterine growth retardation, cerebellar hypoplasia, immune deficiency, and retinal bleeding; this was previously described as Revesz syndrome but is in fact a severe form of dyskeratosis congenita. For a comprehensive listing of mutations see telomerase.asu.edu/diseases.html. *L1 and L2*, Linker regions; *NLS*, nuclear localization singnal; *Sf*, frameshift; *TruB and PUA*, pseudouridine synthase domains. *(**B**, Modified from Rashid R, Liang B, Baker DL, et al: Crystal structure of a Cbf5-Nop10-Gar1 complex and implications in RNA-guided pseudouridylation and dyskeratosis congenita.* Mol Cell *21:249–260, 2006.)*

The functional consequences of a subset of pathogenic *DKC1* mutations have been investigated in fibroblasts or mononuclear cells from patients with X-linked DC[208,259] and in XY mouse embryonic stem cells harboring a mutated *Dkc1* allele.[260,261] Analysis of fibroblasts or mononuclear cells from patients with X-linked DC showed a reduced level of *TERC* RNA and decreased telomerase activity, whereas analysis in mouse cells revealed that not only the level of *Terc* RNA but also the levels of other specific snoRNAs were affected and that overall pseudouridylation activity—in particular, site-specific pseudouridylation involving the affected guide snoRNA—was markedly decreased. Controversy continues as to whether X-linked DC is due solely to defective telomere maintenance or whether defective ribosome biogenesis contributes to the pathogenesis.[262] If the latter is the case, other relevant genes may not be effectively translated and thereby contribute to the pathophysiology (see later discussion of Diamond-Blackfan Anemia [DBA]).

Biochemical and structural studies of the assembly and structure of H/ACA RNPs, including telomerase, have so far failed to explain the structural and functional relationships in the effects of the *DKC1* mutations. Early in the biogenesis of H/ACA RNPs, prior to introduction of NAF1 and before assembly with the RNA component, dyskerin forms a tight connection with a protein SHQ1 and many *DKC1* mutations are in the region of dyskerin that interacts with SHQ1, suggesting that the mutations may affect biogenesis of the complex. It will be interesting to see if telomerase is preferentially affected by these mutations.[256,263]

***NOP10* Mutations.** Autozygous *NOP10* mutations have been identified in one consanguineous family. The mutation leads to replacement of a highly conserved arginine with tryptophan at amino acid position 34 (R34W).[226] The three affected family members were autozygous for the mutation. Heterozygous mutation carriers did not show obvious signs of disease but had shortened telomeres and decreased levels of *TERC* RNA in their peripheral blood cells. The inheritance of hypomorphic gene mutations may mimic a recessive inheritance pattern. The shortened telomeres and reduced levels of *TERC* RNA in heterozygous mutation carriers, however, suggest that both *NOP10* alleles are expressed codominantly. Whether the mutation affects the function of only telomerase RNP or also other H/ACA RNP complexes remains to be determined. In vitro experiments showed that the mutated NOP10 protein was not defective in the formation of the dyskerin-NAF1-NOP10-NHP2 tetramer that assembles with the nascent H/ACA RNA; rather, the mutation severely affected association with the H/ACA domain of TERC and a subset of other H/ACA RNAs.[264]

***NHP2* Mutations.** Two families with *NHP2* mutations have been reported. In one kindred affected children were homozygous for a Y139H mutation, while in the other the affected child was a compound heterozygote for a V126M and a mutation in the stop codon, X154RextX*52, resulting in an elongated protein.[265] In both cases parents were heterozygous and asymptomatic. Patients had low *TERC* levels and short telomeres. In vitro experiments showed that the V126M and the Y139H mutations impaired association with NOP10 and led to defects in H/ACA RNP assembly with all H/ACA RNAs tested, including *TERC*.[264]

***TERC* Mutations.** The *TERC* mutations found in patients with DC are shown in Figure 7-12, *C*. Investigation of family members revealed that in all patients with clinical manifestations of DC the *TERC* gene mutations were inherited. Many mutations are found in the pseudoknot domain of the molecule, a domain that mediates binding to the telomerase reverse transcriptase TERT and is essential for telomerase activity. Other mutations are found in the CR7 and H/ACA domains and affect the stability and nuclear accumulation of the *TERC* molecule. In many of these mutations it has been demonstrated that the mutations act by disrupting the secondary structure of the *TERC* molecule rather than by altering nucleotide sequences crucial for function[266-268]; this finding is in agreement with the fact that although the secondary structure of *TERC* is highly conserved among different species, the primary sequence is not.[269] Large gene deletions encompassing the *TERC* gene have been identified in two independent families with AD DC.[228,268] Along with the pathogenic mutations, which have been shown by several groups to severely reduce telomerase activity, there are two polymorphisms that are relatively frequent in people of African descent. These sequence changes, G58A and G228A, do not affect telomerase activity, and individuals carrying these sequence alterations have normal telomere length independent of the presence of BMF.[267,268,270]

The existence of large deletions and in vitro transfection studies led to the conclusion that haploinsufficiency of *TERC* is the mechanism underlying AD DC.

***TERT* Mutations.** Heterozygosity for nonsynonymous mutations in the *TERT* gene have been identified in individuals with aplastic anemia and short telomeres and also in individuals in whom pulmonary fibrosis was originally diagnosed.[177,229-231] A number of such mutations identified are shown in Figure 7-12, *D*. Mutations located in the N-terminal critical for binding of *TERC* RNA and within the central region of *TERT* defining the catalytic region of the reverse transcriptase impair telomerase activity to a variable degree. Mutations in the C-terminal region affect in vivo telomere synthesis after the assembly of a catalytically active enzyme.[271] Disease manifestations in families with *TERT* gene mutations are highly variable, and frequently disease does not segregate with the mutation in these families, indicating incomplete disease penetrance. No correlation has emerged between site of mutation and disease manifestations, but severity does increase in succeeding generations due to anticipation (see Box 7-11). Thus earlier generations may present as adults with pulmonary fibrosis or liver cirrhosis, whereas later generations may present as children with aplastic anemia or dyskeratosis congenita.[272] Interestingly, individuals with a de novo *TERT* gene deletion secondary to a large chromosomal deletion on chromosome 5p

Box 7-11 Anticipation

Genetic anticipation occurs when an inherited disease develops at increasingly younger ages or with increased severity in each succeeding generation. Thus if the disease develops in the offspring of a patient, it will tend to do so at an earlier age and display more severe clinical manifestations than in the parent. Disease anticipation is found in patients with autosomal dominant dyskeratosis congenita and is caused by the inheritance of increasingly shorter telomeres in subsequent generations.

(5p– syndrome, cri du chat syndrome) show accelerated telomere shortening but no clinical features characteristic of DC.[273] In contrast, biallelic *TERT* gene mutations have been identified in patients with classic manifestations of DC and in one individual with HHS, which suggests that the mutant alleles are expressed codominantly and the residual telomerase activity determines the clinical phenotype.[274,275] Biallelic *TERT* or *TERC* null mutations have never been described, suggesting that in contrast to mice, complete lack of telomerase activity is not compatible with life in humans.

***TINF2* Mutations.** *TINF2* mutations causing DC are strikingly clustered in the TIN2 protein (see Fig. 7-12, *E*).[211,212] Two thirds of the mutations affect a single amino acid residue, Arg282, changing it to a histidine or cysteine residue. Nearly all of the other identified mutations affect the same small region of the protein, encoded in exon 6. In vitro experiments have shown that TIN2, along with TPP1, is involved in recruitment of telomerase to telomeres,[276] and overexpression of the pathogenic TINF2 mutants disrupts the association between TIN2 and telomerase. Intriguingly the cluster of mutations in TIN2 overlaps with the HP1-gamma binding site in the protein that is essential for cohesion, the physical association of newly replicated telomeres.[244] It has been speculated that telomere cohesion may be necessary for efficient telomere elongation by the dimeric telomerase enzyme.[277]

***TCAB1* Mutations.** Two unrelated cases of DC caused by *TCAB* mutations have been reported. In both families the probands were compound heterozygotes for single point mutations causing amino acid substitutions, F164L and R398 in one case and H367Y and G435R in the other.[245] All the affected amino acids are highly conserved. The mutations result in decreased accumulation of TCAB1 and its mislocalization in the cytoplasm rather than the Cajal body. This causes accumulation of *TERC* in the nucleolus rather than the Cajal body, which presumably affects the subsequent trafficking of telomerase to the telomere.[245] In keeping with this premise, iPSC from TCAB1 DC patients shows normal levels of telomerase activity but defects in telomere lengthening.[210]

***CTC1* Mutations.** Most patients are compound heterozygotes, and the mutations include missense, nonsense, frameshift, and inframe deletions and splicing mutations.[247,248] *CTC1* mutations have also been implicated in a rare syndrome known as Coats plus syndrome or cerebroretinal microangiopathy with calcifications and cysts.[278] Null mutations are never found on both alleles of a single patient, suggesting that some CTC1 activity is essential for life. The pathogenetic mechanism by which these mutations cause a DC-like phenotype is unknown. Telomere length is normal[248,279] or short.[247,278]

***RTEL1* Mutations.** Biallelic missense mutations are the major cause of the severe DC (HHS) caused by *RTEL1* mutations,[251,280] though dominant mutations have been reported.[250] Mutations are mainly missense but include a splicing mutation and a nonsense mutation. An Arg981Trp mutation is recurrent. Examination of patients' cells using an assay that measures telomeric circles demonstrated that levels of these circles are higher than in controls. RTEL1 is responsible for disassembly of T loops, which are produced during faulty processing of telomere ends and cause telomere shortening.[251,280] In mice a biallelic *Rtel1* deletion is not compatible with life,[252] and no biallelic null mutations have been indentified in humans, suggesting that some RTEL1 function is essential. No defects in homologous recombination, which also involves RTEL1, have been found.

Genotype-Phenotype Correlation

The disease phenotype of patients with DC is highly variable and is influenced by the gene mutated, the pattern of inheritance (X-linked, autosomal dominant, or autosomal recessive), the nature of the mutation, and the history of inheritance (number of generations that the mutations have been passed on to). A distinctive feature of DC is that clinical manifestations differ with the age of disease onset (see Fig. 7-13).

***DKC1* Mutations.** The severity of disease in individuals with X-linked DC varies from hardly detectable to a severe form (HHS; see later) that causes death in early childhood (see Fig. 7-12, *A*). *DKC1* maps to the X chromosome, so the majority of affected individuals are therefore males. The most frequent clinical manifestation is classic DC with mucocutaneous findings in early childhood and progressive BMF in adolescence.

Female carriers of the *DKC1* mutation rarely show signs of hematologic disease. Cutaneous manifestations may be found in female carriers but are usually mild. Female carriers of X-linked DC show a phenomenon known as skewed or biased X-inactivation. In females, inactivation of one X chromosome must occur at random early in development to achieve the same dosage of X-linked genes as in male cells. When X-inactivation has been established in a particular cell, it is faithfully passed to all progeny. In women, tissues are therefore a mosaic in which half the cells have the active maternal and half the active paternal X chromosome. In female carriers of DC, blood consists entirely of cells in which the X chromosome containing the normal dyskerin gene is active[204,205]; this finding indicates that after random X-inactivation, cells with wild-type dyskerin have a growth or survival advantage (or both) and outgrow mutant cells. Whether this outgrowth takes place in embryogenesis or childhood and whether all tissues are affected are not known. Telomere length in peripheral blood cells in female DC

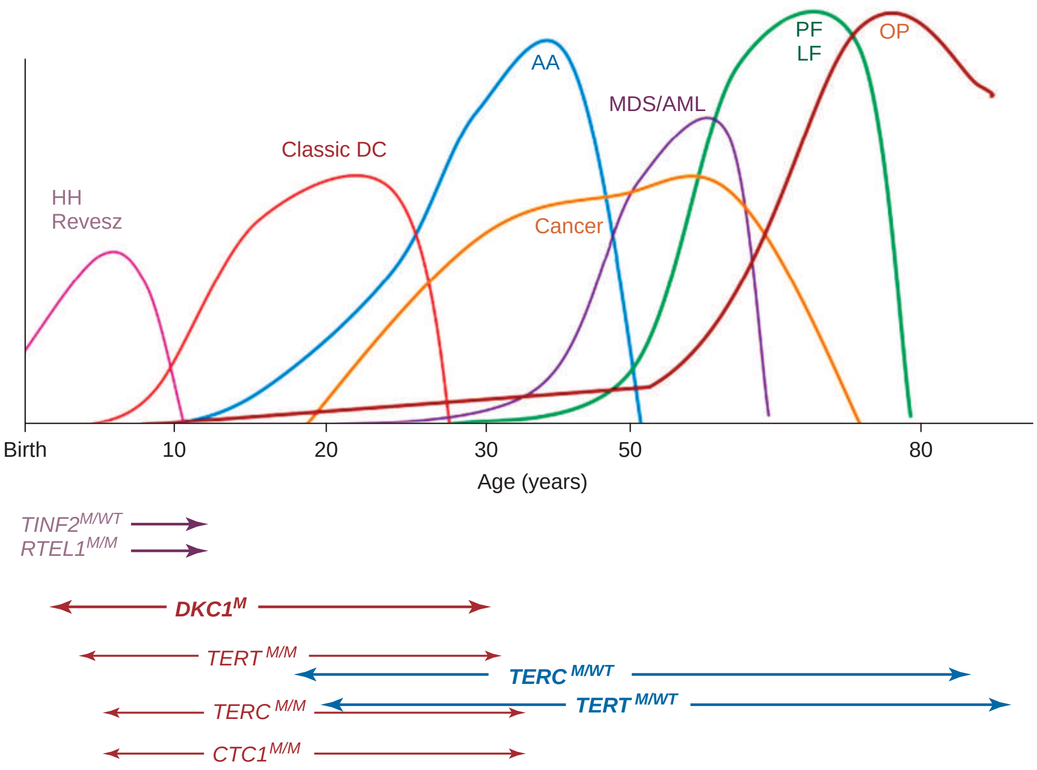


Figure 7-13 Clinical presentation of dyskeratosis congenita differs with age. The most severe forms of the disease, Hoyeraal-Hreidarsson syndrome and Revesz syndrome, manifest in early childhood and are mainly caused by *TINF2*, *RTEL1*, and *DKC1* mutations. Dyskeratosis congenita with the classic cutaneous features occurs in the teens and 20s and is mainly caused by *DKC1* mutations or homozygous *TERT*, *TERC*, or *CTC1* mutations. Aplastic anemia in the adolescent and young adult as well as pulmonary fibrosis and liver fibrosis and osteoporosis later in life are mainly caused by heterozygous *TERC* and *TERT* gene mutations. The risk of cancer and leukemia is highest in the young adult with moderate to severe disease.

carriers varies from within normal limits to very short.[163] Neither MDS nor MDS/AML has been described in a female *DKC1* mutation carrier. Whether female carriers are at increased risk for the development of cancer in other organs remains to be determined. Biased X-inactivation can be used to determine whether DC in a family is X-linked.

***TERC* Mutations.** The clinical phenotype in patients with AD DC caused by *TERC* gene mutations is usually mild. Analysis of family members of individuals with AD DC showed that mutation carriers frequently have only subtle signs of BMF but lack the mucocutaneous features of classic DC (atypical DC; see also Fig. 7-7). Interestingly, the only patient reported to be a compound heterozygote for two different *TERC* gene mutations showed signs of classic DC and early onset of disease, whereas family members with only one of the two mutations did not.[281] *TERC* gene mutations were identified in a subset of adult patients in whom familial or acquired idiopathic aplastic anemia was originally diagnosed and in patients in whom idiopathic pulmonary fibrosis was originally diagnosed.[177,282-284] Investigation of family members revealed that in all patients the *TERC* gene mutations are inherited and that all mutation carriers have short (<10th percentile) or very short (<1st percentile) telomeres. However, short and very short telomeres were additionally found in individual family members who did not carry the mutation and had no signs of disease.[285] *TERC* gene mutations were likewise identified in some patients with cytopenic MDS or MDS/AML.[282,284] The mutations identified were associated with reduced telomerase activity in vitro and very short telomeres in the mutation carriers with BMF. The presence of *TERC* gene mutations in patients in whom pulmonary fibrosis, aplastic anemia, MDS, or MDS/AML was originally diagnosed indicates that the underlying problem in these patients is the pathology of DC and that the variable expressivity and penetrance of the clinical manifestations in DC lead to a much broader clinical picture than originally anticipated when investigating only individuals with classic DC. Interestingly, *TERC* gene alterations were rarely found in children with aplastic anemia or MDS in the absence of a family history, consistent with the later onset of disease in individuals with AD DC.[196] Pulmonary fibrosis and liver cirrhosis were frequent and sometimes even the first clinical finding in individuals or families with DC and *TERC* gene mutations.

Anticipation. AD DC is one of a small number of diseases that show genetic anticipation (i.e., the disease becomes more severe and has an earlier age at onset in later generations). Anticipation became apparent when asymptomatic parents of affected individuals were found to also have *TERC* gene mutations (see Box 7-11).[228,286] In several cases, examination of these asymptomatic

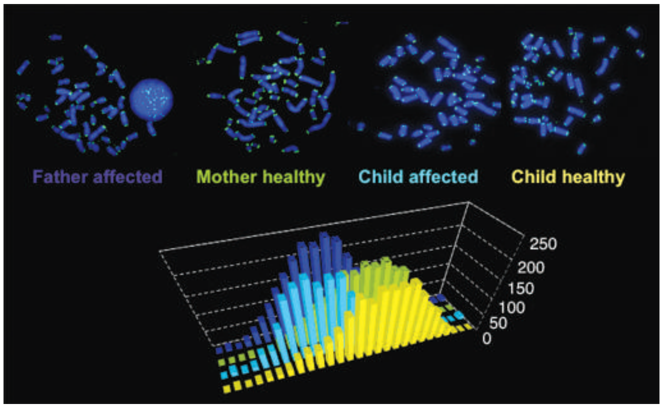


Figure 7-14 Inheritance of preshortened telomeres in a family with autosomal dominant dyskeratosis congenita due to a *TERC* gene deletion. Metaphase spreads of peripheral blood lymphocytes. The red signals at the end of chromosomes show the telomeric repeats hybridized to a fluorescent probe. Below, the graphic representation of the telomere length distribution of each family member. Note that both the affected and the nonaffected child have inherited preshortened telomeres; according to their young age their telomeres should be longer than the telomeres in their parents. *(From Goldman F, Bouarich R, Kulkarni S, et al: The effect of TERC haploinsufficiency on the inheritance of telomere length.* Proc Natl Acad Sci U S A *102:17119–17124, 2005.)*

parents revealed that they had signs of mild anemia, such as raised mean corpuscular volume. In other pedigrees, anemia develops in the parents at an older age than in the children. The mechanism of anticipation appears to be that telomere shortening increases in later generations carrying a defective *TERC* allele.[285,286] Figure 7-14 shows the inheritance of preshortened telomeres in a nuclear family with AD DC caused by a *TERC* gene deletion. Thus AD DC develops if an individual inherits a defective *TERC* or *TERT* allele and shortened telomeres. It is not known how many generations are needed to produce telomeres short enough to cause the disease. Studies in individuals with a de novo deletion of 5p that includes the *TERT* gene (5p– syndrome) indicate that in the first generation with *TERT* haploinsufficiency, telomere shortening is minimal.[273] Interestingly, in individuals with DC and BMF, the telomeres are all equally short, suggesting that there is a minimal length of telomeres required for a progenitor cell to contribute to the peripheral blood cell pool.[273,285]

***TERT* Mutations.** Nonsynonymous mutations, small deletions, and splice-site mutations in the telomerase reverse transcriptase *TERT* gene have been identified in individuals in whom aplastic anemia with short telomeres was diagnosed and in individuals in whom pulmonary fibrosis was diagnosed,[177,229,287] indicating that *TERT* gene mutations may account for cases of atypical DC. Anticipation has also been described in families with AD DC secondary to *TERT* gene mutations.[229] Individuals with heterozygous *TERT* gene mutations rarely have the classic form of DC; the majority have atypical DC. Pulmonary fibrosis is a frequent finding in patients and families with atypical DC caused by *TERT* gene mutations. Mutation carriers with no signs of disease (silent DC) are often found in families with heterozygous *TERT* gene mutations. Whether telomere length may identify silent *TERT* gene mutation carriers remains to be determined. Although one would expect that the disease in AD DC secondary to *TERC* or *TERT* mutations would be similar, initial family studies indicate that penetrance and expressivity differ in families heterozygous for *TERC* or *TERT* gene mutations. The most likely explanation is that TERC or TERT is limiting for different tissues and that expression of a functional TERT protein is regulated differently from regulation of *TERC* gene expression. Biallelic *TERT* gene mutations may lead to the classic manifestations of DC and have also been identified in one individual with HHS, which suggests that the mutant alleles are expressed codominantly and that residual telomerase activity determines the severity of the clinical phenotype.[274,275] Biallelic null mutations of *TERT* or *TERC* have never been described, suggesting that in contrast to mice, in humans complete lack of telomerase activity is incompatible with life.

***NOP10* and *NHP2* Mutations.** The one family with DC due to a homozygous R34W mutation in NOP10 contained three affected children who all showed the classic mucocutaneous features of DC.[226] Only one of the children had BMF. Affected individuals had very short telomeres and low TERC levels, whereas heterozygous family members were asymptomatic but did have shorter than normal telomeres and lower levels of TERC than healthy controls. Whether the lack of BMF in two of the three affected children is due to different genetic backgrounds or mosaicism due to mutations in bone marrow cells is not known. Two individuals with DC due to biallelic mutations in *NHP2* have been reported.[265] One, who was a homozygote, had nail dystrophy, thrombocytopenia, and mental retardation, while the other, a compound heterozygote, followed a classic DC course with pancytopenia at 12 years.

***TINF2* Mutations.** Patients with *TINF2* mutations are amongst the most severely affected of all DC patients.[211,212,288,289] Most patients present in infancy with very short telomeres and often with features of HHS or Revesz syndrome (retinopathy as well as pancytopenia, cerebellar hypoplasia, and developmental delay). *TINF2* mutations are dominant in that only one allele is mutated, but they are generally sporadic in that they are due to de novo mutations, patients being so severely affected that they do not live to reproduce (see also Fig. 7-12, *E*).

***TCAB1* Mutations.** Two unrelated cases of DC due to compound heterozygous TCAB1 mutations have been reported. Both patients had a classic DC presentation, with the mucocutaneous triad and BMF.[245]

***CTC1* Mutations.** Patients with *CTC1* mutations present with a variety of abnormalities, which include many of the characteristic DC features.[247,248] *CTC1* mutations are also found in patients diagnosed with Coats plus syndrome or cerebroretinal microangiopathy with calcifications and cysts.[278] This is a rare multisystem disorder characterized by progressive intracranial calcifications and leukoencephalopathy, often with the development of intracranial cysts. Retinopathy, BMF, and, less frequently, nail dystrophy, graying or sparse hair, and skin pigmentation abnormalities are also seen. Many of these features overlap with those seen in patients with DC, and *CTC1* mutations have in fact been found in patients diagnosed with DC. Patients included those with BMF or some of the mucocutaneous DC features with presence or absence of intracranial calcifications and retinopathy. Telomere length has been reported as short or normal.[248]

***RTEL1* Mutations.** A group of seven families with biallelic mutations in *RTEL1* was reported by Walne and colleagues.[251] These patients had a consistent type of HHS, characterized by BMF, cerebellar hypoplasia, microcephaly, intrauterine and extrauterine growth retardation, B-cell immunodeficiency, and early death. Abnormal skin pigmentation was absent, and less than half the patients had nail dystrophy or leukoplakia. Le Guen and colleagues[280] reported on three patients from two families with HHS. Affected children in both families had compound heterozygosity for an *RTEL1* missense mutation.[280] Fibroblasts from children with RTEL1 gene deletions showed telomere shortening, increased spontaneous 53BP1 foci formation, anaphase bridges, sister telomere losses, and terminal deletions. Ballew and associates[250] described three families with *RTEL1* mutations. One kindred had HHS due to biallelic *RTEL1* mutations. The other two families harbored heterozygous *RTEL1* mutations.[250] In one family, two children with HHS inherited an *R1010X* nonsense mutation from their healthy mother. The other patient was a man in his 20s who was diagnosed with DC-like disease and had an *A645T* mutation. Thus it seems that biallelic *RTEL1* mutations are a common cause of HHS, and time will tell how frequently dominant mutations are found in BMF syndromes.

Molecular Diagnosis of Dyskeratosis Congenita

Currently, molecular diagnostic tests are not routinely performed as the first line of investigation for DC because they are expensive, labor intensive, and informative in only half of patients. Nevertheless, identification of pathogenic mutations in one of the DC genes is the only way to definitively establish the diagnosis of DC. Furthermore, molecular diagnostic tests enable the identification of mutation carriers, facilitate prenatal diagnosis for an existing pregnancy, and allow preimplantation genetic diagnosis for partners identified as mutation carriers. In addition, because the clinical manifestations, severity of disease, prognosis, and response to treatment may vary, depending on the affected gene and the nature of the individual mutation, molecular diagnostic tests may also influence medical management not only for the individual carrying the mutation but also for unaffected family members. Currently, an educated candidate gene approach, in which the gender ages at disease onset and family history of the affected individual are taken into account, is used to identify the affected gene (see also Fig. 7-13). In an affected male, after exclusion of the most frequent *DKC1* mutation (A353V), it might be necessary to perform a mutational screen of the entire coding sequence of all three DC genes (usually only *TERC* and *TERT* in an affected female). For *TERC* and *TERT* the screening methods should include techniques that will detect large deletions. In addition to the large number of mutations in DC genes associated with DC, a number of nonpathogenic mutations and mutations of unknown pathogenic significance have been identified. Determining the pathogenicity of a particular mutation may be difficult.[290]

Telomere measurement in peripheral blood cells and peripheral blood lymphocytes has emerged as a sensitive screening tool for DC in patients with BMF.[162,163] Whether telomere measurements performed on peripheral blood cells may also identify DC mutation carriers or individuals with DC but nonhematopoietic disease manifestations is controversial.[162,163,248,291]

Diseases Related to Dyskeratosis Congenita

Idiopathic Aplastic Anemia. A proportion of patients with idiopathic aplastic anemia or hypoplastic MDS were found to have mutations in *TERC* or *TERT*.[282-284] These patients therefore have AD DC. The prevalence of *TERC* mutations among individuals in whom idiopathic aplastic anemia or hypoplastic MDS is diagnosed appears to be between 2% and 10%.[163] *TERC* mutations are rare in children with severe aplastic anemia or MDS consistent with later onset of the AD disease.[196] Because parents are often asymptomatic as a result of the anticipation phenomenon and because of incomplete penetrance of *TERC* and, in particular, *TERT* gene mutations, a family history is often not apparent and a diagnosis of idiopathic aplastic anemia or idiopathic MDS is made. As a consequence patients below the age of 50 years with acquired aplastic anemia or hypoplastic MDS are now routinely screened for telomere length to identify those who have BMF due to DC. Due to natural telomere shortening with age, telomere length measurements are less sensitive for the identification of patients with underlying DC mutations who are older than 50 years.

Hereditary Pulmonary Fibrosis. Pulmonary fibrosis, especially in patients with AD DC, may be an initial and sometimes prominent feature of those with atypical DC. Families with hereditary pulmonary fibrosis of adult onset should be investigated for an underlying *TERT* or *TERC* gene mutation.[177,178] The exact pathogenesis of pulmonary fibrosis in patients with DC is not understood.[179] Families with pulmonary fibrosis and aplastic anemia almost always have an underlying *TERT* or *TERC* gene mutation. Interestingly, pulmonary fibrosis is frequently found in the older generation, whereas aplastic anemia manifests earlier in life and manifests in subsequent generations (see also Fig. 7-8).[292] Thus pulmonary fibrosis in the 50s in the first generation and aplastic anemia in the 20s not only illustrates disease anticipation but also the unique features of age-specific disease manifestation characteristic for AD DC (see Figs. 7-8 and 7-13).

Hoyeraal-Hreidarsson Syndrome. Hoyeraal-Hreidarsson syndrome (HHS; MIM 300240) is a rare, severe variant of DC that presents in early childhood. It is characterized by intrauterine growth retardation, microcephaly, cerebellar hypoplasia, mental retardation, progressive combined immune deficiency, and aplastic anemia.[293,294] Delayed myelination and hypoplasia of the corpus callosum have been described in patients with HHS.[295] HHS may be caused by a mutation in the *DKC1* gene (see Fig. 7-12, *A*).[296] It is also notable that several cases of HHS are due to the recurrent mutation (A353V) that is responsible for a third of the X-linked *DKC1* cases.[215] In addition, HHS has been found in one individual autozygous (homozygosity in a consanguineous family) or compound heterozygous for a *TERT* gene mutation[290] and in an individual heterozygous for a *TERT* gene mutation and anticipation, suggesting that HHS may also be a consequence of a severe telomerase defect alone. In fact, most, although not all, of the HHS patients have very short telomeres at the time of diagnosis.[215,291] Recently biallelic mutations in *RTEL1* have been identified as responsible for a proportion of patients with HHS with short telomeres.[250,251]

Revesz Syndrome. The association of progressive bilateral exudative retinopathy (Coats retinopathy), intrauterine growth retardation, fine sparse hair, fine reticulate skin pigmentation, ataxia secondary to cerebellar hypoplasia, cerebral calcifications, extensor hypertonia, and progressive psychomotor retardation with progressive BMF and death in early childhood is also known as Revesz syndrome (MIM 268130), another severe form of DC.[297] Similar to patients with HHS, individuals with Revesz syndrome have very short telomeres. *TINF2* exon 6 mutations have been identified in patients with Revesz syndrome.[211,212]

Ataxia-Pancytopenia Syndrome. Ataxia-pancytopenia syndrome is a BMF syndrome originally described with several similarities to severe forms of DC. In patients with this syndrome, BMF is associated with severe cerebellar hypoplasia and chromosomal instability without an increase after DEB or MMC testing.[298] MDS and MDS/AML with monosomy 7 are often associated with this syndrome.[298,299] A *TINF2* mutation and short telomeres were identified in one patient, suggesting that the ataxia-pancytopenia syndrome patients have severe DC, HHS, or Revez syndrome.[300]

Coats Plus Syndrome. Coats plus syndrome (MIM 612199), or cerebroretinal microangiopathy with calcifications and cysts, is caused by compound heterozygous mutations in *CTC1*. Many features of these related disorders overlap with those of DC. These features include hair, skin, and nail changes; anemia; thrombocytopenia; osteopenia; intracranial calcifications; exudative retinopathy; and developmental delay. Telomere length in individuals with *CTC1* mutations are more variable.[251]

Poikiloderma with Neutropenia. Poikiloderma with neutropenia (Clericuzio-type poikiloderma with neutropenia, MIM 604173) is a rare AR disease characterized by early-onset poikiloderma, pachyonychia, palmoplantar hyperkeratosis, skeletal defects, and noncyclic neutropenia.[301] The disease is caused by mutations n the *USB1* gene (alias C16orf57 and MPN1)[302,303] and shares some of the clinical characteristics with DC, including abnormal skin pigmentation (poikiloderma, seen in all of the patients), nail dystrophy, and neutropenia and sometimes other characteristics such as leukoplakia, epiphora, short stature, and pulmonary problems.[302] *USB1* encodes a protein that is essential for the processing and stability of U6 snRNA, a molecule with a crucial role in RNA splicing; whether USB1 also plays a role in telomere biology remains to be determined. Patients with poikiloderma with neutropenia have normal telomere lengths in their circulating blood cells.[302] It is therefore controversial as to whether poikiloderma with neutropenia should be included in the group of patients classified as having DC or rather represents a distinct disease category. Patients with poikiloderma with neutropenia have a predisposition to developing MDS and squamous cell carcinoma of the skin.[304]

Animal Models. Dyskerin is highly conserved throughout evolution in its role in rRNA modification and processing but only interacts with telomerase in vertebrates. Thus yeast mutants of Cbf5 show defects in rRNA processing, ribosome biogenesis, and growth; null mutations are lethal.[221] The dyskerin orthologue in *Drosophila* is Nop60B, also known as minifly, and null mutations are again lethal, while partial loss of functional mutations cause pleiotropic effects, including extreme reduction in body size, developmental delay, and reduced female fertility.[219,305,306] These effects have similarities to minute mutants in *Drosophila* , which are caused by ribosomal protein mutations.[307] They are also similar to features of DC, strengthening the idea that ribosomal defects may contribute to the spectrum of abnormalities in DC. In zebrafish, knockdown or mutagenesis of dyskerin or other H/ACA snRNP proteins (GAR1 or NOP10) leads to defects in ribosome biogenesis and p53-dependent inhibition of hematopoiesis without affecting telomerase activity, making extrapolation to the human disease difficult.[308,309]

In mice, models of X-linked DC have been hampered by the fact that laboratory mice have very long telomeres that do not become critically shortened in the presence of a compromised dyskerin protein. Nevertheless mutations that are copies of human *DKC1* mutations do cause increased DNA damage and defects in hematopoietic stem cell repopulation that are partially corrected by treatment with the antioxidant N-acetyl cysteine.[261,310] The most promising mouse models have involved deficiency of telomerase itself or defects in components of shelterin. Laboratory mice can thrive without either TERT or TERC, but after several generations of breeding, telomeres shorten and eventually mice show many of the features of DC, notably degenerative effects in tissues with high turnover such as hematopoietic cells, testicular cells, and cells of the skin and gastrointestinal tract.[311,312] In wild CAST/EiJ mice, which have shorter telomeres, haploinsufficiency for *Tert* and *Terc* genes causes DC-like features in early generations.[313-315] Defects similar to those in DC are also seen in mice lacking shelterin component Pot1b in combination with reduced telomerase activity. These mice show rapid telomere shortening, skin and nail defects, and fatal BMF.[316,317] Pot1b is a variant form of POT1 found in mice but not humans. These findings illustrate that the mouse can faithfully model telomere diseases and demonstrate the importance of dysfunctional telomeres in DC pathogenesis.

iPS Cells. iPS cells are pluripotent cells derived from differentiated cells such as fibroblasts by reprogramming with a cocktail of transcription factors.[130,318] They have been heralded as having the potential for tissue renewal and regeneration and are immediately useful as models for inherited diseases as they carry the patient's genome and can be cultured to undergo differentiation into many mature cell types. iPS cells have been isolated from DC patients with disease caused by *DKC1*, *TERC*, *hTERT,* and *TCAB1* mutations.[209,210] The efficiency of reprogramming was low compared with that found in other diseases, perhaps reflecting the fact that stem cell defects are one of the cardinal features of DC. In contrast to other cell lines obtained from patients with DC, iPS cell lines express telomerase activity and therefore allow the study of telomere dynamics in the disease that cannot be studied otherwise. Whether undifferentiated iPS cell lines derived in DC are able to maintain their telomeres over time is controversial and awaits further studies. Nevertheless iPS cells from DC patients are promising, powerful, and unique tools for studying human telomere biology and the molecular pathogenesis of DC. However, there is likely a long path before iPS cell technology fulfills its potential in tissue regenerative therapeutic approaches, the goal that makes this technology so attractive in BM research.[319]

Mechanism of Disease Pathogenesis in Dyskeratosis Congenita

Dysfunctional telomeres are the likely central theme in the pathogenesis of DC. Figure 7-11 summarizes our current model of the pathogenesis of disease in DC.

Clinical Management

Most of what we know regarding therapy and prognosis comes from our experience with patients with classic DC. Care must be taken when extrapolating such experience to all DC patients. The prognosis for patients with classic or severe DC is usually poor. In males with X-linked and sporadic DC, the median age at death was 20 years.[27] Patients with AD DC have milder disease with a better survival rate. Deaths were usually due to complications from aplastic anemia, BMT, pulmonary fibrosis, or cancer.[41]

Only identification of a pathogenetic mutation can confirm the diagnosis. Once confirmed, the family should be referred to a clinical geneticist familiar with new developments in DC for counseling and careful examination of family members. Genetic testing and possibly telomere measurement should be offered to all family members. Early diagnosis is important for correct management of hematologic complications; appropriate treatment of associated pulmonary, gastrointestinal, ocular, dental, and bone disease; identification of affected presymptomatic family members and unaffected family members or pregnancies that may serve as potential hematopoietic stem cell donors; and targeted cancer surveillance, not only in the patient with DC but also in mutation carriers.

Unfortunately, there is currently no consensus on how to manage patients with DC. Management depends on the age at diagnosis, the presence or absence of clinical signs, the gene affected, the molecular characteristic of the mutation identified, and possibly the telomere length. All patients with DC and hematologic manifestations should have a full hematologic assessment, including examination of bone marrow, and in anticipation of possible HSCT, HLA typing should be performed. Other investigations at diagnosis should include pulmonary evaluation and examination of the oropharynx and anal region; in females, cytologic examination of the vagina and cervical epithelium is needed. The prognosis and life expectancy of family members who have inherited only the short telomeres but not the gene mutation are unknown. Whether these family members should be excluded as potential sibling donors of an affected family member is also unknown.

Management of Hematologic Disease

Treatment of BMF in patients with classic DC is similar to that in patients with FA. Initially, in the absence of hematologic abnormalities or clinical and laboratory signs of mild BMF, monitoring of peripheral blood values might be sufficient. In the presence of hematologic abnormalities, yearly bone marrow examination should include a trephine biopsy and marrow aspiration for the examination of marrow cellularity and morphologic changes characteristic of MDS, as well as for cytogenetic examination by G banding and FISH analysis; testing should include probes that identify the most frequent cytogenetic abnormalities seen in patients with DC and progression to MDS or AML, or both (see earlier also). Plans for possible HSCT should be in place because hematologic complications may develop rapidly. With progression of BMF it will become necessary to increase the frequency of hematologic monitoring, and it may have to be individualized. The anabolic steroid oxymetholone can produce improvement in hematopoietic function. Approximately two thirds of patients with DC will respond to oxymetholone; in some cases this response can last several years and involve all lineages. Patients with DC may respond to a dose as low as 0.25 mg of oxymetholone/kg/day, which can be increased, if necessary, to 2 to 5 mg/kg/day.[320,321] Hematopoietic growth factors usually, if at all, have only a transient effect and should not be used in combination with androgens because of the risk of peliosis and splenic rupture.[320,321] Unlike idiopathic aplastic anemia, immunosuppressive agents such as antithymocyte globulin and cyclosporine are ineffective or only have a transient response in cases of DC and BMF.

The ultimate treatment of BMF is allogeneic HSCT. However, HSCT for DC has been associated with high morbidity and mortality rates, more so than in other BMF syndromes. Pulmonary and vascular complications are the major causes of death associated with HSCT in patients with DC.[176,322-324] Reduced-intensity nonmyeloablative fludarabine-based conditioning regimens with no or minimal irradiation are associated with lower toxicity and fewer complications.[325-328] Long-term survival and late complication rates after HSCT in patients with DC remain to be determined.

Management of DC patients with MDS or AML (or both) is even less standardized. The significance of transient clonal cytogenetic abnormalities is unclear, but if persistent or complex, such abnormalities usually predict a poor prognosis. Success is unlikely with early hematopoietic stem cell harvest for autologous transplantation at a later time because of the limited repopulating ability of the affected stem cells.

Management of Pulmonary Disease

There is currently no treatment of pulmonary disease in DC. Cigarette smoke is known to accelerate disease onset and severity. Thus patients with DC should refrain from smoking, avoid drugs with pulmonary toxicity (e.g., bleomycin), and have their lungs shielded from irradiation during BMT or cancer treatment. Pulmonary fibrosis in patients with DC does not respond to immunosuppressive therapy. Lung transplantation might be considered in individuals with otherwise mild manifestations of disease.[329]

Cancer Surveillance and Treatment

Patients with confirmed DC should avoid excessive exposure to ultraviolet light and use barrier sun creams. Avoidance of smoking and alcohol should be emphasized, particularly in patients with oral leukoplakia.[170,330] DC patients should also undergo regular dental and hygienist review to prevent early tooth loss and reduce the occurrence of leukoplakia. It is important to screen for malignant disease, especially of the gastrointestinal system. Management of malignant disease may be complicated by a number of factors. The diagnosis may be delayed in some cases because of malignant change occurring within an area of persistent leukoplakia, resulting in a delay in treatment. There is also a high potential for local recurrence and multiple cancers in these patients. In addition, the presence of BMF makes chemotherapy very difficult and can limit the indications for surgery. The mucosa also has high sensitivity to irradiation, which can lead to severe mucosal damage and may result in early termination of radiotherapy.

Management of oral malignant disease usually includes a regular review and biopsy of the premalignant hyperkeratotic lesion and surgical laser excision of any suspicious areas. Neoadjuvant radiotherapy or surgical resection of confirmed malignant tumors, along with ipsilateral node clearance with or without adjuvant radiotherapy in accordance with staging of the disease, is the mainstay of treatment.

Gene Therapy

HSCT may improve or cure hematologic disease in DC patients, but HLA-matched sibling donor cells are available for only a minority of patients; furthermore, immune reconstitution is often delayed. Gene therapy to correct hematopoietic stem cells followed by autologous transplantation might offer an attractive alternative for the treatment of hematologic disease in DC patients. Biased X-inactivation in female *DKC1* mutation carriers suggests that DC cells corrected by gene therapy are likely to have a selective growth and survival advantage. Indeed, gene transfer of *TERT* or *TERC* (or both) corrects the telomere defect in most DC cell lines.[331] However, as in many other IBMFSs, the few surviving hematopoietic stem cells might have already acquired secondary somatic and possibly oncogenic mutations, so correction by gene therapy could promote rather than prevent leukemogenesis in these patients. Interestingly, in rare patients with DC due to *TERC* gene mutations, gene reversion by mitotic recombination in hematopoietic stem cells leads to two normal copies of the *TERC* genes in the majority of circulating blood cells.[332] Revertant cells contribute to the myeloid and B lymphocyte lineages but were not detectable in T lymphocytes, suggesting that mitotic recombination occurred in a stem cell committed to myeloid and B-cell differentiation or that the selective advantage for gene revertant cells is less in T cells than in myeloid and B cells. Indeed, T cells are less severely affected by DC mutations (see also above) and have a longer survival time in circulation. Revertant mosaicism in DC seems to be less frequent than reported in FA and currently has only been described in DC patients with *TERC* gene mutation.[332] However, as in FA, DC revertant mosaicism might mask an inherited *TERC* gene mutation when genetic testing is performed in peripheral blood cells. DNA from skin fibroblasts or buccal swabs might have to be sent for genetic testing in patients with classic DC features but no BMF. Revertant mosaicism in patients with DC but no BMF powerfully illustrates the feasibility of gene therapy and shows that the restoration of a normal *TERC* gene in a single hematopoietic stem cell in these patients can restore blood cell production.

Shwachman-Diamond Syndrome

Shwachman-Diamond syndrome (SDS, MIM 260400) is a rare, recessive multisystem disorder characterized by exocrine pancreatic insufficiency, bone marrow dysfunction, and skeletal abnormalities, first described in the 1960s (Box 7-12).[333,334]

Clinical Manifestations

SDS is a IBMFS with an estimated incidence of 1 per 75,000 live births.[335] SDS is more commonly diagnosed in boys, with a male-to-female ratio of 1:7.[336] The major

Box 7-12 **Shwachman-Diamond Syndrome**

Shwachman-Diamond syndrome (MIM #260400) is a rare, recessively inherited bone marrow failure syndrome characterized by exocrine pancreatic insufficiency, bone marrow dysfunction (including increased risk for malignant transformation), and skeletal abnormalities.

TABLE 7-7 Major Clinical Features of Shwachman-Diamond Syndrome

Clinical Feature	Aggett (1980)[337]	Mack (1996)[341,†]	Smith (1996)[342]	Ginzberg (1999)[336]	Cipolli (1999)[338]	Kuijpers (2005)[340,‡]	Hashmi (2011)[339,§]	Total/ Average
Number of subjects	21	25	21	88	13	23	34	225
Hematologic*								
Neutropenia	95%	88%	100%	98%	77%	78%	74%	90%
Severe (≤500/μL)	67%	60%	33%		23%	48%	38%	46%
Anemia	50%	52%	66%	42%	46%	17%	58%	46%
Thrombocytopenia	70%	60%	24%	34%	23%	56%	38%	42%
Pancytopenia		44%	10%	19%	23%	13%		21%
Gastrointestinal								
Exocrine pancreatic insufficiency*	100%	100%	100%	100%	100%	100%	85%	98%
Liver (elevated transaminases)		56%		60%	100%	69%	45%	61%
Skeletal abnormalities	100%	76%	100%	49%	100%	65%	73%	70%
Metaphyseal dysostosis	62%	44%	100%	46%	54%		41%	53%
Rib cage abnormalities	52%	32%		32%	92%		12%	35%
Short stature (<3rd percentile)	95%	67%		56%	85%	52%	76%	66%

[*] Hematologic abnormalities and exocrine pancreatic insufficiency are defining features of SDS, hence the 100% incidence of these findings.
[†] Short stature was defined as below the 5th percentile for height.
[‡] Severe neutropenia is less than 600/μL. Fifteen of 20 patients (75%) tested had *SBDS* mutations.
[§] Twenty-five of 31 patients (81%) had *SBDS* mutations.

clinical features associated with SDS have been defined in several large cohort studies; they are summarized in Table 7-7.[336-339,340-342] The two most recent studies included genotyping, which showed that approximately 80% of patients in these studies with clinically defined SDS had biallelic mutations of *SBDS*.[339,340] Whether patients without *SBDS* mutations have distinct clinical features is unclear, though one study suggested that patients with wild-type *SBDS* may have more severe hematopoietic disease but milder pancreatic dysfunction.[339]

Hematologic Abnormalities

Neutropenia is present in the majority of patients with SDS (range, 74% to 100%); severe neutropenia (as defined by an absolute neutrophil count [ANC] of less than 500/μL) was observed in a mean of 46% of patients (range, 23% to 67%; see Table 7-7). Neutropenia is intermittent in about two thirds of patients and chronic in the remaining one third. Although defects in neutrophil chemotaxis are present in most patients with SDS,[343] the clinical significance of this finding is unclear, since neutrophils are recruited to sites of infection.[344] Anemia is present in approximately 50% of cases and is usually associated with a low reticulocyte count and mild macrocytosis.[339] Thrombocytopenia is present in approximately 50% of cases, sometimes without concomitant neutropenia. Pancytopenia has been reported in 10% to 44% of patients. Presentation with or progression to severe aplastic anemia has been reported.[345-347] Defects in B-lymphocyte and/or T-lymphocyte function have been described, including impaired specific immunoglobulin response and altered CD4 or CD8 T-cell subsets.[348] Bone marrow findings are variable and nondiagnostic. Although bone marrow hypoplasia is common, normal or even increased cellularity has been reported.[336,337,339,342] Left-shifted granulopoiesis is common. Mild dysplastic changes are commonly seen and may fluctuate, but prominent myelodysplasia is uncommon and may suggest malignant transformation.

Transformation to MDS and AML

Similar to other congenital BMF syndromes, there is a strong predilection to develop MDS or MDS/AML in patients with SDS. Acquired clonal cytogenetic abnormalities are frequently seen; in a prospective study of 14 patients with SDS followed for a maximum of 5 years with annual bone marrow evaluations, clonal cytogenetic abnormalities were detected in 29% of patients.[349] Abnormalities of chromosome 7 are the most common and include monosomy 7, isochromosome 7q, and deletion of 7q. The clinical significance of these cytogenetic abnormalities is uncertain. In particular, isochromosome 7q appears to have a benign course with a low risk of transformation to MDS or MDS/AML.[349-351] Likewise, the loss of the long arm of chromosome 20 [del(20)(q11)], which is common in patients with SDS as an isolated chromosomal anomaly, is not associated with progression to MDS.[352,353] The reported rate of transformation to MDS/AML for patients with SDS is highly variable. One of the difficulties is the definition of early MDS in the presence of clonal hematopoiesis. In an observational study of 29 patients with SDS enrolled in the North American Severe Chronic Neutropenia Registry, two cases (7%) of MDS/AML were reported.[354] In contrast, no cases of MDS/AML were reported in 17 patients with SDS (representing 274 person-years of observation) in the National Cancer Institute IBMFS cohort study.[41] Finally, a cumulative incidence of MDS/AML of approximately 10% at 20 years of age was reported in a French study of 102 patients with SDS.[345] In this study, the severity of cytopenias at presentation correlated with risk of transformation. Of interest, in contrast to a prior report,[355] no increased male gender bias with respect to risk of MDS/AML transformation was observed in this study. The malignant clone

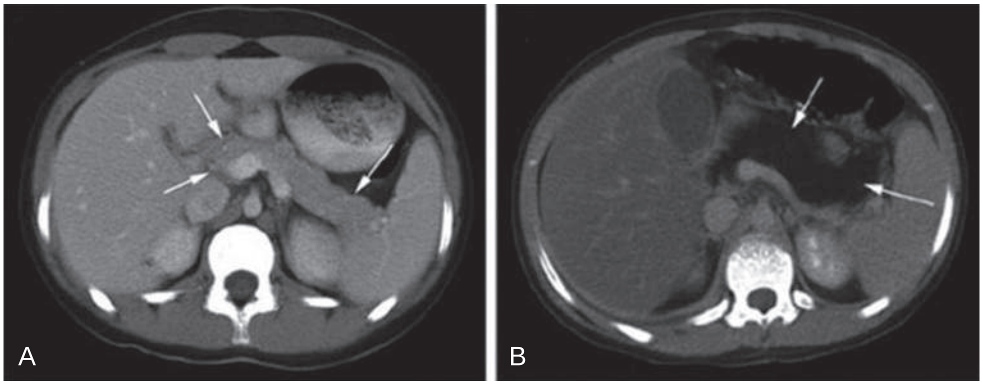


Figure 7-15 Fatty infiltration of the pancreas in Shwachman-Diamond syndrome shown by transverse computed tomography images of a normal individual (**A**) and a patient with Schwachman-Diamond syndrome (**B**). The density of the normal pancreas (arrows) is similar to that of the liver. In contrast, the density of the pancreas in the Schwachman-Diamond syndrome patient (arrows) is reduced, consistent with fatty infiltration. *(From Nijs E, Callahan MJ, Taylor GA: Disorders of the pediatric pancreas: imaging features.* Pediatr Radiol *35:358–373, 2005.)*

often displays complex cytogenetic abnormalities frequently involving chromosome 7.

Gastrointestinal Abnormalities

After cystic fibrosis, SDS is the most common cause of congenital exocrine pancreatic insufficiency. Patients classically present in early infancy with steatorrhea and failure to thrive. Pathologic analysis shows extensive fatty replacement of pancreatic acini with relative preservation of pancreatic ducts and islets of Langerhans.[337] Exocrine pancreas insufficiency can be assessed by measurement of serum trypsinogen, serum isoamylase, or fecal fat.[356] In addition, imaging of the pancreas may reveal characteristic fatty infiltration[357,358] (Fig. 7-15). For unclear reasons, the pancreatic insufficiency improves with increasing age in many patients; by 4 years of age, nearly 50% of patients no longer require pancreatic enzyme supplements.[341] Mild (2- to 3-fold) elevation of liver transaminases is often seen in patients younger than 2 years of age. It is usually of little clinical significance and tends to improve with age.[341]

Skeletal Abnormalities

Most patients with SDS have bony abnormalities, with rib cage abnormalities, metaphyseal dysostosis, and osteopenia being the most common features (see Table 7-7).[359,360] A recent report suggested that impaired osteoclast formation may contribute to the skeletal abnormalities in SDS.[361] Growth retardation also is common in SDS patients, but it is not thought to be secondary to malnutrition. Over half of patients with SDS are below the 3rd percentile for height at 1 year.[336,362] With adequate pancreatic enzyme replacement, most patients show a normal growth velocity but remain below or at the 3rd percentile for height.[336]

Miscellaneous Abnormalities

Many patients with SDS suffer from neurocognitive deficits and pyschosocial dysfunction.[363-365] Dental abnormalities are common.[366,367] While there are reports of endocrine abnormalities such as diabetes mellitus[337,341] and growth hormone deficiency in SDS,[368] a recent comprehensive analysis of 43 patients with SDS showed no consistent endocrine phenotype.[362] Cardiomyopathy has been reported in patients with SDS.[369] In a recent study of 105 patients with SDS (mostly with *SBDS* mutations), the incidence of cardiomyopathy was 6%.[370] An increase in other congenital heart disorders also was observed in this study.

Differential Diagnosis

Diagnostic criteria for SDS are outlined in Box 7-13[371] both exocrine pancreatic insufficiency and hematopoietic abnormality must be documented. Other features of SDS, including short stature or skeletal or hepatic

Box 7-13 **Clinical Diagnostic Criteria for Shwachman-Diamond Syndrome**

HEMATOPOIETIC ABNORMALITIES (at least one is required)
Neutropenia (<1500/µL); can be intermittent or chronic but must be documented on at least two occasions over at least 3 months
Anemia or thrombocytopenia below that for the age-adjusted norm documented on at least two occasions over at least 3 months
Tests that support the diagnosis but require corroboration:
Persistent elevation of hemoglobin F (on at least two occasions over at least 3 months)
Persistent red blood cell macrocytosis (on at least two occasions over at least 3 months), not resulting from other causes such as hemolysis or nutritional deficiency
EXOCRINE PANCREATIC INSUFFICIENCY* (one is required)
Reduced levels of pancreatic enzymes adjusted to age (serum trypsinogen, serum isoamylase, or fecal elastase)
Test that support the diagnosis but require corroboration:
Abnormal 72-hour fecal fate analysis
Reduced levels of at least two fat-soluble vitamins (A, D, E, or K)
Evidence of pancreatic lipomatosis by ultrasound, computed tomography scanning, or magnetic resonance imaging
*Other causes of pancreatic insufficiency should be excluded
ADDITIONAL SUPPORTIVE EVIDENCE OF Schwachman-Diamond syndrome
Bone abnormalities
Behavioral problems
Presence of a first-degree relative with Schwachman-Diamond syndrome

abnormalities, while supportive, are not required for the diagnosis. Genetic testing for *SBDS* mutations is merited because their presence confirms the diagnosis. However, their lack does not exclude the diagnosis, because in 10% to 20% of cases of clinically defined SDS, no mutations of *SBDS* are present.[339,340,372,373] Other causes of exocrine pancreatic insufficiency should be considered, including cystic fibrosis, which can be excluded by performing a sweat test. *Pearson syndrome* (PS, MIM 557000) is characterized by exocrine pancreatic insufficiency, sideroblastic anemia, vacuolization of hematopoietic cells in the bone marrow, and metabolic acidosis.[374,375] It is caused by deletions in mitochondrial DNA.[376] *Cartilage-hair hypoplasia* (CHH, MIM 250250) is characterized by short stature, metaphyseal dysostosis, cytopenias, and hypoplastic hair.[377] It is secondary to mutations of *RMRP* encoding the RNA subunit of the RNase mitochondrial RNA-processing (RMRP) complex.[378]

Genetic Characteristics

SDS is an AR disorder.[379] The gene responsible for SDS was identified by genetic linkage analysis.[372] Boocock and colleagues[372] reported that compound heterozygous mutations of the *SBDS* (Shwachman-Bodian-Diamond syndrome) gene located on chromosome 7 were present in the majority of patients with SDS. Most of these mutations resulted from gene conversion with a neighboring pseudogene (*SBDSP*). Subsequent studies have confirmed that *SBDS* mutations are present in 80% to 90% of clinically diagnosed cases of SDS.[339,340,345,373,380,381] The majority of mutations occur within exon 2 and are predicted to result in the production of a truncated protein. The genotypes p.Lys62X/Cys84fs and p.Lys62X;Cys84fs/Cys84fs are found in 60% and 6% of the patients, respectively. The remaining mutations are missense and nonsense mutations scattered throughout the protein. The majority of mutations result in a dramatic reduction in SBDS protein production.[345,372,373,382] However, patients carrying homozygous null alleles for *SBDS* have not been reported, suggesting that SBDS is essential for life. Consistent with this conclusion, mice lacking the murine orthologue of *SBDS* die in the early embryonic stage.[383] Interestingly, in the subset of patients who do not have *SBDS* mutations, SBDS protein expression in leukocytes is normal, indicating that other genetic mutations may be responsible for these cases of SDS.[373]

Recent studies suggest that there may be a spectrum of disease associated with *SBDS* mutations. Nishimura and colleagues[384] reported that two unrelated patients with neonatal spondylometaphyseal dysplasia carried compound heterozygous mutations of *SBDS*. Clinical features included severe metaphyseal dysplasia, platyspondyly, and neonatal respiratory failure, resembling spondylometaphyseal dysplasia of the Sedaghantian type. Likewise, *SBDS* mutations are present in a subset of patients diagnosed with asphyxiating thoracic dystrophy (Jeune syndrome).[385] Thus it is important to consider the diagnosis of SDS in newborns presenting with thoracic dystrophy.

There also is evidence that haploinsufficiency for *SBDS* may predispose to the development of aplastic anemia. Calado and colleagues[386] reported that 4 of 91 patients with apparently acquired aplastic anemia had heterozygous mutations of *SBDS*. Interestingly, affected patients had no evidence of exocrine pancreatic insufficiency or bony abnormalities but did have short stature. However, no *SBDS* mutations were found in another study of 96 Japanese children with aplastic anemia.[387] Similarly, parents and siblings of patients with SDS are not known to have an increased risk of BMF, although no controlled studies have been performed.

The *SBDS* gene encodes for a protein of 250 amino acids that is highly conserved throughout evolution. The primary function of SBDS appears to be to regulate ribosome biogenesis. Consistent with this observation, SBDS protein prominently localizes to the nucleolus of cells in a cell cycle–dependent fashion.[388] Current studies support a model in which SBDS cooperates with the GTPase elongation factor–like-1 (ELFL1) to facilitate final stages in the translational activation of ribosomes.[389-391,392] Specifically, SBDS and ELFL1 catalyze the removal of eukaryotic initiation factor 6 (eiF6) from the 60S ribosome subunit, allowing for its assembly into an actively translating 80S subunit.[389-391] Together, these observations place SDS in a growing list of bone marrow disorders caused by impaired ribosome biogenesis. SBDS has also been implicated in non–ribosome related functions that may contribute to disease phenotype. For example, SBDS stabilizes the mitotic spindle,[393] and loss of SBDS in hematopoietic progenitors in patients with SDS may contribute to the high rate of chromosomal abnormalities in SDS.

The mechanism for the increased susceptibility to MDS and MDS/AML in SDS is unclear. There is evidence that *SBDS* mutations disrupt hematopoiesis through both cell intrinsic and non–cell intrinsic mechanisms. Hematopoietic colony formation is reduced in the bone marrow of patients with SDS,[394] and inhibition of *SBDS* expression in primary hematopoietic progenitors results in impaired hematopoietic stem cell function[395] and erythroid differentiation.[396] These data are supported by the observation that hematopoietic cells with an increased expression of the SBDS protein as it occurs with i(7)(q10)[397] or with copy number–neutral loss of heterozygosity of 7q[398] outgrow SBDS hematopoiesis, explaining the benign nature of these specific chromosomal anomalies in patients with SDS. Interestingly, loss of 20q results in deletion of the *EIF6* gene whose product competes with SBDS.[399] On the other hand, bone marrow stromal cells from patients with SDS have reduced capacity to support hematopoietic cell growth.[394] Intriguingly, loss of SBDS in bone marrow stromal cells produces an MDS-like phenotype in mice.[400] Thus both altered hematopoietic progenitor and bone marrow stromal function may contribute to the increased rate of transformation to MDS/AML.

Clinical Management

The management of patients with SDS was summarized recently in an updated clinical consensus guideline.[371] Treatment of SDS includes pancreatic enzyme replacement and fat-soluble vitamins for pancreatic insufficiency. Regular assessments of exocrine pancreatic function are recommended because in approximately 50% of patients there is spontaneous resolution of steatorrhea with increasing age.[341] Blood levels of fat-soluble vitamins should be checked every 6 to 12 months.

Regular blood counts should be measured every 3 to 6 months to monitor for cytopenias. The role of surveillance bone marrow biopsies is controversial. Whereas i(7q) has questionable prognostic significance, other chromosomal abnormalities, in particular monosomy 7, are associated with poor outcomes and may be an indication for HSCT. Current guidelines suggest annual bone marrow biopsies for patients treated with G-CSF and biopsies every 1 to 3 years for patients with stable blood counts who are not receiving G-CSF.[371] For patients with severe neutropenia or neutropenia and persistent or recurrent infections, a trial of G-CSF treatment is warranted; response rates are generally high.[401] Similar to severe congenital neutropenia, current recommendations are that the dose of G-CSF should be titrated to achieve and maintain an ANC of 1000 to 1500/μL. As discussed in the section on severe congenital neutropenia, the association of G-CSF with malignant transformation is controversial and has not has been demonstrated to date. Nonetheless, patients with SDS should receive the lowest dose of G-CSF required to maintain an acceptable neutrophil count.

Allogeneic HSCT is the only curative therapy for SDS. It is generally reserved for patients with severe cytopenias, MDS with excess blasts, or frank leukemia. Only small case series of HSCT in patients with SDS are available. HSCT in patients with SDS is associated with increased regimen-related toxicity, in particular of the heart, lung, and liver. The pathobiology of this increased toxicity is not understood, although impaired cardiac function is present in some patients with SDS.[402] The French neutropenia registry reported a 5-year event-free survival rate of 60% in 10 patients with SDS following HSCT when transplanted before transformation to MDS or MDS/AML.[403] Similarly, the European Group for blood and bone marrow transplantation reported a 64.5% overall survival rate with a median follow-up of 1.1 years in 25 patients with SDS following allogeneic HSCT.[404] From these studies, it is clear that patients transplanted for BMF do much better than those transplanted for MDS or AML. Combining these studies, the overall survival rate for patients transplanted for BMF was 86% (18 of 21) compared with 36% (5 of 14) for those transplanted for MDS/AML. Although the number of patients is small, two studies reported excellent results using reduced-intensity conditioning regimens for HSCT in SDS.[405,406]

Cartilage-Hair Hypoplasia

Cartilage-hair hypoplasia (CHH, MIM 250250), also known as *metaphyseal chondrodysplasia, McKusick type,* is an AR chondrodysplasia.[407]

Clinical Manifestations

CHH is characterized by metaphyseal dysostosis, short-limbed dwarfism, and fine, sparse hair that lacks the central pigment core. Clinical manifestations are highly heterogeneous and vary between and within families. Other skeletal findings include chest deformity, varus lower limbs, lordosis, and scoliosis. Gastrointestinal problems include Hirschsprung disease and other physical anomalies. The disease is more frequent in the Amish and Finnish populations because of a founder effect, with a carrier frequency of 1 in 19 among the Pennsylvania Amish (incidence 1.5 in 1000) and 1 in 76 (incidence 1 in 23,000) among Finns.[377] Mild to severe macrocytic anemia and lymphopenia are present in the majority of patients, whereas neutropenia is seen in only about a fourth. In the majority of individuals the anemia is mild and resolves spontaneously during early childhood. In more severe cases, the anemia resembles Diamond-Blackfan anemia (DBA) or may be accompanied by severe combined immunodeficiency. The severity of the anemia inversely correlates with height. Similar severe combined immunodeficiency is associated with more severe and persistent anemia. Immunodeficiency in CHH is variable. More than 30% of patients have significant CD4 and CD8 lymphopenia, abnormal lymphocyte proliferation upon mitogen stimulation, and impaired humoral immunity with efficiencies in IgG and IgA.[408] Immune dysregulation is thought to be the cause of autoimmune diseases in patients with CHH. The frequency of lymphoid malignant diseases, in particular non-Hodgkin lymphoma, and of basal cell carcinoma is increased about 7-fold. Extended follow-up of persons with CHH revealed that approximately 11% develop malignant disease in adolescence and as young adults. The prognosis of these patients after development of malignant disease is poor.[130,409,410]

Differential Diagnosis

Excluding DBA from the differential diagnosis might be difficult in some cases. Thrombocytosis, increased erythropoietin levels, and increased levels of red cell adenosine deaminase (ADA), characteristic of DBA (see later), may be present in some patients with CHH. Metaphyseal dysplasia has not been reported in patients with DBA. Mutation analysis may be necessary to consolidate the diagnosis.

Milder forms of *Schimke immunoosseous skeletal dysplasia* (MIM 242900) may be difficult to distinguish from CHH. Schimke immunoosseous skeletal dysplasia is characterized by disproportionate short stature, nephropathy, and T-cell deficiency. The disease is an AR disorder caused by mutations in *SMARCAL1*.

Combined immunodeficiency syndromes, in contrast to CHH, are not associated with skeletal abnormalities. *Omenn syndrome* includes short stature and hematologic and immunologic changes; however, Omenn syndrome (MIM 603554) caused by *RAG1* or *RAG2* mutations is more severe and includes ichthyosiform skin.

Congenital neutropenias (see later) caused by mutations in *ELANE*, *HAX1*, *GFI1*, or *WAS* are usually not associated with a skeletal phenotype or have additional systemic manifestations that should distinguish them from CHH.

Genetic Characteristics

CHH is an AR disease. Genetic mapping and DNA sequencing identified the gene encoding the RNA component of *RMRP*, a 265-nucleotide, noncoding RNA gene on 9p21-p12.[378] Mutations responsible for CHH are predominantly found in the transcribed and promoter regions of the *RMRP* gene.[411] The most frequently found mutation is the A70G transversion, which is the major mutation in Finnish and Amish CHH patients. Other mutations include insertional mutations and duplications between the TATA box and the transcription start site that silence transcription. No patient homozygous or compound heterozygous for the promoter region duplications or insertions has been identified, suggesting that these mutations may be lethal. *RMRP* mutations have also been found in a variety of other forms of inherited metaphyseal dysplasia that share features with CHH, including metaphyseal dysplasia without hypotrichosis (MIM 250460), anauxetic dysplasia (MIM 607095), and kyphomelic dysplasia (MIM 211350).[410,412,413]

Gene Function

MRPR is the RNA component of the RNP complex RMRP that is involved in several cellular processes, including mtDNA replication, ribosome biogenesis and pre-rRNA processing, and mRNA degradation of cyclin B2 (*CLB2*), thereby regulating cell cycle progression.[413] MRPR has been described to complex with TERC and possibly is involved in siRNA synthesis; however, these data need further confirmation.[414] Which of these pathways is responsible for BMF is unclear; however, differences and similarities to other ribosomal diseases suggest that the defects in ribosome biogenesis might be responsible for the anemia and that degradation of CLB2 contributes to the increased development of lymphoid tumors. Genotype-phenotype correlation studies suggest a strong correlation between the decrease in rRNA cleavage during ribosomal assembly and the degree of bone dysplasia, whereas reduced mRNA cleavage and cell cycle impairment correlate with the presence of hair hypoplasia, immunodeficiency, hematologic abnormalities, and cancer risk.[412] The clinical manifestations may also be the result of failure of translation of a number of normal genes, as seems to be the case in DBA (see below).

Clinical Management

Skeletal dysplasias might require corrective osteotomies later in childhood or adolescence. Low bone density may complicate orthopedic surgery. Severe anemia requiring transfusion is seen rarely in CHH (6%) and portends a poor prognosis. Transfusions and glucocorticoid may improve the anemia. However, possible benefits of the treatment should be carefully weighed against the potential side effects in a condition associated with immune deficiency and short stature. Although many of the patients outgrow their anemia, in some cases the anemia is severe and persistent, as in patients with DBA.[409] With symptoms of varicella infection, immediate antiviral treatment must be considered to prevent complications. Prophylactic antibiotic therapy in patients with recurrent infections or immunoglobulin replacement therapy if immunoglobulin levels are low may be considered. In patients with severe combined immunodeficiency, significant autoimmune complications, or malignant disease, HSCT with or without myeloablation has been shown to provide long-term immune reconstitution and resolution of autoimmune disease; growth failure remained unaffected, however.[415,416] Thus HSCT should be considered in CHH patients with severe immunodeficiency and/or autoimmunity before the development of severe infections, major organ damage, or malignant disease affects HSCT outcome.

Pearson Syndrome

Pearson marrow-pancreas syndrome (PS, MIM 557000) is a refractory sideroblastic anemia characterized by vacuolization of bone marrow precursors as a result of deletion of mtDNA (Figs. 7-16 and 7-17). Pancreatic dysfunction is frequent but not mandatory for the diagnosis of PS.

Clinical Manifestations

PS is a multisystem disorder that usually becomes evident in early infancy.[374,417] Pancytopenia is frequently associated with dysfunction of the exocrine pancreas, hepatic failure, and renal tubulopathy, which may lead to lactic

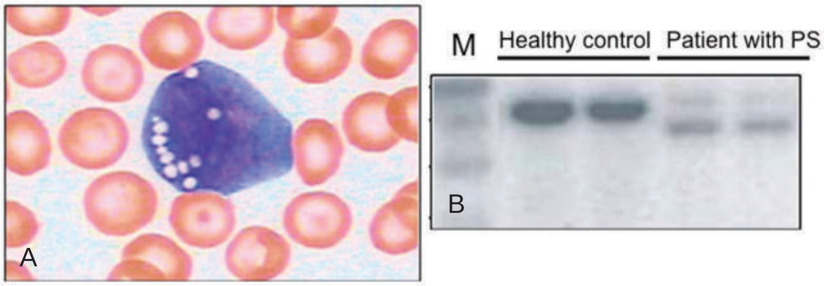


Figure 7-16 Characteristics in Pearson syndrome. A, Myeloid bone marrow precursor cell exhibiting prominent cytoplasmatic vacuolization. B, Southern blot analysis of mitochondrial DNA of a Pearson syndrome patient and a healthy control. The 16.6-kb wild-type mtDNA of the healthy control (lanes 2 and 3) is shown in comparison with the mitochondrial DNA of the patient carrying the 4503-bp deletion (lanes 4 and 5) in approximately 70% of the mitochondrial DNA molecules as quantified densitometrically. *(From Giese A, Kirschner-Schwabe R, Blumchen K, et al: Prenatal manifestation of pancytopenia in Pearson marrow-pancreas syndrome caused by a mitochondrial DNA deletion.* Am J Med Genet *143A:285–288, 2007, Figure 1A and D).*

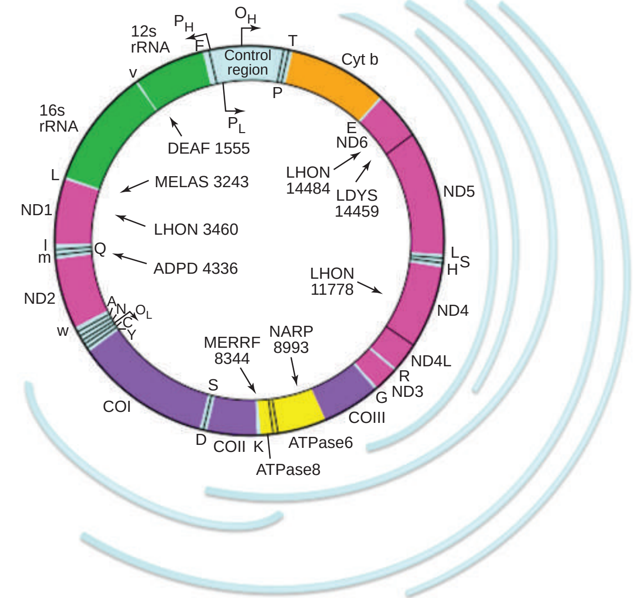


Figure 7-17 Schematic diagram of mitochondrial DNA, indicating an example of the type of deletion found in Pearson syndrome and Kearns-Sayre syndrome. Pink complex 1 genes (nicotinamide adenine dinucleotide dehydrogenase); orange complex III genes (ubiquinol: cytochrome c oxidoreductase); green ribosomal RNA genes; light blue transfer RNA genes; single letters inside are abbreviations of the 22 tRNAs; purple complex IV genes (cytochrome c oxidase); yellow complex V genes (ATP synthase); blue arches indicate the mitochondrial DNA deletions identified in patients with Pearson syndrome and Kearns-Sayre syndrome. *(Modified from* mitomap.org.*)*

acidosis. Vacuolization of hematopoietic precursor cells is characteristic (see Fig. 7-16). Most PS patients die before the age of 3 years. The major cause of death is metabolic acidosis, followed by liver and renal failure. The few surviving children showed hematologic improvement, but typical features of Kearns-Sayre syndrome (MIM 530000) eventually developed.

The male-to-female ratio of PS is 0.7 : 1. Patients from all racial and most ethnic groups have been reported.

Physical anomalies in patients with PS are rare. Approximately a third of patients have exocrine pancreatic insufficiency, and insulin-dependent diabetes develops frequently. Anemia (usually macrocytic) is present in the majority of patients in whom PS is diagnosed before the age of 6 months. Half of patients have neutropenia and thrombocytopenia. Vacuolated myeloid or erythroid precursors, often with decreased erythroblasts, and ringed sideroblasts are present in almost all patients (see Fig. 7-16).

Differential Diagnosis

Vacuolization of the hematopoietic precursor, fibrosis of the pancreas rather than fatty replacement, and the lack of skeletal anomalies distinguishes PS from SDS. PS has to be differentiated from other refractory anemias in children, in particular DBA (see later). Similar to DBA, PS may present with macrocytic anemia, increased fetal hemoglobin, and even an elevated erythrocyte ADA level. However, precursor vacuolization and sideroblasts are characteristic for PS and are not seen in DBA. Mitochondrial enzyme defects and mtDNA deletions usually establish the diagnosis.

Genetic Characteristics

PS is maternally inherited and follows a mitochondrial inheritance pattern (Box 7-14). PS is caused by deletions or duplications of mtDNA.[375,376,418,419] The size of the deletion varies from 2.7 to 7.767 kb[418-420] (see Fig. 7-17). The respiratory enzymes involved in the deletions are relevant to oxidative phosphorylation, and alterations include reduced nicotinamide adenine dinucleotide, cytochrome oxidase, adenosine triphosphatase, tRNA, and rRNA.

The severity of the clinical manifestations and the rate of disease progression are variable. The expression of disease phenotype does not depend on the size of the mtDNA deletion but rather correlates with the ratio of mutated to wild-type mtDNA (heteroplasmy) and with the tissue distribution of mutated mtDNA molecules.[420,421]

Clinical Management

Treatment is mainly symptomatic, with red cell transfusions, alkalizing medications, reduction of further oxidative stress, and carbohydrate restriction. Erythropoietin and G-CSF have been used for the treatment of BMF. The development of leukemia has been reported in one case after HSCT and aggressive immune suppression.[423] The major cause of death is metabolic acidosis, followed by renal and liver failure. The projected median survival time is 4 years, with a plateau rate of 36% at age 10 years. Interestingly, in two patients who received an unrelated HSCT for progressive pancytopenia, there was resolution not only of hematologic complications but also of metabolic acidosis and liver anomalies,[423,424] suggesting that HSCT might be a feasible option in management of PS in the early stages of the disease, before significant organ and tissue damage appear.

Genetic counseling is complicated by the unique genetic features of mtDNA that distinguish it from mendelian disorders, including maternal inheritance, heteroplasmy,

Box 7-14 **Mitochondrial DNA Disorders**

Mitochondria are under genetic control of the mitochondrial and nuclear genomes. Defects in either genome result in mitochondrial disorders. The mitochondrial genome is 16.6 kb in size and encodes 13 enzymes of the respiratory chain, as well as 24 RNA molecules needed for intramitochondrial protein synthesis. Within a cell the mitochondrial genome is present in multiple copies. In healthy individuals, all copies are identical (homoplasmy), whereas in patients with genetic defects of the mitochondrial genome, a mutation can be present in all copies (homoplasmic mutation) or only in some copies (heteroplasmic mutation). Mitochondrial DNA (mtDNA) is usually maternally inherited. For heteroplasmic disorders such as Pearson syndrome, the outcome of a specific pregnancy is difficult to predict, mainly because of considerable variation in the mutated DNA inherited by the offspring and because many of the clinical features correlate with the ratio of mutated to wild-type mtDNA.[417,422]

the threshold effect, the mitochondrial bottleneck, tissue variation, and selection (see Box 7-14).[417,422,425]

Familial Bone Marrow Dysfunction

A large group of patients with BMF do not fit any of the classifications described. If more than one family member is affected, in the absence of environmental exposure explaining BMF, these individuals are classified as having familial BMF, not otherwise specified.

Familial BMF therefore represents a heterogeneous group of individuals and families. In many, BMF is found in various family members and may include aplastic anemia, MDS, leukemia, and immunodeficiency. The inheritance pattern may suggest dominant, recessive, or X-linked disease. In others, the condition may be manifested as sporadic BMF but with associated physical anomalies suggesting an IBMFS not characteristic of FA or any other known IBMFS.

Pathogenesis

A significant number of individuals or families in whom familial BMF is diagnosed most likely have disease caused by one of the major IBMFSs discussed earlier. However, because of variable disease penetrance and expressivity, a possible atypical manifestation, failure to identify the pathogenic mutation, and unawareness of the responsible physician, the correct diagnosis has not been made. For example, BMF secondary to *TERC* or *TERT* gene mutations is certainly underdiagnosed because affected individuals often lack the mucocutaneous features characteristic of classic DC (see earlier). In others, a syndrome specific for particular clinical findings might have been diagnosed, but association with one of the major forms of BMF has not yet been made. Examples from the past are the VATERL syndrome in individuals with vertebral defects, anal atresia, tracheoesophageal fistula, renal defects, and radial limb defects, some of whom turned out to have FA (see the section on FA); HHS, which turned out to be a severe form of DC (see the section on DC); or Kearns-Sayre syndrome, which includes patients with PS who outgrew their BMF (see the section on PS). A small proportion of patients might have an IBMFS resulting from causes that are exceedingly rare and therefore have been missed (see Table 7-1), or the BMF may be due to a mutation in a gene that is not usually associated with BMF. For example, specific mutations in *NBS1*, the gene mutated in Nijmegen breakage syndrome, may manifest as an FA-like phenotype including pancytopenia and a positive DEB or MMC test (also see the section on FA). Finally, a proportion of individuals with familial BMF are presumed to have disease caused by mutations in genes that have not yet been identified. With the availability of whole-genome next-generation sequencing, a number of new and “old genes” will be identified to be associated with the development of BMF.[426,427] One of the challenges will be to determine their significance in the pathogenesis of BMF.

Clinical Management

The overall prognosis in patients with familial BMF is generally less favorable than that in patients with nonfamilial (acquired) BMF. A DEB or MMC test to rule out FA (see the section on FA) and telomere measurements to rule out DC (see the section on DC) are the minimal diagnostic evaluations that should be performed early in the investigation of individuals with familial BMF. *MPL* or *RUNX1* mutation analysis might be considered in patients with a history of thrombocytopenia after birth. Whether *MPL* or *RUNX1* gene testing should be performed in all children presenting with familial or sporadic aplastic anemia is controversial.[428] Cytogenetic investigations should be performed in all patients with aplastic anemia and might reveal constitutional or acquired anomalies that pinpoint or are characteristic of an underlying IBMFS.[429,430] Treatment and supportive care, in general, are the same as those described earlier for FA, DC, or acquired aplastic anemia. Antithymocyte globulin, cyclosporine, androgens, and hematopoietic growth factors have been used with variable success. HSCT from an HLA-matched sibling donor has to be considered carefully because the potential donor may have the same genetic lesion even in the absence of disease. Failure of engraftment has been reported in individuals with familial BMF receiving a transplant from an apparently healthy sibling donor, and development of donor cell–derived malignant disease (MDS/AML) has also been reported in these patients.[284,431] In addition, HSCT may be associated with an unexpected toxicity in patients with familial BMF because the underlying genetic defect may render them hypersensitive to radiotherapy or other agents used for HSCT.

INHERITED BONE MARROW FAILURE SYNDROMES ASSOCIATED WITH ISOLATED CYTOPENIAS

Diamond-Blackfan Anemia

Diamond-Blackfan anemia (DBA, MIM 105650), also known as *congenital erythroid hypoplastic anemia*, is an IBMFS that is usually diagnosed in early infancy (Box 7-15). The disease is named after Louis K. Diamond and Kenneth D. Blackfan, two pediatric hematologists in Boston who first recognized the disease as a distinct clinical entity.[432] DBA is genetically and phenotypically heterogeneous.

Clinical Manifestations

Clinically, DBA is characterized by failure of red cell production and variable congenital anomalies. DBA is a rare disease, with a frequency of 2 to 7 per million

Box 7-15 **Diamond-Blackfan Anemia**

Diamond-Blackfan anemia is a pure red cell aplasia that is usually recognized in early infancy. Diamond-Blackfan anemia is associated with a reduction in or an absence of erythroid precursors in bone marrow, variable congenital anomalies, and a predisposition to malignant disease. Diamond-Blackfan anemia red cells characteristically have increased adenosine deaminase activity.

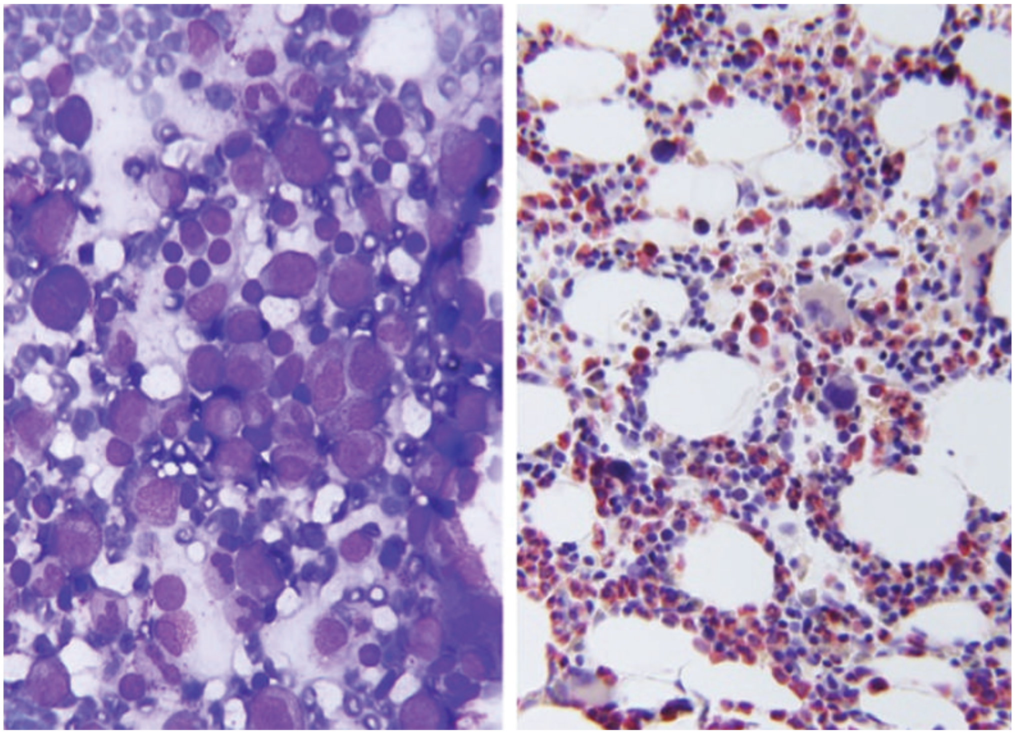


Figure 7-18 Bone marrow aspirate and biopsy obtained from a patient with Diamond-Blackfan anemia. May-Grünwald-Giemsa staining of the aspirate and Leder staining of the biopsy specimen.

live births, and has no ethnic or gender predilection.[433] Much of our knowledge of the disease has been gained from patient registries in North America,[434,435] England,[433,436] Italy,[437,438] France,[439] and the Czech Republic.[440] Classically, patients with DBA present in early childhood with profound macrocytic or normocytic anemia, reticulocytopenia, and a reduction or an absence of erythroid precursors in bone marrow (Fig. 7-18). In more than 90% of patients with DBA the diagnosis is made before the age of 1 year, but in rare cases severe anemia may develop in utero (nonimmune hydrops fetalis) or later in life. The median age at diagnosis is 8 weeks of age.

Congenital Abnormalities

In about 50% of patients DBA is associated with physical anomalies and growth retardation. In some patients a physical anomaly is the only clinical manifestation of DBA. Table 7-8 summarizes the prevalence and range of congenital abnormalities found in patients with DBA. Midline craniofacial defects (e.g., cleft palate), renal anomalies, cardiac malformations, and thumb anomalies (e.g., triphalangeal thumbs) are common.

Short Stature. Although short stature is an integral feature of DBA and is frequently seen in association with other congenital anomalies,[433,441] final height is strongly influenced by glucocorticoid therapy, iron overload, and chronic anemia. Intrauterine growth retardation has been documented in about 20% of individuals with DBA, and characteristically there is an absence of catch-up growth after birth.

Hematologic Abnormalities

Anemia, macrocytosis, reticulocytopenia, and absent or decreased numbers of erythroid progenitor cells in bone marrow are the major diagnostic criteria for DBA. The erythroid marrow may be cellular, albeit with a "leftward shift" toward immature erythroblasts. In 13% to 20% of individuals, anemia may be present at birth.[433,437] Macrocytosis is evident in the majority of cases; however, it may be masked by iron deficiency or thalassemia trait. Increased hemoglobin F levels and persistent "i" antigen are additional features of stress hematopoiesis frequently seen in patients with DBA.[433] Elevated levels of erythrocyte ADA, a critical enzyme in the purine salvage pathway, are characteristic of DBA erythropoiesis and may be found in 80% to 89% of patients.[442,443] Whether macrocytosis, increased fetal hemoglobin, and elevated erythrocyte ADA are integral features of DBA hematopoiesis or rather reflections of stress hematopoiesis remains to be determined. Macrocytosis, increased fetal hemoglobin, persistent "i" antigen, and elevated red cell ADA may be masked in transfusion-dependent patients. In some individuals, macrocytosis or increased red cell ADA levels are the only manifestations of DBA. Parents or siblings of an affected child who have increased red cell ADA levels are likely to be carriers of the pathogenic mutation even in the absence of classic disease (nonclassic DBA). In some individuals with DBA the anemia is transient; however, the macrocytosis and elevated red cell ADA levels often persist. Spontaneous remission of anemia in DBA patients has been reported even after years of steroid or transfusion dependence (also see later). Platelet counts are

TABLE 7-8 Congenital Abnormalities in Diamond-Blackfan Anemia

		FREQUENCY			
Anomaly	**Range of Congenital Anomalies**[452]	**(%)**[433]	**(%)**[435]	**(%)**[437]	**(%)**[439]
No. of patients		420	229	65	88
Head, face, palate	Hypertelorism, cleft palate, high-arched palate, microcephaly, micrognathia, microtia, low-set ears, low hairline, epicanthus, ptosis, flat broad nasal bridge	24	21	36	
Upper limb	Triphalangial, duplex or bifid, hypoplastic thumb; flat thenar eminence; syndactyly; absent radial artery	21[†]	9	18	
Renal, urogenital	Absent kidney, horseshoe kidney, hypospadias	19[†]	7	na	
Cardiopulmonary	Ventricular septal defect, atrial septal defect, coarctation of aorta, complex cardiac anomalies	15	7	na	
Other					
Eyes	Congenital glaucoma, strabismus, congenital cataract	na	12	na	
Neck	Short neck, webbed neck	na	4	na	
Neuromotor	Learning difficulties	na	7	na	
Short stature		na	30	33	na
At least one anomaly[*]		47	41	37	32
Two or more anomalies		25	24	na	na
Short stature alone		na	na	11	16

[*] Not including low birth weight or short stature. Many patients had more than one abnormality.
[†] As found by routine carial and renal imaging.

usually normal or mildly increased, although platelet counts greater than $1 \times 10^6/\mu L$ and decreased platelet counts have also been reported.[433] White blood cell counts are generally normal but in some cases decrease with age. In rare cases DBA progresses to the full clinical picture of severe aplastic anemia.[435]

Bone marrow examination of children with DBA usually shows normal cellularity, myeloid cells, and megakaryocytes, whereas the bone marrow of adult patients with DBA is often hypocellular (MB personal observation). Lymphocyte counts are often increased. A paucity of erythroid progenitor cells is characteristic. Dyserythropoietic morphology, including ringed sideroblasts, has been reported. Increased iron deposition is evident in patients who have received multiple red cell transfusions. Serum levels of erythropoietin are elevated, and ferrokinetic studies show a delay in plasma iron clearance and low red cell utilization. The slightly shortened autologous red cell survival times and low haptoglobin levels suggest mild hemolysis.[444]

Bone marrow progenitor–derived colony assays show a severe maturation defect in DBA erythroid precursors, whereas other blood cell lineages are unaffected or only moderately impaired.[445] Erythroid colony size and maturation, as well as colony numbers, are impaired. The defect is intrinsic to the erythroid progenitor cell[445] and is partially corrected by interleuklin-3 (IL-3) and stem cell factor[446-448] but not by erythropoietin.[445] The rate of apoptosis of erythroid progenitor cells in cultures from patients with DBA is increased and accentuated after erythropoietin deprivation.[449] More detailed culture analysis pinpoints the defect to the erythropoietin-dependent phase of expansion and differentiation of erythroid progenitors with no significant defect in the preceding erythropoietin-independent stages.[450] Mutations in *RPS19* were shown to be associated with a decrease in proliferation of progenitor cells but normal terminal erythroid differentiation, whereas mutations in *RPL11* were shown to be associated with a decrease in progenitor cell proliferation and a delayed erythroid differentiation with a marked increase in apoptosis.[451] Glucocorticoid treatment increases the erythropoietin sensitivity of both normal and DBA erythroid progenitors, but DBA erythropoietic differentiation and maturation remain suboptimal.[450]

Diagnosis

Table 7-9 summarizes the diagnostic and major and minor supporting criteria. Patients with “classic DBA” have all of the major diagnostic criteria.[452] Patients with “nonclassic DBA” have at least three of the diagnostic criteria features plus one major or two minor supporting features; two diagnostic features and two major or three minor supporting features; or two major supporting features.

Predisposition to Malignant Disease

Patients with DBA have a predisposition to malignant disease with a reported frequency of 1.9% to 6.6%.[435,439] AML (AML/MDS) is the most frequently reported

TABLE 7-9 Diagnostic Criteria for Patients with Diamond-Blackfan Anemia[452]

	SUPPORTING CRITERIA	
Diagnostic Criteria	**Major Criteria**	**Minor Criteria**
Age younger than 1 year	Pathogenic mutations*	Elevated red cell adenosine deaminase
Macrocytic anemia	Positive family histroy	Congenital anomalies of Diamond-Blackfan Anemia
Reticulocytopenia		Elevated fetal hemoglobin
Paucity of bone marrow erythroid precursors		No evidence for other inherited bone marrow failure syndromes

From Vlachos A, Ball S, Dahl N, et al: Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. Br J Haematol *142:859–876, 2008.*

*See Table 7-10.

malignant disease, followed by osteosarcoma.[28,453] In a recent report from the Diamond-Blackfan Anemia Registry of North America, only 6 out of 608 DBA patients developed AML or MDS. Among three of the MDS/AML patients tested, none had a ribosomal gene mutation, raising the possibly that at least some of the patients reported to transform from DBA to MDS/AML might have a different disease pathogenesis.[454] Patients who develop osteosarcoma frequently have a history of growth hormone therapy. Other reported malignant diseases include hepatocellular carcinoma, gastric carcinoma, fibrous histiocytoma, vaginal melanoma, Hodgkin and non-Hodgkin lymphoma, breast cancer, and ALL. The medium age at diagnosis of malignant disease was 15 years (range, 1 to 43 years), as opposed to 68 years in a non-DBA control group. The relative risk of all cancers (excluding MDS) in DBA was increased significantly (5.4-fold), with a cumulative incidence of 22% by age 46 years.[453] A phenotype-genotype correlation for the development of cancer is not yet available.

Differential Diagnosis

Clinically, DBA has to be distinguished from other IBMFSs such FA, DC, SDS, CHH, and PS. Clinical manifestations, associated physical abnormalities, family history, a negative chromosome fragility test (DEB or MMC test), and normal telomere length measurements are helpful in strengthening the diagnosis of DBA. Distinguishing DBA from CHH may be difficult. The anemia in CHH is usually less severe but may be similar to that seen in patients with DBA.[409] In the absence of distinguishing features, only identification of the responsible mutation may differentiate between the two conditions. Interestingly, both the RMRP responsible for CHH and the ribosomal proteins responsible for DBA participate in ribosome biogenesis, which may explain the similarities in hematologic manifestations. In patients with PS, BMF is classically accompanied by characteristic vacuolization of hematopoietic precursor cells.[374] Finally, congenital anemia, neutropenia, and trilineage dysplasia caused by *GATA1* gene mutations causes a congenital macrocytic anemia; however, it is not associated with congenital anomalies. Indeed, in some cases *GATA1* gene mutations produce a phenotype that is indistinguishable from ribosomal gene mutations caused by DBA (see below and Table 7-8)(Box 7-16).

DBA has to be distinguished from acquired forms of anemia, including transient erythroblastopenia of childhood (TEC; see later and Chapter 6); viral infections, including infection with human immunodeficiency virus and infectious mononucleosis; drug-related anemia; anemia caused by exposure to toxic chemicals; severe renal failure; and anemia after an ABO-incompatible transplant (for a comprehensive list of possible causes of anemia in childhood, see Vlachos and associates[452]).

Genetic Characteristics

DBA occurs in both familial and sporadic forms. Gene mutations identified in patients with DBA are summarized in Table 7-10. Approximately 70% of patients have identifiable mutations in one of several ribosomal protein genes.[455] Genes encoding proteins of both the small (RPS) and large (RPL) ribosomal subunits may be affected. Large deletions of ribosomal genes account for about 20% to 26% of patients with DBA and may be overlooked when gene sequencing is the only method used for mutation detection.[456,457] In approximately two thirds of cases the mutation occurs de novo; in about one third the mutation is inherited and typically follows AD inheritance.[458] Rare cases of AR inheritance have been reported.[436,459,460] It remains to be determined whether these cases are indeed due to biallelic mutations or are caused by incomplete penetrance of an otherwise dominant mutation mimicking sporadic or AR disease.[435,436] Finally, in two families *GATA1* mutations were identified to be the cause of congenital red cell aplasia with an X-linked inheritance pattern affecting only male family members.[461]

Diamond-Blackfan Anemia Genes and Their Functions

Following from the observation that a DBA patient had an X;19 chromosomal translocation,[462] the first gene to be identified as being mutated in DBA was the *RPS19* gene at 19q13.2.[463] Subsequently, it was established that a fourth of DBA patients harbor a mutation in *RPS19*,[464,465] which encodes the small subunit ribosomal protein 19. The mutations are always heterozygous and include

Box 7-16 Ribosomes

Ribosomes are intracellular organelles about 200 Å in diameter that consist of RNA (ribosomal RNA [rRNA]) and protein (ribosomal proteins). In all organisms, ribosomes are the site of protein synthesis and translation of messenger RNA molecules into continuous chains of amino acids (polypeptides or proteins). The ribosome is a ribozyme, which means that it is the rRNA that catalyzes decoding of the message and the formation of peptide bonds. Ribosomal proteins, on the other hand, are important for maturation of rRNA precursors and ensure their correct structure in the ribosome.

TABLE 7-10 Genes Mutated in Patients Diagnosed with Diamond-Blackfan Anemia

	MIM*					Percentage of DBA Patients		
Gene	**Gene**	**Morbid map**	**Phenotype**	**Subunit**	**Locus**		**Comments**	**References**
RPS19	603474	DBA1	105650	40S	19q13.2	25		463
RPS24	602412	DBA3	610629	40S	10q22-23	≈2		726
RPS17	180472	DBA4	612527	40S	15q25.2	≈1		727
RPS10	613308	DBA9	603632	40S	6p21.31	≈2-6		728
RPS26	613309	DBA10	603701	40S	12q13.2	≈2-6		728
RPS7	612563	DBA8	603658	40S	2p25.3	≈1		475
RPS29	603701	NA	NA	40S	14q21.3	1 family		729
RPL35a	180468	DBA5	612528	60S	3q29-qter	≈2-4		730
RPL5	612561	DBA6	603634	60S	1p22.1	7		475
PRL11	612562	DBA7	604175	60S	1p36.1-35	11		475
RPL26	603704	NA	NA	60S	17p13.1	1 patient	Multiple severe anomalies	731
RPL15	604174	NA	NA	60S	3p24.2	1 patient		753
GATA1	305371	NA	300835	—	Xp11.23	2 families	No extrahematopoietic manifestations	461

From Gazda HT, Grabowska A, Merida-Long LB, et al: Ribosomal protein S24 gene is mutated in Diamond-Blackfan anemia. Am J Hum Genet *79:1110-1118, 2006.*

MIM, Mendelian Inheritance of Man; *NA*, not yet assigned.

*DBA2 MIM 606129: A genome-wide search in 2001 suggested the presence of a DBA locus on 8p; however, no gene mutation was identified and there is no ribosomal protein on 8p.

missense, nonsense, frameshift, and splice-site mutations, as well as small and large gene deletions and chromosomal translocations with the breakpoint in the *RPS19* gene. Whereas the nonsense and frameshift mutations are spread evenly throughout the coding region, the missense mutations are clustered. RPS19 haploinsufficiency is thought to be responsible for disease.[466] Analysis of the crystal structure of RPS19 from *Pyrococcus abyssi* suggests that missense mutations in DBA primarily affect the capacity of the protein to be incorporated into preribosomes, thus blocking maturation of pre-40S particles.[467]

Since then pathogenic mutations have been found in other ribosomal proteins from either the large or small subunits. Ribosomal proteins are an integral component of ribosomes (Box 7-17). Although ribosomal proteins do not participate in the protein synthesis activity of the ribosome, they are important for maintaining the structure and conformation of the ribosome during biosynthesis and for its function in translation (see Boxes 7-16 and 7-17 and Figs. 7-19 and 7-20). The finding that the great majority of mutations causing DBA affect ribosomal proteins led to the concept that for most patients, DBA is a disorder of ribosome biogenesis or a ribosomopathy.

The spectrum of RP gene mutations in DBA range from single amino acid substitutions to small deletions or insertions to nonsense mutations, splicing mutations, and large deletions. This pattern suggests that loss of function leading to haploinsufficiency is the mechanism of pathogenesis for all the RP loci involved. Mutations in ribosomal genes identified in patients with DBA, along with their functional consequences and phenotype, are catalogued in the DBA Mutation Database (www.dbagenes.unito.it).[458,470]

The only mutations leading to DBA not in an RP gene have been found in the *GATA1* gene.[461] The GATA1 patients diagnosed with DBA present with an overlapping phenotype that includes macrocytic anemia, increased fetal hemoglobin, and a responsiveness to glucocorticoids (Fig. 7-21). In contrast to DBA caused by ribosomal protein mutations, individuals with *GATA1* mutations lack increased erythrocyte ADA and congenital anomalies.[461,471] The *GATA1* mutations associated with a DBA-like phenotype are located at the end of exon 2 and favor the production of a short form of GATA1 (GATA1s) that lacks amino acids 1 to 83.[472] This region, the NH_2-terminal activation domain (NAD), binds the retinoblastoma tumor suppressor protein and may regulate the capacity of GATA1 to control cell survival or proliferation. Whether a link exists between RP haploinsufficiency

Box 7-17 **Ribosome Biogenesis and Preribosomal RNA Processing**

Ribosome biogenesis is the new production of ribosomes. In eukaryotes, ribosome biogenesis takes place in the nucleolus, a specialized compartment within the nucleus. Ribosome biogenesis is a complicated, highly regulated process that begins with the synthesis of large preribosomal RNA. Structural rearrangements and cleavage occur as the ribosomal proteins are incorporated to ultimately give rise to mature ribosome subunits. Final maturation of the ribosome subunits takes place in the cytoplasm before they become assembled to form the mature ribosome (see Figs. 7-19 and 7-20).[468,469]

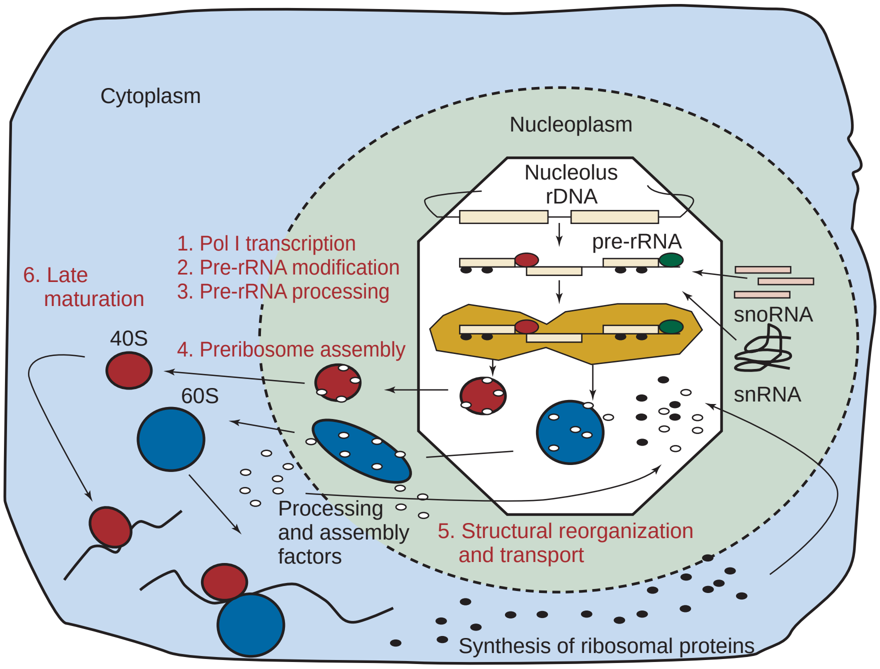

Cytoplasm
Nucleoplasm
Nucleolus
rDNA
pre-rRNA
1. Pol I transcription
2. Pre-rRNA modification
3. Pre-rRNA processing
6. Late maturation
40S
4. Preribosome assembly
60S
snoRNA
snRNA
Processing and assembly factors
5. Structural reorganization and transport
Synthesis of ribosomal proteins

Figure 7-19 Diagram of the six major steps in ribosome biogenesis. 1. Pol I transcription: The process starts with transcription of the multiple copies of the genes for rRNA (rDNA) to form the large preribosomal RNA primary transcript. 2. Preribosomal RNA modification: The preribosomal RNA is methylated and pseudouridylated under the guidance of the small nucleolar RNAs. Nonribosomal proteins *(open circles)* and small nucleolar RNAs *(open rectangles)* associate with the nascent transcript. 3. Preribosomal RNA processing: The preribosomal RNA undergoes a series of cleavages, ultimately resulting in 18S, 5.8S, and 28S ribosomal RNAs. 5S ribosomal RNA, a component of the 60S subunit, is added to the maturing complex. The complex is split into the two precursor particles for the small (40S) and large (60S) ribosomal subunits. 4. Preribosome assembly: Ribosomal proteins *(black circles)* are added to the precursor complexes at various stages of assembly. 5. Structural reorganization and transport: The nearly mature subunits are exported to the cytoplasm through the nuclear pore complexes with the aid of adaptor proteins. 6. Late maturation: The small and large subunits are eventually incorporated into ribosomes in the cytoplasm. Dyskeratosis congenita affects ribosome biogenesis at the preribosomal RNA modification step (step 2); Diamond-Blackfan anemia and cartilage-hair hypoplasia affect ribosome biogenesis at the preribosomal RNA processing step (step 3).

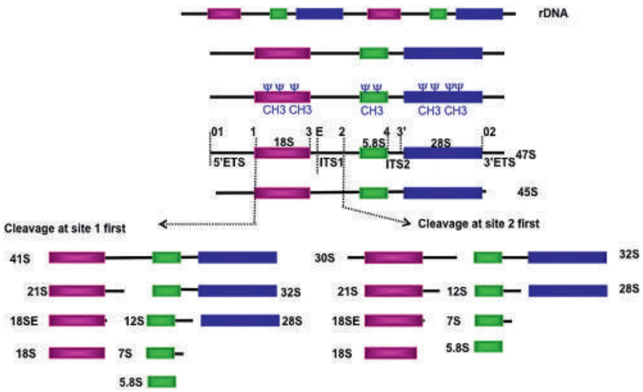

rDNA
ΨΨ Ψ
ΨΨ
ΨΨ ΨΨΨ
CH3 CH3
CH3
CH3 CH3
01
1
18S
3
E
2
5.8S
4
3'
28S
02
5'ETS
ITS1
ITS2
3'ETS
47S
45S
Cleavage at site 1 first
Cleavage at site 2 first
41S
30S
32S
21S
32S
21S
12S
28S
18SE
12S
28S
18SE
7S
18S
7S
18S
5.8S
5.8S

Figure 7-20 Preribosomal RNA processing. The 18S, 5.8S, and 28S ribosomal RNAs are transcribed as a single precursor (45S), which is subsequently processed into mature species by a complex series of cleavage and modification reactions. In addition to the mature ribosomal RNA species, the primary precursor (47S) contains two external transcriber spacers at its 5′ and 3′-ends (5′-ETS and 3′-ETS, respectively), as well two internal transcriber spacers (ITS1 and ITS2). After synthesis of the primary preribosomal RNA molecule is completed, the transcriber spacer sequences are removed by a series of endonucleolytic cleavage and exonucleolytic degradation steps. Two alternative preribosomal RNA processing pathways, A and B, have been described in HeLa cells. *(From Hadjiolova KV, Nicoloso M, Mazan S, et al: Alternative pre-rRNA processing pathways in human cells and their alteration by cycloheximide inhibition of protein synthesis.* Eur J Biochem *212:211–215, 1993; Mullineux ST, Lafontaine DL: Mapping the cleavage sites on mammalian pre-rRNAs: where do we stand?* Biochimie *94:1521–1532, 2012.)*

and *GATA1* expression remains to be determined.[473] Recent studies of Sankaran and colleagues[461] suggest that failure of GATA1 translation (and presumably the translation of other genes) may account for the variable manifestations of DBA. This highlights the importance of correlating molecular and clinical diagnoses in the management of rare diseases as more genetic causes are described.[473]

Genotype-Phenotype Correlation

No striking correlation has been found between the mutated gene and the severity of the BMF in DBA. Disease severity is highly variable among patients with mutations in the same gene and even among affected members of the same family. There is a clear phenotype-genotype correlation, however, in terms of the expression of physical malformations and small stature.[440,455,458,474] Patients with no phonotypical anomalies are most frequently *RPS19* gene mutation carriers or patients with *GATA1* mutations, whereas patients with *RPL5* or *RPL11* mutations more frequently have thumb and congenital heart defects. Indeed, thumb anomalies are not found in patients with *RPS19* gene mutations. Patients with *RPL5* mutations seem to be more frequently affected with craniofacial malformations, while patients with *RPL11* mutations more frequently have hand abnormalities.[440,475-477] Patients with *RPS26* mutations have no thumb anomalies or

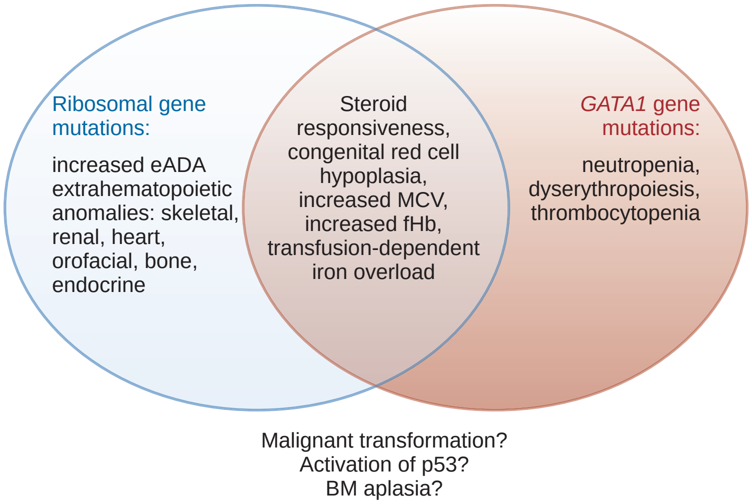

Ribosomal gene mutations:
increased eADA
extrahematopoietic anomalies: skeletal, renal, heart, orofacial, bone, endocrine
Steroid responsiveness, congenital red cell hypoplasia, increased MCV, increased fHb, transfusion-dependent iron overload
GATA1 gene mutations:
neutropenia, dyserythropoiesis, thrombocytopenia
Malignant transformation?
Activation of p53?
BM aplasia?

Figure 7-21 Common and distinct phenotypes in congenital red cell aplasia caused by mutations in RP genes and in GATA1. *eADA*, Erythrocyte adenosine deaminase activity; *Hb*, hemoglobin; *MCV*, mean corpuscular volume. *(Modified from Weiss MJ, Mason PJ, Bessler M: What's in a name?* J Clin Invest *122:2346–2349, 2012.)*

craniofacial dysmorphisms; instead they have skeletal abnormalities, including microcephaly, Sprengel deformity, Klippel-Feil syndrome, or rib anomalies.[440]

Molecular Diagnosis of Diamond-Blackfan Anemia

Currently, molecular diagnosis is not routinely performed for DBA, because it is expensive. If deletion detection is included, genetic testing can currently provide a definite diagnosis in about 70% of cases (about 50% to 60% if gene deletion detection is not included).[455] Furthermore, molecular diagnostic testing enables the identification of mutation carriers, facilitates prenatal diagnosis for an existing pregnancy, and allows preimplantation genetic diagnosis for partners identified as mutation carriers. Anomalies in addition to the ones characteristic for DBA, in particular, mental retardation, are suggestive of a large deletion causing haploinsufficiency for additional genes in the vicinity of the deleted DBA gene.

Diseases Related to Diamond-Blackfan Anemia

Aase Syndrome. Congenital red cell aplasia with a triphalangeal thumb has been described by Aase and Smith as a distinct syndrome.[478] Cleft palate and cardiac anomalies have been associated with this syndrome. Its clinical manifestations have significant overlap with those characteristic of DBA; thus Aase syndrome most likely represents a subset of patients with DBA who have a particular phenotype.

Myelodysplasia with Deletion of Chromosome 5q (5q– Syndrome). Patients with a deletion of the long arm of chromosome 5 (5q– syndrome) have severe macrocytic anemia, elevated platelet counts, erythroid hypoplasia in the bone marrow, and hypolobulated megakarocytes. Delineation of the critical deletion region on chromosome 5q– includes the *RPS14* gene at position 5q33.1 and about 40 additional genes.[479] Reducing RPS14 expression in normal CD34+ cells leads to a gene expression profile similar to that found in bone marrow cells from patients with 5q– MDS and results in selective impairment of erythroid differentiation with only minor effects on megakaryopoiesis or myelopoiesis. Similarly, enforced expression of RPS14 restores the erythroid phenotype in bone marrow cells from patients with 5q– MDS, suggesting that erythroid hypoplasia is caused by *RPS14* haploinsufficiency.[479] In contrast to patients with DBA, the deletion of *RPS14* in patients with 5q– MDS is not inherited but is acquired in early hematopoietic progenitor cells (somatic). Heterozygous loss of the *RPS14* gene leads to activation of p53 in erythroid cells and explains the macrocytic anemia in 5q– MDS. The megakaryocytic and platelet phenotype of 5q– MDS has been attributed to heterozygous deletion of miR145 and miR146a. Murine models have implicated heterozygous loss of *APC*, *EGR1*, *DIAPH1*, and *NPM1* in the pathophysiology of 5q– MDS. These findings indicate that haploinsufficiency or loss of heterozygosity for additional genes affected by the deletion causes the growth advantage of the mutant cells.[480] Interestingly, MDS with 5q– shows a remarkable response to the thalidomide analogue lenalidomide, which is thought to be in part mediated through the inhibition of Cdc25C, resulting in G_2- and M-phase arrest and apoptosis of 5q– cells, in part through the stabilization of HDM2, leading to the degradation of p53 and the restoration of erythropoiesis.[481]

Transient Erythroblastopenia of Childhood. Transient erythroblastopenia of childhood (TEC, MIM 227050) is a rare pure red cell aplasia that occurs most commonly between 6 months and 4 years of age (see Chapter 6).[482] Laboratory findings consist of normocytic normochromic anemia and reticulocytopenia, sometimes associated with thrombocytosis and erythroblastopenia of bone marrow.[482] The course is characterized by complete recovery, usually within 1 to 2 months after diagnosis. No therapy is usually necessary, although some children may require red cell transfusion. The absence of macrocytosis, increased fetal hemoglobin, and increased erythrocyte ADA levels in individuals with TEC are used to distinguish TEC from DBA.[482] In addition, congenital abnormalities are not present in patients with TEC. The etiology of TEC is not entirely clear, but is thought to be postinfectious and possibly autoimmune mediated. The majority of TEC cases are sporadic and acquired, but there are a few reports of familial TEC. Linkage analysis suggested allelism for TEC at the *RPS19* gene locus; however, no mutations in the *RPS19* coding region were identified.[483]

Animal Models

Mice haploinsufficient for *Rps19*, due to heterozygosity for a deletion mutant, are asymptomatic and have normal levels of Rps19 mRNA and protein, indicating that mice can compensate for the gene deficiency but humans cannot.[484,485] Missense mutations in Rps19, isolated in an experiment selecting for a dark skin phenotype, had a mild macrocytic anemia, which was reversed, as was the dark skin, in a $p53^{-/-}$ background.[486] Induced haploinsufficiency for Rps6 produced a mild macrocytic anemia with granulocytopenia, thrombocytosis, lymphopenia, and elevated eADA levels. The anemia responded to lenalidomide treatment.[487] The most faithful model of DBA in mice was produced by making mice transgenic for Rps19 shRNA, whose presence decreases the level of Rps19 mRNA.[488] These mice showed macrocytic anemia with leukocytopenia and variable platelet count. They also had ribosome profile abnormalities and increased apoptosis in hematopoietic progenitors. The phenotype could be rescued by either expression of Rps19 or depletion of p53. Treatment of these mice with leucine produced beneficial effects, with increases in hemoglobin concentration and erythrocyte number.[489]

A number of *Drosophila* mutants called minutes are due to haploinsufficiency for various ribosomal proteins and share some features with DBA.[307] Affected flies have developmental delay and small size. Phenotypes in DBA and *Drosophila* minutes affect cells and tissues with a high rate of protein synthesis (bone marrow cells in humans and sensory bristles in flies). In both species the phenotype is only produced by haploinsufficiency of certain ribosomal proteins but not all. This may indicate that ribosomal protein expression, which varies between different proteins, needs to fall below a critical level for the phenotypes to develop.

In zebrafish several ribosomal protein knockdowns, including those for RPS19 and RPL11, have produced developmental, morphologic, and hematopoietic defects some of which are similar to those seen in DBA,[490-493] though it is difficult to accurately produce haploinsufficiency in this organism. Concomitant knockdown of p53 often rescued the phenotypes; in one case developmental, but not hematopoietic, defects responded to p53 deficiency.[494]

Mechanism of Disease Pathogenesis in Diamond-Blackfan Anemia

The mechanism whereby mutations in *RPS19* or the other ribosomal proteins cause specific defects in red cell maturation is not fully understood and is the focus of current research in the field. The fact that different ribosomal proteins from both subunits can be mutated in DBA suggests that impaired ribosome biogenesis is important in pathogenesis. But why should this process, which is important in all cells, specifically affect erythropoiesis? One prevailing hypothesis amongst investigators is that in a cell that is synthesizing ribosomes at a high rate, shortage of a ribosomal protein leads to an excess in the nucleus of other ribosomal proteins that are not incorporated into ribosomes (ribosome stress).[495] These include RPL5 and RPL11, which bind 5S rRNA in the nucleus[496] and can specifically interact with MDM2 and prevent it from ubiquitinating p53. The concentration of p53 not marked for degradation increases and signals a cell cycle arrest and apoptosis (Fig. 7-22, *A* and *B*). There is good evidence for many of the steps in this process. Levels of the tumor suppressor p53 are maintained as part of a feedback loop whereby MDM2 is an E3 ubiquitin ligase that leads to p53 degradation while it is itself

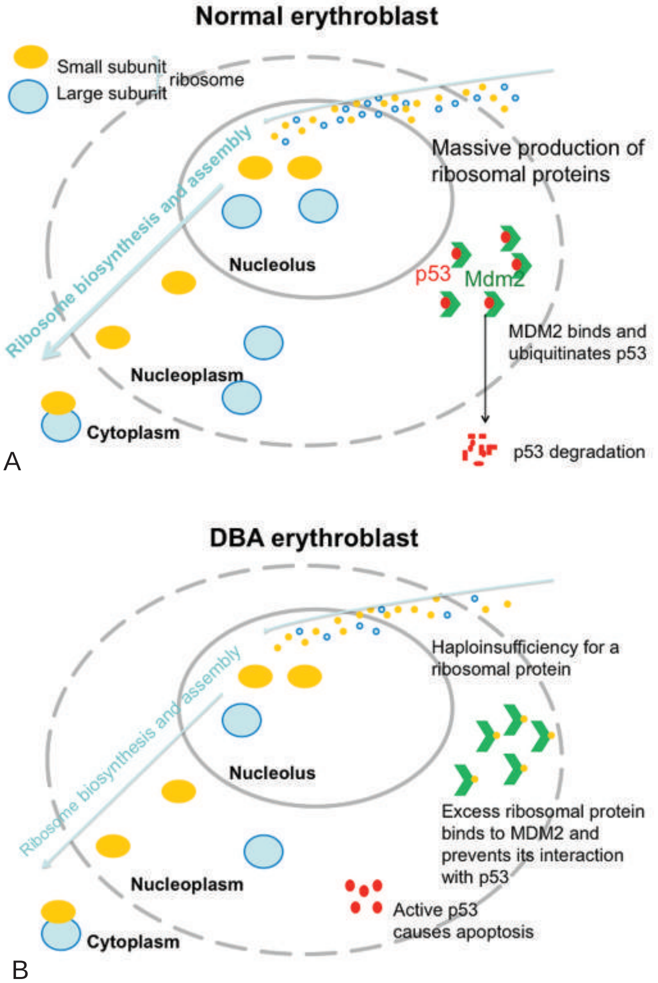

Normal erythroblast
Small subunit
Large subunit
ribosome
Massive production of ribosomal proteins
Ribosome biosynthesis and assembly
Nucleolus
p53
Mdm2
MDM2 binds and ubiquitinates p53
Nucleoplasm
Cytoplasm
p53 degradation
A
DBA erythroblast
Haploinsufficiency for a ribosomal protein
Ribosome biosynthesis and assembly
Nucleolus
Excess ribosomal protein binds to MDM2 and prevents its interaction with p53
Nucleoplasm
Active p53 causes apoptosis
Cytoplasm
B

Figure 7-22 Current model for how RP haploinsufficiency causes Diamond-Blackfan anemia. **A,** Normal erythroblasts produce large numbers of ribosomes for protein synthesis. Levels of p53 remain low via a feedback loop whereby MDM2, a transcriptional p53 target, ubiquitinates p53 to promote its degradation by proteasomes. **B,** Haploinsufficiency for specific RPs causes accumulation of other RPs, which bind to MDM2, thereby inhibiting its ability to promote p53 degradation. Consequently, p53 accumulates and triggers cell cycle arrest and apoptosis. *(Modified from Weiss MJ, Mason PJ, Bessler M: What's in a name?* J Clin Invest *122:2346–2349, 2012.)*

a transcriptional target of p53.[497-499] MDM2 binds RPL11 and RPL5, and their binding inhibits the action of MDM2 in p53 turnover.[500-502] The mechanism by which RPL11 and RPL5 accumulate under conditions of ribosome stress has not been clearly delineated, but it was recently suggested, with experimental evidence, that under these conditions most ribosomal proteins are degraded in the nucleus but RPL11 and RPL5 mutually interact and protect each other from degradation.[503] The scheme also accounts for the role played by p53 in the response to ribosome stress as demonstrated by increased p53 in cells undergoing ribosome stress and the rescue of DBA-like phenotypes in animal models of DBA by p53 depletion. Unanswered questions remain. Why are some mutations in some ribosomal protein genes frequent causes of DBA but others infrequent causes, and why have some never been found? Why are the two proteins that bind to MDM2 in response to ribosome stress, RPL5 and RPL11, encoded by the most frequently mutated large subunit genes?

An alternative hypothesis is that maturing erythrocytes are producing vast amounts of globin and vast amounts of heme. When ribosome synthesis is faulty, the amount of globin produced decreases but the decrease does not affect the rate of production of heme, which then accumulates and is toxic to the cells. A mouse in which the gene encoding FLVCR, a heme export protein,[504] can be inactivated develops many of the features of DBA, including macrocytic anemia.[505] Further investigation is needed to clarify whether these mechanisms operate in DBA.

Clinical Management

The actuarial survival rate at 40 years of age is 75%.[435] Survival is significantly better for steroid-responsive patients than for transfusion-dependent patients.[433,435,438-440] The major causes of death are treatment related and include infection (in particular, *Pneumocystis jiroveci* pneumonia), complications of iron overload, and complications of HSCT. Other causes of death include evolution to severe aplastic anemia and malignant disease.

Glucocorticoid Therapy. Glucocorticoids and red cell transfusions are the main forms of therapy. Prednisone (or prednisolone) therapy is usually initiated at a dosage of 2 mg/kg/day. For glucocorticoid responders, the prednisone dosage is slowly tapered until the patient is taking an alternate-day dosage that maintains a reasonable hemoglobin level. Many patients remain on low, alternate-day doses of steroids for years. Seventy percent to 80% of patients with DBA are initially steroid responsive, but only 60% to 70% achieve transfusion independence.[452,506] Response to treatment is usually seen within 1 month. The likelihood of response cannot be predicted. The steroid dose is tapered to the lowest dose necessary to maintain transfusion independence. The maintenance dose of steroids is highly variable, with some requiring only minimal doses. The mechanism of glucocorticoid-induced remission is as yet unknown.

Side effects of steroid therapy include growth retardation, increased risk of infection, diabetes mellitus, hypertension, cushingoid features, pathologic fractures, cataracts, avascular osteonecrosis, osteopenia without fracture, pseudotumor cerebri, and, in infants, delay in neuromotor development.[433,452] Steroid therapy is often not recommended in infants younger than 6 months.[452,506] Individuals taking high doses of steroids require *P. jiroveci* prophylaxis. The steroid maintenance dosage should not exceed 0.5 mg/kg/day (or 1 mg/kg every other day) and should be discontinued in the absence of response (for more detailed information, see the consensus document regarding diagnosis and management of DBA[452]).

Transfusion Therapy. Red cell transfusion remains the mainstay of therapy for steroid-resistant disease, as well as for treatment of DBA in early infancy (<6 to 12 months of age). Leukocyte-depleted packed red cells should be given every 3 to 6 weeks to maintain a hemoglobin level of about 8 g/dL.[452] The major complication from transfusions is iron overload. Complications associated with iron overload are the second most common cause of death in patients with DBA.[433,435,438-440] Complications of iron overload include endocrinopathy, diabetes, growth failure, and failure to enter puberty, as well as cardiac failure and liver disease. Serum ferritin is not a reliable indicator of the body's iron load, in particular in patients with DBA. There is evolving evidence for an increased cardiac and liver iron uptake in patients with DBA compared with patients with thalassemia.[507-510] Measurements taken from a liver biopsy specimen are the gold standard for estimating hepatic iron concentration. Noninvasive methods of iron measurement include magnetic susceptometry (superconducting quantum interference device) and magnetic resonance imaging. Iron chelation should begin as soon as patients have increased iron stores (ferritin >1500 mg/dL, hepatic iron >6 to 7 mg/g, which corresponds to a transfusion history of about 170 to 200 mL of packed red blood cells/kg). Subcutaneous infusion of deferoxamine had been the standard chelation therapy in patients with DBA for many years. More recently the oral iron chelator deferasirox, a tridentate, has supplanted deferoxamine as the standard chelation therapy.[511] Deferasirox may be given in combination with deferoxamine.

The oral iron chelator deferiprone, a bidentate chelator available in Europe for quite some time, has become available recently in the United States. Side effects of deferiprone include neutropenia (6%) and agranulocytosis (0.6%). Fatal agranulocytosis has been described in one patient with DBA taking deferiprone in addition to deferoxamine.[512] The use of deferiprone in patients with DBA should therefore be avoided. Iron chelation is contraindicated during pregnancy and should cease when pregnancy is planned or confirmed.[513,514]

Hematopoietic Stem Cell Transplantation. HSCT is curative in patients with DBA and may be considered in transfusion-dependent patients, in particular those who have an HLA-matched sibling donor, patients with DBA progressing to severe aplastic anemia, or patients in whom MDS/AML develops. Data from the DBA registry showed survival rates of 90% in individuals below the age of 9 years and 70% in individuals 9 years and older with transplants from sibling donors. Survival rates for DBA patients receiving a transplant from an alternative donor were less encouraging in the past; with more extensive HLA typing, improved iron chelation, and pretransplant and posttransplant care, the rate was found to be 86%.[515] The survival rate is similar with unrelated marrow and cord blood donors, but a report from the Aplastic Anemia Committee of the Japanese Society of Pediatric Hematology suggests that marrow might be superior to cord blood cells.[516] In a recent report from the Eurocord and the Severe Aplastic Anemia Working Party from the European Group for Blood and Marrow Transplant, following an umbilical cord transplant in 21 patients with DBA, 13 received a related cord and 8 an unrelated cord. Of those who received a related graft all were alive at a median follow-up of 65 months. Of the five patients with DBA who received an unrelated cord, three were alive at a median follow-up of 31 months, suggesting that umbilical cord transplantation is an excellent option for children with hereditary BMF syndromes such as DBA when the donor is an HLA-matched sibling donor.[517] The decision to perform HSCT in patients with DBA is confounded by the possibility of spontaneous remission. HSCT is generally more successful in young patients who have received relatively few transfusions.[435]

Alternative Therapies. Several other therapeutic approaches used over the last 30 years include androgens, erythropoietin, IL-3, stem cell factor, antilymphocyte globulin, cyclosporine, and valproic acid. Although occasional hematologic responses were observed, none of these therapies became a major player in the treatment of DBA. Treatment with metoclopramide, a dopamine antagonist, elicited transfusion independence in a small number of individuals with DBA.[518-520]

Splenectomy was reported in approximately 40 patients, with no beneficial effect noted except in those who had hypersplenism related to transfusions. More than half of the splenectomized patients died, often from infections. Splenectomy is no longer recommended but with vaccination and antibiotic prophylaxis might be reconsidered for specific patients.

More recently, leucine, an amino acid that increases protein synthesis, has been reported to be beneficial for some DBA patients studied in a small series in the Czech Republic.[521] Leucine is a branched-chain amino acid that is a substrate for protein synthesis but also serves as a nutrient that regulates protein metabolism through activation of mTOR.[522] Studies in mouse and zebrafish models of DBA showed that leucine treatment resulted in improvement of anemia and that leucine treatment of RPS19-deficient erythroblasts increased cell proliferation, erythroid differentiation, and mRNA translation in culture.[489,523] Clinical trials employing leucine in the treatment of DBA are currently in progress.

Spontaneous Hematologic Remission. About 20% to 30% of patients experience spontaneous hematologic remission, defined as a stable, physiologically acceptable hemoglobin level maintained for at least 6 months, independent of glucocorticoid treatment, transfusion, or other therapy.[434,435] In the majority of cases hematologic remission occurs within the first or second decade of life in patients who were steroid responsive. The hematologic is often stable. However, relapse of hematologic disease has been observed, in particular during pregnancy or in women using estrogen-based oral contraception.[435] The molecular basis of the hematologic remission is not fully understood. In two patients, one with an *RPS17* gene mutation and one with an *RPL35* gene mutation, somatic mosaicism for copy-number neutral loss of heterozygosity of chromosomes 15q and 3q, respectively, has been identified in peripheral blood cells.[456] This suggests that somatic mitotic recombination occurring in early hematopoietic progenitor cells may be responsible for the normalization of peripheral blood cell counts in these two patients.

Pregnancy in Diamond-Blackfan Patients

Pregnancy in women with DBA is associated with an increased frequency of complications in the mother and child, including preeclampsia, fetal loss, intrauterine death, premature delivery, intrauterine growth retardation, and congenital anomalies.[433,524] It has been suggested that these complications are of vascular-placental origin, and treatment with aspirin has been reported to be beneficial in some cases.[524] Almost half of patients had transient worsening of anemia during pregnancy. The maternal hemoglobin level should be maintained at greater than 9 to 10 g/dL to avoid maternal anemia, which might lead to intrauterine growth retardation, preterm delivery, or fetal distress. Women planning to become pregnant should be evaluated for conditions that might compromise the outcome of pregnancy, including bloodborne infectious diseases, iron overload and related diabetes mellitus, hypothyroidism, and cardiomyopathy. Iron chelation is contraindicated during pregnancy and should cease when pregnancy is planned or confirmed. Deferoxamine is teratogenic in rodents but deferasirox is not; however, neither medication is licensed for use in pregnant women.[452]

Cancer Surveillance and Treatment

In addition to an increased risk for AML, there appears to be an association of osteosarcoma and other malignant diseases with DBA.[453] Bone marrow biopsy plus cytogenetic evaluation is indicated when abnormalities suggestive of MDS or MDS/AML are noted or when the disease progresses to aplastic anemia.[452] In contrast to other IBMFSs routine yearly bone marrow biopsies are not recommended in DBA.[452] Growth hormone therapy is not usually recommended in individuals with DBA and small stature, because of the potential increased risk for osteosarcoma.[453] Whether malignant diseases occurring in patients with DBA differ in their biology and response to treatment from similar diseases occurring in non-DBA individuals remains to be determined. Similarly, levels of toxicity in DBA patients with cancer to certain chemotherapy agents remain to be determined.

Box 7-18 Ineffective Erythropoiesis/Dyserythropoiesis

Ineffective erythropoiesis results when in intact, stimulated bone marrow, red blood cell precursors either fail to mature or die in the bone marrow before delivery to the circulation as erythrocytes. Dyserythropoiesis usually defines a qualitative defect in red cells or red cell precursors, or both. Dyserythropoiesis often leads to ineffective erythropoiesis with intramedullary destruction of red cells and their precursors and often a decreased half-life of circulating red blood cells.

Congenital Dyserythropoietic Anemias

Congenital dyserythropoietic anemias (CDAs) are a group of rare genetic disorders characterized by ineffective erythropoiesis and distinctive morphologic abnormalities in bone marrow erythroblasts.[525] Dyserythropoiesis is the major cause of anemia in patients with CDA, although a shortened half-life of the mature cells in circulation may also contribute to anemia (for the definition of dyserythropoiesis see Box 7-18). CDAs are often not considered to belong to the group of IBMFSs. Nevertheless, CDAs are genetic conditions that affect red blood cell production in bone marrow and lead to anemia associated with an inadequate reticulocytosis.

Four major forms of CDA and several minor subgroups have been identified. The main characteristics of the four major forms of CDA are summarized in Table 7-11 (for review see also Iolascon et al, 2011,[526] and Iolascon et al, 2012[527]). Shared clinical symptoms include anemia of variable degree, intermittent jaundice, hepatomegaly, splenomegaly, cholelithiasis, and iron overload. Additionally, extramedullary hematopoiesis presenting as a paravertebral mass may be observed in all types of CDAs.

Differential Diagnosis

CDA should be considered when the reticulocyte response is suboptimal for the degree of anemia in a patient with erythroid hyperplasia or when there is unexplained hyperbilirubinemia or iron overload. Other congenital anemias and acquired disorders associated with dyserythropoiesis that should be excluded include thalassemia syndromes, hemoglobin C, certain unstable hemoglobins, hereditary sideroblastic anemias, hereditary persistence of fetal hemoglobin, red blood cell membrane defect, vitamin B_{12} or folate deficiency, iron deficiency, alcohol abuse, liver disease, heavy metal poisoning, and MDS.

Clinical Management

Treatment is essentially symptomatic. Prevention of severe tissue damage secondary to iron overload is important. Splenectomy results in hematologic improvement in some patients with CDA type II[528]; transfusion independence was also achieved in the one patient with CDA type IV in whom splenectomy was performed.[529] Splenectomy in CDA does not have a beneficial effect on iron overload. The benefit of splenectomy in other forms of CDA is controversial. Patients with CDA type I show hematologic improvement and a reduction in iron overload in response to recombinant interferon alfa.[530,531] Successful treatment with allogeneic HSCT in combination with intensive iron chelation therapy has been reported in severely affected patients with CDA.[532,533] In contrast to other IBMFSs, CDA is not associated with a significantly increased risk for leukemia or cancer.

Congenital Dyserythropoietic Anemia Type I

Clinical Characteristics. The incidence of CDA type I is about 1 per 100,000 births per year, and more than 150 patients have been described. Consanguinity may be the cause of an increased incidence in some areas such as the Middle East. In most patients the diagnosis is made during childhood and adolescence. Anemia is moderate, with a mild macrocytosis and frequent eliptocytes. Clinical manifestations in newborns include small stature, hepatosplenomegaly, jaundice, pulmonary hypertension, abnormal liver parameters, and, in some cases, transient thrombocytopenia. Red blood cell transfusions are often required for newborns and infants but are only occasionally necessary later in life. Splenomegaly is evident in 80% to 90% of adult patients. Occasionally, severe erythroid hyperplasia may cause skeletal deformities such as frontal bossing or paravertebral tumors secondary to extramedullary hematopoiesis.

Iron overload with increased ferritin levels is frequent even in untransfused patients. Liver cirrhosis, increased skin pigmentation, and endocrine dysfunction as a result of iron overload have been reported in occasional patients.[534,535] The increased iron loading is due to the enhanced absorption of iron that usually accompanies ineffective erythropoiesis. In fact, hepcidin levels are reduced in CDA type I patients.[536]

Congenital abnormalities are associated with some CDA type I cases and include abnormalities of the fingers, toes, and wrists; short stature; pigeon chest deformity; deformity of the hips, vertebral bodies, and ribs; abnormal skin pigmentation; congenital ptosis; and deafness.[537-539]

Laboratory Findings. The anemia in CDA type I is moderate, with hemoglobin levels between 6.6 and 11.6 g/dL. Red cell morphology includes macrocytosis, severe anisopoikilocytosis, and basophilic stippling. Inappropriate suppression of hepcidin, high levels of serum hemojuvelin, and high levels of growth differentiation factor 15 (GDF15) have been described in patients with CDA I and II, probably contributing to secondary hemochromatosis. The role of GDF15 in iron absorption is uncertain. The bone marrow in patients with CDA I shows erythroid hyperplasia with megaloblastic changes. Diagnostic of CDA type I is the finding of internuclear chromatin bridges between nearly completely separated erythroblasts, which is seen in about 0.6% to 2.8% of the early and late polychromatic erythroblasts (Fig. 7-23).[539] Though characteristic of CDA type I, internuclear chromatin bridges may also be found in acquired dyserythropoietic states, such as MDS. The characteristic electron microscopic abnormality in CDA type I is a spongy "Swiss-cheese" appearance of heterochromatin in up to 60% of intermediate and late erythroblasts. Additional findings characteristic of CDA type I are summarized in

TABLE 7-11 Features of Congenital Dyserythropoietic Anemias

Feature	Type I (see Fig. 7-23) MIM #224120	Type II (HEMPAS, see Fig. 7-24) MIM #224100	Type III (see Fig. 7-25) MIM #105600	Type IV MIM #613673
Male/female ratio	1:1	0:9	0:8	
Incidence	<1/100,000/year; founder effect in Switzerland, Israel	<1/100,000/year; most common form; founder effect in Italy	Very rare; founder effect in Sweden, Argentina	Very rare (4 patients reported)
Anemia	Mild to moderate	Moderate	Mild to moderate	Severe hemolytic
Red cell size	Macrocytic	Normocytic or macrocytic	Macrocytic	Normocytic
Bone marrow erythroblasts				
Light microscopy	Megaloblastic, binucleated (2% to 5%), chromatin bridges	Normoblastic, binucleated (10% to 40%)	Gigantoblasts (10% to 40%)	Binucleate forms, nuclear budding, rare karyorrhexis
Electron microscopy	Spongy "Swiss-cheese" appearance of heterochromatin (60%)	Peripheral double membranes	Nonspecific, intranuclear clefts, karyorrhexis	Rare immature erythroid cells with marked heterochromatin
Altered red cell proteins	Low levels of protein 4.1, impaired globin synthesis	Abnormal glycosylation of bands 3 and 4.5, reticular endoplasmic proteins		Markedly increased red cell adenosine deaminase, absence of In(Lu) unique blood group phenotype, Co(a-b-), In(b-), and LW(ab-) and Lu(b+), low/lack CD44, low/lack AQP1, low BCAM and ICAM4
Acid serum hemolysis (Ham test)	Negative	Positive	Negative	Negative
Agglutinability				
Anti-i	Normal	Strong	Slight	
Anti-I	Slight	Strong	Slight	
Glycosylation	Some abnormalities	Markedly abnormal	Some abnormalities	
Fetal hemoglobin				Markedly elevated
Congenital abnormalities	(+/–) limb[732-734]			Multiple anomalies in 1 patient
Red blood cell transfusion requirement	In children, rarely in adults		Rarely	Frequent
Iron overload	Frequent, independent of red blood cell transfusions	Frequent, independent of red blood cell transfusions	No	Frequent, independent of red blood cell transfusions
Extramedullary erythropoiesis	Sometimes			Erythroid expansion
Gallstones	Frequent			
Hepatosplenomegaly	Mild	Mild		Hepatomegaly, severe splenomegaly
Splenectomy	Usually not recommended	96%	100%	Performed in 1 patient associated with clinical improvement
Interferon-alfa	Effective in majority of patients			

TABLE 7-11 Features of Congenital Dyserythropoietic Anemias (Continued)

Feature	Type I (see Fig. 7-23) MIM #224120	Type II (HEMPAS, see Fig. 7-24) MIM #224100	Type III (see Fig. 7-25) MIM #105600	Type IV MIM #613673
HSCT	Effective (1 case report[735])			
Inheritance	Autosomal recessive	Autosomal recessive	Autosomal dominant and sporadic	Autosomal dominant
Gene locus*	15q15.1-15.3	20q11.23	15q21-25	19p13.2
Gene mutated (MIM gene number)	Codanin-1 (*CDAN1*)† MIM #607465	*SEC23B (CDAN2)* MIM #610512	*KIF23MKLP1*‡ *(CDAN3)* MIM #605064	*KLF1 (CDAN4)* MIM #600599

Modified Ayas M, al-Jefri A, Baothman A, et al: Transfusion-dependent congenital dyserythropoietic anemia type I successfully treated with allogeneic stem cell transplantation. Bone Marrow Transplant *29:681–682, 2002; Holmberg L, Jansson L, Rausing A, Henriksson P: Type I congenital dyserythropoietic anaemia with myelopoietic abnormalities and hand malformations.* Scand J Haematol *21:72–79, 1978; Le Merrer M, Girot R, Parent P, et al: Acral dysostosis dyserythropoiesis syndrome.* Eur J Pediatr *154:384–388, 1995.*

HEMPAS, Hereditary erythroblastic multinuclearity with a positive acidified serum lysis test; *HSCT*, hematopoietic stem cell transfusion; *MIM*, Mendelian Inheritance in Man.

*Gene locus for some, but not all, patients in each category.

†Mutated in the majority of patients but not all patients with CDA type I.

‡Mutated in the familial form but not the sporadic form of CDA type III.

Table 7-11. The acidified serum lysis test is negative, and red cells usually show normal agglutinability with anti-i. The cause of the unbalanced globin chain synthesis in some patients with CDA type I is not known.[535]

Patients with CDA type I mount a substantial hematologic response to recombinant interferon alfa-2a or alfa-2b. Long-term interferon alfa therapy has been shown to reduce iron overload. The basis of the response to interferon is not known.[530,531]

Genetic Characteristics. CDA type I is inherited as an autosomal recessive disease. Homozygosity mapping of Israeli Bedouin families localized the gene responsible for CDA type I to chromosome 15q15.1-15.3 and led to identification of the responsible gene, named *CDAN1*.[540] *CDAN1* has 29 exons and encodes a putative 1226–amino acid protein termed codanin-1. The function of codanin-1 is part of a cytosolic complex regulating S-phase histone supply and acts as a negative regulator of chromatin assembly. Depletion of codanin-1 accelerates the rate of DNA replication, whereas overexpression arrests S-phase progression.[541] CDAN1 mRNA is found at low levels in a wide variety of tissues. The majority of mutations in *CDAN1* are missense mutations causing single–amino acid substitutions in the mutant protein. Nonsense mutations, splice-site mutations, and nucleotide insertions or deletions causing a shift in the open reading frame are rare. Homozygosity for 3238 C→T mutation causing an arginine-to-tryptophan substitution at amino acid position 1040 (R1040W) was identified in 45 Bedouin and 11 Israeli Arab families, which suggests a founder effect in these populations. No homozygotes or compound heterozygotes for *CDAN1* null mutations have been identified, indicating that residual codanin-1 function is essential during development. In some families with classic CDA type I, the disease does not segregate with chromosome 15q15.1-15.3, suggesting that the disease is genetically heterogeneous.[539,542]

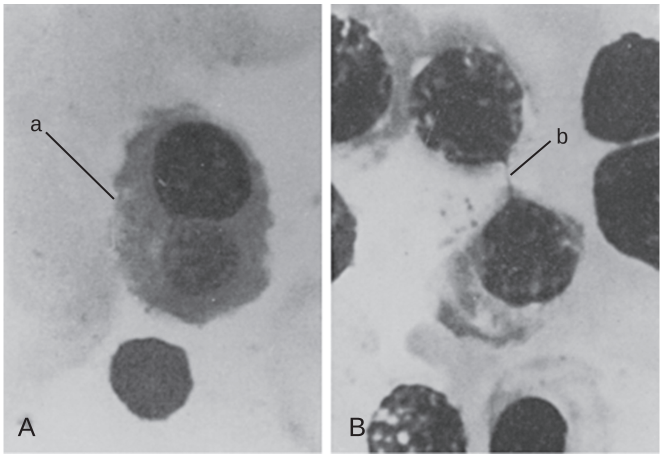


Figure 7-23 Bone marrow from a patient with congenital dyserythropoietic anemia type I. **A,** Small letter a indicates binucleate erythroblast, with nuclei of different size and maturity. **B,** Small letter b indicates internuclear chromatin bridges connecting two erythroblasts. *(From Lewis SM, Verwilghen RL, editors:* Dyserythropoiesis *London, 1977, Academic Press.)*

Congenital Dyserythropoietic Anemia Type II

Clinical Characteristics. The incidence of CDA type II is about 1 in 100,000 births per year, and with more than 300 reported cases, CDA type II is the most frequent form of CDA. The prevalence of CDA II varies widely, with the highest prevalence in Italy (2.49 cases/million).[543] CDA type II is also known as hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS, see later).[525,544] The extent of anemia in patients with CDA type II varies from mild to severe. About 10% of patients require red cell transfusions in infancy and childhood but rarely thereafter.[545] Splenomegaly and the development of gallstones are common.

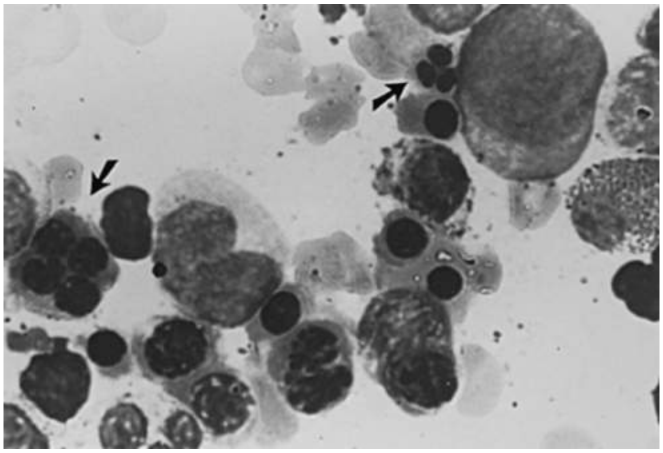


Figure 7-24 Bone marrow from a patient with congenital dyserythropoietic anemia type II, showing binucleate and multinucleate erythroblasts. *(Courtesy of Dr. Gail Wolfe; From Alter BP: The bone marrow failure syndromes. In Nathan DG, Oski FA, editors:* Hematology of infancy and childhood, *3rd ed. Philadelphia, 1987, WB Saunders, p. 159.)*

Extramedullary hematopoiesis with the development of paravertebral tumors is rare.[545,546] Progressive iron overload is seen even in untransfused patients, and liver cirrhosis secondary to iron overload develops in about 20%.

Dysmorphic features appear to be less common than in CDA type I. Mental retardation has been reported in some patients with CDA type II.

Laboratory Findings. Hemoglobin in patients with CDA type II is generally between 8 and 11 g/dL, with a normal mean corpuscular volume.[545] Red cells are usually normocytic, with moderate to marked anisocytosis, anisochromasia, and poikilocytosis (including teardrop-shaped poikilocytes); occasional basophil stippling cells; and a few circulating erythroblasts. There is normoblastic erythroid hyperplasia in the bone marrow, with 10% to 35% of binucleate erythroblasts and, rarely, multinucleate late polychromatic erythroblasts (Fig. 7-24). Pseudo-Gaucher macrophages with birefringent needles may be found in the majority of cases.[545] Electron microscopy shows stretches of double membrane parallel to the inner surface of the erythroblast cell membrane that represent excess proteins containing smooth endoplasmic reticulum, which are normally found in this organelle (i.e., calreticulin, glucose-regulated protein [GRP78], and protein disulfide isomerase).

The red cells of most patients with CDA type II are lysed in the acidified serum lysis test (Ham test) when mixed with 30% to 60% of fresh ABO-compatible normal sera but not the patient's own serum.[544] The reactive sera contain a naturally occurring IgM antibody that recognizes an antigen present on CDA type II cells but not on normal cells. CDA type II red cells show increased agglutinability with anti-i antibody. In contrast to red cells from patients with paroxysmal nocturnal hemoglobinuria, which lyse in the acidified serum lysis test even with autologous serum because of complement activation, CDA type II cells lyse because of persistence of the "HEMPAS antigen" on their surfaces. Thus CDA type II cells do not lyse in the sucrose lysis test.

Incomplete processing of N-linked oligosaccharides leads to defective glycosylation of several usually highly glycosylated membrane proteins such as band 3 (the anion transport protein), band 4.5 (glucose transporter 1), and glycophorin A.[547,548] It has been speculated that an abnormality in the endoplasmic reticulum may be responsible for the defective glycosylation of red cell membrane constituents in CDA type II. Interestingly, abnormal glycosylation has also been found in other proteins such as serum transferrin (synthesized by hepatocytes).

Genetic Characteristics. Inheritance of CDA type II has an AR pattern. Genome-wide linkage analysis has localized the disease gene (*CDAN2*) to a 5-cM region of chromosome 20q11.2. This has led to identification of the *SEC23B* gene encoding the SEC23B component of the coat protein (COP) II complex as the gene responsible for the vast majority of CDA II cases.[549,550] Over 60 different mutations have been identified in patients with CDA II, including missense, frameshift, nonsense, and splice-site mutations.[549-552] There is no clear phenotype-genotype correlation. No patients with biallelic *SEC23B* null mutations have been identified, suggesting that complete protein deficiency is lethal. COP II protein complex–coated vesicles transport proteins from the endoplasmic recticulum to the Golgi apparatus. Mutations in SEC23B result in a misglycosylation and impaired clearance of the endoplasmic recticulum, accounting for the accumulation of endoplasmic reticulum proteins on the periphery. However, the specificity of the disease phenotype to erythropoiesis and its effect on cell division leading to the multinuclearity in erythroid cells remain to be determined. Interestingly, SEC23B and codanin-1 have been shown to colocalize, suggesting a shared disease pathway.[553]

In rare families, the disease gene is not linked to chromosome 20q11.2, a finding indicative of genetic heterogeneity.[554]

Congenital Dyserythropoietic Anemia Type III

CDA type III is rare, with about 60 cases reported, the majority in Sweden. Both familial and sporadic cases have been reported. In familial cases the inheritance pattern is AD. Splenomegaly was present in some patients but not in others. In contrast to CDA types I and II, iron overload seems not to be clinically significant in patients with familial CDA type III, most likely because of intravascular hemolysis and iron loss by hemosiderinuria. Visual disturbances with macular degeneration and angioid streaks, monoclonal gammopathy of undetermined significance, and myeloma have been described in individual families.[555] Sporadic CDA type III cases are clinically heterogeneous. Hepatosplenomegaly and significant iron overload have been described in some.[556]

Laboratory Findings. Anisopoikilocytosis, basophilic stippling of red cells, and some very large red cells may be found in the peripheral blood smear. Serum thymidine

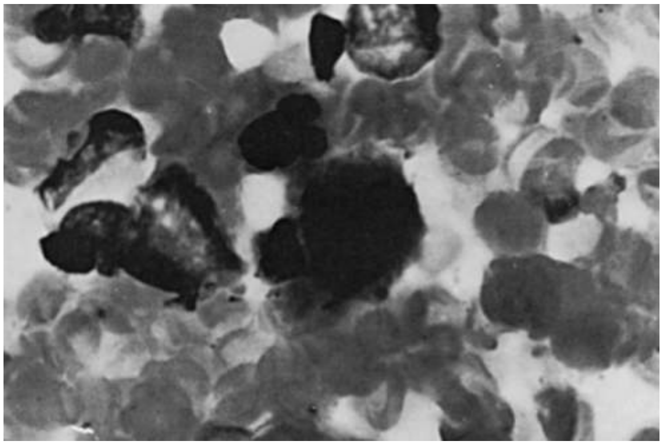


Figure 7-25 Bone marrow from a patient with congenital dyserythropoietic anemia type III, showing multinucleate erythroblast. *(Courtesy of Dr. Gail Wolfe; From Alter BP: The bone marrow failure syndromes. In Nathan DG, Oski FA, editors:* Hematology of infancy and childhood, *3rd ed. Philadelphia, 1987, WB Saunders, p. 159.)*

kinase activity is elevated in most patients with CDA III. In families with hemosiderinuria, iron deficiency may be found. The bone marrow contains multinucleate and mononucleate erythroblasts (Fig. 7-25).[557,558] Other dysplastic changes include basophilic stippling, nuclear lobulation, and karyorrhexis. Erythroblasts show a variety of nonspecific ultrastructural abnormalities, intranuclear clefts, karyorrhexis, abnormalities in the nuclear membrane, and large autophagic vacuoles.[558] Giant multinucleate and mononucleate erythroblasts may also be seen in MDS and erythroleukemia.

Linkage analysis in one large multiplex family localized the disease gene (*CDAN3*) to chromosome 15q22, distal to the *CDAN1* gene.[559] Array-based sequence capture identified a point mutation (c.2747C→G p.P916R) in *KIF23* encoding mitotic kinesin-like protein 1 (MKLP1) that segregated with disease and seems to be responsible for CDA III in all Swedish and American families.[560] MKLP1 plays critical roles in cytokinesis, accounting for the large multinucleated erythroid progenitor cells characteristic of CDA III.[560]

Congenital Dyserythropoietic Anemia Type IV

CDA IV is exceedingly rare; only four patients have been described. All have severe hemolytic anemia with hyperbilirubinemia, a markedly elevated fetal hemoglobin of 40% or higher, iron overload, and marrow showing moderate dyserythropoiesis. Additional features include splenomegaly and growth delay.

Laboratory Findings. In one case CDA IV was associated with additional complex congenital anomalies (see Table 7-11). Patients with CDA IV have a specific red cell phenotype that is Co(a-b-), In(b-), and LW(ab-) and not the In(Lu). Additional characteristics of CDA IV are the low expression of or lack of expression of CD44 and AQP1, as well as low expression of BCAM and ICAM4. Some patients also have persistent expression of embryonic globins.

Genetic Characteristics. All four reported patients are heterozygous for a G-to-A transition in exon 3 of the *KLF1* gene, resulting in an E325K substitution in erythroid-Krüppel–like factor (EKLF), an erythroid specific transcription factor essential for the patterns of erythroid gene expression. KLF1 expression plays a critical role in the switch from fetal to adult globin[561] and also regulates CD44 and AQP1 expression on red cells. *KLF1* mutations lead to a number of distinct human disorders, including the lack of the Lutheran group antigen, hereditary persistence of the fetal hemoglobin, and CDA II. The mutation E325K is thought to have a dominant negative effect.[561] The Nan (severe neonatal anemia) mouse has a hemolytic anemia that displays many of the features of CDA III, and hereditary spherocytosis carries a mutation in the same conserved EKLF1 amino acid.[562]

Additional Variants of Congenital Dyserythropoietic Anemia

A number of forms of CDA cannot be classified as type I, II, III or IV. In a tentative phenotype-based classification, some of them have been assigned to four groups designated CDA group V, VI, and VII, consisting of three or more unrelated families.[556,563] In addition, there are several reports of individual families who have not been further classified.

There are a number of other conditions that have congenital dyserythropoietic anemia as part of their syndromic manifestations, such as Majeed syndrome caused by mutations in *LPIN2* (18p11.31),[564] a syndromic case of dyserythropoiesis associated with exocrine pancreatic insufficiency caused by *COX4I2* mutations,[565] and a patient with two causative mutations in the mevalonate kinase gene (*MVK*).[565]

Congenital Dyserythropoiesis Associated with Thrombocytopenia

X-linked dyserythropoiesis with thrombocytopenia (MIM 305371) is not usually considered a form of CDA; nevertheless, it is associated with mild dyserythropoiesis. X-linked congenital dyserythropoiesis with thrombocytopenia results from mutations in the transcription factor *GATA1*, which cause several hematologic conditions with overlapping phenotypes. These include recessive X-linked thrombocytopenia and mild dyserythropoiesis, X-linked thrombocytopenia with the thalassemia minor phenotype, X-linked Lu(a-b-) phenotype, and mild dyserythropoiesis and red cell aplasia similar to DBA (see DBA section).[473] Because bleeding secondary to thrombocytopenia plus impaired platelet function is the major clinical feature, X-linked dyserythropoiesis with thrombocytopenia is discussed in more detail with the inherited thrombocytopenias (see Chapter 30).

Severe Congenital Neutropenia

Severe congenital neutropenia (SCN, MIM 202700) is a heterogeneous group of disorders first described in an extended consanguineous family by the Swedish physician Rolf Kostmann (Box 7-19).[566,567] The disease has thus also been known as *Kostmann syndrome*, although this eponym has since been used to define a subset of patients with AR inheritance of SCN. The incidence of

Box 7-19 **Severe Congenital Neutropenia Associated with *ELANE* Mutations**

Approximately 60% of cases of severe congenital neutropenia are associated with heterozygous mutations of *ELANE* (MIM #202700). This disorder is characterized by chronic severe neutropenia (absolute neutrophil count <500 cells/µL) and is associated with an arrest in myeloid maturation at the promyelocyte/myelocyte stage of granulocytic differentiation. Affected patients have marked susceptibility to infection and a very high risk of progression to acute myeloid leukemia.

SCN is estimated to be approximately 1 to 10 cases per million, and both genders are affected equally. SCN has been reported in a broad range of ethnic groups, although there is evidence that the incidence of SCN may be lower in American blacks than in the general U.S. population.[401]

Clinical Manifestations

SCN is characterized by severe neutropenia at birth, with ANCs generally below 200 cells/µL. With infection there may be an increase in neutrophils in blood, but this is always transient and usually modest. Other hematologic parameters are generally normal, although peripheral monocytosis and eosinophilia are often observed.[568] Bone marrow cellularity is normal to slightly decreased.[569] In the majority of patients, there is an arrest in myeloid maturation, with a relative, but not absolute, increase in promyelocytes or myelocytes (Fig. 7-26). The promyelocytes often display vacuolization.[570] Bone marrow eosinophilia and monocytosis are common.[569] The number of progenitors committed to the granulocyte lineage is reduced, and they display decreased responsiveness to G-CSF in vitro.[571-574]

Patients with SCN are prone to infections from birth and may initially have omphalitis.[575] In half of patients, infections develop during the first month of life and in 90% by 6 months. Common types of infection include mouth ulcers, gingivitis, cellulitis, skin abscesses, and otitis media. Gingivitis is a frequent problem during early childhood and can lead to premature tooth decay and loss.[576] Pneumonia and deep-tissue abscesses occur frequently and may be life threatening. Indeed, despite therapy with G-CSF, death due to infection remains a major concern in SCN, with a 12% cumulative incidence of death due to sepsis after 15 years of G-CSF therapy.[354,577] The most common causes of infection are *Staphylococcus* and *Streptococcus* species.

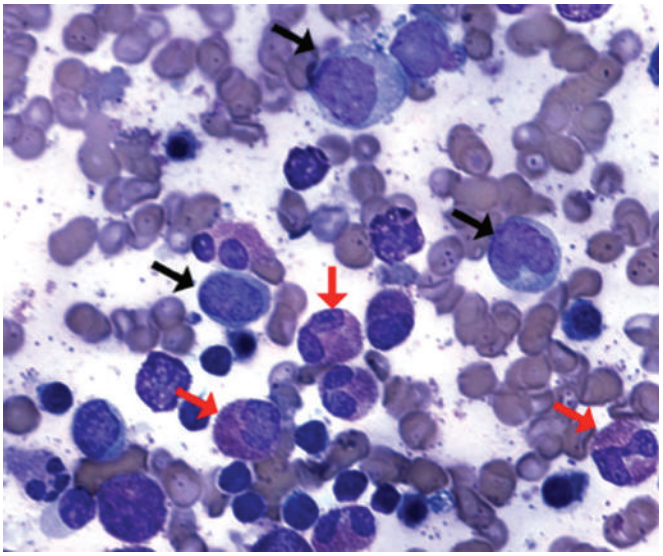


Figure 7-26 Bone marrow aspirate from a patient with SCN showing a paucity of mature neutrophils, relative accumulation of promyelocytes *(black arrows)*, and marrow eosinophilia *(red arrows)*.

There is a high incidence of osteopenia in patients with SCN. In the 422 patients with SCN enrolled in the Severe Chronic Neutropenia International Registry (SCNIR), 28% had bone mineral density measurements or radiographic examinations performed as part of routine care.[401] In this subgroup of patients there was evidence of osteopenia in nearly half. Moreover, pathologic fractures, particularly in vertebrae, have been reported in a substantial minority of patients.[578-580] Although chronic G-CSF treatment has been shown to induce osteopenia in mice,[581] the relationship of long-term G-CSF treatment in humans to osteopenia remains uncertain. A small trial suggested that treatment with bisphosphonates improves the osteopenia in patients with SCN.[579] Given the high incidence of osteopenia and potentially effective therapy, it is reasonable to perform periodic assessment of bone mineral density in all patients with SCN.

Predisposition to Malignant Disease

Patients with SCN have a markedly increased risk for MDS or AML.[354,577,582-584] The North American SCNIR has an ongoing prospective observational study of 374 patients with SCN. The most recent update from this study showed that the cumulative incidence of MDS/AML was 22% after 15 years of G-CSF therapy (Fig. 7-27). Of note, the estimated hazard rate of MDS/AML is approximately 2% per year even after 10 years of G-CSF therapy. Thus the total percentage of patients at risk for MDS/AML is likely to be very high. Similar high rates of transformation to MDS/AML were reported in a Swedish cohort of 21 patients with SCN (31% at 15 years)[582] and a French cohort of 101 patients with SCN (11% at 20 years).[583] Approximately half of patients in whom myeloid malignant tumors develop are found to have MDS, typically classified as refractory anemia with excess blasts.[354,584] A consistent feature of myeloid transformation in SCN is the high rate of chromosome 7 abnormalities. In the largest series, nearly 60% of AML/MDS samples displayed complete or partial loss of chromosome 7.[584]

Both the dose of G-CSF and the neutrophil response to G-CSF are predictive of transformation to MDS/AML. In the subgroup of patients that received more than the median dose of G-CSF (>8 µg/kg/day) but achieved less than the median neutrophil response (ANC <2188/µL), the cumulative incidence of MDS/AML was 34% at 15 years of G-CSF therapy.[577] This compares with a cumulative risk of MDS/AML of 15% at 15 years in patients who responded well to a lower dose of G-CSF (ANC

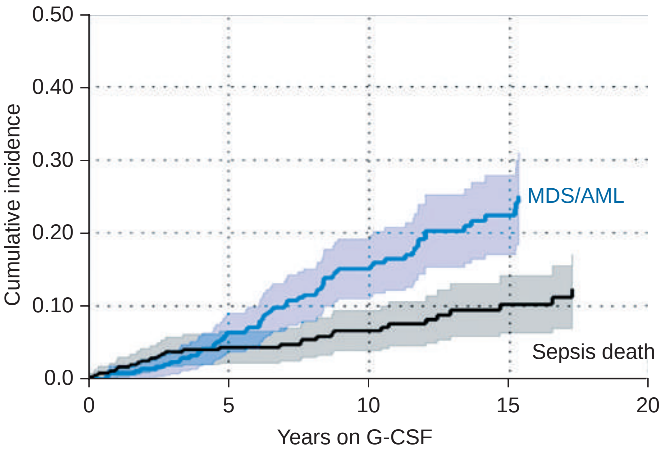


Figure 7-27 Cumulative incidence (cumulative proportion experiencing each event as initial cause of failure, in patients at risk for each adverse event) of myelodysplastic syndrome/acute myeloid leukemia or death from sepsis by years on therapy with granulocyte colony-stimulating factor. The 95% confidence intervals are indicated by the shaded regions. *(From Rosenberg PS, Zeidler C, Bolyard AA, et al: Stable long-term risk of leukaemia in patients with severe congenital neutropenia maintained on G-CSF therapy.* Br J Haematol *150:196–199, 2010.)*

≥2118/μL taking G-CSF ≤8 μg/kg/day). MDS/AML has been reported in patients with SCN associated with *ELANE* or *HAX1* mutations and in SCN of unknown etiology.[577,582] Of note, the risk of MDS/AML may be less in patients with *G6PC3* mutations. In a cohort of 28 patients with *G6PC3* mutations and SCN, no cases of MDS/AML were observed with 291 person-years of observation.[585] Malignant transformation is rare (<1%) in individuals with cyclic neutropenia or idiopathic neutropenia.[354]

Differential Diagnosis

A detailed discussion of secondary neutropenia, including immune-mediated, drug-related, and infection-related neutropenia, is provided in the section on the phagocytic system. Inherited causes of moderate to severe neutropenia are shown in Table 7-12. Cyclic neutropenia (MIM 162800), which shares the same gene mutation (*ELANE*) as most cases of SCN, is characterized by 21-day oscillations in the number of circulating neutrophils.[586,587] *WHIM syndrome* (MIM 193670) (warts, hypogammaglobulinemia, infections, and myelokathexis) can be distinguished from SCN by noting a normal to increased number of mature neutrophils in bone marrow and the presence of *CXCR4* mutations in most cases.[588-590] Both cyclic neutropenia and WHIM syndrome are covered in

TABLE 7-12 Inherited Disorders Associated with Severe Chronic Neutropenia

	MIM				
Disorder	**Phenotype**	**Morbid**	**Gene**	**Inheritance**	**Genetic Mutation**
Severe congenital neutropenia	202700	SCN1	130130	AD, sporadic*	*ELANE*
	610738	SCN3	605998	AR	*HAX1*
	612541	SCN4	611045	AD, sporadic	*G6PC3*
	613107	SCN2	600871	AD	*GFI1*
	162830	NA	138971	AR	*CSF3R*
	300299	NA	300392	XLR	*WAS*
Cyclic neutropenia	162800	NA	130130	AD	*ELANE*
WHIM syndrome/myelokathexis	193670	NA	162643	AD, AR	*CXCR4* (AD)
Shwachman-Diamond syndrome	260400	NA	07444	AR	*SBDS*
Barth syndrome	302060	NA	300394	XL	*TAZ*
Pearson syndrome	557000	NA	mtDNA	Mitochondrial	Variable deletions
Glycogen storage disease type Ib	232220	NA	602671	AR	*SLC37A4* (G6PT1)
Chédiak-Higashi syndrome	214500	NA	606897	AR	*LYST*
Griscelli syndrome (type 2)	607624	NA	603868	AR	*RAB27A*
Cartilage-hair hypoplasia	250250	NA	157660	AR	*RMRP*
Dyskeratosis congenita	305000	NA	300126	XL	DKC1
	127550		602322	AD/AR	TERC
	613989		187270	AD/AR	TERT
Congenital neutropenia with p14 (MABPIP) deficiency	610798	NA	610389	AR	*LAMTOR2* (p14)
Poikiloderma with neutropenia	604173	NA	613276	AR	*USB1*
Hyper-IgM syndrome	308230	NA	300386	XL, AR	CD40LG (XL), *CD40* (AR)

AD, Autosomal dominant; *AR*, autosomal recessive; *mtDNA*, mitochondrial DNA deletion; *NA*, not yet assigned; *XLR*, X-linked recessive.
*Sporadic cases are thought to usually represent de novo mutations of *ELANE*.

detail in the phagocytic section (see Chapter 22). The other disorders can be distinguished by the presence of associated clinical features, including exocrine pancreatic insufficiency (SDS), cardiomyopathy (Barth syndrome), lactic acidosis (PS), hypoglycemia (glycogen storage disease type Ib), partial albinism (Chédiak-Higashi, Griscelli, or p14 deficiency syndromes),[591] rash/poikiloderma (poikiloderma with neutropenia),[592] and dysgammaglobulinemia (hyper-IgM syndrome)[593-595]; each of these disorders is discussed in detail elsewhere in this text. Genetic testing is becoming an increasingly important tool in the diagnosis of congenital neutropenia.[596] Clinical genetic testing for mutations of many of the genes listed in Table 7-12 is available.

Genetic Characteristics and Pathophysiology

SCN demonstrates multiple modes of inheritance, including AR, AD, X-linked, and sporadic patterns. Accordingly, recent genetic studies have identified multiple gene mutations in SCN, including *ELANE* (formerly *ELA2*) (MIM 202700), *HAX1* (MIM 610738), *G6PC3* (MIM 611045), *GFI1* (MIM 613107), *CSF3R* (MIM 162830), and *WAS* (MIM 300299). In approximately 30% of cases of SCN, the genetic basis has not yet been identified. By far the most common mutations affect the *ELANE* gene encoding neutrophil elastase, which accounts for approximately 60% of all cases (all in AD or sporadic SCN).[597-599,600] To date, more than 100 distinct mutations of the *ELANE* gene have been reported in patients with cyclic neutropenia or SCN. Most of the mutations (≈70%) are missense mutations, although mutations that lead to alternative splicing (≈10%), frameshifts (≈10%), and premature stop codons (≈10%) have also been observed. Although specific *ELANE* mutations are typically associated with either SCN or cyclic neutropenia, some specific *ELANE* mutations are associated with both, suggesting that modifying genes determine the severity of neutropenia.[601]

The molecular mechanisms by which *ELANE* mutations disrupt granulopoiesis are unclear. Genetic studies provide two important clues. First, in all cases the *ELANE* mutations are heterozygous, which suggests a dominant mechanism of action. Second, a case report of paternal mosaicism for an *ELANE* mutation provides evidence that expression of mutant neutrophil elastase inhibits granulopoiesis in a cell-intrinsic fashion because no toxic paracrine effects of mutant neutrophil elastase on wild-type granulocytic cells in this mosaic individual were observed.[602] Neutrophil elastase is a serine protease stored in the primary (azurophilic) granules of neutrophils and (at a much lower level) in monocytes. Although the physiologic substrates have not been fully defined, there are many recognized and potential substrates for neutrophil elastase, including coagulation proteins, growth factors, and extracellular matrix components.[603] However, no consistent effect of *ELANE* mutations on neutrophil elastase proteolytic activity, substrate specificity, or serpin inhibition has been observed.[604] Though still controversial, the current model of disease pathogenesis suggests that *ELANE* mutations disrupt granulopoiesis through induction of the unfolded protein response (UPR).[605-607] In this model, *ELANE* mutations result in the production of misfolded neutrophil elastase protein, which accumulates in the endoplasmic reticulum, activates the UPR, and ultimately induces apoptosis of granulocytic precursors. The propensity of individual neutrophil elastase mutants to misfold may determine the magnitude of UPR-induced apoptosis and ultimately the clinical phenotype. Thus SCN may represent one of a growing number of disorders caused by protein misfolding.

Biallelic mutations of *HAX1* are associated with autosomal recessively inherited SCN (i.e., Kostmann syndrome).[608] The reported incidence of *HAX1* mutations in SCN varies widely in different cohort studies, ranging from 0% to 41% (mean 8.9%).[582,599,609-612] Of note, HAX1 is alternatively spliced to produce two major transcripts. Mutations that affect both types of HAX1 transcripts are associated with neurologic abnormalities, including seizures and developmental delay.[609] HAX1 is a Bcl-2–related protein that has antiapoptotic activity and is localized to mitochondria.[613,614] Thus increased susceptibility of granulocytic precursors to apoptosis is one likely mechanism of disease pathogenesis in *HAX1*-mutant SCN. However, HAX1 also has been implicated in the activation of lymphoid enhancer–binding factor 1 (LEF-1),[615] a transcription factor regulated by the canonical Wnt signaling pathway. Since reduced LEF-1 expression in granulocytic precursors from patients with SCN has been reported,[616] these data suggest that loss of LEF-1 activity also may contribute to the block in granulocytic differentiation in SCN.

Bi-allelic mutations of *G6PC3* are another cause of autosomal recessively inherited SCN.[617] The overall incidence of *G6PC3* mutations in unselected patients with SCN is approximately 4%.[599,612,618] SCN associated with *G6PC3* mutations is often, but not always,[619] associated with nonhematopoietic features, including congenital heart disease, prominent superficial veins, abnormal facial appearance, urogenital abnormalities, and intermittent thrombocytopenia.[585,617] *G6PC3* encodes for glucose-6-phosphatase catalytic subunit 6. G6PC3 deficiency is associated with increased granulocytic precursor apoptosis, possibly through activation of the UPR.[617]

An X-linked form of SCN linked to germline mutations in the *WAS* gene has been identified.[620-623] Unlike classic WAS, which results from loss-of-function mutations, WAS-associated X-linked neutropenia appears to result from gain-of-function mutations of *WAS* that disrupt an autoinhibitory domain of the WAS protein. In contrast to most cases of SCN where monocytosis is common, SCN associated with *WAS* mutations presents with monocytopenia.[599,623] Thus monocytopenia in a patient with SCN should prompt a search for *WAS* mutations. Activating mutations of WAS result in enhanced actin polymerization and genomic instability.[624,625]

Heterozygous mutations of *CSF3R* and *GFI* are rare causes of sporadic SCN. *CSF3R* encodes the G-CSF receptor. The *CSF3R* mutations in SCN are thought to act in a dominant negative fashion by inhibiting receptor trafficking to the cell surface and heterodimerization in response to G-CSF.[626-628] SCN associated with *CSF3R* mutations presents with unusually severe neutropenia that is refractory to suprapharmacologic doses of G-CSF. *GFI1* encodes a zinc-finger domain transcriptional repressor. The *GFI1* mutations are in the zinc-finger domains and are thought to generate a dominant negative GFI1 mutant protein.[629] Once again, the phenotype of SCN associated with *GFI1* mutations is distinct from that observed in classic SCN in that perturbations in lymphocyte production and function are not typically seen in SCN associated with *ELANE* mutations.

The genetic factors that contribute to transformation to MDS/AML in patients with SCN are not well defined. Indeed, the spectrum of mutations found in SCN-associated AML is distinct from that seen in de novo AML; whereas mutations in *NPM1, FLT3, KIT*, and *CEBPA* are relatively common in de novo AML, they are rare in SCN-related AML.[630] Conversely, mutations of the G-CSF receptor (*CSF3R*) are uniquely associated with the development of AML or MDS in patients with SCN.[631-634] Of note, these mutations are distinct from the previously discussed germline mutations that affect the extracellular domain of the G-CSF receptor and are thought to inhibit G-CSF signaling in a dominant negative fashion. Instead, these are acquired mutations that introduce a premature stop codon that results in truncation of the distal cytoplasmic portion of the G-CSF receptor and enhanced signaling. In the largest published series, the incidence of G-CSF receptor mutations was 78% (18/23) in individuals with SCN and monosomy 7, MDS, or AML versus 34% (43/125) in individuals without signs of malignant transformation.[633]

Studies in mice show that these mutations enhance proliferative signaling by the G-CSF receptor,[635,636] confer a clonal advantage to hematopoietic stem cells,[625] and cooperate with another oncogene to induce AML.[637] Moreover, a recent study showed the sequential acquisition of two distinct G-CSF receptor mutations in a patient with SCN who evolved to AML.[619] Together, these data strongly implicate G-CSF receptor mutations in the leukemic transformation in patients with SCN.

Clinical Management

Historically, patients with SCN had a poor prognosis and often succumbed in the first or second decade of life with recurrent severe bacterial infections. The use of G-CSF has changed the natural history of this disease; the results of a randomized phase III trial comparing G-CSF therapy with no treatment in patients with SCN demonstrated that the majority (>90%) of patients had a significant increase in circulating levels of neutrophils and a reduction in the incidence and severity of bacterial infections.[638] There is substantial patient-to-patient variability in the dose of G-CSF that is required to achieve an acceptable neutrophil count, with some children requiring only 1 to 2 μg/kg on alternate days and others responding only to doses of 50 to 100 μg/kg/day. The SCNIR currently recommends a starting dose of 5 μg/kg/day with upward twofold increases in dose until the ANC level in blood is 1000 to 1500/mL.[401] Recognized adverse effects of chronic G-CSF therapy include splenomegaly, bone pain, and vasculitis. There are case reports of patients who are refractory to G-CSF treatment alone but respond to combination therapy with G-CSF and glucocorticoids.[626] Of note, GM-CSF treatment is not effective in SCN; it increases circulating eosinophils but not neutrophils.[568] In addition to G-CSF treatment, patients with SCN should receive regular and frequent dental care because the rate of periodontal disease remains high despite G-CSF treatment.[576]

Hematopoietic Stem Cell Transplantation. Allogeneic stem cell transplantation is the only current curative therapy for SCN. A recent review of HSCT in SCN identified 65 patients transplanted without malignant transformation; overall and event-free survival rates were 89% and 75%, respectively.[639] Although highly dependent on the donor source and conditioning regimen, the incidence of acute and chronic GVHD was 34% and 11%, respectively. As expected, results of HSCT in SCN patients with MDS/AML are much poorer, with an overall survival rate of only 44%.[517,639-641] Current widely accepted indications for HSCT in patients with SCN include inadequate neutrophil response to G-CSF and the development of MDS or AML. A more difficult question is when to perform transplantation in SCN patients who are doing well with G-CSF and have an HLA-matched sibling donor. The SCNIR data show that in the subgroup of patients that received more than the median dose of G-CSF (>8 μg/kg/day) but achieved less than the median response (ANC <2188/μL), the cumulative incidence of MDS/AML was 34% at 15 years of G-CSF therapy.[577] Moreover, patients who acquire a G-CSF receptor mutation or monosomy 7 appear to have an elevated risk for the development of MDS or AML.[584,633] Though not confirmed in prospective studies, these criteria may help identify patients who should undergo transplantation.

Leukemia Surveillance and Controversy Regarding Leukemia Promotion by G-CSF. It should be emphasized that there is no definitive proof that G-CSF directly contributes to leukemic transformation. Indeed, reports of AML/MDS in SCN predate the use of G-CSF. Moreover, myeloid malignant diseases are rare in children and adults with cyclic neutropenia despite many years of G-CSF treatment.[354] Thus the contribution of G-CSF to the development of AML/MDS in patients with SCN remains controversial, and therapy with G-CSF should not be withheld from patients with newly diagnosed SCN on this basis. Because of the risk of leukemic transformation in SCN, patients should receive the lowest dose of G-CSF that is required to maintain an acceptable neutrophil count and should undergo yearly bone marrow examination with cytogenetic analysis.

Inherited Thrombocytopenias

Inherited forms of thrombocytopenia are rare. They are summarized in Table 7-13 (for a more detailed discussion see Chapter 30).

Two major forms of inherited thrombocytopenia are discussed in the following sections because of their association with aplastic anemia and because they have to be considered as a possible differential diagnosis in

TABLE 7-13 Inherited Thrombocytopenia Syndromes

Syndrome	**Inheritance**	**Gene Mutation**	**Chromosomal Location**	**Platelet Size MPV (7-11 fl)**	**Thrombocytopenia**	**Associated Findings**
Large Platelets						
MYH9-related thrombocytopenia						Rare progression to MDS/AML
May-Hegglin anomaly (MIM #155100)	AD	*MYH9*	22q11	>11 fl	Mild	Neutrophil inclusion
Fechter syndrome (MIM #153640)	AD	*MYH9*	22q11	>11 fl	Mild	Neutrophil inclusion Sensorineural hearing loss, nephritis, cataract
Epstein syndrome (MIM #153650)	AD	*MYH9*	22q11	>11 fl	Mild	Sensorineural hearing loss, nephritis
Sebastian syndrome (MIM #605294)	AD	*MYH9*	22q11	>11 fl	Mild	Neutrophil inclusion
Bernard-Soulier syndrome (MIM #231200) type A	AR	*GP1BA* (MIM 606672)	17p13	>11 fl	Moderate to severe	Bleeding tendency
Mediterranean thrombocytopenia/ monoallelic Bernard-Soulier carrier (MIM #153670)[737]	AD	*GP1BA* (Bolzano mutation: 515C→TAla156Val)	17p13	>11 fl	Mild	Bleeding tendency
Bernard-Soulier syndrome (MIM #231200) type B	AR	*GP1BB* (MIM 138720)	22q11.21	>11 fl	Mild	Bleeding tendency
Bernard-Soulier syndrome type C (MIM #231200)	AR	*GP9* (MIM 173515)	3q21.1	>11 fl	Moderate to severe	Bleeding tendency
Velocardiofacial/DiGeorge syndrome (MIM #192430)	AD	*GP1BB**	22q11.21	>11 fl	Mild	Cardiac, facial, parathyroid, and thymus anomalies; cognitive/learning impairment
Platelet-type von Willebrand disease (PTVWD, MIM #177820)	AD	*GPIBA*	17pter-p12	>11 fl		Occasional giant platelets, platelet count drops under stress
GATA1 mutations (MIM #305371)	X-linked	*GATA1*	Xp11.23	>11 fl	Moderate to severe	Anemia dyserythropoiesis, thalassemia
FLNA-related thrombocytopenia (FLNA-RT, nd) (MIM #300049)[738]	X-linked	*FLNA*	Xq28	>11 fl		Platelets from small to giant; usually associated with periventricular nodular heterotopia
Paris-Trousseau thrombocytopenia/Jacobsen syndrome (MIM #188025)	AD	*FLI1** Large deletion	11q23	>11 fl Giant alpha granules	Mild to moderate	Psychomotor retardation, facial anomalies

Gray platelet syndrome (MIM #139090)[739-741]	AD or AR	*NBEAL2*	3p21.1	>11 fl absent granule	Mild to moderate	Giant and "pale" platelets, risk of myelofibrosis, splenomegaly, high serum vitamin B_{12}
TUBB1-related thrombocytopenia (TUBB1-RT, nd)[742]	AD	*TUBB1*	6p21.3	>11 fl	Moderate	Giant platelets
ITGA2B/ITGB3-related thrombocytopenia	AD	*ITGA2B/ITGB3*	17q21.32/17q21.31	>11 fl	Moderate	Macrothrombocytopenia with platelet dysfunction
Normal-Sized Platelets						
Familial platelet disorder/acute myeloid leukemia (FPM, MIM #601399)	AD	*CBFA2/AML1/ RUNX1*	21q22.2	7-11 fl	Mild to moderate	MDS/AML in 40% of patients
ANKRD26-related thrombocytopenia (ANKRD26-RT or THC2, MIM #313900)[743,744]	AD	*ANKRD26*	10p2	7-11 fl	Moderate	Risk of leukemia
Congenital amegakaryocytic thrombocytopenia (MIM #604498)	AR	*MPL*	1p34	7-11 fl	Severe	Aplastic anemia in the second decade, MDS/AML
Thrombocytopenia and absent radii (MIM #274000)	AR	*RBM8A microdeletion plus polymorphism*	1q21.1	7-11 fl	Severe	Shortened/absent radii bilaterally
Thrombocytopenia and radial synostosis (MIM #605432)	AD	*HOXA11*	7p15-p14.2	7-11 fl	Severe	Fused radii, incomplete range of motion
CYCS-related thrombocytopenia (CYCS-RT or THC4, MIM #612004)[745]	AD	*CYCS*	7p15.3	7-11 fl		None
Small Platelets						
Wiskott-Aldrich syndrome (MIM #301000)	X-linked	*WAS*	Xp11.23-p11.22	<7 fl	Moderate to severe	Immunodeficiency, eczema, lymphoma
X-linked thrombocytopenia (MIM #313900)	X-linked	*WAS*	Xp11.23-p11.22	<7 fl	Moderate to severe	None

Modified from Balduini CL, Savoia A, Seri M: Inherited thrombocytopenias frequently diagnosed in adults. J Thromb Haemost *11:1006–19, 2013.*
AD, Autosomal dominant; *AML*, acute myeloid leukemia; *AR*, autosomal recessive; *MDS*, myelodysplastic syndrome.
*Contiguous gene deletion.

Box 7-20 Congenital Amegakaryocytic Thrombocytopenia

Congenital amegakaryocytic thrombocytopenia (CAMT, MIM #60448) is a rare inherited bone marrow failure syndrome of infancy characterized by isolated thrombocytopenia, paucity or absence of megakaryocytes in bone marrow, and no physical anomalies. Congenital amegakaryocytic thrombocytopenia exhibits autosomal recessive inheritance and is caused by mutations in the thrombopoietin receptor (TPO-R), more commonly known as the protooncogene *MPL*.

individuals with one of the major forms of IBMFSs discussed in this chapter.

Amegakaryocytic Thrombocytopenia

Congenital amegakaryocytic thrombocytopenia (CAMT, MIM 60448) is a rare IBMFS characterized by isolated thrombocytopenia and a paucity or absence of megakaryocytes in bone marrow (Box 7-20).[642] The isolated thrombocytopenia progresses to severe aplastic anemia in the majority of affected children.

Clinical Manifestations

Thrombocytopenia is usually diagnosed within the first week of life because of petechiae and bleeding. The thrombocytopenia is usually severe, but platelet size and morphology are normal. The diagnosis is established by bone marrow examination. Thrombopoietin levels are high, consistent with the lack of megakaryocytes and low platelet counts.[643] Physical anomalies may be present but are generally nonspecific. An increased incidence of psychomotor retardation has been reported.[644] A less severe form of CAMT is associated with moderate or transient thrombocytopenia. In contrast to thrombocytopenia with absent radii (TAR) (see later), the thrombocytopenia in patients with CAMT worsens over the first 10 years of life and progresses to severe aplastic anemia by the second decade. Transformation to MDS or MDS/AML has been reported.[644]

Differential Diagnosis

Severe CAMT has to be distinguished from other IBMFSs associated with thrombocytopenia, such as TAR and WAS (see Chapter 30). CAMT lacks the forearm abnormalities characteristic of TAR and the microthrombocytes characteristic of WAS. Thrombocytopenia and amegakaryocytosis have been reported in patients with Noonan syndrome (MIM 163950), caused by mutations of *PTPN11* (see Chapter 30). Patients with CAMT who progress to aplastic anemia may resemble those with other IBMFSs such as FA and DC. The lack of increased chromosomal fragility in the DEB or MMC test performed on cultured peripheral lymphocytes or fibroblasts, or both, is helpful in strengthening the diagnosis of CAMT. Whether telomere length distinguishes CAMT from DC remains to be determined. Severe CAMT has to be distinguished from a long list of acquired thrombocytopenias (see Chapter 30), in particular, intrauterine infections, immune thrombocytopenia of the newborn caused by antiplatelet antibodies (neonatal alloimmune thrombocytopenia), or antiplatelet antibodies secondary to maternal idiopathic thrombocytopenic purpura.

Genetic Characteristics

CAMT is an AR disorder. Mutations in the thrombopoietin receptor gene *c-MPL* are responsible for the disease.[645] Mutations have been identified throughout the *c-MPL* gene, including nonsense, missense, and splicing mutations. Affected individuals are usually compound heterozygous for *c-MPL* mutations, although homozygous mutations have been reported in consanguineous families. Because of consanguinity in affected families, homozygous *c-MPL* mutations in an affected individual are not uncommon.[644]

Genotype-Phenotype Correlation

Patients with CAMT can be divided in two groups: type I patients have complete loss of functional c-Mpl, whereas type II patients have some residual c-Mpl function. Patients with type II CAMT often show transient improvement in the platelet count and have a later onset of pancytopenia than individuals with type I CAMT.[642,644,646] Pancytopenia may be the initial clinical manifestation of individuals with type II CAMT. Heterozygous mutation carriers may have impaired megakaryopoiesis despite normal platelet counts.[647]

Clinical Management

The initial treatment of individuals with CAMT is supportive therapy consisting primarily of transfusion of irradiated leukocyte-depleted single-donor platelets to prevent serious bleeding. Hematopoietic growth factors and cytokines do not elicit a sustained response in patients with CAMT. HSCT is the only curative treatment of CAMT and should be considered in affected children with a matched sibling donor.[648] Sibling donors heterozygous for a *c-MPL* mutation have been used successfully.[649] HSCT with an unrelated transplant was historically associated with a poor outcome because of regimen-related toxicity and engraftment failure.[644,650,651] Low-intensity conditioning may improve transplant-related mortality and the outcome of HSCT in patients with CAMT.[652] A CAMT patient is likely to be an excellent candidate for future gene therapy because restoration of normal c-MPL in combination with the high levels of endogenous thrombopoietin would provide a selective growth advantage to the corrected hematopoietic stem cell.[653]

Thrombocytopenia with Absent Radii

Thrombocytopenia with absent radii (TAR, MIM 274000) is a rare but well-characterized cause of neonatal thrombocytopenia. The hallmarks of this disorder are shortened forearms as a result of bilateral defects in development of the radii and severe thrombocytopenia at birth.[654]

Clinical Manifestations

Patients with TAR have absent radii but may have normal or hypoplastic thumbs (Fig. 7-28). Other skeletal anomalies may accompany the absent radii, including ulnar shortening, thumb hypoplasia, and scapular

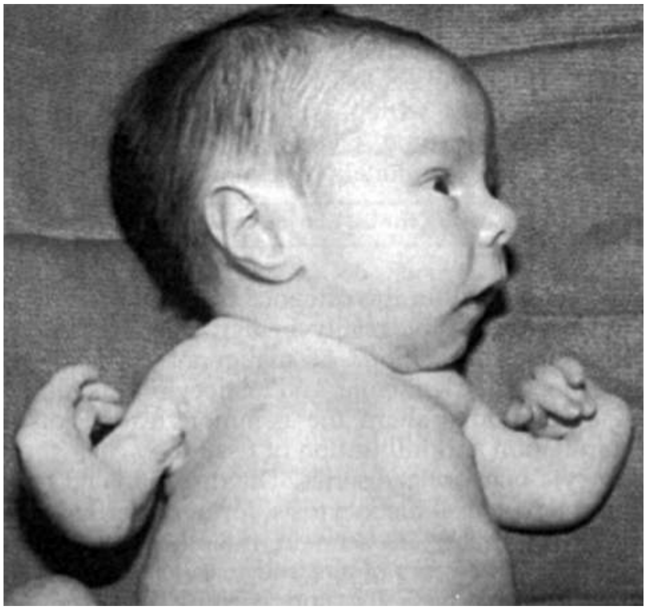


Figure 7-28 Patient with thrombocytopenia with absent radii syndrome. *(From Luchtman-Jones L, Schwartz AL, Wilson DB: The blood and hematopoietic system: diseases of the fetus and infant. In Fanaroff AA, Martin RJ, editors:* Neonatal-perinatal medicine, *7th ed. Philadelphia, 2000, Mosby-YearBook, p. 1235, Figure 44-16.)*

changes. The hands and fingers, however, are not affected. Lower limb abnormalities are frequent but usually less severe and include hip dislocation, patellar anomalies, absence of tibia and fibula joints, and phocomelia, present in about 50% of individuals.[654-657] Common nonskeletal anomalies observed in individuals with TAR include gastroenteritis and cow's milk allergy,[658] genitourinary malformations, cardiac defects, facial dysmorphisms, short stature, macroencephaly, and capillary hemangiomas.[657,659]

Platelet counts in neonates with this condition are generally below 50,000/µl. Platelet size and morphology are normal. Usually, the degree of thrombocytopenia is greatest at the time of birth. Patients demonstrate mucocutaneous bleeding, and platelet transfusions are frequently required in the first year of life. However, thrombocytopenia becomes less severe during the first year of life, and most affected individuals with TAR will not require platelet transfusions after infancy.[656,660] Levels of thrombopoietin are elevated in patients with TAR, yet such patients fail to respond to recombinant thrombopoietin.[661,662] Bone marrow aspiration reveals a decrease in megakaryocytes, although this is not required to make the diagnosis.[663] Eosinophilia and leukemoid reactions have been reported and are usually transient. The development of aplastic anemia has not been observed, but AML and acute lymphocytic leukemia have been reported in patients with TAR.[664-667]

Differential Diagnosis

Although the phenotypes of TAR and FA overlap, several features distinguish these conditions. FA patients are rarely thrombocytopenic in the neonatal period; rather, progressive BMF develops in these patients as children or adults. Only about 30% of patients with FA have a radial defect, whereas this skeletal anomaly is the sine qua non of TAR. In a patient with TAR, thumbs may be present in the absence of radii, whereas in FA, absence of the radius is always accompanied by thumb abnormalities. Despite these differences in clinical features, it is prudent to test patients with a clinical picture of TAR for increased chromosome fragility to formally rule out FA.

Amegakaryocytic thrombocytopenia with radioulnar synostosis (ARTUS, MIM 605432), caused by a mutation in the *HOXA11* gene, is usually associated with mild deformity of the forearms, the inheritance pattern is AD, and severe thrombocytopenia persists, which distinguishes this condition from TAR syndrome.[667,668] Progression to aplastic anemia has been observed in individuals with ARTUS but not those with TAR.

Trisomy 18 can be manifested as radial hypoplasia and thrombocytopenia but is usually associated with esophageal atresia.

Genetic Characteristics

TAR syndrome is caused by compound inheritance of mutations in the *RBM8A* gene, encoding Y14 protein, a component of the exon junction complex that is involved in a variety of cellular functions, including nuclear export of transcripts, translational enhancement, and nonsense-mediated decay.[669] Most individuals with TAR syndrome harbor one null *RBM8A* allele, caused by an interstitial deletion of chromosome 1q21.1,[670] plus one hypomorphic *RBM8A* allele, due to a regulatory SNP in either the 5′-UTR or first intron of the gene.[669] This compound inheritance mechanism results in Y14 protein deficiency, presumably in a cell type– and developmental stage–dependent manner.[669]

Clinical Management

TAR has a much better prognosis than CAMT; the survival curve for TAR plateaus above 70% by 4 years of age. As infants, these patients often require transfusions of single-donor irradiated platelets to maintain a platelet count above 10,000/µL. After the first year of life, platelet transfusion dependence usually diminishes. DDAVP may be helpful for minor bleeding. Recombinant IL-6 has also been reported to lessen the degree of thrombocytopenia in these patients; however, because of considerable side effects, including fever and chills, IL-6 is no longer used for individuals with TAR.[671] Splenectomy improved persistent thrombocytopenia in an adult patient with TAR. In general, HSCT is not indicated. Treatment of nonhematologic manifestations, in particular, orthopedic interventions, dominates the management of TAR later in life. For a detailed discussion of congenital thrombocytopenias, see Chapter 30.

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CHAPTER 8

Principles of Bone Marrow and Stem Cell Transplantation

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CHAPTER OUTLINE

- **HISTORY AND OVERVIEW**
- **CONDITIONING REGIMENS**
- **SOURCES OF STEM CELLS**
  - Allogeneic Stem Cell Transplantation
  - Autologous Stem Cell Transplantation
- **PERITRANSPLANT SUPPORTIVE CARE**
  - Transfusion Support
  - Nutrition Support
  - Infection Prophylaxis and Treatment
  - Veno-Occlusive Disease of the Liver
- **IMMUNE RECONSTITUTION**
- **GRAFT REJECTION**
- **GRAFT-VERSUS-HOST DISEASE**
  - Acute Graft-versus-Host Disease
  - Chronic Graft-versus-Host Disease
- **ORGAN TOXICITY ASSOCIATED WITH HEMATOPOIETIC STEM CELL TRANSPLANTATION**
- **CLINICAL APPLICATIONS OF HEMATOPOIETIC STEM CELL TRANSPLANTATION**
- **GLOBAL HEALTH AND HEMATOPOIETIC STEM CELL TRANSPLANTATION**

Hematopoietic stem cell transplantation (HSCT) is an accepted treatment for a wide variety of diseases afflicting children, including hematologic malignancies, bone marrow failure syndromes, immunodeficiency disorders, congenital hematologic defects, and select solid tumors. HSCT generally involves a conditioning phase with administration of chemotherapy with or without radiation therapy followed by the infusion of autologous (self) or allogeneic (other) hematopoietic stem cells (HSCs). The conditioning damages the patient's immune system, allowing engraftment of the new stem cells, and, in malignant diseases, can eradicate residual tumor cells, but at the cost of normal organ injury. Patients are profoundly immunocompromised as a result and are at risk for opportunistic infections. These infectious risks, together with other transplant-related complications such as graft rejection and graft-versus-host disease (GVHD) (Box 8-1), and primary disease recurrence have been the main barriers to success in HSCT. However, advances in understanding transplant biology coupled with advances in the supportive care and clinical management of HSCT recipients have made this approach an increasingly attractive modality for a broadening range of indications.

HISTORY AND OVERVIEW

Infusion of bone marrow in an effort to restore hematopoiesis was first reported in 1939 when a patient with aplastic anemia was treated by infusion of a small aliquot of fraternal bone marrow.[1] Subsequently, Jacobson and colleagues demonstrated that shielding the spleen allowed lethally irradiated mice to recover normal hematopoiesis.[2,3] Later, it became clear that the hematopoietic reconstitution of irradiated animals originated from elements in the bone marrow or spleen[4] and that these HSCs could survive cryopreservation and thawing.[5] Investigators subsequently demonstrated that animals could recover only when histocompatible HSCs were infused but suffered from lethal GVHD if given cells from a histoincompatible donor.[6] Better understanding of the essential mediators of the immune response coupled with the observation that administration of methotrexate was effective both in the prophylaxis and treatment of GVHD[7] provided the theoretical and practical tools necessary for HSCT to be attempted in humans.

The first semi-successful attempt at a human bone marrow transplant (BMT) was carried out in 1957 by E. Donnall Thomas, whose body of work in this area was later recognized by a Nobel Prize. These first experiments demonstrated that chemotherapy followed by intravenous marrow infusion could result in a transient graft, although all patients subsequently died of progressive disease.[8] In 1959, lethal doses of total-body irradiation (TBI) and bone marrow from an identical twin were used to in an attempt at transplant in two patients with advanced acute lymphoblastic leukemia.[9] Hematopoiesis

Box 8-1 **Complications of Bone Marrow Transplantation**

SHORT TERM

- Graft rejection
- Infection
- Bleeding
- Acute graft-versus-host disease
- Veno-occlusive disease of the liver
- Idiopathic pneumonitis
- Side effects of radiation therapy, chemotherapy, and immunosuppressive therapy (e.g., radiation nephritis, azotemia)

LONG TERM

- Late graft failure
- Chronic graft-versus-host disease and its sequelae (e.g., bronchiolitis obliterans)
- Pulmonary disorders
- Infection
- Altered intellectual and growth development in children
- Reduced stamina
- Endocrine dysfunction
  - Hypothyroidism
  - Growth retardation (children)
  - Pubertal delay; gonadal failure
- Sexual dysfunction (both sexes)
- Complications secondary to radiation therapy (e.g., cataracts, radiation nephritis)
- Complications secondary to immunosuppressive therapy (e.g., aseptic necrosis of bone, hemolytic uremic syndrome)
- Dental problems
- Psychosocial problems
- Increased risk for second malignancies

was established within weeks, although again both patients experienced relapse and died. The first successful allogeneic HSCT were performed in 1968 and 1969 with the survival of three patients who underwent transplants for congenital immunodeficiencies.[10-13]

HSCT is currently used in a variety of malignant and nonmalignant disorders in which replacement of HSC-derived populations of cells provides or restores normal hematopoiesis or other marrow-derived elements. In malignant diseases, infusion of previously collected autologous HSC significantly shortens the period of myelosuppression and allows for dose escalation as an approach to decreasing relapse rates in malignant diseases. Although bone marrow was initially considered the only source of pluripotent HSC, more recently it has become clear that other sources exist. For example, self-renewing HSC can be collected from umbilical cord blood (UCB). HSCs also circulate in the peripheral blood, albeit in very low numbers; they can be increased after chemotherapy or administration of hematopoietic growth factors and collected as mobilized peripheral blood stem cells (PBSCs). Exploration of the potential for cell populations derived from tissues such as liver, nervous system, and muscle to provide HSCs has been initiated.[14-18]

Reinfusion of a patient's own HSCs after administration of high-dose chemotherapy, radiation therapy, or both is referred to as autologous HSCT. Both tumor contamination and preexisting damage to HSC from prior therapy limit its application. Moreover, the lack of allogeneic immunologic response against any residual or recurrent tumor further impacts success. Despite these limitations, autologous HSCT remains the standard approach to patients with refractory or recurrent lymphoma and other select solid tumors of transplant that demonstrate a response to dose escalation of chemotherapy. In the case of congenital conditions or acquired bone marrow failure syndromes, the abnormalities of the HSCs prevent autologous HSCT from being a meaningful option for most patients. Allogeneic HSCT involves the infusion of HSCs from a related donor (RD) or an unrelated donor (UD). Complications of allogeneic HSCT, such as graft rejection and GVHD, are generally more common or severe with increasing histoincompatibility between donor and host; and a histocompatible sibling, if available, remains the preferred donor. However, it is estimated that only 15% to 40% of patients will have a matched RD.[19,20] Available alternatives include HSCT from an RD that is mismatched[21,22] or from donors in the international UD registries,[23-25] including previously collected and cryopreserved unrelated UCB.[26-28]

Specific conditioning regimens used to prepare patients are reviewed in the next section. In brief, the preparative regimen must reduce or eliminate tumor burden and/or recipient hematopoietic cells and provide sufficient immunosuppression to permit engraftment. Reduced-intensity regimens were developed in the late 1990s to decrease acute transplant toxicity and thus make HSCT possible for a broader demographic. These regimens use intense immunosuppression and harness graft-versus-tumor effect instead of cytotoxicity to address residual malignant cells. Early and late graft failure and relapse remain problematic in this setting. After conditioning, HSCs are infused intravenously into the host; the day of HSC infusion is termed day 0. Neutrophil engraftment occurs 10 to 24 days after infusion, with red blood cell and platelet recovery somewhat more delayed. A number of peritransplant and posttransplant complications influence not only survival but also quality of life after transplant. There is a risk for early graft rejection and for late graft failure. Early complications of HSCT are due to profound pancytopenia, regimen-related toxicity, immunologic reaction of the graft against host tissues (acute GVHD) if the transplant is allogeneic, and protracted immune incompetence. Late complications of HSCT are due to chronic end organ damage from drug and immune insults, ongoing or de novo manifestations of immune dysregulation such as poor immune function or chronic GVHD, and the consequences of disease recurrence.[29] These factors and complications are considered in detail later in this chapter.

Given these obstacles, the evolution of HSCT into a practical and curative therapy has been dependent on a number of important advances. These include an improved appreciation of the human histocompatibility system and development of more exact methods to establish the degree of histocompatibility between the donor and recipient. The ability to deliver the preparative regimen with greater accuracy, as illustrated both by improvements in TBI dosimetry and by the ability to monitor busulfan pharmacokinetics, have contributed to a decrease in regimen-related toxicity. Early and aggressive use of antibiotics, antifungal agents, and antiviral agents and more sophisticated screening for viral reactivation have improved survival during the time of neutropenia and the period of profound and persistent immunosuppression after engraftment. Advances in transfusion support, including viral screening and irradiation of blood products and availability of leukopheresed platelet products, have had enormous impact. Nutritional and other supportive therapies, including the ability to establish long-term vascular access with indwelling lines significantly changed the experience and comfort of HSCT recipients. Although HSCT was originally offered to a small number of patients who had no alternative therapeutic option, improved outcomes due to these and other advances have markedly increased the indications for which populations for whom HSCT can provide benefit. Analysis of registry data suggests that the lifetime probability of undergoing HSCT in the population may reach almost 1% for currently accepted indications if universal donor availability and an increase in the number of eligible patients who actually underwent transplant were to occur.[30] Indeed, given ongoing scientific advances the indications for HSCT and cellular therapies such as gene therapy that utilize a backbone of HSCT are predicted to expand. The increasing population of HSCT patients and HSCT long-term survivors will be best served by improved infrastructure and research into lessening the burden of long term effects.

CONDITIONING REGIMENS

The term *conditioning regimen* refers to the preparative drugs and/or radiation that are administered to an HSCT recipient before the graft is infused. The purpose of conditioning is threefold: first, in the setting of HSCT for malignant conditions, to eradicate any residual malignant cells; second, to be sufficiently immunosuppressive to prevent the immune system of the recipient from rejecting the incoming allogeneic cells; and third, to induce enough marrow aplasia that the donor HSCs have a competitive advantage in reconstituting hematopoiesis. The regimens that are capable of achieving all three goals are usually associated with significant toxicity. Therefore a major goal in HSCT is the development of conditioning regimens that provide an optimal balance between effective myeloablation and manageable toxicity.

The first human BMT by Thomas and colleagues in 1957,[9] and a subsequent experience using UD marrow to treat victims of a radiation accident in Belgrade in 1959,[31] established the basic principle of TBI-based conditioning. Work initiated by Santos, Owens, and Sensenbrenner in the 1960s first demonstrated the use of cyclophosphamide as a conditioning agent.[32] TBI combined with cyclophosphamide has remained the standard conditioning regimen for the past 30 years. More recently, the alkylating agent busulfan has become more widely used as an alternative to TBI, in part because of limited access to costly radiation facilities.[33]

The wide range of indications for HSCT has led to considerable variation in the design of conditioning regimens. For example, high relapse rates after HSCT stimulated attempts to increase antileukemic efficacy of conditioning regimens by employing other high-dose chemotherapy agents in place of or in addition to cyclophosphamide. In contrast, the problem of graft failure/rejection in patients receiving grafts that are T cell depleted or are from genetically disparate donors has led to regimens that increase recipient immunosuppression. Patients with aplastic anemia or with severe combined immunodeficiency (SCID) have preexisting marrow aplasia or immunosuppression, and thus conditioning regimens in these diseases do not require the same degree of ablation as regimens for patients with intact immune function. Similarly, patients with benign hematologic diseases have no need of the antileukemic effect of conditioning, and thus specialized regimens have been developed specifically for these diseases.[34] In addition, as autologous HSC support after high-dose chemotherapy became more common, many new conditioning approaches were developed, emphasizing maximal antitumor effects. Although individual studies demonstrated acceptable or even favorable outcomes related to some of these changes, no consensus as to optimal recipient preparation for either autologous or allogeneic SCT has emerged. More recently, alternative concepts regarding the relative roles of conditioning regimens and the donor immune system in achieving recipient hematopoietic ablation have been applied in a nonmyeloablative approach. Some key issues are highlighted in the next sections.

Total-Body Irradiation

Several parameters associated with TBI can be adjusted to deliver different amounts of radiation and different degrees of end organ effect. Total dose, dose rate, fraction size, interfraction interval, and shielding are among the TBI parameters most manipulated in SCT. An ideal schedule would maximize malignant cell kill, hematopoietic ablation, and, in the case of allogeneic SCT, immunosuppression while limiting acute and chronic toxicity. In principle, higher total dose, higher dose rate, and larger fraction size are associated with greater hematopoietic ablative effect (and therefore potentially greater antileukemic efficacy) and with better immunosuppression. Fractionation, the division of the total dose of radiation over time usually in a twice-daily schedule, theoretically provides improved tolerance for nonhematopoietic tissues, leading to decreased acute toxicity and reduced late effects, but carries the risk for decreased antileukemic efficacy. However, a series of studies from the 1980s supports the use of fractionated radiation, and this remains the standard today.[35,36] Studies from Seattle have demonstrated increased antileukemic efficacy with increased total dose, but lung, liver, and kidney tolerances were limiting.[37,38] Parameters of total dose, fractionation, dose rate, and schedule have been largely extrapolated to pediatric HSCT from adults.[39,40]

Busulfan

Developed in the mid-1970s, busulfan (BU)/cyclophosphamide (CY) rapidly became an established regimen for patients with acute myelogenous leukemia (AML) receiving either allogeneic[41] or autologous SCT.[42-44] The original regimen, called BU/CY4 (“big BU/CY,” BU 4 mg/kg/day

× 4 days followed by CY 50 mg/kg/day × 4 days) was joined by BU/CY2 ("little BU/CY," in which the CY was delivered as 60 mg/kg/day × 2 days),[43-47] and this regimen has been used in patients with acute lymphocytic leukemia (ALL) or chronic myelogenous leukemia (CML).[48,49] The BU/CY regimen has been used extensively in pediatric patients, particularly those with nonmalignant diseases,[50-55] in an attempt to avoid radiation-associated toxicity. The current practice of using an intravenous formulation of BU and dose adjustment to achieve a target level has decreased toxicity; effects on efficacy are more difficult to demonstrate.[56] Treosulfan, a related agent already approved in Europe,[57] has limited nonhematologic toxicity, and pediatric trials are ongoing in national trials.

TBI versus BU

Several randomized studies and meta-analyses have compared BU/CY with TBI-based regimens.[58-66] Overall, results are similar although suggestive that both relapse rates and hepatic veno-occlusive disease (VOD) may be less in the TBI-containing regimens. Although recent data support these observations, variability of the chemotherapy component of TBI-based regimens and potential differences in the antileukemic efficacy of BU/CY4 and BU/CY2 regimens[67] render definitive generalizations difficult. Reports of decreased bioavailability, increased volume of distribution, and increased clearance rate of BU in children raise the question of how to interpret studies in which such data are unavailable.[68-71] Alternative BU dosing, based on plasma levels, has been suggested,[72,73] and comparison of "optimal" BU/CY with CY/TBI has not been reported. BU-containing regimens have often been reported to result in greater toxicity when compared with TBI-based regimens; thus increases in BU dose may further increase regimen-related morbidity.[58,59,61,64,74,75] Monitoring of BU pharmacokinetics has generally, but not always, demonstrated an association of increased area under the curve with increased volume of distribution.[72,76] Individualizing BU dosing may possibly increase the risk-benefit ratio, and the recent availability of an intravenous form of BU with more dependable pharmacokinetics now makes this achievable.[77-79,80]

Alternative Myeloablative Regimens

The addition or substitution of a variety of chemotherapeutic agents to the backbone of CY/TBI, and less commonly BU/CY, has met with mixed success. Etoposide, cytarabine, and BU have been added to or substituted for CY in TBI-based allogeneic regimens.[62,81-88] Etoposide (VP-16) and melphalan have also replaced CY in some BU-based protocols.[89-92] Clofarabine, a purine nucleoside analogue, is a relatively new drug known to be very effective in relapsed/refractory pediatric ALL; ongoing studies have shown promise in using it in combination with BU to decrease relapse in advanced cases of pediatric leukemia.[93] Fludarabine has become an increasingly used drug in allogeneic transplant preparative regimens. It has potent immunosuppressive properties and minimally extramedullary toxicity. Combined with BU (BU/FLU) it provides adequate ablation for full donor engraftment,[94] and in the pediatric population it appears to have similar efficacy but less transplant-related toxicity and faster neutrophil engraftment.[95]

Fluorouracil, high-dose methotrexate, doxorubicin, cisplatin, carboplatin, thiotepa, melphalan, and many other agents have been used in a variety of high-dose chemotherapeutic regimens supported by autologous SCT. Common conditioning regimens referred to by acronyms are CBV (CY, carmustine, VP-16); BACT (BCNU [carmustine], cytarabine [Ara-C], CY, 6-thioguanine); BEAM (BCNU, etoposide, cytarabine [Ara-C], melphalan); and CBP (CY, BCNU, cisplatin). In the allogeneic setting, alternative regimens have generally shown equivalent benefit in terms of disease control but often have increased toxicity. No regimen has established unequivocal superiority in terms of disease-free or overall survival when compared with "standard regimens." Given the more limited follow-up and the extraordinary diversity of diseases and regimens, establishment of relative efficacy in the realm of autologous SCT is likely to be a prolonged process.[96-100]

Nonmyeloablative Regimens

One of the target tissues of allogeneic T lymphocytes is the hematopoietic precursors. As a result, the infusion of donor lymphocytes can be sufficient to cause marrow aplasia. Indeed this is the basis of the marrow aplasia seen in inadvertent cases of transfusional GVHD. Observations made initially in the 1980s by the Seattle group have led to a new approach to conditioning based on the immunologic elimination of normal and malignant hematopoiesis. Because this technique minimizes the doses of conventional conditioning drugs and radiation, it has been called "nonmyeloablative" or reduced-intensity conditioning.[101-107] Although allogeneic graft-versus–bone marrow can cause the necessary marrow aplasia and antileukemic efficacy, it does not supply the immunosuppression needed to avoid graft rejection. This is usually achieved by preinfusion conditioning with purine-analogue chemotherapy and/or low-dose TBI. A variety of agents including lower-dose BU, melphalan, and cytarabine have also been used for additional effect and partial HSC depletion.[101-103,105-107] A major advantage of this approach is that the toxicity of conventional myeloablative approaches can be reduced.[108] Comparisons of conventional versus nonmyeloablative regimens have shown reduced nonrelapse mortality, especially in older adults or patients with preexisting illnesses for whom HSCT is associated with significant toxic death rates.[109,110] The safety and effectiveness of this approach has led to its wider application in recent years. However, the low toxicity of nonmyeloablative regimens is balanced against the increased risk for relapse in acute leukemias and the increased risk for chronic GVHD.[111] The indications for nonmyeloablative transplant may therefore remain principally for slower-growing tumors. Because childhood hematologic malignancies rarely fall into this category, the use of nonmyeloablative transplant in children has been more limited.[103,112,113] Moreover, the increase rates of graft rejection after standard HSCT for nonmalignant conditions such as thalassemia and sickle cell anemia have precluded the broad use of nonmyeloablative transplants in these conditions.

SOURCES OF STEM CELLS

Selection of an appropriate source of HSCs for the patient undergoing HSCT is a complex undertaking. HSCs can be obtained from the bone marrow, peripheral blood, or the umbilical vein of neonates. The majority of HSCTs in the pediatric setting are performed from allogeneic donors, but autologous peripheral blood is also used as a stem cell source for transplants, particularly in patients with solid tumors. For each disease entity, the clinician must consider what sources of stem cells are available and the relative risks and benefits associated with each potential stem cell source. For allogeneic donors, the principal consideration in donor selection is the genetic similarity between donor and recipient.

Typing

Genetic disparity between host and donor can lead to graft rejection or GVHD. The genes encoding the antigens most associated with these complications lie in a group of genes known as the major histocompatibility complex (MHC). In humans, the MHC maps to a region of the short arm of chromosome 6, known as the human leukocyte antigen (HLA) system. The MHC codes for many genes are still being identified, and the exploration of the functional importance of some of these gene products is still ongoing. Among the best characterized gene products are proteins comprising the class I and class II antigens. In humans, MHC class I molecules include HLA-A, HLA-B, and HLA-C and MHC class II molecules are called HLA-D. There are at least five subregions, including DR, DQ, and DP, but the number of genes transcribed differs between different HLA class II haplotypes.

The MHC class I antigens are present on nearly all human cells (with rare exceptions such as the erythrocyte and corneal endothelium). MHC class II antigen expression is more restricted, with expression limited to "professional" antigen-presenting cells, including dendritic cells, B cells, monocytes, macrophages, and Langerhans cells. Class II antigens can also be induced on activated T cells and endothelium. MHC genes are codominantly expressed, that is, each parent contributes half of the MHC antigens expressed on each cell of their offspring. MHC class I and II antigens normally function to present partially degraded products of intracellular proteins to T cells. In this way, damaged or virally infected cells can be identified by the immune system. However, in the setting of transplantation, the recognition of MHC molecules by T cells is known as alloimmunity and is the basis for GVHD and graft rejection.

The genes of the MHC region are highly polymorphic, and less than 30% of potential recipients of HSCs have HLA-identical siblings. Moreover, even HLA-identical sibling transplants can be associated with GVHD because of differences in the sequence of the intracellular proteins associated with MHC molecules caused by polymorphisms in non-MHC genes. These polymorphisms are mostly unidentified, and those that are known to elicit alloreactive T-cell responses are termed *minor histocompatibility antigens*.[114] Although these antigens may not be directly responsible for organ and bone marrow rejection, human typing studies suggest that these minor antigens contribute to the generation of GVHD.[115,116]

Historically, HLA typing was performed by using antibodies derived from postpartum sera, from persons who have undergone transplants or transfusions, or even from persons immunized for the purpose of generating specific serologic reagents. These antibodies were used in a standardized complement-dependent, microcytotoxicity assay with purified T or B lymphocytes as their target cell.[117] The extensive cross-reactivity among products coded by the alleles of the HLA-A and HLA-B loci, known as cross-reactive groups, made precise delineation of the MHC class I antigens by this technique difficult. In contrast, HLA-D specificities were originally defined by their ability to stimulate T-lymphocyte proliferation in a mixed leukocyte culture.[118]

Because of the difficulty in precise delineation of HLA-type by serologic methods, modern HLA typing now uses high-resolution techniques that resolve the DNA sequence of specific alleles. The first attempts to use molecular genetic techniques were based on application of restriction fragment length polymorphism analysis of genomic DNA by Southern blotting using HLA region probes. This soon gave way to current technology in which sequence-specific oligonucleotide probes and, in some cases, direct sequence analysis are performed. This has also led to a great increase in the number of identified HLA specificities. This fine analysis is increasingly being used to determine the haplotypes of patients and potential donors. The implications of more exact typing within families, where the haplotypes are "conserved," are probably confined to the potential identification of minor histocompatibility antigen differences, allowing for better selection between possible donors.[115,116,119] In the UD setting, high-resolution HLA matching has significantly contributed to the improved outcomes reported in UD HSCT in the past decade.[120,121,122] Historically, an allogeneic donor must match the patient at five of the six major loci (e.g., A, B, DR) for a transplant to be feasible. Stem cells obtained from UCB are more permissive, and four of six matches are acceptable.

Allogeneic Stem Cell Transplantation

Most allogeneic transplants use HSCs from a matched sibling donor. However, with improvements in immunosuppression and ability to prevent or treat GVHD, UDs, UCB, and partially MHC-mismatched family donors are now increasingly used as sources of HSCs. There are now approximately 16 million UDs listed in over 72 registries worldwide, including registries of banked UD UCB collections.

The selection of an allogeneic HSC source is dependent on many variables. In malignant disease, the ease and speed with which a stem cell source can be obtained are important factors. For patients with aggressive malignancies, being able to proceed rapidly to transplant is particularly desirable. Increasing genetic disparity between donor and host—termed *histoincompatibility*—has historically contributed to both an increased rate of graft rejection and severe acute and chronic GVHD and is a critical factor in the choice of donor. Even in the setting of HLA-identical siblings, selection can be further refined according to donor gender and age, history of donor parity, donor infectious disease status, and other variables, all of which can contribute to the success of transplant.

For the majority of patients who do not have a matched sibling donor, potential sources include a family member who is phenotypically adequately matched at the HLA loci but is genotypically distinct or a volunteer UD donor who shares the majority of HLA alleles with the recipient. The likelihood of locating an appropriate UD donor is dependent both on the patient's ethnicity (in as much as it determines histocompatibility antigen and haplotype frequency) and on the composition of the donor pool. For example, for whites of mid- and northern European descent the likelihood of finding a six-antigen matched UD approaches 50% to 70%.[123] In contrast, the extreme genetic heterogeneity of the African-American population coupled with underrepresentation of this population in volunteer registries makes the likelihood of finding a similar matched donor 10% to 15%. Molecular typing techniques demonstrate that the allelic disparities in both whites and nonwhites is much higher than previously expected, thus making the ascertainment of truly "matched" UD less likely even as the donor pool expands.[124] Although the implications of specific HLA mismatches are incompletely understood, most studies suggest that high-resolution HLA matching is associated with improved outcome in UD HSCT.[120-122,125] Thus additional strategies to broaden the donor base by using alternative HSCs such as UCB that exhibit different biologic properties and allow greater HLA disparity have allowed for more patients with uncommon typing to find an acceptable donor.

Sources of HSC

In the past, most HSCT has used bone marrow mononuclear cells as a source of HSCs. Bone marrow is usually obtained by repeated aspiration of the posterior iliac crests while the donor is under general anesthesia. Multiple aspirations are performed to a total volume of 10 to 15 mL/kg recipient body weight. The marrow is filtered through a fine wire mesh to rid the product of aggregates and bony spicules. This is a surprisingly well-tolerated procedure and serious complications are rare, probably because the donor is generally a healthy individual undergoing an elective procedure. Potential short- and long-term complications include risks of anesthesia, blood loss and potential transfusion, pain, neurologic deficits, and psychosocial complications. In the most truly altruistic setting, UD marrow harvest, only 5% to 8% of donors later expressed any ambivalence about their participation. This was most marked if the transplant was subsequently unsuccessful.[126] Once harvested, the red cells must be removed from the bone marrow if there is ABO incompatibility before infusion into the recipient.[127] The marrow may be manipulated ex vivo to remove donor T lymphocytes (see later) or tumor cells. It is estimated that only 1% of the donor pluripotent HSC population is removed in a typical marrow harvest.

HSCs continuously recirculate from the marrow into the peripheral blood; therefore HSCs can also be harvested by collection of peripheral blood mononuclear cells, usually by leukapheresis. The frequency of cells that express the surface marker CD34 is widely used as a proxy for actual HSC frequency and allows the enumeration of stem cells. HSCs in the peripheral blood are rare, and granulocyte colony-stimulating factor (G-CSF) is usually administered to increased the detachment of HSCs from bone marrow niches and promote their egress into the peripheral blood, increasing their frequency.[128-132] Moreover, studies in adults suggest that additional strategies to further mobilize marrow HSCs are possible. AMD3100 is a reversible inhibitor of the CXC chemokine receptor (CXCR4), a receptor on the surface of HSCs that promotes adhesion to the bone marrow milieu. Combining AMD3100 with G-CSF increases HSC yield in adults,[133] but this approach has not been investigated in children. Complications of PBSC harvest have included bone pain, as a side effect of G-CSF, and inability to collect via peripheral access, necessitating central line placement.[134,135] There is an increased risk for splenic rupture resulting from G-CSF administration in healthy individuals, although this is an extremely rare complication.[136] The implications of mobilizing and harvesting PBSCs from pediatric donors have not been fully explored,[137,138] although cytopenias and hemostatic changes of unknown short- and long-term significance have been observed in healthy donors.[139,140]

Since the first description of its use in a child with Fanconi anemia in 1988,[141] UCB has become an increasingly used HSC source. Placental blood is recovered from the umbilical vein by drainage or catheterization, and techniques to optimize UCB HSC collection are being investigated.[27,142] Cord blood is immediately frozen and stored until needed. If indicated, in the related setting, determination of the HLA type of the fetus can be performed before delivery. Otherwise, typing and analysis is performed on intrapartum or postpartum samples.

Selection of a UD, whether bone marrow or UCB, is facilitated by the availability of epidemiologic information (e.g., age, sex) as well as HLA typing stored in a database accessible via the Internet. By comparing a patient's HLA type with donors listed and categorized in the registries, one can estimate the likelihood of a successful search, devise search strategies for patients with uncommon HLA types, and sort among potential HSC sources. Although stored UCB can be readily supplied after confirmatory typing, the completion of a UD bone marrow donor search is more involved. Potential donors must be contacted and consent to proceed, and the donor center must arrange for appropriate confirmatory studies and medical and other evaluations and schedule the harvest. The time from formal search to BMT averages 4 months for UD, whereas compatible UCB, where typing occurs at time of cryopreservation, can be found in days to weeks.

Engraftment

After allogeneic HSCT, donor stem cells must expand to repopulate hematopoietic niches and differentiate to form mature blood cells. HSCT recipients show accelerated telomere shortening in peripheral blood cells, which is presumably due to increased replicative demand for donor stem cells.[143] Donor hematopoietic engraftment after allogeneic HSCT can be documented both by analysis of chimerism as well as by the recovery of peripheral blood cell counts. However, sensitive DNA amplification technologies have demonstrated residual host hematopoiesis for variable periods of time, certainly up to 1 year.[144-146] Mixed lymphoid chimerism is also well documented.[144-147] The implications of mixed chimerism with respect to subsequent relapse remain controversial, probably because the current extremely sensitive assays generate data that are difficult to interpret uniformly and because outcome may well differ by disease type.[148] For example, chimerism after BMT for CML is quite reliably related to subsequent relapse while this may not be true for other diseases.[147-149]

The rate and probability of engraftment after allogeneic BMT varies with the source of HSCs, the number of HSCs given, the extent of preinfusion cell manipulation, and the GVHD prophylactic regimen used. In general, neutrophil recovery is achieved within 2 to 3 weeks and platelet recovery occurs 1 to 2 weeks thereafter. PBSC transplants are associated with a more rapid rate of engraftment than with bone marrow, most likely a result of the higher CD34+ cell dose obtained.[150] Neutrophil engraftment occurs between 1 and 6 days earlier with PBSC transplant than with BMT and platelet engraftment between 4 and 7 days earlier.[151] Neutrophil and platelet engraftment after UCB transplantation is significantly delayed in comparison with that seen in other stem cell sources.[152-156] It is a consequence of the lower nucleated cell and CD34+ cell dose in UCB products. This has limited the application of this modality in adults, and currently using two unrelated UCB units for a given patient is being studied.[157]

The use of methotrexate as GVHD prophylaxis is associated with delay in count recovery; conversely, recovery after T-cell–depleted BMT is more rapid. Use of colony-stimulating factors after BMT is associated with more rapid neutrophil recovery after related BMT, a finding confirmed in small pediatric series.[147-149,158-163] No change in platelet recovery has been noted. Interestingly, preliminary data do not show any colony-stimulating factor–mediated acceleration of engraftment in patients undergoing UD BMT.[163]

The number and quality of T cells that are present in grafts from peripheral blood, bone marrow, and UCB confer different rates of GVHD. GVHD, particularly chronic GVHD is more frequent in PBSC transplant than in bone marrow–derived HSCT, although this risk may be offset by an increased graft-versus-leukemia effect.[164] In adults this increased rate of GVHD has not diminished the efficacy of PBSC transplant. However, in children, PBSC transplant has been associated with a worse outcome than when bone marrow is used as a cell source.[165] The precise indications of PBSCs versus bone marrow as a source in pediatric HSCT therefore remains to be defined. Conversely, the rate of GVHD in UCB is lower than with either PBSC or bone marrow sources and allows for less stringent HLA-typing requirements.[166]

Autologous Stem Cell Transplantation

The fundamental rationale supporting autologous HSCT is that very high doses of cytotoxic agents (dose intensification) increase tumor cell death and that a high-dose treatment regimen can be devised in which the major dose-limiting toxicity is myelosuppression. This toxicity can be ameliorated by infusion of previously collected and cryopreserved autologous stem cells obtained either by bone marrow harvest or by apheresis of peripheral blood. Using autologous cells to support and reconstitute the host after aggressive ablative or near-ablative therapy has been most thoroughly explored in both Hodgkin and non-Hodgkin lymphomas, less well developed in acute and chronic leukemias, and still considered experimental, albeit relatively widely applied, for a growing number of pediatric and adult solid tumors. As well, the relative merits and demerits of each autologous cells collected peripherally or from marrow are still not firmly established.

Contamination by Tumor Cells

A major limitation of autologous HSCT remains contamination of the stem cell product with residual malignant cells. Although the relative contributions of either residual tumor within the host or tumor reinfused with stem cells to subsequent relapse remain unknown, increasing evidence from gene-marking studies suggests that reinfused cells contribute to relapse.[167] In addition, in vitro assays of tumor cell colony formation and minimal residual disease (MRD) detection by polymerase chain reaction suggest that contamination of the stem cell source by tumor cells is associated with an increased relapse rate after HSCT.[168] No large randomized clinical trial of PBSC versus bone marrow with respect to disease-free survival (DFS) has been reported. However, paired samples of PBSCs and bone marrow have been examined in a variety of settings (e.g., breast cancer, non-Hodgkin lymphoma) in which PBSCs have revealed lower levels of tumor cell contamination in the steady-state setting.[169] More recent data suggest that the advantage of PBSCs may be abrogated after mobilization,[170-174] although impact on DFS remains unclear.

A variety of methodologies have been developed to eliminate, or purge, tumor cells from stem cell collections. These are based on either positive or negative selection. The major clinically applied positive selection strategy is that of stem cell enrichment, most often selection of CD34+ cells. Negative selection is based on technologies that deplete tumor cells to a greater degree than normal stem cells. Strategies include the use of pharmacologic agents (e.g., mafosfamide), immunologic reagents targeting tissue specific antigens (e.g., neural crest antigens in neuroblastoma), and manipulation of physical or culture properties of cells. Combinations of both negative and positive selection strategies may improve both the yield and purity of stem cell collections.[175,176] Increasing evidence in multiple clinical settings demonstrates an association between minimal residual tumor contamination and relapse[168] and, conversely, suggests that more effective purging strategies may be associated with improved DFS. However, randomized studies comparing purged and unpurged stem cells have yet to be reported.

Engraftment

Trilineage hematologic reconstitution has been achieved after the infusion of autologous bone marrow, after bone marrow and PBSCs, and after PBSC infusion alone. The most mature studies are of patients reconstituting hematopoiesis with autologous marrow where engraftment after bone marrow infusion is both reliable and durable; durability of PBSC-reconstituted hematopoiesis, undertaken more recently, is less fully evaluated. The best predictor of engraftment and rate of hematologic recovery has not been established. Although colony-forming units, burst-forming units-erythroid (BFU-E), nucleated cell count, and other measures have correlated with time to engraftment in some studies, correlations have not been universal and may additionally depend on the patient population, method of cell procurement, and subsequent cell manipulation. CD34+ counts have emerged as the most reliable and practical method for predicting engraftment after PBSC infusion, with retrospective analyses demonstrating that the risk for graft failure is substantially higher in patients who received less than 2×10^6/kg CD34 + cells per kg.[177-179]

Reconstitution of hematopoiesis after autologous bone marrow infusion has shown some tendency to be delayed when compared with the allogeneic setting. This delay is presumed due to the extent, nature, and duration of prior therapy. In addition, cell manipulation ex vivo (purging) may also contribute to delayed engraftment. Reconstitution after HSCT with autologous bone marrow can be hastened somewhat by the addition of hematopoietic growth factors to the post-SCT supportive care regimen, although the extent and nature of prior therapy may limit response to growth factors. Preliminary information in both the preclinical and clinical arenas suggests that prior aggressive chemotherapy may limit potential for hematologic recovery.[180-182] Prior intensive chemotherapy supported by use of growth factors may also contribute to poor engraftment after autologous transplantation. In fact, despite recovery of peripheral blood counts, hematologic marrow reserves as assessed by in vitro measures of hematopoiesis may be blunted for many years after autologous HSCT.[183]

Addition of mobilized PBSCs to bone marrow has resulted in a dramatic decrease in days of neutropenia and thrombocytopenia in most series with or without subsequent growth factor support. Further, use of mobilized PBSCs as the sole stem cell source has been almost universally associated with more rapid hematologic recovery. It is still debatable whether the use of hematopoietic growth factors in this setting further enhances the rate of engraftment, but available data suggest that it may. Whether the process of PBSC transplantation will result in durable hematopoiesis over decades is not yet evaluable, but reports of late graft failure are rare. Most studies suggest that the increased rate of engraftment observed with PBSCs results in decreased days in hospital, use of antibiotics, and associated supportive services. The overall impact of PBSCs versus bone marrow as a stem cell source on the outcome or cost of transplantation has yet to be determined.

Myelodysplasia Syndrome as a Late Complication

Chemotherapy, particularly with alkylating agents, may result in an increased risk for subsequent myelodysplasia syndrome (MDS). Many patients who come to autologous transplantation have received treatment with such agents. Over the past several years, increasing numbers of patients developing MDS after autologous transplantation have been reported, usually occurring from 4 to 7 years after HSCT.[184] The actuarial incidence ranges from 5% to 10% in most series,[185-187] although it has been reported to be as high as 18% at 5 to 6 years after HSCT in single center experiences.[188,189] Identified risk factors have included etoposide use for stem cell mobilization,[187] conditioning regimens utilizing TBI,[185,190,191] and the amount of chemotherapy received before HSCT and/or the interval between diagnosis and HSCT.[191,192] Whether the MDS results from previously damaged reinfused hematopoietic cells or from residual hematopoietic cells sustaining further injury during conditioning is unresolved. Cytogenetic analysis of bone marrow before autologous HSCT can reveal preexisting abnormalities and should be a standard component of pre-SCT evaluation.[193] However, most patients developing MDS have been reported to demonstrate normal cytogenetics at the time of transplant.[186] With more sensitive techniques currently available, such as fluorescence in situ hybridization, the majority of patients developing MDS post-SCT have been shown to harbor the same cytogenetic abnormality in pre-SCT specimens.[194] Similarly, using an X-inactivation–based clonality assay in female patients, clonal hematopoiesis was demonstrated in a small percent of patients before HSCT; those patients were at significant risk for developing MDS after HSCT.[184] This suggests that many cases of post-SCT MDS evolve from an abnormal clone already present before HSCT. The appropriate evaluation of hematopoietic status before embarking on autologous HSC collection and transplantation requires further study.

Immune Reconstitution

The period after HCST is characterized by profound immunodeficiency, and death from infectious complications is a major obstacle to the success of transplantation. Myeloablative conditioning results in the wholesale loss of immune cells that are necessary to confer protective immunity, including T, B, and natural killer (NK) cells. Although hematopoiesis can return within weeks of transplant, recovery of a functional immune system can take much longer and the risk for opportunistic infection remains high for up to a year even in uncomplicated transplants.

The degree of immunodeficiency varies with time, reflecting different contributions of donor and recipient immune function at different stages after transplant. Mature T, B, and NK cells are present in the donor inoculum of bone marrow, peripheral blood, or UCB and can contribute to some degree of host immunity in the immediate posttransplant period. The ability of these transferred cells to provide immune protection is dependent on how immunologically "experienced" the donor is. For instance, recipients of grafts from donors who are immune to cytomegalovirus (CMV), that is who have previously been infected with the virus and have mounted a protective immune response, have a lower rate of CMV disease, presumably because of transferred immunologic memory to that virus.[195] Similarly, recipients whose donors have been vaccinated with a pneumococcal vaccine respond to pneumococcal vaccines more rapidly and with higher antibody titers after transplant, again suggesting that donor immunity can be transferred to the recipient.[196] However, full immune reconstitution, with the ability to mount a protective immune response to new pathogenic challenges, is dependent on the differentiation of new immune cells from precursors in the donor graft inoculum.

In most patients, NK cells are the first lymphoid population to recover. In the first month after transplant, NK cells can represent the main lymphoid cell in the peripheral blood. T cells recover much more slowly, and normal numbers of CD4 and CD8 T cells are not achieved for 6 to 12 months after HSCT.[197] The delay in T-cell neogenesis is because the development of new T cells is a complex process that takes months to complete. During the early period after transplant, T cells with a naïve phenotype predominate in the peripheral blood, suggesting repopulation of the T-cell compartment with recent thymic emigrants.[197-199] Increased thymic output contributes to the expanding peripheral T-cell pool after transplant for 12 to 24 months after HSCT.[200] This thymic output occurs even in adult patients whose thymuses have presumably involuted. The rate of increase in T cells is usually much more rapid in pediatric HSCT patients, consistent with the presence of more robust thymic function.[197] The advantage of a functional thymus in immune reconstitution has been demonstrated by the marked decrease in the fraction of naïve peripheral blood T cells in a 15-year-old HSCT patient who had had a previous thymectomy compared with thymus-bearing HSCT patients.[201] By using quantitative molecular measures of T-cell repertoire, several studies have shown that it can take as long as 12 to 24 months after HSCT for a full range of T-cell receptor diversity to be present in the T-cell compartment.[198,199,202] This underscores the critical role of naïve thymic emigrants in restoring the full complement of T-cell receptor specificities.

Several factors influence the length of time that immunologic recovery takes. Age of BMT recipients profoundly affects the rate of immune reconstitution, with an inverse correlation between recipient age and the absolute number of T cells 1 year after HSCT.[203] Using mobilized PBSCs as a source of stem cells appears to be associated with a faster rate of recovery than using HSCs from bone marrow, possibly as a result of a larger cell dose.[129,204] Increasingly intensive conditioning regimens are delaying immune recovery in autologous recipients to a degree comparable with allogeneic HSCT.[203] Although hematopoietic engraftment appears to be delayed in UCB recipients, immune reconstitution in children after UCB HSCT is comparable to those receiving unrelated BMT.[205] The presence of GVHD can significantly delay immune reconstitution both as a result of disruption of normal T-cell development and from the added immunosuppression that is required for management of the disease.[206] The consequence of factors that delay immunologic recovery is significant: slow recovery of immune function is associated with an increase in the cumulative incidence of infections and nonrelapse mortality.[129,207]

PERITRANSPLANT SUPPORTIVE CARE

Transfusion Support

Virtually all patients undergoing HSCT require red blood cell and platelet transfusions during transplant, and some patients require transfusions for several months after transplant. All blood products should be gamma irradiated to prevent transfusion-induced GVHD that can result from donor T lymphocytes in the cellular product.[208,209] Multiple processes including psoralens photochemical treatment and leukocyte filtering have been investigated for prevention of transfusion-associated GVHD, but gamma irradiation remains the standard of care.[209-214] Additionally, it is recommended that blood products are either filtered to achieve white blood cell reduction or CMV seronegative. Prior studies indicated that leukocyte-reduced blood products are equivalent to CMV-seronegative blood products for the prevention of transfusion-transmitted CMV.[215,216] However, a meta-analysis of the available literature suggests that the risk for transfusion-transmitted CMV is greater with white blood cell–reduced blood products when compared with CMV-seronegative products.[217]

Whether platelet and red blood cell transfusions should be given prophylactically during the period of cytopenia remains an area of controversy. There are no studies in the modern transplant era evaluating the use of prophylactic platelet transfusion compared with therapeutic transfusions. More recent studies have addressed different therapeutic triggers for prophylactic transfusions, specifically 10×10^9/L versus 30 or 20×10^9/L.[218,219] A review of studies of prophylactic platelet transfusion triggers found no statistically significant differences between groups with regard to mortality or severe bleeding events.[220] Although no statistical significance was found, these studies were small and no recommendations can supplant the need for clinical judgment in individual situations.

Nutrition Support

Provision of nutritional support during HSCT requires an appreciation of the metabolic needs of the individual patient, which is influenced by the body composition and complications of transplant. Patients who are underweight have been shown to be at an increased risk for death compared with patients of normal weight in the early period after transplant.[221] The impact of obesity on transplant outcome is debated, with some studies noting no effect and others reporting lower survival in obese patients.[221-223] Both the direct effects of conditioning regimens (e.g., mucositis) and complications of HSCT (e.g., GVHD, VOD, infection) can induce gastrointestinal damage that compromises the nutritional status of HSCT patients. Enteral feeding has been shown to be effective and safe and to have fewer complications than parenteral nutrition during HSCT but is not always practical for children.[224-228] Several studies have demonstrated the efficacy of parenteral nutrition when enteral nutrition is no longer possible.[229-231] However, in addition to expense, parenteral nutrition has attendant potential complications, including vascular access difficulties, abnormal liver function, and episodes of hyperglycemia and hyperlipidemia. A review of literature does not support the uniform use of parenteral nutrition as nutritional support during HSCT.[232,233] The authors propose that the use of parenteral nutrition be reserved for patients unable to tolerate enteral feeding. Some patients may be able to maintain their nutritional needs orally throughout transplant, and both enteral and parenteral nutrition routes may be used on occasion. Resumption of enteral feeding should be encouraged, and continued monitoring of nutritional intake and weight is necessary not only through engraftment but also after discharge from the hospital. Malnutrition appears to be relatively frequent after HSCT, and even patients with no or limited GVHD may have significant nutritional problems.

INFECTION PROPHYLAXIS AND TREATMENT

Infection risk is greatest in the allogeneic and T-cell–depleted settings, where, despite phenotypically normal circulating lymphocytes, cellular immunity is depressed for at least 1 year after HSCT.[234,235] Guidelines for infection prophylaxis and treatment are published regularly by the American Society of Blood and Marrow Transplantation[236] and provide a standard approach to an ever-evolving area.

Viral infections, both newly acquired and reactivating from a pre-HSCT exposure, present a major risk to patients. Both CMV-seropositive patients and CMV-seronegative patients who have received grafts from seropositive donors are at risk for CMV, although the risk for reactivation is greatest for seropositive recipients with a seronegative donor, owing to patient reactivation of latent virus in the context of naïve donor T cells.[237,238] A large multicenter trial found that patients receiving a UD HSCT from CMV-positive donors had an improved overall 5-year survival compared with patients receiving cells from CMV-negative donors.[239] T-cell depletion abrogated this effect, which suggested that the improved survival was due to transfer of donor immunity. The clinical manifestations of CMV after HSCT include pneumonia/interstitial pneumonitis, colitis, hepatitis, and CMV-related cytopenias. Before the use of ganciclovir for CMV prophylaxis and treatment, more than a third of allogeneic SCT patients developed CMV infections.[237] Almost 20% developed pneumonia, with a mortality rate of 85%.[237] With the availability of effective antiviral therapies, almost all patients at risk for CMV disease receive prophylaxis during the HSCT admission. Randomized studies have found acyclovir, valacyclovir, and ganciclovir prophylaxis initiated at engraftment to be effective at reducing CMV disease before day 100.[240-243] The use of prophylactic acyclovir has been shown to decrease overall mortality for at-risk patients.[244,245] These studies found that the use of prophylactic ganciclovir after engraftment was limited by drug-induced cytopenias and other toxicities. There are multiple studies of preemptive CMV treatment based on the results of positive culture, polymerase chain reaction assay, antigen testing, or bronchoalveolar lavage fluid evaluation.[246-249] A review of the published CMV literature since 1995 found no advantage to prophylactic treatment over preemptive treatment in response to screening tests.[249] Additionally, this review found that preemptive therapy based on polymerase chain reaction tests or antigen assay was superior to culture or bronchoalveolar lavage–based strategies. Both ganciclovir and foscarnet are considered to be first-line therapy for CMV reactivation or treatment of disease.[250] Ganciclovir and foscarnet are similar in efficacy and primarily differ in terms of side-effect profile.[251] CMV-seropositive patients undergoing autologous HSCT have not generally received either screening or prophylaxis,[250] although CMV disease occurs in a small percent of these patients.

Up to 50% of patients undergoing either autologous or allogeneic HSCT experience varicella-zoster virus reactivation, usually within the first year after SCT.[252] The vast majority of patients respond to acyclovir treatment. In children, the onset may be earlier, with fewer cases of visceral involvement. In general, pediatric patients respond well to antiviral therapy and fatalities are rare.[253,254] Treatment with acyclovir for 1 year after HSCT is the current recommendation for any patients with a pre-HSCT history of varicella infection.[255] Varicella vaccination is currently considered optional after autologous or allogeneic HSCT. A pilot study of the live-attenuated vaccine in autologous patients found the vaccine to be safe, but efficacy data are still pending.[256]

Fungal infection remains a significant problem, especially in those receiving corticosteroids for treatment or prophylaxis of GVHD. A randomized trial demonstrated that administration of prophylactic fluconazole for 75 days after allogeneic HSCT yields ongoing protection against disseminated candidal infections and improved survival.[257] However, prophylactic fluconazole has been associated with the emergence of resistant species such as *Candida krusei*.[258] Low-dose amphotericin B has also been used prophylactically and is as effective as fluconazole but more toxic.[259] The use of liposomal amphotericin has improved the toxicity profile.[260] Voriconazole has a broader spectrum of antifungal activity than fluconazole, but a randomized trial comparing the two agents as prophylactic therapy failed to show a survival difference in allogeneic HSCT patients.[255]

All HSCT patients are at risk for the development of *Pneumocystis jirovecii* after transplant. Standard practice is to administer prophylaxis to recipients of autologous HSCT for 6 months after day 0 and for 6 months after discontinuation of immunosuppression for recipients of allogeneic transplant.

Veno-Occlusive Disease of the Liver

Veno-occlusive disease, also known as sinusoidal obstruction syndrome, is a clinical syndrome occurring in the early posttransplant period and characterized by hepatomegaly, right upper quadrant pain, jaundice/increased bilirubin level, and fluid retention.[261-263] VOD can occur after either autologous or allogeneic SCT[262,264-266] and results from chemotherapy/irradiation-induced damage to endothelium and hepatocytes.[262,265,267,268] Injury to the sinusoidal endothelial cells and hepatocytes in zone 3 of the liver acinus appears to be the initial event, resulting in subendothelial edema and endothelial cell damage and subsequent microthromboses, fibrin deposition, and the expression of factor VIII/vWF within venular walls.[269] Later features include collagen deposition within venular lumens with eventual luminal obliteration and hepatocyte necrosis.[267,270,271] Identified risk factors for developing VOD include previous chemotherapy regimens containing gemtuzumab ozogamicin, BU-based conditioning regimens (especially when combined with CY and methotrexate), prior intensive chemotherapy (as seen in advanced-stage malignancies), total parenteral nutrition for more than 30 days, and transplantation from UDs.[264,265,270,272-281] Pretransplant iron overload may also potentiate the development of VOD, and a pre-HSCT ferritin level of more than 1000 is an additional identified risk factor in some studies.[282]

The first clinical signs of VOD are hepatomegaly and right upper quadrant pain with fluid retention, typically occurring 10 to 20 days after chemotherapy administration.[265] Other clinical signs include hyperbilirubinemia, peripheral edema, ascites, sodium avidity, and pulmonary infiltrates/effusion/edema.[262,265] Patients with VOD are at increased risk for developing renal insufficiency and failure.[265,283] It is hypothesized that portal hypertension from sinusoidal injury leads to decreased renal perfusion and tubular injury, resulting in renal damage.[265,283,284] Hepatorenal syndrome occurs in approximately half of patients with severe disease[265] and is associated with changes in mental status.

It is essential to exclude other causes of liver disease (e.g., sepsis, portal vein thrombosis) before a diagnosis of VOD can be made. Liver biopsy is the gold standard for diagnosis and along with hepatic venous pressure measurements can still provide valuable diagnostic information, although it is associated with considerable morbidity in this critically ill population.[285,286] Doppler ultrasonography can help confirm the diagnosis, but classic findings are not always present early in course of the disease. If clinical concern is high, repeating the ultrasound evaluation after several days may be helpful.[287,288] However, the presence of ascites, gallbladder wall thickening, hepatomegaly, decreased or reversed flow in the portal vein, and increased mean hepatic artery resistive index are support of the diagnosis.[262,287-291] Recent clinical guidelines suggest that clinical criteria are sufficient to make the diagnosis while ultrasonography or liver biopsy can be reserved for cases with high levels of diagnostic uncertainty.[292]

Reported overall survival rates for pediatric patients with established VOD range between 53% and 100%.[264,273,279,280,293-296] Predictors of mortality in children with VOD include the use of allogeneic donors other than a matched sibling, concomitant moderate-to-severe hepatic or cutaneous GVHD, and higher VOD severity (e.g., higher peak bilirubin, higher maximal weight gain, presence of pleural effusion, requirement for intensive care unit admission).[264,273]

Treatment and prophylactic options for patients with VOD have evolved over the past decade. The most straightforward preventative technique is to identify patients at highest risk and to consider alternative conditioning regimens when feasible.[261-263] Ursodeoxycholic acid has been used as a preventative agent with inconclusive results.[297-303] Other strategies such as the use of fresh-frozen plasma, prostaglandin E_1, *N*-acetylcysteine, and antithrombin III are potentially effective but require further study before being considered a standard approach.[304-311] Because the majority patients can recover spontaneously, supportive care with meticulous fluid and blood product management remains the mainstay of therapy for moderate VOD.[312] Based on histologic observations of microthrombi and fibrin deposition coupled with laboratory evidence of abnormalities in the coagulation cascade, many therapeutic strategies have been directed at promoting thrombolysis or fibrinolysis.[311,313-317] Multiple small studies have investigated the use of thrombolytic agents and anticoagulants (typically tissue plasminogen activator and heparin) for VOD prevention and/or treatment.[311,313-315,318,319] Although these strategies appear to be generally safe, they have not shown significant reduction in the incidence or outcome of patients with severe disease and have been associated with serious bleeding in some cases.[304,313-315,320-327]

Defibrotide is a single-stranded polydeoxyribonucleotide that has antithrombotic and thrombolytic activity. It has been demonstrated to be effective with minimal toxicity for both the prevention and treatment of VOD.[292,328-334] The mechanism of action is unclear, but it appears to promote angiogenesis and promote sinusoidal endothelial regrowth and has thrombotic/thrombolytic properties.[335] Defibrotide prophylaxis has been shown to be safe in adults and children; efficacy in preventing VOD has been demonstrated in small studies, but there is still a paucity of large studies or randomized trials.[336,337] Response rates between 35% and 50% have been reported for patients with severe VOD treated with defibrotide, and it is not associated with increased risk for major bleeding.[331,338-340]

Refractory VOD portends poor outcome with limited therapeutic options. Liver transplant has resulted in clinical improvement in about 30% of the small numbers of patients stable enough to undergo the procedure; the immediate- and long-term issues are formidable, however.[341] Transjugular intrahepatic portosystemic shunting (TIPS) has been performed safely in patients with severe VOD, although it has not been shown to improve overall survival and should be reserved for instances in which fluid retention and ascites are relatively isolated issue.[342-347]

In summary, VOD is an early complication of HSCT that occurs more frequently in patients with prior hepatic injury, intensive prior therapy, or exposure to conditioning regimens with known hepatotoxicity. The diagnosis is made on clinical criteria, including hepatomegaly, ascites, right upper quadrant pain, and increased bilirubin level; ultrasound findings can be used to further support the diagnosis. There is no standard approach to prophylaxis. Moderate VOD can be self-limiting, but multiple studies have shown a decrease in mortality from severe VOD when defibrotide therapy is employed.

IMMUNE RECONSTITUTION

The period after HCST is characterized by profound immunodeficiency, and infection complications remain an obstacle to improved HSCT outcomes. A common feature of transplant regimens, particularly for unrelated HSCT, is the use of chemotherapeutic agents and/or antibodies that effectively deplete the host of many lineages of the immune system. Myeloablative conditioning therefore results in the loss of immune cells, including T, B, and NK cells that are necessary to confer protective immunity, although some host immune populations such as resident macrophages persist after conditioning.[348] Although hematopoiesis can return within weeks of transplant, recovery of a functional immune system can take much longer, and the risk for opportunistic infection remains high for up to a year even in uncomplicated transplants. The rapidity and extent of immune reconstitution after transplantation is therefore an important determinant in outcome of HSCT.

The kinetics of recovery of the cells in the immune system after HSCT tends to follow a relatively predictable pattern. Recipients of autologous HSC rapidly recover CD3 T-cell counts within 6 to 8 weeks after transplant, whereas allogeneic HSCT recipients do not normalize their T-cell counts until 12 weeks or later. CD8+ T cells recover more rapidly than do CD4+ T cells,[349] resulting in an inversion of the normal CD4+:CD8+ T-cell ratio, although the clinical significance of this is not fully understood. NK cells reappear early after HSCT and may represent the major lymphocyte population present in the initial weeks after HSCT.[350] This timetable of immune recovery dictates the vaccination schedule of patients after HSCT, and most centers do not commence reimmunization for HSCT patients until 6 months after transplant. The rate of recovery of the immune system also has significant implications for the rate of infection, because slow recovery of immune function is associated with an increase in the cumulative incidence of infections and nonrelapse mortality.[129,207]

The recovery of the T-cell compartment in HSCT patients is from both expansion of mature T cells delivered in the donor inoculum and from de novo differentiation of T cells from early stem cell and lymphoid progenitors. During the early period after transplant, T cells with a naïve phenotype predominate in the peripheral blood, suggesting repopulation of the T cell compartment with recent thymic emigrants.[197-199] The relative numbers of recent thymic emigrants can be quantified in the peripheral blood T-cell pool by the quantitative detection of genomic DNA that is present only in thymocytes (T-cell receptor excision circle [TREC] DNA). These methods have shown that increased thymic output contributes to the expanding peripheral T-cell pool after transplant for 12 to 24 months after HSCT.[200] This thymic output occurs even in adult patients whose thymuses have presumably involuted. The rate of increase in T cells is usually much more rapid in pediatric HSCT patients, consistent with the presence of more robust thymic function.[197] The advantage of a functional thymus in immune reconstitution has been demonstrated by the marked decrease in the fraction of naïve peripheral blood T cells in a 15-year-old HSCT patient who had had a previous thymectomy relative to thymus-bearing HSCT patients.[201] The delay in recovery of the T-cell compartment is reflected in a prolonged time to manifest a normal range of T-cell diversity. Several studies have shown that it can take as long as 12 to 24 months after HSCT for a full range of T-cell receptor diversity to be present in the T-cell compartment.[198,199,202]

Mature T, B, and NK cells are present in the donor inoculum of bone marrow, peripheral blood, or UCB and can contribute to some degree of host immunity in the immediate posttransplant period. The ability of these transferred cells to provide immune protection is dependent on how immunologically "experienced" the donor is. For instance, recipients of grafts from donors who are CMV immune, that is, who have previously been infected with the virus and have mounted a protective immune response, have a lower rate of CMV disease, presumably because of transferred immunologic memory to that virus.[351] Similarly, recipients whose donors have been vaccinated with a pneumococcal vaccine respond to pneumococcal vaccines more rapidly and with higher antibody titers after transplant, again suggesting that donor immunity can be transferred to the recipient.[196] However, full immune reconstitution, with the ability to mount a protective immune response to new pathogenic challenges, is dependent on the differentiation of new immune cells from precursors in the donor graft inoculum.

Several factors influence the length of time that immunologic recovery takes. Age of BMT recipients profoundly affects the rate of immune reconstitution, with an inverse correlation between recipient age and the absolute number of T cells 1 year after HSCT.[203] The source of HSC also has a marked effect on the rate of immune reconstitution. The use of PBSC is associated with a faster rate of recovery than using HSC from bone marrow, possibly as a result of a larger cell dose.[129,204] However, increasingly intensive conditioning regimens are delaying immune recovery in autologous recipients to a degree comparable with allogeneic HSCT.[203] Although early data suggested that immune reconstitution in children after UCB HSCT is comparable to those receiving unrelated BMT,[205] subsequent studies have shown that UCB HSCT is associated with a marked delay in the recovery of T-cell numbers and function.[352-355] The presence of GVHD can significantly delay immune reconstitution both as a result of disruption of normal T-cell development and from the added immunosuppression that is required for management of the disease.[206]

GRAFT REJECTION

Primary graft rejection, defined as the failure to recover hematopoietic function of the stem cell graft by day (+) 100, is an uncommon, life-threatening event.[356] Rejection occurs after approximately 4% of UD stem cell transplants and is even less common in RD transplants.[357,358] It is an immune-mediated process that occurs because of genetic disparity between the donor and recipient.[22,359,360] Although both NK and T cells are considered mediators of graft rejection, it is characterized by the presence of recipient T cells and the gradual loss of donor cells.[361-369] The risk for graft rejection is increased in patients who are mismatched at HLA-A, HLA-B, or DRB1 loci, receive a low cell dose, have a positive crossmatch for antidonor lymphocytotoxic antibody, receive T-cell depletion as GVHD prophylaxis, have aplastic anemia, especially if previously multiply transfused, or have storage disorders or osteopetrosis.[357,358] An altered host microenvironment can also contribute to graft rejection.

Adjustments of both chemotherapeutic and radiotherapeutic components of conditioning regimens, the use of monoclonal antibodies targeting selected populations of recipient T cells, and increases in cell dose (including the use of PBSCs in addition to bone marrow) are among the interventions that have shown some promise in preventing graft rejection. Depletion of host T cells with antithymocyte globulin or the use of CD52 monoclonal antibodies to deplete residual recipient lymphocytes are commonly used options to decrease graft rejection.[370,371] A number of approaches have been exploited to overcome the increased incidence of graft failure associated with T-cell depletion. First, simply increasing the number of stem cells in the graft has been successful, implying that residual host hematopoietic elements are involved in mediating graft rejection. However, such intensification is associated with increased GVHD disease. Alternatively, approaches to increasing the number of stem cells in the graft using manipulation of Notch[372] or prostaglandin[373] pathways are being explored. Additionally, because the risk for graft failure appears to increase with more exhaustive T-cell depletion, less extensive T-cell removal from the graft with and without T-cell add-back has been attempted.[374-377] More recently attempts to reduce rejection with the use of mesenchymal stem cells have been explored.[378,379]

Therapeutic options for patients with graft rejection include second allogeneic transplant,[380] the infusion of frozen autologous stem cells, the use of hematopoietic growth factors, particularly granulocyte-macrophage colony-stimulating factor (GM-CSF), modification of the host's immunologic status, and the infusion of donor leukocytes.[256,381-386] Infusion of stem cells with or without additional conditioning can be an effective intervention for the treatment of primary graft failure.[387] It has been studied using bone marrow or PBSCs, although recently the use of unrelated UCB units[387-390] has been explored. The use of allogeneic PBSCs is appealing in the second transplant setting because PBSCs have been associated with shorter duration of the neutropenic phase and a higher neutrophil recovery rate.[381] Graft rejection is a potentially morbid, uncommon complication of HSCT with limited effective therapies.[391]

GRAFT-VERSUS-HOST DISEASE

Acute Graft-versus-Host Disease

One of the most significant barriers to successful HSCT is acute graft-versus-host disease (aGVHD). aGVHD is a syndrome caused when immunologically competent cells contained in the bone marrow or peripheral blood recognize host tissues, resulting in inflammation and tissue damage. Normally, allogeneic cells contained in simple blood transfusions are rapidly eliminated by the host immune system. However, in the setting of HSCT, the immune system of the recipient is suppressed, allowing donor immune cells to persist. The syndrome of aGVHD was first reported in 1954 by Barnes and Loutit,[6] who observed that lethally irradiated animals given allogeneic spleen cells developed skin abnormalities and diarrhea and died of a wasting illness. It became clear that for aGVHD to develop, certain criteria had to be in place, and these were summarized by Billingham in 1966[392]: (1) "the graft must contain immunologically competent cells"; (2) "the host must possess important transplantation alloantigens that are lacking in the donor graft, so that the host appears foreign to the graft and is, therefore, capable of stimulating it antigenically"; and (3) "the host itself must be incapable of mounting an effective immunological reaction against the graft, at least for sufficient time for the latter to manifest its immunological capabilities; that is, it must have the security of tenure."

In general, the greater the degree of genetic difference between donor and recipient, the higher the risk for and severity of aGVHD. The main genetic loci that determine the compatibility of donor and recipient are HLA classes I and II located on chromosome 6. Each of these loci encode polymorphic glycoproteins—major histocompatibility antigens—that are expressed on the surface of human tissues in a complex with small peptides from degraded intracellular proteins. HLA class I molecules (HLA-A, HLA-B, and HLA-C) are expressed on all nucleated cells, whereas class II antigens (HLA-DR, HLA-DQ, and HLA-DP) are primarily expressed on hematopoietic cells. T-cell receptors on the surface of T cells engage with allogeneic MHC molecules, resulting in the activation and expansion of alloantigen-specific T cells. The recognition of allogeneic MHC molecules is bidirectional: recipient T cells recognize donor antigens and can cause graft rejection; conversely, donor T cells can recognize recipient antigens and are responsible for the syndrome of aGVHD.

The mechanisms by which T cells cause GVHD include both direct effector mechanisms (i.e., killing of host cells by alloantigen-specific T cells) and also the release of inflammatory mediators that cause tissue damage. The presence of tissue damage either from immunologic events or from direct effects of conditioning can propagate the inflammatory response, triggering further immune responses, in a positive feedback loop that has been called a "cytokine storm."[393-395]

TABLE 8-1 Glucksberg Criteria for Staging and Grading of Acute Graft-Versus-Host Disease

	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4
Skin	No rash	Rash <25% body surface area	25-50%	>50% generalized erythroderma	Bullae and desquamation
Intestines	<10 mL/kg/day	10-19.9 mL/kg/day	20-30 mL/kg/day	>30 mL/kg/day	Severe abdominal pain, ileus, blood or melena
Upper Gastrointestinal Tract	Severe nausea/ vomiting				
Liver	Bilirubin ≤2 mg/dL	Bilirubin 2.1-3 mg/dL	Bilirubin 3.1-6 mg/dL	Bilirubin 6.1-15 mg/dL	Bilirubin >15 mg/dL

Despite the pathologic effects of aGVHD, the allogeneic immunologic effect may also confer benefits. Patients with hematologic malignancies who develop mild-to-moderate GVHD have a decreased risk for disease relapse.[396-399] This is believed to be because graft inocula contain immunologic cells[400] that are capable of recognizing malignant cells and mediating a graft-versus-leukemia and, perhaps, a graft-versus-tumor effect,[401-403] although whether T cells that recognize tumor cells are distinct from those that simply recognize allo-MHC is not fully understood. Conversely, T-cell depletion from the marrow inocula reduces the risk for GVHD but can lead to an increased risk for both graft rejection and disease recurrence.[404]

The clinical syndrome of aGVHD develops within 100 (and usually within 60) days of allogeneic SCT. Chronic GVHD (cGVHD) is a distinctive clinical syndrome that occurs after day 100 and is discussed later. aGVHD classically involves the skin, liver, and lower gastrointestinal tract, although the degree of involvement of each organ varies. The first manifestation of aGVHD is often a rash, presenting as a maculopapular eruption, commonly involving the palms and soles, back of neck and ears at first, and later the trunk and extremities. Biopsy specimens from involved areas demonstrate epidermal basal cell vacuolization, followed by epidermal basal cell apoptotic death with lymphoid infiltration.[405] Characteristic eosinophilic bodies may be seen. The specificity of skin biopsy is not high, and it is often difficult to differentiate aGVHD from drug allergy or reaction to the conditioning regimen.[406] For this reason, skin biopsy specimens obtained early on in the transplant course are often nondiagnostic. Bullae formation with epidermal separation and necrosis is seen in advanced stages of skin GVHD.[407]

Hepatic GVHD often presents as cholestatic jaundice, which must be differentiated from other causes of liver disease frequently seen in HSCT patients, including VOD, infection, and drug toxicity.[408] Liver biopsy may be helpful because characteristic pathologic changes can be observed that aid diagnosis,[409,410] although the risk for bleeding complications is often high.

Gastrointestinal involvement presents as watery diarrhea with a “seedy” component and may progress to include crampy abdominal pain, bleeding, and even ileus.[408] By convention, stool volume is used to quantitate the severity of gut involvement. Rectal or colonic biopsy may reveal crypt cell necrosis with lymphocytic infiltration, and, with increasing severity, crypt abscess or loss and mucosal denudation.[411]

The clinical signs of aGVHD are not pathognomonic and overlap with a broad range of other complications frequently encountered during HSCT. For that reason, tissue biopsy samples of affected organs are usually obtained to confirm diagnosis by histology and exclude other infectious causes. Several groups have sought to develop better diagnostic tests for aGVHD, focusing on proteins found in the plasma that correlate with and predict the presence of GVHD; however, none has yet become integrated into clinical care.[412-415]

The severity of aGVHD is graded based on staging the involvement of individual organs and the clinical performance of the patient and then determining an overall grade for the disease. The modified Glucksberg criteria, introduced in 1974,[416] is the system historically used for assessing aGVHD (Tables 8-1 and 8-2). The International Bone Marrow Transplant Registry (IBMTR) constructed a severity index based on organ involvement alone with no need for subjective assessment of performance (Table 8-3). This has been shown to correlate well with outcome.[417] Skin, liver, and gut are staged individually for

TABLE 8-2 Glucksberg Criteria for Staging and Grading of Acute Graft-Versus-Host Disease—Overall Grade

Overall Clinical Grade	Organ System	Clinical Stage
I (mild)	Skin Liver Gastrointestinal	1-2 1 0
II (moderate)	Skin Liver Gastrointestinal	1-3 1-2 1
III (severe)	Skin Liver Gastrointestinal	2-4 2-4 2-4
IV (life threatening)*	Skin Liver Gastrointestinal	2-4 2-4 2-4

*With severe constitutional symptoms.

TABLE 8-3 International Bone Marrow Transplant Registry Severity Index for Acute Graft-Versus-Host Disease

Severity Index	Skin	Liver	Gastrointestinal	Upper Gastrointestinal
A	1	0	0	0
B	2	1-2	1-2	1
C	3	3	3	—
D	4	4	4	—

severity of involvement, after which an overall grade of GVHD is assigned.

Several risk factors have been identified for the development of aGVHD. Increasing genetic disparity between the donor and host increases the incidence and severity of GVHD[418-421] and is the most important single predictor of aGVHD. A large retrospective study of adults undergoing HSCT showed that risk factors for aGVHD grades II through IV included transplantation from UDs, mismatched RDs or UDs, the use of a TBI-based conditioning regimen, and the use of a female donor.[422] More recently, other genetic risk factors have been evaluated. The existence of polymorphisms in genes between donor and host offers a plausible mechanism to explain variations in magnitude of the inflammatory response to tissue injury and hence GVHD. Indeed, specific polymorphisms in the recipient interleukin (IL)-10 promoter region and IL-10 receptor beta gene *(IL10RB)* have been found to be associated with a lower rate of GVHD.[423] Many other candidate genes are currently being examined for similar associations.[424] Moreover, the falling cost and growing scale of genomic sequencing technologies suggest that an increasingly complex and accurate genomic prediction of GVHD risk is likely to evolve over the coming years.

Almost all patients receiving an allogeneic HSCT receive immunosuppressive therapy as prophylaxis to prevent GVHD. Prophylactic regimens originally developed in the early years of the development of HSCT included methotrexate, cyclosporine, and corticosteroids.[396,425,426] A common prophylactic regimen includes cyclosporine given intravenously at a dose of 1.5 mg/kg every 12 hours until oral administration at a dose of 6.25 mg/kg every 12 hours is tolerated. Doses are adjusted to achieve desired blood levels. Cyclosporine is continued on a tapering schedule through day 180 after transplant. Methotrexate is given at 15 mg/m^2 on day 1 and at 10 mg/m^2 on days 3, 6, and 11 after marrow infusion. Tacrolimus, which, like cyclosporine, is a calcineurin inhibitor, was tested in combination with methotrexate and found to decrease aGVHD but not overall survival.[427,428] Mycophenolate mofetil (MMF) inhibits T-cell proliferation by interfering with the purine synthetic pathway and is sometimes used instead of methotrexate to minimize toxicity (especially mucositis).[429] Sirolimus, originally identified as an antifungal agent, has potent immunosuppressive properties and, in combination with tacrolimus, is an effective prophylactic regimen.[430,431] However, the risk for VOD is increased by the use of sirolimus in some settings.[432]

Despite prophylaxis, 10% to 30% of children undergoing HSCT will develop aGVHD. For these patients, frontline therapy consists of systemic corticosteroids, typically at a dose of 2 mg/kg/day of methylprednisolone, together with calcineurin-inhibition with cyclosporine or tacrolimus (Fig. 8-1). There is no advantage to initial therapy with higher doses of corticosteroids.[433] For patients who respond to initial therapy, corticosteroids are tapered by 10% every 3 to 5 days and then more slowly after doses are decreased to less than 1 mg/kg/day. However, because approximately 35% of patients do not improve with initial corticosteroid therapy,[434] several other agents have been used in combination with corticosteroids to increase the efficacy of frontline treatment.[435] A recent, randomized phase II study in adults undergoing HSCT demonstrated that the addition of MMF showed improved efficacy compared with etanercept (which inhibits tumor necrosis factor [TNF]), denileukin (which targets cells expressing the IL-2 receptor), or pentostatin (which inhibits purine synthesis).[436]

If aGVHD progresses or fails to improve after 5 to 7 days of initial corticosteroid therapy, it is considered to be corticosteroid refractory and additional immunosuppressive therapies are added. A variety of other pharmacologic agents have been tried, including methotrexate, MMF, IL-2 receptor–targeting agents, etanercept, infliximab, antithymocyte globulin, and sirolimus.[435] However, none is clearly superior; and, instead, most clinicians select second-line aGVHD therapy based on the effects of previous treatment regimens used in a given patient

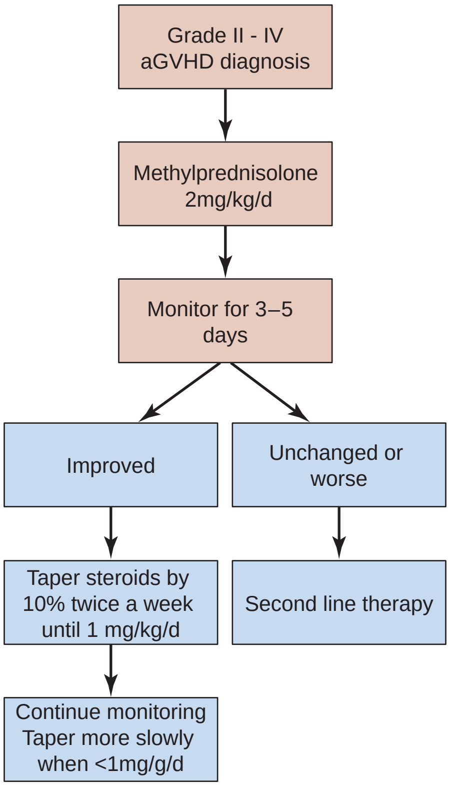
Grade II - IV aGVHD diagnosis
Methylprednisolone 2mg/kg/d
Monitor for 3–5 days
Improved
Unchanged or worse
Taper steroids by 10% twice a week until 1 mg/kg/d
Second line therapy
Continue monitoring Taper more slowly when <1mg/g/d

Figure 8-1 Management of acute graft-versus-host disease.

TABLE 8-4 Comparison of Acute and Chronic Graft-Versus-Host Disease

	Acute GVHD	**Chronic GVHD**
Onset Day	7-60 (up to 100)	Day >100 (may be seen from day 60 on)
Manifestations		
Skin	Erythematous rash	Sclerodermatous changes
Gastrointestinal	Secretory diarrhea	Dry mouth; sicca syndrome
Liver	Hepatitis/cholestasis	Cholestasis/cirrhosis
Other	Fever	Pulmonary dysfunction, alopecia, thrombocytopenia, xerostomia

and on the spectrum of toxicities associated with each agent.[435]

Chronic Graft-Versus-Host Disease

GVHD was initially characterized as occurring in two phases: aGVHD generally presented within the first 100 days after transplant, whereas cGVHD occurred after day 100 (Table 8-4). cGVHD, defined by both clinical and histopathologic criteria, can present as early as day 50 to 60 days after marrow infusion.[437] The pathogenesis of cGVHD is not completely elucidated. It is believed to involve donor T-cell recognition of minor histocompatibility antigens or loss of peripheral T-cell tolerance, leading to symptoms that are very similar to those present in autoimmune disorders. The morbidity and mortality of cGVHD is most severe in patients who have progressive disease after aGVHD and tends to be less severe in those in whom it occurs de novo.[438] Risk factors for development of cGVHD include HLA disparity between donor and host, prior aGVHD, increasing patient age, and use of a TBI-based conditioning regimen. Age is also a risk factor: patient age older than 15 years or donor age older than 5 years is associated with significantly increased risk.[439] In addition, the use of adult female donors for male recipients increases the likelihood of cGVHD, likely related to donor antibody responses to antigens coded for by genes on the recipient Y chromosome.[440] The impact of pediatric female donors on the incidence of cGVHD has not been evaluated.

cGVHD is a disease with a constellation of clinical and pathologic features. The syndrome resembles autoimmune systemic collagen vascular diseases with protean manifestations potentially involving almost every organ (Table 8-5). Pathologic changes in the skin result from epithelial cell damage characterized by basal cell degeneration and necrosis, and, later, epidermal atrophy and dermal fibrosis.[441] Skin involvement may present as lichen planus, areas of local erythema, and areas of hyperpigmentation or hypopigmentation. The skin may be dry, freckled, or ulcerated; and findings are aggravated by exposure to sunlight. Sclerodermatous changes may evolve to flexion contractures that can greatly impair function and may be helped by surgical release procedures. Hair follicles can be involved with resulting partial or total alopecia. Ocular abnormalities are common, and patients present with sicca syndrome, including dry eyes and photophobia; decreased tearing is quantitated by a Schirmer test. Dry mouth is a common presenting complaint of oral GVHD, and decreased saliva production predisposes to caries. Tongue depapillation or scalloping

TABLE 8-5 Clinicopathologic Features of Chronic Graft-Versus-Host Disease

Organ System	**Symptoms and Signs**	**Biopsy Findings**
Systemic	Recurrent infections with immunodeficiency	
Skin	Lichen planus, scleroderma, hyperpigmentation or hypopigmentation, dry scale, ulcerated, erythema, freckling Flexion contractures	Epithelial cell damage, basal cell degeneration and necrosis, epidermal atrophy and dermal fibrosis
Hair	Alopecia	
Mouth	Sicca syndrome, depapillation of tongue with variegations, scalloping of lateral margins	Lichenoid changes with mononuclear infiltrates, epithelial cell necrosis, salivary gland inflammation, lymphocyte infiltration, fibrosis
Joints	Decreased range of motion, diffuse myositis/tendonitis	
Eyes	Decreased tearing, injected sclerae, conjunctivae	
Liver	Increased alkaline phosphatase > aminotransferases and bilirubin Cholestasis, cirrhosis	Focal portal inflammation with bile ductile obliteration, chronic aggressive hepatitis, bridging necrosis, cirrhosis
Gastrointestinal	Failure to thrive (children), weight loss (adults) Esophageal strictures, malabsorption, chronic diarrhea	Crypt destruction, single-cell dropout, fibrosis of lamina propria
Lung	Cough, dyspnea, wheezing Bronchiolitis obliterans, chronic rales, pneumothorax	
Hematology	Refractory thrombocytopenia, eosinophilia, Howell-Jolly bodies	

of the lateral margins of the tongue can be seen. The tongue, buccal mucosa, and gums may have lichen planuslike lesions, which must be differentiated from oral candidiasis. A biopsy specimen typically shows lichenoid changes with mononuclear infiltrates, epithelial cell necrosis, and salivary gland inflammation, lymphocytic infiltrate, and fibrosis. Involvement of the liver with cGVHD is manifest by obstructive jaundice, which may progress, if untreated, to bridging necrosis and cirrhosis. Gastrointestinal signs include inanition with progressive weight loss, chronic malabsorption, diarrhea, anorexia, nausea, and vomiting. Biopsy demonstrates single cell apoptosis with crypt destruction and may show fibrosis of the lamina propria. Early cGVHD may present as diffuse myositis and tendonitis. Progressive joint involvement, which may result from the myositis and tendonitis or simply from overlying skin fibrosis, results in decreased range of motion and flexion contractures. Perhaps the most debilitating effect of cGVHD occurs with pulmonary involvement, manifest as obstructive lung disease progressing to bronchiolitis obliterans. Persistent immunodeficiency with profoundly depressed B- and T-cell responses predisposes to recurrent and severe infections. Patients may have reduced IgG2 and IgG4, decreased mucosal IgA, diminished delayed-type hypersensitivity, and functional asplenia. They are susceptible to opportunistic infections with encapsulated organisms (primarily pneumococcus), *P. jirovecii,* varicella-zoster, and herpes simplex and may have a variety of chronic infections, including sinusitis, bronchitis, and conjunctivitis.

Staging of cGVHD is based on degree of organ involvement (Box 8-2). Limited cGVHD has localized skin involvement with or without hepatic dysfunction. All other manifestations of cGVHD are classified as extensive cGVHD.[441] Limited cGVHD may resolve spontaneously without specific therapy, and these patients have a favorable outcome.[438] Patients with lichenoid skin changes, progressive presentation of cGVHD, and hyperbilirubinemia have a worse prognosis.[442,443] Recent work has focused on developing a more detailed comprehensive scale to measure symptoms of cGVHD so that response to therapy can be more clearly assessed.[444]

It is essential that patients with cGVHD are managed by a multidisciplinary team with expertise in this complicated and often frustrating condition. Corticosteroids are usually the first approach to therapy, and a regimen of alternating-day cyclosporine and prednisone was shown to be effective in more than half of patients and to have an acceptable toxicity profile.[445,446] There is no standard approach for patients who are refractory to initial therapy or whose disease flares during taper of immunosuppression.[447] A number of other therapies are being tested, including high-dose pulse corticosteroids,[448] extracorporeal photopheresis,[449-452] daclizumab (a humanized IL-2 receptor antagonist),[453] mycophenolic acid,[454,455] rapamycin,[456,457] rituximab,[458-461] imatinib,[462] and pentostatin.[463] Recent studies have also examined the use of IL-2 in the treatment of GVHD, which may work by selectively expanding regulatory T cells.[464]

All of the therapeutic options significantly impact immune function, and most fatalities occur from infectious complications versus specific organ toxicity from cGVHD. Meticulous and preemptive management of the complications associated with cGVHD and its therapy is a crucial component of care. Recommendations for patient education, prophylactic interventions, and follow-up are extensively outlined in the National Institutes of Health Ancillary Therapy and Supportive Care Working Group document.[465]

Box 8-2 **Staging of Chronic Graft-Versus-Host Disease**

LIMITED CHRONIC GVHD

Localized skin involvement *and/or*
Hepatic dysfunction due to chronic GVHD

EXTENSIVE CHRONIC GVHD

Generalized skin involvement *or*
Localized skin involvement and/or hepatic dysfunction due to chronic GVHD, *plus:*
  Liver histology showing chronic aggressive hepatitis, bridging necrosis, or cirrhosis, *or*
  Eye involvement: Schirmer test (<5 mm wetting), *or*
  Involvement of minor salivary glands or oral mucosa demonstrated by buccal biopsy, *or*
  Involvement of any other target organ

ORGAN TOXICITY ASSOCIATED WITH HEMATOPOIETIC STEM CELL TRANSPLANTATION

Ophthalmologic Complications

A number of different ophthalmologic complications can occur as sequelae of HSCT.[466-468] Decreased lacrimation secondary to cGVHD may contribute to ocular sicca syndrome and the related complications of photophobia, punctate keratopathy, scar formation, and corneal perforation. Dry eye syndrome can occur independent of GVHD and is attributed to immunosuppression and conditioning with TBI.[469-471] Therapy includes topical or systemic immunosuppression, lubrication, artificial tears, and lacrimal duct (punctual) plugging, which can be useful even in young children.[469-471] Fluid-ventilated scleral lenses have been used to treat adolescents and adults with sicca syndrome and have decreased corneal abrasions and improved vision.[472] Patients remain at risk for ocular infections, particularly with herpes simplex viruses. Cyclosporine use is associated with a number of eye complaints, including a well-described syndrome of headache, hypertension, seizures, visual impairment, or any combination of these symptoms, as well as temporary blindness (see "Neurologic Complications" for a detailed description).[178,473,474] In addition, transplant-related retinopathy can be manifested as optic disc edema, either asymptomatic or accompanied by visual blurring, in patients taking cyclosporine. Ischemic fundal lesions, detected as cotton-wool spots and, in some patients, as optic disc edema, have been reported in patients treated with both TBI and cyclosporine and were not observed in patients who did not have concordance for these risk factors.[475,476] Lesions appeared 3 to 6 months after SCT, with complaints of decreased visual acuity or other visual disturbances. Most patients discontinued cyclosporine, and half were treated with systemic corticosteroids. The majority recovered visual acuity, and their ischemic lesions resolved over time, but little further information is available about the course or long-term prognosis of this complication.

Retinal hemorrhages are relatively common after transplantation and in one study were found in 12% of children undergoing scheduled ophthalmologic examination after transplant.[467] In this study, the hemorrhages were located intraretinally, did not threaten vision, and developed in the acute transplant period in the setting of thrombocytopenia. In all cases the hemorrhages ultimately resolved when the platelet count recovered.

Occlusive microvascular retinopathy has also been described after autologous SCT in patients receiving high-dose cytarabine and TBI, thus suggesting that particular high-dose chemotherapy regimens may predispose to vascular injury at otherwise safe radiation doses.[477] Other infrequent complications of transplantation include chorioretinal lesions, vitritis, increased ocular pressure, and retinal hemorrhages.

Cataracts are a common complication of HSCT. Radiation is a well-described predisposing factor. GVHD and treatment with corticosteroids are inconsistent risk factors across studies.[478-481] Although historically the incidence was as high as 80% in patients receiving single-dose TBI, patients receiving fractionated TBI or chemotherapy-only regimens still have a 20% to 50% incidence of cataracts 5 to 6 years after transplantation.[482,483] The relative risk for cataracts and the need for subsequent cataract surgery may also be related to previous therapy or to glucocorticoid use for prophylaxis or management of GVHD.

Overall, reports of large pediatric populations have shown that some type of ophthalmologic abnormality occurs in up to 50% of patients, although visual function remains excellent in virtually all patients.[484,485] Thus yearly examination by an ophthalmologist is essential, including a Schirmer test to rule out xerophthalmia and a thorough retinal evaluation.

Dental Complications

Disturbances in dental development have been described in children conditioned with TBI, particularly if HSCT took place before 6 years of age.[486,487] Characteristic findings include short dental roots, absence of root development, and microdontia. A review of 52 pediatric patients younger than 10 years at HSCT found that all patients had disturbances in dental root growth, with the most pronounced changes occurring in those 3 to 5 years old at the time of transplantation.[488] Subsequent decreased alveolar bone growth may lead to further compromise of dental development.

Oral sicca syndrome (occurring as a complication of either TBI or cGVHD, or both) may result in significant morbidity, including chronic oral mucosal injury, poor oral hygiene, and subsequent dental decay.[489-491] Patients report frequent thirst, altered taste, difficulty eating and swallowing, and oral sensitivity.[489] The salivary glands are a common site of cGVHD and results in both quantitative and qualitative deficiencies of the major and minor salivary glands resulting in decreased salivary flow.[489,492,493] Decreased salivary production alters the biochemical and immunologic composition of the saliva and can contribute to the development of caries.[489,492,493] The primary treatment of sicca syndrome includes sialogogue therapy—stimulants such as sugar-free gum or prescription medications. Patients with oral sensitivity should avoid foods that cause discomfort and heavily flavored toothpastes or alcohol-based mouthwashes.[494]

Children with oral GVHD and certain underlying diseases such as Fanconi anemia are at heighted risk for oral cancers.[495-498] These patients should receive routine evaluation by an oral medicine specialist comfortable with oral cancer screening. The effects of prophylactic interventions are not well reported, but routine oral hygiene including brushing and flossing and twice-yearly evaluation by a dentist knowledgeable about potential HSCT-related issues is recommended.[494]

Pulmonary Complications

Acute and late noninfectious pulmonary complications are common and occur in as many as 50% of pediatric HSCT patients.[499] Pulmonary illness complicates both autologous and allogeneic HSCT; however, these toxicities are more common after allogeneic transplant.[500] Patients with pulmonary complications have a higher overall mortality compared with patients who do not develop pulmonary complications.[500,500a,501] The spectrum of noninfectious lung disease includes idiopathic pneumonia syndrome (IPS), diffuse alveolar hemorrhage (DAH), bronchiolitis obliterans syndrome (BOS), bronchiolitis obliterans organizing pneumonia (BOOP), and periengraftment respiratory distress syndrome (PERDS).

IPS is a potentially fatal acute complication of transplantation that is characterized by respiratory compromise, hypoxia, and diffuse radiographic change in the absence of an identifiable infectious cause. In 2010 the American Thoracic Society updated the definition of IPS as an idiopathic syndrome of pneumopathy after HSCT with evidence of widespread alveolar injury and in which infectious causes and cardiac dysfunction, acute renal failure, or iatrogenic fluid overload have been excluded.[502] They further classified it into specific entities based in large part on the primary anatomic sites of inflammation and dysfunction. Subtypes of IPS include acute interstitial pneumonitis, DAH, delayed pulmonary toxicity syndrome (chemotherapy induced), and PERDS.[499,502] IPS typically presents between 6 to 7 weeks after allogeneic HSCT, but studies have reported cases presenting earlier and later.[503-505] Risk factors for IPS allogeneic HSCT include allogeneic transplant, full-intensity conditioning, the use of TBI in the conditioning regimen, the presence of aGVHD, older recipient age, and an underlying diagnosis of acute leukemia or MDS.[502,505-509] The treatment of IPS includes supportive respiratory care with oxygen, diuretics, and often mechanical ventilation. The mainstay of medical therapy is systemic corticosteroids. Recently the soluble TNF-binding agent etanercept has shown promise if started early in the course of illness along with a corticosteroid.[504,510,511] Despite advances in ventilatory management, supportive care, and medical therapy the mortality rate for IPS remains high. It is reported to be as high as 80% for all patients and 95% for ventilated patients.[499,502,505,507,511] It should be noted that most reports of IPS include adult patients and it is likely that mortality rates are lower when children are considered independently.

DAH is an acute complication that typically develops in the periengraftment period in both allogeneic and autologous transplant patients.[502,512-516] It is characterized by shortness of breath, cough, and hypoxemia, often with fever. The diagnosis of DAH is based on progressively bloodier return of bronchoalveolar fluid in the appropriate clinical setting. Frank hemoptysis is rare.[502,512] Chest imaging demonstrates bilateral, diffuse infiltrates with potential areas of consolidation typically in the central lung.[517] Risk factors for DAH include older age, transplantation for metabolic disease or solid tumor, severe mucositis, and renal insufficiency.[503,512,517-520] DAH is treated with respiratory supportive care, transfusional support, and high-dose systemic corticosteroids (2 mg/kg/day to 1 g/m^2/day) with slow taper.[513,514,518,521] Even in the setting of rapid and complete treatment the mortality from DAH ranges from 60% to 100%, with death usually the result of multiorgan failure.[513,514,518,521]

PERDS is a subset of IPS that occurs within 5 days of engraftment and is the respiratory component of clinical engraftment syndrome. Other components of engraftment syndrome include rash, fever, capillary leak, and diarrhea. Diagnostic criteria for PERDs include fever, hypoxia, and/or pulmonary infiltrates identified by imaging that occurs within 5 days of engraftment without evidence of infection or pulmonary dysfunction.[522] There can be overlap with DAH, and approximately 30% of patients with PERDS have DAH.[522,523] PERDS that occurs independently of DAH responds well to therapy with systemic corticosteroids (1 mg/kg/day), and death is uncommon.[502,522,523]

BOOP (also called cryptogenic organizing pneumonia) presents as dry cough, fever, and dyspnea and is pathologically characterized by presence of granulation tissue within the alveolar ducts and alveoli.[499,523,525,526] BOOP can present in the immediate posttransplant course or as late as 2 years after HSCT.[523,527] Pulmonary function testing shows a restrictive defect, decreased diffusing capacity for carbon monoxide (DLCO), and hypoxemia.[523,527] Chest imaging demonstrates patchy airspace consolidation and ground-glass and nodular opacities in a bilateral or unilateral pattern.[528,529] Although clinical findings may support the diagnosis of BOOP, the diagnostic standard requires tissue either obtained by transbronchial or surgical biopsy.[523] Unlike BOS, described next, BOOP responds well to systemic corticosteroids and most patients are long-term survivors.

Obstructive lung disease has been reported in up to 20% of children after allogeneic HSCT, which is similar to the incidence in adult patients. BOS is a noninfectious, potentially life-threatening complication of HSCT. It is a pathologic process that leads to the obstruction and eventual obliteration of the small airways[530] and is characterized by chronic airflow obstruction that is unresponsive to bronchodilator therapy.[531] The etiology of BOS is not fully understood. It is believed that immunomodulatory and inflammatory mechanisms play roles in its development.[532] This is supported by an association between BOS and cGVHD.[533] BOS has been associated most firmly with cGVHD, although associations with viral infections prior to day +100 and gastroesophageal reflux have also been reported.[531,533-535] Pulmonary function tests reveal an obstructive pattern with reduction in the forced expiratory volume in 1 second (FEV$_1$) and ratio of FEV$_1$ to forced vital capacity (FVC). High-resolution computed tomographic examinations with inspiratory and expiratory views have been shown to be sensitive and specific indicators of BOS in HSCT patients.[536-538] Characteristic findings include air trapping, a mosaic pattern of lung attenuation, and bronchiectasis.[522,536-539] BOS is traditionally treated with immunosuppressive agents, with corticosteroids being first-line treatment, although corticosteroid-resistant disease is common and portends a poor prognosis. Azithromycin has been shown to be a useful adjunct to customary immunosuppression in some patients.[540,541] Published mortality rates for BOS after HSCT range between 21% and 100%.[531,542,543] The prognosis after developing BOS depends in part on the tempo of the disease and the response to first-line therapy.[531,544] Rapid onset BOS and lack of response to first-line immunosuppression are poor prognostic indicators.[544]

Restrictive and diffusion abnormalities have been reported in pediatric patients after SCT.[545,546] These findings improved over time but do not normalize.[545] Risk factors for restrictive lung disease include sclerodermatous type GVHD, single-fraction TBI, abnormal chest wall growth due to radiation, and certain chemotherapeutic agents.[546-551] Treatment depends on the underlying cause of the pulmonary disease. Because restrictive changes can be asymptomatic, assessment of the frequency of this complication depends on the intensity of post-SCT follow-up. Pulmonary compromise can influence the morbidity and mortality of survivors of HSCT. When developmentally appropriate, all HSCT recipients should have pulmonary function testing at 1-year post transplant. Providers should have a low threshold for screening patients who have risk factors for pulmonary complications earlier and more frequently. High-resolution chest tomography should be considered in high-risk patients who are unable to successfully complete pulmonary function testing.

Hematologic Complications

Patients who undergo ABO-incompatible allogeneic HSCT are at risk for immune hemolytic anemias. In the setting of major ABO incompatibility (e.g., recipient O and donor A or B), preexisting patient isohemagglutinins can cause lysis of donor red cells. This can potentially cause an acute hemolytic reaction during stem cell infusion, and thus anti-A or anti-B isoagglutinins must be removed from the patient before infusion via pheresis or the stem cell product must undergo red cell depletion. Although the acute issues surrounding transplantation in the setting of major ABO incompatibility are easily managed and not associated with any impact on outcome, pure red cell aplasia may develop in up to a fourth of at-risk patients in the months after HSCT.[552] This occurs when long-lived patient lymphocytes continue to produce antibodies capable of destroying red cell precursors in the graft. Patients who are type O and donors who are type A are at greatest risk, presumably related to the level of isohemagglutinin titers.[553] A relationship of red cell aplasia to posttransplant immunosuppression with cyclosporine alone has been suggested because calcineurin inhibitors permit longer persistence of patient B cells than methotrexate does.[554] Some cases of aplasia will resolve spontaneously over time; plasma exchange with donor-type plasma replacement has also been used, and, more recently, rituximab, a chimeric anti–B-cell antibody, has been used with success.[555] Minor ABO incompatibility (e.g., patient A, donor O) is managed by plasma depletion of the graft before infusion. This situation can also lead to low-level hemolysis in the first months after HSCT as newly formed B lymphocytes produce isohemagglutinins targeting residual patient red cells. Occasionally, in the months to years after transplantation, autoimmune hemolytic anemia, autoimmune thrombocytopenia, or autoimmune neutropenia develops as a result of immune dysregulation after HSCT. Corticosteroids and intravenous immune globulin are usually the first therapeutic intervention, but rituximab has been used in this setting as well.[556,557]

Renal Complications

Chronic kidney disease (CKD) is a common complication in long-term survivors of HSCT, occurring in 20% to 60% of adult transplant recipients and as many as 62% of pediatric HSCT survivors.[558,559,560,561] The incidence of chronic nephropathy is probably underestimated. Up to 25% of adults undergoing allogeneic HSCT were affected at 10 years; posttransplant hypertension was the most significant predictor of CKD.[562] Assessment of renal function by determination of the glomerular filtration rate revealed renal impairment in 41% of pediatric patients 1 year after HSCT and in 11% at 7 years, with exposure to TBI being the most significant risk factor.[563] HSCT survivors with CKD can progress to end-stage renal disease (ESRD). Survivors of HCT who develop ESRD have decreased survival compared with other patients with ESRD who did not did not undergo HCT.[564] Factors that contribute to CKD and ESRD may begin during the acute transplant period.

The long-term impact of transplantation on renal function in pediatric patients is not known, but given the natural decline in renal function with age, monitoring function over time should be part of routine care for survivors of pediatric HSCT.

It has been demonstrated that the presence of proteinuria, a marker of nephropathy, at transplant day 100 increases the risk for nonrelapse mortality by as great as sixfold by 1 year after HSCT.[558] Albuminuria is considered to be a marker of endothelial dysfunction and/or inflammation.[565] It is postulated that albuminuria reflects a systemic, generalized endothelial injury and is associated with increased risk for nonrelapse mortality in the first transplant year. Albuminuria has been identified as a risk factor for cardiovascular and noncardiovascular mortality in the general and other patient populations.[566-570] However, little is known about risk factors for CKD progression or why CKD and proteinuria increase nonrelapse mortality in the HSCT patient population. As this is explored in prospective studies it is suggested that children with albuminuria are considered for referral to a nephrologist experienced with posttransplant care.

HSCT-associated thrombotic microangiopathy including hemolytic uremic syndrome and thrombotic thrombocytopenic purpura are complications that occur in 25% to 35% of HSCT.[571] These entities are characterized by vascular endothelial cell injury resulting in microangiopathic hemolytic anemia, thrombocytopenia, fibrin deposition in the microcirculation, and tissue damage.[571] Hemolytic uremic syndrome may develop at a median of 5 months after HSCT.[572,573] This disorder is generally manifested as moderate hemolysis and renal dysfunction, although some patients have more aggressive findings consisting of severe hypertension and seizures. Factors implicated in the development of this syndrome include exposure to radiation and use of calcineurin inhibitors.[574,575] The majority of reported cases appear to resolve spontaneously over time, although modest persistent anemia and decrements in renal function have been described. Thrombotic thrombocytopenic purpura is rare in pediatric patients and may result from more pervasive endothelial damage than seen in hemolytic uremic syndrome. It usually occurs in the first months after HSCT and can have associated fever, bloody diarrhea, and neurologic symptoms. Unlike classic thrombotic thrombocytopenic purpura, HSCT-associated thrombotic thrombocytopenic purpura is not accompanied by deficient von Willebrand multimer proteolysis and plasma exchange therapy is generally unsuccessful.[576]

Neurologic Complications

Patients undergoing HSCT may experience a variety of neurologic complications either directly related to treatment or the underlying diagnosis, as a consequence of immunosuppression, or as a result of medications used in supportive care.

Neurologic side effects are well described when cyclosporine and tacrolimus are used for GVHD prophylaxis.[577,578] Commonly described side effects include essential tremor and paresthesias, both of which typically resolve when the dose is decreased or formulation changed.[577] Seizures, visual disturbances, and encephalopathy are more significant but rarer neurologic complications.[579] Posterior leukoencephalopathy is a syndrome associated with seizure, transient blindness, visual hallucinations, and motor deficits.[578,580] It has been postulated that both seizures and acute visual loss arise from vasogenic edema accompanying cyclosporine-associated hypertension. Hypomagnesemia and elevated calcineurin inhibitor levels can be aggravating factors. These changes affect white matter, most commonly in the occipital area, and are accompanied by characteristic radiologic findings on magnetic resonance imaging (MRI).[581,582] In general, symptoms abate rapidly on discontinuation or decreases in the dose of cyclosporine.[583] A rare, severe complication of cyclosporine is manifested by parkinsonian motor features, ataxia, and/or quadriparesis.[577,584] These changes may improve with discontinuation of the medication or may be permanent. There are fewer neurologic side effects reported for corticosteroids. However, mood swings or even frank psychosis accompanying corticosteroid therapy are well described.[585]

Acyclovir is used for both prophylaxis and treatment of infection with herpes simplex viruses. Confusion, tremors, delusion, and psychosis have been associated with this drug.[586] Similarly, mental status changes, paresthesias, and, uncommonly, seizures have been reported with the antiviral agents ganciclovir and foscarnet.[587,588] Impaired renal function, often seen in HSCT patients, may potentiate the occurrence of these symptoms.

Cerebrovascular accidents, including cerebral hemorrhage, may also be observed after SCT and are usually associated with refractory thrombocytopenia. Large intracranial hemorrhages are generally fatal; patients with isolated subdural hematomas typically do well.[589,590]

Central nervous system (CNS) infectious complications related to immunosuppression, cGVHD, or both can arise after HSCT. The greatest risk for CNS infection is between the second and sixth months after transplantation.[577] The manifestations of CNS infection can differ from those in an immunocompetent patient in that localizing signs and symptoms may be less dramatic. Autopsy series demonstrate that up to 15% of necropsy patients harbor CNS infections, often clinically unsuspected.[591,592] *Aspergillus* species and *Toxoplasma gondii* are the most common organisms found.[593] Bacterial meningitis or encephalitis/meningoencephalitis caused by a variety of viruses, including CMV, herpes simplex types 1 and 6, adenovirus, and varicella-zoster, can also occur.[594] Routine antiviral prophylaxis and CMV screening and treatment of reactivation have reduced the incidence of viral CNS infections.[595,596] Opportunistic CNS infections in HSCT recipients are often fatal, and a high level of suspicion is warranted in patients with even minimal neurologic symptoms.[593]

Leukoencephalopathy is a poorly understood sequela of HSCT that is manifested as progressive neurologic deterioration in the months after transplantation. Clinical findings include lethargy, slurred speech, ataxia, seizures, decreased sensorium, dysphagia, spasticity, or even decerebrate posturing. Consistent white matter changes are found on MRI. In a series of 415 patients from Seattle, leukoencephalopathy was observed in 7% of patients, but only in those who had received CNS radiation, intrathecal therapy, or both before HSCT in conjunction with intrathecal methotrexate after HSCT.[597] More recently, a 4% incidence of irreversible leukoencephalopathy was reported in children receiving cyclosporine; a third of the patients died during the period of neurologic decline.[598] Cyclosporine levels were usually therapeutic in the affected patients, and it remains unclear what the risk factors are for this rare but devastating complication.

CNS involvement in GVHD is highly debated.[599] There are case reports of potential clinical and pathologic cases of possible CNS GVHD involvement in the literature.[577,600-603] The clinical presentation is variable and can include seizure, encephalopathy, and focal neurologic dysfunction.[577,600] MRI may show infarction, leukoencephalopathy, and/or hemorrhages.[604] Definitive diagnosis requires pathologic findings in the brain similar to GVHD changes in systemic organs.[577] Systemic immunosuppression is the mainstay of treatment.

The effects of different conditioning regimens on neuropsychologic functioning have been incompletely investigated. In particular, analysis of very young patients is limited. Nonetheless, it is clear that a significant proportion of young children, including those younger than age 2 years, can undergo HSCT with chemotherapy and TBI and subsequently have normal neurologic development, perform well in areas such as sensorimotor and cognitive functioning, and have no decrement in academic achievement.[605-607] Age at HSCT appears to be the most consistent risk factor predicting subsequent cognitive decline.[607,608]

Endocrine Disorders

Much of the endocrine dysfunction after HSCT results from irradiation and is attenuated in both incidence and severity when radiation is fractionated or when non–radiation-containing conditioning regimens are used. Thyroid dysfunction is one of the most frequent late complications of HSCT reported in between 0% and 52% of transplant survivors.[609-612] After fractionated TBI, the incidence of compensated hypothyroidism ranges from 13% to 39% and that of overt hypothyroidism is around 15%, with diagnosis occurring at a median of 4 years after HSCT.[613-615] Patients treated with BU-based regimens have a lower, but still significant incidence of thyroid complications: 20% compensated and 9% overt hypothyroidism.[616,617] Undergoing transplant when younger than age 10 years is a risk factor for thyroid dysfunction, suggesting that the developing thyroid has increased susceptibility to toxicity.[609,618] Given that the incidence of thyroid dysfunction increases over time, clinical suspicion and yearly monitoring of thyroid function tests are an important part of long-term care for these patients.

Growth disturbance is a late effect specific to pediatric transplantation and is a common problem. However, the incidence of growth disturbance is unclear because multiple different definitions are used in the literature with published incidences between 20% and 82%.[619-621] Linear growth after HSCT is affected by many factors: nutritional status, thyroid and/or growth hormone disturbance, previous therapy, particularly corticosteroids or cranial or spinal radiation therapy, age at transplantation, conditioning regimen, and development of cGVHD with associated long-term corticosteroid exposure. Growth is most disrupted in children who underwent cranial irradiation before HSCT, followed by a TBI-based conditioning regimen due to disturbance of the pituitary axis.[622] This group of children is also at greatest risk for growth hormone (GH) deficiency. If the GH levels are deficient, GH replacement can have a positive impact on final height, particularly in children younger than 10 years at the time of HSCT. Therapy is not associated with an increase in relapse; however, a higher incidence of osteochondromas/exostosis was seen in GH-treated patients.[623] Nonetheless, many children do not demonstrate GH deficiency, and the role of exogenous hormone in these children or in children older than 10 at the time of diagnosis of GH deficiency is not clear.[624,625] Overall, the mean loss of height appears to be approximately 6 cm, as predicted by both genetic height and height at transplantation.[615] Still, nearly 80% of those undergoing HSCT in childhood will achieve a height that is within 2 standard deviations of adult norms.[626]

Gonadal failure after HSCT is common; predictors include previous gonadotoxic therapy and the preparative regimen used for transplantation. For example, children with aplastic anemia who receive CY as their only cytotoxic agent almost always progress normally through puberty, whereas those receiving a BU- or TBI-based ablative regimen are very likely to have delayed development.[625] Thus these children must be carefully monitored by a pediatric endocrinologist as they approach adolescence so that hormone replacement can be appropriately administered. Females who are postpubertal at the time of HSCT also need to be evaluated for hormone replacement therapy. In addition, they are at risk for gynecologic complications, including reduced uterine size, decreased vaginal and cervical size, atrophic vulvovaginitis, introital stenosis, and loss of pubic hair.[627] No comparable data regarding these complications exist for those prepubertal at the time of HSCT. In postpubertal males, HSCT with or without radiation-based conditioning often results in azoospermia, and sperm banking should be offered to all patients with sufficient spermatogenesis.[628] Significant Leydig cell damage resulting in decreased testosterone and decreased testicular volume is less common.[629] In both sexes, spontaneous recovery of gonadal function has occurred years after HSCT. Fertility resulting in liveborn infants has been reported for both male and female SCT patients, particularly after conditioning with CY alone.[630,631] Successful pregnancies are much less common after BU- and TBI-based regimens, and there is an increased risk for spontaneous abortion, preterm labor, and delivery of low- or very-low-birth-weight infants. There does not, however, appear to be an increased incidence of congenital anomalies in the offspring.[629] Concern regarding fertility is one of the major issues for HSCT survivors up to a decade after transplantation.[632]

Bone Problems

The most prominent bony complications after HSCT are decreased bone mineral density (BMD) and osteonecrosis. HCT may result in decreased bone mineral density, which may lead to osteopenia and osteoporosis with associated increased risk for fracture, loss of independence, immobility, chronic pain, and an overall decreased quality of life. Because many patients are asymptomatic, the incidence of osteopenia/osteoporosis in children and the impact on future bone health are not well understood. Small series of pediatric patients indicate that up to 50% of survivors of childhood allogeneic HSCT will have abnormal BMD and thus have an excess risk for fractures and other skeletal complications as they age.[633-635] The Bone Marrow Transplant Survivor Study reported a prevalence of osteoporosis of 9% in long-term survivors (median age 36.5 years) of HSCT for acute leukemia.[636] This was significantly higher than the prevalence of 2.2% in the siblings of the transplant patients. Allogeneic transplant and female sex was associated with increased risk for osteoporosis. There are few reports of posttransplant BMD changes in children, and most are from small, single-center, and retrospective studies.[635,637-639] The single prospective analysis of 29 children after HSCT demonstrated that most bone mineral loss occurs within the first 6 months after transplantation.[638] Studies of adult HSCT patients demonstrate that posttransplant bone turnover is characterized by increased resorption and decreased bone formation and that alteration in BMD is preceded by changes in markers of bone turnover such as serum osteocalcin, serum alkaline phosphatase, and urine amino-terminal telopeptide (N-telopeptide, NTX). It is not known if these patterns are the same in children, and a detailed understanding of how bone mineral status changes during and after HSCT is lacking.

Children are at risk for decreased BMD after HSCT due to factors related to their diagnosis and treatment including the underlying disease, pre-HSCT therapy (especially with corticosteroids), conditioning chemotherapy and TBI, osteotoxic medications, and presence of and treatment for GVHD.[640] Studies of adult patients have shown that the chemotherapy and radiation used in HSCT conditioning can mediate skeletal toxicity in two ways. First, damage can occur through direct insult to osteoprogenitor cells.[641-643] Second, cytokine release during the initial weeks after HSCT can activate osteoclasts resulting in increased bone resorption.[644,645] The RANK/RANKL/OPG pathway is believed to be the primary mediator of osteoclastic resorption after conditioning.[643,646] Receptor activator of the nuclear factor-κB ligand (RANKL) and RANKL/OPG (osteoprotegerin) ratio increase after transplant-stimulating osteoclastogenesis.[643,646]

Osteonecrosis or avascular necrosis, the death of a segment of bone, is a potentially debilitating manifestation of bone disruption and can result in significant pain or fractures. Corticosteroid use and the presence of cGVHD appear to be the major risk factors, and children older than age 10 years appear to be at more risk than younger children.[647,648] Joints of the lower limb, particularly the hip, are most often involved, and up to half of patients ultimately require joint replacement.[649] Radiation-induced bone abnormalities, including osteochondromas and metaphyseal growth abnormalities, can occur after TBI-containing transplant regimens; younger patients appear to be at greater risk.[650] Childhood survivors of HSCT should be evaluated regularly to ensure that hormonal and nutritional status is optimized. The role of bisphosphonates in the treatment of these conditions is being investigated. Early data indicate that bisphosphonates can improve BMD, but long-term effects of this intervention in the pediatric population are unknown.[639]

CLINICAL APPLICATIONS OF HEMATOPOIETIC STEM CELL TRANSPLANTATION

Myelodysplasia/Juvenile Myelomonocytic Leukemia

The myelodysplastic syndromes (MDSs) are a heterogeneous group of clonal stem cell disorders that can progress to acute leukemia. These disorders are much more common in adults but also account for approximately 3% of childhood hematologic malignancies. This figure may be an underestimate because a subset of children presenting with de novo AML presumably had an antecedent MDS.[55] The FAB classification system is based on the proportion of leukemic blasts in the marrow; most pediatric patients have either refractory anemia (RA), refractory anemia with excess blasts (RAEB), or RAEB in transformation (RAEB-T). Juvenile myelomonocytic leukemia (JMML), a rare disorder of infants and young children, shares similarities with MDS, and a new classification system has been proposed that allocates pediatric patients with clonal myeloproliferative diseases into three groups: MDS, JMML, and myeloid leukemia of Down syndrome.[651] MDS and JMML are disorders arising from pluripotent stem cells, and thus allogeneic HSCT remains the only curative approach.

Even though patients with JMML are generally very young and should tolerate therapy, induction chemotherapy prior to HSCT is generally not recommended because remission is unlikely to be attained and toxicity is unexpectedly high.[652] Thus proceeding straight to HSCT is the recommended course. A BU- versus TBI-based conditioning regimen has been associated with a lower rate of relapse.[653,654] As transplant-related mortality has decreased, survival has improved, with recent consortium data reporting an approximately 50% 5-year DFS; relapse remains the greatest obstacle to cure.[654] *Cis*-retinoic acid appears to decrease the spontaneous proliferation of leukemic progenitor cells in JMML as well as abrogate their selective hypersensitivity to GM-CSF.[655] It is often given after HSCT as therapy for MRD, although efficacy has not been firmly established.[656] A small subset of patients may exhibit a milder phenotype of the disease, and rare reports exist of improvement without specific intervention. *RAS* mutations may be more common in this group,[657,658] or children may have had a viral illness mimicking JMML. The vast majority of children, however, exhibit persistent or progressive disease and should move expeditiously to HSCT once other diseases in the differential diagnosis have been excluded.

The role of induction therapy before HSCT for MDS is unclear and has never shown a convincing impact on outcome. Up to one half of patients can achieve remission after AML-directed therapy[659]; however, and this approach may decrease relapse in patients with increased blasts at presentation.[652] Conditioning with both TBI- and BU-based regimens has been used, with most studies showing no difference in outcome,[660] although some favor BU.[661] Overall, the strongest predictor of survival is stage of disease.[662,663] A child undergoing transplantation for RA/RAEB has an event-free survival (EFS) of around 70%; a patient with RAEB-T was five times more likely to experience relapse.[664] Currently, approximately half of pediatric patients with MDSs can be cured, and thus allogeneic HSCT should be considered early in the disease course.[665] A retrospective review of pediatric patients from a single institution demonstrated a 80% DFS for those receiving no induction therapy and going to HSCT within 140 days of diagnosis.[666]

Acute Lymphoblastic Leukemia

Improvements in chemotherapy regimens and supportive care have resulted in a steady increase in the cure rate for ALL, and approximately 85% of children are now cured by current risk-adapted chemotherapy regimens.[667,668] However, for patients with ALL who cannot be cured by standard-dose chemotherapy, HSCT remains the mainstay of treatment. Because HSCT can be very effective for patients with relapsed disease, there has been an increasing focus on identifying the clinical and biologic characteristics of patients who are at highest risk for relapse after standard-dose chemotherapy so that they can be referred for HSCT at the earliest possible point in their course before relapse occurs.

In newly diagnosed children with ALL, there are many clinical and biologic characteristics, referred to as "risk features," that are used to adapt the duration and intensity of chemotherapeutic regimens (Fig. 8-2).[669] However, a smaller number of features are used to identify newly diagnosed patients for whom allogeneic SCT should be the standard approach once remission first remission has been achieved. Patients with leukemia marked by a cytogenetic abnormality resulting from a reciprocal translocation of chromosome 9 and 22 (i.e., t[9;22]) known as Philadelphia chromosome–positive ALL (Ph+) have historically had a dismal prognosis when treated with standard-dose chemotherapy.[670,671] For these patients, matched-sibling HSCT in first remission significantly improved survival. A large European cohort found a relative risk of 0.3 for relapse or any adverse event with

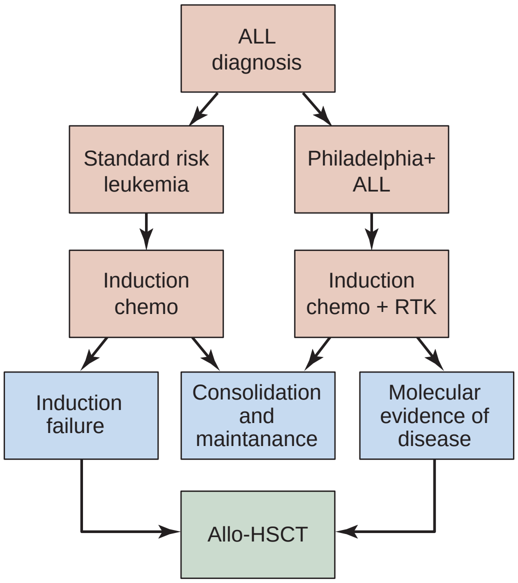

Figure 8-2 The role of hematopoietic stem cell transplantation *(HSCT)* in newly diagnosed acute lymphoblastic leukemia.

transplantation versus conventional chemotherapy[672] and a DFS of more than 60%.[673] Currently, most patients with Ph+ ALL undergo transplant in first remission using the best available donor, with 5-year EFS of 50% after mismatched family or UD transplant.[674] However, the treatment approach to Ph+ ALL has been evolving since the advent of tyrosine kinase inhibitor (TKI) therapy (see Chapter 51),[675,676] and the current recommendations for pediatric Ph+ ALL in first remission now suggest treatment with a combination of intensive CT plus TKI.[677] An emerging consensus is that HSCT should be reserved for newly diagnosed patients with Ph+ ALL who do not show molecular evidence of response.[678]

The role of transplant in first remission is less clear for other adverse cytogenetic abnormalities, including patients with t(4;11) translocation or hypodiploid ALL. Extreme hypodiploidy of less than 44 chromosomes is uncommon but results in an EFS of only 30% with standard chemotherapy.[679] This has led to the inclusion of patients with hypodiploidy in trials of HSCT in first complete remission,[673,680] although the numbers of hypodiploid cases are too small to make definitive statements about the impact of HSCT on outcome.

Historically, the outcome of patients with the T-cell phenotype of ALL has been worse than patients with precursor-B cell ALL, but those differences have largely been abrogated by the intensive chemotherapy used in modern protocols.[681] However, an attempt is ongoing to define high-risk patients within this group, usually in those with a very high white blood cell count at diagnosis. Some reports suggest that the outcome for this subgroup may be improved by transplant in first remission,[682,683] but this has not become standard of care in pediatric patients. It is not certain that the presence of a t(4;11) translocation is an adverse risk factor for children older than 1 year of age,[684] but infants who harbor this translocation have an almost fivefold increase in the risk for an adverse event compared with infants without an MLL rearrangement.[685] Even within this group further distinctions can be made: very young infants with MLL + ALL and a high white blood cell count at diagnosis have an EFS of less than 5%.[686] An international study of infant leukemia found that a poor prednisone response in addition to the previously mentioned factors identified those at the highest risk.[687] It is not definitively known if HSCT in first complete remission can improve the outcome of this group; however, single-center studies have reported an EFS of more than 60%[688,689] and the current version of the international consortium trial recommends HSCT in first remission for the highest-risk group of infants.[690,691]

Many studies have demonstrated the prognostic importance of the rapid clearance of leukemic cells in the peripheral blood or bone marrow during induction chemotherapy, and patients who have induction failure have a poor outcome. Historically, the presence of blasts was assessed histologically; however, more sensitive methods of flow-cytometric detection of leukemic blasts[692] or polymerase chain reaction amplification of transcripts for clonal immunoglobulin, T-cell receptor, or oncogenic fusion genes[693] has become standard. In a large European study, induction failure, defined as the persistence of leukemic blasts 4 to 6 weeks after induction chemotherapy, occurred in only 2.3% of newly diagnosed patients, but the overall prognosis for this group was poor, with a 10-year overall survival of 32%.[694] In this study, patients with induction failure and either T-cell ALL, MLL rearrangement, or age greater than 10 years had a particularly poor outcome and a clear benefit of HSCT over chemotherapy was demonstrated. However, for younger patients (age <6 years) with B-cell phenotype ALL and no high-risk genetic features, no benefit of HSCT was shown.

In summary, it appears that ALL patients with some high-risk features at diagnosis have a decreased incidence of relapse and improved survival when taken to HSCT from a matched RD in first complete remission and that HSCT confers the greatest advantage in the patients at highest risk.[682] However, it is likely that increasing numbers of targeted therapies for specific molecular subtypes of ALL will enter the early stages of therapy in the coming years, making the indications for HSCT in newly diagnosed ALL an evolving field.

Once ALL has recurred, the chance of cure drops dramatically.[695] A landmark study in the late 1980s concluded that HSCT from a matched RD cured approximately 40% of children with relapsed ALL. In contrast, conventional chemotherapy resulted in subsequent relapse over 90% of the time, establishing HSCT as the mainstay of therapy for relapsed ALL.[696] However, just as for newly diagnosed patients, clinical and biologic features strongly affect the likelihood of cure with HSCT versus further chemotherapy. The strongest predictors of outcome in patients with recurrent ALL are the duration of the first remission, T-cell phenotype, and the site(s) of relapse.[695] These features are most commonly used to identify the patients with relapsed disease most likely to benefit from HSCT.

Patients who experience an early relapse (defined variably as relapse occurring on therapy or within 18 to 36 months from diagnosis) present one of the most difficult challenges in ALL therapy. It is clear that chemotherapy alone cannot cure these patients, and most studies show a survival advantage for sibling HSCT.[697] However, the results for HSCT in patients with early relapse ALL are also poor: a report of children with a bone marrow relapse within 12 months of completing therapy found a 3-year EFS of approximately 25% regardless of treatment modality.[698] Most of the earlier discussed studies have been with matched sibling donors; as outcomes with alternative donors have improved, equivalent results with UDs are now being reported.[699,700] In summary, it has not been determined which patients with ALL in the second complete remission conclusively benefit from allogeneic HSCT. Patients with late isolated extramedullary relapses should not undergo transplantation in most circumstances. For the remaining groups there are no prospective randomized trials to guide therapy.

Some children are unable to tolerate the months of intensive therapy given in relapse protocols and then should be considered for HSCT,[700] because most studies reveal a lower incidence of relapse with transplant that often but not always translates into an improved DFS.[680,701] The risks and benefits of transplant must therefore be carefully considered in each situation, balancing type and match of available donor, medical condition of the patient, and patient and family preference.

Patients experiencing an isolated bone marrow relapse fare more poorly than those with either an isolated extramedullary relapse (usually involving the CNS or testes) or those with a combined extramedullary and bone marrow relapse. As a result, most children with an isolated extramedullary relapse occurring after chemotherapy has been completed are treated with intensive chemotherapy and irradiation to the site of relapse rather than allogeneic SCT. This strategy results in very good outcomes, with a recent consortium trial showing that approximately 75% of children in this situation can be cured using systemic and intrathecal chemotherapy and cranial irradiation.[702]

Acute Myeloblastic Leukemia

Successive clinical trials over the past 30 years have firmly established the role of intensive postremission therapy in the treatment of AML. However, there remains debate as to whether allogeneic HSCT or intensive chemotherapy without stem cell support is the optimum postremission therapy. Allogeneic HSCT offers dose-intensity and the possibility of graft-versus-leukemia effect but is not an option for all patients and comes with heightened risks for acute and long-term toxicities. Intensive chemotherapy without stem cell support is feasible for most patients and may have less toxicity. A recent retrospective assessment of 233 children with cytogenetically defined high-risk AML did not demonstrate a survival difference between those who received chemotherapy alone and those who received allogeneic HSCT.[703] In this study the risk for relapse was lower in the transplanted population and the transplant-related mortality rate was higher. The study included patients who underwent transplants between 1989 and 2006. It is likely that the transplant-related mortality is lower in the modern era owing to improvements in supportive care. It is possible that the reduced risk for relapse after transplant may outweigh the risk for transplant-related mortality in the modern era.

Allogeneic HSCT in first complete remission was first addressed in a series of prospective trials in the mid-1980s. In 111 consecutive patients, the Seattle group undertook matched sibling allogeneic HSCT for patients who had a donor whereas the remaining patients received conventional intensification. The probability of 5-year DFS was 49% for HSCT compared with 20% for chemotherapy alone.[704] However, since that time, more intensive postremission chemotherapeutic regimens have been associated with improved disease control in children and adults with AML.[705,706] This has again raised the question of whether allogeneic HSCT is more effective than intensive chemotherapy in the postremission setting. To address this issue the Children's Cancer Study Group conducted a large, randomized trial of allogeneic HSCT versus chemotherapy or autologous HSCT in 652 children and adolescents with AML who achieved remission. At the end of induction, patients with five or six HLA-antigen matched relatives were allocated to allogeneic HSCT. All others were randomized between autologous HSCT with 4-hydroperoxycyclophosphamide (4-HC) purging or four courses of chemotherapy. The results of this trial showed that the outcome with allogeneic HSCT was superior to that of either chemotherapy or autologous HSCT.[705] For patients treated with an intensive induction regimen, the DFS with allogeneic HSCT was 66%, compared with 53% with chemotherapy 48% with autologous HSCT.[705] These data underscore the benefit both of intensive induction chemotherapy and of first-remission matched sibling donor HSCT in the management of newly diagnosed AML for many patients.

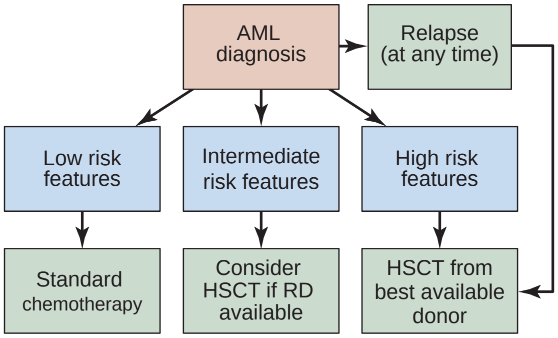

Figure 8-3 The role of hematopoietic stem cell transplantation *(HSCT)* in newly diagnosed acute myeloblastic leukemia.

Studies have attempted to identify groups of patients that are at increased risk for poor outcomes with chemotherapy alone in first complete remission for which HSCT would be beneficial. Investigators have shown that allogeneic transplant in first complete remission in patients can improve the outcome for children with high-risk prognostic features (Fig. 8-3).[707] In the pediatric population, consistent prognostic indicators are the response to initial induction therapy and cytogenetic markers.[708,709] Additionally, the presence of MRD before transplant has gained prognostic importance.[710-713] The Medical Research Council (MRC) AML-10 trial found 5-year survival rates of 67%, 65%, and 23% in patients who achieved complete remission, partial remission (5% to 15% blasts), and had residual disease, respectively.[708] The relapse rates in these groups were 34%, 38%, and 87%. This and other studies stratified patients based on favorable, intermediate, and adverse karyotypic abnormalities. Favorable karyotypic abnormalities include t(8;21), inv(16), t(15;17), and, in some studies, t(9;11) and t(16;16).[708,709,714,715] Adverse abnormalities include −5, −7, del(5q), abn(3q), and complex karyotypes.[715] Patients who have neither favorable nor high risk are considered of intermediate risk. In the MRC AML-10 trial, patients with favorable and intermediate karyotypic abnormalities had survival rates of 76% and 52% with relapse rates of 34% and 44%. This was compared with survival and relapse rates of 40% and 61% in patients with adverse karyotypes.[708] The MRC further designated risk groups of good, standard, and poor risk in the MRC AML-10 trial. The good-risk group included patients with favorable karyotypes or FAB type M3, irrespective of response status after one course of chemotherapy or the presence of other karyotypic abnormalities. The standard-risk group included patients in neither the good- nor poor-risk groups. The poor-risk group included patients with adverse karyotypic abnormalities or with resistant disease in the absence of favorable karyotypic abnormalities and not FAB type M3.[708] There was no significant difference in the percentage of deaths during first complete remission between any of the groups.[706] There were significant differences between the good, standard, and poor groups in regard to relapse-free survival (65%, 62%, and 36%), DFS (59%, 54%, and 32%), survival from relapse (61%, 17%, and 0%), and 7-year survival from complete remission (78%, 60%, and 33%). The 61% salvage rate after relapse in the good prognosis group suggested that HSCT in the first complete remission may be inappropriate for this group. Although not assessed in the MRC trial, patients with an activating *FLT3* mutation have been shown to have a poor prognosis and benefit from HSCT in the first complete remission.[373,716]

Several studies have suggested that the presence of high MRD at any point before HSCT leads to increased relapse rate.[710,711,713,717,718] Researchers at the Fred Hutchinson Cancer Research Center studied 253 patients and found that patients with even small levels of MRD before allogeneic myeloablative HSCT has a negative impact on overall survival in patients transplanted in both first and second complete remissions.[710] Rosenberg and associates investigated the impact of MRD on survival in 89 pediatric patients after myeloablative allogeneic transplant. The 3-year overall survival for patients with MRD positivity before transplant was 15%, compared with 67% in children without MRD. A prospective multicenter study is currently investigating the impact of MRD assessed before and at two time points after transplantation.

Autologous HSCT has been studied for patients who have no allogeneic donor. In many trials, autologous marrow was purged of residual leukemic cells with 4-HC, although no direct benefit of this technique has been proven. Multiple trials in adults and adolescents with AML suggest that autologous HSCT might offer additional therapeutic efficacy over chemotherapy.[659,719] Pediatric trials and a recent meta-analysis have not demonstrated any advantage to this modality in patients in first complete remission, and there are insufficient data to assess the influence of AML subclass on outcome.[720-724]

In most studies of patients with relapsed AML, the duration of first remission is strongly predictive of outcome, with the DFS of patients experiencing relapse less than 1 year after diagnosis 10% to 20% regardless of treatment modality used.[725,726] Early studies reported DFS of patients transplanted in untreated first ("early") relapse to be as high as 25% to 30%.[727,728] In contrast, a more recent report of 379 adults with relapsed or refractory AML found the DFS of patients transplanted in untreated first relapse to be no better than 13%.[729] Patients with treatment-refractory disease had an equivalently poor prognosis. However, the DFS for patients who were are able to go into a second remission was significantly better at 32%. The outcome of children with relapsed AML was studied by the MRC Childhood Leukemia Working Party.[725] Of the 81 patients in whom curative therapy was attempted, 69% went into a second remission. Approximately two thirds of these patients were consolidated with allogeneic HSCT, and the overall survival of the group was 44% at 3 years. Although this was not a randomized study, patients treated with HSCT had a longer duration of second remission than those treated with chemotherapy alone.[725] These data support the use of allogeneic HSCT in the treatment of relapsed AML, especially for those patients who can achieve a second remission.

Chronic Myelogenous Leukemia

CML is a proliferative stem cell disorder arising from a translocation between chromosomes 9 and 22 (t(9;22)) known as the Philadelphia chromosome. The only curative therapy for this condition is allogeneic HSCT. However, with the advent of targeted molecular therapies such as imatinib, a TKI, daily administration of an oral agent can control the disease for years, if not longer, with minimal side effects. As a result, the role of HSCT with the attendant short- and long-term toxicities is being redefined. The majority of the literature on both HSCT and TKI therapy reflects studies in adults in which current practice is to use TKI as initial therapy in virtually all patients presenting with chronic-phase CML.[730] A French prospective trial has shown that imatinib has similar efficacy in the pediatric population.[731] However, the risk-benefit profile remains different in the pediatric versus adult population; the results of transplant are better than in adults owing to both decreased transplant-related mortality and GVHD and the implications for both compliance and toxicity of decades of daily therapy are not yet well understood. In the largest pediatric transplant series to date patients receiving either matched sibling donor (n = 182) or UD (n = 132), HSCT had a 63% and 56% DFS, respectively. The best outcome was observed in those going to transplant in the first chronic phase and within 6 months of diagnosis; DFS with a sibling donor for that cohort was 73%.[732] Given these data and newer studies showing outcomes to be virtually identical with sibling donors or UDs for pediatric leukemia patients, it still may be reasonable to offer HSCT as the first therapeutic intervention for select pediatric patients with either a sibling donor or fully matched UD. However, the most common approach is to begin TKI therapy at the time of diagnosis with careful monitoring of disease response. Robust data now allow response to imatinib at specific time points to be categorized as "failures," thus identifying patients who need alternative therapies.[730,733] In young patients, whether the next intervention should be allogeneic HSCT or a trial of second-generation TKIs depends on donor availability and physician and family and patient assessment of the risks and benefits attendant on both strategies. However, given the paucity of efficacy and safety data on second-generation TKIs in the pediatric population, proceeding to allogeneic HSCT is currently the most conservative approach.[734] The rare patients who present in accelerated phase or myeloid or lymphoid blast crisis of CML have a much worse prognosis and require lymphoid- or myeloid-directed chemotherapy to try to attain a chronic phase followed by HSCT. Allogeneic HSCT historically offered curative therapy for approximately 40% of such patients.[63,735] The addition of TKI therapy both before and after HSCT has favorably impacted relapse in early studies.[736]

For those patients undergoing HSCT, careful monitoring of disease status remains an essential component of posttransplant care.[737] Relapse detected when disease is confined to cytogenetic or molecular positivity responds extraordinarily well to infusions of donor lymphocytes (DLI). Currently, most patients receive escalating doses of CD3+ T cells in an attempt to achieve disease control without inducing GVHD.[738] TKI administration for post-HSCT recurrences—either alone or in combination with infusions of donor lymphocytes—is still being evaluated but seems to be generally well tolerated and may favorably impact outcome.[739-741]

Hodgkin and Non-Hodgkin Lymphoma

Autologous HSCT has been utilized in children with Hodgkin disease (HD) or aggressive non-Hodgkin lymphoma (NHL) in the setting of refractory or recurrent disease after conventional therapy. Both TBI-based and chemotherapy-only approaches have been used with equal success. Autologous PBSCs as compared with bone marrow are the preferred stem cell source based on earlier engraftment for both neutrophils and platelets.[742] The ability of high-dose therapy with autologous rescue to salvage pediatric patients with Burkitt lymphoma was established in 1986, when a 5-year French experience demonstrated approximately 50% DFS.[743] Similar results in children with Burkitt and large cell lymphoma as well as HD have been reported more recently from other European centers and in larger combined pediatric and adult series.[744-748] Paralleling the findings in adult patients with lymphoma, the DFS is most consistently affected by the presence of chemotherapy-sensitive disease before HSCT; long-term survival is unlikely in those with resistant bulky disease at the time of HSCT.[749] Length of first remission (< or >12 months) also appears to impact survival.[750] The addition of rituximab before and/or after HSCT for B-cell lymphomas may further improve outcome in this group of patients,[751,752] although a more recent study of almost 500 adult patients failed to show a decrease in relapse with the addition of rituximab maintenance therapy after autologous HSCT.[753] Refractory or relapsed T-cell lymphoblastic lymphoma is treated identically to T-cell ALL, and allogeneic HSCT from the best available donor is the standard approach; achieving remission with salvage therapy is essential to have the potential for cure.[111]

There has been long-standing interest in the role of allogeneic BMT in the treatment of relapsed/refractory NHL and HD, although the balance of risk and benefit is still frustratingly unclear because most series show a decreased rate of relapse but increased transplant-related mortality.[754] Retrospective studies in adult/pediatric series suggest that ultimately the results are comparable.[755] A prospective trial confirmed lower relapse rates in allogeneic BMT but no overall survival advantage, owing to significant transplant-related mortality. As seen in patients undergoing autologous HSCT, chemosensitivity before the transplant procedure was the major prognostic factor affecting outcome.[756] The advantage conferred by an allogeneic stem cell source may be dependent on the type of lymphoma; a recent study of pediatric patients with anaplastic large cell lymphoma reported a remarkable 75% DFS, with successes even in patients experiencing relapse after autologous HSCT.[757] Whether the decrease in relapse rate reported in these studies reflects a graft-versus-lymphoma effect or merely the infusion of uncontaminated HSCs is not clear.[758] The introduction of reduced-intensity approaches can decrease transplant-related mortality, but this is often at the expense of an increased incidence of relapse.[759] Characterization of patients at particularly high risk for failure should allow better delineation of which patients could benefit from an allogeneic transplant—either alone or as a tandem approach after autologous transplant[760]—despite the increase in transplant-related mortality and the risk for cGVHD.

Neuroblastoma and Other Solid Tumors

The biology and treatment of pediatric solid tumors are discussed elsewhere in this text (see Section XV). The most extensive HSCT experience in pediatric solid tumors is in patients with advanced-stage neuroblastoma. Historically, patients receiving conventional doses of chemotherapy almost always succumbed to disease. In 1999, a landmark trial showed a significant improvement in DFS (34% vs. 22%) for patients randomized to consolidation with autologous BMT compared with continued intensive nonmyeloablative chemotherapy.[761] Regimen-related toxicity was acceptable, and dose intensification appeared to impact DFS; thus sequential or “tandem” transplants were investigated utilizing two[762] or three[763] cycles of non–cross-resistant agents over a short period of time with autologous PBSC support after each cycle. It appears that such approaches can further improve outcome, with reported 3-year DFS of 58%[762] and an estimated DFS of 45% at 7 years from diagnosis.[764] Relapse remains a significant problem primarily at diffuse osseous sites. Neuroblastoma cells marked in the stem cell product have been identified at sites of relapse,[167] and a variety of positive and negative selection techniques have been employed to purge the stem cells before reinfusion, although no technique has been definitively shown to impact outcome; profound depletion of T cells from the graft has, however, resulted in an increase in life-threatening viral infections.[765] Current efforts have focused on selecting a conditioning regimen that provides the greatest additive effect for disease control. Early results from a European randomized trial suggested that the incidence of relapse was lower in children receiving a BU/melphalan approach as compared with the carboplatin-based approach used in the United States. The ongoing national Children’s Oncology Group trial is evaluating this regimen. There are insufficient data to address efficacy; toxicity has been acceptable, although the incidence of severe VOD appears to be increased.[766] There has been long-standing interest in the role of allogeneic transplant for high-risk neuroblastoma. A retrospective study from the international registry did not demonstrate any impact on relapse rate or survival.[767]

Therapy for MRD after HSCT is essential. The use of *cis*-retinoic acid as a differentiating agent adds minimal toxicity and favorably impacts relapse.[761] A recent randomized trial unequivocally demonstrated the role of anti-GD2 antibody therapy after HSCT with significant improvement in both overall survival and DFS.[768] The trial met criteria for early stopping owing to demonstrated efficacy, and the ethical issues surrounding this relatively rare event are nicely reviewed by Unguru and colleagues.[769]

Most children undergoing autologous HSCT for neuroblastoma are very young, and there have been concerns about their quality of life after this intensive therapy. However, it appears that most survivors of this previously almost uniformly fatal disease are neuropsychologically intact. Survivors are at risk for a variety of medical complications, the most common being short stature, gonadal failure, and hearing loss (seen in three fourths of patients)[770,771] and must be closely followed by appropriate specialists to optimize outcome.

The role of high-dose therapy with autologous PBSC rescue for brain tumors is still being evaluated. The literature is most extensive for those tumors with a clear chemotherapy dose-response curve such as medulloblastoma/primitive neuroectodermal tumor and germ cell tumors.[772-775] HSCT is most often employed in the setting of recurrent disease but is occasionally used at the time of first remission in patients with adverse prognostic features or in very young children in an attempt to avoid radiation therapy.[776,777] The EFS ranges from 25% to 60%; and, as is the case for most conditions treated with autologous HSCT, those with no detectable disease at the time of transplant fare best. Ongoing consortium trials hope to standardize therapy and better delineate which patients can benefit most from this therapeutic approach.

Small single-institutional series have been published on the use of either allogeneic or autologous HSCT for other nonhematologic malignancies. Some promise in terms of response rate has been observed for patients with sarcomas who have both MRD and chemosensitive disease,[778,779] but the generalizability and durability of these responses will need to be established before widespread application can be supported.

Immunodeficiency Disorders

Since the late 1960s, allogeneic BMT has been successfully employed to cure children with a number of lethal immunodeficiency diseases, including severe combined immunodeficiency disease (SCID) and Wiskott-Aldrich syndrome.

Severe Combined Immunodeficiency Syndromes. SCID is a clinical syndrome arising from a variety of genetic abnormalities that all result in profound deficiencies in T-cell function.[780] At least 18 types of SCID have been identified that vary in the affected gene but share as a common feature a profound effect in T-cell development or function. Differences in the genetic basis of SCID results in a spectrum of abnormalities that may also affect B cell[781] and NK cell number and function.[782] In classic X-linked SCID there is profound T-cell lymphopenia and a variable number of nonfunctioning B cells whereas patients with adenosine deaminase deficiency lack B cells, T cells, and NK cells. Autosomal recessive SCID phenotypes have different combinations of B-cell and NK-cell presence.

Without effective therapy, the outcome of SCID is invariably fatal[782] and HSCT has become the mainstay of therapy for most types of SCID, with long-term survival as high as 90% of patients.[783-786] Unlike most transplant settings, owing to the underlying immunologic defect, SCID patients with a matched RD do not necessarily require conditioning.[787] After infusion of genotypically matched donor HSCs without cytoreduction, immune reconstitution will take place within several weeks and evidence of good lymphocyte function will be evident by 2 to 3 months.[788] Significant aGVHD or cGVHD is uncommon. Dramatically improved long-term survivorship from the range of approximately 50% in the 1970s to over 90% currently appears attributable to more rapid and precise diagnosis, followed by transplant of patients at a younger age and in better condition, and to improved supportive care for the associated infectious complications.[784,787]

The optimal approach for children lacking a matched sibling donor is less clear, and two different strategies have been employed: T-cell–depleted haploidentical transplant usually using a parent as the donor or transplant from a matched UD or UCB unit. Early experience with haploidentical HSCT was hampered by graft rejection, frequent severe GVHD, and opportunistic infection, resulting in poor long-term survival.[784,789] The advantage of this approach is that neither cytoreductive therapy nor GVHD prophylaxis is needed and thus peritransplant mortality is low. However T-cell function usually takes several months to appear, patients generally do not have B-cell function mandating lifelong intravenous immune globulin replacement, and graft rejection necessitating a second or even third transplant occurs in approximately 20% of patients.[790] T-cell function normalized and was most robust in children diagnosed early and undergoing transplant within the first 28 days of life,[791] thus increasing the importance of screening initiatives to diagnose SCID in the neonatal period.[792,793]

Given the concerns about haploidentical transplantation, there is increasing interest in using of matched UDs as a stem cell source for those patients lacking a matched sibling donor. Although cytoreductive conditioning is required with this approach, it may allow the more rapid reconstitution of a fully-functional donor immune and hematopoietic system.[789] These advantages must be weighed against the time taken to identify a UD and the risk for GVHD, the increased risk for acute infectious morbidity and mortality associated with myeloablative therapy, and the long-term sequelae, including the likelihood of infertility. Recent reports indicate that use of a UD confers a survival advantage compared with mismatched RDs (80% vs. 52%) as well as improved reconstitution of a full T-cell repertoire.[784] The role of nonmyeloablative HSCT and the ability of this approach to decrease acute toxicity and mitigate long-term sequelae is being investigated but may be a particularly attractive option in children with severe organ dysfunction at the time of HSCT.[794]

Wiskott-Aldrich Syndrome. Wiskott-Aldrich syndrome is an X-linked disorder in which progressive T-cell immunodeficiency is associated with eczema, small platelets, and absolute thrombocytopenia. The pathogenesis of this disorder is associated with aberrant expression of the WAS protein, which is involved in cytoskeletal organization as well as T- and NK-cell activation.[795] (See Chapter 24.) Both TBI-containing and BU-based regimens have successfully been used in conditioning patients with Wiskott-Aldrich syndrome, and the reported survival rates of almost 80% to 90% in patients are among the best of any patient cohort undergoing allogeneic SCT.[786,796] A recent collaborative study demonstrated 84% survival in 194 patients with Wiskott-Aldrich syndrome treated with HSCT,[796] although almost a fourth of patients did not have full donor chimerism at a year, suggesting that additional improvements in outcome may accrue from optimized conditioning regimens. Thus most children afflicted with Wiskott-Aldrich syndrome are now expeditiously referred for transplantation using the best available donor, with a palliative approach including splenectomy and chronic intravenous administration of immune globulin being utilized only in rare cases.

In summary, outcomes for patients with primary immunodeficiencies treated with HSCT are improving, as a result of better donor selection, optimal supportive care, and early diagnosis, but the field is still an evolving one, and the relative roles for HSCT from different donor sources—and the use of gene therapy for some diseases—are likely to change with time.

Hemoglobinopathies

Sickle Cell Disease. Allogeneic HSCT offers a curative approach for patients with sickle cell disease (SCD). Because the clinical course of SCD is highly variable and, particularly with the advent of pneumococcal prophylaxis, childhood morbidity and mortality is low, HSCT as a treatment modality has been relatively slow to gain widespread acceptance. Initial studies offered HSCT to the subgroup of patients with the most significant symptoms: stroke, recurrent acute chest syndrome, or frequent painful crises. In the seminal report by Walters, DFS after BU-based myeloablative conditioning was 73% and overall survival was 91% at 4 years; end organ damage was stabilized in patients with sustained donor engraftment.[797] Improved management of transplant complications in general and increased attention to SCD specific toxicities (e.g., additional immunosuppression to decrease graft rejection, antiseizure prophylaxis, strict management of hypertension) have resulted in consistent gains in cure rates,[798] with EFS of more than 95% in patients undergoing a transplant after January 2000.[799,800] Meticulous long-term assessment of these patients has demonstrated that successful engraftment will protect against progressive pulmonary and CNS disease but significant gonadal toxicity is sustained.[801] Newer approaches such as the use of hydroxyurea to decrease the risk for graft rejection by impacting pretransplant bone marrow cellularity[802] or the use of a nonmyeloablative approach to decrease transplant-related morbidity and mortality[803] or even preserve fertility require both larger numbers of patients and longer follow-up before being considered as a standard approach. Early results suggest that reduced-intensity conditioning results in mixed chimerism for most patients but is associated with sufficient erythroid engraftment to reverse the sickle phenotype.[804,805] HSCT has historically been considered only for patients who have an available matched sibling donor. A multiinstitutional survey of factors impacting the use of HSCT in this population found that less than 15% of affected children had an acceptable sibling donor.[798,806] The role of UD HSCT in patients with the most severe manifestations of disease or in those unable to receive long-term transfusion therapy is unknown, but multiinstitutional trials are currently ongoing with a reduced-intensity conditioning platform.[800] The use of unrelated UCB is a theoretically attractive option given the decreased incidence of GVHD and the ability to identify acceptable donors for patients with less common HLA typing by tolerating greater HLA disparity. Small numbers of transplants have been reported with successful outcomes, but graft rejection remains an obstacle to widespread use and resulted in early closure of the UCB arm of the ongoing national trial.[807,808]

The choice to proceed to matched-sibling HSCT remains complicated, particularly if complications of the disease are moderate or intermittent. There are no prospective randomized trials to inform the decision and therapy with hydroxyurea, chronic transfusions and HSCT each have associated risks and benefits. HSCT clearly has the highest upfront risk but also offers the only curative modality. The Belgian experience illustrates that children undergoing transplants at a young age before significant complications occur do best, with a DFS of 93% and overall survival of 100%,[809] and serious manifestations of SCD impacting on long-term survival such as pulmonary hypertension continue to be identified. Unfortunately, patients undergoing HSCT and who are cured of SCD remain at risk for complications from the transplant process: infertility is likely,[810] and the development of extensive cGVHD is a rare but potentially devastating possibility.[799] Patients (when of an appropriate age) and parents must be aware of the possible therapeutic options, thus allowing them to be educated and active participants in the decision-making process.[811-813]

Thalassemia. Thalassemia major (or Cooley anemia) is the most common transfusion-dependent anemia in the world. Chronic transfusion therapy and chelation to prevent the associated iron overload have modified the natural history of thalassemia and transformed a diagnosis that was uniformly fatal in early childhood into a chronic disease that is usually manageable, although progressive end organ dysfunction still develops.[814,815] Over time, even medically compliant patients are at risk for complications, including endocrine dysfunction, hepatic cirrhosis, cardiac disease, and transfusion-associated viral infections. In the developed world with sophisticated medical management, life expectancy is 25 to 55 years; compliance with chelation therapy is the most important factor impacting survival. Although advances have been made in supportive care over the past decade with resulting increases in life expectancy, in the developing world the majority of patients still die before the third decade of life.[814,816]

The successful application of HSCT as a curative approach for thalassemia major was first reported in 1982.[817] Subsequently, a very large experience reported by Lucarelli and coworkers[51] unquestionably established the feasibility of HSCT for thalassemia, and more than 3000 patients worldwide have now received transplants,[818] with the overwhelming majority using a matched sibling donor and a BU-based conditioning regimen.[819] The Pesaro classification system, based on the presence of hepatic fibrosis as assessed by liver biopsy, degree of hepatomegaly, and a history of inadequate chelation, allows risk stratification of potential transplant patients younger than 17 years of age: class I patients have no risk factors, class 2 patients have one or two risk factors, and class 3 patients have all risk factors. The risk factors used by Lucarelli and coworkers may be difficult to reproduce. The probabilities of overall survival, thalassemia-free survival (TFS), and graft rejection for patients undergoing HSCT from a matched family donor were 94%, 94%, and 0%, respectively for class I patients and 80%, 77%, and 9% for class 2 patients. Class 3 patients had only a 53% TFS owing to both increased graft rejection and transplant-related mortality.[51,820] Newer approaches based on increased immunosuppression using hydroxyurea, azathioprine, and/or fludarabine have yielded improved results for class III patients, with a TFS of 80% in patients younger than age 17 years.[821] Post-HSCT management of preexisting hepatic and cardiac iron overload and viral infections is essential for optimal outcome. Positive effects on bone mineral density in children may be seen after successful engraftment,[822] and even reversal of cirrhosis has been reported.[823,824] Impressively, a large study reporting on the quality of life of patients older than age 20 years after HSCT demonstrated that overall quality of life was identical to that of the general population for the majority of patients. Older age at HSCT and the presence of GVHD predicted a poorer quality of life. Interestingly, a matched cohort of thalassemic patients treated with transfusion/chelation reported a significantly worse quality of life when compared with transplanted patients.[825]

Although matched sibling HSCT has been shown to provide curative therapy for more than 90% of patients, the decision to accept the short- and long-term risks associated with HSCT remains complex and should occur on an individual basis after consultation with both hematologists and transplant physicians. Historically, patients lacking a matched sibling donor were not offered the option of HSCT because transplants using alternative donors conferred a dismal prognosis in this disease.[826] However, improvements in HLA typing, approaches to pre-HSCT preparative regimens, and supportive care have globally impacted transplant outcomes; thus alternative donor transplantation is now a reasonable option for selected patients. After mirroring results using sibling donors, it was shown that the best results occur in those with less-advanced disease, and a TFS of 80% to 100% for class I or II patients has been reported in small series.[827,828] Progress has occurred in the use of alternative donors in patients with advanced-stage disease as well. A recent small study using mismatched RDs and a preparative regimen emphasizing immunosuppression achieved a TFS of 94%.[826] A larger Italian study of 60 patients, two thirds of whom received transplants from UDs, employed a treosulfan-based preparative regimen and had a 5-year TFS of 84%, likely owing at least in part to the decreased hepatotoxicity of treosulfan compared with BU. There was no effect of Pesaro class or donor type (sibling vs. unrelated) on outcome.[829]

Thalassemia is a major global health problem because it is now most prevalent in resource-poor countries, with more than 100,000 affected children born annually in the Middle East and Southeast Asia. These countries lack the infrastructure to provide safe transfusions and/or chelation therapy, and the majority of children die before reaching adulthood.[830] The development of safe and affordable transplant strategies that can provide a cure for these patients is one approach to this important problem.

Osteopetrosis

Infantile malignant osteopetrosis is a rare disorder of osteoclast function that affects 1:200,000 to 1:300,000 children.[831-834] Osteoblast function is preserved, and the osteoclast dysfunction leads to an inability to resorb and remodel bone. The resulting osteoblast-osteoclast imbalance results in the formation of excessive mineralized bone and cartilage. Infantile malignant osteopetrosis is inherited in an autosomal recessive or autosomal dominant manner and causes severe phenotypes.[831,834,835] There is significant phenotypic variability dependent on the patient's genetic mutation. Commonalities in all phenotypes are poor growth and bony encroachment on optic and other cranial nerve foramina causing vision and hearing impairment. Eradication of the marrow space ensues, with progressive pancytopenia, failure to thrive, and hepatosplenomegaly.[831] In addition, patients with *CLCN7* autosomal recessive mutations suffer from progressive neurodegeneration and do not benefit from HSCT.[831,835] Murine studies revealed that osteoclasts arise from hematopoietic precursors of the monocytic lineage, and disease reversal was accomplished by engraftment of bone marrow from unaffected animals.[836] Successful HSCT in humans was first reported in 1977, and its applicability has subsequently been shown in many reports mostly using myeloablative BU-based conditioning regimens.[831,834,837,838] The European Group for Blood and Marrow Transplantation (EBMT) reported the long-term outcome of 122 children undergoing allogeneic HSCT between 1980 and 2001.[839] The 5-year DFS rate was 73% in those receiving transplants from an HLA-identical sibling versus approximately 40% in those receiving transplants from mismatched family donors or matched UDs. The major causes of failure were lack of engraftment, attributed in part to disruption of the underlying marrow microenvironment, and pulmonary complications, particularly pulmonary hypertension. Conservation of vision was most likely in those who underwent transplantation before 3 months of age. Patients, especially those older than 2 years at transplantation, are at risk for hypercalcemia as engraftment occurs, and therefore meticulous monitoring of calcium levels is essential in the peritransplant period.[838,839] At present, HSCT is the only curative option, although calcitriol, interferon-γ, and prednisone have been used to slow progression, ameliorate symptoms, or both.[840-842] Donor identification and subsequent HSCT should occur expeditiously as soon as the diagnosis is confirmed. Unless an unaffected, matched sibling is readily available a UCB unit may be preferred because it can be obtained in a more expeditious manner compared with a UD search because neurologic impairment can occur rapidly and may be irreversible, even with successful hematopoietic engraftment.

Transplantation for Lysosomal and Perioxisomal Diseases

Allogeneic transplantation is a therapeutic option for children with select inherited lysosomal and perioxisomal disorders.[843] These are rare diseases, and there are fewer than 100 transplants performed for these disorders annually in the United States.[4] Two key principles for transplanting children with lysosomal and perioxisomal diseases are that in most cases neurologic function lost before HSCT cannot be restored and because of this children with advanced disease do not benefit from transplant.

The mucopolysaccharidoses (MPSs) are inherited diseases caused by gene defects that result in the deficiency of lysosomal enzymes needed to degrade glycosaminoglycans (GAGs).[844,845] This causes cellular accumulation of GAGs and end organ tissue damage.[844,845] Treatment of MPS can include therapy of organ-specific defects, enzyme replacement therapy when available, and HSCT. Enzyme replacement therapy is currently available for MPS types I, II, and VI, is typically given as monthly infusions, and can be complicated by infusional reactions.[846-848] Enzyme replacement can improve the nonneurologic performance status and organ function but does not cross the blood-brain barrier and is ineffective for CNS disease.

The success of transplantation for the MPSs is based on correction of the enzyme deficiency, enzyme cross-correction, and the ability of hematopoietic cells to engraft and distribute as macrophage-derived tissue cells (e.g., microglial cells in the brain and Kupffer cells in the liver).[845,849,850,851] The correction of enzyme deficiency is mediated by donor leukocytes that engraft in the host tissue and secrete the missing enzyme. The leukocytes may enter the tissue directly, or secreted enzyme may enter the circulation. CNS therapy relies on donor cells crossing the blood-brain barrier. Enzymatic cross-correction is based on the principle that healthy cells secrete enzyme and can correct the biochemical consequences of enzymatic deficiency in neighboring cells.[851]

Clinically, the MPSs are characterized by CNS compromise and potential involvement of the skeletal system, heart and heart valves, connective tissue, facial structure, cornea, retina, upper airway, and liver.[844] Adverse effects on growth and development are common.[851] The pattern of organ damage is specific to each disorder, as is the response to transplant; and transplant is not effective for all diseases in this class.[843,845,849,850]

Allogeneic transplantation is best established and most successful for MPS I (Hurler syndrome) and is considered investigational for the other diseases.[850] Transplantation does not correct all manifestations of MPS I. It can correct upper airway obstruction, hepatosplenomegaly, and corneal clouding and may improve hydrocephalus, hearing dysfunction, and abnormalities of growth and development.[852] It is least effective for skeletal disease. The majority of published reports of transplantation for MPS I use non–radiation-containing myeloablative regimens.[843,845,849-851,853] There is debate about the most appropriate stem cell source to use for transplants in children with MPS I. A recent investigation of the transplant outcomes of 258 children with MPS I found that chimerism and enzyme levels were superior when an HLA 6/6 UCB unit was used compared with all other donor sources combined, including matched sibling donors.[853] However, siblings who were disease carriers were included in the analysis and may contribute to lower enzyme levels in the matched sibling group.

Sphingolipidoses are a group of lipid storage disorders related to sphingolipid metabolism and include Tay-Sachs, Niemann-Pick, Fabry, Gaucher, and Krabbe diseases and metachromatic leukodystrophy (MLD).[849] Enzyme replacement therapy is available for Fabry and Gaucher diseases, and with this therapy patients with these diseases can live into adulthood.[848] Despite having similar pathophysiology to the other sphingolipidoses, enzyme replacement therapy is not available for Krabbe disease or MLD and HSCT is the only potentially curative option for these patients.[854-857] Both Krabbe disease and MLD have early onset/infantile forms that can be successfully treated if children undergo a transplant when asymptomatic or with few symptoms.[858-860] Late onset forms of the diseases can be stabilized if symptoms are present, but not in the setting of advanced disease.[859,860]

X-linked adrenoleukodystrophy (ALD) is a perioxisomal disorder characterized by adrenal gland dysfunction, progressive inflammatory demyelination of the brain, and chronic axonal degeneration of the spinal cord.[861,862] The gene responsible for the clinical manifestations of ALD is mapped to Xq28 and encodes for the perioxisomal half-transporter ABCD1. The transporter plays a key role in the metabolism of very long chain fatty acids (VLCFA). The dysfunction of ABCD1 prevents the degradation of VLCFA and results in their tissue accumulation.[862]

Cerebral ALD is the most severe phenotype of the disease. Boys with ALD have a 60% lifetime risk for developing the phenotype, which is asymptomatic in the early stages. Progression is characterized by MRI changes, emotional lability, impaired visuospatial function, cognitive deterioration, hemiplegia or quadriparesis, and a vegetative state followed by death within 2 to 5 years after the onset of symptoms.[861,863] MRI change is evaluated by the Loes severity system, with lower scores having less disease burden.[864]

Therapeutic strategies in cerebral ALD include metabolic correction of the VLCFA accumulation with non-VLCFA (e.g., Lorenzo's oil and walnut oil), adrenal replacement, antioxidant supplementation aimed at reducing CNS inflammation that accompanies the disease, and allogeneic HSCT.[863] Allogeneic HSCT is currently the only nonexperimental, potentially curative option for boys with progressive cerebral ALD.[865,866] The precise mechanism of effect is not clear. Proposed mechanisms include correction of the metabolic defect in the CNS, the presence of donor microglial cells in the brain, and decreased inflammatory and immunologic reactions that contribute to disease progression. Similar to MPS disorders, the neurologic deterioration present before transplant cannot be recovered and the disease can progress for months after successful HSCT. Overall survival is best when transplant is performed in boys with few or no neurologic deficits, low Loes score, and use of an RD.[843,865-867] Because of this it is essential that transplant occurs as soon as disease progression is detected by MRI screening or symptom development.

Hemophagocytic Lymphohistiocytosis

Hemophagocytic lymphohistiocytosis (HLH) is a potentially life-threatening condition of severe inflammation caused by uncontrolled proliferation of lymphocytes and macrophages. HLH can be classified as primary or secondary based on the presence of a genetic mutation in primary disease.[868] Secondary disease develops in response to an infectious, rheumatologic, malignant, or other stimulus.[869-872] HLH typically presents as fever, hepatosplenomegaly, and cytopenias.[873] Other features include hypofibrinogenemia, hypertriglyceridemia, hepatic dysfunction, hyperferritinemia, neurologic symptoms, low NK cell activity, and elevated soluble IL-2 receptor levels.[873-878] Allogeneic HSCT is the only curative option for children with primary HLH. The estimated overall 3-year probability of survival after HSCT for patients treated on protocol HLH-94 was 64%; 71% ± 18% with matched RDs, 70% ± 16% with matched UDs, 50% ± 24% with family haploidentical donors, and 54% ± 27% with mismatched UDs.[879] Transplant conditioning has historically been with BU-based myeloablative regimens.[880-884] Unfortunately, fully myeloablative regimens are associated with transplant-related mortality rates of approximately 25%.[880,881,883,884] Reduced-intensity regimens are being used by some centers in an attempt to decrease acute toxicity and currently are being investigated in the multiinstitutional setting.[885-887] Reduced-intensity regimens are associated with an increased risk for mixed chimerism and frank graft failure and thus may not be appropriate when UCB is the only potential stem cell source.[885-887]

Treatment of Bone Marrow Failure—HSCT in Congenital and Acquired Bone Marrow Failure Syndromes

Acquired Aplastic Anemia. Severe aplastic anemia (SAA) is a syndrome characterized by peripheral pancytopenia and marrow hypoplasia (see Chapter 6). Mortality associated with this disorder in children was historically as high as 50% in the first 6 months.[888] Although a number of immunosuppressive and marrow stimulatory therapies have shown reasonable activity, the only curative treatment remains SCT. The potential efficacy of allogeneic SCT for patients with SAA was established with the initial publication in 1972 of results achieved in four patients (Fig. 8-4).[889] Randomized studies have since established the superiority of matched allogeneic SCT over supportive care (androgens in the 1970s, immunosuppressive therapy with antithymocyte globulin in the 1980s and 1990s) in both children and, more recently, adults.[890-893] In the setting of a matched sibling donor, the role of pre-HSCT conditioning is mainly to provide immunosuppression. CY in combination with antithymocyte globulin is the standard regimen, with cyclosporine and short-course methotrexate for GVHD prophylaxis. This approach cures almost 90% of patients with none of the sequelae associated with radiation-containing regimens.[894,895] Stem cell source has traditionally been bone marrow, but many centers have switched to peripheral blood progenitor cell grafts for ease of collection and faster engraftment kinetics. In a retrospective study of European and American registries, patients younger than 20 years of age had increased risk for cGVHD and higher overall mortality with peripheral blood cell versus bone marrow grafts.[896] Immunosuppressive therapy before matched sibling HSCT leads to an inferior outcome, mostly owing to increased rates of graft rejection.[897]

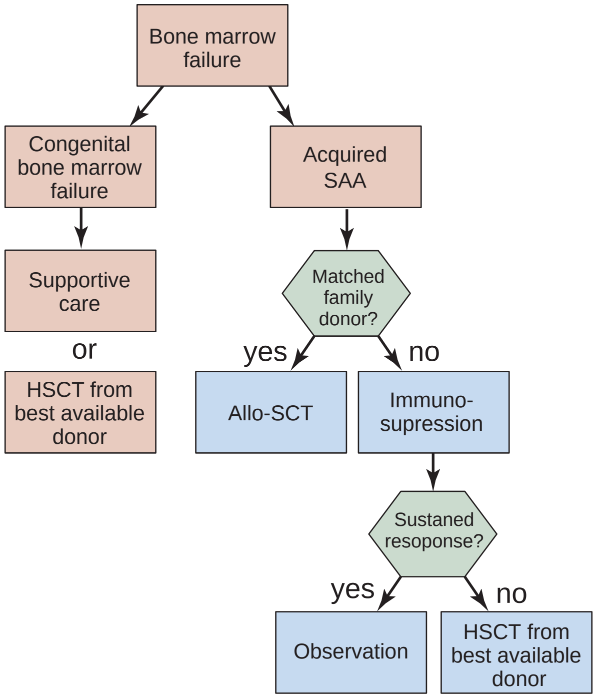

Figure 8-4 The role of hematopoietic stem cell transplantation *(HSCT)* in bone marrow failure syndromes.

Young patients with SAA receiving a non–radiation-containing regimen should have preserved fertility after HSCT.[898] In addition, long-term survivors exhibited few late effects[899] and were indistinguishable from healthy controls in terms of reported health, educational achievement, and psychosexual functioning.[900] Given these data and the expected excellent outcomes, patients with SAA and a matched sibling donor should be referred expeditiously for BMT, thus minimizing the number of pre-HSCT transfusions and the risk for acquiring serious infections.

Children who lack a matched sibling donor receive immunosuppressive therapy usually consisting of antithymocyte globulin, cyclosporine, and corticosteroids in an attempt to restore normal hematopoiesis. Up to 70% of patients will respond at 6 months, but only approximately 30% will have durable responses and remain transfusion independent. Additionally, a proportion of patients do not respond and responders remain at risk for both relapse of SAA and for evolution to MDS/AML.[901,902] The first reports of UD transplants for SAA used preparative regimens similar to those used with sibling donors. Results were very discouraging with graft rejection, transplant-related toxicity, and GVHD all being significant problems and survival rates less than 40%.[903] The incorporation of TBI into the conditioning regimen decreased the incidence of graft rejection and led to better results in some reports at least in part related to differences in patient condition, number of transfusions, and time from diagnosis to transplant.[904] In the era of improved HLA typing and better transfusion strategies, attempts have been focused on decreasing the intensity of the conditioning regimen and encouraging results have been reported with CY, antithymocyte globulin, and TBI doses as low as 200 cGY.[905] The addition of fludarabine or alemtuzumab with potent immunosuppressive properties and minimal organ toxicities may allow a radiation-free approach that could decrease both short- and long-term toxicities.[906,907] However, the experience with these approaches is still limited and long-term results, including durability of donor engraftment, remain to be evaluated. As conditioning regimens become less intense and outcomes using alternative donors approach those seen with sibling donors,[908] the optimal time to refer SAA patients for alternative donor transplant requires ongoing evaluation. As suggested by the results of a Japanese prospective trial, proceeding to well-matched UD HSCT at the time of failure of first immunosuppressive therapy is a reasonable choice for pediatric patients.[909] Alternative donor transplant as primary therapy for SAA in children is attractive, but, ideally, recommendations would result from a randomized trial so that robust data would illuminate whether the befits of early HSCT (fewer infections/fewer transfusions/decrease in graft rejection/less end organ toxicity) would outweigh subjecting the 30% of children who have durable response to immunosuppressive therapy to the risks for HSCT (early mortality/end organ damage/potential of significant GVHD).[910]

Congenital Bone Marrow Failure Syndromes. It is essential to exclude inherited causes for bone marrow failure particularly in the pediatric population. Such a diagnosis has a major impact on both therapy and outcome. Children with these syndromes rarely have a sustained response to immunosuppressive therapy and in general it should not be administered. HSCT provides the only curative approach for the bone marrow failure. Before transplant, associated abnormalities in the kidneys, liver, or lungs must be identified and the preparative regimen chosen in the context of the specific syndrome. Donor selection mandates the exclusion of the same disease in any RD; this can be complicated in those patients lacking a specific genetic marker. Virtually all patients with congenital bone marrow failure have an associated cancer predisposition syndrome. Although successful HSCT may abrogate the risk for secondary leukemia/myelodysplasia, the risk for solid tumors is unaffected or can even be increased owing to exposure to chemotherapy and/or radiation therapy in the preparative regimen and the insult of cGVHD. The most common of these syndromes are discussed next.

Fanconi Anemia/Dyskeratosis Congenita. Fanconi anemia is an autosomal recessive disorder clinically characterized by a highly variable physical phenotype, progressive marrow failure, and a marked propensity to develop AML and certain solid tumors (see Chapter 6). HSCT can be curative for the hematologic manifestations of Fanconi anemia, but early experience revealed excessive regimen-associated morbidity and mortality and in vitro studies confirmed hypersensitivity to alkylating agents and radiation.[911,912] Development of specialized conditioning regimens mostly employing low doses of both CY and radiation has spurred the application of HSCT as a treatment option, and results using HLA-identical sibling donors are excellent if performed early in life before the development of MDS or leukemia,[913,914] with 2-year DFS of as high as 95%.[915] In addition, the first reported transplant using UCB as a stem cell source was successfully undertaken for a child with Fanconi anemia using cells from an HLA-identical sibling.[141]

In contrast, the outcome using alternative donors had historically been very poor, with survival rates of approximately 30%[916-918]; graft failure and GVHD were the major causes of failure. The addition of fludarabine to the conditioning regimen and the removal of TBI have led to significant improvements in outcomes. In a recent review of patients with Fanconi anemia receiving UD, HSCT patients younger than 10 years old who had fewer than 20 transfusions and who received a fludarabine-containing regimen and T-cell–depleted marrow allografts had better outcomes, with 3-year survival of 52%.[919] Thus the prognosis for patients with Fanconi anemia receiving alternative-donor SCT has greatly improved over time and early referral for discussion of HSCT is indicated in patients with marrow failure.[920] There are limited data on the use of HSCT in patients with Fanconi anemia who have developed frank leukemia. Anecdotal reports suggest that there are occasionally long-term survivors, but death from regimen-related toxicity or leukemic relapse is frequent. A recent report from the Minnesota group on 21 patients with Fanconi anemia treated over the past two decades with HSCT for advanced MDS or acute leukemia reported a 5-year overall survival of 33%. Whether pre-HSCT chemotherapy adds more benefit than toxicity remains unclear.[921] Although HSCT can restore normal hematopoiesis, meticulous follow-up of these patients is essential because they retain a significant risk for developing solid tumors, particularly squamous cell carcinomas of the upper gastrointestinal tract and anogenital regions. The peak incidence occurs in the second and third decades of life. A European report on almost 800 patients with Fanconi anemia identified older age (>10 years) at time of HSCT, use of PBSCs, and the development of cGVHD as risk factors for secondary malignancy.[922] The outcome in patients developing these cancers is poor, so it is essential to try to decrease the incidence (through avoidance of radiation in the preparative regimen and prevention of cGVHD) and monitor patients closely so diagnosis can be made at an early stage of disease.[923,924]

Dyskeratosis congenita is a rare congenital bone marrow failure syndrome inherited in an autosomal dominant or recessive fashion. It is caused by changes in telomere biology and associated changes in chromosomal stability. The diagnostic criterion is the presence of extremely short (<1st percentile) telomere length in the lymphocyte population; two thirds of patients will also have an identifiable mutation in a gene affecting telomere function. The characteristic clinical findings are oral leukoplakia, nail dystrophy, and reticular pigmentation of the neck and upper chest. By age 30, more than 80% of patients exhibit signs of bone marrow failure and HSCT can restore normal hematopiesis. Dyskeratosis congenita is a disease of defective DNA repair, and although toxicity is not as severe as that seen in Fanconi anemia, patients have increased transplant-related toxicity when receiving full-dose conditioning. There is currently no standard approach, but the majority of patients in the modern era have received a combination including fludarabine and low-dose TBI. In 34 patients reported to the international registry, 5-year survival after HSCT was not quite 60%. Reduced-intensity regimens caused less acute toxicity but late deaths, the majority of which were from graft failure or pulmonary fibrosis assumed to be related to the underlying dyskeratosis congenita phenotype, remain a challenging problem.[925] The development of post-HSCT malignancies is not as common as in Fanconi anemia but still represents a major cause of morbidity and mortality. In an analysis of more than 500 patients with dyskeratosis congenita at the National Institutes of Health, the risk for cancer was elevenfold greater than that of the general population. The most common malignancies were squamous cell carcinomas of the head and neck, followed by skin and anorectal cancer.[926] Thus, as for all patients with congenital bone marrow failure syndromes, lifelong follow-up by specialists dedicated to the care of these rare diseases is mandatory.

Diamond-Blackfan Anemia. Diamond-Blackfan anemia is a rare, heritable condition manifesting as pure red cell aplasia due to an intrinsic erythroid progenitor defect. Approximately one third of patients have associated physical abnormalities, including short stature, cardiac or urogenital abnormalities, cleft palate, and microcephaly.[927] Haploinsufficiency of ribosomal protein S19 (RPS19) occurs in 20% to 25% of patients. Patients are generally diagnosed in the first months of life, presenting with anemia, macrocytosis, and reticulocytopenia (see Chapter 6). Although approximately two thirds of children initially respond to corticosteroid therapy, many fail to respond or lose response over time.[927] However, response to corticosteroids may wax and wane and 15% to 25% of children will attain a durable spontaneous remission, which makes it difficult to know when to pursue an intense intervention such as HSCT.[928] However, there can be significant toxicity associated with a conservative approach whether from sequelae of chronic transfusions or of prolonged corticosteroid use. Diamond-Blackfan anemia is a cancer predisposition syndrome, and patients are at risk for developing neoplasias in early to mid adulthood.[929] In the Diamond-Blackfan anemia registry the cumulative incidence of solid tumor/leukemia was 20% by age 46.[930] Allogeneic HSCT can offer a curative option when corticosteroids are no longer efficacious or associated with unacceptable toxicity. The first transplant was performed in 1976.[931] Children receiving transplants from matched sibling donors with a BU-based myeloablative regimen have historically done well, with EFS greater than 90%. Patients receiving alternative donor transplants fared poorly, with a 14% survival in early reports,[932] but outcomes are now approaching those seen in the sibling donor setting.[933] The Japanese Aplastic Anemia Registry reported a 100% 5-year survival in 13 patients with Diamond-Blackfan anemia receiving an allogeneic bone marrow transplant from either sibling donors or UDs; however, 3 of 3 patients receiving unrelated UCB transplants experienced graft failure.[934] Thus transplantation from a suitable bone marrow donor provides a reasonable option for those patients who fail to have a sustained response to medical therapy.[935]

GLOBAL HEALTH AND HEMATOPOIETIC STEM CELL TRANSPLANTATION

The field of HSCT has made impressive advances over the past 3 decades, particularly in the reduction of acute toxicity, through the use of less intense conditioning regimens and advances in supportive care. In addition, experience with alternative stem cell sources such as UCB facilitates donor identification for an increasingly diverse group of patients. Although HSCT was initially only performed in highly developed countries, the past decade has seen marked growth in the global application of transplant.

There is unquestionably a need for HSCT to be an available therapeutic option in the developing world, particularly for pediatric patients. The incidence of the major hemoglobinopathies is rising rapidly in the Middle East and Southeast Asia, and there is a critical lack of infrastructure for long-term provision of safe transfusion support, infection prevention, and iron overload management.[936] Lack of adequate supportive care also makes the curative approach of HSCT attractive in many of the other common nonmalignant transplantable conditions in children: bone marrow failure syndromes, immunodeficiency diseases, and storage diseases. In the malignant arena, there is difficulty and expense involved in procuring and administering lifelong TKI therapy in patients with CML and the intense (AML) or protracted (ALL) therapy needed for other hematologic malignancies raises the question of whether HSCT may be a preferable approach in the resource-poor setting if it could be done performed safely and effectively.

However, the obstacles to providing the intense, prolonged, and sophisticated care required to support a platform of HSCT cannot be understated. Although there is a greater probability of identifying a matched family donor in the Middle East than the United States, owing to larger family size and marriages between relatives,[937] it will be difficult to identify donors in the existing registries for those lacking a family donor given the underrepresentation of ethnic minority populations. UCB offers an attractive alternative because matching requirements are more permissive, but unrelated UCB units are expensive and the delayed engraftment and increased risk for graft rejection pose challenges. The technology necessary to maintain an acceptable cell processing facility is costly and requires specific expertise. Other problematic issues include having the consistent ability to provide sophisticated supportive care during the acute transplant period, including management of nutritional issues and pain, readily available transfusion support, and, most fundamentally, the provision of a guaranteed drug supply. Housing and social support must be available to families who may be away from home and work for prolonged periods. Infections must be expeditiously identified and appropriately treated. The incidence of infectious exposures varies between geographic regions and individual countries and is generally higher in the developing world. In Egypt, for example, 15% of the population has had prior exposure to hepatitis C, as manifested by antibody positivity, and 3% are positive for hepatitis B surface antigen. This impacts both donor availability and the risk for patient complications during transplant, including VOD and GVHD. There are also cultural differences impacting issues as diverse as allowing a sibling to donate marrow or choosing a therapy that impacts fertility, and transplant approaches must be viewed as part of a broader cultural context. Even if HSCT is successfully navigated, patients are at risk for long-term complications, particularly in the setting of cGVHD, that may be more significant than those associated with the primary disease for which transplant was performed.

There are currently major transplant centers in the Middle East, India, Southeast Asia, and South America, although there are still many individual countries in these areas without an HSCT program. The majority of centers limit activity to autologous and matched family donor transplants. Some financial data exist, and it appears unsurprisingly that once a transplant center is established the cost (in U.S. dollars) of individual procedures is relatively low—about $15,000 in India (2001) and Egypt (2008) and $50,000 in Chile (2008). Few centers report outcomes to international registries,[938] but published series show DFS of 50% to 80%,[939-941] indicating the possibility of cure for a subset of patients.

Future initiatives include developing geographic centers of excellence in central regional locations, perhaps with a focus on a limited number of diseases where the efficacy of HSCT is well understood. Collaborations with well-established centers can provide virtual and onsite training and ongoing support, including the development of a robust quality management program that will support programmatic improvements.[942] Finally, academic collaborations and publications must occur, because a well-functioning research infrastructure will enhance meticulous data collection, establish international credibility, and allow a presence at international meetings. Ultimately, the entire field of HSCT will benefit as global differences in epidemiology and genetics and differing approaches to supportive care practices inform ongoing advances in transplantation.

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CHAPTER 9

Diagnostic Approach to the Anemic Patient

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CHAPTER OUTLINE

INTRODUCTION
DEFINITION OF ANEMIA
CLASSIFICATION OF ANEMIAS
EVALUATION OF THE ANEMIC PATIENT
Initial Encounter
Selection of Appropriate Laboratory Tests
Automated Cell Counting
The Blood Film

INTRODUCTION

In this chapter we attempt to provide a useful guide to the rapid and accurate diagnosis of anemia in children and young adults. We do not dwell on each diagnosis because they are described in detail throughout the book.

Though productive methods of differential diagnosis of anemia vary greatly with the age of the pediatric patient, the most valuable tools at all ages remain the history and physical examination. Even in the neonate the history of the pregnancy, the family history, and the events surrounding birth are paramount, and the appearance of the newborn baby strongly influences diagnosis. In fact, the history and physical examination may well be more useful than examination of the blood because the morphology of the red cells of normal neonates may be strikingly abnormal. Morphology of neonatal blood can be very deceiving. As mentioned below, anemia is due to either hemorrhage, failure of erythropoiesis, accelerated hemolysis, or combinations of the three. The symptoms of anemia are well established. They are due either to sudden blood volume loss or to chronic redistribution of a low blood volume with a high fraction sequestered in the central blood volume, both complicated by low oxygen carrying capacity. The results are weakness, dyspnea and fatigue, cold intolerance, dizziness, and depression. If the blood volume loss is substantial and sudden, the patient may present in a state of collapse. If anemia develops slowly, low oxygen carrying capacity may be compensated by a higher cardiac output due to the rise in central blood volume and a lowering of blood viscosity and by increased erythrocyte 2,3-DPG. In fact, patients with slowly developing anemia to as low as 1 g of hemoglobin (Hb) per 100 mL of blood have been observed to walk into clinics. But this is surely a feat of the young. Older patients with less accommodating vascular trees are not capable of such gymnastics. Woe to such a patient who encounters a physician determined to transfuse with red cells without removing an equal volume of watery blood. The maneuver will surely drive the victim's massively expanded central blood volume into pulmonary edema.

The rate of development of symptoms provides strong etiologic clues because the normal red cell life span is 3.5 months, and cardiac compensation for a falling oxygen carrying capacity can blunt symptoms. Thus a patient who suddenly complains of severe fatigue and is noticeably pale has probably lost red cells rapidly. This must be due to hemolysis or bleeding. If the symptoms accumulate slowly, this is likely due to failure of erythropoiesis that may be complicated by malnutrition, endocrine deficiencies such as thyroid or adrenal failure, and/or by renal insufficiency or inflammatory disease with concomitant inhibition of iron absorption.

The physical examination of the anemic patient may be particularly revealing of etiology. Pallor is nonspecific, but when tinged with jaundice, it may suggest increased hemolysis. This is readily confirmed if the foam of shaken urine remains white. Yellow skin or sclera without biliuria strongly indicates that the circulating bilirubin has NOT been conjugated and is the direct product of rapidly hemolyzed red cells. Indeed, in the absence of liver disease, the level of unconjugated bilirubin in the peripheral blood is a direct function of red cell turnover. The relationship is confusing in the neonate because of its physiologic defect in the conjugation pathway and is not accurate in liver disease.

Examination of the tongue and oral mucosa will reveal vitamin, iron, and corticosteroid deficiencies, while stature and the skeleton, particularly the forehead, face, and skull, may indicate the chronicity of hemolysis and concomitant energy consumption and marrow space expansion. The digits may reveal boney abnormalities seen in congenital anemias. Enlarged lymph nodes and their texture, together with hepatosplenomegaly, may suggest invasive or infectious diseases, as do petechiae or other skin lesions. The pulse rate and blood pressure and the heave of the heart against the chest may reveal the length and severity of anemia, while the lungs may demonstrate the incipiency of pulmonary edema. A walk up the stairs with comparison of patient and caretaker pulse rates may be highly revealing. Neurologic examination may show the characteristic slow return of Achilles tendon reflexes seen in hypothyroidism or the posterior column defects of vitamin B_{12} deficiency.

DEFINITION OF ANEMIA

Anemia is generally defined as a reduction in red cell mass or blood Hb concentration. The limit for differentiating anemic from normal states is usually set at 2 standard deviations below the mean for the normal population. This definition will result in 2.5% of the normal population being classified as anemic. Conversely, values for Hb-deficient individuals are distributed in such a fashion that some are placed within the normal range. These individuals, who have the potential for an Hb concentration in the upper part of the normal range, may be recognized only after a response to treatment. Definition of the lower limit of normal for both pediatric and adult patients is critically dependent on the population used to establish the normal range of values.[1] Hematocrit (Hct), Hb, and mean corpuscular volume (MCV) are significantly lower in African Americans than in Caucasians. A third of this difference can be accounted for by the α-globin 3.7 kb deletion.[2] In the absence of a race-specific normal range, the fact that African Americans generally have lower Hb levels than Caucasians (approximately 0.5 g/dL) could result in 10% of normal African-American children being considered anemic just because the proper normal range is not used.[3,4] Figure 9-1 presents pediatric reference ranges for Hb in Caucasians, African Americans, and Mexican Americans that were obtained from the National Health and Nutrition Examination Survey (NHANES) III database.

Because the primary function of the red cell is to deliver and release adequate quantities of oxygen to tissues to meet their metabolic demands, it is apparent that some measures of both body oxygen metabolism and accompanying cardiovascular compensation are required to complement the current laboratory definition of anemia. The fact that Hb concentration alone is insufficient to judge whether a patient is "functionally anemic" is best illustrated in a patient with cyanotic congenital heart disease or chronic respiratory insufficiency or in one with mutant Hbs that alter Hb's affinity for oxygen (see Chapter 19).

With these caveats in mind, a useful statistical definition of anemia that recognizes the effect of age, gender, and ethnicity on the designation of anemia is provided in Figure 9-2.[5,6]

CLASSIFICATION OF ANEMIAS

Anemias may be classified on the basis of physiology or morphology. A combination of both approaches is often used in the initial differential diagnosis.

To determine and distinguish the multiple disorders capable of producing anemia, one must separate the causes of anemia into two categories of functional disturbances:

1. Disorders of effective red cell production, in which the net rate of red cell production is depressed. This can be due to disorders of erythrocyte maturation, in which erythropoiesis is present but ineffective, or to an absolute failure of erythropoiesis. In the former the bone marrow may contain many erythroblasts that die in situ before reaching the reticulocyte stage. In the latter there is absolute erythroblastopenia.
2. Disorders in which rapid erythrocyte destruction or red cell loss is primarily responsible for the anemia.

These two categories are not mutually exclusive. More than one mechanism may be present in some anemias, but one functional disorder is generally the major reason for the patient's anemia. Box 9-1 lists the anemias most commonly encountered in infancy and childhood and classifies them into three categories of functional disturbance.

Anemias may also be classified on the basis of red cell size and then further subdivided according to red cell morphology. In this type of classification, anemias are subdivided into microcytic, normocytic, and macrocytic anemias. This classification is also arbitrary, and categories are not mutually exclusive. For example macrocytic reticulocytes abound in the hemolytic anemias. Therefore, although mature erythrocytes in the various hemolytic anemias may be normocytic, the MCV of all the cells may be larger than normal because of the contribution of reticulocytes to the volume measurement.[7] Volume distribution curves may reveal the contribution of a subset of large cells to the MCV. Furthermore, during the course of a disease, classification of the patient's anemia may change from one category to another as a result of other clinical or pathologic variables. In Box 9-2 the more common anemias of infancy and childhood are classified on the basis of their characteristic cell size.

EVALUATION OF THE ANEMIC PATIENT

Initial Encounter

The initial diagnostic approach to an anemic patient includes a detailed history and physical examination and

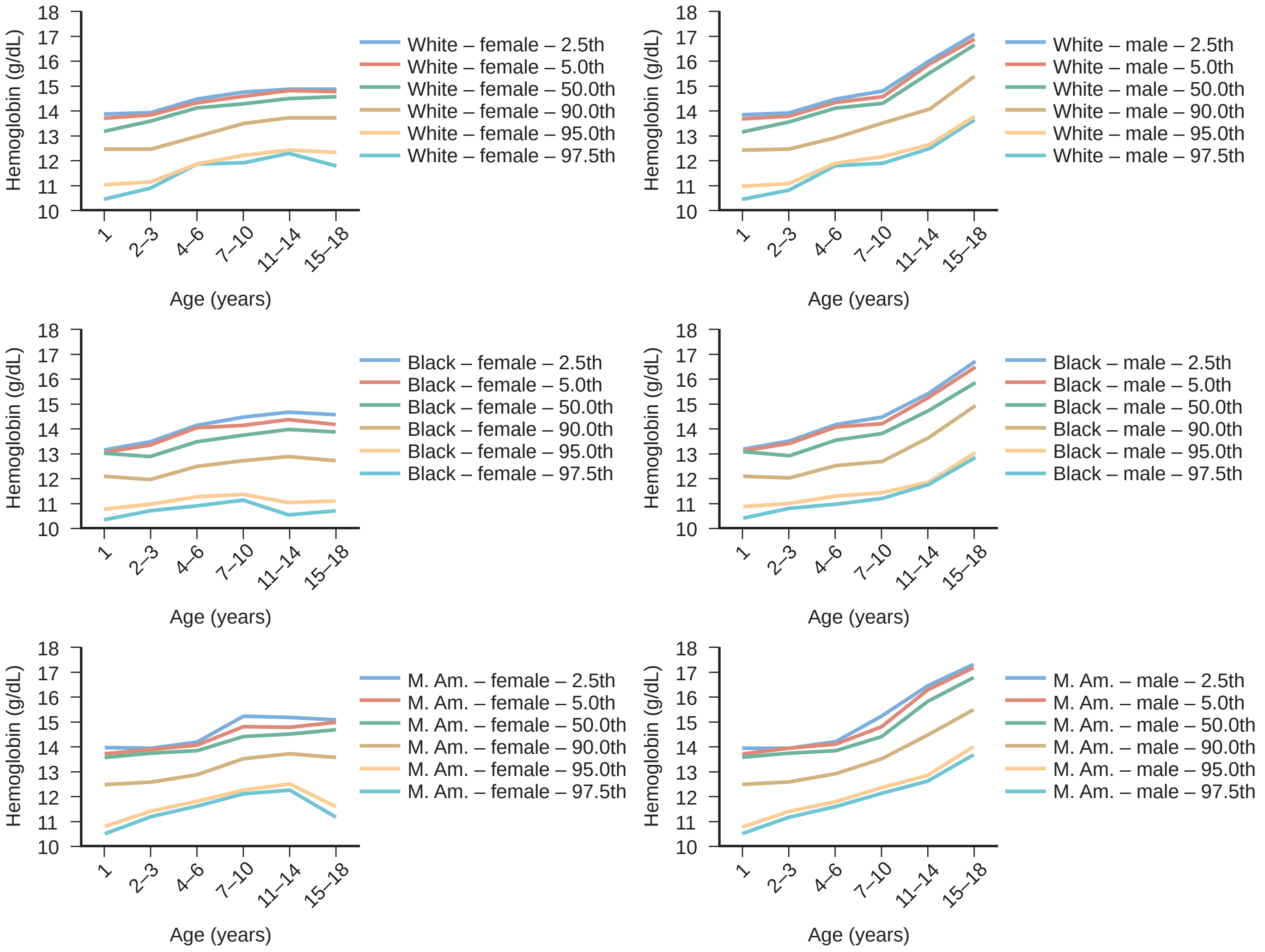


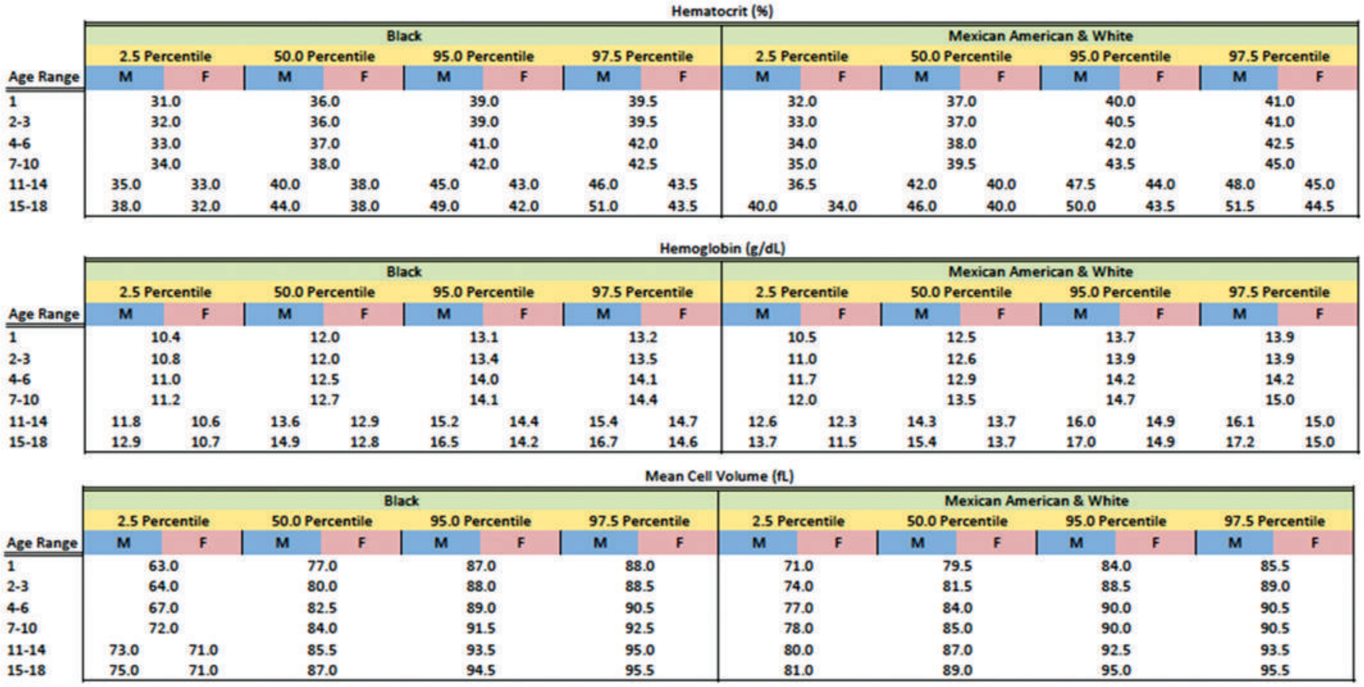
Figure 9-1 Pediatric reference ranges for hemoglobin in Caucasions *(top row)*, African Americans *(middle row)*, and Mexican Americans *(bottom row)*. *(From Cembrowski GS, Chan J, Cheng C, Bamforth FJ: NHANES 1999-2000 data used to create comprehensive health-associated race-, sex- and age-stratified pediatric reference intervals for the Coulter MAXM.* Lab Hematol *10:245–246, 2004. Presented at the annual meeting of the International Society for Laboratory Hematology, Barcelona, Spain, May 2004. Numerical summary provided by GS Cembrowski, MS Cembrowski, KA Versluys, 2013. See Appendices 13 to 14 for complete data sets.)*

a minimum of essential laboratory tests. Box 9-3 and Table 9-1 list the features of the history and physical examination that are most helpful in providing clues to the cause of the anemia. Often they may be more diagnostic than the laboratory studies.

Family History: Because the prevalence of hemoglobinopathies and thalassemias varies greatly among different ethnic backgrounds, it is important to inquire about ethnicity. A family history of anemias or symptoms that may be related to anemias, like jaundice, gallstones, cholecystectomy/splenectomy, and transfusion, in family members should be investigated. A family history of blood loss (menorrhagia, epistaxis) may suggest that the anemia is due to the presence of an inherited bleeding disorder.

Personal History: With almost universal newborn screening in the United States and other developed countries, many patient encounters are generated by the identification of a hemoglobinopathy by newborn screening in the absence of any symptomatology. For all other circumstances, the history should focus on the characterization of possible hemolytic crises or episodes of blood loss. Menstrual history should be obtained in all girls. If the patient has recently moved from or traveled to an underdeveloped country, intestinal parasite infestation (hookworm, whipworm) should be considered, as well as malaria and infectious hepatitis. A dietary history is crucial to identify conditions favoring folate deficiency or iron deficiency. A detailed medication history is of great importance to identify drugs that may directly produce anemia or may be contributing to anemia via increased blood losses (e.g., aspirin, nonsteroidal antiinflammatory drugs).

Physical Examination: The presence of congenital defects is an important feature of congenital anemias, such as Fanconi's anemia (FA). Skeletal abnormalities (hypoplastic or absent thumb/radius) are present in at least half of patients with FA. Many of these patients also have short stature.



Hematocrit (%)

	Black								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	31.0		36.0		39.0		39.5		32.0		37.0		40.0		41.0	
2-3	32.0		36.0		39.0		39.5		33.0		37.0		40.5		41.0	
4-6	33.0		37.0		41.0		42.0		34.0		38.0		42.0		42.5	
7-10	34.0		38.0		42.0		42.5		35.0		39.5		43.5		45.0	
11-14	35.0	33.0	40.0	38.0	45.0	43.0	46.0	43.5	36.5		42.0	40.0	47.5	44.0	48.0	45.0
15-18	38.0	32.0	44.0	38.0	49.0	42.0	51.0	43.5	40.0	34.0	46.0	40.0	50.0	43.5	51.5	44.5

Hemoglobin (g/dL)

	Black								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	10.4		12.0		13.1		13.2		10.5		12.5		13.7		13.9	
2-3	10.8		12.0		13.4		13.5		11.0		12.6		13.9		13.9	
4-6	11.0		12.5		14.0		14.1		11.7		12.9		14.2		14.2	
7-10	11.2		12.7		14.1		14.4		12.0		13.5		14.7		15.0	
11-14	11.8	10.6	13.6	12.9	15.2	14.4	15.4	14.7	12.6	12.3	14.3	13.7	16.0	14.9	16.1	15.0
15-18	12.9	10.7	14.9	12.8	16.5	14.2	16.7	14.6	13.7	11.5	15.4	13.7	17.0	14.9	17.2	15.0

Mean Cell Volume (fL)

	Black								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	63.0		77.0		87.0		88.0		71.0		79.5		84.0		85.5	
2-3	64.0		80.0		88.0		88.5		74.0		81.5		88.5		89.0	
4-6	67.0		82.5		89.0		90.5		77.0		84.0		90.0		90.5	
7-10	72.0		84.0		91.5		92.5		78.0		85.0		90.0		90.5	
11-14	73.0	71.0	85.5		93.5		95.0		80.0		87.0		92.5		93.5	
15-18	75.0	71.0	87.0		94.5		95.5		81.0		89.0		95.0		95.5	

Figure 9-2 Sex- and age-stratified pediatric reference ranges for hematocrit, hemoglobin, and mean corpuscular volume. *(From Cembrowski GS, Chan J, Cheng C, et al: NHANES 1999-2000 data used to create comprehensive health-associated race-, sex- and age-stratified pediatric reference intervals for the Coulter MAXM.* Laboratory Hematol *10:245–6, 2004. Presented at the annual meeting of the International Society for Laboratory Hematology, Barcelona, Spain, May 2004. Numerical summary provided by GS Cembrowski, MS Cembrowski, KA Versluys, 2013. See Appendices 13 to 14 for complete data sets.)*

Box 9-1 **Physiologic Classification of Anemia**

1. Disorders of red cell production in which the rate of red cell production is less than expected for the degree of anemia
   a. Marrow failure
      - Aplastic anemia
        Congenital
        Acquired
      - Pure red cell aplasia
        Congenital
          Diamond-Blackfan syndrome
          Aase's syndrome
        Acquired
          Transient erythroblastopenia of childhood
          Human parvovirus B19 infection
      - Marrow replacement
        Malignancies
        Osteopetrosis
        Myelofibrosis
        Chronic renal disease
        Vitamin D deficiency
        Granulomatous infection
      - Pancreatic insufficiency–marrow hypoplasia syndrome
      - Fanconi's anemia
   b. Impaired erythropoietin production
      - Chronic renal disease
      - Hypothyroidism, hypopituitarism
      - Chronic inflammation
      - Protein malnutrition
      - Hemoglobin mutants with decreased affinity for oxygen
   c. Anemia of chronic disease
2. Disorders of erythroid maturation and ineffective erythropoiesis
   a. Abnormalities in cytoplasmic maturation
      - Iron deficiency
      - Thalassemia syndromes
      - Sideroblastic anemias
      - Lead poisoning
   b. Abnormalities in nuclear maturation
      - Vitamin B_{12} deficiency
      - Folic acid deficiency
      - Thiamine-responsive megaloblastic anemia
      - Hereditary abnormalities in folate metabolism
      - Orotic aciduria
      - Zinc-induced copper deficiency
   c. Congenital dyserythropoietic anemias (types I, II, III, IV)
   d. Erythropoietic protoporphyria
   e. Refractory sideroblastic anemia with vacuolization of marrow precursors and pancreatic dysfunction/deficiency
3. Hemolytic anemias
   a. Defects in hemoglobin
      - Structural mutants
      - Synthetic mutants (thalassemia syndromes)
   b. Defects in the red cell membrane
   c. Defects in red cell metabolism
   d. Antibody mediated
   e. Mechanical injury to the erythrocyte
   f. Thermal injury to the erythrocyte
   g. Oxidant-induced red cell injury
   h. Infectious agent–induced red cell injury
   i. Paroxysmal nocturnal hemoglobinuria
   j. Plasma lipid–induced abnormalities in the red cell membrane

Box 9-2 Classification of Anemias Based on Red Cell Size

MICROCYTIC ANEMIAS

Iron deficiency (nutritional, chronic blood loss)
Chronic lead poisoning
Thalassemia syndromes
Sideroblastic anemias
Chronic inflammation
Some congenital hemolytic anemias with unstable hemoglobin

MACROCYTIC ANEMIAS

- With megaloblastic bone marrow
  - Vitamin B_{12} deficiency
  - Folic acid deficiency
  - Hereditary orotic aciduria
  - Thiamine-responsive anemia
- Without megaloblastic bone marrow
  - Aplastic anemia
  - Diamond-Blackfan syndrome
  - Hypothyroidism
  - Liver disease
  - Bone marrow infiltration
  - Dyserythropoietic anemias

NORMOCYTIC ANEMIAS

- Congenital hemolytic anemias
  - Hemoglobin mutants
  - Red cell enzyme defects
  - Disorders of the red cell membrane
- Acquired hemolytic anemias
  - Antibody mediated
  - Microangiopathic hemolytic anemias
  - Secondary to acute infections
- Acute blood loss
- Splenic pooling
- Chronic renal disease (usually)

There is substantial variability in the effectiveness of the compensatory mechanisms for anemia: While in some cases the severity of the anemia is evident based on the presence of tachycardia, lethargy, or pallor, in other cases a very severe, chronic anemia may be associated with a paucity of symptoms. Short stature and skeletal changes induced by expansion of the erythroid mass should also be noted because they are often the result of anemia and expansion of erythroid marrow.

Selection of Appropriate Laboratory Tests (Box 9-4)

First-Tier Tests

- The initial laboratory tests should always include a complete blood count (CBC) with white blood cell differential, a reticulocyte count, and an examination of a peripheral blood smear. If a test for the identification of Hb variants is included in this initial panel, high-performance liquid chromatography, capillary electrophoresis, or isoelectrofocusing should be preferred over classic cellulose acetate and acid-citrate Hb electrophoresis. A glucose-6-phosphate dehydrogenase screening test, total and direct bilirubin, lactate dehydrogenase (LDH), and iron studies (serum iron, total iron binding capacity, and ferritin concentrations) should also be considered as part of the initial laboratory evaluation of the anemic child. It should be noted that LDH is not a marker of hemolysis: Elevated LDH is an indication of *intravascular* hemolysis. With reliable clinical grade assays now in place, serum hepcidin might be included if iron deficiency is unexplained.[8,9]

Box 9-3 Historical Factors of Importance in Evaluating Patients with Anemia

AGE

Nutritional iron deficiency is never responsible for anemia in term infants before 6 months of age and rarely seen in premature infants before the time that they have doubled their birth weight. Anemia occurring in the neonatal period is generally the result of recent blood loss, isoimmunization, or initial manifestation of a congenital hemolytic anemia or congenital infection. Anemia first detected at 3 to 6 months of age suggests a congenital disorder of hemoglobin synthesis or hemoglobin structure.

GENDER

Consider X-linked disorders in males (G6PD deficiency, pyruvate kinase deficiency).

RACE

Hemoglobins S and C more common in African Americans; β-thalassemia more common in Caucasians; α-thalassemia trait most common among African Americans and Asians

ETHNICITY

Thalassemia syndromes most common among patients of Mediterranean origin. G6PD deficiency observed more often among Sephardic Jews, Filipinos, Greeks, Sardinians, and Kurds

NEONATAL

A history of hyperbilirubinemia in the newborn period suggests the presence of congenital hemolytic anemia, such as the hereditary spherocytosis of G6PD deficiency. Prematurity predisposes to the early development of iron deficiency.

DIET

Document sources of iron, vitamin B_{12}, folic acid, or vitamin E in the diet. A history of pica, geophagia, or pagophagia suggests the presence of iron deficiency.

DRUGS

Oxidant-induced hemolytic anemia, phenytoin (Dilantin)-induced megaloblastic anemia, drug-induced aplastic anemia

INFECTION

Hepatitis-induced aplastic anemia, infection-induced red cell aplasia, hemolytic anemia

INHERITANCE

Family history of anemia, jaundice, gallstones, or splenomegaly

DIARRHEA

Suspect small bowel disease with malabsorption of folate or vitamin B_{12}. Suspect inflammatory bowel disease with blood loss. Suspect protein-losing enteropathy with blood loss.

G6PD, Glucose-6-phosphate dehydrogenase.

Second-Tier Tests

- Based on the initial evaluation of the patient and the first round of laboratory tests, additional disease-specific tests should be considered. In the presence of macrocytosis, plasma B_{12}, folate, and perhaps homocystein and serum/urine methylmalonic acid should be determined together with a measurement of thyroid hormone function. Osmotic fragility (nonincubated and incubated)

TABLE 9-1 Physical Findings as Clues to the Cause of Anemia

	Finding	Cause
Skin	Hyperpigmentation	Fanconi's aplastic anemia
	Petechiae, purpura	Autoimmune hemolytic anemia with thrombocytopenia, hemolytic-uremic syndrome, bone marrow aplasia, bone marrow infiltration
	Carotenemia	Suspect iron deficiency in infants
	Jaundice	Hemolytic anemia, hepatitis, aplastic anemia
	Cavernous hemangioma	Microangiopathic hemolytic anemia
	Ulcers on lower extremities	S and C hemoglobinopathies, thalassemia
Facies	Frontal bossing, prominence of the malar and maxillary bones	Congenital hemolytic anemias, thalassemia major, severe iron deficiency
Eyes	Microcornea	Fanconi's aplastic anemia
	Tortuosity of the conjunctival and retinal vessels	S and C hemoglobinopathies
	Microaneurysms of the retinal vessels	S and C hemoglobinopathies
	Cataracts	Glucose-6-phosphate dehydrogenase deficiency, galactosemia with hemolytic anemia in the newborn period
	Vitreous hemorrhages	S hemoglobinopathy
	Retinal hemorrhages	Chronic, severe anemia
	Edema of the eyelids	Infectious mononucleosis, exudative enteropathy with iron deficiency, renal failure
	Blindness	Osteopetrosis
Mouth	Glossitis	Vitamin B_{12} deficiency, iron deficiency
	Angular stomatitis	Iron deficiency
Chest	Unilateral absence of the pectoral muscles	Poland's syndrome (increased incidence of leukemia)
	Shield chest	Diamond-Blackfan syndrome
Hands	Triphalangeal thumbs	Red cell aplasia
	Hypoplasia of the thenar eminence	Fanconi's aplastic anemia
	Spoon nails	Iron defiency
Spleen	Enlargement	Congenital hemolytic anemia, leukemia, lymphoma, acute infection, portal hypertension

should be considered in the presence of possible hereditary spherocytosis (HS)/xerocytosis, although the presence of cells with high mean corpuscular Hb concentration (MCHC) is usually diagnostic of HS in the absence of hemoglobinopathy-induced cation loss, antibody-induced hemolysis, hereditary xerocytosis, and transient-hypoproliferative states. Serum haptoglobin, serum/plasma Hb, and urine hemosiderin should be considered in working up possible chronic hemolytic conditions as well as a direct and indirect antiglobulin test or Coombs test and red-cell or white-cell based flow cytometric assays for paroxysmal nocturnal hemoglobinuria (PNH).

- Supravital staining of erythrocytes (crystal violet to detect Heinz bodies or denatured Hb, brilliant cresyl blue for unstable Hb), a screening test for the presence of unstable hemoglobins like heat or isopropanol stability, should be considered if there are indications of possible unstable hemoglobinopathy or oxidant-induced hemolysis.
- Erythrocyte protoporphyrin is of limited value in the diagnosis of iron deficiency or lead poisoning. However, increased levels of erythrocyte protoporphyrin help to distiguish X-linked sideroblastic anemia type A (XLSA/A) due to deficiency in either ABCB7, SLC19A2, or other mitochondrial defects from classic XLSA due to ALAS2 mutations, which characteristically has low or normal levels.[10]

Box 9-4 Laboratory Investigation of Anemia

INITIAL STUDIES

Complete blood count with differential, reticulocyte count, and peripheral smear review
Hemoglobin variants identification (high-performance liquid chromatography or isoelectric focusing preferred)
Iron studies: Serum iron, serum transferrin, serum ferritin
Hemolysis indicators: Bilirubin (total and direct), serum lactate dehydrogenase (for intravascular hemolysis)
Glucose-6-phosphate dehydrogenase deficiency screening test

SECOND-TIER

Serum B_{12}, folate, red cell folate, and serum/urine methylmalonic acid
Osmotic fragility
Serum haptoglobin, serum/plasma hemoglobin, urine hemosiderin
Direct antiglobulin test and indirect antiglobulin test
Unstable hemoglobin screen (isopropanol or thermal stability), Heinz bodies prep
Paroxysmal nocturnal hemogloburinemia testing by flow cytometry (red blood cell and white blood cell)
Erythrocyte adenosine deaminase activity
Human parvovirus B19: Immunoglobulin M and G serum titers, viral DNA detection tests
Serum erythropoietin
Serum α-fetoprotein, mitomycin, or diepoxybutane test
Acid serum lysis test with ABO-compatible serum

THIRD-TIER

Bone marrow aspirate/biopsy
Electron microscopy
Complementation test (Fanconi's anemia subtyping)
Molecular genetic studies (congenital dyserythropoietic anemia types I and II, Blackfan-Diamond, and others)

- Erythrocyte adenosine deaminase, serum α-fetoprotein, mitomycin, or diepoxybutane tests are indicated for Diamond-Blackfan anemia (DBA) and FA, respectively.
- Family studies are helpful in the presence of challenging combinations of hemoglobinopathies/thalassemias and in HS/xerocytosis. Specialized red blood cell (RBC) enzyme studies should be considered with the caveat that only a handful of reference laboratories in the United States offer functional studies for red cell enzymopathies.
- To diagnose erythroid suppression induced by human parvovirus B19 infection, immunoglobulin M (IgM) and immunoglobulin G (IgG) serum titers and viral DNA detection tests should be considered.
- Serum erythropoietin (EPO) levels are indicated only when there is a question of inappropriately low EPO for the degree of anemia, provided that the laboratory has generated an EPO normal range as a function of Hct/Hb values.

Third-Tier Tests

- Bone marrow studies should be performed when there is clear evidence of a hypoproliferative anemia, except for cases of transient virus-induced erthroblastopenia, in which they are not indicated. Bone marrow studies are required to diagnose the presence of ringed sideroblasts, which are a distinguishing sign of sideroblastic anemias, to identify megaloblastic erythropoiesis and when there is a suspicion of an associated hematologic malignancy based on peripheral blood morphology.
- DNA-based diagnosis: Ordering of molecular studies should be the result of a proper balancing between the curiosity of the physician and the needs and possible benefits for the patient. Cost of these tests should also be considered, and whether they are associated with significant cost borne by the patient or family or by insurance carriers. For most of red cell membrane and red cell enzyme disorders, molecular testing rarely results in measurable benefits for the patients and their families and for the clinical management of the disease. Genetic testing has a value in congenital dyserythropoietic anemias (CDAs) and in DBA.

After the laboratory tests have been performed, the results may be used in an initial attempt to diagnostically characterize the patient's anemia, as illustrated by the simple algorithm in Figure 9-3. This algorithm provides additional diagnostic steps after the initial characterization of anemia based on the CBC, reticulocyte count, and cellular indices.

The most common cause of rapid red cell loss with reticulocytosis is hemorrhage. In infants and children, unusual causes include hemorrhage beneath the scalp and intraabdominal, urinary tract, and pulmonary hemorrhage. In the latter two situations, hyperbilirubinemia is not present. Indeed, if the hemorrhage is chronic and leads to iron deficiency, the plasma will be pale.

Sequestration in an abnormal spleen will usually cause spherocytosis, but if the hypersplenism is associated with liver disease, target cells will also be present. In this condition the osmotic fragility test will reveal a sensitive and a resistant population of erythrocytes.

Vascular damage to erythrocytes may be associated with thrombocytopenia and can be caused by hemangiomas (Kasabach-Merritt syndrome); intravascular coagulation, often secondary to sepsis; damaged artificial heart valves; and renal vascular disease and severe hypertension. Schistocytes, which resemble military helmets of various nationalities, are usually present, and siderinuria and hemoglobinuria are often detected.

Abnormalities in plasma are frequent causes of hemolysis. Antibodies usually induce spherocytosis by causing splenic sequestration, but sometimes they merely fix complement and cause hemoglobinuria without morphologic change. Many drugs and toxins can damage red cells either by oxidant injury with resultant schistocytosis or by direct lysis, as illustrated by bacterial lipases in septic shock or inoculation of venoms. Acute hemolysis may also be caused by excessive release of hepatic copper in Wilson's disease. No morphologic change is noted.

Primary membrane abnormalities that cause hemolysis are usually congenital and are associated with specific morphologic changes. An exception is PNH, which is an acquired membrane defect associated with no morphologic change. The congenital membrane defects are usually due to abnormalities in structural membrane proteins, such as in HS, but they may involve cation channels (hydrocytosis and xerocytosis) and, rarely, membrane lipids (abetalipoproteinemia).

When the bone marrow fails, as in many hypoproliferative anemias, the red cell volume is frequently increased above 100 fL, in conjunction with an increase in fetal Hb above 2%. Macrocytosis on the peripheral smear may be accompanied by neutropenia or thrombocytopenia or both.

Automated Cell Counting

The classic work of M.M. Wintrobe in 1930-1934 established the criteria for anemia classification based on cellular indices.[11-13] This approach is still in use today. In less than a minute, automated hematology analyzers provide a full CBC with measurements of MCV, MCHC, which also include estimates of the heterogeneity in the distributions of these parameters (red cell distribution width [RDW] for MCV and Hb distribution width [HDW] for MCHC). In automated cell counters using the electrical impedance principle (Abbott, Abbott Park, Ill.; ABX, Kyoto, Japan; Beckman-Coulter, Miami, Fla.; Sysmex, Kobe, Japan), cells passing through an aperture cause changes in electrical resistance that are counted as voltage pulses, which are proportional to cell volume. The electrical pulses are amplified and counted during the time that an accurately metered volume of the suspension is drawn through the aperture. These devices can directly measure MCV and compute the Hct from the MCV and red blood cell count.

In automated cell counters using the laser light scatter principle (Abbott, Abbott Park, Ill.; Siemens Diagnostic

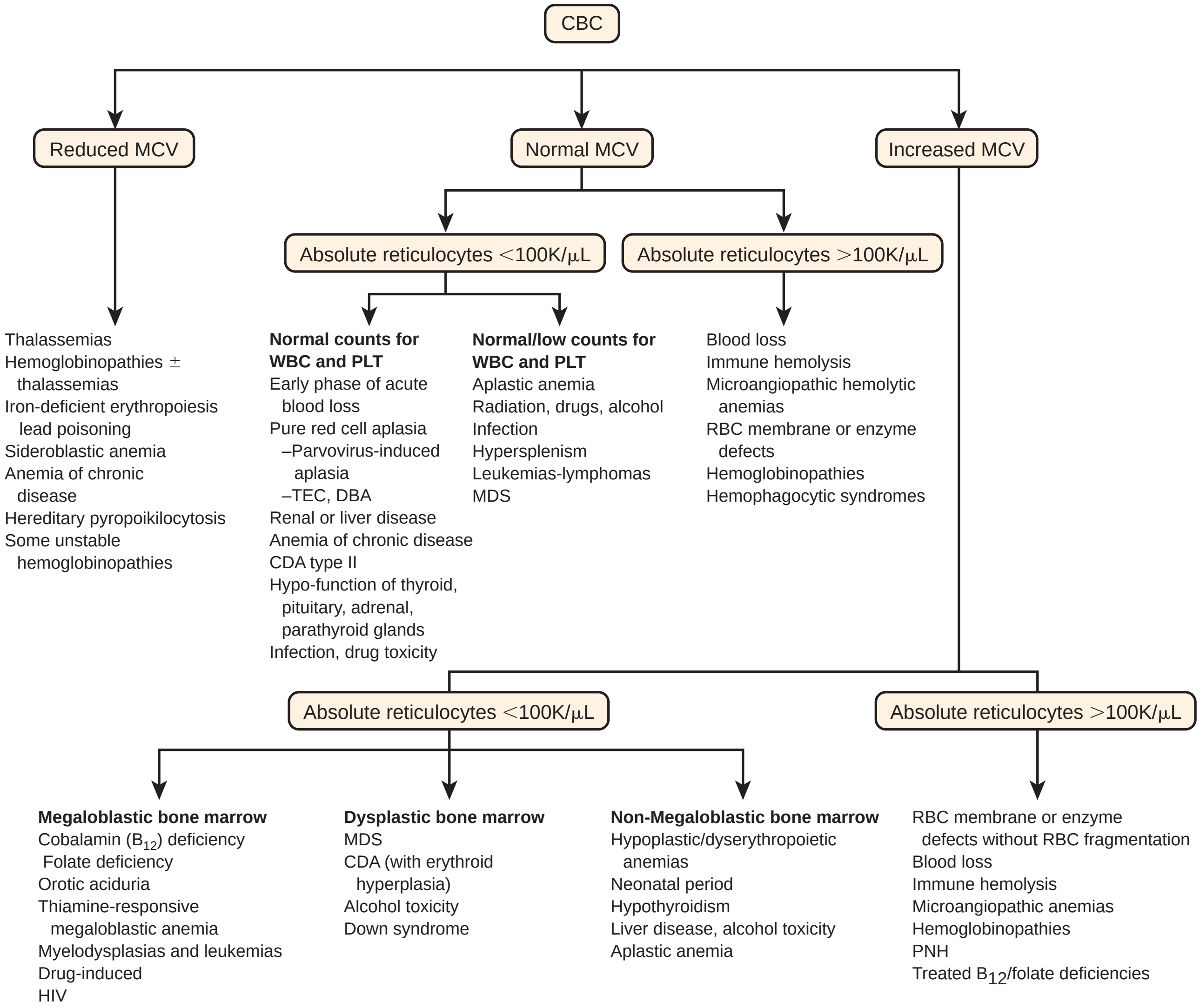

CBC
Reduced MCV
Normal MCV
Increased MCV
Absolute reticulocytes <100K/μL
Absolute reticulocytes >100K/μL
Thalassemias
Hemoglobinopathies ± thalassemias
Iron-deficient erythropoiesis lead poisoning
Sideroblastic anemia
Anemia of chronic disease
Hereditary pyropoikilocytosis
Some unstable hemoglobinopathies
Normal counts for WBC and PLT
Early phase of acute blood loss
Pure red cell aplasia
–Parvovirus-induced aplasia
–TEC, DBA
Renal or liver disease
Anemia of chronic disease
CDA type II
Hypo-function of thyroid, pituitary, adrenal, parathyroid glands
Infection, drug toxicity
Normal/low counts for WBC and PLT
Aplastic anemia
Radiation, drugs, alcohol
Infection
Hypersplenism
Leukemias-lymphomas
MDS
Blood loss
Immune hemolysis
Microangiopathic hemolytic anemias
RBC membrane or enzyme defects
Hemoglobinopathies
Hemophagocytic syndromes
Absolute reticulocytes <100K/μL
Absolute reticulocytes >100K/μL
Megaloblastic bone marrow
Cobalamin (B12) deficiency
Folate deficiency
Orotic aciduria
Thiamine-responsive megaloblastic anemia
Myelodysplasias and leukemias
Drug-induced
HIV
Dysplastic bone marrow
MDS
CDA (with erythroid hyperplasia)
Alcohol toxicity
Down syndrome
Non-Megaloblastic bone marrow
Hypoplastic/dyserythropoietic anemias
Neonatal period
Hypothyroidism
Liver disease, alcohol toxicity
Aplastic anemia
RBC membrane or enzyme defects without RBC fragmentation
Blood loss
Immune hemolysis
Microangiopathic anemias
Hemoglobinopathies
PNH
Treated B12/folate deficiencies

Figure 9-3 Algorithm for the differential diagnosis of anemia. *CBC*, Complete blood count; *CDA*, congenital dyserythropoietic anemia; *DBA*, Diamond-Blackfan anemia; *HIV*, human immunodeficiency virus; *MCV*, mean corpuscular volume; *MDS*, myelodysplastic syndrome; *PLT*, platelets; *PNH*, paroxysmal nocturnal hemoglobinuria; *RBC*, red blood cell; *TEC*, transient erythroblastopenia of childhood; *WBC*, white blood cell.

Solutions, Tarrytown, N.Y.), single cells passing through a laser beam induce scattering of laser light, which can be detected and translated into measurements of cell size. In the instruments produced by Siemens, red cells first undergo isovolumetric sphering, and then MCV and the MCHC are measured from the low-angle forward light scattering and the high-angle (refractive index) light scattering, respectively.[14-16]

Some of the potential errors that can be generated by automated cell counters for Hb, Hct, and red cell indices are listed in Box 9-5.[17] Different Hct values can be obtained on the same sample simply due to the fact that MCV is measured with different technologies. Impedance-based instruments overestimate MCV in erythrocytes with increased MCHC and underestimate it in hypochromic erythrocytes. An artifactually low Hct can be obtained in impedance-based systems for samples containing significant quantities of hypochromic cells, leading to overestimation of anemia in conditions like anemia of chronic disease or chronic renal failure. Therefore Hb should be used instead of Hct to define anemia, because the former is an extremely reliable parameter in all types of analyzers. Although RDW is widely understood to represent the coefficient of variation for the distribution of MCV in a given blood sample, some analyzers calculate RDW from the width of this distribution, resulting in different normal range for this parameter. MCH is the only erythrocyte cellular parameter that is reliable across various analytical platforms because it is derived from direct and precise measurements of Hb and red cell count. Hematologists should train themselves to focus on that parameter instead of MCV, and distributions around MCH should be made available on analyzers. Table 9-2 provides a list of the red cell and reticulocyte parameters provided by the major hematology analyzers.

Cold agglutinins in high titer tend to cause spurious macrocytosis with low red cell counts and very high MCHCs. Warming either the blood or the diluent may eliminate this problem. In hyperglycemic samples (greater than 20 mmol/L), flow cytometric methods provide

Box 9-5 Sources of Spurious Results in Hemoglobin and Red Cell Count Parameters

ERRONEOUS INCREASE IN Hb

Lipemia (hypertriglyceridemia, fat emulsion, type I and IV hyperlipoproteinemias)
Elevated WBC counts (threshold varies from 50 to 250 × 10^9/L)
Immunoglobulins (monoclonal IgG, IgM, IgA in Waldenström's or multiple myeloma)
Hemolysis (when free plasma Hb is markedly elevated because of intravascular hemolysis)

ERRONEOUS INCREASE IN RBC COUNTS

High WBC count (especially if greater than 100 × 10^9/L)

ERRONEOUS INCREASE IN MCV

Hyperglycemia (secondary to dilution of a severe hyperglycemic sample)
Hypernatremia
Sample storage at room temperature

ERRONEOUS DECREASE IN RBC COUNTS

Cold agglutinin (in association with high MCV and high MCHC)
Clotting (abnormal mixing, overfilling of sample)

From Zandecki M, Genevieve F, Gerard J, et al: Spurious counts and spurious results on haematology analysers: a review. Part II: white blood cells, red blood cells, haemoglobin, red cell indices and reticulocytes. Int J Lab Hematol *29:21–41, 2007.*
Hb, Hemoglobin; *MCHC,* mean corpuscular hemoglobin concentration; *MCV,* mean corpuscular volume; *RBC,* red blood cell; *WBC,* white blood cell.

falsely elevated MCV and Hct and inaccurately low MCHC values. This is the result of cell swelling produced when the instrument dilutes the blood sample with a diluent that is not isotonic compared with the hyperglycemic sample.[18]

The classification based on MCV and RDW suggested by Bessman and associates[19] should not be used because it is an oversimplification that has been shown to be limited in its diagnostic yield and erroneous in several conditions. Contrary to what is suggested in this classification schema, a normal RDW can be seen in iron-deficient anemia, and increased RDW in α- and β-thalassemia traits. RDW changes are usually confirmative of a specific diagnosis obtained with other means, but they should

TABLE 9-2 Red Cells and Reticulocyte Indices Provided by Various Hematology Analyzers

	Siemens	Beckman-Coulter	Sysmex	Abbott
MCV	MCV	MCV	MCV	MCV
		MSCV		
RDW	RDW	RDW and RDW-SD	RDW-SD and RDW-CV	RDW
	%MICRO		%MicroR	%MIC
	%MACRO			%MAC
MCHC	MCHC and CHCM	MCHC	MCHC	MCHC
HDW	HDW			HDW
	%HYPER			%HPR
	%HYPO	LDH%	%HYPO-He	%HPO
		MAF		
MCH	MCH	MCH	MCH	MCH
	CH		RBC He	
CHDW	CHDW			
	Micro/hypo ratio		M-H index	
			M/H ratio	
Schistocytes	RBC fragments %		FRC%	
Reticulocytes				
			IRF	
	MCVr	MRV		MCVr
	CHCMr			CHCr
	CHr	RSF	Ret-He	MCHr
			Δ-He	

From Brugnara C, Mohandas N: Red cell indices in classification and treatment of anemias: from M.M. Wintrobes's original 1934 classification to the third millennium. Curr Opin Hematol *20(3):222–30, 2013.*
CH, Cell hemoglobin; *CHr,* reticulocyte hemoglobin content; *CHCM,* cell hemoglobin concentration mean; *CHDW,* cell hemoglobin distribution width; *CHCMr,* CHCM of reticulocytes; *CHCr,* reticulocyte MCHC; *Δ-He,* Ret-He minus RBC He; *FRC%,* fragmented red cells %; *HDW,* hemoglobin distribution width; *%HYPO-He,* % hypochromic erythrocyes; *%HPO,* % hypochromic RBC; *IRF,* immature reticulocyte fraction; *LDH%,* low hemoglobin density %; *MAF,* microcytic anemia factor; *%MACRO,* % macrocytic RBC; *%MAC,* % macrocytic RBC; *%HPR,* % hyperchromic RBC; *MCH,* mean corpuscular hemoglobin; *MCHC,* mean corpuscular hemoglobin concentration; *MCHC/CHCM,* ; *MCV,* mean corpuscular volume; *MCHr,* reticulocyte MCH; *MCVr,* MCV of reticulocytes; *MRV,* mean reticulocyte volume; *MSCV,* mean sphered corpuscular volume; *%MicroR,* % microcytic erythrocytes; *%MICRO,* % microcytic erythrocytes; *%MIC,* % microcytic RBC; *M-H index,* %MicroR-%Hypo-He; *M/H ratio,* %MicroR/%Hypo-He; *RBC,* red blood cell; *RDW,* red blood cell distribution width; *RDW/RDW-SD,* RDW/RDW-standard deviation; *RBC-He,* red blood cell hemoglobin equivalent; *RSF,* red blood cell size factor; *RDW-SD/RDW-CV,* RDW-standard deviation/RDW-coefficient of variation; *Ret-He,* reticulocyte hemoglobin equivalent.

not be used for a primary diagnosis in the absence of other data.

Visual analysis of the red cell histograms generated by automated cell counters provides essential clues for the diagnosis of anemias. The presence of either microcytes or macrocytes and increased RDW can readily be appreciated. Even red cell fragments can now be precisely quantified.[20,21] Histograms for MCHC are particularly useful because they allow prompt identification of dehydrated hyperchromic cells in sickle cell disease, HS, hereditary xerocytosis, and immune hemolytic anemias. A plethora of discriminant functions has been proposed to distinguish β-thalassemia trait and iron deficiency, resulting in numerous and conflicting publications.[22] The Mentzer index (MCV/RBC) is still widely used, but it is inferior to some of the more modern parameters generated by automated cell counters. A more contemporary approach should take into account MCHC and HDW as shown in Figure 9-4.[23] A careful study of volume and Hb concentration histograms allows rapid differentiation of iron deficiency and β-thalassemia trait. But inspection of a well-stained smear provides the most reliable assay because morphology is considerably more abnormal at higher Hb levels in β-thalassemia trait than in iron deficiency. The plasma is very watery instead of yellow in iron deficiency, probably due to low levels of plasma Hb. Wintrobe pointed that out decades ago. Unfortunately the color of the plasma is no longer available to physicians or technicians.

Because MCV and MCHC are the average value obtained from counting large numbers of red cells, changes in red cell subpopulations may not result in changes in these average values: Hydroxyurea treatment of patients with sickle cell disease results in macrocytosis and increased MCH. However, due to the fact that both reticulocyte (low MCHC) and irreversibly sickle cells (high MCHC) are reduced by this treatment, MCHC may show little or no variation, while its distribution width (HDW) shows a significant decrease. Hyperchromic cells are a distinguishing characteristic of HS. In the absence of hemoglobinopathies like sickle Hb (Hb S) or Hb C, or autoimmune or traumatic hemolysis, presence of hyperchromic cells is diagnostic for HS, without the need of performing additional tests like osmotic fragility or more sophisticated flow cytometry studies. In sickle cell anemia the presence of dense dehydrated red cells (measured with a phthalate density gradients) seems to be associated with specific manifestations of the disease like skin ulcers, priapism, or renal dysfunction.[24] An increase in the proportion of dense hyperchromic cells can be seen in conditions of suppression of erythropoiesis: The reduction in the output of newly produced erythrocytes results in a shift in the distribution of the circulating red cells toward an "older" red cell population. A population in which older red cells prevail over younger ones is characterized by an increase in the proportion of hyperchromic cells and an increased in glycated Hb (Hb A_{1c}). This is typically seen when erythropoiesis is suppressed by viral infections or chemotherapy.

Microspherocytes and increased osmotic fragility are frequently seen in congenital dyserythropoietic anemia type II (CDAII), a chronic hemolytic disorder that can be confused with HS. CDAII is characterized by more prominent anisocytosis (increased RDW), while anisochromia (increased HDW) is more prevalent in HS; a RDW/HDW ratio greater than 5.34 has 89% sensitivity and 70% specificity to distinguish CDAII from HS.[25]

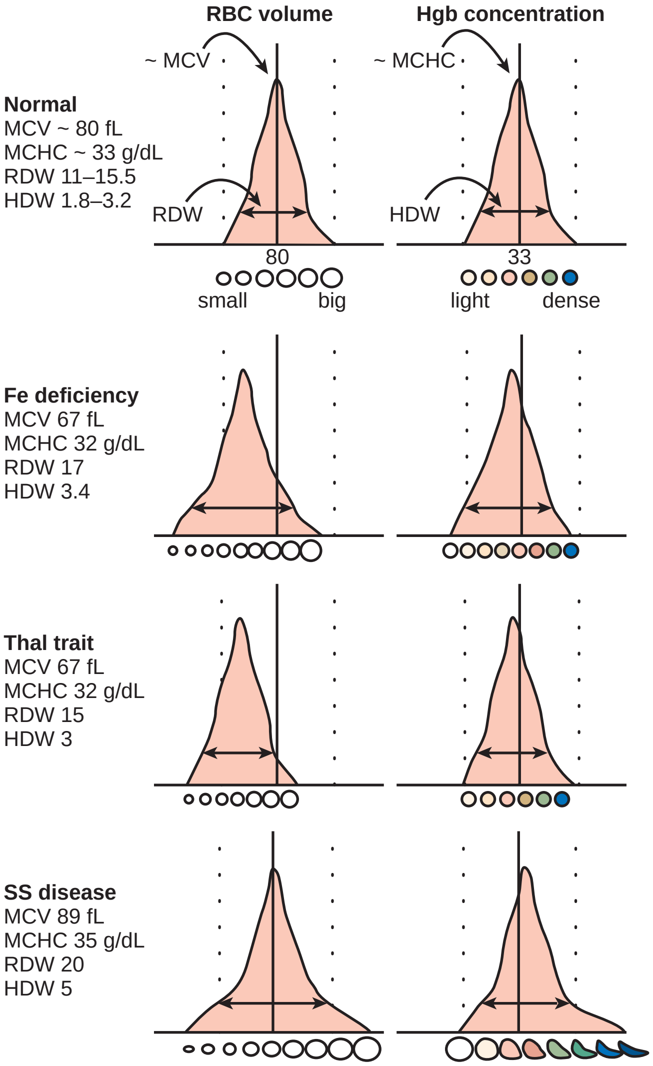


Figure 9-4 Histograms for mean corpuscular volume (MCV, *left column*) and mean corpuscular hemoglobin concentration (MCHC, *right column*) in a normal control subject *(first row)*, a patient with iron (Fe) deficiency *(second row)*, a subject with heterozygous β-thalassemia (Thal) *(third row)*, and a patient with homozygous hemoglobin S (SS) disease *(bottom row)*. Goal posts are placed at 60 and 120 fL for MCV and at 28 and 41 g/dL for MCHC. *HDW,* Hemoglobin distribution width; *Hgb,* hemoglobin; *RBC,* red blood cell; *RDW,* red cell volume distribution width.

Recent work has substantially expanded our understanding of how cell volume, cell Hb content, and concentration are dynamically linked in reticulocyte maturation, red cell aging, and removal. The work of Higgins and Mahadevan[26] has identified a volume of clearance (V_c) below which red cells are cleared from the circulation: V_c is approximately 72 fL in normal cells and 59 to 56 fL for hypochromic cells like those of β-thalassemia trait and iron deficiency anemia. Clearance of red cells from circulation is preceded by a reduction in both Hb content and cell volume, which is associated with fluctuations or drift (D) around the average MCHC for individual cells. These variations in Hb content (D_h) and cell volume (D_v) can be extracted from the data collected with a regular CBC and reticulocyte count. D_h is markedly elevated in iron deficiency anemia and has a 98% diagnostic accuracy in distinguishing this condition from β-thalassemia trait (D_h threshold of 0.0045) in one 50-patient test cohort.

Almost all analyzers are capable of providing automated counting of reticulocytes.[27,28] Automated counting is more precise and accurate than manual counting.[29] Absolute reticulocyte counts are easily obtained with these instruments, thereby obviating the limitations of counting reticulocytes only as a percentage or correcting for changes in Hct (corrected reticulocyte count).

Some analyzers can perform direct measurements of reticulocyte Hb content. Reticulocyte indices like CHr have better diagnostics power than traditional biochemical parameters for diagnosing iron-deficient states and are an early indicator of therapeutic response to hematinic supplements like iron, B_{12}, or folate.[30] CHr[31,32] has been shown to be a valuable parameter in identifying iron deficient states in young children and toddlers.[33,34]

An additional useful feature of automated reticulocyte counters is that stress reticulocytes can easily be identified by their increased volume and RNA content.[35] Immature reticulocyte fraction (IRF) quantifies changes in reticulocyte maturity based on the increased fluorescence associated with the more immature reticulocyte forms. IRF has been show to be an early indicator of engraftment following hematopoietic stem cell transplantation.

The Blood Film

Automatic slide maker/stainer instruments are widely used in clinical laboratories; however, films made on coverslips are preferable to those made on glass slides because a greater proportion of blood on the coverslip film is technically suitable for microscopic examination. Proper processing of blood films on coverslips is fast becoming a lost art. The details of preparation and examination can be found in a variety of manuals of laboratory hematology, but the lucid and succinct instructions of Wintrobe deserve reproduction here[36]:

1. Use a small drop of blood, only 2 to 3 mm in diameter, taken either from a stylet wound, as described earlier, or from a syringe or needle tip used in venipuncture immediately after the venipuncture has been performed (anticoagulants are not to be used because they will alter the morphologic appearance).
2. Hold the coverslips only by their edges, placing one crosswise over the other, and allow the blood to spread between them for about 2 seconds.
3. Quickly but gently separate the coverslips by pulling them laterally, in opposite directions to one another but in the plane of the spreading film, just before the film reaches the edges (do not squeeze or lift the coverslips from one another).
4. Quickly air-dry the films, either by placing them face up on a clean surface if the humidity is low or by moving them through the air while holding them by their edges with your fingertips.

If the procedure has been carried out successfully, the blood will be spread evenly, and there will be no holes or thick areas in the preparation. A multicolored sheen will be seen on the surface of the dried, unstained film if light glances off it at the proper angle because the thin layer of closely fitting cells acts like a diffraction grating. Later under the microscope after staining, the red cells will be seen next to each other, but neither overlapping nor in rouleau formation, and central pallor will be visible; lymphocytes will have a readily distinguished cytoplasm rather than a minimal zone bearing closely on the nucleus, as occurs with thick films or those that dry too slowly.

Examination of a peripheral blood film is an essential step in the initial evaluation of a patient with anemia. The blood film should first be examined under low power to determine the adequacy of cell distribution and staining. Signs of poor blood film preparation include loss of central pallor in red blood cells, polygonal shapes, and artifactual spherocytes. Artifactual spherocytes, in contrast to true spherocytes, show no variation in central pallor and are larger than normal red cells. One should never attempt to interpret a poorly prepared blood film.

After the adequacy of the blood film is determined by low-power examination, the blood film should be examined first under 50× magnification and then under 1000× magnification. Low-power examination is crucial to identify the presence of rare cells like nucleated red cells.

Cells should be graded for size, staining intensity, variation in color, and abnormalities in shape. The blood film should also be examined for the presence of basophilic stippling and red cell inclusions. The significance of some of these findings is described in Table 9-3. Table 9-4 provides a comprehensive description of the morphologic abnormalities that can be observed in a blood film. Some representative smears of the most common hematologic disorders are presented in Figure 9-5.

Red cell hemolytic disorders can be classified according to their predominant morphology. An approach to such a classification is presented in Box 9-6, and it is discussed in detail in Chapters 14 through 17. Examination of the other cell lines may provide additional useful information: Neutrophil hypersegmentation (at least 5% of neutrophils with five lobes or at least 1% with six lobes) is suggestive of folic acid deficiency.

In addition to careful history and physical exam, examination of the blood smear by an experienced hematologist is an essential step in the proper diagnosis of all anemias.

TABLE 9-3 Diagnostic Significance of Red Cell Inclusions

Inclusion	Staining Agent	Diagnostic Significance
Basophilic stippling	Wright stain	Represents aggregated ribosomes; may be observed in thalassemia syndromes, iron deficiency, syndromes accompanied by ineffective erythropoiesis and pyrimidine 5′-nucleotidase deficiency; particularly prominent in unstable hemoglobinopathies and lead poisoning
Howell-Jolly bodies	Wright stain	Represent nuclear remnants; observed in asplenic and hyposplenic states, pernicious anemia, dyserythropoietic anemias, and severe iron deficiency anemia
Cabot's rings	Wright stain	Appear as basophilic rings, circular, or twisted figures-of-eight; considered to be nuclear remnants or artifacts; observed in lead poisoning, pernicious anemia, and hemolytic anemias
Heinz bodies	Brilliant cresyl blue, methyl violet	Represent denatured or aggregated hemoglobin; observed in patients with thalassemia syndromes or unstable hemoglobins, after oxidant stress in patients with enzyme deficiencies of the pentose phosphate pathway, and in patients with asplenia or chronic liver disease
Siderocytes	Prussian blue counterstained with safranin O	Represent nonhemoglobin iron within erythrocytes; seen in increased numbers in the peripheral circulation after splenectomy; observed in increased numbers in patients with chronic infection, aplastic anemias, or hemolytic anemias

TABLE 9-4 Common Red Blood Cell Morphologic Findings

RBC Morphology	Morphologic Definition	Clinical Associations
Acanthocyte (spur cell)	RBC has irregularly distributed, variably sized, pointy projections off its surface	Advanced liver disease, hyposplenism, some dyslipidemias, pyruvate kinase deficiency, McLeod phenotype
Anisochromia	Variation in the amount of central pallor among a population of RBCs	Iron deficiency, myelodysplasia, hypochromic anemia posttransfusion
Anisocytosis	Variation in size among a population of RBCs	Common nonspecific finding; seen in iron deficiency, moderate or severe thalassemia, megaloblastic anemia, partially treated anemia of several causes, posttransfusion
Basophilic stippling: coarse	RBC has variably sized (up to large), basophilic granular discolorations across its entire cytoplasm, on a Wright-stained film	Thalassemia, lead poisoning, myelodysplasia, pyrimidine 5′ nucleotidase deficiency, post chemotherapy
Basophilic stippling: fine	RBC has small, uniform, punctate basophilic dots across its entire cytoplasm, on a Wright-stained film	Reticulocytosis, normal finding
Bite cell/blister cell	RBC has a semi-circular indentation in its outer cytoplasmic border; there may be a "roof" to this indentation (blister cell) or no roof (bite cell)	Oxidative hemolysis
Dimorphism	Two distinct populations of RBC are present, for example, microcytic and normocytic, or hypochromic and normochromic	Myelodysplasia, posttransfusion, partially treated iron deficiency
Echinocyte (burr cell)	RBC has regularly distributed, equally sized, rounded projections off its surface	Artifact, renal failure, posttransfusion, phosphate deficiency, burns
Elliptocyte	RBC is oval shaped	Iron deficiency, megaloblastic anemia, hereditary elliptocytosis, post chemotherapy
Heinz body	RBC has a submembranous or epimembranous small round mass, which can only be seen by supravital or specialized Heinz body stains; this body is not visible on a routine Wright-stained film	Oxidative hemolysis, hyposplenism

TABLE 9-4 Common Red Blood Cell Morphologic Findings—cont'd

RBC Morphology	Morphologic Definition	Clinical Associations
Howell-Jolly body	Solitary round mass, relatively large (e.g., approximately 10%-20% of the diameter of the RBC), within the hemoglobinized portion of the RBC; appears dark blue or purple on a Wright-stained film	Hyposplenism, erythroblastosis, myelodysplasia, megaloblastic anemia, post chemotherapy
Hypochromia	The zone of central pallor is > $^1/_3$ the diameter of the RBC	Iron deficiency, thalassemia, anemia of chronic disease
Irregularly contracted cell	The RBC is small, dark, and lacks a zone of central pallor; its outer margin is not spherical: it may appear dented, compressed, or otherwise "contracted"	Nonspecific finding seen in a variety of conditions including G6PD deficiency, hemoglobinopathies, and normal neonates
Pappenheimer body	Usually multiple small dark blue or purple granular inclusions, all within the hemoglobinized portion of the RBC; these occupy only one portion or region of the RBC, unlike basophilic stippling, which is more global throughout the entire RBC	Iron overload, hyposplenism, myelodysplasia
Piscocytes/fish cells	Teardrop-like cells with tapered end that resembles a fish's tail	Thalassemia trait/thalassemias
Polychromasia	RBCs show color variability as a population: some (usually the majority) are the usual red color, while others are bluish	Reticulocytosis, normal neonate
RBC agglutination	Some RBCs aggregate into multicellular masses resembling a bunch of grapes	Cold agglutinin, cold autoimmune hemolytic anemia
Rouleaux	Some RBCs aggregate into linear patterns, said to resemble a stack of coins	Normal finding in the thick part of the blood smear, hypergammaglobulinemia (monoclonal or polyclonal)
Schistocyte	The RBC appears to have been fragmented: it lacks the usual circular shape, instead showing a triangular or other angulated morphology; the zone of central pallor is often missing	RBC fragmentation syndromes, for example, microangiopathic hemolytic anemia and hemolysis secondary to cardiac valve
Sickle cell	There are several sickle RBC morphologies, including the classic sickle (crescentic with two sharply pointed ends) or boats (linear with two tapered, if somewhat rounded, ends)	Severe sickling syndrome, for example, Homozygous Hb S disease, Hemoglobin SC disease, Hemoglobin SD disease
Spherocyte	The RBC is smaller and darker than normal; there is no zone of central pallor; the outer edge must be almost perfectly round (to differentiate this cell from irregularly contracted cells)	Autoimmune hemolytic anemia, alloimmune hemolytic anemia (e.g., hemolytic disease of the newborn), hereditary spherocytosis
Stomatocyte	The zone of central pallor is linear rather than circular; usually the line of pallor runs parallel to the long axis of the RBC; if the latter is ovoid, but in certain variants (e.g., South East Asian ovalocytosis), the line may run across the long axis or may be nonlinear, for example, bifurcated or trifurcated	Artifact, obstructive liver disease, hereditary stomatocytosis, South East Asian ovalocytosis, Rh null syndrome
Target cell	The RBC has a central red area within the zone of central pallor	Thalassemia, liver disease, hyposplenism, Hgb C disease or SC disease, hereditary xerocytosis; may be seen in iron deficiency
Teardrop cell	The RBC is tapered to a point at one end, resembling the classic artist's rendition of a drop of water	Nonspecific finding seen in several conditions, including myelofibrosis

Modified from Ford J: Red blood cell morphology. Int J Lab Haematol *35:351–357, 2013.*
G6PD, Glucose-6-phosphate dehydrogenase; *Hgb,* hemoglobin; *RBC,* red blood cell.

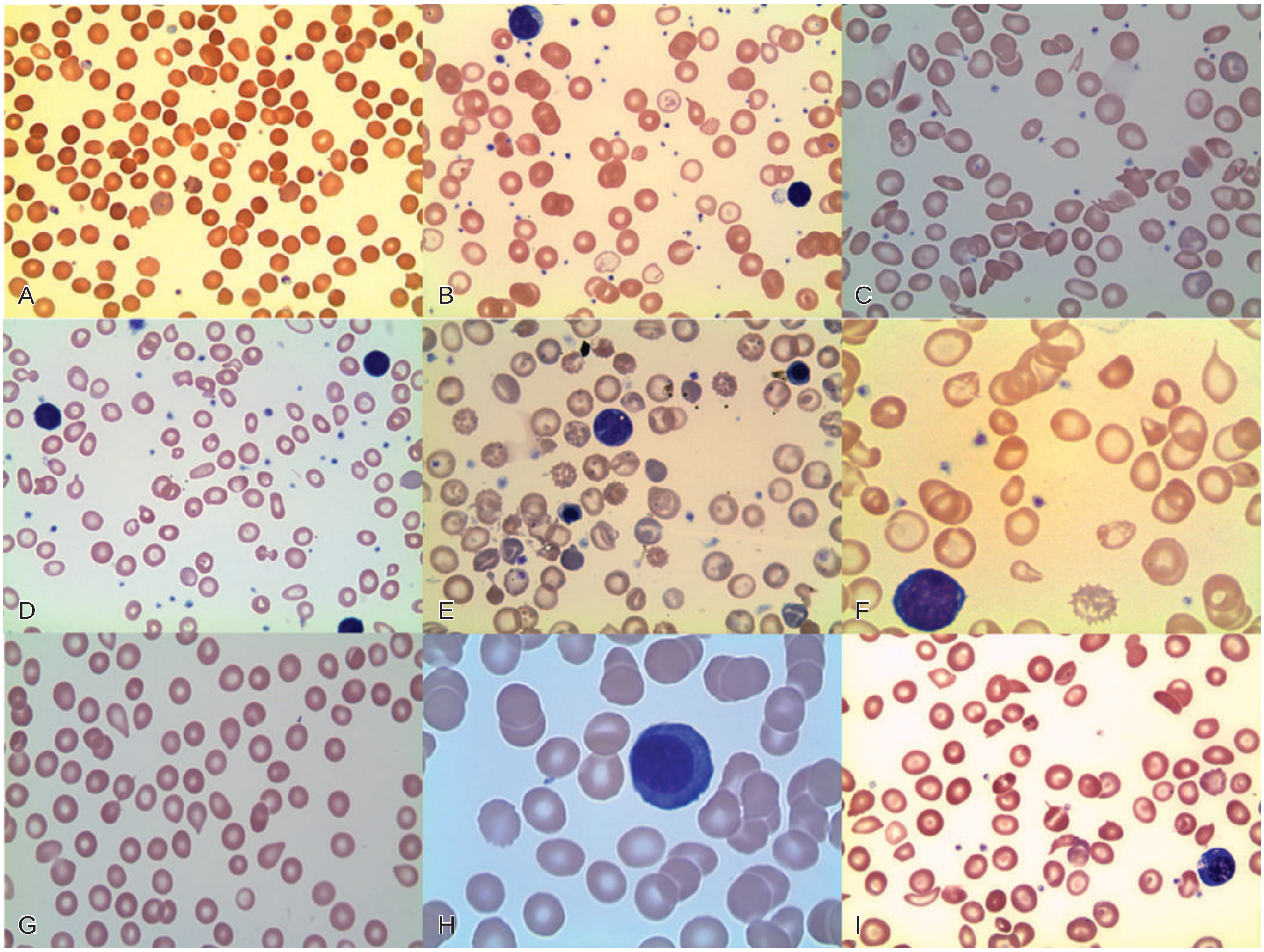


Figure 9-5 Peripheral smear, anemias, and red cell morphology. **A,** 5-year-old female with hereditary spherocytosis. **B,** Hemoglobin (Hb) E disease. **C,** 5-year-old male with sickle cell disease. 2+ sickle cells, %retic 13.1%. **D,** 2-year-old female with severe iron deficiency. **E,** 2-week-old male with hemolytic anemia of unknown origin. 56% retics, 3+polychromasia, schistocytes, spherocytes. **F,** 19-month-old with homozygous Hb E disease and iron deficiency. **G,** 2-year-old female leukemia patient with many teardrop cells. **H,** 7-year-old male with medulloblastoma. See early red cell precursor. **I,** 3-year-old female with homozygous Hb S disease. Many sickly cells, targets, and schistocytes and a large nucleated red blood cell.

Box 9-6 Classification of Red Cell Hemolytic Disorders by Predominant Morphology[*]

SPHEROCYTES

Hereditary spherocytosis
ABO incompatibility in neonates
Immunohemolytic anemias with IgG- or C3-coated red cells[†]
Acute oxidant injury (hexose monophosphate shunt defects during hemolytic crisis, oxidant drugs, and chemicals)
Hemolytic transfusion reactions[†]
Clostridium welchii septicemia
Severe burns, other red cell thermal injury
Spider, bee, and snake venom
Severe hypophosphatemia
Hypersplenism[‡]

BIZARRE POIKILOCYTES

Red cell fragmentation syndromes (microangiopathic and macroangiopathic hemolytic anemias)
Acute oxidant injury[‡]
Hereditary elliptocytosis in neonates
Hereditary pyropoikilocytosis

ELLIPTOCYTES

Hereditary elliptocytosis
Thalassemias
(Other hypochromic-microcytic anemias)
(Megaloblastic anemias)

STOMATOCYTES

Hereditary stomatocytosis
Rh_{null} blood group
Stomatocytosis with cold hemolysis
(Liver disease, especially acute alcoholism)
(Mediterranean stomatocytosis)

Box 9-6 **Classification of Red Cell Hemolytic Disorders by Predominant Morphology**—cont'd

IRREVERSIBLY SICKLED CELLS

Sickle cell anemia
Symptomatic sickle syndromes

INFRAERYTHROCYTIC PARASITES

Malaria
Babesiasis
Bartonellosis

SPICULATED OR CRENATED RED CELLS

Acute hepatic necrosis (spur cell anemia)
Uremia
Red cell fragmentation syndromes[‡]
Infantile pyknocytosis
Embden-Meyerhof pathway defects[‡]
Vitamin E deficiency[‡]
Abetalipoproteinemia
Heat stroke[‡]
McLeod blood group
(After splenectomy)
(Transiently after massive transfusion of stored blood)
(Anorexia nervosa)[‡]

TARGET CELLS

Hemoglobins S, C, D, and E
Hereditary xerocytosis
Thalassemias
(Other hypochromic-microcytic anemias)
(Obstructive liver disease)
(After splenectomy)
(Lecithin-cholesterol acyltransferase deficiency)

PROMINENT BASOPHILIC STIPPLING

Thalassemias
Unstable hemoglobins
Lead poisoning[‡]
Pyrimidine 5′-nucleotidase deficiency

NONSPECIFIC OR NORMAL MORPHOLOGY

Embden-Meyerhof pathway defects
Hexose monophosphate shunt defects
Unstable hemoglobins
Paroxysmal nocturnal hemoglobinuria
Dyserythropoietic anemias
Copper toxicity (Wilson's disease)
Cation permeability defects
Erythropoietic porphyria
Vitamin E deficiency
Hemolysis with infections[‡]
Rh hemolytic disease in neonates[†]
Paroxysmal cold hemoglobinuria[*‡]
Cold hemagglutinin disease[†]
Hypersplenism
Immunohemolytic anemia[†‡]

[*] Nonhemolytic disorders of similar morphology are enclosed in parentheses for reference.
[†] Usually associated with a positive Coombs test.
[‡] Disease sometimes associated with this morphology.

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CHAPTER 10

Megaloblastic Anemia

Ralph Carmel, David Watkins, and **David S. Rosenblatt**

CHAPTER OUTLINE

DEFINITIONS
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FOLATE
Chemistry of Folates
Folate Sources, Requirements, and Stores
Physiology of Folates
Biochemistry of Folates
COBALAMIN (VITAMIN B_{12})
Chemistry of Cobalamins
Cobalamin Sources, Requirements, and Stores
Physiology of Cobalamin
Biochemistry of Cobalamin
MANIFESTATIONS OF DEFICIENCY
Overview of Folate and Cobalamin Deficiencies
Megaloblastic Anemia of Folate and Cobalamin Deficiencies
Nonhematologic Manifestations of Cobalamin Deficiency
Nonhematologic Manifestations of Folate Deficiency
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Biomarkers of Metabolic Status
Response to a Trial of Specific Therapy
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Acquired Disorders of Cobalamin Absorption
Acquired Disorders of Cellular Cobalamin Metabolism
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Management of Acquired Folate Deficiencies
GENETIC DISORDERS CAUSING MEGALOBLASTIC ANEMIA
Inborn Errors of Folate Transport and Metabolism
Inborn Errors of Cobalamin Transport and Metabolism
Thiamine-Responsive Megaloblastic Anemia
Hereditary Orotic Aciduria
3-Phosphoglyceride Dehydrogenase Deficiency

The metabolism of folate and that of cobalamin are intimately intertwined, and deficiency of either vitamin can manifest as megaloblastic anemia. The two deficiencies must be rigorously differentiated and treated specifically because their causes, mechanisms, nonhematologic expressions, courses, and prognoses differ. This is as true in pediatric medicine as in adult and geriatric medicine, from which much of the existing hematologic information originated. Recent advances in molecular biology, genetics, and epigenetics have increased our knowledge of cobalamin and folate metabolism. In addition, as biochemical tools to diagnose deficiencies have become more accurate and precise, the way that we define *deficiency* has expanded to include biochemically defined subclinical deficiency. Epidemiologic advances have connected disorders not traditionally linked to vitamin deficiency, such as neural tube defects (NTDs) and cardiovascular disease, suggesting potential means of preventing their development. Even if some of the epidemiologic information has still unsettled implications for clinical practice and public health, the wealth of new data deserves attention. In this chapter we review the relevant clinical and scientific knowledge from all these streams, emphasize its applications in pediatric hematology, and specify wherever possible the known distinctions between infants, older children, and adults.

DEFINITIONS

Megaloblastic anemia is a specific, relatively small subset of *macrocytic anemia*. It is defined by a characteristic megaloblastic appearance of nucleated cells that suggests maturational nuclear-cytoplasmic asynchrony. Despite the name "anemia," all three hematopoietic cell lines are affected noticeably as the deficiency advances. Pancytopenia can ultimately become extreme. The term *pernicious anemia,* sometimes misapplied to the megaloblastic anemia in cobalamin deficiency, is used here in its preferred, specific meaning: the absence of gastric intrinsic factor (IF) that produces cobalamin malabsorption and, ultimately, deficiency.

Deficiency is an elusive term with surprisingly limited conceptual and pragmatic consensus on its definition(s). Where relevant, in this chapter we will use *clinical deficiency* of cobalamin or folate to denote the presence of vitamin-specific clinical signs and symptoms that require medical intervention. However, most cobalamin and folate deficiencies identified today are *subclinical*. They are clinically silent and expressed only by (often minimal) biochemical manifestations; most importantly, the likelihood for progression is usually uncertain, and consensus does not yet exist for medical intervention or for according them the same weight as clinical deficiency.

The term *cobalamin* refers to all corrins with coenzyme activity in humans. *Vitamin* B_{12} originated as the name for synthetic *cyanocobalamin* but is widely used as a generic synonym for cobalamin. The term *folate* refers to synthetic folic acid and to the various natural folate coenzymes, which are reduced dihydrofolates and tetrahydrofolates and their single-carbon substituted forms. Most folates in nature are reduced and substituted *folate polyglutamates*.

The term *newborn* is used in this chapter for a child in the first month of life and the term *infant* for a child in the first 2 years of life.

HISTORY

The story of megaloblastic anemia began with Addison's description in 1849 of a puzzling disease that he believed had an adrenal origin[1,2] and whose lethality Biermer immortalized by calling it "pernicious anemia." A series of clinical and pathologic descriptions from the 1870s through the 1920s identified the hematologic and neurologic features of the disease, setting the stage for one of the landmark clinical investigations in hematology. Using reticulocyte counts as evidence, Minot and Murphy reversed the anemia by feeding patients large amounts of liver in 1926,[3] and for their findings they were awarded a Nobel Prize. Castle then proved with elegant clinical experiments that the disease was caused by the loss of an "intrinsic factor" in gastric juice that allowed the internalization of an extrinsic factor.[4] In the 1940s, the "extrinsic factor" was crystallized, identified, and synthesized as cyanocobalamin (vitamin B_{12}), which could then be injected intramuscularly. Readers are referred to very readable accounts of the compelling history of pernicious anemia.[5-7]

Another important story was developing on a separate track, in which somewhat similar clinical syndromes involving megaloblastic anemia ("pernicious anemia of pregnancy" and "tropical anemia") were described and shown by Wills to respond to yeast extract.[8] Folic acid was synthesized in 1948 and shown to reverse folate deficiency. A notable self-experiment in a single subject proved that folate deficiency induced by dietary restriction produced megaloblastic anemia and mapped the evolution of the hematologic changes prospectively.[9] After the synthesis of the two vitamins, work by many investigators solidified accurate biochemical testing for cobalamin and folate status and delineated the clinical differences between folate and cobalamin deficiencies. New tests with improved sensitivity and accuracy have been developed, although a gold standard for the diagnosis of deficiency remains elusive, especially for cobalamin deficiency.

Equally important have been the continuing advances over decades in delineating the complex transport system of each vitamin and the modern molecular and genetic understanding of the proteins and receptors involved. These advances are especially encouraging and important to pediatric care of cobalamin deficiency, which is not as common and had not heretofore been studied as intensively as in adults. The major advances in the past two decades and the explosion in diagnostic, biochemical, genetic, and therapeutic advances in identifying and treating new genetic disorders and the pathways they illuminate are reviewed within this chapter.

FOLATE

Chemistry of Folates

Folates are heterocyclic compounds consisting of a pteroyl group conjugated with one or more glutamic acid units (Fig. 10-1). Folic acid, or pteroylglutamic acid, is an oxidized folate consisting of pteroic acid conjugated with a single glutamate residue. Biologically active folates are derived from folic acid by reduction of its pterin ring to form 7,8-dihydrofolate (DHF) and 5,6,7,8-tetrahydrofolate (THF); by addition of single carbon units at different redox states to nitrogen 5 or 10 of the pterin ring; and by conjugation of up to seven total glutamate residues by γ-carboxyl bonding. Intracellular folates exist primarily as one-carbon group substituted tetrahydrofolate polyglutamates.

Folate derivatives that play an important role in cellular metabolism include 5-methylTHF, 5,10-methyleneTHF, 5,10-methenylTHF, 10-formylTHF, 5-formylTHF, and 5-formiminoTHF. Folic acid itself is available commercially as a stable yellow powder. It has low solubility in aqueous solutions and at acid pH. 5-FormylTHF (folinic acid, leucovorin) is a stable reduced folate that is available commercially. Other reduced folates are photolabile and susceptible to destruction by oxidation, readily

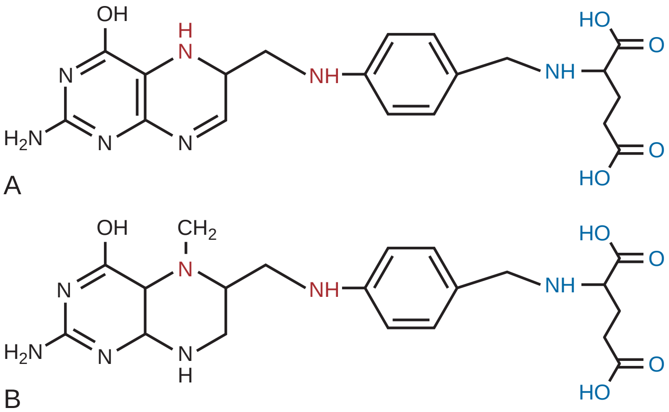

Figure 10-1 Structure of folic acid (A) and 5-methyltetrahydrofolic acid (B). Folic acid consists of pteroic acid *(black)* conjugated to a glutamic acid *(blue)*. Cellular folates are derived from this molecule by reduction to 7,8-dihydrofolate (DHF), further reduction to 5,6,7,8-tetrahydrofolate (THF); by addition of one carbon groups to THF at nitrogens 5 or 10 *(shown in red)*, and by addition of up to six additional glutamic acid residues γ-carboxyl bonding. Forms active in cellular metabolism include 5-methylTHF *(shown)*, 5-formiminoTHF, 5-formylTHF (folinic acid), 5,10-methyleneTHF, 5,10-methenylTHF, and 10-formylTHF.

breaking at the carbon 9—nitrogen 10 bond to produce inactive catabolites.[10]

Folate Polyglutamates

Most intracellular folates exist as polyglutamylated derivatives, containing up to six or seven glutamate residues attached by γ-peptide bonds.[11] Synthesis of these folate polyglutamates depends on activity of the enzyme folylpoly-γ-glutamate synthetase (EC 6.3.2.17), which adds glutamate residues one at a time to reduced folates, using adenosine triphosphate (ATP) as an energy source.[12] Folate polyglutamates have higher affinity for most folate-dependent enzymes than the corresponding monoglutamates and are efficiently channeled between active sites in multifunctional enzymes.[13] They do not readily cross the cell or mitochondrial membranes, and polyglutamylation of folates within cells thus contributes to the ability of cells to retain reduced folates in the presence of low extracellular concentrations. Separation of cytosolic and mitochondrial folate polyglutamate pools has important consequences for cellular folate metabolism.

Folate Sources, Requirements, and Stores

Folate is synthesized de novo by bacteria, plants, fungi, and certain protists. Animals are dependent on their diets for adequate supplies of the vitamin. Foods with very high folate content include dark green vegetables, orange juice, nuts, legumes, and liver. However, the reduced folates present in food are labile to light and oxidation and are partly destroyed during processing and cooking. The recommended daily allowance for folate is expressed in dietary folate equivalents to take into account the greater bioavailability of folic acid than the reduced polyglutamylated forms present naturally in food. Values recommended by the Institute of Medicine of the United States[14] and the World Health Organization[15] are listed in Table 10-1.

The average content of body stores in adults has been estimated to be 12 to 28 mg folate, based on a content of 6 to 14 mg (or 4.5 to 10 μg/g) in liver, the major storage organ.[14,16] It is relevant to note here that the ratio of body folate stores to recommended daily intakes is less than 100 : 1, whereas the ratio for cobalamin approximates 1000 : 1. As a result, the folate depletion necessary to produce megaloblastic anemia is achieved much more rapidly than depletion of cobalamin. Storage data are sparse in children, but infants of normal birth weight who died in the perinatal period had hepatic folate contents of 0.76 mg[16]; low-birth-weight infants' hepatic content averaged only 0.13 mg. These data, although not fully representative, suggest much lower stores–to–recommended intake ratios in newborns and infants than in adults.

Recognition that supplementation in the periconceptional period with folic acid results in decreased occurrence and recurrence of NTDs in children of women with no clinical evidence of folate deficiency[17,18] led to recommendations that all women of childbearing years should take folate supplements (200 to 400 μg daily), because the critical period for prevention of NTDs is early in pregnancy before most women are aware that they are pregnant. Since January 1998, folic acid fortification of all enriched cereal-grain products has been mandated in the United States,[19] Canada, and parts of South America. As a result, serum and red blood cell (RBC) folate concentrations have increased in these populations and the incidence of NTD-affected pregnancies has decreased by 26% in the United States and by 48% in Canada.[20,21] Concerns about folate fortification delaying the diagnosis of cobalamin deficiency, promotion of cancer growth, and issues of free choice have delayed mandating folate addition to the diet in Europe and elsewhere.[22]

TABLE 10-1 Recommended Daily Intakes for Folate

Age/Condition	Institute of Medicine (1998) μg/day (DFE)	World Health Organization (2004) μg/day (DFE)
0-6 mo	65*	80
7-12 mo	80*	80
1-3 yr	150	160
4-8 yr	200	4-6 yr: 200 7-9 yr: 300
9-13 yr	300	300
>14 yr	400	400
Pregnancy	600	600
Lactation	500	500

DFE, Dietary folate equivalent.

*Values are expressed as "adequate intake" rather than recommended daily allowance.

The median serum folate level of 5.5 μg/L in the total National Health and Nutrition Examination Survey (NHANES) study population before fortification increased to 11.9 μg after fortification[23]; in children, folate level increased from 8.8 to 15.6 μg/L for ages 4 to 11 years and from 5.1 to 11.0 μg/L for ages 12 to 19 years. The mildly lower folate status in adolescents, albeit still within the reference range,[14] is unexplained. The median RBC folate level increased from 174 to 256 μg/L in the total population,[23] and in children it increased from 200 to 256 μg/L at ages 4 to 11 years and from 150 to 224 μg/L at 12 to 19 years. NHANES data after fortification estimated that daily folate intake averaged 626 μg in men and 534 μg in women.[24] Intakes in children averaged 425 μg at ages 1 to 3 years, 642 μg at 4 to 8 years, 600 μg at 9 to 13 years, and 606 μg at 14 to 18 years[24]; the total homocysteine (tHcy) levels in the age groups between 4 and 18 years old were within the expected reference range (4.6 to 5.2 μmol/L).

Physiology of Folates

Cellular Folate Uptake

Three distinct systems for cellular uptake of folates have been identified in mammals. The reduced folate carrier (RFC) supports a low-affinity, high-capacity system for uptake of reduced folates and methotrexate (an antifolate drug) at high concentrations (in the micromolar range) with a neutral optimum pH; it is less effective in uptake of folic acid. It appears to be an important folate transporter in many types of cells, including hematopoietic cells. RFC is encoded by the *SLC19A1* gene on chromosome 21q22.3.[25-27] Inactivation of *Slc19a1* in mice results in neonatal lethality owing to failure of erythropoiesis.[28]

A second, high-affinity low-capacity system for folate uptake, active at nanomolar folate concentrations, is mediated by members of the folate receptor family. Several different receptors have been identified, encoded by genes on the long arm of chromosome 11 (*FOLR1, FOLR2, FOLR3,* and *FOLR4*), with different forms expressed at different times in different tissues.[29-31] Folate receptor-α and folate receptor-β are attached to the cell surface by a glycosylphosphatidylinositol anchor.[32] The circulating folate-binding protein that carries some of the circulating folate is derived from folate receptor-α by cleavage from this anchor. Binding of folates to folate receptors initiates carrier-mediated endocytosis. The level of cell surface folate receptors increases greatly in cultured mouse cells grown in very low folate concentrations, suggesting that these receptors play an important role in cellular folate uptake under these conditions.[33]

The third folate transport protein, the proton-coupled folate transporter (PCFT), has an acidic optimal pH, and transports reduced and oxidized folates with similar efficiency.[34] It is expressed at high levels in liver, kidney, and small intestine, with lower expression in other tissues. It was originally identified as a low-affinity heme transport protein[35] but was subsequently shown to have higher affinity for folates. PCFT is encoded by the *SLC46A1* gene on chromosome 17q11.2.[34]

Absorption of Folates from the Gut

Dietary folates are principally folate polyglutamates. These are hydrolyzed within the gut lumen or brush border, or in the lysosomes of enterocytes, by the enzyme γ-glutamylhydrolase (EC 3.4.22.12),[36,37] to generate folate monoglutamates that cross cell membranes much more easily than the corresponding polyglutamates. Adequate absorption is impossible without this hydrolysis. Uptake of folate monoglutamates occurs primarily in the proximal small intestine and is mediated by PCFT.[34] RFC is also expressed in enterocytes, but its role in this tissue remains unknown. The efficiency of absorption of folic acid, folate polyglutamates, and food folate varies in different subjects. In general, absorption of synthetic folic acid is about twice as efficient as that of folate in food whereas folic acid from folic acid–fortified cereal grain food is absorbed about 1.7 times as efficiently as food folate.[38] The carrier-mediated process in the proximal small intestine for reduced folates is saturable, with an upper limit approximating 200 μg of food folate. The absorption of unreduced folic acid, when taken in large doses, occurs more distally by nonsaturable high-capacity mechanisms,[4,39] which helps explain both the higher bioavailability of folic acid than food folates and the frequent response to oral folic acid by patients with malabsorptive disease.

Folate appears in the portal venous blood within 15 minutes of entering the stomach and reaches a maximum level in portal venous plasma about 1 hour later. Reduced folates, such as 5-formylTHF, are rapidly converted to 5-methylTHF during absorption.[40] A second potential source of folate is that synthesized by bacteria in the colon, some of which exists as monoglutamates. There is evidence for a folate transport system, with the same properties as the small intestine system, in the colon.[41,42] This may contribute to whole-body folate homeostasis, or at least support folate requirements of colon cells.

It has been estimated that up to 100 μg of folates, including folate delivered to the liver and some hepatic folate, is excreted into the bile and subsequently reabsorbed.[43-45] This enterohepatic recirculation mediates a slower and more even absorption than would occur in its absence. There is evidence that enterohepatic recirculation of folates is impaired with alcohol ingestion,[44,46] contributing to folate deficiency.[47,48]

Transport of Folates in Plasma

Plasma folate typically consists almost entirely of 5-methylTHF, but significant amounts of folic acid may be present in individuals who have consumed folic acid supplements or food supplemented with folic acid.[49-51] Most folate circulates unbound, although some may be bound to albumin and a small proportion is bound to a folate-binding protein derived from the cell surface protein folate receptor-α.[52-54] Plasma folate-binding protein levels are increased in pregnancy, in folate deficiency, in renal failure, with breast and ovarian cancers, and with some types of inflammation. Its function in plasma is unclear.[55-57] It is present in milk, where it may function to improve the absorption of folate by the newborn.[58,59]

Folate Transport Across the Blood-Brain Barrier

The concentration of folate (primarily 5-methylTHF) in the cerebrospinal fluid (CSF) is twofold to threefold higher than in blood, indicating active transport occurs across the blood-brain barrier.[60] Both PCFT and folate receptor-α are expressed at the choroid plexus, and mutations at either *SLC46A1* or *FOLR1* are associated with decreased cerebral 5-methylTHF levels. It has been suggested that folate receptor-α mediates endocytosis of folates and that PCFT is required for export of endocytosed folates from acidified endosomes.[61]

Folate in the Kidney

Folate, free and bound to folate-binding protein or albumin, is filtered at the glomerulus and reabsorbed in the proximal tubule, with the result that little folate appears in the urine. Both folate receptor-α and folate receptor-β are expressed in the brush border of the proximal tubule and are responsible for reabsorption of free folate.[62,63] Reabsorption of folate bound to albumin is carried out by the large multiligand endocytic receptors megalin and cubilin, whereas folate-binding protein is reabsorbed by megalin.[64]

Folate Transport in the Placenta

Levels of plasma folate are higher in cord than maternal blood.[65] Perfusion of the human placental cotyledon revealed a two-step maternal-to-fetal transfer of methylTHF, with binding of folate to placental folate-binding proteins on the maternally facing chorionic surface and gradual release generating an intervillous blood folate level three times that in the maternal circulation, followed by passive transfer to the fetal circulation along a downhill concentration gradient.[65] There is expression of folate receptor-α, PCFT, and RFC in placenta, with folate receptor-α and PCFT localizing together to the syncytiotrophoblast microvillous plasma membrane, where they may function together in the same way that they do in the choroid plexus.[66-69]

Biochemistry of Folates

Intracellular folate metabolism involves the transfer of one-carbon units derived from breakdown of a variety of metabolites. A scheme of cellular folate metabolism is illustrated in Figure 10-2. Metabolism can be divided into reactions involved in transfer of one-carbon units to THF, generating one-carbon substituted reduced folates; interconversion of one-carbon substituted THF derivatives; and utilization of one-carbon units in amino acid and nucleic acid metabolism.

The primary source of one-carbon units for folate metabolism is catabolism of serine to form glycine, which generates 5,10-methyleneTHF (reaction 4 in Fig. 10-2). Additional sources include breakdown of glycine (reaction 5), dimethylglycine and sarcosine, which also

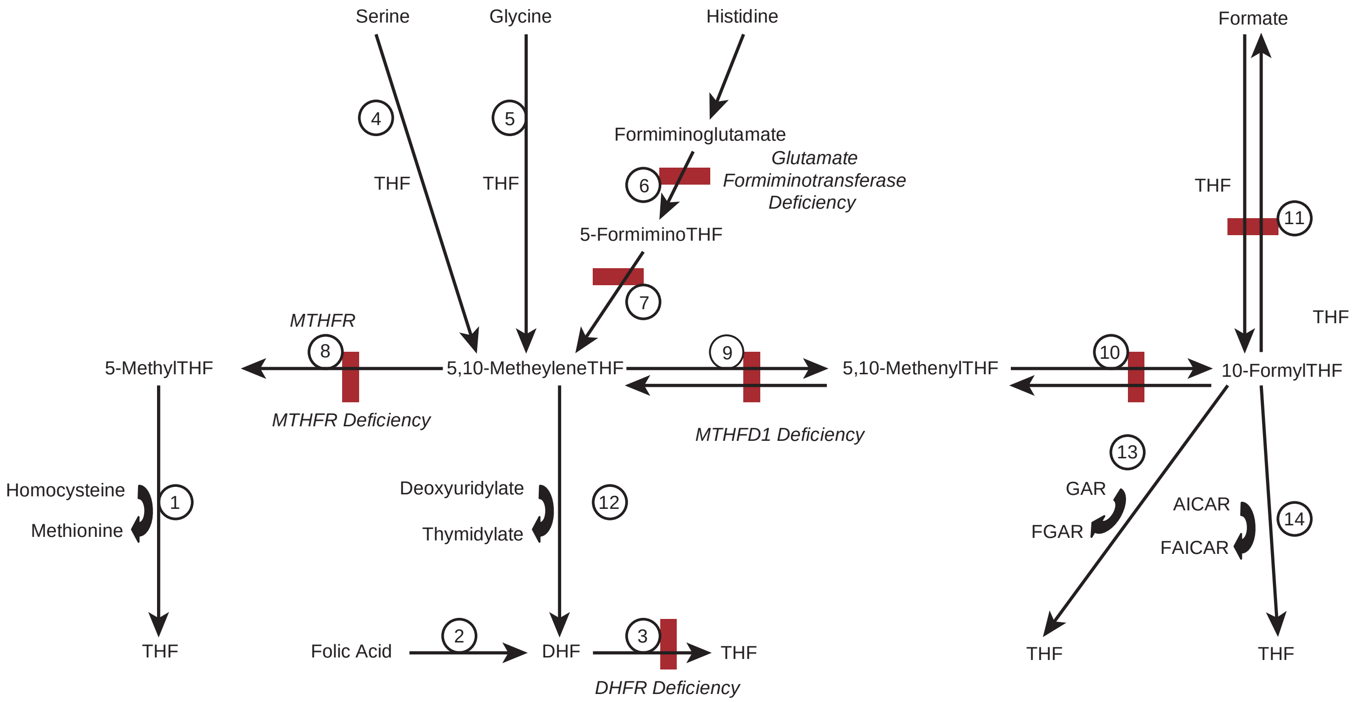

Figure 10-2 Cellular folate metabolism. Sources of one-carbon units are shown in the upper part of the figure, and reactions that consume one-carbon units are shown in the lower part. Steps blocked in the four confirmed disorders of folate metabolism are shown by *red bars;* note that glutamate formiminotransferase deficiency affects both glutamate formiminotransferase (reaction 6) and formiminotetrahydrofolate cyclodeaminase (reaction 7) activities, which are carried on a single protein; and MTHFD1 deficiency affects methyleneTHF dehydrogenase (reaction 9), methenylTHF cyclohydrolase (reaction 10), and formylTHF synthetase (reaction 11). Reactions are numbered as in the text. *1,* Methionine synthase; *2* and *3,* dihydrofolate reductase; *4,* serine hydroxymethyltransferase; *5,* the glycine cleavage system; *6,* glutamate formiminotransferase; *7,* formiminoTHF cyclodeaminase; *8,* methyleneTHF reductase; *9,* methyleneTHF dehydrogenase; *10,* methenylTHF cyclohydrolase; *11,* formylTHF synthetase; *12,* thymidylate synthase; *13,* GAR transformylase; *14,* AICAR transformylase.

generate 5,10-methyleneTHF, and breakdown of histidine, which generates 5,10-methenylTHF (reactions 6 and 7). ATP-dependent condensation of formate with THF to form 10-formylTHF is catalyzed by methylTHF synthetase (reaction 11). Once one-carbon units have been attached, the various forms can be interconverted to ensure that the cell has the folate derivatives required for metabolism. 5,10-MethyleneTHF can be reduced to form 5-methylTHF by the enzyme methylenetetrahydrofolate reductase (MTHFR; reaction 8). Alternatively, it can be oxidized to form 5,10-methyleneTHF and 10-formylTHF by the multifunctional enzymes encoded by *MTHFD1* in the cytoplasm or *MTHFD2/MTHFD2L* in mitochondria (reactions 9 and 10).

One-carbon substituted forms of cobalamin are required for several critical steps in metabolism.

1. Methylation of homocysteine to form methionine is catalyzed by methionine synthase and utilizes 5-methylTHF as methyl group donor (see Fig. 10-2, reaction 1); this reaction also requires cobalamin and S-adenomethionine to function and represents the point in cellular metabolism where folate and cobalamin metabolism interact. Methionine is not only required for protein synthesis; it can also be converted to S-adenosylmethionine, which is the methyl group donor in a large number of transmethylation reactions that are involved in DNA and RNA methylation and in neurotransmitter synthesis, among other processes.
2. Conversion of deoxyuridylate to deoxythymidylate, the final step in thymidine synthesis, utilizes 5,10-methyleneTHF as its one-carbon unit donor (see Fig. 10-2, reaction 12). This reaction generates DHF, which must be reduced to THF before it can be used again as a one-carbon unit recipient. This reduction is accomplished by DHF reductase (see Fig. 10-2, reaction 3).
3. Two separate steps in de novo purine biosynthesis use 10-formylTHF as a one-carbon donor, generating carbons 2 and 8 of the purine ring structure (see Fig. 10-2, reactions 13 and 14).

Folates and Arteriosclerotic Vascular Disease

Homocysteine was first implicated as an atherogenic factor by the observation of premature arteriosclerotic disease in children with severe hyperhomocysteinemia due to inborn errors of metabolism.[37] It was subsequently suggested that moderate elevations of tHcy were associated with development of atherosclerotic disease in adults. A common thermolabile variant of MTHFR, c.677C→T (p.A222V), results in an approximately 50% decrease in enzyme activity when present in the homozygous state[70] and in elevated tHcy in the presence of low folate concentration.[71] Many observational studies showed an association between elevated tHcy and cardiovascular disease and a modest, but statistically significant, increased risk for cardiovascular disease in patients with the T allele.[72] However, suggestions that lowering tHcy might improve cardiovascular outcomes in patients have not been supported in several large epidemiologic studies.[73-75]

Folates and Neural Tube Defects and Other Birth Defects

As noted previously, folate supplementation during the periconceptional period (from initiation of pregnancy to closure of the neural tube at about 1 month of gestation) reduces the occurrence and recurrence[17,18] of NTDs, leading to fortification of flour and other enriched grain products with folic acid in the United States and other countries. This has been associated with a decrease in prevalence of NTDs.[76-80] Studies have also suggested a role for folate supplementation in preventing nonsyndromic cleft lip with or without cleft palate[81-83] and congenital heart defects,[84,85] but the evidence for these associations is not as strong.[86-88]

Folates and Cancer

Folate status appears to have an inverse relationship with the risk for cancers of various types. However, epidemiologic studies have also suggested an association between higher folate status and progression of premalignant lesions in several tissues,[89-91] and increased folate concentrations have been associated with growth of some malignant cells.[92] The effects of folate have been most thoroughly investigated in colorectal cancer, where results of meta-analysis were consistent with a small protective effect of folate against risk for cancer.[93] There is less convincing evidence for a protective role for folate against cancers of the oropharynx, esophagus, stomach, pancreas, lungs, cervix, ovary, and breast as well as for neuroblastoma and leukemia.[92] A transient increase in the rate of colorectal cancer in the United States and Canada following the start of folic acid fortification of grains may reflect the effect of increased folate concentration on proliferation of existing adenomas, however.[94] Meta-analyses of studies of folic acid supplementation (at levels tenfold higher than those used in folic acid fortification of grain) showed no effect on the rate of cancer.[73,95]

Folate Antimetabolites

Antifolates are analogues of folic acid that block folate metabolism, interrupting DNA synthesis and causing cell death. Methotrexate, a potent inhibitor of mammalian DHF reductase, is effective in combination chemotherapy of carcinomas of the breast, lung, bone, ovary, head and neck, and elsewhere and contributes to cure of choriocarcinoma and childhood acute lymphoblastic leukemia. It is also used for its immunosuppressive and antiproliferative properties in the treatment of psoriasis, rheumatoid arthritis, and graft-versus-host disease after bone marrow transplantation. Newer antifolates have been synthesized and tested over the past 40 years. Some are discussed in Chapter 46, "Chemotherapy in the Pediatric Cancer Patient."

Pyrimethamine is an antimalarial drug with greater affinity for parasitic than human DHF reductase. Trimethoprim is an antifolate with antibacterial activity and a higher affinity for bacterial than human DHF reductase. In combination with sulfisoxazole, which interferes with bacterial synthesis of folic acid, there is a double synergistic antifolate attack on bacteria. Such combinations are often used to treat urinary tract and other infections and as prophylaxis against pneumonia resulting from

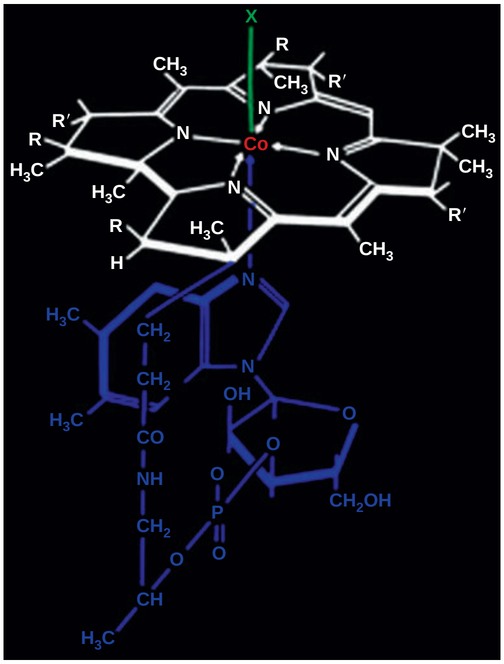

Figure 10-3 Structure of cobalamin. Cobalamin (vitamin B_{12}, Cbl) consists of a planar corrin ring *(black)* that coordinates with a central cobalt atom *(red)*, with a 5,6-dimethylbenzimidazole base *(blue)* covalently attached to the corrin ring and coordinating with the cobalt atom. X *(green)* represents the upper axial ligand, which varies in different biologic and pharmacologic cobalamins. When X is methyl, the compound is methylcobalamin (MeCbl); when it is adenosyl, the compound is 5-deoxyadenosylcobalamin (AdoCbl); when it is CN, the compound is cyanocobalamin (CNCbl); and when it is OH, the compound is hydroxocobalamin (OHCbl).

infection with *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*) in immunocompromised patients.

COBALAMIN (VITAMIN B_{12})

Chemistry of Cobalamins

Cobalamins, members of the class of molecules called corrins, have the chemical structure shown in Figure 10-3. They consist of a planar corrin ring, similar to but distinct from that of porphyrins such as heme, with a central cobalt atom that coordinates with four nitrogen atoms of the corrin ring. A nucleotide, 5,6-dimethylbenzimidazole, coordinates with the cobalt atom below the plane of the corrin ring and attaches covalently as well to the corrin ring. The central cobalt atom may exist in three different oxidation states: the oxidized trivalent state (cob[III]alamin), the divalent state (cob[II]alamin), and the fully reduced monovalent state (cob[I]alamin). Several different groups may be coordinated to the cobalt atom in the upper axial position (position "X" in Fig. 10-3) when it is in the cob(III)alamin state. Corrins that have coenzyme activity or can be converted to coenzyme forms within the cell include cobalamins with cyanide (cyanocobalamin [CNCbl]), the hydroxyl radical (hydroxocobalamin [OHCbl]), the methyl radical (methylcobalamin [MeCbl]), or 5′-deoxyadenosyl (adenosylcobalamin [AdoCbl]) bound in the upper axial position, as well as cob(II)alamin and cob(I)alamin.[96,97] Under certain conditions, for example at acidic pH or when bound to certain proteins, cobalamin can exist in a "base off" conformation, in which the dimethylbenzimidazole base no longer coordinates with the central cobalt atom.

Cobalamin Sources, Requirements, and Stores

Nutritional Sources and Requirements

Although prokaryotes, animals, and many algae require cobalamin for metabolism, only prokaryotes synthesize it.[98] Humans depend on microbial cobalamin, usually obtained second-hand through consumed animal-derived products, for their cobalamin requirement.[16,99,100] Multicellular plants or fungi do not synthesize or accumulate cobalamin and thus do not contribute it to the diet. Many prokaryotes synthesize and utilize corrinoids other than cobalamin, containing a lower axial ligand other than dimethylbenzimidazole or lacking a lower ligand entirely that cannot be utilized by humans.[101] The human cobalamin transport system largely excludes these analogues from internalization by cells.

The estimated daily cobalamin loss is 1 μg in adults,[99] with wide individual variations. Much of the loss is biliary.[102] Estimates of daily intake requirement have varied, in part because of different end points for sufficiency. The Institute of Medicine[99] reviewed available dietary data to set requirements for children of all ages, adults, and pregnant and lactating women (Table 10-2). The recommended daily intake of cobalamin for adults is set at 2.4 μg/day, on the assumption that the expected 50% absorption rate of such an amount from food will replace the estimated daily losses while leaving a cushion for individual variability. A subsequent Scandinavian study of adults found that metabolic status leveled off with ascending daily intakes that approximated 4.2 μg or even 6 to 7 μg,[103,104,105] but interpretations should be

TABLE 10-2 Recommended Intakes of Cobalamin Throughout Life

	Age Group	Recommended Daily Intake* (μg Cobalamin)
Infancy	0-6 mo 7-12 mo	0.4 0.5
Childhood	1-3 yr 4-8 yr 9-13 yr 14-18 yr	0.9 1.2 1.8 2.4
Adulthood	19-50 yr	2.4
Pregnancy		2.6
Lactation		2.8

Data from the recommendations of the Institute of Medicine review and analysis of dietary reference intakes.[99]

*The amounts reflect recommended dietary allowances, which are derived from estimated average requirements by adding twice the latter's coefficient of variation in order to include individuals whose requirements may lie at the extremes. For infants, however, the shown values represent adequate intake, based on evaluation of published data on breastmilk intakes that appeared to provide normal cobalamin status.

tempered by the limitations of the short-term dietary assessments for cobalamin, the biomarker end points, and the study populations (e.g., a striking overrepresentation of vegetarians in one study).

Analysis of NHANES data from 2003 through 2006 showed that median daily cobalamin intake was 6.4 µg (95% confidence interval [CI], 4.7 to 8.6 µg) in males and 5.0 µg (95% CI, 3.7 to 6.9 µg) in females.[24] It is useful to note that the daily intakes reported by NHANES[24] exceeded the Institute of Medicine's recommendations in children (see Table 10-2). The intakes were 5.1 µg at ages 1 to 3 years, 6.1 µg at 4 to 8 years, 5.6 µg at 9 to 13 years, and 5.9 µg at 13 to 19 years. The 95% confidence intervals for these intakes exceeded the Institute's estimated requirements, and the children's 95% confidence levels for methylmalonic acid (MMA) levels were within the expected reference ranges.[24] Data were unavailable for infants.

Foods vary widely in cobalamin content.[100] Liver and some seafood are among the richest in cobalamin but are not eaten often, beef has relatively rich cobalamin content, and cobalamin content of eggs and milk is smaller. However, bioavailability can vary as much as tenfold among different food sources,[100] and food cobalamin content, whose measurement can be imprecise, does not always predict effectiveness. Thus cow's milk and fortified cereal, as well as fish, proved to be much more efficient providers of cobalamin than meat, despite the latter's high cobalamin content.[100,104,106] The specific receptor-mediated absorption of cobalamin via IF allows efficient absorption of more than 50% of this micronutrient while also excluding unutilizable corrinoid analogues. However, this mechanism is also easily saturable, and when more than 1 to 2 µg is ingested in a meal, much of the excess is relegated to nonspecific diffusion in which absorptive bioavailability falls to approximately 1%.[16,107] The implications of such a small margin between the desirable level of intake and the total capacity of receptor-specific absorption have not been explored sufficiently. However, the existence of haptocorrin, a ubiquitous, relatively abundant, high-affinity cobalamin-binding protein that does not mediate specific delivery to intestinal cells or other tissue, raises the obvious question whether such an upper limit on cobalamin internalization serves a positive but still undefined biologic purpose, perhaps tied to corrin analogue exclusion from cells.

Cobalamin Requirement in Infancy. Most of the information on cobalamin physiology and economy has been obtained in adults, which may apply to older children and adolescents also. However, data are limited in newborns and infants, who may differ considerably. Current estimates of the cobalamin requirement for infants are based on the 0.3 to 0.4 µg of cobalamin provided daily in breastmilk from mothers with apparently normal cobalamin status.[16,99] However, recent data suggest that breastmilk may be a poor model.[108] Cow's milk contains not only much more cobalamin[16,109] but its cobalamin is also carried by transcobalamin and may therefore be more bioavailable than the haptocorrin-bound cobalamin of human breastmilk.[109]

Cobalamin Stores

Estimates of stores vary with assessment methods and subject selection. The current consensus is that cobalamin contents in adults average 2 to 3 mg, with wide spreads.[99] The liver contains approximately 1.5 mg of those stores, with concentration ranges of 0.7 to 1.5 µg/g.[16] By comparison, liver concentration is 0.02 to 0.14 µg/g in patients with megaloblastic anemia caused by pernicious anemia, which is a reduction of more than 80%. Store estimates are scarce in children. Maternal-fetal cobalamin transfer favors the fetus even when maternal stores are limited, and cord blood cobalamin levels exceed maternal serum levels, 370 versus 240 ng/L,[99] although maternal serum cobalamin levels tend to be spuriously low during pregnancy. Full-term newborns have a mean liver cobalamin content of 27.3 µg[110]; the total liver content is only 10.7 µg in premature newborns and 4.1 µg in the fetus. The total body cobalamin store in newborns is approximately 50 µg, which illustrates how greatly stores need to expand between infancy and adulthood. As will be discussed, the approximately 1000 : 1 stores-to-daily intake ratio in adults and adolescents may be only 100 : 1 in newborns.

Physiology of Cobalamin

Cobalamin-Binding Proteins

Cobalamin does not exist free in the body outside the gastrointestinal tract, where its carrier-free existence is transient. Cobalamin traffic, transport, and access to cells depend on three cobalamin-binding proteins: IF, transcobalamin, and haptocorrin. Only IF and transcobalamin interact with specific cell receptors, allowing efficient cellular internalization of cobalamin at low concentrations, but these systems have relatively low capacity.[107] These binding proteins have similar primary sequences, and their genes have similar intron/exon structure, suggesting that they arose by duplications of an ancestral gene.[111,112] They show 33% overall homology with each other, and specific regions demonstrate as much as 60% to 80% identity.[113]

IF is a 45-kD glycoprotein synthesized by gastric parietal cells in humans; under some circumstances, ectopic IF synthesis may also occur in chief cells, which are the normal synthetic site in many other mammals.[114] IF mediates efficient uptake of cobalamin in the intestine. It binds cobalamins less tightly than does transcobalamin or haptocorrin[115] but has far greater binding affinity for cobalamins than for nonfunctional corrin analogues, which helps limit the analogues' intestinal absorption. IF is encoded by the *GIF* gene on chromosome 11q13.[116]

Transcobalamin (also called transcobalamin II) is synthesized by endothelial and other cells, and mediates entry of cobalamin into most types of peripheral cells.[117] It is a nonglycosylated protein with a molecular weight of 43 kD[118] that, like IF, binds cobalamin tightly but has low affinity for cobalamin analogues. Transcobalamin content is small in many secretions.[117] The *TCN2* gene is on chromosome 22q12-13.[112]

Haptocorrin (also called transcobalamin I) differs from IF and transcobalamin in having a high affinity for nonfunctional corrinoid analogues as well as for cobalamin and in having no specific cellular receptors for its internalization. These characteristics suggest an important role in excluding analogues, and perhaps excess cobalamin, from cells. The protein, encoded by the *TCN1* gene on chromosome 11q12.1,[119] is approximately 46 kD, but its heavy glycosylation creates glycoproteins of different sizes, isoelectric points, and plasma clearance rates. The glycosylation variability, which is sometimes secretion specific, led to artificial distinctions and names for haptocorrin variants (e.g., transcobalamin III and R binder), which are no longer used. Haptocorrin is synthesized in myeloid precursors at early stages when specific granules form[120]; this appears to be the chief source of plasma haptocorrin. Epithelial or ductal cells of exocrine glands also synthesize haptocorrin and are its source in secretions, where haptocorrin achieves concentrations much higher than in plasma.[117,121,122] The secretions include bile, saliva, tears, breastmilk, bronchial secretions, amniotic fluid, and seminal fluid[117]; and their high haptocorrin content suggests a still unspecified role in relation to the gut microbiome and its abundant content of cobalamin and corrinoid analogues.

Typically, more than 70% of the cobalamin in plasma at any moment exists bound to haptocorrin, but this seeming preponderance is misleading because haptocorrin is cleared slowly from plasma ($t\frac{1}{2}$, 9.5 to 12 days), whereas transcobalamin ($t\frac{1}{2}$, only 90 minutes) carries 40 times as much cobalamin daily in plasma as haptocorrin does. Study of haptocorrin interactions with each of the many corrinoid analogs has been difficult, but levels of plasma analogues, whose sources are unknown, approach or exceed cobalamin levels.[123-125]

Absorption of Cobalamin

Cobalamin in food is almost entirely protein bound. It is released by the action of acid and pepsin in the stomach, where it binds to salivary or gastric haptocorrin in preference to IF when gastric pH is low (Fig. 10-4, *1*). In the duodenum, the haptocorrin-cobalamin complex from the stomach, and from bile, is broken down by pancreatic proteases, releasing cobalamin, which subsequently binds to IF (Fig. 10-4, *2*).[126] The IF-cobalamin complex binds to receptors in the apical brush border of enterocytes; the receptors are most abundant in the distal ileum (Fig. 10-4, *3*). The absorbed fraction varies inversely with the amount of ingested cobalamin. Absorption efficiency is more than 50% when the amount is 1 to 2 μg, which approximates some meal contents. High intake in a meal or supplement (e.g., 5 to 10 μg) exceeds the capacity of IF receptors and the excess free cobalamin is internalized only by nonspecific diffusion, a process with only approximately 1% efficiency.

The ileal receptor consists of two proteins, cubilin (the product of the *CUBN* gene on chromosome 10p12.1) and amnionless (the product of the *AMN* gene on chromosome 14q32), and has been named “cubam.”[127] Cubilin, a 460-kD protein that binds many ligands other than IF, such as transferrin and vitamin D–binding protein,[128] is anchored externally to the enterocyte membrane by amnionless, a 48-kD membrane-spanning protein.[129] Both components must be synthesized for cubam to be expressed at the cell surface.[130,131]

The IF-cobalamin complex is rapidly internalized by endocytosis after binding to cubam.[132] Cobalamin bound to transcobalamin (holotranscobalamin [holo-TC]) appears in the portal circulation 4 hours after ingestion. Although some studies have suggested that attachment of cobalamin to transcobalamin occurs within enterocytes after its release from IF,[133,134] studies in transgenic mice were consistent with export of free cobalamin from enterocytes by the ABCC1 (MRP1) membrane transporter with subsequent attachment to transcobalamin outside the enterocyte.[135]

Enterohepatic Recirculation

Approximately 1.5 μg of cobalamin and varying amounts of cobalamin analogues bound to haptocorrin are excreted in the bile daily.[102] Cross-species experiments in rabbits suggest that plasma haptocorrin uptake by asialoglycoprotein receptors on hepatocytes delivers cobalamin and analogues to bile,[136] but reservations exist and direct data are unavailable. Pancreatic proteases degrade the biliary haptocorrin (see Fig 10-4, *2*). Released cobalamin binds to IF and is reabsorbed in the ileum, whereas released analogues, unavailable to IF, are excreted in the feces.[137] This process salvages about two thirds of the cobalamin secreted in bile.[102]

Renal Cobalamin Reabsorption

The amount of cobalamin filtered daily is approximately 1.5 μg.[138] Little cobalamin is detected in urine under normal physiologic conditions because more than 99.8% is reabsorbed in the proximal tubules.[139] Cubilin and a second large multiligand receptor, megalin, are expressed in the proximal renal tubule,[140,141] where they reabsorb a variety of filtered proteins. Filtered holo-TC is reabsorbed by megalin.[64] Unbound cobalamin does not normally exist in plasma, but if it is introduced by parenteral injection, the filtered free cobalamin cannot be reabsorbed in the proximal tubule.

Cobalamin in Pregnancy

Cobalamin is efficiently transported across the placenta during pregnancy, and cobalamin concentration is higher in cord blood than in the maternal circulation, even in cases of maternal cobalamin insufficiency.[142-145] The mechanism of cobalamin transport remains poorly understood. Both cubilin and amnionless are expressed in the visceral yolk sac of rodents,[129,146] where they support uptake of nutrients (although not cobalamin).[146] It is not clear that the visceral yolk sac plays any role in nutrition of the postimplantation embryo in humans.[147] The transcobalamin receptor is present in human placenta, but its role in transplacental cobalamin uptake has not been studied.[148]

Biochemistry of Cobalamin

Cellular Cobalamin Metabolism

Cobalamin functions as a coenzyme in two reactions in humans (Fig. 10-5). MeCbl is required for methylation

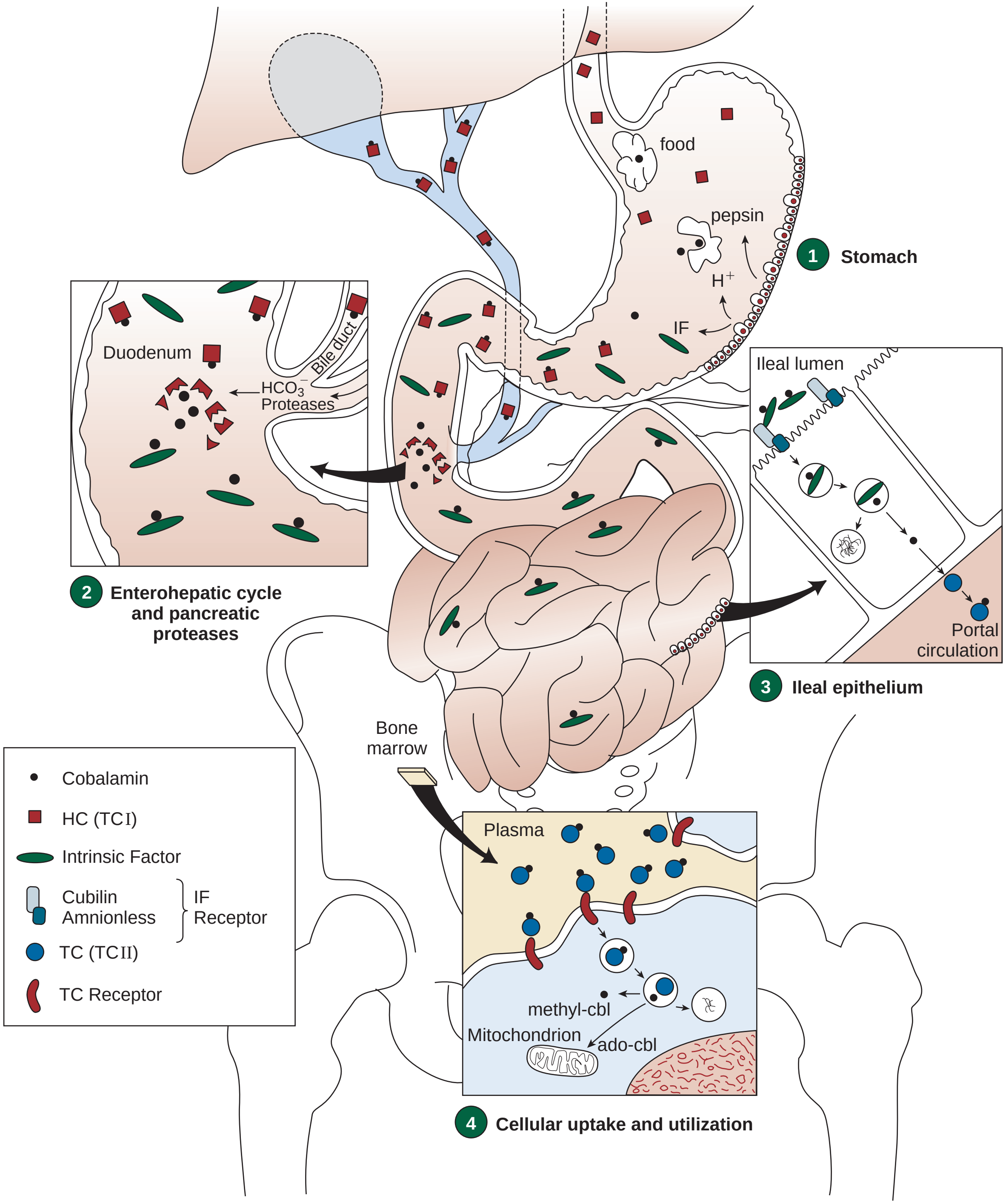
food
pepsin
H+
IF
1 Stomach
Duodenum
Bile duct
HCO3−
Proteases
2 Enterohepatic cycle and pancreatic proteases
Ileal lumen
Portal circulation
3 Ileal epithelium
Bone marrow
Plasma
methyl-cbl
Mitochondrion
ado-cbl
4 Cellular uptake and utilization
Cobalamin
HC (TC I)
Intrinsic Factor
Cubilin
Amnionless
IF Receptor
TC (TC II)
TC Receptor

Figure 10-4 The events of cobalamin absorption, transport, and internalization. The events at four key sites of cobalamin transport are shown: the stomach, the duodenum, the ileum, and cellular sites, such as the bone marrow. *ado-cbl,* 5′-Deoxyadenosylcobalamin; *HC,* haptocorrin; *IF,* intrinsic factor; *methyl-cbl,* methylcobalamin; *TC,* transcobalamin; *TC I,* transcobalamin I; *TC II,* transcobalamin II. *(Modified from Carmel R: Megaloblastic anemias: disorders of impaired DNA synthesis. In Greer JP, Arber DA, Glader B, et al, editors:* Wintrobe's clinical hematology, *13th ed. Philadelphia, 2013, Lippincott Williams & Wilkins, p. 927–953.)*

of homocysteine to form methionine, catalyzed by the cytoplasmic enzyme methionine synthase (EC 2.1.1.13). This reaction utilizes 5-methyltetrahydrofolate as methyl group donor and represents the point in cellular metabolism where cobalamin and folate metabolism intersect.[149] AdoCbl is required in the conversion of methylmalonyl-coenzyme A (CoA) to succinyl-CoA, which is catalyzed by the mitochondrial enzyme methylmalonyl-CoA mutase (EC 5.4.99.2).[150] In microorganisms, a variety of other enzymes utilize cobalamin for reactions that appear not to occur in mammalian cells.

Intracellular cobalamin concentrations are low, and many of the forms that are involved in metabolism are not stable in aqueous solution. This has led to the

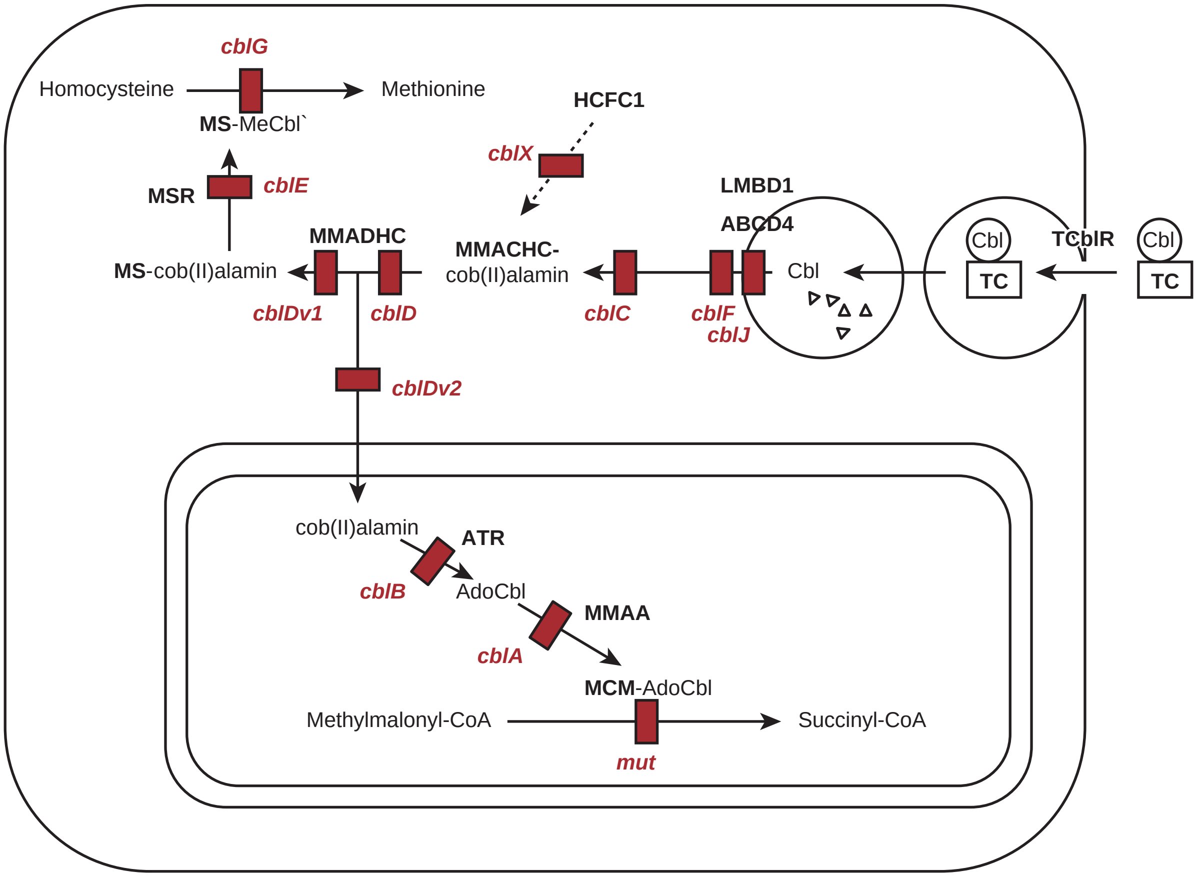

Figure 10-5 Scheme of cobalamin metabolism. The sites affected by methylmalonyl-CoA mutase deficiency *(mut)* and the inborn errors of cobalamin metabolism (*cblA-cblG, cblJ,* and *cblX*) are shown in *red*. The cblA disorder is caused by defects in the MMAA protein; the cblB disorder is caused by defects in the cob(I)alamin adenosyltransferase *(MMAB)* protein; the cblC disorder is caused by defects in the MMACHC protein; the cblD, cblD variant 1 *(cblDv1)*, and cblD variant 2 *(cblDv2)* disorders are caused by defects in the MMADHC protein; cblE disorder is caused by defects in the methionine synthase reductase *(MSR)* protein; cblG disorder is caused by defects in the methionine synthase *(MS)* protein; cblJ disorder is caused by mutations in the ABCD4 protein; cblX disorder is caused by mutations in the HCFC1 protein, and the mut disorder is caused by defects in methylmalonyl-CoA mutase *(MCM)*. The protein affected in the cblF disorder is unknown. *MCM-AdoCbl,* Holomethylmalonyl-CoA mutase (mutase with bound adenosylcobalamin); *MS-cob(II)alamin,* methionine synthase with bound cob(II)alamin; *MS-MeCbl,* holomethionine synthase (synthase with bound methylcobalamin; *TC,* transcobalamin.

suggestion that once cobalamin enters cellular metabolism it must be bound to protein at all times. Most cellular cobalamin exists bound to proteins, primarily methionine synthase in the cytoplasm and methylmalonyl-CoA mutase in the mitochondria.[151] It appears that cobalamin is not retained in cells unless it is bound to protein.

Uptake of circulating holo-TC is mediated by the transcobalamin receptor, which initiates endocytosis (see Figs. 10-4 and 10-5).[148] Transcobalamin is degraded in lysosomes, releasing cobalamin that is transferred across the lysosomal membrane by a process involving the lysosomal membrane proteins LMBD1 and ABCD4.[152,153] Once in the cytoplasm, cobalamin becomes associated with the molecular chaperone protein MMACHC, which catalyzes reduction of the central cobalt to the Co^{2+} state and removal of the upper axial ligand, if one is present.[154-156] Subsequently cobalamin may enter the mitochondria, where cobalamin is converted to AdoCbl by cobalamin adenosyltransferase and becomes associated with methylmalonyl-CoA mutase; a third protein, MMAA, is required for transfer of AdoCbl from adenosyltransferase to methylmalonyl-CoA mutase. Alternatively it can remain in the cytoplasm, where it becomes associated with methionine synthase and is converted to MeCbl.

Biochemical Effects of Cobalamin Deficiency

The biochemical consequences of cobalamin deficiency result from decreased activity of the cobalamin-dependent enzymes methionine synthase and methylmalonyl-CoA mutase. Decreased activity of methionine synthase results in elevated levels of its substrate, homocysteine, in the blood and excretion of homocystine in the urine. Decreased activity of methylmalonyl-CoA mutase results in accumulation of MMA in the blood and urine.

Methionine Metabolism

Methionine is an essential amino acid in humans. Methionine synthase remethylates the homocysteine generated from methionine during cellular metabolism, helping to maintain adequate methionine levels as well as preventing accumulation of potentially toxic homocysteine.

A cobalamin-independent enzyme, betaine : homocysteine methyltransferase, catalyzes homocysteine remethylation in liver,[157] but its activity is insufficient to prevent homocysteine accumulation when cobalamin-dependent methionine synthase activity is impaired. Methionine levels, however, often remain normal in cobalamin deficiency.

Cellular methionine may be utilized in protein synthesis or adenosylated to form S-adenosylmethionine, which acts as a methyl group donor in several dozen different transmethylation reactions, including methylation of DNA, which plays a role in regulation of gene expression, as well as methylation of RNA, lipids, proteins (including histones), neurotransmitters, and thiols.[158] These reactions generate the homocysteine that is acted on by methionine synthase. Homocysteine may also react with serine to form cystathionine, in a reaction catalyzed by cystathionine β-synthase.

Interaction of Folate and Cobalamin Metabolism

Deficiency of either folate or cobalamin, when substantial, produces megaloblastic anemia. The mechanisms leading to the anemia are complex. Folate deficiency directly affects the one-carbon substituted THF derivatives required for de novo thymidine and purine biosynthesis, resulting in abnormal DNA synthesis in bone marrow cells. The explanation for the hematologic similarity in cobalamin deficiency is believed to be the result of a secondary deficiency of these THF derivatives, best explained by the "methyl trap" hypothesis.[159,160] Because methionine synthase is the only reaction in human cells that can convert 5-methylTHF to THF, which can be utilized to form one-carbon substituted THF derivatives, and because the reaction that converts 5,10-methyleneTHF to 5-methyTHF is irreversible under physiologic conditions, cobalamin deficiency results in "trapping" of folate in the form of now unusable 5-methylTHF.[159,160]

MANIFESTATIONS OF DEFICIENCY

Overview of Folate and Cobalamin Deficiencies

Cobalamin and folate deficiencies can cause similar clinical manifestations. The most frequent manifestation, megaloblastic anemia, is identical in both deficiencies and is therefore described here for both. Indeed, the megaloblastic anemia caused by cobalamin deficiency may respond initially if folic acid is mistakenly given, especially in doses above the bare minimum,[161,162] although the hematologic improvement is often only partial and is rarely permanent. In contrast, the neurologic manifestations differ for the two vitamin deficiencies in adults, although cerebral and developmental consequences can be similar in infants with either deficiency. They will be discussed separately in sections devoted to each vitamin. Finally, the disorders causing each vitamin deficiency are very different. For all these reasons, rigorous differentiation between the two vitamin deficiencies must precede selection of the treatment.

Megaloblastic Anemia of Folate and Cobalamin Deficiencies

Three key features distinguish megaloblastic anemia from other anemias. First, there are the distinctive morphologic changes. Cells are not only macrocytic but their nuclei are also often larger. More specifically, a maturational nuclear-cytoplasmic dissociation defines megaloblastosis: the retarded chromatin condensation imparts an unusually immature, dispersed appearance to the nucleus, whereas the cytoplasm appears unremarkable (Fig. 10-6). Myeloid cells have megaloblastic nuclei that can be abnormally shaped and too large for their already large cells ("giant" metamyelocytes and band cells). Megaloblastic changes in mature neutrophils are characterized by nuclear hypersegmentation, which is usually defined by the presence of at least one six-lobed nucleus or suspected when several five-lobed neutrophils are seen in a blood smear. Lobe-count averages above 3.0 to 3.3 lobes per neutrophil also suggest megaloblastosis, but lobe counts are tedious and their subjectivity requires that each observer establishes a personal normal range. When florid, megaloblastic changes are dramatic and obvious in peripheral blood and marrow (see Fig. 10-6) but mild changes in the early stages of deficiency are easily missed.

Second, megaloblastic anemia is the prototype of ineffective hematopoiesis. Precursors are actively generated in the bone marrow, but they are defective. As the dyspoiesis worsens, precursors increasingly undergo cell death in the bone marrow and are phagocytosed before they can exit into the blood as reticulocytes. As erythropoietin secretion increases, the bone marrow becomes more hypercellular, but the reticulocyte count fails to increase. Evidence of the intense intramedullary cell death includes increases in markers of cell turnover, such as serum lactate dehydrogenase, and the consequences of increased hemoglobin breakdown, such as indirect hyperbilirubinemia and decreased or absent haptoglobin. The decreased survival of megaloblastic RBCs in the blood adds an element of peripheral hemolysis.

Finally, the megaloblastic process affects platelets and myeloid cells also. The anemia is the earliest and, for some time, the only cytopenia. However, as the megaloblastic anemia progresses, granulocyte and platelet counts decline, and pancytopenia can eventually mimic aplastic anemia. In rare cases, an erythroblastopenia mimicking pure red cell aplasia may be superimposed.[163,164]

Incidence and Evolution of Megaloblastic Anemia

Although the megaloblastic anemia is identical in folate and cobalamin deficiencies, its appearance after the dietary or malabsorptive onset of vitamin depletion in adults is measured in 2 to 3 months for folate deficiency[9] but in years for cobalamin deficiency[16] because of their disparate stores-to-daily intake ratios. The time course has not been documented as well in children, and onset may occur sooner in infants because of their smaller vitamin stores.

The earliest RBC changes are a rise in mean corpuscular hemoglobin followed shortly by a rise in mean corpuscular volume (MCV). These cell content and size changes precede the overt evidence of anemia,[9,165] in part because the early phases of the anemia involve declining RBC count but little change in blood hemoglobin and packed cell volume levels, which are compensated by the increasing RBC sizes and contents. Clinicians must pay

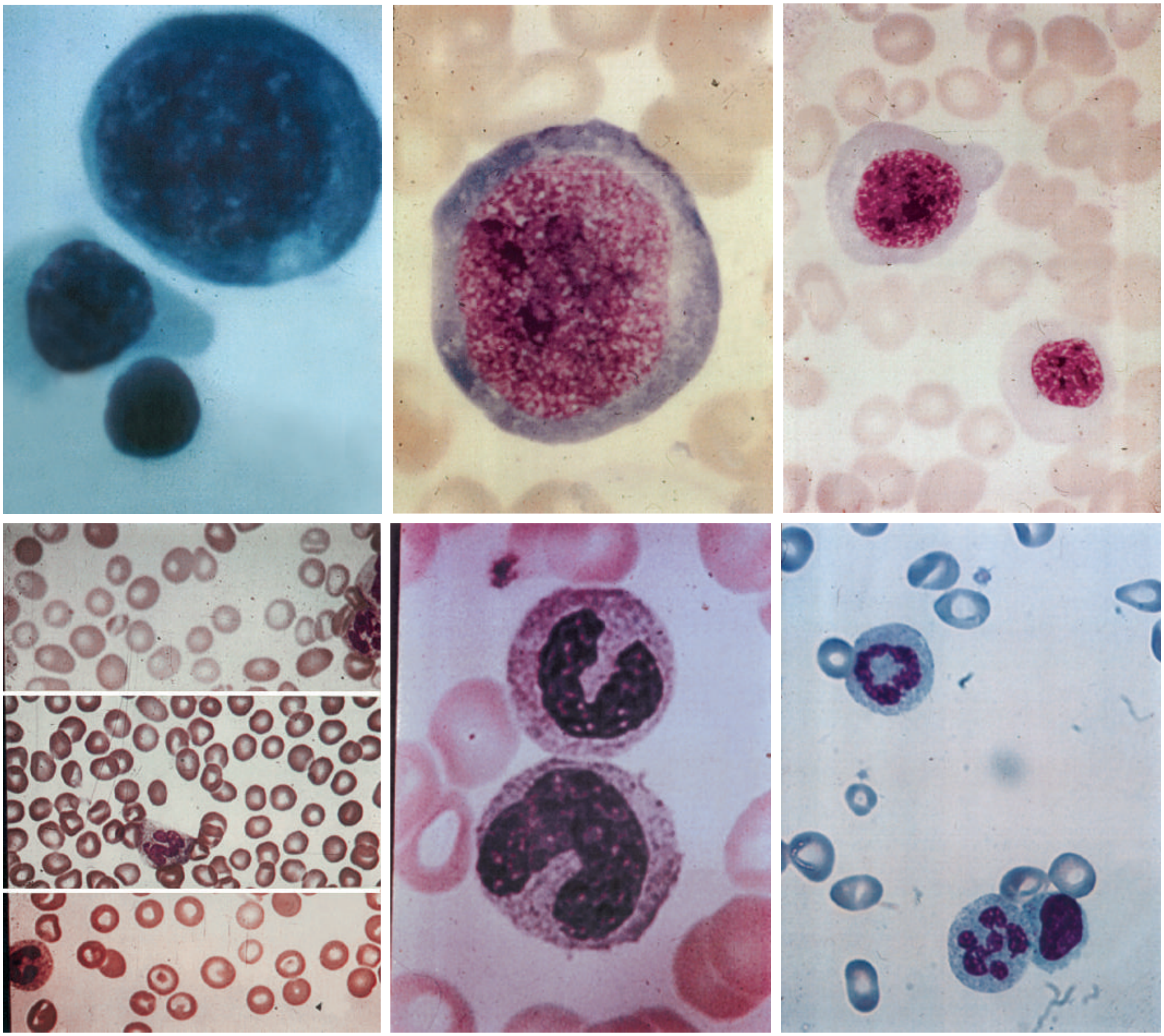

Figure 10-6 Megaloblastic changes in precursor cells in the bone marrow and in mature cells in the peripheral blood, compared with normal cells. *Top left,* Pronormoblast and two late normoblasts. *Top center,* Megaloblastic equivalent of pronormoblast in first panel. *Top right,* Megaloblastic equivalents of the two normoblasts in the first panel. *Bottom right,* Macrocytes, some of them macroovalocytes, in peripheral blood from a patient with megaloblastic anemia *(top),* and macrocytes that are uniform in size and shape, without ovalocytes, in a patient with chronic liver disease *(bottom),* can be compared with the normocytes from a healthy person *(middle). Bottom center,* Normal band cell *(top)* and the larger megaloblastic band cell with more dispersed chromatin *(bottom). Bottom right,* Hypersegmented neutrophil (at 6 o'clock position). *(Modified from Carmel R: Folate deficiency. In Carmel R, Jacobsen DW, editors:* Homocysteine in health and disease. *Cambridge UK, 2001, Cambridge University Press, p. 271–288.)*

attention to changes in RBC indices regardless of hemoglobin and packed cell values. Although macrocytosis has many causes (Table 10-3), early megaloblastic anemia must be considered whenever a patient's MCV, even if still within the normal reference interval, has changed as little as 3 to 5 fL compared with a previous value.[165] The onset of macrocytosis can be documented even sooner by reticulocyte-specific indices of macrocytosis.[166] However, macrocytosis is sometimes masked if a microcytic disorder, most often iron deficiency or thalassemia trait, coexists. These combinations can produce normal, low, or high MCV, but the MCV is always higher than normal for that particular patient and it always decreases after the megaloblastic anemia is treated. The frequency of nonelevated MCV was 7% in one study of adults with pernicious anemia.[167] The macrocytosis typically features macroovalocytes, which suggest but are not specific to megaloblastic anemia. Anisocytosis and admixture of macrocytes with normocytes elevate the relative distribution of RBC width. As megaloblastic anemia progresses,

TABLE 10-3 Causes of Red Blood Cell Macrocytosis

Causative Conditions	Accompanying Hematologic Features
Megaloblastic Anemia	
Cobalamin deficiency Folate deficiency Antifol drugs (e.g., methotrexate) Cytotoxic drugs (e.g., hydroxyurea; 5-FU) Immunosuppressive drugs (e.g., azathioprine) Thiamine-responsive anemia Hereditary orotic aciduria	Megaloblastic changes, including hypersegmented neutrophils Macrocytosis can become severe Mild reticulocytopenia Pancytopenia (when the megaloblastic process is severe)
Disorders of Erythroid Production	
Aplastic anemia; PRCA; Blackfan-Diamond anemia Some sideroblastic anemias CDA; non-CDA dyserythropoiesis; Fanconi anemia Myelodysplasia; myeloproliferative diseases	Nonmegaloblastic Some disorders feature dyserythropoiesis and sometimes hyposegmented neutrophils Macrocytosis can often be severe (e.g., aplastic processes) Reticulocytopenia (often severe)
Reticulocytosis	
Chronic hemolytic anemia	Nonmegaloblastic; no hypersegmented neutrophils
Drugs and Toxins	
Alcohol abuse Some antiviral drugs (e.g., nucleoside RT inhibitors) Some anticonvulsant drugs	Mechanism of macrocytosis is often unknown No hypersegmented neutrophils
Nonhematologic Diseases	
Chronic liver diseases Hypothyroidism Copper deficiency	Nonmegaloblastic; no hypersegmented neutrophils Macrocytes are rarely oval
Artifacts	
RBC clumping by cold agglutinins; some warm RBC antibodies Severe hyperglycemia Hyponatremia	Nonmegaloblastic; no hypersegmented neutrophils Disparity between high MCV and normal morphologic examination

CDA, Congenital dyserythropoietic anemia; *MCV,* mean corpuscular volume; *PRCA,* pure red cell aplasia; *RBC,* red blood cells; *RT,* reverse transcriptase; *5-FU,* 5-fluorouracil.

poikilocytosis, including teardrop cells, becomes prominent. With severe anemia, circulating nucleated erythroid precursors, Cabot rings, and Howell Jolly bodies can appear.

The other early manifestation preceding anemia is nuclear hypersegmentation in neutrophils (see Fig. 10-6).[9,16,168] It is also a much more specific feature of megaloblastic change than is macrocytosis. Unexplained hypersegmentation[169] and other dysplastic granulocytic changes[170] may also follow high-dose corticosteroid treatment of idiopathic thrombocytopenic purpura, however. The mechanism of hypersegmentation is unknown.

Neutropenia and thrombocytopenia are late features, appearing only infrequently before the hemoglobin level decreases below 10 g/dL; cytopenias that accompany mild anemia usually have independent explanations. The pancytopenia can ultimately become severe enough to mimic severe aplastic anemia. The two can be differentiated by the serum lactate dehydrogenase elevation (often exceeding 1000 units) and indirect hyperbilirubinemia in megaloblastic anemia.

Macrocytic anemia, the most common clinical feature of cobalamin or folate deficiency in adults, can be mild but it affects more than 70% of patients with clinically expressed deficiency, such as in pernicious anemia[171,172]; comparable data are not available in children. The reason why some patients with pernicious anemia do not develop anemia, even if they may have mild megaloblastic changes in the bone marrow that often escape detection, is unknown. Interestingly, a roughly inverse relationship between the severity of the hematologic and neurologic expressions has been described[173,174] and the dominant expression tends to dominate again with relapses.[173,175,176] A comparison of predominantly hematologically versus neurologically affected patients showed the predominantly anemic patients to have lower plasma glutathione, S-adenosylmethionine, cysteine, and folate levels, whereas a higher cysteine level was the most significant predictor of neurologic dysfunction.[174]

Biochemistry of Megaloblastic Anemia

In deficiency of either folate or cobalamin, all dividing cells, especially bone marrow precursors and epithelial cells, show megaloblastic changes. MethyleneTHF, which mediates the methylation of deoxyuridylate to deoxythymidylate (see Fig. 10-2), is directly depleted in folate deficiency but can also be secondarily depleted in cobalamin deficiency, as best explained by the methylTHF trap hypothesis. Nevertheless, de novo thymidylate synthesis disruption, although necessary, may not by itself suffice to produce megaloblastic changes.[177-179]

The misincorporation of excess uracil into DNA may exceed the capacity of excision repair, leading to single-strand breaks as well as occasional double-strand breaks. The latter may help explain the nuclear defects.[180] Arrest at various stages of interphase occurs in megaloblastic precursors even while maturation proceeds,[181] but the details are still unclear.

Mistaken folic acid therapy for clinically expressed cobalamin deficiency can bypass the methylTHF trap and improve the megaloblastic anemia.[161,162] However, the response is often only partial and is usually impermanent. Concerns exist that neurologic manifestations may progress in the meantime and become irreversible as the cobalamin deficiency remains unrecognized and untreated. Systematic evidence is unavailable, and mistaken folic acid therapy may occasionally even improve neurologic status.[162] However, ethical constraints prevent prospective studies and it is agreed that folate treatment must be avoided in cobalamin deficiency.[16] These concerns prompted restriction of folic acid doses, as well as folic acid–fortified food intake, to 1 mg/day or less.

Ancillary Features of Megaloblastic Anemia

The ineffective hematopoiesis of megaloblastic anemia disrupts iron metabolism, raising serum iron, transferrin receptor, and ferritin levels, along with sideroblast counts and the ferritin content in erythroid precursors and macrophages.[182,183] These changes can occur even in the presence of a coexisting iron deficiency, which may remain hidden until the ineffective erythropoiesis is corrected.[16] Severe megaloblastic anemia also has a still unexplained element of intravascular hemolysis. Membrane protein changes, including spectrin, have been described in megaloblastic RBCs, but their impact is unknown.

Nonhematologic Manifestations of Cobalamin Deficiency

Neurologic Manifestations

Neurologic dysfunction is common, but its frequency is unclear because neurologic disease is not easily quantified, unlike the probably more common hematologic abnormalities. Whereas 13% to 27% of adults with pernicious anemia have had neurologic symptoms without anemia,[171,172] the proportion with anemia without neurologic abnormalities is higher.

The classic neurologic manifestations feature a symmetric myelopathy, largely limited to the posterior and lateral columns ("subacute combined degeneration") and neuropathy.[184,185] The myelopathy typically begins with sensory and proprioceptive changes in the feet and ascends at variable rates to involve hands and arms as deficiency progresses. If not caught early, the changes may become partially or completely irreversible and can evolve to spasticity, ataxia, and incontinence. Other manifestations include mental changes of varying severity (mild electroencephalographic abnormalities can occur even without overt symptoms), autonomic dysfunction, and, rarely, optic neuritis.[16,173,186-188]

In newborns and infants, deficiency and its manifestations appear sooner than in adults because of the smaller storage reserves, because hereditary defects of cellular metabolism often act more quickly and directly on tissues than dietary or malabsorptive disorders, and most especially because the developing nervous system is at greater risk than the mature nervous system. Often, the manifestations include impaired growth and development. Thus the neurologic changes common to older children and adults are overshadowed in infants by failure to thrive, poor brain growth, developmental delay, seizures, motor retardation, and movement disorders[189-192]; the movement disorders, which sometimes increase transiently after cobalamin therapy, can be mistaken for seizures.[193] The diagnostic connection to cobalamin deficiency is often diverted or delayed for months and, even in nonhereditary disorders, developmental and neurologic residua may persist long after therapy.[191]

The mechanisms responsible for the demyelination in the central nervous system, followed by axonal degeneration and gliosis, in clinical cobalamin deficiency, or for the nondemyelinative peripheral neuropathy, are unknown.[184,185] Still unproven etiologic hypotheses for neurologic dysfunction have included abnormal myelin lipid synthesis because of abnormal propionate metabolism, defective neurotransmitter metabolism, corrinoid analogue accumulation, and cytokine abnormalities. Evidence from the clinical patterns in diverse *cbl* mutations and other findings favors a central, but perhaps not sufficient, role for methionine synthase inactivation, which disrupts methionine metabolism and S-adenosylmethionine–dependent methylation reactions. However, folate deficiency, which also inactivates methionine synthase, rarely produces neurologic abnormalities of comparable severity to cobalamin deficiency in adults,[16] although hereditary disorders of folate metabolism in children often do.

Other Clinical Manifestations

Cobalamin deficiency can cause atrophic glossitis and stomatitis, which can be painful.[16] Somewhat similar changes occur in folate deficiency, but the oral changes respond only to the specific vitamin whose deficiency caused them. Unexplained weight loss, sometimes substantial, can occur with severe cobalamin deficiency in adults and children, and it reverses after cobalamin therapy. Infants sometimes feature prominent gastrointestinal dysfunction, such as diarrhea. Severely deficient infants can also present with recurrent infections that mimic immune deficiency states.

Young patients with severe deficiency, especially nonwhites, sometimes display pigmentary changes that reverse with cobalamin therapy: skin hyperpigmentation that overlies joints, dark hair that turns a light reddish hue, or blue discoloration of fingernails and toenails.[189,194-196] The pigmentary changes should not be confused with the unrelated, irreversible autoimmune vitiligo that occasionally coexists with pernicious anemia. Adults with pernicious anemia display reduced levels of osteoblastic proteins, such as osteocalcin and bone alkaline phosphatase, which respond to cobalamin.[197] Reports of clinical bone complications in adults await systematic proof. Bone effects have not been studied in children.

Subclinical Cobalamin Deficiency

Unexplained low serum cobalamin levels that were unaccompanied by clinical manifestations, did not progress to clinical manifestations, and displayed no clinical benefits after cobalamin treatment were originally regarded as spurious. However, biochemical studies established that some of these represented mild metabolic cobalamin insufficiency and, unlike more than 90% of cases with megaloblastic anemia and other clinical dysfunction, they were rarely associated with the loss of IF or IF-mediated absorption by ileal cells.[178,179] This often purely biochemical abnormality became known as "subclinical cobalamin deficiency." As accurate, sensitive, automated tests of cobalamin metabolism, such as MMA and tHcy, became widely available, epidemiologic studies established that subclinical deficiency affected many adults, especially the elderly, and indeed was several times as common as pernicious anemia and other clinical deficiency states.[198,199] The causes of subclinical deficiency are often unknown, but a plurality of 30% to 50% of patients have malabsorption of food-bound cobalamin, which is usually associated with chronic gastritis, achlorhydria, or gastric surgery.[200] Because poor dietary intake alone cannot usually deplete cobalamin stores sufficiently in adults and older children to cause clinical deficiency, most vegetarians, especially adults, display only subclinical deficiency.

The health consequences of subclinical cobalamin deficiency are unknown, and progression to clinically expressed deficiency seems infrequent.[201] In the absence of compelling data from randomized clinical trials, the benefits of intervention to reverse isolated biochemical changes of still unproven likelihood of progression to clinical deficiency remain unsettled.[202,203,204]

Subclinical deficiency has not been studied in children and was believed rare. However, a transient, purely metabolic cobalamin deficiency affecting many normal infants in the first year of life has been documented in Scandinavia[108,205,206] and is discussed in the section on dietary cobalamin insufficiency. The metabolic phenomenon must also be borne in mind when using current cobalamin status–related reference intervals in infants, and hematologists and geneticists must consider this transient phenomenon when evaluating MMA elevations in infants.

Nonhematologic Manifestations of Folate Deficiency

Neurologic Manifestations

Folate deficiency in adults does not cause the severe demyelinative myelopathy characteristic of cobalamin deficiency. Forgetfulness and mood changes, such as irritability, have been described,[9,207] as has neuropathy.[208] However, their attribution and specificity remain unsettled because folate deficiency is often not an isolated deficiency. Nevertheless, the neurologic consequences can be severe in some folate deficiency disorders in newborns and infants. Children with hereditary folate malabsorption, cerebral folate deficiency, and severe MTHFR deficiency (the last two of which do not display megaloblastic anemia) often have seizures, microcephaly, developmental delay, mental retardation, and myelopathy.

Other Clinical Manifestations

Mucosal changes of the tongue and mouth can sometimes resemble those of cobalamin and other deficiencies. However, because folate deficiency is often part of a broader nutritional inadequacy, responsiveness specifically to folic acid provides the best proof of causation.

Subclinical Folate Deficiency

Subclinical deficiency, definable as isolated abnormalities of folate and tHcy levels without megaloblastic changes or other clinical expressions of folate deficiency, has not been studied or defined as formally as subclinical cobalamin deficiency. Certain subpopulations are at special risk because of marginal dietary folate intake, increased folate requirements, or both, such as children (including adolescents), pregnant women, people with homozygosity for common polymorphisms of the *MTHFR* gene, and alcoholics. Although not studied formally, subclinical folate deficiency is almost certainly more common than clinically expressed deficiency.

Countries such as the United States and Canada have mandated fortification of cereal and grain products with folic acid to reduce NTD risks. This policy has nearly eliminated clinical folate deficiency in the general populations, sharply reduced the prevalence of isolated low folate levels, and lowered tHcy levels.[23,209,210] The influence of folate status on hyperhomocysteinemia and on NTDs is clear, but the record on vascular disease, cancer, and cognitive decline in the elderly is mixed or ambiguous, as discussed elsewhere in the chapter. It merits emphasis that many of the public health risks, including NTDs, have generally been associated with subclinical folate deficiency and possible genetic or acquired alterations of folate demand or utilization, rather than with clinically overt maternal folate deficiency.

DIAGNOSIS OF DEFICIENCY

Once detected, megaloblastic anemia and other clinical expressions of deficiency require confirmation that a vitamin deficiency is responsible and differentiation between folate and cobalamin deficiency. Measuring vitamin levels directly, the usual way to accomplish this, must be done before instituting therapy. Measuring metabolic biomarkers of vitamin adequacy, such as MMA and tHcy, is valuable whenever vitamin levels or the clinical picture are equivocal. Both metabolic biomarkers must be tested whenever a hereditary metabolic disorder that may not depress serum vitamin levels is suspected.

Biochemical tests are now accurate, automated, widely available, and generally quite sensitive, especially when the deficiency is advanced enough to have produced macrocytic anemia or neurologic symptoms. However, none of the tests qualifies as a diagnostic "gold standard," largely because all of them lack the requisite diagnostic specificity for either folate or cobalamin deficiency. The innate specificity shortcomings have been exacerbated by inconsistent and sometimes controversial selections of cutpoints that demarcate "normal" from "abnormal" results, choices that have major, but often hidden, impact.[211] Clinical judgment must always take precedence whenever a test result conflicts with the clinical picture. Pediatricians must also be aware that ranges of biochemical biomarker values appear to fluctuate during childhood, especially in early infancy and perhaps adolescence, where the fluctuations may sometimes hide subclinical deficiency rather than reflecting normal physiologic variability (Table 10-4).[205,212]

The biochemical tests can also help evaluate the response to therapy.[16,202] Measuring MMA or tHcy, depending on the deficiency in question, 1 to 2 weeks after therapy begins is useful because these metabolic intermediates improve only if therapy was effective. In contrast, serum vitamin content (i.e., cobalamin, holo-TC, and folate levels) rises automatically with vitamin therapy irrespective of therapeutic effectiveness. The timing of blood sampling relative to the most recent dose also determines the degree of automatic vitamin content change.

TABLE 10-4 Median (and Interquartile Range) Values for Plasma Cobalamin, Folate, Homocysteine, and Methylmalonic Acid Throughout Normal Childhood

Age	Cobalamin (ng/L)	Folate (μg/L)	tHcy (μmol/L)	MMA (nmol/L)
4 days	425 (322-634)	11.9 (9.0-16.0)	6.2 (5.0-7.5)	290 (240-390)
6 wk to 6 mo	294 (199-393)*	13.9 (9.4-19.1)*	7.5 (6.1-9.2)*	780 (360-1510)*
1 to 10 yr	747 (618-925)	6.6 (5.3-9.3)	5.2 (4.7-6.0)	130 (110-170)
10.5 to 15 yr	591 (400-717)	5.3 (4.0-6.7)	6.5 (5.7-7.8)	170 (130-220)
15.5 to 19 yr	500 (398-612)	4.3 (3.4-5.6)	7.8 (6.6-9.1)	140 (120-180)

Modified from Bjorke Monsen AL, Refsum H, Markestad T, et al: Cobalamin status and its biochemical markers methylmalonic acid and homocysteine in different age groups from 4 days to 19 years. Clin Chem *49:2067–2075, 2003.*

MMA, Serum methylmalonic acid; *tHcy,* plasma total homocysteine.

*These values reflect distortions by the high frequency of transient subclinical cobalamin deficiency in breastfed infants. Breastmilk, although relatively rich in folate, can be limited in cobalamin content. (Cow's milk is rich in cobalamin.)

Vitamin Content Assays

Cobalamin Assay

Serum cobalamin measurement, although problematic, is the traditional test of choice for diagnosing cobalamin deficiency. Microbiologic assay, based on growth of cobalamin-dependent microbes on exposure to the test serum, is unavailable outside select reference and research laboratories. Clinical laboratories, which adopted isotopic, competitive-binding assays in the 1970s, have come to rely almost exclusively on automated, competitive-binding, chemiluminescence-based immunoassays since the late 1990s. Most laboratories define subnormal levels as below 200 or 250 ng/L (150 or 200 pmol/L). However, some authors raised cutpoints to 300 to 350 ng/L to capture nearly all elderly patients with otherwise isolated, mild MMA and tHcy elevations.[198,213] This redefined reference interval led to claims that 40% or more of the elderly have cobalamin deficiency, but most of the redefined cases lacked clinical or metabolic evidence of deficiency,[214] in part because abnormal MMA and tHcy themselves lack specificity.[211] The cutpoint expansion worsened the already poor diagnostic specificity of a low cobalamin level.[202] Most laboratories continue to use the 150-ng/L cutpoint in children, but levels, as shown in Table 10-4, fluctuate during childhood, especially in early infancy,[205,212,215] when cobalamin stores are small relative to intake and when maternal influences dominate.

Adults have many causes of falsely low cobalamin levels, including levels less than 100 ng/L sometimes. A major cause is pregnancy, with levels decreasing below 150 ng/L in almost half of women by the last trimester.[16,216,217] The explanation is uncertain but may reflect maternofetal cobalamin transfer in part. Metabolic or clinical evidence of deficiency is rare, and cobalamin treatment is unnecessary. The levels become normal spontaneously a few weeks after delivery.

Folate deficiency often decreases serum cobalamin levels also, which become subnormal in 10% of patients.[16] Folate therapy restores cobalamin levels to normal, but alertness is warranted because occasional patients, especially those with gastrointestinal disease, have concurrent deficiencies of both vitamins. Interestingly, hepatic cobalamin content is also somewhat low in folate deficiency.[218] Other conditions associated with unexplained low cobalamin levels include oral contraceptive use, obesity, infection with human immunodeficiency virus, and multiple myeloma.[219-222] Falsely low cobalamin levels also accompany hereditary or, less often, acquired deficiency of haptocorrin.[223] Finally, the likelihood of falsely subnormal cobalamin levels rises sharply when overly liberal cutpoints, such as 300 ng/L, are applied.[211]

Elevated cobalamin levels are more common than low levels in adults[224] and can occasionally hide a coexisting cobalamin deficiency.[225] Examples relevant to children include defects in cellular cobalamin metabolism, in which serum cobalamin (and holo-TC) levels remain normal or increase; these metabolic defects can be hereditary or acquired, as with nitrous oxide toxicity. A troubling malfunction peculiar to modern chemiluminescence-based cobalamin assays often causes spuriously normal (or elevated) levels in IF antibody–positive sera from cobalamin-deficient patients with pernicious anemia.[226,227] Children with the juvenile form of pernicious anemia are often antibody positive and thus probably susceptible to the assay error. Renal failure can also cause spuriously elevated serum cobalamin.[224] Race affects cobalamin levels; blacks have significantly higher levels at all ages than whites or Asians.[228] Homozygosity for a common polymorphism of the *FUT2* gene, affecting fucosylation, also causes slightly higher cobalamin levels.[229,230] Finally, clinicians faced with unexpectedly normal or high cobalamin levels must consider recent cobalamin therapy as a possible cause.

Red cell cobalamin is rarely measured today because levels depend largely on the proportion of reticulocytes, which are rich in cobalamin, rendering results unreliable.

Serum Holotranscobalamin Assay

Holo-TC, the cobalamin fraction attached to transcobalamin, contains 10% to 30% of the total cobalamin in plasma at any moment in time (although the very high turnover of transcobalamin obscures the fact that transcobalamin carries much more cobalamin daily than haptocorrin does). Because holo-TC is the only cobalamin fraction that is available for delivery to cells, it is regarded by some authors to be a more reliable indicator of cobalamin status than serum (total) cobalamin.[231] However, head-to-head receiver operator characteristics-based comparisons have shown holo-TC to be only marginally superior to total cobalamin (areas under the curves of 0.66 to 0.90 vs. 0.60 to 0.86 in six studies) in recognizing deficiency defined by MMA levels[232]; its specificity and positive predictive value are as poor as that of serum total cobalamin,[233,234] but the causes await careful study. A recent study reported low holo-TC levels in folate-deficient patients also[235]; as noted earlier in this chapter, serum total cobalamin and hepatic cobalamin levels are also low in folate deficiency. Holo-TC testing also suffers from considerable cutpoint variability.[232]

It is unclear whether the 90-minute half-life of plasma holo-TC leaves its levels too susceptible to transient changes in cobalamin flow from the gut to reliably reflect cobalamin stores and status, which change slowly. Evidence of relatively decreased holo-TC levels in patients with pernicious anemia in clinical and metabolic remission[236] suggests that daily absorption flux, not just cobalamin adequacy, influence holo-TC levels, whose turnover is so great. More studies are needed to identify relevant influences that explain the poor specificity of low holo-TC levels.

Serum Folate Assay

As with cobalamin, folate testing began with microbiologic assay and, after 25 years of isotopic competitive-binding assay, is currently done by automated chemiluminescent assay. Current cutpoints approximate 2.5 to 3.0 μg/L (5.7 to 6.8 nmol/L) in adults. Normal levels fluctuate during childhood, with higher levels in early infancy that decline thereafter, reaching low-normal values in adolescence (see Table 10-4).

Serum folate has high sensitivity for folate deficiency. However, specificity is poorer because the serum levels disproportionately reflect recently ingested and absorbed folate. Transiently poor intake lowers the serum levels within 1 to 3 weeks, long before actual deficiency of stores.[9,16]

A relevant cause of serum folate elevation is clinically expressed cobalamin deficiency, such as pernicious anemia, which causes RBC methylTHF to increase; however, the poor retention of methylTHF in RBC rapidly produces low RBC folate, along with high serum folate levels in 20% of cases.[16] It is unknown if mild or subclinical cobalamin deficiency affects folate levels similarly. Dietary fortification with folic acid in the United States raised serum folate levels,[209,237,238] and the frequency of levels below 3 μg/L decreased from 18% to 0.2%.[238] Plasma tHcy levels improved commensurately.[210]

Red Blood Cell Folate Assay

RBC folate content does not change after the reticulocyte stage and is thus less susceptible to short-term fluctuation than serum folate.[16,239] The RBC level is a 120-day reflection of folate status, not a transient 1- to 3-week snapshot. However, RBC folate is subnormal in 60% of patients with pernicious anemia because the methylTHF accumulation caused by the folate trap in cobalamin deficiency is poorly retained by the RBC.[16] Measuring serum folate, which is low in folate deficiency but high in cobalamin deficiency, can resolve the confusion. Other distortions of RBC folate content include blood transfusion, iron deficiency (which elevates RBC folate), and hemolytic anemia (reticulocytes have increased folate content). The choice between RBC and serum folate testing has generated disagreement,[240,241] and clinicians should base their choice on the diagnostic question being asked.

Cutpoint selection has varied considerably. In adults, RBC folate cutpoints defined by megaloblastic anemia were set at 140 or 150 μg/L[120] but later cutpoints based on metabolic criteria approximated 190 to 200 μg/L. A study of normal Scandinavian children identified age-related variation, with highest RBC folate in newborns (breastmilk has high folate content) and a steady decline from the first 6 months to the lowest levels in adolescents.[205]

Biomarkers of Metabolic Status

Methylmalonic Acid Assay

Current gas chromatography/high-performance liquid chromatography/mass spectrometry methods are accurate and, with automation, serum MMA assay has become increasingly available; urine assays are done less often. Serum MMA elevation has 98% sensitivity for clinically expressed cobalamin deficiency,[242-244] although its sensitivity in subclinical deficiency is unknown because no diagnostic gold standard exists for comparison.[232] Being the only test unaffected by folate deficiency, MMA assay is widely considered the best available metabolic test for cobalamin deficiency, but its specificity is limited (Table 10-5).[211,232,245] A survey in adults found that cobalamin status, renal status (MMA values correlate directly with serum creatinine, even within the normal range), age, and sex explained only 16% of the MMA level.[245] Creatinine is the major known confounder and should be measured when MMA is elevated.

Most laboratories use a cutpoint approximating 270 nmol/L (0.27 μmol/L) in adults, but cutpoint selection has varied between 221 to more than 400 nmol/L, with predictably inverse impact on the frequency of cobalamin "deficiency."[232,246] MMA reference intervals have been lower in children than in adults, but they fluctuate in early infancy (see Table 10-4). Many infants display transient MMA elevations, now believed to reflect a mild subclinical cobalamin deficiency among exclusively breastfed infants[108,205,206,212] but which had distorted pediatric MMA reference intervals for years.

MMA is also an effective, albeit more expensive, biochemical tool for monitoring response in cobalamin deficiency. Levels begin to decrease several days after cobalamin therapy (but not after folate therapy) and become normal within 2 to 3 weeks.[243]

TABLE 10-5 Causes of Elevated Homocysteine and Methylmalonic Acid Levels, with Emphasis on Conditions Encountered in Children

Elevation of tHcy	Elevation of MMA
Acquired cobalamin deficiency[*]	Acquired cobalamin deficiency[*]
Hereditary cobalamin transport and absorption disorders[*]	Hereditary cobalamin transport and absorption disorders[*]
Nitrous oxide toxicity[*]	Nitrous oxide toxicity[*]
Probable subclinical cobalamin deficiency in first year of infancy[*,†]	Probable subclinical cobalamin deficiency in first year of infancy[*]
Some hereditary cbl mutations that impair the methionine synthase reaction[‡]	Some hereditary cbl mutations and MM-CoA mutase deficiencies[‡]
Renal insufficiency (borderline, mild, or severe)	Renal insufficiency (borderline, mild, or severe)
Acquired or hereditary folate deficiency; antifol drugs	—
Folate-related polymorphisms (e.g., homozygous MTHFR C677T polymorphism)[§]	—
Disorders of homocysteine metabolism unrelated to cobalamin or folate (e.g., cystathionine β-synthase)	—
Miscellaneous conditions: hypothyroidism, acute lymphocytic leukemia, psoriasis, "life style" factors	—
Vitamin B_6 deficiency[‖]	—
Drugs: anticonvulsants, trimethoprim, levodopa, cyclosporine, azauridine, niacin	—
Severe alcohol abuse	—
Assay of serum instead of plasma	—
Inappropriately low tHcy assay cutpoint for abnormality	Inappropriately low MMA assay cutpoint for abnormality
Unknown causes	Unknown causes

MM-CoA, Methylmalonyl coenzyme A; *MMA,* methylmalonic acid; *tHcy;* total homocysteine.

[*] Some cobalamin-deficient patients may have only MMA or tHcy elevation, not both, for unknown reasons; this is seen especially often in mild subclinical cobalamin deficiency.

[†] The tHcy elevation in infancy presumably caused by exclusive breastfeeding is milder than the MMA elevation, and levels are often high normal rather than elevated.

[‡] See sections on cobalamin mutations for the specific entities.

[§] Folate deficiency and tHcy elevation may not become expressed in some cases unless dietary intake is also limited.

[‖] tHcy levels are normal in most cases, but abnormality can be elicited by methionine loading.

Plasma Total Homocysteine Assay

Plasma tHcy elevation is highly sensitive for folate deficiency and nearly as sensitive as MMA elevation for cobalamin deficiency.[243,244] The degree of tHcy elevation in severe cobalamin or folate deficiency can mimic that seen in severe cystathionine β-synthase deficiency.[247] However, inability of tHcy to distinguish between folate and cobalamin deficiencies limits its diagnostic utility. The many influences that elevate tHcy include serum creatinine, age, gender, and, uniquely among tests of cobalamin and folate status, preanalytic influences, especially using serum instead of plasma and failing to separate plasma from cells quickly (see Table 10-5).[247] Cutpoint selection also affects diagnostic reliability. Reference intervals for tHcy fluctuate during childhood,[205] peaking during the transient subclinical cobalamin deficiency affecting many healthy infants (see Table 10-4). A second, mild tHcy elevation occurs during adolescence, presumably reflecting a downward trend in folate status.[205,210]

The high sensitivity of tHcy assay for cobalamin and folate deficiency and its relative inexpensiveness provide two valuable diagnostic roles: a normal tHcy level largely rules out the presence of clinically relevant folate deficiency, and the tHcy assay is the most cost-effective metabolic monitor of response of either cobalamin or folate deficiency to specific therapy. tHcy begins to decrease within a week and becomes normal within 1 to 3 weeks.[248]

Miscellaneous Tests

Plasma cysteine increases along with tHcy in cobalamin and folate deficiencies, but methionine levels show little change.[174,248] Measuring 2-methylcitric acid[249] provides little diagnostic advantage over MMA measurement. The deoxyuridine suppression test, an older, indirect means of evaluating thymidylate adequacy, allows diagnosis of folate and cobalamin deficiencies at the cellular level when specific vitamins are added to cells in vitro[178,250,251] but requires bone marrow cells and is labor intensive. Formiminoglutamic acid excretion lacks specificity and is no longer used diagnostically.

Response to a Trial of Specific Therapy

Objective evaluation of response to either cobalamin or folic acid provides the ultimate diagnostic confirmation.[16,202] It is especially helpful in patients whose diagnostic results conflict with clinical judgment or are inconclusive. A convincing therapeutic trial requires giving the suspected vitamin alone, preferably as a single dose, and giving enough vitamin to produce a response but not so much that therapeutic specificity is lost. Approximate suggestions in older children and adults include 10 to 50 μg of cobalamin or 100 μg of folic acid; parenteral injection is advisable if malabsorption is present or suspected. Trial recommendations are less uniform in young children and must be tailored by size, age, and the nature of suspected diseases.

If anemia was present, reticulocytosis should begin within 3 days and peak at 7 days. Potential sources of diagnostic error include using more than one hematinic or using large doses of folic acid, which may elicit a hematologic response even in patients with cobalamin deficiency. With the correct vitamin, tHcy or MMA levels begin to improve within a week and become fully normal within 2 to 3 weeks, except in some severe metabolic disorders[242,248]; neither metabolite improves if the wrong vitamin is given. Serum vitamin and holo-TC have little monitoring value because vitamin content almost always increases, even when therapy is ineffective.

ACQUIRED CAUSES OF COBALAMIN DEFICIENCY

The diagnostic task does not end with identifying the responsible vitamin deficiency. Many management and prognostic decisions require knowing the underlying disorder. This section discusses the acquired causes of cobalamin deficiency, generally following the sequence of events from intake to cellular metabolism (see Fig. 10-4). Hereditary disorders will be discussed in detail in separate sections.

An important distinction between folate and cobalamin physiologies helps explain a major difference between the two deficiencies' frequencies and expressions. The two vitamins' body stores–to–daily intake ratios are strikingly disparate, especially in older children and adults. This difference means that adult folate stores can become depleted sufficiently within 2 to 3 months to cause clinical deficiency whereas cobalamin store depletion in adults requires several years.

A comparison gleaned from Chanarin's summaries in 1979 of the scarce data of stores in infants[16] and the Institute of Medicine's recent estimates of daily intake requirements[14,99] provides a perspective on the reduced cushions for dietary adequacy in infants. The infants' daily cobalamin intake requirements are one fifth of that for adults but stores are only one fiftieth as great in infants as in adults. For folate, the infants' daily requirements are one fifth that for adults, whereas stores are one twelfth as great as in adults. These estimates suggest that infants' stores, while reduced for folate, may be especially precarious for cobalamin.

The underlying causes of cobalamin deficiency are listed in Box 10-1. In adults and older children, clinically expressed deficiency usually occurs only if the highly specific absorption system fails and if the failure is both substantial and persistent enough to nearly exhaust stores. Therefore severe malabsorption, the most common form being pernicious anemia (loss of IF), accounts for more than 90% of clinical cases of deficiency in adults and older children[244] but not in newborns and infants.

Box 10-1 Causes of Cobalamin Deficiency*

- Inadequate nutrition
  - Strict vegetarians and vegans
  - Exclusively breastfed infants of cobalamin-deficient mothers
  - Exclusively breastfed infants of mothers with normal cobalamin status
  - Exclusive reliance on goat's milk
- Defective absorption
  - Inadequate gastric intrinsic factor
    - Acquired pernicious anemia (adult and juvenile)
    - Pernicious anemia associated with agammaglobulinemia or common variable immunodeficiency
    - Hereditary intrinsic factor deficiency
    - Total gastrectomy
  - Food-bound cobalamin malabsorption
    - Chronic gastritis (without loss of intrinsic factor)
    - Gastric surgery (e.g., partial gastrectomy; bariatric surgery)
    - Drugs that inhibit gastric acid secretion
  - Disease of the small intestine:
    - Surgical resection or bypass of the terminal ileum†
    - Bacterial overgrowth in the intestine (motility disorders, strictures, etc.)
    - Diseases involving the ileum†
      - Tropical sprue
      - Regional enteritis (Crohn disease)†
      - Competition by parasites (fish tapeworm)
    - Imerslund-Gräsbeck syndrome
- Defects in transport and delivery to cells‡
  - Transcobalamin deficiency
- Defects in cellular metabolism
  - Nitrous oxide toxicity
    - Chronic ("recreational") abuse
    - Anesthetic exposure in the presence of unrecognized pernicious anemia, veganism, etc.
  - Hereditary metabolic disorders
    - Cbl disorders§
- Disorders of uncertain relevance
  - Pancreatic insufficiency ; cystic fibrosis
  - Miscellaneous drugs affecting absorption by unknown mechanisms¶

*Red type indicates progressive disorders that usually cause clinically expressed cobalamin deficiency, often of malabsorptive origin.

Normal type indicates slowly progressive or nonprogressive disorders that usually cause only mild, isolated biochemical changes (subclinical deficiency).

Blue type indicates disorders that can cause either clinical or subclinical cobalamin deficiencies.

†Development of clinical deficiency depends on the extent of ileal involvement.

‡Haptocorrin (transcobalamin I) deficiency is not included in this box because it causes low cobalamin levels but does not affect cobalamin uptake by cells or cause cobalamin deficiency.

§Patients with cblC, cblD, cblE, cblF, cblG, and cblJ diseases have megaloblastic anemia, but patients with the other cbl disorders do not.

Intrinsic factor–dependent malabsorption is frequent but is surprisingly seldom translated into cobalamin deficiency or clinical signs of deficiency.

¶Many drugs, such as colchicine and paraaminosalicylic acid, inhibit intestinal absorption of cobalamin, but they are too infrequently taken long enough or consistently enough to deplete cobalamin stores and cause cobalamin deficiency.

Dietary Cobalamin Insufficiency

Dietary Insufficiency in Adults and Older Children

A narrowly focused cobalamin inadequacy, ranging from strict veganism to mild ovolactovegetarianism, in which dairy products and eggs are consumed, can produce cobalamin deficiency. However, the process of depleting 80% of the very high storage cushion found in adults and older children to induce clinical deficiency with megaloblastic anemia or myeloneuropathy[16,99] takes years and requires uninterrupted dietary restriction. Indeed, depletion takes longer than in pernicious anemia and other malabsorptive diseases because vegetarians continue to reabsorb biliary cobalamin normally. Subclinical deficiency limited to mild abnormalities of serum cobalamin and MMA levels can be seen, but clinical deficiency is surprisingly infrequent, even in strict vegans, unless other events, such as malabsorption or increased metabolic requirements, supervene.[252]

Dietary Insufficiency in Infants

Intake adequacy poses greater problems in infants than in older children. The much reduced stores–to–daily intake ratio in newborns requires expansion during infancy and the demands of growth and development. Despite maternofetal transfer of cobalamin that favors the fetus even when mothers have borderline cobalamin status,[16] the small cobalamin stores at birth must then be expanded to accommodate the demands of growth, neurologic maturation, and cognitive development. In early infancy, much (and often, all) of the diet consists of breastmilk. The severity and duration of any maternal restrictions, whether in cobalamin transfer to the fetus, breastfeeding, or both, can influence the degree of deficiency in the infant. Indeed, maternal cobalamin status at delivery correlates significantly with the infant's status not just at birth but also 6 months later.[253] Breastmilk cobalamin content is also influenced by maternal status.

TABLE 10-6 Relationships between Breastmilk Cobalamin Content and Maternal and Infant Cobalamin Status*

	Birth/2 wk Postpartum	At 4 mo	At 9 mo
Mean breast hindmilk	561 (760)	214 (290)	325 (440)
Cobalamin concentration (n = 25)			
Paired mean plasma			
Cobalamin levels:			
Mothers (n = 60)	295 (400)	288 (390)	310 (420)
Infants (n = 60)	325 (440)	177 (240)	236 (320)
Mean serum MMA			
Levels (nmol/L):			
Mothers (n = 60)	180	170	160
Infants (n = 28)	270	440	240

Data modified from Greibe E, Lildballe DL, Streym S, et al: Cobalamin and haptocorrin in human milk and cobalamin-related variables in mother and child: a 9-mo longitudinal study. Am J Clin Nutr *98:389–395, 2013.*

*Cobalamin concentrations are shown in both pmol/L and (in parentheses) ng/L. Methylmalonic acid (MMA) data are shown in nmol/L.

Mild Dietary Insufficiency in Breastfed Infants. A series of Norwegian studies beginning in 2001 documented unexplained elevations of serum MMA levels in 60% of normal, asymptomatic 4- to 6-month-old Norwegian infants whose mothers were not cobalamin deficient.[205,206,212,254] The MMA elevation exceeded 1000 nmol/L in 30% of the infants,[254] and median serum MMA was 780 nmol/L in infants aged 6 weeks to 6 months, compared with 290 nmol/L in newborns and 130 nmol/L in children aged 1 to 10 years.[205] As Table 10-4 shows, plasma tHcy was also higher in the 6-week to 6-month age group, although not as strikingly abnormal as MMA.[205] Cobalamin levels tended to be low-normal or, in only 8% of cases, slightly subnormal.[254] These biochemical changes, which could also be easily reversed with cobalamin supplements,[206] usually normalized spontaneously after several months.[205] The absence of clinical signs of deficiency suggests that these normal infants had subclinical deficiency.

The explanation for the transient cobalamin status changes in early infancy has now become clearer. A recent longitudinal Danish study[108] documented an unexplained, apparently spontaneous decline in breastmilk cobalamin levels in healthy, omnivore mothers 4 months after giving birth, often despite their taking cobalamin supplements. The authors associated this decline in breastmilk cobalamin with the transiently worse cobalamin status in exclusively breastfed infants compared with infants eating mixed diets (Table 10-6). At month 9, the breastmilk cobalamin content had improved spontaneously, as had the cobalamin status of the infants (whose diet had also become mixed). The mothers' cobalamin status remained unchanged throughout these fluctuations of breastmilk cobalamin. It remains possible that additional factors play a role because breastfed infants who also ingest a mixed diet showed somewhat similar cobalamin findings as exclusively breastfed infants at 4 and 9 months.[108] The findings are compelling, but their surprisingly high frequency in seemingly normal mothers and infants requires clarification. More studies outside Scandinavia are needed as well. It is also important to note that this transient cobalamin deficiency of early infancy is subclinical. Convincing clinical accompaniments, including changes in hemoglobin and MCV levels, have not been reported. Finally, it is noteworthy that plasma folate levels remained unchanged throughout these transient cobalamin deficiencies but decreased sharply after the age of 1 year and continued to decline throughout childhood (see Table 10-4).[205] The relatively high folate content of human breastmilk provides breastfed babies significantly better folate status than nonbreastfed infants.[255,256]

Severe Nutritional Deficiency Caused by Significant Maternal Deficiency. At the other extreme of the spectrum of dietary cobalamin insufficiency in infants lies a clinically severe deficiency. It is dominated especially by neurologic, cognitive, and developmental manifestations, probably because the first 6 months of life are crucial for brain development.[257] The syndrome affects children who are exclusively breastfed by mothers who have longstanding compromised cobalamin status that probably also compromised maternofetal cobalamin transfer. To date, most of the mothers have had clinically minimal, usually latent pernicious anemia, significant dietary insufficiency, or tropical sprue.[16,258,259] The mothers' extremely low breastmilk cobalamin content, found to be only 0.05 to 0.09 μg/L in a representative study,[196] compared with the more normal 0.29 to 0.76 μg/L concentrations in the healthy mothers summarized in Table 10-6, then converted the infants' already precarious cobalamin state at birth into clinically severe deficiency by exclusively breastfeeding them.

The affected children display megaloblastic anemia, myeloneuropathy, and tongue atrophy, but their less specific but prominent failure to thrive, vomiting, developmental delay, motor and social retardation, hypotonia, seizures, poor growth and myelination of the brain, and, less often, tremor can obscure the true diagnosis.[16,189-192,196,257-264] Diagnostic delays can take months or even a year or more; a single-institution survey of 27 cases diagnosed between 2000 and 2008 reported a median age at diagnosis of 10.4 months.[192] Cobalamin therapy, which can be oral but should preferably include parenteral injections in the beginning, quickly reverses the anemia and improves neurologic function, but neurologic, mental, and developmental residua often persist.[191,262,263] It is illustrative of the differences in consequences of cobalamin depletion that the infants are often catastrophically ill whereas their mildly cobalamin-deficient mothers are usually asymptomatic, often with only mild biochemical abnormalities. Occasional mothers display megaloblastic changes and increased MCV, which are mild.

Initially reported in India,[16,189,196] where the children often also showed hyperpigmented skin changes, the severe deficiency has been increasingly documented around the world.[192,263,264] The syndrome is believed to be the most common cause of clinical cobalamin deficiency in infancy, but its frequency is unknown. Two limited studies of state-run newborn screening laboratories that used tandem mass spectrometry estimated frequencies of 0.88 and 3.02 per 100,000 births.[265,266] Whether additional factors influence who develops the syndrome is unclear. A report of the syndrome in only one of two exclusively breastfed monozygotic twins of a vegan mother whose breastmilk cobalamin was only 0.037 μg/L illustrates the unresolved questions.[261] Prospective studies are awaited to identify all the risk factors, determine if less dramatically clinical deficiencies are common, and establish criteria for prevention.

Nutritional Deficiencies Unrelated to Maternal Influence

Poor cobalamin intake can occur worldwide but its impact is greatest wherever societal vegetarianism is prominent. Less well appreciated is poor intake in generally impoverished regions.[255,256,267] Most regional surveys have documented increased rates of mild, biochemically defined cobalamin insufficiency. However, systematic data are sparse in young children, and the frequency of clinically overt consequences is unknown. Low cobalamin levels were especially frequent in exclusively breastfed infants in India and elsewhere,[255,256,268] resembling the observations in normal, breastfed infants in Scandinavia. Deficiency appears to be subclinical, perhaps because maternal cobalamin status is only mildly or moderately compromised.

Once established, deficiency may persist despite dietary liberalization. Young Indian adults retain MMA and tHcy abnormalities without anemia even years after emigrating to the United States and liberalizing their diets.[269] Clinical sequelae may occur in children in macrobiotic communities, with residual cognitive changes in adolescents even years after their diet was partially liberalized.[270] However, the broad inadequacies of macrobiotic diets make specific attribution to cobalamin deficiency difficult. The benefits of routine supplementation of vegetarians, especially children, need to be studied systematically. Folate and cobalamin supplementation of Indian infants did not protect them against diarrhea and respiratory infections[271]; indeed, folic acid use may have increased diarrheal risk.

Protein-restricted diets, such as phenylketonuric diets, can produce varying degrees of MMA and tHcy elevation at all ages but clinical signs of cobalamin deficiency are infrequent.[272-274] Isolated low cobalamin levels or isolated mild MMA and tHcy elevations were reported in 11% and 13%, respectively, of diet-restricted patients of all ages.[273] Another study of adults, in whom phenylketonuric restrictions often ease, found that 29% had cobalamin levels less than 271 ng/L but only 3% had levels less than 203 ng/L.[274] The plasma tHcy elevations in 29% of subjects may have been influenced by the frequently low vitamin B_6 levels. Borderline or mild macrocytosis was apparent in 68% of cases but was unexplained and appeared to be unrelated to MMA abnormality.[274]

Cobalamin deficiency can develop in children raised on goat's milk, which contains only 0.07 to 0.18 μg/L cobalamin, a small fraction of the contents of cow's milk, breastmilk, and even sheep's milk.[16] Folate deficiency is a more common consequence.

The possibility that inadequacies of cobalamin, folate, or both contribute to the anemia in premature infants or blunt its responsiveness to erythropoietin and iron therapy seems plausible. However, objective evidence of cobalamin deficiency remains marginal. The few available clinical trials,[275,276] although frequently cited, are hard to interpret because many uncontrollable variables confound the infants' laboratory data, management, and course; for example, even the control subjects in the studies received folic acid. Megaloblastic changes have not been observed.

Acquired Disorders of Cobalamin Absorption

Pernicious Anemia (Loss of Intrinsic Factor)

Acquired pernicious anemia, a relatively infrequent disease, is by far the most common cause of clinically overt cobalamin deficiency in adults.[16,244] It arises from chronic atrophic gastritis, a common inflammatory disorder with reduced or absent acid and pepsin secretion, only when such gastritis progresses to also stop secreting IF. Most often, the precursor atrophic gastritis for pernicious anemia is not the more common, so-called type B pangastritis arising from *Helicobacter pylori* infection[277] but the less common type A atrophic gastritis that spares the antrum and has frequent autoimmune features.[16,278-280] Autoantibodies against gastric antigens are frequent and chiefly directed against IF (50% to 70% frequency in pernicious anemia) or against the membrane H^+,K^+-ATP pump subunits of parietal cells (80% to 90% frequency). A strong association also exists with other autoimmune disorders, especially those involving endocrine glands embryologically associated with the stomach, such as the thyroid gland.[16,281]

The poorly understood progression from gastritis to pernicious anemia, which often displays a familial predisposition, is infrequent.[16,282] Several years or decades may pass before the pathognomonic IF loss and cobalamin malabsorption appear that define pernicious anemia. A few years later, more than 80% of the cobalamin stores become depleted, leading to megaloblastic anemia and neurologic dysfunction. Therefore pernicious anemia is typically diagnosed in people in their 60s or older. The disease predominates in women, and it is most common in northern Europeans but occurs worldwide. It is worth noting that the absence of IF prevents both absorption of ingested cobalamin and reabsorption and salvage of biliary cobalamin. Moreover, the achlorhydria of pernicious anemia may allow bacterial colonization of the upper gastrointestinal tract,[283] whose additional effects deserve elucidation. Although the IF loss is irreversible, the cobalamin deficiency, anemia, epithelial changes, and, if treatment is started in time, neurologic symptoms respond to cobalamin therapy.

Acquired Pernicious Anemia in Children. Only infrequently does acquired pernicious anemia affect children, in whom it is sometimes called “juvenile pernicious anemia.” Affected children are usually adolescents, but patients as young as 8 years old have been described.[16] Juvenile pernicious anemia differs from hereditary IF deficiency, in which gastritis is not usually evident but is indistinguishable from the adult form of acquired pernicious anemia, some of whose ancillary features are magnified. A family predisposition is more obvious than in adult pernicious anemia, and autoimmune features such as antibody to IF and coexisting endocrinopathies are more prominent. In these characteristics, juvenile pernicious anemia closely resembles the acquired pernicious anemia in blacks, who are disproportionately affected in their 20s, 30s, and 40s and in whom antibody to IF is nearly universal[284]; curiously, however, the usually very frequent antibody to parietal cells is less often present in these patients.[285]

All children with acquired pernicious anemia should be screened for coexisting endocrinopathies, especially immune thyroiditis, but also hypoparathyroidism and adrenal insufficiency, and for iron deficiency as well because achlorhydria reduces iron absorption. Whether the children also have the increased risk for gastric cancers seen in adults,[16,279,286] especially for carcinoid tumors, which complicate 7% of cases of adult pernicious anemia,[286] is unknown but seems likely. If true, endoscopy may become advisable when the children become older. It is not yet clear whether the four reported cases of acquired pernicious anemia in young adults with sickle cell disease[287,288] bespeak a true association. Nevertheless, it suggests considering coexisting pernicious anemia when blood cell counts decrease or MCV increases unexpectedly in patients with sickle cell disease. It also suggests the importance of screening cobalamin status before giving folic acid supplements.

An uncommon variant of acquired pernicious anemia can affect young adults and children with agammaglobulinemia or common variable immunodeficiency.[289] Bacterial overgrowth of the upper intestine is frequent, and the risk for gastric cancer is high.[289] Pernicious anemia can be hard to diagnose because agammaglobulinemic patients do not display diagnostic antibodies and the pangastritis characteristic of this syndrome prevents diagnostically helpful hypergastrinemia, which requires antral sparing.

The diagnosis of pernicious anemia has become difficult in general since the disappearance of the Schilling test of cobalamin absorption.[290] Testing for antibody to IF, which has greater than 90% specificity for pernicious anemia (as long as blood is not sampled within 1 to 2 days after cobalamin injection) is the best diagnostic tool left, and it is especially useful in juvenile pernicious anemia in which IF antibody is found much more frequently than the 50% to 70% sensitivity in adult pernicious anemia. Both parietal cell antibody, a marker of chronic immune gastritis, and serum gastrin elevation[279] have 80% to 85% sensitivity for pernicious anemia but lack specificity. Because of the low frequency of pernicious anemia, false abnormal results outnumber true abnormal results in both of those tests.

Other Acquired Gastric Disorders

Total gastrectomy eliminates IF secretion but is a rare procedure. Partial gastrectomy leads to eventual IF loss in 15% to 30% of adult patients, especially if chronic gastritis coexisted.[16,291] Preventive cobalamin treatment is advisable because megaloblastic anemia and neurologic abnormalities may develop after a few years. Most partial resections, as well as many current bariatric procedures, do not affect IF but induce a mild malabsorption limited to food-bound cobalamin that is usually associated with only subclinical, biochemical deficiency.[199,292-294] It is reasonable to institute preventive cobalamin supplementation and annual monitoring. Nonsurgical causes of food-bound cobalamin malabsorption, in which reduced acid and pepsin secretion and other poorly understood influences impair release of cobalamin from its binding proteins in food to IF, include chronic gastritis, which is much more common than pernicious anemia, and chronic use of acid-suppressing drugs, such as gastric proton-pump inhibitors.[199] Subclinical cobalamin deficiency often occurs, but clinical deficiency with signs and symptoms is unusual. These common forms of limited malabsorption in adults have not been studied in children.

Acquired Intestinal Malabsorption

Because cobalamin absorption is maximal in the terminal ileum, deficiency is likeliest in diseases that affect the ileum, such as tropical sprue. Sprue affects natives and visitors in endemic regions, but it can be latent, and diarrhea and vitamin deficiencies may not appear in visitors until much later. The megaloblastic anemia of sprue may arise from folate deficiency, cobalamin deficiency, or both; and the deficiencies and disease characteristics can vary not only among India, Southeast Asia, and the Caribbean but also locally[295]; neurologic consequences of cobalamin deficiency are unusual. The mechanism of tropical sprue is unknown, but a microbial component appears likely. The sprue often remits with tetracycline, especially in the Caribbean region where bacterial overgrowth has been observed[295]; sulfa drugs can be substituted in children, who may develop sprue less often than adults.[296] Folic acid itself sometimes augments the reversal. The frequency of tropical sprue has decreased since the 1980s, perhaps because of improved hygiene.

Ileal resection, such as after necrotizing enterocolitis[297] or Crohn disease, can cause cobalamin malabsorption. Clinically overt deficiency results if enough ileum is resected. Celiac disease and dermatitis herpetiformis primarily affect the upper small intestine, causing low cobalamin levels in only 11% to 12% of cases and rarely producing cobalamin-deficiency megaloblastic anemia.[298] More often, patients have folate and iron deficiencies, as discussed in the section on folate malabsorption.

Acquired Malabsorptive Disorders Originating in the Intestinal Lumen. Accumulation of bacteria in the small intestine accompanies some primary intestinal malabsorptive disorders, but its relevance is uncertain. Nevertheless, overgrowth of bacteria that utilize cobalamin can sometimes compete with IF for cobalamin and cause cobalamin deficiency.[299] One survey attributed 1% of cobalamin-deficiency megaloblastic anemias in adults to this intestinal disorder.[244] Causes of major overgrowth include blind loops, strictures, and abnormal intestinal motility.

Cobalamin-avid parasites, such as *Diphyllobothrium latum,* which parasitizes freshwater fish in northern countries, especially Finland, compete for ingested cobalamin in direct proportion to the gastrointestinal parasite load.[16] Severe cobalamin deficiency and megaloblastic anemia, which improve after worm expulsion, affect only 9% of carriers; neurologic abnormalities may also occur. Older adults are affected most often, but children as young as 9 years have been described.[16] The frequency of *D. latum* infestation has decreased greatly today, perhaps from pollution of northern freshwater lakes. The nature and relevance of the occasional association between *Giardia lamblia* and cobalamin malabsorption, especially in children and immunocompromised patients, and the low cobalamin levels seen in patients with heavy *Strongyloides stercoralis* infection are unclear.

The low intestinal pH seen in the Zollinger-Ellison syndrome interferes with IF uptake by ileal receptors, which requires a neutral pH.[300] Pancreatic insufficiency often causes abnormal results in the Schilling test of cobalamin absorption because pancreatic proteases must degrade haptocorrin to make cobalamin available to IF.[301] However, cobalamin deficiency has not been reported convincingly in adults. Cobalamin malabsorption also occurs in children with cystic fibrosis,[302] and cobalamin-responsive anemia was described in at least one such child 12 years after cystic fibrosis was diagnosed.[303]

The long list of drugs and toxins associated with cobalamin malabsorption includes colchicine, neomycin, paraaminosalicylic acid, and alcohol. The mechanisms are largely unknown. The absence of confirmed reports of clinical consequences is probably best explained by the several years of unremitting malabsorption needed to deplete stores in adults, whereas most drugs are taken too briefly or intermittently. The potential for drug effects in infants, with their limited cobalamin stores, is unknown. Metformin has been associated with low cobalamin levels, but the low levels are unexplained and are not associated with known cobalamin deficiency.[304,305]

Acquired Disorders of Cobalamin Metabolism

Nitrous Oxide Toxicity

On exposure to transition-metal complexes such as cobalamin, nitrous oxide can become an oxidizing agent, converting reduced cob(I)alamin to cob(III)alamin. One result is rapid, irreversible inactivation of methionine synthase for which cobalamin is a cofactor[306]; methylmalonyl-CoA mutase activity is less severely affected. In the usual intraoperative use of nitrous oxide lasting a few hours at most, a mild, transient metabolic abnormality occurs without clinical impact as the degraded enzyme is soon replaced.[307-309]

Clinical consequences arise only in special settings. One setting involves chronic, recurrent exposure: either as chronic occupational exposure to nitrous oxide[310] or as chronic inhalational abuse, which is especially popular among teenagers and young adults.[311,312] A quite different setting arises when a surgical patient who has unrecognized clinical cobalamin deficiency, such as pernicious anemia[313-315] or veganism,[316] receives nitrous oxide, especially when the anesthetic exposure is lengthy or repetitive. The consequences of nitrous oxide toxicity because of unrecognized clinical cobalamin deficiency appear within a few weeks of exposure; the timing of cumulative toxicity from repetitive nitrous oxide abuse is less clear and may depend on the extent and timing of exposure. The manifestations include megaloblastic anemia, but the neurologic and mental consequences are especially prominent. MMA and especially tHcy abnormalities are evident, but cobalamin levels are usually normal unless the patient had antecedent cobalamin deficiency, such as pernicious anemia. The diagnosis should be suspected if surgery was done recently, whereas chronic inhalational abuse may be denied. Response to cobalamin is often prompt, but neurologic residua can persist despite therapy.

Postoperative deterioration has been reported in two infants who received nitrous oxide and had severe neurologic dysfunction.[317,318] One child was exclusively breastfed and his mother was suspected to have pernicious anemia, a combination that may have contributed as much to his deterioration as his 80-minute exposure to nitrous oxide; he had severe megaloblastic anemia a mere 6 days after the surgery.[317] The role of nitrous oxide appeared more dominant in the other child, whose surgery took 3 hours and whose feeding and maternal history was less dramatic, although the mother had somewhat limited intake of animal products.[318] The child was not evaluated for hereditary or other contributors to cobalamin deficiency. A third report described a child with seizures and brain atrophy who died with respiratory distress 46 days after prolonged nitrous oxide exposure and was found to have severe MTHFR deficiency.[319] The mechanisms underlying the fatal outcome are not clear but raise concerns that nitrous oxide may worsen folate metabolism as well as cobalamin metabolism, especially in infancy. However, an attribution of neurologic deterioration after nitrous oxide exposure to common, mild MTHFR polymorphisms[320] was unconvincing because the single patient on which it was based also had a very low cobalamin level, probably caused by pernicious anemia, and the patient's effective treatment had included cobalamin.

Nitrous oxide should be avoided in any surgical or dental patient with suspected cobalamin deficiency, although the risk for adverse effects in the large number of healthy elderly persons and vegetarians with subclinical cobalamin deficiency is unknown. The value of routine preoperative screening of cobalamin status or routine preoperative supplementation requires analysis. Caution about using nitrous oxide seems prudent in children, especially infants with hereditary disorders of cobalamin and folate metabolism.[321] If nitrous oxide must be used, patients found to have cobalamin deficiency before surgery can be given cobalamin in advance, although the most effective timing and regimens await definition.

Increased Cobalamin Requirements

Increased requirements have been described in hyperthyroidism and some malignant diseases, but modern metabolic evidence is sparse. Given the large stores-to-daily intake ratio for cobalamin in adults and older children, clinical consequences are unlikely in the absence of contributing dysfunction, such as poor intake or malabsorption. Infants may be more susceptible to increased cobalamin requirements, but evidence is limited.

DIAGNOSTIC APPROACH TO ACQUIRED CAUSES OF COBALAMIN DEFICIENCY

Diagnosing Cobalamin Malabsorption

In adults, malabsorption accounts for more than 90% of cases of clinically expressed cobalamin deficiency.[244] Comparable data are not available in children, in whom malabsorption is less common. Moreover, the disappearance of reliable absorption testing with the Schilling test has left a major diagnostic gap.[290] A new test measuring the rise in plasma holo-TC after three oral doses of nonradioactive cyanocobalamin over 2 days[322] has been studied mostly in normal subjects, but validation in patients with proven malabsorption has been scarce. The current absence of reliable means to test cobalamin absorption leaves only indirect tests for some absorptive disorders. Anti-IF antibody is highly specific for pernicious anemia, but sensitivity is only 50% to 70%; sensitivity may be higher in juvenile pernicious anemia. Tests of gastritis, such as serum gastrin, lack specificity for pernicious anemia. A combination of IF antibody and gastrin testing may improve detection. No tests exist for intestinal causes of cobalamin malabsorption.

Diagnosing Dietary Cobalamin Deficiency

Dietary deficiency can be serious in infants, and diagnostic attention can focus on three major causative elements: maternal cobalamin deficiency, exclusive breastfeeding, and vegetarian diet (i.e., specific, rather than broad, dietary inadequacy). Maternal evaluation should be done routinely whenever any infant suffers from cobalamin deficiency or has elevated MMA, especially when cobalamin deficiency is severe or the infant was exclusively breastfed. The mother herself is usually asymptomatic, even when cobalamin deficient, so dietary history, serum cobalamin and MMA levels, and a blood cell count should be obtained. If maternal deficiency is suspected, IF antibody testing of the mother is warranted and the possibility of bowel disease should also be considered.

In older children, maternal influence is unlikely, but other contributory causes should be considered in the child before attributing any clinically expressed cobalamin deficiency to poor diet.

Diagnosing Acquired Metabolic Disorders

Disorders of cellular uptake or metabolism deserve consideration whenever serum cobalamin levels are normal in a child, especially an infant with megaloblastic anemia, failure to thrive, and developmental delay. MMA and tHcy levels should be measured in all cases. The most frequent acquired metabolic disorder is nitrous oxide toxicity, which can be suspected in children with recent anesthetic exposure and in adolescents who abuse inhalants; the latter often deny exposure. Hereditary disorders, although more common in young children, should be considered even in older children because clinical expression can be delayed or overlooked, especially when masked by multivitamin supplementation.

ACQUIRED CAUSES OF FOLATE DEFICIENCY

The underlying causes of acquired folate deficiency are listed in Box 10-2. Folate deficiency, formerly affecting more than 16% of adults and children of all ages, has nearly disappeared in countries that have mandated fortification with folic acid,[238,323] in large part because diet usually plays the major causative role in folate deficiency.

Dietary Folate Insufficiency

The stores–to–daily losses ratio for folate is relatively small at all ages, and poor intake for any reason can deplete stores quickly. Cooking and processing of food can also degrade folate content.[16] The wide distribution of folate in all food groups often means that folate deficiency can be accompanied by a relatively broad dietary inadequacy and that other nutrients may also be at risk. Alcoholism is a major contributor to folate deficiency in adults, often accompanied by a poor diet. However, alcohol and its breakdown products also directly affect folate absorption, transport, and catabolism.[324]

Infants, with their small folate stores and large demands for growth and development, are at risk if intake decreases. All these factors are magnified in premature

Box 10-2 Causes of Folate Deficiency in Adults and Children[*]

INADEQUATE NUTRITION[†]

- Poor diet[‡]
- Poor food preparation methods
- Exclusive feeding with goat's milk

DEFECTS IN ABSORPTION

- Gastric achlorhydria[§]
- Diseases of the upper small intestine
  - Tropical sprue
  - Celiac disease
  - Dermatitis herpetiformis
  - Inflammatory bowel disease
- Oral pancreatic replacement therapy[§]
- Hereditary folate malabsorption

INCREASED REQUIREMENTS OR LOSSES

- Pregnancy
- Lactation[§]
- Prematurity[§,‖]
- Chronic hemolytic anemia[§]
- Dialysis
- Hyperthyroidism[§]
- Lesch-Nyhan syndrome

DISORDERS OF TRANSPORT

- Cerebral folate deficiency (genetic or acquired)[¶]

DISORDERS OF CELLULAR METABOLISM

- Drugs inhibiting folate metabolism
  - Antifolates (methotrexate and others)
  - Pyrimethamine[§]; trimethoprim[§]
  - Sulfasalazine[§]
  - Valproic acid[§,#]
- Inherited defects
  - Methylenetetrahydrofolate reductase deficiency
  - Methionine synthase deficiency (cblE and cblG diseases)
  - Dihydrofolate reductase deficiency
  - MTHFD1 deficiency
  - Others

MULTIFACTORIAL OR UNCERTAIN MECHANISMS

- Alcohol abuse[‡]
- Anticonvulsants[§]
- Oral contraceptives (?)[§]

[*] Folate deficiency often has multifactorial causes. Many of these causes may be mild and incapable by themselves of producing deficiency or overt clinical expression, unless additional causes augment the deficiency or limit the compensation.

[†] Relative dietary insufficiency (i.e., intake that is adequate under usual circumstances) is a particularly important cofactor that can convert borderline folate deficiency to clinically overt deficiency when other conditions, such as increased requirements for folate or mild malabsorption, coexist.

[‡] Poor intake is often associated with alcoholism in adults.

[§] Megaloblastic anemia rarely results unless other limitations of folate status coexist.

[‖] Neonatal stores are low in premature infants.

[¶] Cerebral folate deficiency can occur on a genetic basis or an autoimmune basis; megaloblastic anemia does not occur because folate deficiency appears not to exist outside the central nervous system.

[#] Disrupts mitochondrial folate metabolism in utero.

newborns,[275] and megaloblastic anemia appearing at 6 to 11 weeks of life was described in 13% to 19% of premature infants.[16] Although breastmilk is relatively rich in folate (approximately 85 µg/L),[14] folate bioavailability from cow's milk is limited[16]; nonbreastfed infants often receive folic acid–fortified formulas and foods. Reliance on goat's milk poses another risk in infants.[16] The poor folate content of goat's milk (5 µg/L) may be a greater factor than the low cobalamin content in causing the megaloblastic anemia that appears at 18 to 20 weeks in infants dependent on goat's milk. Other dietary restrictions, such as the protein-restricted diet for phenylketonuria, occasionally induce folate deficiency. Finally, because folate deficiency (unlike cobalamin deficiency) is often part of a broader malnutrition, it is the usual cause of the megaloblastic anemia in disorders such as kwashiorkor and marasmus. Such children often require very high oral folic acid doses,[325] whereas cobalamin deficiency is rare. For unclear but probably largely dietary reasons combined with a growth spurt, folate status is somewhat lower (although usually normal) in adolescents than in younger children and remains so despite its dramatic improvement after fortification of the American diet.[323]

Acquired Disorders of Folate Absorption

Intestinal Disorders

Malabsorption accounted for only 2% of folate-deficient megaloblastic anemia in a clinical survey of adult patients in the prefortification era,[244] and it usually arises from diseases of the jejunum. Thus celiac disease and dermatitis herpetiformis cause folate deficiency much more often than cobalamin deficiency. Megaloblastic anemia is nevertheless relatively uncommon.[298] Inflammatory bowel disease, such as Crohn disease, affects ileum predominantly, and folate deficiency is infrequent in newly diagnosed children.[326]

Tropical sprue, discussed in the section on intestinal cobalamin malabsorption, can involve enough of the small intestine to disrupt both folate and cobalamin absorption, and folate deficiency is often an early feature.[295,296,327] In some regions of the world or at some stages of the disease, one deficiency predominates over the other. In florid cases, megaloblastic anemia is prominent. As mentioned previously, tropical sprue often responds to antibiotics, and folic acid not only improves megaloblastic anemia caused by folate deficiency but also sometimes reduces the severity of the sprue itself. Because cobalamin and folate deficiencies can coexist, testing for both deficiencies is advisable before instituting therapy.

It is unknown if fortification of the diet with folic acid, which can bypass the carrier-specific jejunal absorption of reduced, polyglutamated folates and is readily absorbed by nonspecific mechanisms in the ileum,[14,39] has lessened the clinical deficiency caused by malabsorptive diseases in general. These nonspecific, secondary absorption processes may also explain why oral folic acid given in high doses, such as 2 to 5 mg once or twice daily, can be effective in malabsorptive folate deficiencies.

Drug-Induced Disorders of Folate Absorption and Metabolism

Drug-induced malabsorption has been described with various medications, such as sulfasalazine and anticonvulsants, especially hydantoins.[16,328] The normally smaller stores–to–daily intake ratio makes folate theoretically easier to deplete than cobalamin by drug-induced mechanisms. Nevertheless, clinical consequences, such as megaloblastic anemia, have been infrequent. Hydantoins sometimes cause mild macrocytosis, but only potent antifolates, such as methotrexate, induce megaloblastic anemia. Valproic acid has been shown to inhibit fetal folate metabolism.[328] Oral contraceptives can lower serum folate levels, but the mechanism is unknown and no metabolic or clinical consequences are apparent.

An alkalinized gastrointestinal environment, whether caused by atrophic gastritis, drugs that reduce gastric acid secretion, or alkalinizing pancreatic replacement therapy for pancreatic insufficiency, reduces folate absorption but rarely induces clinical folate deficiency.[329-331]

Increased Folate Requirement

Requirements can rise in some situations[14,16] and may occasionally compromise folate status, especially when combined with marginal folate intake that limits dietary compensation. Pregnancy and lactation are common settings of increased folate demand.[332-334] High cell turnover rates in some diseases, most prominently chronic hemolytic anemias such as sickle cell disease and thalassemia,[14,335] provide another setting. Folic acid supplementation is therefore given to sickle cell patients routinely in clinical practice. However, megaloblastic changes have been unusual in most conditions of increased requirement, unless additional causes for folate deficiency coexist, such as poor diet.[332-335] A randomized clinical trial of folate supplementation reported equivocal clinical benefits in patients with sickle cell disease.[336] tHcy elevation is not unusual in patients with sickle cell diseases, but it appears to be unrelated to folate deficiency.[337] It appears prudent to screen folate-supplemented patients for pernicious anemia, which affects blacks disproportionately often at a young age, and has been found in at least four patients with sickle cell disease.[287,288] Psoriasis has been associated with possibly increased folate demand. Folate is lost during dialysis, and folate supplementation is usually provided routinely.[14]

DIAGNOSTIC APPROACH TO ACQUIRED CAUSES OF FOLATE DEFICIENCY

A careful history can usually identify absolute or relative insufficiency of dietary folate intake and can also identify the known conditions that increase folate requirements. Direct tests of folate absorption are not available, which makes independent search for individual malabsorptive disorders necessary. A contemporary clinical reassessment in the postfortification era is needed to determine which causes of folate deficiency persist and who remains at risk for folate deficiency.

TREATMENT OF ACQUIRED DEFICIENCIES

Reversal of Megaloblastic Anemia and Other Clinical Manifestations

The sequence of improvement after therapy is outlined in Table 10-7. The earliest clinical response in patients with megaloblastic anemia, whether caused by cobalamin or folate deficiency, can be gauged by the reticulocyte count, which begins to rise within 3 days and usually peaks by day 7. The intensity of the reticulocytosis varies inversely with the severity of the anemia. If pancytopenia existed, leukocyte and platelet counts also rise. Neutrophil hypersegmentation, however, persists for several weeks. Full normalization of all blood changes, including MCV elevation, is completed by 6 to 8 weeks. An incomplete hematologic response, detectable within a week by a poor reticulocyte response, requires assessment. Either the diagnosis was incorrect, including the possibility that the wrong vitamin was chosen, or coexisting disorders are present. Iron deficiency, for example, may not be recognized until the megaloblastic anemia is treated.[16,202] Neurologic and cognitive dysfunction arising from cobalamin deficiency begins to improve in the first week as well, but the speed and completeness of the response vary. Reversibility tends to vary inversely with the severity and duration of the original symptoms[16,173] and may be especially problematic in infants with developmental consequences.

TABLE 10-7 Sequence of Clinical and Hematologic Improvement with Effective Treatment of Cobalamin or Folate Deficiency

Time	Change*
Day 1	*Ineffective hematopoiesis reverses.* Serum iron and bilirubin levels fall. Serum unsaturated iron-binding capacity increases. Marrow iron stores decrease. *Patient may begin feeling better.* In cobalamin deficiency, serum folate decreases transiently.
Day 2	Increases begin in reticulocytes, WBC (if low), and platelets (if low). *Erythropoiesis becomes normoblastic.* Serum iron may become transiently low. Serum potassium decreases transiently (influx into new cells). *Tongue soreness (if present) begins to improve.*
Day 3	Serum lactate dehydrogenase level decreases. In folate deficiency, serum cobalamin level begins to increase.
Day 5	*Reticulocyte count begins to peak.* RBC count begins to increase, and MCV may begin to decrease. *WBC and platelet counts usually become normal.* RBC folate level increases. Any iron deficiency that may have coexisted can be identified reliably. *MMA and tHcy levels begin to improve.*
Week 2	*Tongue and gastrointestinal symptoms clear.* *Neurologic abnormalities improve.* *Neutrophil hypersegmentation reverses and giant metamyelocytes disappear from bone marrow.* MCV improves.
Weeks 6-8	*Blood cell count and RBC indices (e.g., MCV) become completely normal.*

Modified from Carmel R: Megaloblastic anemia. In Brain MC, McCulloch PB, editors: Current therapy in hematology/oncology 1983-1984. *Philadelphia, 1983, BC Decker.*

MCV, Mean corpuscular volume; *MMA,* methylmalonic acid; *RBC,* red blood cell; *tHcy,* total homocysteine; *WBC,* white blood cell.

*Changes that have special clinical relevance are shown in italics.

Box 10-3 **Management of Acquired Cobalamin Deficiency**

MANAGEMENT TASKS

1. Reverse the cobalamin deficiency.* A single injection ensures assimilation and can reverse deficiency but must be followed by further therapy to consolidate reversal (see below).
2. Identify and, if possible, treat the underlying causative condition.
3. Determine dose and route of cobalamin repletion and maintenance regimens. The details usually depend on the underlying cause and often on the patient's age.
4. Determine duration of therapy, which is usually dictated by the underlying disorder and its reversibility.
5. Monitor completeness of clinical and metabolic response to therapy to confirm that the diagnosis was correct and to identify coexisting, sometimes obscured pretreatment complications.
6. Determine the need for periodic follow-up if the underlying cause of cobalamin deficiency is irreversible.
7. Educate the patient and family about management goals, underlying causes, and prognosis.

*Clinically expressed cobalamin deficiency must always be treated. However, the need to treat subclinical, purely biochemical cobalamin deficiency is not established in many, if not most, cases. An exception may exist if the patient has an underlying cause (e.g., malabsorptive disease, such as pernicious anemia) that predicts the likelihood of progression to clinically expressed deficiency.

Patients with subclinical deficiency (i.e., isolated biochemical changes), who far outnumber patients with clinical deficiency, have no clinical signs and symptoms that can improve.[201,232] If clinical improvement occurs, the deficiency must be reclassified as having been clinical, and the likelihood of underlying malabsorption or other causes that persist or progress increases.

Management of Acquired Cobalamin Deficiencies

Management does not end with the identification and reversal of the cobalamin deficiency (Box 10-3). It is important to define why the deficiency occurred, which helps determine many of the management steps.[202]

The initial cobalamin dose is usually given parenterally to ensure its assimilation in case of undetected malabsorption and thus begin the reversal of the deficiency. Either cyanocobalamin or hydroxocobalamin can be used[16,107,202]; the therapeutic details of methylcobalamin have been studied less intensively. Adults and adolescents usually receive an intramuscular 1000 μg cobalamin dose, which ensures retention of approximately 150 μg when cyanocobalamin is used (retention varies inversely with the dose size); the rest is excreted immediately in the urine. Retention of hydroxocobalamin is greater, allowing less frequent doses,[104,338] but some hydroxocobalamin preparations induce antibody to transcobalamin, which inflates cobalamin levels without compromising cobalamin status.[338] For young children, whose stores (and thus the absolute deficit to be repleted) are much smaller, 100-μg or even 50-μg doses suffice and can mean a smaller intramuscular injection volume. The dose amount does not determine the rapidity and extent of response[16] but may affect its duration.

Despite its immediate effectiveness, a single dose never suffices in treating clinically expressed cobalamin deficiency, which reflects severe depletion and often arises from a serious, persistent underlying cause. Subsequent doses are needed to consolidate the response, replete the stores, and prevent relapse. The latter maintenance can be oral in many cases but requires more frequent, high doses in patients with malabsorption.[107] The dose, schedule, and route details depend on whether the underlying causative disorder involves malabsorption, whether the cause is reversible, and whether the immediate goal is repletion of stores (which may require 1 to 2 months of weekly injections or large daily oral doses) or later steady-state maintenance, which can be achieved with an injection of cyanocobalamin monthly or with hydroxocobalamin injection every 2 months[202]; it cannot be predicted whether some patients require more frequent maintenance).[202] The details also depend on the child's and family's acceptance of injections. Adults with pernicious anemia or other malabsorptive diseases can be maintained with daily oral doses of 1000 μg, but effective, age-appropriate doses in young children with malabsorption have not been systematically defined. Cobalamin is considered nontoxic, but the long-term effects of daily oral doses of 1000 μg are unknown, especially in children. Increased risk for some cancers, for example, has been reported in elderly persons chronically receiving high cobalamin and folic acid doses.[339]

Irreversibility of the underlying cause of cobalamin deficiency (e.g., pernicious anemia) or strong cultural strictures (e.g., veganism) require lifelong cobalamin therapy. The underlying causes themselves can also carry risks and complications and require independent management. For example, children with acquired pernicious anemia remain at lifelong risk for autoimmune endocrinopathies, gastric carcinoid tumors, and perhaps gastric cancer.

Clinical monitoring of the response to therapy, including occasional metabolic ascertainment, serves many purposes.[202] As discussed in the previous section, therapeutic improvement confirms whether the deficiency was correctly diagnosed and can also unmask coexisting conditions. Full correction of cobalamin deficiency can be detected by a normal tHcy or MMA level by the second or third week. Vitamin levels (e.g., cobalamin and holo-TC) can be misleading monitors of therapeutic response, as discussed previously.[202,211] Metabolic monitoring can also provide early evidence of impending relapse after remission has been achieved. Monitoring can be continued annually in older children and more often in infants and those with severe underlying disorders.

Education of the patient and parents about the underlying disorder, its nature, and its prognosis is crucial. Patients and families too often discontinue treatment, even in pernicious anemia, and suffer clinical relapse.[175] Children also often discontinue maintenance therapy once they become independent.

Management of Acquired Folate Deficiencies

Folate deficiency is now rare in countries with folic acid–fortified diets.[23,209,238] Therefore management of the few adults and older children who do become folate deficient merits special inquiry. Dietary deficiency is easily treated by daily oral 1-mg doses of folic acid in adults and older children. In infants, megaloblastic anemia responds to doses as small as 10 to 50 μg, and 50 μg daily can maintain remission.[16] Parenteral folic acid is rarely needed unless the patient cannot take oral medications. Patients with malabsorptive disorders often respond to oral therapy, although larger folic acid doses up to 5 mg are usually given, depending on the child's age and size; however, monitoring of the response is advisable. Treatment with a reduced folate form, such as folinic acid or methylTHF, does not provide documented advantages in acquired deficiency unless a metabolic defect is suspected. Coexisting cobalamin deficiency should always be excluded before treatment, especially when large folic acid doses are used.

As in cobalamin deficiency, clinical improvement occurs within a week in clinically folate-deficient patients (see Table 10-7), whereas patients with subclinical deficiency show only biochemical change. Stores usually become repleted within 1 to 3 months, and continuation of folic acid therapy beyond that period is needed only if the underlying cause of the deficiency is irreversible. Continued status can be monitored more effectively and reliably with tHcy than with measurements of serum or red cell folate levels, which increase regardless of effectiveness.

Management of Subclinical Folate Deficiency

Folate deficiency continues to be a problem in countries without folic acid fortification of the diet. Even in countries with fortification and widespread self-supplementation, some infants and adolescents have marginal folate status, often without clinical expression. The health impact of subclinical folate deficiency, including the potential for it in persons with *MTHFR* polymorphisms, has been associated with a wide variety of disorders in the maternofetal setting (e.g., NTDs and other malformations), in infancy, and in adults (e.g., cognitive dysfunction, thrombosis, and cancer risk). However, aside from NTDs, evidence of causality and benefit from intervention has often proved disappointing in randomized clinical trials, with thrombotic risk being the most notable example.[73]

Folate Toxicity

In countries with fortified diets, many normal adults exceed the recommended 1-mg intake ceiling,[14] and self-supplementation augments intake further. Although folic acid is generally regarded as safe, the risk for masking the megaloblastic anemia of untreated cobalamin deficiency has been a long-standing clinical concern whose facts remain incompletely understood,[161,162] high oral doses reduced the effectiveness of epileptic control in some patients,[340] animals given very high intravenous doses displayed nephrotoxicity, and a clinical trial of high oral doses in patients with diabetic nephropathy found increased renal and vascular complications.[341] Moreover, broader questions of unrecognized long-term risks of high folate status are beginning to be examined. Unreduced folic acid, which can act as an oxidizing agent, accumulates in plasma and cells, but its impact is unknown.[342,343] The seemingly binary relationship between folate intake and cancer is discussed in another section, but a few specific cancer risks have been reported.[339] An Indian clinical trial comparing preventive effects of cobalamin and folic acid supplementation on infection risks in young children found that diarrhea and some respiratory tract infections actually increased with folic acid supplementation.[271] Many of the reports of toxicity need confirmation, but so do many claims of preventive benefits. Caution and more firm data are needed to determine the preventive benefits and risks for exposing normal individuals, especially children, to chronically high intakes of vitamins.

GENETIC DISORDERS CAUSING MEGALOBLASTIC ANEMIA

In addition to dietary and acquired deficiencies of folate and cobalamin, several rare genetic disorders can cause megaloblastic anemia. These include disorders affecting uptake and metabolism of folate or cobalamin, as well as thiamine responsive megaloblastic anemia, orotic aciduria, and 3-phosphoglyceride dehydrogenase deficiency.

Inborn Errors of Folate Transport and Metabolism

Hereditary Folate Malabsorption

Hereditary folate malabsorption (MIM 229050) has been reported in approximately 30 patients.[344-346] It is characterized by megaloblastic anemia, diarrhea, mouth ulcers, failure to thrive, and usually progressive neurologic deterioration. Megaloblastic anemia and low serum folate levels appear in the first few months of life. Patients fail to absorb folates across both the intestinal epithelium and the choroid plexus, resulting in decreased folate levels in blood and CSF. The uptake of folate by cultured and uncultured cells is within reference ranges. Mutations in the *SLC46A1* gene on chromosome 17q11.2, which encodes PCFT, have been identified in a number of patients.[34,347,348]

Hematologic changes respond to parenteral therapy with folic acid at doses used to treat dietary folate deficiency, or to pharmacologic doses of oral folic acid (up to 60 mg daily).[349] However, folic acid has proven ineffective in raising CSF folate levels even when given parenterally, and all successful treatments of hereditary folate malabsorption have utilized folinic acid. Successful therapeutic regimens have typically utilized parenteral folinic acid, although some patients have been successfully treated using oral folinic acid.[346] Doses and schedules have varied. Monitoring of CSF folate levels is critical, with modification of dose to ensure that it is maintained within the normal range (typically >15 ng/mL). Prompt diagnosis and commencement of therapy is important because neurologic deficits, if untreated, may become irreversible.

Cerebral Folate Deficiency

A second disorder of folate uptake results in isolated decreased cerebral methylTHF uptake and levels (MIM136430), resulting in neurologic findings (developmental delay or regression, hypotonia, seizures, visual disturbances, and autistic features), but it does not cause megaloblastic anemia.[350] This syndrome can therefore be difficult to connect to folate because all expected systemic features of folate deficiency are lacking: besides the hematologic normality, plasma tHcy and serum and red cell folate levels are normal, and intestinal folate absorption is normal. The disorder is caused by decreased function of folate receptor-α at the choroid plexus, as a result of either antibodies against folate receptor-α[351] or mutations in the *FOLR1* gene on chromosome 11q13.4.[352,353] Treatment with folinic acid (0.5 to 1 mg/kg) relieves many symptoms, although seizures may remain resistant to treatment. Folic acid therapy should be avoided.

Severe Methylenetetrahydrofolate Reductase Deficiency

Severe MTHFR deficiency (see Fig. 10-2, reaction 8; MIM 236250) is the most common inborn error of folate metabolism, with more than 100 cases known. Most patients come to medical attention as infants, with developmental delay, breathing disorders, gait abnormalities, seizures, and microcephaly. Other patients have presented later in childhood or as adults, and some remain clinically unaffected.[354-357] Elevated tHcy levels and homocystinuria are always present, whereas the serum methionine level is in the low-normal range or decreased.[358] This contrasts to classic homocystinuria due to cystathionine β-synthase deficiency, which is characterized by higher homocysteine levels and elevated methionine. In contrast to other disorders affecting homocysteine remethylation, megaloblastic anemia is rarely present, reflecting the lack of trapping of folate as methylTHF. Because methylTHF is the major circulating form of folate, MTHFR deficiency may result in functionally low levels of folate in the brain. MTHFR deficiency is inherited as an autosomal recessive trait. More than 55 mutations in the *MTHFR* gene on chromosome 1p36.3 have been identified in patients with severe MTHFR deficiency.[359-363]

The diagnosis of severe MTHFR deficiency is established by measurement of enzyme activity in the liver, leukocytes, or cultured fibroblasts or lymphocytes. The disorder has been difficult to treat, with a poor prognosis once neurologic involvement is evident. The most successful treatment has been with oral administration of betaine, which is a substrate for the liver enzyme betaine:homocysteine methyltransferase and has the double advantage of raising methionine and decreasing homocysteine levels. Treatment is most effective when it is started at an early age before neurologic damage becomes established.[364,365]

Dihydrofolate Reductase Deficiency

Three families have been described with deficiency of dihydrofolate reductase (MIM 613839), the enzyme responsible for regenerating THF from dihydrofolate formed during the reaction catalyzed by thymidylate synthase (see Fig. 10-2, reaction 3).[366,367] Patients have been characterized by megaloblastic anemia (in some cases pancytopenia) and neurologic problems including seizures and, in severely affected individuals, cerebral and cerebellar white matter atrophy. There were decreased cerebral folate levels in the presence of normal serum folate but decreased red cell folate levels. Dihydrofolate reductase activity was decreased in cell extracts, and mutations in the *DHFR* gene on chromosome 5q14.1 were identified in all affected individuals. Treatment with folinic acid resulted in correction of hematologic abnormalities, although neurologic problems persisted.

Glutamate Formiminotransferase Deficiency

Glutamate formiminotransferase deficiency (MIM 229100) is caused by mutations in the *FTCD* gene on chromosome 21q22.3 that encodes the bifunctional enzyme containing glutamate formiminotransferase and formiminoTHF cyclodeaminase activities. These sequential reactions remove the formimino group from formiminoglutamate, formed during catabolism of histidine in the liver and kidney, to form formiminoTHF, which is converted to 5,10-methenylTHF (see Fig. 10-2, reactions 6 and 7).[368] Formiminoglutamate excretion, either constitutively or in response to a histidine load, is the one constant feature of the disorder. It is not clear whether this disorder, described in fewer than 20 patients, is associated with any consistent clinical findings. Two distinct phenotypes have been described: a severe form characterized by mental and physical retardation, abnormal electroencephalographic activity, and dilatation of the cerebral ventricles with cortical atrophy; and a mild form, with no mental retardation but higher levels of formiminoglutamate excretion.[358] Several patients had macrocytosis and hypersegmentation of neutrophils. Because the relationship between clinical expression and formiminoglutamate excretion is uncertain, the value in reducing formiminoglutamate excretion is unknown. *FTCD* mutations have been identified in two families with this disorder.[369]

MTHFD1 Deficiency

A single patient has been described with deficiency of the trifunctional enzyme encoded by the *MTHFD1* gene, which catalyzes synthesis of 10-formylTHF from THF and mitochondrially derived formate (formylTHF synthetase) and the conversion of 10-formylTHF to 5,10-methenylTHF (methenylTHF cyclohydrolase) and 5,10-methyleneTHF (methyleneTHF dehydrogenase; see Fig 10-2, reactions 9, 10, and 11).[370,371] The patient presented with megaloblastic anemia, atypical hemolytic uremic syndrome, and severe combined immune deficiency, which was partially responsive to therapy with hydroxocobalamin and folates. The patient has been shown to be heterozygous for two missense mutations in the *MTHFD1* gene.[370]

Inborn Errors of Cobalamin Transport and Metabolism

Inborn Errors of Cobalamin Absorption

Cobalamin absorption can be impaired by mutations affecting either IF production (hereditary IF deficiency; MIM 26100) or the ileal cubam receptor that recognizes the IF-cobalamin complex (Imerslund-Gräsbeck syndrome; MIM 261100). Symptoms typically appear after the first year of life but before age 5 years, reflecting time required for sufficiently extensive depletion of cobalamin stores, although onset as late as adolescence has been observed.[372] In addition to megaloblastic anemia there may be failure to thrive, weight loss, and recurrent gastrointestinal or respiratory tract infections.

Fewer than 100 cases of IF deficiency have been published, but the disorder is more frequent than this number suggests. Unlike in classic pernicious anemia, patients have no evidence of gastritis; gastric acid secretion is normal, there is no hypergastrinemia, antibodies against gastric parietal cells and against IF are absent, and increased risk for gastric cancer or carcinoids has not been reported. However, occasional patients show basal achlorhydria,[372] and reports have appeared of adult relatives who develop classic pernicious anemia with antibodies. There may be no detectable IF or production of an immunologically reactive but nonfunctional IF.[373-375] Mutations have been described in the *GIF* gene on chromosome 11q12.1 in several patients with this disorder,[376,377] including some patients who received a mistaken clinical diagnosis of Imerslund-Gräsbeck syndrome.

At least 300 cases of Imerslund-Gräsbeck syndrome (megaloblastic anemia 1), which is caused by deficiency in the ileal cubam receptor, have been described. This suggests that it is the most common hereditary cause of cobalamin deficiency. Many patients have proteinuria that is neither of the classic glomerular or tubular type and does not progress to kidney failure,[378] reflecting the role of cubam in reabsorption of filtered proteins, including albumin, in the kidney.[379] The disorder was initially recognized among Finns and Norwegians, but it is now more frequently diagnosed in patients from eastern Mediterranean countries. Imerslund-Gräsbeck syndrome may be caused by mutations affecting either the *CUBN* (chromosome 10p13) or *AMN* (chromosome 14q32.32) gene.[380-384] A mutation in the *CUBN* gene has been shown to cause isolated proteinuria in one family,[385] and a specific missense variant in the gene has been described as a risk factor for albuminuria and possibly for end-stage renal disease.[386,387]

With the disappearance of the Schilling test for malabsorption,[290] which can usually distinguish between loss of IF and failure of its uptake in the intestine, clinical differentiation between IF deficiency and Imerslund-Gräsbeck syndrome has become more problematic, leading to the suggestion that sequencing of the causative genes (*GIF, AMN,* and *CUBN*) represents the optimal first-line approach strategy for accurate diagnosis.[377] Treatment of both IF deficiency and Imerslund-Gräsbeck syndrome involves intramuscular injection of cobalamin, bypassing the block in intestinal cobalamin absorption. The goals are reversal of the deficiency, replenishment of body stores, and lifelong maintenance therapy. The details are similar to those discussed in the section on acquired malabsorptive disorders.

Transcobalamin Deficiency

More than 50 patients with transcobalamin deficiency (MIM 275350) are known. Because the direct compromise of cellular delivery bypasses cobalamin stores, patients develop severe anemia at a much earlier age than do patients with malabsorption, usually in the first months of life. Other symptoms include failure to thrive, weakness, and diarrhea. The anemia is clearly megaloblastic, but some patients present with pancytopenia or even isolated erythroid hypoplasia.[388] The presence of immature white cell precursors in a marrow that is otherwise hypocellular can lead to the misdiagnosis of leukemia.[389,390] For unknown reasons, neurologic disease is less prominent than in other cobalamin disorders but can appear from 6 months to 30 months after initial presentation,[391-394] in some cases because diagnostic delay led to therapeutic delay as well as sporadic treatment with folic acid.[395] Severe immunologic deficiency with defective cellular and humoral immunity has been reported, as has defective granulocyte function. Serum cobalamin levels are usually normal,[392] because most circulating cobalamin exists bound to haptocorrin, which is not affected, but occasional patients have low serum cobalamin levels.[392,396] Patients may have no detectable transcobalamin, or immunologically reactive transcobalamin that is unable to mediate vitamin delivery into cells.[397,398]

The diagnosis of deficiency requires measuring plasma transcobalamin levels by immunoassay or by transcobalamin-specific cobalamin-binding capacity assay; neither test is widely available. Transcobalamin is synthesized by cultured cells, including fibroblasts and amniocytes[399]; deficiency can be diagnosed by measurement of ability of cultured cells to take up labeled cobalamin in the absence of exogenous transcobalamin. Mutations in the *TCN2* gene on chromosome 22q12.2 have been identified.[400,401] Cobalamin treatment involves high-dose parenteral hydroxocobalamin (1000 mg); once cobalamin body stores have been replenished, injections at least once weekly are required to maintain health.[401] Monitoring of hematologic or biochemical parameters is necessary to ensure effectiveness of therapy. Therapy with cyanocobalamin instead of hydroxocobalamin and oral instead of intramuscular administration may be associated with poorer outcome.[401]

Haptocorrin (Transcobalamin I) Deficiency

Severe hereditary deficiency of haptocorrin (MIM 193090) is characterized by absent or near-absent haptocorrin in plasma, granulocytes, and all exocrine secretions.[402-404] Because haptocorrin, unlike transcobalamin, does not deliver its cobalamin to cells, cobalamin deficiency does not develop. However, because it carries more than 70% of cobalamin in plasma at any given moment, haptocorrin deficiency causes low serum cobalamin levels, often less than 100 ng/L in severe haptocorrin deficiency and only slightly low or low-normal in mild haptocorrin deficiency, in which haptocorrin levels are mildly low. Deficiency is typically identified in adulthood, when a patient might undergo cobalamin testing for unrelated reasons and typically receives an initial misdiagnosis as cobalamin deficiency and unnecessary treatment.

Only nine cases of severe haptocorrin deficiency have been published, which represents underreporting of an easily overlooked, symptom-free condition. A prospective survey reported absent haptocorrin in 3 of 537 (0.6%) sera with low cobalamin levels.[223] The latter study also found mild haptocorrin deficiency in another 15% of such blood samples, compared with 2.8% to 5.4% of various control groups. All nine known patients with severe deficiency to date have been nonwhite, and all who were genotyped were homozygotes or compound heterozygotes for nonsense or missense *TCN1* mutations, most often the nonsense mutation 270delG. Heterozygosity for this or other mutations has been found in more than half of unrelated persons with mild haptocorrin deficiency and in all mildly affected relatives of propositi with severe deficiency, but not in unaffected relatives.[288,405,406]

Several patients with haptocorrin deficiency have also had neurologic problems that appear to be unrelated to the low cobalamin levels or the haptocorrin deficiency[402-404,407]; for example, cobalamin therapy did not prevent progression of neurologic disease in the equally severely haptocorrin-deficient sibling of the original propositus.[402,403,407] A child with hereditary deficiencies of both IF and haptocorrin (both encoded by genes on the long arm of chromosome 11) has been described.[408] The original family with severe haptocorrin deficiency[402] was later shown to have combined haptocorrin and lactoferrin deficiencies[407]; these two proteins are coexpressed in neutrophil specific granules and secretions, but the family had the *TCN1* 270delG mutation, leaving the coexisting lactoferrin deficiency unexplained. Finally, it is noteworthy that globin mutations, especially hemoglobin S and C, coexist disproportionately often with haptocorrin deficiency in blacks, but the mutational dosages do not always match.[288]

Mild haptocorrin deficiency may be responsible for many unexplained low cobalamin levels without clinical or subclinical deficiency.[223] However, it is more difficult to diagnose than severe deficiency in which haptocorrin is absent. The diagnosis should not be made without objective evidence, using haptocorrin measurement or genetic testing, as happened in one poorly documented case,[409] where improvement of neuropathy and mild macrocytosis after cobalamin treatment was also overlooked.

Acquired haptocorrin deficiencies appear to be limited to plasma and can accompany severe neutropenic conditions.[407] Other proposed associations of acquired haptocorrin deficiencies include human immunodeficiency virus disease, multiple myeloma, and metformin use and require further study.

Inborn Errors of Cellular Cobalamin Metabolism

Several inborn errors of cobalamin metabolism, designated cblA to cblG, cblJ, and cblX, have been identified, each representing mutations at a different gene.[153,410-414] These disorders result in isolated hyperhomocysteinemia and homocystinuria, isolated methylmalonic acidemia and aciduria, or combined homocystinuria and methylmalonic aciduria, depending on whether synthesis of MeCbl, AdoCbl, or both cobalamin cofactors is affected. Only those disorders in which synthesis of MeCbl is impaired, either alone (cblE, cblG, cblD variant 1) or in combination with AdoCbl synthesis (cblC, cblD, cblF, cblJ, cblX), are characterized by megaloblastic anemia. Disorders in which synthesis of AdoCbl alone is impaired (cblA, cblB, cblD variant 2) are not associated with megaloblastic anemia. Traditionally, these disorders have been differentiated on the basis of studies of cultured fibroblasts.[415,416] With the identification of the genes underlying each of these disorders, specific diagnosis can now be achieved by mutation analysis of potentially causative genes in patient genomic DNA. All of these disorders are inherited as autosomal recessive traits, with the exception of cblX, which is X-linked.

Functional Methionine Synthase Deficiency. Functional methionine synthase deficiency is characterized by elevated tHcy, homocystinuria, and hypomethioninemia without methylmalonic aciduria. On the basis of complementation analysis, three distinct groups of patients have been identified: cblE (MIM 236270); cblG (MIM 250940); and cblD variant 1 (MIM 277410). The clinical and biochemical findings in these disorders are virtually identical. Patients usually present in the first 2 years of life, but some have not been symptomatic until adulthood. Patients typically present with megaloblastic anemia; neutropenia, thrombocytopenia, and pancytopenia have been reported. Other findings have included developmental delay, cerebral atrophy, electroencephalographic abnormalities, nystagmus, hypotonia, hypertonia, ataxia, seizures, microcephaly, and blindness.[417] Renal problems, including atypical hemolytic uremic syndrome and an atypical glomerulopathy, have been described in cblE patients.[418,419] Findings in patients with adult onset of symptoms have typically involved neurologic or psychiatric symptoms. One patient with onset at 21 years of age was diagnosed as having multiple sclerosis.[420]

Fibroblasts from all three groups of patients show decreased synthesis of MeCbl and decreased methionine synthase function in the presence of normal AdoCbl synthesis and methylmalonyl-CoA mutase activity.[417,421] Methionine synthase specific activity in cell extracts is decreased in cblG, which is caused by mutations in the *MTR* gene that encodes methionine synthase.[422,423] In the cblE disorder, methionine synthase specific activity is normal when the assay is performed in the presence of adequate reducing agent, but unlike in cblG, activity is decreased when the assay is performed using suboptimal levels of reducing agent.[417] The cblE disorder is caused by mutations in the *MTRR* gene, which encodes methionine synthase reductase; this protein maintains the enzyme-bound cobalamin cofactor in its active reduced form.[424] The cblD disorder is discussed in the next section on combined deficiency of AdoCbl and MeCbl.

Generally, systemic therapy with OHCbl, at first daily and then once or twice weekly, has been used to treat these disorders. Treatment with betaine, which increases homocysteine remethylation by the liver enzyme betaine homocysteine methyltransferase, has also been recommended.[365] Treatment corrects the anemia and metabolic abnormalities. The neurologic findings have been difficult to reverse, particularly in cblG disease; this stresses the importance of early diagnosis and therapy. A mother carrying a fetus identified by prenatal diagnosis as having the cblE disorder was treated from the second trimester with systemic OHCbl.[425] The infant was treated from birth and has done very well. It is uncertain how much prenatal therapy contributed to this favorable outcome.

Combined Deficiency of Adenosylcobalamin and Methylcobalamin. The cblC, cblD, cblF, cblJ, and cblX disorders are characterized by decreased synthesis of both MeCbl and AdoCbl, with resultant deficient activity of both methionine synthase and methylmalonyl-CoA mutase. Affected individuals present with combined homocystinuria and methylmalonic aciduria. These disorders affect early steps in cobalamin metabolism subsequent to hydrolysis of the holo-TC complex in lysosomes. In the cblF and cblJ disorders, transfer of free cobalamin from lysosomes to the cytoplasm is affected (see Fig. 10-5). The cblC, cblD, and cblX disorders affect metabolism of cobalamin after it enters the cytoplasm and before it becomes associated with either methionine synthase in the cytoplasm or methylmalonyl-CoA in the mitochondria.

The cblC Disorder. The cblC disorder (MIM 277400) is the most common of the inborn errors of cobalamin metabolism, with more than 550 patients identified. Patients have homocystinuria and methylmalonic aciduria, with hypomethioninemia and cystathioninuria. Presentation is usually in the first year of life, with lethargy, poor feeding, failure to thrive, and developmental delay.[426-428,429] Most patients have megaloblastic anemia, and some have hypersegmented neutrophils and thrombocytopenia. Others have onset later in childhood[430,431] or in adulthood,[432-434] with spasticity, myelopathy, delirium, dementia, or psychosis. Some patients have had onset of problems prior to birth.[428,435] A characteristic pigmentary retinopathy with perimacular degeneration has been observed in several patients,[431,436-438] and a number of patients have had an atypical hemolytic uremic syndrome.[429,439] A high frequency of structural heart defects has been reported.[440]

Fibroblasts show decreased synthesis of both MeCbl and AdoCbl and decreased function of both methionine synthase and methylmalonyl-CoA mutase. Endocytosis of the transcobalamin-cobalamin complex and release of cobalamin from lysosomes into the cytoplasm occur normally,[441] but total intracellular cobalamin levels are markedly decreased in most cases, reflecting inability of cells to retain cobalamin that is not bound to intracellular proteins. The cblC disorder is caused by mutations in the *MMACHC* gene on chromosome 1p34.1.[442] The MMACHC gene product binds cobalamin after its release from the lysosome and appears to play a role in removal of its upper axial ligand, generating a partially reduced form that can be converted to MeCbl or AdoCbl.[154-156]

Therapy can be difficult, particularly in patients with early onset. In one series, about one third of patients with onset in the first month of life died.[426] Parenteral OHCbl therapy, 1 mg/day, has been shown to reduce MMA and homocystine excretion. OHCbl rather than CNCbl should be used, and parenteral OHCbl treatment is much more effective than oral therapy.[443,444] In some cases, frequency of dosage has been reduced as the patient's condition stabilizes, although this may be associated with suboptimal outcome.[428] One study found that extremely high doses of OHCbl (up to 20 mg/day) were necessary to maintain biochemical control.[445] Betaine (250 mg/kg/day) appears to be helpful when used in combination with OHCbl. Neither folinic acid nor carnitine has any effect.[443] Therapy with daily oral betaine and biweekly injections of OHCbl reduced MMA levels, normalized serum methionine and tHcy concentrations, and resolved lethargy, irritability, and failure to thrive. However, complete reversal of the neurologic and retinal findings did not occur; this emphasizes the need for early diagnosis and treatment. Even with good metabolic control, surviving patients usually have moderate to severe developmental delay.[426,429] The prognosis appears to be better in patients with a later age at onset.

The cblD Disorder. The cblD disorder (MIM 277410) was initially described in a patient who presented at age 14 years with a history of mild mental retardation, severe behavioral pathology, and a poorly defined neuromuscular problem, with no hematologic findings. His younger brother, clinically asymptomatic, was shown to have the same disorder[446,447]; interestingly, deoxyuridine suppression testing demonstrated cobalamin deficiency in bone marrow cells despite their normal morphologic appearance.[447] Both boys had methylmalonic aciduria and homocystinuria.[446] Fibroblasts showed a biochemical phenotype indistinguishable from that of cblC fibroblasts, but their disorder could be differentiated from cblC by complementation analysis.[411] Subsequently identified cblD patients have presented with isolated homocystinuria in the absence of methylmalonic aciduria (cblD variant 1), whereas others presented with methylmalonic aciduria in the absence of homocystinuria (cblD variant 2). All cases of this disorder are caused by mutations in the *MMADHC* gene on chromosome 2q23.2.[448] The product of the *MMADHC* gene interacts with the MMACHC protein and appears to be involved in determining whether cobalamin becomes associated with methionine synthase in the cytoplasm or with methylmalonyl-CoA mutase in the mitochondria. Mutations affecting its carboxy terminal region were associated with isolated homocystinuria, and mutations affecting the amino-terminal region were associated with isolated methylmalonic aciduria, whereas truncating mutations were associated with combined homocystinuria and methylmalonic aciduria.

The cblX Disorder. Approximately a dozen patients have been described with decreased synthesis of both AdoCbl and MeCbl as a result of mutations affecting the *HCFC1* gene on chromosome Xq28.[414] These patients had received a diagnosis of cblC disorder on the basis of complementation analysis, but no *MMACHC* mutations could be identified. Clinically they have been characterized by a particularly severe neurologic disorder, while at the cellular level cobalamin coenzyme synthesis appears less severely affected than in the cblC disorder. The *HCFC1* gene encodes a transcriptional coregulator that in conjunction with a second factor, THAP11, is required for expression of several genes, including *MMACHC*.

Expression of MMACHC is markedly decreased in fibroblasts from cblX patients.

The cblF and cblJ Disorders. Fifteen patients with the cblF disorder (MIM 277380) and three with the cblJ disorder (MIM 614857) have been described. Clinical presentation has been variable, with no findings shared by all the patients who have been studied. Frequent findings include being small for gestational age, poor feeding, failure to thrive, growth retardation, and persistent stomatitis. Macrocytic anemia, neutropenia, thrombocytopenia, and pancytopenia have been observed. Two patients had minor facial abnormalities, and at least three have had cardiovascular defects.[152,449-453] Typically, there is hyperhomocysteinemia and methylmalonic aciduria, although elevated tHcy could not be detected in the blood of the first patient with cblF disorder who was described. Cobalamin absorption and serum cobalamin levels have been decreased in several patients.[454-456]

Fibroblasts from patients with cblF and cblJ disorders show decreased function of both cobalamin-dependent enzymes and decreased synthesis of both MeCbl and AdoCbl. Unlike cblC and cblD fibroblasts, there is accumulation of large amounts of intracellular cobalamin, with almost all of it being unmetabolized free CNCbl present within lysosomes.[153,449,457,458] The defects in both disorders appear to impair transport of cobalamin that has been released from transcobalamin across the lysosomal membrane into the cytoplasm. The gene affected in the cblF disorder is *LMBRD1* on chromosome 6q13, which encodes a lysosomal membrane protein of unknown function.[152] The cblJ disorder is caused by mutations in the *ABCD4* gene on chromosome 14q24.3, which encodes a lysosomal membrane ATP-binding cassette transporter.[153] Abnormal intestinal absorption of cobalamin has been documented in two patients with cblF disorder, suggesting that the same lysosomal transport system plays a role in transport of cobalamin across ileal cells.[454,455]

The patients with these disorders appear to respond to systemic therapy with OHCbl, although the first patient responded to oral cobalamin. A concern is that patients may accumulate cobalamin in lysosomes to such an extent that it impairs other lysosomal functions, although there is no evidence to date that this has occurred.

Thiamine-Responsive Megaloblastic Anemia

The thiamine-responsive megaloblastic anemia syndrome (TRMA; MIM 249270) is a rare autosomal recessive disorder characterized by the triad of megaloblastic anemia, diabetes mellitus, and sensorineural deafness that responds to pharmacologic doses of thiamine.[459-463] The bone marrow is characterized by megaloblastic changes and ringed sideroblasts. In some cases, pancytopenia has been reported.[464,465] Additional clinical findings have included visual impairment (optic atrophy, retinal dystrophy, Leber congenital amaurosis), cardiomyopathy, neurologic problems (strokelike episodes, seizures), and short stature.[462,466] More than 70 affected individuals have been reported. Presentation is typically within the first 5 years of life. Megaloblastic anemia and diabetes have responded to therapy with oral thiamine hydrochloride, although in some cases thiamine therapy became ineffective at puberty and patients required ongoing insulin therapy and blood transfusion.[462,467] Hearing loss appears to be irreversible even with thiamine treatment.[468]

Thiamine (vitamin B_1), essential for cellular metabolism, is synthesized by bacteria, fungi, and plants. Humans must acquire the vitamin in their diets. Uptake of thiamine by cells is mediated by two high-affinity thiamine transporters: thiamine transporter 1, encoded by the *SLC19A2* gene on chromosome 1q24.2; and thiamine transporter 2, encoded by the *SLC19A3* gene on chromosome 2q36.3. Once taken up by cells, thiamine is converted to its active coenzyme derivative, thiamine pyrophosphate, by the enzyme thiamine pyrophosphokinase (EC 2.7.6.2). Thiamine pyrophosphate is required for the activity of four known mammalian enzymes: transketolase (EC2.2.1.1), a component of the nonoxidative pentose phosphate pathway; pyruvate dehydrogenase (EC1.2.4.1), which converts pyruvate to acetyl-CoA; oxoglutarate dehydrogenase (EC1.2.4.2), which converts α-ketoglutarate to succinyl-CoA; and branched-chain α-ketoacid dehydrogenase (EC1.2.4.4), which is involved in catabolism of branched-chain amino acids. Impaired activity of transketolase has been suggested to underlie megaloblastosis in thiamine-responsive megaloblastic anemia.[469]

All cases of TRMA that have been investigated are caused by mutations affecting the *SLC19A2* gene.[462,463,470-472] Fibroblasts from patients with TRMA have decreased thiamine uptake relative to control fibroblasts and, unlike control cells, undergo apoptosis when incubated in medium lacking thiamine.[473] More than 30 mutations in the *SLC19A2* gene have been reported in patients with TRMA.[462,463] The tissue specificity of the clinical findings in TRMA may be the result of patterns of expression of the two thiamine transporter proteins, with tissues that do not express thiamine transporter 2 *(SLC19A3)* being affected.

Hereditary Orotic Aciduria

Fifteen well-established cases of hereditary orotic aciduria (MIM 258900) have been described over the past 40 years.[474-476] Patients are characterized by a macrocytic hypochromic megaloblastic anemia that does not respond to therapy with either folate or cobalamin and by orotic acid crystalluria. Additional findings observed in some patients have included some degree of physical and intellectual delay, urinary tract obstruction (due to orotic acid crystals), cardiac malformations, strabismus, and immune problems. Biochemical studies have demonstrated decreased function of uridine monophosphate synthase, a bifunctional enzyme that contains the two terminal enzymes involved in de novo synthesis of uridine monophosphate: orotate : pyrophosporyl transferase (EC2.4.2.10) catalyzes conversion of orotic acid to orotidine monophosphate; and orotidine monophosphate decarboxylase (EC4.1.1.23), which catalyzes conversion of orotidine monophosphate to uridine monophosphate. Analysis of the *UMPS* gene on chromosome 3q21.2 in two patients with hereditary orotic aciduria demonstrated the presence of causative mutations.[477]

Patients have generally responded well to treatment with oral uridine (50 to 300 mg/kg/day) with resolution of megaloblastic anemia and reduction of orotic acid excretion, although a single patient required intramuscular uridine.[478] Requirement for uridine supplementation is lifelong.

3-Phosphoglyceride Dehydrogenase Deficiency

Megaloblastic anemia has been reported in some, but not all, patients with 3-phosphoglycerate dehydrogenase (EC 1.1.1.95) deficiency (MIM 601815),[479,480] a rare disorder that results in impaired ability to synthesize serine from 3-phosphoglycerate. Although serine is a nonessential amino acid, impairment of this pathway results in severely decreased levels of serine in the serum and CSF,[480] demonstrating that the diet alone does not provide sufficient serine to support normal metabolism. Patients with this disorder are characterized by microcephaly beginning prior to birth, with severe psychomotor delay and intractable seizures developing during the first year of life. Cataracts, hypogonadism, adduction of the thumbs, and thrombocytopenia were present in some patients.

The disorder is characterized biochemically by markedly decreased levels of serine in CSF and by decreased plasma serine after an overnight fast. The low levels of serine in the CSF may reflect relatively low affinity of the neutral amino acid transporter for serine, leaving the central nervous system particularly dependent on de novo serine synthesis to meet its metabolic needs.[480] The level of methylTHF in CSF is also reduced.[479] Because under normal conditions serine represents the major source of one-carbon units for cellular folate metabolism, serine deficiency may thus result in decreased levels of folate coenzyme forms required for nucleotide synthesis. Patients have been treated with high doses of serine, and in some cases with glycine as well. Treatment has resulted in correction of biochemical deficits and decreased frequency of seizures, but no progress in psychomotor development has been observed in patients when treatment was started after 1 year of age.[481] One affected individual who was identified by prenatal diagnosis and who was treated with serine beginning in the prenatal period has shown normal development.[482]

Mutations in the *PHGDH* gene on chromosome 1q12 have been identified in several patients with 3-phosphoglyderate dehydrogenase deficiency.[483,484] Seven different mutations have been identified, including six missense and one deletion that results in a frameshift. All mutations resulted in enzymes with very low residual activity in vitro.[483,484]

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CHAPTER 11

Disorders of Iron and Copper Metabolism, the Sideroblastic Anemias, and Lead Toxicity

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CHAPTER OUTLINE

PHYSIOLOGIC CHEMISTRY OF IRON
Iron and Oxidation
Iron-Protein Complexes
Iron Toxicity
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LEAD TOXICITY
Sources of Lead
Biochemical and Hematologic Effects of Lead
Treatment of Lead Poisoning

Iron lacks the glitter of gold and the sparkle of silver but outshines both in biologic importance. This plebeian metal is vital to the function of a wide variety of critical enzymes, including catalases, aconitases, ribonucleotide reductase, peroxidases, and cytochromes, which exploit the flexible redox chemistry of iron to execute a number of chemical reactions essential for our survival (Box 11-1). In addition, we depend on hemoglobin, another iron-containing protein, to transport inhaled oxygen from the lungs to peripheral tissues. Human existence is inextricably linked to iron, and disturbances in its metabolism may have dire consequences.

PHYSIOLOGIC CHEMISTRY OF IRON

Iron and Oxidation

The key to the biologic utility of iron is its ability to exist in either of two stable oxidation states: Fe^{2+} (ferrous) or Fe^{3+} (ferric). This property permits iron to act as a redox catalyst by reversibly donating or accepting electrons. An excellent example is the electron transport chain of oxidative phosphorylation, in which adenosine triphosphate (ATP) is generated from glucose by the orderly transfer of electrons through a network of iron-containing mitochondrial cytochromes.

When dissolved in aqueous solution, ferrous iron rapidly oxidizes to ferric iron, which is insoluble at physiologic pH. The resulting ferric hydroxide salts (rust) are of no metabolic utility. To achieve stable solubility under physiologic conditions, iron complexes with iron-binding agents termed chelators. Chelators are synthesized by all organisms, ranging from microbes (e.g., deferoxamine produced by *Streptomyces pilosus*) to humans (e.g., transferrin in human plasma). These molecules are important to environmental iron acquisition and transport as well as intracellular storage.

Iron-Protein Complexes

Iron-protein complexes capitalize on the properties of the metal to perform metabolic functions, the iron acting as the chemical workhorse and the protein dictating biologic specificity. Individual iron atoms can interact directly with amino acid side groups in proteins, as in ribonucleotide reductase. Alternatively, iron may form coordination

Box 11-1 **Proteins That Contain Iron***

- HEME PROTEINS
  - Hemoglobin
  - Myoglobin
  - Cytochrome *a*, *b*, *c*
  - Cytochrome P-450
  - Tryptophan 1,2-dioxygenase
  - Catalase
  - Myeloperoxidase
- IRON-DEPENDENT ENZYMES
  - Aldehyde oxidase
  - Reduced nicotinamide adenine dinucleotide dehydrogenase
  - Tyrosine hydroxylase
  - Succinate dehydrogenase
  - Prolyl hydroxylase
  - Tryptophan hydrolase
  - Xanthine oxidase
  - Ribonucleotide reductase
  - Aconitase
  - Phosphoenolpyruvate carboxykinase

From Griffin IJ, Abrams SA: Iron and breastfeeding. Pediatr Clin North Am *48:401–413, 2001.*

*Partial list.

complexes with other small molecules. For example, protoporphyrin IX donates four of the six electrons needed to form a stable coordination complex with iron. Heme, the iron–protoporphyrin IX complex, is so stable that removal of the iron moiety requires an enzyme, heme oxygenase. The functional properties of heme are determined by the nature of the associated protein or small molecule ligands supplying the remaining two electrons. The best-characterized heme protein is hemoglobin, in which a globin histidine residue donates the fifth electron and the sixth comes from molecular oxygen.[1] This configuration enables hemoglobin to transport oxygen safely throughout the body.

Iron and sulfur atoms can also form complexes (clusters) that catalyze enzymatic reactions. The Krebs cycle enzyme aconitase, for example, contains an iron-sulfur (Fe/S) cluster. As discussed later in this chapter, the iron content of the Fe/S cluster of a related aconitase-like molecule allows it to “sense” iron concentrations within the cell and to act as an iron regulatory protein (IRP) to modulate the translation of genes of iron metabolism.

Iron Toxicity

The ability of iron to catalyze redox reactions also accounts for its toxicity. As an enzymatic cofactor, the metal is involved in the restructuring of cellular components. Unchelated iron has unbridled redox activity, however, and may contribute to cellular degeneration. We live in an oxygen-rich atmosphere, and our bodies require oxygen for many metabolic processes. However, oxygen is highly reactive and its interaction with iron potentiates its toxicity. Normal cellular reactions, including respiration, generate the reactive oxygen intermediates superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Oxidative stress develops when production of reactive oxygen species exceeds the body’s processing capacity. Under these circumstances, reactive oxygen intermediates may be converted to injurious free radicals by the iron-catalyzed Fenton reaction[2]: Hydroxyl radicals promote the peroxidation of proteins, DNA, and membrane lipids, problems that are exacerbated by iron overload. Certain organelles are particularly susceptible to iron-dependent peroxidation. In iron-overloaded cells, injured mitochondria and lysosomes become leaky.[3] Mitochondrial damage and release of lysosomal proteases cause further cell injury and may ultimately lead to cell death. This process contributes to the severe tissue damage seen in the liver, heart, and endocrine organs of patients with iron overload disorders (see later).

Iron is not normally present in cell membranes. However, in both sickle cell disease and thalassemia, iron, heme, ferritin, and denatured hemoglobin adhere to the inner surface of the red cell plasma membrane and thereby contribute to the pathogenesis of these congenital anemias.[4,5] The membrane complex containing denatured hemoglobin has been termed *hemichrome*.[4,5] The red cell anion transport protein band 3 appears to nucleate the formation of these iron aggregates.[6,7] Injured cells decorated with membrane iron deposits are removed by a functioning spleen in patients with thalassemia and sickle cell–hemoglobin C disease (hemoglobin SC disease), but they circulate in functionally asplenic patients with homozygous sickle cell–hemoglobin S disease (hemoglobin SS disease). Membrane-associated iron promotes free radical formation and further membrane damage, marked by generation of the lipid peroxidation product malonyldialdehyde and by cross-linking of membrane proteins.[5,8] Membranes become rigid and thus contribute to the formation of irreversibly sickled cells that occlude the microcirculation.

Sometimes reactive oxygen intermediates can be beneficial. Neutrophils contain a membrane-associated reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that produces superoxide to kill ingested microorganisms (reviewed by Clark[9]). Superoxide and secondary reactive oxygen intermediates are potent antimicrobial agents. Congenital defects in this NADPH oxidase, collectively termed *chronic granulomatous disease* (see Chapter 22), are characterized by a serious defect in defense against bacterial pathogens.

Neutrophils and iron also injure tissues in inflammatory diseases such as rheumatoid arthritis.[10] Synovial macrophages ingest red cell hemoglobin introduced by intermittent joint hemorrhage. Iron is deposited in the synovial membrane, proximate to superoxide and hydrogen peroxide generated by neutrophils and macrophages participating in the inflammatory reaction. Iron catalyzes the conversion of these compounds to free radical species, which promote lipid peroxidation. Iron therapy exacerbates this process. In contrast, antioxidants and iron chelators retard free radical generation, thereby affording some theoretic protection against injury in rheumatoid arthritis.[11,12]

These deleterious properties of iron are threatening only when the element is in a “free” state or in an abnormal form within the cell. Protection of cell structures from iron-dependent free radical damage is therefore of utmost importance. When iron is bound to protein either directly or indirectly, the generation of free radicals is largely abrogated. Thus, tight chelation of iron is a means of controlling its reactivity. As discussed later, cytoplasmic ferritin allows iron to be stored safely within cells by sequestering it in an innocuous form. Expression of ferritin is induced by oxidative stress.[13] Both prokaryotic and eukaryotic cells contain ferritins, and mice lacking one of the two ferritin genes do not develop past the early embryonic stages.[14] Thus, ferritin appears to be necessary for most, if not all, living cells.

ACQUISITION AND DISTRIBUTION OF IRON

The average adult has 4 to 5 g of iron. To maintain systemic iron homeostasis, a meticulous balance exists between dietary uptake and loss. Approximately 0.5 to 1 mg of iron is lost each day through sloughing of cells from skin and mucosal surfaces (Fig. 11-1).[15] Because menstruating females lose an average of an additional 1 mg of iron daily, their dietary iron requirement is increased.[16] Neither the liver nor the kidney has a significant physiologic capability to excrete iron. Consequently, absorption is the primary means of regulating body iron stores.[17] During neonatal and childhood growth spurts, iron requirements increase in response to augmentation of body, and particularly red blood cell, mass.

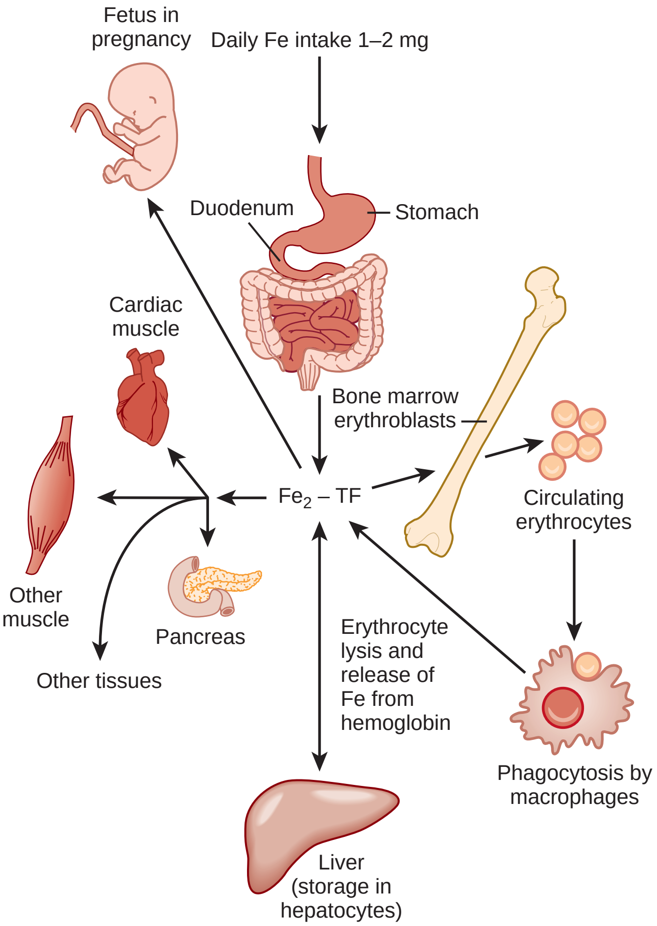

Figure 11-1 The body's iron economy. Although the average adult has 4 to 5 g of body iron, only 1 mg of dietary iron enters and leaves the iron economy on an average day. Dietary iron enters through the duodenum and becomes bound to plasma transferrin for delivery to tissues. The erythron is the largest site of iron use, but all cells require the metal. Storage iron is found primarily in the liver. Reticuloendothelial macrophages carry out iron recycling. Iron is lost from the body with bleeding and with exfoliation of skin and mucosal cells. *(Modified from Andrews NC: Iron homeostasis: insights from genetics and animal models.* Nat Rev Genet *1:208–217, 2000.)*

Iron Absorption

Iron absorption occurs predominantly in the proximal duodenum.[18,19] The physical state of iron entering the duodenum greatly influences its absorption. At neutral pH, ferrous iron is rapidly converted to the insoluble ferric form. Acid produced by the stomach serves to lower the pH in the duodenum and thereby enhance the solubility and uptake of iron (see later). Heme is absorbed separately from and more efficiently than inorganic iron,[18] independent of duodenal pH. Consequently, meat is an excellent nutritive source of iron. Heme iron absorption is, however, very poorly understood, but a heme oxygenase inhibitor has been shown to block heme catabolism in the intestine and result in an iron-deficient state.[20]

A number of dietary factors influence inorganic iron absorption.[21] Ascorbate and citrate increase iron uptake, in part by acting as weak chelators to help solubilize the metal in the duodenum. Iron is readily transferred from these compounds to the absorptive epithelium. Conversely, plant phytates, bran, and tannins inhibit iron absorption.[21-23] These compounds chelate iron and prevent its uptake by the absorption machinery.

Through a combination of genetic and biochemical approaches, much has been learned about the absorption of nonheme iron over the past two decades. Nonheme iron arrives at the apical surface of the absorptive duodenal enterocyte in its ferric (Fe^{3+}) form. It is reduced through the action of a brush border ferric reductase. This enzyme may be duodenal cytochrome *b*, a heme protein that is homologous to *b*561 cytochromes.[24] Expression of duodenal cytochrome *b* is significantly greater in the proximal duodenum than elsewhere and increases in iron deficiency.

Ferrous (Fe^{2+}) iron is then taken up by the enterocyte through the action of divalent metal transporter 1 (DMT1, also known as Nramp2, DCT1, and SLC11A2).[25,26,27] Transport requires the coordinated movement of protons in the same direction (symport). Thus, DMT1 functions only at low pH, and it has little or no activity at neutral pH. DMT1 is widely expressed, but duodenal levels increase dramatically in iron deficiency.[27,28]

DMT1 can also transport other divalent metal ions, including Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Pb^{2+}, and Zn^{2+}.[27] Competition studies have shown that lead, manganese, cobalt, and zinc can share the intestinal absorption pathway used by iron. Increased iron absorption induced by iron deficiency also enhances the uptake of these elements. Because iron deficiency often coexists with lead intoxication, this interaction has vast public health significance and can produce particularly serious medical complications in children.[29] As discussed later, copper absorption and metabolism are handled by an entirely different mechanism.

After iron enters the absorptive enterocyte through the action of DMT1, it has at least two possible fates. It can be retained by the cell and subsequently be lost when the enterocyte dies and is sloughed into the intestinal lumen, or it can be transported across the basolateral membrane to enter the body. Iron retained by the enterocyte is used for cellular metabolism or incorporated into ferritin. Exported iron leaves the cell by way of a unique basolateral transmembrane iron transporter, ferroportin (FPN1, also known as SLC40A1, MTP1, and IREG1).[30,31,32,33]

Basolateral iron transfer also requires a change in the oxidation state of the metal, likely facilitated by the homologous multicopper ferroxidases hephaestin,[34,35] and ceruloplasmin, which are membrane associated and present in the plasma, respectively. A comprehensive model of intestinal iron absorption, primarily derived from studies in mice, is shown in Figure 11-2. This model pertains only to nonheme iron transport; details of heme iron uptake have not yet been worked out. A candidate intestinal heme transporter was reported but subsequently proven to be a folate transporter, instead.[36]

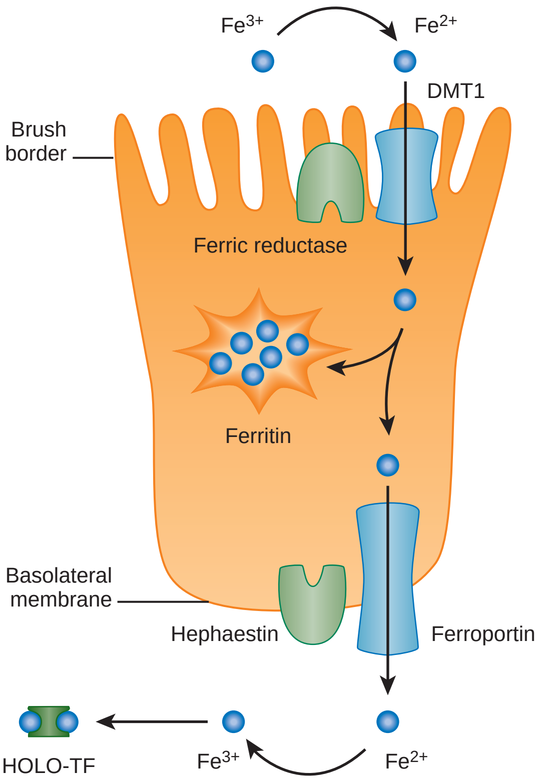

Figure 11-2 Duodenal iron transfer. The microstructure of the iron absorption apparatus is depicted in this cartoon. Iron is taken up by enterocytes lining the duodenal villi. These absorptive cells start out as undifferentiated precursors in the intestinal crypts. Crypt cells appear to be programmed for an iron absorption "set point" that is determined in response to iron needs. As the cells differentiate, they migrate up the villi and begin to express iron transporter proteins. According to current models, nonheme iron uptake occurs in mature enterocytes through the enzymatic reduction of iron, transmembrane import into the cell by DMT1, transmembrane export from the cell by ferroportin, and enzymatic oxidation by hephaestin before loading onto apotransferrin to produce diferric transferrin (HOLO-TF). *(Modified from Andrews NC: Iron homeostasis: insights from genetics and animal models.* Nat Rev Genet *1:208–217, 2000.)*

Normally, only about 10% of dietary nonheme iron entering the duodenum is absorbed. However, this value increases significantly with iron deficiency.[37] In contrast, iron overload reduces, but does not completely eliminate, absorption. Finch designated this modulation the "stores regulator."[37] In addition, both iron deficiency anemia and forms of anemia associated with ineffective erythropoiesis induce a marked increase in iron absorption. This effect has been designated the "erythroid regulator."[37] The erythroid regulator appears to exert a dominant effect over the stores regulator, as individuals with chronic persistent ineffective erythropoiesis can become profoundly iron overloaded even in the absence of transfusion. Hypoxia also increases iron absorption, independent of anemia.[38,39]

Hepcidin, an iron-regulated peptide hormone, links the actions of all these regulators through a unifying molecular mechanism. Hepcidin is a 25–amino acid peptide produced in the liver from a larger precursor (reviewed by Nemeth and Ganz[40]). It circulates in the plasma and binds to the iron exporter ferroportin on the basolateral surface of absorptive enterocytes, causing ferroportin to be internalized and degraded in a ubiquitin-dependent manner.[41] In this way, hepcidin controls cellular iron egress. Hepcidin thus regulates iron absorption at the level of the intestinal epithelium in that any iron unable to leave the enterocytes is lost when these cells turn over.

Expression of hepcidin is induced in response to iron overload[42,43] and inflammation[44,45] and is repressed in response to increased erythropoietic activity[45,46] and hypoxia.[45] Regulation of hepcidin levels appears to be at the level of gene transcription. However, the signals that govern hepcidin transcription in response to these stimuli are incompletely understood. Only the inflammatory cytokine interleukin-6 has been definitely shown to be involved in regulation of hepcidin expression by physiologic changes outside the liver.[47-49]

Most of the total body iron is ultimately incorporated into hemoglobin in erythroid precursors. An average adult produces 200 billion red cells daily to achieve a red cell renewal rate of 0.8% per day. Each red cell contains more than 1 billion atoms of iron, and each milliliter of packed red blood cells contains approximately 1 mg of iron. To meet this daily need for 2×10^{20} atoms (or 20 mg) of elemental iron, iron is recycled from senescent red cells and returned to the circulation. Plasma iron turnover (PIT) represents the mass turnover of transferrin-bound iron in the circulation (expressed as milligrams per kilogram per day).[50] Accelerated erythropoiesis increases PIT and enhances iron uptake from the gastrointestinal tract.[51] Although hepcidin appears to be the effector molecule that alters intestinal absorption, an upstream, circulating factor—the erythroid regulator—that communicates the iron needs of the erythron must exist.[41,52] There are some data to indicate that circulating bone morphogenetic protein (BMP) signaling pathway inhibitors (e.g., GDF15 and TWSG) may play a role in mediating this effect.[53,54]

This erythroid control of hepcidin expression is particularly apparent in patients with thalassemia intermedia (non–transfusion-dependent thalassemia), in whom marked iron overload develops even without transfusions. The accelerated, but ineffective, erythropoiesis substantially boosts iron absorption by inhibiting hepcidin expression.[55-58] By contrast, hepcidin is increased in patients with thalassemia and other anemias receiving chronic transfusion therapy as a consequence of iron overload and the suppression of erythropoiesis.[59]

Intercellular Iron Transport

Only a small proportion of total body iron enters and leaves the body each day. Consequently, intercellular iron transport is quantitatively more important than intestinal absorption. The greatest mass of iron is found in erythroid cells, which make up about 60% to 80% of the total body endowment in normal individuals. To accommodate ongoing erythropoietic needs, the reticuloendothelial system recycles approximately 20 to 25 mg of iron from effete red cells each day.

A small fraction, 0.1% or 4 mg, of total body iron circulates in plasma in an exchangeable pool. In normal individuals, nearly all of the circulating plasma iron is bound to transferrin. Transferrin serves three purposes: (1) it renders iron soluble under physiologic conditions; (2) it prevents iron-mediated free radical toxicity; and (3) it facilitates transport into cells. Transferrin is by far the most important physiologic supplier of iron to red cells.[60] In fact, plasma transferrin serves to deliver iron to most tissues of the body. It is an 80-kd glycoprotein that has homologous N-terminal and C-terminal iron-binding domains.[61] The molecule is related to several other proteins, including ovotransferrin in bird and reptile eggs,[62] lactoferrin in extracellular secretions and neutrophil granules,[63,64] and melanotransferrin (p97), a protein produced by melanoma cells.[65] The functions of these related proteins are incompletely understood. The human transferrin messenger RNA (mRNA) is 2.3 kilobases (kb) in length and encodes 679 amino acids, including a 19–amino acid leader sequence. It is located on chromosome 3q, near genes for the transferrin receptor and melanotransferrin. The liver is the major site of synthesis and secretion of transferrin.[66] Other tissues, including Sertoli cells of the testis, oligodendrocytes of the central nervous system, lymphocytes, muscle cells, and mammary cells, can, however, also produce the protein.[67-72] Local synthesis within the brain and testis apparently provides transferrin for these tissues because serum transferrin does not penetrate their unique capillary barriers.

Transferrin production is regulated at multiple levels. Several *cis*-acting control regions exist upstream of the gene. The transferrin promoter contains binding sites for tissue-specific nuclear factors that activate transcription differentially in the liver and other tissues (e.g., Sertoli cells).[73] Iron, hormones, and inflammatory stimuli also modulate transferrin gene expression.[74-78] In the setting of iron deficiency, serum transferrin levels rise substantially as a result of enhanced synthesis of transferrin mRNA by the liver.[76] In contrast, inflammation depresses levels of both serum transferrin and serum iron, the latter through the action of the iron regulatory hormone hepcidin. The plasma concentration of diferric transferrin has been proposed to modulate expression of the iron regulatory hormone hepcidin.[79] Liver-derived serum transferrin levels fall modestly in patients with genetic or acquired iron overload, although mRNA levels have been reported to be unchanged.[80] This suggests that hepatic transferrin is also controlled at the level of translation or secretion. The quantity of transferrin mRNA in nonhepatic tissues (including the testis, kidney, and spleen) is not affected by iron deficiency. The liver-derived transferrin, therefore, appears to have the unique responsibility of responding to iron status.

X-ray crystal structures have been determined for transferrin and related proteins (reviewed elsewhere[81,82]). All members of the transferrin protein superfamily exhibit similar polypeptide folding. The N-terminal and C-terminal domains are globular moieties of about 330 amino acids; each of these domains is divided into two subdomains, with the iron- and anion-binding sites located in the intersubdomain cleft on either side of a central plane of symmetry, suggesting an origin by gene duplication from a primordial protein containing a single iron-binding site. The binding cleft opens with iron release and closes with iron binding.[83] The N-terminal and C-terminal binding sites are very similar. No cooperativity exists in binding of iron by the two sites, and the protein can be proteolytically cleaved into two halves, each of which retains iron-binding capability.[84,85] Transferrin binds ferric iron much more avidly than ferrous iron.[86]

The precise mechanism by which iron is loaded onto transferrin as it leaves intestinal epithelial cells or reticuloendothelial cells is unknown. The copper-dependent ferroxidase ceruloplasmin and its homologue hephaestin likely play central roles in reducing ferrous iron exported by ferroportin 1 (FPN1) from the reticuloendothelial cells and intestine, respectively, although neither is absolutely required due to the tendency of iron to oxidize in aqueous environments.[87-89] Transferrin binds ferric iron exceptionally avidly, with a dissociation constant (K_d) of approximately 10 to 22 mol/L in the company of an anion (usually carbonate), which serves as a bridging ligand between the metal and protein and excludes water from two coordination sites.[86,90,91] Without the anion cofactor, iron binding is negligible; with it, ferric transferrin is resistant to all but the most potent chelators. Release of iron from transferrin involves protonation of the carbonate anion to loosen the metal-protein association.

The sum of all iron-binding sites on transferrin constitutes the total iron-binding capacity (TIBC) of plasma. Thus, on a molar basis, TIBC is twice the concentration of transferrin protein because each transferrin molecule can bind two iron atoms. The laboratory method used to measure the TIBC can either be truly the total iron-binding concentration of the plasma measured by iron binding or a calculation based upon measurement of the concentration of transferrin. Whatever the method, under normal circumstances, about a third of transferrin iron-binding sites are filled. Consequently, except for the situation of extreme iron overload, in which all transferrin-binding sites are occupied, non–transferrin-bound iron (NTBI) in the circulation is present at very low concentrations.[92] The distribution of plasma and tissue iron can be traced with the use of ^{59}Fe as a radioactive tag by reinfusing a subject with autologous transferrin loaded with radiolabeled iron. Blood samples can be analyzed at timed intervals to determine the rate of loss of the radioactive label. Such ferrokinetic studies indicate that the normal half-life of iron in the circulation is about 75 minutes.[50] The absolute amount of iron released from transferrin per unit time is the PIT (see earlier).

Such radioactive tracer studies indicate that at least 80% of the iron bound to circulating transferrin is delivered to the bone marrow and incorporated into reticulocytes.[93,94] Other major sites of iron delivery include the liver and the spleen, which are the primary depots for iron storage. Hepatic iron is found in both the reticuloendothelial cells and hepatocytes. Reticuloendothelial cells acquire iron primarily by phagocytosis and breakdown of effete red blood cells; they catabolize heme and return iron to the circulation, where it binds transferrin. Hepatocytes take up iron by at least two different mechanisms, which are transferrin-dependent and transferrin-independent. The relative amounts of iron in each of these cell types depend on clinical circumstances, as discussed in detail later.

Given the preeminent role of bone marrow in the clearance of labeled iron from the circulation, ferrokinetic studies provide a window on erythropoietic activity. Conditions that augment erythrocyte production increase PIT. For example, hemolytic anemias such as hereditary spherocytosis and sickle cell disease induce rapid delivery of transferrin-bound iron to the marrow. In contrast, disorders that reduce red cell production, such as Diamond-Blackfan anemia and aplastic anemia, prolong PIT.

When erythrocytes are produced and released into the circulation in a normal fashion, the process of erythropoiesis is termed *effective*. In patients with certain anemias, however, the abnormal, nascent red cells apoptose or are destroyed before they leave the marrow cavity. In this situation, erythropoiesis is *ineffective*, which means simply that the erythropoietic precursors have failed to accomplish their primary task: delivery of mature, enucleated erythrocytes to the circulation. The ferrokinetic profile in this case shows rapid removal of iron from transferrin with delayed entry of label into the pool of circulating red cell hemoglobin. β^+-thalassemia is an important example of this pattern. In β^+-thalassemia, ineffective erythropoiesis is associated with a markedly enhanced PIT.

Intracellular Iron Metabolism

Transferrin and the Transferrin Cycle

Although transferrin was first characterized more than 60 years ago,[95] its receptor eluded investigators until the early 1980s, when monoclonal antibodies prepared against tumor cells were found to recognize the transferrin receptor glycoprotein.[96] Subsequently, receptor-mediated endocytosis of iron bound to transferrin has been characterized in detail. A diagram showing key features of the transferrin receptor is presented in Figure 11-3. Each subunit of the disulfide-linked homodimer contains 760 amino acids.[97-99] Oligosaccharides account for about 5% of the 90-kd subunit's molecular mass.[100] Four glycosylation sites (three *N* linked and one *O* linked) are found in the protein.[101,102] Glycosylation-defective mutants have fewer disulfide bridges, less transferrin binding, and less cell surface expression. The transmembrane domain, located between amino acids 62 and 89, functions as an internal signal peptide because there is none at the N-terminal end.[103] A molecule of fatty acid (usually palmitate) is also covalently linked to each subunit at the internal edge of the transmembrane domain and may play a role in membrane localization. The crystal structure of the ectodomain of the transferrin receptor has been reported.[104] Although no one has yet succeeded in cocrystallizing the receptor with transferrin, molecular modeling and binding studies of mutant transferrin receptor proteins have yielded important insights, including the amino acid residues important for receptor binding to transferrin.[105]

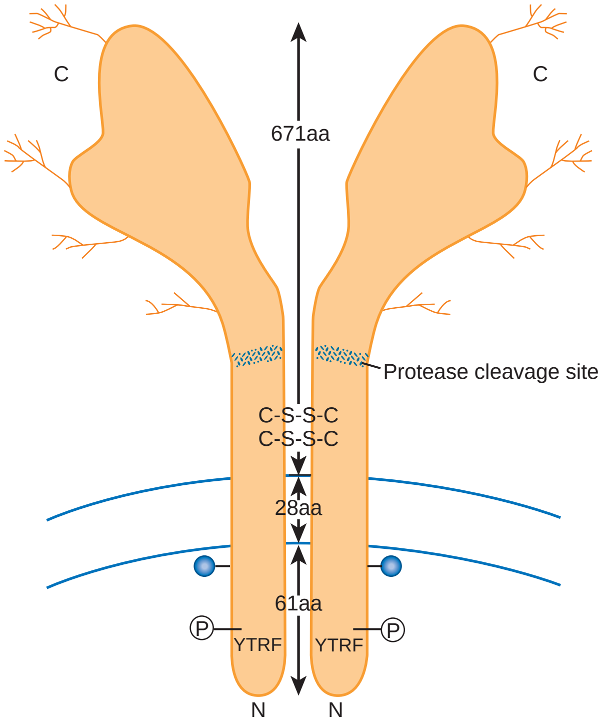

Figure 11-3 Structure of the dimeric transferrin receptor. The N-terminals of both subunits are inside the cell, and the C-terminals are outside. The 61–amino acid *(aa)* intracellular domain has three structural features that appear to play a role in endocytosis: a tyrosine-threonine-arginine-phenylalanine *(YTRF)* amino acid motif, a phosphorylated serine residue *(encircled P)*, and a covalently linked molecule of palmitic acid *(solid circle)*. The transmembrane domain is 28 amino acids long. The extracellular domain has 671 amino acids, including disulfide linkages *(C-S-S-C)*, as well as four glycosylation sites *(branched lines)*. A potential protease cleavage site is located between amino acids 100 and 101.

Iron is taken into cells by receptor-mediated endocytosis of diferric transferrin (Fig. 11-4).[106-109] Receptors on the outer face of the plasma membrane bind iron-loaded

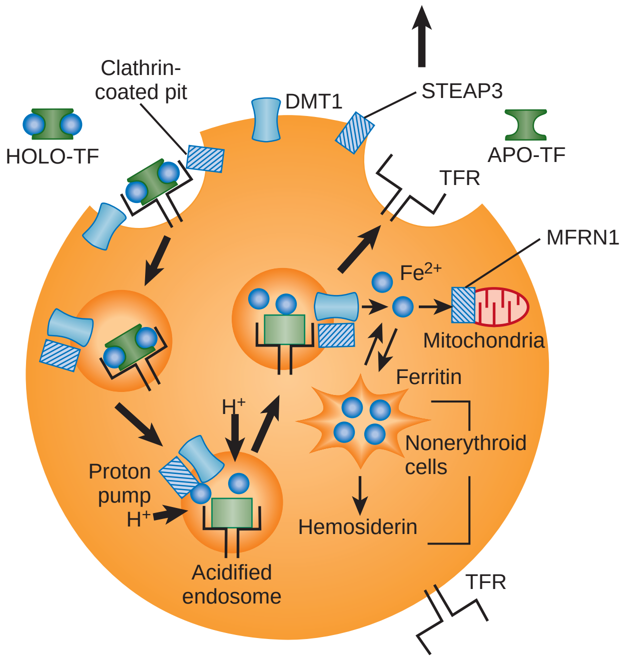

Figure 11-4 The endocytotic transferrin cycle. Apotransferrin *(APO-TF)* binds two atoms of iron per molecule to form diferric transferrin *(HOLO-TF)*. Diferric transferrin binds to the transferrin receptor *(TFR)* on the cell surface. The complex is internalized by invagination of clathrin-coated pits to form specific endosomes. The endosomes import protons, thus lowering the pH within the organelle and decreasing the affinity of transferrin for iron. Liberated iron is translocated through the endosome membrane to the cytoplasm by DMT1 after iron has been reduced from the ferric to the ferrous form by STEAP3. The iron released is shuttled to the mitochondria by mitoferrin 1 (*MFRN1*) for synthesis of heme and to ferritin for storage. The apotransferrin-transferrin receptor complex recycles to the cell surface, where neutral pH promotes release of apotransferrin into serum for reuse. Details are given in the text. *(Modified from Andrews NC: Iron homeostasis: insights from genetics and animal models.* Nat Rev Genet *1:208–217, 2000.)*

transferrin with very high affinity. The C-terminal domain of transferrin appears to mediate receptor binding.[110] Diferric transferrin binds with much higher affinity than monoferric transferrin or apotransferrin does.[111-113] The dissociation constant (K_d) for bound diferric transferrin ranges from 10-7 to 10-9 mol/L at physiologic pH, depending on the species and tissue. The K_d of monoferric transferrin is approximately 10-6 mol/L. The concentration of circulating transferrin is about 25 µmol/L. Therefore, cellular transferrin receptors are ordinarily fully saturated.

After binding to its receptor on the cell surface, transferrin is internalized through a constitutive mechanism that begins with invagination of clathrin-coated pits and the formation of endocytic vesicles. This process requires the short, 61–amino acid intracellular tail of the transferrin receptor molecule.[114-117] Receptors with truncated N-terminal cytoplasmic domains do not recycle properly. This portion of the molecule contains a conserved tyrosine-threonine-arginine-phenylalanine (YTRF) sequence that functions as a signal for endocytosis.

An unknown ATP-dependent proton pump lowers the pH of the internalized endosome to about 5.5.[118-120] Acidification of the endosome weakens the association between iron and transferrin and promotes a conformational change in the transferrin receptor to enhance binding of apotransferrin and facilitate release of iron.[121] An endosomal ferrireductase, STEAP3, reduces iron from the Fe^{3+} state to Fe^{2+}, either at the same time that it is released from transferrin or soon afterward. STEAP3 is a membrane-bound NADPH-dependent oxidoreductase localized to transferrin cycle endosomes and expressed at high levels in erythroid precursors.[122] Related proteins may serve similar functions at other sites.[123]

The iron released from transferrin must exit the endosome and enter the cytoplasm and mitochondria for use in heme biosynthesis, Fe/S cluster biosynthesis, and storage and for other purposes. This transmembrane transport step is also mediated by DMT1.[124,125] DMT1 is unusual in that it is expressed on two very different types of membranes—the apical membrane of intestinal enterocytes and the endosomal membrane of transferrin uptake vesicles in nonpolarized cells. Once transported out of the endosome or across the plasma membrane, iron must be delivered to sites of use or stored in the form of ferritin. The cytosolic chaperone that traffics iron to ferritin appears to be PCBP1, a protein that also functions in the control of mRNA stability and translation, particularly in erythroid cells. In erythroid cells, iron is delivered across the mitochondrial inner membrane by mitoferrin 1 (MFRN1 or SLC25A37)[126]; once across the membrane it is incorporated into heme by the enzyme ferrochelatase.

The fate of transferrin and the transferrin receptor is distinct from that of iron. Rather than entering lysosomes for degradation, as do the ligands in other receptor-mediated endocytosis pathways, intact receptor-bound apotransferrin recycles to the cell surface, where the neutral pH promotes detachment into the plasma. Thus, preservation and reuse of transferrin are accomplished by pH-dependent changes in the affinity of transferrin for iron and the transferrin receptor.[109,118,120] Exported apotransferrin binds additional iron atoms and undergoes further rounds of iron delivery to cells. Thus, a single transferrin molecule, with a half-life of 8 days, may be used hundreds of times for delivery of iron.

It was once a widely accepted belief that the transferrin cycle was important, if not necessary, for the uptake of iron by all mammalian cells. However, two lines of evidence indicate that such is not the case. First, mice and humans with severe deficiencies of plasma transferrin have iron deficiency anemia but vastly increased amounts of iron in nonhematopoietic tissues.[60,127-133] This finding indicates that uptake of iron bound to transferrin is important for red blood cell hemoglobinization and maturation but that transferrin is not essential for delivery of iron to most other tissues. Second, mouse embryos lacking transferrin receptor die midway through gestation as a result of severe anemia, but most nonhematopoietic tissues appear generally intact.[134] Aside from the erythron, only the developing central nervous system shows an apparent requirement for transferrin receptor. In its absence, primitive neuroepithelial cells undergo apoptosis at a greatly increased rate. Taken together, these observations indicate that the transferrin cycle is of primary importance in erythropoiesis and neurogenesis but of lesser importance in other mammalian tissues, at least early in embryonic development.

Non–Transferrin-Bound Iron Uptake

Both hypotransferrinemia and iron overload lead to complete saturation of available plasma transferrin, and non–transferrin-bound iron (NTBI) circulates in a chelatable, low–molecular-weight form (reviewed by Cabantchik and colleagues[135]). This iron is weakly complexed to albumin, citrate, amino acids, sugars, and other small molecules, and it behaves differently from iron associated with transferrin. Nonhematopoietic tissues (particularly the liver, endocrine organs, kidneys, and heart) preferentially take up NTBI. Radiolabeled iron administered to mice with and without available transferrin-binding capacity is distributed in markedly different patterns.[60] In normal animals with excess plasma iron-binding capacity, hematopoietic tissues are the primary sites of uptake. When free transferrin iron-binding sites are absent, however, most iron is deposited in the liver and pancreas, thus indicating that these organs serve as the initial iron reservoirs in the situation of iron overload. Notably, this pattern of distribution is similar to that seen in idiopathic hemochromatosis. NTBI is highly toxic to cells.[136] Plasma NTBI can potentiate the formation of free radicals through the Fenton reaction (see earlier) and thereby induce cell membrane damage. Cardiac cells are particularly susceptible to this damage. Hence, therapeutic chelation must effectively remove plasma NTBI.

The identity or identities of the protein(s) responsible for NTBI uptake are of considerable interest due to the clinical impact of NTBI uptake in hemochromatosis, iron-loading anemias, and chronic transfusion therapy. NTBI uptake activities have been characterized in tissue culture cells, but the physiologically relevant transporters are only beginning to be understood. Topologically, the cell exterior and the endosome interior are equivalent compartments. The primary role of the transferrin–transferrin receptor interaction is to sequester iron near the cell membrane, thereby increasing the likelihood of iron uptake. DMT1 may reside on the plasma membrane of the cell before endocytosis. If so, it should be oriented to transport iron directly into the cell, without the assistance of transferrin (as diagrammed in Fig. 11-4). However, the fact that DMT1 functions only at acidic pH suggests that it probably does not, by itself, account for all NTBI transport. Indeed, hepatocyte DMT1 expression was recently shown not to be essential for hepatocellular iron accumulation in the setting of iron overload.[137] There is some evolving in vivo evidence to suggest that L-type calcium channels,[138,139] or the zinc transporter family (ZIP) proteins ZIP8 and ZIP14 can transport NTBI.[140,141]

ROLE OF IRON IN CELL PROLIFERATION AND DIFFERENTIATION

Iron is indispensable for DNA synthesis and a host of metabolic processes. Iron starvation arrests cell proliferation, presumably because ribonucleotide reductase and other enzymes require the metal.[142] Although transferrin receptors are expressed on all dividing cells in numbers that roughly reflect their growth rate,[143] as discussed in detail earlier, the erythron is the tissue that relies most heavily on delivery of iron by transferrin,. However, the transferrin cycle also appears to play a significant role in other cell types.

Studies of mature T lymphocytes exemplify the general relationship between expression of transferrin receptor and cell proliferation. Transferrin receptors, absent from resting T cells, have long been recognized as markers of T-cell activation. Initiation of cell division by a mitogen such as phytohemagglutinin dramatically increases surface expression of both transferrin receptor and iron uptake.[144] Furthermore, tumor cells upregulate transferrin receptor expression to optimize acquisition of iron for proliferation, and certain monoclonal antibodies against the transferrin receptor curb proliferation of tumor cells in vitro and in vivo.[145-150] Interestingly, some reports have suggested that the transferrin receptor may have an additional role in activated T cells, apart from its iron uptake function. Anti–transferrin receptor monoclonal antibodies have been described that can trigger T-cell activation and secretion of interleukin-2. These antibodies presumably activate a signal transduction pathway beginning with the transferrin receptor but independent of iron trafficking. The transferrin receptor also appears to have a role in early lymphocyte development. Embryonic stem cells in which both transferrin receptor genes have been inactivated fail to differentiate into circulating lymphocytes in vivo in chimeric mice.[151] It is not yet clear whether this is due to defective iron delivery or to perturbation of some other, as yet unknown function of the transferrin receptor.

Chelators that deplete intracellular iron and limit its bioavailability in extracellular fluids further demonstrate the central role of iron in cell proliferation. Agents such as deferoxamine, desferrithiocin, and pyridoxal isonicotinoyl hydrazone inhibit the growth of a variety of tumor cells in culture[152,153] and greatly reduce T-cell proliferation.[154-157] A likely inhibitory mechanism is iron deprivation, with reduced ribonucleotide reductase activity and lower levels of deoxyribonucleotides. This, in turn, leads to mitotic arrest in the S phase of the cell cycle.[158] The addition of iron to the medium reverses the growth inhibition. Chelators may also induce apoptosis, or programmed cell death, through other mechanisms that are not yet understood.

Above all cells, erythroid precursors need an extraordinary amount of iron to support hemoglobin synthesis and differentiation into mature red cells. The density of transferrin receptors on the cell surface is modulated during erythroid development. Transferrin receptors first appear in measurable numbers on colony-forming unit–erythroid (CFU-E) cells and increase to 300,000 per cell on proerythroblasts and as many as 800,000 per cell on basophilic erythroblasts at the time of maximal iron uptake. Numbers then fall to 100,000 per cell on circulating reticulocytes and to negligible levels on mature red cells.[159] A precise correlation exists between iron requirement and transferrin receptor number, thus indicating that the abundance of transferrin receptors on the cell surface is a major determinant of erythroid iron uptake.

In culture, a monoclonal antibody to the transferrin receptor that permits ligand binding but subsequently slows receptor recycling partially blocks erythroid iron uptake. The level of iron uptake is sufficient for cell division but not hemoglobin synthesis.[160]

A wealth of literature demonstrates that oxidized heme (hemin) promotes differentiation of erythroleukemia cell lines in tissue culture.[161-164] Conversely, deficient heme biosynthesis abrogates chemical induction of differentiation in an erythroleukemia cell line subclone.[164] Heme regulates the transcription of globin, transferrin receptor, and ferritin genes.[165-167] Some of these effects may be mediated by the heme-dependent transcriptional repressor BACH1.[168,169] A second, major mechanism in which heme regulates terminal erythroid differentiation is mediated by a heme-dependent elongation factor 2a kinase termed heme-regulated kinase (HRI), which globally suppresses translation in erythroid cells in the absence of heme (reviewed by Chen[170]). Although some of the precise mechanisms remain to be elucidated, it is clear that iron uptake, heme biosynthesis, and globin protein production are coordinately regulated. Interrelated regulatory networks apparently allow red cell precursors to maximize hemoglobin formation without accumulating excess globin proteins, unbound iron, or toxic protoporphyrin intermediates.

REGULATION OF PROTEINS OF IRON METABOLISM

Ferritin

Once inside the cytoplasm, iron is almost certainly bound by carrier molecules that assist in delivering iron to intracellular locations, including mitochondria (for heme biosynthesis) and ferritin (for storage). The identities of the intracellular iron carrier molecules by and large remain unknown. However, there is recent evidence to suggest that poly-C–binding protein 1 (PCBP1), which is involved in mRNA stability and translation, also serves as an iron chaperone specific for iron acquisition by ferritin and the hypoxia-inducible factor (HIF) prolyl hydroxylase.[171-173] The amount of iron in transit within the cell at any given time is small and difficult to measure. This minute pool of transit iron, which is believed to be in the Fe^{2+} oxidation state, is the biologically active and potentially toxic form of the element. Metabolically inactive iron, stored in ferritin and hemosiderin, is nontoxic and in equilibrium with this exchangeable transit iron.

Both prokaryotes and eukaryotes produce ferritin molecules for iron storage. Mammalian ferritin molecules are complex 24-subunit heteropolymers of H (for heavy or heart) and L (for light or liver) protein subunits. L subunits are 19.7 kd in mass, with isoelectric points of 4.5 to 5; H subunits are 21 kd in mass, with isoelectric points of 5 to 5.7. The subunits of the ferritin molecule probably arose by gene duplication, although the degree of nucleotide sequence homology between the two is only about 50%. They assemble to form a sphere with a central cavity in which up to several thousand atoms of crystalline iron can be stored in the form of poly-iron-phosphate oxide.[174] Eight channels through the sphere are lined by hydrophilic amino acid residues, and six more are lined by hydrophobic residues.[175] Strong interspecies amino acid conservation is seen in the residues that line the hydrophilic channels, whereas marked variation is seen in those along the hydrophobic passages. The hydrophilic channels terminate with aspartic acid and glutamic acid residues and are lined by serine, histidine, and cysteine residues, all of which potentially bind metal ligands.

Although the two ferritin chains are homologous, only H ferritin has ferroxidase activity, which is essential for iron incorporation into the ferritin polymer.[176,177] Isoelectric focusing shows that ferritin polymers have a variable composition of H and L subunits. These isoferritins, as they are called, show tissue-specific variation. Ferritin from liver, for instance, is rich in L subunits, as is that from the spleen. In contrast, the heart has ferritin rich in H subunits. Increased H-subunit content correlates with increased iron use, whereas increased L-subunit content correlates with increased iron storage.[178] The ratio of H to L subunits increases with increased cell proliferation.[179] Thus, ferritin provides a flexible reserve of iron.

Despite the fact that it does not, itself, have enzymatic activity, a mutation in L ferritin has been shown to cause a distinctive iron deposition disease in humans termed *neuroferritinopathy*. In patients carrying a nucleotide insertion altering the carboxy-terminus of L ferritin, iron overload develops in the basal ganglia and leads to a neurodegenerative disease with variable extrapyramidal features.[180] By contrast, pathologic accumulation of apo-L-ferritin leads to a distinctive form of hereditary cataracts (hereditary hyperferritinemia-cataract syndrome) that is unassociated with iron metabolism abnormalities (see below). A number of other disorders, including aceruloplasminemia (see below), as well as pantothenate kinase deficiency, can also lead to pathologic iron deposition in distinctive regions of the brain.

Ferritin molecules aggregate into clusters that are engulfed by lysosomes and degraded. The end product of this process, hemosiderin, is an amorphous agglomerate of partially denatured protein and lipid interspersed with iron oxide molecules.[181] In cells overloaded with iron, lysosomes accumulate large amounts of hemosiderin, which can be visualized by Prussian blue (Perls) staining. Although the iron enmeshed in this insoluble compound constitutes an end-stage product of cellular iron storage, it remains in equilibrium with cytosolic ferritin. Ferritin iron, in turn, is in equilibrium with iron complexed to low–molecular-weight carrier molecules. Therefore, introduction of an effective chelator into the cell captures iron from the low–molecular-weight "toxic" iron pool, draws iron out of ferritin, and eventually depletes iron from hemosiderin as well, though only very slowly. As might be expected, the bioavailability of hemosiderin iron is much lower than that of iron stored in ferritin.

The large number of processed pseudogenes that exist for each subunit initially confounded pinpointing the chromosomal location of ferritin genes. Functional ferritin genes for the H and L subunits are located on human chromosomes 11 and 19, respectively.[182] In addition, there is an intronless gene encoding a mitochondrial, H-like ferritin molecule located on human chromosome 5q.[183] The function of mitochondrial ferritin remains uncertain, although the fact that its expression is increased in erythroid precursors from patients with sideroblastic anemia suggests that it is involved in mitochondrial iron management.[183,184]

Ferritin formation is controlled at multiple levels—transcription, message stabilization, translation, and subunit assembly. In the liver and in HeLa cells, iron rapidly induces the synthesis of L-subunit mRNA, with no effect on H-subunit transcription.[185,186] In contrast, induced differentiation of HL-60 promyelocytic leukemia cells and mouse erythroleukemia cells increases production of H-subunit mRNA.[187,188] Tumor necrosis factor induces H-chain transcription in human myoblasts.[189] Iron, heme, reactive oxygen species, and oxidative stress all enhance transcription and translation of the ferritin heavy chain (reviewed by Rouault[190]).

Cytoplasmic ferritin mRNA forms a stable complex with several proteins. Both iron and interleukin-1β enhance translation of ferritin mRNA.[191] Influx of iron into cells shifts the message onto the ribosomes, thereby enhancing the synthesis of ferritin subunits.[192] This translational control mechanism involves an RNA-protein interaction that links the expression of genes encoding ferritin, the transferrin receptor, enzymes of heme biosynthesis, DMT1, ferroportin, and other proteins. Zahringer, Baliga, and Munro[192] initially showed that ferritin synthesis was regulated at the level of message translation. Comparison of the 5′untranslated regions of ferritin mRNA encoding both heavy and light chains showed striking conservation of a 28-bp sequence motif that was predicted to form a stable RNA stem-loop structure and was necessary for translational control of ferritin (Fig. 11-5, inset).[193,194]

Subsequently designated the iron response element (IRE), this RNA stem-loop is recognized by at least two specific RNA-binding proteins called IRP1 and IRP2 for iron regulatory proteins 1 and 2. IRP1 is a 98-kd soluble polypeptide with homology to the mitochondrial tricarboxylic acid–cycle enzyme aconitase (ACO1).[195,196] Indeed, IRP1 is the cytoplasmic isozyme of aconitase. Like mitochondrial aconitase, the enzymatic active site of IRP1 contains a 4Fe/4S cluster. When the iron concentration in the cytosol is high, the cluster is complete and the protein is in its holoenzyme form and possesses aconitase activity and cannot bind mRNA. Under these conditions, the ferritin message is translated efficiently. Conversely, when the iron concentration is low, the protein is in its apo-form and aconitase activity is absent, RNA binding is avid, and ferritin message translation is blocked because translation initiation complexes cannot form properly.[197-202] Thus, IRP1 is a dual-function protein, with ambient iron controlling the switch by assembly and disassembly of a 4Fe/4S cluster. Other molecules that modulate the switch include ascorbic acid, nitric oxide, and heme, all of which contribute to the oxidation status of the cell.[203-205] This mechanism allows ferritin protein expression to be rapidly

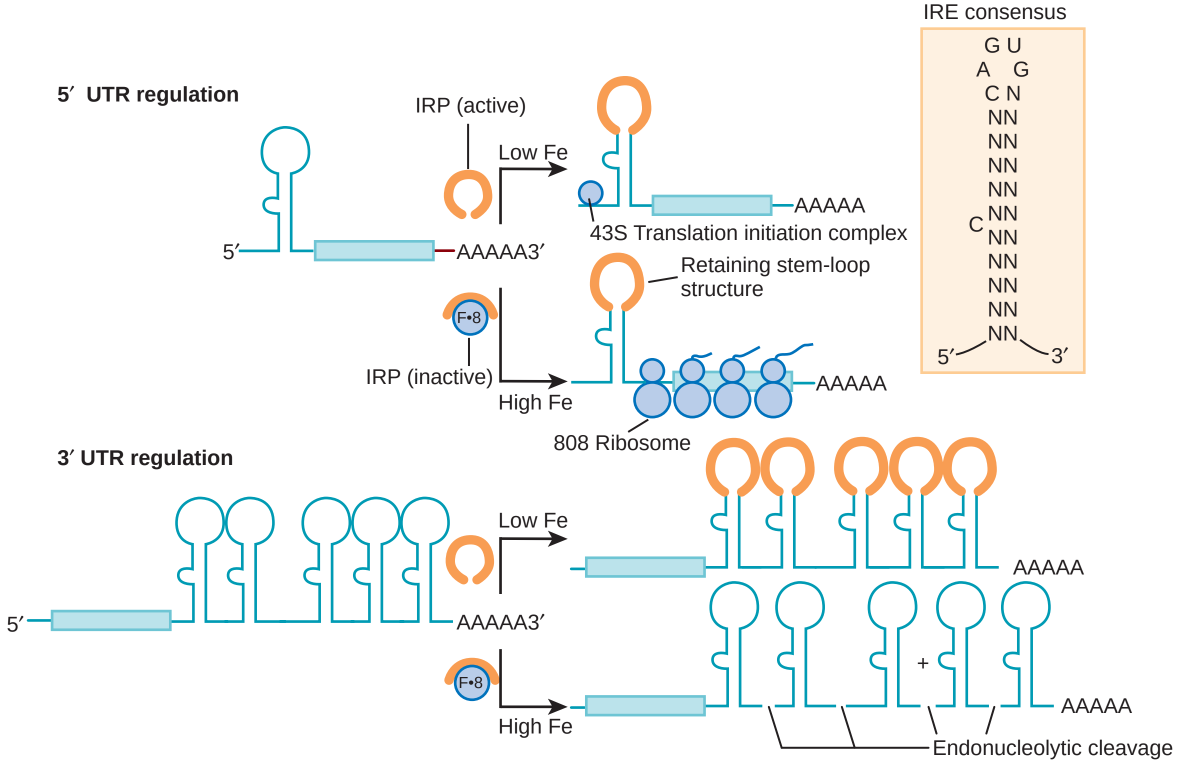

Figure 11-5 Iron response element *(IRE)*/iron regulatory proteins *(IRP)* regulation. Two mechanisms of action of IRPs are shown. An IRP molecule binds to an IRE stem-loop structure located in the untranslated regions *(UTRs)* of messenger RNA sequences. A consensus IRE structure is shown in the *inset*. Under low-iron or heme conditions, IRPs bind avidly to RNA. With abundant iron, no binding occurs. Binding of IRP to IRE elements in the 5′ UTR blocks translation. In contrast, binding of IRPs to 3′ UTR IRE elements protects messenger RNAs from degradation.

downregulated without degradation of mRNA. The importance of IRP-mediated translational control is underscored by the fact that plasma hyperferritinemia and ferritin-containing cataracts develop in patients with mutations in the L-ferritin IRE.[206,207] Nevertheless, these patients with hereditary hyperferritinemia cataract syndrome do not have abnormalities in iron homeostasis.[208,209] Furthermore, it appears that an iron overload disorder resembling hereditary hemochromatosis develops in rare patients with mutations in the H-ferritin IRE, presumably because increased H-ferritin expression leads to increased cellular iron assimilation or retention, or both.[210]

A second IRE-binding protein, IRP2, also modulates posttranscriptional expression of mRNA by binding to IREs in much the same way. Initially isolated in the liver, IRP2 has been found in all tissues examined and is more abundant than IRP1 in some.[211,212] IRP2 lacks aconitase activity[211] and does not contain an Fe/S cluster to function as an iron sensor. Rather, IRP2 protein levels are modulated directly by iron or by heme and the molecule is actively degraded when iron or heme (or both) is abundant.[213-216] Recently, it has been shown that an E3 ubiquitin ligase complex that contains FBXL5 controls IRP2 protein abundance by targeting it for proteasomal degradation. FBXL5 contains an iron- and oxygen-binding hemerythrin domain that acts as a ligand-dependent regulatory switch that mediates the stability of FBXL5 under iron- and oxygen-replete conditions.[217,218]

The Transferrin Receptor

IRPs also modulate expression of the transferrin receptor gene. The gene encoding the transferrin receptor is located on human chromosome 3q26.2-qter, near the gene encoding transferrin at 3q21.[219] It consists of 19 exons spread over 31 kb.[220] The transferrin receptor mRNA is approximately twice the length needed to encode the receptor protein. Its lengthy 3′untranslated region contains five RNA stem-loop structures that are structurally similar to those of the ferritin IREs.[221-223] IRP binding to these sites increases the stability of the transferrin receptor message by obscuring an endonucleolytic cleavage site (see Fig. 11-5).[224] The result is a larger amount of transferrin receptor mRNA in the cell. A deficit of cellular iron raises transferrin receptor mRNA levels at least in part through enhanced message stability. However, IRE regulation is probably not responsible for the primary increases in transferrin receptor number in erythroid cells, because this occurs at the transcriptional level and is unaffected by iron status. Relatively little is understood about the tissue-specific transcriptional regulation of the transferrin receptor.[225]

The versatile regulatory functions of IREs may also confer iron-dependent regulation on other proteins. IREs are present in the 5′ untranslated regions of the mRNA encoding the erythroid form of the heme biosynthetic enzyme δ-aminolevulinic acid synthase, mitochondrial aconitase, and ferroportin.[30,31,33,226-228] A single 3′ IRE is found in one splice isoform of DMT1 mRNA.[229] As the rate-limiting enzyme in heme biosynthesis, the IRE in the erythroid form of δ-aminolevulinic acid synthase produces a conceptually satisfying link between iron and heme production. However, the functions of the IREs in the other mRNAs are not yet understood. It is postulated that all IREs serve the common purpose of coupling changes in the iron status of the cell with its ability to use and store the element (see Fig. 11-5). When the intracellular iron concentration is low, the IRE of ferritin mRNA binds IRP and reduces ferritin synthesis because additional iron storage capacity is not needed in this circumstance. Simultaneously, the level of transferrin receptor mRNA increases as the IRP stabilizes the message, thereby increasing expression of the transferrin receptor. Together, these events increase the flow of iron into cells while protecting against iron toxicity. When iron levels in the cell are high, the opposite scenario is operative. Although the IRPs accomplish these regulatory feats under extremes of iron status, the importance of IREs and IRPs in modulating gene expression in normal humans and animals is not fully understood. Recently, novel IRE-containing genes have been identified, with roles less directly related to iron homeostasis.[230-232]

In an attempt to sort out the unique functions of IRP1 and IRP2 and to evaluate their roles in vivo, targeted mutagenesis was used to generate mutant mice lacking each of the proteins.[233-238] Initial characterization of IRP1 knockout mice indicated that they had no identifiable defects, but more recent studies have shown that the animals have a transient polycythemia that is associated with an elevated erythropoietin level due to dysregulation of HIF-2α translation, providing further evidence for crosstalk between hypoxia-sensing and iron-regulatory pathways.[239-241] IRP2 knockout mice have abnormalities in several tissues. Initially, the most striking aspect of the IRP2 knockout phenotype was a late-onset neurologic disorder associated with abnormal deposition of iron in white matter tracts and nuclei throughout the brain.[236] The mice demonstrated ataxia, bradykinesia, and tremors. However, these findings could not readily be explained by known activities of IRP2, and a second group reported that an independently targeted line of mice did not have obvious neurodegeneration.[235] It is possible that the discrepancy may be explained by differential severity of a neurologic phenotype on different genetic backgrounds. More recently, both groups have reported that IRP2-deficient mice have mild microcytosis and erythropoietic protoporphyria as well as subtle perturbations in systemic iron homeostasis.[233,242]

Serum Ferritin and Soluble Transferrin Receptors

Although most ferritin is located within cells, a measurable amount of the protein exists in serum. Intracellular concentrations, particularly in the liver, are several orders of magnitude higher than serum concentrations. Therefore, a small amount of cellular lysis could liberate a relatively large amount of ferritin. However, nearly all serum ferritin appears to be secreted. There are probably several cell types that are sources of secreted extracellular ferritin, including iron-recycling macrophages and hepatocytes. Circulating ferritin consists almost exclusively of L-chain subunits.[243] In contrast, intracellular ferritin contains a mixture of H and L subunits. In addition, unlike intracellular ferritin, circulating ferritin is glycosylated, thus suggesting that it passes through the endoplasmic reticulum and Golgi apparatus in a manner similar to that of other secreted proteins.

Serum ferritin levels decline with iron deficiency and rise with iron overload.[244] In the absence of severe liver disease, infection, or chronic inflammation, serum ferritin is roughly proportional to total body iron stores. The correlation between serum ferritin levels and body iron stores is useful in the evaluation of patients with possible iron deficiency or iron overload. A low serum ferritin level (<12 μg/L) invariably represents iron deficiency, and high serum ferritin levels are found in patients with iron overload. Extremely high serum ferritin levels should be interpreted cautiously, however, because the correlation between ferritin levels and body iron stores is approximately linear only for storage reserves of iron ranging between 1 and 3 g. In addition, normal circulating ferritin values vary with sex and age. These considerations must be factored into any evaluation of iron stores based on ferritin values, particularly in children.

Various conditions modify serum ferritin levels. Inflammation increases the serum ferritin concentration severalfold.[245] Infections, particularly chronic conditions such as tuberculosis or osteomyelitis, may also increase levels substantially, presumably because of the associated inflammatory response. Autoimmune disorders, chronic renal disease, and chronic liver disease are likewise associated with elevated levels. A number of tumors are variably associated with an increased level of circulating ferritin. For example, ferritin is an important prognostic factor in childhood neuroblastoma, in which serum ferritin levels correlate with disease severity.[246]

The mechanisms by which inflammation and tumors increase the quantity of plasma ferritin are not fully understood. Treatment with interleukin-1β, a prime mediator of the inflammatory response, increases synthesis of ferritin in human hepatoma cells,[191] whereas tumor necrosis factor has been shown to increase ferritin mRNA levels in murine cells in culture.[189] These cytokines may also increase ferritin synthesis in cells secreting the protein. In addition, the inflammatory cytokine interleukin-6 induces hepcidin expression,[47] which results in increased macrophage iron storage and probably contributes to increased serum ferritin levels. Furthermore, serum ferritin elevated out of proportion to iron stores and the transferrin saturation is a hallmark feature of ferroportin disease (see later).[247]

Soluble transferrin receptors are also found in serum. Small vesicles containing transferrin receptors are shed from reticulocytes during their maturation to erythrocytes.[248] In addition to these vesicle-associated receptors, the extracellular portion of the transferrin receptor lacking its transmembrane anchor can be found in the circulation.[249-251] One mechanism by which soluble transferrin receptors are generated is a membrane-associated protease activity that clips the molecule between amino acids 100 and 101, at the base of its extracellular stem.[252,253] This cleavage is potentiated by mutation of an *O*-linked glycosylation site at amino acid 104, thus suggesting that differential glycosylation may play a regulatory role.[254] The transferrin-binding domain is intact in these soluble receptors, and they are likely to be complexed with transferrin in serum.

Because maturing red cells shed their transferrin receptors, the amount of soluble transferrin receptor in plasma reasonably reflects the degree of erythropoiesis. Measurement of plasma levels of soluble transferrin receptor provides a means of estimating erythropoietic activity that is simpler than the relatively cumbersome PIT determination.[255] Soluble transferrin receptor (sTfR) is present in substantially lower amounts in patients with aplastic anemia relative to normal individuals. In contrast, values in patients with anemia caused by ineffective erythropoiesis are markedly increased. Patients with iron deficiency also have increased levels of circulating soluble transferrin receptors. This may result in part from the increase in cellular transferrin receptor expression produced by iron starvation and in part from the increased erythroid turnover associated with the ineffective erythropoiesis of iron deficiency. sTfR and serum ferritin values can be considered together as the ratio of sTfR to the log of ferritin (sTfR-F index). Values greater than 1.5 suggest iron deficiency alone or in combination with an inflammatory condition; values less than 1.5 are characteristic of the anemia of chronic inflammation.[256] The sTfR-F index also appears to be sensitive enough to detect iron deficiency before iron-restricted erythropoiesis is clinically apparent.[257] sTfR and the sTfR-F index are both decreased in patients with iron overload.[258,259]

EXTREMES OF IRON BALANCE

Iron disorders are invariably abnormalities in iron balance or distribution, or both. Iron deficiency is primarily due to acquired causes. In contrast, primary iron overload usually results from genetic abnormalities that perturb the regulation of intestinal iron absorption. Disorders affecting other steps in transport may be manifested as inappropriate iron accumulation in some tissues and iron deficiency in other tissues.

Iron Deficiency

Epidemiology of Iron Deficiency

Iron deficiency is the most frequent and widespread nutritional deficiency in the world[260] because it is common in developing and developed countries alike. In fact, iron deficiency is the only micronutrient deficiency that is prevalent in virtually all developed countries.[260] To this end, one of the U.S. national health objectives for 2010 was to reduce iron deficiency in vulnerable populations such as toddlers and women of childbearing age by 3% to 4%.[261]

Increasing rates of breast-feeding and the availability of iron-fortified formula (see later), in conjunction with initiatives such as the U.S. Special Supplemental Food Program for Women, Infants and Children and the American Academy of Pediatrics' promotion of formula in place of cow's milk, have greatly reduced the prevalence of iron deficiency *anemia* in infants in developed countries. Nonetheless, iron deficiency, both with and without anemia, remains relatively common. According to the Fourth National Health and Nutrition Examination Survey (NHANES IV), iron deficiency without anemia exists in 7% of toddlers aged 1 to 2 years, 9% of adolescent girls, and 16% of women of childbearing age.[262] Adolescents participating in strenuous training are another pediatric subpopulation at risk for iron deficiency. For example, young military recruits and elite adolescent athletes have an increased risk of nonanemic iron deficiency.[263-265]

Socioeconomic factors are associated with iron deficiency anemia in children. For example, infants and children of low-income and minority backgrounds have higher documented rates of iron deficiency anemia.[266,267,268] Although food insecurity is known to be associated with iron deficiency anemia,[269] other factors such as bottle-feeding patterns may also play a role in the prevalence of iron deficiency.[270] The iron status of young children correlates closely with the iron status of their mothers, thus indicating that a constellation of factors in the child's environment influence iron intake and therefore iron status.[271]

Phases of Development of Iron Deficiency

Because most of the body's iron is directed toward synthesis of hemoglobin, erythrocyte production is among the first casualties of iron deficiency to become clinically apparent in usual laboratory evaluations. However, it actually represents a late stage of iron depletion. Iron deficiency progresses through three discernible phases:

1. *Prelatent iron deficiency* occurs when tissue stores are depleted, without a change in hematocrit or serum iron levels. This stage of iron deficiency may be detected by low serum ferritin measurements.

2. *Latent iron deficiency* occurs when reticuloendothelial macrophage iron stores are depleted. The serum iron level drops and TIBC increases without a change in hematocrit. This stage may be detected by a routine check of fasting, early-morning transferrin saturation. Erythropoiesis begins to be limited by a lack of available iron, and sTfR levels increase. The reticulocyte hemoglobin content (CHr) decreases because newly produced erythrocytes are iron deficient.[272] The bulk of the erythrocyte population appears normal. For this reason, sole reliance on indicators derived from the entire erythrocyte population frequently fails to detect this stage of iron deficiency.[272,273]

3. *Frank iron deficiency* anemia is associated with erythrocyte microcytosis and hypochromia. It is detected when iron deficiency has persisted long enough that a large proportion of the circulating erythrocytes were produced after iron became limiting.

Etiology of Iron Deficiency

The development of iron deficiency is a result of the interaction between iron intake, physiologic iron requirements, and the potential for blood loss.

Inadequate Iron Intake

Dietary Sources. Heme, derived from animal tissues, is the most readily absorbed form of iron. Uptake occurs independently of gastric pH and, like the uptake of nonheme iron, is increased in patients with high marrow erythroid activity. Much of the world's population eats little or no meat; for these peoples, nutrition is derived from cultivated grasses such as rice, plants that are relatively poor sources of iron.[22] Eating such a diet is one of the factors that make iron deficiency anemia the most common nutritional anemia worldwide.

In developing countries, nutritional iron deficiency is often compounded by chronic blood loss from parasitic infections and malaria (see later). In industrialized countries, however, iron deficiency is usually due to insufficient dietary intake to meet physiologic needs. Because of their rapid growth and increased need for iron, infants, toddlers, female adolescents, and pregnant women are especially vulnerable.

Consumption of cow's milk may contribute to iron deficiency through several mechanisms. Cow's milk and human milk both have low iron content, but the bioavailability of iron in human milk is greater.[274] Therefore, the transition from human milk to cow's milk may place toddlers at risk for iron deficiency. Indeed, the prevalence of iron deficiency increases with the duration of bottle-feeding of cow's milk.[270] Cow's milk also compounds iron deficiency by replacing iron-rich foods in the diet. In addition, components of cow's milk, such as calcium and caseinophosphopeptide, can directly interfere with iron absorption.[275,276] Furthermore, whole cow's milk contains proteins that may irritate the lining of the gastrointestinal tract in infants, and even low-grade but chronic hemorrhage may produce significant iron deficiency.[277] The neonatal growth spurt requires a tremendous quantity of iron, thus compounding the disadvantages of cow's milk. For these reasons, current recommendations exclude cow's milk from the infant's diet in the first year of life, limit subsequent consumption of cow's milk to 24 ounces per day, suggest that nonbreastfed infants should receive iron-fortified formulas, and advise an additional source of iron for infants who are breastfed after 4 to 6 months of age, when they have depleted the excess iron that was present in newborn stores and erythrocytes.[278,279]

Poor Bioavailability. Although iron is abundant in the environment, it is nearly insoluble in aqueous solution, which makes acquisition of the common element a major challenge. Most environmental iron exists as insoluble salts. Gastric acidity assists in conversion to absorbable forms, but the efficiency of this process is limited.[280] Many plant products contain iron, but absorption frequently is limited both by low solubility and by powerful natural chelators that bind ambient iron. Phytates (organic polyphosphates) found in wheat products, for example, bind iron with tremendous avidity. The challenge of acquiring sufficient iron from the environment may have been an important factor in the spread of genes for hemochromatosis disorders.

High gastric pH reduces the solubility of inorganic iron and thereby impedes absorption. Surgical interventions, such as vagotomy or hemigastrectomy for peptic ulcer disease, formerly were the major causes of impaired gastric acidification with secondary iron deficiency, particularly in adults. The histamine H_2 blockers and the more recently introduced acid pump blockers, used to treat peptic ulcer disease and acid reflux, may cause defective iron absorption.[281,282] There is, however, little evidence that they substantially alter iron status in those without preexisting iron deficiency.[283-285]

The impaired function of gastric parietal cells associated with pernicious anemia not only reduces the production of intrinsic factor but also lessens gastric acidity and hence compromises absorption of iron. In addition, megaloblastic enterocytes absorb iron poorly. Frank iron deficiency can accompany the anemia produced by cobalamin deficiency.[286-288] However, the rarity of pernicious anemia in children makes this complication uncommon in pediatric populations.

A number of environmental factors can produce dietary iron deficiency by interfering with iron absorption, including metals such as cobalt, which share the iron absorption machinery. Lead may fall into this category, although it remains to be definitively established whether DMT1 is the primary intestinal lead transporter in vivo.[26] Regardless of the mechanism, it is clear that iron deficiency increases the rate of uptake of both iron and lead from the gastrointestinal tract. Iron deficiency and lead intoxication frequently coexist.

Malabsorption. Some disorders disrupt the integrity or surface area of the enteric mucosa and thereby hamper iron absorption (Box 11-2). Consequently, in the absence of blood loss (see below), iron deficiency unresponsive to oral iron therapy should prompt an investigation for intestinal diseases that affect the duodenum. Inflammatory bowel disease, particularly Crohn disease, may injure extensive segments of the small intestine, including the jejunum and duodenum. Invasion of the submucosa by inflammatory cells and disruption of the tissue architecture impair iron absorption and uptake of dietary nutrients.[289] Occult gastrointestinal bleeding exacerbates the problem. The result is iron deficiency anemia complicated by the anemia of chronic inflammation. Furthermore, Crohn disease frequently involves the terminal ileum, with the concurrent development of cobalamin deficiency. These disorders are not diagnostic enigmas. Patients with extensive bowel involvement are very ill. Diminishing the underlying inflammatory process best treats the iron deficiency.

Tropical sprue and celiac disease (gluten-sensitive enteropathy) can also interfere with iron absorption.[289] Loss of the villous absorptive surface along with chronic inflammation causes profound malabsorption, although some patients with deranged iron absorption lack gross or even histologic changes in the structure of the bowel mucosa. The anemia in these patients is often complicated by a superimposed, generalized nutritional deficiency.[290] Celiac disease frequently improves with ingestion of a gluten-free diet, with secondary correction of the anemia.

Iron absorption may be disrupted when substantial segments of bowel, particularly the proximal duodenum, are removed surgically. Intractable inflammatory bowel disease, traumatic abdominal injury, and structural complications such as intestinal volvulus or intussusception all may necessitate intestinal resection. Likewise, obesity surgeries that bypass the proximal duodenum may limit iron absorption. Iron deficiency develops slowly afterward and may not become evident for several years after the surgical procedure.

Box 11-2 **Causes of Iron Deficiency Anemia**

- INADEQUATE ABSORPTION
  - Poor bioavailability (absorption of heme Fe > Fe^{2+} > Fe^{3+})
  - Antacid therapy/high gastric pH
  - Bran, tannins, phytates, starch
  - Other metals (e.g., cobalt, lead)
  - Loss or dysfunction of absorptive enterocytes (e.g., gluten-sensitive enteropathy, gastric bypass, Crohn disease)
- INSUFFICIENT OR INACCESSIBLE IRON STORES
  - Physiologically Increased Requirements
  - Pregnancy, particularly repeated
  - Rapid growth (e.g., infancy)
  - Gastrointestinal Blood Loss
    - Epistaxis
    - Gastritis
    - Ulcer
    - Meckel diverticulum
    - Milk-induced enteropathy
    - Parasitosis
    - Varices
    - Tumor or polyps
    - Inflammatory bowel disease
    - Arteriovenous malformation
    - Colonic diverticula
    - Hemorrhoids
  - Vaginal Blood Loss
    - Increased menstrual blood loss
    - Tumor
  - Urinary Blood Loss
    - Chronic intravascular hemolysis (e.g., paroxysmal nocturnal hemoglobinuria)
    - Chronic glomerulonephritis
    - Chronic infection
    - Tumor
  - Pulmonary Blood Loss
    - Pulmonary hemosiderosis
    - Tuberculosis
    - Bronchiectasis
  - Inflammation or Infection
  - Defects in intestinal iron uptake (e.g., *TMPRSS6* mutations)
- INADEQUATE PRESENTATION TO ERYTHROID PRECURSORS
  - Atransferrinemia
  - Anti–transferrin receptor antibodies
- ABNORMAL INTRACELLULAR TRANSPORT OR UTILIZATION
  - Erythroid iron-trafficking defects (e.g., *DMT1* mutations)
  - Defects in heme biosynthesis

Malabsorption of iron is rare in the absence of gross or microscopic structural defects of the intestine or the anemia of chronic inflammation (see later). However, multiple families with an autosomal recessively inherited predisposition to iron deficiency and systemic iron malutilization have been described.[291-293] These individuals exhibit iron deficiency anemia at an early age that is characterized by a nearly complete lack of response to oral iron supplementation and a slow, incomplete response to parenteral iron therapy—so-called iron-refractory, iron deficiency anemia (IRIDA). Individuals with IRIDA have biallelic inactivating mutations in the TMPRSS6 protein, a transmembrane serine protease expressed in the liver, that leads to inappropriate hepcidin overexpression and eventuates in impaired iron absorption and iron recycling (Fig. 11-6).[294-296] Importantly, a

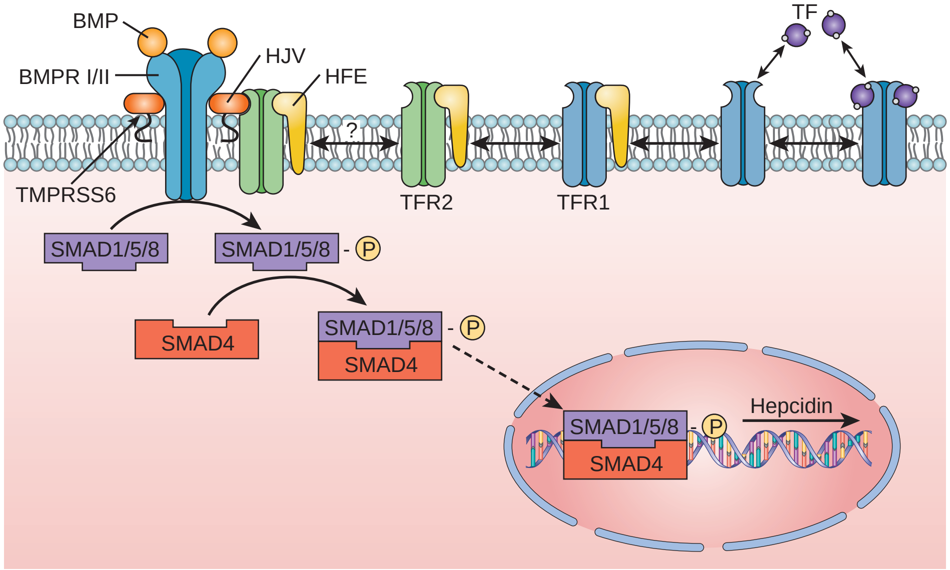

Figure 11-6 Iron-dependent BMP signaling. Transferrin receptor 1 *(TFR1)*; the autosomal recessive hereditary hemochromatosis proteins HFE, transferrin receptor 2 *(TFR2)*, and hemojuvelin *(HJV)*; as well as the bone morphogenetic protein receptor type I and II *(BMPR I/II)* complex are associated with the cell surface. HFE associates with TFR1 and TFR2 in an equilibrium modified by the concentration of holotransferrin *(TF)*. It has been suggested that HFE and TFR2 can further associate with the HJV-BMPR I/II signaling complex to modify BMPR signaling mediated by phosphorylation upon sons of mothers against decapentaplegic homologues *(SMADs)* 1, 5, and 8. Association of phosphorylated SMAD1/5/8 with a common co-SMAD, SMAD4, leads to translocation of the complex into the nucleus and stimulation of hepcidin *(HAMP)* gene expression. TMPRSS6 cleaves HJV to down-regulate the hepcidin response to any given plasma holotransferrin concentration. *(Modified from Fleming MD: The regulation of hepcidin and its effects on systemic and cellular iron metabolism.* Hematology Am Soc Hematol Educ Program *151-8, 2008.)*

common single-nucleotide polymorphism (SNP) in the protein-coding region of TMPRSS6 (a substitution of a valine for an alanine at amino acid position 736) is strongly associated with lower plasma iron and ferritin levels as well as several red blood cell parameters,[297-300] and has been shown to modify the severity of disorders of iron overload.[301] Although mutations in DMT1 might also be expected to, and in model organisms do indeed,[25,125,302] lead to a somewhat similar congenital iron deficiency anemia phenotype as mutations in TMPRSS6; in fact, reported patients with DMT1 mutations have iron-restricted erythropoiesis in the setting of systemic iron overload.[303-307]

Systemic Iron Loss

Gastrointestinal Blood Loss. Blood loss is the leading cause of iron deficiency worldwide. The gastrointestinal tract is both the site of iron uptake and the most common site of blood loss, particularly when bleeding is not readily apparent. However, bleeding from other sites should also be considered if iron deficiency has not otherwise been explained.

Anatomic defects of the gastrointestinal tract commonly cause blood loss and consequent iron deficiency. The most frequent congenital defect is Meckel diverticulum, which results from a persistent omphalomesenteric duct. This abnormality can produce abdominal pain and, occasionally, intestinal obstruction in young children. Meckel diverticulum may equally be associated with clinically overt or occult blood loss and secondary iron deficiency.[308] Peptic ulcer disease is extremely uncommon in children but should be considered as a cause of gastrointestinal blood loss when the clinical setting suggests it.

Other structural defects of the gastrointestinal tract may cause bleeding as well. Arteriovenous malformations involving the superficial blood vessels occur in patients with hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome). These defective vessels frequently bleed to a degree that engenders iron deficiency. Although the disorder is transmitted as an autosomal dominant trait, the characteristic vascular lesions rarely attain clinical significance before young adulthood. The condition is easily recognized because the mucosal lining of the oropharynx and nasal cavity exhibits characteristic telangiectasias.

The leading cause of gastrointestinal blood loss worldwide is parasitic infestation. Hookworm infection, caused primarily by *Necator americanus* or *Ancylostoma duodenale*, is endemic in much of the world and is often asymptomatic. Microscopic blood loss leads to significant iron deficiency, particularly in children.[309] More than 1 billion people, most in tropical or subtropical areas, are infested with parasites. Combined, their total daily blood loss exceeds 11 million liters. The larvae spawn in moist soil and penetrate the skin of unprotected feet. Hookworm infection, once prevalent in the southeastern United States, declined precipitously with better sanitation and routine use of footwear when outside.

Trichuris trichiura, the culprit in trichuriasis or whipworm infection, is believed to infest the colon of 600 to 700 million people. Only 10% to 15% of these individuals have worm burdens sufficiently great to produce clinical disease. However, most are children between the ages of 2 and 10 years. Growth retardation, in addition to iron deficiency, occurs with heavy infestation.[310] Trichuriasis is the most common helminthic infection encountered in Americans returning from visits to tropical or subtropical regions of the world.

Urinary Blood Loss. Occasionally, blood loss into the urinary tract outstrips iron absorption. However, urinary blood loss is usually apparent and sufficiently alarming that patients seek medical attention before substantial iron deficiency develops. Iron deficiency resulting from hematuria secondary to renal disease is relatively uncommon.

The best characterized cause of significant renal blood loss is Berger disease, which produces relapsing episodes of gross or microscopic hematuria. The disorder occurs most commonly in older children and young adults. It is characterized by diffuse mesangial proliferation or focal and segmental glomerulonephritis. Diffuse mesangial deposits of IgA are the hallmark of the disorder. Although the disease spontaneously remits in some children, progression to end-stage renal disease may occur. Occasionally, Goodpasture syndrome produces substantial urinary blood loss. Immunofluorescent staining of biopsy specimens reveals the characteristic antibodies to basement membrane lining the glomeruli. Blood loss into the urinary bladder can occur in association with infectious cystitis. Hematuria to the point of iron deficiency is extremely uncommon, however.

Patients with chronic intravascular hemolysis, such as those with paroxysmal nocturnal hemoglobinuria or arterial jet lesions from congenital or acquired heart disease with or without endovascular appliances, may also develop significant renal blood loss.

Pulmonary Blood Loss. Although pulmonary blood loss may be sufficiently severe to produce iron deficiency, it is distinctly rare. Idiopathic pulmonary hemosiderosis primarily affects children and young adolescents (reviewed by Specks[311]). Any child with iron deficiency and unexplained pulmonary disease should be evaluated for this potentially deadly disorder, which is characterized by slow but intractable hemorrhage into the bronchioles and alveoli. Iron deficiency anemia is a major part of the clinical picture of pulmonary hemosiderosis inasmuch as pulmonary macrophages are distinct from the normal iron-recycling circuit and iron trapped within these cells is effectively lost from the functional systemic pool. Consequently, a paradoxic situation evolves in which iron deficiency anemia develops in the presence of a surfeit of total body iron. Bronchoalveolar lavage reveals hemosiderin-laden macrophages that are characteristic of the disorder. Gastric aspiration, which is a less invasive procedure, may also reveal these cells due to swallowing of sputum. Chronic pulmonary infection with bronchiectasis, once considered to be a frequent cause of pulmonary hemorrhage, is now rare. Iron that accumulates in these cells induces the formation of toxic products, which leads to the activation of macrophages. These cells begin to damage the delicate lining of the bronchoalveolar tree and produce severe fibrosis. Oxygen exchanges poorly across the damaged alveolar surfaces, thereby lowering the efficiency of oxygen/carbon dioxide exchange. Late in the course of pulmonary hemosiderosis, the radiographic picture of the lungs is striking and frequently shows marked retraction and scarring.[312] The restrictive lung disease and substantial pulmonary arterial shunting across the lung may eventually be fatal.

Most often the cause of pulmonary hemosiderosis evades elucidation. In a number of patients, it occurs in conjunction with disorders of immune dysfunction, including celiac disease[313] and Goodpasture syndrome.[314,315] Treatment of the associated disorder can lead to remission of the pulmonary process, consistent with an immunologic mechanism of the lung injury.[316] Chloroquine treatment has been used with modest success in some patients.[317] Cyclophosphamide has produced responses in a number of patients.[318] A combination of prednisone and azathioprine has also been used.[319] Other immunosuppressive agents may prove to be even more effective as immunologic suppressers in the treatment of this condition, but the infrequency of the disorder means that a large, multicenter trial is needed to collect definitive treatment information. Although the prognosis was once believed to be very poor for all patients with idiopathic pulmonary hemosiderosis, more recent data suggest that such may not be the case.[316]

Menstrual Blood Loss. Blood loss associated with menstruation predisposes women to iron deficiency. In a sample of healthy, menstruating, nonpregnant Danish women 18 to 30 years of age not receiving iron supplementation, serum ferritin levels correlated inversely with the duration and intensity of menstrual bleeding.[320] Menorrhagia or dysfunctional uterine bleeding may lead to frank iron deficiency anemia. In many cases, dysfunctional uterine bleeding is due to anovulation, a common phenomenon in pubertal females. Progestational agents or oral contraceptives may ameliorate the dysfunctional uterine bleeding or menorrhagia. If this approach does not resolve the abnormal bleeding, other causes such as pelvic abnormalities or disorders of hemostasis, particularly von Willebrand disease, should be considered. Women with menorrhagia, especially menorrhagia present since menarche, have a high incidence of von Willebrand disease (13%), which in the general population is 1% to 2%.[321,322]

Consequences of Iron Deficiency

Although anemia is the most prominent manifestation of iron deficiency, there may be other clinically significant sequelae of iron deficiency, even in the absence of anemia. Organ and tissue dysfunction, including impaired immunity, decreased muscle performance and neurocognitive function, and poor weight gain, may occur with iron deficiency. Significant inroads in understanding some of these effects, particularly cognitive dysfunction in young children, have been made in the past few years.

Neurocognitive Effects. The association between iron deficiency anemia and impaired neurocognitive function is well established, and this association holds even when potential confounders such as psychosocial and environmental factors are taken into account. Infants and toddlers, who are undergoing critical neurocognitive development, may be at particular risk for such effects.[323]

Iron deficiency that has not yet progressed to anemia is also associated with impaired mental and motor functioning.[324,325] For example, when tested with the Bayley Scales of Infant Development, nonanemic iron-deficient infants had lower developmental scores than iron-sufficient infants did, and these abnormalities were detected in children as young as 9 to 12 months.[324] In addition, the degree of developmental deficits in infants may correlate with the level of iron deficiency.[325,326] Older populations of children, such as nonanemic iron-deficient preschoolers, have lower mental development scores,[327] and school-age children and adolescents have lower math and verbal scores than their iron-sufficient peers do.[327-330]

In some investigations, dietary iron supplementation has reversed the cognitive dysfunction.[324,325,328] For example, in the study of 9- to 12-month-old infants cited earlier, iron replacement increased Mental Development Index scores substantially in only 7 days.[324] Numerous other studies, however, failed to show improvement in depressed performance.[326,331-333] Lozoff and colleagues[334] have also demonstrated that these effects remain evident as long as 19 years later and that children of low socioeconomic status may be disproportionately affected. Older patients with iron deficiency may express a subjective state of cognitive impairment, likely reflecting central nervous system iron deficiency, that is rapidly reversed by treatment.

Iron deficiency may increase the risk for lead exposure through either pica or increased absorption. Concomitant lead toxicity can further hamper the psychological development of these children.[335] Information on the long-term effects of iron deficiency during infancy highlights the importance of early detection and intervention. Health care providers must therefore work actively to prevent and detect iron deficiency during infancy.

The mechanism by which iron deficiency impairs neurologic function is unknown. Iron deficiency could impair neurotransmitter mechanisms. It has been shown to decrease expression of dopamine receptors in the rat brain.[336] Iron deficiency may also interfere with myelination and alters myelin proteins and lipids in oligodendrocytes.[337] In addition, several enzymes in neural tissue require iron for normal function.[338] The cytochromes involved in energy production, for example, are predominantly heme proteins.

Pica. Pica, the compulsive consumption of nonnutritive substances, is a recurrent symptom in patients with iron deficiency. The precise pathophysiology of pica is unknown, but it is probably attributable to central nervous system iron deficiency. Patients often consume starch, ice, soil, foam, or clay. Both clay and starch can bind iron in the gastrointestinal tract and exacerbate the deficiency.[339,340] A dramatic example of the problems produced with clay consumption occurred in the 1950s in iron-deficient children along the border between Iran and Turkey.[341,342] These youngsters had other peculiar abnormalities, including massive hepatosplenomegaly, poor wound healing, and a bleeding diathesis. Presumably, the children initially had simple iron deficiency associated with pica, including geophagia. The soil contained compounds that bound both iron and zinc.[342] Secondary zinc deficiency was thought to cause the hepatomegaly and other unusual features.[341]

Epithelial Changes. Iron deficiency produces significant gastrointestinal tract abnormalities, reflecting the enormous proliferative capacity of this tissue. Angular stomatitis and glossitis with painful swelling of the tongue may develop. The flattened, atrophic, lingual papillae make the tongue smooth and shiny. A rare complication of iron deficiency is Plummer-Vinson syndrome, with the formation of a postcricoid esophageal web. Long-standing, severe iron deficiency affects the cells that generate the fingernails and produces koilonychia, or spooning. The nail substance is soft, so ordinary pressure on the fingertips, as occurs with writing, for instance, produces a concave deformity. Most of these abnormalities are now uncommon in industrialized nations and are particularly rare in children even given the relative frequency of iron deficiency.

Iron Deficiency Anemia. On physical examination, pallor, tachycardia, splenomegaly and a systolic murmur may be appreciated. As is true of all anemias, the anemia of iron deficiency impairs tissue oxygenation and produces the symptoms of pallor, weakness, fatigue, and lightheadedness. The morphologic features of the peripheral blood smear in patients with iron deficiency anemia vary greatly according to the severity and duration of the condition (Fig. 11-7). The earliest morphologic features of iron-deficient erythropoiesis in the peripheral blood are subtle variations in red blood cell size and the degree of hemoglobinization, with occasional smaller, less well hemoglobinized cells recognizable. These parameters are reflected by changes in the red cell distribution width (RDW) and the hemoglobin distribution width (HDW) and are usually present before overt hypochromia and

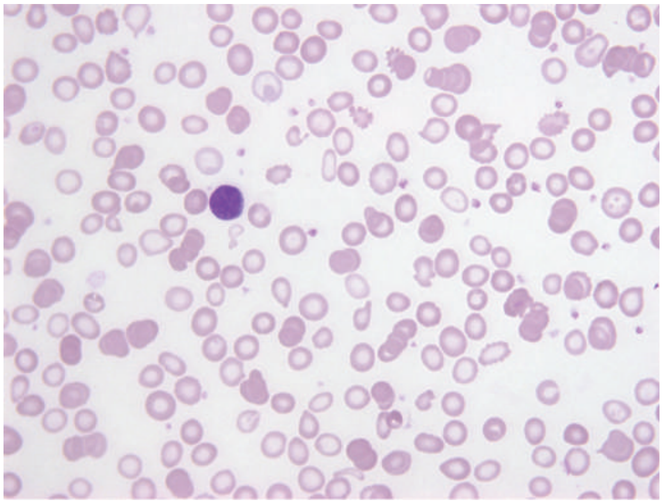

Figure 11-7 Peripheral blood smear in iron deficiency. Note the small, pale (microcytic, hypochromic) red blood cells with variable sizes and shapes (anisopoikilocytosis). Occasional target cells with central hemoglobin pooling as well as several somewhat elongated hypochromic microcytes (pencil cells) are present.

microcytosis are seen. Indeed, at this point, the hemoglobin and the mean cell volume (MCV) may be in the normal range. As the severity and duration of iron-deficient erythropoiesis increase, so do the extent of morphologic changes, with increasing variation in cell size (anisocytosis) and hypochromia and distinctive misshapen hypochromic microcytes with irregular, often angulated borders present (poikilocytes). The plasma membranes of iron-deficient red cells are abnormally stiff, and such cells are more prone to hemolysis.[343] This rigidity may contribute to poikilocytic changes, seen particularly with severe iron deficiency. Small, stiff, misshapen cells are cleared by the reticuloendothelial system, thereby further contributing to low-grade hemolysis. The cause of this alteration in membrane fluidity is unknown. Very thin, elongated hypochromic cells, so-called pencil cells, are said to be characteristic of iron deficiency. Occasional target cells, in which hemoglobin pools in the center of the erythrocyte, may be seen, but these cells, as well as polychromasia, indicative of a reticulocytosis, are not as prevalent as in β-thalassemia. Basophilic stippling, nucleated red blood cells, or nuclear remnants (i.e., Howell-Jolly bodies) are generally not present, distinguishing severe iron deficiency from thalassemias or hemoglobinopathies. β-thalassemia trait (see Chapter 21) also produces microcytic cells and borderline anemia and is sometimes confused with early iron deficiency. Iron deficiency, however, alters red cell size unevenly, resulting in a high RDW, which is typically normal in β-thalassemia trait.[344] Other common features of β-thalassemia trait are rare cells with basophilic stippling and target cells, which are generally not present in the earliest phases of iron deficiency. These characteristics are not sufficiently distinctive to be diagnostically definitive, however, and any suspicion should be confirmed with more objective tests such as a hemoglobin electrophoresis. Importantly, iron deficiency may lead to an elevated hemoglobin A2, confounding the interpretation of hemoglobin electrophoresis results. Given the not uncommon coexistence of iron deficiency and β-thalassemia trait, any abnormal hemoglobin electrophoresis should be interpreted in the context of iron studies (see below) and repeated after iron supplementation in the iron-deficient patient.

Thrombocytosis with platelet counts in the range of 500,000 to 700,000 cells/fL occurs frequently in iron-deficient patients. Megakaryocytes and normoblasts are derived from a common committed progenitor cell, the colony-forming unit–granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM). Thrombopoietin, the molecule that stimulates the growth of megakaryocytes and the production of platelets, is homologous to erythropoietin (see Chapter 6). The high levels of erythropoietin produced by iron deficiency anemia conceivably could cross-react with megakaryocyte thrombopoietin receptors and modestly raise the platelet count. However, this hypothesis has not been proved experimentally.

Bone marrow examinations are no longer routinely used to assess iron stores. However, when they are performed, in severe cases, late erythroblasts often have ruffled or frayed edges, not unlike other hypochromic anemias. Iron staining is best performed on aspirate smears rather than biopsies, as decalcification can leach iron out of the sample, resulting in false-negative results, and the cytologic preservation is typically inadequate to resolve minute, intraerythroid iron granules (sideroblasts, see below) indicative of erythroid iron sufficiency. Iron-stained marrow particles should have readily identifiable cells with coarse iron positivity, representing macrophages with abundant ferritin and hemosiderin storage iron. If particles are absent, careful microscopic examination of normal, iron-replete bone marrows under oil immersion should demonstrate up to 30% to 40% of the erythroid precursors with several fine, peripherally placed iron deposits that represent iron in cytosolic ferritin. Such cells are termed *sideroblasts*, are entirely normal, and are absent or decreased in iron deficiency. In the anemia of inflammation, where there is a defect in iron recycling, macrophage iron stores may be present but intraerythroblast iron will be decreased or absent (see below).

Diagnosis of Iron Deficiency

Because they are inexpensive and widely available, hematologic markers are frequently used to assess iron status. A hemoglobin content less than the fifth percentile in a reference population is often used to define anemia. Changes in erythrocyte parameters accompanying iron deficiency anemia include a decrease in MCV, reflecting microcytosis, and an increase in RDW, reflecting anisocytosis, as described earlier. Because these parameters represent averages of the entire red blood cell population, iron deficiency must be present for some time before it deviates from these measures.

The American Academy of Pediatrics and the U.S. Centers for Disease Control and Prevention recommend the use of hemoglobin to screen children at risk for iron deficiency. Because only the later stages of iron deficiency result in anemia, relying on hemoglobin (or hematocrit) to screen for iron deficiency misses many children who are, in fact, iron deficient and in whom adverse consequences, such as potentially irreversible neurocognitive impairment, may have already begun to occur. For example, based on NHANES data, a hemoglobin concentration of less than 11 g/dL has a sensitivity of 30% for detecting iron deficiency, as defined by ferritin, erythrocyte protoporphyrin, and transferrin saturation (see below).[345] That adverse consequences of iron deficiency may occur in the absence of anemia highlights the importance of diagnostic approaches that detect iron deficiency early, without relying on hematologic indicators that are altered only after iron deficiency has progressed.

Serum or plasma biochemical parameters based on iron metabolism are often used to diagnose iron deficiency. The tests that are commonly employed, as well as their physiologic relevance and confounding variables, are listed in Table 11-1. Plasma iron concentrations, largely reflecting the iron recycled from effete red blood cells, fall as iron is depleted, but they are also affected by diurnal variation and dietary intake. The availability of plasma iron-binding sites, or TIBC, increases as iron stores fall. Serum iron concentration divided by TIBC yields the transferrin saturation, which is expressed as a percentage of transferrin iron-binding sites occupied by

TABLE 11-1 Common Clinical Laboratory Tests to Assess Iron Status[*]

Test	What It Indicates	Result in Iron Deficiency	Result in Iron Overload	Confounding Conditions
Iron	Iron recycling/usage, stores (indirect)	↓	↑-↑↑	↓ in inflammation
Total iron-binding concentration (TIBC)	Serum/plasma transferrin	↑	Normal	↓ in inflammation
Transferrin saturation	% plasma iron-binding sites on transferrin that are occupied	↓-↓↓	↑-↑↑	↓ in inflammation
Free or zinc protoporphyrin	Functional mitochondrial iron status	↑	Normal	↑ in reticulocytosis
Soluble transferrin receptor (sTfR)	Erythropoietic mass	↑	Normal	Ineffective erythropoiesis
Ferritin	Iron stores (indirect)	↓-↓↓	↑-↑↑	↑ in inflammation
Bone marrow iron	Macrophage iron stores/retention, red blood cell iron uptake	↓	Normal to ↑	↑ in inflammation
Liver iron	Iron stores	↓-↓↓	↑-↑↑	Secondarily increased in cirrhosis
Hepcidin[†]	Hepatocyte iron	↓-↓↓	↑-↑↑	↑ in inflammation

[*] All measures are in serum or plasma unless otherwise indicated.
[†] Hepcidin is not yet routinely available as a clinical test.

iron. A transferrin saturation of less than 10% is a stringent definition of iron deficiency that is often employed as the "gold standard" against which other tests are judged. However, erythropoiesis is to some extent iron restricted at a transferrin saturation of 15% or less. Serum ferritin is a glycosylated form of the intracellular iron storage protein L-ferritin that is incapable of binding iron. The precise ontogeny and cells of origin of this circulating form of the protein are uncertain. Nevertheless, the serum ferritin is a good biomarker of total body iron stores and is roughly linearly related to this parameter over a wide range—from iron deficiency to moderate iron overload. A low serum ferritin level is a very specific indicator of iron deficiency, but ferritin is an acute-phase reactant and is elevated in chronic inflammatory states, independent of body iron status, thus reducing the sensitivity of the test. Ferritin has in fact been shown to be a poor indicator of iron deficiency in the pediatric population.[272] Whereas ferritin is a "positive" acute-phase reactant and may lead to an overestimate of iron stores in the setting of inflammation, serum iron and TIBC are "negative" acute-phase reactants and are reduced in this context.

Because of the limitations of both hematologic and biochemical tests, novel methods of diagnosing iron deficiency have been sought. Measures of red blood cell protoporphyrin IX, particularly zinc protoporphyrin (ZPP), assess the functional availability of iron to the erythrocyte mitochondrion, where iron is incorporated into protoporphyrin IX by ferrochelatase to form heme. In the context of mitochondrial iron insufficiency, zinc, an alternative ferrochelatase substrate, is inserted into the protoporphyrin ring. Measurement of the absolute level of red blood cell ZPP or, more specifically, the ratio of ZPP to iron protoporphyrin IX (i.e., heme), which is increased in iron deficiency, therefore provides an assessment of delivery of iron to erythroid mitochondria for heme synthesis.

sTfR is a truncated form of the transferrin receptor that is cleaved from reticulocytes and erythroid cells and circulates in plasma largely bound to transferrin. sTfR is increased in iron deficiency but is also increased in other anemias characterized by ineffective erythropoiesis (e.g., thalassemia). Though not uniformly available in all clinical laboratories, it may be a particularly important adjunct in distinguishing iron deficiency anemia from the anemia of inflammation, in which it is not increased.[346-348]

The cellular hemoglobin of the reticulocyte (CHr) is another test available only in selected laboratories because it is unique to instruments manufactured by Bayer (Tarrytown, NY). CHr has been evaluated prospectively as a means of routinely screening infants and toddlers.[273] In this setting, a CHr cutoff of 27.5 pg had 83% sensitivity and 72% specificity, whereas a hemoglobin concentration of less than 11 g/dL was a poor predictor of iron deficiency, with only 26% sensitivity. CHr has also been used to accurately assess the iron status of potential adult blood donors.[349] A summary of the relative sensitivity and specificity and approximate cost of each of the tests commonly employed to screen for iron deficiency is shown in Table 11-2.

Although commercial tests for urinary or plasma hepcidin are not yet currently in clinical use, measurement of this analyte may well have a role in assessing iron status, whether it be deficiency or overload, in the future. This will certainly be the case if algorithms for determining if the hepcidin is appropriate, or inappropriately high or low, for the clinical context, permitting some insight into the pathophysiology of the disorder under investigation. However it is ultimately implemented, analysis of hepcidin levels will, similar to the serum ferritin, be

TABLE 11-2 Sensitivity, Specificity, and Cost of Common Tests to Screen for Iron Deficiency

Method	Age	Cost	Sensitivity	Specificity	Reference
Transferrin saturation (<10%)	any	≈\$85	100%	100%	"Gold standard" defined as 100% sensitive and specific
Hb (<11 g/dL)	9-12 months	≈\$30	26%	95%	273
Hb (<10.7 g/dL)	9-12 months	≈\$30	67%	87%	623
MCV (<71 fL)	9-12 months	≈\$30	86%	83%	623
CHr (<27.5 pg)	9-12 months	≈\$50	83%	72%	273
Spun Hct (<33%)	9-36 months	≈\$15	16%	92%	624
Ferritin (<10 µg/L)	4-15 months	≈\$65	71%	57%	346
Ferritin (<10.9 µg/L)	9-12 months	≈\$65	83%	80%	625
ZPP (>50 nmol/mol heme)	9-36 months	≈\$150	81%	88%	624
FEP (>2.12 µmol/L RBC)	4-15 months	≈\$150	82%	57%	346
sTfR (>13.5 mg/L)	4-15 months	≈\$90	31%	95%	346

confounded by concomitant inflammation, or, like sTfR, ineffective hematopoiesis.

Treatment of Iron Deficiency

The most important steps in the evaluation and treatment of iron deficiency are determining the cause and correcting the abnormality. Malignant disease of the gastrointestinal tract, the most worrisome cause in adults with iron deficiency, is vanishingly rare in children. Whereas in children, growth spurts, poor dietary patterns, menstrual losses, and non-malignant causes of gastrointestinal bleeding are more often the cause of iron deficiency. After initial diagnostic investigations, oral iron supplementation usually replaces stores most efficiently.

Oral Supplementation. Iron salts offer inexpensive, effective therapy for iron deficiency. Although ferrous sulfate is most often recommended, patients frequently complain of gastrointestinal discomfort, constipation, and bloating, as well as stool discoloration, thus making its use unacceptable to many. Interestingly, in a randomized, controlled trial comparing 3 mg/kg/day of ferrous sulfate drops with placebo in 278, 12-month-old infants, there was no difference in the frequency of vomiting, diarrhea, or fussiness, and infants in the placebo group had a higher incidence of constipation (95% vs. 1%).[350] Iron is frequently provided to children at a dose of 3 mg/kg/day divided into three-times-a-day dosing. Single-dose daily regimens, however, are tolerated as well as three-times-a-day regimens.[351] Administration of iron on an empty stomach at night will lessen the gastrointestinal difficulties. The decreased gastrointestinal motility of sleep will also enhance absorption.

The absorptive capacity of the normal duodenum for iron is essentially saturated with about 25 mg of elemental iron in ionic form. Ferrous gluconate, which is roughly equivalent in cost, contains about 50 mg of elemental iron per tablet. This form of replacement may produce fewer problems than ferrous sulfate and is excellent as the initial treatment of iron deficiency. Ascorbic acid supplementation enhances iron absorption, although it has a relatively minor effect in individuals ingesting normal, balanced diets.[352] Combination tablets containing iron salts and ascorbic acid are significantly more expensive than separate tablets for each. Even with faithful use of oral iron, adequate replacement of body stores in patients with moderate iron deficiency anemia requires several months. With ongoing blood loss, replacement of stores with oral iron becomes very difficult.

Polysaccharide/iron complexes differ from iron salts. Polar oxygen groups in the sugars form coordination complexes with iron atoms. The well-hydrated microspheres of polysaccharide iron remain in solution over a wide range of pH values. Most patients tolerate this form of iron better than the iron salts, even though the 150 mg of elemental iron per tablet is substantially more than that provided by iron salts.

Iron supplements must be stored in a childproof manner. Unintentional iron poisoning from iron supplements is a leading cause of injury related to poisoning in young children and frequently occurs when children obtain improperly stored iron.[353]

Box 11-3 lists potential causes of a poor response to oral iron supplementation. A variety of barriers, including treatment-associated factors and system-wide barriers, contribute to the shortcomings in iron deficiency screening and correction that have been documented.[354]

Parenteral Iron Replacement. Parenteral iron is now available in the United States in four intravenous forms: iron dextran, iron gluconate, iron sucrose,[355] and iron oxide coated with polyglucose sorbitol carboxymethylether.[356-360] Only some of these products are approved specifically for use in the pediatric population. Intravenous iron therapy is indicated when (1) oral iron is poorly tolerated; (2) rapid replacement of iron stores is needed; (3) gastrointestinal iron absorption is compromised; or

Box 11-3 Causes of Poor Response to Oral Iron Supplementation

- Noncompliance
- Ongoing blood loss
- Insufficient duration of therapy
- High gastric pH
  - Antacids
  - Histamine$_2$ blockers
  - Gastric proton pump inhibitors
- Inhibitors of iron absorption or utilization
  - Lead
  - Aluminum intoxication (hemodialysis patients)
  - Chronic inflammation
  - Neoplasia
- Incorrect diagnosis
  - Thalassemia disorder
  - Sideroblastic anemia
  - Anemia of chronic inflammation
- Genetic disorder of iron absorption or utilization (e.g., iron-refractory, iron deficiency anemia [IRIDA])

(4) erythropoietin therapy is necessary, particularly in renal dialysis patients. Iron dextran can also be administered by intramuscular injection, but this route is discouraged because it can be painful and leakage into subcutaneous tissue produces long-standing skin discoloration. A "Z-track" injection into the muscle minimizes the chance of subcutaneous leakage. The suboptimal muscle mass frequently associated with nutritional deficiency further complicates this mode of replacement. Intravenous infusion circumvents these problems altogether. With either route of administration, a small test dose should be given and the patient observed by a physician for 30 minutes to rule out an anaphylactic reaction to the medication. Such reactions do occur but are infrequent. The newer iron sucrose and iron gluconate preparations are believed to be less likely to cause complications, but there is less accumulated experience with these preparations, and many practitioners still use iron dextran routinely.

Ten percent to 15% of patients experience transient mild to moderate arthralgias the day after intramuscular or intravenous administration of iron dextran. Acetaminophen usually relieves the discomfort effectively. Iron dextran is generally avoided in patients with rheumatoid arthritis because it may provoke painful flares of their disease.[12] The symptoms may result from release of inflammatory cytokines such as interleukin-1 and tumor necrosis factor. Iron dextran is cleared from the circulation by macrophages, which are probably activated to release proinflammatory peptides.

In uncomplicated cases of iron deficiency, intravenous replacement may produce subjective improvement in symptoms within a few days. Peak reticulocytosis occurs after about 10 days, and complete correction of the anemia takes 4 to 6 weeks, although the hematocrit rises sufficiently in 1 or 2 weeks to provide symptomatic relief for most patients.[352] CHr is a very sensitive measure of response.[361] Brugnara and colleagues[361] have demonstrated that a rise in CHr is appreciated within 2 to 4 days of the administration of intravenous iron.

Refractory bleeding that cannot be prevented, as occurs, for example, with hereditary hemorrhagic telangiectasia, presents a management problem. Oral iron supplementation often fails to keep pace with losses. Blood transfusions replace red cells in the short term and iron in the long term, but infection and alloimmunization make such therapy unacceptable for many patients. Intermittent iron replacement with infusions of intravenous iron is the most reasonable alternative. Replacements should occur over short intervals two or three times a year, with the aim of repleting body stores. A simple formula can be used to determine the replacement dose of iron dextran:

$$\begin{aligned}\text{Dose (mL)} = {} & 0.0442 \times (\text{Desired Hb} - \text{Observed Hb}) \\ & \times \text{Lean body weight} \\ & + (0.26 \times \text{Lean body weight})\end{aligned}$$

Alternatively, there is a calculator provided on the Internet at infed.com/calcltor.htm. The maximum adult dose of iron dextran is 14 mL.

Over many years this treatment can produce massive deposition of hemosiderin (from iron dextran) in macrophages, the liver, and the pancreas despite the fact that plasma transferrin is iron deficient because of increased iron delivery to erythroid precursors. This supports the concept that tissue iron content is unimportant for erythropoiesis and that erythropoiesis relies exclusively on iron from transferrin.[94,134]

Iron Replacement in Infants. Because the fetus accumulates iron at the mother's expense in the third trimester of pregnancy, full-term infants are born with adequate iron stores to last approximately 6 months. Preterm infants are at greater risk for iron deficiency, with their stores depleted by 3 to 4 months of age.

Human breast milk initially contains relatively high amounts of iron, but the level decreases, independent of maternal iron status, to approximately 0.3 mg/L after 5 months of lactation (reviewed by Griffin and Abrams[362]). The iron is present in several forms: in casein, in whey, in fat, and to a small extent, in the abundant protein lactoferrin. The rate of absorption of iron from human milk has been estimated to be 20% to 50%, which is adequate early in life when iron stores are still high but inadequate to meet an older infant's needs. Based on all available information, the Committee on Nutrition of the American Academy of Pediatrics has recommended the following[279]:

1. Breast milk should be provided for at least 5 to 6 months when feasible. During that period, young infants can mobilize iron from their large stores. Iron supplementation of 1 mg/kg/day should be provided to infants who are exclusively fed breast milk beyond 6 months of age.
2. Infants who are not breastfed should be nourished with an iron-supplemented formula (at least 12 mg/L) until the end of the first year of life.
3. Iron-enriched cereals should be among the first foods introduced with a solid diet.
4. Cow's milk should be avoided during the first year because it contains substances that chelate iron and

it sometimes induces occult gastrointestinal hemorrhage in young infants.

Maternal Iron Stores and Effects on the Fetus

When the mother is iron deficient, the placenta is concerned only with the welfare of the fetus and will continue to remove iron from the mother to support fetal development, even at the cost of negative maternal iron balance. For years, investigators debated the effect of maternal iron deficiency on the fetus. Early findings by Cook and colleagues[363] indicated that the iron storage status of the mother does not affect the infant's iron status as measured by ferritin. The controversy was fueled by the indirect and imprecise assays of neonatal iron status, such as the cord blood ferritin level or transferrin saturation. These parameters in cord blood are subject to all the vicissitudes that affect their values in later life. Accurate determination of neonatal iron status was difficult, if not impossible. The one point on which nearly universal agreement existed was that neonatal anemia secondary to maternal iron deficiency is rare.

It is likely that in fact maternal iron status over a wide range of values does affect the iron stores of the neonate. The most compelling data have come from a study of women who underwent elective abortions.[363,364] The iron status of the mothers was determined from transferrin saturation, plasma ferritin levels, and hemoglobin values. This information was correlated with the iron status of the fetuses, which was determined by atomic absorption spectroscopy of the complete abortus. This procedure determined the absolute fetal iron content. Fetal iron stores varied linearly with maternal hemoglobin, which ranged between 6 g/dL (severe iron deficiency) and 13 g/dL (adequate, though possibly low, iron stores).

Fetal iron stores, in turn, may influence neonatal outcomes. Maternal iron deficiency before midpregnancy is associated with an increased risk for preterm delivery. Maternal anemia detected during the last trimester of pregnancy is not associated with an increased risk for preterm birth. This anemia probably reflects the expansion of maternal plasma volume rather than actual maternal iron stores.

Neonatal iron stores are also influenced by other maternal factors apart from iron stores. Maternal diabetes is associated with decreased fetal stores, as evidenced by lower cord blood ferritin and an increased demand for iron reflected by higher soluble transferrin receptor levels.[365] This may be due to neonatal polycythemia and increased erythropoietic drive associated with maternal diabetes. Similarly, maternal smoking is also associated with increased cord hemoglobin and decreased iron stores.[366]

The infant's gestational age is related to the development of adequate iron stores, as described earlier. The timing of umbilical cord clamping at birth may also affect neonatal iron stores. A randomized, controlled trial of delayed clamping (2 minutes after delivery) versus usual clamping found that infants in the group with delayed umbilical cord clamping had higher ferritin and total body iron stores at 6 months of age.[367] The effect of delayed clamping was particularly pronounced in infants otherwise at risk for iron deficiency, such as infants not receiving iron-fortified milk or formula, infants of mothers with low ferritin levels at birth, and lower–birth-weight infants.

Iron supplementation for pregnant women during the first 28 weeks is associated with a higher mean birth weight and a lower incidence of low–birth-weight infants, but not maternal anemia or higher preterm delivery rates.[368] Consequently, at this time there is insufficient evidence to support or reject the routine practice of prophylactic iron supplementation during pregnancy as opposed to supplementing iron-deficient pregnant women or those at risk for iron deficiency.

Iron Overload

The Evolutionary Advantage of Iron Overload

The fact that multiple mutations in diverse populations all lead to iron overload (see later) raises the possibility that these conditions once conferred a selective advantage. As previously noted, iron deficiency is the most common nutritional cause of anemia in the world. Anemia was probably a survival or reproductive disadvantage earlier in human history. A genetic change that increased the efficiency of iron absorption may therefore have been an advantage. A person who survived into the third decade would have reproduced and passed genes to the next generation, including those that increase iron absorption. Survival into the fifth decade, when severe iron overload begins to produce problems, was uncommon until recently. However, a problem develops with this tidy theory when the inhabitants of Asia are considered. The incidence of iron overload is reportedly quite low in this region of the world, the home of most of the earth's population.

Hereditary Hemochromatosis

Hereditary hemochromatosis is a condition that originates from a genetic predisposition to iron overload as a result of mutations in one of several genes involved in iron metabolism (see Fig. 11-6). It is caused by an alteration in the iron absorption mechanism that fractionally increases the uptake of dietary iron.[369,370] Although the rate and extent of iron loading vary widely among patients, dangerous levels of tissue iron develop in many people by middle age. Iron deposits in cells of the liver, heart, and endocrine tissues. It promotes oxidative cell damage, which leads to fibrosis and, when unrecognized, to the classic manifestations of "bronze diabetes," including liver dysfunction, increased melanization of the epidermis, and pancreatic insufficiency.[371] Because erythropoiesis is normal, iron can be removed through phlebotomies performed regularly. If untreated, hemochromatosis may be a lethal disease and result in death from cirrhotic liver failure or, less often, from cardiomyopathy.

Pediatricians are often asked to evaluate children who have close relatives with hemochromatosis. This is a complicated task because iron loading occurs gradually over a period of many years and may not be apparent before adulthood. Although the genes responsible for most forms of hemochromatosis have been identified, at this

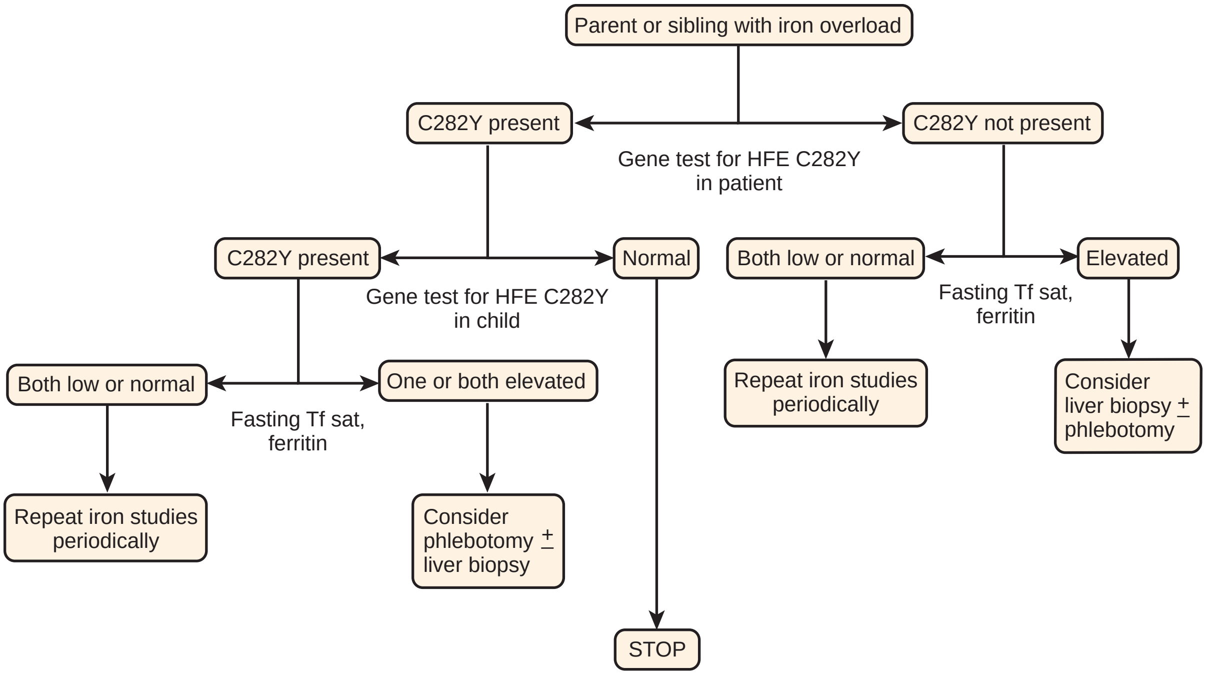
Parent or sibling with iron overload
C282Y present
Gene test for HFE C282Y in patient
C282Y not present
C282Y present
Normal
Gene test for HFE C282Y in child
Both low or normal
Fasting Tf sat, ferritin
Elevated
Repeat iron studies periodically
Consider liver biopsy ± phlebotomy
Both low or normal
Fasting Tf sat, ferritin
One or both elevated
Repeat iron studies periodically
Consider phlebotomy ± liver biopsy
STOP

Figure 11-8 Algorithm for the workup of children at risk for iron overload. *(Modified from Andrews NC: Inherited iron overload disorders.* Curr Opin Pediatr *12:596–602, 2000.)*

time commercial laboratories test for only one form of the disease. However, this situation is likely to change over the next few years and simplify presymptomatic diagnosis. Until then, assessment should take into account the family history, serum transferrin saturation, serum ferritin levels, and, if indicated, quantitative liver iron determined by analysis of a biopsy sample (Fig. 11-8).

In the United States, Canada, Australia, and the northwestern part of Europe, most patients with hemochromatosis are homozygous for a unique missense mutation designated C282Y (for cysteine 282 to tyrosine) in the gene encoding HFE, a protein that resembles class I major histocompatibility (MHC) molecules.[372] The *HFE* gene lies on chromosome 6p, near the HLA-A locus. It was discovered through a positional cloning approach that took advantage of the fact that patients with the C282Y mutation are descended from a common ancestor (i.e., founder effect) who probably lived in a Celtic population in the British Isles 60 to 70 generations ago.[373]

Like class I MHC proteins, HFE forms a heterodimer with β2-microglobulin and is expressed on the cell surface. However, unlike other family members, HFE cannot bind a small peptide. Its structure, as determined by x-ray crystallography, does not allow enough space within the groove that is involved in peptide binding by MHC class I molecules.[374] Some insight into the function of HFE was gained from observations that it forms a high-affinity complex with the transferrin receptor.[374] HFE binds to a site that overlaps the binding site for diferric transferrin, which results in competition between HFE and transferrin for occupancy of the transferrin receptor.[375] Studies in transfected cells overexpressing HFE indicate that the HFE–transferrin receptor association alters cellular iron uptake. However, the effect of HFE on iron homeostasis is not directly attributable to its effect on cellular iron uptake. As discussed later, we now know that HFE and several other forms of hemochromatosis result from aberrant expression of the iron regulatory hormone hepcidin.

In patients with HFE hemochromatosis, serum transferrin becomes saturated with iron before clinical signs appear. The increased serum iron is deposited in parenchymal cells of the liver, heart, pancreas, and other tissues. In knockout mice lacking murine Hfe, an iron overload disorder develops that closely resembles human hemochromatosis.[376,377] The mutant allele for HLA-linked hemochromatosis is prevalent in the United States. Studying a cross section of the American population, Steinberg and colleagues[378] found that 9.5% of non-Hispanic whites, 2% of non-Hispanic blacks, and approximately 5% of the population overall were C282Y carriers. Testing for the HFE C282Y mutation allows identification of patients in whom clinical hemochromatosis may develop. Heterozygotes rarely have clinically significant iron overload, but homozygotes are at risk. Published data give conflicting estimates of the proportion of patients homozygous for C282Y in whom complications of the disease will develop; such estimates range from less than 1% to greater than 20%. It is clear, however, that some individuals have severe iron loading as young adults whereas others may never manifest signs or symptoms of hemochromatosis. There must be both environmental and genetic modifiers that affect the extent of iron loading. Current efforts are aimed at defining these modifiers to aid in predicting in which genetically at-risk individuals clinically significant iron overload will develop.

Other polymorphisms and potential mutations have been described in the HFE coding sequence. One of these polymorphisms, H63D (histidine 63 to aspartic acid), is prevalent throughout the world and found in as many as a quarter of asymptomatic patients, often in combination with the C282Y allele. Although clinical laboratories currently test for and report the H63D polymorphism, most patients with one or two copies of the H63D allele are probably not at risk for iron loading. Other polymorphisms associated with altered amino acids or altered mRNA splicing have also been reported (reviewed by Camaschella and coworkers[379]). Some of these polymorphisms do appear to be associated with iron overload, but most clinical laboratories do not test for them.

Since the identification of HFE, it has become clear that there are at least four other types of hemochromatosis, and that most forms are associated with loss of a protein that regulates hepcidin gene transcription in the hepatocyte through a bone morphogenetic protein/sons of mothers against decapentaplegic (BMP/SMAD)–dependent signaling cascade that is modified by TMPRSS6 and the interaction of the HFE protein with TRFC (see Fig. 11-6). A disorder that may be clinically indistinguishable from HFE-associated hemochromatosis results from homozygosity for mutations in the gene encoding transferrin receptor 2 (TFR2).[380] TFR2 is highly homologous to the transferrin receptor, particularly in its extracellular domain, but it binds transferrin with much lower affinity.[381,382] It is primarily expressed in the liver and in erythroid cells. Its function remains incompletely understood, but, similar to the transferrin receptor, TFR2 forms a protein complex with HFE, which is believed to be involved in regulation of hepcidin expression.[383]

Two other types of hemochromatosis are easier to distinguish from HFE-associated hemochromatosis. Juvenile hemochromatosis, also called type 2 hemochromatosis, is a more severe disorder with accelerated iron loading that typically leads to death by the third decade.[384] Patients have iron deposition in the same pattern as that seen in those with HFE-associated hemochromatosis, but the rapid accumulation of metal leads to a different clinical picture dominated by cardiac disease and endocrine dysfunction. Patients are often identified when they fail to enter puberty or develop secondary amenorrhea. If these patients are recognized before end-stage organ disease develops, they can be treated by aggressive phlebotomy. Juvenile hemochromatosis is inherited in an autosomal recessive pattern and is caused by mutations in *HAMP*, the gene encoding hepcidin,[385] or in an unrelated gene encoding hemojuvelin (*HJV*, also called *HFE2*, *RGMC*).[386] Hemojuvelin is a cell surface protein that acts as a bone morphogenetic protein (BMP) coreceptor to stimulate hepcidin expression, presumably in response to BMP signals.[387] BMP6 appears to be the primary, but not the only, BMP ligand, relevant to this iron-dependent signal transduction pathway. Targeted disruption of the murine hepcidin or hemojuvelin genes results in a phenotype very similar to human juvenile hemochromatosis, with a complete or near absence of hepcidin mRNA expression.[388-391]

Although all of the forms of hemochromatosis discussed thus far (caused by mutations in *HFE*, *TFR2*, *HAMP*, and *HJV*) are associated with inappropriately low hepcidin production, particularly when compared with the degree of iron stores, there is one hemochromatosis disorder that is not. A subset of patients carrying mutations in *SLC40A1*, the gene encoding ferroportin, has autosomal dominant hemochromatosis that is otherwise clinically similar to other forms of the disease.[392-394] Apparently, these mutations render ferroportin resistant to hepcidin regulation; these individuals have appropriately high levels of hepcidin. A second class of ferroportin mutations cause a distinct autosomal dominant disorder characterized by macrophage iron loading, an early rise in serum ferritin levels, comparatively less saturation of transferrin with iron, and no major increase in total body iron stores.[247,395,396] These alleles appear to be mutations leading to partial loss of function and particularly impair macrophage iron recycling. Both forms of "ferroportin disease" are autosomal dominant because functional ferroportin exists in cells as a dimer and mutation of one subunit in a dimer is sufficient to alter for transport or hepcidin responsiveness.[397]

African Iron Overload Syndrome

During the 1960s, several groups of investigators reported that iron overload occurred at a very high rate among sub-Saharan Africans.[398-401] Many patients had massive iron stores approaching or exceeding those seen in individuals with hemochromatosis. Some indigenous inhabitants of the region prepared alcoholic beverages in nongalvanized steel drums, thus raising the possibility that iron overload resulted from the high iron content of these beverages. Iron loading was thought to be exacerbated by an increase in gastrointestinal iron absorption induced by alcohol. The term *Bantu siderosis* was coined to describe the condition.[401]

Later reports suggested that African iron overload syndrome was not simply the result of increased intake.[402-404] In only a fraction of the group who drank the beverage did iron overload develop. In fact, a more extensive study showed that similar degrees of iron overload occurred in people who consumed little, if any, of this brew. Although the pattern of inheritance has not been definitively worked out, the disorder may be autosomal codominant, with obligate heterozygotes expressing an intermediate level of iron loading. African iron overload syndrome is not associated with *HFE* mutations.[405]

In contrast to other forms of hereditary hemochromatosis, in which the iron is almost entirely confined to parenchymal cells, reticuloendothelial macrophages display massive iron deposition in the African variety of iron overload.[398] A common missense mutation in ferroportin, Q248H, has been implicated as a possible genetic factor.[406,407] However, the ultimate clinical consequences of iron overload are similar to those seen in all other forms of hemochromatosis, irrespective of the cause of the accumulation. African iron overload syndrome is not confined to any ethnic group, although it appears to be restricted to the inhabitants of sub-Saharan Africa. In some areas, the incidence may be as high as 10%, which makes it one of the most common, clinically significant iron disorders in any population.

An important, unanswered question is whether mutated genes causing this syndrome are found in people of African descent elsewhere in the world. No comprehensive study of iron overload has been performed in African Americans, but preliminary data suggest that a substantial portion of this population may be at risk.[408,409] There has not yet been an exhaustive examination of hemochromatosis disease genes in this group.

Neonatal Hemochromatosis

Neonatal hemochromatosis (NH) is a rare condition characterized by neonatal liver failure associated with systemic iron overload, parenchymal iron deposition, thrombocytopenia, and coagulopathy in the absence of evidence of any known causative agent or condition, including Down syndrome, metabolic liver disease (e.g., tyrosinemia, galactosemia), congenital infection (e.g., herpesviruses), or congenital anemia (e.g., α-thalassemia, hydrops fetalis secondary to ABO/Rh incompatibility) (reviewed elsewhere[410-413]). In addition to fetal diseases, NH has been associated with maternal autoimmune disorders, including lupus erythematosus and Sjögren syndrome. Although iron chelation and antioxidant cocktails have been studied and occasionally have led to some clinical improvement, the outcome in NH is nearly always fatal in the absence of prompt hepatic transplantation.[414-416] The rare individuals who have survived without transplantation have recovered full organ function,[417,418] and the disease usually does not recur in those who have undergone transplantation successfully, thus suggesting that the hepatic insult is developmental or transient in nature.

Mutations in several of the "adult" hemochromatosis genes have not been identified in NH patients, and there is substantial evidence of a nonmendelian or otherwise unconventional hereditary predisposition to NH.[411,412,419] Specifically, the possibility of mitochondrial inheritance or inheritance of a maternally imprinted gene has been raised by the discovery of several kindreds in which women have borne affected children fathered by different men. However, neither of these types of inheritance can account for the tendency for a mother's first pregnancy to be spared or mildly affected by the disorder whereas subsequent pregnancies have a greater than 50% risk for the development of clinically overt NH. These latter observations, in particular, have spawned the current leading hypothesis that NH is the consequence of prenatal alloimmune liver injury.[413,420] In this model, which parallels the paradigm of neonatal alloimmune thrombocytopenia (see Chapter 34), the mother is sensitized to a fetal antigen during a first pregnancy and subsequent pregnancies provoke an amnestic immune response that results in a severe clinical phenotype. What the antigen might be or how it perturbs iron metabolism is unknown. Nonetheless, a recent clinical trial demonstrated that clinically significant liver disease can be avoided in the vast majority of at-risk fetuses by treating the expectant mother with intravenous immunoglobulin beginning in the early second trimester of gestation, thus further substantiating the alloimmune hypothesis.[420]

Iron Overload Caused by Erythroid Hyperplasia and Ineffective Erythropoiesis

The connection between hematopoiesis and iron absorption is most strikingly apparent in patients with ineffective erythropoiesis. With disorders such as nontransfused β+-thalassemia (thalassemia intermedia), X-linked sideroblastic anemia, and congenital dyserythropoietic anemias, the fractional absorption of ingested iron is increased. Patients with these disorders can manifest iron overload without transfusion.[421]

The mechanism by which bone marrow activity modulates gastrointestinal iron absorption is only partially understood. A soluble factor appears to be released that depresses hepcidin production.[422] Recently, it was reported that GDF15, a member of the transforming growth factor β (TGFβ) superfamily, is induced in patients with thalassemia and that GDF15 suppresses expression of hepcidin by human hepatocytes.[53] Another TGFβ superfamily member, twisted gastrulation 1 (TWSG1) is reported to have similar properties.[54] It is, however, highly likely that there are other soluble factors that contribute to the "erythroid regulator."

In humans, depressed hepcidin levels and, consequently, robust absorption of iron from the gastrointestinal tract are associated most often with ineffective erythropoiesis.[58] Some patients with pyruvate kinase deficiency, a condition frequently associated with reticulocyte counts as high as 70%, manifest iron overload without transfusion. Hemolytic anemias with effective erythropoiesis rarely lead to iron loading. Without some compounding variable, such as hereditary hemochromatosis trait, iron loading without transfusion is not generally seen with conditions such as sickle cell anemia or hereditary spherocytosis, in which the erythroid cells mature and leave the bone marrow before they are destroyed.

Consequences of Iron Overload

Iron-induced injury to certain organs, including the liver and heart, can be fatal. Few notable symptoms precede advanced injury. Abdominal discomfort, lethargy, depression, arthralgias, erectile dysfunction, and fatigue are common but nonspecific complaints. Dyspnea with exertion and peripheral edema indicate significant cardiac compromise and reflect advanced iron loading.

As the major site of iron storage, the liver is a conspicuous target for excessive iron deposition. Mild to moderate hepatomegaly develops early, followed by shrinkage produced by fibrosis and micronodular cirrhosis. Hepatic tenderness may be present, but hemosiderotic liver damage produces little inflammation. Consequently, significant hepatic iron deposition and even fibrosis can occur with very little increase in serum transaminase levels. Disturbances in liver synthetic function indicate advanced disease. Liver biopsy is still the "gold standard" for determining the extent of hepatic iron deposition, although noninvasive imaging techniques do have a role. Hematoxylin-eosin staining reveals a brownish pigment in the hepatocytes that staining with Perls Prussian blue unmasks as iron. Large amounts of iron are also deposited in Kupffer cells in patients with ferroportin disease, African iron overload syndrome, or transfusional iron overload. Electron microscopy reveals substantial hemosiderin aggregates in addition to large quantities of ferritin, often in lysosomes.

Congestive cardiomyopathy is the most common heart problem that occurs with iron overload, but other abnormalities have been described, including pericarditis, restrictive cardiomyopathy, and angina without coronary artery disease.[423-426] There is strong correlation between the cumulative number of blood transfusions and functional cardiac derangements in children with thalassemia.[427] Findings on physical examination are surprisingly benign, even in patients with heavy cardiac iron deposition. Once evidence of cardiac failure appears, however, heart function deteriorates rapidly, and medical intervention is frequently ineffective. Biventricular failure produces pulmonary congestion, peripheral edema, and hepatic engorgement. These potentially lethal cardiac complications have, in some cases, been reversed by vigorous iron extrication.[428]

Iron deposition in the bundle of His and the Purkinje system can lead to conduction defects.[429,430] Sudden death is common in these patients, presumably as a result of arrhythmias. At one time, patients treated with the chelator deferoxamine for transfusional iron overload received supplements of ascorbic acid in the range of 15 to 30 mg/kg/day to promote iron mobilization. Reports of sudden death prompted cessation of this practice.[431] Given carefully at lower doses (2 to 4 mg/kg) by an experienced practitioner, ascorbic acid is a safe adjunct to chelation therapy in patients with transfusional iron overload.

Cardiac dysfunction can also occur with very little tissue iron deposition. The total quantity of iron is less important than the unbound, or “toxic,” iron fraction in the circulation. The concentration of unbound iron in tissues is extremely low and virtually impossible to measure. This toxic iron is precisely the component that is bound and neutralized by iron chelators. Therefore, cardiac damage is best prevented in patients with transfusional iron overload by maintaining a constant, low level of chelator in the circulation (and consequently in the tissues where the protection is rendered).

Dysfunction of the endocrine pancreas is common in patients with iron overload.[432,433] Overt diabetes mellitus requiring insulin therapy develops in some patients. The disturbances in carbohydrate metabolism may be more subtle, however. An oral glucose tolerance test often unmasks abnormal insulin production. Vigorous removal of excess iron may reverse the islet cell dysfunction.[434] Exocrine pancreatic function, in contrast, is usually well preserved.

Pituitary dysfunction may produce several endocrine disturbances.[435] Reduced gonadotropin levels are common. When coupled with primary reductions in gonadal synthesis of sex steroids, sexual maturation is delayed in children with transfusional iron overload.[436] Secondary infertility is common. Although Addison syndrome is uncommon with iron overload, production of adrenocorticotropic hormone is occasionally deficient. A metapyrone stimulation test shows delayed or diminished pituitary secretion of this hormone.[437] Thyroid function is usually well preserved in patients with iron overload. In contrast, parathyroid activity is frequently compromised. Functional hypoparathyroidism can be demonstrated in many patients by inducing hypocalcemia with an intravenous bolus of ethylenediaminetetraacetic acid (EDTA) while monitoring the production of parathyroid hormone.[438]

Hyperpigmentation is a nonspecific skin response to a variety of insults, including excessive exposure to ultraviolet light, thermal injury, and drug eruptions. Cutaneous iron deposition enhances melanin production by melanocytes. Ultraviolet light exposure and iron are often synergistic in the induction of skin pigmentation, and, consequently, many patients with siderosis tan very readily. Fair-skinned people who tan poorly may never exhibit hyperpigmentation despite very large body iron burdens. In contrast, a striking, almond-colored hue frequently develops in patients with moderate baseline pigmentation. With a particularly heavy iron overload, visible iron deposits sometimes appear in the skin as a grayish discoloration.

Arthropathy is a common problem in patients with hereditary hemochromatosis but rare in patients with secondary iron overload.[439,440] Patients with hemochromatosis typically exhibit involvement of small joints, particularly in the hands, but large joints, such as the hip, may also be involved. Chondrocalcinosis is a late but characteristic feature of the arthropathy seen in hereditary hemochromatosis. Other troubling musculoskeletal problems include severe, recurrent cramps and disabling myalgias. Muscle biopsy shows iron deposits in myocytes, but the pathophysiologic connection to the pain and cramps is unclear.

Iron Overload and Opportunistic Infections

Withholding iron from potential pathogens is believed to be a natural part of the host defense repertoire.[441] Because transferrin binds iron with extremely high affinity and two thirds of the iron-binding sites of the protein are normally unoccupied, there is very little free iron available in plasma and extracellular fluids. Not surprisingly, both transferrin and its homologue lactoferrin are bacteriostatic in vitro for many bacteria.[442-444] However, the very high transferrin saturations attained in patients with iron overload compromise the bacteriostatic properties of the proteins, and transferrin can serve as a source of iron for some microorganisms.[445] Various infections with unusual organisms have been reported in patients with iron overload (Box 11-4).[446-449] Host defense is compromised even further in patients with sideroblastic anemia and secondary iron overload because of abnormal neutrophil function. Although aggressive antimicrobial therapy is often successful, some infections, such as the mucormycosis produced by *Rhizopus oryzae*, are usually fatal.

Acute Iron Poisoning

Acute iron poisoning from the accidental ingestion of large doses of medicinal iron is a medical emergency. Significant gastrointestinal symptoms occur with doses as

Box 11-4 Infectious Agents Associated with Iron Overload

BACTERIAL AGENTS
- *Listeria monocytogenes*
- *Yersinia enterocolitica*
- *Salmonella typhimurium*
- *Klebsiella pneumoniae*
- *Vibrio vulnificus*
- *Escherichia coli*

FUNGAL AGENTS
- *Cunninghamella bertholletiae*
- *Rhizopus oryzae*

low as 20 mg/kg of elemental iron, and systemic toxicity becomes evident with ingestions of 60 mg/kg (reviewed by McGuigan[450]). Treatment consists of removing as much residual iron as possible from the gastrointestinal tract and administering intravenous deferoxamine, which chelates the toxic NTBI.[450,451] It is appropriate to consult a toxicologist or poison center for assistance.

DISORDERS OF IRON UTILIZATION

Iron Abnormalities in the Anemia of Inflammation

The anemia of inflammation, also called the anemia of chronic disease, is a well-recognized condition associated with a broad spectrum of maladies. It was elegantly described in a classic review by Cartwright almost half a century ago,[452] and is covered in greater depth in Chapter 37. This chapter will focus on the iron abnormalities associated with the disorder.

The anemia of inflammation typically manifests as a mild to moderate, normochromic and normocytic anemia, with a slightly elevated RDW. A mild anisocytosis is often the only hint on the blood smear that the anemia is due to insufficient iron available for erythropoiesis. Serum iron and serum transferrin levels are usually low. Serum ferritin levels are generally increased but may not be when coexistent with iron deficiency. Similarly, sTfR levels are normal unless iron deficiency accompanies the disorder. Not surprisingly, the diagnosis of anemia of chronic inflammation may be challenging to make. Inflammation is the key to the condition.

Bone marrow examination with Perls Prussian blue stain usually reveals deposits of iron in macrophages. This characteristic feature of the anemia of chronic inflammation reflects ineffective iron recycling. Erythroblasts that fail to mature properly and effete erythrocytes are engulfed by macrophages in the marrow, liver, and spleen. The phagocytes degrade the protein and lipid and recycle the iron from hemoglobin onto transferrin for reuse. The anemia of chronic inflammation disturbs this cycle, with iron left trapped in the reticuloendothelial macrophages.

Much has been learned about the pathogenesis of the anemia of chronic inflammation over the past few years. Initially, studies of patients with glycogen storage disease type 1 complicated by anemia and hepatic adenomas provided a clue that increased hepcidin expression might account for both the reticuloendothelial block and the decreased intestinal iron absorption characteristic of the anemia of inflammation.[46] Subsequent investigations confirmed that hepcidin expression was elevated in patients with inflammation associated with a variety of infectious and noninfectious conditions.[44,453] Hepcidin production is induced by the inflammatory cytokine interleukin-6 through a direct effect on the hepcidin promoter.[47-49,454]

Patients with the anemia of chronic inflammation may have unusually low plasma erythropoietin levels for their degree of anemia.[455] This finding was previously attributed to proinflammatory cytokines, which were shown to suppress production of erythropoietin by cells in culture.[456] However, erythropoietin responsiveness is also suppressed in an animal model of anemia of inflammation developed by transgenic expression of hepcidin in noninflamed animals.[457] In addition, hepcidin has been reported to have a direct effect on erythroid colony formation in vitro.[458] However, a decreased response to erythropoietin is likely also a consequence of direct effects of inflammatory cytokines on erythropoiesis. Taken together, these findings suggest that induction of hepcidin in response to inflammation results in most, but not all, of the characteristic features of the anemia of inflammation.

Control of the inflammation (e.g., by colectomy in patients with ulcerative colitis) usually eliminates the anemia and corrects the disturbances in iron metabolism. In general, the anemia is recalcitrant when the inflammation is uncontrolled. However, it is likely that new treatment strategies will be developed that take advantage of our current understanding of the pathogenesis of the anemia of inflammation and regulation of hepcidin production.

SIDEROBLASTIC ANEMIAS

The sideroblastic anemias are a heterogeneous group of congenital and acquired bone marrow disorders defined by the presence of pathologic iron deposits within the mitochondria of erythroid precursors. On Prussian blue, or Perls, iron-stained bone marrow aspirate smears or biopsies, these iron-encrusted mitochondria appear as coarse granules that typically form a halo or "ring" around the nucleus, thus giving rise to the term "ringed sideroblast," which is, by definition, an abnormal cell[459] (Fig. 11-9). Mitochondrial localization of the iron deposits can be confirmed by electron microscopy, but it is not used routinely for diagnostic purposes. "Siderocytes" are mature, enucleated erythrocytes that contain stainable iron, which may be the consequence of residual mitochondria from ringed sideroblasts or cellular ferritin, organelle, and nucleic acid concretions left over from terminal erythroid maturation.[459] Both are recognized as "Pappenheimer bodies" on conventional Wright-Giemsa–stained peripheral blood smears and are abundant in patients with sideroblastic anemia and a number of other anemias, including thalassemia syndromes and hemoglobinopathies.

In addition to the obligatory ringed sideroblasts, the bone marrow in patients with sideroblastic anemia is typically hypercellular with an erythroid hyperplasia. Ineffective erythropoiesis frequently eventuates in elevated body iron stores and may be so severe that it results

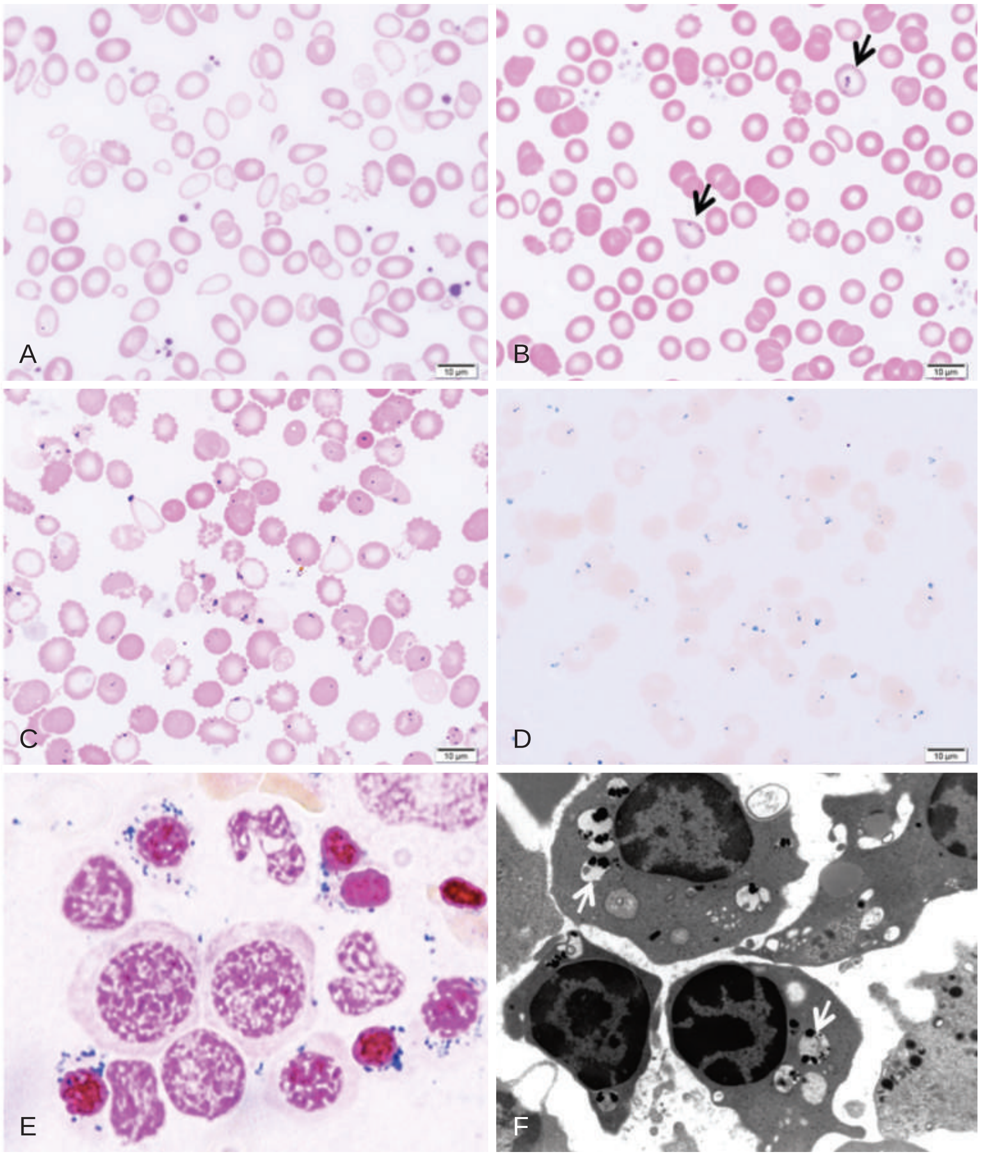

Figure 11-9 **A**, Peripheral blood smear from a man with X-linked sideroblastic anemia showing the anisocytosis, hypochromia, and misshapen cells typical of the disorder. **B**, Peripheral blood smear from a female carrier of XLSA (mother of individual in **A**) showing a dimorphic red blood cell population that includes occasional hypochromic cells, including some with Pappenheimer bodies (i.e., siderocytes) indicated by *arrows*. **C**, Peripheral blood smear of the affected brother of the patient shown in **A**, following splenectomy, demonstrating a profusion of siderocytes containing Pappenheimer bodies that are highlighted by Prussian blue staining in panel **D**. **E**, Prussian blue staining of a bone marrow aspirate from a patient with sideroblastic anemia. The granules that circle the nucleus of the normoblasts are iron-laden mitochondria. **F**, Electron microscopy of erythroblasts from a patient with sideroblastic anemia showing electron-dense inclusions in mitochondria, indicative of iron deposits *(arrows)*.

in clinically significant iron overload with secondary organ dysfunction. All patients are anemic, and thrombocytopenia and neutropenia develop in some, particularly acquired, neoplastic forms. The anemia may be normocytic, microcytic, or macrocytic and is typically characterized by a relative reticulocytopenia (Table 11-3, reviewed by Fleming[460] and Bottomley[461]).

Congenital Sideroblastic Anemias

Genetically defined, congenital sideroblastic anemias (CSAs) may be inherited in an autosomal recessive, an X-linked recessive, or, infrequently, a maternal fashion (reviewed elsewhere[460-462]). Many cases of congenital sideroblastic anemia are considered to be inherited conditions but defy genetic characterization at the present time. In classifying the known causes of CSA, it is helpful to consider the MCV of the anemia, whether other hematopoietic cell lineages are affected, and whether syndromic associations or other distinctive clinical findings are present (see Table 11-1). Currently, the molecular genetic basis of approximately 60% of cases of CSA can be ascertained.

X-Linked Sideroblastic Anemia

X-linked sideroblastic anemia (XLSA) is the prototype of all congenital sideroblastic anemias (CSAs). As the name implies, the disorder is linked to the X chromosome and disproportionately affects men and boys, although females

TABLE 11-3 Clinical and Genetic Features of Congenital Sideroblastic Anemias

	XLSA	SLC25A38	XLSA/A	GLRX5[*]	PMPS	MLASA/PUS1	MLASA/ YARS2	SIFD	TRMA
Inheritance	X-linked	Autosomal recessive	X-linked	Autosomal recessive	Maternal[†]	Autosomal recessive	Autosomal recessive	Autosomal recessive	Autosomal recessive
Chromosome	Xp11.21	3p22.1	Xq13	14q32.2	mtDNA	14q24.33	12p11.21	?	1q23.3
Gene	*ALAS2*	*SLC25A38*	*ABCB7*	*GLRX5*	Variable	*PUS1*	*YARS2*	?	*SLC19A2*
Gender distribution	M > F	M = F	M	M	M ≈ F	M = F	M = F	M = F	M = F
Carrier phenotype	+	–	+	?	NA	–	–	–	–/+
Mean cell volume	⇓⇓[‡]	⇓⇓	⇓/NMA	⇓⇓	⇑	Normal/⇑	Normal/⇑	⇓	⇑
Iron overload	+/++	++	–	++	–/+	–/+	–/+	+	–
Vitamin response	Pyridoxine	–	–	–	–	–	–	–	Thiamine
Transfusion	–/+	++	–	+	+	+/–	+/–	+/–	–
Associated phenotypes	–	–	Cerebellar hypoplasia, ataxia	–	Exocrine pancreas insufficiency, cytopenias, lactic acidosis, myopathy	Myopathy, lactic acidosis, craniofacial abnormalities, intellectual disability	Myopathy, lactic acidosis	Immunodeficiency, fevers, developmental delay, other	Diabetes mellitus, deafness

Modified from Fleming MD: Congenital sideroblastic anemias: iron and heme lost in mitochondrial translation. Hematology Am Soc Hematol Educ Program *2011:525–53, 2011.*

+, Present; –, absent; ⇑, increased; ⇓, decreased; *NA*, not applicable; *mtDNA*, mitochondrial DNA.

[*] A GLRX5 mutation has been described in only one patient.

[†] Essentially all cases of PMPS are sporadic, but rare maternally inherited cases are reported.

[‡] Mean cell volume is typically normal or increased in female carriers.

may be affected because of skewed X-chromosome inactivation or Turner syndrome.[463-465] The cardinal features of XLSA include (1) a microcytic, hypochromic anemia with a large RDW and erythrocyte dimorphism (particularly in carrier females) and Pappenheimer bodies apparent on the peripheral blood smear; (2) an abundance of ringed sideroblasts, especially in late erythroid precursors; and (3) a variable clinical response to pharmacologic doses of pyridoxine (vitamin B_6).[461] Systemic iron overload may also develop as a result of chronic, severe, ineffective erythropoiesis.[466,467] Importantly, clinically affected females often present at a later age and most commonly have a macrocytosis, rather than a microcytosis, necessitating a distinction between this disorder and acquired neoplastic sideroblastic anemia (see below). Nearly all patients with XLSA have missense mutations in the protein-coding regions of the erythroid-specific isoform of the heme biosynthesis enzyme δ-aminolevulinic acid synthase (ALAS2) (reviewed elsewhere[460,461]). Clinically affected females may harbor nonsense or frameshift mutations.[468] A minor subset of patients have mutations in a GATA transcription factor–binding site within a transcriptional enhancer element located in intron 1 that results in decreased *ALAS2* transcription.[469,470] Rare individuals are reported to have promoter mutations, but this element has not been well validated and the variant occurs at a low frequency in normal populations.[471] ALAS2 missense mutations tend to cluster in amino acids surrounding the catalytic site or the subunit interface of the homodimer, thereby leading to enzymes with decreased stability or decreased affinity for the cofactor pyridoxal phosphate, which can account for the clinical response to large doses of pyridoxine.[472] Because ALAS2 is the rate-limiting step in heme biosynthesis in the erythron, mutations inevitably impair the production of heme in developing erythroblasts. How heme deficiency leads to the hallmark ringed sideroblast is uncertain; however, the characteristic hypochromia and microcytosis are almost certainly the result of the protean effects of heme on erythroid transcription, mRNA stability, and translation.

The age at clinical onset of XLSA varies greatly. Anemia develops in some individuals within a few months of birth, whereas in others it occurs later in childhood and often even in adulthood. Generally, the anemia is progressive, with transfusions often required to maintain an adequate hemoglobin level. Frequent transfusions can hasten the development of iron overload. Oral pyridoxine therapy is useful in many patients with XLSA. Often, however, correction of the anemia is incomplete. Some patients become more pyridoxine responsive after their excess body iron stores are depleted.[473] A few children who failed pyridoxine therapy have responded to the parenteral administration of pyridoxal-5-phosphate.[474] Unfortunately, many children with XLSA improve little, if at all, with either of these interventions. Because pyridoxine is inexpensive and lacks deleterious side effects, all children with sideroblastic anemia should receive a trial of vitamin therapy. Splenectomy is ill advised because of thrombotic risks.[461] There is one case report of successful allogeneic peripheral stem cell transplantation for refractory sideroblastic anemia.[475]

X-Linked Sideroblastic Anemia with Ataxia

X-linked sideroblastic anemia with ataxia (XLSA/A) is a very rare, distinct, X-linked form of sideroblastic anemia caused by missense mutations in the mitochondrial ATP-binding cassette transporter ABCB7.[476-478] Patients with XLSA/A typically have static or slowly progressive cerebellar ataxia and only later are found to have a mild sideroblastic anemia with morphologic features similar to those of classic XLSA.[479,480] However, they do not require transfusion therapy, systemic iron overload does not develop, and they do not respond to pyridoxine therapy. ABCB7 and its yeast orthologue Atm1p are essential genes involved in the biogenesis of cytosolic Fe/S clusters.[481-487] Interestingly, another protein implicated in Fe/S cluster biogenesis in yeast and zebrafish, GLRX5, has been shown to be mutated in a single patient with severe sideroblastic anemia and iron overload.[488-490] In all cases, these Fe/S cluster biogenesis mutants appear to disrupt regulation of IRP1 RNA-binding activity,[487,489] thus raising the possibility that this is a common mechanism for the development of sideroblasts.

Thiamine-Responsive Megaloblastic Anemia

Children with thiamine-responsive megaloblastic anemia (TRMA), also known as Rogers syndrome, exhibit a characteristic triad of megaloblastic anemia with ringed sideroblasts, diabetes mellitus, and deafness.[491,492] The clinical manifestations of TRMA are progressive and may also include optic atrophy and nonthrombotic, nonembolic strokes. The anemia is characteristically macrocytic, and erythroid maturation in bone marrow, as the name implies, is megaloblastic and associated with variable numbers of ringed sideroblasts. Patients may also have leukopenia. The gene responsible for TRMA encodes a transmembrane thiamine transporter designated SLC19A2.[493-496] TRMA patients are not thiamine deficient but rather have a defect in high-affinity thiamine uptake in a number of cell types.[497] The megaloblastic marrow maturation is probably related to defective thiamine-dependent steps in nucleic acid synthesis[498]; however, the pathogenesis of sideroblasts remains obscure. Therapy with pharmacologic doses of thiamine ameliorates the anemia and may abrogate progression of the neurologic and pancreatic phenotypes.[491,499,500]

Pearson Marrow-Pancreas Syndrome

Pearson marrow-pancreas syndrome (PMPS) is a fatal, multisystem disorder of infancy with marked disturbances in bone marrow and pancreatic function.[501] The initial manifestation is often a severe, transfusion-dependent macrocytic anemia associated with a variable degree of neutropenia and thrombocytopenia. Marrow examination shows vacuolization in the erythroid and myeloid precursors and ringed sideroblasts.[502] Exocrine pancreatic dysfunction as a result of pancreatic fibrosis is characteristic, and hepatic, renal, and neurologic dysfunction develops in some patients as well. The variability and pleiotropy of the disorder can be accounted for by the fact that PMPS is a mitochondrial cytopathy; nearly half of the patients share heteroplasmy for a common deletion of approximately 5 kb of mitochondrial DNA (mtDNA) that is associated with other mitochondrial disorders, particularly Kearns-Sayre syndrome; the distribution of defective mitochondrial genomes will necessarily influence expression of the disorder.[503,504] Interestingly, nearly all mtDNA deletions associated with PMPS include the deletion of one of the mitochondrial transfer RNA (tRNA) genes, emphasizing the effect that the pathogenetic mutations could have on the synthesis of multiple mitochondrially-encoded proteins. Whatever the specific target proteins, defective energy production appears to be the central disturbance, and no treatment other than supportive care currently exists.

Mitochondrial Myopathy with Lactic Acidosis and Sideroblastic Anemia

Mitochondrial myopathy with lactic acidosis and sideroblastic anemia (MLASA) is a recently recognized autosomal recessive disorder of mitochondrial dysfunction caused by mutations in the genes encoding pseudouridine synthase 1 (PUS1) or the mitochondrial tyrosyl-tRNA synthase (YARS2).[505-512] Most cases of PUS1 deficiency are associated with a unique founder mutation present in the Persian-Jewish population, and several cases of YARS2 deficiency have been reported in individuals of Lebanese-Christian descent. Insufficient numbers of patients with MLASA have been characterized to distinguish individuals with one or the other mutated gene; however, even within genotypes and allelotypes, patients with MLASA may have highly variable neurologic abnormalities that most characteristically consist of exercise intolerance and lactic acidosis but may include severe hypotonia, muscle wasting, developmental delay, and mental retardation. The anemia, which is typically slightly macrocytic, is equally variable despite a common genotype, with some patients having asymptomatic anemia and others requiring transfusional support. PUS1, which localizes to mitochondria, the cytosol, and the nuclei, is one of several enzymes that modify uridine residues in long-lived RNA (e.g., tRNA) to the alternate base pseudouridine. This modification is thought to enhance the stability of these molecules and enhance protein translation. YARS2, by contrast, is solely a mitochondrial enzyme and appears to uniquely have effects on mitochondrial protein synthesis.[511]

Other Forms of Congenital Sideroblastic Anemia

At the present time, the genetic basis of approximately 40% of cases of CSA remains unexplained. Doubtless there are other syndromic and nonsyndromic sideroblastic CSAs to be molecularly characterized. Numerous patients with syndromic associations with other phenotypes associated with mitochondrial cytopathies, such as neurodevelopmental impairment, myopathy, hepatopathy, hypotonia, and failure to thrive, have been described. These patients most often have macrocytic sideroblastic anemia, often associated with other cytopenias, and very much resemble Pearson marrow-pancreas syndrome. Very recently, a syndromic form of microcytic sideroblastic anemia associated with recurrent, aseptic, febrile inflammatory episodes, hypogammaglobulinemia, and developmental delay has also been described (sideroblastic anemia, immunodeficiency, fever, and developmental delay [SIFD]).[513]

Box 11-5 Acquired Sideroblastic Anemias

- Toxic Causes of Anemia
  - Ethanol
  - Lead
- Drugs Causing Anemia
  - Chloramphenicol
  - Isoniazid and other antituberculous drugs
  - Penicillamine
  - Lincomycin
- Nutritional Causes
  - Copper deficiency
- Neoplastic Causes
  - Myelodysplastic syndromes
  - Refractory anemia with ringed sideroblasts
  - Refractory anemia with ringed sideroblasts and thrombocytosis

From Ammus S, Yunis AA: Drug-induced red cell dyscrasias. Blood Rev *3:71–82, 1989.*

Acquired Sideroblastic Anemias

A number of drugs and toxins that impair heme biosynthesis or perturb mitochondrial iron metabolism can produce sideroblastic anemia (Box 11-5) (reviewed by Ammus and Yunis[514]). Whereas chronic alcohol abuse is the most common toxin associated with sideroblastic anemia in adults, the most important of these agents in the context of pediatrics is lead. This element inhibits several enzymes in the heme biosynthetic pathway, including δ-aminolevulinic acid dehydratase, coproporphyrinogen oxidase, and ferrochelatase.[515] Lead screening, therefore, is mandatory in any child suspected of having sideroblastic anemia because lead poisoning can be treated. Isoniazid also produces sideroblastic anemia by causing functional depletion of pyridoxine, which inhibits heme biosynthesis.[516,517] Prophylactic treatment with pyridoxine is now part of the standard therapeutic regimen with this drug.

Acquired, clonal neoplastic sideroblastic anemias such as refractory anemia with ringed sideroblasts (RARS) and other myelodysplastic syndromes (MDSs) are exceptionally uncommon in the pediatric age group, except perhaps in children who have previously received chemotherapy for cancer.[518] In fact, RARS is so rare in individuals younger than 40 years of age that a thorough investigation of potential congenital conditions or toxic causes of sideroblastic anemia should be made in persons of this age group, particularly in the absence of clonal cytogenetic abnormalities or specific somatic mutations (see below). The extent to which the considerable experience with MDS in adults might be applicable to children is uncertain; however, when compared with other categories of MDS, RARS has a lesser propensity to evolve to acute leukemia and may often be associated with iron overload in the absence of significant transfusional support.[519] Intriguingly, some patients have been shown to have mutations within the mitochondrial genome that alter the coding sequence for cytochrome *c* oxidase.[520]

High throughput genomic sequencing technologies have recently showed that approximately two thirds of patients with RARS or other forms of MDS and myeloproliferative neoplasms with smaller numbers of ringed sideroblasts have heterozygous somatic mutations in splicing factor 3 subunit B1 (SF3B1).[521-524] All mutations, including the most common variant that substitutes a glutamic acid for a lysine at position 700, cluster in the region of the protein, indicating that it is highly likely that this is a gain-of-function mutation. Which, if any, of the CSA genes are involved in the pathogenesis of this disorder is uncertain; however, early reports suggest that ABCB7 is among the mRNAs that are abnormally processed and expressed in RARS.[525-527]

COPPER METABOLISM

Copper Physiology and Transport

A total of 100 to 150 mg of copper is present in an adult human. As with iron, the fetus accumulates large amounts of copper near the end of gestation, particularly in the liver, where concentrations are 5 to 10 times greater at birth than in adulthood. These stores do not decrease to adult levels until 5 to 15 years of age. The liver has the highest copper content, which together with muscle and bone, accounts for 50% to 75% of total body copper. The normal net copper intake is 2 to 5 mg in an adult and 0.04 to 0.15 mg/kg in a growing child. After about 6 months of age, normal plasma copper levels range between 70 and 140 mg/dL. Values are lower in early infancy.[528] Depressed plasma copper levels are a good indicator of early copper deficiency; however, functional tests of enzymatic activity (e.g., ceruloplasmin) may be better.

Like iron, copper is essential for normal cell growth and metabolism but toxic in excess. Unlike iron, however, copper balance is regulated by excretion as well as by absorption.[529,530] The gastrointestinal tract absorbs about 30% of the copper present in an average diet. The liver promptly disposes of much of that copper in bile, whereas a lesser amount is lost through the intestinal mucosa.[531]

Plasma copper, which is held much more constant than plasma iron, exists in two forms. Albumin and amino acids loosely bind 10% to 15% of circulating copper. Ceruloplasmin, a multicopper ferroxidase, tightly binds the remaining copper in plasma. Ceruloplasmin was once thought to be a copper transport protein; however, it is now clear that it functions primarily to change the oxidation state of iron, thereby aiding in release of iron from storage cells, including hepatocytes and macrophages (as discussed earlier). In human patients[532-535] and mice[89] lacking ceruloplasmin, a distinct clinical syndrome characterized by neurodegeneration, hepatic iron overload, diabetes, impaired iron recycling by reticuloendothelial cells, and mild iron deficiency anemia develops.

Copper is an essential component of cytochrome oxidase, the terminal oxidase of the electron transport chain required for combining hydrogen with oxygen to form water. As such, the element is essential for the oxidative generation of ATP. The enzyme tyrosinase is a copper-containing protein required for the synthesis of melanin. Thus, copper-deficient animals have depressed levels of cytochrome oxidase in most tissues, along with loss of hair pigment. Another copper metalloenzyme, lysyl oxidase, plays a role in the cross-linking of collagen and elastin. Lysyl oxidase deficiency may cause the vascular defects, including aortic aneurysm, seen in copper-deficient animals and patients with Menkes disease (see later).[529]

Copper trafficking is relatively well understood (reviewed by Kim and colleagues[536]). A multi–transmembrane domain protein, CTR1, appears to be the major transporter that brings copper into mammalian cells.[537-539] Knockout mice lacking CTR1 die early in gestation, with findings that may be attributable to severe copper deficiency. Once copper is inside cells, it moves to its sites of use escorted by specific chaperone proteins (reviewed elsewhere[540,541]). These proteins appear to be conserved from yeast to mammals. Targeted disruption of one of these proteins, Atox1, in mice leads a poorly viable, copper-deficient phenotype, thus indicating that its copper chaperone function is important but not essential.[542]

Two inherited defects of human copper metabolism have given insight into copper transport within cells. Menkes disease, or Menkes kinky hair syndrome, is a congenital disorder characterized by a low serum copper concentration, reduced activity of copper metalloenzymes, slow growth, hypopigmented kinky hair, cerebral degeneration, and an X-linked pattern of inheritance.[543] Parenteral copper administration, like all other attempts to treat the disease, is ineffective, and death before 3 years of age is typical. By contrast, in the autosomal recessive disorder Wilson disease, the predominant clinical phenotypes—liver disease, hemolysis, and neurodegeneration—are due to copper cytotoxicity.

Positional cloning led to identification of the genes mutated in Menkes and Wilson diseases.[544-546,547,548,549] Each is a P-type adenosine triphosphatase transmembrane copper transporter (Cu-ATPase) containing Gly-Met-Thr-Cys-X-X-Cys copper-binding motifs at their N-termini. The Menkes protein, ATP7A located on chromosome Xq21.1, is involved in transport of copper in intestinal enterocytes, as well as a number of other cell types. Deficiency of the Menkes proteins leads to paradoxic systemic copper deficiency as a result of impaired intracellular intestinal copper transport accompanied by intracellular copper accumulation in certain cell types, particularly the kidney. By contrast, deficiency of the Wilson disease Cu-ATPase, ATP7B located on chromosome 13q14.3, leads to cellular copper accumulation in the liver, basal ganglia, and kidney, along with impaired production of ceruloplasmin and hepatobiliary copper excretion. Interestingly, although Menkes disease patients are copper deficient, anemia is not a prominent feature of the syndrome (see below). Wilson disease may be manifested in children as fulminant hepatic failure associated with severe acute intravascular hemolysis (see below).[550,551]

Hematologic Aspects of Copper Deficiency

Copper deficiency commonly causes hypochromic, microcytic anemia among farm animals in areas of the world where the soil is poor in copper.[552,553,554,555] In humans, the predominant clinical feature of copper deficiency is peripheral neuropathy. Because of milk's low copper content (about 0.12 mg/L), infants who consume a diet consisting largely of milk may be at risk for marginal copper nutrition or deficiency.[556] Nutritional copper deficiency can be due to extraordinary dietary circumstances, such as inadvertent omission of copper from intravenous alimentation formulations for an extended time or treatment of severely malnourished patients with a copper-deficient milk diet.[529,530,557] Impaired copper absorption may be a consequence of a variety of renal and gastrointestinal disturbances, including nephrotic syndrome, inflammatory bowel disease and other forms of malabsorption, or bariatric or other intestinal surgery.[558,559] Excessive zinc supplementation or ingestion of coins containing zinc may also lead to copper deficiency because zinc readily induces intestinal epithelial metallothionein, which preferentially chelates intestinal copper and prevents absorption.[529,560] Coexisting iron deficiency or severe malnutrition can obscure the manifestations of copper deficiency.

In humans, the anemia of copper deficiency is most typically normocytic, but it may be microcytic or macrocytic. Leukopenia with very marked neutropenia is particularly prevalent; thrombocytopenia is unusual. Bone marrow aspirates from copper-deficient individuals are often hypocellular and show a relative erythroid hyperplasia with prominent vacuolization of erythroid precursors and variable degrees of dyserythropoiesis or megaloblastoid changes (or both), with or without the presence of ringed sideroblasts. As many recent reports indicate, resistance to treatment with typical hematemics combined with the morphology may suggest the errant diagnosis of MDS (reviewed by Haddad and associates[561]). The coexistence of peripheral neuropathy, also typical of copper deficiency, may suggest the correct diagnosis. Osteoporosis may occur with severe copper deficiency.

Oral treatment with 0.1 to 0.3 mg/kg of copper per day (two or three times the estimated daily requirement) as 0.5% copper sulfate (containing about 2 mg of copper per milliliter) produces prompt reticulocytosis and correction of the anemia and neutropenia. Intravenous copper may also be given in severe deficiency states. Generally, the dietary imbalance that produced the deficiency can be corrected when the problem is recognized.

Hematologic Aspects of Acute Copper Intoxication and Wilson Disease

Oxidant damage is incurred by the red cell membrane in a variety of inherited disorders involving abnormal hemoglobins that create defects in the cells' endogenous system for detoxification of oxidants. These include sickle cell disease, thalassemias, unstable hemoglobinopathies, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and disorders of glutathione metabolism. Membrane oxidant damage may also result from encounters with exogenous oxidants, such as copper. Acute copper-induced hemolysis has been reported in humans after accidental or suicidal ingestion of copper or copper-containing compounds, copper sulfate therapy for burns, copper contamination of hemodialysis units, and Wilson disease.[562-568] In the first three instances, acute intoxication is characterized by flushing, chills, nausea and vomiting, diarrhea, abdominal pain, a metallic taste in the mouth, excessive salivation, headache, weakness, and acute intravascular hemolysis.[562-567,569]

Wilson disease is an autosomal recessive disorder of copper metabolism, presenting between 3 and 52 years of age, and characterized by defective biliary excretion of copper, low plasma levels of ceruloplasmin, and toxic accumulation of copper in the liver, erythrocytes, kidneys, cornea, and brain.[570,571] The clinical consequences of toxic copper overload include cirrhosis, hemolysis, corneal Kayser-Fleischer rings, and a progressive neurologic syndrome.[572-585] Early diagnosis followed by effective treatment can lead to reversal of clinical symptoms, including hemolysis.

Hemolysis is an early feature of Wilson disease; it is seen in about 20% of patients with liver disease[572] and may be the presenting manifestation of the disorder.[573-580,586-590] Because the disease is treatable and because hemolysis may presage fulminant liver disease by days to years,[576,578,579] the clinician must always consider Wilson disease in children and young adults with Coombs-negative hemolytic anemia both with and without overt liver disease. Thrombocytopenia may also occur,[591] and patients have been misdiagnosed as having thrombotic thrombocytopenic purpura or hemolytic uremic syndrome. Typically, these previously well children have chemical evidence of liver disease, mild hepatosplenomegaly, a low ceruloplasmin concentration, increased erythrocyte and hepatic copper levels, and increased urinary copper excretion. Jaundice is often seen and may be severe. The hemolysis observed in Wilson disease is acute, but the clinical features, although similar to those of acute copper poisoning, are usually more subtle. Hemoglobin may be detected on urinalysis, but gross hemoglobinuria is unusual. Peripheral blood smears do not usually show characteristic changes, although spur cells and target cells have been documented in patients with concurrent severe liver disease.

The Wilson disease gene, the copper-transporting ATPase, ATP7B, and the Wilson disease protein, WNDP, belong to the large family of cation-transporting P-type ATPases.[591-596] A wide variety of mutations have been described in this gene in patients with Wilson disease.[597-600] The belief is that, in Wilson disease, copper initially accumulates in hepatocytes because of the absence of the copper-transporting protein, which is required for the incorporation of copper into ceruloplasmin. Copper eventually reaches a toxic level in the liver and is released into the bloodstream by hepatocellular necrosis.[578] Plasma copper is rapidly taken up by red cells.[601] Intravascular hemolysis occurs as a consequence of oxidative damage. The oxidative effects of copper are manifold. In vitro, copper inactivates numerous red cell glycolytic and hexose monophosphate shunt enzymes; directly oxidizes reduced NADPH and glutathione; oxidizes and denatures hemoglobin; damages membrane ATPases, fatty acid acylase, and probably other membrane enzymes; generates lipid peroxides; and cross-links membrane skeletal proteins into disulfide-bonded high–molecular-weight complexes, increasing membrane permeability and rigidity.[579,602] However, many of these effects have not been demonstrated at concentrations of copper observed in hemolyzing erythrocytes (150 to 400 mg/dL of red cells = 2.4 × 10–5 to 6.3 × 10–5 mol/L).[580,584] In vivo studies have indicated a marked inhibition of hexokinase and G6PD from red blood cells in patients with untreated Wilson disease compared with patients taking penicillamine and healthy children.[603] In addition, decreased glutathione levels were found in patients with untreated Wilson disease, as well as decreased antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Although it has so far not been possible to determine the exact cause of the demise of red cells, initial studies indicate that free copper (non–ceruloplasmin bound) leads to oxidative injury resulting in altered erythrocyte metabolism and antioxidant status in patients with Wilson disease. Whatever the pathophysiology of the hemolysis may be, several case reports suggest a role for therapeutic plasma exchange in acute wilsonian hemolytic crisis, if only as a bridge to hepatic transplantation.

LEAD TOXICITY

Sources of Lead

Lead is a toxic metal without any function in the human body. Because of its useful properties, lead has been mined for several centuries. As a result, progressively larger amounts have been removed from the depths of the earth and introduced into the biosphere. Enough lead has found its way into humans that at least in the industrialized parts of the world, all people have some lead in the body and hence in the blood. It has thus become customary to refer to the blood lead values found in apparently healthy populations as “normal” blood lead levels. This term is an obvious misnomer because it implies some kind of minimum physiologic requirement for lead.

Airborne lead enters the human body through inhalation; about 35% is absorbed and 15% to 18% is retained.[604] Intake of airborne lead is greater in children because they breathe at a lower height, where the lead concentration is greater, and have a greater respiratory rate per unit of body weight.[605] Sources of inhaled lead include combustion engines that burn leaded fuel and primary and secondary (recycling) lead smelters. Lead can also be ingested through the intake of contaminated food, water, and beverages. Lead absorption is increased by deficiencies of essential minerals, such as iron and calcium.[606] High maternal lead levels can result in fetal lead toxicity.[607]

Children living in older houses are exposed to the additional risk of lead in paint. Although lead in air, food, and beverages can be considered *low-dose sources*, lead in paint is by far the most concentrated source. As much as 40% of lead by weight is present in dried paint used before World War II. Since then, lead levels in paint have been progressively reduced. For many years it was believed that lead poisoning in children occurred primarily as a result of pica (the urge to ingest nonfood material), with consequent ingestion of paint chips.[608] Although this is not an uncommon occurrence, it is neither the only nor even the prevailing mechanism of ingestion of lead paint. Small children, through their ordinary hand-to-mouth activities (such as thumb sucking) directly ingest dust and thus take in large amounts of lead. Despite legislative efforts and scientific advances in the United States, millions of dwellings still contain leaded paint. These dwellings present an obvious risk, particularly to children of the poor, who tend to live in the oldest houses. The state of repair of the dwelling is almost as important as the lead content of the paint; peeling or flaking paint is obviously more accessible to small children. Because iron deficiency often induces pica, lead poisoning is more common in iron-deficient children.

Less common sources of lead include fumes from outdated plumbing; old battery casings; illegally distilled whiskey; printed paper, particularly colored magazines; and lead-painted toys. The traditional Asian, African, and Middle Eastern eye cosmetic *kohl*, as well as a number of traditional or herbal remedies including *azarcon* (*greta*), are substantially composed of lead. An uncommon source of lead that may result in severe poisoning is improperly glazed earthenware vessels used to store acidic beverages such as fruit juices.

Biochemical and Hematologic Effects of Lead

Lead has strong affinity for the sulfhydryl groups of cysteine, the amino group of lysine, the carboxyl groups of glutamic and aspartic acids, and the hydroxyl group of tyrosine. Lead binds to proteins, modifies their tertiary structure, and inactivates their enzymatic properties. Enzymes that are rich in sulfhydryl groups appear to be most sensitive. Mitochondria are particularly sensitive to lead. Many prominent effects of lead on heme synthesis are mediated through alterations of the mitochondrial membrane. Heme synthesis starts and ends inside the mitochondria; intermediate steps take place in the cytoplasm. In all cells, heme is an essential component of molecules of the cytochrome system, also located inside the mitochondria. Additionally, in erythropoietic precursors, very large amounts of heme are formed to make hemoglobin. Lead interferes at several points in the heme synthetic pathway in all cells. The two most important steps affected are those catalyzed by δ-aminolevulinic acid dehydratase (a cytoplasmic enzyme) and ferrochelatase (an intramitochondrial enzyme). Both of these enzymes depend on critical sulfhydryl groups for their activities. The inhibition by lead of ferrochelatase, which catalyzes the ultimate insertion of iron into protoporphyrin, results in the accumulation of protoporphyrins in maturing erythrocytes. Ultimately, despite the presence of a normal or an increased intracellular content of iron, the mitochondria are starved for iron. Metalloporphyrins inhibit several mitochondrial enzymes, and the increased concentration of zinc protoporphyrin resulting from lead toxicity further impairs mitochondrial function.[609] There is a relationship between the concentration of lead that inhibits ferrochelatase in mitochondria and the ambient concentration of iron in the medium. At any concentration of lead, inhibition of ferrochelatase is most marked when the iron concentration is the lowest.[610] This biochemical mechanism explains the greater elevation of erythrocyte protoporphyrin observed, at equal lead exposure, in children with concomitant iron deficiency.[611] A progressive, exponential increase in erythrocyte protoporphyrin concentration is observed at blood lead levels of 5 to 90 μg/dL.[612] The erythrocyte protoporphyrin level is an indicator of adverse metabolic effects. It is very effective as a screening test for severe lead poisoning, but it cannot be used to screen for blood lead levels below 40 to 50 μg/dL.

The bulk of lead in blood (>90%) is contained in erythrocytes, but blood lead content is independent of the hematocrit. Basophilic stippling results from deposition of ribosomal RNA and mitochondrial fragments.[613] Moderate basophilic stippling may be seen in iron deficiency and thalassemia trait, but stippling is particularly prominent in the polychromatophilic erythrocytes in lead poisoning, unstable hemoglobinopathies, including severe β-thalassemia, and pyrimidine-5′-nucleotidase deficiency. Anemia is observed in children with lead poisoning, primarily in severe cases or in those with concomitant iron deficiency.[610] This is not an unexpected finding, because the defect in heme synthesis affects only a very small fraction of the total protoporphyrin production and clinically significant anemia usually develops in the late stages. It is not uncommon to observe severe neurologic toxicity in the absence of anemia in children. On the other hand, lead intoxication and iron deficiency anemia tend to occur in the same lower socioeconomic population and are aggravated by each other. Iron-deficient children are more severely affected because iron deficiency increases lead ingestion through pica, increases lead absorption, enhances the defect in heme synthesis, and increases the toxicity of lead.

Other enzymes of heme biosynthesis are also affected by lead, but less severely. Uroporphyrinogen synthetase activity is inhibited only by a rather high (10^{-4} mol/L) lead concentration.[609] Coproporphyrinogen decarboxylase, another mitochondrial enzyme, also is inhibited by lead.[609] There is a marked increase in urinary coproporphyrin levels in lead intoxication. Measurement of urinary coproporphyrin has been widely used for the diagnosis of childhood lead poisoning, particularly in emergency departments. Although this test has been superseded by the easier measurement of erythrocyte protoporphyrin, it is important to recognize its usefulness in discriminating between recent and past lead exposure. Because of the protracted persistence of protoporphyrin within erythrocytes, significant elevations may be observed as long as 2 to 3 months after exposure to lead.[614] In contrast, increased coproporphyrin production, as a result of its high solubility, is immediately reflected in excess urinary excretion. Hence, when exposure to lead is current, both the erythrocyte protoporphyrin level and the urinary coproporphyrin levels are increased; when exposure to lead is no longer present, there is a discrepancy between the elevated erythrocyte protoporphyrin level and normal urinary coproporphyrin excretion.

The process of heme synthesis is not exclusive to the erythron. All cells contain essential heme proteins, particularly in the respiratory cytochrome system, also located in the mitochondria. The most visible effects of lead on heme synthesis are those detectable in peripheral blood because this is the easiest tissue to sample. However, the ubiquitous presence of active heme synthesis in the mitochondria of all body cells and the affinity of lead for mitochondria result in widespread impairment of cellular function. Lead intoxication inhibits the synthesis of cytochrome P-450, the key enzyme in the mixed oxidase system. It appears likely that the toxic effects of lead, particularly in the nervous system, reflect the derangement in heme synthesis.[615]

In adults, peripheral neuropathy, abdominal colic, and anemia are the most common initial signs of lead poisoning. In children, the clinical effects of excessive exposure to lead are apparent primarily in the central nervous system; alterations in renal function and anemia occur only in the most severe cases. Acute encephalopathy can be the first manifestation of severe childhood lead poisoning. The onset is usually marked by seizures, often intractable, followed by coma and cardiorespiratory arrest. The syndrome may be fatal within 24 to 48 hours. Survivors often suffer permanent neurologic damage.[616] Acute encephalopathy may be accompanied by Fanconi renal tubular syndrome with aminoaciduria, glycosuria, and hyperphosphaturia.[617]

Chronic symptoms of lead toxicity are vague and include abdominal pain, vomiting, general malaise, and behavioral changes. These symptoms are nonspecific and of little diagnostic help, particularly in the youngest children, aged 1 to 3 years, who are the most frequently affected. The threshold blood lead level at which encephalopathy occurs in children is difficult to define. *Acute lead poisoning* with clinical signs other than encephalopathy has been observed at blood lead levels ranging between 60 and 450 μg/dL. On the other hand, it is possible to observe blood lead levels as high as 200 μg/dL in children without overt neurologic symptoms. The probability of overt neurologic problems increases with the blood lead level and becomes significant when the blood lead level rises above 70 μg/dL. For these reasons and because of the impending risk of an unpredictable clinical deterioration, children found to have blood lead levels of 70 μg/dL or greater should be treated as medical emergencies, even in the absence of symptoms.[618]

As a result of the greater awareness of childhood lead poisoning by physicians, extensive screening programs in large cities, and the reduction of lead in the air subsequent to the wider use of lead-free gasoline, severe lead poisoning is extremely rare. Additionally, there is a greater awareness of the subclinical effects of lead and the fact that there is no threshold below which body lead levels can be considered safe. In view of the evidence of adverse effects of exposure to lead, even at low levels, there has been progressive lowering of the "acceptable" blood lead level. Therefore, it is important to not confuse "intervention levels" (decided on pragmatic considerations) with "safe" levels.

The insidious nature of lead toxicity mandates the detection of excessive exposure in apparently asymptomatic children before irreparable damage occurs. Screening for lead poisoning is made particularly difficult by the fact that the most commonly affected children are those of preschool age, who are also the least accessible, as a group, to mass testing. Screening in this age group is most often performed by pediatricians at routine office visits. It would be desirable, instead, for screening programs to reach out to the community and focus on the most economically deprived children, who have the least access to medical care, as well as the highest risk.

Nationwide programs for screening children must be directed primarily at identifying those in need of medical attention. The definition of *lead poisoning* now includes the category of children with blood lead levels of 10 to 19 μg/dL. Yet it has been shown that at this level the potential loss of IQ is so low that most people would agree that it is trivial.[619,620] Although medical intervention is not necessary for this group, educational and dietary advice is recommended. Children in need of medical attention are those with blood lead levels of 20 μg/dL or higher. Above this level, cognitive issues become significant.[619,620] Severe lead poisoning is defined as levels above 45 μg/dL.

Treatment of Lead Poisoning

The first and most essential step in treating childhood lead poisoning is identification and removal of the source of lead. Without elimination of the exposure, any treatment is futile. Environmental analysis, including home visits, is recommended to ascertain and remove the source of lead. Although complete removal of lead from the environment remains a formidable task for modern society, it offers the only viable solution to prevent lead toxicity in children. Multiple studies have shown that neurotoxicity is unlikely to be reversible, regardless of treatment, once lead intoxication is discovered. Because lead exposure is additive, removal of automobile-generated lead from the air has already had a profound impact on the problem of urban lead poisoning. However, residual lead-based paint continues to be a major problem.

Children who are symptomatic require urgent treatment. However, because the symptoms of lead poisoning may be elusive, treatment is indicated even in their absence if there is clear evidence of an excessive body burden of lead. The goal of treatment is prevention of subclinical damage without producing complications of the therapy itself; it is necessary to exercise judicious compromise. Two parenteral treatments are used in severe lead poisoning: dimercaprol (formerly called BAL, for British anti-Lewisite) and calcium disodium-ethylenediaminetetraacetic acid (CaNaEDTA). Both these agents chelate lead and induce its excretion. Dimercaprol is an oily, malodorous compound that is unpleasant to patients. It forms an emetic compound with iron, so iron therapy should not be given to a patient receiving dimercaprol.[621] It is dissolved in peanut oil and thus is contraindicated in children allergic to peanuts. Approximately half of patients treated with dimercaprol have side effects, many of which can be ameliorated by the concurrent use of antihistamines.[618]

CaNaEDTA is water soluble and best administered by continuous intravenous infusion. When administered intramuscularly (a less preferable and much more painful option), 2% procaine should be added to avoid severe local pain. CaNaEDTA may itself be toxic, particularly to the kidneys. Overdosage may induce tubular necrosis directly or renal toxicity as a result of hypercalcemia. CaNaEDTA is also able to chelate zinc, thus presenting another risk. Because δ-aminolevulinic acid dehydratase, a zinc-dependent enzyme, is already damaged by lead, the additional removal of zinc leads to its complete paralysis. This results in a burst of neurotoxic δ-aminolevulinic acid into plasma, which may aggravate (and even induce) convulsions. For these reasons, CaNaEDTA should never be given alone to symptomatic children or to asymptomatic children with blood lead levels of 70 μg/dL or greater; instead, it should be given after administration of dimercaprol to prevent these acute effects.[618] An additional note of caution is necessary with regard to the use of EDTA. Besides CaNaEDTA, a commercial preparation of disodium EDTA is also available and occasionally used for hyperparathyroidism. Disodium EDTA should never be used for the treatment of lead poisoning because it may induce acute hypocalcemia, which may be fatal. Only CaNaEDTA should be used for the treatment of lead poisoning. Hospital pharmacies should exercise maximum caution in dispensing disodium EDTA, which could be fatal if erroneously given to a child with lead poisoning but without hypercalcemia.

Because of their different pharmacologic properties, dimercaprol and CaNaEDTA are advantageously used in combination to treat children with symptomatic lead poisoning, with or without concomitant encephalopathy, as well as asymptomatic children with blood lead levels greater than 70 μg/dL.[618] The combined use of dimercaprol and CaNaEDTA is both safer and more effective than the use of CaNaEDTA alone. Treatment should always start first with dimercaprol to avoid precipitating encephalopathy.[621] Involvement of an experienced toxicologist is strongly advised for safe, effective management of these patients.

Succimer is a derivative of dimercaprol in which the methyl groups are replaced by carboxylic groups. This results in excellent water solubility, allowing it to be used orally. Although the advantages of an oral chelator are obvious, it is crucial to be cautious in the use of succimer because with its great water solubility and affinity for lead, it may result in increased absorption if used in a lead-laden environment. For this reason, succimer should be used only when it is absolutely certain that the child has been removed from the lead-containing environment and is in a safe home. In a recent trial, succimer was shown to be effective in reducing blood lead levels in patients with levels of 20 to 45 μg/dL.[622] Importantly, however, it did not reverse neurologic and cognitive deficits. For this reason, the authors concluded that it is not indicated in children with moderate lead levels (20 to 45 μg/dL). Experience with this drug in treating children with blood lead levels of 70 μg/dL or higher is very limited. In these cases, as well as in symptomatic children, it may still be prudent to treat with a combination of dimercaprol and CaNaEDTA.

Regardless of the treatment, it is important to remember that improvement in blood lead levels does not mean that all lead has been removed. Blood levels are likely to rebound 1 to 2 weeks after therapy as tissue lead (particularly from bone) enters the circulation. Children with a large body burden of lead, such as those with clinical symptoms or blood lead levels in excess of 70 µg/dL, invariably require several cycles of chelation therapy before a substantial reduction in the body burden of lead is obtained.[621] The need for additional cycles of therapy can be established by measurement of urinary lead excretion during the first days of treatment. The lead excreted should be related to the amount of EDTA given. Children who excrete lead in larger proportions are in greater need of additional cycles of therapy. It is not uncommon for a child to require several months of chelation therapy, initially with both dimercaprol and CaNaEDTA and later with CaNaEDTA alone, before urinary lead excretion declines and the blood lead level stabilizes below 30 µg/dL. Only at that point can chelation therapy be stopped.[621]

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CHAPTER 12

Porphyrias

John D. Phillips and **James P. Kushner**

CHAPTER OUTLINE

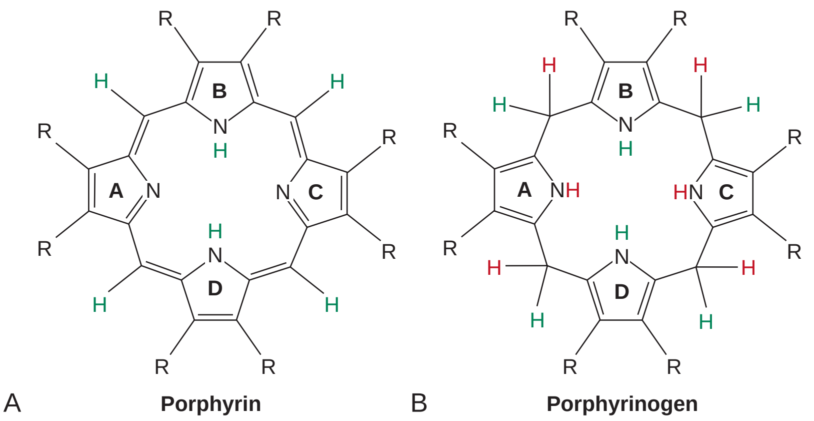
FORMATION OF THE PYRROLE
The δ-Aminolevulinate Synthases
δ-Aminolevulinate Dehydratase
ASSEMBLY OF THE TETRAPYRROLE
Porphobilinogen Deaminase
Uroporphyrinogen III Synthase
MODIFICATION OF THE TETRAPYRROLE SIDE CHAINS
Uroporphyrinogen Decarboxylase
Coproporphyrinogen Oxidase
ENZYMATIC OXIDATION OF PROTOPORPHYRINOGEN IX TO PROTOPORPHYRIN IX AND INSERTION OF IRON
Protoporphyrinogen Oxidase
Ferrochelatase

The porphyrias are caused by reduced activity of enzymes that constitute the heme biosynthetic pathway. With the exception of porphyria cutanea tarda (PCT), all are. Porphyrins are planar molecules comprised of four pyrrole rings connected by four methene groups. Porphyrins are also referred to as *tetrapyrroles* (Fig. 12-1, *A*). Porphyrins are intensely colored compounds (the word *porphyria* is derived from the Greek *porphuros,* meaning red-purple). All porphyrins avidly absorb light in a region of the spectrum known as the Soret band (around 400 nm) because of the alternating single-double bond structure of the porphyrin macrocycle. Once excited by light, porphyrins emit photons in the red region of the spectrum, a phenomenon responsible for their fluorescent properties. The emitted photons mediate the skin damage, which occurs in sun-exposed skin in some of the porphyric disorders.

The porphyrin precursors δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) (a monopyrrole) are colorless compounds that do not fluoresce. Recent evidence suggests that these compounds are responsible for the neurovisceral manifestations of several of the porphyric disorders. A convenient classification scheme divides the porphyric disorders into three groups according to their clinical manifestations (Box 12-1). Alternate schemes have been proposed by which the porphyrias are classified according to the organ in which the enzymatic defect is most apparent (hepatic porphyrias versus erythropoietic porphyrias), but these schemes are less useful to the clinician. Most heme synthesis takes place in erythroid precursors for the generation of hemoglobin, but approximately 15% of the daily production takes place in the liver,[1] mainly for the formation of the cytochrome P450 enzymes. Heme is also a component of numerous other cellular proteins, including cytochromes, myoglobin, catalase, peroxidase, guanylate cyclase, and others, and the heme biosynthetic pathway is functional in all cells.

With the exception of ferrochelatase (FECH), the terminal enzyme in the pathway that catalyzes the insertion of iron into protoporphyrin IX (proto IX), all enzymes in the pathway that utilize cyclic tetrapyrroles require fully reduced porphyrinogens as substrates. Porphyrinogens are flexible, colorless, reduced compounds with hydrogen atoms added to each of the bridge carbons and to nitrogens of the pyrrole rings (Fig. 12-1, *B*). Porphyrinogens are very sensitive to oxidation. When the heme biosynthetic intermediates uroporphyrinogen and coproporphyrinogen are oxidized to the corresponding porphyrins, they can no longer serve as substrates (Fig. 12-2) and are excreted in urine and stool. Protoporphyrinogen oxidase (PPO) also requires the reduced compound protoporphyrinogen as substrate.

Hans Fischer[2] originally developed the currently utilized porphyrin nomenclature. Proto IX was the ninth isomer of protoporphyrin synthesized by Fischer and corresponded to the only isomer of protoporphyrin found in



A Porphyrin

B Porphyrinogen

Figure 12-1 Porphyrin and porphyrinogens. Porphyrins are tetrapyrroles. **A,** Porphyrins are the oxidized form of porphyrinogens. The alternating single-double bond sequence of the molecule imparts the color and fluorescent properties of porphyrins and produces a rigid planar shape. **B,** Porphyrinogens, the reduced form, are colorless, do not fluoresce, and are highly flexible.

nature. Uroporphyrinogen and coproporphyrinogen could occur in four isomeric forms, but only the I and III forms occur in nature. The I and III isomers of uroporphyrinogen differ only in the positions of the acetate and propionate side chains on the D ring (see Fig. 12-1), but only the III isomer can ultimately be converted to proto IX and heme.

In eukaryotic cells, the heme biosynthetic pathway is divided between three compartments: (1) the mitochondrial matrix, (2) the mitochondrial intermembrane space, and (3) the cytosol (see Fig. 12-2). The pathway enzymes have been intensively characterized. All of the genes have been cloned, and many mutations responsible for the porphyrias have been identified. The crystal structures of all of the enzymes have been determined, providing models for the mechanisms of catalysis and the effects of specific mutations (Table 12-1).

Here we will dissect the complex heme biosynthetic pathway into four basic processes, and each porphyric disorder will be described in the context of the process that is defective:

- Formation of the pyrrole
- Assembly of the tetrapyrrole
- Modification of the tetrapyrrole side chains
- Enzymatic oxidation of protoporphyrinogen IX (proto'gen IX) to proto IX and insertion of iron

Box 12-1 Clinical Classification of the Porphyrias

PHOTOSENSITIVITY SYMPTOMS

Congenital erythropoietic porphyria (4)*
Porphyria cutanea tarda (5)†
Hepatoerythropoietic porphyria (5)*
Erythropoietic protoporphyria (8)*
X-linked erythropoietic protoporphyria (1)*

NEUROVISCERAL SYMPTOMS

Aminolevulinate dehydratase porphyria (2)*
Acute intermittent porphyria (3)‡

PHOTOSENSITIVITY AND NEUROVISCERAL SYMPTOMS

Hereditary coproporphyria (6)‡
Variegate porphyria (7)‡
Numbers in parentheses designate the enzyme defect responsible for the disorder (see Fig. 12-2)

*Clinical manifestations usually recognized in childhood.
†Clinical manifestations rarely recognized in childhood.
‡Clinical manifestations rarely occur before puberty except in children with homozygous or compound heterozygous genotypes.

FORMATION OF THE PYRROLE

The δ-Aminolevulinate Synthases

Regulatory mechanisms controlling heme synthesis in erythroid precursors and in the liver differ. In the liver, heme biosynthesis must respond rapidly to changing metabolic requirements. In erythroid cells, the pathway is regulated to permit a high steady state level of heme synthesis, and regulation is tied to the availability of iron. The first and rate-limiting enzyme in the pathway in all cells condenses glycine and succinyl-coenzyme A to yield ALA (Fig. 12-3). This highly exergonic reaction occurs in the mitochondrial matrix and involves cleavage of the thioester bond of succinyl-coenzyme A. Two ALA-synthase (ALA-S) enzymes have evolved to accommodate the requirements of the liver (and other tissues) and the unique demands of the erythron. The first, ALA-S1, is expressed ubiquitously but at very low levels in erythroid precursors. The dominant form of ALA-S in erythroid cells is ALA-S2. The gene encoding ALA-S1 maps to the short arm of chromosome 3,[3] whereas ALA-S2 is encoded on the X-chromosome.[4] The two forms of ALA-S are 62% identical at the amino acid level.[5] Although these two forms of ALA-S are regulated differently, both forms require pyridoxal-phosphate (PLP) as a cofactor. Pyridoxine deficiency is therefore associated with hypochromic anemia.[6] Both ALA synthases function as homodimers.

ALA-Ss are highly conserved between species, and the crystal structure of a bacterial ALA-S (from *Rhodobacter capsulatus*) has been determined (Fig. 12-4).[7] The crystal structure supports the enzyme mechanism that had been suggested by biochemical studies.[8]

In eukaryotes, both forms of ALA-S are synthesized in the cytosol and then translocated to the mitochondrial matrix, the site of succinyl-coenzyme A generation by the tricarboxylic acid cycle. Both forms contain an amino-terminal mitochondrial targeting sequence that also contains heme-binding domains designated as heme regulatory motifs (HRM).[9] Binding of heme to the HRM inhibits mitochondrial import of both forms of ALA-S,

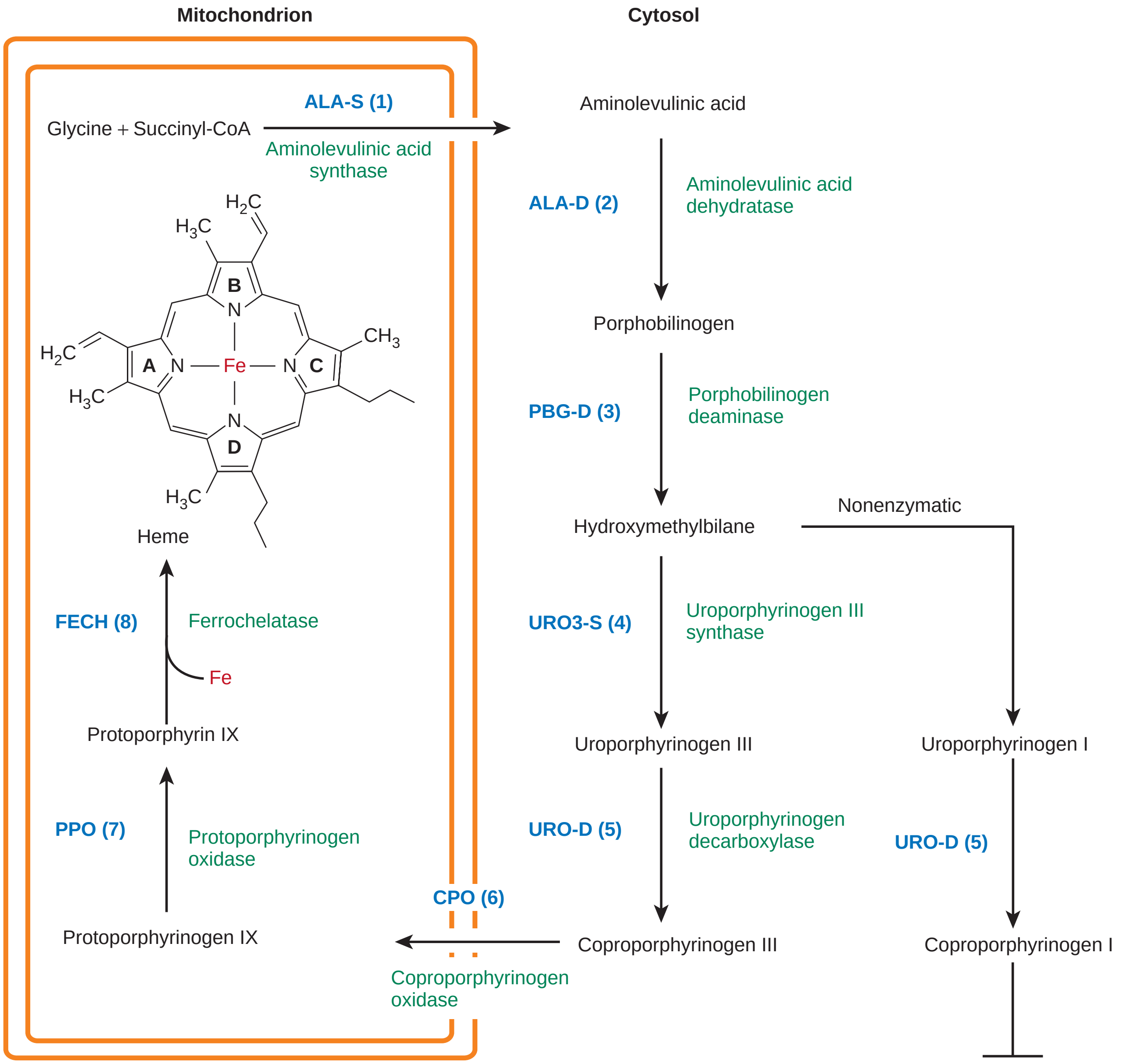

Mitochondrion
Cytosol
Glycine + Succinyl-CoA
ALA-S (1)
Aminolevulinic acid synthase
Aminolevulinic acid
ALA-D (2)
Aminolevulinic acid dehydratase
Porphobilinogen
PBG-D (3)
Porphobilinogen deaminase
Hydroxymethylbilane
Nonenzymatic
URO3-S (4)
Uroporphyrinogen III synthase
Uroporphyrinogen III
Uroporphyrinogen I
URO-D (5)
Uroporphyrinogen decarboxylase
URO-D (5)
Coproporphyrinogen III
Coproporphyrinogen I
CPO (6)
Coproporphyrinogen oxidase
Protoporphyrinogen IX
PPO (7)
Protoporphyrinogen oxidase
Protoporphyrin IX
FECH (8)
Ferrochelatase
Fe
Heme
B
A
C
D
N
Fe
H_2C
H_3C
CH_3

Figure 12-2 Heme biosynthetic pathway. The eight enzymes required to synthesize heme are named *(green)*. Abbreviations used throughout this chapter are in *blue*. Synthesis starts in mitochondria, depicted by the *double orange line*, continues in the cytosol, and terminates in mitochondria. Enzyme 6, coproporphyrinogen oxidase, is located in the mitochondrial intermembrane space. Enzyme 7, protoporphyrinogen oxidase, is an integral membrane protein associated with the inner membrane. Enzyme 8, ferrochelatase, an integral membrane protein, is located on the matrix side of the inner mitochondrial membrane.

TABLE 12-1 Diseases Linked to Steps in Heme Biosynthesis, Causative Genes, Chromosomal Locations, and Protein Codes

Disease	Gene	Location	OMIM	E.C. #	PDB
None	ALA-S1	3p21.1	125290	2.3.1.37	2BWN*
X-Linked Sideroblastic Anemia, X-Linked EPP	ALA-S2	Xp11.21	301300	2.3.1.37	
ALA-D Deficiency Porphyria	ALA-D	9q34	125270	4.2.1.24	1PV8
Acute Intermittent Porphyria	PBG-D	11q23.3	609806	4.3.1.8	3ECR
Congenital Erythropoietic Porphyria	URO3-S	10q25.2	263700	4.2.1.75	1JR2
Porphyria Cutanea Tarda, Familial Hepatoerythropoietic Porphyria	URO-D	1p34	176100	4.1.1.37	1URO
Hereditary Coproporphyria	COP-OX	3q12	121300	1.3.3.3	1R3Q
Variegate Porphyria	PROTO-OX	1q22	600923	1.3.3.4	3NKS
EPP	FECH	18q21.3	177000	4.99.1.1	2HRC

E.C. #, Enzyme Commission number; *EPP*, erythropoietic protoporphyria; *OMIM*, Online Mendelian Inheritance in Man; *PDB*, Protein Data Bank (http://www.rcsb.org/pdb/home/home.do).

*Structure of bacterial homologue.

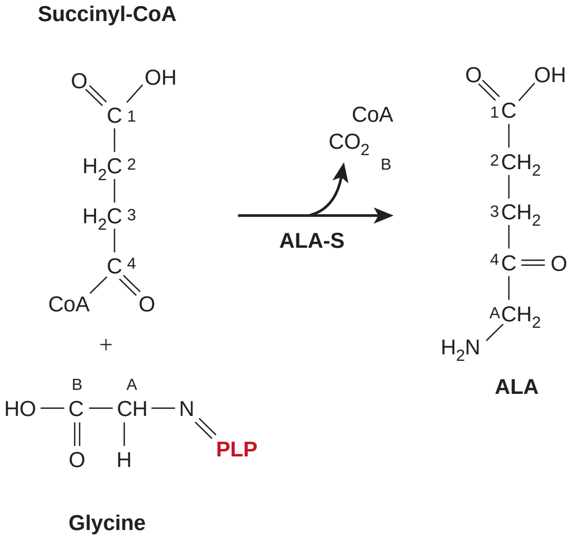


Figure 12-3 Synthesis of δ–aminolevulinic acid (ALA). In mitochondria, glycine is decarboxylated after forming a Schiff base with the cofactor pyridoxal phosphate (PLP). The reaction continues by condensation with succinyl-CoA, followed by release and recycling of CoA. The origin of the carbon atoms in ALA and CO_2 is indicated with either numbers or letters.

representing a posttranslational point of end-product feedback inhibition of the pathway.[10] The effect is greater for ALA-S1 than ALA-S2. In addition, developing erythroid cells express a heme transporter that prevents the accumulation of unbound heme in the cytosol (feline leukemia virus C receptor [FLVCR]).[11]

Both ALA-S1 and ALA-S2 are transcriptionally regulated but by quite different mechanisms. Extensive studies in avian embryonic liver cells indicated that hemin, a chloride salt of heme, repressed transcription of ALA-S1,[12] but the precise mechanism responsible for this effect has not been defined. Transcription of ALA-S1 is upregulated by the PGC-1α,[13] a coactivator of nuclear receptors and transcription factors.[14] Transcriptional regulation of ALA-S1 by PGC-1α is mediated by interaction of nuclear regulatory factor 1 (Nrf-1) and FOXO-1 (a forkhead family member) with the ALA-S1 promoter.[15] When cellular glucose levels are low, transcription of PGC-1α is upregulated,[16] leading to increased levels of ALA-S1 and creating conditions capable of precipitating acute attacks of the neurovisceral porphyrias. Upregulation of PGC-1α likely explains the clinical observation that fasting may precipitate attacks whereas glucose infusions are useful therapeutically.[17,18]

There are two splice forms of ALA-S1 in humans. The major form lacks exon 1B (Fig. 12-5) and is destabilized by heme.[19] The minor form includes exon 1B and is resistant to heme-mediated destabilization.[20] It is apparent that heme regulates ALA-S1 by multiple mechanisms, including transcription, translation, and translocation of the protein from the cytosol to the mitochondria. The regulatory effects of heme are mediated by the concentration of heme in small "pools" of heme not bound to hemoproteins.[21] Factors depleting unbound heme pools result in induction of ALA-S1, an effect that is the prime factor in precipitating acute attacks of the neurovisceral porphyrias.[22] No disease-causing mutations of ALA-S1 have been identified in humans, but down-regulating ALA-S1 with infusion of glucose and/or hemin forms the basis of therapy for acute porphyric attacks.[17,23]

In contrast to ALA-S1, ALA-S2 is transcriptionally regulated by erythroid specific factors (including GATA1) that interact with promoter sequences found in many genes differentially regulated in red cells.[24] Translational regulation also plays a major role in regulating expression of ALA-S2. The ALA-S2 transcript contains a 5' iron responsive element (IRE), which interacts with an IRE-binding protein (IRP).[25] The IRE-IRP complex prevents translation of the ALA-S2 mRNA. Addition of an iron-sulfur cluster (Fe/S) to IRP1 changes the conformation of the protein, reducing its affinity for the IRE. Fe/S clusters are generated and exported by mitochondria, linking regulation of heme biosynthesis in the red cell to the availability of iron and mitochondrial function.[26] Loss-of-function mutations in ALA-S2 are causative for

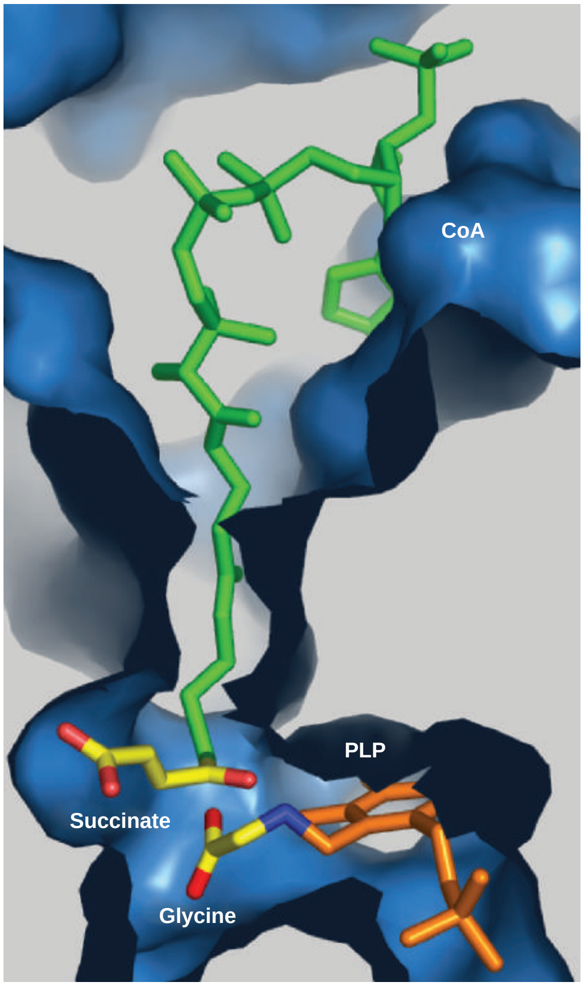


Figure 12-4 The active site of δ-aminolevulinate synthase (ALA-S) from ***Rhodobacter capsulatus***. Pyridoxal phosphate (PLP) *(orange)* forms a covalent linkage with a lysine residue. Glycine is bound through a transient Schiff base with PLP. Condensation with the succinate moiety of succinyl CoA, *(CoA in green)* allows the release of CoA, ALA, and PLP. The PLP cofactor is then bound again for subsequent rounds of catalysis.

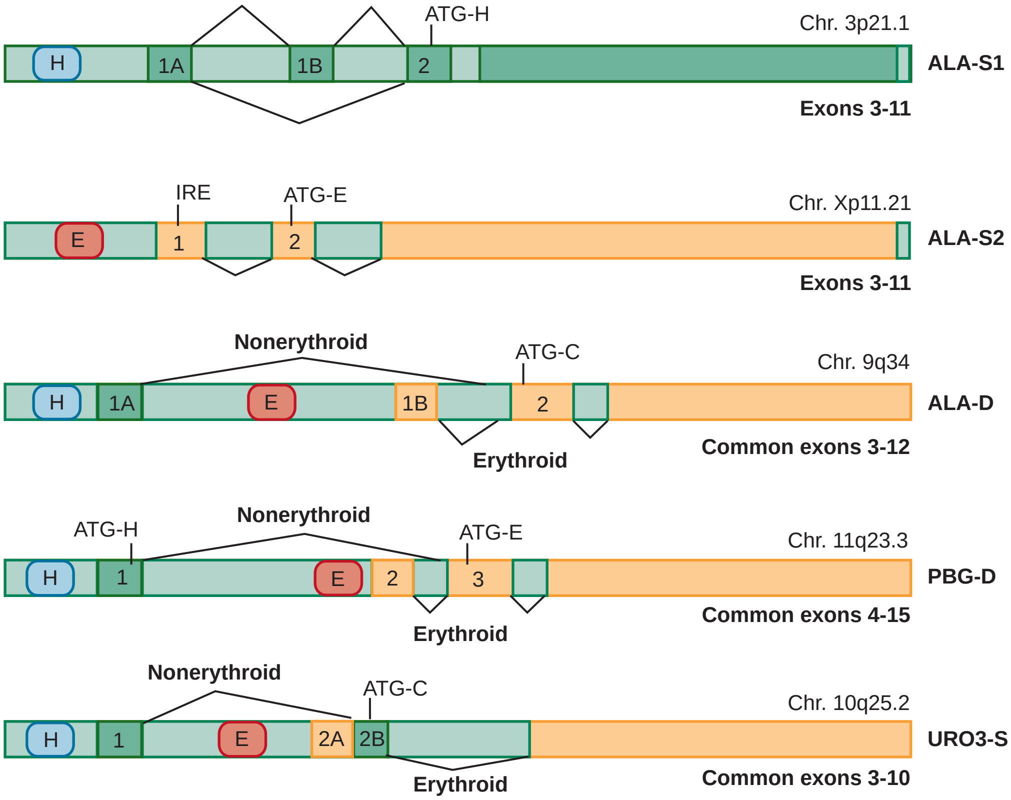

ATG-H
Chr. 3p21.1
H
1A
1B
2
ALA-S1
Exons 3-11
IRE
ATG-E
Chr. Xp11.21
E
1
2
ALA-S2
Exons 3-11
Nonerythroid
ATG-C
Chr. 9q34
H
1A
E
1B
2
ALA-D
Erythroid
Common exons 3-12
ATG-H
Nonerythroid
ATG-E
Chr. 11q23.3
H
1
E
2
3
PBG-D
Erythroid
Common exons 4-15
Nonerythroid
ATG-C
Chr. 10q25.2
H
1
E
2A
2B
URO3-S
Erythroid
Common exons 3-10

Figure 12-5 Genomic organization of the promoters for early steps in heme synthesis. Chromosomal location for the gene *(Chr.)*. Two distinct genes encode δ-aminolevulinate synthase (ALA-S), a housekeeping form (ALA-S1) and an erythroid-specific form (ALA-S2). An iron responsive element (IRE) in exon 1 of ALA-S2 controls posttranscriptional gene expression. The next three genes utilize specific promoters for erythroid *(E)* or nonerythroid tissues *(H)*. The ATG start sites are designated *C*, common; *E*, erythroid; or *H*, housekeeping.

X-linked sideroblastic anemia (see Chapter 11). Gain-of-function mutations in ALA-S2 have been recognized as causative for X-linked erythropoietic protoporphyria (EPP) (see later discussion).[27]

δ-Aminolevulinate Dehydratase

Once synthesized, ALA exits mitochondria by an unknown mechanism likely involving a transporter. In the cytosol, two molecules of ALA undergo a condensation reaction catalyzed by δ-aminolevulinate dehydratase (ALA-D) (also known as *PBG synthase*) to form the monopyrrole PBG (Fig. 12-6). The crystal structure of human ALA-D and ALA-Ds from many other species indicate that the enzyme functions as a tetramer of homodimers formed from eight identical subunits.[28] Each of the homodimers contains one active site.[28] Each of the eight subunits binds a zinc atom, and the enzyme is inactivated by replacement of the zinc by lead. Four zinc

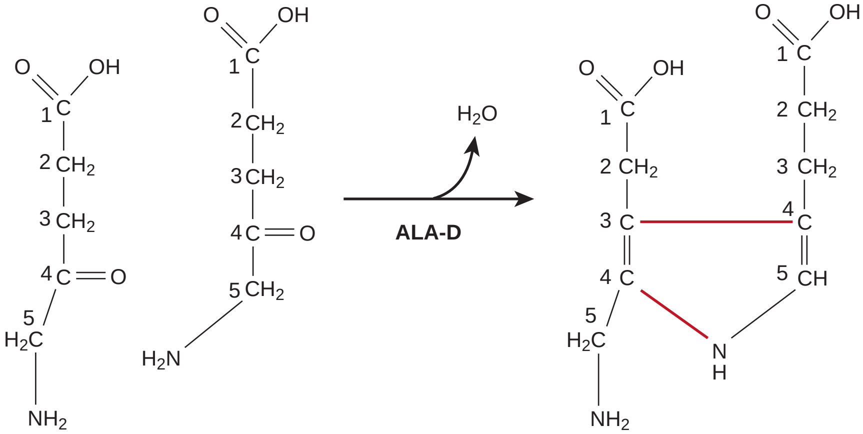


Figure 12-6 Reaction catalyzed by δ-aminolevulinic acid dehydratase (ALA-D). In the cytosol, two molecules of δ-aminolevulinic acid (ALA) condense to form porphobilinogen. The carbon atoms of each molecule of ALA are numbered for reference.

molecules are essential for catalysis. The remaining four zincs serve to stabilize the tertiary structure of the enzyme.[28] Each active site binds two molecules of ALA through the formation of Schiff base linkages with active site lysine residues, one for each of the two substrate molecules. One molecule of ALA contributes the acetate side chain and the aminomethyl groups of PBG. The other contributes the propionate side chain and the pyrrole nitrogen (see Fig. 12-6).

In contrast to ALA-S, there is a single ALA-D gene located on chromosome 9q34 (see Table 12-1).[29] The gene consists of two alternatively spliced noncoding exons (1A and 1B) and 11 coding exons (see Fig. 12-5).[30] The translational start site is located in exon 2. A housekeeping promoter is located upstream of exon 1A, and a second erythroid-specific promoter is located between exons 1A and 1B. The erythroid promoter contains binding sites for erythroid specific transcription factors including GATA1 and Krüppel-like factor 1.[31] Although the housekeeping and erythroid transcripts differ, they both encode identical proteins because they share the same translational start site in exon 2.

A common polymorphism of ALA-D, K59N, has been detected in approximately 10% of Caucasians, 3% of African Americans, and 6% to 11% of Asians.[32] This polymorphism has been designated the ALA-D2 allele. The ALA-D2 allele retains normal enzyme activity and appears to have a higher affinity for both zinc and lead than the ALA-D1 allele.[33] Lead-entering plasma is rapidly taken up by red cells and binds to ALA-D by displacing zinc and inactivating the enzyme.[34] Multiple studies have shown an association between the ALA-D2 allele and high blood lead levels,[33] but it remains unclear whether or not this allele is associated with increased susceptibility or increased resistance to lead toxicity.[34]

Although the quaternary structure of human ALA-D is that of a homo-octamer, a homo-hexamer form of ALA-D composed of three homodimers has also been detected. The homo-hexamer has low enzyme activity. The octameric and hexameric forms of ALA-D are described as morpheeins, indicating that dissociation of the high activity octamer is followed by a conformational change and then reassembly as the structurally different low-activity hexamer.[35] Conformational changes in the component dimers may result from substrate binding, but the most dramatic effects result from ALA-D mutations causative for ∂-aminolevulinate dehydratase deficiency porphyria (see later).[36]

In all tissues, the enzymatic activity of ALA-D greatly exceeds the activity of ALA-S. The abundance of ALA-D in comparison with all of the heme biosynthetic enzymes has been ascribed to the finding that ALA-D plays several roles, serving as a proteasome inhibitor affecting the ATP/ubiquitin-dependent degradation of proteins, playing a role in chaperone assisted protein folding, and as a heme biosynthetic pathway enzyme.[37,38]

δ-Aminolevulinate Dehydratase Deficiency Porphyria

ALA-D deficiency porphyria (ADP) is caused by homozygosity or compound heterozygosity for mutations of the ALA-D gene.

Frequency. ADP is an autosomal recessive disorder resulting from loss of function mutations of ALA-D. The disorder is exceedingly rare, and fewer than 10 cases have been reported, two in one family.[39] Most of the cases have represented compound heterozygotes. Eleven disease causing ALA-D mutations have been described, 10 of which are point mutations leading to amino acid substitutions.[40,41] The sole exception is the deletion of two sequential bases resulting in a frame shift yielding a premature stop codon.[42] Estimates of the frequency of heterozygosity for loss-of-function alleles of ALA-D, based on half-normal or less enzyme activity in erythrocytes of asymptomatic northern Europeans range from 1% to 2%.[43,44] The extreme rarity of homozygosity or compound heterozygosity for ALA-D mutations suggests a high incidence of embryonic lethality when two mutant alleles of ALA-D are present.

Clinical Manifestations. The clinical manifestations of ADP resemble those of the neurovisceral porphyrias but vary greatly in severity. The first two cases reported by Doss and colleagues in Germany were unrelated 15-year-old boys.[45,46] Both suffered recurrent attacks of abdominal pain, nausea, vomiting, constipation, tachycardia, and a motor neuropathy causing paralysis. A third German case reported by Doss and colleagues[47] also developed symptoms at age 15, including abdominal pain and a severe polyneuropathy. All three German patients were compound heterozygotes for ALA-D mutations.

The youngest reported case of ADP was a Swedish boy in whom muscle hypotonia was recognized at birth. At the age of 3, he developed recurrent attacks of abdominal pain, vomiting, and a motor polyneuropathy. This child also proved to be a compound heterozygote for ALA-D mutations.[48] A liver transplant was performed at the age of 6 with the assumption that the metabolic defect was most profound in the liver.[49] No change in the excretion of ALA was observed after the transplant, strongly suggesting that the red cells were the major source of this porphyrin precursor. The child died at the age of 9 of pneumonia and respiratory deficiency.[50]

The oldest reported case of ADP was a Belgian man who developed a motor neuropathy and markedly increased urinary ALA excretion at the age of 63.[51] ALA-D activity in erythrocytes was dramatically reduced to 1% of normal controls. The syndrome developed together with a clonal myeloproliferative disorder, namely polycythemia vera. Molecular studies revealed heterozygosity for a loss-of-function mutation (G133R) present in the polycythemia clone, which became clinically manifest with clonal expansion.[52] This represents the only case in which a fully penetrant phenotype occurred in a heterozygote and supports the suggestion that the source of excess ALA in ALA-D porphyria is the erythron.

In two other cases, partial biochemical evidence of ALA-D deficiency were detected in heterozygotes but by a completely different mechanism. The first was a healthy Swedish newborn undergoing neonatal screening for hereditary tyrosinemia.[53] Erythrocyte ALA-D activity was 12% of normal, and there were slight elevations of urinary ALA and coproporphyrin, as well as an increase in erythrocyte protoporphyrin. The child proved to be heterozygous for an F12L mutation, a catalytically inactive mutant that also affects secondary structure of the protein that favors formation of (with wild type protein) the low activity hexameric form of ALA-D.[35,41] In spite of the ALA-D defect, the child remained healthy and developed normally.

The second case of partial biochemical expression in a heterozygote ALA-D mutant occurred in a 23-year-old American man and was also due to the F12L mutation.[54] This patient presented with an acute porphyric attack clinically and biochemically compatible with hereditary coproporphyria (HCP). Indeed, a mutation of coproporphyrinogen oxidase (CPO) was identified. Because of a greater than usual increase in urinary ALA excretion and an increase in erythrocyte zinc protoporphyrin, an additional defect in ALA-D was suspected. Subnormal erythrocyte ALA-D activity was detected, and molecular studies revealed heterozygosity for the F12L mutation.

Pathogenesis. The pathogenesis of the symptom complex associated with ADP is poorly understood. It has been suggested that ALA may act as a neurotoxin based on structural similarities between ALA and glutamate and ALA and γ-aminobutyric acid (GABA).[55] High concentrations of ALA may interact with GABA receptors or inhibit glutamate uptake. ALA has been demonstrated to bind to GABA receptors in vitro, and GABA receptors are highly expressed by oligodendrocytes.[56] In addition, oligodendrocytes are highly sensitive to glutamate-dependent metabolic pathways, which could be impaired by the effect of ALA on glutamate uptake.[57] A possible role for ALA as a neurotoxin is supported in patients with hereditary tyrosinemia, who develop neurovisceral symptoms associated with marked inhibition of ALA-D by succinylacetone.[58]

Laboratory Diagnosis. Although ADP is rare, it should be suspected when an acute attack of abdominal pain suggests the possibility of a porphyric attack, especially when a prominent distal motor neuropathy is present. The dramatic reduction of ALA-D activity (to levels of 5% or less) results in the urinary excretion of large amounts of ALA and normal or minimally increased levels of PBG, a finding which distinguishes ADP from other neurovisceral porphyrias. Urinary excretion of coproporphyrin is markedly increased. The mechanism responsible for the increase in urinary coproporphyrin excretion is not known, but similar findings occur when ALA-D is inhibited in lead poisoning and when ALA is administered orally to normal individuals.[59] Zinc-protoporphyrin accumulates in erythrocytes in patients with ADP, again by a poorly understood mechanism.

Heterozygous parents and siblings of probands with ADP have approximately half-normal ALA-D activity as measured in red cell lysates. Erythrocyte ALA-D assays are available in reference laboratories in the United States and Europe. Gene sequencing is available for ALA-D mutation analysis through the Department of Human Genetics at the Mount Sinai School of Medicine, New York, N.Y.

It is important to emphasize that ALA-D activity can be subnormal in disorders other than ADP. Patients with lead poisoning often develop clinical signs and symptoms similar to those found in patients with ADP and other neurovisceral porphyrias. Lead in plasma is taken up by red cells and inhibits ALA-D activity by displacing zinc atoms from the enzyme.[34] The result is excess ALA in blood and urine, excess urinary excretion of coproporphyrin, and accumulation of zinc protoporphyrin in erythrocytes, findings also associated with ADP. The abnormal accumulation and excretion of porphyrins may be due, in part, to inhibitory effects of lead on CPO and FECH[60] but is more likely due to disposition of the excess ALA, as occurs in ADP. Lead poisoning can be distinguished from ADP by measuring the blood lead level.

Patients with hepatorenal tyrosinemia type I, an autosomal recessive disorder due to deficiency of fumarylacetoacetate hydrolase (FAH), manifest acute episodes of porphyric-like neurologic crises and secrete large amounts of ALA in the urine.[61,62] The FAH defect results in the generation of succinylacetone, a structural analog of ALA and a potent inhibitor of ALA-D.[63]

A single patient with an acquired reversible, unidentified inhibitor of ALA-D, high urinary ALA levels, and a porphyria-like acute attack has been reported.[64] In addition, both styrene and bromobenzene have been shown to inhibit ALA-D in rats.[65,66] Humans exposed to these compounds have moderately decreased erythrocyte ALA-D activity but do not suffer clinical consequences.

ADP is a rare disorder and must be differentiated from other causes of ALA-D deficiency. Biochemical and molecular evidence is essential to establish the diagnosis of ADP.

Treatment. Clear recommendations for the treatment of ADP are difficult because the number of reported cases is small, and the results of therapy have been variable. Suppression of ALA-S1 by the infusion of heme (heme arginate) proved effective in three German cases (all adolescent males),[46,47] and infusions of glucose, narcotic analgesics, and hemin were effective in a 14-year-old American boy.[40] Hemin infusion proved ineffective in the Swedish infant previously described.[48] There have been no reports of marrow transplantation in a patient with ADP. Avoidance of drugs known to induce acute attacks in other neurovisceral porphyrias, generally by depleting hepatic heme pools and inducing ALA-S1, is suggested but has not been proven to be useful.

ASSEMBLY OF THE TETRAPYRROLE

Porphobilinogen Deaminase

A polymer of four molecules of PBG is generated by PBG deaminase (PBG-D) (also called *hydroxymethylbilane synthase*), a cytosolic enzyme. The polymer, an unstable tetrapyrrole, is designated hydroxymethylbilane (HMB) (Fig. 12-7). PBG-D functions as a monomer, and the crystal structure of the human enzyme has been determined.[67] Purification of PBG-D from both mammals and bacteria has yielded an enzyme with a dipyrrole (dipyrromethane) in the active site. The dipyrrole serves as a

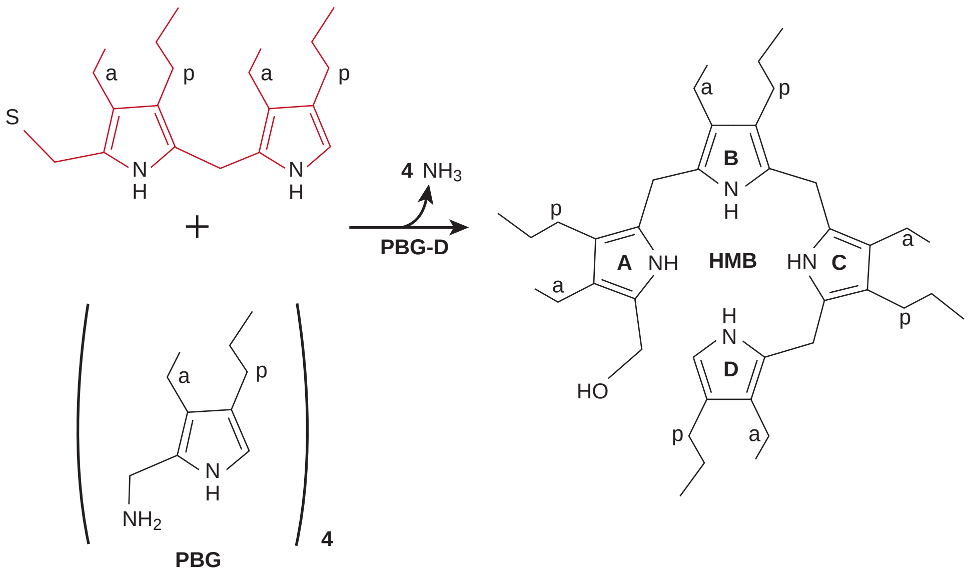


Figure 12-7 Reaction catalyzed by porphobilinogen deaminase (PBG-D). A dipyrrole cofactor, dipyrromethane *(red),* is covalently linked to the enzyme via a thioester bond. Four molecules of PBG are added, each releasing an amino group. Once a hexapyrrole has been formed, the terminal tetrapyrrole is cleaved and released as hydroxymethylbilane (HMB). The acetate *(a)* and propionate *(p)* side chains of the monopyrroles are labeled.

cofactor and is required for enzyme activity. A novel mechanism has been proposed to explain how PBG-D acquires the dipyrrole.[68] PBG-D first binds HMB and then deaminates and polymerizes two additional molecules of PBG to form a hexapyrrole. The distal tetrapyrrole is cleaved and released as HMB. The proximal dipyrrole remains covalently bound through a thioester linkage and serves as the cofactor for subsequent rounds of catalysis.[69] The source of the first HMB molecule to bind to PBG-D remains unexplained. Polymers of PBG can be formed nonenzymatically,[70] but incubation of apo-PBG-D with PBG yields very little holoenzyme with the dipyrrole cofactor bound.[71] It seems likely that the sequence of events involves nonenzymatic condensation of four molecules of PBG to form the amino-methylbilane, which interact with PBG-D. Although a poor substrate, amino-methylbilane is deaminated to form HMB, which is capable of forming a covalent thioester bond with PBG-D, leading to assembly of the initial hexapyrrole.[68]

The human PBG-D gene has been cloned and mapped to chromosome 11q23-11qter.[72,73] The gene spans 10 Kb and contains 15 exons.[74] Two separate promoters control transcription and allow tissue specific regulation of gene expression, a mechanism also used by ALA-D and URO3-S (see Fig. 12-5). The housekeeping promoter lies upstream of exon 1 and is active in all tissues. The erythroid-specific promoter lies upstream of exon 2 and resembles other erythroid-specific promoters.[75] Processing of the transcript initiated by the housekeeping promoter results in splicing of exon 1 to exon 3 and loss of exon 2. Transcription initiated by the erythroid promoter results in a transcript containing exons 2 through 15. Translation of the erythroid mRNA is initiated at an AUG located in exon 3, as exon 2 lacks an AUG codon.[74] The AUG translational start codon in exon 1 of the housekeeping transcript is spliced in frame with the AUG in exon 3, resulting in a protein 17 amino acids longer at the amino terminus compared to the erythroid protein.[76]

Acute Intermittent Porphyria

Acute intermittent porphyria (AIP) is caused by mutations of the PBG-D gene. Over 385 PBG-D mutations have been described.[77] The spectrum of mutations includes point mutations, deletions, duplications, and insertions and has been found in the housekeeping promoter, exons 1 and 3 through 15, and in each of the 14 introns. Exon 12 appears to be a mutational hot spot with 46 identified mutations.[78]

Frequency. AIP is transmitted as an autosomal dominant trait, but five symptomatic children homozygous for disease–causing PBG-D mutations have been reported.[55,79] Symptoms in these children began in infancy and included ataxia, dysarthria, psychomotor delay, and both central and peripheral neurologic manifestations. AIP is the most common of the acute neurovisceral porphyrias, but estimates of incidence vary according to the screening method used (clinical manifestations, erythrocyte PBG-D assays, molecular methods). Estimates based on a clinical diagnosis suggest an incidence of 0.13 per 1,000,000 in Europe.[80] The incidence in Sweden is approximately four times higher, [80] mainly due to a founder effect in northern Sweden.[81] When subnormal erythrocyte PBG-D activity is used for screening, the incidence of AIP in European populations is much higher,[82,83] but estimates based on erythrocyte PBG-D activity must be interpreted with caution. The normal range is wide, with considerable overlap between low-normal and high activity carrier values. Additionally, improper sample processing, storing, and shipping leads to decreased enzymatic activity.[17] Molecular screening for PBG-D mutations on a large scale in a general population has not been done. It is likely that the incidence of undetected gene carriers in the population is much higher than the incidence of clinically evident AIP because most gene carriers remain symptom free throughout their lives.[80] Recent estimates suggest a penetrance of only 1%.[84] In all series, clinical symptoms occur more commonly in women than men.

Clinical Manifestations. Clinical manifestations of AIP may occur before puberty in homozygotes or compound heterozygotes for PBG-D mutations, but most cases occur after puberty.[85-87] Pediatricians are likely to encounter adolescent patients with symptomatic AIP, particularly adolescent girls following menarche. Virtually all of the clinical signs and symptoms that characterize attacks of AIP are due to neurovisceral dysfunction (Table 12-2). Diffuse abdominal pain, often accompanied by nausea, vomiting, abdominal distention, and constipation, is the most common symptom of the acute attack.[17,88] Abdominal pain is usually unremitting and severe but occasionally may be cramping. Decreased bowel sounds and even signs of ileus are generally present, but some attacks may be associated with enhanced gut motility and diarrhea. Tachycardia is often present.[89] Other symptoms due to sympathetic over-activity or autonomic neuropathy include hypertension, tremors, sweating, and restlessness. Anxiety is a particularly common symptom, both during acute attacks and between attacks.[90] A variety of other neuropsychiatric symptoms associated with AIP have been described, ranging from hypomania to overt psychotic behavior,[91,92] and psychiatric symptoms may be the only manifestation of biochemically evident AIP.[93,94]

TABLE 12-2 Signs and Symptoms of the Acute Porphyric Attack

Signs and Symptoms	Frequency*	Comments
Gastrointestinal		
Abdominal pain	Common	Generally constant and not localized
Nausea and vomiting	Common	Accompanies abdominal pain
Constipation	Common	Urinary retention may occur also.
Diarrhea	Uncommon	
Neurologic		
Pain in back, chest, neck, head, extremities	Occasional	Pain may migrate to abdomen; may be accompanied by a sensory neuropathy
Paresis	Common	More proximal than distal; arms involved more often than legs
Respiratory paralysis	Uncommon	Occurs with progressive motor neuropathy and paresis
Mental symptoms	Occasional	Ranges from agitation to psychosis
Convulsions	Uncommon	Mainly occurs with hyponatremia
Cardiovascular		
Tachycardia	Common	Rate control with β-blockers may be needed.
Systemic arterial hypertension	Occasional	May become chronic and affect renal function

*Common: greater than 61%; Occasional: 21%-60%; Uncommon: less than 20%

A peripheral neuropathy may occur with or without an acute attack of abdominal pain. The neuropathy, due to axonal degeneration, is manifest mainly by muscle weakness in the extremities.[95] Nerve conduction abnormalities may be minor, even with symptomatic weakness, but sophisticated studies of slow motor fiber conduction have been abnormal, even in the absence of symptoms.[96,97] Cranial neuropathies are uncommon, but blindness due to optic nerve involvement or occipital lobe involvement has been reported.[98,99]

Seizures may occur during an acute attack and are often associated with hyponatremia.[100] When serum sodium levels are normal it is difficult to determine if the seizure is due to porphyria or to coexistent idiopathic epilepsy. In one large study of 268 patients, the lifetime prevalence of seizures was 5.1% in those who had suffered at least one acute attack and 2.2% in known carriers.[100] Seizures occurring during latent periods are likely to be related to AIP. It is essential to establish a firm diagnosis of idiopathic epilepsy in AIP patients who experience seizures because many antiseizure medications may precipitate acute attacks (see later; Table 12-3). If seizures are associated with the hyponatremia of an acute attack, prolonged antiseizure therapy is not necessary.

Hyponatremia is a common finding during acute attacks.[89] Hyponatremia maybe due to inappropriate secretion of antidiuretic hormone,[101] but renal salt wasting has been implicated in patients with the lowest serum sodium vales.[89] Serum sodium values below 120 nmol/L are generally found only in severe attacks.[102]

The likelihood of full recovery after an acute attack has increased dramatically over the last 25 years. This is undoubtedly due to the introduction of effective therapy with hemin or heme arginate (see "Treatment" section). The risk of death, however, has not been eliminated. A review of 136 patients hospitalized with acute attacks at the University of Minnesota between 1940 and 1998 (i.e., both before and after the introduction of hemin therapy) found that 29 (21%) had died during an acute attack.[103] In a study from Finland and Russia, 7 of 55 patients (13%) died as the result of an acute attack between 1966 and 2002. (Hematin therapy was first employed in 1971,[104] but not widely used until 1978.[105]) In another study of 47 Finnish patients ascertained between 1967 and 1989, 6 deaths (12.7%) occurred as the result of an acute attack.[106]

An unexplained finding in patients with AIP, variegate porphyria (VP), and HCP is a 35- to 70-fold increased risk of developing hepatocellular carcinoma. Most of these tumors occurred in individuals with a history of acute attacks. It has been suggested that DNA damage induced by ALA may be responsible for these observations.[107,108] An unusual feature of the hepatocellular carcinomas arising in porphyric patients is the absence of associated liver cirrhosis.[107,109] Chronic sustained hypertension is also prevalent in AIP and may be associated with renal impairment.[108]

Pathogenesis. Heterozygotes for PBG-D mutations have approximately half-normal enzyme activity in all tissues, which is usually sufficient for heme homeostasis. The enzymatic defect becomes manifest when demand for hepatic heme is increased, and ALA-S1 is induced. The result is increased production of ALA and PBG, creating a situation where the half-normal activity of PBG-D becomes rate limiting, and PBG (and ALA) accumulate in hepatocytes, circulate in plasma, and are excreted in the urine. Drugs are the most commonly recognized factors that increase the demand for hepatic heme, especially those metabolized by hemoprotein enzymes of the cytochrome P450 family.[22] Barbiturates, anticonvulsants, and steroid hormones are well recognized precipitating factors in patients with AIP, but other factors not directly connected with P450 metabolism have also been incriminated, including fasting, dieting, and the stress of an acute illness.[110,111] Most drugs taken by patients with AIP and other neurovisceral porphyrias have been classified as unsafe, potentially unsafe, probably safe, or safe. Isolated case reports have resulted in many drugs being classified as unsafe or potentially unsafe, resulting in a need for caution in drawing conclusions. Some drugs, however, have repeatedly been associated with acute porphyric attacks and must be avoided. A selection of these is shown in Table 12-3.

The current prevailing view is that symptoms of the porphyric attack result from the toxic effects of ALA and/or PBG rather than from a deficiency of heme in neurons.[55,112] The most striking evidence in support of this is the beneficial effect of allogeneic liver transplant in patients with repetitive and disabling acute attacks refractory to more conservative treatments.[113,114,115] An allogeneic liver transplant rapidly normalizes urinary excretion of ALA and PBG and eliminates recurrent neurovisceral attacks. Furthermore, when explanted livers from AIP patients were transplanted into nonporphyric patients, the recipients promptly developed biochemical and clinical evidence of AIP.[116] These impressive reports support the causative role of hepatic overproduction of ALA and PBG in acute neurovisceral attacks, but it is inappropriate to consider liver transplantation as a broadly applied treatment, especially with the efficiency of hemin or heme arginate infusions (see later).

TABLE 12-3 Selected Drugs Unsafe for Patients with Neurovisceral Porphyrias*

ACE inhibitors	Mephenytoin
Barbiturates	Metaclopramide
Carbamazepine	Primidone
Chlorpropamide	Progesterone
Danazol	Rifampin
Diphenylhydantoin	Succinimides
Ergots	Sulfasalazine
Estrogens	Sulfonamides
Glutethimide	Sulfonylureas
Griseofulvin	Valproic acid

*A complete list of unsafe and safe drugs is available at two websites: (1) www.porphyriafoundation.com and (2) http://www.porphyria-europe.com.

Laboratory Diagnosis. The signs and symptoms of an acute porphyric attack are quite nonspecific, and the correct diagnosis requires both consideration of a neurovisceral porphyria and a highly specific laboratory test. An acute porphyria is often not considered until fruitless, expensive imaging studies (and often unnecessary surgical exploration) are complete.

Increased urinary excretion of PBG is the key diagnostic finding in patients suffering an acute attack of AIP, HCP, or VP. Most methods used to quantify urinary PBG excretion rely on formation of a violet pigment when the sample is reacted with p-dimethyl aminobenzaldehyde (Ehrlich's aldehyde). Typical reference ranges for PBG excretion are 0-18 μmol/day (0 to 4 mg/day). Values for ALA range from 0 to 60 μmol/day (0 to 8 mg/day). In contrast, urinary PBG excretion during an acute attack generally range from 220 to 880 μmol/day, and ALA excretion ranges from 100 to 450 μmol/day.[17] Recently liquid chromatography-tandem mass spectroscopy methods have been developed that are rapid, specific, highly sensitive, and simultaneously measure ALA and PBG in urine and plasma.[117]

A simplified method for measuring PBG at the bedside or in the emergency department employs a small anion exchange column packed in a kit that includes all of the required reagents and a color chart. The trace PBG kit (Thermo Trace/DMA, Arlington, TX) can detect PBG at concentrations greater than 6 mg/L,[118] and results correlated well with standard quantitative assays.[119] The kit does not measure ALA.

Urinary porphyrin excretion (mainly uroporphyrin) is increased during acute attacks of AIP, mainly from nonenzymatic polymerization of PBG, but this is a much less specific finding than increased excretion of PBG and ALA. Fecal porphyrin levels are generally normal, but plasma levels may be increased. In contrast, increased urinary porphyrin excretion during an acute attack of HCP and VP consists mainly of coproporphyrin, and stool porphyrin excretion is also increased. Markedly increased stool coproporphyrin and protoporphyrin are found in VP. Fluorescence scanning of diluted plasma at neutral pH readily distinguishes VP from the other neurovisceral porphyrias, even during asymptomatic periods.[120]

A word of caution is required in interpreting urinary porphyrin values. Minimal elevations of urinary coproporphyrin may be found in patients with liver disease, marrow disorders, and a host of other illnesses. An incorrect diagnosis of porphyria is commonly made by overinterpreting minimal elevations of urinary coproporphyrin, leading to a delay in establishing the true cause of abdominal pain and other neurovisceral symptoms.

Assays of PBG-D activity in erythrocyte lysates have been widely employed to confirm the diagnosis of AIP and to detect asymptomatic family members once an index case has been identified. Erythrocyte PBG-D activity is approximately half-normal in most patients with PCT. Normal PBG-D activity, however, does not exclude a diagnosis of AIP because of differential splicing of the PBG-D transcript in the liver and the red cell. Mutations in exon 1 of the PBG-D gene affect activity of the enzyme in the liver, but the erythrocyte enzyme is encoded only by exons 2 through 15.[74] It is important to emphasize that a valid half-normal measurement of erythrocyte PBG-D activity does not distinguish between asymptomatic (latent) and clinically manifest cases of AIP. Only urinary PBG levels can make this distinction.

PBG-D mutation detection by DNA analysis has proven to be more reliable than PBG-D enzyme assays and is now the preferred method for confirming a diagnosis of AIP and for identifying asymptomatic carriers. DNA studies have demonstrated that enzymatic assays may misclassify up to 15% of individuals tested.[121,122] Mutation detection for AIP patients and their relatives is now readily available through the Department of Human Genetics at the Mount Sinai School of Medicine, New York, N.Y.

Treatment. Treatment of an acute attack of AIP (and the other neurovisceral porphyrias) is designed to repress the activity of hepatic ALA-S1 and to offer supportive care and relief of symptoms. Intravenous infusions of heme result in a rapid reduction in levels of ALA and PBG in plasma and urine.[17] Heme binds to plasma hemopexin and albumin and is then taken up by hepatocytes, where it expands the heme pool and represses ALA-S1. Many small, uncontrolled trials have demonstrated both biochemical and clinical responses to infusion of heme.[17] In one study involving 22 patients suffering 57 acute attacks, all patients responded favorably, but therapy was most beneficial when administered early in the attack,[123] a finding confirmed by others.[124,125] A single, placebo-controlled trial found dramatic biochemical benefits of heme infusion, but clinical benefits were more difficult to confirm.[126]

Heme is not soluble at neutral pH, but solubilized hemin (heme-chloride) and heme arginate have been administered to many patients. In the United States a lyophilized preparation of hemin, (Panhematin, Recordati Rare Diseases, Lebanon, NJ) has been approved by the U.S. Food and Drug Administration for the treatment of acute porphyric attacks. Hemin is unstable when reconstituted with sterile water, and degradation may cause an anticoagulant effect and phlebitis at the site of infusion.[127] Phlebitis can be avoided by the use of intravenous catheters that extend to larger veins (e.g., the superior vena cava). Instability and the locally irritating effects of lyophilized hemin can be attenuated by solubilizing hemin in 25% albumin, a method now recommended.[17] The recommended regimen for heme therapy is 3 to 4 mg/kg infused once daily on 4 consecutive days. Clinical improvement generally occurs within 1 or 2 days, but when neuronal damage is severe, complete recovery may take months. The keys to success are to initiate therapy as soon as an attack is recognized and to complete a 4-day course. Rare side effects of therapy include constitutional symptoms such as fever, myalgias, and malaise, but one case of marked hypotension[128] and another of transient renal failure[129] have been reported. The latter case was associated with an excessive dose (1000 mg) of hemin.

Heme arginate (Normosang, Orphan Pharmaceuticals, Paris, France) is more stable in solution than hemin, is equally effective, and is widely available in Europe and South Africa but remains an experimental drug in the United States. Phlebitis is a rare complication with heme arginate,[130] and the shelf life is considerably longer than that of hemin. Accidental overdosing with heme arginate has been associated with acute hepatic failure.[131] Adenoviral-mediated hepatic expression of PBG-D corrects the metabolic defect in mouse models of AIP,[132,133] but no studies have been done in humans. Intravenous infusions of recombinant human PBG-D have been administered to normal subjects and asymptomatic PBG-D mutation carriers.[134] Transient decreases in plasma concentrations of PBG were noted, and no serious side effects occurred. No trials of recombinant PBG-D for the treatment of symptomatic AIP have been reported.

Intravenous infusions of glucose (300 to 500 g/day, administered as 10% glucose) may be effective when an attack is mild or until hemin is obtained,[17] but glucose is far less effective than hemin. Infusions of glucose in water solutions are contraindicated because they aggravate hyponatremia, which can prove fatal.[135] The mechanism of the glucose effect relates to the previously described finding that transcription of ALA-S1 is upregulated by the peroxisome PGC-1α and transcription of PGC-1α is controlled by glucose availability.[13] Insulin may downregulate PGC-1α,[13] and it has been suggested that the combination of glucose and insulin may be more effective than glucose alone.[18]

Acute attacks require treatment of symptoms in addition to therapy directed at ALA-S1. Pain is often severe, and full doses of morphine or other opioids are both safe and effective. Phenothiazines in modest doses are safe and are useful for the control of nausea and anxiety.[17] Hypertension and tachycardia may respond to β-adrenergic blocking agents,[136] but these agents should be used with caution when patients are hypovolemic because increased catecholamine secretion is often an important compensatory mechanism. Seizures during an acute attack can be a particularly vexing problem because most antiseizure drugs have the potential to worsen the intensity of the attack.[137] Clonazepam and gabapentin may be safe,[138,139] and benzodiazepines are probably safe.[17] Hyponatremia and hypomagnesemia should be corrected because these abnormalities are common and may be causative for seizures.

The long-term management of patients with acute neurovisceral porphyrias is focused on the prevention of future attacks. Avoidance of unsafe drugs (see Table 12-3) is critical as is maintenance of a balanced, calorically adequate diet. In some women acute attacks occur only in the luteal phase of the menstrual cycle. In these cases gonadotropin-releasing hormone (GnRH) analogues can be highly effective.[140,141,142] Prolonged use of GnRH analogues in young women may cause irreversible bone loss, but low-dose transdermal estradiol may safely prevent this complication.[143,144] Both cyclic and noncyclic attacks may be prevented by frequent infusions of hemin,[17] but the expense and the potential for iron overload (8 mg of iron per 100 mg of hemin) makes this an unattractive option.

Levels of progesterone, an inducer of hepatic heme synthesis, are elevated in pregnancy, but pregnancy is well tolerated by most women with acute porphyrias.[106,145] In a large series reported from Finland of 176 pregnancies in women with AIP or VP, porphyric symptoms occurred in fewer than 10%.[106] Worsening symptoms during pregnancy may be due to harmful drugs,[146,147] inadequate nutrition, or both.[17]

Uroporphyrinogen III Synthase

The product of the PBG-D reaction, HMB, serves as the substrate for uroporphyrinogen III synthase (URO3-S). URO3-S catalyzes the asymmetrical cyclization of HMB to the III isomer of uroporphyrinogen (uro'gen III). The reaction involves "flipping" of the D ring to yield the asymmetric product (Fig. 12-8). Rearrangement of the D ring occurs by a complex mechanism involving cleavage and formation of carbon–carbon bonds.[148] Spontaneous cyclization of HMB can also occur, but the product, uroporphyrinogen I (uro'gen I), cannot ultimately be converted to heme. URO3-S functions as a monomer. Although the primary sequence is highly divergent, the crystal structures of human URO3-S and a bacterial URO3-S are nearly identical.[149,150] The structure of URO3-S from the bacterium *Thermus thermophilus* has been determined with the uro'gen III product bound.[150] The structure suggests that the enzyme prevents formation of uro'gen I by positioning the A and D rings to favor formation of uro'gen III.

The human URO3-S gene is localized to chromosome 10q25.2-26.3[151] (see Table 12-1) and contains 10 coding exons.[152] The translational start site is located in exon 2B (see Fig. 12-5). Both housekeeping and erythroid-specific transcripts are produced. The housekeeping promoter lies upstream of exon 1.[153] The housekeeping transcript results from a splicing event that joins exon 1 and 2B. The erythroid-specific promoter lies within intron 1 and contains eight GATA1 binding sites. The erythroid-specific transcript contains all of exon 2. Although the housekeeping and erythroid transcripts are expressed from different promoters, they produce identical proteins.

Congenital Erythropoietic Porphyria

Congenital erythropoietic porphyria (CEP) (Günther disease) is a rare form of porphyria in humans due to mutations of URO3-S. About 200 cases have been reported.[154] The disorder is of historical interest as it was the first porphyria to be recognized as an inherited trait and the first to be characterized biochemically.[155] CEP also occurs in squirrels, cows, and other species.[156] Over 40 URO3-S mutations have been reported, including exonic and promoter point mutations, deletions, insertions, and splice-site mutations.[157]

Frequency. The rarity of CEP makes frequency estimates difficult, but the disorder has been described in multiple ethnic groups.[158] The frequency of heterozygosity for URO3-S mutations is not known because heterozygotes have no phenotype, and population-based sequencing screens have not been done. Although rare, CEP is recognized more frequently than ALA-D porphyria, perhaps because of the dramatic cutaneous phenotype.

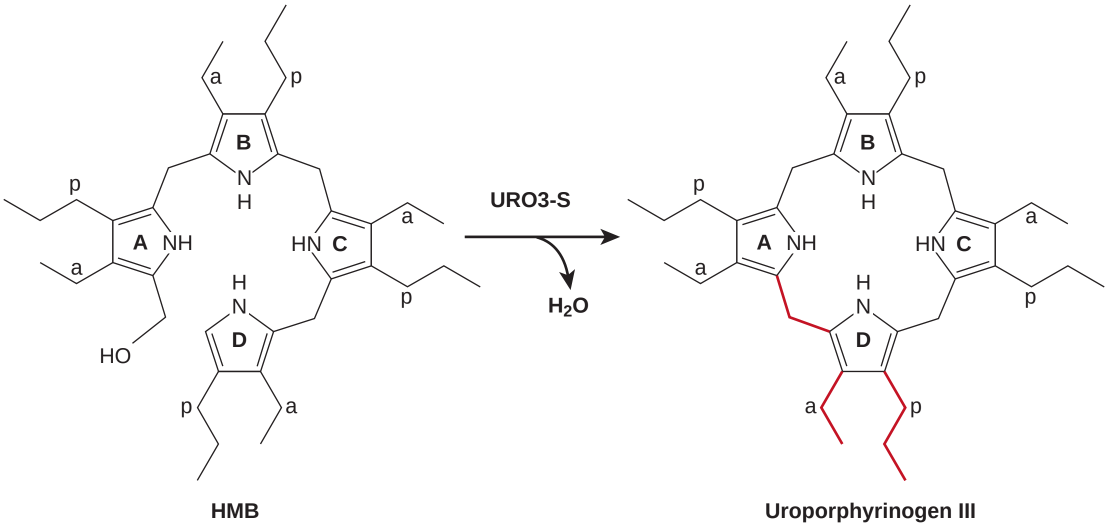


Figure 12-8 Reaction catalyzed by uroporphyrinogen III synthase (URO3-S). URO3-S enzymatically closes the tetrapyrrole ring with concomitant "flipping" of the D ring. The acetate *(a)* and propionate *(p)* side chains are labeled. In the uroporphyrinogen III product, the acetate and propionate groups of the D ring are reversed.

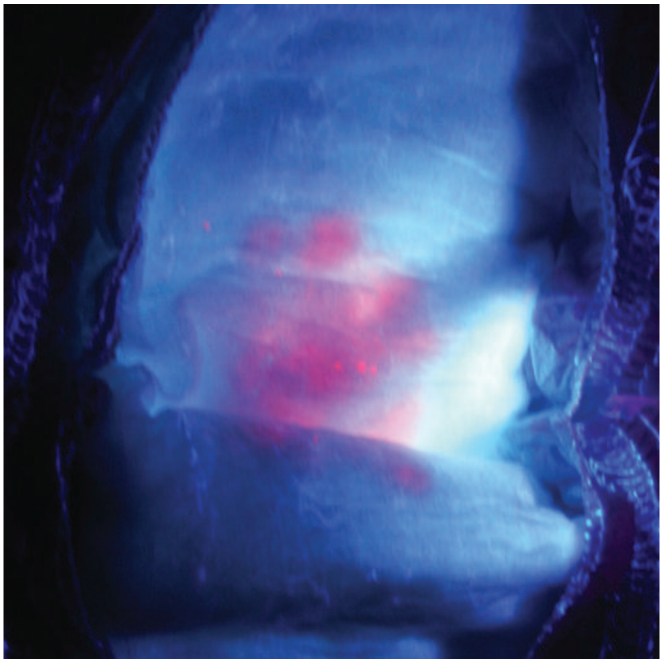


Figure 12-9 The diaper from a child with congenital erythropoietic porphyria. Urine porphyrins, primarily uroporphyrin I, fluoresce with a brilliant pink color when illuminated with a Wood's light.

Clinical Manifestations. Cutaneous photosensitivity is the most dramatic feature of CEP and is usually recognized in early infancy. The diagnosis may be suspected because of the dark brown color of urine soaked diapers that demonstrate a distinctive, intense red-pink fluorescence when illuminated with long-range ultraviolet light (e.g., a Wood's light) (Fig. 12-9).[154] The severity of the skin lesions varies widely and is related to the amount of residual URO3-S activity,[159] which in turn determines the magnitude of porphyrin accumulation and excretion. In most cases photosensitivity is severe and is characterized by skin fragility, bullous lesions, ulcerations, and secondary infections.[158] If exposure to sunlight is not rigorously avoided, mutilation of the face and hands is the ultimate outcome (Fig. 12-10). A common finding in CEP and the other porphyric disorders associated with photosensitivity is the presumably protective response of extensive hypertrichosis.[154,158] Photoinduced damage of nails and ocular structures may also occur.[158] A unique finding in children with CEP is red-brown discoloration of the teeth, due to deposition of porphyrins into dentine and enamel during tooth development.[160] The teeth fluoresce when illuminated with a Wood's light.

Hemolytic anemia of varying severity is found in nearly all cases, but detailed morphologic findings in the blood and bone marrow have been reported infrequently. Ineffective erythropoiesis may also be present. Poikilocytosis, nucleated red cells, and basophilic stippling are common, and the marrow demonstrates erythroid hyperplasia and may manifest marked dyserythropoiesis.[161-163] Fluorescence microscopy may demonstrate fluorocytes in the peripheral blood, but this is more dramatic in the bone marrow.[156,161,164] Splenomegaly generally accompanies the hemolytic anemia, and the magnitude of splenic enlargement reflects the severity of hemolysis. Transfusion dependence may develop.[154] Leukopenia and symptomatic thrombocytopenia may occur as a result of splenic sequestration.[165]

A variety of osteolytic skeletal lesions have been described when the disease reaches the stage of mutilation.[158] Vitamin D deficiency as a result of sun avoidance

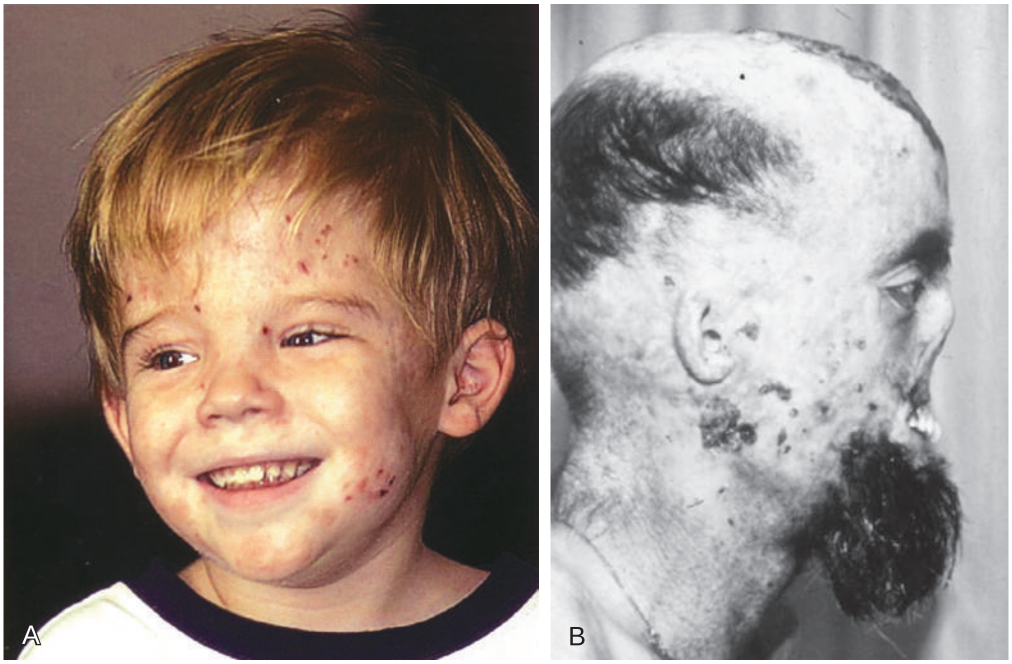


Figure 12-10 Skin changes in congenital erythropoietic porphyria (CEP). **A,** A 3-year-old boy with early facial photodermatitis. **B,** A severe case of CEP in an adult. Sun exposure has lead to blistering, erosions, secondary infections, and subsequent photomutilation.

may play a role in the development of osteolysis.[166] At postmortem examinations the skeleton is fluorescent under long-range ultraviolet light.[167]

Pathogenesis. Loss-of-function mutations of URO3-S lead to the accumulation of the HMB substrate in erythrocyte precursors, which is readily converted nonenzymatically to uro'gen I. Residual activity of URO3-S is generally less than 10% of normal. Uro'gen I may be auto-oxidized to uroporphyrin I or may serve as a substrate for uroporphyrinogen decarboxylase (URO-D) (Fig. 12-2), ultimately generating coproporphyrinogen I (copro'gen I). This results in a metabolic "dead end" as the next enzyme in the pathway, CPO, is stereospecific and will only accept coproporphyrinogen III (copro'gen III) as a substrate. Uroporphyrin I and to a lesser extent coproporphyrin I accumulate in erythroid precursors. Erythrocyte fragility and hemolysis result by an undefined mechanism, liberating porphyrins into the plasma. Uroporphyrin I mediates the cutaneous photosensitivity[168] and is excreted in the urine. Coproporphyrin I is excreted in the stool and to a lesser extent in the urine.

Two phenotypic copies of CEP have been reported. In one, a partial defect in URO-D combined with a congenital dyserythropoietic anemia produced mutilating photosensitivity and massive uroporphyrinuria.[169] The pattern of urinary porphyrin excretion differed from CEP as large amounts of heptacarboxyl porphyrin were found in addition to even larger amounts of uroporphyrin. In the other, a mutation in the erythroid specific transcription factor GATA1 resulted in subnormal URO3-S in red cells and a phenotype of CEP, thalassemia intermedia, and thrombocytopenia.[170]

The clinical manifestations of CEP vary greatly, but a unique case has been reported in which a gain-of-function mutation of ALA-S2 coexisted with compound heterozygosity for loss-of-function mutations of URO3-S.[171] The result was a severe CEP phenotype compared to other family members with the same URO3-S genotype.

Late Onset CEP. CEP may become evident in adult life, and clinical symptoms are generally less severe than in the more typical early-onset cases. In most of the adult-onset cases, moderate URO3-S defects became evident only when an acquired clonal marrow disorder developed.[172,173]

Laboratory Diagnosis. Urinary porphyrin excretion is massively increased in CEP, in some cases up to 1000 times normal. The pattern of porphyrin excretion, determined by high-performance liquid chromatography is characteristic. The dominant urine porphyrin is uroporphyrin I with smaller amounts of coproporphyrin I.[174,175] Assays of URO3-S have been performed in erythrocytes and cultured cells, but the assays are not widely available.[176,177] The most practical way to confirm the diagnosis of CEP is through direct sequencing of the gene, a procedure available through the Department of Human Genetics at the Mount Sinai School of Medicine in New York, N.Y. URO3-S mutations can be detected in chorionic villi or cultured amniotic cells.[178] This may be useful in subsequent pregnancies when parents have had a child with CEP.

Treatment. The only curative therapy for CEP is an allogeneic marrow transplant. Sixteen patients with CEP who have undergone allogeneic transplants have been reported.[179] The published experience suggests that early transplant is preferred, particularly when hematologic manifestations are prominent, but this approach is not universally applicable because of potential donor availability, treatment-related morbidity, and financial constraints. Allogeneic transplants should probably be restricted to the more severe cases of CEP because conservative management can yield longevity of 40 to 60 years.

The morbidity associated with marrow allografts could be avoided by autologous hematopoietic stem cell transplant if stem cells could be stably transfected with a normal URO3-S cDNA. This has been accomplished in a murine model of CEP using a lentivirus vector[180] and in isolated keratinocytes from a CEP-affected individual.[181] In the latter case, the lentiviral vector was excised, yielding induced pluripotent stem cells free of viral reprogramming genes. Complete correction of the phenotype was achieved in both the murine model and in the human pluripotent stem cells. No clinical trials in humans have yet been reported.

Avoidance of sunlight is at the core of conservative management.[179] This can be achieved with protective clothing and effective sunscreens. Conventional sunscreens containing para-aminobenzoic acid are ineffective and opaque "pancake" makeup is preferred. Splenectomy may reduce the severity of hemolysis and correct leukopenia and thrombocytopenia, but beneficial effects on photosensitivity are often transient.[182] Erythropoiesis and endogenous porphyrin production can be suppressed with aggressive transfusion regimens,[183] but transfusion-induced iron overload results.[184]

MODIFICATION OF THE TETRAPYRROLE SIDECHAINS

Uroporphyrinogen Decarboxylase

Uro'gen III is converted to copro'gen III by the sequential removal of the carboxyl groups of the four acetate side chains yielding four methyl groups. This reaction is catalyzed by the cytosolic enzyme URO-D (Fig. 12-11). Decarboxylation of uro'gen III begins at the asymmetric D ring and proceeds in a clockwise manner.[185] Uro'gen III is the preferred substrate. The enzyme will accept uro'gen I as a substrate, but the product, copro'gen I, cannot be converted to heme.

Human URO-D is encoded by a single gene located on chromosome 1p34 (see Table 12-1).[186] Unlike ALA-D, PBG-D, and URO3-S, there is no erythroid specific promoter in the URO-D gene.[187] The URO-D mRNA is highly expressed in developing erythrocytes, but the mechanism responsible for erythroid-specific upregulation is not known.[188]

URO-D has been purified from human erythrocytes,[189,190] bovine liver,[191] and from yeast and bacteria.[192]

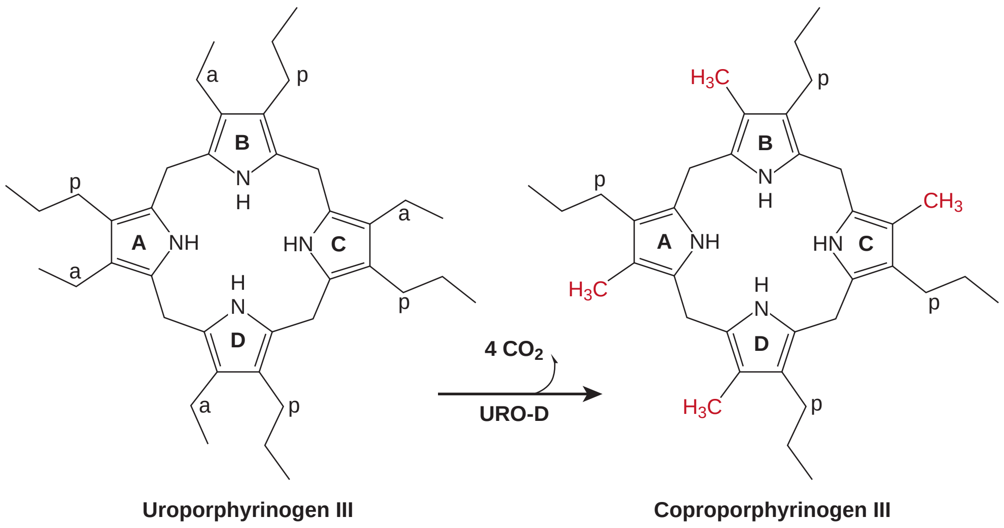


Figure 12-11 Reaction catalyzed by uroporphyrinogen decarboxylase (URO-D). Uroporphyrinogen undergoes four successive decarboxylations, starting at the asymmetric D ring. The acetates side chains *(a)* are decarboxylated to form methyl groups. URO-D will decarboxylate both the I and III isomers of uroporphyrinogen, but coproporphyrinogen I is not a substrate for coproporphyrinogen oxidase.

Unlike most decarboxylases, URO-D does not require cofactors or prosthetic groups for activity. The crystal structure of human URO-D has been determined, revealing that URO-D functions as a homodimer.[193,194] Each monomer contains an active site cleft. The active site can only accommodate the flexible porphyrinogen macrocycle (see Fig. 12-12).[195] Oxidation of the bridge carbons connecting the pyrrole rings renders the molecule rigid and planar and may explain why uroporphyrin is not a substrate for URO-D.

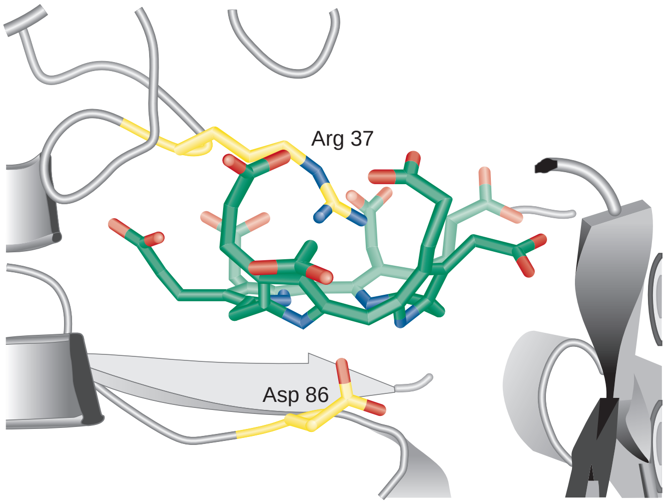


Figure 12-12 Crystal structure of uroporphyrinogen decarboxylase with uroporphyrinogen III (uro'gen III) bound in the active site. Uro'gen III *(green)* is shown in the active site. The fully reduced bridge carbons permit uroporphyrinogen to adopt the domed configuration positioning the 4-pyrrole nitrogens for hydrogen bonding with aspartic acid 86 *(Asp 86)*. The positive charge of arginine 37 *(Arg 37)* interacts with the negatively charged propionate groups of uroporphyrinogen to correctly position the substrate in the active site. *(Adapted from the coordinates 1R3Y, available from the Protein Data Bank.)*

Porphyria Cutanea Tarda

PCT is due to subnormal activity of URO-D in the liver. The disorder is characterized clinically by a photosensitive dermatosis that is usually less severe than in CEP. Expression of the clinical phenotype is associated with one or more risk factors: iron overload, often due to homozygosity for the C282Y mutation of the hemochromatosis gene (HFE); hepatitis C; alcohol abuse; and the use of medicinal estrogens.[196-198] The frequency of these risk factors is far higher than observed incidence of PCT, suggesting that susceptibility factors, either genetic or environmental, yet to be identified are important determinants of disease expression.[196,199]

Two major variants have been identified. The first, designated familial PCT (F-PCT), is associated with heterozygosity for mutations at the URO-D locus. F-PCT is transmitted as an autosomal dominant trait. Affected individuals display approximately half-normal URO-D activity and half-normal URO-D protein in all tissues.[200,201] There is considerable variation in URO-D activity because different mutations have varied effects on enzyme activity.[202] F-PCT accounts for approximately 25% of the clinically observed cases.[196,203] Most carriers of a mutant URO-D allele do not express a porphyric phenotype unless one or more of the risk factors mentioned above are also present.[196] Clinical expression of F-PCT in childhood is rare.[204-209] The authors have evaluated three children with F-PCT, two of whom were brothers, who were also homozygous for the C282Y HFE mutation.

The second major variant, and the most prevalent form of PCT, is designated sporadic PCT (S-PCT) and accounts for approximately 70% of cases.[196] In S-PCT, reduced activity of URO-D is restricted to the liver. No URO-D mutations have been identified in patients with S-PCT, and there is no evidence of a liver-specific URO-D transcript.[210] Nevertheless, S-PCT may cluster in families suggesting the presence of predisposing genetic factors.[211]

The concentration of URO-D protein is normal in all cells, including the hepatocytes, yet when the disease phenotype is expressed, enzyme activity is markedly reduced in the liver. Normal URO-D protein concentration coupled with subnormal URO-D enzymatic activity strongly suggests the presence of an enzyme inhibitor.[212-214] Risk factors identified when S-PCT is clinically evident are identical to those associated with F-PCT.[196,215]

A third rare variant of PCT occurs in children who have inherited two mutant alleles of URO-D.[215-219] This variant has been designated hepatoerythropoietic porphyria (HEP) because porphyrins accumulate in both the liver and the red cells. Severely reduced levels of URO-D resulting from the genetic defect become rate limiting. Activity in red cell lysates is generally less than 10% of normal[220] but may be as high as 20% in cases with a mild phenotype.[218]

A fourth variant of PCT, designated toxic PCT, has been associated with exposure to halogenated aromatic hydrocarbons. An epidemic of toxic PCT occurred in Turkey between 1956 and 1961 following ingestion of wheat treated with the fungicide hexachlorobenzene.[164,221] Many of the Turkish cases were children. Later studies in animals established that the halogenated aromatic hydrocarbons diminished hepatic URO-D activity.[222-226] PCT in humans has also been reported following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)[227,228] and dichlorophenols and trichlorophenols.[229]

Frequency. PCT is the most common of the porphyric disorders, but the incidence varies in different populations. In Caucasian populations of northern European origin, the incidence may be as high as 2 to 5 per 20,000, likely reflecting the high incidence of hemochromatosis gene mutations in this group.[196] In contrast, PCT is uncommon in African Americans but does occur in the Bantus of South Africa, who develop iron overload from beer brewed in iron pots.[230] PCT occurred commonly in patients undergoing chronic hemodialysis,[231-233] but substitution of erythropoietin for red cell transfusions (hence avoiding transfusion-induced iron overload) has reduced the incidence.

The high frequency of PCT, with one quarter of cases associated with URO-D mutations, predicts that HEP should not be rare. Instead fewer than 40 cases have been reported.[218] This is best explained by embryonic lethality of extremely low residual activity of mutant URO-D enzymes or the lethal effect of null alleles.

Clinical Manifestations. Skin fragility on the dorsal aspects of the hands is often the initial finding, but blistering lesions (bullae) then appear on all sun-exposed areas. The fluid-filled bullae rupture, and the denuded area heals slowly, often forming thick eschars and eventually hyperpigmented patches (Fig. 12-13). Facial hypertrichosis is common, particularly in children and women. Facial hypertrichosis may be localized to periorbital regions (Fig. 12-14*A*) or can be manifest in a lanugo-like diffuse facial pattern with or without pigmentation (Fig. 12-14*B*). Some patients develop scleroderma-like skin changes, mainly in light-exposed areas.[234,235] When PCT becomes

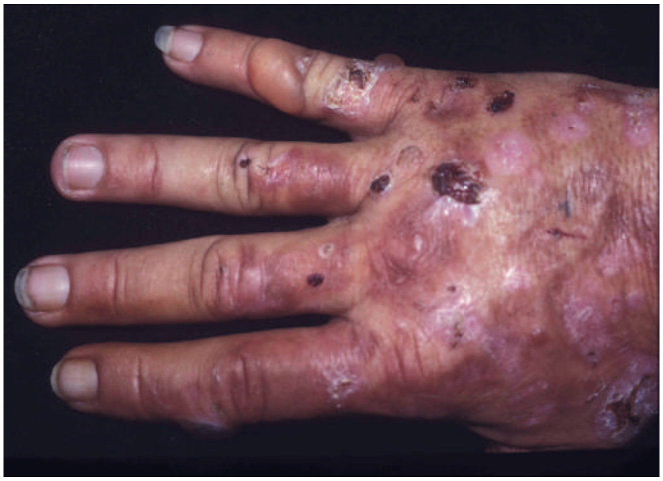


Figure 12-13 Hand of a patient with porphyria cutanea tarda. Bullous lesions form (note large blister on fifth digit) and rupture, leaving ulcerations that heal slowly. The resultant scars are both hypopigmented and hyperpigmented.

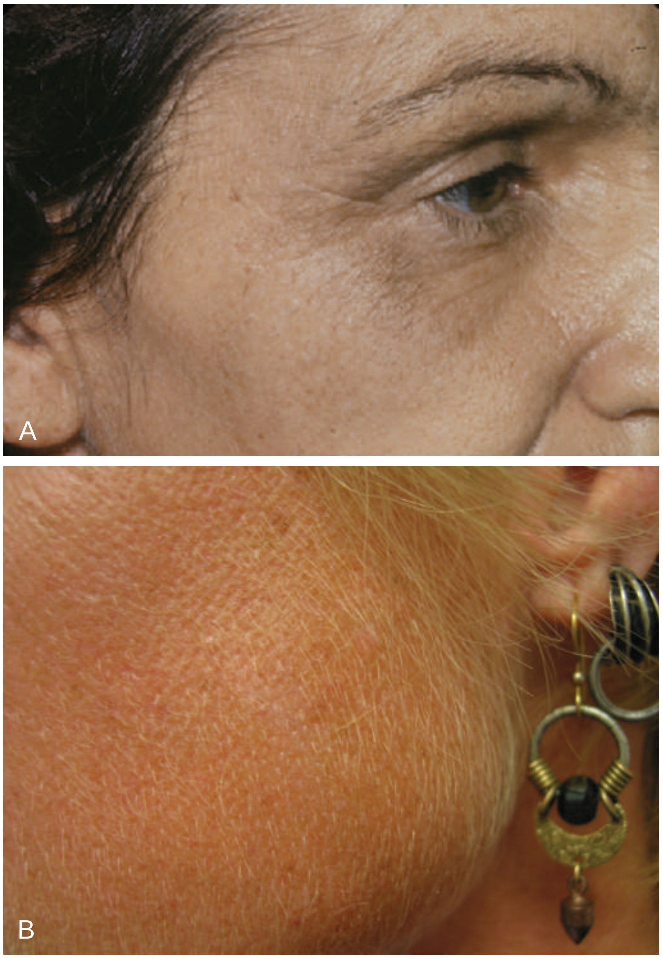


Figure 12-14 Facial hypertrichosis with porphyria cutanea tarda (PCT). **A,** Characteristic periorbital facial hair in a 60-year-old woman in whom PCT developed while receiving postmenopausal oral estrogen replacement. **B,** Diffuse lanugo-like facial hair in a 26-year-old woman in whom PCT developed while using an oral contraceptive.

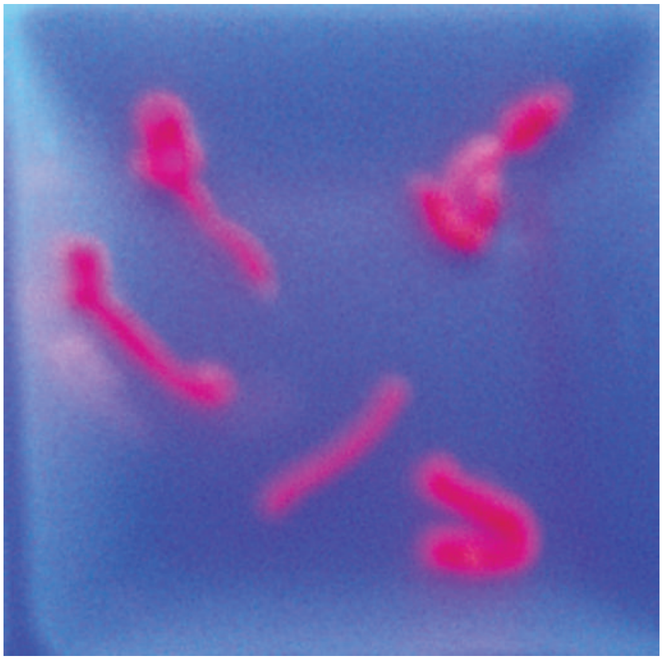


Figure 12-15 Percutaneous liver biopsy specimens from five patients with porphyria cutanea tarda. The massive amount of porphyrins in hepatocytes results in striking fluorescence under illumination with a Wood's light.

chronic, milia (small, cystlike collections of keratin) are common on the hands.[236] Similar photocutaneous findings have been seen in patients with pseudoporphyria, a disorder in which plasma and urine porphyrins are normal. The skin findings are likely due to other photosensitizing compounds. Nonsteroidal antiinflammatory drugs have been implicated in most cases.[237]

The cutaneous manifestations of HEP can be very dramatic and may resemble those seen in CEP.[238] Recurrent blisters and erosions complicated by infection can lead to severe scarring, scleroderma-like changes, and photomutilation.[85,220] In other cases the skin is less severely damaged and appears more like mild PCT.[239,240] Hemolytic anemia and splenomegaly may be present, findings not associated with PCT.[220]

Evidence of abnormal liver function is nearly always present in patients with PCT, even in those without hepatitis C. In some reports, approximately 80% of males with PCT have been infected with hepatitis C[241,242] but the association of hepatitis C with PCT is lower in northern Europe.[241,243] The high concentration of porphyrins in the liver results in brilliant fluorescence of liver biopsy samples illuminated with a Wood's light (Fig. 12-15). Microscopic examination reveals needle-shaped, fluorescent porphyrin crystals in hepatocytes.[244] Other histologic findings are not specific,[245] but increased hepatocellular iron stores are a nearly constant finding.[246-248]

Pathogenesis. Over 100 URO-D mutations have been identified in patients with PCT.[78] The functional consequences of many of these mutations have been studied,[249] but heterozygosity for mutant URO-D alleles alone cannot explain the phenotype, as half-normal URO-D activity is not rate-limiting. Immunoreactive URO-D in the liver of patients with clinically evident F-PCT is half-normal, yet enzyme activity is 25% of normal or less.[250] No URO-D mutations are found in patients with S-PCT and immunoreactive URO-D in all tissues is normal. When S-PCT becomes clinically evident, immunoreactive URO-D in the liver remains normal, yet enzyme activity is reduced to approximately 25% of normal, the same level of activity found in F-PCT.[251,252] These findings indicate that the specific activity of hepatic URO-D is affected. Furthermore, catalytic activity of URO-D returns to normal after phlebotomy-induced iron depletion,[250] suggesting that an iron-dependent process is responsible for the reduction in the specific activity of hepatic URO-D. Similar findings, a reduction in URO-D activity without a change in URO-D protein, have been noted in several animal models of PCT.[253-257] Collectively, these data suggest a common mechanism in the pathogenesis of PCT, namely generation of an inhibitor of URO-D by an iron-dependent mechanism.

The inhibitor of URO-D in humans with PCT and in animal models of PCT has been identified as uroporphomethene.[258] Uroporphomethene differs from uroporphyrinogen due to oxidation of a single bridge carbon between adjacent pyrrole rings. Uroporphomethene is sufficiently flexible to adapt to the spatial requirements of the active site of URO-D, but the variance from the structure of uroporphyrinogen prevents the inhibitor from serving as a substrate.

Generation of the uroporphomethene inhibitor likely requires liver-specific isozymes of cytochrome P450,[259] but the mechanism of the iron effect remains unidentified. Iron clearly plays a central role because iron depletion through phlebotomy therapy reverses the clinical and biochemical phenotype, even when liver iron content is not clearly elevated.[260] Replacement of iron removed through phlebotomy therapy produces a prompt relapse.[261] Iron depletion prevents or dramatically attenuates the porphyric phenotype in animal models of PCT, and iron supplementation accelerates development of the phenotype.[226,262] Hepcidin, a peptide that regulates systemic iron homeostasis, has been studied in patients with conflicting results. In one study, hepatic expression of hepcidin mRNA was significantly reduced,[263] but in another study, serum hepcidin levels were increased.[264] In spite of these data, very few patients with hemochromatosis or other disorders associated with iron overload develop PCT. Hepatic iron loading, therefore, is not sufficient to produce the PCT phenotype. Other factors, both genetic and environmental, appear to be required to create susceptibility to PCT.[199]

The cutaneous findings in PCT, as in other porphyrias associated with photosensitivity, result from interaction of light with a wavelength of approximately 400 nm (Soret band) with porphyrins that accumulate in the skin.[265] The photoexcited porphyrin returns to a stable state by emitting energy as a photon that disrupts membranes and intracellular organelles.[266] The photodynamic reaction activates inflammatory mediators, which damage the epidermal basement membrane, causing fragility of the skin and blistering.[267] Another prominent site of photodamage is at the vascular level of the skin, where thickening of superficial dermal vessel walls is a constant finding.[268,269]

Laboratory Diagnosis. Laboratory testing is essential for confirmation of the clinical diagnosis of PCT and HEP. The appearance of the skin and skin biopsy findings resemble those of other photosensitizing porphyrias and pseudoporphyria. A useful initial approach to establishing that a porphyric disorder is present is to measure the total porphyrin content of plasma.[270] A normal value excludes the diagnosis of PCT and HEP. If the plasma porphyrin level is high, additional testing is required to distinguish PCT and HEP from HCP and VP. Chromatographic separation and quantification of urinary and stool porphyrins serves this purpose. In PCT and HEP, greatly increased amounts of uroporphyrin and heptacarboxyl porphyrin are excreted in the urine (Fig. 12-16). Urinary excretion of coproporphyrin may be slightly increased.[247,271] Values for daily urine porphyrin excretion average 3000 μg (normal is less than 50 μg) and may be considerably higher. Cutaneous symptoms are usually mild or absent when uroporphyrin excretion is less than 1000 μg/day. A distinctive tetracarboxylic porphyrin, isocoproporphyrin, is excreted in feces, but total fecal porphyrin excretion is normal or minimally increased.[272] HEP can be distinguished from PCT by the presence of anemia and an increase in erythrocyte zinc protoporphyrin.[273]

Erythrocyte URO-D assays can be useful but are not widely available. Furthermore, distinguishing between F-PCT and S-PCT is not critical for patient management. Very low activity of URO-D in erythrocyte lysates can distinguish HEP from F-PCT and would affect therapeutic choices, but directly determining URO-D mutation status is more precise and facilitates genetic counseling.

In patients with PCT, additional laboratory testing is indicated to determine body iron burden (serum iron, total iron binding capacity, and serum ferritin), liver functional status, hemochromatosis genotype, and the presence or absence of antibodies to hepatitis C and human immunodeficiency virus.

Treatment. Two effective forms of therapy have been widely used in patients with PCT, phlebotomy and low-dose chloroquine. Phlebotomy treatment is preferred in patients with marked increases in hepatic iron stores, determined by liver biopsy and serum ferritin values. Removal of 500 mL of blood every 2 weeks for 3 to 4 months will usually suffice,[247,274] but additional treatment is required for patients with hemochromatosis. The objective is to produce iron depletion, not iron deficiency. A useful target is a serum ferritin value of approximately 50 μg/L. Skin lesions clear when urine porphyrin excretion falls below 1000 μg/day, but it may take a year or more for the urine to become completely normal. Relapses are infrequent, especially if risk factors (alcohol and oral estrogens) are avoided. Transdermal estrogen

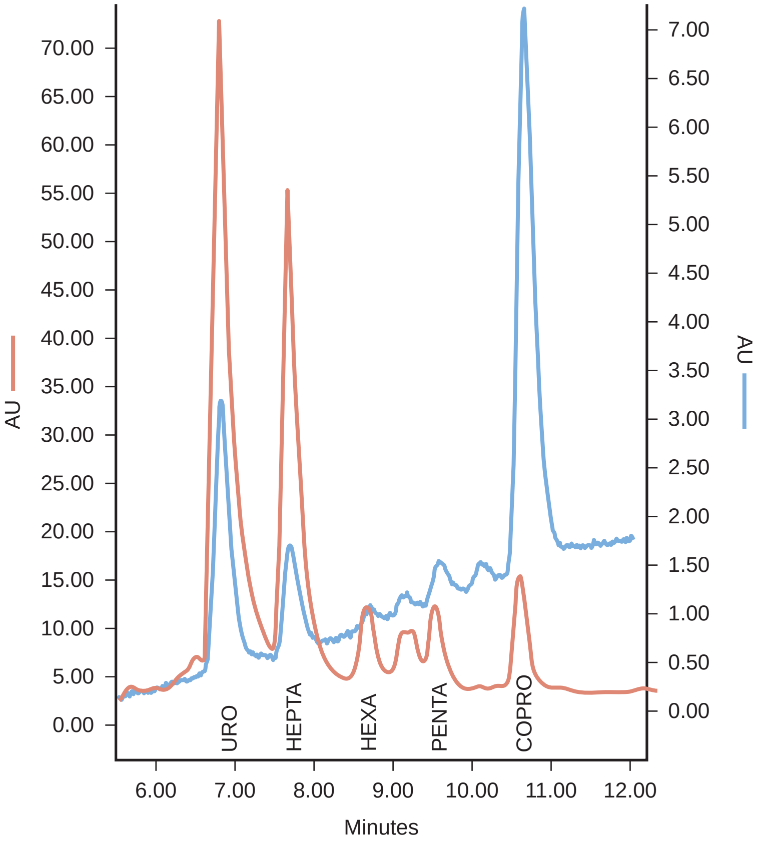


Figure 12-16 Urine porphyrin profile in porphyria cutanea tarda (PCT). Urine porphyrins were separated by high-performance liquid chromatography and quantified fluorometrically. The tracing from a normal person is shown in blue with the scale on the right (0 to 7 absorbance units). The profile of urine porphyrins from a patient with PCT is shown in red with the scale on the left (0 to 70 absorbance units). Uroporphyrin (URO) and 7-carboxyl porphyrin (HEPTA) predominate in the PCT sample, whereas coproporphyrin (COPRO) is the main porphyrin present in normal urine. The intermediate porphyrins with 6- and 5-carboxyl groups (HEXA and PENTA) are also increased in the PCT sample.

administration following phlebotomy-induced remission of PCT in women, however, appears to be safe.[275]

Low-dose chloroquine therapy is an effective alternative treatment and at some centers is preferred for patients with modest iron overload.[276,277] The time required to produce clinical remission is longer than with phlebotomy treatment, but ultimately the results are equivalent. Chloroquine in a dose of 125 mg by mouth twice weekly (or hydroxychloroquine in a dose of 100 mg by mouth twice weekly) will gradually reduce plasma and urinary porphyrins.[278-280] Chloroquine concentrates in the liver, where a water-soluble complex with uroporphyrin is formed, promoting urinary excretion.[281] Higher doses of chloroquine should be avoided because marked hepatotoxicity may occur, an effect that is unique to patients with PCT.[282,283] There is some risk of retinopathy with low-dose regimens,[284] and transient, modest elevations of transaminase values may occur.[247] Because of these potential reactions to low-dose chloroquine regimens, many centers prefer phlebotomy therapy unless comorbid conditions render phlebotomy unsuitable.

In contrast to PCT, phlebotomy therapy is not effective for HEP,[220] and responses to chloroquine are infrequent and suboptimal.[285] Treatment for HEP, as with CEP, is focused on symptomatic relief by avoiding sunlight and the use of protective clothing and topically applied opaque sun screens.

Coproporphyrinogen Oxidase

Copro'gen III, the product of the URO-D reaction, is transported from the cytosol across the outer mitochondrial membrane to the intermembrane space, where CPO catalyzes the sequential oxidative decarboxylation of the propionate groups of pyrrole rings A and B of copro'gen III to form proto'gen IX (Fig. 12-17). CPO in humans, all eukaryotes, and some prokaryotes is an oxygen-dependent enzyme.[286] CPO utilizes two molecules of O_2 and releases two molecules of CO_2 and H_2O.[287,288] It has been suggested that oxygen reacts directly with the targeted pyrrole ring, yielding a hydroperoxide which acts as the electron acceptor.[289] The nuclear-encoded CPO protein possesses an unusually long leader sequence that is required for mitochondrial localization.[10]

The crystal structures of both human and yeast oxygen-dependent CPO have been determined.[290,291] The enzyme functions as a homodimer.[286] The structure of CPO allows the enzyme to maintain the substrate as the reduced porphyrinogen yet permits access of oxygen to the active site for the oxidative decarboxylation reaction and the release of H_2O_2.[290,291] The human CPO gene has been cloned and is located on chromosome 3q12.[292] The CPO promoter region encodes an Sp-1-like element, a GATA site and a novel regulatory element that interacts in a synergistic manner in erythroid cells.[293] The GATA site is not required for transcription in nonerythroid tissues, but the novel regulatory element is necessary. There is increased expression of CPO in erythroid cells, a finding consistent with erythroid upregulation mediated by the GATA site.[294]

Hereditary Coproporphyria

Mutations of CPO are the cause of HCP. The disorder is transmitted as an autosomal dominant trait with low penetrance. Affected individuals and nonpenetrant mutation carriers have half-normal activity of CPO. Acute neurovisceral attacks are identical to those that occur in patients with AIP, but photoinduced skin lesions are occasionally present. Presentation before puberty is extremely rare, but several early-onset cases due to homozygosity or compound heterozygosity for CPO mutations have been reported.[295,296] Homozygosity for a specific CPO mutation is responsible for a rare form of homozygous HCP, designated harderoporphyria, with onset in infancy and with a prominent erythroid phenotype.[297] Harderoporphyrin is a tricarboxylic porphyrin in which the propionate on the A ring is oxidized to a vinyl group, but the propionate on the B ring is not. Harderoporphyrin was named for an unusual porphyrin found in the rat Harderian gland. It was later learned that the porphyrin found in the rat was a glycoconjugate of protoporphyrin and not a tricarboxylic porphyrin, rendering the term *harderoporphyrin* a misnomer.[298]

Frequency. HCP is the least common of the acute neurovisceral porphyrias.[299] Homozygous HCP is very uncommon but important to the pediatrician because clinical

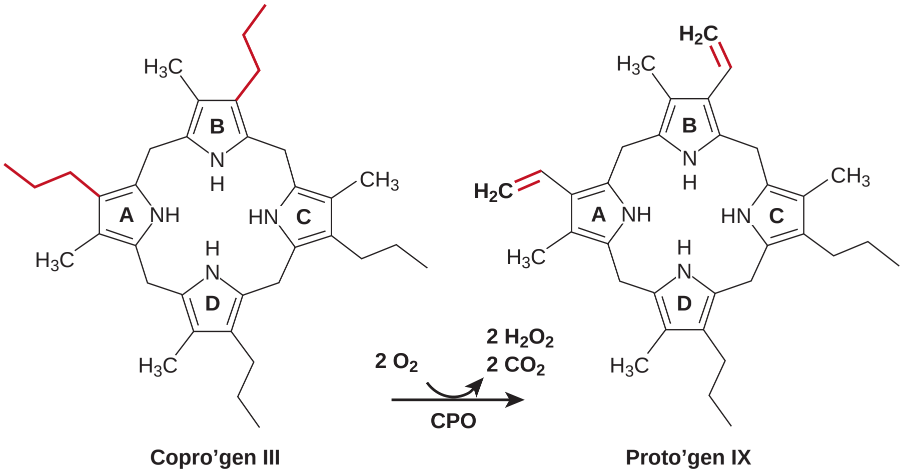


Figure 12-17 Reaction catalyzed by coproporphyrinogen oxidase (CPO). The propionate groups on rings A and B of the tetrapyrrole are sequentially oxidatively decarboxylated, forming vinyl groups. The reaction consumes two molecules of molecular oxygen and produces protoporphyrinogen IX (proto'gen IX) plus two molecules of carbon dioxide and hydrogen peroxide. *Copro'gen,* coproporphyrinogen.

manifestations occur in children. Harderoporphyria is even more rare, with only seven cases reported.

Clinical Manifestations. Acute neurovisceral attacks in patients with HCP are clinically indistinguishable from the acute attacks in patients with AIP. In one series of 53 German patients, abdominal pain was the most common symptom (89%).[300] Neurologic, psychiatric, and cardiovascular symptoms occurred in 25% to 33% of cases. Cutaneous symptoms occurred in only 14%. Similar findings were reported in an earlier series from the United Kingdom, but the incidence of cutaneous findings was slightly higher.[301] The cutaneous findings are the sole feature of HCP that distinguishes an attack from AIP. Most acute attacks of HCP are precipitated by drugs (see Table 12-3),[302] but attacks may also occur in association with menstrual cycles. The cutaneous photosensitivity is manifested mainly as bullous lesions and is generally present only with acute attacks. The bullae resemble those seen in PCT, but the chronic scarring reactions are uncommon because the photosensitivity is intermittent. Rarely, intermittent photosensitivity may be the only clinical sign of HCP.[301] In children who are homozygotes or compound heterozygotes for CPO mutations, the disorder is an early onset phenocopy of clinically affected adults who are simple heterozygotes. Growth retardation, hypertrichosis, and skin pigmentation are additional findings in early onset cases.[295,296]

The clinical manifestations of harderoporphyria are quite different. Hemolytic anemia, intense jaundice, and hepatosplenomegaly are present at or shortly after birth.[297,303] Bullous skin lesions develop if photo therapy for neonatal jaundice is employed. The urine appears red. Nucleated erythrocytes are present on the peripheral blood smear. Exchange transfusions have been helpful. With time, hepatomegaly resolves but splenomegaly remains. The hemolytic anemia becomes partially compensated, and additional transfusions are not necessary. Cutaneous photosensitivity persists and by the teenage years the skin resembles that seen in PCT. Acute neurovisceral attacks occurred in only one of the reported cases, the child homozygous for a H327R mutation.[304]

Four of the seven reported cases of harderoporphyria occurred in three families.[304] In four cases homozygosity for a mutation present in exon 6 (K404E) was detected.[305] In a fifth case the K404E mutation was present on one allele, but a different mutation was found on the other allele (IVS6-+3A>G).[304] In the sixth case a Japanese newborn with jaundice, hemolytic anemia, hepatomegaly, and photosensitivity proved not to be homozygous for a CPO mutation. This child was heterozygous for the K404E mutation, inherited from the father, but enzyme activity was not measured in the child or the parents. A promoter mutation in one maternal allele was suspected as the cause of the harderoporphyria phenotype in the child.[306] A seventh case proved to be homozygous for a H327R mutation.[304]

Pathogenesis. The accumulation and excretion of coproporphyrin is best explained by loss-of-function mutations of CPO but acute neurovisceral attacks, as in AIP, require induction of hepatic ALA-S. Over 50 CPO mutations have been described in patients with HCP and homozygous HCP[307] including large deletions.[78,299,308] The mutation responsible for harderoporphyria, K404E, is of special interest as it mainly effects the second decarboxylation reaction in the conversion of copro'gen III to proto'gen IX. One other mutation, R401W, that was found in a patient with HCP was expressed in vitro and was also found to have a defect in the second decarboxylation.[307] These findings suggest that a region around residues 401 and 404 is important for retaining the tricarboxylic intermediate (harderoporphyrin) in the active site and permitting the second oxidative decarboxylation. The distinctive erythroid phenotype of harderoporphyria is difficult to explain but must be related to a peculiar toxic effect of harderoporphyrin. The accompanying photosensitivity is likely due to the photodynamic properties of coproporphyrin. The roles of ALA and PBG in the pathogenesis of the acute neurovisceral attacks are as described for AIP.

Laboratory Diagnosis. Increased levels of fecal coproporphyrin III are the most prominent findings and are always found in clinically affected cases.[300] The ratio of coproporphyrin III to coproporphyrin I in feces is markedly increased, even in asymptomatic heterozygotes.[300] Urinary excretion of coproporphyrin is markedly increased during acute attacks but generally returns to normal during remission. Similarly, urinary excretion of ALA and PBG is increased during attacks but then returns to normal. When PBG excretion is increased, determining the coproporphyrin isomer ratio and plasma fluorescence scanning serve to establish a diagnosis of HCP.[299] The laboratory findings in homozygous HC are the same, but the abnormalities are more dramatic.

The laboratory findings in harderoporphyria are characterized by the presence of harderoporphyrin in feces along with increased amounts of coproporphyrin III. Urinary excretion of coproporphyrin is also increased, and trace amounts of harderoporphyrin may be found. There is no increase in the urinary excretion of ALA or PBG. Modest increases in erythrocyte protoporphyrin are present, but harderoporphyrin is not present in red cells.[303,305] Iron overload as a consequence of chronic hemolysis and ineffective erythropoiesis has also been reported.[305]

Assays for CPO activity have been utilized but are difficult to perform and are not widely available. Red cells are not a ready source of the enzyme because CPO is localized in the mitochondria. Lymphocytes or cultured cells are required.[70] DNA sequencing is the most direct approach, but the biochemical phenotype is so distinctive that a secure diagnosis can be made by simply analyzing fecal and urine porphyrins. Misdiagnosis of HCP is a common problem because urinary coproporphyrin excretion may be minimally increased in many nonporphyric conditions.

Treatment. Acute neurovisceral attacks are treated as in AIP. Hemin therapy is useful, and regular infusions as maintenance therapy have been successful.[309,310] Although the skin lesions resemble those of PCT, phlebotomy, and chloroquine are not effective. The chronic photosensitivity associated with homozygous HCP and harderoporphyria are best managed with avoidance of sunlight, protective clothing, and opaque sunscreens. The extreme jaundice and hemolytic anemia in newborns with harderoporphyria responds to exchange transfusions. Phototherapy of newborns should be avoided. The chronic compensated hemolytic anemia requires supplementation with folic acid and may require orally administered iron chelators.

ENZYMATIC OXIDATION OF PROTOPORPHYRINOGEN IX TO PROTOPORPHYRIN IX AND INSERTION OF IRON

Protoporphyrinogen Oxidase

Proto'gen IX is oxidized to proto IX by PPO (Fig. 12-18). PPO is functionally conserved in eukaryotes despite a low sequence identity. PPO is an intrinsic protein of the inner mitochondrial membrane and requires molecular oxygen and the cofactor flavin adenine dinucleotide (FAD). Three molecules of oxygen serve as the terminal acceptors in the six-electron oxidation reaction, generating hydrogen peroxide rather than water.[311-313] The reaction proceeds via three two-electron oxidations rather than one six-electron oxidation.[314]

The crystal structure of PPO from multiple species, including human, has been determined.[315-317] In all cases, the enzyme functions as a homodimer. Each monomer contains one noncovalently bound FAD. PPO is synthesized in the cytosol and locates to the inner mitochondrial membrane. It has been proposed that PPO resides on the outer surface,[318] but recent evidence indicates a localization on the mitochondrial matrix side of the inner membrane.[319] The human PPO protein does not contain a cleavable leader sequence but contains a mitochondrial targeting sequence within the first 175 residues. The 28 N-terminal residues have some features of a presequence, which function as an independent mitochondrial targeting sequence.[320,321] Deletion of these residues, however, does not prevent translocation to mitochondria, indicating more distal regions are required for efficient import and insertion into the inner mitochondrial membrane.[321] There is a single 1.8 Kb transcript of PPO in all tissues.[318] The human PPO gene is located on chromosome 1q22-q23.[322,323] The 5' flanking region of human PPO contains a GATA1 binding site, suggesting the potential for erythroid specific regulation. Induction of erythroid differentiation in mouse erythroleukemia cells with dimethylsulfoxide leads to increased expression of PPO.[323]

Variegate Porphyria

VP is transmitted as an autosomal dominant trait resulting from subnormal activity of PPO.[324] The clinical and biochemical features of the disease were first described in 1937,[325] but VP was not recognized as a distinct porphyric disorder until the description by Dean and Barnes in 1955.[326] The term *variegate* was applied when it was recognized that patients had either cutaneous photosensitivity, a predisposition to acute neurovisceral attacks, or both. Presentation before puberty is extremely rare, but early onset with marked photosensitivity has been reported in children homozygous for PPO mutations or with compound heterozygous genotypes.[85] The speculation that VP affected members of the British royal family, including King George III, has been recently discounted.[327]

Frequency. The prevalence of VP in Europe is established at 0.5 per 100,000.[328] The prevalence is higher in Finland, at 1.3 per 100,000,[329] but the disease is extraordinarily common in South Africa, where prevalence is estimated at 3 per 1000 in the white population.[89] The high prevalence in South Africa is due to a founder mutation introduced from the Netherlands in 1688.[330] The connection with the Netherlands has been confirmed by haplotype association studies in South African and Dutch families with VP.[331] Clinical penetrance in earlier reports was estimated at approximately 90%,[332] but the advent of molecular genotyping and sensitive biochemical markers has reduced the estimate to approximately 5% to 10 %.[89,299]

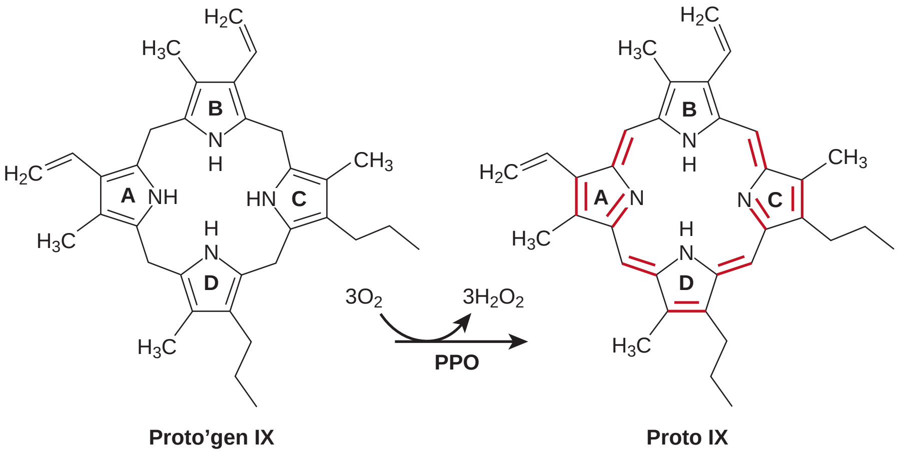


Figure 12-18 Reaction catalyzed by protoporphyrinogen oxidase (PPO). Protoporphyrinogen IX (proto'gen IX) is oxidized to protoporphyrin IX (proto IX) with the loss of six protons. Three molecules of molecular oxygen are consumed, and three molecules of hydrogen peroxide are formed in the reaction.

Clinical Manifestations. The acute attacks in patients with VP are identical to those seen in AIP but occur with a lower frequency and a later age.[89] Skin manifestations in VP are similar to those in PCT[333] and are of long duration. In a heterozygous Western European population of 103 clinically affected patients with VP, 59% had skin lesions only, 20% had acute neurovisceral attacks only, and 21% had both.[334] This distribution is similar to that observed in a 1980 report from South Africa.[332] In contrast recent reports from South Africa and Finland indicate a marked reduction in acute attacks and a less dramatic decrease in photosensitivity—changes thought to be due to systematic detection of mutant PPO alleles in families and the avoidance of potentially harmful drugs.[89,335]

Homozygous VP in two young siblings was first described by Korda and colleagues in 1984.[336] The clinical phenotype differed dramatically from adult onset VP. Marked photosensitivity from birth, mental retardation, and seizures were described, but no acute neurovisceral attacks occurred. Since then, over a dozen additional cases have been described.[337-342] Severe cutaneous manifestations beginning in infancy have been a constant finding. Short stature is common, but mental retardation and seizures are not. A peripheral sensory neuropathy and deformities of the hands were noted in some cases, but in no case was a neurovisceral attack described.

Double heterozygosity for mutant alleles of PPO and CPO has been reported.[343] Surprisingly, the phenotype was mild and restricted to dermal photosensitivity.

Pathogenesis. A wide variety of PPO mutations in families with VP has been reported in different ethnic groups.[334,335,344] A single mutation, R59N, is found in almost all cases in South Africa.[345,346] There appears to be no correlation between specific mutations and the severity of the clinical syndrome.[299] In all cases, the activity of PPO is approximately half-normal.[347,348] The cutaneous manifestations of VP are due to the photodynamic properties of protoporphyrin and coproporphyrin which are produced in excess in the liver. The acute neurovisceral attacks are due to the episodic overproduction of ALA and PBG. The extreme photosensitivity seen in early onset VP is mediated by very elevated plasma porphyrins.[349] Most of the early onset cases have been heteroallelic for PPO mutations (i.e., compound heterozygotes) with markedly reduced PPO activity.[341]

Laboratory Diagnosis. When acute attacks of VP occur, fecal protoporphyrin and coproporphyrin and urinary coproporphyrin are markedly elevated.[335] VP can be distinguished from HCP and AIP by detection in plasma of a characteristic fluorescence emission spectrum with an emission maximum at 626 nm.[350] This emission spectrum results from a dicarboxyl porphyrin bound to plasma proteins.[351] Detection of the 626 nm peak in plasma is a useful method of detecting asymptomatic gene carriers and is more sensitive than fecal porphyrin analysis.[331,352] Urinary ALA and PBG are increased during acute attacks but may be normal during remissions.

Homozygous VP is characterized by markedly increased erythrocyte zinc protoporphyrin levels and elevated stool protoporphyrin and coproporphyrin levels.[85] PPO activity can be measured in cells that contain mitochondria,[353] but enzyme assays are not widely available. Direct fluorometric measurement of plasma porphyrins is probably the most rapid method of establishing the diagnosis of VP. Sequencing of the PPO gene is being applied more widely and is available through the Department of Human Genetics at the Mount Sinai School of Medicine in New York, N.Y.

Treatment. Treatment of acute attacks is as described for AIP and HCP.[354] Protection from sunlight is the only practical way to manage the photosensitivity. Phlebotomy therapy or chloroquine is not effective. A single patient with symptomatic VP has been treated with a liver transplant.[355] The indication for transplantation was advanced alcohol-induced cirrhosis. Following the transplant, fecal porphyrin values returned to normal and no further cutaneous or neurovisceral symptoms occurred. Improved treatment, detection of latent family members, and avoidance of potentially harmful drugs have improved the outcome and reduced disease-related morbidity.[89,331]

Ferrochelatase

The final step in the heme biosynthetic pathway, the insertion of iron into proto IX, occurs on the matrix side of the inner mitochondrial membrane, a reaction catalyzed by FECH (Fig. 12-19). Human FECH has been crystallized, and the enzyme functions as a homodimer.[356] Each monomer of FECH contains a nitric-oxide sensitive 2Fe-2S Fe/S bound to a cysteine-rich site at the C-terminus.[357] It has been suggested that a direct interaction occurs between PPO and FECH.[319] This arrangement would permit direct transfer of proto IX from PPO to the active site of FECH. This mechanism has been questioned, however, as experiments using isolated mitochondria indicate that a complex between enzymes is not required for heme synthesis.[358]

The human FECH gene has been cloned and mapped to chromosome 18q21.3.[359] The same transcriptional start site is used in erythroid and nonerythroid tissues and contains binding sites for both housekeeping and erythroid-specific promoter elements.[360] FECH is synthesized in the cytosol and targeted to mitochondria by sequences in the leader peptide,[361] followed by cleavage to produce the active, mature protein. The first 62 amino acids in the leader sequence are sufficient for mitochondrial targeting, but an additional 11 residues are required for efficient proteolytic processing.[362] Expression of FECH is regulated by intracellular iron levels, likely related to the enzyme's requirement for Fe/Ss.[294] Additional regulatory effects occur through interactions with hypoxia-inducible factor.[363]

Erythropoietic Protoporphyria

EPP is an inherited disorder of heme biosynthesis characterized clinically by the onset in childhood or infancy of acute photosensitivity of sun-exposed skin. The disorder was first recognized in 1953 by Kosenow and Treibs[364]

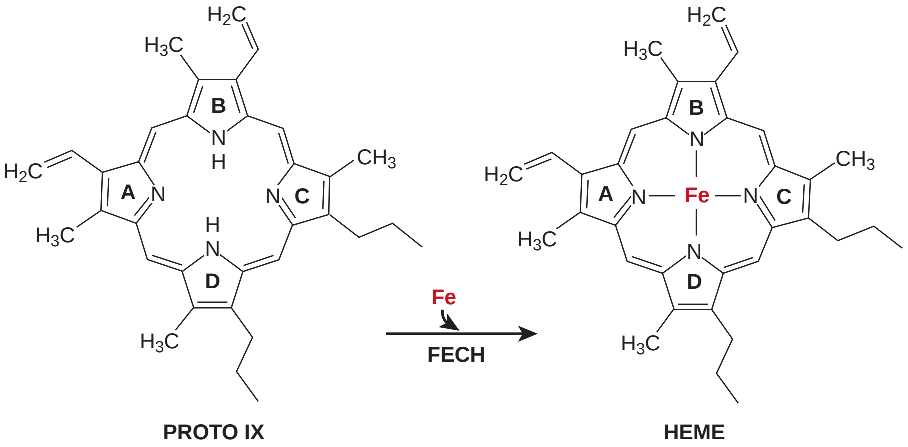


Figure 12-19 Reaction catalyzed by ferrochelatase (FECH). The final step in the synthesis of heme is insertion of ferrous iron into the protoporphyrin IX (proto IX) macrocycle with the release of two protons.

but was clinically characterized and named EPP by Magnus and colleagues in 1961.[365] The inherited nature of the disease was first reported by Haeger-Aronsen in 1963.[366] In most cases EPP is an autosomal recessive disorder due to deficient activity of FECH associated with loss-of-function mutations of one FECH allele coupled with a low expression abnormality (hypomorph) on the other allele.[367] In 2% to 10% of cases no FECH mutations can be identified.[299,368] Most cases of EPP without FECH mutations are due to gain of function mutations of ALA-S2 and are now referred to as X-linked protoporphyria (XLPP) (see later). In one large series from the United Kingdom, 6% of patients with the EPP phenotype had no mutations in FECH or ALA-S2,[369] suggesting other genetic causes for the EPP phenotype are possible. Rare cases with neither FECH nor ALA-S2 have been associated with abnormal expression of mitoferrin-1,[370,371] a protein required for mitochondrial iron assimilation. Excess proto IX generated in erythrocytes causes liver damage in approximately one third of patients with EPP,[372] but severe cholestatic liver disease requiring liver transplant is much less common.[373]

Frequency. The prevalence of clinically manifest EPP has been difficult to estimate but is probably close to that of AIP.[80] In the European immigrant population of South Africa, clinically manifest EPP is estimated to occur in 6.6 individuals per million.[374] The prevalence in the United Kingdom is approximately 7.7 per million.[375] Prevalence estimates for Northern Ireland and the Netherlands have approached 13 per million,[376,377] a finding attributed to a founder effect.[378] A report from Slovenia estimated an even higher prevalence at 17.5 per million.[379] EPP is pan-ethnic but is extremely rare in individuals of African descent.[374]

Clinical Manifestations. The dominant clinical feature of EPP is cutaneous photosensitivity in response to sun exposure with onset in infancy or childhood.[380,381] The clinical picture is very different in comparison to the other photosensitizing porphyrias. Patients develop a characteristic burning pain, erythema, and edema as an acute response to sun exposure.[375] Early in the course of EPP the burning discomfort may occur in the absence of objective cutaneous findings.[382,383] Bullous lesions, pigment changes, and scarring are very uncommon. Instead the skin develops a leathery characteristic that mimics premature aging. The symptoms and objective skin findings change little over time, but increased sun tolerance has been reported during pregnancy.[384,385]

A mild, microcytic anemia is common,[381,386-388] presumably due to impaired heme synthesis. Iron may accumulate in the cytosol and mitochondria of erythroblasts, but this is uncommon.[389] A similar microcytic anemia develops in a mouse model of EPP due to homozygosity for mutant FECH alleles.[390] Serum ferritin values in humans with EPP are reduced, but erythropoiesis does not appear to be iron limited.[387]

Gallstones occur in approximately 10% of patients with EPP and tend to occur early in life.[381,391] The gallstones contain proto IX, which becomes insoluble in bile and alters the solubility of other components of the bile.[392,393] In a mouse model of EPP a strain that sequestered protoporphyrin in the cytosol of hepatocytes and Kupffer cells was protected from cholestatic liver disease.[394] When gallstones occur in children, a diagnosis of EPP should be considered.[395] Minor abnormalities in liver function tests, mainly transaminase elevations, are common,[380] and liver biopsies may demonstrate protoporphyrin deposition and periportal fibrosis.[381,396-398]

Between 1 and 5% of patients with EPP will develop severe cholestatic liver disease progressing to portal hypertension and fatal decompensated cirrhosis.[399-401] Rarely, acute liver failure may be the presenting feature of EPP.[402,403] Over 50 fatal cases of EPP-related liver disease have been reported,[376,399,404,405] including two teenagers and an 11-year-old child.[376]

Pathogenesis. Over 120 mutations in the FECH gene have been detected.[78] The mode of Mendelian transmission had been described as autosomal dominant with incomplete penetrance, but heterozygotes expressing the clinical phenotype have consistently shown FECH activity between 15% to 20% of normal.[406] This was explained by a proposed dominant negative effect in which both mutant–mutant homodimers and mutant–wild type homodimers are catalytically inactive, with only wild type–wild type homodimers responsible for the measured enzyme activity. This theory was discarded with the discovery of a hypomorphic FECH allele, and EPP is now considered an autosomal recessive disorder.[407-409] An intragenic polymorphism (IVS 3-48C) in intron 3 is responsible for low expression of the hypomorphic allele. This polymorphism favors the use of a cryptic acceptor splice site 63 bases upstream of the normal splice site. The aberrantly spliced mRNA contains a premature stop codon and is degraded by a nonsense-mediated decay mechanism.[409] The result is a lower steady state level of wild type FECH mRNA. Most clinically evident cases of EPP are due to coinheritance of the hypomorphic allele in trans to a loss-of-function mutant allele. This genetic combination was found in 98% of French cases with EPP[410] and with a similar frequency in South African patients.[374] The frequency of the IVS 3-48C hypomorphic allele varies widely in different populations and relates to the observed differences in the prevalence of EPP.[410,411]

A rare exception to the compound heterozygous mutant/hypomorph genotype is inheritance of two loss-of-function FECH alleles. This autosomal recessive form of EPP was found in 4% of patients with EPP in the United Kingdom[369] and in occasional cases from other countries.[374,406,412-414] This form of EPP may carry a higher risk of severe liver disease.

Laboratory Diagnosis. The free erythrocyte protoporphyrin concentration is markedly increased in symptomatic EPP.[381,415] Values ranging from 6- to 100-fold greater than normal have been reported. Elevated erythrocyte protoporphyrin levels are also found in patients with iron deficiency and lead poisoning, but in these conditions the elevation is less dramatic, and it is zinc protoporphyrin that is present.[416] Zinc protoporphyrin can easily be distinguished spectrally from the free protoporphyrin found in EPP.[417] Zinc protoporphyrin can also be distinguished from free protoporphyrin by chromatographic techniques.[70] An elevated plasma porphyrin level confirms the presence of a porphyric disorder, but false negative results may be obtained in patients with EPP because protoporphyrin is particularly susceptible to photodegradation.[418] Care must be taken to shield the plasma sample from sunlight or fluorescent lighting.

Fecal protoporphyrin excretion may be increased up to fifteenfold, but values vary widely from patient to patient.[419] Urinary excretion of ALA and PBG are normal. Protoporphyrin, a highly hydrophobic compound, is not excreted in the urine.

Assays for FECH activity are not widely available. FECH mutation detection is the most direct approach to confirm the diagnosis of EPP. Molecular studies are also the only reliable method for identifying asymptomatic cases in families with an affected proband.

Treatment. The therapy of EPP has two objectives. First an attempt should be made to minimize the harmful effects of exposure to sunlight. Protective clothing and opaque sunscreens can be helpful as with other photosensitizing porphyrias. Tolerance to sunlight can be improved in most patients with EPP by inducing carotenemia. This can best be accomplished by administering β-carotene beadlets (Lumitene, Tishcon Corp., Westbery, N.Y.) in doses ranging from 120 to 300 mg/day.[420,421] The only side effect is a dose-related, yellow-orange discoloration of the skin.[422,423] The mechanism of action of β-carotene likely involves quenching of singlet oxygen species or free radicals.[424] A clinical trial of an α-melanocyte–stimulating hormone to darken skin color demonstrated increased tolerance to sun exposure and improved quality of life in patients with EPP.[425,426]

The second objective of therapy is to minimize the hepatotoxic effects of protoporphyrin. Cholestyramine can interrupt the enterohepatic circulation of protoporphyrin excreted in bile and promote fecal excretion. This has been moderately effective in some patients.[392] A variety of other measures have been employed when liver function deteriorates, including plasmapheresis, hemodialysis, exchange transfusions, and suppression of erythropoiesis.[399] In the small proportion of patients with EPP that develop liver failure, liver transplantation is the only effective therapy.[115,373,399,427,428] Liver transplantation, however, does not address the underlying overproduction of protoporphyrin in the native marrow, leading to a high risk of recurrent disease in the transplanted liver.[373,429,430] This complication can be avoided with a bone marrow allograft from an appropriate donor.[428,431,432]

Late-Onset Erythropoietic Protoporphyria. Over a dozen cases of late-onset EPP have been reported.[433-436] Most were associated with myelodysplastic syndromes, but EPP and myeloproliferative disorders have also been reported.[436] All have been due to somatic mutations in hematopoietic clones that result in markedly reduced FECH activity. Two cases were associated with hepatic failure.[436,437]

X-Linked Protoporphyria. Incorporation of gene sequencing in the diagnostic approach to the porphyrias has resulted in several unexpected findings. FECH sequencing and gene dosage analysis of a large number of EPP patients referred to centers in the United Kingdom and South Africa revealed FECH mutations or deletions in most, but no FECH gene abnormalities were found in approximately 7% of cases.[27] Whatley and colleagues studied eight families with mutation-negative EPP.[27] These families were unusual because they displayed a dominant pattern of transmission with an absence of father-son transmission, a finding suggesting X-linkage. Erythrocyte protoporphyrin concentrations were higher than in other EP patients, and the percentage of the zinc chelate was markedly higher (median 44% versus 8%). The cause of the EPP phenotype in these families proved to be increased activity of ALA-S2 due to mutations affecting the carboxy-terminal amino acids.

In six families, deletion of codons 1706 to 1709 (delAGTG) altered the last 19 carboxy-terminal amino acids and elongated the mutant enzyme by four residues. In two families, deletion of codons 1699 to 1700 (delAT) resulted in a frame shift that deleted the 20 terminal residues. Expression of these mutant ALA-S2 enzymes in a bacterial system revealed an increase in enzyme activity compared to wild-type ALA-S2. The end effect of these gain-of-function ALA-S2 mutations is an increase in the production of protoporphyrin that exceeds the capacity of the FECH reaction to generate heme. The generation of large amounts of zinc protoporphyrin suggests that the metal chelating properties of FECH have not been exceeded and that mitochondrial iron delivery limits heme synthesis.

The findings of Whatley and colleagues have been confirmed by Balwani and colleagues in a study of 155 North American patients with the EPP phenotype.[368] FECH sequencing and dosage analysis revealed FECH gene abnormalities in 140 cases. The remaining 15 cases (10% of the EPP patients studied) had ALA-S2 gain-of-function mutations. Thirteen had the previously described ΔAGTC mutation. Two new mutations were found. A single base deletion at codon 1734 (delG) altered the nine carboxy-terminal amino acids and elongated the enzyme by five residues. A nonsense mutation at codon 1642 (C>T) resulted in a Q548X change, which deleted the terminal 40 amino acids. Ducamp and colleagues also detected the Q548X genotype in an Australian patient and the ΔAGTC mutation in two European cases.[438] A novel 26 bp exon 11 deletion affecting codons 1651 to 1677 was also found in a Dutch patient whose parents were from Afghanistan. All of the cases described by Ducamp and colleagues were women.

To-Figueras and colleagues studied four unrelated Spanish patients with CEP with the same URO3-S genotype (C73R/P248Q). The clinical phenotype was similar in three, but the fourth, a young girl, had a more severe phenotype.[171] She proved to have a novel gain-of-function mutation (Y586F) affecting the carboxy-terminus in one allele of ALA-S2.[171] This case suggests that ALA-S2 can act as a modifier gene influencing the phenotype of other erythropoietic porphyrias.

Pathogenesis. The mechanism by which alterations in the carboxy-terminus of ALA-S2 results in increased enzymatic activity has not been clarified, but several hypotheses have been suggested. Structural studies demonstrated that the carboxy-terminus of mammalian ALA-S2 forms a loop over the active site of the enzyme.[8] The open position favors dissociation and release of ALA and thus affects the overall catalytic activity.[8] It has also been suggested that the open position increases substrate access to the active site, resulting in increased enzymatic activity.[439] Finally, the carboxy-terminus of ALA-S2 affects the stability of the enzyme, and certain carboxy-terminal mutations can increase the stability and enzymatic activity.[440] The regulatory effects of the carboxy-terminus are complex because this region of ALA-S2 is responsible for binding to the β subunit of succinyl-CoA synthase, an enzyme that generates one of the substrates for ALA-S2.[441] Mutations in the carboxy-terminus that ablate the succinyl-CoA synthase binding site cause loss-of-function changes in ALA-S2 and X-linked sideroblastic anemia,[441] yet the Q548X mutant enzyme, which does not bind succinyl-CoA synthase, has increased activity and causes XLPP.[439] These findings suggest that conformational changes associated with the carboxyl-terminus of mammalian ALA-S1 are the main mechanism determining specific activity. A number of diverse site-directed mutations of nonconserved loop residues yield hyperactive ALA-S2 variants.[442]

Clinical Manifestations. The clinical findings in patients with XLPP, with one exception, resemble those in FECH-deficient EPP. Erythrocyte and plasma protoporphyrin levels are higher and protoporphyric liver disease is more common.[27,368] Disease expression in females is similar to males, although one asymptomatic obligate female carrier has been described.[27] The single exception is the level of zinc protoporphyrin, which is strikingly higher (30% to 40% of total porphyrin) in XLPP.

Treatment. Strong consideration should be given to bone marrow transplantation when signs of progressive liver disease are recognized.

Late-Onset X-linked Erythropoietic Protoporphyria. Acquired clonal hematopoietic disorders such as myelodysplastic syndromes and myeloproliferative disease have been associated with late-onset cases of CEP and EPP. A case of late-onset X-linked EPP has recently been reported.[443] An 89-year-old French man with a myelodysplastic disorder developed a somatic mutation of ALA-S2 (Q548X) in a hematopoietic stem cell clone. In addition to photosensitivity, the patient had gallstones and cholestasis.

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CHAPTER 13

Autoimmune Hemolytic Anemia

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CHAPTER OUTLINE

PRIMARY AUTOIMMUNE HEMOLYTIC ANEMIA
Incidence
Natural History
Clinical Manifestations
Routine Laboratory Evaluation
Serologic Laboratory Evaluation
Differential Diagnosis
Characteristics of Erythrocyte Autoantibodies
Therapy
Pathogenesis of Autoantibody Formation
SECONDARY AUTOIMMUNE HEMOLYTIC ANEMIA
Evans Syndrome
Acute Lymphoproliferative Syndrome
Immunodeficiency
Other Systemic Disorders
Infections
Drugs

Autoimmune hemolytic anemia (AIHA) is an immunologic disorder in which antibodies are produced that target red blood cells (RBCs).[1,2] AIHAs can be classified into four major types of disease: warm-reacting AIHA, cold agglutinin disease (CAD), paroxysmal cold hemoglobinuria (PCH), and mixed-type AIHA (Box 13-1). Patients with no underlying associated diseases are classified as having primary or idiopathic AIHA. AIHA in patients with associated autoimmune diseases, immunodeficiencies, or infectious diseases is classified as secondary. Drug-induced and alloantibody-mediated immune hemolytic anemias are other important causes of antibody-mediated RBC destruction.

Antibodies produced are of two major classes: immunoglobulin G (IgG) and immunoglobulin M (IgM). IgG-coated RBCs are recognized by macrophages with specific receptors on their surface that are efficient in binding and stimulating the ingestion of the IgG-coated cells.[3-5] In addition to the clearance of IgG-coated RBCs by macrophages, patients with AIHA can also have IgM antibodies or, rarely, immunoglobulin A (IgA) antibodies directed at the RBC. The macrophage Fcγ receptors primarily responsible for the clearance of IgG-coated cells are FcγRI, FcγRIIIA, and FcγRIIA.[6] IgM antibodies bound to the RBC surface can initiate activation of classic complement pathway, resulting in the deposition of complement components—usually C3b. C3b-coated RBCs are recognized by macrophage C3b receptors, which then internalize complement-coated RBCs. IgG antibodies, when present on the RBC surface at a sufficient antigen density, can also induce complement activation.[7,8]

Finally, both IgG and IgM antibodies can induce the clearance of the antibody/complement-coated RBCs by tissue macrophages. The tissue macrophages responsible for the clearance of IgG-coated RBCs are present in the spleen. Hepatic macrophages can also bind C3b-coated RBCs and initiate their clearance. Rarely, RBCs coated with other antibody subclasses such as IgA can be present and induce the accelerated clearance of these RBCs, which occurs in a rare subset of patients with AIHA.

PRIMARY AIHA

Incidence

Primary AIHA is relatively uncommon, with an annual incidence of about 1 per 75,000 to 80,000 persons.[2,9] Within the pediatric population, AIHA is primarily a disease of young children,[10,11] with a median age of diagnosis of 3.8 years in a recent national observational study.[11] Primary AIHA also affects infants and toddlers, typically after a recent infection,[10-12] whereas adolescents with AIHA are more likely to have an associated underlying systemic illness and to have secondary disease.[13] Gender preference is unclear; some studies show an equal male to female ratio,[11,14] whereas other authors report a slightly higher prevalence in young boys but equal

Box 13-1 Classification of Immune Hemolytic Anemias

AUTOIMMUNE HEMOLYTIC ANEMIAS

- Warm-reacting AIHA
  - Idiopathic
  - Secondary (systemic lupus erythematosus, immunodeficiency)
- Cold agglutinin disease
  - Idiopathic
  - Secondary (infection, malignancy)
- Paroxysmal cold hemoglobinuria
  - Idiopathic
  - Secondary (infection)
- Mixed-type AIHA

DRUG-INDUCED IMMUNE HEMOLYTIC ANEMIA

- Drug-induced AIHA
- Drug-related antibody

ALLOANTIBODY-INDUCED IMMUNE HEMOLYTIC ANEMIA

- Hemolytic disease of the fetus and newborn
- Hemolytic transfusion reaction

AIHA, Autoimmune hemolytic anemias.

prevalence between the sexes during adolescence.[15] No evidence exists that AIHA affects persons of any particular racial background.

Warm-reactive antibodies are primarily of the IgG class and account for the majority of AIHA cases in children.[11,13] IgM-mediated CAD is uncommon in children and mostly affects middle-aged adults and elderly persons.[16] In contrast, PCH primarily presents in children, with the median age at diagnosis being 5 years.[17]

Natural History

Young children with IgG warm-reactive AIHA can have an acute illness only but tend to have a chronic clinical course characterized by intermittent remissions and relapses. In contrast, patients with cold-reactive autoantibodies, a condition most commonly associated with PCH, may have severe but acute self-limited illness requiring short-term supportive care with transfusion.

Overall mortality rates of 11% to 18% were reported in older series of pediatric AIHA cases,[10,13,18] but in a recent study of 265 pediatric patients with AIHA, only 4% of children died.[11] The improved mortality rate is likely a result of improvement in supportive care and new immunosuppressive treatment options, such as rituximab.[19,20,21] Pediatric patients who have primary AIHA associated with an infectious etiology have the most favorable outcome, with very low mortality and a high complete remission rate.[11] In this largest cohort of 265 pediatric patients with AIHA, the median evaluable period was 3 years, and it was longer than 1 year for 198 children. More than one third (37%) were diagnosed with Evans syndrome. At the last follow-up, 4% had died, 6% had no response or were in partial remission, and 90% were in complete remission while either receiving or not receiving therapy.[11] Because AIHA is a rare disease, data describing the incidence of acute versus chronic disease are lacking.

Several studies have attempted to define prognostic factors for pediatric AIHA. A positive mixed IgG/C3 direct antiglobulin test (DAT) is more likely associated with chronic disease as an independent prognostic risk factor.[11,13,18] In earlier retrospective studies it was observed that younger children with an abrupt onset of symptoms have a better prognosis.[10,13] In the context of current therapies, sex, age, and the triggering infection were not associated with continuous complete remission.[11]

Clinical Manifestations

Patients with AIHA typically present with symptoms of anemia such as pallor or fatigue, and they sometimes present with jaundice and scleral icterus as a result of rapid erythrocyte destruction and increased bilirubin turnover. Dark urine is reported in up to 80% of cases.[10,11,13] Hemoglobinuria is attributed to intravascular hemolysis of erythrocytes and therefore may occur in cases of cold AIHA as a result of IgM-mediated complement activation, but it is uncommon in persons with IgG-associated warm autoantibodies. The onset of symptoms can be rapid but the child is usually well compensated from a cardiovascular standpoint, and symptoms of congestive heart failure or circulatory collapse are rare. Occasionally fever or abdominal pain is reported by the patient.

Children with AIHA often have a benign medical history, but questions should be asked to determine whether a previous similar episode has occurred or if the child has had a recent viral illness. A history of infections, exposure to drugs or vaccination, or signs of immune disease may suggest secondary AIHA. The review of systems should include questions that address a possible underlying autoimmune disease, primary immunodeficiency, inflammatory disorder, or malignancy. The family history is often not revealing, but familial cases of AIHA have been reported[10] and likely represent a familial predisposition to an autoimmune disorder such as systemic lupus erythematosus (SLE).

The physical examination can be normal in patients with mild anemia, but children with moderate to severe AIHA are usually pale and icteric. Jaundice, which may be particularly prominent when ongoing hemolysis is severe, is caused by the breakdown of bilirubin from the destroyed erythrocytes. However, it should be emphasized that complement-coated RBCs are cleared extravascularly and that RBCs are difficult to lyse after complement activation. With less brisk hemolysis, jaundice may be best appreciated in the eyes or in the palms or soles. A heart examination will show that most children are tachycardic and, depending on the degree of anemia, an early systolic flow murmur may be appreciated as a result of the high output anemic state. A gallop may be heard in severely anemic patients and is a sign of impending cardiac failure. Mild to moderate tender splenomegaly is common in patients with severe hemolytic anemia, and occasionally hepatomegaly may occur as well.

Intravascular hemolysis results in hemoglobinemia and hemoglobinuria when hemoglobin is released into the blood and immediately bound by haptoglobin for clearance in the liver. The rate of hemolysis may exceed haptoglobin production, and extra hemoglobin circulates freely, causing hemoglobinemia. Free hemoglobin is typically filtered in the kidneys and reabsorbed in the form of iron. However, if the hemolysis exceeds the reabsorptive capacity of the renal tubules, hemoglobinuria occurs and urinary hemosiderin is detected. Patients with intravascular hemolysis may experience acute abdominal pain, costovertebral angle tenderness, and fever. Extravascular hemolysis occurs via antibody-coated RBC clearance by splenic and liver macrophages. Patients often present with tender splenomegaly, and although hyperbilirubinemia and bilirubinuria are common, hemoglobinemia and hemoglobinuria are not characteristic.

Routine Laboratory Evaluation

By definition, patients with AIHA present with anemia that ranges from mild to life-threatening. Children with primary AIHA often present with marked anemia, with a hemoglobin concentration of 4 to 7 gm/dL and no apparent cardiovascular compromise. Most patients have a normal platelet count, mild leukocytosis, and neutrophilia, but occasionally patients have leukopenia, neutropenia, or thrombocytopenia.[10,11,13,18] If the thrombocytopenia is significant, a diagnosis of Evans syndrome is possible and antiplatelet antibodies should be assayed.[22] However, microangiopathic hemolytic anemias including thrombotic thrombocytopenic purpura (TTP) or hemolytic uremic syndrome (HUS) must also be considered with concurrent thrombocytopenia. The reticulocyte count is usually elevated to compensate for the anemia, but more than one third of patients have a transient reticulocytopenia despite having normal or hyperplastic erythroid marrow.[18,23] The mechanism for reticulocytopenia is not well understood, but it has been suggested that a distinct autoantibody directed at erythroid progenitor cells leads to their selective destruction.[24] Bone marrow examination is not typically indicated but shows erythroid hyperplasia in the majority of patients.[18,23]

Evaluation of the peripheral blood smear can reveal important diagnostic hallmarks of AIHA. Spherocytes are usually present in IgG-mediated warm-reactive AIHA (Fig. 13-1), which suggests an immune hemolytic process in the absence of hereditary spherocytosis. Spherocytes result from spleen-induced damage to antibody-coated erythrocytes and are most commonly seen with warm, IgG-mediated AIHA.[25] Spherocytes are uncommon in cold-reactive AIHA, but complement-mediated immune spherocytosis can also result from surface microfragmentation of erythrocytes, and occasional to rare spherocytes are noted on the blood smear.[26] Polychromasia can be appreciated and usually correlates to the degree of reticulocytosis. Other morphologic abnormalities include fragmented and nucleated RBCs. Erythrophagocytosis is a

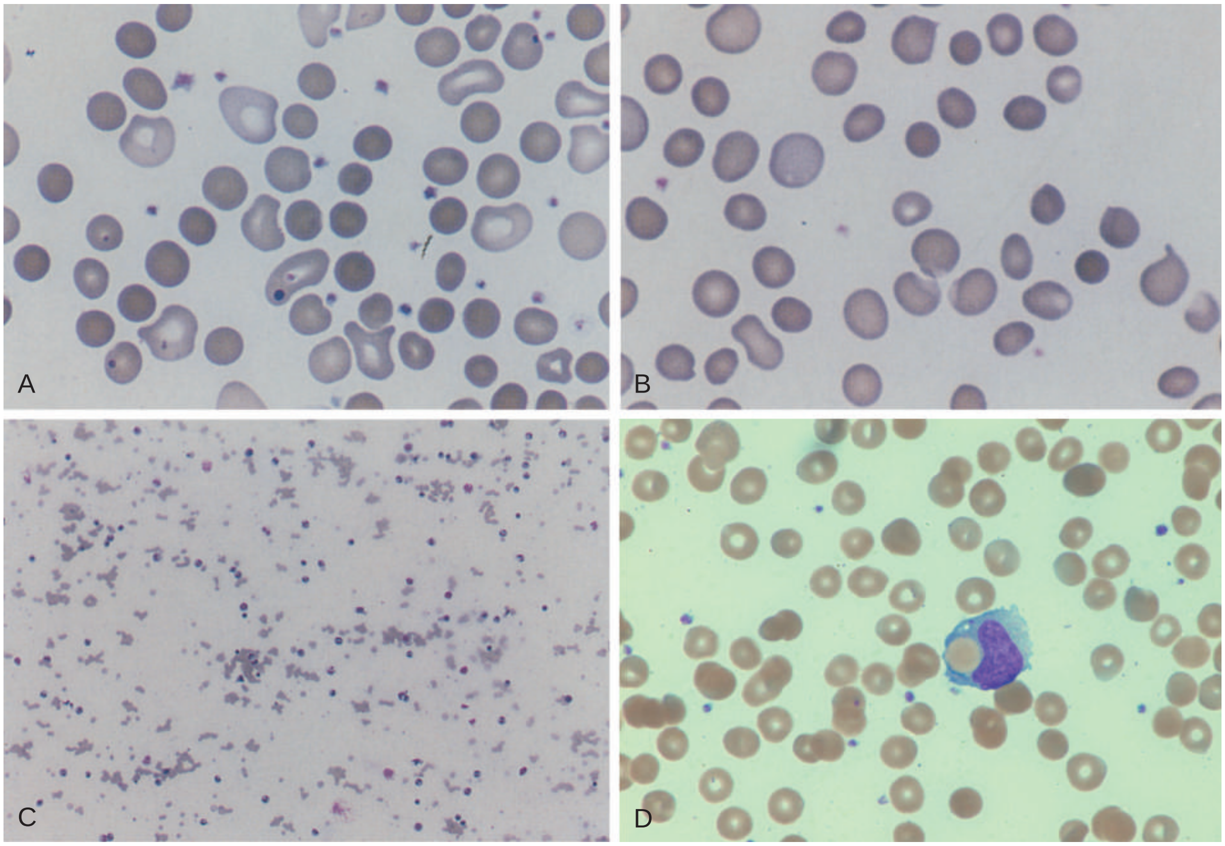

Figure 13-1 Examination of peripheral blood from persons with autoimmune hemolytic anemia (AIHA). **A,** Blood from a patient with immunoglobulin G (warm-reactive) AIHA illustrates many small microspherocytes and larger reticulocytes (×1000), and **B,** blood from a patient with hereditary spherocytosis illustrates the morphologic similarities between these two conditions. **C,** Agglutinated erythrocytes from a patient with immunoglobulin M (cold-reactive) AIHA are clearly visible at low power (×400). **D,** Erythrophagocytosis by a peripheral blood monocyte (×1000). *(From Ware RE: Autoimmune hemolytic anemia. In Orkin SH et al., editors:* Nathan & Oski's hematology of infancy and childhood, *7th ed. Philadelphia, 2009, Saunders, p. 613–658.)*

rare finding on the peripheral blood smear but demonstrates monocytes engulfing erythrocytes. Agglutination of RBCs is frequently seen in IgM-mediated cold-reactive AIHA when the binding affinity of the antibody reaches room temperature.

Serum chemistries may be abnormal as a result of erythrocyte hemolysis but are not essential to the diagnosis of AIHA. Elevated levels of lactate dehydrogenase and aspartate aminotransferase reflect erythrocyte destruction and release of cellular enzymes. In contrast, the serum level of hepatic enzymes alanine aminotransferase and gamma glutamyl transferase should not be elevated in persons with AIHA. The total serum bilirubin level is elevated in most patients with AIHA and is primarily unconjugated as a result of accelerated erythrocyte destruction rather than intrinsic hepatic disease. A decreased haptoglobin level is indicative of rapid intravascular hemolysis when the rate of hemolysis exceeds haptoglobin production by the liver. However, haptoglobin levels will be low in newborn infants who do not synthesize it well or high with concurrent infection or inflammation as an acute-phase reactant.[27] Urinalysis may be unremarkable, but when hemoglobinuria is present, urine dipstick analysis shows the presence of blood, although microscopic examination reveals few if any erythrocytes. Urine hemosiderin is detected in patients with hemoglobinuria as a result of intravascular hemolysis and can be used to distinguish hemoglobinuria from hematuria and bilirubinuria.

Serologic Laboratory Evaluation

The DAT (formerly named the Coombs test) is a simple but important test to establish a suspected diagnosis of AIHA. The DAT detects immunoglobulin and/or complement on the surface of RBCs and aids in the classification of hemolysis as immune or non–immune mediated. The test was first reported in the early 1900s but its use became widespread after it was described in 1945 by Coombs and colleagues.[28] Initially this test was used to demonstrate antibody in the serum, but it was later adapted to demonstrate the in vivo coating of RBCs with antibody or complement components.

IgM autoantibodies are pentameric and can bridge and agglutinate adjacent RBCs (Fig. 13-2). In contrast, IgG autoantibodies are smaller and unable to bridge adjacent RBCs because of the surface repulsion between cells, known as the zeta potential. Antihuman globulin (AHG; Coombs reagent) binds to IgG autoantibodies on the RBCs, bridges the zeta potential, and causes agglutination.

The main principle of the DAT is that AHG agglutinates antibody-coated cells, as evidenced by erythrocyte clumping. Ethylenediaminetetraacetic acid (EDTA)–anticoagulated blood samples are preferred because EDTA prevents in vitro fixation of complement.[29] The DAT is optimally performed with use of freshly washed RBCs; washing is necessary to remove proteins and complement that are present in the surrounding plasma and could neutralize the AHG reagent, causing a false-negative result. Typically a polyspecific AHG containing both anti-IgG and anticomplement is used first. A positive DAT result with polyspecific AHG leads to testing with monospecific IgG and complement.[30] Commercial reagents of anti-IgG recognize the gamma heavy chain portion of IgG, and monospecific anticomplement reagents usually contain anti-C3d, which is indicative of bound IgM.[31] The DAT is typically performed at room temperature (25°C) but can be performed at temperatures between 0°C and 37°C to determine thermal reactivity of the antibody. The DAT may be performed with the conventional test tube method, a gel microcolumn technique, or solid-phase methods (Fig. 13-3).

The DAT results describe the pattern of agglutination with the polyspecific AHG reagent and, if positive, the results of subsequent testing with the specific IgG and C3 reagents. The DAT is positive when agglutination is observed either after immediate centrifugation or after centrifugation following a room-temperature incubation.[29] IgG-coated RBCs usually agglutinate immediately, whereas complement-coated RBCs may be more easily demonstrable after incubation.[29] The DAT may also be performed at different temperatures including 4°C, 10°C, 25°C (room temperature) and/or 37°C to distinguish warm and cold-reactive autoantibodies and to characterize their thermal reactivity and amplitude. DAT agglutination results are scored on a scale of 1 to 4.

The presence of IgG on the erythrocyte in a patient with suspected AIHA who has not undergone transfusion is good evidence for an IgG autoantibody. Warm-mediated AIHA is the most common form of AIHA in children and

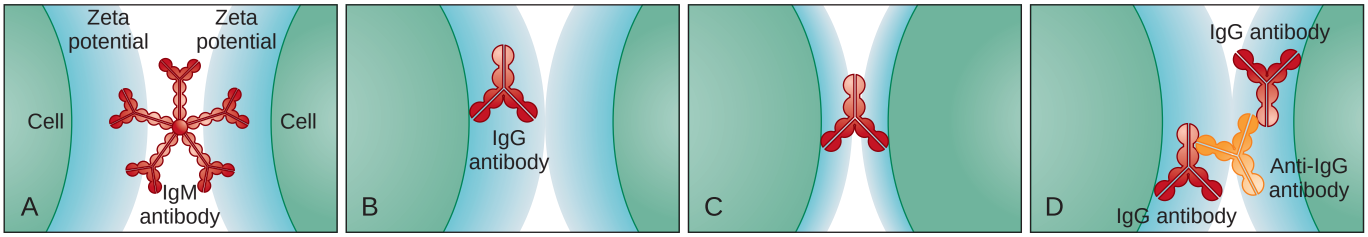

Figure 13-2 Interactions between immunoglobulin molecules and the erythrocyte during agglutination tests and the direct antiglobulin test. **A,** An immunoglobulin M *(IgM)* autoantibody can simultaneously bind two erythrocytes because of its multiple antigen-binding sites. The large size of the IgM molecule allows it to bridge the zeta potential between erythrocytes and cause agglutination. **B,** An immunoglobulin G *(IgG)* autoantibody is too small to bridge the zeta potential. **C,** There is no agglutination of erythrocytes unless the zeta potential is reduced. **D,** Upon addition of the Coombs reagent (a rabbit antiglobulin that recognizes human IgG), the zeta potential is successfully bridged and red cells agglutinate. *(From Ware RE: Autoimmune hemolytic anemia. In Orkin SH et al., editors:* Nathan & Oski's hematology of infancy and childhood, *7th ed. Philadelphia, 2009, Saunders, p. 613–658.)*

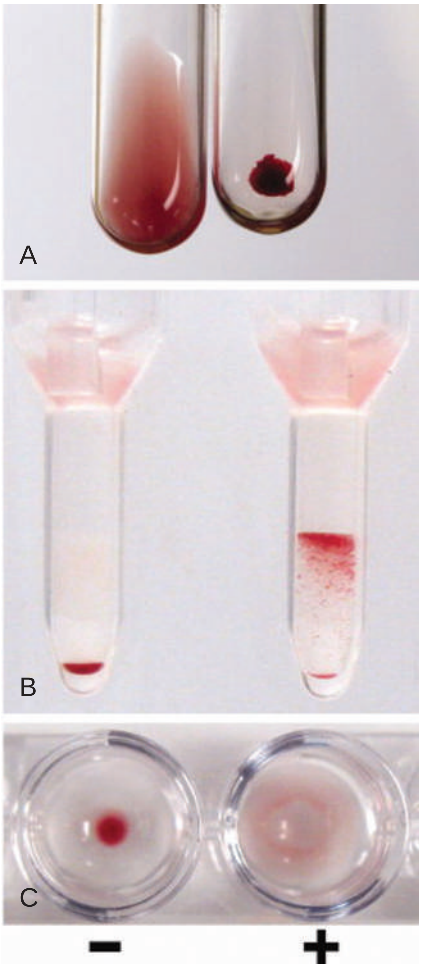

Figure 13-3 Negative and positive direct antiglobulin test (DAT) reactions. The DAT detects immunoglobulin and/or complement on the surface of red blood cells, which agglutinate with the addition of antihuman globulin. The agglutination is detected visually as clumps of red blood cells by the conventional test tube method (**A**), by the failure to filter through a gel matrix in the gel microcolumn method (**B**), or by the adherence of red blood cells onto the surface of a well in solid phase methods (**C**). *(From Zantek ND, Koepsell SA, Tharp DR, Jr, et al.: The direct antiglobulin test: a critical step in the evaluation of hemolysis.* Am J Hematol *87:707–709, 2012.)*

is usually caused by IgG autoantibodies that react at room temperature or 37°C. The DAT is positive for IgG, and simultaneous detection of complement indicates that the antibody can fix complement as well. Cold AIHA is typically caused by IgM autoantibodies that bind to RBCs at temperatures between 0°C and 37°C.[16] A DAT that indicates the presence of complement alone suggests a cold-reactive antibody that fixes complement at lower temperatures but binds poorly to the erythrocyte at 37°C. Further serologic evaluation for the presence of an IgM autoantibody or the IgG Donath-Landsteiner autoantibody would be indicated (described in detail later in this chapter).

The DAT results may be negative despite good clinical evidence of autoimmune hemolysis.[32] DAT-negative AIHA represents 5% to 10% of all AIHAs.[33,34] One explanation is that the quantity of RBC-bound IgG is below the threshold of the DAT with standard reagents,[35-37] which currently detects as few as 150 IgG molecules per erythrocyte. More sensitive assays to detect RBC-bound IgG may be helpful and include enzyme-linked immunosorbent assays,[38] gel card analysis,[39] and flow cytometry.[40] Autoantibodies of the IgA or IgM subtypes may cause hemolysis with a negative DAT because the test is designed to detect IgG and C3d.[41] Reagents that react with IgA or IgM are commercially available but are performed in reference immunohematology laboratories only. Clinically relevant low-affinity antibodies may be removed by washing during the DAT procedure.[32] Washing with low ionic strength solution or with ice-cold saline solution rather than room temperature saline solution may help retain the antibody on the RBCs.[29] Other technical issues may lead to false-negative DAT results. If clinical suspicion for AIHA is strong despite negative DAT results, specialized immunohematology reference laboratories such as those at the American Red Cross offer a DAT-negative AIHA evaluation that tests for IgA, IgM, and low-affinity antibodies.

Importantly, positive DAT results are not necessarily diagnostic. The interpretation and significance of positive DAT results must be correlated with the patient's history, including recent drug or transfusion exposure. Positive DAT results are found in 1 in 1000 to 14,000 healthy donors without hemolysis[42] and in 7% to 8% of hospitalized patients.[30]

Differential Diagnosis

The differential diagnosis of warm AIHA includes other causes of spherocytic anemias; for example, hereditary spherocytosis (HS; see Chapter 16) has many common clinical features. HS is a hemolytic anemia caused by deficient attachments of the lipid bilayer to the underlying membrane skeleton that is most often due to partial deficiency of ankyrin or band 3 or beta spectrin and is less often due to deficiency of alpha spectrin or protein band 4.2. Most diagnoses of HS are made in childhood on the basis of a positive family history or as a result of a similar clinical presentation to AIHA of anemia, jaundice, and splenomegaly. DAT results are always negative in persons with HS. A flow cytometry assay directly targeting the HS molecular defect (the eosin-5′-maleimide binding test) distinguishes spherocytosis due to HS versus other causes, including AIHA.[43,44] Clostridial sepsis,[45] ABO hemolytic disease in neonates (Chapter 3), severe burns or RBC thermal injuries, severe hypophosphatemia, and sometimes the hemolytic anemia associated with Wilson disease[46] (Chapter 11) or congenital dyserythropoietic anemia type 2 are other diseases characterized by spherocytic anemias.

In patients with hemolytic anemia and positive DAT results, several diagnoses should be considered (Box 13-1). Serologic characterization of an autoantibody will help distinguish warm-reacting AIHA, cold-reactive autoantibody syndromes such as CAD and PCH, and mixed-type AIHA. In a patient who has undergone a recent transfusion, positive DAT results may reflect a newly formed alloantibody to donor erythrocytes, indicative of a delayed hemolytic transfusion reaction. Further

TABLE 13-1 Typical Serologic Findings in Autoimmune Hemolytic Anemia

Characteristic	Warm Reactive	Paroxysmal Cold Hemoglobinuria	Cold Agglutinin Disease
Immunoglobulin	IgG	IgG	IgM
Thermal reactivity	37°C	4°C	4°C
Fixes complement	±	Yes	Yes
DAT			
4°C	Not performed	IgG, C3	C3
37°C	IgG ± C3	C3	C3
Plasma titer	Low/absent	Moderate	High
Hemolysin	No	Yes	Variable
Antigenic specificity	Rh and others	P	I/i
Site of RBC destruction	Spleen	Intravascular	Liver, intravascular

DAT, Direct antiglobulin test; *IgG,* immunoglobulin G; *IgM,* immunoglobulin M; *RBC,* red blood cell.

characterization of the antibody can distinguish whether it is directed at donor or autologous RBCs.

Drugs can cause hemolytic anemia through an immune or nonimmune mechanism. Drug-induced immune hemolytic anemia (DIIHA) is rare, but more than 100 drugs have been implicated.[47] Common drugs causing hemolytic anemia are piperacillin, cefotetan, and ceftriaxone, and they can mimic AIHA or delayed hemolytic transfusion reactions in some patients. Drug-induced hemolysis requires laboratory demonstration of (1) the patient antibody binding only to RBCs coated with the drug in vitro or (2) the patient antibody supporting deposition of complement on RBCs only when the drug, patient serum containing the antibody, and a source of fresh complement are simultaneously present with RBCs. Hemolysis ceases within days of discontinuation of the drug. Serologic evaluation for DIIHA can be performed by immunohematology reference laboratories; the Blood Center of Wisconsin and the American Red Cross (Southern California Blood Services Region) have particular expertise in this type of evaluation.

Spherocytes are typically found on blood smears in patients with warm AIHA, occasionally in patients with PCH, and rarely in patients with CAD. In other forms of acquired hemolytic anemia, spherocytes are rare on the peripheral blood smear, the DAT results are negative, and these disorders will be distinguished through the history and appropriate laboratory studies. Dark urine or hemoglobinuria may be a feature of paroxysmal nocturnal hemoglobinuria (Chapter 14), which is distinguished by decreased levels of CD55 and CD59 on blood cells as detected by flow cytometry. Peripheral blood erythrocytes derived from the mutant clone lack the capacity to restrict cell-surface activation of the alternative pathway of complement and to block formation of the cytolytic membrane attack complex because of a deficiency of CD55 and CD59, respectively.[48] Microangiopathic hemolytic anemias such as TTP and HUS are characterized by schistocytes and minimal spherocytosis and thus are easily distinguished on a peripheral blood smear. Moreover, thrombocytopenia is a common feature of TTP and HUS, whereas it is uncommon in persons with AIHA unless it is associated with Evans syndrome.

Characteristics of Erythrocyte Autoantibodies

Erythrocyte autoantibodies are generally categorized as "warm" or "cold" based on their thermal reactivity or amplitude (Table 13-1). Warm AIHA is usually caused by IgG autoantibodies that are optimally reactive at 37°C, and CAD is typified by IgM autoantibodies that are optimally reactive at 4°C but react at higher temperatures of 30°C to 37°C as well. Some patients have mixed-type AIHA with both IgG warm and IgM cold autoantibodies. PCH is a form of cold-reacting AIHA caused by IgG autoantibodies; conversely, rare cases of warm AIHA caused by IgM autoantibodies have been described.

Warm-Reactive Autoantibodies

Warm-reactive autoantibodies are maximally reactive at 37°C and typically react with a patient's own RBCs, as well as with all donor RBCs against which they are tested. In some cases a relative or absolute specificity is identified, of which Rh blood group system antigens are the most common. Warm-reactive autoantibodies are usually polyclonal IgG antibodies. The strength of the antiglobulin reaction and the rate of RBC destruction is variable but appears to be influenced by IgG subtype. Four subtypes of IgG antibodies exist and are designated IgG_1, IgG_2, IgG_3, and IgG_4. IgG_1 is the most commonly encountered subclass, either alone or in combination with other IgG subtypes.[49,50] IgG_1 and IgG_3 autoantibodies activate C1 more readily, bind strongly to FcγRI, FcγRII, and FcγRIII, and increase RBC destruction in comparison with IgG_2 and IgG_4 subtypes.[51-53]

DAT results in persons with warm reactive AIHA may be positive for IgG plus complement (67%); IgG only (20%); or complement only (13%).[54] If the autoantibody has been adsorbed by the patient's RBCs in vivo, the serum may not contain detectable free antibody, resulting in a negative indirect antiglobulin test. However, the presence of an IgG autoantibody bound to the erythrocytes may be confirmed by elution of antibodies from the patient's RBCs. Either autoantibodies eluted from the patient's RBCs or those present in serum typically bind to all common RBCs in blood bank test panels. This condition is described as a "panagglutinin" or having no antigenic specificity. The eluate will usually have no serologic reactivity if the only protein coating the RBCs is complement.

In many cases, warm autoantibodies demonstrate a relative specificity for a blood group antigen, most commonly a member of the Rh blood group system.[55,56] The patient's serum or eluate often has broad specificity in the Rh system but may be weaker or nonreactive if tests are performed with cells of rare Rh phenotypes, such as Rh_{null} RBCs that lack the entire Rh complex. Apparent or clear specificity for one of the common Rh antigens (anti-e, anti-E, or anti-c) is occasionally seen, or more commonly a "relative" specificity is demonstrated in which reactivity is stronger with certain Rh phenotypes. Warm autoantibodies in the Kell, LW, Kidd, Duffy, and Diego systems are rare but have also been reported.[57] Autoantibodies against non–blood group antigens are extremely rare and include protein 4.1[58] and band 3.[59] When a relative specificity is identified, transfusion of RBCs lacking the antigen may be useful.

RBC autoantibodies of the IgA class are found in up to 14% of patients with warm AIHA.[60] In cases of DAT-negative AIHA, an isolated IgA autoantibody on the RBCs can occur.[32,41] However, autoimmune hemolysis due to IgA antibodies alone is rare and more commonly acts synergistically with other immunoglobulins (usually IgG) and complement.[61] AIHA due to warm IgM autoantibodies is rare but is associated with severe hemolysis that often fails to respond to standard therapies in both adults and children and may be fatal.[62-64] These cases are difficult to diagnose because the routine DAT does not detect IgM antibodies, and thus misdiagnosis with CAD or PCH may occur if the DAT is positive for C3 only. Serologically, these IgM antibodies appear to have high affinity, resisting RBC dissociation and causing irreversible autoagglutination at 37°C. RBC autoagglutination of the sample should raise the suspicion of IgM autoantibodies, which do not disaggregate despite washing with warm (37°C) saline solution. Dithiothreitol (a reducing agent that inactivates IgM antibodies) does not affect IgG, and treatment of RBCs can be used to define the immunoglobulin type.[62,63] Reference laboratories can distinguish IgM warm autoantibodies by routine tube methods with anti-IgM reagents or by flow cytometry.[65]

Of three cases of pediatric AIHA attributed to warm IgM autoantibodies in contemporary literature, two were fatal despite treatment with corticosteroids, intravenous immunoglobulin (IVIG), RBC transfusions, whole blood exchange transfusions, rituximab anti-CD20 antibody, cyclophosphamide, mycophenolate mofetil (MMF), and cyclosporin A.[62,63] Successful treatment of severe warm-reactive IgM-mediated AIHA with rituximab in a child with common variable immunodeficiency (CVID) has been reported.[64] In patients who have warm IgM-mediated AIHA that does not respond to standard therapies, resulting in severe AIHA, the anti-C5-9 monoclonal antibody eculizumab is now available; however, results of its use in persons with AIHA have not yet been published. Notably, Salama and Mueller-Eckhardt reported 12 cases of warm AIHA associated with non–complement binding IgM autoantibodies that were referred to their reference immunohematology laboratory for serologic evaluation as a result of observed hemolysis that could not be explained by an underlying RBC abnormality; standard DAT results were negative.[66] These patients were treated with blood transfusions and prednisone only, and definitive improvement or remission of hemolysis occurred in 11 of 12 cases; none was fatal.[66]

Cold-Reactive Autoantibodies

Cold-reactive autoantibodies are primarily associated with CAD[67,68] and PCH.[16] Most commonly, CAD affects adults and PCH affects young children. PCH is the most common form of cold-reactive AIHA in children, but postinfectious CAD also occurs in childhood and adolescence, albeit rarely. Both are caused by autoantibodies that bind RBCs optimally at temperatures below 37°C and usually below 31°C. The associated hemolysis occurs through fixation of complement. In general, CAD and PCH may be idiopathic or secondary to an underlying systemic illness such as neoplasm or infection. Cold agglutinins are IgM autoantibodies that primarily cause extravascular hemolysis but can mediate intravascular hemolysis as well, whereas PCH is IgG mediated and causes intravascular hemolysis (Table 13-1).

The pathogenesis of a cold-reactive autoantibody is dependent on its ability to bind host RBCs and activate complement. Although in vitro agglutination may occur maximally at 4°C in vitro, complement fixation by these antibodies occurs at 20°C to 25°C or higher.[69,70] Complement fixation may cause RBC destruction via opsonization by macrophages in the reticuloendothelial system or rarely by direct lysis. With direct lysis, the Fc portion of the immunoglobulin interacts with C1q, the first component of the complement cascade. One IgM molecule is sufficient to bind C1q and activate the propagation from C1 to C9 sequence on the RBC membrane. Such direct lysis results in intravascular hemolysis, leading to hemoglobinemia and hemoglobinuria. Extravascular hemolysis occurs when the complement cascade leaves opsonic fragments of C3 and C4 on the cell surface, which is then ingested by macrophages in the liver or spleen. The ingested RBC may be engulfed entirely or released back into the circulation as a spherocyte after losing some of its membrane, providing an explanation for the observation of occasional spherocytes with IgM-mediated AIHA.

Cold Agglutinin Disease. Cold agglutinins were first described by Landsteiner in 1903, but CAD was not distinguished as a distinct hemolytic anemia until the 1950s.[71,72] CAD is uncommon in children, but when it does occur it is acute and often a result of *Mycoplasma pneumoniae* infection.[16] Postinfectious cold agglutinins can also be seen with Epstein-Barr virus (EBV),[73] varicella,[74] and adenovirus.[75] Postinfectious CAD is caused by polyclonal IgM antibodies, in contrast to monoclonal IgM that is seen classically in older adults with chronic CAD that commonly occurs as a result of an underlying lymphoproliferative disorder.[67,68] CAD typically occurs in the second or third week of illness, and a rapid onset of hemolysis causes the patient to experience jaundice and pallor. Hemoglobinuria and acrocyanosis are not characteristically present in persons with postinfectious CAD. The hemolysis is self-limiting, and the degree of anemia is typically mild to moderate. The cold autoantibody is an IgM with a characteristic anti-I specificity associated with *M. pneumoniae* and anti-i specificity associated with EBV. Patients with postinfectious CAD will demonstrate high (≥256) cold agglutinin titers (although not nearly as high as in the chronic, adult form of CAD, with titers ≥1000), and they will have negative Donath-Landsteiner test results.[16,67] Treatment of postinfectious CAD is supportive and includes keeping the patient warm and using a blood warmer if the degree of anemia warrants RBC transfusion.

In contrast, the chronic form of CAD is seen in elderly patients and is associated with lymphoma, chronic lymphocytic anemia, or Waldenström macroglobulinemia. The autoantibody is of the IgM subtype, is often monoclonal, and is maximally reactive in the cold but has reactivity up to at least 30°C. Patients with the chronic form experience hemoglobinuria from intravascular hemolysis and acrocyanosis of the ears, nose, fingers, and toes from autoagglutination of RBCs in the skin capillaries, particularly in cold weather.[72] Autoagglutination of anticoagulated blood that occurs as the sample of blood cools to room temperature is characteristic and is frequently the first observation made to suggest the diagnosis (Fig. 13-1, C). Autoagglutination can be enhanced by cooling the blood to 4°C and is reversed by warming to 37°C.

Cold-reactive IgM binds to RBCs at lower temperature in the peripheral circulation and causes complement fixation to the RBCs. However, as the blood circulates to warmer regions of body, the IgM dissociates but the complement remains. Thus complement is the only protein detected on the erythrocytes in almost all cases of CAD (postinfectious or chronic). If the RBCs have been collected properly, maintained strictly at 37°C until the serum and red cells are separated, and washed at 37°C, there should be no immunoglobulin detected on the RBCs and no reactivity in the eluate.[76] The majority of IgM cold-reactive autoagglutinins associated with immune hemolysis should have reactivity up to at least 30°C in saline solution or albumin and have a cold agglutinin titer of greater than or equal to 256 when tested at 4°C.[16] Cold agglutinin IgM molecules are typically directed against the I/i RBC antigens. Importantly, the observation of a cold agglutinin alone is not sufficient to diagnose CAD.

Paroxysmal Cold Hemoglobinuria. Historically, PCH was associated with syphilis and was named for paroxysms of constitutional symptoms and hemoglobinuria precipitated by cold temperatures.[77,78] PCH now occurs primarily in children as an acute transient condition following an upper respiratory or viral infection[15,17,78,79] and is detected by the Donath-Landsteiner test. PCH is the second most common form of AIHA in children, with a median age of 5 years.[17,80] The precise incidence and prevalence of PCH in children is unknown. The transient nature of the Donath-Landsteiner antibody, the availability of the Donath-Landsteiner test, and the level of awareness among physicians all affect the recognition and correct diagnosis of PCH.

In children, PCH classically presents within several weeks after a viral infection with the sudden onset of hemoglobinuria, accompanied by fever, pallor, and jaundice. Symptoms of PCH in children are not precipitated by cold exposure, in contrast to the chronic, syphilitic PCH in adults. A specific etiologic agent is not usually identified, but an upper respiratory tract infection precedes the hemolysis in the majority of cases.[17,78] The intravascular hemolysis and a rapidly progressing anemia may also cause headache, abdominal pain, nausea, vomiting, or diarrhea. Hepatomegaly and splenomegaly were reported in up to 25% of cases.[77] The degree of anemia is variable in children but can be severe. Approximately one third of patients have hemoglobin levels of 5 gm/dL or lower at presentation,[17,78] which may further decrease during their clinical course. Reticulocytosis is characteristic, but reticulocytopenia can be observed early in the course of disease and may result from viral suppression of the bone marrow or destruction by the autoantibody. The peripheral blood smear demonstrates RBC agglutination, polychromasia, nucleated RBCs, and occasional spherocytes. The DAT is usually positive for complement only but can be negative for both C3d and IgG. Other laboratory findings include increased levels of lactate dehydrogenase and unconjugated bilirubin, decreased or absent haptoglobin, and increased levels of blood urea nitrogen and serum creatinine.

The Donath-Landsteiner antibody is an IgG biphasic hemolysin that binds to RBCs and fixes complement at lower temperatures but does not cause hemolysis until the complement-coated RBCs are warmed to near 37°C.[80] Between these temperatures, the autoantibody may dissociate. As a result, the DAT is positive with anti-C3 but is usually negative with anti-IgG unless performed at colder temperatures. Donath-Landsteiner antibodies do not interfere in routine pretransfusion and compatibility testing because the autoantibody rarely causes RBC agglutination above 20°C. For the same reason, the antibody screen is usually negative. The autoantibody may agglutinate normal red cells at 4°C, but in contrast to CAD, rarely to titers greater than 64. The Donath-Landsteiner antibody usually has specificity for the P blood group antigen,[16] but other antibody specificities have been reported.[81,82] Many patients with unexplained hemolytic anemia, often with negative DAT results, probably have undiagnosed PCH.[83] The biphasic antibody may be transient and detectable only during the phase of acute hemolysis, and thus in suspected cases the Donath-Landsteiner test should be carried out as early as possible.

To confirm the diagnosis of PCH, the Donath-Landsteiner test can be performed by the hospital blood bank or reference immunohematology laboratory. A fresh sample of blood from the patient must be collected and maintained at 37°C until the serum is separated from the RBCs. Three sets of test tubes, labeled A1-A2-A3,

TABLE 13-2 Positive Donath Landsteiner Test*

	Tube 1 Patient Serum	Tube 2 Patient + Normal Serum	Tube 3 Normal Serum
Tube A Ice bath and then 37°C	+	+	0
Tube B Ice bath only	0	0	0
Tube C 37°C only	0	0	0

+, With hemolysis; 0, without hemolysis.

*The patient's sample should be kept at 37°C until the serum and red cells are separated to avoid the loss of autoantibody by cold autoadsorption prior to testing.

B1-B2-B3, and C1-C2-C3, are used to mix serum with washed group O RBCs that express the P antigen. Tubes 1 and 2 of each set contain the patient's serum, and tubes 2 and 3 contain fresh normal serum that has normal complement levels. One volume of 50% suspension of washed P^+ RBCs is added to each tube, and all the tubes are mixed. After mixing, the three "A" tubes are placed in a bath of melting ice for 30 minutes and then at 37°C for 1 hour. The three "B" tubes are placed in a bath of melting ice for 30 minutes and kept in melting ice for 90 minutes. The three "C" tubes are placed and kept at 37°C for 90 minutes. After the 90 minutes, the tubes are centrifuged and the supernatant fluids are examined for hemolysis.[29] The test is considered positive when the patient's serum, with or without added complement from normal serum, causes hemolysis in the tubes that were first incubated in the melting ice and then at 37°C (tubes A1 and/or A2) and there is no hemolysis in any of the tubes maintained at 37°C (tubes C1 and C2) or in melting ice (tubes B1 and B2) (Table 13-2). Tubes A3, B3, and C3 serve as controls with normal serum and should not demonstrate hemolysis.

PCH usually resolves spontaneously within several weeks and does not recur. Treatment is supportive with transfusion if the anemia is severe. Although the specificity of the Donath-Lansteiner antibody is almost always directed at the P antigen, RBCs lacking the P antigen are rare and not available for urgent transfusion. Most patients requiring transfusion achieve an adequate post-transfusion hemoglobin and hematocrit with P^+ RBCs despite the presumed or documented incompatibility. Although children with PCH do not typically have cold-induced hemolytic symptoms, the patient should be kept warm and the use of a blood warmer for RBC transfusions is prudent despite the lack of data to support this practice. Steroids are often initiated empirically for treatment of AIHA, but because no evidence exists to support their benefit, they can be discontinued once the diagnosis of PCH is confirmed.

Immune Clearance of Sensitized Erythrocytes. The role of antibodies, complement, monocytes, and the reticuloendothelial system in the immune-mediated clearance of RBCs was elucidated more than four decades ago.[25,84-87] In the 1970s, a guinea pig model for AIHA was developed that allowed the study of RBC clearance as a result of antibody or complement.[7,88] IgG-coated erythrocytes were removed predominantly by the spleen, and the amount of surface IgG correlated with the rate of RBC clearance. In contrast, IgM-coated erythrocytes were sequestered and cleared by the liver. Again, the amount of bound IgM correlated with the rate of RBC clearance. These studies established that clearance of IgM-bound erythrocytes was dependent on the interaction of erythrocyte-bound complement fragments with specific receptors on hepatic macrophages.[7] Human monocyte monolayer assays have been developed to correlate in vivo hemolysis with RBC autoantibodies, but these tests have shown limited utility.[89,90] Flow cytometry can also be used to measure the amount of RBC-bound IgG but are also unable to define a clear quantitative threshold to correlate in vivo hemolysis.[91]

Therapy

AIHA is a potentially fatal condition, and the decision regarding treatment depends on the severity and rapidity with which the anemia develops. Because of the relative rarity of the disease, evidence-based treatment of AIHA is limited,[92] particularly evidence regarding pediatric management. No randomized studies and only a few prospective phase 2 trials have been conducted in adults or children. Most reports describe retrospective studies or small series of patients or are single case reports. Comparison of different treatment modalities is challenged by the fact that no formal consensus exists with regard to the definition of complete or partial hematologic response, remission, or refractoriness. Thus most treatment decisions, including whether to provide treatment, are made on the basis of experience and on an individual patient basis. The two major determinants for treatment are the type of antibody involved (IgG vs. IgM mediated) and whether the AIHA is primary or secondary. Hospitalization is almost always required.

Treatment of Acute AIHA

Transfusion Therapy. In the patient with acute, newly diagnosed, or recurrent AIHA, the clinician must first decide whether the patient needs a transfusion immediately. A child who has relatively mild anemia with a hemoglobin level of 9 to 12 gm/dL may require observation alone. If the patient has a moderate to severe degree of anemia with a hemoglobin level of 6 to 9 gm/dL or is symptomatic with lethargy or shortness of breath, or if the rate of decline is precipitous, transfusion therapy is indicated. For patients who have very severe anemia with hemoglobin levels lower than 6 gm/dL, RBC transfusion should be instituted as soon as possible. In cases of warm, idiopathic AIHA, the antibody is typically directed against blood group antigens that are present on most RBCs. Therefore no truly matched RBC units are possible, but transfusion can be safely performed if underlying alloantibodies are excluded. In children and adolescents, clinically significant alloimmunization should only have occurred if they previously underwent transfusion, were pregnant, or received an organ transplant. A patient who has not previously undergone transfusion should have no risk of underlying alloantibodies and thus that lack of risk should be weighed against the risk of withholding transfusion for a time-consuming blood bank alloantibody evaluation in a severely ill child.

In a patient who has previously undergone a transfusion or been pregnant, the presence of an underlying alloantibody must be assessed to provide appropriate RBCs. Patients with AIHA that is associated with warm autoantibodies have an alloimmunization rate between 15% and 45%, but these studies focused primarily on adult patients.[93,94,95] If IgG autoantibodies are demonstrable in the serum, they will typically interfere with routine pretransfusion antibody screening and compatibility testing. IgG antibodies in persons with PCH do not interfere with routine compatibility testing because the autoantibodies do not cause agglutination above 20°C. Autoadsorption or alloadsorption procedures are performed to remove the patient's autoantibody from their serum sample by adsorption with autologous or allogeneic RBCs, thereby allowing identification of any remaining alloantibody in the adsorbed serum.[29] Autoadsorption is generally preferred because removal of antibody activity by adsorption with autologous RBCs confirms that the antibodies removed are autoantibodies rather than alloantibodies. Autologous adsorption is not recommended for patients who have undergone a transfusion within the past 3 months because the blood sample may contain some transfused RBCs that could adsorb alloantibody, even if only a small amount of transfused RBCs are remaining.[96] The patient's RBCs are typically warmed to dissociate some of the bound autoantibodies and then treated with a mixture of enzymes to enhance their capacity to adsorb autoantibody from the serum sample in vitro.[29]

Alloadsorption techniques are indicated when insufficient autologous RBCs are available from a very anemic patient or if the patient has undergone a transfusion in the past 3 months.[96] Allogeneic RBCs used for autoantibody adsorption must lack the antigen(s) against which the alloantibodies react. Because the underlying antibody specificity is usually unknown, a panel of RBCs with different phenotypes must be used to adsorb several aliquots of the patient's serum. RBC selection is based on antigens for which alloantibodies of clinical significance are likely to be present. These antigens include the common Rh antigens (D, C, E, c, and e), K, Fy^a and Fy^b, Jk^a and Jk^b, and S and s. Adsorption techniques are time-consuming and require a minimum of 2 to 3 hours to exclude the presence of alloantibodies but could take up to 4 to 6 hours because each aliquot of serum may need to be adsorbed two to three times to remove all of the autoantibody. Moreover, many hospital blood banks do not perform adsorption techniques and rely on reference laboratories, particularly for allogeneic adsorptions that require a supply of fully phenotyped RBC reagents.

Because adsorption procedures are labor-intensive, some transfusion services choose to prophylactically provide RBCs that are phenotypically matched with the patient's Rh, Kell, and Kidd system antigens rather than searching for new alloantibodies. However, although the most clinically significant antigens may be matched, the potential exists to miss newly developed alloantibodies against other blood group antigens.[93] Shirey and colleagues[94] reported that prophylactic antigen-matched donor RBCs for patients with warm AIHA can obviate the need for adsorption studies in some but not all cases and that provision of extended antigen-matched RBCs is feasible and safe. In 12 of 20 adult patients with autoantibodies (including six with existing alloantibodies), a serologic phenotype was successfully obtained, a total of 149 prophylactic antigen-matched RBC units were transfused, and adsorption studies were avoided on 51 pretransfusion samples. Autoantibodies in the remaining eight patients interfered with determination of a complete RBC phenotype, and adsorption studies were required before transfusion.[94] In both groups, Shirey and colleagues reported expected rises in hemoglobin and hematocrit values and no clinical signs of hemolysis as a result of transfusion, but these signs are probably difficult to assess in patients for whom RBC survival is already expected to be shortened. With increasing availability of molecular methods to type RBCs, which have the potential for a short turnaround period, prophylactic extended antigen-matched transfusions using RBC blood group genotyping is a reasonable option for patients with autoantibodies.[97,98]

Once the blood bank has determined the desired RBC phenotype for a particular patient, the patient's serum will be used to perform crossmatches with the RBC units. If the antigenic specificity is panreactive, the crossmatch will likely identify no units of blood that are fully compatible. Units that are "least incompatible" with the patient's serum are typically provided for transfusion. Thus to prevent a hemolytic transfusion reaction, the most important consideration is to exclude potentially clinically significant alloantibodies before selecting RBCs for the patient.[93,99] In an urgent situation, the clinician must balance the risk of delaying transfusion to complete an alloantibody evaluation against the benefit of an RBC transfusion in a severely anemic patient.[100]

Corticosteroid Therapy. Corticosteroids are the mainstay of treatment for persons with newly diagnosed primary idiopathic warm AIHA (Table 13-3).[101,102] Corticosteroids improve hemolysis in patients with warm AIHA via several mechanisms. Patients who respond to corticosteroids show a decrease in serum antibody concentration, which rises if the dose of prednisone is insufficient.[103] The guinea pig model of AIHA demonstrated that corticosteroids increase the survival of IgG-sensitized RBCs by decreasing sequestration in the spleen.[104] The ability of corticosteroids to suppress sequestration of RBCs by splenic macrophages may be the result of the reduced number of Fcγ receptors observed on peripheral blood monocytes of patients with AIHA during steroid treatment.[105]

In a patient with acute warm AIHA or PCH, intravenous methylprednisolone at a dose of 1 to 2 mg/kg is typically given every 6 hours for 1 to 3 days. In an adolescent, adult guidelines for warm AIHA recommend

TABLE 13-3 Therapy for Autoimmune Hemolytic Anemias

	Acute First Line	Second Line	Third Line	Refractory Cases
Primary warm AIHA	Corticosteroids	Rituximab IVIG Splenectomy	Cyclosporin Azathioprine Mycophenolate mofetil Cyclophosphamide Danazol	Campath-1H High-dose cyclophosphamide
Paroxysmal cold hemoglobinuria	Supportive care	Corticosteroids		Rituximab
Cold agglutinin disease	Avoidance of cold Plasma exchange	Rituximab		Eculizumab Bortezomib

AIHA, Autoimmune hemolytic anemia; *IVIG,* intravenous immunoglobulin.

1 mg/kg/day of oral prednisone or intravenous methylprednisolone,[102] because higher doses result in hypertension, hyperglycemia, acute irritability, or psychoses. Once the patient's hemoglobin level begins to rise and is clinically stable, oral prednisone should be used at a dose of 2 mg/kg/day for children and 1 mg/kg/day for adolescents. High doses of steroids are usually required for 2 to 4 weeks, and then the amount of corticosteroids must be reduced gradually to prevent relapses. For example, once the patient is taking 1 to 2 mg/kg/day of prednisone, a 10% to 20% taper with each dose change is reasonable. Some physicians first switch to an every other day schedule without reducing the total dose by administering a higher dose on even days and a lower dose on odd days. They then taper slowly on the alternate day schedule. Tapering of the corticosteroids is guided by the patient's hemoglobin concentration and reticulocyte count and, to a lesser degree, the DAT results. Most importantly, the steroid taper must be performed slowly over a long period to avoid relapse. When a patient relapses, a very high dose of prednisone is usually required to achieve remission again. Ultimately, the goal is to maintain a stable hemoglobin count with a relatively low dose of corticosteroids and, preferably, an every-other-day regimen. An overall clinical response is achieved in approximately 80% of patients with warm AIHA.[1,106] The total duration of corticosteroid therapy can vary from 3 to 12 months after remission is achieved.

Adverse effects of prolonged corticosteroid use should be considered. These adverse effects include irritability or even psychoses, sleep disturbances, hypertension, hyperglycemia that may require insulin therapy, weight gain, impaired longitudinal growth, and osteoporosis. Patients who are treated with prolonged courses of glucocorticoids should also take calcium and vitamin D supplements and be screened for osteopenia or osteoporosis if their diet and lifestyle place them at particular risk for these conditions.

Intravenous Immunoglobulin. Although IVIG provides potent blockade of the reticuloendothelial system in persons with immune thrombocytopenic purpura (ITP),[107-109] an inconsistent benefit has been shown for patients with AIHA.[110,111,112] The largest cohort of patients studied included 37 cases treated with IVIG in pilot studies from three institutions, combined with a review of 36 cases in the literature, both adult and pediatric.[111] Overall, all but four patients had received or were receiving prednisone therapy at the time of IVIG treatment, and nearly two thirds had received some previous therapy for AIHA. Patients received a range of IVIG doses, including 0.5 gm/kg/day for 5 days to 1 gm/kg/day for 5 to 7 days in the pilot studies, and a range of 0.4 to 2 gm/kg/day for 2 to 5 days in published cases. Overall, 40% had a response as measured by a rise in the hemoglobin level of greater than 2 gm/dL within 10 days of IVIG initiation. Six of 11 pediatric patients (55%) responded to IVIG, but whether they were receiving combined corticosteroid therapy is not reported.[111] Although based on a small number of cases, high-dose IVIG (a 5 gm/kg total dose over 5 days) has been shown to have limited efficacy for the treatment of AIHA.[111,113] In general, IVIG is not considered first-line treatment for AIHA in children, but it may be considered in a patient who is not responding to steroids.

Therapeutic Plasma Exchange. The role of therapeutic plasma exchange (TPE) in patients with warm AIHA is considered a category III intervention by the American Society of Apheresis, meaning it may be beneficial but insufficient evidence exists to establish efficacy.[114] Individual cases have been reported in which plasmapheresis has been efficacious.[115-117] Whether TPE increases the survival of transfused RBCs in patients with severe hemolysis is controversial.[118,119] Because IgG is mostly extravascular and is absorbed onto RBCs at body temperature, TPE may not efficiently remove the RBC autoantibodies. In contrast, IgM is mostly intravascular and binds poorly to RBCs at body temperature, and thus in persons with CAD, TPE can significantly reduce antibody titers, which are higher, particularly in adults.[114] Whether TPE increases the efficacy of RBC transfusions in patients with severe hemolysis is controversial.[118,119] However, plasmapheresis may be considered as a temporizing measure in a severely ill child who has a suboptimal response to RBC transfusion and may not have had time to respond to corticosteroid therapy.

Treatment of Chronic or Refractory AIHA

For a child with chronic or refractory AIHA, more aggressive therapy is often required to maintain a stable hemoglobin and quality of life. Long-term use of corticosteroids is not desirable given the numerous adverse effects.

Several additional therapeutic options should be considered and should be individualized for each patient based on his or her hematologic response and the adverse effects. Splenectomy and rituximab are the only second-line treatments with proven short-term efficacy.

Rituximab. Rituximab is a chimeric human IgG1/K monoclonal antibody specific for the CD20 antigen, which is expressed on B lymphocytes. It induces rapid in vivo depletion of both normal B lymphocytes and lymphoma B cells.[120,121] Its mechanism of action includes complement-mediated cytotoxicity, inhibition of B-cell proliferation, and induction of apoptosis.[120] Because of its clinical efficacy in B-cell lymphoma and its limited toxicity profile, its use has expanded to many autoimmune diseases, as well as benign hematologic diseases.[122,123] Three cohort studies have described the use of rituximab for refractory AIHA in 25 children, all of whom failed to respond to conventional therapies.[19-21] The standard dose of rituximab (375 mg/m^2) was administered weekly for 1 to 6 weeks (a median of four doses). More than 90% of patients had a complete response that lasted 7 to 28 months (a median of 16 months), and only three responders experienced a relapse. All three children who experienced a relapse were treated with another course of rituximab and achieved remission. Numerous single case reports and case series regarding the use of rituximab for AIHA in pediatric patients have been published, and nearly all reported a complete response.[122,124-134] In the largest study by Zecca and colleagues,[19] it was reported that 13 of 15 children (87%) with refractory AIHA responded to treatment with at least a 1.5 gm/dL increase in hemoglobin level observed at a median of 12 days from the time the first dose of rituximab was administered (range, 5 to 72 days).[19] In the responders, a decrease in the absolute reticulocyte count of more than 50% occurred at a median of 21 days (range, 5 to 82 days). Most patients responded within 2 to 4 weeks from the time of the initial dose. Other therapies should be considered if no hematologic response occurs within 2 months.[19,20,124,134] Given the high degree of efficacy of rituximab in treating refractory AIHA in pediatric patients, early use should be considered, and its use should also be considered for patients who respond to steroids but have significant adverse effects. Patients taking corticosteroids before the initiation of rituximab should continue to take steroids until a response to rituximab is clearly established. Although published data that compare rituximab or splenectomy as a second-line therapy are lacking, the trend has been to use rituximab first.

From a safety perspective, rare complications of rituximab include severe infusion reactions, infection, progressive multifocal leukoencephalopathy, and reactivation of hepatitis B and fulminant hepatitis.[123] Untreated hepatitis B infection is a contraindication to rituximab therapy. Because treatment with rituximab causes marked B-cell depletion, with levels remaining low for 2 to 6 months and returning to pretreatment levels after 6 to 12 months,[135] immunizations should be delayed until the B-cell levels return to baseline. After rituximab treatment younger children appear to have a higher risk of transient hypogammaglobulinemia, which can be treated with prophylactic administration of IVIG.[123]

Splenectomy. Splenectomy results in short-term complete and partial response in approximately two thirds of patients.[2,102,136] No long-term follow-up data are available for patients with AIHA who were treated with splenectomy, but approximately 50% of patients will remain in remission for years. In 53 patients with primary and secondary AIHA who underwent splenectomy, 63% had a hematologic remission defined by a hematocrit of greater than 30% without concurrent corticosteroid therapy after a mean follow-up of 33 months.[137] The perioperative risk of splenectomy is relatively low, because most cases can be performed laparoscopically. In a large national study, the mortality and morbidity of laparoscopic splenectomy for hematologic indications was 0.6% and 18%, respectively.[138] Complications included fever (7%), pleural effusion (4%), and actual or suspected hemorrhage (4%).

Because of the risk of postsplenectomy sepsis with encapsulated bacterial organisms (see Chapter 16), prior to surgery children should be immunized with the polyvalent polysaccharide vaccines against *Streptococcus pneumoniae* and *Neisseria meningitides* in addition to the routine infant immunizations against *S. pneumoniae* and *Haemophilus influenzae*. After undergoing a splenectomy, children should also receive penicillin prophylaxis twice a day for at least several years after surgery. Penicillin V potassium, 250 mg twice daily, is recommended for children older than 3 to 5 years, and a dose of 125 mg twice daily is recommended for younger patients. Some clinicians recommend lifelong penicillin prophylaxis because sepsis can occur years after a splenectomy.[139,140] Patients and families should be educated to seek prompt medical evaluation for fever because of the risk of bacteremia or sepsis in a person who has undergone a splenectomy. Relatively recent evidence suggests that splenectomy in patients with AIHA may increase the long-term risk of thromboembolism and pulmonary hypertension[141-144] (also see Chapter 16), and these possibilities should be considered and discussed with the patient and family. The timing of a splenectomy is controversial, because other approaches such as rituximab have become available and are relatively safe.

Alternative Immunotherapeutic Agents. Immunosuppressive and cytotoxic drugs are indicated in patients who do not respond to corticosteroids, rituximab, and splenectomy or for patients who have contraindications to those therapies. Cyclophosphamide, cyclosporine, azathioprine (6-mercaptopurine), and Campath-1H have been used to treat refractory AIHA, but published data on their efficacy are limited to case reports.[145-153] A response to many of these agents may take months, and thus treatment should be continued for up to 6 months before it is considered to have failed.[1] High-dose cyclophosphamide without stem cell rescue for refractory AIHA has induced complete and partial remissions.[154-156] Moyo and colleagues[156] treated nine adult patients who had severe refractory AIHA with cyclophosphamide, 50 mg/kg/day for 4 days. Six patients achieved complete remission with normal hemoglobin levels for their age and sex, and three patients had partial remissions, measured as a hemoglobin level of at least 10 gm/dL without transfusion support. Patients became RBC transfusion–independent after a median of 19 days (range, 15-31 days) after treatment.[156] High-dose cyclophosphamide was well tolerated, but common adverse effects included nausea, vomiting, and transient myelosuppression.

Cyclosporine, 6-mercaptopurine, and MMF (mycophenolate mofetil, Cell-Cept) are additional immunosuppressive agents that have induced clinical remission in some patients with refractory AIHA.[145,148,150,151] By affecting T-lymphocyte function, these agents interfere with autoantibody synthesis. Although cyclosporine has improved the course of AIHA in a few patients with steroid-refractory AIHA, it is not routinely used because of the adverse effects of nephrotoxicity, hypertension, and hirsutism.[145,149] Single-agent therapy with 6-mercaptopurine in pediatric patients with AIHA was reported as part of a case series of autoimmune cytopenias. All seven pediatric patients with refractory AIHA (five cases of primary AIHA and two cases of secondary AIHA) responded, with response defined as an increase in the hemoglobin level of at least 1.5 gm/dL and to a level of 10 gm/dL or greater.[151] The time required to achieve a hematologic response was not reported. The adverse effects of 6-mercaptopurine include leukopenia, thrombocytopenia, and elevated transaminase levels. MMF also inhibits lymphocyte proliferation and was assessed prospectively in a single-center study for adult refractory autoimmune cytopenias.[148] The study included three patients with AIHA that was resistant to high-dose steroids; two of these patients had also responded poorly to IVIG, and one had responded poorly to high-dose cyclophosphamide. All three patients with refractory AIHA responded and achieved a hemoglobin level greater than 10 gm/dL; they were transfusion independent after 4 to 6 months, and the drug was well tolerated. After this response was achieved, MMF was discontinued in one patient and tapered for the other two patients. None had relapsed at a follow-up of 49, 40, and 13 months.[148] Lastly, Campath-1H monoclonal antibodies may improve refractory disease but do not appear to induce a prolonged remission,[152,153] and the adverse effects include significant immunosuppression.

Danazol. Danazol, a semisynthetic androgen, has been efficacious for some adults with AIHA,[157-159] but it has not been studied in children. Synergistic effects of danazol and corticosteroids have been suggested in two studies.[157,159] A rise in the hematocrit level is usually evident within 1 to 3 weeks.[160] The mechanism of action for danazol is not clear, but decreased titers of cell-bound IgG and complement have been reported in patients who had a response.[160] Because of its potential androgenic adverse effects, it is not typically recommended for young females, and the potential liver toxicity makes its long-term use in young children difficult.

Hematopoietic Stem Cell Transplantation. Complete lymphoid and myeloablation with hematopoietic stem cell transplantation (HSCT) is a potential option. HSCT has been explored to treat a variety of autoimmune diseases.[161] Its use in patients with AIHA has been reported with limited success.[162-164] In a study of 36 patients with severe refractory autoimmune cytopenia, two patients underwent allogeneic HSCT and five patients underwent autologous HSCT. The two patients who underwent allogeneic HSCT achieved continuous remission, and of the five patients who underwent autologous HSCT, one died of treatment-related causes, one died of progressive disease, two had no response, and one had a transient response.[162] A single case report describes successful autologous HSCT in a child with refractory AIHA.[163] Despite significant advances in the management of high-dose chemotherapy and HSCT, this treatment option should be reserved for patients with severe life-threatening disease for whom all other therapies have failed.

Treatment Considerations for Cold-Reactive AIHA. In children, CAD typically occurs after an infection, is associated with *M. pneumoniae* or viral infections, and usually only requires supportive care with RBC transfusion (Table 13-3).[15-17,78,79] Importantly, the patient should be kept warm and instructed to avoid cold stimuli. Patients may have a mild, compensated anemia that may not require treatment or transfusions. For persons with moderate to severe anemia, transfusion is necessary. In contrast to the serologic problems encountered in persons with warm AIHA, cold IgM-mediated antibodies do not interfere with pretransfusion testing, and it is usually easy to find compatible donor erythrocytes. If transfusion is indicated, a blood warmer must be used to warm the RBCs as they are infused. In severe cases of cold, IgM-mediated AIHA, plasmapheresis can efficiently remove autoantibodies and improve the clinical course.[114] Exchange procedures should also be performed with an in-line blood warmer for infusion of the plasma replacement.

The treatment of PCH is also supportive with transfusion, because the anemia is often moderate to severe at presentation.[17,78] Although the specificity of the Donath-Lansteiner antibody is directed at the P antigen, P^- donor RBCs are rare, and most patients achieve an adequate posttransfusion hematocrit level with P^+ RBCs. Although cold-induced hemolytic symptoms are not characteristic, the patient should be kept warm and the use of a blood warmer for RBC transfusions is prudent despite the lack of data to support this practice. Steroids are often initiated empirically for treatment of AIHA, but because no evidence exists to support their benefit, they can be discontinued once the diagnosis of PCH is confirmed with the Donath-Landsteiner assay.[80]

Pathogenesis of Autoantibody Formation

The pathogenesis of AIHA is far from clear, and many mechanisms underlying autoantibody production have been proposed. These mechanisms involve autoantigens, the lack of effective presentation of autoantigens, and functional abnormalities of B and T cells.[1] Most evidence suggests that the basis of disease is due to a breakdown in self-tolerance mechanisms toward RBC antigens.

The most common targets of RBC autoantibodies are the Rh blood group proteins, but other blood group antigen specificities, including antibodies directed at glycophorins, band 3, and the RBC anion channel protein have been documented. Cross-reactivity between environmental antigens and autoantigens, termed *molecular mimicry*, has been proposed as the mechanism to explain such autoantibodies.[165,166] However, although deletion of the dominant autoantigen in New Zealand Black mice with AIHA demonstrated a defect in self-tolerance directed at the RBC, the autoantibodies were not specific to one RBC antigen.[167] In New Zealand Black mice with AIHA, this T-helper cell cross-reactivity between the helper-dominant epitope and an exogenous antigen illustrates how specific therapy may be needed to induce immune tolerance.

Self versus nonself discrimination is dependent on induction of tolerance in the thymus and bone marrow by deleting, receptor-editing, or anergizing self-reactive T- and B-cell clones.[168,169] Antigen-presenting cells such as dendritic cells and macrophages present self-antigens to induce T-cell tolerance. One explanation for the lack of effective presentation of autoantigens is that many self-epitopes may be inefficiently processed and presented by antigen-presenting cells. The T cells stay naïve and remain part of the T-cell repertoire rather than being eliminated by the thymus.[170] A genetic predisposition for AIHA has also been proposed involving human leukocyte antigen (HLA) genes that encode the cell surface antigen–presenting proteins. Although no large study has examined the role of HLA genetic polymorphisms and AIHA, an association between the development of AIHA and HLA-B8 and HLA-B27 has been reported.[171,172]

RBC autoantibodies in patients with AIHA are polyclonal with multiple IgG isotypes and, sometimes, IgA antibodies. Sequence analysis of the immunoglobulin gene rearrangements used by B-cell clones from patients with cold agglutinins suggest that typical in vivo polyclonal antigen-driven selection and mutation occurs.[173] Of note, Stahl and colleagues[174] analyzed the self-reactive IgG and IgM antibody repertoires of patients with warm AIHA using quantitative immunoblotting on a panel of whole-tissue extracts representing sources of self-antigens. They demonstrated that the self-reactive IgG repertoires purified from plasma and from RBC eluates did not differ between healthy donors and patients with warm AIHA, whereas the IgM repertoires from patients showed broadly altered patterns of reactivity compared with those of control subjects. The observation that the IgG autoantibodies of patients with warm AIHA are similar to natural-occurring anti-RBC autoantibodies of healthy donors suggests that dysregulation of IgG autoreactivity may be an underlying mechanism for AIHA.

The role of CD4+ regulatory T cells in AIHA has also been studied, because T regulatory cells have been shown to play a key role in immunologic tolerance and in autoimmune diseases.[175] In patients, autoantibodies and activated autoreactive CD4+ T-helper cells that secrete interferon-γ are specific for Rh proteins on the RBC.[166] It has also been demonstrated that T regulatory cells specific for epitopes on the Rh proteins are present in the peripheral blood and spleens of patients with AIHA and that these cells can inhibit T-helper cells by secretion of the suppressive cytokine interleukin (IL)-10.[176,177] This role for regulatory T cells has been corroborated in murine models of AIHA in which C57/Bl6 mice depleted of CD4+CD25+ regulatory T cells developed AIHA at a very high incidence after repeated immunization with rat RBCs.[178] Adoptive transfer of purified CD4+CD25+ regulatory T cells but not CD4+CD25− T cells from immunized mice into naïve recipients prevented the induction of AIHA.

The roles of complement activation and cytokine production in the pathophysiology of AIHA have also been investigated. One study has reported that patients with primary or secondary AIHA may have a deficiency of CD59 expression on their RBCs, which may stimulate abnormal complement activation.[179] Patients with AIHA have also been shown to have high serum levels of IL-1α, IL-2, IL-4, IL-2R, IL-6, IL-10, and IL-13.[180-182] One proposed mechanism is that an imbalance between IL-10 and IL-12 production leads to autoantibody production.[180] Taken together, the mechanism of autoantibody production in persons with AIHA is likely complex and involves polyclonal autoreactive B cells that may be present in healthy persons but expand in genetically susceptible persons or in persons with dysregulation of T regulatory cells or antigen presentation, with subsequent loss of self-tolerance mechanisms.

SECONDARY AUTOIMMUNE HEMOLYTIC ANEMIA

Secondary AIHA can occur as a manifestation of an underlying pathophysiologic disorder. All patients with AIHA should be evaluated for the presence of concurrent autoimmune disease, immunodeficiency, lymphoproliferative disorders, connective tissue diseases, malignancy, infection, and drugs that may induce immune-mediated hemolytic anemia (Box 13-2). In pediatric studies, AIHA was classified as secondary in 24% to 63% of cases.[10-13,183] In the largest and most recent study, 26 of 265 cases (10%) were purely postinfectious, and in 141 of 265 cases (53%), an underlying immunologic disorder was found.[11] Notably, immunologic conditions were discovered after a median follow-up of 2 years in 23 of 49 cases (47%) that were associated with a well-defined infection at time of AIHA presentation. Underlying autoimmune diseases included Evans syndrome in the majority but also included cases associated with autoimmune hepatitis, SLE, thyroiditis, Graves disease, vitiligo, giant cell hepatitis, rheumatic disease, type 1 diabetes mellitus, and Crohn disease. Underlying primary immunodeficiencies included CVID, combined immunodeficiency, autoimmune lymphoproliferative syndrome (ALPS), adenosine deaminase deficiency, Wiskott-Aldrich syndrome (WAS), and HLA class 2 deficiency.[11] The diagnosis of an autoimmune disease such as AIHA in children younger than 1 year of age warrants evaluation for an underlying immunodeficiency. Because many of the diagnostic tests may be affected by corticosteroid or immunosuppressive agents, the sample for laboratory evaluation should be

Box 13-2 **Etiology of Secondary Immune Hemolytic Anemias**

AUTOIMMUNE DISEASE

Evans syndrome
Systemic lupus erythematosus
Autoimmune hepatitis
Autoimmune thyroiditis
Graves disease
Vitiligo
Giant cell hepatitis
Rheumatic disease
Type 1 diabetes mellitus
Crohn disease
Ulcerative colitis

IMMUNODEFICIENCY

Common variable immunodeficiency
Combined immunodeficiency
Adenosine deaminase deficiency
HLA class 2 deficiency
HIV/AIDS
Wiskott-Aldrich syndrome

LYMPHOPROLIFERATIVE DISORDERS

Autoimmune lymphoproliferative disease

MALIGNANCY

Acute leukemia
Lymphoma
Myelodysplasia

INFECTION

Epstein-Barr virus
Mycoplasma pneumonia
Parvovirus B19
Cytomegalovirus
Varicella
Hepatitis C
Rubella

DRUGS

Piperacillin, cefotetan, ceftriaxone (most common)

AIDS, Acquired immunodeficiency syndrome; *HIV,* human immunodeficiency virus; *HLA,* human leukocyte antigen.

sent prior to the initiation of therapy if the patient is clinically stable.

Evans Syndrome

Evans syndrome was first described by Evans and colleagues more than 60 years ago as a disorder of ITP and AIHA, in the absence of known underlying systemic disease.[22] Although the definition of Evans syndrome for many past studies had been restricted to RBC and platelet destruction, the diagnosis can be any combination of ITP, AIHA, and/or autoimmune neutropenia, or immune pancytopenia. Autoantibodies are directed at specific antigens on erythrocytes, platelets, or neutrophils but are not cross-reactive.[184] The exact frequency of Evans syndrome is unknown, but it accounts for 13% to 73% of published pediatric AIHA cases.[11,185-187] A high proportion of overlapping cases of Evans syndrome and ALPS has been suggested.[188,189] Evans syndrome appears to be a disorder of immune regulation with abnormalities in both cellular and humoral immunity,[187,190-192] but the exact pathophysiology is unknown.[193]

Patients may present with AIHA, ITP, and/or autoimmune neutropenia either concurrently or sequentially. Evans syndrome is a chronic disorder, characterized by frequent exacerbations and remissions. Neutropenia occurs in up to 55% of patients at presentation.[22,185-187,192] Often the second cytopenia occurs months to years after the first and may delay the diagnosis. The clinical presentation includes the typical features of hemolytic anemia (jaundice, pallor, lethargy, or dark urine), thrombocytopenia (easy bruising, mucocutaneous bleeding, or petechiae), and/or neutropenia (fever). Physical examination may reveal splenomegaly, hepatomegaly, and/or lymphadenopathy.

Laboratory examination should include a complete blood cell count to determine the presence of cytopenias and a peripheral blood smear not only to evaluate for spherocytes consistent with AIHA but also to exclude other causes. A reticulocyte count, DAT, and total and unconjugated bilirubin levels will aid in the diagnosis, revealing similar findings as in isolated AIHA. Antiplatelet and antineutrophil antibody assays can evaluate for cell-specific autoantibodies in the patient's sera, but these tests may be unreliable, and a negative result does not exclude the presence of the respective autoantibodies. Conversely, anti-HLA antibodies can cause false-positive results. Serum immunoglobulin levels and subclasses should be measured in all patients, because acquired cytopenias can develop in patients with immunodeficiencies.[194,195] Screening tests for SLE and other autoimmune diseases should be strongly considered, and include the measurement of antinuclear antibody, double-stranded deoxyribonucleic acid, and rheumatoid factor. Finally, Evans syndrome can be distinguished from ALPS by determination of peripheral blood T-cell subsets by flow cytometry. Elevation of double-negative (CD4–/CD8–, CD3+, TCRαβ+) T cells is a relatively sensitive screening test for ALPS.[189]

Importantly, Evans syndrome is a diagnosis of exclusion, and by definition, other underlying disorders must not be present.[22] In pediatric cases, the causes of acquired cytopenias to consider in the differential diagnosis include ALPS, SLE, CVID, IgA deficiency, and human immunodeficiency virus/acquired immunodeficiency virus syndrome, all of which require therapies distinct from that for Evans syndrome. Evans syndrome may also develop after autologous or allogeneic stem cell transplantation[196,197] or be associated with Castleman disease.[198,199]

The management of Evans syndrome remains a challenge. No evidence-based studies have established treatment guidelines, but it is reasonable to treat symptomatic cytopenias including moderate to severe anemia, thrombocytopenia with bleeding, or prolonged and/or severe neutropenia (absolute neutrophil count <250 to 500/μL). No randomized, controlled studies have been performed in persons with Evans syndrome, and most published studies include small numbers of patients. RBC transfusions may be needed prior to the initiation of therapy (see the previous discussion of AIHA treatment). The most commonly used first-line therapy is corticosteroids with or without IVIG. In two small studies with a total of 17 children with Evans syndrome, all responded initially to prednisolone, 1 to 2 mg/kg/day, but nearly all experienced a recurrence upon cessation or tapering of the steroid dose.[186,187] Therefore, although steroids are useful in the acute setting, most patients will require adjuvant or alternative therapy to sustain a remission because long-term high-dose steroid use has many significant adverse effects (see the previous discussion of AIHA treatment). IVIG is also a common first-line therapy, particularly for patients who do not respond well to steroids or who experience steroid toxicity. IVIG as a single agent has not been reported for treatment of Evans syndrome; its use has been described in conjunction with steroids or after steroid failure. In a national survey of 42 patients with a median age of 7.7 years, single or multiple courses of IVIG were given with steroids to 40 patients; responses were varied.[185] Nine patients had a complete remission with responses lasting up to 2 years (all of these patients also continued to receive corticosteroids), two patients initially responded but then the disease became refractory after two courses, 24 patients had transient responses lasting up to 4 weeks, and five patients had no response.[185] These limited data suggest that corticosteroids and IVIG have a role as first-line management of Evans syndrome, which is common practice. Repeated courses of IVIG may be helpful for some patients.

Rituximab, a chimeric anti-CD20 monoclonal antibody, is an effective second-line therapy for persons with Evans syndrome, with most published experience in patients who either experienced a recurrence or failed to respond to corticosteroids, IVIG, or other immunosuppressive agents. The efficacy of rituximab was evaluated prospectively in a multicenter study for children with refractory AIHA, including five patients with Evans syndrome.[19] Treatment consisted of weekly rituximab, 375 mg/m^2, for three to four doses. All five patients also received concomitant therapy with steroids, cyclosporin, or azathioprine. All five patients responded with a greater than 1.5 gm/dL rise in hemoglobin level and an increase in platelets from a median of 27×10^9/L before treatment to 140×10^9/L 2 months after treatment. The other immunosuppressants were tapered and discontinued within 25 weeks. Three patients had continued remission with a mean follow-up of 13 months. Two patients experienced a relapse at 7 and 8 months, but both achieved a second remission with a subsequent course of rituximab.[19] In a retrospective study of 17 children with Evans syndrome who also were treated with weekly doses of rituximab—375 mg/m^2 for three to four doses associated with prednisone alone (14 patients) or other immunosuppressive agents—complete or partial remission of at least one cytopenia was achieved in 13 patients (76%).[200] The mean time to complete or partial remission of AIHA and ITP was 1.5 months and ranged from 0.2 to 4 months. Eleven of the 13 patients had sustained responses with a mean follow-up of 2.4 years (range, 0.5 to 7 years), with discontinuation or a greater than 50% tapering of corticosteroids. Depletion of peripheral CD19+ B cells occurred in all 12 patients who were tested, from a baseline mean of 28% to less than 1%, including three nonresponding patients. This depletion lasted 2 to 7 months in five patients who were tested.[200] In both of these studies, therapy was well tolerated. Nearly all patients received replacement IVIG for 4 to 10 months to prevent therapy-induced hypogammaglobulinemia.[19,200] Given the relatively rapid response in most patients who did not respond to corticosteroids, IVIG, and/or other immunosuppressants, along with the safety profile of this drug, rituximab should be considered as second-line treatment early in the course of patients with Evans syndrome who do not respond to steroids. Immunoglobulin levels should be monitored, and replacement IVIG should be administered for low IgG levels.

Additional second-line therapies for Evans syndrome include splenectomy, cyclosporine, MMF (mycophenolate mofetil, Cell-Cept), vincristine, and danazol. Most of the data are anecdotal and difficult to interpret because corticosteroids or other agents were also used. Common second-line agents are cyclosporin or MMF. Combination therapy with cyclosporin (5 to 10 mg/kg/day initially) and prednisone has been successful in patients with refractory Evans syndrome.[201,202] Liu and colleagues[203] found an improved complete response rate in a large series of adult patients with AIHA and Evans syndrome who were treated with prednisone and danazol when cyclosporin was added to the regimen.[203] Several case reports suggest that MMF is well tolerated, can result in reduced use of steroids, and may be used as an alternative therapy for persons with refractory Evans syndrome.[148,204,205]

The role of splenectomy in persons with Evans syndrome is not clearly established. In general, the response rate is lower than in persons with uncomplicated ITP. Splenectomy can achieve improvement of cytopenias, but the response is often transient and relapse occurs in most cases 1 to 2 months later.[185-187] Nonetheless, sustained complete remission has occurred[187] and may also reduce the frequency of relapses and allow lowering of maintenance doses of steroids.[186,187] Given the relatively poor response rates to splenectomy, the inherent risks (see the previous discussion of AIHA treatment), and increasing data and experience with rituximab, splenectomy should be reserved for patients who do not respond to these other second-line therapies.

For patients with severe, relapsing Evans syndrome who do not respond to first- and second-line therapies, remaining options include cyclophosphamide, alemtuzumab, and hematopoietic stem cell transplantation. Cyclophosphamide has induced remission of thrombocytopenia in patients with Evans syndrome at low doses (1 to 2 mg/kg/day oral),[187] intermediate doses,[206] and high doses without stem cell support in an adult with refractory disease.[155] Alemtuzumab (Campath-1H) is an IgG monoclonal antibody directed against the CD52 antigen on T and B lymphocytes. Three patients with refractory Evans syndrome were treated with Alemtuzumab, 10 mg/day for 10 days, and two of three responded initially but subsequently experienced a relapse within 3 months.[153] HSCT has been used to treat severe refractory autoimmune cytopenia.[162,207] In a recent analysis of long-term outcomes of pediatric patients registered in the European Group for Blood and Bone Marrow Transplantation database, of 22 patients who underwent HSCT for refractory autoimmune cytopenias, 11 had Evans syndrome.

Two underwent autologous HSCT; one patient had an initial response and then experienced a relapse, and the other patient had no response. Nine patients received an allogeneic HSCT; five achieved continuous remission, one experienced a relapse, and three died of treatment-related causes.[207] These limited data suggest that a role exists for allogeneic HSCT in the treatment of severe refractory Evans syndrome, but treatment-related toxicity remains high.

Acute Lymphoproliferative Syndrome

ALPS, an inherited disorder of abnormal lymphocyte survival caused by dysregulation of the Fas apoptotic pathway, was first described in 1967.[208] Children with ALPS have defective lymphocyte apoptosis as a result of the failure of developing lymphocytes to undergo Fas-mediated cell death, resulting in an excess of T-cell receptor αβ+CD3+CD4−CD8− T (double-negative T [DNT]) lymphocytes that accumulate in the lymph nodes, spleen, and peripheral blood.[209] This disorder of disrupted lymphocyte homeostasis results in chronic lymphoproliferation, autoimmune disease, and an increased risk of malignancy. The clinical presentation includes childhood onset of chronic lymphadenopathy, splenomegaly, multilineage cytopenias resulting from sequestration and autoimmune destruction, and an increased risk of B-cell lymphoma.[209-211]

This presentation resembled two related mouse strains with lymphoproliferative phenotypes, the *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) mice, which were found to have mutations in Fas and Fas ligand, respectively.[212,213] Subsequently, *FAS* mutations were identified in human patients with lymphoproliferative disorders and a failure of lymphocyte apoptosis.[214,215] Laboratory evaluations demonstrated polyclonal hypergammaglobulinemia and expansion of DNT lymphocytes.[209,211] Most patients with ALPS (60% to 70%) carry heterozygous germline mutations in *FAS*, which are inherited in an autosomal-dominant fashion,[216,217] but approximately 10% of *FAS* mutations are acquired.[218,219] Mutations in caspase 8, caspase 10, and neuroblastoma RAS (NRAS) have also been identified in fewer than 5% of patients with ALPS.[220-222] No genetic defect has been found in up to 30% of patients with ALPS.[223]

In 2009, the National Institutes of Health International Workshop for ALPS revised the diagnostic criteria and classification for ALPS,[224] with less focus on a functional apoptosis assay and expanded incorporation of genetic defects and biomarkers. Previously a triad of criteria including chronic nonmalignant lymphadenopathy and/or splenomegaly, increased peripheral DNT cells, and a lymphocyte apoptosis assay defect were used to make the diagnosis of ALPS. Supporting criteria included a family history of ALPS, classic histopathologic features, and autoimmune manifestations. The 2009 revised criteria for ALPS divides diagnostic criteria into two required criteria and six accessory criteria (Box 13-3).[224] For a definitive diagnosis, a patient must meet both required criteria and at least one of the accessory criteria. Required criteria include chronic nonmalignant, noninfectious lymphadenopathy and/or splenomegaly, as well as an elevated level of DNTs (≥1.5% of total lymphocytes or 2.5% of CD3+ lymphocytes). Accessory criteria are divided into primary and secondary. Primary accessory criteria include an abnormal lymphocyte apoptosis assay and the presence of pathogenic mutations in genes of the FAS pathway. Secondary criteria include the presence of elevated circulating biomarkers, characteristic histopathologic features, the combined presence of cytopenias, polyclonal hypergammaglobulinemia, and a family history consistent with ALPS. An abnormal lymphocyte apoptosis assay is no longer considered essential for the diagnosis of ALPS because patients with somatic *FAS* mutations and germline *FASLG* mutation have demonstrated normal results in FAS-induced apoptosis assays.[218,225,226]

Patients with unexplained autoimmune disease, unexplained lymphoproliferation, combined cytopenias, a single cytopenia and hypergammaglobulinemia, or autoimmune disease of a second organ system should be evaluated for ALPS.[227] Recommended screening tests include DNT cell, IgG, and vitamin B12 levels. If screening tests are suggestive of ALPS, molecular testing to distinguish the different subtypes of ALPS should be performed. The current classification system includes five subtypes, categorized by the genetic defect: ALPS-FAS

Box 13-3 **2009 Revised Diagnostic Criteria for Autoimmune Lymphoproliferative Disease***

REQUIRED

1. Chronic (>6 months), nonmalignant, noninfectious lymphadenopathy or splenomegaly or both
2. Elevated CD3+TCRαβ+CD4−CD8− DNT cells (≥1.5% of total lymphocytes or 2.5% of CD3+ lymphocytes) in the setting of normal or elevated lymphocyte counts

ACCESSORY

PRIMARY

1. Defective lymphocyte apoptosis (in two separate assays)
2. Somatic or germline pathogenic mutation in *FAS, FASLG*, or *CASP10*

SECONDARY

1. Elevated plasma sFASL levels (>200 pg/mL) OR elevated plasma interleukin-10 levels (>20 pg/mL) OR elevated serum or plasma vitamin B12 levels (>1500 ng/L) OR elevated plasma interleukin-18 levels (>500 pg/mL)
2. Typical immunohistologic findings as reviewed by an experienced hematopathologist
3. Autoimmune cytopenias (hemolytic anemia, thrombocytopenia, or neutropenia) AND elevated immunoglobulin G levels (polyclonal hypergammaglobulinemia)
4. Family history of a nonmalignant/infectious lymphoproliferation with or without autoimmunity

Modified from Oliveiera JB, Bleesing JJ, Dianzani U, et al: Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. Blood *116:e35–e40, 2010.*

DNT, Double-negative T cells.

*A definitive diagnosis is based on the presence of both required criteria plus one primary accessory criterion. A probable diagnosis is based on the presence of both required criteria plus one secondary accessory criterion.

(germline homozygous or heterozygous mutations in *FAS*), ALPS-sFAS (somatic mutations in *FAS*), ALPS-FASLG (germline mutations in the FAS ligand), ALPS-CASP10 (germline mutations in caspase 10), and ALPS-U (undetermined genetic defect and *FAS*, *FAS*L, or *CASP10* mutations excluded). The revised classification system considers patients with mutations in caspase-8 (*CASP8*) and NRAS as separate entities given their distinct phenotypes.[224] Patients with *CASP8* mutations present with lymphadenopathy, splenomegaly, marginal elevation of DNTs, and defective FAS-induced lymphocyte apoptosis, but they also show defective T-cell, B-cell, and natural killer–cell activation and are at risk for recurrent bacterial and viral infections.[220] Patients with the *NRAS* mutations may not show elevated DNT cells and may have elevated myeloid cells and a clinical presentation that overlaps with juvenile myelomonocytic leukemia.[224]

The treatment of ALPS is distinct from the treatment of other autoimmune cytopenias. Rituximab and splenectomy are often the treatments of choice for chronic refractory cytopenias such as ITP or AIHA, but these treatments have been shown to have unexpected ALPS-specific toxicities and thus should not be first-line therapies.[223] Patients with ALPS who receive rituximab may experience prolonged and significant hypogammaglobulinemia that may result in prolonged intravenous IgG replacement therapy.[228,229] Splenectomy should be avoided in patients with ALPS because of a very high risk of sepsis after the splenectomy, even with prophylactic antibiotic administration and proper immunizations.[230,231] The typical initial management of patients with ALPS-related cytopenias is immune suppression with corticosteroids. High-dose pulse therapy with intravenous methylprednisolone (5 to 10 mg/kg) followed by oral prednisone (1 to 2 mg/kg) maintenance therapy that is then tapered over several weeks (8 to 12 weeks) has been suggested.[230] IVIG in combination with corticosteroids may be used for severe cases of AIHA, and low-dose granulocyte colony-stimulating factor (1 to 2 μg/kg) two to three times weekly is indicated for patients with isolated chronic neutropenia and associated infections. Subsequent long-term, steroid-sparing therapy is often needed. MMF (CellCept) and sirolimus (rapamycin) are two immunosuppressants that have been relatively well studied and are effective in patients with ALPS.[232-234] More than 80% of patients treated with MMF demonstrate measurable responses for their cytopenias, but MMF does not necessarily improve lymphoproliferation or reduce DNTs.[223,230] Sirolimus, a mammalian target of rapamycin inhibitor, has also been used to successfully treat patients with ALPS,[234] with improved cytopenias, lymphoproliferation, and reduction of DNTs.[230] Despite the successful responses with MMF and sirolimus, the risk-benefit ratio of long-term immunosuppression must be considered for each individual patient with ALPS.

Immunodeficiency

Immune cytopenias can occur in patients with a congenital or primary immunodeficiency as a result of immune dysregulation. Patients with CVID can present with AIHA or ITP.[235-237] In a large cohort of patients, the estimated frequency of AIHA in adult patients with CVID was 5.5% (14 of 252 patients total). CVID was diagnosed before the onset of AIHA in only two patients (11%); the remainder presented with AIHA first, and the diagnosis of CVID was confirmed later.[236] CVID, diagnosed by reduced serum IgG and reduced serum IgA and/or IgM, is characterized by recurrent infections but also an increased risk of autoimmune disease. Autoimmune disease, most commonly ITP and AIHA, develops in approximately 20% to 22% of patients with CVID.[195,238] Treatment of AIHA in patients with CVID is similar to treatment of primary AIHA, with the majority of patients responding to steroids (with and without IVIG in combination), splenectomy, or rituximab.[236,237] However, severe infections have occurred in patients with CVID after treatment with a splenectomy or rituximab.

Patients with WAS may also have immune-mediated cytopenias, including AIHA and ITP. In a large single-institution study, AIHA was diagnosed in 20 of 55 patients (36%) who had WAS by the age of 5 years.[239] Other immunosuppressed populations are also at risk of AIHA, including patients with human immunodeficiency virus[240,241] and patients receiving immunosuppressive therapy after solid organ or hematopoietic transplants.[242,243]

Other Systemic Disorders

A common form of secondary AIHA occurs in patients with SLE.[127,244] Patients with other generalized autoimmune disorders have also been associated with AIHA, including Sjögren syndrome,[245,246] scleroderma,[247,248] dermatomyositis,[249,250] ulcerative colitis,[251,252] Crohn disease,[253,254] and autoimmune thyroiditis.[255] AIHA can also occur in the setting of malignancy, which, in children and adolescents, is associated with acute leukemia,[115,256] lymphoma,[257,258] and myelodysplasia.[259] It is presumed that the underlying immune dysregulation in these systemic disorders causes production of erythrocyte autoantibodies.

Infections

The most common infectious agents identified with childhood AIHA are EBV, mycoplasma, parvovirus, and cytomegalovirus.[11] Varicella,[74] hepatitis C,[260] and rubella[261] have also been documented. The majority of children with PCH have a history of a recent upper respiratory infection or other viral illness with fever, but an infectious pathogen is often not found.[78] *M. pneumoniae* is often associated with anti-I, and EBV is commonly associated with anti-i. Of note, RBCs typically lose i antigen and gain I antigen during the first 18 to 24 months of life.[262] Therefore anti-i autoantibodies do not typically cause significant hemolysis in adults but can be clinically significant in some pediatric cases.[263]

Drugs

Drugs can cause hemolytic anemia through an immune or a nonimmune mechanism. Drug-dependent antibody and autoimmune induction are the primary immune causes, and drug adsorption is the major nonimmune mechanism; all three mechanisms may cause hemolysis.

DIIHA is a relatively uncommon cause of AIHA in childhood, but it is important to recognize because the main therapy is discontinuation of the drug. The three most common drugs causing DIIHA are piperacillin, cefotetan, and ceftriaxone, but more than 130 drugs have been implicated.[47]

Piperacillin is a semisynthetic penicillin that has caused more than 25 reported cases of immune hemolysis, most commonly in patients with cystic fibrosis.[264] In the majority of cases, the hemolysis was acute and severe, and it was fatal in at least one patient.[264-266] In a case series of eight patients, the DAT was positive in all cases, and six of the eight patients demonstrated a serologic profile (positive IgG and C3d DAT, negative antibody screening test, and negative eluate) that is more typical for DIIHA than for any other type of immune hemolysis.[264] Cefotetan is a second-generation cephalosporin that binds firmly to RBCs and can induce IgM or IgG-mediated hemolysis. Cefotetan-induced hemolytic anemia can occur with the first dose and can cause severe and life-threatening episodes.[267-269] Ceftriaxone, a third-generation cephalosporin, is characterized by a rapid acute intravascular hemolysis occurring within minutes to hours after drug administration. The first several cases reported were fatal.[270-273] Arndt and colleagues[274] reported the serologic characteristics of ceftriaxone antibodies in 25 patients with DIIHA. Seventeen (68%) of the patients were children, and the hemolysis was dramatic and severe in pediatric cases. Thirty-six percent of the cases were fatal. The antibodies were primarily IgM, all of the DATs were reactive with anti-C3, and approximately half were reactive with anti-IgG.

Piperacillin, cefotetan, and ceftriaxone-induced immune hemolytic anemia can mimic AIHA and delayed hemolytic transfusion reactions in certain patients, both clinically and serologically. Diagnostic confusion is less common with ceftriaxone because the onset is usually rapid and occurs within hours of receiving the drug. The mimicking effect can be distinguished by testing for the antidrug antibodies, and discontinuation of the drug leads to rapid resolution of the hemolytic anemia and disappearance of the serologic reactions with untreated allogeneic RBCs and the patient's serum.

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CHAPTER 14

Paroxysmal Nocturnal Hemoglobinuria

Charles J. Parker and **Russell E. Ware**

CHAPTER OUTLINE

HISTORY

EPIDEMIOLOGY

MOLECULAR PATHOGENESIS

Paroxysmal Nocturnal Hemoglobinuria and Complement

Decay Accelerating Factor

Membrane Inhibitor of Reactive Lysis

Inherited Mutations of CD55 and CD59

Congenital Abnormalities of Genes Involved in Glycosyl Phosphatidylinositol–Anchored Protein Synthesis

Paroxysmal Nocturnal Hemoglobinuria and Deficiency of Glycosyl Phosphatidylinositol–Anchored Protein

A Two-Step Model of Paroxysmal Nocturnal Hemoglobinuria Pathogenesis

CLINICAL MANIFESTATIONS

Hemoglobinuria

Episodic Hemolysis

Paroxysmal Nocturnal Hemoglobinuria and Bone Marrow Failure

Thromboembolic Complications

Renal Abnormalities

Dysphagia and Male Impotence

Infections

Physical Examination

DIAGNOSIS

Management Based on Classification

COURSE AND PROGNOSIS

Course and Prognosis in the Pre-Eculizumab Era

Course and Prognosis in the Eculizumab Era

CONCLUSIONS AND FUTURE DIRECTIONS

Medical textbooks usually include paroxysmal nocturnal hemoglobinuria (PNH) among the hemolytic anemias (as is the case herein). Doing so is not wrong because hemolysis is a defining feature of the disease. However, placing too much emphasis on the hemolytic component of the disease risks obscuring the essence of PNH. Two features distinguish PNH from all other hemolytic anemias. First, and most importantly, the abnormality that underlies the pathobiology of PNH is not confined to the erythrocyte. Rather, PNH is a disease of the hematopoietic stem cell. Secondly, PNH differs from all other intrinsic red cell abnormalities in that the defective process is acquired rather than inherited.

PNH arises as a consequence of somatic mutation of a gene (phosphatidylinositol glycan class A [*PIGA*]) whose protein product is a glycosyl transferase that is an essential component of the biosynthetic pathway that generates glycosyl phosphatidylinositol (GPI) (Fig. 14-1).[1] This moiety serves as the anchoring mechanism for a functionally diverse group of membrane-bound proteins, more than 20 of which are expressed on hematopoietic lineage cells in humans (Box 14-1). The PIG-A mutations that give rise to PNH cause loss of enzyme function, the end result of which is the complete or near-complete absence of expression of all proteins that are GPI-anchored (Fig. 14-2). Among the cellular membrane constituents that are GPI-anchored are the complement inhibitory proteins, CD55 (decay accelerating factor) and CD59 (membrane inhibitor of reactive lysis). It is deficiency of these two proteins that underlies the complement-mediated intravascular hemolysis characteristic of PNH (Fig. 14-3).[2] Despite the loss of all GPI-anchored proteins (GPI-APs) from all hematopoietic lineage cells (erythrocytes, granulocytes, monocytes, lymphocytes, and megakaryocyte/platelets) derived from the *PIGA* mutant stem cell, there is no compelling evidence that deficiency of GPI-APs other than CD55 and CD59 contribute significantly to the pathophysiology of PNH.

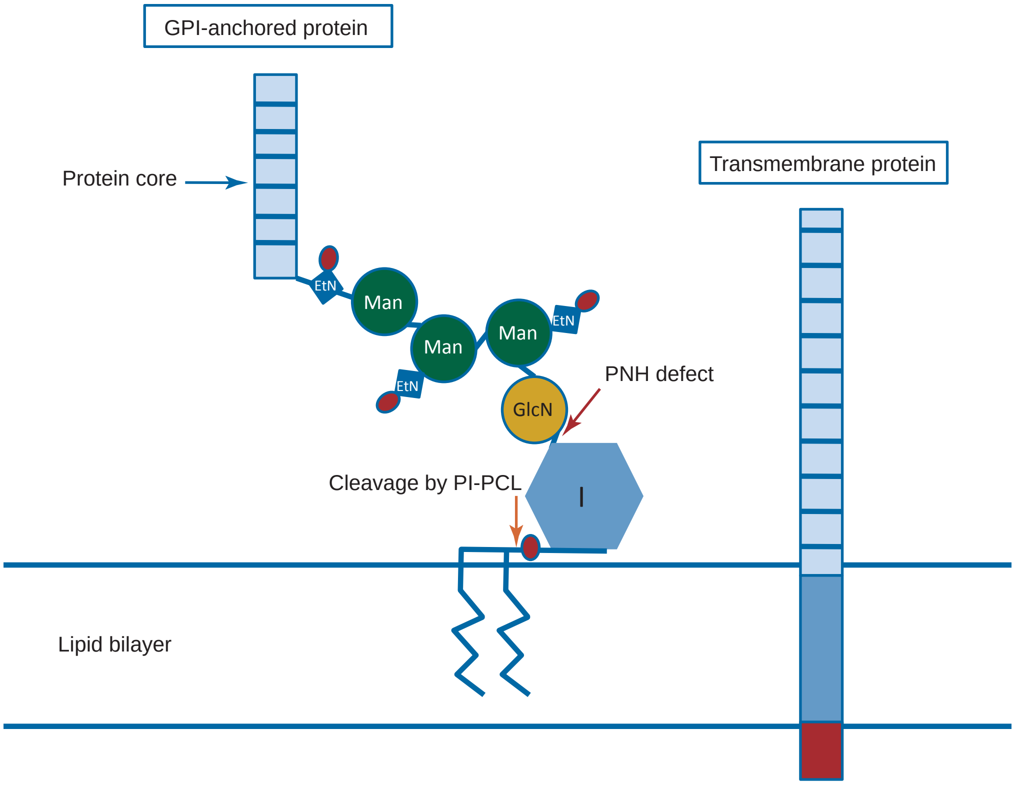


Figure 14-1 Glycosyl phosphatidylinositol–anchored proteins (GPI-APs). The prototypical cellular transmembrane protein *(right)* has an ectoplasmic domain (depicted by the *stacked series of light blue rectangles*, in this case representing the short consensus repeats that characterize proteins that bind complement C3 such as complement receptor type 1 [CD35]), a transmembrane domain composed of a short stretch of ~25 hydrophobic amino acids *(dark blue rectangle)*, and a cytoplasmic tail *(red rectangle)* that usually has signaling properties. In contrast, GPI-APs replace the transmembrane and cytoplasmic domains with a GPI-anchor *(left)*. There are a number of iterations of the GPI-anchor, but the general structure is similar. In the example depicted, two fatty acid residues anchor the inositol *(I)* phosphate *(red circle)* into the lipid bilayer. The glycolipid moiety is attached to the C-terminus of the protein (*light blue stacked rectangles* depicting the short consensus repeats of CD55) by a carbohydrate linker consisting of glucosamine *(yellow circle)* and three molecules of mannose *(green circles)* with ethanolamine *(blue squares)* phosphate *(red circles)* side-chains. The C-terminus of the protein core is covalently bound to the GPI moiety through ethanolamine. A property that distinguishes GPI-APs from transmembrane proteins is release from the cell by treatment with phosphatidylinositol specific-phospholipase C (cleavage site shown by the *orange arrow*). Paroxysmal nocturnal hemoglobin cells are deficient in GPI-APs because *PIGA*, the gene that encodes the glycosyl transferase that mediates transfer of N-acetyl glucosamine to inositol phosphate *(brown arrow)*, is mutant.

In addition to complement-mediated intravascular hemolysis, the other major clinical manifestations of PNH are bone marrow failure and thrombophilia.[3] While the relationship between somatic mutation of *PIGA* and the hemolysis of PNH is understood in detail, the relationship between somatic mutation of *PIGA* (and the consequent deficiency of GPI-APs) and the bone marrow failure and thrombophilia of PNH remain largely speculative. Especially in the case of bone marrow failure, the *PIGA* mutation/deficiency of GPI-APs may not be causal. In fact, the prevailing hypothesis posits that the process that causes the bone marrow failure (proposed as immune attack on the hematopoietic stem cell) selects for the *PIGA* mutant stem cell because absence of one or more GPI-APs bestow a survival advantage by allowing the mutant stem cell either to evade or to better weather immune attack. Whether the thrombophilia of PNH is due to absence of a GPI-AP that is directly involved in regulation of hemostasis/thrombosis (e.g., the receptor for urokinase plasminogen activator) or to indirect consequences of GPI-AP deficiency (e.g., generation of procoagulant microparticles as a result of complement-mediated lysis) remains unresolved.[4]

PNH is a clonal disease (mutant *PIGA* serves as the clonal marker), but it is not a malignant disease in the sense that acute myeloid leukemia (AML) or chronic myelogenous leukemia (CML) are malignant clonal diseases of the hematopoietic stem cell. Unlike AML or CML, in which the mutant clone expands unrelentingly unless antileukemic therapy is initiated, the extent to which the PNH clone expands varies greatly among patients. For example, some patients have small PNH clones that can be detected only by using high-sensitivity flow cytometry to identify GPI-AP peripheral blood cells. Other patients may have large clones in which essentially all hematopoiesis is derived from the mutant stem cell; but even when large, the PNH clone does not have malignant properties in that it does not invade sites outside of the bone marrow, nor does it behave autonomously (i.e., its functional properties appear to be governed by the same regulatory factors as those that control the functional properties normal stem cells), and transformation

Box 14-1 **Glycosyl Phosphytidylinositol–Anchored Proteins Deficient in PNH***

COMPLEMENT REGULATORY PROTEINS†

- CD55 (decay accelerating factor)
- CD59 (membrane inhibitor of reactive lysis)

PROTEINS WITH IMMUNOLOGIC SIGNIFICANCE

- CD58 (lymphocyte function antigen-3)
- CD16b (Fc receptor gamma IIIb)
- CD14 (endotoxin binding protein)

RECEPTORS

- CD87 (urokinase plasminogen activator receptor)
- Folate receptor
- Cellular prion protein *(on resting platelets)*

ENZYMES

- Leukocyte alkaline phosphatase
- Acetylcholinesterase
- 5′-ectonucleotidase

MISCELLANEOUS PROTEINS

- CD24
- CD48
- CD52 (Campath-1)
- CD66c
- CD66b (formerly CD67)
- CD90 (Thy-1)
- CD108 (JMH-bearing protein)
- p50-80, GP109, GP157, GP175, GP500

PNH, Paroxysmal nocturnal hemoglobinuria.
*Partial list.
†Deficiency of complement regulatory proteins underlies the hemolytic anemia of PNH.

into acute leukemia is rarely observed. In fact, an unsolved mystery of PNH is what determines the extent to which the *PIGA*-mutant clone expands. This question is framed by in vitro and in vivo studies demonstrating that mutant *PIGA* alone is insufficient to account for clonal expansion.

More than 25 proteins are involved in the synthesis of the GPI-anchor moiety. Hypothetically, somatic mutation of any of the genes that encode these proteins would produce the PNH phenotype (i.e., deficiency of GPI-APs). Why then is PNH essentially always due to mutation of *PIGA*? The putative answer is based on mathematical probability. Among the genes involved in GPI-AP synthesis, *PIGA* alone is located on the X chromosome.[1] Therefore only one mutational event is required to produce the PNH phenotype because males have one X chromosome, and in females, only one of the two X chromosomes is active in somatic tissues (see Fig. 14-2). Because all other genes involved in GPI-AP synthesis are autosomal, two mutational events would be required to eliminate synthesis of the GPI-anchor and produce the PNH phenotype. Thus, based on mutational frequency ($\sim 1.1 \times 10^{-8}$ per nucleotide position per haploid genome), the probability that deficiency of GPI-APs in PNH would be due to somatically mutated *PIGA* is many orders of magnitude greater than the probability that the deficiency would be due to somatic mutation of both of the alleles of any of the more than 25 autosomal genes involved in GPI-anchor synthesis. Conceivably, germline mutation of one of the alleles of an autosomal gene involved in GPI-AP synthesis could predispose an individual to develop an autosomal form of PNH, and a recent report supports this mechanism as a rare cause of the disease.[5] In essence, however, this situation is analogous to somatic mutation of the X-chromosome gene *PIGA* because the patient was functionally hemizygous for the autosomal gene (*PIGT*) because of the germline mutation of one of the two alleles. This observation adds additional support to the hypothesis that mutant *PIGA* underlies PNH in essentially all cases of PNH because of its unique property among the genes involved in GPI anchor synthesis of structural or functional hemizygosity in somatic tissues.

Understanding the molecular basis of the hemolysis of PNH led to development of targeted therapy in the form of eculizumab (Soliris, Alexion Pharmaceutical), and this treatment has had a major impact on the natural history the disease.[6,7] Nonetheless, management of patients with PNH remains challenging because of the heterogeneous nature of the disease and its close association with bone marrow failure.[8]

This chapter is intended to illuminate for the reader the pathobiology of PNH and to provide guidance on the diagnosis and management of this fascinating disease.

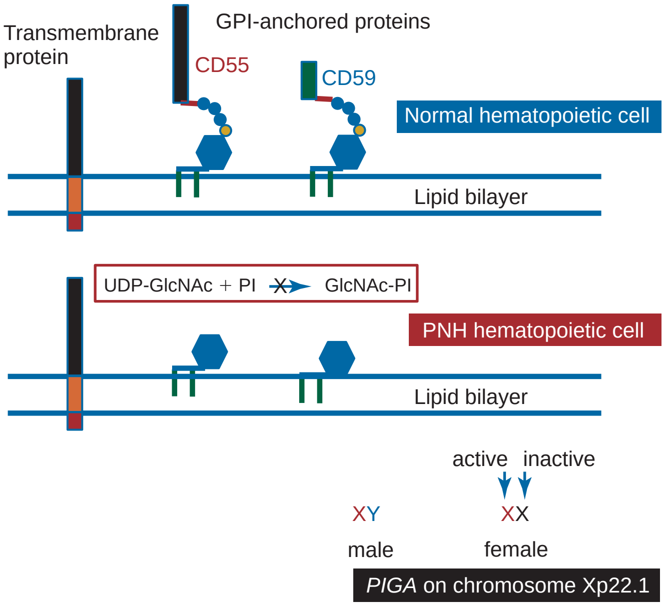


Figure 14-2 The molecular basis of paroxysmal nocturnal hemoglobinuria (PNH). Normal hematopoietic stem cells express both transmembrane and glycosyl phosphatidylinositol–anchored proteins (GPI-APs) *(top panel)*. PNH stem cells express transmembrane proteins normally but fail to express GPI-APs because the first step in synthesis of the anchor is inactivated because the gene *(PIGA)* that encodes the enzyme that is required for transfer of the nucleotide sugar *(UDP-GlcNAc)* to phosphatidylinositol *(PI)* is mutant *(middle panel)*. Of the more than 25 genes involved in synthesis of the GPI anchor, only *PIGA* is located on the X chromosome (all others are autosomal). Location on the X chromosome accounts for the observation that essentially all cases of PNH are due to somatic mutation of *PIGA* because inactivation of only one allele is required to produce the PNH phenotype because males have one X chromosome and in females only one of the two X chromosomes is active in somatic tissues *(bottom panel)*.

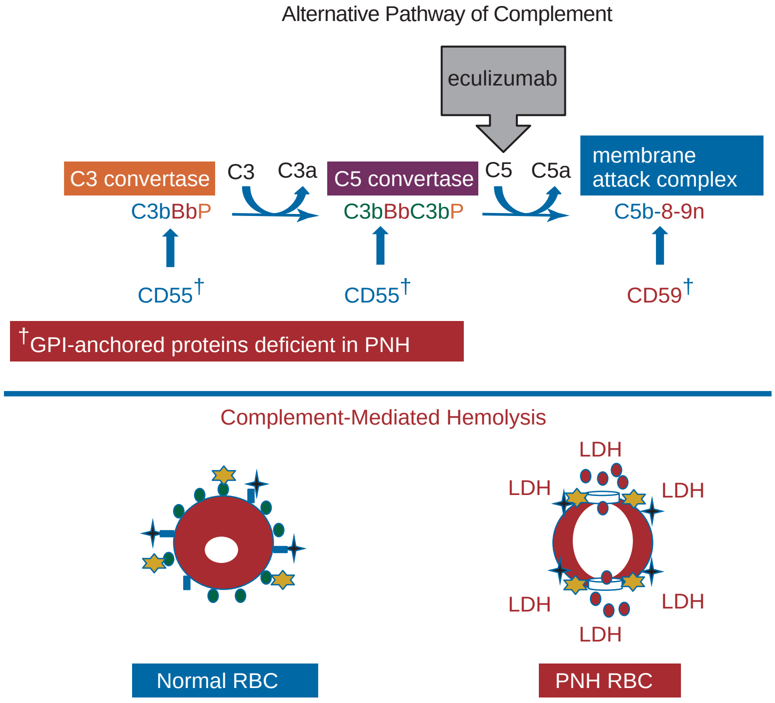


Figure 14-3 Complement and paroxysmal nocturnal hemoglobinuria (PNH). The hemolysis of PNH is due to aberrant regulation of the alternative pathway of complement (APC). The APC is a component of the innate immune system. Unlike the classical pathway of complement that requires a recognition factor such as antibody to activate the pathway, the APC is continuously active. Therefore, safeguards have evolved to protect host cells against APC-mediated injury. In the case of erythrocytes, two glycosyl phosphatidylinositol–anchored proteins, CD55 and CD59, serve this function. Two enzymatic convertases amplify the activity of the APC *(top panel)*. The C3 convertase consists of activated C3 (C3b); activated factor B (Bb), the enzymatic subunit of the complexes; and factor P (formerly called *properdin*). Factor P stabilizes the C3 convertase, allowing each convertase to activate many molecules of C3, and in the process, generate the weak anaphylatoxin C3a. Formation of the C3 convertase is favored at acid pH, explaining the basis of the hemolysis of PNH erythrocytes in acidified serum (Ham's test). The C5 convertase is similar in structure to the C3 convertase except that two molecules C3b are required to position C5 for cleavage by activated factor B (Bb). Many molecules of C5 are cleaved by the C5 convertase, and this process generates many molecules of the potent anaphylatoxin and neutrophil chemoattractant, C5a. Activated C5 (C5b) is the nidus for formation of the membrane attack complex (MAC) of complement consisting of C5b, C6, C7, C8, and multiple molecules of C9. The MAC inserts into the lipid bilayer of the cell, forming a torus that results in osmotic lysis. CD55 blocks the formation and stability of both the C3 and C5 convertases, while CD59 blocks formation of the cytolytic MAC, primarily by inhibiting binding and multiplicity of C9. Eculizumab is a humanized monoclonal anti-C5 antibody that prevents activation of C5 by the C5 convertase. Consequently, the MAC cannot form (and C5a is not generated), accounting for the inhibition of the intravascular hemolysis of PNH. However, eculizumab does not inhibit formation of the C3 convertase, accounting for the opsonization by activation and degradation products of C3 observed in patients with PNH treated with eculizumab. Normal red blood cells *(RBCs)* are protected against APC-mediated injury (*black crosses* represent APC C3 and C5 convertase formation and yellow stars represent MAC formation) by CD55 *(blue ovals)* and CD59 *(green ovals) (bottom panel)*. PNH cells lacking the complement inhibitory proteins CD55 and CD59 undergo complement-mediated lysis, releasing cellular contents including hemoglobin *(red circles)* and lactated dehydrogenase into the plasma.

HISTORY

Comprehensive, scholarly reviews of the history of PNH have been published.[9,10,11,12] The renown Victorian physician, William Gull, may have provided the first published clinical description in 1866, although his brief report failed to distinguish unequivocally PNH from paroxysmal cold hemoglobinuria, which was relatively common at that time because of its relationship to syphilis.[12] In 1882, Paul Strübing of Greifswald, Germany clearly recognized PNH as a distinct entity and undertook prescient experiments designed to test his hypothesis that the nocturnal hemoglobinuria was a consequence of acidification of plasma that occurred when carbon dioxide and lactic acid accumulated because of slowing of respiration during sleep.[9] In 1911, the prolific Dutch physician A.A. Hijmans van den Berg demonstrated that the hemolysis of PNH is caused by a defect in the red cell rather than by the presence of an abnormal plasma factor (as is the case with paroxysmal cold hemoglobinuria that is mediated by the Donath-Landsteiner antibody).[12] Thomas Hale Ham, then in Boston, is credited with discovering, in the late 1930s, that complement mediates the hemolysis of PNH erythrocytes, although it was not until the alternative pathway of complement (APC) was identified and characterized in the mid-1950s by Louis Pillemer of Case Western Reserve University in Cleveland, Ohio, that the basis of Ham's original observations became apparent.[10,11] Notably Ham was on the faculty of Case Western when the alternative pathway was discovered, and Pillemer used PNH erythrocytes in some of his experiments that demonstrated the antibody-independent nature of the alternative pathway and its dependence on magnesium.[10,11] While still in Boston, Ham developed the acidified serum lysis test (Ham's test) that, along with the sucrose lysis test (sugar water test) of Robert Hartmann and David Jenkins of Vanderbilt University in Nashville, Tennessee, were used as the standard diagnostic tests for PNH until being supplanted in the mid-1990s by flow cytometry.[11] Both Hartmann and William Crosby brought attention to the important role that thrombosis (particularly the Budd-Chiari syndrome) plays in the natural history of PNH, and the eminent English physician, John Dacie, and his pupil and colleague S.M. Lewis first systematically characterized the relationship between PNH and marrow failure.[11] Other landmark events in the history of PNH include characterization of the remarkable phenotypic mosaicism, based on complement sensitivity in the late 1960s and early 1970s; demonstration that PNH is a disease of the hematopoietic stem cell in the late 1960s; proof of the clonal nature of the disease in the early 1970s; characterization of the erythrocyte complement defects in the 1970s and 1980s; identification of the complement regulatory proteins that are deficient in PNH in the 1980s; recognition that the membrane proteins that are deficient in PNH are GPI-anchored in the 1980s; identification of *PIGA* as the gene that is mutant in PNH in the 1990s; characterization of the genetic basis of the phenotypic mosaicism in the 1990s; and clinical testing and government approval of the complement C5–inhibitor eculizumab for treatment of the hemolysis of PNH in the first decade of the twenty-first century.[10,11]

EPIDEMIOLOGY

Interpretation of prevalence studies of PNH must take into account the heterogeneous nature of the disease. The blood of patients with PNH is a mosaic of normal and abnormal cells, and the degree of mosaicism varies widely among patients. Those with small PNH clones have few or no symptoms related to hemolysis, and those with clones of less than 1%, detected by high-sensitivity flow cytometry, lack even biochemical evidence of hemolysis. Thus, an argument can be made that asymptomatic patients with small clones do not have clinically significant PNH and should be excluded from prevalence estimates. Others, however, may argue that any patient with flow cytometric evidence of a population of GPI-AP–deficient (GPI-AP–) cells, regardless of clone size, has PNH and should be included in prevalence statistics. Studies of prevalence that address the issue of disease heterogeneity are needed, but by any definition PNH is a rare disease. The prevalence of clinically significant PNH is likely in the order of less than 1 case per 200,000 population, easily fulfilling criteria (less than 1 case per 50,000 population) for classification as an ultraorphan disease.[13]

There is a close association between PNH and aplastic anemia (AA). Environmental factors, drugs, and toxins that cause AA concordantly increase the risk of developing PNH.[14] Although PNH has been reported in all age groups, the peak incidence is in the third and fourth decades of life, similar to that observed for AA. PNH is an acquired disorder, and there is no known inherited risk for developing the disease. A number of cases have been reported in which only one of a pair of identical twins was affected. Inherited mutations in autosomal genes involved in GPI anchor biosynthesis have been identified, but patients with such germline mutations (homozygotes or compound heterozygotes) are characterized by developmental and neurologic abnormalities, including mental retardation and seizures, and by hyperphosphatasia. A patient with mutant *PIGA* died in early infancy because of multiple developmental defects without evidence of hemolysis. Based on this observation and animal studies, *PIGA* mutations whose consequences are sufficiently severe to render the red cells susceptible to complement-mediated hemolysis are embryonic lethal.

MOLECULAR PATHOGENESIS

Careful in vivo studies of red cell survival and in vitro characterization of the complement sensitivity of peripheral blood erythrocytes hinted at the molecular basis of PNH. Red cell survival studies suggested that two populations of erythrocytes coexisted in the peripheral blood of patients with PNH.[15] One population had an abnormally short half-life of about 6 days, whereas the other population had a half-life similar to that of normal erythrocytes (about 60 days). Those observations dovetailed with the results of rigorous experiments that quantified the sensitivity of peripheral blood erythrocytes to complement-mediated lysis, showing that the peripheral blood of PNH patients is a mosaic composed both of erythrocytes that are normally sensitive to complement-mediated lysis and of red cells that are abnormally sensitive to complement-mediated lysis.[16] Together these observations lead to the hypothesis that the abnormal cells were the progeny of a mutant hematopoietic stem cell clone that produced the abnormal erythrocytes.[17] According to this hypothesis, the mutant stem cell coexisted within the bone marrow with normal stem cells from which the erythrocytes of normal complement-sensitivity were derived. Further, that PNH is an acquired rather than an inherited disease implied that the abnormal clone arises as a consequence of somatic mutation rather than germline mutation. In 1970, Oni and colleagues[18] analyzed glucose-6-phosphate dehydrogenase (G6PD) isoforms in both the complement-sensitive and the complement-insensitive erythrocytes of a female PNH patient who was heterozygous for the two G6PD isoforms. The complement-insensitive cells expressed both G6PD isoforms, indicating a polyclonal origin for this cohort. In contrast, the complement-sensitive cells expressed only one G6PD isoform, a finding consistent with monoclonality. These studies provided the first experimental evidence in support of the clonal hypothesis of PNH. In 1969, Aster and Enright[19] reported that a portion of both the platelet and granulocyte populations from patients with PNH was abnormally sensitive to complement-mediated cytolysis. This publication represented another watershed event in the understanding of the origins of PNH because it indicated that the mutation arose in a primitive hematopoietic stem cell that has the capacity to differentiate along myeloid lines. Subsequent studies showed that monocytes are also affected, and, in most patients, affected lymphocytes can be demonstrated. Thus, characterization of PNH contributed substantial early experimental evidence in support of the concept of the pluripotent stem cell.

A defining feature of PNH is phenotypic mosaicism based on sensitivity of the erythrocytes to complement-mediated lysis. This remarkable characteristic was first clearly elucidated by Rosse and Dacie in 1966,[16] and Rosse further refined the analysis in 1973.[20] Using an in vitro test that quantitates the sensitivity of erythrocytes to complement-mediated lysis, three phenotypes of PNH erythrocytes were identified. One of the phenotypes (designated PNH I) was characterized by normal or near-normal sensitivity to complement, whereas another phenotype (designated PNH III) was 15 to 25 times more susceptible to lysis. A third phenotype (PNH II) was of intermediate sensitivity, about 3 to 5 times more susceptible than normal cells. Most patients have a mixture of type I and type III cells, but mosaics of type I, type II, and type III, as well as type I and type II, are also observed. The biochemical and molecular basis of the phenotypic mosaicism of PNH was elucidated by Holguin,[21,22] respectively (see "Membrane Inhibitor of Reactive Lysis" and "Somatic Mutation of PIGA Underlies the Absence of GPI-AP in PNH").

Elegant, rigorous in vitro studies of the aberrant interactions of complement with PNH erythrocytes indicated that two defects underlie the complement-mediated hemolysis that characterized the disease. One abnormality resulted in greater activity of the amplification C3 convertases of the classical and alternative pathways of complement,[23,24] and the other caused PNH erythrocytes to be markedly more sensitive to the cytolytic activity of the membrane attack complex (MAC) of complement.[23,25,26] The basis of these two complement regulatory abnormalities remained a mystery until the 1980s, when PNH erythrocytes were shown to be deficient in both CD55 and CD59 (Figs. 14-2, 14-3).

Paroxysmal Nocturnal Hemoglobinuria and Complement

The chronic intravascular hemolysis that is the hallmark clinical manifestation of PNH is mediated by the APC (see Fig. 14-3).[2] The APC is a component of innate immunity.[27] This ancient system evolved to protect the host against invasion by pathogenic microorganisms. Unlike the classical pathway of complement that is part of the system of acquired immunity and requires antibody for initiation of activation, the APC is in a state of continuous activation, armed at all times to protect the host. The APC cascade can be divided into two functional components: (1) the amplification C3 and C5 convertases and (2) the cytolytic MAC (see Fig. 14-3). The C3 and C5 convertases are enzymatic complexes that initiate and amplify the activity of the APC and ultimately generate the MAC (the MAC is the common cytolytic subunit of the classical and lectin pathways of complement as well as the APC).

Because the APC is primed for attack at all times, elaborate mechanisms for self-recognition and for protection of the host against APC-mediated injury have evolved. Both fluid-phase and membrane-bound proteins are involved in these processes. Normal human erythrocytes are protected against APC-mediated cytolysis primarily by CD55[28,29] and CD59 (see Fig. 14-3).[21,30] These proteins act at different steps in the complement cascade. CD55 regulates the formation and stability of the C3 and C5 convertases, whereas CD59 blocks the formation of the MAC. Deficiency of CD55 and CD59 on the erythrocytes of PNH is the pathophysiologic basis of the direct antiglobulin test–negative, intravascular hemolysis that is the clinical hallmark of the disease.

Decay Accelerating Factor

In 1983, two groups reported that PNH erythrocytes were deficient in CD55.[29,31] CD55, first identified by Hoffmann[32,33] in 1969 and subsequently purified by Nicholson-Weller and colleagues in 1982,[28] is a 70 kD protein that inhibits the formation and stability of the C3 convertases of complement. Thus, the absence of CD55 provided a plausible explanation for the greater binding of activated C3 to PNH erythrocytes that had been observed in in vitro experiments (see Fig. 14-3). Detailed studies, however, demonstrated that CD55 does not regulate the activity of the MAC of complement.[34] Those results implied that PNH erythrocytes were deficient in a second complement regulatory protein that was functionally distinct from CD55.

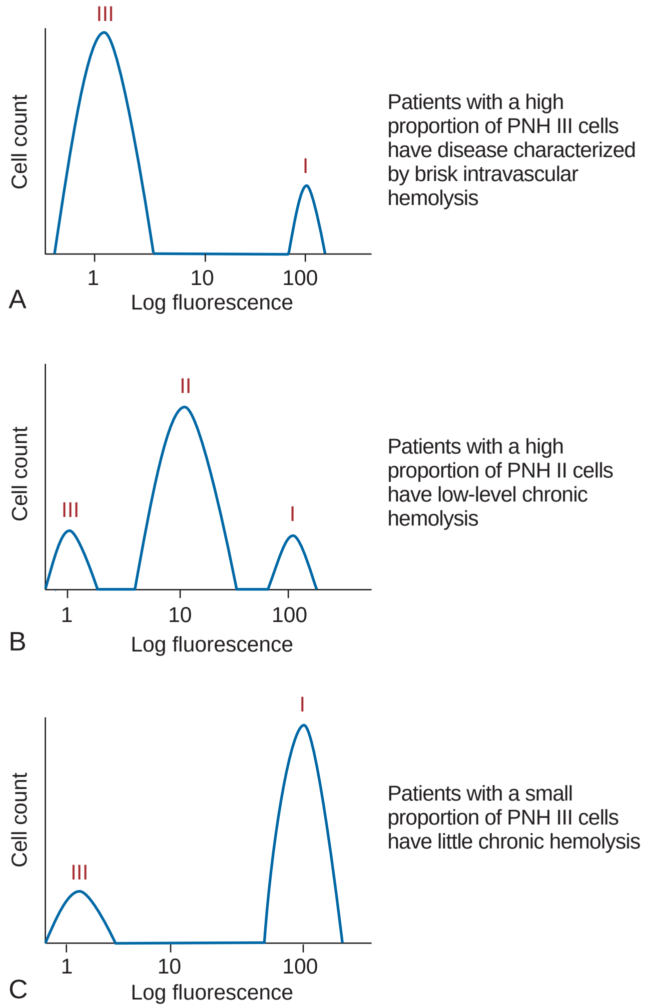
Membrane Inhibitor of Reactive Lysis

In 1989, Holguin and colleagues[30] reported the isolation from normal erythrocytes of an 18 kD protein called *MIRL* (CD59) that protected PNH red cells against complement-mediated lysis. As anticipated, PNH cells were found to be deficient in CD59, and additional studies by those investigators and others demonstrated that CD59 inhibits complement-mediated lysis by blocking the assembly of the MAC.[30,35,36]

By comparing expression of CD55 and CD59 on PNH I, PNH II, and PNH III erythrocytes, the functional basis of the different complement-sensitivity phenotypes was determined.[21] Those studies showed that PNH III cells are completely deficient in both CD55 and CD59, whereas PNH II cells are partially deficient in the two complement-regulatory proteins, and PNH I cells have normal expression. Thus the variability in sensitivity to lysis among the different phenotypes is explained by quantitative differences in expression of CD55 and CD59 (Fig. 14-4). Additional experiments demonstrated that the combined deficiency of CD55 and CD59 was sufficient to explain the enhanced susceptibility of PNH erythrocytes to hemolysis in acidified serum.[37]

Inherited Mutations of CD55 and CD59

Of the two complement regulatory proteins, CD59 is more important than CD55 in protecting cells from complement-mediated lysis in vivo. Antigens of the Cromer-related blood group complex are located on CD55, and rare cases of a null phenotype called *Inab* have been reported.[38] Like PNH cells, Inab erythrocytes are deficient in CD55, but unlike PNH erythrocytes, CD59 expression is normal on Inab red cells.[39] Although Inab erythrocytes bind more activated C3 when exposed to acidified serum,[39] they undergo little or no hemolysis. Further, subjects with the Inab phenotype have no known hematologic abnormalities, and in particular, they have



Patients with a high proportion of PNH III cells have disease characterized by brisk intravascular hemolysis

Patients with a high proportion of PNH II cells have low-level chronic hemolysis

Patients with a small proportion of PNH III cells have little chronic hemolysis

Figure 14-4 Hemolysis in paroxysmal nocturnal hemoglobinuria (PNH) is determined by the erythrocyte phenotype and the size of the clone. Mock, one-color histograms are used to illustrate features of PNH that influence the extent of hemolysis in individual patients. This illustration depicts phenotypes that are observed using anti-CD59 as the fluorescently labeled antibody.

no clinical evidence of hemolysis.[38,40,41] These observations show that isolated deficiency of CD55 does not produce the PNH syndrome.

In contrast, a patient with an inherited, isolated deficiency of CD59 had a syndrome that was indistinguishable from PNH.[42,43] Clinically the patient experienced recurrent episodes of hemoglobinuria, suggesting that CD59 is essential for protecting erythrocytes against complement-mediated lysis in vivo. Recurrent thromboembolic events were also observed in this patient. That patient had normal CD55 expression, but in vitro, his cells were susceptible to hemolysis in acidified serum, implying that CD59 deficiency accounts primarily for the positive Ham's test in PNH.[37]

More recently, Nevo and colleagues reported on five patients from four unrelated families, who presented in infancy with symmetric muscle weakness accompanied by hypotonia and absent deep tendon reflexes involving the legs more than the arms.[44] The neurologic disease was classified as chronic inflammatory demyelinating polyneuropathy. Symptomatic episodes were accompanied by hemolytic anemia characterized by reticulocytosis, negative direct antiglobulin test, elevated serum lactate dehydrogenase (LDH) concentration, and low serum haptoglobin concentration. Leukopenia and anemia were not observed. There was no report of thrombotic complications, although the oldest of the patients in the study was 5 years old. Whole exome sequencing of DNA from one of the patients identified a homozygous missense mutation in the gene that encodes CD59. Subsequent studies identified the same mutation in the other four patients. The mutation segregated with the disease in the families and had a carrier rate of 1 per 66 among Jewish subjects of North-African origin. Cell surface expression of CD59 was absent from the erythrocytes of patients, while expression of CD55 was normal. Together, these studies confirm the importance of CD59 in the pathophysiology of the complement-mediated hemolysis of PNH and suggest an important pathologic role for aberrant regulation of the cytolytic MAC of complement in chronic inflammatory demyelinating polyneuropathy and perhaps other diseases characterized by loss of myelin and axonal integrity.

While the above studies provide interesting insights into the individual function of CD55 and CD59 in vivo, it is important to remember that, in PNH, both proteins (as well as all other GPI-APs on the abnormal hematopoietic clone) are deficient. Therefore it is the combined deficiency of CD55 and CD59 that results in the markedly abnormal susceptibility of the red cells of PNH to complement-mediated lysis.

Congenital Abnormalities of Genes Involved in Glycosyl Phosphatidylinositol–Anchored Protein Synthesis

Germline mutations in genes (*PIGN*, *PIGM*, *PIGO*, *PIGL*, *PIGV*, and *PIGA*) involved in biosynthesis of the GPI-anchor moiety have been reported.[45-50] Notably, hemolysis and bone marrow failure are not observed in patients with germline mutation of GPI-anchor assembly proteins even in the case of germline mutant *PIGA* (see "Epidemiology").[50] The phenotype of patients with germline mutation of genes involved in GPI-AP assembly varies but often involves skeletal anomalies; neurologic abnormalities, including mental retardation and seizures; and hyperphosphatasia.

Paroxysmal Nocturnal Hemoglobinuria and Deficiency of Glycosyl Phosphatidylinositol–Anchored Protein

Beck and Valentine reported in 1951 that neutrophils from patients with PNH were deficient in leukocyte alkaline phosphatase.[51] Thus leukocyte alkaline phosphatase was the first protein found to be deficient in PNH, but the first erythrocyte membrane protein found to be deficient was acetylcholinesterase. In 1959, Auditore and Hartmann[52] presented evidence that the extent of the acetylcholinesterase deficiency correlated with the severity of the hemolysis. More detailed studies by others[53] showed that PNH I red cells had a relatively normal amount of acetylcholinesterase, while PNH III erythrocytes were profoundly deficient. Although the deficiency of acetylcholinesterase plays no role in the abnormal susceptibility of PNH red cells to complement-mediated lysis, ultimately the observations that the red cells lack acetylcholinesterase and that the neutrophils lack alkaline phosphatase provided important insights into the fundamental defect that underlies PNH because both proteins were eventually shown to be GPI-anchored.

If only CD55 or only CD59 were deficient in PNH, it would have been logical to hypothesize that the disease was due to mutations affecting the gene that encodes the particular protein. That PNH cells were deficient in multiple proteins (CD59, CD55, and acetylcholinesterase on red cells and leukocyte alkaline phosphatase on neutrophils), however, eliminated (based on mathematic probability) the possibility that the gene for each protein was mutant. Rather, a more plausible hypothesis was that the PNH defect involved a posttranslational modification common to all of the proteins that are deficient in PNH. In 1984, Medof and colleagues[54] reported that isolated CD55 spontaneously reincorporated into erythrocyte membranes. This property of CD55 had been appreciated by Hoffmann in 1969.[32,33] Working with the butanol-saturated aqueous phase of a crude extract prepared from normal human erythrocyte stroma, Hoffmann showed that the sample contained a factor capable of inhibiting complement-mediated lysis by accelerating the decay of the C3 convertase. He further showed that this substance (that he called *DAF* [CD55] *of stroma* [DAF-S]) had the capacity to reincorporate into red cells and remain functionally active. In 1980, Low and Zilversmit[55] demonstrated that alkaline phosphatase that had been solubilized from cells by butanol extraction exhibited the capacity to bind to phospholipid vesicles. In this case the incorporation was thought to be due to the attachment of a phosphatidylinositol moiety to the enzyme. Subsequent experiments demonstrated that isolated acetylcholinesterase also spontaneously reincorporated into cell membranes. Previous studies had shown that alkaline phosphatase, acetylcholinesterase, and 5′-ectonucleotidase (another protein that is deficient in PNH) were released from the cell surface by treatment with phosphatidylinositol-specific phospholipase C (PIPLC) (see Fig. 14-1). The cumulative work of a number of investigators showed that both the capacity to reincorporate into lipid membranes and susceptibility to cleavage by PIPLC was characteristic of a group of amphipathic membrane proteins that shared the common structural feature of being anchored to the cell surface through a GPI moiety (see Fig. 14-1). The structural link between CD55 and acetylcholinesterase, alkaline phosphatase, and 5′-ectonucleotidase (CD73) was made in 1986 by Davitz and colleagues[56] and Medof and colleagues[57] when those investigators presented evidence that CD55 is a GPI-AP. Subsequent studies confirmed that CD59 is also a GPI-AP.[58] The results of those studies suggested the following paradigm: All proteins that are deficient in PNH are GPI-anchored, and all GPI-APs that are expressed by normal hematopoietic cells are deficient in PNH (see Fig. 14-2). All data to date are consistent with this postulate.

GPI-APs are functionally diverse. In addition to the complement regulatory proteins (CD55 and CD59) and enzymes (acetylcholinesterase, alkaline phosphatase, and 5′-ectonucleotidase [CD73]) discussed above, proteins with a variety of receptor, adhesion, and immune modulatory functions (e.g., FcγRIIIb [CD16b], urokinase plasminogen activator receptor [CD87], endotoxin binding protein receptor [CD14], and LFA-3 [CD58]) are also GPI-anchored. Further a number of proteins whose function is unknown are GPI-anchored. Over 80 eukaryotic proteins have been shown to be GPI-anchored, and approximately 30 of these are found in humans. Currently, the list of proteins deficient in PNH numbers at least 23 (see Box 14-1). This number is less than the total number of GPI-anchored human proteins because PNH is an acquired disease that affects only hematopoietic cells. Thus GPI-APs that are present on somatic tissues other than hematopoietic cells are expressed normally in patients with PNH. Despite the fact that PNH cells lack a number of functionally diverse membrane constituents, the only pathologic component of the disease that is unequivocally causally related to GPI-AP deficiency is the abnormal susceptibility of the erythrocytes to complement-mediated lysis due to deficiency of CD55 and CD59.

The GPI anchor is a complex structure with at least 25 proteins being essential for assembly of the moiety (see Fig. 14-1).[1,59] Hypothetically the PNH phenotype would result if any of these proteins were nonfunctional because if the GPI-anchor is not synthesized, GPI-APs are not expressed. Accordingly, it seemed probable that PNH would be found to be heterogeneous at the molecular level because mutations affecting any one of several genes that encode elements critical for GPI-anchor assembly would produce the disease phenotype. In 1992[60] and 1993,[61] however, two groups published the surprising finding that GPI-protein deficient lymphocyte cell lines derived from different patients with PNH all belonged to the same complementation class (Class A) of GPI-AP–negative cell lines. Additional experiments confirmed that the PNH cell lines had the same biochemical defect as the complementation Class A mutants. Like the Class A mutants, the PNH cell lines failed to synthesize N-acetylglucosaminyl-phosphatidylinositol, the first intermediate in the pathway of GPI-anchor assembly (see Fig. 14-2).

Somatic mutation of PIGA *underlies the absence of GPI-AP in PNH.* A gene that restores normal expression of GPI-APs in the complementation Class A PNH cell lines was identified by Kinoshita and colleagues in 1993.[62,63] As predicted by the studies cited above, the gene, called *PIGA* (for phosphatidylinositol glycan, class A), encodes a protein that is essential for the normal synthesis of GlcNAc-PI (see Fig. 14-2).[62] Subsequently, Takeda and colleagues[63] showed that *PIGA* complements the deficient expression of GPI-APs in PNH lymphoblastoid cell lines and that those cell lines harbored somatic mutations in *PIGA*. Together those studies defined both the biochemical and the molecular basis of the deficiency of GPI-APs in PNH.[64]

Additionally Takeda and colleagues observed that a heterozygous mutation was sufficient to produce the PNH phenotype.[63] As presaged by the studies of Hyman and colleagues,[65] the dominant expression of the somatic mutation was explained when *PIGA* was mapped to chromosome Xp22.1. Because males have a single X chromosome, any functionally significant mutation affecting *PIGA* is expressed. Females are functionally haploid due to X-inactivation. Therefore, somatic mutations in *PIGA* appear dominant when they occur on the active X chromosome (see Fig. 14-2).

Analysis of *PIGA* has revealed that the same mutation can be identified in isolated neutrophils, monocytes, and lymphocytes from individual patients with PNH, confirming that the disease involves the hematopoietic stem cell.[22,63] More than 150 *PIGA* mutations have been identified in affected cells of patients with PNH. Only a few large deletions have been observed.[66,67] The remaining mutations consist of nucleotide substitutions of the missense or nonsense types, or small deletions or insertions that cause frame-shifts and introduce premature termination codons. The mutations are distributed randomly over the entire coding region and at splice junctions. Although some mutations have been observed in more than one patient, most are unique, indicating absence of mutational hot spots in *PIGA*. These observations suggest that clonal selection depends on complete or nearly complete inactivation of PIGA such that expression of GPI-APs is below a critical threshold. Therefore all mutations in PNH cause loss of function (partial or complete) of PIGA.

Studies of *PIGA* mutations have also provided insights into the molecular basis of the phenotypic mosaicism of PNH.[22] Cloned lymphocyte cell lines were established from the peripheral blood of a patient whose erythrocytes were a mixture of PNH I, PNH II, and PNH III cells. Based on expression of GPI-APs, lymphocyte clones with four different phenotypes were observed. Analysis of clones with normal expression of GPI-AP revealed no somatic *PIGA* mutations. In contrast, between lymphocyte clones with two distinct phenotypes based on the pattern of GPI-AP expression (i.e., PNH II and PNH III), four discrete *PIGA* mutations were identified. In the lymphocyte clones with the PNH II phenotype, a missense mutation that changed a highly conserved amino acid was found.[22] This finding suggests that cells with partial expression of GPI-APs (PNH II) are derived from stem cells with mutations that incompletely inactivate PIGA. In the case of the lymphocyte clones with the PNH III phenotype, three separate mutations were identified, each of which was expected to inactivate completely the *PIGA* gene product.[22] Collectively these experiments demonstrate that the phenotypic mosaicism that is characteristic of PNH is a consequence of genotypic mosaicism. Further, because any mutation that completely inactivates PIGA results in PNH III cells, phenotypically identical cells can have different *PIGA* genotypes.

Studies of the pattern of X chromosomal inactivation indicated that, in the female patient with four different *PIGA* mutations, the abnormal clones were not derived from a common ancestor.[22] Further, that each of the mutations was discrete demonstrated that the mutational events occurred independently rather than by clonal evolution. These results demonstrate that PNH is not strictly a monoclonal process and have important implications for the origins of the disease.

Studies of mutational frequency in PNH have produced conflicting results. Some of the disparity is almost certainly due to differences in experimental design and interpretation of data. While an abnormally high mutational rate could contribute to generation of multiple *PIGA* mutant clones, invoking such a process is not necessary to explain the oligoclonality observed in some patients with PNH. Rather, a growth/survival advantage for any *PIGA*-mutant GPI-AP− stem cells likely accounts for the outgrowth of multiple PNH clones. In this sense PNH can be viewed as an example of Darwinian evolution occurring in the bone marrow. According to this hypothesis, hematopoietic stem cells with a randomly acquired mutant *PIGA* exist in the marrow in a quiescent state and become apparent only when deficiency of one or more GPI-APs bestows upon those mutant cells a proliferative or survival advantage. Putatively the selection pressure that is applied to the marrow, such as immune attack, involves one or more GPI-APs such that hematopoietic stem cells with mutant *PIGA* that lack expression of GPI-APs have a survival advantage.

What is the basis of clonal selection and clonal expansion? Despite the progress that has been made in determining the basis of the abnormal sensitivity of the erythrocytes to complement-mediated lysis and the global absence of GPI-APs from hematopoietic cells, an issue that is fundamental to a more complete understanding of PNH remains largely enigmatic. In order for PNH to become clinically evident, the hematopoietic stem cells bearing the mutant *PIGA* must expand so that progeny sufficient to produce symptoms and signs of the disease are generated. In many instances GPI-AP− cells dominate hematopoiesis in patients with PNH, suggesting that the mutant stem cell has a greater proliferative capacity, a survival advantage relative to GPI-AP–sufficient (GPI-AP+) stem cells, or both. That *PIGA* mutations are necessary for the development of PNH is incontrovertible. At issue is whether *PIGA* mutations are both necessary and sufficient to account for the PNH syndrome and whether the *PIGA* mutation provides an absolute or a conditional growth/survival advantage.

PNH differs from monoclonal hematopoietic stem cell disorders such as CML, in which the t(9;21) that generates the fusion protein BCR–ABL is sufficient to account for the proliferative advantage of the mutant cell and in which all normal hematopoiesis is progressively and invariably displaced as a consequence of the uncontrolled proliferation of a transformed clone. In PNH the peripheral blood is a mosaic of normal and abnormal cells and the proportion of GPI-AP+ to GPI-AP− cells varies greatly among patients. Further, that ratio tends to remain fixed over long periods of observations. PNH is, therefore, more aptly characterized as a nonmalignant, clonal process, but the basis of the clonal selection and clonal expansion are incompletely understood.

The oligoclonal nature of PNH[22] suggests that a powerful selection process that is most likely based on phenotype is at work in the bone marrow. According to this hypothesis, stem cells with mutant *PIGA* have an advantage because of some pathologic process (perhaps immune mediated) that involves a GPI-AP. For example an autoimmune process could arise in which the target antigen is a GPI-AP expressed on hematopoietic stem cells. Under those circumstances *PIGA* mutant stem cells (lacking GPI-APs) would escape immune-mediated destruction because the target antigen is absent.

Hematopoietic cells express a relatively large number of functionally diverse GPI-APs (see Box 14-1). Thus absence of all GPI-APs is probably not required for the clonal selection of the mutant stem cells. Rather the selective advantage may be dependent on the absence of a single protein that is GPI-anchored, and the reason for the global deficiency of GPI-AP is that *PIGA* is located on the X chromosome. According to this supposition, an autosomal gene encodes the GPI-AP that is conditionally detrimental (e.g., an antigen targeted for immune destruction or a receptor for a negative growth regulator). Inasmuch as two alleles rather than one must be mutated, the probability of inactivating an autosomal gene through somatic mutagenesis is remote compared to the probability of inactivating an X-linked gene. Therefore stem cells with a deficiency of the detrimental GPI-AP are most likely to arise as a consequence of *PIGA* mutations, as is the case in patients with chronic lymphocytic leukemia treated with Campath1H (anti-CD52).[68] Assuming that the GPI-AP complement regulatory proteins are not the targets of the underlying pathologic process, the hemolytic anemia that is the clinical hallmark of PNH may represent an epiphenomenon that is a consequence of the chromosomal location of *PIGA*.

Although a compelling case can be made in support of the paradigm that the *PIGA* mutation bestows a conditional survival advantage upon the affected stem cell, the hypothesis that the mutant stem cells have an absolute growth advantage must also be considered. Data that both support and challenge this hypothesis can be found.[69-71] The most compelling argument against an intrinsic growth or survival advantage for *PIGA* mutant cells is made by the results of studies using transgenic mice.[72] By using homologous recombination, Kawagoe and colleagues[73] disrupted *Piga* (the murine homologue of *PIGA*). Only mice with a low degree of chimerism survived. Among those animals, the percentage of GPI-AP– erythrocytes ranged from about 1% to 5%. During 10 months of observation, the ratio of GPI-AP– to GPI-AP+ peripheral blood cells did not increase. Studies by others using conditional knockout technology have confirmed these observations.[72] Together these observations indicate that the *PIGA* mutation does not confer an absolute growth or survival advantage.

What Is the Origin of the PIGA-Mutant Stem Cells? Patients with PNH often have more than one *PIGA* mutant clone.[22,74] A tenet of the Darwinian selection hypothesis as it applies to PNH is that *PIGA* mutant hematopoietic stem cells are present during the time when the selection pressure is applied to the bone marrow. *PIGA*-mutant hematopoietic elements have been identified in the peripheral blood and bone marrow of normal volunteers,[75] providing experimental support for this concept, although whether the GPI-AP– cells identified in a normal individual originate from hematopoietic stem cells with mutant *PIGA* has been questioned.[76] While there are experimental data that suggests that the mutational frequency of the gene is abnormally high,[76] that *PIGA* is located on the X chromosome (so that only one allele need be mutated for the phenotype to become apparent) likely accounts primarily for the existence of multiple discrete clones in many patients with PNH.

A Two-Step Model of Paroxysmal Nocturnal Hemoglobinuria Pathogenesis

Studies by Inoue and colleagues suggest that a two-step mechanism may account for the unique pathophysiology of PNH.[77] Those investigators identified two patients whose *PIGA*-mutant cells had a concurrent, acquired rearrangement of chromosome 12. Detailed analysis showed that in both cases, the chromosome 12 rearrangement resulted in disruption of the 3′ untranslated region (3′UTR) of *HMGA2*. As a consequence, a negative regulatory region of the locus was disrupted, resulting in ectopic expression of the gene. *HMGA2* is a member of the high mobility group of proteins (HMGA1a, HMGA1b, HMGA2) that function as architectural transcription factors. HMG members possess no intrinsic transcriptional activity. Instead these nonhistone phosphoproteins orchestrate assembly of stereospecific transcriptional regulatory proteins into enhanceosomes. The cellular targets of *HMGA2* are incompletely defined but appear to include cyclin A and E2F1.

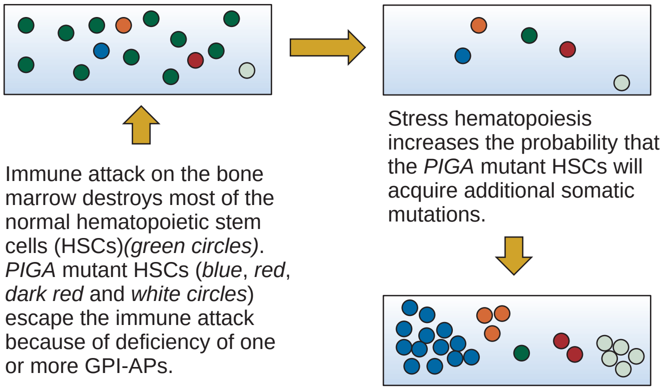
Molecular studies established a causal role for *HMGA2* in benign mesenchymal tumors. Rearrangement of 12q13-15 is observed in these neoplasms, but tumorigenesis does not depend on generation of chimeric proteins derived from fusion of *HMGA2* with specific translocation partners. Rather, clonal expansion induced by *HMGA2* appears to result from deregulated expression of the protein. For the two PNH patients, ectopic expression was a consequence of deregulation of expression caused by truncation of the 3′UTR-containing elements that are the target of microRNA let-7a that negatively regulates HMGA2 expression.[78,79] Additional studies will be required to determine whether aberrant expression of *HMGA2* underlies clonal expansion in PNH patients without structural abnormalities of 12q13-15, but studies by Murakami and colleagues support this hypothesis.[80]

Recently, Sugimori and colleagues reported concurrent mutation of *JAK2* (*JAK2V617F*) in three patients with PNH.[81] This mutation drives clonal expansion in myeloproliferative disorders including polycythemia vera, primary myelofibrosis, and essential thrombocytosis. Thus the JAK2V617F mutation likely contributes to clonal expansion of hematopoiesis in this subgroup of patients with PNH.

In contrast to a disorder characterized by cellular transformation and malignant cellular growth, PNH manifests many of the characteristics of a benign tumor because there is limited expansion of *PIGA* mutant clones (the peripheral blood of patients is a relatively stable mosaic of normal and abnormal cells), *PIGA* mutant cells respect tissue boundaries (there is no invasion of nonhematopoietic tissues), *PIGA* mutant cells respond appropriately to signals that normally regulate hematopoiesis (function is not autonomous), and transformation into acute leukemia occurs rarely (PNH is not a premalignant condition).[6,82] The studies of Inoue and colleagues[77] suggest the concept of PNH as a benign tumor of the bone marrow with aberrant expression of *HMGA2* acting in concert with mutant *PIGA* (and the consequent deficiency of GPI-APs) to produce the proliferative phenotype that underlies clonal expansion. These studies did not define whether (or if) the aberrant expression of *HMGA2* works additively or synergistically with mutant *PIGA* to produce the proliferative phenotype.

The findings of Inoue and colleagues[77] provide new insights into the etiology of the nonmalignant clonal hematopoiesis of PNH. These studies support a two-step process consisting of (1) clonal selection based on phenotype (i.e., GPI-AP deficiency resulting from mutant *PIGA*) and (2) clonal expansion as a consequence of a second somatic mutation that bestows an additional growth/survival advantage. Clonal selection may induce exit of *PIGA* mutant stem cells from a dormant state, thereby favoring acquisition of the second mutation that underlies clonal expansion. The variable expansion of *PIGA*-mutant clones among patients and among mutant clones in the same patient could be explained by heterogeneity involving the second genetic event that partners with mutant *PIGA*. According to this hypothesis, mutant *PIGA* is promiscuous with respect to the second mutant gene with which it partners. But the benign nature of PNH suggests that genes involved in clonal expansion of *PIGA* mutant stem cells are different from those that underlie malignant clonal diseases such as acute leukemia. In fact, concurrent mutation of *PIGA* with mutation of genes that drive tumorigenesis may be synthetically lethal, accounting for the low incidence of transformation of PNH into a malignant clonal process such as acute leukemia.

In summary, the basis of the clonal dominance in PNH is incompletely understood, however, available evidence supports a two step-model of pathogenesis (Fig. 14-5). Step 1 of this model is clonal selection. In this case, a loss-of-function mutation affecting *PIGA* causes deficiency of GPI-APs on affected stem cells. These mutant stem cells have a relative survival advantage in the setting of immune-mediated bone marrow failure (e.g., AA) (see Fig. 14-5). Clonal expansion, step 2 of the PNH pathogenesis model, is envisioned as a consequence of clonal evolution in which mutation of a second gene, working in concert with the consequences of mutant *PIGA*, bestows upon the double mutant stem cell an absolute proliferative/survival advantage (see Fig. 14-5).[77] The extent of expansion of a particular mutant clone and the dominance of one *PIGA* mutant clone in the presence of other *PIGA* mutants may be mediated by the nature of the second event that may be molecularly heterogeneous (see Fig. 14-5). Alternatively, a limited number of second mutations may partner with mutant *PIGA,* and the extent of clonal expansion may be determined by how the second mutation affects gene function.



Immune attack on the bone marrow destroys most of the normal hematopoietic stem cells (HSCs)*(green circles)*. *PIGA* mutant HSCs (*blue*, *red*, *dark red* and *white circles*) escape the immune attack because of deficiency of one or more GPI-APs.

Stress hematopoiesis increases the probability that the *PIGA* mutant HSCs will acquire additional somatic mutations.

The extent to which each *PIGA* mutant HSC expands depends upon the effects of the second somatic mutation on the proliferation/survival characteristics of the affected cell.

Figure 14-5 Hypothetical model for selection and expansion of *PIGA*-mutant, glycosyl phosphatidylinositol–anchored proteins–deficient (GPI-AP–) hematopoietic stem cells. A two-step model is proposed. Step 1 is clonal selection based on phenotype (i.e., deficiency of GPI-APs due to mutant *PIGA*. Step 2 is clonal expansion based on acquisition of additional somatic mutations that provide a growth/survival advantage to the *PIGA* cell. The extent to which the *PIGA* mutant clone expands depends upon the effects of the second mutation on the proliferative and survival characteristic of the GPI-AP– cell.

CLINICAL MANIFESTATIONS

Most commonly, patients with classic PNH complain initially of malaise, lethargy, fatigue, and asthenia (Box 14-2). Other frequently reported symptoms include dyspnea, abdominal pain, and chest pain. PNH usually begins insidiously because clinically apparent hemoglobinuria is the presenting symptom in only about 25% of cases.[6,83] The disease course is chronic with a generally stable clinical pattern in a given individual. The illness ranges in severity from a mild, clinically benign process to a chronically debilitating, potentially lethal disease. The diagnosis is made most frequently in the third to fifth decades of life,[6,82,84] but PNH is also encountered in childhood[85] and in old age.[6,82,84] Both genders are affected with perhaps a slight female predominance, and PNH has been described in many racial groups. The disease has no familial tendency. A patient with complete global deficiency of GPI-AP has not been reported, suggesting that germline mutations of PIGA are embryonic lethal. This hypothesis is supported by the lack of success in producing a homozygous or hemizygous knockout mouse using targeted mutation of Piga.[73] Observations in humans also support the hypothesis. An X-linked lethal disorder characterized by cleft palate, neonatal seizures, contractures, and central nervous system structural malformations was investigated using whole exome sequencing.[50] In that case, a germline mutation of PIGA was identified, but the PNH phenotype was not observed. In vitro studies indicated that the mutant PIGA had partial function, thereby accounting for the absence of hemolysis. Similarly, two unrelated kindreds with germline mutations in the

Box 14-2 Signs and Symptoms of Paroxysmal Nocturnal Hemoglobinuria

SUBJECTIVE

- Fatigue, lethargy, asthenia, loss of sense of well-being
- Abdominal pain
- Dyspnea
- Chest pain
- Odynophagia
- Male impotence
- Headache

OBJECTIVE

- Hemoglobinuria*
- Scleral icterus, jaundice
- Thromboembolic events†

LABORATORY ABNORMALITIES

- Cytopenias (anemia, thrombocytopenia, leukopenia, pancytopenia)
- Evidence of intravascular hemolysis (elevated lactate dehydrogenase, elevated indirect bilirubin, low haptoglobin)
- Flow cytometry demonstrates deficiency of glycosyl phosphatidylinositol–anchored protein on a portion of red cells, granulocytes, and monocytes
- Iron deficiency
- Hemosiderinuria

*A presenting symptom in approximately 25% of cases.

†May involve an unusual site (skin, splanchnic veins [Budd-Chiari syndrome], cerebral veins).

promoter of the autosomal gene PIGM were identified.[46] Homozygotes were characterized by a propensity toward venous thrombosis and seizures, but clinically significant hemolysis was not observed. The lack of hemolysis was explained by flow cytometric analysis that showed only partial deficiency of GPI-anchored complement regulatory proteins on the red cells. Together, these observations indicate that germline deficiency of GPI-APs sufficient to produce the PNH phenotype is not compatible with life (see "Congenital Abnormalities of Genes Involved in Glycosyl Phosphatidylinositol–Anchored Protein Synthesis"). As previously noted (see "Inherited mutations of CD55 and CD59"), patients with an inherited deficiency of CD59 have a syndrome that includes complement-mediated intravascular hemolysis similar to that observed in PNH due to mutant PIGA, but other GPI-APs were expressed normally in these patients.[43,44] In addition to complement-mediated intravascular hemolysis, the group of patients with homozygous germline mutation of CD59 of Jewish North-African origin described by Nevo and colleagues presented with signs and symptoms of chronic inflammatory demyelinating polyneuropathy.[44]

Hemoglobinuria

Although essentially all patients with classic PNH have episodes of hemoglobinuria some time during their illness, this defining symptom is reported as part of the initial evaluation in only one quarter of all patients. Whether nocturnal hemoglobinuria is the result of an increase in the rate of hemolysis that occurs during sleep or simply the result of overnight accumulation of urine in the bladder is unresolved. Hemoglobinuria is not related to time of day, however, because the pattern can be reversed if the patient is kept awake at night and allowed to sleep during the day. In patients with nocturnal hemoglobinuria, the urine is usually darkly discolored in the morning and clears gradually over the course of the day (Fig. 14-6, *A*). When hemolysis is intense, however, hemoglobinuria persists throughout the day.

The cause of the nocturnal exacerbation (if it truly exists) is poorly understood. Retention of carbon dioxide, causing a slight fall in plasma pH sufficient to activate the APC, is a possible explanation, but this hypothesis has been challenged.[86] That the first voided urine of the morning is representative of the concentrated filtrate that has accumulated and remained in the bladder over a prolonged period of time (i.e., during the period of sleep) likely contributes to the impression that hemolysis worsens overnight.

Episodic Hemolysis

In addition to the sleep-related pattern, most patients with classic PNH experience irregular exacerbations of hemolysis and hemoglobinuria. Paroxysms can be precipitated by a variety of events including infections (even minor types), surgery, transfusions, iron supplementation, vaccinations, and menstruation. Attacks of hemoglobinuria are unrelated to cold exposure, thus distinguishing PNH clinically from paroxysmal cold hemoglobinuria.

Mild hemolytic episodes often pass without significant symptoms, but more severe attacks may be associated with substernal, lumbar, or abdominal pain, together with malaise and headaches. The abdominal pain may be colicky and last for 1 to 2 days. The abdomen may be painful to palpation, especially in the left upper quadrant, with guarding and rebound tenderness. The back pain resembles that noted in patients with other types of intravascular hemolysis (such as in an ABO incompatible transfusion reaction) and is most severe in the lumbar region. Headaches may be excruciating and sometimes last for days.

Paroxysmal Nocturnal Hemoglobinuria and Bone Marrow Failure

Although the marrow of patients with classic PNH appears fairly normal morphologically, numerous in vitro studies have shown that the growth characteristics of marrow-derived stem cells are aberrant.[14,71,87] Moreover, when stem cells are sorted into GPI-AP– and GPI-AP+ populations, compared to the GPI-AP+ population, the growth characteristics of the GPI-AP– population more closely approach those of normal control cells.[71] One plausible explanation for this observation is that the GPI-AP– cells are relatively protected from the pathophysiologic process that mediates the marrow injury, thereby providing a basis for natural selection of the *PIGA* mutant clone. In this view of PNH, outgrowth of the *PIGA* mutant clone is seen as an example of Darwinian evolution occurring within the microenvironment of the marrow. Although intellectually appealing, rigorous experimental support for this hypothesis is lacking.

Using high-resolution flow cytometry with the capacity to detect less than 0.003% GPI-AP– erythrocytes and

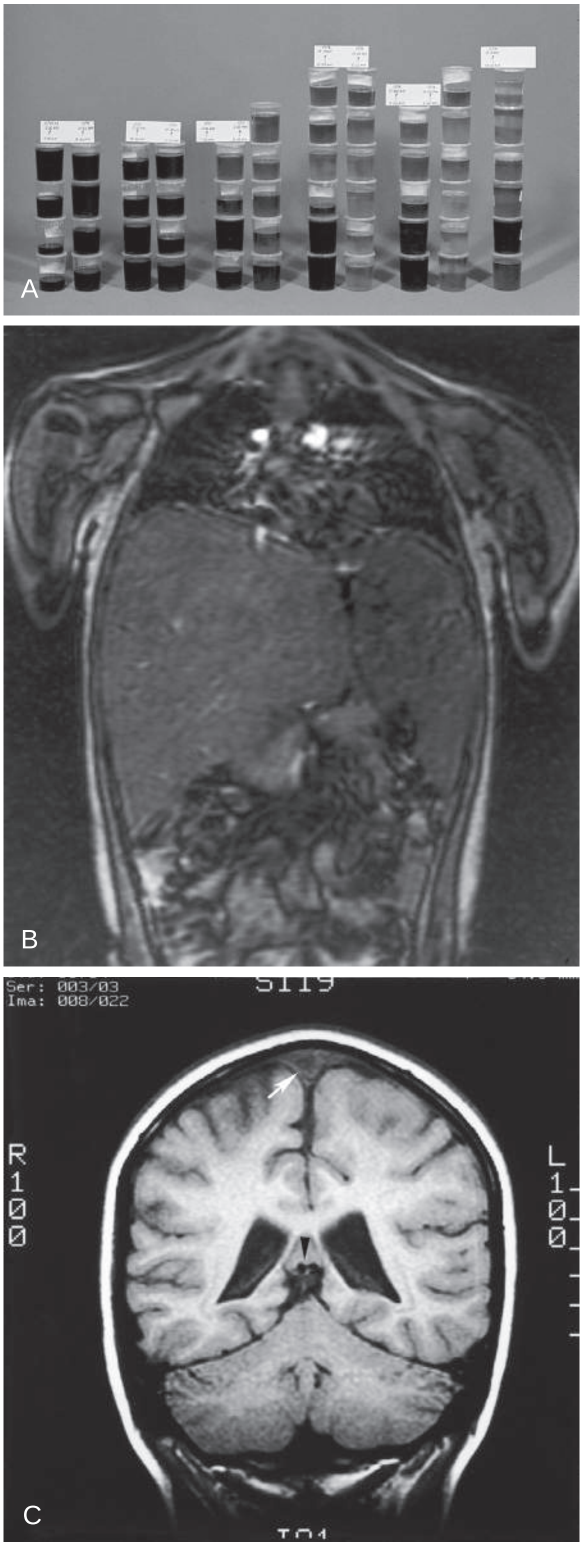


Figure 14-6 Clinical manifestations of paroxysmal nocturnal hemoglobinuria (PNH). **A,** Hemoglobinuria produced over a period of several days, with urine samples ranging in appearance from normal yellow to black. **B,** Massive hepatosplenomegaly that developed in a 14-year-old patient with PNH and Budd-Chiari syndrome. **C,** A fatal superior sagittal sinus thrombosis that developed in a 16-year-old girl with PNH. In this T1-weighted coronal image at the level of the posterior internal cerebral veins, the normally expected black flow signal void in the superior sagittal sinus *(white arrow)* is replaced with an intermediate signal as a result of the thrombus. This appearance contrasts with the normal black venous flow void that is preserved in the internal cerebral veins *(black arrowhead). (A, Courtesy of Dr. Wendell Rosse; C, Courtesy of Drs. Frank Keller and Jeffrey Hogg.)*

granulocytes, 50% to 60% of patients with AA can be shown to have a population of PNH cells at diagnosis;[88-92] however, only 10% to 15% of patients with AA treated with immunosuppressive therapy subsequently develop clinically apparent PNH, and development of clinical disease, when it occurs, typically follows diagnosis of AA by several years.[93] In the remainder, GPI-AP– cells persist subclinically or disappear,[90] suggesting that mutant *PIGA* (and the consequent deficiency of GPI-APs) is necessary for clonal selection but is insufficient to account for the clonal expansion required for clinical manifestations of PNH to become apparent. One interpretation of these observations is that factors in addition to mutant *PIGA* determine the clinical phenotype of the disease by affecting the extent to which the *PIGA* mutant stem cells expand (see "A Two-Step Model of Paroxysmal Nocturnal Hemoglobinuria Pathogenesis"). Conceivably, a second genetic event that works additively or synergistically with mutant *PIGA* is required for clonal expansion.[77] That the extent of clonal expansion varies markedly among patients, however, suggests that the second event may have diverse etiologies and could involve somatic mutations, epigenetic phenomena, or stochastic processes.

The basis of the relationship between PNH and AA is speculative. Essentially, all patients with PNH have some evidence of marrow failure (e.g., thrombocytopenia, leukopenia, or both) during the course of their disease. Therefore, marrow injury may play a central role in the development of PNH by providing the conditions that favor the growth/survival of *PIGA*-mutant, GPI-AP– stem cells. Finding a population of GPI-AP– erythrocytes in patients with AA is clinically relevant because some studies suggest that these patients have a particularly high probability of responding to immunosuppressive therapy, and the onset of the response appears to be more rapid compared to patients with AA without a population of GPI-AP– erythrocytes.[89,92]

The presence of PNH cells has also been observed in patients with myelodysplastic syndrome (MDS).[90,92,94,95] Of note, the association between PNH and MDS appears to be confined to low-risk categories of MDS, particularly the refractory anemia (RA) variant and to a lesser extent refractory cytopenias with multilineage dysplasia (RCMD). Using high-sensitivity flow cytometry in which at least 0.003% or greater GPI-AP– red blood cells (RBCs) or polymorphonuclear leukocytes (PMNs) was classified as abnormal, Wang and colleagues reported that 21 of 119 (18%) patients with RA MDS had a population of PNH cells, whereas GPI-AP– cells were not detected in patients with RA with sideroblasts (RARS), RA with excess of blasts (RAEB), or RAEB in transformation.[92] Compared to patients with RA without a population of PNH cells (RA-PNH$^-$), patients with RA with a population of PNH cells (RA-PNH$^+$) had a distinct clinical profile characterized by the following features: (1) less-pronounced morphologic abnormalities of blood cells; (2) more severe thrombocytopenia; (3) lower rates of karyotypic abnormalities; (4) higher incidence of HLA-DR15; (5) lower rate of progression to acute leukemia; and (6) higher probability of response to cyclosporine therapy.

That a population of PNH cells is associated only with low-risk MDS variants in Japanese patients was confirmed in a North American study of 137 patients classified by World Health Organization criteria.[94] The study found a population of PNH cells in 1 of 5 (20%) patients with 5q– syndrome, in 6 of 17 (35%) patients with RA, and in 2 of 37 (5%) patients with RCMD, whereas no patient with RARS (0 of 9), RCMD-ringed sideroblasts (0 of 6), RAEB (0 of 26), MDS unspecified (0 of 10), myelodysplastic/myeloproliferative disease (0 of 10), primary myelofibrosis (0 of 5), chronic myelomonocytic leukemia (0 of 5), or AML (0 of 6) had a detectable population of GPI-AP– blood cells.

When combined with evidence of polyclonal hematopoiesis (based on the pattern of X-chromosome inactivation in female patients), the presence of a population of PNH cells in patients with MDS predicts a relatively benign clinical course and a high probability of response to immunosuppressive therapy in patients.[88] A relatively good response to immunosuppressive therapy for patients with MDS and AA was also predicted by expression of HLA-DR15 in studies of both North American and Japanese patients.[91,96] Together, these observations provide compelling indirect evidence that AA and a subgroup of low-risk MDS are immune-mediated diseases and that the immune pathophysiologic process provides the selection pressure that favors the outgrowth of *PIGA*-mutant, GPI-AP– stem cells.

Because many patients with PNH/AA have only a small proportion of complement-sensitive cells, few of the changes usually associated with hemolytic anemia are obvious. Therefore the PNH/aplastic syndrome is probably underdiagnosed. Clinicians need to maintain a high degree of suspicion, and any evidence of hemolysis (e.g., elevated serum LDH) in a patient with AA warrants evaluation for PNH. In one series of 80 European patients with PNH, AA was the first diagnosis in 23 cases (29%);[82,83] the same was true of 26 of 85 patients (31%) from Thailand[97] and 65 of 220 French patients (30%).[84] In some instances, the diagnosis of PNH is made first, and pancytopenia develops subsequently.

Thromboembolic Complications

PNH is associated with a predisposition toward intravascular thromboses, especially within the venous circulation.[4,6,82,84,98] Intraabdominal veins are the most commonly affected (Fig. 14-6, *B*). Cerebral vein (Fig. 14-6, *C*) and superficial dermal vein thrombosis also appear to be represented disproportionately. Thrombotic disease accounts for about 50% of all deaths in patients with PNH. Fatal thromboses usually involve the portal system or the brain (see Fig. 14-6, *B* and *C*).

Recurrent abdominal pain is the dominant clinical manifestation in some patients with PNH. The cause of the pain is often obscure but may be severe enough to suggest an acute abdomen warranting emergency surgery. The possibility that a thrombosis in the portal or mesenteric veins is the cause of the pain should be considered in this setting. Both transient intestinal ischemia and intestinal infarction due to thrombosis involving the microcirculation are other possible causes.

Hepatic venous thrombosis (Budd-Chiari syndrome) is a serious, potentially fatal complication of PNH (see Fig. 14-6, *B*).[4,99,100] In various series, 15% to 30% of patients with PNH had hepatic venous thrombosis, and this complication might be even more common because affected individuals can be asymptomatic when Budd-Chiari syndrome is in its early stage.[101] The clinical manifestations include nausea, abdominal pain, variable degrees of ascites, variceal bleeding, and signs of liver failure. Often, the liver increases abruptly in size, but hepatomegaly is not always noted. Three pathophysiologic stages of hepatic venous thrombosis have been defined.[101] In the early or mildest stage, only venules or small hepatic veins are involved. Patients may be asymptomatic, and therefore the condition may go unrecognized. Mild, easily controlled ascites is detected in some patients. In the second stage, larger hepatic veins are partially occluded. Ascites is noted in most patients with such abnormalities. Some individuals develop variceal bleeding, and a few become jaundiced. The third or advanced stage is characterized by complete occlusion of large hepatic veins. Ascites is almost always present, jaundice is common, and variceal bleeding occurs in a few patients. This stage is often fatal.

An increase in the level of serum LDH, alanine aminotransferase, and aspartate aminotransferase may be an early clue to the presence of hepatic vein thrombosis. Plasma concentration of conjugated bilirubin also may increase, but other biochemical tests of liver function are of limited value. Ultrasonography is the most effective, noninvasive method for early detection of hepatic vein thrombosis with a sensitivity and specificity of 85% or more.[102,103] Computed tomographic and magnetic resonance imaging (MRI) scans are more sensitive than ultrasound. MRI is better for visualizing the whole length of the vena cava and may permit differentiation of the acute form from the subacute form of the disease.[103] Radioactive isotope scanning demonstrates patchy uptake in most of the liver, except for a normal functioning, hypertrophied caudate lobe that is spared because of its separate venous drainage. Hepatic venography is definitive, but this procedure carries more risk than noninvasive techniques. Biopsy may demonstrate congestion and liver cell loss, but the procedure is hazardous and not always informative.

Small-vessel thromboses may cause severe, refractory headaches that may presage progressive cerebrovascular involvement, most commonly superior sagittal sinus thrombosis that is fatal in more than 30% of cases (see Fig. 14-6, C)[4,104,105] Signs and symptoms of cerebral sinus thrombosis include headache, nausea and vomiting, cognitive dysfunction including alterations in consciousness, and papilledema. Hemorrhagic infarction may accompany the thrombotic event, making anticoagulation untenable. Given the potentially devastating consequence of central nervous system thrombosis, a high index of suspicion is warranted, and MRI techniques are recommended for diagnosis in symptomatic patients.

Retrospective studies suggest that the monoclonal ant-C5 antibody, eculizumab (Soliris), ameliorates the risk of thrombosis in patients with PNH.[4,6,106] For this reason, patients with PNH with cerebral venous sinus (CNS) thrombosis, Budd-Chiari syndrome, or refractory splanchnic vein thrombosis are candidates for emergency treatment with eculizumab.[4]

Renal Abnormalities

Both acute and chronic renal insufficiency occurs in patients with PNH.[107,108] Acute renal insufficiency is associated with hemoglobinuric crises and may resolve without residual damage. In one series of 19 patients with PNH, however, 12 individuals had reduced values for creatinine clearance while their underlying disease was stable. Furthermore at least three of these patients had progressive renal insufficiency. Patients with PNH may also have hematuria, proteinuria, hypertension, an inability to concentrate their urine, or some combination of these abnormalities. The kidneys usually are enlarged when examined radiographically and MRI may reveal hemosiderin deposition. The renal abnormalities likely result from repeated thrombotic episodes involving small venules.

Dysphagia and Male Impotence

When closely questioned, many patients with PNH complain of painful or difficult swallowing. The symptom is often worse in the morning and appears to be exacerbated during hemolytic episodes. Studies of peristalsis have shown that the esophageal contractions that occur in this setting have 9 to 10 times the normal force. The pathogenesis of these esophageal symptoms is speculative. Inasmuch as hemoglobin binds nitric oxide, it has been proposed that the plasma free hemoglobin that is a consequence of the chronic intravascular hemolysis characteristic of PNH acts as a sump for nitric oxide.[109] Esophageal and intestinal spasm may ensue due to consumption by plasma free hemoglobin of the smooth muscle relaxing activity of nitric oxide. This hypothesis is supported by observations that patients receiving artificial hemoglobin also experience dysphagia and odynophagia.[110]

Male impotence is common in patients with PNH and is worse during hemolytic exacerbations. As is the case with dysphagia, nitric oxide deficiency that is a consequence of the sump effect of plasma free hemoglobin may underlie the erectile dysfunction.

Infections

The apparent increased incidence of infections in patients with PNH may be attributable to leukopenia, to treatment with corticosteroids, or to functional defects in leukocytes (although compelling experimental support is lacking). Even mild infections may constitute a serious hazard because they may precipitate a hemolytic exacerbation.

Physical Examination

Findings are largely nonspecific (related to anemia, thrombocytopenia, or neutropenia). Jaundice may be observed in patients with brisk hemolysis. Moderate splenomegaly and mild to moderate hepatomegaly are sometimes observed and should raise concerns about hepatic or splenic vein thrombosis.

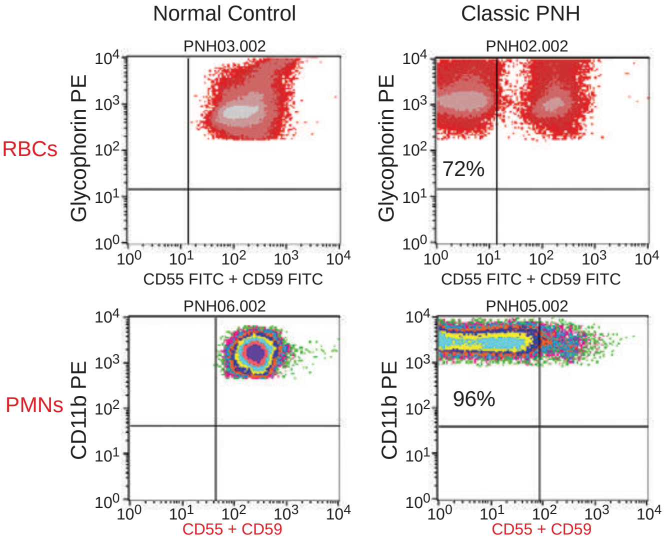


Figure 14-7 Flow cytometry diagnosis of paroxysmal nocturnal hemoglobinuria (PNH). In this example, two-color flow cytometry is used to analyze red blood cells (RBCs) *(top panels)* and neutrophils (PMNs) *(bottom panels)*. RBCs are gated on based on staining with phycoerythrin (PE)–conjugated antiglycophorin, and glycosyl phosphatidylinositol–anchored proteins (GPI-APs) are gated on using a combination of fluorescein isothiocyanate (FITC)–conjugated anti-CD55 and anti-CD59. PMNs *(bottom panels)* are gated on using PE-conjugated anti-CD11b. The top and bottom panels on the left show analysis of RBCs and PMNs from a normal volunteer (control). All the normal RBCs and PMNs express CD55 and CD59. The top and bottom panels on the right show analysis of RBCs and PMNs from a patient with classic PNH. The percentage of cells that are deficient in expression of GPI-APs is shown the inner-upper quadrant of the histograms. The percentage of deficient RBCs is almost always greater than the percentage of deficient PMNs because GPI-AP– RBCs are selectively destroyed by complement-mediated lysis, whereas GPI-AP– PMNs have a normal life span. For this reason, the size of the PNH clone is determined by the percentage of GPI-AP– PMNs rather than by the percentage of GPI-AP– RBCs.

DIAGNOSIS

Once suspected, diagnosing PNH is straightforward as deficiency of GPI-APs on peripheral blood cells is readily demonstrated by flow cytometry (Figs. 14-7 and 14-8).[111] Analysis of both RBCs and PMNs is warranted because clone size will be underestimated if only RBCs are examined because GPI-AP– red cells are selectively destroyed by complement (see Fig. 14-7). Recent transfusion will also affect the estimate of clone size if only RBCs are analyzed, but delineation of PNH phenotypes (i.e., the percentage of type I, type II, and type III cells) requires flow cytometric analysis of the erythrocyte population (see Fig. 14-4).

In addition to flow cytometric analysis, the basic initial evaluation of a patient with PNH should include complete blood count to assess the effects of the disease on production of leukocytes and platelets as well as erythrocytes; biochemical markers of hemolysis (serum concentration of LDH, bilirubin [fractionated], and haptoglobin); determination of iron stores; and bone marrow aspirate, biopsy, and cytogenetics (Box 14-3). These diagnostic studies will allow classification of patients into three

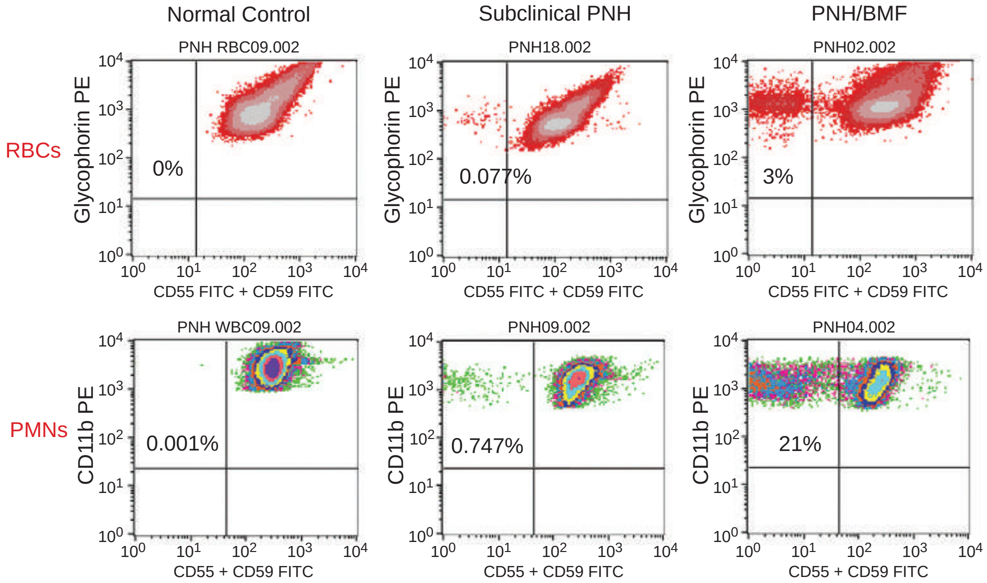


Figure 14-8 Flow cytometry of peripheral blood cells from a patient with subclinical paroxysmal nocturnal hemoglobinuria (PNH) and a patient with PNH/bone marrow failure (BMF). Please see the legend to Figure 14-6 for an explanation of the techniques used to analyze glycosyl phosphatidylinositol–anchored proteins expression on erythrocytes and neutrophils (PMNs). Patients with subclinical PNH have very small clones, typically less than 1%, and they have no biochemical evidence of hemolysis. Patients with PNH/BMF have at least biochemical evidence of hemolysis, but clone size is generally relatively small. In the case illustrated, the clone size of 21% is at the lower limit for biochemical detection of hemolysis.

groups based on the recommendation of the International PNH Interest Group (Table 14-1).[3]

In patients with classic PNH (see Fig. 14-7, Table 14-1), the leukocyte and platelet counts are usually normal or nearly normal, whereas leukopenia, thrombocytopenia, or both invariably accompany PNH in the setting of another bone marrow failure syndrome (see Fig. 14-8, Table 14-1). The reticulocyte count is needed to assess the ongoing capacity of the bone marrow to respond to the anemia. Although the reticulocyte count is elevated in patients with classic PNH, it may be inappropriately low for the degree of anemia, reflecting underlying relative insufficiency of hematopoiesis that is characteristic of the disease. Serum LDH is always markedly elevated in classic PNH. Patients with classic PNH may be iron deficient due to chronic hemoglobinuria and hemosiderinuria. Nonrandom cytogenetic abnormalities are uncommon in PNH.[77,112] The degree of serum LDH elevation is variable in patients with PNH in the setting of another bone marrow failure syndrome; however, in a large majority of patients with PNH/bone marrow failure, the clone size is less than 10%, with less than 10% of patients with PNH/bone marrow failure having a clone size of greater than 50% (see Fig. 14-8, Table 14-1).[90,113,114] Nonetheless, bone marrow aspirate and biopsy are needed to distinguish classic PNH from PNH in the setting of another bone marrow abnormality. By definition, patients with subclinical PNH (PNH-sc) have neither clinical nor biochemical evidence of hemolysis (see Fig. 14-8, Table 14-1).

Box 14-3 Basic Studies for Evaluation and Management of Paroxysmal Nocturnal Hemoglobinuria

- Complete blood count
- Reticulocyte count
- Serum concentration of lactate dehydrogenase, bilirubin (fractionated), haptoglobin
- Flow cytometric analysis of erythrocytes and granulocytes for expression of glycosyl phosphatidylinositol–anchored proteins
- Bone marrow aspirate and biopsy and cytogenetic analysis
- Serum erythropoietin concentration
- Serum concentration of blood urea nitrogen and creatinine
- Serum iron studies (iron concentration, total iron binding capacity, transferrin saturation, ferritin concentration)
- Urine hemosiderin*

*Urine hemosiderin is indicative of chronic hemolysis but provides no quantitative information.

Management Based on Classification

Completing the above diagnostic evaluation (see Box 14-3) will allow development of a systematic treatment plan (Fig. 14-9) based on disease classification (see Table 14-1).

Subclinical Paroxysmal Nocturnal Hemoglobinuria

A close association exists between PNH and AA and to a lesser extent between PNH and low-risk MDSs (see *Paroxysmal Nocturnal Hemoglobinuria and Bone Marrow Failure*). By using high-resolution flow cytometry, approximately 50% to 60% of patients with AA and 15% to 20% of patients with low-risk MDS have been found to have a detectable population of GPI-AP– erythrocytes and granulocytes.[88-90,92,94] In approximately 90% of these cases the proportion of GPI-AP– peripheral blood neutrophils is less than 25% of the total.[90]

TABLE 14-1 Classification of PNH

Category	Rate of Intravascular Hemolysis*	Bone Marrow	Flow Cytometry	Benefit from Eculizumab
Clinical PNH	Florid (markedly abnormal LDH, often with episodic macroscopic hemoglobinuria)	Cellular marrow due to erythroid hyperplasia and normal or near-normal morphology†	Large population (>50%) of GPI-AP– PMNs§	Yes
Classic				
PNH in the setting of another bone marrow failure syndrome‡	Mild (often with minimal abnormalities of biochemical markers of hemolysis)	Evidence of a concomitant bone marrow failure syndrome‡	Although variable, the percentage of GPI-AP– PMNs is usually relatively small (<50%)	Typically no, but some patients have relatively large clones and clinically significant hemolysis and may benefit from treatment
Subclinical	No clinical or biochemical evidence of intravascular hemolysis	Evidence of a concomitant bone marrow failure syndrome‡	Small (<1%) population of GPI-AP– PMNs detected by high-resolution flow cytometry	No

Modified from International PNH Interest Group: Diagnosis and management of paroxysmal nocturnal hemoglobinuria. Blood *106:3699–3709, 2005.*

GPI-AP–, Glycosyl phosphatidylinositol–anchored protein–deficient; *LDH,* lactate dehydrogenase; *PMN,* polymorphonuclear leukocyte; *PNH,* paroxysmal nocturnal hemoglobinuria; *RBC,* red blood cell.

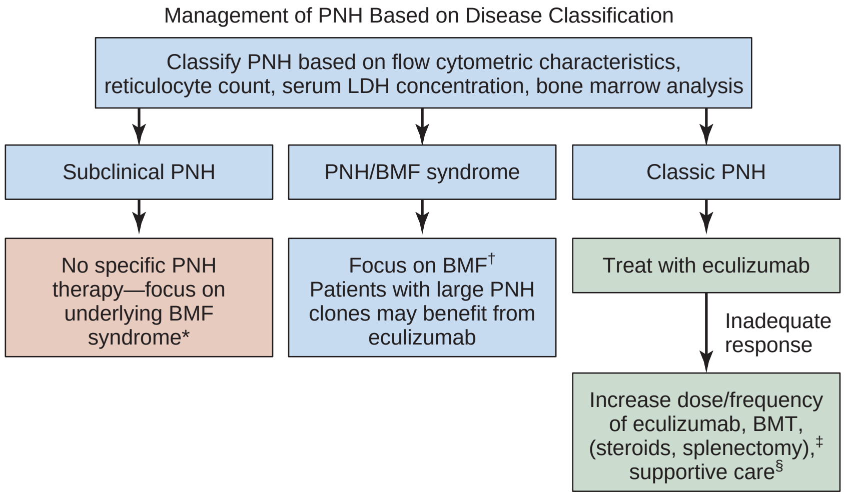
*Based on macroscopic hemoglobinuria, serum LDH concentration and reticulocyte count.

†Karyotypic abnormalities are uncommon.

‡Aplastic anemia or low-risk myelodysplastic syndrome.

§Analysis of PMNs is more informative than analysis of RBCs due to selective destruction GPI-AP– RBCs.

Recent studies have investigated the natural history of PNH clones in the setting of bone marrow failure.[90,113,114] The threshold that separates PNH-sc from clinical PNH is reached when the neutrophil clone size is in the range of 20% to 25% with a corresponding GPI-AP– erythrocyte population of 3% to 5%.[113] Longitudinal studies indicate that clonal expansion occurs in 15% to 50% of cases.[90,113,114] In 10% to 25% of cases the clone disappears, and in 25% to 60% of cases the clone size persists unchanged.[90,113,114] Importantly, available evidence indicates that patients who present with subclinical PNH do not necessarily progress to clinical PNH.[90,113,114] In approximately 80% of the cases in which GPI-AP– cells are detected in the setting of bone marrow failure, the


Management of PNH Based on Disease Classification
Classify PNH based on flow cytometric characteristics, reticulocyte count, serum LDH concentration, bone marrow analysis
Subclinical PNH
PNH/BMF syndrome
Classic PNH
No specific PNH therapy—focus on underlying BMF syndrome*
Focus on BMF†
Patients with large PNH clones may benefit from eculizumab
Treat with eculizumab
Inadequate response
Increase dose/frequency of eculizumab, BMT, (steroids, splenectomy),‡ supportive care§

BMF, Bone marrow failure (aplastic anemia and low risk MDS); *BMT*, bone marrow transplant.
*Some, but not all, studies suggest a favorable response to immunosuppressive therapy (IST).
† BMT eradicates the PNH clone, and typically, treatment with IST does not affect PNH clone size.
‡ Consider for patients with clinically significant extravascular hemolysis.
§ Transfusion.

Figure 14-9 Management of paroxysmal nocturnal hemoglobinuria (PNH). A management scheme based on classification of PNH into three categories (subclinical, PNH in the setting of another bone marrow failure syndrome [PNH/BMF], and classic PNH [see Table 14-1 for characteristics of each category]) is illustrated. *LDH,* Lactate dehydrogenase.

proportion of GPI-AP– cells is less than 1% of the total circulating granulocytes. These patients (designated PNH-sc) with very small populations of GPI-AP– cells have no clinical or biochemical evidence of hemolysis and require no specific treatment for PNH (see Fig. 14-9). Finding a population of GPI-AP– erythrocytes in patients with AA, however, may be clinically relevant because some,[89,92] but not all,[96] studies suggest that these patients have a particularly high probability of responding to immunosuppressive therapy with a more rapid rate of onset of response compared to patients with AA without a population of GPI-AP– erythrocytes.[90]

The presence of PNH cells has also been observed in patients with MDS.[90,92,94,95] As noted above (see *Paroxysmal Nocturnal Hemoglobinuria and Bone Marrow Failure*), the association between PNH and MDS appears to be confined to low-risk categories of MDS, particularly the RA variant and, to a lesser extent, the category of RCMD.[88-90,92,94]

PNH in the setting of another bone marrow failure syndrome. Patients with a bone marrow failure syndrome (AA or MDS) and a PNH clone with clinical/biochemical evidence of hemolysis are classified as PNH in the setting of another bone marrow failure syndrome (see Table 14-1). In these patients bone marrow failure dominates the clinical picture, and hemolysis is primarily an incidental finding.[90,113,114] The majority of patients with PNH/AA and PNH/MDS have relatively small PNH clones (less than 10%) and require no specific PNH therapy. In these cases treatment should focus on the underlying bone marrow failure syndrome (see Fig. 14-9). Among patients who present with clinical PNH in the setting of bone marrow failure, treatment for complications of PNH (eculizumab for hemolysis or anticoagulation for thrombosis) is required in approximately 50% of cases.[114] There is no evidence that treatment with immunosuppressive therapy influences clonal expansion either positively or negatively.

The PNH clone will be eradicated by the conditioning regimen in combination with graft-versus-host disease in patients undergoing allogeneic transplant for AA or MDS. In most instances, the size of the PNH clone is unaffected by treatment with immunosuppressive therapy, and the presence of a PNH clone should not deter immunosuppressive therapy if that approach to treatment of the underlying bone marrow failure syndrome is considered appropriate.[113,114] In instances in which, following immunosuppressive therapy, the size of the PNH clone is sufficiently large so as to produce clinical symptoms, the patient can be managed using the same approach as for patients with classic PNH.

Classic Paroxysmal Nocturnal Hemoglobinuria

Patients with classic PNH have a large clone (greater than 50%) (see Fig. 14-7), and consequently this disease subcategory is characterized by florid intravascular hemolysis as indicated by a markedly elevated serum LDH (see Table 14-1). Patients may complain of episodic hemoglobinuria, and most experience ongoing constitutional symptoms dominated by lethargy, malaise, and asthenia that can be debilitating (see Box 14-3). The complement-mediated intravascular hemolysis of PNH can be inhibited by blocking formation of the MAC (see Fig. 14-3). The MAC consists of complement components C5b, C6, C7, C8, and multiple molecules of C9. Eculizumab is a humanized monoclonal antibody that binds complement C5, preventing its activation to C5b by the APC C5 convertase and thereby inhibiting MAC formation (see Fig. 14-3).[115] In 2007, eculizumab was approved by both the U.S. Food and Drug Administration (FDA) and the European Medicines Agency for treatment of the hemolysis of PNH. Treatment of patients with classic PNH with eculizumab reduces transfusion requirements, ameliorates the anemia of PNH, and improves quality of life by resolving the debilitating constitutional symptoms associated with chronic complement-mediated intravascular hemolysis (see Fig. 14-9).[116,117,118] Following treatment, serum LDH concentration returns to normal or near normal, with approximately one half to two thirds achieving transfusion independence, but mild to moderate anemia, hyperbilirubinemia, and reticulocytosis persist in essentially all treated patients.[6,117,118]

Eculizumab appears to reduce the risk of thromboembolic complications.[106] For patients being treated with eculizumab who have no prior history of thromboembolic complications, prophylactic anticoagulation may be unnecessary, while it is recommended that anticoagulation continue for those patients who experienced a thromboembolic event prior to initiating therapy with eculizumab.[4,6]

Eculizumab is expensive (around $400,000/year in the United States), and it has no effect either on the underlying stem cell abnormality or on the associated bone marrow failure. Consequently, treatment must continue indefinitely, and leukopenia, thrombocytopenia, and reticulocytopenia, if present, persist. Treatment with eculizumab appears to have a favorable impact on survival, as a recent study of 79 patients treated between 2002 and 2010 showed the same survival as that of age- and sex-matched controls from the general population.[6,7] The contribution of eculizumab to survival cannot be quantified definitively, however, as a control patient group (PNH patients not treated with eculizumab) was not included in that study.

Reasons for Eculizumab Failure. The recommended maintenance dose of eculizumab is fixed (900 mg every 2 weeks ± 2 days) rather than being based on weight or body surface area. Some patients may show evidence of breakthrough intravascular hemolysis (i.e., a rise in LDH and development of constitutional symptoms) near the end of a treatment cycle. In these cases, breakthrough hemolysis can be ameliorated by reducing the length of the treatment cycle from 14 days to 13 days or 12 days, and in some instances, the maintenance dose of eculizumab may also need to be increased (see Fig. 14-9).

All patients with PNH have an element of bone marrow failure, and patients treated with eculizumab who have higher degrees of relative reticulocytopenia may remain anemic or even transfusion-dependent despite excellent control of intravascular hemolysis. Iron stores and serum erythropoietin concentration should be quantified in these patients, and if iron stores are adequate, and serum erythropoietin concentration is inappropriately low, a trial of recombinant erythropoietin is warranted in patients who have symptomatic anemia or who are transfusion dependent.

Following treatment with eculizumab, serum LDH returns to normal or near normal, but mild to moderate anemia and laboratory evidence of hemolysis persist in essentially all treated patients.[117,118] A subgroup of eculizumab-treated patients experience little or no improvement in either anemia or constitutional symptoms. In these patients hemolysis is mediated by opsonization of the PNH erythrocytes by activation and degradation products of complement C3, and when tested, they are found to be direct antiglobulin test–positive for C3 but not IgG.[119-121] The known pathophysiology of the PNH predicts that CD55 deficiency would result in ongoing extravascular hemolysis of PNH erythrocytes as a consequence of C3 opsonization (see Fig. 14-3) because eculizumab does not block the activity of the APC C3 convertase that is unregulated because of CD55 deficiency (see Fig. 14-3). Support for this hypothesis is provided by the studies of Risitano and colleagues,[121] who showed that in patients treated with eculizumab, a portion of the PNH erythrocytes (i.e., the CD59-deficient population) had complement C3 bound. Those studies also confirmed the direct antiglobulin test–negative designation of PNH as no C3 was found bound to PNH erythrocytes prior to initiation of treatment with eculizumab, implying that PNH erythrocytes upon which complement has been activated are destroyed directly as a consequence of MAC-mediated cytolysis. Thus those studies provide a plausible explanation for the persistent hemolytic anemia observed in PNH patients treated with eculizumab. By inhibiting formation of the MAC, eculizumab prevents direct cytolysis of PNH erythrocytes, allowing the manifestations of CD55 deficiency to become apparent in the form of aberrant regulation of the APC C3 convertase and the consequent deposition of activated C3 on the cell surface (see Fig. 14-3).[122] Covalently bound activation and degradation products of C3 then serve as opsonins that are recognized by specific receptors on reticuloendothelial cells, resulting in extravascular hemolysis.

The extravascular hemolysis of patients with PNH receiving eculizumab does not require treatment in the absence of constitutional symptoms, symptoms of anemia, or transfusion dependence. Because the process is extravascular, splenectomy or corticosteroids may ameliorate the hemolysis in symptomatic or transfusion-dependent patients by removing or inhibiting the function of phagocytic cells (see Fig. 14-9).[123] Long-term use of corticosteroids is associated with significant toxicity, however, and concerns about both postoperative and late complications temper enthusiasm for splenectomy. It is also conceivable that the primary site of phagocytosis is hepatic rather than splenic. In such cases, response to splenectomy would likely be inadequate. Based on experience in the treatment of refractory autoimmune hemolytic anemia, a trial of danazol can be considered; however, rituximab is not indicated because the process is mediated by C3 opsonization rather than opsonization by IgG antibody.

Recently, Nishimura and colleagues identified a polymorphism of C5 in 9 Japanese patients with PNH who had a poor response to treatment with eculizumab.[124] An anti-C5 antibody directed against an epitope different from that recognized by eculizumab completely blocked the hemolytic activity in the sera of the patients with the C5 polymorphism. The prevalence of the polymorphism in the Japanese population appears to be in the range of 2% to 3%.

Hematopoietic Stem Cell Transplant. Prior to the availability of eculizumab, the primary indications for transplant for PNH were bone failure; recurrent, life-threatening thrombosis; and uncontrollable hemolysis.[3,125,126] The latter process can be eliminated by treatment with eculizumab, and the thrombophilia of PNH may also respond to inhibition of intravascular hemolysis by eculizumab.[4,106] Nonetheless, transplant is the only curative therapy for PNH, and the availability of molecularly defined, matched, unrelated donors; less toxic conditioning regimens; reduction in transplant-related morbidity; and mortality and improvements in posttransplant supportive care make this option a viable alternative to medical management. The decision of who and when to transplant is complex, however, and requires an understanding of the unique pathobiology of PNH and the input of physicians experienced in transplantation and medical management of PNH.[3,125,126] The recent studies of Kelly and colleagues[6] that indicate normal survival for patients with PNH treated with eculizumab make the decision concerning medical management versus transplant even more challenging.

Other Treatment. Based on anecdotal experience, a portion of patients with classic PNH responds to danazol as first line therapy.[127,128] The basis of this response is unknown but likely involves complement inhibition because reduction in hemolysis is observed quickly (within a few days) following initiation of therapy, and PNH white blood cell clone size does not change during treatment.* Why some patients respond dramatically to danazol while others do not is unknown, but it seems plausible to suggest that responders produce a metabolite that inhibits complement, while nonresponders do not (or they produce a subtherapeutic concentration of the putative inhibitory metabolite).

Although hemolysis is ameliorated in some patients by treatment with glucocorticoids, the harm that can accrue from long-term use of prednisone cannot be overemphasized.[3] The main value of glucocorticoids may be in attenuating acute hemolytic exacerbations. Under these circumstances, brief pulses of prednisone may reduce the severity and duration of the crisis while avoiding the untoward consequences associated with long-term use.

Because the hemolysis is a consequence of a defect intrinsic to the patient's erythrocytes, the anemia of PNH responds to red cell transfusion. In addition to increasing the hemoglobin concentration, transfusion may lessen hemolysis by suppressing erythropoiesis. Concerns about inducing a hemolytic exacerbation as a consequence of infusion of small amounts of donor plasma that may contaminate red cell preparations appear unwarranted.[129]

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*Courtesy Parker CJ. University of Utah School of Medicine. Unpublished observation.

Patients with classic PNH frequently become iron deficient as a result of renal loss (both hemoglobinuria and hemosiderinuria).[127] Clinically important iron loss from hemosiderinuria can occur even in the absence of gross hemoglobinuria. Concern for inducing a hemolytic exacerbation should not deter iron repletion because iron deficiency not only limits erythropoiesis but also exacerbates the hemolysis of PNH.[127] If a hemolytic exacerbation occurs in the setting of iron repletion, the episode can be controlled by treatment with corticosteroids or by suppression of erythropoiesis by transfusion. There is no concern about iron-replacement therapy inducing a hemolytic exacerbation in patients being treated with eculizumab because the drug inhibits hemolysis. Further, patients treated with eculizumab should not become iron deficient because treatment will resolve the hemoglobinuria and hemosiderinuria that causes the iron deficiency by blocking intravascular hemolysis.

Management of Thrombophilia. Thromboembolic complications are the leading cause of morbidity and mortality in PNH.[4] Prophylaxis against thromboembolic events in patients with PNH is an issue of active debate.[3] Current estimates of risk are based on retrospective analysis, but risk may correlate with size of the PNH clone (based on flow cytometric determination of the percentage of GPI-AP– PMNs), leading to the recommendation that patients with greater than 50% to 60% GPI-AP– PMNs be offered prophylactic anticoagulation.[4,130-132]

Although arterial thrombosis may be observed,[4,106] thromboembolic events in patients with PNH usually involve the venous system. In the absence of an absolute contraindication (e.g., hemorrhagic infarction involving the CNS), acute thrombotic events require anticoagulation with heparin. Systemic thrombolytic therapy, or thrombolytic therapy delivered via canalization directly to the affected site, can be considered in patients with acute onset of Budd-Chiari syndrome.[133-136] If there is no contraindication, anticoagulation should continue indefinitely for a patient with PNH (even if that patient is treated with eculizumab) who has experienced a thromboembolic complication. For PNH patients on long-term anticoagulation, vitamin K antagonists such as Coumadin have been used customarily. Whether low–molecular-weight heparins or novel oral anticoagulants such as direct thrombin inhibitors and factor Xa inhibitors are more, less, or equally efficacious has not been determined.

Thrombocytopenia often complicates PNH, and this issue must be addressed when formulating an anticoagulation management plan. Thrombocytopenia is a relative, but not an absolute, contraindication to anticoagulation, and transfusions should be given to maintain the platelet count in a safe range rather than withholding therapy. Patients with PNH who experience a thromboembolic event are candidates for indefinite anticoagulation, although recommendations for anticoagulation duration may be modified as more experience with the effects of eculizumab on the thrombophilia of PNH accumulates.[4]

Eculizumab appears to reduce the risk of thromboembolic complications.[4,6,106] For patients being treated with eculizumab who have no prior history of thromboembolic complications, prophylactic anticoagulation may be unnecessary, although it is recommended that anticoagulation continue for those patients who experienced a thromboembolic event prior to initiating therapy with eculizumab.[4]

Pregnancy

Women with PNH can have serious morbidity and increased mortality during pregnancy.[137,138] Because of concerns about fetal/maternal risks from exposure to potentially toxic therapy, anticoagulation and transfusion support have been the mainstay of management of patients with PNH who are pregnant. Eculizumab has been assigned to pregnancy category C (risk cannot be ruled out) by the FDA. There are no controlled studies of the use of eculizumab in human pregnancy; however, anecdotal reports and small series have identified no significant adverse effects when eculizumab is used during pregnancy, including early in gestation and, in one case, from the time of conception.[139-141] Nonetheless, until more is known about the safety of eculizumab in pregnancy, it seems prudent to restrict use of the drug to the third trimester and then only for patients who are at high risk for thrombosis and who have no acceptable therapeutic alternatives.

Moderate to severe thrombocytopenia may complicate the pregnancy, and clinically significant bleeding in this setting necessitates platelet transfusion. The incidence of clinically apparent venous thromboembolism during pregnancy in women with PNH is approximately 10%,[137] and these events are associated with a high risk of mortality.[137,138] Similar to nonpregnant patients with PNH, cerebral and hepatic veins are commonly involved sites of thrombosis. Patients who develop thrombosis should be therapeutically anticoagulated, and thrombolytic therapy and treatment with eculizumab should be considered for those with Budd-Chiari syndrome. Concurrent thrombocytopenia may necessitate platelet transfusion in patients who require anticoagulation. The role of prophylactic anticoagulation for pregnant women with PNH has not been studied systematically; however, because of the significant morbidity and mortality associated with thromboembolism in this setting, prophylaxis is recommended. Coumadin is contraindicated because of both teratogenic potential in the first trimester and hemorrhagic risks later in gestation. Anticoagulation with heparin should begin immediately once the combination of pregnancy and PNH is documented. Low–molecular-weight heparin has a hypothetical advantage over unfractionated heparin because of a lower incidence of drug-induced thrombocytopenia. Careful monitoring of the platelet count is required because thrombocytopenia may worsen during the period of anticoagulation. Anticoagulation can be discontinued briefly around the time of delivery. However, it should be restarted as soon as is feasible and continued for 6 to 12 weeks into the postpartum period because thrombosis during the puerperium is a major concern.[137] Most deliveries can be accomplished vaginally, although premature delivery may be necessary. Despite the many concerns surrounding PNH and pregnancy, successful outcomes appear to be the rule rather than the exception. Management, however, is complicated and should involve the combined efforts of an experienced hematologist and an obstetrician who specializes in dealing with high-risk pregnancies.[3]

Pediatric Paroxysmal Nocturnal Hemoglobinuria

Despite usually being considered an adult disorder, PNH can develop in children and adolescents. PNH has become a better recognized disorder over the past several years and is increasingly identified by pediatric hematologists who have become familiar with its presentation in association with bone marrow failure syndromes and its straightforward diagnosis using flow cytometry. Early studies suggested that about 10% of all patients with PNH are younger than 21 years of age at the time of diagnosis.[85,132] Current data on the incidence and prevalence of PNH in the pediatric age range are not available but might be higher due to better awareness and increased testing.

An older retrospective analysis of 26 cases underscored both similarities and differences between childhood and adult PNH.[85] In young patients the signs and symptoms of marrow failure, thrombosis, and laboratory evidence of hemolysis (e.g., elevated LDH) dominated the clinical picture. In many children PNH was initially misdiagnosed, with an average of almost 2 years from initial symptoms to the correct diagnosis. Gross hemoglobinuria as a presenting symptom appeared to be less common among children and never occurred in one third of the patients. Thrombosis occurred in approximately a third of patients, and some died of this complication. All patients had laboratory evidence of defective or ineffective hematopoiesis, either at diagnosis or over the course of their disease. The survival curve for these patients indicated that the 10-year survival rate was only 60%, although several deaths were due to complications of pancytopenia in an era before modern antibiotic and transfusion therapy.

The peculiar relationship between PNH and AA was first recognized almost a half-century ago,[142] and this association holds true for pediatric PNH. Recent case series confirm that the majority of PNH in young patients arise in the setting of bone marrow failure, usually AA. A study from the Netherlands confirmed the common presentation of marrow failure in 11 children with PNH.[143] The authors reported that 5 patients eventually underwent hematopoietic cell transplant (3 matched unrelated donors and 2 matched family donors), of whom 4 were long-term survivors. A more recent study of 12 young patients over an 18-year period documented that 10 presented with evidence of bone marrow failure and only one with hemoglobinuria.[61] Importantly, there were six children with thrombosis and five with myelodysplastic features, suggesting that the clinical presentation may be more similar to adult PNH than previously recognized.

A strong index of suspicion is needed to ensure the correct laboratory tests are ordered, which can help establish the diagnosis of PNH in children. The clinical presentation of unexplained cytopenia, particularly beyond a single lineage, or the new onset of thrombosis in an unusual location, should prompt the consideration of PNH. Flow cytometry assays for PNH are now becoming a standard part of the laboratory evaluation.[144] Most testing will include fluorescent aerolysin to identify the absence of all GPI-linked surface proteins on the surface of neutrophils and monocytes,[75] along with CD59 monoclonal antibodies to identify GPI-AP− cells among the erythrocytes.

All children with documented AA or other forms of bone marrow failure syndromes should have laboratory evaluation for PNH. However, similar to the situation for adults, the presence of small PNH clones has been documented in this setting,[145,146] but the significance of PNH-sc remains to be defined. Unless there is clinical evidence of hemolysis or a sizable clone that portends an increased risk of thrombosis, periodic monitoring of the PNH clone should be sufficient. Currently there is no predictive value to having PNH clones detected in the setting of AA, although their percentages may increase following immunosuppressive therapy.

Based on data that suggested poor long-term survival for childhood PNH, hematopoietic cell transplantation has been the recommended treatment and only available curative therapy. Matched sibling donors offer the best results, and myeloablative conditioning regimens will eradicate the PNH clones. Matched unrelated donors remain a high-risk experimental therapy, although cures have been reported.[86] Over the past 5 years, however, the availability of eculizumab is modifying the therapeutic approach to young patients with PNH. Although eculizumab is not yet formally approved for PNH patients younger than age 18 years, it has been used anecdotally with good results in the pediatric/adolescent population.[61] A recent prospective study of seven adolescents with PNH documented the safety and efficacy of short-term eculizumab treatment.[147] These data suggest that eculizumab will emerge as a therapeutic option for pediatric PNH, although further experience will be necessary to determine whether its long-term effects (and cost) make it a realistic alternative to matched transplantation. The availability of eculizumab for pediatric PNH may be particularly advantageous as a bridge prior to implementation of more definitive therapy.

COURSE AND PROGNOSIS

The clinical course of PNH is enormously variable. In rare instances, the patient may succumb to this disease within a few months of the first onset of symptoms; however, most patients experience a stable, chronic course. Rarely, the abnormal clone disappears altogether. Transformation to acute leukemia is uncommon (in the range of 1%). In some instances, but not in others, leukemic blasts are GPI-AP−.[148]

As with many other diseases, initial reports on PNH tended to emphasize the more severely affected patients, so the prognosis was generally deemed to be grave. As physicians developed a higher index of suspicion for diagnosing PNH and as diagnostic studies became more sensitive and specific, milder cases were diagnosed, and these tended to have the better long-term outlook. Nonetheless, even today, the disease must be considered a serious one in which some patients eventually succumb to its complications. The most commonly lethal of the complications of PNH are thrombotic events such as Budd-Chiari syndrome and cerebral sinus vein thromboses,[4] but complications of pancytopenia may also lead to death,[82] and in a few patients the terminal episode has been the development of acute leukemia.[148]

Course and Prognosis in the Pre-Eculizumab Era

In a study of 220 patients with PNH, from the pre-eculizumab era, followed for up to 46 years, the Kaplan-Meier survival estimate was 65% at 10 years and 48% at 15 years after diagnosis.[84] Eight-year cumulative incidence rates of the main complications of pancytopenia, thrombosis, and MDS were 15%, 28%, and 5%, respectively. Poor survival was associated with age older than 55 years at the time of diagnosis, the occurrence of thrombosis as a complication, evolution to pancytopenia, an MDS or acute leukemia, and thrombocytopenia at diagnosis. The prognosis of patients in whom AA antedated PNH was better than in those in whom it did not. In another pre-eculizumab era study of 80 consecutive patients, the median survival after diagnosis was 10 years, with 28% of patients surviving for 25 years.[82]

Course and Prognosis in the Eculizumab Era

In addition to symptomatic benefit, treatment with eculizumab appears to influence the natural history of PNH. In a retrospective study, Kelly and colleagues reported on the clinical history of 79 patients with classic PNH or PNH/BMF treated with eculizumab.[6] The median age at diagnosis was 37 years (range, 12 to 79 years) with the median age at the time of initiation of treatment with eculizumab being 46 years (range 14 to 84 years). The mean duration of treatment with eculizumab was 39 months (range, 1 to 98 months). Based on flow cytometric analysis of peripheral blood neutrophils, the average clone size among the treated patients was 96.4% (range 41.8% to 100%). Twenty-four patients (30%) had a history of a bone marrow failure syndrome at the time of diagnosis (23 with AA and 1 with MDS). Eleven patients had a history of long-term cyclosporine treatment prior to initiating therapy with eculizumab, and three patients developed aplasia requiring immunosuppressive therapy subsequent to starting treatment with eculizumab. Thrombotic episodes were reported in 27% of patients prior to starting eculizumab (including 12 cases of Budd-Chiari syndrome, 4 cases of mesenteric vein thrombosis, and 3 cases of cerebral vein thrombosis).

The investigators found that treatment with eculizumab reduced the mean yearly transfusion requirement from 19.3 units to 5.0 units. Of 61 patients who had been on eculizumab for more than 1 year, 40 (66%) became transfusion independent. Thrombosis was observed in two patients while on eculizumab (a retinal vein thrombosis in a patient also on warfarin and thrombosis of an arteriovenous fistula in a patient on hemodialysis). No thrombotic events were reported in 21 patients who discontinued prophylactic anticoagulation after starting treatment with eculizumab.

Survival of the 79 patients treated with eculizumab was the same as that of a group of age- and sex-matched controls from the general population. Three eculizumab-treated patients died. None of the deaths were deemed by the investigators to be treatment related or to be a consequence of complications of PNH. Two patients, ages 71 and 75 years, developed MDS 56 and 76 months, respectively, after starting treatment with eculizumab. One patient, age 50 years, developed AML 27 years after the initial diagnosis of PNH.

Because patients with congenital deficiency of complement C5 are at risk for meningococcal infections, all patients treated with eculizumab must be vaccinated prior to initiation of therapy.[115] However, the currently available vaccine does not protect against infection with serogroup B organisms. Two eculizumab patients developed documented meningococcal infection with *Neisseria meningitidis* serogroup B, and thereafter, a program of antibiotic prophylaxis was instituted by the investigators. There was no evidence that eculizumab affected the bone marrow failure component of the disease, as a change in platelet count was not observed in 12 patients who were thrombocytopenic prior to initiation of treatment (platelet count less than 100 × 109/L). The mean platelet count for this group was 67 × 109/L at the initiation of therapy compared to 66 × 109/L after 12 months of treatment with eculizumab, respectively.

A multicenter study involving 195 patients followed for 66 months[7] largely confirmed the findings of Kelly and colleagues.[6]

Together with the data from the natural history studies from the pre-eculizumab era, these results suggest that treatment with eculizumab alters the natural history of PNH both by reducing or eliminating transfusion requirements through inhibition of intravascular hemolysis and by virtually eradicating thromboembolic complications.[6,7] Eculizumab treatment may also reduce disease-related mortality, although the extent to which the drug enhances survival cannot be accurately determined from the studies of Kelly and colleagues[6] because the experimental design did not include a randomized control group of patients. Eculizumab does not appear to affect either the bone marrow failure component of the disease or the clonal hematopoiesis that underlies disease pathophysiology. Although PNH is a clonal disease, it is not a malignant disease, and it is this unique characteristic of PNH that allows for successful long-term symptomatic management in the absence of a treatment strategy aimed at eradicating the *PIGA*-mutant hematopoietic stem cells.[149]

CONCLUSIONS AND FUTURE DIRECTIONS

Systematic investigation of the molecular basis of PNH has provided a framework for management based on an understanding of disease pathophysiology and has led to development of targeted therapy that has improved the lives of patients and changed the natural history of the disease. Nonetheless continued investigation of new approaches to therapy aimed at obviating the extravascular hemolysis that limits eculizumab efficacy in some patients is warranted.[122,150] A better understanding of the pathobiology that underlies the thrombophilia of PNH is needed,[4,98] and defining the complex relationship between PNH and bone marrow failure syndromes that determine clonal selection and clonal expansion[77,78] may lead ultimately to therapy that targets the disease at the level of the hematopoietic stem cell. In particular, an understanding of the molecular basis of clonal expansion will be facilitated by the availability of next-generation sequencing that will allow comparison between the genomes of GPI-AP–positive and GPI-AP–negative cells from individual patients with PNH.

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CHAPTER 15

Red Cell Membrane

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CHAPTER OUTLINE

MEMBRANE LIPIDS
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Glycolipids and Cholesterol
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Lipid Mobility
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INTEGRAL MEMBRANE PROTEINS
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Glycophorins
Other Integral Membrane Proteins
MEMBRANE SKELETON PROTEINS
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Actin
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Protein p55
Adducin
Dematin (Protein 4.9)
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Tropomodulin
Ankyrin
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Ankyrin Complex
Actin Junctional Complex
Distribution of Band 3
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Vesiculation and Microparticle Formation
CD47 “Don’t Eat Me versus Eat Me” Switch
Eryptosis

The erythrocyte membrane is the most thoroughly studied biologic membrane. It is a small structure, less than 0.1% of the cell’s thickness and only about 1% of its weight, but its easy accessibility has enabled researchers to characterize both its primary structure and a number of its important functions. It was the original example of the fluid mosaic membrane model, a breakthrough concept proposed by Singer and Nicolson, more than 40 years ago.[1] In this model the erythrocyte membrane is composed of mobile, asymmetrically distributed proteins and lipids that interact in a variety of ways. The model has been revised in the intervening four decades, because many membrane proteins are not freely mobile but are instead diffuse in complicated patterns or are confined to discrete domains.[2,3] In this chapter I will discuss normal membrane structure, emphasizing aspects that are most involved in the genesis of membrane disease. Diseases of the red cell membrane are covered in Chapter 16.

MEMBRANE LIPIDS

Lipid Composition

Lipids compose about 50% of the weight of the red cell membrane (Table 15-1)[4-7,8,9-12] or about 1.6% of the weight of the hemoglobin. Phospholipids and unesterified (free) cholesterol predominate and are present in nearly

TABLE 15-1 Composition of Normal Human Red Cell Membranes

Component	Wt%	Gm/Membrane $(\times 10^{13})$	Approximate No. of Molecules/ Membrane $(\times 10^{6})$	% in Outer Half of Bilayer[*]	% in Inner Half of Bilayer[*]
Proteins and glycoproteins[†]	55	5.7[‡]	6		
Lipids	45	4.7	475		
Phospholipids[§]	28	3.0	250[‖]		
Sphingomyelin (SM)	6.8	0.73	60	85	15
Phosphatidyl choline (PC)	7.0	0.75	65	70	30
Phosphatidyl ethanolamine (PE)	7.4	0.79	65	20	80
Phosphatidyl serine (PS)	4.3	0.46	40	0	100
Phosphatidyl inositols (PI)	1.0	0.10	8	0	100
PI	0.34	0.036	3		
PI-4-P	0.22	0.024	2		
PI-4.5-PP	0.39	0.042	3		
Phosphatidic acid (PA)	1.0	0.10	8	10	90
Lysophosphatidyl choline (LPC)	0.3	0.03	3	Unknown	Unknown
Lysophosphatidyl ethanolamine (LPE)	0.3	0.03	3	Unknown	Unknown
Cholesterol[§]	13	1.3	195	~50	~50
Glycolipids[¶]	3	0.3	10	100	0
Free fatty acids[**]	1	0.1	20	Unknown	Unknown
	100	10.4	481		

[*] Based on data in Blau and Bittman,[4] Low and Finean,[5] Verkleij et al,[6] and Zachowski.[7]

[†] An average of three reported values in Fairbanks et al.[8]

[‡] Calculated from data in Table 15-2.

[§] Based on data in Ferrell and Huestis.[9]

[‖] Number of phospholipids per membrane based on an average molecular weight of 723 calculated from the average red cell phospholipid polar head group and fatty acid composition.[10]

[¶] Based on data in Sweeley and Dawson.[11]

[**] An average of two reported values compiled in Cooper.[12]

equal proportions (cholesterol/phospholipid molar ratio = 0.80).[4,13,14] Small amounts of glycolipids, principally globoside (73% of the glycosphingolipids) (Fig. 15-1) are also present.[11] The average red cell contains about 250 million phospholipid molecules, 195 million cholesterol molecules, 10 million glycolipid molecules, and 6 million protein molecules in a membrane whose total surface area[15] is about 140 μm^2.

Phospholipids

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and phosphatidylserine (PS) are the predominant phospholipids (see Table 15-1). Their structures are shown in Figure 15-1. Small amounts of phosphatidic acid (PA), phosphatidylinositol (PI), and lysophosphatidylcholine (Lyso-PC) are also present.

At physiologic pH, PS, PA, and PI have a net negative charge, whereas the other phospholipids are electrically neutral. The polar head groups of PC and SM tend to lie parallel to the membrane surface (Fig. 15-2). The head groups of PE and PS are smaller and more cone shaped, which may help them fit in the inner bilayer.

With the exception of SM and Lyso-PC, the phospholipids have two fatty acids attached to a glycerol backbone. These are usually of similar length and attached through an ester linkage, although sometimes, particularly in PE, one of the fatty acids is a vinyl ether (plasmalogen).[10] Typically, the fatty acid attached to glycerol farthest from the polar head group (*sn*-1 position) is saturated and the nearer (*sn*-2 position) one is unsaturated.[16] PE and PS are more unsaturated than PC. In contrast, both acyl side chains are usually saturated in the SMs and the amide-linked chain (see Fig. 15-1) is often very long and interdigitates with the hydrocarbon chains of the opposite side of the bilayer. The SM amide linkage also makes its interface region more polar. The lysophospholipids have only one fatty acid and are named for the hemolysis induced because of their detergent-like properties.

Modern lipid analysis reveals hundreds of phospholipid molecular species in the red cell membrane. The majority of these are in a planar bilayer with their polar head groups exposed at each surface and their hydrophobic fatty acyl side chains buried in the bilayer core. The phospholipid head groups are probably organized in regular patterned arrays.[17] The interactions that determine these patterns probably also influence the proportion of different phospholipids in the membrane.[17]

Glycolipids and Cholesterol

Glycolipids and cholesterol are intercalated between the phospholipids in the bilayer with their long axes perpendicular to the bilayer plane (see Fig. 15-2). Red cell glycolipids are located entirely in the external half of the bilayer with their carbohydrate moieties extending into the aqueous phase. They carry many important red cell antigens, including A, B, H, Le^a, Le^b, and P.

Cholesterol is present on both sides of the bilayer[4,18] and is nearly saturated in the red cell membrane, that is, its concentration is just below the concentration where major changes in its organization occur.[19] Its distribution

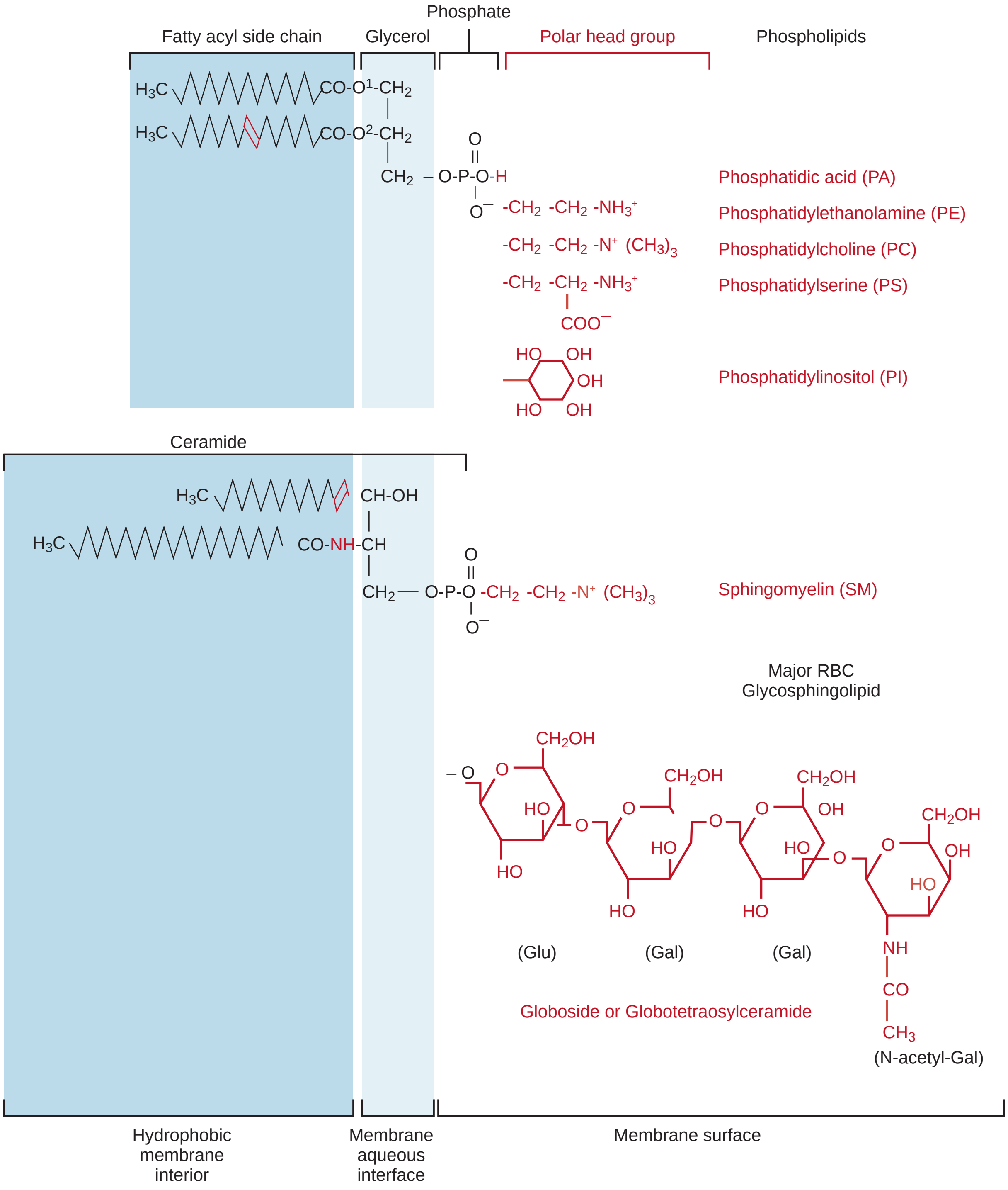

Fatty acyl side chain
Glycerol
Phosphate
Polar head group
Phospholipids
Phosphatidic acid (PA)
Phosphatidylethanolamine (PE)
Phosphatidylcholine (PC)
Phosphatidylserine (PS)
Phosphatidylinositol (PI)
Ceramide
Sphingomyelin (SM)
Major RBC Glycosphingolipid
(Glu)
(Gal)
(Gal)
(N-acetyl-Gal)
Globoside or Globotetraosylceramide
Hydrophobic membrane interior
Membrane aqueous interface
Membrane surface

Figure 15-1 Chemical structures of the major phospholipids and the major glycosphingolipid (globoside or globotetraosylceramide) of the red blood cell *(RBC)* membrane. Note that phosphatidylcholine and sphingomyelin share the same polar moiety (choline) and that sphingomyelin and globotetraosylceramide share the same nonpolar moiety (ceramide).

is not known, but it is probably slightly more prevalent in the outer half,[20,21] owing to its high affinity for sphingomyelin, which is concentrated in the outer bilayer. Cholesterol can flip from one side of the bilayer to the other in a fraction of a second.[22] This facilitates membrane bending, because the movement of cholesterol can offset the difference in surface pressure caused by bending and compressing one of the bilayers relative to the other. Cholesterol depletion promotes inward curvature of the membrane, whereas cholesterol enrichment favors outward deflection.[19]

Cholesterol molecules are relatively buried, because the 3-OH group, the part of the molecule that is at the aqueous interface, lies approximately at the level of the

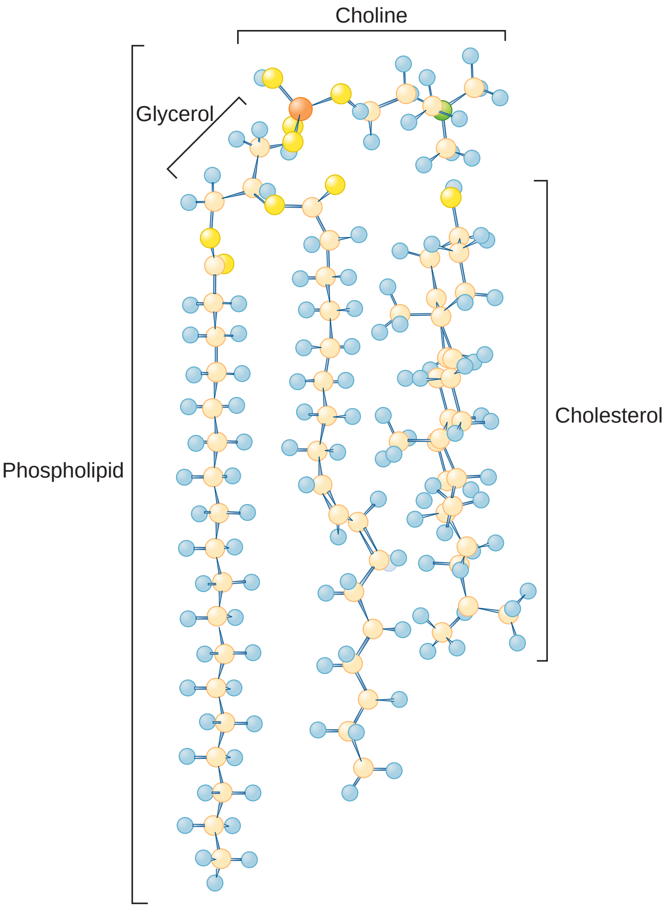


Figure 15-2 Approximate orientation of cholesterol and phospholipid molecules to each other in the membrane bilayer. Here the phospholipid is phosphatidylcholine, although often it is sphingomyelin. On average the cholesterol rings are believed to associate with the first 8 to 10 carbons of the phospholipid acyl chains. Note that the fatty acyl chain attached to the sn-1 position of the glycerol on phosphatidylcholine is saturated and straight whereas the sn-2 side chain is unsaturated and kinked (*tan,* carbon; *light blue,* hydrogen; *yellow,* oxygen; *orange,* phosphorus; *green,* nitrogen).

carbonyl group of the phospholipid fatty acids[15,23] (see Fig. 15-2). Cholesterol has important functions in buffering the fluidity of phospholipid side chains and in forming "lipid rafts," as will be described.

Asymmetric Organization of Phospholipids in the Bilayer

Trans Asymmetry

Red cell membrane phospholipids, like membrane proteins (see later), are asymmetrically distributed across the bilayer plane.[24-26] Choline phospholipids (PC and SM) are concentrated in the outer half of the bilayer and amino phospholipids (PE and PS) and PI are largely confined to the inner half (see Table 15-1).

The asymmetric distribution of phospholipids reflects a steady state involving a constant exchange (flip-flop) of phospholipids between the two bilayer hemileaflets.[25] In the red cell membrane, spontaneous transmembrane diffusion is slow, lasting several hours to a day.[27] In contrast, the enzymatic translocation of phospholipids, described in the following paragraphs, is much faster (minutes). At least three distinct activities are involved in regulating red cell membrane phospholipid asymmetry. They are discussed in several relatively recent reviews.[28-31]

Flippase. The magnesium adenosine triphosphate (Mg^{2+}-ATP)-dependent aminophospholipid translocase, or "flippase," is responsible for localization of PS in the inner leaflet by rapidly translocating it from the outer to inner leaflet. The half-time for translocation is about 5 minutes at 37°C.[32] A similar activity exists in all mammalian red cells[33] and probably in all cells. The flippase contains a critical cysteine, because it is inhibited by sulfhydryl-modifying reagents.[34] It is also inhibited by the pseudosubstrates of P-type ATPases, such as vanadate, and by high concentrations of Ca^{2+}.

Unfortunately, despite almost two decades of effort, the flippase has not been definitely identified.[28] An assortment of phospholipid transporters have been characterized in cells and organelles from bacteria to humans,[35] and some of these have been identified in red cell membranes, including ATP8A1.[36-38] ATP8A1 is an Mg^{2+}-ATPase first described in bovine chromaffin granules.[39] It is a P4-type ATPase, has the size and characteristics of the red cell membrane flippase, and translocates fluorescent PS in reconstitution experiments.[38] Yeasts lacking a homologous protein, Drs2, are defective in aminophospholipid transport.[39] But, an accessory CDC50 family protein needed for proper expression of P4-type ATPases is not found in red cells. More definitive proof—such as a gene knockout—is needed to be sure ATP8A1 is the authentic red cell flippase in mature red cells.

Floppase. The Mg^{2+}-ATP–requiring phospholipid translocase, or "floppase," is responsible for movement of phospholipids from the inner to outer leaflet of the bilayer[40] and is less specific than the flippase.[41,42] The outward lipid movement by the floppase is also much slower than the inward movement of PS by the flippase. Available evidence suggests the floppase is one of the family of ABC transporters, of which the multidrug resistance transporter (ABCB1 or MDR1 or P-glycoprotein) is the best known. The best candidate is ABCC1 (MRP1).[43,44] Long-term (48-hour) inhibition of the activity in red cells leads to loss of PC and SM from the outer bilayer but no change in the distribution of PE or PS.[45] Additionally, red cells from mice lacking the gene equivalent to ABCC1 can no longer translocate short-chain fluorescent phospholipid analogues of PC, SM, and PS from the inner to the outer bilayer.[43] However, it is not yet certain that ABCC1 can transport full-length, native phospholipids, which is a required property of the physiologic floppase.

Scramblase. The Mg^{2+}-ATP–independent, Ca^{2+}-dependent phospholipid "scramblase" causes a rapid, bidirectional movement of all phospholipids in the bilayer, resulting in the collapse of phospholipid asymmetry,[46] which may occur in less than a minute under some circumstances.

The exposure of PS on the cell surface causes red cells to adhere to each other and to endothelial cells and phagocytes and triggers clotting (see later).

After several frustrated attempts, it appears the scramblase has been cloned,[47] or at least part of the scramblase.[48] A protein called anoctamin 6 or TMEM16F (transmembrane protein 16F) is mutated and absent from patients with Scott syndrome,[47,49] a very rare, autosomal recessive disorder characterized by decreased scramblase activity (see Chapter 30).[50,51] As a consequence, activated platelets fail to expose sufficient PS on their surface to assemble prothrombinase and affected individuals have a bleeding tendency.[52] Erythrocytes and other cells also lack scramblase, but without apparent consequence. However, although anoctamin 6 is responsible for Scott syndrome, it does not account for all scramblase activity. Knockout of anoctamin 6 in the mouse impairs but does not abolish scramblase activity,[48] and other experiments show that there are at least two pathways of scramblase induction. In platelets, anoctamin 6 is not required for, but enhances, the scramblase pathway induced during apoptosis but is required by the pathway induced by thrombin and the rattlesnake venom convulxin.[49] The latter pathway is the one that is defective in Scott syndrome. This may be explained by the fact that at least 5 of the 10 members of the anoctamin/TMEM16 family have lipid scramblase activity.[53] More needs to be learned about which members are expressed in different cells and how they are triggered.

Loss of Phospholipid Asymmetry

When Ca^{2+} concentrations rise due to injury, disease, or cell death, red cells lose phospholipid asymmetry owing to activation of the scramblase and inhibition of the flippase. Because normal red cells maintain intracellular Ca^{2+} concentrations of less than a micromolar, their membranes preserve an asymmetric phospholipid distribution through their entire life span.

Phospholipid Asymmetry is Lost in Sickle and Thalassemic Red Cells. Phospholipid flip-flop is increased and phospholipid asymmetry is lost in sickle cells,[54-56] presumably owing to decreased phospholipid translocase activity[56] and uncoupling of the lipid bilayer from the underlying skeleton in the sickle cell spicules.[57] Consequently, subpopulation of circulating sickle red cells expose PS on their surface.[58] There is large variability in the numbers of such cells among sickle cell patients (0.5% to >10%). A subpopulation of thalassemic red cells also expose PS on their surface,[59] and phospholipid asymmetry is altered in diabetic red cells[60] which, like sickle cells,[61,62] tend to adhere to endothelial cells.[60,63]

Externalized PS Triggers Clotting and Phagocytosis. Cells sequester aminophospholipids on the cytoplasmic side of the plasma membrane because the exposed lipids, particularly PS, trigger the conversion of prothrombin to thrombin and promote intravascular clotting.[64-66] Loss of phospholipid asymmetry also activates the alternate complement pathway.[67]

In addition, PS is an “eat me” signal. Macrophages bind and ingest cells, or even liposomes, that have PS exposed on their outer surfaces.[68-70] Exposure of PS is one of the earliest events in apoptosis, and the PS receptor seems to have evolved to scavenge dead or dying cells that have activated scramblase during apoptosis. Expelled erythroblast nuclei also display PS on their surface and are removed by this mechanism.[71]

A protein called PSR was initially believed to be the PS receptor,[72,73] however that proved incorrect.[74-76] Three different PS receptors have been identified that appear to be real: Tim4,[77] Bal1,[78] and stabilin-2.[79] Bal1 functions as the receptor for the ELMO/Dock/Rac pathway that directs the actin cytoskeleton to engulf apoptotic corpses.[80] Stabilin-2 interacts with the intracellular adaptor GULP to activate Rac1 and coordinates with Bal1.[81] Tim4 does not appear to signal but serves primarily as a tethering receptor.[82] The interplay between these receptors and other parts of the phagocytic system is fascinating but complex, and its discussion is beyond the scope of this chapter.

Cis Asymmetry

Protein-Bound Lipids. There is evidence that some transmembrane proteins interact with neighboring “boundary” lipids in a dynamic fashion[83] whereas others bind lipids so tightly that they resist detergent extraction.[84] Presumably the latter proteins bear high-affinity lipid-binding sites. In either case, but particularly when lipids are tightly bound, they must slow the next layer of lipids that they interact with and so on, so that integral proteins exert a kind of “crowd control” on the lipids in the vicinity.

The structure, and in some cases the function, of integral membrane proteins is dependent on the surrounding phospholipids.[83] Membrane proteins can be parochial in their choice of lipid partners. For example, specific lipids are required for the enzymatic functions of the sodium and calcium pumps whereas glycophorin A (to be discussed), binds anionic (PS, PI) but not choline (PC, SM) phospholipids, and those lipids are relatively tightly bound.[85] Positively charged amino acids are concentrated on the cytoplasmic side of the bilayer-spanning domains of membrane proteins, in part because their position determines the orientation of the protein.[86,87] Although it is not well documented, anionic phospholipids probably cluster near these regions of positive charge. Surface-bound membrane proteins such as spectrin and protein 4.1R, which bind preferentially to anionic phospholipids,[88,89] may also contribute to the nonrandom topography of inner membrane phospholipids. And, conversely, the lipids appear to contribute to the stability of the membrane skeleton.[90,91]

Membrane Lipid Rafts. Lipid rafts are domains within the lipid bilayer that were initially defined by being insoluble in cold, nonionic detergents but have since been defined by multiple other techniques.[92] They are present in all cells and have been implicated in a variety of sorting and signaling processes.[92-96] They are rich in cholesterol, sphingomyelin, and glycosylphosphatidylinositol (GPI)-

anchored proteins; have more ordered acyl side chains; and are thicker than nonraft bilayer domains.[93,97] Cholesterol prefers the straighter and stiffer acyl chains of sphingomyelin to more disordered phospholipid side chains and is enriched in the rafts.[96] The extra length of glycosphingolipid side chains increases the thickness of the rafts and promotes further segregation. Various measurements indicate that lipid rafts are dynamic, nanoscale (10 to 20 nm) structures that assemble reversibly into larger structures, often for functional reasons.[92] Red cells lipid rafts are 100 to 300 nm and have an irregular shape.[98]

Red cell lipid rafts contain a complex mixture of membrane proteins, including stomatin, flotillin-1 and flotillin-2, the Duffy protein receptor (DARC), heterotrimeric Gα_s GPI-anchored proteins such as CD55, CD58, and CD59, and at least some band 3 molecules.[98-101] Proteins with myristate or palmitate side chains also tend to partition into the rafts,[102-104] whereas proteins with prenyl side chains are excluded.[103]

Because the glycolipid anchor of GPI-linked proteins is only embedded in the outer leaflet of the bilayer, GPI-linked proteins are much more laterally mobile than transmembrane proteins that interact with the membrane skeleton (discussed later). This high mobility may be important in recruiting complement regulatory proteins to sites of complement activation. Defective biosynthesis of the GPI anchor precludes attachment of GPI-linked proteins to the membrane, causing increased susceptibility to hemolysis by complement, as clinically manifest by paroxysmal nocturnal hemoglobinuria (see Chapter 14).[105]

Depletion of cholesterol from the red cell membrane blocks raft formation and the ability of raft-associated proteins to form membrane microdomains. It appears there are different kinds of lipid rafts in the membrane, with different protein components and different fates,[101] but this is not well defined. Although the function of the rafts in normal red cell physiology remains to be determined, they seem to be critical for the invasion of red cells by malarial parasites.[106]

Bilayer Couple Effect

The shape of the lipid bilayer is responsive to very slight variations (<0.4%)[107,108] in the surface area of its inner and outer halves. This is termed the *bilayer couple effect.*[109] It reflects the tight packing of membrane lipids, the independent motion of lipids in each half of the bilayer, and the extreme thinness of the membrane (<0.1% of the diameter of the cell). Processes that expand the outer bilayer (or contract the inner) will produce uniform membrane spiculation, called *echinocytosis* (Fig. 15-3). Conversely, relative expansion of the inner bilayer will lead to membrane invagination and cup-shaped red

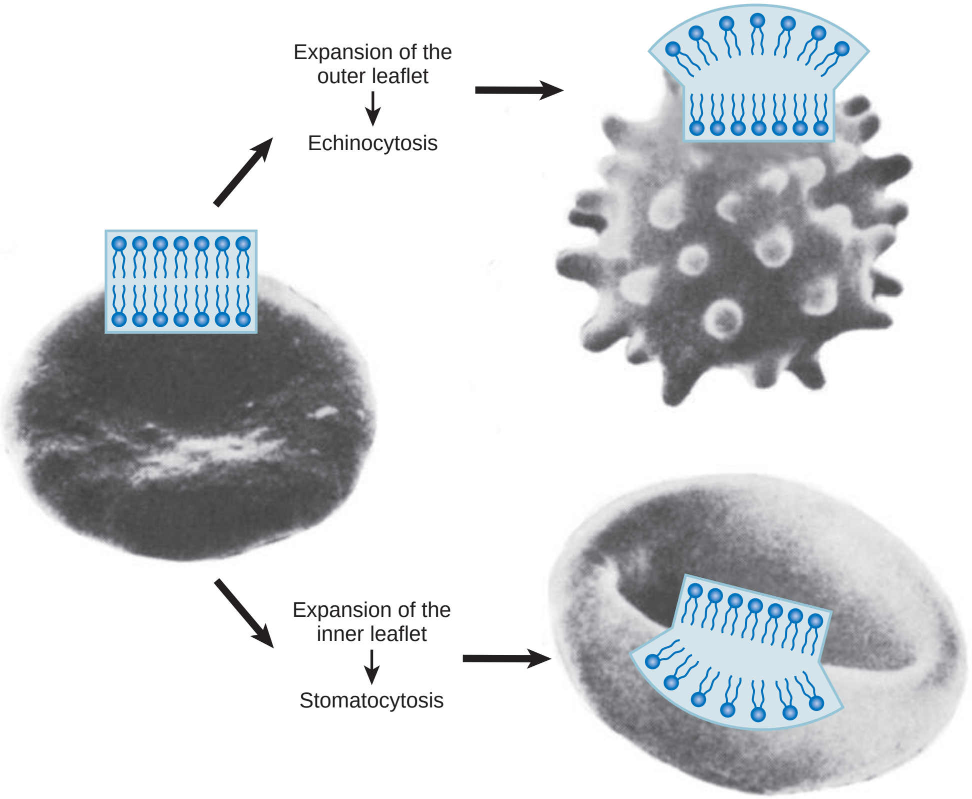

Expansion of the outer leaflet
Echinocytosis
Expansion of the inner leaflet
Stomatocytosis

Figure 15-3 Bilayer couple effect. Compounds or processes that selectively expand the outer leaflet of the lipid bilayer produce uniform membrane spiculation (echinocytosis). Selective expansion of the inner leaflet of the bilayer leads to membrane invagination and cup-shaped red cells (stomatocytosis). *(Modified from Palek J, Jarolim P: Clinical expression and laboratory detection of red cell membrane protein mutations.* Semin Hematol *30:249, 1993.)*

cells (i.e., *stomatocytosis*). Strongly charged amphipathic compounds, such as phospholipids, cause echinocytosis.[108,109] They are trapped in the outer bilayer by their fixed charge. Permeable amphipaths (e.g., weak acids and bases that can cross the membrane in their uncharged form) will cause the membrane to extend toward the side of greater accumulation.[110-112] In general, cationic compounds will accumulate in the negatively charged inner bilayer and anionic compounds will partition to the neutral outer bilayer. Even subtle effects, such as differences in the cross-sectional area of lipid head groups or fatty acyl tails, can lead to changes in membrane curvature.[112]

The bilayer couple effect predicts that shape changes resulting from expansion of one lipid leaflet can be reversed by a commensurate alteration in the other. For example, the intensely spiculated red cells of patients with abetalipoproteinemia can be almost completely converted to biconcave disks by the addition of small amounts (0.1 mM) of chlorpromazine, a cationic amphipath.[113] Unfortunately, this simple test has not been applied to other pathologic red cells, so it is difficult to estimate how often bilayer couple effects dictate cell shape in vivo. But shape is not totally explained by the bilayer couple hypothesis, because protein scaffolds, insertion of proteins into the lipid bilayer, the intrinsic shape of proteins that attach to the bilayer, and crowding of proteins on the membrane surface can all cause membranes to curve.[114] The contribution of these factors to red cell shape is not well studied, but it seems likely that the spectrin-actin protein scaffold and the many integral membrane proteins must be important.

Membrane Phosphoinositides

The polar head group of this interesting class of phospholipids contains phosphatidylinositol (PI), or its phosphorylated forms, PI 4-monophosphate and PI 4,5-bisphosphate (PIP and PIP_2), respectively. In mature erythrocytes, phosphoinositides represent 2% to 5% of the total phospholipids. They reside at the inner membrane surface and undergo rapid phosphorylation and dephosphorylation.[115] In nucleated cells, phosphoinositides are precursors of important intracellular second messengers such as inositol-1,4,5-triphosphate and diacylglycerol. In red cells they help regulate Ca^{2+} transport, interact with membrane proteins such as glycophorin C, spectrin, and protein 4.1R (see later), and may influence discocyte-echinocyte shape transformation.

Lipid Mobility

Acyl Side Chains

Purified Lipids Exist in Gel or Liquid Crystalline States. Purified phospholipids exhibit discrete, liquid crystalline to gel phase transitions that are dependent on the length and degree of unsaturation of their acyl side chains. The presence of a double bond in the acyl chain increases the disorder and flexibility all along the hydrocarbon chain,[116,117] especially toward the terminal methyl end. Below the temperature of the liquid crystalline to gel transition, acyl chains of purified lipids are extended in stiff, parallel, hexagonally packed arrays that are more nearly solid than liquid.

Cholesterol Creates an Intermediate State between Gel and Liquid Lipids. Cholesterol buffers the extremes of the gel and liquid crystalline states. At temperatures above the gel-liquid phase transition, cholesterol partially immobilizes the acyl chains of phospholipids, particularly the proximal eight to ten carbons that are adjacent to the bulky steroid nucleus.[118] Below this transition, cholesterol disrupts the ordering of the acyl chains, in effect preserving membrane fluidity.[119,120] The net result is that cholesterol tends to abolish the gel to liquid crystalline transition and create a condition of intermediate fluidity in which the proximal ends of the hydrocarbon chains are extended and relatively rigid and the distal ends are disordered and fluid.

The red cell membrane contains relatively large amounts of cholesterol (cholesterol/phospholipid = 0.8 mol/mol) and is relatively less fluid than other plasma membranes.[121] The inner bilayer is somewhat more fluid than the outer,[122] probably because it contains more unsaturated fatty acids.

Lateral Diffusion

Phospholipids move about rapidly within each plane of *model* lipid bilayers.[121,123,124] From the measured lateral diffusion constants (2 to 5×10^{-8} cm^2/s), one can calculate that phospholipids exchange places with their neighbors about 10^7 times per second. This rate is somewhat damped in the red cell membrane (estimated diffusion constant of 1.4×10^{-8} cm^2/s),[125] but the difference is about what would be expected, considering the effects of cholesterol on the lateral mobility of phospholipids.[124]

Lipid Renewal Pathways

Mature Erythrocytes Cannot Synthesize Lipids. Mature erythrocytes are unable to synthesize fatty acids, phospholipids, or cholesterol de novo and depend on lipid exchange and fatty acid acylation as mechanisms for phospholipid repair and renewal (Fig. 15-4). Damaged chains are removed by phospholipase A_2, producing a lysophospholipid. Plasma-derived fatty acids are esterified to acyl-CoA by acyl-CoA synthetases, especially ACSL6,[126] and transferred to the lysophospholipids by acyl-CoA: lysophospholipid acyltransferases.[127] In theory this enzyme should facilitate the renewal of lost or damaged fatty acid side chains and should prevent the accumulation of deleterious lysophosphatides within the membrane; however, it is not certain that the phospholipases necessary to remove damaged fatty acids operate in the red cell.

This renewal pathway, although limited, permits a slow replacement of membrane lipid components. Approximately 30 days are required before erythrocyte lipids reach equilibrium after a change in dietary fatty acids.[128] Theoretically this could be pathologically significant in persons on unusual diets (e.g., infants receiving a high intake of medium-chain triglycerides); however, to date, no pathologic consequences have been reported.

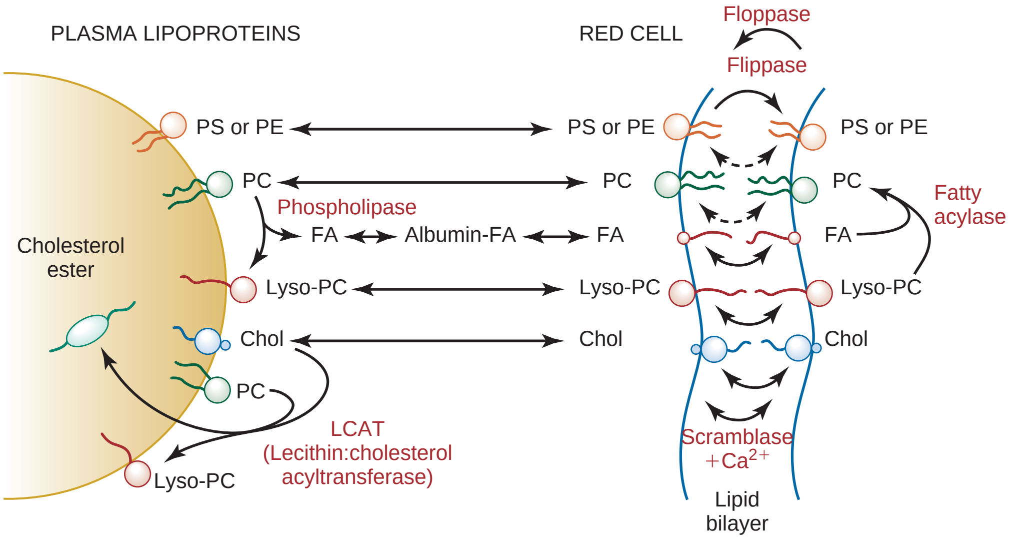


Figure 15-4 Pathways for lipid exchange and fatty acid replacement in the red cell membrane. *Chol,* Cholesterol; *FA,* fatty acid; *Lyso-PC,* lysophosphatidylcholine; *PC,* phosphatidylcholine; *PS,* phosphatidylserine; *PE,* phosphatidylethanolamine.

Esterification Depletes Red Cell Cholesterol. Red cell membrane cholesterol (unesterified) exchanges readily with the unesterified cholesterol in plasma lipoproteins ($t_{1/2}$ = 7 hr), where it is partially converted to *esterified* cholesterol by the action of lecithin:cholesterol acyltransferase (LCAT). Because the newly formed cholesteryl ester cannot return to the red cell membrane, LCAT catalyzes a unidirectional pathway (see Fig. 15-4) that depletes the membrane of cholesterol and decreases its surface area. Conversely, if LCAT is absent or inhibited, excess membrane cholesterol accumulates, expanding the membrane surface area and resulting in target cells.[129]

MEMBRANE PROTEINS

Composition

The red cell membrane (or "ghost") contains nearly 20 major proteins and more than 300 minor ones (Table 15-2).[130-145,146,147-161] Even defining what is a red cell membrane protein is difficult because plasma and cytoplasmic proteins bind to membrane constituents to varying degrees under different conditions. The major proteins of the red cell membrane (Fig. 15-5; see Table 15-2) and their disorders have been intensively studied,[162,163] and, in most cases, their genes have been cloned.

The protein constituents of the red cell membrane are usually defined by hypotonic hemolysis of red cells[164] followed by polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS).[8] In general, membrane proteins are classified as either *integral* or *peripheral.* Integral membrane proteins penetrate or traverse the lipid bilayer and interact with the hydrophobic lipid core. They include band 3, the anion exchange channel, and the glycophorins, which generate a negative charge at the cell surface, and other proteins that function as channels or receptors and display surface antigens (Fig. 15-6). Integral membrane proteins form the randomly distributed 8- to 10-nm intramembranous particles seen on freeze-cleave electron microscopy of red cell membranes.[165] Peripheral proteins interact with integral proteins or lipids at the membrane surface but do not penetrate into the lipid bilayer core. In the red cell, the major peripheral membrane proteins are located on the cytoplasmic membrane face and include enzymes such as glyceraldehyde-3-phosphate dehydrogenase (G3PD) and the structural proteins of the membrane skeleton. The red cell membrane skeleton is operationally defined as the insoluble proteinaceous residue that remains after extraction of red cells[166] or their ghosts[167] with the nonionic detergent Triton X-100. The membrane skeleton contains roughly half of the membrane protein mass and includes such proteins as spectrin, actin, protein 4.1R, p55, adducin, dematin, tropomyosin, tropomodulin, ankyrin, and part of band 3 and protein 4.2 (see Fig. 15-6).

INTEGRAL MEMBRANE PROTEINS

Band 3 (SLC4A1, AE1)

Band 3 is the major integral membrane protein of the red blood cell, comprising 25% to 30% of cell membrane protein (~1.2 million copies per cell). It is also called anion exchange protein 1 (AE1), but its official appellation is solute carrier family 4 (anion exchanger), member 1 or SLC4A1. It is expressed early in erythropoiesis and peaks at the orthochromic erythroblast stage.[168] It encodes a 911-amino acid polypeptide of 102 kD.[169,170] Band 3 migrates as a diffuse band on SDS gels (see Fig. 15-5)

Text continued on p. 467

TABLE 15-2 Some Important Erythrocyte Membrane Proteins

Protein	Blood Group Name(s)	Human Gene Name	Mr on SDS Gel (kD)	Actual Mr (kD)*	No. of Amino Acids*	Monomer Molecules/Cell† × 10^3	Oligomeric State	Peripheral or Integral‡	Chromosome Location	OMIM No.§	NCBI Reference Protein Sequence Accession No.‖	Associated Red Cell Conditions
ABCB6	Lan	*ABCB6*	80	93.9	842			I	2q35	605452	NP_005680.1	FP
Acetylcholinesterase	Yt	*ACHE*	70	64.6	583	3-10		I (G)	7q22.1	100740	NP_000656.1	
β Actin		*ACTB*	43	41.6	374	500	Oligomer (~14)	P	7p22.1	102630	NP_001092.1	
α Adducin		*ADD1*	103	81.0	737	30	Heterodimer/tetramer	P	4p16.3	102680	NP_001110.2	HS (mouse)
β Adducin		*ADD2*	97	80.7	725	30		P	2p13.3	102681	NP_001171983.1	HS (mouse)
ADP-ribosyltransferase (CD297)	Dombrock	*ART4*	47-58	27.8	239			I (G)	12p12.3	110600	NP_066549.2	
Aldolase A		*ALDOA*		39.4	364	20	Tetramer	P	16p11.2	103850	NP_001121089.1	
Ankyrin¶		*ANK1*	210	206.0	1880	124 ± 11¶	Monomer	P	8p11.21	612641	NP_065209.2	HS
Aquaporin 1	Colton	*AQP1*	28	28.4	268	120-160	Tetramer	I	7p14.3	107776	NP_932766.1	
Aquaporin 3	GIL	*AQP3*	25, 45	31.5	292		?Tetramer, ?Dimer	I	9p13	600170	NP_004916.1	
Band 3 (AE1, CD233)	Diego, Wright, Ii, numerous minor antigens	*SLC4A1*	90-100	101.8	911	1200	Dimer or tetramer	I	17q21.31	109270	NP_000333.1	HS, SAO, HAc, OHSt. HC
Basigin (EMMPRIN, CD147)	OK	*BSG*	45-75	27.2	248			I	19p13.3	109480	NP_940991.1	Malaria resistance
Ca^{2+}-ATPase 1		*ATP2B1*		134.7	1220	2-3		I	12q21.3	108731	NP_001673.2	
Ca^{2+}-ATPase 4		*ATP2B4*		133.9	1205			I	1q32.1	108732	NP_001675.3	
Carbonic anhydrase II		*CA2*	29	29.1	259	100		P	8q21.2	611492	NP_000058.1	
Carbonic anhydrase IV		*CA4*	35	33.1	293			I (G)	17q23.1	114760	NP_000708.1	
CD44	Indian	*CD44*	80	37.1	311	6-10		I	11p13	107269	NP_001001391.1	
CD47 (IAP)		*CD47*	50	33.3	304	17	Monomer	I	3q13.12	601028	NP_001768.1	
CD55 (DAF)	Cromer	*CD55*	70	37.8	347	10-20		I (G)	1q32.2	125240	NP_000565.1	
CD58 (LFA3)		*CD58*	55-70	28.1	250	3-8		I	1p13.1	153420	NP_001770.1	
CD59 (Protectin, MIRL)		*CD59*	18-20	9.0	77	20-40		I (G)	11p13	107271	NP_000602.1	PNH
CD99	Xg	*CD99*	25, 30	16.7	163	1		I	Xp22.33	313470	NP_002405.1	
CR1 (CD35)	Knops	*CR1*	190, 220	219.7	1998	0.02-1.5		I	1q32.2	120620	NP_000564.2	Malaria resistance

Continued on following page

TABLE 15-2 Some Important Erythrocyte Membrane Proteins (Continued)

Protein	Blood Group Name(s)	Human Gene Name	Mr on SDS Gel (kD)	Actual Mr (kD)*	No. of Amino Acids*	Monomer Molecules/Cell† × 10^3	Oligomeric State	Peripheral or Integral‡	Chromosome Location	OMIM No.§	NCBI Reference Protein Sequence Accession No.‖	Associated Red Cell Conditions
Dematin (band 4.9)		*EPB49*	48 & 52	43.1 & 45.5	383, 405	130	Monomer	P	8p21.3	125305	NP_001107610.1	Mild HS (mouse)
Duffy (DARC, CD234)	Duffy (Fy)	*DARC*	35-45	35.6	336	6-13		I	1q23.2	613665	NP_001116423.1	Malaria resistance
ERMAP	Scianna/ Radin	*ERMAP*	60-68	49.6	446			I	1p34.2	609017	NP_001017922.1	
Flotillin 1		*FLOT1*	47	47.4	427			P	6p21.33	606998	NP_005794.1	
Flotillin 2		*FLOT2*	42	47.1	428			P	17q11.2	131560	NP_004466.2	
G3PD		*GAPDH*	35	35.9	334	500	Tetramer	P	12p13.31	138400	NP_002037.2	
Gárdos channel (SK4)		*KCNN4*	45	47.7	427	0.12		I	19q13.31	602754	NP_002241.1	
Glucose transporter 1 (Glut1)		*SLC2A1*	45-65	54.1	492	200-700	Dimer & tetramer	I	1p34.2	138140	NP_006507.2	HC
Glucose transporter 4 (Glut4)		*SLC2A4*		54.8	509			I	17p13.1	138190	NP_001033.1	
Glycophorin A (CD235a)	MNSsU	*GYPA*	36	14.3	131	1000	Dimer	I	4q31.21	111300	NP_002090.4	Malaria resistance
Glycophorin B (CD235b)	MNSsU	*GYPB*	20	7.7	72	170-250	Dimer?	I	4q31.21	111740	NP_002091.3	
Glycophorin C (CD236)	Gerbich	*GYPC*	32	13.8	128	143	Dimer?	I	2q14.3	110750	NP_002092.1	HE, Malaria resistance
Glycophorin D (CD236)	Gerbich	*GYPD*	23	11.5	107	82	Dimer?	I	2q14.3	110750	NP_001243513.1	HE
Glycophorin E**		*GYPE*		6.5	59			I	4q31.21	138590	NP_941391.2	
K^+-Cl^--cotransporter 1 (KCC1)		*SLC12A4*		120.7	1085			I	16q22.1	604119	NP_005063.1	
K^+-Cl^--cotransporter 3 (KCC3)		*SLC12A6*		127.6	1150			I	15q14	604878	NP_598408.1	
K^+-Cl^--cotransporter 4 (KCC4)		*SLC12A7*		119.1	1083			I	5p15.33	604879	NP_006589.2	
Kell (CD238)	Kell	*KEL*	93	82.8	732	3-18	Heterodimer with KX	I	7q34	613883	NP_000411.1	
Kx	Xk	*XK*	37	50.9	444		Hetrodimer with Kell	I	Xp21.1	314850	NP_066569.1	McLeod syndrome
Lutheran (BCAM, CD239)	Lutheran, Auberger	*BCAM*	85 & 78	64.3	597	1.5-8		I	19q13.32	612773	NP_005572.2	

LW glycoprotein (CD242)	Landsteiner-Wiener	*ICAM4*	37-43	27.0	249	3-5		I	19p13.2	614088	NP_001535.1	
Monocarboxylate transporter 1 (MCT1)		*SLC16A1*	54	53.9	500			I	1p13.2	600682	NP_003042.3	
Na$^+$-H$^+$ exchanger 1 (NHE1)		*SLC9A1*		90.8	815			I	1p36.1-p35	107310	NP_003038.2	
Na$^+$-H$^+$ exchanger 8 (NHE8)		*SLC9A8*		65.4	581			I	20q13.13	612730	NP_056081.1	
Na$^+$-H$^+$ exchanger 9 (NHE9)		*SLC9A9*		72.6	645			I	3q24	608396	NP_775924.1	
Na$^+$,K$^+$-ATPase, α chain		*ATP1A1*	112	112.4	1018	0.23-0.47	Heterotrimer	I	1p13.1	182310	NP_001153705.1	
Na$^+$,K$^+$-ATPase, β chain		*ATP1B1*	35	35.1	303			I	1q24.2	182330	NP_001668.1	
Na$^+$,K$^+$-ATPase, γ chain		*FXYD2*	7	7.3	66			I	11q23.2	601814	NP_001671.2	
Na$^+$-K$^+$-Cl$^-$ cotransporter 2 (NKCC1)		*SLC12A2*		131.4	1212			I	5q23.3	600840	NP_001037.1	
Nucleoside transporter		*SLC29A1*	55	50.1	455	10		I	6p21.1	602193	NP_001071645.1	
p55		*MPP1*	55	52.3	466	80	Dimer?	I (P)	Xq28	305360	NP_002427.1	
Peroxiredoxin 1		*PRDX1*	23	22.0	198		Dimer	P	1p34.1	176763	NP_002565.1	OH (mouse)
Peroxiredoxin 2		*PRDX2*	23	21.8	197	200	Dimer or decamer	P	19p13.2	600538	NP_005800.3	OH (mouse)
Phosphofructokinase—liver		*PFKL*		85.0	780	6	Heterotetramer	P	21q22.3	171860	NP_002617.3	
Phosphofructokinase—muscle		*PFKM*		85.2	780			P	12q13.11	610681	NP_001160160.1	
Piezo 1		*FAM38A*	275	286.8	2521		?Tetramer	I	16q24.3	611184	NP_001136336.2	HX, FP
Protein 4.1R		*EPB41*	80 & 78	66.4	588	~240	Monomer	P	1p35.3	130500	NP_004428.1	HE
Protein 4.2		*EPB42*	72	79.8/76.9[††]	721/691[‡]	~280	Unknown	P	15q15.2	177070	NP_000110.2	HS
RhAG (CD241)		*RHAG*	45 & 50	44.2	409	100-200		I	6p12.3	180297	NP_000315.2	Rh null
RhCE polypeptide (CD240CE)	CcEe	*RHCE*	30-32	45.4	416	100-200	Heterotrimer with RhAG, RhD, & RhCE?	I	1p36.11	111700	NP_065231.3	Rh null
RhD polypeptide (CD240D)	D	*RHD*	30-32	45.1	416	100-200		I	1p36.11	111680	NP_057208.2	
SMIM1	Vel	*SMIM1*	18	8.7	78		Dimer (S-S linked)	I	1p36	615242	NP_001157196.1	

Continued on following page

TABLE 15-2 Some Important Erythrocyte Membrane Proteins (Continued)

Protein	Blood Group Name(s)	Human Gene Name	Mr on SDS Gel (kD)	Actual Mr (kD)*	No. of Amino Acids*	Monomer Molecules/ Cell[†] × 10^3	Oligomeric State	Peripheral or Integral[‡]	Chromosome Location	OMIM No.[§]	NCBI Reference Protein Sequence Accession No.[‖]	Associated Red Cell Conditions
α Spectrin		*SPTA1*	240	280.0	2419	242	Heterodimer/ tetramer/ oligomer	P	1q23.1	182860	NP_003117.2	HE, HS
β Spectrin		*SPTB*	220	246.4	2137	242		P	14q23.3	182870	NP_000338.3	HS, HE
Stomatin (band 7.2)		*STOM*	29	31.7	288	200-400?	Oligomer (9-12)	I	9q33.2	133090	NP_004090.4	
Tropomodulin 1		*TMOD1*	43	40.6	359	60	Monomer	P	9q22.33	190930	NP_003266.1	Mild HE (mouse)
Tropomyosin 1 (hTm5b)[‡‡]		*TPM1*	27+29	32.9	284	140[‡‡]	Homodimer	P	15q22.2	191010	NP_001018020.1[‡‡]	
Tropomyosin 3 (hTm5)[‡‡]		*TPM3*		32.9	285			P	1q21.3	191030	NP_689476.2	
Urea transporter	Kidd (Jk)	*SLC14A1*	36	42.5	389	10-14		I	18q12.3	613868	NP_056949.4	
XG glycoprotein	Xg	*XG*	22-29	17.3	159	0.1-9		I	Xp22.33	314700	NP_780778.1	

AE1, Anion exchanger 1; *BCAM*, basal cell adhesion molecule; *CD*, clusters of differentiation antigen; *CR1*, complement receptor 1; *DAF*, decay accelerating factor; *DARC*, Duffy antigen receptor for chemokines; *EMMPRIN*, extracellular matrix metalloproteinase inducer; *ERMAP*, erythroblast membrane-associated protein; *FP*, familial pseudohyperkalemia; *G3PD*, glyceraldehyde-3-phosphate dehydrogenase; *HAc*, hereditary acanthocytosis; *HC*, hereditary cryohydrocytosis; *HE*, hereditary elliptocytosis; *HS*, hereditary spherocytosis; *HX*, hereditary xerocytosis; *IAP*, integrin-associated protein; *LFA3*, lymphocyte function-associated antigen type 3 (CD2 ligand); *MCT1*, monocarboxylic acid transporter 1; *MIRL*, membrane inhibitor of reactive lysis; *OH*, oxidant hemolysis; *OHSt*, overhydrated hereditary stomatocytosis; *PNH*, paroxysmal hemoglobinuria; *RTA*, renal tubular acidosis; *SAO*, Southeast Asian ovalocytosis; *SDS*, sodium dodecyl sulfate.

*Size of the mature protein chain without any signal peptide, propeptides, or cleaved initiator methionine, but not including glycosylation, phosphorylation, fatty acid acylation, and other posttranslational modifications.

[†] Copies of proteins 4.1 and 4.2 and G3PD were based on an estimate of 5.7×10^{-13} g protein/ghost[8] and on the approximate proportion of each protein, estimated from densitometry of SDS gels. Numbers of spectrins and ankyrins were measured directly by radioimmunoassay.[131] The copy number of other proteins were taken or calculated from data in the following references: AChE,[132,133] β actin,[134] α and β adducin,[135] aquaporin[136] band 3,[137] carbonic anhydrase 2,[138] Ca^{2+}-ATPase,[139] CD44,[140] CD47,[141] CD55,[122,142] CD58,[140] CD59,[143] CD99,[157] CR1,[142] dematin,[144] Duffy,[145] Gárdos channel,[146] glucose transporter 1 (Glut1),[142] glycophorins A and B,[147] glycophorins C and D,[148] Kell,[149] Lu,[133,150] LW,[151] Na^+,K^+-ATPase,[146,152] nucleoside transporter,[142] p55,[153] RhAG,[154] Rh polypeptide,[154] tropomodulin,[155] tropomyosin,[156] urea transporter,[133,158] and Xg glycoprotein.[133,142]

[‡] Glycosylphosphatidylinositol (G)-linked proteins and heavily palmitoylated (P) proteins such as p55 are classified as integral proteins.

[§] OMIM: http://www.ncbi.nlm.nih.gov/omim.

[‖] NCBI Protein database: http://www.ncbi.nlm.nih.gov/protein.

[¶] Data shown are for full-length ankyrin (band 2.1), the major isoform, except for the number of copies/cell, which includes the smaller isoforms: band 2.2 = 195 kD, band 2.3 = 175 kD, and band 2.6 = 145 kD.

[**] Glycophorin E mRNA has been identified, but it is not certain that the mRNA is translated.

[††] The full-length form of protein 4.2 is 721 amino acids (isoform 1, 79,797 daltons). Features of the protein, such as the band 3–binding site or the palmitoylation site, are numbered using this sequence. However, the common isoform is only 691 amino acids long (isoform 2, 76,878 daltons, NP_001107606.1). It lacks amino acids G4 to D33.

[‡‡] The number of tropomyosins includes the contribution of both the *TPM1* and *TPM3* genes. Tropomyosin has many isoforms.[159] Red cell tropomyosin is composed of the hTm5b isoform of *TPM1* (also called TM5b) and the hTM5 isoform of *TPM3* (also called Tm5NM1).[160] The larger band on SDS gels of red cell membranes is the hTM5 isoform. The author was unable to link the sequence of the hTM5b isoform to any of the reference sequences in the NCBI Protein Database. The sequence listed is the generic 284-amino acid sequence.

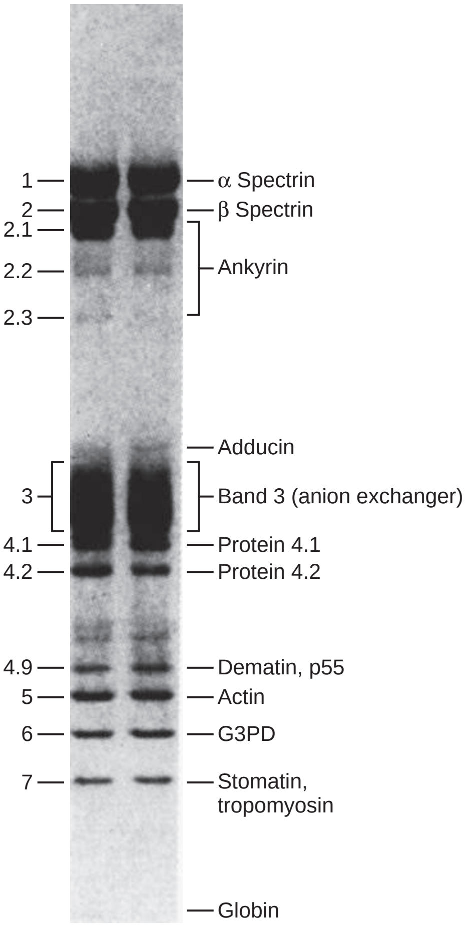


Figure 15-5 Major proteins of the red blood cell membrane separated by SDS gel electrophoresis. The glycophorins and Rh proteins do not bind the Coomassie Blue stain and are not visible. *G3PD,* Glyceraldehyde-3-phosphate dehydrogenase. *(Modified from Costa FF, Agre P, Watkins PC, et al: Linkage of dominant hereditary spherocytosis to the gene for the erythrocyte membrane-skeleton protein ankyrin.* N Engl J Med *323:1046, 1990.)*

owing to heterogeneous *N*-glycosylation.[171] It is divided into a cytoplasmic domain that is a key attachment site for the membrane skeleton, glycolytic enzymes, and hemoglobin and a membrane domain anion exchange channel that aids carbon dioxide (CO_2) transport.

Cytoplasmic Protein Binding Domain

The 43-kD amino-terminal (N-terminal) domain of band 3 (amino acids 1 to 359) resides in the cytoplasm and is released when red cell membranes are treated with chymotrypsin or trypsin.[172] The first 45 amino acids are highly acidic and stretch into the cytoplasm (Fig. 15-7).[173-176] The remainder of the domain is folded but flexible, expanding at high pH and contracting at low pH.[177]

The N-Terminal Sequence Binds Glycolytic Enzymes, Peroxiredoxin-2, and Deoxyhemoglobin and is an Oxygen-Regulated Membrane Metabolon. The first 23 amino acids of band 3 contain two tandem repeats terminating in the sequences DDY_8ED and $EEY_{21}ED$. G3PD,[178] phosphofructokinase (PFK),[179] aldolase[180,181] and deoxyhemoglobin[182] all bind in this region. So does peroxiredoxin-2.[183] Aldolase requires both sites to bind, G3PD binds separately to each, and PFK and deoxyhemoglobin bind to the more distal site.[182,184] In confirmation, the red cells from a child with a mutant band 3 that lacks the first 11 amino acids (Bd3 Neapolis) contain no membrane bound aldolase.[185]

The glycolytic enzyme complex also binds to a site near the junction of the cytoplasmic and membrane domains of band 3 (amino acids 356 to 384), which lies close to the N-terminus in the folded protein.[186] Enzymes in the glycolytic complex can be crosslinked to proteins in both the ankyrin complex and the actin junctional complex,[186] which suggests the glycolytic enzymes attach to band 3 dimers in both complexes (see Fig. 15-6).

There are more than enough band 3 molecules to bind all the peroxiredoxin-2 (~200,000 copies), G3PD (~500,000 copies), aldolase (~20,000 copies), and PFK (~6,000 copies). About 65% of G3PD, 40% of aldolase, and 50% of PFK are membrane bound in the intact red cell.[178,179] PGK may also attach to band 3,[187] but the numbers of bound molecules are small (~230 copies).

Deoxyhemoglobin binding is well documented.[182,188] Its affinity is weak ($kD_{(deoxy\ Hb)} = 0.3$ mM), but because the concentration of hemoglobin is so high in red cells, roughly half of the band 3 molecules are bound to deoxyhemoglobin under physiologic conditions.[188,189] Hemichromes, a partially denatured form of hemoglobin, bind more avidly than hemoglobin and cause band 3 to cluster,[189-191] which is believed to target the cell for phagocytosis (see later section, "Membrane Changes Associated with Aging and Senescence of Red Cells").

G3PD, PFK, and aldolase are inhibited when attached to the membrane.[180,181,192,193] In addition, lactic dehydrogenase (LDH) and pyruvate kinase (PK) also localize to the membrane, even though they *do not* bind to band 3.[193] All five enzymes are displaced into the cytoplasm by an antibody to the N-terminus of band 3, by deoxyhemoglobin, or by $p72^{syk}$ phosphorylation of tyrosines 8 and 21, which lie in the middle of the two binding sites.[193,194] Similarly in mice, even though the acidic N-terminal domain of band 3 is not well conserved, glycolytic enzymes bind to the red cell membrane in a band 3–dependent manner and redistribute to the cytoplasm when hemoglobin is deoxygenated and bound to band 3 or band 3 is deleted.[195,196]

The data suggest that many of the enzymes in the glycolytic pathway form a complex—or more accurately a series of complexes because the wide variation in numbers of enzyme molecules means they cannot form a single stoichiometric complex. The complexes (or "metabolons") are bound to band 3, and their interaction can be regulated by tyrosine phosphorylation and the state of hemoglobin oxygenation,[193,194,197,198] and perhaps by substrates and cofactors of the enzymes (Fig. 15-8).[178,180,187] The enzymes are in discrete clumps along the membrane, which also supports the idea of a metabolon (see Fig. 15-8, *A*). In addition, when metabolic fluxes are measured in *intact* normal red cells, activating glycolysis by displacing the glycolytic enzymes from band 3 increases

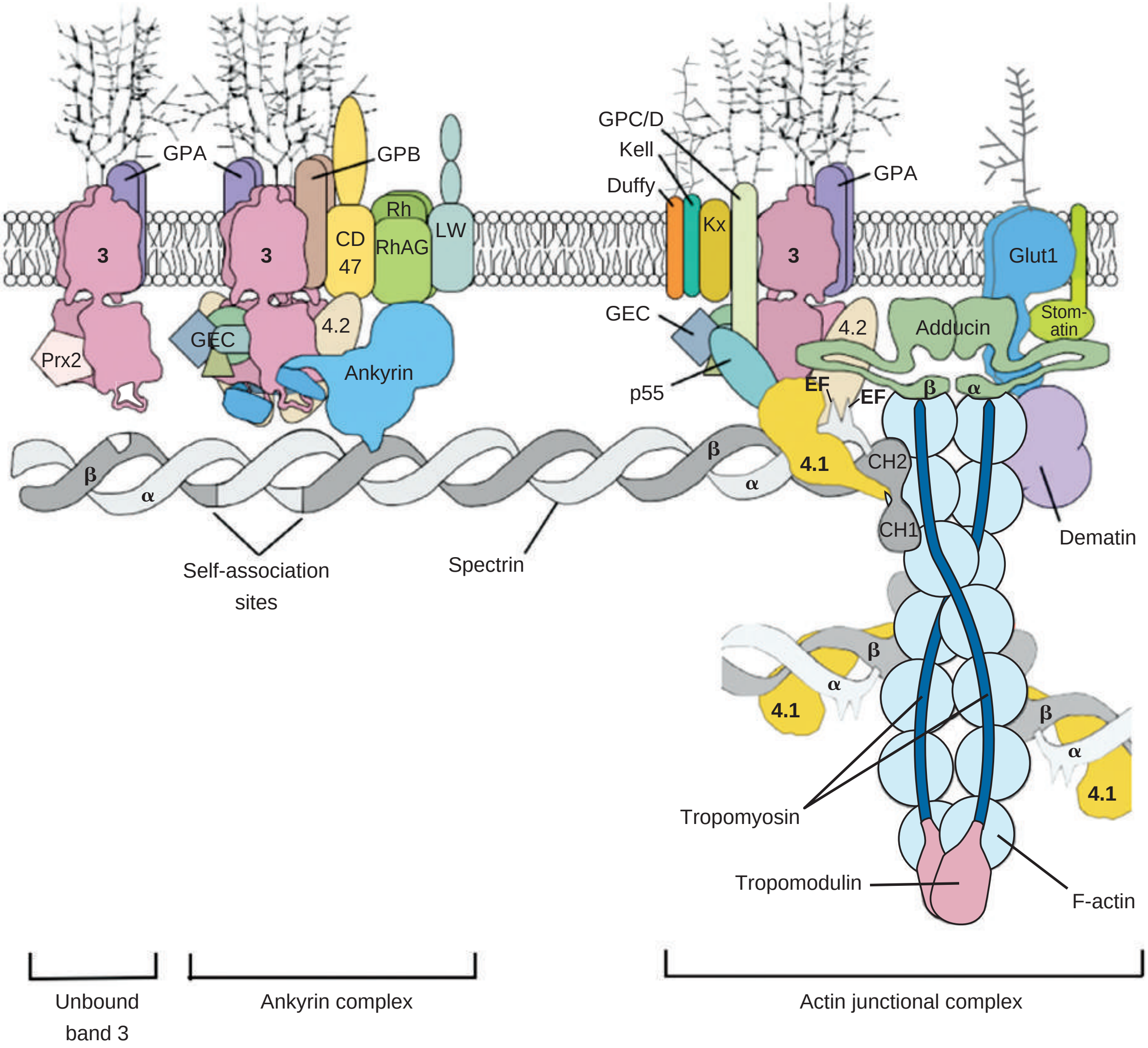

GPA
GPB
Rh
RhAG
CD 47
LW
3
3
4.2
GEC
Prx2
Ankyrin
GPC/D
Kell
Duffy
Kx
GPA
3
Glut1
GEC
4.2
Adducin
Stom-atin
p55
EF
EF
β
α
4.1
CH2
CH1
Dematin
β
α
β
α
Self-association sites
Spectrin
β
α
4.1
β
α
4.1
Tropomyosin
Tropomodulin
F-actin
Unbound band 3
Ankyrin complex
Actin junctional complex

Figure 15-6 Schematic model of the red cell membrane. Most of the known protein contacts are shown, but the relative positions of the proteins to each other within the various complexes are mostly not known. The major proteins are drawn roughly to scale, but the shapes are mostly imaginary. For clarity, less than the expected six spectrin/protein 4.1 complexes and their attached proteins are shown. Roughly 40% of the band 3 molecules are tetramers in a complex with ankyrin and other integral proteins near the spectrin self-association site (ankyrin complex). A fraction of the band 3 dimers are located near the spectrin/actin junction and bind to spectrin via protein 4.1, protein 4.2, and adducin (actin junctional complex). It is likely these two complexes, with their associated proteins, are large enough that they sometimes contact each other. The remaining band 3 dimers float untethered within the lipid bilayer (unbound band 3). To show its various interactions, the actin protofilament is drawn perpendicular to the plane of the lipid bilayer, jutting into the cytoplasm. However, it actually lies parallel to the membrane. The complexes of proteins associated with band 3 are not constant—some proteins are present in much smaller numbers than others (e.g., Rh, Kell, Kx, Duffy, LW, phosphofructokinase, and aldolase). The amounts of p55, adducin, and dematin are also insufficient to interact with all the spectrin/protein 4.1/actin complexes. As a consequence, the ankyrin and actin junctional complexes must vary in composition. Dematin is shown here as a trimer, though recent evidence suggests it is probably monomeric. For visual clarity, peroxiredoxin-2 is shown attached just to unbound band 3 but there is no evidence for this selectivity. *CH1* and *CH2,* Actin-binding domains of β spectrin; *EF,* Ca^{2+}-binding EF hand domain of α spectrin; *GEC,* glycolytic enzyme complex (G3PD, phosphofructokinase, and aldolase); *GPA,* glycophorin A; *GPB,* glycophorin B; *GPC,* glycophorin C; *Prx2,* peroxiredoxin-2; *RhAG,* Rh-associated glycoprotein. *(Modified from Korsgren C, Peters LL, Lux SE: Protein 4.2 binds to the carboxyl-terminal EF-hands of erythroid alpha-spectrin in a calcium- and calmodulin-dependent manner.* J Biol Chem *285:4757–4770, 2010.)*

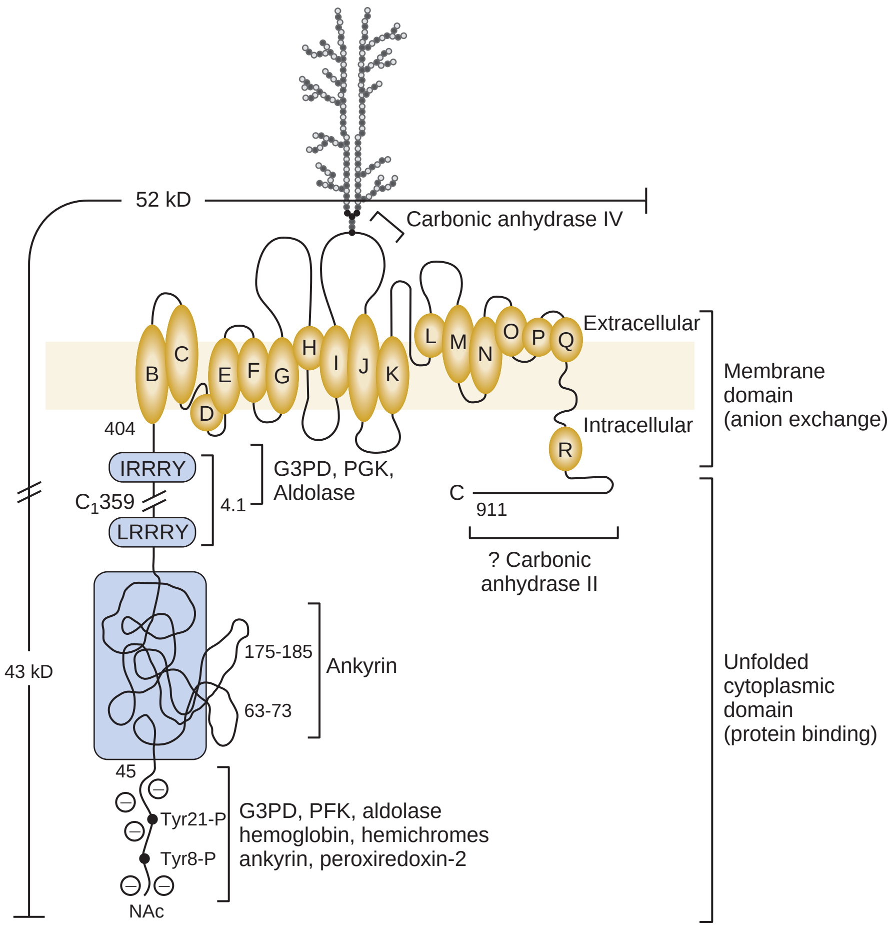


Figure 15-7 Organizational model of human erythrocyte band 3 (anion exchange protein, AE1). The protein is divided into two structurally and functionally distinct domains: an approximately 43-kD cytoplasmic domain (amino acids 1 to 359) that contains binding sites for several cellular proteins and an approximately 52-kD transmembrane domain (amino acids 360 to 911) that forms the anion exchange channel. The two regions can be separated by chymotrypsin cleavage at the inner membrane *(C_1)*. *Cytoplasmic domain:* The highly acidic N-terminus (amino acids 1 to 45) binds deoxyhemoglobin, hemichromes, and the glycolytic enzymes phosphoglycerate kinase *(PGK)*, aldolase, and glyceraldehyde-3-phosphate dehydrogenase *(G3PD)*. Enzyme attachment is blocked by phosphorylation of tyrosines 8 and 21. Antibodies to the N-terminus also interfere with ankyrin binding, but the main ankyrin sites are amino acids 63 to 73 and 175 to 185. Protein 4.1 binds to two sites with the (I/L)RRRY motif, near the end of the domain. The binding site for protein 4.2 has not been clearly identified. *Membrane domain:* The best current model[173] is based on homology with the structure of the ClC bacterial chloride channel.[174-176] The intramembrane helices are lettered as they are in the ClC channel. A complex carbohydrate structure is attached to Asn642. Carbonic anhydrase IV (CA-IV) binds to the extracellular loop of band 3 between helices I and J, and there is evidence, although disputed, that CA-II binds to the C-terminal segment. Both are perfectly positioned to shuttle HCO_3^- ions to or from the plasma through band 3.

glycolytic fluxes by 45% and decreases utilization of the pentose phosphate pathway by 66%.[198]

The obvious reason for a complex of glycolytic enzymes would be to increase the efficiency of glycolysis by channeling glycolytic intermediates from one enzyme to the next (see Fig. 15-8, C). The complex is conveniently located near band 3, which transports the phosphate needed to make ATP. Band 3 also transports phosphoenolpyruvate, pyruvate, and lactate, all substrates or products of the putative complex. The immediate modification of substrates on the inner membrane surface also prevents back-flux through transport channels, increasing the efficiency of transport.

There is strong evidence that human red cell membranes compartmentalize ATP (see Fig. 15-8, *B*).[199,200] The membrane ATP pool is in contact with β spectrin, ankyrin, band 3, and G3PD,[200] and it fuels the Ca^{2+}-ATPase and Na^+,K^+-ATPase pumps[201] and presumably the phospholipid flippase.

Interestingly, the existence of a membrane bound complex of glycolytic enzymes was postulated more than 45 years ago[202] and was indirectly observed more than 35 years ago,[203,204] but the interpretation was not generally accepted. The membrane ATP pool is another discredited old idea[205,206] that has been revived.

The Cytoplasmic Domain Is Where the Membrane Skeleton Attaches. *Ankyrin* links the major membrane skeletal protein spectrin to the plasma membrane of the red cell by binding to a pair of adjacent beta hairpin loops in the cytoplasmic domain of band 3 formed by amino acids 63-73 and 175-185 (Fig. 15-9; see also Fig. 15-7).[207-209] There is considerable evidence that ankyrin also interacts with the acidic N-terminal domain of

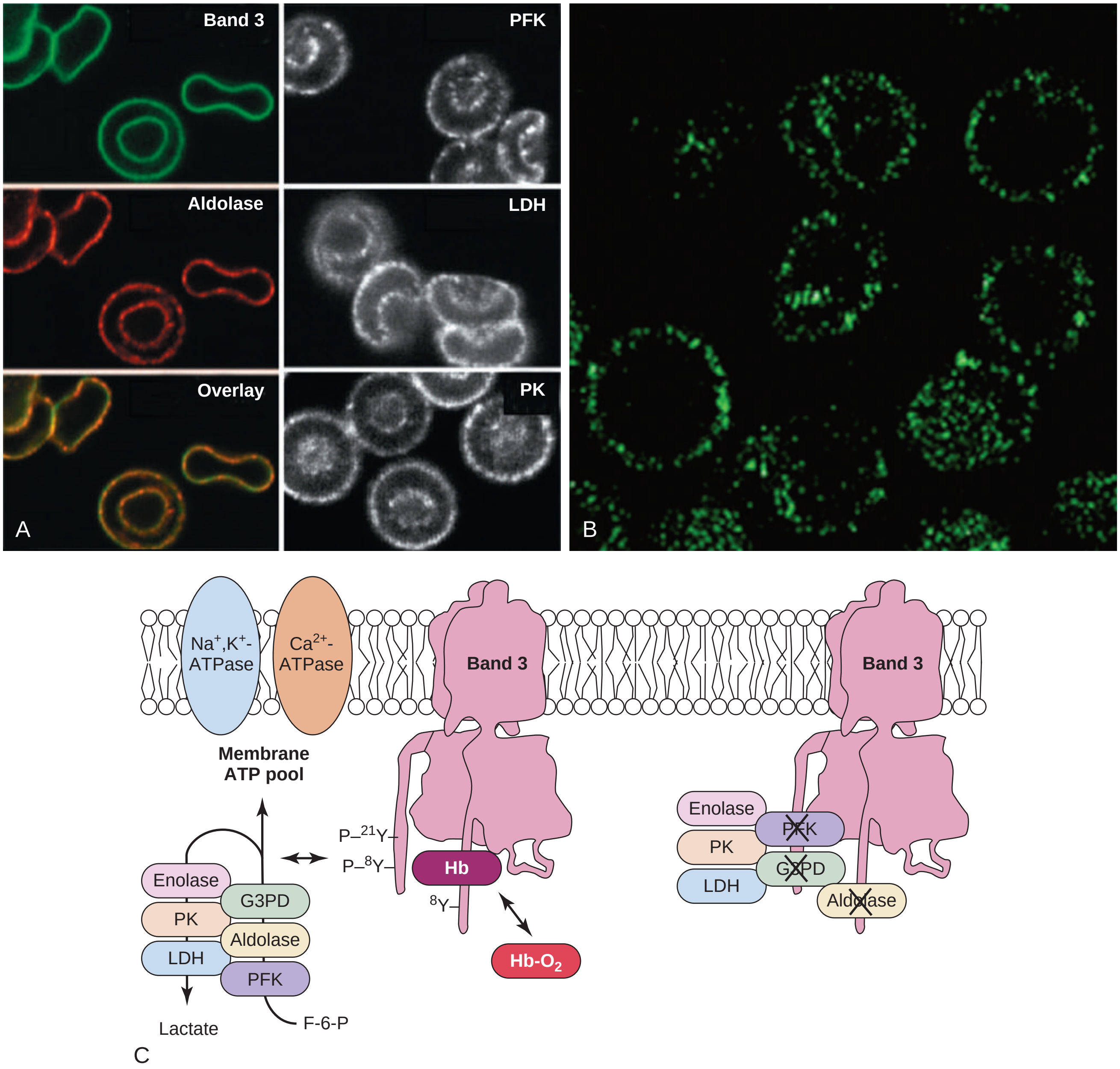


Figure 15-8 Glycolytic metabolon. **A,** Fluorescent imaging of band 3 and aldolase on the *left* and phosphofructokinase *(PFK)*, lactate dehydrogenase *(LDH)*, and pyruvate kinase *(PK)* on the *right*. Note that band 3 is uniformly and exclusively in the membrane whereas the five enzymes are mostly attached to the membrane (some cytoplasmic staining, especially for LDH) and have a punctate appearance as though they are clustered. Glyceraldehyde-3-phosphate dehydrogenase *(G3PD)* stains similarly but is not shown. Aldolase, PFK, and G3PD bind to band 3; PK and LDH do not. However, all are displaced by a fragment of the band 3–binding site, by phosphorylation of the site, or by deoxyhemoglobin, plus all can be cross-linked to one another, indicating that all are bound in a complex or "metabolon." **B,** Discrete, discontinuous submembrane pools of a fluorescent analogue of adenosine triphosphate *(ATP)*, synthesized by phosphoglycerate kinase. **C,** Schematic of the postulated glycolytic metabolon. The enzyme complex binds to the amino terminus of band 3 *(right)* and to a site near the junction between the cytoplasmic and membrane domains *(not shown)*. When the complex is bound to band 3 *(right)*, the enzymes are inactive. The complex can be displaced and activated *(left)* by phosphorylation of Tyr8 and Tyr21 on band 3 or by binding of deoxyhemoglobin. Some of the ATP produced is retained at the membrane and used by the Na^+,K^+-ATPase and Ca^{2+}-ATPase. *F-6-P,* Fructose-6-phosphate; *Hb,* hemoglobin. *(**A,** From Campanella ME, Chu H, Low PS: Assembly and regulation of a glycolytic enzyme complex on the human erythrocyte membrane.* Proc Natl Acad Sci U S A *102:2402–2407, 2005; **B,** From Chu H, Puchulu-Campanella E, Galan JA, et al: Identification of cytoskeletal elements enclosing the ATP pools that fuel human red blood cell membrane cation pumps.* Proc Natl Acad Sci U S A *109:12794–12799, 2012.)*

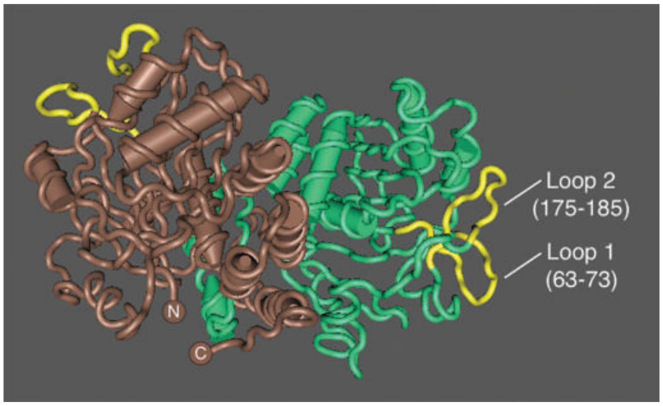


Figure 15-9 Structure of the band 3 cytoplasmic domain. The amino acid backbone of the band 3 dimer is shown. One monomer is *brown*, the other *green*. Note how the monomers interlock in the center. The two loops that protrude from the surface of the molecule to form the ankyrin-binding site (amino acids 63 to 73 and 175 to 185) are *yellow*.

band 3. First, blocking the N-terminus with an antibody inhibits ankyrin binding. Second, association of ankyrin with band 3 is blocked by phosphorylation of tyrosines 8 and 21, and, vice-versa, ankyrin binding prevents phosphorylation of these tyrosines. As mentioned earlier, deoxyhemoglobin also binds to the N-terminus. In doing so it displaces ankyrin and weakens the membrane.[210] Stefanovic and colleagues postulate that this could improve blood flow during brief periods of deoxygenation but might promote unwanted membrane vesiculation during prolonged deoxygenation, as in sickle cell disease.[210]

Ankyrin preferentially binds to band 3 "tetramers."[211,212] Whether band 3 spontaneously forms tetramers and then attracts ankyrin, stabilizing the tetramers, or whether ankyrin, which has two binding sites for band 3, independently binds two band 3 dimers (i.e., a "dimer of dimers") is unresolved. The fact that a stable subpopulation of band 3 tetramers can be isolated from nonionic detergent extracts of ghosts[213,214] argues for the former. The fact that at least one structure of the band 3/ankyrin complex precludes a tetramer[215] argues for the latter. It is estimated that 40% of band 3 is bound to ankyrin as tetramers (120,000 tetramers) whereas 60% exists as dimers (360,000 dimers).[213]

Protein 4.1R, which links the spectrin-actin–based skeleton to the plasma membrane in part via interactions with glycophorin C, binds to the cytoplasmic domain of band 3 at specific (I/L)RRRY sequences near the inner membrane surface (see Fig. 15-7).[216] The interaction is important because zebrafish with a severe hemolytic anemia due to lack of band 3 can only be rescued by band 3 that contains IRRRY sites.[217] Rescue is markedly reduced if mouse band 3 containing a mutation in either the LRRRY or IRRRY site is used and is nearly abolished if both sites are mutated. Because protein 4.1R and ankyrin compete for binding to band 3[218] and ankyrin binds exclusively to band 3 tetramers, it seems likely that protein 4.1R binds preferentially to dimeric band 3 (see Fig. 15-6).

Protein 4.2, a 77-kD peripheral membrane protein of the red cell, also interacts with the cytoplasmic domain of band 3. The binding site is not well defined. Red cells from patients with the cytoplasmic band 3 mutations Glu40→Lys, Gly130→Arg, and Pro327→Arg show some deficiency of protein 4.2, and the mutant band 3 molecules bind protein 4.2 poorly in vitro.[219] Pro327 and Gly130 are not too close to each other in the crystal structure of band 3 so either there is more than one binding site or one of the mutations induces more global effects on band 3 structure. It is not clear whether protein 4.2 preferentially binds to the ankyrin-linked band 3 tetramers or to band 3 dimers, but, as discussed later in the section on protein 4.2, it probably binds to both. Because protein 4.2 binds to the carboxy-terminus (C-terminus) of α spectrin, near the spectrin-actin junction, it appears that band 3 may attach the skeleton to the lipid bilayer at both the head end of spectrin, where ankyrin binds, and at the tail end, where actin, proteins 4.1R and 4.2, and adducin bind (see Fig. 15-6). Whatever the arrangements, protein 4.2 depends on band 3 to be expressed in the membrane. Membranes from band 3 knockout mice completely lack 4.2,[220] and membranes from 4.2 knockout mice are partially deficient in band 3.[221]

Adducin, is an actin-binding protein that caps actin filaments and enhances the binding of spectrin to actin. It is discussed later in the chapter. Adducin binds to the cytoplasmic domain of band 3 via its extended, flexible tail domain (see Fig. 15-6).[222] The tails of both α and β adducin bind band 3, but the free β-adducin tail domain disrupts red cell membrane morphology and stability more than the α-adducin tail,[222] suggesting it is the more important interaction physiologically. Mice lacking all adducins have a mild spherocytic hemolytic anemia but have normal amounts of band 3 and spectrin.[223]

Band 3 Tyrosine Phosphorylation Weakens Ankyrin Binding. Band 3 is phosphorylated on tyrosine residues 8, 21, 359, and 904 by p72Syk kinase[224-226] and to a less extent by Lyn kinase.[227] This displaces the glycolytic enzyme complex,[193,194] as noted earlier, but it also greatly weakens band 3/ankyrin binding, which in turn promotes band 3 diffusion within the lipid bilayer and red cell spiculation and vesiculation.[228] The effect on the interactions of band 3 with its other binding partners is unknown. In the basal state, band 3 tyrosines are unphosphorylated, owing to the action of phosphatases,[228] but they become phosphorylated after malaria invasion[225] and by oxidative damage, such as occurs in hemoglobinopathies[229] and glucose-6-phosphate dehydrogenase (G6PD) deficiency.[224,230]

Flexible Link between the Cytoplasmic and Membrane Domains. Kurtz and his colleagues have analyzed hundreds of high-resolution electron micrographs of single band 3 dimers and find that cytoplasmic and membrane domains orient differently in different molecules, strongly suggesting that the connecting region is flexible.[231] The fact that loss of a portion of this linker in Southeast Asian ovalocytosis (see Chapter 16) results in very rigid red cell membranes suggests that the link may be important in regulating red cell elasticity.

Kidney Band 3. Band 3 is also expressed in the basolateral membrane of the type A intercalated cells in the kidney collecting ducts where it collaborates in the excretion of H^+ and reclamation of HCO_3^-. The kidney isoform lacks the first 65 amino acids and does not bind ankyrin.[232]

Membrane Domain

The 52-kD Membrane Domain (Amino Acids 360 to 911) Contains an Anion Exchange Channel. The anatomy of the membrane domain of band 3 has been an active area of interest, with the goal of research efforts focused on understanding how the membrane domain's topology enables the 1.2 million channels in each red cell to exchange 10 to 30 billion HCO_3^- or Cl^- anions per second.[233,234] The 6 nm long × 11 nm wide × 7 nm deep[174] membrane domain contains numerous transmembrane and intramembrane helices, some of which are tilted in a V shape like they are in the bacterial ClC chloride channel.[174-176] Multiple topology models of the membrane domain have been derived over the years using different techniques to see which amino acids are accessible from the exterior and interior of the cell.[173,235-238] The latest and most plausible one is based on homology between band 3 and the ClC channel (see Fig. 15-7).[173]

Despite considerable effort, we still do not know which transmembrane segments form the anion channel or exactly how the channel functions. Residues in helices D, F, H, I, N, and R are predicted to contribute to the transport process.[173] The clustering of mutations responsible for various disorders of red cell membrane permeability between helices K and N suggests that region is also involved (see Fig. 16-35).

Carbonic anhydrase IV (CAIV), a GPI-linked protein, binds to the outside of the membrane domain,[239] probably just beyond the glycosylation site (Asn642) in the protein loop that connects helices I and J (see Fig. 15-7).

Carbonic anhydrase II (CAII) may[240] or may not[241] bind to band 3, on the C-terminal extension at the inside surface of the channel (see Fig. 15-7). Assuming it does, there is evidence that it increases HCO_3^-/Cl^- exchange[138,242,243] and helps create an acidic microdomain around band 3 during HCO_3^- transport.[244] The two carbonic anhydrases potentially form a CO_2/HCO_3^- transport metabolon with band 3.[138,239]

The Transport Channel Exchanges Chloride and Bicarbonate Rapidly (~20,000 to 50,000 Times per Second per Channel), Facilitating CO_2 Transport from the Tissues to the Lungs. In the peripheral tissues, CO_2 is either rapidly converted to HCO_3^- at the surface of band 3 by CAIV and enters the red cell via band 3 or it diffuses across the red cell membrane and is then converted to HCO_3^- by CAII. The H^+ by-product of the carbonic anhydrase reaction binds to hemoglobin and *facilitates* O2 release to the tissues (i.e., the Bohr effect). The whole process is reversed in the lungs (Fig. 15-10). As the red cell circulates, intracellular HCO_3^- is exchanged for extracellular chloride. As a result, HCO_3^- is transported in the plasma as well as within the red cell, which increases CO_2 transport from the tissues to the lungs by approximately 60%.[234] The HCO_3^-/Cl^- exchange is believed to occur by a "ping-pong" mechanism, whereby an intracellular anion enters the transport channel and is translocated outward and released, with the channel remaining in the outward configuration until an extracellular anion enters and activates the reverse cycle.[174,245]

Although HCO_3^- and Cl^- are the predominant physiologically relevant anions exchanged by band 3, the specificity of the channel is actually quite broad and larger anions such as sulfate, phosphate, pyruvate, and superoxide are also transported, albeit at much slower rates.[246]

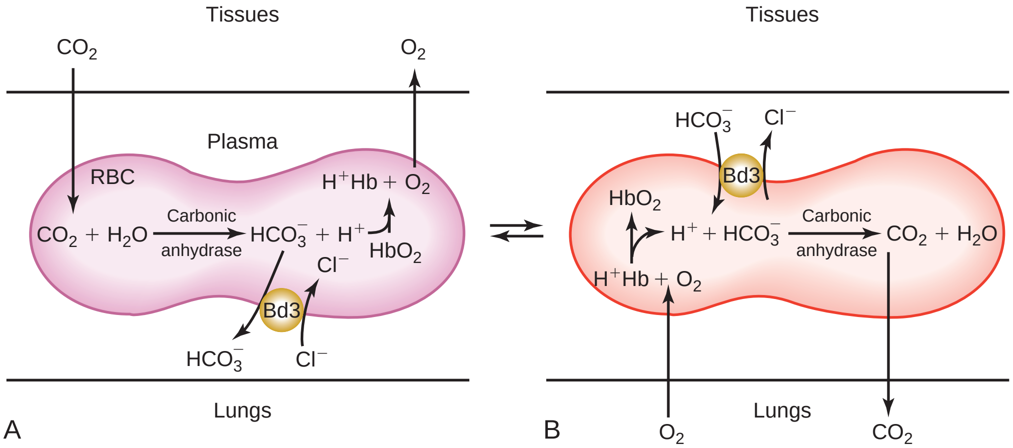


Figure 15-10 Function of band 3 in O_2 and CO_2 transport. **A,** Tissue CO_2 is converted into HCO_3^- and H^+ in the red cell by carbonic anhydrase. Band 3–mediated exchange of the HCO_3^- for Cl^- (Hamburger shift) allows HCO_3^- to be carried in the plasma as well as the red cell and increases CO_2 uptake by about 60%. H^+ binds to hemoglobin and facilitates O_2 release (Bohr effect) and tissue oxygenation. **B,** These processes are reversed in the lungs, which promotes hemoglobin oxygenation and release of CO_2. *(Modified from Weith JO, Anderson OS, Brahm J, et al: Chloride-bicarbonate exchange in red blood cells: physiology of transport and chemical modification of binding sites.* Philos Trans R Soc Lond [Biol] *299:383, 1982.)*

Several potent and experimentally useful inhibitors of band 3 anion transport are also known, with the most commonly employed being stilbene disulfonates, such as 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS).[246]

The Extracellular Surface of the Band 3 Membrane Domain is the Site of Specific Blood Group Antigens. Asn642, located in the extracellular loop between helices I and J, is derivatized with a complex lactosaminoglycan that is variable in length and contains the I/i blood group antigens (see Fig. 15-7).[247] In fetal cells, the carbohydrate chain is unbranched and has i specificity,[248] whereas in adults there is a branched structure that yields I reactivity.[249] Extracellular domains of band 3 also contain the antigens of the Diego and at least 13 other blood groups.[250-258]

The Integral Membrane Protein Glycophorin A (GPA) and the Membrane Domain of Band 3 are Functionally Associated. The Wright (Wr) blood group antigen is formed by the association of GPA and band 3.[258] Glu658 in band 3 is required for normal expression of this blood group antigen,[259] and an Ala65→Pro mutation in GPA causes aberrant Wright antigen expression.[260] Furthermore, a monoclonal antibody directed against the Wr^b antigen immunoprecipitates both proteins,[261] and anti-GPA antibodies immobilize both GPA *and* band 3 in the membrane.[262]

GPA also has a chaperone-like role in bringing band 3 to the plasma membrane. The coexpression of GPA and band 3 facilitates the expression of band 3,[263] red cells lacking band 3 also lack GPA,[264] and mutant band 3 molecules that are stuck in the endoplasmic reticulum can be rescued by coexpression of the mutant band 3 with GPA.[265] GPA also enhances band 3 transport.[263] SO_4^{2-} transport is diminished 60% in the absence of GPA.[266]

Organization of Band 3 in the Membrane

A variety of evidence supports the concept that band 3 exists in three populations: one tethered to spectrin via ankyrin, one attached to the spectrin/actin junctional complexes via adducin and protein 4.1R, and a freely diffusing population.

Band 3 Mutations

Mutations in band 3 are known to cause hereditary spherocytosis, hereditary stomatocytosis, hereditary cryohydrocytosis, Southeast Asian ovalocytosis, hereditary distal renal tubular acidosis and, in one case, hereditary acanthocytosis. These are discussed in the various sections of Chapter 16 and illustrated in Figures 16-10 and 16-35. Incomplete processing of the band 3 N-linked lactosaminoglycan moiety occurs in congenital dyserythropoietic anemia type II (CDA II or hereditary erythroblastic multinuclearity with positive acidified serum lysis test [HEMPAS]), which is discussed in Chapter 7.

Band 3 and Malaria

Plasmodium falciparum parasites are believed to invade red cells via two distinct pathways, one that is dependent on the sialic acids of GPA or glycophorin C (GPC), and a second, a sialic-acid independent, pathway that is linked to band 3. Expression of a parasite protein called PfRh4 correlates with switching to the sialic acid–independent pathway.[267] Liposomes containing purified band 3 inhibit invasion,[268] as does a monoclonal antibody against extracellular epitopes of band 3.[269] Peptides corresponding to the two of the surface loops of band 3 also partially block invasion,[270] suggesting that these regions serve as receptors. MSP1 (*P. falciparum* merozoite surface protein 1) furnishes the ligand.[270]

Glycophorins

The glycophorins[271] are a family of integral membrane glycoproteins that account for about 90% of the surface charge of the red cell. The extracellular domains of glycophorins contain abundant negatively charged sialic acid molecules that keep red cells from adhering to each other and to vessel walls. Surface epitopes of the glycophorins generate the blood group antigens in the MNSs system. Glycophorins also serve as receptors for infective agents, such as *P. falciparum,* and function as chaperones, facilitating the targeting of specific integral membrane proteins to the plasma membrane. The glycophorins belong to the broad family of integral membrane proteins that pass through the lipid bilayer as a single α helix.

Glycophorin A

The glycophorin A (GPA) gene *(GYPA)* is located on the long arm of chromosome 4 (4q31) and is increasingly expressed throughout erythroid development.[168] It encodes a 36-kD protein that is the major sialoglycoprotein of the red cell (~10^6 copies per cell). It was the first membrane protein to have its complete amino acid sequence determined. Amino acids 1 to 61 form the extracellular N-terminal domain, which contains a complex oligosaccharide attached to Asn26[272] and 15 serine or threonine O-linked tetrasaccharides with sialic acid moieties (Fig. 15-11, *A*).[273] The carbohydrate component of GPA accounts for 60% of its molecular mass. Experimentally, the glycophorins run anomalously on SDS gels because the abundant carbohydrate interferes with detergent binding. In addition, the carbohydrate interferes with Coomassie Blue staining, such that special stains (e.g., periodic acid–Schiff) are required to visualize the glycophorins. The N-terminus of GPA contains the site of the MN blood group antigens. Amino acids 1 and 5 along with the sialic acid residues on adjacent O-linked tetrasaccharides dictate the antigenicity.[274] Amino acids 1 and 5 are serine and glycine in MM red cells; leucine and glutamic acid in the corresponding positions characterize NN cells. Amino acids 72 to 95 form the transmembrane domain of GPA, which traverses the lipid bilayer as a single α helix. The membrane domain interacts with negatively charged lipids, such as PS and PI at the internal end (Fig. 15-11, *A*).[275,276] The α-helical transmembrane domains self-associate to form a dimer[277,278]: Ile76, Val80, and Val84 on the α helix of one monomer interface with Leu75, Gly79, Gly83, and Thr87 on the second monomer.[279] The extracellular domain also contributes to this dimeric interaction,[280] which is strong enough to resist dissociation by detergents.

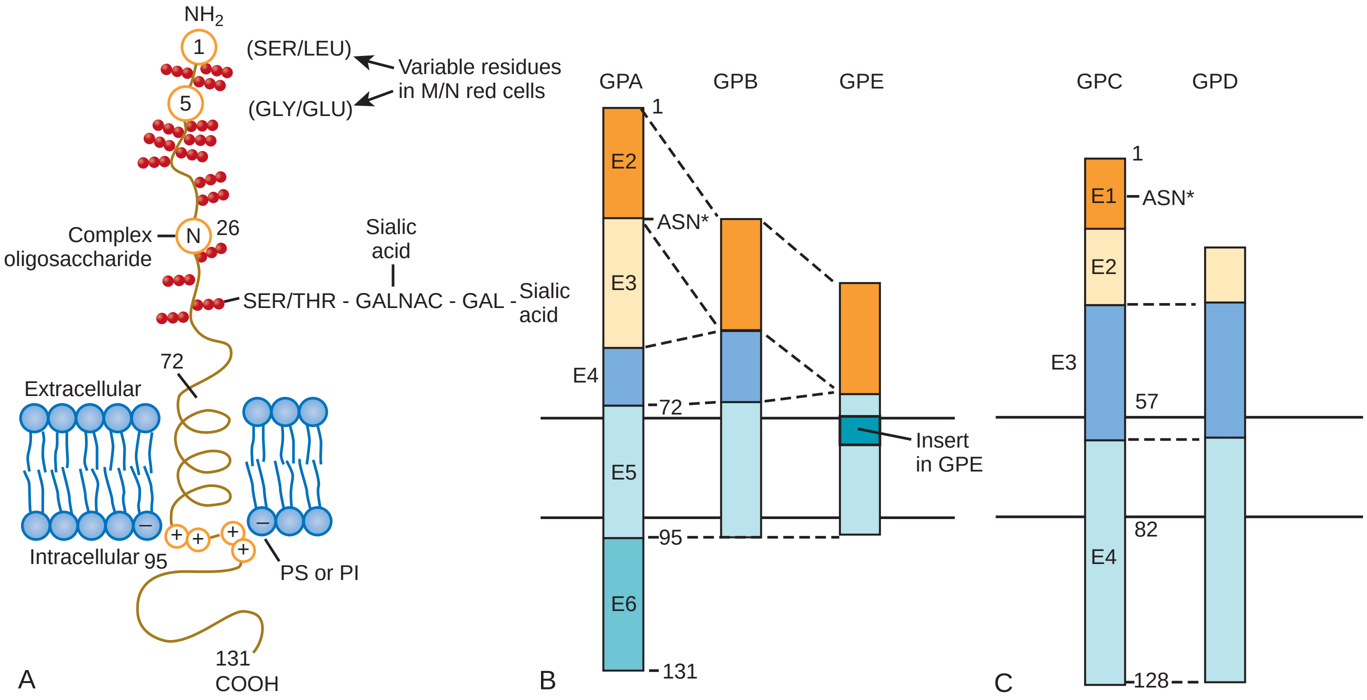


Figure 15-11 Structural model of red cell glycophorins. **A,** Organization of glycophorin A *(GPA)*, the major red cell sialoglycoprotein. The external domain has 15 tetrasaccharides attached to serine or threonine residues and one complex oligosaccharide attached to asparagine 26. Residues 1 and 5 are Ser and Gly in M red cells and Leu and Glu in N cells. **B,** Schematic representation of the domain combinations of GPA, glycophorin B *(GPB)*, and glycophorin E *(GPE)*. Each *box* represents the portion of each protein encoded by a specific exon or insertion sequence. ASN* is the site of attachment of the complex carbohydrate. **C,** Schematic representation of the domains contributing to glycophorin C *(GPC)* and glycophorin D *(GPD)*. *PI,* Phosphatidylinositol; *PS,* phosphatidylserine. *(Modified from Becker PS, Lux SE: Disorders of the red cell membrane skeleton: hereditary spherocytosis and hereditary elliptocytosis. In Scriver CR, Beaudet AL, Sly WS, et al, editors:* The metabolic basis of inherited disease, *7th ed. New York, 1995, McGraw-Hill; and Fukuda M: Molecular genetics of the glycophorin A gene cluster.* Semin Hematol *30:138, 1993.)*

As noted in the earlier section on band 3, the cytoplasmic domain of GPA (amino acids 96 to 131) enhances the trafficking of band 3 to the membrane[280] and increases band 3 anion transport.[266] Conversely, GPA is dependent on band 3 for trafficking or for stable incorporation in the red cell membrane, because mice that lack band 3 also lack GPA.[264]

Interestingly, treatment of red cells with antibodies (or monovalent Fab fragments) to GPA causes GPA to bind to the membrane skeleton and makes the cells rigid.[281] These changes depend on the cytoplasmic domain of GPA, suggesting that it binds to the membrane skeleton in response to a transmembrane signal initiated by antibody treatment.[282] Hypothetically, cellular rigidity induced by adherence of pathogens to glycophorins might enhance reticuloendothelial clearance of infected cells[282] or impede invasion by parasites, but whether it does so is untested.

GPA is a specific marker for the erythroid lineage and is detectable from the proerythroblast stage.[168]

Glycophorin B

The glycophorin B (GPB) gene *(GYPB)* lies just downstream of GPA on chromosome 4.[283,284] The first 26 amino acids of the extracellular N-terminal domain are almost identical to the N blood group form of GPA except that GPB lacks the N-linked complex oligosaccharide (see Fig. 15-11, *B*).[274] In addition to N–blood group reactivity, GPB carries the S, s, and U blood group antigens, with the S and s antigens differing by substitutions at residue 29 (S = Met, s = Thr).[285] Distally, GPB is missing a portion of the extracellular domain corresponding to exon 3 of GPA and lacks almost all of the cytoplasmic domain. Whether GPB exists as homodimers, as heterodimers with GPA, or as both is not known.

GPB may interact with the RhAG protein in the Rh complex (to be discussed) because RhAG is hyperglycosylated in GPB-null individuals[286] and because GPB expression is decreased in Rh_{null} patients.[287]

Glycophorins C and D

Glycophorins C and D (GPC, GPD) have a general domain structure similar to GPA and GPB (see Fig. 15-11, C) but are encoded by a distinct gene *(GYPC)* located on chromosome 2 (2q14-2q21).[288] The gene is maximally expressed in mid-erythropoiesis.[168] GPD is generated from the same mRNA as GPC by means of an alternate initiation codon.[289] GPC is a 32-kD protein bearing one N-linked oligosaccharide and approximately 12 O-linked oligosaccharides in the N-terminal, extracellular domain. Amino acids 41 to 50 of the extracellular domain contain the Gerbich blood group antigens. Like GPA and GPB, the transmembrane domain passes through the lipid bilayer as a single α helix. The cytoplasmic domain of GPC forms a ternary complex with protein 4.1R and p55

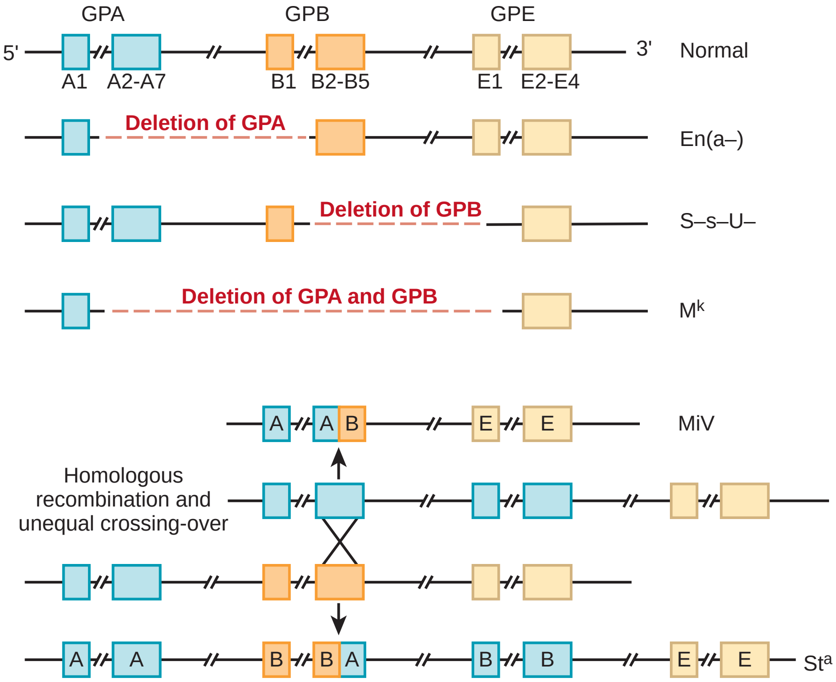


Figure 15-12 Glycophorin variants. Organization of the glycophorin A *(GPA)*, B *(GPB)*, and E *(GPE)* genes on chromosome 4 and probable deletions in the En(a–), S–s–U–, M^k, Miltenberger type V *(MiV)*, and Stones *(St^a)* variants. The MiV and St^a variants arise from unequal crossing over, analogous to the Lepore hemoglobins. In MiV red cells the normal GPB gene is replaced by a fusion gene (Lepore-type recombination). In St^a erythrocytes the product of the reciprocal exchange is inserted between the GPA and GPB loci but no normal genes are lost (anti–Lepore-type recombination). *(Modified from Vignal A, London J, Rahuel C, et al: Promoter sequence and chromosomal organization of the genes encoding glycophorins A, B, and E.* Gene *95:289–293, 1990.)*

and helps attach the spectrin-actin–based skeleton to the plasma membrane (see Fig. 15-6). The 30-kD membrane-binding domain of protein 4.1R interacts with amino acids 82 to 98 of GPC and the corresponding residues 61 to 77 of GPD.[290] p55 is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins and contains a single PDZ domain that binds to GPC at residues 112 to 128 and to GPD at the corresponding amino acids 91 to 107.[290,291] The ternary complex of GPC/GPD, protein 4.1R, and p55 aids in maintaining red cell membrane deformability and mechanical stability. Unlike GPA and GPB, GPC and GPD are expressed in a variety of tissues, although at lower levels than in erythroid cells and with different glycosylation patterns.[292,293] They may have analogous functions in the membrane mechanics of nonerythroid cells.

Glycophorin E

The glycophorin E (GPE) gene *(GYPE)* lies just downstream of GPB on chromosome 4 and encodes a putative 78-amino acid protein with structural similarities to GPB (see Fig. 15-11, *B*).[294] The extracellular domain of GPE corresponds to exon 2 of GPB, but the amino acid sequence specifies blood group M rather than N.[294] The transmembrane domain contains an 8-amino acid insertion. Like GPB, GPE has a markedly truncated cytoplasmic domain. GPE is believed to have arisen by gene duplication of GPB, which in turn derived from ancestral duplication of GPA.[284] Thus GPA, GPB, and GPE define an erythroid-specific family of integral membrane sialoglycoproteins. The level of expression of GPE and its function, if any, are unknown and it may only be a pseudogene.

Glycophorin Mutations

Defects in GPA, GPB, and GPE Give Rise to Nearly 40 Variant Phenotypes of the MNS Blood Group System.[295] Complete loss of GPA, GPB, or both, often by gene deletion from unequal recombination of the neighboring loci, gives rise to En(a–), (S–s–), and M^kM^k red cells, respectively (Fig. 15-12). En(a–) red cells, for example, are completely deficient in GPA and have weak MN blood group antigens.[296,297] The affected red cells compensate for a potential 60% loss of surface charge by increasing the glycosylation of band 3, such that the overall surface charge is only reduced by approximately 20%.[298] Given the proximity and homology of GPA, GPB, and GPE genes, hybrid glycophorin molecules may be generated by recombination events during meiosis. In the MiV variant, for example, Lepore-like recombination yields a fusion gene containing the N-terminus of GPA and C-terminus of GPB.[299] In the St^a variant, anti-Lepore recombination produces the reverse hybrid (N-terminal GPB with C-terminal GPA), which is inserted between the normal GPA and GPB genes, neither of which is lost.[299] Interestingly, none of the GPA and GPB variants produces detectable changes in erythropoiesis or in the shape, function, or life span of the affected red cells. Rare individuals with complete absence of GPA and GPB apparently exhibit no erythrocyte abnormalities.[300]

Similarly, GPA knockout mice have a normal phenotype except that they have 60% less O-linked oligosaccharides than normal red cells.[301] Thus either GPA, GPB, and GPE do not serve any critical basal functions in red cells or such functions are subsumed by other protein(s), such as GPC or band 3, when GPA, GPB, and GPE are deficient. If GPA really has no nonredundant function, it is surprising that deletions and other mutations are not more common, because GPA is a major receptor for *P. falciparum* malaria (see later) and must be under intense evolutionary pressure.

Variations in GPC and GPD Affect the Gerbich Antigen System. Mutations in the extracellular domain of GPC and GPD alter the antigenicity of red cells (Gerbich antigen system) but do not affect cellular mechanical properties.[293,302] In contrast, in the Leach phenotype there is complete loss of GPC and GPD secondary to deletion of exons 3 and 4[303,304] or a frameshift mutation.[304] Affected individuals do not have significant hemolysis, but their red cells are slightly elliptocytic[305,306] and exhibit decreased membrane deformability and mechanical stability,[302,307,308] probably because GPC-deficient cells are partially deficient in protein 4.1R and p55.[309] Individuals with homozygous protein 4.1R deficiency have a more profound deficiency of GPC (~70%) in their elliptocytic red cells,[310,311] underscoring that the cellular content of each component of the ternary complex depends on the expression level of the others.

Glycophorins as Receptors for Malarial Invasion

Invasion of red blood cells by *P. falciparum* malaria occurs by multiple different pathways, depending on the species, and involves all of the different glycophorins. GPA serves as the receptor for the parasite ligand EBA-175.[312] A membrane fraction containing GPA or antibodies to GPA inhibits malarial invasion,[313] and erythrocytes lacking GPA resist invasion.[314,315] The binding specificity of EBL-175 is determined by the Neu5Ac-(α2,3)-Gal moiety on the O-linked tetrasaccharides of GPA[312] and on the amino acid sequence, which places the sugars in the proper configuration.

GPB-dependent invasion is also sialic acid dependent but is believed to utilize another parasite ligand.[316]

GPC appears to be the receptor for a third sialic acid–dependent pathway for *P. falciparum* malaria.[317-320] GPC/GPD-deficient red cells (Leach blood group phenotype) and protein 4.1R–deficient red cells (which partially lack GPC) resist malarial invasion in vitro,[317] and up to 50% of the Melanesian population in the malarious coastal areas of Papua New Guinea have red blood cells that are Gerbich blood group negative, owing to a deletion in the third exon of the GPC gene.[318] GPC binds the *P. falciparum* ligand EBA-140, which is also called BAEBL.[319-321]

In addition, there are *P. falciparum* strains that invade neuraminidase-treated red cells (i.e., cells lacking sialic acid),[322,323] so a sialic acid/glycophorin-independent mechanism(s) for malarial invasion also exists.[267] One such mechanism may involve merozoite adhesion to band 3.[268]

The existence of so many ways for *P. falciparum* to invade erythrocytes and the ability to switch between invasion pathways obviously complicates efforts to create a malarial vaccine.

Other Integral Membrane Proteins

Although band 3 and the glycophorins are the major integral membrane constituents of the red cell membrane, a large number of other integral membrane proteins with specific functions have also been identified and characterized.

Basigin

Basigin, or CD147, is a glycosylated integral membrane protein belonging to the immunoglobulin superfamily.[324] The common 269-amino acid isoform basigin-2 has two external immunoglobulin domains and a single transmembrane domain. It binds to and regulates the expression of monocarboxylic acid transporters (MCTs). A complex between basigin, MCTs, and CD44 occurs in breast cancer cells and probably in erythroid cells. Basigin has many functions in cancer, infection, inflammation, and development, but in the red cell its known functions are that it carries the common OK blood group antigen and serves as the receptor for the *P. falciparum* protein PfRh5.[324,325] Unlike the glycophorins and band 3, which are required for the adhesion of some parasites but not others, it appears that basigin and PfRh5 are required for all *P. falciparum* strains.[320,324] PfRh5 cannot be deleted from the pathogen,[326] and antibodies to PfRh5 block invasion of all strains,[327] as do antibodies to basigin or knockdown of the protein in red cells.[325] This makes the PfRh5/basigin complex a prime target for a malarial vaccine.

Rh Proteins (RHD, RHCE, and RhAG)

The Rh blood group system comprises at least 40 independent antigens carried by two nonglycosylated, palmitoylated proteins encoded by two homologous genes, *RHD* and *RHCE*, located on chromosome 1.[328-330] *RHCE* encodes the CcEe set of antigens, and *RHD* encodes the D antigen. Rh antigen expression at the red cell surface requires the presence of the Rh-associated glycoprotein (RhAG), which exhibits 36% sequence identity with Rh proteins and is encoded by a gene located on chromosome 6. There are 100,000 to 200,000 copies of RhD, RhCE, and RhAG per cell, and they probably form a heterotrimer based on the structure of the kidney isoform of RhAG.[331] They are each predicted to have 12 membrane-spanning domains. In addition to the interaction between the three Rh subunits, the Rh core complex also contains several other proteins[330]—LW glycoprotein (ICAM-4), integrin-associated protein (IAP, CD47), GPB, and protein 4.2. The core complex is part of the ankyrin complex (see Fig. 15-6).[332]

The RhAG protein functions as an NH_4^+-NH_3 transporter in mouse and human red cells[333-337] and can complement defects in ammonium transport in yeast.[338] It may also be partly responsible (along with aquaporin-1) for the exceptionally high permeability of CO_2 in the erythrocyte.[339,340] And some researchers speculate that it may serve as a channel for nitric oxide.[341] The importance of the Rh complex in regulating red cell membrane structure is revealed by the altered phenotype of Rh_{null} red cells, which exhibit stomatocytic morphology with loss of membrane surface area, cell dehydration, cation permeability abnormalities, and shortened red cell survival, leading to a mild compensated hemolytic anemia.[342] The Rh_{null} syndrome is discussed in Chapters 16 and 36.

Defects in RhAG that block the ammonium channel and make it highly permeable to Na^+ ions are responsible for overhydrated hereditary stomatocytosis (see Chapter 16).[343,344]

Kidd Glycoprotein

The Kidd glycoprotein (Jk antigen, SLC14A1) is the urea transporter in the red cell membrane and the kidney.[345] The human Kidd glycoprotein gene is located on chromosome 18q12.3 and encodes a 389-amino acid polypeptide with 10 membrane-spanning domains. There are roughly 10,000 copies of Kidd glycoprotein per cell, and the protein carries the antigens of the Kidd blood group system.[346] The Kidd glycoprotein forms the urea transporter in red cells, which functions by rapidly transporting urea into and out of red cells as they pass through the high urea concentration in the renal medulla.[347] Urea transport across $Kidd_{null}$ red cell membranes is approximately a thousand times slower than across normal membranes.[348] However, no changes in red cell shape or survival have been noted in persons who lack the Kidd glycoprotein.

Kell Glycoprotein

Kell glycoprotein is a 93-kD (732 amino acids) single-pass type II membrane protein encoded by a gene on chromosome 7q34.[349] It is a zinc endopeptidase that functions as an endothelin-3–converting enzyme on the erythrocyte surface.[350] It can also cleave the precursors of endothelin-1 and endothelin-2, but much less effectively. Because endothelins are potent vasoactive peptides, it is believed the Kell glycoprotein may be involved in regulation of vascular tone. There is evidence that endothelin-3 may also activate Ca^{2+}-ATPase in the red cell.[351] The Kell glycoprotein carries the antigens in the Kell blood group system. Isolated Kell glycoprotein deficiency does not alter red cell life span or morphology.[352]

Kx Protein

The Kx protein is a 37-kD protein (444 amino acids) with 10 membrane-spanning domains that is encoded by a gene *(XK)* on the short arm of the X chromosome,[353] The Kx protein carries the Kx blood group antigen. In the red cell membrane the Kx protein is covalently linked to the Kell glycoprotein by a disulfide bond.[354] The Kx/Kell complex is part of a larger complex of proteins that are located at the spectrin/actin/protein 4.1R junctions (see Fig. 15-6). The Kx protein has structural but not sequence similarity with a family of proteins involved in transport of neurotransmitters; however, its transport substrate(s) have not been defined. Experiments on red cells from Kell, Kx, and Kell/Kx knockout mice suggest that Kell and Kx affect Ca^{2+} and Mg^{2+} homeostasis and red cell volume.[351] Whether this is a direct or indirect effect is not known. Unlike isolated Kell deficiency, which has no evident phenotype, lack of the Kx protein in McLeod syndrome results in acanthocytic red cells, compensated hemolytic anemia, and, in later life, muscle wasting and choreiform movements. McLeod syndrome is discussed in Chapter 36.

Duffy Antigen/Chemokine Receptor

The Duffy blood group, atypical chemokine receptor (DARC or Fy antigen), is a promiscuous chemokine receptor[355] that binds a variety of proinflammatory cytokines of both the C-X-C class (acute inflammation) and the C-C class (chronic inflammation), including interleukin-8, MGSA (melanoma growth stimulatory activity), MCP-1 (monocyte chemotactic protein 1), and RANTES (regulated on activation, normal T-cell expressed and secreted). The *DARC* gene is located on chromosome 1q23.2 and encodes a 338-amino acid polypeptide with seven membrane-spanning domains.[356] There are 6,000 to 13,000 copies of DARC per cell and the protein carries the antigens of the Duffy (Fy) blood group system. DARC is the receptor for the human malarial parasites *Plasmodium vivax* and *Plasmodium knowlesi.* Duffy-deficient red cells, Fy(a–b–), which are common in individuals from Central and West Africa, are refractory to invasion by most,[357] but not all,[358] strains of *P. vivax*. The Duffy phenotype Fy(a+b–) also provides considerable protection.[359] On the other hand, DARC is necessary for platelet killing of *P. falciparum* malaria.[360] Platelet factor 4, a cytokine released from activated platelets, binds to the DARC and kills the parasite. The function of DARC in normal red cell physiology remains to be defined.

Lutheran Glycoprotein/Basal Cell Adhesion Molecule

The Lutheran blood group (Lu) resides on the basal cell adhesion molecule (BCAM), which belongs to the immunoglobulin superfamily and is the receptor on erythroid cells for the extracellular matrix protein laminin.[361-364] The Lu/BCAM glycoprotein consists of five disulfide-bonded extracellular immunoglobulin superfamily domains, a single hydrophobic transmembrane domain, and a short cytoplasmic tail. Two isoforms of this membrane protein (85-kD and 78-kD isoforms) are expressed by alternative splicing of a single gene located on human chromosome 19q13.32 and are distinguished by differences in their cytoplasmic domains—the 78-kD isoform has a truncated cytoplasmic tail. Both isoforms bind laminin. Binding is stimulated by epinephrine and is dependent on phosphorylation of Lu/BCAM by protein kinase A.[365,366] There are 1500 to 8000 copies of Lu glycoprotein on the mature red cell. Lu/BCAM directly interacts with the fourth spectrin repeat of α spectrin.[367,368] Disruption of this linkage enhances red cell adhesion to laminin[364] and is observed in hereditary spherocytes.[369] Increased laminin binding by red cell Lu/BCAM also seems to be important in the adhesion of sickle red cells.[370] The interaction between the $\alpha 4\beta 1$ integrin on sickle cells and the Lu/BCAM protein on endothelial cells may also be important.[370,371] Hydroxyurea decreases the phosphorylation of Lu/BCAM and decreases the laminin adhesion of sickle cells.[366] Constitutive Lu/BCAM phosphorylation mediates the increased adhesion of red cells to laminin in polycythemia vera and may contribute to the enhanced risk for thrombosis in that disease.[372] The Lu/BCAM-spectrin interaction is also involved in actin cytoskeleton dynamics in nonerythroid cells and is required for actin stress fiber formation.[373]

About 1 person in 5000 inherits the In(Lu) genotype,[374] which partially suppresses expression of Lu^a and Lu^b and is the most common cause of the null Lutheran phenotype, Lu(a–b–). In(Lu) is not a dominantly acting inhibitor as originally believed but is caused by inactivating mutations in one of the two *KLF1* genes or rarely GATA-1.[375,375a] KLF1 encodes EKLF, a transcription factor that controls many erythroid genes and is critical in erythroid development. Patients with the Lu(a–b–) phenotype due to In(Lu) have abnormally shaped red cells but no hemolysis.[376] The morphology varies from normal or mild poikilocytosis to acanthocytosis.

LW Glycoprotein

The LW glycoprotein (also termed ICAM-4)[377,378] also belongs to the immunoglobulin superfamily.[379] The glycoprotein carries the Landsteiner-Weiner (LW) blood group and consists of two extracellular immunoglobulin-like domains that show very strong sequence homology with the protein superfamily known as intracellular adhesion molecules (ICAMs). The 37- to 43-kD LW glycoprotein is encoded by a gene on chromosome 19p13.2. There are approximately 4000 copies of LW glycoprotein on the mature red cell. Preliminary evidence suggests that extracellular domains of LW glycoprotein can interact with integrins LFA1 (CD11/CD18)[380] and α4β1 and αV integrins.[381] Like Lu/BCAM, LW glycoprotein is activated by epinephrine and may also play a role in adhesion of sickle cells by directly increasing the binding of sickle cells to endothelium[382] and by indirectly activating lymphocytes and monocytes and increasing their attachment to vessel walls.[383]

CD47

CD 47, or integrin-associated protein (IAP), is a ubiquitously expressed, highly glycosylated five-span transmembrane protein with a single extracellular immunoglobulin domain and a small intracellular domain. It is widely distributed and is involved in a range of cellular functions, including apoptosis, proliferation, phagocytosis, adhesion, angiogenesis, inflammation, and cell migration.

There are about 17,000 CD47 molecules per erythrocyte.[141] Part of the CD47 is attached to the membrane skeleton and part is mobile and able to cluster.[384] Human CD47 interacts with the Rh protein complex and with protein 4.2. Reduction of protein 4.2 or band 3 leads to marked reduction in CD47 (see later section on protein 4.2).[384-386] The human RhCE protein is also crucial for CD47 expression because red cells that lack Cc and Ee antigens have a 75% reduction in CD47.[385]

In the mature red cell, CD47 may function as a marker of “self.” It interacts with the inhibitory receptor SIRPα (signal regulatory protein α, CD172a) on macrophages and inhibits phagocytosis.[387] Mouse red cells that lack CD47 are rapidly cleared from the blood by splenic macrophages.[387] The CD47-SIRPα interaction blocks phagocytic signaling from complement receptors, Fcγ receptors, and low-density lipoprotein (LDL) receptor–related protein-1. It does not inhibit scavenger receptors.[388,389] Enhancing the interaction offers therapeutic promise in autoimmune hemolytic anemias and thrombocytopenias but so far has not been achieved. Interestingly, attaching peptides that contain the CD47 “self” signal to nanoparticles prolongs their life in the circulation, enhancing their utility in drug delivery or imaging.[390]

Aquaporins

Aquaporin-1 (AQP1), a 28-kD channel-forming protein, is the major water channel in red cells.[383] The human aquaporin-1 gene *(AQP1)* is located on chromosome 7p14 and encodes a 269-amino acid polypeptide with six membrane-spanning domains. Besides red cells, AQP1 is also expressed in the kidney and in the endothelial cells in lung. There are 120,000 to 160,000 copies of AQP1 per cell, and the protein carries the antigens of the Colton blood group. It exists as a tetramer in the red cell membrane, and the crystal structure of the protein is known.[391] AQP1 can be cross-linked to stomatin in red cell membranes,[392] which suggests at least some of it resides in lipid rafts. Human red cells lacking AQP1 exhibit markedly reduced osmotic water permeability. Humans with complete deficiency of AQP1 have defective urinary concentrating ability as well as decreased pulmonary vascular permeability.[393,394] AQP1 knockout mice appear normal but become severely dehydrated and lethargic compared with control mice after water deprivation for 36 hours.[395] Interestingly, implanted tumors grow very poorly in AQP1(–/–) mice because of reduced vascularity and very poor cell migration.[396] These studies show that AQP1 also has an unexpected role in cell migration and thus in angiogenesis and wound healing. There is evidence that AQP1 also facilitates CO_2[397-400] and perhaps NH_3[400] transport across the red cell membrane.

Aquaporin-3 (AQP3) is the glycerol permeability channel in human red cells and carries the high-frequency blood group antigen GIL.[401] Humans lacking the channel are asymptomatic.[401] In mice, AQP3 is not expressed in red cells and aquaporin-9 (AQP9) is the glycerol channel.[402] AQP9-deficient mice survive longer during the initial phase of mouse malaria,[402] perhaps because plasma glycerol is an important substrate for lipid biosynthesis in malarial parasites.

Stomatin

Stomatin or band 7.2b (gene name *EPB72*), is a 31.5-kD integral membrane protein (approximately 28-kD on SDS gels).[403-406] It is a positively charged, homooligomeric (estimated 9 to 12 subunits), phosphorylated (cyclic adenosine monophosphate [cAMP]–dependent kinase), palmitoylated integral membrane protein with a single transmembrane helix, a small external segment, and a relatively large cytoplasmic domain.[403,405-408] It is present in all tissues and is found throughout evolution. As noted earlier, it is located, at least in part, in membrane lipid rafts.[99,100] Stomatin is a member of a protein superfamily—the PID superfamily, which stands for proliferation ion and death. Red cells contain other members of this family, such as SLP-2[409] and STORP,[410] but nothing is known about their functions or interactions.

Stomatin normally binds to the major glucose transporter, Glut1 (or SLC2A1), which also resides in lipid rafts,[411,412] and to band 3 and AQP1.[392] The site of binding on band 3 is unknown. In human red cells it switches Glut1 to an L-dehydroascorbic acid transporter (see following section).[413] It may also interact with peroxiredoxin-2 (see later section),[414] which itself attaches to band 3 (see Fig. 15-6).[183] Stomatin is interesting because it is diminished by 95% to 98% in the classic type of hereditary stomatocytosis, a severe hemolytic anemia characterized by red cell membranes that are extremely leaky to Na^+ and K^+ ions. The protein and its relationship to the disease are discussed in "Overhydrated Hereditary Stomatocytosis" in Chapter 16.

Glucose Transporter 1

Glucose transporter 1 (Glut1, SLC2A1)[415,416] is a 492-amino acid integral membrane glycoprotein that runs as a diffuse 45- to 65-kD band on SDS gels owing to heterogeneous glycosylation. It is one of the most abundant proteins in the red cell membrane, with more than 200,000 copies per cell arrayed as homodimers and homotetramers.[416a] The protein has a typical transport channel structure with 12 membrane spanning domains and a large hydrophilic cytoplasmic loop in the center.[417] In humans, Glut1 transports dehydroascorbic acid (DHA, the oxidized form of vitamin C) as well as glucose.[418,418a] Remarkably, Glut1 is only expressed in the mature red cells of a small subset of mammals that have lost the ability to synthesize ascorbic acid from glucose, including humans, some of the other higher primates, guinea pigs, and fruit bats.[413] In other mammals, Glut1 is expressed in fetal red cells but is lost soon after birth.[419] In mice, Glut4 synthesis is upregulated as Glut1 disappears and presumably takes over the job of supplying the glucose needed to fuel the red cell. The change from transport of glucose to transport of DHA in humans is tied to the binding of stomatin to Glut1.[413] Under physiologic conditions adequate glucose is transported because the concentration of glucose in the plasma (~5 mM) greatly exceeds the concentration of DHA (<2 μM). This may not be so in the leakiest hereditary stomatocytes where the cells need prodigious amounts of ATP to pump out a torrent of Na^+ and water and survive (see Chapter 16).

Glut1 also interacts with dematin and adducin (see Fig. 15-6)[420] and perhaps band 3 (unpublished comment[421]). These interactions contribute to connecting the membrane skeleton to the lipid bilayer. It will be interesting to see whether they also regulate the function of Glut1.

MEMBRANE SKELETON PROTEINS

Spectrin

Erythroid spectrin is the major constituent of the red cell skeleton, accounting for 50% to 75% of the skeletal mass.[166,167] Its discovery by Marchesi and Steers in 1968 launched the modern era of investigation of the plasma membrane.[422] The spectrin-based skeleton is anchored to the plasma membrane by an ankyrin-mediated linkage to band 3 and its associated proteins at one end of spectrin[423] and by a protein 4.1R–mediated attachment to band 3 and glycophorin C and other proteins in another multiprotein complex at the opposite end of spectrin (see Fig. 15-6). Spectrin is composed of two large polypeptide chains: a 280-kD α chain and the 246-kD β chain that are structurally similar but functionally distinct (Fig. 15-13, *A*). The two chains associate side by side in an opposing (antiparallel) orientation[424] and are twisted about each other in a right-handed double helix.[425]

The Spectrin Family in Humans is Encoded by at Least Seven Distinct Genes. Two genes encoding for α spectrin and five genes encoding for β spectrin have been characterized.[426] The αI-spectrin gene *(SPTA)* is located on chromosome 1q22-q23 adjacent to the Duffy blood group and encodes the erythroid-spectrin α chain.[427] The αII-spectrin (also called α-fodrin) gene *(SPTAN1)* encodes an α-spectrin homologue found in nearly all cells except mature red cells.[428] The βI-spectrin gene *(SPTB)* is located on chromosome 14q23-q24.2 and encodes the β subunit of erythroid spectrin,[429] designated βI, isoform 1 (or βIΣ1). The βI gene promoter region contains specific GATA1 and CACCC-related protein binding sites that account for specific, high-level expression in erythroid cells.[430] Differential 3′-processing of the βI-spectrin pre-mRNA generates an alternative, longer βI-spectrin subunit (βI, isoform 2 or βIΣ2) that is found in brain and muscle.[431] The βII-spectrin (also called β-fodrin) gene *(SPTBN1)* encodes the generally expressed β subunit of spectrin, which shares 60% amino acid identity with βI spectrin.[432] Thus erythrocyte spectrin is composed of αI and βI (isoform 1) chains and the widely expressed spectrin (called fodrin, tissue spectrin or brain spectrin) contains αII and βII subunits. Muscle spectrin is believed to contain predominantly αII and βI (isoform 2) subunits.

βIII and βIV spectrins are predominantly expressed in the central nervous system. Gene mutations cause neurologic diseases.[433-435] A truncated isoform of βIV spectrin is associated with a subnuclear structure (promyelocytic leukemia bodies) and may be part of a nuclear scaffold.[436] βV spectrin is mostly in retinal rods and cones and in gastric epithelial cells.[437] Other spectrin-related proteins—dystrophin, utrophin, actinins, plakins, and dystonin among others—are not relevant to red blood cells.

Spectrin Repeats

Spectrins Consist Mostly of Tandem 106-Amino Acid Repeats. Each spectrin chain is composed of a series of approximately 106-amino acid repeats. Each repeat is formed by three α-helices that are folded into a triple helical bundle.[438-440] The α and β spectrins contain 20 and 16 full repeats, respectively.[427,429] An SH3 domain within the α chain is conventionally called repeat α10, although it is not a true spectrin repeat (see Fig. 15-13, *A*). As will be discussed, the α0 and β17 repeats are complementary partial repeats that join to form a full repeat and link spectrins together. The primary sequence of each repeat

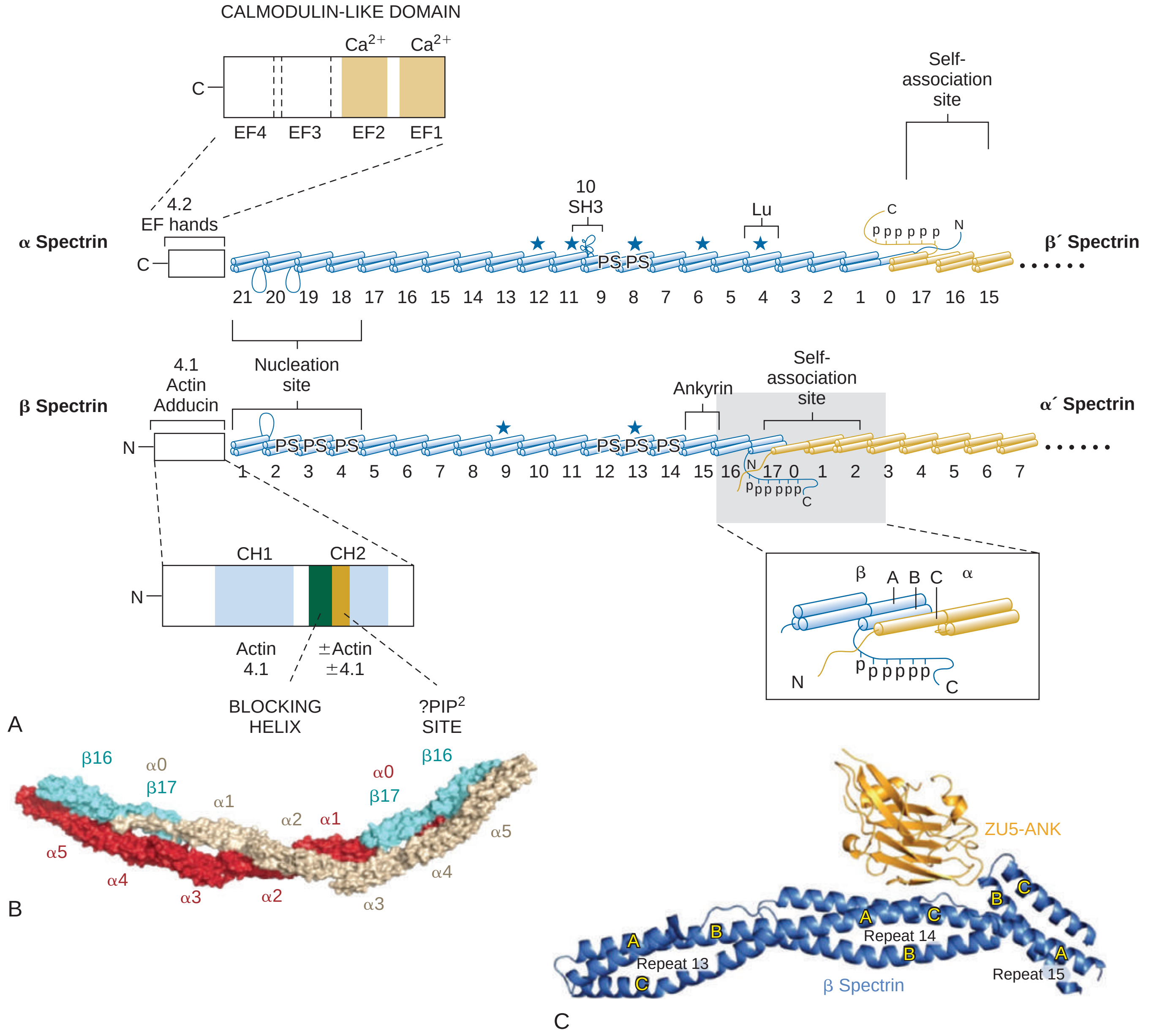

CALMODULIN-LIKE DOMAIN
Ca2+
Ca2+
C
EF4
EF3
EF2
EF1
4.2
EF hands
α Spectrin
C
10
SH3
Lu
Self-association site
C
p p p p p p
N
β´ Spectrin
PS PS
21 20 19 18 17 16 15 14 13 12 11 9 8 7 6 5 4 3 2 1 0 17 16 15
4.1
Actin
Adducin
Nucleation site
Ankyrin
Self-association site
β Spectrin
α´ Spectrin
N
PS PS PS
PS PS PS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 0 1 2 3 4 5 6 7
N
p p p p p p
C
CH1
CH2
N
Actin
4.1
±Actin
±4.1
BLOCKING HELIX
?PIP2 SITE
β
A B C
α
N
p p p p p p
C
A
β16
α0
β17
α1
α2
α1
α0
β17
β16
α5
α4
α3
α2
α5
α4
α3
B
ZU5-ANK
A
B
C
Repeat 13
A
C
B
Repeat 14
B
C
A
Repeat 15
β Spectrin
C

Figure 15-13 Spectrin structure. **A,** Overall organization. The α and β chains are aligned antiparallel with respect to their N- and C-terminal ends. The approximately 106-amino acid "spectrin repeats" are numbered from the N-terminal of each chain. Each repeat is composed of three α helices (A, B, and C). The SH3 domain, which extrudes from between helix B and helix C of repeat 9, is conventionally numbered as a repeat 10, although it is not a spectrin repeat. Similarly, by convention the partial spectrin repeat that begins the α-spectrin chain is called α0, whereas the partial spectrin repeat at the end of the β-spectrin chain is called β17. Note that the spectrin self-association site is formed by joining these two complementary partial repeats (α0 and β17) at the "head" ends of the two chains *(inset)*. The α chain contributes helix C and the β chain helices A and B. Some repeats are specialized, such as β14-β15, which forms the ankyrin-binding site; α4, where the Lu blood group glycoprotein attaches; the several repeats marked "PS," which bind phosphatidylserine; and parts of the first four repeats at the "tail" of each chain, which nucleate interchain, heterodimer interactions. Repeats marked with a *blue star* are relatively unstable at physiologic temperatures and may unfold when spectrin is deformed, increasing flexibility. *Rectangles* denote peptide segments that differ from the repeats and the SH3 domain. They include the actin/protein 4.1/adducin-binding sites and a calmodulin-like Ca^{2+}-binding region (EF hands) that also binds protein 4.2. The structures of these two domains are shown in more detail in the *insets*. **B,** Space-filling model of the spectrin heterotetramer at the self-association site. One α chain is *red*, the other is *tan*. The β chains are *light blue*. **C,** Spectrin-ankyrin interaction. Note how ZU5-ANK, the spectrin-binding subdomain within ankyrin, binds in the notch created by the sharp angle between spectrin repeats β14 and β15. The primary contacts are with helix C of repeat 14 and the loop between helices B and C of repeat 15. *(B, from Li D, Harper SL, Tang HY, et al: A comprehensive model of the spectrin divalent tetramer binding region deduced using homology modeling and chemical cross-linking of a mini-spectrin.* J Biol Chem *285:29535–29545, 2010; C, from Ipsaro JJ, Mondragón A: Structural basis for spectrin recognition by ankyrin.* Blood *115:4093–4101, 2010.)*

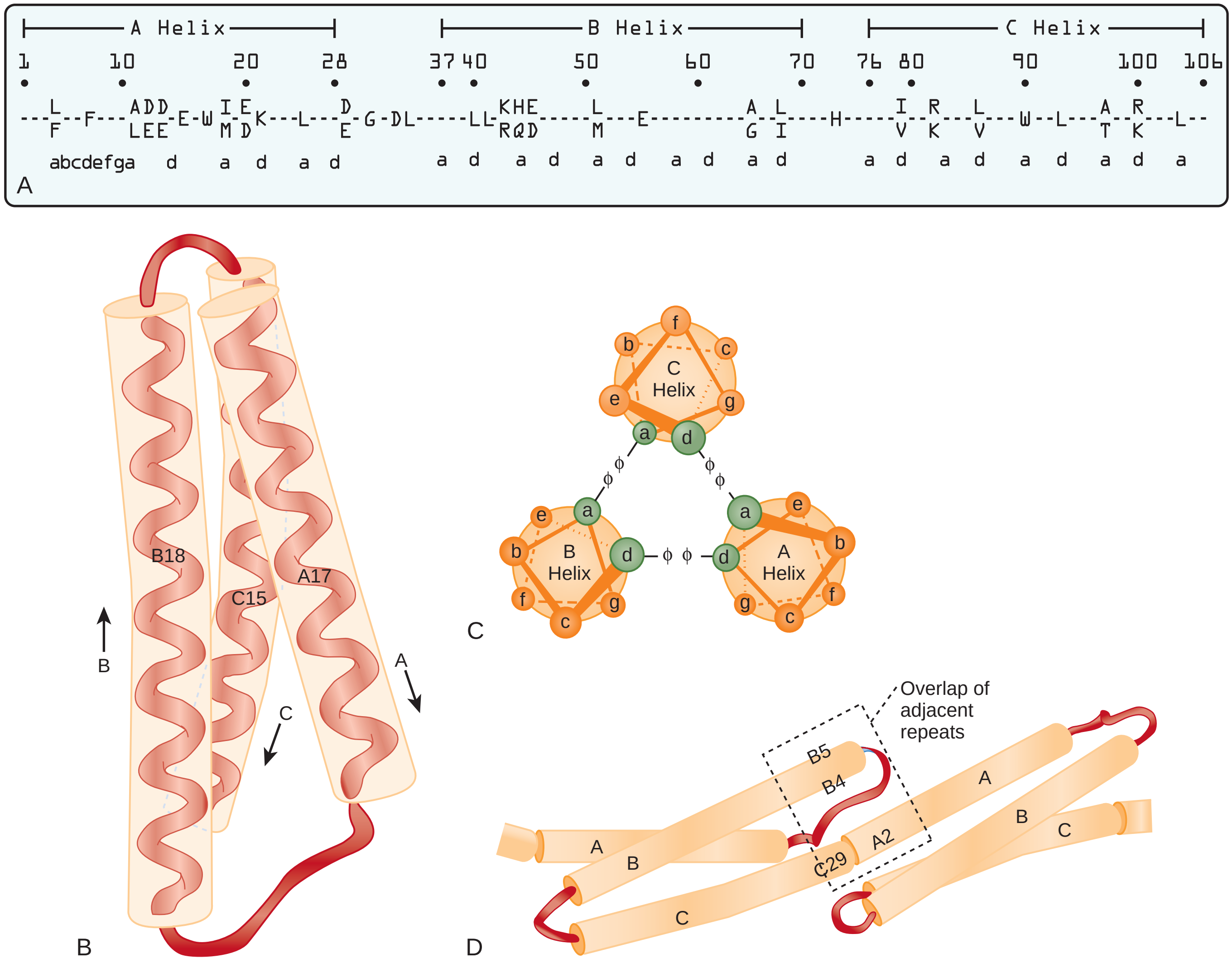


Figure 15-14 Structure of spectrin repeats. **A,** Consensus sequence of a typical 106-amino acid, human erythrocyte spectrin repeat phased according to crystallographic data, which identifies three α helices per repeat. Less-conserved amino acids are marked with a *dash*. The amino acids in the repeat are designated “a” through “g.” The distance from one “a” to the next corresponds to two turns of the α helix. Residues “a” and “d” are the major contact points between helices. They tend to be hydrophobic and are conserved in most spectrins. **B,** Model of a single typical repeat. The positions of the nearly invariant tryptophans at A17 (Trp at amino acid 17 of the repeat) and at C15 (Trp at position 90) are shown. They interact with surrounding residues, including B18 (repeat amino acid 54), at the central junction where the A helix crosses over from B to C. **C,** Cross-section of a typical repeat. Note that the A, B, and C helices are in a triangular array and that the “a” and “d” residues lie on one face of each helix. The side chains of these amino acids are usually hydrophobic (Φ) and interact with each other to stabilize the triple helical configuration. Salt bonds between the typically polar amino acids at positions “e” and “g” also help attach the BC and AC helices to each other. **D,** Interconnection of two adjacent repeats. The A and C helices are colinear, forming one long helix. The distal repeat of each pair is rotated 60 degrees (right-handed). Note that the B helix of the proximal repeat overlaps the A helix of the following repeat. Interactions in the overlap region among conserved hydrophobic residues, such as C29, B4, and B5 (repeat amino acids 104, 40, and 41, respectively), and the mostly hydrophobic residues at A2 (repeat amino acid 2) probably stabilize the connection and limit mobility at the repeat junction.

exhibits extensive heptad (seven-residue) symmetry, with conserved hydrophobic residues situated in the first and fourth positions of each heptad motif (designated as positions *a* and *d* in Fig. 15-14, *A*).[438,440] Each triple helical spectrin repeat is approximately 5 nm long and 2 nm wide (see Fig. 15-14, *B*) and is rotated 60 degrees (right-handed) relative to the neighboring repeats.[440] Details of the structure are presented in Figure 15-14 and in catalogs of spectrin repeat structures.[441]

Although spectrin is often depicted as a highly flexible molecule, the repeats themselves appear rigid and the crystal structures of multiple repeats do not show significant flexibility at the junction between repeats (see Fig. 15-14, *D*). However, the stability of individual repeats varies and some are unstable at physiologic temperatures, which would introduce flexibility.[442] Two of the less stably folded repeats, α13-α14 and β8-β9, are adjacent to each other in the spectrin molecule (see Fig. 15-13, *A*) and may form a hinge.[443,444] Measurements of the force required to unfold a spectrin repeat show that the repeats function as elastic springs.[445] When intact red cells deform, certain spectrin repeats partially unfold, which presumably contributes to the elasticity.[446]

Spectrin Heterodimerization

Side-to-Side Interactions Between α and β Chains Results in Zipperlike Dimerization. Nucleation sites located at repeats 18 to 21 of the α chain and 1 to 4 of the β chain are implicated in triggering the zipping up of α and β chains (see Fig. 15-13, *A*).[447] The key interactions are between the A and B helices of β spectrin repeat 1 and the C and A helices of α spectrin repeat 21 and between the C and A helices of β2 and the A and B helices of α20.[448] These helices form complementary charged surfaces that draw the two chains together.[449] After the initial tight association of these nucleation sites, a conformational change is propagated, causing the remainder of the α and β chains to pair together.[450] The two chains twist around each other with a periodicity of a complete turn approximately every four[425] to eight[450] repeats. The side-to-side interactions beyond the nucleation site are relatively weak,[451] which may be important in allowing the α and β chains to slide past each other when the spectrin molecule flexes in the plane of the membrane. A common polymorphism of the α-spectrin allele, α^{LELY}, encodes α chains that lack one of the nucleation sites and therefore do not form stable heterodimers.[452,453] This clinically relevant allele is discussed in under "Hereditary Elliptocytosis" in Chapter 16.

Spectrin Self-Association

Spectrin Tetramerization Occurs in Head-to-Head Fashion by Association of the Partial Repeats at the Ends of α- and β Spectrin and Side-to-Side Association of the First Three α-Spectrin Repeats.[454,455] The "self-association" sequences required for tetramerization are located at the N-terminus of the α-spectrin chain and the C-terminus of the β-spectrin chain. This is called the "head" end of spectrin and is opposite the "tail" end where binding to actin occurs and where the nucleation site is found (see Fig. 15-13, *A*). The specific structure of the spectrin self-association site has been extensively analyzed.[450,456,457] The free C helix at the N-terminus of α spectrin (α0) associates with the free A and B helices at the C-terminus of β spectrin (β17) to form a stable triple helical bundle repeat (α0/β17). In this manner, spectrin heterodimers associate to form tetramers and higher-order oligomers in head-to-head fashion (see Fig. 15-13, *A*).

In isolated spectrin heterodimers the partial repeats at the ends of the α and β chains are bound to each other. The longer α chain folds back on itself so that the noncovalent α0/β17 repeat lines up and interacts with repeat α4 and so that repeats α3 and α1 interact with each other. In the heterotetramers, there are three such side-to-side interactions between repeats in the two α-spectrins (α and α′): α1-α′3, α2-α′2, and α3-α′1 (see Fig. 15-13, *B*). These additional lateral interactions, although weak, contribute to self-association.[450,457] For dimers to associate into tetramers or vice versa, the bonds involved in self-association must first open. This is the rate-limiting step in dimer-to-tetramer interconversion.[458]

The formation of spectrin tetramers and higher-order oligomers is critical to maintaining the mechanical strength of the membrane skeleton.[459] Mutations in α or β spectrin that interfere with self-association[456] weaken the membrane skeleton. Such defects are by far the most common cause of hereditary elliptocytosis and hereditary pyropoikilocytosis (see Chapter 16).

Spectrin Exists Predominantly as Tetramers in Vivo.[460] Physiologic ionic strength and lower temperatures (25°C) favor tetramer formation; low ionic strength and physiologic temperatures favor dissociation to dimers.[459,461] The self-association of spectrin can be studied in purified spectrin and by manipulating red cell ghosts to alter the degree of spectrin oligomerization.[454,459,461,462] In red cell ghosts, driving the equilibrium toward dimerization produces exceedingly fragile structures, underscoring the importance of spectrin tetramerization in membrane skeletal stability.[459] At 4°C, the dimer-tetramer equilibrium is nearly kinetically frozen owing to its high activation energy.[454] Thus spectrin eluted from ghosts at that temperature retains the degree of oligomerization that existed in vivo. In such eluates, approximately 5% of the spectrin is heterodimers, approximately 50% exists as heterotetramers, and the remainder are divided among higher-order spectrin oligomers and high-molecular-weight complexes of spectrin, actin, protein 4.1R, and dematin.[460]

Spectrin Structure In Situ

Spectrin Molecules are Condensed in Vivo. Electron micrographs of purified spectrin tetramers[455,463] or spectrin filaments in fully stretched skeletons[464] show the molecule has an end-to-end length of 200 nm. But, as discussed later, simple calculations show that spectrin tetramers can only have a length of 62 to 72 nm in vivo. This correlates with the observed length of spectrin filaments in unstretched and partially stretched skeletons.[425] It is not known exactly how spectrin is folded in this physiologic filament. The best morphologic evidence is that the α and β subunits form a double helix and are twisted about each other along a relatively straight common axis (Fig. 15-15).[425] Spectrin molecules extend and contract along this axis by varying the pitch and diameter of the double strand. This springlike behavior serves as a "molecular shock absorber"[465] and is presumably critical to the elastic behavior of the membrane. Native spectrin tetramer has approximately 10 turns with a pitch of 7 nm and a diameter of 5.9 nm.[425] This corresponds to 1 turn every four spectrin repeats. Structural analyses of the spectrin self-association site also show that the chains coil around each other, but with a periodicity of about 1 turn per eight repeats (see Fig. 15-13, *B*).[450] Other structural models suggest there is some bending between spectrin repeats or that helices within some repeats may "melt" and rearrange, shortening the repeat.[466] Whatever the mechanism, it is clear that the ends of a spectrin molecule are normally only about one-third as far apart as they appear in stretched skeletons.

Spectrin Interactions

Spectrin Contains Important Functional Domains Involved in Protein-Protein Interactions. In addition to

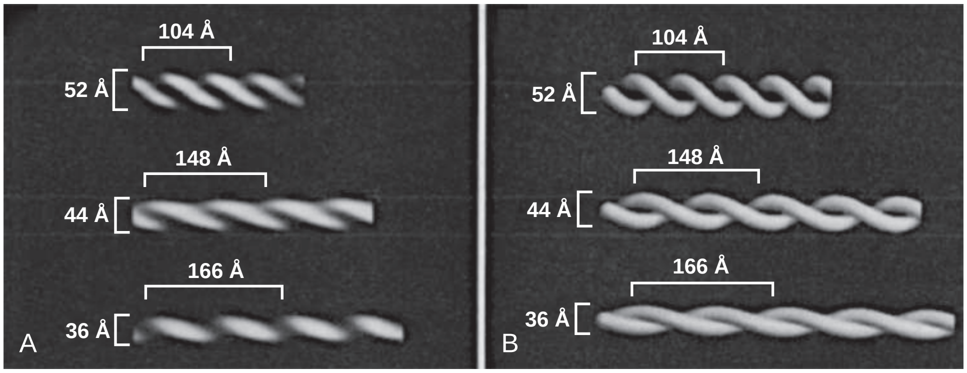


Figure 15-15 Model of spectrin structure in situ on the membrane. **A,** Enhanced images of spectrin molecules captured from negatively stained electron micrographs of variably expanded membrane skeletons. In general, spectrin molecules were relatively straight but of variable lengths and on image enhancement showed a coiled appearance. **B,** Right-handed double helical model derived from the images in **A**. The pitch of the helix varies from 10.4 nm (1 nm = 10Å) to 16.6 nm in the images shown, and the diameter varies from 5.2 nm to 3.6 nm. Like a spring, as spectrin extends, the pitch of the helix lengthens and the diameter narrows. The average pitch and diameter in unstretched skeletons are calculated to be 7 nm and 5.9 nm, respectively. This corresponds to about five turns per spectrin dimer and four repeats per turn for a length of 35 nm per spectrin dimer. This is far less than the length of full extended spectrin dimers (100 nm) and almost exactly to the length of spectrin dimers from calculations based on the number of actin junctions (40,000 in a red cell surface of 140 μm^2) and from direct measurements in unstretched skeletons. *(From McGough AM, Josephs R: On the structure of erythrocyte spectrin in partially expanded membrane skeletons.* Proc Natl Acad Sci U S A *87:5208-5212, 1990.)*

the critical sequences involved in spectrin nucleation and self-association, the α and β chains interface with other proteins, including ankyrin, proteins 4.1R and 4.2, actin, adducin, and dematin, via defined structural domains (see Fig. 15-13, *A*).

Ankyrin-Binding Site. Repeats 14 and 15 of β spectrin are crucial for ankyrin binding, especially the C helix of repeat β14 and the loop between helices B and C of repeat β15.[467-469] Repeat β15 forms an unusual angle with repeat β14, and the spectrin-binding region of ankyrin (called ZU5A or ZU5-ANK, discussed under "Ankyrin") nestles into the corner formed by the two repeats (see Fig. 15-13, *C*).[469] The attachment of β spectrin to ankyrin and ankyrin to band 3 and some of its associated proteins (see Fig. 15-6) creates spectrin's strongest link to the overlying lipid bilayer. In nonerythroid cells, ankyrin homologues mediate the linkage of spectrin to many other proteins of the plasma membrane, including Na$^+$,K$^+$-ATPase[470] and voltage-dependent sodium channels.[471]

Binding Sites for F-Actin, Protein 4.1R, Adducin, and Dematin. The N-terminal 301 amino acid of β spectrin binds protein 4.1R[472,473] and actin[463,473,474] and shares sequence homology with several actin-binding proteins, including calponin, α actinin, dystrophin, filamin, ABP 120, adducin, and fimbrin. There are two of these "calponin homology domains" (CH1 and CH2) in tandem in β spectrin. It appears that protein 4.1R and actin bind to each of these domains.[473] Binding to CH2, the more C-terminal CH domain, is normally inhibited by an α helix at its N-terminus, which may serve a regulatory role (see Fig. 15-13, *A*). PIP$_2$ increases attachment of protein 4.1R to the CH domains.[473]

Adducin binds to the N-terminal actin-binding domain and the first two repeats of β spectrin.[475] Because mutations within the last few repeats of α spectrin markedly reduce membrane adducin content,[476] the C-terminus of α spectrin probably also contributes to adducin binding.

Dematin, but not phosphorylated dematin, binds somewhere in the tail region of spectrin and facilitates binding of spectrin to F-actin. The exact binding site is unknown.[477]

The tail ends of spectrin tetramers and oligomers bind to short, double-helical protofilaments of F-actin. The interaction is greatly enhanced by protein 4.1R.[478-480] Approximately six spectrins can be accommodated on each actin protofilament, leading to the characteristic quasi-hexagonal organization of the membrane skeleton (Fig. 15-16). The spectrin/actin/protein 4.1R junctional complexes contain other proteins, including adducin, dematin, tropomyosin, tropomodulin, and p55. These are discussed in the sections that follow.

EF Hands: Ca^{2+}- and Protein 4.2–Binding Sites and Role in Spectrin-Actin Binding. The C-terminus of α spectrin, like α actinin, contains two pairs of EF hands (see Fig. 15-13), which are structures that bind and mediate the regulatory effects of calcium.[481,482] The N-terminal EF hands (EF$_{1,2}$) are structurally similar to calmodulin[483] and bind Ca^{2+} with low affinity. Ca^{2+} binding to EF$_1$ triggers a conformational change that allows EF$_2$ to bind Ca^{2+}.[484] The C-terminal EF hands (EF$_{3,4}$) are Ca^{2+} insensitive and similar to those in α actinin.[485]

The EF hand domain binds protein 4.2 with high affinity (kD = 3 ×10^{-7} M).[486] The binding capacity is augmented by micromolar concentrations of Ca^{2+}, and the Ca^{2+} effect is blocked by calmodulin, which also binds to the domain. Because protein 4.2 binds to band 3, this adds to the evidence that a portion of band 3 is located near the spectrin-actin junctions and serves as a secondary site for attaching the membrane skeleton to the lipid bilayer. It is not known whether protein 4.2 actually binds to the EF hands in vivo and, if so, what fraction of the protein is located there and what fraction is located in the ankyrin complex (see Fig. 15-6).

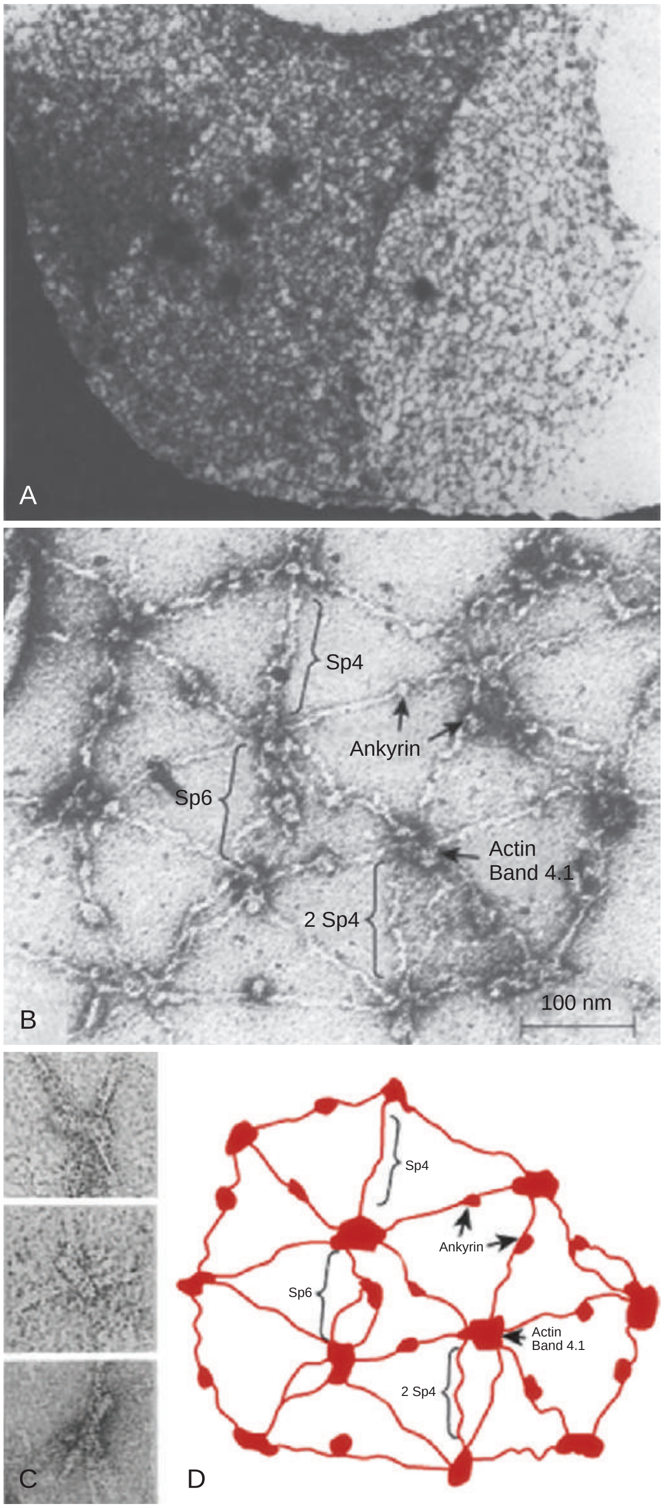


Figure 15-16 Negatively stained electron micrographs of red cell membrane skeletons that have been stretched during preparation to reveal details of their structure. **A,** Low magnification to emphasize the ordered, netlike structure. **B,** High-magnification view and schematic showing the predominantly hexagonal organization of the skeleton. **C,** Detail of the 37-nm long F-actin protofilaments. **D,** Location of various skeletal elements is shown in **B.** *Sp4,* spectrin tetramer; *Sp6,* spectrin hexamer. *(From Palek J, Sahr KE: Mutations of the red blood cell membrane proteins: from clinical evaluation to detection of the underlying genetic defect.* Blood *80:308, 1992; and Byers T, Branton D: Visualization of the protein associations in the erythrocyte membrane skeleton.* Proc Natl Acad Sci U S A *82:6153, 1985.)*

The relationships of the EF hand domain, the calponin homology domain, and F-actin are not known in spectrin but are likely to resemble those in α actinin (Fig. 15-17).[487,488,489] That is, the EF hands probably abut the actin/protein 4.1R–binding CH domains and may help regulate actin binding. Previously the EF hands were believed to be impotent in erythroid spectrin, although they were known to bind Ca^{2+} and affect the function of αIIβII spectrin (fodrin).[481] However, deletion of just the C-terminal 13 amino acids in the EF domain of the sph^{1J} mouse leads to a severe, spherocytic hemolytic anemia and failure of the mutant spectrin to assemble into the membrane skeleton.[490] Also, PfEMP3, a protein from *P. falciparum* that appears on the red cell membrane late in the parasite life cycle, binds to α spectrin in the EF hand domain region and disrupts the spectrin/actin/protein 4.1R complex, perhaps as a precursor to release of new parasites into the circulation.[488] These observations show that the EF hand domain is functionally important, and direct evidence supports this conclusion. A minispectrin composed of the actin binding and EF hand domains and the four adjacent spectrin repeats in each chain that form the nucleation site binds to F-actin with the aid of protein 4.1R, as expected, but this binding is blocked if the EF hands are missing, or even if just the last 13 amino acids of α spectrin are deleted, as in the sph^{1J} mouse.[489]

SH3 Domain. The tenth "repeat" of α spectrin encodes an *src* homology (SH3) domain. SH3 domains generally function as sites of protein-protein attachment at cell membranes. The function of the erythroid α-spectrin SH3 domain and the protein(s) that bind to it in the red cell are not known. One candidate belongs to a

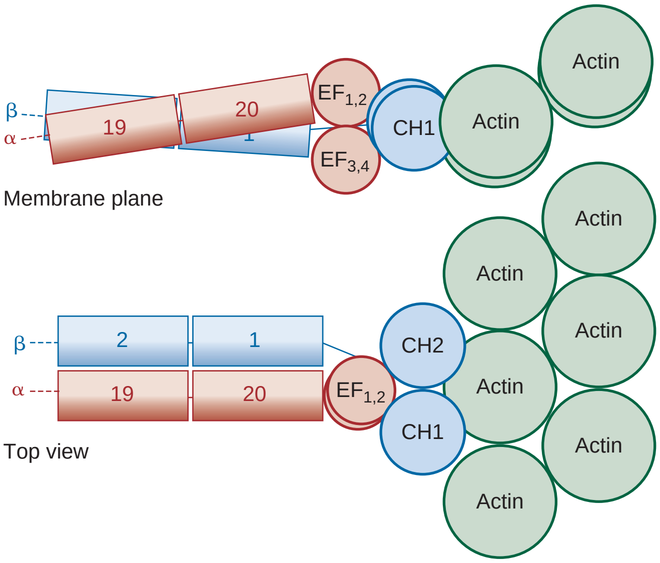


Figure 15-17 Structural model, roughly to scale, of the tail end of spectrin deduced from the structure of α actinin.[487] *Top,* View from within the plane of the membrane skeleton. *Bottom,* View looking down on the membrane. CH1 and CH2 are the actin- and protein 4.1R–binding sites in the N-terminal, actin-binding domain of β spectrin. $EF_{1,2}$ and $EF_{3,4}$ are the N-terminal and C-terminal pairs of EF hands, respectively, in the EF domain, which lies at the C-terminal end of α spectrin. The intimate relationship between the EF and CH domains and recent evidence that the EF domain is required for optimal spectrin-actin binding[488,489] suggest the EF hands may regulate actin binding.

family of tyrosine kinase–binding proteins.[491] Interestingly, plasmepsin II, the aspartic protease of *P. falciparum,* cleaves spectrin mainly within the SH3 domain of α spectrin, suggesting that specific targeting of the SH3 domain by *P. falciparum* may be part of the strategy to dismantle the membrane skeleton.[492]

PH Domain. All β spectrins *except* the major erythroid isoform (βI, isoform 1) have a C-terminal extension that contains a pleckstrin homology (PH) domain.[493,494] The specialized 100-residue PH domain is believed to mediate membrane targeting of proteins.[494,495] The muscle isoform of erythroid spectrin (βI, isoform 2) contains a PH domain,[431] and mature red cells contain a small amount of this isoform, which is clumped in submicron-size patches within the membrane skeleton.[496] The erythroid spectrin PH domain binds with equal affinity to PIP_2 and phosphatidylinositol-3,4,5-trisphosphate (PIP_3).[497,498] The function of the PH domain in red cells is untested. Perhaps it contributes to the attachment of spectrin to the membrane.

Phospholipid Binding. In addition to the PH domain, which binds phosphatidylinositols (PI), other parts of spectrin also bind phospholipids. Several spectrin repeats in each chain bind phosphatidylserine (PS)[89] (Fig. 15-13, *A*). The PS binding sites on β spectrin are located near the site where ankyrin and the junctional complex bind, leading to speculation that PS influences these interactions.[89] Whatever the mechanism, the PS-spectrin interaction increases membrane stability[90] and protects spectrin from glycation.[91]

The β14-β15 spectrin repeats, which form the ankyrin binding site, also bind phosphatidylcholine (PC) and phosphatidylethanolamine (PE).[498a] Spectrin-PE/PC binding is inhibited by ankyrin.[498a] The importance of this is unclear, though it may partly explain how a normal membrane skeleton can form in red cells lacking ankyrin[498b] or band 3.[220] The topic of spectrin-lipid binding was recently reviewed.[498c]

Phosphorylation Sites. Instead of a PH domain, the C-terminal region of erythrocyte (βI, isoform 1) spectrin contains a 52-amino acid extension just beyond the site of self-association that is phosphorylated by a membrane-associated casein kinase-I (see Fig. 15-13).[499] There are five serines and one threonine that are phosphorylated in a sequential fashion.[500] In vitro phosphorylation of β spectrin has no effect on spectrin self-association.[454,501,502] However, by some unknown mechanism, increased phosphorylation of β spectrin in vivo decreases membrane mechanical stability whereas decreased phosphorylation strengthens the membrane.[503]

Ubiquitination Sites. α-Spectrin is ubiquitinated at several sites in the C-terminal region.[504,505] The consequences are uncertain. There is evidence from one group that spectrin contains ubiquitin-conjugating (E2) and ligase (E3) activities,[505] that ankyrin is also ubiqitinatinated,[506] and that ubiquitination favors loss of spectrin from the actin junctional compex,[507] but these observations have not yet been confirmed. The fraction of spectrin molecules that is ubiquitinated in vivo is not clear, and the effect of ubiquitination on the mechanical properties of the intact red cell is not known.

Tryptic Domains. The tryptic domains of spectrin have important historical, structural, and clinical significance. Gentle proteolysis of spectrin with trypsin generates nine structural domains, each composed of several 106-amino acid repeats.[424,508] Five tryptic fragments represent most of the α chain and include the αI (80-kD), αII (46-kD), αIII (52-kD), αIV (52-kD), and αV (41-kD) domains; the β chain is digested into four domains, including βI (17-kD), βII (65-kD), βIII (33-kD), and βIV (74-kD). Many of the molecular defects in hereditary elliptocytosis were initially identified as abnormalities in spectrin domain maps after proteolytic digestion (see “Hereditary Elliptocytosis” in Chapter 16), and many older articles refer to these domains.

Mutations in Erythroid Spectrin Affect the Integrity of the Red Cell and Lead to Clinical Disease

Defects that impair spectrin self-association are the major cause of hereditary elliptocytosis, and spectrin deficiency is an important cause of hereditary spherocytosis (see “Hereditary Elliptocytosis” and “Hereditary Spherocytosis” in Chapter 16).

Actin

The red cell contains β actin, which is the actin subtype found in a wide variety of nonmuscle cells.[134] Whereas red cell actin shares structural and functional similarities with the β actin found in nonerythroid cells, in the membrane skeleton actin is assembled into 30,000 to 40,000 regular *protofilaments* or short, 33- to 37-nm, double-helical F-actin filaments, each containing about 14 monomers.[134,509,510] In contrast to nonerythroid cells, where actin filaments have variable lengths and turnover rapidly, red cell actin protofilaments are a constant size and are extraordinarily stable, persisting for the life of the red cell. They are stabilized by (1) interactions with spectrin, protein 4.1R, and tropomyosin; (2) capping of the pointed or slow-growing end by tropomodulin[511]; and (3) capping of the barbed or fast-growing end by adducin.[512] This is another unique feature of the actin filaments in red cells because capZ is the ubiquitous barbed-end capping protein in nonerythroid cells.[513] Interestingly, this occurs despite the fact that red cells contain capZ.[513] When α or β adducin is deleted in red cells, capZ expression is greatly upregulated, presumably as a compensatory mechanism.[223,514]

The actin protofilaments function as junctional centers connected by spectrin in a more or less hexagonal array (see Fig. 15-16).[464,510] The protofilaments lie parallel to the lipid bilayer within 20 degrees of the membrane plane.[515] Spectrin dimers bind to the side of actin filaments at a site near the tail of the spectrin molecule (see Figs. 15-13 and 15-17).[463] The contact sites of spectrin on the actin protofilaments are not known, although one can make inferences from the way other spectrin-related proteins bind F-actin (see Fig. 15-17). Spectrin tetramers are bivalent and therefore cross-link actin filaments, although binding is weak (kD ~ 10^{-3} M) and ineffectual in the absence of protein 4.1R.[479] Spectrin-actin interactions are specifically enhanced by protein 4.1R, adducin, and dematin (see “Protein 4.1R,” “Adducin,” and “Dematin”).

Defects in Actin Assembly. Not much is known about how the unique actin protofilaments of red cells are assembled, but some hints are emerging from defects in actin assembly. Red cells contain multiple members of the Rho family of guanosine triphosphatases (GTPases), including Rac1, Rac2, Cdc42, and RhoA, RhoG, and RhoH.[516] Rac GTPases are activated by extracellular signals via guanine nucleotide exchange factors, which replace guanosine diphosphate (GDP) with GTP on Rac. GTP-activated Rac then interacts with P21-activated kinase, a diaphanous homologue (DIAPH3), and an assembly of proteins known as the Wiskott-Aldrich syndrome verprolin-homologous protein (WAVE) complex,[517] stimulating ARP2/3 to initiate actin polymerization in many cell types.[518] Erythrocytes probably also utilize this pathway because they contain Rac, RhoA, and all the members of the WAVE complex.[519-521]

Mice that lack both Rac1 and Rac2 develop a microcytic hemolytic anemia with marked anisocytosis and poikilocytosis.[520] Membrane skeletal junctions are aggregated, and there is pronounced irregularity of the spectrin meshwork. These changes are accompanied by decreased cellular deformability, an increase in the actin-to-spectrin ratio, and increased phosphorylation (Ser724) of adducin, an F-actin capping protein. Actin and phosphorylated adducin are more easily extracted by Triton X-100 from Rac1(−/−)/Rac2(−/−) red cells, indicating weaker association to the cytoskeleton.

Hematopoietic protein-1 (Hem-1) is a hematopoietic cell specific member of the WAVE complex. Mice lacking Hem-1 resemble the Rac1/Rac2 double knockout mice, with a moderate microcytic, hemolytic anemia, marked spherocytosis and poikilocytosis, clumped F-actin filaments, and decreased amounts of all membrane skeletal proteins.[521] They also show increased expression of Ser724 on adducin.

Osteopontin phosphorylates or activates multiple proteins in human erythroblasts, including Rac-1 GTPase and adducin. Osteopontin knockout mice are anemic and also show defects in F-actin filaments.[522]

The results indicate that Rac GTPases regulate the organization of the red cell membrane skeleton. It is unclear whether this occurs dynamically in the mature red cell or just during erythropoiesis.

Malaria. *P. falciparum* generates a host-derived actin cytoskeleton within the red cell that appears to be an extension of the normal membrane skeleton. It functions in the transport of vesicles between the parasite and the red cell membrane. This cytoskeleton is abnormal in erythrocytes containing hemoglobins S or C, two hemoglobins that protect against malaria.[523] One possible mechanism is that hemoglobin oxidation products, which are enriched in hemoglobin S and C red cells, inhibit actin polymerization in vitro.

Protein 4.1R

Red cell protein 4.1 (technically the 4.1R paralogue, gene name *EPB41*) is a major component of the erythrocyte membrane skeleton, with roughly 200,000 copies present in each red cell (see Table 15-2). Protein 4.1R confers both stability and flexibility to circulating red cells by potentiating the interaction of spectrin tetramers with F-actin[479,524] and by linking this membrane skeletal scaffold to the plasma membrane through vertical interactions with GPC, band 3, p55, protein 4.2, and perhaps other proteins (see Fig. 15-6). In the mature red cell, protein 4.1R exists as a 588-amino acid protein that has a molecular weight of 66 kD, although it runs as an 80-kD protein on SDS gels. Two forms of the protein are resolved on high-resolution SDS gels, an apparently 80-kD protein designated 4.1Ra and an apparently 78-kD protein designated 4.1Rb. Protein 4.1Ra derives from 4.1Rb by progressive deamidation of Asn502 during the life span of the red cell; thus protein 4.1Ra is more prominent in senescent red cells.[525] Proteolysis of protein 4.1R generates four structural domains: the N-terminal 30-kD membrane binding domain (MBD; also called the FERM domain[526]), a 16-kD linker domain, the 10-kD spectrin-actin–binding domain, and the 22- to 24-kD C-terminal domain (Fig. 15-18).[527]

Spectrin-Actin–Binding Domain

Protein 4.1R Significantly Enhances the Interaction of Spectrin with Actin. In physiologic solvent conditions, spectrin tetramers bind weakly to F-actin (kD ~ 10^{-3} M), whereas the addition of protein 4.1R generates a high-affinity ternary complex (kD ~ 10^{-12} M^2).[479] The 10-kD domain of 4.1R specifically facilitates spectrin-actin interactions[524] and is encoded by alternatively spliced exon 16 and constitutive exon 17.[528,529] A 21-amino acid peptide encoded by exon 16 plus seven amino acids (amino acids 37 to 43) from exon 17 form the spectrin-binding site.[528,529] Protein 4.1R binds to F-actin as well as spectrin.[528,530] The stoichiometry of protein 4.1R/actin binding is 1:1, and the interaction is highly cooperative, such that binding of one protein 4.1R molecule induces a conformational change in the actin protofilament that promotes further protein 4.1R binding.[529,530] The binding site is located within the first 26 amino acids of exon 17, particularly a critical 8-amino acid sequence (LKKNFMES).[528,531] Thus the actin site is straddled by the bipartite spectrin binding site.[528,531] The available data suggest that protein 4.1R facilitates spectrin-actin binding by bridging spectrin and actin in a strong ternary complex.

The critical role of protein 4.1R in maintaining red cell membrane stability is underscored by the erythrocyte membrane fragility and resultant severe hemolysis observed in patients who lack protein 4.1R (see "Hereditary Elliptocytosis" in Chapter 16). Interestingly, normal membrane stability is completely restored when patient red cell ghosts are reconstituted with a 64-amino acid fusion protein containing the spectrin-actin–binding site.[529] This finding highlights the critical functional role of the 10-kD domain of protein 4.1R in red cell function.

Membrane-Binding Domain or FERM Domain

The protein 4.1 paralogues are part of an interesting group of proteins known as FERM proteins.[526,532] These include the four protein 4.1 proteins, plus ezrin, radixin,

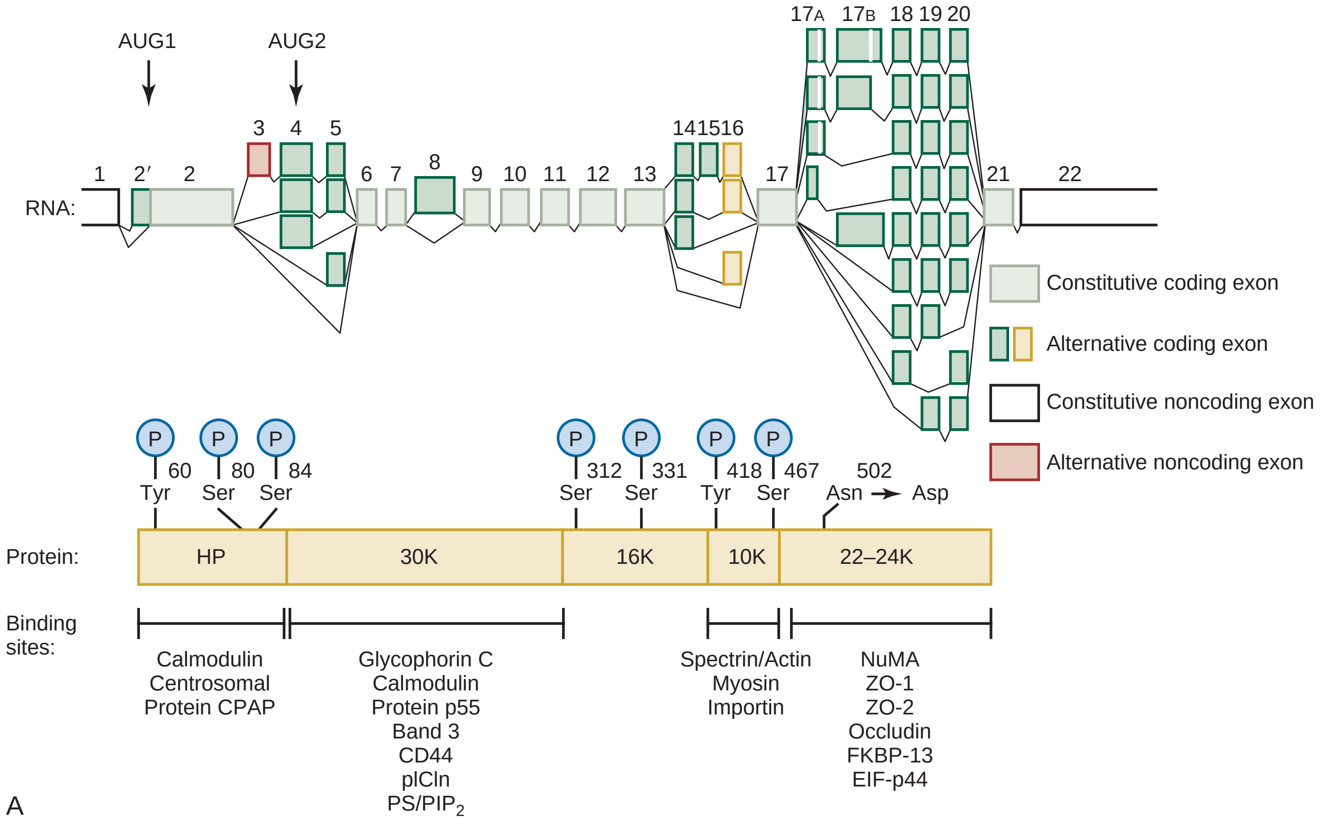
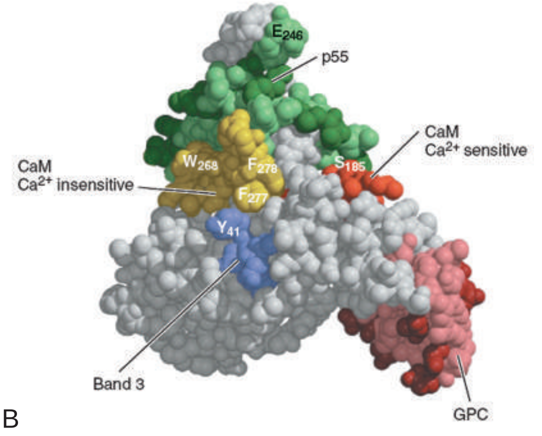

17A 17B 18 19 20
AUG1
AUG2
3 4 5
141516
1 2′ 2
6 7 8 9 10 11 12 13
17
21 22
RNA:
Constitutive coding exon
Alternative coding exon
Constitutive noncoding exon
Alternative noncoding exon
P P P P P P P
60 80 84 312 331 418 467 502
Tyr Ser Ser Ser Ser Tyr Ser Asn → Asp
Protein:
HP
30K
16K
10K
22–24K
Binding sites:
Calmodulin
Centrosomal
Protein CPAP
Glycophorin C
Calmodulin
Protein p55
Band 3
CD44
plCln
PS/PIP2
Spectrin/Actin
Myosin
Importin
NuMA
ZO-1
ZO-2
Occludin
FKBP-13
EIF-p44
A

Figure 15-18 Protein 4.1R. **A,** Alternative splicing map of protein 4.1R mRNA. Erythrocyte 4.1R is translated from AUG-2 and includes a 63-base pair (21-amino acid) erythroid-specific sequence *(orange)* that is critical for spectrin-actin interactions. The nonerythroid protein that begins at AUG-1 contains an additional headpiece *(HP)* domain. Many combinations of exons are expressed, particularly in the C-terminal domain, although many are only observed in nonerythroid tissues. *Middle,* Domain map of protein 4.1R indicating binding sites, phosphorylation sites, and the location of a C-terminal asparagine that is deamidinated in old red cells. Some of the binding partners are only expressed in nonerythroid cells. The deamidation reaction accounts for the variability in the apparent molecular weight of the C-terminal domain. **B,** Structure of the protein 4.1R membrane–binding (30 kD) domain. Various subdomains are marked where specific proteins bind. Critical amino acids in each region are numbered. *GPC,* Glycophorin C. *(Modified from Conboy JG: Structure, function, and molecular genetics of erythroid membrane skeletal protein 4.1 in normal and abnormal red blood cells.* Semin Hematol *30:58, 1993; and Han BG, Nunomura W, Takakuwa Y, et al: Protein 4.1R core domain structure and insights into regulation of cytoskeletal organization.* Nat Struct Biol *7:871, 2000.)*


E246
p55
CaM
Ca2+ sensitive
CaM
Ca2+ insensitive
W268
F278
F277
S185
Y41
Band 3
GPC
B

moesin, and others. Despite their diverse functions, the FERM proteins each utilize the conserved 30-kD MBD, or FERM domain, to facilitate interactions with cellular membranes.

Protein 4.1R Links the Membrane Skeleton to the Plasma Membrane Through Interactions with the Integral Membrane Proteins GPC and GPD (GPC/D). The majority of membrane-associated protein 4.1R is bound to the C-terminus of GPC/D via the 30-kD MBD (see Fig. 15-18).[533] There is considerable evidence that the interaction exists in vivo: (1) protein 4.1R–deficient red cells are also deficient in GPC/D[533]; (2) any residual GPC/D in protein 4.1R–deficient red cells is only loosely bound to the membrane skeleton and becomes tightly bound if the cells are reconstituted with protein 4.1R[533]; (3) stripped, inside-out membrane vesicles from normal red cells bind five times more protein 4.1R than vesicles prepared from red cells lacking GPC/D[534]; (4) treatment of membrane vesicles with antibodies that block the protein 4.1R–binding sites on GPC/D reduces the ability of protein 4.1R to promote spectrin-actin binding to the vesicles by 85%[535]; (5) GPC/D-deficient red cells, like 4.1R-deficient cells, are elliptocytic and mechanically unstable[308]; and (6) GPC sorts to incipient reticulocytes along with protein 4.1R during enucleation of normal red cells but is lost with the nuclei in red cells lacking protein 4.1R.[536] However, absence of GPC/D only causes very mild elliptocytosis and hemolysis, indicating that other interactions compensate for loss of the protein 4.1R–GPC/D link.

Protein 4.1R and GPC/D form a ternary complex with p55, a membrane-associated guanylate kinase (MAGUK) protein (see later). The binding of 4.1R with p55 increases the interaction of protein 4.1R with GPC/D by an order of magnitude.[537,538] Amino acids Tyr94 to Arg166 in the 30-kD MBD of protein 4.1R interact with positively charged residues within a segment (amino acids 82 to 98) of the cytoplasmic domain of GPC/D.[290,537,539] The residues Tyr214 to Glu246 of the protein 4.1R MBD bind to p55 at a positively charged, 39-amino acid segment found between the SH3 domain and the guanylate kinase domain[539]; p55, in turn, binds via its PDZ domain to C-terminal amino acids 112 to 128 of GPC/D.[290,539,540]

Protein 4.1R MBD Also Interacts with Other Red Cell Membrane Proteins

Band 3. Protein 4.1R binds band 3 at two positively charged motifs LRRRY and IRRRY located near the end of the band 3 cytoplasmic domain (see Fig. 15-7).[541] As noted earlier under “Band 3,” this interaction is structurally important, at least in fish.[218]

In vitro, protein 4.1R competes with ankyrin for binding to band 3.[218] This suggests that protein 4.1R and ankyrin bind to separate band 3 molecules and are in separate complexes at opposite ends of spectrin (see Fig. 15-6). There is considerable evidence for this idea,[222,421,486,542-545] but if it is true then displacement of protein 4.1R from band 3 should weaken the membrane. Instead, it increases membrane mechanical stability and decreases membrane deformability.[218] This could be explained if spectrin is folded in situ so that the ankyrin complex and the actin junctional complex are adjacent to each other and the protein 4.1R–band 3 interaction modulates the association of band 3 with ankyrin. Then removal of protein 4.1R from band 3 would increase the more potent ankyrin–band 3 interaction. Further investigation is needed.

CD44. The MBD also binds to CD44, a transmembrane glycoprotein found in erythroid and nonerythroid cells. The red cell only contains about 6,000 to 10,000 molecules of CD44. The binding sites on CD44 are positively charged motifs SRRRC and QKKKL, which are homologous to the interacting site for protein 4.1R found in band 3 (discussed earlier).[546] Analogous to band 3, protein 4.1R competes with ankyrin for CD44 binding and may thus regulate CD44-ankyrin interactions.[546]

NHE1. At least one transporter, the Na^+-H^+ exchanger (NHE1), binds to protein 4.1R via the MBD in the red cell and is inhibited on binding.[547,548] Red cells from mice lacking protein 4.1R are dehydrated and have a high intracellular Na^+ content owing to hyperactivity of NHE1.[549] Some argue that a major function of protein 4.1R is to regulate ion channels.[538,548]

Phosphorylation. Protein 4.1R is phosphorylated in vitro and in vivo by protein kinase A, protein kinase C, and a tyrosine kinase. Protein 4.1R phosphorylation by protein kinase A, which labels Ser331 in the 16-kD domain and Ser467 in the 10-kD domain, inhibits the ability of protein 4.1R to promote the spectrin-actin interaction.[115,550,551] The interaction is also inhibited by tyrosine phosphorylation at Tyr418, which is located in the 10-kD domain.[552] Protein kinase C phosphorylation of protein 4.1R blocks the interaction with band 3[553] and GPC.[550]

Calmodulin Regulation. The MBD contains Ca^{2+}-dependent and Ca^{2+}-independent calmodulin (CaM)-binding sites (see Fig. 15-18, *B*).[554] At physiologic concentrations of Ca^{2+} ($<0.1\ \mu M$), CaM only binds to the Ca^{2+}-independent site. That site stabilizes the MBD.[555] As Ca^{2+} levels rise above 1 μM, CaM binds to the Ca^{2+}-dependent site, where it reduces the affinity of protein 4.1R for band 3,[554] GPC,[537] p55,[537] NHE1,[547] and CD44.[546] The crystal structure of the MBD domain of protein 4.1R (see Fig. 15-18, *B*) localizes the various binding sites.[556]

The proximity of the calmodulin-like EF hands on α spectrin to the calponin homology domains on β spectrin, where protein 4.1R binds (see Fig. 15-17), raises the interesting possibility that the spectrin EF hands interact with either the Ca^{2+}-sensitive or Ca^{2+}-insensitive calmodulin-binding sites on protein 4.1R.

Protein 4.1R Interacts with the Plasma Membrane through Direct Associations with Phosphatidylserine and the Polyphosphoinositides. Protein 4.1R binds to PS in a two-step process in which protein 4.1R first interacts with serine head groups through the positively charged amino acids YKRS in the MBD domain and subsequently forms a tight hydrophobic interaction with fatty acid moieties.[557] Importantly, acyl chain interactions with protein 4.1R impair its ability to interact with band 3, GPC, and calmodulin.

Protein 4.1R also interacts with PIP_2 via its MBD domain.[558] PIP_2 enhances binding of protein 4.1R to the second actin-binding (calponin homology) domain of β spectrin.[473] The lipid also increases binding of protein 4.1R to GPC and inhibits its binding to band 3[558] and apo-calmodulin.[558a] Further, GPC is more readily extracted by Triton X-100 from ATP-depleted red cells, implying that the protein 4.1R–GPC interaction may be regulated by PIP_2 in situ.

C-Terminal Domain

Although the functions of the spectrin-actin–binding domain and membrane-binding domain of protein 4.1R are relatively well studied, the functions of the 16-kD domain and C-terminal domain in the red cells are poorly understood. Several human mutations in the protein 4.1R C-terminal domain are known,[559] but they reduce the amount of functional protein in the red cell rather than affect its function. As previously mentioned, the C-terminal domain is slowly deamidated at Asn502 during the red cell’s life span and is glycosidated (O-linked) with *N*-acetylglucosamine.[560] It also contains a consensus sequence for binding to FKBP13, an immunophilin that is enriched in red cell membranes.[560] The consequences of all these changes are unclear.

4.1 Genes

There are four paralogous genes in the 4.1 family: 4.1R, 4.1G, 4.1N, and 4.1B.[561-564] They have recently been reviewed.[565]

The human 4.1R gene, designated *EPB41*, maps to human chromosome 1p36.1 and is more than 200 kb in length. 4.1R is the erythroid form of protein 4.1 but is expressed in some nonerythroid cells in the brain, kidney, heart, and stomach[566,567] and has a number of interesting nuclear and cytoplasmic functions in nonerythroid cells, a discussion of which that is beyond the scope of this chapter.

4.1G is the closest paralogue to 4.1R and is widely expressed, including at low levels in erythroblasts.[168,568] 4.1N and 4.1B are primarily neuronal homologues of 4.1R and are mostly localized to specific cells within the brain, although some 4.1B is expressed late in erythropoiesis.[168] The 4.1 proteins are required for the correct cell surface accumulation of a very wide variety of membrane proteins and appear to act as hubs for membrane protein organization.

Alternative Splicing of the 4.1R Gene. The 4.1R gene contains at least 24 exons, 13 of which are alternatively spliced.[569-571] This produces a very complex mixture of isoforms (see Fig. 15-18, *A*). The two major isoforms of 4.1R derive from alternate use of two distinct translation initiation codons. The 17-base pair motif at the 5′ end of exon 2 encodes an in-frame ATG start site utilized by early erythroid cells and most nonerythroid cells to produce a 135-kD 4.1R isoform that contains a 209-amino acid N-terminal extension called the headpiece (HP) domain. The upstream translation initiation sequence is spliced out late in erythroid differentiation, and a downstream ATG is used instead, producing the prototypical 80-kD isoform of 4.1R found in mature red cells.[572,573] Selective incorporation of exon 16 late in erythroid differentiation inserts a 21-amino acid sequence into the 10-kD domain of 4.1R, generating the domain essential for spectrin-actin binding. Other alternatively spliced forms of 4.1R exist in nucleated cells. Understanding the functional differences in all the 4.1R spliceoforms is a daunting challenge.

4.1R Mutations

Mutations in the human 4.1R gene cause a subset of hereditary elliptocytosis (see Chapter 16) and underscore the relevance of 4.1R to the cellular physiology of erythrocytes.

Protein p55

Protein p55 (or MPP1) (gene name *MPP1* for membrane-palmitoylated protein 1) is a MAGUK (membrane-associated guanylate kinase) homologue found in the red cell membrane as part of a ternary complex with GPC (and probably GPD) and protein 4.1R.[574] The *MPP1* gene is located on Xq28 just distal to the factor VIII locus.[575] The protein is heavily palmitoylated on cysteine residues (*S*-palmitoylation), which contributes to its tight association with the plasma membrane.[153]

p55 contains an N-terminal PDZ domain, an SH3 domain, a positively charged central (“HOOK”) domain, a tyrosine phosphorylation zone, and a C-terminal guanylate kinase domain (Fig. 15-19).[576] The PDZ domain interacts with the C-terminus of glycophorin C/D.[290,291] The positively charged HOOK segment binds the 30-kD domain of protein 4.1R. Interestingly, this domain is conserved in other MAGUK proteins.[539] It is not known whether p55 shares other functional characteristics with MAGUKs, which participate, for example, in ion channel clustering, signal transduction, and regulation of cell proliferation and tumor suppression. It is clear, however, that p55 forms a tight complex with GPC/D and protein 4.1R,[577] such that red cells lacking either 4.1R or GPC/D are also deficient in p55.[309]

There are roughly 80,000 copies of p55 per red cell,[153] and the molecule is probably a homodimer because some dimer is evident on SDS gels.[153] If so, the number of p55 molecules is approximately the same as the number of F-actin protofilaments (see later section “Organization of the Membrane Skeleton”). Either way, it is much less than the number of GPC/D and 4.1R molecules (~200,000).

p55 is heavily palmitoylated on cysteine residues by DHHC17, the only acyltransferase in red cells.[578] Two rare patients who lack DHHC17 have a mild stomatocytic hemolytic anemia with no detectable palmitoylation of p55 or other membrane proteins.[578] The amount of p55 is normal in their isolated membranes and membrane skeletons, indicating that palmitoylation is not needed to properly localize the protein. But, the “lipid rafts” (i.e., the amount of lipid remaining after extraction of membranes with nonionic detergents) are markedly decreased. Chemical suppression of protein palmitoylation in normal red cells reproduces this finding in vitro. The data indicate that palmitoylation of p55 or some other membrane protein is important in the organization of lipid rafts.

Adducin

Adducin is a heteromeric membrane skeleton protein that caps actin, regulates spectrin-actin assembly, and aids in attaching the membrane skeleton to the lipid bilayer through band 3 and Glut1. Adducin is composed of an 81-kD α subunit that associates with an 80-kD β subunit or, to a lesser extent, with a 70-kD γ subunit.[579,580] The predominant form of adducin in red cells is probably the αβ heterodimer,[135] although the $\alpha_2\beta_2$ heterotetramer may also occur. Whereas α- and γ subunits are widely expressed, β adducin is selectively expressed in brain and hematopoietic tissues.[580-582] Adducin subunits all contain three distinct domains (Fig. 15-20).[580,583] The N-terminal 39-kD domain forms the globular head. It is protease resistant and generally more basic than the remainder of the protein.[584] A 9-kD neck links the N-terminal domain to the 30-kD C-terminal tail. The tail is flexible, protease sensitive, and composed almost entirely of hydrophilic residues. It contains a highly basic 22-residue C-terminal motif homologous to the myristoylated alanine-rich C kinase substrate (MARCKS) protein (see Fig. 15-20).[580] There are multiple alternative

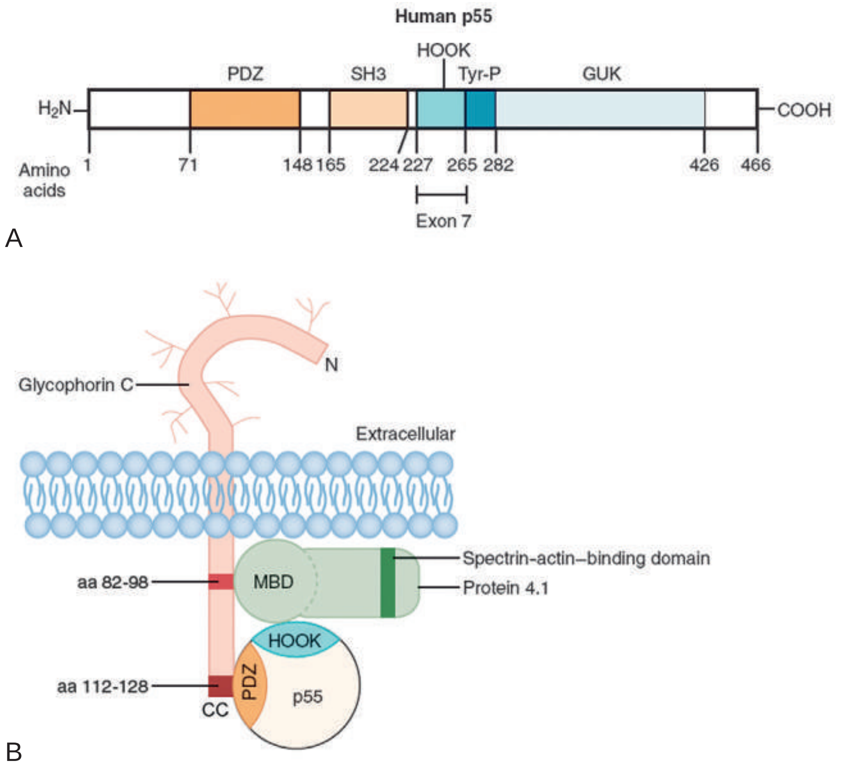

Human p55
HOOK
PDZ
SH3
Tyr-P
GUK
H_2N
COOH
Amino acids
1
71
148
165
224
227
265
282
426
466
Exon 7
A
N
Glycophorin C
Extracellular
Spectrin-actin–binding domain
Protein 4.1
aa 82-98
MBD
HOOK
aa 112-128
PDZ
p55
CC
B

Figure 15-19 Model of p55 and its interactions. **A,** Domain structure of p55. The HOOK domain *(Exon 7)* contains the protein 4.1R binding site. The PDZ (PSD-95/Discs Large/ZO-1) domain contains the binding site for glycophorin C (GPC). Protein p55 belongs to the membrane-associated guanylate kinase (MAGUK) protein family. **B,** Ternary interactions between GPC, protein 4.1, and p55. Note that p55 and GPC bind at two distinct sites on the 30-kD membrane-binding *(MBD)* or FERM domain of protein 4.1R. Although p55 is shown as being distant from the lipid bilayer, it is probably adjacent, because it is heavily palmitoylated. *GUK,* Guanylate kinase domain; *SH3, src* homology 3 domain; *Tyr-P,* site of tyrosine phosphorylation. *(Modified from Chishti AH: Function of p55 and its nonerythroid homologues.* Curr Opin Hematol *5:116, 1998.)*

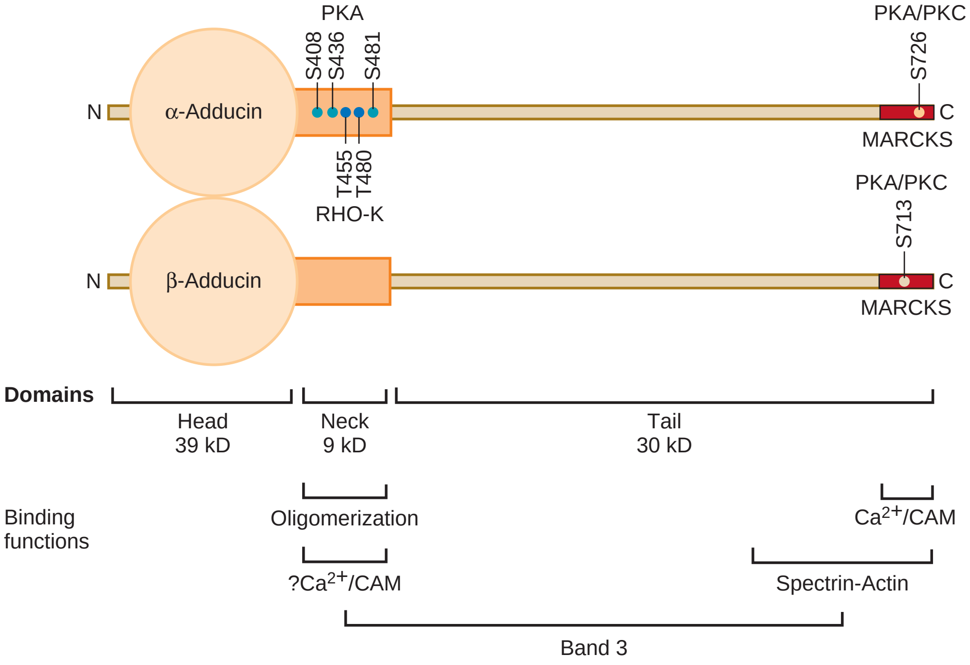

PKA
S408
S436
S481
PKA/PKC
S726
N
α-Adducin
C
MARCKS
T455
T480
RHO-K
PKA/PKC
S713
N
β-Adducin
C
MARCKS
Domains
Head 39 kD
Neck 9 kD
Tail 30 kD
Binding functions
Oligomerization
Ca^{2+}/CAM
?Ca^{2+}/CAM
Spectrin-Actin
Band 3

Figure 15-20 Domain organization of adducin. This model of the structure of the αβ-adducin heterodimer shows the three structural domains, the phosphorylation sites, and the identified binding regions. Although the dimer is shown, the $\alpha_2\beta_2$ heterotetramer also occurs. Binding functions include αβ oligomerization; spectrin-actin binding; binding to band 3, stomatin, chorein, and glucose transporter 1; and Ca^{2+}-dependent calmodulin (Ca^{2+}/CAM) binding. *MARCKS,* Myristoylated alanine-rich C kinase substrate; *PKA,* protein kinase A; *PKC,* protein kinase C; *Rho-K,* Rho kinase. *(Modified from Matsuoka Y, Hughes CA, Bennett V: Adducin regulation: definition of the calmodulin-binding domain and sites of phosphorylation by protein kinases A and C.* J Biol Chem **271**:*25157, 1996.)*

spliceoforms of adducin subunits, many of which generate truncated isoforms that may serve tissue-specific functions.[585-587]

Adducin Binds, Bundles, and Caps Actin, in Addition to Enhancing the Association of Spectrin with Actin. The adducin head domains form a globular core,[583] linked by the neck domains, which contain an oligomerization site.[588] Adducin interacts directly with the fast-growing (barbed) ends of actin filaments and caps filament growth, stabilizing the filaments.[589] Both the C-terminal MARCKS domain and the neck domain are required for actin binding.[588] The numbers of adducin dimers and actin protofilaments are equivalent (~30,000 per red cell), consistent with its capping function.

The adducin tails also recruit spectrin to the fast growing (barbed) ends of actin.[588,590] Adducin binds to the N-terminal actin-binding domain of β spectrin and the adjacent two β-spectrin repeats.[475] However, the fact that mutations within the last few repeats of α spectrin markedly reduce membrane adducin content[476] suggests that the C-terminus of α spectrin also contributes to adducin binding.

Adducin Attaches to Band 3, Stomatin, Chorein, and the Glucose Transporter and Helps Anchor the Membrane Skeleton

Band 3. The C-terminal tail of adducin also binds to the subset of band 3 dimers located near the actin junctions.[222] α-Adducin and β-adducin tails can both bind to band 3 dimers in a 1 : 1 complex.[222] It is not known whether intact adducin can bind two band 3 dimers or just one.

The importance of the band 3-adducin interaction is unclear. Band 3 null mice have a 35% deficiency of adducin and, as noted earlier, some of the mice with α-spectrin C-terminal mutations have an even greater deficiency. This suggests the interaction is important, although care must be taken in deducing the importance of an interaction (or lack thereof) from changes in mouse mutants given the multiple interactions of most membrane skeleton proteins. For example, band 3 null mice have near-normal amounts spectrin[220] but no one would argue that the several attachments of spectrin to band 3 are not important. A better argument is that adducin tail fragments introduced into red cell membranes interfere with the normal adducin–band 3 interaction and weaken the membrane.[222] On the other hand, knockout of all adducin genes produce, at best, a very mild hemolytic anemia (see later).

The band 3–adducin interaction is sensitive to Mg^{2+} over the range of concentrations found in the red cell.[222] Free Mg^{2+} ranges from approximately 4 mM in deoxygenated red cells when 2,3-diphosphoglycerate (2,3-DPG) is bound to hemoglobin to much lower concentrations in oxygenated cells when 2,3-DPG is available to bind Mg^{2+}. This suggests that the mechanical properties of the red cell membrane may be regulated by oxygenation, although whether the binding changes in seconds, as would be needed under normal circulatory conditions, is unknown.

Stomatin and Glut1. As noted earlier, stomatin binds to some of Glut1 molecules and converts them to dehydroascorbic acid channels. Glut1 also binds to adducin[420] and dematin,[420] and there is preliminary evidence, never published in full, that stomatin binds to adducin.[591] The interactions are not as well characterized as the interaction of adducin with band 3, but it is reasonable to assume that they also help attach actin junctions to the membrane.

NHE1. The Na^+-H^+ exchanger (NHE1 or SLC9A1) is markedly deficient in β-adducin knockout mice,[592] suggesting the two molecules interact. As noted earlier, NHE1 also binds to protein 4.1R.[549]

Chorein. Mutations in chorein cause chorea-acanthosis, a disease characterized by striatal neurodegeneration and acanthocytic red cell morphology (see Chapter 37). Chorein is the protein product of the vacuolar protein sorting homolog A gene (gene symbol *VPS13A*). The functions of chorein in the red cell are not understood, but it was recently discovered that it binds to both β adducin and β actin,[592a] which would place it in the actin junctional complex. Since β adducin is only expressed in brain and hematopoietic tissues and the phenotype of chorea-acanthocytosis is limited to those tissues, the interaction of chorein with β adducin may be important.

Adducin Activity is Regulated by Phosphorylation and Ca^{2+}-Calmodulin. The C-terminal MARCKS domain contains a serine phosphorylation site targeted by both protein kinase A (PKA) and protein kinase C (PKC).[593] There are also three additional PKA sites located in the neck domain of α adducin (see Fig. 15-20).[593] PKA phosphorylation reduces both the affinity of adducin for spectrin-actin complexes and the ability of adducin to enhance spectrin-actin binding.[593] Phosphorylation of the MARCKS domain by PKC inhibits actin-capping activity and prevents adducin-mediated recruitment of spectrin to actin protofilaments.[594]

The MARCKS domain also contains a Ca^{2+}-dependent calmodulin binding site.[593] PKA or PKC phosphorylation of the MARCKS domain inhibits calmodulin binding.[593] Calmodulin binding reduces both adducin-actin binding and adducin-mediated enhancement of spectrin-actin binding.[590] Calmodulin also blocks the binding of a second spectrin molecule to an adducin/actin/spectrin complex.[579] Once bound to the MARCKS domain, calmodulin reduces the rate of PKA phosphorylation of β adducin and of PKC phosphorylation of α and β adducin.[593] Despite these many complex in vitro regulatory effects, it appears that PKC phosphorylation of adducin has little effect on red cell membrane mechanical stability.[550]

α Adducin is also subject to Rho kinase phosphorylation (see Fig. 15-20), which enhances the interaction of α adducin with actin filaments and spectrin. Myosin phosphatase dephosphorylates α adducin, but this activity is inhibited by Rho kinase–mediated phosphorylation of myosin phosphatase.[595]

Thus adducin activity is tightly regulated in vivo by Ca^{2+}-dependent calmodulin binding and a complexity of differential phosphorylation states. In general, Rho kinase phosphorylation tends to foster adducin interaction with actin and spectrin and PKA/PKC phosphorylation or calmodulin binding tends to inhibit them.

Red Cells Lacking Adducins Are Similar to Erythrocytes from Patients with Mild Hereditary Spherocytosis. Mice with a targeted deletion of β adducin have a *very* mild anemia with osmotically fragile, spherocytic, and dehydrated red cells.[582] Incorporation of α adducin into the membrane skeleton is decreased by 30%, perhaps owing to decreased α-subunit stability in the absence of its heteromeric partner.[582] Interestingly, there is a compensatory fivefold increase in the amount of γ adducin associated with the red cell membrane skeleton.[582] However, knockout of γ adducin has no hematologic phenotype and the combined βγ-adducin knockout is not worse than loss of β adducin alone.[596] Knockout of α adducin, which eliminates all three adducins (α, β, and γ), still results in only mild hemolysis and moderate spherocytosis, although is slightly more severe than in the β-adducin knockout.[223] There are other problems, including hydrocephalus.[223] However, the loss of both β adducin and the dematin headpiece (see later) does produce a severe spherocytic hemolytic anemia. On the other hand, loss of the dematin headpiece alone has minimal effect.[597] These data, combined with the evidence that red cells lacking GPC have, at most, very mild hereditary elliptocytosis, suggest the various vertical membrane connections involving the actin junctions are largely redundant and only become consequential when more than one is eliminated.

Dematin (Protein 4.9)

Dematin is a widely expressed actin-binding protein that bundles actin filaments into cables[598] (although presumably not within the red cell membrane skeleton) and aids in spectrin-actin binding.[477] Red cells contain both 48-kD and 52-kD spliceoforms.[599,600] The latter form contains an extra 22-amino acid insert. Dematin is composed of two domains: an unstructured N-terminal tail or “core” domain, and a C-terminal actin-binding headpiece that shares homology with villin, an actin-bundling protein in the epithelial brush border.[600] The 22-amino acid insertion contains an ATP-binding site within a conserved 11-amino acid motif that is also found in protein 4.2.[601]

Dematin is generally believed to be a trimer of two 48-kD subunits and one 52-kD subunit held together by disulfide bonds.[598,599,602] However, recent evidence indicates it is monomeric.[602a] Dematin monomers contain two actin filament binding sites: one in the headpiece domain[602b] and one in the tail.[602a] Dematin is phosphorylated by PKA and PKE.[603] PKA phosphorylation of Ser 381 in the headpiece causes the headpiece to bind to the tail, blocking one of the actin binding sites and eliminating actin filament bundling but not actin bonding activity.[602a] PKA phosphorylation at Ser 74 in the dematin tail also abolishes dematin’s actin bundling capability.[602,604] The mechanism of this effect has not been investigated.

Importantly, dematin also has functions in red cells other than actin bundling. First it binds to the cytoplasmic domain of the glucose transporter, Glut1, and presumably helps attach the actin protofilaments to the membrane. Second dematin, but not PKA-phosphorylated dematin, binds to the spectrin tail and facilitates the interaction of spectrin with actin.[477] The interaction sites in these two interactions have not yet been determined.

Rho GTPases regulate actin organization, and dematin interacts with the Ras–guanine nucleotide exchange factor Ras-GRF2 in extracts from brain. This blocks transcriptional activation of Jun by Ras-GRF2 and activates ERK1 via a Ras-GRF2–independent pathway.[605] It is not clear if these pathways operate in mature red blood cells; if they do, dematin may help regulate actin organization within the membrane skeleton.

As noted earlier, knockout of the dematin headpiece results in a *very* mild spherocytic hemolytic anemia,[597] suggesting that the spectrin and actin binding activities of dematin are either redundant or not critical for membrane stability.

Tropomyosin

Tropomyosin (TM) is a dimeric protein that functions in red cells to stabilize and limit F-actin protofilaments to uniform segments measuring 37 nm—the length of tropomyosin molecule.[159,160,606] The α-helical subunits of TM form a rodlike coiled coil. The two major human TM isoforms found in red cells are designated hTM5 (or TM5NM1) and hTM5b (or TM5b) and derive from the *TPM3* (or γ-TM) and *TPM1* (or α-TM) genes, respectively (Fig. 15-21).[160] They migrate as two closely spaced bands in the region of band 7 on SDS gels (see Fig. 15-5).[156] hTM5 is the upper band. hTM5 and hTM5b are present in an equimolar ratio. Both have high affinities for F-actin and tropomodulin. They are shorter than muscle TMs.

Each F-actin protofilament binds two homodimeric[606a] TM molecules in an Mg^{2+}-dependent manner, such that one TM dimer binds to each of the actin filaments contained in the F-actin double helix.[156] Thus all of the actin protofilaments within the red cell membrane skeleton are coated with TM, which strengthens the filamentous network. Each TM dimer is just long enough to cover the estimated seven actin monomers in one of the two strands of a red cell F-actin protofilament. This supports the hypothesis that TM functions as a molecular ruler, along with actin-capping proteins, in dictating the length of the actin protofilaments. There are approximately 140,000 TM monomers per red cell, which corresponds to 70,000 heterodimers and 35,000 double-helical actin filaments. This corresponds to 490,000 actin monomers at a 1 : 7 ratio, which is close to the measured number of 500,000.

The N-terminus of TM binds avidly to tropomodulin, the capping protein for the pointed or slow-growing end of actin. Two TM heterodimers bind per tropomodulin in a cooperative manner.[607] This interaction limits the cooperativity of actin-TM associations, thereby restricting the length of actin filaments beyond the length of TM itself.[608] The binding of TM to actin is unaffected by spectrin, but spectrin-actin interactions are inhibited by saturating concentrations of TM,[609] and red cell membranes that lack TM are markedly more fragile than

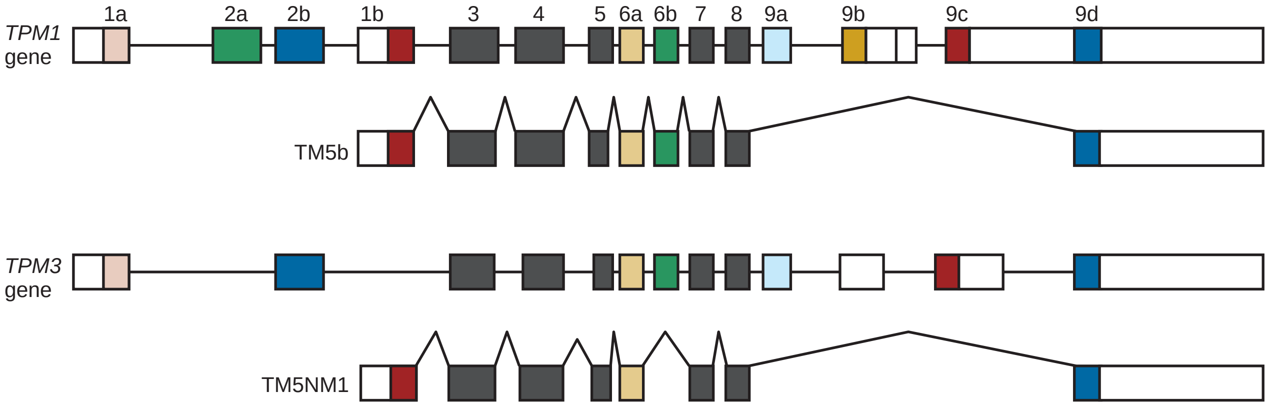


Figure 15-21 Gene structure of the red blood cell tropomyosins *(TM)*. There are more than 40 isoforms of tropomyosin, which derive from four TPM genes.[159] Red cells contain two of these. One is derived from the *TPM1* gene and is called TM5b or hTM5b. The other is derived from the *TPM3* gene and is named TM5NM1 or hTM5.

normal.[610] The actin-binding domain of β spectrin is more easily exchanged into the membrane skeleton of TM-depleted red cell membranes, indicating that TM strengthens spectrin/actin/protein 4.1R binding.[610] Only endogenous TMs, and not the longer muscle isoforms, can restore mechanical stability to the TM-depleted membranes. These findings indicate that actin-TM interactions dictate the site specificity, strength, and/or extent of spectrin binding along the actin filament and that endogenous TMs are required for these effects.

Tropomodulin

Tropomodulin-1 (TMod1) is a 41-kD protein that functions as an actin-capping protein at the pointed or slow-growing end of actin filaments.[611] The C-terminal end of TMod1 has pointed-end actin-capping activity. The N-terminal portion binds with high affinity to TM,[607] which greatly strengthens actin capping.[612] One TMod1 molecule binds to each of the two F-actin/TM strands in the actin protofilament. Among TM homologues, TMod1 binds with highest affinity to the shorter nonmuscle erythrocyte isoforms (hTM5 and hTM5b) and not as well to the longer muscle TMs.[160,608,613] The interaction of TMod1 with TM inhibits the head-to-tail associations of TM molecules along actin filaments, thereby restricting the lengths of TM-stabilized actin filaments.[614] TMod remains attached to the red cell membrane even after removal of spectrin, actin, and TM. The membrane-binding site for TMod1 and the significance of this membrane interaction are unknown.

Mice that lack TMod1 have mild spherocytic elliptocytosis that is more spherocytic than elliptocytic.[615] The phenotype may be ameliorated by an increase in TMod3, which is not normally expressed in mature red cells, to about 20% of the normal level of TMod1. Surprisingly, TMod1-null erythrocytes have normal levels of actin, TMs, adducins, and other membrane skeleton proteins. However, actin filament lengths are variable and the membrane skeleton contains abnormally large and variable pore sizes,[615] proving that TMod1 is critical for maintaining normal actin filament length and skeleton architecture.

Although TMod3 is not normally expressed in mature red cells, it is important in erythropoiesis. Mice that lack the TMod3 gene die in utero due to impaired terminal erythroid differentiation in the fetal liver.[615a] The late erythroblasts have multilobular nuclei, they assemble actin aberrantly during enucleation and enucleate poorly, and they do not form erythroblast islands well.

Ankyrin

Erythrocyte ankyrin (ankyrin-1 or ankyrin-R) is a 206-kD protein[616] that links spectrin to band 3 and thus forms the basis of the skeleton-membrane attachment so critical to the mechanical and viscoelastic properties of the red cell. Ankyrin-R is encoded by the *ANK1* gene, which is located on the short arm of chromosome 8 near the centromere (8p11.2).[617] The protein is composed of three domains: an 89-kD membrane domain (amino acids 2 to 827) that interacts with band 3 (and other integral membrane proteins in nonerythroid cells), a 62-kD spectrin-binding domain (amino acids 828 to 1382), and a 55-kD C-terminal domain (amino acids 1383 to 1881) that is believed to modulate ankyrin's interactions with spectrin and band 3 (Fig. 15-22). Alternative splicing, particularly in the C-terminal domain, generates several ankyrin species in the red cell and accounts for the presence of ankyrin bands 2.1, 2.2, 2.3, and 2.6 on SDS gel electrophoresis (see Fig. 15-5).[616] Disruption of the ankyrin-band 3 interaction by exposing intact red cells to alkaline pH causes marked membrane instability, emphasizing the importance of the ankyrin linkage to red cell physiology.[618] Indeed, defects in ankyrin are the most common cause of hereditary spherocytosis[619] (see section on "Hereditary Spherocytosis" in Chapter 16).

Membrane Domain

Ankyrin Repeats Are Found in Hundreds of Proteins in All Phyla and Are One of the Most Common Protein Sequence Motifs. The membrane domain is composed of a tandem array of ankyrin repeats.[620] Similar repeats function as sites of protein-protein interactions in a broad range of proteins involved in development, cell cycle control, oncogenesis, transport, and many other

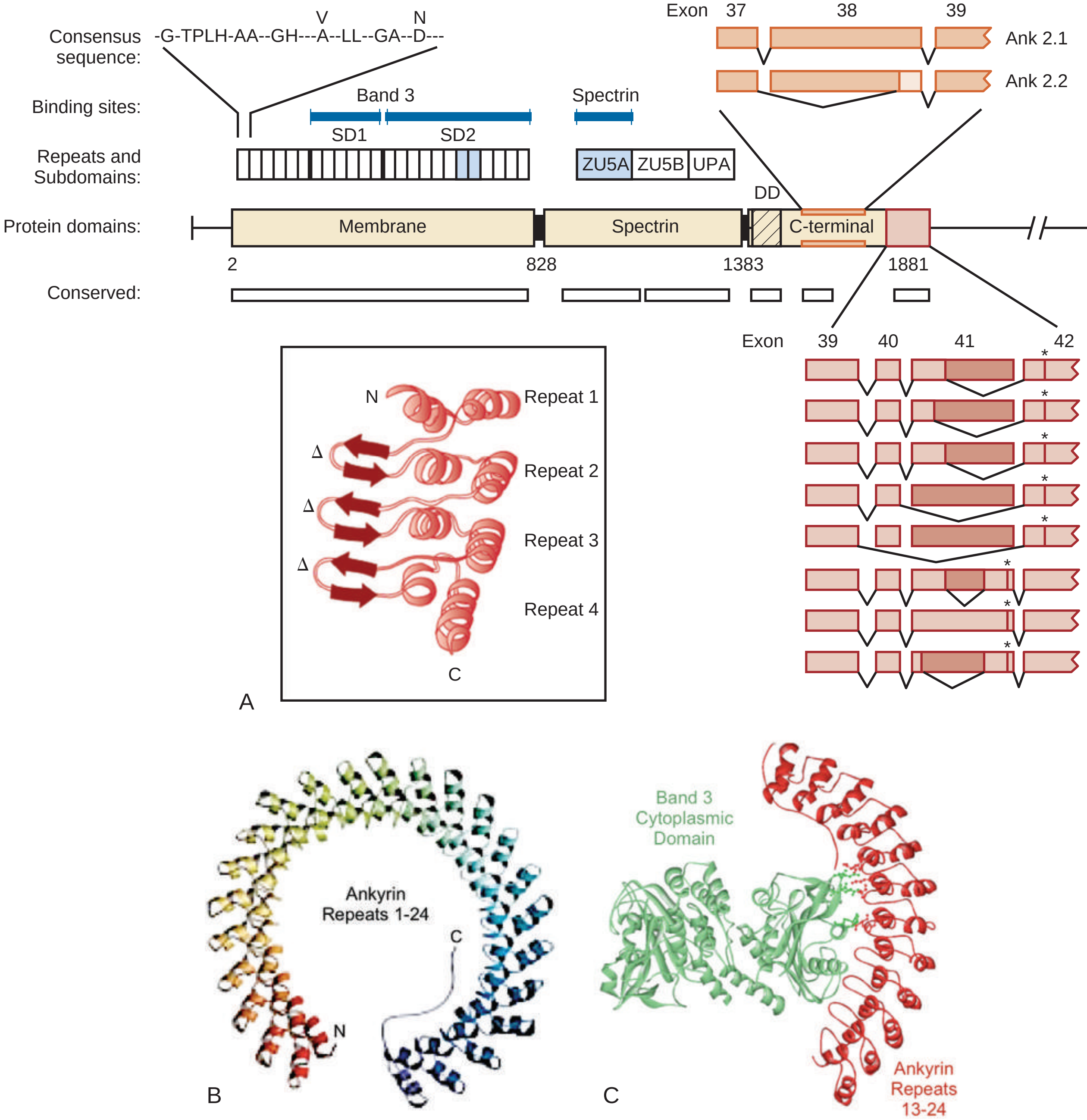

Consensus sequence:
V N
-G-TPLH-AA--GH---A--LL--GA--D---
Binding sites:
Band 3
SD1
SD2
Spectrin
Repeats and Subdomains:
ZU5A
ZU5B
UPA
DD
Protein domains:
Membrane
Spectrin
C-terminal
2
828
1383
1881
Conserved:
Exon
37
38
39
Ank 2.1
Ank 2.2
Exon
39
40
41
42
*
N
Repeat 1
Repeat 2
Repeat 3
Repeat 4
Δ
C
A
Ankyrin Repeats 1-24
C
N
B
Band 3 Cytoplasmic Domain
Ankyrin Repeats 13-24
C

Figure 15-22 Ankyrin structure and interactions. **A,** Model of erythroid ankyrin (Ank1 or AnkR) structure. The *membrane domain* (89 kD) contains 24 "Ank" repeats (33 amino acids each), grouped functionally into subdomains of six repeats. Their consensus sequence is shown at the *top left* in single-letter amino acid code. *Dashes* indicate less-conserved residues. There are two binding sites for band 3, one involving repeats 7 to 12 (SD1, subdomain 1) and one involving repeats 13 to 24 (SD2). The ankyrin-binding loops on band 3 are predicted to interact with ankyrin repeats 19 and 20 *(light blue)* of SD2. The interaction site within SD1 is not known. The *spectrin domain* (62 kD) contains three subdomains (ZU5A, ZU5B, and UPA). ZU5A *(light blue)* forms the binding site for spectrin. The *C-terminal (regulatory) domain* (55 kD) is believed to modulate the binding functions of the other two domains. A conserved death domain (DD) resides at the beginning of this domain. It is similar to death domains in proteins involved in apoptosis, but its function in ankyrins is unknown. In the middle of the domain a highly acidic inhibitory region in exon 38 is spliced out of full-length ankyrin (ankyrin 2.1) to make ankyrin 2.2. At least eight isoforms of the last three exons exist. In addition, isoforms lacking exons 38 and 39, 36 through 39, and 36 through 41 have been detected. The *asterisk* and *vertical line* under it indicate the location of the translation terminator codon. The *inset* shows the structure of four consecutive ankyrin repeats. Each repeat forms an L-shaped structure. A β-hairpin loop *(arrows)* makes up the bottom of the L, and two antiparallel α-helical coils form the vertical stem. The hairpin loops of neighboring repeats interact to create an antiparallel β-sheet, and the α-helices interact to form helical bundles. The *small triangles* in the inset indicate the junction between adjacent repeats. **B,** Model of the whole membrane domain based on the structure of half the domain and viewed along the spiral axis. Repeat 1 is red, and repeat 24 is violet. **C,** Model of the interaction between a dimer of the cytoplasmic domain of band 3 *(green)* and repeats 13 to 24 of ankyrin *(red)*. *(**B,** from Michaely P, Tomchick DR, Machius M, et al: Crystal structure of a 12 ANK repeat stack from human ankyrin*$_R$*.* EMBO J *21:6387–6396, 2002;* ***C,*** *from Grey JL, Kodippili GC, Simon K, et al: Identification of contact sites between ankyrin and band 3 in the human erythrocyte membrane.* Biochemistry *51:6838–6846, 2012.)*

functions.[620-622] Ankyrin repeats form a novel L-shaped structure consisting of a β-hairpin (bottom of the L) followed by two α helices that pack side to side in an antiparallel fashion (stem of the L) (see Fig. 15-21, *A*).[623] In ankyrin, the repeats correspond to individual exons.[624] They start at the tip of the β hairpin and line up side to side like overlapping Ls, with the hairpins forming a β sheet that is perpendicular to the plane of the α helices. The concave and convex surfaces of the connected repeats provide an ideal interface for protein-protein interactions, making the ankyrin repeat a versatile binding partner. A model of the 24 repeats of ankyrin resembles one twist of a spring (see Fig. 15-21, *B*),[623] and mechanical studies show that ankyrin repeats have springlike properties.[625] Some speculate they may link external mechanical forces to signal transduction or changes in ion transport.[625,626]

The 89-kD N-terminal Domain of Ankyrin Contains 24 Consecutive 33 Amino Acid Repeats.[616] In ankyrin, the ankyrin repeats are organized functionally into four subdomains of six repeats each (see Fig. 15-22, *A*)[627] and contain two distinct and cooperative binding sites for band 3.[628] One band 3–binding site occurs within repeats 7 to 12 (subdomain 2) and the other within repeats 13 to 24 (subdomains 3 and 4) (see Fig. 15-22, *A*). It is clear the ankyrin binds four molecules of band 3,[211-213] but whether this is a single band 3 tetramer or two independent dimers is not certain. We favor the former model because stable band 3 tetramers can be isolated from red cell membranes and the numbers correspond to the number of ankyrin molecules.[213,214] The high-affinity binding interface for ankyrin on the cytoplasmic domain of band 3 (kD ~ 10^{-7} to 10^{-8} M) has been localized to a pair of adjacent β-hairpin loops in the cytoplasmic domain of band 3 formed by amino acids 63 to 73 and 175 to 185 (see Figs. 15-7 and 15-9).[207-209] Computer simulations and mutation studies predict that these loops contact residues in ankyrin repeats 19 and 20 within subdomains 3 and 4 (see Fig. 15-22, *C*).[209] A similar prediction was obtained with a spin-label approach.[215] The specific repeats that mediate binding within subdomain 1 have not been identified. There is evidence that ankyrin also interacts with other band 3 residues near the two loops[209] and with the N-terminus of band 3.[207,228,232,535,618] The importance of these interactions is unknown.

Removal of the 75- to 185-amino acid loop of band 3 abolishes affinity for ankyrin in vitro.[207] Surprisingly, however, red cells containing the loop-deleted band 3 are only mildly spherocytic and unstable, much less so than red cells that lack ankyrin or band 3.[629] This suggests that either the other interactions between ankyrin and band 3 are more important in vivo than in vitro or that other interactions compensate. Both explanations are probably true. First, although the single 63-to 73-amino acid loop and its neighboring sites are too weak to maintain the ankyrin/band 3 complex at in vitro concentrations, they will be much stronger under the vastly higher protein concentrations that exist on the membrane. Second, there are other connections between the membrane skeleton and the multiprotein band 3/ankyrin complex (see Fig. 15-6). Both ankyrin and band 3 interact with protein 4.2 (see later), and ankyrin and 4.2 both interact with spectrin and with elements of the Rh complex, especially RhAG.[332,386,630] Ankyrin also interacts with CD44.[546]

Spectrin Domain

The central 62-kD domain of ankyrin binds to β spectrin and attaches the spectrin-actin–based skeleton to the plasma membrane. Ankyrin-spectrin interactions are cooperative, such that ankyrin binding promotes spectrin tetramer and oligomer formation.[631,631a] The attachment of ankyrin to spectrin dimers causes a tenfold increase in the self-association.[631a] However, cleavage of the ankyrin–band 3 linkage also weakens spectrin self-association,[631b] perhaps indirectly by increasing the mobility of the otherwise attached spectrin dimer. This would lead to a lower local two-dimensional concentration of the dimer, diminishing its chances of finding a partner and forming a tetramer. Presumably both of these mechanisms operate to regulate the relatively tenuous self-association interaction.

The spectrin domain is composed of two tandem ZU5 subdomains followed by a UPA subdomain (see Fig. 15-22, *A*). The spectrin-binding site occurs at a conserved, positively charged surface patch within ZU5A.[467-469] This site attaches to repeats 14 and 15 of β spectrin, especially to the C helix of repeat β14, and the loop between helices B and C of β spectrin repeat 15 (see Fig. 15-13, *C*). Repeat β15 forms an unusual angle with repeat β14, and the ZU5A domain nestles into the corner formed by the two repeats.[469]

The ZU5B domain extends away from ZU5A on the side opposite the spectrin binding face. It does not directly interact with ZU5A and does not influence spectrin binding.[632-633] The location of the UPA subdomain has not been studied in red cell ankyrin, but in another ankyrin (ankyrin-B) it folds back and lies adjacent to ZU5A.[633] Like ZU5B, the UPA subdomain does not affect spectrin binding.[633] The functions of ZU5B and UPA in red cells are unknown, but they have conserved binding surfaces that suggest they bind to as yet undiscovered proteins. A few missense mutations in these subdomains have been found in patients with hereditary spherocytosis,[633] which supports the idea that they are functionally important.

C-Terminal (Regulatory) Domain

The 55-kD C-terminal domain contains sequences that regulate ankyrin interactions[634,635] and has been dubbed the "regulatory domain."[616] Alternative splicing within the C-terminal domain yields at least 15 ankyrin isoforms differing in size and, presumably, functions (see Fig. 15-22, *A*).[624] For example, one isoform of ankyrin, represented by band 2.2, lacks an acidic 162-amino acid peptide present in full-sized ankyrin (band 2.1). Omission of this sequence generates an activated ankyrin, which exhibits enhanced binding to band 3 and spectrin[635] and facilitates the binding of ankyrin to additional targets in nonerythroid membranes.[636] The identification of a membrane-associated protease that cleaves ankyrin suggests that cleavage of a repressor region within the C-terminal domain may activate ankyrin.[636] The C-terminal domain also contains a region homologous to the "death domain" of proteins implicated in apoptosis,[637] suggesting that ankyrins may additionally play a role in this signaling pathway. The death domain lies just beyond the spectrin domain but does not interact with it.[633] Its function in red cell ankyrin is unknown.

Phosphorylation and Hydroxylation

The effect of phosphorylation on ankyrin function has not been studied in recent years, but there are old studies showing that the protein can be phosphorylated by casein kinase I[638] and casein kinase II.[639] Unphosphorylated ankyrin preferentially binds to spectrin tetramers and oligomers rather than spectrin dimers.[631,638,640] This preference is abolished by phosphorylation, which also reduces the capacity of ankyrin binding to band 3.[641]

The protein called factor-inhibiting hypoxia-inducible factor catalyzes β-hydroxylation of several asparagine residues erythrocyte ankyrin and multiple other proteins containing ankyrin repeats.[642] The modification stabilizes the membrane domain and inhibits band 3 binding. Its physiologic significance is unknown.

Ankyrin Genes

There are three human ankyrin genes: *ANK1, ANK2,* and *ANK3*. The corresponding proteins, ankyrin-R (ankyrin-1),[616] ankyrin-B (ankyrin-2),[643] and ankyrin-G (ankyrin-3),[644,645] have multiple spliceoforms and distinct patterns of expression in the body.

Ankyrin-R is primarily expressed in erythrocytes, but it is also found in muscle cells, macrophages, endothelium, and specific neuronal populations, particularly Purkinje and granule cells of the cerebellum.[646] Ankyrin-B and ankyrin-G are expressed in most cells other than red cells, but they have distinct functions. A discussion of the large literature on these two proteins is beyond the scope of this chapter but reviews are available.[426,647,648]

Overall, the nonerythroid ankyrins link the spectrin-actin–based skeleton to a large number of structurally diverse integral membrane proteins, mostly through their versatile ankyrin repeats.[622,649,650] Examples include many transporters and channels, various receptors, and a number of adhesion molecules. There is no specific motif that specifies an ankyrin-binding site in these proteins. They seem to have developed by convergent evolution. Ankyrin-B and ankyrin-G not only attach integral proteins to the spectrin skeleton but in many cases also localize them to distinct domains and subcellular compartments. This may be why so many spliceoforms exist.

Ankyrin-1 Mutations

Mice With Normoblastosis (nb/nb) Produce a Defective Ankyrin That Causes a Severe Hemolytic Anemia. Red cells from nb/nb mice contain a nonsense mutation in ankyrin just between the spectrin binding and C-terminal domain that leads to production of decreased amounts of a shortened (157-kD) ankyrin.[651,652] As a result, *nb/nb* red cells are moderately spectrin-deficient despite normal spectrin synthesis.[651,653] The resultant red cells exhibit severe osmotic fragility and shortened life span owing to a defective membrane skeleton. Mature nb/nb mice also develop ataxia secondary to a dropout of Purkinje cells in the cerebellum.[654]

Ankyrin-1 Mutations Are the Most Common Cause of Hereditary Spherocytosis in Humans. Erythrocyte ankyrin mutations lead to combined deficiencies of ankyrin and spectrin. The defective cells produce a variable clinical presentation ranging from mild hemolysis to severe transfusion-dependent anemia[639] (see "Hereditary Spherocytosis" in Chapter 16).

Protein 4.2

Protein 4.2 is an 80-kD peripheral membrane protein that participates in stabilizing the red cell membrane skeleton through interactions with band 3, CD47, spectrin, ankyrin, and the lipid bilayer. Protein 4.2 shares homology with transglutaminases and is part of a transglutaminase gene cluster on chromosome 15q15, but it lacks transglutaminase activity.[655,656] Like most red cell membrane proteins, its expression increases progressively throughout erythropoiesis.[168] There are eight spliceoforms that arise from alternative splicing of exon 3, exon 5, or 90 bp in exon 1.[657,658] The major form lacks the 90-bp segment. The others are all minor species in the mature red cell. The protein is found mostly in erythroid tissues[659] and in the lens.[660] Reports of protein 4.2 detected in other tissues by immunologic methods[661] are not adequately controlled for antibody cross-reactivity with tissue transglutaminases. The protein has recently been reviewed.[662]

Protein 4.2 Structure

Band 3 Binding. Protein 4.2 binds to band 3 with a kD of 10^{-6} to 10^{-7} M. The interaction increases band 3–mediated chloride transport.[663] The band 3–binding site on protein 4.2 has been mapped to amino acids 187 to 210 (Fig. 15-23).[664] This sequence encompasses a palmitoylation site on Cys203. Palmitoylation enhances band 3 binding.[219,664] The binding site for protein 4.2 on band 3 has not been clearly identified. Band 3 binding is absolutely required for 4.2 expression. Red cells that lack band 3 completely lack protein 4.2, despite normal 4.2 synthesis.[220] Conversely, band 3 is partially deficient in red cells that lack protein 4.2.[221] It is not clear whether protein 4.2 binds to the ankyrin-linked band 3 tetramers or to the "free" band 3 dimers. Studies of band 3 mobility suggest 4.2 binds to band 3 dimers, but 4.2 also binds to ankyrin, which attaches to band 3 tetramers.[665,666] Because only a fraction of the protein 4.2 remains attached to the membrane skeleton (which retains ankyrin-linked band 3 tetramers) after band 3 dimers are extracted with Triton X-100,[166] it probably binds to both.

Ankyrin Binding. Protein 4.2 and ankyrin associate in solution, and there is indirect evidence that they interact on the membrane.[667] Mice that completely lack ankyrin-1 have only a small amount of protein 4.2 on their red cell membranes.[668] Red cells expressing the unstable protein 4.2 Komatsu (Asp175→Tyr) lack 4.2, and the

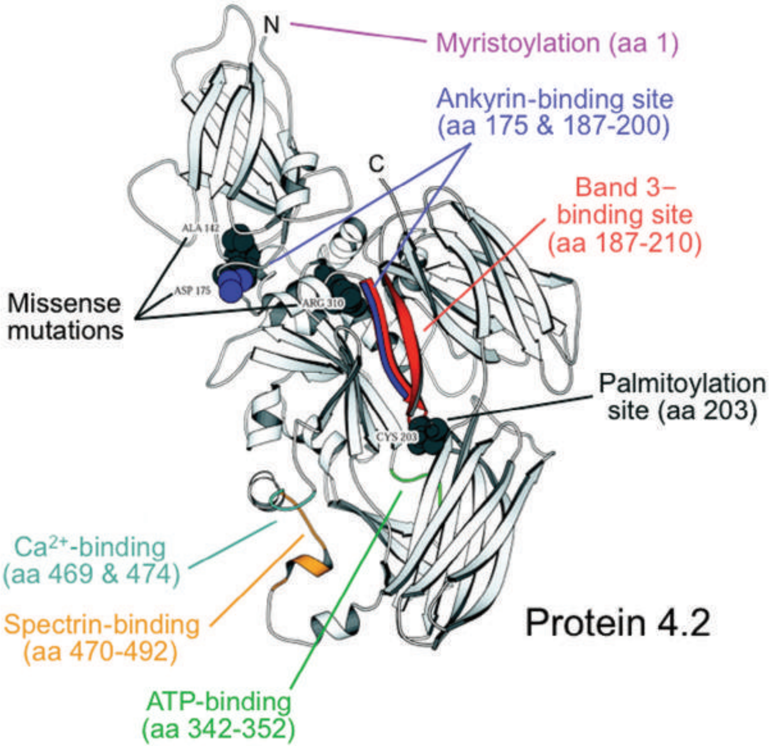


Figure 15-23 Homology model of protein 4.2 based on the known structure of a transglutaminase, with putative binding and posttranslational modification sites mapped onto the structure. The band 3– and spectrin-binding sites and the palmitoylation site are the most certain. Note that the band 3– and putative ankyrin-binding sites overlap, which raises the possibility that one influences the binding of the other. Palmitoylation, which occurs on Cys203 *(shown as a space-filled residue)*, just beyond the band 3–binding site, enhances band 3 binding. Note also that there is a likely Ca^{2+}-binding site just upstream of the spectrin-binding site. Spectrin binding to protein 4.2 is enhanced by Ca^{2+}. Patients with missense mutations of Asp175 and Arg310 *(shown as space-filled residues)* have impaired (R310Q) or absent (D175Y) ankyrin binding to protein 4.2. The location of the common 4.2^{Nippon} mutation (A142T) is also depicted. *Note:* amino acids (aa) are numbered using the full 721 amino acid sequence, even though the common spliceoform of 4.2 is only 691 amino acids long. *(Modified from Toye AM, Ghosh S, Young MT, et al: Protein-4.2 association with band 3 [AE1, SLC4A1] in* Xenopus *oocytes: effects of three natural protein-4.2 mutations associated with hemolytic anemia.* Blood *105:4088–4095, 2005.)*

recombinant protein fails to bind ankyrin.[663,669] Mapping studies locate the ankyrin-binding site to a neighboring region (amino acids 187 to 200)[670] that overlaps the first half of the band 3–binding site (see Fig. 15-23). This raises the possibility that binding of band 4.2 to ankyrin inhibits its binding to band 3. There is some evidence for this in early studies of Korsgren and Cohen, who found that coincubation of ankyrin and band 4.2 in solution partially blocked the binding of both proteins to the membrane.[667] But the question needs to be reexamined.

CD47 Binding. Protein 4.2 interacts with the Rh complex through CD47.[384-386] Red cells that lack protein 4.2 nearly lack CD47,[385,386] and patients with partial deficiency of 4.2 have less CD47 attached to the membrane skeleton. However, mice lack this association[385] (and, interestingly, protein 4.2 is easier to extract from the red cell membrane in mice). On the other hand, mice and humans with absent CD47 or low expression of the protein, do not have reduced protein 4.2.[385] This, and other observations,[332] show that the band 3 complex (band 3, ankyrin, GPA, and protein 4.2) interacts with the Rh complex (RhAG, Rh polypeptides, GPB, LW, and CD47).

Spectrin Binding. Korsgren first showed that protein 4.2 binds spectrin.[665] She identified both high-affinity (kD ~ 10^{-9} M) and low-affinity (kD ~ 10^{-7} M) sites. Reconstituting 4.2-stripped membrane vesicles with protein 4.2 increased spectrin-binding capacity from approximately 50% to approximately 80% of normal.[665] The binding site on protein 4.2 has been mapped to amino acids 470 to 492 (see Fig. 15-23).[671] This peptide contains a segment homologous to ERM proteins, like protein 4.1R. As noted in the section on spectrin, protein 4.2 binds to the EF hand domain of α spectrin in vitro.[486] Whether protein 4.2 binds to this site in vivo is not yet known, but if it does it creates an interesting dilemma because ankyrin is at one end of the spectrin heterodimer and the EF hands are at the other. An apparent Ca^{2+}-binding site lies next to the spectrin-binding site (see Fig. 15-23) and is probably functional because micromolar concentrations of Ca^{2+} triple the binding of the EF hand domain of spectrin to protein 4.2.[486]

Protein 4.1R Binding. Unpublished studies show that protein 4.2 also binds to protein 4.1R (kD ~ 1.6×10^{-6} M) in a ratio of one 4.1R molecule for every two molecules of 4.2.[672] The binding affinity is reduced about 75% by

Ca^{2+}-calmodulin. This is more evidence in favor of protein 4.2 participating in the band 3/spectrin/actin junctional complex as well as the band 3/ankyrin/spectrin complex.

Posttranslational Modifications

Protein 4.2 has a nucleotide-binding P-loop (residues 342 to 352) and binds ATP with a kD of approximately 10^{-6} M.[601] This is well below the concentration of ATP in the red cell (1.5 to 1.9 mM), so protein 4.2 should be saturated with ATP under physiologic conditions. The consequences of this binding are unknown.

Protein 4.2 is myristoylated on the N-terminal glycine[673] and is palmitoylated on Cys203,[674] which suggests the protein binds independently to the lipid bilayer. It is likely that most of the 4.2 molecules are myristylated.[673] The extent of palmitoylation is unknown. The myristoyl group seems to influence cellular localization because nonmyristoylated 4.2 does not go to the plasma membrane.[675] As noted earlier, the palmitoyl group enhances binding of protein 4.2 to band 3 at least threefold.[664]

Protein 4.2 Deficiency Is a Cause of Hereditary Spherocytosis and Ovalostomatocytosis

Mutations in the human protein 4.2 gene are associated with the production of dysmorphic and osmotically fragile red cells, resulting in hemolytic anemia (see "Hereditary Spherocytosis" in Chapter 16). Protein 4.2 null mice also have mild hereditary spherocytosis.[221]

Peroxiredoxins

There are six known mammalian peroxiredoxins (PRDX1 to PRDX6). Peroxiredoxin-2 is the most common in red blood cells and is the third most abundant protein in the cell. Peroxiredoxin-1 and peroxiredoxin-6 are present in smaller amounts. The peroxiredoxins are widely distributed and conserved in evolution. They protect cells from damage by reactive oxygen species and regulate signal transduction pathways that influence cell growth and apoptosis.[676]

Peroxiredoxin-2

Peroxiredoxin-2, also known as thioredoxin peroxidase B, calpromotin, thiol-specific antioxidant protein, protector protein, natural killer–enhancing factor B, acidic peroxidoxin, torin, and band 8, is 198 residues in length (~22 kD) and is a soluble cytoplasmic protein that translocates to the membrane in the presence of Ca^{2+}. There are approximately 14 million copies per cell,[677,678] but only about 200,000 molecules appear to be associated with the membrane in normal red cells, judging from the intensity of band 8 on SDS gels. The protein binds to the N-terminus of band 3,[183] where it remains active and presumably competes for a "parking space" with deoxyhemoglobin, multiple glycolytic enzymes, and ankyrin (see Fig. 15-7). The expression of peroxiredoxin-2 begins in early erythroid development, prior to the start of hemoglobin synthesis.[168] In common with the glutathione peroxidases, peroxiredoxins are able to reduce hydrogen peroxide and lipid hydroperoxides to water or the corresponding alcohol, respectively. The activity of peroxiredoxin 2 depends on a pair of highly conserved cysteine residues that catalyze the peroxide reduction step. Thioredoxin is the hydrogen donor.

In the red cell, peroxiredoxin-2 activates the Gárdos channel, a charybdotoxin-sensitive Ca^{2+}-dependent K^+ channel, which leads to the loss of K^+ and water.[677] This activation is associated with increased Ca^{2+}-dependent binding of dimeric peroxiredoxin (calpromotin) to the red cell membrane as a pentamer of the dimers.[414,679] Peroxiredoxin-2 is especially abundant on the red cell membranes of sickle cells,[680] particularly the dense, dehydrated fraction of sickle cells. Thalassemic red cells also contain increased peroxiredoxin-2 but less is bound to the membrane, perhaps because the binding sites on band 3 are blocked by oxidized hemoglobin (hemichromes).[681] Interestingly, peroxiredoxin-2 binds stomatin,[414] a protein that is located in lipid rafts and is mistrafficked in hereditary stomatocytosis (see Chapter 16).

Peroxiredoxin-2 is extremely efficient at scavenging hydrogen peroxide (H_2O_2).[682] It can rapidly detect and remove even the small amounts generated endogenously by hemoglobin autooxidation. In the process the enzyme is oxidized to a disulfide-linked homodimer. Because there is very little thioredoxin reductase activity in red cells, the oxidized enzyme is recycled slowly. However, the concentration of peroxiredoxin-2 is high enough to competently protect the red cell against low concentrations of H_2O_2. Mice lacking peroxiredoxin-2 have a hemolytic anemia that appears spontaneously by about 2 weeks of age and is characterized by Heinz bodies, burrs, spherocytes, and dense red cells.[683,684]

Peroxiredoxin-1

Despite its low concentration, peroxiredoxin-1 also seems to play an important oxidant-protective role in the red cell. Mice with a Prdx1 knockout develop a severe oxidant-type hemolytic anemia beginning at about 9 months of age and later develop several types of cancer.[685] The anemia features reactive oxygen species, protein oxidation, hemoglobin instability, and Heinz bodies.

ORGANIZATION OF THE MEMBRANE SKELETON

Models of the membrane skeleton, such as that shown in Figure 15-6, have existed, with increasing complexity, since 1979.[686] Current models* all have three major features: (1) a network of spectrin interconnecting short F-actin filaments (protofilaments) and associated proteins with the aid of protein 4.1R, which is attached to (2) a complex of ankyrin linked to band 3 tetramers and other associated proteins (the "ankyrin complex," Table 15-3), and (3) a complex of proteins linking the actin junctions to the overlying lipid bilayer (the "actin junctional complex," Table 15-4). Fowler has recently published a superlative review of this topic.[606a]

Spectrin/Actin/Protein 4.1R Membrane Skeleton

- Electron micrographs of spread membrane skeletons show a roughly hexagonal network of spectrin tetramers and some hexamers interconnecting F-actin protofilaments (see Fig. 15-16).[464,509,510]

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*References 163, 222, 421, 486, 542, 543, 606a, 687.

TABLE 15-3 Composition of the Ankyrin Complex

Protein	**Oligomeric State**	No. of Oligomers Complex*	No. of Oligomers Unit Cell†	**Interactions in Mature Erythrocyte Membranes**
Ankyrin	Monomer	1	3	Spectrin, band 3, protein 4.2, RhAG, CD44
Aldolase	Tetramer	Unk*	Unk*	PGK, PGK, enolase, PK, LDH
Band 3	Dimer	2	6	Ankyrin, GPA, GPB, Glut1, protein 4.2, CAII, CAIV, G3PD, PGK, aldolase, PRDX2, adducin, protein 4.1R, stomatin, deoxyhemoglobin
CD47	Monomer	Unk*	Unk*	Protein 4.2, RhD/RhCE/RhAG
G3PD	Tetramer	Unk*	Unk*	PGK, aldolase, enolase, PK, LDH
GPA/B	Homodimer or heterodimer	2	6	Band 3, RhAG (GPB)
LW (or ICAM-4)	Monomer	Unk*	Unk*	RhD/RhCE/RhAG
PFK	Tetramer	Unk*	Unk*	G3PD, aldolase, enolase, PK, LDH
Protein 4.2	?Monomer	2	6	Band 3, ankyrin, spectrin, CD47, protein 4.1R
RhAG/RhD/RhCE	Heterotrimer	1	3	CD47, LW, ankyrin, CD47, GPB
Spectrin (α/β)‡	Heterodimer	2	6	Spectrin, ankyrin, protein 4.1R, protein 4.2, actin, adducin, dematin, Lu/BCAM

CAII, Carbonic anhydrase II; *CAIV,* carbonic anhydrase IV; *G3PD,* glyceraldehyde-3-phosphate dehydrogenase; *Glut1,* glucose transporter 1; *GPA,* glycophorin A; *GPB,* glycophorin B; *LDH,* lactic dehydrogenase; *Lu/BCAM,* Lutheran antigen/BCAM protein; *LW,* Landsteiner-Wiener glycoprotein; *PFK,* phosphofructokinase; *PK,* pyruvate kinase; *PRDX2,* peroxiredoxin 2; *RhAG,* Rh-associated glycoprotein; *Unk,* unknown.

*G3PD, PFK, and aldolase bind to band 3 as a glycolytic enzyme complex, but there are too few molecules of each to have one such complex per ankyrin complex. Similarly the amounts of CD47 and LW are much less than stoichiometric, so only some ankyrin complexes contain these proteins.

†There are three ankyrin complexes per unit cell (i.e., six complexes, each shared with two adjacent cells).

‡The spectrin dimers in the ankyrin complex are shared with the actin junctional complex.

- There is one double-helical tropomyosin heterodimer containing seven actin-binding sites attached to each F-actin strand,[156] one adducin heterodimer capping the fast-growing end of the actin,[589] and two tropomodulin monomers capping the slow-growing end.[607]
- The numbers of adducins and tropomodulins (see Table 15-2) indicate that there are 30,000 to 40,000 actin protofilaments per red cell. This translates to six to eight G-actins in each of the double-helical F-actin strands. A two-stranded protofilament seven actins long would be approximately 37 nm, which is what is seen in vivo (see Fig. 15-16),[425,464,509,510] and is also the length of a tropomyosin molecule.
- Forty thousand protofilaments also corresponds with the numbers of spectrins and proteins 4.1R (240,000) if six of each bind per actin filament.
- Because the red cell surface area[15] is approximately 140 μm^2, the 40,000 hexagonal unit cells each cover 3,500 nm^2. This means spectrin dimers are only about 32 nm long in vivo (Fig. 15-24, *A*), which is only one third of their contour length (100 nm) in stretched skeletons or in vitro after extraction from the membrane.
- It is not certain how spectrin folds in vivo, but the best data[425] indicate the two spectrin chains are tightly coiled about each other and are relatively straight. They stretch and retract by changing their pitch and diameter (see Fig. 15-15).
- Spectrin heterodimers must occupy two actin subunits when bound, either directly or due to steric interference. Details of the attachment of spectrin to F-actin and where protein 4.1R fits in the interaction are not known. Spectrin is likely to mimic other spectrin-family proteins such as α actinin (see Fig. 15-17),[487,688] dystrophin,[689] utrophin,[690] fimbrin,[691] and plectin,[692] but these proteins interact with F-actin in more than one way[688,690] and do not require protein 4.1R. In the case of spectrin, there must be some flexibility in the linkage or in spectrin because binding sites must wind around the helical actin filaments but the spectrin skeleton and its actin protofilaments[515] are planar and parallel to the membrane.

Ankyrin Complex

- Only one ankyrin binds to the two neighboring ankyrin sites on a spectrin tetramer, presumably because of steric interference.
- Two band 3 dimers bind to two different sites on the membrane-binding domain of ankyrin,[623,628] but it is not clear whether these are a true band 3 tetramer or two unconnected dimers.
- Band 3 is required for protein 4.2 expression on the membrane,[220] and the two proteins bind in vitro, but the binding site on band 3 and the stoichiometry (1:1) are not well defined.
- A separate Rh complex also binds to ankyrin[332] and probably to band 3. It contains a trimer of RhAG,

TABLE 15-4 Composition of the Actin Junctional Complex

Protein	Oligomeric State	No. of Oligomers in Complex and Unit Cell[*]	Interactions in Mature Erythrocyte Membranes
Actin	Protofilament (14 monomers)	1	Spectrin, protein 4.1R, tropomyosin, tropomodulin, adducin, chorein
Adducin (α/β)	Heterodimer	1	Actin, spectrin, band 3, Glut1, stomatin, NHE1, chorein
Aldolase	Tetramer	Unk[†]	PGK, PGK, enolase, PK, LDH
Band 3	Dimer	1-6[‡]	Ankyrin, GPA, GPB, Glut1, protein 4.2, CAII, CAIV, G3PD, PGK, aldolase, PRDX2, adducin, protein 4.1R, stomatin, deoxyhemoglobin
DARC	Unknown	Unk[†]	?Protein 4.1R
Dematin	Monomer	3	Actin, spectrin, Glut1
G3PD	Tetramer	Unk[†]	PGK, aldolase, enolase, PK, LDH
Glut1	Dimer and tetramer	1	Stomatin, dematin, band 3, adducin
GPA	Dimer	1	Band 3
GPC/D	?Monomer	6	Protein 4.1R, p55
Kell	?Monomer	Unk[†]	Kx
Kx	?Monomer	Unk[†]	Kell, ?protein 4.1R
PFK	Tetramer	Unk[†]	G3PD, aldolase, enolase, PK, LDH
Protein 4.1R	Monomer	6	Spectrin, actin, band 3, GPC/D, p55, protein 4.2, ?Kell/Kx, ?Duffy
Protein 4.2	?Monomer	1	Band 3, ankyrin, spectrin, CD47, protein 4.1R
Protein p55	?Dimer	1	GPC/D, protein 4.1R
Spectrin (α/β)[§]	Heterodimer	6	Spectrin, ankyrin, protein 4.1R, protein 4.2, actin, adducin, dematin, Lu/BCAM
Stomatin	Oligomer (9-12)	1	Glut1, band 3
Tropomodulin	Monomer	2	Actin
Tropomyosin	Homodimer	2	Actin

CAII, Carbonic anhydrase II; *CAIV,* carbonic anhydrase IV; *DARC,* Duffy antigen /chemokine receptor; *G3PD,* glyceraldehyde-3-phosphate dehydrogenase; *Glut1,* glucose transporter 1; *GPA,* glycophorin A; *GPC/D,* glycophorin C/D; *LDH,* lactic dehydrogenase; *Lu/BCAM,* Lutheran antigen/BCAM protein; *NHE1,* Na^+/H^+ exchanger 1; *PFK,* phosphofructokinase; *PK,* pyruvate kinase; *PRDX2,* peroxiredoxin 2; *Unk,* unknown.

[*] There is one actin junctional complex per unit cell.

[†] G3PD, PFK, and aldolase bind to band 3 as a glycolytic enzyme complex, but there are too few molecules of each to have one such complex in every actin junctional complex. Similarly the amounts of Kell and Kx are less than stoichiometric, so only some junctional complexes contain these proteins.

[‡] As noted in the text, it is not clear whether one band 3 associates with the actin junctional complex, which would fit with the stoichiometry of band 3-binding proteins like adducin, or whether as many as six band 3 molecules are present, which would fit with the stoichiometry of band 3-binding proteins like protein 4.1R.

[§] The spectrin dimers in the actin junctional complex are shared with the ankyrin complex.

RhD, and RhCE[330] and probably GPB.[287] CD47 and the LW are trace proteins that are sometimes part of this complex[330] (about 1 in 9 complexes contain CD47, about 1 in 30 contain LW). CD47 is linked to protein 4.2 and to the Rh proteins.[384-386]

- Similarly, some ankyrin complexes contain the glycolytic metabolon,[186] described earlier, bound to band 3, but there are only 6000 copies of the least prevalent member, phosphofructokinase,[179] so if the metabolon has constant stoichiometry, there can only be about 1 for each 20 ankyrin complexes.
- The ankyrin complexes are each shared with two unit cells so there are only three per unit cell (see Fig. 15-24, *B*).

Actin Junctional Complex

- This complex is less well defined. It seems clear that, on average, six spectrins and six proteins 4.1R bind per actin, and there is enough band 3, GPA, GPC/D, and probably enough glucose transporter-1, and stomatin to supply six complexes per actin filament. But there is only one copy of adducin per actin

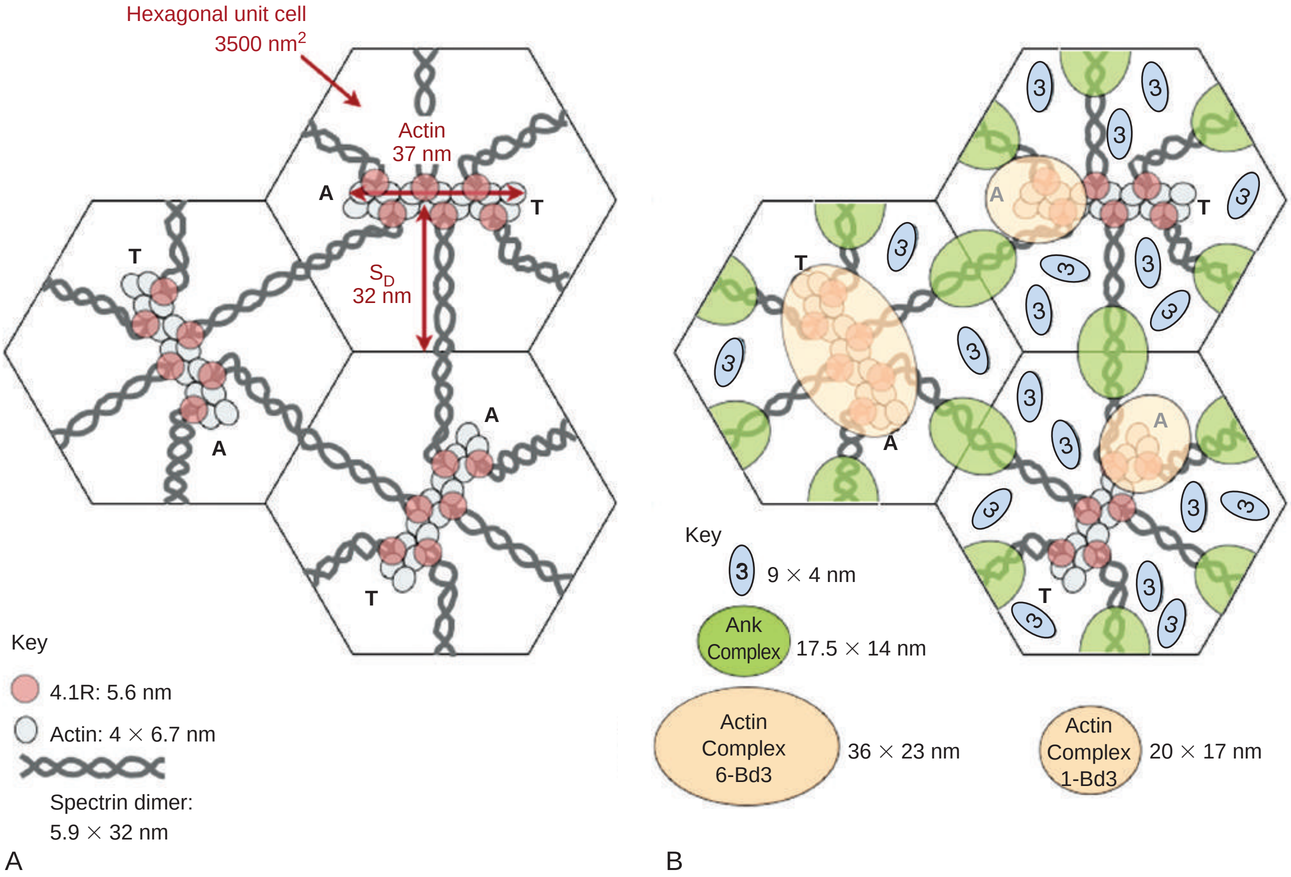

Figure 15-24 Scale model of the membrane skeleton unit cell. **A,** The membrane skeleton is a hexagonal lattice centered around about 40,000 F-actin protofilaments. Each 37-nm protofilament is capped by adducin *(A)* and tropomodulin *(T)* and contains about 14 G-actin subunits. The red cell surface area is about 140 µm^2, so each hexagonal unit cell is 3500 nm^2. Simple calculations show the average length of a spectrin dimer must be about 32 nm, which is one third of its length when fully stretched. The folding of the spectrin chains in this shortened configuration is not known for certain, but the best data indicate that they are relatively straight, are coiled about each other, and expand and contract by changing their pitch and diameter (see Fig. 15-15). **B,** Burton and Bruce[421] have modeled the structures of red cell membrane proteins and conclude that the cross-sectional area of the ankyrin complex *(green ovals)* is approximately 17.5 nm × 14 nm if the proteins are closely packed. The cross-sectional area of the actin junctional complex is about 20 nm × 17 nm if it only contains a single band 3 dimer *(small tan ovals)*. Such a complex would likely lie toward the end of the actin protofilament where adducin resides. This is shown in the two unit cells on the right. Alternatively, if the actin junctional complex contains six band 3 dimers and all six 4.1R proteins, it would probably be more centered and approximate the *large tan oval* in the unit cell on the left. In either arrangement it is likely the ankyrin complex and actin junctional complex would be close to each other and probably often touch, particularly during red cell deformation. Band 3 dimers that are not associated with either the ankyrin or actin junctional complexes *(small blue ovals)* diffuse in the lipid bilayer within the spectrin corrals. There will be three to eight of these per unit cell depending on the number of band 3 dimers in the actin junctional complex.

filament and only three copies of dematin if it is a monomer as recent studies indicate (see Table 15-4). Similarly, whereas protein 4.1R, GPC/D, and protein p55 are usually pictured as a three-part complex (see Fig. 15-19), there is only enough p55 for one copy per actin filament if it is a dimer, as some data suggest. And there is only about one copy of protein 4.2 available if 240,000 copies (one per band 3 dimer) are tied up in the ankyrin complex as is generally believed.

- Because the glucose transport channel (Glut1) binds to adducin,[420] dematin,[420] and stomatin,[413] and perhaps to band 3,[421] there is at least one and possible more copies of Glut1 and stomatin in the actin junctional complex.
- There is also evidence that the complex contains enzymes in the glycolytic metabolon[186] and the blood group proteins Kx/Kell and DARC,[542] although the amounts of these proteins are insufficient for even one copy per complex.
- It is unclear at the moment whether the actin junctional complex contains only a single band 3 dimer, interacting with just one or two of the six spectrin/protein 4.1R dyads or whether it is a larger complex containing three to six band 3 dimers that interact with all the spectrins and proteins 4.1R. In the latter case only a subset of the band 3 molegules would also bind to adducin. Both possibilities are depicted in Figure 15-24.
- In either case, one of the interesting and greatly underappreciated facts is that the *ankyrin and actin junctional complexes are quite close to each other in situ on the membrane and must often come in contact,* especially during red cell deformation, but

perhaps even when the cell is at rest. This raises the interesting possibility that proteins such as band 3, proteins 4.1R and 4.2, and adducin, which have binding partners in both complexes, may sometimes switch allegiances. Because some proteins compete for the same binding sites—protein 4.1R displaces ankyrin from band 3, for example—this could be a regulatory process.

Distribution of Band 3

- There are about 1.2 million band 3 molecules per red cell (average of many published measurements). Forty percent of the total are bound by ankyrin because each ankyrin binds two band 3 dimers.
- Depending on whether the actin junctional complex contains one or six band 3 dimers, an additional 7% to 40% of the band 3 dimers are located there.
- The remaining band 3 dimers (20% to 53%) are believed to be diffusing freely in the lipid bilayer, constrained for the most part by the boundaries of the spectrin corrals (see Fig. 15-24), although occasionally hopping from one corral to a neighboring one.[693] This is within the range of 25% freely diffusing band 3 measured in normal red cells using fluorescence-imaged microdeformation[384] and 46% measured using single particle tracking methods.[693]

Modulation of Membrane Skeletal Structure

Red cell membrane proteins are subject to a large number of posttranslational modifications or other regulatory effects: phosphorylation, fatty acid acylation, methylation, glycosylation, deamidation, ubiquitination, calpain cleavage, polyphosphoinositide and calmodulin regulation, oxidation, and modification by polyanions. A detailed discussion of all these pathways is beyond the scope of this chapter. A thorough review was published some years ago.[115] Table 15-5 lists the better characterized modifications and their effects.

Phosphorylation

In general, most of the membrane skeleton proteins are phosphorylated by one or more protein kinases—including band 3, protein 4.1R, ankyrin, α and β adducin, and dematin—and the added phosphate interferes with one or more protein interactions. Specifically, phosphorylation weakens band 3 binding to glycolytic enzymes, hemoglobin, and ankyrin; ankyrin binding to band 3; protein 4.1R binding to spectrin, band 3, and GPC; adducin capping actin and binding to spectrin; and the ability of dematin to bundle actin and promote spectrin-actin binding (see Table 15-5).

Ca^{2+}-Calmodulin

Similarly, Ca^{2+} in collaboration with calmodulin (CaM) influences the functions of several proteins (see Table 15-5) and these seem to be meaningful effects because physiologic concentrations of CaM, sealed in red cell ghosts, destabilize membranes in the presence, but not absence, of micromolar concentrations of Ca^{2+}.[695] The effects of Ca^{2+} begin at 10^{-6} to 10^{-7} M, which is relatively low, but still higher than the free Ca^{2+} concentration in the erythrocyte (20 to 50 nM[139,696]). Protein 4.1R, adducin, and α-spectrin functions are regulated by CaM. Ca^{2+}-CaM reduces the affinity of protein 4.1R for band 3, GPC, p55, and CD44, and it reduces adducin binding to F-actin and adducin-mediated enhancement of spectrin-actin binding.

Free Ca^{2+}

Among other things, free Ca^{2+} augments the binding of protein 4.2 to the EF hand domain. Another effect of free Ca^{2+} involves calpain, one of a family of Ca^{2+}-stimulated neutral proteases that are present in red cells[115] and other tissues. Susceptible membrane substrates include adducin, ankyrin, protein 4.1R, and, to a lesser extent, spectrin and band 3. The Ca^{2+}-induced K^+-channel (Gárdos channel),[697] described later in the chapter, is also triggered by Ca^{2+}. This unidirectional channel causes K^+ and water loss, producing cellular dehydration. Finally, Ca^{2+} also induces phospholipid scrambling, as discussed earlier in the chapter.

The mechanisms of Ca^{2+} entry into red cells are not well-understood, but investigations implicate the mechanosensitive Piezo1 cation channel and another nonselective cation channel called TRPC6 (both discussed later).

Polyanions

Physiologic concentrations of organic polyanions such as 2,3-DPG and ATP weaken and dissociate the membrane skeleton[698,699] and increase the lateral mobility of band 3 in ghosts.[700] Because hemoglobin F does not bind 2,3-DPG, free 2,3-DPG concentrations are much higher in fetal red cells. This may contribute to the increased severity of hereditary elliptocytosis during infancy in some patients (see Chapter 16).[699]

Biogenesis of the Membrane Skeleton

Surveys of the expression of membrane proteins during red cell development in mice[168] distinguish two patterns. Proteins such as band 3, ankyrin, RhAG, dematin, and the glycophorins are expressed early in erythropoiesis and often decline by the reticulocyte stage, whereas proteins such as spectrin, adducin, and proteins 4.1R, 4.2, and p55 are increasingly expressed throughout erythroid development (Fig. 15-25). Actin expression is nearly constant.[168]

Not much is known about how the membrane skeleton is put together in erythroblasts. Measurements of the sequential assembly of membrane protein complexes using modern proteomic techniques are badly needed. Fortunately, the necessary techniques for isolating pure populations of human erythroblasts are now available.[701] Early studies suggested that spectrin and ankyrin were made early, turned over rapidly, and did not assemble into a stable skeleton until band 3 and protein 4.1R were synthesized.[702,703] However, analyses show that the diffusion of band 3 in the membrane is gradually restricted throughout erythropoiesis and is not suddenly limited by rapid, coordinated assembly of the membrane skeleton.[704] In addition the early observations do not fit with data showing that band 3 is expressed before spectrin (see Fig. 15-25) or with the observation that mice lacking all band 3 assemble a functional, and nearly structurally normal spectrin-based membrane skeleton.[220] Clearly

TABLE 15-5 Regulation of Membrane Skeleton Proteins

Protein	Modification	Location	Effect	References
α Spectrin	Ca^{2+} binding	EF hands	Increases binding of protein 4.2.	486
	Calmodulin	EF hands	Blocks Ca^{2+} effect on 4.2 binding.	486
	Ubiquitination	α-Spectrin C-terminus	Impairs association with F-actin junctional complex.	507
β Spectrin	Phosphorylation	CKI: T2110, S2117, S2123, S2125, S2128	Phosphorylated by CKI. No effect on spectrin interactions in vitro but in vivo increased phosphorylation decreases RBC membrane stability.	454, 499, 500, 502, 503
Band 3	Phosphorylation	TK: Y8, Y21, Y359, Y904	Phosphorylated by $p72^{syk}$ and Lyn kinases. Blocks binding of glycolytic enzymes, and hemoglobin and increases glycolysis. Weakens ankyrin binding.	194, 224-227, 694
Ankyrin	Phosphorylation	Unknown	Phosphorylated by CKI and CKII. Sites unknown. Abolishes ankyrin preference to bind to spectrin tetramers instead of dimers. Decreases binding to band 3.	631, 638, 640, 641
	Hydroxylation	Membrane domain	FIH catalyzed. Modifies Asn residues in several ankyrin repeats. Stabilizes membrane domain and inhibits band 3 binding.	642
Protein 4.1R	Phosphorylation	PKC: S312 PKA: S331 & S467 TK: Y418	Phosphorylated by PKA, PKC, and a TK. Each decreases spectrin binding. PKC also blocks the interaction with band 3 and GPC.	115, 550, 553
	Calmodulin	A264-L280	Stabilizes membrane-binding domain.	555
	Ca^{2+}-calmodulin	A181-L197	Reduces affinity of 4.1R for band 3, GPC, p55, NHE1, and CD44.	537, 546, 547, 554
	PIP_2	Unknown	Enhances binding to the CH2 actin-binding domain of β spectrin and to GPC. Inhibits binding to band 3.	473, 558
α Adducin	Phosphorylation of neck region	PKA: S408, S436, S481 RhoK: T455, T480	RhoK enhances interaction of adducin with F-actin. Effect of PKA phosphorylation unknown.	593, 595
α Adducin & β Adducin	Phosphorylation of MARCKS domain	PKA/PKC: S726 (α) and S713 (β)	PKA decreases binding to and enhancement of spectrin-actin interactions. PKC inhibits actin capping and adducin-mediated recruitment of spectrin to actin. PKA or PKC inhibit calmodulin binding.	593, 594
	Ca^{2+}-Calmodulin	MARCKS domain	Reduces adducin binding to F-actin and adducin-mediated enhancement of spectrin-actin binding. Reduces PKA phosphorylation of β adducin and PKC phosphorylation of α and β adducin.	529, 590, 593
Protein 4.2	Myristoylation	G2	Required for membrane localization	673
	Palmitoylation	C203	Enhances binding of protein 4.2 to band 3.	664, 674
	ATP binding	G342-R352	Binds ATP. Function unknown.	601
p55	Palmitoylation	Unknown	Increases binding to membrane. Probably important for stability of lipid rafts.	153
Dematin	Phosphorylation	PKA: S74 PKC: Unk	Phosphorylated by PKA and PKC. PKA abolishes actin-bundling capability. Phosphorylation also abolishes dematin-spectrin binding and dematin ability to increase spectrin-actin interaction.	477, 602-604
Glut1	Stomatin	Unknown	Changes Glut1 to an ascorbic acid transporter.	413
Lu/BCAM	Phosphorylation	Unknown	Required for laminin binding.	366

ATP, Adenosine triphosphate; *CH,* calponin homology (actin-binding) domain of spectrin; *CKI or CKII,* casein kinases-1 and 2; *GPC,* glycophorin C; *FIH,* Factor-inhibiting hypoxia-inducible factor; *Glut1,* glucose transporter-1; *MARCKS,* myristoylated alanine-rich C kinase substrate; *NHE1,* sodium-hydrogen exchanger-1; *PIP_2,* phosphatidylinositol 4,5-bisphosphate; *PKA,* cAMP-dependent kinase; *PKC,* protein kinase C; *RhoK,* Rho kinase; *TK,* tyrosine kinase; *Unk,* unknown.

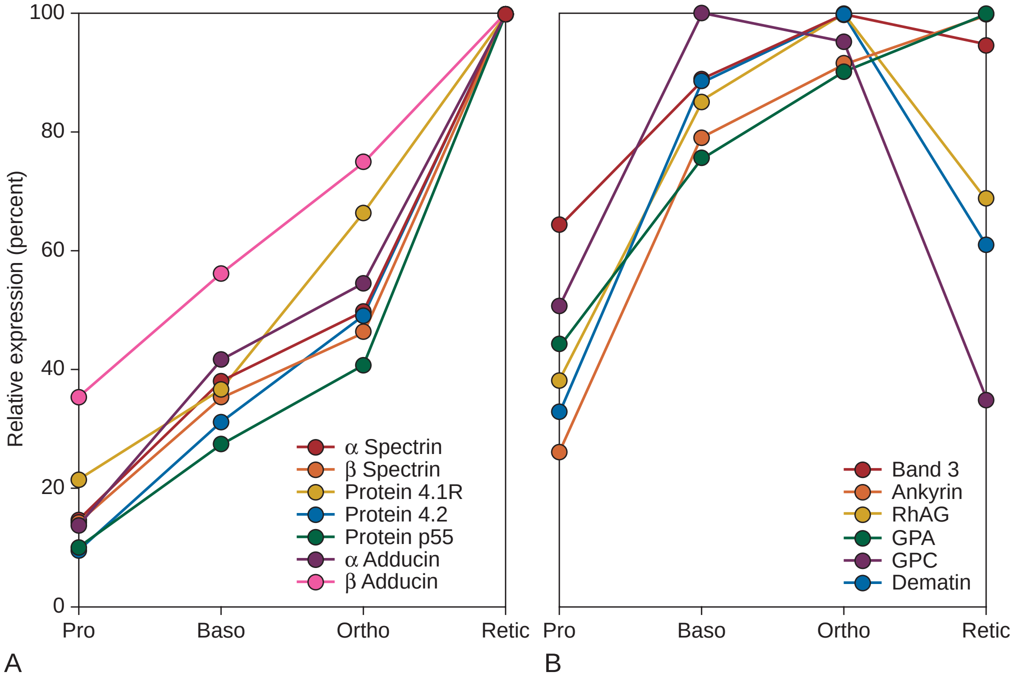

Figure 15-25 Relative expression of mouse red cell membrane proteins at different stages of erythroid development.[168] **A,** Proteins whose expression increases throughout erythropoiesis. **B,** Proteins that are expressed early in erythropoiesis. Actin expression *(not shown)* is nearly constant throughout erythropoiesis. *Pro,* Proerythroblast stage; *Baso,* basophilic erythroblast stage; *Ortho,* mixture of polychromatophilic and orthochromic erythroblast stages; *Retic,* reticulocyte stage.

other membrane sites must bind spectrin and ankyrin in the absence of band 3. The ankyrin/Rh/RhAG, protein 4.1R/p55/GPC, and Glut1/adducin/dematin/actin complexes are good candidates.

Rate-Limiting Steps of Membrane Skeletal Assembly

Spectrin synthesis is highly asymmetric: α-spectrin synthesis exceeds β-spectrin synthesis by at least threefold.[705] Yet the two chains are assembled into the membrane skeleton in equimolar amounts as mixed heterodimers. As a consequence, β spectrin is rate limiting in spectrin assembly. Defects that compromise β-spectrin synthesis are inherited in a dominant pattern, whereas α-spectrin synthetic defects are recessive (see “Hereditary Spherocytosis” in Chapter 16). Individuals missing one complete α-spectrin allele are asymptomatic because sufficient α spectrin is still made to pair with all the β spectrin.

The second rate-limiting step is the availability of ankyrin, which provides the high-affinity binding sites for β spectrin. This is best illustrated by studies of membrane skeletal synthesis in spherocytic mice with ankyrin mutations[653,668] and in a severe form of human hereditary spherocytosis associated with combined deficiency of spectrin and ankyrin.[706] In both disorders ankyrin synthesis is markedly reduced, which leads to decreased assembly of spectrin and ankyrin on the membrane, in spite of normal spectrin synthesis.

Membrane Remodeling During Enucleation and Reticulocyte Maturation

At the orthochromatic erythroblast stage, when membrane biogenesis is nearly complete, the cell membrane undergoes a series of critical remodeling steps. The cell nucleus is isolated by a contractible actin ring, which forms on the cell membrane at the boundary between atoplasm and the nucleus and participates in expelling of the nucleus from the erythroblast. This process requires Rac GTPases and mDia2, one of the formin proteins that nucleate linear actin filaments.[707] Rac1 and Rac2 bind to mDia2 in a GTP-dependent manner, and mDia2 causes the contractile actin ring to form.

During enucleation, the spectrin skeleton, proteins that attach to it, and selected other proteins segregate into the incipient reticulocyte while some surface receptors and presumably excess or unattached membrane proteins cluster in the membrane surrounding the soon to be extruded nucleus and are lost along with the bulk of the endoplasmic reticulum and nuclear proteins (Fig. 15-26, *A*).[708-710] The discarded membrane proteins include part of the band 3, GPC, Glut1, CD44, and stomatin.[536,710] In patients with hereditary spherocytosis due to ankyrin deficiency, GPA and RhAG, which normally partition into the reticulocytes, are also lost.[536] Similarly, in protein 4.1R–deficient red cells, even more GPC is lost with the nucleus.[536]

Further membrane remodeling takes place by exocytosis as the young multilobular reticulocyte attains the biconcave shape (see Fig. 15-26, *B* and *C*).[711-713] This is best studied in the case of transferrin receptors, which enter the reticulocyte by endocytosis and are segregated into vesicles called “exosomes” within large membrane-bounded sacs called multivesicular bodies. These fuse with the plasma membrane, discharging the exosomes and their protein cargo (see Fig. 15-26). The transferrin

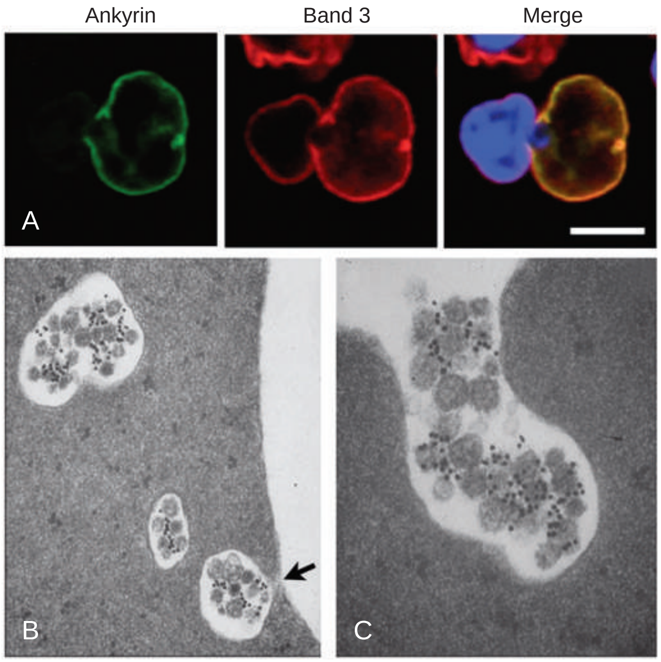

Figure 15-26 Maturation of the reticulocyte membrane. **A,** During enucleation, proteins attached to the membrane skeleton, such as ankyrin, segregate with the incipient reticulocyte, whereas a portion of the unattached membrane proteins, such as band 3, are lost with the nucleus. *Bar* = 5 μM. **B,** As reticulocytes mature, transferrin receptors, labeled with gold-bound antibodies, collect in small vesicles (exosomes) within intracellular sacs called multivesicular bodies, which approach the plasma membrane and begin to fuse *(arrow)*, with eventual release (**C**) of exosomes from the cell. A variety of membrane and cytoplasmic proteins are released by this mechanism. *(**A**, From Bell AJ, Satchwell TJ, Heesom KJ, et al: Protein distribution during human erythroblast enucleation in vitro.* PLoS One *8:e60300, 2013. **B** and **C**, From Johnstone RM: Revisiting the road to the discovery of exosomes.* Blood Cells Mol Dis *34:214–219, 2005.)*

receptor is lost with heat shock protein 70, which binds to the cytoplasmic domain and seems to act as a chaperone.[714] A variety of other membrane proteins are lost by this mechanism, including the Na^+,K^+-ATPase; aquaporin-1; Glut4 glucose transporter; CD47; nucleoside transporter; LFA3; α4β1 integrin, which binds fibronectin; small GTP-binding proteins such as Rab 4p, Rab 5p, and adenosine diphosphate ribosylation factor; GPI-linked lipid raft proteins such as acetylcholinesterase, CD55, and CD59; and other raft proteins such as stomatin and flotillin.[713] Ubiquitination appears to be a signal that directs proteins into the multivesicular bodies via the ESCRT endosomal sorting machinery.[713] Naturally occurring IgM antibodies to Lyso-PC bind the released exosomes, lead to C3 deposition, and are probably involved in exosome clearance.[715]

Cytosolic actin and tubulin are also lost as reticulocytes mature, probably by ubiquitin-proteosome degradation; and in addition to the proteins just mentioned, the amounts of myosin, tropomyosin, NHE-1, LW glycoprotein, GPA, Duffy, and Kell in the membrane are reduced by undefined mechanisms.[716]

Autophagocytosis also plays an important role in removing mitochondria, cytoplasmic organelles, and some membrane receptors. Cholesterol-fed mice that lack the high-density lipoprotein receptor and apoprotein E are anemic with targeted, macrocytic red cells and reticulocytes containing large abnormal autophagolysosomes.[717] The cholesterol-loaded reticulocytes also retain ribosomes and transferrin receptor. Removal of cholesterol, incubation in normolipemic serum, or transfusion into normal recipients leads to expulsion of the autophagolysosomes and reinitiation of reticulocyte maturation.[717] How cholesterol accumulation inhibits reticulocyte maturation remains to be determined.

RED CELL MEMBRANE DEFORMABILITY AND STABILITY

Material Properties

The material properties of the membrane reflect the properties of both the lipid bilayer and the skeleton. During deformation of the red cell membrane, bending is restricted by the incompressibility of the lipid bilayer and is facilitated by rapid translocation of cholesterol from the inner to the outer half of the bilayer.[718] The lipid bilayer cannot expand its surface area more than 3% to 4%. Consequently, when red cells are suspended in hypotonic solutions, such as during osmotic fragility testing, they swell to a spherical shape and then rupture, discharging their hemoglobin into the supernatant.[719]

The membrane skeleton determines both the solid and semisolid properties of the membrane.[720] The solid properties are exemplified by an elastic extension of cells, which completely restore their normal shape after the applied force has been removed. This elastic recovery of the biconcave shape is facilitated by the unique molecular anatomy of the skeletal lattice. In normal red cells individual spectrin molecules are arranged in a quasi-hexagonal array and are twisted in a compact configuration (see Figs. 15-15 and 15-24). The junctional complexes are close to each other and are linked by shortened spectrin tetramers, thus allowing large unidirectional extensions without disruption of the lattice. The skeletal connections are unperturbed during such deformations. On the other hand, application of large or prolonged forces allows the skeletal elements to reorganize and make new connections. This produces a permanent "plastic" deformation (see later). When the force is excessive, membrane fragmentation ensues. An example is the poikilocytosis produced in microangiopathic blood vessels where red cells may adhere to damaged endothelium and be stretched by the vascular torrent or may be "clotheslined" by fibrin strands.[721] After release, many of the cells are either permanently deformed or fragmented.

Cellular and Molecular Determinants of Red Cell Deformability

The need to undergo large deformations is best exemplified in the wall of the splenic sinus where red cells have to "squeeze" through narrow slits between the endothelial cells that line the splenic sinus wall (Fig. 15-27) as the exit the splenic cords and return to the circulation

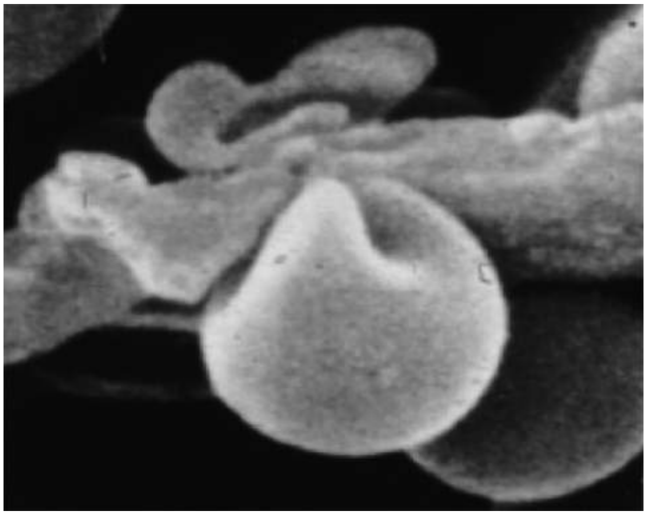

Figure 15-27 Red cell traversing the narrow passage between the endothelial cells separating the splenic cords from the splenic sinuses. Note the marked deformation the cell experiences. *(From Mohandas N, Gallagher PG: Red cell membrane: past, present and future.* Blood *112:3939–3947, 2008.)*

(see discussion of splenic structure and function in Chapter 23). This whole-cell deformability is determined by three factors: (1) cell geometry, that is, a large cell surface-to-volume ratio, which allows cells to undergo large deformations at a constant volume; (2) viscosity of the cell contents, which is principally determined by the properties and the concentration of hemoglobin in the cells; and (3) intrinsic viscoelastic properties of the red cell membrane.[720] Among these factors, the contribution of the surface-to-volume ratio is the most important, as exemplified by the cellular lesion in hereditary spherocytosis. Cellular dehydration, as occurs in hereditary spherocytosis and hereditary xerocytosis (see Chapter 16), sickle cell disease (see Chapter 20), and hemoglobin C disease (see Chapters 19 and 20) is also important because the viscosity of hemoglobin rises extremely rapidly once the concentration of hemoglobin exceeds the physiologic range (Fig. 15-28). Because 97% of the protein in the red cell is hemoglobin,[722] its viscosity has a profound effect on the viscosity of the red cell as a whole.

On the other hand, the intrinsic deformability of the red cell membrane has a relatively small effect on red cell survival. This is best illustrated by the red cell membrane properties of Southeast Asian ovalocytes (SAO; see Chapter 16), which carry a mutant band 3 protein. Both the intact SAO red cells and their membranes are extremely rigid,[723] yet the SAO red cells have a normal or nearly normal survival in vivo.

Some integral proteins may also influence membrane deformability. For example, treatment of red cells by antibodies against GPA, including their Fab fragments, leads to reduced deformability via transmembrane signaling. Red cells that lack the cytoplasmic domain of GPA (e.g., Miltenberger V red cells) are not influenced by such treatment.[262,282]

Membrane Structural Integrity and Fragmentation

The skeleton is the principal determinant of membrane stability. As noted earlier, it is possible to manipulate the proportion of spectrin dimers and tetramers in situ by exposing ghosts to temperatures and salt concentrations that favor or discourage self-association. Ghosts enriched in spectrin dimers are strikingly fragile.[459] Similarly, hereditary elliptocytosis and pyropoikilocytosis is usually due to α- or β-spectrin mutations that weaken spectrin self-association (see later sections). In such cells the quasi-hexagonal skeletal lattice is disrupted,[724] which in severe cases leads to red cell fragmentation and poikilocytosis.

Lipid Bilayer Loss and Microvesiculation. The fragile lipid bilayer is stabilized both by the underlying membrane skeleton and the transmembrane proteins. In vitro, the bilayer can be uncoupled from the skeleton at the tips of spiculated red cells by force or by various treatments (Fig. 15-29).[725,726] The lipids are released in the form of microvesicles that contain integral proteins but lack skeletal components.[726] Such loss of membrane material may underlie the surface area deficiency of red cells in hereditary spherocytosis[727] or red cells subjected to prolonged storage[728,729] or ATP depletion.[730] Aggregation of the band 3–containing intramembrane particles in the membrane also destabilizes the lipid bilayer.[165] The particle-free regions bleb and release lipid microvesicles.

Plastic Deformation of Red Cells

The role of the membrane skeleton in red cell shape is best illustrated by irreversibly sickled cells or hereditary

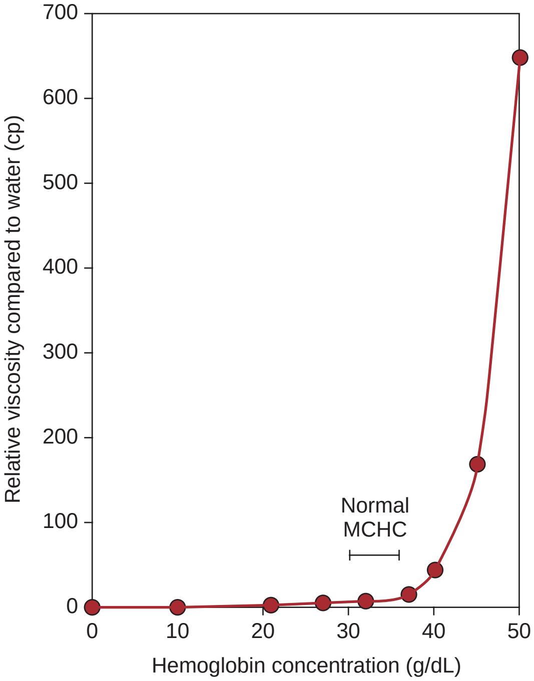

Figure 15-28 Relationship of mean corpuscular hemoglobin concentration *(MCHC)* to the relative viscosity of a hemoglobin solution, where the viscosity of water is 1 centipoise *(cp)*. Note that red cells rapidly become extremely rigid at MCHC values greater than 40 g/dL.

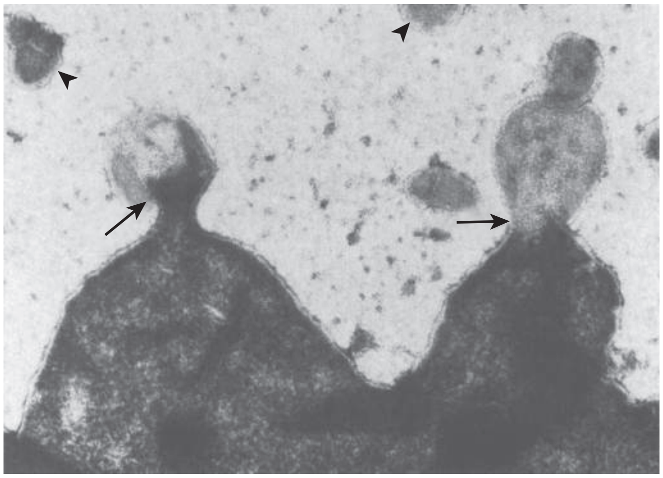

Figure 15-29 Microvesiculation. Red cell ghosts treated with hypertonic saline develop spicules. At the end of the spicules the lipid bilayer often separates from the underlying membrane skeleton. The *arrows* point to the boundary between the skeleton *(dark material below)* and the unsupported lipids *(light material above)*, which are unstable and tend to be released as vesicles *(arrowheads)*. A similar process is believed to occur in a variety of disorders associated with spiculation, such as spur cell anemia and hereditary spherocytosis (see Chapter 16).

elliptocytes, in which the abnormal shape is retained in the ghosts and membrane skeletons.[731,732] This process is probably an example of "plastic deformation," the result of prolonged exposure of red cells to deforming forces, when the proteins of the deformed skeleton rearrange and permanently stabilize the cells in the deformed shape (Fig. 15-30).[720] Existing protein-protein contacts disconnect, and new associations form. In hereditary elliptocytosis, shape transformations may be facilitated by the weakened skeletal protein interactions. In vitro, plastic deformation occurs more rapidly in normal red cells with skeletons weakened by exposure to urea.[733]

In addition both normal and abnormal red cell shapes can be stabilized by intermolecular cross-linking of membrane proteins, either owing to a formation of intermolecular disulfide bridges induced by oxidants or by transamidative protein cross-linking catalyzed by a Ca^{2+}-activated cytosolic transglutaminase.[734] These protein modifications are like endogenous fixatives and permanently stabilize cell shape in vitro.

MEMBRANE PERMEABILITY

Normally the red cell membrane is nearly impermeable to monovalent and divalent cations, thereby maintaining a high potassium, low sodium, and very low calcium content. In contrast, the red cell is highly permeable to water and anions, which are readily exchanged by the water channel and the anion exchange protein (band 3) respectively. Glucose is taken up by the two glucose transporters, whereas larger charged molecules, such as ATP and related compounds, do not cross normal red cell membranes.

The major transport pathways in the human red cell membrane can be divided into four categories (Fig. 15-31):

1. Gradient-driven, passive transporters, which include exchangers, such as the anion exchanger (band 3) and Na^+-H^+-exchanger, and the cotransporters, in which the transmembrane movements of more than one solute are coupled in the same direction, such as the K^+-Cl^- cotransporters and the Na^+-K^+-Cl^- cotransporters.
2. Active transporters, such as the ouabain-inhibitable Na^+,K^+-ATPase pump and the Ca^{2+}-ATPase pump.
3. Channels, such as the aquaporin water channel and Kidd urea channel, discussed earlier, and the Piezo1 K^+ channel and the Ca^{2+}-activated K^+-channel (Gárdos channel), discussed later.

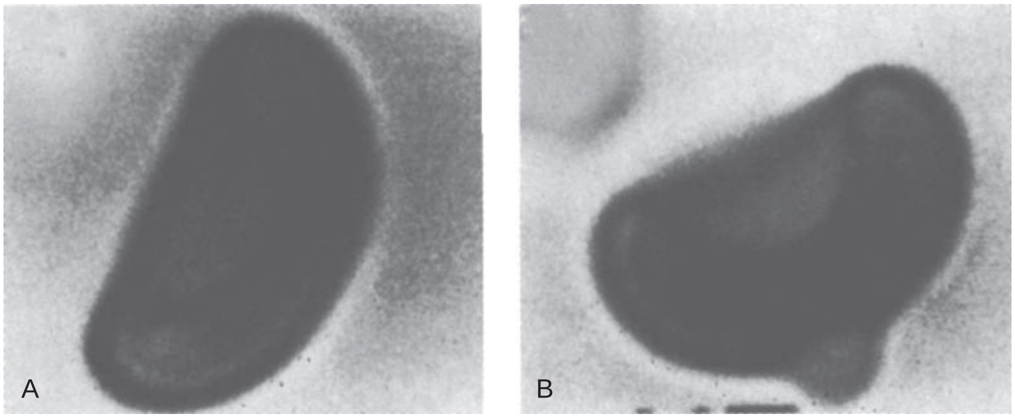

Figure 15-30 Plastic deformation. **A,** Discocytic shape of a red cell at rest in solution. If a nipple of membrane from such a cell is aspirated into a micropipette and released immediately, it snaps back to its original position. However, if the aspirated membrane is deformed for some time, a permanent nipple forms (**B**), as a result of reorganization of the membrane skeleton in the stressed portion of the membrane. This semisolid behavior is called "plastic deformation" and is believed to be responsible for the irregular shape of poikilocytes, especially in diseases such as hereditary pyropoikilocytosis, in which skeletal reorganization is fostered by genetically weakened skeletal interconnections (see Chapter 16). *(From Mohandas N, Chasis JA: Red cell deformability, membrane material properties, and shape: regulation by transmembrane, skeletal, and cytosolic proteins and lipids.* Semin Hematol *30:171-192, 1993.)*

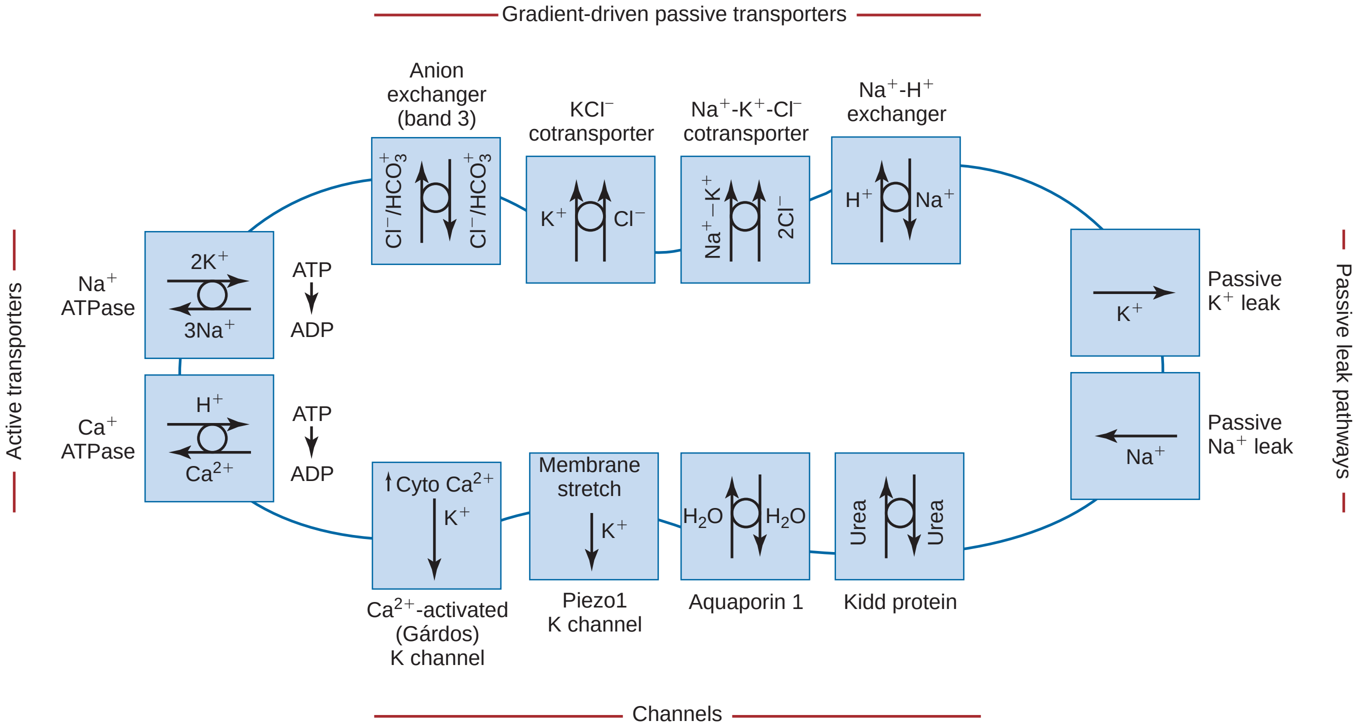
Gradient-driven passive transporters
Anion exchanger (band 3)
Cl⁻/HCO₃⁻
Cl⁻/HCO₃⁻
KCl⁻ cotransporter
K⁺
Cl⁻
Na⁺-K⁺-Cl⁻ cotransporter
Na⁺–K⁺
2Cl⁻
Na⁺-H⁺ exchanger
H⁺
Na⁺
Active transporters
Na⁺ ATPase
2K⁺
3Na⁺
ATP
ADP
Ca⁺ ATPase
H⁺
Ca²⁺
ATP
ADP
Passive K⁺ leak
K⁺
Passive Na⁺ leak
Na⁺
Passive leak pathways
Cyto Ca²⁺
K⁺
Ca²⁺-activated (Gárdos) K channel
Membrane stretch
K⁺
Piezo1 K channel
H₂O
H₂O
Aquaporin 1
Urea
Urea
Kidd protein
Channels

Figure 15-31 Major red cell membrane channels, active transporters, gradient-driven passive transporters, and passive permeability pathways.

4. The passive cation "leak" pathways, which allow Na^+ and K^+ to move in the direction of their concentration gradients.

A detailed review of these pathways is beyond the scope of this chapter. The interested reader is referred to several reviews.[146,696,735]

An important feature of the normal red cell is its ability to maintain a constant volume. One of the very intriguing, yet unanswered, questions is how cells "sense" changes in cell volume and activate appropriate volume regulatory pathways.

Membrane Pumps

To maintain low intracellular concentrations of sodium and calcium and a high potassium concentration, the membrane is endowed with two cation pumps, both utilizing intracellular ATP as an energy source.

Na^+,K^+-ATPase Pump

This well-known, ouabain-inhibitable pump contains a catalytic α-subunit, a β-subunit that interacts with the membrane skeleton, and a small modulatory γ-subunit. The protein extrudes sodium and takes up potassium, with a stoichiometry of three Na^+ ions pumped outward for two K^+ ions pumped inward. This exactly balances the normal passive leaks (Na^+ influx = 1.0 to 2.0 mEq/liters of red blood cells [LRBC]/hr; K^+ efflux = 0.8 to 1.5 mEq/LRBC/hr). The enzyme is activated by intracellular Na^+ (K_m = 25 mM) and extracellular K^+ (K_m = 2.5 mM).[736] Because plasma K^+ is high enough to saturate the extracellular K^+ site, Na^+ and K^+ transport is primarily regulated by increases in red cell Na^+ concentration and cell K^+ losses are rectified only if there is a concomitant Na^+ gain to stimulate active transport. Even then the ratio between the inward Na^+ leak and outward K^+ leak must not be less than the physiologic ratio of 3:2. At lower ratios, the Na^+,K^+-ATPase pump, driven by the Na^+ leak and internal Na^+ concentration will not balance the leaks and will actually exacerbate K^+ loss.[737,738] Consequently, conditions in which K^+ loss approaches or exceeds Na^+ gain lead to irreversible K^+ depletion and cell dehydration.

Ca^{2+}-ATPase Pump

This calmodulin and phosphorylation-activated, Mg^{2+}-dependent protein extrudes Ca^{2+} and maintains a very low intracellular free Ca^{2+} concentration (~20 to 50 nM).[139,696] It protects red cells from the multiple deleterious effects of free Ca^{2+} (echinocytosis, membrane vesiculation, calpain and transglutaminase activation, membrane proteolysis, and cell dehydration), described elsewhere in the chapter. Two of the multiple Ca^{2+}-ATPases are detectable in the red cell (Ca^{2+}-ATPase-1 and Ca^{2+}-ATPase-4).[168] Ca^{2+} extrusion is balanced by H^+ influx.

Transport Pathways Leading to Cellular Dehydration

Several pathways exert a critical volume regulatory effect that can lead to red cell dehydration and destruction. In all cases these pathways lead, directly or indirectly, to a net loss of cations, primarily K^+, which is accompanied by water.

K^+,Cl^- Cotransporter

This typical carrier-mediated cotransport pathway is particularly active in reticulocytes. It is activated by cell swelling, acidification, depletion of intracellular Mg^{2+}, thiol oxidation, and especially by erythrocyte oxygenation, which increases its transport activity about 20-fold.[739] Because band 3 is the only known binding site for deoxyhemoglobin on the membrane, and because a covalent inhibitor of band 3 anion exchange also inhibits the K^+-Cl^- cotransporter,[740] it is likely the K^+-Cl^- cotransporter associates with band 3 to facilitate this O_2-dependent regulation. K^+-Cl^- cotransporter function is increased in sickle red cells and hemoglobin C erythrocytes, accounting, in part, for cellular dehydration of these cells.[741,742]

Gárdos Channel

The second transporter is the Gárdos channel, also called the SK4 or KCa3.1 or KCNN4 channel. The pathway is not active in red cells under normal conditions. It opens and allows K^+ and water loss when intracellular Ca^{2+} rises.[697,743-745] A Ca^{2+} concentration of 0.3 to 2 μM is required for half-maximal activation of the channel. In T lymphocytes and presumably erythrocytes, KCNN4 is also activated by phosphatidylinositol-3-phosphate, which recruits a nucleoside diphosphate kinase-B (gene name *NME2*) that phosphorylates KCNN4 on His358 in the C-terminal region.[746] The channel is inhibited by insect toxins, such as charybdotoxin, and by clotrimazole and other imidazole antimycotics.[146] In sickle cells, the combination of the Gárdos pathway, the K^+-Cl^- cotransporter, and probably the Piezo1 channel (see next section) account for the net loss of K^+ and water, leading to cellular dehydration.

Piezo Channels

The two Piezo proteins, Piezo1 and Piezo2, are very large, widely distributed proteins. They form huge multimeric cation channels, with 120 to 160 transmembrane segments, that are triggered by mechanical distortion of the membrane.[747,748] The channels are nonselective: Na^+, K^+, Ca^{2+}, and Mg^{2+} are all permeable, with a slight preference for Ca^{2+}.[747] Piezo proteins are involved in pain sensing[747] and perhaps in other mechanical sensations such as hearing and touch. The presence of Piezo1 in the red blood cell membrane suggests the channel may also be involved in homeostatic adjustments to changes in volume, responding to membrane stretch. Piezo1 is defective in hereditary xerocytosis (see Chapter 16). Mutations in the protein are present in all 22 families in which sequencing is reported.

Whereas the Piezo1 channel is permeable to K^+, K^+ loss may also be a secondary consequence of excess Ca^{2+} entry through the channel. It is known that Ca^{2+} enters normal red cells when they are deformed.[749] It is likely that this occurs through Piezo1 and triggers Ca^{2+}-stimulated K^+-loss via the Gárdos channel. Indeed, Piezo1 appears to contribute to the dehydration of sickle cells because sickling increases the membrane permeability to Ca^{2+} and other ions, a process, called P^{sickle}, that requires mechanical deformation.[741,742,750,751] P^{sickle} is inhibited by *Grammastola spatulata* mechanotoxin-4,[752] an inhibitor of Piezo1,[753] and the Gárdos channel is a major contributor to K^+ loss and red cell dehydration in sickle cells.[754]

TRPC6 Channel

The TRPC6 channel is another nonselective cation channel that is believed to mediate Ca^{2+} entry into cells. It is activated by diacylglycerol[755] and by binding of phosphoinositides, especially PIP_3, to a C-terminal segment of the protein.[756] PIP_3 disrupts calmodulin binding to the protein. It is also activated by osmotic shock, ATP or chloride depletion, and oxidative stress.[757] Free Ca^{2+} concentration and the extent of red cell shrinkage and phospholipid scrambling are lower in stressed TRPC6 null red cells than in normal mouse erythrocytes,[757] which suggests this channel is physiologically important and participates in Ca^{2+}-induced red cell dehydration, at least under some conditions.

Other Pathways

As noted earlier, hyperactivity of the Na^+,K^+-ATPase pump, triggered by increased intracellular Na^+, can dehydrate red cells because three Na^+ ions are extruded in exchange for only two K^+ ions.

MEMBRANE CHANGES ASSOCIATED WITH AGING AND SENESCENCE OF RED CELLS

Pleiotropic Effects of Red Cell Aging

The removal of senescent red cells from the circulation is a subject of a long-standing interest and has recently been reviewed.[757a] As discussed in a comprehensive review of the literature published prior to 1988,[758] the concept of red cell senescence is based on results of radioactive labeling of a cohort of reticulocytes and bone marrow erythroblasts. In many species, including humans, the fraction of labeled cells remains constant for a defined time period, followed by a rapid decline of radioactivity, suggesting that the red cells are removed from the circulation in an age-dependent manner. Many techniques have been employed to isolate senescent red cells, including methods based on differences in cell density, osmotic fragility, and cell size.[758] Density separation is most widely used but is imperfect. Red cells exhibit a progressive increase in density as they age in vivo, but not all dense red cells are senescent.[758-760]

In addition to in vitro separation techniques, various in vivo animal models have been employed to study red cell aging.[758] For example, red cells can be labeled with biotin and then removed at defined times with avidin, which complexes tightly to biotin.[761] This is the best technique for obtaining a pure population of old red cells, but it has only been applied to humans recently.[762]

The senescent cells defined by these techniques exhibit numerous membrane abnormalities.[758,759,761] Of note is the loss of potassium and water, leading to cell dehydration and increased cell density, and the loss of surface area, presumably owing to gradual release of membrane microvesicles.[758,763] In addition, membrane proteins and lipids in old red cells are oxidized, as evidenced by high-molecular-weight aggregates containing hemoglobin, spectrin, and band 3,[764] and by adducts of proteins and malonylaldehyde, a product of lipid peroxidation. Red cell aging is also associated with a decline of many red cell enzyme activities and in the activity of certain channels, such as the K^+-Cl^- cotransporter.[762,765] However, red cell ATP concentration is normal[758] and there is no evidence that PS is exposed on the red cell surface.[762] Likewise no major abnormalities have been detected in Ca^{2+} transport and there is no compelling evidence that Ca^{2+} accumulates in senescent cells.[758] Importantly, numerous studies show that IgG accumulates on older red cells[762] and may help trigger the removal of senescent cells (see later).

Red cells stored for transfusion show many of the changes of aged red cells—the so-called "storage lesion." The protein alterations are mostly due to oxidation.[766] Degradation of membrane skeleton proteins is particularly prominent, especially protein 4.2. Cross-linking of α spectrin and β spectrin is also observed.[766] Many other cellular changes occur. The topic is beyond the scope of this chapter but has been reviewed elsewhere.[767]

Senescent Autoantibodies

An Autologous Autoantibody Targets Senescent Red Cells for Destruction by Macrophages. Although some of the alterations described above in old red cells may compromise red cell microcirculatory flow, most do not destroy the cells. Kay and colleagues first showed that senescent red cells have small amounts (a few hundred molecules per cell) of autologous IgG on their surface that target the cells for destruction by macrophages.[768] The topic was recently reviewed.[769] Studies suggest the senescent autoantibody arises from a distinctive subset of B cells, termed B-1, that express CD5.[770] The "senescent cell antigen" target appears to be part of the external surface of band 3 that is normally sequestered but becomes exposed when the molecule aggregates.[191,761,771-776] A few believe the epitope resides on the band 3 carbohydrate.[777,778] Band 3 clusters are seen by immunofluorescence microscopy and detected by biochemical methods in aged normal red cells.[191,772]

Aggregation probably results from cumulative oxidative damage because similar damage, including the binding of autologous IgG, takes place on oxidized red cells or red cells containing Heinz bodies, a product of oxidative denaturation of hemoglobin.[191,772,779-781] Both protein peroxidation and methemoglobin formation are needed for band 3 to cluster.[781a] The senescent cell antigen is also exposed on red cells infected with *P. falciparum*.[782,783] Intramembrane particles, which are mostly band 3, are not reproducibly clustered in cells that bear senescent IgG,[784] but this is probably because the clusters of band 3 are rare enough that they are missed by electron microscopy.

Band 3 autoantibodies also trigger complement deposition on senescent cells,[781,785] which accelerates their removal via CR1 receptors on phagocytes. As noted later, this process may be facilitated by microvesiculation and loss of GPI-linked complement regulatory proteins such as CD55 and CD59 as red cells age.

Although the role of autologous IgG in the destruction of senescent erythrocytes is widely accepted, two observations suggest that other mechanisms must be considered. First, blockade of the Fc portion of autologous "senescent" IgG with protein G fails to inhibit red cell phagocytosis.[786] Second, red cell survival is the same in agammaglobulinemic mice as in controls.[786] However, the latter data may not apply to humans, because the removal of mouse red cells from the circulation is random.[758]

Red cells uniquely express the LW blood group antigen or ICAM-4, which interacts with various β2 integrins, including CD11c/CD18 on macrophages. Antibodies to either component inhibit erythrophagocytosis and removal of senescent red cells, suggesting that this interaction contributes to the process.[787,788]

Vesiculation and Microparticle Formation

Blood microparticles or "blood dust" are small, membrane-derived vesicles of less than 1 μm released into the blood by endothelial cells, platelets, white blood cells, and red cells. They are believed to be involved in processes as diverse as inflammation, hemostasis, apoptosis, and vascular and immune function,[789] although it is hard to prove that they play a pathologically important role in any of these processes in vivo.

Microvesiculation occurs during reticulocyte maturation and to a lesser degree throughout the red cell's life span. It is triggered by damage to the connections between the lipid bilayer and the membrane skeleton and by processes such as shear stress, oxidative damage, complement, and eryptosis (see later). The vesicles display the senescent cell antigen (see earlier) as well as PS, which is recognized by scavenger receptors.[790] The fact that the vesicles are enriched in removal signals suggests their release may postpone the untimely removal of functional cells by phagocytosis.[791]

Microvesiculation may serve a housekeeping function, cleansing the red cell membrane of damaged proteins. Low and his colleagues find that oxidative cross-linking of band 3 causes the tyrosine kinase Syk to bind and selectively phosphorylate the oxidized protein.[224] This greatly weakens binding of band 3 and ankyrin, which in turn promotes band 3 diffusion within the lipid bilayer and collection of the damaged band 3 in clumps and membrane spicules, where it is believed to be lost by vesiculation.[228] Or perhaps it is "pitted" from the red cell membrane by the spleen because pitting may just be the coordinated release of vesicles from the membrane and their simultaneous phagocytosis by reticuloendothelial cells.

Red cell–derived microvesicles are also enriched in GPI-linked proteins, such as CD55 and CD59,[792-794] and in other lipid rafts components. Although the loss of complement defense proteins such as CD55 and CD59 would make older red cells more sensitive to complement attack,[795] there is also evidence that the membrane attack complex, C5b-C9, is actively shed in microvesicles when complement is activated.[796]

CD47 "Don't Eat Me Versus Eat Me" Switch

As noted in the earlier section on CD47, the protein serves as a marker of "self" in the mature red cell, interacting with the inhibitory receptor SIRPα on macrophages and inhibiting phagocytosis via complement and

IgG receptors but not via the scavenger receptor.[387-389] Mouse red cells that lack CD47 are rapidly cleared from the blood by normal splenic macrophages.[390] Old red cells have about 30% less CD47 than young red cells, and there is evidence that this may contribute to removal of aged red cells,[797] although reality must be more complicated because red cells lacking CeEe antigens have a normal life span despite diminished expression of CD47 and red cells lacking protein 4.2 nearly lack CD47 and yet experience only mild hemolysis.

Interestingly, there is recent evidence that a conformational change occurs in the CD47 of aged erythrocytes that causes the CD47 to bind thrombospondin-1. This converts CD47 from a “don’t eat me” to an “eat me” signal and leads to removal of the damaged red cells.[798] The stimulus for the conformational change is unknown, but oxidation seems a likely culprit based on what happens to many membrane proteins during red cell storage.[766] Although this is a very promising avenue of research, much more information is needed to understand whether CD47 really contributes to red cell demise.

Eryptosis

Red Cells May Undergo a Kind of Apoptosis in Response to Oxidative Damage. After an initial report by Bratosin in 2001,[799] a “blizzard” of brief papers, virtually all by Lang and his colleagues, have described a process resembling apoptosis in damaged erythrocytes that they term *eryptosis* and that can be triggered by a menagerie of insults and agents.[800,801] The process seems to be a forme fruste of apoptosis because it is not induced by classic apoptotic agents, such as staurosporine, and because mature red cells lack mitochondria and nuclei.[799] It is characterized[800,801] by decreased red cell volume, membrane blebbing, activation of the protease calpain, and PS exposure at the outer membrane leaflet that leads to phagocytosis by macrophages without cell membrane rupture and to adherence of the eryptotic cells to vascular endothelium.[802] Eryptosis is triggered by oxidative stress, an influx of Ca^{2+} ions, red cell dehydration, osmotic shock, prostaglandin E_2, ATP depletion, and a huge variety of other substances and stimuli[801] and is inhibited by erythropoietin and endothelin-1. It is not clear whether each of these stimuli induces the full panoply of eryptosis or just some of the aspects. At a minimum, eryptosis seems to require red cell shrinkage and externalization of PS.

The process resembles that induced by caspases. Investigations show that mature red cells contain Fas, Fas ligand, Fas-associated death domain, caspase 8, and caspase 3 and that oxidatively stressed cells recapitulate apoptotic events, including translocation of Fas into lipid rafts (see later), formation of a Fas-associated complex, and activation of caspases 8 and 3.[803-805] Activation of Fas is also associated with inhibition of the aminophospholipid translocase (flippase) and activation of the scramblase.[806] Thus exposure of PS in red cells, as in nucleated cells, may often be the consequence of apoptotic pathways. However, caspases are not required for eryptosis after Ca^{2+} entry.[801]

These findings raise the possibility that hemolysis in many conditions may be mediated by eryptosis, especially conditions associated with increased oxidant damage and/or cell dehydration, such as sickle cell anemia, thalassemia, Heinz body hemolytic anemias, hemoglobin CC disease, G6PD deficiency, and hereditary xerocytosis. If this is true, interference with the process should be therapeutic. Suicidal death of red cells may also be a defense mechanism against malaria and other erythrocytic parasites, promoting the clearance of infected cells.[807]

FoxO (Forkhead O) transcription factors regulate the cellular response to oxidative stress,[808,809] and the absence of FoxO3, in particular, leads to a marked decrease in expression of antioxidant enzymes in erythroid precursors and a marked susceptibility of mature red cells to the reactive oxygen species that red cells generate during their daily oxygen-carrying duties or to exogenous oxidants. Similarly, lack of the α_1 isoform of AMP-activated protein kinase found in erythrocytes lowers FoxO3 levels and triggers an oxidant-induced hemolytic anemia.[810] It will be interesting to see whether these red cells also die of eryptosis, as would be predicted from the earlier studies, and whether manipulation of the FoxO pathway can be used to treat anemias characterized by oxidant damage.

Finally, other corrosive processes, such external exposure of PS,[811] Ca^{2+}-induced transamidation,[812] or nonenzymatic glycosylation[813] or carboxymethylation[814,815] of membrane proteins are postulated to contribute to red cell demise, but there is little evidence for these speculations.

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CHAPTER 16

Disorders of the Red Cell Membrane

Samuel E. Lux

CHAPTER OUTLINE

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Hereditary Xerocytosis
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Classification Based on the Temperature Dependence of Cation Leaks
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ACQUIRED DISORDERS OF RED CELL MEMBRANE LIPIDS
Spiculated Red Cells: Echinocytes and Acanthocytes
Spur Cell Anemia
Obstructive Liver Disease: Target Cells

During erythropoiesis the red cell membrane responds to erythropoietin and imports the billion or so iron atoms each red cell needs to complete hemoglobin synthesis. It sequesters the reductants required to protect hemoglobin and other cell proteins from corrosion by oxygen, and selectively retains other vital components such as organic phosphates, manufactured with precious adenosine triphosphate (ATP) energy, but it lets metabolic detritus escape. It even helps regulate metabolism by reversibly binding and inactivating many glycolytic enzymes.[1] In the circulation it carefully balances cation and water concentrations so red cells do not shrivel or burst. Simultaneously, it exchanges tremendous numbers of bicarbonate and chloride anions (≈10 to 30 billion/second/red cell), which aids transfer of carbon dioxide from the tissues to the lungs.[2] It also maintains a slippery exterior so that red cells do not adhere to endothelial cells or aggregate and clog capillaries. Finally, buttressed by the "membrane skeleton," a protein scaffold that lines the inner membrane surface, the membrane achieves the critical combination of strength and flexibility needed to survive four months in the circulation. Failure of any of these, or numerous other, functions shortens red cell survival, a process termed hemolysis. In fact, all hemolysis is ultimately due to membrane failure. This chapter will focus on inherited disorders of red blood cell membranes, particularly hereditary spherocytosis and hereditary elliptocytosis, the two most common and best understood diseases.

HEREDITARY SPHEROCYTOSIS

Hereditary spherocytosis (HS) (Table 16-1) is a common, inherited hemolytic anemia in which defects of spectrin

TABLE 16-1 Diseases in which Spherocytosis Is the Predominant Morphology[*]

Common Conditions
Hereditary spherocytosis
Immunohemolytic anemias (warm antibody type)
ABO hemolytic disease in neonates[†]
Uncommon to Rare Conditions
Hereditary stomatocytosis[†]
Hereditary cryohydrocytosis[†]
Clostridial sepsis
Hemolytic transfusion reactions
Severe burns and other red cell thermal injuries
Spider, bee and snake venoms[†]
Severe hypophosphatemia[†]
Acute red cell oxidant injury (e.g., glucose-6-phosphate dehydrogenase deficiency during hemolytic crisis, Wilson disease)[†]
Congenital dyserythropoietic anemia, type II[†]

[*] Some spherocytes may be seen in other diseases where they are not the predominant red cell morphology (e.g., hereditary pyropoikilocytosis, Heinz body hemolytic anemias, severe liver disease, microangiopathic hemolysis, hexokinase deficiency).

[†] Spherocytes are the predominant morphology in only a subset of patients.

or of proteins that attach spectrin to the membrane—ankyrin, protein 4.2, or band 3—lead to spheroidal, osmotically fragile cells that are selectively trapped in the spleen, resulting in a shortened red cell life span.

History

HS was first recognized more than 140 years ago by two Belgian physicians, Vanlair and Masius, who gave a remarkably thorough account of the disease.[3] They described a woman who suffered from the clinical symptoms now known as hallmarks of HS—anemia, jaundice, splenomegaly, and recurrent abdominal pain. The authors noted that most of the woman's red cells were spherical (Fig. 16-1) and hypothesized that a combination of splenic enlargement and liver atrophy led to their rapid destruction and the patient's anemia. They also noted that the patient's sister had suffered from an identical illness. In the 1890s, the British physicians Wilson and Stanley recognized the hereditary nature of the disease and were the first to note the characteristic pathologic appearance of the spleen engorged with red cells.[4,5] Subsequently, a report by Minkowski in 1900, in the German literature,[6] received wide attention, and many additional papers soon appeared, including Chauffard's classic description of osmotic fragility[7] and reticulocytosis[8] as hallmark laboratory features of the disease. The first successful splenectomy for HS was unintentionally performed by Wells in England 20 years before splenectomy became widely accepted[9] and 3 years before Wilson's description of HS appeared. While operating on a jaundiced woman for a supposed uterine fibroid, Wells encountered and bravely removed an enormous spleen. The patient recovered and her jaundice disappeared. Forty years after the splenectomy performed by Wells, Dawson[9] found the abnormal erythrocyte osmotic fragility during an examination of the woman and her son. The major clinical features of HS were defined by the 1920s, although nothing was known about the pathophysiology of the disease. Readers interested in more details about these and other aspects of the history of HS should consult the chapters by Dacie, Crosby, and Wintrobe in the wonderful book Blood, Pure and Eloquent.[10] Recent reviews of HS are also available.[11,12,13,14,15,15a]

Prevalence and Genetics

HS occurs in all racial and ethnic groups. It is particularly common in Europeans and their descendants in the New World. The classic estimate is that 1 person in 5000 is affected,[16] but this is almost certainly an underestimate. Surveys of red cell osmotic fragility suggest that mild forms of the disease may be four or five times more common than that.[17-19] In addition, testing of Swiss patients identified as having hyperchromic red cells on a routine complete blood count led to a diagnosis of HS in 1 of every 1700 individuals, most of whom were asymptomatic.[20] So the prevalence in Europeans is probably closer to 1 in 1500 to 1 in 2000. The disease is also common in Japan[21] and most other countries. Clinical experience suggests it is less common in African Americans and Southeast Asians.

HS exhibits both dominant and nondominant inheritance. About 75% of patients have typical autosomal dominant disease.[16,22-24] Homozygotes for dominant HS are very rare, with only a handful of reported cases.[23,25-28] The remaining 25% of HS patients show nondominant inheritance.[29,30] In these patients, both parents are clinically normal but some of them exhibit subtle laboratory abnormalities that suggest a carrier state.[22,30,31] The only available molecular survey suggests that the majority of nondominant HS patients have new dominant mutations, which tend to occur at CpG dinucleotides and are associated with small insertions or deletions.[29,30] This study suggests that true recessive HS is quite rare, perhaps less than 5% of the total,[30] but larger-scale studies are needed.

HS is caused by unique mutations in the membrane skeletal proteins ankyrin, β-spectrin, α-spectrin, band 3, and protein 4.2.

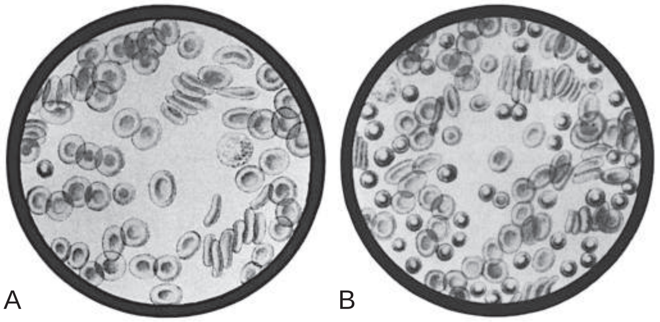

Figure 16-1 Lithograph of normal blood cells (**A**) and cells from a patient with "microcythemia" (**B**) described by Vanlair and Masius in 1871. This was the first description of hereditary spherocytosis. *(From De la microcythémie.* Bull R Acad Med Belg *5:515–613, 1871.)*

TABLE 16-2 Characteristics of Hereditary Spherocytosis

Clinical Features
Pallor
Splenomegaly
Intermittent jaundice
From hemolysis
From biliary obstruction
Anemic crises: hemolytic, aplastic or megaloblastic
Inheritance
Dominant ≈ 75%
Nondominant ≈ 25%
Rare manifestations
Leg ulcers
Chronic dermatitis
Extramedullary hematopoietic tumors
Angioid streaks
Spinocerebellar degeneration
Myocardiopathy
Excellent response to splenectomy
Laboratory Features
Anemia
Reticulocytosis
Spherocytosis
Elevated MCHC and elevated percentage of hyperchromic or hyperdense cells
Increased RDW (red cell distribution width, a measure of poikilocytosis)
Abnormality in one or more "HS tests"
Decreased band 3 (decreased fluorescence of red cells labelled with eosin-5′-maleimide)
Increased osmotic fragility (especially in the incubated osmotic fragility test)
Abnormal acidified glycerol lysis time or Pink test
Abnormal cryohemolysis test
Characteristic osmotic gradient ektacytometry profile
Normal Coombs (direct antiglobulin) test
Decreased red cell spectrin, or spectrin and ankyrin, or band 3 and protein 4.2, or absent protein 4.2 and decreased CD47

Clinical Presentation

Typical Hereditary Spherocytosis

The typical clinical picture of HS combines evidence of hemolysis (anemia, jaundice, reticulocytosis, gallstones, and splenomegaly) with spherocytosis and a positive family history (Table 16-2; Fig. 16-2). Mild, moderate, and severe forms of HS have been defined according to differences in various clinical and laboratory parameters under baseline conditions (Table 16-3).[13,22,32,33] Unfortunately, patients don't fit neatly into these categories: a person my be classified as "mild" by one criterion and "moderate" by another.[33a]

Initial assessment of a potential HS patient should include questions about neonatal or subsequent jaundice, anemia, splenomegaly, or gallbladder symptoms; transfusions; and a family history of these things and of splenectomies or cholecystectomies. The physical examination should seek signs of pallor, scleral icterus, and splenomegaly. Pediatricians should also examine the parents and siblings if they are present, particularly for splenomegaly, which is more evident in older patients. Scleral icterus is usually subtle and best perceived in daylight and at the edge of the conjunctivae (Fig. 16-3). Similarly, a spleen that cannot be palpated in a supine patient can sometimes be felt when the patient is placed on his or her right side with knees bent.

HS typically presents in infancy or childhood, but it may present at any age.[34] In children, anemia is the most common presenting complaint (50%), followed by splenomegaly, jaundice, or a positive family history.[35] No comparable data exist for adults. The majority of patients with HS have incompletely compensated hemolysis and mild to moderate anemia.[24] The anemia is often asymptomatic except for fatigue and mild pallor or, in children, nonspecific parental complaints such as irritability. Jaundice is seen at some time in about one half of patients,[24] usually in association with viral infections. When jaundice is present it is acholuric (i.e., unconjugated [indirect] hyperbilirubinemia without detectable direct bilirubinuria). Palpable splenomegaly is detectable in about one half of infants and most (70% to 95%) older children and adults.[24,35-37] Typically, the spleen is modestly enlarged (2 to 6 cm below the costal margin), but it may be massive.[38,39] There is no proven correlation between the size of the spleen and the severity of HS, although, given

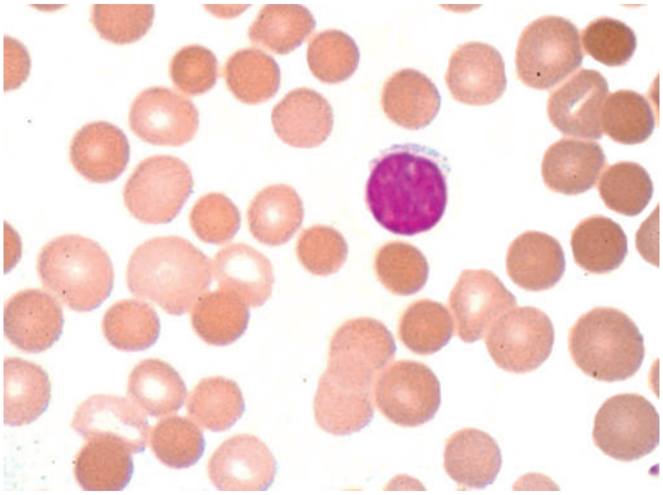

Figure 16-2 Peripheral blood smear from a patient with typical hereditary spherocytosis. Small, dense round microspherocytes are scattered throughout the smear, and most of the remaining, larger red cells have a smaller area of central clearing than normal.

TABLE 16-3 Clinical and Laboratory Classification of Hereditary Spherocytosis

	Normal	Mild Spherocytosis	Moderate Spherocytosis	Moderately Severe Spherocytosis	Severe Spherocytosis[*]
Inheritance	—	Autosomal dominant	Autosomal dominant, de novo mutation	Autosomal dominant, de novo mutation	Autosomal recessive
Proportion of hereditary spherocytosis cases	—	≈20% to 30%	≈60% to 70%	≈10%	<5%
Hemoglobin (g/dL)[†]	11.5-16[‡]	10.5-15	8-12	6-8	<6
Reticulocytes (%)[†]	0.5-1.5	1.5-6	≥6	≥10	≥10
Bilirubin (mg/dL)[†,‖]	0-1	0.5-2	≥2	≥2	≥3
Peripheral smear[†]	Normal	Mild spherocytosis	Spherocytosis	Spherocytosis	Spherocytosis ± poikilocytosis
Osmotic fragility (fresh)	Normal	Normal or slightly increased	Increased	Increased	Greatly increased
Osmotic fragility (incubated)	Normal	Usually increased	Increased	Increased	Greatly increased
MCHC (g/dL)[§]	32-36	34-37	34-38	35-39	
RDW (%)[§]	11-14	12-19	16-23	20-30	
Hb/MCHC[*]	0.38-0.41	0.35-0.40	0.29-0.33	0.18-0.28	
Hb/RDW[§]	0.95-1.05	0.7-1.0	0.48-0.74	0.16-0.35	
Serum transferrin receptor (nmol/L)[§]	18-25	30-65	80-125	100-150	
Erythropoietin (mIU/mL)[§]	7-16	9-30	25-90	30-300	
Membrane protein patterns (SDS-PAGE)[¶]	—	"Normal"[#] Slight ↓ spectrin Slight ↓ spectrin and ankyrin Slight ↓ band 3 and 4.2 Absent protein 4.2 and ↓ CD47	↓ Spectrin ↓ Spectrin and ankyrin ↓ Band 3 and protein 4.2 Absent protein 4.2 and ↓ CD47	↓ Spectrin ↓ Spectrin and ankyrin ↓ Band 3 and protein 4.2	↓↓ Spectrin ↓↓ Spectrin and ankyrin ↓↓ Band 3 and protein 4.2[**]
Transfusions	—	No	Sometimes required in infancy or with aplastic crisis	Occasionally with crises	Regular[*]
Splenectomy	—	Rarely, partial splenectomy[††]	Sometimes; consider partial splenectomy	Usually (6-9 years)	Yes (>3 years)

MCHC, Mean corpuscular hemoglobin concentration; *RDW*, red cell distribution width (measure of variation in shape); *SDS-PAGE*, SDS polyacrylamide gel electrophoresis; ↓, decreased.

[*] Patients with severe disease are transfusion dependent by definition. Values are in untransfused patients or at nadir before transfusion.

[†] *Data modified from Eber SW, Armbrust R, Schröter W: Variable clinical severity of hereditary spherocytosis: relation to erythrocytic spectrin concentration, osmotic fragility and autohemolysis.* J Pediatr *177:409–416, 1990.*

[‡] Varies with age.

[§] Ranges shown encompass the majority of individuals in each category. *From Rocha S, Costa E, Rocha-Pereira P, et al: Complementary markers for the clinical severity classification of hereditary spherocytosis in unsplenectomized patients.* Blood Cells Mol Dis *46:166–170, 2011.*

[‖] Multiply by 17.1 to convert to μmol/L.

[¶] Indicates common patterns observed on SDS gels. Decreased spectrin alone is seen in α-spectrin or β-spectrin defects. Decreased spectrin and ankyrin are observed with ankyrin defects. Decreased band 3 and protein 4.2 occur with band 3 defects. Absent protein 4.2 and decreased CD47 occur with protein 4.2 defects.

[#] Patients with mild spherocytosis who appear normal probably have small deficits (10% to 15%) that cannot be distinguished from normal findings on SDS gels.

[**] Rare patients with severe spherocytosis who are homozygous or compound heterozygous for band 3 defects.

[††] Consider in adolescents and adults who require a cholecystectomy or have disfiguring chronic jaundice.

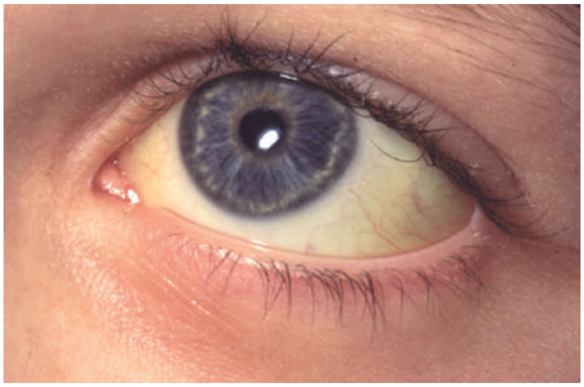

Figure 16-3 Scleral icterus in child with hereditary spherocytosis (HS). The jaundice in HS is often subtle and is more evident toward the posterior of the conjunctiva. *(From Bolton-Maggs P: Hereditary spherocytosis. Best Practice website, British Medical Journal Evidence Centre. BMJ Publishing Group Ltd. Accessed August 2013 at* http://bestpractice.bmj.com/best-practice/monograph/1143/resources/images/print/6.html.*)*

the pathophysiology and the response of the disease to splenectomy, such a correlation probably exists.

Typical HS is associated with both dominant and nondominant inheritance. The nondominant disease tends to be more severe, but there is considerable overlap.[22,29,31] Clinical severity and the response to splenectomy roughly parallel the degree of spectrin (or ankyrin) deficiency in patients with defects in either protein.[22,31,32,40-42] Disease in such patients can vary from mild to severe. Patients who are heterozygotes for band 3 defects have typical, moderate HS and moderate band 3 deficiency (15% to 35%).[42] Severe HS is rare in this population unless the patient carries two defective band 3 genes. Protein 4.2 deficiency is also associated with milder disease. Overall, patients with deficiency of spectrin or both spectrin and ankyrin have more severe disease than patients with band 3 or protein 4.2 deficiency,[24] but the differences are not great because true "severe" HS is rare.

In general, members of the same family will have a similar clinical picture, but there are exceptions, presumably due to varying compensation by the normal allele in patients who are heterozygous for a mutant gene, or by the coinheritance of another deleterious allele.[43-45] Such alleles should be sought in patients whose disease is much more severe than that of the rest of the family.

Silent Carrier State

The parents of patients with "nondominant" HS are clinically asymptomatic and do not have anemia, splenomegaly, hyperbilirubinemia, or spherocytosis on peripheral blood smears.[31] However, some have subtle laboratory signs of HS, including slight reticulocytosis (average, 2.1 ± 0.8%), diminished haptoglobin levels, slightly elevated osmotic fragility, or slightly shortened times in the acidified glycerol lysis test (AGLT).[22] Screening of normal Norwegian[17] and German[18] blood donors with the osmotic fragility test or AGLT showed a 0.9% to 1.1% incidence of previously unsuspected "very mild" HS. Presumably, some of these individuals were silent carriers of recessive HS genes, whereas others just had mild dominant HS. Unfortunately, no systematic molecular survey of this group of patients has been conducted.

Mild Hereditary Spherocytosis

Red cell production and destruction are balanced or nearly balanced in 20% to 30% of patients with HS.[24,36,46,47] These persons are considered to have "compensated hemolysis" and are usually asymptomatic. In some patients, the diagnosis may be difficult because hemolysis, splenomegaly, and spherocytosis are unusually mild. In this group of patients, reticulocyte counts are generally less than 6%, and only 60% of patients have obvious spherocytosis on peripheral blood smears.[22] About 10% of HS patients have very few (≤2%) or no detectable spherocytes.[24] In addition, red cell spectrin and ankyrin levels are typically more than 80% of normal.[22,42] As noted, many of these patients have band 3 or protein 4.2 defects.

Hemolysis may become severe with illnesses that cause splenomegaly, such as infectious mononucleosis.[48] Hemolysis may also be exacerbated by pregnancy[49,50] or exercise,[50] to the point where it may impair athletic performance in endurance sports. In many of these patients, HS is diagnosed during family studies or discovered when adult patients are diagnosed with splenomegaly or gallstones. Although mild HS is usually familial, it develops sporadically in families with more severe disease.[51] Presumably this is due to the coinheritance of modifying genes, such as those affecting spectrin or ankyrin synthesis or splenic function.

How to Explain "Compensated Hemolysis"

It is unclear why patients with HS and "compensated hemolysis" (i.e., normal hemoglobin levels) continue to have erythroid hyperplasia. Erythroid stimulation should disappear once hemoglobin levels reach normal if hemoglobin functions normally, as it does in HS. That it does not is shown by the fact that erythropoietin is inappropriately elevated (up to eight times normal) in HS patients with compensated hemolysis and no anemia,[33,47,52] and the fact that the serum transferrin receptor concentration, a measure of erythropoiesis,[53] is elevated.[42] The levels of 2,3-diphosphoglycerate (2,3-DPG) are reportedly low in hereditary spherocytes before splenectomy,[54] which could explain it; however the P_{50} of blood from HS patients is normal,[55] which does not make sense. A more likely possibility is that the dehydrated HS red cells are rheologically impaired and do not adequately perfuse the juxtaglomerular renal vessels, where erythropoietin is produced, even when the hematocrit is normal. If this hypothesis is correct, then erythropoietin production should correlate inversely with red cell deformability in any disorder where red cells are dehydrated (e.g., sickle cell anemia, hereditary xerocytosis). Clinically, the high level of erythropoietin and persistent erythroid hyperplasia in patients with HS and a normal hemoglobin level indicate that physiologically the patients are still anemic. *Indeed, it is possible that all HS patients are physiologically more anemic than their hemoglobin values suggest.*

Moderate and Severe Hereditary Spherocytosis

A small fraction of patients with HS (5% to 10%) have moderately severe to severe anemia. Patients with “moderately severe” disease typically have a hemoglobin value of 6 to 8 g/dL, a reticulocyte count of about 10%, a bilirubin level greater than 2 mg/dL, and 40% to 70% of the normal red cell spectrin content. The mean corpuscular hemoglobin concentration (MCHC) is high and the red cell distribution width (RDW) is very high (typically >20%) (see Table 16-3). Such patients are more susceptible to the dangers of hemolytic and aplastic crises (see later) and may occasionally need a transfusion. This category includes patients with both dominant and recessive HS and a variety of molecular defects in spectrin and ankyrin. Occasional patients with band 3 defects may fall into this category.

Patients with “severe” disease, by definition, have life-threatening anemia and are transfusion dependent. They often have recessive HS. Most of these patients probably have isolated, severe spectrin deficiency (<40% of normal) resulting from a defect in α-spectrin,[56] but some have ankyrin defects[42,57,58] or are homozygous or compound heterozygous for band 3 mutations.[28,43,44,59] Patients with severe HS are also distinguished by red cell morphologic findings. They often have some irregularly contoured or budding spherocytes and bizarre poikilocytes, in addition to typical spherocytes.[58-60] Such cells are rare before splenectomy in patients with less severe disease, although some may be seen after splenectomy. In addition to the risks of recurrent transfusions, patients with “severe” disease often suffer from an aplastic crisis and may develop growth retardation, delayed sexual maturation, or aspects of thalassemic facies.[38,39,60]

Hereditary Spherocytosis in Pregnancy

In general, unsplenectomized patients with HS have no significant complications during pregnancy except for anemia, which worsens because of plasma volume expansion[61] and sometimes because of increased hemolysis.[49,50] Hemolytic crises during pregnancy requiring transfusion have been reported.[50] Folic acid deficiency is also a risk.[62,63] One group reported that 20% of patients with HS received transfusions during pregnancy,[50] but in our experience, pregnant patients with HS rarely need transfusions.

Hereditary Spherocytosis in the Neonate

HS often presents as jaundice in the first few days of life.[24,64-67] Roughly one half of all patients with HS have a history of neonatal jaundice,[24,68] and 91% of infants discovered to have HS in the first week of life are jaundiced (bilirubin level >10 mg/dL).[66] In a French study of 402 jaundiced neonates requiring phototherapy, 1% were found to have HS.[69] Hyperbilirubinemia usually appears in the first 2 days of life and bilirubin levels may rise rapidly,[64,67,68] driven by the combination of hemolysis and the reduced capacity of the neonatal liver to conjugate bilirubin. Newborns with both HS and Gilbert syndrome, a common polymorphism in the promoter region of the uridine diphosphate–glucuronosyltransferase gene (UGT1A1) (see Chapter 4), develop neonatal jaundice more frequently[70] and may develop severe hyperbilirubinemia.[71] Kernicterus is a risk[64,65,71] and exchange transfusion or treatment with barbiturates are sometimes necessary, but in most patients the jaundice is controlled with phototherapy.

Only 43% of neonates with HS are anemic at birth (hemoglobin level <15 g/dL), and severe anemia is rare,[66] but those who present at birth tend to have a more severe course.[42] Most have a normal hemoglobin level at birth, which then decreases sharply over the first 3 weeks of life, leading to a transient, severe anemia.[72] In up to three fourths of HS infants, the anemia is severe enough to warrant transfusion or treatment with erythropoietin (see below). This anemia appears to be aggravated by an inability of the infant’s bone marrow to mount an appropriate erythropoietic response to anemia and to the development of splenic function.

Hydrops fetalis has been reported in a few patients and is associated with homozygosity or compound heterozygosity for spectrin or band 3 defects.[27,28,73] If hydrops fetalis is detected in utero, the fetus may require intrauterine transfusions. No instances of hydrops fetalis have been associated with ankyrin deficiency. Perhaps infants with ankyrin defects, similar to ankyrin-deficient *nb/nb* mice,[74] are partially protected in utero by the expression of ankyrin-related proteins in embryonic and fetal erythroblasts.

The diagnosis of HS is sometimes more difficult in the neonatal period than later in life. Splenomegaly is uncommon—at most the spleen tip is palpable—and reticulocytosis is variable and usually not severe.[67,68] Only 35% of affected neonates have a reticulocyte count greater than 10%.[66] In addition, the haptoglobin level is not a reliable indicator of hemolysis during the first few months of life.[75] An even greater problem is that 33% of neonates with HS do not have significant numbers of spherocytes on their peripheral blood smears.[66] Moreover, because fetal red cells are more osmotically resistant than adult cells when fresh and more osmotically sensitive after incubation at 37°C for 24 hours,[66,76] the osmotic fragility test occasionally gives false-positive (incubated) or false-negative (unincubated) results unless fetal controls are used. Fortunately, these results have been published,[66] and they appear to reliably differentiate neonates with HS, particularly when the incubated osmotic fragility test is used.[66,67] However, in our experience it is rarely necessary to use fetal controls. The standard osmotic fragility test usually suffices to make the diagnosis in conjunction with the blood smear, Coombs test, and other data. Tests such as the eosin-5′-maleimide–binding test, AGLT, Pink test, and cryohemolysis test and osmotic gradient ektacytometry (all discussed later) are more sensitive and specific than the osmotic fragility test in older children and adults, but they have not been systematically tested in the neonatal period. Studying the erythrocytes from the parents is also very useful in the diagnosis of HS, particularly if the infant has received a transfusion.[77]

However, it is clear that many infants with HS are not detected in the neonatal period on clinical grounds alone, even infants with hyperbilirubinemia,[78] probably because the disease is not considered or is not obvious. Ascertainment is greatly enhanced if all infants with a high MCHC and a high RDW are evaluated for HS.[78]

As noted above, some infants with HS become progressively more anemic in the first month or two of life and require transfusion. Fortunately, the problem is transient, except in rare patients with severe HS, and usually remits after one or two transfusions. Almost all infants with HS will outgrow the need for transfusion by the end of the first year of life.[72] If the child is otherwise well, we allow the hemoglobin level to fall to 5.5 to 6.5 g/dL before giving transfusions to try to stimulate the marrow and only raise the hemoglobin level to 9 to 11 g/dL after transfusion to avoid suppressing the desired marrow response.

Administration of recombinant human erythropoietin (rHuEpo) to infants with HS was beneficial in reducing blood transfusions in an uncontrolled, open-label study.[79] In this study, 13 of 16 infants given weekly subcutaneous injections of rHuEpo at a dosage of 1000 IU/kg had increases in the absolute reticulocyte count and hemoglobin values. The infants were given the same dosage of rHuEpo weekly based on their initial weight (at a range of initial ages from 16 to 119 days), a weekly hemoglobin level of less than 8 g/dL, and an absolute reticulocyte count of 200×10^9/L or less. Subsequent studies show similar effectiveness with different dosages and different frequencies.[80] Therefore erythropoietin therapy in infants with HS has become an alternative to transfusion, although weekly erythropoietin injections can be challenging for parents. Also, erythropoietin is twice as expensive as a single transfusion and erythropoietin-treated infants don't always avoid a transfusion.[80a] Further studies of recombinant erythropoietin therapy for neonatal anemia in HS are needed to determine the optimal time to initiate treatment, the optimal dosage, and the optimal duration of therapy.

Close observation of infants with HS is necessary to avoid complications of severe anemia. All infants with HS should have hemoglobin and reticulocyte measurements at least monthly during the first 6 months of life to avoid unnecessary side effects of severe anemia. The intervals may be increased thereafter, depending on the severity of anemia, but care must be taken to instruct parents on the signs and symptoms of an aplastic crisis.

Etiology: Membrane Protein Defects

The primary molecular defects in HS reside in membrane skeleton proteins, particularly the proteins whose *vertical* interactions connect the membrane skeleton to the lipid bilayer: spectrin, ankyrin, protein 4.2, and band 3 (Fig. 16-4). Because each of these proteins has more than one vertical attachment (ankyrin to RhAG as well as band 3, protein 4.2 to CD47 as well as band 3, spectrin to phospholipids and to the band 3 complex near actin), missense mutations that only impair a single protein-protein interaction are not pathologic, whereas reductions in the amounts of the four proteins are. In contrast, missense mutations predominate in hereditary elliptocytosis, which is caused by diminished *horizontal* interactions between

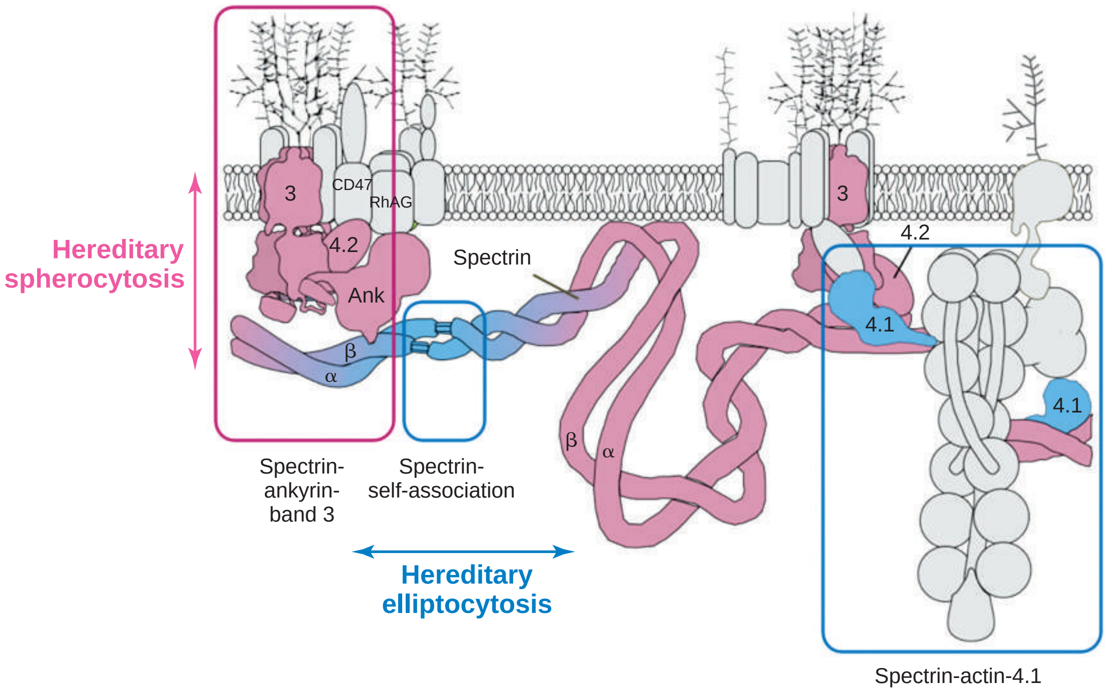

Figure 16-4 Membrane defects in hereditary spherocytosis and elliptocytosis. Hereditary spherocytosis is caused by a quantitative deficiency of spectrin, ankyrin, band 3, or protein 4.2, proteins involved in the "vertical" interactions that attach the membrane skeleton to the overlying lipid bilayer with its integral membrane proteins. Hereditary elliptocytosis is caused by functional defects in the "horizontal" protein interactions that hold the skeleton together. These are either defects near the head end of spectrin that impair self-association of spectrin dimers, or deficiency of protein 4.1R, a cofactor protein that bolsters the attachment of spectrin to actin. A more accurate model of the membrane is shown in Figure 15-6 of Chapter 15.

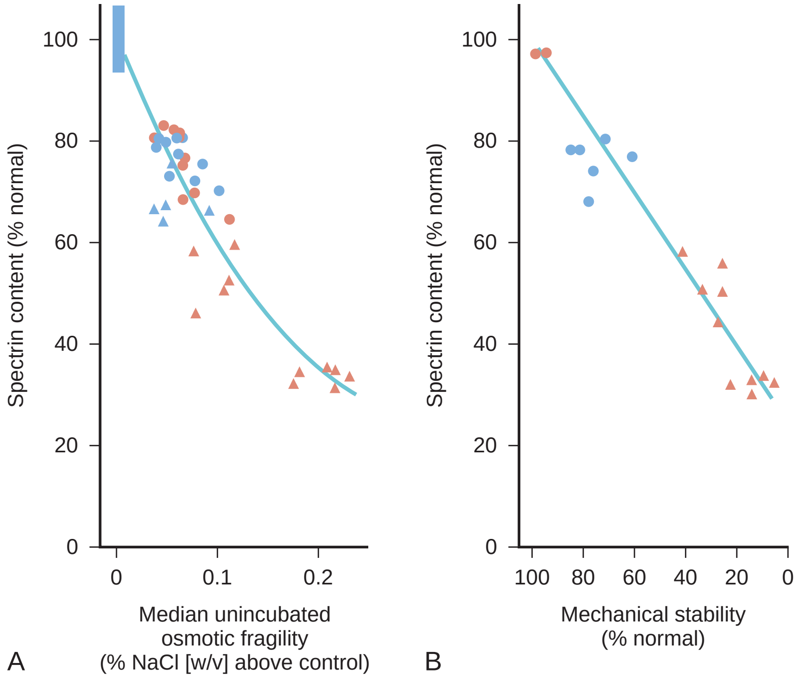

Figure 16-5 Correlation of spectrin content with (**A**) unincubated osmotic fragility and (**B**) mechanical stability in hereditary spherocytosis. *Circles* are patients with typical autosomal dominant hereditary spherocytosis. *Triangles* are patients with nondominant spherocytosis. *Reddish brown symbols* denote patients who have had a splenectomy. The *blue rectangle* in **A** is the normal range. *(**A**, Modified from Agre P, Asimos A, Casella JF, et al: Inheritance pattern and clinical response to splenectomy as a reflection of erythrocyte spectrin deficiency in hereditary spherocytosis.* N Engl J Med *315:1579–1583, 1986; **B**, Modified from Chasis JA, Agre PA, Mohandas N: Decreased membrane mechanical stability and in vivo loss of surface area reflect spectrin deficiencies in hereditary spherocytosis.* J Clin Invest *82:617–623, 1988.)*

individual proteins that hold the membrane skeleton together, especially defects in spectrin self-association (see Fig. 16-4).

Patterns of Membrane Protein Deficiency in Hereditary Spherocytosis

Four patterns predominate: isolated, partial spectrin deficiency, seen with defects in α-spectrin or β-spectrin; combined, partial spectrin and ankyrin deficiency, seen with primary ankyrin defects; combined, partial band 3 and protein 4.2 deficiency, seen with defects in band 3; and nearly complete loss of protein 4.2 and CD47, seen in primary defects of protein 4.2. In Europeans and Americans ankyrin and band 3 defects are most common, roughly 45% and 30%, respectively; β-spectrin defects account for perhaps 20%. Protein 4.2 and α-spectrin defects are both recessive and are rare. However, the pattern of membrane protein defects varies in different populations. In Japan, deficiencies of band 3 and protein 4.2 are the most common forms of HS.[21] In Korean patients, ankyrin and spectrin defects are most common, but protein 4.2 deficiency occurs more often than in European or American patients.[81]

Because most mutations are unique within each family and the mutation itself does not usually predict the phenotype, it is rarely clinically useful to determine the specific molecular defect in patients with HS. Readers who are interested in tables listing many of the mutations described to cause HS should consult lists in various chapters and reviews,[82-85] including the previous edition of this text,[86] or one of the two versions of the Human Gene Mutation Database: HGMD Professional,[87] a subscription database that is available through many medical libraries, and the public version of HGMD,[88] which is free but lags 3 years behind the subscription version.

For patients whose red cells are spectrin deficient, the degree of deficiency correlates well with the spheroidicity of HS red cells, the severity of hemolysis, and the response to splenectomy (Fig. 16-5, *A*).[22,31,89] The mechanical properties of the cells, particularly their ability to withstand shear stress, also correlate with their spectrin content (see Fig. 16-5, *B*).[89,90] Microscopically, HS red cells with spectrin deficiency show fewer spectrin filaments interconnecting spectrin–actin–protein 4.1R junctional complexes, but overall skeletal architecture is preserved,[91] except in the most severe forms of the disease.

α-Spectrin Defects: Recessive Hereditary Spherocytosis

In humans, α-spectrin synthesis exceeds β-spectrin synthesis by about four to one.[92] Heterozygotes for α-spectrin synthetic defects still produce enough normal α-spectrin chains to pair with all of the β chains that are made. Thus α-spectrin defects are only apparent in the homozygous or compound heterozygous state and exhibit recessive inheritance. Moreover, there may only be a limited spectrum of mutations that cause clinical disease. Production-defective or “thalassemia-type” mutants that only moderately affect expression, such as α-spectrin$^{\text{LELY}}$ (to be discussed under hereditary elliptocytosis), have no

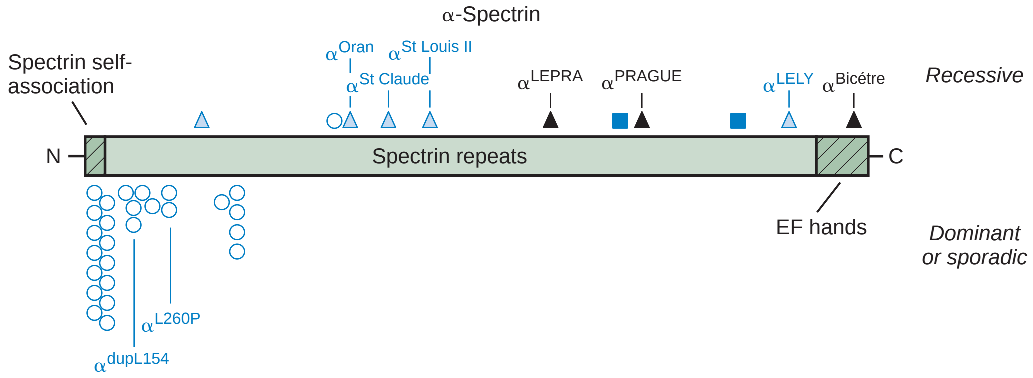

Figure 16-6 α-Spectrin mutations in hereditary spherocytosis (HS; *black*) and hereditary elliptocytosis (HE; *blue*). Missense mutations and in-frame insertions and deletions are indicated by *circles*. Splicing mutations that abolish or nearly abolish α-spectrin expression are indicated by *solid triangles*. Splicing mutations that partially decrease expression are indicated by *shaded triangles*. Note that HE mutations cluster near the N-terminus and tend to be amino acid substitutions that impair function (spectrin self-association), whereas HS mutations are defects that greatly decrease α-spectrin expression. The HS mutations are recessive because α-spectrin synthesis exceeds β-spectrin by a factor of 3 to 4, so that the expression of both α-spectrin genes must be greatly impaired to cause disease. α^{LELY} is a very common polymorphism of α-spectrin that diminishes synthesis by about 50% (see section on hereditary elliptocytosis).

phenotype, even in the homozygous state. Based on the few reported cases, it appears that the output of the α-spectrin gene must be less than 25% of normal to cause disease and may be as low as 8% of normal and still be compatible with life.[93] No homozygous null mutations have been reported, so it is possible they are lethal in the embryo.

To date, only 2% of all the mutations reported to cause HS involve α-spectrin (Fig. 16-6). Wichterle et al[94] reported a patient with severe HS who was a compound heterozygote for two different α-spectrin gene defects: in one allele, there was a splicing defect associated with an upstream intronic mutation, α^{LEPRA} (low-expression allele Prague); in the other allele, there was a null mutation, α^{PRAGUE}. The α^{LEPRA} allele produces one-sixth as much correctly spliced α-spectrin mRNA as normal. It is a relatively common allele, estimated to be 5% among Caucasians,[11] and is in linkage dysequilibrium with $\alpha^{Bughill}$, which is an innocent amino acid polymorphism (Ala970Asp).[56] Delaunay reported a similar family combining $\alpha^{LEPRA}/\alpha^{Bughill}$ on one chromosome with a double mutant $\alpha^{LELY}/\alpha^{Bicêtre}$ on the other, where the latter mutation essentially abolished α-spectrin output.[93] In both cases the combination of the severely impaired α^{LEPRA} allele with a null mutation of α-spectrin in *trans*, leads to significant, spectrin-deficient spherocytic anemia. As expected, even in the most severe forms of α-spectrin–linked recessive HS, obligate heterozygotes are asymptomatic and have little or no spectrin deficiency.[31,40,60]

Clinical Features. Less than 5% of patients with HS have primary defects in α-spectrin, but most of those patients have severe HS. Their red cells only contain 25% to 50% of the normal amount of both spectrin chains (Table 16-4).[31,40,60] Many are transfusion dependent, and even after splenectomy they may experience only a partial recovery. Blood smears are more bizarre than is typical for HS and show intense spherocytosis and irregular contracted cells (Fig. 16-7, *A*).

β-Spectrin Defects: Dominant Hereditary Spherocytosis

β-Spectrin synthesis limits formation of $\alpha\beta$-spectrin heterodimers, so even modest defects in the expression of β-spectrin are apparent in the heterozygous state and exhibit a dominant pattern of inheritance. Of the reported HS mutations 17% are in β-spectrin,[87] a finding that fits with the general impression that β-spectrin defects account for 15% to 25% of cases. Some groups report a much higher proportion of HS patients with isolated spectrin deficiency when sodium dodecyl sulfate (SDS) gels are used for membrane protein analysis, but that may reflect problems with assessing ankyrin deficiency on SDS gels (see later). The characteristics of HS due to β-spectrin deficiency are listed in Table 16-5.

The described mutations in β-spectrin include initiation codon disruptions, frameshift and nonsense mutations, gene deletions and rearrangements, and splicing defects (Fig. 16-8). Most patients have defects that impair or abolish β-spectrin production from the affected allele. All of the β-spectrin is generated from the normal allele

TABLE 16-4 Hereditary Spherocytosis Due to α-Spectrin Mutations
<5% of hereditary spherocytosis
Autosomal recessive since four-fold more α-spectrin is made than β-spectrin
A low expression, “thalassemia-type” defect in α-spectrin is often paired with a second presumably null allele on the opposite chromosome (in *trans*). The combination must reduce α-spectrin expression by at least 75% to impair $\alpha\beta$-spectrin assembly enough to cause HS. Homozygous null mutations may be lethal
Severe hemolysis and anemia, often transfusion dependent
50%-75% spectrin deficiency in red cells
Red cell morphology not well defined but dense, irregular poikilocytes as well as spherocytes in some patients

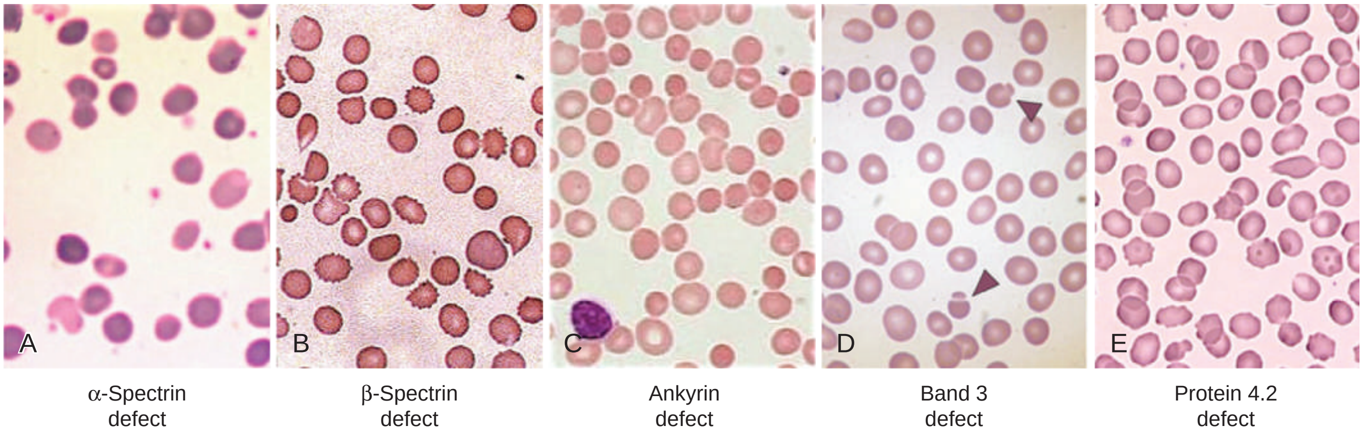

Figure 16-7 Red cell morphologic findings in hereditary spherocytosis (HS). **A**, Severe HS due to a homozygous α-spectrin defect. The spherocytosis is intense, and some cells have an irregular shape. Few cells have any central pallor. **B**, HS due to a heterozygous β-spectrin null mutation. Echinocytes and acanthocytes (5% to 15%) are often observed in addition to typical spherocytes. **C**, Heterozygous ankyrin-deficient HS. Typical microspherocytes are evident among red cells with some central pallor. **D**, Heterozygous band 3–deficient HS. A few button mushroom–shaped (or "pincered") red cells *(arrowheads)* are found in about 80% of patients with this form of HS. **E**, HS due to a homozygous protein 4.2 defect and nearly complete lack of the protein. Red cell morphologic findings are variable in this form of HS and, as here, there may be relatively few spherocytes. Echinocytes, slightly elongated elliptocytes (ovalocytes), stomatocytes, and other poikilocytes may be seen. *(A and E, From Delaunay J: The molecular basis of hereditary red cell membrane disorders.* Blood Rev *21:1–20, 2007; D, from Bolton-Maggs P, Crary S:* Hereditary spherocytosis. *Epocrates Online. BMJ Publishing Group, Ltd. Accessed August 2013 at* https://online.epocrates.com/u/29111143/Hereditary+spherocytosis/Summary/Highlights.*)*

TABLE 16-5 Hereditary Spherocytosis Due to β-Spectrin Mutations

15%-25% of hereditary spherocytosis cases
Almost all individual, dominant null mutations
Spectrin content decreased 15%-40%
Mild to moderately severe anemia
Blood smear with spherocytosis plus 5%-15% spiculated red cells in most patients
Spherocytosis plus elliptocytosis (i.e., spherocytic elliptocytosis) in some patients with C-terminal mutations that impair spectrin self-association *and* decrease spectrin content

in *trans*, which compensates to varying degrees, so that the red cell spectrin content is only decreased by 15% to 40% instead of the expected 50%. This suggests that β-spectrin is also synthesized in excess, though much less so than α-spectrin. The degree of compensation probably accounts for much of the variation in clinical severity among patients with β-spectrin HS.

Patients with deletions of the whole β-spectrin locus,[95] or with gross rearrangements,[96] also have autism[95] and learning disabilities,[96] indicating that genes close to β-spectrin on chromosome 14 are involved in cognition. There are HS patients with potentially instructive missense mutations, including a group clustered in the actin

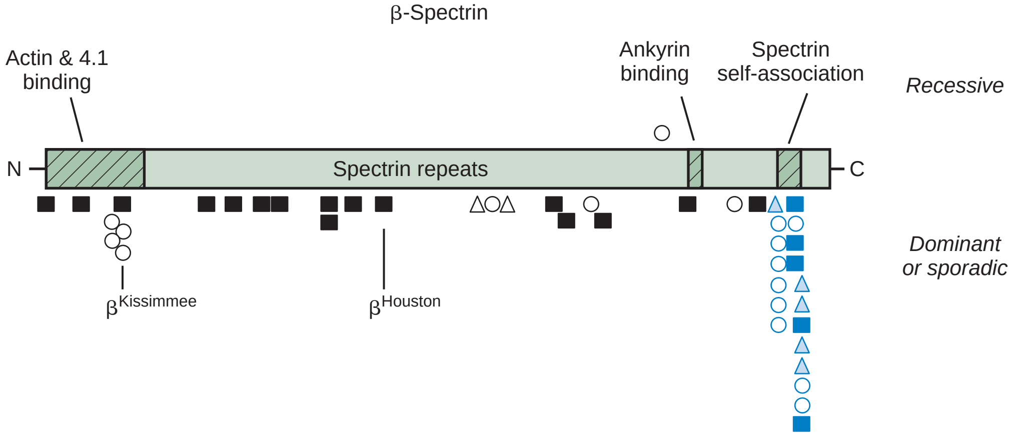

Figure 16-8 β-spectrin mutations in hereditary spherocytosis (HS; *black*) and hereditary elliptocytosis (HE; *blue*). Missense mutations and in-frame insertions and deletions are indicated by *circles*. Nonsense and other null mutations are indicated by *squares*. *Partially shaded triangles* denote splicing mutations that decrease but do not abolish expression. Patients with deletions of the whole β-spectrin locus[95] and with gross rearrangements[96] are also known but are not shown here. Note that HE mutations cluster near the C-terminus of β-spectrin and impair function (spectrin self-association) by altering amino acids or prematurely terminating the peptide chain. In contrast, most HS mutations abolish expression of the affected β-spectrin allele.

and protein 4.1R binding domain (spectrins Atlanta,[97] Kissimmee,[98,99] and Oakland[97]) and in the spectrin repeat domain (spectrins Columbus,[97] Birmingham,[97] and Sao Paulo[85]). It would be interesting to know whether these amino acid substitutions cause HS because they impair a specific function or whether the missense mutations simply destabilize β-spectrin and act like null mutations. In the case of spectrin$^{\text{Kissimmee}}$ (Trp202Arg), which is the only mutation where function was examined, both things were true. The mutant protein lacked the ability to bind protein 4.1R and, as a consequence, bound poorly to actin,[98] but it was also unstable and decreased in amount.[98,99]

All of the reported defects of β-spectrin are limited to single families with the exception of spectrin$^{\text{Houston}}$, a null mutation that has been found in several unrelated kindreds.[97]

Clinical Features. Patients with HS caused by β-spectrin mutations have hemolysis and anemia that vary from mild to moderately severe (see Table 16-5). Interestingly, in HS patients in whom peripheral blood morphologic characteristics have been described, those with β-spectrin mutations have had a small population (5% to 15%) of acanthocytes or spiculated spherocytes (spheroechinocytes) as well as spherocytes (see Fig. 16-7, *B*).

Ankyrin Defects

Spectrin heterodimers are only stable when bound to the membrane, and ankyrin, the high-affinity binding site, is normally present in limiting amounts. As a result, ankyrin defects are typically expressed as a dominant defect, although recessive mutations have been described. Ankyrin mutations are estimated to account for 30% to 60% of HS cases in Northern European populations and 5% to 10% of HS cases in Japan. Of all the known HS mutations, 35% are Ank1 defects.[87] The characteristics of HS due to ankyrin defects are listed in Table 16-6.

A role of ankyrin in HS was initially suggested by two patients with an atypical, unusually severe form of HS characterized by bizarre-shaped microspherocytes and red cell spectrin and ankyrin levels that were only 50% of normal.[58] Ankyrin synthesis was half normal; spectrin synthesis was normal, but only 50% of the synthesized spectrin attached to the membrane, presumably owing to the lack of ankyrin sites.[41] These results were the initial indication that ankyrin deficiency or dysfunction leads to concomitant spectrin deficiency in HS.

Additional studies showed that ankyrin deficiency is common in typical, dominant HS. When measured by radioimmunoassay, red cell membrane spectrin and ankyrin levels were less than the normal range in a large fraction of American kindreds, and the two proteins were proportionally decreased in nearly all the deficient kindreds.[100] Similar data have been obtained in multiple populations.[32,81,101-105]

Genetic analyses supported the hypothesis that a defect of the ankyrin gene could cause HS. Initially, studies of atypical patients with HS and karyotypic abnormalities, including translocations and interstitial deletions, defined a locus for HS at chromosomal segments 8p11.2-21.1.[106-110] After the ankyrin gene was cloned, it was localized to this same region, 8p11.2.[106] Fluorescence-based in situ hybridization of metaphase spreads from a patient with HS and an interstitial deletion of chromosome 8p11.1-p21 provided direct evidence that one copy of the erythrocyte ankyrin gene was deleted.[106] Ankyrin content in the patient's red blood cell membranes was reduced by 50%. Gene mapping then linked ankyrin to HS in a large family with typical dominant HS.[111] Further studies showed that ankyrin mutations are common in HS[57] and that many HS patients express only one of their two ankyrin alleles.[112]

Ankyrin mutations occur in both dominant and nondominant HS. Frameshift and nonsense mutations, which abolish the normal ankyrin product, are particularly common in dominant HS (Fig. 16-9). They produce mild to moderately severe hemolytic anemia, depending on the output of the remaining normal ankyrin gene and, perhaps, other modifier genes. For example, ankyrin$^{\text{Marburg}}$ and ankyrin$^{\text{Einbeck}}$ are frameshift mutations that occur in the NH_2-terminal (membrane) domain. Neither produces a detectable product in mature red cells. They would be expected to have a similar phenotype, but patients with ankyrin$^{\text{Einbeck}}$ have very mild disease and normal ankyrin levels, whereas patients with ankyrin$^{\text{Marburg}}$ have moderate to severe HS and moderate ankyrin deficiency (64% of normal). Understanding such phenotypic variations is one of the critical issues in HS research.

The most common ankyrin variant in patients with recessive HS is a T→C nucleotide substitution in the ankyrin gene promoter −108 bp from the translation start site. The allele occurs in 29% of German HS patients and 3% of normal individuals.[57,113] It is usually in *cis* with a G→A nucleotide substitution 153 bp from the start site[114] and impairs ankyrin synthesis in transgenic animals.[115] A −72/73 dinucleotide deletion in the ankyrin promoter also impairs expression by disrupting the recognition site for the TATA-binding protein and interfering with transcription initiation.[116] These "thalassemia-like" promoter mutations are silent in obligate heterozygotes; thus patients with nondominant HS must have a second mutation.[114] The promoter mutations aggravate ankyrin mutations if

TABLE 16-6 Hereditary Spherocytosis Due to Ankyrin Mutations

30%-60% of hereditary spherocytosis in the United States and Europe; 5%-10% in Japan
Autosomal dominant and autosomal recessive
Dominant mutations are mostly null mutations and are nearly all unique
Recessive mutations are missense and promoter defects: −108T→C promoter mutation is common
Both spectrin and ankyrin are equally decreased by 15%-50%
Mild to moderately severe anemia. Occasionally causes severe disease
Blood smear shows spherocytosis without other abnormal morphology

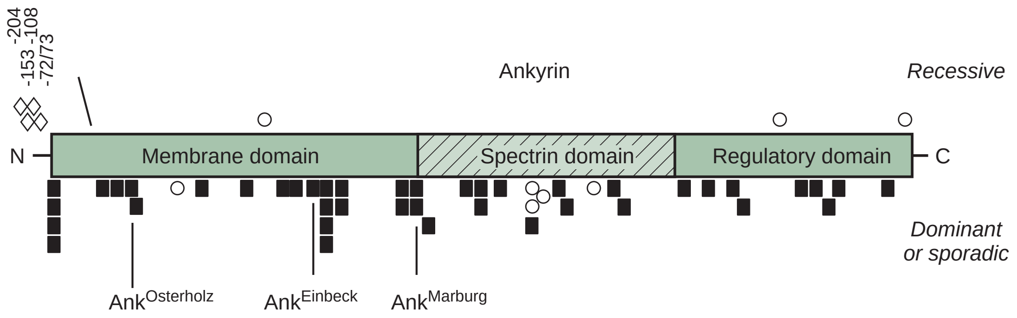

Figure 16-9 Ankyrin mutations, all of which cause hereditary spherocytosis (HS; *black*). Missense mutations and in-frame insertions and deletions are indicated by *circles*. Nonsense and other null mutations are indicated by *squares*. Four mutations in the 5′-untranslated region that are believed to decrease expression are indicated by *diamonds*. Note that the majority of mutations are dominant and abolish expression of the affected ankyrin allele.

they are inherited in *trans*, on the "normal" allele, particularly the −108 mutation, which is relatively common. For example,[116] in a family with ankyrin$^{\text{Osterholtz}}$, which is a null mutation,[57] the daughter, who has a combination of ankyrin$^{\text{Osterholtz}}$/ankyrin$^{-72/73}$, has more severe disease than her mother, who only carries ankyrin$^{\text{Osterholtz}}$.

Patients with gene deletions[106-110] involving the ankyrin locus at 8p11.2 often have features both of HS and Kallmann syndrome (hypogonadotrophic hypogonadism, type 2, due to FGFR1 defects) because the two loci are less than 4 Mb apart. Most of these patients also have psychomotor developmental delay, resulting from loss of another still undefined gene in the region.[117]

Clinical Features. Peripheral blood smears demonstrate only prominent spherocytosis (see Fig. 16-7, C). Hemolysis can be mild to severe, and the patients with recessive mutations typically have a more severe clinical course. Almost all dominant ankyrin mutations are private; that is, each individual kindred has a unique mutation. There is one exception, ankyrin$^{\text{Florianopolis}}$, a frameshift mutation that has been identified in HS patients from three unrelated kindreds.[118] Differences in a linked ankyrin gene polymorphism show that the three ankyrin$^{\text{Florianopolis}}$ alleles are different and arose independently.

Band 3 Defects: Dominant Hereditary Spherocytosis

Band 3 deficiency is present in approximately 25% to 45% of European and American patients with dominant HS,[57,119] and band 3 defects account for 37% of the known HS mutations.[87] Erythrocyte membranes from these patients are equally deficient in band 3 and protein 4.2,[120] which requires band 3 to bind to the red cell membrane. The characteristics of HS due to band 3 deficiency are listed in Table 16-7.

Defects in band 3 produce a variety of red cell phenotypes (Fig. 16-10), including stomatocytosis, cryohydrocytosis, Southeast Asian ovalocytosis (SAO), and acanthocytosis, as well as HS. These are discussed in later sections of the chapter. The HS mutations are spread throughout the band 3 gene, occurring in both the cytoplasmic and membrane-spanning domains. They include nonsense mutations, frameshift mutations, partial gene duplications, other missense mutations, and splicing defects. Many of the mutations cause messenger RNA (mRNA) instability or a misfolded protein that is degraded or not inserted into the membrane.[120] Conserved arginine residues are frequent sites of missense mutations in the transmembrane domain of the band 3 gene.[121,122] The affected arginine residues are all located in the same position on the inside edge of membrane-spanning segments and are believed to interact with negatively charged phospholipids in the inner bilayer and help orient the segments. As predicted, in vitro studies show that these arginine substitutions, as well as several other HS-associated band 3 missense mutations, exhibit defective cellular trafficking from the endoplasmic reticulum to the plasma membrane,[123,124] perhaps due to misfolding. Determination of the structure of the cytoplasmic domain of band 3[125,126] and detailed studies of the topography of the transmembrane domain[127,128] are beginning to provide insights into the biologic significance of the missense mutations.

Similar to the other molecular defects that cause HS, band 3 mutations are unique familial defects with no common mutations among affected groups.

Clinical Features. Clinically, most patients with band 3 mutations have mild to moderate hemolysis and anemia. The band 3 protein is reduced by 15% to 35% in HS red cells. On average, band 3 defects are somewhat milder than ankyrin or β-spectrin defects, although there is considerable overlap in severity. Patients with HS and

TABLE 16-7 Hereditary Spherocytosis Due to Band 3 Mutations

25%-45% of hereditary spherocytosis
Individual, dominant functionally null mutations. No common defects
15%-40% decrease in band 3 and a similar decrease in protein 4.2
Mild to moderate hemolysis and anemia
Spherocytosis with a small number of characteristic button mushroom-shaped or "pincered" cells in most patients

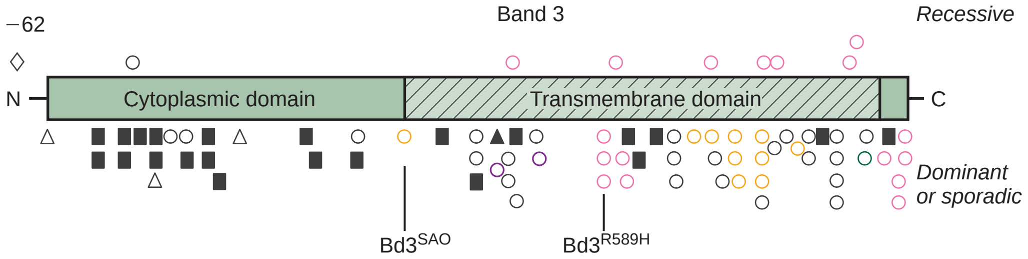

Figure 16-10 Band 3 mutations associated with hereditary spherocytosis (HS; *black*), various inherited disorders of cation permeability *(orange)*, distal renal tubular acidosis without *(pink)* or with *(purple)* hemolytic anemia, and hereditary acanthocytosis *(green)*. Missense mutations and in-frame insertions and deletions are indicated by *circles*. Nonsense and other null mutations are indicated by *squares*. Splicing mutations that abolish or nearly abolish band 3 expression are indicated by *solid triangles*. Splicing mutations that are or are presumed to be less severe are indicated by *open triangles*. One mutation in the 5′-untranslated region that is believed to decrease expression is indicated by a *diamond*. The cation permeability defects include stomatocytosis, cryohydrocytosis, a cryohydrocytosis-like form of HS, and Southeast Asian ovalocytosis. They are discussed later in the chapter.

band 3 deficiencies have a small number (≤1%) of button mushroom–shaped or "pincered" erythrocytes in addition to spherocytes on peripheral blood smears (see Fig. 16-7, *D*). This morphologic characteristic is not observed in the other membrane protein defects.

Distal Renal Tubular Acidosis. Distal renal tubular acidosis (dRTA) is a disorder characterized by the inability of the kidney to acidify the urine below pH 5.5 in the presence of systemic metabolic acidosis. It is caused by failure of H^+ secretion in the distal nephron. Patients present with various combinations of failure to thrive, hyperchloremic metabolic acidosis, hypokalemia, weakness, metabolic bone disease, and kidney stones. Because a shortened form of band 3 lacking the first 65 amino acids is expressed in the intercalated cells of the kidney cortical collecting ducts where its anion exchange capability is coupled to H^+ excretion, defects in band 3 would be expected to produce dRTA, and in fact a number of recessive and dominant missense mutations within the membrane domain of band 3 have been identified in dRTA patients (see Fig. 16-10).[129] The dominant defects tend to cluster in the middle of the membrane domain (Arg589His has been described in multiple kindreds) or at the C-terminus (see Fig. 16-10). The recessive mutations are localized throughout the membrane domain. The dominant mutations seem to be more prevalent in Europe, whereas the recessive mutations are more prevalent in Southeast Asia, but this may be because SAO (band 3^{SAO} or band $3^{\Delta 400\text{-}408}$) is very common in that part of the world (discussed later) and causes dRTA when it is coupled with a recessive dRTA allele.[129-131]

Surprisingly, few patients with HS due to band 3 defects have dRTA.[132-135] There are a few exceptions in patients who are homozygous or compound heterozygous for band 3 defects[28,136-139]; however, the majority of HS patients with mutations in band 3 do not have dRTA. Conversely, few patients with band 3 defects causing dRTA have hemolysis or spherocytosis.[132-133] In general, the dRTA mutations do not impair anion exchange.[132,133] They cause band 3 to either be retained in the endoplasmic reticulum or Golgi apparatus, or to be mistrafficked to the wrong membrane surface in polarized cells.[124,135,136,138-142] Most of the transport and trafficking studies have been done in oocytes, Madin-Darby canine kidney (MDCK) cells, or other epithelial cells and not in erythroid cell lines, so it is not possible to test whether band 3 trafficking is aberrant in kidney cells but not in red cells. This would explain why the dRTA mutants do not cause HS, and there is some evidence supporting the idea.[135] Another possibility is that dominant dRTA results from the association of mutant band 3 and wild-type band 3 into heterooligomers in the kidney, but not in erythroid cells, leading to intracellular retention or mistargeting of both proteins in the kidney and normal expression in red cells.[120]

Carbonic anhydrase II (CAII) binds to the C-terminus of band 3 and aids in chloride-bicarbonate transport.[135] Patients who lack CAII develop dRTA, osteopetrosis, and neurologic disease. This raises the possibility that some C-terminal band 3 dRTA mutations (see Fig. 16-10) may cause dRTA through loss of CAII function instead of, or in addition to, mistrafficking of band 3.

Protein 4.2 Defects: Recessive Hereditary Spherocytosis

HS caused by protein 4.2 deficiency is most common in Japan, although a few patients have been described in other populations. Overall, protein 4.2 mutations account for 9% of the published HS molecular defects.[87] The characteristics of this form of HS are summarized in Table 16-8.

Multiple protein 4.2 mutations have been described (Fig. 16-11). The most common one is 4.2^{Nippon}, in which the red cells contain only a small quantity of a 74/72-kD doublet of protein 4.2 instead of the usual abundant 72-kD species, because of an Ala142→Thr mutation that affects the processing of the protein 4.2 mRNA.[143] Protein 4.2^{Nippon}–deficient membranes lose 70% of their ankyrin with low ionic strength extraction, and the ankyrin loss is blocked by preincubation of the membranes with purified 4.2, which suggests that protein 4.2 stabilizes ankyrin on the membrane.[144,145] This hypothesis is supported by the observations that the amount of protein 4.2 is low in the red cell membranes of ankyrin-deficient *nb/nb* mice and in HS patients who lack one ankyrin gene. In addition, red cells from patients homozygous for 4.2^{Nippon} are fragile and have heat-sensitive skeletons, clumped intramembranous particles, and increased lateral mobility of

TABLE 16-8 Hereditary Spherocytosis Due to Protein 4.2 Mutations

<5% of hereditary spherocytosis in the United States and Europe; 45%-50% of hereditary spherocytosis in Japan
Autosomal recessive
95%-100% deficiency of protein 4.2, decreased amount of CD47
4.2 Nippon is common in Japan
Blood smear with variable red cell morphology: spherocytes, acanthocytes, ovalostomatocytes, normal discocytes. Spherocytes may not be the predominant morphology

band 3.[145] A weakening of ankyrin–band 3 binding due to loss of protein 4.2 has also been observed.[146] However, ankyrin lability is not evident in all patients with protein 4.2 deficiency.[147,148] Patients whose red cells lack protein 4.2 also lack CD47.[149-151] CD47 connects the band3–ankyrin–4.2 complex to the Rh complex of which CD47 is a part. Loss of CD47 may also be important because CD47 is recognized by an inhibitory receptor on macrophages that blocks phagocytosis.[152]

Finally, partial erythrocyte protein 4.2 deficiency has also been associated with mutations of band 3.[43,153,154] Presumably, these mutations affect sites of band 3–protein 4.2 interactions.

Clinical Features. Clinically, patients with HS due to protein 4.2 deficiency have mild to moderate HS. The peripheral smear sometimes contains typical spherocytes, but it may also contain ovalocytes, stomatocytes, or spiculated cells and relatively few spherocytes (see Fig. 16-7, *E*). For this reason the diagnosis of HS may be missed in patients with protein 4.2 deficiency. The mechanism behind this unique and variable red cell morphologic picture is currently unknown.

Pathophysiology

Loss of Membrane Surface by Vesiculation

The primary membrane lesions in HS all involve vertical interactions between the skeleton and the bilayer and fit the theory that HS is caused by local disconnection of the skeleton from the bilayer and its integral membrane proteins, followed by vesiculation of the unsupported surface components. This, in turn, leads to progressive reduction in membrane surface area and to a shape called a spherocyte, although it usually ranges between a thickened discocyte and a spherostomatocyte.[155]

Biomechanical measurements show that HS membranes are fragile. The force required to fragment the membrane is diminished and is proportional to the density of spectrin on the membrane.[89,90] Membrane elasticity and bending stiffness are also reduced and are proportional to spectrin density.[156] In addition, HS red cells lose membrane more readily than normal red cells when metabolically deprived or when their ghosts are subjected to conditions facilitating vesiculation.[157,158] This has not been shown to occur in metabolically healthy HS spherocytes, perhaps because it occurs slowly (1% to 2% per day) under such conditions. However, massive vesiculation is evident in mice and zebrafish with severe spherocytic hemolytic anemias (Fig. 16-12).[159,160,161,162,163]

Because budding red cells are rarely observed in typical blood smears from patients with HS, the postulated vesiculation may either involve microscopic vesicles or occur in the reticuloendothelial system. Vesiculation may also occur in the bone marrow of patients with HS because similar decreases in the red cell surface area have been found in HS reticulocytes and in mature red blood cells.[164] When membrane vesicles are induced in normal red cells, they originate at the tips of spicules, where the lipid bilayer uncouples from the underlying skeleton (see Fig. 15-29).[165] The vesicles are small (≈100 nm) and devoid of hemoglobin and skeletal proteins and would be invisible by light microscopy. Tiny (50- to 80-nm) bumps have been detected by atomic force microscopy, on the surface of red cells obtained from patients with HS whose red cells are actively hemolyzing (Fig. 16-13).[166] The bumps could be microvesicles, although this needs to be proven. They are less than the length of a spectrin molecule (100 nm) and are not present on red cells from splenectomized patients.

Hypothesis 1. The observation that spectrin or spectrin/ankyrin deficiencies are common in HS has led to the suggestion that they are the primary cause of spherocytosis. According to this hypothesis (Fig. 16-14, hypothesis 1), interactions of spectrin with bilayer lipids or proteins are required to stabilize the membrane. Spectrin-deficient

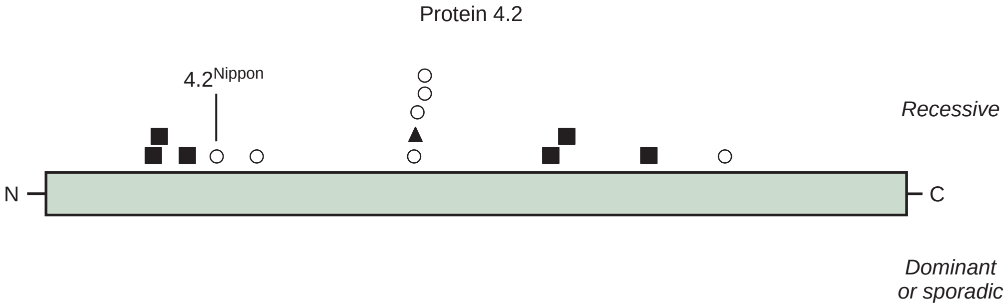

Figure 16-11 Protein 4.2 mutations associated with recessive hereditary spherocytosis (HS; *black*). Missense mutations and in-frame insertions and deletions are indicated by *circles*. Nonsense and other null mutations are indicated by *squares*. One splicing mutation that nearly abolishes protein 4.2 expression is indicated by a *solid triangle*. 4.2$^{\text{Nippon}}$ is a common mutation in Japanese cases.

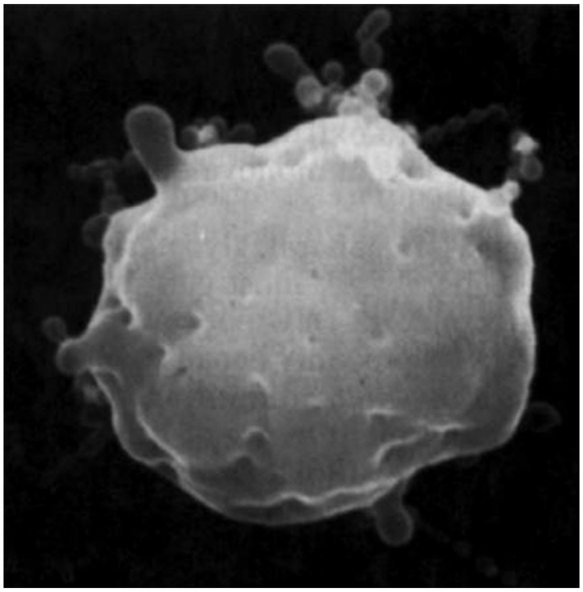

Figure 16-12 Scanning electron micrograph of a mouse red cell that lacks α-spectrin and has only trace amounts of β-spectrin. Note the extraordinary membrane instability and microvesiculation. It is assumed that similar vesiculation and membrane loss occur in human HS but at a much slower rate, consistent with the milder degree of spectrin deficiency in the human disease.

areas would tend to bud off, leading to spherocytosis. However, this conjecture does not explain how spherocytes develop in patients whose red cells are deficient in band 3 but have normal amounts of spectrin.

Hypothesis 2. An alternate hypothesis argues that the bilayer is stabilized by interactions between lipids and the abundant band 3 molecules (see Fig. 16-14, hypothesis 2). Each band 3 molecule contains about 12 hydrophobic transmembrane helices, many of which must interact with lipids. These interactions presumably spread beyond the first layer of lipids and influence the mobility of lipids in successive layers. In deficient red cells the area between band 3 molecules would increase, on average, and the stabilizing effect would diminish. Transient fluctuations in the local density of band 3 could aggravate this situation and allow unsupported lipids to be lost, resulting in spherocytosis. This hypothesis is supported by targeted disruption of the band 3 gene in mice. Erythrocytes from these mice lose massive amounts of membrane surface despite a normal membrane skeleton.[161] This concept is also consistent with early studies of intramembrane particle aggregation, which leads to particle-free domains, as discussed in Chapter 15. These domains are unstable, giving rise to surface blebs that are subsequently released from the cells as vesicles.[167]

It is most likely that both of these hypotheses operate to a different degree depending on the membrane skeletal protein which is deficient—hypothesis 1 dominating in spectrin and ankyrin disorders and perhaps in protein 4.2 deficiency, and hypothesis 2 dominating in band 3 defects. Hypothesis 1 predicts that released microvesicles will contain band 3 and other skeleton-linked integral membrane proteins; hypothesis 2 predicts such vesicles will contain mostly lipids. This should be testable but has not yet been examined.

Loss of Cellular Deformability

Hereditary spherocytes hemolyze because of the rheologic consequences of their decreased surface-to-volume ratio. The red cell membrane is very flexible, but it can only expand its surface area about 3% before rupturing.[168] Thus erythrocytes become less and less deformable as surface area is lost. Clever experiments show that the spleen begins to sense and restrain red cells that have lost as little as 2% to 3% of their membrane surface and that loss of more than 18% of the red cell surface leads to complete splenic sequestration.[169] This corresponds to the 15% to 20% loss of membrane lipids and, presumably, membrane surface in HS red cells.[157,170-172] For HS red

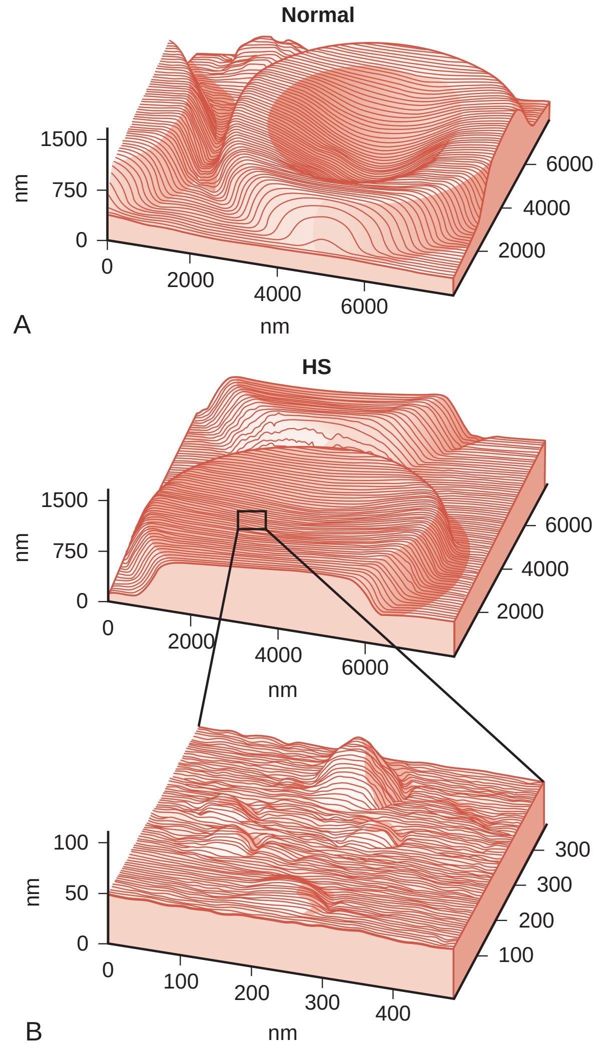

Figure 16-13 Comparison of the surface of a normal red cell (**A**) and a hereditary spherocyte (HS) (presplenectomy) (**B**) by atomic force microscopy. Note that at high magnification, 50 nanometers (nm) to 80 nm pseudopodia are evident on the surface of the spherocyte (bottom of **B**). These probably represent membrane microvesicles. They were not observed following splenectomy.

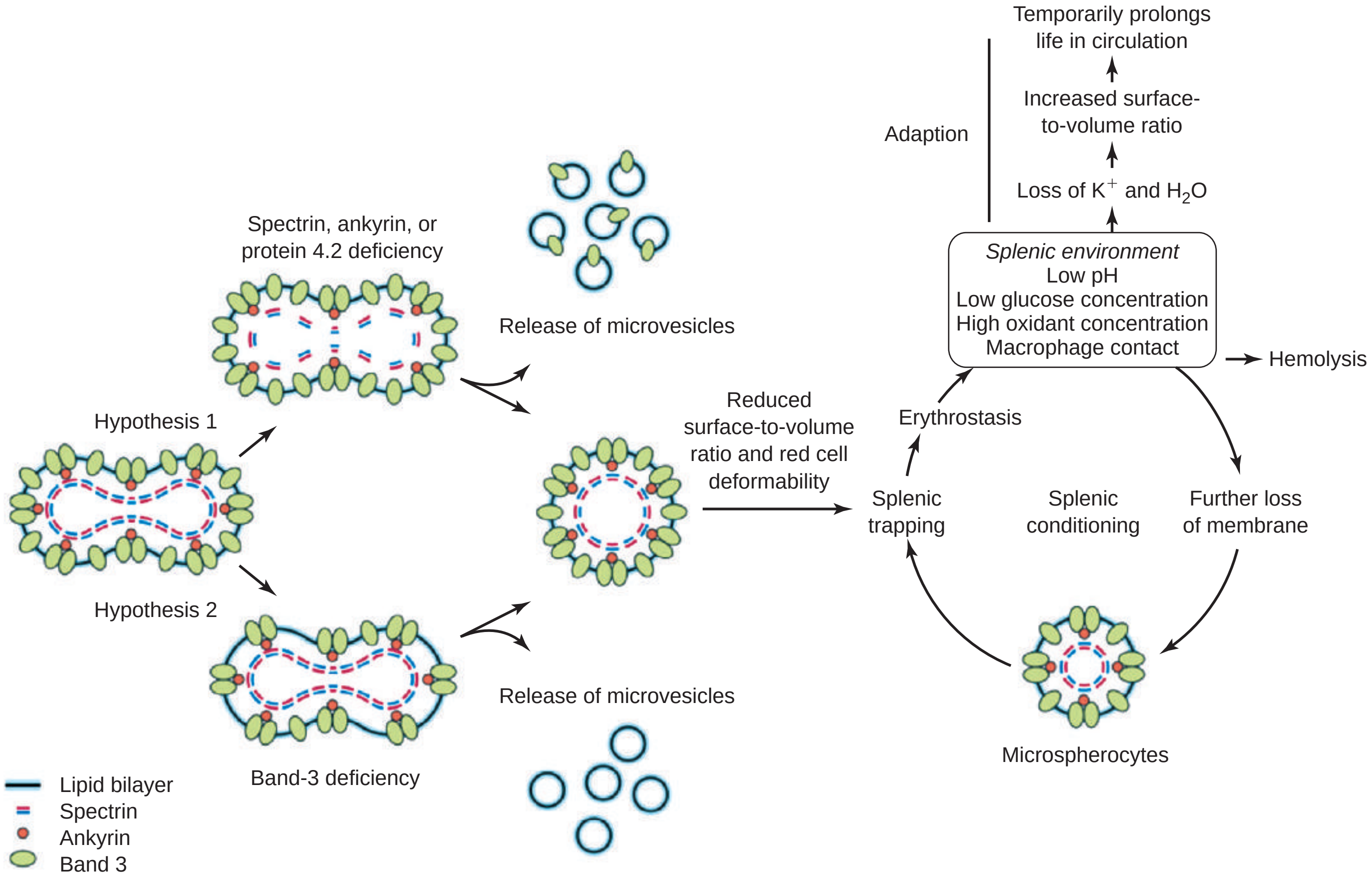
Temporarily prolongs life in circulation
Increased surface-to-volume ratio
Adaption
Loss of K^+ and H_2O
Splenic environment
Low pH
Low glucose concentration
High oxidant concentration
Macrophage contact
Hemolysis
Spectrin, ankyrin, or protein 4.2 deficiency
Release of microvesicles
Hypothesis 1
Reduced surface-to-volume ratio and red cell deformability
Erythrostasis
Splenic trapping
Splenic conditioning
Further loss of membrane
Hypothesis 2
Release of microvesicles
Microspherocytes
Band-3 deficiency
Lipid bilayer
Spectrin
Ankyrin
Band 3

Figure 16-14 Pathophysiology of hereditary spherocytosis (HS). Hypothesis 1 assumes that the "membrane" (i.e., the lipid bilayer and the integral membrane proteins) is directly stabilized by interactions with spectrin or other elements of the membrane skeleton. Spectrin-deficient areas, lacking support, bud off, leading to spherocytosis. This may be the dominant mechanism in spectrin, ankyrin, and perhaps protein 4.2 deficiency. Hypothesis 2 assumes the membrane is stabilized by the interactions of band 3 with neighboring lipids. The influence of band 3 extends into the lipid milieu because the immobilized lipids adjacent to band 3 interact with and slow the lipids in the next layer, and so on. In band 3–deficient cells, the lipid area between band 3 molecules increases, and unsupported lipids are lost. This may be the primary mechanism in band 3 deficiency. Both mechanisms lead to loss of membrane surface, spherocytosis, reduced red cell deformability, and splenic trapping, where the unfavorable splenic environment shown in the box augments membrane loss, a process called splenic conditioning. Some red cells hemolyze, some escape as microspherocytes, susceptible to recapture, and some adapt by loss of cell volume (K^+ and H_2O), leading to a temporary reprieve before more membrane loss reinitiates the process. *(Modified from Perrotta S, Gallagher PG, Mohandas N: Hereditary spherocytosis.* Lancet *372:1411–1426, 2008.)*

cells, poor deformability is only a hindrance in the spleen, because the cells have a nearly normal life span after splenectomy.[173,174]

Splenic Sequestration and Conditioning

In the spleen most of the arterial blood empties directly into the splenic cords, a tortuous maze of interconnecting narrow passages formed by reticular cells and lined with phagocytes (see discussion of splenic structure and function in Chapter 23).[175-178,179,180] Histologically, this is an "open" circulation, but most of the blood that enters the cords normally travels by fairly direct (i.e., functionally closed) pathways. If passage through these channels is impeded, red cells are diverted deeper into the labyrinthine portions of the cords, where blood flow is slow and the cells may be detained for minutes to hours. Whichever route is taken, to reenter the circulation, red cells must squeeze through spaces between the endothelial cells that form the walls of the venous sinuses (Fig. 16-15). Even when maximally distended, these narrow slits are always much smaller than red cells, which are greatly distorted during their passage.[177,178] Experiments have shown that spherocytes are selectively sequestered at this cordal-sinus juncture.[181-185] As a consequence, spleens from patients with HS have massively congested cords and relatively empty sinuses.[175,182-184] In electron micrographs, few spherocytes are seen in transit through the sinus wall,[175,183,185] which contrasts to the situation in normal spleens, where cells in transit are readily found.[179]

During detention in the spleen, HS red cells undergo additional damage marked by further loss of surface area and an increase in cell density. Many of these "conditioned" red cells escape the hostile environment of the spleen and reenter the circulation. In unsplenectomized patients with HS, two populations of spherocytes are detectable: a minor population of hyperchromic "microspherocytes" that form the "tail" of very fragile cells in the unincubated osmotic fragility test and a major population that may be only slightly more spheroidal than normal.

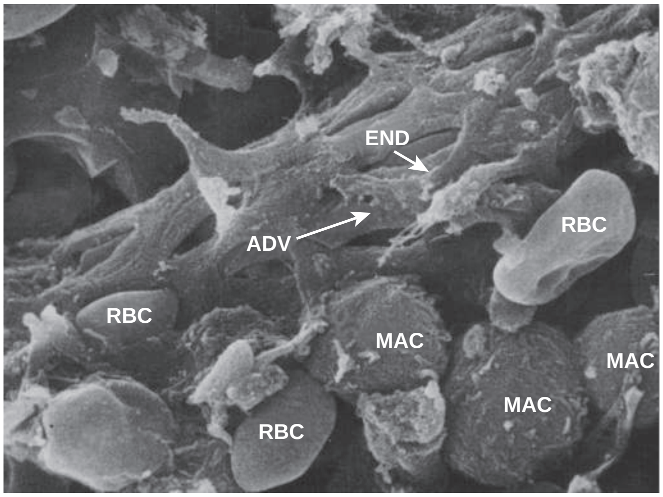

Figure 16-15 Scanning electron micrograph of a splenic sinus viewed from within the splenic cords. The narrow transmural slits between the endothelial cells *(END)* and adventitial cells *(ADV)* of the sinus are evident. The edges of these slits probably touch each other in the normal spleen, so the openings are potential structures rather than fixed pores. They are revealed here because of a drying artifact. Note that the adjacent erythrocytes *(RBC)* are considerably larger than the slits and hence must be flexible to pass into the splenic sinuses and return to the venous circulation. If they are unable to pass into the sinuses, macrophages *(MAC)* lie in wait. *(From Weiss L: A scanning electron microscopic study of the spleen.* Blood *43:665–691, 1974.)*

By 1913, it was known that HS red cells obtained from the splenic vein were more osmotically fragile than those in the peripheral circulation.[186] However, the significance of this finding was not clear until the classic studies in the 1950s by Emerson and Young, who showed that osmotically fragile microspherocytes are concentrated in and emanate from the splenic pulp (Fig. 16-16).[181,187] After splenectomy, spherocytosis persists, but the tail of hyperfragile red cells is no longer evident on osmotic fragility testing. These and other data led to the conclusion that the spleen detains and "conditions" circulating HS red cells in a way that increases their spheroidicity and hastens their demise.[181,187-189] The kinetics of this process were beautifully illustrated in vivo by Griggs, who found that a cohort of ^{59}Fe-labeled HS red cells gradually shifted from the major, less fragile population to the more fragile, conditioned population 7 to 11 days after entering the circulation.[189] Although most conditioned HS red cells that escape the spleen are probably recaptured and destroyed, the damage incurred is sufficient to permit extrasplenic recognition and removal, because conditioned spherocytes, isolated from the spleen and reinfused postoperatively, are rapidly eliminated.[189,190]

The mechanism of splenic conditioning is less certain. It is difficult to obtain accurate information about the cordal environment, but the existing data suggest it is metabolically inhospitable. Crowded red cells must compete for limited supplies of glucose[191] in acidic surroundings (pH 6.8 to 7.2)[192,193] where glycolysis is inhibited.[194,195] The acidic environment induces Cl^- and water entry and cell swelling[196] but also stimulates the K^+,Cl^- cotransporter, which produces a net loss of K^+ and water from the cells. The adverse effects of the cordal environment are further compounded by the presence of oxidant-producing macrophages. It has also been suggested that methylation of erythrocyte membrane proteins could contribute to the splenic conditioning of spectrin-deficient HS red cells.[197] Hence, the spherocyte, detained in the splenic cords because of its surface deficiency, is stressed by erythrostasis in a metabolically threatening environment.

Erythrostasis

The spherocyte is particularly vulnerable to erythrostasis. This is the basis of the well-known autohemolysis test.[198] During prolonged sterile incubation in the absence of supplemental glucose, red cells undergo a series of changes that culminate in hemolysis. The sequence of the changes is the same for HS and normal red cells; however, because HS cells are abnormally leaky and bear unstable membranes, their degeneration is accelerated.

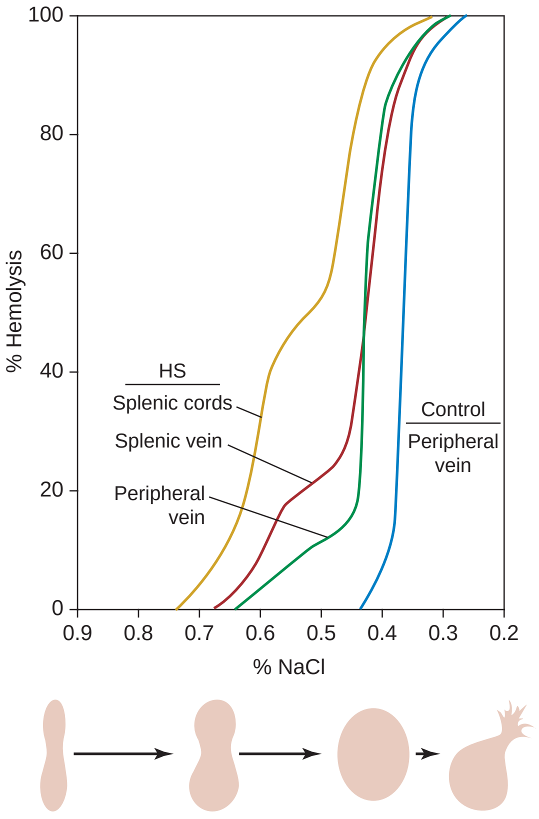

Figure 16-16 Osmotic fragility in hereditary spherocytosis *(HS)*. In the osmotic fragility test, red cells are suspended in salt solutions of varying tonicities between isotonic saline (0.9% NaCl) *(left)* and distilled water *(right)*. Normal red cells swell in hypotonic media and eventually reach a limiting spherical shape, beyond which they hemolyze *(bottom)*. Spherocytes, which begin with a lower than normal surface-to-volume ratio, can swell less before they reach their limiting volume; hence, their osmotic fragility curve is shifted to the left (higher salt concentrations). They are said to be osmotically fragile. Before splenectomy, a small proportion of the red cells are extra fragile "microspherocytes" and produce a "tail" on the osmotic fragility curve. The cells have been conditioned by the spleen, as shown by their higher concentration in the splenic vein and especially the splenic cords.

HS red cells are initially jeopardized, because their membrane permeability to Na^+ is mildly increased.[199] Their propensity to accumulate Na^+ and water is normally balanced by increased Na^+ pumping; however, the increased dependence on glycolysis is detrimental in erythrostasis, where substrate is limited.[199] HS red cells exhaust serum glucose and become ATP depleted more rapidly than normal red cells. As ATP levels fall, ATP-dependent Na^+,K^+ and Ca^{2+} pumps fail, and the cells gain Na^+ and water and swell. Later, when the Ca^{2+}-dependent K^+ (Gárdos) pathway is activated, K^+ loss predominates and the cells lose water and shrink. The Na^+ gain is accelerated in HS red cells but is insufficient by itself to induce hemolysis. However, HS red cells are doubly jeopardized. As noted earlier, they are inherently unstable and fragment excessively during metabolic depletion.[157,158,200] Membrane lipids (and probably integral membrane proteins) are lost at more than twice the normal rate.[158] At first this surface loss is balanced by cell dehydration, but eventually (within 30 to 48 hours) membrane loss predominates, the cells exceed their critical hemolytic volume, and autohemolysis ensues.

Consequences of Splenic Trapping

Calculations indicate that the average normal red cell passes through the splenic cords about 14,000 times during its lifetime and has an average transit time of 30 to 40 seconds,[201] surprisingly close to measured transient times in normal human spleens in vivo.[202] The calculated residence time of the average HS red cell in the splenic cords is much longer, perhaps as long as 15 to 150 minutes, but still far short of the time required for metabolic depletion to occur.[201] This conclusion is supported by direct analysis of splenic red cells. HS red cells obtained from the splenic pulp and containing 80% to 100% conditioned cells are moderately cation depleted and show changes in adenosine diphosphate (ADP) and 2,3-DPG concentrations consistent with metabolism in an acidic environment, but their ATP levels are normal.[203] Others have reported similar results.[204,205]

The data suggest that splenic conditioning is caused by mechanisms other than ATP depletion. For example, K^+ loss and membrane instability may be exacerbated by the high concentrations of acids and oxidants that must exist in a spleen filled with activated macrophages lunching on trapped HS red cells. In vitro, oxidants from activated phagocytes can diffuse across the membranes of bystander red cells and damage intracellular proteins within minutes. Red cells moving through the rapid transit pathways in the spleen might escape damage, but those caught in cordal traffic would be vulnerable. Oxidants, even in relatively low concentrations, cause selective K^+ loss by a variety of mechanisms[206,207] and also damage membrane skeletal proteins.[208,209] Finally, there is some evidence that HS red cells may be abnormally sensitive to oxidants.[209,210] When exposed to peroxides they undergo remarkable blebbing and, presumably, vesiculation. If a similar process occurs in the spleen, it could be responsible for the excessive surface loss observed in conditioned cells.

The possibility that macrophages may directly condition HS red cells should also be considered. It is well known that spherocytosis often results from the interaction of immunoglobulin G (IgG)–coated red cells with macrophages, but HS red cells do not have abnormal levels of surface IgG.[211] Macrophages also bear receptors for oxidized lipids (scavenger receptor) and phosphatidylserine, but there is no evidence at present that HS red cells expose the relevant ligands. The involvement of macrophages is supported by observations that large doses of corticosteroids markedly ameliorate HS in unsplenectomized patients.[212-214] The effects are similar to those produced by splenectomy. It is well known that similar doses of corticosteroids inhibit splenic processing and destruction of IgG- or C3b-coated red cells in patients with immunohemolytic anemias, probably by suppressing macrophage-induced red cell sphering and phagocytosis.[215,216] Electron microscopy shows that splenic erythrophagocytosis is common in HS, particularly in the splenic cords.[183,185,217] In addition, phagocytes expressed from the cords of patients with HS contain bits of ghost-like "debris,"[218] presumably resulting from membrane fragmentation.

Adaption: Benefits of Red Cell Dehydration for the HS Red Cell

It has been known for many years that HS red cells intrinsically leak more Na^+ and K^+ ions than normal cells.[199,219] All the Na^+ transmembrane movements are increased, including passive diffusion, Na^+,K^+-ATPase pump, Na^+,K^+,$2Cl^-$ cotransport, and Na^+, Li^+ countertransport.[220] The excessive Na^+ influx activates the Na^+,K^+-ATPase pump, and the accelerated pumping, in turn, increases ATP turnover and glycolysis. At one time, it was believed that this modest Na^+ leak was responsible for the hemolysis of hereditary spherocytes,[199] particularly those cells trapped in the unfavorable metabolic environment of the spleen, but it now is clear that this is incorrect, because the magnitude of the Na^+ flux does not correlate with the extent of hemolysis in HS.[220,221]

The dehydration of HS red cells, as reflected in their high MCHC, is likely to be caused, at least in part, by the adverse environment of the spleen. This assumption has been made on finding that spherocytes from surgically removed spleens are the most dehydrated.[203] The pathways causing HS red cell dehydration have not been clearly defined. One likely candidate is the Gárdos channel, a Ca^{2+}-activated K^+ channel, which is known to contribute to erythrocyte dehydration in sickle cell anemia and thalassemia. One study showed that the Gárdos channel is functionally upregulated in a form of mouse HS caused by lack of erythrocyte protein 4.1R.[222] When these mice were treated with clotrimazole, a known Gárdos channel blocker, hemolysis and anemia worsened, indicating that red cell dehydration protected the hereditary spherocytes, presumably by preserving their surface-to-volume ratio and therefore their flexibility in the face of surface loss.[222]

Another possible cause of red cell dehydration in HS is increased K^+,Cl^- cotransport, which is activated by acid pH.[223] HS red cells, particularly from unsplenectomized subjects, have a low intracellular pH,[54] reflecting the low pH of the splenic environment. The K^+,Cl^- cotransport pathway is also activated by oxidation,[224] which is likely to be caused by splenic macrophages.

Hyperactivity of the Na^+,K^+ pump, triggered by increased intracellular Na^+, can dehydrate red cells directly, because three Na^+ ions are extruded in exchange for only two K^+ ions. The loss of monovalent cations is accompanied by water.

A fourth possible cause is the recently described mechanosensitive Piezo1 channel, which responds to membrane stretch and is thought to be involved in homeostatic adjustments to changes in volume.[225,226] It is defective in hereditary xerocytosis,[227,228,229] a disease characterized by red cell dehydration.

Whichever transport pathways cause HS red cells to lose K^+ and water, it is likely that the dehydration is a form of "adaption" (Fig. 16-14) that prolongs the life of the HS red cell. How much it does so is unknown. Polymorphisms in cation channels like Piezo1 and calcium ATPase 4, in the globin loci, in major membrane skeletal proteins, and in genes like *TAF3*, *DNAJA4*, *WDR61* and *HBS1L-MYB* are associated with variation in human MCHC.[230-231b] It will be interesting to see if these genes affect the severity of HS.

Laboratory Characteristics

Most of the classic laboratory features of HS are those found in other forms of hemolytic anemia, such as reticulocytosis, erythroid hyperplasia of the bone marrow, indirect hyperbilirubinemia, and increased fecal urobilinogens.[35,36,232] The plasma hemoglobin level is often normal,[233] and the haptoglobin value is only variably reduced,[234] because most of the hemoglobin that is released when HS red cells are destroyed is catabolized at the site of destruction, so-called extravascular hemolysis. The spherocytic morphology combined with laboratory tests that measure a loss of surface area, such as the osmotic fragility test or eosin-5′-maleimide–binding test, distinguish HS from most other forms of hemolytic anemia. Membrane protein analysis and molecular genetic testing are only performed in research laboratories, as these tests are expensive and not very useful clinically. This is likely to change in the near future, though, as the cost of whole-exome and even whole-genome sequencing is falling very rapidly.

Red Cell Morphology

Spherocytes (see Fig. 16-2) are the hallmark of HS. They are dense, round, and hyperchromic; lack central pallor; and have a decreased mean cell diameter. They are always evident in blood smears from patients with moderate or moderately severe HS but are obvious in only 25% to 35% of patients with mild HS.[22,35,36] Hereditary spherocytes are technically misnamed, as they range in shape from thickened discocytes to spherostomatocytes when examined under the scanning electron microscope.[155]

In patients with HS, spherocytes and microspherocytes are the predominant abnormal cells on the peripheral blood smear (other than polychromatophils). However, there are morphologic variations that occur with some of the specific membrane defects in HS (see Fig. 16-7). Patients with ankyrin defects, the most common subgroup, have typical smears (see Fig. 16-7, *C*),[57] but many patients with β-spectrin defects also have a subpopulation of acanthocytes or hyperchromic echinocytes (see Fig. 16-7, *B*)[97,98] and most patients with band 3–deficient HS also have a small number of button mushroom–shaped red cells on their smears (see Fig. 16-7, *D*).[235] In severe HS, caused by homozygous or compound heterozygous α-spectrin mutations or by severe ankyrin defects, misshapen spherocytes, spiculated red cells, and bizarre poikilocytes may be seen and even dominate the blood smear (see Fig. 16-7, *A*). Red cell morphologic findings are variable in protein 4.2 deficiency. Some patients have typical spherocytosis,[144] but acanthocytes, echinocytes, ovalostomatocytes, and other poikilocytes have been observed in others[236] (see Fig. 16-7, *E*). Finally, some patients with truncated β-spectrin chains have spherocytic elliptocytosis (see later).

In contrast to red cells in IgG-mediated autoimmune hemolytic anemias, where spherocytosis also dominates the blood picture, HS red cells lose most of their surface area in the bone marrow instead of in the circulation.[164] Nucleated red cells are uncommon in blood smears,[35] except in the most severe forms of HS.[60] Howell-Jolly bodies are also uncommon before splenectomy (4% of patients)[35] and suggest reticuloendothelial blockade.

Red Cell Indices

Most patients have mild to moderate HS with mild anemia (hemoglobin level of 9 to 12 g/dL) or no anemia at all (so-called compensated hemolysis). In moderate to severe HS, the hemoglobin concentration ranges from 6 to 9 g/dL. In patients with the most severe disease, the hemoglobin concentration may drop to as low as 4 to 5 g/dL. The reticulocyte count is elevated without a comparable increase in the immature reticulocyte fraction.

The MCHC of HS red cells is increased due to relative cellular dehydration.[36] The average MCHC exceeds the upper limit of normal (36%) in about one half of patients with HS, but all patients have some dehydrated cells.[36] The Technicon H1 blood counter and its successors, which use the principle of flow cytometry (i.e., dual-angle laser light scattering), measure the mean corpuscular volume (MCV) and MCHC directly and provide histograms of both parameters. There is a right shift of the MCHC histogram in HS with an increased (>4%) population of hyperdense cells (MCHC > 41 g/dL) due to red cell dehydration, and a broadening of the distribution of MCVs, due to the mixture of microspherocytes and reticulocytes (Fig. 16-17). This excess proportion of hyperdense cells is accurate enough to identify nearly all patients with HS[237-240] and is one of the easiest and most accurate ways to diagnose the disease when one member of a family is already known to have HS.

The MCV usually falls within the lower normal range in HS,[24,33,36] but it is low in about 8% of adults and 16% of children, especially those with more severe HS.[24,33] It is rare for the MCV to be less than 70 fL unless there is concomitant thalassemia or iron deficiency. However, the MCV is low relative to the age of the cells (reticulocytes

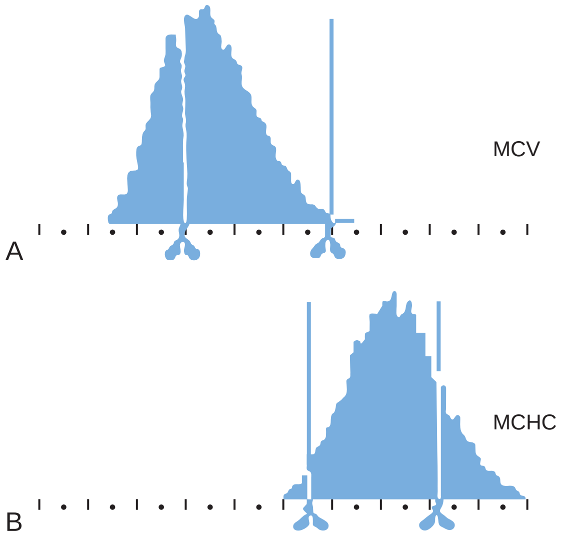

Figure 16-17 Histogram of the distribution of mean cell volume *(MCV)* (**A**) and mean cell hemoglobin concentration *(MCHC)* (**B**) in red cells of a patient with hereditary spherocytosis (HS) before splenectomy. The vertical lines mark the normal limits of the distributions. For the volume distribution, they separate microcytic cells (<60 fL) and macrocytic cells (>120 fL) from normocytic red cells (60 to 120 fL). For the hemoglobin concentration distribution, they separate hypochromic cells (<28 g/dL) and hyperdense cells (>41 g/dL) from normochromic cells (28 to 41 g/dL). The data were collected with a laser-scattering blood counter. Note that the HS patient has subpopulations of microcytes (low MCV) and dehydrated cells (high MCHC). All 21 HS patients in this study had similar subpopulations. *(Modified from Pati AR, Patton WN, Harris RI: The use of the Technicon H1 in the diagnosis of hereditary spherocytosis.* Clin Lab Haematol *11:27–30, 1989.)*

have a high MCV) in all patients with HS, reflecting the dehydrated state of HS red cells. The reticulocyte MCV is also low, which contrasts with IgG-mediated autoimmune hemolytic anemia, where spherocytes are present but the reticulocyte indices are normal.

The RDW of HS erythrocytes is also very high, particularly in patients with more severe disease (see Table 16-3).[33]

Various studies show that the MCHC or the percentage of hyperdense cells and the high RDW discriminate HS patients from normal individuals,[240-242] though less so following splenectomy.[243] An MCHC greater than 35 g/dL has a sensitivity of 70% and a specificity of 86% in diagnosing HS; an RDW greater than 14 has 85% sensitivity and 97% specificity.[241] Combining the MCHC with the erythrocyte distribution width leads to a specificity approaching 100%.[240] The RDW, MCHC, hemoglobin : MCHC ratio, and hemoglobin : RDW ratio all correlate with the severity of disease (see Table 16-3).[33,238]

Fragility and Autohemolysis Tests

Osmotic Fragility Test. Osmotic fragility testing (see Fig. 16-16) is performed by suspending red cells in increasingly hypotonic buffered NaCl.[188,244,245] In hypotonic solutions, normal erythrocytes increase their volume by swelling until they become spherical and burst, releasing hemoglobin into the supernatant. Cells that begin with a decreased surface-to-volume ratio, like spherocytes or stomatocytes, reach the spherical limit at a higher NaCl concentration than normal cells and are termed osmotically fragile. Freshly drawn red cells from approximately one fourth of individuals with HS will have a normal or nearly normal osmotic fragility curve, approximating the number of HS patients with few spherocytes seen on their peripheral blood smears.[37,246] However, after incubation at 37°C for 24 hours, HS red cells lose membrane surface area more readily than normal red cells, because their membranes are leaky and unstable. This incubation accentuates the defect in HS erythrocytes and brings out the defect of osmotic fragility. When the spleen is present, a subpopulation of very fragile erythrocytes that have been conditioned by the spleen form the "tail" of the osmotic fragility curve. This tail disappears after splenectomy.

The osmotic fragility test detects as abnormal (i.e., osmotically sensitive) any red cells with a decreased surface-to-volume ratio. This includes red cells in hereditary stomatocytosis (HSt), hereditary pyropoikilocytosis (HPP), autoimmune hemolytic anemia, and, sometimes, congenital dyserythropoietic anemia, type II (CDAII). Red cells with a large surface-to-volume ratio, whether owing to an increase in membrane surface, like target cells, or to cellular dehydration, like hereditary xerocytes, have decreased osmotic fragility (i.e., are osmotically resistant).

The incubated osmotic fragility test is thought to be more sensitive than the unincubated osmotic fragility test, although this is not well proven. For a long time the osmotic fragility test, particularly the incubated test, was the gold standard test for diagnosing HS,[246] but recent comparisons[24,247,248] show that other tests are more sensitive and specific.

Acidified Glycerol Lysis Test and Pink Test. The glycerol lysis test was developed as an alternative to the osmotic fragility test and is widely used in Europe. In this test, red cells are incubated in glycerol-sodium phosphate–buffered hypotonic saline solution. The glycerol slows the entry of water into the cells, so that the time for the cells to lyse is prolonged and can be measured accurately. The glycerol lysis time is shortened for HS red cells because of the reduced surface-to-volume ratio.

The original glycerol lysis test lacked sensitivity and specificity,[24,247] but the acidified glycerol lysis test (AGLT),[24,247,249] and especially its incubated variant,[250-252] have a high sensitivity (82% to 98%) and specificity (91% to 95%). Another variation achieves 100% sensitivity and specificity by changing the concentration of the sodium phosphate buffer from 5.3 to 9.3 mmol/L.[18] This eliminates the need for preincubation. The modified test is easy to perform and requires very little blood, though accurate control of pH is critical. The simplicity of this test allows it to be used for rapid screening of a large number of blood samples.

Another adaptation of the original glycerol lysis test is the Pink test.[253] Modifications of the test have made it adaptable to microsize samples (e.g., fingerstick blood samples).[254,255] However, direct comparisons suggest that it is less specific than the osmotic fragility test or incubated AGLT.[250]

The AGLT, like the osmotic fragility test, is often abnormal in patients with autoimmune hemolytic anemia, but it is also sometimes abnormal in a variety of other conditions, including CDAII, paroxysmal nocturnal hemoglobinuria, hereditary elliptocytosis, myelodysplastic syndromes, mechanical hemolysis, and chronic renal failure, and in pregnancy.[247,249-252]

Hypertonic Cryohemolysis Test. This method is based on the fact that HS red cells are particularly sensitive to cooling at 0°C in hypertonic solutions, which causes the cells to lyse and release hemoglobin.[256,257] The molecular basis for this sensitivity is not understood, which is a disadvantage, but the test is reported to have a high sensitivity (90% to 100%)[256-258] with one exception (79%),[248] and to have good specificity (94% to 100%).[248,256-258] It is very easy to perform, requires very little blood (a capillary blood sample is sufficient[256,259]), and is compatible with the common blood specimens anticoagulated with ethylenediaminetetraacetic acid (EDTA). These qualities make it a good test for large-scale screening. The hypertonic cryohemolysis test is not widely used but deserves more attention.

Autohemolysis Test. The autohemolysis of erythrocytes after 48 hours at 37°C is normally less than 5% in the absence of glucose or less than 1% in the presence of glucose. Autohemolysis of spherocytes increases to 15% to 45% in the absence of glucose.[37,198,260] In HS, the degree of autohemolysis is reduced by the addition of glucose,[37,260,261] whereas in red cell glycolytic disorders and acquired disorders such as immune-mediated anemias, the degree of autohemolysis is not reduced. Although this differentiation may be occasionally helpful, the autohemolysis test is time consuming and cumbersome; gives variable results, with a sensitivity of only 70% to 80% and a specificity of 80% to 90%[248]; and is rarely performed.

Eosin-5′-Maleimide–Binding Test

When red blood cells are treated with the fluorescent probe eosin-5′-maleimide (EMA), 80% of the fluorophore binds to Lys430 on the outside surface of band 3. The other 20% binds to three proteins in the Rh complex: the Rh blood group proteins, RhAG, and CD47.[262] Thus the measurement of EMA fluorescence detects loss of membrane surface if that loss includes band 3 or the Rh complex. The EMA test is usually done with a flow cytometer so the fluorescence of individual cells is quantified.[263] Because membrane vesicles containing band 3 are believed to be lost in HS due to spectrin and ankyrin deficiency (see Fig. 16-14) and CD47 and, perhaps, band 3 molecules are lost in HS due to protein 4.2 deficiency,[149-151] the EMA test detects all the different forms of HS, not just HS due to primary band 3 deficiency. The test is both highly sensitive (92% to 100%) and highly specific (94% to 100%) for HS.[247,263-267] The only exception is one study that reported only 70% specificity.[248] The test requires very little blood, does not require preincubation, takes little time to perform, is not affected by storage of red cells for a week,[247] and is probably the single best test for diagnosing HS at the present time. It is recommended by the General Haematology Task Force of the British Committee for Standards in Haematology,[14,267a] and when combined with the AGLT achieved 100% sensitivity in one recent comparison of tests for HS.[247] In particular, the test is normal in autoimmune hemolytic anemias, which give false-positive results in the osmotic fragility test, AGLT, and cryohemolysis test. The EMA test is abnormal in disorders where membrane surface is decreased or band 3 is abnormal, such as HPP, SAO, cryohydrocytosis, congenital dyserythropoietic anemia, type II, and $\mathrm{Rh}_{\mathrm{null}}$ disease. However, these are all rare disorders and are fairly easy to distinguish by red cell morphologic findings and other criteria.

Osmotic Gradient Ektacytometry

The ektacytometer is a laser diffraction viscometer, a device that measures how much red cells are deformed by shear stress. The device can be used to measure membrane mechanical strength by determining the shear stress needed to fragment isolated red cell membranes,[268,269] or it can be used to assess red cell surface area, surface-to-volume ratio, and cellular hydration by measuring red cell deformation as a function of osmolarity at a constant shear stress.[270,271] The latter method is termed osmotic gradient ektacytometry and is the gold standard method for diagnosing HS (Fig. 16-18). One study demonstrated a 100% detection rate of HS using osmotic gradient ektacytometry and only a 66% detection rate of HS using the osmotic fragility test in the same population.[271] Unfortunately, only a few laboratories in the world are equipped with an ektacytometer and the instrument is no longer commercially available.

Membrane Protein, RNA, and DNA Measurements

Specialized tests are occasionally used to study patients with complicated disease or those for whom additional information is desired. Useful tests for these purposes include structural and functional studies of erythrocyte membrane proteins, such as protein quantitation, limited tryptic digestion of spectrin, membrane protein synthesis and assembly, and ion transport studies.

Membrane protein concentrations are usually assessed using SDS gels. Individual stained bands are quantified by densitometry or by eluting the dye from an excised band and measuring its concentration spectrophotometrically. This technique is satisfactory for detecting spectrin deficiency (expressed as a spectrin-to–band 3 ratio),[32] although it is not as accurate as a radioimmunoassay[100] or enzyme-linked immunoassay.[32] With densitometry, spectrin and ankyrin deficiencies may be underestimated because they are normalized to band 3, which is partially lost along with membrane lipids as spherocytes circulate. As a result, the spectrin-to–band 3 ratio is lower after splenectomy. This is even more true for ankyrin, which is present in smaller amounts, migrates close to β-spectrin on SDS gels, and is elevated in reticulocytes.[272] It is likely that patients with ankyrin defects, who have combined spectrin and ankyrin deficiency, are often misdiagnosed as having pure spectrin deficiency on SDS gels. SDS gels are satisfactory for detecting band 3

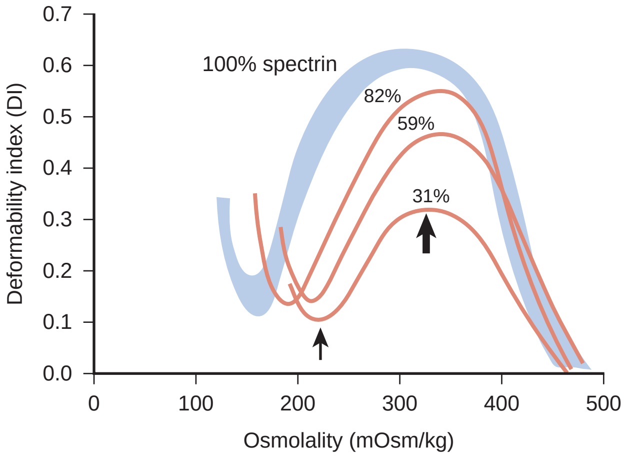

Figure 16-18 Osmotic gradient ektacytometry of hereditary spherocytosis (HS) erythrocytes with differing degrees of spectrin deficiency. In this technique, red cells are stretched by flow in a viscometer in a solution of gradually increasing osmolarity and the degree of red cell deformation is measured by laser diffraction. The deformability index *(DI)*, an arbitrary measure of deformation, is plotted as a function of the osmolarity. In the HS red cells *(red-brown lines)*, the minimum DI observed in the hypotonic region *(thin arrow)* is shifted to the right of the control region *(blue shaded area)*, indicating a decrease in the cell surface area–to-volume ratio. The maximum DI (DI_{max}) associated with the spectrin-deficient HS red cells *(thick arrow)* is less than that of control cells due to the reduced surface area of the HS cells. The more pronounced the spectrin deficiency, the greater is the loss of surface area and the lower is the DI_{max}. The osmolality in the hyperosmolar region at which the DI reaches half of its maximum value is a measure of the hydration state of the red cells. It is decreased in the HS patient with the lowest spectrin content, indicating cellular dehydration. *(Modified from Tse WT, Lux SE: Hereditary spherocytosis and hereditary elliptocytosis. In Scriver CR, Beaudet AL, Sly WS, Valle D, editors:* The metabolic and molecular bases of inherited disease, *8th ed. New York, 2001, McGraw-Hill p. 4665–4727.)*

deficiency (elevated spectrin-to–band 3 ratio) or protein 4.2 deficiency, and they are useful in combination with antibody staining (Western blots) for detecting mutant proteins of altered size.

Moreover, SDS gels do not always identify the abnormal protein in HS, because missense mutations may cause functional defects rather than quantitative defects and because protein deficiencies may be as small as 10%. Therefore several other approaches have been described in an attempt to define specific molecular defects. In families with multiple affected members, linkage analysis has been performed to include or exclude the candidate genes. In addition, suspected proteins can be sequenced using direct nucleotide sequence analysis of polymerase chain reaction (PCR)–amplified complementary DNA (cDNA) or genomic DNA. Currently, no commercial laboratories identify the molecular defects in HS membrane proteins, and these analyses are performed only by a few research laboratories. However, with the rapidly falling costs of both whole-exome and whole-genome sequencing it is likely that DNA sequencing may become the most cost-effective way to diagnose HS (and many other inherited diseases) in the near future. Because HS membrane protein mutations are mostly restricted to specific families and do not predict the severity of the disease, in most cases it is not clinically important to know the exact molecular defect. More detailed studies, sometimes including DNA sequencing, should be considered in patients where the diagnosis is unclear after standard testing and in patients whose HS is more severe than in other family members, possibly due to coinheritance of another deleterious allele.

Differential Diagnosis

In general, HS is easily diagnosed if there are typical laboratory findings of spherocytosis, Coombs negative hemolysis, increased osmotic fragility, high MCHC, high RDW, and a positive family history. However, HS must be differentiated from other causes of spherocytosis, and there are several situations in which diagnosis can be difficult. Other causes of spherocytosis are listed in Table 16-1.

ABO Incompatibility in Neonates

In the neonatal period it may be challenging to differentiate HS from ABO incompatibility.[273] Anemia, hyperbilirubinemia, circulating microspherocytes, and altered osmotic fragility are found in both conditions, and results of the direct Coombs test are sometimes negative in ABO incompatibility. However, in most infants with ABO incompatibility and significant hemolysis, anti-A (or anti-B) antibodies can be eluted from the red cells, and free anti-A (or anti-B) IgG antibodies can be detected in the infant's serum. The combination of HS and ABO incompatibility, though rare, is particularly severe.[274]

Immunohemolytic Anemias

Occasionally, patients with immunohemolytic anemias and spherocytosis also have so few antibody molecules attached to their red cells that results of the direct Coombs test are negative. In these rare cases, the differentiation of the disease from HS is possible only with the use of radioactive antiglobulin reagents, the so-called super–Coombs test.[275]

Heinz Body Hemolytic Anemias

Spherocytosis is also seen in Heinz body hemolytic anemias during an acute hemolytic crisis and occasionally in the steady state. However, in this situation, bite cells and/or blister cells and various dense, irregular cells are always present on the peripheral blood smear, and Heinz bodies can often be detected in red cells supravitally stained with methyl violet.

Aplastic Crises

Diagnostic difficulties may also arise in HS patients who are seen during an aplastic crisis (see below). Early in the crisis, the acute nature of the symptoms may suggest an acquired process and the absence of reticulocytes may divert the physician from a diagnosis of hemolytic anemia. Later, as marrow function returns, physicians may occasionally be misled by the properties of the emerging young HS red cells, which are less spherocytic and osmotically fragile than usual.

Congenital Dyserythropoietic Anemia, Type II

In a preliminary report, Mariani et al[276] noted that 13% of cases referred to their reference laboratory with a provisional diagnosis of HS were found to have CDAII, a disorder featuring underglycosylated band 3 and prominent binuclearity of late erythroblasts that is caused by defects in the secretory pathway gene *SEC23B* (see Chapter 7). Although CDAII patients typically display a variety of morphologic findings, a subset have prominent spherocytosis or elliptocytosis.

Hereditary Stomatocytosis and Xerocytosis

As discussed later in this chapter, patients with disorders of cation permeability, especially hereditary stomatocytosis (HSt) and hereditary xerocytosis (HX), may be misdiagnosed as having HS because they have a dominant hemolytic anemia with an abnormal osmotic fragility test. Care must be taken to exclude these disorders in any HS patient who is to be treated with splenectomy because there is a high risk of thromboembolic complications following splenectomy in patients with HSt and HX.[277]

Conditions That Camouflage Hereditary Spherocytosis

HS may be camouflaged by disorders that increase the surface-to-volume ratio of the red cells, such as iron deficiency,[278] vitamin B_{12} or folate deficiency,[279] obstructive jaundice,[170] β-thalassemia,[280] or sickle cell–hemoglobin C disease (hemoglobin SC disease).[281] In these difficult cases, additional tools, such as red cell membrane protein analysis, or exome or genome sequencing may be necessary to confirm the diagnosis of HS.

Iron Deficiency

Iron deficiency corrects the abnormal shape, fragility, and high MCHC of hereditary spherocytes but does not improve their life span.[278] Megaloblastic anemia can mask the morphologic characteristics of HS.[279] Osmotic fragility is also improved. The masking effect is observed in both vitamin B_{12} and folate deficiencies and is rapidly reversed after correction of the nutritional deficit.

Obstructive Liver Disease

When obstructive jaundice develops, spherocytosis and osmotic fragility transiently improve and hemolysis abates.[170] This is due to the expansion of red cell membrane surface area that follows the increased uptake of cholesterol and phospholipids from the abnormal plasma lipoproteins that are released by the obstructed liver. In normal cells, this increase in surface area leads to the formation of target cells, but in spherocytes, it leads to the appearance of discocytes. For example, we have seen a 6-year-old child who developed jaundice and symptoms of biliary obstruction. She had a palpable spleen tip, evidence of mild compensated hemolysis (hemoglobin concentration of 14 g/dL; reticulocyte count of 3.3%), a normal peripheral blood smear, and normal results for an osmotic fragility test (fresh and incubated cells). Abdominal radiographic studies showed calcified stones in the gallbladder and common bile duct. After cholecystectomy and relief of the partial biliary obstruction, the child's hemolysis worsened (reticulocyte count of 10.8%) and she developed anemia (hemoglobin concentration of 10.2 g/dL), spherocytosis, and abnormal results on an incubated osmotic fragility test. She subsequently underwent splenectomy with clinical improvement.

β-Thalassemic Trait

The coexistence of HS and β-thalassemia trait has been described in a few case reports and has been reported to worsen,[282,283] ameliorate,[280,284] or have no effect on the clinical status of the patient. In a large French family with independently segregating HS and β-thalassemia trait, patients with both traits had signs of both diseases: small, hemoglobin A_2-rich, osmotically fragile cells and some spherocytes on peripheral blood smears. However, the HS phenotype in these patients was less severe than that of family members with HS but without β-thalassemia trait.[280]

Hemoglobin SC

Coinheritance of HS and hemoglobin SC disease may exacerbate the hemoglobinopathy.[284] A 15-year-old male with both diseases was much more anemic than his siblings with hemoglobin SC disease and experienced five splenic sequestration crises. However, the two diseases may also disguise each other morphologically. Only a few spherocytes or target cells were evident on the boy's blood smear, and the surface-to-volume ratio of his red cells (and probably their osmotic fragility) was normal. This is likely due to the balancing effects of HS (loss of surface area) and hemoglobin SC (loss of cell volume) on red cells.

Hemoglobin AS

Sickle trait may also be worsened by HS.[285,286] Yang and colleagues[287] reported two patients with the combination who suffered multiple splenic sequestration crises. Splenic infarcts also occur.[287,288] On the other hand, spontaneous regression of HS, presumably due to development of a hyposplenic state, has also been observed in two family members who had HS and sickle cell trait.[289]

Complications

Most patients with HS have reasonably well-compensated hemolysis and are rarely symptomatic. These patients only seek medical attention when complications occur. Complications are generally related to chronic hemolysis and anemia.

Gallstones

The formation of bilirubinate gallstones is one of the most common complications of HS and is a major impetus for splenectomy in many patients. Instances of gallstones occurring in infancy[290] have been reported, but most appear in adolescents and young adults. In a retrospective study of 152 consecutive patients with HS, conducted before the development of ultrasonography, gallstones were detected in only 5% of children younger than age 10 years who were adequately examined and in approximately 50% of HS patients between 10 and 30 years of age (Fig. 16-19).[291] More recent data are similar; about 15% of patients less than 18 years old develop gallstones.[24,292] The increase in the incidence of gallstones after age 30 years parallels the incidence in the general

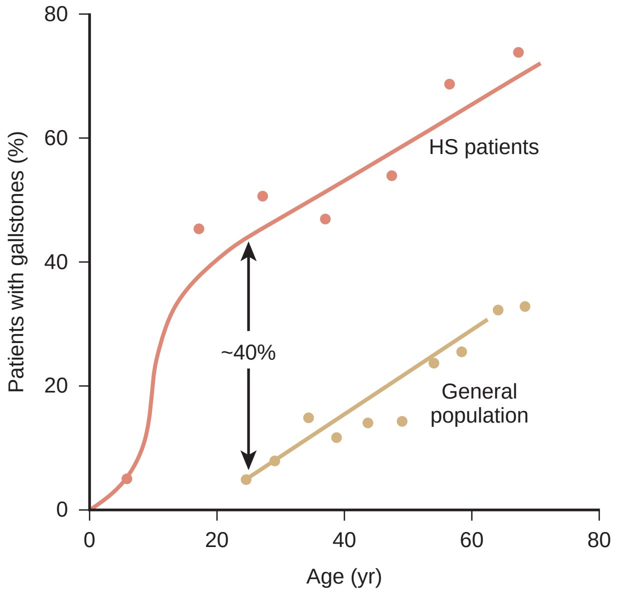

Figure 16-19 Proportion of normal and hereditary spherocytosis (HS) patients with gallstones as a function of age. The graph is redrawn from data in a study of gallbladder disease in 152 consecutive patients seen at The Cleveland Clinic before 1952. Only patients whose gallbladders were examined at surgery or by cholecystography are included. Data for the general population are from an autopsy series of patients who did not have hemolytic anemia. Note that the prevalence of gallstones rises sharply between the ages of 10 and 30 years and parallels the general population after age 30 years. *(Bates GC, Brown CH: Incidence of gallbladder disease in chronic hemolytic anemia [spherocytosis].* Gastroenterology *21:104–109, 1952; Mentzer SH: Clinical and pathologic study of cholecystitis and cholelithiasis.* Surg Gynecol Obstet *42:782–793, 1926.)*

population,[293] which suggests that cholelithiasis owing to HS is primarily manifested in the second and third decades.

The incidence of gallstones in HS patients is related to the ability of the liver to metabolize increased amounts of bilirubin. A common polymorphism in the promoter of the uridine diphosphate-glucuronyl transferase gene (*UGT-1A*) causes decreased enzyme production and, in the homozygous form, causes Gilbert syndrome. Coinheritance of Gilbert syndrome with HS increases the risk for cholelithiasis.[294-297] One retrospective study of 103 children with HS showed that the rate of gallstone formation was 2.2 times higher in those patients with a homozygous mutation in the *UGT-1A* gene than in those with a heterozygous mutation and 4.5 times higher than in healthy patients.[294] HS patients with a homozygous mutation in the *UGT-1A* gene also develop gallstones at a younger age.[298] Therefore analysis of this allele is useful in predicting the risk of cholelithiasis in HS.

Because the pigment stones typical of HS are easily detected by ultrasonography,[294] all patients with HS should have ultrasound examinations about every 5 years if the spleen is intact and just prior to splenectomy. Unfortunately, longitudinal prospective studies using modern techniques (i.e., liver and biliary tree ultrasonography) are not available, making the true incidence and natural history of gallstones in this population unknown. One recent retrospective study showed that patients with HS (or other hemolytic anemias) were much more likely to develop symptomatic disease (i.e., pain, emesis, obstructive jaundice) than children with gallstones for other reasons.[299] In that study, 68% of HS patients with gallstones had symptoms and all of them required cholecystectomy. This coincides with earlier reports.[300,301] The most common complication was biliary obstruction, followed by acute cholecystitis.[299] However, studies that include large numbers of patients with mild HS show a much lower incidence of symptomatic gallbladder disease.[302]

More accurate, longitudinal studies are needed to determine the optimal treatment of patients with mild to moderate HS and cholelithiasis, particularly asymptomatic cholelithiasis that is detected on routine screening. Only about 15% to 20% of adults with asymptomatic stones (all types) develop symptoms over a prolonged period,[303] but the retrospective data above suggest the complication rate may be much higher for bilirubinate stones. Surgery is indicated for recurrent symptoms or signs of biliary obstruction. It is unclear whether children who are undergoing cholecystectomy for symptomatic cholelithiasis should also have a splenectomy or partial splenectomy if it is not otherwise indicated (see later). Most authorities recommend it,[14,304,305] arguing that the risk of stones persists, presumably in the remaining biliary radicals or cystic duct stump. However, cholelithiasis after cholecystectomy for typical gallbladder disease is rare unless a gallbladder remnant remains.[306] Presumably the risk would be higher in someone with continuing hemolysis, but it is not clear how much higher.

If an HS patient requires a splenectomy, an ultrasound study of the gallbladder is indicated prior to surgery to assess the need for concomitant cholecystectomy.[14,307] Cholecystotomy (removal of stones but not the gallbladder) is an option in children with silent stones because the risk of new stones forming is markedly reduced after splenectomy.[308] For this reason, prophylactic cholecystectomy at the time of splenectomy solely to prevent the formation of gallstones is not indicated.[308]

Crises

Patients with HS, like patients with other hemolytic diseases such as sickle cell disease, face a number of potential crises. Table 16-9 summarizes the types of crises that occur in patients with HS.

Hemolytic Crises. Hemolytic crises are probably the most common type in HS. They usually occur with viral syndromes, particularly in children younger than 6 years of age, and are presumed to be due to activation of the reticuloendothelial system.[309] They are characterized by anemia, vomiting, abdominal pain, an increase in reticulocytes, a mild transient increase in jaundice, and tender splenomegaly. Severe hemolytic crises are rare and medical intervention with hospitalization and transfusions is seldom required.

Aplastic Crises. Aplastic crises are less common than mild hemolytic crises but are more serious and may lead to severe anemia, resulting in congestive heart failure or even death.[38] They tend to occur in infants and children.[24]

TABLE 16-9 Classification of Crises

Type	Anemia	Reticulocytes	Jaundice	Cause	Comment
Hemolytic	Increased	Increased	Increased	Viral infections leading to splenomegaly	Frequent, usually mild
Aplastic	Increased	Decreased	Decreased	Parvovirus B19	Occurs once. Severe. Risk to pregnant contacts
Megaloblastic	Increased	No change or decreased	No change or increased	Relative folic acid deficiency	Rare, preventable

Aplastic crises are caused by parvovirus B19, the etiologic agent in erythema infectiosum (fifth disease).[310] Parvovirus B19 infection may present as fever, chills, lethargy, vomiting, diarrhea, and myalgias.[311] Normal children may also develop a maculopapular rash on the face (slapped cheek appearance), trunk, and extremities—the rash of fifth disease—but this is less common in children with hemolysis and an aplastic crisis. In addition, patients with an aplastic crisis experience pallor and weakness from their worsening anemia.[312,313] Jaundice, if present, may decrease as the number of abnormal red cells that can be destroyed declines.

Parvovirus B19 selectively infects erythropoietic precursors and inhibits their growth by inducing apoptosis and cell cycle arrest at G_2.[314-317] Parvovirus infections are often associated with mild neutropenia (≈20%) or thrombocytopenia (≈40%), and instances of transient pancytopenia have been reported.[310,318-320] Parvovirus may infect several members of a family simultaneously.[320-324]

The sequence of events in an aplastic crisis was well described by Owren[312] (though he confusingly called the crisis "hemolytic," reflecting the recovery phase, instead of aplastic) and is illustrated in Figure 16-20. During the aplastic phase, the hematocrit level and reticulocyte count fall, marrow erythroblasts disappear, and unused iron accumulates in the serum. As production of new red cells declines, the cells that remain age and microspherocytosis and osmotic fragility increase. The return of marrow function is heralded by a fall in the serum iron concentration and the emergence of granulocytes, platelets, and, finally, reticulocytes. During this recovery phase, the serum phosphorus can drop to dangerously low levels.[325] Furthermore, the lack of reticulocytes early in the recovery phase should not eliminate hemolytic anemias from diagnostic consideration.

Infection with parvovirus B19 is a particular danger to susceptible pregnant women because it can infect the fetus, leading to fetal anemia, nonimmune hydrops fetalis, and fetal demise.[326-328] The risk of nonimmune hydrops fetalis to the fetus of a woman who becomes infected with B19 during pregnancy is probably low. Nevertheless, because the virus is highly contagious and is easily transmitted to patients and staff[329,330] and because only one half to two thirds of pregnant women have acquired protective antibodies,[331] patients who have or are suspected of having an aplastic crisis should be placed under respiratory and contact precautions while hospitalized and IgG-negative contacts who are pregnant should be tested for evidence of seroconversion. Nonimmune hydrops fetalis can be detected by ultrasonography and treated with intrauterine transfusions.[332]

Many asymptomatic patients with mild HS and compensated hemolysis first come to medical attention during an aplastic crisis.[333-335] It is important to recognize that

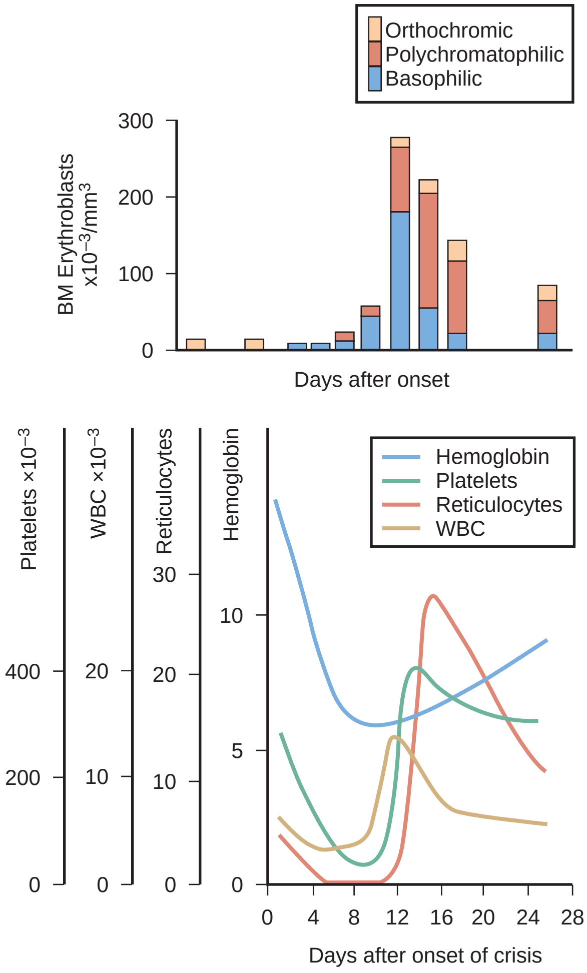

Figure 16-20 The temporal sequence of a severe aplastic crisis in a patient with hereditary spherocytosis who previously had well-compensated hemolysis. Note the profound reticulocytopenia and the mild leukopenia and thrombocytopenia in the early phases of the reaction. Note also that the reemergence of reticulocytes is heralded by the sequential return of peripheral blood granulocytes and platelets and bone marrow normoblasts. *(Data from Owren PA: Congenital hemolytic jaundice: the pathogenesis of the "hemolytic crisis."* Blood *3:231–248, 1948.)*

other family members with HS are susceptible and need to be tested. Diagnostic confusion may arise during reemergence of marrow function, when the physician may mistake an aplastic crisis for a hemolytic one. Because aplastic crises usually last for 10 to 14 days[312] (about one half the life span of HS red cells), the hemoglobin value typically falls to about one half its usual level before recovery occurs. Thus aplastic crises are a serious threat to young children with HS, particularly children with more severe forms of the disease.

The diagnosis of parvovirus can be made using serum PCR or immunologic tests. Bone marrow pathology demonstrates giant pronormoblasts, which are a hallmark of the cytopathic effects of parvovirus B19.[336,337] Treatment is supportive until the aplastic episode self-resolves. For severe anemia, red cell transfusions may be necessary. In immunocompromised patients, intravenous immunoglobulin assists in clearing the virus. Immunity after parvovirus infection is lifelong.

Megaloblastic Crises. Rarely, HS patients may have megaloblastic crises due to folate deficiency. These crises occur in patients whose dietary intake of folic acid is inadequate for the increased needs of the red cell precursors in the bone marrow. Therefore megaloblastic crises typically occur in patients with HS who are recovering from an aplastic crisis or who are pregnant.[62,63] A megaloblastic crisis in pregnancy has been reported as the first manifestation of HS. All patients with moderate to severe HS should routinely receive folic acid supplements (about 1 mg/day) prior to splenectomy to prevent this complication. Folate supplementation is unnecessary in mild HS or in HS following splenectomy except during pregnancy.[14]

Other Complications

Gout and Leg Ulcers. Rarely, adults with HS develop gout,[338,339] indolent leg ulcers,[340,341] or a chronic erythematous dermatitis on the legs.[342,343] Splenectomy appears to be curative.

Extramedullary Hematopoiesis. Adults with HS may also develop extramedullary masses of hematopoietic tissue, particularly alongside the posterior thoracic, lumbar, or sacral spine[9,339,344-349] or in the hila of the kidneys[9] or the adrenal gland.[350,351] These masses may spontaneously bleed, leading to hemothorax.[347] They may also cause spinal cord compression.[349] Surprisingly, these tumors often arise in patients with mild HS, perhaps because they often do not undergo splenectomy, and may be the first manifestations of HS.[346] The masses gradually enlarge and are sometimes mistaken for neoplasms.[339,348] Biopsy may lead to extensive bleeding. If a biopsy is necessary, an open biopsy should be performed. These bone marrow tumors can be diagnosed by magnetic resonance imaging,[352] which may make biopsies unnecessary. The masses stop growing and undergo fatty metamorphosis after splenectomy, but they do not shrink in size.[344,345]

Hemochromatosis. Iron overload is uncommon in HS. Only 4% of unsplenectomized, untransfused HS patients have a serum ferritin greater than 500 ng/mL.[24] However, untreated HS may exacerbate hemochromatosis in patients who are heterozygous for the hereditary disease.[353-356] Several patients with HS subsequently died of liver failure or hepatoma.

Angioid Streaks. These brownish or gray streaks resembling veins in the optic fundus have been described in adult members of several HS kindreds.[357,358] The rate of occurrence of this association is unknown. Angioid streaks are relatively common in some other hematologic disorders, notably sickle cell disease and thalassemia,[357,359] and may be complicated by retinal vascular proliferation that requires treatment.[360]

Pseudohyperkalemia. Because HS red cells leak potassium ions more rapidly than normal red cells, serum samples from HS patients may demonstrate pseudohyperkalemia if allowed to sit for a long period of time before electrolytes are analyzed.[361]

Splenic Rupture. Although splenomegaly is a common symptom in HS patients, cases of splenic rupture are rare.[362,363] In one large recent study, the risk of blunt splenic injury from trauma was only 1 in 20,000 to 50,000 per person per year, a rate that did not differ from the risk in the general population.[363] This contrasts significantly with the splenomegaly that occurs during acute viral infections, such as Epstein-Barr virus, in which splenic rupture occurs with a higher frequency. It is unclear why this difference exists, although it may reflect the unique pathophysiology of splenic enlargement in the different conditions. Children with HS and splenomegaly should not be restricted from normal activities. However, older children and adults with splenic enlargement that extends below the rib cage should probably avoid activities that may inflict a powerful, direct blow to the abdomen.

Nonerythroid Manifestations. Kindreds with HS and neurologic manifestations have been described. In the older literature, multiple case reports describe patients with HS and various neurologic abnormalities, such as a Friedrich ataxia-like disease, cerebellar disturbances, muscle atrophy, and a tabeslike syndrome.[364,365,366,367] More recently, patients with certain ankyrin gene deletions have been reported, who have psychomotor retardation and various neurologic manifestations with or without hypogonadism.[58,106-109,368] Additional reports describe patients with ankyrin-deficient HS and slowly progressive spinocerebellar disease.[364] Erythroid ankyrin and β-spectrin are expressed in the cerebellum and spinal cord, and mice that lack ankyrin develop delayed cerebellar ataxia owing to a slow loss of Purkinje cells.[369] This suggests that selected ankyrin and β-spectrin mutations may affect the neurologic system in addition to red blood cells.

A three-generation Russian family with co-segregating HS and hypertrophic cardiomyopathy has been described,[370] and two brothers have HS, a movement disorder, and myopathy.[371] These cases are interesting because the erythroid forms of spectrin and ankyrin are known to be expressed in muscle.

Splenectomy

Splenectomy cures all but the most severe cases of HS, eliminating anemia and hyperbilirubinemia and reducing the reticulocyte count to nearly normal levels (1% to 3%). In most patients, red cell survival becomes normal or remains only slightly shortened. Spherocytosis and altered osmotic fragility persist, but the "tail" of the osmotic fragility curve, created by conditioning of a subpopulation of spherocytes by the spleen, disappears. After splenectomy, patients with the most severe forms of HS still have some hemolysis, but their clinical improvement is striking.[31,60] Splenectomy appears to have a more beneficial effect on the deformability of spectrin/ankyrin-deficient erythrocytes than band 3–deficient erythrocytes.[372]

Currently, the major decisions about treatment are who should have a splenectomy, what kind of operation should be performed, and how patients should be cared for postoperatively. The indications for splenectomy have to be weighed carefully because a small fraction of patients will die of overwhelming postsplenectomy infections.[373-377,378,379] Because the risk of postsplenectomy sepsis is very high in infancy and early childhood, splenectomy should be delayed until the age of 5 to 9 years if possible and to at least age 3 years in all children, even if transfusions are required chronically in the interim. There is no evidence that further delay is useful, and it may be harmful because the risk of cholelithiasis increases substantially after age 10 years (see Fig. 16-19).[291]

Risks of Splenectomy

Immediate Postsplenectomy Complications. Early complications of splenectomy include local infection such as a subphrenic abscess, bleeding, and pancreatitis due to intraoperative injury to the tail of the pancreas.[380]

Postsplenectomy Sepsis. Overwhelming serious bacterial infection with encapsulated bacterial organisms is one of the most significant risks of splenectomy. The risk is highest with encapsulated organisms, such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae*. Postsplenectomy sepsis can be rapidly fatal, and it is unclear how the risk of postsplenectomy sepsis changes over time. Some studies have suggested that the risk is highest immediately after splenectomy, and decreases over time. Other studies conclude that the risk of sepsis is present indefinitely after splenectomy.[377-381]

It is difficult to estimate the risk of postsplenectomy infections after infancy.[382] The surveys of Schwartz and colleagues,[376] Green and coworkers,[373] and Schilling[377] are limited to adults and largely predate immunization for *S. pneumoniae* and other bacteria. These surveys show an incidence of fulminant sepsis in adults of 0.2 to 0.5 per 100 person-years of follow-up and a death rate of about 0.1 per 100 person-years. Three of the four deaths in the Schilling study occurred 18 years or more after the operation, and none of the patients who died had received pneumococcal vaccine or prophylactic antibiotics. In addition, other serious bacterial infections (e.g., pneumonia, meningitis, peritonitis, and bacteremia) in adults were much more common (4.5 per 100 person-years) than normal, particularly in the first few years after the operation. The incidence of postsplenectomy sepsis appears to be higher in children, particularly younger children.[378,379,382-385]

Unfortunately, the majority of studies of postsplenectomy sepsis in adults and children have serious methodologic problems. Most are case reports or retrospective reports subject to recall bias. The patients included in the studies have heterogeneous diseases and have often undergone splenectomy for a variety of indications. Furthermore, in most of the studies, the adult patients are not fully immunized and do not receive antibiotic prophylaxis. Further research is necessary to quantitate the risk over time of postsplenectomy sepsis in HS patients treated according to current practice guidelines.

With the development of the *Haemophilus influenzae* type B conjugate vaccine, meningococcal conjugate and nonconjugate vaccines, and pneumococcal conjugate and nonconjugate vaccines, the risk of postsplenectomy sepsis should be reduced. However, Grace found that only 26% of adults with HS who were splenectomized prior to 1990 had received all of these vaccines and most did not continue to take their antibiotic prophylaxis as adults.[386] A second study came to similar conclusions.[387] Few patients were adequately educated about antibiotic prophylaxis and fever management. These studies highlight the necessity for quality improvement in the care of HS patients after splenectomy.

Babesiosis and Malaria. Splenectomized HS patients are at increased risk for serious parasitic infections, such as babesiosis[388-390] and malaria.[391,392] Babesiosis is a zoonotic infection in which an intraerythrocytic protozoan is transmitted by ticks; the vector is endemic in Europe and in the northeastern, upper Midwestern, and coastal northwestern United States.[393] Babesiosis can also be transmitted by transfusion. About 20 cases per year are reported.[394] In the United States, the etiologic agent of babesiosis is *Babesia microti*. The clinical symptoms usually begin with chills, anorexia, and fatigue and are followed by intermittent fever and symptoms including myalgias, arthralgias, headache, and vomiting.[393] In healthy patients, the infection is often asymptomatic or mild. In patients with asplenia, severe illness with hemolytic anemia can occur, including fulminant illness resulting in death. Diagnosis is made through microscopic identification on Giemsa- or Wright-stained thick or thin blood smears. Serologic tests for *Babesia* antibodies and PCR are also available. Because the primary vector for the parasite is the *Ixodes scapularis* tick, which can also transmit diseases such as Lyme disease and granulocytic anaplasmosis (previously called granulocytic ehrlichiosis), these infections should be considered as well.[393] Current treatment for severe babesiosis uses clindamycin and oral quinine for 7 to 10 days.[393] Azithromycin and atovaquone are effective in milder disease. Exchange blood transfusions should be considered for severely ill patients, especially those with parasitemia of more than 10%. Splenectomized HS patients,

TABLE 16-10 Risk of Adverse Vascular Events Years after Splenectomy for Hereditary Spherocytosis

	Cumulative Incidence of First Event by Age 70[*]	Hazard Ratio[†]	95% Confidence Interval	P-value
Arterial events[‡]				
Normals (male)	17%			
Normals (female)	10%			
Hereditary spherocytosis (HS) spleen out (male)	32%			
HS spleen out (female)	22%			
HS spleen in (male)	2%			
HS spleen in (female)	3%			
Male gender		1.73	1.02-2.93	0.04
HS with spleen in versus normals		0.22	0.08-0.58	0.002
HS with spleen out versus normals		1.56	0.90-2.68	0.11
HS with spleen out versus HS with spleen in		7.15	2.81-18.2	<0.0001
Venous events[§]				
Normals (male)	12%			
Normals (female)	4%			
HS spleen out (male)	19%			
HS spleen out (female)	20%			
HS spleen in (male)	4%			
HS spleen in (female)	7%			
Male gender		0.86	0.42-1.75	0.68
HS with spleen in versus normals		0.90	0.26-7.13	0.87
HS with spleen out versus normals		3.00	1.26-7.13	0.01
HS with spleen out versus HS with spleen in		3.33	1.13-9.79	0.03

From Schilling RF, Gangnon RE, Traver MI: Delayed adverse vascular events after splenectomy in hereditary spherocytosis. J Thromb Haemost *6:1289–1295, 2007.*

[*] Splenectomy prior to 18 years of age.

[†] From Cox proportional hazards regression model.

[‡] Arterial events include myocardial infarction, stroke, coronary artery surgery, and carotid artery surgery.

[§] Venous events include thrombophlebitis, deep vein thrombosis, and pulmonary embolism.

when traveling in endemic areas, should attempt to avoid tick bites by wearing long pants and using tick repellants.

Animal experiments demonstrate that the spleen is essential in eliminating malaria parasitemia,[395] and human observations show that in the absence of the spleen parasitemia is greater and more mature parasites (schizonts and trophozoites) are observed in the peripheral blood[396] (reviewed[391]). However, only anecdotal reports or small series exist that demonstrate an increased risk of *Plasmodium falciparum* and *Plasmodium vivax* malaria in asplenic individuals, some of whom had unusually severe disease and others of whom did not.[392,397-400] This may reflect the fact that in previously immune patients the spleen is almost nonessential for protection against severe malaria.[392] We conclude that the risk of malaria in splenectomized individuals is not well documented. Nevertheless, the author knows experienced hematologists practicing in areas endemic for malaria who will not allow their patients to be splenectomized. This "local knowledge" suggests the risk is higher than the limited number of reports indicates. Accordingly, we believe that splenectomized HS patients should strictly adhere to malaria chemoprophylaxis when traveling in endemic areas or avoid such areas altogether, and should immediately seek medical advice if a fever develops.

Thrombosis and Thromboembolic Diseases. Many studies demonstrate an increased risk of thrombosis in splenectomized patients where hemolysis persists after the operation,[277,401,402] but it is not clear to what extent this risk applies to patients with HS. Major large-vessel and organ thrombosis is a common finding in murine models of severe HS,[403,404] but thrombotic complications are rare in the milder forms of HS that are typical in humans, at least in the short term. Several studies from Schilling indicate that there is a risk in human HS as well, but it is not manifest for decades.[405,406,407,408] Schilling has a unique experience, with up to five decades of follow-up of a cohort of 634 HS patients. His observations are summarized in Table 16-10.

First, splenectomized HS patients have a significantly increased risk (threefold) of venous thrombotic events compared with normals.[407] These included episodes of thrombophlebitis, deep vein thrombosis, and pulmonary embolism. There is also a modest (1.5-fold) but not quite statistically significantly increased risk of arterial events such as myocardial infarction, stroke, coronary artery disease requiring surgery, and carotid artery disease requiring surgery compared with normals.[407] By age 70 years, 32% of men and 22% of women have experienced an arterial event and about 20% of each gender have had a venous event (see Table 16-10). These numbers are two to five times greater than in normal family members. In HS patients splenectomized in childhood or adolescence, venous thrombotic events begin to occur after age 30 years but arterial complications do not increase significantly until 55 to 60 years of age.[407] The incidence of arterial events occurring following splenectomy shown in Table 16-10 is comparable to the 1.86-fold greater incidence of death from ischemic heart disease following splenectomy that Robinette and Fraumeni observed in an excellent case-controlled study of the outcomes of World War II battlefield splenectomies after three decades.[409]

A few instances of pulmonary hypertension have been observed in HS patients years after splenectomy.[408,410-413] Pulmonary hypertension is a known complication of splenectomy for other reasons, including nonhemolytic conditions such as Gaucher disease,[277,411,413-418] but it was not unusually common in Schilling's long-term study of HS patients,[407] which corresponds to the recently reported experience of Crary et al[419] and the unpublished experience of R.F. Grace at Boston Children's Hospital. Portal vein thrombosis,[402,420,421] priapism,[422-425] and osteonecrosis[426] also seem to be more prevalent after splenectomy for conditions such as thalassemia and Gaucher disease but do not seem to be a complication of splenectomy for HS.

The most interesting and surprising finding in Schilling's studies is that HS patients who avoid splenectomy have about fivefold *fewer* arterial events than *normals* and sevenfold fewer events than HS patients who have had their spleens removed (see Table 16-10).[406,407,427] This is probably because they have lower hemoglobin levels and higher bilirubin levels than normal. A low hemoglobin level is known to correlate with a lower risk of myocardial and cerebral infarction in the Framingham study.[428,429] This may be because anemia of all types is associated with a low cholesterol level.[430-432] This fact has been known for over 100 years[433] but is little studied, is not understood, and has largely been forgotten. Whether the lower cholesterol level accounts for all of the vascular benefits of a low hemoglobin level is not known. Homocysteine concentrations rise after splenectomy for HS and may also be a contributing factor.[432] Higher bilirubin levels also correlate with a low risk of coronary artery disease,[434] perhaps because bilirubin is an antioxidant. It is possible that this benefit accounts for the prevalence of the Gilbert polymorphism.

Thus most of the sevenfold increased risk of deleterious arterial events following splenectomy in HS seems to result from relief of the anemia and hemolysis, whereas most of the threefold increased risk of venous thromboembolism seems to be due to the splenectomy itself (see Table 16-10). The factors potentiating thromboembolism are not well understood. Potential factors include (1) a chronically high platelet count, (2) decreased splenic clearance of free hemoglobin, which decreases serum nitric oxide and potentially causes vasocontriction, and (3) diminished splenic clearance of "blood dust" (microvesicles released from HS red cells and platelets). These particles are assumed to have phosphatidylserine exposed on their surfaces, thus activating the clotting cascade.

Although the vascular risks of splenectomy in HS observed by Schilling need to be confirmed by others, a conservative approach to splenectomy is supported by the available data. It is not clear to what extent polymorphisms that are known to raise the risk of hypercoagulation may contribute to the risks imposed by splenectomy, disorders such as factor V Leiden polymorphism, antithrombin III deficiency, deficiency of protein S or C, and the prothrombin G20210A mutation (see Chapter 33), but it is reasonable to assume they do contribute and incorporate them into the decision making as to whether a splenectomy is indicated or not. Further, once a patient with HS has been splenectomized attempts should be made to limit additional hypercoagulable risk factors.

Indications for Splenectomy

Splenectomy should only be performed if there are clear indications. All patients with severe spherocytosis, who are transfusion dependent or have growth failure or skeletal changes, should undergo splenectomy. Full or partial (subtotal) splenectomy (see later section) is also usually recommended for patients with moderate HS if they suffer from reduced vitality or physical stamina due to anemia; if, later in life, anemia compromises vascular perfusion of vital organs; or if leg ulcers or extramedullary hematopoietic tumors develop. Whether asymptomatic patients with moderate HS should have a splenectomy remains controversial. Partial splenectomy may also be indicated in some of these patients. Splenectomy can be deferred, probably indefinitely, in patients with mild HS and compensated hemolysis. In young symptomatic children with HS, splenectomy should be delayed until at least 3 years of age, and, if possible, until age 6 to 9 years of age, due to the risk of infection. In addition, after 6 years of age, the number of febrile infections is reduced, which decreases the frequency of postsplenectomy fever evaluations.

Surgical Procedures

Laparoscopic Splenectomy. When splenectomy is warranted, laparoscopic splenectomy is the method of choice in most centers, even for large spleens.[435-438] The procedure can be combined with laparoscopic cholecystectomy if desired.[304,305,439] Comparisons of the laparoscopic approach to the open surgical approach demonstrate fewer complications, less postoperative discomfort, a quicker return to preoperative diet and activities, shorter hospitalization stays, decreased costs, and smaller scars in the laparoscopic group.[439-445] The drawbacks of a laparoscopic approach are longer operative time, potentially greater difficulty in controlling bleeding, and capsular fracture with subsequent splenosis.[446] Recent innovations include doing the entire laparoscopic procedure through a single incision, which decreases pain and improves the cosmetic result.[444] Nearly all recent studies support the laparoscopic approach to splenectomy as the method of choice in centers in which the surgical staff is experienced.

Partial (Subtotal) Splenectomy. The emergence of antibiotic-resistant pneumococci and increasing evidence suggesting an increased risk of thrombosis after splenectomy has led to reexamination of the role of alternate treatment modalities. Partial splenectomy has been suggested for selected patients with HS and other hematologic conditions.[447,448,449-451] The goal of the operation is to decrease hemolysis while maintaining residual splenic phagocytic function. In practice, at least 80% to 90% of

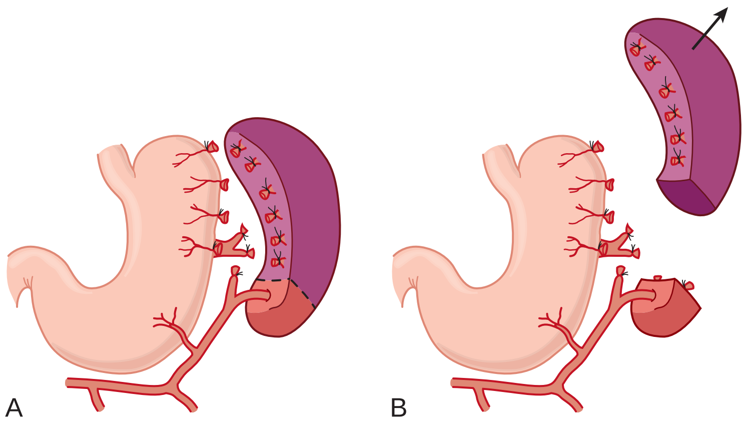

Figure 16-21 Surgical technique used for partial or subtotal (≈80%) splenectomy. **A**, All vascular pedicles supplying the spleen are divided, except those arising from the left gastroepiploic vessels. **B**, The upper pole of the spleen is removed at the boundary between the well-perfused and poorly perfused tissue. *(Modified from Tchernia G, Gauthier F, Mielot F, et al: Initial assessment of the beneficial effect of partial splenectomy in hereditary spherocytosis.* Blood *81:2014–2020, 1993.)*

the enlarged organ is removed (Fig. 16-21). Partial splenectomy was initially an open operation, but laparoscopic and robotic approaches have been developed and are becoming more common.[452-455] The procedure is more time consuming than a total splenectomy and the time for recovery is longer (4 to 7 days of hospitalization), but studies have shown that partial splenectomy is safe and reduces the rate of hemolysis.[447-450,456-458]

Tchernia and colleagues followed 40 patients for up to 12 years after partial splenectomy and found that the mean hemoglobin value increased from 9.2 g/dL to 12.7 g/dL and the absolute reticulocyte count decreased from 523×10^9 cells/L to 267×10^9 cells/L (Fig. 16-22).[448] In the children with at least 10 years of follow-up, no differences in hemoglobin and reticulocyte levels were noticed between the group with partial splenectomy and the group with total splenectomy. Significant splenic regrowth was seen in the first year after partial splenectomy, but, after an initial period, the rate of splenic growth, in this study, was much reduced (see Fig. 16-22, C). Nevertheless, 8% of patients in this study required a total splenectomy after the partial splenectomy, particularly those with severe HS, because of splenic regrowth and resumption of hemolysis. Moreover, 4 of 18 patients in whom the gallbladder was not removed with the initial surgery, required a subsequent cholecystectomy.[448] In a more recent multiinstitutional study of 62 children with a partial splenectomy for HS, only 5% had recurrent hemolysis requiring reoperation and complete splenectomy.[449] Although these results are encouraging, the percentage of HS patients who will require reoperation many years after a partial splenectomy will not be known for several more decades. This is the major risk of the procedure.

A major argument for partial splenectomy is the expectation that patients will be less susceptible to severe sepsis or thromboembolic complications if some splenic tissue remains. This seems logical and is probably true but is impossible to prove without a very large study over a very long time. We are not aware of any reports of overwhelming sepsis following partial splenectomy, and there is some evidence that the risk of sepsis is less.[459] Experimentally, animals are better protected from bacterial challenge if some splenic tissue remains,[460,461] though not as well protected as normal animals,[461] and animals and humans recover immune function more rapidly after partial instead of complete splenectomy.[462,463]

In summary, we believe partial splenectomy is an effective procedure in patients with moderate HS, largely ameliorating hemolysis and anemia while preserving some splenic function. It is important for patients and families to realize that long-term follow-up is encouraging but limited and that splenic regrowth may eventually require a reoperation. For this reason, the procedure cannot yet be recommended as the preferred approach. Some believe that partial splenectomy is the best option for infants with transfusion-dependent HS, who would otherwise be transfused for 3 to 4 years before a complete splenectomy would be done. In such children it is likely that a complete splenectomy will still be needed, however.

Embolization. A few case reports have reported partial splenic arterial embolization as an alternative to splenectomy to treat hemolysis in patients with HS.[464,465] Because the experience with this procedure is limited, it cannot be recommended as routine therapy.

Postsplenectomy Changes

After splenectomy, spherocytosis persists but conditioned microspherocytes disappear, and changes typical of the postsplenectomy state, including Howell-Jolly bodies, target cells, siderocytes, and acanthocytes, become evident in the peripheral blood smear. On average, the MCV and mean red cell surface area increase and the MCHC and osmotic fragility decrease, but the effects are modest (5% to 10%).[466] In typical dominant HS, reticulocyte counts fall to normal or nearly normal levels,[31] although the red cell life span, if carefully measured, remains slightly shortened (96 ± 13 days).[174] In all but the most severe occurrences, anemia and jaundice remit and do not recur.

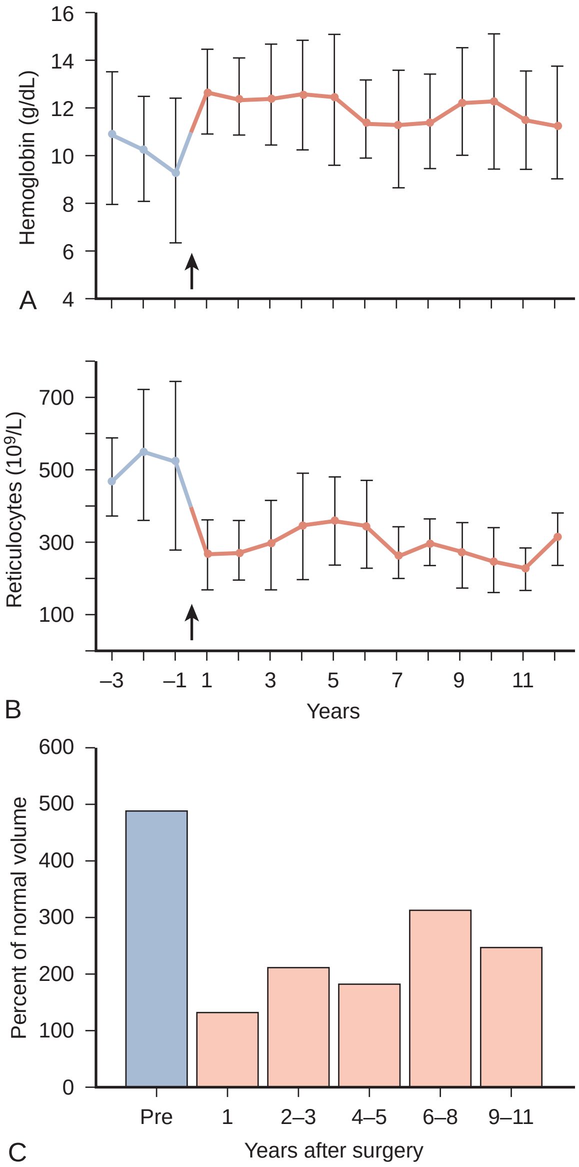

Figure 16-22 Long-term follow-up of partial splenectomy in hereditary spherocytosis (HS). The data are the results of 12 years of experience with the procedure in 40 patients (ages 1 to 25 years). **A** and **B**, Change in hemoglobin and reticulocyte count with time before and after the subtotal splenectomy *(arrow)*. The mean and standard deviations are shown. Both the rise in hemoglobin and fall in reticulocytes have been sustained for more than a decade. **C**, The size of the spleen during the same period. There is some regrowth of the splenic remnant, especially in the first few years. It remains to be seen whether the remnant reaches a stable size or continues to grow. *(Modified from Bader-Meunier B, Gauthier F, Archambaud F, et al. Long-term evaluation of the beneficial effect of subtotal splenectomy for management of hereditary spherocytosis.* Blood *97:399–403, 2001.)*

Splenectomy Failure

The rare splenectomy failure (i.e., recurrence of hemolysis) is usually caused by an accessory spleen that was missed during surgery[456,467] or by another red cell disorder, such as pyruvate kinase deficiency.[468,469] Accessory spleens occur in 15% to 40% of patients and must always be sought.[300,301,380] Occasionally they can be in unusual places: in the chest,[470] across the midline,[471] deeper in the abdomen, and so forth. Recurrence of hemolytic anemia years or even decades after splenectomy should raise suspicion of an accessory spleen,[456,467] particularly if Howell-Jolly bodies are no longer evident on peripheral blood smears. The absence of "pitted" red cells with craterlike surface indentations, readily seen by interference contrast microscopy, is also a sensitive measure of recrudescent splenic function.[472,473] The ectopic splenic tissue can be confirmed by a radiocolloid liver/spleen scan or a scan using ^{51}Cr-labeled, heat-damaged red cells.[467,474]

Other Therapies

Corticosteroid Therapy

It has been known since the 1950s that large doses of corticosteroids markedly ameliorate HS in unsplenectomized patients,[212] similar to the effects produced by splenectomy. More recent studies have confirmed this finding.[213,214] Corticosteroids (prednisolone 1 to 2 mg/kg/day or equivalent for 7 days) can be a useful, temporary alternative to splenectomy in controlling severe hemolytic crises.

Vaccination

All candidates for splenectomy should receive a complete series of vaccinations against encapsulated organisms, including pneumococcus, meningococcus, and *Haemophilus influenzae*. Yearly influenza immunizations are recommended to reduce the chance of secondary bacterial infections. Recent studies have shown that the majority of adults who were splenectomized as children are not up-to-date with their vaccinations.[386] In a study examining the current cases of overwhelming postsplenectomy infection in the era of pneumococcal vaccines, only 31% of 77 patients with such infection received the pneumococcal vaccine.[387] It is important to emphasize the importance of immunizations to the affected parents of children with HS.

Experts differ somewhat on how to protect patients from infection after splenectomy. Our approach follows the 2014 CDC guidelines for vaccination of children[474a] and adults[474b] with high risk conditions. The guidelines change frequently, so the latest version should be consulted. The recommendations of the British Committee for Standards in Haematology have also been published relatively recently.[474c]

If possible, all vaccines should be given at least two weeks and preferably four weeks before splenectomy.

Pneumoccal Vaccines. All children should receive the 13-valent pneumococcal conjugate vaccine (PCV13, Prevnar 13) beginning at two months in the recommended four-dose schedule.[474a] Between two and six years of age, one or two additional doses of PCV13 should be given to children who will have a splenectomy, depending on how many doses of PCV were given in infancy.[474a] The minimum interval between doses of PCV13 is 8 weeks. After 2 years of age, the 23-valent pneumococcal polysaccharide vaccine (PPSV23, Pneumovax 23) should also be administered, assuming it hasn't been given previously.

PPSV23 immunization should be delayed until at least 8 weeks after PCV13.

For adults and children 6 years and older who have not received both PCV13 and PPSV23, one dose of the missing vaccine(s) should be given. If both are needed, PCV13 should be given first and followed at least 8 weeks later by PPSV23.[474a,474b] If PPSV23 is given first, PCV13 administration must be delayed for at least one year.

A single dose of PPSV23 should be given five years after the first dose and again at 65 years of age.[474a,474b]

***Haemophilus influenzae* Type B Vaccine.** Most children will have received the routine Hib vaccine series and a booster dose prior to the age where splenectomy is considered. Whether fully immunized or not, all patients 15 months of age or older should receive a single dose of a Hib-containing vaccine at least two weeks prior to an elective splenectomy. No studies have been performed on the need for reimmunization, which is not recommended at this time.

Meningococcal Vaccines. All patients over the age of nine months who are undergoing splenectomy should be immunized with one of the meningococcal conjugate vaccines: MenACWY-CRM (Menveo) or MenACWY-D (Menactra).[474a,474b] Both vaccines are targeted at serogroups A, C, W-135, and Y. Serogroups B and X are not covered. Children younger than 19 months of age should receive the four-dose infant series of Menveo. Those 19 through 23 months who have not completed this series should receive two primary doses of Menveo at least three months apart. Adults and children older than two years of age should receive two doses of an MenACWY vaccine at least two months apart. Menactra should not be given until at least four weeks after the completion of all PCV13 doses.[474a,474b] Asplenic children whose primary vaccination occurred before 7 years of age should receive an additional dose of either Menveo or Menactra three years later and then every five years.[474d] Asplenic adults and children whose primary vaccination occurred after 7 years of age should be revaccinated with Menveo or Menactra every five years.[474b,474d]

Antibiotic Prophylaxis

Prophylactic antibiotics after splenectomy are recommended, with emphasis on protection against pneumococcal sepsis (i.e., penicillin V or equivalent, 125 mg orally twice daily in children younger than 5 years of age, and 250 mg twice daily in older children and adults). For those patients allergic to penicillin, erythromycin is recommended. Antibiotic prophylaxis is recommended at least through childhood, and, for teenagers and adults, for at least the first 5 years after surgery.

Some physicians recommend lifelong antibiotic prophylaxis because it is unclear how the risk of postsplenectomy overwhelming infection changes over time. The emergence of penicillin-resistant pneumococci (5% in 1989 to ≥35% in 1997) has led many to reconsider this recommendation, particularly because prior antibiotic use is a risk factor for the development of penicillin resistance in pneumococci.[476] Patients who do not take prophylactic antibiotics should have a supply of oral antibiotics on hand, which they should take immediately should fever higher than 101.5°C develop. They should then seek medical attention immediately.

The risk of infection is decreased in immunized patients, because 50% to 70% of postsplenectomy sepsis is due to *S. pneumoniae* and about 80% of pneumococcal disease is due to strains contained in the two vaccines. However, it is likely that strains omitted from the vaccines will rise in frequency as pathogens due to selection. Further risk reduction would be anticipated from the chronic use of prophylactic penicillin. Nevertheless, the risk cannot be reduced to zero. Postsplenectomy infections occur occasionally in successfully immunized patients,[477-479] and compliance with prophylactic medication regimens is a problem, particularly in teenagers.[480]

The occurrence of penicillin-resistant pneumococci is increasing very rapidly all over the world.[481-483] In some countries, more than one half of all isolates are resistant.[484] In the United States, the prevalence is approximately 35%, but it is much higher in some localities and is rising.[481,483,485] Although most of the strains show some sensitivity to penicillin, some are highly resistant and others are multiply resistant.[485-487] The emergence of these strains will greatly complicate the use of antibiotics for pneumococcal prophylaxis during the next decades.

Folic Acid

Patients with moderate or severe HS should take folic acid (0.5 to 1 mg/day or 5 mg/week orally) prior to splenectomy to prevent folate deficiency. Folate supplementation is unnecessary in mild HS or in HS following splenectomy except during pregnancy.[14]

Animal and Fish Models of Hereditary Spherocytosis and Elliptocytosis

Animal models, such as the well-characterized mouse and zebrafish models, have assisted in our understanding of the pathophysiology of HS. Four types of spontaneous spherocytic hemolytic anemia have been identified in the common house mouse, Mus Musculus[488]: ja/ja (jaundice); sph/sph (spherocytosis) and its alleles (sph^{1J}/sph^{1J} [hemolytic anemia], sph^{2J}/sph^{2J} [now lost], sph^{3J}/sph^{3J}, sph^{4J}/sph^{4J}, sph^{IhJ}/sph^{IhJ}, sph^{2BC}/sph^{2BC}, and sph^{DEM}/sph^{DEM}); nb/nb (normoblastosis); and wan/wan. The nomenclature indicates that anemia is observed only in the homozygous state and that the mutants represent four loci: ja, sph, nb, and wan. All the defects are autosomal recessive and cause severe hemolysis, with spherocytosis, jaundice, bilirubin, gallstones, and hepatosplenomegaly,[489] except in the sph^{DEM}/sph^{DEM} mice, which have hereditary elliptocytosis. Another recessive spherocytic hemolytic anemia has been described in the deer mouse, *Peromyscus maniculatus*.[490] This disorder is less severe and closely resembles the autosomal dominant form of human HS. The spectrin content of deer mouse erythrocytes is reduced by about 20% (unpublished data).

Spectrin Mutants

The ja/ja mutant has a defect in the spectrin β-chain (Arg1160→Stop) and produces no detectable spectrin.[491]

The sph/sph variants lack α-chains but have small amounts of β-spectrin. These mice do not produce α-spectrin either due to decreased synthesis, function, or stability. The sph and sph^{2BC} alleles are frameshift mutations and null alleles.[492,493] Four mutations affect the C-terminus of α-spectrin, which was previously thought to be functionless in erythrocytes: (1) sph^{IhJ} is a null mutation (Q1853Stop) in repeat 18[494]; (2) sph^{3J} contains a missense mutation (H2012Y) in repeat 19, near the C-terminus, that alters splicing and results in premature termination of translation[494]; (3) sph^{1J} allele lacks the last 13 amino acids of α-spectrin[495]; and (4) sph^{4J} is a missense mutation (C2384Y).[494] The latter two mutations are in the C-terminal EF-hand domain. All four mutations show marked spherocytosis, and sph^{4J} does so despite minimally decreased membrane spectrin and normal band 3 levels.[494,495] Thus the amino acids in the C-terminus of the spectrin tail are functionally important and, it turns out, are critical in attaching spectrin to actin.[496] The sph^{DEM}/sph^{DEM} mouse is missing exon 11 and 46 amino acids near the amino terminus of α-spectrin repeat 5. These mice have elliptocytes, spherocytes, and poikilocytes, and, therefore clinically appear to be a cross between HS and hereditary elliptocytosis (see later).[495]

Cardiac thrombi, fibrotic lesions, and renal hemochromatosis are found in *ja/ja* and *sph/sph* mice in adulthood.[403,404,497] Transplantation of the hematopoietic cells from *sph/sph* mice is sufficient to induce thrombotic events in the transplant recipients.[498] In mice with α-spectrin mutations, 70% to 100% experience cerebral infarction.[498,499] Studies of red cell adhesion in these mice demonstrate a tenfold higher adhesion to thrombospondin compared with normal red blood cells and an increased adhesion to laminin.[500] Therefore it is hypothesized that changes in the red cell adhesive characteristics contribute to the thrombotic events in these mice.

Riesling (*ris*) is an induced mutation in zebrafish that results in a nearly bloodless phenotype, which causes severe anemia and congestive heart failure.[163] The *ris* phenotype is caused by a null mutation in β-spectrin. The red blood cells of affected zebrafish are spherocytic rather than elliptocytic, the normal zebrafish red cell shape. Scanning electron micrographs show membrane loss, as in other forms of severe HS.

Ankyrin Mutants

Mice with the nb/nb mutation have a primary defect in ankyrin. This is due to a single-nucleotide deletion leading to a frameshift and premature chain termination in the regulatory region of ankyrin.[501,502] The nb/nb red cells have 50% to 70% of normal spectrin levels but only a trace of ankyrin mRNA and protein.[369,489] This is in striking contrast to ankyrin defects in human HS, in which ankyrin and spectrin levels are comparably depressed.[32,100] Fetal nb/nb mice have no anemia and normal reticulocyte counts at birth,[503] possibly because of expression of ankyrin-related proteins in utero.[74] Mice that completely lack Ank1 mostly die in utero.[504] Those that survive have extreme hemolysis and are severely deficient in β-spectrin as well as ankyrin. However, recently a mouse mutation (hema6) has been identified that results in decreased levels of full-length normal ankyrin and more typical HS.[505] This should be useful experimentally.

The *nb/nb* mice also lack ankyrin in cerebellar Purkinje cells.[369] This leads to an age-related loss of Purkinje cells during the first 5 to 7 months of life and the emergence of cerebellar ataxia.[369] Spinocerebellar degeneration and related syndromes have also been reported in a few adult humans with HS,[364-367] although it is not yet known whether ankyrin is affected.

Band 3 Mutants

Complete deficiency of band 3 was first described in cattle with a recessive form of HS. In cows, homozygosity for a nonsense mutation in the band 3 gene leads to absence of band 3 and protein 4.2.[506] Red cell membranes are unstable and show surface area loss, as demonstrated by invagination, vesiculation, and extrusion of microvesicles. The affected cattle have defective anion transport and a mild acidosis but mild hemolysis in comparison with other band 3–deficient species.

In mice, targeted disruption of the band 3 gene causes a severe spherocytic hemolytic anemia, with exuberant loss of the membrane surface as vesicles, tubules, and myelin forms.[161] Red cell membranes also lack protein 4.2 and glycophorin A.[507] Surprisingly, the content of membrane skeletal proteins and the architecture of negative-stained, spread skeletons is normal or nearly normal.[161] Therefore band 3 appears to be required for stabilizing membrane lipids but not for membrane skeleton assembly. For unclear reasons, these band 3–deficient mice have a significant propensity for thrombosis and often die in the neonatal period.[508]

Another mouse model, *wan/wan*, in a C3H/HeJ background, has a null defect in the band 3 gene.[509] These mice have a severe anemia, which is lethal in the neonatal period. However, when *wan/wan* mice are crossed with wild-type mice of a different strain, *Mus castaneus*, the F2 generation shows variable hematologic severity. The genetic modifier causing this variability in hematologic abnormalities has been localized to a small chromosomal region containing the β-spectrin gene.[509]

Finally, zebrafish with band 3 mutations, associated with the *retsina* (*ret*) phenotype, suffer from severe anemia with a complete arrest in erythroid maturation at the late erythroblast stage.[162] These rare *ret/ret* red blood cells are spherocytic rather than elliptical. Many of these arrested erythroblasts have bilobed nuclei that demonstrate defects in cytokinesis.

Protein 4.2

Mice with targeted deletions of the protein 4.2 gene survive normally and have only mildly spherocytic anemia and hemolysis.[510]

Protein 4.1R

Mice lacking the protein 4.1R gene due to targeted deletion have moderate hemolysis and decreased red cell membrane stability.[511] In addition to lacking protein 4.1R, these mice are also deficient in protein 55, glycoprotein

C, ankyrin, and spectrin. The red cell morphology is remarkable for spherocytic shape, rather than elliptocytic shape, likely related to the spectrin deficiency. Interestingly, the knockout mice exhibit specific neurobehavioral deficits in movement, coordination, balance, and learning that correspond with the selective localization of 4.1R in granule cells of the cerebellum and dentate gyrus.[512] Two zebrafish models with protein 4.1R deficiency have been discovered, merlot and chablis, and these mutants have very severe hemolytic anemia, characterized by spherocytosis and increased osmotic fragility.[160]

Adducin and Dematin

Mice lacking just β-adducin[513] or β-adducin and γ-adducin[514] have a very mild, spherocytic hemolytic anemia. Knockout of α-adducin,[515] which eliminates all three adducins (α, β, and γ), still results in only mild hemolysis and moderate spherocytosis, though slightly more severe than in the β-adducin knockout. Similarly, loss of the headpiece of dematin[516] produces, at most, *very* mild hemolysis. However, mice that lack *both* β-adducin and the dematin headpiece have a severe spherocytic hemolytic anemia that resembles severe HS. The data suggest the various vertical membrane connections involving the actin junctions—4.1R-p55–glycophorin C, 4.1R–band 3, alpha spectrin–4.2–band 3, adducin–band 3, adducin–Glut1, and actin-dematin–Glut1—are largely redundant and only become consequential when more than one is eliminated.

HEREDITARY ELLIPTOCYTOSIS AND HEREDITARY PYROPOIKILOCYTOSIS

Hereditary elliptocytosis (HE) is characterized by the presence of elliptical erythrocytes on peripheral blood smears (Fig. 16-23) and defects in the "horizontal" connections that tie the membrane skeleton together (see Fig. 16-4), particularly mutations that impair spectrin self-association. Clinically, these disorders range from the asymptomatic carrier state to severe hemolysis to death in utero due to hydrops fetalis.

History

HE was first reported in 1904 by Dresbach,[517] a physiologist at Ohio State University, who discovered the condition in one of his histology students during a laboratory exercise in which the students were examining their own blood. This report elicited some controversy, because the student died soon thereafter, leading to speculation that the student actually suffered from incipient pernicious anemia.[518] A number of famous pathologists supported Dresbach's view that the elliptocytosis was a primary disorder,[519] and this view was substantiated during the next two decades by the reports of Bishop[520] and Huck

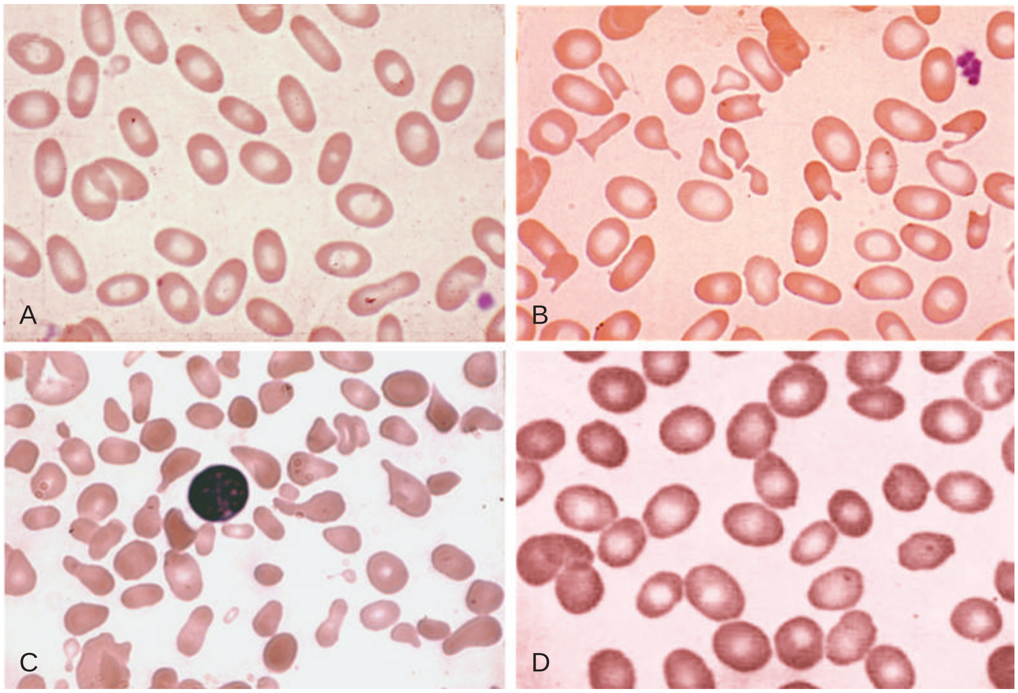

Figure 16-23 Peripheral blood smears from subjects with various forms of hereditary elliptocytosis (HE). **A**, Simple heterozygote with typical HE associated with an elliptogenic α-spectrin mutation. Note the predominant elliptocytosis with some rod-shaped cells and virtual absence of poikilocytes. **B**, HE with infantile poikilocytosis. Note the combination of typical elliptocytes with budding and fragmenting red cells. In some patients the fragmentation is more prominent and may resemble hereditary pyropoikilocytosis. **C,** Severe hereditary pyropoikilocytosis (HPP). Note prominent microspherocytosis, micropoikilocytosis, dense irregular cells, and fragmentation. Relatively few elliptocytes are present. Many poikilocytes are in the process of budding. In patients with less severe HPP, there is less fragmentation and more elliptocytes are evident. **D**, Spherocytic elliptocytosis. Rounded elliptocytes dominate the morphologic picture. The morphologic findings are variable, even within a family, and some patients have more microspherocytes or microelliptocytes than in this example and are more "HS-like."

and Bigelow.[521] The demonstration of the disease in three generations of one family clearly established its hereditary nature.[522,523]

In the 1930s and 1940s, there was some debate about whether HE was a disease or simply a morphologic curiosity.[522-527] Early on, some confusion also existed in the differentiation of HE from sickle cell anemia, from hypochromic elliptocytosis (probably thalassemia), and, later, from HS.[525] These reports emphasize the morphologically deceptive nature of HE and its hemolytic variants. Additional historical features of the disease are found in the reports of Wyandt and associates,[526] Wolman and Ozge,[527] and Dacie.[528]

Prevalence and Genetics

In the United States, HE has been estimated to occur in 1 in 2000 to 4000 of the population.[526,529] The true incidence of HE is unknown because the clinical severity is heterogeneous and many patients are asymptomatic. HE is much more common in areas of endemic malaria, specifically in people of African and Mediterranean ancestry.[159,529-536] The higher frequency of HE in these populations suggests that this disease may confer some resistance to malaria. In a study of HE in Benin, Western Africa, an incidence of 1.6% was observed.[530]

HE variants are inherited predominantly in an autosomal dominant manner. Typically, individuals who are heterozygous for an elliptocytic variant have asymptomatic elliptocytosis without anemia. Individuals who are homozygotes or compound heterozygotes for HE variants may suffer from mild to severe hemolysis with moderate to marked anemia. Spontaneous elliptocytogenic mutations have been reported. Presumably, they are also inherited in an autosomal dominant fashion. In one unique kindred with Alport syndrome, mental retardation, midface hypoplasia, and elliptocytosis, inheritance was X-linked and associated with a submicroscopic chromosome X deletion.[537]

HE is heterogeneous with multiple genetic loci, including reports of mutations in α-spectrin, β-spectrin, protein 4.1R, and glycophorin C genes.[87,88,538-540] Missense mutations in α-spectrin or β-spectrin that produce a functional defect in spectrin self-association are the most common. The mutations described include deletions, point mutations, insertions, and splicing defects. The incidence of one common elliptocytic mutation of α-spectrin, $\alpha^{I/65-68}$, approaches 1% in Central Africa. It has a worldwide distribution in people of African ancestry. Genetic haplotyping studies suggest that this mutation may have a "founder effect," originating in central Africa similar to the Benin type of hemoglobin.[530] This observation further supports the possibility that there has been genetic selection for HE because it imparts some resistance to malaria.[540]

Clinical Syndromes

Most of the reported patients with HE can be classified into one of four clinical categories: typical heterozygous HE, HE with hemolysis/hereditary pyropoikilocytosis (HPP), spherocytic HE, and Southeast Asian ovalocytosis (SAO) (Table 16-11). With the exception of SAO, which is homogeneous in molecular terms and is considered separately (see later), these classifications denote clinical phenotypes and not specific molecular causes, although correlations between the two exist.

Typical Heterozygous Hereditary Elliptocytosis

The clinical characteristics of typical HE trait vary considerably, defining several clinical subtypes. Different clinical patterns may be seen in members of the same family, and the clinical presentation in single individuals may vary over time, so the clinical patterns are probably more useful for illustrating the spectrum of HE than for classifying the disease.

Silent Carrier State. This condition was identified by analyzing asymptomatic members of kindreds with HE or HPP. The affected persons have red cells with normal morphologic characteristics and no evidence of hemolysis, but detailed investigations show a subtle defect in their membrane skeletons, with decreased red cell thermal stability, decreased mechanical stability of isolated skeletons, an increased fraction of spectrin dimers in 0°C spectrin extracts, abnormal tryptic peptide maps of spectrin, or various combinations of these defects.[159,533-536,541,542] Some patients also have α-spectrin^{LELY}, a thalassemia-like splicing defect that impairs production of an otherwise normal α-spectrin protein (see later). It is notable that some patients classified as "silent carriers" have the same molecular defect as patients with mild typical HE,[159,536,542,543] emphasizing the variability of clinical expression.

Typical Hereditary Elliptocytosis (Common or Mild Hereditary Elliptocytosis). This is the most common clinical form of HE.[544-557] Patients are asymptomatic, and HE is often diagnosed incidentally when individuals are undergoing screening for an unrelated condition. These persons are not anemic and rarely have splenomegaly. Red cell survival may be normal,[556] but more often there is very mild, compensated hemolysis with a slight reticulocytosis and decreased haptoglobin level.[548,550,554,558] In these patients, HE is hardly more than a morphologic curiosity. The peripheral blood smear shows prominent elliptocytosis with little red cell budding or fragmentation and no spherocytosis. A subset of patients with more functionally severe defects, usually those most affecting spectrin self-association (see later), have slightly greater hemolysis and anemia together with some red cell fragmentation. Elliptocytes (by definition) usually exceed 30% of the red cells and, in some instances, approach 100% (see Fig. 16-23, *A*).[526,542,543,554,558] Very elongated elliptocytes are common (>10%). These patients are easily separated from normal individuals, who have less than 2% to 5% elliptocytes.[550,552,558] Somewhat higher proportions of elliptocytes are seen in patients with anemias, particularly megaloblastic anemias, hypochromic-microcytic anemias, myelodysplastic syndromes, and myelofibrosis,[559] but even in these individuals elliptocytes do not exceed 35%.[552] Thus the diagnosis of typical HE is rarely difficult.

Typical Hereditary Elliptocytosis with Sporadic Hemolysis. Patients with typical heterozygous HE may develop

TABLE 16-11 Clinical Subtypes of Hereditary Elliptocytosis

Clinical Manifestations	Laboratory Manifestations
Typical Heterozygous HE	
Asymptomatic Dominant inheritance: one parent with HE No splenomegaly Variants: Some neonates with moderately severe hemolytic anemia and HPP-like smear. Converts to typical HE by ≈1 year Some patients with typical HE have mild to moderate chronic hemolysis and some poikilocytosis, due either to coinheritance of the low-expression variant α-spectrin^{LELY}, coexistence of chronic disease producing splenomegaly, or unknown factors	Blood smear: elliptocytes, rod forms, few or no poikilocytes No anemia, little or no hemolysis (reticulocytes = 1% to 3%) Normal osmotic fragility Usually a defect in α-spectrin or β-spectrin leading to decreased spectrin self-association, or a defect in protein 4.1 leading to partial deficiency or dysfunction
Homozygous HE or HPP	
Moderate to severe hemolytic anemia Splenomegaly Intermittent jaundice Aplastic crises Recessive inheritance: typically one parent with HE and one with α-spectrin^{LELY}, or both parents with HE Good improvement after splenectomy	Blood smear: bizarre poikilocytes, fragments, ± spherocytes, ± elliptocytes Reticulocytosis Decreased MCV because of red cell fragmentation Increased osmotic fragility, positive EMA test α-spectrin defects: Decreased red cell and spectrin heat stability Marked defect in spectrin self-association In the more severe variants, partial spectrin deficiency (indicated by more spherocytes on the blood smear)
Spherocytic HE	
Mild to moderate hemolytic anemia Splenomegaly Intermittent jaundice Aplastic crises Dominant inheritance pattern Excellent response to splenectomy	Blood smear: rounded elliptocytes, ± spherocytes. May see variable morphology within a kindred Reticulocytosis Increased osmotic fragility Variable molecular defects: C-terminal truncation of β-spectrin Protein 4.2 deficiency (some patients). Variable morphology: spherocytes, ovalocytes, stomatocytes, or spiculated cells, sometimes resembling spherocytic HE Glycophorin C deficiency (rare)
Southeast Asian Ovalocytosis	
Anemia and jaundice in neonatal period; then asymptomatic Dominant inheritance Lowland aboriginal tribes, especially in Melanesia, Malaysia, and the Philippines Very rigid red cells that resist invasion by malarial parasites in vitro and protect against cerebral malaria in vivo	Blood smear: rounded elliptocytes, some with a transverse bar that divides the central clear space (theta cells) No hemolysis or anemia after neonatal period Normal osmotic fragility, positive EMA test Mutant band 3 that lacks anion exchange function and tends to aggregate, leading to a rigid membrane

Modified from Walensky LD, Narla M, Lux SE: Disorders of the red cell membrane. In Handin RI, Lux SE, Stossel TP, editors: Blood: principles and practice of hematology. *Philadelphia, 2003, JB Lippincott.*

EMA, Eosin-5′-maleimide; *HE*, hereditary elliptocytosis; *HPP*, hereditary pyropoikilocytosis; *MCV*, mean cell volume.

uncompensated hemolysis in response to stimuli that cause hyperplasia of the reticuloendothelial system, particularly if the spleen is involved. Examples include viral hepatitis, cirrhosis, infectious mononucleosis, bacterial infections, and malaria.[555,560-562] Hemolysis has also been observed with thrombotic thrombocytopenic purpura and with disseminated intravascular coagulation, which suggests that elliptocytes may be especially susceptible to microangiopathic damage.[563] For unknown reasons pregnancy and cobalamin (vitamin B_{12}) deficiency may also transiently aggravate the disease.[50,564]

Hereditary Elliptocytosis with Infantile Poikilocytosis. Infants with typical HE sometimes begin life with moderately severe hemolytic anemia, characterized by marked red cell budding, fragmentation, and poikilocytosis (see Fig. 16-23, *B*) and by neonatal jaundice, which may require an exchange transfusion.[534,549,558,565-570] Usually enough elliptocytes are present to suggest the diagnosis, but this is not always so and the disorder is mistaken for sepsis, infantile pyknocytosis, or a microangiopathic or oxidant-induced hemolytic anemia.[567,568] HE with infantile poikilocytosis can easily be distinguished from the latter conditions if the parents' blood smears are examined, because one will show typical HE. However, it is more difficult to distinguish HE with infantile poikilocytosis from severe HE or HPP (see later). Many α-spectrin variants have been associated with the infantile poikilocytosis syndrome. The factors that determine susceptibility are unknown. With time, fragmentation and hemolysis decline, and the clinical picture of typical HE emerges.[571] This transition requires 4 months to 2 years.

Subsequently, the disease is clinically indistinguishable from typical HE. The prevalence is unknown, but in our experience, it is unusual but not rare.

The fragmenting neonatal elliptocytes are very sensitive to heat, as are hereditary pyropoikilocytes (see later), but unlike with pyropoikilocytes, this sensitivity lessens over time.[570] During the conversion, the poikilocytic red cells are dense (old) and rich in hemoglobin F whereas the smooth elliptocytes are less dense (young) and enriched in hemoglobin A (unpublished observations). This finding suggests that the change in the disease corresponds to the change from fetal to adult erythropoiesis. No variations in the primary α-spectrin defect or its functional effects on spectrin self-association occur during the conversion, so other skeletal interactions must differ in fetal and adult red cells. Mentzer and associates[572] made the interesting suggestion that 2,3-DPG is the critical agent. Because it is not bound by hemoglobin F, the concentration of 2,3-DPG is elevated in fetal red cells. The free anion is known to weaken spectrin-actin–protein 4.1R interactions[573,574] but the mechanical properties of *normal* red cells or ghosts are not greatly impaired at physiologic concentrations of 2,3-DPG in vitro.[575,576] Whether they are weakened in intact *elliptocytes*, which are more fragile, is untested. If 2,3-DPG does impair spectrin-actin–4.1R binding in situ, the inherited defect in spectrin self-association would certainly be aggravated. However, if this is the explanation it is not clear why only a subset of infants with HE develop infantile poikilocytosis. Perhaps they are the ones with the more functionally severe mutations.[577]

Hereditary Elliptocytosis with Dyserythropoiesis. In a small number of families with otherwise typical HE, the sporadic occurrence of hemolysis and anemia is at least partially due to the development of dysplastic and ineffective erythropoiesis. All the reported patients with this rare syndrome are from Italy. They have somewhat more rounded red cells than is typical for HE plus the characteristic findings of ineffective erythropoiesis: relatively low reticulocyte counts, indirect hyperbilirubinemia, and high serum iron and ferritin concentrations.[578,579] Erythrocytes from some patients are macrocytic. The patients' bone marrows are hyperplastic, with decreased late erythroblasts and dysplastic features (asynchrony of nuclear-cytoplasmic maturation, binuclearity, internuclear bridges, and small numbers of ringed sideroblasts). Anemia and, presumably, erythroid dysplasia usually commence during adolescence or early adult life and advance gradually over years. Splenectomy is not curative for these patients. The available data suggest that dysplasia and elliptocytosis co-segregate because no individuals with dysplasia have been observed who did not also have elliptocytosis.[578,579] These families must have a unique subtype of HE because none of the typical HE protein defects were observed in one well-studied family.

Typical Hereditary Elliptocytosis with Chronic Hemolysis and Hereditary Pyropoikilocytosis

These two conditions are really variations of the same thing: homozygosity or compound heterozygosity for elliptocytogenic mutations and/or silent alleles that augment elliptocytogenic mutations. In general, the greater the hemolysis the more the evidence of membrane instability on the peripheral blood smear: budding red cells, fragments, and other bizarre poikilocytes.

Combination of Typical Hereditary Elliptocytosis with α-Spectrin^{LELY}. Roughly 10% of HE patients have a more severe variant. Sometimes this occurs within members of the same kindred.[547,580-583] It is often due to coinheritance of a very common α-spectrin polymorphism that leads to decreased α-spectrin expression—the α^{LELY} allele—in *trans* to a severely dysfunctional α-spectrin HE allele.[548,577,584-590] The α^{LELY} allele has the effect of increasing the proportion of the HE α-spectrin that is available to combine with β-spectrin and thereby increasing the fraction of defective spectrin on the membrane (see later). Patients with α^{LELY} opposite a severe α-spectrin HE mutation ($\alpha^{LELY}/\alpha^{Severe\ HE}$) have marked hemolysis and elliptopoikilocytosis compared with their siblings with wild-type α-spectrin opposite the same HE allele ($+/\alpha^{Severe\ HE}$), whereas patients with α^{LELY} combined with a mild HE defect ($\alpha^{LELY}/\ \alpha^{Mild\ HE}$) have more elliptocytes than their siblings with $+/\alpha^{Mild\ HE}$ but no significant hemolysis.[587] Less often hemolysis is caused by heterozygous inheritance of a mutant spectrin that is grossly dysfunctional.[580] This is indicated by dominant transmission of the hemolytic syndrome and by an unusually high fraction of spectrin dimers in 0°C spectrin extracts (see later).

Hereditary Pyropoikilocytosis. This uncommon and unfortunately named disorder presents in infancy or early childhood as a hemolytic anemia (hemoglobin level of 2 to 11 g/dL)[558,591-602] characterized by extreme poikilocytosis with budding red cells, fragments, spherocytes, triangulocytes, and other bizarre-shaped cells, as well as, in some patients, few or no elliptocytes (see Fig. 16-23, *C*). The morphologic characteristics of the blood smear somewhat resemble those seen in patients who have severe thermal burns, which explains the name of the disorder. In fact, it is just a severe variant of HE. Most of the patients are individuals of African origin. Patients typically exhibit hyperbilirubinemia in the neonatal period or marked anemia in the first few months of life,[601] but diagnosis may be delayed in milder variants.[603] Red cell fragmentation, erythroblastosis, and splenomegaly are also characteristic.[601,604] Complications of severe anemia, including growth retardation, frontal bossing, and early gallbladder disease, have been reported.[599,600] The red cells are very osmotically fragile, particularly after incubation.[591,598-600] In the most severely affected patients, there is a significant microcytosis, with very low MCVs (25 to 75 fL), because of the large number of fragmented red cells.[159,537,543,558,598,599] Another characteristic feature of these cells is their remarkable thermal sensitivity. Hereditary pyropoikilocytes fragment at 45°C to 46°C (normal 49°C) after short periods of heating (10 to 15 minutes).[600] After splenectomy, hemolysis is markedly decreased, with the hemoglobin level typically ranging from 10 to 14 g/dL with 3% to 10% reticulocytes.[558,599,600]

Some HPP patients are homozygous or compound heterozygous for a structural variant of spectrin, located in the region of the spectrin αβ heterodimer self-association.[558,591-596] Rare patients with homozygous protein 4.1R deficiency have a similar picture.[605] Other HPP patients are compound heterozygotes for an α-spectrin mutation and a silent allele such as α^{LELY} or an even more severe thalassemia-like defect of spectrin synthesis.[606-608] As noted earlier, this thalassemia-like defect enhances the expression of the mutant α^{HE}-spectrin in the cells. An α°-spectrin mutation opposite the α^{HE}-spectrin allele would functionally be the same as having homozygous HE.

Unlike typical HE, HPP red cells are often deficient in spectrin.[558,604,606,607] Presumably if the defect in spectrin self-association is severe enough spectrin either falls off the membrane or never properly assembles. The degree of spectrin deficiency correlates with the severity of HPP and is presumably responsible for the large number of spherocytes and relative paucity of elliptocytes in some patients.

Spherocytic Hereditary Elliptocytosis

This dominant disorder is a phenotypic hybrid of typical HE and HS. It has only been reported in Caucasian families of European descent.[555,609-612] The prevalence of spherocytic HE is unknown, but judging from the number of published reports, it is relatively rare, probably accounting for no more than 5% of HE cases in patients of European ancestry. Unlike in typical HE, almost all affected patients have some hemolysis. This is usually mild to moderate and is often incompletely compensated. The elliptocytes are fewer and plumper than in typical HE (see Fig. 16-3, D), and some spherocytes, microspherocytes, and microelliptocytes are often present. Poikilocytes and red cell fragments are uncommon, a finding that distinguishes this disorder from typical HE with hemolysis. Some spiculated cells may be present following splenectomy,[609] similar to what is seen in β-spectrin deficient HS. Red cell morphologic findings may vary, even within the same family. Some family members may have relatively prominent spherocytes and as few as 10% to 20% elliptocytes, whereas in others elliptocytes predominate and spherocytes are rare.[524,609] This may cause diagnostic confusion initially, particularly if the propositus has few elliptocytes. Family studies almost always reveal some members with obvious elliptocytosis.

Spherocytic elliptocytes are osmotically fragile,[524,609,611,612] particularly after incubation. Gallbladder disease is common, and aplastic crises are a risk.[609,611] The pathologic course of spherocytic HE mimics that of HS.[613-615] Splenic sequestration is evident, red cells are conditioned during splenic passage, and hemolysis abates after splenectomy.[609,611,614]

The molecular pathology of classic spherocytic HE is not well studied. Patients with C-terminal truncations of α-spectrin (see later) have many of the clinical features of spherocytic HE and probably are a subset of the disorder.[616-624] Patients with such truncations typically have moderate hemolysis and anemia, punctuated by recurrent, severe hemolytic crises.[617,618,621] Blood smears show plump and usually smooth elliptocytes, although in a few instances, poikilocytosis was prominent.[617,618,620]

Patients who lack glycophorin C (see later) also have positive osmotic fragility tests and rounded, smooth elliptocytes.[625,626] They should probably also be classified as having a recessive (and unusually mild) variant of spherocytic HE. Patients who lack protein 4.2 (another recessive condition) sometimes display features of spherocytic HE (ovalocytes, spherocytes, stomatocytes)[236,627]; however, protein 4.2 deficiency more often resembles HS, morphologically and pathophysiologically, and is better classified as a variant of that disorder. Mice that lack tropomodulin-1 have mild spherocytic HE that is more spherocytic than elliptocytic.[628] The anemia may be partly compensated by an increase in tropomodulin-3, which is not normally expressed in mature red cells, to about 20% of the normal level of tropomodulin-1. Tropomodulin deficiency has not been identified in humans but should be considered.

Very rare patients appear to be compound heterozygotes for HS and HE. One Turkish girl is a particularly good candidate.[629] Her mother had mild HS and her father most likely had very mild HE, whereas she suffered from a moderately severe hemolytic anemia (hemoglobin level, 8.4 g/dL; reticulocyte count, 24%; bilirubin level, 1.6 mg/dL), with frontal bossing, osteoporosis, splenomegaly, and a mixture of microspherocytes and rounded elliptocytes.

Southeast Asian Ovalocytosis

This condition, which has a unique phenotype, molecular defect, and geographic distribution, is discussed later in this chapter.

Treatment

Typical HE is mild and splenectomy is not required. Serial interval ultrasound examinations, beginning at approximately 6 years of age, to detect gallstones should be performed in patients with brisk hemolysis. Splenectomy to decrease hemolysis, ameliorate anemia, and avoid the formation of bilirubinate gallstones has been the cornerstone of therapy for patients with severe hemolytic HE and HPP. Most practitioners believe that the indications for splenectomy in HS should also be applied to patients with symptomatic HE and HPP. Patients with HE and HPP who have been splenectomized have experienced increased hematocrit values, decreased reticulocytosis, and improvement in clinical symptoms. The risk of thrombosis after splenectomy for HE is unclear, although cases have been reported.[630] If hemolysis is still active after splenectomy, folic acid should be administered daily. Recommendations for antibiotic prophylaxis, immunization, and monitoring during intercurrent illnesses are the same as those noted for HS patients before and after splenectomy.

Neonates with infantile poikilocytosis should be treated as any patient with hemolytic anemia. Phototherapy and exchange transfusions are warranted in neonates with severe anemia and pathologic hyperbilirubinemia. Splenectomy is never indicated in the neonatal period. HE patients with extreme hemolysis should be transfused until they are 2 to 3 years of age, when immunizations are effective and splenectomy can be performed safely. Partial splenectomy is not as effective as in HS.[631]

Laboratory Methods

Thermal Sensitivity of Red Cells and Spectrin

Red cells heated to temperatures approaching 50°C for short periods of time become unstable and fragment spontaneously,[631-633] probably owing to denaturation of spectrin.[634] Normal spectrin denatures at 49°C (10-minute exposure),[634,635] and normal red cells fragment at the same temperature.[600,636] Almost all patients with HPP and some patients with milder forms of HE have thermally sensitive red cells. Red cells from patients with HPP and infants with HE and infantile poikilocytosis fragment after 10 minutes at 44 to 46°C.[570,600] Red cells from some but not all patients with typical HE fragment at 47 to 48°C.[600,635] As expected, purified spectrin from these red cells is also heat sensitive.[634,635] This test is limited because we do not understand, in molecular terms, why specific mutations are thermally sensitive. However, it remains one of the simplest tests available for diagnosing HPP in laboratories that do not specialize in membrane protein analysis.

Abnormal Spectrin Oligomerization

In many patients with HE and all patients with HPP, spectrin dimers are not properly converted to tetramers and higher oligomers in vitro or on the membrane. This important functional property is easily assessed in low-temperature spectrin extracts. At 0°C, the equilibrium between spectrin dimer and tetramer is kinetically frozen. If spectrin is extracted from the membrane at 0°C and carefully protected from warming during separation of dimers, tetramers, and oligomers (usually on nondenaturing polyacrylamide gels), the proportion of each spectrin species reflects its relative proportion on the membrane.[637] Patients with defects in spectrin self-association have abnormally high proportions of spectrin dimer in 0°C spectrin extracts (i.e., more than 10% of total spectrin dimers and tetramers).[582,636,638] The fraction of spectrin dimers is an important functional assessment in patients with α-spectrin defects. It correlates well with clinical severity and accurately predicts unusually severe mutations.[588] Conversely, discordance between the degree of hemolysis and fraction of spectrin dimers may alert the physician to an underlying, secondary complication.

Ektacytometry

The technique of osmotic gradient ektacytometry (OGE) to measure membrane surface area and cell hydration of intact red cells was discussed earlier, in the section on laboratory tests in HS, but the ektacytometer can also be used to assess red cell membrane mechanical stability.[268,269] Isolated red cell ghosts are subjected to high shear stress in a laser diffraction viscometer, and the DI (a measure of the average elongation of the sheared ghosts) is recorded as a function of time. Fragile ghosts fragment into small spheres more quickly than normal, causing their "deformability" to fall. The technique is a useful screening test because membrane stability is reduced in HE and dramatically so in HPP.[268]

Eosin-5′-Maleimide–Binding Test

EMA binds to band 3 and members of the Rh protein complex and is a measure of the amount of these proteins on the red cell surface, and therefore an indirect measure of membrane surface area (see earlier discussion under Hereditary Spherocytosis). Because red cell surface area is greatly decreased by cell fragmentation in HPP, EMA fluorescence is very reduced on a per cell basis, even more than it is in HS.[639]

Tryptic Maps of Spectrin

Limited tryptic digestion of spectrin extracted from erythrocytes, followed by two-dimensional gel electrophoresis, separates the resulting trypsin-resistant domains of α-spectrin and β-spectrin, including an 80-kD α-I domain peptide ($\alpha^{I/80}$) containing the self-association site of α-spectrin.[640,641] Many of the α-spectrin HE mutations reside in this domain and alter its structure so that it is cleaved into smaller fragments by trypsin. These were given names such as $\alpha^{I/74}$ or $\alpha^{I/65\text{-}68}$. Although the technique is historically important it is rarely used now.

Genetic Analyses

Increasingly HE mutations are identified by DNA sequencing. Unlike HS, some of the HE mutations are quite common and their location and nature relate to their clinical severity, so it is useful to know the specific molecular lesion. It is also important to know whether the patient carries the common spectrin α^{LELY} polymorphism and whether it is in cis or trans to the HE mutation because that can greatly affect the clinical picture (see below). Typically exons are amplified from genomic DNA isolated from peripheral blood leukocytes, but because most HE mutations are missense mutations, reverse-transcribed reticulocyte or bone marrow mRNA can also be amplified. At the moment, DNA testing for HS and HE mutations is not available commercially and only a few research laboratories will do the analysis. However, with the rapid fall in the cost of whole-exome and targeted exome sequencing, it is likely these will become the methods of choice in the near future.

Pathophysiology

Spectrin Defects

Abnormalities of either α-spectrin or β-spectrin are responsible for most cases of HE and HPP. The majority are due to missense mutations near the spectrin self-association site (blue symbols in Figs. 16-6 and 16-8). The spectrin self-association contact site is a combined triple helical "spectrin repeat" (see Fig. 15-13 and Fig. 15-14) in which two of the three helices that make up a typical spectrin repeat, helices A and B, are contributed by the C-terminus of the β-spectrin, whereas the third helix (C) comes from the first portion of the NH_2-terminus of α-spectrin. These three helices pair up[533,642,643] and create the bond responsible for spectrin self-association. HE mutations weaken the ability of spectrin dimers to form tetramers and destabilize the membrane skeleton. The functional damage is manifested by an increased proportion of spectrin dimers in 0°C low–ionic strength extracts of spectrin, and by decreased conversion of spectrin dimers to tetramers in solution and on the membrane. The broken spectrin-spectrin links are visible in electron micrographs and diminish the resistance of the isolated membranes to shear stress.[268,536] The clinical severity of HE correlates with the degree of impairment of spectrin self-association and with the amount of mutant spectrin that is produced.

A few examples of mutations that cause HE or HPP follow. Readers who are interested in a more extensive list of HE/HPP mutations can refer to an earlier chapter by the author[83] or to selected reviews,[11,82,540,644] or consult the paid[87] or free[88] versions of the Human Gene Mutation Database.

Alpha-Spectrin Defects Within the Self-Association Binding Site. α-Spectrin mutations account for 48% of the reported defects in HE and HPP.[87] Many of the mutations are located in the C helix at the beginning of the α-spectrin chain (Fig. 16-24 and see Fig. 16-6), which is a partial spectrin repeat, also called spectrin repeat 0 (see Fig. 16-24). Most of these mutations change amino acids that are part of the contact surfaces between the α-spectrin and β-spectrin chains at the tetramerization site and greatly impair or even abolish spectrin-spectrin binding.[643] As a result, they are among the most severe of the common α-spectrin mutations that cause HE and HPP.

Alpha-Spectrin Defects Beyond the Self-Association Binding Site. Mutations that affect spectrin repeats beyond repeat 0 tend to be less severe and some of them are very common. An example is the unnamed duplication of leucine at residue 154 (Leu154LeuLeu or α$^{\text{dup154L}}$; see Figs. 16-6 and 16-24). This mild mutation is widely distributed in blacks in West Africa, where the prevalence of HE is between 0.7% and 1.6%, and in their descendants in the West Indies and North America.[530,531] It is also seen in Arab populations and is the most common cause of HE in North Africa and southern Italy. It causes very mild HE in North Africans, who sometimes have little or no elliptocytosis (i.e., are silent carriers). Even homozygotes have only mild to moderate hemolysis. Another example is α-spectrin$^{\text{Nigerian}}$ (Leu260Pro; see Figs. 16-6 and 16-24), which has an incidence of 0.5% in Benin and Togo.[530,645] It is more severe than Leu154LeuLeu but less severe than most of the mutations located in repeat 0. A similar variant (Leu207Pro) is common among the Dendi people of West Africa and called α-spectrin$^{\text{St. Louis}}$, a low-expressing allele, when it is combined in *cis* with spectrin α$^{\text{LELY}}$. The prevalence of Leu154LeuLeu, Leu260Pro, and Leu207Pro strongly suggests that these mutations have undergone genetic selection[530,645] and may provide some protection from malaria (see later).

Other examples of distal HE mutations include α-spectrin$^{\text{St. Claude}}$, α-spectrin$^{\text{Oran}}$, and α-spectrin$^{\text{Jendouba}}$ (see Fig. 16-24). These recessive alleles are asymptomatic in the heterozygous state but cause HE or HPP when homozygous. α-Spectrin$^{\text{St. Claude}}$ has an incidence of 3% in Benin, Africa.[646] It is a splice junction mutation that yields two mRNAs,[593] one that encodes an impotent, truncated α-spectrin that fails to assemble into the membrane skeleton, and another that encodes a protein lacking exon 20 that binds ankyrin poorly.[647] Homozygotes have a severe poikilocytic anemia.[646] α-Spectrin$^{\text{Oran}}$ is another interesting splicing variant, which deletes exon 18.[592,595] It is expressed at low levels and causes severe HE in the homozygous state.[592]

The mechanism by which the more distal mutations propagate their effects along the α-spectrin chain and interfere with spectrin self-association is beginning to be dissected. One recent study[592a] shows that the common Leu207Pro defect, which lies in the second alpha spectrin repeat, alters the conformation of the first three repeats. The result is a more compact spectrin dimer structure that is strengthened by several new interprotein contacts, reducing the tendency of spectrin to form tetramers. The common Leu260Pro mutation also causes α-spectrin to form a more compact structure than normal and increases

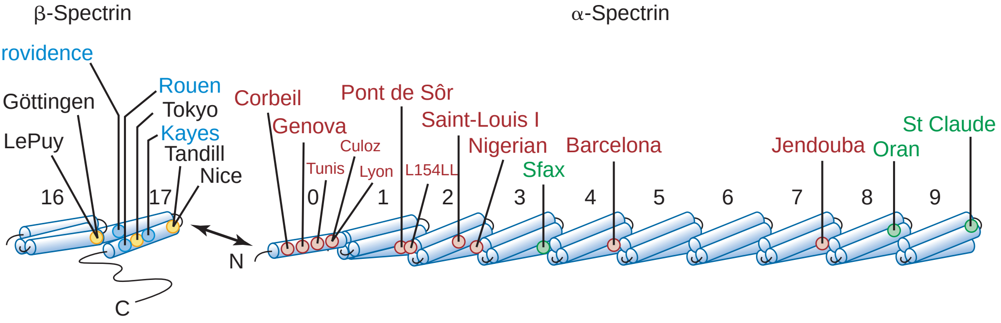

Figure 16-24 Triple-helical model of a subset of the roughly three dozen mutations in α-spectrin or β-spectrin associated with hereditary elliptocytosis (HE) and hereditary pryopoikilocytosis (HPP). Nearly all the α-spectrin mutations are missense mutations or in-frame deletions or insertions *(red circles)* or are splicing defects *(green circles)*. The mutations in the partial spectrin repeats α0 and β17 that comprise the spectrin self-association site tend to be more severe. The mechanism by which the more distal mutations propagate their effects along the α-spectrin chain and interfere with spectrin self-association is unknown. Most β-chain defects are C-terminal trunctations *(blue circles)* or missense mutations directly affecting spectrin self-association *(yellow circles)*. The truncations also impair synthesis of the mutant spectrin and often lead to a spherocytic elliptocytosis phenotype. Common mutations include the unnamed duplication of Leu at codon 154 (L154LL), spectrin Nigerian (L260P), and spectrin St. Claude, a splicing defect.

the stability of the closed dimer state, thereby reducing tetramer formation and destabilizing the membrane.[592b]

Low-Expression α-Spectrin Alleles: α^{LELY} and Related Polymorphisms. An HPP-like clinical picture may result from inheriting two elliptocytogenic genes for either α-spectrin or β-spectrin, but more often it occurs by combining an HE α-spectrin gene with a thalassemia-like defect on the opposite "normal" α-spectrin gene. These patients may also have spectrin deficiency. The parents who transmit the low-expression allele are clinically and biochemically normal. The best characterized of these low-expression alleles is the α^{LELY} allele (low-expression Lyon), which affects approximately 30% of the α-spectrin alleles of Caucasian European patients, 20% of the alleles of Japanese and African patients, and 22% of the alleles of Chinese patients.[587,648] Therefore approximately 42% of Caucasian Europeans are heterozygous for this allele and 9% are homozygous. The genetic advantage of the α^{LELY} allele has not yet been uncovered.

The α^{LELY} allele is caused by a combination of two mutations (Fig. 16-25). The key mutation is a C→T substitution at position −12 of intron 45, immediately upstream of exon 46, which causes the six amino acids encoded by exon 46 to be skipped 50% of the time. The second passenger mutation is in linkage disequilibrium. It is an amino acid substitution, Leu→Val at codon 1857 in exon 40, due to a C→G substitution.[649,650] This mutation alters tryptic cleavage of α-spectrin and creates an increased concentration of the 41-kd αV tryptic peptide, called the $\alpha^{V/41}$ polymorphism.[587]

The α^{LELY} allele is clinically silent by itself, even when in the homozygous state, because α-spectrin is normally synthesized in a fourfold excess.[651] In patients who have α^{LELY} on one chromosome and an α-spectrin mutation causing HE in *trans* on the opposite chromosome, the low expression of α^{LELY} decreases the amount of the otherwise normal spectrin from that allele that is incorporated into the membrane by approximately 50% and increases the relative incorporation of spectrin containing the mutant HE α-chain. α^{LELY} is poorly expressed owing to the mutation in intron 45, described above, which causes defective spectrin nucleation because the six deleted amino acids encoded by exon 46 lie within the nucleation site that joins the α-spectrin and β-spectrin chains.[652,653] α-Chains that lack exon 46 fail to assemble into stable spectrin dimers and are rapidly degraded (see Fig. 16-25).

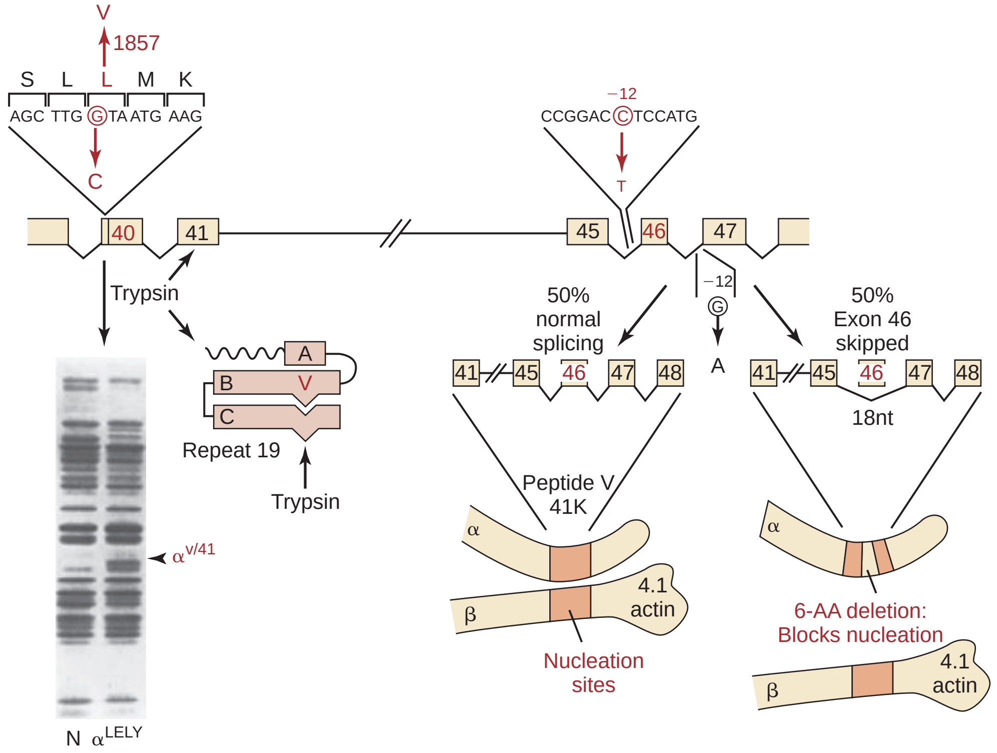

Figure 16-25 Nature of the low-expression α^{LELY} allele. The allele contains two linked mutations: a marker mutation, the $\alpha^{V/41}$ defect, and a splicing mutation, the α^{LELY} defect. *Left*, The $\alpha^{V/41}$ defect is caused by a G→C substitution at the beginning of exon 40, which changes a Leu1857→Val in helix B of spectrin repeat 19. The valine impinges on the neighboring helix C of the repeat and makes it more trypsin sensitive, leading to a more rapid release of the $\alpha^{V/41}$ peptide from the trypsin-treated spectrin derived from the α^{LELY} allele. Sodium dodecyl sulfate gels of trypsin-treated normal spectrin and spectrin containing α^{LELY} are shown at the bottom left. The $\alpha^{V/41}$ peptide is marked. *Right*, The linked splicing mutation is a C→T substitution at position −12 in the acceptor splice site of intron 45 (a second linked mutation G→A, at position −12 of intron 46 may also contribute). This causes exon 46 to be skipped in 50% of α^{LELY} spectrin molecules *(far right)*. Exon 46 is very small, only 6 amino acids, but it lies in the center of the nucleation site required for side-to-side association of the spectrin α and β chains. The half of α^{LELY} spectrin that lacks exon 46 cannot form spectrin heterodimers and is destroyed. Note that the other 50% of α^{LELY} spectrin contains exon 46 and will be functionally normal (though marked by the $\alpha^{V/41}$ tryptic peptide).

The effect of the α^{LELY} allele can be quite dramatic. In one family, a patient who was heterozygous for HE had very mild disease and almost no morphologic abnormality, whereas a relative who had also inherited the α^{LELY} allele in *trans* had severe elliptocytosis.[587] Similarly, in another family, the α^{LELY} allele increased the proportion of mutant spectrin in heterozygotes with a mild form of HE from 45% to 65% of the total spectrin.[587] This was associated with an increase in the proportion of elliptocytes from none or only a few to nearly 100%. Conversely, when the α^{LELY} allele is on the same chromosome as an α-spectrin mutation (i.e., in *cis*), it mutes the elliptocytic phenotype.[535]

The spectrin α^{LELY} should be distinguished from other thalassemia-like defects of α-spectrin synthesis that, when coinherited with some of the α-spectrin mutations, produce a phenotype of HPP. The latter defects are characterized by reduced α-spectrin mRNA levels and diminished α-spectrin synthesis such as occurred in the original HPP patient ($\alpha^{\text{St. Louis II}}$)[607] (see Fig. 16-6) or in two HPP patients who each had a typical HE missense mutation with a null mutation of α-spectrin in *trans*.[608] In contrast, although the synthesis of α^{LELY} subunits is decreased owing to poor incorporation of the peptide lacking exon 46, the production of α^{LELY} mRNA is normal.

A few low-expression alleles have been identified that have a greater effect on α-spectrin synthesis than α^{LELY}. Examples include $\alpha^{\text{St. Louis II}}$, which occurred in the original HPP patient[607] (see Fig. 16-6) and in two other null mutations of α-spectrin.[608] When these are inherited in *trans* essentially all of the spectrin on the membrane contains the mutant HE chains and is dysfunctional. As discussed earlier, three other low-expression alleles, α^{LEPRA}, α^{PRAGUE}, and $\alpha^{\text{Bicêtre}}$ (see Fig. 16-6) cause severe recessive HS and would be expected to augment the effect of a coinherited HE allele, but the combination has not yet been described.

Beta-Spectrin Self-Association Site Defects. Almost 20 mutations in the β-spectrin chain have been associated with HE or HPP (32% of reported HE/HPP defects). These mutations occur at the C-terminal end of β-spectrin (see Fig. 16-8) and tend to be truncations or missense mutations in the partial spectrin repeat (repeat 17) that forms part of the spectrin self-association site (see Fig. 16-24). The truncating defects include frameshift mutations,[618-620,654,655] exon skipping,[616,621-625,656] and a nonsense mutation.[657] Some of the missense mutations directly alter amino acids that form the contact surfaces between α-spectrin and β-spectrin.[643] Others substitute proline residues[658] that disrupt the helices in β-spectrin repeat 17 and indirectly damage spectrin-spectrin binding. Patients with β-spectrin missense mutations have typical HE. However, patients with frameshift mutations leading to truncated β-spectrin have spherocytic HE. Patients who are homozygous for a β-spectrin mutation tend to present with HPP and a more severe clinical course.

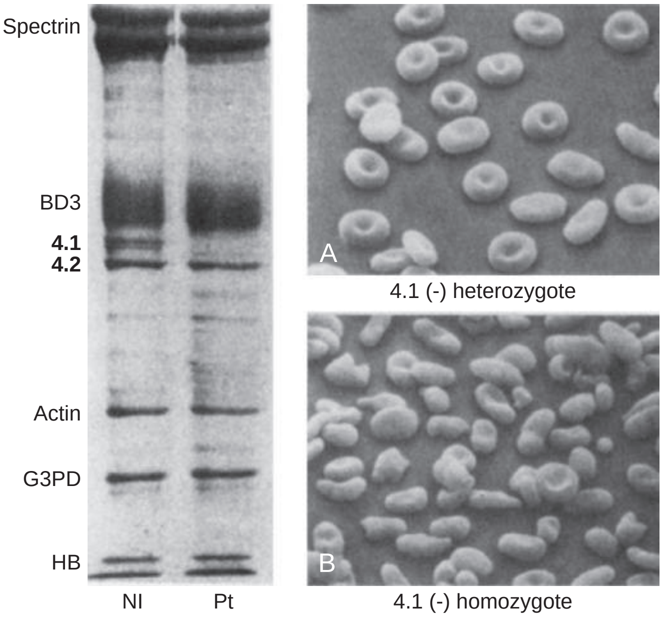

Figure 16-26 Deficiency of protein 4.1R in hereditary elliptocytosis (HE). *Left*, Sodium dodecyl sulfate gels of red cell membrane proteins from a normal individual (*Nl*) and a patient with homozygous HE who lacks protein 4.1R (*Pt*). *Right*, Scanning electron micrographs of red blood cells in 4.1R-deficient patients. **A**, Elliptocytes in a patient with heterozygous HE and 50% protein 4.1R. **B**, Elliptocytes, poikilocytes, and fragmented red cells in a patient with homozygous HE and no 4.1R. *BD3*, Band 3; *G3PD*, glyceraldehyde-3-phosphate dehydrogenase; *HB*, hemoglobin. *(From Tchernia G, Mohandas N, Shohet SB: Deficiency of cytoskeletal membrane protein band 4.1 in homozygous hereditary elliptocytosis: implications for erythrocyte membrane stability.* J Clin Invest *68:454–460, 1981.)*

Protein 4.1R Defects

Approximately 20% of the reported defects that cause HE involve protein 4.1R. The link between protein 4.1R deficiency and elliptocytosis was first described in a consanguineous Algerian family.[605,594] (Fig. 16-26). Partial absence of the protein is found in the 4.1R(−) trait, which appears to be a common cause of elliptocytosis, accounting for 30% to 40% of occurrences in some Arab and European populations.[550,551,659,660] However, this deficiency is not observed in individuals of African ancestry.[531]

In the original Algerian kindred,[605] protein 4.1R(−) mRNA is not translated,[661] owing to a 318-bp deletion, which includes the downstream translation initiation site that is used in reticulocytes (see Fig. 15-18).[662] In other patients, point mutations of the downstream initiator codon have been identified (Fig. 16-27).[594,663,664] Interestingly, expression of protein 4.1R is relatively unimpaired in nonerythroid tissues and early erythroblasts[662] because most of the protein 4.1R isoforms in these tissues initiate translation at an alternatively spliced, upstream translation initiation site.

Protein 4.1R(−) heterozygotes have clinically mild HE with prominent elliptocytosis, often approaching 100%, and little or no hemolysis.[666] These patients have 50% of the normal amount of protein 4.1R.

The very rare homozygous 4.1R(−) patients lack protein 4.1R and have a severe transfusion-dependent, hemolytic anemia with a good response to splenectomy.[605,594,667] Their red cells are elliptical, fragmented,

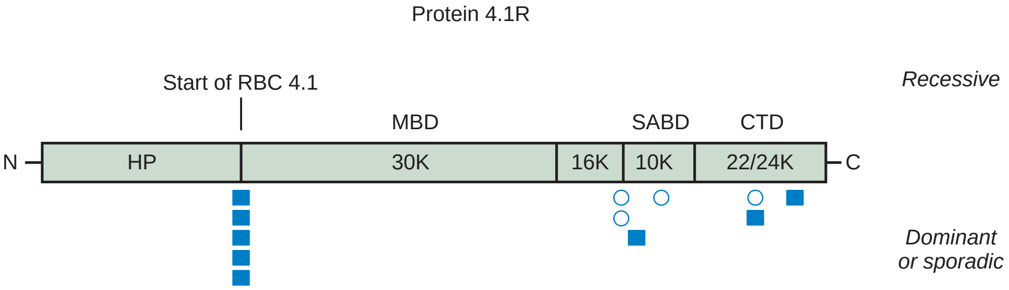

Figure 16-27 Protein 4.1R mutations associated with hereditary elliptocytosis are shown alongside a schematic representation of the protein. Red cell protein 4.1R contains a 30-kD membrane-binding domain *(MBD)*, a 16-kD linker domain, a 10-kD spectrin-actin–binding domain *(SABD)*, and a 22- to 24-kD C-terminal domain *(CTD)*. On the left, four mutations abolishing the translation start site are shown in *solid blue* rectangles. A large deletion and a large insertion affecting the spectrin-actin–binding domain are shown as *open circles*, as is a missense mutation at codon 447. The nucleotide numbers of the mutations are relative to the first nucleotide of the ATG initiation codon, and may differ from the numbering in some published reports.

and poikilocytic, resembling HPP or severe spherocytic HE (see Fig. 16-26, *B*).[605,594,667] They are very osmotically fragile and possess normal thermal stability. Membranes from homozygous protein 4.1R(–) red cells fragment much more rapidly than normal at moderate sheer stresses, an indication of their intrinsic instability.[268] Membrane mechanical stability can be completely restored by reconstituting the deficient red cells with normal protein 4.1R or the spectrin-actin binding site from protein 4.1R.[605,668]

In addition to complete protein 4.1R deficiency, erythrocytes from protein 4.1R(–) homozygotes lack protein p55[669,670] and have only 30% of the normal content of glycophorin C and D.[594,670-672] This strengthens the evidence that glycophorin C is one of the membrane attachment sites for protein 4.1R.[673] Electron microscopic studies of homozygous 4.1R(–) erythrocyte membranes reveal a markedly disrupted skeletal network with disarray of the intramembrane particles, suggesting that protein 4.1R plays an important role in maintaining the organization of integral membrane proteins as well as the skeletal network.[674] Mice that lack protein 4.1R have decreased intestinal calcium absorption due to altered expression of PMCA1b, the plasma membrane calcium ATPase 1b.[674a] This in turn leads to secondary hyperparathyroidism. It's not clear whether the same problem occurs in humans but clinicians should be aware of the possibility.

Protein 4.1R Structural Defects. Variants of protein 4.1R with abnormal molecular weights have also been described in association with HE.[551,675-678] For example, a shortened protein 4.1R that lacks the two exons encoding the spectrin/actin-binding domain was discovered in an Italian family with very mild HE and mechanically unstable red cells. The patients are heterozygous for the mutant protein, which is presumed to be functionally inept. Similarly, a high-molecular-weight variant, protein 4.1R$^{\text{Hurdle-Mills}}$,[551] contains a duplication of three exons that include the spectrin/actin-binding domain and causes very mild HE in heterozygotes.[675,676] Its membrane function appears to be largely preserved because red cell membranes have normal mechanical stability.[679] Other examples of variants of protein 4.1R with abnormal molecular weights have been described.[677,678]

Acquired Protein 4.1R Deficiency. Elliptocytosis with an acquired deficit in protein 4.1R occasionally occurs in patients with myelodysplastic syndromes, particularly in association with a deletion in the long arm of chromosome 20.[680,680a]

Glycophorin C Defects

Elliptocytes are noted on peripheral blood smears of patients whose erythrocytes carry the rare Leach phenotype of Gerbich-negative (Ge–) cells.[625,626,681-686] These erythrocytes are devoid of glycophorin C and glycophorin D and, presumably secondarily, are also deficient in protein p55 and protein 4.1R. It is possible that the protein 4.1R deficiency in Leach erythrocytes is the cause of their elliptocytic shape.

The Leach phenotype is usually due to a deletion of 7 kb of genomic DNA that removes exons 3 and 4 from the glycophorin C/glycophorin D locus.[681,682] Patients carrying the Leach phenotype suffer from mild spherocytic HE with increased erythrocyte osmotic fragility.[625,683-685] In some individuals, no elliptocytes are detected on a peripheral smear.[671]

In contrast to glycophorin C and glycophorin D deficiency, individuals whose erythrocytes lack glycophorin A or glycophorin B, or both, are asymptomatic and have erythrocytes of normal shape and mechanical stability.

Relationship Between Hereditary Elliptocytosis, Hereditary Pyropoikilocytosis, and Spherocytic Hereditary Elliptocytosis

Clinically there is a continuum between the different forms of HE and between spherocytic HE and HS. We hypothesize that there is also a pathophysiologic continuum between these conditions that results from different degrees of loss of spectrin or loss of the spectrin functions involved in holding the membrane skeleton together—specifically, loss of spectrin self-association or the attachment of spectrin to actin with the aid of protein 4.1R (Fig. 16-28).

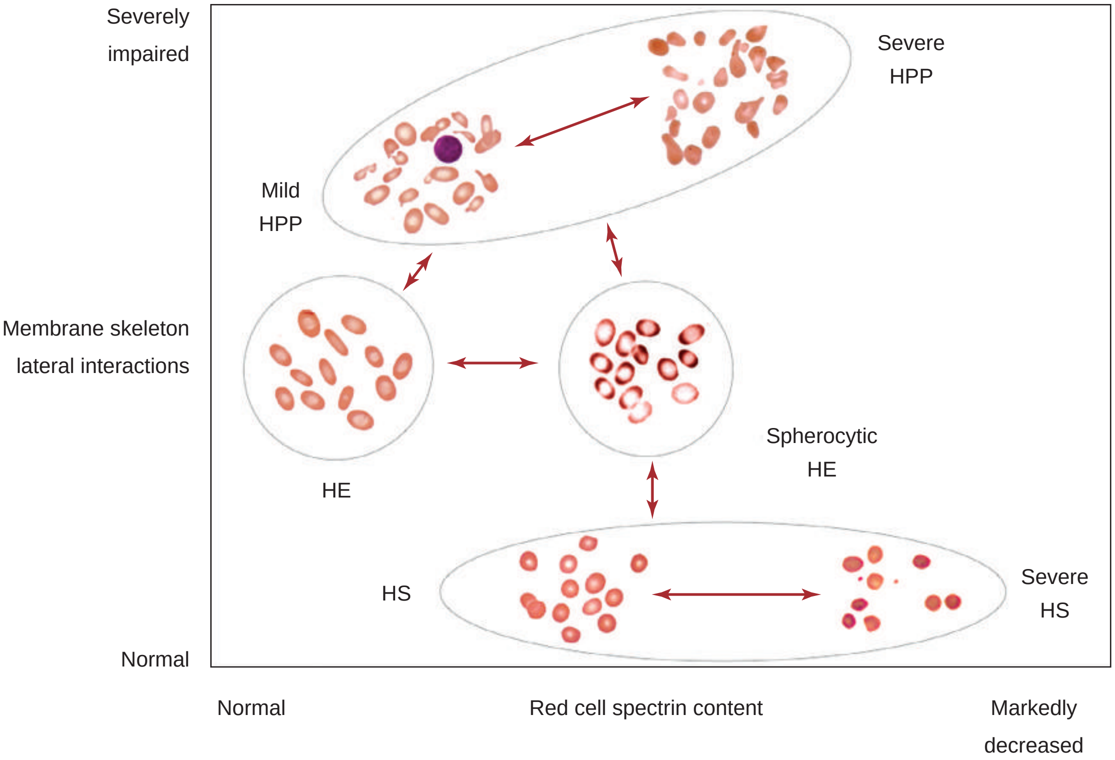

Figure 16-28 Relationships between hereditary elliptocytosis *(HE)*, hereditary pyropoikilocytosis *(HPP)*, hereditary spherocytosis *(HS)*, and spherocytic HE as a function of the degree of spectrin deficiency and the degree of functional impairment of the spectrin self-association or spectrin-actin-4.1R interactions ("lateral" interactions) that hold the red cell membrane skeleton together.

Loss of spectrin without impairment of its function results in HS. Partial loss (≈50%) of either of spectrin's lateral binding functions without loss of spectrin itself leads to typical HE. More severe loss of spectrin binding leads to HPP, but as the functional deficit increases, HPP red cells also tend to lose spectrin from the membrane. This aggravates membrane instability and moves the HPP red cells toward a more spherocytic and less elliptocytic morphology (see Fig. 16-28). It is not clear why spectrin is lost in HPP. It may be that if the proportion of defective spectrin is high enough or the binding defect is severe enough spectrin just cannot bind tightly enough to the membrane to remain attached. Or, it may be that the most deleterious mutations also make spectrin unstable.

Our hypothesis predicts that patients whose heterozygous HE mutation causes the red cells to lose both spectrin content and function will develop spherocytic HE (see Fig. 16-28). Indeed, where measured, spherocytic elliptocytes have been spectrin deficient to a degree similar to that of HS erythrocytes.[624] Spherocytic HE seems to be more linked to mutations that lead to premature termination of β-spectrin than to missense mutations. Shortened molecules would be more likely to be unstable than those with a simple amino acid substitution. Plus, the splicing or nonsense mutations that lead to premature chain termination may trigger nonsense-mediated decay of β-spectrin mRNA. Either process would result in spectrin deficiency.

On the other hand, mutations in α-spectrin would be expected to accentuate loss of spectrin function over loss of spectrin content and lead to HPP rather than spherocytic HE. The reason is because fourfold more α-spectrin is synthesized than β-spectrin. Whether HPP is caused by homozygosity for an HE mutation, or is caused by the combination of an HE defect with a mutation that impairs expression of the "normal" α-spectrin allele, the result is that all or nearly all the spectrin is functionally defective.

Why Elliptocytes?

Why elliptocytes form when the membrane skeleton is weak is less clear. In typical HE, red cell precursors are round and become progressively more elliptical as they age in vivo.[526,552,687] One hypothesis is that the weakened skeletal interactions facilitate skeletal reorganization after prolonged or repetitive cellular deformation and cause red cells to adopt the elliptical shape they sometimes assume when flowing through the microcirculation. However, recent theoretical work argues that the elliptocyte, a biconcave ellipsoid, is the minimum energy shape that the red cell assumes in the absence of shear when the membrane skeleton is weakened.[688]

Hereditary Elliptocytosis and Malaria

The high prevalence of the spectrin $\alpha^{\text{Leu154LeuLeu}}$, $\alpha^{\text{Leu260Pro}}$, and $\alpha^{\text{Leu207Pro}}$ mutations in various tribes of West Central Africa, which is in the range of 0.5% to 1.6%,[530,531,645] suggests that these mutations may have been selected because they provide some benefit against malaria. In fact, in vitro studies show diminished *P. falciparum* parasite invasion or growth in red cells that contain these mutants,[645,689,690] particularly after two to three cycles of growth.[645] Results with protein 4.1R(−) cells were mixed, with one group finding decreased parasite invasion[691] and another group not.[690] Other malarial strains have not been tested and epidemiologic studies have not been done to ascertain whether natives with HE are less likely to be infected or have a milder course.

SOUTHEAST ASIAN OVALOCYTOSIS

Southeast Asian ovalocytosis (SAO) is observed in the aboriginal populations of Melanesia and Malaysia and in portions of Indonesia and the Philippines.[692-698] The abnormality is very common in Melanesia, particularly in lowland and island tribes in whom malaria is endemic,[693,698-701] affecting 1% to 20% of the population.[701-703] It is rare in other populations.[704-707] SAO is inherited in an autosomal dominant pattern.[692-695,699,708] In screening populations where SAO is common, no homozygotes have been identified,[701,709] suggesting that that homozygosity is incompatible with life.

Etiology

SAO is unique among red cell membrane disorders in that all patients have the same defect—a deletion of amino acids 400 to 408 at the beginning of the first transmembrane segment of band 3 (see Fig. 15-7 and Fig. 16-10).[710-715] Many patients also carry an asymptomatic linked polymorphism, Lys56Glu, also known as band 3 Memphis, in cis to the SAO mutation. The mutant SAO band 3 constitutes about half of the band 3 in heterozygotes. It is incorporated into the membrane but is misfolded and is unable to transport anions.[716,717]

Clinical and Laboratory Features

SAO heterozygotes are asymptomatic[692,694,696,697,718] except in the neonatal period. Newborns have significant hemolysis and anemia (the hemoglobin value at 2 days is 14.1 g/dL vs. 16.4 g/dL in normals), and half develop neonatal hyperbilirubinemia,[719] but hematologic values normalize by the age of 2 years.[719] This suggests the mutation uniquely damages fetal red cells. There may be occasional exceptions, as one adult with SAO had compensated hemolysis (no anemia), with mild splenomegaly, hyperbilirubinemia, and gallstones.[720] Homozygous SAO is a near lethal condition. The one known homozygous patient had severe anemia (2.9 g/dL) and hydrops fetalis at 22 weeks gestation. He was saved by intrauterine transfusion and has been transfusion dependent since birth, with distal renal tubular acidosis and marked dyserythropoiesis, especially in late erythroblasts.[720a] The few circulating patient cells were large ovalocytes. The clinical picture is reminiscent of that in zebrafish lacking band 3.[162]

Most SAO red cells are rounded elliptocytes, but a few are traversed by one or two transverse bars that divide the central clear space (Fig. 16-29).[692,696-698,708,718] These "theta cells" are common in SAO (sensitivity 94%) and are rarely seen in normals (specificity 99%) or in other diseases.[718] Thus identification of multiple theta cells on a peripheral blood smear featuring elliptocytosis strongly suggests SAO. Some stomatocytes or elliptical stomatocytes may also be present and the number of hyperdense cells may be increased.[721]

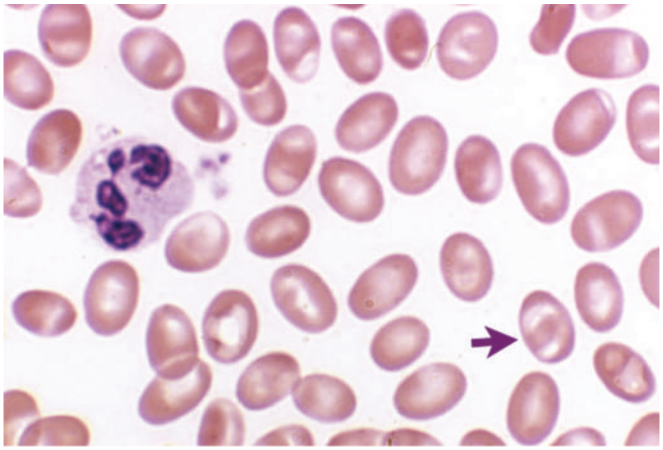
Figure 16-29 Peripheral blood morphologic findings in Southeast Asian ovalocytosis (SAO). The majority of cells are rounded elliptocytes, but some contain a transverse ridge across the central clearing, which gives them the appearance of the Greek letter theta (θ) (*arrow* denotes one example of several). Theta cells are characteristic of SAO. Elliptical stomatocytes are also evident in some patients.

The strong resistance of red cells to crenation, after several days of storage,[722] provides an additional clue to the diagnosis, as does the eosin-5′-maleimide–binding test, which shows low binding, similar to HS or HPP, because SAO band 3 does not react with the reagent.[257] SAO red cells also exhibit cryohemolysis,[723] an influx of cations and water at low temperatures leading to hemolysis, essentially identical to that seen in hereditary cryohydrocytosis (HC), another disorder of band 3 (see later section in this chapter). Thus although SAO band 3 channels cannot exchange anions, the proper function of band 3, they mistakenly transport cations. However, the most specific diagnostic test for SAO is amplification of the deleted region from the band 3 gene in genomic DNA or reticulocyte cDNA. This produces a single band in control red cells and a doublet in SAO cells, with the second band shorter by 27 bp.[710,712,723]

Curiously, many blood group antigens are poorly expressed on the surface of SAO red cells,[698] possibly because the rigid membrane inhibits their clustering and impedes agglutination. Duffy antigen is an exception and is mildly increased.[724]

SAO band 3 is a recessive mutation for distal renal tubular acidosis (dRTA). Patients who are compound heterozygotes for SAO and other band 3 mutations causing dRTA, develop the disease.[129,725,726] The reason is because sufficient heterodimers of normal and SAO band 3 reach the plasma membrane to prevent acidosis, whereas heterodimers of SAO and other dRTA band 3 molecules become trapped in the endoplasmic reticulum and lead to deficient Cl^- excretion.[142]

Resistance to Malaria

The prevalence of SAO increases with age in populations rife with malaria, suggesting that individuals with SAO have a selective advantage.[727] In vivo, there is evidence that SAO protects against all different types of malaria, particularly against heavy infections,[727,728] placental malaria during a first pregnancy,[729] and cerebral malaria in children.[730,731] For example, in infants, SAO reduces the incidence of P. vivax clinical infections by 46%.[728] On the other hand, SAO does not protect against mild malaria, asymptomatic P. falciparum malaria parasitemia, or severe malarial anemia.[728,730,732,733] In fact, SAO erythrocytes may be selectively lost during acute malaria, exacerbating the anemia.[718] In vitro, SAO red cells are resistant to invasion by malarial parasites,[734-737] though the effect is strain dependent.[737] However, population studies show no correlation between the SAO mutation and the prevalence of malaria,[703,732,733,738] with the exception of P. vivax malaria,[739] which suggests that resistance to invasion is not a factor in vivo. The fact that children with SAO are very resistant to cerebral malaria suggests that SAO may alter the endothelial adherence that underlies that complication[730,740]; however, this remains to be proved.

Membrane Rigidity

SAO red cell membranes are very rigid, 10 to 20 times more rigid than normal.[711,712,722,741] Other membrane characteristics reflect this property, including unusually high heat resistance, lack of endocytosis in response to drugs that produce dramatic endocytosis in normal cells, and strong resistance to crenation, even after several days of storage in plasma or buffered salt solutions.[722] In addition, the non-SAO band 3 molecules and other integral proteins in SAO red cells are less laterally mobile than in normal red cells, presumably more confined by interactions with the membrane skeleton.[742,743] The mechanism of the rigidity is unclear. One suggestion is that conformational changes of the cytoplasmic domain of SAO band 3 preclude lateral movement (extension) of the skeletal network during deformation.[711,744] A second possibility is that SAO band 3 itself is rigid due to loss of a flexible linker at the juncture of the cytoplasmic and membrane domains, right where the SAO deletion occurs.[745] A third explanation is that SAO band 3 binds abnormally tightly to ankyrin and thus to the underlying skeleton.[700] The increased propensity of SAO band 3 to aggregate into higher oligomers may be important because the oligomers can strengthen band 3 attachment to ankyrin.[746] Fourth, the tendency of SAO band 3 to form linear arrays would also decrease its mobility within the bilayer.[747] Finally, SAO band 3 may adhere to the skeleton in a nonspecific manner, possibly due to denaturation of the membrane spanning domain,[748] which is known to be misfolded.[749] Whatever the mechanism, it is interesting that such extremely rigid red cells circulate freely and neither clog the circulation nor are removed prematurely.

STOMATOCYTOSIS, CRYOHYDROCYTOSIS, AND XEROCYTOSIS

Red cell hydration is largely determined by the intracellular concentration of monovalent cations. A net increase in Na^+ and K^+ ions causes water to enter, forming “stomatocytes” or “hydrocytes,” whereas a net loss of monovalent cations produces dehydrated red cells or “xerocytes.” In the past 45 years, numerous descriptions of congenital or familial hemolytic anemias associated with abnormal cation permeability and, in some cases, disturbed red cell hydration have been reported. These span the range from severe stomatocytosis to severe xerocytosis. They can be divided into six, somewhat overlapping and artificial clinical categories based on differences of severity, morphology, cation content, lipid and protein composition, genetics, molecular defect, and response to splenectomy: overhydrated stomatocytosis (severe), mild stomatocytosis, cryohydrocytosis, SAO, xerocytosis, and pseudohyperkalemia (Table 16-12 and Fig. 16-30). Each of these diseases is caused by defects in normal red cell membrane transport proteins, defects that pervert the normal functions of the transporters and allow Na^+ or K^+ ions abnormal passage. This is probably a general rule for most disorders of membrane permeability: that mutations cause otherwise normal channels to be distorted and malfunction. Many of the recent advances are covered in reviews published during the past few years.[750,751]

Overhydrated Hereditary Stomatocytosis

Clinical Features

Overhydrated hereditary stomatocytosis (OHSt) is the classic form of hereditary stomatocytosis (HSt) and is characterized by cation permeability and swollen, water-laden red cells. It is a very rare hemolytic anemia. Less than two dozen patients have been reported.[752-768] OHSt red cell membranes are remarkably permeable to Na^+ and K^+ ions, particularly Na^+ ions. Intracellular Na^+ is greatly increased and K^+ is decreased, but the total monovalent cation content (Na^+ plus K^+) is high, which leads to an increase in cell water and cell volume. The resulting “edematous cells” are bowl shaped in suspension, but when they deposit on slides the extra volume encroaches on the normal circular central clearing, yielding the characteristic slit shape (see Fig. 16-30, A). Even more swollen “stomatocytes” appear as large spherocytes (see Fig. 16-30, B).

The disease is inherited in an autosomal dominant manner and presents with moderately severe to severe hemolysis and anemia, soon after birth or in infancy. In most cases the disease is constant throughout life, though we have seen patients who had severe anemia in infancy that became milder with time during the first year of life. There are no reports of how often this occurs. Hemolysis is extravascular, so that jaundice, splenomegaly, and cholelithiasis are features. Anemia may be exacerbated by intercurrent infections that activate macrophages or exacerbate splenomegaly. As with all hemolytic anemias, patients may also suffer aplastic crises if infected with parvovirus B19. In addition, because hereditary stomatocytes leak potassium as well as gain sodium, patients may be mistakenly diagnosed with hyperkalemia if electrolyte measurements are delayed after blood samples are drawn.

TABLE 16-12 Features of Hereditary Stomatocytosis-Xerocytosis Syndromes

Feature (normal value)	Overhydrated Hereditary Stomatocytosis	OTHER HEREDITARY STOMATOCYTOSIS SYNDROMES			HEREDITARY XEROCYTOSIS (DEHYDRATED STOMATOCYTOSIS)	
		Mild Hereditary Stomatocytosis	Hereditary Cryohydrocytosis	Southeast Asian Ovalocytosis	Typical	Pseudohyperkalemia
Hemolysis	Severe	Mild to moderate	Mild to moderate	Mild (neonatal only)	Moderate	Mild or none
Anemia	Moderate to severe	Mild to moderate	Mild to moderate to completely compensated	Mild (neonatal only)	Mild to moderate to completely compensated	None
Blood smear	Stomatocytes; ± spherocytes	Stomatocytes and/or spherocytes	Stomatocytes, sometimes with curved or offset stoma; ± spherocytes; ± target cells	Rounded elliptocytes, some with a double central clearing and a theta (θ) shape; ± stomatocytes	Target cells; sometimes small numbers of echinocytes or stomatocytes	Target cells; few stomatocytes
MCV	Increased	Normal to increased	Normal to increased		High normal to increased	Normal to increased
MCHC	Decreased	Normal to decreased	Normal to increased		Normal to increased	Normal to increased
Unincubated osmotic fragility	Very increased	Variable	Variable	Unknown	Decreased to very decreased	Slightly decreased
RBC Na^+ (5-12 mEq/L)[*]	60-150	30-60	15-100	Normal	5-30	10-30
RBC K^+ (90-105 mEq/L)[*]	20-55	40-85	30-100	Normal	60-90	75-100
RBC $Na^+ + K^+$ (95-110 mEq/L)[*]	110-170	115-145	70-130	Normal	70-100	85-110
RBC passive membrane leak[*,†]	20-40	≈3-10	1-6	2-4	2-4	1-2
Cold hemolysis	No	Unknown	Yes	Yes	No	No
Pseudohyperkalemia	Sometimes	Unknown	Yes	Unknown	Sometimes	Yes
Perinatal ascites	No	Unknown	No	No	Sometimes	No
Stomatin markedly decreased	Yes	No	No (type 1) Yes (type 2)	No	No	No
Effect of splenectomy on hemolysis	Some benefit	Some benefit	Little or no effect	No significant hemolysis	No effect	No significant hemolysis
Thromboembolism risk following splenectomy	Yes	Unknown	?Yes	Unknown	Yes	Unknown
Genetics	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal dominant
Defective gene(s)	RhAG	Band 3 (SLC4A1)	Type 1: Band 3 (SLC4A1) Type 2: Glut 1 (SLC2A1)	Band 3 (SLC4A1) in-frame deletion	Piezo1 (FAM38A)	Piezo1 (FAM38A), ABCB6

MCHC, Mean corpuscular hemoglobin concentration; *MCV*, mean cell volume; *RBC*, red blood cell.

[*] Based on a relatively small number of measurements reported in the literature.

[†] Times normal. Defined as the ouabain- and bumetanide-resistant $^{86}Rb^+$ influx at 37°C, and expressed as the ratio of patient residual leak to normal residual leak (normal = 0.06 to 0.10 mmol/L RBC/hour).

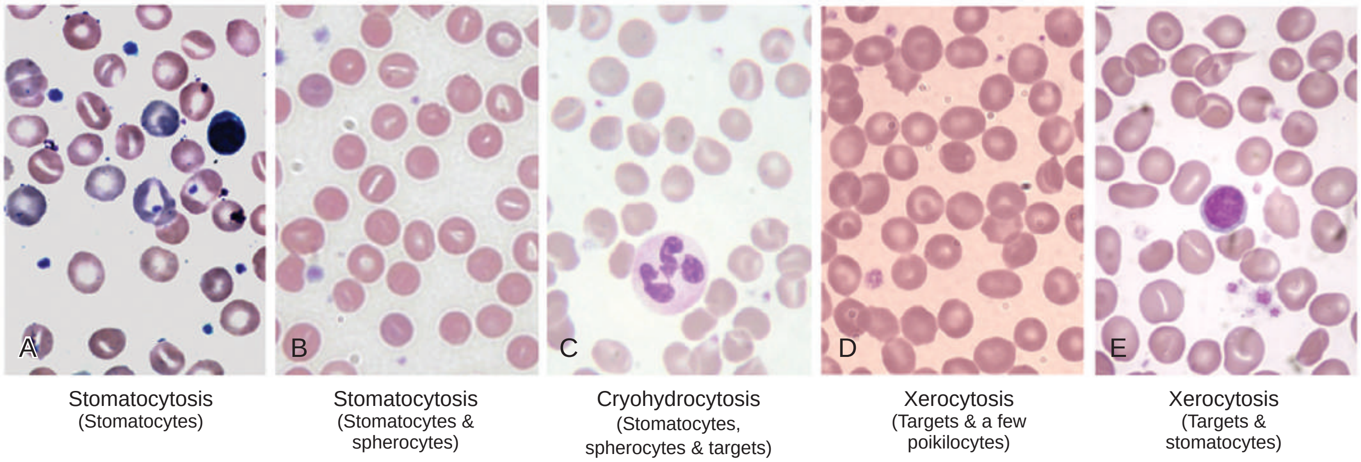

Figure 16-30 Peripheral blood morphologic findings in hereditary stomatocytosis and hereditary xerocytosis (HX). **A**, Overhydrated hereditary stomatocytosis (OHSt). Many stomatocytes and polychromatophilic erythrocytes are present. **B**, OHSt. This patient has a mixture of stomatocytes and large spherocytes. **C**, Cryohydrocytosis, type 1. A mixture of stomatocytes, spherocytes, and target cells is evident. Note that some of the stomatocytes have curvilinear slits, such as those at the upper right and upper left. **D**, HX. Typical blood smear with some target cells but mostly unimpressive morphologic characteristics. **E**, HX with some target cells and stomatocytes. *(**C**, From Bruce LJ, Robinson HC, Guizouarn H, et al: Monovalent cation leaks in human red cells caused by single amino acid substitutions in the transport domain of the band 3 chloride-bicarbonate exchanger, AE1.* Nat Genet *37:1258–1263, 2005; **E**, From Lichtman MA:* Lichtman's atlas of hematology, *New York, 2007, McGraw Hill, Figure I.C.155.)*

The diagnostic laboratory features include the unique red cell morphology (5% to 50% stomatocytes, sometimes with accompanying spherocytes), moderate to severe hemolysis, macrocytosis (95 to 150 fL), *low* MCHC (24% to 30%), and a positive osmotic fragility test. *The combination of macrocytosis and a low MCHC is virtually diagnostic for hereditary stomatocytosis,* especially when stomatocytes are present on the peripheral blood smear and the osmotic fragility test is positive. Occasionally spherocytes can dominate the smear and cause confusion, but patients with true HS *never* have macrocytosis or a low MCHC—just the opposite—so the two diseases should not be confused.

Additional studies in specialty laboratories will show a greatly elevated erythrocyte Na^+ concentration, a reduced erythrocyte K^+ concentration, an increased total Na^+ plus K^+ concentration, and an extremely high passive membrane permeability for Na^+ and K^+. The excess monovalent cations elevate cell water, which is responsible for the low MCHC.

Osmotic gradient ektacytometry, which is described in the sections on HS (see Fig. 16-18), shows a characteristic right-shifted pattern in OHSt due to increased red cell hydration (Fig. 16-31, *A*). The minimum deformability index (DI) observed in the hypotonic region of the ektacytometry curve (called O_{min}) is shifted to the right of the control region, indicating a decrease in the cell surface area–to–volume ratio. The maximum DI is normal or increased due to the increased surface area of the macrocytic cells, and the osmolality in the hyperosmolar region at which the DI reaches half its maximum value is increased because the cells are overhydrated. Osmotic gradient ektacytometry is a sensitive and specific method for diagnosing disorders of membrane permeability. Unfortunately, the equipment required is available at only a few centers.

Finally, OHSt is characterized by a marked decrease or absence of stomatin on SDS gel electrophoresis of red cell membranes or by immunostaining of erythrocytes. As will be discussed, this turns out to be a marker of the disease but is not directly implicated in its pathophysiology, which is due to mutations in the *RhAG* gene.

Pathophysiology

The major physiologic defect in hereditary stomatocytosis is a marked asymmetric increase in passive Na^+ and K^+ permeability (the amount of Na^+_{in} exceeds the amount of K^+_{out}). Permeabilities as great as 15 to 40 times normal are observed.[752,769,770] Because the influx of Na^+ predominates, stomatocytic red cells progressively gain cations and water and swell. As a result, their average density is less than normal (see Fig. 16-31, B) and the swollen stomatocytes are osmotically fragile. Unlike normal cells, aged stomatocytes are less dense and more stomatocytic than stomatocytic reticulocytes.[752]

In stomatocytosis, monovalent cation transporters are greatly stimulated by the tremendous influx of Na^+, particularly the Na^+,K^+ pump and K^+,Cl^- cotransporter.[760] The Na^+,K^+ pump attempts to extrude the Na^+ torrent, consuming much ATP in the process. If the compensatory Na^+ extrusion fails, stomatocytes would be expected to succumb to osmotic lysis, but the lack of evidence for intravascular hemolysis suggests that somehow they are recognized and eliminated by the reticuloendothelial system before they burst. Overhydrated stomatocytes are vulnerable to splenic sequestration[759] because red cell metabolism and ATP production are compromised in the stagnant, acidotic, hypoglycemic splenic cords. Analysis of metabolic intermediates in OHSt erythrocytes is compatible with increased production of ATP and glycolytic exhaustion.[771] Predictably, splenectomy has been beneficial in some patients with severe

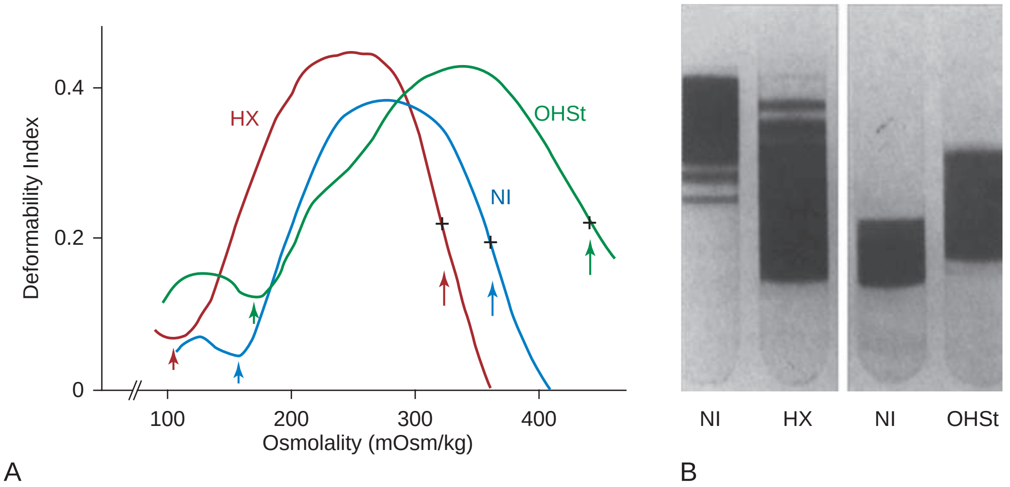

Figure 16-31 **A**, Osmotic gradient ektacytometry of hereditary xerocytosis *(HX)* and overhydrated hereditary stomatocytosis *(OHSt)* showing erythrocytes compared with normal red cells *(Nl)*. The deformability index (DI), an arbitrary measure of deformation, is plotted as a function of the osmolarity. The maximum DI is greater for both HX and OHSt red cells, indicating their greater surface areas. The minimum DI in the hypotonic region (*smaller arrows* to the *left*) is markedly shifted to the left for shrunken HX red cells, indicating their increased surface-to-volume ratio. It is shifted to the right for the swollen OHSt cells the surface-to-volume ratio of which is decreased. The osmolality in the hyperosmolar region at which the DI reaches half of its maximum value (*larger arrows* on the *right*) is a measure of the hydration state of the red cells. It is considerably decreased in the HX sample and massively increased in OHSt. **B**, Separation of xerocytes *(left)* and stomatocytes *(right)* by density on Stractan gradients. The hereditary xerocytes *(HX)* extend to higher densities (toward the bottom) than normal red cells *(Nl)*, whereas many of the overhydrated hereditary stomatocytes are lighter than normal. *(**A**, Modified from Delaunay J: The hereditary stomatocytoses: genetic disorders of the red cell membrane permeability to monovalent cations.* Semin Hematol *41:165–172, 2004; **B**, From Lande WM, Mentzer WC: Haemolytic anaemia associated with increased cation permeability.* Clin Haematol *14:89–103, 1985.)*

hemolysis[752,760,761,763,770] but not without severe risk (see later section on Treatment).

Bifunctional imidoesters, which cross-link proteins, reverse the abnormal shape and permeability of OHSt red cells and normalize their survival in the circulation.[772] The critical proteins involved have not been identified using these agents, because many red cell membrane proteins are cross-linked at the concentrations required to achieve this effect.

Stomatin. The red cell membranes of all or almost all patients with OHSt nearly lack a 31-kD protein called stomatin or band 7.2b (Fig. 16-32).[754,755,760,773-775] This small, widely distributed, integral membrane protein resides in detergent-resistant membrane lipid domains called lipid rafts, and appears to regulate ion channels.[776,777] For a time defective stomatin was believed to cause OHSt. However, the stomatin gene is entirely normal in the disease[778] and a stomatin gene knockout in mice does not cause any Na$^+$ leak in red blood cells or hemolysis.[779] Some OHSt red cells contain small amounts of stomatin; however, in most OHSt red cells stomatin is lost early in erythropoiesis.[764] Surprisingly, it turns out that stomatin normally binds to the major glucose transporter, Glut1, which also resides in lipid rafts. Stomatin switches part of the Glut1 to an L-dehydroascorbic acid transporter in red cells.[780] This only occurs in species such as humans that lack the ability to synthesize ascorbic acid. Because stomatocytic red cells consume so much ATP trying to pump out Na$^+$, it may be that the OHSt red cells that survive and circulate are those that are able to lose stomatin and retain Glut1 as a glucose channel to provide extra metabolic substrate.

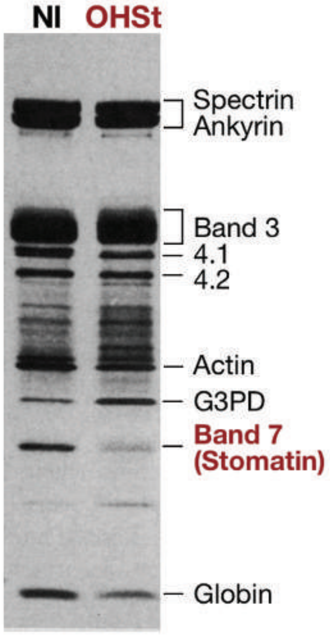

Figure 16-32 Stomatin deficiency in overhydrated hereditary stomatocytosis *(OHSt)*. Sodium dodecyl sulfate gels of normal *(Nl)* and OHSt red cell membranes show that the principal (31-kD) band in the band 7 region (stomatin) is markedly decreased. Immunoblots confirm that stomatin is nearly absent in these cells. *G3PD*, Glyceraldehyde-3-phosphate dehydrogenase. *(Modified from Rix M, Bjerrum PJ, Wieth JO, Frandsen B: Congenital stomatocytosis with hemolytic anemia—with abnormal cation permeability and defective membrane proteins.* Ugeskrift for Laeger *153:724–726, 1991.)*

How stomatin is lost is not clear. About 2% to 5% of OHSt red cells contain immunoreactive stomatin, particularly reticulocytes.[764] This suggests that stomatin is made but is lost from lipid rafts in OHSt red cells as the cells mature in the bone marrow and in the circulation. The amount of actin and tropomodulin associated with lipid rafts is markedly reduced in OHSt erythrocytes,[781] which suggests that there may be a defect in the interaction of stomatin with the membrane skeleton. Potentially that could cause stomatin to be lost in the exocytic vesicles that are released as the red cell matures into a reticulocyte and that eliminate proteins such as the transferrin receptor from normal mature red cells. On the other hand, in cultured erythroid cells from OHSt patients, stomatin is confined to multivesicular complexes and the nucleus and is not seen on the plasma membrane,[782] which suggests that stomatin deficiency may result from a trafficking defect. Stomatin is also deficient in red cells from patients with type 2 cryohydrocytosis (see below) and in dogs whose red cells have a genetic polymorphism that downregulates the Na^+,K^+ pump.[783]

Defect in RhAG in Overhydrated Hereditary Stomatocytosis Erythrocytes. All of the patients with classic OHSt studied so far have mutations in the RhAG protein (Rh-associated glycoprotein; gene name *RHAG*)[765,767] (Fig. 16-33, *A*). RhAG associates with the RhD and RhCE proteins that carry the Rh antigens to form the Rh complex. This complex resides in a larger membrane protein complex that includes the red cell adhesion proteins CD47 and LW, band 3 (the Cl^-–HCO_3^- exchange channel), and glycophorin B, which serves as a chaperone to bring RhAG to the membrane.[784] The complex binds to ankyrin.[785,786] RhAG has the typical structure of a protein channel, with 12 transmembrane helices. It functions as an ammonium ion channel[787-789] and/or a gas (NH_3, CO_2) channel[790,791] in addition to helping display Rh blood groups. So far, all OHSt patients have mutations in one of just two conserved amino acids in the second transmembrane helix, Ile61Arg or Ser65Phe, and all but one of those have the Ser65Phe mutation. The extremely limited spectrum of causative mutations may explain the rarity of this disease. The two mutations above impinge on the presumptive ammonium channel and presumably disrupt the channel structure (see Fig. 16-33, *B*). In fact, the mutant RhAG proteins induce large cation leaks when introduced into oocytes.[767]

Treatment: Splenectomy Is Dangerous

The swollen, water-laden stomatocytes have trouble negotiating the spleen, where the acidotic and hypoglycemic environment impairs their ability to synthesize ATP and expel the inward torrent of Na^+ and water via the Na^+,K^+-ATPase pump.[759] As a consequence, the spleen is a graveyard for stomatocytes and splenectomy is an effective though incomplete therapy (i.e., the anemia is only partially relieved).[752,753,760,761,770]

However, physicians who care for patients with either OHSt or hereditary xerocytosis (HX, to be discussed) agree that there is a high risk of thromboembolic complications following splenectomy, so much so that the operation is contraindicated in these diseases in all but extreme situations. Unfortunately, the data backing up this prohibition are not very extensive. The recommendation flows primarily from a single study, a retrospective compilation of case reports of nine splenectomized adults collected by Stewart and colleagues in 1996.[277] All the patients were labeled as having "hereditary stomatocytosis," but some had OHSt and others had HX. Both groups suffered from serious thrombotic complications, sometimes recurrent over years, including deep venous thrombosis, pulmonary embolism, superficial thrombophlebitis, portal vein thrombosis, intracardiac mural thrombosis, arterial thrombosis, and pulmonary hypertension. Four of the nine patients died, and the author is aware of another splenectomized adult patient

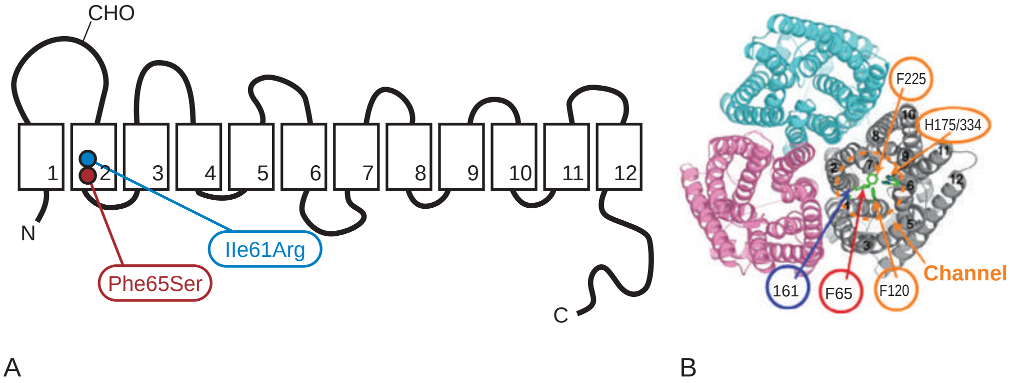

Figure 16-33 **A**, Model of Rh-associated glycoprotein (RhAG) showing the mutations that cause overhydrated hereditary stomatocytosis (OHSt). The extracellular space is on top. Almost all patients have the Phe65Ser defect. **B**, Structural model of an RhAG trimeric complex looking from the outside of the cell. Histidines 175 and 334 and phenylalanines 120 and 225 (marked in orange) are believed to lie within a channel that the RhAG protein uses to transport NH_4^+ ions or NH_3 or CO_2 gas molecules. Note that the amino acids mutated in OHSt (marked in red and blue) lie within this channel, where they could potentially alter its permeability and induce the massive influx of Na^+ ions that causes the disease. *CHO* indicates the location of side chain carbohydrate on RhAG. *(**B**, Modified from Bruce LJ, Guizouarn H, Burton NM, et al: The monovalent cation leak in overhydrated stomatocytic red blood cells results from amino acid substitutions in the Rh-associated glycoprotein.* Blood *113:1350–1357, 2009.)*

with hereditary stomatocytosis[752] who died of thromboembolic complications years after the operation. No such complications were observed in six affected but unsplenectomized adults. There have been six additional reports of individuals with OHSt or HX who developed severe thrombotic complications in the 18 years since this initial paper became available.[792-795] Given the retrospective, incomplete, and anecdotal nature of all these reports; the lack of paired unsplenectomized controls; and other inherent flaws, it is impossible to estimate the precise risk of splenectomy in these two diseases, but it is apparent that it is high and that splenectomy, which is only partially effective in OHSt and ineffective in HX, should be avoided.

We do not clearly understand why thrombotic complications are so much higher after splenectomy in these diseases than in similar conditions such as HS. Stomatocytes have about fourfold more phosphatidylserine (PS) on their surfaces than control cells.[796] PS exposed on the outer surface of cells is highly thrombogenic.[797,798] Such cells are cleared by macrophages, which have PS receptors on their surfaces.[799-801] This function would presumably be impaired after splenectomy. In vitro, erythrocytes from two splenectomized individuals with hereditary stomatocytosis show mildly increased adherence to endothelial monolayers.[796,802] This might also contribute to a hypercoagulable state.

Fortunately, in most cases, patients with OHSt have a tolerable degree of hemolysis and anemia and do not require any dramatic interventions, or at most occasional transfusions for intermittent hemolytic or aplastic crises. In occasional patients the anemia is severe enough (usually a hemoglobin value of ≤5 to 7 g/dL) to slow growth, greatly decrease normal energy, or cause congestive heart failure, in which case the physician is faced with the unpalatable choice of chronic transfusions coupled with iron chelation therapy, splenectomy followed by life-long anticoagulation, or bone marrow transplantation. The propensity for iron overload in this disease further complicates the options for chronic transfusion therapy and bone marrow transplantation. There is insufficient information about the relative risks of these unappealing therapeutic options in OHSt patients to offer rational advice on how to choose among them.

We recommend that patients who have already been splenectomized be placed on chronic coumadin therapy and that they avoid contraceptives and, if possible, pregnancy.

Like all severe hemolytic anemia patients, patients with OHSt should receive regular folic acid supplementation (1 mg/day), and, because iron overload is common in this disorder, even in the absence of transfusions, iron stores should be monitored.

Hereditary Xerocytosis (Dehydrated Hereditary Stomatocytosis, High Phosphatidylcholine Hemolytic Anemia)

Hereditary xerocytosis (HX) is the most common red cell cation permeability disorder. It is inherited as an autosomal dominant disorder. HX also goes by the name of dehydrated hereditary stomatocytosis. This is a very unfortunate name because the blood smears of most patients show few, if any, stomatocytes and the term leads to confusion with OHSt, which is an entirely different disease. In fact, most patient blood smears are surprisingly normal (see Fig. 30, D), with a few target cells, some irregular spiculated cells, and rare stomatocytes.

Clinical Features

HX is characterized by a mild increase in potassium permeability that is sufficient to lead to the gradual loss of red cell K^+ and water, and to red cell dehydration, stiffness, and hemolysis. Multiple families have been described.[200,228,229,803,804,805-814,815-830] The disease is estimated to have a prevalence of 1 in 50,000; however, because blood smears are unimpressive and the anemia is often fully compensated (i.e., hemoglobin levels are normal), and because red cell electrolyte measurements are hard to obtain, it is likely that many, and probably most, patients go undiagnosed.

Patients with HX may present at any age for evaluation of reticulocytosis, stomatocytosis, pseudohyperkalemia, jaundice, gallstones, or splenomegaly. Hemoglobin and hematocrit values are often normal despite evidence for mild to moderate hemolysis, suggesting that *patients are physiologically more anemic than the numbers suggest*. There is some evidence that the viscous, dehydrated red cells do not traverse the kidney well, leading to less oxygen reaching the juxtaglomerular apparatus, which would stimulate more erythropoietin production and lead to a higher hemoglobin level than would be expected for the degree of hemolysis.

Red cell 2,3-DPG concentrations are moderately decreased in HX,[200,803,813,814,832] just as they are in hereditary stomatocytosis. The reasons are unknown. Because loss of the polyvalent DPG is compensated for by an influx of monovalent chloride ions and water, patients with xerocytosis and unusually low DPG levels have fewer dehydrated red cells than expected for the degree of cation loss and in rare cases have no dehydrated cells at all.[832] Patients with low DPG levels also have increased whole-blood oxygen affinity.[823,832] This may also contribute to their relative polycythemia. The bottom line is HX patients are probably more anemic functionally than their hemoglobin levels suggest, a point clinicians need to consider.

HX red cells have a reduced K^+ concentration and reduced total monovalent cations (Na^+ plus K^+) (see Table 16-12). *Red cell morphology is usually unimpressive.* There are typically a few target cells, occasionally a modest number, and sometimes a few echinocytes, a few stomatocytes (<5%), or some dense, contracted, irregular cells (see Fig. 16-30, *D*). Occasionally, stomatocytes are more apparent (see Fig. 16-30, *E*), especially in the very rare patients with homozygous HX.[811] In addition, in the most severely affected patients, particularly after splenectomy, blood smears may display some contracted and spiculated red cells and cells in which the hemoglobin appears to be aggregated in one portion of the cell.[803]

The MCV ranges from high normal to macrocytic and the MCHC is typically elevated. The high MCV is partially an artifact of cellular stiffness. In Coulter-type electronic counters, the measurement of MCV depends on the cell shape. Xerocytes do not deform to the same degree as normal cells, which causes the electronically measured MCV to be about 10% too high[831] and leads to a corresponding increase in the hematocrit, which is calculated using the MCV. However, the MCV of HX red cells is also high in cell counters that measure MCV by light scattering, so it appears the cells really are macrocytic. Because the red cells are dehydrated, the osmotic fragility curve is always shifted toward osmotic resistance, though the effect can sometimes be subtle.[811] *The combination of macrocytic cells that are dehydrated (resistant osmotic fragility and high MCHC) should strongly suggest the diagnosis of HX*.

Nonimmune Hydrops. A constellation of clinical findings, including anemia, jaundice, pseudohyperkalemia, fetal or perinatal edema or ascites, and even hydrops fetalis, has been described in the fetal and newborn period in HX patients.[805,806,825,826,833] Different combinations of these features occur in different patients. Only a subset of infants develop intrauterine or perinatal fluid effusions. The effusions vary remarkably in severity, regress spontaneously, either in utero or soon after birth (a few months at most), and do not reappear. The cause of this complication and how it relates to the red blood cell permeability defect is a complete mystery. It does not seem to be related to anemia or hypoalbuminemia. Although treatments such intrauterine transfusions and albumin infusions have been employed, the efficacy of these interventions is difficult to assess due to the spontaneous resolution of symptoms.[821,826,833]

Iron Overload. The clinical course of HX is also remarkable for iron overload in the absence of transfusions, even more so in patients who are heterozygous for a hemochromatosis gene.[828] The serum iron, serum ferritin, and iron saturation values are usually high in affected adults and often require treatment. The degree of iron overload is greater than that of other hemolytic anemias, except for those characterized by ineffective erythropoiesis. The underlying cause is not understood.

Pathophysiology

Physiologically, the major red cell abnormality is a change in the relative permeability of the membrane to potassium. Efflux of K^+ is increased twofold to fourfold and approximates or slightly exceeds Na^+ influx.[750,803] Na^+,K^+ pump activity is increased appropriately for the slightly elevated Na^+ content, but the pump cannot compensate for K^+ losses in excess of Na^+ gain, because the pump expels three Na^+ ions for every two K^+ ions it returns,[834,835] which is the normal ratio of the Na^+_{in} and K^+_{out} leaks. As a consequence, the K^+ permeability defect, though modest, causes HX red cells to gradually become cation depleted and dehydrated.

Defect in the Piezo1 Mechanotransducer Channel. The defective gene was originally mapped to chromosome 16q23-q24,[805,836,837] but that was then revised to the adjacent telomeric segment (16q24.2-qter),[830] which led to identification of Piezo1 (gene name *FAM38A*) as the responsible protein.[227] Missense mutations in conserved amino acids near the middle and tail end of the protein were found in 24 families (Fig. 16-34).[227,228,229,838] These include families with the features of pseudohyperkalemia and perinatal edema as well hemolysis and red cell dehydration. A total of 14 mutations were identified. One family (kindred Bicêtre)[228] carried two Piezo1 mutations (*G782S* and *R808Q*). One common mutation that added two amino acids to the sequence (*E2496ELE*) was found in 8 of 11 French families but not in families from other countries, suggesting a founder effect.[229] Two other mutations were found in two (*T2127M*) and four (*R2456H*) apparently unrelated families.

There are two Piezo proteins (Piezo1 and Piezo2). They are very large, widely distributed proteins and form huge multimeric cation channels, with 120 to 160 transmembrane segments, which are triggered by mechanical distortion of the membrane[225,226] (reviewed[859]). Piezo proteins are involved in pain sensing[225] and perhaps in other mechanical sensations such as hearing and touch. The presence of Piezo1 in the red blood cell membrane suggests the proteins may also be involved in homeostatic adjustments to changes in volume, responding to membrane stretch. In fact, the HX mutations lengthen the time it takes to activate the Piezo1 channel after membrane deformation and slow the time it takes to deactivate it.[840] How these changes in channel function lead to K^+ loss and cell dehydration in hereditary xerocytes is not yet clear. Perhaps the lost K^+ ions simply flow out through the mutant Piezo1 channel. Alternatively, K^+ loss may be a secondary consequence of excess Ca^{2+} entry through the mutant channel. It is known that Ca^{2+} enters normal red cells when they are deformed. It is likely that this occurs through Piezo1 and triggers Ca^{2+}-stimulated K^+ loss via the Gárdos channel. It is a mystery why defects in the widely distributed, evolutionarily conserved Piezo1 channel do not have more widespread pathologic effects in HX, especially in homozygotes,[811] which some of the sequenced patients were.[227] Perhaps red cells have only Piezo1 whereas other cells have both Piezo1 and Piezo2, providing redundancy.

Red cells from some HX patients have an increased proportion of the phospholipid phosphatidylcholine in their membranes (12 to 20 fmol/cell; normal range, 10 to 12 fmol/cell), and their disorder was initially given the name *high phosphatidylcholine hemolytic anemia*.[808,812,816,819,820,822] It is not clear if this is true of all HX patients or demarcates a subset of the disease, but there seems to be little to differentiate the two diseases.[820] Early studies suggested the excess phosphatidylcholine was due to diminished transfer of phosphatidylcholine fatty acids to phosphatidylethanolamine,[816] a pathway that is normally stimulated by cellular dehydration. It is not clear why this pathway is inhibited in HX or how it relates to the underlying membrane leakiness and hemolysis.

Xerocytes are also shear sensitive[815] and are exceptionally prone to membrane fragmentation in response to metabolic stress.[200] It is interesting to speculate that this

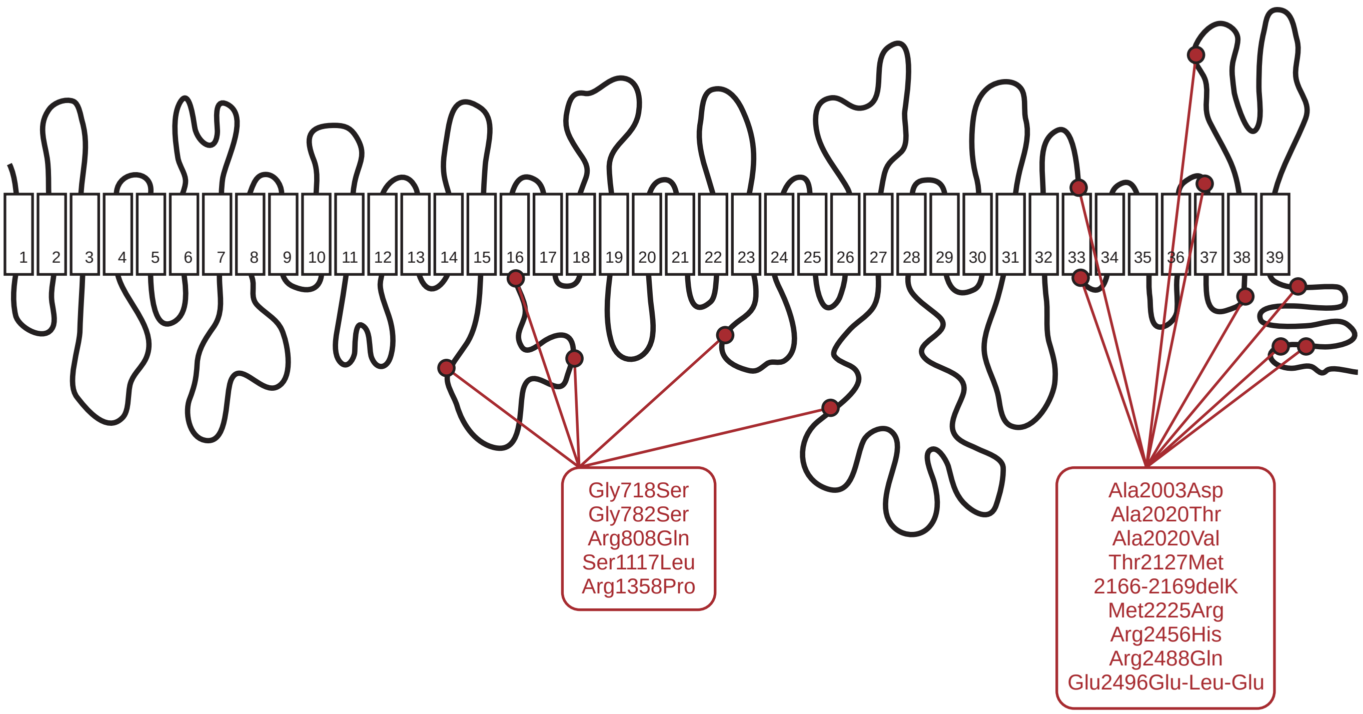

Figure 16-34 Model of Piezo1 (gene name FAM38A), a very large mechanotransducer protein involved in red cell volume regulation, showing mutations that cause hereditary xerocytosis. The extracellular space is on top. The mutations are grouped in the middle and C-terminus of the protein. One mutation (Glu2946Glu-Leu-Leu) was found in 8 of 11 French families, and two others (T2127M) and (Arg2456His) occurred in two and four unrelated families, respectively. *(From Zarychanski R, Schulz VP, Houston BL, et al: Mutations in the mechanotransduction protein PIEZO1 are associated with hereditary xerocytosis.* Blood *120:1908–1915, 2012; Andolfo I, Alper SL, De Franceschi L, et al: Multiple clinical forms of dehydrated hereditary stomatocytosis arise from mutations in PIEZO1.* Blood *121:3925–3935, 2013; and Albuisson J, Murthy SE, Bandell M, et al: Dehydrated hereditary stomatocytosis linked to gain-of-function mutations in mechanically activated PIEZO1 ion channels.* Nat Commun *4:1884, 2013.)*

is somehow related to the defect in Piezo1, a channel that responds to membrane distortions such as shear. However, similar shear sensitivity can be induced in normal red cells by dehydrating them[815] and is also observed in other conditions, such as sickle cell disease,[841] where red cell dehydration occurs. One consequence is that strenuous exercise can exacerbate hemolysis in HX.[815]

Treatment

As discussed in the previous section, there is considerable evidence that life-threatening thromboembolic complications are frequent after splenectomy in HX as well as in OHSt.[277] Because severe hemolysis and anemia requiring treatment are rare in HX, and because splenectomy is ineffective in quelling hemolysis, HX patients should never be splenectomized. Unfortunately many patients are, having been mistakenly diagnosed as having HS. In such instances we recommend life-long anticoagulation, though it is uncertain if this is always protective.

As noted, iron overload is a common though poorly understood complication of HX, more so than in the other disorders of cation permeability.[277,828] In some patients the problem is severe and tissue iron deposition is great enough to damage the liver and other organs.[828] For this reason, *all patients with HX should have their iron status regularly monitored,* and, if necessary, they should be treated with deferasirox or other iron chelators. The treatment of iron overload is discussed in more detail in Chapter 21.

Familial Pseudohyperkalemia

A small number of families are reported with a dominantly inherited disorder characterized by erythrocyte K^+ loss, through a passive K^+ leak across the red cell membrane, which occurs when blood is allowed to stand for a few hours at room temperature or below.[804,842-848] Electrolyte analyses may falsely suggest that affected individuals are hyperkalemic. The leak is insignificant at body temperatures, so familial pseudohyperkalemia (FP) is symptomless and affected patients experience little or no hemolysis and no anemia. Osmotic fragility and osmotic gradient ektacytometry are normal. The MCV is mildly elevated in some cases,[804] and red cell Na^+ and K^+ are mildly increased and decreased, respectively. In particular the patients have a normal electrocardiogram and do not have evidence of acidosis, renal failure, Addison disease, or hypertension. If they do have hypertension, they do not have hyporeninemic hypoaldosteronism (Gordon syndrome), in which hyperkalemia is genuine. The key point clinically is to be sure these patients do not receive hyperkalemic therapy, which could cause severe hypokalemia.

The most common form of FP is apparently a mild form of HX because it maps to the same locus as HX on chromosome 16.[805,836,849] The temperature dependence of the cation leak in FP varies considerably, which may simply reflect different alleles in the same transporter or may reflect defects in different genes.

A second form of FP maps to chromosome 2 (2q35)[850] and is associated with mutations in the ABCB6 transporter.[851] ABCB6 is one of the ATP-binding cassette proteins and functions as a mitochondrial porphyrin transporter, but it is also expressed in the red cell membrane and carries the Lan (Langereis) blood group.

In addition, patients with hereditary spherocytosis, stomatocytosis, xerocytosis, or cryohydrocytosis all have red cells that leak K^+ and may also show pseudohyperkalemia if blood is drawn and is not analyzed quickly.

Other Hereditary Stomatocytosis Syndromes

Between the extremes of OHSt and HX there are a series of disorders characterized by mild to moderate hemolysis and anemia; stomatocytosis or spherocytosis or both; and abnormal cation permeability. All but one of these conditions is caused by missense mutations in band 3, the anion exchanger, which alter its permeability. For the purposes of this chapter these disorders are sorted into categories, but, with the exception of hereditary cryohydrocytosis (HC), type 2, which has a different genetic basis, they are overlapping variations on a phenotypic theme.

Mild Hereditary Stomatocytosis

Clinical Features. Some patients with hereditary stomatocytosis have disease similar to the classic overhydrated variant described above, except that hemolysis and anemia are milder (see Table 16-12).[769,774,852,853-857] The degree of stomatocytosis may be less; in one case no stomatocytes were evident on the peripheral blood smear.[854] The osmotic fragility test may also be normal or may show the expected fragile red cells. One family also had evidence of dyserythropoiesis.[855] Red cell stomatin concentrations are normal or only mildly decreased. The two genetically tested patients had mutations in band 3.[854,855] Some patients with severe permeability defects have had little or no hemolysis.[769] In addition, studies of 44 Japanese patients with a stomatin-sufficient form of stomatocytosis showed that the proportion of stomatocytes and the degree of Na^+ influx did not correlate with each other, and neither correlated with the amount of hemolysis or anemia.[774] This suggests that factors other than the Na^+ leak influence the demise of the stomatocyte. Splenectomy has not been clearly beneficial, and at least one patient died with pulmonary hypertension some years after splenectomy.[857]

Defect in Band 3. Four defects in the membrane domain of band 3 (gene name *SLC4A1*) have been identified in five families with the mild variant of hereditary stomatocytosis (Fig. 16-35)[854,856,858] and in a family with stomatocytosis and dyserythropoiesis.[855] Band 3 is the major erythrocyte membrane protein and is the anion channel responsible for shuttling chloride and bicarbonate ions across the membrane, which aids in the transport of CO_2 from tissues to the lungs. Quantitative deficiency of band 3 causes hereditary spherocytosis, not stomatocytosis. In these stomatocytosis patients, normal amounts of band 3 are made but the defects abolish anion exchange function.[854,858] Two of the mutations convert the putative anion channel[859] to a Na^+,K^+ conductance pathway (Leu687Pro and Gly796Arg).[854,860] The third mutation (Arg730Cys) does not.[854] Perhaps in that case the mutant band 3 perturbs a neighboring channel.

Hereditary Cryohydrocytosis

Hereditary Cryohydrocytosis Type 1: Normal Stomatin and Defect in Band 3. Hereditary cryohydrocytosis, type 1 (HC-1)[766,767,853,858,861-865] is a rare autosomal dominant disorder that is milder than the classic overhydrated form of hereditary stomatocytosis (OHSt) and similar clinically to the mild form of hereditary stomatocytosis described in the preceding paragraph. It differs in having a cold-sensitive monovalent cation leak (see Table 16-12). Na^+ and K^+ concentrations and permeabilities are less abnormal at physiologic temperatures in HC-1 than in OHSt, but instead of declining with temperature, as in OHSt, cation permeability increases below 20°C, exceeding the ability of the red cell to compensate. As a consequence, red cells from patients with HC-1, collected in EDTA or heparin, swell and partially hemolyze when stored in the refrigerator overnight.[853,861,862,864] This is a requirement for the diagnosis of HC and is its signature feature. This temperature sensitivity means that red cell measurements in these patients must be completed before a blood sample is allowed to cool.

Patients with HC-1 have mild to moderate hemolysis, with or without anemia.[853,861,862] Their red cells are mildly macrocytic and slightly osmotically fragile. They tend to be stomatocytic (see Fig. 16-30, C). The author and others[853] have observed a tendency for the slit of the "stoma" to be curved and eccentrically placed, a finding that the authors have not observed in other conditions. Several such cells are seen at the top left and top right of Figure 16-30, C. However, this characteristic is not always present. Spherocytes may also be seen. The MCHC is variable in cryohydrocytosis and is not consistently low as in severe overhydrated stomatocytosis, and the MCV is also not always macrocytic. Unlike HC-2 (see below), HC-1 erythrocytes have normal concentrations of stomatin.

Patients with HC-1 may present at any age with macrocytosis, anemia, reticulocytosis, intermittent jaundice, gallstones, pseudohyperkalemia, a palpable spleen tip, or stomatocytosis, or the diagnosis may be made because cold hemolysis is noted by an astute laboratory technician.

The primary molecular lesions in HC-1 occur in the intramembrane domain of the band 3 (or AE1) anion exchanger encoded by the *SLC4A1* gene (see Fig. 16-35).[767,858] The mutations occur in a region toward the C-terminal end of band 3, a region that is thought to form part of the anion channel. Anion exchange is decreased in the mutants and cation permeability is increased, suggesting that the mutations convert the anion exchanger into a leaky cation channel. At physiologic temperatures the cation leak and hemolysis are modest, but at temperatures below 20°C the leak is exacerbated, leading to Na^+

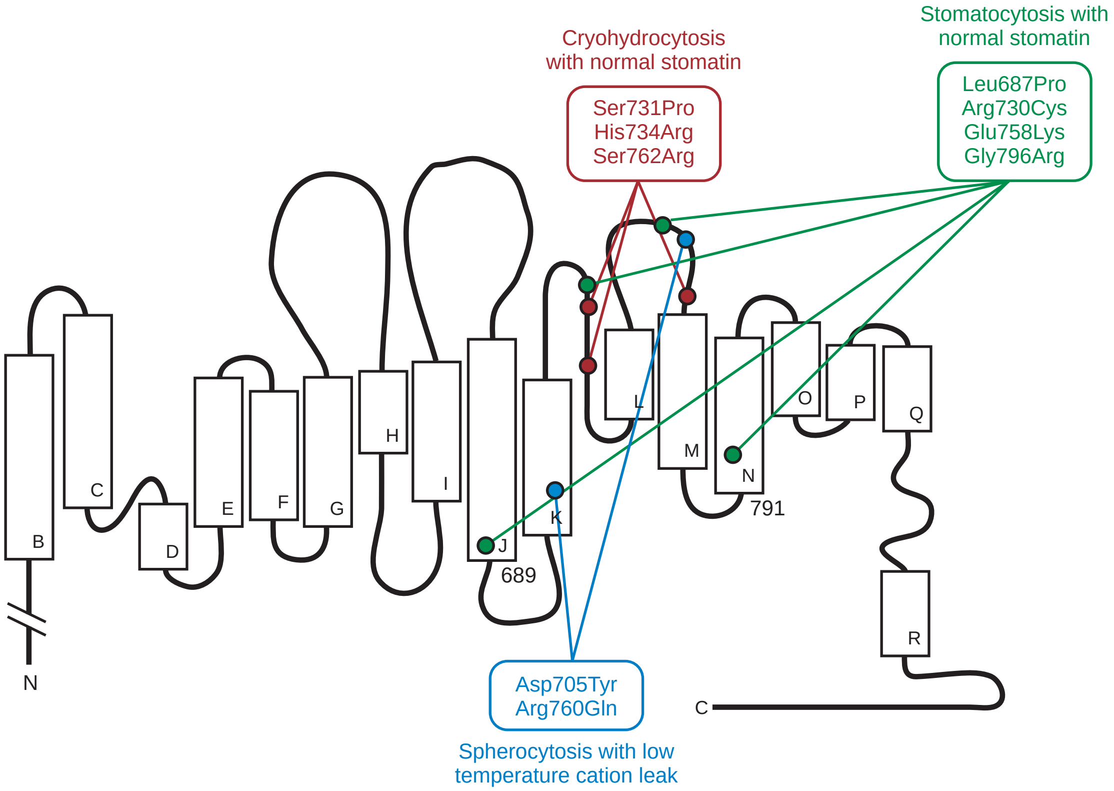

Figure 16-35 Model[859] of band 3 (also called AE1 and SLC4A1), the red cell anion exchanger and the major red cell integral membrane protein, based on the ClC chloride channel (see Fig. 15-7). The mutations shown cause mild hereditary stomatocytosis *(green circles)*, type 1 hereditary cryohydrocytosis *(red circles)*, and a form of hereditary spherocytosis (HS) with a low-temperature cation leak that is better classified as a subset of cryohydrocytosis *(blue circles)*. The extracellular space is on top. Note that all the mutations lie within a segment of the membrane domain between helices J and N. Presumably this portion of the protein must form part of the anion exchange channel. The mutations illustrate the range of phenotypes that can be produced by mutations within the same protein; mutations that presumably all function by altering the permeability of the protein channel in slightly different ways. The Gly796Arg mutation is also associated with dyserythropoiesis.

and water influx and osmotic lysis.[858] Why some band 3 mutations are cold sensitive and others are not is a mystery. Presumably the conformational changes produced by the temperature-sensitive mutations worsen at low temperatures. The sensitivity to low temperatures suggests critical hydrophobic bonds may be involved because hydrophobic bonds become weaker as temperatures fall. There is also evidence that the primary defect in band 3 may indirectly activate other cation channels at low temperatures under certain conditions.[865]

Hematologic improvement has not been reported after splenectomy in HC, but very few patients with this relatively mild disorder have had their spleens removed. Thrombotic complications have also not been reported, but it appears there is little to be gained from splenectomy and it is impossible to be certain that the risk is low.

Hereditary Spherocytosis with a Low-Temperature Cation Leak and Defect in Band 3. On the peripheral blood smears of some patients, spherocytes predominate or are the sole red cells seen.[858,865] These patients are essentially indistinguishable from those with HS except that they have a low-temperature cation leak (LTL) that mimics that seen in HC-1 and their MCHC tends to be lower than in typical HS.[858] Typical HS patients have a small cation leak, but it is much less than in HS-LTL and it does not show any augmentation at low temperatures.[858]

All of the reported patients have missense mutations in band 3 that are very similar to those observed in mild stomatocytosis and cryohydrocytosis (see Fig. 16-35) and that presumably pervert the function of band 3. In typical HS, on the other hand, when band 3 is involved the mutations cause deficiency but not dysfunction of the protein. Perhaps in these patients the missense mutations destabilize band 3, leading to band 3 deficiency and an HS phenotype, yet allow enough of the leaky band 3 to reach the surface to alter Na^+ permeability. In a small survey, 2 of 16 HS pedigrees had the HS-LTL phenotype.[858] It seems doubtful that HS-LTL is actually so prevalent, because band 3 mutations of all types account for only about 25% of HS cases,[57] but the phenotype may not be rare.

The reported families had mild to moderate hemolysis and anemia and benefited greatly from splenectomy. However, one of the four splenectomized members of one kindred had a deep vein thrombosis years after his splenectomy and then developed pulmonary hypertension presumed to be of thrombotic origin.[858] He also carried the factor V Leiden polymorphism, but even so, it seems prudent to avoid splenectomy in this subset of HS/HC-1 patients until more data are available. Findings in these patients support the argument that a test for cryohemolysis should be included in the workup of patients with HS, at least those for whom splenectomy is being considered.

Hereditary Cryohydrocytosis Type 2: Stomatin Deficiency and Defect in Glut1. Type 2 hereditary cryohydrocytosis (HC-2) has been observed in only two families so far and is associated with loss of red cell stomatin, which occurs during reticulocyte maturation. The hematologic picture resembles that of HC-1, except that peripheral red cell morphologic findings are nearly normal, with just a few echinocytes and stomatocytes.[766,866] However, the patients also have cataracts and a variety of neurologic features, including seizures, mental retardation, spastic rigidity, dyskinesias, and ataxia.[755,766] The older patients also had massive hepatosplenomegaly. Pseudohyperkalemia is reported.[866] HC-2 red cells are macrocytic and osmotically resistant.

HC-2 is caused by mutations in glucose transporter 1, or Glut1 (gene name *SLC2A1*) (Fig. 16-36),[866] which, as noted earlier, binds stomatin and is converted to an L-dehydroascorbate transporter in human red cells and other tissues. The mutations block glucose transport and induce a cation leak. The block in glucose transport causes the characteristic neurologic symptoms (seizures, mental retardation, and movement disorders). The hemolysis and cataracts appear to be due to the altered cation permeability of the mutant channel. Many (>60) patients have been described with a similar neurologic phenotype who are heterozygous for defects in Glut1 (so-called *Glut1 deficiency disorder*),[867] but these patients do not have hemolysis or cataracts and their Glut1 mutations do not increase Na^+ or K^+ permeability. They just block glucose transport. Only three HC-2 families with Glut1 mutations have been identified so far, and two have the same mutation (Ile435del). Although the hemolysis is so mild that some HC-2 patients may be misclassified as having simple Glut1 deficiency disorder, it seems more likely that, like OHSt, the disease is rare because only a few mutations allow Na^+ and K^+ to leak through the glucose channel, causing hemolysis. A fourth family is known whose members are heterozygous for a 4–amino acid in-frame deletion of Glut1 that increases the permeability of Ca^{2+} as well as Na^+ and K^+ (see Fig. 16-36).[868] The affected members also have a mild hemolytic anemia, but their red blood cells are dehydrated due to Ca^{2+} stimulation of K^+ egress (the so-called *Gárdos channel*), and are prominently echinocytic instead of stomatocytic or normal appearing.

In patients with HC-2, seizures and other neurologic complications dominate the clinical picture. The neurologic symptoms are believed to be due to inadequate glucose transport into the brain.[866] In other patients with defects in Glut1 who lack the hemolytic component, there is evidence that the seizures improve when the patient is placed on a ketogenic diet[869]; this finding includes one child with HC-2 disease.[866] Hemolysis and anemia are mild to moderate in HC-2 and do not require treatment.

Southeast Asian Ovalocytosis

Southeast Asian ovalocytosis (SAO) was discussed earlier in the chapter as a subtype of HE, but it is mentioned here because recent studies show that SAO red cells have the cation permeability properties of cryohydrocytes, including the cold sensitivity.[723] The primary defect is a deletion of nine amino acids (400 to 408) at the beginning

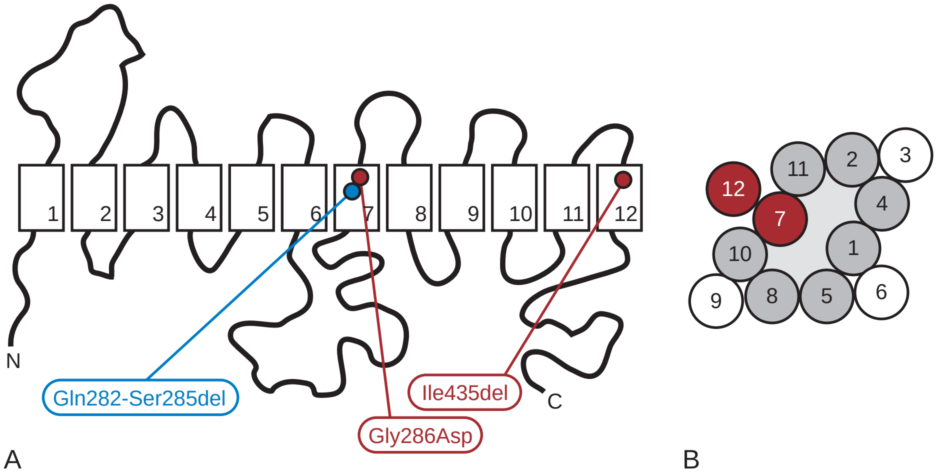

Figure 16-36 **A**, Model of glucose transporter 1 (Glut1) showing the mutations that have been identified in kindreds with hemolytic anemia, neurologic deficits, and either type 2 cryohydrocytosis *(red circles)* or hereditary echinocytosis *(blue circle)*. Two of the three reported HC2 kindreds have the Ile435 deletion. Many other defects in Glut1 are known that cause neurologic disease alone, without hemolysis or anemia. **B**, Model of Glut1 showing a proposed arrangement of the various transmembrane helices. The defects in helix 7 impinge directly on the putative glucose transport pathway. The defect in the adjacent helix 12 is believed to produce large conformational changes that distort the channel indirectly. *(**A**, Modified from Flatt JF, Guizouarn H, Burton NM, et al: Stomatin-deficient cryohydrocytosis results from mutations in SLC2A1: a novel form of GLUT1 deficiency syndrome.* Blood *118:5267–5277, 2011;* ***B****, Modified from Weber YG, Storch A, Wuttke TV, et al: GLUT1 mutations are a cause of paroxysmal exertion-induced dyskinesias and induce hemolytic anemia by a cation leak.* J Clin Invest *118:2157–2168, 2008.)*

of the first transmembrane domain of band 3,[710] which causes the protein to misfold. However, the mutant band 3 can still traffic to the membrane, where it mistakenly conducts cations.[723]

Stomatocytosis with a Defect in Palmitoylation

Two elderly brothers in a Polish family have recently been described with a mild macrocytic, stomatocytic hemolytic anemia (hemoglobin 11 to 13 g/dL, MCV 101 to 104 fL, normal MCHC, and reticulocytosis 7% to 8%), with normal erythrocyte Na^+ and K^+ concentrations, normal stomatin, some basophilic stippling, indirect hyperbilirubinemia, and a positive osmotic fragility test.[870] Biochemical studies revealed a marked defect in the palmitoylation of red cell membrane proteins, especially p55, the major palmitoylated protein, due to a deficiency of DHHC17, the sole acyltransferase in the mature red cell. The numbers of lipid rafts (see Chapter 15) are also markedly diminished. It is unclear how these defects relate to the clinical phenotype.

Classification Based on the Temperature Dependence of Cation Leaks

Gordon Stewart and his colleagues have established a classification system of the disorders of red cell membrane cation permeability based on the effects of temperature on membrane cation leak.[805,852,862,871] The membrane leak is measured by the residual influx of $^{86}Rb^+$ (a radioactive substitute for K^+) across the red cell membrane in the presence of ouabain (to inhibit the Na^+,K^+-ATPase pump) and bumetanide (to inhibit the $Na^+,K^+,2Cl^-$ cotransporter). This measurement is often taken as a measure of the passive permeability of monovalent cations, although it does not exclude the contributions of the Gárdos channel and the K^+,Cl^- cotransporter. These transporters do not significantly contribute to cation permeability in normal red cells but may contribute more significantly in pathologic erythrocytes. Nevertheless, the estimate of monovalent cation leak is useful in understanding the pathophysiology of these permeability disorders.

Normal red cells have a low leak that falls with temperature, reaching a minimum at approximately 10°C (Figs. 16-37 and 16-38).[871] The reason for this minimum is unknown. The capacity of the Na^+,K^+ pump far exceeds the normal leak rate at physiologic temperatures, but pump activity falls sharply below room temperature and approximates the normal leak at 0°C (see Fig. 16-38). The temperature profile of the passive leak varies in each disorder (see Fig. 16-37). In classic OHSt, the leak rate, particularly of the Na^+ leak, is extremely high at body temperatures and exceeds the capacity of the Na^+,K^+ pump, which leads to cell swelling and hemolysis (see Fig. 16-37, *A*). Stomatocytosis patients with milder leaks are better compensated by the Na^+,K^+ pump and experience less hemolysis (see Fig. 16-37, *A*).[852] Patients with cryohydrocytosis have a similar mild to moderate cation leak at body temperature, causing mild to moderate hemolysis. However, in cryohydrocytosis, the cation leak increases dramatically at below-ambient temperatures,

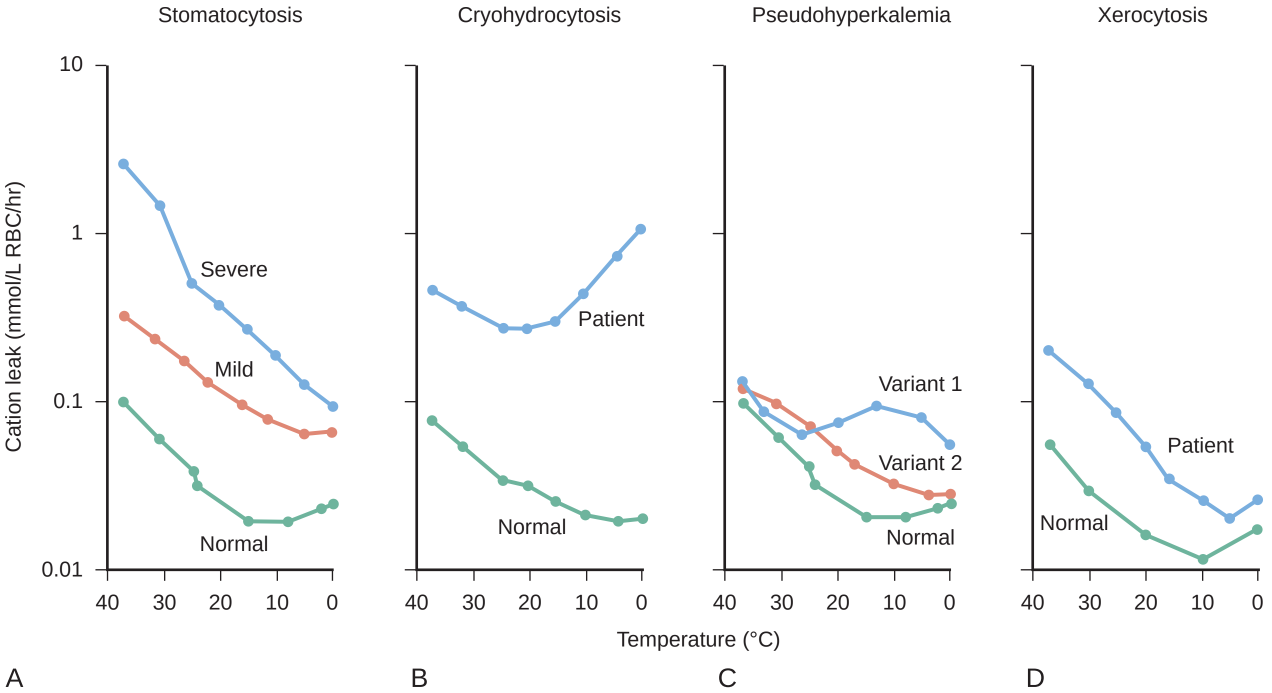

Figure 16-37 Passive permeability of monovalent cations as a function of temperature in the hereditary stomatocytosis-xerocytosis disorders (*blue* and *red circles*) compared with normals *(green circles)*. Stomatocytosis (**A**), cryohydrocytosis (**B**), pseudohyperkalemia (**C**), and xerocytosis (**D**) red cells are shown. Passive permeability is defined as the uptake of $^{86}Rb^+$ across the red cell membrane in the presence of ouabain (to inhibit the Na^+,K^+-ATPase pump) and bumetanide (to inhibit the $Na^+,K^+,2Cl^-$ cotransporter). *RBC*, Red blood cell.

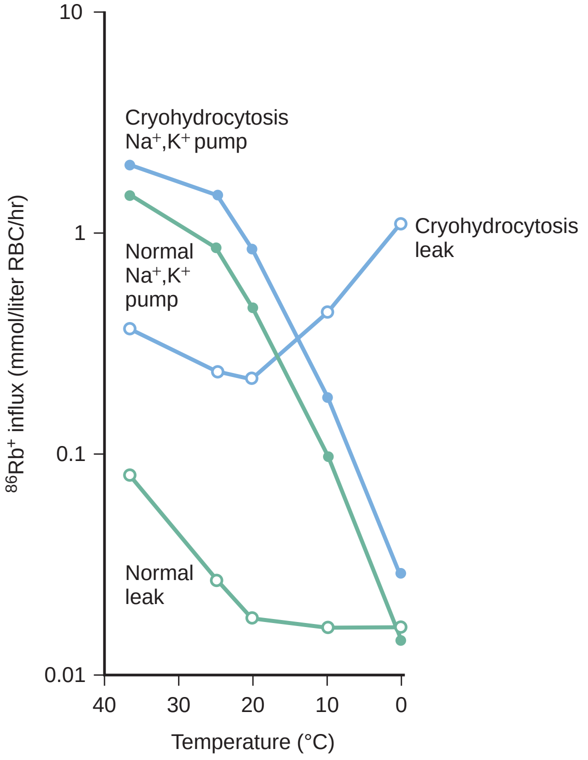

Figure 16-38 Comparison of passive monovalent cation permeabilities *(open circles)* and the capacity of the Na$^+$,K$^+$ pump *(solid circles)* in hereditary cryohydrocytosis *(blue circles)* and normals *(green circles)*. Note that the rate of cation leak exceeds the pump rate in cryohydrocytosis at low temperatures, causing the cells to swell and hemolyze. *RBC*, Red blood cell.

exceeding the capacity of the Na$^+$,K$^+$ pump below 10°C to 15°C and causing severe hemolysis (see Figs. 16-37, *B* and 16-38).

Individuals with pseudohyperkalemia typically have a nearly normal cation leak at physiologic temperatures and, therefore, little or no hemolysis. The slope of the leak is flatter than normal and sometimes even rises at below-ambient temperatures, so that K$^+$ loss occurs during storage room temperature (see Variant 1 in Fig. 16-37, *C*).

The temperature profile of the cation leak in HX parallels the normal temperature profile, but the leak is threefold to fourfold greater (see Fig. 16-37, *D*). Although the leak is relatively small, it favors K$^+$, which creates a problem because the Na$^+$,K$^+$ pump extrudes three Na$^+$ ions for every two K$^+$ ions returned. The red cell cannot compensate for leaks of K$^+_{out}$ relative to Na$^+_{in}$ that exceed the normal 2:3 ratio. Therefore the pump exacerbates the loss of intracellular cations and water and likely contributes to the dehydration of xerocytes.

Other Disorders Characterized by Stomatocytosis

A variety of conditions other than membrane permeability defects are associated with stomatocytosis (Table 16-13).

Acquired Stomatocytosis

In normal individuals, 3% or less of the red cells on peripheral blood smears are stomatocytic,[872,873] although more stomatocytic forms are evident (≤10%) if sensitive techniques such as scanning electron microscopy are used.[874] Because stomatocytes may occasionally occur as a drying artifact in limited areas of the smear, one must take care to examine multiple areas on several smears before diagnosing stomatocytosis. In wet preparations, stomatocytes are bowl shaped (uniconcave). Such preparations are useful in excluding artifactual stomatocytes, but the presence of bowl-shaped cells cannot be used as proof of stomatocytes, because target cells are also bowl shaped in solution.[875]

Drugs. In a prospective study of 4291 peripheral blood smears, Davidson and coworkers[876] found increased numbers of stomatocytes in 2.3% of the preparations. Fifty-nine percent of these smears had 5% to 20% stomatocytes, 35% had 20% to 50% stomatocytes, and 6% had more than 50% stomatocytes. In this and other studies, a wide variety of drugs and diagnoses were associated with stomatocytosis.[811,875-879] Examples of such drugs include phenothiazines, taxanes, vinca alkaloids (e.g., vincristine and vinblastine), and perflurane. Further studies are needed to determine which associations are specific and reproducible and which occur in vivo as well as in vitro. As discussed in Chapter 15 in the section on the bilayer couple hypothesis, amphiphilic, lipid-soluble drugs can cause red cells to assume a stomatocytic shape if the drug partitions preferentially into the inner half of the lipid bilayer. The affected red cells are misshapen but are not cation loaded or overhydrated and do not usually hemolyze. Vinca alkaloids often induce hemolysis, sometimes with increased membrane Na$^+$ permeability and stomatocytosis.[879] This is a particular problem in the rare instances in which these drugs must be given to patients with cancer who also have HS. We have seen two such patients and in both cases very severe hemolysis occurred. Presumably the explanation is that spherocytes and stomatocytes both have a decreased surface-to-volume ratio. Imposing a stomatocytic stress on HS red cells will make them even more spheroidal and hasten their demise.

TABLE 16-13 Conditions in Which Stomatocytosis Is the Predominant Morphology*

Hereditary stomatocytosis
Hereditary cryohydrocytosis
Mediterranean stomatocytosis and macrothrombocytopenia (hereditary sitosterolemia)
Rh$_{null}$ and Rh$_{mod}$ blood groups†
Adenosine deaminase hyperactivity hemolytic anemia†
Tangier disease†
Acute alcoholism†
Certain drugs (phenothiazines, taxanes, vincristine, perflurane)†
Marathon runners†
Hereditary xerocytosis†

*Normal: ≤3% stomatocytes.
†Sometimes the predominant morphology.

Alcoholism. Acquired stomatocytosis is common in alcoholics, particularly in those with acute alcoholism, and may be associated with moderate hemolysis.[872,873] Red cell cation measurements have not been reported; however, severe hydrocytosis is unlikely, because osmotic fragility tests have been normal.[872]

Long-Distance Runners. Some marathon and long-distance runners transiently develop stomatocytosis immediately after completing a race.[880,881] The effect is mild; 13% stomatocytes were seen immediately after a race versus a baseline of 4% stomatocytes.[881]

Rh Null and Rh Mod Disease

The Rh(D) antigen and the other antigens of the Rh group (cCeE) are part of two minor red cell membrane proteins encoded by two closely linked genes, one encoding the D polypeptide and the other encoding the Cc/Ee proteins; they are discussed in detail in Chapter 36. Some patients who lack all Rh antigens (Rh_{null})[882] have a moderately severe hemolytic anemia (^{51}Cr-labeled red cell half-life of 10 to 14 days)[883-886] characterized by stomatocytosis and spherocytosis. The underlying defects are in the Rh genes themselves or in the RhAG gene (also called Rh50), an ammonium transporter that is part of the Rh complex in the membrane.[887-889] Interestingly, as noted earlier, missense mutations in RhAG that alter its transport channel are also responsible for OHSt. Osmotic fragility is mildly increased in Rh_{null} disease,[883,886,890] and ektacytometry analysis shows a significant loss of membrane surface, particularly in denser (and presumably older) cells.[883] Red cell membrane K^+ or Rb^+ (a K^+ analogue) permeability is about twice normal.[883,891] Mild stomatocytosis and hemolysis are also features of Rh_{mod} disease,[892] a related anomaly, in which the expression of Rh antigens is suppressed but not absent, also owing to defects in RhAG.[887,889]

Mediterranean Stomatocytosis with Macrothrombocytopenia (Phytosterolemia or Sitosterolemia)

The hematologic aspects of this disorder—stomatocytosis with mild to moderate hemolytic anemia, splenomegaly, normal red cell cations, a normal osmotic fragility, and macrothrombocytopenia (40,000 to 150,000 platelets of 3 μ^3 to 120 μ^3 in size; Fig. 16-39)[893]—were first observed in Mediterranean individuals who migrated to Australia,[878] the population that gave the disorder its name. Tendon and tuberous xanthomas, a high cholesterol level, and a strong tendency toward premature atherosclerosis (sometimes even symptomatic in adolescents) are also features of the disease. The large platelets aggregate poorly in response to ristocetin, and some patients have a bleeding tendency.[894] We now know that these are manifestations of a rare inherited metabolic condition called phytosterolemia (or sitosterolemia), in which the absorption of all sterols, including both cholesterol and plant sterols (phytosterols, of which sitosterol is one of several that are elevated), is massively increased.[894] Phytosterolemia is caused by homozygous or compound heterozygous mutations in either the ABCG5 or ABCG8 gene.[894,895] The product of these genes normally forms a dimeric-ATP–binding cassette protein in the enteric mucosa, which aids in rejecting sterols into the gut. In retrospect some previously reported patients with phytosterolemia have also had stomatocytosis and hemolysis.[896] Plasma lipoproteins and red cell membranes contain excess plant sterols in this disease,[894] and these are presumably directly responsible for the defects in red cell shape and survival, and in platelet size and function. Diets high in vegetable oils, such as olive oil, aggravate the disease; this finding may explain why it was initially observed in Mediterranean immigrants in Australia.[894]

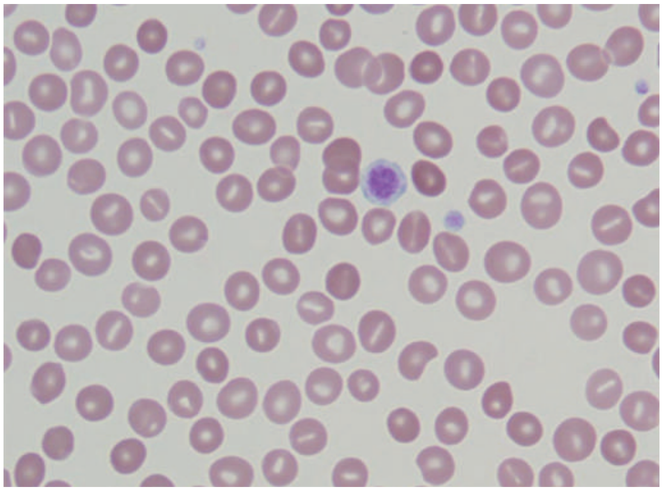

Figure 16-39 Peripheral blood smear from a patient with stomatocytosis and macrothrombocytopenia, so-called Mediterranean stomatocytosis, due to phytosterolemia (or sitosterolemia). Note the giant platelet fragment in the right center. *(From Neff AT: Sitosterolemia's stomatocytosis and macrothrombocytopenia.* Blood *120:4283, 2012.)*

ACQUIRED DISORDERS OF RED CELL MEMBRANE LIPIDS

Spiculated Red Cells: Echinocytes and Acanthocytes

There are two basic types of spiculated red cells, echinocytes and acanthocytes. Echinocytes typically have a serrated outline with small, uniform projections more or less evenly spread over the circumference of the cell, whereas acanthocytes have a few spicules of varying size that project irregularly from the red cell surface. These differences are easily detected in scanning electron micrographs and can usually be discerned in wet preparations, but it is often quite difficult to make the distinction in dried smears. In general, echinocytes appear crenated in smears, that is, as cells with relatively uniform scalloped edges, whereas acanthocytes appear contracted, dense, and irregular (Fig. 16-40).

Echinocytes are readily produced in vitro by washing red cells in saline,[897] by the interaction of red cells with glass surfaces,[897] by amphipathic molecules that partition into and expand the outer one half of the lipid bilayer,[898] and by molecules that inhibit band 3 transport.[898] High pH values, ATP depletion, and Ca^{2+} accumulation also cause echinocytosis.[899-901] In vivo, echinocytes are most often found in patients with advanced uremia[901] or with

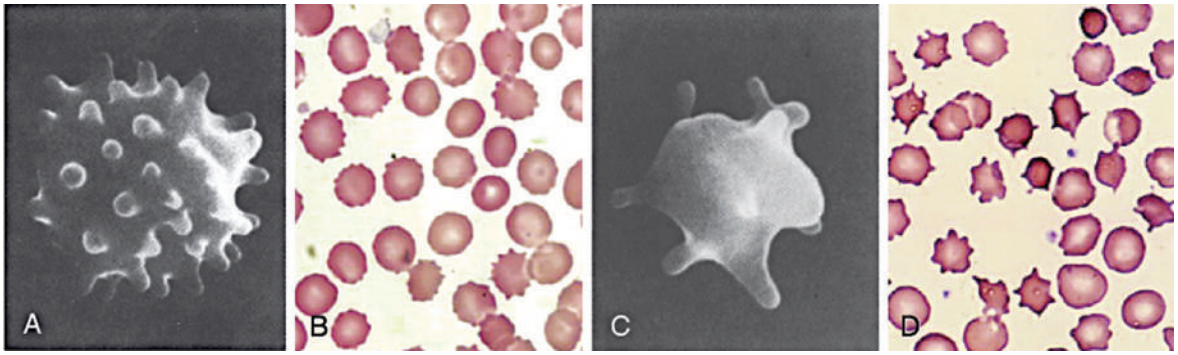

Figure 16-40 Morphology of echinocytes and acanthocytes. **A**, Scanning electron micrograph of a spherocytic echinocyte. **B**, Peripheral blood smear of echinocytes. **C**, Scanning electron micrograph of an acanthocyte. **D**, Peripheral smear of acanthocytes in a patient with spur cell anemia.

defects of glycolytic metabolism,[902] in patients after splenectomy,[897] and in some patients with microangiopathic hemolytic anemias (Table 16-14). Echinocytes are also commonly seen in neonates, especially those who are premature,[903] and in divers after decompression from pressures of more than 3 to 4 bar.[904] They are seen transiently after transfusions with large amounts of stored blood, because red cells become echinocytic after a few days of storage. Acanthocytes and echinocytes may be found in patients with neuroacanthocytoses,[905,906] severe hepatocellular damage,[907] infantile pyknocytosis,[908] anorexia nervosa,[909,910] In(Lu) blood groups,[911] and hypothyroidism (see Table 16-14).[912] Occasionally, they are the predominant morphologic feature in patients with myelodysplasia.[913,914]

The anemia seen in patients with liver disease has a complex etiologic pattern. Common causes include blood loss, hypersplenism, iron deficiency, folic acid deficiency, and marrow suppression from alcohol ingestion, hepatitis virus infection, and other poorly understood factors.[915,916] These conditions are discussed elsewhere in this text.

TABLE 16-14 Conditions in Which Spiculated Red Cells (Echinocytes and Acanthocytes) Are a Predominant Morphology

Acute hepatic necrosis (spur cell anemia)
Embden-Myerhof pathway (glycolytic) defects*
Microangiopathic hemolytic anemias*
Uremia*
McLeod blood group
In(Lu) blood group
Abetalipoproteinemia
Acanthocytosis with neurologic disease and normal lipoproteins (choreoacanthoses)
Postsplenectomy
Anorexia nervosa*
Vitamin E deficiency*
Infantile pyknocytosis
Heat stroke*
Transiently after massive transfusion of stored blood
Hypothyroidism*

*Disorder sometimes associated with spiculated red cells.

In addition, in some patients, acquired abnormalities of red cell membrane lipids may contribute to anemia. Two morphologic syndromes are recognized. In one, target cells predominate. In the other, a syndrome of brisk hemolysis develops in association with acanthocytes or "spur" cells, so-called *spur cell anemia* (see Fig. 16-40, *D*).[907,916-935] Typically, target cells are associated with obstructive liver disease, and acanthocytes are associated with hepatocellular disease. In practice, the situation is more complex. It is not uncommon for both cell morphologic findings to coexist, and some experimental data suggest that they are different stages of the same process. For example, when the bile duct is ligated in a rat, acanthocytes appear within 8 hours, but they convert to target cells if the obstruction persists for 7 days or more.[936] Nevertheless, acanthocytes (spur cells) and target cells differ in many important respects, and will be treated here as if they result from distinct pathophysiologic processes.

Spur Cell Anemia

Spur cell anemia has mostly been described in patients with alcoholic cirrhosis.[907,918-920,931,932] However, it is also reported in those with cardiac cirrhosis,[919] metastatic liver disease,[923] liver transplantation,[933] hemochromatosis,[922] neonatal hepatitis,[926] cholestasis,[916,917] Wilson disease,[937] and severe acute hepatitis.[937] The range of diseases in which spur cells are found suggests that it may occur in any disease in which damage to hepatocytes is severe. Not all patients with hepatocellular disease have spur cells or anemia, but their presence is a bad prognostic sign in adults.[934] Whether that is also true in children is unknown.

Typically, patients have moderately severe hemolysis (hematocrit of 20% to 30%), marked indirect hyperbilirubinemia, splenomegaly, and clinical and laboratory evidence of severe hepatic dysfunction. By definition, more than 20% acanthocytes are evident in multiple areas on several peripheral blood smears. Morphologically, the acanthocytes of spur cell anemia are indistinguishable from those seen in patients with abetalipoproteinemia. In

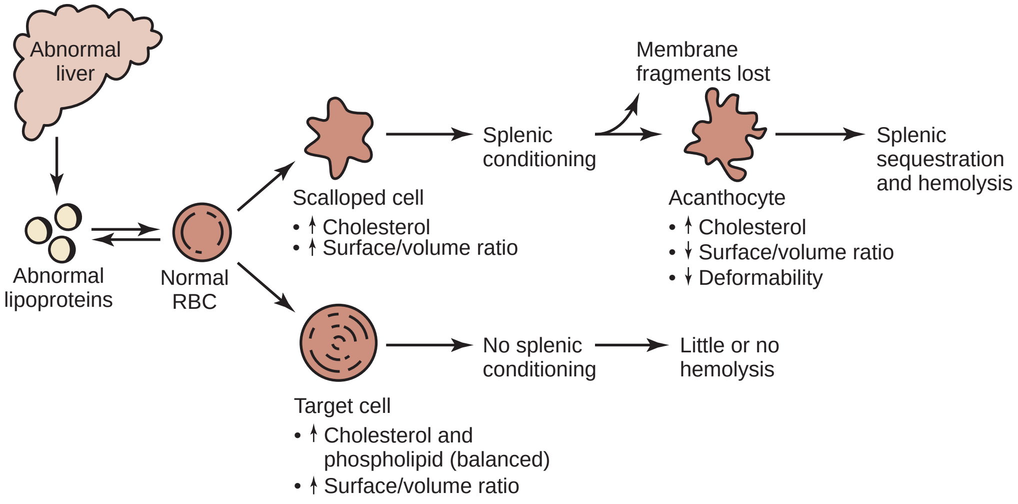

Figure 16-41 Schematic illustration of the pathophysiology of acanthocyte (spur cell) and target cell formation in liver disease.

some patients significant numbers of echinocytes and target cells may be present, and spherocytes may develop, presumably as a result of vesiculation. Occasionally, this may cause some diagnostic confusion. In our experience, however, many of these "spherocytes" have fine spicules, evident on close examination, a finding that distinguishes them from true microspherocytes.

The red cell life span of spur cells is markedly shortened because of splenic sequestration,[907,919,924,927] and, as expected, hemolysis abates after splenectomy.[927] Unfortunately, splenectomy is a dangerous and often fatal procedure in these very sick patients and is not generally recommended.[928] In addition, spur cell anemia has been reported in a splenectomized patient.[921] Some success has been reported in treating the anemia of these patients with phospholipid infusions[928]; flunarizine[929]; or a combination of flunarizine, pentoxifylline, and cholestyramine[938]; however, these approaches are still experimental. Cure of the anemia is obtained through liver transplantation,[939] but the disease recurs if the transplant fails.[940]

The clinical syndrome of spur cell anemia can be produced by at least two different pathogenic mechanisms. In one group, typically consisting of patients with alcoholic cirrhosis,[907,920,925] the disorder is due to an acquired abnormality of red cell lipids. The pathophysiology of spurring in these patients was defined by Cooper and his associates many years ago[907,927,930,931] and is illustrated in Figure 16-41. It occurs in two stages: cholesterol loading and splenic remodeling. In the first stage, abnormal, cholesterol-laden, apolipoprotein AII–deficient lipoproteins, produced by the patient's diseased liver, transfer their excess cholesterol to circulating erythrocytes and increase the membrane cholesterol concentration, the cholesterol-to-phospholipid ratio (Fig. 16-42), and the membrane surface area.[932] This is an acquired process and can be mimicked in vitro by incubation of normal red cells in spur cell plasma[907,927] or in artificial media containing cholesterol-phospholipid dispersions with a cholesterol-to-phospholipid ratio greater than 1. In scanning electron micrographs, these cholesterol-laden cells are flattened and often folded, with an undulating periphery.[931] They appear scalloped or crenated on dried smears.[907,927] In vivo, in the second stage, scalloped cells are converted into spur cells by a process of splenic conditioning (see Fig. 16-41). Over a period of 1 to 7 days, membrane lipids and surface area are lost; cellular rigidity increases, presumably because of the decline in surface-to-volume ratio; and the cell assumes a typical acanthocytic form.[907,927] Splenectomy prevents both the formation of spur cells and their premature destruction; however, as noted earlier, it is a high-risk procedure in someone with severe liver disease and is seldom indicated.

The cholesterol-laden, incipient spur cell is presumably detained and remodeled by the spleen because it is less deformable than normal. The molecular explanation for this change in deformability is unknown. The involvement of band 3 is suggested by the acanthocytic morphologic findings of rare individuals carrying the band 3 HT

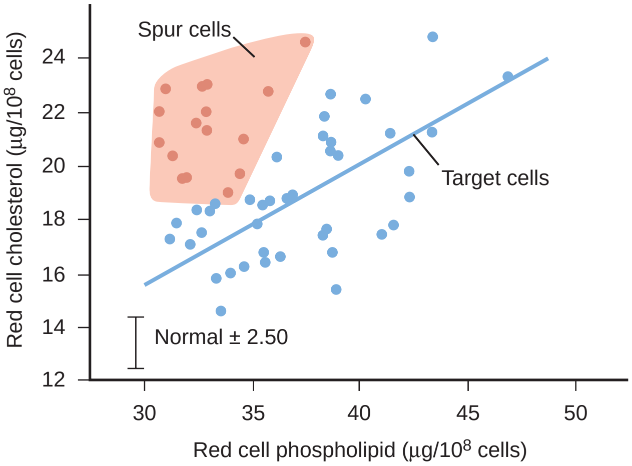

Figure 16-42 The cholesterol and phospholipid content of target cells *(blue circles)* and spur cells *(orange circles)*. Note that target cells tend to have a balanced increase in cholesterol and phospholipid, whereas spur cells are usually selectively enriched in cholesterol. *(Modified from Cooper RA, et al: An analysis of lipoproteins, bile acids, and red cell membranes associated with target cells and spur cells in patients with liver disease.* J Clin Invest *51: 3182–3192, 1972.)*

variant.[941] Wong believes that an increase in inward-facing band 3 molecules contracts the membrane skeleton and causes echinocytosis or acanthocytosis, whereas an increase in outward-facing band 3 molecules causes stomatocytosis,[942] but there is no direct evidence for this interesting hypothesis.

Other studies have identified a defect in phospholipid repair.[943] Spur cells and cholesterol-laden normal red cells have decreased fatty acid acylation of phospholipids. Furthermore, red cell membrane lipid “rafts” are cholesterol-rich microdomains that contain unique proteins.[944,945] Changes in the intracellular electrolytes cause shedding of these lipid “rafts.”[946] It is unclear whether increasing the membrane cholesterol would further increase this process. All of these processes may play a role in this anemia, but further studies are needed to better understand this process.

A second group of patients with liver disease and spur cell anemia differs in that red cell membrane lipids are normal and incubation of normal red cells in the patients’ plasma does not induce spurring.[919,923,924] The pathophysiology of this condition is unknown and largely unstudied. It has been reported in patients with alcoholic cirrhosis[919,924] and metastatic liver disease.[923] This is the form of the disease that the author has most often seen in children with spur cells and severe hepatocellular dysfunction.

Obstructive Liver Disease: Target Cells

Red cells increase their surface area by an increase in membrane lipids. In dried smears the excess surface accumulates and bulges outward in the red cell’s central clearing, producing the characteristic target cell morphologic pattern (Fig. 16-43). Target cells are also seen when red cell volume is diminished, owing to decreased hemoglobin synthesis (e.g., thalassemia or iron deficiency), abnormal hemoglobin charge or aggregation (e.g., hemoglobins S, C, D, and E), or decreased cell cations and water (Table 16-15). In these instances there is a relative increase in the surface-to-volume ratio. With the exception of dehydrated red cells, an increase in membrane surface, whether relative or absolute, has little effect on red cell deformability or life span and hence is usually innocuous.

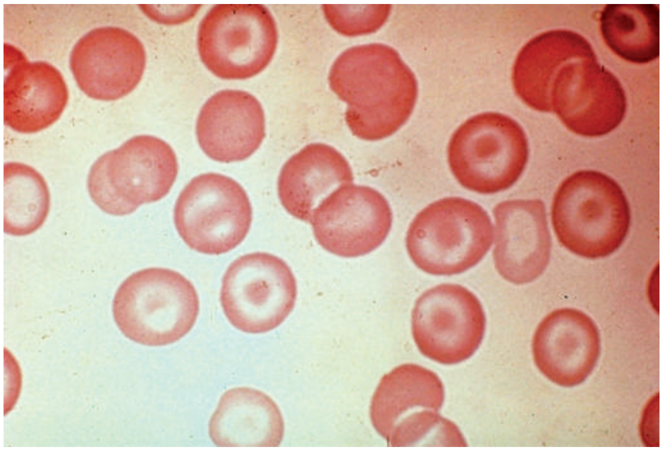

Figure 16-43 Target cells in obstructive liver disease. In contrast to the target cells in hypochromic-microcytic anemias, the target cells in liver disease are normal to large in size and well hemoglobinized.

TABLE 16-15 Conditions in Which Target Cells Are a Predominant Morphology

Hemoglobins S, C, D and E
Obstructive liver disease
Hereditary xerocytosis
Thalassemias
Iron deficiency
Sideroblastic anemias
Postsplenectomy
Lecithin:cholesterol acyltransferase deficiency

Target cells are particularly characteristic of biliary obstruction[875,947] but also occur in other forms of liver disease. Like spur cells, they form when red cells accumulate excess lipids from abnormal lipoproteins (see Fig. 16-41). However, unlike spur cells, target cells are characterized by a balanced increase in both free cholesterol and phospholipids (see Fig. 16-42).[948,949] The phospholipid increase is confined to phosphatidylcholine.

The pathogenesis of lipid accumulation in target cells is relatively clear. The process is extracorpuscular and reversible and is due to an abnormal serum component. Normal cells acquire target cell surface area, morphologic characteristics, and osmotic resistance when transfused into patients with obstructive jaundice or incubated in their serum, whereas target cells from such patients lose their excess lipids and revert to biconcave discs in normal persons or their sera.[947] Cooper and coworkers[950] found a close relationship between the cholesterol-to-phospholipid ratios of target cells and serum lipoproteins, particularly low-density lipoproteins. It is known that in obstructive jaundice a unique, abnormal lipoprotein called Lp-X accumulates in the low-density lipoprotein class,[951,952] possibly caused, at least in part, by an acquired deficiency of the hepatic enzyme LCAT. Lp-X contains approximately equal molar amounts of free cholesterol and phosphatidylcholine, plus small amounts of protein, cholesterol esters, triglycerides, and lithocholic acid.[952] The ratio of free cholesterol to phospholipid in Lp-X approximates the ratio in normal red cells, which may be why the increase in lipid is not pathologic. Normal red cells rapidly acquire excess cholesterol, phosphatidylcholine, and surface area when incubated with Lp-X in vitro,[949] and it seems likely that this may be the source of lipids for membrane expansion and targeting in vivo.

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Classic ultrastructural study of the spleen showing the narrow fenestrations between the endothelial cells of the venous sinus walls that represent the ultimate test of cellular deformability for a red cell attempting to return to the venous circulation.

189. Griggs RC, Weisman R, Jr, Harris JW: Alterations in osmotic and mechanical fragility related to in vivo erythrocyte aging and splenic sequestration in hereditary spherocytosis. *J Clin Invest* 39:89–101, 1960.

Classic study following a cohort of HS red cells marked in vivo with 59Fe. New red cells become osmotically fragile in about 10 days. Also shows that if HS red cells are recovered from the spleen at splenectomy and reinfused, the most fragile cells disappear rapidly but the others circulate normally.

214. Ballin A, Waisbourd-Zinman O, Saab H, et al: Steroid therapy may be effective in augmenting hemoglobin levels during hemolytic crises in children with hereditary spherocytosis. *Pediatr Blood Cancer* 57:303–305, 2011.

Follows up on the classic study by Finch (reference 212) in showing that corticosteroids reduce hemolysis in HS and are an effective short-term therapy in unsplenectomized patients with hemolytic crises.

222. De Franceschi L, Rivera A, Fleming MD, et al: Evidence for a protective role of the Gardos channel against hemolysis in murine spherocytosis. *Blood* 106:1454–1459, 2005.

HS red cells have a high MCHC and are dehydrated. There is some controversy as to whether this is a good or bad thing. This study shows that increasing red cell volume in a mouse model of HS by blocking the Gárdos channel worsens hemolysis, indicating that dehydration benefits HS red cells and suggesting that cation transporters might be important genetic modifiers of HS.

227. Zarychanski R, Schulz VP, Houston BL, et al: Mutations in the mechanotransduction protein PIEZO1 are associated with hereditary xerocytosis. *Blood* 120:1908–1915, 2012.

Important discovery that defects in the deformation-induced Piezo1 cation channel are responsible for the K+ loss and cell dehydration in HX.

228. Andolfo I, Alper SL, De Franceschi L, et al: Multiple clinical forms of dehydrated hereditary stomatocytosis arise from mutations in PIEZO1. *Blood* 121:3925–3935, 2013.

Codiscovery of the molecular defects in Piezo1 in HX.

247. Bianchi P, Fermo E, Vercellati C, et al: Diagnostic power of laboratory tests for hereditary spherocytosis: a comparison study in 150 patients grouped according to molecular and clinical characteristics. *Haematologica* 97:516–523, 2012.

Comparison of the diagnostic effectiveness of various laboratory tests for HS, alone and in combinations.

263. King MJ, Behrens J, Rogers C, et al: Rapid flow cytometric test for the diagnosis of membrane cytoskeleton-associated haemolytic anaemia. *Br J Haematol* 111:924–933, 2000.

Original description of the eosin-5-maleimide (EMA)–binding test for HS. EMA binds primarily to a single lysine residue on band 3 and is lost when membrane microvesicles containing band 3 are shed from all HS red cells. Probably the best test for diagnosing HS at the present time.

267a. Bolton-Maggs PH, Langer JC, Iolascon A, et al: General Haematology Task Force of the British Committee for Standards in Haematology. Guidelines for the diagnosis and management of hereditary spherocytosis–2011 update. *Br J Haematol* 156:37–49, 2012.

Followup to reference 14, with updated recommendations.

277. Stewart GW, Amess JAL, Eber SW, et al: Thrombo-embolic disease after splenectomy for hereditary stomatocytosis. *Br J Haematol* 93:303–310, 1996.

Important study showing a high rate of serious thromboembolic complications in patients with "stomatocytosis" following splenectomy. A flawed study that confusingly lumps OHSt and HX and is anecdotal and lacks controls, but is persuasive and has led to the recommendation that splenectomy be avoided in these diseases.

294. Miraglia del Giudice E, Perrotta S, Nobili B, et al: Coinheritance of Gilbert syndrome increases the risk for developing gallstones in patients with hereditary spherocytosis. *Blood* 94:2259–2262, 1999.

Important study showing that even heterozygosity for the Gilbert polymorphism more than doubles the risk of gallstones in patients with HS.

331. Valeur-Jensen AK, Pedersen CB, Westergaard P, et al: Risk factors for parvovirus B19 infection in pregnancy. *JAMA* 281:1099–1105, 1999.

About 65% of pregnant women have evidence of a past parvovirus infection. The risk of infection is high for susceptible pregnant women exposed to an infected patient. Illustrates the importance of respiratory and contact precautions for HS patients during an aplastic crisis.

363. Hsiao M, Sathya C, Nathens AB, et al: Is activity restriction appropriate for patients with hereditary spherocytosis? A population-based analysis. *Ann Hematol* 92:523–525, 2013.

Important study, which shows the risk of blunt splenic injury due to trauma is very low in unsplenectomized patients with HS (<1 in 20,000 per person per year) and is no greater than the risk in the general population.

366. Schwartz PE, Sterioff S, Mucha P, et al: Postsplenectomy sepsis and mortality in adults. *JAMA* 248:2279–2283, 1982.

One of the best studies of the risk of overwhelming sepsis following splenectomy. Limited in that it largely precedes routine pneumococcal immunization and focuses on adults. Nevertheless, more studies like this one are needed.

378. Eraklis AJ, Kevy SV, Diamond LK, et al: Hazard of overwhelming infection after splenectomy in childhood. *N Engl J Med* 276:1225–1229, 1967.

Classic study of the risk of overwhelming sepsis following splenectomy, especially in young children. Not the first to identify the problem but probably the most influential early report.

382. Hansen K, Singer DB: Asplenic-hyposplenic overwhelming sepsis: postsplenectomy sepsis revisited. *Pediatr Dev Pathol* 4:105–121, 2001.

Follow-up to famous and influential 1973 review (reference 379).

407. Schilling RF, Gangnon RE, Traver MI: Delayed adverse vascular events after splenectomy in hereditary spherocytosis. *J Thromb Haemost* 6:1289–1295, 2008.

The definitive study to date of late adverse arterial and venous events in splenectomized patients with HS.

409. Robinette CD, Fraumeni JF, Jr: Splenectomy and subsequent mortality in veterans of the 1939–45 war. *Lancet* 2:127–129, 1977.

Classic case-controlled study of the consequences of wartime traumatic splenectomies 30 years later. Shows a near doubling of the risk of ischemic heart disease.

448. Bader-Meunier B, Gauthier F, Archambaud F, et al: Long-term evaluation of the beneficial effect of subtotal splenectomy for management of hereditary spherocytosis. *Blood* 97:399–403, 2001.

The best long-term (10- to 15-year) follow-up of HS patients treated by partial splenectomy. Patients had sustained improvement of their hemolysis and anemia and apparent stabilization of spleen size after an initial period of splenic regrowth. However, some patients with severe HS required reoperation and complete splenectomy.

558. Coetzer T, Lawler J, Prchal JT, et al: Molecular determinants of clinical expression of hereditary elliptocytosis and pyropoikilocytosis. *Blood* 70:766–772, 1987.

Severity of hemolysis in HE and HPP directly correlates with the percentage of spectrin dimer (i.e., the degree of functional impairment of spectrin self-association) and with the decrease in spectrin content.

572. Mentzer WC, Jr, Iarocci TA, Mohandas N, et al: Modulation of erythrocyte membrane mechanical stability by 2,3-diphosphoglycerate in the neonatal poikilocytosis/elliptocytosis syndrome. *J Clin Invest* 79:943–949, 1987.

Interesting hypothesis that the unusual severity of HE in some neonates results from fetal hemoglobin, not binding 2,3-diphosphoglycerate (DPG), because free DPG destabilizes the membrane skeleton.

587. Alloisio N, Morlé L, Marechal J, et al: Sp $\alpha^{V/41}$: a common spectrin polymorphism at the αIV-αV domain junction. Relevance to the expression level of hereditary elliptocytosis due to α-spectrin variants located in trans. *J Clin Invest* 87:2169–2177, 1991.

Discovery that a common polymorphism in α-spectrin (later named spectrin αLELY) is associated with decreased expression of the affected allele. If the opposite allele carries an α-spectrin mutation, such as HE, the effect is to increase the severity of the disease.

588. Coetzer T, Palek J, Lawler J, et al: Structural and functional heterogeneity of α spectrin mutations involving the spectrin heterodimer self-association site: relationships to hematologic expression of homozygous hereditary elliptocytosis and hereditary pyropoikilocytosis. *Blood* 75:2235–2244, 1990.

Shows that different α-spectrin mutations differ widely in their severity, with those located in the self-association site more severe than those located more distally.

600. Zarkowsky HS, Mohandas N, Speaker CB, et al: A congenital haemolytic anaemia with thermal sensitivity of the erythrocyte membrane. *Br J Haematol* 29:537–543, 1975.

Discovery and first report of HPP.

605. Tchernia G, Mohandas N, Shohet SB: Deficiency of cytoskeletal membrane protein band 4.1 in homozygous hereditary elliptocytosis: implications for erythrocyte membrane stability. *J Clin Invest* 68:454–460, 1981.

The first report of a defect in protein 4.1 in HE. Homozygotes had nearly complete deficiency and severe fragmentation and hemolysis. Heterozygotes had 50% deficiency and typical HE. Proof of the importance of protein 4.1 in membrane stability.

638. Liu SC, Palek J, Prchal J: Defective spectrin dimer-dimer association in hereditary elliptocytosis. *Proc Natl Acad Sci U S A* 79:2072–2076, 1982.

Classic study showing, for the first time, a defect in spectrin self-association in some patients with HE. HE membranes from these patients contain increased specrin dimer, and membrane skeletons of HE patients or of normals manipulated to convert spectrin tetramer to dimer are highly mechanically fragile.

649. Wilmotte R, Maréchal J, Morlé L, et al: Low expression allele α^{LELY} of red cell spectrin is associated with mutations in exon 40 (α V/41 polymorphism) and intron 45 and with partial skipping of exon 46. *J Clin Invest* 91:2091–2096, 1993.

Showed that spectrin αLELY is a very common polymorphism that causes exon skipping, interfering with spectrin heterodimer assembly and protein expression.

710. Jarolim P, Palek J, Amato D, et al: Deletion in the band 3 gene in malaria resistant Southeast Asian ovalocytosis. *Proc Natl Acad Sci U S A* 88:11022–11026, 1991.

Discovery that all patients with SAO have a deletion of nine amino acids at the boundary between the cytoplasmic and membrane domain of band 3.

722. Saul A, Lamont G, Sawyer WH, et al: Decreased membrane deformability in Melanesian ovalocytes from Papua New Guinea. *J Cell Biol* 98:1348–1354, 1984.

Discovery that Southeast Asian ovalocytes are very rigid, which may explain, at least in part, their resistance to invasion by malarial parasites.

723. Guizouarn H, Borgese F, Gabillat N, et al: South-east Asian ovalocytosis and the cryohydrocytosis form of hereditary stomatocytosis show virtually indistinguishable cation permeability defects. *Br J Haematol* 152:655–664, 2011.

SAO is a form of cryohydrocytosis.

750. Stewart GW: Hemolytic disease due to membrane ion channel disorders. *Curr Opin Hematol* 11:244–250, 2004.

Good review of the stomatocytosis-xerocytosis syndromes by one of the leaders in the field.

751. Gallagher PG: Disorders of red cell volume regulation. *Curr Opin Hematol* 20:201–207, 2013.

Good recent review of stomatocytosis-xerocytosis syndromes by one of the leaders in the field.

752. Zarkowsky HS, Oski FA, Sha'afi R: Congenital hemolytic anemia with high-sodium, low-potassium red cells: I. Studies of membrane permeability. *N Engl J Med* 278:573–581, 1968.

Early description and first detailed physiologic study of OHSt.

780. Montel-Hagen A, Kinet S, Manel N, et al: Erythrocyte Glut1 triggers dehydroascorbic acid uptake in mammals unable to synthesize vitamin C. *Cell* 132:1039–1048, 2008.

Remarkable discovery that interaction of stomatin with the Glut1 glucose transporter converts it into a dehydroascorbic acid transporter in mammals, such as humans, that lack the ability to synthesize ascorbic acid.

803. Glader BE, Fortier N, Albala MM, et al: Congenital hemolytic anemia associated with dehydrated erythrocytes and increased potassium loss. *N Engl J Med* 291:491–496, 1974.

Initial description and investigation of what was initially called hereditary dessicytosis, which we now call hereditary xerocytosis (HX) (or dehydrated hereditary stomatocytosis).

805. Grootenboer S, Schischmanoff PO, Laurendeau I, et al: Pleiotropic syndrome of dehydrated hereditary stomatocytosis, pseudohyperkalemia, and perinatal edema maps to 16q23-q24. *Blood* 96:2599–2605, 2000.

Description of variable features of HX in fetuses and neonates: hydrops, perinatal edema, and pseudohyperkalemia.

815. Platt OS, Lux SE, Nathan DG: Exercise-induced hemolysis in xerocytosis: erythrocyte dehydration and shear sensitivity. *J Clin Invest* 68:631–638, 1981.

A particularly well studied patient with HX, showing that dehydrated red cells are shear sensitive and that dehydration-induced shear sensitivity is not fully reversible if prolonged.

853. Miller G, Townes PL, MacWhinney JB: A new congenital hemolytic anemia with deformed erythrocytes (”stomatocytes”) and remarkable susceptibility of erythrocytes to cold hemolysis in vitro. I. Clinical and hematologic studies. *Pediatrics* 35:906–915, 1965.

Original description of hereditary cryohydrocytosis.

858. Bruce LJ, Robinson HC, Guizouarn H, et al: Monovalent cation leaks in human red cells caused by single amino-acid substitutions in the transport domain of the band 3 chloride-bicarbonate exchanger, AE1. *Nat Genet* 37:1258–1263, 2005.

Important discovery that patients with cryohydrocytosis, some milder forms of hereditary stomatocytosis, and a form of HS with a low-temperature cation leak all have missense mutations in band 3 that pervert the transport properties of the anion channel.

866. Flatt JF, Guizouarn H, Burton NM, et al: Stomatin-deficient cryohydrocytosis results from mutations in SLC2A1: a novel form of GLUT1 deficiency syndrome. *Blood* 118:5267–5277, 2011.

Discovery that patients with cryohydrocytosis associated with stomatin deficiency and mental retardation and movement disorders have unique defects in Glut1 that both impair glucose transport and convert Glut1 into a cation channel.

907. Cooper RA: Anemia with spur cells: a red cell defect acquired in serum and modified in the circulation. *J Clin Invest* 48:1820–1831, 1969.

Classic initial description and characterization of spur cell anemia. Little has been learned since that supersedes these beautiful early studies.

947. Cooper RA, Jandl JH: Bile salts and cholesterol in the pathogenesis of target cells in obstructive jaundice. *J Clin Invest* 47:809–822, 1968.

Classic study showing that target cells arise by acquiring lipids from the plasma, which increase surface area. Cross-transfusion experiments show that normal red cells acquire a target phenotype after transfusion in patients with obstructive jaundice, and vice versa.

950. Cooper RA, Diloy-Puray M, Lando P, et al: An analysis of lipoproteins, bile acids, and red cell membranes associated with target cells and spur cells in patients with liver disease. *J Clin Invest* 31:3182–3192, 1972.

Classic study distinguishing the lipid composition of target cells and spur cells in patients with liver disease.

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CHAPTER 17

Pyruvate Kinase Deficiency and Disorders of Glycolysis

William C. Mentzer, Jr.

CHAPTER OUTLINE

HEXOKINASE DEFICIENCY
Clinical Manifestations
Biochemistry
Genetics
Therapy
GLUCOSE PHOSPHATE ISOMERASE DEFICIENCY
Clinical Manifestations
Biochemistry
Genetics and Inheritance
Therapy
PHOSPHOFRUCTOKINASE DEFICIENCY
Clinical Manifestations
Biochemistry
Genetics and Inheritance
Therapy
ALDOLASE DEFICIENCY
Clinical Manifestations, Biochemistry, and Genetics
TRIOSE PHOSPHATE ISOMERASE DEFICIENCY
Clinical Manifestations
Biochemistry
Genetics and Inheritance
Therapy
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE DEFICIENCY
Clinical Manifestations, Biochemistry, and Genetics
PHOSPHOGLYCERATE KINASE DEFICIENCY
Clinical Manifestations
Biochemistry
Genetics and Inheritance
Therapy
2,3-BISPHOSPHOGLYCERATE MUTASE DEFICIENCY
Clinical Manifestations
Biochemistry
Genetics and Inheritance
Therapy
MONOPHOSPHOGLYCERATE MUTASE DEFICIENCY
Clinical Manifestations, Biochemistry, Genetics, and Therapy
ENOLASE DEFICIENCY
Clinical Manifestations, Biochemistry, Genetics, and Therapy
PYRUVATE KINASE DEFICIENCY
Clinical Manifestations
Biochemistry
Genetics and Inheritance
Therapy
LACTATE DEHYDROGENASE DEFICIENCY
Clinical Manifestations, Biochemistry, and Genetics
ABNORMALITIES OF ERYTHROCYTE NUCLEOTIDE METABOLISM
Pyrimidine 5′-Nucleotidase Deficiency
Adenylate Kinase Deficiency
Adenosine Deaminase Overproduction
ACQUIRED DISORDERS OF ERYTHROCYTE GLYCOLYSIS

The mature erythrocyte, devoid of nucleus, mitochondria, ribosomes, and other organelles, has no capacity for cell replication, protein synthesis, or oxidative phosphorylation. The glycolytic production of adenosine triphosphate (ATP), the sole known energy source of erythrocytes, is sufficient to meet their limited metabolic requirements. The discovery that hemolytic anemia may result from any of several glycolytic enzymopathies has underscored the dependence of erythrocytes on glycolysis. In this chapter the clinical, biochemical, and genetic features associated with abnormalities in erythrocyte glycolysis are described in detail.

Hereditary hemolytic anemias resulting from altered erythrocyte metabolism are distinguished from hereditary spherocytosis by the absence of spherocytes on the peripheral blood smear, by normal osmotic fragility of fresh erythrocytes, by a partial therapeutic response to splenectomy, and by a recessive mode of inheritance. Hemoglobin structure and synthesis are normal. Because no specific morphologic abnormality is associated with

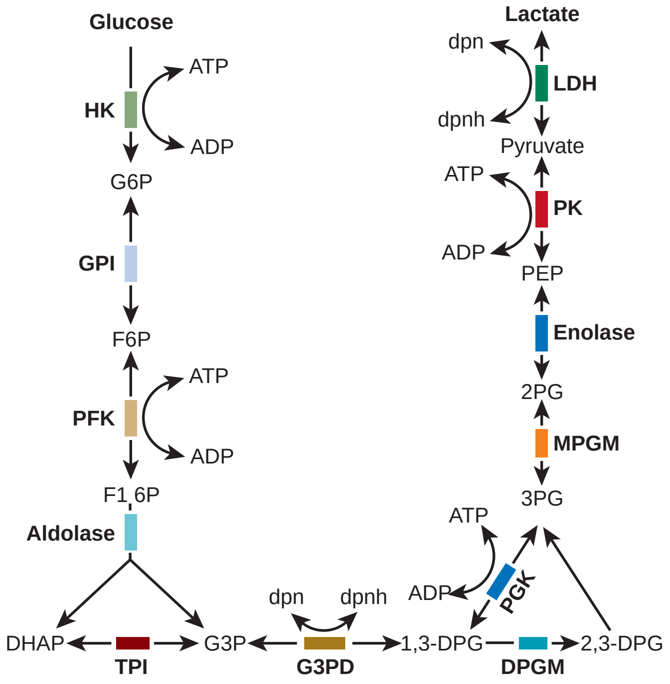

Figure 17-1 The Embden-Meyerhof pathway. Recognized enzyme defects are indicated by *solid bars*. *1,3-DPG*, 1.3-Diphosphoglycerate; *ADP*, adenosine diphosphate; *ATP*, adenosine triphosphate; *DHAP*, dihydroxyacetone phosphate; *DPGM*, 2,3-disphosphoglycerate mutase; *dpn*, nicotinamide adenine dinucleotide; *dpnh*, reduced nicotimamide adenine dinucleotide; *F1 6P*, fructose 1,6-phosphate; *F6P*, fructose 6-phosphate; *G3P*, glucose-3-phosphate; *G3PD*, glucose-3-phosphate dehydrogenase; *G6P*, glucose 6-phosphate; *GPI*, glucose phosphate isomerase; *HK*, hexokinase; *LDH*, lactate dehydrogenase; *MPGM*, monophosphoglycerate mutase; *PEP*, phosphoenolpyruvate; *PFK*, phosphofructokinase; *PG*, phosphoglycerate; *PK*, pyruvate kinase; *TPI*, triose phosphate isomerase.

these disorders, they have become known as congenital nonspherocytic hemolytic anemias (CNSHAs).[1] Although CNSHAs are usually transmitted in an autosomal recessive fashion, phosphoglycerate kinase (PGK) deficiency is an X-linked abnormality, and adenosine deaminase (ADA) overproduction is an autosomal dominant disorder. Symptoms and signs may be limited to the manifestations of hemolysis or, if the enzymopathy is present in other tissues, may involve other organ systems. The pattern of involvement of nonerythroid tissues may be of assistance in diagnosis.[2]

Initial attempts to classify these anemias were based on the autohemolysis test, in which saline-washed erythrocytes were incubated without glucose in vitro at 37° C under sterile conditions and the percentage of hemolysis was determined after 48 hours.[3,4] In 1961, Valentine and associates[5] identified a deficiency of erythrocyte pyruvate kinase (PK) in three patients with CNSHA. Subsequently abnormalities of other glycolytic enzymes have also been associated with CNSHA, as indicated in Figure 17-1. Specific alterations in protein structure underlie many of the enzyme deficiency states, and many of the underlying mutations have been identified.[6,7,8,9]

The presence of a glycolytic enzymopathy should be suspected when chronic hemolysis occurs in the absence of marked abnormalities in erythrocyte morphology or osmotic fragility. An exception to the usually unremarkable red cell morphology in CNSHA is the pronounced basophilic stippling found in pyrimidine-5′-nucleotidase (P-5′-N) deficiency.[10] Hemoglobin electrophoresis, stains for inclusion bodies, hemoglobin heat stability, acid hemolysis, and appropriate studies for immune hemolysis are normal. The autohemolysis test, previously used to screen for mutations in the glycolytic pathway, has at best limited value in the evaluation of CNSHA.[11] Unfortunately no other simple, convenient laboratory screening test has been developed that will unequivocally reveal the presence of a glycolytic enzymopathy. Therefore the appropriate diagnostic strategy for the evaluation of a suspected enzymopathy is first to eliminate easily identified causes of hemolysis, such as autoantibodies, hemoglobinopathies, or spherocytosis, before proceeding to tests for enzyme disorders.[12] Definitive diagnosis depends on quantitative assay of the activity of the suspected enzyme or identification of a specific mutation by deoxyribonucleic acid (DNA) analysis. The availability of such assays is limited, but screening tests for deficiencies in PK, triose phosphate isomerase (TPI), and glucose phosphate isomerase (GPI) can be carried out in any well-equipped clinical laboratory.[13] The in vitro properties of mutant enzyme proteins vary, and characterization of such properties has improved understanding of the genetics and pathogenesis of anemias associated with defective glycolytic enzymes (Table 17-1). Measurement of glycolytic intermediates extracted from freshly obtained erythrocytes, although potentially useful, has little clinical applicability other than the measurement of erythrocyte 2,3-bisphosphoglycerate (BPG). In contrast, measurement of intracellular metabolites is the most convenient way to screen for abnormalities in nucleotide metabolism. Red-cell ATP levels are below normal with overproduction of ADA, whereas P-5′-N deficiency is associated with increased concentrations of red-cell ATP and reduced levels of glutathione. The apparent increase in ATP is in fact the result of the presence of large amounts of cytidine

TABLE 17-1 Parameters Commonly Used In Vitro to Characterize Mutant Enzyme Protein

V_{max}	Maximal enzyme velocity obtainable with saturating substrate concentrations
K_m	The substrate concentration yielding half-maximal activity; an index of catalytic efficiency
pH optimum	The pH at which maximal enzyme activity is present
Heat stability	Resistance of enzyme protein to heat denaturation
Electrophoretic mobility	Migration of enzyme protein in an electric field
Specific activity	Enzyme activity per defined amount of enzyme protein (e.g., milligram); enzyme protein is measured immunologically with antienzyme antibodies

and uridine nucleotides, which are also measured in the enzymatic assay for ATP. Spectral analysis of a deproteinized extract of red cells provides a straightforward means of identifying such nucleotides, and it is a simple method to screen patients for suspected P-5′-N deficiency.[10]

Caution is needed in interpreting the results of quantitative assays of enzyme activity. First, only surviving cells are available for sampling in circulating blood, and the metabolic circumstances of these favored cells cannot necessarily be extrapolated to indicate the status of cells already hemolyzed. Second, assay in vitro under optimal conditions may not adequately reflect the performance of an enzyme under less favorable circumstances in vivo. Third, the high specific activity of certain enzymes in leukocytes may result in spurious normal values for erythrocyte enzyme activity unless contaminating leukocytes are removed before assay or their contribution to total activity is compensated for by appropriate calculations. Fourth, transfusion therapy with normal erythrocytes within several months before assay may obscure the presence of an enzyme defect. Fifth, the mean enzyme activity that is determined fails to portray the distribution of activity within individual erythrocytes. The endowment of intracellular enzymes is fixed, and protein synthetic ability disappears at the reticulocyte stage; thereafter the inevitable denaturation of enzyme protein that accompanies cell aging reduces enzymatic activity at a rate characteristic for each enzyme. Therefore transient accentuation of reticulocytosis is often accompanied by rising mean enzyme activity. Certain glycolytic enzymes (notably hexokinase [HK] and PK) are strikingly more active in reticulocytes than in postreticulocyte red cells, and the majority of this excess activity is rapidly lost coincident with reticulocyte maturation.[14-16] The true magnitude of an enzyme deficiency may not be apparent unless comparison is made to blood that is equally rich in reticulocytes[15] or corrections are applied that eliminate the contribution of the reticulocyte subfraction to total enzyme activity.[16]

Finally there is evidence that reversible binding of glyceraldehyde-3-phosphate dehydrogenase (G3PD), HK, and phosphofructokinase (PFK) to the band 3 membrane protein is involved in the regulation of glycolysis, and altered binding of mutant forms of these enzymes (and perhaps others) is not assessed in conventional assays performed on hemolysates.[17,18]

HEXOKINASE DEFICIENCY

Clinical Manifestations

CNSHA has been attributed to deficient erythrocyte HK activity in 24 patients (Table 17-2). Severely affected individuals may exhibit neonatal hyperbilirubinemia and thereafter require transfusion at regular intervals for intractable anemia. In patients with mild disease, the hemolysis is fully compensated for and anemia is absent. However, jaundice, reticulocytosis, and splenomegaly are usually present in such patients. Gallstones may be evident, even in early childhood.[27] Hyperhemolytic episodes are not a feature of the disorder. The results of red cell morphologic examination are usually unremarkable, but occasional burr cells, target cells, stippled cells, and densely stained spiculated cells may be observed after splenectomy. The osmotic fragility of fresh erythrocytes is normal, but a fragile population of cells may appear after incubation at 37° C.

Biochemistry

In human red cells, HK is a monomer (relative molecular mass: 112,000).[37] It is encoded by a gene (*HK1*) located on chromosome 10q22.[22] HK activity declines as red cells age. Loss of activity is particularly striking during reticulocyte maturation. In human reticulocytes, two major isoenzymes of HK have been identified by chromatographic techniques.[38] One (HKR) has an apparent half-life in vivo of only 10 days, whereas the other (HK1) has a longer half-life of 66 days. These two proteins are the products of two closely similar messenger ribonucleic acids (mRNAs) that are transcribed from a single gene (*HK1*) by the use of alternate promoters. Exon 1 is unique to each mRNA species, but the remaining 17 exons are identical.[39] Differential loss of these two isoenzymes appears to explain the biphasic character of the decay in HK activity during erythrocyte aging. An ATP- and ubiquitin-dependent proteolytic system capable of degrading about 80% of HK activity may explain the rapid loss of HK in rabbit reticulocytes,[40] or the loss may be secondary to an intrinsic property of the HK molecule itself.[41] Previous oxidative injury appears to be necessary for recognition and destruction of HK by the ubiquitin-dependent system.[42] Because ATP- and ubiquitin-dependent proteolysis is limited to reticulocytes,[40] it cannot be responsible for the loss of HK in aging human red cells.

HK is the glycolytic enzyme with the lowest activity in normal red cells, and a variety of observations indicate that it plays a rate-limiting role in erythrocyte glycolysis.[43-47] The maximal activity of erythrocyte HK from deficient patients has varied from 13% to 91% of normal (see Table 17-2). In evaluating these findings, comparisons of enzyme activity between red cell populations of equivalent age must be made. In the case described by Valentine and associates[46] (Fig. 17-2), although HK activity was 62% of the normal value for mature erythrocytes, it was only 14% of the activity found in blood with high reticulocyte counts. Separation of young and old red cell populations by centrifugation revealed only the expected moderate diminution (to 0.11 mol/min/10^{10} red blood cells) of HK activity in older cells from this patient.[47] HK activity was even lower (0.075 mol/min/10^{10} red blood cells) in an asymptomatic brother, yet no evidence of undue hemolysis was present. However, Figure 17-2 shows that the brother's cells are actually far less deficient with respect to cell age than the immature cells of the propositus. The impact of HK deficiency is clearly greater in energetic young erythrocytes, whereas cells that survive to an older age can meet their limited metabolic requirements even at very low HK levels.[48] As the erythrocyte ages, in vivo changes in stability or kinetics peculiar to mutant HK may also render older cells liable to undergo premature hemolysis. In rats and rabbits, HK from immature erythrocytes has a higher Michaelis constant (K_m) for

TABLE 17-2 Hexokinase Variants Associated with Hemolytic Anemia

	CLINICAL FEATURES			PROPERTIES OF RBC HEXOKINASE			
Reference	**Inheritance**	**Anemia**	**Other**	**Activity**	**Kinetic Abnormalities**	**Stability**	**Mobility**
19	—	+	Congenital malformations	13-24*	0	—	—
20	Recessive	++		15-20*	+	Normal	Abnormal
21	Recessive	++		16*	0	—	Abnormal
22	Recessive	+++	Hydrops fetalis	17			
23	Recessive	+		20*	0	Normal	Normal
24, 25	Recessive	++	Low platelet and fibroblast HK activity	20*	0	Low	Normal
26	Recessive	++	Low platelet HK activity	25*	+	Normal	Abnormal
27	Recessive	+		25*	0	Low	Normal
28	Dominant	+	Spherocytes, ovalocytes	30*	0	Low	Normal
29	Recessive	+	Psychomotor retardation	45†	+	Normal	Normal
30	Recessive	+		50*	0	Normal	Normal
31	—	+	Congenital malformations	33*	+	—	—
32	Recessive	+		40-53*	+	Low	Normal
33	—	+		50*	+	—	—
34				53			
35	Dominant	+		45-91†	+	Normal	Abnormal
36	Dominant	++	WBC HK activity low	75*	+	Normal	Abnormal
37	Recessive	±		77†			

HK, Hexokinase; *RBC*, red blood cell; *WBC*, white blood cell.
*Maximal enzyme activity (V_{max}) compared with reticulocytosis controls.
†Maximal enzyme activity (V_{max}) compared with normal red cells.

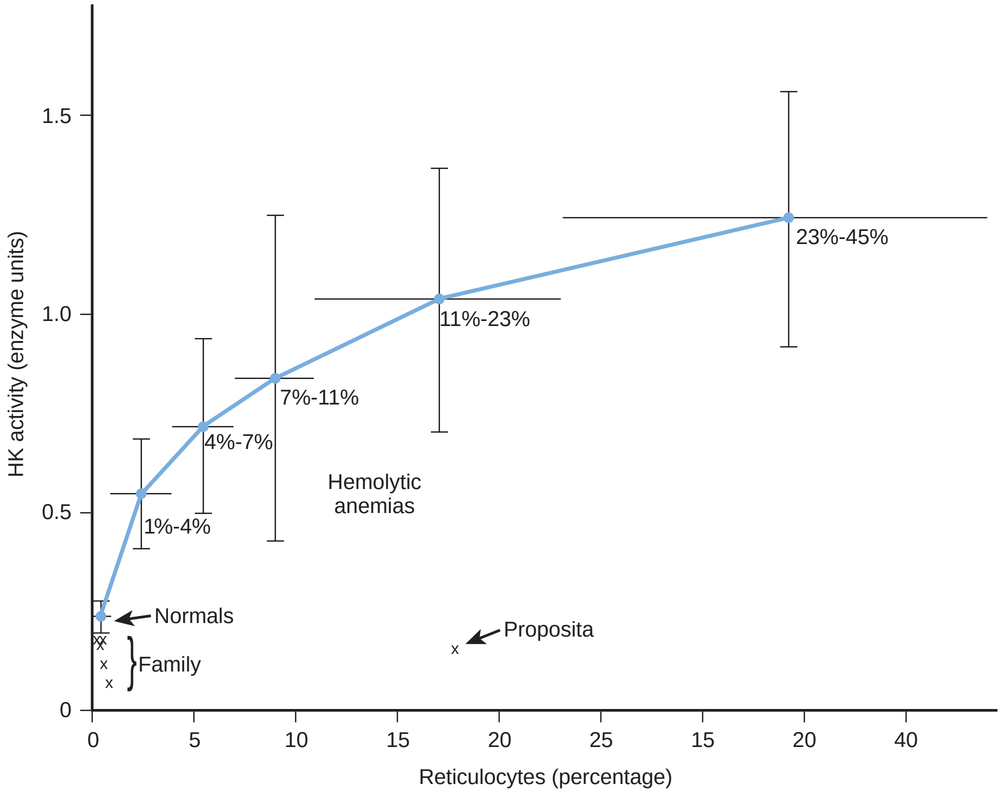

Figure 17-2 Hexokinase (HK) activity observed in 54 patients with hemolytic anemia of various causes plotted against percentages of reticulocyte in cells assayed. Patients are grouped according to reticulocyte levels. Mean HK activity for each group is plotted against the mean reticulocyte percentage in cells of that group. Standard deviations are indicated by *vertical bars*. Values for a single HK-deficient patient *(proposita)* and her family are designated separately. *(From Valentine WN, Oski FA, Paglia DE, et al: Erythrocyte hexokinase and hereditary hemolytic anemia. In Beutler E, editor: Hereditary disorders of erythrocyte metabolism, New York, 1968, Grune & Stratton, p. 288–300.)*

glucose than mature cells do, but such is not the case in normal human erythrocytes.[48]

In keeping with their enzymatic defect, HK-deficient erythrocytes have usually demonstrated subnormal glucose consumption and lactate production in vivo. Such cells also metabolize fructose poorly but utilize mannose or galactose normally,[47] because these substrates are not metabolized by HK. Some HK-deficient erythrocytes are capable of normal glucose consumption at the glucose concentrations (5 mmol/L) customarily found in plasma but utilize glucose poorly or not at all at lower glucose concentrations, either because of an abnormally low affinity for glucose[36] or because of enzyme instability under conditions of low substrate availability.[32] Such erythrocytes may encounter a particularly unfavorable metabolic environment within the spleen. The concentration of glucose in normal splenic homogenates has been found by Necheles and coworkers[36] to be only 5 to 11 mmol/g of tissue, and in a patient with HK deficiency the concentration was even lower (1.1 mmol/g of tissue). Furthermore, because splenic tissue metabolizes glucose rapidly,[49] prolonged vascular pooling in the spleen is probably accompanied by profound local hypoglycemia. Erythrocytes with unfavorable kinetics for glucose metabolism are clearly at a disadvantage in competing with reticuloendothelial cells for such a reduced glucose supply. Another disadvantage of the splenic environment is its relative acidity. The optimal pH of erythrocyte HK is approximately 8; at lower pH values, diminished enzyme activity may be expected. Possibly of greater importance, at low pH values, glucose 6-phosphate, a potent inhibitor of HK, accumulates because of PFK inhibition.[50] An erythrocyte that has diminished HK activity under optimal pH conditions will be further compromised in the acidic environment of the spleen. The clinical improvement that follows splenectomy attests to the importance of this organ in the pathogenesis of hemolysis. No splenic sequestration of chromated autologous erythrocytes was noted in two patients.[27,33] Thus in this disorder the red cells may be damaged by the spleen and die elsewhere.

Significant alterations in intracellular metabolites are associated with defective HK function. The erythrocyte ATP concentration is sometimes,[33,37] but not always[23,26,31,35] subnormal. The glucose-6-phosphate concentration is reduced to approximately half normal, and the concentrations of other more distal intermediates, most notably 2,3-BPG, are also usually reduced. These metabolites may exert a significant regulatory influence on glycolysis. For example, Brewer has shown that concentrations of 2,3-BPG in the physiologic range inhibit HK,[51] so the low 2,3-BPG levels in HK-deficient red cells may facilitate the performance of available HK. The increased affinity of hemoglobin for oxygen expected to be associated with subnormal 2,3-BPG levels has been documented in one patient with anemia and HK deficiency by Delivoria-Papadopoulos and coworkers[52] and by Oski and associates.[53] This patient, whose hemoglobin–oxygen affinity (P50) was 19 mm Hg (normal, 27 ± 1.2), was capable of just minimal exercise despite only moderate anemia (hemoglobin, 9.8 g/dL). On exercise, her central venous partial pressure of oxygen (pO_2) promptly fell to minimal levels as oxygen consumption rose. Increased oxygen delivery was achieved primarily by an increase in cardiac output, because the unfavorable oxygen affinity curve precluded any substantial further desaturation of hemoglobin.[53] Thus the altered concentration of intracellular metabolites induced by HK deficiency may, as in this patient, accentuate symptoms associated with anemia.

Because of its reactive sulfhydryl group, HK is susceptible to oxidant inactivation in the absence of sufficient glutathione.[54] Both normal and low glutathione levels have been reported in HK deficiency, but Heinz bodies have not been observed. Resting hexose monophosphate (HMP) shunt activity, measured with glucose-1 (C^{14}), was quantitatively normal in one patient despite subnormal glucose consumption, but stimulation with methylene blue was suboptimal.[47] Failure of the shunt at low glucose concentrations has also been noted in a patient with HK mutancy with a high K_m for glucose.[34] Methylene blue–stimulated methemoglobin reduction was subnormal in the single instance that it was evaluated.[19] When HK-inactivating antibodies are incorporated into normal red cells by the process of hypotonic lysis and isotonic reannealing, enzyme-deficient cells exhibit a greatly impaired response to HMP shunt stimulation by methylene blue.[44] These studies, though not indicating a central role for defective shunt activity in the pathogenesis of hemolysis, do suggest that under unusual circumstances, HK-deficient cells might be compromised by a limited shunt. Such circumstances might arise on exposure to a potent oxidant in the low glucose environment of the spleen.

Genetics

Inheritance of HK deficiency is autosomal recessive. Biochemical identification of asymptomatic carriers is not always possible, because enzyme activity often falls within the low-normal range. In a few pedigrees (see Table 17-2), the heterozygous state appears to be severe enough to result in hemolytic anemia. One such heterozygote appeared to be doubly heterozygous for HK and glucose-6-phosphate dehydrogenase (G6PD) deficiency.[55]

The qualitative abnormalities characteristic of mutant HK (see Table 17-2) may reflect either a structural or a regulatory gene mutation. On electrophoresis, mutant HK lacks one or more of the normal bands of activity, but no bands migrating in an abnormal position have been observed. The various bands represent the presence of several isozymes of HK. Diminished synthesis of one or more isozymes with predominance of the remaining isozymes accounts for the electrophoretic differences observed (see Table 17-2). DNA analysis has defined two separate *HK* mutations in a compound heterozygote who exhibited nonspherocytic hemolytic anemia.[22,56,57] One was a missense mutation (*1667T→C*) and the other was a deletion of exon 5 (96 base pairs [bp]). Expression of each *HK* mutation in a bacterial system allowed recovery of sufficient purified enzyme to determine that the missense mutation completely abolished HK activity, whereas the deletion reduced activity to about 10% of normal.[57] In another individual,[26] a homozygous missense mutation in the active site of *HK1* (*C2039→G* in exon 15) was discovered.[58] In a Japanese family, a stillborn fetus with HK deficiency was found to be homozygous for a large deletion that removed exons 5 through 8 in *HK* mRNA and led to premature termination of translation.[22] A mutation in the erythroid-specific promoter of *HK-1* inherited along with a missense mutation in exon 3 that caused aberrant splicing of both HK-R and HK-1 led to HK deficiency and mild chronic hemolytic anemia.[59] The kinetic abnormalities noted in many other mutant forms of HK undoubtedly also reflect as yet undefined structural abnormalities in enzyme protein.[*]

Studies of the tissue distribution of HK deficiency have been performed in blood cells and cultured fibroblasts. Electrophoresis of leukocyte or platelet HK reveals an anodal isozyme (HK-3) distinct from those of the erythrocyte (HK-1 and HK-2), as well as a shared isozyme (HK-1).[60,61] Leukocyte HK activity has been normal in some patients,[32,47] but the qualitative abnormality in leukocyte HK described by Necheles and colleagues,[36] as well as the case of generalized HK deficiency in all blood cells reported by Rijksen and coworkers,[26] indicates that to some extent the enzyme is under common genetic control in different tissues. When platelet HK activity has been low, platelet function has been normal despite subtle defects in in vitro energy metabolism.[62] Cultured fibroblasts from two individuals with different HK mutants contained HK with properties and activity like those found in red cells,[25] thus suggesting that this source of fetal tissue could be used for prenatal diagnosis.

A murine model of HK deficiency, termed *Downeast anemia,* is caused by homozygous inheritance of a mutation that markedly decreases the expression of HK-1 in erythroid tissues, spleen, and kidney. The mutation is the result of insertion of a transposon into intron 4 of the *HK1* gene. Mice exhibit nonspherocytic hemolytic anemia, marked reticulocytosis, splenomegaly, and striking accumulation of iron in the liver and kidney. Echinocytes and red cells split into two poles of hemoglobin connected only by thin strands of membrane are features of erythrocyte morphology in affected homozygous mice that are of interest because they have not been reported in human HK deficiency.[63]

Therapy

Treatment consists of red cell transfusion as indicated, supplemental folic acid, and close observation for cholelithiasis. Splenectomy may alleviate but does not eliminate the anemia.[†]

GLUCOSE PHOSPHATE ISOMERASE DEFICIENCY

Clinical Manifestations

A deficiency of erythrocyte GPI has been reported in more than 60 patients with congenital hemolytic anemia.[64] Hemolytic anemia usually appears in infancy and often requires red cell transfusion therapy. Hyperbilirubinemia,[65] hydrops fetalis,[66,67] or death[68] may occur during the neonatal period. Several patients have experienced hyperhemolytic crises after infections or drug exposure.[67,69,70] In some of these individuals G6PD, as well as GPI, has been deficient.[69,71] Hemolytic anemia is usually the sole clinical manifestation of GPI deficiency. A subset of patients also exhibit neurologic dysfunction.[72-75] In one patient, neuromuscular abnormalities were correlated with a severe reduction in muscle and cerebrospinal fluid GPI activity.[73] Blockade of glycolysis at the GPI step may divert the flow of glucose metabolism in the direction of glycogen synthesis. Several patients with GPI deficiency have been reported to have increased hepatic glycogen stores,[76,77] and in one, muscular fatigue was severe enough to suggest a diagnosis of glycogen storage disease.[77] Animal models of GPI deficiency and chronic hemolytic anemia have been developed in the mouse. The clinical and biochemical features of the disease in mice closely resemble those found in humans.[78,79]

Red cell morphologic characteristics are generally similar to those seen in other types of CNSHA. In patients with severe anemia, dense, spiculated, or "whiskered" microspherocytes have been noted after splenectomy. In one instance, sufficient numbers of such cells were present before splenectomy to suggest the diagnosis of hereditary spherocytosis.[80] In another, the predominant morphologic abnormality was stomatocytosis.[81] The reticulocytosis may be profound. Mean corpuscular volume is elevated (97 to 139 fL). With incubation at 37° C, a variable fraction of erythrocytes may exhibit abnormally increased osmotic fragility, whereas fresh cells are usually normal. Survival of chromated autologous red cells is reduced, often[82-84] but not invariably,[68,82,85] with evidence of splenic sequestration.

Biochemistry

GPI is a dimer[86] composed of two identical subunits, each with a relative molecular mass of approximately 60,000.[84,86] Subunit synthesis is directed by a single genetic locus on chromosome 19.[87] The crystal structure of human GPI has been defined.[88] GPI is a multifunctional protein. The dimeric form exhibits glycolytic enzyme activity, whereas the monomeric form functions as a cytokine. Monomeric GPI is 100% homologous to neuroleukin, a neurotrophic growth factor secreted by lectin-stimulated T cells[64,89,90]; to autocrine motility factor, which stimulates cell motility and the growth of metastases[91]; and to a differentiation and maturation mediator for human myeloid leukemia cells in tissue culture.[92] Altered neuroleukin function may explain the neurologic disorder associated with several GPI mutations.[64,75] Alternatively, induced GPI mutations in Chinese hamster ovary cell lines have an adverse effect on glycerolipid biosynthesis, suggesting that deficient GPI activity affects important non-glycolytic pathways.[93]

The metabolic events that precede hemolysis of GPI-deficient erythrocytes are poorly understood. Erythrocyte glycolysis is impaired in vivo, as reflected by an increased ratio of substrate to product (i.e., glucose 6-phosphate to fructose 6-phosphate) in freshly obtained GPI-deficient cells.[71,80,94-99] Paradoxically, with only occasional exceptions,[80,100] such cells are fully capable of glycolysis in vitro.[71,85,101-104] In contrast to HK deficiency, in which 2,3-BPG levels are low, sufficient glycolysis generally occurs in GPI deficiency to maintain the 2,3-BPG concentration at or above the normal level.[91,94,96,100,104] Except for three patients with diminished ATP concentrations, two of whom also exhibited reduced in vitro glycolysis for cell age,[81,87,105] the erythrocyte ATP concentration has been normal.

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[*] References 19, 20, 24, 29, 30, 34.

[†] References 20, 24, 26, 32, 36, 47.

A profound defect in recycling of fructose-6-phosphate through the pentose phosphate pathway has been observed repeatedly in GPI-deficient red cells.[80,103,104] The increased formation of Heinz bodies[68,81] and glutathione instability[99,106] after exposure to acetylphenylhydrazine, an abnormal ascorbate cyanide test, and diminished concentrations of red cell glutathione[81,85,93,95,96] in fresh erythrocytes all suggest that diminished shunt activity in vivo may contribute to hemolysis.

With rare exceptions,[105] mutant forms of red cell GPI exhibit considerable thermal lability in vitro, thus making it likely that enzyme lability in vivo as red cells age will lead to premature metabolic collapse and hemolysis. Separation of red cells by centrifugation into young and old subpopulations has usually demonstrated accelerated decay of enzyme activity in the older cells.[85,107] Arnold and coworkers[107] simulated the in vivo process of aging by incubating red cells in vitro at 37° C for 8 days and changing the incubation medium often to ensure that glucose availability and pH remained constant. GPI activity declined by 66% in GPI-deficient red cells during incubation, with a level of only 6% of normal being reached, and lactate production, normal at the onset, was reduced to 11% of normal. In contrast, normal reticulocyte-rich blood lost only 6% of the original GPI activity after 8 days, and lactate production fell only 7%. If mannose rather than glucose was used, GPI-deficient and normal red cells made equivalent amounts of lactate, and there was little or no loss in lactate production after 8 days. The normal glycolytic rate noted with mannose, which is isomerized by mannose phosphate isomerase and thus bypasses the GPI reaction, clearly pinpoints defective GPI activity as the cause of glycolytic failure in GPI-deficient cells. ATP depletion with consequent erythrocyte rigidity and reticuloendothelial entrapment would be anticipated to follow failure of glycolysis. GPI-deficient red cells are indeed less deformable than normal cells, particularly when comparison is made to young, reticulocyte-rich populations of cells.[92,94,108,109] Goulding studied a 9-year-old patient with GPI deficiency and priapism and concluded that the priapism was the consequence of abnormal erythrocyte deformability.[110] Studies by Coetzer and Zail revealed aggregation of membrane spectrin in GPI-deficient erythrocytes.[111] The extent of the aggregation is a function of cell age.

Even reticulocytes may be severely GPI deficient.[76] Deficient reticulocytes with limited anaerobic glycolytic capability may also be incapable of effective oxidative phosphorylation in the acidic, hypoglycemic splenic environment (see Pyruvate Kinase Deficiency), thereby leading to metabolic failure and hemolysis. Large numbers of reticulocytes were found when specimens from the spleen of a patient with GPI deficiency were examined by transmission electron microscopy.[76] Furthermore, the reticulocyte count often increases after splenectomy.[68,76,83,85] Because hemoglobin levels also increase, this observation suggests survival of a population of reticulocytes that would otherwise be hemolyzed almost immediately after their release from bone marrow.

Genetics and Inheritance

Like most other glycolytic enzymopathies, GPI deficiency is inherited as an autosomal recessive trait. Heterozygotes are hematologically normal but exhibit reduced erythrocyte GPI activity (usually to about 50% of normal). They inherit one mutant and one normal GPI allele, which results in the synthesis of two unlike GPI subunits that may combine in one of three ways to form a normal homodimer, a mutant homodimer, or a heterodimer that contains both normal and mutant subunits. Electrophoresis of GPI from the erythrocytes of heterozygotes demonstrates one to three bands depending on the extent to which the charge or activity of the mutant subunit is altered. Posttranslational events, such as oxidation of enzyme protein, may alter the electrophoretic pattern and confuse its interpretation.[112,113]

GPI mutations associated with hemolytic anemia are listed in Table 17-3. Of the 31 known mutations, 2 are splice site, 3 are nonsense, and 31 are missense mutations involving a single amino acid substitution.[64,114-116] The mutations are found in 12 of the 18 exons and are generally unique to one or, at most, a few families. The rarity of GPI deficiency and the heterogeneity of the mutations identified indicate that no selective advantage is conferred to affected individuals. Half of the homozygotes or compound heterozygotes for missense mutations have mild to moderate hemolytic anemia, and half have severe anemia. In contrast, all compound heterozygotes for missense mutations and either splice site or nonsense mutations have severe hemolytic anemia. Hemolytic anemia occurs in individuals when GPI activity drops below about 40% of the normal mean activity. Substrate kinetics and the optimal pH of mutant enzymes have almost always been normal, but most mutant enzymes have exhibited varying degrees of thermal instability. Study of recombinant GPI enzyme protein generated from 16 different human mutations has clarified the relation between specific mutations, their location within the three-dimensional structure of GPI, and enzyme properties (catalytic activity, enzyme kinetics, and thermostability). Mutations at or near the active site have the most severe consequences, whereas areas more distant generally have lesser effects. Thermostability is affected by different mutations to varying degrees.[117] Short of direct detection of the mutation at the DNA level, the most useful means of classifying the numerous GPI variants reported has been on the basis of stability, electrophoretic mobility, or residual enzyme activity in red cells and leukocytes.[‡] Until DNA mutation analysis has been completed on these variants, it will not be clear how many are truly unique. Immunologic titration of functionally inactive enzyme protein in red cells indicates that some mutant GPI alleles are "silent" and

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[‡] References 67, 68, 71, 73, 81-85, 94-103, 105, 106, 118-121.

TABLE 17-3 Mutations Associated with Glucose Phosphate Isomerase Deficiency and Hemolytic Anemia

Variant	Ethnic Origin	Genotype	Exon	Amino Acid Change	Red Cell GPI Activity*
Homozygotes: Missense Mutations					
Matsumoto	Japanese	*14C→T*	1	Thr5Ile	27
Unnamed	American Indian	*247C→T*	3	Arg83Trp	16.4
Sarsina	Italian	*301G→A*	4	Val101Met	7.2[†]
Iwate	Japanese	*671C→T*	7	Thr224Met	10.2
Unnamed	Turkish	*970G→A*	12	Gly324Ser	49.7[†]
Morcone	Italian	*1028A→G*	12	Gln343Arg	7.2[†]
Narita	Japanese	*1028A→G*	12	Gln343Arg	32.4
Mount Scopus	Ashkenazi	*1039G→A*	12	Arg347Cys	14.8[†]
Unnamed	Spanish[‡]	*1040G→A*	12	Arg346Hist	18[†]
Unnamed	Hispanic	*1415G→A*	16	Arg472His	15.4
Unnamed	Turkish	*1415G→A*	16	Arg472His	24.9
Unnamed	Indian[‡]	*1459C→T*	16	Leu487Phe	8.6
Unnamed	? English	*1574T→C*	18	Ile525Thr	3[†]
Fukuoka	Japanese	*1615G→A*	18	Asp539Asn	6.4
Compound Heterozygotes: Nonsense/Missense Mutations					
Stuttgart	German	*43C→T/1028A→G*	1, 12	Gln15Stop/Gln343Arg	27.9[†]
Elyria	Caucasian	*223A→G/286C→T*	3, 4	Arg75Gly/Arg96Stop	4[†]
Bari	Italian	*286C→T/584C→T*	4, 6	Arg96Stop/Thr196Ile	14.9[†]
Unnamed	Russian	*286C→T/1039C→T*	4, 12	Arg96Stop/Arg347Cys	19
Zwickau	German	*1039C→T/1538G→A*	12, 17	Arg347Cys/Trp513Stop	21[†]
Catalonia	Spanish[‡]	*1648A→G*	18	Lys550Glu	<7[†]
Compound Heterozygotes: Splice Site/Missense Mutations					
Mola	Italian	*584C→T*/del1473-IVS16(+2)	6, 16	Thr195Ile/splice site	12.9[†]
Nordhom	German	*1028A→G/IVS15(–2)A→C*	12/IVS15	Gln343Arg/splice site	11.4[†]
Compound Heterozygotes: Missense/Missense Mutations					
Homburg	German	*59A→C/1016T→C*	1, 12	His20Pro/Leu339Pro	3.5[†]
Barcelona	Spanish[‡]	*341A→T/663T→G*	4, 7	Asp113Val/Asn220Lys	20
Unnamed	? English	*475G→A/1040G→A*	5, 12	Gly159Ser/Arg347His	6[†]
Unnamed	Black American	*671C→T/1483G→A*	7, 17	Thr224Met/Glu495Lys	12.2
Unnamed	Black American	*818G→A/1039C→T*	10, 12	Arg273His/Arg347Cys	15.9
Unnamed	Caucasion	*833C→T/1459C→T*	10, 16	Ser278Leu/Leu487Phe	9.8
Unnamed	Hispanic	*898G→C/1039C→T*	11, 12	Ala300Pro/Arg347Cys	25.4[†]
Kinki	Japanese	*1124C→G/1615G→A*	13, 18	Thr375Arg/Asp539Asn	3.7[†]
Calden	German	*1166A→G/1549→G*	13, 18	His389Arg/Leu517Val	12[†]

Modified from Kugler W, Lakomek M: Glucose-6-phosphate isomerase deficiency. Baillieres Clin Haematol *13:89–101, 2000.*

*International units per gram of hemoglobin.

[†]Severe disease (splenectomy, frequent red cell transfusions, and/or reticulocyte count >15%).

[‡]The Spanish patients are described by Repiso et al[114,115] and the Indian patient by Warang et al.[116]

produce no detectable enzyme protein, whereas others produce structurally altered protein with varying degrees of activity and stability in vivo.[118-120]

A single GPI isozyme is present in all human tissues.[87] GPI deficiency is usually less severe in nonerythroid tissues than in erythrocytes because nonerythrocytic tissues retain the ability to synthesize GPI subunits. Clinical abnormalities outside the hematopoietic system are rare and, if present, are neuromuscular in nature. Leukocytes are capable of normal phagocytosis and chemotaxis despite a reduction in GPI activity to 25%[94] to 73%[102] of normal, but if GPI activity is more severely depressed, granulocyte function is impaired.[73,121] Similarly, platelet GPI may be only 20% to 30% of normal; however, clot formation, platelet aggregation, and other clotting studies are normal.[94,102] Prenatal diagnosis is feasible by DNA analysis if the mutations are known or by measurement of GPI activity in amniotic fluid fibroblasts[66] or chorionic villus trophoblasts.[122]

Therapy

Transfusion requirements are usually eliminated by removal of the spleen, but the anemia persists.[68,76,83,102] The postsplenectomy hemoglobin levels of 6.7 to 10.3 g/dL and reticulocyte counts of 36% to 73% observed in three siblings by Paglia and coworkers[104] reflect the magnitude of the continued hemolysis that may be present. Attempts by Arnold and colleagues[102] to enhance glycolysis in a GPI-deficient patient by intravenous administration of methylene blue or inorganic phosphate (P_i) did not have a lasting benefit.

PHOSPHOFRUCTOKINASE DEFICIENCY

Clinical Manifestations

Inherited deficiency of PFK can involve erythrocytes, muscle, or both, depending on the PFK subunit affected and the nature of the biochemical defect (Table 17-4). Although low erythrocyte PFK activity and mild hemolytic anemia are commonly found in type VII glycogen storage disease (Tarui disease), the dominant clinical feature of this disorder is exertional myopathy caused by deficient muscle PFK activity.[127,128] Physical activity is limited not by anemia but by weakness, easy fatigability, and severe muscle cramps associated with the myopathy. The disease may be evident at birth and cause death during infancy as a result of respiratory insufficiency and other complications,[129] or it may be so mild that it is not manifested until old age.[130] However, in most affected individuals the disorder is first detected during adolescence or young adulthood. The diagnosis may be suspected if no lactate is produced during an ischemic (anaerobic) forearm exercise test, but confirmation requires muscle biopsy for determination of PFK activity or noninvasive magnetic resonance imaging (MRI) studies of muscle carbohydrate metabolism.[131]

When PFK deficiency is confined to erythrocytes, there are no symptoms of myopathy, and the blood lactate response to anoxic exercise is normal.[132-134] Such patients may be hematologically normal[125,135,136] or exhibit mild to moderate hemolytic anemia.[132,136] In general, red cell morphologic characteristics are not strikingly abnormal, although prominent basophilic stippling has occasionally been noted.

Biochemistry

PFK is one of several glycolytic enzymes that reversibly bind to the inner aspect of the erythrocyte membrane. Binding, which is thought to occur between the amino-terminal position of the transmembrane protein band 3 and the adenine nucleotide binding site, located in a cleft between the two dimers that form the PFK tetramer, may serve to both activate the enzyme and protect it against proteolytic degradation during erythrocyte aging.[137]

The active form of human erythrocyte PFK is a tetramer (relative molecular mass: 380,000) composed in varying combinations of two different subunits, one identical to the M subunit found in muscle PFK and the other identical to the L subunit found in liver PFK.[138,139] The relative molecular mass of the M subunit is 85,000, and that of the L subunit is 80,000.[138] About 50% of the erythrocyte enzyme is formed from M subunits,[133,138] whereas muscle PFK is composed entirely of M subunits.[133,140] A deficiency in M subunits severely depresses muscle PFK activity and results in myopathy, but it has a lesser effect on erythrocyte PFK because residual L subunits, under separate genetic control, combine to form an active L4 tetramer of PFK. However, PFK formed entirely from L subunits is unstable to heat or dilution in vitro and is more sensitive to ATP inhibition than muscle (M4) PFK.[132] At the in vivo concentrations of ATP that are present within normal erythrocytes, the enzyme activity of L4 PFK tetramers is severely inhibited, thus possibly explaining the presence of hemolytic anemia even when enzyme activity, as measured in vitro, is approximately 50% of normal. Mice that lack the M subunit but retain

TABLE 17-4 Various Forms of Human Phosphofructokinase Deficiency

			RED BLOOD CELL		MUSCLE		
Type	**Patients**	**Affected PFK Subunit**	**Hemolysis**	**PFK***	**Myopathy**	**PFK***	**Other**
I	18	M (absent or unstable)	Present	29-64	Present	0-5	Hyperuricemia, arthritis
II	3	NA	NA	17	Present	0-6	
III	3	M (unstable)	Present	8-62	Absent	100	
IVa	2	M (unstable)	Absent	28-50	Absent	78†	Asymptomatic
IVb	3	L (unstable)	Absent	60-65	Absent	NA	Asymptomatic
V	3	NA	NA	75†	Present	2-6	Arthritis

Data from Tani et al[123,124] and Vora et al.[125,126]; From Mentzer WC, Glader BE. Disorders of erythrocyte metabolism. In Mentzer WC, Wagner GM, editors: The hereditary hemolytic anemias, *New York, 1989, Churchill Livingstone, p. 269–319.*

NA, Data not available; *PFK,* phosphofructokinase.

*Percentage of normal value.

†Studied in only one patient.

the L subunit have a disease similar to Tarui disease in humans and exhibit chronic hemolysis.[141] Most recognized examples of PFK deficiency are the result of either missing[125,127] or structurally altered[134] M subunits. An interesting exception was found in a clinically normal individual, fortuitously discovered when he volunteered to serve as a "control" during studies of red cell PFK carried out by Vora and colleagues.[125] Normal M subunits but mutant, unstable L subunits were found in his red cells. There was no myopathy, and although erythrocyte PFK was only 65% of normal, hemolytic anemia was not present, presumably because residual enzyme activity within the red cell was entirely the result of the presence of M4 tetramers of PFK. The relatively greater stability and lesser susceptibility to ATP inhibition of this form of PFK apparently allowed adequate enzyme activity under conditions normally found within the red cell in vivo.

Because of the central role of PFK in regulation of erythrocyte metabolism, it is not surprising to find that a deficiency of this important enzyme is associated with hemolysis, but the mechanism of hemolysis is not well understood. Erythrocyte sodium and potassium concentrations, sodium influx, and lactate production were normal in one patient.[131] Despite their normal glycolytic capability in vitro, deficient cells were incapable of maintaining normal ATP concentrations in vivo. The low (73% of normal) intracellular ATP concentration in these cells, though indicative of an abnormality in cellular metabolism, may also exert a positive influence by partially relieving the inhibitory influence of ATP on PFK. Extensive in vitro study of erythrocytes obtained from a Swedish family with Tarui disease and mild compensated hemolytic anemia found elevated intraerythrocytic calcium levels associated with increased calcium permeability of the erythrocyte membrane. High intracellular calcium levels activated the Gardos channel with subsequent ATP depletion, potassium depletion, increased mean corpuscular hemoglobin concentration (MCHC), and diminished erythrocyte deformability. The authors point out that the PFK and Ca^{2+} channel genes are in close proximity to one another on chromosome 12q, but the reason for abnormal Ca homeostasis remains undefined.[142,143]

The complex interaction between metabolites that may dictate actual PFK activity in vivo is illustrated by physiologic studies performed on four individuals with Tarui disease.[144] At usual levels of physical activity, the pattern of erythrocyte glycolytic intermediates clearly reflected inhibition at the PFK step, and the concentration of the important downstream metabolite 2,3-BPG was only 50% of the level found in normal red cells. After the patients had 2 days of bed rest, their red cell 2,3-BPG levels sank to just one third of normal. Subsequently ergometric exercise on a bicycle eliminated the glycolytic intermediate pattern of PFK inhibition and allowed downstream intermediates, including 2,3-BPG, to increase toward normal. Release of large amounts of inosine and ammonia from enzymopathic muscle during exercise into plasma was observed in these patients. Ammonia is a powerful activator of PFK; inosine can be metabolized to lactate by glycolytic pathways (i.e., the HMP shunt) that bypass the PFK reaction. Thus the muscle metabolic abnormalities created by PFK deficiency generate metabolites that alleviated the enzymopathy in erythrocytes. (Diversion of the flow of erythrocyte glycolysis through the HMP shunt by the block at PFK may also generate increased amounts of purines and pyrimidines from 5-phosphoribosylpyrophosphate [PRPP].) The hyperuricemia sometimes noted in individuals with PFK deficiency (see Table 17-4) may be explained on this basis.[126]

An inherited deficiency of PFK found in English springer spaniels, whippets, and wachtelhunds allows interesting comparisons to be made with the human condition.[145-149] As in humans, canine PFK deficiency is an autosomal recessive disorder. Red cell PFK levels are only 7% to 22% of normal in homozygotes because the muscle subunit, which is lacking, accounts for the majority of the available subunits in normal dog red cells.[150] PFK deficiency in dogs is associated with severe hemolytic anemia. Newborn dogs are not anemic, because there is a greater abundance of L subunits and thus of functional PFK enzymes in their red cells. Hemolytic anemia appears because the normal developmental pattern of replacement of L by M subunit synthesis occurs in a setting in which M subunits either are not synthesized or are defective.[151]

A unique feature of PFK deficiency in dogs is episodic hemolysis induced by hyperventilation during exercise, mating, barking, or other similar activities.[147] Dog red cells with high sodium levels exhibit spontaneous hemolysis at alkaline pH; even the small pH change induced by hyperventilation is sufficient to generate the effect in PFK-deficient animals. Underlying the susceptibility of PFK-deficient dog red cells to hyperventilation-induced hemolysis may be their low 2,3-BPG levels, which increase intracellular pH. Raising 2,3-BPG levels to normal in vitro normalizes the response to alkalinity.[152] In comparison to humans, there is less evidence of exertional myopathy, even though dog muscle PFK activity is nearly absent,[153] because dogs do not rely on anaerobic glycolysis for generation of energy during exercise.[154] During exercise, PFK-deficient dogs do exhibit less extraction of oxygen from hemoglobin than normal dogs do, either because the affinity of hemoglobin for oxygen is high (caused by low 2,3-BPG levels in their erythrocytes) or because oxidative metabolism is impaired.[155]

PFK activity in erythrocytes from newborn infants is about 50% to 60% of that in normal adult cells.[156] PFK deficiency is more evident in older cells made earlier in gestation, perhaps because of accelerated enzyme decay.[157] Vora and Piomelli[158] showed that 25% to 30% of newborn erythrocyte PFK consists of L4 isozyme, with the remainder being divided equally between three hybrid isozymes of L and M subunits (LlM3, L2M2, and L3Ml). The L4 isozyme, not found in normal adult red cells, is unstable and presumably accounts for the reduced PFK activity of older cord red cells. The demonstration that PFK deficiency may result in hemolytic anemia in adults suggests that the enzyme deficiency characteristic of normal newborn red cells may contribute to their shortened survival.

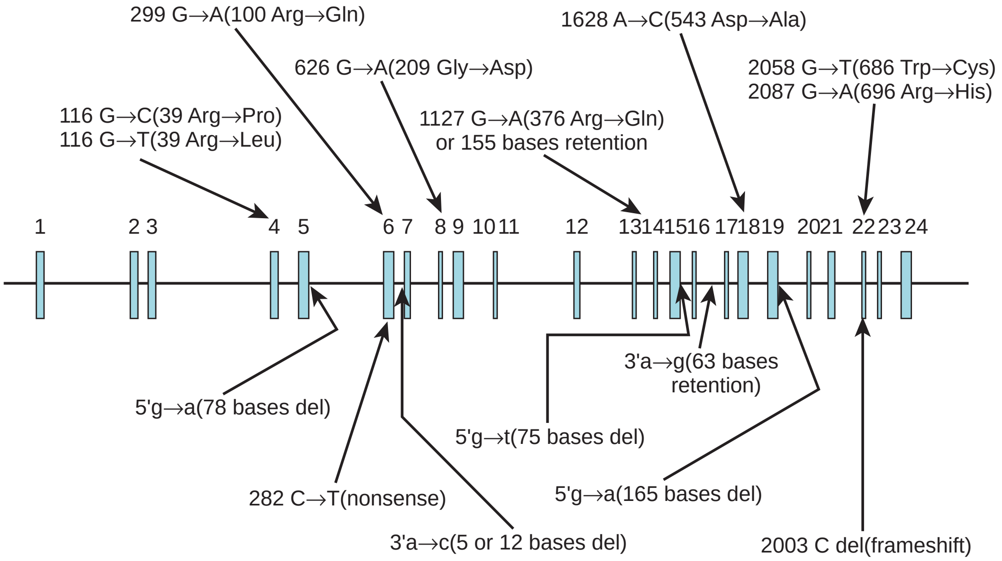
299 G→A(100 Arg→Gln)
1628 A→C(543 Asp→Ala)
626 G→A(209 Gly→Asp)
2058 G→T(686 Trp→Cys)
2087 G→A(696 Arg→His)
116 G→C(39 Arg→Pro)
116 G→T(39 Arg→Leu)
1127 G→A(376 Arg→Gln)
or 155 bases retention
1
2 3
4
5
6 7
8 9
10 11
12
13 14 15 16
17 18 19
20 21
22 23 24
3'a→g(63 bases
retention)
5'g→a(78 bases del)
5'g→t(75 bases del)
282 C→T(nonsense)
5'g→a(165 bases del)
3'a→c(5 or 12 bases del)
2003 C del(frameshift)

Figure 17-3 Mutations in the muscle phosphofructokinase gene. *del,* Deletion. *(From Fujii H, Miwa S: Other erythrocyte enzyme deficiencies associated with nonhaematological symptoms: phosphoglycerate kinase and phosphofructokinase deficiency.* Baillieres Clin Haematol *13:141–148, 2000.)*

Genetics and Inheritance

The gene locus for the L subunit of PFK has been assigned to chromosome 21, whereas the M subunit locus is on chromosome 12.[126,159] The erythrocytes, but not the leukocytes and platelets of individuals with trisomy 21, consistently contain increased PFK activity.[160] Increased erythrocyte PFK activity is caused by increased amounts of L subunit, consistent with a simple gene dosage effect.[126]

Inheritance is autosomal recessive. Type I PFK deficiency (Tarui disease), which is found predominantly in Ashkenazi Jews and Japanese individuals, is the result of mutations involving the *PFKM* gene.[161,162] The 15 reported mutations[159,163] (5 splice site, 8 missense, 1 nonsense, and 1 frameshift) are shown in Figure 17-3.[163] The widely scattered location of the mutations makes it difficult to correlate genotype and phenotype. In PFK-deficient dogs, a missense mutation involving 120 nucleotides from the 3′ end of the coding region produces a truncated mRNA and an unstable PFKM subunit tetramer with altered kinetic properties.[164,165]

Therapy

Supportive care with red cell transfusions as needed and daily folic acid supplementation are the mainstays of therapy for hemolytic anemia.

ALDOLASE DEFICIENCY

Clinical Manifestations, Biochemistry, and Genetics

Aldolase A is found in erythrocytes, muscle, and the fetal brain. Only six individuals with CNSHA and erythrocyte aldolase deficiency have been reported. Their clinical course has varied, with one also exhibiting mental retardation, three an associated myopathy, and two no abnormalities other than hemolytic anemia (Table 17-5). Two members of a Japanese family who had a severe

TABLE 17-5 Mutations Associated with Aldolase A Deficiency and Hemolytic Anemia

Ethnicity	Genotype	Amino Acid Change	Aldolase Activity[‡]	Thermolability	Anemia	Myopathy	Mental Retardation	Reference
Jewish	*	Unknown	15	NR	Yes	No	Yes	172
Japanese	†	Unknown	7	Increased	Yes	No	No	167
Japanese	Homozygote	Asp128→Gly	5	Increased	Yes	No	No	167, 169
German	Homozygote	Glu206→Lys	4	Increased	Yes	Yes	No	170
Sicilian	Compound	Cys338→Tyr	23	Increased	Yes	Yes	No	171, 173
Italian	Heterozygote	Gly346→Ser	NR	Normal	No	Yes	No	168, 174

NR, Not reported.
*Presumed homozygote because the parents were first cousins.
†Homozygote or compound heterozygote.
‡Aldolase activity in erythrocytes expressed as percentage of normal.

deficiency of red cell aldolase[166] exhibited severe chronic hemolytic anemia, sometimes exacerbated by infections. In vitro, red cell glycolysis and HMP shunt activity were depressed, thus indicating that the deficiency of aldolase was of functional significance. Both parents were hematologically normal but had intermediate reductions in red cell aldolase A activity. The mutant aldolase was strikingly thermolabile. A missense mutation (**GAT→*GGT*) giving rise to a single amino acid substitution (Asp→Gly) at position 128 was present in the mutant enzyme.[167] Transfection of *Escherichia coli* with an expression plasmid containing normal, mutant, or modified (by site-directed mutagenesis) aldolase A–complementary DNA (cDNA) generated functional aldolase molecules that were used to confirm that the amino acid substitution at position 128, a site distant from the catalytic site but within an exposed hinge region, was responsible for enzyme thermolability.[168]

In a German child, homozygosity for a point mutation at residue 206 (Glu→Lys) in the coding region of aldolase A also led to thermolability and severe enzyme deficiency in both erythrocytes and skeletal muscle.[169] Unlike the Japanese subjects, this child had a metabolic myopathy, as well as mild to moderate hemolytic anemia. The mutation at residue 206 would be predicted to disrupt the main subunit interface of the aldolase tetramer and cause thermolability. Why myopathy was noted in one setting but not in the other is unknown.

A girl of Sicilian ancestry whose red cell aldolase activity was less than a quarter of normal also exhibited myopathy and chronic hemolytic anemia.[170] Elevated plasma creatine phosphokinase activity was noted with febrile illnesses. She died at the age of 54 months of rhabdomyolysis and hyperkalemia during an acute febrile illness. Like the German patient, her aldolase A enzyme was thermolabile, which offered an explanation for the exacerbation of myopathy during fever.[171] DNA analysis revealed two mutations in the aldolase A gene in this patient: one was a premature stop codon mutation (*931*C→T*), and the second was a missense mutation (*1037*G→A*) that conferred thermolability on the enzyme.[170,171]

Another patient with aldolase deficiency exhibited CNSHA, mental retardation, mild glycogen storage disease, intestinal lactase deficiency, growth retardation, and peculiar facial features. Enzyme activity was approximately 15% of normal in erythrocytes and cultured skin fibroblasts.[172] No structural abnormality of residual erythrocyte aldolase was detected by electrophoresis, isoelectric focusing, heat stability studies, or kinetic examination. The patient was the offspring of a consanguineous marriage, but both parents were hematologically normal and had normal red cell aldolase activity.

A sixth patient, heterozygous for a thermostable mutation of aldolase A, had myopathy, arthrogryposis multiplex congenita, and hypopituitarism but normal IQ and no evidence of hemolytic anemia.[173,174] Because both parents were clinically normal, the aldolase mutation in the patient may have no relationship to his clinical abnormalities.

TRIOSE PHOSPHATE ISOMERASE DEFICIENCY

Clinical Manifestations

An association with TPI deficiency has been documented in approximately 50 patients with congenital hemolytic anemia.[175-185,186-191] In addition to chronic hemolysis, a severe neuromuscular disorder characterized initially by spasticity and psychomotor retardation and often progressing to weakness and hypotonia has been found in nearly all patients surviving beyond the neonatal period. Increased susceptibility to bacterial infection has also been noted. The neurologic abnormalities are not usually manifested before 6 to 12 months of age and are progressive, with death occurring before the age of 5 years. Occasionally, these abnormalities may stabilize during childhood[192] or adolescence.[176] In a Hungarian family, one adult son with severe TPI deficiency had extrapyramidal neurologic symptoms, whereas his older brother, who was equally TPI deficient, remained free of symptoms.[188,193] Further investigation has shown that these brothers both inherited the same two TPI mutations (see “Genetics and Inheritance” section) but that levels of dihydroxyacetone phosphate (DHAP), methylglyoxal, and advanced glycation end products were much higher in the neurologically affected brother.[194] The basis for the prolonged survival of both brothers and their different neurologic status is unknown, but epigenetic factors are suspected.

Anemia is variable, but most patients require at least occasional blood transfusions. Macrocytosis and polychromatophilia are evident on the blood smear because of the presence of reticulocytosis, which may reach 50% on occasion. Aside from occasional small, dense spiculated cells, no striking changes in erythrocyte morphologic characteristics are present.

Biochemistry

TPI is a homodimer whose two subunits (relative molecular mass: 26,750[195]) are the product of a single locus on the short arm of chromosome 12.[196] Posttranslational modification of one or both subunits may occur by deamidization of aspartines at positions 15 and 71, thereby resulting in multiple forms of the enzyme.[197,198] The 248–amino acid sequence of human (placental) TPI has been determined directly[195] and is nearly, but not completely, identical to the sequence predicted by nucleotide analysis of adult human liver cDNA.[181]

TPI is a classic housekeeping gene, present in all tissues, with an amino acid sequence that has been remarkably well conserved during evolution.[199] The eight-stranded αβ-barrel structure of the human enzyme has been confirmed at 2.8-nm resolution by x-ray crystallography.[199] TPI has no requirement for cofactors or metal ions, and there is no evidence of cooperativity or allosteric interactions between subunits.

When measured in vitro, erythrocyte TPI activity is approximately 1000 times that of HK, the least active glycolytic enzyme. Even TPI-deficient erythrocytes exhibiting only 2% to 35% of normal TPI activity in vitro possess far more TPI than HK activity. Not surprisingly, TPI-deficient erythrocytes are capable of normal glycolysis in vitro, even when compared with reticulocyte-rich normal blood.[176] Furthermore, mathematical modeling shows that increased activity of glycolytic kinases can lead to normal conversion of glucose to lactate in TPI-deficient cells.[200] Nonetheless, DHAP, the substrate for TPI, accumulates to high levels in TPI-deficient erythrocytes,[200] and the ATP concentration is usually low for cell age. These results indicate the presence of a substantial impairment in glycolysis in vivo. Reduction of residual TPI activity in deficient cells by binding to the red cell membrane or to brain microtubulin has been proposed to account for some of the differences between in vitro determinations of TPI activity and the evidence of a more severe impairment in enzyme function in vivo.[201-203] The defect can be partially bypassed by way of the HMP shunt, which generates glyceraldehyde 3-phosphate from glucose without the participation of TPI. Methylene blue stimulation of the shunt produces a lesser increase in glycolysis relative to baseline in TPI-deficient erythrocytes than in reticulocyte-rich control blood.[176] This has been interpreted as indicating a markedly greater "resting" shunt rate in the deficient cells, consistent with the proposed reliance of such cells on the shunt. Evidence indicates that TPI-deficient red cells are relatively deficient in antioxidant capabilities and that this deficiency may contribute to their shortened life span.[177,204]

Rare electrophoretic variants of TPI, not associated with reduced enzyme activity or hemolysis, have been described.[198,199,205,206] In anemic patients, enzyme kinetics and electrophoretic mobility are usually normal, but in vitro evidence of enzyme lability after heating is often obtained,[179-181,183] thus suggesting that instability and rapid loss of enzyme protein play an important role in the pathogenesis of hemolysis in vivo.[179,181] A single amino acid substitution (Glu→Asp) at position 104 has been identified in at least 17 geographically widely distributed families with clinically affected children and is the most commonly encountered mutation responsible for TPI deficiency.[185,190] Its relative rate of occurrence is thought to be the result of a founder effect. The Glu104→Asp TPI gene product is thermolabile.[207] Computer modeling indicates that a substituted amino acid at position 104, which is buried in a hydrophilic side pocket of the normal enzyme, will reduce the stability of the pocket, promote unfolding, and enhance thermolability.[207] Comparison of the recombinant mutant TPI enzyme to the recombinant wild type indicates that enzyme kinetics are normal but that dimer formation and thermal stability are greatly decreased. Analysis of the crystal structure of the mutant TPI suggests that a mutation-triggered disruption of the water network in the vicinity of the dimer–dimer interaction is the underlying basis for enzyme instability and ultimately for the clinical abnormalities.[208]

Fourteen TPI mutations have been defined.[185] Most clinically affected individuals are homozygous for a single mutation or compound heterozygous for two different mutations. In the Hungarian pedigree mentioned earlier,[193] a point mutation at codon 240 (Phe→Leu) produced a moderately thermolabile TPI with abnormal substrate kinetics and electrophoretic migration.[209] Phe240 is near the active site of the enzyme and appears to be essential for maintaining its correct geometry.[199] Furthermore, both anemic brothers in this pedigree inherited a nonsense mutation at codon 145 that terminated TPI protein synthesis, thereby producing a truncated protein and also reducing the output of TPI mRNA from the affected allele by 10- to 20-fold.[210] The red cells of the brothers had less than 10% of the TPI activity found in normal cells.[193,209]

The enzyme deficiency is manifested not only in red cells but also in leukocytes, platelets, muscle, serum, and cerebrospinal fluid. Histologic examination of muscle from a girl with TPI deficiency and myopathy revealed marked degenerative changes of the contractile system, altered mitochondria, and absent TPI by histochemical staining.[211] Brain and nerve tissue have not yet been analyzed, but the cerebrospinal fluid deficiency suggests that deficient TPI activity in neural tissue is responsible for the neurologic abnormalities observed in enzymopenic patients. Orosz and coworkers have advanced the hypothesis that mutant TPI is aggregation prone and that such aggregates might be neurotoxic, but direct evidence for this is not available.[191] Although increased susceptibility to infection might be the consequence of defective function by TPI-deficient leukocytes,[182] functional studies carried out on such cells in vitro have often been normal.[178,192] A functional defect in TPI-deficient platelets has been described.[212]

Genetics and Inheritance

Several large pedigrees display an autosomal recessive mode of inheritance of TPI deficiency.[178,181,185] Obligate heterozygotes are clinically normal, but their erythrocytes contain only approximately half the TPI activity of control erythrocytes. As is often the case in other glycolytic enzymopathies, no clear boundary exists between heterozygous deficient and low-normal enzyme activity. Several upstream polymorphisms are found at a high rate of occurrence in African-American populations.[185,213] Although they may be associated with mild reductions in erythrocyte TPI activity, they do not seem to be associated with clinical abnormalities, even in homozygotes.[185] At the other extreme, the homozygous state in mice for a TPI null allele is lethal early in embryogenesis.[214] A more informative mouse model results in an Asp→Gly substitution at codon 49 located in the functional domain of TPI. The mutant enzyme is highly thermolabile, resembling in this regard the human Glu104→Asp TPI mutant. Homozygous mice have only 13% of normal TPI activity and exhibit hemolytic anemia.[215] Simultaneous heterozygous inheritance of TPI deficiency and either G6PD deficiency or sickle cell trait has not altered the typical clinical pattern of the disorders when either is present alone.[177] TPI deficiency can be diagnosed prenatally by analysis of fetal blood cells,[184] cultured amniocytes,[183] or trophoblastic cells[189] with suitable precautions.[216] If the mutation is known, mutation analysis can be performed directly on fetal DNA.[189,210]

Therapy

Transfusions and folic acid supplementation are the therapies presently available. Splenectomy in one patient did not alter the intensity of hemolysis.[178] In vitro studies point to the possibility of transfer of TPI enzyme protein from normal to deficient cells with transient improvement in enzyme activity and a reduction in DHAP levels.[217] There have been no attempts to deliver normal TPI enzyme to patient tissues, but in other disease settings direct enzyme transfer from normal hematopoietic stem cell–derived microglial cells to enzyme-deficient nervous tissue after allogeneic stem cell transplantation has been successful.[217]

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE DEFICIENCY

Clinical Manifestations, Biochemistry, and Genetics

Study of a large kindred in which three members exhibited a reduction in erythrocyte G3PD activity to 50% of normal levels yet were hematologically normal have clearly established that G3PD deficiency need not result in hemolysis.[218] The clinical severity of hemolytic anemia in four members of this pedigree who inherited both hereditary spherocytosis and G3PD deficiency was no greater than that associated with spherocytosis alone. Affected members of this kindred were presumed to be heterozygotes, because the amounts of both G3PD enzyme activity and enzyme protein were reduced equally to about 50% of normal and the amount of residual G3PD was qualitatively normal. Two of three patients who had anemia with G3PD deficiency in other kindreds had even lower levels of enzyme activity (20% to 30% of normal) and conceivably were either homozygotes or doubly heterozygous for two mutant G3PD genes.[219,220] However, as with the spherocytosis pedigree, a cause-and-effect relationship between hemolytic anemia and G3PD deficiency was not established.

PHOSPHOGLYCERATE KINASE DEFICIENCY

Clinical Manifestations

PGK deficiency is a sex-linked disorder that largely affects males. Nonspherocytic hemolytic anemia may occur alone or in combination with neurologic abnormalities ranging from emotional instability to seizures, movement disorders, psychomotor retardation, aphasia, or tetraplegia.[163,221] In a subset of individuals with PGK deficiency, myopathy is the dominant and sometimes the only clinical manifestation (Table 17-6). Individuals with one form of mild PGK deficiency (PGK München) exhibit no clinical abnormalities (see Table 17-6). In females, erythrocyte PGK activity is less depressed than it is in males, hemolytic anemia is mild or absent, and neurologic and muscle abnormalities are not seen (see Table 17-6).

Hematologic findings in individuals who have anemia with PGK deficiency have been those customarily associated with hemolysis, namely, jaundice and reticulocytosis. Hemolytic episodes are often associated with acute febrile illnesses. No changes in erythrocyte morphologic characteristics have been seen, and osmotic fragility has usually been normal.

Biochemistry

PGK is a monomeric enzyme with a primary structure consisting of 417 amino acids.[255] Horse and pig muscle PGK has a primary structure that is highly homologous to that of human PGK.[256,257] Crystallographic studies show that its tertiary structure consists of two lobes (the C and N domains) connected by a hingelike structure that allows considerable conformational change to occur during substrate binding (Fig. 17-4).[256,257] Because the nucleotide (adenosine diphosphate [ADP] and ATP) combining site is located within the C domain and the phosphoglycerate (1,3-BPG or 3-phosphoglyerate) binding site is within the N domain, bending of the enzyme is required to bring the several substrates required for the PGK reaction into close proximity.[257] Mutant PGKs often exhibit abnormal enzyme kinetics and diminished stability in vitro, properties that are likely to impair enzyme function in vivo (see Table 17-6).

PGK-deficient red cells are capable of normal glycolysis in vitro.[222,253] Intracellular ATP concentrations are normal or slightly low,[222,227,253] whereas the 2,3-BPG concentration is elevated, sometimes to two or three times the normal level.[208,213,218,223,237] These results reflect increased flow through the 2,3-BPG cycle (see Fig. 17-1) at the expense of the ATP-generating PGK reaction. Despite the availability of an alternative pathway (the Rapoport-Luebering shunt or the 2,3-BPG cycle) to bypass PGK, substantial accumulation of glycolytic intermediates proximal to the enzyme defect is found in fresh red cells, thus indicating that the normal flow of erythrocyte glycolysis is impeded in vivo.

Little is known about the mechanism of hemolysis of PGK-deficient red cells. Most PGK activity is membrane associated.[258] It has been suggested that the ATP for membrane adenosine triphosphatase (ATPase)–mediated cation transport is mostly (or entirely) generated by membrane-bound PGK. Indeed, ADP derived from membrane ATPase exerts an important regulatory influence on glycolysis by its participation in the PGK reaction.[259] However, the implication of a special role for PGK in cation transport has been challenged.[260] Active transport of Na^+ and K^+ by PGK-deficient red cells with residual PGK activity only 10% to 15% of normal was not impaired, even with the challenge of an increase in intracellular Na^+ concentration.[261] Thus it seems unlikely that hemolysis of PGK-deficient red cells is related to premature cation pump failure as a direct result of inadequate PGK activity.

PGK activity in leukocytes is consistently subnormal in affected males, but white cell function is not usually compromised.[233,262]

Genetics and Inheritance

The major structural gene for PGK, located on the long arm of the X chromosome, is 23 kb in size and composed of 11 exons and 10 introns.[263-266] PGK-deficient male hemizygotes demonstrate very little active enzyme and have more symptoms than heterozygous females do, who have higher levels of PGK activity (see Table 17-6). A second functional PGK gene, expressed only in spermatozoa, is found on chromosome 19.[267] This autosomal PGK gene lacks introns but is otherwise similar to the X-chromosomal PGK gene. A single isozyme of PGK is found in all human nonhematopoietic tissues except

TABLE 17-6 Characteristics of Reported Cases of Phosphoglycerate Kinase Deficiency

Variant	Mutation[*]	RBC PGK Activity (% of Normal)	Stability In Vitro	Kinetic Abnormalities	Hemolytic Anemia	Neurologic Abnormalities	Myopathy
Hemizygotes (Male): Hemolytic Anemia or No Clinical Manifestations							
New York[222,225]	Asp164→Val	2-2.7	Normal		+	+	0
Alabama[226]	Lys190 or 91 del	4			+	0	0
Matsue[227-229]	Leu89→Pro	5	Low	+	+	+	0
Uppsal[230,231]	Arg206→Pro	5-10	Low	+	+	+	0
Cincinnati[232,233]		8-11			+	+	0
Tokyo[234]	Val266→Met	10	Low	+	+	+	0
Michigan[235]	Cys315→Arg	10	Low	+	+	+	0
Barcelona[236,237]	Ile147→Asn	10			+	+	0
San Francisco[238]		12	Normal	+	+	0	0
München[239,240]	Asp267→Asn	21	Low	0	0	0	0
Murcia[236]	Ser319→Asn	49.0			+	+	0
Herlev[241]	Asp285→Val	50			+	0	0
Hemizygotes (Male): Muscle Disease							
Shizuoka[242]	Gly158→Asp	0.7	Normal	0	+	0	+
North Carolina[243]	10–amino acid insert	3	Normal	+	0	+	+
Creteil[244]	Asp315→Asn	3	Low	+	0	0	+
Trondheim[245]		5			0	0	+
Antwerp[245]	Glu252→Ala[†]	5.6			0	0	+
Fukui [246]	4-bp del, exon 6[§]	5.6			0	0	+
+Kyoto[247]	Ala354→Pro	6			+	+/–	+
Hammamatsu[248,249]	253Ile→Thr	8	Normal	0	0	+	+
New Jersey[250]		18	Normal	+	0	0	+
Alberta[251]		1.5[‡]	Normal	+	0	0	+
PGK Afula2[252]	Thr378→Pro	2			0	0	+
Heterozygotes (Female)							
Piedmont[253]		27			+	0	0
Memphis[254]		78			+	0	0
Amiens[222-224]		77			+	0	0

[*] Mutation position as corrected by Beutler.[221]

[†] This mutation also adversely affects mRNA splicing efficiency to about 10% of normal.

[‡] Measured in muscle.

[§] Frameshift at codon 706-709 resulting in a stop codon truncating phosphoglycerate kinase to 231 amino acids in length.

spermatozoa.[268] It is therefore not surprising that nonerythroid tissues may be compromised in patients with erythrocyte PGK deficiency.

In 1972, Yoshida and coworkers[269] succeeded in purifying and sequencing both normal erythrocyte PGK and a clinically normal but electrophoretically distinct human mutant PGK, the New Guinea variant. The mutant enzyme differed from normal PGK by the substitution of arginine for threonine at position 352.[255] Subsequently, the structure of 17 other PGK mutants, most associated with clinical manifestations, has been determined by peptide or nucleotide sequencing (see Table 17-6). Fifteen of these mutants involve only a single amino acid (14 missense mutations and 1 deletion). The 16th activates a cryptic splice site that leads to the insertion of 10 additional amino acids into the PGK polypeptide, and the 17th, a 4-amino acid deletion, also affects splicing and produces a truncated polypeptide. The majority of the mutants are found within the C domain, as shown in Figure 17-4. It is not clear how the particular spectrum of clinical abnormalities associated with each mutation is related to its position within the molecule. For example, PGK Michigan, a point mutation at amino acid 316, is associated with hemolytic anemia and

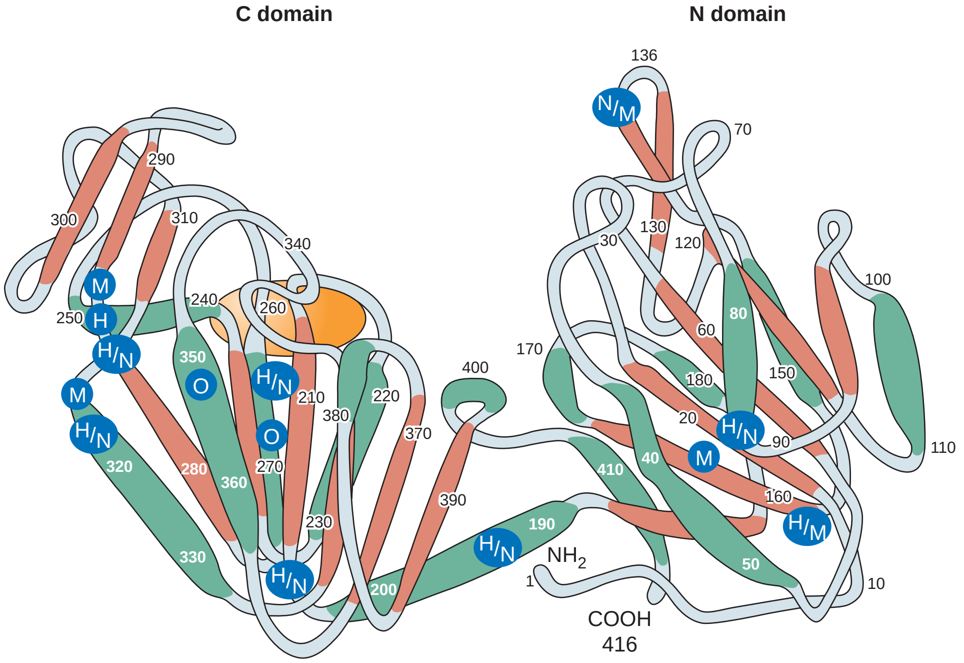

Figure 17-4 Three-dimensional model of human PGK. This figure is based on the three-dimensional model of horse PGK published by Banks et al.[256] Positions of the amino acid substitutions and the clinical features associated with 14 PGK point mutations are indicated by *filled circles*. The *orange shaded ellipse* in the C domain indicates the location of the ATP and ADP binding site. Random coil is *light blue*, β strands are *orange*, and α helices are *green*. *H*, Hemolytic anemia; *M*, muscle disease; *N*, neurologic manifestations; *O*, no clinical abnormalities. *(Modified from Fujii H, Kanno H, Hironi A, et al: A single amino acid substitution [157 Gly→Val] in a phosphoglycerate kinase variant [PGK Shizuoka] associated with chronic hemolysis and myoglobinuria.* Blood *79:1582–1585, 1992.)*

neurologic abnormalities, whereas individuals with PGK Creteil, a point mutation of the adjacent amino acid at position 315, do not have anemia and neurologic abnormalities but exhibit rhabdomyolysis.[270] Both mutants are thermolabile and demonstrate kinetic abnormalities in vitro, and both have similarly reduced activity in erythrocytes (see Table 17-6). Random inactivation of the mutant X chromosome may produce differing proportions of enzyme-deficient cells in female heterozygotes. Some may be anemic (see Table 17-6), whereas others are clinically and hematologically normal.[228] In the latter, the population of PGK-deficient red cells may be so small that erythrocyte PGK activity will be completely normal.[227]

Therapy

In one patient, splenectomy had no beneficial effect on anemia,[254] but in several others, surgery has decreased or eliminated the need for transfusions, reduced the degree of reticulocytosis, and sometimes resulted in an increase of several grams in the hemoglobin level.

2,3-BISPHOSPHOGLYCERATE MUTASE DEFICIENCY

Clinical Manifestations

Absence of erythrocyte BPGM activity does not affect red cell life span but may have other hematologic consequences. In one pedigree, four siblings who completely lacked functional red cell BPGM had polycythemia but were otherwise clinically and hematologically normal. All three children of these individuals had about 50% of the normal amount of red cell BPGM and were hematologically normal except for polycythemia.[271,272] In another patient with polycythemia, BPGM activity was only 4% of normal, 2,3-BPG was almost undetectable, and hemoglobin's oxygen affinity curve was shifted to the left. Despite the concomitant inheritance of severe erythrocyte G6PD deficiency, this individual did not have evidence of hemolytic anemia.[273]

Biochemistry

Human red cell BPGM is a homodimer whose identical subunits (relative molecular mass: 29,840) each consist of 258 amino acids.[274] Nearly all 2,3-BPG phosphatase activity also resides in the BPGM molecule, so 2,3-BPG metabolism is controlled by a single, multifunctional enzyme. In fact, purified BPGM is also capable of performing as an MPGM, though at a low rate of activity.[275] Considerable structural homology exists between red cell BPGM and MPGM,[276,277] and the enzymes to some extent exhibit overlapping functions. However, both biochemical[276] and genetic[278] evidence indicates that they are each unique and under separate genetic control.

The BPGM molecule has been modified in vitro by site-directed mutagenesis in an attempt to model a potential therapeutic approach to sickle cell disease.[279] Replacement of Gly13 by Arg enhances phosphatase activity at the expense of the mutase. The effect of such an alteration in vivo, achieved by gene therapy or by pharmacologic means, would be to lower erythrocyte 2,3-BPG levels, increase the affinity of hemoglobin for oxygen, and in this way retard the polymerization of hemoglobin S.

BPGM deficiency results in reduced synthesis of 2,3-BPG. In complete BPGM deficiency, there is virtually no 2,3-BPG within the red cell.[271-273] The affinity of whole blood for oxygen is increased because of lack of 2,3-BPG, thereby accounting for the polycythemia noted in individuals with BPGM deficiency.[271-273] The pattern of glycolytic intermediates is disturbed,[271] with a crossover at PFK sometimes being exhibited,[272] which is consistent with relief of the inhibitory influence of 2,3-BPG on PFK. The erythrocyte ATP concentration is usually normal or slightly increased, compatible with diversion of 2,3-BPG into the PGK reaction as a consequence of reduced flow through BPGM. Erythrocyte glycolysis and pentose phosphate pathway activity have been normal in vitro.[271] Probably as a consequence of the large amount of 1,3-BPG present in BPGM-deficient red cells, hemoglobin A may undergo posttranslational modification by glycerylation at a82. The modified hemoglobin, about 3% of the total, has a lower isoelectric-electric point than hemoglobin A_{1C} and is easily identified by isoelectric focusing.[280]

Genetics and Inheritance

The BPGM locus is on chromosome 7 (7q22-34).[281] The gene is fully expressed only in erythroid tissue.[272] BPGM deficiency has the expected autosomal recessive mode of inheritance.[271-273] Individuals from the large pedigree with polycythemia and virtually no red cell BPGM activity have been shown to be compound heterozygotes for two different BPGM mutations.[282] One, BPGM Creteil I, is a point mutation (Arg89→Cys) at or near the BPGM active site,[283] and the other, BPGM Creteil II, is a frameshift mutation caused by a deletion of nucleotide 205 or 206.[284] Only BPGM Creteil I enzyme protein is found in red cells.[285] It is catalytically inactive and thermolabile in vitro and exhibits altered electrophoretic mobility.[285] Although BPGM and 2,3-BPG phosphatase activity was virtually absent in compound heterozygotes, MPGM activity was nearly normal, thus illustrating the complex nature of this multifunctional enzyme.[283] The individual with severe BPGM and G6PD deficiency was homozygous for a missense mutation (*185*G→A*; Arg 62→Gln) in exon 2.[273]

Therapy

Polycythemia, if symptomatic, may require phlebotomy.[272]

MONOPHOSPHOGLYCERATE MUTASE DEFICIENCY

Clinical Manifestations, Biochemistry, Genetics, and Therapy

A woman with hereditary spherocytosis whose erythrocyte MPGM activity was reduced to about 50% of normal was found to have inherited two copies of a missense mutation (230met→isol) in the MPGM-BB isoenzyme, which is the form that contributes most of the MPGM activity in erythrocytes.[286] The mutant enzyme exhibited reduced thermal stability but normal kinetics.[287] Family members who inherited a single copy of the mutation were healthy, and no evidence was presented to indicate that in homozygotes MPGM deficiency influences the hemolysis associated with hereditary spherocytosis.[288]

ENOLASE DEFICIENCY

Clinical Manifestations, Biochemistry, Genetics, and Therapy

A woman in whom red cell enolase activity was only 6% of normal exhibited a modest reduction in the survival of ^{51}Cr-labeled autologous erythrocytes (half-life of 18 days; normal, 25 to 30 days) but no overt chronic anemia.[289] A severe, life-threatening acute hemolytic episode occurred when nitrofurantoin was administered for the treatment of a urinary tract infection. Enzymes of the HMP shunt all exhibited normal or increased activity, and glutathione stability was normal. Such drug-induced hemolysis is distinctly uncommon in disorders of the Embden-Meyerhof pathway, although it is theoretically possible in enzyme-deficient reticulocytes.[290] In this patient, in whom sudden massive hemolysis involved predominantly mature red cells, no biochemical mechanism has yet been defined, and the association between enolase deficiency and hemolytic anemia may be coincidental. A sister was hematologically normal, even though her red cells were as deficient in enolase activity as those of the propositus.

Partial red cell enolase deficiency was inherited in an autosomal dominant manner by six members of a second pedigree that spanned four generations. With the exception of the propositus, a 13-day-old boy with profound hemolytic anemia, affected family members were not anemic and had little or no evidence of hemolysis. Spherocytes were present on the peripheral blood smear, and the MCHC was usually elevated.[291]

A girl with Diamond-Blackfan anemia had near absent erythrocyte enolase activity pre–bone-marrow transplant with normal enolase activity 1 year after the transplant. Both parents also had subnormal erythrocyte enolase activity, but no hematological abnormalities. An autosomal recessive inheritance was suggested. The relationship, if any, between enolase deficiency and DBA was undefined.[292]

PYRUVATE KINASE DEFICIENCY

Clinical Manifestations

PK deficiency is the most commonly encountered glycolytic enzymopathy associated with anemia. Its incidence has been estimated to be 51 cases per million in white individuals.[293] Of the approximately 500 human patients thus far reported,[6,7] the majority have been of Northern European extraction. Sporadic instances have been seen in other ethnic groups. A hemolytic anemia resembling that seen in human PK deficiency has also been described in basenji dogs,[294,295] beagles,[296] cats,[297] and inbred mice[298] that inherited mutant forms of erythrocyte PK with subnormal activity. Anemia, jaundice, and splenomegaly are regularly present in PK deficiency. The anemia may be profound, occurring in utero[299,300] or in early infancy,[65] and require regular blood transfusions for survival. Conversely, the anemia may be mild enough to evade discovery until adulthood. In a few patients, anemia is absent, hemolysis is fully compensated for, and jaundice may be the sole clinical abnormality. When present, anemia is a lifelong condition and its intensity usually varies little, although it may become more severe during pregnancy.[301,302] Exacerbations of anemia are uncommon and generally result from transient erythroid hypoplasia after infections[303] or, rarely, from increased hemolysis of unknown cause. Iron overload is a common problem, particularly in adults. Transfusion, splenectomy, and coinheritance of hereditary hemochromatosis genes are factors that make iron overload more likely in PK deficiency.[304,305] Serum hepcidin levels are inappropriately low[306] or normal,[305] and ferritin levels are high,[306] indicating dysregulation of iron homeostasis, possibly related to ineffective erythropoiesis as is the case in the thalassemias. Iron chelation may be required.[304] Manifestations of PK deficiency outside the hematopoietic system are uncommon. In several pedigrees, chronic leg ulcers have been observed in family members with PK deficiency and hemolytic anemia.[307,308]

Hyperbilirubinemia is often encountered in newborns with PK deficiency,[309] and these infants may require exchange transfusions.[65] Serum unconjugated bilirubin levels remain elevated in later life, and gallstones are common. Unconjugated bilirubin levels greater than 6 mg/dL are occasionally seen[310]; one brother and sister regularly had levels greater than 20 mg/dL.[311] These patients have abnormal hepatic function in addition to hemolysis. Whether abnormalities in liver PK contribute to hyperbilirubinemia is unknown.[312,313]

Macrocytosis, occasional shrunken, spiculated erythrocytes, and rarely, acanthocytes may be observed on examination of the blood smear; these changes may be accentuated by splenectomy. More extreme alterations in erythrocyte morphologic characteristics are sometimes encountered (Fig. 17-5).[314,315] Such abnormalities in shape may result from the inadequate ATP synthesis characteristic of PK-deficient erythrocytes.[316] A paradoxical increase in the reticulocyte count often follows splenectomy despite evidence of a beneficial reduction in the rate of hemolysis. Reticulocyte counts may exceed 90%, and in many patients counts of 40% to 70% are maintained for years. Conversely, in other patients the expected reduction in reticulocyte count is seen after splenectomy. The osmotic fragility of fresh and incubated erythrocytes is most often normal, although minor populations of fragile or resistant cells may be encountered in occasional patients after incubation. The results of an autohemolysis test are generally, but not invariably[317] abnormal, with hemolysis of as many as 50% of erythrocytes after 48 hours of incubation in saline. Prior addition of glucose may reduce the hemolysis in some instances, but glucose usually has little or no effect. In fact, if the reticulocyte count exceeds 25%, incubation with glucose regularly accentuates the hemolysis. This phenomenon has been attributed to inhibition of oxidative phosphorylation by glucose (the Crabtree effect), with unfavorable consequences in PK-deficient reticulocytes because of their reliance on oxidative phosphorylation for synthesis of ATP.[318]

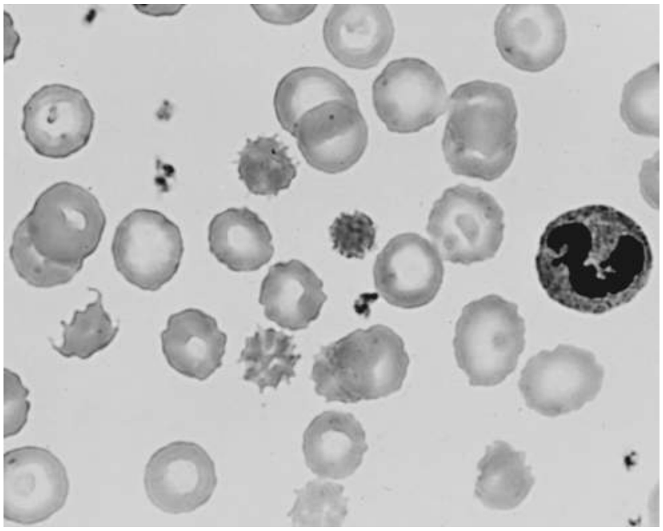

Figure 17-5 Postsplenectomy blood smear from a patient with severe PK deficiency. *(From Nathan DG, Oski FA, Sidel VW, Diamonk LK: Extreme hemolysis and red cell distortion in erythrocyte pyruvate kinase deficiency. I. Morphology, erythrokinetics, and family enzyme studies.* N Engl J Med *270:1023–1030, 1964.)*

Biochemistry

The active form of PK is a tetramer formed from one of four different tissue-specific subunits. The R subunit is found in red cells; the L subunit in liver; the M1 subunit in muscle, heart, and brain; and the M2 subunit in all early fetal tissues and most adult tissues, including leukocytes and platelets.[319] The R and L subunits derive from a common gene (*PKLR*) located on chromosome 1 (1q21),[320] whereas the M1 and M2 subunits are generated by a second gene (*PKM*) located on chromosome 15 (15q22).[321] In the rat, the *PKM* gene is 20 kb long and consists of 12 exons and 11 introns.[322] M1- or M2-specific mRNA is formed from a common primary transcript by alternative spicing involving the removal of either exon 9 (M2) or exon 10 (M1).[323] Because human and rat M2 cDNA is highly homologous,[321] alternative splicing probably also accounts for the differences in M1 and M2 mRNA in humans. The *PKLR* gene also consists of 12 exons.[324] Tissue-specific expression of one of two different promoters generates a transcript containing either an R or an L exon at the 5′ end.[325,326] The remaining 10 exons are identical. L-type cDNA encodes a polypeptide of 543 amino acids,[325] whereas R-type cDNA encodes a product that is longer by 31 amino acids.[327]

The three-dimensional structure of cat and rabbit muscle PK has been studied by x-ray crystallography.[328] There is extensive sequence homology among species and between M and R subunits, particularly in the vicinity of the active site,[329] thus indicating that the enzyme structure has been conserved during evolution. Crystallographic analysis of recombinant human erythrocyte R-PK (Fig. 17-6) indicates a close homology to the three dimensional structure of muscle PK.[330] As shown in Figure 17-6, each PK subunit consists of a short aminoterminal region and three distinct domains (A, B, and C). Domain A is

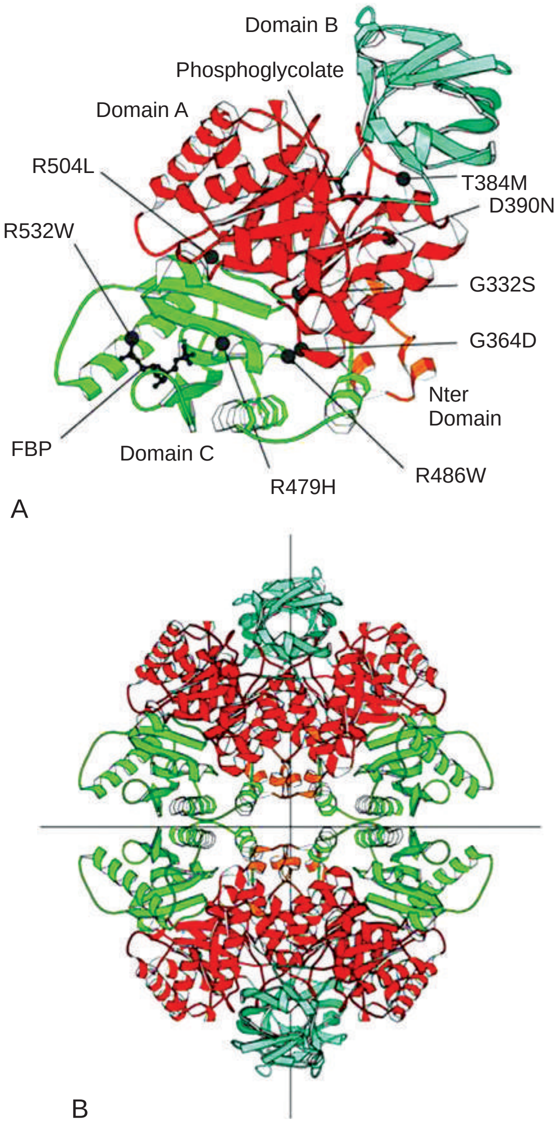

Figure 17-6 Three-dimensional crystal structure of erythrocyte PK (R-PK). The N-terminal domain is *orange,* the A domain is *red,* the B domain is *cyan,* and the C domain is *green.* The 49 N-terminal amino acids are not shown. **A,** Subunit structure. The active site lies in a pocket between domains A and B, the potassium and magnesium binding sites are in domain A, and allosteric modulation of PK function primarily involves interaction with domain C (the FDP binding site is indicated by *FBP*). Shown are the position of 8 mutations found in individuals with PK deficiency and hemolytic anemia *(gray spheres).* Analysis of recombinant enzyme proteins showed that 4 (G332S, G364D, R504L, and R532W) were associated with strongly perturbed kinetic and thermostability parameters as well as severe anemia. However, even enzymes (T384M) with only mild abnormalities in vitro could nevertheless be associated with a severe clinical phenotype, making predictions about the consequences of a particular mutation difficult. **B,** The RPK tetramer, formed of four identical RPK subunits. *(From Valentini G, Chiarelli LR, Fortin R, et al: Structure and function of human erythrocyte pyruvate kinase.* J Biol Chem *277:23807–23814, 2002.)*

cylindrical and formed by eight parallel strands of b sheet encased by an outer coaxial cylinder of eight a helices; domain B consists of a closed antiparallel b sheet; and domain C is a five-stranded b sheet connected by a helices. The active site lies in a pocket between domains A and B, the potassium and magnesium binding sites are in domain A, and allosteric modulation of PK function primarily involves interactions with domain C.

In erythroid cells, PK is a tetramer (relative molecular mass: 230,000[329]) with subunits that may vary in type. In erythroid precursors, M2 homotetramers are the predominant PK isoenzyme. With erythroid maturation, synthesis of M2 subunits declines and is replaced by production of R-type subunits.[331-333] The mature red cell enzyme may exist in either of two physical conformations, analogous to the R and T forms proposed by Monod and colleagues[334] for allosteric proteins. Partially purified enzyme preparations usually exhibit sigmoid kinetics in the presence of increasing concentrations of substrate (phosphoenolpyruvate). Small amounts of phosphoenolpyruvate facilitate further binding of substrate by the enzyme in a manner analogous to that seen with heme–heme interactions. Fructose 6-diphosphate (FDP) induces a transition from sigmoid to hyperbolic kinetics, probably by acting directly at the phosphoenolpyruvate binding site.[335]

A number of factors may result in posttranslational modification of the enzyme. Transition between an FDP-sensitive conformation with sigmoid kinetics and an insensitive form with hyperbolic kinetics has been achieved by varying pH,[336] temperature,[336,337] and conditions of storage. Aging of the enzyme in vivo appears to favor the FDP-sensitive conformation.[338] These transitions may play a significant role in modulation of PK activity in vivo. Posttranslational modification of enzyme properties mediated by oxidation of exposed thiol groups on the surface of the molecule may explain some abnormalities previously ascribed to genetic or acquired alterations in the primary structure of the enzyme.[338-343] In several instances it has been possible with the use of sulfhydryl reagents to restore to normal the altered stability and abnormal kinetics of mutant PK from individuals with hemolytic anemia.[339,344] On the other hand, it has often not been possible to implicate oxidation of enzyme thiol groups as a cause of abnormal enzyme properties.[345,346]

The enzyme is subject to numerous other regulatory influences. ATP is a competitive inhibitor ($K_i = 3.5 \times 10^{-4}$ mol/L)[347]; at physiologic ATP concentrations (approximately 1 mmol/L), erythrocyte PK activity should be significantly constrained by ATP. Both potassium[335,337] and magnesium[337,348] activate PK; rubidium (Rb^+) or ammonium (NH_4^+) substitute for potassium (K^+), and manganese (Mn^{2+}) or cobalt (Co^{2+}) can replace magnesium (Mg^{2+}).[341] Activation of purified PK by FDP has been demonstrated at concentrations normally found within the erythrocyte.[349] At a higher concentration (0.5 mmol/L), another glycolytic intermediate, G6PD, activates PK.[350] The glycolytic intermediate 2,3-BPG, of particular interest because of its high concentration in PK-deficient erythrocytes, has no influence on PK in hemolysates[351] but has variously been reported to inhibit[352] or activate[353] purified PK. Phosphorylation of PK, mediated by cyclic adenosine monophosphate, alters its kinetic properties and may regulate 2,3-BPG levels and thus oxygen transport by red cells.[353,354] It is clear that intracellular PK activity is determined by the complex interplay of a number of regulatory factors and may bear little relationship to measures of activity determined in vitro under optimal conditions.

When erythrocyte PK (R homotetramer) is abnormal, hepatic PK (L homotetramer) may also be affected because the R and L subunits are derived from a common gene. In two anemic individuals with PK deficiency, liver PK was reduced to 59%[355] and 46%[356] of normal. Residual liver PK, measured in the latter patient, was mostly of the M2 type. No disorders of hepatic function appear to result from such partial deficiency of PK.[357] In another individual, total liver PK activity was only 22% of normal, virtually no L-type enzyme was detectable, and there were abnormalities in serum aminotransferases.[312] Paradoxically, in still another individual, liver L-type PK exhibited entirely normal activity and properties despite abnormalities in the supposedly identical isoenzyme in the red cells.[313]

The metabolic capabilities of PK-deficient erythrocytes in vitro vary considerably. Although resting HMP shunt activity may be slightly to moderately low for cell age,[358] no significant effect on either oxidized or reduced glutathione (GSH) levels has been observed,[359,360] even after incubation with acetylphenylhydrazine.[359,361,362] However, results of the ascorbate cyanide test are abnormal in patients with PK deficiency, and stimulated HMP shunt activity is modestly depressed.[360] In many instances, glycolysis, as measured by the glucose consumption or lactate production of incubated erythrocytes, is markedly subnormal.[311,318,358] Such diminished glycolysis is relative rather than absolute because the glycolytic rate of enzymopenic cells can be increased substantially by incubation in a high-P_i medium.[318,363] A reduction of residual PK activity within the erythrocyte to 10% of normal still leaves sufficient enzyme to support normal glycolysis if potential enzyme activity is fully used. Such considerations indicate that intracellular regulators of PK function must play an important role in the reduced glycolysis characteristic of enzymopenic cells. Often, particularly with kinetic variants of PK, glycolytic rates characteristic of mature normal erythrocytes are achieved.[364-367] However, such rates are clearly subnormal in comparison to those attained by reticulocyte-rich control blood of an equivalent mean cell age.[364] Furthermore, the glucose consumption of incubated normal hemolysate is unchanged when supplemental purified PK is added, whereas the addition of supplemental PK to hemolysate from PK-deficient erythrocytes produces a substantial rise in glucose consumption.[364]

Accumulation of glycolytic intermediates proximal to the enzyme defect has customarily,[364,367] though not invariably,[364] been observed.[366] Detection of elevated 2,3-BPG or 3-phosphoglycerate levels in red cells may help confirm a clinical diagnosis of PK deficiency,[368,369] and the degree of elevation in 2,3-BPG or glucose 6-phosphate levels is directly correlated with clinical severity.[107,370] An alteration in the normal ratio of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD), as well as complex changes in the substrates governing the rate of PK and 2,3-BPG mutase, appears to be responsible for triose phosphate accumulation when glycolysis is accelerated by P_i in normal red cells.[371] Such striking elevations in triose phosphate intermediates can be returned to normal in both control and PK-deficient red cells by the addition of exogenous pyruvate or another oxidant.[362] Concentrations of both NAD and NADH are low in PK-deficient erythrocytes,[357,372] or if the level of NAD is normal in fresh cells, it falls with undue rapidity on incubation in vitro.[363] The concentration of 2,3-BPG in PK-deficient erythrocytes may be greater than three times normal values, thereby leading to a rightward shift of the oxyhemoglobin dissociation curve.[52,53] The ability to extract a greater percentage of available oxygen from hemoglobin at any given pO_2 associated with such a right-shifted curve increases the exercise tolerance of patients with PK deficiency.[53] Such patients, though anemic, may exhibit none of the expected symptoms of fatigue and exercise intolerance.

The level of erythrocyte ATP and the formation of PRPP[373] are often abnormally low in PK deficiency, although patients with reticulocyte counts greater than 25% usually have normal ATP levels. In blood with such high reticulocyte counts, ATP is unstable on incubation with glucose, in contrast to normal reticulocyte-rich blood. When incubated without glucose, however, the PK-deficient reticulocyte conserves ATP more successfully than the normal cell does.[318] The reticulocyte, able to generate ATP from substrates other than glucose via oxidative phosphorylation, can circumvent its glycolytic defect. However, in a high-glucose environment, ATP levels plummet. The PK-deficient reticulocyte is thus exquisitely dependent on oxidative phosphorylation for maintenance of ATP, as was first shown by Keitt.[318] The greater oxygen consumption of PK-deficient reticulocytes than normal reticulocytes (3.75 ± 1.55 versus 0.56 ± 0.5 mL of $O_2/10^9$ reticulocytes/hr[312]) underscores the reliance of such cells on oxidative phosphorylation. Reticulocyte oxygen consumption is abolished by hypoxia in vitro at approximately venous pO_2 levels (Fig. 17-7). Therefore, when exposed to prolonged periods of hypoxia in vivo or on maturation with consequent loss of mitochondria, the PK-deficient immature erythrocyte becomes reliant on its inadequate glycolytic apparatus, with loss of cell ATP being the inevitable consequence. In contrast, the reduced ATP needs of the mature erythrocyte may be marginally, but adequately served for a time by the diminished glycolytic activity of the PK-deficient cell.

ATP depletion greatly increases the cation permeability of PK-deficient erythrocytes.[311] In part, this is the consequence of failure of the ouabain-inhibitable ATPase cation pump, which transports approximately 1 to 2 mEq of K^+ per hour per liter of erythrocytes.[374] Although adequate membrane ATPase is present,[364,375] a net loss of 0.2 to 6.3 mEq of K^+ per hour per liter of cells occurs in freshly obtained PK-deficient blood.[316,364,374] After ATP depletion, net K^+ loss may exceed 20 mEq/hr/L of cells.[311] Failure of the cation pump cannot explain such large losses of K^+. The effect of ATP depletion on K^+ permeability, first described by Gardos and Straub,[376] is a feature of all metabolically depleted red cells and is not unique

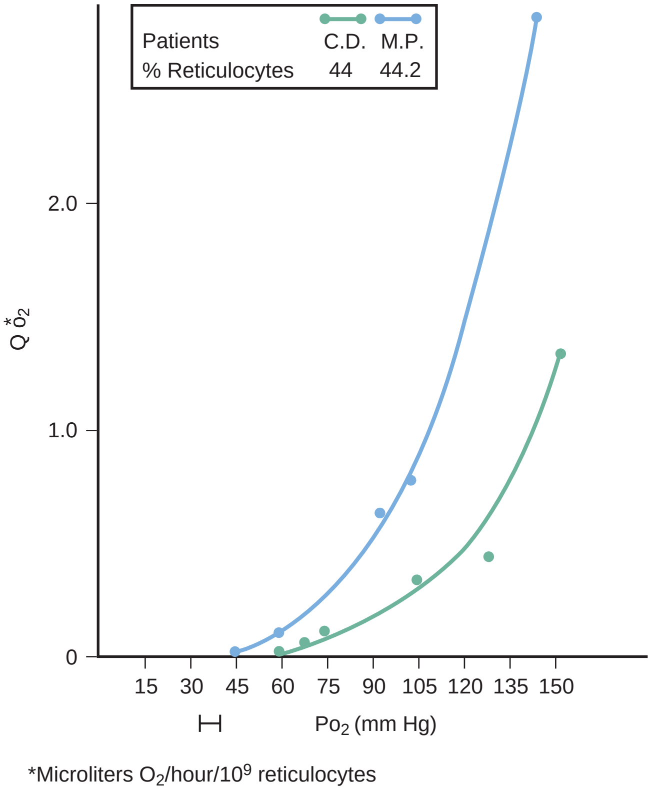

Figure 17-7 Influence of pO_2 on oxygen consumption by PK-deficient reticulocytes. The normal range for venous pO_2 is indicated by the *solid bar*. *(From Mentzer WC, Baehner RL, Schmidt-Schonbein H, et al: Selective reticulocyte destruction in erythrocyte pyruvate kinase deficiency.* J Clin Invest *50:688–699, 1971.)*

to PK deficiency. It is thought to be related to altered binding of membrane-associated Ca^{2+} and can be partially prevented by ethylenediaminetetraacetic acid or quinine, even though these agents have no direct influence on the rate or extent of ATP depletion.[311,377]

Initially in the ATP-depleted cell, potassium loss exceeds sodium gain. The resultant net loss of cations is accompanied by an obligate osmotic loss of water and a reduction in cell volume. The shrunken, crenated cells produced by ATP depletion in PK-deficient reticulocytes are shown in Figure 17-8. These spiculated cells pass, with difficulty, through 8-mm Millipore filters, and cell suspensions demonstrate increased viscosity in the Wells-Brookfield viscometer.[311] The destiny of such ATP-depleted erythrocytes, then, is to become dehydrated, rigid “desicytes” or “xerocytes,” with unfavorable characteristics that may well prematurely terminate their existence.[378] Membranes prepared from PK-deficient or normal ATP-depleted red cells are more dense than normal as a result of absorption of cytoplasmic components—in particular, an as yet unidentified 50,000-dalton protein—on the inner membrane surface.[379] Such changes in the cell membrane may contribute to the increased rigidity of these cells. There is evidence that membrane abnormalities not related to ATP depletion may also exist in PK-deficient red cells, but the role, if any, of such abnormalities in the hemolytic process is not established.[380] Not all workers have found abnormalities in the red cell membrane protein profile on sodium dodecyl sulfate–polyacrylamide gel electrophoresis,[381] and results of several other types of membrane analyses (spectrin extractability and membrane fluidity) have been normal.[382]

As enzyme-deficient erythrocytes age, a progressive reduction in glycolysis should accompany the inevitable gradual degradation of enzyme protein. Such deteriorating glycolysis eventually results in ATP depletion and subsequently, hemolysis. However, centrifuge studies have not revealed dramatic differences in the PK activity of enzymopenic young and old cells,[311] with the exception of one unstable PK variant[383] in which accelerated denaturation of enzyme protein was present in vivo, as well as in vitro. Deterioration in catalytic efficiency, reported to occur in both normal[338] and variant[384] enzymes on aging in vivo, may also hasten the demise of enzymopenic cells.

The normal or nearly normal survival of radiolabeled, severely PK-deficient erythrocytes reported by several investigators[311,365,366] indicates that diminished PK activity need not significantly curtail the life span of affected erythrocytes. Biphasic erythrocyte survival curves are sometimes obtained[385,386] and suggest that two populations of cells are present, one destined for almost immediate destruction and the other having a considerably better outlook for survival. Ferrokinetic studies[311,386] indicate that destruction of newly made erythrocytes in the bone marrow, spleen, or liver may be the major source of hemolysis in this disorder. Organ monitoring has shown that as reticulocytes are released from the marrow, some are almost immediately sequestered in the spleen. The paradoxical reticulocytosis that follows splenectomy is

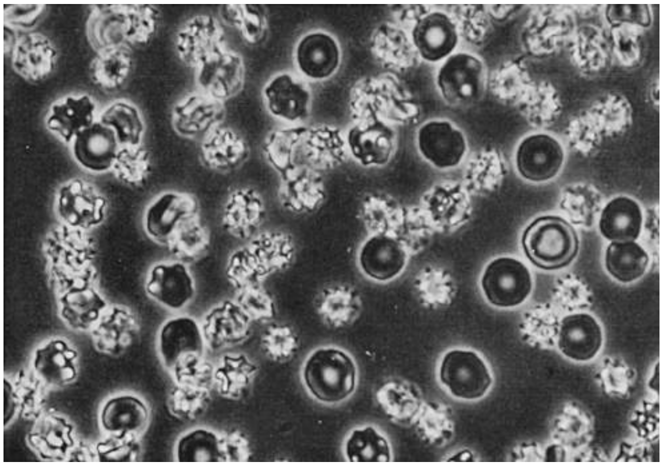

Figure 17-8 Phase-contrast micrograph of PK-deficient blood (patient C.D., whose anemia was severe after a 2-hour exposure to 5 mmol/L of cyanide to deplete ATP). The spiculated cells are, for the most part, reticulocytes (magnification ×6600). *(From Mentzer WC, Baehner RL, Schmidt-Schonbein H, et al: Selective reticulocyte destruction in erythrocyte pyruvate kinase deficiency.* J Clin Invest *50:688–699, 1971.)*

probably the consequence of improved survival of this population of reticulocytes.

Spleens removed from patients who have anemia with PK deficiency contain an unduly large number of reticulocytes.[361,387] Splenic histologic analysis shows the following results that contrast with those seen in hereditary spherocytosis: 1) the pulp spaces are empty rather than packed with erythrocytes; 2) erythrophagocytosis of reticulocytes and mature red cells by reticuloendothelial histiocytes is prominent in PK deficiency but rare in spherocytosis; and 3) many more crenated, deformed cells are seen in PK deficiency.[387,388] Studies of such cells obtained from peripheral blood have demonstrated them to be poorly deformable.[389] The hypoxic, acidic environment of the spleen would be expected to produce just such crenation in reticulocytes through the sequence of events outlined earlier: inhibition of oxidative phosphorylation, ATP depletion, selective K^+ leakage, loss of cell water, and resultant loss of cell volume. These rigid desicytes should negotiate the 3-mm fenestrations between the splenic cords and sinuses only with difficulty. Thus they are doomed to a stay of uncertain duration in the metabolically unfavorable splenic environment, and further deterioration in cell capabilities would seem inevitable. Isotope studies show that the final *coup de grace* often occurs in the liver.[390,391]

Splenic destruction of reticulocytes is a variable feature of PK deficiency; in some instances either bone marrow or liver destruction predominates. Why some reticulocytes are destroyed whereas others survive to reach maturity and thereafter have a nearly normal existence despite their enzyme defect is unclear. It is possible that chance determines which reticulocytes are detained in unfavorable metabolic circumstances. On the other hand, there is some evidence for actual variation in PK activity among reticulocytes.[311] Those with the most PK activity would be more likely to survive.

Although cellular dehydration has been given a central mechanistic role in the destruction of PK-deficient human red cells, it appears to be unimportant in the hemolytic process in PK-deficient basenji dogs. When PK-deficient dog red cells with high sodium levels are exposed to cyanide in vitro, they rapidly lose ATP. However, the ensuing loss of K^+ is balanced by an equivalent gain in Na^+, so no cellular dehydration occurs. Other mechanisms must explain the hemolysis in this setting.[390] Of possible interest in this regard is the finding of apoptosis of erythroid progenitors within the spleen of PK-deficient mice, thus suggesting a role for ineffective erythropoiesis in the anemia seen in this species[391] and perhaps in humans as well.[392]

Genetics and Inheritance

Autosomal recessive transmission of the enzyme defect is usually observed in PK deficiency. Homozygotes and compound heterozygotes exhibit hemolytic anemia. Simple heterozygotes can be distinguished from compound heterozygotes or homozygotes by the degree of reticulocytosis, the extent of accumulation of red cell glucose 6-phosphate, and the in vitro properties of the mutant PK.[393,394] Although simple heterozygotes usually remain clinically normal despite an approximately 50% reduction in erythrocyte PK activity, some may exhibit evidence of mild hemolysis.[394,395] Population surveys in different ethnic groups based on assays of red cell PK activity have estimated the incidence of PK heterozygosity to be 0.14% to 6%.[396-399,400,401] Because activity assays may sometimes falsely identify heterozygotes as normal and vice versa, DNA analysis should provide a more accurate estimate of the prevalence of PK mutations. A survey based on DNA analysis reported the rate of occurrence of heterozygosity in white individuals for the *C1456→T* mutation, the most common PK mutation in southern Europe, to be 3.5×10^{-3} and that for the *G1529A* mutation to be 2.03×10^{-3}. In African-Americans, the rate of occurrence of heterozygosity was 3.90×10^{-3} for the *C1456→T* mutation, and in Asians, it was 7.94×10^{-3} for the *C1468→T* mutation.[293] Evidence in PK-deficient mice with chronic hemolytic anemia suggests that PK deficiency may play a protective role against malaria.[402] In vitro studies with *Plasmodium falciparum*-infected red cells from human PK-deficient homozygotes and heterozygotes show reduced parasite invasion in homozygotes and preferential clearance of infected cells by macrophages in both homozygotes and heterozygotes. Using fluoride to inhibit enolase and reduce ATP levels in normal human erythrocytes, thereby simulating the metabolic defect that characterizes PK deficiency, a relationship was found between the degree of ATP depletion and reduced invasion by *P. falciparum* as well as enhanced clearance by macrophages. Thus a similar protective effect to that seen in mice, possibly mediated by ATP deficiency, may be present in humans.[399,403] Emerging population studies from malarial regions of Africa report a higher than expected incidence of PK deficiency, consistent with the hypothesis that, as in G6PD deficiency and sickle cell trait, PK heterozygotes are protected from malaria but confirmation requires much larger sample sizes.[404,405]

There is little correlation between the severity of anemia in homozygotes and the level of erythrocyte PK activity as measured by the conventional in vitro assay system,[406] unless enzyme activity is corrected for the degree of reticulocytosis present.[370] Variable clinical severity is explained, at least in part, by the existence of numerous mutant forms of the enzyme whose differing properties result in variable degrees of hemolysis. Mutants are distinguishable in vitro on the basis of maximal activity, electrophoretic mobility, substrate kinetics, stability, immunologic properties, and response to the activator FDP. In general, mutants with unfavorable kinetics are usually associated with more severe hemolysis.[370,407,408] Diminished thermal stability of the enzyme in vitro also seems important in determining clinical severity.[406] Homozygous frameshift, promoter, or deletion mutations that greatly reduce or completely abolish PK activity generally lead to such severe phonotypes as intrauterine death or life-threatening neonatal anemia, as does inheritance of a single copy of such deleterious mutations along with another severe PK gene.[409-413]

International standards for the characterization of mutant PK phenotypes have facilitated the comparison of mutants studied in different laboratories.[414] In compound heterozygotes, full characterization is difficult because tetramers formed from varying proportions of the two different mutant subunits are present, each with unique properties.[407] The best-defined phenotypes are those found in true homozygotes,[415] who are usually the offspring of consanguineous matings. The kinetic and electrophoretic abnormalities characteristic of each variant PK reflect underlying structural changes in the enzyme secondary to point mutations or deletions. A total of at least 190 mutations in the *PKLR* gene associated with chronic hemolytic anemia have been defined at the nucleic acid level,[406,413,415-418] Single-nucleotide missense mutations (including 2 in the promoter region) account for the majority of the total. The rest are splice site mutations, stop codon mutations, large and small deletions, frameshifts, or insertions. Additional PK mutations are known, but because they are present only as single copies in patients with chronic anemia, their role in the pathophysiology of hemolysis has not been defined.[406,416,417] In the basenji dog, PK deficiency is attributable to a frameshift mutation (deletion of C at nucleotide 433),[419] and in PK-deficient mice, a missense mutation in R-PK (338Gly→Asp) is responsible for the enzymopathy.[298] Most PK mutations are extremely rare and usually limited to a single family. Exceptions are the *G1529→A* and the *C1456→T* substitutions, common in European patients with PK deficiency,[406,420-423] and the *C1151→T* substitution, common in Japanese patients.[424] The majority of the mutations are located within exon 8 or 9 (A domain), where components of the active site and the K^+ binding site are located, or in exons 10 or 11 (C domain), a region responsible for the allosteric regulatory properties of the enzyme. Although important information about genotype–phenotype correlation has been developed,[370,406,413,425-427] detailed analysis of the way in which each mutation disturbs the function of PK and produces the clinical phenotype observed has only recently become available after ascertainment of the crystalline structure of human R-type PK and the use of recombinant ribose-phosphate pyrophosphokinase (RPPK) generated in *E. coli* to study enzyme kinetics and stability.[330,406,413,428] For example, generation of the *G1529→A* (510 Arg→Gln) mutant, the most common mutation found in northern Europe, by site-directed mutagenesis produced a recombinant enzyme with normal substrate kinetics and activation by FDP but with strikingly decreased stability.[428] These studies suggest that the hemolytic anemia seen in *G1529→A* homozygotes, which can be moderate to severe, is the result of low PK activity caused by enzyme instability in vivo rather than unfavorable enzyme kinetics. The variable clinical severity seen in different *G1529→A* homozygotes remains unexplained by these molecular studies and suggests a role for epigenetic or environmental factors.[406,413] DNA analysis has indicated that several PK variants thought to be unique because of differing enzyme properties are in fact caused by a single shared mutation. For example PK Maebashi, PK Fukushima, and PK Sendai are each the consequence of a substitution of A for C at nucleotide *1261*.[406] DNA analysis has also made possible the prenatal diagnosis of PK-deficient fetuses.[429]

When kinetic abnormalities have been discovered in anemic patients, at least one parent has exhibited similar abnormalities. The other parent may also have kinetically aberrant PK[430] or is found to have a low-activity variant with normal kinetics.[431] Occasionally, it has not been possible to demonstrate an abnormality of erythrocyte PK in one[416] or both[383] parents of patients with PK activity in the homozygous deficient range. Staal and coworkers[383] have speculated that in some heterozygotes, a compensatory increase in synthesis of enzyme protein by the normal allele may result in enzyme activity indistinguishable from normal. The "classic" form of PK deficiency is associated with severe enzyme deficiency, persistence of the M2 isoenzyme in mature erythrocytes, and little or no R isozymes. The persistence of M2 isoenzyme may represent an attempt to compensate for the lack of R subunit–containing forms of PK.[315] An analogy to the persistence of fetal hemoglobin synthesis in β-thalassemia has been made by Miwa.[432] When M2 compensation is incomplete or absent,[411] PK activity in mature red cells is low and hemolytic anemia ensues. When M2 isoenzyme is synthesized at a higher rate, greater than normal amounts of PK activity can accumulate in mature red cells.[333,433-436] Such patients do not have hemolytic anemia, and in fact the increased glycolytic flow through PK at the expense of the 2,3-BPG–generating pathway may lower 2,3-BPG levels, increase the affinity of hemoglobin for oxygen, and result in erythrocytosis. Similarly, mice bearing a mutant form of R-type PK exhibit a delayed onset of hemolytic anemia after birth that coincides with delayed switching from the M2 isoenzyme to the mutant R isoenzyme.[437]

A kindred in which hemolytic anemia occurred in heterozygotes for PK mutants was studied by Etiemble and colleagues.[438] PK activity was well below the 50% activity (17% to 45%) usually encountered in the presence of one normal and one mutant PK allele. It was thought that perhaps the presence of only one mutant subunit in the PK tetramer might be sufficient to reduce its catalytic function, with greater impairment found in the presence of additional mutant subunits. Similar considerations were raised in another kindred (PK Greensboro), in which heterozygotes exhibited less than 50% of the normal red cell PK activity but no hemolytic anemia.[439] However, in neither kindred was the presence of multiple combinations of mutant and normal subunits (i.e., M4, M3N, M2N2, MN3, and N4, where M is the the mutant subunit and N is the normal subunit) actually confirmed.

To date no evidence suggests interaction between PK deficiency and most other disorders of the erythrocyte such as β-thalassemia minor,[440] band 3 deficiency,[441] or G6PD deficiency.[314] Markedly increased involvement of the kidneys was noted at autopsy in a patient with Gaucher disease who also exhibited hemolytic anemia and deficiency of erythrocyte PK. It was suggested that cerebroside production was enhanced as a result of hemolysis.[442] Several individuals who inherited both severe PK deficiency and sickle cell trait had symptoms suggestive of sickle cell disease, possibly related to changes in oxygen affinity associated with high 2,3-BPG levels.[443,444]

Therapy

Although complete cure is not achieved by splenectomy, elimination or amelioration of transfusion requirements, a decrease in bilirubin level, and an increase in hemoglobin concentration are seen quite often. Even though splenectomy may be lifesaving in individuals with severe anemia,[361,366] the procedure may have no effect in individuals with mild anemia. When significant morbidity exists, it would seem reasonable to recommend splenectomy, bearing in mind the fact that the degree of benefit cannot be predicted with certainty.[445] The results of therapeutic intervention with agents that either circumvent the metabolic aberrations induced by the defective enzyme or directly modify enzyme activity have generally been disappointing, although a recent abstract reports normalization of metabolic activity in red cells from PK deficient patients by a small molecule, AG-348.[344,383,446-448,449a]

Although hemolytic crises are uncommon in patients with PK deficiency and have not been associated with drug ingestion, Glader[290] demonstrated a potential hazard with the use of large doses of salicylate in patients with severe PK deficiency. Salicylates inhibit oxidative phosphorylation and thus cause ATP depletion and cellular dehydration in severely PK-deficient reticulocytes in vitro. The salicylate doses used by Glader were high but were equivalent to serum levels achieved with chronic salicylate therapy for disorders such as rheumatoid arthritis. It is prudent to select alternative therapy for such patients when possible or to monitor them carefully for signs of increased hemolysis.

Bone marrow transplantation has permanently corrected the hemolytic anemia seen in PK-deficient basenji dogs,[449] in PK-deficient mice,[450,451] and in a single human with PK deficiency.[452] The potential feasibility of gene therapy has been demonstrated in PK-deficient mice.[453,454,455]

LACTATE DEHYDROGENASE DEFICIENCY

Clinical Manifestations, Biochemistry, and Genetics

Partial or complete absence of the H subunit of lactate dehydrogenase (LDH) in erythrocytes, leukocytes, platelets, and serum is not associated with anemia or hemolysis.[456] M-subunit deficiency, though associated with exertional myoglobinuria and a characteristic skin eruption, also does not lead to hemolytic anemia.[457] In contrast to humans, mice homozygous for a low-activity mutation of the skeletal muscle subunit of LDH have less than 10% of normal enzyme activity in erythrocytes and exhibit severe lifelong hemolytic anemia.[458]

ABNORMALITIES OF ERYTHROCYTE NUCLEOTIDE METABOLISM

In individuals with CNSHA, low erythrocyte ATP levels often play a central role in the pathogenesis of hemolysis. Hemolytic anemia associated with an unusually high erythrocyte ATP level has also been described.[459] Red cell PK levels were normal in the affected pedigree, so the mechanism for ATP elevation would appear to differ from that found in other families with high red cell ATP levels, low 2,3-BPG, erythrocytosis, and a twofold or greater elevation in PK activity.[433-435] In yet another family, two infants with hemolytic anemia, high erythrocyte ATP levels, and low 2,3-BPG levels had reduced 2,3-BPG phosphatase activity.[460] The relationship of this enzyme abnormality to the unusual elevation in erythrocyte ATP level or to hemolysis is uncertain. In the three disorders of erythrocyte nucleotide metabolism to be described in the text that follows, evidence of a relationship between the abnormal enzyme and hemolytic anemia is more convincing, although even in these disorders much remains to be learned about the pathogenesis of hemolysis.

Pyrimidine 5′-Nucleotidase Deficiency

Clinical Manifestations

Deficiency of erythrocyte P-5′-N, the most common inherited abnormality of nucleotide metabolism causing hemolytic anemia, is associated with lifelong anemia of moderate severity.[10,461-470,471,472] Splenomegaly is usually present, and iron overload may occur in adults.[471] Developmental delay is sometimes seen, but whether it is a direct result of P-5′-N deficiency has not been determined. Pronounced basophilic stippling, which may occur in as many as 5% of all erythrocytes, is an important and useful finding and an exception to the usual lack of distinguishing morphologic abnormalities in erythroenzymopathies. Definitive diagnosis requires assay of red cell P-5′-N activity.

Biochemistry

Reticulocyte maturation requires disposition of intraerythrocytic ribosomal RNA, which is no longer required for protein synthesis. The hydrolysis of pyrimidine nucleotides (cytidine monophosphate [CMP] and uridine monophosphate [UMP]) formed by the action of ribonucleases on ribosomal RNA, an essential step in RNA degradation, is catalyzed by P-5′-N. The cytidine and uridine formed can diffuse across the cell membrane, whereas the pyrimidine nucleotide substrates of the nucleotidase reaction are incapable of diffusion and accumulate within the cell when activity of the nucleotidase is subnormal.[10]

Hirono and associates[473] found two different isoenzymes of P-5′-N in hemolysates from normal subjects and those with P-5′-N deficiency. Only one isoenzyme, CMP responsive, was deficient in all five subjects evaluated with P-5′-N deficiency, whereas the other, deoxythymidine monophosphate (dTMP) responsive, was normal. Kinetic and thermostability abnormalities were seen in the CMP-responsive isoenzyme from subjects with P-5′-N deficiency but not in the dTMP-responsive isoenzyme. Apparently, the lack of overlapping substrate sensitivities makes it impossible for the residual normal isoenzyme to substitute for its deficient partner in enzymopenic individuals.

The amount of nucleotides in P-5′-N–deficient red cells is increased by 1.3 to 5.0 times, chiefly as a result of an increase in pyrimidine derivatives (CMP, cytidine diphosphate [CDP], cytidine triphosphate [CTP], UMP, uridine diphosphate, and uridine triphosphate [UTP]).[10,463,464,474,475] The most abundant derivatives are the diphosphodiesters CDP-choline and CDP-ethanolamine.[473] Accumulation of pyrimidine derivatives is undoubtedly caused in part by RNA degradation,[464] but it may also reflect de novo synthesis of these compounds from the uridine and orotate transported into the red cell.[476] Classification of P-5′-N deficiency as a high-ATP syndrome was based on early observations of unusually high ATP levels in cells, as measured with an enzymatic assay of ATP content that also reflected the presence of other nucleotide triphosphates, notably CTP and UTP. More specific assays have subsequently shown that cell adenine nucleotide levels are normal or even low rather than elevated.[10,465,475]

The extraordinary accumulation of pyrimidine nucleotides within P-5′-N–deficient red cells is easily detected by subjecting cellular extracts to MRI[477] or to ultraviolet spectroscopy. Extracts from normal cells exhibit an absorbance peak at 255 to 260 nm (caused almost entirely by the presence of adenine nucleotides). In P-5′-N deficiency, a higher absorbance peak slightly shifted in position (266 to 270 nm) is observed as a result of the presence of large amounts of pyrimidine nucleotides and provides a relatively simple means of screening patients for the disorder.[10,464] Measurement of inorganic phosphorus liberated from uridine monophosphate by P-5′-N is an equally simple approach to screening.[478] The secondary effects of P-5′-N deficiency are complex. For example, the concentration of GSH is regularly increased by a factor of 1.5 to 2.3,[468] a finding that may help confirm the diagnosis in patients suspected of having P-5′-N deficiency. Red cell PRPP synthetase activity is markedly low (15% to 35% of normal) in P-5′-N–deficient red cells.[479,480] In fact, PRPP synthetase deficiency was originally thought to be the primary defect responsible for the hemolysis. It is now regarded as a secondary phenomenon.[470] Pyrimidine nucleotides have been shown to bind and sequester magnesium, a cofactor required for subunit aggregation and maximal activation of PRPP synthetase.[481] Magnesium depletion (by the addition of pyrimidine nucleotides) also inhibits PK and pentose phosphate pathway activity in hemolysates. On the other hand, red cell magnesium levels are normal or even elevated in P-5′-N–deficient red cells, and incubation of these cells with exogenous magnesium does not reduce their susceptibility to autohemolysis or to Heinz body formation.[482] The role of magnesium deficiency (or unavailability) in the central abnormalities that limit the life span of P-5′-N–deficient red cells is not clear.

In fact, it is not known why P-5′-N–deficient red cells are destroyed prematurely. Perhaps the pyrimidine nucleotides that accumulate interfere with the normal function of key glycolytic enzymes by competing for available binding sites with the adenine nucleotides that are the normal enzyme substrate. The possible importance of pyrimidine nucleotides in hemolysis is underscored by the report of an individual with chronic hemolysis, basophilic stippling, and normal P-5′-N activity, in whom the only biochemical abnormality detected was a striking elevation in erythrocyte CDP-choline.[483] Accumulation of pyrimidine nucleotide has been shown to lower red cell pH in P-5′-N–deficient red cells as a result of shifts in Donnan equilibrium caused by the increase in fixed intracellular anion.[468,474] This drop of 0.1 to 0.2 pH unit is sufficient to increase the red cell's oxygen affinity above the normal range[475] and may also explain the slightly subnormal glycolytic rate of P-5′-N–deficient red cells.[484] Tomoda and colleagues[435] demonstrated a moderate impairment in stimulated pentose phosphate pathway activity in P-5′-N–deficient erythrocytes, which they attributed to inhibition of G6PD by pyrimidine nucleotides. This impairment in shunt activity is noted in both light and dense red cells separated by centrifugation and is accompanied by a parallel decrease in G6PD activity.[485] It is hard to visualize how pentose phosphate pathway failure could result in the unusually high levels of GSH characteristic of P-5′-N deficiency because the opposite effect, a deficiency in GSH, would be predicted.[468]

Study of individuals with lead poisoning has confirmed the central role of P-5′-N deficiency in the origin of hemolytic anemia. P-5′-N is markedly inhibited in vitro by low concentrations of lead, and the red cells of patients with significant lead poisoning have depressed levels of P-5′-N activity.[486] The basophilic stippling that is present in some patients with lead poisoning has been attributed to nucleotidase deficiency.[487] Although accumulation of pyrimidine nucleotides within erythrocytes is not found in all patients with lead poisoning in which P-5′-N activity is reduced, it is regularly found in patients exhibiting acute lead-induced hemolytic anemia.[486,487] Perhaps because of the shorter duration or less severe character of the enzyme deficiency, the red cells of lead-poisoned individuals do not exhibit RPPK deficiency.[487] GSH levels are normal[487] or elevated.[464] Despite the less-than-perfect homology between congenital and acquired P-5′-N deficiency, study of the latter may be expected to provide important insight into the mechanisms of hemolysis in this disorder.

Genetics and Inheritance

The gene for P-5′-N is located on chromosome 7 and consists of 11 exons. Alternative splicing leads to the production of three proteins: one is 286 amino acids long (P-5′-N I), the second is 297 amino acids long, and the third, found only in reticulocytes, is 285 amino acids long.[488,489] P-5′-N I is the major isoenzyme expressed in erythrocytes. Twenty-seven different mutations in P-5′-N I have been identified in patients with P-5′-N deficiency and chronic hemolytic anemia.[472] Nine are missense mutations, two are in-frame deletions that lead to loss of a single amino acid, and the remaining 16 are nonsense mutations, splice site alterations, insertions, or larger deletions.[471]

Approximately 100 patients with P-5′-N deficiency and hemolytic anemia have been described.[470-472,490] The disorder has been found in many ethnic groups.[471] Inheritance is autosomal recessive.[464,471] Nearly all patients who have P-5′-N deficiency and exhibit hemolytic anemia are homozygous for a single P-5′-N mutation; the remainder are compound heterozygotes for two different mutations.[471] Heterozygotes have no clinical manifestations but exhibit a reduction of about 50% in red cell P-5′-N activity.[10,464,470,471] The wide range of enzyme activity encountered in normal individuals makes detection of heterozygotes difficult in some families.[491] Variation in the severity of disease is, in part, the result of heterogeneity in the molecular nature of the defective enzyme.[462,473,492] This has been shown most clearly by study of the biochemical properties of purified recombinant mutant P-5′-N.[472,493] These studies also revealed that despite profound abnormalities in P-5′-N kinetics or stability in vitro, erythrocytes containing certain P-5′-N mutations exhibited only a moderated reduction in P-5′-N activity, thus suggesting that other pathways or other P-5′-N isoenzymes could compensate for P-5′-N I deficiency. P-5′-N has been found in the spleen,[492] kidney, and brain.[494,495] The presence of the enzyme in the brain may be relevant to the mental retardation noted in several individuals who have P-5′-N deficiency with hemolytic anemia.[465]

Therapy

The congenital form of P-5′-N deficiency must be distinguished from the acquired form associated with lead poisoning because specific therapy is available for the latter. The benefit of splenectomy in congenital P-5′-N deficiency is limited.[470] Investigational therapeutic use of allopurinol in one affected individual had no effect on hemolysis and actually increased erythrocyte pyrimidine nucleotide levels.[476]

Adenylate Kinase Deficiency

Clinical Manifestations, Biochemistry, and Genetics

Controversy exists regarding the exact role of adenylate kinase (AK) deficiency in hemolytic anemia. Beutler and colleagues[496] studied two African-American siblings whose red cells lacked measurable AK activity. One had hemolytic anemia, but the other did not. It was suggested that in this family the hemolysis was unrelated to AK deficiency or that another coexistent defect was required for hemolysis to occur. The latter seemed to be the case in an Arab family in which two siblings had severe red cell AK deficiency but only the sibling who had also inherited severe G6PD deficiency exhibited hemolysis.[497] However, further investigation of this large pedigree disclosed six more individuals with severe AK deficiency and mental retardation.[498] All had chronic nonspherocytic hemolytic anemia. Three had also inherited G6PD deficiency and were severely anemic, with red cell transfusions required 6 to 10 times annually. The other three, who did not have G6PD deficiency, were moderately anemic and only occasionally required transfusions. Family members who had carrier levels of red cell AK activity (50% of normal), either with or without G6PD deficiency, and individuals with G6PD deficiency alone did not have chronic hemolytic anemia. These results suggest that either AK deficiency has a direct role in hemolysis or the enzyme defect is a marker for another genetically linked but as yet unidentified defect that is the primary cause of the anemia.[499] Lachant and colleagues[499] found no AK activity in the red cells of a Syrian girl who had chronic hemolytic anemia. Only modest impairment in the formation of ADP from adenosine monophosphate was observed in intact cells, thus suggesting that alternative pathways might be available to substitute for the missing AK. Alternatively, it was proposed that AK itself might be present in intact cells but somehow became inactivated during preparation of a hemolysate for assay of enzyme activity. Eight additional patients with AK deficiency associated with hemolytic anemia have been reported in France,[500] Japan,[501,502] Spain,[503] and Italy.[504-506] Molecular study of these patients has defined three missense, two in-frame single-codon, and two nonsense mutations. Site-directed mutagenesis of wild type AK1 cDNA followed by expression of mutant enzyme proteins in an *E. coli* system and purification of enzyme protein allowed characterization of five of these mutations. Marked abnormalities in enzyme stability and catalytic properties were found.[507] Modeling studies using the structure of porcine AK, which is highly homologous to human AK, have begun to define the structural basis for mutation-associated defects in enzyme function.[503] The Japanese subject inherited a missense mutation (Arg128→Trp) in the coding region of the *AK1* gene from her mother and a normal *AK1* allele from her father. Although both mother and child were heterozygous for AK deficiency, only the child exhibited hemolytic anemia, and the mother was hematologically normal.[502] An Italian girl homozygous for a missense mutation at codon 164 had virtually undetectable erythrocyte AK activity and moderate hemolytic anemia.[504,505] Another Italian girl, who also had moderate hemolytic anemia, was homozygous for a deletion that created a frameshift with a premature stop codon. Even though no erythrocyte AK mRNA was detectable, thus implying rapid degradation of the truncated mRNA, a low level of AK enzyme activity was present in hemolysates from the propositus. Evidence of activity of other AK isozymes, not usually present in erythrocytes, was obtained, and this residual AK activity was thought to have mitigated the clinical severity of the enzymopathy.[506] Two siblings in a third Italian family who were homozygous for a nonsense mutation at codon 107 (*CGA*→*TGA*) had no detectable erythrocyte AK activity and mild to moderate chronic hemolytic anemia.[505] Both siblings also exhibited psychomotor retardation. Similar findings in other families[498,500] suggest that AK deficiency may be shared by the red cell and the brain.

Therapy

Splenectomy led to prompt disappearance of anemia and hemolysis in five of six Arab patients.[498]

Adenosine Deaminase Overproduction

Clinical Manifestations, Biochemistry, and Genetics

Fourteen individuals in three families have been found to have hereditary hemolytic anemia, sharply diminished amounts of intraerythrocytic adenosine nucleotides (to less than 50% of normal), and a remarkable 45- to 110-fold increase in red cell ADA activity.[508-511] In contrast to virtually all other erythroenzymopathies, ADA excess is transmitted as an autosomal dominant trait. Hemolysis is apparently the consequence of diminished red cell

ATP content, which is caused by the diversion of nearly all adenosine metabolism through the unusually active ADA reaction at the expense of the competing adenosine kinase reaction. The former results in irreversible loss of adenosine nucleotides, whereas the latter conserves such nucleotides and preserves cell ATP stores. The finding of abnormalities of lesser magnitude in other enzymes of nucleotide metabolism in affected individuals suggests that the excess ADA activity may be caused by an as yet undefined primary defect. The physical properties of the mutant ADA are normal,[510,512] which indicates that the great excess of ADA activity is caused by overproduction rather than an increase in catalytic efficiency. In one subject, an increase in red cell ADA protein equivalent to the striking increase in ADA activity was demonstrated by immunoblotting.[513] Synthesis of ADA by erythroid progenitors grown from bone marrow cells was 11-fold greater in an affected individual.[514] Ribonuclease (RNase) mapping techniques and Northern blotting have revealed at least a 100-fold increase over normal in the ADA mRNA content of affected reticulocytes. Sequencing of ADA cDNA showed no abnormalities in the coding region and 5′- and 3′-untranslated regions of the parent mRNA. Examination of genomic DNA by Southern blotting did not disclose evidence of gene amplification, deletion, or gross rearrangements. Thus although the basis for ADA excess appears to be an overabundance of apparently normal mRNA, the mechanism underlying this abnormality remains obscure.[515-517] Much lower increases in ADA activity (approximately fourfold) are seen in congenital hypoplastic anemia,[518,519] arthrogryposis multiplex congenita,[520] acquired immunodeficiency syndrome,[521] and cartilage-hair hypoplasia.[522] The origin and implications of these increases are obscure, but they are not associated with hemolysis.

ACQUIRED DISORDERS OF ERYTHROCYTE GLYCOLYSIS

Alterations in the external chemical milieu may profoundly influence erythrocyte metabolism. The rate of glycolysis, for example, is governed by the availability of P_i. High concentrations of P_i augment and low concentrations impede the glycolytic synthesis of ATP and 2,3-BPG. Erythrocytes from uremic patients with hyperphosphatemia contain an average of 70% more ATP than normal erythrocytes do, and a lesser but significant increase in 2,3-BPG concentration is also found in the hyperphosphatemic cell.[523] Conversely, hypophosphatemia is associated with a reduction in erythrocyte organic phosphates. Red cell glucose metabolism is compromised in patients with severe hypophosphatemia (serum phosphorus level less than 0 to 0.3 mg/dL).[523-525]ATP and BPG levels decline and hemoglobin oxygen affinity increases.[526,527] If the red cell ATP concentration falls to very low levels (10% to 20% of normal), a severe hemolytic anemia characterized by marked microspherocytosis, spheroacanthocytosis, and red cell rigidity results[525,528-530] Hemolysis is reversible after phosphate repletion and ATP regeneration. Severe hypophosphatemia can occur with gastrointestinal malabsorption, use of phosphate binders,[528] starvation, diabetes mellitus, increased urinary losses resulting from tubular dysfunction, hyperalimentation with low-phosphate nutrients,[524] and after nutritional repletion (e.g., refeeding in anorexia nervosa[531] or in severe malnutrition[532]).

Some investigators have found that experimental magnesium deficiency in the rat resembles hypophosphatemia in that erythrocyte glycolysis is inhibited, ATP and 2,3-BPG levels are subnormal, and red cell rigidity is increased. Spherocytes are evident on the blood smear, and red cell survival is reduced.[533] Magnesium is essential for the normal function of a variety of glycolytic and nonglycolytic enzymes. However, using a different diet to induce magnesium deficiency, Piomelli and coworkers[534] showed that although hemolytic anemia accompanies magnesium depletion in rats, erythrocyte glycolysis and the activity of red cell glycolytic enzymes remain normal. Magnesium deficiency also influences other red cell components, notably the membrane,[535] and the relative contribution of such abnormalities to shortened red cell life span remains undefined.

Iron deficiency not only decreases the production of red cells but also accelerates their destruction.[536] Studies of the metabolic properties of iron-deficient red cells have revealed several abnormalities that might reduce cell viability. Although erythrocyte glycolysis in vitro is normal for cell age,[537] cell ATP is unstable on incubation. The ATP concentration in freshly obtained red cells may be either normal[538] or low.[539] The spontaneous autohemolysis of iron-deficient red cells incubated at 37° C is increased.[540] The increased rigidity characteristic of an ATP-depleted cell is present in iron-deficient cells as well.[540] These findings suggest that a defect in energy metabolism may contribute to the shortened survival of iron-deficient red cells.

Alterations in erythrocyte enzyme activity are often found during the course of either acute or chronic leukemia,[541] nonmalignant pancytopenia,[541,542] congenital dyserythropoietic anemia,[543] acquired dyserythropoietic anemia,[541,544] myeloid metaplasia,[541] and polycythemia vera.[541] One, several, or many enzymes may be involved, and activity may be either increased or decreased. The unusual pattern of enzyme activity may, on occasion, be useful in diagnosis, for example, to distinguish congenital hypoplastic anemia from transient erythroblastopenia of childhood.[545]

Several quite different processes appear to be responsible for the development of acquired enzymopathies.[546] Reversion to fetal hematopoiesis may alter enzyme function.[547] Posttranslational changes in normal enzyme protein have been demonstrated.[548] Synthesis of enzyme protein by developing erythroid cells may be impaired, perhaps as a result of alterations in chromosome number or disorderly and dyssynchronous nuclear maturation. Although some components of the abnormal pattern of enzymes (such as low PFK or high enolase activity) may suggest a reversion to fetal erythropoiesis, others do not. The possibility that these enzyme abnormalities may be familial and antedate the onset of an acquired blood disorder has not been fully explored.

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CHAPTER 18

Glucose-6-Phosphate Dehydrogenase Deficiency

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CHAPTER OUTLINE

G6PD IN RED BLOOD CELL METABOLISM
Structure and Biochemistry of G6PD
Genetics of G6PD
Features of G6PD in Red Blood Cells
Molecular Basis of G6PD Deficiency
Epidemiology of G6PD Deficiency
CLINICAL MANIFESTATIONS OF G6PD DEFICIENCY
Acute Hemolytic Anemia
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LABORATORY DIAGNOSIS OF G6PD DEFICIENCY
Tests for G6PD Deficiency
GENOTYPE-PHENOTYPE CORRELATIONS
G6PD DEFICIENCY AND PREVENTIVE MEDICINE
G6PD DEFICIENCY IN NONERYTHROID CELLS
G6PD DEFICIENCY COEXISTING WITH OTHER DISORDERS
Association with Other Hematologic Diseases
Association with Nonhematologic Diseases
G6PD POLYMORPHISM AND MALARIA

Most hemolytic anemias can be categorized, at least at first approximation, as being either inherited or acquired as a result of either intracorpuscular or extracorpuscular causes. Hemolytic anemia associated with deficiency of glucose-6-phosphate dehydrogenase (G6PD) glaringly defies this categorization. Indeed, most persons with inherited G6PD deficiency have no anemia and almost no hemolysis; both develop only as a result of challenge by exogenous agents. Because the metabolic role of G6PD in red blood cells is primarily related to its reductive potential, the threat to G6PD-deficient red blood cells is oxidative damage. Therefore to understand hemolytic anemia associated with G6PD deficiency, we first need to define the physiologic role of G6PD[1-3] and pinpoint why the red blood cells are deficient in G6PD.[4,5,6]

G6PD IN RED BLOOD CELL METABOLISM

In metabolic maps, G6PD is commonly referred to as the first enzyme of the hexose monophosphate shunt, or the pentose phosphate pathway (Fig. 18-1). Although these time-honored phrases persist in textbooks, it is now clear that the main role of G6PD is not glucose utilization (G6PD accounts for less than 10% of that function); rather, its main role is production of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH has a crucial role in preventing oxidative damage to proteins and other molecules in all cells (Fig. 18-2).[7,8] This role is particularly crucial in red blood cells because, being oxygen carriers par excellence, they have a literally built-in danger of damage by oxygen radicals generated continuously in the course of methemoglobin formation.[9] The highly reactive oxygen radicals either decay spontaneously or are converted by superoxide dismutase to hydrogen peroxide (H_2O_2), which is still highly toxic. Detoxification of H_2O_2 to H_2O is effected by catalase[10] and glutathione peroxidase[11] (GSHPX). NADPH is crucial for the function of both these enzymes; it is a structural component of catalase[12] and is required as a substrate by glutathione reductase, which regenerates reduced glutathione (GSH) when it has been oxidized to oxidized glutathione by GSHPX (see Fig. 18-2). G6PD-deficient red blood cells are highly vulnerable to oxidative damage (discussed later in this chapter), even though G6PD deficiency is never complete in humans. When complete G6PD deficiency was produced in mouse embryonic stem cells by targeted inactivation of the G6PD gene,[13] the G6PD-null cells thus obtained were viable, but they formed colonies only in a low-oxygen environment; even so, they had an impaired capacity to form erythroid colonies.[14] When G6PD-null embryonic stem cells were injected into mouse blastocysts, chimeric embryos were obtained, and germline transmission was achieved; however, only female heterozygous mice were obtained because hemizygous male embryos died.[15] Thus a G6PD-null mutation is an embryonically lethal condition.

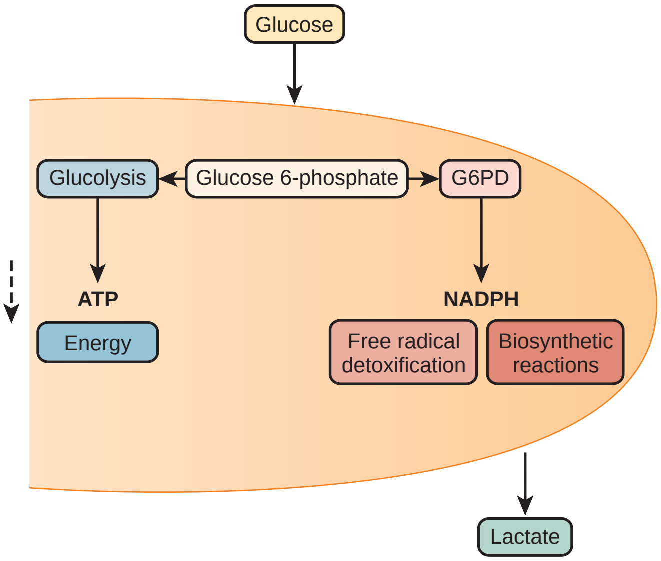

Figure 18-1 The role of glucose-6-phosphate dehydrogenase *(G6PD)* in red blood cell metabolism. As a somewhat crude oversimplification, after phosphorylation to G6P, glucose can be visualized as being at the bifurcation between two major pathways: glycolysis, which produces adenosine triphosphate *(ATP)*, and the pentose phosphate pathway, which generates reducing power (reduced nicotinamide adenine dinucleotide phosphate *[NADPH]*). Under "normal" conditions, probably less than 1% of G6P enters the pentose phosphate pathway, whereas under maximal oxidative stress, the amount may reach 10%.

Structure and Biochemistry of G6PD

G6PD is a ubiquitous enzyme that must be quite ancient in evolution because it has been found in all organisms, from prokaryotes to yeasts, protozoa, plants, and animals.[2,16,17] In mammals, G6PD is a good example of a housekeeping enzyme produced by a housekeeping gene; indeed, the gene is expressed in all cells of the body. G6PD is a typical cytoplasmic enzyme, although some G6PD activity is associated with peroxisomes in liver and kidney cells.[18] This finding is consistent with the view that these organelles have evolved as part of the need of early eukaryotes to defend against oxygen, which is germane to the role of G6PD today.

The enzymatically active form of G6PD is either a dimer or a tetramer (Fig. 18-3) of a single polypeptide subunit of about 59 kD.[19] The complete primary structure of the human enzyme has been deduced from the sequence of a full-length complementary deoxyribonucleic acid (DNA) clone.[20] The amino acid sequence of rat liver G6PD shows 94% homology to the human sequence and provides evidence that the N-terminal amino acid is *N*-acetylalanine, which must result from posttranslational cleavage of the N-terminal methionine. The same is probably true of the human enzyme.[21]

Extensive data are available on the kinetics of G6PD. Its coenzyme specificity is exquisite; human G6PD has practically no activity with nicotinamide adenine dinucleotide. Its substrate specificity is also very high because activity on other hexose phosphates (e.g., mannose 6-phosphate or galactose 6-phosphate) is negligible. The affinity for nicotinamide adenine dinucleotide phosphate (NADP) is about one order of magnitude higher than the affinity for glucose 6-phosphate (G6P).[2,16]

In the three-dimensional structure of G6PD (see Fig. 18-3),[22-26] one sees that the G6P binding site is near K205, and the critical role of this amino acid in electron transfer[27] has been confirmed by showing that replacing it with threonine nearly abolishes catalytic activity.[28] The NADP binding site is located near a fan of β-sheet structures, with a critical G-X-X-G-X-X peptide motif corresponding to amino acids 38 to 43 encoded in exon 3. Interestingly, the presence of a molecule of "structural" NADP, which had been suggested by early biochemical work,[29] has been confirmed.[24]

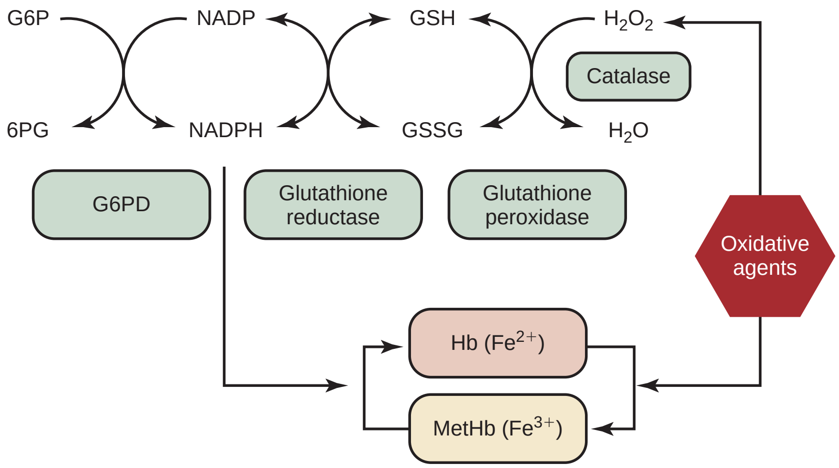

Figure 18-2 Glucose-6-phosphate dehydrogenase *(G6PD)* and the glutathione *(GSH)* cycle. The front-line defense against oxidative damage by hydrogen peroxide *(H_2O_2)* is GSH by means of GSH peroxidase. GSH peroxidase uses up GSH, and its regeneration can be effected in the red blood cell only through GSH reductase (GSSGR) by reduced nicotinamide adenine dinucleotide phosphate *(NADPH)*, which is ultimately provided by G6PD. *G6P,* Glucose-6-phosphate; *Hb (Fe^{2+}),* ferromethemoglobin; *MetHb (Fe^{3+}),* ferrimethemoglobin; *NADP,* nicotinamide adenine dinucleotide phosphate; *6PG,* 6-phosphogluconate. *(Modified from Luzzatto L, Mehta A, Vulliamy T: Glucose-6-phosphate dehydrogenase deficiency. In Scriver C, Beaudet A, Sly W, et al, editors:* The metabolic & molecular bases of inherited disease, *ed 8, New York, 2001, McGraw-Hill, p. 4517–4553.)*

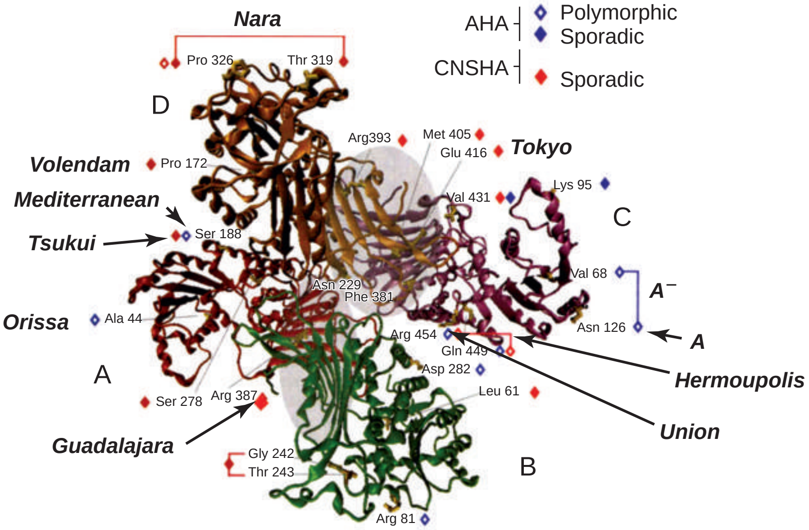

Figure 18-3 The three-dimensional structure of the human glucose-6-phosphate dehydrogenase (G6PD) tetramer. The structure of the dimer was modeled first,[22] and then the tetramer structure was solved[23,24] and the details of substrate binding were elucidated.[25,26] A to D are the four identical monomers. The tetramer is a dimer of a dimer, and both the dimer and the tetramer are enzymatically active, whereas the monomer is not enzymatically active. The monomer consists of two domains—a smaller coenzyme domain encompassing residues 1 to 198 and a larger β+α domain consisting of residues 199 to 514. The *gray background* outlines, within each dimer, the critical area of the monomer-monomer interface, in which 57 amino acid residues are involved. Only some of the many mutations known are indicated; most of those in the dimer interface cause severe G6PD deficiency with congenital nonspherocytic hemolytic anemia (CNSHA). *AHA,* Acute hemolytic anemia (see Table 18-1). *(Modified from Mason PJ, Bautista JM, Gilsanz F: G6PD deficiency: the genotype-phenotype association.* Blood Rev *21:267–283, 2007.)*

Although many natural and nonnatural substances can affect the activity of G6PD, it is not certain which ones may be important physiologically. NADPH, one product of the G6PD reaction, is a potent quasicompetitive inhibitor,[30] and because most of the coenzyme in cells is in the reduced form,[31] it can be assumed that G6PD is normally under strong inhibition. Because the normal intracellular concentrations of both G6P and NADP are lower than their respective Michaelis constant (K_m) values, it is likely that these two substrates themselves are the main regulators of intracellular G6PD activity, together with NADPH. Any oxidative event affecting the cell will alter the NADPH/NADP ratio in favor of NADP. The simultaneous increase in NADP and decrease in NADPH act additively to increase G6PD activity by increasing the substrate drive on the reaction rate and decreasing product inhibition.[8] Under most conditions this may be the most important short-term regulatory signal, although it is, of course, possible that other regulatory effects play a role as well. Recently, as part of a study of the role of microribonucleic acids in Duchenne muscular dystrophy myoblasts, it has been discovered that miR-1 is a powerful negative regulator of G6PD synthesis.[32] Although not relevant to mature red blood cells, this phenomenon may be important in determining the G6PD level in nucleated cells both physiologically and under stress conditions.

Notes on Terminology

G6PD is the accepted abbreviation for the enzyme glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49); the G6PD gene is designated *G6PD* or *Gd*.[4,16] Because *Gd* is X-linked (discussed later in this chapter), males can be only normal hemizygotes (*Gd*+) or deficient hemizygotes (*Gd*–); females can be normal homozygotes (*Gd*+/*Gd*+), deficient homozygotes (*Gd*–/*Gd*–), or heterozygotes (*Gd*+/*Gd*–). The phenotype of the last group is often referred to as "intermediate" because their overall red blood cell G6PD level usually lies in between the normal and the deficient range; however, exceptions do occur and will be discussed later in this chapter. Consequently, G6PD deficiency should not be regarded as a recessive but rather as a codominant trait. In this chapter, by *G6PD deficient,* we mean Gd^{-} males and Gd^{-}/Gd^{-} females; Gd^{+}/Gd^{-} females will have some degree of G6PD deficiency, but for clarity, we will always specify heterozygotes. According to a classification introduced in 1966 (Table 18-1), G6PD-deficient variants that result in congenital nonspherocytic hemolytic anemia (CNSHA) are designated class I; G6PD-deficient variants that do not result in CNSHA are designated class II or class III, depending on the severity of the reduction in enzyme activity in red blood cells. The separation between class II and class III is blurred and probably no longer useful. Class IV variants are those with normal activity. Class V was reserved for variants with increased activity, but after an initial report (G6PD Hektoen[33]), none has been found. In practice, because the majority of G6PD-deficient persons are mostly asymptomatic, their G6PD deficiency is referred to as mild, simple, or common (corresponding to class II or III); the minority of persons who have CNSHA are referred to as having rare, sporadic, or severe G6PD deficiency (corresponding to class I).

When a diagnostic test for G6PD is carried out, the phrase "positive result" is sometimes used to indicate that the test has shown G6PD deficiency. Use of this phrase has caused confusion, and its use should be avoided;

TABLE 18-1 Heterogeneity and Clinical Expression of Glucose-6-Phosphate Dehydrogenase Deficiency

Class*	Clinical Manifestations†	G6PD Activity (% of Normal)	No. of Known Mutant Alleles‡	Examples	Comments
IV	None	>85	2	A,B	G6PD B is the normal "wild type"
II + III	Asymptomatic in the steady state, but risk for NNJ, AHA, favism	<30	75	Med, A^-, Orissa, Mahidol, Canton Vanua Lava, Seattle	Most of these variants are known to be polymorphic
I	NNJ (severe), CNSHA	<10 in most cases	61	Sunderland, Nara, Guadalajara	Never polymorphic, but the same mutation can recur

AHA, Acute hemolytic anemia; *CHSHA,* congenital nonspherocytic hemolytic anemia; *NNJ,* neonatal jaundice.

*The separation between class II and class III is blurred and, in our opinion, is no longer useful.

†In brief, variants in class II and III can be regarded as having a *mild* phenotype confined to *acute* episodes; variants in class I have a *severe* phenotype with *chronic* illness.

‡See Fig. 18-4, *B*. We have counted only variants for which the mutation is known and the clinical expression is clearly reported.

instead, one should indicate that the test has shown either a *normal* result or a *deficient* result.

Genetics of G6PD

Gd, the G6PD gene, is located near the telomeric region of the long arm of the X chromosome[34-36] (band Xq28); historically, *Gd* has been a valuable X-linked genetic marker and a precious tool for studying the X-chromosome inactivation phenomenon[37,38] and clonal populations of somatic cells.[39-41] The *Gd* region was also one of the first regions of the human genome to be fully sequenced.[42]

X-linkage of *Gd* has three major consequences: (1) *Gd* mutations display the typical pattern of mendelian X-linked inheritance[43]; (2) severe G6PD deficiency is much more common in males than in females; and (3) as a result of X-chromosome inactivation, females who are heterozygous for two different *Gd* alleles exhibit somatic cell mosaicism.[37,44] As a result, if one of the alleles entails enzyme deficiency, about half the cells will be G6PD(+) and the other half will be G6PD(–), although there is a large range of variation around that average[45,46] for various reasons, not all of them fully known. At first approximation it can be assumed that X-inactivation takes place at random, and therefore a binomial distribution would be expected; the width of this distribution depends on the number of cells in the embryo or in individual embryonic tissue at the time of X-inactivation. If this number is 32 to 64, a fraction of about 2% of women with an "extreme phenotype" is predicted, that is, with less than 5% of one of the two cell types; this prediction is in good agreement with observation in an unselected sample of *Gd*+/*Gd*– heterozygotes,[46] although some studies have suggested an even larger proportion.[47]

Thus in many cases unbalanced or skewed phenotypes may arise simply by chance, according to the laws of statistics. However, in certain cases evidence exists of selection at the somatic cell level after X-chromosome inactivation. This finding was first well established in women who are heterozygous for hypoxanthine phosphoribosyltransferase deficiency,[48] and it has also been observed in several women heterozygous for severely deficient G6PD variants.[49,50] In these cases one must infer that the G6PD(–) state is a selective disadvantage for hematopoietic stem cells; interestingly, this disadvantage is cell lineage–specific because it does not affect, for instance, fibroblasts.[49] Finally, because selection might take place at other X-linked loci,[51] heterozygotes exhibiting extreme phenotypes by analysis of G6PD may arise from selection acting on an allele at another locus (a "hitchhiking effect"), as has been suggested in a family with G6PD Ilesha.[52-54] Evidence indicates that skewing may increase with age.[55]

At the genomic level, the *Gd* gene (Fig. 18-4, *A*) consists of 13 exons, the first of which is noncoding.[56] The total length of the gene is about 18.5 kilobases (kb), much of which (about 12 kb) consists of intron 2. The significance of this large intron is unknown; it may be important for efficient transcription or for processing because it is still the largest intron, even in the compressed version of the *G6PD* gene found in the puffer fish *Fugu rubripes*.[57] The promoter region has a high proportion of guanine (G) and cytosine (C) residues (i.e., it is GC-rich), as found characteristically in other housekeeping genes.[58] Deletion analysis has revealed that the "essential" portion of the promoter is only about 150 base pairs long.[59] Within this region, two Sp1 binding sites have been identified, either of which is essential for promoter activity.[60] This region and other regions are highly conserved between human and mouse,[61] thus supporting the notion that they are important for gene regulation.

Features of G6PD in Red Blood Cells

Only one structural gene exists for G6PD, although a related autosomal hexose dehydrogenase also exists.[62,63] Biochemical evidence is in keeping with the notion that the G6PD protein in red blood cells is the same as that in other somatic cells; thus when red blood cells are severely deficient in G6PD, this deficiency is also found to a greater or lesser degree in other somatic cells.[16] However, a significant difference in the metabolism of G6PD arises from the characteristic inability of mature red blood cells to synthesize protein. As a result, whereas in most somatic cells G6PD is subject to turnover, in red blood cells any G6PD molecule undergoing denaturation or proteolytic breakdown cannot be replaced (this

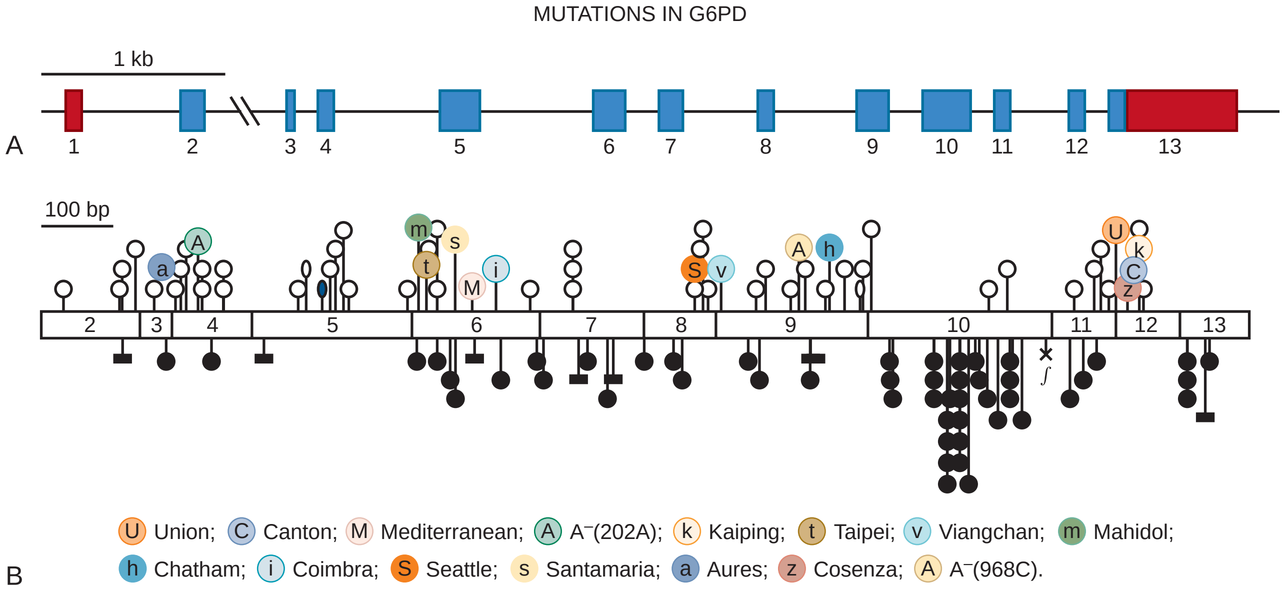
MUTATIONS IN G6PD
1 kb
A
1
2
3
4
5
6
7
8
9
10
11
12
13
100 bp
B
U Union; C Canton; M Mediterranean; A A⁻(202A); k Kaiping; t Taipei; v Viangchan; m Mahidol;
h Chatham; i Coimbra; S Seattle; s Santamaria; a Aures; z Cosenza; A A⁻(968C).

Figure 18-4 The human glucose-6-phosphate dehydrogenase *(G6PD)* genomic gene (**A**) showing exon-intron organization and a map of many of the known mutations (**B**). All variants shown, except G6PD A, are associated with enzyme deficiency. Mutations below the diagram are associated with a severe phenotype (class I), all of which are sporadic and rare. Mutations above the diagram are associated with a mild phenotype (class II or III), many of which are polymorphic, and some of those that have been more extensively investigated are shown by *initialed lollipop symbols*. A– is heterogeneous because it can result from a combination of the N126D replacement with any of three additional mutations. However, in the large majority of instances (probably more than 90%), the second mutation of G6PD A– is V68M.

condition is true, of course, not only for G6PD but also for most other red blood cell enzymes[64]). In normal red blood cells, the decay of G6PD approximates an exponential (see Fig. 18-6) with a half-life of about 60 days,[65] although it has been claimed that it may more closely approximate a two-slope curve with very fast breakdown when reticulocytes mature to erythrocytes and much slower breakdown subsequently.[66] The age dependence of red blood cell G6PD activity is so characteristic that it can be almost regarded as a marker of red blood cell age. In normal blood, reticulocytes have about five times more activity than the 10% oldest red blood cells.[67]

Molecular Basis of G6PD Deficiency

In principle, genetically determined deficiency of G6PD, like that of any other protein, might be attributed either to quantitative changes, such as mutations that affect the amount of the enzyme but not its structure, or to qualitative changes, such as mutations that affect the structure of the enzyme and hence its stability or catalytic efficiency. Extensive investigations of G6PD from G6PD-deficient cells, mostly carried out before the sequence of G6PD was known, revealed that (1) enzyme activity, even when severely reduced (sometimes to less than 1% of normal), is never completely absent and (2) enzymic properties (K_m, K_i, activity on substrate analogues, and thermostability, for example) are often different from those of the normal enzyme[8] (i.e., G6PD deficiency is associated with qualitative abnormalities). These data are consistent with point mutations in the coding region. In fact, from a database[68] of 186 mutant alleles that are now known, a reasonably clear pattern has emerged (see Table 18-2).

1. In nearly all G6PD variants there is a *single amino acid replacement* caused by a single missense point mutation.
2. In several of the cases in which two amino acid replacements are found, one of them is that of G6PD A (N126D). Because this nondeficient G6PD variant is polymorphic in Africa, the most likely explanation for the presence of two mutations in *cis* is that a second point mutation has taken place in a *Gd*^{A} gene.
3. Ten internal gene deletions have been discovered, all of them in frame (see Table 18-2); we can presume that deletions causing frameshifts would be lethal.
4. Only two mutations affecting splicing[68] have been discovered thus far.
5. The majority of the mutations (see Fig. 18-4, *B*) in the database[68] are sporadic, and most of them have been detected because they result in G6PD deficiency and CNSHA by causing sufficient loss of activity in red blood cells to become limiting for their in vivo survival. Sporadic variants associated with CNSHA are not likely to spread. However, in several instances the same variant has been encountered recurrently (for instance, we have found G6PD Tokyo in Scotland,[69] G6PD Guadalajara has turned up in Japan[70] and Belfast,[69,71] and G6PD Nara has been observed at least three times).[72] That the same mutation may be found recurrently in people who are almost certainly not ancestrally related is not trivial; these observations corroborate the notion that specific subtle constraints must exist whereby a particular G6PD variant has a distinctly

TABLE 18-2 Classification of 186 Known Human Glucose-6-Phosphate Dehydrogenase Mutations

Type of Mutation	Type of Mutation	No. of Mutants	Comments
Missense point mutations	Single	159	At least 35 of these mutations are polymorphic
	Two	13	6 of these mutations share the N126D mutation found on its own in the polymorphic nondeficient G6PD A: all of these are polymorphic, and 3 of them are the so-called G6PD A– common in Africa and in people of African descent 1 has the G6PD Canton mutation plus another mutation 1 of these has two mutations, one of which alone is known to cause CNSHA, and the other may aggravate the clinical picture[243]
	Three	2	One of these has two mutations characteristic of G6PD A– plus a third mutation causing CNSHA[244]
In-frame deletions	1 amino acid	7	None of these are polymorphic; all are associated with CNSHA
	2 amino acids	1	
	6-8 amino acids	2	
Affecting splicing		2	None of these are polymorphic; all are associated with CNSHA

Data from Minucci A, Moradkhani K, Hwang MJ, et al: Glucose-6-phosphate dehydrogenase (G6PD) mutations database: review of the "old" and update of the new mutations. Blood Cells Mol Dis *48:154–165, 2012.; Database courtesy of Ettore Capoluongo.*
CHSHA, Congenital nonspherocytic hemolytic anemia; *G6PD*, glucose-6-phosphate dehydrogenase.

severe clinical expression but remains compatible with life.

6. The majority of known polymorphic mutations (Fig. 18-5) again are associated with G6PD deficiency, and overwhelming evidence indicates that they have become polymorphic as a result of malaria selection (discussed later in this chapter). For these mutations we can visualize more stringent constraints; indeed, although still causing deficiency in red blood cells, they must not affect them so severely that the advantage with respect to malaria is outweighed.
7. The pattern of G6PD mutations, whereby null mutations (such as nonsense mutations, frameshifts, or large deletions) are conspicuously absent, is in striking contrast to that seen in other inherited disorders, such as the thalassemias, hemophilia, and muscular dystrophy. An important functional difference between these conditions and G6PD deficiency is that the former result from mutations in tissue-specific genes, whereas *Gd* is a housekeeping gene. When a tissue-specific gene is totally inactivated by a mutation, it may cause severe disease but will not necessarily have interfered with embryonic development. By contrast, we know that G6PD-null mutations are lethal to the mouse embryo[15]; they probably occur in humans, but we never see them because they must be lethal in the human embryo as well. Some clue regarding what mutation can cause G6PD deficiency has come from alignment of the amino acid sequence of G6PD from 42 different organisms. A striking correlation was found between the amino acid replacements that cause G6PD deficiency in humans and the sequence conservation of G6PD.[17] Interestingly, two thirds of such replacements are in highly and moderately conserved (50% to 99%) amino acids, and relatively few are in fully conserved amino acids (where they might be lethal) or in poorly conserved amino acids, where presumably they simply would not cause G6PD deficiency.
8. The biochemical mechanism of G6PD deficiency is strongly contingent on the fact that in normal red blood cells, G6PD decreases exponentially as red blood cells age, with a half-life of about 50 days (as discussed earlier in this chapter). Because G6PD is an oligomeric globular protein, it is not surprising that many point mutations producing replacement of individual amino acids can further decrease the stability of G6PD; in fact, for most mutants, enzyme instability is the main mechanism of enzyme deficiency (Fig. 18-6).[6,68,73] In a few cases (e.g., G6PD Orissa[74] and G6PD Mahidol[75]), altered catalytic function (instead of or in addition to protein instability) may be the main mechanism of G6PD deficiency. The instability is most extreme when the binding of structural NADP is compromised[76] or when the amino acid involved is at the interface between the two subunits of G6PD; in such cases the dimer does not form or falls apart.[24] These situation apply to G6PD mutants that cause the most severe clinical phenotypes (that of CNSHA, as will be discussed later).

Recent studies on the rare disease incontinentia pigmenti have revealed the remarkable fact that the underlying gene, called *IKBKG/NEMO*, overlaps with *Gd,* is transcribed in the opposite direction, and shares one of its two promoters with *Gd*. Some patients with incontinentia pigmenti have large deletions that remove four exons of *G6PD*[77]; however, no G6PD deficiency has been reported in this unique situation because all affected patients are heterozygotes and presumably in the blood there is overwhelming selection in favor of the cells in which the active X chromosome has the normal Gd allele.

Epidemiology of G6PD Deficiency

The geographic distribution[78] of G6PD deficiency is rather extraordinary in two ways. First, it has a very high prevalence overall, with more than 500 million people estimated to be involved (most of them mostly asymptomatic). Second, it has a high prevalence in many populations in most tropical and subtropical parts of the world

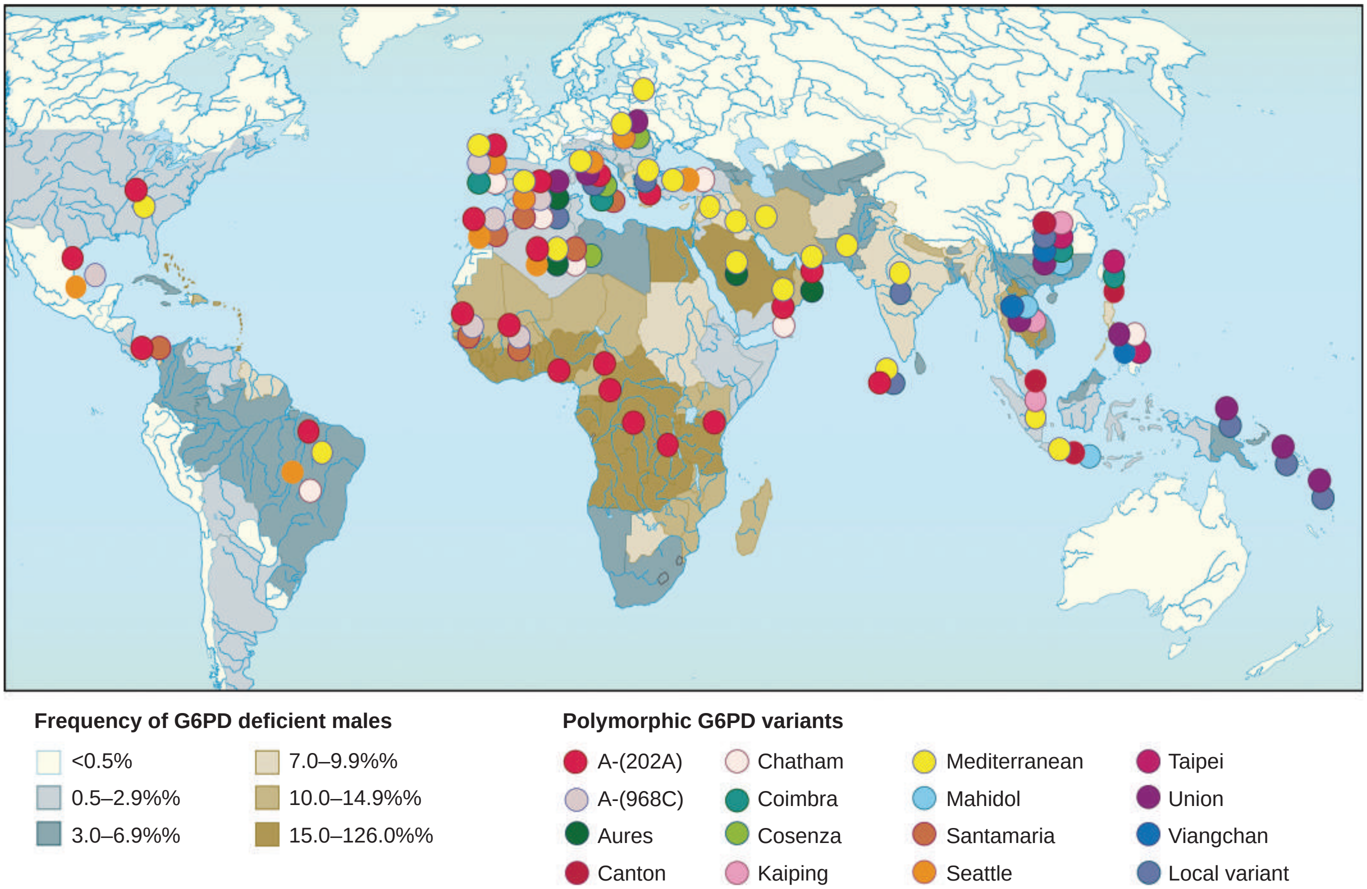

Figure 18-5 A world map of glucose-6-phosphate dehydrogenase *(G6PD)* deficiency. The shadings indicate the overall prevalence of G6PD deficiency in individual countries; the distribution of individual G6PD variants is shown by colored symbols. *(Modified from WHO Working Group: Glucose-6-phosphate dehydrogenase deficiency.* Bull World Health Organ *67:601–611, 1989; and Tan KL, Boey KW: Clinical experience with phototherapy.* Ann Acad Med Singapore *18:43–48, 1989.)*

because of malaria selection (see Fig. 18-5 and the last section in this chapter). Thus G6PD is prevalent in all five continents; however, it has never been reported in Amerindians, perhaps because they have been exposed to malaria only during the past three centuries. Very recently a new map of G6PD deficiency in malaria-endemic areas has been produced.[79]

CLINICAL MANIFESTATIONS OF G6PD DEFICIENCY

The most classic manifestation of G6PD deficiency is acute hemolytic anemia (AHA); in infants, however, another syndrome of great clinical and public health importance is neonatal jaundice (NNJ). CNSHA is a much rarer manifestation of G6PD deficiency and is the result of a lifelong hemolytic process. These different clinical manifestations are discussed in turn.

Acute Hemolytic Anemia

Clinical Picture

A child with G6PD deficiency is clinically and hematologically normal most of the time, and this status can be designated as a steady-state condition. However, a rather dramatic clinical picture can develop upon ingestion of fava beans (favism),[80] during the course of infection, or after exposure to certain oxidative agents (see Table 18-3).

Favism. After a lag of hours since eating fava beans, the child may become fractious and irritable or subdued and even lethargic. Within 24 to 48 hours the child's temperature is often moderately elevated. Nausea, abdominal pain, diarrhea, and rarely vomiting may be present. In striking contrast to these relatively nonspecific symptoms, the patient or a parent will observe, within 6 to 24 hours, the telltale and rather frightening sign of discolored urine (Fig. 18-7). The urine will be reported as dark; as red, brown, or black; or as "passing blood instead of water." Depending on experience, culture, and socioeconomic background, it will be said to resemble Coca-Cola, strong tea, or port wine. At about the same time, jaundice will become obvious. Physical examination may reveal little more than the signs corresponding to these symptoms. The child will be pale and tachycardic, and in severe cases evidence may be seen of hypovolemic shock or, less likely, heart failure. The spleen is usually moderately enlarged, and the liver may also be enlarged; either or both may be tender.

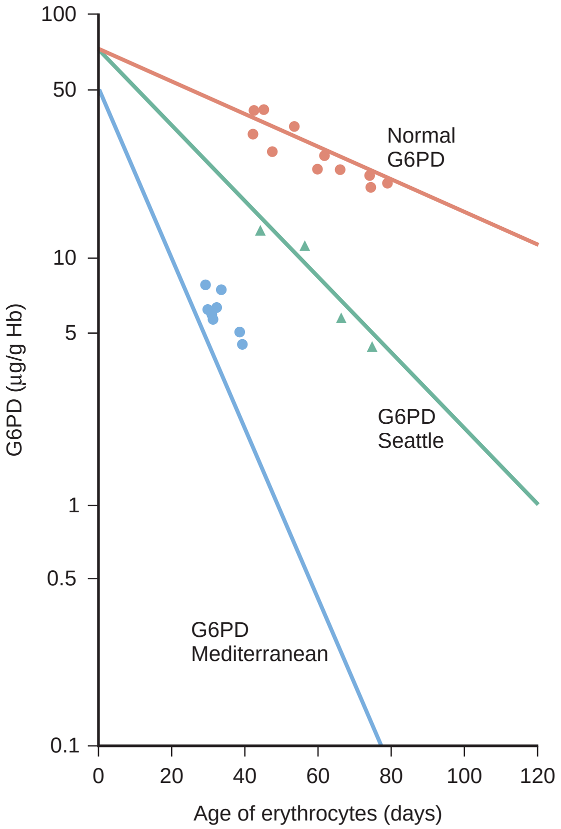

Figure 18-6 The main mechanism of glucose-6-phosphate dehydrogenase *(G6PD)* deficiency in red blood cells is in vivo instability of mutant enzyme. For many G6PD variants, such as the two studied here, this is essentially acceleration of a process that takes place normally with the aging of red blood cells in the circulation. *Hb,* Hemoglobin. *(Modified from Morelli A, Benatti U, Gaetani GF, et al.: Biochemical mechanisms of glucose-6-phosphate dehydrogenase deficiency.* Proc Natl Acad Sci U S A *75:1979–1983, 1978.)*

Drugs. G6PD deficiency was first discovered by investigating a possible genetic basis for sensitivity to primaquine, and early studies have documented the clinical manifestation of primaquine-induced AHA in G6PD-deficient volunteers who were adults.[81] In children with G6PD deficiency the largest reported series of AHA is not with primaquine but with dapsone.[82] The children were from different parts of Africa, but because they all received the same dose, quantitative data could be obtained for the first time. In 119 G6PD-deficient (hemizygous or homozygous) children with malaria who received 7.5 mg/kg of dapsone over 3 days, the drop in hemoglobin averaged about 2 g/dL, with a nadir on day 7. An increase in neutrophils and a brisk reticulocyte response were noted, and the bilirubin peaked on day 3.

Laboratory Findings

Anemia may range from moderate to extremely severe (hemoglobin values of 2.5 g/dL have been recorded). In the absence of other preexisting hematologic abnormalities, the anemia is normocytic and normochromic. The structure of the red blood cells may be striking (Fig. 18-8, *A*). Marked anisocytosis is often noted (reflected as a wide red blood cell size distribution on the electronic counter) because of the coexistence of large polychromatic cells and "contracted" cells, some of which can be frankly classified as spherocytes. Marked poikilocytosis is also noted with the presence of distorted red blood cells, "irregularly contracted" red blood cells, and red blood cells with apparently uneven distribution of the hemoglobin inside them (hemighosts).[83] Although some of these appearances are probably smearing artifacts, electron micrographic evidence suggests that in some of the cells, opposing surfaces of the membrane have become "cross-linked."[84] Probably the most characteristic poikilocytes are those in which the cell margin literally appears dented, as though a portion has been plucked out or bitten away ("bite cells"; see Fig. 18-8, *A*). The reticulocyte count is increased and may reach peaks of 30% or greater, with an elevated percentage of the immature reticulocyte fraction, reflecting a prompt and effective bone marrow response. Careful inspection of reticulocyte preparations may reveal inclusion bodies different from those normally seen in reticulocytes; these bodies are

TABLE 18-3 Drugs That Can Trigger Hemolysis in Glucose-6-Phosphate Dehydrogenase–Deficient Subjects*

Category of Drug	Definite Hemolysis	Possible Hemolysis
Antimalarial	Dapsone Primaquine Methylene blue†	Chloroquine Quinine
Analgesic	Phenazopyridine	Aspirin (high doses)
Antibacterial	Sulfamethoxazole/ co-trimoxazole‡ Sulfadiazine Ciprofloxacin Moxifloxacin Norfloxacin Ofloxacin Nalidixic Acid Nitrofurans: Nitrofurantoin	Sulfasalazine Chloramphenicol Isoniazid
Other	Rasburicase Toluidine blue	Ascorbic acid Glibenclamide Vitamin K

*The literature on this subject was critically reviewed by Youngster et al.[245] in 2010, and we took this review into account when revising the table that appeared in previous editions. However, we believe that the notion of levels of evidence used to assess the efficacy of drugs cannot be applied in the same way to assess the safety of drugs. Therefore we believe that even individual case reports, if convincingly documented, must be taken seriously, and thus this table has some differences with respect to the article by Youngster et al.

†There has been a revival in the use of methylene blue (MB), together with artemisinin-based combinations, in the treatment of *Plasmodium falciparum* malaria, because MB also has a gametocytocidal action. At the doses used, MB has caused significant hemolysis in children in Africa[246]; most cases were not very severe.

‡Atovaquone has been found to be a safe alternative in a glucose-6-phosphate dehydrogenase–deficient patient with *Pneumocystis jiroveci* infection[247]; this might apply to other protozoal infections.

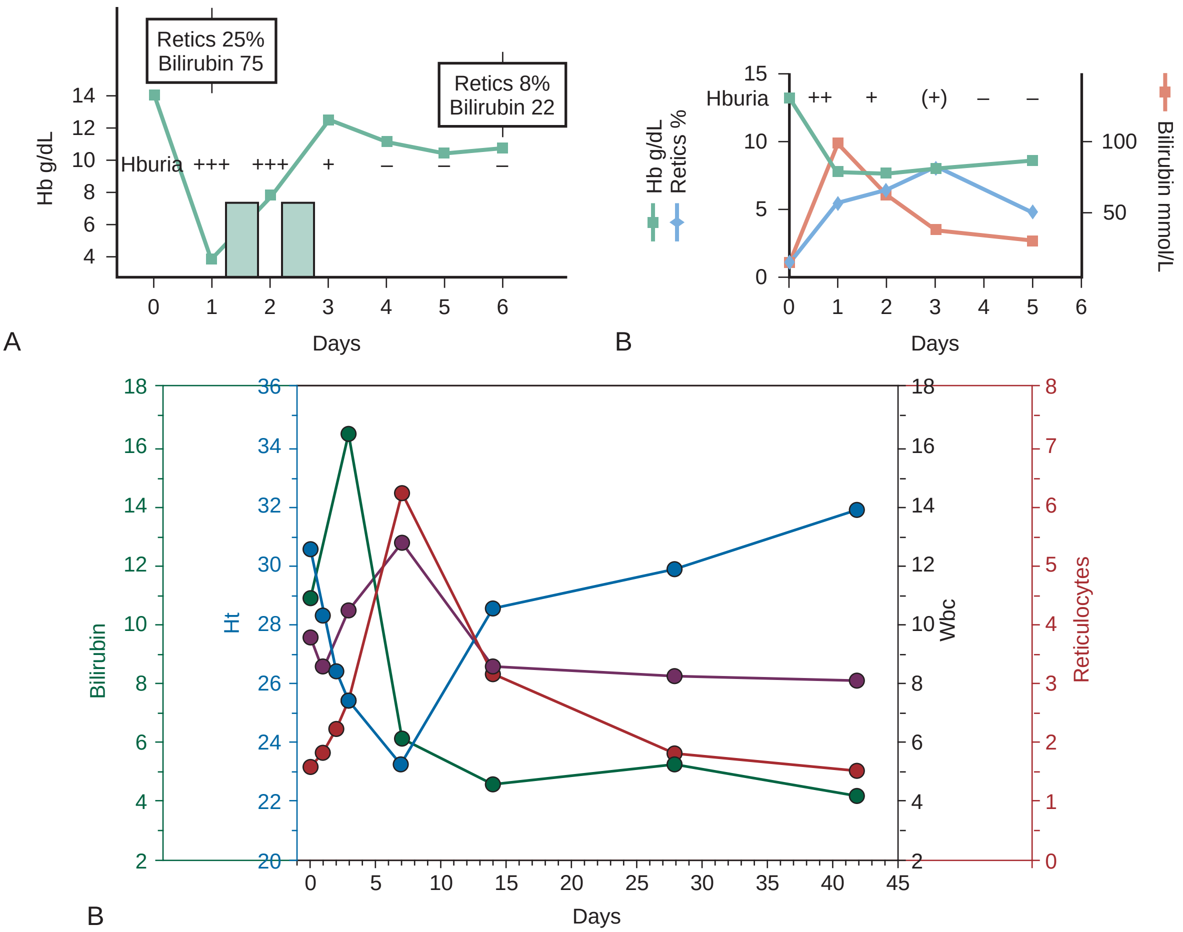

Figure 18-7 Clinical course of acute hemolytic anemia in glucose-6-phosphate dehydrogenase (G6PD) deficient children. **A,** A severe attack of favism in a 21-month-old boy, who required two blood transfusions *(bars)* because of life-threatening anemia after the ingestion of fava beans. The second blood transfusion was administered because of persistent hemoglobinuria (Hburia). The boy had severe G6PD deficiency (red blood cell G6PD activity less than 3% of normal; the underlying mutation was G6PD Mediterranean). The Hb value on day 0 is presumed. **B,** Hemolytic anemia caused by the administration of an antimalarial drug containing dapsone (2.5 mg/kg/day for 3 days) to children with acute (uncomplicated) *Plasmodium falciparum* malaria. Each curve represents the arithmetic mean of data from 95 male children with G6PD deficiency. The extent of the drop in hematocrit value indicates that, on average, about one fourth of red blood cells have undergone hemolysis; this is associated with a sharp increase in serum bilirubin. There is a brisk bone marrow response, with a peak on day 7 of both reticulocytes and leukocytes (mainly neutrophils). Although it is seen that with this dose of dapsone and with this G6PD variant the hemolytic anemia is on average less severe than that depicted in part **A,** 12 of the 95 children required a blood transfusion. Note that by the end of 6 weeks the blood counts are back to normal, with a hematocrit (Ht) value slightly higher than before treatment, presumably as a result of successful treatment of malaria. *Hb,* Hemoglobin; *Retics,* reticulocytes; *Wbc,* white blood cell. *(***A,** *Courtesy of Professor Tullio Meloni, Sassari, Sardinia.* **B,** *Modified from Pamba A, Richardson ND, Carter N, et al: Clinical spectrum and severity of hemolytic anemia in glucose 6-phosphate dehydrogenase-deficient children receiving dapsone.* Blood *120:4123–4133, 2012.)*

discrete, round, and 1 to 3 μm in diameter, and they usually appear to be leaning, from the interior, against the cell membrane. These inclusions are more clearly displayed by supravital staining with methyl violet and are referred to as *Heinz bodies* (see Fig. 18-8, *B*). They consist of precipitates of denatured hemoglobin and are the vivid manifestation of the oxidative insult that this protein and the cell itself has experienced. However, Heinz bodies are a very transient finding because they tend to be promptly "pinched off" by the spleen[85] (thus giving rise to bite cells), and the red blood cells that contain them are very rapidly removed from the circulation. Haptoglobin is reduced to the point of being undetectable. In severe cases it is possible to demonstrate free hemoglobin in plasma (*hemoglobinemia*). The white blood cell count is usually moderately elevated, with a predominance of granulocytes. The platelet count may be normal, increased, or moderately decreased. The unconjugated bilirubin level is elevated, but "liver enzyme" levels are generally normal. Test results of the dark urine are strongly positive for blood because of the presence of free hemoglobin (see the differential diagnosis of this sign in Table 18-4).

Clinical Course

In the majority of cases the hemolytic attack, even if severe, is self-limited and tends to resolve spontaneously (see Fig. 18-7, *B*). Depending on the proportion of red blood cells that have been destroyed, the anemia will be more or less severe, but the hemoglobin level may be back to normal in 3 to 6 weeks. However, in some cases the decrease in hemoglobin is so precipitous that blood

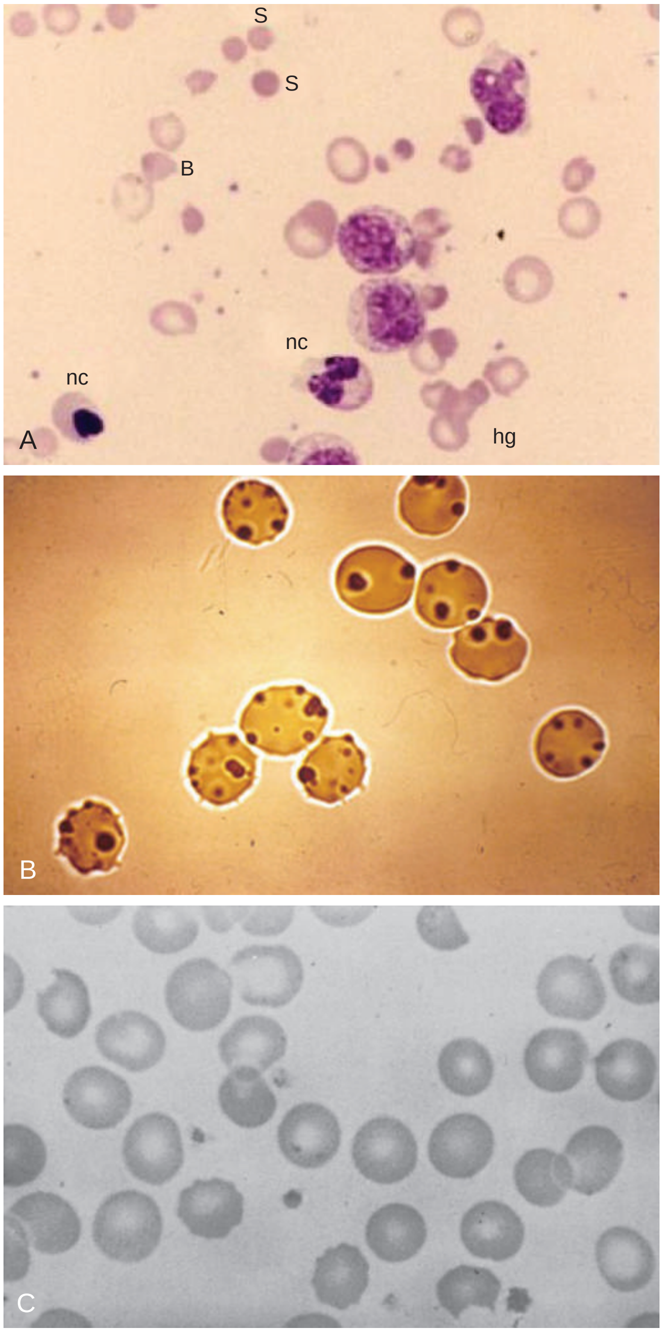

Figure 18-8 Blood smears in glucose-6-phosphate dehydrogenase (G6PD) deficiency. **A,** Acute hemolytic anemia (favism) characterized by marked morphologic abnormalities of red blood cells with anisocytosis, polychromasia, bizarre poikilocytes, "bite cells," and "hemighosts." Note the nucleated red blood cell and polymorphonuclear leukocytosis with a marked shift to the left. **B,** A supravital stain with methyl violet carried out at an early stage during an attack of favism demonstrates one or more Heinz bodies in individual red blood cells. **C,** Chronic nonspherocytic hemolytic anemia. The morphologic abnormalities are much less pronounced, but several poikilocytes and occasional bite cells are seen. *B,* Bite cells; *hg,* hemighosts; *nc,* nucleated cells; *S,* spherocytes.

transfusion is required to save the child's life. The proportion of such cases will depend primarily on (1) the dose of the offending agent, (2) concomitant pathology, and (3) the previous hemoglobin level. Although the blood urea level may be transiently elevated, the development of renal failure in children is exceedingly rare, even in the presence of massive hemoglobinuria (see Fig. 18-7, *A*).

Diagnosis

With a history of fava bean ingestion or of administration of a potentially hemolytic drug (see Table 18-3) followed by hemoglobinuria (see Table 18-4), the diagnosis is almost always straightforward, and it can be made quite confidently even before obtaining the final proof that the patient is deficient in G6PD (discussed later in this chapter). If the hemoglobinuria has already subsided and the history is uncertain, one is faced instead with the much wider differential diagnosis of AHA. Negative findings of a direct antiglobulin test will militate against autoimmune hemolytic anemia. In endemic areas it will be important to exclude malaria or the much rarer babesiosis. In persons with hemolytic-uremic syndrome, the red blood cell structure is different, and evidence of impaired renal function will be seen. In all cases, demonstration of G6PD deficiency will be conclusive, and in uncertain cases it will be crucial.

Pathophysiology

The clinical picture of AHA in a G6PD-deficient child is a vivid expression of the fact that, as we know already, hemolysis results from the action of an exogenous factor on latently but intrinsically abnormal red blood cells. Hemoglobinemia and hemoglobinuria unambiguously indicate that the hemolysis is at least in part intravascular. At first approximation we can visualize the following sequence of events: (1) an oxidative agent causes conversion of GSH to oxidized glutathione; (2) because of the limited capacity of G6PD-deficient red blood cells to regenerate GSH, their GSH reserve is rapidly depleted; (3) once GSH is exhausted, the sulfhydryl groups of hemoglobin and probably other proteins are oxidized to disulfides or sulfoxides; and (4) coarse precipitates of denatured hemoglobin cause irreversible damage to the membrane, and the red blood cells lyse or become prey to macrophages.

TABLE 18-4 Hemoglobinuria in Children

Condition	Circumstances	Diagnostic Approach
G6PD deficiency	Exposure to a trigger of hemolysis	Test for G6PD activity
Blackwater fever	Relatively rare complication of malaria	Blood slide for malaria parasites
Paroxysmal cold hemoglobinuria	Usually associated with viral infection	Test for Donath-Landsteiner antibody
Mismatched blood transfusion	Usually ABO incompatibility	Repeat crossmatch
Paroxysmal nocturnal hemoglobinuria	Tends to recur	Flow cytometry for CD59
Clostridium welchii septicemia	Burns, severe open trauma, transfusion of contaminated blood	Culture of blood or appropriate patient material

G6PD, Glucose-6-phosphate dehydrogenase.

Not all of these steps from oxidative attack to final hemolysis have been fully documented in vivo, in part because of one major difficulty: in the course of AHA the red blood cells sampled from the patient are obviously, at any given stage, those that have not yet hemolyzed. However, GSH depletion is a most characteristic finding when red blood cells are subjected to oxidative challenge,[86,87] and in one careful study it has been demonstrated that during an attack of favism, the first measurable biochemical change is a decrease in NADPH, followed by a decrease in GSH,[88] in keeping with stages 1 and 2 described earlier. Heinz bodies (see Fig. 18-8, *B*) are the visible expression of stage 3. Stage 4 is less clearly defined, although studies suggest that the abnormal proteolytic activity is associated with increased levels of intracellular calcium,[89,90] as well as binding of hemichromes (arising from denaturation of hemoglobin) to band 3 molecules.[91]

Even though intravascular hemolysis in persons with AHA that is associated with G6PD deficiency is important in pathophysiology and diagnosis, a substantial proportion of the hemolysis is extravascular (as shown by the enlarged spleen). Probably the most severely damaged red blood cells hemolyze in the bloodstream on their own, whereas less severely damaged red blood cells will be recognized as abnormal by macrophages and will undergo extravascular hemolysis in the reticuloendothelial system. This process has been referred to as an example of an "innocent bystander" phenomenon[92] (although the red blood cells, by virtue of being deficient in G6PD, are not that innocent), in which complement and immunoglobulins may be involved.[93] Finally, it is important to remember that the red blood cell destruction in AHA associated with G6PD deficiency is an orderly function of red blood cell age (see Fig. 18-6). The oldest red blood cells with the least G6PD are the first to hemolyze, and the hemolytic process progresses upstream toward cells with more and more G6PD.[94] As a result there is a selective enrichment in red blood cells that, despite being genetically G6PD deficient, have relatively higher levels of G6PD. This phenomenon can be so marked with certain G6PD variants that patients in the posthemolytic state are found to be relatively resistant to further challenge; thus the patient may be in a state of compensated hemolysis.[81]

Triggers and Mechanism of Hemolysis

After primaquine, numerous other drugs have been reported as being potentially dangerous in G6PD-deficient persons (Table 18-3). There is no obvious similarity in chemical structure among all of these substances, but they have in common the ability to stimulate the pentose phosphate pathway in red blood cells,[95] which must mean that they are able to oxidize NADPH directly or indirectly. The components of fava beans responsible for hemolysis have been identified as vicine and convicine, two β-glycosides that generate the aglycones divicine and isouramil.[96] These compounds, in the course of their auto-oxidation, produce free radicals, which in turn oxidize GSH and thereby activate the chain reaction of events previously outlined.[97] The drugs listed in Table 18-3, or their metabolites, act in a similar way. Henna dyes, which are used as cosmetics in several populations, mainly in Asia, can also cause serious hemolysis after absorption through the skin[98-101] or ingestion (apparently to induce an abortion[102]).

An intriguing feature of AHA associated with G6PD deficiency is its erratic character, which is more conspicuous with certain agents than with others. For instance, it is estimated that in adults, ingestion of fava beans does not trigger AHA in more than 25% of cases, and even in the same person, favism may occur on one occasion but not on another.[93] One obvious factor must be the amount of fava beans ingested (in relation to body mass). Another is the quality, with raw fava beans more likely to cause favism than cooked, frozen, or canned fava beans. Perhaps even more important is the finding that the glycoside content is a function of the maturity of the beans, with young, small beans being much richer (as well as more tasty!). With respect to drugs, primaquine causes hemolysis regularly, but aspirin does so only sometimes[103]; once again, the dosage must be important, but additional genetic or acquired factors affecting the metabolism of the drug may play a role. With regard to infection, the best documented precipitants are hepatitis, pneumonia, typhoid fever, and recently brucellosis[104] and *Clostridium difficile* (in newborns[105]); however, viral infections of the upper respiratory or gastrointestinal tract may also cause hemolysis.[106] Apparently trauma also can be a trigger. It has been reported that in a group of patients who had been involved in major road traffic accidents, the frequency and severity of infection were significantly higher in patients who were G6PD deficient, and more severe anemia also occurred in this group.[107]

Release of peroxides during phagocytosis of bacteria by granulocytes might explain the hemolytic process in persons with bacterial infections.[108] In this respect it is possible that hemolysis has sometimes been attributed to drugs used for treating infection when it should have been blamed on the infection itself.

Treatment

AHA in a child may be a diagnostic problem that, once solved, does not require any specific treatment at all; in other cases, the child's condition may be considered a medical emergency requiring immediate action. The most urgent question is whether a blood transfusion is needed. It is difficult to give absolute directives, but the following guidelines may be useful[109]:

1. If the hemoglobin level is less than 7 g/dL, the child should undergo a transfusion immediately.
2. If the hemoglobin level is less than 9 g/dL and evidence indicates persistent brisk hemolysis (hemoglobinuria), immediate blood transfusion is also indicated.
3. If the hemoglobin level is greater than 9 g/dL but hemoglobinuria persists or if the hemoglobin level is between 7 and 9 g/dL but the child has no hemoglobinuria, the child should be kept under close observation for at least 48 hours and should undergo a transfusion if either condition 1 or 2 develops.

The most important complication that may require treatment is acute renal failure, which is exceedingly rare in children.

Neonatal Jaundice

From an epidemiologic point of view, it is noteworthy that the frequency of NNJ varies widely in different populations; in many populations in which G6PD deficiency is prevalent, the rate of pregnancies at risk for Rhesus incompatibility happens to be low.[110,111] In addition, because Rhesus-related hemolytic disease of the newborn (HDN) is disappearing thanks to the implementation of appropriate prophylaxis, one can expect G6PD-related NNJ to be generally on the increase, at least in relative terms. G6PD deficiency has been well recognized as a major cause of NNJ in countries as disparate as Greece,[110] Nigeria,[112] and Taiwan.[113] During the past decade in the United States it has been increasingly recognized that G6PD deficiency is an important etiologic factor of severe NNJ, including kernicterus.[114,115]

Clinical Features

NNJ related to G6PD deficiency is very rarely present at birth; it has a peak incidence between day 2 and day 3.[116] Jaundice is present more often than anemia, and the anemia is very rarely severe.[117] For this reason the terms *HDN* and *NNJ* cannot be regarded as interchangeable, at least in the context of G6PD deficiency. The severity of G6PD-related NNJ varies from being subclinical to imposing the threat of kernicterus if not treated, and thus prompt recognition of the problem is extremely important to avoid crippling neurologic sequelae.[118-121]

Nature of the Association between G6PD Deficiency and Neonatal Jaundice

It is not fully explained why NNJ develops in some but not all G6PD-deficient newborns. Several clinical studies have established beyond any possible doubt that the association is statistically much higher than could be expected by chance (Table 18-5).[122] However, because not *all* G6PD-deficient newborns have NNJ (Table 18-6), it is likely that genetic or environmental factors (or both) are involved in addition to G6PD deficiency and that the same factors that are more extreme can also make the NNJ more severe. NNJ is not the prerogative of some G6PD variant because it is prevalent in widely remote parts of the world (e.g., Nigeria,[122] Sardinia,[123] Singapore,[124] and China[125]) in which the *Gd* alleles underlying G6PD deficiency are different; moreover, within both Sardinia[126] and Taiwan,[127] NNJ has been found in babies with any of several different G6PD variants. The possibility that NNJ correlates with the quantitative level of residual G6PD activity is controversial.[128,129] Specific features of erythrocytes in newborns such as elevated levels of ascorbic acid, depressed activity of glutathione reductase,[122] and low levels of vitamin E, GSHPX, and other enzymes[130] could contribute to the degree of jaundice. A dissociation has been reported between hyperbilirubinemia and anemia in G6PD-deficient neonates.[117,123] Indeed, in one series there was no difference in the distribution of hematocrit values in cord blood and on day 3 between G6PD-deficient newborns with and without jaundice, thus suggesting that to a large extent this jaundice may be of hepatic origin rather than hemolytic. In keeping with this notion, measurements of the bilirubin production-conjugation index have demonstrated that the NNJ in otherwise healthy G6PD-deficient neonates depends more on inefficient bilirubin conjugation than on hemolysis.[131] The *UGT1A* mutation characteristic of Gilbert syndrome is associated with a much higher risk for NNJ and kernicterus in G6PD-deficient babies.[132,133] In addition, environmental factors can certainly exacerbate NNJ, in some cases by causing hemolysis in individual G6PD-deficient newborns; such factors include prematurity, breastfeeding,[134] inhalation of naphthalene (camphor balls), acidosis, hypoxia, an infection such as viral hepatitis,[135] use of oxidant drugs, and ingestion of

TABLE 18-5 Association Between Glucose-6-Phosphate Dehydrogenase Deficiency and Jaundice in Newborns

	No.	% G6PD Deficient
Normal	500	22.5
Mild jaundice (bilirubin 150-200 μmol/L)	38	45
Severe jaundice (bilirubin >230 μmol/L)	70	60
Admitted with kernicterus	20	78

Data collected in Ibadan, Nigeria, on consecutive babies born in or admitted to a teaching hospital; see Bienzle U, Effiong CE, Luzzatto L: Erythrocyte glucose 6-phosphate dehydrogenase deficiency (G6PD type A–) and neonatal jaundice. Acta Paediatr Scand *65:701–704, 1976.*

G6PD, Glucose-6-phosphate dehydrogenase.

TABLE 18-6 Features of Neonatal Jaundice in 500 African-American Male Babies

	G6PD Normal (*n* = 436)	G6PD Deficient (*n* = 64)
PTB, mmol/L	139 ± 48	157 ± 43
Median end-tidal CO, ppm 2.1 (1.7-2.5)	2.1 (1.7-2.5)	2.4 (2.0-2.9)
Babies with PTB >75th percentile (%)	23.4	48
Babies with PTB >95th percentile (%)	6.7	22
Mean age at which the highest PTB occurs (h)	64	55
Babies requiring phototherapy (%)	5.7	20.3

Data from Kaplan M, Herschel M, Hammerman C, et al: Hyperbilirubinemia among African American, glucose-6-phosphate dehydrogenase–deficient neonates. Pediatrics *114: e213–e219, 2004.*

CO, Carbon monoxide; *G6PD,* glucose-6-phosphate dehydrogenase; *PTB,* plasma total bilirubin.

drugs[136] or fava beans (favism in utero[137]) by the mother before delivery.

In summary, it is likely that two different types of NNJ are associated with G6PD deficiency: (1) a more common type (see Table 18-6) that can best be visualized as a marked exaggeration of "physiologic jaundice" (this type is usually clinically benign and not greatly influenced by the environment) and (2) a rarer, frankly hemolytic type that is more severe and can be visualized as AHA occurring in a newborn baby because the baby happened to be exposed to one of the same agents that could cause AHA even in an adult.

Severe NNJ can occur in girls heterozygous for G6PD deficiency.[138] In addition, in geographic areas where G6PD deficiency is very common, female newborns might be homozygous for the trait, thus behaving like a hemizygous G6PD-deficient male newborn.

Treatment

Management of NNJ associated with G6PD deficiency does not differ from that recommended for other causes. Thus mild cases do not require treatment; intermediate cases require phototherapy; and severe cases require exchange transfusion, just as in NNJ caused by "classic" HDN. Kernicterus is still an impending threat, especially when severe NNJ is associated with anemia, hypoxia, or infection. Clinical practice guidelines for the management of hyperbilirubinemia in newborns quote: "measurement of the glucose-6-phosphate dehydrogenase (G6PD) is recommended for a jaundiced infant who is receiving phototherapy and whose family history or ethnic or geographic origin suggest the likelihood of G6PD deficiency or for an infant in whom the response to the phototherapy is poor."[139] G6PD-deficient newborns must be considered as being "at high risk"[134] and therefore requiring greater surveillance and more intensive treatment of hyperbilirubinemia. Specifically, it is recommended that in term newborns, exchange transfusion be carried out if the serum bilirubin level exceeds 15 mg/dL in the first 2 days of life or 19 mg/dL at any time in the first week of life.

Congenital Nonspherocytic Hemolytic Anemia

A small minority of children with G6PD deficiency have hemolytic anemia not only when it is triggered by an exogenous factor but even in the steady state (Fig. 18-9). These children have special G6PD mutations (class I); all of them are rare, but they are scattered worldwide, regardless of whether G6PD deficiency is endemic in the region.

The patient is almost invariably male (with exceptions, discussed later) and in general is evaluated because of unexplained jaundice, frequently presenting at birth (NNJ). Unfortunately, anemia recurs and the jaundice fails to clear completely, thus requiring further investigation. In other cases, NNJ may have been overlooked or forgotten and the patient is reinvestigated only much later in life (e.g., because of gallstones in a boy or in a young adult). The severity of the anemia ranges from being borderline to being transfusion-dependent in different patients (Table 18-7). The anemia is usually normochromic but somewhat macrocytic because a large proportion of reticulocytes (up to 20% or more) will cause an increased mean corpuscular volume and a shifted, wider

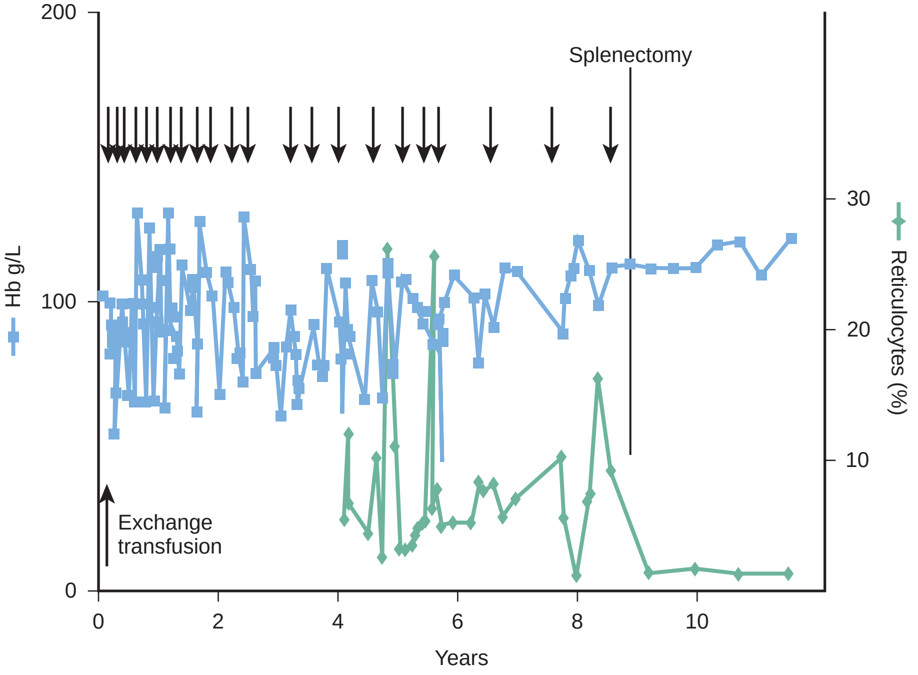

Figure 18-9 The clinical course of a boy with congenital nonspherocytic hemolytic anemia (CNSHA) caused by severe glucose-6-phosphate dehydrogenase (G6PD) deficiency. Severe neonatal jaundice was the initial manifestation; subsequently, the child developed normally but required numerous blood transfusions. The transfusion dependence remitted after splenectomy. Currently, the patient, now in his early twenties, has had a cholecystectomy; he has moderate anemia and is still jaundiced. The patient's *Gd* mutation was characterized as G6PD Harilaou (F216L).[47,131] *Hb*, Hemoglobin.

TABLE 18-7 Clinical Spectrum of Chronic Nonspherocytic Hemolytic Anemia Caused by Severe Glucose-6-Phosphate Dehydrogenase Deficiency

Patients Studied	25 (All Male)
% with neonatal jaundice	100
% requiring exchange transfusion	24
% with splenomegaly	68
Steady-state hemoglobin, range (g/dL)	6-14
Lowest hemoglobin known, range (g/dL)	3.8-9.3
Reticulocytes, range (%)	3-51
% requiring multiple blood transfusion	72

than normal red blood cell size distribution curve. The red blood cell structure is mostly not characteristic, and for this reason it is referred to in the negative as being "nonspherocytic." The bone marrow shows normoblastic hyperplasia, unless the increased requirement for folic acid associated with the high red blood cell turnover has caused it to become megaloblastic. The patient has chronic hyperbilirubinemia, a decreased level of haptoglobin, and an increased level of lactate dehydrogenase. Hemoglobinuria is rare, but hemosiderinuria sometimes may be detected. The spleen is usually moderately enlarged in small children, and subsequently it may increase in size sufficiently to cause mechanical discomfort, hypersplenism, or both. Although most patients with CNSHA are diagnosed when they are children or young adults, patients have been known to be diagnosed in their fifties.[140]

Pathophysiology

The pattern of hemolysis in these patients is different from that described earlier for patients who have AHA associated with G6PD deficiency and instead is more reminiscent of the chronic hemolysis seen in persons with hereditary spherocytosis. However, oxidative stress caused by exposure to the same agents that cause AHA will cause acute exacerbation of hemolysis in persons with CNSHA. Unlike the situation in persons with thalassemia major, even in severe cases no evidence of ineffective erythropoiesis is found. The fact that no hemoglobinuria is present, at least in the steady state, suggests that the hemolysis is mainly extravascular and therefore that its mechanism is different from that of AHA. Studies of red blood cell membrane proteins have revealed the presence of high molecular weight aggregates[141,142] consisting largely of spectrin, which seem to make the membrane abnormally susceptible to shear-induced fragmentation.[143] These abnormalities in the red blood cells of G6PD-deficient patients with CNSHA are not found in asymptomatic G6PD-deficient subjects, thus suggesting that whereas in the latter group the reductive potential of residual G6PD is adequate in the steady state, in the former group, continuous oxidation of sulfhydryl groups takes place, followed by irreversible changes in the configuration of membrane proteins. The reason why the severity of CNSHA associated with G6PD deficiency is so variable is that almost every case is attributed to a different mutation (discussed later in this chapter) and each mutation will have a different effect on the stability of the enzyme, on its kinetic properties, or both. In exceptional cases, CNSHA has been seen in women: two heterozygotes (G6PD Volendam[144] and G6PD Tondela[145]) had an extremely skewed X-inactivation pattern favoring, paradoxically, the X chromosome with the abnormal G6PD allele. Two women have been genetic compounds for a polymorphic variant and a rare variant associated with CNSHA; they had evidence of chronic hemolysis but no anemia.[140]

Diagnosis

Laboratory diagnosis of G6PD deficiency is discussed next, but a special problem in relation to CNSHA is to firmly establish the causal link between G6PD deficiency and CNSHA. For example, if the patient is a Swede of Swedish ancestry or a Japanese person of Japanese ancestry, the link will be taken for granted, and this approach is generally justified, given the rarity of G6PD deficiency in these populations. On the other hand, if the patient is from a population in which G6PD deficiency is common, its presence in a patient with CNSHA might be a mere coincidence, and the cause of the CNSHA might be something else altogether. In such cases, while other causes of CNSHA are being ruled out, it becomes essential to characterize the G6PD of the patient. If it is a common variant known to be asymptomatic in other subjects, it can certainly be exonerated, whereas if it is a new unique variant, it is likely to be the culprit.

Treatment

In general terms, management of CNSHA that is associated with G6PD deficiency does not differ from that of CNSHA related to other causes (e.g., pyruvate kinase deficiency). If the anemia is not severe, regular folic acid supplements and regular hematologic surveillance suffice. It is important to avoid exposure to potentially hemolytic drugs, and blood transfusion may be indicated when exacerbations occur, mostly in concomitance with intercurrent infection. In rare patients, the anemia is so severe that it must be regarded as transfusion-dependent. In these cases blood transfusion will be probably needed at approximately 2-month intervals to keep the hemoglobin in the 8 to 10 g/dL range. A hypertransfusion regimen aiming to maintain a normal hemoglobin level is not indicated because there is no ineffective erythropoiesis in the bone marrow. However, depending on the extent of the blood transfusion requirement, appropriate iron chelation should be instituted from the age of 2 years onward and must be continued as long as transfusion treatment is necessary (the transfusion requirement may sometimes decrease after puberty).

A special problem is that of splenectomy.[69] No evidence exists of selective red blood cell destruction in the spleen, as in hereditary spherocytosis. However, the fact that the spleen is usually enlarged suggests that its role in hemolysis is not negligible. In practice, the following three indications exist for splenectomy: (1) if splenomegaly becomes a physical encumbrance; (2) if the patient exhibits evidence of hypersplenism; and (3) if the anemia is severe, even in the absence of the first two indications. A splenectomy may reduce the overall rate of hemolysis (see Fig. 18-9) just enough to make a transfusion-dependent child become transfusion-independent.[146] Becoming transfusion-independent is doubly important because it makes it possible to dispense with iron chelation. Active immunizations before a splenectomy and penicillin prophylaxis after a splenectomy must be started as indicated by guidelines such as those produced by the British Committee for the Standards in Haematology (http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2141.2011.08843.x/pdf).

When a diagnosis of CNSHA is made, genetic counseling must be provided to the family, and it is advisable to establish whether the mother is a heterozygote. If she is a heterozygote, the chance of recurrence is 1 : 2 for every subsequent male pregnancy. Prenatal diagnosis should be offered; although it could be carried out on amniotic fluid cells, it would be much preferable to determine the mutation involved and test for it on DNA from chorionic villi.[147] G6PD-deficient patients with severe CNSHA (see Fig. 18-9)—because their clinical manifestations are limited to blood cells—would in principle be good candidates for gene therapy by gene transfer into hematopoietic stem cells. This approach has not yet reached the stage of clinical application, but preclinical experiments have been carried out successfully in mice[148] and rhesus monkeys.[149]

LABORATORY DIAGNOSIS OF G6PD DEFICIENCY

The final diagnosis of G6PD deficiency must rely on an enzyme essay, which is fortunately easy to perform, and numerous "screening tests" can be used as substitutes if a spectrophotometer is not available. However, a number of potential pitfalls and sources of error must be understood, and the use of commercial kits is not a substitute for such understanding.

Tests for G6PD Deficiency

The classic spectrophotometric method[150] directly measures the rate of formation of NADPH through its characteristic absorption peak in the near-ultraviolet spectrum at 340 nm. Red blood cell activity is expressed in international units (micromoles of NADPH produced per minute) per gram of hemoglobin; therefore it is best to assay the enzyme activity and the hemoglobin concentration in the same hemolysate and work out the ratio. Because G6PD activity is much higher in leukocytes (particularly in granulocytes) than in erythrocytes, for accurate measurements it is essential to remove all leukocytes by using the Ficoll-Hypaque method or by filtration through cellulose powder[151] rather than by using the cruder approach of sucking off the buffy coat; however, for the purpose of clinical diagnosis of G6PD deficiency, it is not strictly necessary to remove all leukocytes. In normal red blood cells, the range of G6PD activity, measured at 30° C, is 7 to 10 international units/g of hemoglobin.

Several "screening tests" for G6PD deficiency are useful and reliable provided they are run properly and their limitations are understood. The dye decolorization test[152] and the methemoglobin reduction test[153] are not used much anymore; a fluorescence spot test[151] and a formazan-based spot test[154] are available, and the latter has been field tested.[155] All these methods are semiquantitative and are meant to classify a sample simply as "normal" or "deficient." The cutoff point can be set by following the appropriate instructions and by trial and error in the individual diagnostic laboratory. One should aim to classify as deficient any sample having less than 30% of normal activity, because above this level it is unlikely that serious clinical manifestations will occur. Screening tests are of course especially useful for testing large numbers of samples. They are also perfectly adequate for diagnostic purposes in patients who are in the steady state but *not* for patients in the posthemolytic period or with other complications; in addition, they cannot be expected to identify all heterozygotes. Finally, an ideal screening test ought not to give "false-negative" results (i.e., it should not misclassify a G6PD-deficient subject as normal), but it can be allowed to give a few "false-positive" results (i.e., a G6PD-normal subject might be misclassified as being deficient in G6PD). Ideally, in every patient found to be deficient in G6PD by screening, the finding should be confirmed with a spectrophotometric assay.

The Effect of Red Blood Cell Age and Selective Hemolysis

Because G6PD decreases gradually as red blood cells age,[64] any condition associated with reticulocytosis will entail an *increase* in G6PD activity (Table 18-8), which means that if a subject is genetically G6PD normal, in the course of hemolysis, red blood cell G6PD activity will now be *above* the normal range. This situation does not affect the diagnosis because G6PD deficiency will be correctly ruled out. However, if the subject is genetically G6PD deficient, red blood cell G6PD may now be raised to the extent of being near or even within the normal range, and the patient might therefore be misclassified as being G6PD normal[156] (even though at the onset of the attack the level may have been low).[157] Thus after a hemolytic attack, two circumstances occur to cause a risk of misdiagnosis: first, the older cells have been destroyed selectively, and second, the marrow response has caused a sudden outpouring of young cells into the peripheral blood. (A third confusing factor may be admixture of G6PD-normal red blood cells if the patient has undergone a transfusion.) Although the reticulocyte count is a good warning to avoid this mistake, it must be realized that because reticulocytes turn into morphologically "mature" erythrocytes within 1 to 2 days, their count is not a sensitive index of mean red blood cell age; in other words, mean red blood cell age may be significantly younger than normal even when the reticulocyte count is normal. These problems may be circumvented in several ways. First, a G6PD level in the low-normal range (as opposed to higher than normal) in the presence of reticulocytosis is always suspicious; indeed, this finding suggests that the patient is actually G6PD deficient. Second, if the

TABLE 18-8 Red Blood Cell Glucose-6-Phosphate Dehydrogenase Levels in Various Clinical Situations

Clinical Condition	Gender	Result of Screening Test	Result of G6PD Assay	Interpretation
Normal	M or F	Normal	8.1	Normal
Normal	M	Abnormal	0.4	G6PD deficiency, steady state
Normal	F	Abnormal	2.1	Heterozygote for G6PD deficiency
Normal	F	Normal	4.9	Heterozygote for G6PD deficiency
Acute hemolysis	M	Abnormal	2.3	AHA in a G6PD-deficient boy
Acute hemolysis	F	Normal	7.2	AHA in a G6PD heterozygote girl
Chronic hemolysis	M	Normal	15.5	Hemolysis unrelated to G6PD deficiency
Chronic hemolysis	M	Abnormal	1.4	CNSHA, probably caused by G6PD deficiency

AHA, Acute hemolytic anemia; *CNSHA,* congenital nonspherocytic hemolytic anemia; *G6PD,* glucose-6-phosphate dehydrogenase.

patient is experiencing or recovering from AHA, the suspicion generated from the finding just mentioned can be simply kept in store for a few weeks, when the situation will be evolving toward the steady state, and a repeat test will prove whether the patient is indeed deficient in G6PD. Third, if either the urgency of some clinical decision or academic curiosity demands a more prompt solution of the problem, the presence of severely G6PD-deficient red blood cells can be demonstrated either by an enzyme assay of the oldest cells (fractionated by sedimentation) or by a cell-based methodology (described later).

G6PD Deficiency in Heterozygotes

For special purposes, formazan-based cytochemical methods are also available.[158,159] For hemolysis to be clinically significant in heterozygous girls, at least 50% of the red blood cells must be deficient, and therefore the G6PD level will be about 50% of normal or less. This level of deficiency can be diagnosed by a quantitative test; however, the problems associated with current or recent hemolysis outlined for male patients will be compounded in the case of heterozygous females, and they can usually be overcome by a similar approach, particularly with the use of a cytochemical test. A different matter is the biologic diagnosis of heterozygosity, regardless of immediate clinical implications, as may sometimes be required for appropriate genetic counseling. In cases of “extreme phenotypes” (sometimes referred to as arising from “imbalanced lyonization”), the G6PD-deficient red blood cells may be so few that the only way to identify heterozygous G6PD deficiency will be by DNA analysis: for this approach the underlying mutation must be known. Recently, based on methemoglobin reduction,[153] a cytofluorometric G6PD assay has been developed,[160] which ought to be ideal for assessing the phenotype of heterozygotes. A special situation involves heterozygotes for G6PD variants associated with CNSHA. In our experience, the mothers of (male) patients with this condition are often G6PD normal, either because the variant in the offspring is due to a de novo mutation[53] or because the mother is a heterozygote but is phenotypically normal, presumably because somatic selection has favored the hematopoietic progenitor cells with the normal G6PD allele.[50]

Molecular Analysis of the G6PD Gene

Because mutation analysis at the DNA level is currently easy, and because in many populations one knows which mutations are most common, it may be tempting to adopt this approach and to bypass a G6PD assay altogether. This approach is fine as long as a known mutation is found; however, if it is not found, it may be that the patient in question has an unexpected mutation, in which case it would be unsafe to presume that the patient is G6PD normal. On the other hand, in special cases (see the previous section for heterozygote diagnosis), and particularly in the case of CNSHA, identification of mutations with reference to the large numbers of mutations already known (see the Human Gene Mutation Database at http://www.hgmd.cf.ac.uk/ac/gene.php?gene=G6PD) is recommended, and it could also discover new mutations. Of course, it may not be long before complete sequencing of all exons and intron-exon junctions may be sufficiently inexpensive to become routine.

GENOTYPE-PHENOTYPE CORRELATIONS

A rigorous definition of a polymorphic allele is one with a frequency higher than can be accounted for by recurrent mutation; however, for convenience, a conservative practical criterion is any allele with a frequency of at least 1% in at least one population. According to this criterion, 26 well-mapped *Gd* polymorphic alleles have already been identified (see Figs. 18-4, *B*, and 18-5).[68] The ratio of subjects having G6PD deficiency associated with CNSHA to those having “simple” G6PD deficiency varies in different populations. For instance, in Japan, where G6PD deficiency is on the whole very rare, the majority of patients with G6PD deficiency have been reported to have CNSHA.[161] This finding suggests that CNSHA that is caused by rare sporadic variants, many of which may result from recent mutations, reflects the intrinsic mutation rate of the human *Gd* gene, which is likely to be uniform throughout the world; in contrast, “simple”

G6PD deficiency almost always results from common variants that have arisen many generations ago and spread through biologic selection.[68]

The three variants mentioned previously—G6PD A, G6PD Mediterranean, and G6PD Mahidol—are probably those for which clinical expression has best been characterized.[162] Although G6PD A is often quoted as having fewer clinical manifestations than the others, the differences are marginal, and they are unlikely to be relevant with respect to patient management. The severity of hemolysis and whether it is "self-limited" probably depend on the offending agent, its dose, and the time course of exposure more than it depends on the G6PD variant involved. Notably, favism has been unambiguously documented with G6PD A–.[163,164,165]

In terms of clinical expression, the demarcation between class I variants and all others is, by definition, much more clear-cut. Indeed, one of the outstanding questions in the biochemical genetics of G6PD is why a particular variant can cause CNSHA rather than just AHA. In certain cases the level of residual enzyme activity is not the whole answer because qualitative differences are also important, as first suggested by Kirkman and Riley.[166] The most likely way in which a structural change can significantly alter the function of G6PD, *given the same level of deficiency*, is by affecting the binding of one of the main ligands (i.e., G6P, NADP, and NADPH). For instance, G6PD Mediterranean and G6PD Coimbra both have mutations near the G6P binding site, and both have *increased* affinity for G6P. Perhaps because of this characteristic, although they are both severely deficient, they belong to class II and not to class I. G6PD Orissa, the main polymorphic variant in tribal Indian populations,[74] also belongs to class II despite having *decreased* affinity for NADP. Thus the affinity for G6P appears to be of greater importance. Indeed, comparison of the distribution of K_m^{G6P} values of class I variants with that of class II and III variants shows that they are significantly lower in the latter group.[8]

However, in most cases the main factor responsible for causing CNSHA is a very low level of residual activity in red blood cells, which in turn is due to marked in vivo instability. It is quite remarkable that although G6PD mutations as a whole are evenly spread throughout the gene's coding sequence, the majority of class I mutations are clustered in exons 10 and 11 (see Fig. 18-4, *B*). The three-dimensional model of the human G6PD dimer has, at long last, provided a reasonable explanation for this finding.[68,167] Indeed, these two exons encode the protein region that constitutes the interface between the two identical subunits of the enzyme. As a result, any mutation in this region causes the respective amino acid replacements in the two subunits to be quite near each other in the dimer structure, thus potentially increasing their deleterious effects. Even more important, because no covalent link exists between the subunits, it stands to reason that any interference in the shape of the interface surfaces may affect their mutual fit and therefore dramatically destabilize the active form of the enzyme. Defects in protein folding have been studied in detail for certain G6PD variants.[168,169]

G6PD DEFICIENCY AND PREVENTIVE MEDICINE

Because NNJ and AHA are the most common manifestations of G6PD deficiency, it is most important to consider how they can be prevented. The first step is to identify G6PD-deficient persons, which is where screening is most pertinent. Of course, whether population-wide screening is both desirable and feasible depends primarily on the prevalence of G6PD deficiency in any particular community, which will determine the cost-benefit ratio. For instance, there has been experience with screening in Sardinia[170] and Malaysia,[171] and recently screening has been considered as a possibility in the United States.[115] If screening is performed at all, it is best performed with cord blood. Once a person is known to be G6PD deficient, the two main implications are the risk for NNJ and the importance of avoiding exposure to agents that can cause AHA. NNJ cannot be prevented as yet, but awareness of G6PD deficiency must entail surveillance for NNJ until at least day 4 and special recommendations with respect to factors such as naphthalene that can cause it or make it worse. By contrast, at least one type of AHA—namely, favism—is completely preventable (Fig. 18-10). Prevention of infection-induced hemolysis is obviously more difficult. Prevention of drug-induced hemolysis is possible in most cases by choosing alternative drugs, but that may be difficult when none is available. The most common problem is the need to administer primaquine for the eradication of malaria caused by *Plasmodium vivax* or *Plasmodium malariae*. In these cases, administration of a lower dosage for a longer time is the recommended approach. Hemolysis will still occur, but under appropriate surveillance it will be of an acceptably mild degree.

A special problem in prevention is what to do about new drugs, the hemolytic potential of which is unknown. Although in vitro methods to test drugs in this respect do exist,[172,173] such tests are unfortunately not routinely carried out before drugs are released on the market, and thus their hemolytic potential will become apparent only as a result of clinical observation. Recently a combination of dapsone and chlorproguanil that was used as an antimalarial agent in several African countries[174,175] was withdrawn from the market because of life-threatening hemolytic complications.

G6PD DEFICIENCY IN NONERYTHROID CELLS

As mentioned earlier, because nucleated somatic cells have the capability to synthesize G6PD constitutively, they are affected by G6PD deficiency to a lesser degree than are red blood cells.[176] For instance, subjects with G6PD Mediterranean have less than 5% G6PD activity in red blood cells but about 30% of normal in granulocytes, whereas subjects with G6PD A– have about 12% G6PD activity in red blood cells but nearly normal activity in granulocytes. On the other hand, if deficiency results from a drastic change in catalytic efficiency or in substrate affinity, deficiency may be more universal. In practice, the only well-documented pathologic effect is expressed in granulocytes. Very few of the class I G6PD

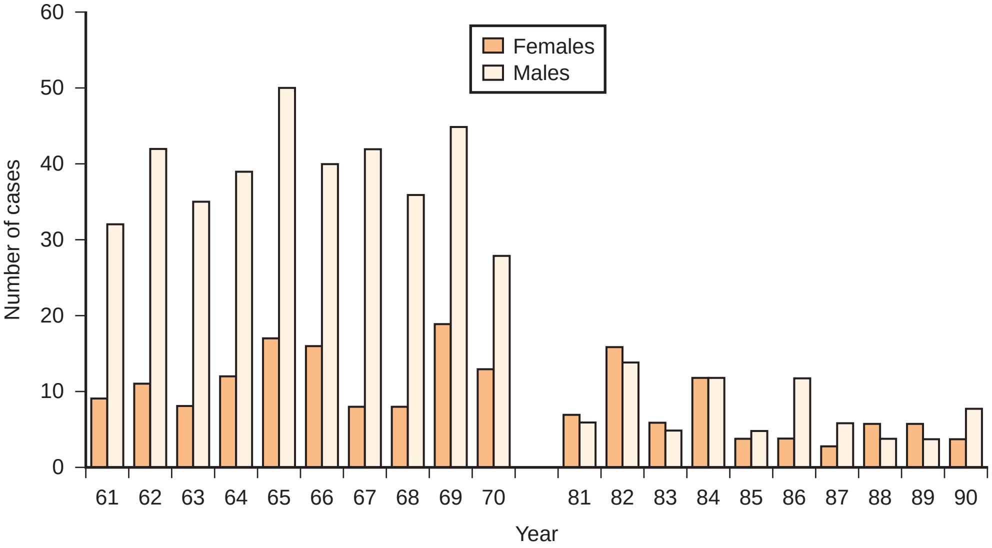

Figure 18-10 Control of favism in a community. The data show the decrease in the number of yearly admissions for favism in the children's department of the main city and university hospitals in Sassari, northern Sardinia. During the 10-year period intervening between the two sets of data, two preventive measures were adopted: (1) education through the media and (2) screening of all newborns for glucose-6-phosphate dehydrogenase (G6PD) deficiency. Note the dramatic reduction in the incidence of favism in boys and the more modest decrease in girls. The data suggest that (1) preventive measures are effective; (2) the increased proportion of girls can be attributed to failure of the screening method used to pick out many of the heterozygotes; and (3) in view of (2), the dramatic reduction observed in boys can perhaps be credited to screening more than to the educational campaign. *(Courtesy of Professor Tullio Meloni, Sassari, Sardinia.)*

variants cause not only CNSHA but also granulocyte dysfunction, mainly in the manner of impaired killing of phagocytosed bacteria (an example is G6PD Barcelona).[177] Patients with these variants have increased susceptibility to bacterial infection, particularly with *Staphylococcus aureus*. Recently, in a group of patients who sustained major trauma in road traffic accidents, Spolarics et al. (2001)[107] made the interesting observation that the frequency and severity of infection were significantly higher in patients who were deficient in G6PD. Thus neutrophil function may be affected in extreme situations, even in persons with the common type of G6PD deficiency (the patients in the clinical study all had G6PD A–). The mechanism whereby G6PD deficiency impairs phagocytosis is a defect of the oxidative burst caused by a shortage in NADPH supply, similar to that observed in chronic granulomatous disease, in which one of the components of the cytochrome b_{245} system is defective.[178] Functional abnormalities have also been demonstrated in macrophages from G6PD-deficient mice.[179]

Erythrocytes are not the only nonnucleated cells in the body. Another example is in the eye lens, and juvenile cataracts have been reported occasionally in subjects with G6PD deficiency.[180,181] Whether G6PD deficiency is more generally associated with a higher frequency or earlier onset of cataracts still appears to be controversial.[182-185]

G6PD DEFICIENCY COEXISTING WITH OTHER DISORDERS

In areas where G6PD deficiency is common, it is not infrequent that it may be encountered in the same patient together with another hematologic or nonhematologic condition.

Association with Other Hematologic Diseases

The combination of G6PD deficiency with the sickle cell trait has been no more frequent than could be expected by chance.[186,187] Several studies have shown that no significant differences exist in a variety of clinical and hematologic parameters between two otherwise comparable groups of patients with sickle cell anemia (SCA), those with and those without G6PD deficiency.[156,188-190] It has been reported that G6PD deficiency is associated with increased cerebral blood flow velocity[191] in children with SCA, but this finding has not been confirmed by other investigators,[192] and a significantly increased risk of stroke does not appear to exist.[193] Overall, G6PD deficiency does not seem to make much difference to SCA in the steady state; however, acute intravascular hemolysis that can occur in G6PD-deficient patients must be regarded as more dangerous when it is superimposed on the chronic hemolytic anemia of SCA. The combination of G6PD deficiency with the β-thalassemia trait has been found to cause a significant increase in mean corpuscular volume,[194] which nonetheless remains below the normal range. Association of G6PD deficiency with thalassemia major is unlikely to be a problem because patients with the latter condition are treated with regular blood transfusions or with bone marrow transplantation. However, the association may be significant in patients with various forms of thalassemia intermedia syndromes (e.g., hemoglobin E-β-thalassemia).[195]

Occasionally G6PD deficiency has been observed in association with much rarer red blood cell abnormalities, such as pyruvate kinase deficiency,[196,197] congenital dyserythropoietic anemia type II,[198-200] and hereditary elliptocytosis.[201] In these cases the two abnormalities seem to produce only additive clinical effects, but in at least one family, G6PD deficiency was synergistic with hereditary spherocytosis in causing moderately severe chronic hemolytic anemia.[202]

Association with Nonhematologic Diseases

Several studies have reported variable degrees of association between diabetes and G6PD deficiency,[203-209] and various underlying mechanisms have been hypothesized.[210,211] In the course of diabetic decompensation, AHA may develop in G6PD-deficient patients with diabetes[212,213]; on the other hand, downregulation of G6PD has been reported in some West African patients with type 1 diabetes who are predisposed to ketoacidosis.[214] The prevalence of proliferative retinopathy may be increased in G6PD-deficient patients with type I diabetes.[215] In the area of eye disease, it has also been reported that G6PD deficiency is a risk factor for the development of pterygium.[216] Apart from the fact that hepatitis can precipitate hemolysis in G6PD-deficient children, ribavirin, which is now often used in the treatment of hepatitis C, can itself cause hemolytic anemia (regardless of whether the patient is deficient in G6PD). On the other hand, it has recently been shown that G6PD-deficient patients with hepatitis C can be treated safely with ribavirin and pegylated interferon.[217] During the past few years extensive studies have been performed with regard to the influence of G6PD deficiency on cardiovascular physiology and pathology and even cardiac surgery.[218-220]

Recently a unique case has been reported of a Turkish child who has co-inherited the rare condition hypohidrotic ectodermal dysplasia, together with G6PD deficiency, on the same X chromosome.[221]

Trauma and G6PD Deficiency

Although G6PD deficiency protects against malaria, some reports indicate that it worsens the clinical course of traumatic patients.[202] Altered cytokine response would be the underlying pathogenetic mechanism predisposing to infections in G6PD-deficient persons.[222]

G6PD-Deficient Donors

Thousands of units of G6PD-deficient blood have been used in blood transfusion practice without problems. However, for babies with NNJ, it is advisable to test donor blood and to only use blood with a normal level of G6PD.[223] G6PD-deficient subjects have also served as bone marrow transplant donors; the recipient has, of course, become G6PD deficient, but engraftment has been normal.[224]

G6PD POLYMORPHISM AND MALARIA

The striking correlation between the worldwide distribution of G6PD deficiency (see Fig. 18-5) and that of *Plasmodium falciparum* prompted formulation of the "malaria hypothesis" half a century ago.[225,226] Since that time numerous more detailed epidemiologic studies (which can be referred to as "micromapping"),[227] as well as clinical studies,[228] have supported the notion that G6PD deficiency confers some degree of resistance to the potentially lethal malaria parasite *P. falciparum* and that in malaria-endemic areas, G6PD alleles associated with enzyme deficiency have therefore been subjected to positive darwinian selection.[229-231] A geographic correlation certainly does not amount to proof, but four large clinical studies carried out in Africa have tested the malaria hypothesis directly in the field. Although it is puzzling that in two of these studies[232,233] increased resistance to the parasite was significant only in heterozygous females, in one study[234] a protective effect was seen in both males and females, and in another study[235] a protective effect was noted only in males. All four studies concur in supporting the notion of malaria selection for G6PD deficiency. As for the mechanism of this phenomenon, in vitro studies have shown that invasion of G6PD-deficient red blood cells by *P. falciparum* takes place normally, but parasite growth is affected by previous passage through G6PD– cells.[236] On the other hand, parasitized red blood cells are recognized more promptly by macrophages when they are G6PD deficient[237]; thus accelerated removal of parasitized red blood cells (suicidal infection)[238] may be the most important mechanism of protection, as it is for subjects with the sickle cell trait.[239] An additional strong argument in favor of malaria selection is the genetic heterogeneity of polymorphic *Gd*$^-$ alleles in itself. Indeed, each one of these alleles, having arisen through an independent mutational event, must have increased in frequency on its own in a particular geographic area[240] where malaria was or still is endemic—a good example of convergent evolution driven by the same selective force.[241] Recent work from Thailand suggests that G6PD deficiency may confer resistance not only against *P. falciparum* but also against *P. vivax*.[242]

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CHAPTER 19

Normal and Abnormal Hemoglobins

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Studies of hemoglobin (Hb) structure, function, and expression over more than 50 years have produced many paradigms in biology and medicine. The first part of this chapter describes the structural and biochemical properties of Hb, emphasizing those that underlie its physiological roles. The conserved globin protein fold with the heme prosthetic group confers the basic function of reversible oxygen (O_2) binding, which is common to myoglobin (Mb), Hb, and numerous other recently discovered proteins such as neuroglobin and cytoglobin. The tetrameric quaternary structure of Hb allows for cooperative O_2 binding to achieve efficient loading of O_2 in lung capillaries at high partial pressure of O_2 (P_{O2} ≈100 mm Hg), and effective O_2 delivery to peripheral tissues at low P_{O2} (≈40 mm Hg). Several small molecules, including 2,3-diphosphoglycerate (2,3-DPG), chloride anion (Cl^-), and carbon dioxide (CO_2)/bicarbonate dynamically regulate O_2 binding to Hb in response to varying physiological conditions. Hb also facilitates transport of CO_2 in the form of bicarbonate anion and participates in oxidation reactions, both beneficial and deleterious. The second part of this chapter reviews the biochemistry and pathophysiology of abnormal Hbs that give rise to human diseases. Globin gene mutations that alter the properties of Hbs are common, affecting about 7% of the world's population,[1,2] and present a significant global health problem.[3]

This chapter reviews Hb structure, biochemistry, and mutations that produce abnormal globin proteins. These "Hb variants" are usually caused by single amino acid substitutions, but also by amino acid insertions and/or deletions, antitermination mutations and altered post-translational processing. The most common pathological Hb variants, HbS and HbC are reviewed in Chapter 20. Other variants, discussed in the second part of this chapter, number more than a thousand and are rare individually but common collectively. Globin gene mutations that impair protein subunit production (i.e., thalassemias) are discussed in Chapter 21. Several excellent references review the concepts discussed in this chapter.[4-12,13]

HEMOGLOBIN STRUCTURE AND FUNCTION

The study of Hb and related proteins has contributed immensely to the understanding of protein function in general. In 1957, Kendrew and colleagues used X-ray diffraction to solve the structure of Mb at 6-Å resolution[14] and a few years later refined this structure to 2 Å, making it possible to build models at near atomic resolution.[15] These remarkable achievements showed for the first time what a protein actually "looks like." Almost simultaneously the structure of Hb was determined at 5.5-Å resolution by Perutz and colleagues.[16] Since then numerous biophysical studies have further refined the structures of normal and mutant Hbs to resolutions approaching 1 Å.[17]

HbA, the major adult form of Hb, is a tetramer of two α polypeptide chains and two β polypeptide chains ($\alpha_2\beta_2$),

Box 19-1 Hemoglobin Structure

- The globin fold is an evolutionary conserved structure made up of seven or eight α helices folded into a globular shape.
- HbA is a tetramer made up of two α- and two β-globin subunits, each linked to a heme moiety that binds O_2 reversibly via a central iron molecule. Only reduced iron (Fe[II]) can bind O_2.
- Hb can bind other diatomic ligands via heme iron, including CO and NO.
- The α1β1 (and α2β2) dimer is the basic structural unit of hemoglobin.
- Various α- and β-like globins are expressed during human ontogeny. These include:
    - Embryonic: Gower 1 ($\zeta_2\varepsilon_2$), Gower 2 ($\alpha_2\varepsilon_2$), Hb Portland I ($\zeta_2\gamma_2$), Hb Portland II ($\zeta_2\beta_2$)
    - Fetal: HbF ($\alpha_2\gamma_2$)
    - Adult: HbA ($\alpha_2\beta_2$), ~95%; HbA_2 ($\alpha_2\delta_2$), 1.5%-3.5%; HbF ($\alpha_2\gamma_2$), 1%-2%.

CO, Carbon monoxide; *Hb,* hemoglobin; *NO,* nitric oxide; *O_2,* oxygen.

each bound to a heme prosthetic group (Box 19-1). The α and β globins are believed to have arisen from the duplication of a common ancestral gene approximately 450 million years ago,[18] around the time that the lineages of cartilaginous fish and bony fish diverged. Subsequent gene duplications produced additional globin genes that are expressed at different times during ontogeny. During early human embryogenesis, the α-like (ζ) and β-like (ε and γ) chains produce embryonic Hbs ($\zeta_2\varepsilon_2$, $\zeta_2\gamma_2$, and $\alpha_2\varepsilon_2$). During midgestation, fetal Hb (HbF; $\alpha_2\gamma_2$) is predominantly expressed. From birth until around postnatal week 12, β-globin expression gradually increases and γ globin decreases as HbA replaces HbF. Another β-like globin (δ) is also expressed postnatally, and normal hemolysates contain approximately 2% HbA_2 ($\alpha_2\delta_2$). The properties of embryonic and fetal globins are tuned to the physiological conditions experienced in utero, particularly low O_2 tension, although the functions of all human Hb isoforms can be understood as variations on the mechanism of adult HbA. The onset of symptoms caused by deleterious globin gene mutations varies according to the developmental expression pattern of each particular gene, as discussed later in this chapter.

Hemoglobin Primary Structure

Primary structure refers to the linear sequence of amino acids within a protein. The primary structures of the α-like and β-like globin chains are shown in Table 19-1. Translation of the polypeptide chains on the ribosome is initiated with methionine, which is subsequently removed by methionine aminopeptidase before assembly of mature Hb tetramer. The α (141 amino acid residues) and β (146 amino acid residues) chains share only 41% amino acid

TABLE 19-1 Primary Amino Acid Structure of Hemoglobin*

Helix	α	ζ	Helix	β	δ	γ	ε
NA1	1 Val	Ser	NA1 NA2	1 Val 2 His	Val His	Gly His	Val His
NA2	2 Leu	Leu	NA3	3 Leu	Leu	Phe	Phe
A1	3 Ser	Thr	A1	4 Thr	Thr	Thr	Thr
A2	4 Pro	Lys	A2	5 Pro	Pro	Glu	Ala
A3	5 Ala	Thr	A3	6 Glu	Glu	Glu	Glu
A4	6 Asp	Glu	A4	7 Glu	Glu	Asp	Glu
A5	7 Lys	Arg	A5	8 Lys	Lys	Lys	Lys
A6	8 Thr	Thr	A6	9 Ser	Thr	Ala	Ala
A7	9 Asn	Ile	A7	10 Ala	Ala	Thr	Ala
A8	10 Val	Ile	A8	11 Val	Val	Ile	Val
A9	11 Lys	Val	A9	12 Thr	Asn	Thr	Thr
A10	12 Ala	Ser	A10	13 Ala	Ala	Ser	Ser
A11	13 Ala	Met	A11	14 Leu	Leu	Leu	Leu
A12	14 Trp	Trp	A12	15 Trp	Trp	Trp	Trp
A13	15 Gly	Ala	A13	16 Gly	Gly	Gly	Ser
A14	16 Lys	Lys	A14	17 Lys	Lys	Lys	Lys
A15	17 Val	Ile	A15	18 Val	Val	Val	Met
A16	18 Gly	Ser					
AB1	19 Ala	Thr					
B1	20 His	Gln	B1	19 Asn	Asn	Asn	Asn
B2	21 Ala	Ala	B2	20 Val	Val	Val	Val

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TABLE 19-1 Primary Amino Acid Structure of Hemoglobin (Continued)

Helix	α	ζ	Helix	β	δ	γ	ε
B3	22 Gly	Asp	B3	21 Asp	Asp	Glu	Glu
B4	23 Glu	Thr	B4	22 Glu	Ala	Asp	Glu
B5	24 Tyr	Ile	B5	23 Val	Val	Ala	Ala
B6	25 Gly	Gly	B6	24 Gly	Gly	Gly	Gly
B7	26 Ala	Thr	B7	25 Gly	Gly	Gly	Gly
B8	27 Glu	Glu	B8	26 Glu	Glu	Glu	Glu
B9	28 Ala	Thr	B9	27 Ala	Ala	Thr	Ala
B10	29 Leu	Leu	B10	28 Leu	Leu	Leu	Leu
B11	30 Glu	Glu	B11	29 Gly	Gly	Gly	Gly
B12	31 Arg	Arg	B12	30 Arg	Arg	Arg	Arg
B13	32 Met	Leu	B13	31 Leu	Leu	Leu	Leu
B14	33 Phe	Phe	B14	32 Leu	Leu	Leu	Leu
B15	34 Leu	Leu	B15	33 Val	Val	Val	Val
B16	35 Ser	Ser	B16	34 Val	Val	Val	Val
C1	36 Phe	His	C1	35 Tyr	Tyr	Tyr	Tyr
C2	37 Pro	Pro	C2	36 Pro	Pro	Pro	Pro
C3	38 Thr	Gln	C3	37 Trp	Trp	Trp	Trp
C4	39 Thr	Thr	C4	38 Thr	Thr	Thr	Thr
C5	40 Lys	Lys	C5	39 Gln	Gln	Gln	Gln
C6	41 Thr	Thr	C6	40 Arg	Arg	Arg	Arg
C7	42 Tyr	Tyr	C7	41 Phe	Phe	Phe	Phe
CE1	43 Phe	Phe	CD1	42 Phe	Phe	Phe	Phe
CE2	44 Pro	Pro	CD2	43 Glu	Glu	Asp	Asp
CE3	45 His	His	CD3	44 Ser	Ser	Ser	Ser
CE4	46 Phe	Phe	CD4 CD5	45 Phe 46 Gly	Phe Gly	Phe Gly	Phe Gly
CE5	47 Asp	Asp	CD6	47 Asp	Asp	Asn	Asn
CE6	48 Leu	Leu	CD7	48 Leu	Leu	Leu	Leu
CE7	49 Ser	His	CD8	49 Ser	Ser	Ser	Ser
CE8	50 His	Pro	D1 D2 D3 D4 D5 D6	50 Thr 51 Pro 52 Asp 53 Ala 54 Val 55 Met	Ser Pro Asp Ala Val Met	Ser Ala Ser Ala Ile Met	Ser Pro Ser Ala Ile Leu
CE9	51 Gly	Gly	D7	56 Gly	Gly	Gly	Gly
E1	52 Ser	Ser	E1	57 Asn	Asn	Asn	Asn
E2	53 Ala	Ala	E2	58 Pro	Pro	Pro	Pro
E3	54 Gln	Gln	E3	59 Lys	Lys	Lys	Lys
E4	55 Val	Leu	E4	60 Val	Val	Val	Val
E5	56 Lys	Arg	E5	61 Lys	Lys	Lys	Lys
E6	57 Gly	Ala	E6	62 Ala	Ala	Ala	Ala
E7	58 His	His	E7	63 His	His	His	His
E8	59 Gly	Gly	E8	64 Gly	Gly	Gly	Gly
E9	60 Lys	Ser	E9	65 Lys	Lys	Lys	Lys

TABLE 19-1 Primary Amino Acid Structure of Hemoglobin (Continued)

Helix	α	ζ	Helix	β	δ	γ	ε
E10	61 Lys	Lys	E10	66 Lys	Lys	Lys	Lys
E11	62 Val	Val	E11	67 Val	Val	Val	Val
E12	63 Ala	Val	E12	68 Leu	Leu	Leu	Leu
E13	64 Asp	Ser	E13	69 Gly	Gly	Thr	Thr
E14	65 Ala	Ala	E14	70 Ala	Ala	Ser	Ser
E15	66 Leu	Val	E15	71 Phe	Phe	Leu	Phe
E16	67 Thr	Gly	E16	72 Ser	Ser	Gly	Gly
E17	68 Asn	Asp	E17	73 Asp	Asp	Asp	Asp
E18	69 Ala	Ala	E18	74 Gly	Gly	Ala	Ala
E19	70 Val	Val	E19	75 Leu	Leu	Ile, Thr	Ile
E20	71 Ala	Lys	E20	76 Ala	Ala	Lys	Lys
EF1	72 His	Ser	EF1	77 His	His	His	Asn
EF2	73 Val	Ile	EF2	78 Leu	Leu	Leu	Met
EF3	74 Asp	Asp	EF3	79 Asp	Asp	Asp	Asp
EF4	75 Asp	Asp	EF4	80 Asn	Asn	Asp	Asn
EF5	76 Met	Ile	EF5	81 Leu	Leu	Leu	Leu
EF6	77 Pro	Gly	EF6	82 Lys	Lys	Lys	Lys
EF7	78 Asn	Gly	EF7	83 Gly	Gly	Gly	Pro
EF8	79 Ala	Ala	EF8	84 Thr	Thr	Thr	Ala
F1	80 Leu	Leu	F1	85 Phe	Phe	Phe	Phe
F2	81 Ser	Ser	F2	86 Ala	Ser	Ala	Ala
F3	82 Ala	Lys	F3	87 Thr	Gln	Gln	Lys
F4	83 Leu	Leu	F4	88 Leu	Leu	Leu	Leu
F5	84 Ser	Ser	F5	89 Ser	Ser	Ser	Ser
F6	85 Asp	Glu	F6	90 Glu	Glu	Glu	Glu
F7	86 Leu	Leu	F7	91 Leu	Leu	Leu	Leu
F8	87 His	His	F8	92 His	His	His	His
F9	88 Ala	Ala	F9	93 Cys	Cys	Cys	Cys
FG1	89 His	Tyr	FG1	94 Asp	Asp	Asp	Asp
FG2	90 Lys	Ile	FG2	95 Lys	Lys	Lys	Lys
FG3	91 Leu	Leu	FG3	96 Leu	Leu	Leu	Leu
FG4	92 Arg	Arg	FG4	97 His	His	His	His
FG5	93 Val	Val	FG5	98 Val	Val	Val	Val
G1	94 Asp	Asp	G1	99 Asp	Asp	Asp	Asp
G2	95 Pro	Pro	G2	100 Pro	Pro	Pro	Pro
G3	96 Val	Val	G3	101 Glu	Glu	Glu	Glu
G4	97 Asn	Asn	G4	102 Asn	Asn	Asn	Asn
G5	98 Phe	Phe	G5	103 Phe	Phe	Phe	Phe
G6	99 Lys	Lys	G6	104 Arg	Arg	Lys	Lys
G7	100 Leu	Leu	G7	105 Leu	Leu	Leu	Leu
G8	101 Leu	Leu	G8	106 Leu	Leu	Leu	Leu
G9	102 Ser	Ser	G9	107 Gly	Gly	Gly	Gly
G10	103 His	His	G10	108 Asn	Asn	Asn	Asn

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TABLE 19-1 Primary Amino Acid Structure of Hemoglobin (Continued)

Helix	α	ζ	**Helix**	β	δ	γ	ε
G11	104 Cys	Cys	G11	109 Val	Val	Val	Val
G12	105 Leu	Leu	G12	110 Leu	Leu	Leu	Met
G13	106 Leu	Leu	G13	111 Val	Val	Val	Val
G14	107 Val	Val	G14	112 Cys	Cys	Thr	Ile
G15	108 Thr	Thr	G15	113 Val	Val	Val	Ile
G16	109 Leu	Leu	G16	114 Leu	Leu	Leu	Leu
G17	110 Ala	Ala	G17	115 Ala	Ala	Ala	Ala
G18	111 Ala	Ala	G18	116 His	Arg	Ile	Thr
G19	112 His	Arg	G19	117 His	Asn	His	His
GH1	113 Leu	Phe	GH1	118 Phe	Phe	Phe	Phe
GH2	114 Pro	Pro	GH2	119 Gly	Gly	Gly	Gly
GH3	115 Ala	Ala	GH3	120 Lys	Lys	Lys	Lys
GH4	116 Glu	Asp	GH4	121 Glu	Glu	Glu	Glu
GH5	117 Phe	Phe	GH5	122 Phe	Phe	Phe	Phe
H1	118 Thr	Thr	H1	123 Thr	Thr	Thr	Thr
H2	119 Pro	Ala	H2	124 Pro	Pro	Pro	Pro
H3	120 Ala	Glu	H3	125 Pro	Gln	Glu	Glu
H4	121 Val	Ala	H4	126 Val	Met	Val	Val
H5	122 His	His	H5	127 Gln	Gln	Gln	Gln
H6	123 Ala	Ala	H6	128 Ala	Ala	Ala	Ala
H7	124 Ser	Ala	H7	129 Ala	Ala	Ser	Ala
H8	125 Leu	Trp	H8	130 Tyr	Tyr	Trp	Trp
H9	126 Asp	Asp	H9	131 Gln	Gln	Gln	Gln
H10	127 Lys	Lys	H10	132 Lys	Lys	Lys	Lys
H11	128 Phe	Phe	H11	133 Val	Val	Met	Leu
H12	129 Leu	Leu	H12	134 Val	Val	Val	Val
H13	130 Ala	Ser	H13	135 Ala	Ala	Thr	Ser
H14	131 Ser	Val	H14	136 Gly	Gly	Gly, Ala	Ala
H15	132 Val	Val	H15	137 Val	Val	Val	Val
H16	133 Ser	Ser	H16	138 Ala	Ala	Ala	Ala
H17	134 Thr	Ser	H17	139 Asn	Asn	Ser	Ile
H18	135 Val	Val	H18	140 Ala	Ala	Ala	Ala
H19	136 Leu	Leu	H19	141 Leu	Leu	Leu	Leu
H20	137 Thr	Thr	H20	142 Ala	Ala	Ser	Ala
H21	138 Ser	Glu	H21	143 His	His	Ser	His
HC1	139 Lys	Lys	HC1	144 Lys	Lys	Arg	Lys
HC2	140 Tyr	Tyr	HC2	145 Tyr	Tyr	Tyr	Tyr
HC3	141 Arg	Arg	HC3	146 His	His	His	His

From Bunn HF, Nagel RL: Hemoglobins: normal and abnormal. In Nathan DG, Orkin SH, Ginsburg D, et al, editors: Nathan and Oski's hematology of infancy and childhood, *vol 7, Philadelphia, 2009, Saunders, p. 911–948; and modified from Bunn HF, Forget BG:* Hemoglobin: molecular, genetic, and clinical aspects, *Philadelphia, 1986, Saunders.*

*The α and α-related (ζ) subunits are shown at the left. The β and β-related (δ, γ, and ε) chains are at the right. The amino acids' relationship to the eight globin helices (A to H) is also shown. Thus A16 is the sixteenth amino acid in the A helix. Interhelical elbows are named for the two adjacent helices (e.g., AB1 is the first amino acid between helices A and B). The N- and C-terminal residues are labeled NA and HC, respectively. The residues are aligned to maximize the homology between subunits, which causes some gaps.

sequence identity, yet they adopt very similar three-dimensional (3D; tertiary) structures. Numerous naturally occurring mutations alter the primary sequence of globin proteins[12,13] (see the second part of this chapter).

Hemoglobin Secondary Structure

Secondary structure refers to the organization of primary structure into sections of regular polypeptide backbone geometry. Hb chains are comprised primarily of α-helical secondary structure joined by turns or short sections with irregular polypeptide backbone conformation. The geometry of regular secondary structure is dictated by steric constraints (clashes between atoms in neighboring residues) and maximization of hydrogen bonding between amide and carbonyl groups in the backbone. Although hydrogen bonds are relatively weak individually, their repeating pattern in α helices has a cooperative effect to make the structure comparatively rigid. Through this property, α helices transmit forces in proteins and protein complexes to alter their structure and modulate function, as occurs for Hb. Most globin chains contain seven or eight α helices labeled *A* through *H* (from amino to carboxyl terminus), with the intervening links referred to as *corners* or *loops*. The position of each residue is described with reference to these structural elements. For example, the heme-coordinating histidine occurs at position F8 (residue 8 in helix F) in all globins (see Table 19-1).

Hemoglobin Tertiary Structure

Tertiary structure refers to the 3D structure of a polypeptide that is formed by packing secondary structural elements together. In general, hydrophobic side chains occupy the interior of a protein or protein domain, and polar and charged residues are arranged on the exterior (Fig. 19-1, *A* and *B*). Thus mutations converting a nonpolar interior amino acid to a polar one commonly produce an unstable protein (see the second part of this chapter). The α-helical segments of globin chains fold together into a globular shape with a cavity that is occupied by the O_2-binding heme prosthetic group[16,17] (see Fig. 19-1, *B*-*D*). The basic tertiary structure, called the *globin*

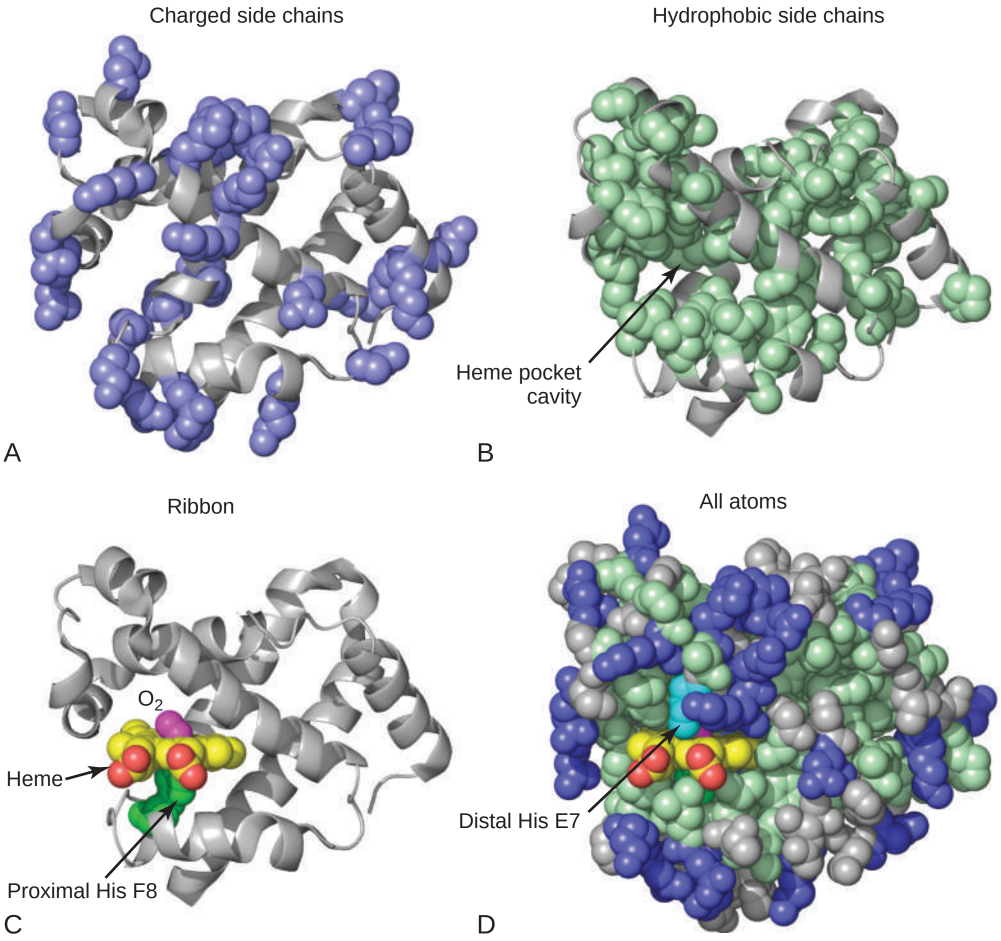

Figure 19-1 The secondary structure and amino acid composition of the β chain of Hb. **A**, Charged side chains (*blue*: Lys, Arg, Glu, Asp) are all located on the surface of the structure exposed to the polar solvent (water). His side chains can be charged or uncharged depending on solvent and local protein environment and are not shown. **B**, Hydrophobic (nonpolar) side chains (*pale green*: Ala, Val, Leu, Ile, Pro, Met, Phe, Trp) are clustered in the center of the structure, forming a hydrophobic core. Other polar, uncharged residues are not shown. **C**, A ribbon diagram showing the eight α helices, the location of the heme group *(yellow/red spheres)*, heme-coordinating proximal His *(green)*, and bound O_2 *(magenta)*. **D**, All atoms showing how O_2 *(magenta)* is buried in the heme pocket and stabilized by distal His E7. Rotation of the distal His E7 side chain is required to allow O_2 entry/exit from the distal heme pocket.

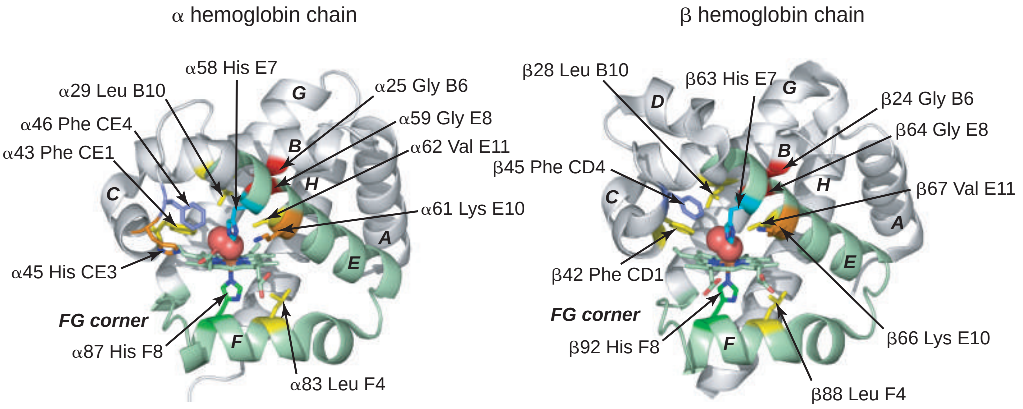

Figure 19-2 Structural features of the globin fold. Ribbon representations of the α and β Hb chains in the O_2-bound form, with the α helices A–H in the β chain and A–C and E–H in the α chain labeled. Note the conserved tertiary structure of the two globin subunits. Conserved residues in α and β are shown in stick representation, and carbon atoms are colored according to their function as follows. The proximal His F8 *(green)* coordinates the heme iron. The distal His E7 *(cyan)* makes electrostatic interactions that stabilize bound O_2 *(red spheres)*. Hydrophobic side chains in the distal heme pocket *(yellow)* make steric interactions with O_2 and other diatomic ligands. Hydrophobic Leu F4 prevents water from entering the proximal heme pocket and disrupting the His F8–Fe bond.[26] Hydrophobic αPhe CE1/βPhe CD1 make important stabilizing interactions with the heme, help orient bound O_2, and together with αPhe CE4/βPhe CD4 help position the distal His E7. Basic side chains *(orange)* interact with the negatively charged propionates of the heme group. The small side chains of Gly E8 and Gly B6 *(red)* are required to allow close packing of the E and B helices. Upon O_2 binding, tertiary structural changes in the globin chains are most significant in helices E and F and in the FG corner, which together with the heme group form the “allosteric core” *(pale green)*.

fold, occurs in a large, ancient protein family with members expressed in animals, plants, and bacteria.[19,20] Figure 19-2 shows the tertiary structures of α and β globin with conserved α-helical arrangements and amino acid residues that mediate Hb function. Importantly, these structures are not static or rigid. Rather the tertiary structures of α and β globins (and the quaternary structure of the tetramer) change according to binding of O_2 or other ligands and also through interactions with allosteric effectors. These dynamic structural properties underlie the physiological function of Hb as a gas transporter and the pathologic features of many Hb variants.

The Prosthetic Heme Group

Each α and β chain binds one heme b group (Fig. 19-3), which forms the binding site for O_2 (see Fig. 19-1, C). Heme is an amphipathic molecule that is highly insoluble in water, so it must be bound to protein carriers or protein active sites under physiologic conditions. Biosynthesis of protoporphyrin IX occurs through eight well-characterized steps that take place either in the cytosol or the intermembrane space of mitochondria.[21] The final step, insertion of an iron ion, is catalyzed by the enzyme ferrochelatase.[21]

In the folded α and β chains, heme is bound into a deep pocket that is lined with hydrophobic residues (see Fig. 19-1, *B* and *D*). Interactions between the protein and heme are critical for stabilizing the native globin fold.[22-25] Thus mutations that destabilize heme–protein interactions lead to increased globin precipitation and the release of free heme, which has pathologic consequences (see the second part of this chapter). A coordinate covalent bond between the heme iron and the Nε atom of a histidine residue in the co-factor binding pocket, termed the *proximal histidine (F8)*, is of primary importance for globin

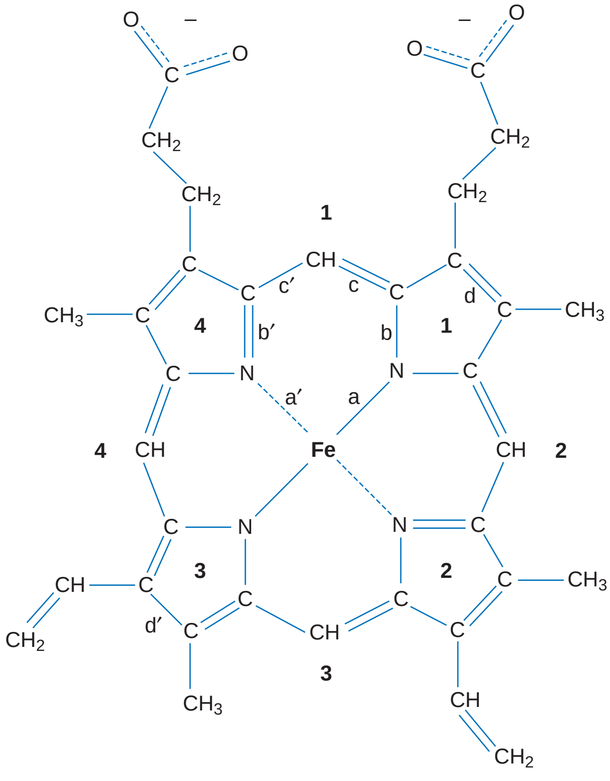

Figure 19-3 The heme group. The chemical structure of heme b, Fe(II)–protoporphyrin IX. The organic component, protoporphyrin IX, is a conjugated system of four pyrrole rings joined by methene bridges, with four methyl, two vinyl, and two propionate side chains. A central iron, the O_2-binding molecule in Hb, is coordinated in a square-planar arrangement by lone pairs from the four pyrrole nitrogens. *(From Bunn HF, Nagel RL: Hemoglobins: normal and abnormal. In Nathan DG, Orkin SH, Ginsburg D, et al, editors:* Nathan and Oski’s hematology of infancy and childhood, *vol 7, Philadelphia, 2009, Saunders, p. 911–948.)*

structure and O_2 binding function (see Fig. 19-1, *C* and Residues of the Heme Pocket). A sixth iron–coordination site on the opposite (distal) side of the planar heme is available for reversible O_2 binding (see Fig. 19-1, *C*). In addition to direct iron coordination to the proximal histidine, the following heme–globin interactions are important for maintaining the Hb tertiary structure: 1) hydrophobic interactions between nonpolar residues in the pocket and the porphyrin ring; 2) hydrogen bonding between residues in the distal heme pocket and diatomic ligands; and 3) electrostatic interactions between the heme propionates and amino acids on the surface of the protein.[22,26-28] An asymmetric distribution of methyl and vinyl side groups on the heme ring defines a stereospecific orientation of heme binding. Heme iron can exist in a range of oxidation states in vivo, most commonly as Fe(II) or Fe(III), but the ferryl Fe(IV) is also biologically relevant (see Production of Ferryl Heme and Hemoglobin Protein Radicals). Importantly, only Fe(II) (ferrous) heme can bind O_2. The distal histidine (E7) of globin chains typically stabilizes bound O_2, but under certain circumstances can also bind the sixth coordinate position of heme iron to form structures termed *hemichromes* or *hemochromes* when the iron is oxidized or reduced, respectively.

Hemoglobin Quaternary Structure

Quaternary structure refers to the packing of protein subunits into a multimeric complex. During Hb assembly, α and β monomers join to form a very high-affinity αβ dimer (association equilibrium constant $[K_A] > 10^{10}\ M^{-1}$) (Fig. 19-4, *A*). Two identical α1β1 and α2β2 dimers interact to form a tetramer, in which the constituent dimers are related by a two-fold rotational (C2) symmetry (see Fig. 19-4, *B*). This interaction between dimers is coupled to O_2 ligand binding, with deoxygenated dimers having a higher affinity for self-association to form tetramers ($K_A \approx 4 \times 10^{10}\ M^{-1}$) than fully oxygenated dimers ($K_A \approx 10^6\ M^{-1}$).[29,30] The major dimer–dimer contacts involve nonidentical chains at the α1β2 and α2β1 interfaces. Changes in the interactions between α1β1 and α2β2 dimers are central to the mechanism of cooperative O_2 binding (see Fig. 19-4, *C*).

Free globin subunits, particularly the apo forms lacking heme, are unstable and toxic as monomers[31] and must be assembled rapidly and efficiently into Hb tetramers during erythropoiesis. The order of globin subunit assembly into mature Hb tetramers is unknown, but probably involves parallel pathways.[32-34] For example, heme may be incorporated into apo α or β globins before or after their assembly into αβ dimers. In mammals, nascent apo or met α globins may be bound and stabilized by the molecular chaperone alpha Hb stabilizing protein (AHSP) during the early steps of Hb assembly in vivo.[33,34,35,36]

Dioxygen Binding

The Oxygen Carrying Capacity of Hemoglobin

The solubility of O_2 in water (≈0.3 mM at 20° C and 1 atm) is insufficient to achieve full tissue oxygenation in most animals. Consequently animals have evolved to use heme or other respiratory pigments to transport O_2. However, O_2 rapidly oxidizes free heme from the Fe(II) to Fe(III) state, which cannot bind O_2 (see Redox Reactions of Hemoglobin). To overcome this potential limitation, globin proteins protect the heme group from oxidation and fine tune ligand binding to allow for reversible O_2 uptake and delivery. Hb carries O_2 from the lungs to peripheral tissue capillaries and CO_2 in the reverse direction. The concentration of Hb tetramers in red blood cells (RBCs) is typically about 5.2 mM (equivalent to ≈21 mM heme), and RBCs typically account for 45% of blood volume, giving a theoretical O_2 concentration of approximately 9 mM. O_2 is 21% by volume in air, which corresponds to roughly 8.75 mM in the gas phase at 1 atm and 20° C (1 mol gas under these conditions occupies 24 L by the ideal gas law). Hence Hb raises the O_2 concentration in blood to the same level as in air.

Residues of the Heme Pocket

Conserved, functionally important residues in the heme pockets of α and β globin are shown in Figure 19-2. The

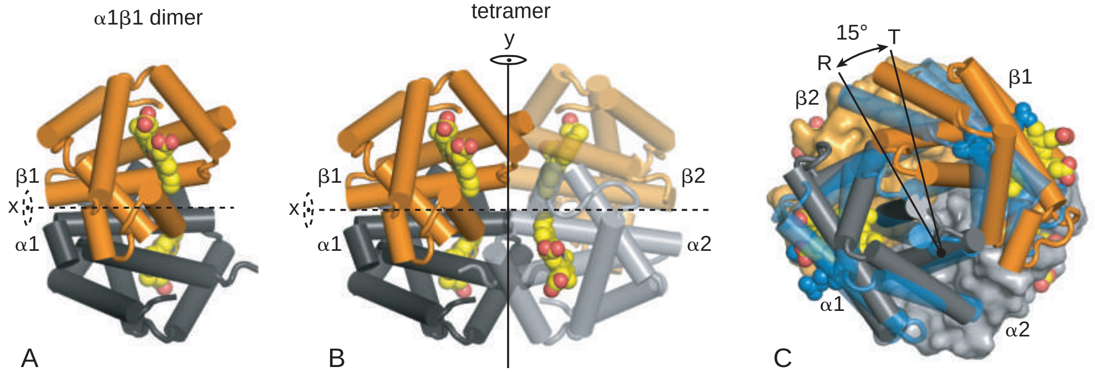

Figure 19-4 Quaternary structure of Hb. **A**, Schematic of the α1β1 dimer with helices shown as solid cylinders and the heme groups in space-fill representation. **B**, The Hb tetramer is comprised of two αβ dimers, related through a twofold rotational (C2 symmetry) axis *(y)*. A pseudo twofold axis *(x)* perpendicular to *y*, relates the positions of the α and β chains in each dimer. **C**, Looking down the pseudo twofold x axis, the van der Waals surface of the α2β2 dimer is shown. The position of the α1β1 dimer in the R (oxy) quaternary state is shown as *gray/orange cylinders* and *yellow hemes*. The position of the same α1β2 dimer in the T (deoxy) quaternary state is shown in *blue*. The α1β1 dimer undergoes a rotation of 15 degrees with respect to α2β2 about an axis that passes through both α chains.

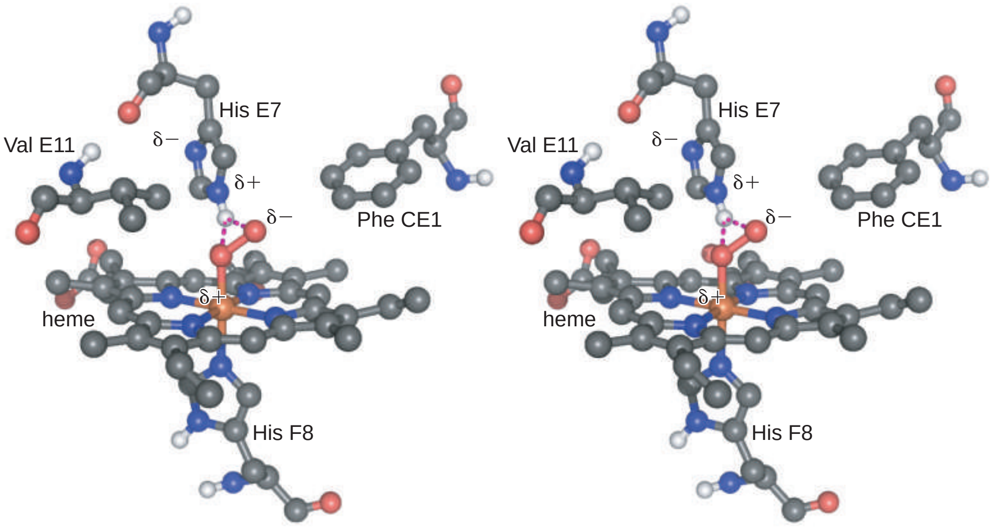

Figure 19-5 Binding of O_2 in the heme pocket. Stereo (cross-eye) diagram of the heme pocket of Hb β subunit. The heme Fe(II) *(orange sphere)* is coordinated by Nε of the proximal His F8. The iron–O_2 complex is polar, with net transfer of negative charge to O_2 *(red spheres)*.[37] The polar Fe–O_2 interaction is stabilized by hydrogen bonding *(magenta)* with the distal His E7. Phe CE1 helps hold the heme in place and orients both bound O_2 and His E7 for more efficient hydrogen bonding. Val E11 contacts the bound O_2 and helps restrict solvent access to the distal pocket.

proximal His-F8 stabilizes an axial iron ligand, which is essential for establishing the octahedral metal coordination geometry that is necessary for O_2 binding. Electrostatic interactions between bound O_2 and the distal His-E7 are of primary importance for ligand selectivity (Fig. 19-5)[37] and inhibiting heme oxidation (autoxidation).[38] In both α and β globin, hydrogen bonding between His-E7 and O_2 has been identified through high resolution (1.25 Å) x-ray crystal structures,[17] nuclear magnetic resonance,[39] electron paramagnetic resonance (EPR),[40] and site-directed mutagenesis.[41] This interaction contributes around 1.9 kcal mol^{-1} to O_2 binding affinity.[41] The importance of this interaction is illustrated by an approximate thousandfold increase in the O_2 dissociation rate from Mb when the distal His is replaced with a nonpolar amino acid.[37,41] A number of nonpolar side chains in the proximal heme pocket make important steric interactions with bound O_2 (see Fig. 19-2, *yellow*). In particular, αPhe CE1/βPhe CD1, which is highly conserved in all globins, stabilizes the heme group and helps orient bound O_2 and His-E7 for efficient hydrogen bonding. βPhe-CD4 (αPhe-CE4) also plays a role in positioning of the distal His side chain to enable O_2 binding (see Fig. 19-2).[42] In the proximal heme pocket, Leu F4 prevents water from entering and disrupting the His F8–iron bond.[26]

The heme pocket of globins is relatively enclosed (see Fig 19-1, *D*), and multiple routes of O_2 entry have proposed, based on binding studies of xenon gas into nonpolar cavities of Mb[43-45] and Hb.[46] X-ray crystallographic and kinetic analyses of Mb[47,48] and Hb[41,49,50] indicate that O_2 enters via a channel caused by outward rotation of the distal His-E7 (see Figs. 19-1, *D*, and 19-2, *cyan*), which allows direct entry into the pocket rather than migration through other cavities in the protein.

In addition to O_2 binding, ferrous Fe(II) Hb also binds carbon monoxide (CO) and nitric oxide (NO) (see Nitric Oxide–Hemoglobin Reactions). Studies with model porphyrin systems show that the Fe–CO bond is intrinsically stronger than the Fe–O_2 bond by a factor of 2×10^4.[37,51] If this ratio of affinities were true in the context of an Hb tetramer, then humans would be rapidly poisoned by endogenously produced CO, which is generated by heme catabolism, neuronal function, and cell signaling in the vasculature. High-level environmental CO exposure can also occur from cigarette smoke and internal combustion engines. Thankfully the relative affinity of CO and O_2 is reduced from 2×10^4 in free porphyrins to about 220 in Hb,[52] largely because of the different electrostatic properties of the Fe–CO and Fe–O_2 complexes.[37] The Fe–O_2 complex is highly polar and makes strong hydrogen bonding interactions with the distal His E7 (see Fig. 19-5). In contrast, the nonpolar Fe–CO complex interacts only weakly with the distal His.

Cooperative Oxygen Binding

Binding of O_2 to Mb can be described by a simple one–binding-site model that yields a hyperbolic curve (Box 19-2 and Fig. 19-6):

$$Y = P_{O2}/(P_{50} + P_{O2})$$

In this equation, Y is the fractional O_2 saturation, P_{O2} is the partial pressure of O_2, and P_{50} is the value of P_{O2} at which 50% of the O_2-binding sites are occupied. For the simple one–binding-site equilibrium, the dissociation constant, K_d, is equal to P_{50}. In contrast, the O_2 equilibrium curve for Hb is sigmoidal (see Fig. 19-6). The initial portion of the curve has a small slope, indicating that fully deoxygenated Hb (deoxy-Hb) has a relatively low affinity for O_2. As O_2 loading proceeds, the slope of the curve steepens, reflecting increasing Hb affinity for O_2. Ultimately, the slope levels off again as Hb approaches O_2 saturation. The shape of this curve indicates that O_2

Box 19-2 **Cooperative Binding of Oxygen to Hemoglobin**

- Cooperative O_2 binding results when the binding of O_2 to one site in Hb increases the O_2 affinity of the remaining sites. Similarly, release of O_2 from one site increases the probability that O_2 will be released from the remaining unbound sites. The overall effect enhances the amount of O_2 that can be bound and released over the narrow range of P_{O2} values that characterize arterial and venous blood (see Fig. 19-6).
- Hb tetramers can bind four O_2 molecules (one per subunit). Binding of several O_2 molecules to deoxy-Hb causes a switch to a high affinity conformation. The high–O_2 affinity Hb quaternary structure is referred to as *relaxed (R),* and the low-affinity Hb structure is referred to as *tense (T).*
- O_2 binding to the heme iron of one Hb subunit alters quaternary structure at the $\alpha1\beta2$ and $\alpha2\beta1$ interfaces of the tetramer, leading to structural changes in the heme pockets of unliganded Hb subunits to enhance their O_2 affinity. As a result, some mutations that affect the $\alpha1\beta2$ and $\alpha2\beta1$ interfaces alter the O_2 equilibrium curve, causing right or left shifts and different amounts of O_2 transport in vivo.

deoxy-Hb, Deoxygenated hemoglobin; *Hb,* hemoglobin; *O_2,* oxygen; *P_{O2},* partial pressure of oxygen.

binding to one or more sites causes an increase in the O_2 affinity of the remaining sites. This property, called *positive cooperativity,* is essential for Hb function.

Conversely, deoxygenation at one or more Hb sites decreases O_2 affinity for the remaining sites, favoring its release in peripheral tissues where O_2 concentration is relatively low. In this way, approximately 1.7 times more O_2 can be delivered to the tissues than would be possible with a hypothetical noncooperative O_2 binding protein with the same intrinsic O_2 affinity. An empirical relationship that described cooperative O_2 binding activity was proposed by A.V. Hill in 1910.[53]

$$Y = (P_{O2})^n / (P_{50})^n + (P_{O2})^n$$

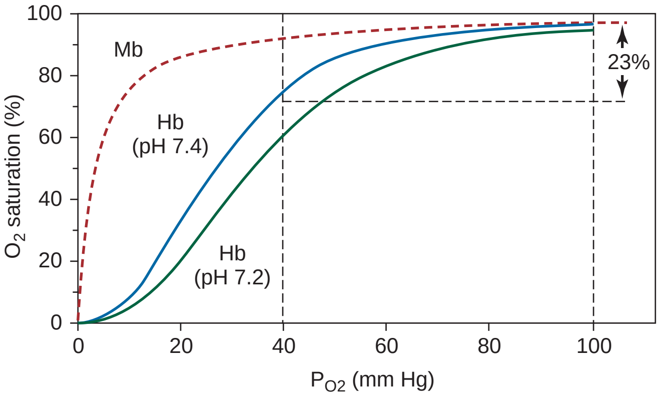

Figure 19-6 O_2 equilibrium curves of sperm whale Mb and Hb in human red blood cells. Myoglobin binds O_2 noncooperatively as a monomer with high affinity. The sigmoidal shape of the O_2 equilibrium curves reflect cooperative O_2 binding. In arterial blood (pH 7.4, 37° C, P_{O2} 100 mm Hg), O_2 saturation is 98%. In mixed venous blood (pH 7.2, 37° C, P_{O2} 40 mm Hg) O_2 saturation is 75%. The HbO_2 saturation difference between arterial and venous blood reflects the extent of O_2 delivery to peripheral tissues. *(From Imai K:* Allosteric effects of haemoglobin, *Cambridge, 1982, Cambridge University Press.)*

The exponent, *n,* termed *Hill coefficient*, measures cooperativity. A value of $n > 1$ represents positive cooperativity. A value of $n = 1$ yields no cooperativity. A value of $n < 1$ results in negative cooperativity. The Hill coefficient for HbA is approximately 2.8. Note that for the cooperative binding reaction, the apparent K_d is equal to $(P_{50})^n$ and is not the ligand concentration at half saturation.

Although the Hill equation reproduces the observed O_2 binding of Hb reasonably well, it implies simultaneous binding of multiple O_2 ligands.[54] G.S. Adair determined that Hb contained four binding sites for O_2[55] and formulated a sequential binding model in which each O_2 binds with progressively higher affinity to each of the four sites.[56] Adair's equation gives an accurate fit to the experimental O_2 equilibrium curve and uses a general and physically plausible binding model. However, the generality of the model means that it does not provide any mechanistic insight into Hb cooperativity. In fact, cooperative binding implies some communication between the heme molecules in each globin chain. Before the structure of Hb (or any protein) was determined, Linus Pauling formulated a model based on a direct chemical interaction between heme groups[57] and showed that O_2 binding characteristics could be reproduced, assuming that subunits of Hb were symmetrically arranged such that O_2 at one heme could influence binding at two adjacent sites.[56] Given the distant spacing between the heme groups, communication must be mediated by the Hb protein matrix, with binding at one site sterically affecting O_2 binding to a heme group at another ("allo-") site on an adjacent subunit.

Allostery and the Perutz–Monod, Wyman, Changeux Model of Hemoglobin Function

The term *allosteric* was first coined in 1961 by Monod and Jacob[58] to described how regulatory ligands could influence the activity of enzymes by binding to distinct sites that did not overlap with the substrate binding site.[59] Allostery, an extremely important concept in biochemistry, has been described as "the second secret of life," second only to the genetic code.[60] The Monod, Wyman, Changeux (MWC) model, developed in the early 1960s,[61] still forms a theoretical basis for understanding cooperative O_2 binding to Hb. The theory itself was influenced by the structural view of Hb provided by Perutz.[59] A key finding from Perutz and colleagues was the elucidation of the human deoxy-Hb structure at 5.5 Å,[62] which provided an opportunity for comparison with the earlier structure of horse oxygenated Hb (HbO_2), also at 5.5 Å.[16] At this resolution no difference in the structures of the individual subunits could be clearly identified. However, there was a clear difference in the quaternary structure. Perutz and colleagues noted that, "the two β chains in human reduced (deoxy-)Hb have moved apart, increasing the distance between their two haem groups 7 Å compared with that in horse oxyhaemoglobin."[62] This quaternary structural rearrangement of Hb in response to O_2 binding is at the heart of the MWC model.

The MWC model is shown in Figure 19-7, *A*. Its basic principle is that quaternary structural changes in Hb regulate the O_2-binding affinity of the subunit chains. The

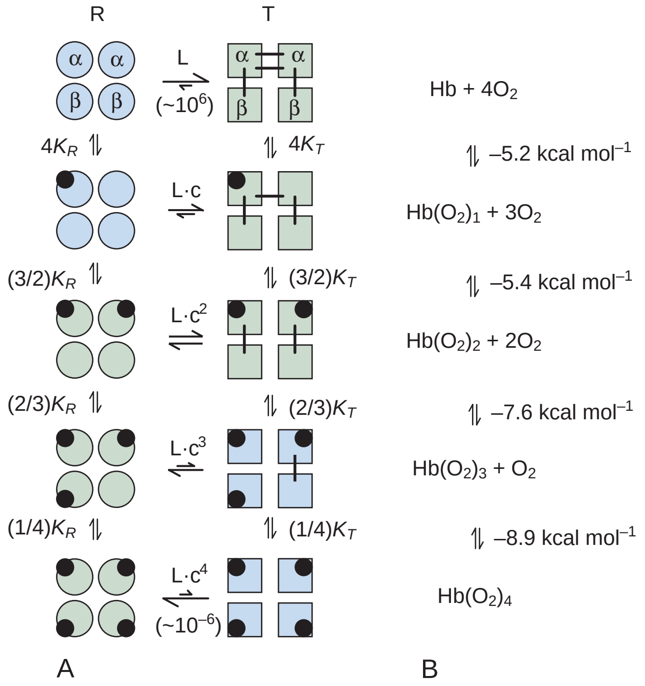

Figure 19-7 Model for cooperative O_2 binding. **A,** A schematic of the MWC-Perutz model[61,465] based on Eaton, et al.[56] The four subunits of the R or T quaternary structure are indicated as *four circles* or *four squares*, respectively. A *solid black sphere* represents an O_2 molecule bound to one subunit. The affinity constant for O_2 binding to any one subunit in the R or T state is K_R or K_T, respectively, hence there is no cooperativity within the R or T quaternary state. With no O_2 bound, the ratio of the T and R state is $L = [T_0]/[R_0] \approx 10^6$. Specific molecular interactions (e.g., salt bridges) between T state subunits (represented by *joining lines*) are broken upon conversion to the R state. Binding of each O_2 molecule weakens these T state quaternary interactions by a factor $c = K_T/K_R \approx 10^{-3}$,[94,95,465] shifting equilibrium towards the R state. The highly populated quaternary states at each oxygenation level (accounting for >90% of Hb molecules) are *green*; the minor quaternary species are *pale blue*.[465] **B,** The free energy change for successive O_2 binding events, obtained by fitting O_2 equilibration data obtained under standard conditions (21.5 °C, pH 7.4, 0.18 M Cl^{-1}) to the four Adair binding constants, after adjustment for dimer-tetramer equilibrium.[69] Note that binding becomes more favorable (*ΔG* more negative) as O_2 saturation increases.

MWC theory describes Hb as a symmetrical oligomer that can adopt two distinct quaternary structures, termed the *tense (T)* and *relaxed (R)* states or conformations. The T and R states are in equilibrium, and in the absence of O_2, the T state is much more stable than the R state (see Fig. 19-7, *A, top row*). The equilibrium between the T and R states of unliganded Hb is defined by the MWC parameter, $L = [T_0]/[R_0]$, where *[T₀]* and *[R₀]* are the molar concentrations of the zero-liganded T and R states. In the T state, all the subunits have a tertiary conformation with low–O_2 affinity (small association equilibrium constant K_T or high P_{50}), whereas in the R state, all subunits have a tertiary conformation with high–O_2 affinity (large association equilibrium constant K_R or low P_{50}). Note that O_2 binding to the individual T (or R) state tetramers does not increase the affinity of the T (or R) state for binding additional O_2 molecules, so there is no cooperativity within the T (or R) quaternary state. However, because O_2 preferentially binds to empty sites in the R quaternary state rather than the T state ($K_R > K_T$), the T→R equilibrium must shift towards R with the addition of O_2, according to the Le Châtelier principle. The basis for cooperativity is that the T→R quaternary switch involves a concerted switch of all subunits from the low–O_2 affinity tertiary structure to the high–O_2 affinity structure (*right-to-left shifts* in Fig. 19-7, *A*). The level of cooperativity is determined by the parameters *L* and *c* = K_T/K_R. The MWC model provides a good fit to experimental equilibrium O_2-binding data, yielding typical parameters of $L \approx 5\times10^6$ and $c \approx 1.5 \times10^{-3}$ and $1/K_T \approx 80$ torr[56,63] for pH 7.4, 0.15 M NaCl, 21.5° C. These parameters are significantly modulated by pH, salt, and organic phosphate concentration (see Heterotropic Effectors of Oxygen Binding to Hemoglobin). The parameter *c* reveals that the quaternary T→R switch corresponds to a 500- to 1000-fold increase in O_2 affinity. The MWC model neglects differences between α and β affinities, but these are likely to differ by less that twofold.[41,64] Many excellent reviews of the MWC model with expanded and refined models are available.[56,65-67]

In order to move from the unliganded T_0 state to the fully O_2-liganded R_4 state, the bonding and nonbonding interactions that stabilize the T_0 relative to R_0 quaternary structures (see *lines joining T-state subunits* in Fig. 19-7, *A*) must be broken using free energy provided by O_2 binding. Consequently the free energy for binding of each successive O_2 becomes more favorable (see Fig. 19-7, *B*). In the unconstrained R state, each binding event would yield −8.35 kcal mol⁻¹.[68,69] The difference between the free energy for binding four O_2 molecules in HbA (−27.1 kcal mol⁻¹) compared with binding in the unconstrained R state (−33.4 kcal mol⁻¹) is called the *cooperative free energy of binding* ($\Delta G_c = +6.3$ kcal mol⁻¹).[68,69] To provide a molecular explanation for how O_2 binding is coupled to changes in tertiary and quaternary structure, Perutz developed his stereochemical model for cooperative O_2 binding,[70] which is described in the following sections.

The High- and Low-Oxygen Affinity Quaternary Structures of Hemoglobin

The stereochemical mechanism proposed by Perutz identifies the T and R states within the MWC model as two discrete quaternary structures that differ in the relative orientation of the α1β1 and α2β2 dimers within the context of the Hb tetramer.[70,71] The T-to-R quaternary switch of the tetramer involves about a 15-degree rotation of the α1β1 dimer relative to α2β2 (see Fig. 19-4, C).[71] The rotation occurs around an axis perpendicular to the C2 symmetry axis and passes through both α subunits. The β chains experience greater relative movement, moving closer together in the R state (as originally observed by Perutz).[62] At the same time, the central cavity between the four globin chains, particularly the cleft between the β subunits, narrows in the R state and inhibits binding of 2,3-DPG, an important modulator of O_2 affinity (see *2,3-Diphosphoglycerate*).

Interactions between α1β1 and α2β2 dimers (i.e., α1β2 interactions) are weaker in the R (oxy) state compared with the T (deoxy) state, which accounts for the observation that R-state tetramers dissociate into $\alpha 1\beta 1$ dimers at low protein concentrations, whereas T state Hb remains a tetramer and emphasizes the role of $\alpha 1\beta 2$ interactions in cooperative O_2 binding. In contrast there is little or no change in the $\alpha 1\beta 1$- (or $\alpha 2\beta 2$-) dimer interface between the T and R quaternary states. Consequently residues at these interfaces do not contribute significantly to allostery. Accordingly, free α and β monomers[72] and $\alpha 1\beta 1$ dimers[73] bind O_2 noncooperatively with a high affinity similar to that of R-state tetramers. Conversely, when crystallized[74,75,76] or embedded in a silica gel,[77] T-state Hb cannot undergo a quaternary T→R transition and binds O_2 noncooperatively at low affinity. These latter findings demonstrate that interactions at the $\alpha 1\beta 2$ interface reduce O_2 affinity in the T state and to a first approximation account for the tetramer's cooperativity.

In tetrameric Hb, the α_1 chain contacts both the α_2 and β_2 chains. Each of these interfaces plays a role in the allosteric mechanism. Interactions between nonidentical chains ($\alpha 1\beta 2$ and $\alpha 2\beta 1$ contacts) account for the majority of the buried surface area. A more restricted set of $\alpha 1\alpha 2$ contacts are present in the T and R states. The $\beta 1\beta 2$ interface is present in the R state but is lost as the β chains move apart in the T structure.

Shown in Figure 19-8, *A*, is the $\alpha 1\beta 1$ dimer with the contact face for the $\alpha 2\beta 2$ dimer facing up out of the page (the $\alpha 2\beta 2$ dimer has been removed to reveal the contact surface). The $\alpha 1\beta 2$ and $\alpha 2\beta 1$ interfaces include the C helix and the FG corner (residues between the F and the G α helices) in each chain. These elements are colored *blue* and *magenta* in the α_1 and β_1 subunits shown in Figure 19-8. The C helix from each Hb chain contacts the FG corner from the nonidentical chain across the $\alpha 1\beta 2$ (or $\alpha 2\beta 1$) interface. This interaction creates four pairs of contacts, as shown in Figure 19-8, *B*. In this panel, the C and FG segments of the α_2 and β_2 chains are shown as "bent tubes" overlaying the $\alpha 1\beta 1$ dimer, with the rest of the chain omitted to allow the interface to be seen. Notably, the behavior of the C helix in α and β chains is different throughout the T→R quaternary change and is indicated by their different coloring in Figure 19-8. Specifically, the molecular contacts between the C helices of the β chains (βC) and the FG corners of the α chains (αFG) change very little during the T→R quaternary transition (see Fig. 19-8, *B*, *dark* blue). Therefore the βC–αFG contacts are known as the *pivot* (or *joint*) contacts. In contrast, the αC helix moves relative to βFG corner, experiencing a translation of approximately 6 Å in the direction of the helix axis, equivalent to approximately one helical turn (5.4 Å) relative to the βFG corner (see Fig. 19-8, *B*, *magenta*). The two alternative positions of the βFG loop relative to the αC helix are dictated by different side-chain packing interactions. Hence βAsp-99 in the βFG corner fits between the side chains of αThr-41 and αTyr-42 from the αC helix in the T state (Fig. 19-9, *B*). In the R state, βFG is shifted such that the side chain of βHis-97 fits between αThr-38 and αThr-41 in the αC helix (see Fig. 19-9, *A*). These two alternative positions of the αC–βFG contact have led to this contact being described as the "switch" region of the $\alpha 1\beta 2$ interface. It is thought that steric hindrance makes intermediates between these two positions unstable,[71] giving rise to the two-state TR quaternary switch. The $\alpha 1\beta 2$ interface is also characterized by different inter–subunit-hydrogen bonding patterns in the T and R states (Fig. 19-10). The intricate nature of the switch region allows mutations at these residues to significantly perturb the stability of the T state (*L* value in the MWC model) and therefore Hb function, sometimes with clinical consequences (see the second part of this chapter).

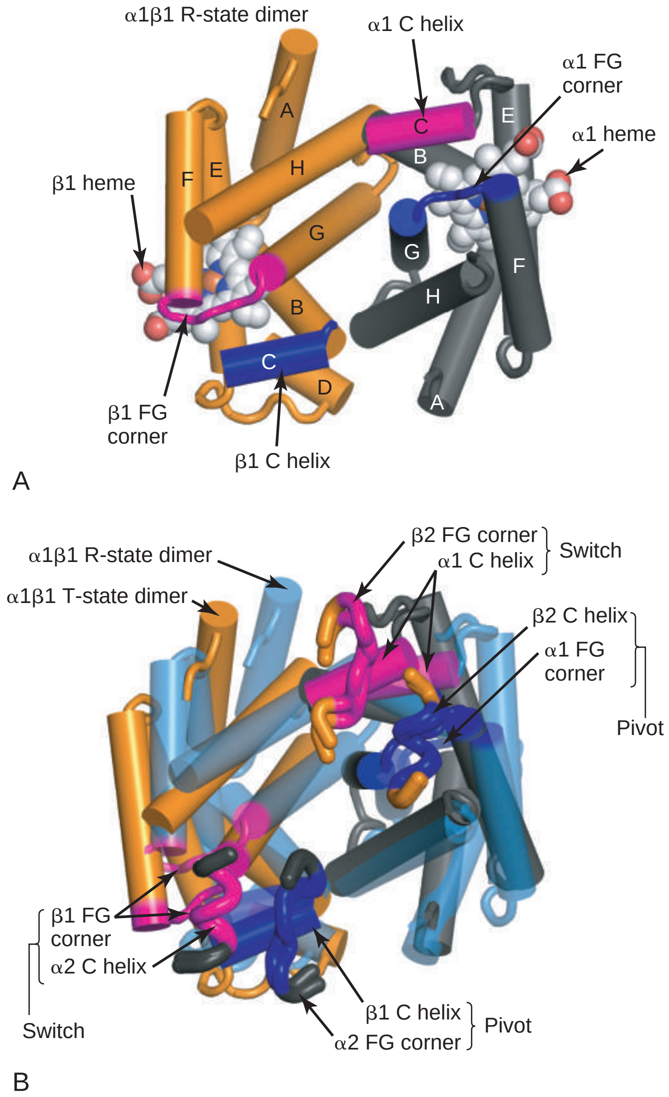

Figure 19-8 The $\alpha 1\beta 2$ interface: switch and pivot interactions. **A**, The $\alpha 1\beta 1$ dimer from R-state HbA (Protein Data Bank [PDB] 2DN2) is shown with the $\alpha 1\beta 2$ dimer removed to reveal the interdimer interface, looking down the pseudo twofold axis, *x* (see Fig.19-4, *A*). Major contacts between the dimers are mediated by the C helices and FG loops (*blue* and *magenta*). **B**, The $\alpha 1\beta 1$ dimer from T-state HbA (PDB 2DN1) is shown *(orange, dark gray)*, superposed with the R-state structure from **A** *(light blue transparency)*. The positions of the $\alpha 2\beta 2$ dimers has been fixed to reveal the rotation of $\alpha 1\beta 1$ between T and R structures. Only the C and FG segments of the $\alpha 2\beta 2$ chains are shown (flexible "tubes"). Contacts between αFG and βC *(blue)* experience little change upon conversion from T to R ("pivot" contacts). In contrast, the αC helix moves relative to βFG loop (*magenta*; "switch" contacts).

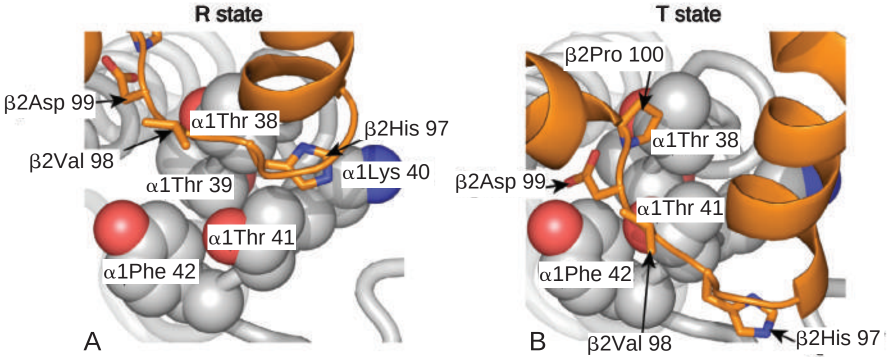
R state
β2Asp 99
α1Thr 38
β2His 97
β2Val 98
α1Thr 39
α1Lys 40
α1Thr 41
α1Phe 42
A
T state
β2Pro 100
α1Thr 38
β2Asp 99
α1Thr 41
α1Phe 42
B
β2Val 98
β2His 97

Figure 19-9 The α1β2 switch contact. Side chains of the βFG loop *(orange)* pack into grooves that are defined by side chains on the αC helix *(gray)*. To switch from the R state (PDB 2DN1) to the T state (PDB 2DN2), the βFG loop must move between two "valleys" that are separated by a "hill," which is αThr 41.

Although the switch region helps define distinct T and R structures, a range of R quaternary structures have been observed. For example, the C2 symmetry axis in liganded Hb that is crystallized under low-salt conditions at pH 5.8 is distinct from the T or R state structures determined at a neutral or high pH.[78,79] This additional quaternary structure, R2 HbO_2 has been proposed to be either an intermediate in the T→R transition[78] or a true end-point for fully oxy-Hb.[80,81] The current understanding is that in the thermodynamic R state (fully oxy-Hb) proposed by the MWC model, Hb exists in a range of conformations, rather than as a single rigid structure. Crystal structures of the R, R2, and other quaternary variations[82] provide information about the range of structures that are energetically accessible to HbO_2. In support of this interpretation, recent measurements of O_2 binding to crystallized R- and R2-state Hb reveal that these are thermodynamically equivalent states,[83] and a nuclear magnetic resonance (NMR) study of Hb in solution suggests that the average structure of liganded Hb lies approximately halfway between the crystallographic R- and R2-states.[84] Despite considerable variation in the C2 symmetry axis between different R-state crystal structures, a set of common interactions between residues at the α1β2, α1α2, and β1β2 interfaces can be used as structural markers that identify the thermodynamic R state.[17]

Altered Oxygen Binding at the Heme Site in the T and R States

In the unliganded state, pentacoordinate heme iron (Fe[II]) makes four bonds to pyrrole nitrogens and one axial bond with proximal His F8 (α87 or β92). A water molecule sits in the distal pocket of the α chain but does not coordinate the heme iron. The distal pocket of β chains appears empty, or there is no discrete position for a water molecule. The heme site in T-state deoxy-Hb has a high-spin Fe(II) electronic configuration, and the iron is displaced 0.4 to 0.6 Å out of the plane of the four pyrrole nitrogen atoms in the direction of the proximal His F8 ligand. Studies on model porphyrins indicate that an out-of-plane iron is the preferred structure for high-spin Fe(II) porphyrin and hence the iron site in T-state deoxy-Hb is unstrained. Importantly, O_2 binding requires a change in electronic configuration to a low-spin, hexacoordinate, in-plane iron. This configuration is seen in R-state HbO_2 where the heme iron is only 0.09 and 0.06 Å out of the porphyrin plane in α and β chains, respectively.[17]

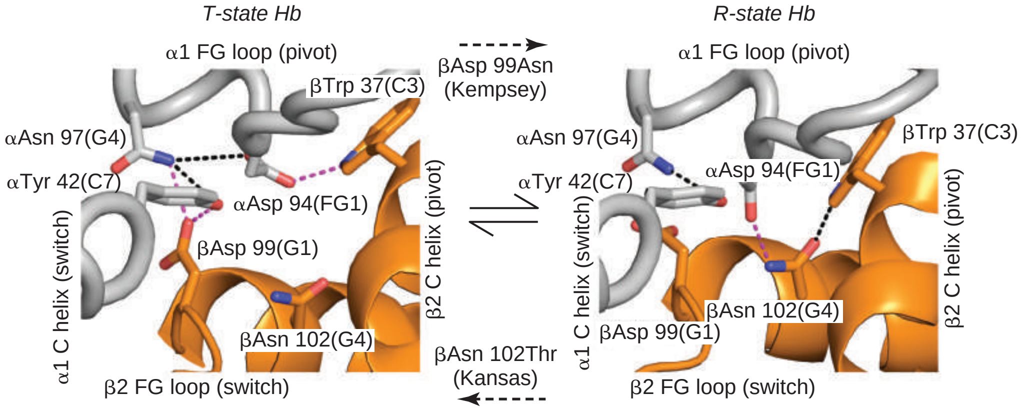
T-state Hb
α1 FG loop (pivot)
βTrp 37(C3)
αAsn 97(G4)
αTyr 42(C7)
αAsp 94(FG1)
βAsp 99(G1)
βAsn 102(G4)
α1 C helix (switch)
β2 C helix (pivot)
β2 FG loop (switch)
βAsp 99Asn
(Kempsey)
βAsn 102Thr
(Kansas)
R-state Hb
α1 FG loop (pivot)
βTrp 37(C3)
αAsn 97(G4)
αAsp 94(FG1)
αTyr 42(C7)
α1 C helix (switch)
βAsn 102(G4)
βAsp 99(G1)
β2 C helix (pivot)
β2 FG loop (switch)

Figure 19-10 Detail of the α1β2 interface in the deoxy T state (PDB 2DN2) and oxy R state (PDB 2DN1). Hydrogen bonding networks in each state employ nonoverlapping sets of side chains (intrachain hydrogen bonds, *black*; interchain hydrogen bonds, *magenta*). Hb variants at these residue positions can alter the T→R equilibrium. Thus β99 (G1) Asp→Asn (Hb Kempsey) compromises electrostatic interactions only in the T state, thereby favoring the R state and resulting in high–O_2 affinity. Conversely, β102 (G4) Asn→Thr (Hb Kansas) disrupts the R state leading to low–O_2 affinity.

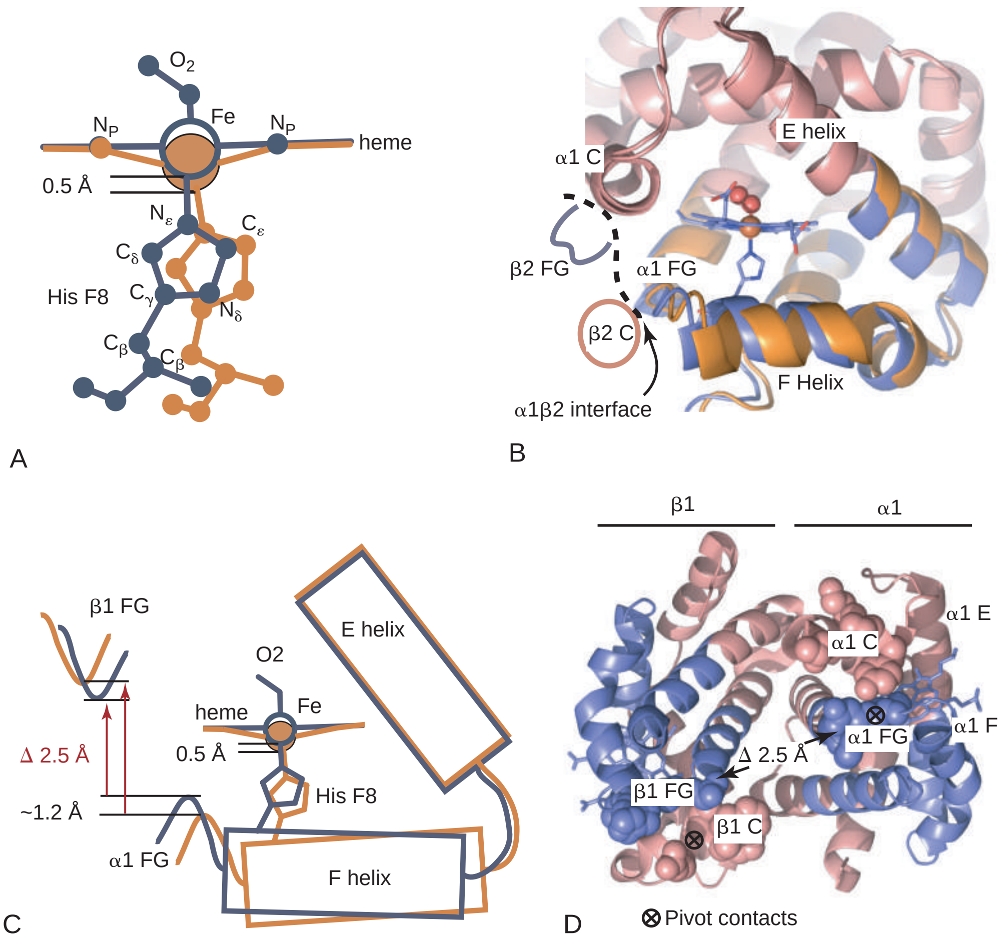

Figure 19-11 O_2 binding is coupled to tertiary structural changes in globin chains. **A,** Schematic of the heme and proximal His F8 in T-state deoxy-Hb *(orange)* and R-state oxy-Hb *(blue-gray)*, looking along the plane of the porphyrin. The central iron atom (*orange sphere* in deoxy Hb) moves about 0.5 Å into the plane of the porphyrin ring upon O_2 ligation. Steric repulsion between His F8 Cε and heme pyrrole nitrogens (N_P) introduces an energetic penalty against iron moving into the heme plane in the T state. Atoms are not drawn to scale. **B,** The most significant tertiary structure changes between α-globin chains from T- and R-state Hb occur at the F helix and EF loop, with smaller changes in parts of the E, G, and H helices (T state, *orange*; R state, *blue*). Other regions are essentially unchanged *(salmon)*. **C,** Movements of the F helix and FG corner are linked to movement of the heme iron, coupled through the His F8 side chain. Combined motions of the α and β chains from a single α1β1 dimer results in the FG loops moving approximately 2.5 Å closer together (T state, *orange*; R state, *blue-gray*). **D,** The α1β1 dimer is the base structural unit of hemoglobin. The stable portion of the dimer, including the α1β1 interface *(pink)*, supports the allosteric core of each chain *(blue)*, which includes the heme group *(sticks)*, EF corner, F helix, and FG corner. Changes in the allosteric core, including movement of the FG corners, couple O_2 binding to quaternary structural changes.

Hence the iron site in R-state HbO_2 is also unstrained. However, accommodating the O_2-liganded heme in the T-quaternary structure introduces strain into the Hb structure because movement of iron into the porphyrin plane requires upward movement of the F-helix away from the α1β2 interface and consequently reduces the free energy of O_2 binding.[75,85-89] Thus in effect, the low–O_2 affinity of the T state can be explained, because the O_2-liganded heme is a poor fit in the T-state structure. In the R-state oxy-Hb, the His F8 imidazole ring is positioned over the heme such that the Fe–Nε bond is normal to the plane of the porphyrin (Fig. 19-11, *A*, *blue*). This geometry is important to minimize steric repulsion between the imidazole and porphyrin rings when iron is in the porphyrin plane.[88] However, in the T-state structure, the His F8 imidazole is rotated relative to heme plane (see Fig. 19-11, *A*, *orange*). In this position there is a greater energetic cost for moving iron into the heme plane because of steric repulsion between Cε of His F8 and the pyrrole nitrogens of the porphyrin.[88]

Determining how strain, introduced into T-state Hb by O_2 binding, is distributed across the tertiary and quaternary T-state structure is the subject of ongoing research. In Perutz's stereochemical model, the strain energy is at least partly compensated (or "stored") by salt bridges present only in the quaternary T state, as described in the following sections.

Quaternary Interactions Contribute to Cooperativity

Salt bridges are electrostatic interactions between oppositely charged groups. These can involve the side chains of basic amino acid residues (Arg or Lys), acidic residues (Glu and Asp), N-terminal amino groups, or C-terminal carboxyl groups of polypeptide chains. Perutz identified eight specific salt bridges present in the T state (but not the R state) that stabilize the low-affinity quaternary conformation.[70] More recent, higher-resolution crystal structures of T-state (deoxy-Hb) and R-state (oxy- and carbonmonoxy-Hb) Hb suggest minor modifications of this set of interactions (Table 19-2).[17,90] In the R state, these interactions are largely replaced by weaker hydrogen bonding interactions between polar residues or to water molecules. For example, the C-terminal residues αArg 141 (HC3) and βHis 146 (HC3) participate in T-state–specific salt bridges (Fig. 19-12).[89-91] Removing both αArg 141 and βHis 146 with carboxypeptidase B increases O_2 affinity and reduces cooperativity for the resultant modified Hb molecule.[91] Similar conclusions were obtained using T-state Hb trapped in gels. Removal of αArg 141 alone caused a 15-fold increase in O_2 affinity of the T state,[92] compared with a threefold increase for the β146 His→Leu mutation.[93]

TABLE 19-2 Salt bridges and Hydrogen Bonding Interactions of the T- and R-State Quaternary Structure

Donor	Acceptor
T-State Quaternary Structure	
α1 Lys127 (H10) ε-amino group (+)	α2 Arg141 (HC3) carboxyl terminus (–)*
α1 Arg141 (HC3) guanidinium (+)	α2 Asp126 (H9) side chain carboxyl (–)*
α1 Lys40 (C5) ε-amino group (+)	β2 His146 (HC3) carboxyl terminus (–)*
β1 His146 (HC3) imidazolium (+)	β1 Asp94 (FG1) side chain carboxyl (–)*
α1 Arg92 (FG4) guanidinium (+)	β2 Glu43 (CD2) side chain carboxyl (–)*
α1 Asn97 (G4) side chain amine	β2 Asp99 (G1) side chain carboxyl (–)
α1 Tyr42 (C7) phenol	β2 Asp99 (G1) side chain carboxyl (–)
α1 Lys127 (H10) ε-amino group (+)	α1 Val1 (NA1) backbone carbonyl
β1 Val1 (NA1) amino terminus (+)	β1 Leu78 (EF2) backbone carbonyl
β1 Trp37 (C3) indole amino group	α2 Asp94 (FG1) side chain carboxyl (–)
R-State Quaternary Structure	
β1 Val1 (NA1) amino terminus (+)	β2 His146 (HC3) carboxyl terminus (–)*
α1 Asn97 (G4) side chain amide	α1 Tyr42 (C7) phenol
α1 Val1 (NA1) amino terminus (+)	α2 Ser138 (H21) backbone carbonyl
β1 Trp37 (C3) indole amino group	β1 Asn102 (G4) side chain carbonyl
β1 Asn102 (G4) side chain amine	α2 Asp94 (FG1) side chain carboxyl (–)
β1 Arg40 (C6) guanidinium (+)	α2 Thr41 (C6) hydroxyl
β1 Arg40 (C6) guanidinium (+)	α2 Leu91 (FG3) backbone carbonyl

*Rows containing _asterisks_ indicate salt bridges. No _asterisk_ indicates hydrogen bonding interactions.

Beginning with Szabo and Karplus,[94] several groups created mathematical models suggesting that T-state–specific salt bridges could account for the free energy of cooperativity. Consistent with experimental evidence, some models suggested that ligand binding in the T state strains but does not break the salt bridges.[85,86,95] Importantly, it seems that the lower O_2 binding affinity of T-state Hb is the result of effects from salt bridges and other interactions at the α1β2 interface, which transmits ligand-induced strain in the allosteric core (heme, proximal His, F helix and FG corner).[85,86,95] Crystallized T-state deoxyHb can be oxygenated without switching to the R structure. This procedure has revealed changes in the stereochemistry of the heme, shifts in the FG corners towards the R-state quaternary structure, and lengthening (i.e., weakening) of the T-state–specific salt bridges.[75,87] For example, the C-terminal salt bridge between β_1 His 146 (HC3) and α_2 Lys 40 (C5) (see Fig. 19-12) is lengthened by 0.9 Å in the oxy T state compared to the deoxy T state.[87] However, more recent computational approaches conclude that the free energy of cooperativity is also stored across many other molecular interactions.[88]

Hydrogen bonds at the α1β2 interface may also be strained when the T state is liganded.[88,96-98] For example, Hb variants with nonconservative switch-region substitutions at βAsp 99[99] or αTyr 42[100,101] show increased O_2 affinity and reduced cooperativity (see Fig. 19-10 and discussion of Hb Kempsey in High–Oxygen affinity Hemoglobin Variants). In contrast, conservative substitutions of Tyr or Asp gave less marked effects.[98-100] Similarly, at the α1β2 hinge region, the side chain of βTrp37 makes an inter-subunit hydrogen bond in the T state that stabilizes the α1β2 interface in the T relative to the R state,[98,102,103] and mutations of βTrp 37 decrease cooperativity.[97,104]

Coupling of Oxygen Binding to Structural Changes in Hemoglobin

Superposition of the α1β1 dimers from T- and R-state Hb reveals a set of residues that have invariant tertiary structure. A group of 74 residues are used as a reference frame to describe structural changes in the α and β chains, as well as the α1β1 and α2β2 dimers, during T→R shifts.[105] These include α-chain residues 23 through 42 (helices B and C), α 57 through 63 (helix E), α 101 through 111 (helix G), and α 118 through 125 (helix H); and β-chain residues β 51 through 57 (helix D), β 110 through 116 (helix G), and β 119 through 132 (helix H).[17] These reference-frame residues predominantly map to the α1β1 interface, reinforcing the concepts that relative orientation of the α1β1 and α2β2 dimers represents the major quaternary rearrangement associated with cooperative O_2 binding.

The concerted changes in iron coordination and globin tertiary structure are shown diagrammatically in Figure 19-11. During the T-to-R switch, helix F moves closer to the center of the globin, the gap between helices E and F narrows, and the heme iron moves toward the porphyrin ring (see Fig. 19-11, *A*-C). In addition (not shown in Fig.

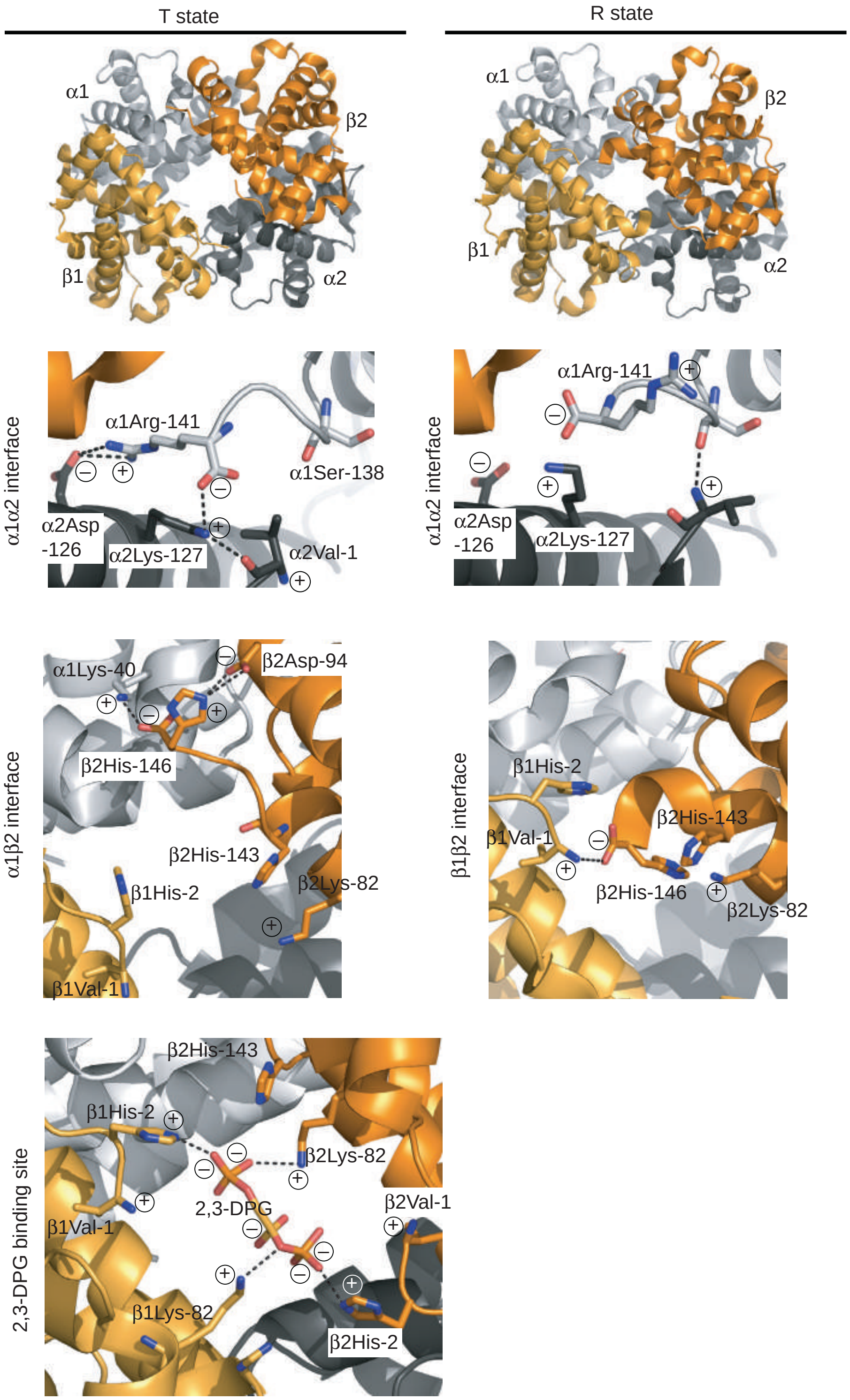
T state
R state
α1
β2
β1
α2
α1α2 interface
α1Arg-141
α1Ser-138
α2Asp -126
α2Lys-127
α2Val-1
α1β2 interface
α1Lys-40
β2Asp-94
β2His-146
β2His-143
β1His-2
β2Lys-82
β1Val-1
β1β2 interface
2,3-DPG binding site
2,3-DPG
β2Val-1
β1Lys-82
β2His-2
+
−

Figure 19-12 Electrostatic interactions at the HbA dimer–dimer interfaces. α1β1 *(light gray, gold)* and α2β2 *(dark gray, orange)* dimers make different sets of interactions in the T *(left)* and R *(right)* quaternary structures. These interactions are summarized in Table 19-2 and depicted in the lower panels of this figure. Note that the central cavity of the Hb tetramer widens in the T state *(top left)* allowing 2,3-DPG to bind at the β1β2 interface *(bottom panel)*.

19-11) the α heme group moves about 0.5 Å towards the back of the heme pocket, and the β heme undergoes a larger translation of 1.5 Å and a 9-degree rotation. Motion of the heme iron in and out of the porphyrin plane upon O_2 binding and/or dissociation is transmitted through the proximal His, causing the F helix to pivot. The relatively rigid α-helical structure allows a small movement of the heme iron in or out of the porphyrin (≈0.5 Å) to be amplified, resulting in relatively large motions at the FG corners. The combined tertiary structural changes in the α and β subunits from the same high- affinity (α1β1 or α2β2) dimer bring the αFG and βFG corners approximately 2.5 Å closer together in the R state (see Fig. 19-11, *D*, *blue*). In contrast, the relative positions of the αC and βC helices are little changed (see Fig. 19-11, *D*, *pink*). The net result of contracting the distance between the FG corners, while keeping the distance between the C helices fixed, is that some of the C-to-FG contacts across the α1β2 interface must altered. As described previously, the α_1C-to-β_2FG contacts break and reform (switch), whereas the α_1FG-to-β_2C contacts pivot, resulting in the 15-degree rotation of α1β1 relative to α2β2 (see Fig. 19-4, C). Although the αC helix (hinge region) does not undergo substantial conformation changes, it is believed to store significant strain upon T-state oxygenation.[86,88,97,102,106]

Several lines of evidence confirm that the bond between iron and the proximal His-F8 plays key roles in transmitting the effect of O_2 binding to the F helix and in promoting structural rearrangements at the tertiary and quaternary level. For example, when deoxy-Hb was crystallized in the T-quaternary state and the crystals were oxidized and soaked with sodium cyanide (CN), the binding of the strong CN ligand to the T-state α chains forced the iron-His–F8 bond to rupture, indicating that introducing ligands into the T-state structure places the iron-His–F8 bond under strain.[107] In another experiment, Ho, Barrick, and colleagues specifically ablated the iron-His–F8 bond using an F8 His-to-Gly mutation and introduced free imidazole, which bound into the proximal heme pocket and coordinated the heme iron. They found that this complex exhibited increased O_2 affinity and reduced cooperativity.[108] This experiment suggested that the proximal heme ligand in wild-type Hb transmits approximately two-thirds of the cooperative free energy of O_2 binding. Steric interactions between the pyrrole rings and heme pocket residues are also involved.[88]

Changes in Tertiary Structure Precede Quaternary Switching

Gibson, Eaton, and their colleagues employed time-resolved spectroscopy to study internal ligand movement and conformational events that occur after laser photolysis of the Fe–CO bond but before bimolecular rebinding of the ligand from solvent.[109] This approach has shown that tertiary structural changes after ligand binding or dissociation occur within a much shorter time frame (hundreds of nanoseconds [ns] to 1-2 microseconds [μs])[110-112] compared with quaternary structural changes (on the order of milliseconds [ms]).[109,112] Computational methods indicate that fast tertiary T→R structural transitions precede slow quaternary switching[85,86] and that tertiary structural changes induced by oxygen binding to multiple subunits increases the probability that Hb will make the T→R switch.[65] These studies show that the "concerted" quaternary switch, integral to the MWC model, is more accurately viewed as a coupling between rapid tertiary and slow quaternary events. Efforts to develop quantitative biophysical models to fully explain the coupling of tertiary and quaternary structural changes to ligand binding, incorporating all equilibrium O_2 binding and kinetic data, are ongoing.[66,67,113-115]

Box 19-3 Allosteric Regulators of Hemoglobin

- Allosteric regulation refers to changes in the structure and activity of a protein that results from binding of regulatory substances outside of the protein's active site.
- Allosteric regulators of Hb modulate O_2 binding by affecting the T-to-R transition.
- Cooperative O_2 binding is a form of allosteric regulation.
- Other allosteric regulators of Hb include H^+, Cl^-, and 2,3-DPG, which bind outside of the heme pocket to alter Hb O_2 affinity.
- CO_2 produced in peripheral tissues generates H^+, which binds Hb to reduce its O_2 affinity, facilitating tissue oxygenation. This is the alkaline Bohr effect.
- 2,3-DPG, a by-product of glycolysis, binds Hb to reduce its O_2 affinity. Hypoxia stimulates 2,3-DPG production to enhance O_2 delivery to tissues.
- Fetal Hb ($\alpha_2\gamma_2$) exhibits low affinity for 2,3-DPG, which facilitates transfer of O_2 from mother to fetus.

2,3-DPG, 2,3-Diphosphoglycerate; *Cl^-,* chloride anion; *CO_2,* carbon dioxide; *H^+,* hydrogen cation; *Hb,* hemoglobin; *O_2,* oxygen.

Heterotropic Effectors of Oxygen Binding to Hemoglobin

In addition to O_2, other small molecules and ions known as *heterotropic allosteric effectors* bind with different affinities to T- and R-state Hb and as a result impact cooperative O_2 binding (Box 19-3). The physiologically important species are CO_2, hydrogen cation (H^+), Cl^- and 2,3-DPG. In general, these molecules bind more tightly to the T state, thereby reducing the O_2 binding affinity of Hb. This binding causes a "right shift" in the O_2 saturation curve (Fig. 19-13). Heterotropic effectors have their most significant effect at low P_{O2} values, because they augment O_2 release in the tissues. In the lungs, at relatively high P_{O2} (≈95 mm Hg), the slightly decreased O_2 affinity caused by allosteric effectors has little effect on O_2 uptake.

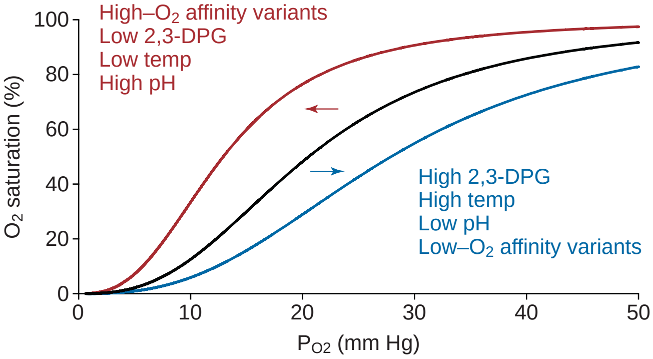

Figure 19-13 Heterotropic effectors alter the Hb-O_2 equilibrium curve. The O_2 equilibrium curve for HbA in cells *(black line)* was simulated using P_{50} = 20 mm Hg and n_{Hill} = 2.7. High pH (low H^+), low 2,3-DPG, low temperature, and high–O_2 affinity Hb variants all cause a "left shift" in the HbO_2 saturation curve (*red line*; simulated with P_{50} = 13 mm Hg, n_{Hill} = 2.7). Low pH, high 2,3-DPG, high temperature, and low–O_2 affinity Hb variants cause a "right shift" (*blue line*; simulated with P_{50} = 28 mm Hg, n_{Hill} = 2.7). All curves are sigmoidal due to the enhanced affinity of Hb for O_2 upon binding successive O_2 molecules (see Box 19-2). *(Modified from Thom CS, Dickson CF, Gell DA, Weiss MJ: Hemoglobin variants: biochemical properties and clinical correlates.* Cold Spring Harb Perspect Med *3:a011858, 2013.)*

Carbon Dioxide Transport and the Bohr Effect

In 1904 the physiologist Christian Bohr showed that CO_2 reduced the O_2 affinity of Hb.[56] Inside RBCs, carbonic anhydrase converts dissolved CO_2 to carbonic acid, which dissociates to bicarbonate and H^+ (i.e., $CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$). Upon conversion from the R to the T state, ionizable chemical groups within Hb bind H^+ ion. Hence increasing [H^+] or decreasing pH shifts equilibrium in favor of the low-affinity T state. The decreased O_2 affinity as pH changes from about 7.4 in the lungs to 7.2 or less in tissue capillary beds is known as the *alkaline* (or *physiological*) *Bohr effect.* Below pH 6.0, the affinity for O_2 increases again. This is called the *acid Bohr effect,* which does not occur in vivo. The physiologic importance of the Bohr effect is that increased pCO_2 (acidification) occurs at sites of high metabolic activity, promoting O_2 release from Hb where it is most required. For example, pCO_2 rises from approximately 35 torr in lung alveoli to about 50 torr in exercising muscles, causing the pH of blood to fall from about 7.4 to 7.2 or lower. This translates into a right-shifted O_2 saturation curve (see Fig. 19-13) with a 25% increase in the P_{50} for O_2 by the alkaline Bohr effect ($\partial \log P_{50}/\partial$ pH = –0.5 in 0.1 M Cl^-, pH 7.4).[71]

Hb facilitates the transport and disposal of CO_2 in several ways. As O_2 is released in actively respiring tissue capillaries, T-state Hb takes up H^+, which promotes conversion of more CO_2 to bicarbonate via deprotonation of carbonic acid in red cells, thereby increasing the CO_2 carrying capacity of blood. Approximately 70% to 80% of CO_2 transport occurs as bicarbonate. In the lungs, the formation of R-state Hb releases bound H^+, converting bicarbonate back to carbonic acid and CO_2, which exchanges with air in the alveoli. An additional 15% to 20% of CO_2 is carried as carbamino-Hb, which is formed by reaction of CO_2 with the terminal amine groups of α and β chains (CO_2 + Hb-NH_2 ⇌ Hb-NH-COO^- + H^+).[116-118] Finally, about 7% of CO_2 gas is simply dissolved in the blood.

The Bohr effect is largely attributed to formation of specific salt bridges involving chemical groups that can bind and release H^+ in response to physiological changes in pH. His residues have basic side chains with pKa of about 6.5, meaning that they can adopt different charge states in the physiological pH range, depending on their chemical environment. In the equilibrium His^+-to-His + H^+ reaction, equal amounts of the charged and neutral His side chains are present when pH equals pKa. When pH is greater than pKa, the neutral state predominates, and when pH is less than pKa, the positively charged state predominates. A key point is that local electrostatic interactions have a significant effect on the pKa value. Salt bridges in the T or low-affinity quaternary conformation stabilize the charged His^+ side chain, increasing its pKa. In other words, the affinity of these His side chains for H^+ is higher in the T state compared with the R state (the necessary condition for an allosteric effector). Then these salt bridges are broken during the transition to the R state when O_2 binds, causing a decrease on pKa and loss of the His protons.

The side chain that contributes most significantly to the alkaline Bohr effect is βHis-146 (HC3), which forms a salt bridge with βAsp-94 (FG1) in T-state Hb[119-121] (see Fig. 19-12). Ho and colleagues measured proton dissociation by NMR to determine the pKa of all His side chains in T- and R-state Hb. They found that the pKa of βHis-146 increases from 6.4 in the R state to 7.9 in the T state and accounts for 60% of the Bohr effect (similar to the finding of Kilmartin et al[122]). Meanwhile, the sum of the contributions from all 13 surface histidyl residues in the αβ dimer accounted for 86% of the alkaline Bohr effect in 0.1 M Cl^-, pH 7.4,[121] the remaining effect being the result of other basic groups including the N-terminal amines.[123] A tertiary Bohr effect has also been described (independent of quaternary bonds), which may involve protonation of buried His side chains including βHis-103 and the distal His side chains in the α and β heme pockets.[98,124,125]

Cl^- is another physiologic inhibitor of O_2 binding that plays a role in the Bohr effect. As bicarbonate ions move out of the RBC down a concentration gradient, they exchange for Cl^-, which is imported into the red cell to maintain charge balance. This exchange is called the *Hamburger* or *chloride shift,* and is mediated by the anion exchange protein Band 3 on the erythrocyte plasma membrane. The alkaline Bohr effect is reduced by approximately 50% in the absence of Cl^-.[68] Virtually all of the chloride-independent contribution of the Bohr effect occurs through βHis-146. Multiple basic groups contribute to the chloride-dependent Bohr effect. The pKa of the amino terminal group of αVal-1 (NA1) and a number of His side chains show O_2-linked pKa changes that are dependent on Cl^- concentration.[126,127] Specific Cl^- binding sites may exist, but these have not been reliably identified through crystallographic studies.[128] A more global network of electrostatic interactions of Cl^- with multiple His side chains,[126] the amino termini, and the positively charged central channel[129,130] may ultimately be involved.

2,3-Diphosphoglycerate

2,3-DPG (also known as *2,3-bisphosphoglycerate* or *2,3-BPG*) is produced from 1,3-DPG as a by-product of glycolysis in a reaction called the *Rapoport-Luebering shunt.* The concentration of 2,3-DPG in RBCs is about 5 mM, approximately equimolar with Hb tetramers. 2,3-DPG is an allosteric inhibitor of O_2 binding and increases the P_{50} of HbA to achieve more efficient O_2 delivery.[131,132] 2,3-DPG carries 5 negative charges that interact with cationic groups lining the entrance to the central Hb cavity between the amino termini of the β chains in the 2,3-DPG binding site[133,134] (see Fig. 19-12). The 2.5 Å crystal structure of HbA containing bound 2,3-DPG reveals the major electrostatic interactions to be salt bridges between the βHis-2 and βLys-82 side chains and the carboxyl and phosphate groups of the effector (see Fig. 19-12). βHis-143 also lies in the binding pocket, but this side chain appears to neutral at pH 7 and may not be essential for 2,3-DPG binding.[121,133] In the R-state structure, movement of the β chains closes the central channel and occludes that binding site for 2,3-DPG. Thus 2,3-DPG binding to Hb causes a large shift in equilibrium towards the T state (15- to 30-fold increase in the MWC parameter, $L = [T_0]/[R_0]$, and a significant decrease in K_T, see Allostery and the Perutz–Monod, Wyman, Changeux Model of Hemoglobin Function).[113,135] Kinetic and equilibrium measurements at O_2 binding suggest that up to 50% of the decrease in P_{50} caused by 2,3-DPG is the result of changes in K_T and perhaps even K_R.[136]

2,3-DPG provides a means for physiological regulation of Hb O_2 affinity under conditions of hypoxia that occur at high altitude. Similarly 2,3-DPG synthesis increases in anemia, favoring O_2 dissociation to counter decreased Hb concentration (see Fig. 19-13). In pregnancy 2,3-DPG can be increased by 30%. Fetal Hb (HbF, $\alpha_2\gamma_2$) shows weak binding to 2,3-DPG compared with HbA,[137] which is presumably an adaptation to the P_{O2} gradient between the maternal and fetal tissues. A number of sequence changes in the N- and C-termini of γ globin, compared with β globin, have been investigated with the conclusion that the diminished 2,3-DPG binding is complex and cannot be attributed to single residue changes.[138,139] HbF also shows limited CO_2 binding as carbamates[140] but retains the chloride independent Bohr effect.[141]

Glycolytic enzyme defects affect 2,3-DPG production and therefore HbO_2 dissociation.[142] In general, glycolytic defects affecting enzymes downstream of 2,3-DPG such as pyruvate kinase result in a higher level of 2,3-DPG, causing a right-shifted O_2 dissociation curve with increased O_2 unloading by red cell HbA in peripheral tissues (see Fig. 19-13). Clinically this reduces the erythropoietic drive. In contrast, glycolytic defects upstream of 2,3-DPG such as hexokinase deficiency cause a reduction in 2,3-DPG concentration and lead to increased Hb O_2 affinity and increased erythropoietic drive. The physiologic differences exhibited in anemic glycolysis defective patients with right-shifted (pyruvate kinase deficiency) versus left-shifted (hexokinase deficiency) O_2-saturation curves are described in a classic study from Oski et al.[143] The patient with hexokinase deficiency (left-shifted curve) demonstrated rapidly decreased central venous P_{O2} tension and increased cardiac output in response to exercise. In contrast, the patient with pyruvate kinase deficiency (right-shifted curve) was able to offload more O_2 to tissues, reflected by a more gradually decreased central venous P_{O2} and a relatively slower rise in cardiac output. Thus at the same Hb levels a patient with pyruvate kinase deficiency (increased 2,3-DPG) has greater exercise tolerance than one with hexokinase deficiency, in which 2,3-DPG production is inhibited.

Redox Reactions of Hemoglobin

Physiologically, iron can exist in the II, III, and IV oxidation states. However, only Hb containing Fe(II) heme is capable of reversible binding to molecular O_2. Oxidation of the heme-iron to the III and IV states can trigger a range of reactions that lead to Hb denaturation and the production of reactive oxygen species (ROS) that damage erythroid cells.

Autoxidation

In the presence of O_2, α and β chains can spontaneously oxidize in a process called *autoxidation*, generating Fe(III) methemoglobin (met-Hb) and superoxide.[144-146] The resulting Fe(III) met-Hb is unable to bind to O_2, exhibits ~60-fold decrease in resistance to denaturation,[22] is prone to form toxic insoluble globin precipitates containing dissociated hemin,[147] and contributes to iron redox cycling and the generation of ROS (see Section on Production of ferryl heme and Hb protein radicals). To counter this, erythrocytes contain high levels of superoxide dismutase and catalase that convert superoxide to H_2O and O_2. Erythrocytes also have high levels of the reducing agents ascorbate and glutathione, as well as a soluble form of cytochrome b5 reductase (also known as met-Hb reductase) that converts met-Hb back to the Fe(II) state. Despite these mechanisms, approximately 1% of Hb exists in the non-functional, oxidized Fe(III) state at any time, probably due in part to reactions with NO. The mechanisms of spontaneous Hb autoxidation have been the subject of much investigation. Early studies suggested that an electron might is transferred from Fe(II) to bound O_2, leading to direct dissociation of superoxide anion[144,146]:

$$\text{Hb–Fe(II)–O}_2 \rightarrow \text{Hb–Fe(III)}^+ + \text{O}_2^{\bullet -}$$

The normal Fe(II)–O_2 bond involves partial transfer of electron density from the iron to the O_2, such that the HbO_2 can be described as $\text{Fe(III)}^+\text{–O}_2^{\bullet -}$.[148] This charge transfer is reversed when molecular O_2 dissociates. The activation barrier for dissociation of negatively charged $O_2^{\bullet -}$ from the positively charged iron center makes the barrier to direct superoxide anion dissociation high. An alternative reaction is an outer sphere electron transfer from the Fe(II)–porphyrin to an O_2 molecule that is not coordinated to the iron:

$$\text{Hb–Fe(II)} + \text{O}_2 \rightarrow \text{Hb–Fe(III)}^+ + \text{O}_2^{\bullet -}$$

However, these reactions by themselves do not explain the dependence of autoxidation on O_2 concentration. It can be shown that both reactions cause autoxidation rate to increase up to a maximum asymptotic value with increasing $[O_2]$.[149,150,151] However, a plot of autoxidation rate against O_2 concentration is bell-shaped, with the maximum rate occurring at approximately P_{50} and an asymptotic value at very high O_2 concentrations.[150,151] Work with various globins and model heme compounds suggested that electron transfer can occur by an outer sphere reaction for hexacoordinate Fe(II)-porphyrin that has a solvent molecule or other anionic ligand (L) at the sixth coordination site to facilitate formation of stable ferric iron complex[145,149-151]:

$$\begin{aligned} &\text{Hb–Fe(II)–O}_2 + \text{L} \rightleftharpoons \text{Hb–Fe(II)} + \text{L} + \text{O}_2 \\ &\quad \rightleftharpoons \text{Hb–Fe(II)–L} + \text{O}_2 \rightarrow \text{Hb–Fe(III)}^+\text{–L} + \text{O}_2^{\bullet -} \end{aligned}$$

Under physiologic conditions, this nucleophilic ligand in the previous reaction is probably water. In the Hb α chain[17,125] and Mb,[152] a fully occupied water molecule is hydrogen-bonded to the distal His in the observed deoxygenated crystal structures. These structural studies show that water can enter the deoxy-Hb heme pocket. An ordered water molecule is not observed in the β heme pocket,[17,125] but fluctuations of the protein conformation in solution almost certainly allow some water to enter.[153]

Consistent with this, β chains have been reported to auto-oxidize seven times more slowly than α chains at pH 6.5.[154] The reaction shown accounts for the drop in auto-oxidation rate at high [O_2] (the bell-shaped curve),[149-151] because O_2 competes with solvent, inhibiting formation of the Hb–Fe(II)–L complex. However, this reaction shown immediately before does not explain the strong pH dependence of the auto-oxidation reaction, the increase in rate when the distal histidine is replaced with nonpolar amino acids, or the limiting value at high [O_2]. The latter three phenomena are accounted for by a dissociative mechanism in which H^+ binds to the coordinated O_2 and neutral superoxide dissociates (c.f., same with bound O_2 protonated):

$$\text{Hb–Fe(II)–O}_2 + \text{H}^+ \rightarrow \text{Hb–Fe(II)–OOH}^+ \rightarrow \text{Hb–Fe(III)}^+ + \cdot\text{OOH}$$

The stabilizing effect of the neutral distal histidine is the result of hydrogen-bond donation to the bound O_2, which inhibits its protonation and prevents dissociation of the protonated superoxide radical. Both processes appear to occur during autoxidation of Mbs and Hbs, with the dissociative mechanism (reaction described immediately before) dominating under aerobic conditions and the outer sphere mechanism dominating under hypoxic conditions.[149-151] The dissociative mechanism readily explains the increased autoxidation and resulting ROS produced under conditions of acidosis after hypoxia and tissue damage, which are likely to be clinically significant. Other autoxidation mechanisms have been proposed, including direct displacement of superoxide from HbO_2 by hydroxide ions at high pH[155] or through interactions of the distal histidine with the iron center caused by fluctuations in the protein conformation during denaturation.[156]

Mutations of the distal His E7 to a nonpolar side chain abrogates the electrostatic stabilization of bound O_2 and leads to 100-fold decrease O_2 binding affinity,[37] along with a 100-fold increase in autoxidation rate.[150,157] Mutations of hydrophobic residues that line the heme pocket, such as E11[158] and B10,[159] to larger or smaller side chains tend to have reciprocal effects on O_2 affinity and autoxidation, consistent with similar direct steric interactions with O_2 or ·OOH. Importantly, autoxidation of Hb is also strongly influenced by oligomerization state. Dissociation of Hb into dimers, as occurs by dilution after hemolysis, leads to a sixfold increase in auto-oxidation.[160] Separated α and β chains show even more rapid auto-oxidation,[154] presumably because of loss of structural integrity and increased hydration of the heme pocket. Consequently the presence of free α and β chains in β-thalassemic erythroid cells leads to oxidative damage (see Ineffective Erythropoiesis in Thalassemia).[161,162]

Nitric Oxide–Hemoglobin Reactions

It has been known for well over 150 years that NO is toxic to most animals, can form an ultra-high affinity complex with deoxy-Hb, and rapidly oxidizes HbO_2 to met-Hb[163,164] (Box 19-4). Until the 1980s, most studies of the reaction of NO with Hb involved binding of the ligand to deoxy-Hb under anaerobic conditions.[165] In the absence of O_2, an Hb(Fe[II])–NO complex is formed rapidly, with a bimolecular rate constant that is almost diffusion controlled ($10^8 M^{-1}s^{-1}$),[41,164,166] and has a distinct free radical Fe(II)–NO EPR signal. This EPR signal is often used to detect the synthesis of NO in endothelial cells or in activated macrophages during inflammation.[167,168] However, exposure of the reduced HbNO complex to air leads to oxidation and met-Hb formation with a rate that is equal to that of thermal dissociation of NO, which is on the order of 10^{-4} to 10^{-5} s^{-1} (half-time of 2 to 20 hours).[169,170] In 1981 Doyle and Hoekstra[171] showed that the products of the reaction of HbO_2 with NO are exclusively met-Hb and nitrate, and later Arnold and Bohle[172] showed that the same products are generated when the reduced HbNO complex is exposed to air. A summary of these reactions of Hb with NO is given in Figure 19-14, *A*.

Box 19-4 Nitric Oxide and Hemoglobin

- HbO_2 can rapidly and irreversibly oxidize NO to NO_3^- in a reaction called *dioxygenation*.
- NO produced by nitric oxide synthetase in endothelial cells relaxes arterial smooth muscle, causing vasodilation.
- During massive hemolysis or after infusion of Hb-based artificial blood substitutes, free plasma Hb can cause hypertension by degrading endothelial cell produced NO.

Hb, Hemoglobin; *NO_3^-,* nitrate ion; *NO,* nitric oxide; *O_2,* oxygen.

The kinetics of NO-induced oxidation of HbO_2 were first examined by Eich and colleagues.[173] The observed bimolecular rate constant is very large (≈1×10^8 $M^{-1}s^{-1}$) and almost identical to that for simple NO binding to deoxy-Hb (see Fig. 19-14, *A*). In both cases, the rate-limiting step is ligand entry and capture in the active site, which can be inhibited by placing large aromatic amino acids in the interior portion of the distal pocket.[140] Gardner and colleagues[174] showed that both atoms of the originally bound O_2 are incorporated into nitrate using $^{18}O_2$ labeling experiments. Thus they verified the process as *NO dioxygenation*, a term he coined in 1998 when he discovered that microbial flavohemoglobins carry out this reaction to detoxify NO produced by macrophages during pathogen-induced inflammation.[175] He also showed that these flavohemoglobins are true enzymes with a built-in flavin reductase that resembles the met-Hb reductase of red cells.[176] Thus both the microbial flavohemoglobins and red cell adult Hb can be considered NO dioxygenases that rapidly remove NO by generating a nontoxic nitrate product (upper reactions in Fig. 19-14. *A*).

The mechanism of the NO dioxygenation reaction is shown in Figure 19-14, *B*. As soon as NO is captured in the distal pocket of Hb, it reacts with bound O_2 to form an oxidized peroxynitrite-Fe(III) transition state that immediately isomerizes to an Fe(III)-nitrate complex that is unstable because of the large size of the nitrate anion.[177-179] At neutral pH and 37° C, this transient intermediate is not observed in rapid-mixing experiments, indicating that the nitrate dissociates in less than 1 ms. The speed and efficiency of the NO dioxygenation reaction suggests strongly that Hb evolved to retain this

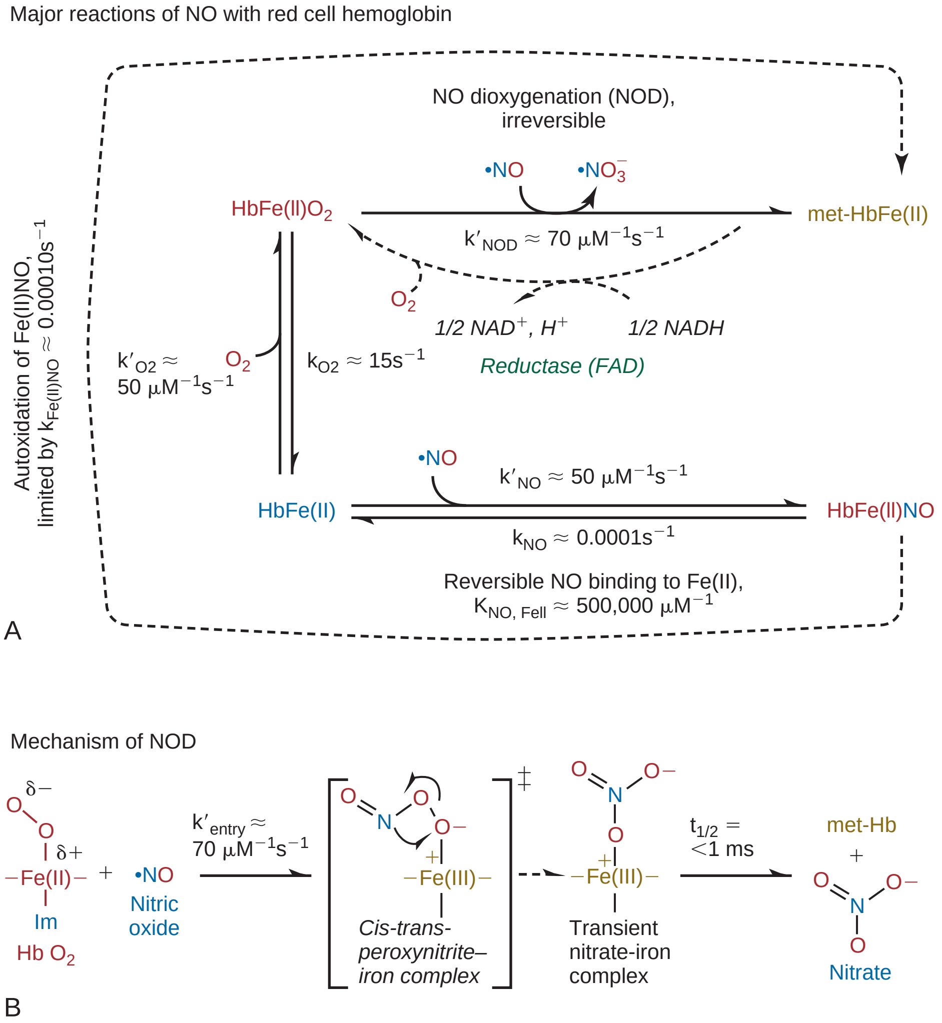
Major reactions of NO with red cell hemoglobin
NO dioxygenation (NOD), irreversible
•NO
•NO3−
HbFe(II)O2
met-HbFe(II)
k′NOD ≈ 70 μM−1s−1
O2
1/2 NAD+, H+
1/2 NADH
Reductase (FAD)
Autoxidation of Fe(II)NO, limited by kFe(II)NO ≈ 0.00010s−1
k′O2 ≈ 50 μM−1s−1
O2
kO2 ≈ 15s−1
•NO
k′NO ≈ 50 μM−1s−1
HbFe(II)
HbFe(II)NO
kNO ≈ 0.0001s−1
Reversible NO binding to Fe(II), KNO, FeII ≈ 500,000 μM−1
A
Mechanism of NOD
δ−
O
O
δ+
−Fe(II)−
Im
Hb O2
+
•NO
Nitric oxide
k′entry ≈ 70 μM−1s−1
Cis-trans-peroxynitrite–iron complex
‡
−Fe(III)−
Transient nitrate-iron complex
t1/2 = <1 ms
met-Hb
+
Nitrate
B

Figure 19-14 Key reactions of NO with red cell Hb. **A**, NO can react with either ferrous deoxyhemoglobin (HbFe[II], *blue*) to form a reversible nitrosyl–ferrous complex (Hb[FeII]NO) or with oxyhemoglobin, which leads to oxidation of hemoglobin and NO, generating met-Hb (met-HbFe[III]) and nitrate. The bimolecular rate constants for these processes are similar, very large (50-100 $\mu M^{-1}s^{-1}$), and limited by the rate of NO movement into the distal pocket.[178] Even when HbFe(II)NO is formed, the ultimate fate of the NO is nitrate. During autoxidation of nitrosylhemoglobin, NO slowly dissociates from ferrous Hb, O_2 binds to HbFe(II), and the resultant HbFe(II)O_2 then rapidly reacts with the newly liberated NO to generate met-Hb(FeIII) and nitrate.[178] **B**, The mechanism of NO dioxygenation involves capture of NO in the distal pocket and rapid reaction of this radical with bound O_2 to generate a *cis*-peroxynitrite-oxidized iron intermediate that quickly isomerizes to a transient Fe(III)-nitrate complex, which can be seen at high pH.[179] Normally, the Fe(III)–NO_3^- complex quickly dissociates yielding the final products met-Hb(FeIII) and nitrate. Both oxygen atoms of the originally bound O_2 are incorporated into nitrate with 95% fidelity or greater.[174] *FAD,* Flavin adenine dinucleotide.

activity and keep a large empty space in the interior of the distal pocket to both capture ligands efficiently and allow formation of the peroxynitrite-Fe transition state.

The physiologic relevance of the NO scavenging reactions shown in Figure 19-14, *A*, is clear. The presence of free Hb in plasma, caused either by hemolytic disease or infusion of acellular simple tetrameric Hb-based O_2 carriers, leads to a rapid increase in blood pressure by attenuating the vasodilatory effects of NO synthesized by endothelial cells. Using a library of recombinant Hbs, Olson and colleagues[178] showed that there is a linear correlation between the increase in mean arterial blood pressure in animal transfusion models and the bimolecular rate of NO dioxygenation measured in vitro. These results explain in part the toxicity of plasma Hb, which can move up to and into the vessel walls, scavenge NO, and inhibit vasodilation of the surrounding smooth muscle. In contrast, NO dioxygenation in red cells has an important protective effect. NO is a potent inhibitor of cytochrome c oxidase and can inhibit mitochondrial respiration at nanomolar concentrations by binding to either the reduced or oxidized form of the enzyme.[180] Similarly if NO just displaced bound O_2 it would form a long-lived, high affinity HbFe(II)–NO complex, which would greatly reduce the O_2 carrying capacity of blood.

These toxic effects are prevented by the efficient removal of elevated levels of NO in the blood stream by the NO dioxygenase activity of intracellular Hb. Gladwin and coworkers[181] have unambiguously demonstrated this activity in humans. When six volunteers breathed in 80 ppm NO gas, their levels of met-Hb and nitrate rose together in a one-to-one fashion up to approximately 80 μM after 1 hour. There was little increase in Hb(Fe[II])NO, S-nitrosylhemoglobin, or nitrite, which were all at levels of 2 μM or less. NO dioxygenation by Mb plays a similar role in striated muscle cells by preventing inhibition of respiration during exposure to high levels of NO caused by inflammation or high induction of endothelial nitric oxide synthase (eNOS) for vasodilation.[182,183]

Over the past 15 years, numerous groups have been studying the reactions of nitrite with Hb, both in vivo and in vitro.[184-187] Depending on the presence or absence of molecular O_2, the products of these reactions can range from S-nitrosylhemoglobin with an NO group covalently attached to the thiol side chain of βCys 93 or NO coordinated to a reduced-heme iron atom.[188,189] All of these processes are several thousand times slower than any of the direct NO reactions with HbO_2 and deoxy-Hb shown in Figure 19-14, *A*. S-nitrosylation does not involve free NO, but instead occurs by disproportionation of N_2O_3 into ON^+ and NO_2^-, with the cation reacting rapidly with strong thiol nucleophiles. N_2O_3 is generated either by the reaction of NO with O_2 or protonation and dehydration of two nitrite molecules, and these two reaction pathways are interconnected.[188,189] Thus S-nitrosylation is indicative of either the presence of nitrite or the products of the reaction of NO with molecular O_2, which occur during inflammation.[188,190] Some of these processes are clearly important in vasoregulation; however, it is also important to note that high levels of nitrite (≥100 μM), unlike nitrate, are toxic and cause methemoglobinemia and heme degradation.[191,192] Inside RBCs, where the concentration of Hb subunits is roughly 10^{-2} M, the lifetime of free NO is less than or equal to 1 μs because of the high bimolecular rates of NO dioxygenation by HbO_2 and binding to deoxy-Hb, both of which lead to met-Hb and nitrate. Thus the physiology of NO and intracellular Hb is dominated by the reactions in Figure 19-14, *A*.

Interestingly, Isakson and coworkers recently reported that endothelial cells of resistance arterioles express free α globin, which degrades NO to promote vascular contractility.[193] Suppression of endothelial α globin expression by short hairpin ribonucleic acids (shRNAs) enhanced NO accumulation and reduced contractility in isolated blood vessels. How endothelial α globin regulates blood pressure and local tissue blood flow in vivo remains an open question.

Production of Ferryl Heme and Hemoglobin Protein Radicals

The superoxide anions generated from Hb autoxidation undergo rapid enzymatic and nonenzymatic conversion to hydrogen peroxide (H_2O_2) and O_2. H_2O_2 is a key mediator of oxidative stress that can react with either reduced or oxidized globins to produce highly reactive oxoferryl (HbFe[IV] = O) or oxoferryl radical (Hb•Fe[IV] = O) containing heme groups.[194,195] Simple ferryl iron can subsequently be reduced by second molecule of H_2O_2 to produce Fe(III) and superoxide.

$$\mathrm{HbFe(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{HbFe(IV)} = \mathrm{O} + \mathrm{H_2O}$$

$$\mathrm{HbFe(IV)} = \mathrm{O} + \mathrm{H_2O_2} \rightarrow \mathrm{HbFe(III)} + \mathrm{O_2}^{\bullet -} + \mathrm{H_2O}$$

Superoxide and H_2O_2 can also degrade heme directly, producing fluorescent pyrroles and free iron.[195,196] The reaction of H_2O_2 with Fe(II) is complicated by reduction of ferryl Hb with H_2O_2 to produce superoxide (which in turn can dismute to generate $\frac{1}{2}H_2O_2 + \frac{1}{2}O_2$), comproportionation (the reaction of Fe[II] and Fe[IV] = O species to form two Fe[III] species), and autoreduction involving electron transfer from Hb side chains (producing protein radicals). Fe(III) heme reacts more rapidly than Fe(II) heme with H_2O_2 to produce the oxoferryl radical species.[156,197,198]

$$\mathrm{HbFe(III)} + \mathrm{H_2O_2} \rightarrow \mathrm{Hb{\bullet}Fe(IV)} = \mathrm{O} + \mathrm{H_2O}$$

One electron has to be removed from either the porphyrin ring or the surrounding protein to generate water and the Fe(IV) = O species, and as a result a protein or porphyrin radical is generated. Another H_2O_2 can react with the oxoferryl Hb radical to produce O_2 without heme degradation and to regenerate HbFe(III). These two reaction can consume H_2O_2 with peroxidase or catalase-like activity.[195]

$$\mathrm{Hb{\bullet}Fe(IV)} = \mathrm{O} + \mathrm{H_2O_2} \rightarrow \mathrm{HbFe(III)} + \mathrm{O_2} + \mathrm{H_2O}$$

However, the protein radical and the oxoferryl heme species are highly reactive, and cycling between the Fe(III) and Fe(IV) oxidation states increases the probability of reactions that degrade Hb or other cellular components.[197-199]

Prooxidant Reactions of Hemoglobin

Oxidative reactions of Hb, such as lipid oxidation, have been well studied in vitro. Ferryl heme and Hb radicals can initiate lipid oxidation by abstraction of hydrogen to form a lipid radical. In the presence of O_2, lipid radicals can participate in an autocatalytic cycle that generates further lipid oxidation products.[198] Lipid oxidation products produced at an artery wall can interfere with inflammatory signaling and may contribute to the pathogenesis of atherosclerosis.[199-201]

Evidence for the oxidative reactions of Hb include the detection of globin radicals[202] and degradation products of heme[203] in blood. A greater level of oxidized Hb has been found in venous compared with arterial blood, as detected by EPR.[156] However, in general the involvement of heme proteins in oxidative damage in vivo is hard to establish because of the transient nature of many of the reaction products. Two markers that are considered reliable indicators of oxidative stress in vivo are isoprostanes[204] and cross-linked heme-globin products.[198] Isoprostanes are potent vasoactive compounds that are generated nonenzymatically from lipids found in low-density lipoproteins by radical oxygen species[205] and in vitro can be produced through the reaction of low-density lipoproteins with heme proteins.[206] Heme-Hb cross-links are thought to be exclusively generated by reaction of ferryl heme and a protein-based radical and are therefore stable markers of ferryl heme production from met-Hb and H_2O_2 in vivo.[198]

Detection of these markers has implicated redox reactions of globin proteins under two significant clinical conditions. First, in rhabdomyolysis, muscle breakdown releases Mb, which causes kidney damage from lipid radical formation and the vasoconstrictive action of isoprostanes.[199] Second, after subarachnoid hemorrhage, isoprostanes[199,207] and heme-protein cross-links indicative of ferric–ferryl redox cycling[208] are detected in cerebrospinal fluid. Accumulation of these metabolites may contribute to delayed vasospasm and consequent ischemic brain injury that occurs in about 30% of surviving patients.

In addition to oxidative reactions mediated by heme proteins, the formation of met-Hb promotes heme release because the Fe(III)–His F8 interaction is significantly weaker than the Fe(II)–His F8 bond.[22,209] Folding of Hb chains is stabilized by interactions with heme,[22] and heme loss leads to rapid denaturation of Hb chains. Free iron can be released after peroxide or heme oxygenase breakdown of protoporphyrin IX and participate in the Fenton reaction, which converts H_2O_2 to a hydroxyl anion and a hydroxyl radical. Reduction of Fe(III) by superoxide and subsequent reaction with H_2O_2 generates highly damaging hydroxyl radicals.[198,199,210]

$$\mathrm{Fe(II)} + \mathrm{H_2O_2} + \mathrm{H^+} \rightarrow \mathrm{Fe(III)} + \mathrm{HO\cdot} + \mathrm{H_2O}$$

$$\mathrm{Fe(III)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(II)} + \mathrm{HOO\cdot} + \mathrm{H^+}$$

Ineffective Erythropoiesis in Thalassemia

Ineffective erythropoiesis (IE) is a failure to produce mature RBCs because of apoptosis and/or maturation delay of erythroid progenitors, often compounded by premature hemolysis of circulating RBCs. Compensatory increase in erythropoietic drive leads to accumulation of erythroid progenitors in the bone marrow, liver, and spleen. IE is one of the primary pathologies of thalassemia (especially β type) and arises from the toxic effects of excess free αHb.[31] Free globins, particularly α globin, are toxic for several reasons. They have greater susceptibility to denaturation and auto-oxidation,[154] heme loss,[25] and production of ROS.[161,162,211] Levels of ROS are elevated in erythroid cells of β-thalassemic mice.[212-215] These data suggest that ROS production caused by imbalanced Hb synthesis is likely to contribute to cellular damage in thalassemia. However, the role of oxidative stress to IE and hemolytic anemia is still only partly understood. Damage to cell membrane structures through Hb precipitation appears to be a trigger for apoptosis contributing to IE. However, defective cell signaling through erythropoietin (EPO) signaling with consequent increase in proliferation and failure to differentiate also contributes to IE.[216]

ABNORMAL HEMOGLOBINS

Introduction to Hemoglobin Variants

Mutations in globin genes can affect their expression, causing thalassemias (see Chapter 21), or produce structurally abnormal globin proteins (i.e., Hb variants). Many human Hb variants are discovered incidentally and have minimal effects on the Hb molecule or patient. However, naturally occurring Hb mutations can cause biochemical abnormalities with consequent phenotypes ranging from clinically benign to severe. The most common and medically important globin gene mutations include HbS (Chapter 20), HbC (Chapter 20), HbE, and the thalassemias (Chapter 21), most of which are under positive genetic selection because they confer survival advantages against malaria.[1] These abnormalities are therefore most prevalent in populations where malaria is endemic, although global migration is increasing the incidence of such mutations in the United States and Northern Europe. In addition to the common globin gene abnormalities, there are also over 1000 other mutations that are rare individually, but common collectively. Most known Hb mutants are catalogued on the Globin Gene Server[12,13] (http://globin.cse.psu.edu). By convention, these variants are named after the geographic origin of the affected individual. Analyses of Hb variants, which can be considered "experiments of nature,"[217] have generated valuable insights into structure–function relationships within the Hb molecule and the associated clinical consequences. This section provides a conceptual framework for understanding the biology and clinical implications of Hb variants, and it discusses how these variants are ascertained, analyzed, and classified broadly according to their effects on Hb physiology. Selected examples of Hb variants that reinforce basic principles are then described in greater detail.

Identification of Hemoglobin Variants

Unexplained clinical signs and laboratory findings indicative of altered Hb physiology should prompt physicians to consider the possibility of globin gene mutations (Box 19-5). These include pallor and hemolytic anemia (unstable Hbs; see Hemolytic Anemia: Unstable Hemoglobin Variants), ruddy complexion with polycythemia (Hbs with increased O_2 affinity; see Erythrocytosis: High–Oxygen Affinity Hemoglobin Variants), or bluish skin (cyanosis caused by Hbs with decreased O_2 affinity or enhanced oxidation; see Cyanosis: Low–Oxygen Affinity and M-Type Hemoglobin Variants). Routine laboratory testing can also reveal Hb variants incidentally. For example, in many countries, Hb analysis (typically by high-performance liquid chromatography [HPLC]) is performed on all neonates to screen for sickle cell anemia and thalassemia syndromes. This testing can also reveal less common Hb variants by virtue of their abnormal chromatographic mobility. Although such variants may warrant further investigation, they are often clinically

Box 19-5 **Typical Clinical Presentations of Hemoglobin Variants**

Cyanosis with normal arterial P_{O2}
Cyanosis in the absence of cardiopulmonary disease
Methemoglobinemia
Erythrocytosis with normal or elevated erythropoietin levels
Hemolytic anemia
Thalassemia phenotypes
Family history consistent with an Hb variant
Incidental finding on routine laboratory testing or neonatal screening

Hb, Hemoglobin; *P_{O2},* partial pressure of oxygen.

silent. Routine testing in adults can also reveal abnormal Hbs. For example, several Hb variants were discovered because they migrate similarly to HbA_{1c} in chromatographic assays that are used to monitor long-term glycemic control in patients with diabetes (Fig. 19-15).[218-221] Such variants can interfere with diabetic management by artificially elevating or reducing HbA_{1c} measurements. The fact that relatively benign Hb variants can reveal themselves through altered skin color or incidentally through laboratory testing explains why so many have been discovered.

Because Hb variants are so common, they are also likely to be identified via global exome or whole genome sequencing, increasingly common approaches used to investigate many acquired and germline genetic disorders. Incidental findings revealed through such testing, termed the *incidentalome*,[222,223] are likely to include benign Hb variants or reveal carrier status for more clinically significant ones.

Clinical Manifestations of Hemoglobin Variants Reflect Their Physiological Effects

Hb variants are classified according to their effects on the molecule, which influence clinical manifestations (Table 19-3). These classifications include unstable, high–O_2 affinity, low–O_2 affinity and M (met-Hb) type. General clinical issues associated with Hb variants are discussed in this section, and the structural and biochemical effects of such mutations are discussed in Pathophysiology of Hemoglobin Abnormalities.

Hemolytic Anemia: Unstable Hemoglobin Variants

Globin mutations that destabilize Hb tetramers are among three major categories of inherited disorders that cause hereditary hemolytic anemia through erythrocyte-intrinsic abnormalities[224-229] (Table 19-4). Most of these disorders are manifested by neonatal jaundice and/or chronic anemia with reticulocytosis, although lesions in the β-globin gene may present later in the first year of life as adult Hb replaces fetal Hb. With the exception of HbS, unstable variants are a relatively rare cause of hemolytic anemias but must be considered in unexplained cases. The clinical severity ranges from mild to extreme, depending on the nature of the mutation and environmental factors. The most severely affected patients are dependent on RBC transfusion. Fever and/or transient acidosis during intercurrent illness can aggravate hemolysis by increasing denaturation of unstable Hb variants. Patients with unstable Hbs may display dark urine caused by hemoglobinuria and/or bilirubinuria, although these findings do not indicate the degree of hemolysis,[230,231] nor does the absence of pigmenturia rule out this condition.

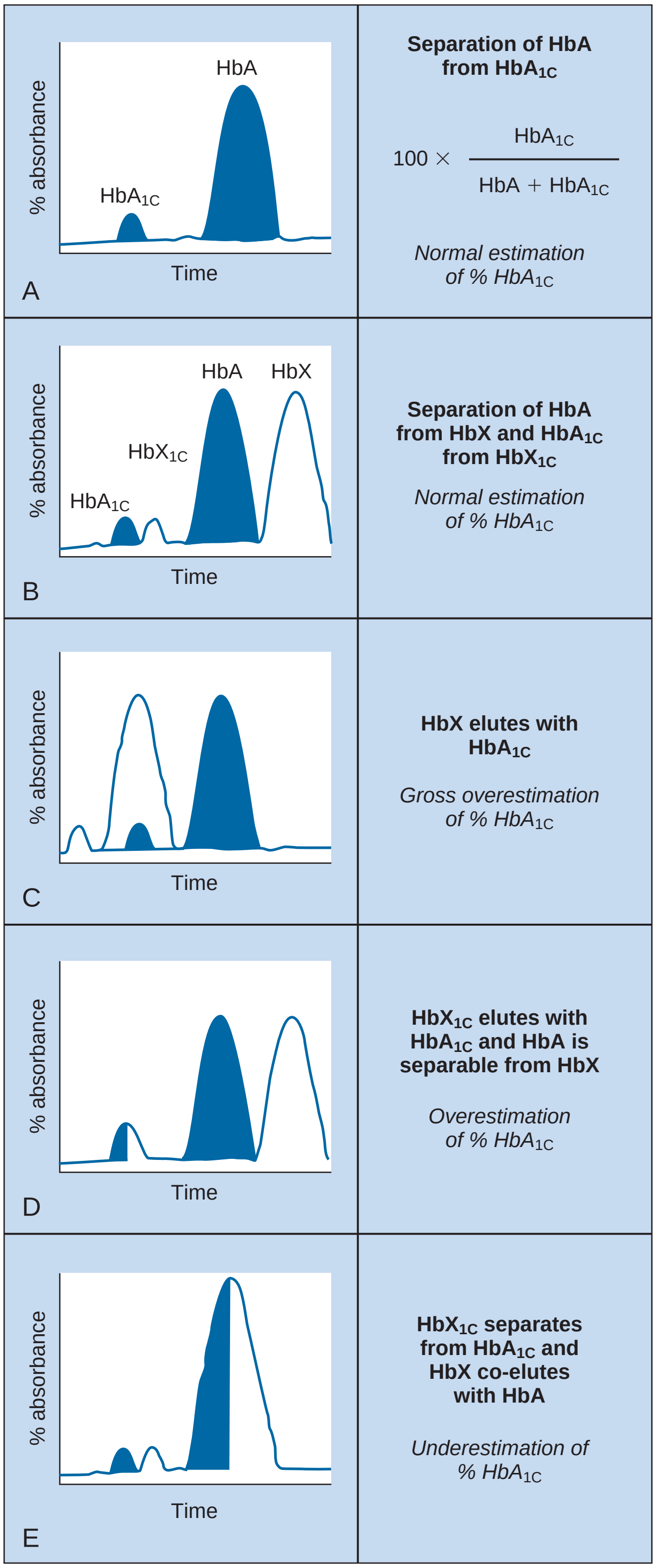

Figure 19-15 Chromatographs showing how Hb variants affect HbA_{1C} quantification. HbA and HbA_{1C} are indicated by *shaded areas*; HbX and HbX_{1C} (variant Hb) are indicated by *unshaded areas*. **A**, Subject with no variant Hb. **B**, HbX and its glycated form (HbX_{1C}) fully separate from HbA and HbA_{1C}, respectively. **C**, HbX comigrates with HbA_{1C}, causing overestimation of the percentage of HbA_{1C}. **D**, HbX_{1C} and HbA_{1C} comigrate, whereas HbA and HbX separate. This result produces a mild, spurious increase in the percentage of HbA_{1C}. **E**, HbX_{1C} and HbA_{1C} separate, while HbA and HbX co-elute. This produces a spurious decrease in the calculated percentage of HbA_{1C} value. *(From Bry L, Chen PC, Sacks DB: Effects of hemoglobin variants and chemically modified derivatives on assays for glycohemoglobin.* Clin Chem *47:153–163, 2001.)*

TABLE 19-3 Classification of Hemoglobin Variants

Hb Variant Class	Signs and Symptoms	Structural Alteration
Unstable	Hemolytic anemia, dominant thalassemia	Destabilized 2°, 3°, or 4° structure Impaired heme–globin interactions
High–O_2 affinity	Erythrocytosis	Stabilized R state Impaired Bohr effect Impaired 2,3-DPG binding site
Low–O_2 affinity	Cyanosis	Stabilized T state
M (Met) Hb	Cyanosis, methemoglobinemia	Altered heme pocket
Multiple effects	Varied	Combinations of the above

2,3-DPG, 2,3-Diphosphoglycerate; *Hb*, hemoglobin; *O_2*, oxygen; *R*, relaxed; *T*, tense.

Other associated signs or symptoms of hemolytic anemia with unstable Hb variants (or other etiologies) include persistent or intermittent jaundice, gall bladder disease, and splenomegaly. In many forms of congenital hemolytic anemia, including those associated with unstable Hb variants, parvovirus B19 infection can cause red-cell aplasia with severe, sometimes life-threatening anemia. In undiagnosed patients, this clinical presentation can be mistaken for disorders of red-cell hypoplasia, such as transient erythroblastopenia of childhood or Diamond Blackfan anemia. The aforementioned signs and symptoms suggestive of a congenital hemolytic anemia can be diagnostically useful in these patients.

Of note, the laboratory and clinical findings associated with unstable Hbs can be relatively nonspecific, overlapping with other etiologies of congenital hemolytic anemia, such as membranopathies (see Chapter 16), enzyme defects (see Chapters 17 and 18), and thalassemias (see Chapter 21). Indeed, their intrinsic transient nature renders many unstable Hbs difficult to detect, isolate, or study.[232,233] Thus globin gene sequencing should be performed to identify potential unstable Hb variants in any patient with unexplained hemolytic anemia.

TABLE 19-4 Differential Diagnosis of Erythrocyte-Intrinsic Hereditary Hemolytic Anemia[225,227]

Classification	Common Examples	More Information
Membrane disorder	Hereditary spherocytosis Hereditary elliptocytosis	Chapter 16
Enzyme deficiency	Pyruvate kinase deficiency and glycolytic disorders Glucose-6-phosphate dehydrogenase deficiency	Chapter 17 Chapter 18
Hemoglobinopathy	Unstable hemoglobin variants Sickle cell disease Thalassemias	This chapter Chapter 20 Chapter 21

Modified from Jaffe ER: Hereditary hemolytic disorders and enzymatic deficiencies of human erythrocytes. Blood *35:116–134, 1970; and Valentine WN: The Stratton lecture: hemolytic anemia and inborn errors of metabolism.* Blood *54:549–559, 1979.*

Box 19-6 Polycythemia versus Erythrocytosis

Erythrocytosis is defined as increased concentration of blood erythrocytes, measured as the number of cells per volume, total hemoglobin concentration, or packed red cell volume (hematocrit). This can be the result of reduced plasma volume, typically caused by dehydration or polycythemia ("many cells"). *Polycythemia* describes an increase in the total quantity or mass of red blood cells in the body and is caused by numerous abnormalities, including reduced tissue O_2 delivery caused by high–O_2 affinity Hb variants (see Table 19-5).

Hb, Hemoglobin; *O_2,* oxygen.

Erythrocytosis: High–Oxygen Affinity Hemoglobin Variants

The Hb tetramer must bind and deliver O_2 properly in response to environmental conditions. Hb variants that increase the O_2 affinity of Hb typically inhibit O_2 delivery to tissues, which stimulates erythropoietic drive, causing erythrocytosis with a resultant ruddy complexion.[234] Note that although the terms *polycythemia* and *erythrocytosis* are often used interchangeably, they are not equivalent (Box 19-6). High–O_2 affinity Hb variants are associated with elevated EPO levels, distinguishing them from erythroid-cell autonomous mechanisms of erythrocytosis that are independent of EPO[4] (Table 19-5). Erythrocytosis caused by high–O_2 affinity Hb variants does not usually produce dangerous vaso-occlusive events, in contrast to myeloproliferative disorders such as polycythemia vera. Patients with erythrocytosis caused by high–O_2 affinity Hb variants are reported to have mistakenly received radioactive ^{32}P for presumed polycythemia vera.[4,6] Conditions of low ambient O_2, such as unpressured airplane cabins or exposure to high altitude, do not represent a risk to patients with high-O_2 affinity Hbs. In fact, such individuals are better equipped than an average person to handle such situations, because their Hbs bind O_2 avidly.[235] After giving birth, women with high–O_2 affinity Hb mutants exhibit no increases in morbidity or mortality for mother or child, even when the mother's variant Hb has higher O_2 affinity than that of HbF.[236] This suggests that compensatory mechanisms other than the differences in O_2 affinity must exist in pregnancies to help deliver O_2 to the fetus.

Cyanosis: Low–Oxygen Affinity and M-Type Hemoglobin Variants

Cyanosis is defined as a bluish discoloration of the skin and mucous membranes as a result of excessive levels of deoxy-Hb. In general, cyanosis is either central, involving the core, lips and tongue, or peripheral, involving the extremities. Central cyanosis usually becomes apparent at about 2 to 5 g/dL of deoxy-HbFe(II), 1.5 to 2 g/dL of

TABLE 19-5 Differential Diagnosis of Polycythemia

Classification	EPO Level	Etiology
Primary (erythrocyte intrinsic)	Low	Myeloproliferative disorders (polycythemia vera) EPO receptor mutations
Secondary (erythrocyte extrinsic)	Appropriately high	Hypoxemia Cardiopulmonary disease Sleep apnea Obesity Living at high altitude Red cell defects High–O_2 affinity Hbs* M Hbs* Absent or low 2,3-DPG mutase Chronic CO poisoning
Secondary (erythrocyte extrinsic)	Inappropriately high	EPO-producing neoplasms EPO-producing renal lesions Defects in oxygen sensing including VHL, HIF2α, EGLN1 mutations
Idiopathic		

Modified from Bunn HF, Nagel RL: Hemoglobins: normal and abnormal. In Nathan DG, Orkin SH, Ginsburg D, et al, editors: Nathan and Oski's hematology of infancy and childhood, *vol 7, Philadelphia, 2009, Saunders, p. 911–948.*

2,3-DPG, 2,3-Diphosphoglycerate; *CO*, carbon monoxide; *EPO*, erythropoietin; *Hb*, hemoglobin; *O_2*, oxygen.

*Abnormalities discussed in detail in this chapter.

met-HbFe(III), or 0.5 g/dL sulfhemoglobin (sulf-Hb), and can be more difficult to detect in individuals with darker skin pigments. Cyanosis is caused by many conditions affecting numerous organ systems[237] (Box 19-7), most commonly heart or lung problems. However, chronic cyanosis can also be caused by globin mutations that reduce Hb O_2 affinity or favor oxidation of Hb iron to the Fe(III) (met-Hb) form (M Hbs), which cannot bind O_2. Cyanosis caused by these Hb variants is usually associated with normal arterial O_2 saturation, in contrast to most other etiologies, which induce arterial hypoxia.

M-type Hbs cause the blood to appear chocolate brown because of characteristic light absorbance properties of met-Hb (visible peaks at 510 and 630 nm), which looks brown in solution. This coloration forms the basis for diagnostic testing (see Clinical Laboratory Tests to Identify Hemoglobin Variants). The variant HbM Iwate, first observed more than 200 years ago in Japan, was described as *black blood* or *hereditary nigremia.*[238-241] Clinically, the M Hb variants must be distinguished from methemoglobinemia induced by drug and chemical exposures, as well as genetic and developmental abnormalities in the erythrocyte met-Hb reduction system (Table 19-6).

In contrast to many disorders associated with cyanosis and/or methemoglobinemia, low–O_2 affinity and M Hb variants are not usually life- or health-threatening, although they can produce cosmetic effects that are deleterious to patients. As stated by Bunn and Forget, "the primary reason for establishing the diagnosis [of M Hbs] is to prevent iatrogenic misadventures that might arise under the mistaken impression that the patient has a cardiac or pulmonary disorder."[5] Thus establishing the correct diagnosis of low–O_2 affinity and M Hbs reduces the potential risk for unnecessary and potentially dangerous medical procedures such as cardiac catheterization for presumed cyanotic heart defects. For example, Bunn and Nagel discuss a 1-week-old infant who underwent a Blalock procedure because of pseudotruncus arteriosus.[4] Only when a family study was completed many years later was the individual identified as a carrier of Hb M Boston. Crowley et al. described a newborn infant of healthy appearance in whom cyanosis was caused by a low–O_2 affinity Hb variant.[242] The patient received aggressive medical support including mechanical ventilation and arterial catheterization that, in retrospect, was unnecessary. Her father also exhibited cyanosis at birth, which prompted an extensive workup that included a liver biopsy. In general, distinguishing low–O_2 affinity and M Hb variants from more dangerous conditions can be achieved through straightforward clinical assessment and laboratory testing. However, diagnostic dilemmas can occur as noted, particularly in newborns and infants where the potential for more common life-threatening situations must be addressed urgently and the clinician does not have the benefit of extended observation.

Ontogeny of Globin Gene Expression Influences the Clinical Presentation of Hemoglobin Variants

The α- and β-like globin gene families are regulated temporally during fetal and postnatal life, which influences

TABLE 19-6 Differential Diagnosis of Congenital Methemoglobinemia

Class	Description
Type I	Erythroid cytochrome b5 reductase deficiency
Type II	Total cytochrome b5 reductase deficiency
M hemoglobins*	Hb variants*
Infections	Gastrointestinal infections with dehydration
Toxins and Drugs	Dapsone, benzocaine, lidocaine, nitrites, others[463,464]

Modified from Beutler E: Methemoglobinemia and other causes of cyanosis. In Lichtman MA, Beutler E, Coller BS, Kipps TJ, editors: Williams hematology, *New York, 1995, McGraw-Hill; and Ash-Bernal R, Wise R, Wright SM: Acquired methemoglobinemia: a retrospective series of 138 cases at two teaching hospitals.* Medicine *83:265–273, 2004.*

*Abnormalities discussed in detail in this chapter.

Box 19-7 Differential Diagnosis of Central Cyanosis

Decreased atmospheric O_2 (rapid ascents to high altitude)
Hypoventilation secondary to central nervous system disease
Cardiac disorders
- Right-to-left shunt
- Heart failure

Pulmonary disorders with decreased O_2 exchange
Hb abnormalities
- Low–O_2 affinity Hb variants
- Methemoglobinemia: M Hbs, other (see Table 19-6)
- Sulfhemoglobinemia

Modified from Loscalzo J: Hypoxia and cyanosis. In Longo DL AF, Kasper DL, Hauser SL, editors: Harrison's principles of internal medicine, *New York, 2012, McGraw-Hill.*
Hb, Hemoglobin; *O_2,* oxygen.

the timing at which Hb variants first manifest clinical symptoms (see also Chapter 1). HbF ($\alpha_2\gamma_2$) is the predominant form during late fetal life. Over several months after birth, expression gradually switches to HbA ($\alpha_2\beta_2$).[243] Thus symptomatic mutations affecting α or γ globins present prenatally or at birth, whereas the symptoms of β-globin mutations are typically delayed until a few months after birth. Importantly, γ-globin gene mutations that are apparent at birth, most typically reflected by cyanosis or hemolytic anemia, usually abate over a few weeks as Hb production switches from F ($\alpha_2\gamma_2$) to A ($\alpha_2\beta_2$). Examples include HbF Toms River and HbF Poole, which are discussed later in this chapter. In contrast, symptoms associated with α- or β-chain variants persist throughout life.

Clinical Laboratory Tests to Identify Hemoglobin Variants

Several routine blood tests are used to identify and characterize Hb variants (Table 19-7). Most of these methods, including electrophoresis, ultraviolet (UV)-visible light spectroscopy, and O_2 association studies, have been used for decades. These methods are reliable and relatively inexpensive. However, many of these tests are becoming increasingly unavailable for routine clinical testing. Newer technologies such as deoxyribonucleic acid (DNA) sequencing and mass spectrometry are now used increasingly to characterize Hb variants. These tests are now becoming less expensive and importantly, can pinpoint causative mutations in the globin-gene DNA or protein amino acid sequences. However, they do not reveal the functional effects of the mutation. The most comprehensive understanding of Hb variants comes from combined clinical observation, functional testing, and mutational analysis. Commonly used tests are reviewed in this section.

Physical Methods of Hemoglobin Separation

Many amino acid substitutions alter the physical properties of variant Hbs, allowing them to be distinguished by electrophoresis or chromatography. These methods examine the mobility of α1β1 dimers or individual globin subunits. Typical standards include normal Hbs A, F, and A_2, as well as the common variants S and C. Some Hb variants exhibit distinct migration patterns in these assays, although many are "electrophoretically silent." Unstable Hbs often appear as a diffuse band or, in some circumstances, simply as precipitated material at the gel origin.[244] This finding probably arises from partial denaturation of Hb molecules during electrophoresis or manipulation of the hemolysate. Historically, cellulose acetate and citrate agar electrophoresis were most commonly used to detect variant Hbs. Current clinical testing usually employs more sensitive techniques such as isoelectric focusing and HPLC. These methods are now used for routine neonatal screening in North American and European countries to identify common hemoglobinopathies.[245,246] Rare Hb variants can also be identified coincidentally through neonatal screening, sometimes necessitating follow-up testing to ascertain their clinical significance.

Oxygen Equilibrium Binding Curves

In this test, Hb in whole RBCs or hemolysate is deoxygenated and then exposed to gradually increasing P_{O2} with simultaneous measurement of percent (%) HbO_2 saturation. Results are plotted graphically as % HbO_2 saturation versus O_2 pressure (Fig. 19-16). The P_{O2} at which normal HbA becomes half-saturated (P_{50}) is 26 mm Hg using undialyzed hemolysate at pH 7.2,[247] and the shape of the saturation curve is sigmoidal, reflecting cooperative O_2 binding (see Cooperative Oxygen Binding). High–O_2 affinity variants become saturated at

TABLE 19-7 Laboratory Tests for Hemoglobin Variants

Test Category	Variant Types	Test Descriptions
Physical Hb separation	Any	Cellulose acetate or citrate agar electrophoresis Isoelectric focusing HPLC
O_2 equilibrium curve (see Fig. 19-13)	High– and low–O_2 affinity variants, M Hbs	Vary hemolysate exposure to O_2 and/or allosteric regulators (H^+, pH, 2,3-DPG)
UV-visible light spectroscopy	Any, especially M Hbs	UV-visible spectroscopy yields characteristic spectra for different M Hbs
Hb stability	Unstable	Heat, isopropanol, mechanical agitation, zinc acetate
Heinz body staining	Unstable	Supravital staining of red cells for precipitated globins
DNA sequencing	Any	Direct sequencing of patient's globin genes
Mass spectrometry	Any	Obtain amino acid sequence of patient's Hb

2,3-DPG, 2,3-Diphosphoglycerate; *DNA,* deoxyribonucleic acid; *H^+,* hydrogen cation; *Hb,* hemoglobin; *HPCL,* high-performance liquid chromatography; *UV,* ultraviolet.

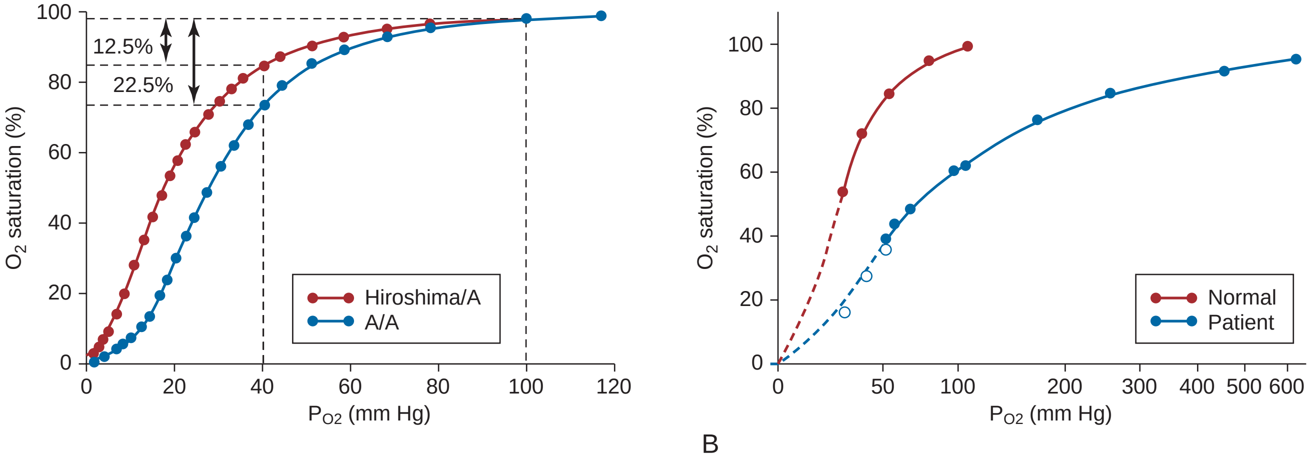

Figure 19-16 Oxygen saturation curves from normal and variant Hbs. **A**, Oxygen saturation curves of red blood cells from a healthy individual (Hb A/A) and one who is heterozygous for the high–O_2 affinity variant Hb Hiroshima (Hb Hiroshima/A). Oxygen pressure is plotted against oxygen saturation. *Vertical dotted lines* mark the typical P_{O2} of the lungs (100 mm Hg) and tissues (40 mm Hg). *Horizontal dotted lines* mark O_2 extraction (lungs minus tissues) in normal red cells (22.5%) and the reduction observed in high–O_2 affinity red cells (12.5%). **B**, Hb O_2 equilibrium curves of red cells from a healthy person *(Normal)* and those from a person heterozygous for the low–O_2 affinity variant Hb Kansas *(Patient)*. P_{O2} (in mm Hg) is plotted against percent O_2 saturation. Note that the curve for Hb Kansas is right-shifted, causing a higher P_{O2} at which half of the hemoglobin is oxygenated (P_{50}). *(**A**, From Hamilton HB, et al: Hemoglobin Hiroshima [beta-143 histidine→aspartic acid]: a newly identified fast moving beta chain variant associated with increased oxygen affinity and compensatory erythremia.* J Clin Inv *48:525–535, 1969; **B**, From Reissman, et al: A human hemoglobin with lowered oxygen affinity and impaired heme-heme interactions.* J Clin Inv *40:1826–1830, 1961.)*

lower O_2 pressures and demonstrate a left-shifted O_2 equilibrium curve (see Fig. 19-16, *A*). Mutations that reduce O_2 affinity cause a right shift, because less Hb is oxygenated at a given P_{O2} (see Fig. 19-16, *B*). Because cooperative regulation of Hb is an important functional property (see Allostery and the Perutz–Monod, Wyman, Changeux Model of Hemoglobin Function), investigation of O_2 affinity responses to allosteric regulators, particularly 2,3-DPG or H^+ (pH changes), can provide insight into the structural causes of the observed phenotypes.

Ultraviolet-Visible Wavelength Light Spectroscopy

The heme moiety of Hb absorbs visible light and therefore is responsible for the distinct color of the protein. Alterations in the electron configuration of heme, such as those caused by changes in the iron oxidation or ligand states, produce characteristic color changes that are reflected in the visible light absorption spectrum. These different absorbance peaks explain why HbO_2 (Fe[II]–O_2 heme) is red (two peaks at 541 and 577 nm but transmits red light at 600 nm), whereas deoxy-Hb (five-coordinate Fe[II] heme) is a darker purple-bluish color (one broad peak at 555 nm that absorbs some red light at 600 nm). Met-Hb (Fe[III] heme with a weakly coordinated H_2O) forms a characteristic absorbance peak at 630 nm, which forms the basis for its detection by cooximetry in the clinical laboratory and accounts for its brownish color. The M Hbs exhibit complex absorbance spectra containing the normal red peaks for wild-type HbO_2 subunits along with new ferric bands in the 600 to 630 nm region for the mutated met-Hb subunit. These spectra are readily distinguished from “normal” met-HbA formed by extrinsic abnormalities, such as deficiencies in the met-Hb reductase system or toxin exposures.[5] Visible light spectroscopy can also distinguish deoxy-Hb and met-Hb from sulf-Hb, a rare cyanotic condition in which environmental exposure to sulfur compounds causes a sulfhydryl to be added to the pyrrole B ring,[248] rendering sulf-Hb incapable of binding O_2.[249] Sulf-Hb has a characteristic peak at 620 nm, which is not altered significantly by reduction.

Pulse oximetry is used routinely to clinically assess blood oxygenation. This is a noninvasive spectrophotometric test that measures absorbance ratios for oxygenated (660 nm) and deoxygenated (940 nm) blood at these single wavelengths.[250] Since M Hbs and sulf-Hb alter light absorbance in unique ways across the visible spectrum, absorbance measurements calculated from these two discrete light wavelengths may be misleading for these conditions where pulse oximetry usually calculates high levels of deoxy-Hb (i.e., hypoxia), although arterial blood O_2 levels are normal. In this setting, analysis of Hb by light absorbance throughout the full visible wavelength spectrum can provide diagnostic information.

Hemoglobin Stability Testing

Hemolytic anemia results when erythrocyte stability is compromised. Unstable Hbs precipitate within cells and are deposited on the inner plasma membrane, making the erythrocyte rigid, brittle, and prone to lysis. Thus hemolytic anemia is associated with variants that destabilize Hb. Hb stability tests measure the propensity for Hb to denature upon exposure to various stresses including heat,[251] isopropanol,[252,253] mechanical agitation,[254,255] and zinc acetate.[256] In all of these tests, unstable variants denature at lower thresholds than normal Hb.

Examination of the Blood Smear

Erythrocyte morphology observed in Wright-Giemsa–stained blood smears from patients with unstable Hbs is

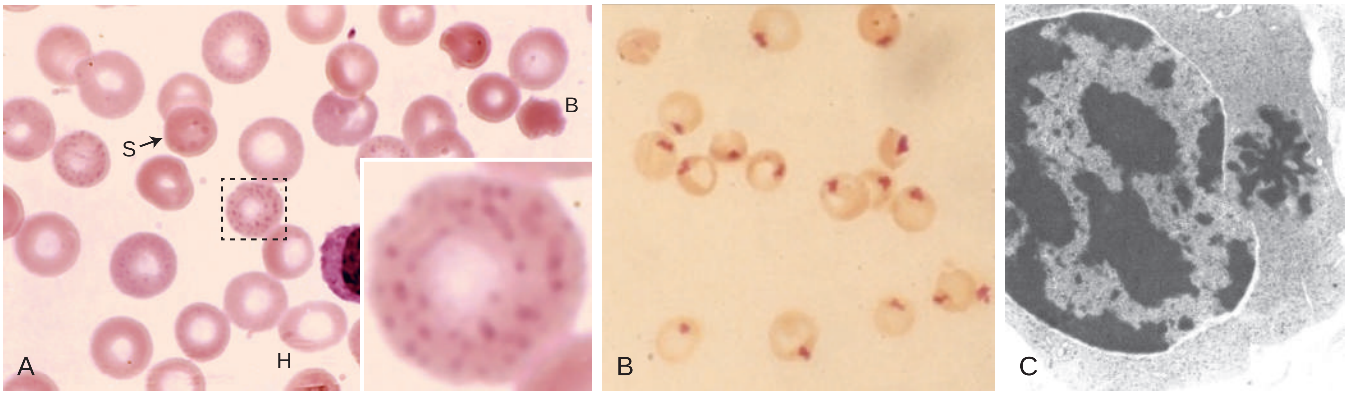

Figure 19-17 Abnormal blood smear findings in patients with unstable Hbs. **A**, Blood smear from a patient with Hb Nottingham (presplenectomy), stained with Wright-Giemsa. The inset shows an enlarged view of the boxed cell to illustrate basophilic stippling. Other abnormalities include hypochromic cells (*H*), spherocytes (*S*), and degmacytes ("bite" cells, *B*). **B**, Methyl violet stain illustrating erythrocyte Heinz bodies in a patient with an unstable Hb. **C**, Electron micrograph showing Hb precipitate in a bone marrow erythroid precursor from a patient with Hb Constant Spring. *(From Derry S, et al: Hematologic and biosynthetic studies in homozygous hemoglobin Constant Spring.* J Clin Inv *73:1673–1682, 1984;* ***A*** *and* ***B*** *Courtesy of Dr. Samuel Lux, Boston Children's Hospital.)*

variable depending on the nature of the mutation. Basophilic stippling, reflecting accumulated ribosomal RNA aggregates, is common (Fig. 19-17, *A*). Spherocytes, hypochromia, bite cells, blister cells, and altered red-cell size can also occur. All of these morphological abnormalities tend to be more prominent postsplenectomy and/or during hemolytic crisis.

The Heinz body test utilizes supravital stains to detect unstable precipitated globins within erythrocytes.[257-260] Heinz bodies are increased in the setting of unstable Hbs, oxidative damage, and thalassemias. They appear as irregular, pale purple inclusions, present as singular or grouped bodies that are often attached to the membrane[261] (see Fig. 19-17, *B*). Cells containing precipitated Hbs are rapidly removed by the spleen and therefore may be apparent only after splenectomy or by staining of bone marrow erythroblasts.[257,259,260] In patients who have not undergone splenectomy, Heinz bodies become more prominent after 24-hour incubation without glucose. Brilliant cresyl blue is a stain with mild oxidant activity that enhances the sensitivity of this test by causing precipitation of unstable Hbs. In contrast, methyl violet stains only preformed Hb precipitates without inducing their formation. When testing for Heinz bodies in hemolytic anemia, a sample of normal blood is run in parallel, because nonspecific staining can yield false-positive results. Unstable Hbs can also precipitate in bone marrow erythroid precursors, which can be detected by Heinz body staining or electron microscopy (see Fig. 19-17, C).

Biochemical Testing

Specialized biochemical tests can readily differentiate different causes of cyanosis.[4] A simple test to distinguish met-Hb from cardiopulmonary cyanosis or cyanosis caused by low–O_2 affinity Hbs is to expose blood from the patient to pure O_2. This should turn the purple deoxy-Hb in venous blood to bright red HbO_2. In patients with methemoglobinemia, sulfhemoglobinemia, or M Hbs, the blood will retain its brown color. The presence of an M Hb variant can then be distinguished from other causes of methemoglobinemia and sulfhemoglobinemia through the addition of potassium CN. Hemolysates containing "normal" met-Hb (with wild-type globin chains) will turn red immediately after addition of potassium CN. In contrast, those with M Hb variants often change color more slowly, although the rate of conversion varies with different M Hbs.[262] Sulf-Hb, an inert compound that does not react with CN, will not change color.[263]

Biochemical tests can also discern erythrocytosis caused by high–O_2 affinity variants from conditions caused by certain congenital enzyme deficiencies. At physiologic pH and temperature, comparing the O_2 affinity of whole red cells to their phosphate-free hemolysates readily differentiates whether a perturbation is the result of intrinsic Hb alterations or extrinsic causes, such as congenital deficiency of 2,3-DPG mutase. Variant Hbs will show altered O_2 affinity in both the hemolysate and whole red cells. In contrast, erythrocytosis resulting from a deficiency of an allosteric regulator of Hb (e.g., 2,3-DPG) will demonstrate high–O_2 affinity in whole cells and normal O_2 affinity in the hemolysate.

Specialized Testing

Historically, mass spectrometry, X-ray crystallography, and DNA sequencing have been used in research laboratory settings to confirm and characterize the biology of interesting Hb variants that were identified clinically using the methods described. However, DNA sequencing is becoming more convenient to perform at reduced costs. Directed analysis of globin genes is widely used to diagnose Hb variants in specific patients and in high-risk populations.[220,264,265] As noted in Identification of Hemoglobin Variants, whole exome and genome sequencing, which are increasingly being performed for a variety of reasons, are likely to discover incidental globin gene mutations of varying significance.[222,266] Mass spectrometric analysis has been used extensively to identify amino acid alterations in globin proteins,[267,268] although DNA sequencing is becoming a more common test to diagnose

Hb variants. However, mass spectrometry remains useful, particularly in unusual cases where posttranslational modifications of globin chains are suspected to contribute to disease pathology[242,269] (see the discussion on Hbs Bristol-Alesha and Toms River in Hemoglobin Variants that Cause Multiple Biochemical Alterations). Crystallographic analysis has been an extremely fruitful approach to determine how amino acid substitutions alter the structure and function of Hb variants.[270-272]

Clinical Management of Patients with Hemoglobin Variants

Management issues arising from unique circumstances associated with specific Hb variants were previously discussed (see *Clinical Manifestations of Hemoglobin Variants Reflect Their Physiological Effects*). In contrast to sickle-cell disorders and many forms of thalassemia, most Hb variants are either asymptomatic or produce mild clinical effects that require only counseling and supportive care. Indeed, one important reason to diagnose and characterize rare Hb variants is to prevent unnecessary invasive procedures.

Hemolytic anemia caused by unstable Hb variants is associated with a wide range of clinical severity, depending on the nature of the mutation and environmental factors such as infection and oxidant exposure. In moderate cases, folic acid supplementation and/or intermittent RBC transfusion during hemolytic crises may be required. The most severe unstable Hbs, including the “hyperunstable” variants (discussed later in this chapter), can cause severe anemia requiring regular transfusions with attendant problems, including iron overload, risk of infection, and alloimmunization. For severely affected patients, splenectomy may be beneficial but must be weighed against potential toxicities such as increased risk for infection and thrombosis,[273,274] including priapism.[275,276] Rose and colleagues reported clinical improvement after hydroxyurea treatment to induce HbF in two cases of hemolytic anemia caused by unstable Hb variants.[277] In the most severe refractory cases of Hb variant–associated hemolytic anemia, bone marrow transplantation may be indicated.

Most patients with high–O_2 affinity Hbs require no treatment. Rarely, erythrocytosis caused by high–O_2 affinity Hbs can cause symptoms reflecting vaso-occlusion, including headaches,[234] angina,[278] or hypoxemia.[279] In these cases phlebotomy may be considered, although there are conflicting reports on the benefits.[280,281] Low–O_2 affinity Hbs almost never require treatment, because tissue oxygenation is adequate. In fact, one Hb variant (Hb Hope identified in a Tunisian athlete) is reported to increase performance by enhancing O_2 delivery.[282] Publicly available databases (i.e., the Globin Gene Server[12,13]) can serve as valuable resources because many rare Hb variants have been previously encountered and described.

Pathophysiology of Hemoglobin Abnormalities

This section reviews the biochemical mechanisms by which globin-gene mutations alter Hb function and physiology (see Table 19-3). Selected examples of Hb variants that illustrate general mechanistic principles are discussed in detail and listed in Table 19-8. Standard Hb variant nomenclature denotes the mutated chain, amino acid residue number, residue position within the helical globin fold, and substituted amino acid residue type (normal→substituted), for example, β92 (F8) His→Tyr (Hb M Hyde Park).

TABLE 19-8 Hemoglobin Variants Discussed in this Chapter

Variant	Mutation Site (Fold)	Clinical Findings	Laboratory Findings	Molecular Mechanism
Unstable Variants				
Brockton	β138 (H16) Ala→Pro	Hemolytic anemia, reticulocytosis		Δ 2° structure
Singapore	α_1 or α_2 141 (HC3) Arg→Pro	Normal	Δ Mobility	Δ 2° structure
F Poole	γ130 (H8) Trp→Gly	Hemolytic anemia		Δ 3° structure
Moscva	β24 (B6) Gly→Asp	Hemolytic anemia, reticulocytosis	↓ O_2 affinity	Δ 3° structure
Savannah	β24 (B6) Gly→Val	Hemolytic anemia, reticulocytosis		Δ 3° structure
J Calabria	β64 (E8) Gly→Asp	Normal	↑ O_2 affinity	Δ 3° structure
Zurich-Albisrieden	α59 (E8) Gly→Arg	Microcytic, hypochromic erythrocytosis		Δ 3° structure
Borås	β88 (F4) Leu→Arg	Hemolytic anemia	Heinz bodies	Δ Heme pocket
Olmsted	β141 (H19) Leu→Arg	Hemolytic anemia	Heinz bodies	Δ Heme pocket

Continued on following page

TABLE 19-8 Hemoglobin Variants Discussed in this Chapter (Continued)

Variant	Mutation Site (Fold)	Clinical Findings	Laboratory Findings	Molecular Mechanism
Himeji	β140 (H18) Ala→Asp	Normal	↑ O_2 affinity	Δ Heme pocket
Gun Hill	β91 (F7) –95 (FG2) Leu-His-Cys-Asp-Lys→0	Hemolytic anemia, reticulocytosis	Heinz bodies, ↓ cooperativity	Heme pocket deletion
Philly	β35 (C1) Tyr→Phe	Hemolytic anemia, reticulocytosis	↓ Cooperativity, ↑ O_2 affinity	Δ α1β1 interface
Peterborough	β111 (G13) Val→Phe	Hemolytic anemia, reticulocytosis	↓ O_2 affinity	Δ α1β1 interface
Stanmore	β111 (G13) Val→Ala	Hemolytic anemia	↓ O_2 affinity	Δ α1β1 interface
J Guantanamo	β128 (H6) Ala→Asp	Hemolytic anemia	Target cells	Δ α1β1 interface
Khartoum	β124 (H2) Pro→Arg	Normal	Δ Mobility	Δ α1β1 interface
Prato	α_1 or α_2 31 (B12) Arg→Ser	Anisocytosis, hypochromia	Mildly unstable in isopropanol	Δ α1β1 interface
Lombard	α_2 103 (G10) His→Tyr	Anemia		Δ α1β1 interface
Contaldo	α_1 or α_2 103 (G10) His→Arg	Hemolytic anemia		Δ α1β1 interface
Foggia	α_2 117 (GH5) Phe→Ser	Microcytosis	Rapidly degraded α chains	Δ α1β1 interface
Groene Hart	α_1 119 (H2) Pro→Ser	Hemolytic anemia, microcytosis		Δ α1β1 interface, Δ AHSP binding
Turriff	α_1 or α_2 99 (G6) Lys→Glu	Normal	Comigrates with HbA_{1C}, rapidly degraded α chains	Δ α1β1 interface, Δ AHSP binding
Beziers	α_1 99 (G6) Lys→Asn	Normal	Comigrates with HbA_{1C}, rapidly degraded α chains	Δ α1β1 interface, Δ AHSP binding
Hirosaki	α_2 43 (CE1) Phe→Leu	Hemolytic anemia	Heinz bodies, hyperunstable	Δ Heme pocket
Terre Haute	β106 (G8) Leu→Arg	Hemolytic anemia, dominant inclusion body thalassemia	Heinz bodies, hyperunstable	Δ Heme pocket
Constant Spring	α_2 142 (HC3) Stop→Gln	Microcytosis	↓ mRNA stability	Elongated (antiterm)
Cranston	β145 (HC3) + CT	Hemolytic anemia	↑ O_2 affinity, ↓ cooperativity	Elongated (frameshift)
Auckland	α97 (F8) His→Asn	Hemolytic anemia		Δ Heme pocket
High–O_2 Affinity Variants				
Chesapeake	α92 (FG4) Arg→Leu	Erythrocytosis	↑ O_2 affinity, ↓ cooperativity, Δ Mobility	Δ α1β2 interface
Kempsey	β99 (G1) Asp→Asn	Erythrocytosis	↓ Cooperativity	Unstable T state
Hiroshima	β146 (HC3) His→Asp	Erythrocytosis	↓ Cooperativity, ↓ Bohr effect	Δ Bohr proton donor
York	β146 (HC3) His→Pro	Erythrocytosis	↓ Cooperativity, ↓ Bohr effect	Δ Bohr proton donor

TABLE 19-8 Hemoglobin Variants Discussed in this Chapter (Continued)

Variant	Mutation Site (Fold)	Clinical Findings	Laboratory Findings	Molecular Mechanism
Cowtown	β146 (HC3) His→Leu	Erythrocytosis	↓ Bohr effect	Δ Bohr proton donor
San Diego	β109 (G11) Val→Met	Erythrocytosis	↓ Cooperativity	Δ α1β1 interface, comigrates with HbA_{1C}
Pôrto Alegre	β9 (A6) Ser→Cys	Normal	↑ O_2 affinity, ↓ cooperativity	Functional polymers
Rahere	β82 (EF6) Lys→Thr	Erythrocytosis		Δ 2,3-DPG binding site
Providence	β82 (EF6) Lys→Asn	Erythrocytosis	↓ O_2 affinity	Δ 2,3-DPG binding site
Helsinki	β82 (EF6) Lys→Met	Erythrocytosis	↓ Bohr effect	Δ 2,3-DPG binding site
Low–O_2 Affinity Variants				
Kansas	β102 (G4) Asn→Thr	Cyanosis	↓ Cooperativity	Δ α1β2 interface
Beth Israel	β102 (G4) Asn→Ser	Cyanosis	↓ Cooperativity	Δ α1β2 interface
St. Mandé	β102 (G4) Asn→Tyr	Cyanosis		Δ α1β2 interface
Yoshizuka	β108 (G10) Asn→Asp	Anemia		Δ α1β1 interface
Chico	β66 (E10) Lys→Thr	Anemia	Unstable	Steric hindrance of heme for O_2
M Type Variants				
M Iwate	α_1 or α_2 87 (F8) His→Tyr	Cyanosis (met-Hb)	Δ Visible spectrum, ↓ O_2 affinity, ↓ Bohr effect, ↓ cooperativity	Oxidized heme
M Boston	α58 (E7) His→Tyr	Cyanosis (met-Hb)	Δ Visible spectrum, ↓ O_2 affinity, ↓ Bohr effect, ↓ cooperativity	Oxidized heme
M Saskatoon	β63 (E7) His→Tyr	Cyanosis (met-Hb)	Δ Visible spectrum	Oxidized heme
M Hyde Park	β92 (F8) His→Tyr	Cyanosis (met-Hb)	Δ Visible spectrum, unstable	Oxidized heme
M Milwaukee-I	β67 (E11) Val→Glu	Cyanosis (met-Hb)	Δ Visible spectrum, ↓ O_2 affinity, ↓ cooperativity	Oxidized heme
M F Fort Ripley	Gγ92 (F8) His→Tyr	Cyanosis (met-Hb)	Δ Visible spectrum	Oxidized heme
M F Osaka	Gγ63 (E7) His→Tyr	Cyanosis (met-Hb)	Δ Visible spectrum, ↓ O_2 affinity	Oxidized heme
Variants with Multiple Effects				
E	β26 (B8) Glu→Lys	Microcytosis		Unstable, reduced synthesis
Bruxelles	β42 (CD1) Phe→0	Hemolytic anemia, cyanosis, splenomegaly, reticulocytosis	Heinz bodies, ↓ cooperativity	Δ Heme pocket
Warsaw	β42 (CD1) Phe→Val	Hemolytic anemia, cyanosis	Heinz bodies, ↓ cooperativity	Δ Heme pocket
Hammersmith	β42 (CD1) Phe→Ser	Hemolytic anemia, cyanosis	Heinz bodies, ↓ cooperativity	Δ Heme pocket
Buccuresti-Louisville	β42 (CD1) Phe→Leu	Hemolytic anemia, cyanosis	Heinz bodies, ↓ cooperativity	Δ Heme pocket
Zurich	β63 (E7) His→Arg	Normal, but hypersensitive to oxidative stress	↓ Cooperativity, ↑ O_2 affinity, ↑ CO affinity	Δ Heme pocket

Continued on following page

TABLE 19-8 Hemoglobin Variants Discussed in this Chapter (Continued)

Variant	Mutation Site (Fold)	Clinical Findings	Laboratory Findings	Molecular Mechanism
Hb Köln	β98 (FG5) Val→Met	Hemolytic anemia	Heinz bodies, ↑ O_2 affinity	Δ Heme pocket
Bristol-Alesha	β67 (E11) Val→Met	Hemolytic anemia, reticulocytosis	Heinz bodies, ↓ cooperativity, ↓ Bohr effect, ↓ O_2 affinity	Δ Heme pocket
F Toms River	γ67 (E11) Val→Met	Anemia, cyanosis	Unstable, ↓ O_2 affinity	Δ Heme pocket
Jamaica Plain	β6 (A3) Glu→Val and β68 (E12) Leu→Phe	Hemolytic anemia, cyanosis, splenomegaly, splenic sequestration	Heinz bodies, sickle cell phenotype, ↓ O_2 affinity, unstable	Δ 2° structure
Variants that Genetically Interact with HbS				
D Ibadan	β87 (F3) Thr→Lys	Normal	Inhibits HbS polymerization	Δ Interaction with HbS polymer
Korle-Bu	β73 (E17) Asp→Asn	Normal	Inhibits HbS polymerization, ↓ O_2 affinity, Δ mobility	Δ Interaction with HbS polymer
C Harlem	β6 (A3) Glu→Val and β73 (E17) Asp→Asn	Normal	Inhibits HbS polymerization, unstable	Δ Interaction with HbS polymer
Quebec-Chori	β87 (F3) Thr→Ile	Normal	Promotes HbS polymerization	Δ Interaction with HbS polymer

2,3-DPG, 2,3-Diphosphoglycerate; *AHSP,* Alpha hemoglobin stabilizing protein; *CO,* carbon monoxide; *Hb,* hemoglobin; O_2, oxygen; *T,* tense.

Assembly of Mutant Hemoglobins

The relative proportions of normal and mutant Hbs in the red cells of heterozygotes provides insight into Hb assembly and hemoglobinopathies.[283,284] Most β-globin variants caused by missense mutations are synthesized at the same rate as normal β globin (βA) and have normal stability. Therefore heterozygotes might be expected to have equal amounts of normal and mutant Hbs, at least the stable ones. However, Bunn, McDonald, and colleagues found that the proportions of normal and mutant globin chains in heterozygotes vary according to the charge difference conferred by the mutation.[285,286] Figure 19-18 compares the proportion of different stable variant β chains to βA in heterozygotes. Note that the more positively charged mutants, such as Hbs S, C, D Los Angeles, and E, account for significantly less than half of the total Hb in heterozygotes. The proportions are further reduced in the setting of α-thalassemia, when the α chain is limiting for Hb assembly. In contrast, many of the negatively charged variants are present in amounts exceeding that of βA. The proportion of mutant Hb was further increased in two heterozygotes for negatively charged mutants (HbJ Baltimore or HbJ Iran) combined with α-thalassemia. These studies suggest that globin subunit surface charges contribute to the rate of Hb tetramer assembly. Since normal α chains exhibit a net positive charge (basal isoelectric point at pH 8.72), they bind more rapidly to β-variant chains with increased negative charge. This electrostatic model for Hb assembly is supported by in vitro mixing experiments[287,288] and also has clinical implications. For example, differences in rates of Hb assembly explain why patients with HbSC disease have about 50% HbS and 50% HbC in their RBCs, whereas patients with HbAS (sickle cell trait) have about 35% to 40% HbS and 60% to 65% HbA. The electrostatic model also can account for differences in levels of HbA_2[284] and HbF[289,290] that accompany certain hematologic disorders.

Unstable Hemoglobin Variants

Unstable globins tend to denature and precipitate, forming Heinz bodies that damage erythrocytes through several mechanisms and cause hemolytic anemia. Examples are listed in Table 19-8, and the location of variant amino acid residues are denoted in Figure 19-19. For example, unstable globins can release heme, unfold, and interfere with other cellular proteins through hydrophobic interactions. Partially denatured oxidized Hbs can also form hemichromes, which are low-spin, unstable forms of ferric Hb in which the sixth coordination position of iron is aberrantly bound by globin amino acids via hydroxyl or histidyl groups.[291-296] Hemichromes are unstable and lose hemin, which in turn readily produces ROS.[297] Denatured, hemichrome-associated globins and hemin–globin precipitates can associate with the plasma membrane,[298,299] causing peroxidation and cross-linking of membrane

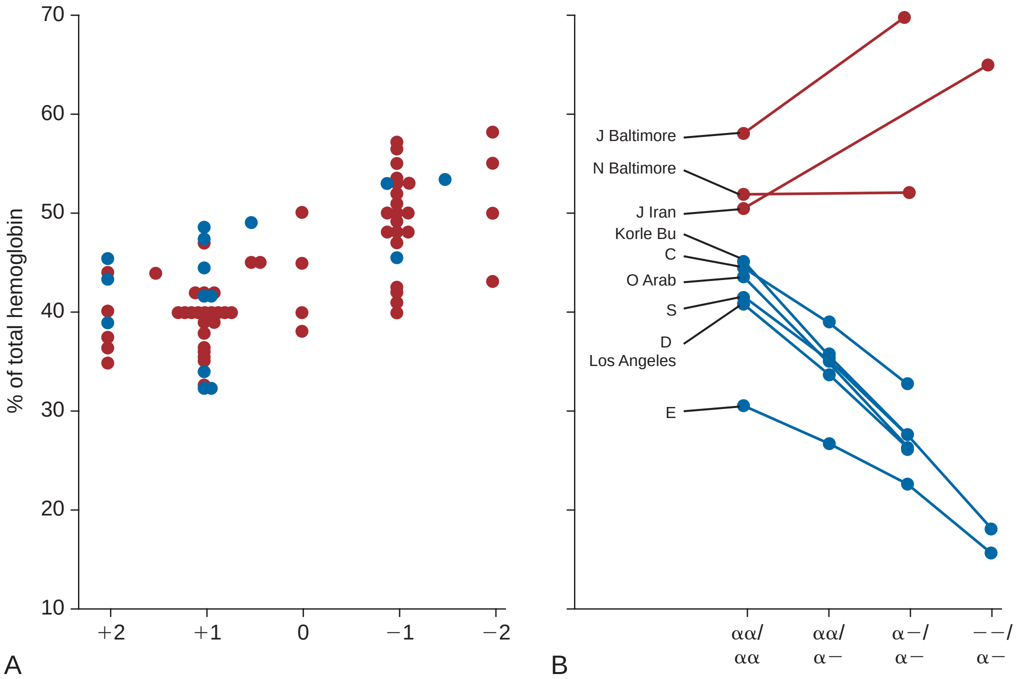

Figure 19-18 Charge affects Hb folding and expression. **A**, Effect of charge on the proportion of abnormal Hb in individuals heterozygous for 72 stable β-globin variants. Each point denotes a mean value for a given variant. *Blue points* denote high-resolution chromatography measurements. Histidine residue substitutions were scored as a change of one-half charge. The "–1" group differs significantly from the "+1" group ($P < .001$) and from the "0" group ($P < .05$). **B**, Effect of α-thalassemia on the proportion of six positively charged β-chain variants *(blue)* and three negatively charged variants *(red)*. *(A, From Huisman TH: Percentages of abnormal hemoglobins in adults with a heterozygosity for an alpha-chain and/or a beta-chain variant.* Am J Hematol *14:393–404, 1983; B, From Bunn HF, McDonald MJ: Electrostatic interactions in the assembly of haemoglobin.* Nature *1306:498–500, 1983 and Rahbar S, Bunn HF: Association of hemoglobin H disease with Hb J-Iran [beta 77 His→Asp]: impact on subunit assembly.* Blood *70:1790–1791, 1987.)*

proteins including Band 3.[231,300-305] This process can also inhibit membrane transport processes.[231,306,307] Precipitated globins may also interfere with general protein quality-control mechanisms that are required for normal erythropoiesis.[31]

Association of precipitated globins with plasma membranes can also inhibit the deformability of erythrocytes. These changes causes the cells to be "pitted" and/or captured by the spleen, the organ responsible for maintaining erythrocyte quality control.[306] *Pitting* refers to the mechanism by which Heinz bodies along with a portion of the overlying plasma membrane are excised from erythrocytes by splenic macrophages. Over time, repeated pitting and membrane loss converts biconcave erythrocytes into spherocytes, ultimately producing a rigid remnant that is eliminated from the circulation.

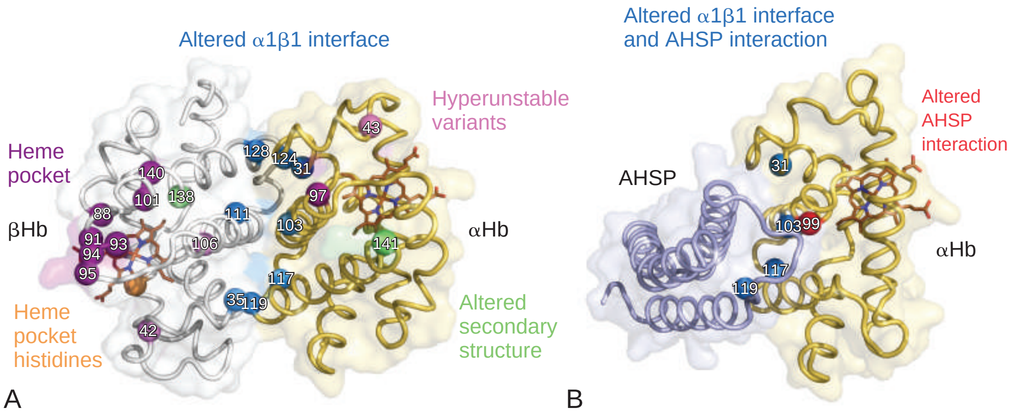
Altered α1β1 interface
Hyperunstable variants
Heme pocket
βHb
Heme pocket histidines
αHb
Altered secondary structure
128
124
31
43
140
101
138
97
88
111
103
91
94
93
106
141
95
117
35
119
42
A
Altered α1β1 interface and AHSP interaction
AHSP
Altered AHSP interaction
31
103
99
117
119
αHb
B

Figure 19-19 Diagram of the α1β1 dimer showing amino acid substitutions in unstable Hb variants discussed in this chapter. See also Table 19-8. **A**, *Spheres* indicate the locations (Cα positions) and amino acid residue number of substitutions giving rise to the unstable Hb variants. Positions are color coded as indicated according to abnormal biochemical/structural properties conferred by the substitution. **B**, Some variants in α globin (substituted amino acids shown in *blue*) inhibit interactions with the α-globin chaperone, AHSP, and with β globin at the α1β1 interface. Mutations of α99 (G6) Lys *(red)* destabilize the interaction of α globin with AHSP but not with β globin.

Altered Secondary Structure of Hemoglobin Subunits. More than 75% of Hb is α helical.[17,308] This structure is particularly susceptible to disruptions by proline substitutions because proline lacks an amide proton and so does not contribute to the regular hydrogen bonding pattern, and steric hindrance from the proline side chain requires the helix to kink. Indeed, about 15% of mutations responsible for unstable Hbs involve the introduction of a proline residue into the middle of an α helix.[4] For example, Hb Brockton (β138 [H16] Ala→Pro) results in an unstable Hb and mild hemolytic anemia[309,310] (see Fig. 19-19, *A*, *green sphere*; see also Fig. 19-17, *A*). X-ray crystallographic analysis showed that this mutation causes structural perturbations localized only in the vicinity of helix H.[310] Accordingly, Hb Brockton does not exhibit altered O_2 binding affinity or electrophoretic mobility shifts. In contrast, Hb Singapore (α_1 or α_2 141 [HC3] Arg→Pro) contains a substituted proline that does not destabilize the molecule because the substitution occurs at α141 Arg, the last amino acid in the chain, which is not part of an α helix[311] (see Fig. 19, *A*, *green sphere*). As a result, this latter mutation has no clinical consequences. Hb Singapore was discovered through its altered electrophoretic mobility during the workup of a child with anemia that was eventually attributed to iron deficiency.

Altered Tertiary Structure of Hemoglobin Subunits. As described in sections on hemoglobin structure, mutations that alter the polarity or size of buried hydrophobic residues can destabilize the tertiary structure. For example, HbF Poole ($^G\gamma$130 [H8] Trp→Gly) was identified in a newborn with hemolytic anemia.[312] The replacement of the larger aromatic side chain with glycine, which has no carbon side chain, renders this globin unstable by creating a cavity in the middle of the γ subunit. As the mutation occurred within a γ-globin gene, the condition resolved spontaneously after the first few months of life. Information on protein stability is available from the Globin Gene Server[12,13] for 29 Hb variants with X_{aa}-to-Gly mutations in α or β Hb chains. Of these, eight are annotated as unstable variants, with six being Gly replacements of Val or Trp side chains in the globin interior. All 21 variants with normal to mildly unstable phenotypes have substitutions on the surface of the α/β-globin chain.

Conversely, substitution of the conserved pair of Gly residues at positions B6 and E8 (see sections on hemoglobin structure) with more bulky residues can also destabilize Hb. Variants at positions β24 (Hb Moscva β24 [B6] Gly→Asp[313] and Hb Savannah β24 [B6] Gly→Val[314]), β64 (HbJ Calabria [E8] Gly→Asp[315]) and α59 (Hb Zurich-Albisrieden [E8] Gly→Arg[316]) destabilize the globin fold, giving rise to unstable Hb phenotypes, presumably by forcing the B and E helices slightly apart. The unstable Hb Savannah variant (β24 [B6] Gly→Val), which causes hemolytic anemia,[314] supports the conclusion that destabilization is the result of steric effects and probably not changes in electrostatic interactions.

Altered Heme–Globin Interactions. Mutations that impair interaction with the heme group also produce unstable Hbs (see Fig. 19-19, *A*, *purple spheres*). There are several ways in which globin mutations can interfere with heme incorporation. For example, introduction of a charged side chain into the heme pocket where a nonpolar side chain normally exists occurs in unstable Hbs Borås (β88 [F4] Leu→Arg),[317] Olmsted (β141 [H19] Leu→Arg),[275,318,319] and Himeji (β140 [H18] Ala→Asp).[320] Unstable Hb variants may also occur via deletions of residues that directly interact with heme, such as Hb Gun Hill (β91 [F7]–95 [FG2] Leu-His-Cys-Asp-Lys→0).[321,322] This class of variants also includes nontyrosine substitutions of the proximal (F8) or distal (E7) histidines, which in the wild-type subunits either directly coordinate the heme iron or donate a stabilizing H-bond to bound dioxygen, respectively (see Fig. 19-19, *A*, *orange spheres*). Hb Zurich (β63 [E7] His→Arg) is a distal histidine substitution that both destabilizes the molecule and alters interaction with allosteric regulators[323,324] (see Hemoglobin Variants that Cause Multiple Biochemical Alterations). Hb Zurich exhibits enhanced susceptibility to drug-induced oxidation, because the charged guanidino group of the Arg E7 side chain swings out of the distal pocket, exposing the distal pocket to water, and affected patients can experience sulfonamide-induced hemolytic anemia.[325,326] Tyrosine substitutions of the proximal and distal His residues, which cause increased levels of met-Hb (see Methemoglobin [M Type] Hemoglobin Variants), can also be destabilizing.

Altered Globin Subunit Interactions (Quaternary Structure). Globins typically exist as tightly associated α1β1 dimers or tetramers that are formed through α1β2 interactions (see Hemoglobin Quaternary Structure). Hb monomers (α) or homotetramers (β_4) are unstable. Therefore substitutions of amino acids that participate in α1β1 association to form the initial dimer can cause the accumulation of unstable free subunits (see Fig. 19-19, *A*, *blue spheres*). For example, Hb Philly (β35 [C1] Tyr→Phe) prevents formation of a hydrogen bond that normally occurs between β35 Tyr and α126 Asp at the α1β1 interface.[244] Electrophoretic analyses of Hb Philly hemolysate demonstrated free α chains and an abnormal "smear" indicative of denatured Hb. Other examples of Hb variants that impair globin chain assembly by altering the α1β1 interface include Hb Peterborough (β111 [G13] Val→Phe),[327] Hb Stanmore (β111 [G13] Val→Ala)[328] and HbJ Guantanamo (β128 [H6] Ala→Asp).[329] Hb Khartoum (β124 [H2] Pro→Arg) contains a substitution at the α1β1 interface that is mildly destabilizing in vitro.[311,330] This variant was discovered incidentally and does not usually cause clinical symptoms in isolation, but it can exacerbate concurrent thalassemia.[331]

Disrupted Hemoglobin–Alpha Hemoglobin Stabilizing Protein Interactions. Some α-globin gene mutations that disrupt the α1β1 interface also inhibit binding of α globin to the AHSP, which is a molecular chaperone that binds and stabilizes free α-globin chains to facilitate efficient and correct HbA synthesis.[332,333] These variants include Hbs Prato (α_1 or α_2 31 [B12] Arg→Ser),[334] Lombard (α_2 103 [G10] His→Tyr),[335] Contaldo (α_1 or α_2 103 [G10] His→Arg),[336] Foggia (α_2 117 [GH5] Phe→Ser),[337] Groene

Hart (α_1 119 [H2] Pro→Ser),[338-341] and others[342,343] (see Fig. 19-19, *B*, *blue sphere*). Certain α-globin variants with amino acid substitutions at position α99 Lys, including Hb Turriff (α_1 or α_2 99 [G6] Lys→Glu)[219] and Hb Beziers (α_1 99 [G6] Lys→Asn),[344] bind β globin normally but exhibit impaired interaction with AHSP and may be mildly destabilizing (see Fig. 19-19, *B*, *red sphere*).[33,34,343] Antitermination mutations may also destabilize α globin by impairing its binding to AHSP.[345]

Elongated Hemoglobin Variants

Antitermination and frameshift mutations that extend the open reading frame beyond the normal carboxyl terminus of the globin chain yield unstable proteins that are underrepresented in the hemolysate of affected patients. The most prevalent example is Hb Constant Spring (α_2 142 [HC3] Stop→Gln), caused by an antitermination mutation at the α_2 stop codon.[346-349] Hemolysates contain about 5% variant globin that is elongated by 31 amino acids (each α-globin chain in an HbA tetramer is normally present at 25%). Hb Constant Spring erythroblasts and reticulocytes contain cytoplasmic inclusions, similar to what occurs in β thalassemia and some hyperunstable Hb variants (see Fig. 19-17, C).[350] In addition, abnormal ribosome entry past the termination codon into the 3′ untranslated region of Hb Constant Spring mRNA displaces RNA-stabilizing proteins from this region, causing mRNA degradation.[350-353] For these reasons, Hb Constant Spring contributes to α-thalassemia syndromes. When combined with two α-globin deletional alleles ($—/\alpha^{CS}\alpha$), this variant produces a relatively severe hemoglobinopathy in which β_4 (HbH) tetramers accumulate and damage erythrocytes.[354] In pure heterozygous ($\alpha\alpha/\alpha^{CS}\alpha$) or homozygous ($\alpha^{CS}\alpha/\alpha^{CS}\alpha$) forms, Hb Constant Spring results in more severe anemia than occurs when the same α alleles are deleted ($\alpha\alpha/–\alpha$) or ($–\alpha/–\alpha$) as a result of the combined cytotoxic effects of the unstable Constant Spring protein and its mRNA instability.[355] Although it is most common in Southeast Asia, Hb Constant Spring is increasingly identified in other geographic regions, largely through global migration.[356] In fact, this variant was first discovered in a Chinese family living in Constant Spring, Jamaica.

Hb Cranston (β145 [HC3] + CT)[357] is an example of a β-globin chain elongation mutation. This mutation introduces a frameshift at the normal stop codon to generate a β chain that is extended by 11 amino acids, resulting in an unstable Hb tetramer with high–O_2 affinity and diminished cooperativity.[285,358] Affected patients exhibit compensated hemolytic anemia. Interestingly, studies on the structure of Hb Cranston occurred simultaneously with investigations aimed to determine the β globin mRNA 3′ untranslated sequence.[359] Cross-comparison mRNA-sequencing data between Hb Cranston and normal β globin allowed Bunn, Forget, and colleagues to more rapidly define normal β-globin gene structures and ascertain that the Hb Cranston mutation likely arose from nonhomologous crossover of two normal β-globin genes.

Hyperunstable Hemoglobin Variants

Some Hb variants are rapidly degraded after synthesis and therefore termed *hyperunstable* (see Fig. 19-19, *A*, *pink spheres*). Patients with these variants exhibit autosomal dominant "inclusion body thalassemia" resulting from both the precipitated variant globin and consequent chain imbalance with accumulation of the unaffected globin, which is unstable in its free form (particularly α chains).[360] This syndrome is characterized by significant anemia, splenomegaly, reticulocytosis, and a blood smear with prominent hypochromia, microcytosis, and basophilic stippling.[361,362] The bone marrow typically shows considerable erythroid hyperplasia and evidence of IE.

Weatherall, Thein, and colleagues characterized several hyperunstable mutations in the last exon (exon 3) of the β-globin gene, which produces the C-terminal portion of the protein.[361] All of the mutations were frameshifts or nonsense codons that produced relatively long proteins (>120 amino acid) with carboxyl-terminal truncations. The authors proposed that truncated globins causing dominantly inherited thalassemia are long enough to bind heme posttranslationally, which rendered them relatively resistant to proteolytic degradation, allowing for subsequent precipitation of heme-containing aggregates detected as Heinz bodies. Missense mutations can also cause hyperunstable Hb variants through extreme effects that are discussed earlier in this section.

Hyperunstable Hbs are usually difficult to detect at the protein level. Hb Hirosaki (α_2 43 [CE1] Phe→Leu) was discovered in a family with autosomal dominant hemolytic anemia.[232,233] After tests failed to identify a soluble variant Hb within erythrocytes, DNA sequencing identified a missense mutation affecting an amino acid in the α-globin heme pocket. Analogous mutations in β chains such as Hb Buccuresti-Louisville (β42 [CD1] Phe→Leu)[363,364] cause instability via reduced heme–globin interactions and accelerated autoxidation (see Altered Heme–Globin Interactions). These mutations affecting the "CD corner" of the heme pocket also show reduced O_2 affinity (see Hemoglobin Variants that Cause Multiple Biochemical Alterations).

Hb Terre Haute (β106 [G8] Leu→Arg) is another hyperunstable variant associated with severe Heinz body hemolytic anemia and globin-chain imbalance.[365] The Leu (G8) side chain is highly conserved in all mammalian Hbs and is an Ile in most Mbs. It is located in the interior of the heme pocket, and replacement with charge residues almost certainly prevents folding and heme binding. In initial studies of erythroid cells in patients, performed in 1979, abnormal Hb tetramers were not detected and peptide mapping of radiolabeled nascent globins identified a β112 (G14) Cys-to-Arg substitution, originally termed *Hb Indianapolis*.[257] However, the β112 Cys-to-Arg mutation was subsequently identified in unrelated individuals with much less severe disease. In 1991, reevaluation of the original pedigree by DNA analysis discovered a β106 Leu-to-Arg mutation, which was renamed *Hb Terre Haute*.[365] Most likely, incomplete tryptic cleavage of the abnormal β globin peptide in the earlier studies led to misidentification of the causative mutation. This work reflects the interesting historical point that many Hb variants were initially identified through laborious and technically challenging protein studies performed some time ago, before DNA sequence analysis of patient

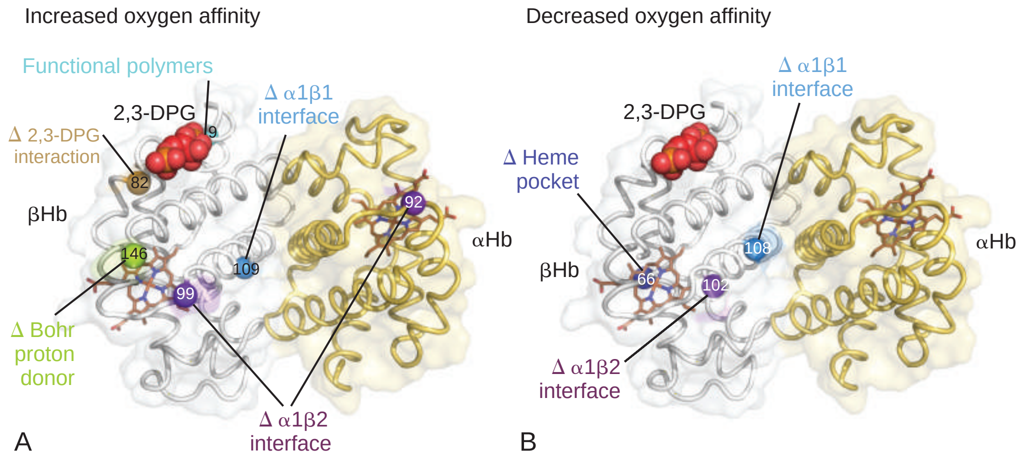

Figure 19-20 Amino acid substitutions in Hb variants with altered O_2 affinity discussed in this chapter. See also Table 19-10. **A**, High–O_2 affinity Hb variants. The α1β1 dimer is shown with spheres indicating the locations (Cα positions) and amino acid positions of substitutions. Positions are color coded according to biochemical/structural properties. **B**, Low–O_2 affinity Hb variants. The α1β1 dimer is shown, labeled similar to panel **A**.

globin genes was feasible. Reevaluation of these mutations though genetic testing has yielded some interesting surprises (see the discussion on Hb Bristol-Alesha in Hemoglobin Variants that Cause Multiple Biochemical Alterations).

High–Oxygen Affinity Hemoglobin Variants

In general, Hb variants that preferentially stabilize the R (oxygenated) state relative to the T (deoxygenated) state cause increased Hb O_2 affinity with a consequent decrease in tissue O_2 delivery (Table 19-8 and Fig. 19-20, *A*).[290] This decrease in delivery results in heightened EPO production and erythrocytosis. Increased O_2 affinity is indicated by a characteristic left-shifted O_2 equilibrium binding curve (see Figs. 19-13 and 19-16, *A*). Four classes of mutations enhance Hb O_2 affinity.

Altered α1β2 Interface. As described in the first part of this chapter, the T and R states are characterized by different sets of molecular interactions at the α1β2 interface. Mutations at the α1β2 interface that either stabilize the R state or destabilize the T state yield high–O_2 affinity Hb variants (see Fig. 19-20, *A*, *purple spheres*). Hb Chesapeake (α92 [FG4] Arg→Leu) was one of the first such Hb variants to be identified.[278] The propositus was an 81-year-old patient complaining of mild angina. He was found to have erythrocytosis, an abnormal Hb detected on electrophoresis, and a left-shifted Hb O_2 equilibrium curve. Affected relatives had similarly high hematocrit levels. Discovery and analysis of this family was remarkable at the time, because it demonstrated that an Hb variant could generate a distinct clinical syndrome. Whether the index patient's angina resulted from his erythrocytosis is unclear, however, as most high-affinity O_2 variants do not produce clinical consequences. The amino acid replacement in Hb Kempsey (β99 [G1] Asp→Asn) perturbs α1β2 interactions by preventing the formation of a hydrogen bond between β99 Asp and α42 Tyr, which normally stabilizes the deoxygenated low–O_2 affinity T state[366-369] (see Fig. 19-10).

Altered α1β1 Interface. Whereas mutations at the α1β2 interface have classically been associated with a perturbed T-to-R transition, variants affecting α1β1 interface residues have also been implicated in altering Hb cooperativity (see Fig. 19-20, *A*, *blue sphere*). For example, Hb San Diego (β109 [G11] Val→Met) was identified in a Filipino family with autosomal dominant erythrocytosis. The hemolysate demonstrated an increased O_2 affinity (P_{50}, 16.4 mm Hg; normal 26 mm Hg) with a normal Bohr effect and slightly reduced cooperativity.[234] Tryptic digestion and protein sequencing identified the amino acid replacement, which occurs on the α1β1 interface.[234] Crystallographic analysis of deoxy-Hb San Diego demonstrated that the substituted Met side chain produces steric effects that weaken the α1β1 interaction and destabilize the variant T state.[370]

Altered Cooperativity Through Modifications in Quaternary Structure. Hb Pôrto Alegre (β9 [A6] Ser→Cys) was identified during a population study as an abnormal electrophoretic variant in a healthy child (see Fig. 19-20, *A*, *light blue sphere*).[371] Hb Pôrto Alegre showed increased O_2 affinity and slightly decreased cooperativity. The sulfhydryl group of the mutant cysteine participates in intermolecular cross-linking reactions to form Hb Pôrto Alegre polymers.[371-374] This polymerization occurs during prolonged storage of blood samples and is suppressed in vivo, likely by increased erythrocyte glutathione reductase activity in subjects who carry the mutation.[375] Surprisingly, however, Hb Pôrto Alegre polymerization does not contribute to its altered O_2 binding characteristics.[373]

Altered Response to Allosteric Regulators. Several high-O_2 affinity Hb variants are caused by substitutions that inhibit interaction with allosteric activators such as 2,3-DPG (see Fig. 19-20, *A*, *brown sphere*), which normally binds between the β-globin chains to stimulate O_2 release (see *2,3-Diphosphoglycerate*). For example, in Hb Rahere (β82 [EF6] Lys→Thr) the invariant lysine in the

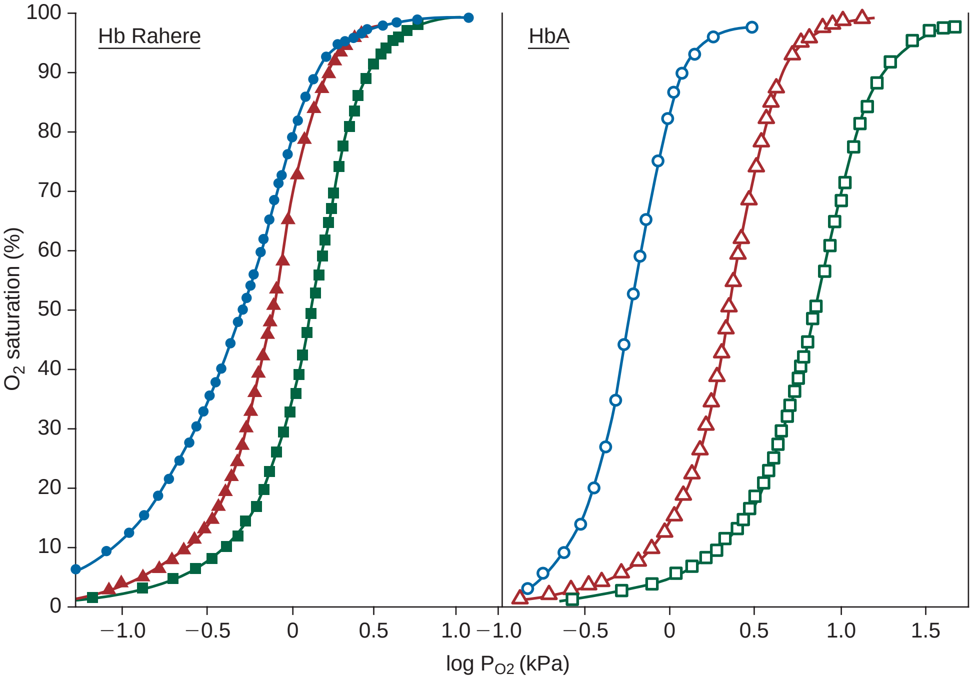

Figure 19-21 Hb Rahere is a high–O_2 affinity variant with impaired binding to 2,3-DPG. The graphs show O_2 dissociation curves for purified Hb Rahere *(closed symbols, left)* and HbA (*right, open symbols*) with no allosteric effector *(circles)* or in the presence of 2 mM 2,3-DPG *(triangles)* or 2 mM inositol hexaphosphate *(squares)*. Note that the right shift caused by organic phosphates is smaller in Hb Rahere versus HbA. *(From Lorkin PA, et al: Haemoglobin Rahere [beta Lys-Thr]: a new high affinity haemoglobin associated with decreased 2,3-diphosphoglycerate binding and relative polycythaemia.* Br Med J *4:200–202, 1975.)*

2,3-DPG binding site of β globin is replace by a smaller uncharged side chain, thereby reducing the affinity for this allosteric regulator.[376,377] Consequently, Hb Rahere exhibits blunted O_2 release in response to added 2,3-DPG in vitro.[376] The blunted response of this variant to 2,3-DPG is illustrated by the O_2 equilibrium curves shown in Figure 19-21. In vivo O_2 release in peripheral tissues is inhibited, resulting in erythrocytosis. Similarly, Hb Providence (β82 [EF6] Lys→Asn/Asp)[378,379] and Hb Helsinki (β82 [EF6] Lys→Met)[380,381] are high–O_2 affinity variants caused by different amino acid substitutions at the 2,3-DPG binding site on β82.

The C-terminal β146 His contributes significantly to the Bohr effect by forming a salt bridge with β94 Asp[272] (see Fig. 19-12 and *Carbon Dioxide Transport and the Bohr Effect*). This interaction is disrupted in several high–O_2 affinity variants with β146 substitutions, including Hb Hiroshima (β146 [HC3] His→Asp),[382,383,384,385] Hb York (β146 [HC3] His→Pro),[386] and Hb Cowtown (β146 [HC3] His→Leu)[272,387] (see Fig. 19-20, *A*, *green sphere*). Disruption of the Bohr effect contributes significantly to the high–O_2 affinity of these variants.

Low–Oxygen Affinity Hemoglobin Variants

Low–O_2 affinity variants preferentially stabilize Hb tetramer in the T (deoxygenated) state versus the R (oxygenated) state (see Table 19-8 and Fig. 19-20, *B*), typically resulting in cyanosis. Decreased O_2 affinity is indicated by a right-shifted O_2 equilibrium curve (see Figs. 19-13 and 19-16, *B*). Commonly mutated regions include the α1β2 interface, although mutations involving amino acid residues at the α1β1 interface have also been associated with low–O_2 affinity variants. Steric hindrance in the heme pocket can also reduce O_2 affinity.

Altered α1β2 Interface. Several low-O_2 affinity variants involve replacements at the α1β2 interface, which plays an important role in Hb cooperativity (see Fig. 19-20, *B*, *purple spheres*). Hb Kansas (β102 [G4] Asn→Thr) is a well-studied variant with low–O_2 affinity.[388-391] Affected individuals are markedly cyanotic, although clinically well. Replacement of β102 Asn at the α1β2 interface inhibits the formation of a hydrogen bond with α94 Asp that normally participates in R-state stabilization (see Fig. 19-10). A similar mechanism causes low–O_2 affinity in two other Hb variants with different substitutions at β102: Hb Beth Israel (β102 [G4] Asn→Ser)[392] and Hb St. Mandé (β102 [G4] Asn→Tyr).[393,394] Of note, one child with Hb Beth Israel exhibited cyanosis of the fingers, lips, and nail beds that was not detected by his parents. In fact, he had been disciplined in the past for having “dirty hands.” A surgeon who was about to perform herniorrhaphy discovered that the patient had cyanosis.[4]

Altered α1β1 Interface. β108 Asn lies near the α1β2 interface with its side chain projected into the solvent-filled central cavity. Substitution of β108 Asn with a negatively charged Asp Hb Yoshizuka (β108 [G10] Asn→Asp)[395] or positively charged Lys in Hb Presbyterian (β108 [G10] Asn→Lys)[396] alters interactions with the allosteric effectors Cl^- and H^+ (see Fig. 19-20, *B*, *light blue sphere*). Hb Yoshizuka has diminished Cl^- and Bohr effects, whereas Hb Presbyterian has enhanced Cl^- and Bohr effects.[397] Interactions with Cl^- and the Cl^--dependent Bohr effect are some of the least well-understood features of Hb.[68] Studies on Hb Yoshizuka and Hb Presbyterian proteins suggest that the distribution of positive and negative charges in the central cavity of the Hb tetramer can alter O_2 affinity and cooperativity, either directly through stabilizing the T state quaternary structure or indirectly through interactions with allosteric effectors.[397,398] However, the consequent low–O_2 affinity variant causes mild anemia resulting from globin instability but no apparent cyanosis.

Altered Charge at the Entrance to the Heme Pocket. Hb Chico (β66 [E10] Lys→Thr) displays a significantly reduced O_2 affinity, with normal cooperativity and anion sensitivity[399-401] (see Fig. 19-20, *B, dark purple sphere*). Biochemical studies suggest that the heme pocket in Hb Chico adopts a conformation that inhibits O_2 binding by reducing the reactivity of the iron atom even when ligands are in the active site.[400,401] Consistent with a mechanism involving an altered tertiary structure, isolated β Chico chains also have reduced O_2 affinity because of higher dissociation rate constants. In addition, the rate of CO binding to isolated β Chico subunits is at least twofold lower than that of wild-type β chains. One possible mechanism is that removal of the native positively charged Lys weakens interactions with the heme propionate, allowing the iron atom to move further out of the plane of the porphyrin ring, particularly in the T-state conformations.[389] This mutation also promotes autoxidation with consequent mild instability.

Methemoglobin (M-Type) Hemoglobin Variants

This class of Hb variants contains amino acid substitutions that alter the heme environment and stabilize the oxidized (ferric, Fe[III]) state over the reduced one (ferrous, Fe[II]), normally by coordination of the mutant side chain to the Fe(III) atom (see Table 19-8). Since oxidized met-Hb cannot bind O_2, patients exhibit cyanosis, although arterial oxygenation is normal. The blood usually appears brown in patients with met-Hb from numerous etiologies (see Table 19-6). However, the M Hb variants exhibit absorption spectra that are distinct from those for fully oxidized HbA and some unstable Hb variants with a tendency to oxidize, such as Hbs Saint Louis,[402] Bicetre,[403,404] I Toulouse,[405] and Seattle.[406] The differences are because of the presence of a native reduced subunit in the M Hb and also iron coordination to the mutant side chain. Comparative studies of the M Hbs have contributed greatly to understanding the biochemical properties of the heme iron, including its interactions with O_2 and ferric ligands. All of the M Hbs are caused by missense mutations in which the side chain of the substituted amino acid forms an abnormal coordination bond with ferric heme iron. Six out of the seven known M Hbs contain tyrosine replacements of either the α- or β-globin proximal (F8) or distal (E7) histidine residues present in the normal active site (Fig. 19-22).[407,408] In M Hbs, these mutations stabilize the ferric state to various extents via several mechanisms.

Heme Pocket Perturbations. In HbM Iwate (α_1 or α_2 87 [F8] His→Tyr), the proximal histidine is replaced by tyrosine[240,241] (see Fig. 19-22, *A*). Whereas Fe(III)-heme coordinated to the native proximal His F8 is readily reduced by met-Hb reductase, tyrosine (F8) coordination stabilizes the oxidized ferric state and decreases reactivity with the reductase system. This interaction also distorts the position of heme and helix F, creating a "green" α subunit with spectral properties similar to catalase, which also has a proximal Tyr ligand.[409-411] In native HbA, movement of the proximal His F8 and helix F away from the heme group stabilizes the deoxygenated T state and reduces the O_2 affinity of the native β-subunit partners. Hence substitution of the proximal His F8 side chain for a longer Tyr F8 in HbM Iwate also stabilizes the deoxygenated T state, possibly by positioning the F helix away from the heme group (see Fig. 19-22, *B*) and therefore also reduces O_2 affinity of the native β subunit within the mutant tetramer.[412,413] These biochemical and structural alterations underlie the lack of cooperativity and severe cyanosis in patients with HbM Iwate, where met-Hb levels can exceed 20% (normal <2%).[414] Nonetheless, O_2 delivery to tissue is not dramatically compromised because of the presence of native tetramers in heterozygotes and the native β chain in the mutant Hb.

HbM Saskatoon (β63 [E7] His→Tyr), which contains a distal His-to-Tyr substitution, is unusual amongst the M Hbs in that it is readily reduced by methemoglobin reductase (see Fig. 19-22, *C*). However, the heme in HbM Saskatoon is primarily hexacoordinated with Tyr E7 being in equilibrium between the protonated and deprotonated state, with the latter anionic state binding preferentially to Fe(III) but not to Fe(II) (see Fig. 19-22, *C and D*). These features presumably allow reduction.[415-417] Consequently, patients with HbM Saskatoon have lower levels of circulating met-Hb compared to those with HbM Iwate. HbM Boston (α58 [E7] His→Tyr) also substitutes the distal His with Tyr,[271,417,418] which again preferentially binds to the sixth coordinating position of the iron on the distal side of the porphyrin ring (see Fig. 19-22, *A*). In α subunits, Tyr E7 coordination can lead to breaking the Fe–His F8 bond in the T-state conformation. Thus the new Tyr E7–Fe(III) bond can alter the heme plane enough to disrupt the normal interaction between proximal His F8 and heme iron when strain is already present with significant consequences on the O_2 affinity of adjacent subunits (see Oxygen Binding Properties of M Hemoglobins). It should be noted that NO binding to α subunits stabilized in the T quaternary state can also lead to disruption of the His F8–Fe(II) bond.[165]

In contrast to all other M Hbs, HbM Milwaukee-I (β67 [E11] Val→Glu) does not involve a replacement of the proximal or distal His.[367,413,416,419,420] Instead, β67 Val is replaced by Glu in a position directly adjacent to bound O_2. The anionic carboxylate side chain in this variant coordinates directly to the sixth coordinate position of heme iron to stabilize the ferric state, and the proximal His F8 remains bound to the fifth coordinate position.[419,421] The proximity of this anionic residue to the iron atom facilitates the outer sphere autoxidation mechanism (see Autoxidation). As a result, the speed of spontaneous oxidation is dramatically increased, and the

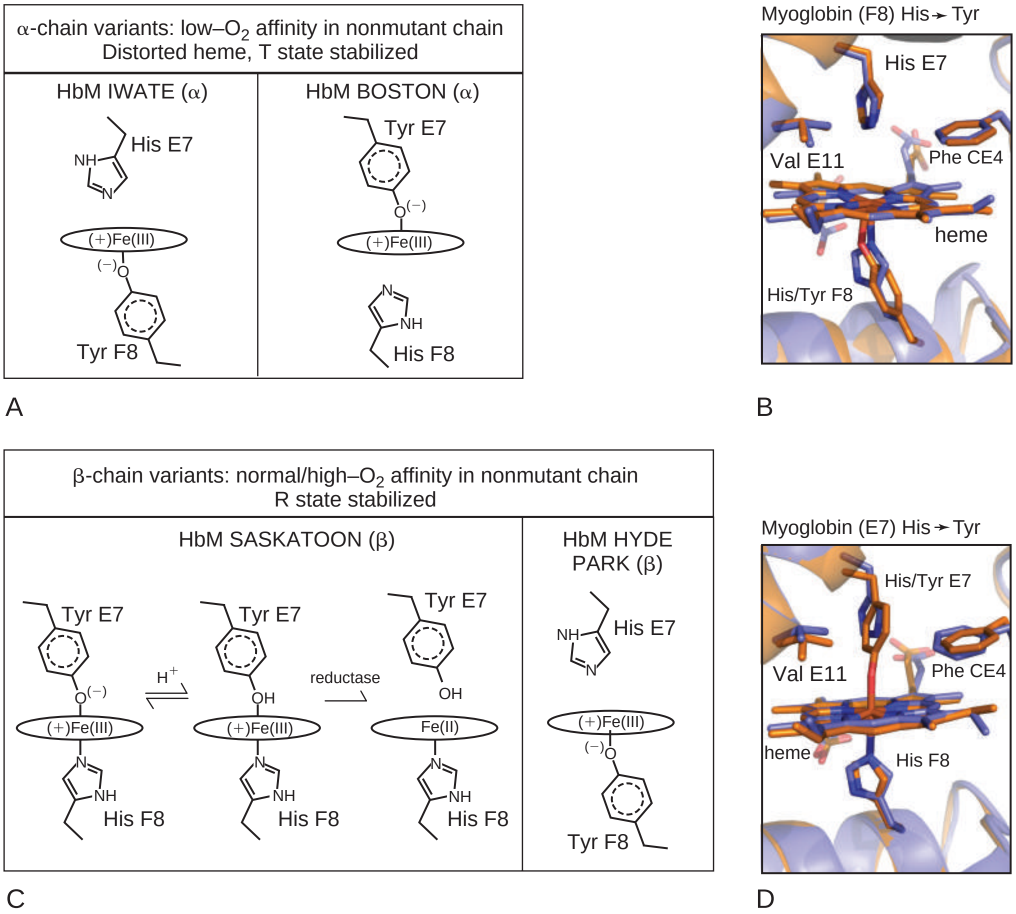
α-chain variants: low–O_2 affinity in nonmutant chain
Distorted heme, T state stabilized
HbM IWATE (α)
His E7
NH
N
(+)Fe(III)
(−)O
Tyr F8
HbM BOSTON (α)
Tyr E7
O(−)
(+)Fe(III)
N
NH
His F8
A
Myoglobin (F8) His→Tyr
His E7
Val E11
Phe CE4
heme
His/Tyr F8
B
β-chain variants: normal/high–O_2 affinity in nonmutant chain
R state stabilized
HbM SASKATOON (β)
Tyr E7
O(−)
(+)Fe(III)
NH
His F8
H+
Tyr E7
OH
(+)Fe(III)
His F8
reductase
Tyr E7
OH
Fe(II)
His F8
HbM HYDE PARK (β)
His E7
(+)Fe(III)
(−)O
Tyr F8
C
Myoglobin (E7) His→Tyr
His/Tyr E7
Val E11
Phe CE4
heme
His F8
D

Figure 19-22 Examples of M-type Hbs. **A**, Hb M variants with His-to-Tyr substitutions in the α chain. In HbM Iwate, the proximal His is replaced by Tyr, which is deprotonated and favors Fe(III) oxidation. In Hb M Boston, the distal His is replaced by Tyr, which coordinates bound O_2 and leads to disruption of the Fe–His F8 bond in the T-state conformation. These ferric α subunits are not reduced by met-Hb reductase. **B**, The heme pocket of wild-type Mb *(blue)* and an F8 (proximal) His-to-Tyr mutant (*orange*, PDB 1HRM), which serves as a model for HbM Iwate. The mutation causes displacement of helix F away from the heme moiety. **C**, HbM variants with His-to-Tyr substitutions in the β chain. In Hb Saskatoon (distal His→Tyr substitution) the hexacoordinate ferric iron can be reduced by met-Hb reductase, possibly owing to a transient protonation of Tyr E7. HbM Hyde Park replaces proximal His F8 with Tyr, resulting in heme loss and instability. **D**, Substituting the distal His E7 side chain in Mb for a larger Tyr E7 (*orange*, PDB 1MGN), as occurs in Hb Saskatoon, brings the Tyr hydroxyl group within binding distance of the iron, forming an hexacoordinate iron. Mb is used as a model for Hb subunits in **B** and **D**, whereas panels **A** and **C** are based on spectroscopy of mutant Hbs.[412,413,466]

met-Hb form of the mutant β subunit is markedly stabilized.

Weak Heme Attachment. Heme loss and consequent instability contribute to the pathophysiology of Hb M Hyde Park (β92 [F8] His→Tyr; see Fig. 19-22, *C*).[422-424] X-ray crystallography of deoxy-Hb Hyde Park showed 20% to 30% heme loss with consequent restructuring of nearby residues.[270] Electrophoresis detected a minor component (5%) of the hemolysate with altered mobility, which contained heme-deficient β Hyde Park chains.[425] Interestingly, although Hb Auckland (α97 [F8] His→Asn) mutates the same residue as HbM Hyde Park, the nontyrosine substitution causes accelerated heme loss, instability, and a mild compensated hemolytic anemia, but not methemoglobinemia because the ferric form of the mutant rapidly loses heme and denatures.[426]

Oxygen Binding Properties of M Hemoglobins. The two-state model for cooperativity predicts that the Hb tetramer will bind 2-3 molecules of O_2 in the low-affinity T (deoxy) state and then switch to the R (oxy) conformation (see Allostery and the Perutz–Monod, Wyman, Changeux Model of Hemoglobin Function). This facilitates cooperativity by increasing the O_2 affinity of the remaining deoxygenated globins. In M Hbs, the stabilized ferric heme groups in the mutant subunit cannot become oxygenated, but there are still two normal heme groups in the native subunits within any HbM tetramer. Interestingly the physiologic properties of HbM tetramers with His substitutions in α globin (HbM Boston and HbM Iwate) and β globin (HbM Saskatoon and HbM Hyde Park) differ. [413,424] The normal α subunits in HbM Saskatoon have increased O_2 affinity, and tetrameric HbM Saskatoon and HbM Hyde Park display significant cooperativity and Bohr effect when these two normal α chains are oxygenated.[424,427] In contrast, the normal β subunits in HbM α-globin variants have low–O_2 affinity, reduced cooperativity, and decreased Bohr effect.[424,428] HbM Iwate and HbM Boston remain in the low-affinity T state

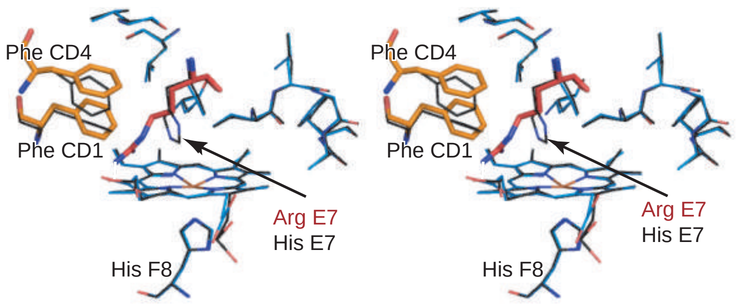

Figure 19-23 Hb Zurich is a variant with an altered heme pocket. Hb Zurich (β63 [E7] His→Arg). Stereo diagram model of the deoxy-βHb Zurich heme pocket *(blue)* overlaid with the wild-type βHb heme pocket (*black*, PDB 2DN2). This model, based on the structures solved by Phillips et al[323] and Tucker et al,[324] was generated with the macromolecular modeling program Coot[467] by mutating the distal His of deoxy-β (PDB 2DN2) to Arg. The distal Arg E7 *(red)* is oriented toward the CD corner, disturbing the position of Phe CD1 and Phe CD4 *(orange)*. The heme pocket entrance of Hb Zurich is much wider than normal, allowing increased access to ligands. However, unlike normal His E7, the mutant Arg is unable to stabilize bound O_2 via hydrogen bonding. *(From Thom CS, et al: Hemoglobin variants: biochemical properties and clinical correlates.* Cold Spring Harb Perspect Med *3:a011858, 2013.)*

even when the two normal β hemes are liganded, probably because of disruption of the α His (F8)–Fe(III) bond.[271,412,413,428] Moreover, these variant α subunits show extraordinary resistance to enzymatic or chemical reduction. The exact biochemical mechanisms responsible for all of these observed differences between for α and β M Hbs are not yet completely understood.

Hemoglobin Variants that Cause Multiple Biochemical Alterations

Not surprisingly, amino acid substitutions within critical regions of globin proteins can produce multiple effects (see Table 19-8). For example, HbE (β26 [B8] Glu→Lys), a common variant in Southeast Asia, contains an amino acid substitution that renders β chains mildly unstable in vitro with minimal clinical significance.[429-431] However, this mutation also creates an alternate splice site in the β-globin mRNA, leading to reduced synthesis of productive transcripts with resultant thalassemia.[432] HbE is particularly deleterious when co-inherited with a more severe β-thalassemic allele, a common occurrence in Southeast Asia.

Mutations that alter the heme pocket can also produce multiple biochemical effects. For example, the ability of HbM Hyde Park to generate an M-type and unstable Hb was discussed in the previous section. Other examples include Hb Bruxelles (β42 [CD1] Phe→0, deletion),[433,434] Hb Warsaw (β42 [CD1] Phe→Val),[435] Hb Hammersmith (β42 [CD1] Phe→Ser),[436] and Hb Buccuresti-Louisville (β42 [CD1] Phe→Leu),[363,364] which all cause both congenital Heinz body hemolytic anemia and cyanosis.

The latter effects are the result of severely reduced cooperativity, rapid rates of autoxidation with heme loss, and consequent unfolding of these unstable Hb variants.[434] After His F8, the Phe residue at the CD1 helical region is the most conserved residue in all known globins, from microorganisms to plants and animals, and its presence in a sequence is often used to define a globin fold.[423] The aromatic side chain is in direct contact with the heme group and helps orient the distal histidine and bound O_2. Thus its replacement with a smaller or polar amino acid markedly destabilizes the affected globin, altering its resistance to heme loss and also its O_2 affinity.

Hb Zurich (β63 [E7] His→Arg) is another interesting heme pocket variant.[230,323,324,437-440] In this variant, the distal His is replaced by Arg. Its large, highly polar side chain swings out of the distal heme pocket, and the positively charged guanidino group forms a salt bridge with a heme propionate (Fig. 19-23). This outward conformation enlarges the ligand binding pocket, destabilizes O_2 binding, and causes iron autoxidation through loss of stabilizing hydrogen bonds with the bound ligand as well as exposure to water (see Autoxidation). Affected individuals exhibit increased sensitivity to oxidants, including sulfa drugs, which more easily enter the enlarged heme pocket. Loss of the distal histidine markedly decreases O_2 affinity but has little effect on CO binding. As a result, individuals with Hb Zurich tend to have supranormal levels of HbCO, which protects the Hb molecule from oxidation and subsequent denaturation. Affected subjects who are cigarette smokers accumulate even higher levels of HbCO, which ironically tends to protect against hemolysis by inhibiting oxidation, heme loss, and unfolding of the mutant subunit. Thus "the pathology of a mutant protein is ameliorated by a normally toxic pollutant."[5]

Hb Köln (β98 [FG5] Val→Met), another heme pocket variant, is the most commonly diagnosed unstable Hb variant in the West.[7,441] This mutation disrupts the interactions between the proximal histidine and iron in the heme pocket, leading to enhanced heme dissociation.[442,443] Hb Köln is present at 10% to 25% of normal levels and has also been noted to have high–O_2 affinity. Affected individuals exhibit reticulocytosis, splenomegaly, and pigmenturia.[231] Of note, splenectomy is relatively contraindicated in Hb Köln, because erythrocytosis and thromboembolic complications, including priapism, have been linked to this procedure in affected patients.[276,444] Clusters of aggregates containing denatured Hb Köln and Band 3 have been shown to be immunogenic,[445] which may serve as a mechanism for rapid splenic clearance.[304,446]

Hb Bristol-Alesha (β67 [E11] Val→Met)[447,448] and HbF Toms River (γ67 [E11] Val→Met),[242] which contain the same amino acid substitution in β and γ chains respectively, represent interesting globin variants with multiple biochemical defects. Hb Bristol-Alesha was initially observed in patients with moderately severe hemolytic anemia. Structural analyses of the mutant protein in patient erythrocytes revealed a β67 Val-to-Asp substitution, predicted to render the protein unstable by introducing a highly charged polar residue into the hydrophobic heme pocket. However, subsequent DNA analysis of the same patient identified a Val-to-Met codon substitution.[448] The authors concluded that the mutant Met residue was converted to Asp posttranslationally, probably through oxidative mechanisms. More recently, the analogous variant was identified in fetal (γ) Hb (HbF Toms River).[242] The affected patient was a newborn with both cyanosis and anemia. DNA testing revealed the codon change (Val→Met at E11). Mass spectrometry of patient hemolysate indicated a mixture of variant γ globins containing either Met or Asp at position E11. Biochemical studies suggested that the HbF Toms River Met substitution produced a relatively stable variant γ globin that takes up O_2 too slowly to become saturated in the lungs, causing cyanosis. Its gradual posttranslational conversion to Asp destabilizes the molecule, causing denaturation and hemolytic anemia. This variant provides an example of how posttranslational modifications of variant globins can modify phenotypes. The reason that Hb Bristol-Alesha causes predominantly hemolytic anemia but HbF Toms River causes mainly cyanosis probably reflects different rates of Met conversion to Asp in the variant globin chains.

One recently identified example illustrates how multiple biochemical defects within a single molecule can produce unique phenotypes. Hb Jamaica Plain (β6 [A3] Glu→Val and β68 [E12] Leu→Phe) contains two defects in the same β chain: a β6 Glu-to-Val substitution that causes sickle cell anemia (see Chapter 20) and a β68 amino acid substitution that alters electrophoretic mobility and reduces O_2 affinity, probably by destabilizing the oxygenated conformation through steric effects introduced into helix E.[449] The affected patient, who was heterozygous for the mutant allele, exhibited symptoms of sickle cell anemia that were precipitated by infection or airplane travel. Thus an amino acid substitution that reduces O_2 affinity exacerbates the effects of a sickling mutation in the same globin chain.

Hemoglobin Variants that Modulate the Sickle Cell Anemia Phenotype

Several Hb variants modulate the severity of sickle cell anemia when co-inherited with HbS (see Table 19-8, see also Chapter 20). For example, γ globin inhibits polymerization of HbS.[450] This effect is attributable to differences in several amino acid residues compared with the corresponding β chain, including γ80 and γ87.[450-452] HbD Ibadan (β87 [F3] Thr→Lys), which introduces a lysine residue at the β87 position, is presumed to have decreased interaction with the mutant Val residue at HbS β6.[450,453] Thus HbD Ibadan inhibits HbS polymerization. Hb Korle-Bu (β73 [E17] Asp→Asn) was initially identified in an asymptomatic individual through its altered electrophoretic mobility.[454-456] The variant HbC Harlem is a double mutant that harbors the Hb Korle-Bu mutation and HbS on the same β chain.[457] Interestingly, purified HbC Harlem forms polymers less readily than HbS, suggesting that the Hb Korle-Bu mutation (β73 [E17] Asp→Asn) inhibits sickling.[457-459] Further biochemical studies showed that this mutation interferes with lateral contacts between HbS fibers by inhibiting abnormal interactions created by the β6 Val mutation of HbS.[460,461]

In contrast, Hb Quebec-Chori (β87 [F3] Thr→Ile) was identified in a compound heterozygous patient with mild to moderately severe sickle cell anemia.[462] The introduction of an isoleucine at β87 causes Hb Quebec-Chori to promote deoxygenated HbS polymerization.

CONCLUSION

The extent and level of sophistication to which normal Hb and its variants are understood is truly remarkable. Studies of these molecules over more than 50 years have elucidated paradigms related to protein and gene structure, allosteric regulation, heme/iron chemistry and the genetic basis of inherited disease. More than 1000 Hb variants are identified.[12,13] With the exception of HbS and HbC, Hb variants are rare individually, although relatively common collectively. These variants alter Hb function and structure in interesting ways, causing clinical manifestations that include cyanosis, erythrocytosis, and hemolytic anemia. Associated morbidities range from severe to minimal, although they tend toward the latter. However, symptoms caused by Hb variants, particularly cyanosis and polycythemia, are often mistaken for more common and serious disorders. Recognizing Hb variants in these circumstances can provide reassurance to patients and spare them from potential complications associated with unnecessary diagnostic procedures and therapies. Of equal importance, identification and characterization of new Hb variants continues to occur, illustrating new features of erythrocyte biology, structural insights to guide the design of Hb-based blood substitutes, and general biochemical principles that lead to improved understanding of many medically relevant proteins.

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CHAPTER 20

Sickle Cell Disease

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CHAPTER OUTLINE

PATHOPHYSIOLOGY
Hemoglobin S Mutation
Hemoglobin S Polymer
Effects on the Erythrocyte Membrane
External Membrane Interactions
Hemolysis and Nitric Oxide Depletion
Acute and Chronic Inflammation
Genetic Modifiers
DIAGNOSIS
Fetus
Newborns
Older Children
CLINICAL MANIFESTATIONS
Acute Sickle Cell Crises
Infection
Chronic Organ Damage
MORTALITY
APPROACHES TO THERAPIES
Routine Health Maintenance
Painful Crisis Management
Reduction in Polymer Formation
Transfusion
Hematopoietic Stem Cell Transplantation
Gene Therapy
Preparation for Surgery
OTHER SICKLE SYNDROMES
Sickle Cell Trait ($\alpha_2\beta_2$, $\alpha_2\beta_2^{6\text{ Val}}$)
Hemoglobin SC Disease ($\alpha_2\beta_2^{6\text{ Val}}$, $\alpha_2\beta_2^{6\text{ Lys}}$)
Hemoglobin S β-Thalassemia
Hemoglobin SO_{Arab} ($\alpha_2\beta_2^{6\text{ Val}}$, $\alpha_2\beta_2^{121\text{ Lys}}$)
Hemoglobin SD ($\alpha_2\beta_2^{6\text{ Val}}$, $\alpha_2\beta_2^{121\text{ Glu}}$)
Hemoglobin S Korle Bu ($\alpha_2\beta_2^{6\text{ Val}}$, $\alpha_2\beta_2^{73\text{ Asn}}$)
Hemoglobin C_{Harlem} ($\text{C}_{\text{Georgetown}}$) ($\alpha_2\beta_2^{6\text{ Val,73 Asn}}$)
Hemoglobin S Antilles ($\alpha_2\beta_2^{6\text{ Val,23 Ile}}$)
Hemoglobin S Québec-CHORI ($\alpha_2\beta_2^{6\text{ Val}}$, $\alpha_2\beta_2^{87\text{ Ile}}$)
Hemoglobin SE ($\alpha_2\beta_2^{6\text{ Val}}$, $\alpha_2\beta_2^{26\text{ Lys}}$)
Hemoglobin C Disease ($\alpha_2\beta_2^{6\text{ Lys}}$, $\alpha_2\beta_2^{6\text{ Lys}}$)

The first report of sickle cell anemia (SCA) in the medical literature described a dental student from Grenada, Walter Clement Noel. It was by Herrick and Irons in 1910 and introduced the word *sickle* in the title of the case report.[1] Sydenstricked described the first cases in children, recognized the association with hemolytic anemia, and introduced the term *crisis* to describe periodic acute episodes of pain.[2] The pathologic basis as a disorder of hemoglobin (Hb) was defined in 1927,[3] confirmed 2 decades later by electrophoresis that separated sickle Hb from normal Hb.[4] Neel[5] defined the genetics of the disorder and clearly distinguished sickle cell trait—the heterozygous condition (AS)—from SCA, the homozygous state (SS).

Further understanding of the molecular basis of the disorder was made possible by the finding that normal human Hb is composed of two pairs of globin subunits. Beyond the embryonic period, one globin pair is invariant (α chain), and the other pair is variable (γ, δ, or β chain). The amino acid substitution in sickle cell Hb is now known to be valine for glutamic acid in the number 6 position of the β chain (β^S). Figure 20-1 provides an overview of pathophysiology of β^S mutation).

In this chapter, *sickle cell disease (SCD)* refers to the broad group of disorders that feature erythrocyte sickling under physiologic conditions. SCD genotypes include homozygous HbSS and compound heterozygous conditions including HbS β^0-thalassemia, HbS β^+-thalassemia, and HbSC disease. HbSS, the most prevalent genotype, and HbS β^0-thalassemia are clinically similar and therefore are commonly referred to as *SCA*; these genotypes are associated with the most severe clinical manifestations. Persons with sickle cell trait (HbAS), the carrier state, rarely have clinical problems and are not considered to have a form of SCD.

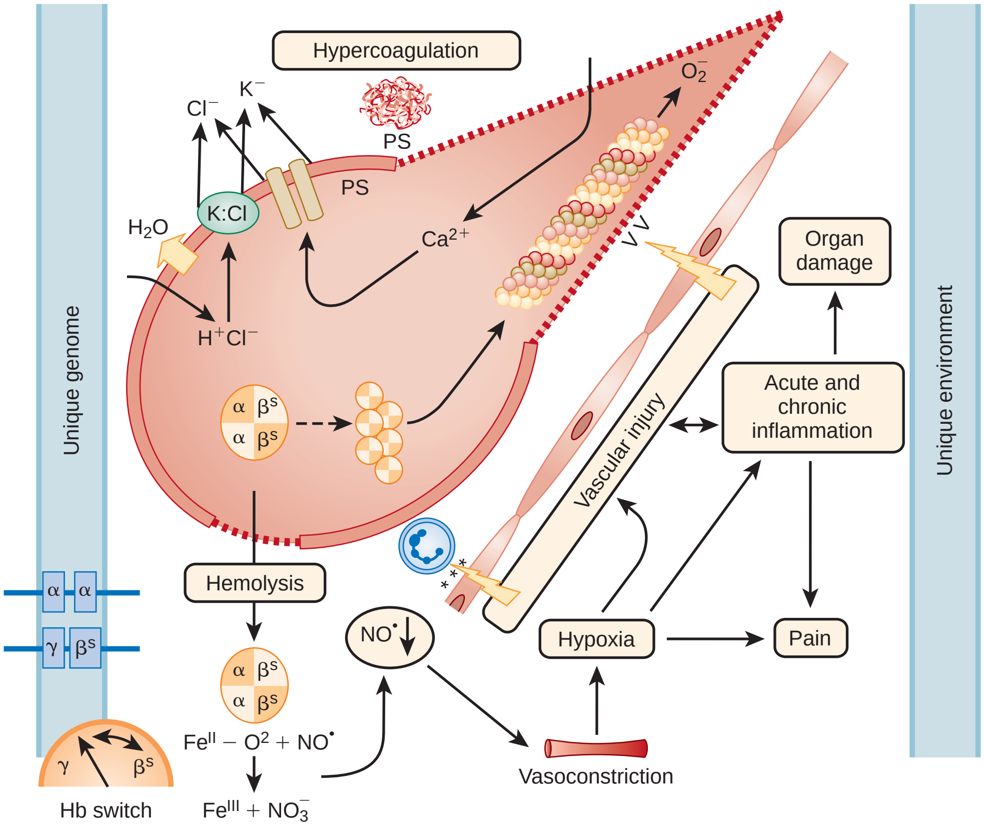

Figure 20-1 The pathophysiology of sickle cell disease. The β^S mutation results in an abnormal β globin with valine substitution for glutamic acid at the sixth position. The hydrophobic valine is exposed when the HbS tetramer assumes the deoxygenated confirmation, leading to polymerization of HbS tetramers and increased red cell deformation/rigidity. The instability of the HbS polymer leads to oxidative damage (O_2^-) of the red cell membrane. Damaged red cell membrane exposes phosphatidyl serine (PS) on the outer surface of the cell, which activates the coagulation system and leads to activation of coagulation. Membrane damage also leads to increased Ca^{2+} influx that activates the Gardos channel, leading to K^+ and Cl^- efflux. To compensate for the K^+ and Cl^- loss, water leaves the cell and it becomes more acidic/dehydrated. Acidosis activates the K^+,Cl^- cotransporter, leading to further efflux of K^+ and Cl^- and more dehydration and intracellular acidosis. Some intravascular hemolysis caused by red cell membrane damage leads to free Hb in the plasma that consumes nitric oxide (NO). Depletion of plasma NO leads to vascular endothelial dysfunction and vasoconstriction, which along with increased red blood cell (RBC) rigidity and increased adherence of sickle RBC to activated vascular endothelium leads to decreased blood flow, hypoxia, and further damage to the endothelial cells. Endothelial damage *(lightning bolts)* leads to expression of adherent proteins on the surface of the endothelial cells *(∗∗∗, VV)*, thus increasing adherence of white blood cells and RBCs to the vessel wall. Hypoxia and reperfusion leads to acute and chronic inflammation vascular injury and ultimately results in organ damage. Each patient has a unique environment (e.g., exposure to infections), and unique genetic makeup (e.g., fetal Hb expression) influences the disease phenotype, thereby making the clinical severity extremely variable.

PATHOPHYSIOLOGY

Hemoglobin S Mutation

The sickle gene mutation is common in sub-Saharan Africa and other parts of the world with endemic malaria. The allele frequency of the β^S mutation closely tracks the distribution of malaria worldwide.[6] Persistence and expansion of such a presumably deleterious mutation is explained by the benefits conferred in the heterozygous state; persons with SCT have a survival advantage from malaria.[7]

Polymorphisms that flank the β^S mutation indicate that this mutation developed independently and spontaneously at least five times.[8,9] Within sub-Saharan Africa, there are four major sickle haplotypes, each associated with a particular geographic region: Senegal, Benin, Central African Republic (CAR), and Cameroon.[10] Among North American patients of African heritage with sickle cell, 50% to 70% of chromosomes are Benin, 15% to 30% are Bantu-CAR, and 5% to 15% are Senegal.[11,12] Benin and Senegal haplotypes are associated with higher levels of fetal Hb (HbF) and fewer dense cells compared with CAR, but most African Americans are compound heterozygotes. As a result, these haplotypes have only limited predictive value for overall clinical severity, with CAR being the most severe and the Senegal variety being the least severe.[13]

A different haplotype is found in India and eastern parts of Saudi Arabia and is known as the Arab-Indian haplotype. Patients from these regions have long been recognized as having milder disease, particularly in childhood, with elevated levels of HbF.[14,15] However these patients develop chronic organ damage that may reflect, in part, their higher Hb concentrations.

Heterozygotes with HbS, HbC, HbE, α- and β-thalassemia and glucose-6-phosphate deficiency (G6PD), particularly infants, are partially protected from cerebral falciparum malaria relative to the normal population.[16]

The mechanistic basis for this survival advantage from the sickle gene is not fully understood, but as recently reviewed, the effect is multifactorial with impaired parasite growth and development, enhanced clearance of infected cells from the circulation, and altered adherence of damaged erythrocytes to the capillary endothelium.[17]

The Hemoglobin S Polymer

Polymer Structure

In 1927, Hahn and Gillespie showed that red blood cell (RBC) sickling, the change from a biconcave disk to the sickle form, was dependent on deoxygenation.[3] Harris subsequently demonstrated that cellular sickling was associated with the formation of "tactoids" of HbS that appeared as the Hb became deoxygenated.[18] Electron micrographs of sickled red cells reveal long, thin bundles of HbS fibers that run parallel to the long axis of the cell.[19,20] The ultrastructure of HbS fibers, as detailed by electron microscopy and image reconstruction, reveal a complex solid-core structure 21 nm in diameter composed of 14 filaments arranged as seven pairs of double filaments.[21,22]

The detailed crystal structure suggested by Wishner and Love and colleagues[23,24] identified several critical intermolecular contact sites. Contacts along the axis of the filament are made by α and β chains, whereas lateral contacts between filaments of a pair are largely between β chains and contacts between filament pairs are largely through α chains. Within each Hb tetramer one abnormal β chain contributes the β^S mutation, whereas the other contributes a critical receptor region around the Phe β85 residue. In this critical receptor region lie residues where mutants affect fiber formation: β73 (Korle Bu), β66 (I Toulouse), β83 (Pyrgos), and β87 (D Ibadan). Within the acceptor pocket the mutant valine closely contacts four different hydrophobic residues (β70, β73, β84, and β85). In addition to the hydrophobic interaction, water molecules that form hydrophilic interactions in the lateral contact region are present near the mutant valine.[25]

Polymer Formation

The polymerization of deoxygenated HbS (deoxy-HbS) is a highly complex process that results in the formation of gelled, aggregated HbS tetramers in equilibrium with Hb tetramers in solution. Perturbations in oxygen levels,[26] temperature,[27] pH,[28] ionic strength,[29] 2,3-diphosphoglycerate (2,3-DPG),[30] and carbon monoxide affect the formation of HbS gels. This intracellular polymerization of HbS is the sine qua non of the disorder and leads to distorted cell morphology, altered blood viscosity, circulatory sludging with occlusion of blood flow, and ultimately tissue damage and organ infarction that cause clinical manifestations of SCD.

The kinetics of HbS polymerization can be explained by a double nucleation mechanism.[31] Gelation is initiated by a process called *homogeneous nucleation,* in which single deoxy-HbS molecules aggregate. Aggregation of a few molecules is thermodynamically unstable, but once a certain number of molecules aggregate, a condition termed the *critical nucleus,* addition of further molecules produces a more stable aggregate or polymer. Thus homogeneous nucleation is highly dependent on the concentration of deoxy-HbS molecules. Gelation continues with heterogeneous nucleation that occurs on the surface of preexisting polymer. The same intermolecular contacts between the mutant valine and its receptor on the surface of the polymer are responsible for heterogeneous nucleation and cross-linking between strands.[32] While polymerization progresses, more surface area becomes available and therefore the reaction becomes autocatalytic.

The result of this double nucleation mechanism is a measurable delay time between the initiation of polymerization and the exponential rise in polymer formation. In solution, the delay time varies as the thirtieth power of the Hb concentration: $1/t_d = K\ (C/C_s)^n$ where t_d = delay time, C = Hb concentration, C_s = Hb solubility, and $n = 30$ (the number of Hb tetramers in the critical polymer). Because n is so large, very small changes in Hb concentration have a profound effect on the delay time. This phenomenon of delayed gelling of HbS in solution is also observed in erythrocytes containing HbS.[33] The distribution of observed delay times within intact RBCs is consistent with the distribution of the mean corpuscular Hb concentration (MCHC). Slow deoxygenation causes large aligned polymers, which results in significant distortion of cell morphology.[34]

A kinetic model of HbS gelation that incorporates the concept of a critical delay time led Eaton and co-workers to propose that polymerization kinetics play an important role in the pathophysiology of SCA (Fig. 20-2).[35] Erythrocytes exposed to oxygen within the lungs are quickly "degelled" so the arterial circulation contains little polymerized HbS and few sickled cells. But as the cells enter the capillary circulation, the oxygen saturation (SpO_2) decreases rapidly and the HbS solubility also drops. If the transit time within the capillary (analogous to the delay time) is longer than 1 second, the cell will sickle and likely occlude the capillary, whereas a fast transit time means sickling will not take place in the capillary and obstruction will not occur. Patients who become hypoxic, acidotic, dehydrated, and febrile are likely to experience vaso-occlusive episodes caused in part by a shorter delay time. The hypertonic renal medulla and the acidotic, high-hematocrit environment of the spleen make these organs prime targets for sickling.

Relatively little is known about the uniformity of the depolymerization process within erythrocytes, but failure to fully melt the polymer within the lungs may lead to residual polymer.[36-38] Important variables that affect HbS polymer formation (HbF, 2,3-DPG levels, SpO_2, and pH) are heterogeneously distributed among erythrocytes, and these factors (along with membrane abnormalities and red endothelial interactions) likely play a role in the variable clinical severity of SCD.

Interactions of Hemoglobin S with Hemoglobin A and Hemoglobin F

The study of the interaction between HbS and other Hbs supports a rational basis for understanding the clinical manifestations of various sickle syndromes and provides a rationale for therapy. Investigators have extensively studied mixtures of HbS with HbF or HbA to determine

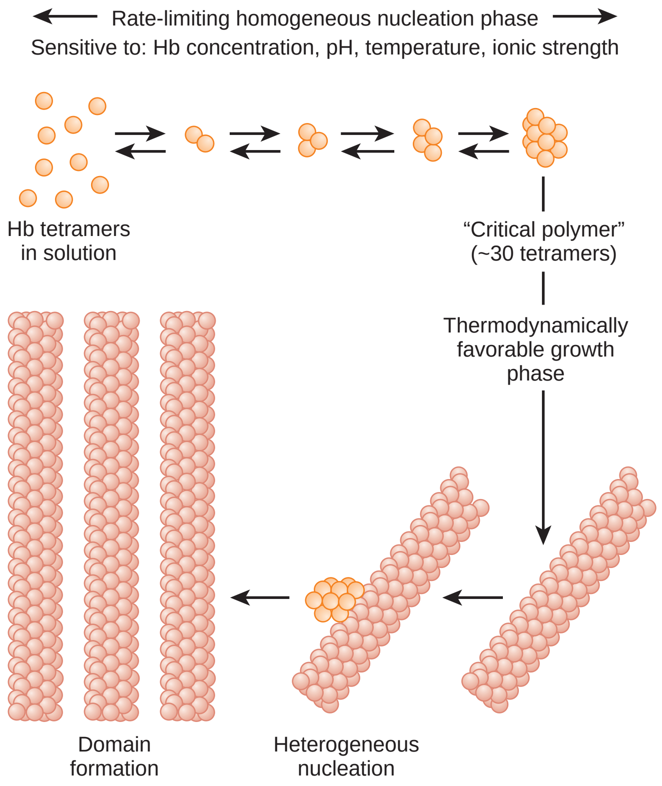
Rate-limiting homogeneous nucleation phase
Sensitive to: Hb concentration, pH, temperature, ionic strength
Hb tetramers in solution
"Critical polymer" (~30 tetramers)
Thermodynamically favorable growth phase
Domain formation
Heterogeneous nucleation

Figure 20-2 Model for the polymerization and alignment of deoxy HbS. *Hb,* Hemoglobin. *(Modified from Hofrichter J, Ross PD, Eaton WA: Super saturation in sickle cell hemoglobin solutions.* Proc Natl Acad Sci U S A *73:3035–3039, 1976; and Ferrone FA, Hofrichter J, Eaton WA: Kinetics of sickle hemoglobin polymerization. II. A double nucleation mechanism.* J Mol Biol *183:611–631, 1985.)*

the effects on gelation[39,40] and solubility.[41] The kinetic data show that both HbA and HbF have a profound, dose-related effect, increasing the delay time and decreasing HbS polymer content within erythrocytes (Fig. 20-3), but the effects of HbF are greater than HbA. Compared with pure HbS solutions, mixtures with 15% to 30% HbA (as found in HbS β^+-thalassemia) have delay times that are up to 100 times longer; mixtures with 20% to 30% HbF (as found in HbS–hereditary persistence of fetal hemoglobin [HPFH]) have delay times that are up to 10^4 times longer, and mixtures with 60% HbA (as found in sickle trait) have delay times that are 10^6 times longer.[39] HbA and HbF also increase the solubility of HbS, with HbF again being more effective than HbA. Very little if any HbF is incorporated into the polymer, suggesting that asymmetric hybrids of HbS and HbF ($\alpha_2\beta^S\gamma$) are not incorporated into polymer under most physiologic conditions,[41] whereas asymmetric hybrids of HbS and HbA ($\alpha_2\beta^S\beta^A$) are readily incorporated into the HbS polymer.

Effects on the Erythrocyte Membrane

The basic pathophysiology of SCA is directly related to HbS polymerization. However effects of polymerization on the erythrocyte membrane, which is in direct contact with this intracellular process, alter the pathophysiology of the disease. For example when the damaged membrane breaks, then intravascular hemolysis will ensue. When the altered membrane interacts with other circulating blood cells, soluble plasma factors, the vascular endothelium, and the reticuloendothelial system (RES), a wide range of additional events can lead to the highly variable but often severe phenotypic expression.

Mechanisms of Membrane Damage

In normal erythrocytes, HbA binds to the membrane around band 3 protein[42,43] and also to phospholipids on the inner surface of the membrane.[44] Hb at physiologic concentrations also stabilizes the configuration of spectrin heterodimers.[45] HbS binds to membranes more readily than HbA[46] and membrane-associated sickle Hb is a major determinant of erythrocyte rigidity.[47] Membrane rigidity has been associated with a small amount of high–molecular-weight spectrin-Hb complex in the membrane.[48]

HbS readily denatures[49] and forms small aggregates, so-called *micro-Heinz bodies* that attach with high

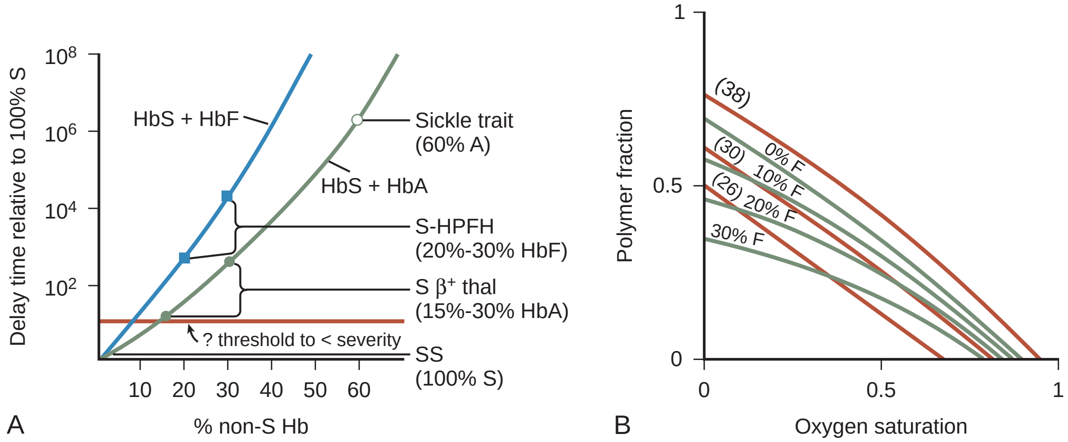

Figure 20-3 **A**, Effect of increase in concentrations of HbF and HbA on delay time of polymerization of HbS. HbS polymer formation. **B**, HbS polymer formation as a function of Spo$_2$ for different levels of HbF and for different concentrations of pure HbS at concentrations of 26, 30, and 38 g/dL. *S-HPFH,* HbS and hereditary persistence of HbF; *thal,* thalassemia. *(**A**, From Sunshine HR, Hofrichter J, Eaton WA: Requirement for therapeutic inhibition for sickle hemoglobin gelation.* Nature *275:238–240, 1978.* ***B****, From Noguchi DT, Torchia DA, Schechter AN: Intracellular polymerization of sickle hemoglobin: effects of cell heterogeneity.* J Clin Invest *72:846–852, 1983.)*

affinity to the cytoplasmic portion of band 3 protein at the HbA binding site.[50] Both band 3 protein and glycophorin are clustered above these micro-Heinz bodies,[51] and clustering of band 3 protein is associated with the deposition of specific IgG antibodies onto the cell,[50,52] which are linked to normal cell senescence and may contribute to shorten the circulating half-life of HbS erythrocytes.[53] Ankyrin is also abnormally clustered around the denatured HbS, and like band 3 protein, may be damaged in the process.[51]

Membrane damage also occurs when HbS autooxidizes to form methemoglobin (met-Hb), thereby generating superoxide and losing heme.[54] Intracellular iron from different compartments (e.g., denatured Hb, free heme, hemichromes, and nonheme iron) can bind to the inner membrane and serves as a catalyst for production of highly reactive hydroxyl radicals that cause oxidative damage to the membrane.[55] Erythrocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was recently identified as a source of reactive oxygen species (ROS) that contribute to erythrocyte rigidity and fragility in SCA.[56] Therapies directed toward ROS may therefore be beneficial in SCA by reducing oxidative stress.[57]

Membrane Deformability

While sickle erythrocytes become deoxygenated and fill with polymerized HbS they become less deformable.[58,59] The viscoelastic properties of sickled erythrocytes can be measured by ektacytometry, which allows investigation of the deformability of HbSS erythrocytes in aggregate and has shown that decreased deformability is directly related to the amount of intracellular Hb concentration.[60] Using micropipette techniques to measure membrane deformability of individual cells, two studies have shown that the mean corpuscular hemoglobin concentration (MCHC) is the major contributor to cell rigidity, although cellular dehydration independently increases membrane rigidity.[61,62]

Cell rigidity with lack of deformability is further evidence that a structural membrane lesion is present. Most erythrocytes undergo repeated HbS polymerization/depolymerization that leads to repeated shape change from the characteristic sickled form to the normal biconcave disc, and thus represent reversibly sickled cells (RSCs). However some erythrocytes retain the sickled conformation even upon reoxygenation; the cytoskeleton of such irreversibly sickled cells (ISCs) has a variety of abnormalities including aberrant cross linking of actin,[63] dissociation of spectrin tetramers,[64] and clustering of band 3 protein and glycophorin.[65] Disordered mobility of the cytoskeleton relative to the overlying membrane may affect protein–protein interactions including binding of normal spectrin to sickle ankyrin.[66] With proteomic techniques four groups of proteins are increased in sickle erythrocyte membranes: actin accessory proteins, components of lipid rafts, scavengers of oxygen radicals, and protein repair participants.[67] All of these observations support the hypothesis that membrane rigidity is the result of cytoskeletal damage associated with HbS polymer formation and oxidative damage.

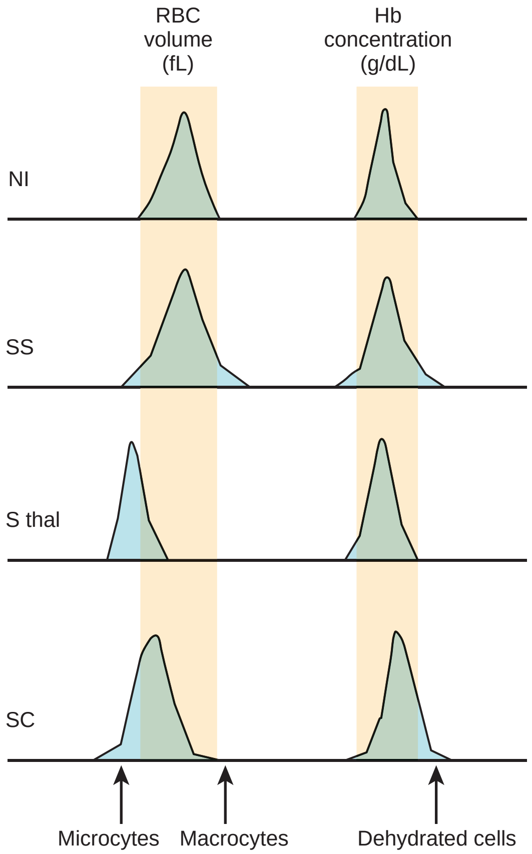

Figure 20-4 Typical distribution patterns of corpuscular volume and Hb concentration in different sickle syndromes. Note that in HbSS disease, the cells fall below and above both the volume and concentration normal *(shaded)* range. The large (microcytic) cells are the low-density reticulocytes. The dehydrated population is enriched in ISCs and low HbF. In HbS β-thalassemia syndromes, the entire volume curve is shifted toward the left, and few dense cells are seen. In HbSC disease, the microcytes are often spherocytic, very dehydrated cells. *Hb*, Hemoglobin; *Nl*, normal; *RBC*, red blood cell; *SC*, HbSC disease; *SS*, HbSS disease; *S thal*, HbS β thalassemia.

Hemoglobin Concentration

Patients with SCA have a wide variation in their erythrocyte density, including many light cells (reticulocytes) and more dense cells (MCHC >37 g/dL) than normal erythrocytes. This is reflected in the histograms of mean corpuscular volume (MCV) and Hb from the sickle genotypes (Fig. 20-4). Sickle erythrocytes separated by density (and therefore MCHC) obstruct flow in an artificially perfused capillary system in proportion to their intracellular Hb concentration.[68] However there appears to be no correlation between the percentage of dense cells and the frequency or onset of vaso-occlusive events.[69] A newly described in vitro system with microfluidics and endothelialized channels[70] may represent an improved method to study the rheological characteristics of sickled erythrocytes and will aid the investigation of pathologic consequences of ISC and dense cells as they interact with vascular endothelium.

The heterogeneity of red-cell shape and density in SCA is influenced significantly by the distribution of HbF within the erythrocytes. For unexplained reasons, HbF is not evenly distributed among erythrocytes (both in normal persons and patients with SCA) and defines a subpopulation known as F cells.[71] Because HbF helps prevent HbS polymerization, F cells sickle less often and thus survive longer in the circulation.[72] The densest cell fraction in SCA contains the oldest erythrocytes that contain the lowest amounts of HbF and include the ISC fraction.[73]

Erythrocyte Shape Changes

RSCs have normal shape and normal viscosity when oxygenated. The vaso-occlusive complications of SCD may be because of the fact that these cells are able to circulate into the microvasculature because of their normal rheologic properties when oxygenated but then become distorted and more viscous as they become deoxygenated within the small capillaries. In contrast ISCs are slender, elongated cells with blunt ends that are visible even on an oxygenated peripheral blood smear (Fig. 20-5). These cells are extremely dense with MCHC of greater than 44 g/dL, have little HbF to dilute their concentrated HbS, and survive in the circulation for only a few days.[73] ISCs can be formed in vitro by prolonged deoxygenation[74] and by repeated oxygenation–deoxygenation cycles. Reversible oxidative damage to β-actin and loss of ubiquitination of α-spectrin leads to formation of an ISC membrane skeleton that is "locked" because it cannot disassemble or reassemble.[75] Antioxidants can reduce the formation of ISCs in vitro.[76] ISC numbers vary widely from patient to patient and have been correlated with hemolysis and splenomegaly[77] but only poorly with vaso-occlusive severity.

Ion Transport and Cell Volume

Abnormal cation permeability of the sickle erythrocyte is characterized by calcium loading, potassium depletion, acidosis, and dehydration, all of which enhances the tendency for HbS polymerization (see Fig. 20-1). HbS polymer formation upon deoxygenation causes a reversible potassium loss and sodium gain in the cell.[78] Abnormal water and cation movements across the cell membrane have been associated with a variety of pumps, channels, and leaks involving potassium (K^+), sodium (Na+), chloride (Cl^-), calcium (Ca^{2+}), and magnesium (Mg^{2+}). Although the pathogenesis of these pathways remains

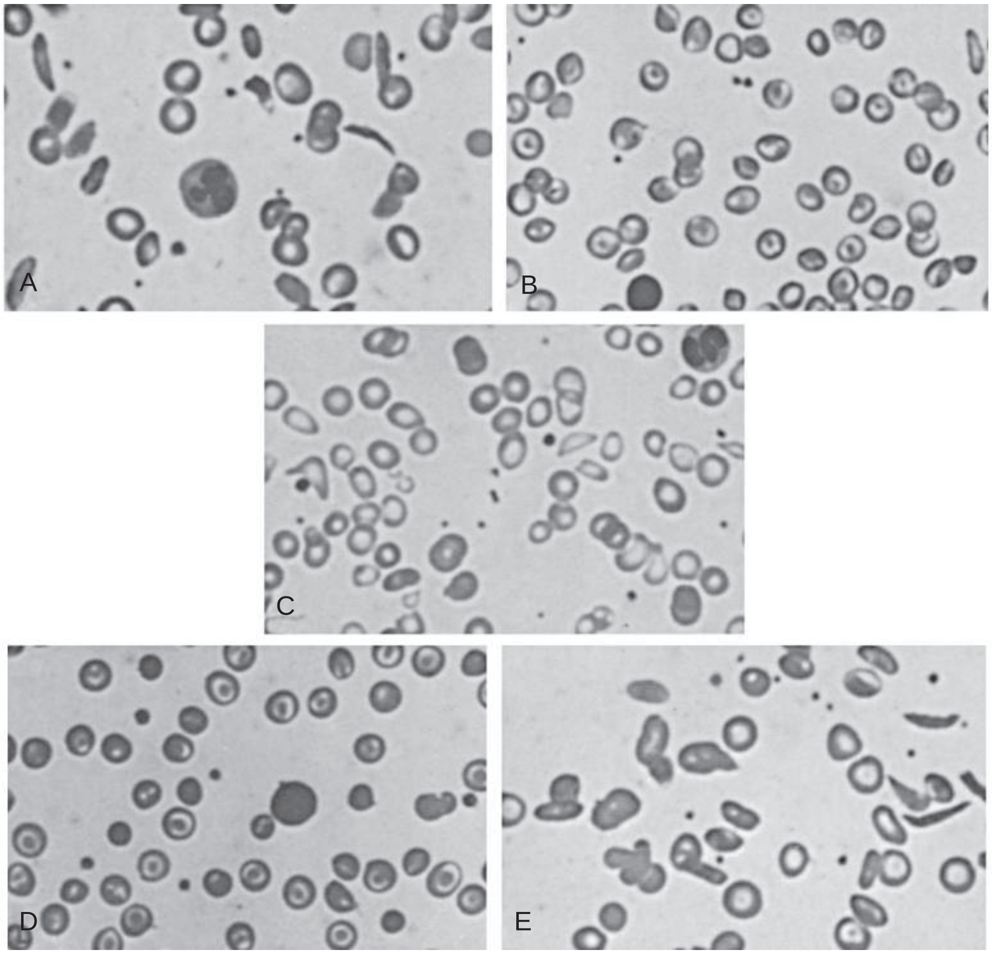

Figure 20-5 Oxygenated peripheral blood smears from individuals with SCD (**A**), HbSC disease (**B**), HbS β^0 thalassemia (**C**), homozygous HbC disease (**D**), and HbSD disease (**E**).

incompletely understood, they are undoubtedly related to the physical distortion associated with sickling,[61,62,78-85] oxidant damage,[86] and the Hb mutation itself.[87]

At least two major erythrocyte membrane channels are responsible for cellular dehydration: the calcium-activated potassium-selective process (Gardos channel, encoded by the *KCNN4* gene) and the K^+Cl^- cotransporter (encoded by *KCC* with multiple isoforms). These channels are critical for cation flux and water regulation in the sickle erythrocytes, and thus influence Hb concentration and cell volume. Sickle reticulocytes exposed to deoxygenation and HbS polymerization accumulate sufficient Ca^{2+} to activate the Gardos channel,[88] which results in osmotic-driven water loss and rapid RBC dehydration to a much greater extent than with other transporters. The Gardos channel can be inhibited by the antimycotic agent clotrimazole, and a short-term study demonstrated benefits on cellular hydration.[89] A novel Gardos channel inhibitor (senicapoc, ICA-17043) entered into formal clinical trials, and although having substantial effects on cellular hydration,[90] a definitive phase III double-blinded placebo-controlled trial demonstrated no efficacy for reducing vaso-occlusive events.[91]

A second pathway involved in RBC dehydration is the potassium chloride channel (KCC). The KCC is active in reticulocytes as part of normal volume reduction during cellular maturation but has increased activity in sickle reticulocytes with deleterious consequences. The KCC pathway is stimulated to lose K^+, Cl^-, and water when predominantly young sickle erythrocytes are exposed to a low pH environment, as occurs in areas of poor perfusion.[92] The KCC pathway is also activated by deoxygentation of Hb[76,93]and by low levels of Mg^{2+}.[94] Genes for four KCC isoforms have been identified; *KCC1*, *3*, and *4* isoforms are present in normal and sickle erythrocytes, but the latter may have N-terminal splicing variants of these isoforms.[95] The KCC pathway can be inhibited by magnesium in vivo,[94] and Mg^{2+} is depleted in sickle erythrocytes possibly through an abnormal Na–Mg exchange.[96] Preliminary studies demonstrated the effectiveness of oral magnesium in the prevention of cellular dehydration,[97] and a pilot trial suggested utility for intravenous (IV) magnesium in the setting of acute vaso-occlusive pain.[98]

External Membrane Interactions

Increased Erythrocyte Adhesion to the Endothelium

In 1979 investigators first discovered that sickle erythrocytes have an abnormal propensity to adhere to vascular endothelial cells with an adhesive force that results from numerous attachment sites that withstand detaching forces found in low shear vascular beds.[99-101] While cells flow through vessels of critical dimension, abnormal flow caused by endothelial interactions increases the odds that polymerization and obstruction will occur (see Fig. 20-1). Support for this hypothesis comes from studies that suggest a correlation between adhesiveness and clinical severity.[102]

A variety of surface adhesion molecules on sickle erythrocytes are involved in this abnormal endothelial interaction. Sickle reticulocytes are especially prone to adhesion, having recently exited the marrow (where adhesion is required during erythroid maturation) and without any splenic filtrative circulation to allow “grooming” of the sticky young erythrocytes. Sickle erythrocytes bind to both the endothelial cells and to subendothelial extracellular matrix proteins such as laminin, thrombospondin, and fibronectin, which can become exposed after adhesion. Sickled cells have the highest affinity for laminin because of two isoforms of the protein that bear the Lutheran blood group antigens (B-CAM and LU).[103,104] Adhesion proteins to thrombospondin on reticulocytes include CD47 and VLA-4 ($\alpha_4\beta_1$ integrin, which also bind to VCAM-1), whereas CD44 is the adhesion protein that binds to fibronectin.

Endothelial cells have different surface molecules depending on their location. For example, CD36 is expressed on microvascular but not large-vessel endothelium.[105] Endothelial cells also express differing amounts of surface molecules and become more or less adherent depending on environmental stimuli. Cytokines such as interleukin-18 and tumor necrosis factor[106] promote endothelial adherence, largely because of up-regulated VCAM-1 expression. In the setting of infection and inflammation, endothelial cells also modify their adhesion molecule profile.

Circulating endothelial cells can be identified in patients with SCA, and they exhibit an activated phenotype including surface expression of ICAM-1, VCAM-1, E-selectin, P-selectin, and tissue factor.[107] Increased production of endothelial derived adhesion molecules (VCAM-1, ICAM-1, and E-selectin) was observed in 10 adult patients during acute SC pain crises.[108] Soluble levels of VCAM-1, ICAM-1, and E-selectin were elevated and independently associated with organ damage, pulmonary hypertension (PHT), and mortality.[109]

Membrane Lipid Orientation and Coagulation Defects

Normally phospholipids of the erythrocyte membrane are partitioned with amino phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) located on the inner cytoplasmic surface, whereas sphingomyelin and phosphatidyl choline (PC) are exposed on the outer surface (see Fig. 20-1). This asymmetry is not unique to the red cell and probably reflects a general structural pattern that keeps the amino phospholipids from activating soluble coagulation factors. In RSC, PS and PE flip back and forth from the inner leaflet to the outer leaflet during oxygenation and deoxygenation. Exposure of PS or PE on the outer surface during sickling may lead to increased phagocytosis by PS-binding proteins secreted by macrophages[110] and may bind to specific PS receptors on endothelial cells.[111] Alteration of the fatty acyl groups in PC results in changes in cell shape and deformability in SS cells, suggesting that the species composition of PC can affect membrane permeability and cellular deformability.[112]

Abnormal lipid asymmetry accelerates clotting in vitro, and PS-positive sickle erythrocytes are correlated with markers of thrombin generation (prothrombin fragment F1.2, thrombin–antithrombin complexes) and fibrin degradation (D-dimers and plasmin–antiplasmin complex).[113]

Other markers of thrombophilia observed in patients with SCA are reduction in proteins C and S,[114] platelet activation, and microparticles from endothelial cells, platelets, monocytes, and red cells that promote coagulation and may increase during crises.[115]

Procoagulant and anticoagulant proteins as well as platelets play an important role in sickle erythrocyte adhesion to the endothelium. With sickle erythrocytes already having increased adhesiveness, the additional effects of increased levels of von Willebrand factor,[116] activated platelets,[117] and thrombospondin[118,119] further increase RBC adhesiveness and make SCA a legitimate hypercoagulable state. Investigation with anticoagulants and antiplatelet agents are just now beginning, and may have a role in the prevention or amelioration of vaso-occlusive events.

Hemolysis and Nitric Oxide Depletion

The erythrocyte life span is shortened in SCA to approximately 10 to 20 days. The premature destruction of sickle erythrocytes includes both extravascular clearance by the RES (two thirds contribution) and intravascular processes (one third contribution). Extravascular hemolysis is enhanced by externalized PS exposure on the erythrocytes, which is recognized by macrophages with PS-specific receptors.[110] The membrane changes found on sickle erythrocytes are similar to those seen in nucleated cells undergoing apoptosis (externalization of PS, annexin binding to and blebbing of the membrane, and cell shrinkage). Intravascular hemolysis is probably occurring at a steady-state (with 4 μM of plasma Hb)[120] but with exacerbations caused by infectious or immunologic etiologies that lead to the concept of hyperhemolysis. In this setting, the massive release of Hb into the plasma overwhelms the normal binding and clearance mechanisms of haptoglobin and hemopexin and may lead to pathologic outcomes.

Nitric oxide (NO) is a critically important molecule that regulates vascular tone and functions as a cell-signaling molecule. NO is produced by a specific synthetase found in endothelial cells (endothelial nitric oxide synthase [eNOS]) that use L-arginine in an NADPH-dependent enzymatic reaction. NO increases intracellular cyclic guanosine monophosphate (GMP), which decreases smooth-muscle calcium concentrations, leading to muscle relaxation, vasodilation, and increased regional blood flow. NO also suppresses platelet aggregation, secretion of procoagulants, and reduces expression of endothelial cell adhesion molecules.[121] Patients with SCA have elevated levels of plasma-free Hb, which consumes NO with resulting functional consequences of an "NO-depletion" state.[122,123] Intravascular hemolysis has been postulated to cause a wide variety of clinical events in patients with SCA such as PHT, priapism, leg ulcers, stroke, and renal insufficiency,[124,125] although the validity of this hypothesis has been recently called into question.[126,127] Newer evidence suggests that an entirely different pathway may lead to PHT, one that involves marrow hyperplasia and high levels of circulating placental growth factor, which leads to endothelin-1 release and pulmonary vasoconstriction.[128]

Acute and Chronic Inflammation

The HbS mutation and the pathophysiology of repeated sickling with erythrocyte–endothelium interactions leads to an acute and chronic inflammatory state with widespread endothelial dysfunction and vasculopathy. Markers of inflammation include increased levels of leukocytes, neutrophils, platelets, C-reactive protein (CRP), von Willebrand factor, and fibrinogen, all increased at steady state and during crises.[129,130] Increased adhesion of sickle erythrocytes, reticulocytes, neutrophils, and platelets to vascular endothelium can initiate local inflammatory responses that alter the expression of endothelial surface molecules and lead to release of soluble forms into the plasma. Hypoxia, increased proinflammatory cytokines, and infections can activate vascular endothelium and leukocytes, which in turn can further increase adhesion molecules on both circulating cell surfaces and on endothelial cell surfaces[130] (see Fig. 20-1). Over time, the endothelium becomes damaged enough that a chronic vasculopathy develops, causing clinical complications even without any acute sickling or vaso-occlusive events.

Circulating leukocytes and in particular neutrophils are also involved in the pathophysiology of SCA. Baseline white blood cell (WBC) and neutrophil counts in patients with SCA are elevated in the steady state, usually to twice the normal value. The degree of elevated WBCs is directly related to early mortality[131] and increased acute chest syndrome (ACS)[132] and has been suggested as a predictor of clinical severity in affected infants.[133] Activated neutrophils are common in the steady state and also during vaso-occlusive crises, and they have enhanced adhesion to endothelium during vaso-occlusive events.[134] In a transgenic sickle mouse model, neutrophils are involved in reperfusion inflammation after hypoxia, but the process is abrogated by blocking adherence with an antibody to P-selectin,[135] which has led to therapeutic efforts in humans that target the selectins as a means to reduce vaso-occlusive pain. E-selectin has been identified as a crucial portal for invasive pneumococcal disease in a mouse model of SCA, and its down modulation by hydroxyurea can prevent fatal infection.[136]

Recently two additional inflammatory pathways have been identified that have relevance for the pathophysiology and clinical manifestations of SCD. The first is the invariant natural killer T-lymphocyte (iNKT) cell, which when activated can mediate pulmonary inflammation and has been associated with the development of ACS.[137] The adenosine A2A receptor (A2AR) agonist regadenoson reduces iNKT-cell activation and can decrease inflammation in sickle cell mice,[138] and this agent is currently in human clinical trials. The second pathway is through mast cell activation, which promotes neurogenic inflammation and nociceptor activation in a murine model; specific inhibition with imatinib reduced hyperalgesia and suggests that pharmacologic manipulation of mast cells may influence inflammation and vaso-occlusive pain.[139] General reduction of inflammation using statins may improve endothelial function as well. A pilot study demonstrated that simvastatin was safe, well-tolerated, and had benefits on markers of inflammation suggesting a potential therapeutic role.[140]

Genetic Modifiers

In some ways SCA can be considered a simple disease, at least from the standpoint of pathogenesis, because the precise genetic mutation is known, the selection pressure for the origin and propagation of the sickle mutation is understood, the genetics of inheritance and transmission are straightforward, and the expression of the abnormal β^S allele is predictable and deleterious. Despite this designation as a simple monogenic disorder, however, SCA is highly complex because of its phenotypic variability. Patients have a wide range of laboratory findings, clinical disease expression, and severity outcome that presumably reflect additional genetic and environmental influences.

Given the clarity of the pathogenetic basis for SCA, it is surprising how difficult the search for genetic modifiers of SCA has been. Perhaps because of differences in the definition or measurement of certain phenotypes, or because there are additional confounders, the identification of genetic modifiers for common laboratory and clinical events in SCA has been mostly fruitless, with a few notable exceptions. Even common mutations that originated in Africa with the same survival advantage against malaria do not have a predictable effect. For example G6PD deficiency is a common single-gene variant that also protects against malaria. In the United States, the prevalence of the A variant is about 10% to 13% among males, and there is no increased incidence of G6PD deficiency in SCD.[141] Within the Cooperative Study of Sickle Cell Disease (CSSCD), the presence of G6PD deficiency in 801 males was not associated with more hemolysis or increased anemic episodes.[142] Presumably no effects were observed because this mild variant of G6PD deficiency typically becomes manifest only in older erythrocytes, and the erythrocyte lifespan observed in SCA is very short. More recently G6PD deficiency has been associated with a risk of cerebral vasculopathy among children with SCA,[143] although this association has not been verified by other investigators.

α-Thalassemia trait is another common finding among patients with SCA, with a prevalence of 30% to 40% in the United States and exceeding 50% in some parts of the world. The most common form of α-thalassemia trait is caused by a 3.7-kb deletion involving the entire α_2 gene.[144] Additional deletional and nondeletional forms of α-thalassemia are found but are not as common. The primary effect of concomitant α-thalassemia is to limit the supply of α globins and thus further favor the competition of nonsickle β globin (γ globin) in the intracellular struggle for available α globins.[145,146] This lowers the intracellular HbS content and thus prolongs the delay time for HbS polymerization. The resulting erythrocytes are more deformable because they have higher membrane area to volume ration; hence they have less potassium loss and cellular dehydration and a lower MCHC.[147] Patients with SCA and α-thalassemia tend to have smaller, lighter cells with a higher circulating Hb concentration and lower rate of hemolysis.[147,148] Indeed α-thalassemia trait combined with SCA has been associated with a lower risk of cerebral vasculopathy and stroke, a finding recently confirmed in a large cohort of children with SCA.[149] However this combination does not necessarily translate into a better clinical outcome, because α-thalassemia has been associated with more vaso-occlusive pain,[150] renal infarction, and avascular necrosis (AVN) of the femoral head.[151] This may be the result of increased hematocrit and concomitant increase in viscosity in certain organs.

The baseline HbF levels in patients with SCA are highly variable, ranging from very low values (<5%) to very high values (>30%). Because HbF is known to ameliorate the phenotypic expression by reducing HbS polymerization, the origin of this marked variability has been of great interest. In the CSSCD any increment of HbF above 4% was associated with reduced painful events,[152] and HbF levels greater than 8.6% were associated with decreased mortality.[131]

Three broad categories of genetic mutations have been described that increase HbF postnatally. The first category includes large deletions of the γδβ-globin gene locus region on chromosome 11, which are rare but result either in δβ-thalassemia or pancellular hereditary persistence of fetal Hb (HPFH) as described in Chapter 21. Patients who inherit HbS on one allele and a large pancellular HPFH deletion on the other have a condition that does not sickle under physiologic conditions. Patients with HbS/HPFH represent a unique syndrome with 20% to 35% HbF levels overall and distributed in all red cells, which results in normal Hb concentration, mild microcytosis, no sickled erythrocytes on the peripheral blood smear, and no clinical disease manifestations.[153]

A second category of mutations that increase HbF in patients with SCA is linked to the β-globin gene region but does not feature large deletions of the gene locus and so has historically been termed *nondeletional HPFH*.[154,155] HbF is usually distributed heterogeneously in these disorders, but because the fraction of F cells has a tight but nonlinear association with the %HbF,[156] some of these patients approach a pancellular distribution. Despite the linkage to the β^S mutation, a search within the promoter region of the γ-globin genes typically does not identify any specific mutations.[157] The only nondeletional mutation clearly associated with an increased %HbF is the −158C to T polymorphism (rs7482644, also called the *XmnI polymorphism* because of the creation of a restriction enzyme cutting site) found in the $^G\gamma$ promoter, which is found in the Senegal and Arab-Indian (Saudi) β-globin haplotypes.[14,15] These haplotypes are found commonly in genetic isolates in Western Africa, in Shiite Saudi Arabians, and in the Orissa region of India.[158,159] Among patients in the United States with SCA, fewer than 10% have one chromosome with either of these haplotypes and fewer than 2% are homozygous for the Senegal haplotype.[11,12]

The third category of genetic mutations that influence HbF levels in patients with SCA are those not linked to the β-globin region. Some patients have HbF levels ranging from very low (<3%) to much higher levels (15% to 20%); the latter are sometimes referred to as patients with HbSS/high HbF to distinguish them from HPFH variants. Many nonglobin genetic loci regulating HbF have been postulated and pursued including an elusive X-linked locus,[160] but discovery of the *BCL11A* gene as a potent regulator of %HbF levels has vindicated this pursuit and provides mechanistic insights into HbF regulation and even therapeutic opportunities for patients with thalassemia and SCA. The BCL11A locus on chromosome 2p15 was first identified as a modifier of HbF levels using genome wide-association studies[161,162] and subsequently this locus has been shown to have the greatest genetic influence on baseline HbF levels identified to date. The BCL11A protein is now recognized as a major biologic repressor of γ-globin gene expression,[163] and silencing its expression can yield "de-repression" with high HbF levels and correction of the sickle phenotype in a murine model.[164] In humans at least four single nucleotide polymorphisms affect *BCL11A* expression and thus HbF levels.

An additional gene locus regulating HbF levels on chromosome 6 has been pinpointed to the intergenic region of *HBS1L-MYB* at 6q23.[165] Several noncoding polymorphisms within this region have been associated with higher HbF levels, but to date, no specific mechanisms have been elucidated for this genetic locus, although changes in *MYB* expression seem to be important.[166] It is important to note that all genetic modifiers of HbF have not been identified; together, genetic variants within the *BCL11A*, *HBS1L-MYB*, and β-globin loci account for only 30% of the phenotypic variability of endogenous HbF expression and 50% of the heritable variability.[167] Additional genetic regulators of HbF will be identified, but their effects will likely be smaller and their prevalence will be much lower.

Additional genetic modifiers that influence specific clinical phenotypes have long been sought for patients with SCA, including stroke, pain, ACS, leg ulcers, nephropathy, and AVN, among others. It is likely that networks of genes that respond to or modulate hemolysis, vasoregulation, inflammation, cell adhesion, hemostasis, and oxidative stress play roles in these phenotypes (see Fig. 20-1). Unfortunately most of these efforts have been hampered by the lack of accurate and rigorous phenotypes, small sample sizes, high false-discovery rates, and absence of validation cohorts. Moreover studies that investigate candidate genes are inherently limited to those genes, so unknown genes or networks can only be identified using unbiased "agnostic" approaches. Finally discovering an association between a genetic variant and a phenotype does not necessarily mean causality, and functional studies are needed to confirm the biologic significance and effect of any specific genetic association. The importance of a validation cohort is illustrated by the report that 90% of previously published genetic polymorphisms associated with stroke were not associated with stroke in a new cohort of children with documented stroke phenotype.[149] At this time few genetic variants have been found that reliably influence any specific phenotype in patients with SCA except the UGT1A1 promoter polymorphism,[168] which is also potent in the normal population for bilirubin levels. With improved gene-sequencing techniques including whole exome analysis, it is likely that new genetic variants will be identified that influence clinical phenotypes and even treatment responses to hydroxyurea.[169]

DIAGNOSIS

Fetus

The ability to perform prenatal diagnosis for SCD has progressed significantly since 1978 when Kan and Dozy[170] described the DNA polymorphisms around the β-globin gene that were in linkage disequilibrium with the β^S gene. Techniques for identification of the A-to-T substitution in codon 6 responsible for the glutamic acid-to-valine change in β globin have evolved from the use of restriction enzymes and homologous synthetic oligonucleotides to polymerase chain reaction–based amplification of DNA and sequencing.[171-174] Sufficient DNA can be obtained from a small number of fetal cells obtained by amniocentesis or as early as 8 to 10 weeks' gestation by chorionic villous biopsy.[175] The desire to avoid the small risk of fetal loss associated with these invasive techniques has led to investigation of noninvasive prenatal diagnosis by testing fetal cells or free fetal DNA known to circulate in the plasma of pregnant women.[176] Preimplantation genetic diagnosis (PGD) is now an established reproductive alternative to prenatal diagnosis in many specialized centers. Single-cell polymerase-chain reaction diagnosis of blastomeres followed by selection of unaffected embryos for standard in vitro fertilization has led to the birth of unaffected twins to a couple both with sickle trait.[177] PGD has been used to facilitate selection of an unaffected histocompatible sibling donor for hematopoietic stem-cell transplantation of SCD.[178]

Newborns

The newborn with SCD is generally not anemic or symptomatic until toward the end of the first year of life because of the protective effects of fetal Hb. Screening combined with comprehensive follow-up care was first begun by Pearson in New Haven and Serjeant in Jamaica in the 1970s.[179,180] Because recognition of the disease in a newborn can lead to prevention of mortality and morbidity, the National Institutes of Health (NIH) recommends that all infants be screened in the neonatal period for SCD,[181] and as of 2007 all 50 states and the District of Columbia perform universal screening for SCD.[182] The method used for initial screening varies from state to state with most using isoelectric focusing (IEF) or high-performance liquid chromatography (HPLC) as their primary screening method. These tests can be performed on cord blood or on dried blood specimen blotted on filter paper. False negative screens using these methods have been reported in infants who received perinatal transfusions before screening.[183] Diagnosis can also be performed using polymerase-chain reaction amplification of deoxyribonucleic acid (DNA) extracted from filter paper.[184] Universal screening compared with targeted screening of newborns of parents at risk has been shown to identify more infants with disease, prevent more deaths; and is cost effective in areas where sickle trait is present in 7 to 15 of 1000 births.[185,186] Considerations of logistics and equity have contributed to the decision for

TABLE 20-1 Hematology of Infants with SS Disease, Sickle Cell Disease, and S β+-Thalassemia

		Age (mo)										
	Percentile	2-3	4-5	6-8	9-11	12-14	15-17	18-23	24-29	30-35	36-47	48-60
SS Disease												
Hemoglobin level (g/dL)	5	7.0	7.0	7.1	7.2	7.2	7.2	7.1	6.9	6.7	6.4	6.6
	50	9.3	9.2	9.2	9.2	9.1	9.0	8.9	8.6	8.3	8.1	8.3
	95	11.4	11.3	11.4	11.5	11.5	11.5	11.3	11.1	10.9	10.5	10.4
Mean corpuscular volume (fL)	5	72	69	68	67	67	67	67	68	69	71	72
	50	84	81						95	96	94	94
	95											
Fetal hemoglobin level (%)	5	14.6	12.3	10.8	9.1	7.8	6.7	5.6	4.8	4.5	4.4	3.3
	50	43.5	34.1	29.1	24.3	20.6	17.7	14.8	12.8	12.4	12.4	9.0
	95	68.5	59.0	53.0	47.3	42.7	39.1	35.3	32.5	31.2	29.6	21.9
Reticulocyte count (%)	5	1.0	1.1	1.2	1.3	1.4	1.6	1.9	2.3	2.6	2.7	1.8
	50	4.0	5.1	5.9	6.7	7.4	8.0	8.7	9.3	9.8	10.4	11.8
	95	15.5	17.9	19.4	20.7	21.8	22.5	23.2	23.5	23.6	23.6	25.8
SC Disease												
Hemoglobin level (g/dL)	5	8.0	8.2	8.6	8.9	9.2	9.3	9.5	9.5	9.4	9.3	9.6
	50	9.7	9.8	10.1	10.3	10.5	10.6	10.7	10.8	10.7	10.6	10.6
	95	11.6	11.5	11.7	11.8	12.0	12.0	12.1	12.2	12.2	12.1	11.9
Mean corpuscular volume (fL)	5	68	65	64	64	63	63	63	63	64	66	69
	50	81	78	77	75	74	74	74	74	76	77	77
	95	91	88	86	85	84	84	84	84	86	88	87
Fetal hemoglobin level (%)	5	13.6	2.9	2.9	3.1	3.1	2.9	2.4	1.4	0.5	0.0	2.0
	50	31.6	17.9	14.5	11.6	9.3	7.4	5.5	4.2	3.9	4.4	4.2
	95	54.0	39.1	32.1	25.7	20.9	17.6	14.7	13.8	14.7	15.9	8.3
Reticulocyte count (%)	5	0.8	0.8	0.8	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.9
	50	2.8	2.8	2.7	2.6	2.5	2.5	2.5	2.6	2.7	2.9	2.8
	95	8.2	8.8	8.9	9.0	8.9	8.7	8.4	8.0	7.9	8.8	13.4
S β+-Thalassemia												
Hemoglobin level (g/dL)	5	9.2	9.4	9.1	8.5	9.1	9.1	9.9	10.0	9.8	9.3	10.0
	50	10.8	10.9	11.0	10.8	10.6	11.2	10.9	11.0	10.7	10.6	10.8
	95	12.4	12.7	13.5	11.8	14.1	12.0	12.0	13.0	11.3	11.6	11.2
Mean corpuscular volume (fL)	5	70	64	61	61	63	82	63	61	66	64	66
	50	80	73	72	69	72	70	70	70	72	76	68
	95	88	83	84	75	84	73	77	79	76	76	76
Reticulocyte count (%)	5	1.1	0.0	1.1	0.9	0.8	0.9	0.7	1.5	1.2	1.0	3.0
	50	2.6	1.8	2.5	2.5	3.0	2.5	2.4	2.2	3.4	2.2	4.1
	95	8.5	6.4	2.5	4.6	5.9	7.4	5.1	6.2	7.4	7.6	5.7

Data from Brown AK, Sleeper LA, Miller ST et al. Reference values and hematologic changes from birth to five years in patients with sickle cell disease. Arch Pediatr Adolesc Med *148:796–804, 1994.*

universal screening in the United States and the United Kingdom.[187]

Older Children

After the first few months of life, as β^S-globin production increases and HbF declines, the clinical syndrome of SCD emerges (Table 20-1). Although at 1 week of age the Hb level of infants with HbSS is statistically lower than that of normal HbAA infants, the overlap between the two groups is considerable and does not diverge much before the second month of life.[179] Anemia and reticulocytosis is usually evident by 4 months of age.[179] ISCs are often absent from the peripheral blood of young children, and the morphology is typical of that of normal newborns: target cells, fragments, and poikilocytes. By 3 years of age, the typical peripheral blood smear is seen, including ISCs, target cells, spherocytes, fragments, biconcave disks, Howell-Jolly bodies, and nucleated red cells. The amount of HbF decreases with age, as in normal children, but this occurs much more slowly.[179,188]

CLINICAL MANIFESTATIONS

The clinical manifestations of SCD are extremely variable. Some patients are entirely asymptomatic, whereas others suffer often from acute painful episodes. Most patients fall between these extremes and have relatively long asymptomatic periods punctuated by occasional clinical crises. The complex nature of the clinical variability from patient to patient and over time in each

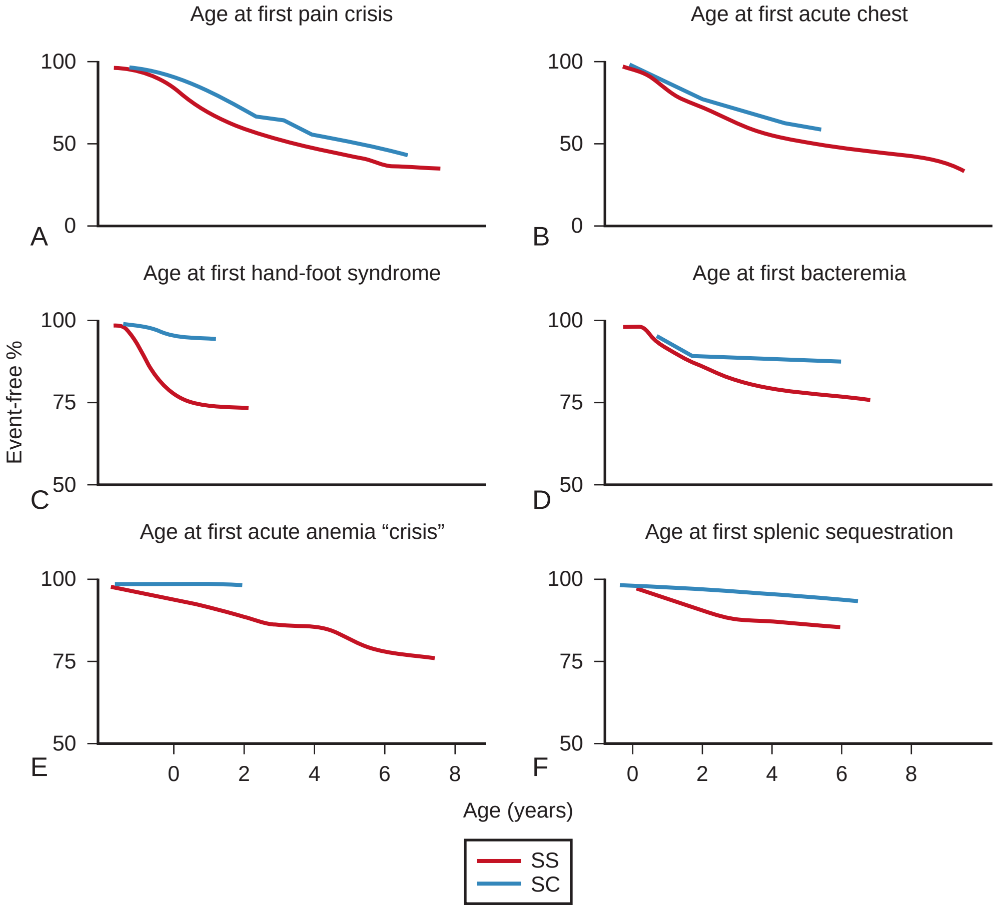

Figure 20-6 Age at first clinical event in patients with SCD, from birth to 10 years of age. **A**, Pain crisis. **B**, Acute chest syndrome. **C**, Hand-foot syndrome. **D**, Bacteremia. **E**, Aplastic crisis. **F**, Splenic sequestration. *(Modified from Gill FM, Sleeper LA, Weiner SJ et al: Clinical events in the first decade in a cohort of infants with sickle cell disease: Cooperative Study of Sickle Cell Diseases.* Blood *86:776–783, 1995.)*

patient has been prospectively studied on a large scale in the CSSCD, under the auspices of the Sickle Cell Disease Branch of the National Heart, Lung, and Blood Institute (NHLBI).[189,190]

Acute Sickle Cell Crises

The term *sickle cell crisis* was defined by Diggs[191] as "any new syndrome that develops rapidly in patients with SCD as a result of the inherited abnormality." There are three categories of sickle crisis: vaso-occlusive, sequestration, and aplastic, and they are covered individually in the next few sections. The best perspective on how these acute events play out in a typical group of children comes from a report by Gill and colleagues describing the experience of almost 700 infants studied for 10 years as part of the CSSCD.[192] The age at first event is displayed in Figure 20-6.

Vaso-occlusive crises are acute, often painful episodes resulting from intravascular sickling and tissue infarction. In a prospective study of children with HbSS disease studied since birth in Jamaica, painful crisis was the first symptom in more than one fourth of the patients and the most common symptom after the age of 2 years.[193] Vaso-occlusive crises (also called *vaso-occlusive episodes* or *events*) are the major clinical manifestations of SCD and occur most commonly in the bones, soft tissues, lungs, liver, spleen, brain, and penis. Sometimes the differential diagnosis is difficult, because there is no definitive objective hallmark of vaso-occlusive crises and they can mimic other pathophysiology.

Acute Painful Crisis

The most common acute vaso-occlusive crisis is pain. Virtually all patients with HbSS disease experience some degree of acute pain. For many these episodes are mild and managed entirely at home, school, or work. Little is known about the extent or nature of pain-coping activities that go on outside the medical setting, but diary studies suggest that they are numerous. The "tip" of the pain "iceberg" is made of those episodes that drive patients to seek medical attention. These episodes vary widely among patients[152] (Fig. 20-7) and represent the most common reason for patients to visit outpatient offices and emergency departments and be admitted for inpatient care. Despite the fact that there is variation in how or why individual patients decide to seek attention for a given episode of pain, epidemiologic evidence strongly indicates that patients with higher rates of medical attention for pain have lower levels of fetal Hb, higher WBC counts, higher steady-state Hbs, and higher

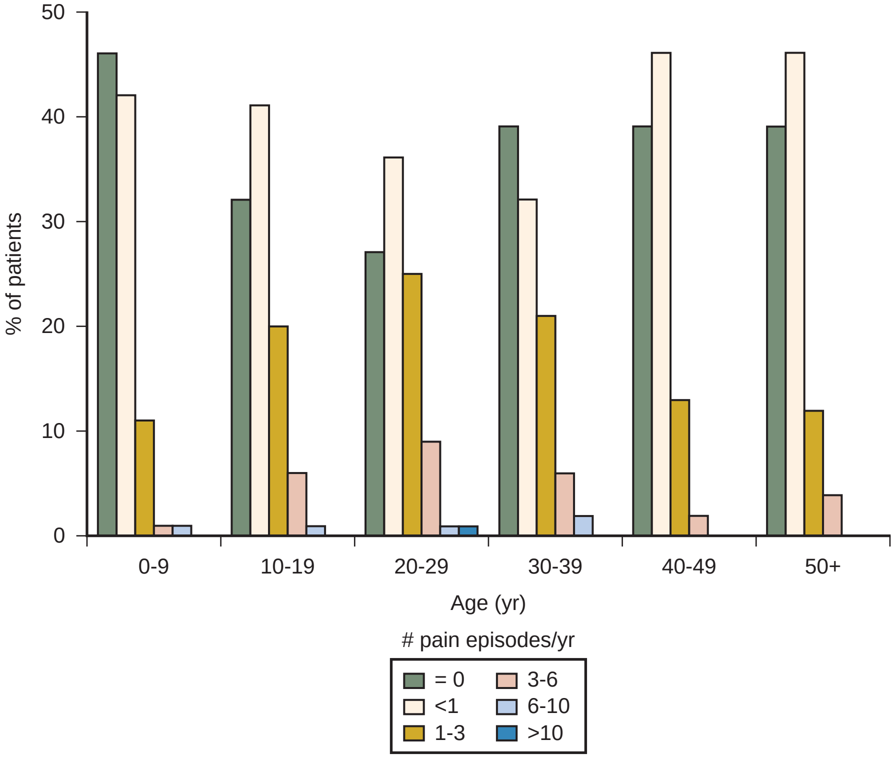

Figure 20-7 Distribution of pain rate among patients with HbSS disease. *(Data from Platt OS, Thorington BD, Brambilla DJ et al: Pain in sickle cell disease: rates and risk factors.* N Engl J Med *325:11–16, 1991.)*

mortality.[131,152] Some studies have suggested that infections, changes in climate,[194,195] and psychological factors[196] may precipitate pain episodes, although commonly no precipitating factors can be identified.

Patients typically experience rapid onset of deep, gnawing, throbbing pain, usually without any abnormal physical or laboratory findings but sometimes accompanied by local tenderness, erythema, warmth, and swelling. The underlying pathology is bone marrow ischemia, sometimes leading to frank infarction with acute inflammatory infiltrate.[197-199] The most commonly involved areas are the lumbosacral spine, knee, shoulder, elbow, and femur. Less often, the sternum, ribs, clavicles, calcaneus, iliac crest, mandible, zygoma, and mandible are involved.[200] Sympathetic joint effusions during acute periarticular episodes are particularly common in the knees and elbows. Typically joint aspiration yields straw-colored fluid, usually with a "noninflammatory" profile. Rarely sterile purulent exudates are found.[201] Given the range of marrow involvement and inflammatory response, it is not surprising that the findings in patients with the most inflammation mimic the findings of osteomyelitis, and those without such findings are at risk of being considered malingerers.

Even in patients with measurable signs of inflammation, the diagnosis of infarction is favored over osteomyelitis. The results of one study suggest that acute long-bone infarction is at least 50 times more common than osteomyelitis.[202] In this study of 41 acute long-bone infarcts, 38% affected the humerus, 23% affected the tibia, and 19% affected the femur. All patients experienced local tenderness, with swelling in 85%, joint findings in 68%, and local heat in 65%. Fourteen percent appeared "toxic," 21% had a temperature greater than 39°C, and 43% had a temperature lower than 38°C. The total WBC count ranged between 7200 and 43,000 cells/mm^3, with a mean leukocytosis of 17,000 cells/mm^3. The mean sedimentation rate was 30.5 mm/h, with a range of 3 to 66 mm/h. Although various radionuclide scans have been suggested to distinguish between infarction and infection,[203-207] in many studies such investigations were inconclusive.[208-210] Magnetic resonance imaging (MRI) of patients with HbSS disease shows decreased intensity of short relaxation time/echo-time pulse-sequence imaging, owing to hyperplastic marrow that converts to high intensity on long relaxation time/echo time images in painful crises,[199] but no definitive series compares infarction to infection. Therefore despite the progress made in the development and use of imaging techniques, a definitive diagnosis of osteomyelitis in SCD still relies more upon clinical assessment together with positive cultures from blood or bone obtained by aspiration or biopsy than upon any single imaging modality.[211]

Except for a positive blood or tissue culture, no laboratory test can differentiate acute infection from painful crisis.[198,212] Needle aspiration and culture of the highly suspicious area is critical for isolating the organism and should be considered before initiating empirical antibiotic therapy. The aspirated fluid may be quite purulent even in the patients with sterile infarcts. In most series, the most common organism causing osteomyelitis is *Salmonella*[213] though *Staphylococcus*, *S. pneumoniae* and Gram-negative enteric bacilli are also common.[214] Initial empiric antibiotic therapy should be chosen to cover these possibilities. Treatment failures are seen when anything but the most aggressive antibiotic regimens are used.

As described earlier, episodes of acute bone pain and impressive signs of inflammation may be difficult to distinguish at outset from osteomyelitis. More common and just as challenging is the evaluation and management of severely painful episodes in patients without obvious signs of inflammation. Some of these patients show laboratory evidence of acute inflammation such as elevated CRP,[215] fibrinolysis such as elevated D-dimers,[216,217] or red-cell trapping such as loss of dense cells.[218] These measurements are not helpful in the management of individual cases, and should not be used as an attempt to "validate" an individual patient's report of symptoms. In a research setting, however, these measurements done on large numbers of patients with and without symptoms provide clues to potential innovative therapeutic interventions. For example the common finding of elevated acute-phase reactants stimulated a trial of methylprednisolone for treating acute bone pain.[219] This preliminary work showed that a short course of high-dose corticosteroid decreased the duration of severe pain; however, it resulted in more "rebound" attacks after treatment was discontinued. Similarly despite the fact that previous trials of aspirin therapy were not encouraging,[220] there is significant interest in the role of platelets,[221,222] soluble procoagulants,[223] anticoagulants,[224,225] and endothelial cells[135] in the precipitation or propagation of vasoocclusion and has reopened these as potential lines of treatment.[226-228]

In children younger than 5 years of age, the small bones of the hands and feet are often affected, even to the distal phalanges, and in contrast to most episodes of bone pain in older children, physical findings are common. This painful dactylitis ("hand-foot syndrome") is typically the first clinical manifestation of SCD. The young child cries with pain; refuses to bear weight; and has puffy, tender, and warm feet, hands, or both. The child may appear acutely ill, be febrile, and have an impressive leukocytosis. At the onset of soft-tissue swelling, there are usually no bony changes on radiographs. After 1 to 2 weeks, subperiosteal new bone, irregular areas of radiolucency, cortical thinning, or complete destruction of bones can be seen. All the bone changes are usually reversible but may persist for as long as 8 months.[229,230] A rare complication, permanent shortening of the digits after hand-foot crisis, has been reported.[231] Dactylitis before age 1 year is a strong predictor of overall severity (stroke, death, high pain rate, or recurrent ACS) by 10 years of age,[133] although recent single-institution evidence suggests dactylitis is not a strong predictor of subsequent pain or ACS.[232] In addition to dactylitis the risk for severe disease is further increased if the child has also experienced an episode in which the Hb dropped below 7 g/dL, and/or the baseline WBC count was elevated.[133]

Acute Chest Syndrome

ACS is an acute illness of lung injury characterized by chest pain, fever, and/or respiratory symptoms, accompanied by a new pulmonary infiltrate on chest radiograph. The diagnostic criteria most commonly include radiographic evidence of a new segmental pulmonary infiltrate and one or more of the following: fever, tachypnea, cough, or new-onset hypoxia; increased work of breathing (intercostal retractions, nasal flaring, accessory muscle use); or chest pain.[233] The term *acute chest syndrome* was introduced by Charache et al[234] and reflects the difficulty of establishing a definitive etiology and emphasizes the fact that in individual patients, knowing the specific etiology is less critical for management than being able to assess the magnitude and pace of the lung injury.

The critical pathophysiology is that only deoxygenated HbS polymerizes and that reoxygenation eradicates the polymer. The lung is the critical organ that protects the arterial side of the circulation from the sludge of sickle polymers. When the lung is injured, underventilated, or inflamed such that a ventilation/perfusion (V/Q) mismatch exists, this protection is inadequate, and downstream tissues including the injured lung itself become increasingly susceptible to sickling and ischemia. In fact, when the flow is eventually restored in a sickle mouse model, reperfusion injury occurs with activation of iNKT cells[137] and p-selectin–inhibitable movement of inflammatory cells into the tissue.[135]

Although some patients, particularly adults, initially can provide a full-blown picture of chest pain, hypoxia, and abnormal x-ray findings, the diagnosis of ACS in young patients can be difficult to make. Often the diagnosis becomes clear only days into an event that started as a fever without source or pain. Children younger than 4 years of age often have few signs at presentation: in one study, 35% to 40% had a normal lung examination; 30% to 40% had no tachypnea; and 30% to 50% had no tachycardia.[235] The radiographic evolution often lags behind the clinical progression. In one series, only 36% of patients had an abnormal x-ray on presentation, although all eventually developed abnormal radiographic findings.[236]

Vichinsky and associates demonstrated that almost half of all patients who developed ACS were initially admitted for other reasons, often with vasoocclusive pain.[233] Opioid analgesics and pain of the spine, ribs, and abdomen can lead to hypoventilation with decreased tidal volume, development of atelectasis, and V/Q mismatch. The subsequent regional hypoxemia results in further intrapulmonary sickling. Secretory phospholipase A2 (sPLA2) and serum CRP have been proposed as possible laboratory predictors of ACS development but not widely adopted.[237-239] Elevated sPLA2 as a trigger for simple transfusion appeared to reduce the risk of developing ACS compared with standard therapy in one small uncontrolled trial,[240] but a larger multicenter feasibility trial was challenged by poor enrollment.[241]

ACS is a leading cause of morbidity and mortality in SCD.[131,242] It represents the second most common acute complication (pain episode is first) with a rate of 12.8

cases per 100 patient-years[132] and the most common condition at the time of death.[131,233] The highest incidence is in children aged 2 to 4 years (25.3 per 100 patient-years in SCA), and lowest in adults (8.8 per 100 patient-years in SCA).[132] Patients who have not experienced an episode of ACS have a longer life expectancy than those who have, and those with a higher level of HbF and lower steady-state WBCs have a lower attack rate for ACS.[132] Induction of fetal Hb with hydroxyurea therapy lowers incidence of ACS in all age groups.[243,244]

Asthma or reactive airways may represent a distinct or overlapping comorbid condition[245] and appears to be a risk factor for vaso-occlusion and for ACS.[246-248] Patients with SCD and asthma were two to four times more likely to develop ACS than those without asthma, had longer hospital stays, and experienced a higher incidence of painful episodes.[249,250] Analysis of the CSSCD cohort revealed that comorbid asthma is a significant risk factor for mortality for patients with SCD[251] and should be treated aggressively.[252]

Vichinsky and colleagues prospectively studied 671 episodes of ACS.[233,253] The most common etiologies were fat/bone-marrow embolus, and bacterial, atypical bacterial (*Chlamydia, Mycoplasma*), viral, or mixed infection. In contrast to patients with infection, those with bone-marrow embolus were more likely to have an associated episode of bone pain, chest pain, abnormal neurologic symptoms, and fall in platelet count.[253] Regardless of etiology ACS is always accompanied by some degree of localized sickling and lung ischemia. There is considerable change in blood counts at the time of presentation in patients with ACS. The Hb concentration drops an average of 0.7 g/dL that likely reflects sludging and intrapulmonary sickling, and WBC counts increase an average of 69%. The mean partial pressure of oxygen (pO_2) was 71 mm Hg with one fifth of the patients having a pO_2 less than 60 mm Hg. Transcutaneous SpO_2 measurements may not be reliable in SCD, and most clinical transcutaneous pulse oximeters overestimate the fractional arterial oxygenated (oxy-)Hb saturation.[254,255] It is unwise, therefore, to place too much reliance on single SpO_2 measurement in isolation.

Several important therapeutic principles emerged from the Vichinsky study.[233,235] First, bronchodilator therapy was remarkably effective in patients with wheezing or pulmonary function tests indicating obstruction. This is consistent with the observation that measurable airway hyperactivity exists in many children with HbSS even without a history of asthma.[256] Second, red-cell transfusion improved oxygenation. Simple transfusion is as effective as exchange transfusion and may indicate that rapid reversal of intrapulmonary sickling is therapeutic. Third, the prominence of *Chlamydia* and *Mycoplasma* underscores the importance of including macrolide antibiotics in the initial broad empiric therapy.

All patients with ACS must be managed in the hospital setting with frequent and close monitoring. Broad spectrum antibiotics (including a macrolide), bronchodilator therapy, frequent incentive spirometry, and simple transfusion represent the mainstay of early intervention. Oxygen therapy should be provided for patients with hypoxia, and exchange transfusion is indicated for worsening hypoxia or clinical deterioration despite simple transfusion. Chest and rib pain often lead to splinting and atelectasis that may exacerbate or even precipitate ACS; therefore analgesic therapy should not be withheld but should be titrated to analgesic effect with careful monitoring and frequent reevaluation. Fluid overload with pulmonary edema/effusion and congestive heart failure can occur, so careful attention to fluid balance is critical. For patients with poor respiratory effort or rising oxygen requirements, the use of noninvasive ventilation such as continuous positive airway pressure (CPAP) or bilevel positive airway pressure (BiPAP) may be useful.[257,258] Progression to respiratory failure is not uncommon, and if the patient is ventilated, bronchoscopy may have both diagnostic and therapeutic benefit. Detection of plastic bronchitis and removal of branching bronchial casts can improve hypoxemia and V/Q mismatch.[259,260] Although most patients with ACS recover uneventfully, the course is unpredictable and can progress to rapid, unexpected deterioration that leads to multiorgan failure and death.

Acute Abdominal Pain

Severe acute abdominal pain is a common presentation that can pose a difficult differential diagnosis. The etiology of abdominal pain in children with SCD is often benign (e.g., secondary to constipation or gastroenteritis) but can reflect mesenteric sickling and vertebral disease with nerve root compression. A true sickle-related abdominal crisis can be accompanied by guarding, tenderness, rebound, fever, and leukocytosis that is indistinguishable from an acute surgical abdomen. Often the patient is the best judge and can state whether the pain is characteristic of a sickle "crisis."

These patients should receive general supportive measures with the omission of high-dose opioid analgesics until the clinical trajectory becomes clear. Patients with severe pain should be given nothing by mouth and followed closely by both medical and surgical personnel. Abdominal films, including upright views, may be helpful in identifying retained stool, air-fluid levels consistent with infection-related hypomotility, or even a perforated viscus. Usually the patient with vaso-occlusive pain remains stable or improves slightly with hydration and mild sedation. In extreme cases in which the clinical situation is deteriorating, emergency surgical exploration may be necessary. Simple or exchange transfusion should be done before surgery if possible.

Acute focal right upper quadrant pain may be a result of acute cholecystitis or intrahepatic sickling.[261,262] Abdominal ultrasonography will indentify cholelithiasis and choledocholithiasis in the hands of a skilled operator. The presence of gallstones and a clinical syndrome characteristic of cholecystitis is common but does not always mean that the symptoms are the result of the stones. However most gallstones in young patients with SCD are symptomatic, and patients benefit from elective cholecystectomy. Patients with acute cholecystitis should have surgery only after the gallbladder has had a chance to "cool down," because perioperative management of emergent surgery can be fraught with complications.[263]

(For further discussion see the Hepatobiliary System section).

On rare occasions there may be intrahepatic sickling and even hepatic sequestration. Intrahepatic sickling can result in acute right upper quadrant pain from acute sickle hepatic crisis, hepatic sequestration, and/or SCD intrahepatic cholestasis. Acute hepatic crisis commonly presents with acute right upper quadrant pain, tender hepatomegaly, nausea, low-grade fever, and jaundice.[262] Transaminases are rarely greater than 300 IU/L and serum bilirubin levels are usually less than 15 mg/dL.[262,264] The crisis usually resolves within 2 weeks with IV hydration and analgesia. Hepatic sequestration also presents with sudden tender hepatomegaly, but in contrast presents with an acute anemia and without cholestasis or transaminitis.[265,266] Treatment is to replace circulating volume with crystalloid or simple transfusion. Transfusion must be undertaken judiciously, because the liver may "release" the endogenous sickle erythrocytes leading to elevated hematocrit consequent problems secondary to hyperviscosity. Exchange transfusion should be considered. SCD intrahepatic cholestasis is a syndrome of right upper quadrant pain, nausea/vomiting, fever, tender hepatomegaly, leukocytosis, and extreme direct hyperbilirubinemia. There is a broad spectrum of severity in intrahepatic cholestasis that can include acute liver failure with bleeding diathesis, encephalopathy, renal failure, and progression to multiorgan failure and is associated with high mortality, particularly in adults.[267-269] These hepatic complications are not to be confused with the "benign hyperbilirubinema" described by Buchanan et al[261] and is characterized by marked conjugated hyperbilirubinemia, only mild transaminitis, and no liver synthetic dysfunction. This entity resolves spontaneously within 2 to 8 weeks and may represent a very mild variant of intrahepatic cholestasis.

Acute Central Nervous System Event

Acute infarction of the brain can result in a devastating stroke, which occurred in approximately 7% to 10% of children with SCA in the era before transcranial Doppler (TCD) screening.[270,271] The incidence is estimated to be 0.7% per year during the first 20 years of life, with the highest rates in children 5 to 10 years of age.[272] Stroke may occur as an isolated event but also appears in the setting of evolving ACS, aplastic crisis with severe anemia, viral illness, painful crisis, priapism, or dehydration.[270,271] The most common underlying lesion is an intracranial arterial stenosis or obstruction, usually in the distal internal carotid, the proximal middle cerebral, or the anterior cerebral arteries.[273-275] Pathologically these vessels whose endothelium has presumably been chronically injured by sickle erythrocytes show hyperplastic intima with proliferation of fibroblasts and smooth muscle.[276] The lumen may be narrowed or completely obliterated by the vascular lesion, suggesting that acute sickling may simply be the "last straw," causing acute infarction in the setting of a chronic vasculopathy. Hemiparesis, focal seizures, gait dysfunction, and speech defects are the most common presenting signs, but persistent cognitive and psychological impairments commonly accompany motor deficits after stroke.

A careful discussion of the evaluation of the child with new neurologic symptoms is provided by Adams.[277] As soon as cerebral ischemia is suspected and the patient stabilized, the best initial diagnostic test is a noncontrast CT scan. However diagnostic imaging should never delay immediate therapeutic intervention. Although CT may not be positive for infarction within the first 6 hours of ischemia, it can rule out hemorrhage or nonischemic etiologies. MRI with diffusion-weighted imaging (DWI) and fluid-attenuated inversion recovery (FLAIR) sequences are highly sensitive and provide better detail of the areas of ischemia within minutes of ischemia and remain abnormal for several days.[278,279,280] The typical findings are infarcts associated with major vessel obstruction or distal obstruction of smaller vessels leading to infarction in the "border zone" area between the anterior cerebral and middle cerebral vessels.[281,282] Assessment of the intracranial and cervical artery vasculature using three-dimensional time-of-flight (TOF) magnetic resonance angiography (MRA) is becoming increasingly useful in the early evaluation of the patient with new symptoms[283] and is nearly as accurate as conventional cerebral angiography.[284]

Patients with suspected acute neurologic deficits should immediately receive IV hydration while blood is being cross-matched, because signs and symptoms of stroke can often be quickly reversed, even with isotonic IV fluids. Simple transfusion to a Hb target of approximately 10 g/dL is also helpful to alleviate acute intracranial sickling and can be accomplished in most clinical settings. In a tertiary hospital the standard approach to treating a patient with acute infarction is immediate exchange transfusion (see "Transfusion" for details). There are no data for the use of recombinant tissue plasminogen activator (tPA) in sickle-associated cerebral ischemia for either children or young adults.

Patients treated with simple or exchange transfusion usually show marked improvement in motor function, although the prognosis is considerably worse for those with multiple infarcts. After the initial transfusions, a chronic maintenance transfusion program with a goal of suppressing HbS to less than 30% reduces the incidence of stroke recurrence to 10% to 15%, representing a substantial reduction in stroke rate compared to no intervention.[274,285-289] Patients whose initial stroke was temporally unrelated to another medical event (e.g., ACS) may be at higher risk for recurrent stroke even while receiving regular transfusions.[290] The appropriate duration of chronic erythrocyte transfusions for secondary stroke prophylaxis has not been firmly established. Two prospective studies documented a high risk of recurrent stroke (60% to 70%) after discontinuation of either a short-term (1 to 2 years) or long-term (5 to 12 years) transfusion regimen.[274,291] Therefore an indefinite transfusion program is currently recommended to prevent secondary stroke. Although clearly beneficial, indefinite maintenance transfusion carries the risks of allosensitization,[292] autoantibody formation,[293] transfusion-borne infections, and the certain progression to iron overload. Bone-marrow transplantation in patients who have had strokes can be curative,[294] although parenchymal brain disease may progress.[295]

Hydroxyurea treatment with increased HbF served as an alternative to chronic transfusion[296] for effectively preventing secondary stroke in a single institution.[297] The subsequent multicenter trial (Stroke With Transfusions Changing to Hydroxyurea study; SWiTCH) compared the efficacy of standard treatment (chronic transfusions and chelation) to alternative treatment (hydroxyurea and phlebotomy) for children with SCA, previous stroke, and iron overload.[298] SWiTCH was terminated early after a scheduled interim analysis indicated statistical futility for reaching the composite primary study endpoint of both recurrent stroke rate and improved iron burden.[299] The alternative treatment arm had an increased stroke recurrence rate compared with the standard treatment arm but within the predicted stroke margin of the study design; however, the iron unloading from serial phlebotomy was not superior to daily deferasirox, so the composite study endpoint was not reached. For the children in SWiTCH who had a recurrent stroke, risk factors included severe cerebral vasculopathy, younger age at first stroke, and history of transient ischemic attack (TIA). The duration of follow-up study may have been too short to demonstrate any differences in cerebral vasculopathy between the transfusion and hydroxyurea arms. Because of the inferior results on SWiTCH's hydroxyurea and phlebotomy arm, chronic transfusion therapy with chelation remains the mainstay of secondary stroke prevention in children with SCA.

Patients who develop severe cerebral vasculopathy (moyamoya syndrome) appear to have a high risk for recurrent stroke despite adequate chronic transfusion. One retrospective evaluation of chronic transfusions for secondary stroke prevention[300] revealed recurrent stroke was more common among patients with moyamoya (11/19 patients, 58%) than without moyamoya (7/25 patients, 28%). Given this increased risk, some advocate for surgical revascularization to restore circulation to the at-risk hemisphere. Although there are no randomized, controlled trials in children with SCA and moyamoya disease to determine the effectiveness of indirect surgical revascularization procedures such as encephaloduroarteriosynangiosis (EDAS)[301] or pial synangiosis,[302] postsurgical angiography has shown promising revascularization of the affected area.

Given the devastating consequences of overt clinical stroke and the difficulties in preventing recurrent stroke, application of radiologic techniques for noninvasive assessment of the brain and its vasculature has focused attention on two areas: identifying children at high risk for primary stroke by TCD ultrasonography and identifying children with asymptomatic "silent" cerebral infarcts (SCI) by MRI.

In the early 1980s the utility of TCD to measure intracranial arterial blood flow was first reported.[303] TCD allows an easy, noninvasive method of measuring cerebral blood velocity in the circle of Willis. The highest time-averaged maximum velocity (TAMV) measured in the distal internal carotid artery (ICA) and proximal middle cerebral artery (MCA) has become the standard reported TCD value. In children with SCA, TCD can identify intracranial arterial vasculopathy,[304,305] although an inverse relationship between hematocrit and TAMV values has been recognized.[306] A landmark prospective single-institution study of untreated children with SCA demonstrated that higher TCD velocities in the distal ICA or proximal MCA were associated with an elevated risk of primary ischemic stroke.[307] A subsequent study with a longer follow-up range confirmed that TCD velocities reflect a continuum of stroke risk, but 200 cm/s represents a potential threshold value, above which the risk of primary stroke is very high. For 315 untreated children with SCA studies for an average of 64 ± 24 months, 25 (8%) had TAMV greater than or equal to 200 cm/s, and 10 of these children (40%) had a primary stroke.[308]

Similar to secondary stroke prevention with chronic transfusion, positive results have also been demonstrated for primary stroke prevention in SCA for children with abnormal TCD velocity. In the multicenter prospective Stroke Prevention Trial in Sickle Cell Anemia (STOP) with 130 asymptomatic children with abnormal TCD measurements (>200 cm/s), subjects were randomized to transfusions or standard care. The study was terminated early when only 1 of 63 transfused children compared with 11 of 67 untreated children had strokes, representing a 92% decrease in the relative stroke rate in the treated group.[309] A subsequent randomized controlled trial (STOP 2) tested whether chronic transfusions for primary stroke prevention based on abnormal TCD could be safely discontinued after at least 30 months in children who had not had an overt stroke and who had reversion to low-risk TCD (<170 cm/s) with chronic transfusion therapy. After 79 of a planned 100 children were randomized, the trial was also terminated early because 16 of 41 subjects randomized to stop transfusion achieved endpoint (14 reversions to high-risk TCD without stroke and 2 ischemic strokes) versus 0 of 38 maintained on chronic transfusion treatment.[310] The current TCD with Transfusions Changing to Hydroxyurea (TWiTCH) clinical trial is testing whether hydroxyurea can be used as an alternative treatment to indefinite transfusions in the setting of abnormal TCD.

A systematic study of MRI in asymptomatic children with SCA revealed that unanticipated SCIs are associated with an increased risk of subsequent clinical stroke.[311] However in older children (mean age, 11 years) without evidence of prior central nervous system (CNS) disease, TCD and MRI results are often discordant, indicating that these tests may be measuring different aspects of CNS pathology.[312] Brain MRI has revealed that 18% to 25% of children in the CSSCD had evidence of SCI.[313-315] These silent infarcts are associated with impaired performance on psychometric tests that measure fine motor skills and cognitive ability.[316-318] Older children (>6 years) with silent infarcts were more likely to have a history of seizures, low Hb level, and high WBC count ($>11.8 \times 10^9$/L).[315] In a study of 392 children studied prospectively up to 10 years, elevated WBC count, dactylits, and low Hb (<7 g/dL) before the age of 2 years were associated with adverse outcomes including strokes.[133] In other studies low hematocrit,[319] ACS[320] and hypertension[321] have been associated with silent infarcts and/or clinical strokes. These observations suggest that MRI might be used to screen older children with poor school performance or evidence of other risk factors.

Clinical trials are underway for treatments of younger children who develop evidence on MRI of silent infarcts or abnormal intracranial Doppler flow measurements including transfusions,[322] hydroxyurea,[323,324] or bone marrow transplant.[325]

Not all acute focal neurologic events in patients with SCA are infarcts. Intracranial hemorrhage is the other major category of sickle-related neurologic event, although the incidence is higher among adults than children.[320] Hemorrhagic stroke presents as a sudden severe headache, sometimes with neck pain, vertigo, syncope, nystagmus, ptosis, meningismus, nausea/vomiting, or photophobia. Many of these episodes are subarachnoid hemorrhages from small bleeding aneurysms that probably arise from intimal damage during childhood. Multiple aneurysms with extensive collaterals (similar to moyamoya disease) are often found during angiographic evaluation of children with cerebrovascular disease. Successful aggressive neurosurgical approaches to these bleeding lesions have been published[326-328] and suggest that angiography should be done to identify patients with surgically amenable lesions.

Priapism

Priapism occurs in males of all ages, but the incidence and prevalence are not well established. In a Jamaican study of 104 males age 10 to 62 years, 42% reported at least one episode of priapism, with a median age of onset of 21 years. [329] The long-term follow-up study of patients in Los Angeles determined that approximately 7% of males with SS and about 2% of those with SC develop priapism, with a median age of onset of about 22 years.[13] A questionnaire survey administered to 98 patients below the age of 20 in Dallas found the mean age at the initial episode to be 12 years, with an average number of episodes per patient of 15.7 and an 89% actuarial probability of experiencing priapism by age 20.[330] Prolonged sexual arousal, fever, cold exposure, nocturnal tumescence (rapid eye movement [REM] sleep), full bladder, dehydration, alcohol, cocaine, and testosterone therapy have all been implicated as triggers.[331-333] Because early intervention and treatment may prevent irreversible penile fibrosis and impotence, patients and parents should be educated in advance of its occurrence to prevent embarrassment and to treat prolonged priapism as a urological emergency.

Four clinical entities are described: stuttering priapism, with short (less than 2 to 3 hours), often multiple episodes; acute prolonged priapism (more than 24 hours), which can persist for weeks; chronic priapism, a painless induration that may last for years; and acute-on-chronic priapism, acute painful episodes complicating chronic induration. Sexual dysfunction was reported by 46% of patients with a history of priapism.[329] In general young patients with brief episodes restricted to the corpora cavernosa (bicorporal priapism) are less likely to become dysfunctional,[334,335] whereas adults with involvement of the corpora cavernosa and corpus spongiosa (tricorporal priapism) tend to have prolonged episodes and higher than a 50% chance of becoming impotent.[336] Urinary retention requring catheterization may complicate such acute episodes. Patients with tricorporal involvement are also particularly susceptible to acute severe neurologic complications and overall increased morbidity, even those treated with exchange transfusion.[337,338] They should be monitored closely for early neurologic symptoms, especially headache.

Diagnostic studies such as technetium scans, MRI, Doppler flow studies, and measurements of corporal pressures, Hb electrophoresis, and blood gases have been used in a variety of settings to help define the anatomy and potential prognostic features of priapism. As yet they have not had an impact on clinical decision making in individual patients.[339-341]

A consensus on treatment of priapism has not been reached, and there are no controlled clinical trials to guide therapy.[342,343] Short episodes of acute priapism usually occur on awakening and can be managed at home. Patients should be instructed to urinate often, perform mild exercise, increase oral fluid intake, and soak in a warm tub and take analgesics. If the episode does not resolve within a few hours, the patient should receive medical treatment for a typical vaso-occlusive episode with aggressive IV fluids and analgesics. If the episode persists beyond 4 hours, intracavernosal aspiration and instillation of an α agonist should be performed and repeated.[344,345] Although the role of RBC transfusion is somewhat controversial,[346] simple red-cell transfusion may relieve priapism and provide safety for urgent surgical intervention.[334] Exchange transfusion may produce results in 6 to 8 hours,[347,348] although most do not show signs of detumescence for 24 to 48 hours, and some do not respond at all.[349] Use of intracorporal and oral α-adrenergic agents has been tried successfully in a small experimental group in Paris in both acute and chronic settings,[345] and in one group of patients with stuttering priapism in Jamaica, stilbestrol was helpful in preventing recurrences.[350] Numerous medical therapeutic options have been attempted, including diethylstilbestrol, gonadotropin-releasing hormone analogs, various adrenergic agonists, antiandrogens, baclofen, gabapentin, phosphodiesterase type 5 (PDE5) inhibitors, and hydroxyurea.[342,351,352]

Surgical shunting procedures should be reserved for patients who have not shown any signs of detumescence 12 to 24 hours after corporal irrigation and/or transfusion, particularly if they are postpubertal. Even partial responses should be considered adequate to postpone surgical intervention, since historically these procedures have carried a high complication rate with subsequent penile deformities and impotence. The most conservative shunt is the Winter procedure or one of its modifications, whereby a needle or scalpel is inserted through the glans into one of the corpora and the viscous blood is aspirated. This temporarily allows for drainage of cavernous blood into the systemic circulation and has been successfully used in patients with SCA.[353] Intermittent compression with a blood pressure cuff is critical to limit refilling. Anecdotal evidence suggests that penile implants to correct impotence may be more easily done soon after an impotence-causing episode.[354,355]

Splenic Sequestration

Along with bacterial sepsis, historically one of the leading causes of death in children with SCA is the acute splenic sequestration crisis.[356] Children with SCA who have not yet undergone autosplenectomy as well as older patients with other forms of SCD[357] may develop sudden, rapid, massive enlargement of the spleen with trapping of a considerable portion of the red-cell mass. Patients suddenly become weak, pale, and dyspneic, with rapidly distending abdomen, left-sided abdominal pain, vomiting, and hypovolemic shock. The tempo of this crisis may be so fast that the patient dies before reaching the hospital. On physical examination there may be profound pallor plus hypotension with cardiac decompensation and massive tender splenomegaly. The Hb concentration is at least 2 g/dL lower than baseline, accompanied by a brisk reticulocytosis with increased nucleated red cells (distinguishing it from the aplastic crisis described below) and moderate to severe thrombocytopenia caused by splenic trapping of platelets.[356] This complication has been described in patients as young as 8 weeks of age.[358]

Emond described the natural history of acute sequestration crisis in a cohort of 308 children with SCA in Jamaica.[359] Eighty-nine patients experienced 113 attacks, with 67 children having their first attack before the age of 2 years. There were 13 fatalities, 10 of which occurred before the age of 2. The most commonly associated clinical problem was upper respiratory tract infection and ACS. Sixteen percent of the patients had positive blood cultures. Recurrences were frequent, occurring in 49% of survivors of the first attack. A parental education program directed toward teaching parents the technique of spleen palpation and the urgency of seeking medical attention for enlarged spleen and pallor led to an increase in the incidence of cases (from 4.6 to 11.3 per 100 patient years of observation) and decreased the fatality rate (from 29.4 per 100 events to 3.1 per 100 events).

Therapy is the emergency restoration of intravascular volume and oxygen-carrying capacity by the immediate transfusion of packed red cells, even if only unmatched blood is available. Once normal cardiovascular status is restored, patients improve rapidly. The spleen usually shrinks within a few days, and the thrombocytopenia resolves. Care must be taken to not transfuse to baseline Hb, however, because with resolution the spleen releases the endogenous trapped erythrocytes and may result in a relative erythrocytosis with hyperviscosity, what is sometimes referred to as the "overshoot phenomenon" after simple transfusion. Recurrence of sequestration is common, usually within 2 to 3 months of the initial episode as the protection afforded by the transfused cells is lost. To eliminate recurrence some have recommended elective splenectomy after the first significant episode,[360] whereas others more concerned with postsplenectomy sepsis have suggested splenectomy only after two episodes of sequestration.[356] Emergent splenectomy during the acute sequestration is not indicated.

Kinney's review from Duke University of splenic sequestration strengthens the position of those who favor elective splenectomy for children who have had one episode of acute sequestration, especially if the initial event was life-threatening.[361] In this series of 23 patients, four underwent early splenectomy, seven were observed carefully, and 12 were placed on a maintenance transfusion program. Of those treated using transfusion, two were well and still receiving transfusion, three had recurrences while still on transfusion, four experienced recurrences within 3 months of the last transfusion, and only three remained well at 11 months (3.9 years after the last transfusion). Fourteen percent of children became alloimmunized, and 1 patient developed non-A, non-B hepatitis. Eventually 14 of the 23 patients underwent splenectomy. All were immunized against pneumococcus and were prescribed penicillin for prophylaxis. None had experienced a life-threatening infection 65.6 patient years later. Serjeant has demonstrated in a 22.5 year follow-up study that children in Jamaica who are postspenectomy are not at greater risks for infection than a matched control group of patients with SCA.[362]

Rao has described a subacute form of splenic sequestration in 11 patients characterized by increased spleen size, 25% drop in hematocrit, less than 100,000 platelets, and reticulocyte count elevation above patient's baseline. All responded to chronic transfusion programs, but seven patients had recurrent episodes after transfusions were stopped and eventually required splenectomy.[363]

Aplastic Crisis

The clinical characteristics of the transient aplastic crisis of SCD result from the abrupt cessation of erythropoiesis, most commonly because of acute parvovirus infection.[364-366] In the normal steady state, patients can compensate for the decreased red-cell survival (15 to 50 days) by increasing the bone marrow output six- to eightfold, with the absolute reticulocyte count sometimes reaching 400 to 500×10^9/L. Temporary cessation of bone marrow activity because of suppression by intercurrent viral or bacterial infection causes the hematocrit value to fall as much as 10% to 15% per day with no compensatory reticulocytosis. The short-lived HbF-poor cells are the first to disappear from the circulation, whereas the cells containing more HbF persist. This natural selection of HbF-containing cells accounts for the apparent increase in the percentage of HbF during aplastic episodes.

Patients usually have pallor and fatigue but are without jaundice and have laboratory evidence of severe anemia below baseline with associated severe reticulocytopenia. Spontaneous recovery is usually heralded by a markedly elevated nucleated RBC count, followed in 1 or 2 days by a brisk reticulocytosis. Spontaneous recovery usually occurs within 7 to 10 days and may require no therapy, but some patients are extremely ill at presentation and many require short-term hospitalization and erythrocyte transfusions. This recovery phase, with the characteristic anemia, nucleated red cells, reticulocytosis, and occasional hyperbilirubinemia, may be responsible for most cases referred to as hyperhemolytic crisis.

In 1981, two groups linked transient aplastic crisis to parvovirus B19 infection.[367,368] The Jamaican group subsequently documented the epidemiology and follow-up treatment of parvovirus in a cohort of infants identified

TABLE 20-2 Bacteremia and Associated Acute Events in a Cohort of 694 Children with Sickle Cell Anemia and Hemoglobin Sickle Cell Disease Monitored Prospectively from Infancy

Organism	Patient	Total No. Cases	Isolated Bacteremia	Acute Chest Syndrome	Meningitis	Bone/Joint
Streptococcus pneumoniae	SS	62	39 (5 dead)	14 (1 dead)	8 (2 dead)	1
	SC	12	9	3	0	0
Haemophilus influenzae	SS	10	6 (2 dead)	3	1	0
	SC	4	2	2	1	0
Staphylococcus aureus	SS	5	2	2	0	1
	SC	1	1	0	0	0
Viridans streptococci	SS	5	4	1	0	0
	SC	1	1	0	0	0
Escherichia coli	SS	5	3	1	0	1
	SC	2	0	1	0	1
Salmonella species	SS	3	2	0	0	1
	SC	2	1	0	0	1
Other	SS	2	1	1	0	0
	SC	0	0	0	0	0

Data from Gill FM, Sleeper LA, Weiner SJ et al. Clinical events in the first decade in a cohort of infants with sickle cell disease. Blood *86:776, 1995.*

SC, HbSC disease; *SS*, HbSS disease.

at birth: 308 with SCA and 239 controls.[369,370] They made a number of important observations: 1) the frequency of infection (about 40%) did not differ between sickle and control groups; 2) 20% of infections in the sickle group did not result in clinically recognizable aplasia; 3) 100% of aplastic episodes were associated with parvovirus; 4) no patients had recurrent aplasia; and 5) 45% of infected patients maintained an elevated parvovirus-specific IgG after 5 years. The secondary attack rate of siblings was 50% to 60%; therefore affected siblings should be monitored for evolving aplastic crisis when a child is diagnosed with an acute parvovirus B19 infection. Parvovirus B19 has also been implicated as the etiologic agent in erythema infectiosum (fifth disease),[371,372] although often the classic rash of fifth disease is absent.[372,373] Parvovirus B19 infection has also been related to several other sickle cell complications including acute splenic sequestration,[374] hepatic sequestration,[375,376] ACS,[233] glomerulonephritis,[377,378] and stroke.[379] Many patients are seropositive for parvovirus B19 without history of a known aplastic crisis, suggesting many infections are subclinical.[380] The relationship between parvovirus and erythropoiesis has been studied in detail. The virus specifically retards late erythroid precursor differentiation[381] and is responsible for temporary erythroid aplasia in a broad array of hemolytic anemias.[373]

Infection

Acute Infection

Bacterial infection is the most common cause of death in children with SCA.[382-384] In one study the risk of acquiring sepsis or meningitis was higher than 15% in children younger than 5 years, with an associated mortality of approximately 30%.[385] In young children the risk of pneumococcal sepsis appears to be 400 times that of normal children, and *Haemophilus influenzae* sepsis appears to be two to four times as common.[386] These two reports are from the period before the licensing of conjugated vaccines to *S. pneumoniae* and *H. influenzae*. In general the organisms responsible for infection are primarily encapsulated bacteria and not unusual pathogens (Table 20-2), but the infections that they cause in such patients occur more often and are more severe.

The major risk factor for this increased vulnerability to infection is splenic dysfunction. The spleen normally serves two separate immunologic functions: 1) clearance of particles from the intravascular space, and 2) antibody synthesis. Both functions are impaired in SCD.

During the first year of life, "functional asplenia" develops in patients with SCD, particularly in those with SCA.[387] This is heralded by the appearance of red cells with micronuclear remants (Howell-Jolly bodies) and intracellular vacuoles that appear as irregular surface characteristics ("pits"). When the percent of pitted red cells exceeds 3.5%, the spleen is generally considered nonfunctional.[388] Splenic dysfunction develops early in life, with 50% of 2068 children with SCA having greater than 3.5% pitted red cells by 2 years of age. Appearance of dysfunction was less rapid and less common in children with HbSC or HbS β^+-thalassemia. Despite palpable splenomegaly, children with elevated pitted RBCs have no splenic uptake of technetium-99m (^{99m}Tc) sulfur colloid and are susceptible to the most serious infectious complication of asplenia and pneumococcal sepsis.[389] Repeated infarction results in a nonpalpable, fibrotic, often calcified spleen.[390] Splenic infarction in young children with SCA is an insidious process (usually with few symptoms) but is also progressive and culminates in splenic dysfunction. In older children and adults, especially those with HbSC and HbSβ^+-thalassemia, severe left upper quadrant pain often accompanies acute splenic infarction. Transfusion,[391] hydroxyurea,[392,393] and bone-marrow transplantation[394] can correct the splenic phagocytic defect, although these treatments are not indicated for splenic dysfunction per se.

Children with functional asplenia fail to respond to intravenously administered antigen vaccines,[395] even when the phagocytic function of the spleen has been restored by transfusion. As with other individuals with asplenia, however, these children do respond normally to intramuscularly/subcutanously administered polysaccharide and conjugated pneumococcal vaccine.[396-398]

Levels of serum immunoglobulins are normal or increased in children with SCA.[397,399,400] However the serum is deficient in heat-labile opsonizing activity[401] related to an abnormality of the properdin pathway,[402,403] which is specific for the phagocytosis of pneumococci. There is an increased activation of complement via the alternate pathway[404] but no intrinsic defect in the complement system.[405] Opsonic activity can be reconstituted in vitro by addition of only the $F(ab')_2$ fragments of capsular antibodies to *S. pneumoniae*.[406] This abnormal opsonic activity and associated functional asplenia may partially explain the propensity for infections with pneumococcus and other encapsulated organisms.

Recurrent pnuemoccocal infections have been described in patients with SCD.[407] In one series patients with serious invasive bacterial infections are significantly more likely to have recurrent infection.[408]

Treatment of Infection

Because of the increased incidence of serious bacterial infections in patients with SCD, the index of suspicion for infection should always be high. In general the higher the temperature[409] and leukocyte count,[410] the higher the probability of a serious bacterial infection. Unfortunately however, the wide variability in the temperature and laboratory values of children with bacteremia does not permit an accurate prediction of whether an individual febrile child is bacteremic. The following guidelines are adapted from the NHLBI guidelines for empiric evaluation and treatment of the febrile child with a temperature higher than 101.3° F (38.5° C)[411]:

- Perform complete blood cell count, reticulocyte count, blood culture. Consider urine and throat culture based on signs or symptoms. Perform chest radiography if pulmonary signs or symptoms are present.
- Prompt administration of broad-spectrum empiric parenteral antibiotics that provide coverage against *S. pneumoniae* and gram-negative enteric organisms before the results of laboratory tests are available or radiographs are taken. Empiric antibiotic selection should be based on the ability to kill both pneumococcus and *H. influenzae* and to penetrate the CNS. In areas where β-lactamase-producing *H. influenzae* or penicillin- and cephalosporin-resistant pneumococci are regularly encountered, these issues should be factored into the choice of antibiotic.
- Typically after antibiotic administration (e.g., ceftriaxone, 50 to 75 mg/kg, parenterally) the patient should be observed over 2 to 6 hours. Subsequent outpatient management is feasible in patients who do not appear to have toxic reactions or other features to consider for admission. Follow-up evaluation the next day and possibly repeat the empiric antibiotic dose.[412] If children do well and culture results remain negative after 24 to 48 hours, antibiotics can be discontinued.
- Admission to hospital and continuation of empiric parenteral antibiotic treatment should be considered for:
  - Appearance of toxicity
  - Temperature higher than 40° C
  - Infiltrate on chest radiograph
  - WBC count higher than $30{,}000/mm^3$ or below $500/mm^3$, platelet count less than $100{,}000/mm^3$, or Hb below 5 g/dL
  - History of prior bacterial sepsis should be treated parenterally and patient should be admitted to the hospital.
  - Previous surgical splenectomy
  - Incomplete vaccination to pneumococcus or *H. influenzae*
- Lumbar puncture should be performed on children having toxic reactions and those with any signs of meningitis. Bacterial meningitis should be treated for a minimum of 10 days parenterally or for at least 1 week after the cerebrospinal fluid has been sterilized.
- Documented sepsis should be treated parenterally for a minimum of 1 week.
- Patients with infiltrate on the chest radiograph should be treated as described for ACS. Because of the high incidence of atypical pathogens, patients should be treated with an appropriate antipneumococcal agent and macrolide antibiotic for at least a week.

Despite advance in laboratory and imaging techniques, the diagnosis of osteomyelitis in SCD is often difficult to distinguish from aseptic bone infarct and still relies heavily upon clinical assessment.[211] Patients with clinical findings that are highly suggestive of osteomyelitis should have needle aspiration and culture of the lesion. After cultures have been obtained, children who appear acutely ill should be started on antibiotics. Antibiotic choice should include agents effective against *Salmonella* species and *Staphylococcus aureus*. Antibiotics should be discontinued or modified when culture reports are available.

Prevention of Infection

Methods of preventing invasive bacterial infection in SCA patients have focused on prophylactic antibiotics and vaccines. Prophylactic penicillin was first shown to be effective in the prevention of pneumococcal disease by John and associates,[413] who used monthly intramuscular injections of long-acting penicillin. However if penicillin prophylaxis was discontinued after 3 years of age, an increase in pneumococcal infections was noted. Gaston and co-workers, in a blinded placebo-controlled clinical trial in the United States, showed that 84% of the pneumococcal infections in children younger than the age of 5 years could be prevented with oral penicillin.[414] As a result of this landmark study, universal newborn screening has emerged throughout the United States, so that all children with SCA can be identified and started on penicillin prophylaxis by 3 months of age. If penicillin is discontinued after 5 years there is no increased incidence in infections compared to those who remained on prophylaxis;[415] and there are conflicting data regarding the increased risks of penicillin-resistant pneumococcal colonization while on prophylaxis.[416,417] Our approach to prophylaxis is as follows:

- Give prophylaxis to all newborns with HbSS, HbS β-thalassemia, and HbSC disease. (Some practitioners prefer not to treat infants with HbSC disease because their splenic dysfunction typically appears later in life.)
- Start as early as possible, optimally by 8 weeks of age but certainly by 3 months.
- Prescribe penicillin, 125 mg orally, twice a day until age 3 years. Then increase the dose to 250 mg orally, twice a day. Penicillin can be stopped after 5 years of age without resulting in an increased incidence of infection. Consider continuation for history of invasive bacteremia or surgical splenectomy.
- For patients allergic to penicillin, prescribe erythromycin, 10 mg/kg orally, twice a day.
- Educate families as to the importance of compliance and the early recognition of signs of infection, particularly temperature of 101.3° F (38.5° C) or higher.
- For vaccination recommendations, consult the Advisory Committee on Immunization Practices (ACIP) statements for detailed up-to-date recommendations for SCD.[418] Complete routine vaccination schedule including complete conjugated vaccine series for *S. pneumoniae* (Prevnar-13; PCV13) and *H. influenzae*. Polyvalent pneumococcal vaccine has been shown to be effective in eliciting a normal antibody response, increases in pneumococcal opsonizing activity, and reducing the incidence of pneumococcal disease.[404,419] The 23-valent polysaccharide pneumococcal vaccine (Pneumovax; PPV23) represents 90% of the common serotypes of pathogenic pneumococci, however, because children younger than 2 years have a poor response to this vaccine[420] it should be given at age 2 years with a booster 3 to 5 years later. *H. influenzae* conjugated vaccine is immunogenic in children with SCD and should be given on the same schedule used for normal infants.[421,422] Seasonal influenza vaccine should be administered to all SCD patients annually. Conjugated meningococcal vaccine (Menactra; MCV4) should be administered to patients at age 2 years.

Despite established strategies of penicillin prophylaxis and conjugated vaccines, invasive bacterial infections continue to occur in patients with SCA, sometimes because of pneumococcal serotypes not included in the vaccines.[423-425] Although the incidence of penicillin-resistant pneumococcus has not been demonstrated in patients receiving prophylaxis, there appears to be an emergence of multidrug resistant pneumococcus in patients treated longer than 5 years.[416] Physicians must continue to emphasize the need for penicillin and vaccination compliance and continue to have a high index of suspicion of sepsis in the febrile child with SCD.

Chronic Organ Damage

Cardiovascular System

Abnormal cardiac findings are present in most patients with SCA[426-429] and are primarily the result of chronic anemia and the compensatory increased cardiac output.[430] On physical examination, the most common finding is systolic ejection murmur, although concomitant S_3, split S_1, suprasternal notch thrill, and diastolic murmur may also be present.[426] Echocardiography may be necessary to diagnose abnormal cardiac structure. Cardiomegaly is common, with electrocardiographic findings of left ventricular hypertrophy in about 50% of patients.[427]

In view of the fact that the hallmark of SCD is vaso-occlusion, it is remarkable that myocardial infarction is an extremely rare event.[427,431,432] In one review of the postmortem literature related to SCD including examination of 153 hearts, only four infarcts were reported.[431] Despite the high oxygen extraction in the coronary circulation, blood in the coronary sinus contains no more sickled forms than the general circulation,[433] presumably because of the short transit time through the coronary vessels.[430] Atherosclerosis is virtually absent in this population, and the SCD heart has normal patent coronary arteries, often of larger caliber than seen in normal hearts.[434]

The most comprehensive prospective examination of cardiac function in an unselected population of patients with SCA was done in the CSSCD.[435] One hundred and ninety one patients ages 13 and older in steady state underwent echocardiography, and all of the measurements were performed centrally by an investigator without access to other patient data. After appropriately adjusting for body surface area, the left and right ventricles, aortic root, and left atrium were found to be larger than normal. Significant wall thickening was found only in the septum. The left ventricular dilation correlated with Hb and age, suggesting that the major cardiac findings are indeed related to the years of increased stroke volume in compensation for anemia and abnormal rheology.

PHT can occur in adult patients with SCD, both as a result of cardiac dysfunction and also from intrinsic pulmonary vasculopathy, with a reported prevalence as high as 20% to 40% based on tricuspid regurgitant jet velocity (TRJV) measurements.[436-439] Multiple retrospective studies report a significantly increased mortality in patients with SCD and elevated TRJV compared with those patients without this finding.[437,440,441] Castro et al. demonstrated that patients with SCD and PHT confirmed by right heart catheterization had median survival of 25.6 months, whereas patients without PHT had greater than 70% survival at the end of a 10-year observation period.[441] A more recent study of 195 patients with SCD reported a significantly higher mortality rate for adult patients with TRJV (>2.5 m/s on echocardiography after a median follow-up length of 18 months).[439] Increased lactate dehydroenase (LDH), bilirubin, and reticulocyte count have been associated with the elevated TRJV findings, which

TABLE 20-3 Blood Pressure in Patients with Sickle Cell Anemia

		Age (yr)										
	Percentile	**2-3**	**4-5**	**6-7**	**8-9**	**10-11**	**12-13**	**14-15**	**16-17**	**18-24**	**25-34**	**35-44**
Females												
Systolic	50	90	95	96	96	104	106	110	110	110	110	110
	90	100	110	110	110	110	118	120	122	122	125	130
Diastolic	50	52	60	60	60	60	62	70	70	64	68	70
	90	62	70	70	70	74	74	80	78	80	80	84
Males												
Systolic	50	90	95	100	100	100	110	108	112	112	114	110
	90	104	110	108	116	112	120	120	128	130	130	132
Diastolic	50	54	60	60	60	60	64	64	70	68	70	70
	90	66	68	68	70	70	72	78	80	80	80	84

Data from Pegelow CH, Colangelo L, Stunberg M et al. Natural history of blood pressure in sickle cell disease. Am J Med *102:171–177, 1997.*

has led to a hypothesis that increased hemolysis is responsible for the vasculopathy.[439,442] Because intravascular hemolysis leads to increased plasma Hb and arginase, which are associated reduced NO bioavailability (see Hemolysis and Nitric Oxide Depletion), subsequent endothelial dysfunction and PHT could result from this pathophysiology.[443] The prevalence of elevated TRJV in children appears similar to that in adults[444-446]; however, a recent French adult study revealed a prevalence of 27%, but confirmation by catheterization revealed that PHT prevalence was only 6%. Therefore the positive predictive value of TRJV greater than 2.5 m/s appears low.[447] Further concerns about the reproducibility of TRJV measurements in children,[448] as well as the prognostic implications that differ from adults, have prevented the development of screening recommendations among children with SCA.[448,449]

The steady-state blood pressure in patients with SCA (and to a lesser extent all forms of SCD) is lower than in a race-, gender-, and age-matched normal population (Table 20-3).[321] This observation may reflect differences in peripheral vascular resistance or be related to the tendency to lose sodium and water in the urine. Higher blood pressure is a risk factor for stroke, early mortality, and nephropathy,[450] but the diagnosis of elevated blood pressure depends on knowing the normal values for this population (see Table 20-3). The clinician should note that blood pressures that appear to be normal based on race, gender, and age could be significantly elevated and clinically ominous in the sickle population. Such patients should be considered candidates for antihypertensive therapy.

Renal System

Hyposthenuria, hematuria, nephrotic syndrome, and uremia are the major renal complications of SCD.[451,452] In addition the production of erythropoietin in response to anemia may be lower in older patients with SCA, possibly because of primary renal disease.[453]

Hyposthenuria develops early in childhood, and as is the case with functional asplenia, may be temporarily reversed with transfusion.[454-457] The hypertonic environment of the renal medulla promotes intraparenchymal sickling even at normal pO_2,[458] which leads to decreased medullary blood flow and derangement of the countercurrent multiplier. Abnormality of the countercurrent multiplier may be the mechanism for hyposthenuria, or as suggested by Buckalew and Someren,[451] it may be the result of decreased flow to nephrons with long loops of Henle and preservation of flow to nephrons with short loops. The obligatory water loss in SCA results in a tendency toward dehydration and invalidates the use of urine volume or specific gravity as an indicator of the patient's state of hydration. Nocturia and enuresis are common complaints of these patients, who excrete large volumes of dilute urine.[459] Urinary sodium losses may be high and result in significant hyponatremia.[460] A renal tubular acidification defect[461,462] as well as hyporeninemic hypoaldosteronism[463] and impaired potassium excretion[464] have been identified. In one review deJong and Van Eps emphasized that renal vasodilating prostaglandins are increased in patients with SCD, leading to a compensatory increase in renal blood flow, glomerular filtration rate (GFR), and proximal tubular activity.[465]

Although hematuria is usually mild, overt bleeding is occasionally severe enough to cause significant blood loss.[466] Papillary necrosis is usually the underlying anatomic defect.[467] Maintenance of high urinary flow with increased fluid intake will eliminate clots from the bladder and decrease medullary osmolality. Urinary alkalinization and diuretics may be beneficial until the bleeding stops. ε-Aminocaproic acid has been suggested to treat severe hematuria that is refractory to supportive care and transfusion,[468] but it must be used cautiously because of the risk of ureteral or pelvic clotting and obstruction. In patients with long-standing hematuria, supplemental iron may be necessary to prevent iron deficiency. Hematuria may also be the presenting symptom of a renal tumor. A surprisingly high incidence of renal medulary carcinoma has been reported in patients with SCD and even persons with sickle trait.[469,470]

Uremia is a rare complication in children with SCD that may follow a symptom complex of nephrotic syndrome[471] with glomerulonephritis. The nature of the glomerular lesion is unknown and may represent response to iron deposition,[472] antigen-antibody complex,[473] or mesangial phagocytosis of fragmented sickled cells.[474,475] Recently an association between parvovirus infection with or without an antecedent aplastic crises has been associated with acute glomerulonephritis.[377,378]

Proteinuria in SCD begins in childhood and teenage years and can progress to nephrotic syndrome[476] and ultimately to renal failure,[475,477] which is a common cause of mortality among adults with SCA.[131,478] Microalbuminuria manifested by a ratio of albumin to creatinine (>20 mg/g Cr) is common; a study of 442 children with SCD studied for 10 years reported proteinuria in 6.2% of children and 12% of teenagers.[479] Proteinuria was associated with lower Hb levels, higher MCV, higher white cell counts, and more clinically severe disease. A study of 381 adults with SCD demonstrated that 7% had elevated serum creatinine levels and 26% had proteinuria.[480] Ten patients with proteinuria underwent renal biopsy, and the glomerular lesions showed perihilar focal segmental sclerosis and glomerular enlargement, similar to findings in an animal model with glomerular hypertension and efferent arteriolar vasoconstriction. To test the animal analogy, these patients were treated with enalapril, an angiotensin-converting enzyme (ACE) inhibitor that has been shown to decrease efferent arteriolar constriction. In all treated patients the level of proteinuria fell during treatment and returned toward abnormal after discontinuation.

The predictive value of microalbuminria screening has not been fully elucidated in children but has been suggested by some.[481,482] Serum cystatin C may be a better screen for GFR than creatinine in SCA, because it appears to rise according to the degree of albuminuria and before significant elevation in serum creatinine is detected.[483]

Treatment of microalbuminuria[484] or proteinuria[480] with ACE inhibitors can be effective, but long-term therapeutic benefits for the prevention of progressive glomerular disease have not yet been demonstrated.[485] Addition of hydroxyurea to ACE inhibitors may further improve renal function.[486]

Bakir and associates estimate that 4% of adult patients with SCA develop nephrosis and that two thirds of these patients go on to develop renal failure.[475] Renal failure can be managed with peritoneal dialysis, hemodialysis, and transplantation. Renal transplantation can be successful in patients with end-stage sickle nephropathy, but three-year allograft and patient survival are significantly decreased compared with other patient populations.[487]

Hepatobiliary System

Liver and biliary tract abnormalities are common in SCD[488,489] and are the result of cholelithiasis, hepatic infarction, and transfusion-related hepatitis and hemosiderosis.

Bilirubin gallstones are common,[490,491] and two large series have studied the incidence of gallstones as detected by ultrasound in children with SCA.[492,493] The percentage of gallstones in 226 patients aged 5 to 13 years selected randomly from a group of children with HbSS disease identified at birth was 13%,[488] a value lower than that found in a survey of clinic patients studied by Sarnaik and associates.[492] In this report, the incidence of gallstones was 12% by 2 to 4 years of age.[492] With advancing age, the incidence increased gradually, reaching 42% in the 15- to 18-year-old age group. Fourteen of the 226 patients were noted to have “sludge” in the gallbladder, and on repeated ultrasonograms up to 2 years later, four had developed stones, 4 had no further evidence of sludge, and 6 remained unchanged. Comorbid Gilbert syndrome (uridine diphosphate [UDP]–glucuronosyltransferase 1A1 [UGT1A1] promoter polymorphism) significantly increases the serum bilirubin levels in children with SCD and the likelihood of requiring early cholecystectomy.[168] Evidence is good that children tolerate elective cholecystectomy with little morbidity if they are prepared properly for surgery.[263,494,495] Laparoscopic cholecystectomy has been particularly effective in reducing the postoperative hospital stay in children with SCD.[496] In contrast operating during the acute phase carries a significant risk of complication. [263,497]

Intrahepatic sickling can result in massive hyperbilirubinemia,[261] elevated liver enzyme values, and a painful syndrome mimicking acute cholecystitis[262] or viral hepatitis.[490] (See “Acute Abdominal Pain”). Fulminant hepatic failure with massive cholestasis and rapidly progressing hepatic encephalopathy and shock has been described as a rare but often fatal complication of SCD that may be amenable to exchange transfusion.[264,498,499]

Eyes

SCD can affect virtually every vascular bed in the eye.[500] Tortuosity and sacculation of conjunctival vessels are seen in more than 90% of patients with SCD. These lesions are best seen in the lower temporal area, disappear after exchange transfusion, and are curiously related to the ISC count in the peripheral blood.[501] They are purely cosmetic, however, and have no deleterious effect on the eye.

Sickle retinopathy is classified as either proliferative or nonproliferative. Nonproliferative retinopathy probably results from retinal arteriolar infarction with adjacent hemorrhage and requires no therapy. Depending on the age, layer, and extension of the hemorrhage, the result can be a salmon patch, schisis cavity, vitreous hemorrhage, or black sunburst.[502] In two patients with documented acute arteriolar occlusion, salmon patches developed in a matter of hours to days, with atrophic schisms cavities evolving in 3 to 4 months.[367] In older patients angioid streaks are common, but the cause is unknown.[503,504]

The more serious complication is proliferative retinopathy, which has been classified by Goldberg[505] as stage 1, peripheral arteriolar occlusions; stage 2, arteriolar-venular anastomoses; stage 3, neovascularization; stage 4, vitreous hemorrhages; and stage 5, retinal detachment. Because these lesions may progress to blindness, laser therapy to occlude feeding vessels of advanced proliferative lesions has been advocated. Unfortunately photocoagulation carries the risks of neovascularization of the choroid[506] and retinal breaks,[507] and complications that can result in blindness. The dilemma of choosing potentially blinding therapy for a potentially blinding lesion is complicated by the observation that some proliferative lesions heal spontaneously by autoinfarction. In one study of untreated sickle retinopathy in Jamaica, 567 eyes were observed for 8 years.[508] Proliferative retinopathy was initially present in 12% of the eyes: another 8% developed retinopathy during the follow-up study, and patients with HbSC were more commonly affected than those with SCA. Blindness resulted in 12% of the eyes with retinopathy. In the original group of eyes with retinopathy, 30% developed progressive retinopathy, 10% showed spontaneous regression, and the remaining 30% showed a mix of regression and progression. In another prospective Jamaican study, treatment of retinopathy was compared with no treatment.[509] No statistical difference in visual acuity between the two groups was reported. Macular ischemia and color blindness have been reported to be prevalent in patients with SCD without evidence on ophthalmologic examination of retinal lesions.[510,511]

Rarely acute painless loss of vision is the result of central retinal artery occlusion. Although such lesions may resolve spontaneously, exchange transfusion has been recommended for bilateral disease.[512]

Blunt trauma to the eye may result in hyphema (bleeding into the anterior chamber). Because the conditions in this chamber overwhelmingly favor sickling, any HbS-containing red cell will sickle (even sickle trait) and may cause obstructive glaucoma and blindness. Common medical treatments that might be considered in patients without SCD may promote sickling and should be avoided (e.g., hyperosmotic/diuretic agents/carbonic anhydrase inhibitors). Initial topical β-adrenergic antagonists (e.g., timolol) or α_2-adrenergic agonists (e.g., brimonidine) may be effective,[513] but a low threshold for surgical evacuation of anterior chamber blood should be observed so that vision is preserved.[514] This condition is one of the true ocular emergencies that occurs in patients with SCD as well as sickle trait.

Skin

Leg ulcers usually do not occur in childhood, but in adolescence and adulthood ulcers may constitute a crippling symptom. This skin lesion is also very rarely seen in other chronic hemolytic anemias such as hereditary spherocytosis, thalassemia, elliptocytosis, and pyruvate kinase deficiency[515] and therefore may not represent a vaso-occlusive phenomenon. Ulceration may result from increased venous pressure in the legs caused by the expanded blood volume in the hypertrophied bone marrow.[516] In tropical areas in which shoes are not usually worn and insect bites are common, leg ulcers are also common.[517] In Jamaica, leg ulcers typically start in the 10- to 20-year-old group and eventually appear in 75% of adults. Koshy and colleagues[518] have reported data from the CSSCD regarding leg ulcers in patients with SCD. Leg ulcers appear to be less common in individuals with two α-globin genes than with those with three or four genes. The incidence of leg ulcers appears to decrease consistently with increases in HbF production.[518] There is decreased venous outflow in in the lower extremities of patients with leg ulcers compared with patients without ulcers, suggesting that venous incompetence may contribute to the development or failure to heal of these lesions.[519] In addition, low steady-state Hb values and markers of high hemolytic rate are associated with increased incidence of leg ulcers and may be associated with decreased NO bioavailability and endothelial dysfunction.[520,521]

Chronic leg ulcers become a major source of morbidity and have a profound negative impact on educational achievement and employment.[522] Usually present over the medial surface of the lower tibia or just posterior to the medial malleolus, they begin as a small depression with central necrosis and, if unattended, widen to encircle the entire lower leg. Debridement, scrupulous hygiene, topical antibiotics, bed rest, and elevation of the leg are the mainstays of therapy. In some patients, protection of the ulcer by the application of a soft sponge-rubber doughnut and a low-pressure elastic bandage seems to be beneficial. One report suggested that an arginine-glycine-aspartic acid (RGD) peptide matrix designed to mimic the normal matrix was beneficial.[523] Close attention to improved venous circulation by the use of above-the-knee elastic stockings may prevent ulceration. If ulcers persist despite optimal care, transfusion therapy may be used and consideration given to split-thickness skin grafts. Transfusion therapy is sometimes effective, but in many patients the ulcers either do not heal or recur after discontinuation of this therapy. Oral zinc sulfate may promote healing of leg ulcers,[524] and peripheral vasodilator therapy appears ineffective.[525]

Skeleton

Skeletal changes in SCD are common[201,211,526,527] and are the result of expansion of the marrow cavity and repeated bone infarction. The expanded marrow is best seen in radiographs of the thickened calvarium with a wide diploic space. Overgrowth of the anterior maxilla may lead to severe orthodontic and cosmetic problems. Vertebrae are generally flattened, with a characteristic biconcave deformity called *codfish vertebrae*. In older patients, vertebral disease may cause chronic back pain. These individuals need to be treated as other patients who have chronic back disease with appropriate exercises, braces, bed rest, muscle relaxants, and moral support.

The major chronic bone complication of SCD is AVN. Indeed, the most common etiology of AVN of the femoral head in children is SCD. As shown (Fig. 20-8), the incidence is relatively low in children, with a prevalence estimated at 3% in children under 15 years, but increases with age and occurs in both patients with SCA and HbSC disease.[528] The incidence is remarkably increased in patients with SCA and α-thalassemia, who also have higher hematocrit levels and rates of pain.[151] Athophysiology of AVN is unknown but hypothesized to result from limited blood supply in the femoral (and humeral) heads that promotes sludging in marrow sinusoids, marrow necrosis, healing with increased intramedullary pressure, articular surface necrosis, bone resorption, and eventually epiphyseal collapse.[151,529] The diagnosis is often made clinically with pain and limited range of motion or radiographically, classically with a spectrum of findings from subepiphyseal lucency and widened joint

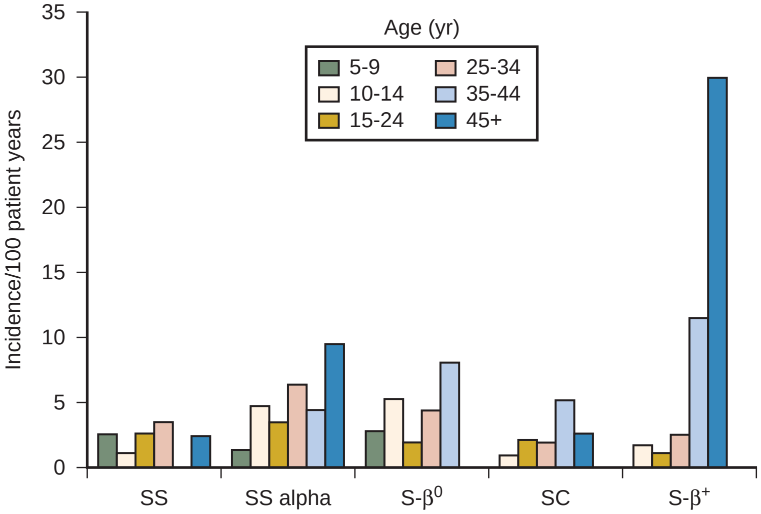

Figure 20-8 Incidence of osteonecrosis of the femoral head per 100 patient years by genotype. *(Data from Milner PF, Kraus AP, Sebes JI et al: Sickle cell disease as a cause of osteonecrosis of the femoral head.* N Engl J Med *325:1476–1481, 1991.)*

space to flattening or fragmentation and scarring of the epiphysis. Although roughly half of the patients diagnosed on the basis of screening radiographs were clinically asymptomatic, significant chronic pain and limited joint mobility plagued the others. MRI can identify AVN changes earlier than conventional films.[528,530]

Managing osteonecrosis of the femoral head in young adults is a challenging problem with limited treatment options and often disappointing outcomes. Strict bed rest and limited weight bearing are helpful to some patients,[531] with little or no evidence to support the use of chronic transfusion therapy, core decompression,[532] bone grafting,[533] or injection of autologous bone marrow[534] or cement.[535] A randomized prospective study of hip core decompression versus physical therapy for the treatment of early femoral head osteonecrosis revealed clinical improvement in both groups, but one was no more effective than the other.[536] Despite interventions to preserve the femoral head, progression is common and may result in the need for total hip replacement arthroplasty for severely compromised patients. In the study of Milner and colleagues, 17% of patients underwent hip replacement, 30% of replaced hips required surgical revision within 4.5 years, and more than 60% of patients continued to have pain and limited mobility postoperatively.[151] The epidemiology of AVN of the humeral head is virtually identical to that of the femur.[537] In general, however, the patients are less symptomatic because of reduced weight bearing, and shoulder arthroplasty is not required.

Lungs

In contrast to ACS, chronic sickle lung disease has been more difficult to define and quantitate, with many conflicting studies showing restrictive or obstructive defects or both. Furthermore the relationship between ACS and chronic lung disease has been difficult to establish reproducibly. In older children, lung volumes are reduced[538,539] when compared with those of a normal white population but are appropriate when compared with those of a black control population.[540] An early restrictive and obstructive pulmonary function pattern has been reported in children with SCD,[541,542] and there appears to be an emerging link between obstructive lung disease and ACS pathogenesis, particularly in children.[247,256,543,544] In a study of infants 3 to 30 months of age restrictive lung disease was present in 3 out of 12 infants and was not related to a history of ACS.[545] These studies suggest that subtle subacute damage to the lungs proceeds even in the absence of acute disease. Hydroxyurea therapy[243] reduces the incidence of recurrent ACS and can improve chronic hypoxemia.[546] In 24 patients with a history of chest syndromes studied after bone marrow transplant, 22 showed no improvement and 2 patients showed worse pulmonary function posttransplant.[547]

Central Nervous System

Acute neurologic abnormalities in SCA were discussed earlier. Kral et al found that children with SCD perform more poorly on measures of neurocognitive functioning with increasing age or lower hematocrit levels[548] and that transcranial Doppler ultrasonography was the only radiologic measure that could predict neurocognitive outcome after controlling for age and hematocrit. It is now clear that cognitive functioning and fine motor skills are also impaired in patients with silent cerebral infarcts as diagnosed by MRI[316,549]; however, no established relationship exists between SCI and abnormal TCD or MRA imaging.[318] Cognitive deficits remain even in the absence of cerebral infarction.[550] Because abnormal TCD and/or MRI studies can be seen in children with decreased neurological and/or cognitive performance, these radiologic studies are being considerd as screening tools to select very young patients for experimental therapies that may prevent further CNS damage.[322-324]

Growth and Development

The birth weight of infants with SCA is normal.[551] Subsequently a pattern of delayed growth emerges. Detailed

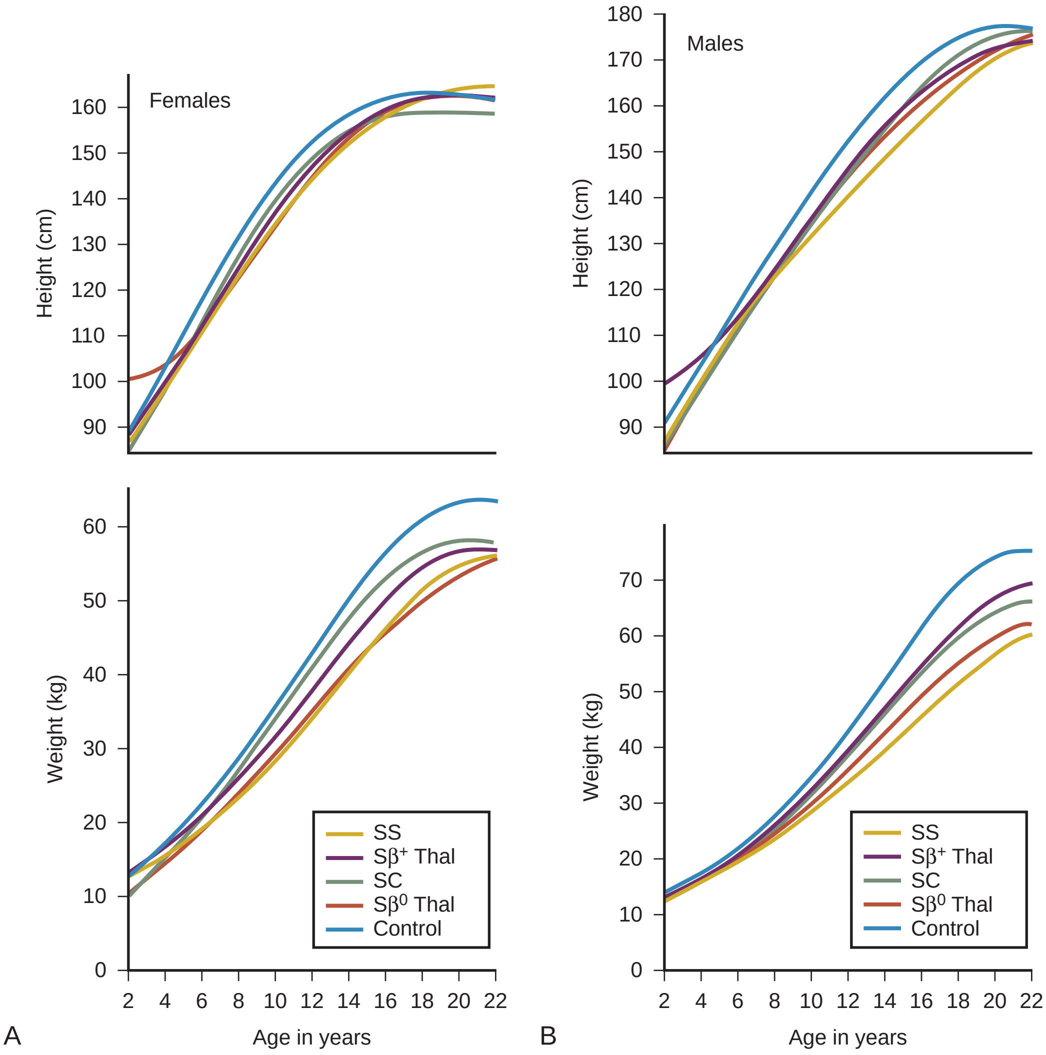

Figure 20-9 Height and weight growth curves for females (**A**) and males (**B**) 2 to 22 years of age, according to type of hemoglobinopathy. Shown are the 50% curves. *(From Platt OS, Rosenstock W, Espeland MA: Influence of sickle hemoglobinopathies on growth and development.* N Eng J Med *311:7–12, 1984.)*

anthropomorphic measurements of Jamaican children reveal decreased limb length, sitting height, and skinfold thickness, with increased chest anteroposterior diameter[552] that is possibly related to cardiomegaly.[553] Height and weight growth curves for individuals with SCD in the United States were generated as part of the CSSCD sponsored by the NHLBI (Fig. 20-9).[554] The important features of these curves are that they are different from those for normal black controls in that weight is more affected than height and that patients with SCA experience more delay in growth than patients with other forms of SCD. In general by the end of adolescence, the patients with SCD have caught up with controls in height but not weight. The reason for this poor weight gain is not understood but is likely to represent increased caloric requirements of elevated resting energy expenditure related to anemia, increased bone marrow activity, and cardiovascular compensation.[555] In a second large prospective study in Jamaica, both height and weight were significantly lower than that of age- and sex-matched controls as early as 2 years of age.[556] A more recent study reveals that body mass index (BMI) percentiles for children with SCA in New England are higher than historically reported, mimicking the rise in BMI in the general pediatric population. The odds of being overweight or obese were increased in the children with higher baseline Hb levels.[557]

Zinc deficiency has been suggested as a cause of poor growth in children with HbSS disease[558,559] but has not been confirmed.[560] Levels of vitamins A, B6, and D have been discovered to be lower than in ethnically matched children and may relate to morbidity.[561,562] Growth hormone deficiency is a rare and reversible cause of growth delay.[563] In general growth hormone levels and growth hormone stimulation studies appear to be normal in children with HbSS disease who have impaired growth.[564] A review of nutritional studies in patients with SCA is available,[565] and one study has suggested that hyperalimentation may be useful in some patients.[566]

Sexual development is also delayed in patients with SCD.[554] This delay is found in both males and females

TABLE 20-4 Estimated Median Age at Attainment of Tanner Stages According to Hemoglobinopathy

	Tanner Stage	Median Age			
		SS	SC	S-β$^+$	S-β^0
Females					
Breasts	2	11.8	9.8	10.6	11.8
	3	13.5	11.9	12.6	12.8
	4	15	13.9	13.8	14.8
	5	17.3	16	16.5	17.2
Pubic hair	2	12	10.1	10	11.5
	3	13.5	11.8	11.2	12.8
	4	15.2	14	13.8	14.2
	5	19.2	17	17	20.8
Males					
Penis	2	12	10.4	11.9	12.4
	3	14.2	13	13	13.2
	4	16	14.1	13.5	15.5
	5	17.6	16.6	16.6	18.8
Pubic hair	2	13.2	11.5	12	13.2
	3	14.8	13.2	13	13.9
	4	16.2	14.2	13.9	16.2
	5	17.9	16.6	17.1	18.5

From Platt OS, Rosenstock MSPH, Espeland MA: Influence of sickle hemoglobinopathies on growth and development. N Engl J Med *311:7, 1984.*

S-β$^+$, S β$^+$-thalassemia; *S-β^0*, S β^0-thalassemia; *SC*, sickle cell disease; *SS*, sickle cell anemia.

and follows the same pattern as height and weight in other hemoglobinopathies. The estimated median age of attainment of Tanner stage for each hemoglobinopathy is listed in Table 20-4. For females (regardless of hemoglobinopathy), menarche status is a function of age and weight. This normal relationship between menarche and weight suggests that in females, delayed sexual maturity is constitutional. Most males have delayed sexual maturation, also suggesting constitutional delay.

Psychological Aspects

Although most patients with SCD handle their illness very well, patients and families require strong, empathetic support and screening for psychological stressors. As with any chronic disease, given the physical and psychosocial burdens that children with SCD and their families experience, it should not be surprising that they may exhibit psychological dysfunction[567-569] and impaired family functioning.[570,571] It is estimated that between one third to two thirds of children with SCD experience significant psychological symptoms and impaired health-related quality of life (HRQoL).[567,572-575] Dampier and associates[576] found that children and their parents scored significantly lower on several HRQoL domains, including general physical, motor, and independent daily functioning. Children with SCD tend to have deteriorations in social and school competence compared with healthy peers, but this is not associated with disease severity or genotype.[573]

Medical stressors in SCD not limited to pain (e.g., inability to keep up with peers, delayed growth/puberty, and fears of premature death) and psychosocial factors (e.g., low socioeconomic status, poor family functioning) will challenge a child's ability to adapt.[575] To manage these stressors, patients and families use a range of approaches and avoidance-oriented coping strategies.[577]

MORTALITY

Although much is known about the risk factors for early mortality in SCA the disease severity remains higly heterogeneous, and risk stratification of individual patients is challenging. Patients with a higher level of HbF have a longer life expectancy,[131] as do those with a low rate of pain crisis,[131] a low rate of ACS,[132] no hand-foot crisis before age 1 year,[133] no severe anemia before age 2 years,[131,133] a low baseline WBC count,[133] and low blood pressure.[321]

Leikin and colleagues examined mortality in a cohort of children in CSSCD.[578] They found that the peak incidence of death was between 1 and 3 years of age and was generally caused by infection. The overall mortality rate was 0.5 deaths per 100 patient years, and for patients with SCA, the probability of surviving to age 29 years was about 85%. For those with HbSC, the probability of surviving to age 20 years was about 95%.

As shown in Figure 20-10, when data on deaths of children from CSSCD are combined with data from adults, the median age at death was 42 years for males and 48 years for females with HbSS but 60 years for males and 68 years for females with HbSC.[131] Among adults, those with the lowest HbF levels, highest steady-state WBC counts, and highest rates of pain and chest syndrome had the highest risk of dying at an early age. There was an age-related pattern in mortality rates with a peak in the patients younger than 5 years of age and a gradual increase starting in late adolescence. Younger patients died primarily of pneumococcal disease and less often of acute splenic sequestration, ACS, or stroke.[382,579] Older patients die of ACS, during pain crises, chronic organ system failure from cancer and other specific diseases of adults, and suddenly without obvious cause.[131]

Quinn and colleagues reported a more recent experience from the Dallas Newborn Cohort[580] with an overall survival of 93.9% and an all-cause mortality rate of 0.52 per 100 patient years for children with SCA who were observed from birth through 18 years of age. In HbSC and S β$^+$-thalassemia their respective figures were 98.4% and 0.10 per 100 patient years. The Dallas cohort also showed trends of decreasing sickle-related mortality, increasing mean age at death, and a smaller proportion of deaths related to infection compared with the earlier CSSCD[192] and Jamaican[581] cohorts.

It is encouraging that coincident with the widespread use of prophylactic penicillin and conjugated vaccines in young children, early education and screening, aggressive treatment of pulmonary disease and infection, careful selection of patients for transfusion therapy, and thoughtful comprehensive care has coincided with

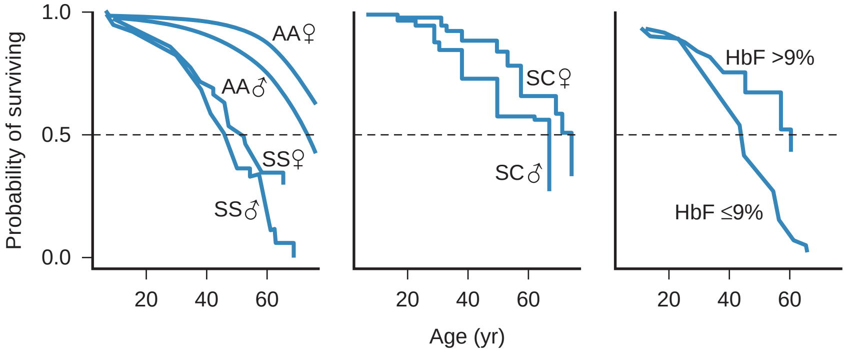

Figure 20-10 Survival of male and female patients with SS disease and SC disease, comparing those in the highest quartiles (>9%) of HbF level with the others. African-American (AA) controls are shown for comparison. *(Modified from Platt OS, Brambilla DJ, Rosse WF et al: Mortality in sickle cell disease: life expectancy and risk factors for early death.* N Eng J Med *330:1639–1644, 1994.)*

an improvement in survival among children,[582] though the long-term mortality of adults with SCD remains foreshortened.

APPROACHES TO THERAPY

The NHLBI published its fourth edition of *The Management of Sickle Cell Disease* in 2002,[411] and an update is expected.[583] The following descriptions are based on these evidence-based guidelines that reflect prospective research findings as well as expert opinion and consensus.

Routine Health Maintenance

Infants diagnosed by newborn screening should be referred to pediatric hematologists for confirmation of the diagnosis and genetic counseling. Health maintenance visits to a pediatric hematologist should occur on a routine basis (Table 20-5). In the first year after diagnosis they should be scheduled for every 3 months with a focus on intensive parental education and provision of written materials. Subsequent routine visits should occur every 6 months, although higher frequency visits may be required for patients with increased educational needs, accumulated complications, and therapeutic monitoring (e.g., hydroxyurea and/or chronic transfusion therapy). Regular visits allow the practitioner to record baseline physical findings (e.g., vital signs, SpO_2, icterus, murmurs, organomegaly) and baseline laboratory studies (e.g., complete blood count, reticulocytes, chemistry). These baseline data are extremely important in the recognition of an acute complications, and routine visits afford the opportunity for education and anticipatory guidance.

Careful evaluation of the interval history may provide insight into factors that provoke painful crises. Continuing disease counselling of patients and families regarding basic pathophysiology, avoidance of triggers, and early recognition/home treatment of the wide variety of complications of SCD will reduce the morbidity and hospitalization rate and will promote the patient's and family's sense of independence. Disease education should also include genetic counseling of the parents with the goal that they understand the risks of SCD in future pregnancies.

Careful attention is paid to prevention of bacterial infections including vaccinations and prophylactic penicillin (see Prevention of Infection). All children with SCD should receive the complete routine immunization schedule according to the Advisory Committee on Immunization Practices (ACIP)[418] that includes conjugated pneumococcal, conjugated *H. influenzae,* and hepatitis B vaccines. Additional vaccines including the 23-valent pneumococcal polysaccharide vaccine (Pneumovax; PPV23), seasonal influenza vaccine, and conjugated meningococcal vaccine (Menactra; MCV4) should be administered. Prophylactic penicillin should be given to all children younger than 5 years of age.[414] Most importantly the patient and family should be educated about the importance of early detection, medical evaluation, and empirical treatment of a possible bacterial infection triggered by a temperature of 101.3°F (38.5°C) or higher.[411]

Although supplemental folic acid (1 mg/day) is recommended in chronic hemolytic anemias to prevent folate deficiency and maintain effective erythropoiesis, no definite advantage has been seen in children with SCA.[584] Given that the United States has been fortifying foods with folic acid for more than 20 years as part of a public health effort to prevent neural tube defects, the need for additional supplementation is less compelling. Iron supplements are used only when iron deficiency is documented or when blood loss is chronic (e.g., hematuria).

Screening for renal disease should begin by age 10 years with assessment for microalbuminuria and proteinuria. If the result is negative, it should be repeated annually thereafter. If the result is abnormal, confirmation testing on first morning void within 1–2 weeks is suggested. If the repeat is abnormal, referral to a pediatric nephrologist should be made for consideration of ACE inhibitor therapy.

Screening for cardiovascular disease is limited to measurement of blood pressure with standard treatment for hypertension based on appropriate normal ranges for children. Routine screening electrocardiogram is not recommended for children. Screening aymptomatic children for elevated TRJV by echocardiography is controversial, and prognostic implications differ from adults; therefore screening recommendations in the pediatric age group have not been established.[448,449]

TABLE 20-5 Routine Health Maintenance for Patients with Sickle Cell Anemia*

	Birth	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Visit Frequency	Q 3 mo	Q 6 mo	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Vital signs (including BP and SaO_2)	Every visit	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Physical examination	Every visit	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Laboratory																						
CBC, differential, Reticulocyte Count	▨	Q 6 mo	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
RBC antigen typing	▨																					
Hb electrophoresis	▨	Q 2-3 yr	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
AST/ALT/LDH, BUN/Cr		Q 6 mo	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Urinalysis/Microalbumin											Annual	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Special Screening Studies																						
Transcranial Doppler ultrasound			Annual	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨					
Pulmonary Function Testing											▨											
Ophthalmology Examination											Annual	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Treatment																						
Folic Acid	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Penicillin prophylaxis	▨	▨	▨	▨	▨	▨																
Hepatitis vaccine	▨																					
Pneumococcal vaccine			▨			▨																
Meningococcal vaccine			▨																			
Influenza vaccine	Annual	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Anticipatory Guidance/ Education																						
Disease education—Parent (Pathophysiology, Inheritance)	▨	▨	▨	▨	▨	▨	▨															
Disease education—Child (Pathophysiology)						▨	▨	▨	▨	▨	▨	▨	▨									
Disease Empowering—Teen/Young Adult												▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Treatment options (Hydroxyurea, Stem Cell Transplant)																						
Medication adherence (penicillin, hydroxyurea)	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Fever/Thermometer	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Hydration/Cold exposure	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Aplastic Crisis	▨	▨	▨	▨	▨	▨																
Outpatient pain plan		▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Splenic sequestration	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨											
Priapism (males)						▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Self-esteem						▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Puberty/Growth												▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨

TABLE 20-5 Routine Health Maintenance for Patients with Sickle Cell Anemia (Continued)

	Birth	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Fertility/Contraception/ Inheritance															▨	▨	▨	▨	▨	▨	▨	▨
Prognosis															▨	▨	▨	▨	▨	▨	▨	▨
Transition to Adult Hematologist																				▨	▨	▨
Social																						
Parent Groups	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨											
Patient Groups												▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
School Performance						▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
College/Career Planning															▨	▨	▨	▨	▨	▨	▨	▨

ALT, Alanine transaminase; *AST,* aspartate transaminase; *BP,* blood pressure; *BUN,* blood urea nitrogen; *Cr,* creatinine; *LDH,* lactate dehydrogenase; SaO_2, oxygen saturation; *Q,* every.

*Shading indicates the time when the specified health maintenance should be performed.

Screening for primary stroke risk in SCA performed by specially trained ultrasonographers using TCD ultrasonography according to the STOP criteria is recommended annually. In children with conditional TCD values (170 to 199 cm/s) the frequency of screening should be increased. If TCD values are abnormal (>200 cm/s), referral to a specialist with expertise in chronic transfusion therapy aimed at preventing stroke is strongly recommended. Routine neurologic screening of asymptomatic children with SCA by brain MRI or CT is not recommended.

Screening for ophthalmologic complications with dilated eye examinations to evaluate for retinopathy should begin at age 10 years. A normal dilated retinal examination should be repeated at 1 to 2 year intervals; however, those with suspected retinopathy should be referred to a retinal specialist to establish the tempo of the lesion and to determine appropriate therapy.

Birth control options should be discussed as with any adolescent patient. Progestin-only contraceptives (pills, injections, and implants), levonorgestrel intrauterine devices (IUDs), and barrier methods have no restrictions or concerns for use in women with SCD.[585,586] If the benefits are considered to outweigh the risks, combined hormonal contraceptives (pills, patches, and rings) may be used in women of childbearing age.

Painful Crisis Management

Most painful crises can be successfully treated at home with increased oral fluid intake, heat to the affected area, and oral analgesics. If the pain is too severe for oral analgesics or if the patient cannot maintain adequate fluid intake, the episode must be treated in the hospital setting (day hospital, emergency room, or inpatient). Hospital management of painful crises must begin with a careful pain history, including recent analgesic use and consideration of nonsickle causes of pain, particularly if the pain is atypical by report of the patient. The mainstays of therapy are local strategies (e.g., heat, massage), IV hydration, and parenteral analgesia. In dehydrated patients, the fluid deficit should be corrected and then the IV fluid rate be set at 100% to 125% maintenance using a relatively hypotonic maintenance solution for age (e.g., D5 1/2 NS or D5 1/4 NS). However some patients cannot tolerate this expansion of intravascular volume, and the hydration rate may need to be tempered, particularly if oral fluid intake is adequate. Frequent monitoring of vital signs during fluid administration is essential to prevent pulmonary complications or cardiac compromise. If the patient is receiving primarily IV fluids, serum electrolyte levels should be checked early and fluid solutions adjusted appropriately. Although theoretically sound based on pathophysiology, the use of alkaline IV fluids has not been shown to be effective in uncomplicated painful crisis.[587]

Face-mask oxygen is of little therapeutic value unless the patient is hypoxic. Furthermore Embury and colleagues have shown that continuous supplemental oxygen can suppress erythropoiesis, with reticulocyte counts falling within days.[588] In addition painful crisis may result from the rebound marrow activity after the abrupt discontinuation of oxygen therapy.[589]

Analgesic selection should be based on pain assessment, associated symptoms, recent outpatient analgesic use, patient knowledge of effective agents and doses, and past experience with side effects. Analgesia must be sufficient to control the pain. Ideally an individualized prescribing and monitoring protocol provided by the patient's sickle cell provider or an SCD-specific practice guideline is available to promote rapid, effective, and safe analgesic management.[590] A general approach to analgesic choice should follow the World Health Organization (WHO) Pain Relief Ladder.[591] An initial trial of oral analgesics is reasonable if none was taken before presentation, with parenteral nonopioids (e.g., the nonsteroidal antiinflammatory drug [NSAID] ketorolac) as a common second tier. For patients in severe pain, rapid initiation of parenteral opioids is indicated, although standard doses may not be adequate to relieve an individual patient's pain, especially if the patient is not opioid naïve on presentation; therefore dosing should be titrated to relief. Analgesics and other adjuvants may be combined to optimize efficacy/safety.

One should choose opioids with knowledge of their side effects, drug interactions, serum half-lives, and previous efficacy. Difficult IV access may necessitate alternate routes of administration (e.g., intranasal, subcutaneous, transdermal) and may require adjustments for equianalgesic dosages. Serial intramuscular injections should be avoided and can lead to the formation of sterile abscesses. Meperidine is contraindicated, because accumulation of the toxic metabolite normeperidine may cause seizures, particularly in the setting of established cerebrovascular disease or compromised renal function.[592]

At the onset of inpatient therapy for severe pain, regular dosage intervals based on half-life should be used instead of an "as-needed" dosage strategy. This approach may avoid the recurrence of pain and anxiety as a result of delays in obtaining the next dose of medicine after being requested. Continuous IV opioid therapy in children has been successfully used,[593] but ACS may be precipitated by respiratory depression.[594] Perhaps an optimal approach is the use of patient-controlled analgesia[595,596] initiated in the emergency department[597] to provide adequate pain relief at a lower total opioid dose.[598] Continuation of NSAID therapy is helpful to most patients. Some have benefitted from nonpharmacologic adjuncts[599] such as acupuncture,[600] biofeedback,[601] cognitive therapy,[602] and hypnosis.[603]

Adverse effects of opioid analgesics include histaminergic (pruritus, hives), nausea, vomiting, constipation, and respiratory depression. Hypoventilation with progression to ACS is the most serious side effect of opioid therapy and must be fastidiously avoided by aggressive use of incentive spirometry,[604] encouragement of early ambulation, careful monitoring of SpO_2, and use of oxygen if the patient becomes hypoxic. In one study, epidural anesthesia was successfully used to alleviate pain and maintain or improve oxygenation.[605] Constipation is a common problem that should be anticipated, and stool softeners/cathartics should be started concommittently with opioids. Oral antihistamines are useful for the treatment of opioid-induced pruritus. Antinauseants and antiemetics may provide some relief if these side-effects are experienced. Urinary retention is not uncommon and may require bladder catheterization.

Sometimes it is virtually impossible to make a patient entirely comfortable with safe doses of opioids. This frustrating situation needs to be approached with care and sensitivity and with involvement of every member of the patient-care team.[606,607] For the rare patient who is incapacitated by recurrent episodes of pain and who spends more time in the hospital than out, chronic outpatient opioid therapy may be useful.[608] Although transfusion (even exchange transfusion) is rarely effective for acute pain, a brief maintenance program may facilitate completion of a school year or an important project at work; however, this must be tempered by risks of allosensitization, iron overload and bloodborne infection. Hydroxyurea is effective at decreasing the frequency of recurrent painful crises, but its favorable effect may be delayed by months or until the maxium tolerated dose (MTD) is reached.[243]

Although opioid dependence or opioid-seeking behavior may be a rare problem for pediatric patients, overestimation of the incidence of this unusual situation must not affect the decision to control severe pain.[609] It is very unfortunate that subtle misconceptions interfere with providing appropriate pain relief. In one study, standardized patients with a chief complaint of pain who gave histories of frequent pain crises were given less opioid by nurses than the identical patients whose scripts included a history of infrequent pain episodes.[610]

Reduction in Polymer Formation

Pharmacologic Induction of Hemoglobin F

Increasing the amount of HbF will decrease the intracellular concentration of HbS and increase the delay time and solubility of HbS, which slows polymer formation and sickling. On the basis of epidemiologic data that showed that increases in HbF from 4% to 16% could decrease vaso-occlusive crises by 50%,[152] as well as numerous CSSCD studies that showed HbF to be the single most predictive factor of clinical severity and even survival, one therapeutic approach given considerable effort over the last 30 years has been the pharmacologic induction of HbF beyond the fetal and newborn period. Several pharmacologic agents have shown promise including demethylating agents such as 5-azacytidine[611] and decitabine,[612-614] and short-chain fatty acids such as butyrate,[615-618] but each has limitations in route of administration, safety, or sustained efficacy. Hydroxyurea, first introduced by Platt and co-workers,[619] has emerged as the single therapeutic agent to gain Food and Drug Administration (FDA) approval for the treatment of SCA, although it is approved only for adults with severe disease.

Hydroxyurea may be an ideal therapeutic agent for use in children with SCA. It has excellent bioavailability after oral administration; requires only once-daily dosing, which improves medication adherence; has few if any immediate side-effects; has predictable hematologic toxicities that are dose-dependent, transient, and reversible; and has sustained benefits against multiple pathophysiologic mechanisms of SCA, including potent HbF induction.

In patients with hemoglobinopathies, the myelosuppressive and cytotoxic effects of hydroxyurea appear to alter erythroid kinetics by inducing erythroid regeneration and the premature commitment of erythroid precursors, with resulting increased production of HbF-containing reticulocytes (F-retics) and total HbF.[620] Additional pharmacologic effects of hydroxyurea that may contribute to its beneficial effects in SCA include increasing erythrocyte HbF through NO-dependent pathways, decreasing the neutrophil count, increasing erythrocyte volume and hydration, increasing deformability of sickle erythrocytes, and altering the adhesion of sickle erythrocytes to the endothelium.[619-623] The potential release of NO directly from the hydroxyurea molecule[624,625] could also provide beneficial local effects on the endothelium, thereby ameliorating the vaso-occlusive process and limiting vascular dysfunction.

Pilot trials in patients with SCA demonstrated that hydroxyurea increased HbF and caused little short-term toxicity.[619-622] In addition to increasing HbF, hydroxyurea was noted to reduce the proportion of dense cells and increase the MCV and MCHC of sickle cells.[619-621,626] On the basis of these favorable results, the NHLBI sponsored the pivotal Multicenter Study of Hydroxyurea (MSH) in adults with SCA.[243] This trial was terminated early when it was obvious that vaso-occlusive crises, ACS, transfusions, and hospitalizations were reduced by almost 50% in the hydroxyurea-treated group compared with controls. However increases in HbF alone did not predict the decrease in painful crises seen in this double-blinded placebo-controlled randomized trial.[243] Multiple factors such as perturbation of WBC or cytokine levels, changes in RBC adhesion to vascular endothelium (see Fig. 20-1), or alteration of NO/intravascular Hb equilibrium may also play a role in hydroxyurea-related decrease in crises.[627,628]

A 9-year follow-up observation of adult MSH subjects taking hydroxyurea revealed an impressive 40% reduction in mortality,[629] and a subsequent assessment after 17.5 years of follow-up study revealed that hydroxyurea continued to be associated with improved survival and without accompanying serious adverse events.[630] In 2008 an NIH Consensus Development Conference was commissioned and performed a systematic review of the efficacy, effectiveness, toxicity, and barriers to the use of hydroxyurea in SCD. The conclusion was, "Strong evidence supports the efficacy of hydroxyurea in adults to decrease severe painful episodes, hospitalizations, number of blood transfusions, and the ACS. Although the evidence for efficacy of hydroxyurea treatment for children is not as strong, the emerging data are encouraging."[631]

After small-scale trials of hydroxyurea therapy in children demonstrated that the drug could be given safely,[296,632,633] the first pediatric multicenter phase I/II trial of SCA (HUG-KIDS)[634] revealed similar hematologic effects as seen in the MSH trial with improved anemia (increased Hb and decreased reticulocytosis) as well as diminished hemolysis (decreased LDH and decreased total bilirubin), macrocytosis, improved erythrocyte hydration, and striking increases in %HbF and F cells. Laboratory toxicities were mild and reversible with no life-threatening clinical events observed. Subsequent evaluation of the HUG-KIDS cohort revealed no adverse effects on growth or pubertal development,[635] and predictors of HbF response included compliance and higher baseline HbF, as well as higher Hb, WBC, and reticulocytes that allowed a higher MTD.[636] Five- and 10-year follow-up studies of hydroxyurea in children with SCA continue to demonstrate increased HbF with minimal side effects and improved growth parameters.[637] It is important to note that in the only large study of 122 children in which MTDs were obtained in the same fashion as in the adult MSH study, very few if any nonresponders were noted. Hydroxyurea therapy at MTD is well tolerated and demonstrates sustained hematologic efficacy with apparent long-term safety.[638]

The efficacy of hydroxyurea to prevent acute complications of SCA in infants as young as 9 months old has recently been evaluated; BABY HUG was the phase III multicenter double-blinded placebo-controlled randomized trial of hydroxyurea in infants as young as 9 months. This study demonstrated the efficacy of hydroxyurea to reduce the rate of acute complications including pain and dactylitis, with additional evidence for decreased hospitalizations, transfusions and ACS, while also demonstrating safety with no increased risk of infection or serious adverse events.[244]

The role of hydroxyurea in preserving organ function in SCA has not yet been determined. From a practical standpoint, these beneficial effects are difficult to assess prospectively because organ damage develops broadly over the whole pediatric age range, beginning with splenic and renal changes in infancy and evolving to pulmonary and neurologic deficits with vasculopathy among older children. In the open-label Hydroxyurea Safety and Organ Toxicity (HUSOFT) study, SCA infants had preserved splenic filtrative function compared with historic controls,[639] and a follow-up study on this cohort identified preservation and even apparent gain of function in some cases.[393] Similar beneficial effects of hydroxyurea on organ function have now been reported anecdotally, usually single-institution experiences for children with proteinuria,[486] priapism,[342,351] hypoxemia,[640] PHT,[641] glomerular hyperfiltration,[642] neurocognitive delay,[643] elevated TCD velocities,[644] primary stroke prevention,[644,645] and secondary stroke prevention.[297] Based on these encouraging but uncontrolled reports, several prospective multicenter randomized trials using hydroxyurea to prevent organ dysfunction in SCA are currently under investigation. Practical guides to initiation and monitoring of hydroxyurea therapy in children have been published,[646,647,648] and the upcoming NHLBI guidelines have strongly worded recommendations about offering hydroxyurea to all young patients with SCA.

Decreased Cell Hemoglobin S Concentration

Because the polymerization of HbS is exquisitely concentration dependent, reduction of MCHC by swelling the cell with water has been considered as a therapeutic maneuver. Two approaches have been tried: lowering plasma osmolarity and increasing intracellular cations. The first approach was used by Rosa and colleagues,[649] who successfully reduced the duration and frequency of painful crises by lowering the MCHC using dietary salt restriction and desmopressin. Subsequently others have found this therapy too difficult to maintain, neurologically toxic, and ineffective.[650-652] The second approach focused on the property of various membrane-active agents to increase intracellular cations. Some of the interesting agents include the antibiotics monensin[653] and gramicidin[654]; calcium channel blockers nitrendipine, nifedipine, and verapamil[655]; and the peripheral vasodilator cetiedil,[656] but none has reached clinical trial. As discussed previously, preliminary evidence suggests that blocking the Gardos channel with clotrimazole[657] and blocking K^+,Cl^- cotransport with oral magnesium[658] may decrease HbS concentrations by preventing erythrocyte dehydration. Only the Gardos channel inhibitor senicapoc entered formal clinical trials, and although it demonstrated substantial effects on erythrocyte hydration,[90] the definitive phase III trial demonstrated no efficacy for reducing vaso-occlusive events.[91]

Increased Hemoglobin S Solubility

The most straightforward approach to preventing the polymerization of HbS is to attack the $\beta^{6\ \text{valine}}$ that is the key to polymer formation. Unfortunately this is an extremely unreactive residue, so efforts to increase the solubility of HbS have hinged on the importance of the other intermolecular contacts. Reagents that increase deoxy-HbS solubility by noncovalent interactions with HbS have been recognized. Chang and colleagues[654] compared the effects of 15 antisickling agents on HbS solubility, oxygen affinity, and cell sickling. Urea is an effective agent but requires concentrations greater than 200 μM, too high for in vivo use. The best studied covalent modifier is cyanate, but clinical trials have not been promising. Unfortunately despite the encouraging clinical and in vitro data, this compound is not usable because of significant neurotoxicity[659] and cataract formation.[660] A major problem with covalent reagents is that they interact with a wide variety of functional groups found in many molecules other than Hb.

Decreased Dwell Time

Any manipulation that would allow a cell to traverse a vessel faster than its delay time will prevent occlusion of that vessel. Therapies aimed at reducing endothelial adherence have only recently been successful. Attempts to develop a vascular lubricant include purified poloxamer 188, a nonionic block copolymer that improves micrvascular blood flow by reducing viscosity and adhesive frictional forces.[661] This has led to an ongoing phase III study of poloxamer 188 to reduce the duration of vaso-occlusive crisis in SCD.[662] Recognition of the role of leukocyte adhesion to the vascular endothelium in the process of vaso-occlusion led to promising investigations of selectin blockade with synthetic glycomimetic molecule[663] and monoclonal antibody[135] in murine models. These have led to initiation of phase II multicenter, randomized, placebo-controlled trials for the treatment/prevention of vaso-occlusive pain.[664-666] Rodgers and co-workers[667] showed that microcirculatory flow in patients with SCD has an unusual periodic pattern, indicating abnormal vasomotor control. Recent work indicates that free plasma HbS binds NO, thus reducing NO availability to maintain vasodilation[125,668] and could lead to increased vasocontriction and dwell time (see Fig. 20-1). Efforts to increase NO bioavailability with use of inhaled NO in a prospective, multicenter, double-blind, randomized, placebo-controlled clinical trial did not improve the time to crisis resolution.[669] Intravascular hemolysis also consumes arginine, the obligate substrate for NO production, thereby contributing to decreased NO bioavailability.[670]

Transfusion

Transfusions improve oxygen-carrying capacity, alleviate anemia, and decrease the proportion of erythrocytes that carry HbS. Although transfusion is typically not used for the treatment of chronic anemia or acute painful crises, it is often necessary for associated comorbidities.[671] Current transfusion practices include episodic or chronic regimens depending on whether the risk reduction for severe clinicopathologic sequelae from SCD (e.g., stroke or recurrent ACS) outweighs the risks inherent to a chronic transfusion regimen.

Episodic transfusions (simple or exchange) are used primarily to treat acute complications of SCD. Simple transfusion is possible when the anticipated posttransfusion Hb will not exceed 10 to 11 g/dL. Sickle trait blood should not be used because of difficulties in monitoring the response by electrophoresis. Exchange transfusion (either by manual technique or automated erythrocytapheresis) is reserved for complications that are either life-threatening or pose risk for irreversible tissue damage (e.g., stroke or severe ACS) or when initial simple transfusion was ineffective. The primary goal of exchange transfusion is to reduce the HbS concentration rapidly to less than 30% of the total Hb content without increasing blood viscosity or expanding the blood volume, thereby reducing potential cardiovascular strain. In those centers in which an automated cell separator is available, a two-volume exchange transfusion can be efficiently accomplished in less than 90 minutes.[347,348] Episodic simple transfusion is indicated for the management of symptomatic episodes of acute anemia (e.g., aplastic crisis triggered by parvovirus infection), severe/progressive ACS, acute stroke, splenic sequestration, priapism unresponsive to medical therapy, and perioperatively.

Chronic transfusion therapy is implemented when the ongoing risk of morbidity from SC complications outweighs the risks inherent to recurrent blood exposure. In a chronic regimen, transfusions are usually scheduled every 4 weeks and use simple or exchange methods with the goal of maintaining the HbS level lower than 30%. Chronic transfusion therapy has been indicated for primary stroke prevention in children identified by abnormal TCD velocity (>200 cm/s) and for secondary stroke prevention in children who have already experienced an overt stroke. Limited duration regular transfusions may be indicated for reducing the frequency and severity of ACS, complicated pregnancy, prolonged hematuria, and chronic vaso-occlusive complications (e.g. nonhealing leg ulcer). Routine pain is not an indication for chronic transfusion therapy.

Chronic transfusion regimens in SCD may increase the risk of transfusion-associated complications of allosensitization, infection, and progressive iron overload. Transfusional iron accumulation is unavoidable, because there is no physiologic mechanism for iron removal. Excess iron is deposited in multiple organs including the liver, heart, pancreas, endocrine glands, and skin, with potential for organ damage, and can go clinically undetected and undertreated if not anticipated. Fortunately iron chelators including parenteral deferoxamine,[672,673] oral deferasirox,[674] and oral deferiprone are available and effective if adhered to as prescribed. Serial assessment of serum ferritin levels may provide insight into ongoing iron balance in SCD[675,676]; however, because infections and vaso-occlusive crises also increase ferritin levels, it is an inaccurate predictor of liver iron burden. MRI estimation of hepatic iron has largely replaced liver biopsy for hepatic iron quantification. MRI has the additional benefit of noninvasively quantifying extrahepatic iron, particularly cardiac iron in transfusional hemosiderosis.[677] The pathophysiology of tissue iron loading in chronically transfused patients with SCD appears to be different than in other transfusion-dependent anemias such as thalassemia; specifically cardiac and endocrine iron loading are not as common in SCD,[678,679] and high levels of liver iron were not associated with as much liver disease compared with similar levels in thalassemia.[680]

The incidence of erythrocyte alloimmunization in the general U.S. population has been estimated to be approximately 0.5% to 1.5%. In contrast the incidence of alloimmunization in patients with SCD ranges from 18% to 76% with ABO and D matching alone; 5% to 14.5% with limited phenotype matching for C, E, and K antigens; and 7% for extended minor RBC antigen-matching beyond C, E, and K.[681-683] The likelihood of alloimmunization is related to the number of transfusions, the racial differences between common donor and recipient erythrocyte phenotype, possibly the age of the recipient, and as yet unknown genetic factors that control the responsiveness of the recipient to transfused antigens.[292,684] Opinions and practices differ regarding the utility of preventing sensitization using antigen-matched cells in all recipients, in contrast to only providing matched cells after sensitization occurs.[685,686] The most common antibody-provoking antigens are C, E, K, Jk^b S, Fy^a, and Fy^b.[684] Ideally patients with SCD should receive antigen-matched red cells beyond ABO and D to include C, E, and K to reduce the hazard of sensitization,[686] and such a strategy has been demonstrated prospectively to reduce sensitization.[687] Some suggest further extension of phenotype matching,[688,689] but the added expense and relative scarcity of the required random donors makes this impractical.[686] Despite C, E, and K matching, many patients with SCD continue to form alloantibodies with common Rh specificities (D, C, E, c, e). Some cases can be blamed on transfusion at outside institutions without extended crossmatching,[683] whereas others have observed higher-than-expected rates of allosensitization despite matching donors with C, E, K, and ethnicity because of unusually diverse Rh system variants revealed by genotyping.[690]

Potential benefits from attempting to prevent allosensitization include prevention of complications from the first delayed transfusion reaction and possibly the prevention of autoantibodies that appear in the same context. Clinically significant autoantibodies do occur in about 10% of patients with SCD, and the incidence appears to be higher in those with alloantibodies.[684,689,691] In 183 children the incidence of autoantibodies was 7.6% ,and when associated with C3 on the red cells, resulted in hemolysis.[293] However many patients with SCD who develop apparent autoantibodies to their Rh antigens using routine serologic reagents may actually have developed Rh alloantibodies, because they harbor RH gene variants detectable by genotyping.[692]

Hematopoietic Stem Cell Transplantation

The only current curative approach to SCD is hematopoietic stem cell transplantation (HSCT). HSCT from sickle trait or normal human leukocyte antigen (HLA)-identical siblings with have been performed in over 250 young patients.[693,694] Preparative regimens have been primarily myeloablative (e.g., busulfan and cyclophosphamide) and involve a spectrum of graft-versus-host disease (GVHD) prophylaxis including antithymocyte globulin, cyclosporine, corticosteroids, irradiation, and methotrexate. HLA-identical sibling HSCT for SCD has a reported overall survival of 93% to 97%, event free survival of 82% to 85%, and rejection/recurrence of SCDs of about 10%.[695] More recently among 87 consecutive patients treated with myeloablative regimens, addition of antithymogyle globulin reduced rejection from 23% to 3%.[696]

Transplant complications have included CNS hemorrhage (particularly in patients with history of stroke), hypogonadal function, graft rejection, and acute or chronic GVHD.[693,696-698] Prophylactic anticonvulsant treatment and maintenance of a high platelet count may result in reduced CNS morbidity.

In patients who have stable donor engraftment, complications related to SCD resolve, and there are no further episodes of pain, stroke, ACS, or sickle-related CNS complications. Stabilization or reversal of SCD-related organ damage has been documented after HSCT.[547] There is evidence of stabilization of CNS disease by MRI,[696] but reversal of cerebral vasculopathy has been variable.[699,700] Improvements in other end-organ function has been observed, including radiologic improvement of AVN of the humeral head[701] and return of splenic phagocytic function.[702] In a recent summary of 53 children with SCA undergoing bone marrow transplant, growth was not impaired in younger children, but diminished growth occurred in patients who received transplants near or during an adolescent growth spurt.[703]

Although HSCT offers the potential for cure, barriers to its wider adoption include relative paucity of HLA-identical sibling donors, eligibility restrictions, and the high risks of regimen-related infertility as well as lower risks of GVHD, mortality, and secondary malignancy. Several novel approaches to improve the safety and availability of HSCT aim to decrease the associated toxicity and expand the pool of potential donors. These approaches include nonmyeloablative or reduced-intensity conditioning regimens[704,705] and the use of haplo-identical[706-708] and HLA-matched unrelated donors.[325]

Gene Therapy

The most direct approach to a cure for SCD may lie in the correction or replacement of the patient's own defective β-globin gene with a normal gene. Genetically engineering autologous hematopoietic stem cells offers two major benefits over HSCT: these stem cells eliminate the need to find a HSCT donor and eliminate the risks of GVHD and immune-mediated graft rejection.

Gene therapy for the β-hemoglobinopathies has been explored as a promising avenue in multiple preclinical and a few clinical studies. The design of integrative vectors for human β-globin gene transfer has been difficult. The genetic elements required for high erythroid specific expression include the β-globin gene with its introns, promoter, and β-locus control region (β-LCR). However murine models of β-thalassemia and SCD have been corrected by using lentiviral vectors encoding either a human γ-globin gene[709] or a normal or modified β-globin gene designed for antisickling activity.[710-712] An ongoing clinical trial in France for patients with SCD or β-thalassemia has reported its first successful case in which the lentiviral vector encodes a modified β-globin with antisickling properties.[713]

Rather than relying on the random insertion of a normal β-globin gene and the potential for insertional mutagenesis and malignant transformation, taking advantage of genomic editing tools is another avenue of onging investigation. Transplantation of embryonic stem cells or induced pluripotent stem cells from donor fibroblasts after homologous recombination replaced the human β^S gene with a normal human β-globin gene has led to the correction of the sickle mutation in transgenic mice.[714,715] The advent of even more precise and specific genomic editing tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based ribonucleic acid (RNA)-guided DNA endonucleases[716] may allow for even more precise correction of the DNA base pairs underlying the sickle mutation.

In contrast to other gene therapy strategies involving the insertion of a normal γ or β globin that requires expression of the transgene to compete with and exceed the endogenous mutant β^S-globin production, another approach under consideration would not alter the β-globin locus but rather take advantage of the normal regulatory machinery that controls the γ-to-β–globin switch that occurs in hematopoietic development. This could be accomplished by delivery of a short-hairpin (sh) RNA-targeting knock-down of BCL11A[164] or genomic editing/disruption of the known intronic *BCL11A* enhancer elements[717] in hematopoietic stem cells. The reduction of BCL11A would result in concurrent increased γ-globin and reduced mutant β^S globin expression and the potential for greater clinical impact by simultaneously harnessing the antisickling effects of HbF and silencing the mutated β^S-globin.

Preparation for Surgery

Children with SCD have increased likelihood of undergoing surgical procedures, in particular cholecystectomy, splenectomy, tonsillectomy/adenoidectomy, and joint replacement. The CSSCD found that 7% of all deaths among patients with SCD were related to surgery.[131] In 1079 surgical procedures on 717 patients of all genotypes the mortality within 30 days of the procedure was 1.1% (12 deaths); however no deaths were reported in children under 14 years of age.[718] That same series illustrated comparable complication rates among SCD genotypes and suggested that patients undergoing regional anesthesia were at higher risk of complications than those undergoing general anesthesia.[718]

The CSSCD "natural history" study did not definitively address the important question of the effectiveness of preoperative transfusion. In a review of records from Dallas Children's Hospital, pulmonary complications were particularly common among patients undergoing laparotomy, thoracotomy, or tonsillectomy and adenoidectomy who did not receive preoperative transfusions (9/29), compared with those who did receive transfusions for those procedures (0/8).[719] Vichinsky and collaborators in the Preoperative Transfusion in Sickle Cell Disease Study Group reported the findings of a prospective, multi-institutional study that randomized over 600 patients with HbSS to preoperatively receive either simple transfusion designed to increase the total Hb to 10 g/dL or exchange transfusion designed to decrease HbS% to less than 30 and increase total Hb to 10 g/dL.[720-724] Several key points emerged from that study:

- There was no significant difference between the two transfusion strategies in terms of complications; however, both strategies reported a high 30% to 35% postoperative complication rate.
- Transfusion-related complications were twice as common (14%) in the exchange transfusion arm, compared with the simple transfusion arm (7%).
- The most common and serious complication was ACS, which occurred in 10% of procedures in both arms. The average time at onset was postoperative day 3; the average length of episode was 8 days; 11% of the patients required intubation, and two died.
- Those with higher surgical risk category, history of pulmonary disease, incidence of ACS, and frequency of hospitalization were at higher risk of complications.

These data suggest that at least a simple transfusion designed to raise the preoperative Hb to 10 g/dL should be given to all patients with SCD before surgery.

The multicenter, unblinded randomized Transfusion Alternatives Preoperatively in SCD (TAPS) trial compared the outcomes of preoperative transfusion versus no preoperative transfusion in patients with SCA undergoing low-risk or medium-risk surgery.[725] The study was terminated prematurely because there was a significantly lower rate of perioperative complications in the transfused group (5/34, 15%) than the untransfused group (13/33, 39%). Two major questions remain: what is the best preoperative management for low-risk procedures, and what is the best preoperative management for patients with Hb too high for simple transfusion (e.g., HbSC or S β^+-thalassemia)?

In addition to transfusion, perioperative morbidity can be minimized safely by careful collaboration between hematologist, anesthesiologist, and surgeon during the perioperative period. Those with prior pulmonary dysfunction, ACS, or history of multiple hospital admissions should receive a minimum of overnight maintenance IV hydration and be monitored perioperatively as high-risk patients. During the procedure and in the postoperative period all patients should have compulsive attention paid to oxygenation, ventilation to prevent atelectasis, hydration, maintenance of warm body temperature, acid-base balance, and positioning that does not impair circulation.[718,726-731] Choice of an anesthetic agent is not as critical as the care with which it is delivered.[728]

OTHER SICKLE SYNDROMES

Sickle Cell Trait ($\alpha_2\beta_2$, $\alpha_2\beta_2^{6\ \text{Val}}$)

Sickle cell trait is the term describing individuals who are heterozygous carriers for the HbS mutation. Sickle cell trait is estimated to affect up to 3 million Americans and hundreds of millions of persons worldwide[732] but is generally not associated with increased morbidity or mortality under normal physiologic conditions.[733-737] There is no associated anemia, abnormal erythrocyte morphology, or decreased red cell survival. Most individuals have approximately 40% HbS and 60% HbA; however, comorbid α-thalassemia can reduce the HbS fraction to 28% to 35%.[738] Decreased levels of HbS can also be seen in patients with comorbid iron[739] and folate[740] deficiencies. Growth, behavior, and educational achievement and pregnancy risks in children with sickle trait are entirely normal.[741-743] Patients diagnosed with sickle trait but developing a clinical and laboratory phenotype consistent with a significant sickling phenotype should be considered for molecular globin diagnostics to assure accuracy of diagnosis.[744,745]

Under certain extreme conditions, however, sickling and even death may occur in individuals with sickle cell trait. These include severe pneumonia,[746] unpressurized flying, and exercise at high altitudes.[747,748] The risk of sickle trait in routine commercial aviation has been overstated.[749] Sickle cell trait has been associated with medical complications[750] including loss of urinary concentrating ability (hyposthenuria),[751] papillary necrosis with persistent hematuria,[452] and rarely renal medullary carcinoma.[469] Sickle cell trait may be a risk factor for end-stage renal disease; however, data is conflicting.[750,752,753] Under extreme conditions such as high altitudes, severe left upper quadrant pain associated splenic infarction can occur.[754] Sickle cell trait has been associated with increased risk of venous thromboembolism.[755] Women with sickle trait have shown an increased risk for preeclampsia; however, their infants showed a minimal decrease in gestational age and birth weight and no increased infant mortality.[756] Traumatic hyphema in sickle trait is a medical emergency, because in the hypoxic conditions of the anterior chamber of the eye HbAS erythrocytes sickle with mechanical obstruction of aqueous humor outflow and development of increased intraocular pressure.[757,758] Careful general anesthesia does not carry a great risk for the individual with sickle trait,[759,760] but tourniquet surgery[728] and deep hypothermia should be avoided.

In a retrospective study of basic training in the U.S. Armed Forces, Kark and colleagues noted a 27 times greater relative risk of sudden unexplained death among recruits with sickle cell trait compared to non-HbS controls.[761] Deaths were associated with exertional heat stress, heat stroke, and rhabdomyolysis in the context of previously poorly conditioned individuals undergoing strenuous physical exertion. After this report universal modifications in basic training drill schedule and improved attention to hydration status improved the rate of sickle cell trait-related sudden death.[762,763] When exertional death was examined in National Collegiate Athletic Association (NCAA) Division I football players, the relative risk of death was 22 times greater in athletes with sickle cell trait compared with those without SCT, and all 5 deaths of patients observed to have SCT occurred during practice and conditioning drills.[764] In 2010 as the result of a lawsuit settlement, the NCAA adopted a policy requiring Division I student athletes to be screened for sickle cell trait, and this has subsequently been extended to Divisions II and III. The American Society of Hematology does not support the screening for sickle cell trait as a prerequisite for athletic participation.[765]

Hemoglobin SC Disease ($\alpha_2\beta_2^{6\ \text{Val}}$, $\alpha_2\beta_2^{6\ \text{Lys}}$)

HbSC disease is a milder chronic hemolytic anemia than SCA and is associated with variable degrees of vaso-occlusive complications. As described in Table 20-1, the patient typically has an Hb concentration of 10 to 12 g/dL, a reticulocyte count of 1% to 13%, and a relative microcytosis for the degree of reticulocytosis. The peripheral blood smear (see Fig. 20-5) is more characteristic of HbC than of HbS, showing impressive target cells, microcytosis, and rarely, ISCs. The clinical course of HbSC is quite variable, with some patients severely affected at an early age and others entirely asymptomatic and identified only as adults on routine screening. Hemolysis is less severe than SCA,[766] resulting in a higher hematocrit level with resulting higher blood viscosity.

The major complications of SCA are not as common in HbSC disease, but all have been reported including recurrent painful bone crises, painful abdominal crises, gallstones, pulmonary infarction, priapism, and CNS infarction.[767-770] As shown in Figure 20-6, the pattern of clinical events is different in HbSC, with most events occurring not only less often but later in life. Certain complications appear to be common and perhaps reflect higher blood viscosity including eye disease,[505,768,771-773] AVN of the femoral heads, renal papillary necrosis,[774] and pregnancy-related problems.[775] Splenomegaly is found in approximately 60% of patients with HbSC disease and has been associated with late-onset splenic infarction[776] and splenic sequestration,[777] particularly at high altitudes. Although infection is not as common as in SCA,[778] splenic hypofunction and an increased risk of pneumococcal and *H. influenzae* sepsis are observed.[779] For this reason, pneumococcal and *H. influenzae* vaccines are indicated, and the use of prophylactic penicillin is recommended for children with HbSC.

The utility of hydroxyurea for patients with HbSC has not been well studied, with only small series and little prospective research. Favorable hematologic and clinical results have been reported,[780,781] but the only prospective trial was terminated because of poor enrollment and so provided few meaningful results.[782] A robust clinical trial using hydroxyurea for HbSC is sorely needed and was identified as an important knowledge gap in the most recent NHLBI SCD guidelines.

Patients with HbSC disease clearly have more sickling complications than individuals with sickle trait, who are essentially free of sickling complications. One would therefore assume that mixtures of HbS and HbA are less likely to polymerize than mixtures of HbS and HbC. Interestingly Bunn and associates[783] have shown that this is not the case, because the kinetic and equilibrium behaviors of mixtures HbS and HbA are essentially identical with the behaviors of mixtures of HbS and HbC. One can resolve this apparent paradox by considering how the Hb mixtures are packaged in SC and AS cells. Two major factors explain why HbSC cells sickle more readily than AS cells: 1) There is more HbS in an HbSC cell, and 2) there is a higher MCHC in an HbSC cell. Because the charge and affinity for β globin differs between β^S globin and β^C globin, individuals with HbSC disease usually have 50% HbS and 50% HbC, whereas those with the sickle trait typically have 60% HbA and 40% HbS.[784] This increased HbS content of SC cells results in an approximately sevenfold increase in the rate of polymerization.[784] The increase in MCHC in HbSC disease, which is quite obvious on density separation of HbSC red cells[783,785,786] (see Fig. 20-5) and even C-trait red cells,[784] is probably the result of the activation by the positively charged HbC molecules of the K^+,Cl^- cotransport system.[787] The cellular dehydration and microcytosis associated with HbC has been well characterized by Brugnara and colleagues[788] and Ballas and associates.[786]

Hemoglobin S β-Thalassemia

Patients heterozygous for HbS and β-thalassemia have clinical severity that depends on the output of the thalassemic β gene.[357,789,790] If no HbA is produced (S β^0-thalassemia), the clinical course is comparable (see Table 20-1) with that of homozygous HbSS; hence both are referred to as SCA. The Hb electrophoresis shows mostly HbS and variable amounts of HbF. These patients are more often of Mediterranean or Middle Eastern origin, have microcytosis, and often have palpable splenomegaly. One parent will have classic sickle trait, whereas the other will have β-thalassemia trait with eleveated HbA_2. Peripheral blood morphology is notable for target cells and microcytosis.

If there is output from the thalassemic β-globin gene (S β^+-thalassemia), patients tend to have a milder clinical course, comparable with that of HbSC disease. The Hb electrophoresis shows predominantly HbS and variable amounts of HbF and HbA. Most African Americans with HbS β^+-thalassemia will have about 15% to 20% HbA, which is enough to modify the clinical course but not enough to abrogate the pathophysiology. The features that distinguish these individuals from those with SCA are the presence of HbA, microcytosis, splenomegaly, and a relatively benign clinical course. The ameliorative effects of HbA (see Table 20-1) are apparent. These patients can be distinguished from individuals with sickle trait, because HbS percentage is greater than that of HbA, as well as microcytosis, hemolytic anemia, abnormal peripheral morphology, and splenomegaly.

Hemoglobin SO_{Arab} ($\alpha_2\beta_2^{6\ Val}$, $\alpha_2\beta_2^{121\ Lys}$)

$HbSO_{Arab}$ compound heterozygotes are quite rare and have a severe sickling phenotype with chronic hemolytic anemia and vaso-occlusive episodes.[791,792] The O_{Arab} β-globin mutation lies within the area critical to the axial contact in the HbS fiber. Routine cellulose acetate electrophoresis at pH 8.6 does not distinguish between HbC and HbO_{Arab}. Citrate agar electrophoresis at pH 6 does separate HbC from HbO_{Arab} and should be done on patients who appear to have HbSC disease on cellulose acetate electrophoresis but who have particularly severe symptoms.[793] The peripheral blood smear typically shows sickled erythrocytes and target cells with polychromasia and nucleated RBCs.

Hemoglobin SD Disease ($\alpha_2\beta_2^{6\ Val}$, $\alpha_2\beta_2^{121\ Glu}$)

HbSD compound heterozygotes have a severe hemolytic anemia with a peripheral blood smear comparable to that seen in SCA. These rare patients have severe vaso-occlusive complications,[794-796] illustrating as those with the HbO_{Arab}, the critical nature of the β^{121} contact site. HbD migrates with HbS on cellulose acetate electrophoresis at alkaline pH but can be distinguished on citrate agar electrophoresis at acid pH. HbD should be suspected when an Hb appears to be HbS on cellulose acetate electrophoresis but gives a negative “sickle prep.” The HbD mutation has been identified worldwide but most commonly occurs in India, so the variant is often called HbD_{Punjab}.

Hemoglobin S Korle Bu ($\alpha_2\beta_2^{6\ Val}$, $\alpha_2\beta_2^{73\ Asn}$)

Hb Korle Bu is a rare Hb mutant.[797] It is mentioned here only because it illustrates an important pathophysiologic point. Korle Bu participates poorly in sickling.[798] This mutation interferes with the lateral contact between HbS fibers by blocking the critical receptor area for the Val-β6. S Korle Bu compound heterozygotes are entirely free of symptoms.

Hemoglobin C_{Harlem} ($C_{Georgetown}$) ($\alpha_2\beta_2^{6\ Val,\ 73\ Asn}$)

Hb C_{Harlem} (also known as $C_{Georgetown}$) has two substitutions on the same β chain: the sickle mutation $\beta^{6\ Val}$ and the Korle Bu mutation $\beta^{73\ Asn}$.[799] Patients with these mutations are asymptomatic. Patients doubly heterozygous for HbS and HbC_{Harlem} have clinical crises resembling SCA.[799,800]

Hemoglobin S Antilles ($\alpha_2\beta_2^{6\ Val,23\ Ile}$)

HbS Antilles also has two substitutions on the same β chain: the sickle mutation $\beta^{6\ Val}$ and a second mutation, $\beta^{23\ Ile}$.[801] This mutation results in a low–oxygen-affinity Hb that in heterozygotes for HbA and HbS Antilles results in a mild hemolytic anemia and the presence of 5% to 7% irreversible sickled cells. A transgenic mouse with HbS Antilles and the HbD (see earlier) mutation exhibits many of the clinical features of SCA.[802]

Hemoglobin S Québec-CHORI ($\alpha_2\beta_2^{6\ Val}$, $\alpha_2\beta_2^{87\ Ile}$)

Hb Québec-CHORI is an electrophoretically silent Hb variant that is indistinguishable from HbA by standard clinical electrophoresis and therefore if in compound heterozygous state with the sickle mutation may present with a newborn screen “FAS” phenotype mimicking sickle trait. Hb Québec-CHORI readily separates by

HPLC. Two cases of compound heterozygous Hb S Québec-CHORI have been reported in the literature.[803,804] Functional studies have revealed that Hb S Québec-CHORI behaves similarly to HbS with respect to nucleation and polymerization,[803] which accounts for the sickle phenotype in the two affected patients. One of the cases had a favorable response to hydroxyurea.[745]

Hemoglobin SE ($\alpha_2\beta_2^{6\ \mathrm{Val}}$, $\alpha_2\beta_2^{26\ \mathrm{Lys}}$)

HbE is found primarily in Sri Lanka, India, and Southeast Asia. Patients homozygous for HbE have a mild anemia and microcytosis and are clinically asymptomatic; however, compound heterozygotes for HbE and a β-thalassemia mutation have variable phenotype from mild to severe, with some virtually indistinguishable from β-thalassemia major.[805] The HbE mutation creates a cryptic splice site and therefore manifests also as a mild β^+-thalassemia mutation.[806] The clinical phenotype of HbSE disease appears to be relatively mild, resembling that of HbSβ^+-thalassemia; however vaso-occlusive crises, ACS, splenic sequestration, rhabdomyolysis, and AVN have been described.[807-809] HbSE will be diagnosed more often while population migration patterns change and racial intermarriages become more prevalent. HbSE may be initially misdiagnosed because of the comigration of HbE with other Hbs under some screening conditions. The Hb ratio is usually 60% to 65% HbS and 30% to 35% HbE. Affected individuals are mildly anemic with microcytosis (MCV 65-75fL).

Hemoglobin C Disease ($\alpha_2\beta_2^{6\ \mathrm{Lys}}$, $\alpha_2\beta_2^{6\ \mathrm{Lys}}$)

Homozygous HbC disease is a mild disorder characterized by hemolytic anemia, microcytosis, and splenomegaly.[810-812] The tendency of HbC to aggregate and crystallize[813-815] is probably responsible for the characteristic target morphology of the stained and dried red cell in homozygous C disease and in HbC trait, although these crystals are not likely to be directly responsible for the hemolytic anemia. In HbC trait, target cell formation and mild microcytosis is the only manifestation of the anomaly. Most patients are identified on newborn screening; the diagnosis should be confirmed, families given proper genetic counseling, education about how HbC disease is not a form of SCD, and that the hemolytic anemia is very mild.[812]

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CHAPTER 21

Thalassemias

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CHAPTER OUTLINE

HUMAN HEMOGLOBINS: COMPOSITION AND GENETICS
Globin Gene Structure
Regulation of Globin Gene Expression
CLASSIFICATION OF THE THALASSEMIAS
Origin of Thalassemia Mutations: The Influence of Malaria
Classes of Mutations That Cause Thalassemia
Identification, Characterization, and Ethnic Distribution of β-Thalassemia Mutations
Ethnic Distribution
Mutations That Affect β-Globin Gene Regulation
β-Thalassemia Mutants Unlinked to the β-Globin Complex
Hereditary Persistence of Fetal Hemoglobin
Mutations That Alter α-Globin Gene Regulation
Deletion Mutations within the α-Globin Gene Cluster
Mutations Not Linked to the Globin Gene Clusters That Alter Globin Gene Expression
CLINICAL HETEROGENEITY OF THALASSEMIA SECONDARY TO DIVERSITY OF MUTATIONS
α-Thalassemia
Mild β-Thalassemia
Severe β-Thalassemia
Interaction of Thalassemia with Globin Structural Variants
PRENATAL DIAGNOSIS OF THALASSEMIA

The thalassemias are a heterogeneous group of inherited forms of anemia caused by mutations that affect the synthesis of hemoglobin.[1-3] Milder forms are among the most common genetic disorders, whereas the severe forms, which are seen less often, lead to significant morbidity and mortality worldwide (Fig. 21-1).

Study of the thalassemias traces the history of the application of recombinant deoxyribonucleic acid (DNA) methods to analysis of inherited diseases and underscores how naturally occurring mutations in humans illuminate genetic principles. In this chapter the genetics of hemoglobin genes are reviewed as background for discussion of the molecular basis of the thalassemia syndromes, their clinical phenotypes, current management, and prenatal diagnosis.

HUMAN HEMOGLOBINS: COMPOSITION AND GENETICS

Normal hemoglobins are tetramers of two α-like and two β-like globin polypeptides. The predominant hemoglobin in normal adult red blood cells is hemoglobin A (HbA), $\alpha_2\beta_2$.[4,5] The α- and β-globins contain 141 and 146 amino acids, respectively. In addition to HbA, adult red blood cells normally contain two minor hemoglobins: HbA_2 ($\alpha_2\delta_2$) and fetal hemoglobin (HbF) ($\alpha_2\gamma_2$). The γ and δ polypeptides are related to β but differ in their primary amino acid sequences; hence they are referred to as β-like globins. HbA_2 normally accounts for 2% to 3.5% of the total hemoglobin. Although it is a minor component in adult red blood cells, HbF is the predominant hemoglobin in fetal red blood cells during the latter two trimesters of gestation. Because it does not bind 2,3-diphosphoglycerate, its affinity for oxygen is higher than that of HbA.[6] As such, HbF enhances the fetus' ability to extract oxygen from the placenta. HbF constitutes a small fraction of the total hemoglobin in adult red blood cells (0.3% to 1.2%), in which it is largely restricted to a small subset of circulating erythrocytes (0.2% to 7% of the total cells), termed *F cells*.[7,8] Production of HbF in normal adults is largely genetically controlled as demonstrated by studies in twins and familes.[9-13] During the first trimester in utero, embryonic hemoglobins with differing subunit composition are found in the yolk sac–derived macrocytic (or primitive) red blood cells.[1]

The genes that encode the globin polypeptides are organized into two small clusters.[2,4,5,14,15] The α-like genes are located near the telomere of the short arm of chromosome 16 (16p13.3), whereas the β-like genes reside on chromosome 11 at band 11p15.5.[16,17] A schematic diagram of the human globin genes and the composition of the various hemoglobins are shown in Figure 21-2.

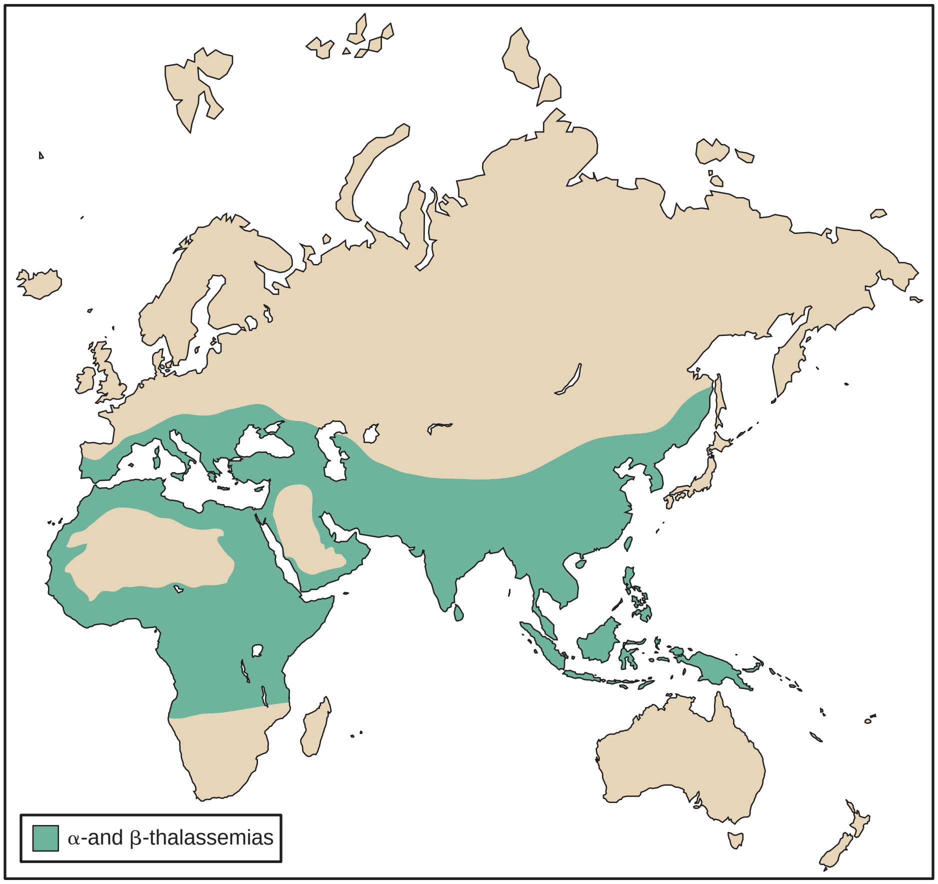

🟩 α-and β-thalassemias

Figure 21-1 Geographic distribution of thalassemia.

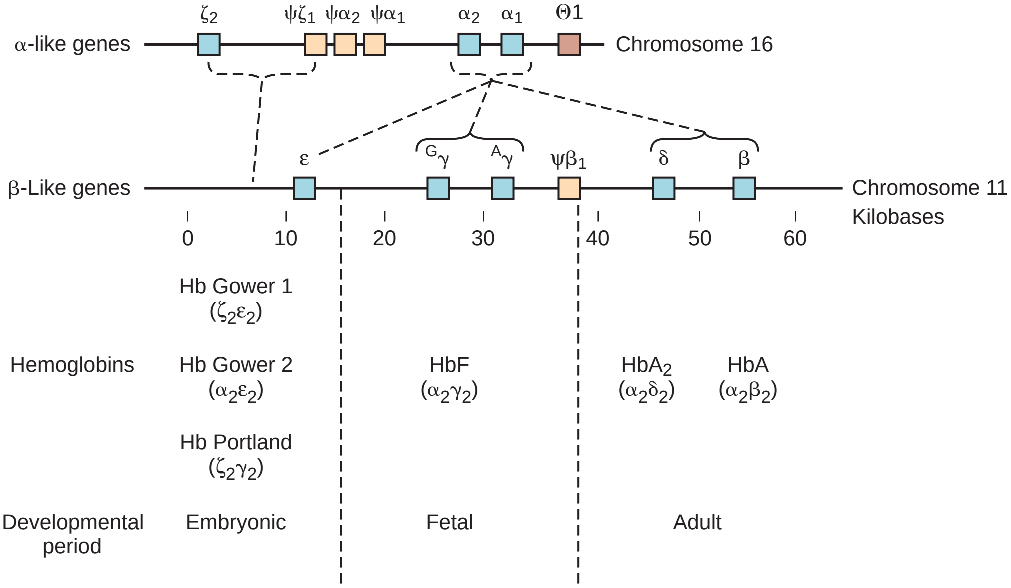

α-like genes
ζ_2
$\psi\zeta_1$
$\psi\alpha_2$
$\psi\alpha_1$
α_2
α_1
Θ1
Chromosome 16
β-Like genes
ε
$^G\gamma$
$^A\gamma$
$\psi\beta_1$
δ
β
Chromosome 11
Kilobases
0
10
20
30
40
50
60
Hemoglobins
Hb Gower 1
($\zeta_2\varepsilon_2$)
Hb Gower 2
($\alpha_2\varepsilon_2$)
Hb Portland
($\zeta_2\gamma_2$)
HbF
($\alpha_2\gamma_2$)
HbA_2
($\alpha_2\delta_2$)
HbA
($\alpha_2\beta_2$)
Developmental period
Embryonic
Fetal
Adult

Figure 21-2 Chromosomal organization of the globin genes and their expression during development. The *blue boxes* indicate functional globin genes, whereas the *yellow boxes* indicate pseudogenes (see text). The Θ1 globin gene is depicted by a *brown box* and is expressed at very low levels compared with the adult α-globin genes. The scale of the depicted chromosomal segments is in kilobases of deoxyribonucleic acid. The switch from embryonic to fetal hemoglobin occurs between 6 and 10 weeks of gestation, and the switch from fetal to adult hemoglobin occurs at about the time of birth. *Hb,* Hemoglobin.

The α-globin gene cluster contains three functional genes, ζ, α_2, and α_1, oriented in a 5′-to-3′ direction along the chromosome.[2,15,18,19] ζ-Globin, encoded by the ζ gene, is found in two embryonic hemoglobins, Hb Gower-1 ($\zeta_2\varepsilon_2$) and Portland ($\zeta_2\gamma_2$).[20] The duplicated α-globin genes (α_1 and α_2) encode identical polypeptides. DNA sequence analysis has revealed three additional globin gene–like sequences in the cluster: pseudo-ζ (ζ_1), pseudo-α_1, and pseudo-α_2.[2,15] Although they resemble the functional genes, sequence differences in coding or critical regulatory regions render these genes inactive, and hence they are referred to as pseudogenes.

Five functional genes, ε, ${}^{G}\gamma$, ${}^{A}\gamma$, δ, and β, are present within the β-like cluster and arranged in a 5′-to-3′ direction as they are expressed during development.[21-24] The product of the embryonic ε gene is found in the embryonic hemoglobins Hb Gower-1 ($\zeta_2\varepsilon_2$) and Hb Gower-2 ($\alpha_2\varepsilon_2$). The fetal γ genes are duplicated but encode globins differing only at amino acid 136; ${}^{G}\gamma$-globin and ${}^{A}\gamma$-globin contain a glycine or alanine residue, respectively. ${}^{G}\gamma$- and ${}^{A}\gamma$-globins are both found normally in HbF ($\alpha_2\gamma_2$). The δ-globin gene encodes a polypeptide differing in only 10 of 146 residues from β, and yet it is expressed at a very low level in adult red blood cells (<3% of β). The poor expression of δ-globin is attributed to differences in critical regulatory sequences[25] within the gene that appear to inhibit messenger ribonucleic acid (mRNA) processing[26] and the inherent instability of δ mRNA.[27] Only a single functional β-globin gene is present in the cluster. β-Globin is the predominant β-like globin in adult red blood cells, in which HbA ($\alpha_2\beta_2$) accounts for more than 95% of the total hemoglobin.

The relative synthesis of individual globin chains, the major sites of erythropoiesis during development, and major regulators of this process (discussed later) are depicted in Figure 21-3.[1,4,28-30] Embryonic hemoglobins are expressed nearly exclusively in primitive nucleated red blood cells differentiating in the yolk sac blood islands. HbF production commences during the next wave of erythropoiesis in the fetal liver. Fetal liver–derived red blood cells lose their nuclei as terminal maturation occurs, whereas the primitive, yolk sac–derived cells remain nucleated. The transition from HbF to HbA coincides approximately with the switch from fetal liver to bone marrow erythropoiesis. Despite this correlation between the site of erythropoiesis and the hemoglobins expressed, careful analysis of tissues derived from experimental animals and human fetuses has shown that embryonic hemoglobins are synthesized in the liver as well as the yolk sac and that HbF is produced in the bone marrow as well as the liver. The developmental switches in hemoglobin expression are related to the time of gestation rather than the anatomic site of erythropoiesis per se.

Globin Gene Structure

The globin genes were among the first eukaryotic genes isolated by recombinant DNA cloning methods in the late 1970s. Subsequent work provided the entire DNA sequences of the human α- and β-globin gene clusters and extensive sequences of other vertebrate globin complexes. These data have been invaluable in determining the mutations underlying thalassemia syndromes and in manipulating gene regulatory regions.

Intervening Sequences or Introns

A remarkable finding was made on initial study of the globin genes: the coding region, rather than being organized in a single continuous unit, is interrupted by noncoding DNA known as intervening sequences (IVSs), or introns. Most eukaryotic genes contain one or more introns. As indicated in Figure 21-4, globin genes are interrupted at two positions. The discontinuous nature of the coding region of globin genes poses a formidable problem for the formation of mRNA that must be translated into globin polypeptides on cytoplasmic ribosomes. Transcription of a globin gene generates a precursor mRNA containing introns. Formation of mature mRNA is accomplished by posttranscriptional processing, termed *RNA splicing*. The pathway of RNA processing is depicted in Figure 21-5.

RNA splicing must be executed with exquisite precision for functional mRNA to be generated. Because translation of mRNA proceeds by the reading of triplets (codons), excision of introns needs to be accurate to the nucleotide; otherwise, shifts in the reading frame of the translated polypeptide will result. RNA processing is guided by specific sequences, known as splice site consensus sequences, located at the 5′ and 3′ boundaries of the introns. The donor site, which marks the 5′ end of the intron, generally conforms to the sequence 5′ (C/A) AG′*GT*(A/G)AGT, where the prime sign indicates the position of splicing and GT is an essentially invariant dinucleotide at the 5′ end of the intron. The acceptor site, which defines the 3′ end of the intron, usually fits the consensus 5′ $(T/C)_n$N(C/T)*AG*′G, where *n* is 11 or greater, N is any nucleotide, the prime sign indicates the site of splicing, and AG is an essentially invariant dinucleotide. Excision of introns generally occurs between the dinucleotides GT and AG, known as the GT-AG rule.

Conserved Features of Mature Globin mRNA

Sequences of human globin genes represented in processed globin mRNA include additional segments located before (5′) and after (3′) the coding region. These untranslated regions are depicted in Figure 21-5. In addition, the mature mRNA is modified at both termini. At the 5′ end, a methylated guanylic acid (m^7G) cap structure is present. A variable number of adenylic acid residues are added at the 3′ end and constitute a poly(A) tail. The 5′ cap structure appears to be important for efficient initiation of mRNA translation, whereas the poly(A) tail contributes to mRNA stability. Overlapping the beginning of the mRNA sequence in genomic DNA are sequences that aid in directing the initiation of transcription to the proper site. In some eukaryotic genes these sequences conform to an initiator (Inr) consensus element.[31] The human β-globin gene has been shown to possess an Inr element that is functional in transcription reactions performed in vitro.[32]

Polyadenylation at the 3′ end of mRNA precursors depends on a signal in the 3′-untranslated region, generally AAUAAA (AATAAA in genomic DNA). The

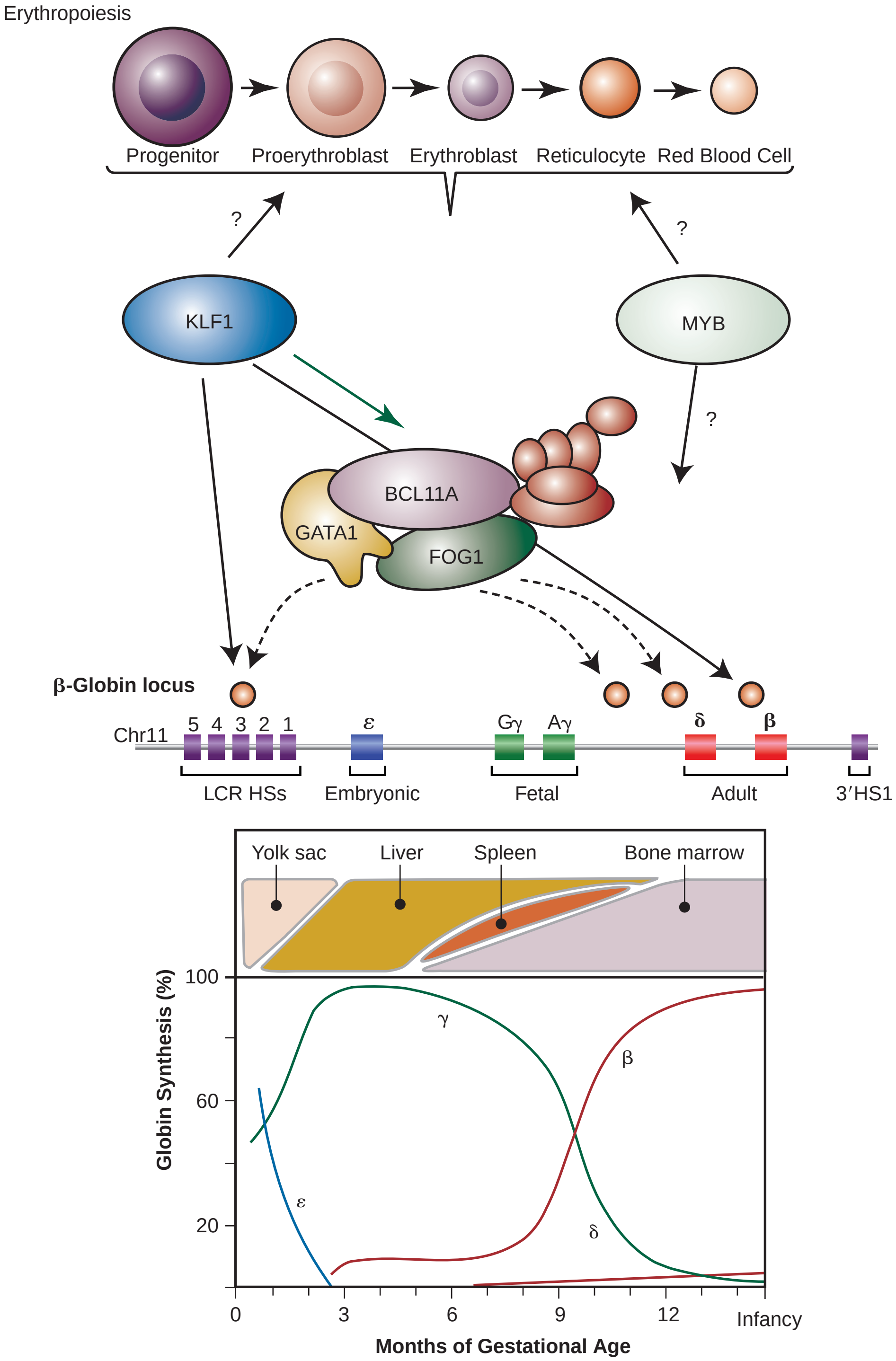

Erythropoiesis
Progenitor
Proerythroblast
Erythroblast
Reticulocyte
Red Blood Cell
?
?
KLF1
MYB
?
BCL11A
GATA1
FOG1
β-Globin locus
Chr11
5 4 3 2 1
ε
Gγ
Aγ
δ
β
LCR HSs
Embryonic
Fetal
Adult
3′HS1
Yolk sac
Liver
Spleen
Bone marrow
Globin Synthesis (%)
100
60
20
γ
β
ε
δ
0
3
6
9
12
Infancy
Months of Gestational Age

Figure 21-3 Erythroid cell differentiation, transcriptional regulators of the globin genes, the β-globin gene locus, sites of erythropoiesis, and the pattern of globin biosynthesis during development. The top panel shows the pattern of differentiation from multipotent progenitors to mature red blood cells. Regulators of β-like globin gene expression, including BCL11A, GATA1, KLF1/EKLF, and MYB, are shown with arrows depicting the site of action of these factors. Some regulators, such as BCL11A, act directly at the β-globin locus shown below the diagram. Other regulators, such as MYB, act through unclear mechanisms to affect globin gene regulation. This may be either an indirect affect upon erythropoiesis or a direct activity at the β-globin locus. Nucleated megaloblasts, which are produced predominantly in the yolk sac, are replaced by macrocytic fetal red blood cells produced in the liver and subsequently in the spleen and bone marrow. The height of the shaded area approximates the proportion of circulating red blood cells produced by each organ. Globin biosynthetic measurements were made to obtain the data shown in the lower part of the figure. *HS,* Hypersensitivity site; *LCR,* locus control region.

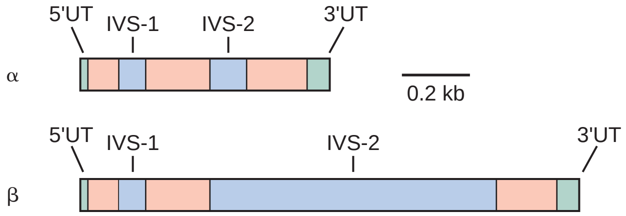


Figure 21-4 Structure of the human α- and β-globin genes. Untranslated (UT) regions, exon, and intervening sequences (IVS, introns) are depicted by *green*, *salmon*, and *blue boxes*, respectively.

mechanism of modification of the 3′ end is complex and involves not only polyadenylation but also cleavage of the precursor RNA because the primary RNA transcript extends several hundred nucleotides past what becomes the position at which the poly(A) tail is added.

Translation of mRNA into a polypeptide proceeds by the reading of triplets (codons) on cytoplasmic ribosomes. The first AUG codon (specifying methionine) present in the mRNA specifies the start site for translation of the mRNA into protein and is embedded in a sequence context (the Kozak consensus sequence, typically CC[A/G] CC*ATG*G) that signals the binding of translation initiation factors and ribosomes to the mRNA. Usually the amino-terminal methionine residue is removed from the growing polypeptide chain even before its synthesis is completed. Termination of polypeptide chain translation is directed by the termination codons UAA, UAG, or UGA. Mutation of these codons allows continued translation into the 3′-untranslated sequences of mRNA, as occurs in selected α-globin chain variants associated with α-thalassemia (discussed later).

As briefly reviewed earlier, the formation of functional mature mRNA demands extraordinary precision and depends on highly conserved sequence elements. As exemplified by the thalassemia syndromes, point (or other) mutations in these signals lead to reduced or absent polypeptide chain synthesis, which is the hallmark of thalassemia. Mutations causing thalassemia involve all phases of gene expression, including gene transcription, RNA splicing, integrity of the coding sequence, 3′ polyadenylation, and translation initiation (discussed later).

Regulation of Globin Gene Expression

Globin genes in all vertebrates are expressed in a tissue-specific and developmentally programmed manner. Their transcription is activated only within developing erythroid precursor cells. Moreover, individual globin genes are expressed at different developmental stages. Hence within the genes of the β cluster, globins are expressed at the embryonic (ε), fetal (γ), or adult (β and δ) stages, whereas within the α cluster, embryonic (ζ) and adult (α) chain expression is seen. A central problem posed by the organization of the globin gene clusters is how these patterns of tissue- and developmental-stage specificity are achieved. Recent findings from human genetic studies have begun to elucidate the molecular control of this developmental switch in the β-globin gene cluster and are discussed later in this chapter.

Proximal Regulatory Sequences and Transcription Factors

Several conserved sequence elements (motifs) in the 5′-flanking sequences of globin genes comprise the promoter, a region required for accurate and efficient transcription of genes by RNA polymerase II.[33-36] Promoters of vertebrate globin genes are similar in overall configuration and subset of motifs present but differ in their detailed organization and sequences. Promoters generally cooperate with more distant regulatory elements, termed *enhancers*, to stimulate transcription.[37-39] As discussed later, globin gene promoters appear to interact in a synergistic fashion with very powerful distant elements known as locus control regions (LCRs).

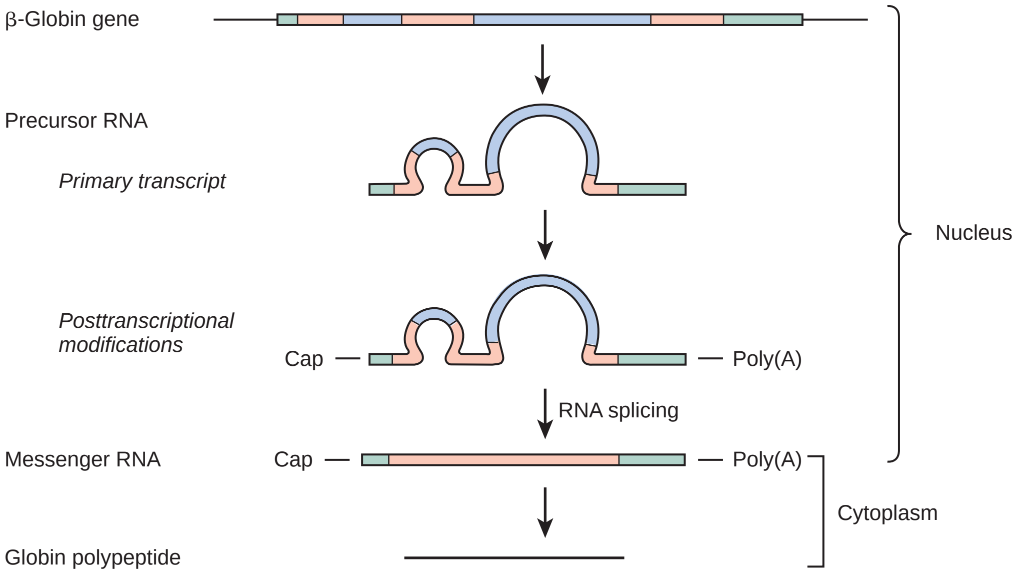


Figure 21-5 Expression of the β-globin gene. Transcription of the gene generates the precursor ribonucleic acid (RNA), which is processed by RNA splicing to form messenger RNA.

The TATA (or ATA) box is a motif seen in nearly all promoters, including those of the globin genes. The TATA box, typically located 20 to 30 base pairs (bp) upstream from the transcription start site, constitutes the binding site for a general transcription factor, the TATA-binding protein (TBP).[40-42] Binding of TBP to the TATA box is the first step in the assembly of a basal transcription complex (often termed *TFIID*) that includes many additional proteins (such as TFIIA, TFIIB, TFIIE/F, and TFIIH) and RNA polymerase II.[36] Mutations within the TATA box, as occur in some types of β-thalassemia,[43-49] decrease the binding of TBP to the promoter and decrease transcription.[40,41,50]

DNA sequence motifs located upstream of the TATA box bind proteins that interact with the general transcription machinery through protein-protein contacts with the TFIID complex and other associated proteins.[35,37,38,42] These promoter-bound proteins may either increase (activate) or decrease (repress) the rate of transcription. A small set of motifs are consistently present in globin gene promoters, including the CCAAT box, the CACC box, and GATA consensus sequences. Each motif may be viewed as a potential binding site for one or multiple transcription factors, which are either tissue restricted or ubiquitous in their cellular distribution.

Transcription factors are typically viewed as modular proteins made up of two domains that fulfill different functions: a DNA-binding domain responsible for sequence-specific DNA recognition and an activation (or repression) domain or domains that interact with components of the basal complex to modulate transcription. Additionally, some transcription factors may function to recruit activities that modify the histones around which DNA is wrapped to make chromatin. These modifications can indirectly alter the activity of the basal transcriptional complex. It is currently believed that transcriptional specificity is achieved by functional cooperation and interaction between cell-restricted and general transcription factors. As background for understanding globin gene control, the presently characterized erythroid-enriched transcription factors are reviewed here. For additional discussion of these proteins, readers are referred elsewhere.[51,52]

The consensus motif (A/T)GATA(A/G), known as the GATA motif, is found in the promoter region of most vertebrate globin genes and binds an abundant erythroid-restricted transcription factor, GATA1. GATA motifs have been identified in the regulatory elements of virtually all erythroid-expressed genes, consistent with the notion that GATA1 should serve a critical role in erythroid gene expression. As noted later, multiple GATA sites are also present within distant regulatory elements. The essential role of GATA1 in erythroid development was formally demonstrated through gene targeting experiments in mouse embryonic stem cells. Disruption of the single X-chromosome *GATA1* gene in totipotent embryonic stem cells prevents their development into normal erythroid cells. Naturally occurring but rare hypomorphic mutations of GATA1 in humans have been shown to cause β-thalassemia or disrupt normal erythroid development.[53-59] The GATA1 protein is a member of a small family of related "GATA factors" that are distinguished by a novel zinc-finger DNA-binding domain. In addition to merely specifying DNA recognition, this domain also mediates protein-protein interactions. Accordingly, GATA1 is able to interact physically with other GATA1 molecules or with other types of zinc-finger proteins, including the ubiquitous CACC- or GC-binding factor Sp1, the transcription factor erythroid Krüppel-like factor (EKLF), and a specific cofactor called FOG-1 (for "friend of GATA1"[60,61]; discussed later). It is envisioned that through its multiple physical interactions, GATA1 cooperates with other transcription factors, perhaps bound to DNA at distant sites, to program erythroid-specific transcription.

CACC motifs, which are represented by diverse sequences within globin and other gene promoters, bind a variety of transcription factors. Many CACC sequences are recognized by Sp1, a ubiquitous zinc-finger activator protein.[62] A particular CACC motif, CCACACCCT, is found in the adult β-globin gene promoter and is recognized with high affinity by the erythroid-specific protein EKLF (also known as KLF1). The functional relevance of this binding site has been established through naturally occurring mutations that lead to β-thalassemia (discussed later). In addition, gene targeting (or knockout) experiments in mice have formally established that EKLF/KLF1 is necessary for efficient β-globin transcription in vivo.[63]

A third erythroid transcription factor, known as NF-E2, binds to an extended motif—(T/C)TGCTGA(C/G)TCA(T/C)—that is found within some distant regulatory elements (discussed later) and a small subset of erythroid promoters but not within globin gene promoters. NF-E2 is a heterodimer of two polypeptides of the basic domain–leucine zipper (or "b-zip") class of transcription factors.[64,65] One subunit of NF-E2 is tissue restricted, whereas the other is ubiquitous. Although NF-E2 is essential for globin gene expression in mouse erythroleukemia cells in tissue culture,[66,67] its role in vivo appears to overlap that of one or more unknown factors that may act through the same target sites in DNA.[68]

Locus Control Regions and Chromatin Domains

How is globin gene transcription activated and developmentally controlled? Inspection of the DNA sequences of globin gene promoters in the early 1980s failed to provide substantive insight. Initial attempts to dissect control elements involved introducing globin genes into the germline of mice by oocyte injection but were plagued by low-level and erratic transgene expression. Nonetheless, it was possible to show that low-level stage specificity was imparted by the human β- and γ-globin gene promoters. For example, when the human β-globin promoter is introduced into transgenic mice, it directs gene expression only in adult erythroid cells,[69-72] whereas the human γ-globin promoter is active only in embryonic erythroid cells (mice do not have an HbF stage).[73,74]

These early globin gene regulation studies suggested that critical regulatory elements required for high-level expression were missing from the immediate vicinity of the genes themselves. When sensitivity to digestion by the enzyme DNase I was used as an indicator of chromatin structure in the mid 1980s, regions of extreme sensitivity (hypersensitivity sites [HSs]) were identified far upstream (≈30 to 50 kilobases [kb]) of the adult human β-globin gene.[75,76] Four subregions were delimited; they are present in the chromatin of erythroid, but not in nonerythroid cells. An additional site located even further upstream was found in all tissues. In a formal test of their functional relevance, the HSs were linked to a human β-globin and introduced into the germline of mice. Remarkably, transgenic mice then expressed the human β-globin gene at a level equivalent to that of the endogenous mouse β gene.[77] Further studies showed that the transgene is expressed not only in a tissue-specific manner but also in a copy number–dependent fashion, independent of the chromosomal site of integration. These HSs comprise an essential distal regulatory domain, now referred to as the LCR (see elsewhere[51,52] for a more detailed history and discussion).

Other globin gene clusters also contain erythroid-specific DNase I HSs. A segment of extreme DNase I hypersensitivity (known as HS-40) located far upstream of the human α-globin genes serves as an enhancer for the α locus. HS-40, however, does not display the full properties of an LCR because it does not direct copy number–dependent transgene expression.[78] Nonetheless, the in vivo relevance of both the β-LCR and HS-40 is underscored by the discovery of patients with thalassemia who have specific deletions in these regions (discussed later).

The human β-LCR, HS-40, and analogous regions studied in other species are composed of cores, each encompassing a DNase I HS. Cores are approximately 200 to 300 bp in length. Remarkably, within the cores three major protein-binding sites are consistently found: GATA, AP-1 (NF-E2), and CACC sequences. The position-independent activity of the β-LCR correlates best with the presence of GATA and CACC motifs, particularly within the subregion known as HS-3. Enhancer activity of the LCR, particularly within subregion HS-2, requires the NF-E2 motif. The protein-binding motifs within the LCR are also found in globin and other erythroid-expressed gene promoters. To date, no protein-binding sites unique to LCR elements have been identified. Hence the distinctive properties of the LCR (or HS-40) appear to reflect the synergistic interactions of more typical transcription factors rather than the action of a new set of regulatory proteins.

The discovery of distant control elements, marked by DNase I hypersensitivity, emphasizes the relationship between chromatin structure and globin gene regulation, an association solidified by the unraveling of a rare syndrome, α-thalassemia with X-linked mental retardation.[79] This condition results from mutations in a gene designated *XH2* (or *ATRX*) that encodes a member of the helicase superfamily.[80] Such proteins, which are often involved in DNA recombination and repair and in the regulation of transcription in *Drosophila*, yeast, and mammals, appear to influence transcription in a global manner by altering chromatin structure.

Regulation at a Distance: Globin Gene Switching

How do LCR sequences influence globin gene transcription over large distances (>50 kb), and how are the individual globin genes developmentally regulated? Two formal possibilities have been considered. On one hand, the LCR might merely provide an environment conducive for activation of the downstream globin genes. The globin genes would be autonomously regulated; that is, the developmental profile of their expression would be intrinsic to the individual genes (and presumably determined by their promoters). The "influence" of the LCR is most simply viewed as reflecting physical association of the LCR with globin genes brought into apposition by chromosomal looping. On the other hand, sequential activation of the particular genes might depend (at least in part) on competition of each gene for influence of the LCR such that only one gene-LCR interaction would be productive on a single chromosome at any time. The outcome of the competition would be dependent on the array of proteins bound not only at each promoter but also at specific sites in the LCR. Data in favor of both autonomous and competitive mechanisms of regulation have been obtained (see Orkin[51] for a detailed discussion).

The human embryonic ζ- and ε-globin genes appear to be largely autonomously regulated. Transgenes that contain LCR are expressed during embryonic erythropoiesis (the yolk sac stage) and then extinguished during the fetal liver stage. The information required for shutoff is contained near the globin genes, and competition by adjacent globin genes is not required. Shutoff is hypothesized to reflect the action of repressors, or silencer proteins, that bind the gene promoters. Motifs within the human ε-globin promoter are involved in silencing bind GATA1 and a ubiquitous factor, YY1.[81]

The competitive model of gene regulation is based on experiments in chicken red blood cells that demonstrate competition between the chicken β- and ε-globin gene promoters for a single enhancer located between the genes.[82] In the chicken it has been proposed that an adult stage–specific factor (NF-E4) favors interaction of the β promoter with the enhancer to the exclusion of the ε promoter. In an analogous fashion, data suggest that the human β-globin gene may be negatively regulated in a competitive fashion by the γ-globin gene. Whereas the γ gene is largely autonomously regulated, the β-globin gene is silenced in the embryonic and early fetal stage by a linked γ gene (a γ gene in *cis* to β). Shutoff of the γ gene, presumably as a result of repressors (or silencers), allows the adult β-globin to be expressed. Although other models are theoretically possible, the capacity of the LCR to act at a distance in regulating activation of globin genes is most compatible with the formation of physical contacts between the LCR (or subregions thereof) and their associated proteins with regulatory elements neighboring the genes themselves. Stage-specific and competitive regulation would therefore reflect engagement of the LCR with genes one at a time. LCR-gene interactions probably have intrinsic stabilities and off-rates such that a single erythroid cell might express more than one globin over time, even from a single chromosome. Experiments examining nascent human globin RNA along the β-gene complex tend to support such models and lend credence to the notion that chromosomal looping brings the LCR and individual genes in apposition. Evidence for LCR interactions with the proximal promoter regions of both the β- and α-globin genes has recently been directly demonstrated by using novel in vivo biochemical approaches.[83-85] Dynamic interactions between the β-LCR and the γ- and β-globin genes appear to underlie the reciprocal expression of these genes in erythroid cells and provide hope that subtle alterations in the nuclear environment may facilitate reactivation of γ-globin genes in patients with hemoglobinopathies such as sickle cell anemia or β-thalassemia. Recent work suggests that looping of the LCR itself in close proximity to the globin genes may be sufficient to allow for at least partial activation of transcription of these genes, as least in mouse models.[86] This group has recently gone on to demonstrate that such modulation of looping is sufficient for activation of embryonic or fetal hemoglobin genes in mouse models and human cells. This offers the prospect that such approaches could be therapeutically useful.[86a]

Recent Insights into Developmental Globin Gene Switching from Human Genetics

The molecular basis of the fetal-to-adult hemoglobin switch had remained an enigma over the course of nearly three decades. Although regulators of globin gene regulation, including GATA1, EKLF/KLF1, and NF-E2, had been identified, none of these regulators appeared to be sufficient to confer the stage-specific expression properties that are observed for the γ- and β-globin genes. By following up on the results of genome-wide association studies (GWAS)—a technique by which associations between common polymorphisms across the genome and various traits are tested—the first developmental stage-specific regulator of the fetal-to-adult hemoglobin switch, BCL11A, was identified (see Fig. 21-3).[87] Initial GWAS had identified a robust signal of association on chromosome 2 with HbF levels in both healthy persons and in patients with sickle cell disease.[88-90] This signal was most robust within a regulatory element of the gene *BCL11A*, which encodes a multizinc finger transcriptional regulator that had previously been implicated in B lymphocyte and neural development. By examining the hypothesis that BCL11A may also regulate HbF expression, it was found that reducing BCL11A expression allowed robust reactivation of HbF in adult cells.[91] Consistent with this finding, BCL11A is developmentally regulated and its expression is maximal in erythroid cells with little or no γ-globin expression. Moreover, BCL11A occupies regulatory elements within the β-globin locus, although it does not bind the promoter of the γ-globin genes. BCL11A holds tremendous promise as a therapeutic target for HbF induction in patients with β-thalassemia or sickle cell disease. Indeed, a proof of concept study has shown that removal of BCL11A in the hematopoietic system of mouse models of sickle cell disease can result in amelioration of disease in this model system.[92] An erythroid-specific enhancer element of BCL11A has recently been identified and holds promise for tissue-specific targeting of BCL11A expression.[92a] Furthermore, recent insight from rare human deletions within the β-globin gene cluster, which will be discussed in more detail later in this chapter, are providing important mechanistic insight into how BCL11A acts to regulate the fetal-to-adult hemoglobin switch.[93] It is likely that further studies of the type described here will lead to targeted approaches by which BCL11A activity can be selectively modulated.

In addition to the insight gained into hemoglobin switching from studies of BCL11A, other work has suggested that rare mutations in KLF1/EKLF may lead to elevations of HbF levels in patients and that this transcription factor may be involved in hemoglobin switching through the regulation of BCL11A (see Fig. 21-3).[94,95] However, other mutations in KLF1 result in either a significant congenital anemia or have little affect upon globin gene regulation, suggesting that other factors may modify the observed phenotypes in the patients.[87,96-98] Therefore further study of KLF1 and its role in this regulatory process is needed. In addition to the GWAS highlighting the role of BCL11A in HbF regulation, these studies have also suggested a role for the *MYB* gene in silencing of HbF.[88,89,99,100] This role has been substantiated through functional studies that have shown that reducing MYB levels can robustly activate γ-globin expression.[101,102] GWAS of β-thalassemia patient populations have shown that variants upstream of *MYB* can have a profound affect on the clinical course, and therefore MYB may be an important therapeutic target.[103,104] However, further studies are needed to better understand the exact mechanism by which MYB regulates globin gene expression (see Fig. 21-3).

CLASSIFICATION OF THE THALASSEMIAS

The hallmark of thalassemia syndromes is decreased (or absent) synthesis of one or more globin chains. The designation α- and β-thalassemia refers to deficits in α- and β-globin production, respectively. The α- and β-thalassemias include clinical syndromes of varying severity (Table 21-1). Knowledge of molecular genetics provides a framework in which to consider their clinical heterogeneity.

Because the structural gene for α-globin is duplicated on chromosome 16, each diploid cell contains four copies of the α-globin gene. The four α-thalassemia syndromes—silent carrier, α-thalassemia trait, hemoglobin H (HbH)

TABLE 21-1 Clinical Classification of the Thalassemias

Clinical Classification	Description
Silent carrier (α or β)	Hematologically normal
Thalassemia trait (α or β)	Mild anemia with microcytosis and hypochromia
HbH disease (α-thal)	Moderately severe hemolytic anemia, icterus, and splenomegaly
Hydrops fetalis (α-thal)	Death in utero caused by severe anemia
Severe β-thalassemia (Cooley anemia)	Severe anemia, growth retardation, hepatosplenomegaly, bone marrow expansion, and bone deformities
Thalassemia major	Transfusion dependent
Thalassemia intermedia	No regular transfusion requirement

HbH, Hemoglobin H; *thal,* thalassemia.

disease, and hydrops fetalis (see Table 21-1)—reflect the inheritance of molecular defects affecting the output of one, two, three, or four of the α-globin genes, respectively. More than 30 different mutations affecting one or both α-globin genes on a chromosome have been described. Some mutations abolish expression of an α-globin gene (α^0), whereas others reduce expression of the gene to a variable degree (α^+). Marked genetic and clinical heterogeneity occurs within the four general categories of α-thalassemia. Heterogeneity arises because the syndrome in any given person may represent a combination (or so-called interaction) of 2 of the 30 or more mutations that have been described.

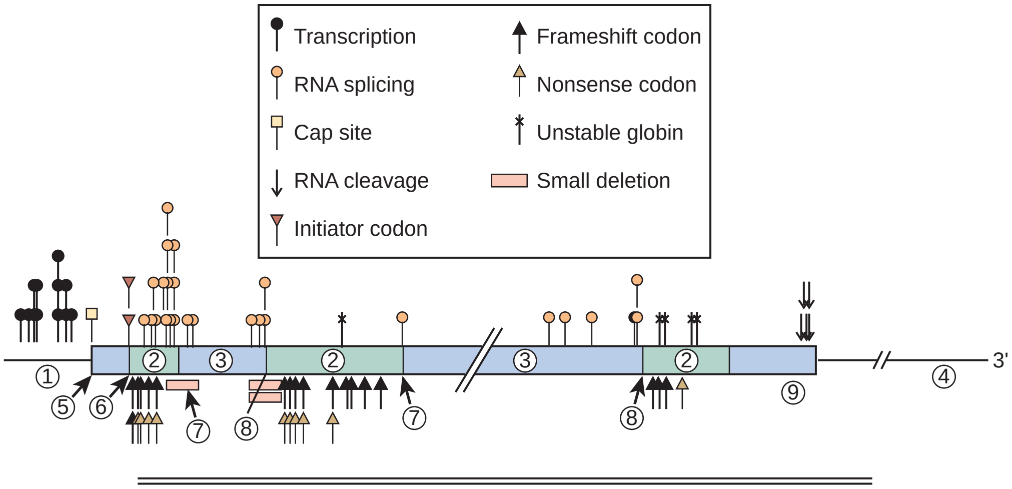
The β-thalassemias also include four clinical syndromes of increasing severity—silent carrier, thalassemia trait, thalassemia intermedia, and thalassemia major (see Table 21-1).[1,3,105,106] In contrast to the α-thalassemias, the four classes of β-thalassemia are not correlated with the number of functioning genes. Because a single functional β-globin gene resides on each chromosome 11, a diploid cell normally has two β-globin genes. The clinical heterogeneity of the β-thalassemias represents the diversity of specific mutations that variably affect β-globin gene expression. Almost exclusively, these mutations involve the β-globin gene rather than an unlinked genetic determinant. Many mutations eliminate β-gene expression (β^0), whereas others cause a variable decrease in the level of β-gene expression (β^+).[3,106] The capacity of individual patients to synthesize γ-globin modulates the clinical severity. Such is the case because the severity of thalassemias is determined by the degree of globin chain imbalance rather than by the absolute level of either α- or ββ-globin synthesis per se.[107-109,110,111] Substantial synthesis of γ-globin in the marrow cells of persons with β-thalassemia lessens the extent of chain imbalance and therefore improves red blood cell production.[112-114] Particular mutations of the β-globin gene in β-thalassemia appear to affect γ-globin gene expression directly. However, some persons with otherwise severe β-thalassemia may co-inherit additional genetic determinants that enhance the synthesis of HbF. Indeed, in patients with β-thalassemia who do not receive transfusions, HbF levels are strongly associated with clinical severity.[115] Coincident inheritance of an α-thalassemia mutation also reduces chain imbalance in patients with homozygous or heterozygous β-thalassemia.[116] Clinical severity in any individual patient represents the outcome of these complex genetic interactions.

Origin of Thalassemia Mutations: The Influence of Malaria

Mutations causing thalassemia have arisen spontaneously. The nearly exclusive distribution of lethal red blood cell disorders such as thalassemia, sickle cell disease, and glucose 6-phosphate deficiency in tropical and subtropical regions led Haldane in 1949 to propose that the heterozygous carrier state for these conditions confers a selective advantage in locations where malaria is endemic.[117] The incidence of these genes in a population is determined by the balance between premature death of homozygotes and increased fitness of heterozygotes. The frequency of β-thalassemia mutations is high (>1%) in regions such as the Mediterranean basin, northern Africa, Southeast Asia, India, and Indonesia but uncommon in northern Europe, Korea, Japan, and northern China.[3,118,119] The incidence of β-thalassemia trait may exceed 20% in some villages in Greece.[120] α-Thalassemia is perhaps the most common single gene disorder in the world.[2] The frequency of α^+-thalassemia alleles ranges from 5% to 10% in the Mediterranean basin,[121] 20% to 30% in portions of West Africa,[122] and 68% in the southwest Pacific.[123] The incidence of α-thalassemia is less than 1% in Britain, Iceland, and Japan.[124,125] Although the incidence of malaria and the rate of occurrence of thalassemia are not always inversely correlated, the anomalies and inconsistencies seem to be the result of genetic drift, migration, and demographic changes that have occurred in the last 10,000 years.[126]

Additional epidemiologic studies have provided further evidence for the validity of the "malaria hypothesis" in both α- and β-thalassemia.[123,127-134] Siniscalco and colleagues[127] showed that β-thalassemia is uncommon in inhabitants of the mountainous areas of Sardinia, where malaria is rare, compared with the incidence in coastal populations. In Melanesia, α-thalassemia is correlated with malaria across both latitude and altitude.[123] β-Thalassemia in Melanesia is also associated with malarious coastal regions.[131] Williams and colleagues[135] found that children with α-thalassemia trait appear to have a higher incidence of malaria in childhood that appears to confer subsequent immunity to more severe malarial infections. Follow-up studies in other populations have demonstrated that although α-thalassemia does not confer a reduced risk of malarial infection, it does dramatically reduce the incidence of severe malarial complications.[136,137]

The cellular mechanisms responsible for the selective advantage of thalassemia heterozygotes remain incompletely defined. Cultured erythrocytes containing high concentrations of HbF retard the growth and development of *Plasmodium falciparum*.[138] β-Thalassemia heterozygotes have a delayed disappearance of HbF in the first year of life,[1] which might provide protection from potentially fatal cerebral malaria early in life as the passive immunity acquired in utero wanes. Until recently, however, investigators were unable to document decreased invasion or growth of *P. falciparum* in red blood cells from thalassemia heterozygotes except under conditions of unusual oxidant stress.[99,139] Using modified tissue culture conditions, Brockelman and colleagues[140] and, more recently, Pattanapanyasat and colleagues[141] demonstrated decreased parasite multiplication in the red blood cells of β-thalassemia heterozygotes. They theorized that *P. falciparum* resistance was a consequence of the inability of the parasite to acquire sufficient nutrients from the digestion of hemoglobin in thalassemic red blood cells. In one study, red blood cells with α- and β-thalassemia trait bound greater levels of antibody than did control cells, which could lead to greater removal of parasitized red blood cells and hence provide protection.[142] Recent studies have suggested that parasitized red blood cells from α-thalassemia heterozygotes may have altered



1. PROMOTER: DNA sequences required for accurate and efficient initiation of transcription.
2. EXONS: DNA that specifies the amino acid sequence of the polypeptide.
3. INTRONS: DNA that interrupts the coding sequence of the gene.
4. ENHANCER: DNA sequences that increase promoter activity at a distance and independent of orientation relative to coding sequence.
5. CAP SITE: Position where transcription of gene into RNA begins.
6. TRANSLATION INITIATION SITE: Position where translation of mRNA into protein begins.
7. SPLICE DONOR SITE } Sequences required for precise and efficient
8. SPLICE ACCEPTOR SITE } removal of RNA transcribed from introns.
9. RNA CLEAVAGE/POLYADENYLATION SIGNAL: Sequence that specifies the 3' end of the RNA transcript and the addition of the poly(A) tail.

Figure 21-6 Location of various classes of point mutations that cause β-thalassemia with respect to important structural elements present in the β-globin gene. *DNA*, Deoxyribonucleic acid; *RNA*, ribonucleic acid. *(Modified from Kazazian HH Jr, Boehm CD: Molecular basis and prenatal diagnosis of beta-thalassemia.* Blood *72:1107–1116, 1988.)*

membrane properties that more readily allow binding of antibody to the red blood cell and may promote more effective antimalarial immune responses.[143] Erythrocytes from persons with HbH disease also appear to inhibit *P. falciparum* in vitro.[140,144] Recently it has been suggested that rosette formation, the binding of uninfected red blood cells to *P. falciparum*–infected red blood cells, is decreased in persons with thalassemia because of reduced red blood cell size and that such impaired rosette formation may hinder the development of cerebral malaria by lessening sequestration.[145]

The difficulty of documenting the cellular mechanism of *P. falciparum* resistance in thalassemic erythrocytes in vitro suggests that the heterozygote advantage may be small. The high mortality associated with malaria in endemic regions is a powerful selective force that may be sufficient to amplify a small increase in fitness.

Classes of Mutations That Cause Thalassemia

Thalassemia is the consequence of mutations that diminish (or abolish) production of either the α or β chain of hemoglobin. Molecular cloning, DNA sequencing, and functional analysis of cloned genes have provided the tools with which to dissect the thalassemia syndromes. This analysis has revealed remarkable heterogeneity in the specific alterations in DNA that lead to these clinical syndromes.

Typically, single nucleotide mutations associated with thalassemia interfere with one of the critical steps in mRNA production (Fig. 21-6 and Table 21-2). Base substitutions alter promoter function, RNA processing, or mRNA translation or modify a codon into a "nonsense codon" that leads to premature termination of translation or substitution of an incorrect amino acid. Insertion or deletion mutations within the coding region of the mRNA create "frameshifts" that prevent the synthesis of a complete, normal globin polypeptide. Large deletions within the α- or β-globin clusters may remove one or more genes and alter regulation of the remaining genes in the cluster. The phenotype that results from the diverse mutations found in thalassemia is determined by the degree of inactivation of the affected gene or genes and the extent of associated increases in expression of other genes within the cluster. In the following section we discuss examples of such mutations. The discussion of various mutations is meant to illustrate different molecular mechanisms that

TABLE 21-2 Point Mutations That Cause Thalassemia

Gene	Position*	Mutation	Classification	Ethnic Group†	Detection‡	References
A. Transcription Mutations						
β	1-101	C→T	β^+	Turkish		146
				Bulgarian		
				Italian		
	2-92	C→T	β^+	Mediterranean		3
	3-88	C→T	β^+	American black	(+) *Fok*I	147
				Asian Indian		
	4-88	C→A	β^+	Kurdish		148
	5-87	C→G	β^+	Mediterranean	(–) *Avr*II	149
	6-86	C→G	β^+	Lebanese		3
	7-31	A→G	β^+	Japanese		43
	8-30	T→A	β^+	Turkish		44
				Bulgarian		
	9-30	T→C	β^+	Chinese		45
	10-29	A→G	β^+	American black	(+) *Nla*III	46
				Chinese		47
	11-28	A→C	β^+	Kurdish		48
	12-28	A→G	β^+	Chinese		49
B. Cap Site Mutation						
β	11	A→C	β^+	Asian Indian		259
C. RNA Splicing Mutations						
1. Splice Junction Change in						
A. 5′ DONOR SITE						
α_2	1 IVS-1 n. 2-6	5-bp deletion	α^0	Mediterranean		156, 157
β	1 IVS-1 n. 1	G→A	β^0	Mediterranean	(–) *Bsp*M1	149
	2 IVS-1 n. 1	G→T	β^0	Asian Indian	(–) *Bsp*M1	158
				Chinese		
	3 IVS-1 n. 2	T→G	β^0	Tunisian		159
	4 IVS-1 n. 2	T→C	β^0	Black		160
	5 IVS-1 5′ end	44-bp deletion	β^0	Mediterranean		161
	6 IVS-2 n. 1	G→A	β^0	Mediterranean	(–) *Hph*I	162
				Tunisian		159
				American black		163
β	1 IVS-1 n. 130	G→C	β^0	Italian		3
	2 IVS-1 n. 130	G→A	β^0	Egyptian		3
	3 IVS-1 3′ end	17-bp deletion	β^0	Kuwaiti		161
	4 IVS-1 3′ end	25-bp deletion	β^0	Asian Indian		164
	5 IVS-2 n. 849	A→G	β^0	American black		46
	6 IVS-2 n. 849	A→C	β^0	American black		165
2. Splice Consensus Sequence Change in						
A. 5′ DONOR SITE						
β	1 IVS-1 n. –3 (codon 29)	C→T	?	Lebanese		158
	2 IVS 1 n. –1 (codon 30)	G→C	Hb Monroe	Tunisian		159
				American black		167
	3 IVS 1 n. –1 (codon 30)	G→A	?	Bulgarian		168
	4 IVS-1 n. 5	G→C	β^+	Asian Indian		158
				Chinese		169
	5 IVS-1 n. 5	G→T	β^+	Melanesian		131
				Mediterranean		170
				American black		171
	6 IVS-1 n. 5	G→A	β^+	Algerian	(+) *Eco*RV	172
				Mediterranean		
	7 IVS-1 n. 6	T→C	β^+	Mediterranean	(+) *Sfa*NI	149
B. 3′ ACCEPTOR SITE						
β	1 IVS-1 n. 128	T→G	β^+	Saudi Arabian		173
	2 IVS-2 n. 843	T→G	β^+	Algerian		174
	3 IVS-2 n. 848	C→A	β^+	Iranian		173
				Egyptian		
				American black		171

Continued on following page

TABLE 21-2 Point Mutations That Cause Thalassemia (Continued)

Gene	Position*	Mutation	Classification	Ethnic Group†	Detection‡	References
3. Mutations within Exons That Affect Processing						
β	1 Codon 19 (Asn-Ser)	A→G	Hb Malay	Malaysian		180
	2 Codon 24 (silent)	T→A	β^+	American black		177
	3 Codon 26 (Glu-Lys)	GvA	Hb E	Southeast Asian European	(−) *Mnl*I	106, 178
	4 Codon 27 (Ala-Ser)	G→T	Hb Knossos	Mediterranean		179
4. Internal IVS Change						
β	1 IVS-1 n. 110	G→A	β^+	Mediterranean		181, 182
	2 IVS-1 n. 116	T→G	β^0	Mediterranean		183
	3 IVS-2 n. 654	C→T	β^0	Chinese		147
	4 IVS-2 n. 705	T→G	β^+	Mediterranean		186
	5 IVS-2 n. 745	C→G	β^+	Mediterranean	(+) *Rsa*I	149
D. RNA Cleavage And Polyadenylation Mutations						
α_2	1 Cleavage signal	AATAAA→ AATAAG	α^+	Middle Eastern Mediterranean		187, 188
β	1 Cleavage signal	AATAAA→AACAAA	β^+	American black		190
	2 Cleavage signal	AATAAA→AATAAG	β^+	Kurdish		148
	3 Cleavage signal	AATAAA→AATGAA	β^+	Mediterranean		191
	4 Cleavage signal	AATAAA→AATAGA	β^+	Malaysian		191
	5 Cleavage signal	AATAAA→A (−AATAA)	β^+	Arab		3
E. Initiation Consensus Sequence Mutations						
α_2:	1 Initiation codon	ATG→ACG	α^0	Mediterranean	(−) *Nco*I	193
α_1:	2 Initiation codon	ATG→GTG	α^0	Mediterranean	(−) *Nco*I	194
$-\alpha$:	3 Initiation codon	ATG→GTG	α^0	Black	(−) *Nco*I	195
$-\alpha^{3.7II}$	4 Initiation consensus	CCACCATGG→CC CATGG	α^+	Algerian		196
				Mediterranean		197
β	1 Initiation codon	ATG→AGG	β^0	Chinese		3
	2 Initiation codon	ATG→ACG	β^0	Yugoslavian		191
	3 Initiation codon	ATG→ATA	β^0	Swedish		
F. Premature Termination Mutations						
1. Substitutions						
α_2	1 Codon 116	GAC→TAG	α^0	Black		215
β	1 Codon 15	G→A	β^0	Asian Indian		158
	2 Codon 17	A→T	β^0	Chinese	(+) *Mae*I	199
	3 Codon 35	C→A	β^0	Thai		201
	4 Codon 37	G→A	β^0	Saudi Arabian		202
	5 Codon 39	C→T	β^0	Mediterranean	(+) *Mae*I	203
	6 Codon 43	G→T	β^0	European		204
	7 Codon 61	A→T	β^0	Chinese	(−) *Hinf*I	205
				Black		171
2. Frameshifts						
−a	1 Codons 30/31	−2 bp (−AG)	α^0	Black		206
β	1 Codon 1	−1 bp (−G)	β^0	Mediterranean		3
	2 Codon 5	−2 bp (−CT)	β^0	Mediterranean		207
	3 Codon 6	−1 bp (−A)	β^0	Mediterranean	(−) *Cvn*I	171
				American black		207
	4 Codon 8	−2 bp (−AA)	β^0	Turkish		208
	5 Codon 8/9	+1 bp (+G)	β^0	Asian Indian		158
	6 Codon 11	−1 bp (−T)	β^0	Mexican		3
	7 Codons 14/15	+1 bp (+G)	β^0	Chinese		209
	8 Codon 16	−1 bp (−C)	β^0	Asian Indian		158
	9 Codons 27/28	+1 bp (+C)	β^0	Chinese		3
	10 Codon 35	−1 bp (−C)	β^0	Indonesian		177
	11 Codons 36/37	−1 bp (−T)	β^0	Iranian		148
	12 Codon 37	−1 bp (−G)	β^0	Kurdish		149
	13 Codons 37-39	−7 bp (−GACCCAG)	β^0	Turkish		210
	14 Codons 41/42	β^0		Asian Indian		158
		−4 bp (−CTTT)		Chinese		211
	15 Codon 44	−1 bp (−C)	β^0	Kurdish		212
	16 Codon 47	+1 bp (+A)	β^0	Surinamese black		3
	17 Codon 64	−1 bp (−G)	β^0	Swiss		213
	18 Codon 71	+1 bp (+T)	β^0	Chinese		3
	19 Codons 71/72	+1 bp (+A)	β^0	Chinese		169
	20 Codon 76	−1 bp (ϖC)	β^0	Italian		281
	21 Codons 82/83	−1 bp (−G)	β^0	Azerbaijani		3
	22 Codons 106/107	+1 bp (+G)	β^0	American black		259

TABLE 21-2 Point Mutations That Cause Thalassemia (Continued)

Gene	Position*	Mutation	Classification	Ethnic Group†	Detection‡	References
G. Termination Codon Mutations						
α_2	1 Codon 142 (ter-Gin)	TAA→CAA	Hb Constant Spring	Chinese		220, 221
	2 Codon 142 (ter-Lys)	TAA→AAA	Hb Icaria	Mediterranean		222
	3 Codon 142 (ter-Ser)	TAA→TCA	Hb Koya Dora	Indian		223
	4 Codon 142 (ter-Glu)	TAA→GAA	Hb Seal Rock	Black		224
β	1 Codon 147 (ter-Gin)		Hb Tak	Thai		225
H. Unstable Hemoglobin Chains						
1. Amino Acid Substitutions						
–α	1 Codon 14 (Trp-Arg)		Hb Evanston	Black		244
α_2	2 Codon 109 (Leu-Arg)	T→G	Hb Suan Dok	Southeast Asian		245, 246
α	3 Codon 110 (Ala-Asp)	T→C	Hb Petah Tikvah	Middle Eastern		247
α_2	4 Codon 125 (Leu-Pro)		Hb Quong Sze	Southeast Asian		242, 243
β	1 Codon 60	T→A	β^+	Italian		248
	2 Codon 110 (Leu-Pro)	T→C	Hb Showa-Yakushiji	Japanese		249
	3 Codon 112 (Cys-Arg)		Hb Indianapolis	European		250
	4 Codon 127 (Gin-Pro)		Hb Houston	British		251
	5 Codons 127/128 (Gin, Ala-Pro)	–3 bp (–AGG)	β^+	Japanese		252
2. Frameshift, Extended Chain						
β	1 Codon 94	+2 bp (+TG)	Hb Agnana (inclusion body)	Italian		253
	2 Codons 109/110	–1 bp (–G)	Hb Manhattan	Lithuanian		3, 251
	3 Codon 114	–2, +1 (–CT, + G)	Hb Geneva (inclusion body)	French-Swiss		254
	4 Codons 128-135	Net-10 bp β^+ (inclusion body)		Irish		255
3. Premature Termination						
β	1 Codon 121	G-T	β^0 (inclusion body)	Greek-Polish French-Swiss British		255-257

From Kazazian H: The thalassemia syndromes: molecular basis and prenatal diagnosis in 1990. Semin Hematol *27:209, 1990; and Huisman TH: Beta-thalassemia repository.* Hemoglobin *14:661, 1990. Courtesy Marcel Dekker, Inc.*

Hb, Hemoglobin; *IVS,* intervening sequence; *RNA,* ribonucleic acid.

*The position specifies the location in the gene at which the point mutation occurs. Positions are specified with reference to the start site for transcription (Cap site), the position within the intron (IVS), or the position of the codon.

†When more than one ethnic group is indicated, the mutation has had more than one origin.

‡Loss (–) or gain (+) of a restriction enzyme site with mutation is indicated; the remainder of the mutations can be detected with allele-specific oligonucleotides (see the section on direct detection of thalassemia mutations).

can lead to thalassemia and in no way is meant to be comprehensive. A comprehensive and regularly updated listing of all described mutations can be found at http://globin.cse.psu.edu/.

Mutations Affecting Gene Transcription

Point mutations within promoter sequences recognized by transcription factors tend to reduce the affinity with which these proteins bind and typically leads to reduced gene transcription. Analysis of the promoter for the β-globin gene in patients with β-thalassemia has identified a variety of mutations clustered in the ATA and CACC motifs (Fig. 21-7; see also Table 21-2).[3,40-46,146-149] These mutations are associated with preservation of some β-globin expression and hence are customarily associated with the phenotype of thalassemia intermedia. The C→T substitution at position –101, which results in a particularly mild defect, is associated with the "silent carrier" phenotype in heterozygous carriers.[146,150] Although the CCAAT box is highly conserved in globin genes, no mutations within this motif have been identified in thalassemia. Rare mutations in transcription factors that result in thalassemia have been detected, and exceedingly rare families have been identified in which a thalassemia mutation is unlinked to the globin clusters (see the section on mutations not linked to the globin gene clusters that alter globin gene expression).

Mutations of the ATA box presumably reduce binding of TBP and therefore lead to decreased transcription initiation. Substitutions in the CACC motifs decrease the affinity of binding by several transcription factors, including the erythroid-specific factor EKLF/KLF1 and the ubiquitous protein Sp1. Studies showing that mice engineered to lack EKLF experience lethal β-thalassemia at the fetal liver stage have established EKLF as a β-globin activator protein in vivo, and patients with mutations in EKLF have reduced production of the adult β-globin gene.[63,87,151] Human β-thalassemias resulting from

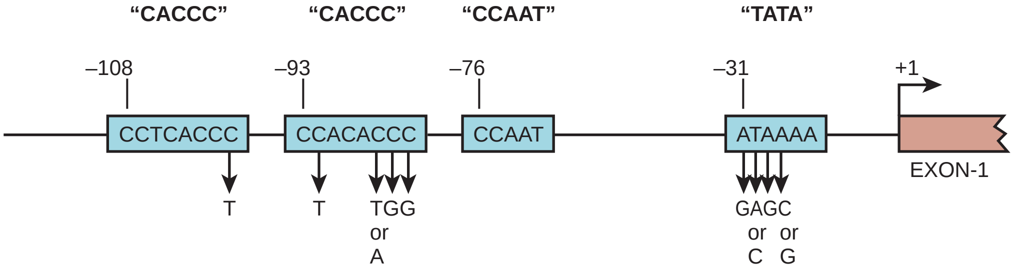


Figure 21-7 Point mutations in the β-globin gene promoter. The sequences of conserved motifs within the promoter and their distance from the transcription start site are indicated. A single base substitution at the indicated positions results in β⁺-thalassemia.

mutation of a single CACC motif are presumably mild because of the presence of one normal CACC motif within the promoter.

In addition to the protein-binding sites in the promoter, proper transcription depends on sequences surrounding the start site of transcription (known as +1). These sequences often display functional activity in in vitro assays and herald the binding of specific protein complexes to this type of element, termed the *Inr*. Mild β-thalassemia has been associated with a base substitution (A→C) at +1. This substitution has been shown to impair the β-globin Inr.[32] The proteins that mediate this effect are unknown.

A novel mechanism by which transcription at the α-globin locus can be disrupted has been described and serves as a paradigm for a unique class of mutations that can cause human disease. Higgs and colleagues[152] found a variant single nucleotide change upstream of the α-globin genes that creates a binding site for the transcription factor GATA1,[152] which in turn produces a novel promoter that competes with the endogenous α-globin promoters for interaction with upstream enhancer elements such as HS-40. As a result of this mutation, α-globin gene synthesis is reduced and α-thalassemia results. This mutation is present in approximately 4% of the Melanesian population.

RNA Processing Defects in Thalassemia

The importance of RNA splicing for the formation of functional mRNA cannot be overemphasized. As discussed earlier, removal of introns must be precise to the nucleotide for a continuous, translatable mRNA to be generated from an mRNA precursor. As soon as introns were discovered, it was hypothesized that mutations affecting RNA splicing would probably be involved in the thalassemia syndromes. Apart from its role in constructing a functional mRNA, RNA splicing also appears to be a determinant of mRNA stability[153,154] and is possibly coupled to RNA transport from the nucleus to the cytoplasm.[155]

Mutations That Alter Splice Junctions or Splice Consensus Sequences

Mutations at the 5′ donor site (GT)[149,156-163] or at the 3′ splice acceptor site (AG)[3,46,160,161,164-166] abolish proper splicing of the pre-mRNA transcript and result in α^0- or β^0-thalassemia (Fig. 21-8; see also Table 21-2). Substitutions at other sites within the splice junction consensus sequence have varied effects; because some correctly spliced RNA is produced, albeit a reduced amount, a β⁺-thalassemia phenotype ensues.[130,148,149,158,159,167-175]

Mutations within the splice site or the splice site consensus sequences favor improper processing of the mRNA precursor. These secondary splicing events, which are not seen under normal circumstances, occur at positions that resemble splice site consensus sequences. Splicing at these "cryptic" sites generates aberrantly processed, nonfunctional globin mRNA (see Fig. 21-8). Mutations within the β-globin IVS-1 splice donor site activate two cryptic donor sites in exon 1 and a third site in IVS-1,[149,161,176] whereas mutation in the IVS-2 splice donor activates a cryptic donor site in IVS-2.[162] Mutation of the IVS-2 splice acceptor site activates an upstream cryptic splice acceptor at position 579 in IVS-2.[46] These incorrectly spliced mRNA molecules sustain either insertions or deletions in the coding region and also shifts in the translational reading frame downstream of the cryptic splice site. The polypeptide synthesized beyond this point bears no resemblance to the globin chain and is often prematurely shortened by a termination codon encountered in the new reading frame.

Mutations within Exons That Create an Alternative Splice Site

RNA from β-thalassemia genes with mutations in the IVS-1 donor splice site may be processed via a cryptic splice donor site GTGG*T*GAGG in exon 1 (codons 24 through 27). Four independent mutations have been identified that activate this cryptic site in the presence of a normal IVS-1 splice donor site (Fig. 21-9; see also Table 21-2).[106,177,178,179] These mutations appear to enhance the ability of the cryptic site to compete with the normal site for binding of the splicing complex. A T→A mutation at codon 24 is "silent" at the translational level, yet approximately 80% of RNA transcripts are spliced at this incorrect site; hence, mild β⁺-thalassemia ensues. Two mutations—GAG→AAG in codon 26 and GCC→TCC in codon 27—lead to amino acid replacements that produce the hemoglobin variants HbE and Hb Knossos, respectively, in normally processed mRNA. Because a proportion of transcripts are aberrantly spliced, mild β⁺-thalassemia results. An analogous mutation in codon 19 produces β⁺-thalassemia with the hemoglobin variant Hb Malay,[180] representing mutations that lead to thalassemic hemoglobinopathies.

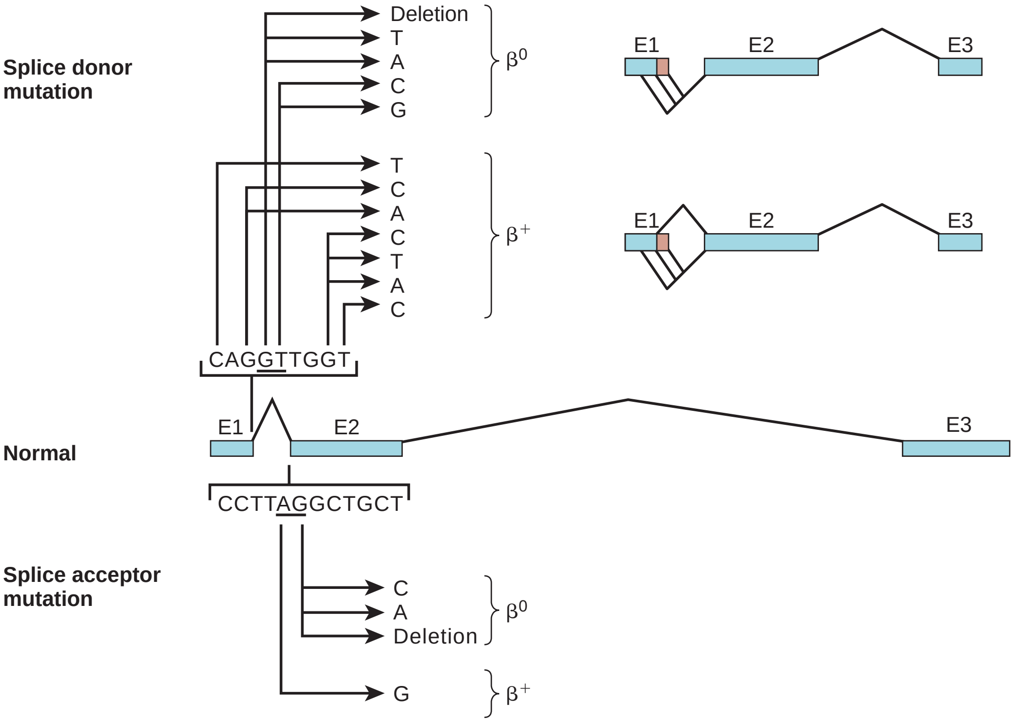


Figure 21-8 Examples of abnormal splicing that result from alterations in the splicing consensus sequences. The three β-globin gene exons are symbolized by *blue boxes*, the normal splicing pattern is illustrated by lines that project above the exons, and the splice donor and splice acceptor sequences of the first intron are shown. Mutations in the invariant GT dinucleotide of the splice donor site abolish normal splicing of the first intron and result in β^0-thalassemia, whereas mutations elsewhere in the consensus sequence preserve some normal splicing and cause β^+-thalassemia. Changes in the splice donor site are associated with abnormal splicing from three cryptic splice donors (lines that project below the exons); one site is within the first intron and results in the addition of intron sequences to exon 1 *(brown boxes)*. Similarly, changes in the invariant AG dinucleotide of the splice acceptor sequence are associated with β^0-thalassemia, whereas a mutation in an adjacent nucleotide causes β^+-thalassemia.

Mutations within Introns That Create an Alternate Splice Site

Mutations within β-globin IVS-1 may create a new splice acceptor sequence (Fig. 21-10; see also Table 21-2).[181-183] In the first of this class of mutations to be characterized, a G→A substitution at position 110 (19 nucleotides upstream of the normal intron/exon boundary), the majority of globin mRNA precursors are spliced at this alternate site.[181,182,184,185] Because the incorrectly spliced mRNA contains 19 nucleotides from IVS-1, a shift in the reading frame leads to premature termination of translation. A T→G mutation at position 116 of IVS-1 creates a new acceptor site that is used exclusively, thereby leading to little or no normal β-globin mRNA production and to β^0-thalassemia.[183]

Three mutations in IVS-2 create new donor sites and activate an upstream cryptic donor site located 579 nucleotides from the exon 2–IVS-2 boundary (see Table 21-2 and Fig. 21-10).[149,169,186] The consequence of these mutations is the insertion of a fourth "exon" derived from sequences within IVS-2. Although the normal donor and acceptor sites are unaffected, little or no correctly spliced β-globin mRNA may be produced.[149,169,186]

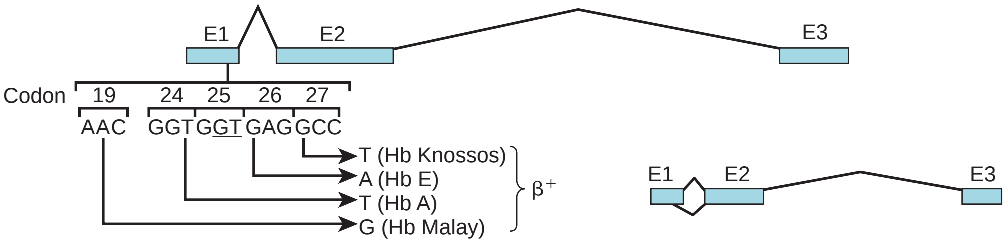


Figure 21-9 Mutations that create an alternate splice donor site in the first exon decrease but do not abolish the occurrence of normal splicing (pattern that projects above the exons) and are associated with splicing from the new site in the first exon (pattern that projects below the exons). Three of these mutations lead to the incorporation of a different amino acid into the β-globin chain derived from the decreased quantity of correctly spliced β-globin messenger ribonucleic acid and generate variant hemoglobins.

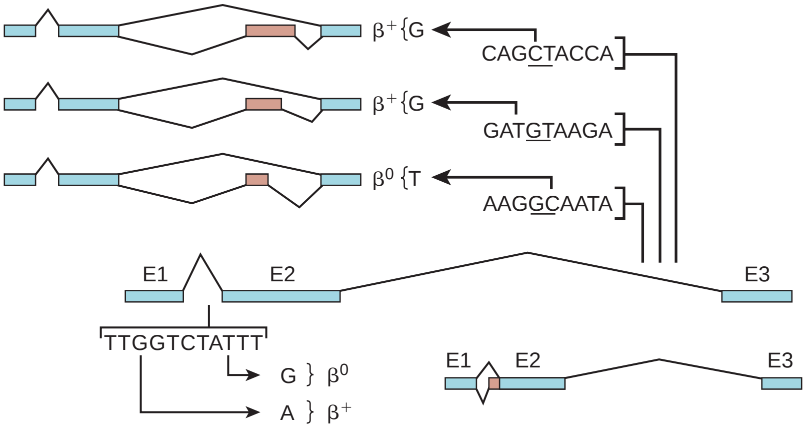


Figure 21-10 Mutations within introns that create a new splice site. Three mutations in the second intron create a new splice acceptor site (the invariant GT is underlined) and activate the identical cryptic splice donor site located just upstream. Abnormal splicing from these sites (pattern that projects below the exons) leads to the creation of a fourth exon *(brown boxes)* derived from sequences within the second intron. Two mutations in the first intron create a new splice acceptor site with the conserved AG dinucleotide, and abnormal splicing from this site (pattern that projects below the exons) adds sequences from the first intron to the beginning of the second exon.

RNA Cleavage and Polyadenylation Defects

Proper cleavage at the 3′ end of the pre-mRNA and subsequent poly(A) addition depend on the integrity of the AAUAAA signal in the 3′-untranslated region. The importance of the polyadenylation signal for efficient production of globin mRNA was first demonstrated in α-thalassemia.[187-189] An AAUAAA→AAUAAG mutation in the α_2 gene reduces the efficiency of cleavage-polyadenylation of precursor RNA and leads to "run-on" transcripts that terminate downstream of the gene (see Fig. 21-12). Mutations in the AAUAAA element have also been described in β-thalassemia,[148,190,191] in which the presence of elongated in vivo transcripts has been demonstrated.[190] The transcripts appear to terminate at the next AAUAAA signal, which is present approximately 900 nucleotides downstream of the normal cleavage site. These mutations lead to a moderate reduction in the level of β-globin mRNA and a β^+ phenotype.

Mutations Affecting mRNA Translation Initiation

Translation begins at an AUG codon that usually lies within the consensus sequence (GCC)GCC(A/G)CC*AT*GG.[192] Substitutions within the AUG codon abolish translation, whereas those in other positions of the consensus often result in less efficient initiation of translation.

Four mutations in α-globin genes alter the consensus sequence and impair translation (see Table 21-2). Three of them affect the AUG Inr.[193-195] No globin polypeptide is produced because the next downstream Inr is in a different reading frame. The fourth α-globin mutation in this class, found on a chromosome in which one α-globin gene was deleted, alters the consensus sequence by the deletion of two bp and reduces mRNA translation to 50% of normal.[196,197] Two AUG Inr mutations of the β-globin gene have been described, and both are of the β^0 type (see Table 21-2).[3,191]

Premature Termination (Nonsense) Mutations

Nucleotide substitutions within the coding region are innocuous if they occur in the third position of a codon and do not alter the amino acid inserted during translation. Substitutions that alter codons from one amino acid to another lead to hemoglobin structural variants. Some substitutions change a triplet coding for an amino acid to a stop codon (UAG, UUA, or UGA). Such chain termination (or nonsense) mutations abort mRNA translation and lead to the synthesis of a truncated polypeptide. Moreover, nonsense mutations also reduce the amount of stable mRNA generated, which is a reflection of coupling between mRNA biogenesis and mRNA translation (Fig. 21-11).[198]

Chang and Kan[199] described the first nonsense mutation in β-thalassemia in which a lysine codon at amino acid position 17 was converted to a stop codon (AAG→UAG). Although no β-globin chains were produced in vivo, complete translation of the abnormal mRNA could be achieved in a cell-free extract capable of protein synthesis by the addition of a "suppressor" transfer RNA (tRNA) that inserts a serine at the UAG codon.[200] Several other nonsense mutations causing thalassemia have been described (Fig. 21-12; see also Table 21-2).[158,171,201-205] In addition, single or dinucleotide insertions or deletions have been observed that alter the translational reading frame and introduce a premature stop codon as a consequence.[3,148,169,172,180,206-214] Two termination mutations have been described in the α-globin genes, one that introduces a stop codon[215] and the other a frameshift.[205] In addition, frameshift mutations have been described that result in abnormal elongation of globin chains (see the section on unstable β-globin chains).

mRNA molecules with termination mutations often do not accumulate to a normal level in vivo.[189,215] The extent of this effect is variable and depends on the specific mutations; deletion of the third nucleotide (C) from codon 41 (Fig. 21-13) leads to complete absence of globin mRNA,[216] whereas a single substitution in the β39 codon allows the accumulation of roughly 5% to 10% of the normal amount of globin mRNA.[215] The basis for the quantitative deficiency in these mRNA species is of considerable interest. Some data suggest that such mutations lead to

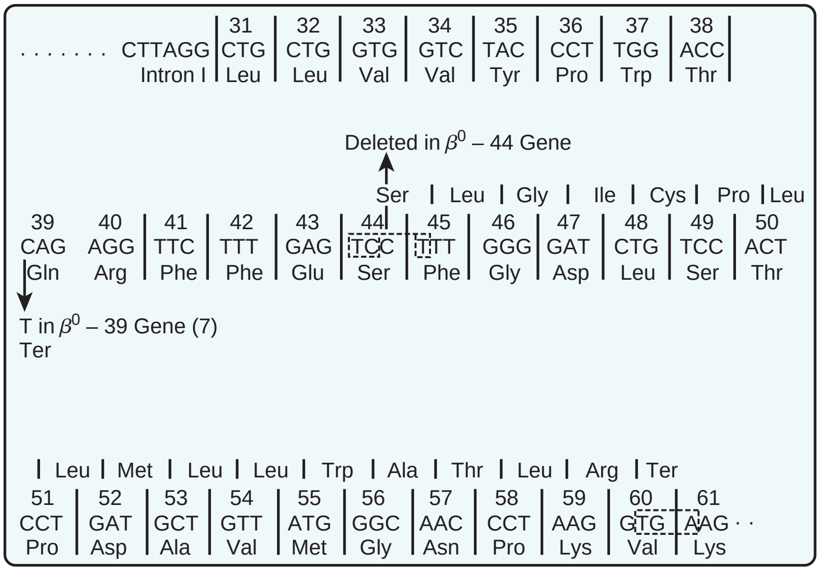

. . . . CTTAGG
Intron I
31 CTG Leu
32 CTG Leu
33 GTG Val
34 GTC Val
35 TAC Tyr
36 CCT Pro
37 TGG Trp
38 ACC Thr
Deleted in β0 – 44 Gene
Ser Leu Gly Ile Cys Pro Leu
39 CAG Gln
40 AGG Arg
41 TTC Phe
42 TTT Phe
43 GAG Glu
44 TCC Ser
45 TTT Phe
46 GGG Gly
47 GAT Asp
48 CTG Leu
49 TCC Ser
50 ACT Thr
T in β0 – 39 Gene (7)
Ter
Leu Met Leu Leu Trp Ala Thr Leu Arg Ter
51 CCT Pro
52 GAT Asp
53 GCT Ala
54 GTT Val
55 ATG Met
56 GGC Gly
57 AAC Asn
58 CCT Pro
59 AAG Lys
60 GTG Val
61 AAG Lys

Figure 21-11 Two of the several thalassemia mutations that destroy gene function by the introduction of a premature translation termination codon in β-globin messenger ribonucleic acid (mRNA). The numbers above the individual codons refer to the position of the encoded amino acid in the β-globin. Replacement of C with T in codon 39 introduces the terminator UAG in β-globin mRNA. Another β^0 gene has a deletion of the third nucleotide (C) in codon 41, which results in a shift in the reading frame of the mRNA; the new amino acid sequence is shown above the line. This new reading frame has an in-phase terminator (UGA) in a position corresponding to codon 60 to 61. *(Modified from Nienhuis AW, Anagnou NP, Ley TJ: Advances in thalassemia research.* Blood *63:738–758, 1984.)*

intranuclear degradation of abnormal globin RNA and suggest a link between mRNA translation and nuclear RNA processing or nuclear to cytoplasmic transport of mRNA.[217,218] Experimental studies in tissue culture systems have shown that the deficiency in β-globin mRNA accumulation is specific for nonsense mutations and is not observed with missense mutations[198]; a suppressor tRNA that allows the abnormal mRNA molecule to be translated completely will correct the quantitative deficiency in globin mRNA.[218] Recent work from a number of investigators has begun to unravel the molecular machinery that mediates this phenomenon, which has been termed *nonsense-mediated decay*, and appears to play an important role in normal physiology, as well as pathologic states.[219]

Termination Codon Mutations

UAA is the normal termination codon for both α- and β-globin mRNA translation. The 3′-untranslated regions are 109 and 132 nucleotides for α- and β-globin mRNA, respectively. A single nucleotide substitution in the termination codon could either create another stop codon (UAG) or permit incorporation of an amino acid at this position and translation of the otherwise untranslated 3′ sequences until the next in-frame stop codon. Four termination codon mutations involving the α_2 gene have been reported (see Table 21-2).[220-225] These mutants differ only in the specific amino acid incorporated at the terminator codon position (see Fig. 21-13). Translation terminates in each instance at a UAA codon in the

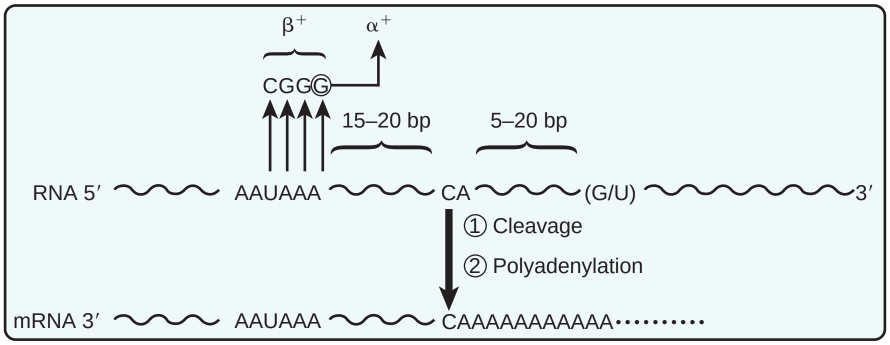

β+
α+
CGGG
15–20 bp
5–20 bp
RNA 5′
AAUAAA
CA
(G/U)
3′
① Cleavage
② Polyadenylation
mRNA 3′
AAUAAA
CAAAAAAAAAAA

Figure 21-12 Ribonucleic acid cleavage and polyadenylation occur 15 to 20 bp downstream from the AAUAAA polyadenylation signal. Mutational analysis in the rabbit β-globin gene has established that sequences located downstream from the polyadenylation site, called the G/U cluster, are also required for efficient cleavage and polyadenylation. Individual point mutations at one of several nucleotides within the AAUAAA polyadenylation signal result in β^+-thalassemia. The same A→G mutation in the last position that causes β-thalassemia has been observed in the α_2-globin gene and results in α^+-thalassemia.

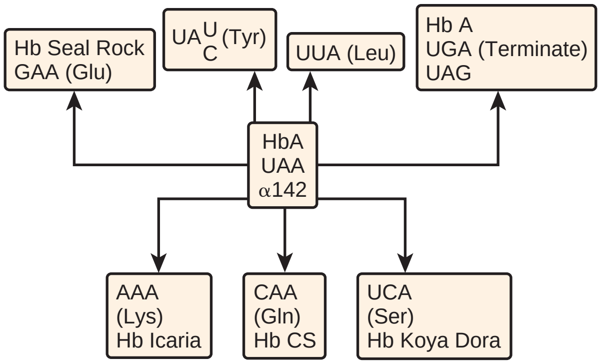


Figure 21-13 Point mutations in the terminator codon of the α-globin gene that lead to the synthesis of elongated α-globins. The normal terminator, UAA, of α-globin messenger ribonucleic acid is shown in the center. Each of the nine possible single nucleotide substitutions is depicted; two would result in the formation of another terminator codon, whereas the other seven would lead to insertion of an amino acid at this position and continued synthesis of the globin chain. Four such mutations have been described; the mutation that causes the synthesis of hemoglobin (Hb) Constant Spring (CS) is the most common. *(Modified from Weatherall DJ, Clegg JB:* The thalassemia syndromes. *Oxford, 1981, Blackwell Scientific, p. 578.)*

polyadenylation signal (AA*UAA*A) downstream, and a 172–amino acid polypeptide is produced. The first of these elongated α chains to be described was found in Hb Constant Spring (CS).[220,221] The α chain in this hemoglobin has a glycine substituted at codon 142. Hb CS produces an associated thalassemia phenotype because of a marked reduction in α_2-globin mRNA stability.[226-228]

Mutations that give rise to elongated β-globin chains have also been described. Hb Tak is a 157–amino acid product of a β-globin mRNA molecule containing two inserted nucleotides in the terminator codon 147.[225] An analogous elongated β-globin with 157 amino acids found in Hb Cranston reflects a dinucleotide insertion in codon 147, but red blood cells containing Hb Cranston are morphologically normal.[229] The mechanism by which the β^{Tak} mutation causes thalassemia has not been elucidated.

Mutations Affecting Globin Chain Stability

Hemoglobin Assembly. Shortly after synthesis is completed, α- and β-globin chains bind a heme moiety and rapidly associate into $\alpha_1\beta_1$ dimers in a noncovalent reaction that is nearly irreversible under physiologic conditions.[230] The majority of the heme contact points are present in the portion of the globin chains encoded by exon 2, whereas most $\alpha_1\beta_1$ contacts are located within the exon 3 domain (Fig. 21-14).[6,231] These dimers may then reversibly associate with other dimers to form the hemoglobin tetramer. Formation of the $\alpha_1\beta_1$ dimer, therefore, is the principal controlling step in the assembly of hemoglobin. This subject is discussed in greater depth in Chapter 19.

Hemoglobin assembly is an important determinant of the final hemoglobin composition of the erythrocyte.[230,232,233] The rate constant of dimer formation depends greatly on the surface electrostatic charge of the subunits.[230] α-Globin has a net positive surface charge, whereas β-globin has a net negative surface charge. The other normal β-like globin chains, γ-globin and δ-globin, dimerize with the α chain at a lower rate. δ-Globin has a lower net negative surface charge than does β-globin, and the significant structural differences between γ- and β-globin presumably account for the differing dimerization rates. When β-globin chains are in limited supply (i.e., β-thalassemia), HbA_2 and HbF levels may increase because of enhanced dimerization with α chains, independent of changes in the production of δ and γ chains. In α-thalassemia or iron deficiency, HbA_2 and HbF levels will decrease because of competition with β chains for the limited number of α chains. Similarly, β-chain variants may have decreased (β^S and β^E) or increased ($\beta^{Baltimore}$) affinity for α chains based on their net surface charge. The net hemoglobin composition of the cell is determined by these simple rules of competition based on the relative affinity of hemoglobin subunits.

An efficient, energy-dependent protein quality control system is present in erythrocytes that rapidly degrades free globin chains while leaving chains that are incorporated into dimers or tetramers unaffected.[230,234,235] Changes in globin chain structure that result from amino acid substitutions, premature chain termination, or chain elongation may slow or block the formation of stable $\alpha_1\beta_1$ dimers and lead to rapid degradation of the globin chain. In some instances, as discussed later, mutations may also enhance association of the free globin chain with the cell membrane and thereby promote oxidative damage to the membrane and shortened red blood cell survival.

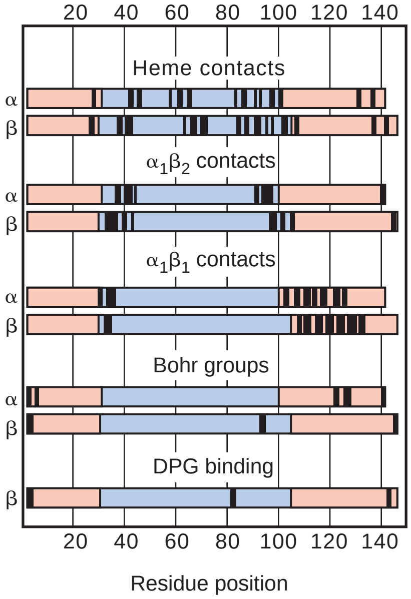


Figure 21-14 Schematic representation of the α- and β-globin chains and the location of residues involved in different hemoglobin functions (*black vertical bars*). Heme contact sites and $\alpha_1\beta_2$ contacts are concentrated in the second exon (*blue*), whereas $\alpha_1\beta_1$ contacts are principally located in the third exon (*salmon*). *DGP,* Diphosphoglycerate. *(Modified from Eaton WA: The relationship between coding sequences and function in haemoglobins.* Nature *284:183–185, 1980.)*

An erythroid-specific α-hemoglobin–stabilizing protein (AHSP) acts as a chaperone for free α-globin chains. In the absence of this protein, mice exhibit mild hemolytic anemia as a result of α-globin precipitation.[236] When this protein is absent in the presence of a β-thalassemia mutation in mice, a much more severe thalassemic phenotype occurs.[237] However, it is currently unclear whether variation in the structure or expression of this gene in humans causes phenotypic variability in patients with β-thalassemia. One study has suggested that this was not the case in patients with HbE–β-thalassemia in Thailand.[238] Another study has suggested that expression levels of this gene vary in humans and that such levels may correlate with the severity of β-thalassemia.[239] Further studies will need to be conducted to better understand whether AHSP plays a role as a modifier gene in β^+-thalassemia.

During hemoglobin biosynthesis it is also important that the globin chains not be produced in excess of heme. A protein called heme-regulated inhibitor (HRI) kinase appears to mediate this function by inhibiting globin chain translation when an erythroid precursor is deficient in heme.[240] When HRI is absent in mice with a β-thalassemia mutation, the thalassemic phenotype is exacerbated and lethality of the mice results.[241]

Unstable α-Globin Chains. One such unstable variant was identified on sequencing of a mutant α-globin gene (see Table 21-2).[242] The $\alpha^{\text{Quong Sze}}$ variant, which contains a Leu→Pro substitution at position 125, is so unstable that the mutant globin chain cannot be detected by biosynthetic studies in intact cells or by conventional hemoglobin electrophoresis.[243] The $\alpha^{\text{Quong Sze}}$ chain appears to be stable once it is incorporated into the hemoglobin tetramer. Three other similar unstable α-globin chain mutations have been described (see Table 21-2).[244-247]

Unstable β-Globin Chains

Several unstable β-globin chains have been associated with thalassemia (see Table 21-2). Five mutations lead to amino acid substitutions in the β-globin chain.[248-252] Frameshift mutations in the third exon result in the synthesis of an elongated β-globin chain with a novel carboxyterminal.[3,251,253-257] A premature termination mutation in exon 3 has also been described.[255-257] Many of the unstable β-globin chain mutations in exon 3 are associated with a dominantly inherited form of thalassemia (see the section on dominant β-thalassemia).[251,252] Thein and colleagues[255] proposed that alterations in β-globin structure in exon 3 interfere with $\alpha_1\beta_1$ dimer formation yet may permit binding of heme to the mutant globin chain through contacts in the exon 2 domain. These free, heme-associated β chains may be more resistant to proteolysis and associate with the cell membrane, where they form "inclusion bodies" and induce oxidative damage.

Thalassemic Hemoglobinopathies

The mutations described in this section, taken together with the RNA-processing mutants HbE, Hb Knossos, and Hb Malay and the termination mutant Hb Tak, include a distinctive set characterized by structural changes in the hemoglobin molecule and a thalassemia phenotype. These mutations are often referred to as "thalassemic hemoglobinopathies" (see the section on thalassemic hemoglobinopathies)[258] and are characterized clinically by a syndrome of ineffective erythropoiesis. Other globin variants may be associated with mild hypochromia, microcytosis, and chronic hemolysis because of instability and degradation of hemoglobin tetramers and are discussed in detail in Chapter 19. Because thalassemia is the consequence of an imbalance in α- and β-globin chains, these variants are not considered part of the spectrum of thalassemia.

Identification, Characterization, and Ethnic Distribution of β-Thalassemia Mutations

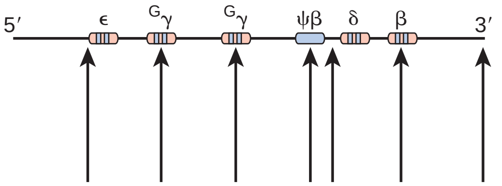
The hemoglobin disorders serve as a paradigm for the molecular analysis of genetic disease. Study of β-thalassemia was aided by the introduction of methods for identifying and characterizing mutant alleles that are now widely used. Accordingly, the identification of β-thalassemia mutations in many ethnic groups is nearly complete.[3,161]

In this section we briefly outline molecular techniques that have been applied to the characterization of β-thalassemia mutations. An understanding of these methods is important not only because they are broadly used in the study of other genetic diseases but also because they are directly relevant to strategies for genetic screening and prenatal diagnosis of β-thalassemia.

Haplotype Analysis

The first several β-thalassemia mutations were identified by cloning and sequencing β-globin genes isolated from persons with β-thalassemia major.[162,181,182,199,203,214,259-263] Because certain mutations are extremely common, a nondirected strategy is inefficient because β genes with common mutations will be repeatedly studied. For example, 95% of the β-thalassemia alleles on the island of Sardinia contain the codon 39 nonsense mutation.[264]

To facilitate the search for new β-thalassemia mutations, Orkin, Kazazian, Kan, and others[149,265,266,268] introduced the concept of haplotype analysis to the study of thalassemia. Naturally occurring, genetically neutral, nonselected sequence differences among individuals constitute polymorphisms, which are estimated to occur roughly once every 100 bp.[267] These sequence differences are heritable, and those residing close to one another on a chromosome tend to be inherited together, a property known as *linkage*. A subset of polymorphisms will alter the cleavage site for a restriction enzyme or create a site where one did not exist. Therefore, when DNA from unrelated individuals is digested with a restriction enzyme and analyzed by Southern blotting, polymorphisms in the restriction enzyme digest pattern may be observed and are referred to as restriction fragment length polymorphisms (RFLPs).[268] Within the 60 kb of the human β-globin cluster, more than such 20 RFLPs are known[5,269]; at least 13 have been identified in the α cluster.[2,270] The pattern of these RFLPs (each based on the presence [+] or absence [–] of a restriction enzyme cutting site) along the chromosome defines a haplotype of associated or linked polymorphisms. Seven RFLPs were used initially to define nine distinct haplotypes (I through IX) of the



Haplotypes	Hinc II	Hd III	Hd III	Hinc II	Ava II	Bam HI	Mutation	Overall frequency
I	+	–	–	– –	+	+	IVS-I n. 110	47%
II	–	+	+	– +	+	+	Codon 39	17%
III	–	+	–	+ +	+	–	IVS-2 n. 1	8%
IV	–	+	–	+ +	–	+		1%
V	+	–	–	– –	+	–	IVS-In. 1 (G→A)	12%
VI	–	+	+	– –	–	+	IVS-I n. 6	6%
VII	+	–	–	– –	–	+	IVS-2 n. 745	6%
VIII	–	+	–	+ –	+	–	–87	1%
IX	–	+	–	+ +	+	+	Codon 39	3%

Figure 21-15 Linkage of chromosomal haplotypes to specific β-thalassemia mutations in Mediterranean populations. A haplotype is defined by the sequential pattern of restriction enzyme sites (present "+" or absent "–") along a chromosome. In this example, seven restriction enzyme sites were used to classify nine haplotypes. Overall frequency refers to the prevalence of the specific haplotype as found in all persons, with or without thalassemia. *IVS,* Intervening sequence. *(Modified from Orkin SH, Kazazian HH Jr, Antonarkis SE, et al: Linkage of beta thalassaemia mutations and beta-globin gene polymorphisms with DNA polymorphisms in the human beta-globin gene cluster.* Nature *296:627–631, 1982.)*

β-gene cluster in the analysis of thalassemia mutations in Greek and Italian populations from the Mediterranean basin (Fig. 21-15).[149]

Close inspection of these haplotypes revealed a nonrandom association of restriction digest patterns within the β-gene cluster (Fig. 21-16).[265,271] The pattern of restriction sites 5′ of the δ-globin gene is inherited as a group, whereas restriction sites downstream (including the β-globin gene) track as another set. In all populations only a few haplotypes predominate.[272] The full spectrum of haplotypes is derived from random association over evolutionary time between the 5′ and the 3′ subhaplotypes, presumably reflecting the presence of a recombination "hot spot" lying between these regions.[162,271,273,274]

The generation of haplotypes appears to be an ancient event predating racial dispersion. Consequently, a specific haplotype may be found in diverse ethnic and racial groups from different geographic locations. The introduction of malaria as a selective pressure for certain random mutations is a more recent phenomenon. A mutation leading to thalassemia would be under positive selection and amplified within a population; accordingly, the mutation resides on the haplotype background in which it originated in that ethnic group. Several conclusions were derived from the study of different racial groups.[106] Within a single population both normal and thalassemia β-globin genes are found on the same haplotype, but specific thalassemia mutations tend to be linked to a single haplotype. Individual thalassemia mutations are generally restricted to a single population (see Table 21-2). In circumstances in which specific mutations are found in different populations, the identical thalassemia mutation may have occurred and been selected independently and will be found on a different haplotype background.[47,167,275] This observation has provided a sound genetic basis for the belief that thalassemia has had multiple distinct origins throughout the world. In circumstances in which a specific mutation is found on more than one haplotype within a population, the 3′ subhaplotypes (where the β-globin gene resides)

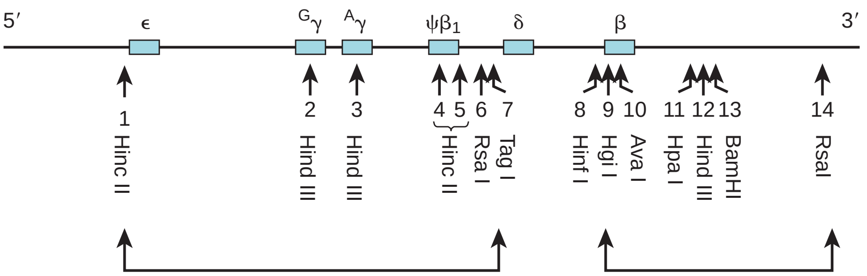


Figure 21-16 Restriction endonuclease sites for which restriction fragment length polymorphisms (RFLPs) have been identified in the β-globin gene cluster. A recombinational hot spot has been identified between the two brackets; the RFLP enclosed in each bracket must often remain associated during recombination (see text).

may be identical, whereas the 5′ subhaplotypes differ because of recombination between the two subhaplotypes (for an example, see codon 39 mutation, see Fig. 21-15). By this mechanism, specific mutations can be distributed to new haplotypes within an ethnic group, and an independent origin of the mutation need not be invoked.

New thalassemia mutations were identified by cloning thalassemia β-globin genes from distinct haplotypes within a population, thereby avoiding the likelihood of repeated cloning of the same common mutation.[149] In this way, the great diversity of thalassemia mutations was elucidated.[164]

Ethnic Distribution

Determining the incidence of specific β-thalassemia mutations in different ethnic groups is particularly relevant to strategies for prenatal diagnosis of or preconception screening for thalassemia.[3] Nearly complete surveys of thalassemia mutations have been performed in Greek and Italian,[266] Asian Indian,[154,276] American black,[43,171] Sardinian,[264] Chinese,[277,278] Lebanese,[155] Turkish,[279] Spanish,[280] Sicilian,[281] Thai,[201] Kurdish Jewish,[148] and Japanese[252] populations. From these studies, several general conclusions can be drawn. First, in each ethnic group a small subset of mutations (as few as four or six) constitute more than 90% of the mutant alleles. This characteristic is particularly striking on the island of Sardinia, where the codon 39 nonsense mutation accounts for 95% of the β-thalassemia genes and a codon 6 frameshift represents another 4%.[264] Second, the remaining 5% to 10% of mutant alleles in an ethic group are divided among a larger number of rarer alleles. For example, four alleles account for 90% of the β-thalassemia genes in Chinese, and 11 rare alleles account for the remaining 10%.[3,277] Third, several mutations appear to have originated independently in different ethnic groups and are present on different haplotype backgrounds, as discussed earlier. For example, the IVS-2 No. 1 (G→A) mutation is present in Mediterranean, Tunisian, and American black populations,[159,161,163] whereas the IVS-1 No. 5 (G→C) substitution is present in Asian Indians, Chinese, and Melanesians.[131,158,169] Finally, as a consequence of the large number of mutations present in each population, most persons with severe β-thalassemia are genetic compound heterozygotes for two different thalassemia mutations.

Mutations That Affect β-Globin Gene Regulation

Deletions within the β-globin gene cluster often lead to thalassemia. Many of these deletions are associated with a significant increase in the level of HbF, a finding that distinguishes them from the common varieties of β-thalassemia. Ordinarily, heterozygous carriers of β-thalassemia have an increase in the level of HbA_2 (to >3% of total hemoglobin) and, at most, a slight increase in the level of HbF. Before detailed molecular analysis was available, these conditions were broadly grouped into two categories, hereditary persistence of fetal hemoglobin (HPFH) and δβ-thalassemia[4,282,283] (Table 21-3). HPFH heterozygotes have normocytic, normochromic red blood cells, whereas δβ-thalassemia heterozygotes have hypochromic and microcytic cells. Many HPFH heterozygotes have high levels of HbF (up to or greater than 30%) with a uniform or pancellular distribution in circulating erythrocytes, whereas in δβ-thalassemia heterozygotes, the amount of HbF is less abundant and it is present in an uneven or heterocellular distribution among red blood cells. In rare persons who are homozygous for either condition, only HbF is found. HPFH homozygotes have normal or slightly elevated total hemoglobin concentrations, their red blood cells are slightly hypochromic and microcytic, and globin synthesis is modestly imbalanced.[284,285] Thus these mutations are appropriately considered along with the thalassemia mutations.

TABLE 21-3 Phenotypes of $^G\gamma^A\gamma$ Hereditary Persistence of Fetal Hemoglobin and $^G\gamma^A\gamma\ (\delta\beta)^0$-Thalassemia Heterozygotes

	HPFH	δβ-Thalassemia
Red blood cell morphology	Normal	Abnormal
MCH	Nearly normal	Decreased
Hematocrit	Normal	Slightly decreased
HbF (%)	15-30	1-15
HbF distribution in red blood cells	Pancellular	Heterocellular

HbF, Fetal hemoglobin; *HPFH,* hereditary persistence of fetal hemoglobin; *MCH,* mean corpuscular hemoglobin.

Deletion mutations of the β-globin gene cluster represent in vivo experiments of nature useful in developing and validating experimental models of gene regulation. Multiple regulatory elements are present within the cluster, and the clinical phenotypes observed with specific deletions relate to removal of one or more such regulatory elements. More than 30 deletion mutations have been described (Table 21-4 and Fig. 21-17)[286]; they vary greatly in size from a few hundred base pairs of the β-globin gene to more than 100 kb with loss of the entire cluster. Recent studies have shown how insight from such deletions and mapping regions that diverge between the HPFH and δβ-thalassemia deletions can help to elucidate key regulatory regions important for HbF expression.[93] For example, a region upstream of the δ-globin gene has been implicated in playing a key role in silencing the γ-globin genes and binds the HbF silencing factor BCL11A. In addition to deletion mutations, significant elevations of HbF in adults may arise as a result of single base substitutions within the γ-globin gene promoters (Table 21-5). Such mutations, also classified as nondeletion HPFH, appear to enable the γ-globin genes to "capture" the influence of the LCR at the adult stage. Persons with these HPFH mutations have HbF values ranging from only slightly elevated to more than 20%, which can be distributed in either a heterocellular or pancellular fashion.

HPFH and δγ-thalassemia mutations are uncommon, and persons who inherit these mutations are asymptomatic or have mild disease. Their importance relates to the insight that they provide into globin gene regulation and

TABLE 21-4 Deletion Mutations of the β-Globin Gene Cluster

Type	Ethnic Group	Deletion Size (kb)	Deletion Coordinates	HbF Level in Heterozygotes (%)	Other Information	References
A. $^{A}\gamma^{0}$						
1		2.5	37.7-40.2	0.2	"Silent"	304
B. δ^{0}						
1	Corfu	7.20	48.9-56.1	1.1-1.6	δ^{0}-Thalassemia	305-307
C. β^{0}						
1	Indian	0.619	63.?-64.0	Normal	β^{0}-Thalassemia	158, 262, 265, 310
2	American black	1.393	61.6-63.0	7.0-7.9	β^{0}-Thalassemia	311, 870
3	Dutch	12.6	59.7-72.3	4-11	β^{0}-Thalassemia	514, 515
4	Turkish	0.29	62.1-62.4	2.7-3.3	β^{0}-Thalassemia	871, 872
	Jordanian			9.4	β^{0}-Thalassemia	873
5	Czech	4.237	58.9-63.1	3.3-5.7	β^{0}-Thalassemia	874
	Canadian					
D. $(\delta\beta)^{0}$						
1	Sicilian	13.377	56.0-69.4	5-15		286, 312, 313
2	Spanish	≅114	52.2-?	5-15		339, 875, 876
3	American black	12.0	52.3-64.1	25	Pancellular[*]	877
4	Japanese	>130	43.1-?	5-7		886, 887
5	Laotian	12.5	56.0-68.5	11.5		878
6	Macedonian	18-23	54-74	7-14		879
7	Mediterranean	7.4	55.3-62.7	1-5	Hb Lepore	290-292
E. $^{G}\gamma^{+}(A\gamma\delta\beta)^{0}$						
1	Indian	Total 8.3 kb	40.1-40.9	10-15		315, 316
	Iranian	Deleted	55.5-63.0			
	Kuwaiti	14.6 kb inverted	40.9-55.5			
2	American black	35.7	40.7-76.4	6-16		286, 317
3	Turkish	36.22	37.1-73.3	10-15		286, 318, 319
	American black					
4	Malaysian (1)	>27	37.0-?	Unknown		880
5	Malaysian (2)	>40	39.1-?	Unknown		320
6	Chinese	≅100	40.5-?	10-15		321, 880
7	German	53.0	37.6-90.6	9.9-12.5		322
8	Cantonese	>43	37.0-?	20		316
F. $(\gamma\delta\beta)^{0}$						
1	Hispanic	39.5	−19.5-10.0			329
2	English	>100	?-35.9			330
3	Dutch	99.4	?-59.8			331-333
4	Anglo-Saxon	95.9	?-62.6			319, 332, 334
5	Mexican	>105	?-?			326
6	Scotch-Irish	>105	?-64-71			327
7	Yugoslavian	>148	?-?			328
8	Canadian	>185	?-?			328
G. HPFH						
1	American black	≅106	51.2-?	20-30 (5% G)[†]	HPFH-1	319, 339-341
2	Black (Ghana)	≅105	47-?	20-30 (30% G)[†]	HPFH-2	304, 339-344
3	Indian	48.5	45.0-93.5	22-23 (70% G)[†]	HPFH-3	58, 337, 339
4	Italian	40.0	50.0-90.0	14-30	HPFH-4	888
5	Italian	12.9	51.6-64.5	16-20 (15% G)[†]		345
6	Kenyan	22.8	40.0-62.8	5-8	Hb Kenya	300-302

Hb, Hemoglobin; *HbF,* fetal hemoglobin; *HPFH,* hereditary persistence of fetal hemoglobin.

[*] In a compound δβ-thalassemia/HbS heterozygote.

[†] Percentage of $^{G}\gamma$ in total γ (Gγ/Gγ + Aγ). Forty percent $^{G}\gamma$ is the normal value for an adult.

the role of HbF in modulating disease severity in patients with severe γ-thalassemia or sickle cell anemia.

Isolated point mutations of the δ-globin gene, similar to those in β-thalassemia, may lead to "δ-thalassemia." δ-Thalassemia is a benign condition with no clinical significance that, when inherited with a β-thalassemia mutation, may lead to a normal or low HbA_2 thalassemia phenotype in heterozygotes.[287,288]

Crossover Globins: Hemoglobin Lepore and Hemoglobin Kenya

Deletion mutations in the α- and β-globin gene clusters arise through unequal homologous recombination or through nonhomologous (illegitimate) recombination. In contrast to the α-globin cluster, in which there are blocks of tandem duplicated sequences (discussed later), the only directly repeated homologous segments of DNA in the β

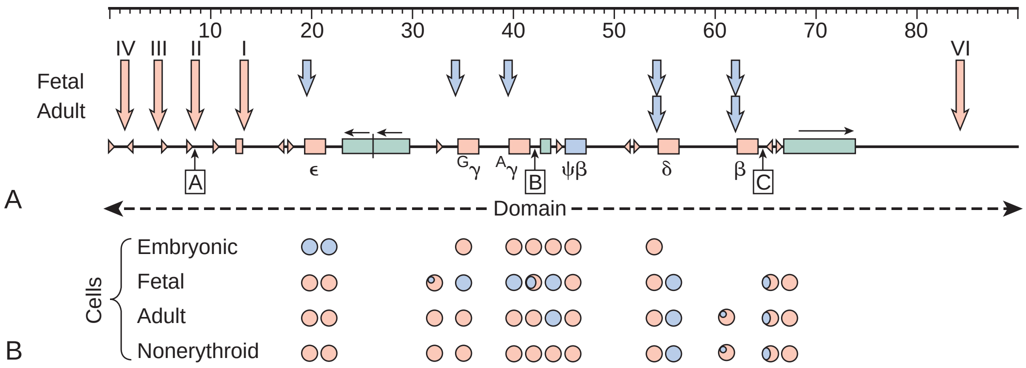


Figure 21-17 **A,** Organization and chromatin structure of the human β-globin cluster. The expressed genes are shown as *salmon boxes* and the single pseudogene in the cluster as a *blue box*. The *arrowheads* in the line figure indicate the location and orientation of Alu repetitive deoxyribonucleic acid (DNA) sequences, whereas the *green boxes* represent members of the L1 family of repetitive DNA. The *salmon downward arrows* mark developmentally stable, erythroid-specific hypersensitive sites that constitute the locus-activating region (LAR) flanking the cluster. The true boundaries of the "active" chromatin domain established by these sites are unknown and extend beyond the cluster. Hypersensitive sites are also found over the promoters of the expressed genes (*blue downward arrows*). The location of the three enhancers in the cluster are marked by letters: A, hypersensitive site II enhancer; B, 3′ $^A\gamma$ enhancer; C, 3′ β enhancer. **B,** The methylation pattern of the locus at different stages of ontogeny is depicted. *Blue circles* show an unmethylated site, *salmon circles* show a totally methylated site, and *circles with blue and salmon* show the degree of site methylation. The coordinates refer to distance in kilobases. *(Modified from Stamatoyannopoulos G, Nienhuis AW: Hemoglobin switching. In Stamatoyannopoulos G, Nienhuis AW, Leder P, Majerus PW, editors:* The molecular basis of blood diseases, *Philadelphia, 1987, WB Saunders, p. 79.)*

cluster are the globin genes themselves. Hence mutations arising from homologous but unequal crossover in the β-globin cluster are relatively uncommon and usually involve two globin genes directly (Fig. 21-18). Two crossover hemoglobins, Hb Lepore and Hb Kenya, are associated with thalassemia.

In 1958, Gerald and Diamond[289] identified a minor hemoglobin component by starch gel electrophoresis in the blood of parents of a patient with thalassemia major. Structural analysis revealed that the hemoglobin was composed of α-globin and a heretofore undescribed globin consisting of fusion of the 80 to 100

TABLE 21-5 Nondeletion Hereditary Persistence of Fetal Hemoglobin Mutations

Type	Molecular Defect	γ Gene	Ethnic Group	Percentage of HbF in Heterozygotes	Chains $^G\gamma$, $^A\gamma$	Distribution of HbF	References
1	C→G at −202	G	Black	15-20	G only	Pancellular	346, 350, 351
2	C→T at −202	A	Black	2-3	93% A	Heterocellular	352
3	+C insertion at −200	G	Tunisian	18-27	G only		809
4	T→C at −198	A	British[*]	4-12	90% A	Heterocellular	353-355
5	C→T at −196	A	Chinese	10-15	90% A	Heterocellular[†]	356, 357
			Italian[‡]	10-20	95% A	Pancellular	358, 359
7	T→C at −175	G	Italian	20-30	90% G		890
			Black	30[†§]	G only	Pancellular	363
8	T→C at −175	A	Black	35-40	80% A	Pancellular	364
9	G→A at −161	G	Black	1-2		Heterocellular	365
10	G→T at −158[‖]	G	Saudi[¶]	2-4	G > A	Heterocellular	366-372
11	G→A at −117	A	Mediterranean[#]	10-20	95% A	Pancellular	360, 361, 373
			Black	10-20	85% A	Pancellular	357
12	C→G at −114	G	Australian	8.5	90% G		891
13	C→T at −114	G	Japanese	11-14	G > A		892
14	C→T at −114	A	Black	3-5	90% A		893
15	Deletion −114 to −102	A	Black	30[†§]	85% A[‡]		374
16	X-Linked	G	"Swiss"[**]	0.8-3.4		Heterocellular	894
17	Unknown[†]	G	German	5-8	A and G	Heterocellular	881
18	Unknown	G	"Georgia"	2.6-6	G > A	Heterocellular	882, 883
19	Unknown	G	"Seattle"	3.7-7.8	G = A	Heterocellular	884, 885

HbF, Fetal hemoglobin.
[*] Also found in whites in North America.
[†] More than 80% of the red blood cells contain HbF.
[‡] Also found in *cis* to codon 39 mutation (Sardinian δβ-thalassemia).
[§] In a β-S heterozygote.
[‖] This determinant appears to increase HbF only in patients with erythropoietic stress.
[¶] Identified in Greek and Sardinian families.
[#] Also found in several other ethnic groups.
[**] Found in all ethnic groups.

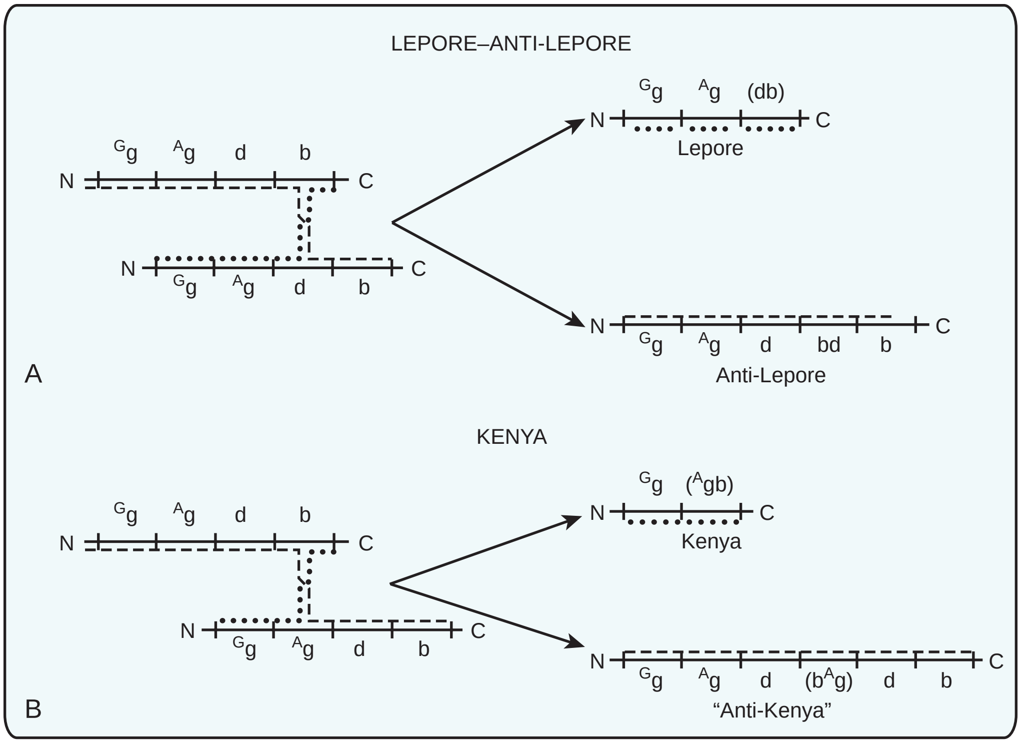


Figure 21-18 Schematic representation of the unequal crossover events that occur during meiosis and result in formation of the Lepore and anti-Lepore (**A**) and the Kenya (**B**) genes. *(Modified from Nienhuis AW, Benz EJ Jr: Regulation of hemoglobin synthesis during the development of the red cell.* N Engl J Med *297:1318, 1977.)*

amino-terminal amino acids of δ-globin with the carboxyl portion of β-globin.[290-293] This "chimeric" globin polypeptide, named Lepore after the family in which it was first described, arose from an unequal, homologous recombination event between the δ-globin and β-globin genes (Fig. 21-18). It is poorly expressed because transcription of the fusion gene is under control of the δ-gene promoter. Other Lepore-type globins have been characterized in which the relative contribution of the δ and β genes to the fusion protein varies as a result of a different point of crossover. Homozygotes for Hb Lepore have 90% HbF and approximately 10% Hb Lepore, but no HbA or HbA$_2$, whereas heterozygotes have mainly HbA, 2% to 4% Hb Lepore, and 3% to 5% HbF.[294,295] In heterozygotes, red blood cells have a very heterogeneous HbF content. Anti-Lepore globins with the amino-terminal sequence of β-globin and the carboxyl sequence of δ-globin have also been described (see Fig. 21-18).[296-299] Persons with anti-Lepore genes also have two normal δ- and two normal β-globin genes; consequently, their red blood cells lack any stigmata of thalassemia. The anti-Lepore globins are produced in very low amounts, perhaps because of sequences within the large intron of the δ-globin gene that may reduce mRNA production.[26]

Hb Kenya, another important crossover hemoglobin, contains a non–α-globin composed of amino sequences of γ-globin and carboxyl sequences of β-globin[300-303] (see Fig. 21-18). Molecular analysis demonstrated that the $^{A}\gamma$-globin gene was involved in the crossover. The crossover occurred approximately at the position of amino acid codon 100. Hb Kenya was first observed in an individual who was heterozygous for a β^{S} gene. This patient had an increased level of HbF (up to 6%) that was uniformly distributed in all his red blood cells. The HbF was entirely of the $^{G}\gamma$ type. Hb Kenya accounted for 17% to 20% of the total hemoglobin. Persons who are heterozygous for Hb Kenya without HbS are clinically healthy. Approximately 10% Hb Kenya and 6% to 10% HbF of the $^{G}\gamma$ type are found homogenously distributed in their red blood cells, in contrast to the distribution of HbF with the Lepore-type deletion.[302]

Silent Deletions

Deletions that eliminate a single γ-globin gene or the δ-globin gene are unlikely to result in a phenotype that would be detected in hematologic screening, and thus few of these mutations have been detected and described. A clinically silent deletion of the $^{A}\gamma$-globin gene was identified through extensive molecular screening of chromosomes in Melanesians.[304] A 7.2-kb deletion involving the δ-globin gene but not the γ- or β-globin gene was reported in Corfu $(\delta\beta)^0$-thalassemia in *cis* to the G→A substitution at IVS-1 position 5.[305] Homozygotes for the IVS-1 position 5 mutation ordinarily exhibit a mild β+-thalassemia intermedia phenotype with 10% to 20% HbA,[172] but homozygotes for Corfu $(\delta\beta)^0$-thalassemia display the clinical phenotype of β^0-thalassemia intermedia with 100% HbF. This finding has been interpreted as evidence that sequences in the deleted region are important in activating the β-globin gene and potentially in suppressing γ-gene expression. Subsequently, however, the identical deletion was identified in *cis* to a normal β-globin gene.[306] In this circumstance, the deletion leads to failure of δ-globin gene expression but has no demonstrable effect on expression of the γ- and β-globin genes on the same chromosome. Recent work has suggested that this deletion may increase γ-gene expression in *cis* to the deletion but that translation of the γ gene may be limiting in certain settings, thus helping explain the discrepancy between carriers of the deletion and homozygotes.[307] The previously discussed mapping studies of HPFH and δβ-thalassemia deletions suggest that the regulatory element upstream of the δ-globin is important for γ-globin silencing, consistent with the idea that the Corfu deletion is removing this critical regulatory element and therefore can result in elevated HbF levels.[93,308]

β^0-Thalassemia Deletion Mutations

Several deletions remove the β-globin gene and are associated with typical high-HbA_2 β^0-thalassemia (see Table 21-4). Interestingly, deletions that remove the promoter region are associated with somewhat higher HbF levels than usually seen in β^0-thalassemia heterozygotes (e.g., see Oner and colleagues[309]). The HbF is distributed in a heterocellular pattern. In contrast, a 0.6-kb deletion at the 3′ end of the β gene that spares the β-globin promoter, seen in Asian Indians, is not associated with an increased level of HbF in heterozygotes.[158,260-262,310] One explanation for the different phenotypes relates to the possibility that an intact β-gene promoter may interact productively with the LCR and thereby effectively compete with an incompletely silenced γ-globin gene at the adult stage. Removal of the promoter may alleviate competition and favor persistent γ-globin expression.

δβ-Thalassemia

Persons heterozygous for these deletions generally have 5% to 20% HbF composed of both $^G\gamma$- and $^A\gamma$-globins and distributed in a heterocellular fashion (see Table 21-4). The erythrocytes are typically hypochromic and microcytic.[1] The level of HbA_2 is characteristically low in comparison with the modest elevations observed in most persons with β-thalassemia trait. Homozygotes for these deletions produce only HbF yet exhibit a mild β^0-thalassemia intermedia phenotype with hemoglobin levels of approximately 10 g/dL. The most common of these deletions is referred to as the Sicilian type.[311-314] The deletion extends from within the δ-globin gene to just beyond the β-globin gene (see Table 21-4). As previously discussed, recent work comparing such deletions with the similar HPFH deletions has provided insight into potential regulatory regions needed for HbF silencing and which are bound by the critical HbF silencing factor, BCL11A.

$^A\gamma\delta\beta$-Thalassemia

The phenotype of these deletions in heterozygotes is identical to that of δβ-thalassemia: hypochromic microcytic red blood cells with low levels of HbA_2 and moderately increased $^G\gamma$-HbF levels (5% to 15%) with a heterocellular distribution (see Table 21-4). Homozygotes have β^0-thalassemia intermedia with 100% $^G\gamma$-HbF. The 5′ breakpoints in these instances lie between the two γ genes or within the body of the $^A\gamma$ gene, thereby resulting in silencing of the $^A\gamma$ gene together with the δ and β genes.[315-324] The 3′ end points of several of these deletions have been precisely mapped. Other deletions are very large and extend well beyond the boundary of the β-gene complex (see Table 21-4). One interesting mutation of this type, the Indian form (see Table 21-4), has two deletions, one involving the γ gene and the second involving a portion of the δ and β genes and intragenic DNA. The segment of DNA between the two deletions is inverted such that the 5′ ends of the γ and δ genes come to be adjacent but in an inverted orientation.[315,316]

γδβ-Thalassemia

The diagnosis of γδβ-thalassemia should be considered in newborns with hemolytic disease associated with hypochromic red blood cells. In adults, it is also a cause of normal-HbA_2, normal-HbF β-thalassemia trait. The syndrome was first identified in a newborn with microcytic, hemolytic anemia.[325] Heterozygous β-thalassemia associated with normal levels of HbA_2 and HbF was identified in the father and many relatives. Decreased γ- and ββ-chain synthesis was demonstrated in the infant's reticulocytes. The anemia was self-limited; as the baby grew older, the hemolytic anemia disappeared and the phenotype of simple heterozygous β-thalassemia developed in the child.

The syndrome of γδβ-thalassemia results from large deletions within the β-globin gene cluster (see Table 21-4). They can be divided into two categories: extremely large deletions that remove the entire β-globin gene cluster or all of the structural genes[326-328] and deletions such as the Hispanic, English, Dutch, and Anglo-Saxon forms in which the structural genes are left intact but the LCR elements are removed.[319,329-334] The remaining genes on the chromosome are not expressed and are found in inactive chromatin that is methylated and resistant to nuclease digestion. Study of these deletions has provided the most convincing evidence that the LCR is required in normal erythroid cells for transcription of the β-like globin genes in vivo. Homozygous γδβ-thalassemia is expected to be incompatible with survival and has not been observed.

β-Thalassemia Mutants Unlinked to the β-Globin Complex

Until recently, the mutations characterized in β-thalassemias resided within or near the β-globin gene itself or the β-globin locus. In principle, defects in *trans*-acting regulatory factors could lead to failure of proper β-globin gene expression. Two examples are now known.

Mutation of the xeroderma pigmentosum disease (XPD) helicase gene, which encodes a component of the general transcription factor TFIIH, results in β-thalassemia in association with trichothiodystrophy. Patients with specific mutations in *XPD* have typical features of β-thalassemia trait, including reduced levels of β-globin synthesis. Inadequate expression of diverse, highly expressed genes is presumed to be the direct consequence of an abnormality in TFIIH.[335]

Mild β-thalassemia trait in association with thrombocytopenia was reported as an X-linked trait in a rare family.[336] Linkage studies demonstrated that the gene mutated in this family resides on the short arm of the X chromosome, near the Wiskott-Aldrich syndrome (*WAS*) gene and the erythroid/megakaryocytic transcription factor GATA1.[337] Subsequently, it was shown that affected boys in this family have a specific amino acid substitution in the amino zinc finger of GATA1 that subtly alters its interaction with DNA and leaves its physical interaction with the cofactor FOG-1 intact.[58] How this change in the properties of GATA1 leads to a slight imbalance in globin gene expression is unknown, although it has been suggested that this mutation may disrupt interactions of GATA1 with other erythroid transcription factors such as TAL1.[338] More recently, similar mutations have been found in other patients. In one case, β-thalassemia was present along with an elevation in HbF to nearly 60%.[56] This patient also had features of erythropoietic porphyria and mild thrombocytopenia. However, another patient with a similar mutation had features of only gray platelet syndrome, and β-thalassemia was not present.[57] These phenotypic differences may be due to differential effects on the TAL1 complex to which GATA1 binds.[338]

Hereditary Persistence of Fetal Hemoglobin

Persons who are heterozygous or homozygous for mutations that enhance HbF are asymptomatic. They are identified incidentally in routine screening programs or during investigation of family members with hematologic disease because of interaction of these variants with other mutations in the β-globin gene cluster. Homozygotes for deletion forms of HPFH have normal hemoglobin concentrations, but their red blood cells are slightly hypochromic and microcytic.[284,285] Minimal globin chain biosynthetic imbalance may be seen. No symptoms or physiologic impairment has been associated with the exclusive production of HbF despite its high oxygen affinity.

Hereditary Persistence of Fetal Hemoglobin Deletion Mutations

Several deletions produce the HPFH phenotype of increased levels of HbF in otherwise normal red blood cells (Table 21-5).* Heterozygotes for these deletions can produce greater than 30% HbF containing both $^G\gamma$ and $^A\gamma$ chains in a pancellular distribution. Differences in the relative percentages of $^G\gamma$ and $^A\gamma$ chains can be detected between different types of HPFH deletions (see Table 21-4). Heterozygotes have normocytic red blood cells. The red blood cells of homozygotes contain HbF exclusively. The higher oxygen affinity of HbF may lead to slightly elevated hemoglobin levels in such individuals. Although homozygotes are clinically healthy, the α-globin to γ-globin chain biosynthetic ratio may be slightly imbalanced, and mild microcytosis and hypochromia may be observed. This finding suggests that γ-chain synthesis occurs at levels below the output of the normal β-globin gene on these chromosomes.

Several hypotheses have been put forward to account for the increased expression and pancellular distribution of HbF in the deletion HPFH syndromes, particularly when compared with the moderate increase and heterocellular distribution in δβ-thalassemia. As previously discussed, recent studies have examined the differences between several deletional forms of HPFH and δβ-thalassemia.[93] These studies have led to the identification of a region upstream of the δ-globin gene that appears to be a critical binding site for BCL11A and several partner proteins. This observation has also been substantiated through the analysis of the patients with deletional forms of β-thalassemia, where variation in the level of HbF expressed appears to be due to the presence or absence of this upstream region.[308] Further studies of this sort are needed to better understand the exact mechanisms by which this region acts to silence the γ-globin genes.[349]

Nondeletion Hereditary Persistence of Fetal Hemoglobin Mutations

Persons with nondeletion hereditary persistence of HbF mutations exhibit elevated levels of HbF with normal red blood cell indices and are identified through hemoglobin screening programs or by study of families in which a segregating high-HbF allele modulates the severity of sickle cell disease or β-thalassemia.[112,113] In many instances, single base changes have been discovered within the promoter region of either the $^G\gamma$- or $^A\gamma$-globin gene.[350-375] These mutations are postulated to alter the binding of nuclear regulatory proteins and lead to a more favorable interaction of the promoter with the LCR at the adult stage.[360,361,376-381] Alternatively, these mutations may disrupt the binding of repressor molecules that act to silence the expression of the γ-globin gene. Typically, only increased expression of the γ-globin gene in which the mutation is found occurs.

The level of HbF observed in patients with this syndrome varies greatly and ranges from a few percent to 30% in the −175 T→C substitution found in either the $^G\gamma$- or $^A\gamma$-globin gene.[362-364] Similar to the deletion forms of HPFH, the HbF may be found in either a heterocellular or a pancellular distribution (Table 21-6 and Fig. 21-19). The −158 C→T substitution in the $^G\gamma$-globin gene is associated with a normal level of HbF in otherwise normal heterozygotes but with a high level of HbF in the presence of erythropoietic stress.[366-372] For example, in Saudi Arabia, patients with sickle cell anemia often have high HbF levels (25% or greater) and mild disease, whereas their parents with sickle cell trait have normal or only slightly elevated levels.[366] Identification of persons with this mutation (or polymorphism) has been aided by the finding that it is linked to a rare subhaplotype.[367,369] The −158 HPFH substitution also improves the clinical course of β-thalassemia. A Chinese person homozygous for the −29 promoter mutation has transfusion-dependent β-thalassemia,[47] yet black patients homozygous for the same mutation who co-inherit the −158 HPFH mutation in the $^G\gamma$ promoter have mild β-thalassemia.[46,114]

Confirmation that the base substitutions in the promoter region are the cause of increased γ-chain synthesis

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*See references 58, 286, 300, 301, 303, 319, and 339-348.

TABLE 21-6 α-Thalassemia Syndromes

Syndrome	Clinical Features	Hemoglobin Pattern	α-Globin Genes Affected by the Thalassemia Mutation
Silent carrier (α-thal-2)	No anemia, normal red blood cells	1%-2% Hb Bart's (γ_4) at birth; may have 1%-2% Hb Constant Spring; remainder HbA	1
Thalassemia trait (α-thal-1)	Mild anemia, hypochromic and microcytic red blood cells	5%-10% Hb Bart's (γ_4) at birth; may have 1%-2% Hb Constant Spring; remainder HbA	2
HbH disease	Moderate anemia; fragmented, hypochromic, and microcytic red blood cells; inclusion bodies may be demonstrated	5%-30% HbH (β_4); may have 1%-2% Hb Constant Spring; remainder HbA	3
Hydrops fetalis	Death in utero caused by severe anemia	Mainly Hb Bart's; small amounts of HbH and Hb Portland also present	4

Hb, Hemoglobin; *HbH,* hemoglobin H; *thal,* thalassemia.

rather than associated random DNA polymorphisms is based on several lines of evidence (reviewed by Ottolenghi and colleagues[382]). As mentioned earlier, they are generally associated with overexpression of the gene in which the mutation is found and typically represent the only sequence change. None of the mutations listed, with the exception of the −158 mutation, have been observed in persons with normal HbF levels. Haplotype analysis in some pedigrees of patients with HPFH has demonstrated that the HPFH determinant is linked to the β-globin cluster. The British type of nondeletion HPFH is quite informative in this regard. The gene has been tracked through three generations, and three homozygous individuals have been observed.[353-355,383] Haplotype analysis using restriction enzyme polymorphisms has established linkage of the British HPFH phenotype to the β-globin gene locus.[354] More than 90% of the γ chains are of the $^{G}\gamma$ type, and there is an associated mutation at −198 of the $^{G}\gamma$ gene.[354] Even the three homozygotes, with approximately 20% HbF, have heterogeneous distribution of HbF among their red blood cells. Two homozygotes for −117 $^{A}\gamma$ HPFH have also been described, and they had 24% HbF in a pancellular pattern.[384] More recent transgenic experiments provide support for the idea that the single base substitutions seen in HPFH can lead to enhanced γ-globin expression in adult life.

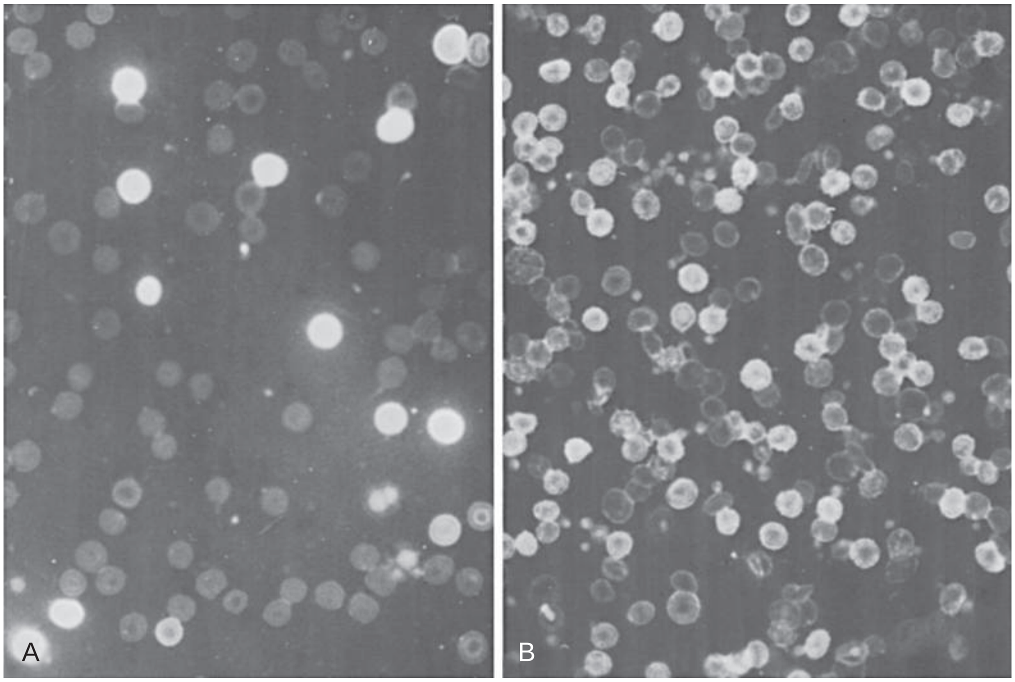

Figure 21-19 **A,** Immunofluorescent staining of erythrocytes with antibody directed against fetal hemoglobin (HbF) shows the distribution of HbF in a heterocellular pattern. **B,** Immunofluorescent staining of erythrocytes with HbF distributed in a pancellular pattern. *(Courtesy Dr. George Stamatoyannopoulos.)*

An interesting aspect of this syndrome is a balanced α-globin to non–α-globin chain synthetic ratio, even in homozygotes,[384] which implies that increased γ-globin synthesis is offset by decreased β-globin synthesis.[373] Expression of the β gene in *cis* to the mutation may be reduced by 20% to 30%.[350,363,364,374,385] It has also been observed that HbA_2 levels are uniformly low in the nondeletion HPFH syndromes and generally correlate inversely with HbF levels.[1] The reduction in δ- and β-globin gene expression in *cis* to the HPFH mutations is compatible with models of competitive regulation of β-globin expression by the linked γ-globin gene.

Mutations That Alter α-Globin Gene Regulation

In contrast to the β-globin cluster, in which single nucleotide substitutions are the most common cause of thalassemia, large deletions within the α-globin cluster are the predominant basis of α-thalassemia. The overall impairment in α-globin chain synthesis that results from defects in the α-globin cluster is determined by the number of genes inactivated (either by deletion or mutation), the type of lesion (deletion or mutation), and whether the lesion affects the α_2 or α_1 gene.

The α-globin cluster on chromosome 16[16,386,387] contains three functional genes (ζ, α_2, and α_1), an expressed gene of no apparent significance, and three pseudogenes[2,15] (see Fig. 21-2). Transcription of the genes depends on the integrity of the distant regulatory element HS-40. An α-thalassemia deletion mutation ($\alpha\alpha^{RA}$) has been reported that removes a large segment of DNA upstream of the ζ_2 gene but spares the remainder of the cluster.[388] In heterozygotes, no expression of the ζ- or α-globin genes from this chromosome is detected in heterozygotes. These findings are formally analogous to those in Hispanic and English γδβ-thalassemias (see Table 21-4),[329,330] in which upstream LCR elements of the β-globin cluster are deleted.

The two human α-globin genes are thought to have been generated through a gene duplication event that occurred approximately 60 million years ago.[389] The nucleotide sequences of the α_2 and α_1 genes have remained remarkably similar and represent an example of concerted evolution[390-392] in which the two genes have exchanged genetic information through crossover fixation and gene conversion events. The coding regions of the two genes are virtually the same and encode identical polypeptides.[390-393] The genes differ only in minor respects within IVS-2, whereas the sequences diverge significantly in the 3′-untranslated region.

The α-globin genes are expressed in embryonic, fetal, and adult-stage erythroid cells. Initially it was believed that the two α genes were expressed at similar levels, but subsequent RNA analysis relying on sequence differences in their 3′-untranslated regions revealed that α_2 RNA predominates over α_1 RNA by a ratio of 3:1.[226,394-396] Because the two α-globin mRNA molecules have the same intrinsic stability, the higher level of α_2 RNA reflects increased transcription of the α_2 gene. "Transcriptional interference" of the α_1 gene by the upstream α_2 gene has been proposed as an explanation for this observation.[397] Ribosome-loading studies suggest that α_2 and α_1 transcripts are translated at equivalent rates.[398] Systematic characterization of the expression of α-globin structural variants at both the α_2 and α_1 loci has confirmed that the α_2 gene has a predominant role in α-globin chain production.[394,395] A direct prediction of this model is that mutations altering the α_2 gene would result in a greater deficiency of α-globin chain production than would mutations of the α_1 gene. Clinical support for this prediction is provided by study of Sardinian patients with HbH disease who are heterozygous for one chromosome with a deletion of both α genes (–/) and a chromosome with a nondeletion mutation affecting the Inr codon of either the α_2 gene ($\alpha^T\alpha$/) or the α_1 gene ($\alpha\alpha^T$/).[194] Patients with the mutation in the α_2 gene (–/$\alpha^T\alpha$) have disease that is clinically more severe. Consistent with its predominance, the α_2 gene is involved in the majority of the reported mutations of the α-globin genes (see Table 21-2).

Deletion Mutations within the α-Globin Gene Cluster

Mutations That Remove One α-Globin Gene

The two α-globin genes are embedded in highly homologous, tandem repeated sequence blocks (called X, Y, and Z) that are separated by nonhomologous segments (Fig. 21-20).[18] Unequal, homologous recombination through the X and Z blocks generates a chromosome with a single α-globin gene and another with three α-globin genes. Homologous recombination in the small Y box has not been observed. The most common type of deletion in this class removes 3.7 kb as a result of misalignment of the Z boxes and is known as "rightward deletion." The products of this crossover are the ($-\alpha^{3.7}$/)[399,400] and ($\alpha\alpha\alpha^{anti\text{-}3.7}$/) haplotypes.[401,402] The $-\alpha^{3.7}$ products may be further subdivided into types I, II, and III by the precise location of recombination within the Z box.[392] Unequal crossover events through the X box leads to "leftward deletion" of 4.2 kb of DNA and the $-\alpha^{4.2}$ chromosome[382] and its triplicated antitype.[403,404] The incidence of the observed recombination products appears to reflect the size of the homologous target sequence within the boxes because $-\alpha^{3.7I}$ (1436 bp) is the most common, followed by $-\alpha^{4.2}$ (1339 bp), $-\alpha^{3.7II}$ (171 bp), and $-\alpha^{3.7III}$ (46 bp).[2,390,391,405] Unequal α-gene recombination through the X and Z boxes has been reproduced in both prokaryotic and eukaryotic experimental systems with episomal vectors.[18,406,407]

The $-\alpha^{3.7I}$ deletion is extremely common and is seen in all populations in which α-thalassemia is prevalent, whereas the $-\alpha^{4.2}$ deletion is most common in Asian populations.[2,15] Frequencies of up 80% to 90% for the single-deletion chromosome have been reported in some populations.[123] The association of these mutations with a variety of α-globin cluster haplotypes and α-globin variants in different populations suggests that they have arisen through numerous independent mutational events.[123,408]

Deletion of one α-globin gene does not affect expression of the ζ-globin gene[409] but has unanticipated consequences on expression of the remaining α-globin gene. The α_1 gene that remains on the $-\alpha^{3.7}$ chromosome is expressed approximately 1.8 times more often than the

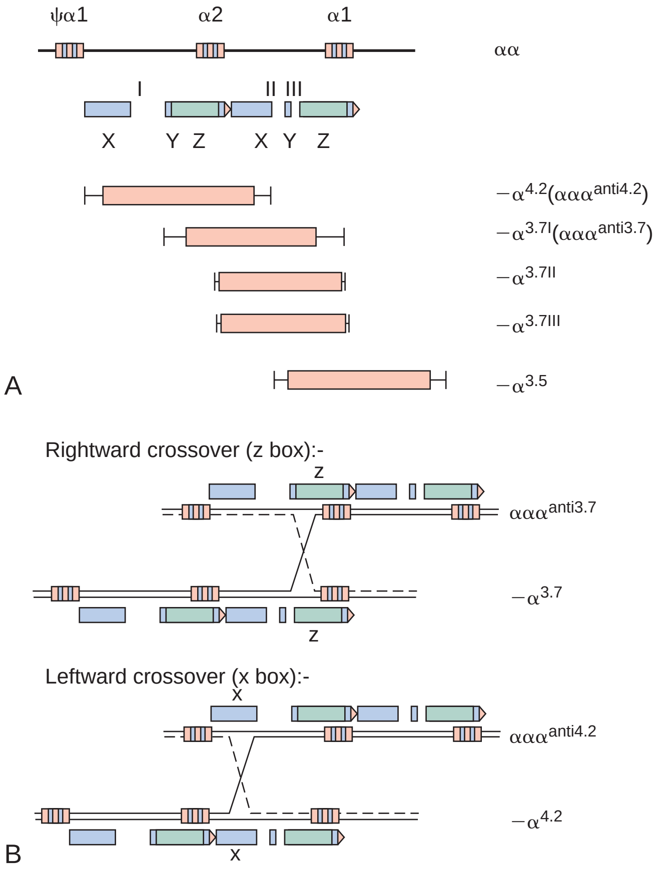

Figure 21-20 **A,** Deletion mutations that give rise to α-thalassemia. The two α-globin genes are embedded in a duplicated segment of deoxyribonucleic acid (DNA) with homologous (X, Y, and Z boxes) and nonhomologous (I, II, and III) regions. The extent of specific mutations that delete a single α-globin gene are indicated by *salmon boxes,* and the limits of the breakpoints are indicated as *solid lines*. These deletions and the reciprocal chromosomes containing three functional α-globin genes result from misalignment and unequal recombination mediated by the homologous blocks. **B,** Unequal crossover through the Z box deletes 3.7 kb of DNA and produces the $-\alpha^{3.7}$ chromosome and its antitype, whereas recombination through the X box produces the $-\alpha^{4.2}$ chromosome and its antitype. The $-\alpha^{3.7}$ deletion can be further subdivided (I to III) by the exact location within the Z box where recombination occurs (see **A**). *(Modified from Higgs DR, Vickers MA, Wilkie AO, et al: A review of the molecular genetics of the human alpha-globin gene cluster.* Blood *73:1081–1104, 1989.)*

α_1 gene on a normal chromosome.[395] Persons homozygous for the leftward deletion ($-\alpha^{4.2}/-\alpha^{4.2}$), who carry two copies of the α_1 gene, express more α-globin than the anticipated 25% of normal.[410] Homozygotes for the $-\alpha^{3.7III}$ deletion ($-\alpha^{3.7III}/-\alpha^{3.7III}$), who carry two α_2 genes, express less than the anticipated 75% of normal.[410] The increased expression of the α_1 gene on the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ chromosomes may be secondary to release of transcriptional interference from the upstream α_2 gene,[397] whereas the lower expression of the α_2 gene in the $-\alpha^{3.7III}$ deletion remains to be explained. An important consequence of this observation, however, is that common mutations that delete the α_2 gene ($\alpha^{3.7I}$ and $\alpha^{4.2}$) may lead to increased expression of the remaining α_1 gene and to a less severe clinical phenotype than predicted from expression studies of the native α-globin genes.[410] Single nucleotide substitutions that inactivate the α_2 gene do not affect α_1 expression and consequently lead to a more severe clinical phenotype than do single gene deletions.[2,15]

Triplicated α-gene chromosomes are observed in populations in which the $\alpha^{3.7I}$ and $\alpha^{4.2}$ chromosomes are present. The third gene is expressed and leads to a slight increase in the α-chain to β-chain ratio in homozygotes for the triplication, but this increase is generally of no clinical consequence.[411,412] However, if a triplicated α-gene chromosome is co-inherited with a β-thalassemia gene, thalassemia intermedia may be seen.[413,414] A unique deletion that removes the α_1 gene and some downstream sequence was recently described as a cause of α-thalassemia and appears to be due to a novel biologic mechanism.[415] As a result of the deletion, a promoter in the antisense orientation is brought into close proximity to the remaining α_2 gene. Antisense transcripts are created and appear to cause methylation of DNA and silencing of the chromatin in the region, thus attenuating transcription of the intact α_2 gene. Recent work in both yeast and human cells has begun to delineate the mechanisms that may be responsible for this phenomenon, which may normally play a role in physiologic gene regulation.[416]

Mutations That Remove Both α-Globin Genes

Illegitimate, nonhomologous recombination within the α-globin cluster results in partial or complete deletion of both α-globin genes and generates an α^0 chromosome.[2,388,417-421] (Note that the term α^+ and α^0 may refer to the expression of α-globin from a single gene or may refer to the presence or absence of an intact α-globin gene on a chromosome.) In some circumstances, the ζ-globin gene may also be encompassed by the deletion. The boundaries of many of the deletions that have been characterized are summarized in Figure 21-21. The geographic distribution of these lesions is more restricted than that of the α^+-thalassemia deletions, the most common being the $-^{SEA}$ (Southeast Asia) and $-^{MED}$ (Mediterranean) deletions, thus suggesting that they are rare genetic events. Theories concerning the mechanisms of these deletions are summarized elsewhere.[2]

Homozygotes for α^0 chromosomes (–/–) experience hydrops fetalis, whereas persons heterozygous for an α^+ chromosome and an α^0 chromosome ($-/-\alpha$ or $-/\alpha^T\alpha$) exhibit HbH disease. Increased expression of minute quantities of ζ-globin into adulthood may accompany some of the mutations in which the ζ gene is left intact by the deletion.[409] Sensitive radioimmunologic assays for the presence of ζ-globin chains in heterozygous adult carriers of these mutations have been devised,[422] and they may prove useful in areas such as Southeast Asia, where the incidence of the $-^{SEA}$ chromosome is as high as 3%.[423]

Mutations Not Linked to the Globin Gene Clusters That Alter Globin Gene Expression

X-Linked α-Thalassemia Associated with Mental Retardation Syndrome

Rare instances of α-thalassemia occurring in persons of Anglo-Saxon origin with phenotypically normal parents

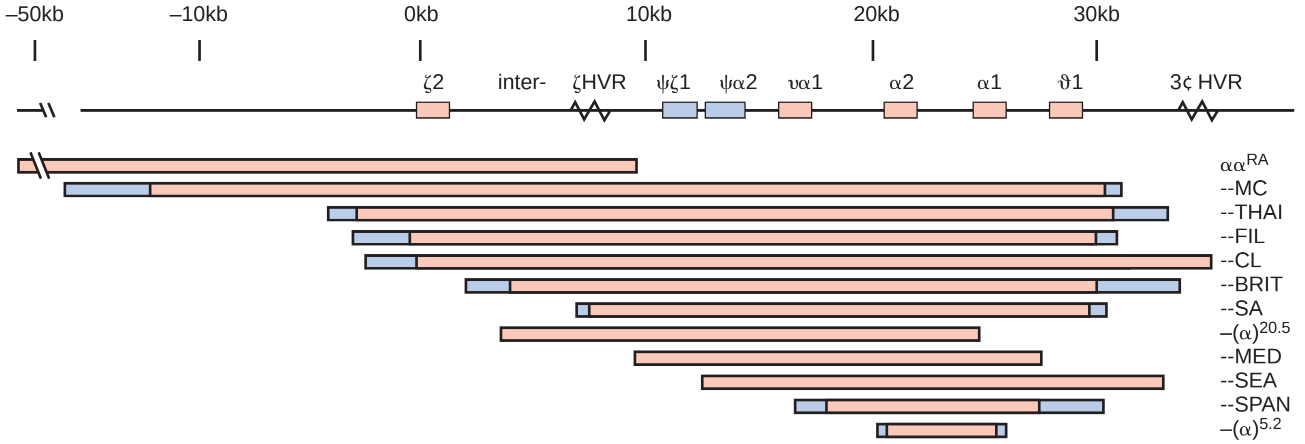

Figure 21-21 Deletion mutations that give rise to α^0-thalassemia. The organization of the α-globin cluster is shown. Expressed genes are represented by *salmon boxes*, whereas pseudogenes are shown as *blue boxes*. Two hypervariable regions (HVRs) are indicated as *zigzag lines*. Coordinates are in kilobase pairs (kb). The extent of each deletion is defined by a *salmon box* and the uncertainty of the breakpoints by *blue boxes*. The designation of the genotype is indicated to the right of each deletion. *(From Higgs DR, Vickers MA, Wilkie AO, et al: A review of the molecular genetics of the human alpha-globin gene cluster.* Blood *73:1081–1104, 1989.)*

first drew attention to a new syndrome.[424] Further investigation revealed the commonly seen association of profound mental retardation, facial anomalies, and genital abnormalities.[79] Important recent studies demonstrated mutation of the *ATRX* gene, a member of the DNA helicase superfamily and presumed global transcriptional regulator, as the underlying basis of this syndrome.[80] The exact function of this gene in globin gene regulation is not exactly clear, although recent studies suggest that it may be involved in binding to tandem repeats and regulating allele-specific expression of genes such as the α-globins.[425]

Acquired Hemoglobin H Disease

A particularly severe, acquired form of HbH disease has been described in elderly men with clonal myeloproliferative disorders.[426-429] In this setting, levels of HbH approaching 60% may be seen.[402] Extremely low α-chain to β-chain synthesis ratios and low α-globin mRNA levels have been documented in bone marrow cells from affected individuals.[427,428] Residual α-globin mRNA expression may be derived from nonclonal erythroid cells. A bimorphic population of cells is present on examination of the peripheral blood smear. The manifestations of hemolytic disease caused by HbH may increase and decrease with the clinical course of the myeloproliferative disorder.[427] This condition has been shown to be due to acquired mutations in the *ATRX* gene, which is also mutated in the syndrome of X-linked α-thalassemia associated with mental retardation.[430] Interestingly, similar mutations that are somatically acquired in this acquired condition confer a much more severe α-thalassemia phenotype in comparison with those acquired in the germline in the syndrome of X-linked α-thalassemia associated with mental retardation.[431]

Silent β-Thalassemia

Hematologically normal “silent carriers” of β-thalassemia are identified through family studies.[1,6,432-435] Some instances of “silent” β-thalassemia may be attributed to mutations that cause a very modest reduction in β-globin gene expression—the −101 promoter mutation[146,259] and the +1 cap site mutation[158]—or attributed to the inheritance of triplicated α-globin gene chromosomes.[413,414] In an Albanian family in which the father was a silent carrier, the mother had high-HbA_2 β-thalassemia trait, and two children had β-thalassemia, the silent carrier determinant did not appear to segregate with either of the paternal β-globin gene clusters.[436] Subsequently, a number of reports of families in which a β-thalassemia mutation appeared to be unlinked to the β-globin complex have been reported.[437-439] These rare instances of unlinked β-thalassemia may be due to mutations in *trans*-acting factors. However, all these patients did not have the syndromic features seen in persons with the previously characterized *trans*-acting regulatory factor mutations (see the section on β-thalassemia mutants unlinked to the β-globin complex). Thus new insight may be gained from genetic analysis of these rare cases. However, caution must be applied in the analysis of such cases, because at least some of these cases may be due to an increase in the number of α-globin genes present on a single α-globin locus of these patients.[440,441]

Hereditary Persistence of Fetal Hemoglobin Unlinked to the β-Globin Locus

Ordinarily, HbF constitutes less than 1% of the total hemoglobin in normal adult erythrocytes and is restricted to a subpopulation of erythrocytes (F cells) that represent between 3% and 7% of the total number of red blood cells.[7,8] Both the percentage of F cells present in the circulation and the amount of HbF are under genetic control, and evidence suggests that the determinants are not linked to the β-globin gene cluster.[442]

Although these genetic determinants were initially thought to be traits that could be inherited in a Mendelian fashion, linkage studies failed to identify such genes. More recent GWAS has uncovered a number of common genetic variants that lead to natural variation in the level of HbF. Although some common variants are linked to the β-globin gene cluster itself, others are unlinked to this region, as discussed previously in this chapter. Rare determinants in specific families that alter HbF levels may still exist, and further work with modern sequencing approaches may help to elucidate the genetic basis for such observations.

CLINICAL HETEROGENEITY OF THALASSEMIA SECONDARY TO DIVERSITY OF MUTATIONS

In the previous sections, the extraordinary array of mutations associated with the clinical syndrome of thalassemia has been described. In populations in which thalassemia is prevalent, different types of mutations that affect genes of either the α-globin, β-globin, or both globin clusters may coexist. Frequently, patients are compound heterozygotes for these mutations. The relative degree to which globin chain synthesis is impaired reflects this genetic heterogeneity and determines the clinical phenotype. In the following clinical description of the thalassemia syndromes, we emphasize such genetic interrelationships.

α-Thalassemia

Nomenclature and Genetics of α-Thalassemia

α-Thalassemia is divided into four clinical subsets that reflect the extent of impairment in α-globin chain production: silent carrier (–α/αα), α-thalassemia trait (–α/–α or –/αα), HbH disease (–/–α), and hydrops fetalis (–/–; see Table 21-6). Before the introduction of molecular analysis, these syndromes were classified on the basis of red blood cell parameters (hemoglobin level, mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], globin biosynthetic ratio, presence of inclusions, and HbH [β_4] levels), as well as the manner in which they interacted genetically to produce different phenotypes. Much of this early work relied on the study of Asian populations in which α-thalassemia is particularly common.[1]

The silent carrier state for α-thalassemia, in which a single α-globin gene on a chromosome is inactivated, has traditionally been designated α-thalassemia-2. Homozygosity for α-thalassemia-2 or heterozygosity for an allele causing more severe disease in which both α-globin genes are inactivated leads to α-thalassemia trait, also termed α-thalassemia-1. According to the historical nomenclature, HbH disease reflects heterozygosity for α-thalassemia-2 and α-thalassemia-1, whereas hydrops fetalis results from homozygosity for α-thalassemia-1.

Advances in molecular characterization of the α-globin gene cluster have clarified the genetic basis of these phenotypes. Weatherall and colleagues proposed a more informative nomenclature for these mutations.[1,2] Each chromosome may be designated as α^+ or α^0 to indicate the presence or absence of any α-globin chain production derived from that chromosome. This designation is referred to as a haplotype and should be distinguished from use of the term haplotype in describing the linkage of DNA polymorphisms on a chromosome. Haplotypes are further subdivided by the status of each α-globin gene on a chromosome. As discussed previously, mutations may be of the deletion or nondeletion type. Normal persons are designated (αα/αα) to indicate the presence of four active α genes, two on each chromosome 16, with the first position corresponding to the α_2 gene and the second position to the α_1 gene. Deletion of a single α-globin gene on a chromosome is designated (–α/), whereas deletion of both genes is indicated as (–/). Further refinement of this nomenclature includes a designation of the specific mutation; for example, $(-^{SEA}/)$ signifies the α^0 deletion mutation that is common in Southeastern Asian populations (see Fig. 21-21), and $(-\alpha^{3.7}/)$ symbolizes the α^+ rightward α-gene deletion that is extremely common in the American black population (see Fig. 21-21). Nondeletion mutations in a haplotype are designated in this scheme by a superscript T ($\alpha\alpha^T$) and can be more precisely described by symbols for the specific mutation and the gene affected. For example, ($\alpha^{CS}\alpha$) designates a chromosome bearing the CS chain termination mutation affecting the α_2 gene (see Table 21-2), and ($\alpha^{QS}\alpha$) designates a chromosome bearing the α gene with a substitution in codon 125 that leads to the synthesis of an unstable globin (see Table 21-2). This shorthand nomenclature is extremely useful when a patient's genotype is known in detail and has simplified description of the many genotypes that interact to generate the heterogeneous clinical syndromes.

At least 30 mutations that may inactivate one or both α-globin genes are known; thus the different combinations of chromosomes bearing α-thalassemia mutations number more than 200. The number of occurrences of the different genotypes and haplotypes define the spectrum of disease observed in a given ethnic group. For example, severe forms of α-thalassemia (HbH disease and hydrops fetalis) are uncommon in blacks. In this group the single-deletion chromosome is very common,[443,444] whereas chromosomes with two defective α genes are quite rare. In some circumstances the phenotype of HbH disease is not readily related to genotype. Indeed, there is a significant spectrum of disease severity in persons from the Southeast Asian area with the same genotype $(-\alpha^{3.7}/-^{SEA})$.[408] The observed phenotype may be further complicated by the inheritance of another hemoglobin disorder (β-thalassemia) or the influence of environmental factors such as iron or other nutritional deficiency.

Silent Carrier

The silent carrier state, or the α-thalassemia-2 defect, is due to the presence of a mutation affecting only one α-globin gene. Most often this defect occurs because of a deletion mutation (–α/αα). Patients who are silent carriers for α-thalassemia have three rather than four functional α-globin genes.[400,445] Thus the impairment in α-globin synthesis is very mild. Significant overlap of their globin biosynthetic ratio occurs with both that of normal subjects and that of persons with only two functional α-globin genes (Fig. 21-22). Similarly, the mean MCV of patients with three functional genes is slightly lower than that in normal subjects, but significant overlap between the two groups exists.[2] Despite these differences

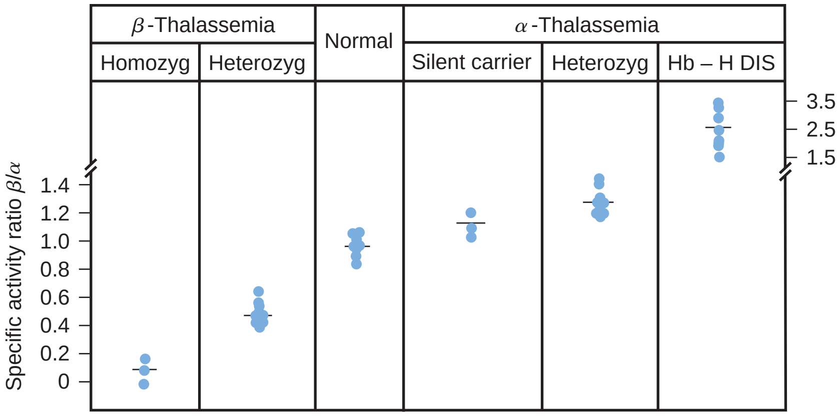

Figure 21-22 Biosynthetic α-globin to β-globin ratio in various forms of thalassemia. Peripheral blood cells were incubated with [^{14}C]leucine for 2 hours in autologous plasma. The globin chains were isolated by ion exchange chromatography. The specific activity of the chains and the ratio of their radioactivities were then calculated. *Hb,* Hemoglobin. *(Modified from Nathan DG. Thalassemia.* N Engl J Med *286:586–594, 1972.)*

that become apparent upon comparison of groups with different numbers of α-globin genes, there is no reliable way to diagnose silent carriers of α-thalassemia by hematologic criteria. Diagnosis has been reported by detection of small amounts of hemoglobin Bart's in cord blood,[1] but additional studies indicate that this approach is unreliable in ascertaining the presence of the silent carrier state.[446,447] However, a more recent study in the United Kingdom using high-performance liquid chromatography (HPLC) and genotyping to verify α-globin genotype on fresh cord blood samples demonstrated that HPLC is a reliable technique for determining the silent carrier state.

Silent Carrier Variant: Hemoglobin CS. Some silent carriers of α-thalassemia produce small quantities of slowly migrating hemoglobins (Fig. 21-23) that contain elongated α-globin chain variants resulting from termination codon mutations (see Table 21-2 and Fig. 21-14).[220-224] The original elongated α-chain variant, hemoglobin Constant Spring, was named for the small Jamaican town that was the residence of the Chinese family in whom these hemoglobins were first discovered.[221] The family had three children with HbH disease; each had a small amount of the abnormal hemoglobins. One parent had typical thalassemia trait, whereas the other had normal red blood cells but, in addition, had approximately 1% of the abnormal hemoglobin. Similar slowly migrating hemoglobins have been found in other racial groups in Thailand and Greece.[448] Persons homozygous for the α^{CS} mutation ($\alpha^{CS}\alpha/\alpha^{CS}\alpha$) have a clinical syndrome similar to HbH disease, although their erythrocytes contain hemoglobin Bart's (γ_4) rather than HbH and the degree of anemia and the extent of abnormalities in red blood cell indices are milder than those in most patients with HbH disease.[449] Compound heterozygotes for the α^0 and α^{CS} haplotypes ($-/\alpha^{CS}\alpha$) exhibit typical HbH disease.

α-Thalassemia Trait

Two genotypes (–/αα and –α/–α) that reflect inactivation of two α-globin genes are associated with α-thalassemia trait. Substitution mutations that affect the predominant α_2 gene ($\alpha^T\alpha/\alpha\alpha$) may also lead to α-thalassemia trait. The extent of the observed changes in red blood cell indices mirrors the reduction in α-globin production seen with each genotype, with the (–α/–α) genotype being less affected than the (–/αα) genotype. α-Thalassemia trait is characterized by marked microcytosis and hypochromia of red blood cells in conjunction with mild anemia and erythrocytosis (see Table 21-6). Levels of HbA_2 and HbF are generally normal or low. Diagnosis of this condition is typically made by family studies or by exclusion of iron deficiency and β-thalassemia trait. The incidence is particularly high in Asian populations and less in African, Mediterranean, and American black populations. Even in the neonatal period, the red blood cells appear hypochromic and microcytic. The proportion of hemoglobin Bart's may be as high as 1% in the blood of normal newborns, but levels of 4% to 6% are attained in infants who are

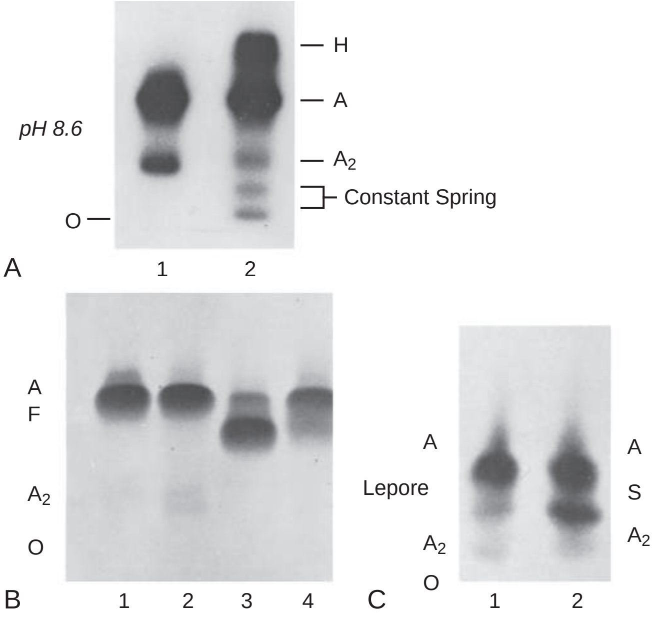

Figure 21-23 Hemoglobin electrophoresis. **A,** Starch gel electrophoresis at pH 8.6; *1,* normal; *2,* hemoglobin H disease with hemoglobin Constant Spring. **B,** Agarose electrophoresis at pH 8.6; *1,* normal; *2,* β-thalassemia trait with increased HbA_2; *3* and *4,* homozygous β-thalassemia with different relative amounts of hemoglobin A and fetal hemoglobin. **C,** Starch gel electrophoresis at pH 8.6; *1,* hemoglobin Lepore trait; *2,* sickle cell trait. "O" indicates the origin. The anode is at the top of the figure.

later shown to have α-thalassemia trait[1] (see Table 21-6). Beyond the neonatal period, biochemical markers such as HbA_2 and HbF in β-thalassemia are not particularly helpful in confirming the diagnosis of α-thalassemia trait. The one exception occurs in circumstances in which the expression of ζ-globin is increased because of deletion of both α-globin genes on a chromosome.[409,421,450] In Southeast Asia, where the $-^{SEA}$ allele is common, a sensitive radioimmunoassay allows detection of ζ-globin chains in adult carriers of this mutation.

Measurement of the β-to-α biosynthetic ratio has little place in the routine diagnosis of α-thalassemia trait. Although detection of impaired α-globin synthesis has been reported,[451,452] measurement is technically difficult because these persons have a low reticulocyte count and the measured values overlap with those found in healthy persons (see Table 21-6). Iron deficiency may raise the measured β-toα biosynthetic ratio, thus further complicating interpretation.[453-456] Consequently, in most patients the diagnosis of α-thalassemia trait is established by red blood cell structure and parameters, coupled with exclusion of β-thalassemia trait and iron deficiency. Gene deletions responsible for deficient α-globin production can be demonstrated by restriction endonuclease mapping; this technique may be applied when an accurate diagnosis is critical. Newer technology allows polymerase chain reaction detection of deletion[457] and triplication[458] of α genes, and it has the same level accuracy as the more cumbersome Southern blotting and DNA hybridization with a ϕζ-globin gene probe.

Hemoglobin H Disease

Genetics. HbH disease occurs in persons who have only a single fully functional α-globin gene. The genotypes leading to HbH disease are diverse.[2] Most common is heterozygosity for the single- and double-deletion chromosomes (–/–α). As a result, HbH disease is observed most often in Southeast Asia ($-^{SEA}/-\alpha^{3.7}$) and the Mediterranean basin ($-^{MED}/-\alpha^{3.7}$), where the incidence of both the α^+ and α^0 haplotypes is significant. Nondeletion mutations may also interact to cause HbH disease. As discussed earlier, the α_2 genes are responsible for the production of approximately 75% of the total α-globin chains. Mutations that decrease expression from the α_2 gene ($\alpha^T\alpha/$) result in an α+ haplotype that produces fewer α-globin chains than a deletion α+ haplotype (–α/) does. Thus homozygosity for mutations in both α_2 genes ($\alpha^T\alpha/\alpha^T\alpha$) is phenotypically equivalent to deletion of three genes and is associated with HbH disease and not α-thalassemia trait.[2,15] Consistent with this observation, nondeletion α+-thalassemia haplotypes that are paired with α^0-thalassemia haplotypes ($-/\alpha^T\alpha$) lead to more severe HbH disease than does the interaction of the deletion α+ haplotypes (–/–α).

Hemoglobin Pattern. Anemia of moderate severity characterized by hypochromia, microcytosis, striking red blood cell fragmentation (Fig. 21-24), and the presence of a fast migrating hemoglobin, HbH (β_4), on electrophoresis (see Fig. 21-23) may be shown to represent an α-thalassemia syndrome by measurement of the β-to-α biosynthetic ratio.[451,459,460] Incubation of peripheral blood reticulocytes with [^{3}H]leucine and subsequent chromatographic resolution of the globins indicate that such patients have a twofold to fivefold excess of β-chain synthesis (see Table 21-6 and Fig. 21-22). These excess β chains form HbH, which accounts for 5% to 30% of the total hemoglobin in patients with HbH disease.[461] In approximately 50% of persons with HbH disease in Southeast Asia, small quantities of hemoglobin CS are found (see Fig. 21-23). HbH disease may be suspected in anemic neonates in whom all the red blood cells are severely hypochromic. At birth, patients with HbH disease also have large amounts of hemoglobin Bart's (γ_4). In patients with HbH disease and an intact spleen, incubation of peripheral blood cells in brilliant cresyl blue produces many small inclusions in most red blood cells (Fig. 21-25). The dye induces precipitation of HbH by a redox reaction. In contrast, large and usually single preformed inclusions (Heinz bodies) may be visualized with methyl violet in the red blood cells of patients who have undergone a splenectomy (see Fig. 21-25).[110,462] These inclusions are composed of precipitated β-globin rendered insoluble by hemichrome formation or by interaction of β-globin sulfhydryl groups with the red blood cell membrane.[110,463]

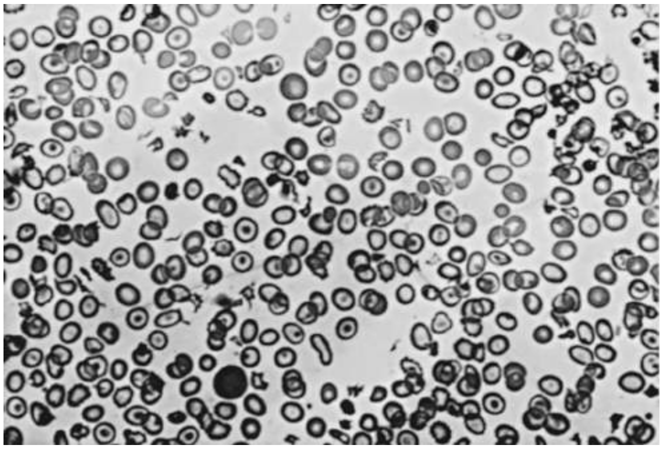

Figure 21-24 Peripheral blood smear from a patient with hemoglobin H disease.

Pathophysiology. HbH exhibits no Bohr effect or heme-heme interaction and thus is not effective for oxygen transport under physiologic conditions.[464] Consequently, patients with appreciable amounts of HbH have a more severe deficit in functional hemoglobin and oxygen-carrying capacity than the measured hemoglobin concentration might suggest. Red blood cells containing HbH are sensitive to oxidative stress, thus accounting for the enhanced red blood cell destruction that may occur with the administration of oxidant drugs such as sulfonamides.[465] As erythrocytes age and lose their capacity to withstand oxidant stress, precipitation of HbH occurs and leads to premature destruction of circulating red blood cells[110]; hence HbH is primarily a hemolytic disorder.[462,466] Inclusions of HbH are rarely seen in bone marrow cells,[467] and erythropoiesis is fairly effective.[466]

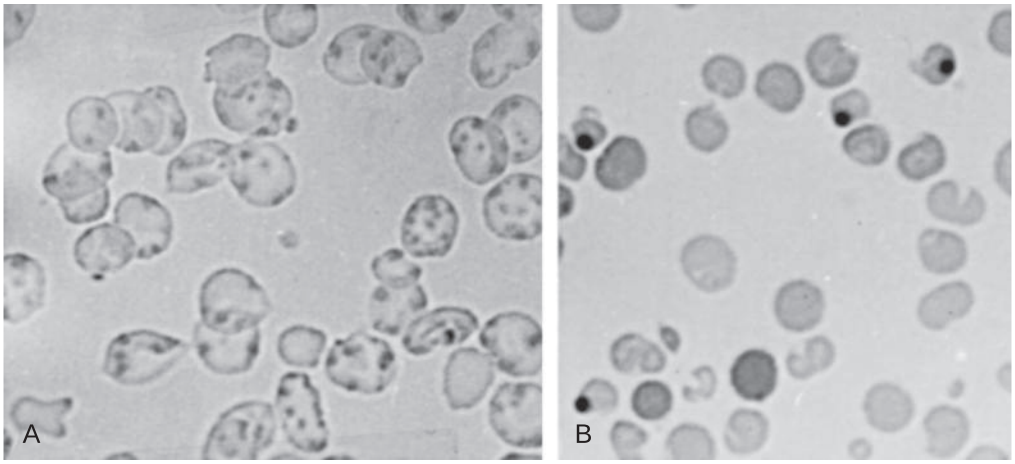

Figure 21-25 Red blood cell inclusions in hemoglobin H disease. **A**, Inclusions induced by incubation of peripheral blood in 1% brilliant cresyl blue and 0.4% citrate for 30 minutes at 37°C (the patient had not undergone a splenectomy). **B**, Preformed inclusions in the peripheral blood of a patient who had undergone a splenectomy stained by a new methylene blue reticulocyte stain.

Clinical Features. Typical patients with HbH disease live quite normally. Generally, the anemia is moderate with a hemoglobin concentration of 7 to 10 g/dL, although occasional patients may have hemoglobin levels as low as 3 to 4 g/dL.[1] The complications of HbH disease are related to chronic hemolysis. Jaundice and hepatosplenomegaly are commonly present. Folic acid deficiency, pigment gallstones, leg ulcers, and increased susceptibility to infection are also observed. Hemolytic episodes may be precipitated by drugs or infection. Iron overload is uncommon but may occur in patients receiving transfusions and those older than 45 years.[468] Development of the characteristic thalassemic facies, which reflects expansion of marrow space equivalent to that seen in patients with severe β-thalassemia, is rare. Usually only moderate bone marrow erythroid hyperplasia is evident.[456]

Therapy. Consistent with the benign course of HbH disease, treatment is primarily supportive. Therapy includes supplementation with folic acid, avoidance of oxidant drugs and iron salts, prompt treatment of infectious episodes, and judicious use of transfusions. Splenectomy should be contemplated in patients with HbH disease only if hypersplenism is present as reflected by leukopenia, thrombocytopenia, and worsening anemia or the development of a transfusion requirement in a patient whose condition was previously stable. Thrombocytosis after splenectomy may be complicated by a clotting diathesis, recurrent pulmonary emboli, and pulmonary hypertension, particularly in patients with severe HbH disease and a fairly normal hematocrit.[468-470] The use of measurements of red blood cell survival and splenic sequestration by chromium-51 labeling of red blood cells is controversial in making a clinical decision regarding splenectomy because the label binds selectively to β-globin chains and may, by virtue of selective removal of HbH inclusions from red blood cells in the spleen, give a falsely high index of splenic destruction.[471] Other studies, however, suggest that results from the ^{51}Cr technique may be valid.[472]

Other Hemoglobin H Syndromes

Syndrome of α-Thalassemia with Mental Retardation. Patients with a syndrome characterized by mental retardation and a form of α-thalassemia that cannot be explained by simple Mendelian inheritance of an α-thalassemia gene have been described.[424,473] In rare patients, the mutation arises as a de novo event in a paternal germ cell. These patients can be divided into two distinct syndromes (deletion and nondeletion) on the basis of their clinical features and the results of molecular analysis of their α-globin gene clusters.

Patients have been described with extensive deletions involving chromosome band 16p3.3 or, alternatively, with deletions resulting from unbalanced chromosome translocations that also lead to aneuploidy in a second chromosome. Patients with deletion mutations exhibit mild to moderate mental retardation and a broad spectrum of dysmorphic features. Other rare patients are characterized by more severe mental retardation and a uniform pattern of dysmorphic features, including genital abnormalities, but no detectable deletions involving the α-globin gene cluster.[79] Such patients fall into the category of the syndrome of X-linked α-thalassemia with mental retardation. As noted earlier, the affected gene is a putative global regulator (*ATRX*).[80]

Although these disorders are seen rarely, they may be underrecognized. This underrecognition is particularly true of ethnic groups in which α-thalassemia is uncommon, and the mild hematologic findings in patients who do not inherit a second α-thalassemia gene might be overlooked. Infants with uncharacterized mental retardation should be studied with techniques suitable for detection of a deficiency in α-globin synthesis.

Acquired Hemoglobin H Disease. HbH is also found rarely in the red blood cells of patients with erythroleukemia or a myeloproliferative syndrome.[426-428] The disorder may be clonal and displays a striking male predisposition (85% of confirmed instances).[424] The clinical features manifested by these patients are those of their primary disorder; the finding of HbH is incidental to the outcome of the disease, although striking red blood cell abnormalities and active hemolysis may be present. The genetic mechanism of this syndrome appears to involve abnormal regulation of α-gene expression because of a mutation affecting the *ATRX* genetic locus (discussed earlier).

Hydrops Fetalis

Genetics. Homozygosity for the α^0 haplotype (–/–) leads to hydrops fetalis, a disorder that is particularly common in Southeast Asia where the α^0 haplotype is seen frequently. In rare infants from Greece and Southeast Asia, hydrops fetalis has been shown to result from the interaction of α^0 haplotypes with nondeletion α^+ haplotypes.[474-476]

Hemoglobin Pattern. The birth of stillborn infants to parents with α-thalassemia trait reflects the most severe form of α-thalassemia.[477,478] Because of the inability to synthesize any α-globin, their blood contains only hemoglobin Bart's (γ_4), HbH (β_4), and small amounts of Hb Portland ($\zeta_2\gamma_2$) within red blood cells displaying morphologic changes characteristic of severe thalassemia.[479,480]

Pathophysiology. Although the hemoglobin concentration averages 6.2 g/dL, both HbH and hemoglobin Bart's have high oxygen affinity and no subunit cooperativity, and therefore a hydropic infant has very little functional hemoglobin. Viability in utero is maintained by the presence of small quantities of hemoglobin Portland 1 and 2.

Clinical Features. Generally, affected infants are delivered stillborn at 30 to 40 weeks' gestation or die shortly after delivery at term.[423,479] Infants are grossly edematous or hydropic as a result of congestive heart failure induced by severe anemia. The blood smear is characterized by large hypochromic macrocytes and numerous nucleated red blood cells. At autopsy, extensive extramedullary hematopoiesis and placental hypertrophy are seen. A high incidence of toxemia of pregnancy and postpartum hemorrhage is observed in mothers of hydropic infants,[456,481] presumably as a consequence of the massive placenta. Hence early recognition of the disorder in at-risk pregnancies by prenatal diagnosis should lead to consideration of termination of the pregnancy.

Therapy. At least five infants born prematurely with hydrops fetalis have survived with the use of chronic transfusion regimens.[482,483] Intrauterine transfusion of fetuses with hydrops fetalis should also be considered if treatment after delivery is contemplated.[484] Marrow transplantation may be considered if the affected infant is delivered safely.

Mild β-Thalassemia

Silent Carrier

A silent carrier state for β-thalassemia was recognized through the study of families in which affected children had a more severe β-thalassemia syndrome than a parent with typical β-thalassemia trait.[1,6,432,435] A study of the "normal" parent often revealed mild microcytosis or a slight impairment in β-globin synthesis on radiolabeling of the globin chains in peripheral blood reticulocytes. Characteristically, silent carriers of β-thalassemia have normal levels of HbA_2. These silent carriers must be distinguished from persons whose red blood cells have all the stigmata of β-thalassemia trait but have normal levels of HbA_2. Several patients who are homozygous for the silent carrier β-thalassemia gene have been described.[1] Anemia is moderate (a hemoglobin concentration of 6 to 7.0 g/dL), and these patients rarely require transfusion. Hepatosplenomegaly may be significant. HbF values range from 10% to 15%, and the HbA_2 level is elevated to the range normally seen in persons with thalassemia trait.

The silent carrier state of β-thalassemia appears to be a distinct clinical and biochemical entity. The underlying molecular defects cause only a modest reduction in β-globin synthesis. Two point mutations in the β-globin gene region have been linked to the silent carrier phenotype. The −101 promoter mutation appears to be a common cause of silent β-thalassemia in the Italian population[150] and has been observed in Bulgarian and Turkish persons,[146] whereas the +1 cap site Inr mutation is associated with a silent carrier phenotype in an Indian family.[158] As noted earlier, rare instances of silent β-thalassemia secondary to mutations unlinked to the β-gene cluster have been reported.

β-Thalassemia Trait

Genetics. In 1925, Rietti[485] described Italian patients with mild anemia whose red blood cells exhibited increased resistance to osmotic lysis. A similar syndrome was later recognized in American patients of Italian descent by Wintrobe and colleagues,[486] who noted that both parents of a patient with Cooley anemia also had this syndrome. Detailed analysis of several pedigrees by Valentine and Neel[487] and others[488,489] established the fact that this form of thalassemia occurs in persons who are heterozygous for a mutation that affects β-globin synthesis. Expression of one β gene is impaired by mutation, whereas the other gene is normal.

Hemoglobin Pattern. Characteristically, HbA_2, HbF, or both are elevated in patients with β-thalassemia trait, although normal levels are observed occasionally. The relative amounts of HbA_2 or HbF have been used to classify thalassemia trait, which has relevance to the molecular lesion in certain instances and also to the severity of the disease in homozygous offspring.

Globin Biosynthetic Ratio. In peripheral blood reticulocytes, the measured β-to-α biosynthetic ratio varies from 0.5 to 0.7, as predicted for inactivation of a single β gene (see Fig. 21-22). However, when bone marrow cells are incubated with [^{3}H]leucine, the measured β-to-α biosynthetic ratio is often 1:1 in patients with heterozygous β-thalassemia.[490-494] The capacity of bone marrow cells to degrade free α-globin by proteolysis may explain why it is difficult to demonstrate unbalanced synthesis of β- and α-globin chains in the marrow cells of heterozygotes. The measured β-to-α ratio is approximately 0.5 in pulses of 10 to 20 minutes, but if the incubation of bone marrow cells is prolonged, proteolysis apparently destroys the excess newly synthesized α-globin.[495,496] Fractionation of bone marrow cells after incubation in [^{3}H]leucine demonstrated that proteolysis is most efficient in the earliest erythroid cells, namely, proerythroblasts and basophilic erythroblasts.[496] The impairment in β-globin synthesis becomes easier to detect as erythroid cells mature, thus accounting for the reduced β-to-α biosynthetic ratio that is usually found in peripheral blood reticulocytes.

δ-Globin Synthesis. Among inherited anemias, the elevation in HbA_2 level is unique to β-thalassemia. The absolute quantity of HbA_2 present in red blood cells is a complex function of the inherent capacity for δ-globin chain synthesis and factors regulating rates of hemoglobin dimer and tetramer assembly. As discussed earlier, δ-globin is normally expressed at a low level, approximately 2.5% that of β-globin. The level may be reduced further by mutations that affect δ-globin expression. Heterozygous[497,498] and homozygous[499] states have been described. δ-Thalassemias reflect point mutations that completely silence the already defective δ-globin genes.[287,288,306,435,500-502]

In heterozygous β-thalassemia, the observed increase in HbA_2 represents a relative as well as an absolute increase in the quantity of HbA_2 per red blood cell from the normal level of 0.6 to 0.7 pg to 1.0 pg.[1] The increased amount of HbA_2 contains δ-globin chains derived from the δ gene adjacent to the mutant β gene, as well as from the δ gene on the opposite chromosome, which has been inferred from the study of β-thalassemia heterozygotes, who also have a variant δ chain.[503] The increase in the HbA_2 level can be accounted for by enhanced incorporation of δ-globin chains into hemoglobin dimers as a consequence of deficient β-globin chain production (see the earlier discussion on hemoglobin assembly).[230]

Environmental factors also influence the absolute level of HbA_2. Iron deficiency leads to a decrease in the level of HbA_2, which may become normal in thalassemia heterozygotes and therefore mask the diagnosis of β-thalassemia trait. In such instances, the level of HbA_2 becomes elevated with iron repletion.[504] An increase in the level of HbA_2 may be seen in acquired megaloblastic disorders associated with either folic acid or vitamin B_{12} deficiency.[504]

Clinical Features. Peripheral blood smears from patients with β-thalassemia trait are shown in Figure 21-26. Microcytosis, hypochromia, targeting, basophilic stippling, and elliptocytosis may be striking features, although the red blood cells may be nearly normal in occasional patients. The bone marrow is characterized by mild to moderate erythroid hyperplasia; red blood cell survival is modestly decreased, and slight ineffective erythropoiesis is present.[505] Inclusions in bone marrow cells and peripheral red blood cells are rare. Similar hematologic parameters appear to characterize Thai, Chinese, Greek, British, and Italian populations with β-thalassemia trait,[506-509] whereas African Americans appear to have a milder syndrome.[510] Hepatomegaly and splenomegaly have been reported in 10% to 19% of Italian and Greek patients but are less common in other groups.[508,509] Iron or folic acid deficiency, pregnancy, or intercurrent illness may exacerbate the anemia in patients with thalassemia trait.

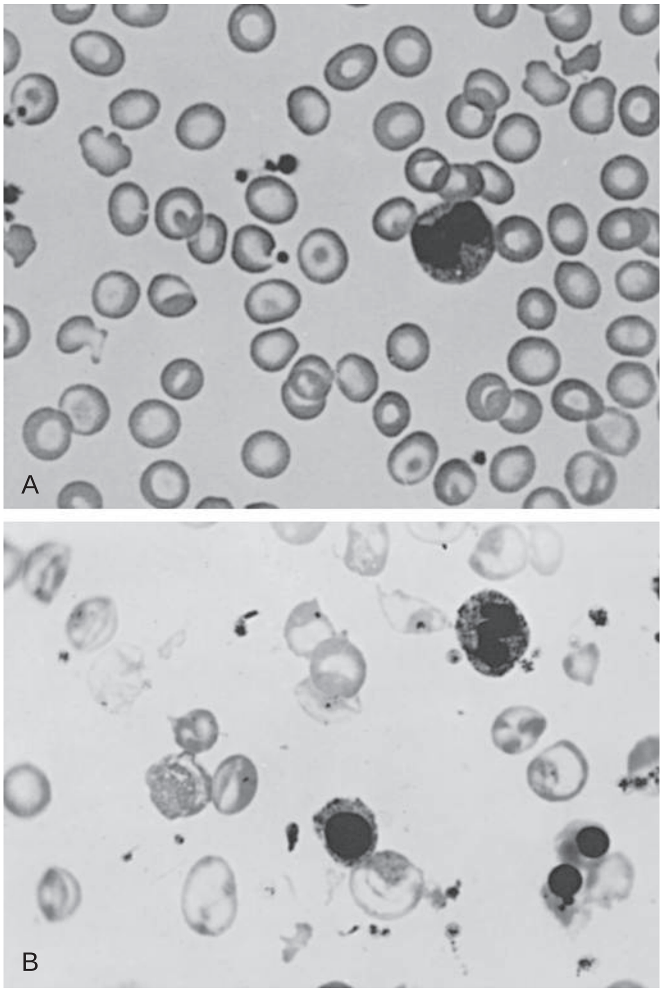

Figure 21-26 Peripheral blood smears from patients with heterozygous β-thalassemia (**A**) and homozygous β-thalassemia (**B**) after a splenectomy.

Therapy. No therapy is required for thalassemia trait, but genetic counseling is imperative. The homozygous offspring of two persons with high-HbA_2 β-thalassemia trait usually have transfusion-dependent anemia and thalassemia major, but they may sometimes have a thalassemia intermedia phenotype. The precise nature of the mutation or mutations in homozygous persons is one factor in determining the clinical phenotype.

β-Thalassemia Trait Phenotypes

High-A_2 β-Thalassemia. High-A_2 β-thalassemia is the most common form of β-thalassemia trait. HbA_2 levels vary from 3.5% to 8.0%, whereas the HbF level varies from less than 1% to 5%.[1,506-510] The vast majority of single-base mutations resulting in β-thalassemia (see Table 21-2) lead to typical high-HbA_2 β-thalassemia trait. Although individual mutations variably reduce β-globin synthesis, efforts to differentiate the effect of specific mutations in heterozygous persons have largely been unrewarding.[1,508,511,512] In blacks, β^0 and β^+ mutations can often be distinguished in individual families by the rate of occurrence of double heterozygotes for a β-thalassemia mutation and the β^S mutation. In this population, MCV and MCH values are higher in β^+ heterozygotes than in β^0 heterozygotes, but circulating hemoglobin concentrations are nearly equivalent.[510,513] The common mutations found in American blacks, most notably residing in the ATA box of the promoter (see Table 21-2), only modestly impair β-globin gene expression,[46,172] which perhaps accounts for the relatively mild phenotype of many persons with heterozygous or homozygous β^+-thalassemia in this population.

δβ-Thalassemia. Persons heterozygous for δβ–thalassemia have increased levels of HbF (5% to 15%) and low HbA_2 levels. This phenotype is most often generated by deletions that remove most or all of the coding sequences of the δ- and β-globin genes (see Table 21-4). The propensity of these deletion mutations for enhancement of the expression of γ-globin genes contributes to a relatively mild phenotype in homozygous persons or those in whom a δβ-thalassemia deletion is inherited along with a thalassemia allele with a typical substitution mutation.

High-A_2, High-F β-Thalassemia. A distinct variant of β-thalassemia trait has been described in which the level of HbA_2 is elevated and the level of HbF is also elevated (5% to 20%).[514-516] This form of thalassemia trait is associated with deletions of the β-globin gene that leave the δ and γ genes intact (see Table 21-4).

Normal-A_2 β-Thalassemia. This form of β-thalassemia trait should be distinguished from the silent carrier state. Although both are characterized by low or normal HbA_2 levels, the red blood cells in persons with normal-HbA_2 β-thalassemia are characteristically hypochromic and microcytic,[434,517] in contrast to silent carriers, whose red blood cells appear near normal.[424,433] Persons who co-inherit this type of β-thalassemia allele from one parent along with a high-HbA_2 β-thalassemia allele from the other usually have severe transfusion-dependent β-thalassemia.

This phenotype is thought to represent co-inheritance of mutations (usually deletions) that decrease β- and δ-gene function. The mutations in the δ-globin gene may be present on the same chromosome or on the opposite chromosome from the β-thalassemia gene.[287,288,306,435,500-502]

Differential Diagnosis of Thalassemia Trait. In clinical practice, thalassemia trait must be distinguished from iron deficiency (see Chapter 11 for additional discussion of the differential diagnosis of microcytosis and iron deficiency). Frequently the differential diagnosis may be suspected from red blood cell indices; mild erythrocytosis and marked microcytosis are characteristic of thalassemia trait (Table 21-7).[518-520] The red blood cell count is usually decreased in patients with iron deficiency, whereas red blood cell volume may be normal or decreased, depending on whether the iron deficiency anemia is acute or chronic,[521-524] but red blood cell volume is rarely as low as in persons with thalassemia trait. The mean corpuscular hemoglobin concentration (MCHC) is usually normal in persons with thalassemia trait, whereas the MCHC may be low in a subset of cells in persons with iron deficiency; in both syndromes the hypochromic appearance of the red blood cells reflects their small size, diminished hemoglobin content, and high surface-to-volume ratio. The red blood cell distribution width, a parameter available in modern automated cell counters, was originally believed to distinguish between iron deficiency and other causes of microcytosis.[525,526] However, the red blood cell distribution width is also increased in the thalassemias and other hemoglobinopathies and is not a useful independent discriminator.[527-531] The absence of stainable iron

TABLE 21-7 Hematologic Parameters in Thalassemia Trait and Iron Deficiency

Parameter	α-Thal	β-Thal	Iron Deficiency
Hemoglobin concentration (g/dL)	12.6 ± 1.1	M—12.6 ± 1.4 (697)*	10.2 ± 1.6 (715)
	12 ± 0.7	F—10.8 ± 0.9 (697)	
		C—11.3 ± 1 (697)	
		Adults—12 ± 1.3	
Red blood cell count (×10^6/μL)	5.6 ± 0.5	M—5.8 ± 0.6 (697)	4.67 ± 0.43 (715)
		F—5.1 ± 0.9 (697)	
		C—4.7 ± 0.6 (697)	
		Adults—6 ± 0.48	
Mean corpuscular volume		64.7 ± 4.3	67 ± 6.6 (715)
88.7 ± 5.3	72.2 ± 3.3	60.8 ± 5.6 (715)	
4 m^3/red blood cell	65.2 ± 3		
Mean corpuscular hemoglobin	23.2 ± 3.3	20.3 ± 2.2	21.8 ± 2.9 (715)
pg/red blood cell	21 ± 1	20.2 ± 1.9 (715)	
Hemoglobin A_2 (2-3.5%)	Normal or decreased	5.2 ± 0.8 (542-545)	Normal or decreased
Hemoglobin F (<1%)	<1%	2.1 ± 1.2 (697)	<1%

C, Children; *F*, females; *M*, males.

*Numbers in parentheses are reference citations.

TABLE 21-8 Formulas for Differentiation of Thalassemia Trait from Iron Deficiency

	Thalassemia Trait	Iron Deficiency
Mentzer index (522)* MCV/RBC	<13	>13
Shine and Lal (532) (MCV) 2 × MCH	<1530	>1530
England and Fraser (533) MCV–RBC–(5 × Hb)–8.4	Negative values	Positive values

Hb, Hemoglobin; *MCV,* mean corpuscular volume; *RBC,* red blood cell.
*Numbers in parentheses are reference citations.

in a bone marrow specimen is highly suggestive of iron deficiency, but the presence of iron does not necessarily exclude an iron-responsive anemia.

Several formulas have been developed to assist in the diagnosis of thalassemia trait during the assessment of microcytic hypochromic anemia (Table 21-8).[522,532,533] None of the discriminant functions is infallible. Nonetheless, the Mentzer index[522] is useful as an office screening test. More accuracy in separating thalassemia trait from iron deficiency may be achieved with the England and Fraser function,[533-535] but when both conditions exist simultaneously, all indices are subject to error. Additional discriminant functions and automated protocols have been proposed that may also prove useful.[536-538] Free erythrocyte protoporphyrin, which is often incorporated into the initial workup of hypochromic microcytic anemia in childhood to screen for lead exposure, is an additional useful test for patients suspected of having thalassemia trait.[539,540] This technique has been especially helpful in evaluating newly arrived Southeast Asian refugees to the United States.[541] Of course, measurement of transferrin saturation or ferritin may also be used to verify or exclude the diagnosis of iron deficiency. The ratio between microcytic (MCV <61 fL) and hypochromic (MCHC <28 g/dL) erythrocytes is a helpful parameter. A ratio greater than 1.0 is highly suggestive of thalassemic trait, whereas a ratio less than 1.0 is mostly associated with iron deficiency.

In office practice the distinction between thalassemia trait and iron deficiency generally depends on examination of peripheral blood smears and, above all, the patient's clinical history, as well as measurement of red blood cell indices in the parents. It is worth remembering that patients with thalassemia trait are not immune to iron deficiency. In these patients, iron deficiency may mask increases in the levels of HbA_2 and HbF that would otherwise suggest a diagnosis of β-thalassemia.

After iron deficiency is excluded, differential diagnosis between α- and β-thalassemia trait depends on measurement of HbA_2 and HbF levels (see Table 21-7) and rarely if ever on measurement of the α-to-β biosynthetic ratio in peripheral blood reticulocytes. In patients with microcytosis, hypochromia, and erythrocytosis but without evidence of iron deficiency or altered HbA_2 and HbF levels, α-thalassemia is the most probable diagnosis. When both α- and β-thalassemia coexist, changes in the levels of HbF and HbA_2 characteristic of β-thalassemia trait may not be present[116] (discussed later). Molecular genetic analysis and family studies are often necessary to define these complex interactions.

Severe β-Thalassemia

Historical Perspective

It was not until Thomas Cooley, a Detroit pediatrician, first described the clinical entity that was to bear his name that thalassemia major was recognized as a distinct disease.[542,543] Cooley recognized similarities in the appearance and clinical course of four children of Greek and Italian ancestry. These children exhibited severe anemia (hemoglobin concentration of 3 to 7 g/dL), massive hepatosplenomegaly, and severe growth retardation. In addition, bone deformities, such as frontal bossing and maxillary prominence, gave the patients characteristic facies. Deformities of the long bones of the legs were also commonly seen and were thought to reflect severe osteoporosis. Thalassemia major, or "Cooley anemia," was and is far more common in Greece and Italy than in the United States, and descriptions of the disease in these countries soon followed Cooley's original report.[544]

Autopsy studies revealed extraordinary expansion of bone marrow at the expense of bony structures. Extramedullary hematopoiesis was often a striking feature and appeared as either isolated massive hepatosplenomegaly or hepatosplenomegaly in association with intrathoracic or intraabdominal masses.[543-545] Children were also noted to have significant iron deposition in almost all organs, the significance of which was not initially appreciated.[545] Before regular blood transfusion regimens were used, these changes progressed inexorably and were invariably fatal during the first few years of life. Patients died of the effects of their anemia: congestive heart failure, intercurrent infection, or complications resulting from all too commonly seen pathologic fractures.

Initial transfusion regimens were mainly palliative; transfusions were recommended only when the anemia interfered significantly with a patient's ability to function on a daily basis. In defense of this regimen, we must realize that the pathophysiologic basis of the disease was not understood and that clinicians first attempted to minimize the transfusional iron administered because they feared that the iron, seen ubiquitously in these patients at autopsy, might itself be the cause of the disease.[545] Palliative transfusion therapy enabled affected persons to live somewhat longer than patients who did not receive transfusions, but the bone deformities and severe anemia forced them to lead markedly restricted lives.

In an attempt to improve the quality of life of these patients, trials using routine transfusion schedules to maintain hemoglobin levels of 8.5 g/dL or greater were initiated.[546-548] The condition of patients improved dramatically, with fewer overt adverse effects of anemia and more normal growth.[549,550] Clinical improvement proved transient, however, because regular transfusions led to the complications of chronic iron overload, including growth retardation from endocrine disturbances, diabetes mellitus, and delayed sexual maturation. Death invariably ensued in the second or early third decade of life, most often as a result of iron-induced cardiac arrhythmias or intractable congestive heart failure.[551]

Pathophysiology

Imbalance in Globin Biosynthesis. That severe β-thalassemia was caused by impaired production of β-globin was established in the mid 1960s by direct measurement of globin biosynthesis in reticulocytes.[552-556] Soon thereafter, impaired production of α chains was demonstrated in the α-thalassemia syndromes.[451] Decreased hemoglobin production is reflected in the peripheral blood smear by hypochromia, microcytosis, and target cells (which are a sign of the severe impairment in total hemoglobin production), as well as by teardrops, fragments, and microspherocytes (see Fig. 21-26). Red blood cell fragmentation is a direct consequence of unbalanced globin chain synthesis. Excess α chains, which have no complementary non–α chains with which to pair, form insoluble inclusions, demonstrable on methyl violet staining of the peripheral blood of asplenic patients with β-thalassemia (Fig. 21-27). In persons who have not undergone a splenectomy, these inclusions are difficult to demonstrate because they are efficiently removed during the passage of red blood cells through the splenic sinusoids, where fragments and teardrops are generated (Fig. 21-28).

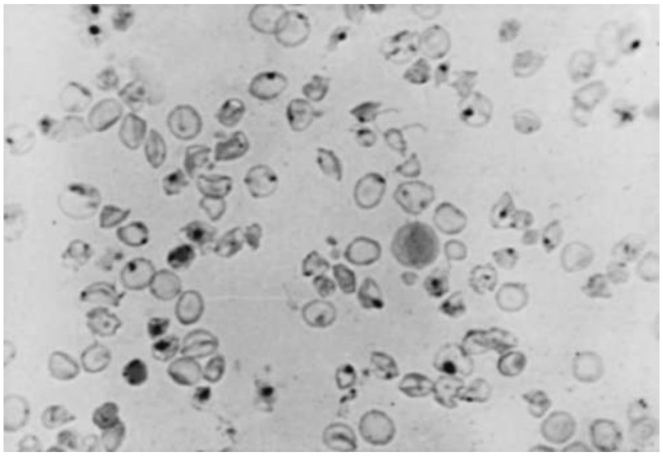

Figure 21-27 Supervital stain (methyl violet) of the peripheral blood cells from a patient with homozygous β-thalassemia after a splenectomy.

α-Globin inclusions in the erythrocytes and nucleated erythroid cells of patients with thalassemia major were first demonstrated by Fessas and colleagues[557-559] and by Nathan.[560] The incidence of the inclusions parallels the degree of impairment in β-globin synthesis. Inclusions have several deleterious effects on erythroid cells and are believed to lead to severe ineffective erythropoiesis. Intranuclear α-globin inclusions, visualized with electron microscopy, have been hypothesized to interfere with cell division.[561,562]

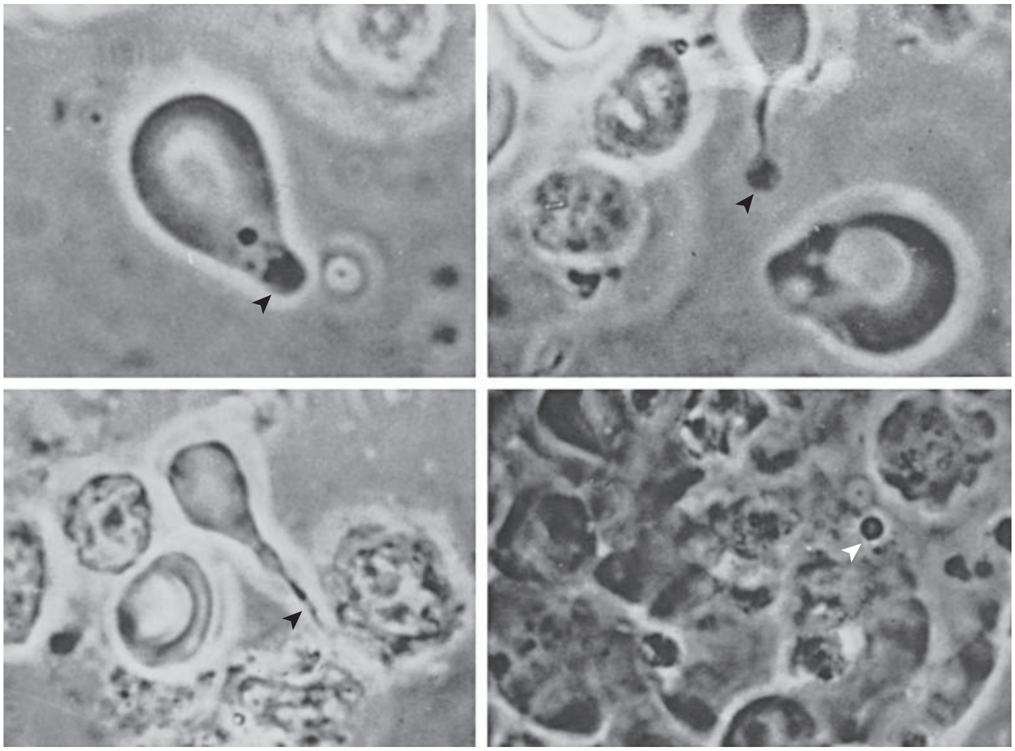

Figure 21-28 Phase-contrast microscopy of a wet preparation of scrapings from the spleen of a patient with homozygous β-thalassemia. Note the chain inclusion bodies *(black arrowheads)* within teardrop-shaped red blood cells; inclusions being pulled out, or "pitted," from the red blood cell by reticuloendothelial cell action *(lower left)*; and inclusions free in the splenic pulp *(white arrowhead)*.

Intracytoplasmic inclusions oxidize and mechanically damage cell membranes, interfere with mitochondrial function, and permit the ingress of calcium ions, which perturb the internal monovalent cationic environment and thereby contribute to the intramedullary death of erythroid cells. Moreover, α inclusions reduce red blood cell deformability and very likely interfere with egress from the bone marrow spaces, as well as from the tortuous vasculature of the spleen. The combined effects of ineffective erythropoiesis and severe anemia account for the extraordinary marrow expansion seen in patients with thalassemia major. In fact, these persons have a nonfunctional cell mass that approximates the 10^{15} tumor cells present in patients with fatal leukemia and is associated with a profound hypermetabolic state that includes fever, wasting, and hyperuricemia.[560]

The Red Blood Cell Membrane. Numerous membrane abnormalities of thalassemic erythrocytes have been described.[463,465,563] Many are attributable to oxidative damage from excess globin chains and the association of heme and iron with the cell membrane after the degradation of precipitated hemoglobin chains.[463,564] Other abnormalities may result from selective interaction of excess α or β chains with the membrane cytoskeleton. After oxidation, globin chains, as well as trace metals such as iron and copper, generate free oxygen radicals that foster oxidative injury to the membrane.[565] Peroxidation of lipids in the red blood cell membrane is reflected by altered membrane lipid composition and distribution; polymerization of membrane components caused by lipid peroxidation leads to excessive calcium influx, monovalent cation loss and dehydration, decreased cell deformability, and increased rigidity of the phospholipid bilayer.[109,110,566] Similar changes are observed in normal red blood cells forced to engulf excess α chains.[567] The reductions observed in the level of serum and red blood cell vitamin E, a natural antioxidant, may result from accelerated consumption secondary to oxidant stress. The abnormal lipid distribution may contribute to enhanced clearance by macrophages. Increased binding of autologous immunoglobulin G with an α-antigalactosyl specificity, originally proposed to reflect a decrease in sialic acid content of the membrane, may also promote clearance of thalassemic red blood cells from the circulation. The increased calcium content of thalassemic red blood cells is a hallmark of membrane damage.

Electrophoresis of red blood cell membrane proteins readily demonstrates an increased association of globin chains with the red blood cell membrane (up to 11% of total protein), and similar findings can be reproduced in nonthalassemic erythrocytes by exposure to experimental oxidative stress.[568] The α- and β-globin chains interact differently with components of the cytoskeleton.[569-571] The specificity of these interactions may contribute to the different phenotypic manifestations of the disorders. Membrane protein 4.1 of β-thalassemia erythrocytes has a diminished capacity to enhance the binding of spectrin to actin. In HbH disease, protein 4.1 appears to be normal, but binding of spectrin to inside-out vesicles is decreased, potentially because of oxidative damage to ankyrin promoted by binding of HbH to protein band 3, with which ankyrin interacts. Spectrin binding is normal in β-thalassemia erythrocytes.

Schrier and colleagues[572] reported differences in the rheologic properties of erythrocytes obtained from patients with HbH disease and β-thalassemia intermedia. Erythrocytes from both groups had a normal surface area but decreased cellular volume and increased membrane rigidity that resulted in decreased cellular deformability under conditions of hypertonic osmotic stress. Although HbH erythrocytes exhibit an increase in mechanical stability and a decrease in cell density, β-thalassemia erythrocytes are prone to cellular dehydration and have markedly decreased mechanical stability. In a murine model of β-thalassemia, these cellular abnormalities correlated with the extent of α-globin associated with the membrane; small increases in β-globin chain expression through the introduction of a β-globin transgene resulted in a marked decrease in membrane-associated globin and significant reversal of the rheologic abnormalities.[573] Thus the slight improvements in chain synthetic balance that accompany increased expression of γ-globin or decreased expression of α-globin may result in dramatic improvements in membrane integrity in patients with β-thalassemia. More detailed understanding of the red blood cell membrane abnormalities in thalassemia may facilitate the development of novel therapies in the future.

Genetic Heterogeneity. In current hematologic parlance, the term *thalassemia major* refers to patients with severe β-thalassemia who require regular blood transfusions to sustain life. In the vast majority of such patients, both β-globin genes are affected by a thalassemia mutation; hence they are described as homozygous β-thalassemics, although in the United States most are actually double heterozygotes. In other patients who also appear to be homozygous for β-thalassemia mutations based on family studies, a hemoglobin concentration of 6 to 10 g/dL is maintained without blood transfusions except during periods of infection, surgery, or other stress.[1,574] Such patients are said to have *thalassemia intermedia*. Thalassemia intermedia is a clinical term that describes the transfusion status of the patient. The term *Cooley anemia* is still often used and applied somewhat indiscriminately to individual patients with either the major or intermedia clinical syndrome. Although other acquired and genetic modifiers exist, disease severity in the β-thalassemias is most directly related to the degree of imbalance between α- and total non–α-globin synthesis. Three major factors emerge as important determinants of the biosynthetic ratio in individual patients: the nature of the specific mutation or mutations, the presence of abnormalities in the α-globin cluster that increase or decrease α-globin expression, and the genetic capacity for synthesis of HbF. Proteolysis of excess α chains, evident on comparison of the α to non-α biosynthetic ratio by short- and long-term incubation of bone marrow cells[490-494] (discussed earlier), is also likely to modulate disease severity, although no meaningful quantitative studies of this mechanism have yet appeared. Recently, a chaperone of free α-globin chains has been described (discussed earlier).

Nature of Specific Mutations. The capacity for production of β-globin chains in a person with thalassemia is directly determined by the specific mutations involving the β-globin gene. Because several different mutations are commonly found in populations with a high incidence of β-thalassemia, most American patients with clinical homozygous β-thalassemia are heterozygous for two different mutations. The effect of specific mutations may range from total loss to only a mild impairment in β-globin synthesis, and thus the potential interactions based on the many different mutations are extraordinary. Indeed, disease severity may be viewed as a continuum, the extremes of which have been shown to correlate with specific mutations.

Mutations in the IVS-1 splice donor site (see Table 21-2 and Fig. 21-10) provide a particularly illustrative example of the variable consequences of specific mutations on β-chain synthesis. These mutations lead to either a complete block in correct splicing of β-globin mRNA or merely a reduction in the abundance of normal mRNA. Patients homozygous for a mutation in the conserved GT splice junction dinucleotide and who have not co-inherited an α-thalassemia gene or a determinant that increases HbF synthesis display severe transfusion-dependent β^0-thalassemia.[149,158-160] Substitution of C or T at position 5 of the splice donor consensus sequence results in severe β^+-thalassemia,[131,158,169-171] whereas substitution of A at the same position results in a more mild phenotype.[172] The T→C substitution at position 6 is also associated with mild β^+-thalassemia.[149]

Dominant β-Thalassemia. In rare circumstances, the heterozygous state of β-thalassemia may be associated with severe disease rather than typical thalassemia trait. Several pedigrees with severe heterozygous β-thalassemia characterized by splenomegaly, cholelithiasis, and leg ulcers have been described.[575,576] A striking association has been noted between mutations involving exon 3 of the β-globin gene and these unusual cases of dominantly inherited β-thalassemia.[251,255] Third-exon mutations typically lead to the production of an unstable β-globin chain. A subset of third-exon β-thalassemia mutations are associated with the presence of inclusion bodies in the normoblasts of affected heterozygotes (see Table 21-2)[253-257] and were first reported as "inclusion-body β-thalassemia," originally defined as a dominantly inherited dyserythropoietic anemia.[577,578] The inclusion bodies, which may contain both α-globin and β-globin, represent an aggregation of precipitated α-globin and unstable β-globin chains with the cell membrane, where they presumably potentiate oxidative damage. The dominantly inherited β-thalassemias are relatively rare and are found in ethnic groups from nonmalarious regions.[251,255] Presumably, there is little selective pressure for these mutations because of the decreased fitness of heterozygous persons.

Increased production of α-globin chains above normal levels may exacerbate the chain imbalance in β-thalassemia heterozygotes; co-inheritance of triplicated α-globin gene chromosomes is another potential cause of severe heterozygous β-thalassemia, and patients usually have the phenotype of thalassemia intermedia.[413,414,579-581]

Interaction with Genetic Determinants That Increase Hemoglobin F Synthesis. Deletions within the β-globin gene cluster and mutations in the γ-globin promoters are sometimes associated with increased expression of γ-globin (see earlier discussion). Relatively small, genetically determined heterocellular increases in HbF ameliorate the clinical course of thalassemia,[112,113,354,366,516,582] thereby raising the prospect that pharmacologic augmentation of γ-chain production may be of particular benefit in the management of thalassemia (discussed later).

An apparent amplification of the capacity for HbF synthesis during erythroid maturation is readily demonstrated in thalassemic patients. In the earliest erythroid precursor cells, γ-globin may be synthesized at a level less than that of β-globin and at only a small percentage of that of α-globin, yet the final proportion of HbF in patients not receiving transfusions may be quite high.[1,109] This effect occurs because the subset of cells expressing γ-globin have less overall globin chain imbalance and therefore preferentially survive in both bone marrow and peripheral blood; hence measurement of steady-state HbF levels provides an imperfect measure of the capacity for γ-globin synthesis in individual patients. More accurate assessment of the HbF program is achieved by measurement of the HbF accumulated in progenitor-derived erythroid colonies produced in vitro.[583]

Interaction of α- and β-Thalassemia Mutations. Because the clinical severity of α- and β-thalassemia correlates with the degree of imbalance in the production of α and non-α chains, co-inheritance of α- and β-thalassemia mutations would be anticipated to yield syndromes of intermediate severity.[584-590] This principle has been validated by clinical experience.[116,591,592] One striking example is the influence of the number of functional α genes in persons with β-thalassemia trait in Sardinia.[116] As shown in Table 21-9, MCV and MCH improve in a stepwise fashion as α-globin production decreases and the α-globin to β-globin ratio approaches 1, but they then deteriorate as the ratio decreases to less than 1. This analysis provides conclusive evidence that the degree of chain synthesis imbalance determines the red blood cell phenotype. Thus a higher incidence of α-gene deletions (–α/αα or –α/–α) has been found in patients with thalassemia intermedia than in patients with thalassemia. Further evidence for the effect of α-gene number on clinical phenotype is the occurrence of thalassemia intermedia in a subset of persons doubly heterozygous for β-thalassemia and triplicated α genes (ααα/αα)[413,521,579-581] or severe β-thalassemia trait in persons also heterozygous for triplicated α genes.[570,571,593-599] Persons heterozygous for β-thalassemia but homozygous for triplicated α genes invariably have thalassemia intermedia.

Clinical Features and Laboratory Values on Initial Evaluation. Severe β-thalassemia is most often diagnosed between 6 months and 2 years of age when the normal physiologic anemia of the newborn fails to improve. γ-Globin production is not impaired in utero; therefore, only when β-globin becomes the predominant β-like globin

TABLE 21-9 Effect of Successive α-Globin Gene Deletion on Hematologic Parameters in a Homogeneous Population with β-Thalassemia Trait

Group/Genotype	Hb (g/dL)	MCV (mm^3)	MCH (pg)	HbA$_2$ (%)	HbF (%)	Ratio
αα/αα (*n* = 20)	12.4 ± 1.2	66.2 ± 3.7	21.2 ± 1.2	4.8 ± 0.7	1.4 ± 1.3	2.2 ± 0.3
α–/αα (*n* = 12)	12.8 ± 1.3	67.3 ± 3.4	22.1 ± 1.1	5.2 ± 0.7	1 ± 0.3	1.3 ± 0.1
α–/α– (*n* = 4)	14.1 ± 1.1	76 ± 3.4	24.8 ± 1.4	4.8 ± 0.6	1.3 ± 0.7	0.8 ± 0.1
α–/— (*n* = 4)	11.7 ± 0.8	54.7 ± 2.5	18.4 ± 0.8	4.6 ± 0.2	—	0.5 ± 0.05

Modified from Kanavakis K, Wainscoat JS, Wood WG, et al: The interaction of alpha thalassemia with beta thalassemia. Br J Haematol *52:465–473, 1982.*

Hb, Hemoglobin; *HbF,* fetal hemoglobin; *MCH,* mean corpuscular hemoglobin; *MCV,* mean corpuscular volume; *RBC,* red blood cell.

does anemia ensue. Occasionally the disease is not recognized until 3 to 5 years of age because prolonged HbF synthesis compensates for the lack of HbA. When first seen, affected infants usually display pallor, poor growth, and abdominal enlargement from hepatosplenomegaly.

Children with severe β-thalassemia are readily distinguished from children with other congenital hemolytic anemias. At initial evaluation, patients who have not received transfusions show 20% to 100% HbF, 2% to 7% HbA$_2$, and 0 to 80% HbA (see Fig. 21-23), depending on the precise genotype. Consistent with severe ineffective erythropoiesis and splenomegaly, the reticulocyte count is characteristically low (often <1%), whereas the nucleated red blood cell count is elevated. Erythrocytes are severely microcytic (MCV of 50 to 60 μm^3) with a hemoglobin content as low as 12 to 18 pg/cell. HbF can be shown to be heterogeneously distributed among the red blood cells either by the Betke-Kleihauer acid elution technique or by anti-HbF fluorescent staining (see Fig. 21-19).

Bone marrow examination reveals marked erythroid hyperplasia, often with an erythroid-to-myeloid cell ratio of 20 : 1 or greater. Before the onset of hypersplenism, the accelerated rate of hematopoiesis may also be reflected by elevated white blood cell and platelet counts in peripheral blood. The serum iron value is markedly elevated, but total iron-binding capacity is usually only slightly increased, thereby resulting in a transferrin saturation of 80% or greater. Serum ferritin levels are generally elevated for age.

If laboratory parameters fail to establish the diagnosis of thalassemia, demonstration of a mild microcytic and hypochromic anemia, indicative of the presence of thalassemia trait in both parents, will allow the diagnosis to be made with confidence. If the diagnosis remains in doubt, it can be confirmed by globin genotyping.

Complications

The cause of the complications of thalassemia is multifactorial and variable and depends on the genotype, other known and unknown phenotypic modifiers, and availability and adherence to therapy. The pathophysiologic features of the complications stem from ineffective and extramedullary hematopoiesis and treatment-related adverse effects, including transfusional iron overload, transfusion-associated infections, and chelation. In addition, more recently recognized but not fully understood is the increased risk for clinically evident and silent thromboses leading to pulmonary hypertension and overt portal vein thromboses. The complications of thalassemia in a large group of North American patients have recently been reviewed.[600]

Bones. To appreciate the full spectrum of bone changes in persons with severe β-thalassemia, one must examine patients who receive transfusions rarely.[542,543] Bone disease in such patients is primarily related to erythroid expansion and not to iron overload or abnormalities in vitamin D metabolism.[601,602] Maintenance of nearly normal hemoglobin levels results in suppression of erythropoiesis and tends to reverse the bone abnormalities,[570,571,593-599,603-605] but osteoporosis is common even in patients who receive regular transfusions.[606-609] Some clinics report a high incidence of vertebral compression fractures.[608] A recent review of more than 700 patients with thalassemia syndromes reported an overall fracture prevalence of 12.1% that ranged from 2.5% in very young patients to 23% in patients older than 20 years. Therapy with excessively high doses and sometimes even standard doses of deferoxamine may also lead to metaphyseal dysplasia.[610-612]

Radiologic abnormalities may be present during the first 6 months of life but are not usually marked until about 1 year of age.[613] In the small bones of the hands and feet the trabecular pattern is coarse, cystic abnormalities are present, and the bones are tubular in appearance. The long bones of the extremities exhibit thinning of the cortices and marked dilation of the medullary cavities. Accordingly, they become extremely fragile and prone to pathologic fractures.[614] The skull is also classically involved, with marked widening of the diploic space and arrangement of the trabeculae in vertical rows, which tends to give a "hair on end" appearance to the skull radiograph (Fig. 21-29). Other radiologic abnormalities in the skull include failure of pneumatization of the maxillary sinuses and overgrowth of the maxilla. These changes lead to maxillary overbite, prominence of the upper incisors, and separation of the orbits—changes that contribute to the classic "thalassemic facies."

Other bone changes caused by medullary overgrowth include widening of the ribs with notching and the development of masses of extramedullary hematopoietic tissue, which may appear as tumors in the chest and mediastinum.

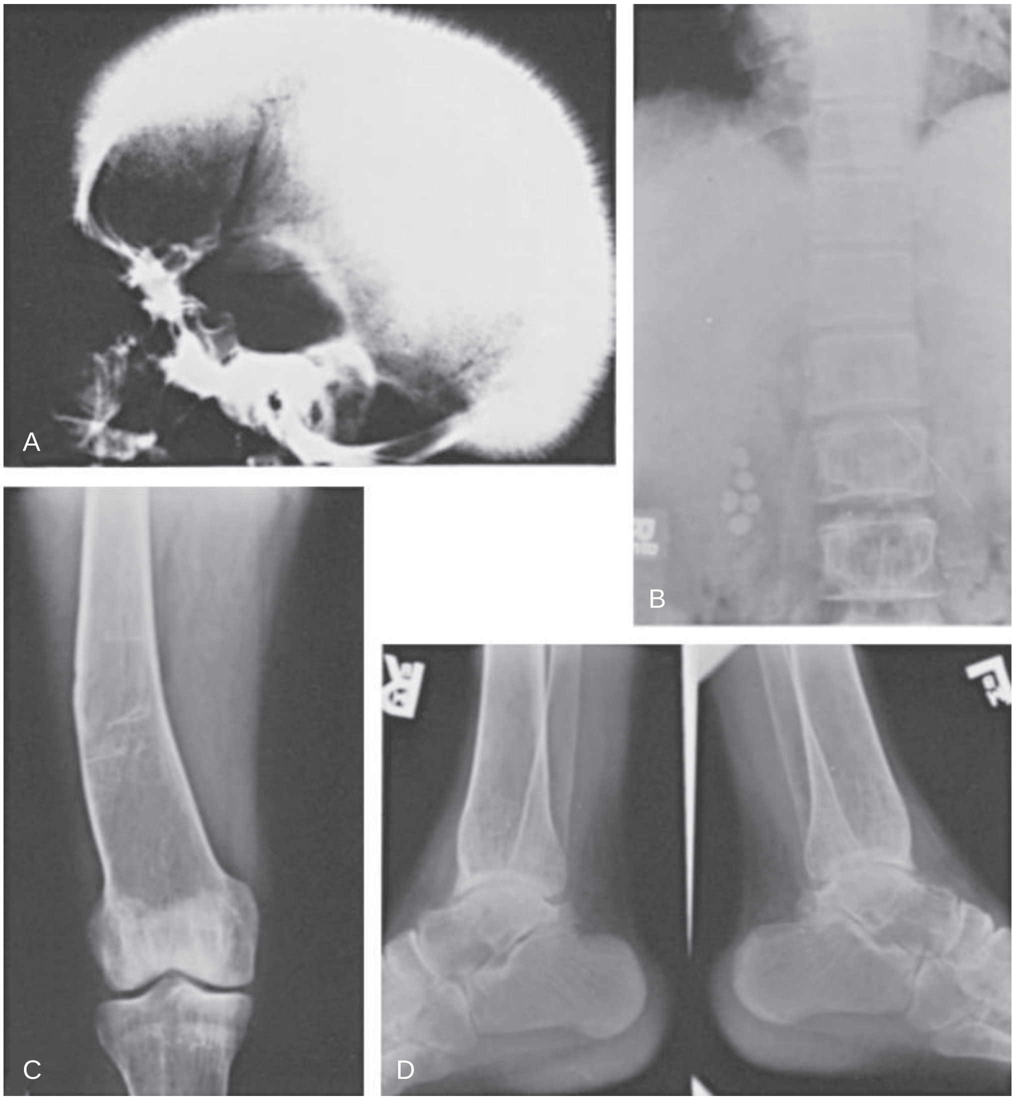

Figure 21-29 Radiologic abnormalities in a patient with homozygous β-thalassemia who receives blood transfusions rarely (thalassemia intermedia). **A**, A skull radiograph illustrating the typical "hair on end" appearance of the diploë and failure of pneumatization of the frontal sinus. **B**, An abdominal film illustrating the coarse trabeculation and osteoporosis within the vertebrae. Multiple calcified gallstones are also seen. **C**, Severe osteoporosis, pseudofractures, thinning of the cortex, and bowing of the femur. **D**, Degenerative arthritis affecting the tibiotalar joint in particular is reflected by loss of the cartilage space and sclerosis in the adjacent bone.

The vertebrae are square with coarse trabeculae. The aforementioned osteoporosis and associated bone pain may be relieved by calcitonin therapy,[615] but the role of vitamin D and calcium supplementation in the prevention of osteoporosis in patients with thalassemia has not been established. Controlled randomized studies have revealed the potential value of bisphosphonate therapy to improve bone density as measured with bone density scans.[616]

Gallbladder. Calcium bilirubinate gallstones resulting from excessive excretion of the products of heme catabolism are common in older patients.

Iron Deposition. A detailed discussion about iron deposition and treatment options is found in the subsequent section on Therapy for β-Thalassemia.

Therapy for β-Thalassemia

Transfusion

Choice of Transfusion Regimen. The mainstay of management of severe β-thalassemia remains blood transfusion.[617-624] Transfusion programs have several aims. By increasing the hemoglobin content, transfusions enhance the oxygen-carrying capacity of blood and thereby decrease tissue hypoxia. The concomitant decrease in erythropoietin levels blunts the massive erythroid expansion associated with the anemia. Furthermore, improved tissue oxygenation and reversal of the hypercatabolic state promote more normal growth and development. Suppression of erythropoiesis is associated with decreased intestinal iron absorption. These benefits must be weighed against the prospects of excessive iron loading, particularly with more intensive transfusion protocols.

Transfusion management and the optimal maintenance level of hemoglobin have evolved. Wolman and associates[546,625] first recommended a pretransfusion hemoglobin level of 8.5 g/dL. This approach improved survival, but chronic illness, bone disease, and anemic cardiomyopathy persisted. To enhance quality of life, Piomelli and colleagues[550] suggested maintaining the hemoglobin level at greater than 10 g/dL with a mean of 12 g/dL. Such "hypertransfusion," if initiated in the first year of life, promotes normal initial growth and development, limits the development of hepatosplenomegaly, prevents disfiguring bone abnormalities, reduces intestinal iron absorption, and decreases cardiac workload.[626-629]

In 1980, Propper and colleagues[630] proposed a "supertransfusion" program with a pretransfusion hemoglobin level greater than 12 g/dL and a mean of 14 g/dL to more effectively eliminate chronic tissue hypoxia. Initial studies reported that the quantity of blood required to maintain a higher hemoglobin level was no greater than that required for maintenance of a lower level because of a decrease in intravascular volume.[630-632] Further data, however, suggested that increasing the pretransfusion hemoglobin level may simply increase the quantity of transfused blood and thus increase iron loading,[633,634] and this regimen is no longer recommended.

Senescence in red blood cells is a function of cell age. Because the iron content of transfused erythrocytes is independent of cell age, attempts have been made to improve the "quality" of transfused blood by infusing the youngest third of red blood cells present in whole blood ("neocytes").[629,635] Administration of these cells would be predicted to decrease the transfusion requirement. Neocytes can be readily prepared with automated cell separators,[636-640] but 3 or more units is required to prepare the equivalent of 1 unit of blood, thus drastically increasing donor exposure for these patients. Although prolonged survival of neocytes has been documented in vivo, the observed sparing of transfusions with such therapy has been disappointing.[641-644] Similarly, methods to remove "gerocytes" (old red blood cells) from the recipient's circulation at the time of transfusion, although technically feasible, have not been widely applied.[643,645] These approaches should be considered experimental at present.

Although specific practices differ among clinical centers, transfusion is indicated both to correct anemia and to suppress erythropoiesis.[617] After diagnosis, a period of observation should be initiated to determine whether transfusion is required to maintain the hemoglobin level at 7 g/dL or greater. The condition of patients with thalassemia intermedia will be stable without transfusion. If the hemoglobin level decreases to less than 7 g/dL, a transfusion program should be initiated to maintain the hemoglobin level at 9.5 to 11.5 g/dL. During the first decade of life, normal growth provides reassurance that the transfusion regimen is adequate. Because the rate of iron absorption parallels the number of nucleated red blood cells in peripheral blood,[646] adequate transfusion should suppress the nucleated cell count to less than 5 per 100 white blood cells[617]; however, in older patients who received inadequate transfusion therapy early in life, it may not be possible to achieve this level. During the teenage years, growth failure may reflect endocrine dysfunction rather than inadequate transfusions; laboratory investigation and appropriate replacement therapy are then indicated. After the epiphyses have fused and growth is complete, a hemoglobin level of 8.0 to 9.0 g/dL may be well tolerated. If the transfusion requirement exceeds 200 mL of packed red blood cells per kilogram per year, splenectomy should be considered (discussed in more detail later).[617] Our current transfusion procedure is outlined in Box 21-1.

Box 21-1 Guidelines for Chronic Transfusions in Patients with Thalassemia

1. Determine the blood type of the patient completely to identify minor red blood cell antigens before the first transfusion.
2. Keep the pretransfusion hemoglobin level between 9.5 and 11.5 mg/dL as needed for suppressing ineffective erythropoiesis and maintaining a reasonable sense of well-being.
3. Give 10 to 20 mL/kg of leukocyte-poor, washed, and filtered red blood cells at a maximum infusion rate of 10 mL/kg over a 2-hour period; transfuse more slowly in patients with heart disease.
4. Avoid raising the posttransfusion hemoglobin level to greater than 16 g/dL.
5. Choose a transfusion interval to maintain pretransfusion levels as outlined above (3 to 5 weeks, depending on individual patient needs). (*Comment*: Some patients tolerate slightly lower pretransfusion hemoglobin levels and need 5 weeks between transfusions, whereas others feel best when they receive a transfusion every 4 weeks. Some patients prefer receiving fewer units and coming every 3 weeks. Some of the younger patients whose weight would require between 1 and 2 units receive a transfusion every 3 or 4 weeks and alternate between 1 and 2 units per transfusion.)
6. Pretransfusion laboratory tests include a complete blood cell count, differential, crossmatch, and red blood cell antibody screen.
7. Height and weight are recorded at least every 3 months.
8. Liver function (AST, ALT, bilirubin, and LDH) is evaluated every 3 months, and serum ferritin is evaluated every 3 to 6 months. A deferoxamine (Desferal) challenge test is performed at irregular intervals to measure the appropriate Desferal dosage and chelatable iron stores.

ALT, Alanine transaminase; *AST,* aspartate transaminase; *LDH,* lactate dehydrogenase.

Complications of Blood Transfusion. The primary long-term complication of blood transfusion is iron loading and the resultant parenchymal organ toxicity, as discussed earlier. Febrile reactions to leukocyte antigenic determinants and allergic reactions to plasma components are commonly encountered in patients who receive transfusions on a long-term basis. The use of prestorage leukoreduction filters has significantly decreased the rate of febrile reactions. Washing of red blood cells in saline solution to remove plasma proteins can be beneficial.[647]

Alloimmunization to minor blood group antigens occurs in 20% to 30% of patients[648-651] and may be manifested as delayed hemolysis. Rare circumstances of multiple alloantibodies may be a potentially life-threatening complication in patients with transfusion-dependent β-thalassemia because it may not be possible to find sufficient appropriately matched blood. Alloimmunization is frequently a less significant problem in patients in whom transfusion is initiated before the age of 3 years.[648-651] The benefit of extended red blood cell phenotyping to minimize alloimmunization has been debated in the literature,[652,653] but crossmatching for the rhesus and Kell systems from the time of initial transfusion may decrease the incidence of alloimmunization.[650] Detailed red blood cell phenotyping should be performed in all patients with newly diagnosed thalassemia before transfusion. The average transfusion regimen in most centers is 12 to 15 mL of leukocyte-poor red blood cells per kilogram per month. Autoimmune hemolytic anemia is a rare but perilous complication of allosensitization. It must be managed aggressively to prevent dangerous iron overload.

Transmission of viral infections by transfusion remains a serious and significant problem in patients receiving transfusions on a long-term basis in countries in which the safety measures of volunteer donors, detailed donor questionnaires, and serologic and nucleic acid testing are not used. In one study, approximately 25% of transfusion-dependent patients with thalassemia had been exposed to hepatitis B, 80% of whom had clinical evidence of hepatitis.[654] Exposure to hepatitis C, with an incidence of approximately 6% per transfusion, was nearly inevitable in thalassemic patients receiving regular transfusions, and this agent accounts for the active hepatitis seen in many older patients. Identification of the hepatitis C agent and the development of a serologic test to screen donors have greatly minimized this risk,[655,656] but the infection can lead to fibrosis, cirrhosis, and hepatic carcinoma if untreated.[657-659] Treatment with interferon alfa and ribavirin is indicated and has now been demonstrated to be safe in patients with thalassemia despite the increased rate of hemolysis and transfusion resulting from the administration of ribavirin.[660] Some studies have suggested that iron overload inhibits a therapeutic response,[661-664] but others have shown little or no such effect.[665,666] A minority of patients with thalassemia have become infected with human immunodeficiency virus, and the rate of progression to symptomatic acquired immunodeficiency syndrome has been proportionately lower than in most infected populations.[667] Although death and complications from these illnesses are uncommon in this patient population, it is prudent to exercise precautions. The most important consideration is the use of blood products screened for the presence of potential infectious agents. Patients should be immunized against hepatitis B upon diagnosis or if they have not acquired immunity. When practical, exposure to multiple donors or units of blood should be minimized. In this regard it is important to recognize that the use of washed or frozen red blood cells may lead to an increase in the number of transfused units.

Splenectomy. Splenectomy may be indicated in some patients in an attempt to decrease transfusion requirements. The role of the spleen must be carefully considered in patients who are treated with transfusion, iron chelation programs, or both. The spleen serves both as a scavenger by increasing red blood cell destruction and iron redistribution and as a storage depot by sequestration of the released iron in a potentially nontoxic pool. Unfortunately, splenic iron may equilibrate with other iron pools throughout the body. In one uncontrolled study, three patients who had undergone a splenectomy exhibited cirrhosis and massive iron deposition, whereas slightly younger patients with an intact spleen had only iron deposition.[668] Risdon and colleagues,[669] however, observed no difference between patients who had and had not undergone a splenectomy with regard to pathologic changes in the liver. If the spleen acts primarily as a storage depot for excess iron, premature removal could theoretically be detrimental. On the other hand, if the splenic pool is a particular target for deferoxamine, the beneficial effects of aggressive chelation therapy might be diminished by preferential removal of iron from this relatively innocuous pool. Perhaps most important is the feasibility of achieving negative iron balance with conventional chelation regimens (discussed later). Eventually, an increased requirement for transfusion because of hypersplenism perturbs the balance and contributes to iron loading.[670]

Splenectomy may be indicated in the management of patients with severe β-thalassemia. Massive splenomegaly with hypersplenism causing leukopenia, thrombocytopenia, and an increasing transfusion requirement is often seen in young patients whose transfusion regimens are sporadic. Early splenectomy is often required. The development of splenomegaly is delayed in patients who are receiving a high number of transfusions.[671] Several factors should be considered in the decision to remove the spleen. Modell and Berdoukas[633] and Modell[672,673] carefully documented the annual blood requirement of patients with thalassemia major who had undergone a splenectomy and suggested that the spleen be removed if the observed requirement exceeds that predicted by 50%. Data from other investigators suggest that the benefits of splenectomy with regard to iron balance are realized if the transfusion requirement exceeds 200 to 250 mL of packed red blood cells per kilogram per year with a minimum hemoglobin level of 10 g/dL.[670,674] Because a huge spleen, irrespective of functional hypersplenism, may account for a large fraction of the total blood volume, its removal often leads to a marked though transient reduction in the patient's requirement for blood.[675,676] Most patients show a moderate but significant reduction in their requirement for transfusion to the predicted 200 mL of packed red blood cells per kilogram per year,[672,673] and it remains stable for many years.[677]

The immediate surgical risk accompanying splenectomy is minimal for experienced practitioners, although reports of acute portal vein thrombosis are increasingly being recognized. Some evidence indicates that iron overload itself may inhibit the immune response.[678,679] Infections with *Yersinia* are sometimes observed in patients with iron overload.[680] The potential for overwhelming infection by *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Neisseria meningitidis*[681-683] should always be considered by the attending physician. Because removal of the spleen may blunt the primary immune response to encapsulated organisms, delay of splenectomy until after approximately 5 years of age is preferable. Patients should be immunized with polyvalent pneumococcal, meningococcal, and *H. influenzae* vaccines.[684-687] Supplemental prophylactic oral penicillin may also be used to prevent colonization by strains not covered by vaccines,[617] particularly in young children. Illnesses accompanied by high fever of uncertain cause should be treated aggressively with parenteral antibiotics until bacterial culture results are available. Patients in regions endemic for malaria should receive prophylactic treatment.[673] Infections appear to be significant causes of morbidity in HbE–β-thalassemia.[688]

Red blood cell survival usually increases immediately after a splenectomy.[675] The peripheral blood smear may reveal increased numbers of hypochromic, microcytic, and nucleated red blood cells. Platelet counts greater than 10^6 are often seen, although correction of anemia by transfusion generally results in suppression of platelet production.[469] White blood cell counts of 15,000 to 20,000 are common; the differential is usually normal.

As was previously discussed in more detail, arterial hypoxemia and evidence of pulmonary vascular disease have been reported in patients with thalassemia, and it has been suggested that splenectomy may exacerbate these problems.[689,690] Accordingly, patients who have undergone a splenectomy should be examined carefully for these findings. Because thrombocytosis may be an inciting factor,[690,691] prophylaxis with low-dose aspirin may be considered, although effective transfusion to correct anemia is probably the best form of preventive therapy.

Therapy for Iron Overload. The economy of iron in the human is discussed in detail in Chapter 11. Briefly, the growing child slowly accumulates iron from his or her diet, using about two thirds for hemoglobin production and one tenth for various enzymes and myoglobin; the remainder is held in storage for later use, largely as ferritin in hepatocytes and macrophages.

Although iron is essential for metabolism and oxygenation, its reactivity would be lethal were it not tightly bound to protein. Nonheme iron is an essential cofactor in many hydroxylases and dioxygenases, and heme iron is required for the function of cytochromes and hemoglobin. "Free" iron (i.e., iron that is unbound to hemoglobin, enzymes, or transport or storage proteins) participates in the generation of reactive oxygen species that rapidly oxidize lipids, nucleic acids, and proteins, injuring cell membranes as well as vital enzymes. To counteract the potential reactivity of excess iron, the absorption of iron from the gastrointestinal tract is limited to daily iron losses and incremental amounts required for growth. Iron is absorbed entirely in the duodenum. The mechanism of absorption of heme iron (largely from meat) is not thoroughly understood. In any case, heme iron absorption is independent of and much more efficient than the absorption of nonheme iron, which must first be reduced to the ferrous form and then transported by a divalent metal transporter called DMT1 on the brush border. Iron from both sources is then transported from the intestinal cell via ferroportin at the basolateral surface into the portal blood. This definitive systemic absorption step is negatively regulated by the peptide hormone hepcidin, which is synthesized in the liver and induced by iron adequacy/overload. Failure of this hepcidin-ferroportin axis is associated with the genetic disorder hereditary hemochromatosis in which the liver produces insufficient hepcidin for the degree of iron overload. Importantly for the discussion of the pathophysiology of non–transfusion-dependent thalassemias, hepcidin synthesis is also dysregulated by anemia, particularly the forms of anemia that are characterized by severe ineffective erythropoiesis. The signal that mediates this link between anemia, the rate of erythropoiesis, and the regulation of hepcidin is presently unknown.

Rate and Extent of Iron Overload. Patients with thalassemia intermedia who have not received transfusions slowly but inexorably accumulate iron from their diets because, in persons with this condition, the level of hepcidin released by the liver is inappropriately low.[692] This complication and its consequences are discussed in more detail below. Patients with severe thalassemia who have received transfusions have high serum levels of hepcidin and consequently do not absorb excess iron from their diets.[692] However, every milliliter of transfused red blood cells contains 1 milligram of iron, and only a tiny fraction of the transfused iron is eliminated in the form of shed skin and intestinal mucosa. Hence the accumulation of iron in epithelial and storage cells in nearly every organ but bone and skeletal muscle, as well as high non–transferrin-bound plasma iron (NTBI), is rapidly progressive, and the complications of iron-induced organ dysfunction are inexorable.[693] As the iron accumulation approaches 15 to 20 g (15 to 20 L of red blood cells), symptoms of iron-induced oxidative tissue damage are nearly universal. Liver damage with cirrhosis, cardiac failure and arrhythmia, diabetes, and endocrine failure (including pituitary, thyroid, and parathyroid insufficiency) are inevitable, and death usually ensues before the age of 18 years in patients who do not undergo chelation. Demise occurs much earlier in patients with growth retardation who do not undergo transfusion or in patients who receive inadequate transfusion; these patients die of heart failure and endure marked bony deformities, particularly facial deformities as a result of massive marrow expansion, before they finally succumb. Acknowledgement of this dilemma influenced a 1960s decision[694] to maintain near-normal hemoglobin levels in transfused patients, recognizing that although disfigurement and a miserable quality of life could be ameliorated, death from iron overload would be assured unless effective chelation could be developed.

Measurement of Iron Overload. Measurements of fasting serum iron and transferrin saturation can be useful in the initial evaluation of iron overload but do not provide an index of the extent of overload. Serum ferritin (actually apoferritin) is also useful but only bears a linear relationship to the liver iron content at low levels of excess iron burden.[695] Ferritin level measurement is useful to determine the effects of chelation therapy.[696]

Until well into the 1980s, the only reliable approach to assessment of iron overload in organs was by quantitative analysis of iron content in a liver biopsy. Unfortunately, liver biopsy does not deserve the "gold standard" that was assigned to it because the hepatic fibrosis that characterizes iron overload causes a spuriously low iron content per milligram of liver tissue. Therefore iron content in the liver from one biopsy specimen to another in a single liver may be highly variable.

The development and application of the superconducting quantum interference device (SQUID susceptometer) in a tiny handful of centers (at Case Western Reserve and Columbia Universities in the United States by Gary Brittenham and his colleagues)[697] did permit a noninvasive analysis of total liver iron, but the equipment is so costly and difficult to use and occupies so much space that it never became widely adopted. Furthermore, the reproducibility of the measurements in a single patient studied with use of different machines is less than reassuring. Nonetheless, much information on the rate of iron loading and the relationship of liver iron load to morbidity and mortality was gained by the few groups that used the machines. Measurements of the effects of phlebotomy after successful stem cell transplant for thalassemia were particularly illuminating.[698]

The most useful advances in organ iron measurement have been those provided by St Pierre and colleagues[699] for assessment of hepatic iron and by Anderson and colleagues[700] with respect to cardiac iron. These magnetic resonance imaging (MRI)-based methods have been assessed in experimental animals and found to be reliable indicators of total organ iron by Wood and colleagues.[701] The relationship of the MRI signal to liver iron is much tighter at lower levels of hepatic iron than at the very high levels observed in poorly chelated patients. The techniques can be applied by skilled MRI technologists wherever the instruments can be suitably employed and if the computational programs developed by St Pierre and others are carefully applied. They are potentially applicable worldwide, as long as appropriate MRI is available. Appropriate evaluation of chelation therapy demands repeated measurements of both hepatic and cardiac iron. The two measurements may be dyssynchronous because iron kinetics are faster in the liver than in the heart during chelation. The measurement of NTBI may provide a better index of impending cardiac damage than MRI.[701a]

Iron Chelation Therapeutics[702]

Deferoxamine. Hope for a successful iron chelator began in the 1950s with the studies of Neilands,[703] who showed that a class of compounds he called siderophores were responsible for the extraction of iron from soil by prokaryotes. Influenced by Neilands' work, Heinrich Keberle, of what was then Ciba-Geigy Pharmaceuticals (now Novartis), discovered deferoxamine in soil samples.[704]

Deferoxamine (Fig. 21-30) is a hexadentate, highly selective iron chelator with an affinity constant for iron that exceeds that of transferrin. One molecule of deferoxamine binds one molecule of iron. The drug is amazingly nontoxic in the presence of high iron stores. Patients who undergo a great many transfusions can and do receive grams of the drug daily for decades without serious toxicity in the vast majority of cases. The major toxicity results from uneven depletion of iron stores and an excess of free deferoxamine in certain tissues. The latter scavenges essential iron and thereby produces various (and sometimes serious) toxicities. Both hearing loss and vision impairment are thought to be related to deferoxamine administration in some patients.[705]

Chelation of iron by deferoxamine converts the molecule into feroxamine, a circular red compound. Approximately half is excreted in the bile and half is excreted in

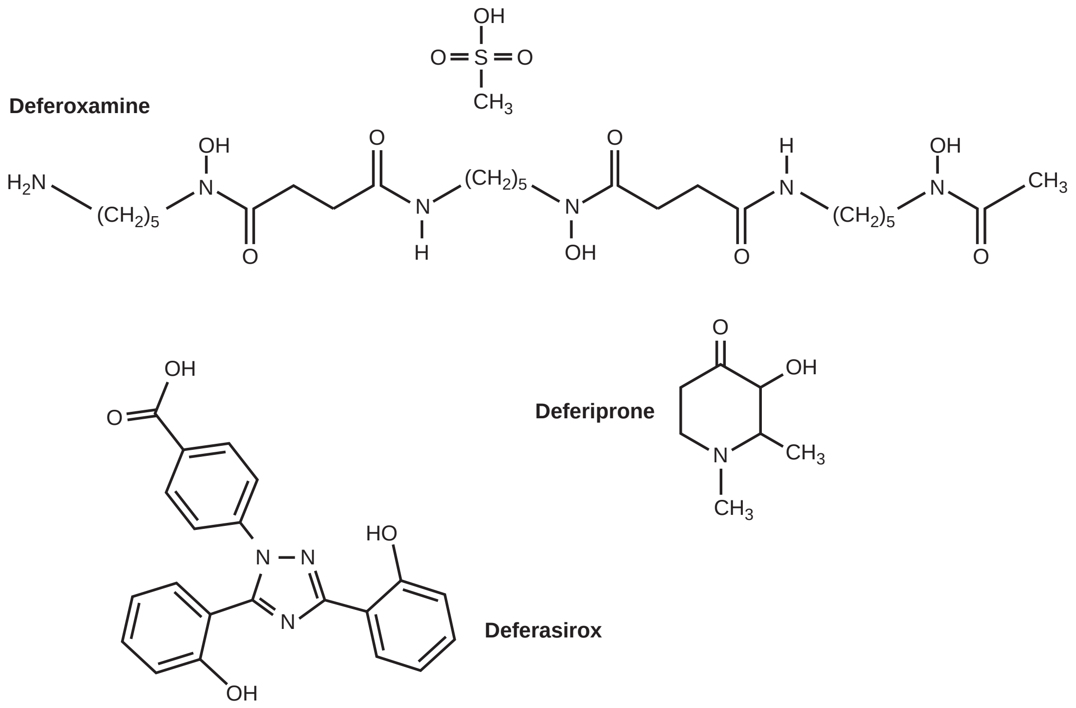

Figure 21-30 Chemical structures of the iron chelators deferoxamine, deferiprone, and deferasirox.

the urine, and thus compliance can be evaluated by simple inspection of urine color. However, the drug has serious drawbacks. It penetrates cells very slowly and is barely if at all absorbed from the gastrointestinal tract. It is rapidly cleared from the blood into stools and urine with a very short half-life of less than 30 minutes. Therefore the drug must be administered parenterally.

Although the introduction of deferoxamine in the early 1960s was enthusiastically greeted by hematologists, it soon became apparent that intramuscular administration of the drug was poorly tolerated by patients. However, Letsky and colleagues,[706] persisted and clearly showed that repeated intramuscular injection of the drug at sufficiently high and frequent doses could lead to diminution of liver iron content and reduced hepatic fibrosis in patients with thalassemia who undergo multiple transfusions. Still, the drug was poorly accepted as a therapeutic agent until the 1970s, when Propper and colleagues[707,708] demonstrated that it could be given as a continuous syringe pump-driven subcutaneous infusion. This discovery and the schedule modifications of Pippard and colleagues[709] utterly changed the future of patients with thalassemia and other patients who required multiple transfusions. During the late 1970s and through the 1980s, several groups, particularly the large and well-managed Italian group of experts,[710,711,712,713] showed that the life span and cardiac disease–free life span of patients with thalassemia was remarkably improved by the administration of deferoxamine via the subcutaneous route. The concept was simple. Transferrin is totally saturated in iron overload, and thus the extra iron that leaks from loaded macrophages spills into the blood and creates a highly reactive plasma pool known as NTBI, a lethal reactive species first described by Hershko and colleagues[714,715] and productively investigated by Porter and colleagues.[716] NTBI attacks cell membranes, particularly the membranes of cardiac myocytes, and creates a flow of electrons that leaves the membranes badly oxidized. The continuous presence of deferoxamine in the plasma binds and removes NTBI, thus preventing further heart damage. However, the short half-life of deferoxamine leaves gaps in the plasma concentration of the drug unless the subcutaneous infusion is maintained 24 hours a day, 7 days a week. Such a schedule, although pharmacologically sensible, is totally unacceptable to the vast majority of patients. Furthermore, some patients experience inflamed nodules at the sites of subcutaneous injection (usually the fat pad of the anterior and lateral abdominal wall). The end result is a compromise in which patients and their physicians settle on a 12-hour subcutaneous infusion 5 nights a week at a dose of 20 to 40 mg/kg. Although such a schedule is reasonably well tolerated, adolescents dislike it not only because of the discomfort of the needle, but also because of the limitation of lifestyle. It is estimated that at least half of the patients "cheat" with regard to this abbreviated schedule and risk the development of cardiac symptoms of iron overload.

Orally Active Iron Chelators. The advent of orally active iron chelators has radically improved the quality of life of patients who undergo transfusion on a long-term basis, particularly those with severe β-thalassemia. However, both of the available drugs—deferiprone and deferasirox—have their limitations. Neither is as efficacious or as free of toxicity as deferoxamine, but freedom from a portable pump and an irritating subcutaneous needle has popularized both of them.

DEFERIPRONE. Deferiprone (see Fig. 21-30) was discovered by Huehns and colleagues[717] in the 1980s and licensed to Ciba Geigy, whose scientists discarded the drug because of lack of efficacy and evidence of rare but severe toxicity. Interest in the drug was then pursued in another London hospital, where it began to be introduced into clinical medicine.[718] This drug is approved by the Food and Drug Administration only for second-line therapy in the United States because of the inadequacy of its clinical trials in North America.

Although deferiprone is very specific for iron and readily penetrates cells, it is a bidentate chelator; therefore, three molecules of deferiprone are required to bind and eliminate one molecule of iron. The drug is promptly absorbed, but its half-life in plasma is less than 2 hours (better than deferoxamine but still very short). Therefore the drug must be administered frequently (every 8 hours is recommended, but that schedule leaves large gaps of inadequate plasma drug concentration), and high doses are required (a total of 75 to 100 mg/kg/day). Some patients (approximately half in a large recently published series in Thailand)[719] respond to the drug on the thrice-daily schedule with a decline in ferritin and in liver iron measured by MRI, but on average, the drug is barely effective at obtaining negative iron balance. This lack of effect on hepatic iron was also seen by the group in London that advanced the use of the drug most vociferously.[720] Some of the lack of efficacy may be due to compliance issues. It is very difficult to take any drug three times a day, let alone the preferable every 4 hours, but the latter schedule is probably required to maintain satisfactory plasma deferiprone concentrations that will eliminate toxic NTBI and achieve negative iron balance.

Deferiprone has a rare but lethal adverse effect. It can produce very severe neutropenia,[702] and the risk of that complication is present at any moment in the course of treatment. This potential adverse effect requires close supervision (in the United States, via weekly blood cell counts) and adds to the inconvenience and cost. Although Olivieri and her colleagues abandoned use of the drug because of its lack of efficacy and what they thought was toxicity expressed as excessive hepatic fibrosis,[702,721] the latter conclusion is uncertain. The observed fibrosis may be an artifact of sampling or simply due to lack of efficacy. Other serious adverse effects include severe joint (particularly knee) pain and swelling that seem to be prominent in South Asian patients[722] and rare drug-induced hepatitis.

In the past decade it has been suggested that deferiprone has a unique salutary effect on the removal of iron from the heart and siderotic heart disease.[723,724] Indeed, it has been claimed that iron is removed from the heart by deferiprone in the absence of any effect of the drug on liver iron stores. If that is true, no other chelator has such a beneficial effect. Proof of that unique capacity would require very careful comparative clinical trials that have not yet been performed. However, it is well worth exploring because such an effect would be most desirable. Unfortunately, the Canadian manufacturer of deferiprone has evinced little appetite for controlled clinical trials of any kind. It is likely that we will learn much more about this controversial drug from prospective trials.[724] It may well prove to be useful for compliant patients in jeopardy of iron-induced heart disease who cannot tolerate either deferoxamine or deferasirox.

DEFERASIROX. Deferasirox (Exjade; see Fig. 21-30) is a rationally designed drug related to a Russian discovery[725] and structurally related to the desferrithiocin class of chelators that was investigated very carefully by Ciba-Geigy and Bergeron and his colleagues in the 1980s and early 1990s.[726] Desferrithiocin was found to be highly effective but nephrotoxic even in the presence of high iron stores. Both deferasirox and desferrithiocin excrete iron entirely through the biliary system, and therefore only a blood level determination can be used to test compliance. Deferasirox was approved by the Food and Drug Administration in 2005. The drug's major attributes are that it is readily (if variably) absorbed,[725,727] it is a tridentate chelator highly specific for iron, and it has a half-life in plasma of more than 8 hours. Thus two molecules of the drug readily bind and eliminate one molecule of toxic NTBI. In addition, the drug persists for hours in the blood, providing excellent protection.[725] It is currently given as a single daily dose of between 20 and 40 mg/kg/day, but at the higher doses, absorption and therefore iron excretion are increasingly variable. Lower doses given twice a day might be more effective, but trials have not yet been performed to evaluate that possibility.

Deferasirox has been carefully investigated by its manufacturer. Many clinical trials have been performed in several iron-loading conditions.[728-730] The drug is effective in all of them, but variable absorption occurs in a substantial fraction of patients, for whom manipulation of dose and schedule is required. An increased level of creatinine is nearly universal, but significant nephrotoxicity has been an infrequent problem. However, gastrointestinal intolerance extending to bleeding duodenal and gastric ulcers has been reported. Rare hepatotoxicity can be severe. Dose and schedule adjustments and the liberal use of food during dosing should be explored in such patients.[731] A very large amount of literature describes clinical trials of deferasirox. Although it is several times as expensive as deferiprone, it is the best choice overall at this time. Data show that it prevents heart disease and other organ damage as well as deferoxamine, and the quality of life of patients who tolerate it well is far superior to that of patients who take either deferoxamine or deferiprone.[728,732]

Iron Overload in Persons with Thalassemia Intermedia. Prior to the advent of oral iron chelators, the management of persons with thalassemia intermedia was extremely difficult. Because of the effort to avoid transfusion, such patients experienced severe bony abnormalities, including short stature and gargoyle facies, as well as debilitating hepatosplenomegaly. In addition, as Musallam and others have pointed out, iron overload still remained a problem over time because of unimpeded iron absorption from the diet in the absence of a normal hepcidin response.[733,734]

The option of prescribing deferasirox has changed the calculus of the management of thalassemia intermedia. If serious growth disturbances occur, the patients should receive transfusions to suppress excess erythropoiesis and take deferasirox to prevent iron overload. Some investigators believe that transfusion and chelation should be offered during growth spurts and to induce sexual maturation. However, intermittent transfusion is a risky endeavor that can readily induce sensitization to minor blood groups, particularly in the United States where thalassemia is found so frequently in non-white persons but the vast majority of blood donors are white. The high incidence of sensitization in African Americans with sickle cell anemia who receive intermittent transfusions is an example of that problem.

Vitamin Supplementation

Ascorbic Acid. The role of ascorbic acid in iron metabolism and chelation therapy is complex and controversial.[735,736] Tissue deficiency of vitamin C often develops in patients with hemosiderosis because of accelerated catabolism; frank scurvy is documented in persons with marginal dietary intake.[737-740] Administration of vitamin C significantly augments iron excretion in response to deferoxamine, particularly in patients who have vitamin C deficiency. Serum iron and ferritin levels may also rise.[740-742] Ascorbic acid retards the rate of conversion of ferritin to hemosiderin[743,744] and presumably allows more iron to remain in a chelatable form. Unfortunately, ascorbic acid also enhances iron-mediated peroxidation of membrane lipids[745,746] and has been shown to increase iron-induced membrane damage in cultured myocardial cells.[747] It has also been observed that patients with iron overloads who are receiving vitamin C may experience cardiac dysfunction that is reversed when the supplementation is discontinued.[748,749]

Some investigators have suggested giving low doses of ascorbic acid (3 mg/kg) at the start of each subcutaneous infusion of deferoxamine. The chelator should be able to block the deleterious effects of ascorbic acid on lipid peroxidation of cellular organelles in vitro. Other clinicians avoid administering vitamin C to patients with iron overload and argue that iron depletion can be achieved without supplemental vitamin C. Patients with significant iron burdens should be cautioned against self-administration of substantial amounts of ascorbic acid because abrupt cardiac deterioration has been observed in this setting (Nienhuis AW, unpublished observations, 1990).

Vitamin E. Deficiency of vitamin E has been noted in many patients with thalassemia major who receive transfusions on a long-term basis.[750-754] Deficiency of vitamin E may contribute to hemolysis as a result of red blood cell membrane damage.[463,755] α-Tocopherol has long been considered to be a potent antioxidant that protects membrane lipids from attack by free radicals formed when excess iron is present. Deficiency in the neonatal period[756] or deficiency caused by malnutrition is associated with varying degrees of hemolysis. Hemolysis and the characteristic increased red blood cell susceptibility to in vitro hydrogen peroxide are readily reversed via the administration of supplemental vitamin E. Of interest, supplemental iron administration increases the hemolytic rate, even in patients with vitamin E deficiency who do not have thalassemia.[756] In patients with iron overload, supplemental vitamin E may lessen iron-mediated cellular toxicity. One study in experimental animals suggests that vitamin E may also inhibit deferoxamine-induced urinary iron excretion.[757] Although vitamin E is used clinically, the efficacy of this therapy remains to be demonstrated.

Folic Acid. Megaloblastic anemia, which is almost invariably due to folic acid deficiency, may occur in patients with severe β-thalassemia.[758-760] In contrast, vitamin B_{12} deficiency is extremely rare in patients with thalassemia.[760] Folic acid deficiency is thought to develop as a result of decreased absorption, low dietary intake, and the enormous demand of bone marrow expansion. Most patients benefit from daily folic acid administration (1 mg), although patients receiving adequate transfusions to suppress erythropoiesis and those living in countries where folic acid is added to many breads and cereals probably do not require supplementation.

Trace Metals. Trace metal deficiency associated with thalassemia or aggressive chelation therapy is not commonly observed.[735,761] Zinc deficiency (acrodermatitis enteropathica) has been reported in a patient receiving diethylenetriaminepentaacetic acid for chelation.[762] The reversible toxic effects seen with high-dose deferoxamine chelation may reflect trace metal deficiency or intracellular chelation of a trace metal. Newer techniques to analyze trace metal concentrations may reveal subtle deficiencies; yearly screening for zinc deficiency is appropriate, but supplementation is not usually required.

Allogeneic Bone Marrow Transplantation. Successful cure of β-thalassemia by bone marrow transplantation was first reported by Thomas and colleagues[763] in 1982. Subsequently, a number of centers have explored the use of this modality as definitive therapy for β-thalassemia. The most extensive published experience with bone marrow transplantation in β-thalassemia is that of Lucarelli and colleagues[764-768] in Italy. The results of their early attempts to use transplantation in this patient population were discouraging.[765] The preparative regimens used were often ineffective and associated with graft failure, toxicity, and high mortality. Their recent experience is considerably more promising.[768] Patients were analyzed to identify clinically important variables predictive of transplant outcome. The probability of survival was decreased in patients with poor chelation status, hepatomegaly (>2 cm), and portal fibrosis. The probability of event-free survival was reduced in the presence of hepatomegaly. Risk factors for hepatomegaly and portal fibrosis were used to divide patients into three classes: class 1, absence of both risk factors; class 2, one risk factor; and class 3, both risk factors. However, such classification of patients may be difficult to reproduce (i.e., hepatomegaly of ≤2 cm and a history of adherence to chelation therapy). The most recent data from Lucarelli for class 3 patients younger than 17 years who received human leukocyte antigen (HLA)-identical matched sibling donor transplants demonstrated significant improvement with the addition of hydroxyurea, azathioprine, and fludarabine to the previous regimen of cyclophosphamide and busulfan.[769] In the 33 patients treated with the new protocol (protocol 26), the survival rate increased to 93% and the rate of graft rejection decreased from 30% to 8%.[769] Adults treated with protocol 26 demonstrated an improved thalassemia-free survival rate of 62% to 67%, and transplant-related mortality was decreased but still significant at 27%, down from 37%.

One of the major reasons for failure of bone marrow transplantation in patients with thalassemia may be the unpredictable pharmacologic effects of the busulfan used in most preparative regimens. Hyperabsorption of the drug is associated with hepatic veno-occlusive disease and hypoabsorption with recurrent thalassemia. Improved control of busulfan levels during induction may provide better clinical results.[770] Whatever the event-free survival estimates, bone marrow transplantation is the only viable option for patients who cannot or refuse to adhere to a well-administered transfusion and chelation program.[771]

On the basis of available data, bone marrow transplantation may be recommended to patients receiving adequate chelation who have no evidence of liver disease. Many of these patients can be cured. Although most such patients are very young, age does not appear to be a significant variable in determining outcome. Chronic graft-versus-host disease is still a potential long-term complication of successful allogeneic transplantation. A current limitation to the general applicability of this therapy is the availability of a related, HLA-matched donor. Only one in four siblings on average will be HLA identical. Improved management of graft-versus-host disease and the development of technologies for bone marrow transplantation from unrelated donors may expand the pool of potential donors in the near future. Some encouraging results have recently been reported.[772] The use of cord blood stem cells may also extend the donor pool.[773,774]

Pharmacologic Manipulation of Hemoglobin F Synthesis. The interaction between β-thalassemia and genetic syndromes that increase γ-globin synthesis has illustrated how even small increases in γ-globin production lead to a significant improvement in the effectiveness of red blood cell production in patients with thalassemia. Although steady-state production of HbF is a genetically determined trait,[12] perturbations in erythropoiesis may be associated with an increased capacity for HbF synthesis. Treatment of experimental animals or patients with a variety of cytoreductive agents including 5-azacytidine (5-Aza), hydroxyurea, vinblastine, and cytosine arabinoside leads to an increase in production of HbF.[775-785] Although the precise mechanism of action of these drugs remains incompletely defined, the increased capacity for γ-globin synthesis appears to be linked to rapid erythroid regeneration. Consistent with this hypothesis, hematopoietic growth factors that promote the expansion and maturation of erythroid precursors, including erythropoietin,[786-788] interleukin-3,[787-789] and granulocyte-macrophage colony-stimulating factor,[789] have also been suggested to enhance HbF synthesis in primate models. Of particular interest is the observation that infants delivered of diabetic mothers exhibit a delayed switch from γ- to

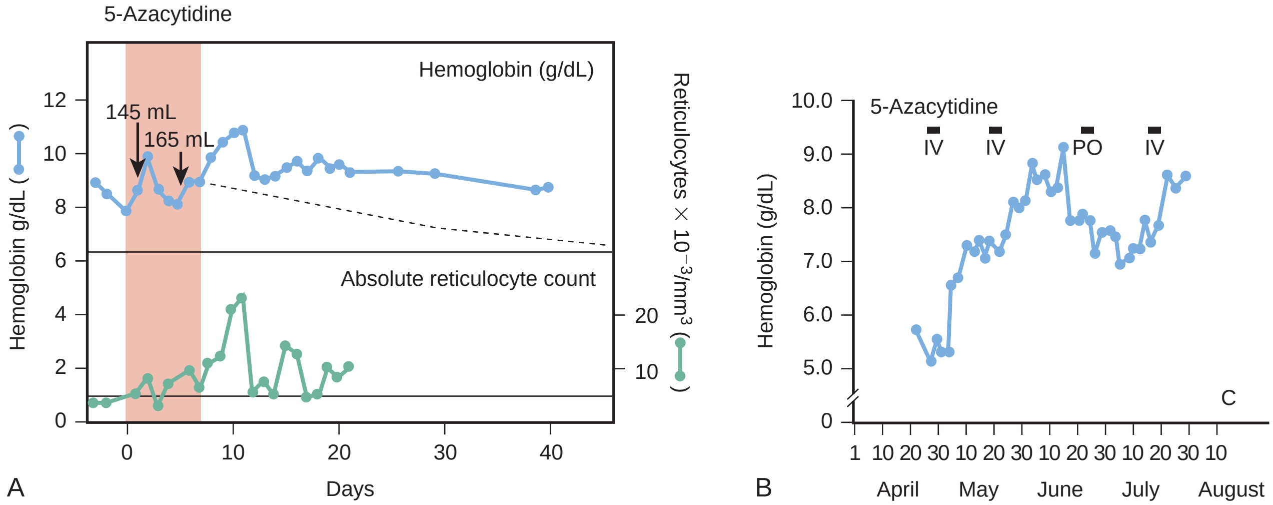

Figure 21-31 **A**, Effects of 5-azacytidine on erythropoiesis in a patient with severe homozygous β-thalassemia after a single administration of the drug by continuous infusion over a period of 7 days. Two small blood transfusions were administered on days 2 and 5. The *dashed line* indicates the projected hemoglobin concentration and reticulocyte count in this patient without treatment based on clinical records. **B**, Effects of sequential courses of treatment with 5-azacytidine in a patient with β-thalassemia who had severe cardiomyopathy and alloimmunization. *(**A**, Modified from information appearing in Ley TJ, DeSimone J, Anagnou NP, et al: 5-Azacytidine selectively increases gamma-globin synthesis in a patient with beta thalassemia.* N Engl J Med *307:1469–1475, 1982. **B**, From Dunbar C, Travis W, Kan YW, et al: 5-Azacytidine treatment in a beta zero-thalassaemic patient unable to be transfused due to multiple alloantibodies.* Br J Haematol *72:467–468, 1989.)*

β-globin.[790] Levels of butyric acid derivatives are elevated in the serum of these mothers and their infants before birth. Similarly, patients with metabolic disorders associated with increased levels of short-chain fatty acids also have elevated HbF levels.[791] Infusions of sodium butyrate or α-aminobutyric acid into fetal sheep markedly delay the perinatal switch,[792] whereas infusions in primates lead to activation of the γ gene (McDonagh KT, Bodine DM, and Nienhuis AW, unpublished observations).[793,794] It has been proposed that butyrate, propionate, or the metabolite acetate promotes increased γ-globin expression by acetylation of histones and alteration of chromatin structure, but other mechanisms cannot be excluded.[795,796] A flurry of articles have described the results of treatment of patients who have sickle cell anemia with butyrate or its derivatives.[797-802] One patient[798] had a sustained increase in hemoglobin, and healing of leg ulcers without an increment in circulating hemoglobin has also been observed.[801] A recent report cited a sustained increase in HbF in persons with sickle cell anemia.[803,804] Caution must be exercised, however, because high doses of butyrate are associated with neurotoxicity in simians.[805]

The most extensive experience with pharmacologic manipulation of HbF synthesis in patients has involved administration of hydroxyurea to patients with sickle cell disease (see Chapter 20).[781,806-810] In this population, the majority of patients appear to respond to the drug with a twofold or greater increase in HbF levels over their baseline. In many patients, HbF levels between 10% to 15% of total hemoglobin are seen. This increase reflects both augmented production and enhanced survival of cells that contain HbF. The clinical results in patients with sickle cell anemia are impressive, as described in Chapter 20. However, the results in patients with thalassemia have shown mixed results to date.[783,811] Of note, almost all studies performed in patients with thalassemia have either been case-control or cohort studies and variable end points have been used, confounding the ability to compare various studies.[811] However, it should be noted that hydroxyurea does show some promise in select patients with thalassemia.

A response to 5-Aza has been seen in several patients with thalassemia[776,812,813] (Fig. 21-31). The dramatic increases in HbF levels associated with administration of this drug may result from a combination of cytotoxicity and inhibition of postsynthetic methylation of DNA.[813-817] Global demethylation of DNA, including the γ-globin gene, is observed after the administration of 5-Aza. Despite its effectiveness,[811] the use of 5-Aza in the treatment of hemoglobinopathies has been limited by concerns about its known carcinogenic potential,[818-820] as well as the demonstrated effectiveness and safety of current treatment strategies involving transfusion and chelation. Because response to the drug is short-lived, long-term therapy would be required.

As previously discussed, the recent mechanistic insight gained into the regulation of HbF production through human genetic studies suggests that targeted and mechanism-based approaches to induce HbF may be developed in the coming years. In particular, targeting of regulators of this process such as BCL11A, KLF1/EKLF, or MYB holds promise for induction of HbF in patients.[821] Either direct targeting of these molecules, modulation of their cofactors that modify chromatin, or disruption of their activity at specific chromatin interaction sites may serve as valuable approaches for HbF induction.

Gene Therapy. As a result of advances in molecular biology, treatment of hematologic disease through the introduction of new genetic material into bone marrow stem cells is a goal for the foreseeable future.[822-825] Application of somatic gene therapy to the treatment of hematologic diseases requires improved efficiency of gene delivery, regulated and sustained expression of the introduced genes, and biologic studies demonstrating expression of the foreign gene. Although active research is ongoing, experimental shortcomings in all these aspects presently preclude the use of gene therapy for the management of thalassemia or sickle cell anemia.

Correction of thalassemia by gene transfer will necessitate the introduction of a normal β-globin gene into hematopoietic stem cells.[822-824] Experimental studies in mice have demonstrated that retroviral vectors are capable of transferring foreign sequences to hematopoietic stem cells.[826,827] Expression of transferred genes has been more problematic but has been improved with use of a new vector containing a portion of the LCR, which has led to 17% to 33% HbF.[828] The efficiency with which primate (or human) stem cells are infected by retroviral vectors appears to be lower than that observed in murine cells. Nonetheless, transfer of foreign sequences to long-term repopulating hematopoietic cells of primates and humans has been demonstrated.[829]

For treatment of β-thalassemia (or sickle cell anemia), it will be necessary to express the transferred gene at high levels in erythroid cells. This task has been a challenge in the field, but vectors that incorporate elements of the LCR have recently been generated. Lentiviral vectors containing such elements have been constructed. Indeed, it has become possible to achieve long-term correction of both sickle cell anemia and β-thalassemia in mouse models with these viruses.[830-832] In addition, a phase I human gene therapy trial has been initiated, and the first patient treated with use of such approaches has been reported.[833] However, concerns exist about the limited clonality of hematopoiesis observed in this patient and an insertion of the lentiviral genome upstream of the *HMGA2* oncogene that is observed in this dominant clone. In addition, it should be noted that the patient has had a high level of HbF expression after the introduction of the corrected cells, bringing into question whether the gene correction itself was sufficient for the independence from transfusion observed.

Interaction of Thalassemia with Globin Structural Variants

In geographic areas in which thalassemia mutations and structural variants of α- and β-globin genes are both commonly seen (such as Africa and Southeast Asia), compound heterozygotes with a thalassemia mutation and a structural variant are common.[1] In such double heterozygotes, disease may be more or less severe than that seen in persons who are heterozygous for only the structural variant. For example, heterozygotes for a β^S gene have 30% to 45% HbS and are usually clinically healthy, whereas patients with a β^S gene and a β-thalassemia mutation (in the *trans* β gene) have 60% to 95% HbS and may have a severe sickling disorder. Persons with a β^S gene and α-thalassemia trait generally have less HbS than those with sickle trait[834,835] and are asymptomatic.[836,837] The interaction of α- and β-thalassemia mutations and the HPFH mutations with the β^S gene are described in detail in Chapter 20.

Hemoglobin E–β-Thalassemia

HbE–β-thalassemia is particularly common in Southeast Asia, where as many as 4000 to 5000 patients may reside in Thailand alone.[838,839] As a result of immigration from Southeast Asia, HbE–β-thalassemia is now a commonly encountered form of transfusion-dependent thalassemia in certain areas of the United States.[840,841] Double heterozygotes not receiving transfusions have hemoglobin levels of 2.3 to 7 g/dL, depending primarily on the output of the β-thalassemia gene. Because α-thalassemia is common in Southeast Asia, more complex phenotypes may be observed.[842] Nucleated red blood cells are found on the peripheral blood smear, whereas they are absent in patients homozygous for β^E. HbE and HbA_2 account for 50% to 70% of the total hemoglobin (5% HbA_2 by HPLC).[841,843] Small quantities of HbA are found in association with a β^+-thalassemia gene. Patients with HbE–β-thalassemia usually require transfusions and exhibit the clinical features of thalassemia major. In areas where intensive treatment is unavailable, the disease resembles classic thalassemia major, with patients exhibiting massive hepatosplenomegaly, hypersplenism, severe skeletal disease, and death from infection in childhood. Iron loading occurs as a result of either transfusion or enhanced intestinal absorption.[844] Proper treatment is the same as that recommended for thalassemia major or intermedia, depending on the requirement for regular blood transfusion. Recent work has also suggested that there are different compensatory responses in HbE–β-thalassemia as patients get older, which may have important implications in the management of this disease.[845] These alterations in the compensatory responses of older patients are also likely to be applicable to patients with other forms of β-thalassemia.

Hemoglobin C–β-Thalassemia

The β^C gene, which encodes a variant β chain with a lysine→glutamic acid substitution at position 6,[846] is common in blacks of West African origin. The only hematologic consequence of simple heterozygosity for the β^C gene is increased target cells on the peripheral smear. Double heterozygotes with a β^C gene and β-thalassemia genes exhibit moderately severe hemolytic anemia with splenomegaly. The peripheral blood smear reveals hypochromia and microcytosis with many target cells. Hemoglobin C accounts for 65% to 95% of the total hemoglobin, depending on whether the thalassemia mutation is of the β^+ or β^0 variety. In the black population the disease is generally mild because of the prevalence of mild β-thalassemia genes (discussed earlier),[510] whereas in Italian,[847] North African,[848] and Turkish patients,[849] the disease is more severe, particularly in those who have a β^0-thalassemia mutation.

Thalassemic Hemoglobinopathies

As discussed in detail in earlier sections, several variant polypeptides can be described as thalassemic hemoglobinopathies. With the exception of HbE and the elongated α-chain variants, these mutations are very uncommon. Interest in these mutations arises from the unique mechanisms by which they produce the thalassemia phenotype.[258,850] The most common thalassemic hemoglobinopathy is HbE disease.

Hemoglobin E Disease. As described earlier, the β^E mutation activates a cryptic splice site in exon 1 (see Fig. 21-9). Because the correct splice site is less efficiently used, production of functional β-globin mRNA that codes for the variant decreases.[168] The incidence of the β^E gene is extraordinarily high in some populations (≈30% in Laos, Cambodia, and Thailand).[838,851] The occurrence of the gene in immigrants from Southeast Asia to the United States reflects these origins.[841]

In the heterozygous form, patients are largely asymptomatic with a hemoglobin level of 12 g/dL or greater, no reticulocytosis, an MCV of 74 ± 10.6 μm^3, and an MCH of 25 ± 2.5 pg. The peripheral blood smear is distinguished by mild microcytosis and occasional target cells. Hemoglobin electrophoresis reveals HbE comigrating with HbA_2 in the range of 19% to 34%. The α to ββ biosynthetic ratio is usually 0.8 or greater, hence the mild nature of HbE trait.

Patients with homozygous HbE disease are also asymptomatic.[839,843] The hemoglobin level is rarely less than 10 g/dL, and significant reticulocytosis is uncommon. The red blood cells, however, are markedly microcytic (MCV of 50 to 66 μm^3) and hypochromic (MCH of 20.1 pg). Target cells and occasional coarse stippling are evident on the smear. HbE accounts for 90% or more of the total hemoglobin, with varying levels of HbF.

The differential diagnosis of microcytic anemia in the Southeast Asian population initially requires the exclusion of iron deficiency. When present, electrophoresis will identify HbE. However, its level may be diminished in the presence of α-thalassemia or iron deficiency because the affinity of normal β chains for α-globin exceeds that of β^E chains. Interaction of α-thalassemia mutations with the β^E gene is usually seen because of the high incidence of each in the Southeast Asian population.[842]

PRENATAL DIAGNOSIS OF THALASSEMIA

The morbidity and mortality associated with severe forms of thalassemia prompted efforts to develop effective prenatal diagnosis more than 2 decades ago. For the vast majority of β-thalassemias, for which point mutations are usually responsible, early efforts focused on determination of globin chain synthesis in fetal blood cells obtained by aspiration of placental vessels or direct visualization of fetal vessels.[852-856] The risk associated with fetal blood sampling at 18 to 20 weeks' gestation proved to be acceptably low when performed by experienced personnel (fetal loss rate of ≈3%; error rate of <0.5%) such that between 1975 and 1985, more than 7900 pregnancies were studied.[855] As molecular methods and the knowledge of mutations leading to thalassemia improved, strategies for prenatal detection of these conditions evolved.

Successful prenatal diagnosis of α-thalassemia of the hydrops fetalis variety by solution hybridization methods to detect deficiency of α-globin genes in amniotic fluid cell DNA was first reported by Kan and colleagues[856] in 1975. Southern blot analysis rapidly supplanted this approach for the detection of gene deletion in either the α- or β-thalassemias.[857,858] Detection of mutations in DNA rapidly became the preferred strategy for prenatal diagnosis as mutations became defined in the β-thalassemias.[3] The introduction of polymerase chain reaction methods further facilitated the detection of mutations and also permitted the use of nonradioactive tests.[859] Coupled with chorionic villus biopsy, accurate and safe diagnosis can be accomplished within the first trimester of pregnancy. More recently, noninvasive methods consisting of examination of fetal erythrocytes in maternal plasma[860] and analysis of nucleic acids in maternal plasma[861] have provided promise for detection of severe thalassemia

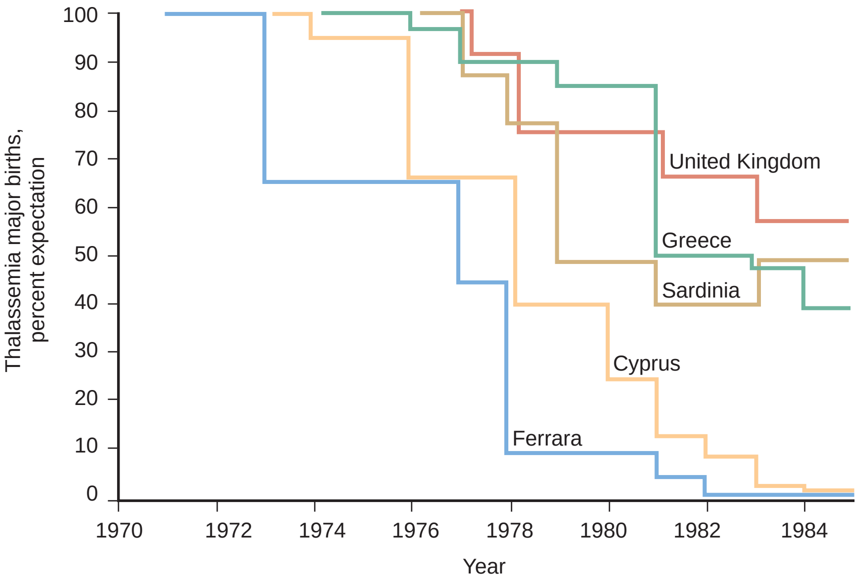

Figure 21-32 A decrease in the birth rate of infants with thalassemia major in Great Britain and several Mediterranean regions after the introduction of effective prenatal diagnosis. *(Modified from Modell B, Bulyzhenkov V: Distribution and control of some genetic disorders.* World Health Stat Q *41:209–218, 1988.)*

syndromes, without risk to the fetus. The recent development of such noninvasive prenatal genetic testing approaches, enabled by newer sequencing approaches, holds promise for their implementation for prenatal diagnosis of severe thalassemia syndromes.[862,863]

Besides molecular biology, prenatal diagnosis of thalassemia has relied on identification of couples at risk, widespread public education, and genetic counseling. In countries in which the incidence of β-thalassemia is high and the burden of disease to the overall population great, such as on the island of Sardinia and in Greece, intensive prevention programs have been established and have proved to be extraordinarily successful. For example, the birth of children with β-thalassemia in these areas has been reduced by more than 90% in recent years[864-869] (Fig. 21-32). These results represent major achievements in the prevention of genetic disease and paradigms for other disorders.[†]

[†] References 304, 311, 339, and 870-885.

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CHAPTER 22

Phagocyte System and Disorders of Granulopoiesis and Granulocyte Function

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CHAPTER OUTLINE

DEFINITION AND CLASSIFICATION OF PHAGOCYTES
PHAGOCYTE DISTRIBUTION AND STRUCTURE
Regulation of Myelopoiesis
Granulocytes
Mononuclear Phagocytes
FUNCTION OF PHAGOCYTES
Humoral Mediators of the Inflammatory Response
Adhesion and Migration into Tissues
Recognition, Opsonization, and Phagocytosis
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QUANTITATIVE GRANULOCYTE AND MONONUCLEAR PHAGOCYTE DISORDERS
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DISORDERS OF GRANULOCYTE AND MONONUCLEAR PHAGOCYTE FUNCTION
Disorders of Adhesion
Disorders of Chemotaxis
Disorders of Opsonization and Ingestion
Disorders of Neutrophil Granules
Disorders of Oxidative Metabolism
Disorders of Cytokines and Impaired Phagocyte Function
Evaluation of Patients with Recurrent Infections

DEFINITION AND CLASSIFICATION OF PHAGOCYTES

Phagocytic leukocytes are bone marrow-derived cells that have the capacity to engulf and digest particulate matter. Phagocytes are essential for the host response to infection and injury and are equipped with specialized machinery enabling them to seek out, ingest, and kill microorganisms. Other functions include the synthesis and secretion of cytokines, pyrogens, and other inflammatory mediators, as well as the digestion of senescent cells and debris. These functions are important for resolution of injury and wound repair as well as linking innate to adaptive immunity.

The phagocyte system has two principal limbs: granulocytes (neutrophils, eosinophils, and basophils) and mononuclear phagocytes (monocytes and tissue macrophages). Both limbs participate in innate immunity and initiation of acquired immune responses. Neutrophils, the rapid effector cells of the innate immune system, circulate in the blood stream until encountering specific chemotactic signals that promote adhesion to the vascular endothelium, diapedesis into tissues, and migration to sites of microbial invasion or tissue injury. Mononuclear phagocytes also function as resident cells in certain tissues, such as lung, liver, spleen, and peritoneum, where they perform a surveillance role. This chapter is divided into three major sections. The first describes the normal distribution, structure, and function of granulocytes and mononuclear phagocytes. The second section reviews the clinical disorders associated with deficient or excessive phagocytic number. The third section focuses on disorders of phagocyte function, including both intrinsic phagocyte defects and conditions secondary to other disease processes.

PHAGOCYTE DISTRIBUTION AND STRUCTURE

Regulation of Myelopoiesis

Granulocytes and monocytes are produced in the bone marrow in a complex, highly regulated, and dynamic process that requires both specific hematopoietic growth

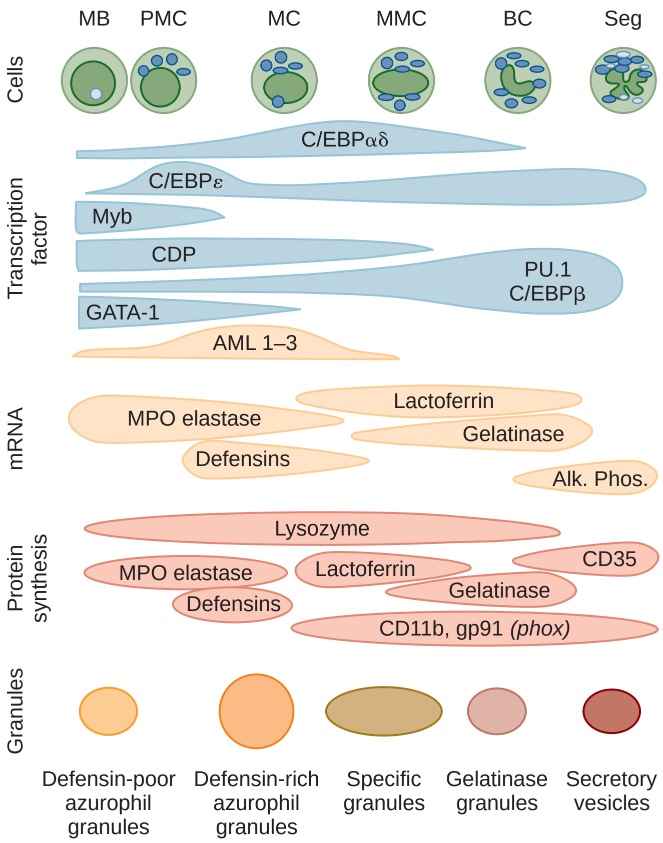


Figure 22-1 Neutrophil maturation. Neutrophil maturation and control of granule protein biosynthesis are achieved by the sequential and combined action of specific transcription factors, including PU.1, the C/EBP, and AML families, CDP, Myb, and GATA-1. The granules formed at any given stage of maturation will be composed of the granule proteins synthesized at that time. The different subsets of neutrophil granules are the result of differences in the biosynthetic windows during maturation and not the result of specific sorting between individual granule subsets. When the formation of granules ceases, it is believed that secretory vesicles will form. It cannot be ruled out that posttranscriptional control occurs, so biosynthesis of proteins may not always be a precise reflection of the corresponding mRNA levels. *AML,* Acute myelogenous leukemia; *BC,* band cell; *CDP,* CCAAT displacement protein; *C/EBP,* CCAAT/enhancer-binding protein; *MB,* myeloblast; *MC,* myelocyte; *MMC,* metamyelocyte; *MPO,* myeloperoxidase; *PMC,* promyelocyte; *Seg,* segmented cell (mature neutrophil). *(Modified from Borregaard N, Cowland JB: Granules of the human neutrophilic polymorphonuclear leukocyte.* Blood *89:3503–3521, 1997.)*

factors and an appropriate bone marrow microenvironment.[1] As reviewed in Chapter 6, multipotent, self-renewing hematopoietic stem cells (HSCs) give rise to lineage-restricted progenitor cells that divide and further differentiate in the bone marrow before their release into the intravascular compartment. Transcription factors of the PU.1 and CCAAT/enhancer binding protein (C/EBP) families play prominent roles in normal myelopoiesis (Fig. 22-1).[2] PU.1 is important for the development of early myeloid precursors and is absolutely essential for subsequent differentiation of the monocyte/macrophage lineage.[3,4] Early steps in the differentiation of granulocytes are dependent upon C/EBPα, whereas C/EBPε activity is required for terminal maturation beyond the metamyelocyte stage.[4] Cytokines that promote the proliferation and differentiation of neutrophils and monocytes from primitive precursor cells include interleukin (IL)-3, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage CSF (M-CSF), and granulocyte CSF (G-CSF).[5-7] The latter two cytokines are relatively specific for the monocyte and neutrophil lineages, respectively. When apoptotic neutrophils are taken up by macrophages in tissues, they deliver a negative feed back via T-helper type (Th17) lymphocytes that results in reduction of G-CSF production.[8,9] During infections, activated macrophages release cytokines such as IL-1, IL-6, and tumor necrosis factor (TNF) that activate stromal cells and T lymphocytes to produce additional amounts of CSFs and increase the production of myeloid cells. This is termed *emergency granulopoiesis* and in mice has been shown to depend on G-CSF activation of *Stat3* and on C/EBPβ expression in granulocyte monocyte precursors (GMPs). However, in vivo feedback is impaired in *Tlr4–/–* and *Trif–/–*, but not *MyD88–/–* animals, thereby directly linking TLR-triggering to granulopoiesis.[10-13] IL-5[14,15] and IL-3 are the principal cytokines inducing human basophil growth and differentiation.[16] In addition to their regulatory role in hematopoiesis, hematopoietic growth factors can act on mature myeloid cells and stimulate their functional activities and survival.[17,18]

Myeloid differentiate also appears to be modulated by retinoic acid receptors and by transcriptional repressors such as Gfi-1.[19-22] The participation of retinoic acid in myeloid development was originally surmised from its ability to induce differentiation of myeloid leukemia cell lines and leukemic promyelocytes in patients with acute promyelocytic leukemia,[23] as discussed in Chapter 11.

Micro–ribonucleic acids (miRs) such as miR21, miR29a, miR125, miR130a, miR146, miR155, miR196b, and miR223 participate at several levels of myelopoiesis and in control of lineage decisions in the bone marrow.[24-27] MiRs are expressed at different stages during myelopoiesis, and several clusters can be identified depending on their expression profile during granulopoiesis.[28] Gfi-1 is a key repressor of miR21 and miR196b expression. Expression of these miRs promote monocytic differentiation and block granulocytic differentiation.[26] In addition to the well-known repression of translation, miRs can also regulate transcription by controlling the accessibility of promoters by epigenetic modifications as demonstrated on the nuclear factor IA promoter, which is silenced by miR223, allowing for terminal granulocytic differentiation.[28,29]

Granulocytes

Neutrophils

The neutrophil life span is traditionally divided into the bone marrow, circulating, and tissue phases. Approximately 14 days are spent in the bone marrow, where proliferation and the early stages of neutrophil differentiation are followed by the final stages of maturation and retention in a large, nonmitotic storage pool that is many times larger than the circulating and tissue neutrophil populations (Table 22-1).[30-33] Release is regulated by

TABLE 22-1 Neutrophil and Monocyte Kinetics

	Transit Time Range (hr)	**Total Cells ($\times 10^9$/kg)**
Neutrophils		
Marrow Mitotic Compartment		
Myeloblast	23	0.14
Promyelocyte	26-78	0.51
Myelocyte	17-126	1.95
Postmitotic Marrow Maturation and Storage Compartment		
Metamyelocyte	8-108	2.7
Band	12-96	3.6
Neutrophil	0-120	*2.5*
Total storage		8.8
Vascular Compartment		
Circulating neutrophils	4-10	0.3
Marginated neutrophils	4-10	*0.4*
Total blood neutrophils		0.7
Tissue compartments	0-3 days (?)	Not known
Neutrophil turnover rate	1.6×10^9/kg/day	
Monocytes		
Marrow mitotic compartment: promonocyte	≈160	0.006
Postmitotic marrow compartment: monocyte	24	0.10
Vascular compartment	36-104	0.024
Tissue compartment	Days-months	Not known
Monocyte turnover rate	1×10^8/kg/day	

chemokines expressed on the cells, and their ligands are expressed by stroma cells. *CXCR4* and its ligand *CXCL12* retain cells,[34] and mutations in the CXCR4 receptor account for the warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome, an inherited neutropenia.[35,36] Whereas *CXCR2* and its ligands *CXCL1* and *CXCL2* promote neutrophil release. Also, the integrin $\alpha_4\beta_1$, known as very late antigen 4 (VLA-4) may tether bone marrow neutrophils to vascular cell adhesion molecule 1 (VCAM-1) expressed on bone marrow stromal cells.[37-40] Once released into the bloodstream, neutrophils have an estimated half-life of 6 to 10 hours and move between circulating and marginated pools in a reversible fashion. These estimates are based on several independent determinations using both in-vivo and in-vitro labeling techniques and transfusions, and they agree well with the estimates of neutrophils production rates of 10^9cells/kg/day and circulating neutrophil counts 2.5 to 4 × 10^9/L.[41] A recent controversial report suggests a half-life of 4 to 5 days both in humans and mice.[42] Neutrophils exit circulation by diapedesis between or through endothelial cells into tissue sites of infection or inflammation.[43-46] Once in the tissues, neutrophils are believed to live for another 1 to 2 days before undergoing apoptosis and engulfment by macrophages[47,48] or they form neutrophil extracellular traps (NETs) consisting of deoxyribonucleic acid (DNA) and antibiotic proteins from nucleus, granules, and cytosol in a regulated process called *netosis* that traps microorganisms.[49,50] This is discussed in more detail later in this chapter.

Myeloblasts are the earliest morphologically recognizable granulocyte precursors in the marrow and are identified by their relatively undifferentiated appearance with a large, oval nucleus, several prominent nucleoli, and few or no granules in a gray-blue cytoplasm in Wright-stained preparations. This stage of neutrophil differentiation is followed by the promyelocyte and myelocyte stages, which are distinguished by the appearance of distinct neutrophil granule populations (Table 22-2). Azurophilic, or primary, granules are formed during the promyelocyte stage and contain myeloperoxidase (MPO), bactericidal peptides, and lysosomal enzymes. The subsequent myelocyte stage is distinguished by the formation of peroxidase-negative specific, or secondary, granules containing lactoferrin. No further cell divisions occur after the myelocyte stage. The metamyelocyte, band, and mature neutrophil exhibit progressive nuclear condensation, accumulation of glycogen, and accumulation of tertiary, gelatinase-rich granules and secretory vesicles that are endocytic vesicles marked by albumin and Complement Receptor 1 (CR1) (CD35).[49] These neutrophil precursors can be identified and isolated by flow cytometry based on their surface antigen profile and forward and side scatter.[51]

In Wright-stained blood smears, the mature neutrophil is 10 to 15 mm in size with a multilobed, polymorphic nucleus that has highly condensed chromatin and a yellow-pink cytoplasm containing numerous granules as well as clumps of glycogen. The mean lobe count is usually slightly less than three. Circulating neutrophils appear round with some cytoplasmic projections and surface ruffling. The morphologic changes seen with neutrophil differentiation are accompanied by temporally coordinated changes in gene expression and protein synthesis (see Fig. 22-1). Transcription and translation of messenger RNAs (mRNAs) for MPO and cathepsin G, which are both primary granule constituents, are restricted to myeloblasts and promyelocytes.[52,53] In contrast, expression of the secondary granule proteins such as lactoferrin and transcobalamin I occurs in myelocytes and metamyelocytes.[53,54] Gelatinase expression occurs even later in maturation and is first detected in bands and bone marrow neutrophils.[55,56] The leukocyte β-integrin subunit CD11b is first detectable in myelocytes, and increases throughout the later stages of neutrophil differentiation. The $gp91^{phox}$ subunit of the respiratory burst oxidase complex is expressed relatively late in neutrophil maturation,[57] consistent with the observation that respiratory burst activity is not detected until the metamyelocyte stage.

The mature neutrophil, previously thought of as an "end-stage" cell, retains the capacity for inducible gene expression and protein synthesis even after release from the marrow cavity.[58-62] Diapedesis and exposure to cytokines induce neutrophil expression of mRNA transcripts for IL-1, IL-6, TNF-α, GM-CSF, M-CSF, and IL-8, which may promote recruitment and activation of both phagocyte and lymphocyte populations in the inflammatory response.[1,61]

TABLE 22-2 Content of Human Neutrophil Granules and Secretory Organelles

Primary: Azurophil Granules	Secondary: Specific Granules	Tertiary: Gelatinase Granules	Secretory Vesicles
Membrane			
CD63 CD68 Presenilin-1 Stomatin V-type H$^+$-ATPase	CD11b/CD18 (Mac-1) CD15 antigens (Lewis X) CD66 CD67 Cytochrome b_{558} Formyl peptide receptor Fibronectin receptor G protein α subunit Laminin receptor NB-1 antigen (CD177) Rap1, Rap2 SCAMP SNAP-23, -25 Stomatin Thrombospondin receptor Tumor necrosis factor receptor Urokinase-type plasminogen activator (uPA) receptor VAMP-2 Vitronectin	CD11b/CD18 (Mac-1) CD67 Cytochrome b_{558} Diacylglycerol-deacylating enzyme Formyl peptide receptor Leukolysin (MMP-25) NRAMP-1 SCAMP SNAP-23, -25 Tumor necrosis factor receptor Urokinase-type plasminogen activator (uPA) receptor V-type H$^+$-ATPase VAMP-2	Alkaline phosphatase CD10 CD11b/CD18 (Mac-1) CD13 CD14 CD16 (FcγIIIR) CD35 (complement receptor type 1) CD45 CD67 Chemokine receptors Cytochrome b_{558} Decay-accelerating factor Ig (G, A, E) FcR Formyl peptide receptor Leukolysin (MMP-25) SNAP-23, -25 Tumor necrosis factor receptor V-type H$^+$-ATPase VAMP-2
Matrix			
Acid β glycerophosphatase Acid mucopolysaccharide α_1 Antitrypsin α Mannosidase Azurocidin/CAP37/heparin-binding protein Bactericidal/permeability-increasing protein (BPI) β Glycerophosphatase β Glucuronidase Cathepsins Defensins Elastase Lysozyme Myeloperoxidase *N*-acetyl-β-glucosaminidase Proteinase 3 Sialidase Ubiquitin-protein conjugate	β_2 Microglobulin Collagenase (MMP-8) Gelatinase (MMP-9) hCAP-18 Heparanase Histaminase Lactoferrin Lipocalin (NGAL) Lysozyme Plasminogen activator Vitamin B$_{12}$–binding protein (transcobalamin I)	Acetyltransferase β_2 Microglobulin Gelatinase (MMP-9) Lysozyme	Plasma proteins

Modified from Borregaard N, Cowland JB: Granules of the human polymorphonuclear leukocyte. Blood *10:3503–3521, 1997.*

Abnormalities in Neutrophil Morphology. Upon neutrophil activation by inflammatory signals, granule fusion can result in vacuolization and toxic granulation (prominent azurophilic granules). These morphological changes reflect a nonspecific response to inflammation and do not necessarily indicate the presence of bacterial infection. Large azurophilic granules but normal specific granules are seen in the Chédiak-Higashi[63] and the Alder-Reilly anomalies (both autosomal recessive [AR] traits), but neutrophil function does not appear to be affected in the latter. Döhle bodies can be seen in normal neutrophils at times of infection. These inclusions represent strands of rough endoplasmic reticulum that are retained from a more immature stage and stain bluish because of their high content of RNA and ribosomes. Döhle bodies in granulocytes and monocytes, in combination with leukopenia, giant platelets, and variable thrombocytopenia, characterize the May-Hegglin anomaly. This autosomal-dominant syndrome, like the similar Fechtner and Sebastian syndromes, is caused by mutations in the gene encoding non–muscle myosin heavy chain 9.[64]

Neutrophil hypersegmentation can be a sign of vitamin B$_{12}$ or folate deficiency. Hypersegmentation is also reported in small subset of neutrophils in the circulation that have reduced expression of L-selectin (CD62L).[65,66] These may represent a more mature subset approaching senescence and also are reported to be capable of suppressing T-cell activation during inflammation. Hypersegmented neutrophils with a mean of four lobes also occur as a rare autosomal dominant (AD) trait that is not associated with disease. Nuclear hyposegmentation is seen in Pelger-Huët anomaly, an AD trait caused by mutations in the gene encoding the lamin B receptor, an integral protein of the nuclear envelope.[67] Typically the nucleus is bilobed (often described as *pince-nez*) but has mature, coarse, and densely clumped chromatin. The nucleus remains round in the rare homozygote. Pelger-Huët anomaly must be distinguished from neutrophil band forms and from the acquired or "pseudo" Pelger-Huët form that can be seen with myeloproliferative disorders. Bilobed neutrophil nuclei are also seen in a rare functional disorder of neutrophil maturation, specific granule deficiency (SGD). In this disorder, the pink-staining specific granules are absent in peripheral blood neutrophils. Giant granules representing defective membrane targeting of proteins in secretory lysosomes are seen in Chédiak-Higashi syndrome (CHS) neutrophils, most prominently in the bone marrow. SGD and CHS are discussed in more detail later in this chapter.

Neutrophil Granule Biosynthesis and Classification. The numerous intracellular granules and vesicles in the neutrophil cytoplasm function as storage pools for cell surface receptors and as reservoirs of sequestered digestive and microbicidal proteins. Many compounds are multifunctional. For example, cathepsin G defensins and azurocidin are both antimicrobial and chemotactic for monocytes and T cells, which helps amplify the inflammatory response and link innate to adaptive immunity. The older classification of granules as either peroxidase-positive (azurophilic or primary) and peroxidase-negative (specific or secondary) has proven to be too simplistic.[51,60,61,68] Instead, neutrophil granules are a continuum from the earliest appearing azurophil granules formed in immature promyelocytes to gelatinase granules formed in band cells. The content of granules reflects the transcriptional profile during terminal neutrophil differentiation in the marrow.[1,69] A current classification of neutrophil granules is shown in Table 22-2, which summarizes the composition of their membranes and luminal (matrix) contents.

Azurophilic (primary) granules are defined histochemically by the presence of MPO, an enzyme in the oxygen-dependent killing pathway. This green heme enzyme lends it color to collections of mature neutrophils (pus) or myeloid leukemia cells in the bone marrow or extramedullary tumors ("chloromas"). Azurophilic granules also contain peptides and proteins that participate in oxygen-independent killing of microbes. Other components of the azurophilic granule matrix include neutral serine proteases and other digestive enzymes characteristic of lysosomes.

Specific (secondary) granules, which are uniquely found in neutrophils, are classically identified by their content of lactoferrin, an iron-binding protein that also has direct bactericidal activity. Specific granules also contain additional antibiotic substances, including lysozyme, lipocalin 2 (also known as neutrophil gelatinase–associated lipocalin; NGAL), a bacterial siderophore-binding protein, and the metalloproteases collagenase and gelatinase. The membrane of the secondary granules contains a major proportion of the neutrophil's supply of flavocytochrome *b558*, the electron carrier of the respiratory burst oxidase.[70,71] Specific granule membranes also contain a pool of receptors for adhesive proteins, TNF, and chemotactic formyl peptides.

Although specific granules contain collagenase and some gelatinase, most of the neutrophil's store of gelatinase is localized to the matrix of gelatinase (tertiary) granules, which also contain the membrane-associated metalloproteinase leukolysin (MMP-25). Tertiary granules are formed relatively late in neutrophil differentiation and are smaller and more easily mobilized for exocytosis than secondary granules. Secretory vesicles are formed in bands and mature neutrophils by endocytosis of the plasma membrane and serve as an important store of leukolysin as well as the adhesive protein Mac 1 (CD11b/CD18) and many other membrane receptors (see Table 22-2). Proteomics has permitted a more global view on neutrophil granule proteins.[72-74]

Neutrophil Cell Surface Receptors. A primary function of the mature neutrophil is to move rapidly into tissue sites to destroy invading microbes and clear inflammatory debris. To respond to inflammatory stimuli, the neutrophil is equipped with an array of cell surface receptors for adhesive ligands, chemoattractants, and cytokines that can be divided into groups based on their structure and the major intracellular signaling pathway to which they are linked (Table 22-3).[75] Many of these surface proteins are pattern-recognition molecules such as Toll-like receptors (TLRs) and formyl peptide receptors,[76-80] reflecting the neutrophil's role in the innate immune response. Microdomains in the plasma membrane also known as lipid rafts, which are enriched in cholesterol, glycosphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins, and CD11b/CD18, can function as pattern recognition structures by virtue of their glycosphingolipids such as lactosylceramide (CDw17), the lipopolysaccharide (LPS)-binding GPI-anchored protein CD14 and the carbohydrate-binding domain of CD11b. These aggregates can then signal intracellularly via the associated integrins but likely also via lipid tail interactions of CDw17 and the tyrosine kinase Lyn attached to the inside of the membrane via its lipid-tail.[81]

The signal transduction cascades triggered upon ligand binding to neutrophil receptors are complex and probably redundant.[82-88] TLRs recognize structures specific for microorganisms, the pathogen-associated molecular patterns (PAMPs) such as LPS, lipoteichoic acid, and flagellin,[89] but they also host molecules associated with cellular stress and injury, the damage-associated molecular patterns (DAMPs) such as heat-shock protein 60 and DNA.[76] A common early event downstream of neutrophil receptor binding is activation of phospholipase C (PLC), which hydrolyzes the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two important second messengers, diacylglycerol and inositol 1,4,5-triphosphate (IP_3), which in turn cause release of calcium from intracellular stores and activate protein kinase C. Changes in intracellular calcium concentration are important for neutrophil degranulation and secretion and for phagolysosome fusion during phagocytosis.[85] Activation of phosphoinositide 3′-kinase (PI3K) is another common early event that catalyzes the phosphorylation of PIP_2 to generate a third important lipid messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP_3).[90] Neutrophil activation is also accompanied by alterations in the phosphorylation status of intracellular proteins, as

TABLE 22-3 Receptors in Neutrophils

Receptor Grouping	Examples	Structural Characteristics
G-protein linked	fMLP, C5a, PAF, LTB_4, IL-8, chemokines	Seven-transmembrane–spanning domains (serpentine); linked to heterotrimeric GTP-binding proteins
Membrane tyrosine kinases	PDGF	Integral membrane protein, intrinsic tyrosine kinase activity; ligation leads to receptor dimerization and cross (“auto”) phosphorylation
Tyrosine kinase linked	FcγRIIa, GM-CSF	FcγRII is a member of the immunoglobulin family of receptors The GM-CSF receptor is an 84-kD transmembrane protein related to receptors for IL-2 and IL-6 Ligation of receptor activates cytosolic tyrosine kinases
GPI linked	FcγRIIIb, DAF, CD14	Receptors with no transmembrane or intracellular domains. May associate with a partner receptor to mediate signal transduction
Adhesion molecules	β_2 Integrins L-selectin	β Integrins are heterodimers with relatively long cytoplasmic tails L-selectin has an extracellular lectin-binding domain and a very short cytoplasmic tail Ligation results in potentiation of the oxidative burst and phagocytosis in adherent cells, calcium signaling, actin cytoskeletal changes, and upregulation of gene expression
Ceramide linked	TNF	Single-membrane-spanning glycoproteins; ligation activates membrane-bound sphingomyelinase with generation of ceramide, which in turn activates a 96-kD protein kinase

Modified from Downey GP, Fukushima T, Fialkow L: Signaling mechanisms in human neutrophils. Curr Opin Hematol *2:76–88, 1995.*
DAF, Decay-accelerating factor; *FcγR,* Immunoglobulin G Fc receptor; *fMLP,* N-formyl-methionyl-leucyl-phenylalanine (formyl peptide); *GM-CSF,* granulocyte-macrophage colony-stimulating factor; *GPI,* glycosylphosphatidylinositol; *GTP,* guanosine triphosphate; *IL,* interleukin; *LTB_4,* leukotriene B_4; *PAF,* platelet-activating factor; *PDGF,* platelet-derived growth factor.

regulated by protein kinase C, tyrosine kinases and phosphatases, and serine/threonine kinases of the mitogen-activated protein (MAP) kinase family.[88,91,92] Guanine nucleotide binding proteins play important roles in neutrophil signal transduction. These include the heterotrimeric guanosine triphosphate (GTP)-binding proteins that are coupled to the seven transmembrane-spanning domain (serpentine or heptahelical) receptors for chemokines and other chemoattractants[93-95] and the low molecular weight guanosine triphosphatases (GTPases) of the Ras superfamily.[82] The latter category includes p21Ras itself, which can be activated via chemoattractant receptors,[96] and the Rho family GTPases Rho, Rac, and Cdc42, which are involved in the regulation of many neutrophil responses, including adhesion, the respiratory burst–reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and actin remodeling during migration and phagocytosis.[82] A dominant-negative form of the Rac2 GTPase has been identified in an infant with recurrent deep-seated bacterial infections and leads to multiple defects in phagocyte function.[97-99]

Signaling through these receptors is subject to positive and negative modulation. Triggering receptors expressed on myeloid cells (TREMs), of which TREM-1 is expressed on neutrophils, potentiate the signaling through TLRs. TREMs signal through an intracellular adaptor protein, DNAx activating protein (DAP) of 12 kD, DAP12.[100-103] DAPs contain an immunoreceptor tyrosine–based activation motif (ITAM), which when phosphorylated, actives the protein tyrosine kinase Syk, which activates PI3K and phospholipase Cγ (PLCγ) via Bruton tyrosine kinase and thus potentiates the signals generated through TLRs, chemokine receptors, and integrins.[103]

Neutrophils express high levels of G-CSF and GM-CSF receptors. These receptors signal through Janus kinases (JAKs),[104] which dock to phosphorylated tyrosine residues in the cytoplasmic tail of the receptors, and from there phosphorylate and activate STAT-3 and -5 that induce transcription of several genes, in particular cytokines. The signaling through receptors activated via tyrosine phosphorylation is modulated by two different mechanisms, suppressors of cytokine signaling (SOCS) and immunoreceptor tyrosine–based inhibitory motifs (ITIMs). SOCS-3 is induced by activated STAT-3 and binds to and blocks phosphotyrosines in the activated growth factor receptor.[105,106] In contrast, activation of receptors such as signal inhibitory receptor on leukocytes-1 and CD300a that contain an ITIM recruits phosphotyrosine phosphatases to dephosphorylate the activated cytokine receptors and reduce signaling.[107-110]

Neutrophil Subsets. Circulating neutrophils are traditionally viewed as one homogenous population. However, it is known that two subsets can be identified based on the presence or absence of CD177. The fraction of neutrophils that express this antigen varies from 0 to 100 amongst individuals but is stable through life.[111] CD177 is the surface receptor for proteinase 3, the antigen associated with Wegener granulomatosis, and signals generated via CD177 are believed to play a major role in this disease.[112] As mentioned, a subpopulation of hypersegmented neutrophils that also have reduced L-selectin expression has been reported, which may indicate a more mature neutrophil population with T-cell suppressive activity.[65,66] Recently, a specific granule protein with antibacterial properties, OLFM4 was found to be expressed in 25% of human granulocytes with wide but constant variation among individuals.[113]

Eosinophils

Like the neutrophil, the eosinophil is compartmentalized in the bone marrow into mitotic and storage pools; these usually constitute no more than 0.3% of the nucleated bone marrow cells. Eosinophils arise from a progenitor cell, the CFC-Eo, that is committed at a relatively early stage to differentiate into eosinophils instead of neutrophils and monocytes in the bone marrow. GATA-1, PU.1, and C/EBPs play critical roles in the transcriptional regulation of eosinophil lineage commitment and differentiation.[114-116] Morphologic differentiation and maturation of the eosinophil parallel that of the neutrophil series, and its characteristic eosin-staining–specific granules are prominent by the myelocyte stage. IL-3, IL-5, and GM-CSF mediate eosinophil production in the marrow; IL-5, IL-13, chemokines (such as eotaxins [CCL11] regulated on activation, normal T cell expressed and secreted [RANTES; i.e., CCL5]), and leukotrienes [LTB4] play key roles in regulating eosinophil differentiation, chemotaxis, and functional activation.[14,15,117-119]

After leaving the circulation, the majority of mature eosinophils reside in tissues, with a blood-to-tissue ratio estimated to be 1:300 to 1:500. The life span of tissue eosinophils is not known but may be several weeks, whereas the half-life in blood is around 24 hours.[120] Eosinophils typically localize in areas exposed to the external environment, such as the tracheobronchial tree, gastrointestinal (GI) tract, mammary glands, and vagina and cervix. As discussed later, eosinophils have both immunoenhancing and immunosuppressive functions and play a role in helminthic infection, allergy, and the responses to certain tumors.[114-116,119,121-123]

The mature eosinophil is slightly larger than the neutrophil, with a diameter of 12 to 17 μm. The nucleus is characteristically bilobed, although multiple lobes can be seen in patients with eosinophilia of diverse causes. The cytoplasm has prominent and morphologically distinctive granules that stain strongly with acid aniline dyes because of their high content of basic proteins.

Like the neutrophil, the mature eosinophil is endowed with the capacity for chemotaxis, phagocytosis, degranulation, and the synthesis of reactive oxidants and arachidonate metabolites.[14,124,125] Eosinophils may also undergo a process similar to neutrophil netosis.[126-128] Eosinophil cell surface membranes express a wide variety of molecules, including receptors for immunoglobulins and members of the immunoglobulin superfamily; cytokine receptors; adhesion molecules; chemokine, complement, and other chemotactic receptors; and major histocompatibility complex (MHC) class I and II and costimulatory molecules.[14,124]

The distinctive mature eosinophil granules are membrane-bound organelles, 0.15 to 1.5 μm in length and 0.3 to 1 μm in width that contain a variety of enzymes and cytotoxic proteins.[14,124] These eosin-staining specific granules are large ovoid bodies that contain an electron-dense crystalloid core surrounded by a less dense matrix. The eosinophil major basic protein (MPB) makes up about 50% of the dense crystalloid core of the eosinophilic specific granule.[129] MBP from eosinophils induces histamine release from basophils and mast cells[130] and also an autocrine degranulation of eosinophils.[116] Release of proteins from these large granules is different from the degranulation process of neutrophils, where intact granules fuse with the surface membrane and empty their entire content and the membrane of granules is incorporated into the surface membrane. Instead, because the eosinophil is active in tissues, its granules swell and a tubulovesicular system extends from the granules to the surface permitting piece meal degranulation (PMD), or the graded release of granule content.[129,131,132] Eosinophil peroxidase plays an important role in the antihelmintic function of eosinophils[133,134] and utilizes bromate to generate hypobromous acid from hydrogen peroxide.[135,136]

Much less common than eosinophil-specific granules are the primary granules characterized by their content of a lysophospholipase, the Charcot-Leyden crystal (CLC) protein that can polymerize to form the bipyramidal hexagons that are CLCs.[14,124] CLCs are typically found in areas of eosinophil degeneration, such as sputum from asthmatic patients, nasal mucous of patients with allergies, stools of patients with parasitic infections, and the pleural fluid of patients with pulmonary eosinophilic infiltrates. The CLC lysophospholipase[137] catalyzes the hydrolysis and inactivation of lysophospholipids generated by phospholipase A_2, thus preventing the generation of proinflammatory arachidonic acid metabolites. The CLC protein composes about 5% of the total protein in eosinophils.

Slightly more numerous but distinctly smaller than primary granules are the small type granules whose content is not well characterized and secretory vesicles, which like secretory vesicles of neutrophils contain the NADPH oxidase flavocytochrome *b558*, and albumin, indicating an origin as endocytic vesicles; however, in eosinophils these may also be part of the tubulovesicular system of PMD.[138]

Eosinophil granule products, particularly MBP, eosinophil peroxidase, and eosinophil neurotoxin, are toxic to tissues, including the heart, lungs, and brain.[14,124] These mediate many of the adverse clinical complications of eosinophilia and hypereosinophilic syndrome (HES), such as Löffler endocarditis and pneumonia.[139,140]

Basophils and Mast Cells

Basophils and mast cells are believed to share a common progenitor cell,[141-146] but mast cells leave the bone marrow to proliferate and mature in tissues, whereas full differentiation of basophils occurs in the bone marrow over 7 days before their release into the bloodstream; they are not normally found in the connective tissues.[147] Basophils account for approximately 0.5% of the total circulating leukocytes and 0.3% of nucleated marrow cells.[148] Mature basophils have a bilobed nucleus. Although basophils are distinctly smaller (5 to 8 μm) than mast cells (20 μm), they both contain large metachromatic granules that stain purple or bluish with Wright stain because of their high content of sulfated glycosaminoglycans. These granules are rich in heparin-type and chondroitin sulfate-type glucosaminoglycans linked to the serglycin protein backbone that is responsible for packing the cationic proteins, histamine, and kallikrein.[149-154] Mast cells and basophils express high affinity receptor for the Fc portion of immunoglobulin E (IgE), which is an important trigger for release of granule contents and production of arachidonic acid metabolites in anaphylactic degranulation on their plasma membrane,[155] and are key effector cells in certain hypersensitivity reactions.[141,149,150,156] However, mast cells lack receptors for IL-2, IL-3, and CD11b/CD18 that are present on basophils.[16] The heparin of basophils appears to have poor anticoagulant activity. Basophil granules also contain small amounts of MBP as well as serine proteases. Basophils synthesize and secrete IL-4 and IL-13 and may thus mediate a link between the innate and adaptive immune systems for generation of Th2 lymphocyte responses.[150,157] Murine mast cells can secrete a wide variety of mitogenic or inflammatory cytokines (including IL-1, IL-3, IL-4, IL-5, and IL-6), chemokines, GM-CSF, and TNF-α, that are likely to play an important role in leukocyte recruitment and inflammation.[158,159]

Mast cells are ordinarily distributed throughout normal connective tissue, where they are often situated adjacent to blood and lymphatic vessels, near or within nerve sheaths, and beneath epithelial surfaces that are exposed to environmental antigens such as the respiratory and GI tracts.[156,158] The c-kit receptor for stem-cell factor is present on mast cells, but absent from the majority of basophils.[158,159] The c-kit ligand, or stem-cell factor (SCF), is the main survival and developmental factor for mast cells, but other growth factors and cytokines such as IL-3, Il-4 and IL-9 are also supportive for mast cell development.[158] Mature mast cells do not circulate in the blood, although circulating mast cell progenitors have been described[141] and retain a limited proliferative capacity in the tissue compartment.[160] In contrast to monocytes and macrophages, a transformation between the circulating and tissue forms of basophils and mast cells has not been observed.

Mast cells can be categorized into several types in mice and humans. In mice, the different types can be distinguished by their main tissue distribution and the major type of proteoglycan in their granules (heparin-containing mast cells are predominantly connective tissue and serosal mast cells, whereas chondroitin sulphate-containing mast cells are associated with mucosal surfaces). In humans two major subsets are identified based on the their content of chymase and tryptase. Both contain tryptase, but a subset that is particularly low in chymase largely secretes cytokines and chemokines and does not degranulate as opposed to the chymase rich type.[154,159]

Mononuclear Phagocytes

The blood monocyte is derived from a bone marrow progenitor cell, the GMP, shared with the neutrophil, and undergoes differentiation through stages as monoblasts and promonocytes in the bone marrow. Monocytic differentiation of GMP is favored by a high level of the transcription factor PU.1 relative to C/EBPα, whereas the opposite drives granulocytic differention.[161-163] The transit time for monocytes in the marrow compartment is briefer than for neutrophils, and the mature monocyte is released into the circulation only 24 hours after the last mitosis (see Table 22-1).[164,165] Consequently, a relative monocytosis in peripheral blood commonly precedes the return of granulocytes during recovery from bone marrow aplasia or hypoplasia. Monocyte production and differentiation are regulated by IL-3, IL-6, GM-CSF, and the more lineage-specific cytokine M-CSF.[2,5-7,166]

The monocyte may spend several days in the intravascular compartment in either circulating or marginated pools.[167] Monocytes migrate into tissues and body cavities to participate in inflammatory processes as exudate macrophages and to replenish the resident tissue macrophage population, which has a relatively long life span. In patients receiving allogeneic bone marrow transplants, host tissue macrophages disappear gradually and are replaced by donor macrophages approximately 3 months after transplantation.[168] Recent studies in mice suggest that at least some tissue macrophages are derived from stem cells that migrate to tissues during fetal development and maintain the macrophage pools in situ,[169-171] provided these stem cells are not destroyed as occurs during allogeneic stem-cell transplantation as shown in mouse models.[172]

The circulating monocyte in Wright-stained blood smears is 10 to 18 μm in diameter with a convoluted surface, a grey-blue cytoplasm, and an indented or kidney-shaped, foamy nucleus. However, some monocytes can be as small as 7 μm in diameter and difficult to distinguish morphologically from lymphocytes. Like neutrophils, monocytes contain secretory vesicles[173] but only a single major class of granules with lysosomal characteristics,[174] of which subsets have been described based on content of transforming growth factor α (TGF-α).[175] Traditionally, two circulating populations of monocytes are identified by flow cytometry as either classical monocytes or the nonclassical monocytes. Classical monocytes have a high expression of CD14 (the LPS receptor) and a low expression of CD16 (the low-affinity immunoglobulin G [IgG] receptor), accounting for approximately 90% of circulating monocytes, whereas nonclassical monocytes have a high expression of CD16. An intermediate type, previously assigned to the nonclassical type, is now recognized as having high expression of CD14 and intermediate expression of CD16. These CD16 expressing types are in general seen as possible precursors of dendritic cells. The relative distribution among these subsets is not fixed and changes with exposure to growth factors and cytokines such as M-CSF and TNFα.[176,177]

After leaving the circulation, monocytes become larger and take on the appearance of tissue macrophages characteristic of the organ in which they reside. The macrophage nucleus is typically oval with more prominent nucleoli, and the cytoplasm stains blue because of an increase in RNA content. Monocytes and macrophages are distinguished histochemically by the presence of a fluoride-inhibitable nonspecific esterase and can be identified immunohistologically by a variety of monoclonal antibodies such as F4/80 in the mouse and anti-CD68 in human tissue.[178,179]

Monocytes and macrophages share many structural and functional features with neutrophils, and are capable of sensing chemotactic gradients, migrating to inflamed sites, ingesting microorganisms, and killing them using a variety of cytocidal products. However, compared to neutrophils, mononuclear phagocytes have a large and diverse developmental potential.[176,180,181] In addition to their protective function as phagocytic cells in host defense, mononuclear phagocytes play a central role in the adaptive immune response by presenting antigens to lymphocytes, elaborate growth factors and cytokines important for lymphocyte function, wound repair, and hematopoiesis, and participate in a variety of scavenger and homeostatic pathways.[180,182-184] Mononuclear phagocytes at inflammatory sites become "activated," displaying morphologic alterations and a variety of enhanced functions. These include a more pronounced ruffling of the plasma membrane and pseudopod formation, an increased capacity for adherence and migration to chemotactic factors, increased microbicidal and tumoricidal activity, and enhanced ability to release cytokines.[183-186]

Monocytes and tissue macrophages are considered to make up a "mononuclear-phagocyte system."[167] Resident tissue macrophages were formerly referred to as *histiocytes,* an imprecise and often loosely applied term. Tissue macrophages are widely distributed and perform specialized functions at portals of entry such as the pulmonary alveoli and in sterile sites such as the bone marrow.[180]

Spleen

Macrophages are distributed in all parts of the spleen, including the germinal centers where they are associated with lymphocytes. Splenic macrophages located in the red pulp and sinuses serve a clearance function, where a sluggish blood circulation maximizes the interaction between blood elements and the macrophages lining the sinus walls.[187]

Liver

The portal circulation percolates through a labyrinthine system, the spaces of Disse, before exiting via the hepatic venous system. This hepatic circulation, although less sluggish than that of the spleen, provides considerable contact between the blood and the resident liver macrophages, known as *Kupffer cells,* that reside within these vascular sinuses.

Lymph Nodes

As in the spleen, macrophages exist through all regions of peripheral lymph nodes. They are most abundant in the medullary zone close to efferent lymphatic and blood capillaries. This location is likely related to the important role macrophages play in the presentation of antigens to T lymphocytes.[182,188]

Lungs

Pulmonary macrophages reside both in the interstitium of alveolar sacs and free within the air spaces, where they participate in the clearance of inhaled microorganisms and particulate matter. The number of lung macrophages increases in many chronic pulmonary inflammatory disorders. Pulmonary macrophages are easily seen in lungs of smokers, where black inclusions mark the macrophage vacuoles.[189] Hemosiderin-laden alveolar macrophages can be indicative of recurrent pulmonary hemorrhage, such as in idiopathic hemosiderosis or Goodpasture syndrome. Gastric aspiration to detect ingested iron-laden macrophages is a useful test for these disorders.

Bone Marrow

Macrophages are found throughout the bone marrow cavity. They are particularly abundant within hematopoietic islands and on the walls of the marrow sinuses.[190] Bone marrow macrophages may have a clearance function in normal or pathologic states of ineffective hematopoiesis.[39,63,191] The clearance function of marrow macrophages is dramatically illustrated by the lysosomal storage diseases such as Gaucher disease.[192] Large inclusions build up within marrow macrophages (as well as hepatic and splenic macrophages) because of the inability of these cells to break down lysosomal contents.[193] Bone marrow macrophages also support hematopoiesis by modulating mesenchymal stem cells and osteoblasts to express retention signals, in particular CXCL12, for HSCs.[194-196]

Other Sites

Mononuclear phagocytes associated with lymphoid cells reside throughout the alimentary tract, particularly in the submucosal tissues and small intestinal villi. They are present as microglial cells in the central nervous system (CNS), where their numbers increase after injury as monocytes emigrate across the blood-brain barrier,[170,197] and they may contribute to the pathogenesis of the CNS manifestations of infection with the human immunodeficiency virus (HIV).[198] Mammary gland macrophages released into milk during lactation have been implicated as a potential source of postnatal transmission of the HIV virus.[199]

Dendritic Cells

Dendritic cells are specialized antigen-presenting cells with long cytoplasmic processes that are located in tissues throughout the body except for brain.[200,201] They develop from a progenitor common with monocyte progenitors, supported by GM-CSF. M-CSF induces monocytic differentiation, and Flt3 ligand induces differentiation into a dendritic cell progenitor that may leave the bone marrow and home to lymphatic tissue and nonlymphoid tissue throughout life. In contrast, Langerhans cells of the epidermis, which are also antigen presenting cells, are self sustained and localize to skin during embryonic development.[181,201,202] Some dendritic cells are also derived from the lymphoid lineage.[201,203] Antigen presentation by dendritic cells, which have a high density of MHC class II molecules, is a particularly potent stimulus for T-cell mediation of the primary immune response.[200,201,204] Plasmacytoid dendritic cells are a specialized population of dendritic cells, although derived from a shared precursor, that respond rapidly to viruses or nucleic-acid–containing complexes by secreting large amounts of type I interferon (IFN).[205,206] NETs have been linked to activation of plasmacytoid dendritic cells and the pathogenesis of lupus.[200,205-208]

Osteoclast

Osteoclasts are large, multinucleated mononuclear phagocytes that resorb mineralized cartilage and bone.[209,210] Rodent transplantation studies have shown that osteoclasts can be derived from granulocyte-macrophage progenitor cells.[211] Defects in osteoclast function result in osteopetrosis, a genetically heterogeneous group of disorders characterized by defective bone resorption.[212,213] The *Op/op* osteopetrotic mouse mutant lacks M-CSF, which results in deficiencies of both osteoclasts and tissue macrophages.[214] However, M-CSF levels and osteoclast numbers are normal in human infantile ("malignant") osteopetrosis,[215] an AR disorder with progressive obliteration of the marrow space.[212,216] This severe form of osteopetrosis is caused by mutations in genes encoding a vacuolar proton pump or the chloride channel 7.[217,218]

FUNCTION OF PHAGOCYTES

Phagocytic leukocytes play a central role in the acute phases of the inflammatory response, where they are rapidly mobilized into sites of tissue infection or injury and release an array of cytotoxic molecules to quickly eliminate the offending substance or microbe, as well as mediators that initiate an adaptive immune response. Phagocytes are also essential for normal repair of tissue injury, as evidenced by the impairment in wound healing in patients with deficits in leukocyte function or number.

The classic signs of the inflammatory response were described by the Roman writer Celsus as *"rubor et tumor, cum calore et delore,"* or, *"redness and swelling with heat and pain."*[219] However, it was not until the late nineteenth century that the cellular events associated with these signs were studied by Virchow and by Cohnheim.[219] The beneficial role of phagocytes in the inflammatory process for host defense and wound healing was championed by Metchnikov. He received a Nobel prize for his work,[219,220] much of which involved studies on the wandering ameboid mesenchymal cells of marine organisms like the larval starfish, for which he coined the term *phagocyte,* after the Greek word, *phagein*, "to eat."

In this section, the principle functions of granulocytes and mononuclear phagocytes in the inflammatory process are reviewed. Although these functions will be discussed as individual components, it is important to recognize that many occur either simultaneously or in rapid succession. An overview of early events in the inflammatory process and cross talk between different leukocyte populations is shown in Figure 22-2. Note that proinflammatory events that are critical for the response to tissue injury, and the effective elimination of microbial challenge also sets into motion the generation of

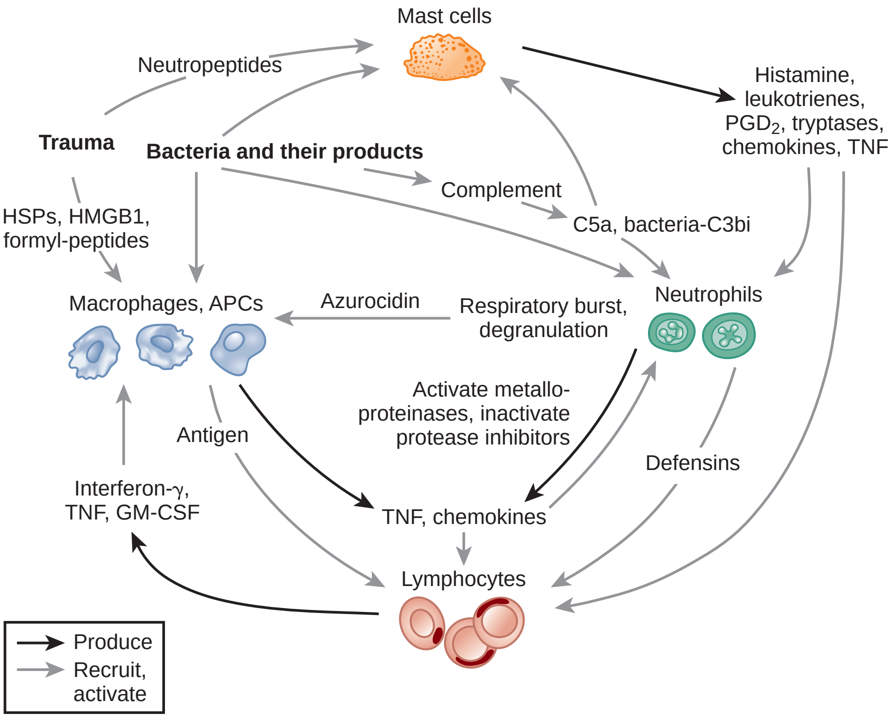

Mast cells
Neutropeptides
Histamine,
leukotrienes,
PGD2, tryptases,
chemokines, TNF
Trauma
Bacteria and their products
Complement
HSPs, HMGB1,
formyl-peptides
C5a, bacteria-C3bi
Neutrophils
Macrophages, APCs
Azurocidin
Respiratory burst,
degranulation
Activate metallo-
proteinases, inactivate
protease inhibitors
Antigen
Defensins
Interferon-γ,
TNF, GM-CSF
TNF, chemokines
Lymphocytes
Produce
Recruit,
activate

Figure 22-2 Early events in the response to infection accompanied by mild tissue injury. During the early inflammatory response, a variety of soluble inflammatory mediators are involved in crosstalk between different leukocyte populations and lead to their recruitment and activation. Multiple signals, generally requiring evidence of both injury and infection, are required before each population fully joins in to amplify the inflammatory process. Not shown are interactions among leukocytes, the endothelium, and the coagulation system; the generation of counter-regulatory signals that dampen the inflammatory response; and signals that regulate the subsequent transition to wound healing. *APC,* Antigen-presenting cell, *GM-CSF,* granulocyte-macrophage colony-stimulating factor; *HMGB1,* high–molecular group box 1 protein; *HSP,* heat shock proteins; *PDG2,* prostaglandin G2; *TNF,* tumor necrosis factor. *(From Nathan C: Points of control in inflammation.* Nature *420:846–852, 2002.)*

counterregulatory signals leading to resolution of the inflammatory response.[221-224]

Humoral Mediators of the Inflammatory Response

The acute inflammatory response reflects an ongoing collaboration between tissue macrophages and mast cells, vascular endothelial cells, and circulating phagocytes.[222,225] The release of soluble inflammatory mediators is crucial for activating and coordinating this process. These molecules can be generated from plasma proteins (e.g., the complement-derived protein fragment C5a), secreted by endothelial cells or inflammatory leukocytes (e.g., lipid metabolites, histamine, cytokines, S100 proteins), derived from invading microbes (e.g., endotoxin or formylated chemotactic peptides), or released from damaged cells (e.g., heat shock proteins [HSPs], the nuclear protein high molecular–group box 1 protein [HMGB1]).[222,226-230]

The proinflammatory cytokines TNF-α and IL-1 have a broad range of activities in the acute inflammatory response.[83,231] Both IL-1 and TNF can cause fever and muscle breakdown and are involved in the cachexia associated with chronic infection and malignancy. The synthesis of acute phase reactants by the liver is induced by IL-6, whose synthesis and secretion is stimulated by IL-1. Proinflammatory cytokines also induce a proadhesive state on the surface of endothelium and increase the production of the chemotactic cytokines (chemokines). IFN-γ is another important proinflammatory mediator that enhances the responsiveness of phagocytes to inflammatory stimuli.[183,184,232] Counterbalancing the activities of these polypeptides are IL-4, IL-10, and TGF-β, which tend to down-regulate the acute inflammatory response.[233-235]

Lipid mediators play both proinflammatory and antiinflammatory roles.[221] As the inflammatory process progresses, a "class switch" is observed in neutrophils such that lipoxygenase activity induces production of antiinflammatory lipoxins instead of proinflammatory leukotrienes. In addition products of ω-3 unsaturated fatty acids, resolvins D1 and D2, are generated by neutrophils, and the related maresins are generated by efferocytosing macrophages.[236] These products inhibit neutrophil transmigration by downregulating surface proteins involved in transepithelial migration, by inducing nitric oxide (NO) production in endothelial cells, and by enhancing the production of antiinflammatory cytokines and inhibiting production of proinflammatory cytokines of macrophages and making them more apt for uptake of apoptotic neutrophils.[223,237]

Vasodilation and increased vascular permeability are two early responses to an inflammatory insult that are elicited in large part by products secreted by granulocytes and mononuclear phagocytes. Activated basophils and tissue mast cells release histamine, which leads to vasodilation of tissue arterioles and microvascular beds through H_1-type receptors.[238,239] The lipid metabolite platelet activating factor (PAF), which is secreted by activated macrophages, mast cells, and endothelial cells, induces platelet degranulation and the release of additional histamine and also serotonin, another vasoactive amine.[230,240] Prostaglandin E and other arachidonic acid metabolites secreted by activated neutrophils and macrophages are another group of potent vasodilators.[221,230] Finally, vasodilation can be triggered by the release of NO from endothelial and smooth muscle cells as well as perhaps from activated macrophages, which may be particularly important in the hypotension seen with gram-negative septicemia.[241,242] The increased vascular permeability that produces the edema of acute inflammation allows plasma proteins such as immunoglobulins and complement to enter tissues to promote phagocyte activation and opsonize microbes. Agents that increase vascular permeability include histamine, serotonin, PAF, and leukotrienes (LTs) C_4, D_4, and E_4.[240] Bradykinin, which is generated as the result of Hageman factor (factor XII) cleavage, also induces enhanced vascular permeability.

Chemokines, Small Lipids and Other Chemoattractants

A wide variety of chemoattractants for neutrophils and other circulating leukocytes are generated at sites of inflammation (Table 22-4).[96,243-245,246,247] These molecules are chemically diverse and are derived from many different sources in response to bacterial products and inflammatory mediators released as a result of tissue necrosis. This diversity provides a functional redundancy and ensures that leukocytes will be attracted to sites of injury or infection. In addition to their role as chemoattractants, the molecules listed in Table 22-4 induce the activation of many other phagocyte functions upon binding to their cognate cell-surface receptors. These include the upregulation and increased affinity of leukocyte integrin adhesion receptors to promote firm attachment to the endothelium, degranulation, and activation of the phagocyte respiratory burst. Many chemoattractants are secreted by activated phagocytes, which act as a positive feedback loop for additional recruitment and activation of inflammatory cells.

The phospholipid PAF, released by both activated phagocytes and endothelial cells, triggers platelet activation and granule release in addition to being a potent chemoattractant for neutrophils and eosinophils.[230,240] Activation of phagocytes also stimulates the phospholipase A_2-mediated cleavage of membrane phospholipids to generate arachidonic acid, which is then converted into a variety of eicosanoid metabolites, including the chemoattractant leukotriene B_4 (LTB_4).[221,229]

Chemokines (named for their combined *chemo*tactic and cyto*kine* properties) are a family of small (8 to 10 kDa) basic heparin-binding proteins that comprise an important group of phagocyte chemoattractants.[94,244,247] Chemokines were first discovered in the late 1980s as molecules that interact relatively specifically with subsets of inflammatory leukocytes and therefore help orchestrate the sequential influx of neutrophils, monocytes, and finally lymphocytes into an inflamed tissue site. Proteoglycans on endothelial cells or in the subendothelial matrix bind chemokines to produce locally high chemokine concentrations at an inflamed site.[243,248,249] As additional chemokines and their receptors have been identified, many other functions have emerged, including regulation of lymphoid homeostasis, hematopoiesis, and angiogenesis.[96,244] Of note, SDF-1 *(CXCl2)*, provides a key

TABLE 22-4 Granulocyte and Monocyte Chemoattractants in Humans

Chemoattractant	Receptor(s)	Source	Upregulators	Target Cells
Lipids				
PAF	PAFR	N, E, B, P, M, endothelium (phosphatidylcholine metabolism)	Calcium ionophores	N, E
LTB$_4$	B-LTR	N, M (arachidonate metabolism)	Microbial pathogens, *N*-formyl peptides	N, M, E
12-HETE	P (arachidonate metabolism)	Platelet activation		E
CXC Chemokines				
IL-8 (CXCL8)	CXCR1, 2	M, N, endothelium, many other cells	LPS, IL-1, TNF, IL-3	N, B
GRO α, β, γ (CXCL1, 2, 3)	CXCR2, 1	M, endothelium, many other cells	IL-1, TNF	N, B
NAP-2 (CXCL7)	CXCR2	P*	Platelet activators	N
PF4 (CXCL4)	CXCR3B	P	Platelet activators	N, M, E
SDF-1 (CXCL12)	CXCR4	Marrow stroma, other		N, M, B, T
Fractalkine (CX$_3$CL1)	CX$_3$CRI	M, endothelium, other	IL-1, TNF, LPS, IFN-γ	M, T, NK
CC Chemokines				
MCP-1, 2, 3, 4	CCR2, 3 (CCL2, 8, 7, 13)	M, endothelium, many other cells	IL-1, TNF, LPS, PDGF	M, B, E, T
RANTES (CCL5)	CCR1, 3, 5	M, E	IL-1, TNF, anti-CD3	M, B, E, T
Eotaxin (CCL11)	CCR3	M, endothelium, other	Allergens	E, B, TH2
Other				
N-formyl peptides	fMLPR	Bacteria, mitochondria	—	N, M, E, B
C5a	C5aR	Plasma complement	Complement activation	N, M, E, B
PDGF	PDGFR	P	Platelet activation	M
TGF-β	TGFR	P, other	Platelet activation	N, M

Modified from Curnutte J, Orkin S, Dinauer M: Genetic disorders of phagocyte function. In Stamoyannopoulos G, editor: The molecular basis of blood diseases, *2nd ed, Philadelphia, 1994, WB Saunders, p. 493–522.*

B, Basophil; *B-LTR,* LTB4 receptor; *CCR,* CC receptor; *CXCR,* CXC receptor; *E,* eosinophil; *F,* fibroblast; *fMLPR,* formyl-methionyl-leucyl-phenylalanin receptor; *GRO,* growth-regulated oncogene; *HETE,* 15(S)-hydroxyeicosatetraenoic acid; *IFN-γ,* interferon γ; *IL,* interleukin; *K,* keratinocyte; *LPS,* lipopolysaccharide; *LTB$_4$,* leukotriene B$_4$; *M,* monocyte; *MCAF,* monocyte chemotactic and activating factor; *MCP,* monocyte chemoattractant protein; *N,* neutrophil; *NAP,* neutrophil-activating protein; *NK,* natural killer; *P,* platelet; *PAF(R),* platelet-activating factor (receptor); *PDGF(R),* platelet-derived growth factor (receptor); *PF4,* platelet factor 4; *RANTES,* regulated upon activation, normal T cell expressed and presumably secreted; *SDF,* stromal cell–derived factor; *T,* T lymphocyte; *TGF(R),* transforming growth factor (receptor); *TH2,* T-helper type 2 lymphocyte; *TNF,* tumor necrosis factor.

*Platelets, when activated, secrete platelet basic protein (PBP) and connective tissue–activating peptide III (CTAP-III), which are cleaved to NAP-2 by cathepsin.

retention signal for neutrophils in the marrow through its interaction with the CXCR4 receptor, and mutations in the *CXCR4* receptor account for the WHIM syndrome, an inherited neutropenia.[35,36]

Members of the chemokine family, which have a conserved structure containing two cysteine pairs, have been divided into two groups based on the disulfide sequence pattern. The CXC family, in which the first cysteine pair is separated by an intervening amino acid, include IL-8 *(CXCL8),* the growth-regulated oncogene (GRO) peptides (*CXCL1, 2,* and *3*), and neutrophil-activating protein 2 (NAP-2; *CXCL7*), which are all potent neutrophil activators and chemoattractants. The IL-8 and GRO chemokines are secreted by phagocytes and mesenchymal cells (including endothelial cells) in response to inflammatory mediators such as IL-1 and TNF.[96] Fractalkine *(CX$_3$CL1)* is unique in having three intervening amino acids between the first two cysteine residues. In addition, rather than being soluble, fractalkine is expressed on the cell surface, because it is linked via a mucinlike stalk to a transmembrane domain.[250] The other major family of chemokines is called the CC *family,* because the first two cysteines are adjacent to each other. CC chemokines include two important inducers of mononuclear phagocyte migration, monocyte chemotactic protein 1 (MCP-1; *CCL2*) and RANTES *(CCL5).*[251-253] MCP-1 is produced by a wide variety of cells, whereas RANTES is secreted by macrophages and eosinophils.[96] RANTES is chemotactic for eosinophils, basophils, and memory T cells as well as monocytes,[252] and both MCP-1 and RANTES induce histamine release from basophils.[238,239,252]

Despite the diverse chemical structures of phagocyte chemoattractants listed in Table 22-4, the corresponding receptors all belong to the seven-transmembrane–spanning receptor (7-TMR) family, also known as *heptahelical* or *serpentine receptors,* that are coupled to heterotrimeric G proteins.[254,255] For chemokines, more than six receptors for CXC chemokines and ten receptors for CC chemokines have been identified.[94,244] Most chemokines bind to more than one receptor, and most chemokine receptors, particularly those for CC chemokines, recognize more than one chemokine. Neutrophils, monocyte/macrophages, eosinophils, basophils, dendritic cells, lymphocytes, and T cells each express a distinctive subset of chemokine receptors. According to one model, specific receptors are used sequentially in successive gradients of chemoattractants. Some transmit desensitizing, rather than activating, signals or even fail to signal and act instead as "decoy" receptors to downregulate inflammatory reactions.[244] Of note, a number of chemokine receptors are coreceptors for HIV-1, including *CCR5* and *CCR3,* whose ligands include RANTES, and *CXCR4,* the major receptor for stromal cell–derived factor 1 (SDF-1), which is a chemoattractant for T lymphocytes, CD34+ hematopoietic progenitor cells, and neutrophils.[35,96]

The signaling through 7-TMR has proven much more complicated (and powerful) than the original signaling mediated via associated heterotrimeric G proteins, which bind to specific intracellular domains of the receptor.[256] Ligand binding to the receptor promotes the exchange of GTP for guanosine diphosphate (GDP) bound to the G protein α subunit, which in turns leads to the dissociation of the β-γ subunits and their interaction with downstream signaling effectors. In addition, 7-TMR associates with β arrestins and signals through these in a G-protein independent way.[257,258] Finally, 7-TMR may transactivate tyrosine kinase growth factor receptors.[259] Signals generated from 7-TMR activate enzymes that catalyze the production of important phospholipid second messengers at the cell membrane.[260]

Adhesion and Migration into Tissues

The discovery that leukocytes migrate from the bloodstream into extravascular sites of inflammation, described by Cohnheim in 1867, was a major milestone in the conceptualization of the inflammatory process.[219] Cohnheim, who used intravital microscopy to study the microvasculature in the frog tongue and mesentery after tissue injury, also first proposed that inflammatory stimuli induce a molecular change in the blood vessel wall that promoted the increased adherence of leukocytes, a concept that was finally proven a century later.

To move from the bloodstream into inflamed sites, leukocytes must attach to the vascular endothelium, migrate between adjacent endothelial cells in a process referred to as *diapedesis,* and penetrate the basement membrane. The molecular mechanisms underlying these events involve a series of sequential adhesive interactions between chemoattractant-activated leukocytes and endothelial cells that are activated by inflammatory mediators (Fig. 22-3).[75,117,243,261-265]

Leukocyte Adhesion and Migration into Tissues

The initial step in emigration from postcapillary venules is a low-affinity interaction between the neutrophil and the endothelium that is often referred to as *rolling* because of its appearance in intravital microscopy. This transient adherence, also called *tethering,* is mediated by the upregulation of selectin expression on endothelial cells.[266] The selectin family of adhesion molecules are membrane-spanning glycoproteins (Fig. 22-4) that bind

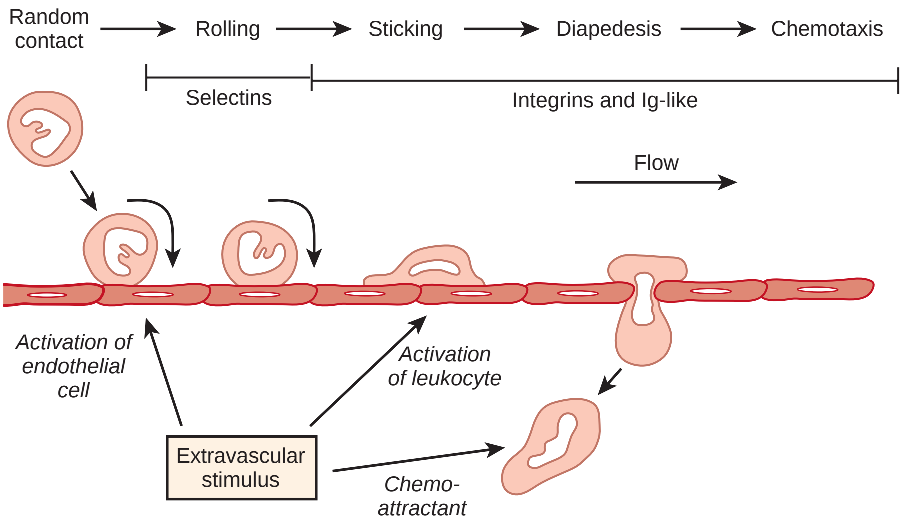
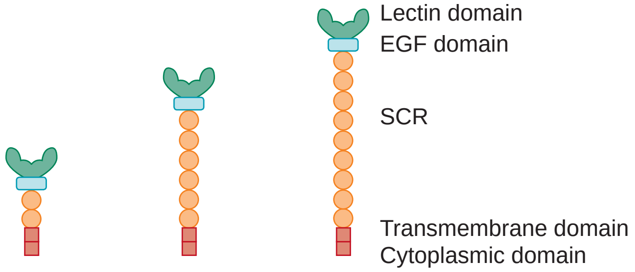


Figure 22-3 Adhesive interactions during phagocyte emigration. Under conditions of flow within postcapillary venules, leukocytes are first observed to roll along the endothelium adjacent to the extravascular site of inflammation. Subsequently, some of the rolling leukocytes adhere firmly, pass between endothelial cells by diapedesis, and then migrate into subendothelial tissue. Leukocyte rolling is mediated by multiple low-affinity interactions between selectin receptors and carbohydrate counter-receptors, and firm adhesion and diapedesis are largely mediated by integrin and Ig-like adhesion proteins. Chemokines or other chemoattractants are important for both upregulating the cell surface expression of leukocyte integrins and triggering inside-out signaling to increase their avidity for endothelial ligands. *(Modified from Carlos TM, Harlan JM: Leukocyte-endothelial adhesion molecules.* Blood *84:2068–2101, 1994.)*



	L-selectin (LAM-1, LECAM-1)	E-selectin (ELAM-1)	P-selectin (PADGEM, GMP-140)
Distribution	Neutrophils Monocytes Lymphocytes	Stimulated endothelium	Stimulated endothelium Activated platelets
Ligands	Sialyated, fucosylated carbohydrates	Sialyated, fucosylated carbohydrates	Sialyated, fucosylated carbohydrates
Function	Lymphocyte homing Inflammation	Inflammation	Inflammation Hemostasis (?)

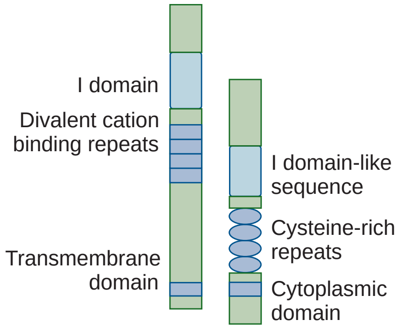
Figure 22-4 Selectin family of adhesion molecules. *EGF,* Epidermal growth factor; *SCR,* short consensus repeats. *(Modified from Kishimoto TK, Rothlein R: Integrins, ICAMs, and selectins: role and regulation of adhesion molecules in neutrophil recruitment to inflammatory sites.* Adv Pharm *25:117–169, 1994.)*

to fucosylated structures such as Lewis X (Galβ1→4 [Fucα1→3] GlcNac→R), Sialyl-Lewis X, and other specific carbohydrates.[262,264,266,267] P-selectin is important for the initial steps of neutrophil adhesion to the endothelium and is stored in the Weibel-Palade bodies and α granules of endothelial cells and platelets, respectively. Upon endothelial cell activation by histamine, thrombin, and other inflammatory molecules, these cytoplasmic storage granules fuse with the cell membrane to rapidly increase the surface expression of P-selectin. E-selectin is expressed on endothelial cells at low levels, but it is upregulated by transcriptional activation and *de novo* protein synthesis in response to inflammatory cytokines.[268] E-selectin binds to three different ligands on neutrophils: P-selectin glycoprotein ligand (PSGL) 1, E-selectin ligand (ESL) 1, and CD44.[269] These ligands allow endothelial cells to capture neutrophils by mediating tethering, rolling, and slowing of neutrophil velocity, respectively. L-selectin is expressed constitutively on the surface of neutrophils, mononuclear phagocytes, and lymphocytes and is shed within minutes of leukocyte activation by a proteolytic cleavage event near the external membrane surface insertion site.[270,271] Circulating L-selectin may modulate leukocyte adhesion during inflammation.[272] PSGL-1 is constitutively expressed on the tip of microvilli on the neutrophil surface and remains associated during activation but moves to the uropod when the neutrophil polarizes.[264,273]

Rolling neutrophils can detach and return to the circulation. Others will come to a halt and within seconds adopt a flattened, adherent morphology and attach firmly to the vessel wall.[262] This firm attachment appears in large part to be mediated by leukocyte integrin adhesion receptors binding to intracellular adhesion molecules (ICAMs) on the endothelium.[262,263] In addition, complement fragments are found on the endothelial surface at inflamed sites and may also function as integrin binding sites. Leukocyte activation by chemoattractants and other inflammatory mediators is critical to the development of these strong adhesive interactions, because it leads to the upregulation of the number and avidity of cell-surface integrins (“inside-out signaling”).[274,275] Exposure to locally high concentrations of chemoattractants may be enhanced by selectin-mediated tethering and by the retention of chemokines on extracellular matrix.[248,249]

The integrins are a large family of adhesion proteins that are glycosylated heterodimers of a noncovalently linked α chain and β chain and are classified into subfamilies according to the type of β subunit.[262,263] Many integrins mediate attachment to extracellular matrices by serving as receptors for matrix proteins. Others are involved in hemostasis, such as glycoprotein IIb/IIIa on platelets. Neutrophil β_2 and β_1 integrins appear to be involved in regulating neutrophil retention and release, respectively, from the bone marrow storage pool into the circulation.[35]

The leukocyte β_2 integrins (Fig. 22-5) play a critical role in mediating adhesive interactions in inflammation, including the attachment of leukocytes to endothelial cells and are also opsonic receptors for complement fragment C3bi-coated particles. There are four different leukocyte β_2 integrins, each having a common 95 kDa β subunit (CD18) but different α subunits, CD11a (177 kDa), CD11b (165 kDa), CD11c (150 kDa), and CD11d (160kDa) (see Fig. 22-5). Lymphocyte function antigen 1 (LFA-1) is expressed on the surface of all leukocytes, including lymphocytes. Mac-1 and p150,95 are



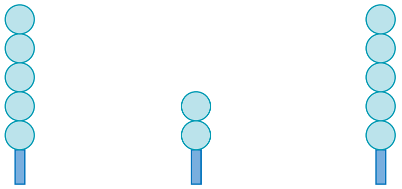
	LFA-1	Mac-1 (CR3)	p150, 95	$\alpha_d\ \beta_2$
α subunit	α_L (CDIIa)	α_m (CD11b)	α_x (CD11c)	α_d (CD11d)
β subunit	β_2 (CD18)	β_2 (CD18)	β_2 (CD18)	β_2 (CD18)
Ligands	ICAM-1 ICAM-2 ICAM-3 JAM-1	ICAM-1 C 3bi Fibrinogen Factor X JAM-2 other	C3bi Fibrinogen other	ICAM-3 other?
Distribution	All leukocytes	Granulocytes Monocytes Macrophages NK cells Dendritic cells	Macrophages Monocytes Granulocytes NK cells Dendritic cells	Macrophages Monocytes Granulocytes T Lymphocytes

Figure 22-5 The b_2 (CD18) family of leukocyte integrins. *ICAM,* Intercellular adhesion molecule; *JAM-1,* junctional adhesion molecule 1; *NK,* natural killer.

expressed by granulocytes, mononuclear phagocytes, some activated T lymphocytes, and large granular lymphocytes.[262,263] Mac-1 is the most prominent β_2 integrin on neutrophils, whereas $\alpha_d\beta_2$ is expressed particularly in tissue macrophages. Mutations in the common β_2 subunit result in an inherited defect in phagocyte function, leukocyte adhesion deficiency type I (LAD I) as discussed in a later section. All β_2 integrins are absent in LAD I, indicating that the stability of each α subunit requires association with the β_2 chain. The β_2 subunit has a large, glycosylated extracellular domain, a single transmembrane-spanning domain, and a short cytoplasmic tail.[276] The extracellular domain has two regions that are conserved among other β subunits.[274] There are four cysteine-rich tandem repeats that appear to be important for the tertiary structure of the β subunit. Another conserved region, located near the N-terminus, is critical for maintenance of the α/β heterodimer and may also bind divalent cations. The α subunit is also a glycosylated integral membrane protein with a single membrane-spanning segment and a short cytoplasmic tail. The external domain contains three divalent cation-binding motifs that must be occupied for ligand binding to occur. A second important extracellular domain, the I domain (for *inserted* or *interactive* domain), can coordinate divalent cations and is also thought to be involved in ligand binding. The intracellular domain of the α subunit includes a conserved sequence that is critical for the modulation of integrin avidity (see "Leukocyte Adhesion Deficiency").[277]

Although β_2 integrins are constitutively expressed on the neutrophil cell surface, a large pool of Mac-1 is stored in intracellular secretory vesicles (see Table 22-2). These vesicles are rapidly mobilized upon neutrophil activation by chemoattractants and fuse with the membrane to increase the cell surface expression of β_2 integrins by about tenfold.[243,278] Signaling through chemoattractant receptors also markedly increases the avidity of β_2 integrins for their ligands, which plays an even more important role in rapidly upregulating integrin activity and promoting firm attachment to the blood vessel wall.[262,263,274] Integrins on inactivated cells have a bent position that does not allow ligand binding. Signals generated from 7-TMRs recruit talins to the cytoplasmic part of the integrin β chains, twisting the α and β chains apart and tethering the integrins to the actin cytoskeleton. This is assisted by actin-binding protein (ABP) 1[279] and by the protein Kindlin 3, which is only expressed in cells of hematopoietic origin. This changes the conformation of the extracellular parts of the αβ heterodimer to an extended form, now capable of ligand binding and hence also of transducing signals from outside in.[275] Defects in Kindlin 3 results in LAD III (see "Leukocyte Adhesion Deficiency Type III").[274,280-285] Ligand binding to integrins results in clustering of the integrins and the transmembrane calcium channel Orai1 that mediates store operated calcium entry[286] and activation of tyrosine kinase and other signaling cascades,[287] which in addition to mediating adhesion, provide important costimulatory signals to enhance migration, respiratory burst, Fcγ-mediated phagocytosis, and degranulation.

The major counterreceptors for the β_2 integrins are the ICAMs (Fig. 22-6), which are members of the immunoglobulin superfamily.[262,263,274] These transmembrane proteins contain anywhere from two to six immunoglobulin



	ICAM1	ICAM2	ICAM3
Structure	Five Ig-like domains	Two Ig-like domains	Five Ig-like domains
Ligands	LFA-1 Mac-1 CD43 Rhinovirus Malaria infected RBC	LFA-1	LFA-1
Distribution	Widely inducible Lymphocytes Monocytes Endothelium Others	Constitutive Endothelium Lymphocytes	Leukocyte-specific Lymphocytes Monocytes Neutrophils

Figure 22-6 Intercellular adhesion molecules (ICAMs). *Ig,* Immunoglobulin; *LFA-1,* lymphocyte function antigen 1; *RBC,* red blood cell. *(Modified from Kishimoto TK, Rothlein R: Integrins, ICAMs, and selectins: role and regulation of adhesion molecules in neutrophil recruitment to inflammatory sites.* Adv Pharm *25:117–169, 1994.)*

domains and are present on endothelial cells, T cells, and a variety of other cell types. ICAM-1 and ICAM-2 are of particular importance in mediating binding of neutrophils and other leukocytes to the endothelium. Endothelial cell expression of ICAM-1 increases in response to inflammatory cytokines, which promotes increased cell–cell interactions with leukocytes at inflamed sites. VCAM-1 is another immunoglobulin superfamily member expressed on endothelial cells that is inducible by cytokines.[263,288] VCAM-1 is the counter-receptor for the β_1 integrin, VLA-4, and appears to be important in promoting the adherence of monocytes and eosinophils during inflammation. The β_2 integrin Mac-1 also has an important role as an opsonic receptor for the complement fragment, C3bi (see "Recognition, Opsonization, and Phagocytosis").

Although leukocyte β_2 integrin-mediated adhesion is clearly important for neutrophil recruitment from the systemic microvasculature into inflammatory sites, neutrophil emigration out of the pulmonary circulation can also be mediated by alternative pathways, depending on the inflammatory stimulus.[289] Whether the alternative pathway in pulmonary capillaries, which are in close proximity to the pulmonary epithelial cells, involves selectins or other adhesion molecules remains to be defined. TREM-1 is essential for pulmonary transepithelial migration of neutrophils.[290]

The final steps in emigration of neutrophils from the blood vessel lumen into inflamed tissue involves squeezing between adjacent endothelial cells (diapedesis) and penetrating the basement membrane (see Fig. 22-3). The presence of a chemotactic gradient is required to induce the directional migration of neutrophils.[248,291,292] Adhesive interactions between the β_2 integrins and endothelial cell ICAM-1 are essential for neutrophil diapedesis, whereas VCAM-1 and E-selectin can mediate the transmigration of monocytes and eosinophils.[117,261] Junctional adhesion molecule (JAM) 1, an immunoglobulin superfamily protein that is expressed at tight junctions of resting endothelial cells and epithelial cells, facilitates leukocyte transmigration via binding to LFA-1 (CD11a/CD18).[293] The tight junction between endothelial cells is weakened by loss of paxillin and focal adhesion kinase in proximity to migrating neutrophils.[294,295] Transendothelial migration of neutrophils is also dependent on homologous binding between neutrophil and endothelial cell platelet–endothelial cell adhesion molecule (PECAM) 1 (CD31), another immunoglobulin superfamily member expressed on the surface of leukocytes, platelets, and endothelial cells, where it is localized at the junctions between cells.[296,297] Signals from ICAM-1 induce vascular permeability,[298] activate PECAM-1 for enhanced adhesivity, and support of neutrophil transendothelial migration.[299-301] The JAM-C expressed by endothelial cells seems important for directing neutrophil migration into tissues and inhibiting migration in the reverse direction.[302] Migrating neutrophils induce increases in endothelial intracellular calcium levels and changes in actin cytoskeleton that facilitates transmigration.[303,304] To find gaps between pericytes sheathing endothelial cells, neutrophils use ICAM-1 to crawl along pericyte extensions, which further guide their migration into tissues.[44-46] Finally, chemoattractant-induced neutrophil degranulation results in the release of digestive enzymes, including collagenase, elastase, and gelatinase, which may facilitate basement membrane penetration.[68,305,306]

Chemotaxis

Chemotaxis is the directional movement of a cell along a concentration gradient.[86,266,307-310] Defects in neutrophil cellular motility or other steps in chemotaxis can result in decreased resistance to bacterial and fungal infections, as discussed later in this chapter. Cells respond to a chemotactic gradient by sensing constantly across their surface, and bound chemotactic receptors are continuously internalized. A migrating neutrophil has a polarized appearance, extending pseudopodia or lamellipodia, thin structures rich in actin filaments and lacking intracellular organelles, at the leading edge.[308,311,312] The pseudopods appear to glide forward, pulling the cell body behind them. The nucleus tends to remain at the posterior half of the moving leukocyte. Migration also requires formation of a uropod at the "tail" of the leukocyte, which detaches from the underlying matrix and retracts the rear of the cell as it moves forward.[313] Rho GTPases play an important role in establishing chemoattractant-induced polarization and migration.[82,314] Ly49Q, a surface-expressed MHC I–associated receptor is associated with the SH2 domain–containing protein tyrosine phosphatase (SHP) 1 phosphatase in nonpolarized neutrophils but exchanges SHP-1 with SHP-2 in activated cells, which results in reorganization of lipid rafts and polarization.[315]

Neutrophil movement is dependent on the dynamic assembly and disassembly of filamentous actin, which is coordinated by various ABPs whose activity is regulated by intracellular signaling molecules.[307,309,316-318] Actin can exist as either a soluble monomer (globular actin) or in needlelike helical filaments (filamentous actin). Actin filaments align spontaneously in parallel bundles, but in the cell are organized into a branching network because of the presence of actin filament cross-linkers such as ABP.[319] Agonists acting via receptors on the cell membrane trigger the generation of second messengers, which interact with ABPs to control dynamic local cycles of filamentous actin assembly.[308] The Rho GTPases (Cdc42 and Rac) are important regulators of actin remodeling and are activated by ligand binding to chemoattractant receptors.[80,314,320,321] Cdc42 activates proteins of the Wiskott-Aldrich syndrome protein (WASP) family, which then bind to a complex of seven proteins known as the Arp2/3 complex to nucleate assembly of new actin filaments at the leading edge of migrating cells.[312,316]

During pseudopod extension in chemoattractant-activated neutrophils, new actin polymerization occurs at the site of membrane protrusion while the filamentous actin in the rear of the cell disassembles.[309,310,322] How actin assembly–disassembly results in membrane extension and formation of pseudopodia is not fully understood, but it may involve localized changes in osmotic pressure caused by changes in actin polymerization.[308,311] Membrane movement and retraction at the rear of the cell is mediated in part by Rho GTPase-regulated contractile proteins such as myosin 1.[309,323,324]

Recognition, Opsonization, and Phagocytosis

The recognition, ingestion, and disposal of microbes, foreign particulate matter, and damaged cells constitutes a major aspect of phagocyte function. To facilitate their recognition by phagocytes, these targets are coated with serum opsonins (the term *opsonin* is from Greek, *to prepare for dining*) that include proteolytic fragments derived from the complement cascade as well as specific immunoglobulins. The key humoral opsonins are the proteolytic cleavage products of C3 (C3b and C3bi), which can be generated in the absence of specific immunity by the alternative and mannose-binding lectin pathways (see later), and the opsonic antibodies, IgM, IgG_1, and IgG_3. Targets are opsonized by the deposition of C3b and C3bi or of IgG onto their surfaces via the specific (Fab) portion of the antibody. Antibacterial IgM antibodies, although not opsonic by themselves, play an important role in phagocytosis by activating complement. Opsonins are recognized by phagocyte cell-surface glycoprotein receptors for immunoglobulin and C3 cleavage products, as described later. Inherited deficiencies of opsonization can result in increased susceptibility to bacterial infections, as discussed in "Disorders of Chemotaxis." In contrast, primary defects in phagocyte receptors for these opsonins appear to be an uncommon cause of recurrent infections.[325]

Phagocytes also have cell surface receptors capable of recognizing targets even in the absence of opsonins. These members of the pattern recognition receptor (PRR) family recognize broad classes of macromolecules.[77,79,80,182,326] PRRs recognize molecular signatures unique to microbes, known as PAMPs (such as LPS or molecules released from damaged or stressed tissue known as DAMPs), such as adenosine triphosphate, as discussed previously in this section. PRRs include scavenger receptors, which have broad binding specificity for polyanionic ligands and participate in the clearance of diverse materials, including modified low-density lipoprotein (LDL) and apoptotic cells.[182] The mannose receptor recognizes carbohydrate structures present in a range of bacteria, fungi, virus-infected cells, and parasites whereas the β-glucan receptor (dectin-1) binds to β-glucan structures in zymosan and other yeast-derived particles. Mammalian TLRs are an important group in the PRR family.[78,79,89,327] At least 12 different TLRs have been described, which recognize conserved peptide, lipid, carbohydrate, and nucleic-acid structures expressed by different groups of microbes. For example, peptidoglycan and LPS are PAMPs associated with gram-positive and gram-negative bacteria and are recognized by TLR-2 and TLR-4, respectively. Binding to PRRs, which often occurs in a combinatorial fashion, activates an array of proinflammatory responses including expression of proinflammatory cytokines and release of oxidants and reactive nitrogen intermediates. TLR signaling activates the nuclear factor κB (NF-κB)– and IFN-regulatory factor (IRF)–dependent pathways, and genetic defects in the protein that couples TLRs to NF-κB lead to recurrent bacterial infections.[328] Some PRRs also trigger phagocytosis, including the mannose receptor, scavenger receptors, and β-glucan receptor.[326]

In addition to cell surface PRRs, soluble PRRs present in serum or tissues serve important roles in alerting phagocytes to the presence of microbes.[329,330] These include the collectins (calcium-dependent lectins) that bind to oligosaccharide or lipid moieties of microorganisms to enhance their opsonization and efficiently activate phagocytes. Members of the collectin family include the lung alveolar surfactant proteins A and D and the mannose-binding lectin (MBL), also known as mannose binding protein.[330-332] The binding of MBL to mannose residues initiates a third pathway of complement activation (in addition to the classical and alternative pathways) by interacting with the MBL-associated serine proteases (MASPs) 1, 2, and 3, which generates opsonic C3 fragments.[329,333] MBL can also function itself as an opsonin for promoting uptake by the CR1 complement receptor.[330,333] Ficolins 1, 2, and 3 are other soluble PRRs that are capable of recruiting MASPs.[334-336] Pentraxin 3 is a soluble pattern recognition molecule stored in neutrophil-specific granules and important for humoral innate immunity.[337]

Inflammasomes

Signaling through the membrane-bound PRRs in the previous paragraph generally leads to activation of the NF-κB pathway or IRF and production of proinflammatory cytokines, in particular IL-1β and IL-18 and type-1 IFNs, respectively.[87] IL-1β and IL-18 are both without signal peptides and stored in the cytosol as proforms, but when processed by proteases, which in turn are activated by inflammasomes, these cytosolic cytokines are secreted from the cells by unconventional secretion mechanisms. IL-1β and IL-18 play a major role in activation of several steps of the inflammatory process, including endothelial cell and B-cell activation. Inflammasomes are structures that are assembled from preformed subunits in the cytosol in response to a variety of stimuli, such as signaling through cell surface (as discussed) or intracellular PRR such as the nucleotide-binding oligomerization domain–like receptors (NLRs) and retinoic acid–inducible gene 1–like receptor. Depending on the activating stimulus, different subunits become engaged in formation of the macromolecular complex known as an inflammasome and activate procaspase 1 to process the cytosolic proIL-1β and proIL-18 to the mature forms for secretion.[89,338-340] A major cytosolic protein of neutrophils, pyrin, forms part of some inflammasomes, and mutations in the pyrin gene *MEVF* cause the inflammatory condition known as familial Mediterranean fever.[341]

Complement Receptors

The activation of complement C3 to generate the cleavage fragments C3b and C3bi is the dominant source of opsonins in the absence of antibodies. Four C3 fragment receptors have been described on phagocytic leukocytes (Table 22-5). The human phagocyte C3b receptor (CR1; CD35) is a high–molecular-weight, single-subunit glycoprotein that shows a substantial heterogeneity in size because of the presence of four distinct alleles in the human population that encode proteins ranging in size

TABLE 22-5 Human Phagocyte Receptors for Complement C3 Fragment

	CR1 (CD35)	**CR3 (CD11b/CD18; Mac-1)**	**CR4 (CD11d/CD18)**	**CRIg**
Cell distribution	Neutrophils Monocytes B lymphocytes	Neutrophils Monocytes Macrophages NK cells	Macrophages Dendritic cells	Kupffer cells Certain other resident tissue macrophages
Structure	Transmembrane protein	Transmembrane, two subunits	Transmembrane, two subunits	Transmembrane protein
Ligands	C3b C4b C3bi C1q MBL (mannose-binding protein)	C3bi ICAM-1, 2 Fibrinogen Factor X	C3bi Fibrinogen	C3b C3bi
Function	Phagocytosis	Phagocytosis Respiratory burst (neutrophils) Adhesion Activation	Phagocytosis Adhesion	Phagocytosis

CR, Complement receptor; *ICAM*, intracellular adhesion molecule; *MBL*, mannose-binding lectin; *NK*, natural killer.

from 160 to 250 kDa. CR1 is responsible for the binding of C3b-opsonized particles and for initiating their ingestion.[333,342] CR1 also recognizes microbes opsonized with MBL.[330,331,333] The other major opsonic receptor, CR3, recognizes particles opsonized with C3bi. This receptor is the same as the β_2 integrin Mac-1 (CD11b/CD18) discussed in detail in an earlier section (see Fig. 22-5). Binding of C3bi-opsonized particles to Mac-1 triggers both phagocytosis and, in neutrophils, the respiratory burst. The CR4 receptor is the same as the β_2 integrin CD11c/CD18 and, on macrophages, can also initiate phagocytosis of C3bi-coated targets. Finally, a newly discovered receptor that binds the complement fragments C3b and C3bi, termed *complement receptor of the immunoglobulin superfamily (CRIg)* is expressed primarily in resident macrophages of the liver (Kupffer cells).[343] CRIg appears to play a critical role in mediating phagocytosis and clearance of C3 fragment-opsonized bacteria from the circulation, at least in mice.[343]

Immunoglobulin Receptors

Immunoglobulins are recognized by Fc receptors (FcRs), which are members of the immunoglobulin gene superfamily. FcRs bind to the "constant domain" of the antibody molecule, these being specific to each class of immunoglobulins (IgA, IgE, IgG, and IgM).[325,344,345] The most important from the standpoint of microbial opsonization are those FcRs that recognize IgG (FCγRs) (Table 22-6). The FcγRs include three distinct classes, FcγRI, FcγRII, and FcγRIII, which are encoded by at least eight genes that have evolved through gene duplication and alternative splicing, although not all give rise to detectable mRNA and/or protein.[346] The low-affinity FcγR genes (FcγRII and III families) and two of the genes for the high-affinity IgE receptor are clustered on chromosome 1q22. The high-affinity FcγRs map to other sites on chromosome 1.

Except for FcγRIIIb, which is anchored in the membrane by a GPI moiety, the FcγR proteins have a single transmembrane domain. FcγRI and FcγRIIIa exist as oligomeric complexes with γ (in phagocytes) or ζ (in lymphocytes) chains that are important both for their stable expression and signaling functions.[346] These accessory chains contain YXXL ITAM motifs that become tyrosine phosphorylated upon receptor cross-linking to initiate downstream signaling cascades.[325,344,345] The cytoplasmic tail of the FcγRIIa receptor contains variant ITAM motifs, and hence this receptor does not require accessory chains for its function. There is no murine equivalent to the FcγRIIa receptor.[345] The GPI-linked FcγRIIIb receptor must be co-ligated to either FcγRIIa or the β_2 integrin CR3 (Mac-1) to initiate signaling functions upon ligation.[325,344,345] FcγRIIb receptors, expressed on monocyte/macrophages, mast cells, and lymphocytes, contain ITIM sequences and instead inhibit cellular activation upon immunoglobulin cross-linking.[344,345]

Each FcγR is expressed at different levels, depending on the type of phagocytic cell (see Table 22-6), and some FcγRII and FcγRIII family members are also expressed on lymphocytes, platelets, thymocytes, natural killer (NK) cells, and mast cells.[345,346] The FcγR1 class includes the products of three highly homologous genes, denoted A, B, and C, although the protein products of the latter two have not been detected in vivo. The FcγR1a receptors are expressed by monocytes and neutrophils or eosinophils stimulated by IFN-γ or G-CSF and have a high affinity for monomeric IgG.[325,344,345] Members of the FcγRII and FcγRIII families have a low affinity for monomeric IgG but a high affinity for clusters of IgG (e.g., several antibodies bound to the same particle) and immune complexes. Only neutrophils and IFN-γ–stimulated eosinophils appear to express FcγRIIIb, and polymorphisms in this receptor correspond to the serologically defined NA1/NA2 antigen system, which is a common antibody target in alloimmune and autoimmune neutropenia (AIN).[347,348] Polymorphisms in FcγRIIa, FcγRIIIa, and FcγRIIIb are associated with an increased incidence or severity of various autoimmune or infectious diseases.[349] For example, the 131H allele of FcγRIIa, which is the only FcγR that mediates efficient phagocytosis of

TABLE 22-6 Human Phagocyte Fcγ Receptors

	FcγRIa (CD64)	**FcγRIIa (CD32)**	**FcγRIIIa, b (CD16)**
Polymorphisms		131R/H	IIIa: 48L/R/H, 158F/V IIIb: NA-1, NA-2
Cell distribution	Monocytes Macrophages IFN-γ– or G-CSF–treated neutrophils IFN-γ–treated eosinophils	Monocytes Neutrophils Macrophages Eosinophils Basophils Platelets	IIIa: macrophages, monocytes (some), T cells, NK cells IIIb: neutrophils, IFN-γ–treated eosinophils
Protein size, type	72 kD, transmembrane	40 kD, transmembrane	50-90 kD IIIa: transmembrane IIIb: GPI anchored
Associated proteins	FcRγ homodimer	—	IIIa: FcRγ homodimer (macrophage, monocyte) IIIb: colligation with FcγRIIa or CR3
Ligands	Monomeric IgG (G1 = G3 > G4 > G2)	Complexed IgG (G1 = G3 > G2, G4) (FcγRIIa 131H-G2)	Complexed IgG (G1 = G3 > G2, G4)
Affinity	High	Low	Low
Function	Phagocytosis Respiratory burst Endocytosis of IC ADCC Antigen presentation	Phagocytosis Respiratory burst Exocytosis Endocytosis of IC ADCC Antigen presentation	Phagocytosis Exocytosis Endocytosis of IC ADCC (IIIa) Antigen presentation (IIIa)

ADCC, Antibody-dependent cellular cytotoxicity; *FcγR,* Fc receptor for IgG; *G-CSF,* granulocyte colony stimulating factor; *GPI,* glycosylphosphatidylinositol; *IC,* immune complex; *IFN-γ,* interferon-γ; IgG, immunoglobulin G; *NA,* neutrophil antigen; *NK,* natural killer.

IgG$_2$-opsonized bacteria, has been linked to a lower incidence of infections with encapsulated bacteria.[325]

Cross-linking of FcγRs can trigger a wide range of functional responses, including phagocytosis of IgG-coated microbes, blood cells, or tumor cells; ingestion of immune complexes; release of inflammatory mediators; activation of the respiratory burst; and antibody-dependent cellular cytotoxicity (ADCC). The relative roles of different phagocyte FcγR classes have not been clearly delineated, although all can function as receptors for opsonized particles or immune complexes. Binding of IgG to FcγRI and FcγRIIa activates the respiratory burst, whereas secretion of granular contents is a prominent response upon binding to FcγRII and FcγRIIIb.[325] Although the ligand-binding domains of different FcγRs all bind IgG, the specificity of the cellular response may be governed by the unique transmembrane and cytoplasmic domains of a particular FcR subtype.[344,345]

Ligation and cross-linking of FcγRs initiates a cascade of biochemical signals that are initiated by activation of nonreceptor protein-tyrosine kinases of the Src family, which phosphorylate ITAM motifs in the FcγR chain and in FcγRIIa to recruit and activate the Syk tyrosine kinase and further amplify ITAM phosphorylation.[83,350,351] Subsequent activation of several parallel pathways, including activation of PLC, PI3K, and MAP kinase (MAPK)-related pathways, and other targets leads to various cellular responses, including actin and membrane remodeling for particle ingestion, NADPH oxidase activation, and cytokine release. The DAP12 adaptor that normally potentiates these activating signaling pathways may signal for FcR and TLRs on macrophages that express TREM-2.[352]

The opsonization of microbes with secretory IgA antibodies is likely to be important in the clearance of microbes from the mucosal surfaces of the respiratory, GI, and urogenital tracts. Neutrophils and monocytes have an FcR for IgA (FcαR) that has close structural similarities with other members of the FcR family.[353] Ligation of the phagocyte FcαR can trigger phagocytosis, degranulation, and superoxide release.[354,355] IgE antibodies can activate eosinophils and mast cells through the high-affinity FcεRI receptors.

Phagocytosis

Engagement of any of the opsonic receptors initiates phagocytosis of an opsonized particle to form a phagocytic vacuole that encloses the particle.[356,357] The molecular details of this process are incompletely understood, but they involve membrane remodeling and the regulated assembly and disassembly of the actin cytoskeleton in a fashion analogous to the mechanisms that result in cell movement when chemotactic receptors are engaged. FcγR-mediated phagocytosis triggers the extension of long pseudopodia that attach in a zipperlike fashion around the particle and then fuse to form the phagosome, which is then drawn into the cell.[350,351] In contrast, complement-opsonized particles attach to the cell surface only at limited points and "sink" into the cytoplasm in the absence of any pseudopod extension.[351] These morphologic differences are associated with differences in the underlying biochemical pathways. FcR-mediated

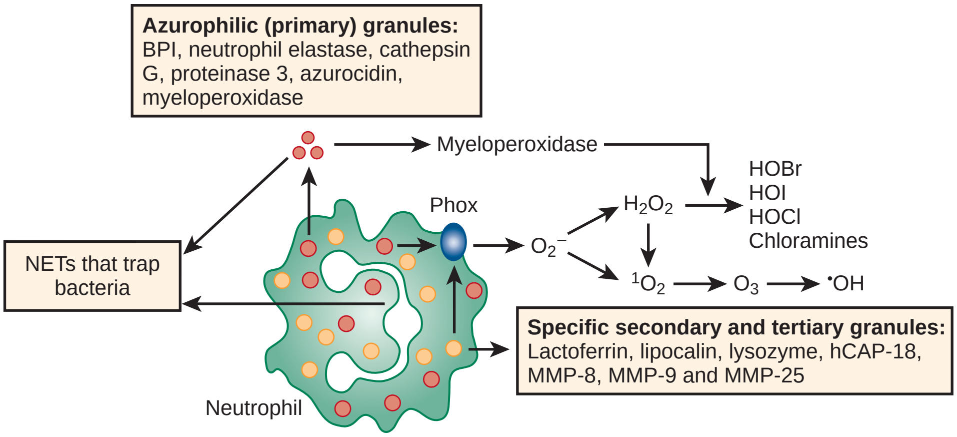

Azurophilic (primary) granules:
BPI, neutrophil elastase, cathepsin G, proteinase 3, azurocidin, myeloperoxidase
Myeloperoxidase
HOBr
HOI
HOCl
Chloramines
Phox
H_2O_2
O_2^-
$^1O_2$
O_3
$^{\bullet}OH$
NETs that trap bacteria
Specific secondary and tertiary granules:
Lactoferrin, lipocalin, lysozyme, hCAP-18, MMP-8, MMP-9 and MMP-25
Neutrophil

Figure 22-7 Neutrophils deliver multiple antimicrobial molecules. Microbicidal products arise from most compartments of the neutrophil: azurophilic granules (also known as primary granules), specific granules (also known as secondary granules), tertiary granules, plasma and phagosomal membranes, the nucleus, and the cytosol. *BPI*, Bactericidal/permeability-increasing protein; *H_2O_2*, hydrogen peroxide; *HOBr*, hypobromous acid; *HOCl*, hypochlorous acid; *HOI*, hypoiodous acid; *MMP*, matrix metalloproteinase; *NETs*, neutrophil extracellular traps; *$^1O_2$*, singlet oxygen; *O_2^-*, superoxide; *O_3*, ozone; *^-OH*, hydroxyl radical; *phox*, phagocyte oxidase. *(Modified from Nathan C: Neutrophils and immunity: challenges and opportunities.* Nat Rev Immunol *6:173–182, 2006.)*

phagocytosis is sensitive to inhibitors of tyrosine kinases and requires the Rac and Cdc42 GTPases, whereas CR3-mediated phagocytosis is dependent upon protein kinase C and the Rho GTPase.

Microorganisms can also enter phagocytes by nonopsonic routes that enable them to evade the cytocidal weapons that are otherwise activated upon phagocytosis. For example, after binding to the macrophage cell surface, *Salmonella typhimurium* induces extensive membrane ruffling that leads to internalization of the bacterium into a macropinosome or "spacious phagosome."[358] *Legionella pneumophila* attachment to macrophages induces the formation of a pseudopod that spirals around the bacterium to form a "coiling phagosome" that does not acidify or fuse with lysosomes.[358]

Cytocidal and Digestive Activity

The binding of ligands to phagocyte chemoattractant and opsonic receptors ultimately leads to the mobilization of phagocyte granules that contain cytotoxic and hydrolytic proteins and to the activation of enzymatic reactions that generate toxic oxygen metabolites (Fig. 22-7). These complementary processes are designed to modify or destroy the inciting object and are often classified as oxygen-independent and oxygen-dependent pathways.

Neutrophil granules are secretory organelles that can be divided into four general classes, as discussed in "Neutrophil Granule Biosynthesis and Classification" (see Table 22-2). Degranulation (also referred to as *exocytosis* or *mobilization*), the fusion of granule membranes with the plasma or phagosome membrane, results in the transfer of granule membrane constituents to a new membrane compartment and the discharge of the granule contents into the extracellular fluid or phagocytic vacuole. Microtubules and microfilaments are involved in granule translocation. Munc13-4, a regulator of vesicle trafficking, is essential for the recruitment of granule membranes to the surface and to the phagosomal membrane.[359] Degranulation is triggered by increases in calcium concentration when neutrophils are activated through chemoattractant and other receptors. Different neutrophil granule populations have marked differences between expression of vesicle-associated membrane protein 2 (VAMP-2), a fusogenic protein involved in exocytosis, which is highest in secretory vesicles, less in gelatinase granules, and still lower in specific granules.[61] Thus different granule classes vary in their responsiveness to calcium, which leads to the mobilization of different granule classes depending on the calcium concentration, which, in turn is proportional to the concentration of the chemoattractant or other activating signal.[51,68,360] Secretory vesicles, whose membranes are storage pools for β_2-integrin–adhesion proteins and other receptors, are mobilized with relatively low concentrations of calcium. The fusion of secretory vesicles with the plasma membranes provides a rapid way of upregulating the cell surface expression of these receptors along with MMP-25, another membrane constituent of secretory vesicles. Gelatinase (tertiary) granules are also easily mobilized for exocytosis at the cell surface to release the gelatinase (MMP-9). These metalloproteases are stored as inactive proforms and become activated by proteolysis after exocytosis to facilitate the breakdown of extracellular matrix during the early phases of neutrophil migration.[305] At the opposite end of the spectrum, azurophil (primary) granules, which contain cytotoxic proteins and hydrolytic enzymes, undergo only limited exocytosis, fusing primarily with phagocytic vacuoles to deliver their contents into a sequestered compartment.

Monocytes and macrophages do not have populations of cytoplasmic storage granules equivalent to those found in neutrophils. Instead, internalized phagosomes fuse sequentially with endocytic vesicles, and subsequently lysosomes, to form a phagolysosome, which has a highly acidic interior rich in hydrolases.[174,350,351]

Certain microorganisms can become intracellular parasites, because they have developed mechanisms to prevent granule fusion with the phagocytic vacuole or otherwise evade the phagocyte digestive and oxidative armamentarium. For example, although mycobacteria exist intracellularly within a phagosome, they produce compounds that inhibit their fusion with lysosomes.[361] *L. pneumophila* and *Toxoplasma* may inhibit acidification and lysosomal fusion.[358] Virulent strains of *Salmonella* engulfed by macrophages produce compounds that prevent translocation of the respiratory burst oxidase to the phagosomal membrane.[362] *Listeria monocytogenes* escapes from the phagocytic vacuole altogether to avoid attack by lysosomal products and can survive in the cytoplasm of relatively quiescent macrophages and hepatocytes.[358] *Yersinia,* group A streptococci, *Helicobacter, Ehrlichia,* and *Francisella* are examples of microbes that have developed similar strategies to survive in neutrophils.[363]

In addition to delivering their antimicrobial granule contents to the interior of phagosomes, dying neutrophils are also capable of extruding weblike extracellular structures, NETs, composed of chromatin and granule proteins that can bind to microbes and may facilitate their killing.[49,50] Formation of NETs appears to involve a novel process of cell death that in some cases is dependent on oxidants generated from the neutrophil NADPH oxidase.[364] Since the discovery of this process, the molecular mechanisms of this process are emerging. It appears that intracellular liberation of elastase from azurophil granules, formation of hypochlorous acid, and decitrullination of histones to loosen the nucleosomes all play a role.[365-370] Experimentally, several agents are used to induce NETs, phorbol myristate acetate being the most common. Not all stimuli induce NETs by the same mechanism and induction by bacteria or by soluble immune complexes are reported to be independent of MPO and the NADPH oxidase.[371,372] Intracellular signals that lead to NETs, depending on the stimulus, include the Ref-MEK-ERK pathway,[373] and protein kinase C isoforms,[374] but also mammalian target of rapamycin–regulated expression of hypoxia-inducible factor 1α is involved in endotoxin-induced NET formation.[375]

Neutrophils from newborns have a reduced capacity to form NETs and a correspondingly reduced capacity to kill bacteria extracellularly.[376] This may relate to a reduction in the content of granule proteins including elastase[376] in neutrophils from newborns.[377]

The view of NETs as an alternative death pathway of neutrophils by which they still serve to defend against microbial invasion[50] is now broadened by observations that demonstrate extrusion of DNA from neutrophils in circulation in a process that requires the participation of platelets to induce NET formation.[378-380] Furthermore, neutrophils may extrude DNA to ensnare bacteria but maintain the capacity to migrate, albeit without the same clear orientation as before becoming anucleate.[381] Formation of NETs are not only to the benefit of the host, because NETs have been shown to promote thrombosis[382-385] and transfusion-related acute lung injury.[386]

Oxygen-Independent Toxicity

Phagocyte granules supply preformed cytotoxic and digestive compounds that play a key role in oxygen-independent killing and digestion of microbes, senescent cells, and particulate debris. In neutrophils, azurophilic (primary) and specific (secondary) granules serve as the main storage reservoir of these compounds. Oxygen-independent pathways complement those dependent on the respiratory burst (see "Oxygen Dependent Toxicity"), and are also important for phagocyte antimicrobial activity under the adverse conditions of hypoxia and acidosis often encountered locally at the site of infection.

Numerous cationic antimicrobial proteins are contained within neutrophil azurophilic granules.[66,360,387,388] Defensins are small (29-25 amino acids) basic peptides that constitute more than 5% of the total cellular protein of human neutrophils, although they are absent in murine neutrophils. These peptides exhibit antimicrobial effects against a broad range of gram-positive and gram-negative organisms, fungi, mycobacteria, and some enveloped viruses. Defensins are also cytotoxic to mammalian cells. Defensins kill target cells by insertion into the cellular membrane and formation of voltage-regulated channels. Defensinlike peptides have also been found in small intestinal Paneth cells and in tracheal epithelium.[387] Bactericidal/permeability-increasing protein (BPI) is a 55-kD cationic protein that has potent cytotoxic effects toward gram-negative bacteria.[389] BPI binds avidly to LPS, leading to both bacterial killing by damaging the cell membrane and to the neutralization of endotoxin associated with the bacterial cell wall and in serum. Serprocidins are a family of 29 kD glycoproteins that are homologous to members of the serine protease superfamily and include azurocidin (CAP37) and four serine proteases (cathepsin G, elastase, proteinase 3, and the recently identified NSP4).[387,390,391] In human neutrophils, serprocidins are even more potent than the defensins in antimicrobial activity and have a broad spectrum of cytotoxicity that is, with few exceptions, unrelated to proteolytic activity. Cathepsin G, elastase, and proteinase 3 are often referred to as neutral proteases because the optimal pH for their proteolytic activity is approximately 7. Azurocidin can bind to endotoxin, which appears to account for its activity for gram-negative bacteria.[392] Azurocidin also is a potent chemoattractant for monocytes as well as fibroblasts and T cells.[392] Exogenous administration of antimicrobial peptides is being studied as an alternative or adjunctive therapy to conventional antibiotics in a number of settings.[387]

Both azurophilic and specific granules contain lysozyme, which hydrolyzes the cell wall of saprophytic gram-positive organisms and may also assist in the nonlytic killing of other organisms.[66] hCAP-18 is a member of the cathelcidin family of antimicrobial peptides, which is cleaved by proteinase-3 after exocytosis. The N-terminal region is homologous to other cathelicidins, whereas the 37-amino acid C-terminal fragment (LL-37) has additional activities, including acting as chemoattractant as well as effects on apoptosis.[61] Specific granules also contain the iron-binding glycoprotein lactoferrin, which has direct bacteriocidal activity both related and unrelated to the chelation of iron compounds required for bacterial metabolism. Lactoferrin may also catalyze the nonenzymatic formation of hydroxyl radicals (OH·)

during the respiratory burst (see “Oxygen-Dependent Toxicity”). Vitamin B_{12} (cobalamin)–binding protein has been proposed to bind the analogous family of compounds found in bacteria to exert an antimicrobial effect.[393] Lipocalin 2, also known as NGAL, interferes with bacterial iron utilization by binding to bacterial ferric-siderophore complexes.[394,395] NGAL-deficient mice demonstrate a major reduction in defense against both *Klebsiella pneumoniae*[396-398] and *Mycobacterium tuberculosis,*[399,400] but NGAL, like most other neutrophil granule proteins, is highly expressed in epithelial cells during inflammation,[61,401,402] and the role of neutrophil-delivered proteins versus epithelial-cell–fabricated proteins has not been determined. Human neutrophil NGAL has, however, been associated with defense against *M. tuberculosis.*[403]

Azurophilic granules contain a variety of hydrolases (see Table 22-2) that have a lower pH optimum (<6), consistent with the lysosomal character of these granules. Studies using indicator dyes and biochemical techniques suggest that after a transient rise, the pH of the phagocytic vacuole falls below 6, which would enhance the activity of these enzymes upon their discharge into the vacuole.[404,405] The acid hydrolases serve primarily a digestive rather than a microbicidal function.[406] Azurophilic granules also contain MPO, which is an important enzyme in microbicidal oxygen-dependent reactions that are described in “Oxygen-Dependent Toxicity.”

Inherited partial or complete deficiency of MPO, which occurs in 0.05% of the population, can occasionally result in increased susceptibility to infection (see “Myelooxidase Deficiency”). Deficiencies in other individual neutrophil granule proteins have not yet been described in humans, but gene-targeted mice lacking the neutrophil granule serine proteases elastase or cathepsin G have impaired host defense against gram-negative sepsis and fungal infections.[407,408] A few rare disorders involving defects in granule formation (SGD) or degranulation (CHS) are associated with recurrent bacterial infections. Inherited mutations in neutrophil elastase are present in patients with cyclic neutropenia (CyN) and severe congenital neutropenia (SCN).[409,410] The mutant forms of elastase may have abnormal properties that exert a toxic effect on granulopoiesis, likely by eliciting an unfolded protein response that induces apoptosis.[411-413]

Oxygen-Dependent Toxicity

The resting neutrophil relies primarily on glycolysis for energy and hence consumes relatively little oxygen.[414] However, within seconds after contacting opsonized microbes or high concentrations of chemoattractants, oxygen consumption increases dramatically, often by more than 100-fold. This “extra respiration of phagocytosis” was first observed in 1933,[415] but it was almost 30 years before it was appreciated that this process was insensitive to mitochondrial poisons and thus not related to increased energy demands.[416] The enzyme complex responsible for this phenomenon, referred to as the NADPH or respiratory burst oxidase, is associated with the plasma and phagolysosomal membranes and catalyzes the transfer of an electron from NADPH to molecular oxygen, thereby forming the superoxide radical (O_2-) (Fig. 22-8).[417-421] Superoxide, although itself a relatively weak microbicidal agent, is the precursor to a family of potent oxidants that are essential for the killing of many microorganisms (see Fig. 22-8).[417,418,422-424] The importance of the respiratory burst to normal host defense is underscored by the recurrent and often life-threatening infections seen in patients with chronic granulomatous disease (CGD), who are genetically deficient in respiratory burst oxidase activity.

The respiratory burst oxidase is a multi-subunit enzyme complex assembled from membrane-bound and soluble

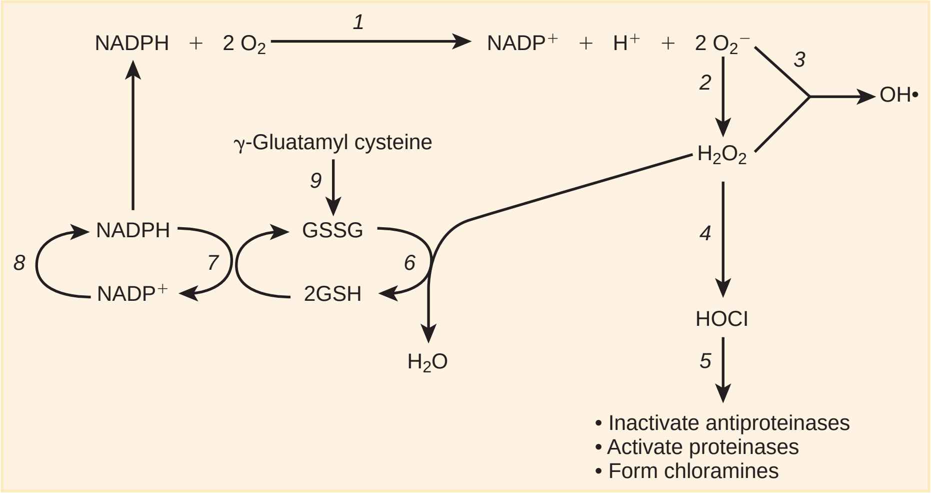


Figure 22-8 Reactions of the respiratory burst pathway. The enzymes responsible for reactions 1 to 9 are as follows: *1,* the respiratory burst oxidase (reduced nicotinamide adenine dinucleotide phosphate *[NADPH]* oxidase); *2,* superoxide dismutase or spontaneous; *3,* nonenzymatic, Fe^{2+} catalyzed; *4,* myeloperoxidase; *5,* spontaneous; *6,* glutathione peroxidase; *7,* glutathione reductase; *8,* glucose-6-phosphate dehydrogenase; *9,* glutathione synthetase. *GSH,* Reduced glutathione; *GSSG,* oxidized glutathione; *NADP,* nicotinamide adenine dinucleotide phosphate. *(Modified from Curnutte J, Orkin S, Dinauer M: Genetic disorders of phagocyte function. In Stamoyannopoulos G, editor:* The molecular basis of blood diseases, *2nd ed. Philadelphia, 1994, WB Saunders, p. 493.)*

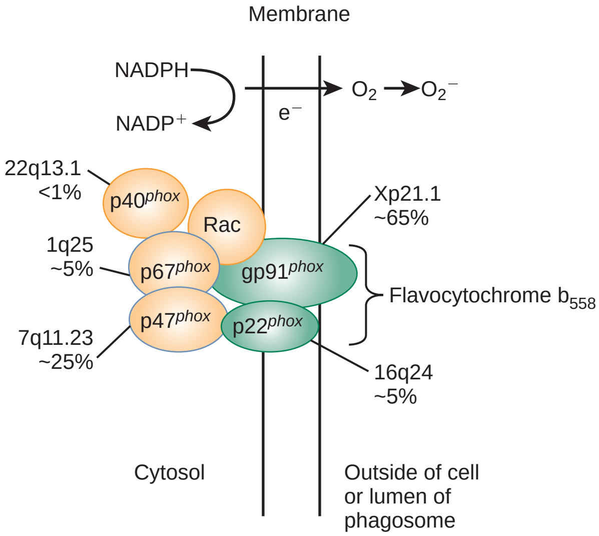


Figure 22-9 Activation of reduced NADPH oxidase. Shown is the assembled form of the enzymatically active NADPH oxidase, along with subunits affected in the four different genetic subgroups of CGD, the approximate incidence, and the chromosomal location of the corresponding gene. Flavocytochrome b_{558}, the redox center of the enzyme, is located in plasma, specific granule, and phagolysosomal membranes. This heterodimer is composed of gp91^{phox} and p22^{phox}, which are affected in X-linked and an AR form of CGD, respectively. Mutations in the genes encoding three soluble regulatory proteins, p47^{phox}, p67^{phox}, and p40^{phox} account for three AR forms of CGD. These subunits are found in the cytosol until phagocyte activation, after which they move as a complex to the membrane to assemble the active NADPH oxidase complex. Membrane translocation of the p47^{phox}-p67^{phox}-p40^{phox} complex is mediated by binding domains on 47^{phox} that are exposed by activation-induced p47^{phox} phosphorylation. Another essential regulatory component of NADPH oxidase is the small GTPase Rac, which in its active GTP-bound state becomes membrane bound and associates with p67^{phox}. By a mechanism that involves an activation domain on p67^{phox}, binding of these multiple regulatory subunits activates the flavocytochrome to catalyze the transfer of electrons from cytosolic NADPH across the membrane via the flavin adenine dinucleotide and heme redox centers to molecular oxygen, thereby forming superoxide in the extracellular or intraphagosomal compartment. No cases of CGD have been reported to result from mutations in Rac. *CGD,* Chronic granulomatous disease; *NADP,* nicotinamide adenine dinucleotide phosphate; *NADPH,* reduced nicotinamide adenine dinucleotide phosphate; O_2, oxygen; O_2^-, superoxide; *phox,* phagocyte oxidase. *(Modified from Dinauer MC:* Chronic granulomatous disease and other disorders of phagocyte function, *Washington, DC, 2005, American Society of Hematology, p. 83–89.)*

proteins upon phagocyte activation (Fig. 22-9). Five polypeptides, gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, and p40^{phox}, that are essential for normal respiratory burst function have been identified (Table 22-7), and mutations in the corresponding genes are responsible for five different genetic subgroups of CGD. The oxidase subunits have been given the designation *phox*, for *ph*agocyte *ox*idase. Neutrophils have the highest expression level of the NADPH oxidase, followed by monocytes, macrophages, eosinophils, and dendritic cells. B lymphocytes also express the NAPDH oxidase, but at very low levels, and even smaller amounts are reported in T lymphocytes.

An unusual b-type flavocytochrome, located in the membrane of secretory vesicles, gelatinase granules, and specific granules, mediates electron transfer in the oxidase complex. It is often referred to as *flavocytochrome* b_{558}, for its spectral peak of light absorbance at 558 nm, or as *flavocytochrome* b_{245} in reference to its midpoint potential of −245 mV, which is extremely low.[425] This flavocytochrome is a heterodimer that contains a 91-kD glycosylated protein, gp91^{phox}, and a nonglycosylated subunit, p22^{phox}.[426-429] The gene for gp91^{phox}, which is the site of mutations in the X-linked form of CGD, was one of the first human disease–associated genes to be identified by positional cloning.[429] gp91^{phox} is the redox center of the oxidase and contains both flavoprotein and heme-binding domains in its cytosolic and membrane-spanning portions, respectively.[419,420] This subunit is also sometimes called *NADPH oxidase (NOX) 2,* referring to its number in a series of homologous flavocytochromes.[430,431] The p22^{phox} subunit is also an integral membrane protein and provides an important docking site for p47^{phox} during NADPH oxidase assembly. Heterodimer formation with gp91^{phox} is essential for heme incorporation and intracellular stability of both flavocytochrome subunits.[432,433]

The p47^{phox}, p67^{phox}, and p40^{phox} subunits are found in the cytosol as a complex that is stabilized by intermolecular interactions that include those mediated by SH3 domains and proline-rich SH3 binding motifs within these proteins.[419,420] Phagocyte activation induces translocation of this complex to the flavocytochrome, which is triggered by the phosphorylation of p47^{phox} to expose additional SH3 domains that bind to a proline-rich target SH3-binding sequence in p22^{phox}.[420,421] The p47^{phox} subunit is necessary for translocation of p67^{phox} to the membrane of activated neutrophils based on studies of p47^{phox}-deficient CGD neutrophils.[419,434] However, substantial amounts of superoxide can be generated from neutrophil membranes in vitro in the absence of p47^{phox}, provided that high concentrations of p67^{phox} and Rac-GTP are supplied.[419] Hence, p47^{phox} acts as an "adaptor" protein to mediate translocation of p67^{phox} and to position it correctly in the active NADPH oxidase complex. The p40^{phox} plays a specialized role in stimulating high levels of superoxide production on phagosome membranes via a domain that binds to the membrane lipid phosphatidylinositol 3-phosphate.[435-439] p40^{phox} may also help with stabilization of p67^{phox} on flavocytochrome b–containing granule membranes as they are recruited to the phagosomes.[440]

The active NADPH oxidase also requires the GTP-bound form of the Rho GTPase, Rac.[82,314,419,420] The main target of Rac-GTP is the p67^{phox} subunit, which contains a Rac-binding domain created by four α-helical tetratricopeptide repeat motifs.[441] The GTP-bound form of Rac also appears to interact with flavocytochrome *b*.[442]

Oxidase assembly is triggered by receptor-mediated binding of many soluble chemoattractants (see Table 22-4), which requires higher concentrations of these molecules compared with the initiation of chemotaxis. The binding of opsonized microbes to Fcγ and complement receptors or of fungi to β-glucan receptors are other major physiologic triggers of the respiratory burst, which is activated at sites of contact.[443,444] Two critical events downstream of receptor binding are the phosphorylation of p47^{phox} and the activation of Rac to its GTP-bound state.[314,419,420] The functional oxidase complex is assembled at the plasma membrane in response to

TABLE 22-7 Properties of the Phagocyte Respiratory Burst Oxidase *(phox)* Components

	gp91^{phox} (NOX2)	**p22^{phox}**	**p47^{phox}**	**p67^{phox}**	**p40^{phox}**
Synonyms	β Chain Heavy chain	α Chain Light chain	NCF-1 SOC II	NCF-2 SOC III	NCF-4
Amino acids	570	195	390	526	339
Gene locus	*CYBB* Xp21.1	*CYBA* 16q24	*NCF1* 7q11.23	*NCF2* 1q25	*NCF4* 22q.13.1
Cellular location in resting neutrophil	Specific granule and secretory vesicle membranes Plasma membrane	Specific granule and secretory vesicle membranes Plasma membrane	Cytosol	Cytosol	Cytosol
Functional domains	Binding sites for heme and FAD NADPH binding sites for cytosolic oxidase components	Proline-rich domain in carboxy-terminal that binds p47^{phox}	9 potential serine phosphorylation sites SH3 domains Proline-rich domains PX domain	SH3 domains Proline-rich domains TPR repeats that bind Rac-GTP PB1 domain	SH3 domain PX domain PB1 domain
Homologies	NOX protein family FNR Yeast ferric iron reductase	Polypeptide I of cytochrome *c* oxidase (weak homology)	NOXO1 Interacts with other NOXs	NOXA1 Interacts with other NOXs	

Modified from Curnutte JT: Molecular basis of the autosomal recessive forms of chronic granulomatous disease. Immunodefic Rev *3:149–172, 1992.*

FAD, Flavin adenine dinucleotide; *FNR,* ferredoxin–nicotinamide adenine dinucleotide phosphate (NADP+) reductase; *GTP,* guanosine triphosphate; *NADPH,* reduced nicotinamide adenine dinucleotide phosphate; *NCF,* neutrophil cytosol factor; *NOX,* NADPH oxidase; *PB,* phox and bem1; *phox,* phagocyte oxidase component; *PX,* phox homology; *SH3,* src homology domain 3; *SOC,* soluble oxidase component; *TPR,* tetratricopeptide repeat.

soluble agonists. During phagocytosis, additional flavocytochrome *b* for NADPH oxidase assembly is delivered to the phagosome by the membrane fusion of specific granules, which contain the majority of the neutrophil's supply of flavocytochrome *b*.[49,66,360,445] Since release of O_2^- occurs largely at the extracellular side of the membrane, oxidants are released at sites of microbial contact or within the phagocytic vacuole, where they can interact with granule contents to potentiate their microbicidal effects.

Based on the redox properties of the flavocytochrome b_{558}, the following pathway has been proposed for transfer of electrons from NADPH to oxygen (O_2) in the respiratory burst (see reaction 1 in Fig. 22-8):

NADPH	flavin	heme	O_2	O_2^-
→	→	→	→	
–330 mV	–256 mV	–245 mV	–160 mV	

The flavocytochrome spans membrane, so that NADPH is oxidized at the cytoplasmic surface and oxygen is reduced to form O_2^- on the outer surface of the plasma membrane (or inner surface of the phagosomal membrane).[417]

Once formed, the O_2^- radical is first converted, either spontaneously or by means of superoxide dismutase, into hydrogen peroxide (H_2O_2; see reaction 2 in Fig. 22-8). Azurophilic granule (derived MPO) in the presence of halides catalyzes the conversion of H_2O_2 to hypochlorous acid (HOCl), the active agent in household bleach (see reaction 4 in Fig. 22-8). H_2O_2 may also be converted into OH· in a nonenzymatic reaction with O_2^- catalyzed by either iron or copper ions (see reaction 3 in Fig. 22-8). H_2O_2, HOCl, and OH· are all strong oxidants that participate in microbial killing within the phagocytic vacuole.[423,424,446] Reactive oxidants also regulate phagocyte proteolytic activity by activating latent phagocyte metalloproteinases (such as collagenase and gelatinase) and inactivating plasma antiproteinases.[68,305] Enhanced phagocyte proteolysis at localized sites may be important for facilitating cellular migration into inflamed tissues, destruction of microbes, and removal of cellular debris.

Other enzymatic pathways related to oxidant generation include the detoxification of H_2O_2 by glutathione peroxidase and reductase (see reactions 6 and 7, Fig. 22-8).[423,424,446] Glutathione is produced from γ-glutamyl cysteine by the enzyme glutathione synthetase (see reaction 9, Fig. 22-8). Other important antioxidant systems in phagocytes and other tissues include catalase, which catalyzes the conversion of H_2O_2 into O_2 and water; ascorbic acid; and α tocopherol (Vitamin E).[447] The generation of NADPH is important in providing a source of reducing equivalents for the glutathione detoxification pathway as well as the respiratory burst itself. NADPH is replenished from $NADP^+$ by leukocyte glucose-6-phosphate dehydrogenase (G6PD; see reaction 8, Fig. 22-8) in the hexose monophosphate shunt.

A second oxygen-dependent pathway with antimicrobial effects involves the generation of NO from the oxidation of L-arginine to L-citrulline. This reaction is catalyzed by NO synthase (NOS), with molecular oxygen supplying the oxygen in NO.[448-450] There are three different NOSs, two of which are constitutively expressed in a variety of tissues, including endothelium, brain, and neutrophils.

Expression of a third, high-output isoform of NOS (inducible NOS [iNOS]) is inducible by inflammatory stimuli in a variety of cells, including macrophages and neutrophils, where it has a wide spectrum of antitumor and antimicrobial activity against bacteria, parasites, helminths, viruses, and tumor cells.[448-450] NO can also interact with superoxide to form peroxynitrite, which mediates tyrosine nitration of cellular and bacterial proteins.[422] High levels of iNOS-catalyzed NO production are readily elicited in normal mouse macrophages by exposure, for example, to IFN-γ and endotoxin. In human monocytes and macrophages, IFN-α/β, IL-4 plus anti-CD23, or chemokines can induce iNOS expression.[449] The expression of iNOS has been detected in a variety of inflammatory and infectious diseases in humans, including malaria, hepatitis C, tuberculosis, tuberculoid leprosy, and acquired immunodeficiency syndrome dementia.[449] Cytokine-activated human neutrophils also exhibit inducible production of NO that leads to nitration of ingested bacteria.[451]

Specialized Functions of Mononuclear Phagocytes

Mononuclear phagocytes, particularly tissue macrophages, participate in a broad range of activities important for tissue homeostasis and repair as well as in the host defense against viruses, bacteria, fungi, and protozoa (Box 22-1).[182-184,188,452,453] From the standpoint of antimicrobial function, activated macrophages play a key role in the ingestion and killing of intracellular parasites, such as mycobacteria, *Listeria, Leishmania, Toxoplasma,* and some fungi,[183,454] although some intracellular organisms have evolved specialized evasion mechanisms.[455] Both oxygen-dependent and oxygen-independent systems are involved in this process, as described. When microbes (most characteristically mycobacteria, but any other organism or particle) cannot be fully ingested or killed, macrophages fuse in a cytokine-mediated process to form giant cells and granulomas.[456,457]

Box 22-1 **Functions of Mononuclear Phagocytes**

INFLAMMATORY RESPONSE AND PATHOGEN CONTROL

Antimicrobial activity
Antiviral activity
Antitumor activity
Secretion of cytokines, eicosanoids, proteases, coagulation factors
Granuloma formation

IMMUNE REGULATION

Antigen processing and presentation
Secretion of cytokines and chemokines

TISSUE HOMEOSTASIS AND REPAIR

- Scavenger function
  - Removal of apoptotic, senescent, or necrotic cells
  - Phagocytosis of debris
- Wound repair
  - Débridement and phagocytosis
  - Secretion of growth factors for endothelial cells and fibroblasts
- Hematopoiesis
  - Secretion of growth factors
- Iron metabolism
- Lipid metabolism

IFN-γ, one of the principal macrophage-activating factors, is secreted by T lymphocytes as well as by macrophages and neutrophils.[102,183,184,232,458] The cytokine induces changes in macrophage gene expression through the JAK–signal transducer and activator of transcription (STAT) pathway, elements of which are shared by many other cytokines, including IL-2, IL-6, and G-CSF.[102,104] Endotoxin, the bacterial LPS derived from gram-negative bacteria, is another important trigger of macrophage activation through pathways involving CD14 and TLRs.[183,184]

The classically activated, or M1 macrophages, are induced and supported by IFN-γ or IFN-β in combination with TNF-α. NK cells or other IFN-γ-producing lymphocytes can initiate this activation, and it may be sustained by an autocrine loop where INF-β and TNF-α are induced by danger signals sensed through TLRs expressed on the macrophage.[183,184] M1 macrophages are indispensable for killing of intracellular pathogens and are also producers of proinflammatory cytokines such as TNF-α, IL-1, IL-6, Il-12, and IL-23. These are also potent activators of T-helper cells (Th1 and Th17).[159,183,184] Macrophages may also develop into the alternatively activated or M2 phenotype that have antiinflammatory functions and promote tissue healing.[459] IL-4 and IL-13 are key inducers of M2 macrophages.[460,461] These cytokines are primarily produced by eosinophil and basophils and CD4+ type 2 helper T cells (Th2 cells); also IL-10, IL-21, and GM-CSF support the M2 phenotype. The M2 macrophages secrete antiinflammatory cytokines, primarily IL-10 and TGF-β. These macrophages are important for resolution of inflammation.[159,183,184,459]

A particular activity of macrophages is the inhibition of antitumor activity of T cells. This is ascribed to myeloid-derived suppressor cells (MDSCs) that share many functional activities with M2 macrophages.[159,462] Neutrophil subsets have also been described that function as inhibitors of T-cell function.[463-466] A common feature of these MDSCs is the ability to deplete T cells of arginase.[467] The development of MDSCs is supported by local factors secreted by tumors, such as TGF-β and GM-CSF. Because many tumors induce a systemic inflammatory response, at least neutrophil MDSCs may represent immature neutrophils liberated from the bone marrow. LPS induces liberation of such immature neutrophils with features characteristic of MDSCs in blood.[66,468]

Interactions of macrophages with T and B cells are essential for the development of cellular and humoral immunity.[78,204,469,470] Macrophage production of IL-1, and subsequent interactions between stimulated T and B cells, leads to B cell production of antigen-specific immunoglobulins. Macrophages are also major physiological sources of cytokines and chemokines that regulate both the innate and adaptive immune systems.[80,182,204,469] As a source of “endogenous pyrogens,” they are responsible for the production of fever in response to infection or inflammation.[471]

Macrophages participate in many aspects of wound repair.[472,473] The early phases of this process are dominated by an influx of neutrophils, followed by the migration of monocytes that differentiate into activated

Box 22-2 **Products Secreted by Mononuclear Phagocytes**

ENZYMES

- Acid ceramidase, lipase, phosphatase, deoxyribonuclease (DNase), ribonuclease (RNase)
- α-Iduronidase, α-L-fucosidase, α-mannosidase, α-neuraminidase
- α-Naphthylesterase
- Angiotensin-converting enzyme
- Arginase
- Aspartylglycosaminidase
- β-Glucuronidase, β-glucosidase, β-galactosidase
- Cathepsins, collagenase, elastase
- Leucine-2-naphthylaminidase
- Lipoprotein lipase
- Lysozyme
- *N*-acetyl-β-galactosaminidase, *N*-acetyl-β-glucosaminidase
- Phospholipase A_2
- Sphingomyelinase
- Sulfatases

OXIDANTS

- Hydroxyl radical, hydrogen peroxide, hypohalous acids, nitric oxide, peroxynitrate, superoxide anion

BINDING PROTEINS

- Avidin, apolipoprotein E, acidic isoferritins, gelsolin, haptoglobin, transcobalamin II, transferrin

COAGULATION SYSTEM PROTEINS

- Factors V, VII, IX, X, XIII
- Tissue factor/procoagulant activity (PCA), plasminogen activator, urokinase, thrombospondin, plasminogen activator inhibitors, plasmin inhibitors

LIPID METABOLITES

- Prostaglandins E_2, F_2, I; thromboxane A_2; leukotrienes B, C, D, E; malonyldialdehyde; mono-dihydroxyeicosatetraenoic acids; platelet-activating factor

COMPLEMENT SYSTEM PROTEINS

- C1, 2, 3, 4, 5; factors B, D, H; properidin; C3b inactivator

CYTOKINES AND GROWTH FACTORS

- Interferons (INFs) α, β, γ
- Interleukin (IL) 1α, 1β, 6, 8
- Tumor necrosis factor (TNF) α
- Fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β)
- Colony-stimulating factors (CSFs) granulocyte (G), granulocyte/monocyte (GM), monocyte (M)
- Macrophage inflammatory proteins (MIPs) 1α, 1β, 2
- Monocyte chemoattractant proteins (MCPs) 1, 2, 3
- Thymosin B_4

MATRIX PROTEINS

- Fibronectin, proteoglycans, thrombospondin

OTHER METABOLITES, PEPTIDES, AND PROTEINS

- Glutathione, purines, pyrimidines
- Sterol hormones, including 1α-25-dihydroxyvitamin D_3

Modified from Nathan C: Secretory products of mononuclear phagocytes. J Clin Invest *79:319, 1987. Reproduced by copyright permission of the American Society for Clinical Investigation.*

macrophages, and finally, the appearance of T lymphocytes. Proliferating fibroblasts secrete collage and other matrix proteins important for wound closure and tissue remodeling, and migrating keratinocytes regenerate the epithelial surface. Both neutrophils and macrophages protect against infection and dispose of phagocytosed debris. Mononuclear phagocytes also elaborate fibroblast, epithelial, and angiogenic growth factors (Box 22-2), which stimulate the normal progression of tissue repair and neovascularization that characterize the later phases of wound healing.[185,472,473]

Macrophages ingest and dispose of apoptotic and necrotic cells.[224,452,474-476] This function not only contributes to cellular processing for antigen presentation but also plays a critical role in tissue remodeling and homeostasis, embryological development, and the resolution of inflammation.[9] A redundant and promiscuous system of receptors including integrins, scavenger receptors, complement receptors, calreticulin, CD14, and Mer receptor recognize and effect the uptake of apoptotic cells. Resident tissue macrophages, particularly those lining the sinusoids of the spleen and liver (formerly known as the *reticuloendothelial system*), clear senescent and antibody-bound blood cells from the circulation.[187,477,478]

Iron from the catabolism of hemoglobin in aged erythrocytes is incorporated into ferritin and hemosiderin, where it accounts for about two-thirds of the body's store of reserve iron. Iron in this macrophage storage pool turns over and returns in a transferrin-bound form to the bone marrow for new red blood cell synthesis (see Chapter 11).[479] Sequestration of iron in macrophages leads to the anemia of chronic disease.[480] This is largely regulated by hepcidin, a small peptide synthesized by the liver in response to Il-6, which binds to and induces degradation of ferroportin, the mediator of iron export from macrophages and enterocytes.[481-485]

Monocytes and macrophages contribute to the pathophysiology of atherosclerosis through the uptake, metabolism, and oxidation of LDL and very low density lipoprotein by receptor-mediated endocytosis.[486,487] Macrophages in blood vessel walls that are exposed to sufficient quantities of cholesterol develop into foam cells characteristic of atherosclerotic plaques[488] and contribute to the important inflammatory component of plaque formation and destabilization.[489]

Specialized Functions of Eosinophils and Basophils

Eosinophils and basophils, which share many of the functional characteristics of neutrophils and mononuclear phagocytes, participate in distinctive aspects of the inflammatory response and interact with each other in the context of certain allergic reactions.[119,122,123] Eosinophils and mast cells are often situated beneath epithelial surfaces exposed to environmental antigens, such as the respiratory and GI tracts, where they may be actively involved in mucosal immune responses. However, the role of eosinophils, basophils, and mast cells are better known in pathologic settings than in normal homeostasis.

Eosinophils appear to have both immunoenhancing and immunosuppressive functions (Table 22-8).[14,117,119,122,123] Although capable of ingesting and killing bacteria, eosinophils are not particularly efficient at this task. Rather, they possess an unusual ability to destroy invasive metazoan parasites, especially helminthic parasites. Eosinophils bind to the surface of both adult and larval helminths and inflict damage through release of cationic granule proteins and by the generation of reactive oxidants, including the eosinophil peroxidase–catalyzed formation of hypohalous acids via the action of the respiratory burst and eosinophil peroxidase.[14,117]

Eosinophil production of the lipid inflammatory mediators, leukotriene C4 and PAF, play a role in the pathogenesis of allergic diseases.[14] PAF and leukotriene C_4 can induce smooth muscle contraction and promote the

TABLE 22-8 Eosinophil Function

Function	Mechanism
Defense against helminths (both larval and adult forms)	Binding of eosinophils to surface Peroxidation of larval surface mediated by eosinophil peroxidase Toxicity to larval surface by released major basic protein (MBP)
Immunosuppression of immediate hypersensitivity reactions	Engulfment of most cell granules Release of prostaglandin E_1/E_2 to suppress basophil degranulation Release of histaminase Oxidation of slow-reacting substance of anaphylaxis Release of phospholipase D to inactive mast cell platelet-activating factor Release of MBP for binding of mast cell heparin Release of plasminogen to reduce local thrombus formation

secretion of mucous, and PAF itself is a potent activator of eosinophils. The release of eosinophil granule contents may also contribute to localized tissue damage. Purified eosinophil MBP, for example, can cause cytopathic changes in tracheal epithelium in vitro that are similar to the changes observed in asthmatic patients.

Eosinophils may also perform an immunosuppressive function in immediate hypersensitivity reactions (see Table 22-8). IgE-activated basophils or mast cells release eosinophilic chemotactic factor of anaphylaxis, which recruit eosinophils to the site. Subsequent eosinophil degranulation releases products that can inactivate inflammatory mediators. For example, histaminase inactivates histamine, phospholipase B inactivates PAF, MBP inactivates mast cell heparin, and lysophospholipase prevents the generation of arachidonic acid metabolites.[490]

Basophils and mast cells are central participants in a variety of inflammatory and immunologic disorders, particularly immediate hypersensitivity diseases, and they may also play a role in host defense against bacterial infections.[122,491,492] Basophils and mast cells express plasma membrane receptors that specifically bind with high affinity the Fc portion of the IgE antibody (Fcε receptors).[346] After active or passive sensitization with IgE, exposure to specific multivalent antigen triggers an almost immediate release of granule contents (anaphylactic degranulation) and the synthesis and release of newly generated chemical mediators such as leukotriene C_4, which stimulates smooth muscle contraction, mucous secretion, and vasoactive changes. Degranulation can also be triggered in response to insect venoms, radiocontrast dye, and other irritants. Studies conducted in 1994 on mutant mice engineered by gene targeting to lack Fcγ and/or Fcε receptors have shown that mast cell FcγRIII receptors are essential in activating the inflammatory response to IgG immune complexes (Arthus reaction), heretofore an unrecognized role for the mast cell.[493] Mast cells can secrete numerous mitogenic or inflammatory cytokines, including many ILs (1, 3, 4, 5, and 6), chemokines, GM-CSF, and TNF-α, that are also likely to regulate leukocyte recruitment and inflammation in IgE-dependent reactions and immune complex injury.[158,159] Inflammatory cytokines and leukotrienes released from tissue mast cells have been recognized to play an important role in neutrophil recruitment during the acute response to bacterial infection.[494-497]

Pathologic Consequences of Phagocyte Activation and Inflammatory Response

Although it normally serves a protective function, the inflammatory response can also result in damage to host tissues. The release of proteases, oxygen radicals, and proinflammatory cytokines by activated phagocytes appears to play a major role in the generation of tissue injury in a wide variety of pathologic inflammatory processes (Box 22-3).[222,266,305,498-500] For example, neutrophil elastase has been implicated in the pathogenesis of emphysema in both adult smokers and individuals with α1-antitrypsin deficiency. Neutrophil granule proteases may contribute to the joint destruction in rheumatoid arthritis and other chronic arthropathies. Neutrophils are also believed to play a key role in the systemic inflammatory response syndrome, a term that has been created to encompass the host response to both infectious (e.g. gram-negative sepsis) and noninfectious (e.g. pancreatitis, trauma) etiologies, and can lead to organ dysfunction and tissue damage.[500] Sequestration of activated neutrophils in the pulmonary capillary bed and subsequent release of tissue-damaging agents is an important component in the development of adult respiratory distress syndrome. Activation of the complement cascade by artificial membrane surfaces during hemodialysis and cardiopulmonary bypass also can result in neutrophil activation, intrapulmonary sequestration, and lung injury. Macrophages are integral to the pathophysiology of atherosclerosis by uptake of serum lipoproteins and contribute an important inflammatory component that influences atherosclerotic plaque development and rupture.[489,501] In addition to their cytotoxic effects, oxidative products released by activated phagocytes are also mutagenic, as documented by plasmid mutagenesis, sister chromatid exchange, and transformation of cells in culture.[502,503] Hence the increased risk of malignancy observed with certain chronic inflammatory states, such as ulcerative colitis or chronic hepatitis, has been postulated to be in part related to oxidant-induced carcinogenesis.

Box 22-3 **Selected Pathologic Inflammatory Reactions Associated with Phagocyte-Induced Tissue Injury**

Arthus reaction
Systemic inflammatory response syndrome
Nephrotoxic and immune complex nephritis
Postischemic myocardial damage
Adult respiratory distress syndrome
Atherosclerosis
Bronchiectasis
Acute and chronic allograft rejection
Malignant transformation with chronic inflammation
Rheumatoid arthritis

The development of antiinflammatory interventions based on agents that block leukocyte adhesion or inhibit the action of specific proinflammatory agents has been an area of intense interest. IL-1 receptor antagonists are used to treat autoinflammatory diseases such as familial Mediterranean fever as well as rheumatoid arthritis and are being evaluated in a broad range of inflammation-associated states.[231] TNF-α antagonists are now widely used for treatment of inflammatory bowel disease, rheumatoid arthritis, and other autoimmune disorders.[231,504] Antagonists of leukocyte integrins have shown benefit in phase II and III studies for inflammatory bowel disease, psoriasis, and multiple sclerosis.[263] However, although protective effects of monoclonal antibodies directed against β_2 integrins, ICAM-1, or selectins were found in various animal models of inflammation including ischemia-reperfusion injury, endotoxic shock, and acute arthritis, results from clinical trials failed to show significant benefit.[263] The contributions of nonphagocytic cells to inflammatory tissue injury must also be kept in mind. For example, the adult respiratory distress syndrome can occur in the presence of severe neutropenia.[505] Moreover, despite the adverse consequences of the acute inflammatory process, these events are also important for normal healing. For example, the use of antiinflammatory agents in myocardial infarction, which can decrease infarct size acutely, results in impaired healing of the myocardium and the formation of fragile scar tissue.[498] Finally, the impact of the new antiinflammatory biologic agents on host defense must be kept in mind, particularly if such agents are being used in combination with other immunosuppressive drugs such as steroids. For example, patients receiving TNF-α antagonists have increased rates of tuberculosis and infections with endemic mycosis or intracellular bacterial pathogens.[506]

QUANTITATIVE GRANULOCYTE AND MONONUCLEAR PHAGOCYTE DISORDERS

This section reviews clinical disorders associated with disturbances in granulocyte number, with particular emphasis on syndromes in which the granulocyte abnormality is a central feature. Several disorders have now been associated with specific genetic defects, although the molecular pathophysiology leading from the gene to the phenotype often remains obscure. This section of the chapter is organized by clinical characteristics of the disorders, with genetic and functional laboratory data provided where available.

Neutropenia

Definition and Classification

Neutropenia is defined as a decrease in the absolute number of circulating neutrophils in the blood. Normal neutrophil levels should be stratified for age, race, and other factors. For whites, the lower limit for normal absolute neutrophil count (ANC; total neutrophils and bands) is 1000 cells per µL in infants between 2 weeks and 1 year of age; then it rises after infancy to 1500 cells per µL. Persons of African descent have somewhat lower neutrophil counts, with ANC less than 1500 cells per µL in 4.5% of African-American participants in one U.S. survey.[507] This is entirely attributable to the lower ANC in the subpopulation with the Duffy negative (Fy–/–) blood group.[508] This blood group, selectively enriched in populations in the malarial belt of Africa, is associated with ANCs 200 to 600 cells per µL less than those who are Duffy positive. Falsely low white blood cell counts can result when counts are done long after blood is drawn or after leukocyte clumping in small clots or in the presence of certain paraproteins.

Neutropenia can represent disturbances in neutrophil production, altered distribution of neutrophils between the circulating and the marginated or tissue pools (pseudoneutropenia), increased peripheral utilization or destruction, or combinations of these causes. However, assays of leukokinetics and myelopoiesis are not routinely available, so mechanisms may be hard to identify. Classifications based on biochemical or functional studies are also difficult because of the paucity of neutrophils in the circulation of neutropenic patients. In general, neutropenic syndromes are broadly classified as intrinsic because of defects in myelopoiesis or as acquired, as a result of extrinsic factors such as drugs, infections, or autoantibodies (Box 22-4). Congenital neutropenias are now classified largely on the basis of known genetic defects.

The clinical significance of neutropenia depends upon the level of depression of the ANC. Mild neutropenia with neutrophil counts of 1000 to 1500 cells per µL and moderate neutropenia with counts of 500 to 1000 cells per µL are generally not clinically significant with respect to host defense, but these situations may warrant evaluation to determine the underlying cause. Severe neutropenia refers to an ANC below 500 cells per µL, with the term *agranulocytosis* usually reserved for counts

Box 22-4 Classification of Neutropenia

NEUTROPENIA CAUSED BY INTRINSIC DEFECTS IN GRANULOCYTES OR THEIR PROGENITORS*

Reticular dysgenesis
Severe congenital neutropenia
Cyclic neutropenia
Myelokathexis/WHIM syndrome
Shwachman-Diamond syndrome
Albinism/neutropenia syndromes (including Chédiak-Higashi syndrome)
Familial benign neutropenia
Bone marrow failure syndromes (congenital and acquired)
Neutropenia associated with immune deficiency disorders
Neutropenia associated with metabolic disorders

NEUTROPENIA CAUSED BY EXTRINSIC FACTORS

Infection
Drug-induced
Autoimmune
Neonatal immune
Metabolic diseases
Nutritional deficiencies
Reticuloendothelial sequestration
Bone marrow infiltration
Chronic idiopathic neutropenia (may also be intrinsic)

WHIM, Warts, hypogammaglobulinemia, infections, myelokathexis.
*Types of neutropenia are listed in order of discussion in the text.

below 200 cells per μL. Children with severe chronic neutropenia can be registered with the Severe Chronic Neutropenia International Registry (SCNIR; http://depts.washington.edu/registry/).

Symptoms of Neutropenia

The hallmark of neutropenia is an increased susceptibility to bacterial and fungal infection; neutropenia by itself does not diminish host defense to viral or parasitic infections. The most common types of pyogenic infections in patients with significant neutropenia are cellulitis, furunculosis, superficial or deep cutaneous abscesses, pneumonia, and septicemia.[509-512] Stomatitis, gingivitis, and periodontitis may be presenting signs of neutropenia that develop into chronic problems. Perirectal inflammation and otitis media (especially in younger children) occur as well. Endogenous bacteria are the most common cause of infections, but colonization or infection may occur with a variety of nosocomial and opportunistic organisms. The most commonly isolated organisms from neutropenic patients are *Staphylococcus aureus* and gram-negative bacteria, but almost any organism can appear, particularly in hospitalized patients or those with frequent or prolonged courses of antibiotics. The usual symptoms and signs of local infection—such as exudates, fluctuation, ulceration, and regional adenopathy—may be less evident in patients with neutropenia than in normal individuals.[513]

Susceptibility to bacterial infection, even with severe neutropenia, can be quite variable, depending on the underlying etiology. For example, some patients with chronic neutropenia resulting from autoantibodies do not experience serious infections over a period of many years even with neutrophil counts as low as 200 cells per μL, most likely because these individuals have rapid production and transit of normal neutrophils with relative preservation of neutrophil delivery to tissues.[514] Many patients with chronic neutropenia also have normal to increased numbers of circulating monocytes, an alternative phagocyte.[515] However, the recruitment of monocytes to inflammatory sites is delayed relative to neutrophils, and monocytes are not as efficient as neutrophils in ingesting bacteria.[516,517] Thus monocytes appear to provide only marginal protection against pyogenic organisms in patients with severe neutropenia. It is likely that the humoral, cell-mediated, and tissue macrophage immune systems also play important alternative or compensatory roles preventing infection in these individuals.

Neutropenia Caused by Intrinsic Defects in Granulocytes or Their Progenitors

These disorders are also discussed in Chapter 8.

Reticular Dysgenesis. The selective failure of stem cells committed to myeloid and lymphoid development leads to reticular dysgenesis, one of the rarest and most severe forms of severe combined immunodeficiency.[518-521] It is characterized by severe leucopenia—including both agranulocytosis and lymphopenia; defective cellular and humoral immunity functions; and absence of lymph nodes, tonsils, Peyer's patches, and splenic follicles—as well as sensorineural hearing loss. Erythroid and megakaryocyte development is normal. All reported infants have died of bacterial or viral infections within the first weeks of life, except a few treated with hematopoietic stem cell transplantation (HSCT).[522-524] The underlying genetic defects in this AR disorder are biallelic mutations in the *AK2* gene, encoding the mitochondrial energy metabolism enzyme adenylate kinase 2.[525,526] As discussed below, other mitochondrial enzyme deficiencies have been associated with isolated neutropenia.

Severe Congenital Neutropenia. SCN was first described by Kostmann in 1956 as an AR disorder associated with severe neutropenia that was identified in the population of an isolated northern parish of Sweden.[527] Other forms of SCN have since been identified with de novo occurrence and AD, AR, or X-linked inheritance.[528-531] *SCN* (Online Mendelian Inheritance in Man [OMIM] # 202700) refers to the entire disorder regardless of inheritance or genotype, whereas Kostmann disease (OMIM # 610738) refers to the AR subtype caused by mutations in the *HAX1* gene[532] (see later in this section). This disorder is also discussed in Chapter 8.

The incidence of SCN is approximately two cases per million population.[533] Affected patients generally maintain ANCs below 200 cells per μL, which has been documented on the first day of life in several cases.[534] Episodes of fever, skin infections (including omphalitis), stomatitis, pneumonia, and perirectal abscesses typically appear during the first months of life. Infections often disseminate to the blood, meninges, and peritoneum, and are usually caused by *S. aureus*, *Escherichia coli,* and *Pseudomonas* species. Before the current era of G-CSF therapy, most patients died in the first 1 to 2 years of life,[530,535] and survivors were known to be at risk for developing myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML).[535-537]

Peripheral blood counts generally show, in addition to agranulocytosis, moderate to marked monocytosis, mild to moderate eosinophilia, and secondary changes such as anemia and thrombocytosis. G-CSF production in patients with SCN appears to be normal, if not elevated.[538,539] Antineutrophil antibodies may be present, and a positive test should not be used to rule out SCN.[540]

Bone marrow examination characteristically shows a myeloid "maturation arrest" at the myelocyte stage of development, with normal to increased numbers of promyelocytes but few if any more mature forms.[528,533] The promyelocytes may show dysplastic morphology, including large size, atypical nuclei, and vacuolated cytoplasm. Marrow eosinophilia and monocytosis is common and does not change during treatment. Cellularity is usually normal or slightly decreased. Megakaryocytes are normal in number and morphology.

The genetic basis of 40% to 80% of SCN cases derives from mutations in the *ELANE* gene (formerly termed *ELA2*),[409,541,542,543] which encodes neutrophil elastase and is also responsible for the related but distinct disorder, CyN (discussed later in this section). *ELANE*-related SCN may arise sporadically by de novo mutation or appear in kindreds with an AD Mendelian inheritance pattern, most

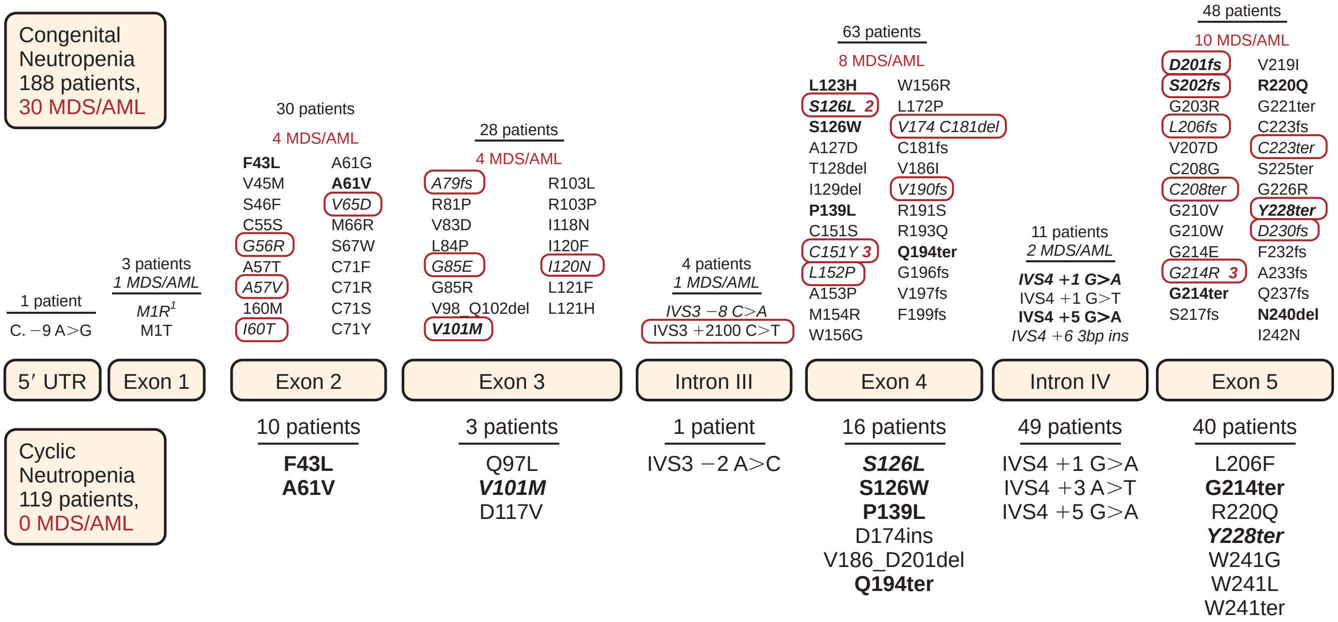

Congenital Neutropenia 188 patients, 30 MDS/AML
1 patient
C. −9 A>G
3 patients
1 MDS/AML
M1R1
M1T
30 patients
4 MDS/AML
F43L
V45M
S46F
C55S
G56R
A57T
A57V
160M
I60T
A61G
A61V
V65D
M66R
S67W
C71F
C71R
C71S
C71Y
28 patients
4 MDS/AML
A79fs
R81P
V83D
L84P
G85E
G85R
V98_Q102del
V101M
R103L
R103P
I118N
I120F
I120N
L121F
L121H
4 patients
1 MDS/AML
IVS3 −8 C>A
IVS3 +2100 C>T
63 patients
8 MDS/AML
L123H
S126L 2
S126W
A127D
T128del
I129del
P139L
C151S
C151Y 3
L152P
A153P
M154R
W156G
W156R
L172P
V174 C181del
C181fs
V186I
V190fs
R191S
R193Q
Q194ter
G196fs
V197fs
F199fs
11 patients
2 MDS/AML
IVS4 +1 G>A
IVS4 +1 G>T
IVS4 +5 G>A
IVS4 +6 3bp ins
48 patients
10 MDS/AML
D201fs
S202fs
G203R
L206fs
V207D
C208G
C208ter
G210V
G210W
G214E
G214R 3
G214ter
S217fs
V219I
R220Q
G221ter
C223fs
C223ter
S225ter
G226R
Y228ter
D230fs
F232fs
A233fs
Q237fs
N240del
I242N
5′ UTR
Exon 1
Exon 2
Exon 3
Intron III
Exon 4
Intron IV
Exon 5
Cyclic Neutropenia 119 patients, 0 MDS/AML
10 patients
F43L
A61V
3 patients
Q97L
V101M
D117V
1 patient
IVS3 −2 A>C
16 patients
S126L
S126W
P139L
D174ins
V186_D201del
Q194ter
49 patients
IVS4 +1 G>A
IVS4 +3 A>T
IVS4 +5 G>A
40 patients
L206F
G214ter
R220Q
Y228ter
W241G
W241L
W241ter

Figure 22-10 Linear representation of the *ELANE* gene showing the locations and types of mutations observed in 188 patients with SCN *(upper panel)* and 119 patients with CyN *(lower panel)* from the Severe Chronic Neutropenia International Registry. *Red boxes* indicate mutations associated with myelodysplastic syndrome and acute myelogenous leukemia *(MDS/AML)*, *red numbers* within the boxes indicate the numbers of affected patients if there are more than one, and *red numbers* in the summary boxes in the left margin indicate total numbers of patients with MDS/AML. Human Genome Variation Society nomenclature was used to describe the mutations. *(Data from Makaryan V, Zeidler C, Bolyard AA, et al: The severe chronic neutropenia international registry and repository, clinical outcomes for patients with severe chronic neutropenia due to mutations in the gene for neutrophil elastase.* Elane *[ASH Annual Meeting Abstracts] 120:3275, 2012.)*

dramatically illustrated by the six affected children from five families, all conceived by sperm from the same anonymous donor.[529,544]

The SCNIR analyzed 188 patients with SCN who had 94 mutations and 119 patients with CyN who had 22 mutations (see Fig. 22-10).[545] Most *ELANE* mutations resulted in only an SCN or the cyclic phenotype (Fig. 22-10), but 12 mutations were observed in both patients with CyN and SCN. A number of recurring mutations were observed only in CyN, including Q97L, D174ins, and W241G and conversely, a number of recurring mutations were observed only in SCN, including A57T, R103P, and G214R.

How mutations in neutrophil elastase result in the cyclical production of neutrophils and other blood cells is not entirely understood. Bone marrow samples from both patients with CyN and patients with SCN demonstrate increased apoptosis of neutrophil precursors.[546-549] Thus expression of mutant neutrophil elastase appears to induce excessive apoptosis, most likely through either aberrant subcellular targeting of the protein[548] or induction of a strong unfolded protein response.[411,412,550]

Additional very rare cases of AD SCN appear to arise from mutations in the *Gfi1* gene,[551] which encodes a transcriptional repressor that inhibits multiple genes involved in neutrophil maturation, including *ELANE*.[17,18] Resultant overexpression of *ELANE* could lead to apoptosis by either of the mechanisms discussed previously.

AR SCN can be caused by defects in several genes, including *HAX1*, an antiapoptotic gene that is responsible for the classic Kostmann syndrome,[532] and *G6PC3*. *HAX1* encodes a multifunctional protein that is involved in the regulation of apoptosis, as well as cell motility, signal transduction, and mRNA processing.[552-554] Other genes associated with AR SCN include *VPS45*, which encodes an endosomal trafficking regulator,[555,556] and *G6PC3*, which encodes a neutrophil-specific catalytic subunit of glucose-6-phosphatase and may thus be considered functionally similar to glycogen storage disease type 1b (see later discussion in this section) but with pathology limited to the neutrophil.[557] In contrast to *ELANE*, which is expressed only in myeloid cells, these genes are broadly expressed in many tissues and organs. As a result, some patients with *HAX1* protein defects have associated neurological deficits; *G6PC3* defects were originally described in a cohort of patients with cardiac anomalies, urogenital abnormalities, and venous angiectasias[558]; and *VPS45* mutations are associated with neutrophil dysfunction, bone marrow fibrosis, and nephromegaly. However, both *HAX1* and *G6PC3* mutations can also be responsible for isolated neutropenia without an extended phenotype.[559,560] *G6PC3* has also been associated with rare cases of atypical CyN,[561] indicating that, as for *ELANE*, the same mutation can produce either the severe or CyN phenotype depending upon the genetic background.

Other rare mutations responsible for an SCN phenotype include gain-of-function mutations in the X-linked Wiskott-Aldrich syndrome (WAS) gene[562,563] and constitutional defects (as opposed to acquired mutations; see later) in the gene encoding the G-CSF receptor.[564,565] Overall, approximately 60% of patients with a diagnosis of SCN can now be given a precise genetic diagnosis.

G-CSF, the standard treatment modality for SCN, has greatly improved both life span and quality of life for these patients.[566-570] Until the availability of this cytokine in the 1980s, severe neutropenia would inevitably lead to fatal infections in the majority of patients, despite supportive care with prophylactic trimethoprim-sulfamethoxazole, aggressive use of antibiotics at the time of documented infections, and scrupulous attention to oral hygiene. More than 95% of patients with SCN respond to G-CSF treatment with an increase in the ANC to greater than 1000 per µL, with documented reduction in infections.[530,566,571,572] There is often a delay of 7 to 10 days before the rise of peripheral blood neutrophil counts at the start of treatment. In patients enrolled in the SCNIR between 1993 and 1999, more than 90% of patients with congenital neutropenia responded to G-CSF treatment with rises in ANC to more than 1×10^9/l and required significantly less antibiotic therapy and fewer hospitalizations.[531] SCN patients required G-CSF doses ranging from 0.01 to 242 µg/kg/day (mean 12.6 µg/kg/day and standard deviation [SD] 19.3 µg/kg/day).[567] These doses are significantly higher than those needed for cyclic or idiopathic neutropenia.[530,567] The percentage of maturing neutrophils in the bone marrow increases with G-CSF, although morphologically abnormal promyelocytes with vacuolized and asynchronous nuclear-cytoplasmic maturation can persist, and the number of monocytes and eosinophils in the bone marrow and peripheral blood may remain high.[533]

Based on data from the SCNIR, a starting daily dose of G-CSF at 5 µg/kg/day is recommended, increasing gradually if necessary to as high as 100 µg/kg/day for at least 14 days at each dose to achieve a neutrophil count of 1000 to 2000 per µL.[572-574] The drug is generally administered subcutaneously but may be given at equal dosage intravenously if clinical conditions warrant or if vehicle volumes are too high to permit comfortable subcutaneous injection. In G-CSF refractory patients, combination therapy with SCF has been reported to be effective[533] but rarely used, because it also leads to mast cell activation with histamine release and anaphylactoid reactions. GM-CSF treatment results in large increases in eosinophils and monocytes, but not neutrophils.[575]

Side effects of G-CSF treatment are usually (but not always) mild and include bone pain, headache, and rashes. Other common but often asymptomatic findings include splenomegaly and osteopenia.[530,576] Splenomegaly has been reported in patients with SCN before and with G-CSF therapy.[530,576] Palpable splenomegaly, with a median spleen measurement of 2 cm below costal margin was recorded in 18.2% of untreated patients registered in the SCNIR. During the first year of G-CSF therapy, the incidence increased to 38.2% and remained at this level of occurrence (26.7% to 44.7%) through 10 years of therapy, with no trend to progression in spleen size on therapy.[530]

Of 128 SCNIR subjects analyzed in 2010,[577] including 40 children younger than age 21 years and 122 subjects receiving G-CSF, 46% of adults met criteria for osteopenia (t score −1), 9% met criteria for osteoporosis (t score <–2.5), and17.5% of children had low bone mineral density for age (Z score <–2.0). In children but not adults, bone mineral density was inversely related to duration of G-CSF therapy. The SCNIR recommends regular monitoring of bone density.[530] Rare patients have also been found to have vasculitis or glomerulonephritis, both before and during G-CSF therapy.[530]

The SCNIR has reported thrombocytopenia (platelet count $<50 \times 10^3/\mu L$) in 26 patients, including 17 with SCN.[530] Among the 19 patients receiving G-CSF, seven developed thrombocytopenia concurrent with conversion to MDS/AML. Thus development of thrombocytopenia in SCN patients requires thorough evaluation as a possible heralding sign of disease conversion.

Conversion to MDS/AML and bacterial sepsis remain the most serious complications of SCN. The predilection to acute leukemia was recognized before the availability of G-CSF,[535,536] when most affected children died early in life from severe infections. Now longer survival and G-CSF itself combine to produce, after 10 years of therapy, an ongoing 2.3% per year risk of developing AML or MDS that almost invariably progresses to AML.[512]

During long-term therapy with G-CSF, some SCN patients acquire mutations in the G-CSF receptor gene (*CSF3R*), particularly C-terminal truncation mutations that confer a clonal growth advantage.[578] Acquisition of these mutations is often, but not always, followed by evolution to myelodysplasia characterized by monosomy 7 and finally myeloid leukemia.[579-581] Acquired mutations in *CSF3R* have been detected in approximately 80% of SCNIR patients who developed MDS/AML, whereas mutations in the *FLT3, KIT,* and *JAK2* tyrosine kinase genes commonly found in de novo AML are uncommon.[581,582] *RUNX1* mutations appear most often upon evolution to AML,[583] but genome-wide analysis has revealed acquired mutations in multiple genes, including *CSF3R*, *LLGL2*, and *ZC3H18* in a subpopulation of progenitor cells early in the process, followed by mutations in known AML-associated genes (*RUNX1* and *ASXL1*) and chromatin remodelers (*SUZ12* and *EP300*) in leukemic clones.[584]

Some patients with SCN and *CSF3R* mutations have not progressed, and there are some who lost the mutated clone upon discontinuation of G-CSF therapy and reacquired it upon resumption of treatment.[581,585,586] Cytogenetic abnormalities, particularly monosomy 7, also precede and predict leukemic conversion. For this reason, yearly bone marrow surveillance, including karyotyping and chromosome 7 fluorescence in situ hybridization (FISH), should be performed on all patients with SCN, whether or not they are treated with G-CSF.[512,572,574] Although clonal cytogenetic abnormalities may spontaneously remit, their appearance should be considered a strong indication for HSCT, including use of a matched unrelated donor if necessary, because transplantation is more likely to be successful when undertaken before progression to MDS/AML.[587,588]

Recent studies by the SCNIR and the French Severe Chronic Neutropenia Study Group[512,545,579,589] have shed light on the incidence and some of the risk factors for sepsis and MDS/AML. Although early reports suggested a rising risk of MDS/AML after 10 years of G-CSF therapy,[579,589] longer follow-up study has shown the risk to be stable at 2.3% per year after 10 years of G-CSF

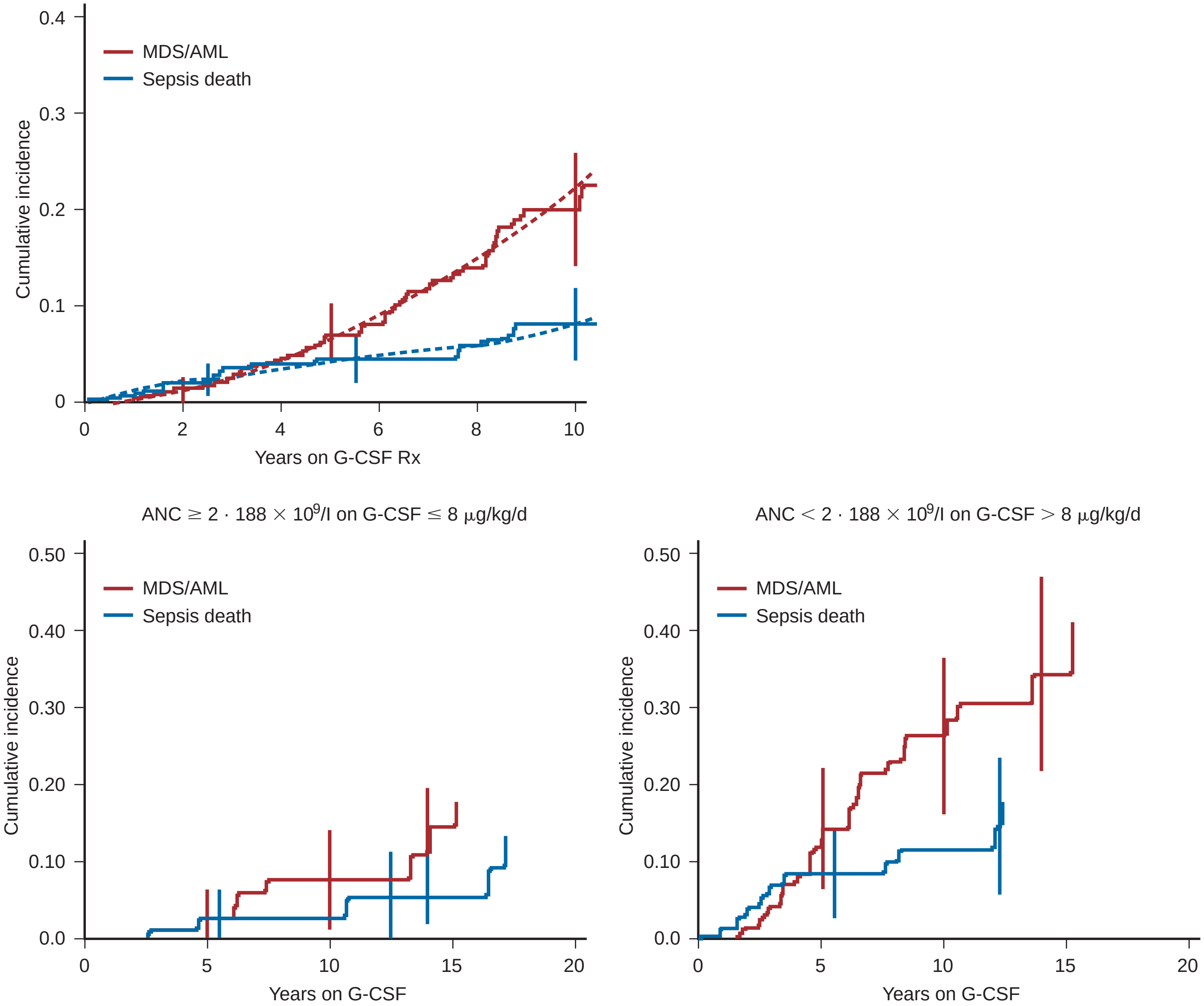


Figure 22-11 Cumulative incidence of MDS/AML and sepsis death in patients with SCN. *Upper panel:* Cumulative incidence (cumulative proportion experiencing each event as initial cause of failure, in patients at risk of each adverse event) for MDS/AML *(red)* and sepsis death *(blue)* by years on G-CSF therapy. Error bars show pointwise 95% confidence intervals. *Lower panels:* Cumulative incidence of MDS/AML and sepsis death in subgroups of patients with SCN. *Left,* Cumulative incidence of MDS/AML *(red)* and sepsis death *(blue)* among patients who achieved mean ANC ≥2.2 × 10^9 cells/μL at doses of G-CSF ≤8 μg/kg/day. *Right,* Corresponding cumulative incidence curves among patients who failed to achieve ANC ≥2.2 × 10^9 cells/μL despite doses of G-CSF >8 μg/kg/day. Error bars show pointwise 95% confidence intervals. *AML,* Acute myelogenous leukemia; *ANC,* absolute neutrophil count; *G-CSF,* granulocyte colony-stimulating factor; *MDS,* myelodysplastic syndrome. *(From Rosenberg PS, Zeidler C, Bolyard AA, et al: Stable long-term risk of leukaemia in patients with SCN maintained on G-CSF therapy.* Br J Haematol *150:196–199, 2010).*

therapy (Fig. 22-11).[512] The SCNIR analysis also identified a subgroup of patients with SCN at greatest risk for MDS/AML.[512,589] The high-risk group with a G-CSF dosage of 8 μg/kg/day or more and ANC less than 2188 cells/μL had a cumulative incidence at 15 years of 34% for MDS/AML and 15% for death from sepsis, compared to 15% and 5%, respectively, for the low-risk group (G-CSF dosage <8 μg/kg/day and ANC ≥2188 cells/μL) (see Fig. 22-11). Importantly, however, even the patients with the best responses to G-CSF were not free of risk for MDS/AML or even death from sepsis. Although data were not available for blood counts at the time of onset of sepsis, these patients had demonstrated ANCs over 1000 cells/μL while receiving G-CSF therapy, suggesting that they were either not fully adherent to the treatment[590] or that their granulocytes, although seemingly adequate in number, may have been limited in function.[591] For patients in the "worst responder" category of high G-CSF dosage requirements and low ANC on therapy, early transplantation should be considered, depending upon donor availability, but the indication is not as strong as for those already showing abnormal cytogenetics.

Analysis of *ELANE* gene mutations has also identified genetic risk factors for conversion to MDS/AML. In an SCNIR study of 188 patients with SCN and *ELANE* gene sequencing, 30 (16%) developed MDS/AML that was

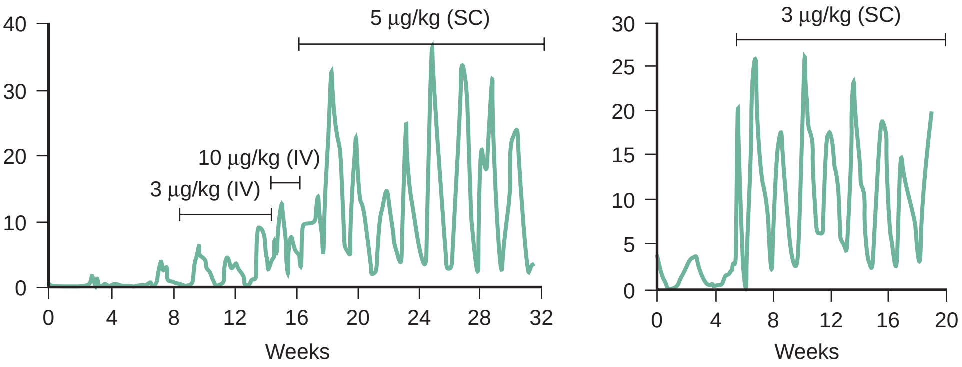


Figure 22-12 Absolute neutrophil counts in CyN and the response to administration of G-CSF. The cycling of neutrophil counts before and during therapy with the indicated doses of G-CSF is shown. Note the approximately 21-day cycle before therapy that is characteristic of CyN. With G-CSF therapy, the cycles shortened to approximately 14 days, peak neutrophil counts rose approximately 10-fold, and nadir neutrophil counts increased to ≥1000/µL. *(From Hammond WP, Price TH, Souza LM, Dale DC: Treatment of cyclic neutropenia with granulocyte colony-stimulating factor.* N Engl J Med *320:1306–1311, 1989.)*

associated with 25 different mutations, but clusters of MDS/AML cases were associated with mutations *C151Y, G214R,* and *S126L,* and both these and other mutations identified in single MDS/AML cases tended to be associated with higher G-CSF dosage requirements and poorer ANC responses.[545] On the other hand, there were no cases of MDS/AML in patients with mutations associated with both SCN and CyN phenotypes.

Cyclic Neutropenia. CyN is an AD disorder characterized by regular periodic oscillations approximately every 21 days in the number of peripheral blood neutrophils, with a nadir of less than 200 cells per µL (Fig. 22-12).[592,593] The incidence of CyN has been estimated at 0.5 to 1 case per million population.[533] During the neutropenic nadir of each cycle, patients may suffer from malaise, fever, oral and genital ulcers, gingivitis, periodontitis, and pharyngitis with local lymph node enlargement. Symptoms typically begin during the first year of life but may not present until adulthood. More serious complications can occasionally occur, including intestinal perforation with peritonitis, mastoiditis, and pneumonia. The severity of the infections tends to parallel the depth of the neutropenia, but some patients escape infections entirely during the neutropenic period. Many patients experience improvement in symptoms as they grow older. In these individuals, the cycles tend to become less noticeable as the neutrophil nadir rises to a more functionally adequate level. Although CyN is commonly viewed as a benign condition, 10% of patients in historical reviews have died of infectious complications.[593,594] Pneumonias and peritonitis, the latter complicated by clostridial sepsis, have been the most common causes of death.[592,593]

Oscillations in the rate of bone marrow production of neutrophils result in neutropenia with nadirs at intervals of 21 ± 3 days in the majority of patients. Neutrophil counts often fall to 0 at some time during the nadir and remain below 200 cells per µL for at least 3 to 5 days. In most patients, monocytosis occurs when neutrophil counts are at their lowest. In the recovery phase, the neutrophil counts rise, often into the low normal range, and monocyte counts drop. Oscillations are subtle in some individuals, particularly older patients, who instead have a pattern more consistent with chronic neutropenia. Oscillations in reticulocyte and platelet counts may parallel those seen for neutrophils,[593,595] hence the alternative term *cyclic hematopoiesis,* but these other cell numbers range between normal and elevated levels and thus have no clinical impact. Marrow aspirates show highly variable morphology, depending upon the phase of the cycle. The myeloid series may show hypoplasia with “maturation arrest” at the myelocyte stage during the declining phase of the neutrophil oscillation, but normal or hyperplastic myelopoiesis during neutrophil recovery.

Patients with CyN have been shown to have mutations in the *ELANE* gene, most often in exons 4 or 5, but not other genes associated with SCN.[409,410,596] How mutations in neutrophil elastase result in the cyclical production of neutrophils and other blood cells is not well understood. As discussed, both CyN and SCN are characterized by increased apoptosis of bone marrow neutrophil precursors,[546-549] probably because of an excessive unfolded protein response to mutant elastase.[411,412,550] Cycling may occur because of partial recovery of myelopoiesis stimulated by endogenous G-CSF production at the nadir, but with oscillation rather than homeostasis occurring as a result of the abnormal kinetics of the feedback loop.[597,598]

CyN must be distinguished from cyclic fevers without neutropenia[599] and from other forms of neutropenia, which can also produce less regular and lower amplitude variations in neutrophil counts. To establish the diagnosis of CyN, neutrophil counts should be monitored twice, or at least once, a week for 6 to 8 weeks. The genetic diagnosis can be confirmed by sequencing of the *ELANE* gene.

The management of patients with CyN includes careful attention to the identification and treatment of infections acquired when these patents are in a neutropenic phase. In general, fevers, upper respiratory symptoms, and cervical lymphadenopathy occurring during neutropenic episodes require no specific therapy. However, the physician must remain alert to symptoms suggesting a specific infection, particularly the sometimes fatal abdominal complications. Careful attention to oral and dental hygiene is important to minimize periodontal disease and ameliorate the discomfort of mouth sores. To prevent the development of severe infections and dental complications, G-CSF (filgrastim) therapy is recommended for patients with CyN with ANC nadirs consistently below 500 cells per μL.

G-CSF has been very effective in improving peripheral blood neutrophil counts in this disorder and alleviating the signs, symptoms, and risks associated with neutropenic nadirs.[530,566,576,596,600] Most patients still experience cycles, and in fact the amplitude of the cycling may increase but with shortened cycle periods and increased neutrophil counts at the nadir (see Fig. 22-7).[597,600] The dose of G-CSF required to maintain peripheral neutrophil counts in the normal range in most patients with CyN is usually 2 to 4 μg/kg/day, administered either daily or on alternate days.[567,589,601] Cases of MDS or leukemia have not been reported for patients with CyN, including those treated long term with G-CSF.[545,601]

Myelokathexis and WHIM Syndrome. Myelokathexis is an uncommon form of moderate to severe chronic neutropenia characterized by granulocyte hyperplasia in the bone marrow, which contains degenerating neutrophils with cytoplasmic vacuoles, prominent granules, and nuclear hypersegmentation with very thin filaments connecting pyknotic-appearing nuclear lobes.[602-604] Similar abnormalities can be seen in the majority of peripheral blood neutrophils. Eosinophils exhibit similar abnormal morphology, but lymphocytes, monocytes, and basophils appear normal,[603] although relative lymphopenia has been noted in some patients.[603] Recurrent warts and hypogammaglobulinemia (low IgG and occasionally low IgM and IgA) have often been reported in patients diagnosed with myelokathexis; hence the acronym *WHIM*.[605]

Recurrent sinopulmonary infections and other bacterial infections in WHIM syndrome probably reflect an increased susceptibility because of both neutropenia and hypogammaglobulinemia. However, early deaths related to infection have not been reported, and affected patients are capable of mobilizing increased numbers of neutrophils into the peripheral blood during infectious episodes.[604] Neutrophil function has generally been reported to be normal.

The genetic basis of WHIM syndrome was first identified as an AD truncating mutation in the cytoplasmic tail domain of the *CXCR4* gene, encoding chemokine receptor-4,[606] a G-protein–coupled receptor for *CXCL12,* (SDF-1). However, in other kindreds with wild-type *CXCR4* genes and probable AR inheritance, including one with homozygous mutations in *G6PC3*,[607] patients' leukocytes displayed similar marked enhancement of G-protein–dependent responses to *CXCR4* ligands.[608,609] Therefore the aberrant dysfunction of the *CXCR4*-mediated signaling constitutes a common biologic trait of WHIM syndromes with different causative genetic anomalies. As the interaction between *CXCR4* and *CXCL12* provides a key retention signal for neutrophils in the bone marrow,[40] myelokathexis has been suggested to result from retention of neutrophils in the marrow (*kathexis* means *retention*), where they undergo eventual apoptosis, evident in their diminished expression of the antiapoptotic factor *bcl-x*.[610]

The neutropenia and excessive granulocyte apoptosis of WHIM syndrome are partially corrected by G-CSF or GM-CSF therapy, with responses typically seen within 4 to 8 hours of administration.[604,610] Immunoglobulin levels also return to normal in patients treated with G-CSF.[604] Specific therapy aimed at the primary molecular defect has recently been reported, using the *CXCR4* antagonist plerixafor (formerly known as *AMD3100*) to correct the leukopenias of WHIM syndrome.[611,612]

Shwachman-Diamond Syndrome. Shwachman-Diamond syndrome, a rare multiorgan disease inherited as an AR trait, includes clinical findings of neutropenia, pancreatic exocrine insufficiency, short stature, and metaphyseal chondrodysplasia.[613-615] This disorder is also described in Chapter 8 with a more detailed discussion of its genetics and pathophysiology. As in other bone marrow failure syndromes, patients are at risk for conversion to MDS/AML.[614] The pancreatic acini are largely replaced by fatty tissue with relative sparing of the pancreatic ducts and islets.[615,616] Growth failure and short stature are usually noted during the first or second year of life, and puberty is often delayed. Although the classic syndrome includes the triad of cytopenias, pancreatic dysfunction, and skeletal abnormalities, clinical presentations can be highly variable, including any number or none of these features.[614,617]

Patients may show symptoms in infancy with eczema, skin infections, and otitis media; pneumonia, osteomyelitis, and sepsis can also be seen. Pancreatic insufficiency typically manifests in early infancy with steatorrhea, weight loss, and failure to thrive. Later in childhood, pancreatic function may improve, rendering the clinical diagnosis elusive.[618] Levels of serum trypsinogen, pancreatic isoamylase, and stool elastase are generally low but not specifically diagnostic,[614] and normal ranges are not well established for infants. Computed tomography scanning may reveal pancreatic lipomatosis. Mild abnormalities of the liver including hepatomegaly or elevated serum liver enzyme, are seen in 50% to 75% of younger patients, but they are usually mild and tend to resolve with age.

Common skeletal abnormalities include short stature and bone dysplasia evident in delayed appearance (but subsequent normal development) of secondary ossification centers as well as metaphyseal widening, first evident in the ribs (which appear short, with flared ends) and later in the proximal and distal femur.[614] Other skeletal findings include clinodactyly, syndactyly, pes cavus, kyphosis, scoliosis, osteopenia, vertebral collapse, slipped femoral epiphysis, and supernumerary digits.[616]

Neutropenia is the most common hematological manifestation of the syndrome, but some patients present with GI or skeletal problems and virtually normal blood counts.[613,614,617] Neutrophil counts fall below 1000 cells per μL in approximately two thirds of patients, with no reciprocal monocytosis, but low counts may be intermittent. Some patients also have defects in chemotaxis or in numbers or function of B, T, and NK cells that may increase susceptibility to pyogenic infection.[619,620] Shwachman-Diamond syndrome leukocytes may also show telomere shortening, which can overlap in severity with that observed in other bone marrow failure syndromes, even including dyskeratosis congenita.[617,621] Anemia (normocytic or macrocytic) is common, and one fourth of patients have mild to moderate thrombocytopenia.[616,618,622] Bone marrow studies have generally shown some degree of myeloid hypoplasia[616,618,622] but are nondiagnostic.

The diagnosis of Shwachman-Diamond syndrome may be based on a combination of neutropenia, possibly other cytopenias, pancreatic insufficiency, and skeletal anomalies. Around 90% of patients who meet the clinical criteria harbor mutations in the Shwachman-Bodian-Diamond syndrome gene, *SBDS*,[622,623] which functions in ribosomal biogenesis and other cellular processes.[613] With increased detection of variant forms of the syndrome by genetic testing, a wider clinical phenotype has been recognized. In a study from the North American Shwachman-Diamond Registry,[617] pancreatic lipomatosis was found in 22 of 23 patients, low fecal elastase in 14 of 17 patients, and low serum trypsinogen or pancreatic isoamylase in all 18 patients tested. Of 31 patients with clinical assessments, 26 had failure to thrive and 17 had diarrhea or steatorrhea; 17 had congenital anomalies. Neutropenia was the most common hematologic abnormality at presentation (77%), but two patients had isolated thrombocytopenia, three had severe anemia, and five had no cytopenias.

Treatment includes pancreatic enzyme replacement, which does not improve the neutropenia or dwarfism. Steatorrhea tends to diminish with time, although pancreatic insufficiency persists.[624] The incidence of bacterial infections can vary among patients, generally depending upon the ANC. Administration of G-CSF increases the neutrophil count to the normal range[616,622] and should be used in patients with persistently severe neutropenia or with recurrent or life-threatening infections.

Patients with Shwachman-Diamond syndrome may develop aplastic anemia, MDS, or leukemia, although usually later in life than patients with Fanconi anemia or SCN.[569,625] These complications generally require treatment by HSCT.[614] There are only very rare reports of solid tumors.[626-628] A study from the French Severe Chronic Neutropenia Registry reported a 24% 20-year cumulative risk of aplastic anemia and MDS/AML in 102 patients; young age (<3 months) at first symptoms and low hematologic parameters both at diagnosis and during follow-up study were associated with severe hematologic complications.[569] Because acquisition of a structural chromosomal abnormality, most commonly isochromosome i(7q) followed by del(20q), often precedes conversion to MDS,[616,622,629,630] regular blood counts are recommended, and bone marrow examinations should be performed annually or more often in the face of evolving peripheral blood or cytogenetic findings.[616] Cytogenetic abnormalities may be transient or unaccompanied by marrow dysplasia, so it remains unclear whether high-risk clonal aberrations constitute indications for HSCT.[614,616]

Albinism-Neutropenia Syndromes

This group of rare primary immunodeficiency syndromes derive from AR defects in the biogenesis or trafficking of lysosomes and related late endosomal organelles.[631,632] As a result, they share varying degrees of overlap in phenotype, including defects in formation of melanosomes (hence partial albinism), abnormal platelet function, and immunological defects involving not only neutrophil number but also the function of neutrophils, B lymphocytes, and cytotoxic T lymphocytes. They share a high risk of hemophagocytic lymphohistiocytosis (HLH) because of defects in T and NK cells.[633] The partial albinism may be sufficiently subtle to require microscopic examination of hair for irregular, clumped melanosomes.

CHS is a rare genetic disorder characterized by partial oculocutaneous albinism, giant lysosomes in many cell types including granulocytes, and neuropathy.[634] Most patients have moderate neutropenia, apparently because of ineffective granulopoiesis, and a high risk of HLH. CHS is described in detail later in this chapter.

Hermansky-Pudlak syndrome, another rare AR disease characterized by oculocutaneous albinism, is best known for associated platelet defects (see Chapter 8). One subtype, Hermansky-Pudlak syndrome type 2, also includes neutropenia and decreased numbers of NK cells, leading to increased susceptibility to infections and HLH.[635,636] The *AP3B1* gene responsible for this disorder,[637] as well as for CyN in grey collie dogs,[638] encodes an adapter protein involved in shuttling granule proteins, including neutrophil elastase, and thus may provide a connection to the molecular pathology of SCN[638] and HLH.[633]

Griscelli syndrome type 2, falls within the spectrum of Griscelli syndrome subtypes that share a phenotype of hypomelanosis with varying degrees of neurological impairment and immunodeficiency.[639] The type 2 variant is characterized by neutropenia, hypogammaglobulinemia, partial albinism, and a predisposition to HLH.[633,640] It is caused by mutations in the *RAB27A* gene,[641] which encodes a small GTPase that serves as a key effector of granule exocytosis.[642]

Cohen syndrome is an AR condition that includes neutropenia, pigmentary retinopathy, microcephaly, intellectual deficiency, and facial dysmorphism.[643] The gene responsible for Cohen syndrome, *COH1* (also termed *VPS13B*), shares homology to a yeast protein that functions in vesicular sorting and intracellular protein trafficking. A case with congenital neutropenia, pigmentary retinopathy, and only subtle dysmorphism due to splicing mutations in *VPS13B* indicates a wider phenotype and a need to consider this gene in the evaluation of patients with neutropenia and retinopathy.[643a]

A single Mennonite family has been described with severe chronic neutropenia combined with short stature, albinism, coarse facial features, and recurrent bronchopulmonary infections by *Streptococcus pneumoniae*.[644] This syndrome is caused by defects in the endosomal adaptor protein p14, which is involved in MAPK signaling to late endosomes.[645]

Familial Benign Neutropenia

Familial benign neutropenia is a nonspecific descriptive diagnosis indicative of mild neutropenia with no tendency to increased infection. AD transmission has been identified in some familial cases.[646] Although “familial benign neutropenia" has been described in Yemenite Jews,[647] and in persons of German, French, American, and South African descent,[648] these kindreds were investigated before the era of current immunological and molecular diagnostic tools, so they might now be classified with more specific diagnoses. For example, "benign ethnic neutropenia" in persons of African origin has recently been found to result from the Duffy negative genotype.[508]

Other Causes of Neutropenia Caused by Intrinsic Defects in Myelopoiesis

Congenital or acquired bone marrow failure syndromes can occasionally present with isolated neutropenia. Fanconi anemia and dyskeratosis congenita should usually be recognized by other features associated with these disorders (see Chapter 8). Acquired aplastic anemia and MDSs may occur as primary processes, or as complications of bone marrow failure syndromes. Mild chronic neutropenia is a minor feature of monocytopenia and mycobacterial infection (MonoMAC) syndrome[649] discussed later in this section.

Neutropenia Associated with Immune Dysfunction

Primary disorders of immunoglobulin production have been associated with neutropenic syndromes.[650-652] One third of males with X-linked agammaglobulinemia have neutropenia at some time during the course of their disease.[650,652] Persistent or CyN is common in patients with the hyper-IgM immunodeficiency syndrome, which in many instances appears to be secondary to the formation of autoantibodies.[650,652,653] Immunoglobulin replacement therapy abolished the neutropenia in some but not all of these patients.[654] AIN and other autoimmune cytopenias can also be seen in common variable immunodeficiency and isolated IgA deficiency.[650,651,653]

Autoimmune lymphoproliferative syndrome (ALPS) reflects a family of defects in lymphocyte apoptosis. The syndrome includes lymphadenopathy, splenomegaly, and a variety of autoimmune disorders including immune thrombocytopenia, anemia, and neutropenia.[655,656] Neutropenia associated with ALPS tends to be chronic, and because of the unique pathophysiology of the underlying disorder, represents one of the few forms of neutropenia treated by immunosuppression with corticosteroids or mycophenolate mofetil.

Cartilage hair hypoplasia, an AR disorder found in the Amish population, is characterized by short-limbed dwarfism, fine hair, moderate neutropenia (100 to 2000 cells per μL), and impaired cell-mediated immunity.[657,658] The disorder arises from mutations in the noncoding RNA gene *RMRP*,[659] which provides the RNA subunit of the pancreatic ribonuclease mitochondrial RNA processing enzyme, a complex involved in multiple cellular RNA processing events.[660] G-CSF therapy has been reported to be effective in a single patient who also had an antineutrophil antibody[661]; bone marrow transplantation has corrected both the immunologic defects and neutropenia.[662]

Schimke immuno-osseous dysplasia, a rare AR syndrome with variable expressivity, is characterized by spondyloepiphyseal dysplasia, steroid-resistant nephritic syndrome, lymphopenia with defective cellular immunity, neutropenia, and other cytopenias.[663,664] Vaso-occlusive processes, including cerebral and generalized atherosclerosis, are a life-limiting complication in the more severely affected patients.[665] The disorder is associated with mutations in the gene *SMARCAL1*, although the genetics may be heterogeneous.[666,667] The immunodeficiencies and cytopenias can be treated with HSCT[668]; neutropenia, seen in 40% of patients, responds to G-CSF therapy.[664]

Poikiloderma with neutropenia, Clericuzio type, is a rare AR genodermatosis characterized by early onset poikiloderma followed by pachyonychia, palmoplantar hyperkeratosis, and skeletal defects, as well as variable degrees of neutropenia and possible predisposition to malignancy.[669,670] The gene mutated in this disorder, *USB1* (also termed *C16orf57*),[669,671,672] encodes a putative phosphodiesterase responsible for oligoadenylation of spliceosomal small nuclear RNA U6.[673] Mutations in *USB1* have also been identified in patients with Rothmund-Thomson syndrome (a phenotypically similar but distinct genodermatosis) and dyskeratosis congenita.[670,672]

Neutropenia Associated with Metabolic Diseases

Significant neutropenia, often with relative monocytosis, occurs in Barth syndrome, a distinctive X-linked disorder caused by mutations in the *TAZ* gene, which encodes tafazzin, a phospholipid-lysophospholipid transacylase involved in the maintenance of mitochondrial cardiolipin.[674-676] Clinical features include dilated cardiomyopathy, growth retardation, and 3-methylglutaconic aciduria.[677,678] Affected boys develop symptoms in infancy or childhood, and neutropenia can precede the development of cardiac abnormalities. Neutrophil counts are variable and occasionally cyclic, ranging from nearly normal to very low, with recurring and sometimes life-threatening bacterial infections occurring in the most severely affected.[676] Bone marrow shows maturation arrest at the myelocyte stage, with myeloblasts and promyelocytes demonstrating abnormal mitochondria.[677]

Neutrophils in patients with glycogen storage disease type Ib (OMIM # 232220) are not only diminished in number, with neutrophil counts commonly less than 500 cells per μL,[679-681] but also functionally defective, with abnormalities reported in chemotaxis, respiratory burst activity, and bacterial killing.[682,683] The disorder derives from mutations in the glucose-6-phosphate transporter, encoded by the *SLC37A4* gene.[678] The defects in neutrophil survival and function are similar to those due to mutations in the myeloid-specific *G6PC3* gene.[558] Recombinant G-CSF has been effective in correcting the neutropenia and reducing serious infections in glycogen storage disease type Ib.[684] Prolonged use of G-CSF has not been associated with any increased risk of MDS/AML in this disease.[579,681]

Macrocytic anemia, often accompanied by neutropenia or thrombocytopenia, is a hematologic hallmark of Pearson syndrome, a rare and fatal congenital disorder involving not only the hematopoietic system but also the exocrine pancreas, liver, and kidneys.[685] The bone marrow shows normal cellularity but striking abnormalities that include vacuolization of erythroid and myeloid precursors, hemosiderosis, and ringed sideroblasts. A mitochondrial defect related to large deletions in mitochondrial DNA, whose integrity depends on a specific DNA polymerase, likely leads to impaired hematopoiesis.[686]

Neutropenia, usually accompanied by metabolic abnormalities including acidosis, ketosis, hyperammonemia, and hypoglycemia, can be a feature of organic acidemias such as propionic acidemia, methylmalonic acidemia, methylmalonic aciduria, and others.[687] Although these congenital disorders are usually evident in the first 10 days of life, variants can present in older children or adolescents. Diagnosis is based on screening of urine organic acids, followed by more specific metabolic or genetic testing. Disorders of cobalamin metabolism can present in the first months to years of life with neutropenia and are usually accompanied by macrocytic anemia and often by methylmalonic acidemia.[688]

Neutropenia Caused by Extrinsic Factors

Infection. The most common cause of transient neutropenia in childhood is viral infection. Viruses commonly causing acute transient neutropenia include influenza A and B, adenovirus, respiratory syncytial virus, enteroviruses, human herpes virus 6, measles, rubella, and varicella.[689-691] Neutropenia develops during the first 24 to 48 hours of the illness and may persist for 3 to 6 days. This period usually corresponds to the time of acute viremia and may relate to virus-induced redistribution of neutrophils from the circulating to the marginated granulocyte pool, sequestration, or increased neutrophil utilization after tissue damage caused by the viruses.[692,693]

Both transient and chronic neutropenia occur with Epstein-Barr virus infection[694,695] and may be related to splenomegaly, direct infection of progenitors,[696] and antineutrophil antibodies.[697,698] Cytomegalovirus (CMV) infection and anti-CMV therapies may cause neutropenia in the setting of perinatal infection or immune suppression (particularly HSCT or solid organ transplant) but can also affect the immunocompetent.[699-702] Parvovirus B19 and hepatitis A and B may also cause neutropenia,[703,704] although they are more commonly associated with pure red cell aplasia and multiple cytopenias, respectively.

Leukopenia, either transient or chronic, is also common in patients with HIV infection as a result of multiple causes, including antiretroviral drugs, cellular immune dysfunction, ineffective hematopoiesis, antineutrophil antibodies, B_{12} or folate deficiency, and hypersplenism.[705-708]

Significant neutropenia may occur during typhoid, paratyphoid, tuberculosis, brucellosis, tularemia, and rickettsial infections.[689,709-712] The mechanisms responsible for neutropenia in these conditions remain ill-defined. During periods of relapsing fever caused by acute malaria, the apparent neutropenia may be secondary to increased neutrophil margination in the spleen.[713,714]

Neutropenia in patients with bacteremia and endotoxemia may result from excessive destruction of neutrophils with depletion of the bone marrow reserve pool and constitutes a dire prognostic sign, particularly in newborns.[715] This consumption can occur after phagocytosis of microbes, from the release of metabolites of arachidonic acid, or from activation of the complement system through either the alternate or classic pathway. The resultant generation of the chemoattractant C5a induces neutrophil aggregation and leads to the formation of leukoemboli, which adhere to and damage endothelial surfaces in the pulmonary capillary bed. Complement-mediated neutropenia may also occur transiently during hemodialysis, during continuous-flow leukapheresis, and after burn injury.[716-719]

Newborns with sepsis may benefit from granulocyte transfusion[720,721] or G-CSF,[722,723] but clinical trials have not yet provided compelling evidence for benefit from either modality.[721,724]

Drug-Induced Neutropenia. Drug-induced neutropenia, defined as an idiosyncratic reaction to the offending drug,[725-739] excludes disorders in which other cell lines are perturbed (e.g. drug-related aplastic anemia) and the predictable neutropenias observed with anticancer therapy. Idiosyncratic reactions tend to develop more often in women than in men and more often in older than in younger persons.[729]

In its most serious form, drug-induced agranulocytosis, neutrophil counts reach levels below 200 cells/μL, with high morbidity (e.g. deep tissue infection and sepsis) and mortality rates as high as 5%.[725] Most deaths occur in patients who are elderly or have metabolic complications such as shock or renal failure.

Almost any drug can cause neutropenia; Table 22-9 lists the agents most commonly associated with isolated, idiosyncratic neutropenia or agranulocytosis. Drugs reported to have been associated with agranulocytosis have been extensively reviewed.[725,726,728,731,736-738] The most common drug classes include antiinflammatories, particularly aminopyrine and sulfasalazine; antimicrobial agents, particularly trimethoprim and sulfamethoxazole; antithyroid drugs; antipsychotics such as clozapine and phenothiazines; and others such as deferiprone and rituximab (see Table 22-9).

Although the underlying mechanisms for most drug-induced neutropenias are unknown, most studies suggest that they can be classified as leading to toxic suppression of neutrophil formation or immune destruction of mature cells.[728,730,737,738] Neutropenia resulting from myelosuppression is usually insidious in onset and may be asymptomatic. Oral mucositis may be the first clinical sign before infectious complications. Individual differences in drug pharmacokinetics can lead to toxic levels of the drug or metabolites in the marrow microenvironment. For example, whereas neutropenia can be observed in any patient taking sulfasalazine, individuals who are slow acetylators show much greater toxicity than do those who are fast acetylators.[736] Alternatively, a susceptible patient's myeloid precursors may be abnormally sensitive to typical drug concentrations, as observed in neutropenia that can appear 20 to 40 days after starting phenothiazines.[728]

Drugs can also induce immune-mediated peripheral destruction of neutrophils by several mechanisms. In one,

TABLE 22-9 Partial List of Drugs Causing Idiosyncratic Drug-Induced Neutropenia*

	Possible Mechanism		
Drug	**Direct Suppression**	**Metabolite Suppression**	**Immune Destruction**
Antiinflammatory			
Aminopyrine			X
Ibuprofen			X
Indomethacin	X		
IV immunoglobulin			X
Phenylbutazone	X		
Sulfasalazine	X		
Anticonvulsant			
Carbamazepine		X	
Phenytoin			X
Valproic acid	X		
Antimicrobial			
Chloramphenicol	X		
Dapsone		X	
Penicillins	X		X
Sulfonamides	X		
Trimethoprim/Sulfa	X		
Antithyroid			
Methimazole			X
Propylthiouracil			X
Cardiovascular			
Hydralazine			X
Procainamide			X
Quinidine			X
Psychotropic			
Chlorpromazine	X		
Clozapine	X		
Olanzapine	X	X	
Phenothiazines	X		
Other			
Chlorpropamide			X
Cimetidine, ranitidine	X		
Deferiprone			
Gold			X
Levamisole			X
Rituximab			
Ticlopidine	X		

IV, Intravenous; *Sulfa,* sulfamethoxazole.

*Partial list of agents capable of causing idiosyncratic drug-induced neutropenia. Italics indicate the drugs with the highest relative risk of neutropenia and agranulocytosis. The mechanism, when unknown, has been left blank; some of the indicated mechanisms are not well established.

the drug serves as a hapten in promoting the synthesis of antibodies that are capable of destroying mature neutrophils. Aminopyrine, penicillin, propylthiouracil, and gold can cause neutropenia by this mechanism.[728,732,735,738] Alternatively, the drug can elicit the formation of circulating immune complexes that attach to the surface of the neutrophil and lead to its destruction, as in the case of quinidine. In addition, immunologic changes induced by drugs can suppress granulopoiesis. For example, activation of both cellular and humoral immune responses has been reported to impair myelopoiesis after therapy with quinidine and phenytoin, respectively.[730,738] Immune-mediated neutropenia is characterized by abrupt onset, often with fever, chills, and prostration, occurring 7 to 14 days after the first use of the drug or immediately after reexposure. Transient, usually mild, neutropenia may follow treatment of immune thrombocytopenia with intravenous immunoglobulin.[740]

The duration of drug-induced neutropenia is highly variable. Myelosuppressive drug reactions may last from a few days to months or years, whereas immune-mediated reactions usually last for 6 to 8 days. Once neutropenia occurs, the most important therapeutic action is, of course, to withdraw all drugs that are not absolutely essential, particularly those most highly associated with neutropenia. G-CSF therapy may hasten neutrophil recovery slightly, but evidence-based data are lacking to justify its routine use[548,741,742]; therefore the clinical indication is questionable unless the patient has a severe infection. During recovery from drug-induced neutropenia, with or without G-CSF therapy, rebound leukocytosis can occur and is accompanied by a left shift in the peripheral blood and bone marrow myeloid series with occasional circulating immature leukocytes.

Autoimmune Neutropenia. AIN can occur as an isolated process, as a manifestation other autoimmune diseases, or as a secondary complication of infection, drugs, or malignancy.[528,741,743] In primary AIN, low circulating neutrophil counts are the only hematological finding, and associated diseases or other factors that cause neutropenia are absent. The peak incidence of AIN occurs in infants and young children,[744,745] where it is a generally benign disorder that remits spontaneously, as discussed in the following section. Many cases of chronic idiopathic neutropenia in children and adults are now recognized as secondary to more generalized autoimmune disorders (Box 22-5).

Patients with AIN may have highly (and rapidly) varying neutrophil counts, ranging from the mild neutropenia to agranulocytosis. Monocytosis is common. Bone marrow examination generally shows normal to increased cellularity with myeloid hyperplasia and normal to increased numbers of mature neutrophils, although diminished neutrophils or maturation arrest at earlier stages of differentiation can also be seen.[528,741,743] Mild splenomegaly is occasionally present. The incidence of pyogenic infection is not always related to the degree of neutropenia[514,528] and is generally limited to cutaneous and respiratory infections. Serious infections are generally uncommon. Spontaneous remissions resolve the

Box 22-5 **Disorders Associated with Immune-Mediated Neutropenia**

AUTOIMMUNE DISORDERS

Autoimmune cytopenias (Evans syndrome)
Autoimmune lymphoproliferative syndrome
Felty syndrome (arthritis, splenomegaly, leukopenia)
Primary biliary cirrhosis
Sjögren syndrome
Scleroderma
Systemic lupus erythematosus

INFECTION

Infectious mononucleosis
Human immunodeficiency virus

MALIGNANCY

Leukemia
Lymphoma (Hodgkin and non-Hodgkin)

HYPOGAMMAGLOBULINEMIAS, DYSGAMMAGLOBULINEMIAS

Common variable immunodeficiency
Hyperimmunoglobulin M syndrome
Immunoglobulin A deficiency
X-linked agammaglobulinemia

ANGIOIMMUNOBLASTIC LYMPHADENOPATHY (CASTLEMAN DISEASE)

DRUG REACTION (see Table 22-9)

process in most infants, but occur less commonly in older children (discussed later).

Neutrophil-specific cell surface antigens identified as targets of autoantibodies include human neutrophil antigen (HNA) 1a, formerly termed *NA1*; HNA-1b, formerly NA2; and HNA-2, formerly NB1.[348,746] The HNA-1a and HNA-1b antigens are glycosylated isoforms of the neutrophil immunoglobulin receptor FcγIIIb (CD16) (see Table 22-6), and the HNA-2 antigen is CD177, the counter-receptor for PECAM-1.[747] Mechanisms that trigger autoantibody production are unknown. In children, primary AIN is usually associated with HNA-allele–specific antibodies, but secondary AIN may be associated with pan-FcγRIIIb antibodies.[748]

The neutropenia of AIN is presumed primarily to be caused by peripheral destruction of antibody-coated neutrophils, which may be also be augmented by the deposition of C3. Phagocytosis of neutrophils in the spleens of patients with AIN has been observed.[749] In some cases, antineutrophil antibodies also appear to interfere with myelopoiesis.[750-752] Impairment of phagocytosis, respiratory burst activity, and adhesion by neutrophil-directed antibodies has also been observed, which may contribute to the risk of infection in AIN.[750,752,753]

Antineutrophil antibodies can be detected by direct or indirect granulocyte immunofluorescence tests (analogous to the direct and indirect antiglobulin tests for erythrocyte antibodies but usually performed by flow cytometry) or by granulocyte agglutination tests.[347,348,754,755] Granulocyte agglutination is less sensitive but may be useful for detecting neutrophil-binding immune complexes in Felty syndrome.[752] All of these tests are subject to false positive results from anti-HLA antibodies or nonspecific binding of immunoglobulins to neutrophil FcRs, as well as false negative results caused by high background levels of bound immunoglobulins. Because of the limited sensitivity and specificity of these assays, even in combination, laboratory tests for antineutrophil antibodies are neither necessary nor sufficient to make the diagnosis of AIN, and their interpretation depends on the specific clinical setting.

Importantly, the standard laboratory test for antineutrophil cytoplasmic antibody does not detect antibodies responsible for AIN.

Treatment of patients with immune neutropenia includes the judicious use of appropriate antibiotics for bacterial infections, plus antimicrobial mouthwashes and regular dental hygiene for mouth sores or gingivitis. Infections tend to be less common in immune neutropenia than with the corresponding degree of neutropenia from other causes, probably because granulopoiesis is in most cases intact and tissue delivery of neutrophils is greater than that with equivalent neutrophil levels caused by impaired production. Prophylactic administration of trimethoprim/sulfamethoxazole may be also helpful for management of recurrent minor infections, although there are no controlled studies addressing the efficacy of this approach.

For definitive therapy, administration of G-CSF starting at low dosages of 1 to 2 μg/kg/day has been successful in increasing the neutrophil count to the normal range or even higher in patients with primary AIN, including secondary immune neutropenia and Felty syndrome.[743,744,756,757] The initial response is usually apparent within days of starting therapy but may require up to 2 weeks. Patients with normal or high ANC during therapy may be weaned to a very low dosage or administration of G-CSF on alternate days. The incidence of bone pain appears to be higher in AIN than in hypoproductive neutropenia, probably because of expansion of the already hyperplastic bone marrow. Because most patients with immune neutropenias do not suffer serious infectious complications, G-CSF therapy should be reserved for the few with serious or recurrent infections or with multiple hospitalizations for febrile neutropenia. When used for AIN and other forms of acquired neutropenia, G-CSF is not associated with development of MDS/AML.

Intravenous immunoglobulin administration can result in normalization of the neutrophil count, but efficacy is variable and often short-lived.[743,744,756] Corticosteroids can raise the neutrophil count but may actually increase the risk of serious infection. Similarly, splenectomy can provide transient benefit that is offset by the risk of sepsis, and the procedure is limited to conditions with clinically significant splenomegaly such as Felty syndrome.[758]

T-cell large granular lymphocyte leukemia (LGL), a clonal disorder of cytotoxic T lymphocytes most common in the elderly, often presents as severe chronic neutropenia with or without accompanying anemia.[547,759] LGL-associated neutropenia, which overlaps clinically with Felty syndrome, may derive from either immune complex– or cell-mediated mechanisms and generally responds to a combination of immunosuppressive and cytotoxic therapy for the underlying disease and G-CSF for the neutropenia.[547]

Neutropenia can be seen as part of a broader spectrum of disease in a variety of other acquired disorders of the immune system. Autoimmune diseases such as systemic lupus and rheumatoid arthritis can be associated with antibody or immune complex-mediated destruction of neutrophils or their precursors.[760] Felty syndrome refers specifically to neutropenia in association with splenomegaly and seropositive rheumatoid arthritis; there is considerable pathophysiologic overlap between Felty syndrome and rheumatoid arthritis–associated LGL.[761] Therapy with G-CSF is effective but may precipitate flares of the underlying disease[762,763]; splenectomy may be effective, as discussed previously.

Chronic Benign Neutropenia and Autoimmune Neutropenia of Childhood. Most cases of what was termed chronic benign neutropenia of infancy and childhood are now believed to represent an AIN that has parallels to childhood idiopathic thrombocytopenic purpura.[743,744,756,764] AIN of childhood occurs predominantly in children younger than 3 years (Box 22-6). The median age of presentation is 8 to 11 months, with a range of 2 to 54 months.[743,744,756,764] There is a slight female predominance. Although AIN of childhood is the most common cause of chronic neutropenia in the pediatric age group, it is still a relatively uncommon diagnosis, with an incidence of approximately 1 in 100,000 children per year.[511,765] However, this figure is probably an underestimate because of the generally benign course of this disorder.

Box 22-6 **Summary of Autoimmune Neutropenia of Childhood**

CONSIDERABLE OVERLAP WITH CHRONIC BENIGN NEUTROPENIA OF CHILDHOOD

Incidence	Most common cause of chronic neutropenia in infancy and childhood; incidence ≥1 per 100,000 children per year.
Clinical features	Median age of diagnosis is 8-11 months of age (range 3-38 months). Relatively minor infections (otitis media, gingivitis, upper respiratory tract, skin) occur with an increased frequency only in some patients and respond well to antimicrobial therapy. Rare patients (usually young infants) have pneumonia or sepsis.
Laboratory evaluation	Median absolute neutrophil count at time of diagnosis ≈200 neutrophils/μL (range 0-500 cells/μL). Hemoglobin, platelet count generally normal. Antineutrophil antibodies can be detected in many patients and are often directed to human neutrophil antigen (HNA) 1 (FcγRIII). Bone marrow test (if performed) shows normal to increased myelopoiesis, sometimes with a decrease in mature neutrophils or maturation arrest at earlier stages.
Therapy	Antibiotics for acute infection; prophylactic antibiotics may be helpful in some patients with recurrent otitis media. Consider use of granulocyte colony-stimulating factor only in the event of serious infection, very frequent minor infections, or very frequent precautionary emergency room visits or admissions.
Prognosis	Excellent. Although the ANC often remains below 500 cells per μL for 12 or more months, spontaneous remission occurs in almost all patients (median of 20 months, range 6-54 months).

The presenting ANC is often in the severe range (<200 cells/μL), usually with a relative monocytosis and/or eosinophilia. Transient increases in circulating neutrophil counts can occur in response to infection. Other peripheral counts are normal for age. Mild thrombocytopenia has been reported,[766] but the presence of other autoimmune cytopenias more likely represents Evans syndrome (see Chapter 13), which is more likely to become chronic and to be associated with underlying immunodeficiency disorders or ALPS.

Antineutrophil antibodies can be detected in many patients by repeated testing with a combination of immunofluorescent and agglutination assays,[754,756,764,767] but absence of detectable antibody does not exclude the diagnosis, and a positive result does not exclude congenital neutropenia.[540] Bone marrow examination generally shows normal to increased cellularity with myeloid hyperplasia and normal to increased numbers of mature neutrophils.

Bacterial infections in AIN of childhood are generally mild (e.g., skin infections, otitis media, gingivitis) and responsive to standard antibiotics.[511,750,768] Cellulitis involving the labia majora was seen in 23% of 26 girls with AIN in one series, often the result of *Pseudomonas aeruginosa*.[511] Pneumonia, sepsis, and meningitis have been reported only occasionally in infants with AIN.[750] Spontaneous remission occurs in almost all patients, with neutropenia persisting for a median duration of 20 months.[768] Children younger than 9 months of age at the time of diagnosis may recover normal peripheral neutrophil counts more rapidly.[766] With increasing age, spontaneous remission becomes less likely.

Febrile episodes should be managed aggressively in the initial period before the diagnosis of childhood AIN is established, including the use of broad-spectrum parenteral antibiotics. However, once the diagnosis is definite and the patient has had no serious infections, subsequent febrile illnesses can be managed more conservatively if clinically warranted. Prophylactic trimethoprim/sulfamethoxazole can be helpful for children with recurrent otitis media and other minor infections. Administration of G-CSF results in an increased peripheral neutrophil count in childhood AIN and is the treatment of choice for the rare child with AIN who manifests very severe or recurrent major infections.[744,756,769] G-CSF doses of 1 to 2 μg/kg/day or less are usually effective, and responses are typically seen within days. Steroids and intravenous gamma globulin have also been used, but have limited efficacy, as discussed above.

Neonatal Immune Neutropenias

Immune-mediated neutropenia in the newborn may be isoimmune because of maternal immune response to fetal alloantigens or maternal autoimmune as a result of passive transfer of maternal autoantibody.

Neonatal isoimmune neutropenia, an immunologic disorder analogous to Rh hemolytic disease, results from maternal sensitization to fetal neutrophils bearing antigens that differ from the mother's. Maternal IgG antibodies cross the placenta and result in an immune-mediated neutropenia that can be severe and lasts from several weeks to as long as 6 months.[348,745,770] The infant bone marrow demonstrates myeloid hyperplasia, usually with depletion of mature neutrophil forms.

Neutrophil antibodies are found in the serum of the mother and the infant and are often directed to the neutrophil-specific HNA antigen system.[746] The HNA-1 antigens most often responsible are isotypes of the neutrophil Fcγ receptor IIIb (see Table 22-6). Affected infants can be asymptomatic or develop omphalitis, cutaneous infections, pneumonia, sepsis, and meningitis. Since neonatal sepsis can be itself associated with profound neutropenia, the underlying immune-mediated neutrophil destruction may not be appreciated immediately in affected newborns who have signs of sepsis.

The initial management of neonatal isoimmune neutropenia should include parenteral antibiotics, even if signs of infection are absent, because of the association of neutropenia with neonatal sepsis. Intravenous γ globulin may sometimes be effective in increasing the neutrophil counts.[771,772] Treatment with G-CSF starting at 5 to 10 μg/kg/day intravenously should be considered for very profound neutropenia or when serious infections develop in infants with isoimmune neutropenia.[745,773,774]

Neonatal immune neutropenia can also occur in infants of mothers with AIN. Transplacental passage of an IgG antineutrophil antibody can react with the infant's neutrophils bearing the inciting antigen and result in a profound neutropenia lasting 2 to 4 weeks.[745,775] Treatment is similar to that for isoimmune neutropenia. Leukoagglutinating antibodies directed against HLA antigens are commonly found in multiparous women and their newborn children's sera[776,777]; however, these antibodies are not associated with neutropenia and thus need to be distinguished from neutrophil-specific antibodies in laboratory assays.[754,770]

Nutritional Deficiencies. Ineffective granulopoiesis is part of the megaloblastic marrow pathology observed in patients with nutritional deficiencies of vitamin B_{12} or folic acid.[778] Neutropenia also occurs with severe protein-calorie malnutrition, as in anorexia nervosa[779] and marasmus. Neutropenia and marrow megaloblastosis have also been observed in copper deficiency[780] Notably, these nutritional deficiencies do not cause isolated neutropenia; anemia is virtually universal, and thrombocytopenia is an occasional additional finding.

Reticuloendothelial Sequestration. Splenic enlargement caused by portal hypertension, intrinsic splenic disease, or splenic hyperplasia can lead to neutropenia that is usually accompanied by similar degrees of thrombocytopenia and anemia. The neutropenia is usually mild and may be ameliorated by successful treatment of the underlying disease. In selected situations, splenectomy may be necessary to restore the neutrophil count to normal.

Bone Marrow Infiltration. Malignancies such as leukemia, lymphoma, and solid tumors that infiltrate the bone marrow result in a myelophthisic picture, producing leukoerythroblastic peripheral blood smears with neutropenia that is usually accompanied by other cytopenias. Tumor-induced myelofibrosis may further reduce the peripheral blood neutrophil count. Neutropenia can also result from bone marrow involvement in granulomatous infections, lysosomal storage disease, or osteopetrosis.[212,781,782]

Chronic Idiopathic Neutropenia

Chronic idiopathic neutropenia represents a group of disorders that by definition are poorly understood and cannot be placed in any of the previously named categories.[547,574] Investigation of the mechanism of neutropenia has suggested that in some cases, decreased or ineffective production of neutrophils may be the result of excessive apoptosis, such as in congenital neutropenia.[783] Overall, the clinical features, bone marrow findings, and natural history of chronic idiopathic neutropenia are variable but generally milder than congenital neutropenia; it is likely that many of the cases described in older studies had antibody-mediated neutrophil destruction and normal bone marrow reserve. Treatment, when necessary, should be based on the severity of symptoms; G-CSF has proven effective at lower doses than in SCN and without any reported increase in risk of MDS/AML.[530]

Evaluation of the Patient with Neutropenia

The basic approach to a patient with neutropenia (Fig. 22-13)[574] includes a history and physical examination with emphasis on: (1) related phenotypic abnormalities; (2) determination of whether bacterial infection is present (including evaluation of gingiva and perineum); (3) evaluation of lymphadenopathy, hepatosplenomegaly, and any other signs of an underlying associated chronic illness; and (4) history of recent infection and drug exposure. The frequency and duration of symptoms is important, and a history of periodontitis, dental abscesses, or tooth loss is particularly suspicious for significant chronic or recurrent neutropenia. The family history may reveal other individuals with recurrent infection. Unexplained deaths and the ethnic background in children younger than 1 year of age should be noted.

The duration and severity of the neutropenia and the presence or absence of significant other symptoms or physical findings greatly influence the speed and extent of laboratory evaluation. If the patient has isolated neutropenia, is asymptomatic, and other findings are absent, clinical observation for several weeks is usually the best approach. Any medications known or suspected to be associated with neutropenia should be discontinued. Any signs of an acute bacterial infection in a patient with moderate to severe neutropenia and fever calls for prompt evaluation and treatment, including the use of intravenous broad spectrum antibiotics.

Patients with persistent neutropenia should have white blood cell and differential counts obtained at least twice weekly for 6 to 8 weeks in order to evaluate for the periodicity of CyN. Direct and indirect antiglobulin tests should be performed to evaluate for the presence of red cell autoantibodies and quantitative serum immunoglobulins (IgG, IgA, and IgM, but not IgE) measured to detect

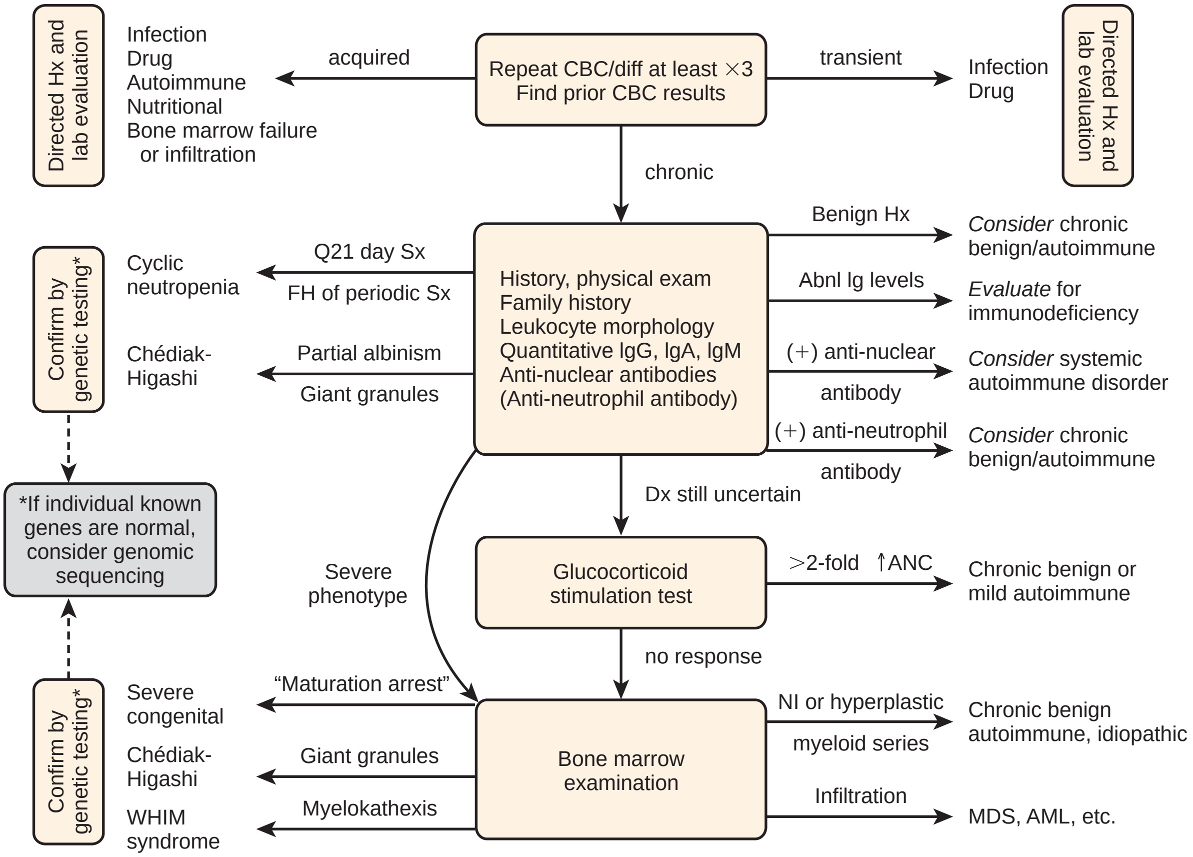

Directed Hx and lab evaluation
Infection
Drug
Autoimmune
Nutritional
Bone marrow failure or infiltration
acquired
Repeat CBC/diff at least ×3
Find prior CBC results
transient
Infection
Drug
Directed Hx and lab evaluation
chronic
Benign Hx
Consider chronic benign/autoimmune
Confirm by genetic testing*
Cyclic neutropenia
Q21 day Sx
FH of periodic Sx
History, physical exam
Family history
Leukocyte morphology
Quantitative IgG, IgA, IgM
Anti-nuclear antibodies
(Anti-neutrophil antibody)
Abnl Ig levels
Evaluate for immunodeficiency
Chédiak-Higashi
Partial albinism
Giant granules
(+) anti-nuclear antibody
Consider systemic autoimmune disorder
(+) anti-neutrophil antibody
Consider chronic benign/autoimmune
*If individual known genes are normal, consider genomic sequencing
Dx still uncertain
Severe phenotype
Glucocorticoid stimulation test
>2-fold ↑ANC
Chronic benign or mild autoimmune
no response
Confirm by genetic testing*
Severe congenital
"Maturation arrest"
Chédiak-Higashi
Giant granules
WHIM syndrome
Myelokathexis
Bone marrow examination
Nl or hyperplastic myeloid series
Chronic benign autoimmune, idiopathic
Infiltration
MDS, AML, etc.

Figure 22-13 Schematic diagram of the approach to a patient with neutropenia. The central flow diagram presents a sequential series of steps in the evaluation, and the labeled arrows to each side indicate probable mechanisms or diagnoses followed by subsequent steps in the evaluation. *Severe phenotype* refers to a history of physical signs of severe, recurrent, or opportunistic infections. *Antineutrophil antibody* is enclosed in parentheses to indicate that the test has limited utility. *Abnl,* Abnormal; *AML,* acute myeloid leukemia; *ANC,* absolute neutrophil count; *CBC/diff,* complete blood count with differential count; *Dx,* diagnosis; *FH,* family history; *Hx,* history; *Ig,* immunoglobulin; *Q21 day,* every 21 days; *MDS,* myelodysplastic syndrome; *Nl,* normal; *Sx,* symptoms; *WHIM,* warts, hypogammaglobulinemia, infections, and myelokathexis.

associated immunoglobulin abnormalities. HIV testing is also indicated in chronic cases. Antineutrophil antibody testing, although technically imperfect (as discussed), is generally included in an initial evaluation because of the frequency of AIN.

Bone marrow aspiration and biopsy with cytogenetics should be part of the initial evaluation if leukoerythroblastosis, anemia, macrocytosis, or thrombocytopenia is present and should be performed eventually if the diagnosis remains obscure despite initial testing. If SCN is suspected, bone marrow analysis with cytogenetic screening for monosomy 7 should be performed both to establish the diagnosis (through demonstration of "maturation arrest") and to rule out MDS before G-CSF therapy. Other laboratory tests that should be considered, depending on the clinical situation, include evaluation for collagen vascular disease, exocrine pancreatic insufficiency, metabolic disorders (e.g. organic acidurias), and measurement of B_{12}, folate, and copper levels. Radiographic studies may be useful in the diagnosis of Shwachman-Diamond syndrome. Assessing the ANC before and 4 to 6 hours after a single dose of glucocorticosteroid (usually prednisone 1 to 2 mg/kg) measures mobilization of the bone marrow reserve pool of mature neutrophils[784,785]; an increase in the ANC to a normal or only moderately low level indicates "chronic benign" or idiopathic neutropenia and may render bone marrow examination unnecessary. The identification of genes responsible for most forms of congenital neutropenias (discussed previously) now permits specific genetic diagnosis in many cases and provides a basis for genetic counseling and prenatal diagnosis.

Principles of Therapy for Neutropenia

The management of neutropenia depends on the underlying cause and severity of the neutropenia.[574] The major concern in patients with neutropenia is the development of serious pyogenic infection. Patients with severe neutropenia (ANC ≤500 cells/μL) with poor marrow reserves are at highest risk for developing progressive infection and septicemia. Fever may often be the only indication of infection, because local signs and symptoms of inflammation may be diminished in the face of neutropenia. Organisms involved are usually from the skin or GI tract. Thus febrile patients with severe neutropenia caused by poor marrow function should be treated promptly with broad-spectrum antimicrobials after blood and other appropriate cultures are obtained, and treatment should be continued as long as the patient is febrile, even if blood cultures are negative. Patients with neutropenia who are febrile for more than 4 days while receiving broad spectrum antibacterial antibiotics are at risk for fungal infection, so empiric treatment with antifungal antibiotics should be considered in this setting.[786] Patients who have neutropenia with documented fungal infection or gram-negative bacterial sepsis who have not responded to appropriate therapy are candidates for granulocyte transfusions.[787-789]

Patients with neutropenia and normal to increased marrow cellularity (e.g., in the setting of AIN) often have a minimally increased risk of pyogenic infection and respond more briskly to appropriate antibiotics. A less aggressive course may be a reasonable approach to a febrile illness in such patients who appear clinically well, even if the ANC is below 500 cells/μL. A child with AIN who develops fever or infection can generally be managed as an outpatient unless the infection is severe.

Chronic gingivitis and periodontitis can be a persistent source of morbidity and result in tooth loss in patients with chronic or CyN. Good dental hygiene and regular professional treatment are necessary to forestall or prevent tooth loss and far outweigh any theoretical risk of bacteremia from local trauma.

The advent of recombinant G-CSF and other hematopoietic growth factors has revolutionized the therapy of neutropenias.[530,547,566,571,741,757] As discussed in previous sections, G-CSF has been successfully used to increase the neutrophil count in a wide variety of conditions including SCN, CyN, and immune-mediated neutropenias. Continuous G-CSF is recommended for all patients with SCN and other conditions with poor marrow production of neutrophils and a high risk of developing serious infection. For disorders with lower risks of infection, such as autoimmune or drug-induced neutropenia, G-CSF therapy should be reserved for specific clinical indications such as serious or repeated infections. In general, more frequent administration (daily or alternate days) of the lowest dose needed to achieve an ANC greater than 1000 cells/μL produces a more steady response and lower toxicity than less frequent administration (e.g., weekly) of higher doses. Polyethylene glycol-filgrastim has been used off label in a few patients who were unresponsive to filgrastim, but it causes considerable bone pain and more serious toxicities.[790,791]

Antibiotic prophylaxis, usually with trimethoprim-sulfa, may be useful for some patients with neutropenia and normal to increased myelopoiesis who do not meet criteria for G-CSF therapy.[757] There are no evidence-based data to indicate the effectiveness of measures beyond careful handwashing to prevent infections, most of which arise from the patients' own skin and gut flora; there is no need for reverse precautions or neutropenic diets in patients who have neutropenia without concurrent immunosuppression. To avoid invasive fungal infection, patients with chronic neutropenia should also avoid mulch, dusty construction or demolition sites, and bird or animal waste.[786,792]

Box 22-7 **Classification of Neutrophilia**

INCREASED PRODUCTION

- Chronic infection
- Chronic inflammation
  - Ulcerative colitis
  - Rheumatoid arthritis
- Tumors
- Postneutropenia rebound
- Myeloproliferative disease
- Drugs (lithium; rarely ranitidine, quinidine)
- Chronic idiopathic neutrophilia
- Familial cold urticaria

ENHANCED RELEASE FROM MARROW STORAGE POOL

- Corticosteroids
- Stress
- Hypoxia
- Acute infection
- Endotoxin

REDUCED MARGINATION

- Stress
- Infection
- Exercise
- Epinephrine
- Leukocyte adhesion deficiency

DECREASED EGRESS FROM CIRCULATION

- Corticosteroids
- Splenectomy
- Leukocyte adhesion deficiency

Neutrophilia

Neutrophilia refers to an alteration in the total number of blood neutrophils that is in excess of about 7500 cells/μL in children and adults. During the first few days of life the upper limit of the normal neutrophil count ranges from 7000 to 13,000 cells/μL for neonates born prematurely and at term gestation, respectively,[793] and is followed by a decrease to adult levels within the first few weeks of life. An increase in circulating neutrophils is the result of a disturbance of the normal equilibrium involving neutrophil bone marrow production and movement in and out of the marrow and circulation. Three primary mechanisms accounting for neutrophilia[31,794] act most often in combination (Box 22-7). First, increased bone marrow production can expand the circulating neutrophil pool through: (1) increased progenitor cell proliferation and terminal differentiation through the neutrophilic series; (2) increased mitotic activity of neutrophilic cell precursors; or (3) shortening of the cell cycle time in the mitotic pool of neutrophil precursors. Second, increased numbers of neutrophils may be mobilized from either the bone marrow storage compartment or the peripheral marginating pools into the circulating pool. Additionally there may reduced neutrophil margination or egress into tissue.

Acute neutrophilia occurs rapidly within minutes in response to exercise or epinephrine-induced reactions and has been attributed to mobilization of the marginating pool of neutrophils into the circulating pool.[795,796] Slower onset of acute neutrophilia can occur after glucocorticoid administration or with inflammation or infection associated with the generation of endotoxin, TNF, IL-1, IL-8, and hematopoietic growth factors such as G-CSF.[6,7,785,797] Maximal response usually occurs within 4 to 24 hours after exposure to these agents and is probably the result of release of neutrophils from the marrow storage compartment into the circulation. The mechanisms that underlie release from the marrow pool involve interactions between neutrophils and the bone marrow stroma mediated by adhesion molecules (such as β2 integrins) and by chemokines such as CXCL12 and its receptor CXCR4.[40] Glucocorticoids may also slow the egress of neutrophils from the circulation into tissue.[798]

Chronic neutrophilia may accompany the prolonged administration of glucocorticoids, persistent inflammatory reactions, infection, chronic blood loss, or chronic anxiety.[799-803] Marked neutrophilia is a hallmark of functional disorders of neutrophils that are caused by impaired adhesion or motility, such as in patients with leukocyte adhesion deficiencies or actin dysfunction (see later). Sustained moderate neutrophilia invariably follows either surgical or functional asplenia,[804] probably because of decreased clearance of circulating neutrophils.[32] Neutrophilia also occurs in familial cold urticaria and periodic fever syndromes.[805,806]

Congenital primary neutrophilia is rare. In one family with AD hereditary neutrophilia, affected individuals maintained ANCs of 14,000 to 164,000 cells/μL along with hepatosplenomegaly, increased leukocyte alkaline phosphatase, and Gaucher-type bone marrow histiocytes.[807] A more recently-described AD neutrophilia kindred of 12 patients in three generations had an activating mutation in the *CSF3R* gene leading to constitutive activation of the G-CSF receptor and increased proliferation and differentiation of neutrophil precursors.[808] Affected family members had a median neutrophil count of 16,900 cells/μL (range: 11,000 to 23,700) and one progressed to MDS.

Leukemoid Reactions

Leukocyte counts greater than 50×10^3 normal cells/μL are referred to as *leukemoid reactions*.[809] The peripheral blood may show small proportions of immature myeloid cells, including occasional myeloblasts and promyelocytes. Leukemoid reactions need to be distinguished from chronic myelocytic leukemia (CML), which features a more extreme "left shift" and basophilia in the peripheral blood differential count, as well as the characteristic clinical, laboratory, and cytogenetics signs of a myeloproliferative disorder (see Chapter 11). Chronic neutrophilic leukemia is a distinct, extremely rare, malignant cause of mature neutrophilia, with a mean age at diagnosis of 62.5 years (range 15 to 86) in 33 reported cases.[810]

Leukemoid reactions are most commonly triggered by pyogenic infections, especially by *S. aureus* or *S. pneumoniae* but can also occur with other infections such as tuberculosis, brucellosis, or toxoplasmosis; with inflammatory syndromes such as acute glomerulonephritis or acute rheumatoid arthritis; and with liver failure or diabetic acidosis.[809,811] Infants with Down syndrome may develop a transient myeloproliferative disorder[812] (see Chapter 11) that is distinguishable by the marked leukoerythroblastosis with circulating blast cells. Leukemoid reactions have also been identified in neonates with thrombocytopenia-absent radius syndrome.[813]

Clinical States Associated with Alterations of Eosinophil Numbers

Eosinophilia

A very broad range of conditions can elicit an eosinophilic response (Box 22-8).[814] Eosinophil stimulation most commonly occurs after repetitive or prolonged antigen exposure, especially when the antigens are deposited in the tissues and elicit hypersensitivity reactions, whether of the immediate (IgE-mediated) or delayed (T-lymphocyte–mediated) type.[490] Stimulation of eosinophilia is T-lymphocyte–dependent and underlies the immune response to metazoan parasites.[14,490]

Allergy is the most common cause of eosinophilia in children in the United States.[815] Acute allergic reactions may cause leukemoid eosinophilic responses, with eosinophil counts exceeding 20,000 cells/μL, whereas chronic

Box 22-8 **Causes of Eosinophilia**

ALLERGIC DISORDERS

Acute urticaria
Allergic bronchopulmonary aspergillosis
Asthma
Atopic rhinitis
Drug reaction

DERMATITIS

Atopic dermatitis
Pemphigus, pemphigoid

PARASITES AND INFECTIONS

Amebiasis
Cat scratch fever
Coccidioidomycosis
Fungal rhinosinusitis
Helminths (particularly if invasive)
Malaria
Scabies
Scarlet fever
Toxoplasmosis
Tuberculosis

GASTROINTESTINAL DISORDERS

Eosinophilic gastroenteritis
Food allergy

HEREDITARY DISORDERS

Familial eosinophilia
Hereditary angioedema
Hyper-Immunoglobulin E syndrome
Omenn syndrome
Thrombocytopenia with absent radius
Wiskott-Aldrich syndrome

NEOPLASMS

Acute lymphoblastic leukemia
Lymphoma (Hodgkin and non-Hodgkin)
Metastatic cancer
Myeloproliferative disorders

RHEUMATOLOGIC DISORDERS

Eosinophilic fasciitis
Polyarteritis nodosa
Scleroderma

MISCELLANEOUS

Adrenal insufficiency
Graft-versus-host disease
Peritoneal dialysis or hemodialysis
Sarcoidosis
Toxins (eosinophilia/myalgia, toxic oil syndromes)

HYPEREOSINOPHILIC SYNDROME

Clonal hypereosinophilic syndrome (HES)
Idiopathic HES

allergy is rarely associated with eosinophil counts of more than 2000 cells/μL.[816] A variety of skin diseases have been associated with eosinophilia,[817] the best documented being atopic dermatitis, eczema, pemphigus, acute urticaria, and toxic epidermal necrolysis. Drug reactions often elicit eosinophilia,[818] including the potentially fatal drug rash with eosinophilia and systemic symptoms (DRESS) syndrome.[819]

Outside the United States, and hence also in immigrants and returning travelers, parasitic infections are the most common causes of eosinophilia.[820-822] Infestations by certain parasites, including helminths, induce greater degrees of eosinophilia than do protozoan infestations.[14,815,817] Other parasites such as *Giardia lamblia*, *Enterobius vermicularis,* and *Trichuris trichiura* fail to elicit an eosinophilic response, probably because they remain localized to the intestinal tract and do not enter somatic tissue.

When parasites invade systemic organs, they may incite clinical symptoms and signs related to the involved organs, such as hepatomegaly and pulmonary infiltrates. Complications include seizures, encephalitis, myocarditis, retinal lesions (that are often difficult to distinguish from retinoblastoma), and skin nodules on the palms of the hands and soles of the feet. Leukocyte counts may exceed 100,000 cells/μL, with marked eosinophilia persisting from months to years after resolution of symptoms. Polyclonal hypergammaglobulinemia is common. Increased anti-A and anti-B titers are commonly observed because of cross-reactivity between red cell and parasitic antigens.

Eosinophilia is also observed in scarlet fever, CMV pneumonia of infancy, cat-scratch disease, infectious lymphocytosis, and occasionally, infectious mononucleosis. Many patients with acute pulmonary tuberculosis show decreased numbers of circulating eosinophils followed by an increase in eosinophil numbers during the convalescent phase, but rarely patients exhibit marked eosinophilia at diagnosis.

In general, fungal diseases do not cause eosinophilia, but important exceptions are allergic and nonallergic fungal sinusitis and rhinosinusitis, coccidioidomycosis, and allergic bronchopulmonary aspergillosis.[817,823-825] The latter two diagnoses fit into the spectrum of pulmonary eosinophilic syndromes, which also include idiopathic eosinophilic pneumonia, Churg-Strauss syndrome (a form of vasculitis), tropical pulmonary eosinophilia (caused by microfilariae and other parasites), and drug- and radiation-induced pulmonary eosinophilia.[817,826,827]

GI disorders may also be associated with eosinophilia.[815,828] Primary eosinophil-mediated disorders usually involve the esophagus and stomach but can affect any or all segments of the GI tract.[828,829] Eosinophilia can also be a secondary sign of food allergy.[830] Inflammatory bowel disease and gluten enteropathy may involve large numbers of tissue eosinophils but show little or no elevation of blood eosinophil numbers.[831-833]

Eosinophilia is a prominent feature of several immunodeficiency disorders, especially hyper-IgE syndrome (HIES; Job syndrome) (see later), Wiskott-Aldrich and Omenn syndromes,[834,835] and hereditary angioedema.[836,837] Approximately 10% of patients with rheumatoid arthritis develop mild eosinophilia during the course of their disease.

AD familial eosinophilia has been reported in several families in which individuals displayed marked eosinophilia, but few had pulmonary, cardiac, or neurologic involvement.[838,839] The disorder has been mapped to chromosome 5q31-q33, a region that contains a cytokine cluster including genes encoding IL-3, IL-5, and GM-CSF.[840] However, no mutations or functional polymorphisms have been identified within the promoter, exons, or introns of any of these genes, suggesting that the primary defect in familial eosinophilia is located in another gene in the cluster. The disorder can be distinguished from HES (see the following section) by the relative absence of eosinophil activation markers and rarity of end organ damage.[839]

Marked eosinophilia can accompany or precede the diagnosis of malignancies including Hodgkin and non-Hodgkin lymphomas, acute lymphoblastic leukemia, and a variety of solid tumors.[817,841,842] Excessive numbers of normal eosinophils may also be produced in response to production of cytokines, primarily IL-5, by abnormal (but only sometimes clonal) T cells with aberrant immunophenotypic markers.[843] About one third of patients undergoing chronic hemodialysis develop blood eosinophilia without an apparent cause.[844] Similarly, chronic peritoneal dialysis may cause an eosinophilic peritoneal effusion and occasionally an elevated number of eosinophils in the blood.[845]

Eosinophilia can produce organ damage, particularly to the heart, lungs, and GI tract, by infiltration and deposition of toxic granule proteins. If treatment of the underlying infection or disease is not sufficient to reduce the eosinophil count, then steroid therapy generally produces a rapid decline in eosinophil production and circulating cell numbers. Steroid-sparing targeted therapies such as monoclonal antibodies directed at IL-5 or IL-5R (e.g., mepolizumab, reslizumab, and benralizumab) or at T cells (e.g., alemtuzumab) have shown efficacy, and a variety of additional biologic agents are in development.[846]

Hypereosinophilic Syndrome

HES is defined as a persistent eosinophilia in patients that meet the following criteria: (1) absolute eosinophil count of 1500 cells/μL or more for longer than 6 months (or fatal termination within 6 months); (2) lack of other diagnoses to explain secondary eosinophilia; and (3) signs and symptoms of organ involvement by infiltrating eosinophils.[847,848] Clinical manifestations result from tissue infiltration by eosinophils and the release of eosinophil granule products that cause tissue damage. Symptoms include nonspecific findings of fever, weight loss, and fatigue. Cardiac damage is the major cause of morbidity and mortality in HES and includes endocardial fibrosis and formation of mural thrombi with infiltrating eosinophils (Loeffler endocarditis).[815,817,849] These findings can also be seen from eosinophilia of multiple other etiologies, including parasitic infection, drug reactions, or secondary to malignancies. Hepatosplenomegaly, pulmonary infiltrates, and skin involvement with urticarial or nodular lesions are common; neuropathies, encephalopathy, and CNS thromboembolism can also occur.

The recognition of specific gene rearrangements has provided a molecular basis for understanding the pathophysiology, developing classification systems, and guiding treatment.[846,848,850-853] The most common involve the gene encoding the platelet-derived growth factor receptor α, *PDGFRA*, which forms fusion products that generate constitutively active tyrosine kinase molecules.[854,855] The most common fusion partner is the Fip1-like 1 gene, *FIP1L1*, resulting from an interstitial chromosome 4q12 deletion that is not cytogenetically detectable; however, multiple rarer fusions and cytogenetic changes have been reported.[851] These rearrangements result in HES with a marked male predominance and a phenotype that includes splenomegaly, elevated serum vitamin B_{12} and tryptase levels, and hypercellular bone marrow with fibrosis and increased eosinophils and mast cells. Other patients with clonal HES have a wide variety of rearrangements involving the *PDGFRB* gene, which encodes platelet-derived growth factor receptor β, and rare cases have been reported with rearrangements of *FGFR1*, which encodes fibroblast growth factor receptor 1, and other genes.[851] Importantly, patients with PDGFR fusion products are likely to respond to imatinib therapy, sometimes at dosages lower than those used for CML.[846,850]

There is still no consensus on classification of HES, in part because of overlap between primary and secondary hypereosinophilia.[814,848,851,853] The most general classification groups the syndromes as: (1) primary neoplastic HES with an underlying clonal abnormality; (2) secondary HES caused by an underlying neoplastic or nonneoplastic condition leading to nonclonal eosinophilia; and (3) idiopathic HES. The diagnosis of chronic eosinophilic leukemia is very difficult to distinguish from clonal HES, although the World Health Organization classification makes this distinction.[848] The lymphoid form of secondary HES is a distinct variant caused by monoclonal expansion of T cells with an indolent course and rare evolution to T-cell lymphoma.[490]

HES can generally be distinguished from the M4Eo variant of AML, which is characterized by myelomonocytic blasts with eosinophilia and inv(16) cytogenetics (see Chapter 11).

Eosinopenia

Eosinopenia is not uncommon but is seldom recognized clinically, because its precise diagnosis requires an absolute eosinophil count.[856] Eosinopenia may be produced by at least two mechanisms: (1) primary elevation of adrenal corticosteroids or epinephrine; and (2) acute inflammation or stress, acting in part through secondary release of adrenal corticoids and/or epinephrine. The immediate eosinopenia after administration of glucocorticoids reflects the destabilization of mRNA encoding cytokines such as eotaxins and inhibition of the cytokine-dependent survival of eosinophils.[14,857] Glucocorticoids also suppress the transcription of a number of genes involved in eosinophil production and trafficking, including IL-3, IL-4, IL-5, and GM-CSF.[14] Acute inflammation is associated with alterations in eosinophil distribution and production; at least part of this response occurs independently of adrenal corticoid release.

Box 22-9 Disorders Associated with Basophilia

MILD BASOPHILIA

- Hypersensitivity reactions
  - Drug and food hypersensitivity
  - Urticaria
- Infection
  - Helminths
  - Chickenpox
  - Influenza
  - Smallpox
  - Tuberculosis
- Inflammation
  - Rheumatoid arthritis
  - Ulcerative colitis
- Hypothyroidism (severe)

MARKED BASOPHILIA

- Chronic myelogenous leukemia
- Myelodysplastic syndrome
- Acute myeloid leukemia (rare)

Basophilia and Basophilopenia

Basophils are associated not only with hypersensitivity reactions of the immediate type but also have increasingly recognized roles in innate immunity, particularly to helminthes and other parasites.[150,858-860] Basophil levels may be elevated in allergic responses; infections with helminthes, viruses, and mycobacteria; and chronic inflammatory diseases (Box 22-9).[157,861,862] Most importantly, basophil levels are increased in myeloproliferative disorders such as CML (see Chapter 11). Basophil counts exceeding 30% can occur during the course of CML; marked basophilia often heralds a poor prognosis,[863,864] and the cells may be morphologically and functionally abnormal.[865,866] Patients with marked basophilia may develop symptoms attributed to the release of biogenic amines or heparinlike material released from degranulated basophils[866] and may benefit from the administration of antihistamines. Increased numbers of marrow basophils may occur in MDS and sideroblastic anemia.[867,868] Peripheral blood or bone marrow basophilia may also accompany acute myeloid leukemias, usually in association with 6p or 12p chromosomal abnormalities,[869] or juvenile myelomonocytic leukemia.[866]

Basophilopenia occurs in conditions that are associated with eosinophilopenia, such as during acute infection or after the administration of glucocorticoids.[870-872] Basophil counts are diminished in thyrotoxicosis and after treatment with thyroid hormones, and conversely they may be increased in myxedema.[870]

Monocytosis and Monocytopenia

The normal absolute blood monocyte count varies with the age of the patient, and this variation must be taken into account when assessing monocytosis. During the first 2 weeks of life, the absolute monocyte count is more than 1000 cells/μL.[873] With increasing age, there is a gradual

Box 22-10 Disorders Associated with Monocytosis

INFECTION

Subacute bacterial endocarditis
Tuberculosis
Syphilis
Protozoal and rickettsial infections (e.g., Rocky Mountain spotted fever, kala-azar)

MALIGNANCY

Preleukemia
Juvenile myelomonocytic leukemia
Acute myelogenous leukemia
Chronic myelogenous leukemia
Lymphoma (Hodgkin and non-Hodgkin)
Solid tumors (usually advanced carcinoma)

RHEUMATOLOGIC DISORDERS

System lupus erythematosus
Rheumatoid arthritis
Sarcoidosis
Inflammatory bowel disease

MISCELLANEOUS DISORDERS

Severe chronic neutropenia
Recovery from transient neutropenia
Postsplenectomy status
Alcoholic liver disease
Tetrachloroethane poisoning

decline in the monocyte count until it reaches a plateau of 400 cells/μL in adulthood. Monocytosis may therefore be defined as a total monocyte count of more than 500 cells/μL. Many clinical disorders give rise to monocytosis (Box 22-10), most typically bacterial, protozoan, and rickettsial infections such as subacute bacterial endocarditis, tuberculosis, syphilis, Rocky Mountain spotted fever, and kala-azar.[167] Monocytosis is a hallmark of juvenile myelomonocytic leukemia (see Chapter 11) and can also be observed in malignant disorders such as preleukemia, AML, CML, lymphomas, and advanced stage carcinomas.[874] Approximately 25% of all patients with Hodgkin lymphoma have monocytosis, which does not correlate with prognosis.[875] Monocytosis has also been noted in a wide variety of inflammatory and immune disorders including lupus, rheumatoid arthritis, sarcoidosis, and inflammatory bowel disease.[876] Finally, as discussed, both relative and absolute monocytosis occurs in some forms of severe chronic neutropenia and in patients recovering from myelosuppressive chemotherapy.

Monocytopenia has been observed after glucocorticoid administration and in infections associated with endotoxemia.[877,878] In the latter case, systemic activation of complement occurs with the deposition of C5a on the surface of monocytes, leading to their aggregation and clearance. Decreased monocyte superoxide production, chemotaxis, and microbial killing have been reported in Gaucher disease, possibly contributing to the risk of infections in this disease.[879-881] The defects are reversed at least partially by enzyme replacement therapy.[879]

Monocytopenia is a primary feature of the recently described MonoMAC syndrome. This pleiotropic disorder also includes, to varying degrees, B- and NK-cell deficiencies; mild neutropenia; susceptibility to severe and chronic nontuberculous mycobacterial, fungal, and viral infections; predisposition to MDS, myeloid leukemias, and autoimmunity; pulmonary alveolar proteinosis; and lymphedema.[649,882-886] Deficient NK-cell function reflects the selective loss of CD56 bright cells.[887] The syndrome includes phenotypes previously reported as Emberger syndrome (AD deafness, lymphedema, and MDS/AML)[888,889] and NK-cell deficiency with severe herpes virus infections.[890] MonoMAC syndrome derives from hypomorphic and regulatory mutations that diminish expression of the GATA-2 transcription factor,[891,892] which regulates development and function in multilineage hematopoiesis and lymphangiogenesis, as well as leukemogenesis.[886,893] The disorder has been treated successfully by HSCT.[894]

Monocytopenia is a feature of another immunological disorder, so far unnamed, that is caused by mutations in the *IRF8* gene and resulting in monocytopenia, absent or deficient dendritic cells, and immunodeficiency presenting as disseminated bacille Calmette-Guérin (BCG) disease.[895]

Infantile (malignant) osteopetrosis is a hereditary disease of bone marrow replacement by bone that is caused by a failure of bone resorption and remodeling by osteoclasts, a form of specialized tissue macrophage.[212,896,897] This disorder presents in infancy and is characterized by the progressive obliteration of the bone marrow space by bone, leading to progressive loss of marrow function, hepatosplenomegaly, growth retardation, and compression of cranial nerves.[212] AR and less severe AD osteopetrosis are caused by mutations in any of multiple genes involved in receptor activator of nuclear factor κB and its ligand (RANK and RANKL) signaling, receptor-mediated endocytosis, vacuolar proton and chloride transport, G-protein degradation, or vesicular sorting.[217,218,898-904]

Generation of superoxide by peripheral blood leukocytes is defective in some patients with AR (malignant) osteopetrosis[905] and recombinant IFN-γ (also used for CGD; see later) has been shown to increase bone resorption and improve hematopoietic marrow function in children with infantile osteopetrosis.[906] Infantile osteopetrosis is generally treated with bone marrow transplantation,[212,907] Promising studies in mouse models suggest that RANKL administration or gene therapy might provide future therapeutic options.[908,909]

DISORDERS OF GRANULOCYTE AND MONONUCLEAR PHAGOCYTE FUNCTION

Abnormalities in one or more steps of phagocyte function—adhesion, chemotaxis, ingestion, degranulation, and oxidative metabolism—are causes of inherited and acquired clinical disorders that can be classified based on the function primarily affected. Consistent with the critical role of phagocyte function in host defense, patients afflicted with these disorders often suffer from recurrent, difficult-to-treat bacterial and fungal infections in skin or mucosa, lung, lymph nodes, or deep-tissue abscesses. Many of these disorders have characteristic clinical and microbiologic features related to the particular functional defect. Finally, inherited disorders involving cytokine or TLRs and their signal transduction are increasingly recognized, many of which affect phagocyte function and result in recurrent infections. These are described near the end of this chapter.

Of note, in clinical practice most physicians encounter a number of patients who suffer from recurrent bacterial infections. Whereas it is true that nearly all patients with well-characterized phagocyte abnormalities have recurrent infections, the converse is seldom the case. Most patients with impressive histories of persistent and repeated infections do not have identifiable qualitative or quantitative phagocyte abnormalities.[910] Therefore the disorders described below account for only a fraction of patients with recurrent infections. This point will be discussed further in the final section describing the laboratory evaluation of phagocyte function.

Disorders of Adhesion

Leukocyte Adhesion Deficiency Type I

LAD I is a rare AR disorder in which phagocyte adhesion, chemotaxis, and ingestion of C3bi-opsonized microbes is impaired because of mutations in the gene for the β subunit of the β_2 integrins, CD18. As a result, the expression of β_2 integrins on leukocyte cell surfaces is reduced or absent.[274,911-914] More than 100 patients with this disorder have been described in the literature. The hallmark of LAD I is the occurrence of repeated, often severe bacterial and fungal infections without the accumulation of pus despite a persistent granulocytosis (Table 22-10). The clinical syndrome is heterogenous and is related to the severity of the reduction in β_2-integrin expression. Most patients have a severe clinical phenotype, in which fewer than 0.3% of the normal amount of β_2 integrins are present, although a more moderate phenotype with levels of 2.5% to 11% of normal has been observed. Patients with the severe form of LAD I generally show signs in early infancy with neutrophilia, omphalitis, and delayed separation of the umbilical cord. Recurrent necrotic and indolent infections of skin, mucous membranes, and the GI tract are also seen, including perirectal abscesses, which often heal poorly. An aggressive form of gingivitis and periodontitis is characteristic, and ulcerative lesions of the tongue and pharynx can be seen. Otitis media and pneumonia are also often encountered. Most infections are caused by *S. aureus* and gram-negative enteric bacteria. Fungal infections also occur, particularly *Candida albicans* and *Aspergillus* species.

The molecular basis for LAD I was first suggested by Crowley and colleagues,[915] who found that neutrophils from a patient with this clinical syndrome lacked a high–molecular-weight membrane glycoprotein. Because the patient's neutrophils neither adhered to plastic surfaces nor underwent an oxidative burst when exposed to serum-opsonized particles, it was hypothesized that the missing glycoprotein was responsible for both adhesion and cell-particle interactions. A similar glycoprotein was also found to be missing in several other patients,[916,917] and that proved to be the α subunit (CD11b) of the Mac-1 β_2 integrin (CD11b/CD18).[918] It was subsequently recognized that the levels of all leukocyte β_2 integrins (see Fig. 22-5) were absent or severely deficient in LAD I.[911]

Deficient β_2-integrin expression impairs a variety of leukocyte adhesion-dependent activities, with neutrophils being the most significantly affected.[274,911,912,914-916] In addition to their role in mediating adhesive interactions during neutrophil emigration and phagocytosis, signaling through β_2 integrins potentiates virtually all functional responses of adherent neutrophils, including production of reactive oxidants and degranulation.[919] Hence LAD I neutrophils have a profound defect in their ability to become fully activated.

A striking finding in LAD I is the failure of neutrophils to migrate to sites of inflammation. The initial phases of neutrophil adhesion to the endothelium (see Fig. 22-3), which is mediated by selectins and their sialylated counter receptors, is normal in LAD. However, because of deficient Mac-1 (CD11b/CD18) expression, LAD I neutrophils are neither able to attach firmly to the endothelium

TABLE 22-10 Clinical Features of Leukocyte Adhesion Deficiency Type I

Chronic Conditions*	Acute Infections	Infecting Organisms	Other
Persistent granulocytosis (12,000-100,000 cells/mm^3) Gingivitis Periodontitis Stomatitis Impaired wound healing	Omphalitis Cutaneous abscesses and cellulitis (possibly invasive) Perirectal abscesses and cellulitis (possibly invasive) Facial cellulitis Sepsis Pneumonia Laryngotracheitis Peritonitis Necrotizing enterocolitis Sinusitis Esophagitis Erosive gastritis Appendicitis Otitis media	*S. aureus* *E. coli* *P. aeruginosa* *Pseudomonas* species *Proteus* *Klebsiella* *C. albicans* *Aspergillus species* Viral (slightly increased risk)	Delayed umbilical cord separation

*Each list is arranged in approximate order of incidence based on reviews summarizing various series of patients with leukocyte adhesion deficiency.

nor undergo transendothelial migration.[920] Neutrophil intravascular survival is prolonged,[921] presumably related to deficient adhesion and migration. In vitro assays of neutrophil adhesion to glass or to cultured endothelial cells and of neutrophil chemotaxis also exhibit marked abnormalities. An exception to these observations is neutrophil adhesion and emigration in the pulmonary capillary bed, which can be mediated by CD11/CD18-independent mechanisms.[289] In autopsy tissue from a patient with severe LAD I, no neutrophils were observed in infected appendiceal and skin lesions, whereas many neutrophils were seen within the alveolar spaces.[289] In contrast to neutrophils, other leukocytes (monocytes, eosinophils, and lymphocytes) express the β_1 integrin, VLA-4 and are able to utilize this adhesion molecule to emigrate into inflammatory sites throughout the body.

Another major defect in LAD I is the inability of neutrophils and monocytes to recognize microorganisms coated with the opsonic complement fragment C3bi, since Mac-1 is a major C3bi receptor.[911,915,922] The binding of C3bi normally triggers neutrophil degranulation, phagocytosis, and activation of the respiratory burst, but these responses are diminished or absent in neutrophils from patients with LAD I. However, these responses can be at least partially activated in LAD I neutrophils by opsonins (e.g., IgG, C3b) that have different cell surface receptors or by soluble agonists that bypass Mac-1.[915,919]

Defects in LAD I lymphocyte functions dependent on LFA-1 (CD11a/CD18) are often reported when assayed in vitro. These include proliferative responses to mitogens, NK-cell function, and lymphocyte-mediated killing.[923-925] Nevertheless most patients with LAD I manifest few if any problems related to lymphocyte dysfunction in vivo. Cutaneous hypersensitivity reactions are normal, and patients are generally not unusually susceptible to viral infections, including varicella. However, one patient has died of an overwhelming respiratory infection with picornavirus, and one or more episodes of aseptic meningitis have been reported in three patients.[926]

Molecular Basis of Leukocyte Adhesion Deficiency. That LAD I involves a deficiency of all leukocyte β_2 integrins focused attention on the common β_2 chain (CD18) of this integrin family as the site of the molecular defect. This hypothesis proved to be correct. Expression of the leukocyte-integrin α subunits is normal in LAD I, but these are not transported to the cell surface because the β_2 chain is absent or contains mutations that disrupt β_2 structure or its interaction with the α subunit.[927,928] More than 80 different LAD I-associated mutations in the β_2 gene *IBTG2*, located on chromosome 21q22.3, are reported.[929] A database is available at http://bioint.uta.fi.[930] Many patients are compound heterozygotes for two different mutant alleles, whereas others are homozygous for a single mutant allele. About one half of patients with LAD I and characterized genetic defects have point mutations that result in single amino-acid substitutions in CD18 that almost invariably reside between amino acids 111 and 361 encompassed by exons 5 to 9.[929] This protein domain is highly conserved among all β subunits, and appears to be important for interaction with the α subunit. In this LAD I subgroup, approximately one half exhibit a low level of CD11/CD18 cell-surface expression and moderate disease, with the remainder having absent expression and the severe phenotype. Other LAD I-associated mutations include mRNA-splicing defects as well as small deletions within CD18 coding sequences, resulting in a premature termination signal. Mutations resulting in nonfunctional but normally expressed β_2 occur, but very rarely.[929]

There are several animal models of LAD I.[912] These include the occurrence of a severe form of the disease in an Irish setter born of a mother-son mating[931,932] and in Holstein cattle.[933,934] In the latter case, affected calves could be traced to a common sire and were homozygous for a point amino-acid substitution in the conserved extracellular domain of CD18.[935] Finally, CD18-deficient mice have produced by gene targeting.[912]

Diagnosis and Treatment of LAD I. The diagnosis of LAD I should be suspected in any infant or child who has unusually severe or recurrent infections and/or periodontitis accompanied by persistently elevated peripheral blood neutrophil counts, particularly if there is a paucity of neutrophils at affected sites and/or there is a delayed separation of the umbilical cord (see Table 22-10). However, a well infant with delayed separation of the umbilical cord and normal blood counts is very unlikely to have LAD I. Although the mean age of cord separation is reported to be 7 to 15 days, 10% of healthy infants can have cord separation at 3 weeks of age or later.[936,937] A rare cause of recurrent, deep-seated tissue infections in association with neutrophilia and poor formation of pus is mutation of the Rac2 GTPase, which leads to neutrophil signaling defects affecting chemotaxis and adhesion (see "Disorders of Chemotaxis").[97-99,938]

The diagnosis of LAD I is established by flow cytometry to assess cell surface expression of any of the β_2-integrin α (CD11) subunits or the shared CD18 subunit (Fig. 22-14). Neutrophil Mac-1 deficiency is more dramatic if neutrophils are first stimulated, which normally upregulates Mac-1 cell-surface expression. Carriers of LAD I can generally be identified by flow cytometry, because they typically express approximately 50% of the normal level of β_2 integrins on leukocyte cell surfaces.[911,939] Commercial genetic testing is also available.

Treatment of LAD I depends on the clinical severity of the disorder. In patients with the moderate clinical phenotype, who typically have residual β_2-integrin expression, cutaneous and oral infections should be treated aggressively. The use of prophylactic trimethoprim/sulfamethoxazole appears to be beneficial. Aggressive prophylactic treatment of periodontal disease is also advisable in the form of frequent dental cleanings and the use of antimicrobial oral rinses such as chlorhexidine. The clinical management of patients with moderate phenotype should be guided by the observation that these patients are still at risk for dying of overwhelming infection.[274,914] Patients with severe LAD I have even a more grim prognosis, with a high incidence of death from infection before the age of 2 years, and therefore bone marrow

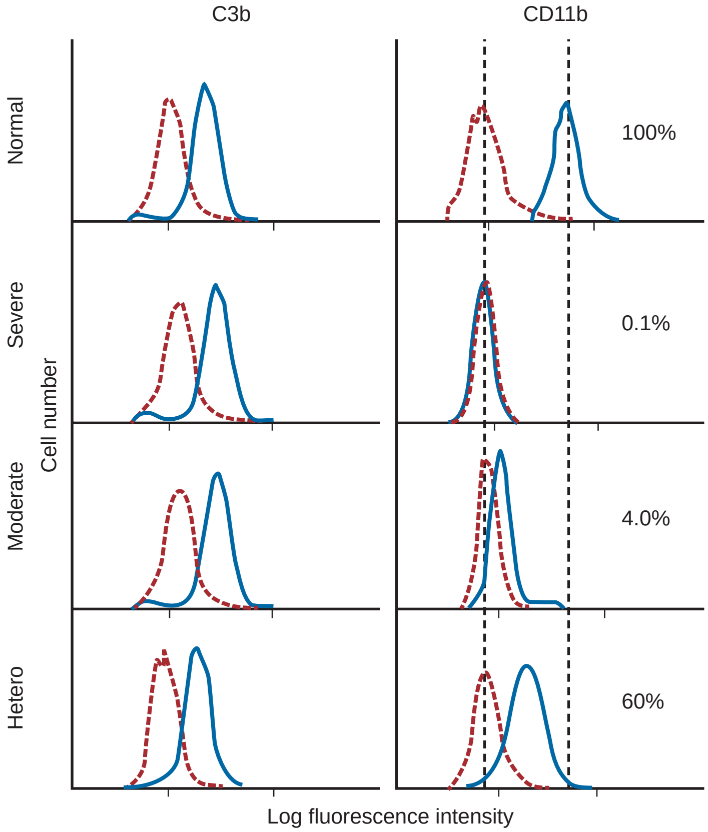


Figure 22-14 Evaluation of adhesion molecule expression for diagnosis of leukocyte adhesion deficiency (LAD). Fluorescence of C3b specific antibody labeled neutrophils *(solid line)* increases compared with a nonspecific control *(dashed line)* after 30-minute exposure to 10-nM fMLP in healthy donors, subjects with severe and moderate forms of LAD I, and a heterozygous LAD I carrier. However, the increase in CD11b fluorescence *(solid line)* is markedly diminished in patients with LAD 1. The respective percentage of normal stimulated mean channel number is shown in the *right column*. Note that these results are expressed as percentage of normal fluorescence intensity, not as a percentage of positive cells, as is the case in most flow cytometry assays. *(Modified from Dinauer MC, Coates TD: Disorders of phagocyte function. In Hoffman R et al, editors:* Hematology: basic principles and practice, *6th ed. Philadelphia, 2012, Saunders, p. 667.)*

transplantation is recommended.[274,914,940] A recent study reported 75% survival in LAD I patients after allogeneic transplantation using either ablative or reduced intensity regimens.[941] Graft rejection or graft-versus-host disease can be problematic with partially compatible human leukocyte antigen (HLA) matching.[941,942]

Gene therapy to correct CD18 expression in a patient's HSCs may be a future therapeutic modality. High-level expression of the transferred CD18 sequence may not be necessary to confer substantial clinical benefits, based on the observed milder course of patients with residual β_2-integrin expression. Studies in canine LAD I suggest that restoring CD18 expression to even 10% of leukocytes will result in clinical improvement,[940,943] and current preclinical efforts are focused on the development of lentivirus or foamy virus vectors to express a normal CD18 gene.[944,945] Obstacles for clinical translation have been the development of techniques that achieve high-level gene transfer into human HSCs and minimizing adverse effects of vector insertion.[940,946,947]

Leukocyte Adhesion Deficiency Type II

LAD II is a very rare AR clinical syndrome similar to LAD I but associated with defective selectin-mediated adhesion resulting from deficiency of Sialyl-Lewis X ligands (Table 22-11).[913,948-952] First described by Etzioni and colleagues,[948] it has since been described in a total of 5 kindreds (4 Arab and 1 Turkish). As in CD11/CD18-deficient LAD, these children suffered from periodontitis, recurrent cellulitis, otitis media, and pneumonia without the formation of pus despite peripheral leukocyte counts of 30,000 to 150,000 cells/μL. However, neutrophils had normal levels of CD18, and infectious symptoms were not as serious as in LAD I. Also in distinction from classic LAD I, the affected children had short stature, a distinctive facial appearance with a flat face and depressed nasal bridge, and severe mental retardation. All had the rare Bombay (hh) blood phenotype, a clue to the molecular defect in which red cells express a nonfucosylated variant of the H antigen. Red cells were also were secretor negative and Lewis antigen negative. The Bombay and nonsecretor phenotypes are caused by deficient formation of Fucα1 to 2 Gal linkages in ABO blood group core antigens, whereas the Lewis antigen-negative phenotype is the result of failure to synthesize Fucα1 to 4 GlcNAc and Fucα1 to 3 GlcNAc moieties.

The defect in fucose metabolism in LAD II cells results from point mutations in the *SLC35C1* gene encoding the

TABLE 22-11 Types of Leukocyte Adhesion Deficiency

	Genetic Defect	Clinical Presentation	Diagnosis
LAD I	CD18 (β_2) subunit of β_2 integrins, resulting in impaired adhesion, chemotaxis, and neutrophil activation	Skin infection, soft tissue abscesses, delayed separation of umbilical cord and omphalitis, periodontal disease	Flow cytometry for leukocyte β_2 integrins
LAD II	GDP-fucose transporter 1, resulting in impaired expression of fucosylated proteins, including SLeX ligand for selectins	Similar infections to LAD I but not as severe; developmental delay, short stature	Flow cytometry for leukocyte CD15s (SLeX) Bombay (hh) phenotype in red blood cell typing
LAD III	Kindlin 3, a regulator of β integrins in blood cells, resulting in defective integrin activation and impaired leukocyte and platelet adhesion	Similar to LAD I; also bleeding tendency	Functional assays for neutrophil and platelet adhesion

GDP, Guanosine diphosphate; *LAD,* leukocyte adhesion deficiency.

Golgi GDP-fucose transporter 1.[951,952] This leads to a generalized loss of fucosylated glycans on cell surfaces, particularly the sialylated and fucosylated tetrasaccharide, SleX (CD15a) on the neutrophil. As a result, LAD II neutrophils cannot bind to E- and P-selectin receptors. The early step of neutrophil rolling on activated endothelial cells is defective in LAD II,[920,948] consistent with the role of selectin-mediated adhesion in this process (see Fig. 22-3). However, chemoattractant-induced tight adhesion and emigration of LAD II neutrophils is normal, both of which are severely impaired in LAD I.[920] Several *slc35c1*-deficient mouse models are available.[274]

All of the Arab patients have the same nucleotide substitution in the Golgi GDP-fucose transporter mRNA, which alters an amino acid in a transmembrane domain, whereas the Turkish patient has a different nucleotide alteration that results in an amino acid substitution in a different transmembrane domain of the transporter.[951,952] The different mutations may explain why the Turkish patient had some response to oral fucose therapy with increased expression of certain fucosylated ligands and improved clinical symptoms except mental retardation, but the Arab children did not.[949,953] It is speculated that the mutation in the Turkish child leads to a decrease in transport affinity for GDP fucose, which can be overcome by higher concentrations of fucose, whereas the mutant protein in Arab children has normal affinity for GDP fucose but decreased transport activity.[950]

Leukocyte Adhesion Deficiency Type III

LAD III is the most recently described subgroup of LAD and is characterized by both severe, recurrent infections and leukocytosis similar to LAD I as well as a severe bleeding tendency similar to Glanzmann thrombasthenia, a β_3-related disorder (see Table 22-11).[954-958] First described in 1997 by Kuijpers and colleagues and originally named LAD-1/variant syndrome, almost a dozen patients have now been reported in the literature, many of whom are descendants of Turkish ancestors.[274,914,954-960]

In contrast to LAD I, integrin expression in LAD III patients is normal or near normal. Instead there is a defect in the inside-out activation of hematopoietic cell integrins to a high-affinity binding state. Gene targeted mice lacking Kindlin 3, a blood cell-specific cytoplasmic protein that cooperates with talin to activate β_1, β_2, and β_3 integrins had a similar phenotype to these patients,[274] leading to the discovery of mutations in the *FERMT3* gene and absence of its Kindlin 3 protein product in patients with LAD III.[280,282,284,961] Additional mutations in a blood cell-specific guanine nucleotide exchange factor, CalDAG-GEFI, were originally implicated as causing LAD III in several Turkish kindreds.[960,962] However, these patients also had mutations in *FERMT3,* and gene rescue experiments showed that restoring Kindlin 3 expression corrects the defects in integrin-mediated adhesion and migration.

As mentioned, patients with LAD III have severe, recurrent infections as well as a bleeding diathesis that can be variable in severity. Kindlin 3 is also reported present in erythrocytes, similar to mice, and its loss is proposed to contribute to anemia and poikilocytosis in some patients with LAD III.[963] Because of defects in osteoclast integrin activation, Kindlin 3–/– mice have osteopetrosis, as do some patients who have LAD III.[274] The diagnosis of LAD III should be considered in patients with the clinical features of LAD I but normal expression of β_2 integrins and, commonly, bleeding with abnormal platelet aggregation. Commercial genetic testing is available. Supportive care includes prophylactic antibiotics and transfusion, as needed. However, because the clinical manifestations are typically severe, early bone marrow transplantation may be indicated.[274,954,958,964]

Acquired Disorders of Adherence

Neutrophils may exhibit decreased adhesiveness after exposure to a variety of drugs, the most common being corticosteroids and epinephrine.[796,965,966] Clinically the diminished adhesiveness induced by these drugs is manifested by a dramatic rise in the total neutrophil count in the blood as cells from the marginated pool are quickly released into the circulating pool. Whereas the mechanisms by which corticosteroids alter adherence are likely complex,[967] epinephrine and other β-adrenergic agonists exert their effect indirectly by causing endothelial cells to release cyclic adenosine monophosphate (cAMP), which in turn impairs the ability of neutrophils to adhere.[796,968]

The adhesiveness in neutrophils can be dramatically increased in a variety of clinical conditions that have in common the formation of biologically active complement fragments: gram-negative bacterial sepsis, severe thermal injury, pancreatitis, trauma, and exposure of neutrophils to artificial membrane surfaces during hemodialysis and cardiopulmonary bypass.[969] In these various conditions, the generation of complement fragments leads to activation of neutrophils and enhanced adhesiveness, possibly owing to enhanced expression of β_2 integrins. Under these conditions, neutrophils undergo increased aggregation with each other and become trapped within capillary beds, such as those in the lungs.[970] It is believed that the aggregated neutrophils then generate toxic oxygen radicals and release proteases that conspire to damage structural protein such as collagen and elastin.[305] Binding of viral proteins to neutrophils can depress adhesion and other neutrophil functions in influenza or HIV infection.[969]

Amphotericin B has been associated with increased neutrophil aggregation, particularly when administered in conjunction with transfusions of neutrophils that have been harvested by filtration (rather than by centrifugation).[971,972] It is possible that the enhanced aggregation is mediated by upregulation of surface β_2 integrins induced both by the amphotericin B and by the filters used to harvest the cells. Liposomal amphotericin causes substantially less in vitro aggregation of mobilized neutrophils and therefore may be easier to use in patients being treated for fungal infections who are also receiving allogeneic neutrophil transfusions.[973]

Disorders of Chemotaxis

The directed migration into sites of infection and inflammation involves a complex series of events. As reviewed in the beginning of this chapter, the generation of chemotactic signals and their binding to specific surface receptors activate leukocyte intracellular signaling pathways that trigger changes in adhesiveness and in the actin cytoskeleton leading to binding to the endothelium and chemotaxis into inflamed tissue. Given the numerous cellular functions involved in chemotaxis, it is perhaps not surprising that impaired phagocyte chemotaxis is observed in a large number of clinical conditions.[969,974] Some of the more important syndromes are listed in Box 22-11. These disorders are classified according to the mechanisms thought to be responsible for defective chemotaxis. In some cases, abnormal chemotaxis is one component of a disorder that involves multiple defects.

It is worth noting that in many of the reports describing defective in vitro chemotaxis of neutrophils from various clinical conditions, it is not clear whether an increased number of infections is a result of the observed chemotactic abnormality or of medical complications of the underlying disorder. Further complicating the interpretation of these reports is that the in vitro assays for chemotaxis are subject to laboratory artifacts and may not accurately reflect the in vivo extracellular environment.[975-977] Although now rarely used in practice, phagocyte migration to an inflamed site in vivo can be measured by the skin window technique of Rebuck and Crowley, in which a superficial dermal abrasion is produced,[978] or in skin blisters induced by suction,[979] and the appearance of inflammatory cells in the lesion are monitored over 24 hours.

Box 22-11 **Clinical Conditions Associated with Impaired Neutrophil Chemotaxis**

DEFECT IN GENERATION OF CHEMOTACTIC AGENTS

Familial deficiency of C1r, C2, C4
Familial deficiency of C3, C5
Other abnormalities of complement pathways (e.g., systemic lupus erythematosus, immature complement system in neonates, diabetes mellitus, C5 dysfunction, chronic hemodialysis, glomerulonephritis)

EXCESSIVE PRODUCTION OF NORMAL CHEMOTACTIC FACTOR INACTIVATORS

Hodgkin disease
Cirrhosis of the liver
Sarcoidosis
Lepromatous leprosy

INHIBITORS OF THE NEUTROPHIL RESPONSE TO CHEMOTACTIC FACTORS

Hyperimmunoglobulin E syndrome
Localized aggressive periodontitis (LPA)
Immune complex diseases (rheumatoid arthritis, systemic lupus erythematosus)
Influenza virus
Human immunodeficiency virus
Immunoglobulin A paraproteinemia states
Solid tumors
Bone marrow transplantation
Drugs (ethanol, antithymocyte globulin, interleukin-2)

DEACTIVATION (DOWNREGULATION) BY INCREASED LEVELS OF CHEMOTACTIC FACTORS

Wiskott-Aldrich syndrome
C5a generation in plasma (hemodialysis)
Bacterial sepsis

PHAGOCYTE DEFECTS

Neutrophil actin dysfunction
Rac2 mutation
LAP
Neonatal neutrophils
Leukocyte adhesion deficiency
Chédiak-Higashi syndrome
Specific granule deficiency
Glycogen storage disease 1b

MISCELLANEOUS DEFECTS

Hypophosphatemia
Shwachman-Diamond syndrome
Burn patients

As outlined in Box 22-11, several of the conditions associated with impaired neutrophil chemotaxis are caused by complement deficiencies and other immunodeficiency syndromes (e.g., WAS). These disorders are discussed elsewhere in this text. In addition, several disorders of phagocyte function (LAD, CHS, and SGD) are reviewed in other sections of this chapter. The following section focuses on well-defined clinical entities in which impaired chemotaxis is the predominant feature of phagocyte dysfunction and contributes to increased risk of infection.

Hyperimmunoglobulin E Syndrome

HIES (see Chapter 24) is a complex multisystem disorder characterized by markedly elevated serum levels of IgE

Box 22-12 Summary of Hyperimmunoglobulin E Syndrome

Incidence	More than 200 cases reviewed in the literature
Inheritance	Autosomal dominant (AD) with variable expressivity, sporadic forms, autosomal recessive (AR; rare)
Molecular defect	Dominant-negative mutations in STAT3 (AD and sporadic cases); Dock8, Tyk2 kinase (AR)
Clinical manifestations	"Cold" cutaneous skin abscesses and furuncles Chronic eczematoid dermatitis Mucocutaneous candidiasis Staphylococcal pneumonia, pneumatoceles Chronic cutaneous viral infections (AR) Fungal superinfection of lung cysts Coarse facies (AD) Delayed shedding of primary teeth (AD) Scoliosis (AD) Recurrent fractures (AD) Hyperextensible joints (AD) Craniosynostosis (AD) Vascular disease (AD) Severe allergies (AR)
Laboratory evaluation	Serum immunoglobulin (IgE) >2500 IU/mL Peripheral blood eosinophilia
Differential diagnosis	Atopic dermatitis Wiskott-Aldrich syndrome, DiGeorge syndrome Hypergammaglobulinemia Chronic granulomatous disease
Therapy	Prophylactic antibiotics for *S. aureus* Aggressive treatment of acute infections with parenteral antibiotics Surgical drainage of deep infections and resection of lung cysts Monitoring for scoliosis, fractures, vascular disease, malignancy
Prognosis	Generally good if managed aggressively

(often greater than 2000 IU/mL), serious recurrent staphylococcal infections of the skin and lower respiratory tract, pneumatoceles, chronic pruritic dermatitis, and skeletal and dental abnormalities.[102,980-986] The key features of HIES are summarized in Box 22-12. Neutrophils from patients with this syndrome exhibit a variable, but at times severe, chemotactic defect.[910,981,987,988] This syndrome was originally known as Job syndrome when it was first reported in 1966 in two red-haired, fair-skinned females who had hyperextensible joints and "cold" abscesses that lacked the usual characteristics of inflammation.[980] It is now appreciated that only a small fraction of patients with HIES have red hair or hyperextensible joints, and it may be that Job syndrome is a variant subset of HIES. AD and sporadic cases account for the majority of cases, and HIES is now known to result from dominant negative mutations in STAT-3.[989,990] The more rare AR forms of HIES result from mutations in genes encoding DOCK8, a guanine nucleotide exchange factor, or less commonly TYK2, a JAK family member, and are associated with more profound impairments in lymphocyte function.[983,984,991-994]

The clinical manifestations of HIES are often severe and usually become apparent during infancy. Staphylococcal furuncles of the head and neck as well as chronic dermatitis are seen most often in younger patients. The skin abscesses in HIES typically lack erythema (cold abscesses). Chronic candidiasis of the mucosa and nail beds is also common and can occur in children. Recurrent staphylococcal pneumonia is a common problem as patients grow older and can be complicated by the formation of persistent pneumatoceles that can become superinfected with *Haemophilus influenzae*, gram-negative bacteria, or *Aspergillus*. Chronic infections of the ears, sinuses, and eyes (keratoconjunctivitis) are seen, and septic arthritis and osteomyelitis have been observed. Nonimmunologic manifestations of HIES include dental and bone abnormalities. Delayed shedding of or failure to shed primary teeth occur in the majority of patients. Hyperextensible joints and scoliosis are common. Osteopenia of unknown etiology is observed many patients, and there is also an increased risk of fractures of the long bones and the vertebral bodies, even in the absence of osteopenia.[983] Vascular abnormalities, including aneurysms, tortuosity of middle-sized arteries, and hypertension are also common. Coarse facial features characterized by a broad nasal bridge and a prominent nose are evident in the majority of patients by the time they reach the teenaged years. Craniosynostosis can also occur.

Patients with AR HIES additionally have recurrent or chronic cutaneous viral infections that include Molluscum contagiosum, human papilloma virus, herpes simplex, and varicella, and increased susceptibility to atypical mycobacterial infection.[986,991,992] Significant neurologic sequelae are reported, ranging from partial facial paralysis to hemiplegia.[991] In contrast to AD HIES, pneumatoceles are not seen, nor are skeletal abnormalities, dental problems, or the characteristic facies. However, unlike in AD HIES, patients who are *DOCK8*-deficient can develop asthma and severe allergies, including to foods.

HIES is associated with increased risk of malignancies, particularly *DOCK8* deficiency including non-Hodgkin lymphoma and cancers of the vulva, liver, and lung.[985,986]

The identification of gene defects affecting cytokine signaling in HIES have helped explain its pathogenesis and multisystem manifestations, particularly in the AD form. The associated dominant-negative mutations in STAT3 are located primarily in regions that interact with other proteins or with DNA.[989,990] This interferes with the activity of wild-type STAT3 and results in a complex pattern of altered cytokine signaling and impaired T-helper type 17 (TH17) cell differentiation.[103,985] Both proinflammatory (IL-6) and antiinflammatory cytokines are regulated by STAT3 and thus explain how both too little (e.g., cold abscesses) as well as excessive responses (e.g., enhanced IgE production) might occur. Defective STAT3 signaling impairs differentiation of TH17 cells, which are important for control of mucocutaneous candidiasis. Keratinocytes and bronchial epithelial cells are particularly dependent on TH17 cytokines to secrete defensins and chemokines, which may explain the frequent skin and lung infections. Skeletal defects that are hallmarks of HIES may reflect defective STAT3 signaling in osteoblasts and osteoclasts. Inactivation of IL-11 signaling may explain the dental abnormalities and craniosynostosis.[985] The etiology of impaired neutrophil chemotaxis often seen in these patients, however, remains uncertain.[910,981,987,988]

Patients with AR HIES who have mutations in Tyk2 exhibit additional defects in IL-12 and IFN-α signaling.[992] AR HIES mutations affecting *DOCK8,* which regulates cytoskeletal rearrangements involved in migration, adhesion and growth, lead to defective CD4+ T-cell priming because of impaired dendritic cell migration, and also impair STAT3 activation in B cells. These differences help explain the broader spectrum of infections and other symptoms seen in AR HIES.[994]

The diagnosis of HIES should be considered in any child with the aforementioned clinical history. Almost all patients have peripheral eosinophilia. A markedly elevated polyclonal serum IgE, or at least 2500 IU/mL, is a constant laboratory finding, although IgE levels can fluctuate over time and occasionally decrease to the normal range.[983] Despite the impressive elevations in serum IgE (up to 150,000 IU/mL), this laboratory finding alone is not diagnostic, because comparably high serum levels of IgE can be seen in patients with atopic dermatitis. Because many patients with atopic dermatitis suffer from superficial skin infections and eczema, this disorder must be considered in the differential diagnosis of HIES. The two can be distinguished from each other because of the severe and recurrent nature of the staphylococcal furuncles and the pneumonias seen in HIES. A HIES scoring system developed by the National Institutes of Health can be helpful.[983] DNA testing should be used to make a definitive diagnosis.

Treatment of HIES is largely supportive.[984,985,994] One of the mainstays of therapy for HIES is the use of prophylactic antibiotics such as dicloxacillin or trimethoprim/sulfamethoxazole. These drugs can help prevent staphylococcal infections and should be prescribed at the time of diagnosis. Bleach baths or chlorhexidine washes can diminish colonization by *S. aureus* and reduce abscess formation. Pneumonias and other deep-seated infections should be treated aggressively with parenteral antibiotics. Patients with HIES are unusually predisposed to developing pneumatoceles as a result of staphylococcal lung infections. If these lesions persist, antifungal prophylaxis should be considered. Intravenous IgG infusions have shown some success in management of HIES.[984,994] Delayed shedding of primary teeth may necessitate extraction. Patients should be carefully monitored for scoliosis, and skeletal fractures can occur even after minor trauma. Patients should also be assessed for hypertension and other vascular disease. If infections and their complications are managed aggressively, the prognosis for patients with AD HIES syndrome is good. HSCT may be a consideration in AR HIES because of its higher morbidity and mortality, and has been used successfully in *DOCK8* deficiency.[994] Several patients with AD HIES also received transplants for treatment of lymphoma, with resolution of both immunologic and nonimmunologic features of HIES reported.[985]

Neutrophil Actin Dysfunction

Primary defects in neutrophil actin polymerization are exceedingly rare. The first such case, described by Boxer and colleagues in 1974, was a male infant who suffered from recurrent skin infections caused by *S. aureus* and a cutaneous-cecal fistula complicated by *Streptococcus faecalis* sepsis.[995] Despite a marked neutrophilia, sites of infections were devoid of neutrophils and healed slowly. The patient's neutrophils showed markedly diminished chemotaxis and a decreased capacity to ingest serum-opsonized particles. He underwent a bone marrow transplantation with transient engraftment of normally functioning neutrophils but succumbed to infectious complications. The underlying defect in this patient appeared to involve neutrophil actin or an ABP. Neutrophils from his parents and a sibling had decreased actin polymerization in vitro. Neutrophils from family members also had intermediate levels of and decreased expression of the Mac1 integrin (CD11b/CD18),[996,997] raising the question of whether the neutrophil actin dysfunction in the proband was a variant of LAD I. However, actin filament assembly is normal in patients with LAD I.[997] Alternatively, a primary actin-associated defect might alter cell surface expression of the integrins.

Coates and coworkers have reported a male infant of Tongan descent who was afflicted with severe skin and mucosal infections.[998] At the age of 2 months, he was found to have hepatosplenomegaly, moderate thrombocytopenia, recurrent pulmonary infiltrates, and a lingual ulcer that grew *Candida tropicalis*. Neutrophils had normal cell surface expression of CD11b but exhibited abnormalities in a wide range of motile behaviors, including chemotaxis, phagocytosis, and spreading on glass. Morphologically, the neutrophils displayed thin, filamentous projections of membrane with an underlying abnormal cytoskeletal structure. Biochemical studies revealed markedly defective actin polymerization, and a severe deficiency of an 89-kD protein along with a markedly elevated level of a 47-kD protein. Hence, this disorder has been referred to as neutrophil actin dysfunction with 47- and 89-kD protein abnormalities (NAD 47/89). The 47-kD protein was identified as lymphocyte-specific protein (LSP) 1,[999] an actin-binding protein present in normal neutrophils. Neutrophils from the patient's parents showed a partial defect in actin polymerization and intermediate abnormalities in the levels of LSP1 and the 89-kD protein. These observations, along with a history of previously affected siblings, suggest that NAD 47/89 is an AR disorder. The patient received an allogeneic bone marrow transplant at the age of 7 months with resolution of thrombocytopenia and neutrophil dysfunction.

Markedly impaired neutrophil chemotaxis associated with a heterozygous point mutation in β actin was reported by Nunoi and colleagues in a female patient with recurrent infections, stomatitis, photosensitivity, and mental retardation.[1000] The defect lies within a domain that binds to profilin and other actin-regulatory molecules, and the mutant β actin may interfere with normal actin function in a dominant-negative manner. Interestingly, formyl peptide-induced superoxide production was also decreased in patient neutrophils, suggesting that actin function is important for normal NADPH oxidase assembly.

Localized Aggressive Periodontitis

Localized aggressive periodontitis (LAP), previously known as localized juvenile periodontitis, is a distinct clinical entity characterized by severe alveolar bone loss localized to the first molars and incisors, with an onset around the time of puberty.[1001-1003] LAP appears to be an acquired disorder in some patients and an inherited disorder in others, clustering in families. Defective neutrophil chemotaxis has been identified in vitro in approximately 70% of patients with LAP.[1001,1004-1007] Neutrophil chemotactic activity is not restored in vitro or after the patient is treated for periodontal infection, suggesting a genetic disorder affecting chemotactic receptors. An approximate twofold decrease in the total number of receptors for formyl peptides and C5a is reported for some patients who have LAP.[1005,1008] Other studies indicate that factors elaborated by periodontopathic bacteria may secondarily alter leukocyte function and depress chemotaxis (e.g., *Actinobacillus actinomycetemcomitans, Capnocytophaga* species, and *Bacteroides* species); susceptibility to these factors may also have a genetic component. Recent studies suggest that inflammatory signaling in LAP neutrophils is dysregulated and can lead to hyperinflammatory responses that may contribute to increased periodontal breakdown.[1003,1009,1010]

The diagnosis of LAP should be suspected in any adolescent in whom there is unusually severe and destructive alveolar bone loss involving the first molars and incisors. From a diagnostic point of view, it is important to bear in mind that many qualitative and quantitative neutrophil disorders are associated with periodontal disease that at times may be severe. The differential diagnosis should include LAD, CGD, CHS, leukemia, chronic neutropenia, and CyN.

Neonatal Neutrophils

Although most infants are able to defend themselves successfully against microbial challenges, they are nonetheless at increased risk for development of severe bacterial infections, particularly sepsis, pneumonia, and meningitis caused by group B streptococci.[1011] The risk of infection and the rate of mortality from pyogenic infections are even greater in premature infants. Thus the development of innate immunity in neonates has been the subject of intense investigation for many years.[1011-1014] There is general agreement that various aspects of the neonatal immune response are impaired, including cellular cytotoxic mechanisms and cytokine generation. Defects in neutrophil adherence, chemotaxis, phagocytosis, and bacterial killing all have been reported.[1012-1014] Formation of NETs is also impaired.[376] Compounding these functional defects in neonates is a deficiency of antibodies directed against organisms that typically infect infants. Furthermore, neonates can easily exhaust bone marrow reserves of granulocytes and develop neutropenia in the midst of severe pyogenic infections, as discussed in the previous section.

The most important of the functional defects, at least from a clinical point of view, appears to be the depressed chemotactic ability of neonatal neutrophils and their adhesion. Compared to adult cells, the directed migration of neonatal neutrophils toward a variety of chemotactic agents (C5a, formyl peptides, and bacterial extracts) is reduced by approximately 50% for the first several weeks of life.[1012,1015-1017] The biochemical basis for the diminished chemotaxis does not appear to be related to abnormalities in the number or affinity of either the C5a or formyl peptide receptor.[1012,1017] Although cell surface expression of the β_2 integrin Mac-1 was found to be normal in neonatal neutrophils, total cellular Mac-1 is decreased in neonatal neutrophils.[1018] Because internal membrane stores serve as an intracellular pool for Mac-1 (see Table 22-3), the overall reduction in cellular Mac-1 could explain its diminished upregulation after exposure to chemotactic concentrations of C5a and formyl peptides.[1019,1020] Preterm infants also have decreased levels of neutrophil PSGL-1, and their endothelial cells exhibit diminished upregulation of ICAM-1, E-selectin, and reduced neutrophil binding.[1021] Additional biochemical defects in neonatal neutrophils that may contribute to impaired chemotaxis and adhesion is diminished polymerization of F-actin after stimulation[1022] and decreased amounts of the Rac2 GTPase.[1023] Finally, defects in chemoattractant-induced NF-κB and calcium responses by neonatal neutrophils are reported.[1012,1024]

Other Disorders of Neutrophil Chemotaxis

A syndrome of severe neutrophil dysfunction can result from dominant-negative mutations in the small GTPase Rac2. This was first characterized in a male infant who was born to unrelated parents and experienced recurrent rapidly progressive soft-tissue infections, including a perirectal abscesses and a necrotic periumbilical infection associated with poor formation of pus and neutrophilia.[97-99] Although suggestive of LAD I, expression of $ß_2$ integrins and fucosylated cell surface proteins were normal. Chemoattractant-induced neutrophil functions, including F-actin formation, chemotaxis, degranulation, and the respiratory burst were markedly defective, along with defects in phagocytosis and L-selectin–mediated adhesion.[97,99] Responses to other agonists were normal. This constellation of functional abnormalities resembled those described for gene-targeted mice lacking the hematopoietic-specific GTPase, Rac2.[321,1025] This patient was heterozygous for a point mutation in the Rac2 gene that affects the guanine nucleotide-binding pocket, which apparently exerts a dominant-negative effect on wild type Rac2 to interfere with normal signal transduction.[97,99] Neither parent had the mutant Rac2 allele. The patient was successfully treated by an HLA-matched marrow transplant from a sibling. The clinical history, neutrophilia, and functional neutrophil defects in this patient are very similar to those seen in a female infant described in an earlier report.[1026] This patient was the first child of nonconsanguineous healthy parents, both originating from India, and suffered from chronic omphalitis and otitis media secondary to *S. aureus*, buccal candidiasis, and an internal cecal fistulation that ultimately lead to death from gram-negative sepsis at 8 months of age. Newborn screening for T-cell lymphopenia using the T-cell receptor excision circle assay recently identified another infant with a dominant-negative mutation in Rac2 who underwent successful cord blood transplantation.[938]

A study of 240 patients with a significant history of recurrent infection generally requiring at least one hospitalization identified 10 patients, all children, with a consistent marked reduction chemotactic activity in vitro that was not associated with any other neutrophil abnormalities.[1027] In 6 of these patients, a partial reduction in chemotactic activity was also seen in either the mother or a sibling. Similar findings were reported in a more recent study.[910] However, other than for the disorders previously discussed, well-defined clinical entities in which defective neutrophil chemotaxis plays a predominant role in impaired host resistance to bacteria and fungi have not been established. In part, this is related to the difficulties in performing and interpreting in vitro assays of adhesion and chemotaxis except in specialized research settings. Biochemical approaches to delineating a specific abnormality are also difficult because of the complex nature of the chemotactic response.

Disorders of Opsonization And Ingestion

Clinical disorders of recognition fall into two major categories: humoral and cellular. In the former, plasma-derived opsonins are deficient or absent and result in incomplete opsonization. In contrast, the cellular disorders are characterized by defective receptors for opsonins or by abnormalities in the actin cytoskeletal system responsible for microbial ingestion.

Humoral Disorders of Opsonization

Primary B-cell deficiencies result in the decreased or absent production of immunoglobulins, most commonly IgG. Patients afflicted with these antibody-deficiency syndromes suffer from recurrent infections with pyogenic bacteria such as *S. aureus*, pneumococci, and *H. influenzae*. One of the major functional abnormalities in these disorders is the defective opsonization of pathogenic microorganisms. As a result, these microbes are not efficiently cleared by the host phagocytic system. A variety of clinical disorders may lead to an antibody deficiency, including deficiencies in specific IgG subclasses; these are discussed in Chapter 24.

Complement deficiencies also can result in recurrent infections, particularly when they involve those factors shared by both the classic and the alternative pathways. Therefore patients with deficiencies of C1, C2, or C4 have relatively minor problems with infections, because the alternative pathway remains intact. In the case of C3, on the other hand, recurrent infections are much more common, since this is the protein that is the direct precursor of two major complement opsonins: C3b and C3bi. Two forms of C3 deficiency have been described, and both are quite rare. In one, there is a congenital deficiency of C3 inherited in an AR manner that has been described in approximately 20 patients.[1028-1032] Heterozygotes contain half the normal levels of C3 but do not suffer from infections. The molecular genetic basis of the C3 deficiency has been identified in several families, and the mutations are heterogeneous.[1031,1032] A second type of C3 deficiency is caused by unchecked catabolism of C3 resulting from the absence of a C3 protease inhibitor.[1033] In both types of C3 deficiencies, the majority of patients suffer from recurrent pyogenic infections caused by encapsulated bacteria such as pneumococci. Patients with deficiencies of the terminal complement components (C5, C6, C7, C8, or C9) are particularly susceptible to infections with meningococci or gonococci. Infections in individuals who are complement deficient should be treated with the aggressive use of antibiotics, and immunization against *H. influenza*, *S. pneumoniae*, and *Neisseria meningitidis* may be helpful.

Approximately 5% of the population have low serum levels of MBL, also known as mannose binding protein. MBL is a serum lectin secreted by the liver and functions as a soluble PRR, binding to mannose sugars present on the surface of bacteria, fungi, and some viruses.[329,331,332] Bound MBL activates the complement cascade to mediate opsonization of a broad range of microbes with C3 cleavage products. The incidence of MBL deficiency is higher in infants with frequent unexplained infections, chronic diarrhea, and otitis media. It has been proposed that MBL serves as a primitive antibody of broad specificity to provide an important defense mechanism during the time period when maternal antibody levels have waned yet the antibody repertoire of the infant is still immature.[329,331,332] MBL deficiency is associated with AD inheritance of point mutations in a collagen-like domain of the MBL polypeptide.[1034-1036] These mutations decrease MBL levels by interfering with the normal polymerization of MBL subunits to form the oligomeric structure required for complement activation. A polymorphism in the promoter of the MBL gene has also been described that can influence MBL levels.[329] Clinical illness associated with MBL deficiency may not be limited to infants. In a recent report, MBL deficiency was the only identifiable immune defect in four adults with recurrent infections as well as in one patient who also had IgA deficiency.[1037] The spectrum of illness included recurrent skin abscesses, chronic cryptosporidial diarrhea, meningococcal meningitis with recurrent herpes simplex, and fatal *Klebsiella* pneumonia. Three of the five adults were homozygotes for mutant MBL alleles. Decreased MBL levels are seen in some populations, for example in sub-Saharan Africa, suggesting that low levels of this protein may be beneficial in certain settings. For example, low levels of MBL may partially protect against mycobacterial infections.[329]

Cellular Disorders of Ingestion

Patients with LAD I show a marked abnormality in phagocytosis of C3bi-opsonized particles, since the Mac-1 β_2 integrin (CD11b/CD18) that is deficient in LAD I functions as the C3bi receptor (see Tables 22-2 and 22-5). Patients with neutrophil actin dysfunction also show abnormal ingestion, since actin assembly plays a critical role in the formation of phagosomes.

A deficiency of FcγRIa has been described in four members of a Dutch family.[1038] The monocytes from the affected family members did not bind IgG with high affinity. However, these individuals did not show an increased susceptibility to infection despite this receptor deficiency. There is an incidence of 4 in 3377 in the

French population for the complete absence of FcγRIIIb, and neutrophils from affected individuals type as neutrophil-specific antigen (NA)-null.[1039] None of these individuals exhibit any increased incidence of infection, although infants of women with the NA-null phenotype can develop alloimmune neutropenia because of placental transmission of anti-FcγRIIIb antibodies. Similar findings were also reported in a study of 21 individuals from 14 families with FcγRIIIb deficiency in the Netherlands.[325] Complete absence of the FcγRIIIb gene was found in all kindreds studied. Three of the women who had multiple pregnancies lacked antineutrophil antibodies, suggesting that neonatal alloimmune neutropenia does not always develop in this setting. Marked deficiency in neutrophil FcγRIIIb has also been observed in patients with paroxysmal nocturnal hemoglobinuria (PNH),[1040] an acquired stem cell disorder caused by a defect in the biosynthesis of GPI membrane anchors. Since neutrophil FcγRIIIb is linked to the plasma membrane by means of a GPI anchor,[1041] this receptor is unable to insert in the cell membranes in patients with PNH. Decay-accelerating factor and acetylcholinesterase are also deficient in the membranes of patients with PNH, because these two proteins are also anchored by GPI moieties. Neutrophils from patients with PNH undergo a normal oxidative burst in response to IgG-coated latex particles.[1041] Thus there appears to be sufficient redundancy in the function of Fc and complement receptors to permit normal phagocytic function in these cases of FcγRI and FcγRIIIb deficiencies.

Allelic polymorphisms in FcγRIIa (131H vs. 131R) and in FcγRIIIb (NA1 vs. NA2) may each contribute to a subtle defect in the host response to encapsulated microorganisms. The polymorphism in amino acid 131 in FcγRIIa affects binding to IgG$_2$, with *H* referring to *high responder* and the ability to bind to this IgG subclass. The 131H versus 131R polymorphism is seen in approximately equal rates in the Caucasian population, but are distributed in a 3 : 1 ratio in Japanese and Chinese populations.[325] FcγRIIa131H allele is the only FcγR able to mediate efficient phagocytosis of IgG$_2$-opsonized bacteria, and the FcγRIIa 131H/131H genotype in children was associated with a lower incidence of infections with encapsulated bacteria.[325] The NA1 polymorphism in the FcγRIIIb receptor, which affects receptor glycosylation, is associated with more efficient phagocytosis of IgG-opsonized particles compared to the NA2 allele.[325,1042] The allelic frequency of the NA1 allele is approximately 0.33 compared to roughly 0.64 for the NA2 allele in Caucasians, but this ratio is reversed in Japanese and Chinese populations. Patients who are homozygous for both the FcγRIIa131R and the FcγRIIIb NA2 alleles appear to be at higher risk for meningococcal meningitis.[1043]

Disorders of Neutrophil Granules

Phagocytes kill microorganisms using a variety of cytotoxic compounds. These include a host of preformed antimicrobial polypeptides that are stored within intracellular granules and released into the phagocytic vacuole upon phagocytosis. The importance of granule contents in host defense is attested to by two clinical syndromes associated with disorders of neutrophil granules, CHS, and SGD.

Chediak-Higashi Syndrome

CHS is a rare, AR multiorgan disease resulting from defects in granule morphogenesis and is characterized by partial oculocutaneous albinism, frequent bacterial infections, giant lysosomes in granulocytes, and (in some patients) a mild bleeding diathesis as well as peripheral and cranial neuropathies associated with decussation defects at the optic chiasm (Box 22-13).[634,1044-1049] Ten types of oculocutaneous albinism have been described in humans, and CHS is one of the tyrosinase-positive forms that has been designated as type VIB. Because granule defects also affect cytotoxic T-cell and NK-cell function,

Box 22-13 Summary of Chédiak-Higashi Syndrome

Incidence	More than 200 cases described
Inheritance	Autosomal recessive
Molecular defect	Defect in granule morphogenesis in multiple tissues as a result of mutations in the *CHS1* gene, which encodes a lysosomal trafficking protein
Pathogenesis	Giant coalesced azurophilic/specific granules in neutrophils resulting in ineffective granulopoiesis and neutropenia, delayed and incomplete degranulation, and defective chemotaxis
Clinical manifestations	Partial oculocutaneous albinism Recurrent severe bacterial infections (usually *S. aureus*) Gingivitis and periodontitis Cranial and peripheral neuropathies (muscle weakness, ataxia, sensory loss, nystagmus) Hemophagocytic lymphohistiocytosis (HLH; accelerated phase)
Laboratory evaluation	Giant granules in peripheral blood granulocytes and in bone marrow myeloid progenitor cells Widespread lymphohistiocytic infiltrates in accelerated phase
Prenatal diagnosis	Demonstration of giant granules in fetal blood neutrophils or cultured amniotic cells
Differential diagnosis	Other genetic forms of oculocutaneous albinism Giant granules can be seen in acute and chronic myelogenous leukemia
Therapy	Prophylactic trimethoprim/sulfamethoxazole Parenteral antibiotics for acute infections Ascorbic acid (200 mg/day for infants; 6 g/day for adults) Steroids, cyclosporine, etoposide for HLH Bone marrow transplantation before or at beginning of HLH
Prognosis	Most patients die of infection or complications of HLH in the accelerated phase during the first or second decade of life unless transplantation performed

CHS is associated with HLH in the "accelerated" phase of the disease that often develops after the first or second decade of life.[634,1048-1050]

CHS is caused by a fundamental defect in granule morphogenesis that results in abnormally large granules in multiple tissues. The most extensively studied of the affected cells are the neutrophils. In the early stages of myelopoiesis, some of the normally sized azurophil granules coalesce to form giant granules[63] that later fuse with some of the specific granules to form huge secondary lysosomes that contain constituents of both granule types.[1051-1053] In addition to this uncontrolled fusion of granules, CHS neutrophils are markedly deficient in neutral proteases,[1054] including two azurophil granule enzymes, cathepsin G and elastase.[1055] In melanocytes, there are giant melanosomes that prevent the even distribution of melanin and result in hypopigmentation of the hair, skin, iris, and ocular fundus. Giant granules are also seen in other leukocytes, Schwann cells, and certain cells in the liver, spleen, pancreas, gastric mucosa, kidney, adrenal gland, and pituitary gland.[634,1047,1049] Disorders similar to human CHS have also been described in many mammalian species, including Aleutian mink, *beige* mice, blue foxes, cats, killer whales, and Hereford cattle.[1046,1047,1056]

Mutations in the *CHS1* gene, believed to normally regulate lysosome biogenesis, accounts for the most cases of CHS.[1057-1059] *CHS1* is on the long arm of chromosome 1 and is homologous to the *bg* locus responsible for the granule defect in *beige* mice.[1060] The *beige* gene was positionally cloned, and termed *LYST* for its presumed function as a lysosomal trafficking regulatory protein.[1057,1059] The encoded CHS/LYST protein is very large (3801 amino acids) polypeptide, and at least one of its functions may be to inhibit lysosome fusion with other intracellular membrane vesicles.[1061] Frameshift and nonsense mutations have been identified in the *CHS1* gene in patients with CHS, with the result of truncated forms of the protein.[1057-1059,1062,1063] Patients with clinically typical CHS that lack identifiable mutations in *CHS1* have been reported, suggesting that additional genetic loci may be affected in some cases.[1064]

Some of the most dramatic examples of the granule defect in CHS are manifested in the various blood cells and are summarized in Box 22-14. A few to a large majority of circulating neutrophils contain giant coalesced azurophil and/or specific granules. These giant granules are often more prominent in the bone marrow than in the peripheral blood, because many of the abnormal myeloid precursors are destroyed before they ever leave the marrow.[1065] Extensive myeloid cell vacuolization, enhanced marrow cellularity, and elevated levels of serum lysozyme all reflect this process of intramedullary granulocyte destruction. As a result, most patients with CHS have a moderate neutropenia with absolute neutrophil counts ranging between 500 and 2000 cells/mm^3.[1044,1045] Monocytes are also affected in CHS and have similar but not identical cytoplasmic inclusions that appear to be ring-shaped lysosomes.[1066] Lymphocytes can also contain giant cytoplasmic granules that contribute to abnormal cytotoxic T-cell and NK-cell function. Finally, CHS platelets have a decreased number of dense granules and a storage pool deficiency of ADP and serotonin.[1067-1070] This abnormality leads to a defect in platelet aggregation and an increased bleeding time manifested clinically as easy bruising, intestinal bleeding, and epistaxis. Most patients with CHS do not have thrombocytopenia until they enter the accelerated phase of the disease (described later in this section).

Box 22-14 Hematologic Manifestations of Chédiak-Higashi Syndrome

STABLE PHASE

NEUTROPHILS

- Giant, coalesced azurophilic specific granules
- Vacuolization of marrow neutrophils (ineffective myelopoiesis)
- Neutropenia (intramedullary destruction)
- Decreased bactericidal activity
  - Decreased chemotaxis in vivo and in vitro
  - Delayed and incomplete degranulation

MONOCYTES/MACROPHAGES

- Ring-shaped lysosomes
- Decreased chemotaxis

LYMPHOCYTES/NATURAL KILLER CELLS

- Giant cytoplasmic granules
- Diminished natural killer function
- Diminished antibody-dependent cell-mediated cytolysis

PLATELETS

- Giant cytoplasmic granules may be seen
- Normal platelet count
- Increased bleeding time because of abnormal aggregation caused by storage pool deficiency of adenosine diphosphate and serotonin

ACCELERATED PHASE (HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS)

- Hepatosplenomegaly
- Bone marrow infiltration
- Hemophagocytosis by histiocytes
- Worsening neutropenia, thrombocytopenia, and anemia as a result of the first three manifestations

The infections usually encountered with CHS involve the skin, respiratory tract, and mucous membranes and are caused by both gram-positive and gram-negative bacteria as well as by fungi. The most common organism is *S. aureus*. These infections are often recurrent and can be fatal. Gingivitis and periodontitis are common. The skin is also susceptible to severe sunburns, and photosensitivity in bright light is common.

The neutrophil defects are primarily responsible for the propensity to bacterial infection. In addition to the moderate neutropenia, several defects in neutrophil function lead to impaired bactericidal activity. First, chemotaxis is markedly depressed, whether measured in vivo by the Rebuck skin window or using in vitro chemotaxis assays.[1045,1071] The large granules appear to interfere with the ability of neutrophils to travel through narrow passages such as those between endothelial cells. Second, degranulation is delayed and incomplete in CHS neutrophils.[1072,1073] Third, the marked deficiency of antimicrobial proteins such as cathepsin G probably contributes to the diminished bactericidal potency of CHS neutrophils.[1073] Finally, decreased expression of

Mac-1 (CD11b/CD18) in CHS neutrophils may also play a role.[1074]

Contributing to the enhanced susceptibility to infection are abnormalities in monocytes, lymphocytes, and NK cells. Monocytes, like neutrophils, exhibit decreased chemotaxis.[1075] Peripheral blood CHS lymphocytes demonstrate diminished ADCC of tumor cells.[1076] Perhaps most important, cytotoxic T-cell and NK-cell cytolytic function is profoundly abnormal in CHS.[1048,1050,1076-1078] This defect not only contributes to the recurrent bacterial infections, but also leads to the development of HLH in the accelerated phase of CHS, which is sometimes precipitated by a herpes-type virus such as Epstein-Barr virus or varicella.[1048,1050,1079]

Those patients who survive the infectious and neurologic problems of the stable phase of CHS have a high risk of progressing to the accelerated phase of the disease, now recognized to be a genetic form of HLH.[1044,1048,1050] HLH results from uncontrolled hyperinflammation and multiorgan dysfunction that can be triggered by viral infections or other stimuli, and typically develops during the first or second decade of life in CHS.[1044,1048,1050] The accelerated phase is characterized pathologically by a diffuse lymphohistiocytic infiltration of the liver, spleen, lymph nodes, and bone marrow, and the outcome for the patient is uniformly fatal if not treated.[1048,1050] The accelerated phase is heralded by hepatosplenomegaly, bone marrow infiltration, and hemophagocytosis that lead to a worsening of the neutropenia and an ever-increasing risk of infection. Thrombocytopenia likewise develops and intensifies the bleeding disorder already present in the platelets.

The diagnosis of CHS should be suspected in any child who shows one or more of the following findings: (1) recurrent bacterial infections of unknown etiology; (2) hypopigmentation of the hair, skin, and eyes; a white forelock can be present; (3) the presence of giant peroxidase-positive lysosomal granules in granulocytes from peripheral blood or bone marrow; (4) mild to moderate neutropenia; (5) easy bruising or nose bleeds despite a normal platelet count; and (6) unexplained hepatosplenomegaly (associated with the accelerated phase of the disease). CHS is usually manifested in infancy or early childhood but may present later when the child is in the accelerated phase. In some patients, the disease may be suspected on the basis of neurologic abnormalities that include ataxia, muscle weakness, decreased deep tendon reflexes, sensory loss, a diffusely abnormal electroencephalogram, and abnormal visual and auditory evoked potentials.[1080,1081] The physician should not be dissuaded from considering the diagnosis of CHS if the patient does not show clear-cut oculocutaneous albinism. Depending on the skin coloration in the family, the only manifestations of the albinism may be a metallic sheen in the hair (which can vary from blond to dark brown) and a lighter skin color than that seen in siblings. In younger patients, there is likely to be a cartwheel distribution of pigment in the iris and an abnormal red reflex.

The diagnosis of CHS is made on the basis of giant peroxidase-positive lysosomal granules found in blood or bone marrow myeloid cells. In some cases, relatively few abnormal granulocytes are seen in the peripheral blood, presumably because of extensive intramedullary destruction of myeloid precursors, and a bone marrow aspirate may be necessary to identify the large lysosomes. Microscopic examination of the hair reveals giant melanin granules. In the accelerated phase, biopsies of the liver, spleen, and lymph nodes reveal diffuse infiltrates of lymphohistiocytic cells. For prenatal diagnosis, the observation of large abnormal granules in neutrophils and larger than normal acid-phosphatase lysosomes in cultured amniotic and chorionic villus cells has been used.[1082] Microscopic examination of fetal hair shafts is also reported.[1083]

The differential diagnosis for CHS includes other genetic forms of partial albinism.[645] These include Griscelli syndrome,[640] which is distinguished from CHS by the lack of giant cytoplasmic granules in neutrophils and the finding of large pigment clumps in hair shafts and accumulation of mature melanosomes in melanocytes. Patients with Griscelli syndrome also typically develop HLH, again because of defective cytolytic T- and NK-cell function.[1048,1050,1084] Hermansky-Pudlak syndrome is another disorder of partial albinism but is characterized by platelet function defects and an associated bleeding diathesis. Giant granules resembling those seen in CHS can be seen in both acute and chronic myelogenous leukemias[1085-1087] and should not be confused with those seen in CHS.

The management of the stable phase of CHS primarily involves treatment of infectious complications. Prophylactic trimethoprim/sulfamethoxazole may be beneficial. Infections should be treated vigorously with appropriate intravenous antibiotics. Treatment with high doses of ascorbic acid (20 mg/kg/day) has been reported to cause clinical improvement as well as improved function of neutrophils in vitro (neutrophil chemotaxis and/or bactericidal function).[1088,1089] Although there is some disagreement in the literature regarding the efficacy of ascorbic acid,[1090] it seems reasonable to try this medication given its safety in moderate doses. NK-cell function appears to remain abnormal even after ascorbate therapy.

The treatment of HLH (accelerated phase) has improved in recent years using combinations of drugs that suppress the function of activated macrophages and T cells, followed by allogeneic HSCT. Regimens for HLH include corticosteroids, cyclosporine A, and etoposide and are recommended for the accelerated phase of CHS.[1048,1050] Active therapy should be maintained until bone marrow transplantation can be performed. The only curative therapy is bone marrow transplantation with matched related or unrelated donor grafts.[1048,1050] This procedure is ideally performed before the onset of the accelerated phase. Transplantation does not prevent the progressive neuropathy associated with CHS.[1091]

Specific Granule Deficiency

SGD is an extremely rare congenital disorder of neutrophil function characterized by recurrent bacterial infections and multiple abnormalities in neutrophil structure and composition, including absent specific granules and bilobed nuclei.[1092,1093] Despite its rarity, SGD is an important part of the differential diagnosis in patients with suspected phagocyte immunodeficiencies and provides unique insights into the transcriptional regulation of granule biogenesis and the functional roles of lactoferrin, vitamin B_{12}-binding protein, and other specific granule constituents in host defense. Six patients have been reported with SGD.[56,1055,1094-1108] The inheritance appears to be AR,[1103,1104,1109] or in one case, AD.[1108]

The key features of SGD are summarized in Box 22-15. Clinically, patients with this disorder suffer from indolent, smoldering cutaneous infections punctuated by episodes (sometimes prolonged) of severe infections involving the lungs, ears, lymph nodes, and the deeper structures of the skin. Lung abscesses and mastoiditis have been reported as complications of these infections. *S. aureus*, *P. aeruginosa*, *Proteus* species, other enteric gram-negative bacteria, and *C. albicans* appear to be the major pathogens. Further complicating the clinical course in some of the patients is the presence of neutropenia that is either intermittent and mild[1094,1096] or prolonged and severe.[1106]

Box 22-15 Summary of Neutrophil Specific Granule Deficiency

Incidence	Six cases reported
Inheritance	Autosomal recessive
Molecular defect	Protein deficiencies in azurophilic granules (defensins), specific granules (lactoferrin, vitamin B_{12}-binding protein, gelatinase), and secretory vesicles (alkaline phosphatase) suggest a common defect in regulation of the production of these proteins in myeloid cells. Eosinophil specific granules also deficient in protein contents. Mutations in the myeloid transcription factor CCAAT/enhancer-binding protein (C/EPB) ε identified in several patients.
Pathogenesis	Recurrent infections result from the combined effect of deficiencies in microbicidal granule protein (e.g., defensins and lactoferrin) and abnormal chemotaxis, perhaps as a result of failure to upregulate surface β_2 integrins and chemotactic peptide receptors from granule stores
Clinical manifestations	Recurrent (sometimes indolent) pyogenic infections of the skin, ears, lungs, and lymph nodes that may have diminished neutrophil infiltration; onset usually during infancy
Laboratory evaluation	Absent or empty specific granule vesicles in neutrophils by electron microscopy (by light microscopy, granules appear absent) Bilobed nuclei resembling the Pelger-Huët anomaly are often seen in neutrophils Severe deficiency of neutrophil lactoferrin, vitamin B_{12}-binding protein, defensins, and alkaline phosphatase (by histochemical assay)
Differential diagnosis	Acquired specific granule deficiency (e.g., thermal burns or myeloproliferative syndromes)
Therapy	Prophylactic antibiotics Parenteral antibiotics for acute infections Surgical drainage or resection of refractory infections
Prognosis	With appropriate medical management, patients can survive into their adult years

Functional defects identified in patient neutrophils include a marked abnormality in chemotaxis that is observed in vivo by the Rebuck skin window technique.[1094,1096,1097,1104] In vitro measurements of neutrophil killing of *E. coli* and *S. aureus* show a moderate impairment despite the presence of a normal respiratory burst.[1094-1097,1104,1107] Patient neutrophils adhere normally to plastic surfaces[1095,1104] but exhibit slightly diminished sticking to nylon fibers and endothelial cells.[1097] Degranulation of both azurophil and (to the extent that they are present) specific granules also appears to be normal.[1095,1097,1104]

At least three cellular compartments are affected by the underlying molecular defect(s) in SGD: the nucleus, specific granules, and azurophil granules. Approximately half or more of the peripheral blood neutrophils in SGD have nuclei that resemble those seen in the Pelger-Huët anomaly. The nucleus has a kidney-shaped, bilobed configuration that is flawed by a series of microlobulations and clefts apparent by electron microscopy.[1096,1097,1104] On a Wright stain of peripheral blood, neutrophils appear to be devoid of the pink-staining specific granules. By electron microscopic analysis, however, it is apparent that the specific granules are not actually absent but are instead present as empty, elongated vesicles that retain their characteristic trilamellar membrane structure and positive staining for complex carbohydrates.[1096,1098,1099,1103] Biochemical measurements of specific granule contents parallel the morphologic studies. Lactoferrin, vitamin B_{12}-binding protein, and gelatinase[1110] are present at levels that are only 3% to 10% of normal.[1095,1097,1104] Thus there appears to be an abortive and incomplete formation of normal specific granules. Azurophil (primary) granules in this disorder are also strikingly abnormal. Although they contain a normal (if not slightly elevated) amount of MPO and are present in normal numbers, they are severely deficient in defensins.[1055] There is also a severe deficiency of alkaline phosphatase activity, which is normally localized to secretory vesicles, in peripheral blood neutrophils[1094,1096,1099,1104] Finally, granule formation also appears to be abnormal in eosinophils. These cells are deficient in three eosinophil-specific granule proteins (eosinophil cationic protein, MBP, and eosinophil-derived neurotoxin), although the corresponding mRNA transcripts are present.[1111]

In view of the multiple deficits in granule matrix contents produced after the promyelocyte stage, it had long been speculated that SGD is caused by an abnormality in biosynthetic regulation of granule (and probably nongranule) proteins during granulopoiesis. This is also consistent with the observation that the SGD neutrophils are severely deficient in defensins. In contrast to other azurophilic granule proteins, which are synthesized at the promyelocyte stage, defensins are produced during the subsequent myelocyte stage (see Fig. 22-1).

At least some cases of SGD result from AR mutations in a transcription factor that regulates gene expression during granulocyte differentiation. Serendipitously, mice generated with a targeted deletion in the myeloid transcription factor C/EBPε exhibit absence of neutrophil secondary granules and their matrix proteins, bilobed neutrophil nuclei, impaired chemotaxis, and increased susceptibility to bacterial infection.[1112-1114] Mutations in the C/EBPε gene have been identified in two patients with SGD to date. In one case, a five base-pair deletion results in a truncated, nonfunctional protein,[1115] whereas a second, unrelated patient was homozygous for a nucleotide insertion in the coding sequence that also leads to the synthesis of a truncated and nonfunctional form of C/EBPε.[1116] However, DNA sequencing of the C/EBPε gene of several other patients with SGD has not revealed abnormalities, suggesting that SGD is a genetically heterogeneous disorder.[1117]

The cellular defects described can explain, at least in part, the observed clinical problems in SGD. The markedly abnormal specific granules and secretory vesicles, which contain intracellular stores of both β_2 integrins[1118-1120] and a chemotactic peptide receptor,[1121] fail to support normal upregulation of these two types of receptors[1101,1104] and may thereby contribute to the chemotactic defect. Furthermore, the severe deficiency of key bactericidal proteins such as lactoferrin and the defensins render the cell less efficient in killing bacteria and *Candida*. Neutropenia, when it does occur, also impairs host defense and appears to be caused by intramedullary destruction of the abnormal neutrophils, as evidenced by their ingestion by marrow macrophages.[1103]

The diagnosis of SGD can be made by light microscopic examination of Wright-stained peripheral blood neutrophils. Electron microscopic studies can confirm the presence of empty specific granule vesicles. Bilobed nuclei and greatly diminished levels of alkaline phosphatase by histochemical analysis also support the diagnosis. In conjunction with these morphologic studies, biochemical and immunologic measurements of lactoferrin, vitamin B_{12}-binding protein, and defensins can be made. As discussed previously, all three of these granule proteins are severely deficient in this disorder. Acquired SGD is observed in myeloproliferative syndromes and after thermal burns.[1092,1122] In these acquired disorders, however, at least a few intact specific granules are still observed microscopically, and the deficiencies of the various granule enzymes are less profound.

As with other neutrophil disorders, prophylactic antibiotics appear to be beneficial based on anecdotal experience. Parenteral antibiotics should be used aggressively for acute infections. With appropriate medial management, patients can survive into their adult years. Malignant transformation of the dysmorphic myeloid cells in this disorder has not been reported.

Disorders of Oxidative Metabolism

The elimination of many pathogens requires oxygen-derived microbicidal compounds that are generated by the phagocyte respiratory burst pathway in response to inflammatory stimuli (see Fig. 22-8). Five clinically recognized defects have been identified in this series of reactions. These include deficiencies in the NADPH oxidase (see reaction 1 in Fig. 22-8), G6PD (reaction 8), MPO (reaction 4), glutathione reductase (reaction 7), and glutathione synthetase (reaction 9).

Chronic Granulomatous Disease

CGD is an inherited disorder of phagocyte function in which the generation of superoxide by the respiratory burst oxidase (see Fig. 22-9) in neutrophils, monocytes, macrophages, and eosinophils is absent or markedly deficient. The disorder occurs with an estimated minimum incidence of approximately 1 in 200,000 individuals in the United States and Europe, making it the most common clinically significant disorder of phagocyte function that is inherited.[1123] CGD is the result of mutations in any one of five essential subunits of the respiratory burst oxidase (see Fig. 22-9; Tables 22-7 and 22-12).[429,438,1124-1126] Although originally described as an X-linked recessive

TABLE 22-12 Classification of Chronic Granulomatous Disease

Component Affected	Inheritance	Subtype[*]	Flavocytochrome *b* Spectrum	NBT Score (% Positive)	Incidence (% of Cases)
gp91^{phox}	X	X91^0	0	0	60
		X91$^-$	Low	80-100 (weak)	5
		X91$^-$	Low	5-10	<1
		X91$^+$	0	0	1
p22^{phox}	A	A22^0	0	0	4
		A22$^+$	N	0	<1
p47^{phox}	A	A47^0	N	0[†]	25
p67^{phox}	A	A67^0	N	0	5
		A67$^+$	N	0	<1
p40^{phox}	A	A40$^-$	N	100	<1

NBT, Nitroblue tetrazolium.

[*] In this nomenclature, the first letter represents the mode of inheritance (X-linked [X] or autosomal recessive [A]), whereas the number indicates the *phox* component that is genetically affected. The superscript symbols indicate whether the level of protein of the affected component is undetectable (^{0}), diminished ($^-$), or normal ($^+$) as measured by immunoblot analysis.

[†] Can be weakly positive.

TABLE 22-13 Infections in Chronic Granulomatous Disease

Infections	% of Infections*	Infecting Organisms	% of Isolates
Pneumonia	70-80	*S. aureus*	30-50
Lymphadenitis†	60-80	*Aspergillus* species	10-20
Cutaneous infections/impetigo†	60-70	*E. coli*	5-10
Hepatic/perihepatic abscesses†	30-40	*Klebsiella* species	5-10
Osteomyelitis	20-30	*Salmonella* species	5-10
Perirectal abscesses/fistulas†	15-30	*B. cepacia*	5-10
Septicemia	10-20	*S. marcescens*	5-10
Otitis media†	≈20	*S. epidermidis*	5
Conjunctivitis	≈15	*Streptococcus* species	4
Enteric infections	≈10	*Enterobacter* species	3
Urinary tract infections/pyelonephritis	5-15	*Proteus* species	3
Sinusitis	<10	*Candida* species	3
Renal/perinephric abscesses	<10	*Nocardia* species	2
Brain abscesses	<5	*H. influenzae*	1
Pericarditis	<5	*P. carinii*	<1
Meningitis	<5	*Mycobacterium* species‡ *C. violaceum* *F. philomiragia* *T. glabrata* *Paecilomyces* species *G. bethesdensis*	<1 <1 <1 <1 <1 <1

*The relative frequencies of different types of infections in chronic granulomatous disease (CGD) are estimated from data pooled from several large series of patients in the United States, Europe, and Japan (see text for references). These series encompass approximately 550 patients with CGD after accounting for overlap between reports. The list of infecting organisms is also arranged according to the data in these reports and is not paired with the entries in the first column.

†Infections most commonly seen at initial evaluation.

‡Incidence is higher in some regions.

disorder affecting boys,[1127-1131] approximately one third of CGD is inherited as an AR trait (see Table 22-12).[1123,1132] In more than 90% of patients, superoxide production by activated phagocytes is below 2% of normal or undetectable. Patients with a respiratory burst of 2% to 10% of normal are sometimes referred to as having "variant" CGD.[1133-1135] Other aspects of phagocyte function are normal in CGD, including adherence, ingestion, and degranulation.[1136-1138]

Clinical Manifestations. CGD is associated with a distinctive clinical syndrome that illustrates the importance of the phagocyte respiratory burst in both innate immunity and the inflammatory response.[1123,1139-1156] Patients suffer from often severe purulent bacterial and fungal infections, in many cases caused by organisms that are not ordinarily considered pathogens. The other and distinctive hallmark of this disorder is the propensity to develop chronic inflammatory granulomas that can be of widespread tissue distribution. The majority of patients with CGD manifest symptoms within the first year of life,[1123,1142] although some may remain relatively symptom-free until later in childhood or even adult life.[1157-1162] As discussed later in this section, current management of CGD patients has markedly reduced the overall incidence of infections and other complications along with disease-associated mortality.

Table 22-13 summarizes the types of infections and infecting organisms encountered in CGD. The major sites of infection are those that come into contact with the external environment—lungs, skin, GI tract, and the lymph nodes that drain these organs. Hematogenous seeding can lead to liver abscesses or osteomyelitis. The most common pathogens include *S. aureus*, *Aspergillus* species (most often *A. fumigatus* but occasionally *A. nidulans* and other species),[1163-1167] *Nocardia*, and a variety of gram-negative bacilli, including *Serratia marcescens* and various *Salmonella* species. Members of the *Burkholderia cepacia* complex are aggressive and potentially lethal pathogen in CGD.[1123,1168-1170] Those causing infection in CGD include *B. cenocepacia*, *B. gladioli*, and *B. mallei*, which causes melioidosis in East Asian countries.[1168,1169] *Burkholderia* and *Nocardia* are less commonly seen in Europe compared to North America.[1146] Tuberculosis is seen in endemic regions,[1171] and vaccination with BCG can result in severe localized, or less commonly, disseminated BCG infection[1146,1153,1172]: infections with other atypical mycobacteria are also reported in

CGD.[1173] *Granulobacter bethesdensis,* a member of the gram-negative *Acetobacteraceae* family and previously not associated with human disease, was first identified in a CGD patient with recurrent fevers and a chronic necrotizing deep lymphatic infection.[1174,1175] In many febrile illnesses, no organism can be identified despite extensive culturing. In these cases, it is important to look carefully for the presence of unusual microbes that can occasionally cause serious infections in CGD (see Table 22-13).[1158,1176-1183]

Pneumonia is most commonly caused by *S. aureus, Aspergillus* species, and enteric bacteria. It is common for an organism not to be identified even in lung biopsy specimens. Complications of pneumonia include empyema or lung abscess. Suppurative lymphadenitis, usually involving the cervical nodes, is especially common in younger patients and is typically caused by *S. aureus* and enteric bacilli such as *Serratia* and *Klebsiella*. Staphylococcal skin infections are also common. Hepatic and perihepatic abscesses, usually caused by *S. aureus*, are surprisingly common and should suggest the diagnosis of CGD if it has not already been made.[1184-1187] Bone infections can be particularly problematic in CGD and arise either from direct spread of infections from contiguous sites or from hematogenous spread from more distant locations.[1123,1188] The former type of osteomyelitis is usually seen in ribs and vertebral bodies as a result of invasion by pulmonary *Aspergillus*.[1188] The latter type is more frequently seen in peripheral long and small bones, and is typically caused by *S. marcescens*, *Nocardia* species, and *S. aureus*.[1189] Perirectal infections are extremely difficult to treat in CGD and can lead to fistula formation.[1189,1190] The rare pathogen *Chromobacterium violaceum* is found in brackish waters and is an uncommon but potentially serious cause of soft-tissue infection, pneumonia, or sepsis in CGD patients from the southern United States.[1178,1179,1191] In northern areas, *Francisella philomiragia* in brackish waters can cause sepsis in CGD.[1180,1192]

It has long been recognized that patients with CGD are particularly susceptible to organisms that contain catalase, which prevents the CGD phagocyte from scavenging microbial-generated H_2O_2 for phagosomal killing.[1193] Thus infections with streptococci or *Haemophilus* species, which are typically catalase-negative, are not problematic in CGD. Another possible link among the organisms with increased virulence in CGD is that they are resistant to nonoxidative killing mechanisms of the phagocyte mediated by antibiotic proteins contained within the various granule compartments.[1194]

TABLE 22-14 Chronic Conditions Associated with Chronic Granulomatous Disease

Condition	% of Cases
Lymphadenopathy	98
Hypergammaglobulinemia	60-90
Hepatomegaly	50-90
Splenomegaly	60-80
Anemia of chronic disease	Common*
Underweight	70
Chronic diarrhea	20-60
Short stature	50
Gingivitis	50
Dermatitis	35
Hydronephrosis	10-25
Ulcerative stomatitis	5-15
Pulmonary fibrosis	<10*
Esophagitis	<10*
Gastric antral narrowing	<10
Granulomatous ileocolitis	<10
Granulomatous cystitis	<10
Chorioretinitis	<10
Glomerulonephritis	<10
Discoid lupus erythematosus	<10

*The relative frequencies of the chronic conditions associated with chronic granulomatous disease were estimated from the series of reports listed in Table 22-13.

Chronic conditions associated with CGD are responsible for many of the major complications seen with this disorder[1156] and are summarized in Table 22-14. These include the granulomas that are a distinctive feature of CGD. These lesions contain a mixture of lymphocytes and inflammatory macrophages, some of which may have a foamy lipoid cytoplasm that has a characteristic yellow-brown color. Although not pathognomonic of CGD, the presence of such macrophages in granulomas or other tissue sites should suggest the diagnosis of CGD.[1128,1129,1131,1195] Granulomatous inflammation in some instances reflects an indolent infection because of inadequate phagocyte killing. However, this manifestation is also believed to in part reflect a dysregulated inflammatory response in the absence of respiratory burst–derived oxidants. This leads to abnormally high level of cytokines and other proinflammatory mediators and delayed neutrophil apoptosis at inflammatory sites.[1156,1196-1198] Inefficient phagocyte degradation of debris is also likely to be a contributing factor to chronic inflammation in CGD.[1198] Granulomatous inflammation can develop in response to fungal cell walls independent of active infection.[1198]

Granuloma formation can lead to obstructive symptoms in the upper GI tract, including gastric outlet obstruction that can be confused clinically with pyloric stenosis.[1184,1189,1190,1199,1200] Inflammation can affect the GI tract from the esophagus to the rectum, is associated with a range of symptoms affecting as many as 30% in a referral center,[1189,1201] and is more common in X-linked CGD. A chronic ileocolitis syndrome resembling Crohn disease is seen in approximately 5% to 10% of patients and can lead to a debilitating syndrome of diarrhea and malabsorption; perirectal abscesses are also problematic.[1184,1189,1202] Chronic inflammatory lesions can develop in the urinary bladder walls and can cause a chronic cystitis that can present as dysuria, penile pain, and decreased urine volume.[1203-1207] In one study of 60 patients with CGD, 11 cases of granulomatous inflammation of the bladder wall lesions, ureters, or urethra were seen and accompanied by stricture formation in the latter two sites.[1208] Hydronephrosis can occur as a complication of obstruction.

Other chronic inflammatory complications of CGD include lymphadenopathy, hepatosplenomegaly, and an eczematoid dermatitis,[1142,1209] along with hypergammaglobulinemia, anemia of chronic disease, and short stature (see Table 22-14). Gingivitis and ulcerative stomatitis can be seen.[1210] Destructive white matter lesions in the brain have been also been described.[1211,1212] In CGD-associated chorioretinitis,[1213,1214] a recent study identified bacterial DNA in the chorioretinal scars, suggesting the possibility of an infectious etiology.[1215] Glomerulonephritis has been reported.[1216,1217] Discoid lupuslike skin lesions develop in up to 20% of patients, and occasionally patients develop systemic lupus erythematosus, juvenile rheumatoid arthritis, sarcoidosis, or other autoimmune disorders.[1218-1223] Subtle defects in CGD memory B cells[1224,1225] may influence the development of autoimmune disease in CGD. Interestingly, gene association studies have identified links between variant alleles encoding cytosolic NADPH subunits and inflammatory bowel disease, rheumatoid arthritis, and systemic lupus erythematosus, consistent with the notion that NADPH oxidase activity normally exerts a suppressive effect on inflammation and autoimmunity.[1156,1226]

The severity and pattern of complications of CGD are heterogeneous. Patients with p47^{phox} deficiency and patients who have X-linked CGD with some residual enzyme activity overall tend to have milder disease that correlates with small but detectable levels of superoxide production.[1123,1145,1227] However, a severe clinical course can occur in some patients with variant X-linked CGD that is indistinguishable from what is observed in patients with complete absence of phagocyte superoxide production.[1228,1229] On the other hand, some individuals with undetectable levels of respiratory burst activity experience relatively few symptoms. Hence polymorphisms in oxygen-independent antimicrobial systems or other components regulating the innate immune response likely modify disease severity in CGD. Specific variants in the MPO, MBL, and FcγRIIa genes are associated with a higher risk for granulomatous or autoimmune/rheumatologic complications in CGD.[1230]

NADPH oxidase subunits, although most highly expressed in phagocytic leukocytes, are also found in other tissues, including endothelial and vascular smooth muscle cells, cardiomyocytes, β cells, and the brain, where they may function in the context of other related NADPH oxidases.[430,431,1231] This may explain an association of diabetes and cardiovascular disease with p47^{phox} deficiency.[1232]

Heterozygous carriers of the CGD trait are generally asymptomatic. Carriers of the AR forms of CGD are free from infection. Carriers of X-linked CGD, on the other had, typically have two populations of circulating neutrophils and monocytes: some with normal respiratory burst activity and others with none—in approximately equal numbers[1233-1239] because of random X-chromosome inactivation.[1240,1241] Up to 20% of female carriers of X-linked CGD are reported to have aphthous ulcers, discoid lupuslike skin lesions, and/or photosensivity.[1220,1242] Polyarthritis, arthralgia, and Raynaud phenomenon are also observed. Serologic testing for systemic lupus erythematosus is generally negative, as are immunofluorescence studies of biopsied lesions. Discoid lupuslike lesions are also occasionally reported in AR CGD patients.[1218,1219,1243] Because the lupuslike skin lesions typically do not appear to develop unless there is at least a subpopulation of nonfunctioning phagocytes, it has been hypothesized that autoantibodies to antigens from incompletely destroyed microbes may be responsible for the syndrome.[1220] Rarely, carriers of X-linked CGD have an unusually small percentage of oxidase-positive neutrophils, which if less than 10% to 15%, can result in infectious complications typical of CGD.[1159,1229,1233,1244]

Molecular Basis of CGD. CGD is classified according to the respiratory burst oxidase subunit that is affected (see Table 22-12 and Fig. 22-9). Also sometimes used is an abbreviated designation within each major genetic subgroup that includes the mode of inheritance and level of *phox* protein expression (see Table 22-12). In two large studies of 140 pedigrees in the United States and Europe, X-linked recessive defects in the gene for the gp91^{phox} subunit of flavocytochrome *b* accounted for approximately two thirds of cases, with the remainder resulting from AR inheritance of defects in genes encoding p47^{phox} (25%), p67^{phox} (5%), and p22^{phox} (5%).[1123,1132] However, deficiency of p47^{phox} accounts for only 7% of CGD in Japan.[1150] In regions where consanguineous marriage is common, the rate of AR CGD can exceed that of X-linked CGD.[1153]

Mutations in Flavocytochrome b. That CGD might result from a genetic defect in an electron-transporting cytochrome was first suspected based on reports that neutrophils obtained from the majority of patients with CGD lacked a low-potential cytochrome *b*.[1245-1247] It is now known that this corresponds to a flavocytochrome comprised of two subunits. One is a 91-kD glycoprotein, gp91^{phox}, encoded by the X-linked *CYBB* gene, which harbors both flavin and heme groups to function as the redox center of the NADPH oxidase. It is closely associated with a 22-kD polypeptide, p22^{phox}, with a corresponding *CYBA* gene that is affected in a subgroup of AR CGD. Formation of the gp91^{phox}/p22^{phox} heterodimer appears to be important for stable expression in phagocytes, and absent expression of both subunits is typically seen in both subgroups of CGD with flavocytochrome *b* mutations.[427,1248,1249]

The *CYBB* gene was mapped to Xp21.1 by its linkage to the X-linked form of CGD[1250] and was the first human gene to be cloned on the basis of its chromosomal location.[429] Defects in X-linked CGD are heterogenous[1150,1251-1253] and with a few exceptions are associated with absence of both the flavocytochrome heterodimer ($X91^0$) and respiratory burst activity. More than 600 distinct *CYBB* mutations have been identified in X-linked

CGD and are distributed across the gene. An international registry is accessible at http://bioinf.uta.fi.[930] In general, each mutation is unique to a given kindred. The findings indicate that the incidence of CGD represents many different mutational events without any founder effect, as expected for a formerly lethal disorder. Approximately 10% to 15% of X-linked CGD is caused by new germ line mutations.[1252]

A small percentage of patients with X-linked CGD express a small amount of residual flavocytochrome *b* (X91–) associated with low levels of superoxide production. Other rare patients have normal levels of a dysfunctional flavocytochrome *b* (X91+), involving mutations in gp91*phox* domains that either participate in oxidase assembly or are involved in its redox function.[1253,1254] Two unusual kindreds were recently reported in which there were point mutations in gp91*phox*, leading to marked reduction of flavocytochrome *b* expression and activity in macrophages compared to neutrophils.[1255] The mutations appeared to impair heterodimer formation with p22*phox*, which in neutrophils could be partially overcome by their higher levels of gp91*phox* expression relative to macrophages. Affected patients were susceptible to mycobacterial infections but did not suffer from other bacterial and fungal infections characteristic of CGD, highlighting the importance of macrophages for controlling mycobacteria.

Relatively large deletions in Xp21.1 involving CYBB and adjacent loci occur occasionally and are associated with complex phenotypes.[429,1250,1256-1259] These include CGD and McLeod syndrome (a mild hemolytic anemia with acanthocytosis that is associated with depressed levels of Kell antigens because of defects in the red cell antigen K_x), with or without concomitant Duchenne muscular dystrophy and retinitis pigmentosa. McLeod phenotype patients may develop anti-Kell antibodies when transfused with red blood cells, which can make future transfusion very difficult. These patients can also develop nerve and muscle disorders related to absent Kell expression in nonerythroid tissues.[1260-1262]

Aberrant regulation of gp91*phox* transcription has been identified in two unusual phenotypes and is associated with point mutations in the *CYBB* promoter near the transcription start site.[1263,1264] In one type, described in two unrelated kindreds, affected males have two distinct populations of neutrophils.[1228] One population, with normal gp91*phox* expression, accounted for 5% to 15% of circulating phagocytes and myeloid progenitor cells, whereas the other entirely lacked gp91*phox* expression. Affected males had a severe clinical course.[1228] Another unusual patient had neutrophils, monocytes, and B lymphocytes with markedly reduced levels of gp91*phox*, but gp91*phox* expression and function in circulating eosinophils was normal.[1265]

Mutations in the *CYBA* gene for the p22*phox* subunit of flavocytochrome *b* are an uncommon cause of AR CGD (see Table 22-12). The genetic defects are heterogeneous,[1266] and in most cases p22*phox* is not expressed (A22° CGD). In one patient, a proline to glutamine substitution in p22*phox* was associated with normal flavocytochrome *b* levels but a nonfunctional oxidase ($A22^+$ CGD).[1267] This mutation disrupted a cytoplasmic proline-rich domain of p22*phox* that otherwise binds to tandem src homology domain 3 (SH3) domains in p47*phox* during oxidase assembly.

Mutations in Cytosolic Subunits. Key in understanding the molecular components of the NADPH oxidase and subsequently unraveling the different genetic subgroups in CGD was the discovery that both the plasma membrane and the cytosol were required to reconstitute a catalytically active oxidase in a cell-free assay.[1268-1271] Complementation analysis using this assay identified two different subgroups of CGD in which the defect involved a cytosolic protein rather than the membrane-bound flavocytochrome *b*.[1272,1273] These proved to involve mutations in the neutrophil cytosolic factor 1 *(NCF1)* gene encoding p47*phox*, and in the *NCF2* gene for p67*phox* (see Table 22-12). The p47*phox* subunit functions as an adaptor protein important for efficient recruitment of cytosolic *phox* proteins to flavocytochrome *b* during NADPH oxidase assembly, whereas p67*phox* binds to gp91*phox* to activate electron transport.

In contrast to other forms of CGD, the most cases of p47*phox*-deficient CGD result from a single type of mutation, a GT deletion at the beginning of *NCF1* exon 2 that results in a frameshift and premature translational termination.[1266,1274,1275] Most patients are homozygous for this mutation without any history of consanguinity, although a few compound heterozygotes have been described with a missense mutation accompanying the GT deletion.[1276] The high frequency of the GT deletion reflects the fact that humans have closely linked pseudogenes for p47*phox* that contain the GT deletion.[1277,1278] Recombination or gene conversion events between the authentic *NCF1* gene and its pseudogene(s) lead to a nonfunctional *NCF1* allele containing the GT deletion.[1279] All reported patients with *NCF1* mutations do not express this protein (A47°). Perhaps because p47*phox* is an adaptor protein rather than directly involved in activating electron transport, A47° neutrophils can still generate very small amounts of superoxide, which may account for the overall milder course of this genetic subgroup.[1123,1145,1227]

Mutations identified in the *NCF2* gene for p67*phox* in patients with CGD are heterogeneous and typically result in absent p67*phox* expression (A67° CGD).[1266,1280-1284] In one patient where a triplet nucleotide deletion results in an in-frame deletion of lysine 58, a nonfunctional form of p67*phox* was expressed, which failed to translocate and was unable to bind to Rac.[1285]

Finally, the first patient with AR p40*phox* defects was recently reported, a compound heterozygote for two null *NCF4* alleles.[438,1286] The p40*phox* subunit plays a specialized role in regulating intracellular NADPH oxidase activity via its binding to phosphatidylinositol 3-phosphate. Although neutrophil NADPH oxidase activity on the plasma membrane was normal in this patient, phagosome oxidant production was profoundly impaired. The main clinical findings were granulomatous inflammation of the intestinal tract rather than the bacterial and fungal infections characteristic of other forms of CGD, perhaps reflecting the more selective role of p40*phox* and residual enzyme activity that may be sufficient for microbial killing.

Diagnosis. A diagnosis of CGD is suggested by the characteristic clinical features (Tables 22-13 and 22-14) or by a family history of the disease. Because the severity and onset of the disease can vary considerably among different patients, CGD should still be considered in adolescents and adults who have an unusual site of infection or carry an organism typical of CGD (see Table 22-13). The diagnosis of CGD is established by demonstrating an absent or greatly diminished neutrophil respiratory burst. It is important to have testing performed on appropriately handled blood samples and by experienced laboratories in order to avoid inconclusive or false normal results.

Many different methods have been used to monitor respiratory burst activity. One of the simplest and most accurate techniques is the nitroblue tetrazolium (NBT) slide test, in which neutrophils in freshly isolated blood are stimulated to undergo a respiratory burst in the presence of NBT.[1234,1235,1287,1288] When reduced by electrons donated from superoxide, the water-soluble yellow tetrazolium dye is converted into deep blue insoluble formazan deposits that precipitate on any cell that undergoes a respiratory burst. As shown in the NBT slide test in Figure 22-15, stimulated neutrophils and monocytes are normally strongly stained with NBT formazan deposits *(A),* whereas cells from a patient with classic X-linked CGD show compete absence of staining *(B).* Nearly 100% of normal cells are NBT-positive, in contrast to the mixed population of NBT-positive and NBT-negative cells seen in the patient's mother (see Fig. 22-15, *C*). In X91– CGD, characterized by a partial deficiency of flavocytochrome *b*, all neutrophils are weakly NBT-positive. Intermediate levels of NBT reduction are not seen in phagocytes of AR carriers. Flow cytometric assays of oxidase activity, such as those based on the conversion of dihydroxyrhodamine (DHR) 123 to rhodamine 123,[1289,1290] are increasing in use for diagnosis of CGD and like the NBT test provide both quantitative measurements of oxidant generation and the cell-by-cell distribution of activity (see Fig. 22-15, *D* to *G*). A small but measurable level of DHR fluorescence can be seen in A47° cells *(G),* which can also show weak staining in the NBT test, reflecting their small amount of residual oxidant production. The DHR assay is available in many referral centers and reference laboratories and can be performed on blood collected in heparin and stored overnight. Notably, neutrophils from patients with MPO deficiency can give a false-positive result for CGD in the DHR test because of weak DHR fluoresence.[1291] This is because although respiratory burst oxidase activity is normal, MPO is required to convert superoxide into the species that reacts with DHR.

Identification of the specific genetic subgroup for a patient with CGD is useful primarily for purposes of genetic counseling and prenatal diagnosis. There is also some clinical predictive value, because patients with p47^{phox} deficiency tend to have less frequent complications. With the exception of classic X-linked disease in a male whose mother is a carrier, determination of the affected gene (see Table 22-12) requires additional studies. Genetic testing for all subgroups of CGD is now available commercially. Other assays, available in research laboratories, include immunoblot analysis of *phox* protein expression in neutrophil extracts and flavocytochrome *b* spectroscopy. Testing for the McLeod red cell phenotype should be done if there is evidence of a large Xp21 deletion in patients with X-linked CGD.[1260] This can cause a mild hemolytic anemia as well as other complications (see "Mutations in Flavocytochrome b"). More importantly, there can be serious problems with development of hemolytic antibodies if these patients are transfused with Kell-positive blood.

Prognosis and Treatment. The prognosis for patients afflicted with CGD has continued to improve since the disorder was first described in the 1950s, at which time almost all patients died in childhood.[1127-1129] In one retrospective review of 38 patients studied between 1964 and 1989, actuarial analysis showed 50% survival through the third decade of life.[1292] In another retrospective study of 48 patients that took place between 1969 and 1985 in Paris, the actuarial survival rate was 50% at 10 years of age, with a prolonged plateau thereafter.[1143] Further refinements in management, the use of prophylactic antimicrobials and more active antifungal and antibacterial agents, and the introduction of IFN-γ therapy have continued to improve the prognosis for CGD.* There is a general consensus that a large majority of patients should survive well into their adult years. The overall mortality for all patients with CGD in the United States including adults was estimated to be 5% per year for X-linked CGD and 2% per year for AR CGD in a retrospective analysis from 2000.[1123] The most common causes of death were pneumonia and/or sepsis caused by the *Aspergillus* species of *B. cepacia*.[1123,1144] A more recent single institution study in 76 younger patients reported an overall mortality of 1.5% per year.[1294] As already noted, patients with deficiency of p47^{phox} typically exhibit a milder disease compared to those with flavocytochrome-negative CGD, likely because of small amounts of residual superoxide produced.[1123,1145,1227]

A multifaceted therapeutic approach is responsible for the greatly improved prognosis in CGD.[1143,1297,1298] The cornerstones of current management include: (1) prevention and early treatment of infections; (2) use of prophylactic antibacterial and antifungal therapy; (3) early use of parenteral antibiotics, including antifungal drugs, and augmented by surgical drainage or resection of recalcitrant abscesses; and (4) the use of prophylactic recombinant human IFN-γ (rIFN-γ).

Several approaches can be used to prevent infections. Patients with CGD should receive all routine immunizations (including live-virus vaccines) on schedule and an influenza vaccine yearly. Cuts and skin abrasions should be promptly cleansed with soap and water and rinsed with a solution of 2% hydrogen peroxide. The frequency and severity of rectal infections can be greatly reduced by avoiding constipation and soaking early lesions in warm, soapy water. Flossing and professional dental cleaning can help prevent gingivitis and periodontitis. The risk of

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*References 1145, 1147, 1164, 1167, 1293-1296.

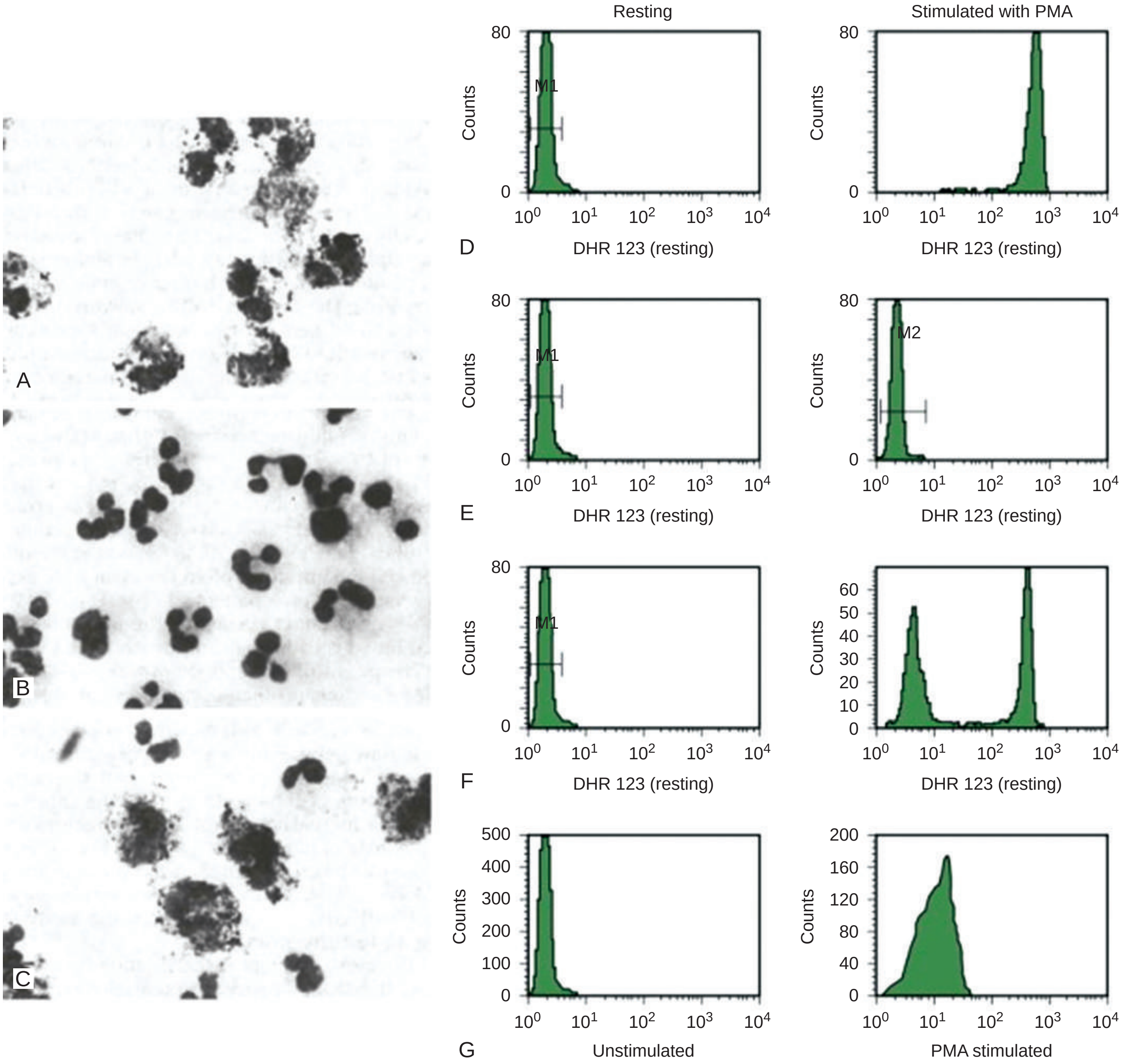

Figure 22-15 Analysis of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activity for diagnosis of chronic granulomatous disease (CGD). **A-C**, Nitroblue tetrazolium (NBT) slide test. Peripheral blood neutrophils and monocytes from a drop of fresh whole blood were made adherent to glass slides and stimulated with phorbol myristate acetate *(PMA)*. **A**, Normal neutrophils and monocytes, all of which are NBT-positive. **B**, Neutrophils and monocytes from an X-linked patient with CGD, which are all NBT-negative. **C**, A mixture of NBT-positive and NBT-negative neutrophils from the X-linked carrier mother of the patient in part **B**. **D-G**, Dihydrorhodamine *(DHR)* 123 flow cytometry test. Nonfluorescent DHR 123 is taken up by neutrophils and becomes fluorescent after reaction with reactive oxygen species produced in the respiratory burst. **D**, Normal neutrophils. **E**, Neutrophils from an X-linked patient with CGD that do not fluoresce after stimulation. **F**, A mixture of nonfluorescent and fluorescent neutrophils from an X-linked CGD carrier. **G**, Neutrophils from a p47^{phox}-deficient patient, which show weak fluorescence after stimulation. *(Modified from Dinauer MC, Coates TD: Disorders of phagocyte function. In Hoffman R, et al, editors:* Hematology: basic principles and practice, *6th ed. Philadelphia, 2012, Saunders, p. 660.)*

Aspergillus infection can be decreased by avoiding marijuana smoke[1299] and decaying plant material (e.g., rotting wood, mulch, sawdust, hay), both of which often contain numerous *Aspergillus* spores.[1300,1301]

Prophylactic antibiotics for bacterial and fungal infections is an important component in the management of CGD. Retrospective studies showed that chronic prophylaxis with trimethoprim/sulfamethoxazole (5 mg/kg/day of trimethoprim given in one or two doses) can decrease the number of bacterial infections in patients with CGD without increasing rates of fungal infections or infections with pathogens resistant to trimethoprim/

sulfamethoxazole.[1143,1296] Dicloxacillin (25 to 50 mg/kg/day) can be used in patients with allergies to sulfa drugs, although there are relatively few data documenting its efficacy. Itraconazole is used for prophylaxis against fungal disease. A prospective study found an incidence of 3.4 *Aspergillus* infections (lung) per 100 patient years compared to 11.5 in a historical control group that did not receive any prophylaxis.[1302] A subsequent randomized, double-blind, placebo-controlled study further documented the efficacy of itraconazole for prophylaxis for fungal infections in CGD.[1295] Daily itraconazole is therefore now recommended for all patients with CGD (200 mg daily if 13 years of age or older or if weighing at least 50 kg, or 100 mg daily if less than 13 years of age or weighing less than 50 kg). Liver function tests should be monitored on patients receiving itraconazole.

rIFN-γ is another cornerstone of current management that reduces the frequency of serious infection in patients with CGD.[1294,1303-1305] The recommended dose of rIFN-γ in CGD is 0.05 mg (50 µg)/M^2 (or 0.0015 mg/kg if <0.05 M^2) given three times a week subcutaneously. A multicenter double-blinded, placebo-controlled phase III trial first established that rIFN-γ was an effective and well-tolerated agent for infection prophylaxis in CGD (Table 22-15).[1303] Patients receiving rIFN-γ had a 70% reduction in the number of serious infections during the study period. These beneficial effects were independent of age, mode of inheritance, and concomitant use of prophylactic antibiotics. The most common side effects were mild fever, headache and other flulike symptoms, which can be ameliorated with acetaminophen. No additional adverse effects, including any increased incidence of chronic inflammatory complications, have been noted with longer periods of prophylactic rIFN-γ (more than 10 years).[1294] On average, these patients are averaging one serious infection per patient every 4 to 5 years. rIFN-γ is used less often in Europe, where a retrospective study suggested no additional benefit over prophylactic antibiotics alone.[1147] However, longer follow-up study of the prospective trials does support a sustained benefit.[1294,1304,1305]

TABLE 22-15 Summary of the Phase III Study Establishing the Efficacy of Recombinant Interferon-γ for Infection Prophylaxis in Chronic Granulomatous Disease[*]

	Treatment Group		
Variable	**Interferon**	**Placebo**	**P Value**
Number of patients	63	65	
Patient years on study	52.1	50.9	
Age ± SD (yr)	14.3 ± 10.1	15.0 ± 9.6	
Number of patients with at least one serious infection	14 (22%)	30 (46%)	.0006
Serious infections per patient-year	0.38	1.1	
Total number of serious infections	20	56	<.0001
Number of hospital days per patient-year	8.6	28.2	
Percentage without Serious Infection[†]			
Age			
<10 yr (52 patients)	81	20	
≥10 yr (76 patients)	73	34	
Inheritance			
X-linked (86 patients)	79	33	
Autosomal (42 patients)	71	39	
Prophylactic antibiotics			
Yes (111 patients)	78	33	
No (17 patients)	69	28	

The International Chronic Granulomatous Disease Cooperative Study Group. A controlled trial of interferon gamma to prevent infection in chronic granulomatous disease. N Engl J Med *324:509–516, 1991.*

SD, Standard deviation.

[*] The table shows a summary of the final results of a phase III randomized, double-blind, placebo-controlled study in which 128 patients with chronic granulomatous disease (GCD) received either recombinant interferon γ (50 mg/m^2 per dose) or placebo by subcutaneous injections three times per week for an average duration of 8.9 months (a controlled trial of interferon γ to prevent infection in CGD). The major endpoints of the study were time to the first serious infection and the number of such infections. A serious infection was defined as an event requiring hospitalization and parenteral antibiotics.

[†] The bottom portion of the table shows the Kaplan-Meier estimates of the cumulative proportion of patients free from serious infections for 12 months (after randomization) with adjustment for stratification factors.

The beneficial effect of rIFN-γ in most patients with CGD is not well understood. Since rIFN-γ increases levels of gp91^{phox} mRNA expression,[1306] increased superoxide production and bacterial killing can be seen in some patients with variant X-linked CGD (X91– in Table 22-12).[1306-1309] However, in most cases the effects of rIFN-γ are likely achieved by its enhancement of nonoxidative antimicrobial pathways and other phagocyte functions.

A common shortcoming in the treatment of patients with CGD is the failure to treat potentially serious infections promptly or long enough with the appropriate parenteral antibiotics. Patients who have CGD do not always have typical symptoms and signs even with serious infections, and fever and leukocytosis can be absent. An elevated sedimentation rate is often present, which can thus be very useful to monitor both as a clue to the presence of a significant infection as well as to observe the response to therapy. Therapy should be directed initially at characteristic pathogens. In the absence of a diagnostic culture, broad-spectrum gram-negative coverage, including for *B. cepacia*, should be administered in conjunction with a potent antistaphylococcal agent (e.g., a combination of nafcillin and ceftazidime). If the site of infection and/or offending pathogen are not known, and if the patient is severely ill and is not responding to the initial therapy within 24 to 48 hours, an aggressive search for the underlying infection should be conducted, including bronchoscopy or open biopsies, if indicated. In ill patients, therapy for fungi, *Nocardia* species, and *B. cepacia* may have to be given empirically.

B. cepacia is notorious for causing persistent fevers followed by bloodstream dissemination from the lung or other sites, resulting in sudden septic shock. *B. cepacia* generally responds to ceftazidime or meropenem,[1168] which are most effective if given before dissemination into the blood stream.

Proven or suspected fungal infections have been treated with amphotericin B in the past, although its use in many cases is being replaced with newer azole antifungal agents such as voriconazole or posaconazole.[1164,1167,1310-1312] Mortality from *A. fumigatus* is now uncommon.[1164] Fulminant "mulch" pneumonitis resulting from a large inhalation exposure to *Aspergillus* includes a substantial component of necrotizing inflammation and benefits from treatment with steroids in addition to antifungals.[1301] Nonfumigatus species of *Aspergillus* and some other fungal species remain difficult to treat, and early therapeutic surgery should be considered.[1164,1167,1313]

Even when appropriate antibiotics are used, infections often respond slowly, and generally should be treated with an antibiotic course that is more prolonged than for a patient with normal neutrophil function. Some infections may require many months of therapy, particularly *Aspergillus*, but with staphylococcal infections as well. Surgical drainage or resection can play a key role in the management of lymphadenitis, osteomyelitis, and abscesses of the liver, lungs, kidney, brain, and rectum. However, recent studies indicate that antibiotics combined with corticosteroids can be effective in curing liver abscesses without surgery.[1314,1315] Finally, granulocyte transfusions have been used in the setting of recalcitrant or life-threatening infections, but their efficacy is uncertain and pose a risk of alloimmunization.[1316]

Granulomatous inflammation in the esophagus, gastric antrum, and bladder can result in symptoms of obstruction, and in the bowel wall, a syndrome resembling inflammatory bowel disease. These complications can cause considerable morbidity in some patients with CGD, and are generally managed with a combination of antibiotics and steroids.[1200,1206,1294,1317-1319] Although the antiinflammatory effects of steroids are often beneficial in this setting even at low oral doses (1 mg/kg/day of prednisone),[1319] their use should be carefully monitored because of the additional immunosuppression associated with their use. TNF-blocking agents should be avoided in CGD as they are associated with a high risk of serious infections in these patients.[1320]

Allogeneic bone marrow transplantation can be used to successfully treat CGD.[1297,1321] Because of the risks associated with this procedure, marrow transplantation had been generally considered only for those patients who have frequent and severe infections despite aggressive medical management and also have a fully HLA-matched sibling donor. However, matched unrelated donors as well as reduced intensity conditioning regimens have now been successfully used in CGD, including several cases with ongoing fungal infections, although graft-versus-host disease can be a significant problem in adult patients.[1321-1324] Despite improved success rates and decreased complications with newer transplant regimens, which CGD patients should undergo transplantation remains an individualized decision, particularly for those with residual NADPH oxidase activity and little or no history of serious infection or other complications.

Gene replacement therapy for CGD using HSCs is under active investigation, and may be a future therapeutic modality.[1321,1325] Observations on carriers of X-linked CGD with skewed X-linked inactivation as well as preclinical studies in murine CGD suggest that partial correction of oxidase activity in as few as 10% of circulating neutrophils will have substantial clinical benefit.[1326-1329] In contrast to lymphocyte immunodeficiencies, there is no selective advantage for gene correction in CGD, and transplantation of gene-corrected cells must be combined with marrow conditioning both to reduce the number of uncorrected HSCs and to facilitate engraftment.[1325] Using a partially ablative conditioning regimen and a conventional γ-retroviral vector to treat three patients with X-linked CGD, 5% to 20% NADPH oxidase-positive neutrophils were detected in the first weeks after transplantation along with resolution of infections. However, 1% or fewer gene-corrected cells were seen over the long term.[1330] Results from another clinical trial, which used a γ-retroviral vector with a very potent viral long terminal repeat to drive expression of the X-linked CGD protein, illustrated the potential for adverse effects of gene therapy.[1331] In two patients reported, much higher numbers of oxidase-positive neutrophils (20% to 50%) were seen, along with resolution of chronic infectious complications. However, this high level of correction resulted from clonal expansion of myeloid precursors that harbored vector insertions in genes that are master regulators of myeloid cell growth, and led to the development of myelodysplasia and leukemia.[1321,1325,1331] Thus in addition to investigating conditioning regimens, current efforts for gene therapy of CGD are focused on evaluating vectors that lack strong enhancer sequences, which reduces the potential for insertional mutagenesis caused by activation of neighboring genes.[1321]

Glucose-6-Phosphate Dehydrogenase Deficiency

The substrate for the respiratory burst oxidase, NADPH, is generated by the first two reactions of the hexose monophosphate shunt, G6PD (see Fig. 22-7, reaction 8) and 6-phosphogluconate dehydrogenase. Since G6PD is the first enzyme in this pathway, its absence results in greatly diminished shunt activity and thus a decrease in the availability of NADPH. Thus severe G6PD deficiency in neutrophils results in an attenuated respiratory burst and, in some cases, a clinical picture somewhat similar to that observed in CGD.[1332-1335] The key features of G6PD deficiency are summarized in Box 22-16. The organisms causing infection in severely deficient patients are similar to those observed in CGD and are predominately catalase-positive bacteria.

In light of the relative high frequency of G6PD mutations in the African-American and Mediterranean populations,[1336] as well as the fact that leukocyte and erythrocyte G6PD are encoded by the same gene, it might be expected that clinically significant neutrophil G6PD deficiency would occur more often than it does

Box 22-16 Summary of Neutrophil Glucose-6-Phosphate Dehydrogenase Deficiency

Incidence	Extremely rare
Inheritance	X-linked
Molecular defect	Poorly characterized mutations that cause congenital nonspherocytic hemolytic anemia (CNSHA) in erythrocytes and functional failure of glucose-6-phosphate dehydrogenase (G6PD) in neutrophils (possibly kinetic mutants); other rare mutants may also be responsible
Pathogenesis	Severe functional failure of neutrophil G6PD (<5% of normal) leading to an extremely low steady-state concentration of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which serves as the substrate for NADPH oxidase
Clinical manifestations	CNSHA (hemolytic anemia that occurs even in the absence of redox stress) Chronic granulomatous disease (CGD)-like syndrome with recurrent bacterial infections
Laboratory evaluation	Neutrophil G6PD activity <5% of normal Severely diminished respiratory burst Associated CNSHA with elevated reticulocyte count and diminished erythrocyte G6PD activity
Differential diagnosis	CGD Glutathione reductase or synthetase deficiency
Therapy	Prophylactic trimethoprim/sulfamethoxazole Aggressive use of parenteral antibiotics Transfusion support for severe anemia
Prognosis	Not clear because too few patients have been reported

From Curnutte JT: Disorders of phagocyte function. In Hoffman R, Benz EF, Shattil S, et al, editors: Hematology: basic principles and practice, *New York, 1991, Churchill Livingstone, p. 577.*

(fewer than 10 cases have been reported in the literature). One of the reasons it does not is that neutrophils must have a severe deficiency (less than 5% of normal G6PD levels) before respiratory burst function is adversely affected. Even low levels of G6PD are apparently sufficient to recycle NADP+ back to NADPH at a rate that permits reasonable levels of respiratory burst activity. Furthermore, studies on patients with variant CGD indicate that surprisingly low levels of respiratory burst activity (5% to 10% of normal) can still provide adequate protection against microbial infection (see “Chronic Granulomatous Disease”). Therefore clinically significant decreases in levels of neutrophil G6PD are rarely encountered. In most types of G6PD deficiency, neutrophil levels are in the range of 20% to 75% of normal.[1337,1338] Since most G6PD mutations cause the enzyme to decay over a period of days and weeks, levels in the short-lived neutrophil usually do not become critically low, even in some of the more unstable G6PD variants. Hence it appears that only a rare (and poorly understood) group of mutations causes extremely low levels of G6PD in neutrophils.

The diagnosis of G6PD deficiency should be considered in any patient with congenital nonspherocytic hemolytic anemia in whom the erythrocyte G6PD level is unusually low or the frequency of infections is high. The diagnosis is established by measuring the level of G6PD activity in neutrophil homogenates. Although not necessary for the diagnosis, neutrophil function tests show a diminished respiratory burst (5% to 30% of normal).[1334,1339] The treatment of neutrophil G6PD deficiency is similar to that of CGD except that the efficacy of rIFN-γ has not been demonstrated in the former. In general, G6PD deficiency appears to be somewhat milder than CGD, although recurrent pneumonias and a fatal infection with *C. violaceum* have been described.[1334,1339]

Myeloperoxidase Deficiency

MPO deficiency is the most common inherited disorder of phagocytes, although is rarely associated with clinical symptoms. Although only 17 known cases had been collected up to 1979, with the widespread use of automated flow cytochemistry in clinical hematology laboratories to enumerate peripheral blood neutrophils using peroxidase activity, it is now recognized to occur much more often. In the United States and Europe, complete deficiency is seen in approximately 1 in 4000 individuals, and partial deficiencies occur in 1 in 2000 people, although MPO deficiency occurs in only 1 in 57,000 people in Japan.[446,1340-1342] MPO deficiency is seen in neutrophils and monocytes. Peroxidase levels in eosinophils, on the other hand, are normal, because eosinophil peroxidase is encoded by a different gene.

MPO plays a pivotal role in amplifying the toxicity of H_2O_2 generated by the respiratory burst, and catalyzes the formation of HOCl from chloride and hydrogen peroxide (see Fig. 22-7, reaction 4).[423,446,1341] HOCl, in turn, reacts with a variety of primary and secondary amines to form chloramines, some of which can be toxic.[305] Moreover, HOCl is capable of activating latent metalloproteinases. Based on these considerations, one would expect that severe MPO deficiency should attenuate important antimicrobial reactions catalyzed by HOCl. MPO-deficient mice have impaired host defense against *Candida* and *Klebsiella*.[1343,1344] Neutrophils from patients with MPO deficiency show markedly abnormal in vitro killing of *C. albicans* and hyphal forms of *A. fumigatus*.[1341,1342] Curiously, however, these abnormalities are rarely reflected in an increased incidence of infection in patients except for rare individuals who also suffer from diabetes mellitus.[1340,1342] In these individuals, disseminated candidiases can be seen (Box 22-17). Several studies have suggested that MPO deficiency may also be associated with an increased susceptibility to malignancy.[1340,1345,1346]

Congenital MPO deficiency is inherited in an AR manner,[1341,1342] and variable expression of the defect is reported.[1346] The gene for MPO has been localized to

Box 22-17 **Summary of Myeloperoxidase Deficiency**

Incidence	1 in 2000 (partial deficiency) 1 in 4000 (total deficiency)
Inheritance	Autosomal recessive
Molecular defect	Myeloperoxidase (MPO) mutations affect posttranslational processing or heme incorporation
Pathogenesis	Partial or complete MPO deficiency leads to diminished production of hypochlorous acid (HOCl) and HOCl-derived chloramines; MPO products are necessary for rapid killing of microbes (especially *Candida*) but not absolutely required
Clinical manifestations	Usually clinically silent Rarely disseminated candidiasis/fungal disease (usually in conjunction with diabetes mellitus) May be increased risk of malignancy
Laboratory findings	Deficiency of neutrophil/monocyte peroxidase by histochemical analysis (eosinophil peroxidase normal) Delayed, but eventually normal killing of bacteria in vitro Failure to kill *C. albicans* and hyphal forms of *A. fumigatus* in vitro
Differential diagnosis	Acquired partial MPO deficiency seen in acute myelocytic leukemia (M2, M3, and M4), myelodysplastic syndromes, iron deficiency, or pregnancy
Therapy	None in asymptomatic patients Aggressive treatment of fungal infections when they occur Control of blood glucose levels in diabetes
Prognosis	Usually excellent

Modified from Curnutte JT: Disorders of phagocyte function. In Hoffman R, Benz EJ, Shattil S, et al, editors: Hematology: basic principles and practice. *New York, 1991, Churchill Livingstone, p. 578.*

the long arm of chromosome 17 at position q22-q23 near the breakpoint for the 15;17 translocation of promyelocytic leukemia.[1347,1348] The primary translation product is a 80-kD protein that undergoes cotranslational glycosylation and subsequent proteolytic cleavage to heavy and light chains, which oligomerize to form a tetramer composed of two heavy and two light chains.[1341] The molecular basis of congenital MPO deficiency reflects defects affecting MPO processing and heme incorporation. A variety of mutations have been reported.[1341] One common cause of complete absence of immunoreactive MPO is a point mutation that results in an arginine to tryptophan substitution at codon 569 (R569W).[1349,1350] The R569W mutation results in a maturation arrest of the MPO apoprotein, which fails to incorporate heme.[1351]

In addition to inherited MPO deficiency, secondary or acquired MPO deficiency can occur. MPO-deficient cells can be seen in the M2, M3, and M4 forms of acute myeloid leukemia as well as in approximately 25% of patients with MDS and chronic myeloid leukemia.[1352,1353] Other clinical situations in which secondary MPO deficiency can occur include pregnancy, iron deficiency, lead poisoning, ceroid lipofuscinosis, and Hodgkin disease.[1340] Attesting to a proinflammatory role for MPO, individuals deficient in MPO may have a decreased susceptibility to neutrophil-mediated vascular dysfunction.[446]

There are several possible reasons for the surprisingly mild clinical phenotype of MPO deficiency. First, neutrophils contain such a large amount of MPO that appreciable levels may be left in the cell even in cases of severe deficiency. These residual stores, coupled with normal levels of eosinophil peroxidase, may provide at least some degree of peroxidative activity at sites of infection. Second, the respiratory burst in MPO-deficient neutrophils is substantially augmented in terms of velocity and duration.[1354,1355] This may be because of the enhanced stability of the respiratory burst oxidase made possible by the absence of HOCl, which normally inactivates the oxidase.[1356] Finally, other oxidants produced by the respiratory burst may work together with the various granule antimicrobial proteins to provide sufficient protection against most microorganisms.[446]

Given the mild clinical nature of MPO deficiency, no treatment is generally required. Infections, when they occur, are treated as in normal individuals. It is important for the clinician to be aware of the increased risk of *Candida* infections in patients with MPO deficiency and to treat them aggressively when they do occur.

Disorders of Glutathione Metabolism

As shown in Figure 22-8 (reaction 6), glutathione peroxidase protects neutrophil proteins (including NADPH oxidase) from the harmful effects of H_2O_2 generated in the course of the respiratory burst by degrading it to water. The reducing equivalents for this reaction are carried by the reduced glutathione (GSH), the intracellular levels of which are maintained by recycling oxidized glutathione back to GSH by means of glutathione reductase (see Fig. 22-8, reaction 7). Glutathione levels are also maintained through de novo synthesis catalyzed by glutathione synthetase (see Fig. 22-8, reaction 9). Severe deficiencies of glutathione reductase and glutathione synthetase in neutrophils have been reported and found to cause moderately severe abnormalities in the respiratory burst.[1357-1361] The key features of each of these extremely rare disorders are summarized in Table 22-16.

Glutathione reductase deficiency has been reported in only a few families.[1362] In one, three siblings were found to have a marked deficiency of this enzyme in their neutrophils (10% to 15% of normal).[1357,1359] The inheritance appears to be AR, because the parents of the children were first cousins and their neutrophils contained 50% of the normal levels of glutathione reductase. The responsible mutation was a large deletion affecting the enzyme's dimerization domain.[1362] A second patient was a compound heterozygote for point mutations leading to a premature stop codon and a missense mutation affecting enzyme stability.[1362]

Clinically, none of the affected patients showed signs of increased infection. Their erythrocytes were also

TABLE 22-16 Disorders of Glutathione Metabolism

Disease Aspect	Glutathione Reductase Deficiency	Glutathione Synthetase Deficiency
Incidence	Several families	More than 50 families
Inheritance	Autosomal recessive	Autosomal recessive
Molecular defect	Diminished glutathione reductase levels in neutrophils (10%-15% of normal) and erythrocytes	Moderate to severe deficiency of glutathione synthetase activity
Pathogenesis	Brief respiratory burst truncated by toxic accumulation of H_2O_2 by glutathione	Same as with glutathione reductase deficiency except that the respiratory burst is normal; elevated 5-oxoproline levels because of lack of feedback inhibition by glutathione
Clinical manifestations	No history of repeated infection	Metabolic acidosis caused by elevated 5-oxoproline Otitis media Hemolysis with oxidant stress Intermittent neutropenia Hemolysis with oxidant stress Decrease in glutathione synthetase level
Laboratory evaluation	Glutathione reductase level diminished Premature cessation of O_2^- production by neutrophils	Normal respiratory burst
Differential diagnosis	Chronic granulomatous disease Glucose-6-phosphate dehydrogenase deficiency	Glutathione reductase deficiency
Therapy	None required	Vitamin E for hemolysis and infections Treatment of metabolic acidosis
Prognosis	Benign disorder	Relatively benign disorder

From Curnutte JT: Disorders of phagocyte function. In Hoffman R, Benz EJ, Shattil S, et al, editors: Hematology: basic principles and practice, *New York, 1991, Churchill Livingstone, p. 578.*
H_2O_2, Hydrogen peroxide; O_2^-, superoxide radical.

deficient in glutathione reductase and were prone to hemolysis in the face of oxidant stress. Two siblings suffered from juvenile cataracts and deafness. In vitro studies of neutrophil function were normal except for a premature termination of the respiratory burst after about 5 minutes.[1357] The probable reason for this defect is that NADPH oxidase is inactivated by the products of the MPO reaction.[1356] If glutathione reductase is deficient, the neutrophils cannot maintain a level of GSH sufficient to detoxify H_2O_2, leading to deactivation of the oxidase. One important insight gained from the study of these patients is that only a brief respiratory burst appears to be necessary for adequate microbial killing. The diagnosis of glutathione reductase deficiency is made by measuring the levels of this enzyme in neutrophil homogenates. No therapy is required, but avoiding foods and drugs containing potent oxidants will decrease the chance of depleting intracellular glutathione.

Several forms of congenital glutathione synthetase deficiency have been described, all of which are inherited in an AR fashion.[1363] Specific mutations have been reported. Only in those cases in which the glutathione synthetase activity is severely deficient in neutrophils (5% to 10% of normal) is a phagocytic abnormality detected.[1358,1360,1361] In contrast to glutathione reductase deficiency, neutrophils deficient in glutathione synthetase have a normal respiratory burst.[1358,1361] For reasons that are uncertain, in vitro bacterial killing is decreased. Despite this in vitro microbicidal defect, patients have only a relatively mild problem resolving infections. However, they suffer from a severe metabolic acidosis resulting from elevated levels of 5-oxoproline, a metabolite formed in one of the early steps in the pathway of glutathione synthesis, as well as progressive neurologic symptoms.[1363] This acidosis may be responsible for the intermittent neutropenia observed in patients with glutathione-synthetase deficiency.[1358] Because the erythrocytes in these patients are also deficient in glutathione synthetase, a hemolytic anemia brought on by oxidant stress is seen. Diagnostic tests include measurements of glutathione synthetase activity and glutathione levels in skin fibroblasts and red blood cells.[1363] Therapy with vitamin E and vitamin C has been found to be beneficial in patients who suffer from hemolysis and infection.[1358,1363]

Glutathione peroxidase (see Fig. 22-8, reaction 6) was found to be decreased to levels 25% of normal in three unrelated patients with a clinical syndrome resembling CGD.[1364,1365] While this enzyme plays an important role in removing H_2O_2, it has not been established whether the degree of deficiency seen in these patients is sufficient to cause functional abnormalities in neutrophils. Two of the affected kindreds were subsequently shown to have a severe deficiency of the respiratory burst oxidase flavocytochrome *b*.[1244] Thus it appears that in at least two of these previously reported cases, CGD is the underlying problem, not glutathione peroxidase deficiency.

Disorders of Cytokines and Impaired Phagocyte Function

Inherited defects in the production and response to inflammatory cytokines by phagocytes or other cells of the immune system can present with recurrent or unusual bacterial and fungal infections (see Chapter 24).[65,1366-1370] All of these disorders are rare. In addition to the disorders described, HIES is also caused by defects in cytokine signaling, as discussed earlier in this chapter.

Defects in a group of genes regulating the production and macrophage response to IFNγ are associated with recurrent and severe atypical mycobacterial infections, as well as salmonellae and viral infections in some subgroups.[1369,1371-1373] The phenotype of this group of disorders is also referred to as Mendelian susceptibility to mycobacterial diseases (MSMD). The distinctive and limited spectrum of infections in affected patients results from defects in the IFNγ pathway. IFNγ is secreted by NK and T cells in response to IL-12 and IL-23 released by macrophages and dendritic cells. Some patients have AR mutations in the p40 subunit of the IL-12 and IL-23 cytokines, leading to their decreased expression; another subgroup of patients have mutations in the β_1 subunit of the receptor for IL-12 and IL-23; in both of these groups, levels of IFNγ are abnormally low. Other patients have either AR mutations affecting the IFNγ receptor, leading either to loss of receptor expression or complete or partial loss of IFNγ binding. Dominant IFNγ receptor mutations leading to partial IFNγ receptor deficiency are also described. The phenotype is milder in patients with partial defects. Very rarely, patients have partial or complete deficiency in the STAT-1 protein that is critical for transduction of IFNγ-mediated signals. Mutations in the *CYBB* gene that selectively impair gp91^{phox} expression and NADPH oxidase activity in macrophages but not neutrophils are also a cause of X-linked MSMD.[1255]

Defects in IL-17–mediated signaling are associated with susceptibility to chronic mucocutaneous candidiasis (CMC) and perhaps cutaneous *Staphylococcus* infections. These include dominant-negative mutations in *STAT3* associated with AD HIES and recessive or dominant-negative mutations involving the IL-17 receptor.[1370] Recessive mutations in the caspase recruitment domain-containing protein 9 (*CARD9*) impair intracellular signaling responses downstream of C-type lectin receptors for fungal cell walls that are necessary to generate TH17 responses to *Candida*. Affected patients can develop CMC as well as systemic candidiasis and also have problems with control of dermatophytes.[1370,1374] Interestingly, human neutrophils have impaired killing of *Candida* but not of *Aspergillus* or bacteria, associated with abnormal phagolysosome structures but normal NADPH oxidase activity.[1375]

Recurrent pyogenic infections are characteristic of a group of genetic disorders involving the NF-κB signaling pathway. These include patients with AR mutations in IL-1 receptor–associated kinase (IRAK) 4, a kinase in the toll and IL-1 receptor signaling pathway.[1369,1376,1377] Affected children have recurrent and sometimes fatal infections with *S. pneumoniae* and *S. aureus*, along with low or absent fever and inflammatory responses. Patients with anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) syndrome have either X-linked recessive mutations in the gene encoding NEMO, a regulatory protein that activates NF-κB, or, much less commonly, an AD defect in IκBA, and NF-κB inhibitor. In addition to widely spaced abnormal teeth, hypohidrosis, and hypotrichosis, children affected with EDA-ID have recurrent pyogenic infections with a minimal system inflammatory response, and can also develop opportunistic infections with atypical mycobacteria, viruses, or *Pneumocystis carinii*.

Evaluation of the Patient with Recurrent Infections

The patient who has recurrent infections can be a diagnostic challenge. Many of the phagocyte disorders present with similar clinical manifestations that can overlap those seen in inherited or acquired disorders of lymphocyte function. Furthermore, the majority of patients with recurrent infections do not have an identifiable granulocyte or monocyte defect. Given this low yield and the lack of ability to characterize many aspects of phagocyte function in routine laboratory studies, physicians are faced with the difficult question of which patients merit a complete evaluation. Those who have at least one of the following clinical features are more likely to have a phagocyte defect: (1) an unusually high frequency of bacterial and fungal infections; (2) the presence of an infection at an unusual site (e.g., a hepatic or brain abscess); (3) infections with atypical pathogens (e.g., *Aspergillus* pneumonia, disseminated candidiasis, lymphadenitis resulting from *Serratia* or *Klebsiella,* disseminated mycobacterial infection); (4) infections of exceptional severity; and (5) childhood periodontal disease. Certain other clinical findings can also be helpful. For example, a child with nystagmus, fair skin, and recurrent staphylococcal infections should be evaluated for CHS, whereas an infant with neutrophilia, bacterial infections, and delayed separation of the umbilical cord should be tested for LAD. Once the physician has decided that a phagocyte evaluation is warranted, the algorithm present in Figure 22-16 may be helpful in organizing the work-up. When coupled with a thorough clinical history and physical examination, the laboratory tests outlined in Figure 22-16 should help the physician to establish the diagnosis and formulate an appropriate therapeutic plan. With the advent of cost-effective genome and exome sequencing, it is also anticipated that new inherited disorders of phagocyte function will be defined in the coming years.[1378]

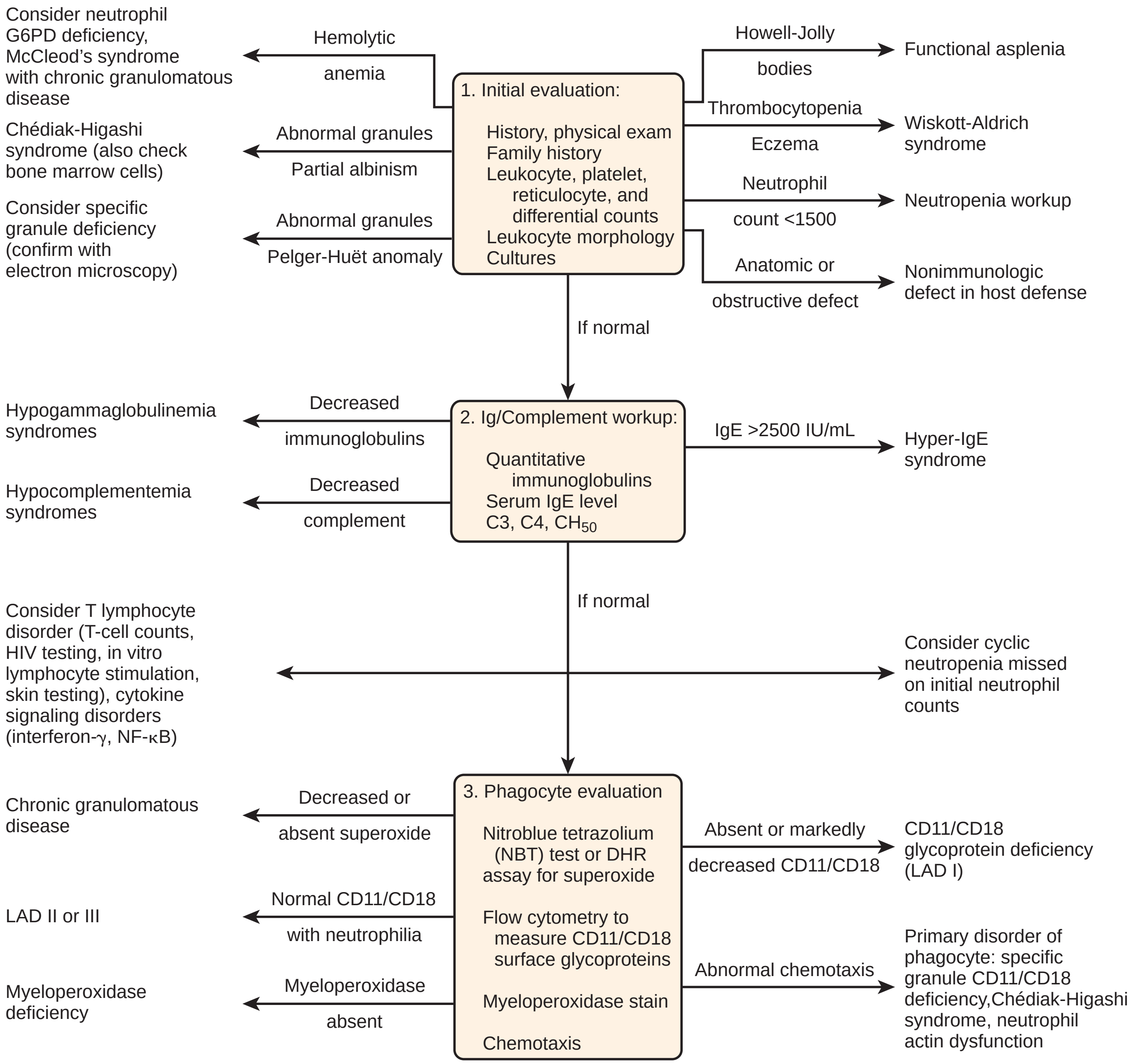
1. Initial evaluation:
History, physical exam
Family history
Leukocyte, platelet, reticulocyte, and differential counts
Leukocyte morphology
Cultures
Hemolytic anemia
Consider neutrophil G6PD deficiency, McCleod's syndrome with chronic granulomatous disease
Abnormal granules
Partial albinism
Chédiak-Higashi syndrome (also check bone marrow cells)
Abnormal granules
Pelger-Huët anomaly
Consider specific granule deficiency (confirm with electron microscopy)
Howell-Jolly bodies
Functional asplenia
Thrombocytopenia
Eczema
Wiskott-Aldrich syndrome
Neutrophil count <1500
Neutropenia workup
Anatomic or obstructive defect
Nonimmunologic defect in host defense
If normal
2. Ig/Complement workup:
Quantitative immunoglobulins
Serum IgE level
C3, C4, CH50
Decreased immunoglobulins
Hypogammaglobulinemia syndromes
Decreased complement
Hypocomplementemia syndromes
IgE >2500 IU/mL
Hyper-IgE syndrome
If normal
Consider T lymphocyte disorder (T-cell counts, HIV testing, in vitro lymphocyte stimulation, skin testing), cytokine signaling disorders (interferon-γ, NF-κB)
Consider cyclic neutropenia missed on initial neutrophil counts
3. Phagocyte evaluation
Nitroblue tetrazolium (NBT) test or DHR assay for superoxide
Flow cytometry to measure CD11/CD18 surface glycoproteins
Myeloperoxidase stain
Chemotaxis
Decreased or absent superoxide
Chronic granulomatous disease
Normal CD11/CD18 with neutrophilia
LAD II or III
Myeloperoxidase absent
Myeloperoxidase deficiency
Absent or markedly decreased CD11/CD18
CD11/CD18 glycoprotein deficiency (LAD I)
Abnormal chemotaxis
Primary disorder of phagocyte: specific granule CD11/CD18 deficiency,Chédiak-Higashi syndrome, neutrophil actin dysfunction

Figure 22-16 Algorithm for the workup of a patient with recurrent infections. The various tests are described in the appropriate sections of the chapter. Units for the neutrophil count *(box 1)* are neutrophils/mm^3. *DHR,* Dihydrorhodamine; *G6PD,* glucose-6-phosphate dehydrogenase; *HIV,* human immunodeficiency virus; *Ig,* immunoglobulin; *LAD,* leukocyte adhesion deficiency; *NBT,* nitroblue tetrazolium; *NF-κB,* nuclear factor κB. *(Modified from Lehrer RI, Ganz T, Selsted ME, et al: Neutrophils and host defense.* Ann Intern Med *109:127–142, 1988.)*

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CHAPTER 23

Immune Response

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CHAPTER OUTLINE

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Peripheral B-Cell Maturation and Differentiation
T-CELL ONTOGENY
T-Cell Receptor Subtypes and Specificity
Early T-Cell Commitment and Seeding
Stages of Intrathymic T-Cell Receptor αβ T-Cell Development and Spatial Considerations
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THE SPLEEN
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B-Cell Tolerance
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T-CELL ACTIVATION AND FUNCTION
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Costimulation of T Cells
PERIPHERAL T-CELL MATURATION
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Control of the Immune Response: Regulation and Termination of T-Cell Activation
NONCONVENTIONAL T-CELL SUBTYPES
Development and Function of CD1-Restricted T Cells or Invariant Natural Killer T Cells
DEVELOPMENT AND FUNCTION OF T-Cell Receptor γδ T CELLS
NATURAL KILLER CELL DEVELOPMENT AND FUNCTION
CONCLUSIONS

The immune system is a complex network of cells that serve to discriminate "self" from "nonself"—that is, to recognize one cell as one's own and another cell as foreign or infected. This capacity helps protect the organism from infection by foreign pathogens (bacteria, viruses, and parasites) and from malignant degeneration. In addition, the cells involved in the immune response mediate rejection of foreign allografts. Although immune cells recognize and respond to all manner of foreign elements, at the same time they must not respond inappropriately to self-antigen. Education of immune cells results in a state of immunologic tolerance—that is, the absence of destructive antiself immune responses. Failure of self versus nonself discrimination, or failure of immunologic tolerance, may result in autoimmunity.

Whereas collectively a number of different kinds of cells are involved in the response to infection, the cells involved in mediating adaptive immunity are lymphocytes (Table 23-1). The adaptive immune response is broadly classified into two categories: (1) humoral immunity, mediated by antibodies produced by B lymphocytes and their progeny; and (2) cell-mediated immunity, dependent

TABLE 23-1 Innate Versus Acquired Immunity

	Innate Immunity	Acquired Immunity
Specificity	Nonself	Random (in the context of self)
Effector cell type	Polymorphonuclear leukocytes (PMNs, neutrophils), natural killer cells, macrophages, eosinophils, etc.	Lymphocytes: B and T cells
Cell surface receptors	Germline	Rearranged
Distribution	Nonclonal	Clonal
Kinetics of the initial response	Rapid and immediate	Slow

on cell-cell interactions and mediated by T lymphocytes. Both T and B lymphocytes are derived from early hematopoietic stem cells (HSCs) and are distinguished not by morphology but by rearrangement of their antigen-specific receptors, by function, and by the expression of different cell surface molecules. In addition to adaptive immunity, natural or innate immune mechanisms also work to defend an individual from pathogens (see Table 23-1). Natural killer (NK) cells, also derived from bone marrow progenitors, serve as principal components of innate immunity and are discussed briefly later in this chapter. In addition, polymorphonuclear leukocytes (neutrophils), eosinophils, macrophages, and other myeloid cells are important components of the body's early defense against microorganisms and are discussed in Chapter 22.

The acquired or adaptive immune response is mediated in large part by the recognition of a variety of cell surface proteins encoded by genes of the major histocompatibility complex (MHC). The MHC, located in the human genome on the short arm of chromosome 6, encodes two classes of proteins, MHC class I and MHC class II, that are highly polymorphic within the population and involved in the immune response. In humans these genes are termed *human leukocyte antigens* (HLA class I and HLA class II), as defined further later. In addition to HLA determinants, the MHC also encodes several secreted proteins of the complement system, selected immune modulators such as tumor necrosis factor (TNF), and specific proteins required for proper processing of antigen.

In addition to self-nonself discrimination, the adaptive immune response is further characterized by immunologic specificity and by memory. Acquired resistance to one antigen does not confer resistance to another unrelated (non–cross-reactive) antigen. The immune response must be capable of eliminating a foreign pathogen and developing mechanisms so that a second exposure to the same agent results not in worse disease but in more rapid elimination of the foreign agent. Cells previously exposed to and specific for one foreign protein or antigen respond more rapidly and more effectively to subsequent exposure to that same antigen, thereby leading to an anamnestic or secondary immune response. The immune response is highly regulated; cooperativity among different cellular elements and between cells and soluble mediators leads to modulation (i.e., amplification or suppression) of the immune response.

Technical advances, including single-cell cloning, monoclonal antibody production, generation of transgenic and knockout animals, and sequencing of the genome, have led to a veritable explosion in the number of cell surface proteins, cytokines, and chemokines discovered. The nomenclature has evolved and can be confusing. Cell surface proteins are often named by a cluster of differentiation (CD) assignment; the website http://www.uniprot.org/docs/cdlist contains links to protein information listed by CD name. Interleukins, the name given to soluble factors secreted by leukocytes or that act on leukocytes, or both, can be found along with their receptors at this site of the Human Gene Nomenclature Committee: http://www.genenames.org/genefamily/il.php. This chapter will focus on specific or acquired immunity and principally on the differentiation and function of B and T lymphocytes.

LYMPHOCYTE DEVELOPMENT

All B cells, T cells, and NK cells derive from pluripotent HSCs that arise first in fetal liver and then postnatally in the bone marrow. HSCs differentiate in the bone marrow and migrate to the appropriate lymphoid organs. These HSCs express cell surface CD34, a heavily glycosylated protein of 105 to 120 kd.[1] Although CD34 serves as a marker for hematopoietic progenitors, its expression is not limited to these cells alone. CD34 is also expressed on early lymphoid (but not pluripotent) progenitors, endothelial cells in small vessels, some embryonic fibroblasts, and some cells in the nervous system.[2] CD34 may be involved in cell-cell adhesion[3] and may be important for inhibition of hematopoietic differentiation.[4] CD34+ hematopoietic precursors give rise to both myeloid and lymphoid early progenitors. The traditional paradigm makes a strong distinction between HSC contained within the CD34+ compartment and lineage committed progenitors, which lack long-term reconstitution potential. Early myeloid differentiation derives from common myeloid progenitors (CMPs), which can give rise to common granulocyte-monocyte precursors (GMPs) or megakaryocyte-erythroid precursors (MEPs). The common lymphoid progenitor (CLP) differentiates into cells of the T (and NK) and B lineage. Dendritic cells can be generated in vitro from CD34+ bone marrow cells exposed to certain cytokines, but the relevant in vivo precursor population remains controversial,[5] and CLP, CMP, and GMP are all shown to contain dendritic cell potential.[5,6,7,8] New evidence from murine studies suggests that in single cell transplants, individual cells defined as HSC can give rise after a single division to one long-term repopulating cell and one myeloid committed cell[9]; thus lineage commitment may occur in individual cells at the HSC stage. How this translates to the human situation has yet to be uncovered. T-lymphocyte differentiation occurs largely in the thymus and is discussed in detail later. B-cell maturation occurs in the bone marrow and peripheral lymphoid organs.

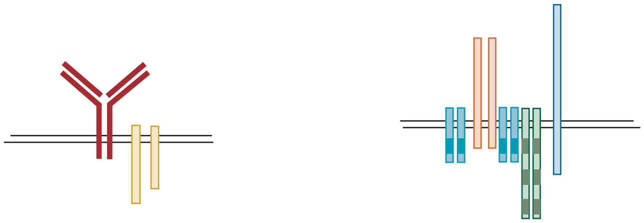
Naïve B and T cells released into the peripheral blood have exquisite specificity. These resting B and T cells circulate in a quiescent state and function only after encountering cognate antigen (i.e., the specific substance that the lymphocyte recognizes). Recognition of antigen alone is generally insufficient to trigger lymphocyte activation; both B and T cells require other so-called *costimulatory signals*, in the form of cell surface receptor engagement or soluble factors, for effector function. Recognition of antigen by T cells and the costimulatory signals for both B and T cells are typically mediated by accessory cells of the innate immune system called *antigen-presenting cells* (APCs). Lymphocytes activated by specific antigen in the appropriate costimulatory context proliferate and mature into effector cells, a portion of which eventually differentiate into long-lived memory cells. The specifics of peripheral maturation, costimulation, and function of B and T cells are detailed in later sections.

LYMPHOCYTE SPECIFICITY

Immunologic specificity is conferred by antigen-specific receptors expressed on the surface of B and T lymphocytes and by specific antibodies (immunoglobulins [Igs]) secreted by B cells and plasma cells (Fig. 23-1). An individual lymphocyte has only one specific antigen to which it can respond. Each T cell expresses a unique T-cell receptor (TCR) and each B cell a unique Ig that can either be displayed on the surface (B-cell receptor [BCR]) or be secreted. The membrane BCR and secreted Ig of the B cell interact with epitopes expressed by native antigens, largely protein and nonprotein antigens such as complex epitopes of glycoproteins, lipids, and nucleic acids. Unlike Ig, the TCR recognizes small (most commonly 9 to 11 amino acid long) processed oligopeptides bound within the cleft of an MHC molecule on the surface of an APC. Dendritic cells, B cells, monocytes-macrophages, epithelial Langerhans cells, and (in humans) endothelial cells function efficiently as APCs. These APCs bear costimulatory molecules on their cell surface (discussed later), secrete cytokines, and process antigen; it is the complex formed between the processed peptide fragment of antigen and MHC proteins that interacts with the TCR. Exposure to antigen induces activation of only lymphocytes that bear Ig receptors or TCRs specific for that antigen. Any individual, however, has approximately 10^7 to 10^9 different T-cell and B-cell specificities. It is this clonal variability and diversity of the lymphocyte repertoire that maintain effective protection against foreign invasion.

B-CELL ONTOGENY

The first phase of B-cell maturation occurs in the bone marrow, where HSCs committed to B-lymphoid lineage develop into immature IgM+ IgD– B cells. The immature B cells exit the bone marrow, circulate in peripheral tissues, and mature further (Fig. 23-2). The most critical steps in B-cell development at this stage involve the expression of Ig molecules on the surface as BCR, whose specificity is the same as the antibody that will ultimately be secreted by activated mature B-cell progeny. The diversity of the B-cell repertoire is due in large part to random rearrangement of multiple germline genes for Ig that are spliced to encode a unique Ig molecule for each cell. Highly regulated checkpoints ensure that only B cells that successfully rearrange Ig genes are selected for survival.[10-12]



	B lymphocyte	T lymphocyte
Antigen-specific receptor	Surface immunoglobulin (IgM, IgD)	T cell receptor (αβ or γδ)
Surface signaling molecules	Ig-α, Ig-β	CD3, ζ family
Surface molecules	CD19, CD22, FcR	CD4, CD8, CD2, CD28
Principal site of development	Bone marrow	Thymus
Effector function	Humoral immunity: antibody secretion	Cell-mediated immunity: cytokine production, cytolysis regulatory role

Figure 23-1 Characteristics of B and T lymphocytes. Both B and T lymphocytes have rearranged immune system receptors. Immunoglobulin (Ig) molecules are expressed on the surface of cells, whereas T cells are characterized by expression of the clonotypic T-cell receptor complexed to CD3 chains. Other important differences are listed.

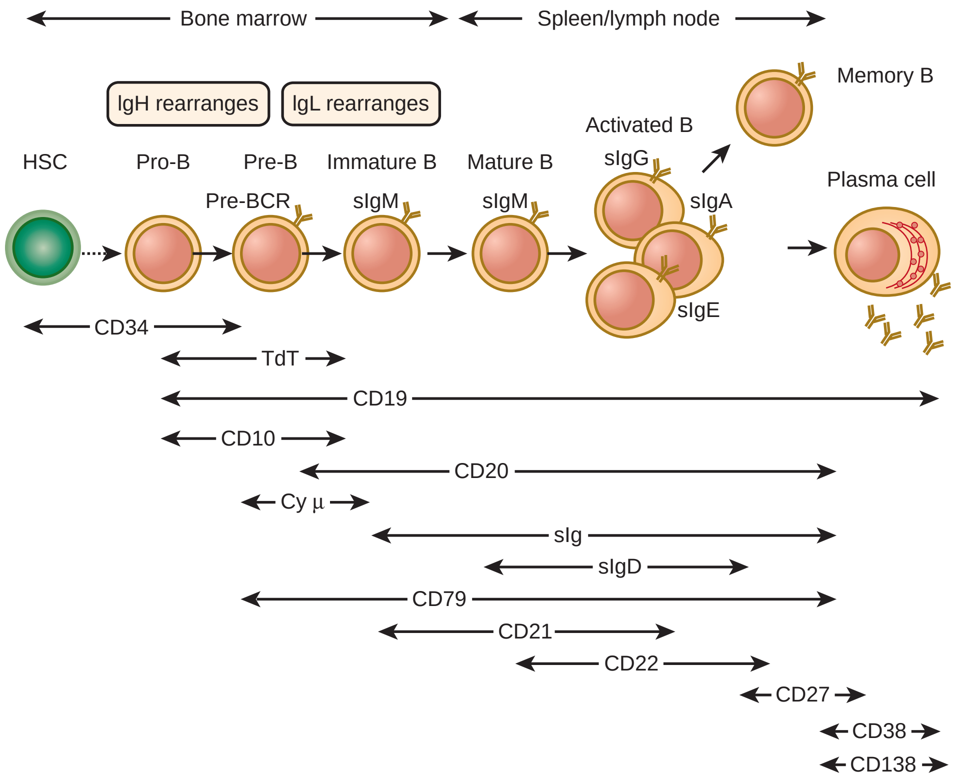


Figure 23-2 Overview of B-cell development. Pro-B cells develop from pluripotent hematopoietic stem cells (HSC) in bone marrow and acquire cell surface markers such as the pan-B-cell marker CD19, CD10, and terminal deoxynucleotidyl transferase (TdT). Serial rearrangement of the heavy- and light-chain loci drives expression of first the pre–B-cell receptor (pre-BCR) and later the B-cell receptor or surface IgM (sIgM). Downregulation of early markers at the pre-B stage gives way to expression of markers of immature and mature B cells, such as CD20, CD21, and CD22. Activation of the B cell with appropriate T-cell help leads to isotype switching and the expression of either IgA, IgG, or IgE. Switched B cells that form memory cells are characterized by expression of CD27 and downregulation of IgD, whereas activated B cells that proceed to the plasma cell stage lose most B-cell markers, upregulate CD38 and CD138, and secrete high levels of soluble specific antibody.

Elimination of B cells whose receptors have high affinity for self-antigens is important to prevent autoreactivity. Finally, immature B cells released from the bone marrow complete their development in the spleen and differentiate into at least two distinct lineages of mature naïve B cells: follicular B cells and marginal-zone B cells.

Immunoglobulin Structure

The structures of antibody molecules all share remarkable conservation of secondary conformation. The Ig-like fold is a domain shared among diverse proteins, each with signature hydrophobic common cores and common disulfide bridges. The antibody molecule is the prototype member of the Ig supergene family, a group that includes not only Ig but also TCR, the MHC, and a number of costimulatory molecules. Each Ig molecule is composed of two light chains of approximately 25 kd and two heavy chains of approximately 50 or 70 kd (Fig. 23-3); each chain is composed of multiple Ig domains. B-cell antibodies can be subdivided by heavy-chain isotype and further subdivided by subclass (Table 22-2).

There are five classes of antibodies—IgM, IgG, IgA, IgD, and IgE—defined by expression of the heavy chain: μ, γ, α, δ, and ε. The Ig-heavy chains contain sites for binding of Fc receptor (FcR) and complement, and each subtype appears to have a particular role in immune defense. The first class of antibody to be produced during development is IgM, which is expressed on the surface as a monomer and secreted as a pentamer. B cells that have undergone class switching (discussed later) express IgG, IgA, IgD, or IgE on the surface as the BCR or secrete a splice form of the Ig with the same heavy-chain isotype and specificity but lacking a transmembrane region (or both). The function of IgD in humans is not clear, although its expression is a marker for later B-cell development. IgA is secreted as a dimer and is found at mucosal surfaces such as the intestine. The bulk of circulating Ig is in the form of IgG, which is monomeric and further subdivided into four subclasses: IgG1, IgG2, IgG3, and IgG4. Persistently low serum levels of one or more IgG subclasses,[13,14] in the presence or absence of IgA deficiency,[15] may cause individuals to be susceptible to recurrent infections.[16] Though controversial, prophylactic use of intravenous Ig has been suggested.[17,18]

There are two subclasses of light chains: κ and λ (see Fig. 23-3). Both subclasses appear to serve similar functions. Monoclonal disorders of terminally differentiated mature B cells or plasma cells (e.g., multiple myeloma) express cells of a given light chain—that is, either κ or λ, but not both.

The structure of each Ig molecule is determined by hydrophobic interactions and disulfide bridges between

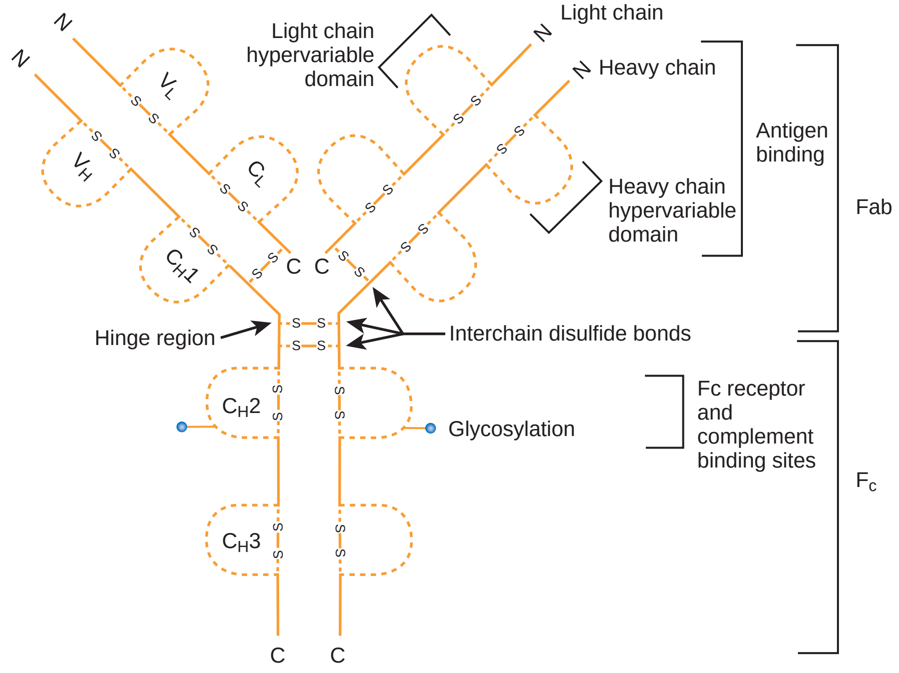

N
N
Light chain hypervariable domain
Light chain
N
N
Heavy chain
V_L
V_H
C_L
C_H1
Antigen binding
Fab
Heavy chain hypervariable domain
S–S
C C
Hinge region
Interchain disulfide bonds
C_H2
Glycosylation
Fc receptor and complement binding sites
F_c
C_H3
C C

Figure 23-3 Diagrammatic representation of the immunoglobulin (Ig) molecule. The Ig molecule has two light chains of approximately 25 kd, each with two Ig domains (C_L and V_L), and two heavy chains of 50 to 70 kd, each composed of four Ig domains (C_H1, C_H2, C_H3, and V_H). The antigen binding region is defined by the variable regions of the heavy and light chains (V_H and V_L). There are five subclasses of the heavy chain: IgG, IgA, IgM, IgD, and IgE.

the two heavy chains and two light chains (see Fig. 23-3). For each chain the N-terminal domain has a variable or polymorphic amino acid structure (V_H for heavy chain, V_L for light chain), whereas the C-terminal portions are constant within each heavy-chain or light-chain class (termed C_H1, C_H2, C_H3 for heavy chain and C_L for light chain). These domains form pairs, V_H with V_L, C_H1 with C_L, and C_H3 with C_H3. Proteolytic digestion of Ig by papain results in two Fab fragments, each composed of the V_H/V_L, C_H1/C_L domains, and an Fc fragment composed of the two C_H2 and two C_H3 domains. The binding region of Ig that recognizes antigen is composed of portions of the V_H and V_L domains. Within each variable region there are three portions with increased amino acid variation, called *hypervariable regions* (HV1, HV2, HV3) or complementarity-determining regions (CDR1, CDR2, CDR3). The highest variability is found in CDR3, and all three CDRs from both chains participate in the binding of antigen. Variability within each variable region and each CDR and pairing of a unique heavy chain with a unique light chain all contribute to the combinatorial diversity of Ig specificity.

Immunoglobulin Rearrangement and B-Cell Maturation in Bone Marrow

The genes encoding the heavy chains of Ig are located on human chromosome 14, whereas the genes encoding κ and λ light chains are located on chromosomes 2 and 22, respectively. The heavy- and light-chain loci are each composed of variable (V), diversity (D), and joining (J) gene segments that recombine to yield the V_H and V_L domains, which in turn form the antigen contact surface of the antibody molecule and confer specificity. In addition, the heavy-chain locus contains nine heavy-chain constant (C) region genes (μ, δ, γ1, γ2, γ3, γ4, α1, α2, and ε constant regions) that define the mature Ig isotype.

Ig rearrangement occurs serially, and cells failing to rearrange the Ig genes successfully are eliminated and do not develop further (Fig. 23-4). B-cell maturation also results in cell surface protein expression patterns that are used to define the pro–B-cell, pre–B-cell, and immature B-cell stages of development. CD34+ CD19– CD10– progenitors that have not yet committed to the B lineage have Ig genes in the germline configuration. The earliest B-cell precursors express the pan B-cell marker CD19 and the early marker CD10. They also express components of the rearrangement machinery such as recombination-activating genes (RAGs) and thus have early gene rearrangements detectable. Rearrangement begins with one heavy-chain allele; one of the 29 D_H exons is spliced to one of the 6 J_H exons, the intervening D and J sequences are eliminated, and a DJ segment is produced. The pro-B cell then splices one of the approximately 50 V_H exons to the DJ segment to form a V(D)J segment. Finally, the μ constant region of the heavy chain (C_μ) is spliced onto the V(D)J segments.

Differentiation to the pre-B stage is triggered by expression of intact μ heavy-chain protein or cytoplasmic μ, which occurs only in cells that bear in-frame productive V(D)J-C_μ rearrangements. If rearrangement of the first heavy-chain locus is unsuccessful, the second allele undergoes rearrangement. Because the light-chain genes have not yet been expressed, cytoplasmic μ complexes instead with the nonpolymorphic surrogate light-chain proteins λ5 and VpreB. Cytoplasmic μ, surrogate light chain, and the accessory signaling molecules Ig-α (CD79a) and Ig-β (CD79b) together form the pre–B-cell receptor. Ensuing signals induce proliferation and instruct the cell to begin rearrangement of one of the κ

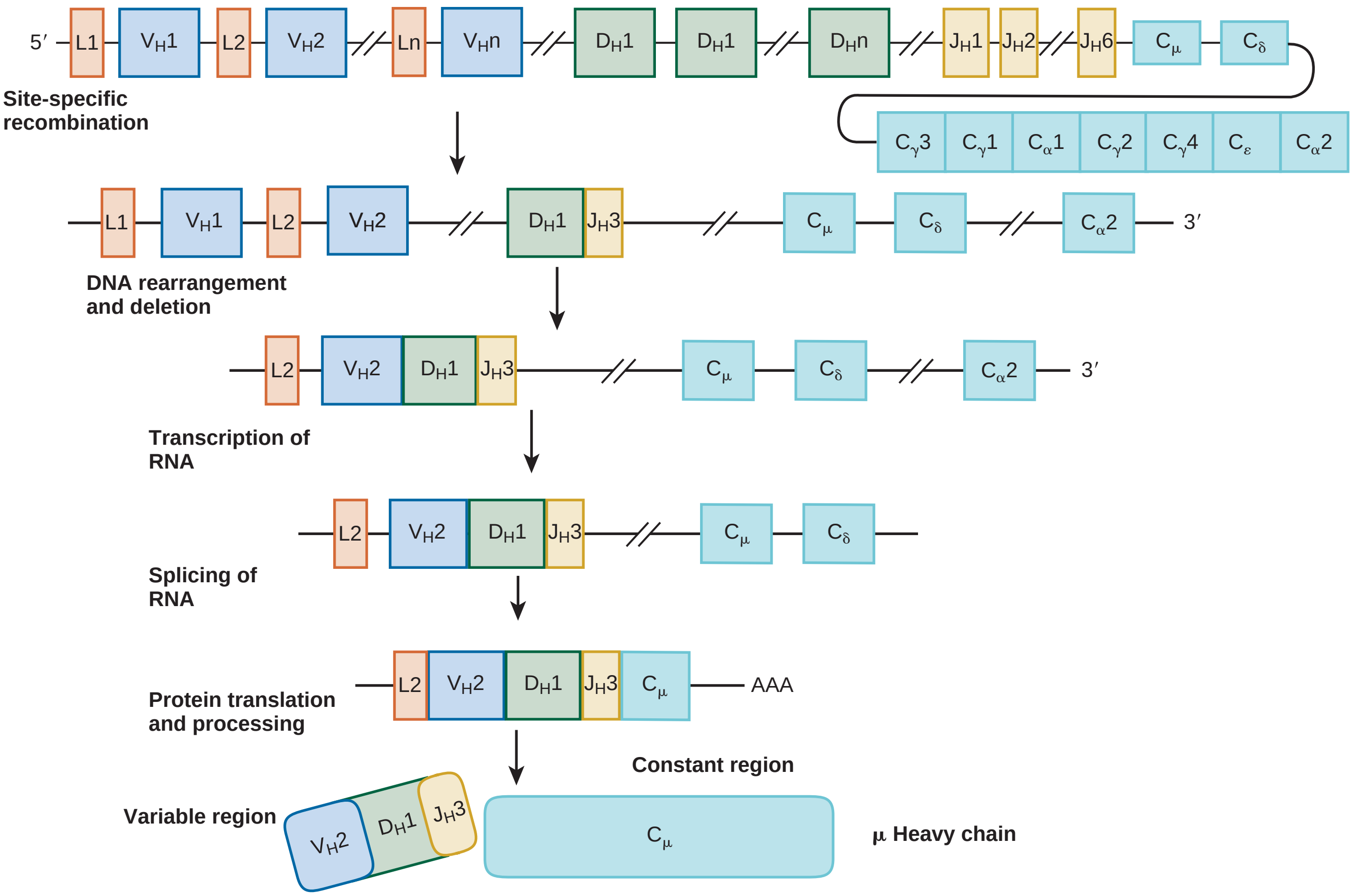


Figure 23-4 Immunoglobulin rearrangement. Immunoglobulin genes are encoded on human chromosome 14. Approximately 100 variable (V) genes, 50 diversity (D) genes, and 4 to 6 joining (J) genes make up the heavy-chain variable locus. The constant region consists of nine constant (C) genes. During immunoglobulin gene rearrangement, gene segments are deleted so that specific regions are joined to form one of many individual and characteristic genes.

light-chain loci. Pre-B cells lose CD34 expression, continue to express CD19 and CD10, and additionally upregulate CD20. The signaling cascade downstream of the pre–B-cell receptor is highly analogous to that of the mature BCR and activates a number of critical kinases, including Bruton tyrosine kinase (Btk) (discussed later). Boys born with mutations in Btk, manifested as X-linked agammaglobulinemia, have normal numbers of pro-B cells but lack pre-B cells and their progeny.[19] Thus formation of the pre–B-cell receptor along with activation of downstream signaling is a critical checkpoint in this stage of maturation.

Light-chain genes rearrange by a process similar to the recombination of heavy-chain genes. The variable region of the κ gene, located on chromosome 2, is spliced to the J gene segment to produce a VJ region, which then splices to the one constant region (C_κ). If nonproductive, the second κ locus will rearrange. If both attempts to rearrange the κ locus are unsuccessful, the λ locus, located on chromosome 22, will begin to rearrange. Productive rearrangement of any light-chain locus prevents further B-cell rearrangement and also generates survival signals. Pre-B cells that successfully rearrange a light-chain gene will then express mature IgM protein, which appears as surface IgM (sIgM) complexed to Ig-α and Ig-β, thus forming the BCR. Immature B cells are sIgM+, CD79+, CD19+, CD10+, and CD20+, but they have not acquired IgD expression, which occurs after peripheral maturation.

Expression of the tissue-specific recombination-activating genes RAG-1 and RAG-2 is required for recombination.[20,21] In addition, diversity is enhanced by the fact that the junctions between the D and J regions and the V and DJ regions are not precise; not only can the nucleotides vary, but one or more nucleotides (termed *N regions*), not encoded in the genome, may also be randomly inserted (N-region diversification). The nuclear enzyme terminal deoxyribonucleotidyl transferase (TdT) is the polymerase that adds random nucleotides to recombination junctions[22] and may have other roles.[23] Recruitment of TdT to the junctions appears to depend on the Ku molecule (specifically Ku80), a heterodimer that binds DNA ends and is required for V(D)J recombination and DNA double-stranded break repair.[24,25] Other proteins critical for nonhomologous end rejoining include Ku70, DNA-dependent protein kinase, DNA ligase IV, XRCC4, and the recently cloned protein Artemis.[26] Defects in the recombination machinery in genes such as RAG1, RAG2, DNA ligase IV, and Artemis all result in severe combined immunodeficiency (see Chapter 24).[27-29]

Peripheral B-Cell Maturation and Differentiation

Immature B cells must undergo further maturation in the spleen before acquiring the phenotype and functions of a naïve mature B cell. Naïve mature B cells are resting cells in G_0 phase; express the pan B-cell marker CD19, the marker CD20, and sIgM; and in contrast to immature bone marrow B cells, have lost CD10 expression and gained IgD expression. A similar population in the mouse is believed to be the common precursor for both follicular and marginal-zone B cells, named for their location within the spleen, discussed later.[30]

The development of early B precursors into immature B cells is a process that requires Ig rearrangement and expression of the pre–B-cell receptor and BCR, but it does not require recognition of antigen. Maturation to the naïve resting B cell involves selection of B cells that recognize antigen (positive selection) while eliminating B cells whose BCR recognize self-antigens with high affinity (negative selection). It has been proposed, on the basis of murine data, that determination of mature B-cell fate relies on the relative signal strength and specificity of the BCR, with higher and lower signal strength driving to the follicular and marginal-zone lineages, respectively.[30] Negative selection in particular is important for the deletion of potentially autoreactive clones and induction of B-cell tolerance. Indeed, it is estimated that 55% to 75% of immature B cells in humans recognize self-antigens,[31] and thus a minority of developing B cells pass the negative-selection test.

T-CELL ONTOGENY

T lymphocytes, like B lymphocytes and all other blood cells, are derived from self-renewing pluripotent HSCs. T cells are unique, however, in that their development also requires passage through a specific organ, the thymus. Indeed, the absolute requirement for the thymus is made clear by human congenital immunodeficiency states such as the DiGeorge syndrome, in which thymic organogenesis and therefore T-cell development are absent (see Chapter 24). Similar to B-cell ontogeny, the generation of millions of diverse T cells is accomplished by serial rearrangement of germline genes encoding the receptor for antigen recognition, the TCR. The bulk of peripheral T cells are of either the CD4+ helper or the CD8+ cytolytic lineages.

T-Cell Receptor Subtypes and Specificity

The TCR is a cell surface receptor capable of recognizing discrete antigens bound to the MHC or to MHC-related molecules expressed on the surface of APCs. TCRs are disulfide-linked heterodimers of either αβ or γδ proteins.[32-34] TCR αβ–bearing T cells constitute the vast majority of circulating T cells, most of which recognize peptide antigens bound to polymorphic MHC class I or class II molecules. A subset of TCR αβ T cells, representing approximately 1% of all T cells in humans, in contrast recognize lipid antigens bound to the MHC class I–related nonpolymorphic molecule CD1 and are termed CD1-restricted or NK T cells, discussed in a later section. TCR γδ–bearing T cells account for a tiny fraction of the circulating T cells in humans but are very prevalent in intestinal tissue and are thought to recognize nonpeptide antigens (discussed later). The functions of CD1-restricted TCR αβ T cells and TCR γδ T cells are somewhat distinct from those of conventional TCR αβ T cells. The discussion of intrathymic T-cell development in a subsequent section will focus on the major conventional subset of T cells.

Early T-Cell Commitment and Seeding

The molecular events associated with and responsible for commitment to the T lineage have been increasingly elucidated in both murine and human models over the last 10 years. The question of whether T-lineage commitment occurs within the thymus or the bone marrow has been actively investigated. In addition to pluripotent CD34+ HSCs, a number of other more committed progenitors in bone marrow with specific cell surface marker characteristics have been identified with T-lymphoid potential, primarily in the mouse, but these progenitors in general also retain the capacity to differentiate into other lymphoid lineages, including the B and NK lineages.[35] That various subsets identified in the human thymus similarly retain bilineage potential (i.e., NK/T potential or NK/dendritic cell potential) has led to the notion that the final steps in T-cell commitment occur in the thymus rather than the bone marrow.[36]

In addition to losing the capacity to differentiate into non–T-lineage cells in vitro, T-lineage commitment is characterized by the upregulation of certain cell surface markers (Fig. 23-5) and T-cell–specific genes important for the execution of downstream developmental programs.[37-39] CD34+ cells in human thymus can be further subdivided by the expression of CD38 and the MHC-like molecule CD1a, with progression from the CD34+ CD38– CD1a– stage to the CD34+ CD38+ CD1a– stage to the CD34+ CD38+ CD1a+ stage. Acquisition of CD1a+ is largely associated with the loss of NK, dendritic cell, and plasmacytoid dendritic cell differentiation in vitro.[36] Likewise, CD1a+ cells express the recombinase-activating gene products RAG1 and RAG2, and early TCR rearrangements are detectable after the acquisition of CD38 and CD1a. Transcription factors upregulated during these stages of early human T-cell commitment, whose requirement for T-cell development have also been demonstrated in gene-deficient mice, include GATA3 and Notch.[40-43]

Interactions between thymocytes and epithelial cells are critical for events in later thymic development, such as positive and negative selection, as detailed later, as well as for early commitment and development. The so-called *nude mouse*, which lacks expression of the epithelial transcription factor *Foxn1*, is born athymic, and humans lacking *FOXN1* have severe T-cell deficiency, alopecia, and nail dystrophy.[44,45] Unlike B cells, which can be induced in vitro to mature from precursors by co-culture on bone marrow stromal cells, T cells generally cannot be differentiated in vitro by this means, only when seeded into a three-dimensional source of primary thymic stroma, such as whole thymic organ culture. This distinction

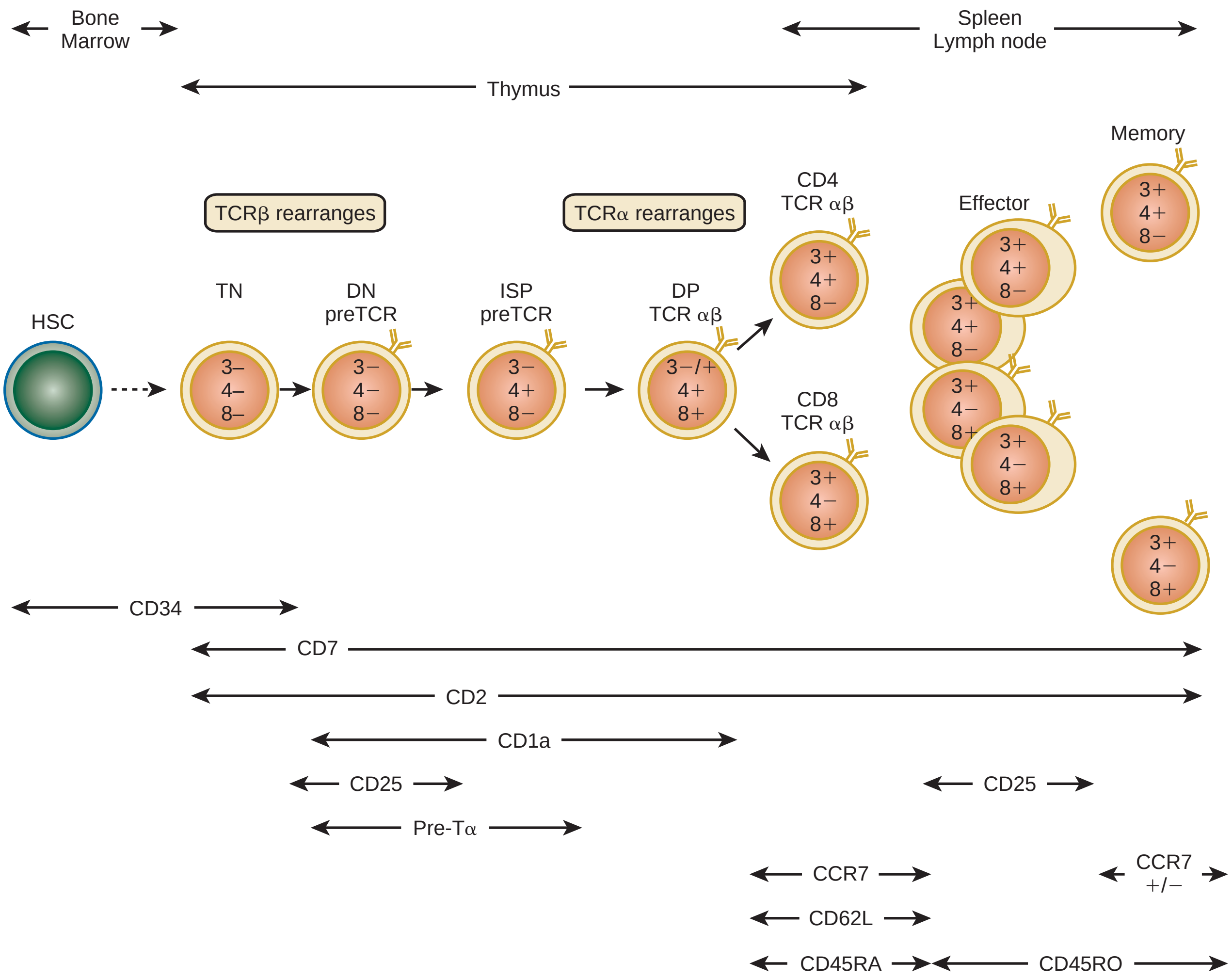
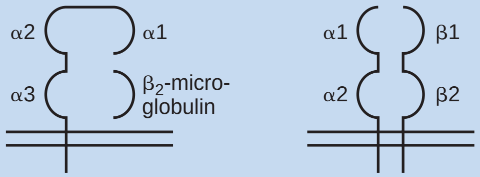


Figure 23-5 Overview of T-cell development. Pluripotent hematopoietic stem cells (HSC) in bone marrow commit to the T lineage, seed the thymus as early triple-negative CD3– CD4– CD8– (TN) thymocytes, and express the T-cell markers CD7 and CD2. T-cell receptor genes rearrange serially (TCRβ then TCRα), which leads to expression of the first pre–T-cell receptor (pre-TCR) and then the mature TCR αβ complex. (The CD3 components of the pre-TCR and TCR are omitted for simplicity.) Double-negative CD4– CD8– (DN) thymocytes upregulate both CD4 and CD8 after passing through an intermediate single-positive (ISP) stage to become double-positive CD4+ CD8+ (DP) thymocytes, which undergo positive and negative selection. During lineage commitment, either CD4 or CD8 is downregulated, thereby resulting in the formation of single-positive (SP) thymocytes with CD4+ thymocytes restricted to major histocompatibility (MHC) class II and CD8+ thymocytes restricted to MHC class I. Mature T cells in the spleen and lymph node that have not encountered cognate antigen express markers associated with the naïve state such as CD45RA and CD62L, and the pattern of surface markers changes with activation, acquisition of effector function, and further differentiation into memory T cells.

implies that specific soluble or cell-cell contact factors present only in the three-dimensional primary organ are required to engage developing thymocytes and induce their differentiation. The Notch signaling pathway, which is activated by the interaction of Notch ligands on epithelial cells and Notch family members on thymocytes, has emerged as one such factor.[46,47] Expression of activated Notch1 in human HSCs is sufficient to induce T-cell development in vitro and in immunodeficient mice,[48] and deletion of Notch1 from mouse thymocytes severely impairs early T-cell development.[49] Interestingly, primary three-dimensional thymic stroma expresses Notch ligands such as Delta-like 1 and 4, and this expression is lost when the architecture is disrupted.[50] Indeed, expression of Delta-like 1 or 4 in the OP9 bone marrow stromal cell line is capable of directing mouse and human uncommitted HSCs and even embryonic stem cells into the T lineage when co-cultured in a two-dimensional format in vitro.[46,51,52] This powerful system underscores the importance of thymocyte-epithelial interactions during intrathymic development and raises the intriguing possibility of differentiating and expanding T cells in vitro for use in human therapeutics,[53] which has been pursued in murine systems[54] and recently in expansion of umbilical cord blood units to expand access to this source of therapeutic stem cells.[55]

Stages of Intrathymic T-Cell Receptor αβ T-Cell Development and Spatial Considerations

T-lymphoid precursors that enter the thymus from the blood mature through a series of ordered developmental stages characterized by changes in cell surface markers,



Structure	MHC class I	MHC class II
	α chain + β_2-microglobulin	α + β chains
Molecular weight (Kd)	42 (α) + 12 (β_2m)	33 (α) + 29 (β)
Expression	All nucleated cells	Antigen-presenting cells, mono/macrophages, B cells, dentritic cells Thymic epithelial cells
Major loci	A, B, C	DR, DQ, DP
Minor loci	E, F, G, H	DM, DN, DO
	>100	>20
Peptide presented to	CD8+ T cells	CD4+ T cells
Peptide bound	8-9 amino acids	2-20 amino acids
Source of peptide	Endogenous antigens	Exogenous antigens

Figure 23-6 Characteristics of major histocompatibility (MHC) class I and class II molecules. MHC class I molecules are single transmembrane molecules noncovalently associated with β_2-microglobulin, whereas MHC class II molecules exist as heterodimers. CD8+ T cells recognize endogenous processed peptides (length 8 to 11 amino acids) embedded in the MHC class I groove, whereas CD4+ T cells recognize exogenous peptides (length 12 to 20 amino acids) embedded in the groove formed by MHC class II molecules. The major and minor loci are listed, as are important differences between the two classes.

sequential expression of the TCR genes, and predictable migration from the cortex to the medulla (see Fig. 23-5). The earliest thymocytes reside in the cortex, are CD34+, express pan-T-cell markers such as CD2 and CD7, but lack the TCR-associated αβ heterodimer and CD3-associated subunits of the TCR complex and the helper and cytotoxic mature T-cell coreceptors CD4 and CD8.[37] Hence, these thymocytes are CD3– CD4–CD8–, or triple negative. During αβ T-cell development, triple-negative thymocytes that succeed in rearranging a functional TCRβ chain express TCRβ complexed to the invariant pre-TCRα protein.[39] Pre-TCR signaling in thymocytes promotes rapid expansion and differentiation into double-positive (DP) thymocytes expressing both the CD4 and CD8 coreceptors and initiates rearrangement of the TCRα gene. In humans, in contrast to mice, there is an additional intermediate stage in which thymocytes express only CD4 and not CD8 (intermediate single positive [ISP]). DP thymocytes account for approximately 80% to 90% of the total thymocyte number and can be found in the cortex and at the corticomedullary junction. Although the majority of DP thymocytes die by apoptosis, those that successfully undergo positive and escape negative selection survive, commit to either the CD4/helper or CD8/cytotoxic lineage, and migrate to the thymic medulla before terminal maturation and export to the periphery.

T-Cell Recognition of Peptide-Major Histocompatibility Complex Complexes

A hallmark of the cellular immune response is antigen specificity, which is conferred by the rearranged and selected TCR on the surface of T lymphocytes. The TCR recognizes processed fragments of foreign proteins embedded in MHC molecules. There are two forms of MHC molecules: MHC class I (the major determinants are HLA-A, HLA-B, and HLA-C in humans) and MHC class II (the major determinants are HLA-DR, HLA-DQ, and HLA-DP in humans) (Fig. 23-6).[56] MHC class I molecules are composed of a 42-kd transmembrane, polymorphic α chain encoded in the MHC and noncovalently associated with a 12-kd soluble, nonpolymorphic (non-MHC) β chain termed β_2-microglobulin. X-ray crystallographic analysis of HLA molecules in which peptide was embedded[56] has demonstrated that the first two domains of the α chain both form α helices that together form a cleft (Fig. 23-7).[57] This cleft forms the binding domain for peptide antigen and accommodates a 9– to 11–amino acid fragment in an extended conformation; residues that interact with the MHC and those that interact with the TCR can be defined.[58,59] Polymorphism within the MHC itself serves to ensure variation in the affinity of peptide binding; the extraordinary MHC polymorphism within the species further ensures that any single microbe is unlikely to mutate such that it is unable to bind all MHC molecules in the population and therefore escape T-cell recognition.[60-63]

MHC class II molecules are formed by the noncovalent association of two transmembrane glycoproteins, αβ, both of which are polymorphic and encoded in the MHC.[56,60] Solution of the structure of MHC class II molecules has shown that a peptide-binding cleft is formed by the first domains of each of the αβ chains; however, the ends of the cleft are open, unlike the situation in MHC class I molecules (see Fig. 23-7).[56] The peptide-binding cleft of MHC class II accommodates processed peptides that are 10 to 30 (mean of 14) amino acids in length.[64,65] As with MHC class I, the genetic polymorphism of MHC class II determines the affinity and specificity of peptide binding and T-cell recognition.[64-66] Although MHC class I molecules are expressed constitutively on all human nucleated cells, MHC class II molecules are constitutively expressed only on B cells, monocyte-macrophages, and dendritic cells. Expression of MHC class II proteins may be induced on the surface

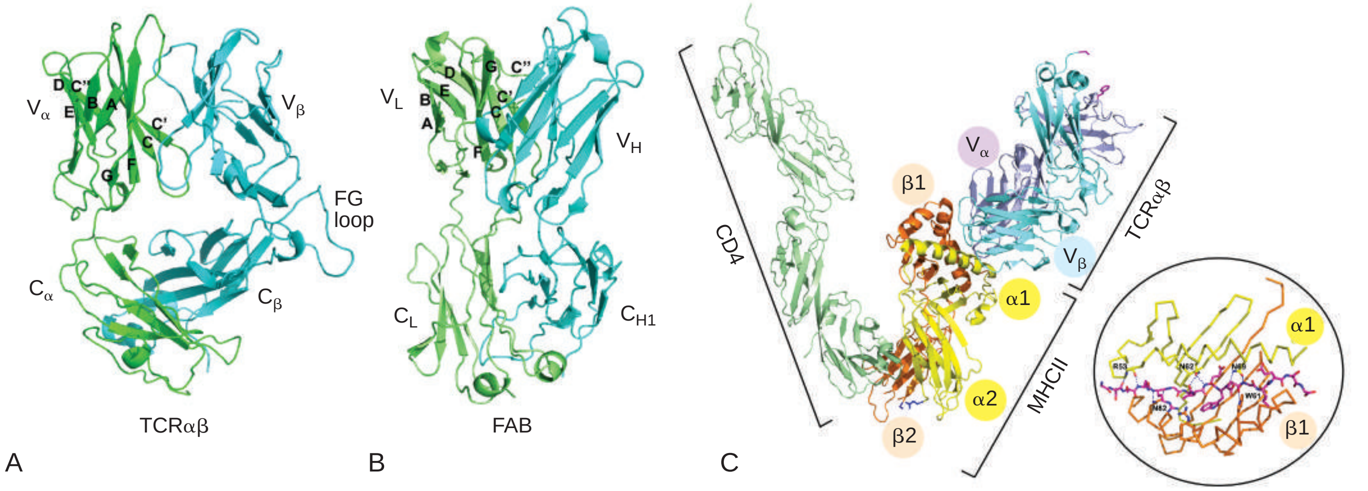


Figure 23-7 Comparison of the TCRαβ heterodimer with an immunoglobulin Fab and overall topology of the CD4/TCR/pMHCII ternary complex. **A** shows the N15 TCRαβ structure taken from the complex with the anti-Cβ FG loop Fab fragment of H57 antibody, the latter depicted in **B** as adapted from Wang et al.[268] The individual Ig-like domains are labeled, and the FG loop of Cβ indicated. The β strands of the Vα and VL domains are designated. Note that the TCRβ subunit and Fab heavy-chain fragment are shown in blue, and the TCRα subunit and Ig light chain are shown in green. **C** shows the overall orientation of the ternary complex (coordinates from PDB 3T0E). The representation shows the overall V-shaped CD4-MHCII architecture and how the MHC class II–restricted TCR must contact the MHCII molecule tangentially from the right in the figure. The TCRαβ will "bump up against the wall" of the α1 domain to provide a mechanical force facilitating mechanotransduction, as described in the text.[72] The *circular insert* shows a class II antigen-presenting platform with a peptide bound in the α1β1 groove. The peptide is in magenta and MHC sidechain to mainchain hydrogen bonds denoted. The view is from the TCR.

of monocyte-macrophages, fibroblasts, endothelial cells, and certain mesenchymal and epidermal cells by a variety of inflammatory mediators and cytokines such as IFN-γ. In humans but not in mice, MHC class II proteins can be inducibly expressed on T lymphocytes as well, thus rendering T cells capable of antigen presentation to other T cells. These two classes of MHC proteins generally interact with different classes of T lymphocytes: CD4+ T cells recognize peptide antigen in association with MHC class II molecules, whereas CD8+ T cells recognize peptide antigen in association with MHC class I molecules.[67,68] CD8 binds to invariant portions of the α3 domain of MHC class I and CD4 to a hydrophobic crevice between α2 and β2 domains[69-73] (see Fig. 23-7).

A growing number of drug allergies have been reported and shown to be a consequence of their drug binding to MHC molecules.[74-76] In general, peptides associate with HLA molecules by inserting parts of their amino acid residues into a set of six binding pockets in the HLA. The structure of these pockets is highly allele-specific, thereby dictating peptide-binding preferences for each HLA molecule. Remarkably, a recent study demonstrated that life-threatening drug-induced immune responses occur during abacavir treatment of HLA-B*57:01+ individuals as well as carbamazepine treatment of HLA-B*15:02+ patients through their binding to a specific pocket of the respective HLA molecules.[75] Drug binding shifts the repertoire of self-peptides normally complexed with that HLA molecule for surface display. As a consequence, self-peptides never before arrayed on the surface of cells within the patient's body are expressed and perceived by the T cell immune system as foreign. These led to the CTL-mediated destruction of such peptide arrayed target cells. It is likely that other drugs, environmental chemicals and toxins, and bacterial products function in a similar way, including interactions with class II MHC molecules. Given the immunologic basis of many cases of severe aplastic anemia (SAA), it is conceivable that the etiology of some or most SAA cases might be linked to such a mechanism.

T-Cell Receptor Rearrangement and Selection in the Thymus

Effective adaptive immunity relies on the random generation of a diverse repertoire of antigen-specific T cells, along with appropriate quality control measures to ensure that nonfunctional T cells are not needlessly allowed to mature fully. To achieve this goal, the TCR genes undergo somatic rearrangement in a serial fashion, and the growth and proliferation of developing T cells are tightly linked to signaling and transcriptional events downstream of functional TCR or TCR intermediates.

The four TCR gene clusters, α, β, γ, δ, are each composed of germline genes encoding discontinuous variable regions (Vα, Vβ, Vγ, Vδ), diversity regions (Dβ, Dδ), joining regions (Jα, Jβ, Jγ, Jδ), and constant regions (Cα, Cβ, Cγ, Cδ).[77] The TCRγ locus is situated on human chromosome 7, and the remaining three are located on human chromosome 14, with the TCRδ locus embedded between the V_α and J_α regions of the TCRα locus. The TCRβ germline genes consist of approximately 50 Vβ, 2 Dβ, 13 Jβ, and 2 Cβ genes, whereas the TCRα germline genes consists of at least 70 Vα, 60 Jα, and a single Cα gene. The TCRβ and TCRα loci have many more genes than the TCRδ or TCRγ loci do; however, the diversity of TCR γδ T cells is increased by the splicing of 2 D_δ regions to each other (VDDJ). Similar to the Ig genes in B cells, rearrangement of the TCR genes is carried out by

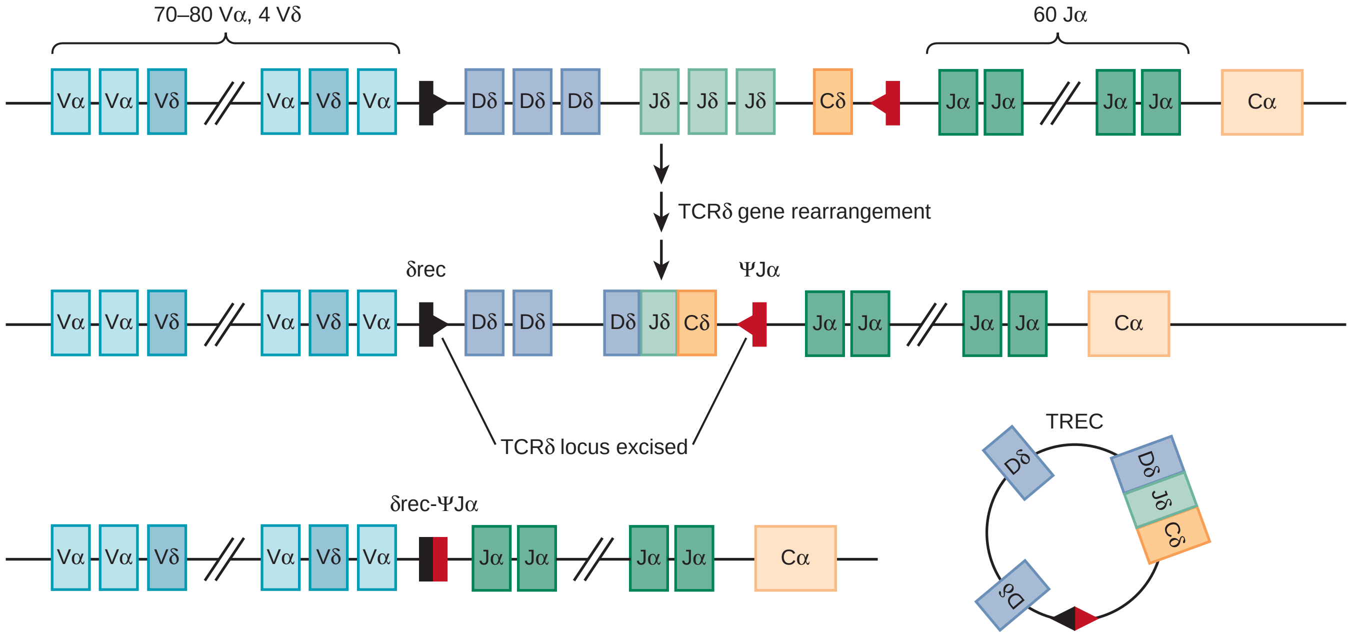


Figure 23-8 Formation of T-cell receptor excision circles (TRECs). The T-cell receptor α and δ (TCRα, TCRδ) loci are schematically depicted to show the basic rearrangement of variable (V), diversity (D), joining (J) and constant (C) regions. V_δ regions are interspersed between V_α regions, whereas the remainder of the TCRδ locus (D_δ, J_δ, C_δ) is embedded within the TCRα locus between the V_α and J_α regions. The *black box* and *red box* represent the δrec and ΨJα sequences, whereas the *black* and *red triangles* represent recombination signal sequences (RSSs) that recombination-activating gene (RAG) products recognize. The *first line* depicts the locus in the germline configuration. After TCRδ rearrangement, the D_δ, J_δ, and C_δ segments are randomly recombined. Thereafter, if the cell undergoes TCRβ and TCRα rearrangement, joining of δrec to ΨJα excises the TCRδ locus and results in an episomal circle of DNA containing the fusion of RSS signals. Polymerase chain reaction primers specific for this joining region can thus be used to detect the presence of the excision circle, an indicator of active rearrangement and T-cell neogenesis.

the RAG1 and RAG2 proteins, with serial somatic rearrangements between the V, D, J, and C regions. Although N-region diversification is found in both the TCRα and TCRβ genes, T cells do not undergo somatic mutation as B cells do and instead undergo limited further rearrangements after emerging from the thymus.[78]

Several major checkpoints regulate the development of TCR αβ and TCR γδ thymocytes.[79-82] First, serial rearrangement of the TCR gene clusters in an ordered fashion ensures that the TCRβ and TCRα genes are not rearranged in developing TCR γδ T cells. Thus in human thymocytes TCRγ and TCRδ loci rearrange first, during the triple-negative stage.[40] That human TCR γδ T cells generally have unrearranged TCRβ genes and that TCR αβ T cells generally have nonproductively rearranged TCRδ genes implies that only thymocytes that do not generate a functional γδ TCR go on to rearrange the TCRβ gene.[83] The molecular mechanisms controlling this process are still being elucidated. The second or so-called *beta selection checkpoint* occurs after TCRβ rearrangement. Cells that undergo productive rearrangement of the TCRβ locus express functional TCRβ protein that complexes to the invariant pre-TCRα protein and CD3 signaling complex, thereby resulting in the expression of pre-TCR. In gene-deficient mice, where beta selection is known to occur during a precise phase of the triple-negative stage of development, loss of a number of signaling molecules or transcription factors downstream of pre-TCR results in defective beta selection, failure of differentiation into DP stage, and lack of proliferation or apoptosis (or both).[84] Beta selection in humans begins during the CD34+ CD38+ CD1a+ stage and continues through the CD4 ISP and early DP stage of development (see Fig. 23-5).[40,83] Thus only thymocytes that express functional TCRβ are allowed to proliferate and proceed to TCRα locus rearrangement.

Before TCRα gene rearrangement, the TCRδ locus is excised by a nonproductive rearrangement between the δRec and ψJα regions[85] that generates an episomal circle of DNA or T-cell receptor excision circle (TREC), which is increasingly being used on both a research and a clinical basis to quantify thymic activity (Fig. 23-8).[86,87] The utility of TREC as a marker of new T cell generation has recently been used to detect severe combined immunodeficiency (SCID) in newborn dried blood spots,[88-90] and universal screening for SCID with TREC was endorsed for addition to the Recommended Universal Screening Panel in the United States by Secretary of Health and Human Services Kathleen Sebelius in May 2010.[91] Unlike the TCRβ locus, rearrangement of the TCRα locus occurs processively; that is, Vα-Jα recombination of 5’ J segments that are nonproductive or out of frame is followed by further rearrangement on the same allele to more 3’ J segments.[92] This process increases the chances of generating a complete TCR αβ protein from any single developing DP thymocyte. Successful rearrangement of the TCRα gene results in expression of the mature TCR αβ heterodimer and noncovalent association with CD3εγ, εδ, and ζζ dimer. This TCR complex appears during the late DP stage. These cells then undergo positive and negative selection (see the next section) and simultaneously commit to either the CD4 or the CD8 lineage to generate CD4 single-positive (SP) and CD8 SP thymocytes (see Fig. 23-5).

Positive Selection, Negative Selection, and CD4/CD8 Lineage Commitment

The massive proliferation of DP thymocytes results in TCR αβ thymocytes with an immense range of specificities, including many that are incapable of binding host MHC, cannot recognize host MHC complexed to endogenous peptide ligands, or have inappropriately high affinity for MHC–self-peptide and hence are autoreactive. Selecting only thymocytes with intermediate MHC–self-peptide affinity (so-called Goldilocks or just-right conditions) is critical for maintaining diversity of the repertoire while promoting self-tolerance.[93]

Positive selection is the term used to describe the process of selecting thymocytes capable of recognizing host MHC complexed to self-peptides generated from the processing of endogenous proteins. DP thymocytes are exquisitely prone to cell death in response to γ-irradiation, corticosteroids, exposure to anti-CD3 antibodies, or simply removal of the thymus from the body, and thus a positive signal via TCR-MHC/peptide interactions is required to rescue the small number of DP thymocytes destined to become SP thymocytes from cell death. Gene-deficient mouse experiments have demonstrated that multiple elements of TCR signaling are required for optimal positive selection, including TCR components (TCRα, CD3δ), early signaling molecules (GADs, ZAP70), late signaling proteins (RasGRP, calcineurin, ERK), and transcription factors (nuclear factor of activated T cells [NF-AT], Egr family members).[92,94-96] Interestingly, humans deficient in CD3δ and ZAP70 have been described who have discrepant development when compared with the knockout mouse counterparts. Deficiency of CD3δ in humans causes an earlier block in development at the CD4− CD8− stage, well before the development of DP thymocytes.[97,98] ZAP70 deficiency results in selective deficiency of CD8 SP thymocytes and mature CD8+ T cells.[99-101] Likewise, although in the mouse interaction of DP thymocytes with MHC on radioresistant cortical thymic epithelium is probably the dominant requirement for positive selection, the source of MHC and the relative contributions of MHC from thymic epithelium, thymic dendritic cells, or other thymic APCs to positive selection in humans are less clear.

Negative selection is the term used to describe the process of removing thymocytes that are self-reactive, also termed *central tolerance,* by clonal deletion.[102,103] This is the first and major mechanism for control of autoimmunity, although clones that escape deletion in the thymus are subject to peripheral tolerizing influences (discussed later). In mice negative selection can be mediated by cortical epithelium, medullary epithelium, and medullary dendritic cells. However, it was never clear how thymocytes reactive against self-antigens expressed only in peripheral tissues, such as islet cells, were selected against in the thymus. In other words, how are these tissue-specific proteins expressed in the thymus? More recently, murine and human studies have revealed that all three cell types, medullary thymic epithelial cells (mTECs) in particular, exhibit promiscuous gene expression and transcribe a broad range of tissue-specific, nonthymic antigens.[104] This insight helps explain how developing thymocytes reactive against nonthymic proteins can be centrally deleted during negative selection. A newly discovered gene, *AIRE* (autoimmune regulator), is a factor shown to control the transcription of tissue-specific antigen expression in murine mTECs.[105] Simultaneously, this gene was shown to be responsible for the autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) syndrome, characterized by chronic mucocutaneous candidiasis, hypoparathyroidism, adrenal insufficiency, and other organ-specific autoimmune manifestations.[106,107] Thus this disease may be the first example of failure of negative selection underpinning a human monogenic autoimmune disorder.

Maturation of CD4 and CD8 SP thymocytes from DP thymocytes occurs concurrently with positive selection and results in "matching" of coreceptor to MHC restriction with downregulation of the opposite coreceptor. The mechanisms controlling the CD4/CD8 lineage decision are separable from those governing positive selection[108] and have been the subject of active investigation in murine models.[109-112] Patients with defects in a number of genes that control MHC class II expression, including *RFXANK, RFX5, RFXAP,* and *CIITA,* all manifest the so-called *bare lymphocyte syndrome*, a rare autosomal recessive disorder characterized by decreased numbers of CD4+ T cells, deficient helper function, and absence of specific antibody production because of lack of CD4-mediated B-cell maturation.[113]

THE SPLEEN

Historical Perspective

A complete understanding of the adaptive immune system requires a discussion of the structure and function of the spleen, a critical secondary lymphoid organ with both immunologic and nonimmunologic functions. Since ancient times the spleen has occupied a mysterious niche among organs within the human body. Its proposed functions and necessity for good health were subjects of debate and discussion for centuries.[114] Hippocrates believed that the spleen helped balance the body's "humors," a process necessary to promote harmony and stability. Plato and Aristotle further suggested, on the basis of its location, that it assisted or balanced the liver. Galen noted the anatomic connections among the spleen, liver, and stomach and concluded that the spleen had true digestive functions that included the filtering of humoral impurities. Subsequently, the spleen gained a reputation for controlling feelings and emotions, including laughter, ill temper, melancholy, and others. Shakespeare often referred to the spleen as an organ of anger, merriment, or other strong emotion. During the Renaissance, when the study of anatomy and physiology became popular, the spleen's microscopic patterns supported a role for filtration; however, only circulatory connections between the spleen and the stomach, liver, and other digestive organs could be identified. Moreover, survival of both animals and humans after surgical splenectomy demonstrated that the spleen was not absolutely required for life. Only in the modern era, with the discovery of individual blood cells, have investigators begun to elucidate the true functions of the spleen. We now recognize that the spleen has vital roles in blood filtration and immunologic competence.

Anatomy and Physiology

The spleen can be identified in human embryogenesis during week 5 of gestation, although the development of characteristic splenic architecture with influx of red and white blood cells occurs much later in gestation. At birth the spleen weighs about 10 g, then 30 g at 1 year of age, 60 g by 5 years, and approximately 200 g in adults.[115] Located in the left anterior aspect of the rib cage, the spleen can have a triangular, crescent, or rhomboidal shape. Spleen size is usually estimated clinically by how far below the left costal margin the tip of the spleen can be palpated. Frequently, an enlarged spleen is most easily palpated at the midclavicular line, but it can extend prominently toward the midline or even to the midaxillary line in some patients.

The spleen receives 3% to 5% of the total cardiac output, thus making it a highly perfused organ.[116] Blood enters and leaves the spleen primarily through the splenic artery and vein, respectively, both located at the hilum on the concave (medial) side of the organ. Within the splenic parenchyma, the splenic arterial branches have little overlap, thereby allowing classification of the spleen into lobes and segments separated by relatively avascular planes.

Histologically, the spleen can be separated into two distinct regions, the red pulp and the white pulp, so named because of the blood cells most commonly found in each area. These two regions provide the vital functions of blood filtration and immunologic response, respectively, which are made possible through a unique circulation pattern found within the spleen. Figure 23-9 illustrates the arterial and venous circulations of the spleen and the close proximity of the red and white pulp.

The white pulp consists primarily of collections of lymphocytes and macrophages that form small white nodules (1 to 2 mm in diameter) distributed throughout the splenic parenchyma. As the largest arteries within the spleen branch progressively into the smallest arterioles, the white pulp can be recognized as a circumferential ring of leukocytes that surround the vessel, also known as the *periarterial* (or periarteriolar) *lymphoid sheath* (PALS). Whereas blood cells tend to continue within the vessel, plasma elements enter the PALS by way of tiny vessels that arise perpendicular to the flow of blood. Soluble antigens are thus herded into the white pulp, where they encounter dendritic cells and macrophages that function as professional APCs and allow proper immune stimulation of T and B lymphocytes.

The PALS consists primarily of T lymphocytes, and this cylindrical cuff of cells becomes thinner as the artery becomes smaller. Spheroidal lymphoid follicles arise at intervals from the PALS, usually where arterial branch points occur, and these follicles contain primarily B lymphocytes (Fig. 23-10). The primary follicles contain naïve mature B cells that co-express IgM and IgD; secondary follicles contain activated B cells within its germinal center (GC), all termed *follicular B cells*. Naïve follicular B cells in the primary follicle are CD27–, in contrast to memory B cells, discussed later, which express CD27.[117,118] In the mouse T cells congregate around the central arteriole in the so-called PALS studded by follicles and GCs containing B cells; the marginal zone delineates

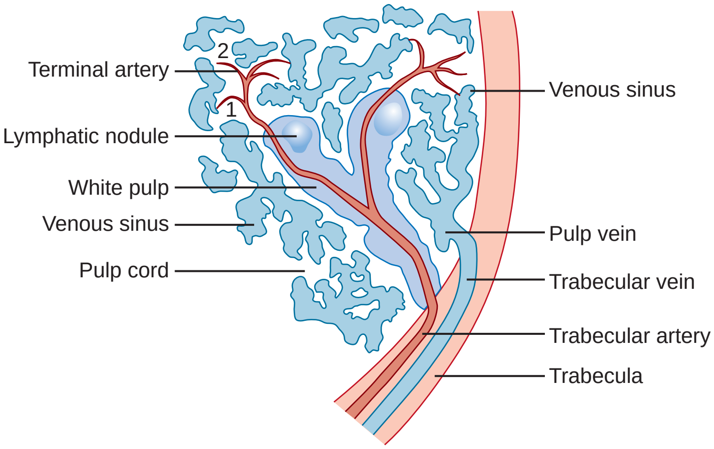


Figure 23-9 Blood circulation in the spleen. As blood enters the spleen, the arterial vessels are surrounded by lymphatic sheaths and nodules that constitute the white pulp. The majority of arterial blood terminates in the venous sinuses (red pulp), where filtration and quality control of erythrocytes take place. Erythrocytes return to the venous circulation via the venous sinuses. *(From Fawcett D:* A textbook of histology, *ed 11, Philadelphia, 1985, Saunders, p. 474.)*

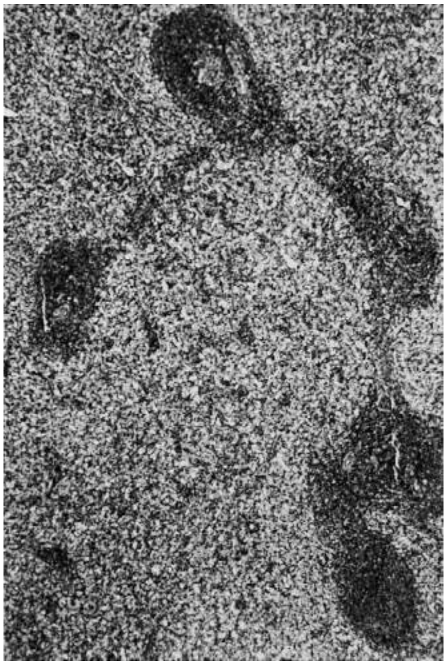


Figure 23-10 Lymphoid follicles in the white pulp appearing as budlike outgrowths from the periarteriolar lymphoid sheath. *(From Neiman RS, Orazi A, editors:* Disorders of the spleen, *ed 2, Philadelphia, 1999, Saunders, p. 10.)*

the white pulp from the red and surrounds both the follicles and PALS. In humans, by contrast, T cells and B cells tend to interdigitate, and the follicles are encompassed within the PALS. The marginal zone in humans is positioned around the follicles, does not envelop the PALS, and is not in direct contact with red pulp.[119] Marginal-zone B cells are IgM+ IgD– CD1c+ CD23– and thus are distinguished from follicular B cells (IgM– IgD+ CD1c– CD23+).[30] Additionally, marginal-zone B cells express high levels of CD21 and as a result are highly responsive to complement. Finally, in contrast to rodent marginal-zone B cells, which are sessile, marginal-zone-like B cells in humans can circulate in blood and have a memory phenotype, being IgM+ IgD+ CD27+.[120]

The red pulp represents a large open circulation within the spleen through which erythrocytes pass slowly for filtration and culling functions. The red pulp contains the sinusoids, which are lined by macrophages, and the splenic cords, which lie between the sinusoids and are composed of connective tissue, epithelial cells, monocytes, and macrophages. After plasma is redirected toward the white pulp, the blood cells that continue through the splenic circulation are mostly erythrocytes, and they form the red pulp as they exit the arteries. Occasionally, erythrocytes pass directly from the arterial to the venous circulation by way of traditional endothelialized capillaries, a process known as the *closed (rapid) splenic circulation*, which is primarily nutritive. Much more commonly, however, erythrocytes leave the arterial circulation and empty into large pools called the *splenic cords*, where they encounter macrophages and other immune effector cells. Erythrocytes must survive these intimate encounters in this hypoxic and acidic environment and then pass from the cords through an endothelialized barrier into the splenic sinuses and finally back to the venous circulation. This open (slow) circulation allows careful filtration of individual erythrocytes, with repair or phagocytosis of cells that cannot successfully negotiate the return trip.

Important Functions

The spleen provides several important immunologic functions. Skimming of plasma into the PALS allows particulate antigens to interact with dendritic cells and other APCs, which initiates the immune response for T- and B-lymphocyte response and proliferation. Polysaccharide antigens in particular require this unique immunologic environment for optimal antibody response.[121,122] Intravenous antigens, such as those found during infection or after immunization, are primarily processed by the spleen.[123] Not surprisingly, normal splenic function is required for optimal early (IgM) response to most intravenous antigens, as well as the secondary (IgG) response.[124] In contrast, antigens administered by the intramuscular or subcutaneous routes do not require normal splenic function for a robust immune response.[125,126] Finally, bloodborne bacteria, particularly encapsulated organisms, are removed by splenic macrophages located in the PALS and in the cords. Without a functioning spleen, patients are susceptible to overwhelming bacterial sepsis and exaggerated parasitemia from *Plasmodium, Babesia,* or other organisms.[127,128] The interaction between specialized T helper cells and follicular B cells in the GC is further described in later sections.

The spleen also has nonimmunologic functions. All RBCs pass through the spleen many times each day.[129,130] The spleen serves as an efficient filter for erythrocytes and is able to perform grooming (removal of antibodies or other surface molecules), culling (destruction of cells), and pitting functions (removal of intracellular material). While moving slowly through the hostile environment of the splenic cords, erythrocytes are carefully examined for abnormalities, a process constituting a biologic form of quality control. Macrophages in the red pulp identify abnormal erythrocytes, such as those that have IgG or complement on their surface, and either remove a portion of their membrane or fully engulf them. Newly made reticulocytes spend their first day or two in the spleen,[131] during which the cells appear to be groomed with removal of cytoplasmic organelles and surface adhesion molecules. Normal mature erythrocytes, in contrast, typically endure macrophage scrutiny without difficulty. To leave the red pulp, erythrocytes must then pass back from the splenic cords through potential spaces in the endothelial lining of the splenic sinuses. To negotiate these tiny apertures, known as the *cords of Billroth*, the erythrocytes must deform themselves substantially (Fig. 23-11). Cordal macrophages phagocytose erythrocytes that cannot deform themselves sufficiently, such as senescent cells; as erythrocytes age, they lose volume, gain density, and eventually lose the deformability that allows reentry into the splenic sinus. In certain pathologic conditions, such as immune-mediated hemolytic anemia, as well as a variety of erythrocyte membrane, hemoglobin, and enzyme disorders, younger erythrocytes will also be trapped in the splenic cords. Erythrocytes with intracellular inclusions, such as nuclear remnants known as *micronuclei* (Howell-Jolly bodies), denatured hemoglobin (Heinz bodies), siderotic granules (Pappenheimer

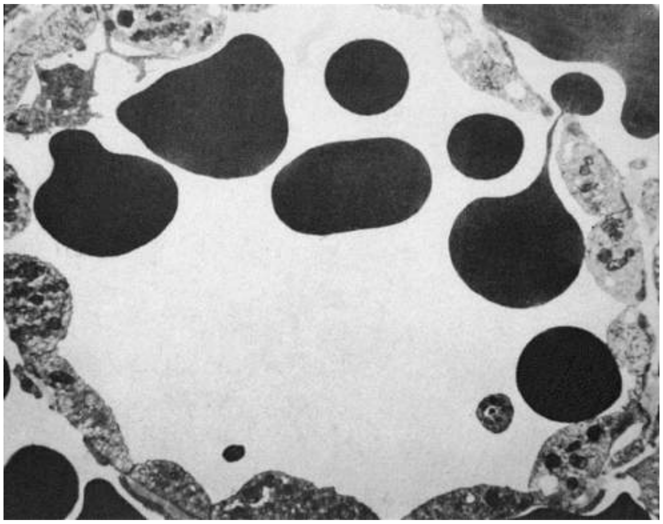


Figure 23-11 Deformation of erythrocytes returning from the splenic cords (red pulp) back into the venous sinus. The erythrocytes must pass through very small potential spaces in the sinus wall known as the *cords of Billroth*. *(From Neiman RS, Orazi A, editors:* Disorders of the spleen, *ed 2, Philadelphia, 1999, Saunders, p. 18.)*

bodies), and malarial parasites, have their inclusions "pitted" out before they pass through the endothelial slits and return to the circulation.

To a much lesser extent, the human spleen can also serve as a reservoir for circulating blood cells. Approximately 50 mL of blood is usually contained in a normal spleen, as well as 30% of the platelet mass. In the abnormal state (discussed later), the spleen can enlarge and trap a larger portion of the circulating blood cells, a condition known as *hypersplenism*. The spleen may also serve a small role in iron metabolism and homeostasis. Unlike the spleen in other species, however, it has no appreciable role in human hematopoiesis.

Abnormalities of the Spleen

Congenital Asplenia and Polysplenia

Congenital asplenia is found in children with the rare Ivemark syndrome, in which the body has bilateral "right-sidedness" with trilobed lungs and a centralized liver.[132] Cardiac defects are common, as well as a risk of infection.[133] In even rarer cases patients have congenital asplenia with no other abnormalities.[134,135] This resembles mice with a targeted deletion of Hox11 (also called *Tlx1*).[136] No defects in Hox11 have been discovered in humans with isolated congenital asplenia. Instead, about half of the patients have a heterozygous inactivating mutations in RPSA (ribsomal protein SA), a component of the small subunit of the ribosome.[137] How haploinsufficiency of a part of the ribosome, which is needed in all cells, specifically blocks development of the spleen remains a mystery. A heterozygous missense mutation in Nkx2-5 has also been found in one kindred.[138] This makes sense because Nkx2-5 interacts with Pbx1 and p15Ink4b to regulate spleen organogenesis in mice.[139]

A number of genes in humans and mice are linked to asplenia combined with multiple defects in other tissues. Examples include: Nkx2-3,[139] Nkx3-2 (or Bapx1),[140,141] Sox11,[142] Wt1,[143] Pbx1,[144] Tcf21 (or Pod1),[144] and HuR.[145] Interestingly, HuR interacts with a putative regulatory element in the Hox11 gene.[146]

Uptake of radionuclide by functioning splenic tissue can be difficult to document because the anatomic features are distorted and liver tissue can obscure any splenic uptake. Circulating erythrocytes with Howell-Jolly bodies or intracellular vesicles that appear as pits or pocks allow the diagnosis of asplenia to be established noninvasively.[147] Figure 23-12 illustrates the Howell-Jolly bodies that are found in the peripheral blood smear of an asplenic person. The converse arrangement of bilateral "left-sidedness" leads to congenital polysplenia with several spleens of varying size. In most patients the splenic tissue in polysplenia has normal function. Cardiac defects are common, as well as hepatobiliary abnormalities.[148,149]

Accessory spleens can be identified in 15% of normal persons.[150] In most of these individuals, a single accessory spleen measuring only 1 to 2 cm in diameter is present either in the splenic hilum or in the tail of the pancreas. Multiple accessory spleens can exist, however, as well as ectopic spleens in locations ranging from the abdomen to the pelvis and scrotum. The function of accessory spleens depends on the quality and quantity of blood flow; in most instances their filtration capabilities are minimal, but antibody responses may be present.

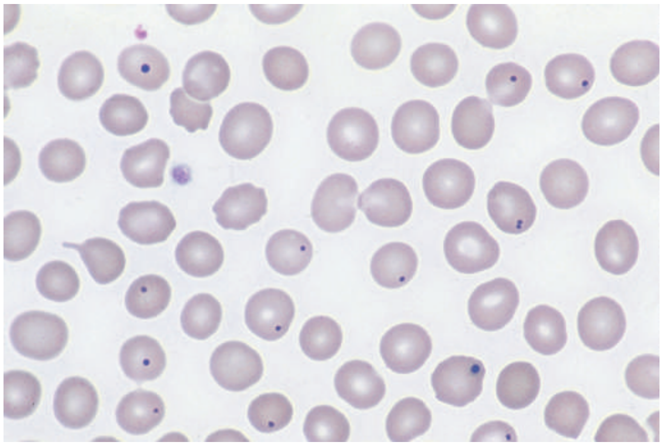


Figure 23-12 Howell-Jolly bodies in peripheral blood erythrocytes. These nuclear remnants are identified as small inclusions within circulating erythrocytes and have several characteristic and required features by light microscopy: (1) they are spherical inclusions 0.5 to 2.0 μm in diameter, about the size of a small platelet; (2) they have a smooth contour and no surrounding halo, which distinguishes them from platelets juxtaposed to an erythrocyte; (3) they are always in the same focal plane as the erythrocyte because they are intracellular; (4) they are never refractile, unlike talc or dust particles; and (5) they are homogeneous in appearance, unlike other intracellular inclusions such as Pappenheimer bodies or malarial parasites. Howell-Jolly bodies are normally removed when erythrocytes pass through the cords of Billroth into the venous sinus. Their presence in peripheral blood indicates lack of splenic filtrative function.

Splenosis and Splenoptosis

Splenoptosis, or wandering spleen, occurs when the spleen is not fixed within the retroperitoneum.[151] The resulting mass can be palpated anywhere in the abdomen and may require reattachment (splenopexy) or even splenectomy if symptoms of sequestration or torsion develop.[152] *Splenosis* refers to the autotransplantation of splenic tissue into the omentum or peritoneal surfaces of the abdominal cavity. Splenosis typically occurs after fracture or rupture of the spleen as a result of spillage and subsequent implantation of splenocytes. Although some protection against infection may be afforded by splenosis,[153] filtering functions and immunologic responses are limited by the relatively small total amount of splenic tissue and reduced blood supply.

Splenic Sequestration

Sequestration refers to enlargement of the spleen that occurs when blood enters the organ but is unable to exit properly. This condition is most often observed in children with sickle cell anemia when venous return of blood is hindered by intrasplenic sickling of erythrocytes within the red pulp. Splenic sequestration also occurs in other settings with abnormal erythrocytes, however, including congenital spherocytosis. Splenic sequestration can develop acutely with sudden pooling of blood within the spleen and result in rapid and painful expansion of the splenic parenchyma and capsule. Children exhibit pallor, fatigue, severe anemia, and occasionally hypovolemic shock.[154] Occasionally, splenic sequestration develops over a period of days or weeks and is then termed *subacute* or *chronic* because it is accompanied by relatively asymptomatic splenomegaly.

Hypersplenism

Hypersplenism refers to the condition of splenomegaly with non–immune-mediated trapping of peripheral blood cells and results in mild to moderate cytopenia.[155] Hypersplenism can develop in patients with abnormal erythrocytes, metabolic storage disorders, or other causes of chronic congestive splenomegaly. In these clinical settings, the spleen first enlarges because of progressive trapping of erythrocytes or storage material and then subsequently becomes a reservoir for all circulating blood cells. Typically, the peripheral platelet count is lower than normal, but the leukocyte count can also be low. If surgery is required, peripheral blood counts normalize after splenectomy.

Surgery Involving the Spleen

Although the spleen has important hematologic and immunologic functions, in some instances surgical removal is beneficial. A variety of congenital anomalies of the spleen, as well as numerous pathophysiologic processes, are indications for splenectomy.

Benign tumors of the spleen are relatively rare in childhood but primarily include congenital cysts (epidermoid or lymphangiomatous) or pseudocysts that result from liquefaction of splenic parenchyma after infarction or hemorrhage. These tumors may require splenectomy, depending on their size. Malignant splenic tumors are almost always lymphomatous or leukemic in origin, with cancerous cells found diffusely or in a nodular pattern throughout the splenic parenchyma. Perhaps somewhat surprisingly, very few solid tumors metastasize to the spleen. As a general guideline, splenectomy is not usually indicated in the setting of malignancy.

Splenomegaly requiring splenectomy can develop in a variety of clinical settings, including metabolic storage disorders such as Gaucher disease, transfusion-acquired iron overload from chronic erythrocyte transfusions, and splenic congestion resulting from portal hypertension. Commonly, splenectomy is the recommended therapeutic option for splenic enlargement in patients with primary erythrocyte abnormalities. Congenital hemoglobin, enzyme, and membrane disorders lead to trapping of the abnormal erythrocytes within the splenic cords and gradual expansion of the red pulp. In these settings splenectomy can relieve the signs and symptoms of splenomegaly, including abdominal discomfort, anemia, hypersplenism, and dependence on transfusions.

Total surgical splenectomy is generally performed by open laparotomy by way of a left subcostal incision. Laparoscopic splenectomy has become a popular and safe alternative in children[156,157] but requires careful attention and may be technically difficult with very large spleens. With the laparoscopic approach, small amounts of residual splenic tissue may be left or accessory spleens may be missed,[158] which can be a problem for patients with immune-mediated hematologic disorders. When splenectomy is recommended for autoimmune hemolytic anemia (AIHA) or immune thrombocytopenic purpura (ITP), all splenic tissue must be removed to prevent recurrent autoantibody production. Over the past decade subtotal (partial) splenectomy has become a viable therapeutic alternative to total splenectomy. The partial splenectomy approach is possible because the spleen can be divided into distinct lobes and segments.[159] Removal of 80% to 90% of the enlarged spleen usually provides relief from splenomegaly while retaining sufficient splenic tissue for immune competence. Partial splenectomy has been used successfully in children with nonparasitic splenic cysts,[160] hereditary spherocytosis,[161] homozygous β-thalassemia[162] and sickle cell disease,[163] although splenic regrowth may limit its long-term efficacy.[164] Even children with massive splenomegaly can successfully undergo partial splenectomy.[165] Animal studies suggest that a third of the normal spleen mass is required for normal plasma filtration and antibody formation.[166] Compared with splenic tissue reimplanted either by surgical autotransplantation or splenosis,[167,168] partial splenectomy provides superior retained immune function. Laparoscopic partial splenectomy combines these two new approaches and may represent an ideal option for selected patients who require surgical splenectomy; it is becoming more commonly available for children.[169,170]

The primary danger after total splenectomy is fatal bacterial sepsis, sometimes referred to as *overwhelming postsplenectomy infection (OPSI)*. First recognized more than 50 years ago,[171] bacteremia evolving rapidly into fatal sepsis with or without meningitis can abruptly develop in a patient without splenic function. Pathogens associated with OPSI are primarily encapsulated bacterial organisms and include *Streptococcus pneumoniae, Haemophilus influenzae* type b, and *Neisseria meningitidis*. The presumed pathophysiologic origin of OPSI is loss of plasma filtration by the spleen, which allows bacteria to multiply within the bloodstream and evolve from low-grade bacteremia to fulminant sepsis. Risk factors for OPSI include younger age at initial clinical evaluation, younger age at splenectomy, and failure to comply with prophylactic antibiotics or vaccinations. The true risk for the development of OPSI has been estimated in two large studies, although many of the patients were from the prevaccination era. First, in a literature review covering 35 years from 1952 to 1987 (5902 patients), the incidence of infection after splenectomy in children younger than 16 years was 4.4% with a mortality rate of 2.2%; for adults the corresponding values were 0.9% and 0.8%, respectively. Children younger than 5 years and especially infants had a much higher risk for sepsis.[172] In a later survey of 226 splenectomized patients with hereditary spherocytosis that included 5461 years of postoperative follow-up, the estimated mortality rate was 0.73/1000 years for the entire group, but somewhat higher for children.[173] The introduction of polysaccharide and now protein-conjugated vaccines has greatly helped decrease the incidence of pneumococcal bacteremia and sepsis and will help offset the lack of splenic filtration function for children undergoing splenectomy. However, even with the use of presplenectomy immunization, prophylactic antibiotics, and prompt medical attention for fever, a splenectomized child still has a finite risk of OPSI developing with substantial morbidity and mortality.

B-CELL ACTIVATION AND FUNCTION

An appropriately diverse population of naïve B cells should collectively be capable of responding to any soluble antigen that the individual encounters, yet there are very few naïve B cells that are capable of recognizing any given antigen. B-cell activation that occurs in the appropriate costimulatory context is the trigger for clonal expansion of the responding B cell. A portion of these activated B cells differentiate into memory cells, which rapidly respond on reencounter with the same antigen, and another portion into plasma cells, which continuously secrete antibodies (see Fig. 23-2). In addition, activated B cells undergo isotype switching from low-affinity IgM to high-affinity IgG, IgA, or IgE and somatic hypermutation, also called *affinity maturation*, thus further ensuring that the humoral response is exquisitely specific, effective, and long lasting. Recognition of antigen by the BCR occurs with or without T-cell help, depending on the type of antigen, so-called *thymus-dependent* (TD) or thymus-independent (TI) antigens. BCR signals that occur during TD responses are modulated and enhanced by cell-cell contact with T cells by way of critical costimulatory molecules, discussed further in the section on T-cell costimulation.

Thymus-Dependent Versus Thymus-Independent B-Cell Responses

Unlike T cells, which recognize either short peptides or small lipid molecules that must be presented in the cleft of an MHC or MHC-like molecule, the BCR is capable of binding a variety of antigens, including soluble proteins and polysaccharides. Recognition of protein antigen generally requires cooperation with T cells, or T-cell help, and thus such antigens are TD antigens. Naïve follicular B cells in lymph node follicles or the spleen recognize their cognate antigen while contacting a helper T cell specific for the same antigen; linked T-B interaction then fuels the formation of a key structure, the GC, where isotype switching, somatic hypermutation, memory cell generation, and plasma cell differentiation occur.

TI antigens are classified as type 1 or type 2 (TI-1 and TI-2).[174] TI-1 antigens are bacterial components that can trigger B-cell activation regardless of Ig specificity and thus lead to polyclonal activation. These components fall into the general category of pathogen-associated molecular pattern (PAMP) molecules, the canonic example being lipopolysaccharide (LPS). PAMP molecules activate B cells by binding to a distinct family of receptors, the Toll-like receptors (TLRs), thereby accounting for their independence from Ig. The Ig secreted in response to TI-1 antigens is typically low-affinity IgM because TI-1 antigen binding does not induce class switching.[175]

TI-2 antigens, in contrast, are molecules that have a repetitive structure and activate B cells by cross-linking of multiple BCRs on a single cell, hence leading to specific clonal antibody production. These antigens are typically bacterially derived polysaccharides, such as those found in the coating of encapsulated organisms. Marginal-zone B cells are important for responses to TI-2 antigens, and the fact that marginal-zone B cells typically take years to mature in children probably underlies the lack of response of children younger than 2 years to polysaccharides.[30] The spleen is the major site of TI-2 antigen recognition, and the antibody produced again is often IgM, although IgG responses also clearly occur. Class switching in this instance does not depend on cognate T-cell help but probably is enhanced by noncontact T-cell factors such as the cytokine interleukin-4 (IL-4).[174]

B-Cell Receptor Triggering and Signaling

B-cell activation involves a complex array of molecular signaling events. The signaling cascades that emanate from the BCR are highly analogous to those triggered in T cells by the TCR and involve a host of kinases, adaptor proteins, and other enzymes.

In brief, B-cell activation commences with engagement of surface Ig, which initiates receptor oligomerization, an event that depends on B-cell coreceptors such as the stimulatory CD19 and CD21 receptors and the inhibitory low-affinity FcR.[176,177,178,179] In addition, aggregation of Ig receptors takes place in specialized membrane microdomains, termed *lipid rafts,* that are characterized by their cholesterol- and sphingolipid-rich content.[180-183] In cellular systems lipid rafts have been postulated to function in intracellular trafficking, apical sorting, and signal transduction.[184,185] In both T and B lymphocytes, lipid raft components may function as a potential signaling platform to foster the aggregation and co-localization of signaling proteins.[180,186-188]

Within the lipid raft, the Ig molecule transmits signals through the associated Ig-α and Ig-β molecules (Fig. 23-13), together termed the *BCR;* the BCR, like other members of the immune recognition receptors, has no intrinsic tyrosine kinase activity. On oligomerization the cytoplasmic domains of Ig-α and Ig-β become tyrosine phosphorylated on so-called immunoreceptor tyrosine–based activation motifs (ITAMs) with the amino acid consensus sequence D/ExxYxxL/Ix7YxxL/I. Phosphorylation of ITAMs is mediated by Src kinase family members, notably Fyn, Blk, and Lyn (see Fig. 23-13),[187,189,190] which then recruit the critical tyrosine kinase Syk by way of its Src homology 2 (SH2) domains. Syk itself, when bound, requires activation either by autophosphorylation or phosphorylation by Src kinases, and thus clustering of multiple ITAM-bearing BCRs, Src kinases, and Syk is required to achieve a threshold of activation that initiates downstream cascades.[191]

Activation of Syk, similar to activation of ZAP70 in T cells (see later), is a crucial linchpin in BCR signaling. Syk itself functions as a tyrosine kinase and is capable of phosphorylating key substrates, which then activate three main signals, the protein kinase C (PKC), intracellular calcium, and Ras pathways. Activation of these pathways occurs by assembly of a macromolecular complex of critical signaling molecules clustered on an adaptor protein known as the *B-cell linker protein* (BLNK) or SLP65.[190] Recruitment of a member of the Tec kinase family, Btk,

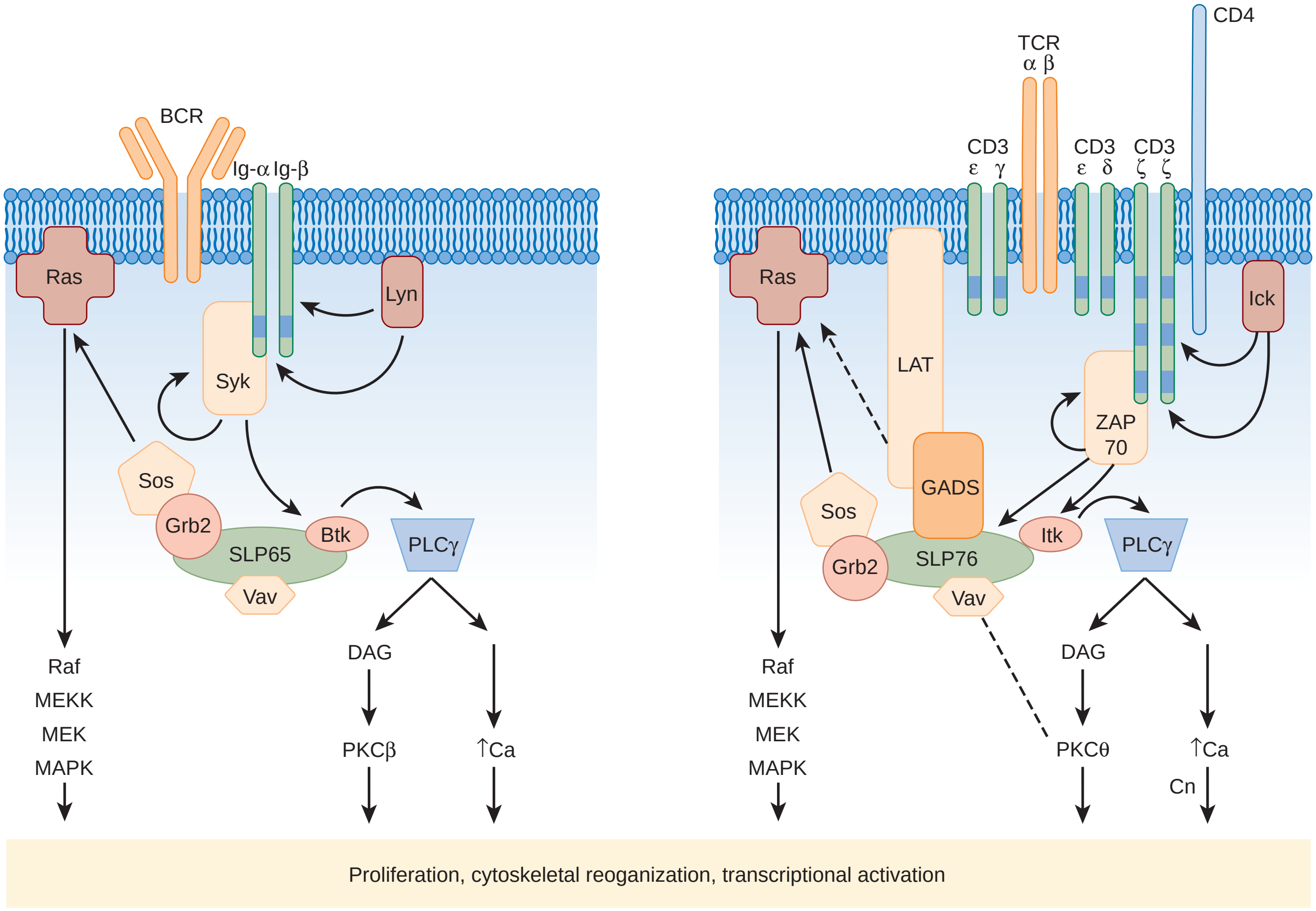


Figure 23-13 Schematic of B- and T-cell signaling pathways. Signaling cascades that emanate after engagement of antigen-specific receptors, the B-cell receptor (BCR) in B cells *(left)* and the T-cell receptor (TCR) in T cells *(right)*, are depicted. Each receptor has short cytoplasmic tails that do not themselves signal; the receptors signal instead via a complex of associated transmembrane molecules (Ig-α, Ig-β for B cells and the CD3 γ, δ, ε, and ζ proteins for T cells) that have immunoreceptor tyrosine–based activation motifs (ITAMs, depicted as *blue boxes*). Phosphorylation of ITAMs by early protein tyrosine kinases such as Lyn and Lck is critical for recruitment of Syk in B cells and ZAP70 in T cells, which go on to trigger the rest of the cascade. Adaptor proteins such as SLP65 and SLP76 nucleate a host of signaling molecules to activate Ras and protein kinase C (PKC) and increase intracellular calcium (Ca). The final effects of proliferation, cytoskeletal reorganization, and transcriptional activation ensue. B and T cells share a number of intermediates such as phospholipase C γ (PLCγ), Grb2, Sos, Ras, and Vav. Related molecules specific to B or T cells are depicted with similar shapes (e.g., SLP65/SLP76, Btk/Itk). *DAG,* Diacylglycerol; *MAPK,* mitogen-activated protein kinase; *MEK,* MAPK kinase; *MEKK,* MEK kinase.

to SLP65 by way of the SH2 and Src homology 3 (SH3) domains and to the membrane by the Pleckstrin homology (PH) domain activates Btk.[192] The critical nature of Btk activation in BCR signaling is underscored again by its role as the gene responsible for X-linked agammaglobulinemia. Btk in turn activates an isoform of phospholipase C (PLCγ2) that hydrolyzes inositol phosphates to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates both an isoform of PKC (PKCβ) and Ras, whereas IP_3 induces an increase in intracellular calcium. Recruitment of Sos by way of the adaptor protein Grb2 also activates the Ras pathway, as does Vav, a guanine nucleotide exchange factor for Rac. Together, activation of these three pathways then triggers activation of mitogen-activated protein kinases (MAPKs); reorganization of the actin cytoskeleton; and nuclear translocation of critical transcriptional activators, including the NF-κB family, that promote survival and clonal expansion (see Fig. 23-13).[189,191]

Coreceptors Involved in B-Cell Activation

Three important coreceptors for B-cell activation are CD21 (also called complement receptor 2 [CR2]), CD19, and CD81. Antigens derived from bacteria or other extracellular microorganisms are more efficiently cleared when opsonized or bound by complement components, a mechanism that links the early innate immune response elicited by activation of complement to the adaptive humoral B-cell response. Binding of C3b to CD21 coclusters CD21, CD19, and CD81 with the BCR within lipid rafts.[179,187,193] Phosphorylation of CD19 by proximity to the activated BCR complex in turn generates docking sites for phosphatidylinositol 3'-kinase (PI3K), for Vav, and for Lyn. PI3K generates phospholipid intermediates that recruit Btk, as described earlier. Vav activates Rac, thereby activating MAPKs and inducing cytoskeletal reorganization. The response to opsonized antigens recognized by the BCR via its specific epitope is thus greatly enhanced by the binding of C3b to CD21.[194] CD81 appears to function primarily as an adaptor and is critically required for BCR and CD21/CD19 to associate with lipid rafts.[195,196]

Stimulation by way of the antigen receptor alone is insufficient to activate B or T cells, and, in fact, binding of receptor-ligand pairs between B and T cells is critical for activation of both. Many costimulatory pathways are shared between B and T cells and are discussed further later; binding of CD40 to its ligand CD40L (CD154) is the most crucial of these pathways for B-cell activation. CD40 is a 48-kd member of the TNF receptor superfamily that is expressed on B cells and other APCs constitutively, whereas CD40L is a 33-kd transmembrane protein with homology to TNF upregulated on activated CD4+ T cells. CD40/CD40L binding has been shown to be essential for B-cell growth and differentiation.[197] In vitro stimulation of B cells by cross-linking by way of monoclonal antibodies is sufficient to promote growth and class switching and is synergized by BCR stimulation or treatment with IL-4, or both. Engagement of CD40 with trimeric CD40L and clustering of the cytoplasmic tails lead to recruitment of a class of adaptor proteins, the TNF receptor–associated factors (TRAFs), which in turn activate a variety of kinases that ultimately culminates in activation of MAPK and NF-κB.[198] In addition to clonal expansion and survival, CD40 signaling also leads to upregulation of other costimulatory molecules such as CD80 (B7) and CD54 (intercellular adhesion molecule 1 [ICAM-1]).[198]

The relatively recently discovered TLR can trigger B-cell activation alone (see earlier discussion) and also can serve a costimulatory function with BCR signals.[175,199] At least 11 TLRs in humans have been found, each recognizing different microbial components, such as LPS (via TLR4), peptidoglycan (TLR2), double-stranded RNA (TLR3), flagellin (TLR5), and CpG DNA (TLR9).[200] The response to intact microbes may involve recognition of microbial components simultaneously by the BCR and TLR, and interestingly, BCR-TLR co-recognition may play a role in the generation of autoantibodies.[201,202]

Follicular B-Cell Differentiation: the Germinal Center

Activation of B cells and differentiation into memory and plasma cells have become increasingly well understood in the context of cellular trafficking and the complex architecture of the specialized anatomic site, the GC.[203,204] B cells, a critical subset of CD4+ T cells (follicular helper T cells or T_{FH}), and specialized follicular dendritic cells (FDCs) must interact through costimulatory contact and soluble factors to generate a long-lasting humoral response.

In the early stages of infection, innate immune cells such as dendritic cells and macrophages capture antigen, migrate to lymph nodes, and present peptides in the cleft of class II MHC to CD4+ T cells. Simultaneously, naïve B cells that recognize the same pathogen can internalize complex protein particles by receptor-mediated internalization of Ig, with the protein fragments being broken down and displayed as peptides by class II MHC. Naïve B cells that encounter their cognate antigen and become activated then migrate through the T-cell zone and contact T cells stochastically during the journey.[205] When T and B cells that recognize the same antigen interact through TCR-MHC interactions, costimulatory signals, namely CD40-CD40L interactions, enhance the interaction and result in clonal expansion of both T and B cells. These activated "helped" B cells then travel to the follicle and infiltrate a network of FDCs. FDCs are thought to have a specialized function consisting of capture of immune complexes and simultaneous binding of FcR to antibody and complement receptor 3 (CR3) to components of complement.[206] The antigen component of the immune complex can then be displayed to passing B cells by way of the BCR. Activated B cells that recognize antigen displayed on FDCs proliferate rapidly after further costimulation by CD4+ T cells and become the centroblasts and centrocytes that form the GC.

The role of T_{FH} in this process has been increasingly elucidated in recent years.[207,208] These specialized CD4+ T cells were first identified on the basis of their expression of CXCR5, a receptor typically found on B cells that binds the B cell homing chemokine CXCL13.[209-211] They can be found in spleen, tonsil, and lymph node, optimally positioned in the B follicles where they function, and phenotypically similar cells circulate in the blood.[212,213] Functionally T_{FH} are the key contributors to B cell help in the secondary lymphoid organs by virtue of the high-level production of IL-21,[214] a pleiotropic cytokine that is critical for induction of class switching along with IL-4, and high surface expression of ICOS.[215-218] The development of T_{FH} depends on IL-21 itself and the master transcription factor BCL6, as further discussed in the section on helper T cell differentiation and function.[217,219,220]

Somatic hypermutation is thought to occur at the centroblast stage and further diversifies the overall antibody pool by introducing mutations in the V_H and V_L regions of Ig in B cells involved in a GC response.[221,222] Nucleotides are thought to be mutated in a somewhat random fashion, in both the rearranged and nonrearranged V regions, but ultimately survival of B cells that have undergone hypermutation is dictated by positive and negative selection based on the affinity of the expressed Ig. In other words, mutations that disrupt affinity to the antigen to which the animal is responding will be selected against, whereas those that enhance affinity will be selected for, hence the term *affinity maturation*.

Isotype or class switching is thought to occur at the centrocyte stage. Because all naïve B cells express IgM, a low-affinity antibody, inducing activated B cells to produce high-affinity IgG or IgA significantly enhances the functional humoral immune response to specific antigen at the time of infection and requires splicing of the V(D)J regions to the C_γ or C_α region. CD40-CD40L interactions are critical for this process, and T-cell–derived cytokines enhance class switching while influencing the Ig class or subclass.[223-225] In the mouse, T-B interactions in the context of interferon-γ (IFN-γ) tend to promote switching to IgG2a and IgG3, whereas IL-4 directs switching to IgG1 and IgE (see Table 23-2).

GC B cells then differentiate into either plasma cells or switched memory B cells. How GC B cells "choose" between the plasma cell and memory B-cell fate remains unclear.[226] Plasma cells are terminally differentiated B cells found primarily in bone marrow and are specialized for high-level secretion of IgG, IgM, and IgA. They have a

TABLE 23-2 Immunoglobulin Subclass Definition

Class	Isotype	Heavy Chain	Molecular Formula	Molecular Mass (kd)	Plasma Concentration (mg/mL)*	Cross Placenta	Fix Complement	Biologic Functions
IgG								Complement activation, opsonization for Fc receptor
	IgG1	γ1	$\gamma1_2\kappa_2$ $\gamma1_2\lambda_2$	150	7-9	+	+	Antitoxins, antibody to most bacterial antigens
	IgG2	γ2	$\gamma2_2\kappa_2$ $\gamma2_2\lambda_2$	150	2-3	+	+	Antipolysaccharide antibodies to bacterial capsules
	IgG3	γ3	$\gamma3_2\kappa_2$ $\gamma3_2\lambda_2$	150	0.7-1	+	+	Cytophilic antibodies (e.g., to Rh antigens)
	IgG4	γ4	$\gamma4_2\kappa_2$ $\gamma4_2\lambda_2$	150	0.3-0.5	+	–	Anticoagulants, such as anti–factor VIII in hemophiliacs
IgM		μ	$(\mu_2\kappa_2)_5$ $(\mu_2\lambda_2)_5$	950	1.5	–	+	Receptor in B-lymphocyte membrane, antibodies to lipopolysaccharides, complement activation, natural antibody
IgA				150 or 300				Present in external secretions, alternative complement activation (?)
	IgA1	α1	$\alpha1_2\kappa_2$ $\alpha1_2\lambda_2$		1-3	–	–	Virus-neutralizing antibody
	IgA2	α2	$(\alpha2_2\kappa_2)_2$		0.5-0.7	–	–	Principal secretory antibody
IgD		δ	$\delta_2\lambda_2$ $\delta_2\kappa_2$	180	0.3	–	–	Unknown, receptor in B lymphocyte membrane
IgE		ε	$\varepsilon_2\lambda_2$ $\varepsilon_2\kappa_2$	190	0.0005[†]	–	–	Anaphylactic antibodies or "reagins," binds to mast cells, mediator of release

*Plasma concentrations of the immunoglobulins vary significantly with age in pediatric populations. Adult norms are given here. IgG at birth reflects maternally transferred immunoglobulin and wanes over several months, with a nadir at approximately 4 months and rising thereafter as the infant's own production takes over. IgM and IgA levels rise with age during infancy and childhood.

[†] Usually expressed as international units; up to 200 IU is considered normal.

characteristic histologic appearance with so-called clockwork chromatin, eccentric nuclei, and markedly expanded endoplasmic reticulum and can be identified by expression of CD138 (also called syndecan-1). Class-switched memory B cells, which generally can be identified by virtue of the loss of IgD and gain of CD27 surface expression,[118] are able to circulate back to bone marrow and secondary lymphoid organs and, like memory T cells discussed later, are responsible for the rapid and effective anamnestic immune response characteristic of acquired immunity. A number of congenital deficiencies that affect T-cell–dependent B-cell maturation are characterized by a lack of GC formation and switched memory B cells; such conditions include CD40L deficiency (discussed later), X-linked lymphoproliferative disease (caused by mutation in SH2D1A or SLAM-associated protein [SAP]), and two molecules recently shown when mutated in humans to cause common variable immunodeficiency, ICOS (inducible T-cell costimulator) and TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor).[227-231] More recently, both somatic hypermutation and class switch recombination have been found to require a newly discovered activation-induced deaminase (AID) that deaminates deoxycytidine to form deoxyuridine; subsequent repair of the deoxyuracil-deoxyguanosine mismatch triggers nucleotide substitution or excision, thereby generating diversity and also joining VDJ regions to a different constant region class.[221,232] Humans born with defects in the *AID* gene have failure of both processes that results in a hyper-IgM phenotype.[233-235]

B-Cell Tolerance

Like T-cell tolerance (discussed later), B-cell tolerance is essential to prevent autoimmune reactions. Immature B cells can be made tolerant in the bone marrow to antigens expressed by the host (i.e., self-antigens) through clonal deletion.[236] If the surface IgM (sIgM) expressed on an immature B cell binds with high avidity to a self-antigen, the cell will be stimulated to undergo apoptosis, a form of cell death characterized by chromosomal fragmentation and membrane blebbing. The B cell may also undergo receptor editing, a process whereby that B cell's Ig gene will further rearrange to express a different, nonautoreactive Ig molecule.[78] The few B cells that escape central deletion may be rendered anergic in the periphery, a state in which the B cell expresses a gene that encodes a self-reactive antibody yet is unable to respond to antigen. The molecular mechanisms responsible for anergy are incompletely understood but may involve alternative signaling when the BCR is activated in the presence or absence of costimulation. For instance, BCR triggering in the context of concurrent TLR signaling (secondary to microbial products in addition to the BCR-specific antigen) leads to upregulation of CD86, which engages CD28 on T cells and recruits T-cell help. In the absence of TLR signals, the upregulation of CD86 is not sustained, and the BCR signals are attenuated.[237] A group of TNF and TNF receptor family members, including BAFF (also known as BLyS, *TNFSF13B*)[238] and APRIL (*TNFSF13A*),[239] whose receptors include TACI (*TNFRSF13B*, CD267),[240-242] BCMA (*TNFRSF17*, CD269)[240,242] and BAFF-R (*TNFRSF13C*, CD268),[243] have been shown to be important in B-cell homeostasis and autoimmunity. Interestingly, mice that overexpress BAFF exhibit autoimmunity,[244,245] and humans with a variety of rheumatologic diseases have been shown to have high BAFF serum levels.[246] The increasing use of rituximab, humanized anti-CD20 antibody, for the treatment of autoimmune disease and, recently, chronic graft-versus-host disease (GVHD) underscores the importance of dysregulated humoral immunity in human disease. More recently, a fully human IgG1-λ monoclonal antibody specific for BAFF/BLyS, belimumab, has been shown to have efficacy in systemic lupus erythematosus[247] and is approved by the U.S. Food and Drug Administration (FDA) for treatment of mild to moderate disease.

Function of Secreted Immunoglobulin

Secretion of a diverse repertoire of Ig is essential for protection of the host from infection and for neutralization of toxin. Once formed, binding of specific antibodies to microorganisms, virally infected cells, toxins, or other pathogens is essential for complement-mediated lysis, antibody-dependent cellular cytotoxicity (ADCC), opsonization, phagocytosis, and neutralization of toxins and viruses (see Table 23-2). Different heavy-chain isotypes perform different functions preferentially. IgM, IgG1, IgG2, and IgG3 activate the complement cascade, whereas only IgG1 and IgG3 bind effectively to phagocytic Fcγ receptors. IgE is able to bind to the Fcε receptors expressed on mast cells and eosinophils. In addition, secreted IgA antibodies play a role in protection from invasion through mucosal surfaces.

Different classes of antibodies not only have different functions but also predominate in different compartments in the body. IgA is predominantly secreted, IgM is primarily intravascular, and IgG is generally the major antibody found in tissues and peripheral blood. IgG is able to cross the placenta to provide some protective immunity to the fetus; passive protection from long-lived IgG molecules confers postnatal immunity. However, although the function and location of the antibody classes differ, subpopulations of B cells are able to migrate and home to specific regions of antigen presentation. Therefore generalized B-cell immunity may result from localized antigen presentation, as paradigmatically demonstrated by the efficacy of successful vaccination programs.

T-CELL ACTIVATION AND FUNCTION

CD4+ and CD8+ TCR αβ T cells exported from the thymus circulate in a naïve or resting state in the blood and lymphoid organs but are poised to be activated by cognate antigen presented by MHC on the surface of APCs. CD8+ cytolytic T cells induce the lysis of foreign cells, such as infected, malignant, or allogeneic cells.[67] CD4+ helper T cells interact with B cells via cell-cell and soluble factors to induce class switching and the generation of antigen-specific antibodies. They also provide "help" to CD8+ cytolytic T cells.[37,248] Activation of resting T cells not only relies on signaling cascades downstream of the TCR-MHC interaction (signal 1) but also requires or is enhanced by the binding of a variety of T-cell surface molecules to their ligands on APCs (signal 2). The lack of a second signal generally results in nonresponsiveness or anergy of the T cell and serves to ensure that activation of the naïve T cell is not only antigen specific but regulated as well. The affinity of the cognate antigen, the engagement of specific costimulatory molecules, and the cytokine milieu collectively influence and modulate the outcome of T-cell activation and thereby result in the generation of distinct effector cell subsets. Although most effector T cells perform their function and expire, a subset of activated cells differentiate into long-lived memory cells capable of rapid anamnestic responses.

T-Cell Receptor Triggering and Activation

The αβTCR is a multimeric transmembrane complex composed of a disulfide-linked antigen-binding clonotypic heterodimer in noncovalent association with the signal-transducing CD3 subunits (CD3εγ, CD3εδ, and CD3ζζ) (Fig. 23-14, *A*).[249,250] TCR signaling via CD3 dimers evokes T cell lineage commitment and repertoire selection during development, maintains the peripheral T cell pool, and further differentiates naïve T cells into effector or memory cell populations upon immune stimulation. Each CD3ε, γ, and δ subunit contains an extracellular Ig-like domain, a membrane-proximal stalk region, a transmembrane segment, and a cytoplasmic tail. The interaction between an αβ TCR heterodimer on the T cell and a pMHC ligand on an antigen-presenting cell (APC) initiates a cascade of downstream signaling events. These events are transmitted via the immunoreceptor tyrosine-based activation motif (ITAM) elements in the cytoplasmic tails of the associated CD3 subunits, whose lengths are substantial relative to those of the TCR α and β tails.[251,252,253,254] The various CD3 chains induce distinct patterns of cellular protein tyrosine phosphorylation on activation to recruit intracellular adaptors and signaling molecules. Early, intermediate, and late gene activation programs ensue.[255] Reviews such as Rudolph and colleagues[249] have focused on the structural nature of immune recognition involving Vα and Vβ domains of a given TCR and its pMHC ligand. How recognition of pMHC by a weakly interacting αβ TCR heterodimer on the T cell surface evokes intracellular signaling by way of the adjacent CD3 components of the TCR complex has remained undefined.

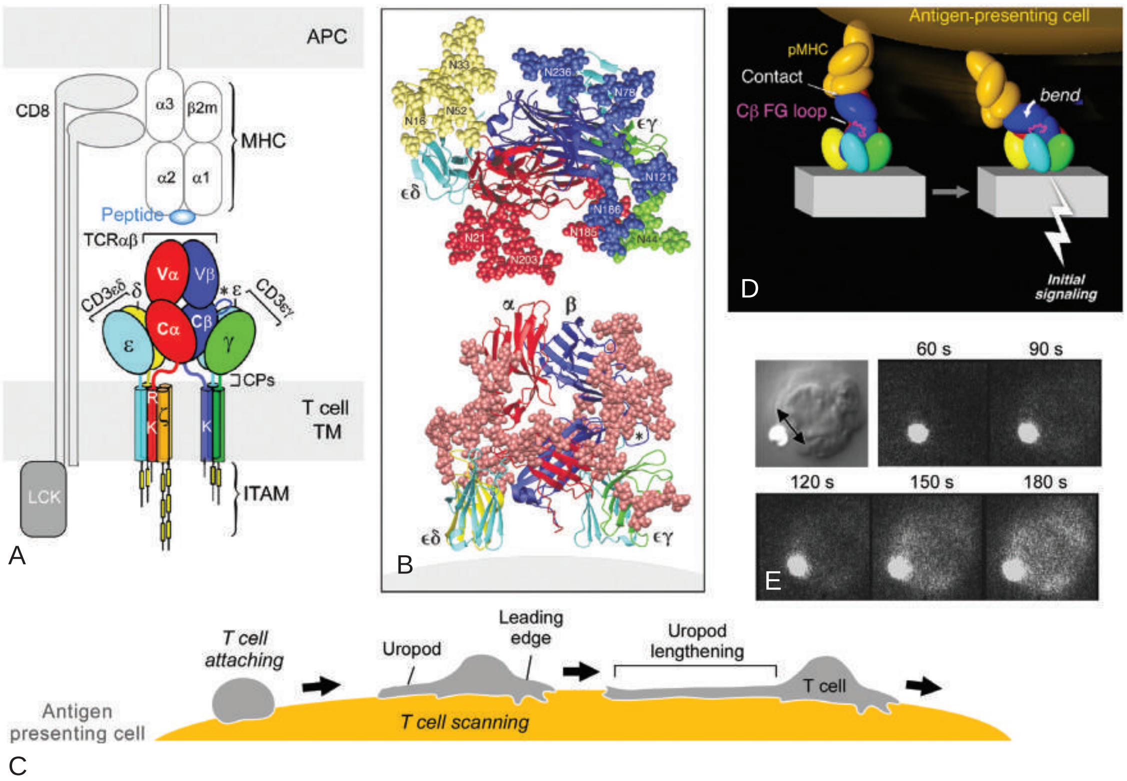


Figure 23-14 TCR complex interaction with pMHC and its activation by mechanical force. **A**, TCR components (ectodomains, stalk CP, TM, and cytoplasmic tails) are labeled and shown in distinct colors. The pMHC on the APC and the interacting CD8αβ heterodimer are not colorized. The relative positions of the positively charged residues in TCR α and β TMs are shown. For simplicity, putative interactions with CD3 acidic residues are not indicated. **B**, Ectodomain structure in ribbon form based on PDB code IFND, 1XMW, and 1JGJ is shown from the perspective of pMHC *(top)* and side *(bottom)* views with the position of T-cell membrane shown in the latter. In the *top* view, adducted glycans in CPK visualization are colored according to subunit, whereas all sugars are denoted in beige in the *side* view. The CB FG loop is labeled by an * in each panel. **C**, T-cell scanning an APC surface in search of specific pMHC. On attachment the TCR assumes a polarized morphology with a leading edge and lengthening uropod. **D**, One pMHC molecule is shown in orange on the APC, whereas the ectodomains of TCR subunits on the T cell membrane below are colored as in **A**. The view is from the CD3εγ side. Initial ligation of the TCR by pMHC *(left)* constitutes a detachable mechanosensor that, as a result of continued T cell scanning, transmits an external torque (bend) into initial signaling (bolt) via the rigid components of the TCR complex before dissociation. The CB FG loop is shown in magenta. **E**, Calcium flux in naïve T cells after application of external mechanical force (low piconewtons) using optical tweezers. 10 to 100 pMHC copies per polystyrene bead are used in such experiments. The white sphere represents the specific pMHC loaded polystyrene bead. Increase in intracellular free calcium is evident from the increase in immunofluorescence of the calcium orange loaded cells. The time intervals after force application are given in the fluorescent images, and the direction of external mechanical force is denoted by the *double-headed arrow* in the bright field image.

Functional TCR αβ heterodimers were first identified by mAbs on antigen-specific T cell clones and then T cell hybridomas.[256,257,258,259] Subsequent sequence analysis of TCRs predicted that they would share with antibodies a common structural basis of ligand recognition, akin to an antibody Fab fragment.[260,261] These results agreed with peptide mapping studies of α and β subunits that identified conserved as well as variable peptides, implying the existence of constant and variable domains in the TCR α and β subunits. The biochemical results were later confirmed and extended by DNA cloning [262,263] and elegantly delineated further by the crystal structure of an intact murine αβ TCR[264] and a complex between a human TCR, viral peptide, and human MHCI molecule that followed.[265]

As noted previously, structures of TCR αβ heterodimers and antibody Fab fragments seem very similar (see Fig. 23-7). While each of the four TCR α and β domains, like those of Fab, has been assigned an Ig fold, deviations are notable in both TCR constant domains[79,263,264] as well as in the Vα domain.[266] These deviations define fundamental differences between the TCR as a cell surface receptor and antibody as a soluble immune molecule. First noted on structural analysis was the striking elongation of the FG loop of the Cβ domain connecting its F and G β-strands. Unlike other Ig-like structures, there is a 13 amino acid (aa) insertion within the FG loop.[267] Figure 23-7, *A* shows a side view of the crystallographically resolved murine N15 MHCI-restricted αβ TCR.[268] The unique protrusion of the Cβ domain's FG loop is apparent (labeled). Despite its length, the FG loop is internally well-structured, and constitutes an integrated component of a rigid structural entity that connects the Vβ and Cβ domains.[268] This feature of the FG loop is conserved in αβ TCRs among all mammalian species studied to date, having co-evolved with the development of distinct CD3γ and CD3δ genes.[269] Consistent with the importance implied by this conservation, removal of the FG loop

(ΔFG) in T cell transfectants as well as TCRs displayed on naïve T cells by transgenesis,[270,271] diminishes T cell activation and alters development. Second, the overall shape of the TCR C domain module was observed to be remarkably asymmetric. Third, unlike Fabs, the αβ TCR heterodimers are heavily N-linked glycosylated (Fig. 23-14, *B*). Such adducts can dynamically modify TCR function.

A model of the TCR complex of αβ, CD3εγ, and CD3εδ ectomains[272] defines a plausible topology and emphasizes its glycan richness (see Fig. 23-14, *B*). Given that CD3ζ has an ectosegment that is only 9 amino acid long, its extracellular segment is omitted from the figure as are the connecting peptides (CPs) of the TCR α and β chains and the stalk regions of CD3ε, CD3γ, and CD3δ. The multiple N-linked glycan adducts of the TCR complex (Fig. 23-14, *B, top panel*) help guide pMHC ligands to the TCR recognition surface, reducing entropic penalties by directing binding to the exposed, glycan-free CDR loops. It is important to note that glycans are large and dynamic. These adducts can affect movement of the TCR subunits, thereby affecting signaling. Immediately evident in Figure 23-14, *B* is the central position of the TCR αβ heterodimer with a vertical dimension of 80Å projecting from the cell membrane, flanked on either side by the shorter (40Å) squat and rigid CD3 heterodimers, CD3εδ on the “left” TCRα side and CD3εγ on the “right” TCRβ side.

As T cells perform immune surveillance by scanning APCs in search of antigenic peptide/MHC complexes (pMHC) (Fig. 23-14, *C*), ligation of a TCR by pMHC will bend the TCRαβ heterodimer and thereby push on CD3 components by way of the FG loop among other structural elements before a stop-movement signal (Fig. 23-14, *D*). Recent data using optical tweezers show that the TCR is activated by force (Fig. 23-14, *E*).[273] Mechanotransduction makes T-cell triggering extremely sensitive, accounting for the small number of pMHC complexes per APC required to activate T cells.[274] Precedent for mechanoreceptors in the hematopoietic system is the von Willebrand factor (VWF) receptor on platelets where tensile stress on bonds between the GPIbα subunit and the VWFA1 domain under fluid dynamic conditions triggers integrin $\alpha_{IIb}\beta_{III}$ activation to support platelet adhesion.[275,276]

The physical force relayed from the ectodomain onto the TM during mechanosensor function may be transmitted to the cytoplasmic tail directly or indirectly (or both) by modification of the membrane lipid organization.[277,278] The CD3ε cytoplasmic tail maintains close interaction with the plasma membrane via basic CD3ε residues and acidic phospholipids enriched in the inner leaflet of the plasma membrane.[279,280] Two key tyrosine residues in the CD3ε ITAM are deeply inserted in the hydrophobic core of the lipid bilayer. Release of sequestered tyrosines must occur for phosphorylation by lck to follow. CD3ζ phosphorylation is also lipid dependent,[281] and the importance of the six ITAMs in the ζζ homodimer for signaling must not be underestimated.[251]

Our understanding of the signaling cascades downstream of the TCR has become increasingly complete and complex. Five major signal transduction pathways are triggered by TCR engagement: phosphotyrosine kinase (PTK) activation, plasma membrane phosphatidylinositol hydrolysis, changes in intracellular calcium concentration $[Ca^{2}+]_{i}$, RAS/MAPK, and PKC pathways (see Fig. 23-13). In recent years a variety of adaptor molecules and other families of proteins, including small Rho guanosine triphosphatases (GTPases), have been implicated in these pathways. Thus there is much crosstalk between the main pathways, which then converge on the activation of nuclear transcription factors critical for the expression of T-cell effector molecules, namely the cytokine genes, particularly IL-2. Finally, the spatial organization of the TCR signaling complex in ordered lipid microdomains, in which an “immune synapse” is formed at the point of contact between the T cell and APC, is thought to enhance T cell–APC interaction and allow directed secretion of cytokines locally to the contacting APC (Fig. 23-15).[282,283]

TCR activation leads to stop movement and formation of the immunologic synapse. Mechanoreceptor function is most likely additionally tunable by inducible actin cytoskeletal interactions with TCR and pMHC on T cells and APCs, respectively, because torque will be greatest in their presence. Within the synapse, force could be generated on the TCR by way of microcluster formation and actin-based trafficking.[284] Mechanosensing can be further amplified at the synapse where intermembrane distances (~150Å) are optimal for TCR/pMHC ligation in conjunction with other signaling molecules to sustain activation from both p-SMAC and c-SMAC components. The facilitating roles of adhesion molecules and CD4 and CD8 coreceptors have been discussed.[273] Rapid transport of TCRs to the immunologic synapse and TCR signaling after disengagement of pMHC likely give rise to serial TCR triggering for high activation efficiency within the immunologic synapse. In this dynamic mechanosensor model, signaling occurs by way of the built-in geometry of the TCR-pMHC complex.

Within seconds to minutes of TCR engagement, PTKs such as Lck and Fyn are activated and phosphorylate the ITAMs on CD3 subunits, particularly CD3 ζ (see Fig. 23-13). The physical association of Lck with the CD4 and CD8 coreceptor molecules, recruitment to the membrane by myristoylation and other modifications, and the multiplicity of Lck molecules in close proximity when the TCR-coreceptor complex is triggered are thought to promote and amplify its rapid activation.[285] Phosphorylated ITAM on CD3 ζ in turn recruits ZAP70, a 70-kd protein kinase whose homolog in B cells is Syk, via the phosphotyrosine-binding SH2 domain.[286] ZAP70 is phosphorylated by Lck, is activated, and then phosphorylates two key substrates, LAT (linker for activation of T cells) and SLP76 (SH2 domain–containing leukocyte protein of 76 kd). A host of downstream proteins, including Grb2, SOS, ITK, PLCγ, GADS, and VAV1, then converge on LAT and SLP76 to activate the phosphoinositol, calcium, and RAS/MAPK pathways.[190]

Recruitment of PLCγ to LAT and SLP76 catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate to generate IP_3 and DAG, which in turn promote the increase in $[Ca^{2}+]_{i}$ and activation of the T-cell–specific isoform

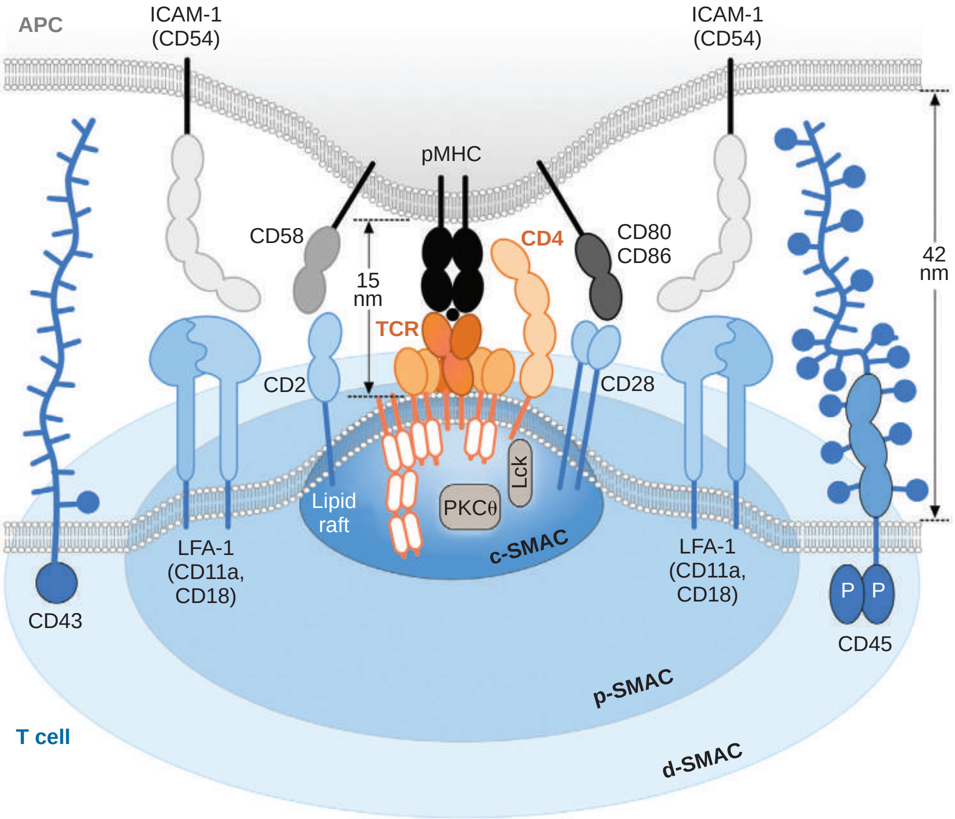


Figure 23-15 Immunologic synapse between a T cell and an antigen-presenting cell. The immunologic synapse is divided into central-supermolecular activation cluster (c-SMAC), peripheral-(p)-SMAC, and distal-(d)-SMAC in the *vertical view* provided. The alignment of receptors and adhesion molecules are ordered according to size. Membrane-membrane distance of approximately 15 nanometers (150Å) in the c-SMAC and 42 nanometers in the d-SMAC.[311,474-476] *(Modified from Yokosuka T, Saito T: The immunological synapse, TCR microclusters, and T cell activation.* Curr Top Microbiol Immunol *340:81–107, 2010.)*

PKCθ, respectively (see Fig. 23-13).[287,288] The critical downstream effect of the rise in $[Ca^2+]_i$ is activation of the calcium- and calmodulin-dependent serine/threonine phosphatase calcineurin, whose key direct substrates in T cells are members of the NF-AT family.[289-291] Dephosphorylation of NF-AT allows its translocation from the cytoplasm to the nucleus, where it binds, often in the presence of AP-1 elements, to the transcriptional regulatory sequences of *IL2* and other cytokine genes. The immunosuppressant agents cyclosporine and tacrolimus (FK506) function by forming macromolecular complexes with intracellular binding proteins (cyclophilins and FK-binding proteins [FKBPs], respectively), which in turn inhibit the phosphatase activity of calcineurin, thereby abrogating the early cytokine elaboration critical for T-cell expansion and function.[290,292,293]

Activation of the RAS pathway primarily occurs by the recruitment of the adaptor proteins Grb2 and Shc to phosphorylated LAT, which leads to recruitment of the guanine nucleotide exchange protein Sos (see Fig. 23-13). Sos catalyzes the conversion of inactive guanosine diphosphate (GDP)–bound RAS to active GTP-bound RAS and activates a series of MAPKs.[294] Activation of PKCθ in response to PLCγ described earlier can crosstalk with this pathway and induce increases in RAS and MAPK activity, although the mechanisms and relative contributions are still unclear. Major downstream effects of PKCθ activation include stimulation of AP-1 nuclear activity, regulation of JNK (the upstream kinase of AP-1), and activation of NF-κB.[287] Induction of these and other transcription factors, which cooperate with NF-AT translocation in response to rises in $[Ca^2+]_i$, strongly stimulates production of IL-2.

The transcriptional program executed in response to TCR stimulation includes the major T-cell cytokine IL-2; other cytokines, including IFN-γ and IL-4, the canonic T-helper type 1 and type 2 cytokines (discussed later); proliferative factors, including c-myc and c-fos; a variety of activation markers such as CD69, CD45RO, CD44; and growth factor receptors, most prominently the high-affinity IL-2 receptor subunit IL2Rα, also called CD25.[255] Binding of IL-2 to its receptor, in both autocrine and paracrine fashion, results in a growth factor–induced signal to the cell and induces the transition from G_1 to S phase of the cell cycle.[295,296,297,298] Interestingly, in recent years CD25 has been found not only to mark activated T cells but also to be expressed on naturally occurring T-regulatory cells, discussed later. The role of IL-2 in expanding activated effector cells and simultaneously its requirement for development and maintenance of the T-regulatory population result in pleiotropic effects of IL-2 in vivo and has complicated the potential use of IL-2 in the clinical setting.[299] However, recently a trial of IL-2 monotherapy has been successful in the treatment of steroid-refractory chronic GVHD, associated with expansion of regulatory T cells.[300] The high-affinity IL-2 receptor is composed of IL-2Rα (*IL2RA*, CD25), IL-2Rβ (*IL2RB*, CD122), and IL2Rγ (*IL2RG*, γ_c, CD132), this last a common signaling component of the cytokine receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Mutations in the *IL2RG* gene[301,302] and the downstream factor *JAK3* (Janus kinase 3)[303,304] result in X-linked and autosomal recessive forms of SCID, respectively. In contrast to the inhibition of early signals downstream of $[Ca^2+]_i$ by cyclosporine and tacrolimus, the late proliferative response of T cells to binding of IL-2 to its receptor are abrogated by the immunosuppressant sirolimus (rapamycin), which complexes with FKBP to inhibit mTOR (mammalian target of rapamycin).[305] All three of these agents have been used in the prevention and treatment of GVHD and solid organ-graft rejection, and the actions of calcineurin inhibitors and rapamycin on serial events in T-cell activation have encouraged their use in combination therapy. Monoclonal antibodies directed against the IL-2Rα protein such as basiliximab and daclizumab have been approved for prevention of rejection of renal allografts, and their use is being explored for treatment of multiple sclerosis and GVHD.[306,307]

Coreceptors Involved in T-Cell Activation and the Immune Synapse

Because the affinity of the TCR for cognate antigen–MHC complexes and the density of TCRs on the naïve T cell are both relatively low, activation of the T cell depends on augmentation of the TCR signal by a complex combination of TCR-associated coreceptors, adhesion molecules, and other costimulatory molecules that transduce distinct signals. Spatial separation of kinases and phosphatases facilitates activation (see Fig. 23-15). Recent techniques that visualize the distribution of TCR and these affiliated molecules in relation to the site of T cell–APC contact have led to a model in which the immune synapse segregates into central, peripheral, and distal supramolecular activation clusters (c-SMAC, p-SMAC, and d-SMAC, respectively). The functional relevance of different synapse morphologies and the dynamics of formation and disassembly of the synapse are actively being investigated.[308,309,310,311]

The CD4 and CD8 glycoprotein coreceptors bind to class II and class I MHC molecules, respectively, and are used to classify T cells into helper and cytolytic subtypes.[37] The proximity of CD4 and CD8 coreceptors to the TCR-pMHC complex via bidentate interaction of pMHC with TCR and coreceptor[34] and the association of both CD4 and CD8 intracellular domains with the Src family tyrosine kinase p56lck bring p56lck into close association with its target CD3 ζ.[312,313] CD4 and CD8 coreceptors are generally found in the c-SMAC with TCR.

CD2 is a T-cell–specific glycoprotein receptor that binds to several ligands on the APC, including CD58 (LFA-3) and CD48 (see Fig. 23-15). Binding of CD2 to its ligand on the APC serves to improve adhesion and the strength of the TCR-pMHC interaction as well as membrane-membrane distances for optimal TCR-pMHC interaction.

CD45, also known as *leukocyte common antigen*, is a phosphotyrosine phosphatase. It is generally excluded from the immune synapse in a manner that suggests that the size of the ectodomain of a given receptor determines its presence or absence in the synapse.[282] The major substrate of CD45 is thought to be p56lck, as well as possibly JAK/STATs, CD3 ζ, and ZAP70[314]; it is suggested that the dynamic repositioning of CD45 during synapse formation and disruption alters its substrate specificity.[315] Deficiency of CD45 has been studied in mice and humans and results in defects in thymic T-cell development, T-cell function, and autoimmunity.[314,316,317] Alternative splicing of the CD45 message results in multiple isoforms, most notably CD45RA, expressed in naïve or resting T cells, and CD45RO, characteristic of activated or previously activated T cells.[318]

Costimulation of T Cells

TCR ligation (signal 1), even in the presence of CD4 or CD8 engagement, is insufficient for activation of resting T cells. In the absence of appropriate costimulation, TCR occupancy alone can result in T-cell unresponsiveness (T-cell clonal anergy, ignorance, or tolerance) on subsequent exposure to antigen. Tolerance is characterized by the inability to proliferate or secrete cytokines in response to secondary stimulation by the same antigen. Anergy is classically defined as this same failure, but it can be rescued or reversed by exogenous IL-2 in vitro.[319] Costimulation (signal 2) typically is mediated by receptors on T cells recognizing ligands on activated APCs (see Fig. 23-15).

CD28 (*CD28*), the first discovered costimulatory molecule that prevents the induction of anergy, is the original defined member of a family of related molecules. Human CD28, a homodimer of two 44-kd glycoproteins, is expressed on essentially all CD4+ cells and approximately 50% of CD8+ cells. CD28 interacts with the B7 family members CD80 (*CD80*, B7-1) and CD86 (*CD86*, B7-2). Interaction of CD28 with CD80 or CD86 increases, quantitatively, the amount of IL-2 produced by both transcriptional and post transcriptional mechanisms,[320] augments T-cell proliferative responses, and enhances T-cell survival by induction of the anti-apoptotic protein Bcl-X_L.[321] Notably, CD28-dependent costimulation appears to be resistant to inhibition by the immunosuppressants cyclosporine and tacrolimus (FK506), inhibitors of the serine/threonine phosphatase calcineurin, thus suggesting that this pathway uses a different cascade of biochemical signals than the calcineurin-sensitive TCR complex.[322-324]

Structurally similar to CD28, CD152 (cytotoxic T-lymphocyte antigen 4, *CTLA4*) is another CD28 family member that also binds to CD80 and CD86. CD152 is expressed only minimally on resting T cells but is induced on recently activated T cells. Mice rendered genetically deficient in *Ctla4* expression die postnatally with progressive lymphoproliferation[325,326]; it is now clear that the endogenous function of CD152 is to downregulate T-cell activation to terminate T-cell responses.[322,327]

TABLE 23-3 Stimulatory Molecules

Receptor	Expression	Ligand	Expression	Major Effect of Engagement
CD28	CD4 T cells, CD8 T cells (50%)	CD80, CD86	Dendritic cells, B cells, monocytes, T cells	Activating
CTLA4	Activated T cells	CD80, CD86	Dendritic cells, B cells, monocytes, T cells	Inhibitory
ICOS	Thymocytes, activated T cells	ICOSLG	Dendritic cells, B cells, monocytes, T cells, fibroblasts, endothelial, epithelial	Activating
PD-1	Activated T and B cells, myeloid cells	PD-L1, PD-L2	Dendritic cells, B cells, monocytes, T cells, tissues, tumors (PD-L1); dendritic cells, monocytes, some B cell lymphomas (PD-L2)	Inhibitory
Unknown		B7-H3	Dendritic cells, B cells, monocytes, T cells, NK cells, bone marrow	Unknown
CD27	T cells, some B cells, some NK cells, hematopoietic progenitors	CD70	Dendritic cells, B cells, T cells, NK cells, mast cells, smooth muscle, thymic epithelium	Activating
OX40	Activated T Cells, NK cells, neutrophils	OX40L	Dendritic cells, B cells, monocytes, T cells, NK cells, mast cells, endothelium, smooth muscle	Activating
4-1BB	Activated T cells, NK cells, mast cells, neutrophils	4-1BBL	Dendritic cells, B cells, monocytes, T cells, NK cells, bone marrow mast cells, smooth muscle cells	

CTLA-4, Cytotoxic T-lymphocyte antigen 4; *ICOS,* inducible T-cell costimulator; *NK,* natural killer; *PD-1,* programmed death 1.

CD278 (inducible costimulator [*ICOS*]) is another member of the CD28 family that was cloned by generating monoclonal antibodies directed against activated T cells.[328] Like CD152, ICOS is expressed on activated T cells. ICOS does not bind CD80 or CD86 but instead binds to a novel, TNF-α–inducible adhesion molecule, CD275 (inducible costimulator ligand [*ICOSLG*], B7h, B7-H2, B7RP-1).[329] Signicantly, engagement of ICOS appears to affect T-helper differentiation by favoring IL-10 and IFN-γ production (discussed later). Studies of deficient mice and humans with *ICOS* mutation demonstrate its critical role in T-cell help, normal humoral responses, and B-cell heavy-class switching.[229,330-332]

The number of CD28/B7 family members that function as costimulatory molecules continues to grow (Table 23-3). Expressed on thymocytes and activated immune cells, programmed death 1 (PD-1) is a CD152-like molecule that also functions as an important negative regulator of immune responses.[333-335] An autoimmune disorder resembling systemic lupus erythematosus develops in PD-1–deficient mice. The molecular mechanism of negative regulation by PD-1, like that by CD152, appears to be recruitment of a phosphotyrosine phosphatase (SHP-2) to the signaling receptor.[336] Two PD-1 ligands have been isolated; both are posited to play a role in the induction and maintenance of peripheral tolerance.[333,337,338] Finally, B7-H3, a distinct B7 family member important for costimulation of IFN-γ production, has been isolated[339]; B7-H3 binds a counter-receptor on activated T cells distinct from any CD28 family member heretofore characterized.

In addition to CD28/B7 family members, members of the TNF family function as important modulators of T-cell effector and T-cell–dependent B-cell responses. Interaction of CD154 (*CD40LG*) with CD40 is essential for B-cell activation, Ig production, and heavy-chain isotype differentiation, as discussed earlier. The interaction of CD154 with CD40 also upregulates CD80 expression on APCs and is thus important for sustaining a T-helper precursor cell (T_H1) response.[340] X-linked hyper-IgM syndrome is an autosomal recessive disorder characterized by a deficiency of CD4+ T-cell function and T-cell–dependent antibody responses; it is caused by mutation in the *CD40LG* gene located on human chromosome X.[341] Affected patients have normal or increased IgM levels and very low to undetectable IgG and IgA levels and are susceptible to opportunistic infections such as *Pneumocystis jiroveci* because of intrinsic CD4□ T-helper dysfunction.[342,343] Allogeneic stem cell transplantation offers a curative therapy for patients with an appropriate donor.[344] Murine models of X-linked hyper-IgM syndrome have been developed by genetic disruption of either the *Cd40* or *Cd40lg* genes, which mimics the phenotype of the human disorder.[345-348]

Other TNF family molecules that are important for modulating the T cell response include CD27 *(TNFRSF7)*, CD134 (OX40, *TNFRSF4*), and CD137 (4-1BB, *TNFRSF9*). All of these molecules are inducible on activated T cells. CD70 (CD27L, *TNFSF7*), a protein of approximately 50 kd that is transiently expressed on recently activated T and B lymphocytes, binds CD27, a homodimer expressed on the surface of naïve CD4+ and CD8+ T cells.[349,350] CD27/CD70 interactions promote TNF-α but not IL-2 production from T cells, as well as expression of CD154 on the T-cell surface. This receptor-ligand interaction also appears to induce B-cell terminal differentiation to plasma cells.[117] Expression of CD134 (OX40, *TNFRSF4*), a glycoprotein of approximately 48 kd, depends on TCR signaling on CD4+ activated cells and binds its ligand CD252 (OX40L, *TNFSF4*) which is expressed on lymphocytes, APCs, and also endothelium and smooth muscle.[351] CD134/CD252 engagement enhances T-cell proliferation and cytokine production at limiting antigenic densities, enhances B-cell and dendritic cell function, and modulates T-cell migration to B-cell follicular areas.[352,353] Thus CD134/CD252 interactions have been suggested to prolong and enhance clonal expansion and immune responses at sites of infection and inflammation. 4-1BB[354-357] is a transmembrane glycoprotein whose expression is induced on T cells after activation and binds to its ligand CD137L (4-1BBL, *TNFSF9*), which has an expression pattern similar to that of OX40L.[351] All these TNF family ligand-receptor pairs share a common signaling pathway via the TNF receptor–associated factors (TRAFs), which bind IκBα and activate the NF-κB pathway.[351] These receptors serve to enhance T cell survival, proliferation, and cytokine production; modulate the inflammatory milieu through cytokine production by APCs; and affect B-cell proliferation and antibody production.[351] The potential effect of blockade or neutralization of OX40 or 4-1BB is complicated to predict given that these receptors are also expressed on regulatory T cells, with some studies showing inhibition and others showing enhancement of regulatory T-cell generation or survival.[351]

Manipulation of T cell stimulation by soluble receptors, ligands, or antibody-mediated blockade is of increasing interest in the treatment of autoimmunity, cancer, and solid organ rejection.[358-360] More recently, the discovery that regulatory T cells (discussed later) are also exquisitely sensitive to costimulatory signals has complicated the potential outcome of such therapeutics. The pleiotropic effects in vivo of our current armamentarium are underscored by the sobering case of six patients in England who suffered life-threatening cytokine storm within hours of receiving an anti-CD28 antibody agonist intended to expand regulatory T cells.[361] Nevertheless, progress has been made in that blockade of costimulation with CTLA4-IgG1 (abatacept; see Table 23-4) has been approved for use in rheumatoid arthritis and juvenile inflammatory arthritis,[362,363] and its use is being explored in other settings.

TABLE 23-4 Selected Monoclonal Antibodies and Fusion Proteins in Therapeutic Use for Treatment of Autoimmune or Inflammatory Disease, or Transplantation

Generic Name*	Trade Name	Type	Target	Approved Use
natalizumab	Tysabri	Humanized mAb	α4 integrin	Multiple sclerosis, Crohn disease
belimumab	Benlysta	Human mAb	BAFF (BLys)	Systemic lupus erythomatosus
rituximab	Rituxan	Chimeric human mAb	CD20	Refractory rheumatoid arthritis
ofatumumab	Arzerra	Human mAb	CD20 membrane proximal epitope	Rheumatoid arthritis
epratuzumab	Lymphocide	Humanized mAb	CD22	Systemic lupus erythromatosus, Sjogren disease
daclizumab	Zenapax	Humanized mAb	CD25	Transplant rejection
basiliximab	Simulect	Chimeric mAb	CD25	Transplant rejection
ipilimumab		Human mAb	CTLA4	Melanoma
abatacept	Orencia	CTLA4 extracellular domain-IgG1 Fc fusion protein	CTLA4	Rheumatoid arthritis refractory to TNF blockade
anakinra	Kineret	IL1RA	IL-1	Rheumatoid arthritis
tocilizumab	Actemra	Chimeric mAb	IL6R	Rheumatoid arthritis
infliximab	Remicade	Chimeric human mAb	TNFα	Psoriasis, Crohn disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis, ulcerative colitis
etanercept	Enbrel	TNF receptor 2-IgG1 Fc fusion protein	TNFα	Psoriasis, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis
adalimumab	Humira	Human mAb	TNFα	Psoriasis, Crohn disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis, juvenile idiopathic arthritis, ulcerative colitis

TNF, Tumor necrosis factor.

*The nomenclature of monoclonal antibodies has evolved to denote the origin of the variable and constant regions. The suffix *mab* denotes a monoclonal antibody. Antibodies that are fully murine have *mo* immediately preceding *mab*. Antibodies in which the constant regions have been replaced with human are denoted as chimeric with *xi* immediately preceding *mab*. Antibodies in which the constant regions and part of the variable regions have been replaced with human are denoted as humanized with *zu* immediately preceding *mab*. Finally antibodies that are fully human with no murine elements are denoted with *u* immediately preceding *mab*.

Progress in the understanding of T-cell stimulation and activation with regard to intracellular signaling has been further exploited to engineer T cells directed against cancer cells, bearing so-called *chimeric antigen receptors* (CARs).[364,365] CARs are fusion receptors in which the specificity resides in single-chain variable fragments (scFv) from monoclonal antibodies directed against a tumor antigen of choice, and the signaling domains are derived from components of the T-cell receptor signaling machinery. The first such molecule used the CD3 ζ signaling domain.[366] These fusion receptors have some key advantages, including that specificity does not require presentation via MHC, that scFv with the appropriate avidity can be chosen or designed, and that CARs do not pair with TCR or compete with TCR for CD3 chains. That CARs can only be directed against cell surface proteins is a significant disadvantage insofar as it obviously limits the tumor antigens that can be targeted. Many technical hurdles must be overcome to make CARs safe and effective, including efficient transduction into human T cells, adjustment of the size of the spacer between scFv moiety and transmembrane region to optimize avidity,[367,368] addition of signaling domains beyond CD3 ζ alone to improve function,[369-371] and persistence of CAR bearing T cells in vivo.[372,373] The signaling moiety of CARs has evolved from CD3 ζ alone, which was insufficient to drive cytokine production and T cell expansion, to dual or multiple signaling modules. Including the cytoplasmic domain of CD28, 4-1BB, OX40, or others significantly enhanced the activity of CARs when expressed in primary T cells.[365] CARs directed against CD19 have had recent success in the treatment of human B cell malignancies, including chronic lymphocytic leukemia and acute lymphoblastic leukemia,[374-376] and a host of other malignancies are being targeted with this novel technology.

PERIPHERAL T-CELL MATURATION

A hallmark of adaptive immunity is the generation of an antigen-specific response that can be rapidly recalled upon reexposure to the same antigen at a later time.[377,378] Naïve T cells released from the thymus have a broad range of TCR specificities, with few clones that recognize any given antigen, and they generally do not proliferate in the absence of cognate antigen. Exposure to antigen results in differentiation and rapid proliferation of the specific naïve precursor population into effector T cells that either kill targets bearing cognate antigen in the case of CD8+ T cells or produce polarized sets of effector cytokines in the case of CD4+ T cells. The expansion of effector cells is rapid and brief, with the majority dying by activation-induced cell death. Long-lived memory T cells that survive after the effector response serve to increase the precursor frequency in the periphery and thus arm the organism for a more rapid and efficient response on subsequent exposure to the same antigen.

Naïve T-Cell Differentiation to Effector and Memory T Cells

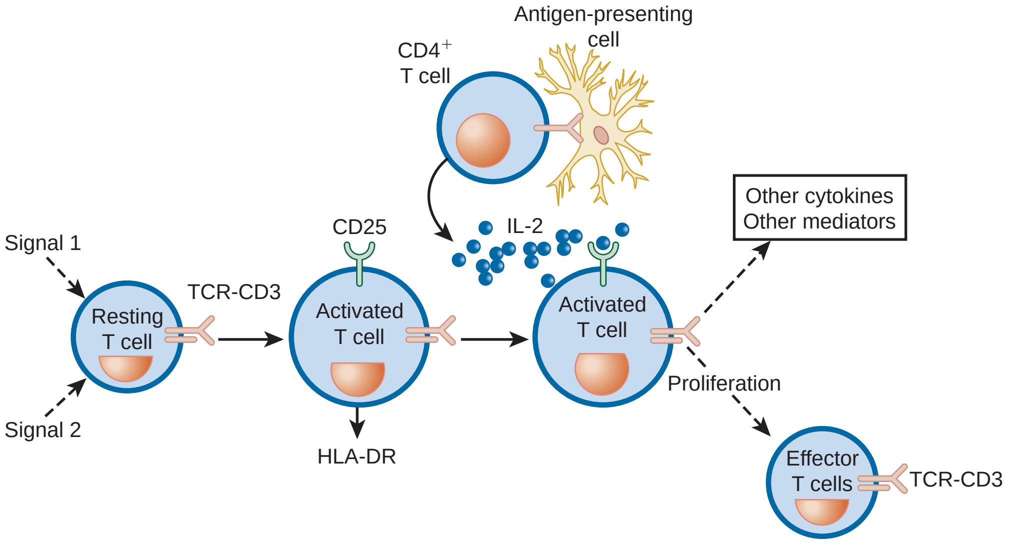
Circulating naïve, effector (activated), and memory T cells can be differentiated by phenotypic differences as well as by genetic changes (Fig. 23-16). With T-cell activation, a number of cell surface receptors, including CD2, CD58, LFA-1, CD29, and CD44, are upregulated and increase the avidity of the interaction between the T cell and the antigen-bearing APC. CD45RA, which is constitutively expressed on naïve T cells, is downregulated and replaced by the CD45RO isoform.[379]

More recently, human T-cell memory populations have been further characterized by the expression of CD62L (L-selectin) and the chemokine receptor CCR7 as either effector memory (CCR7–) or central memory (CCR7+) subsets. CCR7 is a lymph node–homing receptor that interacts with its ligand SLC on endothelial cells.[380] It is proposed that differential CCR7 expression serves to segregate effector memory cells to tissues, where they rapidly release cytokines and kill targets, and central memory cells to lymph nodes, where interactions with dendritic cells and B cells orchestrate effective T-cell help and secondary proliferation.[381,382] In addition to these cell surface markers, tetramer-based technology now allows identification of antigen-specific CD8+ memory cells on a research basis.[383] Four class I MHC molecules are bound covalently and tagged with fluorescent marker and then loaded with specific cognate peptide.[384] T cells that recognize that MHC-peptide complex bind the tetramer and can be identified by flow cytometry.

Helper T-Cell Differentiation and Function

CD4+ helper T cells are now known to further differentiate into distinct subsets with specialized functions. The cytokines elaborated during the normal innate inflammatory response to pathogens results in induction of master transcription factors that drive differentiation into the appropriate pathway, thereby tailoring the immune response to the pathogen in question. Currently 5 T_H subtypes have been identified: T_H1, T_H2, T_H17, inducible Treg (discussed later), and T_{FH}. Although these subsets are frequently referred to as *lineages,* the growing evidence of plasticity between these cells, most notably the conversion of T_H17 cells into iTreg, strengthens the argument that they should be classified as subsets rather than lineages.[207]

Effector helper T cells were first described to differentiate from naïve CD4+ T-helper precursors (T_Hp) into two main classes of T_H cells with distinct functional properties.[385,386] A panoply of factors in the microenvironment where antigen recognition occurs have been shown to influence the differentiation of T_H cells, including the nature and density of ligand, the strength of TCR ligation, the presence and kinds of costimulatory molecules, and the differentiation state of the APC.[387] The cytokines in the immediate milieu are thought to be critical and result in vitro and in vivo in the early upregulation of canonic transcription factors that drive the T_H cell to one subset while suppressing the transcriptional program characteristic of other subsets (Fig. 23-17).[388,389] For instance, under the influence of IL-12 and IFN-γ, the transcription factor T-bet is upregulated and drives the cell to the so-called T-helper type 1 (T_H1) subset, which is characterized by production of the inflammatory cytokines IL-2, IFN-γ, and TNF-α.[390] These cytokines drive



Changes in receptor function

Resting T cells	Qualitative	Quantitative	Memory T cells
CD45RA$^+$	CD8	HLA-DR	CD45RO$^+$
CD29^{low}	LFA-1	CD71	CD29^{high}
CD2^{low}	CD2	CD25	CD2^{high}
CD58^{low}	VLA-4	CD26	CD58^{high}
LFA-1^{low}			LFA-1^{high}
CCR7^{high}			CD44^{high}
CD62L^{high}			CD62L^{low}
			CCR7^{high} (central memory)
			CCR7^{low} (effector memory)

Figure 23-16 T-cell activation resulting in proliferation, acquisition of immunocompetence, and memory. Resting T cells are stimulated by antigen/major histocompatibility complex (MHC) molecules in the presence of a number of secondary signals. Appropriate activation results in an increase in both cell size and entry into the cell cycle. The high-affinity interleukin-2 (IL-2) receptor is expressed on the cell surface. Other activation antigens, such as transferrin receptor (CD71) and HLA-DR, are also expressed on the cell surface. The T cell is stimulated to produce cytokines (e.g., IL-2) that act in an autocrine and paracrine fashion on the T cell to promote cell replication and maturation to effector cells. CD4+ T cells generally differentiate to become helper T cells that are able to secrete IL-2 and other cytokines in response to peptide antigen in the context of MHC class II proteins. These cytokines in turn help B cells and other T cells to proliferate and differentiate. T lymphocytes that express CD8 on their cell surface generally function as cytotoxic T lymphocytes (CTLs) and are able to kill target cells that express a foreign peptide antigen or viral product in the context of MHC class I proteins. In addition, a certain proportion of CD8+ T cells function as suppressor T cells and inhibit the ability of B cells to secrete immunoglobulin, the generation of CTLs, or the production of cytokines by CD4+ cells. A small proportion of circulating, stimulated T cells become immunocompetent memory T cells that are able to respond to antigen exposure in a secondary immune response. The phenotype of resting T cells changes upon T-cell activation, transcriptional activation, and acquisition of the memory phenotype. Certain cell surface receptors are upregulated quantitatively as shown, within days after activation. Although the level of expression of the TCR complex remains constant, that of CD2, CD58, CD29, and CD44 increases. L-selectin expression decreases, and CD45RA expression falls. Expression of CD45RO is characteristic of the memory phenotype. In addition to early (e.g., CD2) and late (e.g., VLA-1) quantitative changes in receptor expression, the avidity of a number of T-cell surface receptors is changed quantitatively after T-cell triggering. The avidity of LFA-1 (CD11a/CD18) for its ligands is upregulated after T-cell stimulation, as is CD8 for MHC class I proteins, CD2 for CD58, and VLA-β_1 receptors for their ligands. The specific and dynamic regulation of T-cell avidity may impart sensitivity and plasticity to the immune response.

paracrine expansion of T cells and activation of macrophages and other innate cells, thus providing help to CD8+ T cells and promoting cell-based immune responses. IL-4 in the environment, in contrast, upregulates the transcription factor GATA-3, thereby directing the cell to a T-helper type 2 (T_H2) cell fate.[391,392] The elaboration of T_H2 cytokines such as IL-4 promotes class switching of B cells and is important for T-cell–mediated help for humoral responses. IL-4 and other T_H2 cytokines such as IL-5 and IL-13 also promote eosinophil differentiation, IgE production, and the expulsion and control of parasites. Imbalance of T_H1 and T_H2 cell responses is thought to play a role in the development of autoimmunity, allergy, and asthma.

T_H17 cells (see Fig. 23-17) are a more recently defined subset, characterized by high-level production of the cytokine IL-17, which participates in the normal immune response to bacteria and fungi, and in pathogenic processes such as autoimmunity.[393-396] Differentiation along the T_H17 pathway is induced by exposure to TGB-β and IL-6, which upregulate the master regulator RORγt (encoded by *RORC*)[397]; the paracrine expansion

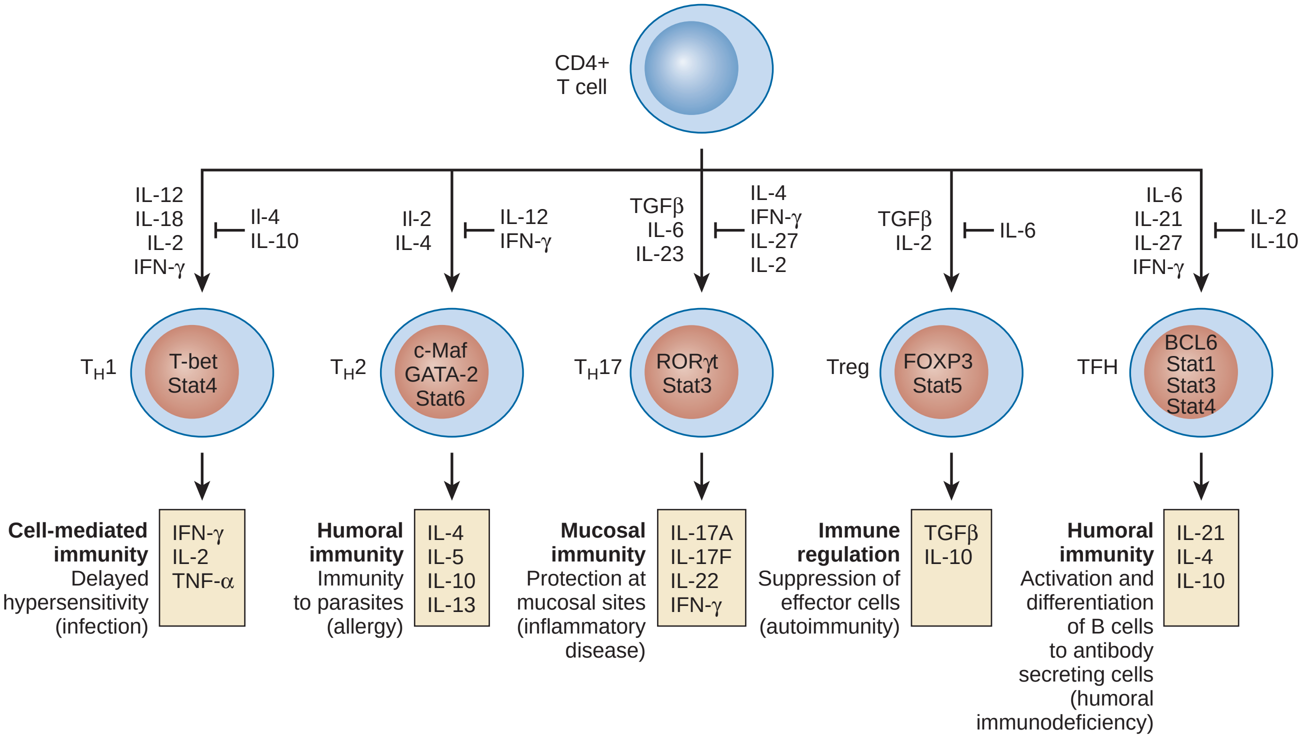

CD4+
T cell
IL-12
IL-18
IL-2
IFN-γ
Il-4
IL-10
Il-2
IL-4
IL-12
IFN-γ
TGFβ
IL-6
IL-23
IL-4
IFN-γ
IL-27
IL-2
TGFβ
IL-2
IL-6
IL-6
IL-21
IL-27
IFN-γ
IL-2
IL-10
T_H1
T-bet
Stat4
T_H2
c-Maf
GATA-2
Stat6
T_H17
RORγt
Stat3
Treg
FOXP3
Stat5
TFH
BCL6
Stat1
Stat3
Stat4
Cell-mediated immunity
Delayed hypersensitivity (infection)
IFN-γ
IL-2
TNF-α
Humoral immunity
Immunity to parasites (allergy)
IL-4
IL-5
IL-10
IL-13
Mucosal immunity
Protection at mucosal sites (inflammatory disease)
IL-17A
IL-17F
IL-22
IFN-γ
Immune regulation
Suppression of effector cells (autoimmunity)
TGFβ
IL-10
Humoral immunity
Activation and differentiation of B cells to antibody secreting cells (humoral immunodeficiency)
IL-21
IL-4
IL-10

Figure 23-17 Differentiation of naïve CD4+ cells to T-helper subsets (T_H1, T_H2, T_H17, T_{reg}, T_{FH}). The mechanisms of development of these subsets have largely been delineated in murine models, and polarization of human CD4+ T cells into these subsets in general is less distinct than murine. In the case of T_{reg}, polarization of in vitro of human T-helper cells using cytokines as described in the mouse does not result in cells with strong suppressive capacity. Naïve CD4+ cells, stimulated by ligation of the T-cell receptor and costimulatory molecules, differentiate into four classes of helper T cells. T_H1 cells produce inflammatory cytokines, characterized by interferon-γ (IFN-γ) and interleukin-2 (IL-2), that are capable of activating macrophages and promoting the further expansion of T cells; they appear to be the principal effectors of cell-mediated immunity. T_H2 cells produce IL-4, IL-10, and IL-5, among others, important for IgE production by B cells and differentiation of eosinophils, and thus are the principal effectors of humoral immunity. The cytokines produced by T_H1 cells (IL-2, IFN-γ, and IL-12) inhibit the induction of T_H2 differentiation, whereas the cytokines produced by T_H2 cells (IL-4 and IL-10) reciprocally inhibit the differentiation of T_H1 cells. T_H17 and T_{reg} cells both arise out of TGF-β rich environments with IL-6 driving towards T_H17 and all-trans retinoic acid (ATRA) driving towards T_{reg}. T_H17 cells produce IL-17A, IL-17F, IL-22, and IFN-γ, which stimulate and recruit innate immune cells to function particularly at mucosal surfaces. There is plasticity between T_H17 and T_H1 cells. T_{reg} cells in the mouse induced from naïve T-helper cells by exposure to TGF-β and ATRA and in vivo–derived human T_{reg} cells expressing FOXP3 exhibit suppressive activity of conventional T cells. T_{FH} cells arise in the secondary lymphoid organs through a combination of exposure to cytokines and cell-cell contact with dendritic cells and B cells, in a two-way interaction that leads to differentiation of T helper cells into T_{FH} and naïve B cells into germinal center B cells. *TNF-α*, Tumor necrosis factor α.

of T_H17 committed cells and further maturation of effector function appears to be driven by IL-21 and IL-23.[398-401] The primary cytokine made by T_H17 cells is IL-17A, but other inflammatory cytokines are produced, including IL-17F, TNF-α, IL-22, and IL-26. T_H17 cells express a variety of chemokine receptors such as CCR6, CCR4, and CXCR3, which in turn induce trafficking of T_H17 cells to skin and mucosal surfaces; the IL-17 elaborated induces epithelial cells to produce IL-8 and other mediators of neutrophil migration; and IL-22 induces the production of antibacterial peptides such as defensins.[402] Thus in murine models T_H17 cells are critical for handling of bacterial infections at mucosal surfaces such as *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, and others.[402] T_H17 cells have been increasingly implicated in a number of pathogenic inflammatory conditions in humans, including arthritis, multiple sclerosis, inflammatory bowel disease, asthma, transplant rejection, and GVHD.

T_{FH} cells are professional providers of T-cell help to B cells in the B-cell follicles of the GC, as reviewed previously. T_{FH} cells are induced to differentiate by the master regulator BCL6, which has been shown to be both necessary and sufficient in murine models.[219,220,403] These cells are primarily localized in GC; express CXCR5, SAP, and high levels of ICOS; and are high producers of IL-21, which in turn is critical for the induction of class switch recombination and differentiation of B cells into antibody-secreting cells.[209-211,213,217,404,405] Cells of a similar phenotype can be found circulating in the peripheral blood of mice and humans, although the relationship of these T_{FH}-like cells to their counterparts in the spleen or tonsil remains to be clarified.[207,212,404,405] The process of complete differentiation into T_{FH} cells is not a one-way interaction with B cells; rather, there are multiple lines of evidence that B cells, particularly antigen-specific B cells, are required.[219,406,407] This fact is underscored by human and murine models of deficiency in B-cell development that are also associated with a lack of T_{FH} cell development.[408]

Cytotoxic T-Cell Differentiation and Function

The primary function of effector CD8+ T cells is direct killing of APCs infected by virus or other intracellular organisms. Cytotoxicity is antigen dependent; is contact dependent; and does not result in destruction of the cytotoxic T cell itself, which can kill multiple targets serially. At least two mechanisms of cytolysis are now recognized: cytotoxic granules and the Fas-Fas ligand pathway. Effector CD8+ T cells release the contents of cytotoxic granules into the immune synapse after contact with an infected cell. The main components of these granules are perforin and the serine esterases granzyme A and granzyme B.[409,410] Perforin molecules form multimerized pores in the target cell and are classically thought to mediate delivery of granzymes into the cytoplasm. Targets then die by a combination of osmotic swelling and proteolytic damage. The importance of perforin in both responses to infection and immunoregulation is apparent by the study of patients with congenital mutations in both alleles of perforin, which results in congenital hemophagocytic lymphohistiocytosis.[411,412] Because perforin is also produced by NK cells (discussed later), this disease is characterized by deficiencies in both CD8 cell and NK cytotoxic function. The second mechanism is induction of caspase-mediated apoptosis by engagement of CD95 (Fas, Apo-1) on target cells with its ligand CD95L (Fas ligand, CD178) on effector CD8+ T cells. CD95L is not constitutively expressed on naïve T cells and is upregulated on activation of T cells and NK cells. That this pathway also has important immunoregulatory function is demonstrated by the fact that mutations in CD95 or CD95L typically cause not overwhelming infection but generalized lymphoproliferation and autoimmunity in humans, the autoimmune lymphoproliferative syndrome.[413,414,415]

Control of the Immune Response: Regulation and Termination of T-Cell Activation

Maintaining a state of unresponsiveness to self-antigens, or self-tolerance, is as important for a healthy immune system as recognition of nonself, or foreign, antigens from infectious agents or tumors. Failure of self-tolerance can lead to autoimmunity. The main mechanisms of tolerance include central or clonal deletion (deletion by negative selection in the thymus, discussed earlier), anergy, activation-induced cell death (AICD; termination of T-cell activation), and cell-mediated suppression or regulation.

A productive T-cell response requires stimulation by antigen-MHC complexes via the T-cell receptor (signal 1) and appropriate costimulation (signal 2). T cells that encounter antigen in the absence of costimulation enter a state of anergy. *Anergy* is generally defined as the inability to proliferate to a specific antigen when rechallenged while maintaining the ability to proliferate in response to cytokine (i.e., IL-2).[319,416] Anergy ensures that autoreactive T cells that encounter peripherally expressed antigens in a noninflamed environment, where costimulatory molecules on APCs have not been upregulated, are not activated. AICD is important to ensure that the robust clonal expansion of antigen-specific T cells is short-lived and contracted. T cells exposed to foreign antigen or autoreactive T cells that escape thymic deletion and encounter self-antigen in the periphery can be killed by the CD95-CD95L pathway, which activates caspases and other death signals. The importance of the CD95-CD95L pathway for control of lymphoproliferation in humans is demonstrated by the autoimmune lymphoproliferative syndrome (ALPS). Patients with ALPS have mutations in CD95 or more rarely CD95L or caspases, which leads to uncontrolled lymphoproliferation and autoimmunity.[415]

T cells that mediate suppression or regulation of other T cells have long been purported to exist. A subset of CD8+ T cells, so-called suppressor cells, have been described, but in recent years a distinct population of mostly CD4+ T cells with regulatory properties has emerged as an important effector of cell-based regulation. This population of T-regulatory (Treg) cells can be distinguished from other CD4+ T cells by the expression of certain cell surface receptors, most commonly CD25,[417] and more recently by expression of a key transcription factor, Foxp3.[418,419,420] Foxp3 expressing Treg can be divided into two types: those that arise continuously during development in the thymus (so-called *natural Treg*, or nTreg) or are formed during the course of an antigen-specific immune response in the periphery (so-called *inducible Treg*, or iTreg).[421,422] FoxP3+ CD4+ CD25+ T cells develop in the thymus and in murine and human models require IL-2 for development and maintenance.[423] Humans deficient in *FOXP3* have the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which is characterized by autoimmune diabetes and thyroiditis, inflammatory bowel disease, allergy, and other organ-specific autoimmunity.[424-426] In vitro, suppressive activity of these cells is defined by the ability to depress proliferation by lymphocytes stimulated via the TCR.[427-429] In vivo depletion of these cells in mice leads to spontaneous autoimmunity, whereas cotransfer suppresses autoimmunity in various models.[423] The iTreg can arise from Thp after exposure to TGF-β and derivatives of vitamin D. They can be distinguished from nTreg on the basis of expression of Helios, and the demethylation state of the *FOXP3* locus.[430,431] The shared requirement for TGF-β for both T_H17 and iTreg development, and the finding that interconversion of these cells can occur has led to a model featuring co-development and plasticity between these two subsets.[432,433] Likewise, perturbation of a coordinated and flexible response of T_H17 and iTreg may underlie the pathogenesis of inflammatory disease.

NONCONVENTIONAL T-CELL SUBTYPES

In addition to conventional TCR αβ–bearing T cells that recognize peptide antigens, other nonconventional smaller populations of T cells exist. The function of these cells is less well characterized, but their conservation through evolution implies nonredundant roles in the immune system. Both populations share the ability to bind to the nonclassic MHC molecules and have restricted TCR diversity. They are generally believed to have functions during the innate immune response. Additionally, a host of innate lymphoid cells have been characterized; these are beyond the scope of this chapter, but they have been reviewed recently. These innate lymphoid cells appear to have critical functions at mucosal surfaces such as lung, gut, and skin.[434-436]

Development and Function of CD1-Restricted T Cells or Invariant Natural Killer T Cells

A subset of T cells that bear αβ TCR but recognize lipid antigens rather than peptide antigens has been defined in several ways over the past couple of decades. These cells were first characterized in the mouse as TCR αβ T cells that co-expressed cell surface markers also found on NK cells such as NK1.1 (CD161) and therefore were termed NK T cells.[437] When it was found that a subset of NK T cells recognize the CD1 family of nonclassic MHC molecules,[438] the term *CD1-restricted T cells* was adopted as a more precise functional definition; conversely, not all CD1-restricted T cells express NK1.1. CD1 is a family of proteins with one isoform in the mouse, CD1d, and five isoforms in humans, CD1a, CD1b, CD1c, CD1d, CD1e, the last of which is not expressed on the cell surface.[439] These MHC class I–like proteins are generally expressed in a complex with β_2-microglobulin. The diversity of TCRα rearrangements used by CD1d-restricted T cells is limited, with many bearing Vα24-Jα18 rearranged TCRα protein, preferentially complexed to a Vβ11 TCRβ protein. Thus the term *invariant NK T cells* is also used to distinguish this main population from those with diverse αβ TCR restricted by CD1 molecules, and most studies have focused on the invariant CD1d-restricted subset.

Invariant NK T cells develop in the thymus, arise from the DP stage of development, and in murine models are selected by other DP thymocytes that express CD1d rather than by thymic stroma.[440] In contrast to conventional T cells, invariant NK T cells recognize lipid presented by CD1.[441] A number of foreign lipids and glycolipids have been identified that are recognized by invariant NK T cells, although the discovery of self-antigens has been more elusive.[442] In the laboratory, α-galactosylceramide, derived from marine sponge, is a highly potent agonist for invariant NK T cells[443] and can be loaded onto tetramers of CD1d molecules tagged to fluorescent molecules to identify human or mouse invariant NK T cells by flow cytometry, similar to MHC tetramer technology.[444,445] The structure of the human NK T-cell receptor complexed to CD1d loaded with α-galactosylceramide has been reported and yields interesting insights into the plasticity of this particular TCR.[446] In humans, about half of these cells do not express either CD4 or CD8 and half express CD4.[447] Invariant NK T cells produce cytokines, such as IL-4 and IFN-γ, more rapidly than conventional TCR αβ T cells do, within hours in mice after in vivo injection with antigen.[448] This early secretion has been proposed to function as a bridge between innate and adaptive immunity in diverse processes, including the response to infection, antitumor immunity, autoimmune disease, and asthma.[449] In sickle cell anemia, invariant NK T cells are both more numerous and hyperactivated; the adenosine A2A receptor agonist regadenoson has recently been shown to modulate invariant NK T cell number and activation state in patients with sickle cell anemia during vaso-occlusive crisis.[450,451]

DEVELOPMENT AND FUNCTION OF T-CELL RECEPTOR γδ T CELLS

TCR γδ T cells can be identified in mammals, chickens, and even cartilaginous fish, though not in jawless fish.[452] Although they develop in the thymus alongside TCR αβ T cells, in contrast to conventional T cells, the variety of γδ receptors is highly restricted, with six V_γ genes used (5 $V_\gamma1$ and 1 $V_\gamma2$) and 8 to 10 V_δ genes, three of which are commonly used in humans.[452,453] TCR γδ T cells make up a very small percentage of cells in the thymus or lymph node, on the order of 1% to 2%, but are much more abundant in mucosal surfaces, particularly the human gastrointestinal tract. Interestingly, the distribution of human TCR γδT cells is skewed toward particular γδ combinations: peripheral blood contains a preponderance of $V_\gamma9V_\delta2+$ T cells, whereas TCR γδ T cells found in intestinal epithelium often bear $V_\delta1+$ or $V_\delta3+$ TCR.[453] Most of these cells do not express either the CD4 or CD8 coreceptor. Although it is known that γδ rearrangement precedes αβ rearrangement and that γδ development occurs early during embryonic life, whether γδ development depends on ligand binding is unclear. Indeed, the natural ligands of TCR γδ T cells remain poorly defined but can be nonpeptides, as shown for circulating human TCR γδ T cells that recognize mycobacterial antigens.[454-456] $V_\gamma9V_\delta2+$ T cells can generally recognize various types of phosphoantigens, whereas non-Vδ2+ T cells have more diverse ligands, including MHC class I–like molecules such as CD1. TCR γδ T cells can be expanded during responses to a variety of infections and tumors.[453]

NATURAL KILLER CELL DEVELOPMENT AND FUNCTION

In the peripheral blood of all human beings are circulating lymphocytes that morphologically resemble large granular lymphocytes and have distinctive physical characteristics that allow enrichment on a Percoll density gradient in vitro. These cells are termed *NK cells* and consist of a distinct subset of lymphocytes that, unlike T lymphocytes, do not rearrange the T-cell antigen receptor genes (see Table 23-1).[457] These cells have the capacity to mediate the spontaneous lysis of sensitive target cells, including certain tumor cells, virus-infected cells, and hematopoietic cells, and to mediate ADCC.[458,459] NK cells appear to lyse their targets by pathways similar to those used by cytotoxic T lymphocytes.[458-460] NK cells have been shown to have a role in allograft rejection and tumor surveillance. Despite the relative homogeneity of their morphologic appearance and function, NK cells are clearly a heterogeneous population of cells. NK cells are derived from a common lymphoid precursor cell. Peripheral blood NK cells may be derived directly from precursors in the bone marrow or may transit through the thymus, although the thymus is not required for their development. Phenotypic and functional NK cells may arise from immature CD7+ CD1– TCR– CD3– CD4– CD8– prothymocyte precursors,[461,462] and they express CD2. Recent data indicate that Flt3L, IL-15, and IL-21, a cytokine closely related to IL-2 and IL-15, are all involved in NK hematopoietic development and expansion.[214] More recent evidence suggests that NK cells in humans can also mature in secondary lymphoid tissue.[463]

In addition to a role in immune surveillance, a role has been documented for NK cells in protection from viral infections, which is particularly important in the early phases of an immune response before T-cell recruitment. Indeed, NK cells are critical for protection against latent DNA viruses of the herpes family inasmuch as individuals with a deficiency in NK cell number or function typically have overwhelming herpesvirus infections.[460] Viral infection can trigger NK cell activity in several ways. First, elaboration of cytokines such as IFN-α, IFN-β, and IL-12 by infected cells, particularly monocytes, macrophages, and dendritic cells, induces NK cells to produce IFN-γ.[464] Second, cells expressing viral antigens that are then coated with specific Ig can bind to FcR on the surface of NK cells and trigger ADCC. Finally, NK cell surface receptors that usually recognize MHC class I and class I–like molecules mediate so-called *missing self-recognition*.

NK cells interact with and are regulated by nonclassic MHC class I molecules. The killer cell Ig-like receptors (KIRs) recognize HLA class Ia and class Ib proteins, including HLA-G.[465] Members of a related family, termed *Ig-like transcripts* or *leukocyte Ig-like receptors (LIRs)*, also recognize HLA class I proteins and are expressed not only by NK cells but also by cells of the myelomonocytic lineage.[466] These families of receptors act as inhibitory receptors that downregulate NK cell activity on recognition of ligand by recruiting negative regulatory signaling proteins (e.g., tyrosine phosphatases) to the receptor complex.[467,468] Thus NK cells can be activated or inhibited by HLA class I–like molecules; net activity depends on expression of these receptors and expression of MHC class I molecules on the target cells. Viruses may use the ability to downregulate MHC class I expression as a means of subverting the immune response; cells with low class I MHC expression are, however, targets of NK cytotoxicity. Significantly, human cytomegalovirus expresses an MHC class I homologue, UL18, which associates with β_2-microglobulin and endogenous host peptides. UL18 binds with high affinity to LIR-1 and related NK molecules, thereby effectively competing with host class I molecules, and escapes immune recognition.[469-471]

CONCLUSIONS

T and B cells play a central role in the acquired immune response, and NK cells are an early and critical effector of the innate immune response. The evolution of an adaptive immune response with the capacity for long-lived anamnestic responses affords exquisite specificity and adaptability to higher animals such that novel infections or arising malignancy can be both cleared initially and protected against upon reexposure. Indeed, the requirement for T-cell function in normal homeostasis is exemplified by the consequences of its suppression. Deficiency of T-cell function associated with acquired immunodeficiency syndrome (AIDS), allogeneic stem cell transplantation, or T-cell–directed immunosuppressive therapy places patients at risk for herpes family viral reactivation (herpes simplex, herpes zoster, and cytomegalovirus) and lymphoproliferation (particularly Epstein-Barr virus–transformed B-cell lymphomas). As the mechanisms of immune control of nascent malignant degeneration become better understood, strategies to improve control of cancer by immunomodulatory therapy may become more tractable.

The same acquired immune response that gives higher animals an advantage in control of infection conversely predisposes these same species to the unique problems of autoimmunity. Failure of self-tolerance may result from the dysregulation of host T-cell responses after exposure to specific antigens in the setting of specific MHC molecules. Self-reactive B-cell clones may also mediate autoimmunity through inappropriate production of self-antigen–specific antibodies directed against a variety of tissue-specific targets. That enhancement of immune responses can precipitate autoimmunity is exemplified by the development of autoimmunity in melanoma patients who experienced tumor regression after peptide vaccination in the context of immunostimulatory anti–CTLA-4 antibody.[472,473] Greater understanding of antigen-specific activation and tolerance should open the way to the development of targeted immunomodulation and carry the field beyond global suppression of T-cell responses.

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CHAPTER 24

Primary Immunodeficiency Diseases

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CHAPTER OUTLINE

- COMBINED IMMUNODEFICIENCIES
  - Severe Combined Immunodeficiency
  - Other Forms of Combined Immunodeficiency with Variable Presentation
- MANAGEMENT OF COMBINED IMMUNODEFICIENCY
  - Diagnostic Approach
  - Hematopoietic Stem Cell Transplantation
  - Gene Therapy
- IMMUNODEFICIENCY SYNDROMES
  - Wiskott-Aldrich Syndrome
  - WAS Protein-interacting Protein Deficiency
  - DiGeorge Syndrome
  - FOXN1 Deficiency
  - Ataxia-Telangiectasia
- OTHER IMMUNODEFICIENCIES ASSOCIATED WITH DEFECTIVE MECHANISMS OF DNA REPAIR
- IMMUNODEFICIENCIES WITH CHROMOSOMAL INSTABILITY
- PIGMENTARY DILUTION DISORDERS
- MUTATIONS OF THE NUCLEAR FACTOR κ-B PATHWAY
- DEFECTS OF TOLL-LIKE RECEPTOR SIGNALING
- THE SYNDROME OF WARTS, HYPOGAMMAGLOBULINEMIA, INFECTIONS, AND MYELOKATHEXIS
- HYPERIMMUNOGLOBULIN E SYNDROME CAUSED BY *STAT3* MUTATIONS
- AUTOSOMAL RECESSIVE HYPER-IMMUNOGLOBULIN E SYNDROME CAUSED BY *TYK2* MUTATIONS
- CHRONIC MUCOCUTANEOUS CANDIDIASIS
- IMMUNODEFICIENCIES WITH SKELETAL ABNORMALITIES
- IMMUNODEFICIENCIES ASSOCIATED WITH DISORDERS OF FOLATE AND COBALAMIN METABOLISM
- *GATA2* DEFICIENCY
- IMMUNODEFICIENCIES WITH IMMUNE DYSREGULATION
- AUTOIMMUNE POLYENDOCRINOPATHY-CANDIDIASIS-ECTODERMAL DYSTROPHY SYNDROME
- IMMUNE DYSREGULATION POLYENDOCRINOPATHY ENTEROPATHY X-LINKED SYNDROME
- DEFECTS OF IL-2–MEDIATED SIGNALING
- AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME
- ITCH DEFICIENCY
- IMMUNODEFICIENCIES WITH IMPAIRED CELL-MEDIATED CYTOTOXICITY
- FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS
- X-LINKED LYMPHOPROLIFERATIVE DISEASE
- IL-2 INDUCIBLE TYROSINE KINASE DEFICIENCY
- CD27 DEFICIENCY
- CD16 DEFICIENCY
- IMMUNODEFICIENCIES WITH SELECTIVE PREDISPOSITION TO PATHOGENS
  - Mendelian Susceptibility to Mycobacterial Diseases
  - Epidermodysplasia Verruciformis
  - Herpes Simplex Encephalitis
  - Susceptibility to Trypanosomiasis
- HUMORAL IMMUNODEFICIENCY
  - The Agammaglobulinemias
  - Other Predominantly Antibody Deficiency Syndromes
  - Management of Humoral Immunodeficiency
- COMPLEMENT DEFICIENCIES
  - Deficiency of Classical Complement Pathway Components
  - Deficiency of Alternative Complement Pathway Components
  - Deficiency of Lectin Pathway Complement Components
  - Deficiency of Terminal Complement Components
  - Deficiency of Complement Regulatory Factors
  - Diagnosis and Management of Complement Disorders

Immune compromise arises either as a heritable genetic defect (primary immune deficiency diseases, PIDDs) or as a consequence of another pathologic process such as infection, malignancy, malnourishment, or iatrogenic immunosuppression (secondary immune deficiencies). Immune dysfunction in patients with PIDDs is most often manifested as recurrent and chronic infections; however, malignancy and autoimmunity are common and may be prominent in some disorders. In many cases, symptoms and signs of immunodeficiency develop soon after birth or early in childhood. However, in some forms of PIDD clinical onset may be delayed into late childhood, adolescence, or adulthood. Even though many patients with severe immunodeficiency suffer dramatic morbidity and mortality in infancy and early childhood, the majority of patients with immunodeficiency have milder forms that permit survival into adulthood, but often with reduced longevity because of infection, autoimmune disease, malignancy, or constitutional debility. Health-related quality of life is significantly impaired in individuals with PIDDs.[1,2]

PIDDs may be classified with respect to the specific immune effector mechanisms disrupted.[3,4] In broadest terms, one may distinguish: (a) disorders that affect both cellular and humoral components of specific immunity (combined immunodeficiencies); (b) defects of antibody production (antibody deficiency syndromes): (c) disorders of innate immunity (phagocytic cell defects, disorders of Toll-like receptor (TLR) signaling, complement deficiencies). Moreover, some forms of PIDD are characterized by prominent immune dysregulation. Finally, in some cases impairment of immune function is part of a broader spectrum of symptoms (syndromic immunodeficiency). Discussed here are a broad range of PIDDs arising from aberrant development and function of T and B cells, disorders with immune dysregulation, immunodeficiency syndromes, and complement deficiencies. Phagocytic disorders are the subject of Chapter 22. Table 24-1 lists several Internet sites with information relevant to primary immunodeficiencies.

TABLE 24-1 Internet Sites with Information Relevant to Primary Immunodeficiency Diseases

URL (http://+)	Name/Description
bioinf.uta.fi/idr/Immunology.shtml	ImmunoDeficiency Resource, University of Tampere, Finland
www.aaaai.org	American Academy of Allergy, Asthma, and Immunology
www.esid.org	European Society for Immunodeficiencies
www.immunodeficiencysearch.com	Searchable database, clinical algorithms, laboratory resources
www.info4pi.org	Primary Immunodeficiency Resource Center (sponsored by the Jeffrey Modell Foundation)
www.ipidnet.org	Immune Phenotyping in Primary Immunodeficiency
www.ipopi.org	International Patient Organization for Primary Immunodeficiencies
www.jmfworld.org	Jeffrey Modell Foundation (connects directly to Primary Immunodeficiency Resource Center)
www.primaryimmune.org	Immune Deficiency Foundation
www.usidnet.org	US Immunodeficiency Network (USIDNET)

COMBINED IMMUNODEFICIENCIES

Severe Combined Immunodeficiency

The term *severe combined immunodeficiency* (SCID) designates a genetically heterogeneous group of PIDDs characterized by impaired development and function of T lymphocytes. In some cases, development of B or natural killer (NK) lymphocytes (or both) is also affected (Fig. 24-1).[3,5] However, because T cells play a critical role also in most B-cell responses, serious T-cell dysfunction precludes effective humoral immunity even in patients in whom the genetic defect does not affect B-cell development. Complete absence of specific cellular and humoral immunity in patients with SCID leads to an extreme infectious diathesis early in life. Mucocutaneous candidiasis is a common finding. Infections with common viral pathogens (e.g., varicella, herpes simplex, measles, adenoviruses, respiratory syncytial virus, influenza, and parainfluenza) are often fatal. Patients are also susceptible to opportunistic infections with commensal organisms that are normally nonpathogenic, such as *Pneumocystis jiroveci*. Even attenuated vaccine organisms such as oral polio vaccine virus, rotavirus vaccine, varicella vaccine, and bacille Calmette-Guérin (BCG) can cause severe or fatal infection. Transfusion of blood products containing viable lymphocytes may lead to fatal graft-versus-host disease. Autoimmunity and other manifestations of immune dysregulation (e.g., skin rash) are frequently observed, especially in patients with residual development of T lymphocytes ("leaky" SCID). Patients with SCID are also at increased risk of malignancies, including lymphoproliferative disorders caused by Epstein-Barr virus (EBV) and other tumors.

Typical symptoms of SCID are recurrent severe infections, chronic diarrhea, and failure to thrive.[4,5] Symptoms are often seen in the first weeks of life but may be delayed by several months. Antibodies derived from the mother by transfer across the placenta may provide some early protection. Physical examination may reveal foci of infection (e.g., thrush) and the absence of discernible lymphoid tissue. Hypogammaglobulinemia is often present but is not universal. Specific antibody responses are almost always severely impaired.

SCID is most often classified according to the peripheral blood lymphocyte phenotype—that is, absence or presence of the major lymphocyte types. T cells are absent (or very low) and nonfunctional in all classic forms of

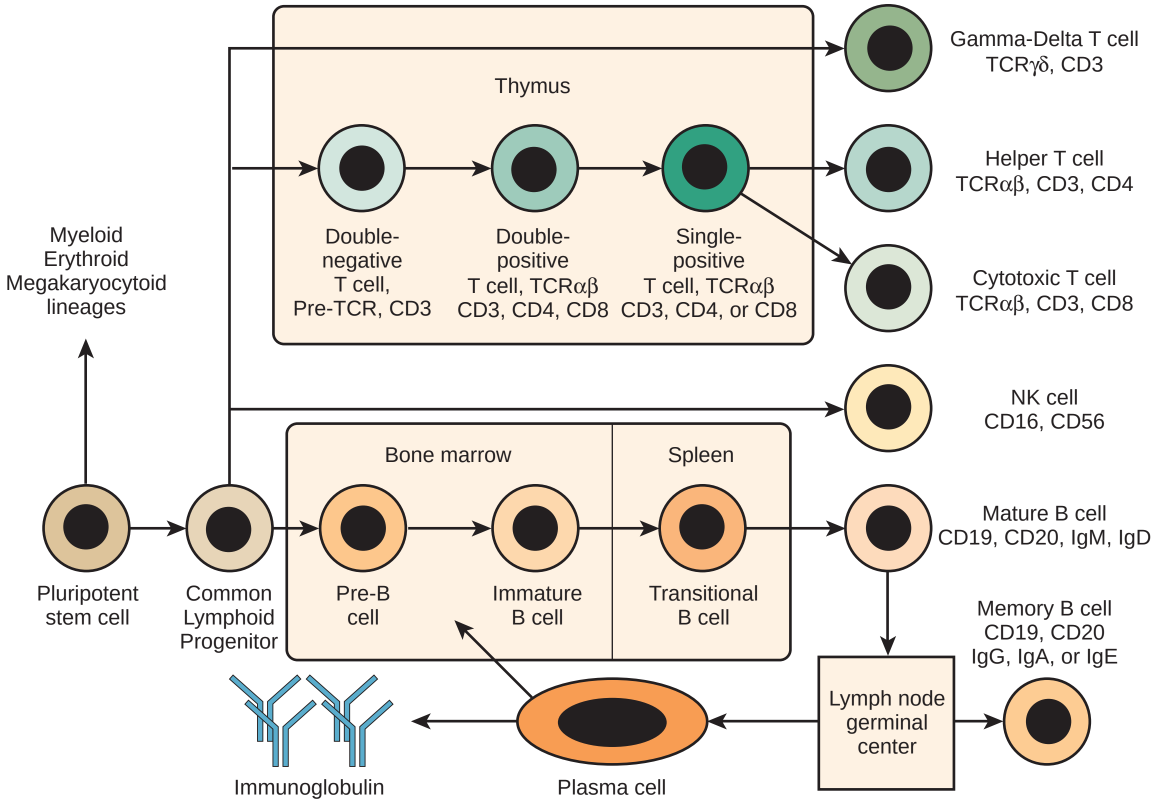
Thymus
Gamma-Delta T cell
TCRγδ, CD3
Helper T cell
TCRαβ, CD3, CD4
Cytotoxic T cell
TCRαβ, CD3, CD8
Double-negative T cell, Pre-TCR, CD3
Double-positive T cell, TCRαβ CD3, CD4, CD8
Single-positive T cell, TCRαβ CD3, CD4, or CD8
Myeloid
Erythroid
Megakaryocytoid
lineages
NK cell
CD16, CD56
Bone marrow
Spleen
Mature B cell
CD19, CD20, IgM, IgD
Pluripotent stem cell
Common Lymphoid Progenitor
Pre-B cell
Immature B cell
Transitional B cell
Memory B cell
CD19, CD20
IgG, IgA, or IgE
Lymph node germinal center
Immunoglobulin
Plasma cell

Figure 24-1 Diagram of lymphocyte development. Pluripotent stem cells give rise to all cellular blood elements. The common lymphoid progenitor gives rise to the three principal lineages of lymphocytes: T cells, B cells, and natural killer *(NK)* cells. Most peripheral T cells express the αβ form of the T-cell antigen receptor *(TCR)* and develop through distinct stages in the thymus. Mature helper T cells express the CD3 antigen coreceptor complex along with the major histocompatibility complex (MHC) class II receptor CD4, whereas cytotoxic cells express the MHC class I receptor CD8. A minority (1% to 5%) of peripheral T cells express the γδ form of the TCR along with CD3, but without CD4 or CD8; less is known about their development. B cells develop initially in the bone marrow and pass through several stages before exiting to complete development in the spleen. Mature B cells express distinct markers (CD19 and CD20) along with surface immunoglobulin M (IgM) and IgD. B cells are activated in germinal centers to undergo class switching and become either memory B cells expressing IgG, IgA, or IgE or Ig-secreting plasma cells. Most IgG-producing plasma cells reside in the bone marrow. NK cells are a distinct lineage of cytotoxic cells; little is known regarding their development from lymphoid precursors.

SCID, a phenotype designated “T$^-$.” The presence or absence of B cells is indicated by adding “B$^+$” or “B$^-$,” respectively. The same pattern applies for NK cells. Table 24-2 lists the various lymphocyte phenotypes of SCID and the molecular defects associated with them. In as many as 40% of patients with SCID, maternal T cells may have engrafted in the fetus during gestation, and this situation may occasionally confuse the diagnostic picture.[6,7] Maternally derived T cells tend to be anergic, but they may also be associated with clinical manifestations similar to those of graft-versus-host disease, such as eczematous rash, eosinophilia, and splenomegaly.[6,7] In rare circumstances, maternal engraftment may lead to even more unusual clinical findings, such as IgA monoclonal gammopathy, autoimmune cytopenias, and allograft rejection.[7,8]

T$^-$ B$^-$ NK$^-$ Severe Combined Immunodeficiency

Adenosine Deaminase and Purine Nucleoside Phosphorylase Deficiencies. Figure 24-2 shows the salvage pathway of purine nucleotide synthesis. Deficiencies of the enzymes adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) are associated with immunodeficiency.[9,10] ADA deficiency accounts for approximately 10% to 15% of all patients with SCID, or approximately a third of SCID cases with autosomal recessive inheritance. PNP deficiency is quite rare, with approximately 70 cases reported.[11] Although PNP deficiency is mainly expressed as a somewhat selective defect in cellular immunity, it is

TABLE 24-2 Lymphocyte Phenotype Classification of Severe Combined Immunodeficiency and Associated Gene Defects

Lymphocyte Phenotype	Genes
T$^-$B$^-$NK$^-$	*ADA, AK2, NP*[*]
T$^-$B$^-$NK$^+$	*DCLRE1C, LIG4, NHEJ1, PRKDC, RAG1, RAG2*
T$^-$B$^+$NK$^-$	*IL2RG, JAK3*
T$^-$B$^+$NK$^+$	*CD3D, CD3E, CD3G,*[†] *CD3Z, IL7R, PTPRC*

[*] B-cell development is variably affected in nucleoside phosphorylase (NP) deficiency.

[†] *CD3G* mutations are rarely associated with a severe T-cell deficiency.

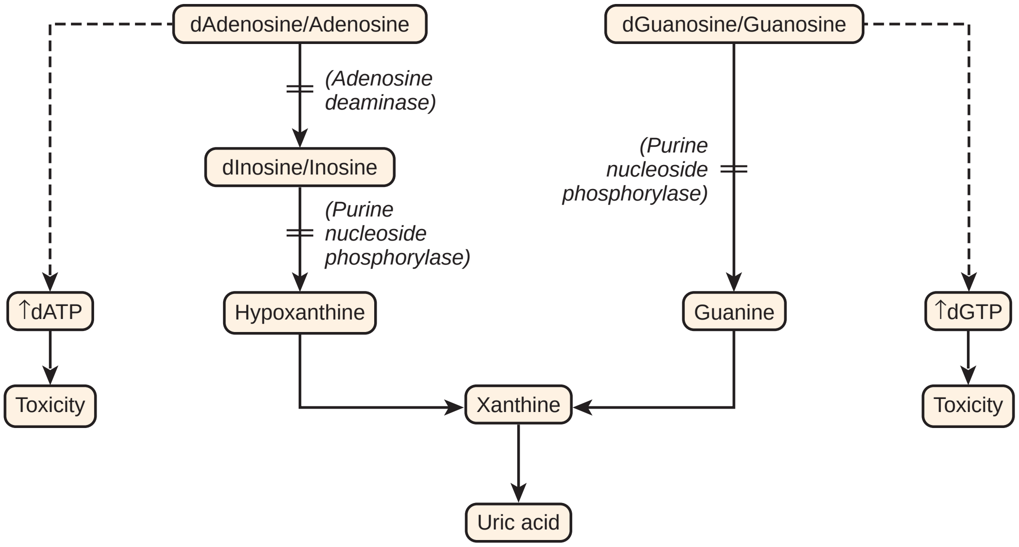

Figure 24-2 Salvage pathway of purine nucleotide biosynthesis. *d,* Deoxy; *dATP,* deoxyadenosine triphosphatase; *dGTP,* deoxyguanosine triphosphatase.

discussed here because of similarities in their biochemical pathophysiology with ADA deficiency. The absence of ADA or PNP leads to intracellular accumulation of deoxyadenosine and deoxyguanosine, respectively. These molecules are not themselves toxic to lymphocytes. However, when they are converted to their 5′-triphosphates (dATP and dGTP), they inhibit ribonucleotide reductase and prevent de novo synthesis of deoxynucleotides. Without building blocks for the replication and repair of DNA, the cells cease to divide. Lymphocytes die to a much greater extent than other cell types do. In both ADA and PNP deficiency, T-cell precursors in the thymus appear to be especially sensitive to death by apoptosis (programmed cell death).[12] B cells are more often depleted in ADA deficiency than in PNP deficiency. In the latter the B-cell phenotype is more variable.

Approximately 85% of individuals with ADA deficiency will display a SCID phenotype with markedly reduced numbers of both T and B cells and low serum antibody levels. NK cells are only rarely found in patients with ADA deficiency. Additional clinical manifestations may include radiographic alterations of the ribs, vertebral bodies, and iliac crests[9] as well as sensorineural deafness,[13] liver dysfunction,[14] lung disease resembling alveolar proteinosis,[15] and cognitive impairment and other neurologic abnormalities.[16,17] Immune dysregulation is common in patients with ADA deficiency[18]; it may manifest with allergy (eczema, asthma) and autoimmunity (cytopenias, hypothyroidism, diabetes) and is associated with loss of regulatory T-cell function.[19] Rarely, ADA deficiency may present with generalized erythroderma and infiltration of skin by activated autologous T cells, resembling Omenn syndrome (OS).[20] Bone marrow hypocellularity is frequently observed in ADA deficiency; myelodysplasia also has been reported.[21] Finally, patients with ADA deficiency are at increased risk for multicentric dermatofibrosarcoma protuberans, a very rare tumor with a characteristic chromosomal translocation (t[17;22][q22;q13]) resulting in the *COL1A1*-platelet-derived growth factor β *(PDGFB)* fusion gene.[22] Between 10% and 15% of patients with ADA deficiency may have a delayed or late-onset form that may be manifested in late infancy or early childhood. These patients initially may have variable numbers of circulating lymphocytes and some humoral immunity that quickly wanes. The severity of the phenotype appears to correlate to some degree with the amount of residual ADA activity.[23] Autoimmunity is particularly common in patients with residual ADA enzymatic activity.[24]

Patients with PNP deficiency are very susceptible to viral and fungal infections. They have decreased numbers of circulating T cells, often initially with normal numbers of B cells and normal serum immunoglobulin (Ig) levels. With time, humoral immunity usually deteriorates. Approximately 50% of children with PNP deficiency have neurologic complications such as spasticity, diplegia, paresis, and other motor disorders, as well as cognitive impairment.[25] Autoimmune manifestations, especially cytopenias, are also very common.[26]

After clinical suspicion is aroused, diagnosis of ADA or PNP deficiency is not difficult. Both ADA and PNP activity is readily measurable in red blood cell or leukocyte lysates. In symptomatic patients, activity is usually 1% or less of that in normal subjects. These methods may be applied also for prenatal diagnosis on cultured amniotic or chorionic villus cells or on fetal blood sampling. PNP is required for the production of hypoxanthine, a precursor of uric acid (see Fig. 24-2). Thus individuals with PNP deficiency will have reduced serum and urine urate levels.

Several therapeutic options are available to patients with ADA deficiency, including hematopoietic stem cell transplantation (HSCT), enzyme replacement therapy (ERT) and gene therapy. Excellent results (survival >85%) have been reported after HSCT from matched sibling donors, but results of HSCT from haploidentical or unrelated donors are much less satisfactory (43% and 66% survival, respectively).[27] Moreover, patients with ADA deficiency surviving after HSCT remain at high risk of neurologic complications.[28] ERT with weekly intramuscular injections of polyethylene glycol–conjugated bovine ADA (PEG-ADA) is usually well tolerated and is effective to normalize levels of toxic phosphorylated (deoxy)adenosine derivatives and to improve immune function.[29] However, immune reconstitution is often incomplete.[30] Encouraging results have been obtained with gene therapy, associated with nonmyeloablative cytoreduction.[31,32] This represents a very important option for patients who lack a human leukocyte antigen (HLA)-identical related donor. HSCT is the only curative therapeutic option for patients with PNP deficiency.

Reticular Dysgenesis Caused by Adenylate Kinase 2 Deficiency. Reticular dysgenesis caused by adenylate kinase 2 (AK2) deficiency is a very rare autosomal recessive form of SCID, associated with agranulocytosis (with a block at the promyelocyte stage of differentiation in the bone marrow) and sensorineural deafness.[33] AK2 regulates levels of ADP in mitochondria. Deficiency of this enzyme is associated with abnormalities of energy metabolism in granulocytes and lymphocytes (especially T cells), resulting in apoptosis.[34,35] The number of circulating B and NK lymphocytes can be variably affected, and NK cell differentiation is usually preserved. Therefore, these patients most often exhibit a phenotype other than T- B- NK- SCID; however, we describe this disorder here because it also affects purine metabolism, similar to ADA and PNP deficiencies. OS with oligoclonal expansion of T cells has been described in a patient with *AK2* hypomorphic (partially functional) mutations.[36] Patients are at increased risk of myelodysplasia.[37] Sensorineural deafness reflects a role of AK2 in the stria vascularis of the inner ear.[34] Treatment is based on HSCT, but results are less satisfactory than in other forms of SCID, and use of myeloablative conditioning is required for engraftment.[38]

T$^-$B$^-$NK$^+$ Severe Combined Immunodeficiency

Defects of Recombinase-Activating Genes (RAG) 1 and 2. *RAG1* and *RAG2* encode for DNA-binding proteins that bind to specific recognition sequences flanking coding gene elements of the Ig and T cell receptor (TCR) loci. RAG1 and RAG2 initiate the VDJ recombination process by inducing DNA double-strand breaks at these loci. Proteins of the nonhomologous end-joining (NHEJ) complex ultimately join coding Ig and TCR elements, allowing expression of Ig and TCR molecules (Fig. 24-3).[39] In patients with RAG defects, B- and T-cell differentiation is arrested at an early stage of development. Therefore patients with functionally null mutations of the

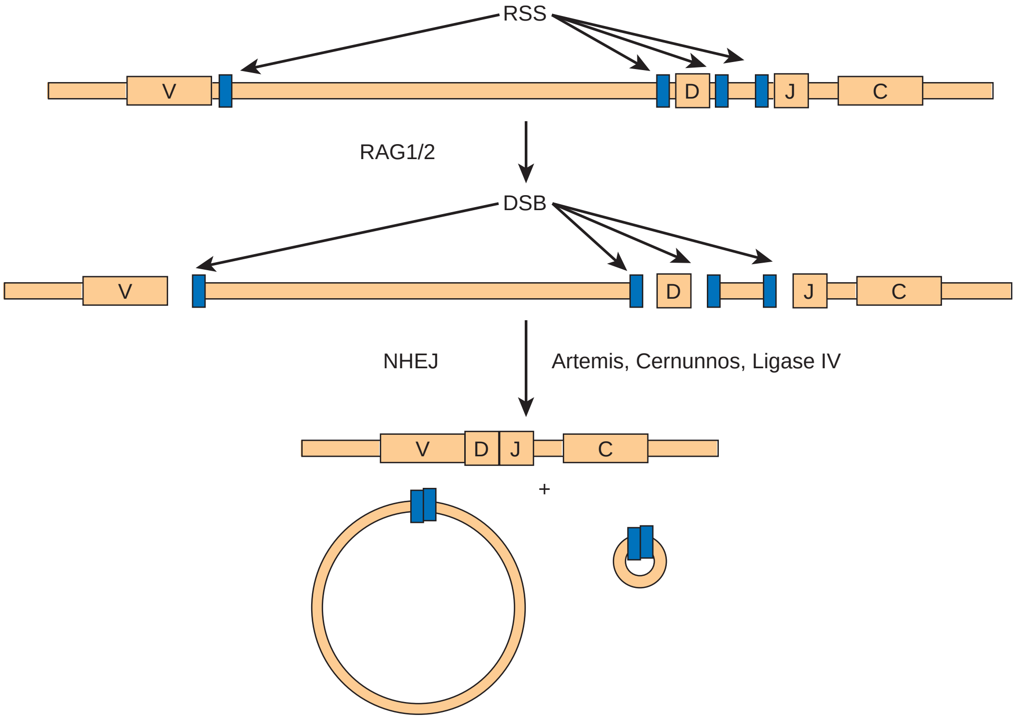

Figure 24-3 Gene defects affecting lymphocyte antigen receptor gene rearrangement and causing T$^-$B$^-$NK$^+$ severe combined immunodeficiency. The genes that encode immunoglobulin and T-cell antigen receptors are assembled from subunit segments that are separated in the germline and undergo assembly or somatic rearrangement during lymphocyte development. In the figure, a TCR or Ig heavy-chain locus is shown. The heavy chains are composed of four distinct segments: *V,* variable; *D,* diversity; *J,* joining; and *C,* constant. Some of the segments are flanked by recombination signal sequences *(RSS)*. RAG1 and RAG2 initiate the process that leads to double-strand breaks *(DSB)* in DNA. The DNA between coding segments is looped out and forms circles, with the coding sequences joining to one another. In the case of T cells, the larger DNA circle is referred to as a *T-cell receptor excision circle*. All these DNA joins are made by a group of molecules that together subserve the function of nonhomologous end joining *(NHEJ)*.

RAG1 and *RAG2* genes have very low numbers of T and B lymphocytes, but NK cell differentiation is preserved.[40] Some patients with hypomorphic *RAG1* or *RAG2* mutations may sustain differentiation of a limited number of T and (less frequently) B lymphocytes, a condition that is also referred to as "leaky" or "atypical" SCID.[41,42] In these patients, T lymphocytes often undergo homeostatic proliferation in the periphery and acquire an activated (CD45RO+) phenotype. Oligoclonal expansion of T lymphocytes that infiltrate target tissues causing damage is observed in patients with OS.[43] Patients with OS present with clinical features suggestive of acute graft-versus-host disease. Symptoms include recurrent severe infections, failure to thrive, chronic severe diarrhea, and erythroderma with exfoliation and exudation. Examination may reveal hepatosplenomegaly and lymphadenopathy. Laboratory studies show anemia, hypogammaglobulinemia with an elevated IgE level, a decrease in the number of peripheral B cells, and presence of activated (CD45RO+) circulating T lymphocytes with a restricted (oligoclonal) T cell repertoire. In vitro proliferation of T lymphocytes to antigens and to anti-CD3 is abrogated, whereas proliferation to phytohemagglutinin (PHA) may be variably affected.[44] Although hypomorphic *RAG1* and *RAG2* mutations are the most common form of OS, the same phenotype may be observed in patients with hypomorphic mutations in other SCID-causing genes. Some evidence suggests that expansion of oligoclonal, tissue-infiltrating T lymphocytes in patients with OS may reflect defects of T-cell tolerance. The study of thymic biopsies from patients with OS has shown abnormalities of medullary epithelial cells, with impaired expression of AIRE (autoimmune regulator, discussed later),[45] a transcription factor that promotes expression of self-antigens, thereby permitting intrathymic deletion of autoreactive T cells or their diversion to FOXP3$^+$ regulatory T (Treg) cells. Consistent with this, absence of FOXP3$^+$ cells has also been demonstrated in thymic biopsies from patients with OS.[45]

Environmental triggers may modify the disease phenotype in patients with RAG gene defects. In at least one case, progression to OS was observed in a patient with a *RAG2* mutation after infection with parainfluenza virus type 3.[46] Cytomegalovirus (CMV) infection has been associated with in vivo expansion of TCR$\gamma\delta^+$ T cells and autoimmune manifestations.[47,48] Finally, somatic mutations may become superimposed on null or "amorphic" mutations and yield mosaicism with revertant or hypomorphic populations of lymphocytes and resultant OS.[49]

Hypomorphic RAG mutations that result in production of mutated proteins with more robust (although subnormal) recombination activity have been identified in patients with delayed presentation characterized by granulomatous lesions and autoimmunity.[50,51] Occasionally, autoimmunity in patients with RAG defects is associated with preserved number of circulating B cells and normal or increased Ig serum levels. One patient with compound heterozygous missense *RAG1* mutations presented with idiopathic CD4 lymphopenia.[52]

Mutations of *DCLRE1C* (Artemis). A form of autosomal recessive SCID with the classic T$^-$B$^-$NK$^+$ phenotype has an associated feature of general radiation sensitivity not seen with *RAG1* or *RAG2* mutations and is caused by mutations in the gene *DCLRE1C* (DNA cross-link repair enzyme 1C), whose protein product is called Artemis.[53] Artemis has a critical role in the function of NHEJ, or the repair of double-stranded DNA breaks (see Fig. 24-3). During VDJ recombination, it opens the hairpins sealing the coding ends at V, D, and J elements that have been targeted by the RAG proteins.[39] Absence or impaired function of Artemis blocks T and B lymphocyte development at an early stage, similar to *RAG1* and *RAG2* mutations. Artemis is ubiquitously expressed, and loss of its function in nonhemopoietic tissues may account for nonimmunologic problems (oral and genital ulcers, dental abnormalities, and malabsorption) and for the increased cellular radiation sensitivity that these patients exhibit. OS has been observed in some patients with Artemis defects.[54] A partial form of the disease, with residual expression and function of the protein, is characterized by reduced number of circulating T and B lymphocytes and increased risk of lymphoma.[55]

DNA Ligase IV Deficiency. DNA ligase IV (LIG4) is also required for NHEJ. Mutations in the *LIG4* gene are associated with microcephaly, cognitive impairment, bone marrow failure, and cellular immunodeficiency that may manifest as T$^-$B$^-$NK$^+$ SCID, "leaky" SCID, or OS. There is profound cellular radiosensitivity. Patients are at increased risk of myelodysplasia and leukemia.[56-59]

Cernunnos Deficiency. Cernunnos (also known as XLF) is another component of the NHEJ pathway[39] and is encoded by the *NHEJ1* (nonhomologous end-joining 1) gene. Mutations of this gene cause lymphopenia, radiation sensitivity, growth retardation, microcephaly and developmental delay, bone marrow failure, and an increased risk of myelodysplasia.[60] The T-and B-cell immunodeficiency of Cernunnos deficiency is less severe than in patients with *RAG* or *LIG4* defects; however, there are quantitative and qualitative alterations of the T-cell repertoire, a severe reduction in the number of invariant NK T cells and mucosa-associated invariant T lymphocytes,[61] and abnormalities of class-switch recombination in B lymphocytes (see section on class-switch defects).[62]

DNA Protein Kinase Catalytic Subunit (DNA-PKcs) Deficiency. A homozygous missense mutation in the *PRKDC* (protein kinase, DNA-activated, catalytic polypeptide) gene, encoding for DNA-PKcs was identified in a patient with T$^-$ B$^-$NK$^+$ SCID and cellular radiosensitivity, but without microcephaly or mental retardation.[63]

T$^-$B$^+$NK$^-$ Severe Combined Immunodeficiency

X-Linked Severe Combined Immunodeficiency, Mutations of the Cytokine Receptor Common γ Chain. In Western countries, approximately 40% of all patients with SCID are males with an X-linked form of the disease, (XSCID), caused by mutations of the *IL2RG* (interleukin-2 receptor γ) gene, that encodes for the common γ chain (γ_c), shared by cytokine receptors for interleukin-2 (IL-2),

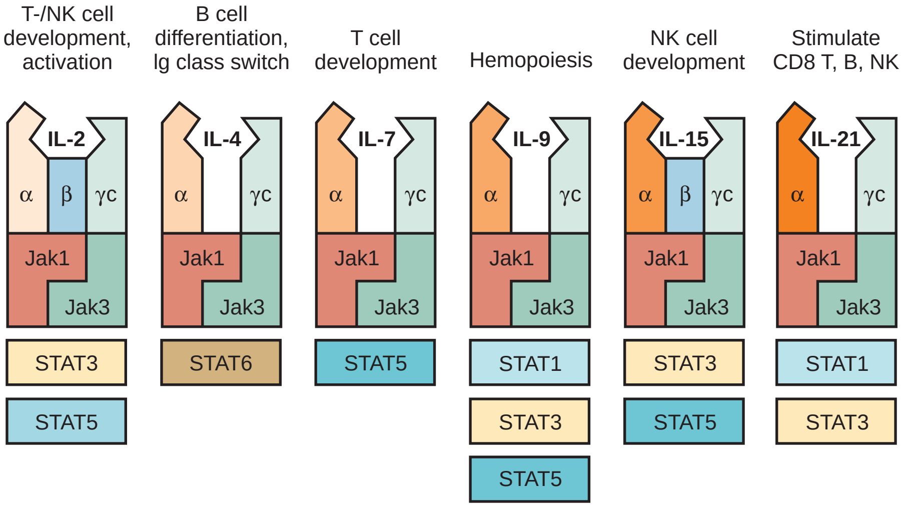

Figure 24-4 Cytokine receptors and signaling via γ_c and Jak3. The γ_c chain participates in signaling by way of all these cytokine receptors; it associates with the Jak3 kinase in every case. The α chains of each receptor are distinct; receptors for IL-2 and IL-15 also share a β chain. The α or β chains or both associate with Jak1. After cytokine binding, Jak kinases recruit STAT (signal transducer and activator of transcription) proteins. These molecules dimerize and then translocate to the nucleus, where they associate with other transcriptional regulators and alter gene expression.

IL-4, IL-7, IL-9, IL-15, and IL-21 (Fig. 24-4).[64,65] Thus a defect in a single molecule interrupts several cytokine pathways, thereby severely disrupting immune function. Typically, patients with XSCID lack T and NK lymphocytes; these defects reflect defective signaling via IL-7R and IL-15R, respectively. However, some patients with XSCID may have variable numbers of their own T or NK cells and may retain partial immune function, reflecting some preservation of γ_c expression and of cytokine-mediated signaling.[66] B lymphocytes are typically present, but they are nonfunctional as a result of impaired IL-21–mediated signaling.[67] Patients with γ_c deficiency have typical clinical features of SCID. In addition, they are unusually susceptible to chronic and severe human papillomavirus (HPV) infections, and this phenotype may not be corrected after HSCT.[68] Delayed presentations have been reported in association with hypomorphic mutations or with somatic mutations in T-cell progenitor cells, allowing generation of a diversified pool of functional T lymphocytes that may persist for years.[69-71] Gene revertant T cells undergoing in vivo expansion may also cause a phenotype resembling OS.[72]

JAK3 Deficiency. A form of $T^-B^+NK^+$ SCID phenotypically very similar to XSCID is associated with defects in the tyrosine kinase JAK3.[73] The similarity with XSCID is easily understood because JAK3 is a critical signal-transducing molecule associated with the γ_c chain (see Fig. 24-4). Hypomorphic mutations that allow residual JAK3 protein expression and function may associate with partial preservation of circulating T cells and delayed clinical onset, with lymphoproliferation and susceptibility to warts and other viral infections.[74]

$T^-B^+NK^+$ Severe Combined Immunodeficiency

Defects of the Interleukin-7 Receptor α Chain. Signaling through the IL-7 receptor (IL-7R) is required for proliferation of early T-cell progenitors and survival of T lymphocytes. The IL-7R is composed of an α chain (IL-7Rα), encoded by the *IL7R* gene, and γ_c (see Fig. 24-4). Mutations of the *IL7R* gene in humans cause $T^-B^+NK^+$ SCID.[75] One case of OS caused by a homozygous missense mutation, permissive for IL-7Rα expression, has been reported.[76]

Mutations Affecting Components of the T-Cell Antigen Receptor Complex

The TCR/CD3 complex is made up of the TCRα and TCRβ (or TCRγ and TCRδ) chains of the receptor making contact with major histocompatibility antigen-peptide complexes, as well as components of the CD3 signal-transducing complex: CD3γ, CD3δ, CD3ε, and CD3ζ (Fig. 24-5).

Defects in the CD3δ (gene *CD3D*) lead to a $T^-B^+NK^+$ SCID phenotype.[77,78] Patients have varying degrees of panhypogammaglobulinemia and are susceptible to disseminated viral infections. In some cases, a normal-sized thymus was visualized, in marked contrast to almost all other forms of SCID, in which the thymus is absent or extremely hypoplastic.[77] In one case, CD3δ deficiency was associated with OS.[79] A multicenter study of 13 patients with CD3δ deficiency treated by HSCT has shown superior immune reconstitution after transplantation from HLA-matched donors: use of conditioning chemotherapy may be needed to achieve engraftment.[79]

Deficiency of CD3ε (gene *CD3E*)[78] and of CD3ζ (gene *CD3Z*)[80,81] are associated with a similar phenotype and markedly reduced levels of the TCR/CD3 complex on the surface of circulating T lymphocytes. In one patient with CD3ζ deficiency, some of the circulating T cells were found to express normal levels of the TCR/CD3 complex as the result of somatic mutations on one of the *CD3Z* alleles, allowing expression of poorly functioning TCR/CD3 complexes.[80]

In contrast, the phenotype of CD3γ (gene *CD3G*) deficiency is heterogeneous. One of two genotypically affected siblings presented with clinical features of SCID and autoimmunity associated with severe T-cell lymphopenia, whereas the other one had a milder immunologic phenotype and remained asymptomatic until 12 years of age,

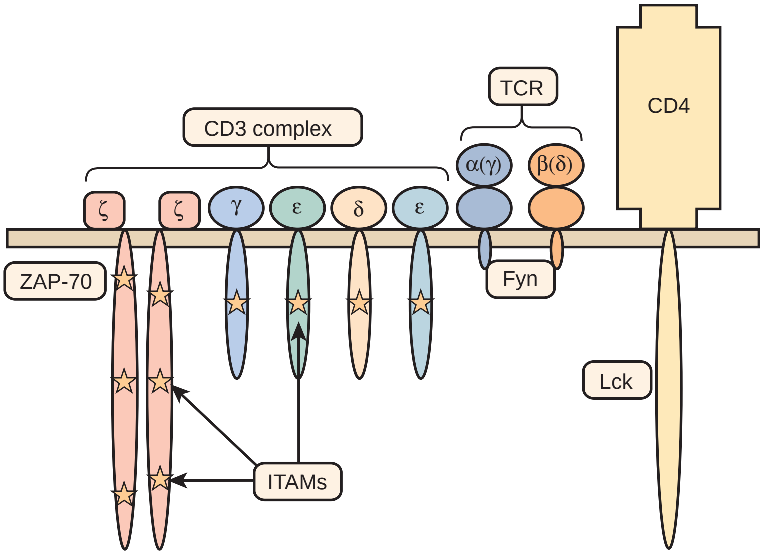

Figure 24-5 Components of the T-cell antigen receptor. The actual receptor making contact with a major histocompatibility complex (MHC)-peptide complex is a heterodimer of α and β (or γ and δ) chains. This heterodimer sits in the cell membrane in association with the signal-transducing CD3 complex. This complex contains two heterodimers, one γε and one δε, and one ζ_2 homodimer. Also shown is the MHC class II coreceptor molecule CD4. Tyrosine kinases Lck, Fyn, and ZAP-70 associate with components of the receptor complex via immunoreceptor tyrosine-based activating motifs *(ITAMs)* and transduce signals after interaction of the receptor with peptide/MHC.

when he developed autoimmunity and recurrent infections.[82,83] This variability of clinical and immunologic phenotype has been confirmed in other patients.[84]

A homozygous splice-site mutation in the TCRα constant region gene has been identified in two patients with a history of recurrent infections and autoimmunity. Development of T cells expressing TCRγδ was not affected. In vitro proliferative responses to mitogens and antigens were decreased, but specific antibody production was preserved.[85]

CD45 Deficiency

A few patients have been found to have a form of SCID resulting from mutations in the gene encoding the protein tyrosine phosphatase CD45 (gene *PTPRC*), which plays an important role in T-cell activation.[86-88] These patients have a SCID phenotype with diminished numbers of poorly functional T cells, normal or increased numbers of B cells, and variable number of NK cells.

Other Combined Immunodeficiencies (with T Cells Present)

Table 24-3 lists disorders of combined immunodeficiency with variable lymphocyte phenotypes.

Relative Deficiency of CD4+ T Cells

Lck Deficiency. The lymphocyte-specific protein tyrosine kinase (Lck) associates with CD4 and CD8 molecules and plays a critical role in T-cell signaling by mediating phosphorylation of immunoreceptor tyrosine activation motifs in the intracytoplasmic domains of CD3 subunits and of the ζ chain–associated protein of 70 kDa (ZAP70). Homozygosity for a missense *LCK* mutation was identified in a child with recurrent respiratory infections, protracted diarrhea, failure to thrive, skin nodules, vasculitis, arthritis, and autoimmune thrombocytopenia. There was a severe defect in the number of circulating CD4+ lymphocytes, and the density of both CD4 and CD8 molecules on the surface of CD3+ cells was markedly reduced. T lymphocytes showed a restricted TCR repertoire. Proliferative responses to anti-CD3 were profoundly impaired.[89]

RhoH Deficiency. The Ras homology family member H (RhoH) is a small GTPase that is phosphorylated in response to TCR stimulation, allowing recruitment of Lck and ZAP-70 to the TCR-associated signaling complex. Mutations of the *RHOH* gene have been identified in two siblings with warts, variably associated with granulomatous lung disease, psoriasiform skin changes, and molluscum contagiosum.[90] One of the affected individuals developed Burkitt lymphoma in childhood. Immunologic abnormalities included reduced number of naïve CD4+ lymphocytes and oligoclonal T cell repertoire, with accumulation of CD8+ effector memory CD45RA+ lymphocytes (TEMRA cells) with an "exhausted" (CD45RA+CCR7−) phenotype. The reduced number of lymphocytes expressing skin-homing receptors (cutaneous lymphocyte antigen, CCR4, CCR6, CCR10,

TABLE 24-3 Combined Immunodeficiencies with T Cells Present and Associated Gene Defects

Syndrome	Gene
Variable T cell lymphopenia	*CORO1A, MST1*
Relative CD4+ T cell deficiency	*LCK, MHCIITA, RFX5, RFXANK, RFXAP, RHOH*
Relative CD8+ T cell deficiency	*CD8A, TAP1, TAP2, TAPBP, ZAP70*
Hyper IgE syndrome type 2	*DOCK8*
Veno-occlusive disease with immunodeficiency	*SP110*
Ion (Ca^{++} and Mg^{++}) transport defects	*MAGT1, ORAI1, STIM1*
Other	*CARD11, IL21R*

β7-integrin) may impair defense against cutaneous viral infections and contribute to development of warts.

Major Histocompatibility Complex Class II Deficiency. The major histocompatibility complex (MHC) in humans is called the HLA complex. The class I and class II HLA molecules expressed on cell surfaces are critical for practically all mechanisms of specific immune activation. Class I molecules are present on most cells of the body. They present antigenic peptides derived principally from intracellular sources to the antigen receptors of T cells expressing CD8. HLA class II molecules are generally restricted to specialized antigen-presenting cells (APCs), including monocytes and macrophages, dendritic cells, and B cells, but are also expressed by thymic epithelial cells. HLA class II molecules mainly present peptides imported from outside the cell to antigen receptors of T cells bearing CD4. Defects in the synthesis, expression, or function of HLA molecules may be expected to have profound consequences on lymphocyte development and on immune competence. In particular, defective expression of HLA class II molecules on the surface of thymic epithelial cells impairs development of CD4+ lymphocytes by impairing their positive selection.

Most patients with MHC class II deficiency present early in life with recurrent and severe infections, chronic diarrhea, and failure to thrive.[91] Chronic opportunistic infections of the gastrointestinal tract caused by *Cryptosporidium* or *CMV* organisms may lead to severe liver disease with sclerosing cholangitis. The number of circulating CD4+ cells is markedly decreased, and delayed hypersensitivity responses are impaired. All forms of MHC class II molecules (DP, DQ, and DR) are not expressed. Most patients have profound hypogammaglobulinemia; however, some patients have normal serum Ig levels, yet specific antibody responses are not elicited. As a rule, these children do not survive without HSCT. However, bone marrow transplantation in these patients has not been consistently successful,[91] probably reflecting inability to correct defective expression of MHC class II molecules on the surface of thymic epithelial cells.

MHC class II deficiency results from defective regulation of gene transcription as a result of mutations in any of four different genes *(CIITA, RFXANK, RFX5, RFXAP)* encoding for components of HLA class II transcription complex.[91] Without a complete transcription complex, no HLA class II mRNA is produced, and class II proteins cannot be synthesized. The same complex activates transcription at all HLA class II gene promoters. *RFXANK* mutations are the main cause of the disease, which is more common in Northern Africa and in the Middle East.[92]

Relative Deficiency of CD8+ T Cells

Defective Expression of HLA Class I. Several patients with defective expression of HLA class I molecules have been described.[93] These patients have mutations in one of two genes encoding either TAP1 or TAP2 (transporter associated with antigen processing 1 or 2) or in the gene encoding tapasin, a TAP-binding protein molecular chaperone. The TAP1/TAP2 heterodimer is required for shuttling of cytosolic peptides across the endoplasmic reticulum to load onto nascent HLA class I molecules for eventual expression at the cell surface and presentation to TCRs (Fig. 24-6). HLA class I molecules are highly unstable without a peptide in their binding cleft, and they are not transported to the cell surface. Patients with defective expression of HLA class I molecules suffer mainly from upper and lower respiratory tract bacterial infections, bronchiectasis, and granulomatous skin inflammation. Occasional asymptomatic individuals have also been described. The main immunologic abnormality is low peripheral blood CD8+ T cells; antibody responses may be normal.

ZAP-70 Deficiency. The ZAP-70 tyrosine kinase has a critical role in TCR-mediated signaling by inducing phosphorylation of the linker of activated T cells and SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) (see Fig. 24-5). Mutations of the *ZAP70* gene in humans are typically associated with severe, early onset infections. Patients have almost complete absence of CD8+ T cells. Although circulating CD4+ cells appear normal, they fail to respond to signals delivered by way of the TCR.[94,95] In a few cases, hypomorphic *ZAP70* mutations have been associated with delayed onset of the disease and manifestations of immune dysregulation (skin rash, wheezing, eosinophilia).[96,97] Treatment is based on HSCT.

CD8α Deficiency. CD8 molecules are expressed on the cell surface as either a CD8α_2 homodimer or a CD8αβ heterodimer. CD8α (gene *CD8A*) deficiency is a rare autosomal recessive disorder characterized by lack of circulating CD8+ lymphocytes and by an increased number of CD3+CD4−CD8− cells that express markers of effector cytotoxic T lymphocytes (CD11b, CD57). The disease is reported here because of some phenotypic similarities (reduced number of CD8+ cells) with ZAP-70 deficiency; however, clinical manifestations are distinct and more similar to MHC class I deficiency, with recurrent respiratory infections and delayed diagnosis.[98]

Other Forms of Combined Immunodeficiency with Variable Presentation

A number of primary immunodeficiencies may have a variable clinical phenotype; in some cases, patients affected with these disorders may present with clinical and laboratory features suggestive of a combined immunodeficiency.

Macrophage Stimulating 1 Deficiency

Macrophage stimulating 1 (MST1), also known as serine threonine kinase 4 (STK4), is a molecule that controls apoptosis and survival of naïve T lymphocytes by inducing expression of FOXO1, a transcription factor that induces expression of the IL-7R. MST1 deficiency is associated with recurrent infections, immune dysregulation, a moderate degree of neutropenia, and congenital heart disease. Viral infections are particularly common and include warts, molluscum contagiosum, and EBV-driven lymphoproliferative disease. The number of naïve T cells is

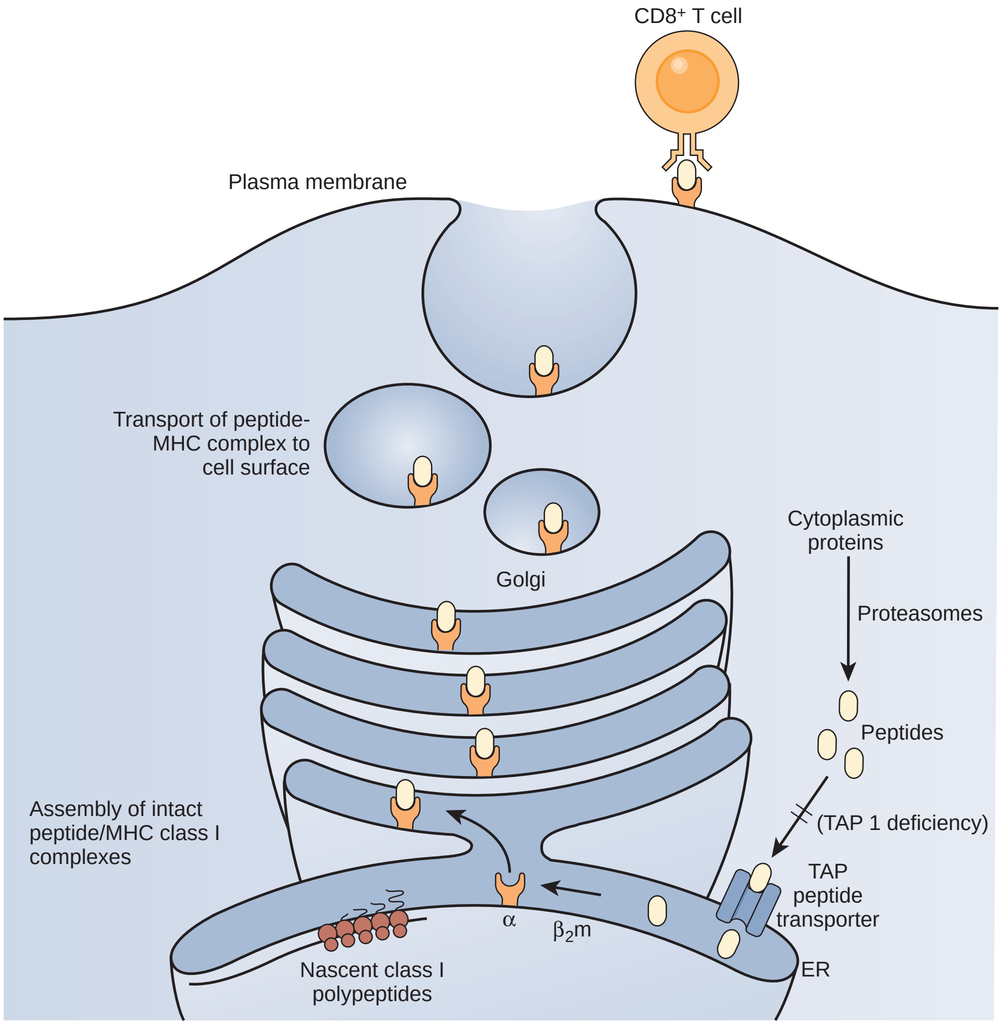
CD8$^+$ T cell
Plasma membrane
Transport of peptide-MHC complex to cell surface
Golgi
Cytoplasmic proteins
Proteasomes
Peptides
(TAP 1 deficiency)
TAP peptide transporter
Assembly of intact peptide/MHC class I complexes
α
β$_2$m
Nascent class I polypeptides
ER

Figure 24-6 Synthesis and assembly of major histocompatibility complex (MHC) class I molecules. *ER*, Endoplasmic reticulum; β2m, β2-microglobulin; *TAP*, transporter associated with antigen processing.

markedly decreased, and there is an expansion of TEMRA cells, associated with a restricted T cell repertoire. T lymphocytes show increased apoptosis and reduced proliferation to mitogens. There is also an increased proportion of transitional B lymphocytes, with reduced number of memory B cells.[99,100]

Dedicator of Cytokinesis 8 Deficiency

Dedicator of cytokinesis 8 (DOCK8) is a guanine nucleotide exchange factor that activates Cdc42 and participates at intracellular signaling in lymphoid and dendritic cells. *DOCK8* mutations cause an autosomal recessive form of combined immunodeficiency with hyper-IgE (this is also known as hyper-IgE syndrome type 2).[101,102] Patients suffer from chronic viral cutaneous infections (warts, molluscum contagiosum), recurrent skin abscesses and cellulitis, respiratory tract infections, candidiasis, eczema and other manifestations of severe allergy, autoimmunity (cytopenias, central nervous system [CNS] vasculitis), and an increased risk of malignancy (epithelial cell carcinoma, lymphomas, leiomyosarcoma). The disease has a severe prognosis with high mortality rate; treatment is based on HSCT.[103]

Immunologic abnormalities include T-cell lymphopenia with impaired thymopoiesis, a low number of naïve CD8+ cells, and accumulation of TEMRA lymphocytes. T cell proliferation to mitogens and antigens is often reduced, especially within CD8+ lymphocytes. Ig abnormalities include elevated serum IgE and low levels of IgM, and B cell responses to TLR agonists are compromised.[104] Immunologic memory is defective in both T- and B-cell compartments. In most cases, the genetic defect is represented by large intragenic deletions.[102]

Coronin-1A Deficiency

The Coronin-1A protein inhibits F-actin formation in T lymphocytes and thereby modulates cytoskeleton rearrangement and T-cell activation. Coronin-1A deficiency causes a combined immunodeficiency with recurrent and severe infections, including severe varicella and

EBV-driven lymphoproliferation.[105,106] Developmental delay has also been observed, possibly reflecting a role for Coronin-1A in the CNS.[105] There is profound T-cell lymphopenia, with reduced survival of T lymphocytes and impaired in vitro proliferation to mitogens and antigens. HSCT can cure the immunologic abnormalities and the increased susceptibility to infections.[105]

Veno-Occlusive Disease with Immunodeficiency Syndrome

Veno-occlusive disease with immunodeficiency (VODI) syndrome is an autosomal recessive condition characterized by severe infections (often including *Pneumocystis jiroveci* and CMV infection) and hepatomegaly with abnormalities of liver function and evidence of veno-occlusive disease on liver biopsy.[107] Cerebrospinal leukodystrophy has been reported in several patients.[108] Immunologic abnormalities include T- and B-cell lymphopenia, poor T-cell function, severe hypogammaglobulinemia, and lack of memory B lymphocytes.[108] If untreated, the disease is often fatal within the first years of life. Administration of intravenous immunoglobulin (IVIG) and antimicrobial prophylaxis may prolong survival, but the only curative approach is represented by HSCT.[109] Recurrence of veno-occlusive disease after HSCT has been reported.[108] VODI was initially described in patients of Lebanese descent, but the disease has been reported in other ethnic groups as well. The disease is caused by mutations of *SP110* encoding for a nuclear body protein that is thought to have a role in regulation of gene transcription.[107]

Immunodeficiencies Caused by Defects of Calcium-Release Activated Channels

On TCR-mediated signaling, Ca^{++} is mobilized from endoplasmic reticulum (ER) stores. This depletion is sensed by stromal interacting molecule 1 (STIM1), which oligomerizes and binds to the ORAI proteins that form the calcium-release activated channels (CRACs) on the cell surface, thereby permitting influx of Ca^{++}, activation of transcription factors (e.g., nuclear factor of activated T cells), cytokine production, T-cell proliferation, and induction of effector T cell responses. Mutations of *STIM1* and *ORAI1* genes in humans account for autosomal recessive combined immunodeficiencies with recurrent infections, signs of ectodermal dystrophy, nonprogressive myopathy (reflecting a role of CRACs in muscle cells).[110,111] Autoimmune manifestations (cytopenias, lymphadenopathy, and splenomegaly) may also be observed, especially in patients with STIM1 deficiency.[111] Kaposi sarcoma due to human herpesvirus 8 (HHV-8) infection has been reported.[112] The absolute lymphocyte count is normal, with preserved distribution of major lymphocyte subsets. However, in vitro proliferation to mitogens and antigens is impaired, and cell-mediated cytotoxicity and cytokine production are also affected.

Magnesium Transporter Protein 1 Deficiency

Magnesium transporter protein 1 (MAGT1) is an X-linked disease characterized by severe chronic viral infections, chronic diarrhea, and EBV-driven lymphoproliferative disease.[113] Immunologic abnormalities include CD4 lymphopenia and impaired T cell proliferation to CD3 stimulation. The *MAGT1* gene encodes for a membrane-associated Mg^{++} transporter. Female carriers have a skewed pattern of X chromosome inactivation in circulating T lymphocytes, suggesting that Mg^{++} signaling plays an important role in T lymphocyte development and survival.

Caspase Recruitment Domain Family Member 11 Deficiency

Caspase recruitment domain family member 11 (CARD11) is a scaffold protein that interacts with B-cell lymphoma 10 and mucosa-associated lymphoid tissue lymphoma translocation gene 1 proteins, thereby inducing activation of the canonical nuclear factor κB (NF-κB) pathway in lymphocytes. Biallelic loss of function mutations of *CARD11* are associated with a combined immunodeficiency, marked by increased susceptibility to opportunistic infections and hypogammaglobulinemia.[114] The number and distribution of T lymphocytes are preserved, other than for a reduced number of Treg cells; by contrast, there is expansion of transitional B lymphocytes and reduction of mature naïve and memory B lymphocytes. Moreover, in vitro activation of T and B cells with phorbol myristate acetate is severely impaired, and proliferation of T lymphocytes in response to stimulation by way of CD3/CD28 is abrogated. On in vitro activation, production of IL-2 by T cells and of interferon IFN-γ by NK cells is decreased.[114]

IL-21 Receptor Deficiency

IL-21 regulates proliferation of lymphocytes, differentiation of T helper 17 (Th17) cells, maturation of B cells into plasmablasts, and induction of NK cell cytotoxicity. IL-21 receptor (IL-21R) defects have been identified in four patients from two kindreds whose clinical phenotype included respiratory infections, chronic diarrhea, failure to thrive, and severe liver and biliary tract disease caused by infection with *Cryptosporidium* organisms.[115] The number of T, B, and NK lymphocytes is normal, but the proportion of switched memory B cells is reduced. Serum IgG levels are low or slightly subnormal, but IgE is elevated. Antibody responses to T-dependent and T-independent antigens are decreased. On in vitro activation with CD40 ligand (CD40L) and IL-21, B lymphocytes from the patients show reduced proliferation and defective class switch recombination. NK cytotoxic activity is markedly decreased. T cells proliferate normally in response to stimulation by CD3 and CD28 but fail to produce IL-17 and IL-22. Proliferation to antigens in vitro is impaired.[115]

MANAGEMENT OF COMBINED IMMUNODEFICIENCY

Diagnostic Approach

Suspicion of SCID or other forms of combined immunodeficiency is prompted by clinical symptoms, family history, or positive results of screening assays. In particular, newborn screening for SCID can be performed by quantifying levels of TCR excision circles (TRECs; see

Fig. 24-3 legend) in dried blood spots collected at birth.[116] TRECs are a by-product of rearrangement of TCR genes during intrathymic T-cell development. In patients with SCID, TREC levels are extremely low or absent as a result of lack of significant thymic T-cell output.[117] Indeed, since newborn screening for SCID has been started in various parts of the United States, several cases of SCID have been identified at birth in otherwise healthy-looking newborns.[118] However, it is not clear yet whether determination of TREC levels at birth may also reliably identify other forms (and which ones) of combined immunodeficiency with residual T-cell development. Tandem mass spectrometry can also be used for newborn screening of ADA deficiency[119] and may also identify subjects with residual levels of ADA activity, who could otherwise be missed by the TREC-based assay.[120]

Quantification of TRECs represents an important, yet nonspecific, indicator of a possible severe T-cell immunodeficiency, the diagnosis of which must be confirmed by appropriate immunophenotypic, functional, and molecular tests. Flow cytometry, looking at the distribution and absolute number of the major T-cell subsets, B and NK lymphocytes, may provide important information. Determination of naïve (CD45RA+CD62L+) and activated/memory (CD45RO+ CD62L−) T lymphocytes should be included in the diagnostic panel to correctly identify patients with maternal T-cell engraftment or with leaky SCID and expansion of in vivo activated, activated T cells. Functional tests include in vitro proliferation to mitogens (PHA, anti-CD3 monoclonal antibody) and antigens (provided that the patient has been exposed to these through immunization or natural infection). Ultimately, identification of the specific disease-causing mutation(s) may permit molecular diagnosis of the disease.

Hematopoietic Stem Cell Transplantation

Identification of a patient with SCID should be considered a pediatric emergency.[121] Children with this disorder are so prone to infections that extreme measures must be taken to protect them from microbial invasion. Before newborn screening for SCID became a reality, most patients were already infected when initially evaluated and required aggressive interventions to keep their clinical course from running inexorably downhill. After full immunologic evaluation has been performed (as expeditiously as possible), these patients should begin receiving IVIG infusions. Prophylaxis for *P. jiroveci* pneumonia is indicated for all patients.

Although alternative therapies may work for some patients (e.g., PEG-ADA for ADA deficiency), the definitive treatment for the great majority of patients with SCID and other combined immunodeficiencies is HSCT. A variety of sources have been used for stem cells, including HLA-identical sibling donors, haploidentical related donors (usually parents), unrelated donors, and umbilical cord blood.[122,123] Transplantation from a matched sibling donor in patients with SCID does not require conditioning and is associated with excellent survival and long-term immune reconstitution. Patients with SCID who lack a matched sibling donor may receive HSCT from one of their haploidentical parents, most often the mother. In this case, mature T lymphocytes must be removed from the graft to prevent graft-versus-host disease. T cell–depleted HSCT from a haploidentical donor for SCID may be performed without or with pretransplant chemotherapy. Unconditioned T cell–depleted haploidentical transplantation leads to long-term T-cell engraftment[124]; however, B- and NK-cell engraftment is rarely achieved, and lifelong use of IVIG may be needed, especially in patients who lack B cells (e.g., defects of VDJ recombination) or those whose genetic defects compromise B-cell function (γc, JAK3 defects).[125] Use of chemotherapy may allow stem cell engraftment (and hence also permit reconstitution of B- and NK-cell immunity); however, it is also associated with toxicity. In a large series, overall survival in SCID patients transplanted after the year 2000 was 90% when HLA-matched siblings served as donors and 66% after haploidentical HSCT.[123] However, when transplantation is performed in infants younger than 3.5 months of age, survival is greater than 95%, even after haploidentical donors,[126] thus justifying newborn screening for SCID based on quantification of TRECs at birth.[118]

HSCT is also the mainstay of treatment for other forms of combined immunodeficiency with presence of residual or normal number of autologous T lymphocytes. In such cases, however, pretransplant conditioning must be used to achieve engraftment. Overall survival after HSCT for these conditions is less satisfactory than in patients with SCID, being approximately 80% from matched siblings and 50% from haploidentical donors.[123]

Gene Therapy

Gene therapy for the treatment of human disease was first attempted in patients with ADA deficiency.[127,128] A functional copy of the *ADA* gene in a retroviral vector was transduced into populations of mature T cells or bone marrow cells. Transduced cells expressed normal levels of ADA and showed normal function in vitro. However, clinical efficacy was difficult to assess in these early attempts because adjunctive therapy with PEG-ADA was mandated on ethical grounds, even after the re-introduction of transduced cells into patients. More recently, gene therapy for ADA deficiency has been performed by transducing hematopoietic stem cells without concurrent in vivo administration of PEG-ADA[31,129]; use of submyeloablative doses of busulfan has permitted robust engraftment and long-term immune reconstitution in the vast majority of the patients.[31,32,129] However, the absolute T-cell count remains suboptimal in many patients, possibly reflecting irreversible thymic damage caused by toxic adenosine derivatives.

The earliest "complete" success of gene therapy is considered to have occurred with XSCID.[130,131] Hematopoietic stem cells were transduced ex vivo with a retroviral vector carrying a functional copy of the γ_c gene and then infused into patients without any conditioning chemotherapy. Twenty patients have been treated in this manner. Most patients have exhibited rapid and persistent reconstitution of T cell–mediated immunity. Reconstitution of B-cell immunity has been achieved only in some patients. The therapy appears to be less effective if performed later in life, probably because of deterioration of the thymic rudiment over time.[132]

Unfortunately, 5 of the 20 infants with XSCID treated by gene therapy have developed leukemia, which was fatal in one. Leukemias were caused by insertional mutagenesis. The vector integrated within or near oncogenes (in particular, *LMO2*) and the strong enhancers contained in the viral long terminal repeats (LTRs) caused oncogene transactivation and uncontrolled cell proliferation.[133,134] Novel clinical trials, based on self-inactivating retroviral and lentiviral vectors in which expression of the γ_c gene is driven by weaker cellular promoters (instead of the strong viral LTRs), are currently under way in Europe and in the United States.

IMMUNODEFICIENCY SYNDROMES

The term *syndromic immunodeficiencies* has been coined to describe the occurrence of abnormal development or function of the immune system leading to susceptibility to infection, autoimmunity, or malignancy in the setting of a genetically determined disorder with characteristic features of altered development or function of other organ systems or the body as a whole. Here we will review several disorders with prominent immune dysfunction. These diseases and their associated genes are listed in Table 24-4. Space does not permit a detailed description of the tremendous variety of syndromes that have been associated with immunodeficiency. Interested readers are referred to an excellent review on this topic.[135]

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS) is an X-linked disease characterized by eczema, immunodeficiency, and thrombocytopenia.[136] Bloody diarrhea is often seen and may be the initial feature. Eczema may be mild or exuberant; staphylococcal superinfection is common. Recurrent otitis media and sinopulmonary infections occur frequently, and opportunistic infections are also seen. In addition, autoimmune hemolytic anemia, vasculitis, inflammatory bowel disease, glomerulonephritis, and other autoimmune processes have been frequently observed in WAS. Patients most often die as a result of overwhelming infection or massive hemorrhage. Those who avoid these complications have a greatly increased risk for lymphoma, many of which are EBV positive. There is also an increased risk of aortic aneurysms.[136,137]

Variability of the clinical phenotype has been reported, and a clinical score has been developed to reflect this phenotypic heterogeneity.[136] A milder form of the disease, characterized by thrombocytopenia with mild eczema and lack of severe infections, is also referred to as *isolated X-linked thrombocytopenia (XLT)*.[138] Although XLT patients have better survival rates compared with patients who have classical WAS, nonetheless they have a significant risk of complications (e.g., autoimmunity, hemorrhage). In some cases, the thrombocytopenia may even be intermittent.[139] On the other hand, patients who present early in life with very profound ($<10 \times 10^9$/L) and persistent thrombocytopenia have very poor outcomes.[140]

TABLE 24-4 Cellular Immunodeficiency Syndromes and Their Associated Gene Defects.

Syndrome	Gene
Wiskott-Aldrich syndrome	*WAS*
DiGeorge syndrome	del22q11, *TBX1*
DNA repair defects:	
Ataxia-telangiectasia	*ATM*
Nijmegen breakage syndrome and others	*ATLD, NBS1, PMS2*
Disorders of chromosome instability:	
Bloom syndrome	*BLM*
ICF syndrome	*DNMT3B, ZBTB24*
Disorders of pigmentary dilution:	
Chédiak-Higashi syndrome	*LYST*
Griscelli syndrome type 2	*RAB27A*
Hermansky-Pudlak syndromes types 2 and 9	*AP3B1, PLDN*
Defects of the NF-κB pathway	*IKBKG, IKBA*
Defect in Toll-like receptor signaling	*IRAK4, MYD88*
WHIM syndrome	*CXCR4*
Hyper IgE syndromes	*STAT3, TYK2*
Chronic mucocutaneous candidiasis	*CARD9, IL17RA, IL17F, STAT1*
Immunodeficiencies with skeletal abnormalities:	
Cartilage-hair hypoplasia	*RMRP*
Schimke immuno-osseous dysplasia	*SMARCAL1*
Disorders of vitamin metabolism	*TCN2, MTHFD1*

ICF, Instability facial anomalies; *WHIM,* warts, hypogammaglobulinemia, infections, and myelokathexis.

Platelets are small, do not function normally, and are also cleared more rapidly (although the number of bone marrow megakaryocytes is normal or even increased).[136] In the appropriate clinical context, measurement of platelet size confirms the diagnosis. In normal individuals platelet volume is 7.1 to 10.5 fL, with a diameter of 2.3 ± 0.12 μm, whereas platelets from patients with WAS have volumes ranging from 3.8 to 5.0 fL with diameters of 1.82 ± 0.12 μm. The low platelet volume distinguishes the thrombocytopenia of WAS from immune thrombocytopenic purpura (ITP), in which platelets are often large. However, ITP may develop in as many as 20% of patients with WAS.

The mainstay of treatment for WAS is HSCT.[141] Without HSCT, survival beyond adolescence is uncommon. The rates for 5-year survival after HSCT for WAS approximates 90% for transplants performed after year 2000. Optimal results (survival >95%) are obtained after HSCT from matched siblings, but significantly improved outcome has been also achieved after HSCT from unrelated donors and even mismatched related donors.[141] Mixed chimerism is associated with increased risk of autoimmunity after transplant.[141]

Pretransplant morbidity and mortality rates may be reduced with regular infusions of IVIG and antibiotic prophylaxis.[136] Blood products, when needed, should be irradiated and tested for CMV. Splenectomy often increases the platelet count, although immune thrombocytopenia may later supervene. Moreover, splenectomy markedly increases the risk of overwhelming infections. Immunosuppressive agents may be needed to treat autoimmune complications of the disease.[136]

The immune defects in WAS are variable.[136,142] Immune function may be normal in early infancy, but it gradually wanes. T cell numbers may be decreased, and T cells have diminished, but not absent, responses to mitogens and antigens in vitro. T cells in WAS will also proliferate in response to nonspecific activating stimuli such as phorbol esters and calcium ionophores but show a striking absence of reactivity to immobilized antibodies directed against the CD3 complex of the TCR. The diversity of the T-cell repertoire appears normal throughout childhood, but it may become more restricted with consequent worsening immunodeficiency in adulthood.[143]

Serum levels of IgA and IgE are often elevated, whereas IgM may be low; however, increased serum IgM is associated with an increased risk of autoimmunity and worse outcome.[144] Isohemagglutinins and specific antibody responses to polysaccharide antigens are also diminished. Although the frequency of class-switched B cells (i.e., those not expressing IgM) appears to be normal, there are fewer memory B cells expressing CD27, thus indicating some alteration in B-cell activation in germinal centers that could underlie or contribute to the humoral immunodeficiency.[145] Finally, dendritic cells and monocytes show impaired formation of podosomes and defective directional migration in response to chemotactic signals.[142]

The gene defective in WAS has been identified; its official name is *WAS,* and its product is designated the WAS protein (WASP). The same gene is defective in XLT,[146] as well as in X-linked congenital severe neutropenia.[147] The latter disease is due to activating *WAS* mutations; its phenotype is very different from WAS and includes chronic neutropenia with a differentiation block in the bone marrow and an increased risk of myelodysplasia.[147,148]

WASP interacts with several intracellular partners, including the WASP-interacting protein (WIP), Rho family guanosine triphosphatases, and the Arp2/3 complex, which leads to reorganization of the actin cytoskeleton in response to activating stimuli in lymphoid and myeloid cells.[142]

There is a good, yet imperfect, genotype-phenotype correlation in WAS.[149] In particular, XLT is more often associated with residual expression of WASP and with missense mutations in exons 1 and 2 of the *WAS* gene that correspond to a domain of WASP that interacts with WIP. This interaction stabilizes WASP, thereby explaining reduced levels of WASP detected in cells from patients with XLT.[150] In contrast, the more severe phenotypes are associated with complete absence of protein or with expression of truncated, nonfunctional forms of WASP. Somatic mutations allowing expression of WASP in some cell populations (more often, in CD8+ T lymphocytes) have been frequently reported in WAS.[151] However, the impact of this phenomenon on clinical phenotype remains unclear.

The diagnosis of WAS is facilitated by analysis of WASP expression by flow cytometry or Western-blotting; however, this approach may miss patients who express a mutant form of the protein. Ultimately, mutation analysis is required to confirm the diagnosis.[136] Female carriers of WAS are generally asymptomatic and have normal platelet counts but show nonrandom X-chromosome inactivation in lymphocytes and granulocytes.[152] Occasionally, occurrence of WAS or XLT in females has been reported as the result of various mechanisms, including extreme lyonization favoring the mutant X chromosome,[153,154] random X-chromosome inactivation,[155,156] or *WAS* gene mutation on both X chromosomes.[157]

WAS Protein-interacting Protein Deficiency

A single case of a female patient with mutations of the *WIPF1* gene, encoding WIP, has been reported. The phenotype included recurrent infections, thrombocytopenia, defective T-cell proliferation and chemotaxis, and impaired NK cell function. No expression of the WIP and WASP proteins was detected in circulating cells.[158]

DiGeorge Syndrome

DiGeorge syndrome (DGS) arises as a result of a failure of migration of neural crest cells into the third and fourth pharyngeal pouches. Patients display a characteristic facies, cardiac defects, parathyroid hormone deficiency, and varied immune defects.[159,160] The facies consists of hypertelorism; micrognathia; short philtrum; and low-set, posteriorly rotated ears with small pinnae. Cardiac defects are varied. Type B interrupted aortic arch is the most common and is associated with DGS in 50% of patients with this defect. Truncus arteriosus and other conotruncal anomalies are also common. Structural anomalies of the airways have likewise been found in some patients, as well as dysphagia secondary to laryngoesophageal dysmotility. The bilateral paired parathyroid glands are normally adherent to the thymus. Thus these organs are affected together in this disease. The parathyroid deficiency is often more pronounced than the thymic defect; hypocalcemic tetany is one of the more common initial symptoms of DGS. Even small islands of ectopic thymus tissue may permit the development of sufficient T cells for immune competence. Neuropsychiatric problems and some degree of cognitive impairment are seen in many patients and become more prominent during adulthood.[161]

Partial and complete forms of DGS have been distinguished on the basis of clinical and immunologic characteristics.[159,160] In the complete form the thymus is absent or severely hypoplastic.[162] These patients have absent or markedly decreased numbers of T lymphocytes. B cells are present, but specific antibody production is impaired. Some patients may have erythroderma, similar to that seen in OS. In this condition, also referred to as *atypical complete DGS,* oligoclonal, activated (CD45RO+) tissue-infiltrating T lymphocytes are detected.[162] If untreated, complete DGS is most often fatal in early childhood; patients die as a result of hypocalcemia, cardiac complications, infection, or a combination of these causes.

The occurrence of partial forms of DGS outnumbers that of the complete form by about 100 : 1. T-cell number and function in most patients are varied and presumably correlate with the amount of ectopic thymus tissue present. Immune function in patients with complete DGS does not improve; patients with partial DGS may have less immunodeficiency as they get older. In some cases, the diagnosis has been made in adults being evaluated for hypoparathyroidism and hypocalcemia.[163] Humoral immunity is generally intact in patients with partial DGS; however, IgA deficiency and defective antibody production have been reported.[160] An increase in atopic dermatitis and asthma has been reported in individuals with 22q11 deletion.[164] Autoimmunity (especially ITP, other cytopenias, juvenile arthritis, and celiac disease) is observed in 10% of the patients.[165]

In various series, between 55% and 90% of patients with DGS have 2 to 3 megabase deletions involving chromosome 22q11.2.[166,167] Deletions in the same region are found in patients with a spectrum of phenotypically overlapping conditions, including velocardiofacial syndrome and conotruncal anomaly face syndrome, and in some patients with isolated cardiac defects. There is heterogeneity in expression of the DGS phenotype, even in monozygotic twins. Approximately 40 genes are present in the deleted regions in DGS, and the contributions of each to the phenotype are only beginning to be understood.[168] *TBX1* encodes a member of a family (T-box) of transcription factors. Mice that are heterozygous for deletion of this gene have a phenotype that is variable but often very similar to that of humans with DGS, including thymic and parathyroid hypoplasia, cardiac outflow tract abnormalities, and abnormal facial structure.[169] Two truncating and three missense mutations in *TBX1* have been found in a few patients with DGS or velocardiofacial syndrome and no detectable 22q11.2 deletion.[170-172] Interestingly, the missense mutations were found to result in gain of function, suggesting that increased TBX1 activity may phenocopy *TBX1* loss of function or haploinsufficiency.[172] Some patients with DGS have deletions on chromosome 10p13-p14.[173]

Although recurrent infections can be observed in up to 25% to 30% of patients with DGS, severe viral and opportunistic infections are very rare, other than in patients with complete DGS. Live viral vaccines may be administered safely to the great majority of patients.[160] By contrast, complete DGS has a severe prognosis, but it can be treated successfully by thymus transplantation. Immunosuppression is needed to control severe immune dysregulation in patients with complete atypical DGS while preparing for thymus transplantation.[174] In a recent study 43 of 60 patients (72%) treated with this procedure were reported to be alive at a median of 4.7 years after transplantation, and they attained an increase in T-cell count with improved function, although absolute naïve CD4+ and CD8+ counts remained below the 10th percentile in most of them.[174] HSCT from matched donors may also provide some immune reconstitution through homeostatic proliferation of mature T lymphocytes contained in the graft; however, in a series of 17 patients with complete DGS treated by HSCT, overall survival rate was only 41% and the count of naïve CD4+ cells remained very low.[175]

FOXN1 Deficiency

The transcription factor FOXN1 plays a critical role in development of the thymus and eccrine glands. *FOXN1* mutations in humans cause athymia, profound T-cell lymphopenia, alopecia totalis, and nail dystrophy, fully resembling the "nude" phenotype observed in *Foxn1*-mutated mice.[176] There is complete lack of CD4+ lymphocytes, whereas a low number of oligoclonal CD8+ cells may be present.[177] Similar to DGS, reconstitution of T-cell immunity can be achieved with thymic transplantation.[178] Alternatively, HSCT from HLA-identical siblings may provide some immune reconstitution through homeostatic proliferation of T lymphocytes contained in the graft.[179]

Ataxia-Telangiectasia

Ataxia-telangiectasia (AT) is an autosomal recessive disorder with an estimated incidence of 1 in 20,000 U.S. Caucasian births (95% confidence interval, 1 in 2500 to 700,000).[180] AT is characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, and immunodeficiency.[181] Associated features are an increased incidence of lymphoma and sensitivity to radiation. Impairment in motor development may be noted early in the disease course and is progressive. Walking may be delayed until 16 to 18 months of age or later. Telangiectases of conjunctival and cutaneous vessels do not appear until children are 3 to 5 years of age. The cutaneous lesions are found mainly on the pinnae and in skin creases. Immunodeficiency will occur in approximately 70% of patients. Manifestation of the immunodeficiency is quite varied, but it is often seen as recurrent sinopulmonary bacterial infections. Granulomatous lesions are frequently seen and may reflect immune dysregulation.[182] Growth retardation is a prominent feature in many patients and has been associated with decreases in insulin-like growth factor I and its binding protein.[183]

The median age at death in patients with AT is approximately 19 to 25 years.[184,185] Many patients die as a result of progressive pulmonary disease caused by repeated infection. Possibly, noninfectious and ultimately fatal interstitial lung disease may also occur in approximately 25% of patients.[186] The lifetime risk for malignancies in AT is 30% to 40%; 85% of the cancers are acute leukemias or lymphomas.[187] Elevated serum levels of α-fetoprotein (AFP) are found in more than 95% of patients with AT and have diagnostic value in the appropriate clinical context; however, levels of AFP are physiologically higher at birth and for the first two years.[188]

T and B cell lymphopenia is a well-recognized, though variable, feature of AT in adulthood but may also be present at birth and be detected by abnormal TREC levels at newborn screening for SCID.[189] T cells bearing the TCRγδ constitute up to 50% or more of the total number of T cells in patients with AT.[190] These cells make up fewer than 10% of peripheral T cells in normal individuals. Cutaneous delayed hypersensitivity responses, as well as results of in vitro assays of T-cell function, are often depressed. Varying degrees of humoral immunodeficiency are common. IgA deficiency occurs in two thirds of patients. Decreased levels of IgG2, IgG4, and IgE are also common, yet approximately 10% to 30% of the patients have increased IgM.[191,192] Impaired specific antibody responses, particularly to pneumococcal polysaccharide antigens, have also been noted in AT patients.[193]

The gene that is defective in AT is designated *ATM* (AT mutated). *ATM* has critical roles in regulation of the cell cycle: It is involved in the detection of DNA damage and in the initiation of repair.[194] There is a marked delay in induction of the tumor suppressors p53 and *BRCA1* after exposure of AT cells to radiation. Even low-dose radiation leads to significantly greater DNA damage in AT fibroblasts and lymphoblasts than in normal individuals.[195] *ATM* mutations are associated with impaired lymphocyte proliferation.[196] Lymphocytes of patients with AT also show a remarkable number of chromosomal translocations and inversions. These changes predominantly involve the loci that rearrange to generate mature Ig (2p12, 22q12, and 14q32) and TCR (7p15, 7q35, and 14q11) genes and reflect defective repair of the double-stranded breaks produced as part of the physiologic gene rearrangement process during lymphocyte development. ATM is also involved in class-switch recombination (CSR), and this may explain reduced levels of IgG, IgA, and IgE seen in patients.[197]

No definitive cure is available for AT. Medical management aimed at preventing and treating infections (antibiotics, Ig), tumor surveillance, and neurorehabilitation represent the mainstays of treatment.[188] Very recently, some improvement of neurologic signs has been observed on administration of betamethasone, possibly reflecting antiinflammatory and antioxidant activity of glucocorticoids in the CNS.[198]

Whether pathologic changes result from the heterozygous carrier state of AT remains a subject of controversy. Carriers heterozygous for *ATM* mutations have none of the classic clinical manifestations of AT; however, the sensitivity of their DNA to radiation is increased, and several reports suggest that AT heterozygotes have an increased rate of a variety of malignancies, such as breast cancer.[199]

OTHER IMMUNODEFICIENCIES ASSOCIATED WITH DEFECTIVE MECHANISMS OF DNA REPAIR

The Nijmegen breakage syndrome is a disorder of chromosome fragility with autosomal recessive inheritance.[200] The phenotype is similar to that of AT and consists of growth retardation, microcephaly, immunodeficiency, sensitivity to radiation, and a high rate of lymphoma. The affected gene is designated *NBS1*. Nibrin, its protein product, is another substrate of ATM in the cellular response to ionizing radiation. Absence of nibrin function leads to disruption of mechanisms of DNA repair, but it does not appear to result in abnormal function of cell cycle checkpoints. Another similar syndrome, AT-like disorder, results from mutations of the gene *MRE11A*, which encodes another ATM substrate.[201]

Postmeiotic segregation 2 (PMS2) and MutS homologue 6 (MSH6) are components of the DNA mismatch repair pathway. PMS2 deficiency is associated with increased occurrence of malignancies, café-au-lait spots, and defective CSR.[202] MSH6 deficiency is also associated with increased risk of cancer and defective maturation of antibody responses, with impairment of CSR and a skewed pattern (higher rate of transitions) of somatic hypermutation.[203]

IMMUNODEFICIENCIES WITH CHROMOSOMAL INSTABILITY

Bloom syndrome is characterized by chromosomal instability with increased sister chromatid exchange and homologous chromosome translocations and is associated with increased frequency of infections, low levels of IgM, and impaired delayed-type hypersensitivity responses. The disease is caused by mutations of the *BLM* gene, encoding for a helicase.[204]

The immunodeficiency-centromeric instability facial anomalies syndrome is characterized by hypogammaglobulinemia, facial dysmorphisms, and branching of chromosomes 1, 9, and 16. The disease may be caused by mutations in the DNA methyl transferase 3B *(DNMT3B)*[205] or in the zinc-finger- and-BTB (bric-a-brac, tramtrack, broad complex)-domain-containing 24 *(ZBTB24)* genes.[206]

Mutations of the mini-chromosome maintenance-deficient 4 *(MCM4)* gene have been identified in patients with recurrent and severe viral infections (in particular, caused by herpes simplex virus 1 [HSV-1], varicella zoster virus [VZV] and CMV), growth retardation, adrenal insufficiency, and selective NK cell deficiency with lack of the CD56^{dim} NK subset.[207,208] The disease is inherited as autosomal recessive trait. MCM4 is a component of a multiprotein complex required for both initiation and elongation of eukaryotic DNA replication.[209] *MCM4* mutations were shown to cause abnormalities of cell cycle, with increased proportion of fibroblasts in G2/M and S phases of cell cycle and an aberrant (>4n) DNA content, and genomic instability.[207]

PIGMENTARY DILUTION DISORDERS

Pigmentary dilution disorders include diseases in which abnormalities in melanosome trafficking within cells lead to characteristic pale skin and silvery-gray or ashen hair. Several forms of pigmentary dilution disorders are also characterized by a variable degree of immune deficiency, in particular impaired cell-mediated cytotoxicity. In these conditions inability to clear virus-infected cells leads to persistent and exaggerated inflammatory responses with macrophage activation, release of proinflammatory cytokines, and continuous activation of infiltrating CD8$^+$ T cells that secrete high amounts of IFN-γ and cause tissue damage. Fever, hepatosplenomegaly, lymphadenopathy, and cytopenia caused by impaired hematopoiesis in the bone marrow and hemophagocytosis are typical manifestations of immune activation during the so-called accelerated phase of the disease. Impaired cytotoxicity of virus-infected cells reflects defective trafficking, docking or release of cytolytic granules in NK cells, and cytotoxic T cells. This defect parallels defects of melanosome trafficking.

Chédiak-Higashi syndrome is characterized by partial albinism, peripheral neuropathy, and increased risk of viral infections. Neutropenia is often present and may facilitate bacterial infections. Recognition of "giant" lysosomes in leukocytes facilitates the diagnosis.[210] The disease is caused by mutation of the lysosomal trafficking regulator *(LYST)* gene.[211] The precise mechanism by which the LYST protein works is not known, although it is thought to regulate trafficking of intracellular vesicles. HSCT is the only definitive treatment, but it does not prevent possible progression of neurologic complications.

Griscelli syndrome (GS) type 2 also associates partial albinism and immunodeficiency with increased susceptibility to infections, fever, and cytopenia. The disease is caused by mutations of the *RAB27A* gene, which encodes for a protein that permits docking of the cytolytic granules to the cell membrane.[212] In contrast, GS type 1 (caused by mutations of the *MYO5A* gene, encoding for the myosin Va protein) and GS type 3 (due to mutations of the gene encoding for the melanin chaperone melanophilin) are characterized by hypopigmentation without immunodeficiency.

Hermansky-Pudlak syndrome type 2 (HPS2) is characterized by oculocutaneous albinism, bleeding diathesis, neutropenia, interstitial lung disease, and recurrent infections.[213] Patients are at increased risk of hemophagocytosis. The disease is caused by mutations of the β subunit of the adaptor-related protein complex 3 (gene *AP3B1*) involved in sorting of proteins to the lysosomes.[214] Patients with HPS9, which is caused by mutations of the pallidin *(PLDN)* gene,[215] present with hypopigmentation and nystagmus; recurrent cutaneous infections also have been reported.[216]

MUTATIONS OF THE NUCLEAR FACTOR κ-B PATHWAY

NF-κB is a transcription factor that is critical for the activation of numerous genes on leukocyte stimulation.[217] Activity of this factor is regulated by an inhibitor known as IκB. Phosphorylation of IκB by IκB kinase (IKK) leads to IκB degradation and liberates active NF-κB. IKK is composed of three subunits, α, β, and γ; the latter is also known as the NF-κB essential modulator (NEMO). The gene encoding NEMO is on the X chromosome. Null mutations in this gene are incompatible with life for male subjects and lead to the X-linked dominant disease incontinentia pigmenti in females. Certain mutations that allow partial NEMO function are associated with an immunologic phenotype that may have some similarities to hyper-IgM. Along with a combined immunodeficiency, these patients may exhibit hypohidrotic ectodermal dysplasia and lymphedema with osteopetrosis.[217-220] Distinct hypomorphic mutations define specific disease characteristics, thus establishing genotype-phenotype correlation, albeit imperfectly.[218] Somatic mosaicism in T lymphocytes has been detected in a significant proportion of males with NEMO deficiency, providing evidence for selective advantage for NEMO-expressing T cells in vivo.[221] However, whether this may affect the disease phenotype is not clear.

Patients may suffer opportunistic-type disseminated viral infections and *P. jiroveci* pneumonia and are also highly susceptible to atypical mycobacterial infections. Inflammatory bowel disease is also frequently seen, reflecting a role of NF-κB in controlling epithelial integrity and the interaction between the mucosal immune system and gut microflora.[222] Finally, lymphedema and osteopetrosis may also be part of the disease phenotype.[220]

Most patients are hypogammaglobulinemic and have impaired vaccine responses (especially to polysaccharide antigens). Peripheral blood lymphocyte subsets and in vitro measures of lymphocyte function may be unremarkable or variably diminished; NK cell cytotoxicity may be impaired. Signaling by way of TLRs is also affected. Therapy with gamma globulin and antibiotic prophylaxis is essential. Despite these measures, infections may still occur. HSCT may cure the immunologic abnormalities and resolve increased susceptibility to infections, but inflammatory bowel disease may persist, along with extra-immune manifestations of the disease.[223]

A few patients have been found to have a similar phenotype as a result of a so-called hypermorphic (gain of function) mutation of the IκBα chain.[224,225] In this situation the IκBα is resistant to phosphorylation by IKK and cannot be dissociated, degraded, and induced to release NF-κB so that it may translocate to the nucleus to activate transcription.

DEFECTS OF TOLL-LIKE RECEPTOR SIGNALING

IRAK-4 and Myd88 are components of a complex mediating signaling downstream of all TLRs (except TLR3).[226] Patients with *IRAK4* or *MYD88* mutations have severe, invasive bacterial infections such as meningitis, bacteremia, septic arthritis, deep-tissue abscesses, and osteomyelitis with gram-positive organisms (predominantly *Staphylococcus aureus* and *Staphylococcus pneumoniae*.[227-229] Less severe skin and respiratory infections also occur frequently. Most patients begin to exhibit infections in early childhood, but these wane significantly in frequency and intensity in the second decade of life. Most patients have impaired antibody responses to pneumococcal polysaccharides, but other routine tests of immune function are normal. Many patients have high levels of all Ig isotypes, possibly owing to immune stimulation by repeated bacterial infections. Signaling is impaired through all TLRs other than TLR3. Management is mainly with antibiotic prophylaxis and IgG therapy.

THE SYNDROME OF WARTS, HYPOGAMMAGLOBULINEMIA, INFECTIONS, AND MYELOKATHEXIS

The syndrome of warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM syndrome) is an autosomal dominant disorder of leukocyte trafficking caused by mutations of the chemokine receptor CXCR4.[230] Patients carry heterozygous mutations in the intracellular tail of CXCR4 that prevent interaction with β-arrestin, thus allowing continuous signaling induced by CXCR4 ligand, CXCL12. This signal retains mature neutrophils in the bone marrow (myelokathexis) and alters trafficking of B and T lymphocytes, causing lymphopenia.[231]

Impairment of cellular immunity and defective number of plasmacytoid dendritic cells (which are the major source of IFN-γ) cause warts, and neutropenia and hypogammaglobulinemia account for recurrent bacterial infections that mostly affect the respiratory tract.[231,232] The number of switched memory B cells is reduced, and there is oligoclonality of the antibody repertoire.[233] Gamma globulin replacement therapy generally reduces the rate of infection. The CXCR4 antagonist AMD3100 (plerixafor) corrects neutropenia and lymphopenia.[234]

HYPERIMMUNOGLOBULIN E SYNDROME CAUSED BY *STAT3* MUTATIONS

Hyper-IgE syndrome (HIES, often designated type 1) was initially described in 1972 in patients with severe sinopulmonary bacterial infections, severe skin superinfections with *S. aureus* on a chronic eczematous eruption (for which the disease is also known as *Job syndrome*), and susceptibility to *Aspergillus fumigatus* pneumonitis with pneumatocele formation.[235] Additional manifestations include asymmetric or coarse facial features, delayed shedding of primary teeth, bone fragility and fractures, scoliosis, and keratoconjunctivitis.[236] Patients show increased susceptibility to nonfilamentous molds, such as *P. jiroveci* pneumonia and in particular chronic mucocutaneous candidiasis (CMC). There is an increased rate of malignancies, especially non-Hodgkin lymphoma.[236] Cardiovascular anomalies have been described in recent years and include aneurysms, in particular of the carotids, cerebral arteries, and coronary arteries, and may lead to additional complications such as myocardial infarction.[237] Inheritance of this form of HIES is autosomal dominant, but many cases represent sporadic presentations. The disease is caused by heterozygous dominant-negative mutations of the *STAT3* gene that encodes for a transcription factor involved in signaling from a multitude of receptors.[238,239] In particular, STAT3 is required for the development of T_h17 cells, which secrete IL-17 and IL-22, two cytokines that play an important role in defense against candida and in promoting secretion of antimicrobial peptides by bronchial epithelial cells.[240]

The major immunologic findings are elevated serum levels of IgE (3000 to >50,000 IU/mL) and eosinophilia. Along with a deficiency of T_h17 cells, a reduced number of memory (CD27+) B lymphocytes also have been reported. The diagnosis is established on clinical and laboratory grounds; a scoring system[241] has been developed that may be helpful, especially if used in combination with demonstration of lack of T_h17 cells.[242] Management of HIES caused by *STAT3* mutations is mainly by skin care and antimicrobial prophylaxis against *S. aureus*. The role of antifungal prophylaxis is controversial, but it may be helpful in patients with CMC. HIES patients may lack classical signs and symptoms of infection, such as fever. Therefore careful physical examination, medical history, and imaging studies are needed to evaluate, diagnose, and treat any infections. Controversial results have been obtained after HSCT.[243,244]

AUTOSOMAL RECESSIVE HYPER-IMMUNOGLOBULIN E SYNDROME CAUSED BY *TYK2* MUTATIONS

Two distinct forms of autosomal recessive HIES are known. Of these, DOCK8 deficiency has been discussed in the preceding section "Other Combined Immunodeficiencies with Variable Presentation." Two patients with autosomal recessive HIES caused by mutations of *TYK2*, a member of the JAK family of tyrosine kinases involved in intracellular signaling, have been reported. The first patient presented with atopy, elevated serum IgE, and susceptibility to infections sustained by *S. aureus*, BCG, *Salmonella* organisms, and viruses.[245] The second patient suffered from disseminated BCG infection, neurobrucellosis, and cutaneous herpes zoster but had normal serum IgE and did not develop atopy.[246]

CHRONIC MUCOCUTANEOUS CANDIDIASIS

CMC affecting the nails, skin, and mucosa is the clinical hallmark of three distinct genetic disorders that affect IL-17–dependent immunity.[247] These include complete, autosomal recessive deficiency of IL-17 receptor α chain *(IL17RA)*,[248] partial autosomal dominant IL-17F deficiency,[248] and heterozygous gain-of-function mutations of signal transducer and activator of transcription 1 *(STAT1)*.[249,250] The latter represents the most common cause of isolated CMC. Mutations of caspase recruitment domain-containing protein 9 *(CARD9)* gene, encoding a cytosolic adaptor molecule involved in intracellular signaling in cells recognizing fungi, also affect development of T_h17 cells and cause CMC; however, CARD9-deficient patients are also at increased risk of disseminated *Candida* infections as well as of other fungal infections.[251] Deficiency of T_h17 cells accounts for CMC also in other forms of primary immunodeficiency, including T-cell deficiencies, HIES, and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

IMMUNODEFICIENCIES WITH SKELETAL ABNORMALITIES

Cartilage-hair hypoplasia (CHH) is an autosomal recessive disease characterized by short-limbed dwarfism with metaphyseal dysplasia, sparse and fair hair, and joint hypermotility. Dyserythropoietic anemia and a variable degree of immunodeficiency are present in 80% of the patients. A minority of subjects also present with Hirschsprung disease. There is a 6% to 10% rate of malignancies, especially lymphoma and basal cell carcinoma. The disease has a higher prevalence among Finnish and Amish populations[252] and is caused by mutations in the *RMRP* gene that encodes for the untranslated RNA component of the mitochondrial RNA-processing endoribonuclease[253] that is involved in multiple functions, including ribosomal and messenger RNA processing, mitochondrial DNA replication, and negative regulation of target RNA transcripts on interaction with TERT (telomerase reverse transcriptase).[252]

The immunodeficiency of CHH is characterized by increased frequency of infections. Severe and persistent viral infections (EBV, CMV, HSV, and VZV) and fungal infections may occur, in addition to recurrent respiratory infections. Granulomatous lesions affecting the skin and other tissues have been described in several patients.[254] Laboratory tests reveal a variable degree of immune dysfunction that predominantly affects T cells. In rare cases, immunodeficiency is very severe and may resemble SCID, "leaky" SCID with reduced number of CD8+ lymphocytes, or OS.[255,256] Irrespective of the severity of the clinical phenotype, thymopoiesis is reduced, and there is T-cell lymphopenia. Moreover, T lymphocytes show abnormalities of cell cycle progression and an increased rate of apoptosis.[257] HSCT is an effective form of treatment for patients with a severe clinical phenotype.[258]

Schimke immuno-osseous dysplasia is an autosomal recessive disease characterized by spondyloepiphyseal dysplasia, focal glomerulosclerosis leading to progressive renal failure, and T-cell immunodeficiency.[259] There is T cell lymphopenia, with an increased proportion of activated/memory T cells and of TCRγδ-expressing T lymphocytes. The disease is caused by mutations of the *SMARCAL1* gene, which encodes for a chromatin remodeling protein.[260]

IMMUNODEFICIENCIES ASSOCIATED WITH DISORDERS OF FOLATE AND COBALAMIN METABOLISM

Inborn errors of folate and cobalamin metabolism may associate with varying degrees of immunodeficiency. In particular, genetically determined defects in vitamin B_{12} metabolism are associated with megaloblastic anemia, neutropenia, and neurologic symptoms with seizures and developmental delay.[261] Transcobalamin II (gene *TCN2*) deficiency causes neutropenia leading to recurrent bacterial infections.[262] Severe lymphopenia is observed in patients with deficiency of methionine synthase, causing a SCID-like phenotype with severe viral and bacterial infections.[263] Mutations of proton-coupled folate transporter that impede folate absorption through the gastrointestinal mucosa are also associated with extreme lymphopenia and clinical features of SCID.[264] Mutations of the *MTHFD1* gene that encodes for a trifunctional protein essential for processing of single-carbon folate derivatives cause SCID with decreased number of T, B, and NK lymphocytes, which is associated with megaloblastic anemia, leukopenia, atypical hemolytic uremic syndrome, and neurologic abnormalities. Parenteral supplementation therapy is beneficial.[265]

GATA2 DEFICIENCY

In 2010 Vinh and coworkers[266] reported on a series of patients with a distinct clinical phenotype characterized by onset in late childhood or adulthood, susceptibility to disseminated nontuberculous mycobacterial disease, recurrent and severe viral infections (especially those caused by papillomavirus and herpesviruses), and fungal infections. There is increased risk of myelodysplasia and malignancies, including acute myeloid leukemia, squamous cell carcinoma, and EBV-positive leiomyosarcoma. Pulmonary alveolar proteinosis and primary lymphedema have been observed in several patients.[266-269]

Typical laboratory features include profound monocytopenia, severe reduction in the number of B and NK lymphocytes (in particular, of the $CD56^{bright}$ subset) and dendritic cells and elevated levels of fms-like tyrosine kinase ligand (Flt3L).[266,267,270] Depletion of regulatory T cells also has been reported.[267] Examination of the bone marrow reveals hypocellularity, often associated with multilineage dysplasia and cytogenetic abnormalities, including monosomy 7 and trisomy 8. In vitro IL-12 and IFN-γ production in response to stimulation with LPS is markedly reduced,[267,268] suggesting that disruption of the IL-12/IFN-γ axis may contribute to the increased occurrence of mycobacterial disease.

The disease has been referred to as *MonoMAC syndrome* (to indicate the monocytopenia and susceptibility to nontuberculous mycobacterial infections)[266,268] or DCML (dendritic cells, monocytes, B and NK lymphocytes) deficiency[267] and has been shown to be caused by heterozygous, loss-of-function mutations of the *GATA2* gene.[271-273] GATA-2 is a transcription factor that contains two highly conserved zinc finger domains that mediate protein-DNA and protein-protein interactions and is required for stem cell homeostasis,[274] lymphatic vessel development,[273] and the maturation and homeostasis of NK lymphocytes.[270] Disease-causing mutations also include intronic deletions and point mutations in regulatory regions of the gene that affect *GATA2* transcript levels.[275] Both autosomal dominant inheritance and sporadic presentation have been reported. If untreated, the disease has a severe prognosis, with death occurring in adulthood as a result of infections or malignancies. Nonmyeloablative HSCT has been successfully performed in several patients, with reconstitution of the number of monocyte, B-cell, and NK-cell populations and reversal of the clinical phenotype.[276]

IMMUNODEFICIENCIES WITH IMMUNE DYSREGULATION

Although autoimmune and inflammatory manifestations may be seen in a large number of primary immunodeficiencies, they represent the main feature in some. The pathophysiology of these disorders may reflect a variety of mechanisms, including impairment of central and peripheral tolerance and chronic immune activation associated with the inability to clear infections. These disorders and their associated genes are listed in Table 24-5.

AUTOIMMUNE POLYENDOCRINOPATHY-CANDIDIASIS-ECTODERMAL DYSTROPHY SYNDROME

APECED syndrome represents the prototypic disorder associated with impairment of central T-cell tolerance.

TABLE 24-5 Immunodeficiency Syndromes Associated with Dysregulation

Syndrome	Gene
Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED)	*AIRE*
Immune dysregulation polyendocrinopathy X-linked (IPEX)	*FOXP3*
Defects of IL-2 signaling	*IL2RA, STAT5B*
Autoimmune lymphoproliferative syndrome (ALPS)	*CASP8, CASP10, FAS, FASLG, KRAS, NRAS*
Other	*ITCH*

APECED is inherited as an autosomal recessive trait and is characterized by autoimmune hypoparathyroidism, Addison disease, and CMC. Patients are also prone to other autoimmune manifestations, such as hepatitis, alopecia, ovarian failure, and hypothyroidism. There is an increased incidence of oral squamous cell carcinoma, and fulminant autoimmune hepatitis. The disease is caused by mutations in the gene *AIRE* (autoimmune regulator) encoding a transcription factor expressed by medullary thymic epithelial cells (mTECs).[277,278] AIRE promotes expression of tissue-specific antigens that are presented by mTECs and thymic dendritic cells to nascent T lymphocytes.[279] High-affinity recognition of self peptides leads to deletion of self-reactive T cells. Therefore mutations of the *AIRE* gene compromise the mechanism of central tolerance and result in APECED, which is associated with production of multiple autoantibody specificities.[280] Interestingly, autoantibody production in patients with APECED is not restricted to AIRE-dependent self-antigens but also includes antibodies to interferon-α and β[281] as well as IL-17 and IL-22.[282,283] Defective T_h17-dependent immune responses may account for chronic mucocutaneous candidiasis in this disease.

IMMUNE DYSREGULATION POLYENDOCRINOPATHY ENTEROPATHY X-LINKED SYNDROME

Treg cells maintain peripheral T cell tolerance by suppressing self-reactive T lymphocytes that have escaped central tolerance.[284] Generation of Treg cells is controlled by the transcription factor FOXP3.[285] Mutations of the *FOXP3* gene cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome.[286,287] IPEX is characterized by severe early-onset autoimmune manifestations that involve the gut (causing intractable diarrhea), pancreas (diabetes), the skin, and other organs. Lymphoproliferation and cytopenias are also very common. Delayed presentations have been also observed.[288] Patients with IPEX require immune suppression, but the only definitive treatment currently available is allogeneic HSCT.[289]

DEFECTS OF IL-2–MEDIATED SIGNALING

IL-2–mediated signaling is important to maintain immune homeostasis and support the function of Treg lymphocytes. Mutations in the IL2RA gene, which encodes CD25, the α chain of the IL-2R, cause immunodeficiency with IPEX-like immune dysregulation.[290,291] The clinical phenotype is marked by severe diarrhea, failure to thrive, CMV infection, chronic lung disease, lymphadenopathy, splenomegaly, and autoimmunity (diabetes mellitus, autoimmune hepatitis). Poor in vitro T-cell function that could not be corrected with exogenous IL-2 was demonstrated. The number of $FOXP3^+$ cells was preserved in patients with CD25 deficiency; however, secretion of IL-10, a cytokine with immunosuppressive activity, was impaired,[290] possibly accounting for the autoimmune manifestations of the disease.

Mutations of the signal transduced and activator of transcription 5B *(STAT5B)* gene, which encodes a transcription factor activated by IL-2 signaling, cause an autosomal recessive form of immunodeficiency with immune dysregulation. Because STAT5B is also activated in response to growth hormone receptor stimulation, patients with STAT5B deficiency also show growth failure with growth hormone insensitivity.[292,293]

AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME

Apoptosis of self-reactive lymphocytes is another important mechanism to prevent autoimmunity in the periphery and may be induced either through the extrinsic, Fas-dependent pathway or through induction of the mitochondrial pathway in response to cell damage and cytokine (e.g., IL-2) starvation. Both pathways converge on caspase 9 and downstream effector caspases 3 and 7, causing apoptosis. Autoimmune lymphoproliferative syndrome (ALPS) is characterized by lymphadenopathy, splenomegaly, and autoimmune cytopenias and an increased risk of developing lymphoma. Infiltrative lymphoproliferative disease may also cause organ-specific complications (glomerulonephritis, hepatitis, encephalitis, uveitis, interstitial pulmonary disease). There is a characteristic expansion of CD3+TCRαβ+CD4−CD8− double-negative T cells (DNTs).[294]

Most patients are heterozygous for a mutation in the *FAS* gene; in such families the disease is inherited as an autosomal dominant trait with variable penetrance. Somatic mutations in *FAS* are another common cause of the disease.[295,296] More rarely, patients may show biallelic mutations.

Other rare causes of ALPS are represented by mutations of genes encoding Fas ligand (FasL),[297] FADD,[298] caspase 8,[299] and caspase 10,[300] all of which are involved in the Fas-mediated signaling pathway leading to apoptosis. The number of DNTs is only marginally elevated in patients with caspase 8 deficiency.

In addition to an increased proportion of DNTs, other biomarkers that may facilitate the diagnosis of ALPS and that are associated especially with *FAS* mutations include elevated serum levels of vitamin B_{12}, soluble FasL, IL-10, and IL-18. Hypergammaglobulinemia is common. Moreover, patients with ALPS caused by *FAS* mutation frequently have a reduced number of memory (CD27+) B lymphocytes.[301]

Defects of the "intrinsic" (mitochondrial) pathway of apoptosis leading to ALPS include gain-of-function mutations of *NRAS*[302] and somatic mutations of *KRAS*,[303,304] but these variants do not result in an increased number of DNTs.

Management of ALPS includes use of immunosuppressive drugs (steroids, mycophenolate mofetil, sirolimus) to control autoimmune manifestations and regular monitoring of lymphoproliferation by computed tomography scanning or positron emission tomography for surveillance of lymphoma. Splenectomy may improve the cytopenias but is associated with a marked increase of sepsis. Chronic cytopenias tend to improve over time; HSCT should be reserved for very severe clinical presentations with autoimmune disease refractory to treatment.[305]

ITCH DEFICIENCY

Mutations of the E3 ubiquitin ligase ITCH have been shown to cause multisystem autoimmune disease in affected individuals from a large Amish kindred.[306] Characteristic clinical features include failure to thrive, hepatosplenomegaly, multiorgan autoimmune manifestations (hypothyroidism, hepatitis, diabetes, enteropathy), chronic lung disease, developmental delay, dysmorphic features, and hypotonia. Dysfunction of E3 ubiquitin ligases have been shown to lead to indiscriminate T cell activation, loss of tolerance to self-antigens and accumulation of autoreactive B lymphocytes.[307]

IMMUNODEFICIENCIES WITH IMPAIRED CELL-MEDIATED CYTOTOXICITY

CD8+ cytotoxic T lymphocytes (CTLs) and NK cells play a critical role in antiviral immune defense through two mechanisms: Fas-mediated apoptosis of virus-infected cells and release of cytolytic granules. In resting CTLs and NK lymphocytes, cytotoxic proteins are contained in endosomal secretory granules. On activation of CTLs and NK lymphocytes, lytic granules polarize toward the immune synapse, dock, and fuse with the cell membrane of the effector cell. Multimers of perforin form pores through which CTLs and NK cells release cytotoxic proteins (granzyme B, granulolysin) into virus-infected target cells, causing activation of caspases and apoptosis of target cells.[308]

Defects in this process are responsible for various forms of familial hemophagocytic lymphohistiocytosis.[309] Defective cell-mediated cytotoxicity is also observed in several forms of pigmentary dilution disorders (discussed previously). Finally, selective susceptibility to EBV infection is at the basis of various forms of lymphoproliferative syndrome. These diseases and their associated genes are listed in Table 24-6.

TABLE 24-6 Immunodeficiency Syndromes with Impaired Cytotoxicity

Syndrome	Gene
Familial hemophagocytic lymphohistiocytosis	*PRF1, STX11, STXBP2, UNC13D*
X-linked lymphoproliferative syndromes	*BIRC4, SH2D1A*
Other	*CD16, CD27, ITK*

FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

Familial hemophagocytic lymphohistiocytosis (FHL) includes a heterogeneous group of monogenic disorders characterized by impaired cell-mediated cytotoxicity. In patients with FHL, the inability to clear viral infections causes persistent activation of CD8+ and NK lymphocytes, with production of high amounts of IFN-α and -β and of proinflammatory cytokines (tumor necrosis factor-alpha [TNF-α], IL-6).[309] Clinical features of FHL include recurrent episodes of high fever, pancytopenia, liver and spleen enlargement, and signs of neurologic involvement. The diagnostic criteria were revised in 2007.[310] Elevated serum levels of triglycerides, ferritin, and soluble CD25 and decreased levels of fibrinogen and poor NK function are typical laboratory findings. If left untreated, FHL is rapidly fatal. Severe bacterial and fungal infections and multiple organ failure are the main causes of death. Treatment should be aimed at eradicating the underlying infection while suppressing immune activation at the same time. Corticosteroids, cyclosporine A, etoposide, and antithymocyte globulin are often effective in tempering immune activation.[311] However, relapses are common, and the only curative treatment is represented by HSCT. In particular, reduced intensity conditioning has shown excellent efficacy and limited toxicity.[312]

The genetic defect has been identified for four forms of FHL so far, and all of them are inherited as autosomal recessive traits. Perforin deficiency accounts for approximately 30% of all cases of FHL. It is caused by mutations of the *PRF1* gene that prevent expression or correct assembly of perforin multimers that form pores through which cytolytic enzymes are released into target cells.[313] Mutations of the *UNC13D* gene, encoding for Munc13-4, impair priming of the cytolytic granules, which is necessary for their fusion with the cell membrane. *UNC13D* mutations represent the most common form of FHL (30% to 40% of all cases).[314] Defects of the Syntaxin 11 *(STX11)* gene affect granule exocytosis.[315] Finally, FHL may also be caused by defects of Munc18-2, a protein partner of STX11 encoded by the Syntaxin binding protein 2 *(STXBP2)* gene.[316]

X-LINKED LYMPHOPROLIFERATIVE DISEASE

X-linked lymphoproliferative (XLP) disease is characterized by unique susceptibility to severe complications after EBV infection in male subjects. Two genetically distinct forms are known. In XLP1 the disease is caused by mutations of the SH2 domain containing protein 1 A *(SH2D1A)* gene, encoding for a small adapter protein (SLAM-associated protein, or SAP) involved in intracellular signaling in T and NK lymphocytes.[317,318] In particular, SAP binds to CD244 (a SLAM family member) expressed by NK lymphocytes. EBV-infected B cells express high levels of CD48, the CD244 ligand. On CD244/CD48 binding, SAP participates at intracellular signaling and promotes NK cytolytic activity against EBV-infected target cells. In the absence of SAP, NK cytolytic activity is impaired.[319] SAP is also important for the function of follicular helper T cells that promote maturation of antibody responses.[320] XLP2 is caused by mutations of the *BIRC4* gene, which encodes for the X-linked inhibitor of apoptosis (XIAP) protein.[321]

XLP1 and XLP2 have partially overlapping clinical features. Hemophagocytic lymphohistiocytosis (HLH) subsequent to primary EBV infection is a prominent sign at clinical presentation in both diseases. XLP1 may manifest with fatal infectious mononucleosis, aplastic anemia, pulmonary lymphoid granulomatosis with vasculitis, and lymphoma.[322] A proportion of XLP1 patients who survive primary EBV infection will develop hypogammaglobulinemia, which in some cases is associated with elevated levels of IgM or IgA. Some patients may have a clinical and laboratory phenotype consistent with common variable immunodeficiency (CVID, discussed later). Functional activity of NK and CTLs is impaired. Finally, patients with XLP1 lack NKT lymphocytes.[323] Without HSCT, 62.5% of the patients survive (and most of them require Ig replacement); however, poor survival rates (less than 20%) have been observed in patients without transplants who have features of HLH. HSCT may cure approximately 80% of XLP1 patients, but poorer outcome (50% survival) has been observed if HLH is present.[322]

In contrast, XLP2 causes recurrent episodes of HLH, with or without EBV infection.[323] Patients with XLP2 are not at increased risk of lymphoma; on the other hand, inflammatory bowel disease is frequently observed.[324] Definitive treatment of XLP2 is also based on HSCT; however, use of a myeloablative conditioning regimen is associated with high mortality.[325]

IL-2 INDUCIBLE TYROSINE KINASE DEFICIENCY

Interleukin-2 inducible tyrosine kinase (ITK) is a nonreceptor tyrosine kinase that modulates the strength of intracellular signaling in T lymphocytes on TCR-induced activation. Patients with ITK deficiency are susceptible to EBV-driven lymphoproliferative disease (often involving the lungs as well), Hodgkin lymphoma, hypogammaglobulinemia, autoimmunity, and HLH.[326] The disease is inherited as an autosomal recessive trait. There is a reduced number of naïve CD4+ lymphocytes and of NKT cells. Allogeneic HSCT may cure the disease.

CD27 DEFICIENCY

CD27 is a member of the TNF receptor family and is broadly expressed on the surface of various lymphocyte subsets. By interacting with CD70, it promotes generation and maintenance of T-cell memory. It is also commonly used as a marker of memory B lymphocytes. CD27 deficiency has been identified in some patients presenting with severe EBV infection. Lymphoma and secondary hypogammaglobulinemia have been reported in some of these patients. HSCT represents a curative form of treatment.[327,328]

CD16 DEFICIENCY

One of the mechanisms by which NK lymphocytes mediate killing of target cells is antibody-dependent cellular cytotoxicity (ADCC), whereby antibody-bound target cells come in contact with NK lymphocytes through binding of the Fc region of IgG molecules to a specific receptor (FcγRIIIa, also known as CD16) expressed on the surface of NK lymphocytes. Homozygosity for the Leu66His amino acid substitution in CD16 causes deficient spontaneous NK cell-mediated cytotoxicity but surprisingly leaves ADCC intact. Expression of CD2, a co-activating NK cell receptor, is decreased, affecting intracellular signaling in NK lymphocytes. Recurrent and severe herpesvirus infections are the clinical hallmark of this condition.[329]

IMMUNODEFICIENCIES WITH SELECTIVE PREDISPOSITION TO PATHOGENS

Most forms of primary immunodeficiencies are characterized by susceptibility to a broad range of pathogens, whose nature (bacteria, viruses, fungi, protozoa) reflects the underlying immune defect. However, in the last two decades it has become clear that certain gene defects that affect the immune system may lead to selective predisposition to a narrow group of pathogens. These findings have supported the genetic theory of infectious diseases, whereby life-threatening infections early in life may in fact be caused by inborn errors of immunity.[330] These disorders and their associated genes are listed in Table 24-7.

Mendelian Susceptibility to Mycobacterial Diseases

The designation *Mendelian susceptibility to mycobacterial diseases (MSMD)* refers to a group of PIDDs characterized mainly by selective susceptibility to infection with mycobacteria including both *Mycobacterium tuberculosis* and many atypical species.[331-334] Patients

TABLE 24-7 Immunodeficiencies with Selective Predisposition to Pathogens

Syndrome	Gene
Mendelian susceptibility to mycobacterial disease	*IFNGR1, IFNGR2, IL12B, IL12RB1, STAT1*
Epidermodysplasia verruciformis	*TMC6, TMC8*
Trypanosomiasis	*APOL1*

are also susceptible to disseminated infections with *Salmonella* species. There are no other consistent clinical manifestations, and all routine screening tests of humoral and cellular immunity are normal. The vast majority of these patients harbor mutations in one of five genes that encode elements in a signaling cascade involving IFN-γ and IL-12. IL-12 is produced by mononuclear cells in response to stimulation by way of TLRs and other pattern recognition receptors. IL-12 stimulates T_h1 differentiation, and mature T_h1 cells and NK cells produce IFN-γ. IFN-γ then acts on mononuclear cells to promote intracellular killing of phagocytosed organisms. In the absence of the normal operation of this sequence, macrophages are not able to kill ingested mycobacteria or *Salmonella* organisms, and severe infection results. Thus far, mutations have been found in the genes encoding the IL-12 p40 subunit *(IL12B),* the β1 chain of the IL-12 receptor *(IL12RB1),* both chains of the IFN-γ receptor (*IFNGR1, IFNGR2),* and the STAT1 signal transducing molecule which mediates signaling of the IFN-γ receptor.[331-334] Most cases show autosomal recessive inheritance, but a few specific mutations of *IFNGR1* may exhibit autosomal dominant inheritance. Acquired forms of susceptibility to mycobacterial infection have been ascribed to autoantibodies to IFN-γ.

Therapy for MSMD is mainly through aggressive treatment of infections when they occur and prophylaxis for selected individuals who may relapse frequently. For patients with hypomorphic (partly functional) mutations of *IFNGR1,* IFN-γ supplementation may be beneficial.

Epidermodysplasia Verruciformis

Mutations in the genes *TMC6* (transmembrane channel-like 6) and *TMC8* give rise to epidermodysplasia verruciformis (EV). These genes were previously called *EVER1* and *EVER2*, respectively. EV exhibits autosomal recessive inheritance, and affected individuals are susceptible to persistent skin infection only by HPV.[335,336] There is a high rate of malignant transformation in the skin, but there are no other infectious susceptibilities or clinical manifestations of the disease. Acquired EV has been associated with human immunodeficiency virus (HIV) infection.[337,338]

Herpes Simplex Encephalitis

HSVs are prevalent in the general population and are capable of causing disseminated severe and even fatal infections in patients with profound T-cell defects (discussed earlier). However, in rare cases individuals are susceptible specifically to encephalitis caused by HSV, in the absence of any other organ or system involvement.[339-343] To date, mutations in five distinct genes have been associated with this clinical entity: TLR3 *(TLR3), UNC93B1, TRAF3, TICAM1* (previously known as *TRIF*), and *TBK1*. All of the proteins encoded by these pathways interact in transducing responses by way of TLR3 (and in some cases also TLR4) with the common result of a failure to produce sufficient amounts of type 1 interferons in response to HSV infection in the brain. Inheritance varies according to the genes and the specific mutations involved. Thus far, mutations in *UNC93B1* have shown exclusively autosomal recessive inheritance, whereas mutations of *TRAF3* and *TBK1* have exhibited exclusively autosomal dominant inheritance. Mutations of *TLR3* and *TICAM1* have shown either dominant or recessive inheritance depending on the specific lesion.

Susceptibility to Trypanosomiasis

Trypanosomes are single-cell (protozoan) organisms causing endemic infections in many parts of the world. *Trypanosoma cruzi* is the causative agent of Chagas disease, and it is estimated that approximately 10 million people in South America harbor this parasite, most being asymptomatic. Subspecies of *Trypanosoma brucei* are responsible for sleeping sickness, and it is estimated that more than 60 million individuals in Africa harbor this organism. Most individuals are protected from progression of disease by apolipoprotein L1 (APOL1), which can lyse the *T. brucei* plasma membrane and kill the organism. Some strains of trypanosomes have become more pathogenic by virtue of their resistance to APOL1 lysis.[344-348] Conversely, individuals who have mutations in their *APOL1* genes are more susceptible to trypanosome infection and severe disease. There are no known clinical consequences of deleterious *APOL1* mutations beyond susceptibility to *T. brucei*. However, isoforms of APOL1 selected through evolution for higher trypanosome lytic activity are risk factors for nondiabetic kidney disease in African-Americans.[349]

HUMORAL IMMUNODEFICIENCY

The humoral immunodeficiencies and their associated gene defects are listed in Table 24-8. These disorders are characterized by hypogammaglobulinemia or dysgammaglobulinemia, often together with relative deficiency of antibody responses to various forms of antigenic challenge.[3,4] Cellular immunity is generally intact. Humoral immunodeficiency is most commonly manifested by recurrent bacterial and viral infections of the upper and lower respiratory tract. Pyogenic skin infections, as well as meningitis, osteomyelitis, and other foci of infection, are also seen frequently. These infections are generally caused by the same organisms that are virulent in immunocompetent hosts, predominantly encapsulated bacteria such as *Streptococcus pneumoniae*, *Hemophilus influenzae, S. aureus*, and *Neisseria meningitidis*. Viral infections usually resolve normally in these patients, although a higher rate of recurrence with the same agent may be seen because there is impaired production of neutralizing antibodies or B-cell memory. The results of physical examination are not specific and show only the presence or sequelae of microbial infections. A relative paucity of peripheral lymphoid tissue may be noted in some patients, especially areas rich in B cells (e.g., tonsils). Studies of peripheral blood lymphocyte subsets are often normal; the number of B cells may be reduced in certain syndromes.

TABLE 24-8 Humoral Immunodeficiencies and Associated Gene Defects

Syndrome	Gene
X-linked (Bruton) agammaglobulinemia	*BTK*
Autosomal recessive agammaglobulinemias	*CD79A, CD79B, BLNK, IGHM, IGLL1, LRRC8, PIK3R2*
Monogenic common variable immunodeficiency	*BAFFR, CD19, CD21, CD81, ICOS*
Common variable immunodeficiency	Undefined
Good syndrome	Undefined
Selective IgA deficiency	Undefined
IgG subclass deficiency	Undefined
Specific antibody deficiency with normal immunoglobulins	Undefined
Transient hypogammaglobulinemia of infancy	Undefined
Class-switch defects: CD40 ligand deficiency (X-linked HIM) CD40 deficiency B-cell–specific defects	 *TNFSF5* *TNFRSF5* *AICDA, UNG*

The Agammaglobulinemias

X-Linked Agammaglobulinemia

X-Linked agammaglobulinemia (XLA) is also known as *Bruton agammaglobulinemia* because Bruton provided the classic description of the disease in 1952.[350] Affected male subjects are often asymptomatic during the first months of life while they are protected by transplacentally acquired maternal antibodies. These antibodies fall to low levels by 6 to 9 months of age, and patients begin to experience recurrent bacterial infections.[351-353] Physical examination at any age often reveals a striking absence of tonsils and palpable lymphoid tissue. Serum Ig is absent or the level is extremely low; in addition, no B cells are found or their numbers are extremely low. Cellular immunity is completely intact. However, these patients are prone to the development of chronic enteroviral meningoencephalitis and vaccine-associated paralytic poliomyelitis, which suggests an important role for humoral immunity in the control of these infections. Moreover, patients may have arthropathy resembling rheumatic disease, which may be due to infection with *Mycoplasma* or *Ureaplasma* organisms. Other opportunistic infections, such as *P. jiroveci* pneumonia, are seen only rarely.

XLA is another example of an immunodeficiency caused by a defect in a signal transduction molecule. In fact, this protein tyrosine kinase is now known as Bruton tyrosine kinase ([Btk], Fig. 24-7).[351-353] It is expressed in B cells at all stages of development, as well as in cell lines derived from monocytes, macrophages, mast cells, and erythroid cells. A variety of *BTK* gene defects such as point mutations, deletions, and insertions have been described in patients with XLA. In most patients B-cell development is impeded at an early stage (the pro–B-cell to pre–B-cell transition).

BTK mutations account for approximately 85% of all cases of agammaglobulinemia.[354] *BTK* mutations have also been described in patients with "atypical" XLA who have low numbers of B cells and low-level antibody production.[351-353] In some patients with atypical XLA, CVID was misdiagnosed (discussed later) before discovery of the *BTK* defect.[355] In general, *BTK* mutations that permit the expression of small amounts of functional or partially functional Btk protein are associated with milder clinical phenotypes and slightly higher numbers of B cells in the circulation. [351-353] However, even siblings with identical mutations may show quite divergent phenotypes. Female carriers of XLA show nonrandom X-chromosome inactivation in their B cells.

Autosomal Recessive Agammaglobulinemias

Female patients with agammaglobulinemia and no B cells and males with a similar phenotype lacking *BTK* mutations have been described. These patients have forms of agammaglobulinemia with autosomal recessive inheritance that result from several distinct molecular defects,[354] including mutations of the Igμ heavy-chain locus, which prevents formation of the IgM heavy chain required for formation of the Ig receptors on pre-B cells and mature B cells (see Fig. 24-7). Defects of λ5 (a component of the

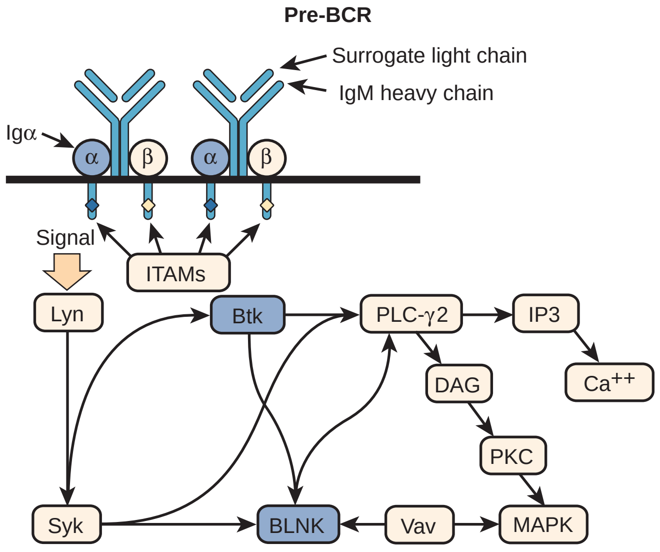

Figure 24-7 Signaling via the pre–B-cell receptor (Pre-BCR). The Pre-BCR is composed of an IgM heavy chain with a surrogate light-chain heterodimer (VpreB and λ5), along with a signaling heterodimer of Igα and Igβ. The immunoreceptor tyrosine-based activating motifs (*ITAMs*, see Fig. 24-5) recruit tyrosine kinases *(Lyn, Syk, Btk)* that initiate downstream signaling. The adapter proteins BLNK and Vav recruit additional signaling intermediates. Defects in components shown in blue lead to agammaglobulinemia by blocking pre-BCR signaling and interrupting B-cell development. *DAG,* Diacylglycerol; *IP3,* inositol triphosphate; *PKC,* protein kinase C; *PLC,* phospholipase C; *MAPK,* mitogen-activated protein kinase.

surrogate light chain) also prevent formation of the pre–B-cell Ig receptor required for B-cell development. Igα (CD79a) and Igβ are transmembrane molecules required for signal transduction by way of the pre–B-cell and mature B-cell Ig receptors. Lesions in these genes prevent Ig receptor signaling and B-cell development.[356] Defects in the signal transduction proteins BLNK (B-cell linker protein) and the p85 (β-regulatory) subunit of phosphoinositide 3′ (PI3) kinase (gene *PIK3R2*) have also been found.[354,357] Mutations in these molecules prevent intracellular signaling after ligation of the B-cell receptor. All these mutations arrest B-cell development at early stages in bone marrow.

One patient has been described with an apparent dominantly expressed translocation abrogating the function of the *LRRC8*) gene.[358] The function of the LRRC8 protein is not yet known; it is a member of a growing family of proteins of unknown function called the LRRC8 family, within the much larger and very diverse superfamily of leucine-rich repeat proteins. The female patient has agammaglobulinemia with arrest of B-cell development in bone marrow.

Other Predominantly Antibody Deficiency Syndromes

Common Variable Immunodeficiency

The diagnostic label of CVID encompasses an unknown number of conditions that are genetically and etiologically distinct but have in common late-onset humoral immunodeficiency, most often in the first or third decades of life.[359-363] Hypogammaglobulinemia and impaired specific antibody production are universal, by definition. Deficiency of IgA is very common in CVID.[364] IgM is also frequently affected; the pattern varies from one individual to another. The levels of particular isotypes in any patient are often static over time, but fluctuations may occur.

Patients with CVID resemble individuals with XLA (and other humoral immunodeficiencies) because they also have recurrent sinopulmonary bacterial infections. Chronic enteroviral infections, including meningoencephalitis, may also be seen in CVID. A form of granulocytic/lymphocytic interstitial lung disease occurs in a subset of patients and has been linked in some patients to HHV-8 infection.[365,366] More diffuse noncaseating granulomatous disease resembling sarcoidosis may be encountered in the skin or viscera. Additional manifestations include lung disease resembling asthma, chronic rhinitis, chronic giardiasis, and recurrent or chronic arthropathy. The apparent "atopic" symptoms usually occur in the absence of allergen-specific IgE.[367] Malabsorptive inflammatory bowel disease may occur.[368] A broad spectrum of autoimmune diseases may be associated, including a variety of cytopenias, arthritides, and vasculitides.[369,370] Widespread lymphoproliferation may cause splenomegaly, adenopathy, and intestinal lymphonodular hyperplasia.[371-373] Patients with CVID also have a higher incidence of gastrointestinal and lymphoid malignancies.[364] The relative risk for lymphoma has been estimated to be as much as 30- to 400-fold greater than that in the general population.

Memory B-cell subpopulations may be useful diagnostic and potentially prognostic markers in CVID.[363,374-379] Memory B cells express the CD27 (TNFRSF7) molecule. Naïve B cells express IgM and IgD but not CD27. Unswitched memory B cells (also called marginal zone cells) express IgM, IgD, and CD27. After class-switching in germinal centers, B cells cease expressing IgM and IgD and subsequently express "downstream" Ig isotypes such as IgG, IgA, and IgE (Fig. 24-8). Memory B cells after class-switching (switched memory B cells) are IgM^{-}IgD^{-}CD27^{+}. Additional subsets of B cells include transitional B cells (IgM^{high}CD38^{high}) and CD21^{low} B cells. Proportions of these peripheral B cell subtypes enumerated by flow cytometry correlate with clinical phenotypes. A more severe clinical phenotype with lower Ig and worse infections and increased granulomatous disease correlates with a decreased number of marginal zone and class-switched B cells. Granulomatous disease and splenomegaly also correlate with an expansion of CD21^{low} B cells. Increase in transitional B cells is associated with lymphadenopathy. Specific B cell subsets are developmentally regulated, and age-adjusted values should be used in these instances.[380-382]

Although cellular immune function is grossly preserved in the majority of patients with CVID, a variety of

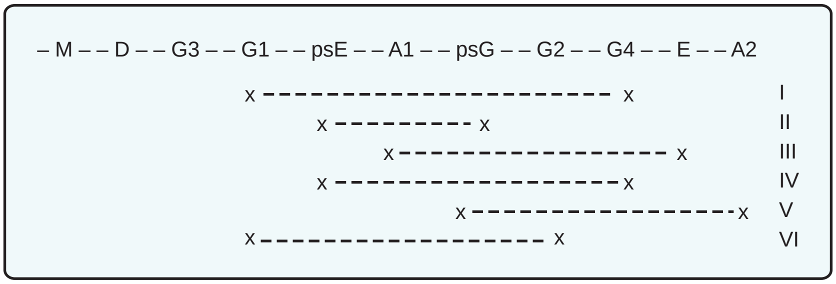
– M – – D – – G3 – – G1 – – psE – – A1 – – psG – – G2 – – G4 – – E – – A2
x x I
x x II
x x III
x x IV
x x V
x x VI

Figure 24-8 Immunoglobulin (Ig) heavy-chain gene deletion haplotypes. The top line shows the order of the Ig constant region genes (e.g., M = IgM, etc.). The letters *ps* designate a pseudogene that is not expressed; numbers indicate subclasses. Six haplotypes have been defined (Roman numerals). In each haplotype the entire region indicated by the Xs is missing. *(Data from Lefranc MP, Hammarstrom L, Smith CI, Lefranc G: Gene deletions in the human immunoglobulin heavy chain constant region locus: molecular and immunological analysis.* Immunodefic Rev *2:265–281, 1991.)*

functional and phenotypic T-cell abnormalities are found in some.[359,382-385] These include reductions in peripheral blood T-cell populations, as well as functional defects such as reduced in vitro proliferative responses, defects in cytokine production, decreased T-helper cell function, abnormalities in T-cell signaling, diminished expression of the costimulatory molecule CD40L, decreased numbers of naïve T cells, increased suppressor T-cell function, and decreased numbers of regulatory T cells.

The genetic heterogeneity of CVID is beginning to yield to molecular biologic analysis. Several single-gene defects have been found in varying (small) proportions of patients with CVID (discussed later). It may also happen that individuals classified as having CVID (or a phenotype consistent with CVID) on clinical and laboratory grounds may be found to have mutations in genes characteristically associated with other primary immunodeficiencies. Such has been the case with some individuals with mutations in *BTK* (causative in XLA),[355] *SH2D1A* or *XIAP* (XLP disease),[386,387] and *TNFSF5* (CD40L, X-linked hyper-IgM syndrome).[388]

Functionally relevant polymporphisms in *TNFRSF13B* (encoding TACI) occur in approximately 1% of the general population. The most common are Cys104Arg (cysteine 104 replaced by arginine) and Ala181Glu. Approximately 10% of patients with CVID are heterozygous, and 1% are homozygous for these.[389-392] TACI is expressed on B cells and interacts with the ligands BAFF and APRIL expressed on macrophages and dendritic cells. These interactions have important roles in B-cell activation and Ig class switching. TACI polymorphisms are not entirely disease causing by themselves, but the presence of one of these TACI polymorphisms does appear to confer increased risk of lymphoproliferation and autoimmunity.[393]

The genetic determinants of CVID in the majority of patients may be more complex. A recent genome-wide association study revealed complex patterns.[394] The strongest associations were with the MHC locus and a family of disintegrin/metalloproteinase genes. However, there were at least 16 associated gene duplications or deletions together with many (≈100) unique intraexonic duplications or deletions. Furthermore, a complex genetic signature composed of more than 1000 single nucleotide polymorphisms was highly predictive of the CVID phenotype. How this heterogeneous genetic picture relates to the clinic manifestations of CVID is unknown.

Deficiency of Inducible T-Cell Costimulator

The inducible T-cell costimulator (ICOS) is expressed on T cells after stimulation. It interacts with a member of the B7 family of molecules known as ICOS ligand. Fewer than 20 patients worldwide have been found to have CVID associated with ICOS deficiency.[395] These patients may manifest recurrent respiratory and gastrointestinal infections in adulthood, panhypogammaglobulinemia and impaired vaccine responses, autoimmune disease (neutropenia), lymphoproliferation, and cancer (carcinoma of the vulva). Lymphoid tissues exhibit poorly formed germinal centers, and circulating switched memory B cells are markedly reduced.

CD19, CD21, and CD81 Deficiencies

CD19 is expressed on B cells and is a component of a receptor complex that contains CD21, a receptor for the complement fragment C3dg (it is also called complement receptor 2, or CR2, and it is the cellular receptor for EBV) along with a tetraspan molecule called TAPA1 (also CD81). Simultaneous cross-linking of the CD19/CD21/CD81 complex and the B-cell Ig receptor lowers the threshold for B-cell activation. Patients with defects of the *CD19*,[396,397] *CD21*,[398,399] or *CD81*[400] genes exhibit recurrent infections, hypogammaglobulinemia, and low levels of switched memory B cells. Autoimmunity and lymphoproliferation do not appear to be elements of the clinical presentations of these disorders.

BAFF Receptor Deficiency

Two siblings have been described with homozygous deletions in the *BAFFR* gene encoding the BAFF (B cell activating factor) receptor.[401] Both had low IgG, poor polysaccharide antibody response, and low memory B cells. However, only one sibling had recurrent infections. It is not clear why only one sibling was affected clinically, but variable penetrance is frequently observed in all forms of immunodeficiency.[4]

Good Syndrome

The occurrence of thymoma in the context of a clinical and immunologic picture very similar to that of CVID with low numbers of B cells and generally more severely impaired T-cell function has been designated Good syndrome.[402-406] The spectrum of bacterial sinopulmonary infections and pathogens is similar to those associated with the more prevalent forms of CVID. However, Good syndrome is associated more frequently with opportunistic infections, including mucocutaneous candidiasis, severe varicella-zoster virus infection, *P. jiroveci,* CMV, and recurrent HSV. Lymphadenopathy and splenomegaly, commonly seen in CVID, are not characteristic features of Good syndrome. Autoimmune disease is a frequent complication of Good syndrome, most notably pure red cell aplasia and neutropenia. Patients with Good syndrome also often suffer from chronic diarrhea of unclear etiology.

Panhypogammaglobulinemia is a consistent finding in Good syndrome.[402-406] Unlike the majority of patients with CVID, immunophenotypic analysis of peripheral blood lymphocytes frequently shows absent or very low numbers of B cells, reduced CD4+ T cells, absent cutaneous delayed hypersensitivity responses, and reduced in vitro T-cell response to mitogen. It is not clear whether patients with Good syndrome exhibit as yet undiscovered specific genetic or immunologic abnormalities that clearly distinguish them from CVID.

Selective IgA Deficiency

There are two subclasses of human IgA: IgA1 and IgA2. They are encoded by separate genes on the heavy-chain C region locus on chromosome 14 (see Fig. 24-8). IgA1 predominates in serum (80% to 90%), whereas IgA1 and IgA2 are equally prevalent in secretions. Both subclasses are equally affected in selective IgA deficiency (SIGAD). SIGAD is defined as a serum IgA level of less than 0.07 g/L with normal serum IgG and IgM in a child older than 4 years of age in whom other causes of hypogammaglobulinemia have been excluded.[4] Note that this definition is restricted to the absence of serum IgA. There is no known clinical association with IgA levels between the lower limit of detection and the lower limit of the normal range. It is not appropriate to refer to patients with these nonzero low IgA values as being IgA-deficient.

SIGAD is seen in approximately 1 in 700 Caucasians in the United States.[407] In contrast, its incidence among Japanese is only 1 in 18,500. This presumably reflects population genetic differences, possibly related to the HLA complex. SIGAD has been associated with extended HLA haplotypes similar to those observed in CVID.[408,409] The majority of individuals with SIGAD are completely asymptomatic. Some may have a clinical course similar to that seen in CVID or in IgG subclass deficiency (discussed later). The proportion of patients with SIGAD is increased in a population of people with chronic lung disease, and 20 years' observation of blood donors with SIGAD has documented an increased incidence of respiratory infections and autoimmune disease.[410-413]

The spectrum of autoimmune syndromes associated with SIGAD is similar to CVID.[410-413] In addition, atopic diseases appear to be more prevalent among those with SIGAD, and some have also noted an increased incidence of the type of malignancies encountered in CVID. In rare cases, SIGAD has evolved into CVID, or levels of IgA have increased over time.[414,415] Treatment of SIGAD has generally focused on management of complications as they occur. In some instances, gamma globulin infusions may be helpful, especially if an associated IgG subclass or specific antibody deficiency is present (discussed later). However, researchers in one study did not find an association between a history of infections and lower antibody responses to pneumococcal polysaccharides.[411] Some studies do show an association of increased infections if there is an IgG subclass deficiency or mannose-binding lectin (MBL) deficiency together with SIGAD.[416]

Apart from the absence of IgA itself and its occasional association with abnormalities of IgG subclasses, there are few other characteristic immunologic abnormalities. Some researchers have found low levels of switched memory B cells in some patients and observed correlations with disease severity and autoimmune complications similar to those in CVID.[417]

Molecular genetic defects have not been described in SIGAD. Lesions in IgA C region genes are not found, except in rare instances associated with other deletions (discussed later). SIGAD and CVID have been associated with the HLA haplotype A1, B8, DR3 in Caucasians.[409,418] A SIGAD susceptibility locus (IGAD1) has been mapped to the boundary region between HLA class II and class III.[419,420] A more detailed analysis identified a positive association of SIGAD with HLA-DRB1*0301, DQB1*02 and a negative association with DRB1*1501, DQB1*0602. Another study identified an association of SIGAD with DRB1*0102, DQB1*0501.[421] The significance of these associations requires clarification.

SIGAD may occur as a drug side effect and has been reported with phenytoin, carbamazepine, valproic acid, zonisamide, sulfasalazine, gold, penicillamine, hydroxychloroquine, and nonsteroidal antiinflammatory drugs.[418] The IgA level may normalize after cessation of the drug.

Immunoglobulin G Subclass Deficiency

Four subclasses of IgG exist in humans: IgG1, IgG2, IgG3, and IgG4. Their designations order their prevalence in serum, with each constituting roughly 67%, 23%, 7%, and 3% of the total, respectively. A different gene encodes the heavy-chain constant region of each subclass (see Fig. 24-8). Each subclass has unique structural properties and plays a different role in immune responses as a result of different interactions with the complement system and with receptors for IgG on phagocytic cells and lymphocytes. In addition, IgG subclasses are produced in different relative amounts depending on the antigenic stimulus.[422] Mainly IgG1 is produced in response to soluble protein antigens. Viral antigens also elicit predominantly IgG1 responses and often significant amounts of IgG3 as well. Particular viruses (e.g., hepatitis, herpes simplex, and varicella) also elicit IgG4. The pneumococcal capsular polysaccharide response is almost exclusively of the IgG2 subclass, whereas *H. influenzae* type B (HIB) polysaccharide elicits mainly IgG2, with some IgG1 (the latter is found in younger children particularly). Note that the current HIB and pneumococcal (Prevnar) vaccines are protein conjugates and elicit responses characteristic of protein antigens.

IgG subclass deficiency (IGGSD) is defined as a level of one or more IgG subclasses greater than 2 SD below the age-adjusted mean with a normal total level of IgG. Precise criteria for diagnosis are difficult to establish because of variability in Ig measurements by various techniques (e.g., nephelometry and radial immunodiffusion) and the wide variability in normal ranges with respect to age and ethnicity. The significance of decreased levels of IgG subclasses in patients with recurrent infections is further clouded by the fact that the majority of individuals with isolated subclass deficiencies are asymptomatic. These factors make the diagnosis of IGGSD controversial.[423]

Patients with IGGSD commonly present with recurrent sinopulmonary infections of varying severity caused by common respiratory bacterial pathogens.[424-428] Some may also have recurrent diarrhea, often of infectious origin. Furthermore, atopic diseases such as asthma and allergic rhinitis are more prevalent in patients with IGGSD. Deficiency of the IgG2 subclass is detected most commonly in symptomatic patients. It may occur in isolation but is also usually associated with deficiency of IgG4, IgA, or both. Selective deficiency of IgG3 alone is likewise observed (most often in adult women) and is associated with the same clinical picture described earlier.[424,425] The lowest IgG4 levels are usually associated with IgG2 deficiency. Recurrent infections have been described in some patients with only IgG4 deficiency, although a diagnosis of isolated IgG4 deficiency is considered most controversial because normal ranges for IgG4 are poorly defined.

Several studies have shown an increased proportion of individuals with subclass deficiencies among patients with recurrent infections.[4] Not all of these patients have demonstrably impaired responses to vaccines, however. Thus the relationship between lack of an antibody isotype and susceptibility to infection is far from straightforward. Assessing the response to antigen challenge is an important element in the evaluation of these patients. Assessment of memory B-cell subsets may become a useful diagnostic tool for IGGSD; some studies suggest that reduced switched memory B cells have better a correlation with risk for infection than antibody responses do.[429]

The C region genes determining antibody classes and subclasses are arranged in the same transcriptional orientation on chromosome 14 (see Fig. 24-8). As with SIGAD, only rarely is clinically significant IGGSD associated with genetic lesions of IgG C region genes. This has been observed in a single reported patient with IgG2 deficiency, in whom mutation prevented expression of cell surface IgG2.[430] In fact, large deletions of Ig C region genes are surprisingly common; six multigene deletion haplotypes have been identified (see Fig. 24-8).[431] In one population with a high degree of consanguinity, individuals were found to be homozygous for one haplotype or heterozygous for two different haplotypes. Fifteen of 16 people with deletions on both chromosomes were entirely healthy despite a complete lack of one or more IgG subclasses or IgA1; one person had recurrent infections.[432] Similarly, two healthy siblings have been described, each having homozygous deletions of IgA1, IgG2, IgG4, and IgE.[433] Another group described a healthy 50-year-old man who had deletions encompassing the heavy-chain genes encoding IgG1, IgG2, IgG4, and IgA1.[434] This individual did not produce polysaccharide antibody but did produce IgG3 to protein antigens, including pneumococcal surface proteins. Finally, an analysis of the Ig heavy-chain loci of 33 patients with CVID revealed only two patients who were heterozygous for C gene deletions.[435]

Specific Antibody Deficiency with Normal Immunoglobulins

A population of patients with recurrent infections and poor antibody responses (mainly to polysaccharide antigens) have completely normal levels of antibody classes and subclasses. This condition has been called *specific antibody deficiency with normal immunoglobulins* (SADNI) or *functional antibody deficiency*.[436-438,439-441] In one retrospective compilation of 90 patients in a tertiary-care institution with newly diagnosed immunodeficiency, specific antibody deficiency with normal Igs was the most common diagnosis, made in 23% of patients.[442] Repeated immunization may lead to measurable antibody responses, although even protein conjugate pneumococcal polysaccharide vaccines may not be as effective as in healthy individuals.[440] Measurement of memory B-cell populations may correlate better with infection risk in these patients, although further study is required.[429] SIGAD, IGGSD, SADNI, and a subset of CVID may be entities in a spectrum with similar pathophysiologic characteristics. The natural history of IGGSD and specific antibody deficiency is usually slow spontaneous resolution.[415] Most children have a greatly reduced incidence of infections by late childhood.

Transient Hypogammaglobulinemia of Infancy

Transient hypogammaglobulinemia of infancy (THI) is, as its name suggests, an antibody deficiency beginning in infancy that resolves spontaneously, usually by 4 to 5 years of age.[443-447] It is defined as the occurrence of an IgG level more than 2 SD below the age-adjusted mean. Many patients also have low IgA, and some also have low IgM. Vaccine antibody responses are usually intact, but this is not always the case. Peripheral lymphoid tissue is sparsely populated with small or no germinal centers and markedly reduced plasma cell contents. Numbers of circulating B and T cells are generally normal, as are in vitro assays of lymphocyte function (e.g., mitogen response). One recent study documented higher B-cell counts than in an age-matched group.[448]

Ig levels in THI gradually increase over time, and the majority of patients have normal levels by the age of 4 or 5, rarely later.[443-447] Thus the diagnosis can be confirmed only after Ig levels normalize. Prospective studies show a preponderance of the diagnosis in male subjects and normalization of Ig levels at a median of approximately 2 years, with an upper limit of approximately 5 years. Female subjects and patients with lower IgG levels tend to take longer to normalize.

Immunoglobulin Class-switch Defects

As mentioned previously, naïve B cells express IgM and IgD on the cell surface. These different isotypes are generated by way of differential splicing of mRNA transcripts. When B cells are activated by antigen and interaction with helper T cells in germinal centers, they initiate a process of class-switching whereby the DNA intervening between the variable region gene and a downstream isotype is excised. In this manner, B cells begin to express IgG, IgA, or IgE. When this process is impeded, the amount of these isotypes produced is very low, while the amount of IgM may be normal or very high. These disorders are often referred to as *hyper-IgM syndromes*. However, there are many important differences among the disorders often placed in this group.[449]

X-Linked Immunodeficiency with Normal or Elevated IgM

As its name implies, X-linked immunodeficiency with normal or elevated IgM (XHIM, also HIM1) denotes a form of selective hypogammaglobulinemia G and A, together with normal or elevated amounts of IgM.[450-454,455] These patients are seen in the first 1 or 2 years of life with the recurrent sinopulmonary bacterial infections often found in those with hypogammaglobulinemia, together with opportunistic infections by organisms such as *P. jiroveci*, *Cryptosporidium*, and *Histoplasma*. Affected patients are also prone to the development of neutropenia, anemia secondary to parvovirus, stomatitis, sclerosing cholangitis, and liver and hematologic malignancies. A variety of autoimmune manifestations may be seen, including cytopenias, arthropathy, and inflammatory bowel disease.[456]

The number of circulating B cells is normal, but they express IgM exclusively (in association with IgD) on their surface. Immune responses are varied. Isohemagglutinins are often present, and vaccination may elicit specific IgM responses. No other isotypes are produced; there is no B-cell memory. Secondary lymphoid tissues are sparsely populated and do not contain germinal centers. In spite of the important defects in T-cell function for B-cell help, screening studies such as flow cytometric measurement of T-cell populations or in vitro proliferation tests are often normal.[450-455]

The genetic lesion in XHIM is in *TNFSF5* (tumor necrosis factor superfamily member 5), also known as CD40 ligand (CD40L) or CD154. [450-455] This is an integral membrane protein that is mainly expressed on T cells after an activating stimulus, such as interaction of the TCR with an MHC-peptide complex on the surface of a B cell or APC (Fig. 24-9). The signal delivered by T-cell CD40L to CD40 is critical for Ig class switching, the development of B-cell memory, affinity maturation of the antibody response (somatic hypermutation of Ig V regions), and the expression of important costimulatory molecules such as those of the B7 family by B cells and APCs (see Fig. 24-9).

The CD40 system has additional roles in the interaction of T cells with monocytes. Several T-cell–derived cytokines (granulocyte-macrophage colony-stimulating factor, IL-3, and IFN-γ) induce the expression of CD40 on monocytes. Furthermore, engagement of monocyte CD40 induces cytokine synthesis and activates tumoricidal mechanisms. These phenomena suggest an important interaction, the lack of which may underlie the predisposition to opportunistic infection and malignancy seen in patients with CD40 ligand deficiency.

Perhaps not too surprisingly, a clinically and immunologically identical immunodeficiency syndrome arises from mutations in the gene *TNFRSF5*, which encodes CD40.[451,454,457,458] Only a few patients with this defect have been found so far.

These defects in CD40L and CD40 (*TNFSF5* and *TNFRSF5*) are discussed here together with the humoral immunodeficiencies because of the prominence of the class-switch defect in the clinical expression. However, they are truly combined immunodeficiencies with important defects of T-cell function. In addition to IgG therapy and infection prophylaxis, these patients are candidates for HSCT.

Mutations in Activation-Induced Cytidine Deaminase and Uracil Nucleoside Glycosylase

Activation-induced cytidine deaminase (AID) and uracil nucleoside glycosylase (UNG) defects exclusively affect B-cell function while T-cell number and function are completely normal. They were originally defined in the medical literature as forms of hyper-IgM (HIM) syndrome, which leads to some confusion insofar as other forms of HIM are combined immunodeficiencies with important T-cell dysfunction (discussed previously). AID deficiency has been called HIM2[454,459-461] and UNG deficiency has been called HIM5[454,462] (see Table 24-8). The AID and UNG enzymes are RNA modifiers expressed in B cells that act during class-switch recombination. In the

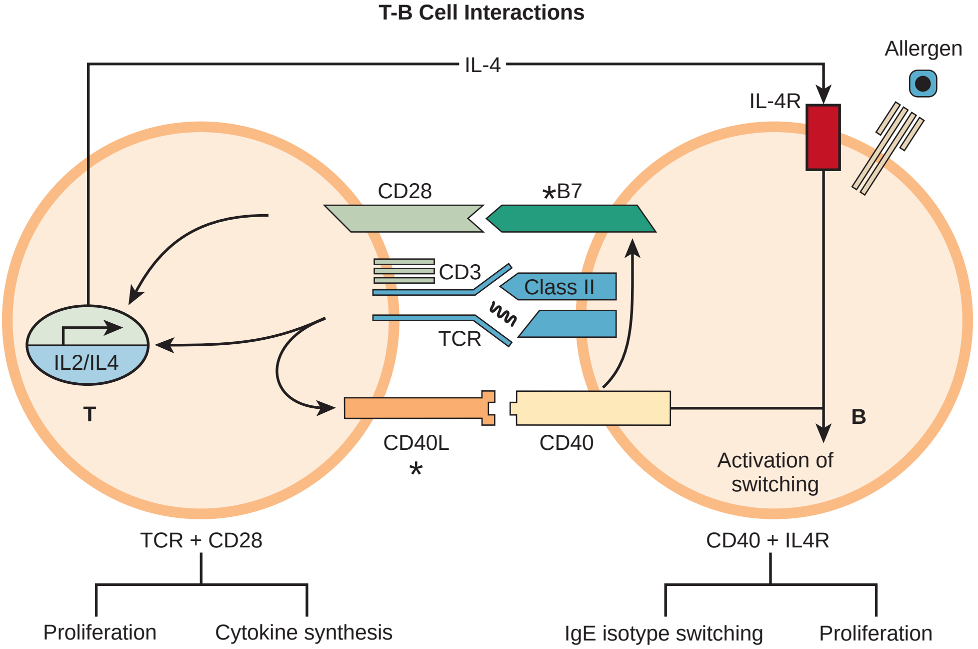

Figure 24-9 Signals required for T-cell activation by antigen-presenting cells (APCs), B-cell activation by the T cell, and B-cell immunoglobulin class-switching. The T-cell receptor (TCR) interacts with MHC + peptide. Subsequent to this interaction, the T cell expresses CD40 ligand (CD40L) and begins producing IL-2 and IL-4. CD40L interacts with CD40 on the APC (or B cell) inducing expression of B7-1 (also called CD80) and B7-2 (CD86). This interaction leads to full activation of APC function, macrophage killing capacity, or B-cell activation, depending on the cells involved. Binding of B7 molecules to CD28 on the T-cell surface provides an important costimulatory signal leading to full T-cell activation and increased cytokine production. IL-4 produced by the T cell interacts with its receptor on B cells to induce class-switching. Other cytokines act on other cell types to modulate their activities in the immune response.

absence of their activity, B cells cannot switch from production of IgM and IgD to production of downstream Ig isotypes (see Fig. 24-8). Somatic hypermutation is also affected. These processes normally occur during B-cell activation in germinal centers.

Another form of HIM syndrome with an unknown gene defect leads to impaired Ig class-switching recombination.[463-466] This has been called HIM4. All these disorders are very similar clinically and immunologically. Patients suffer mainly from respiratory tract bacterial infections and lymphadenopathy, and cellular immunodeficiency is not seen. IgG, IgA, and IgE levels are all low or absent; IgM levels tend to be higher than in the X-linked HIM syndrome. Patients tend to do well with IgG replacement.

Management of Humoral Immunodeficiency

Diagnostic Approach

Patients with recurrent infections, regardless of antibody class or subclass levels, should undergo thorough evaluation to rule out other potential predisposing factors (e.g., anatomic defects or environmental allergies).[4] The amounts of one or more antibody isotypes are reduced in many of these diseases.[3,4] The severity of the phenotype often correlates with the degree of hypogammaglobulinemia. The predisposition that patients have to particular types of infections may also depend on the affected isotypes (discussed later). It is always of the utmost importance to use age-adjusted reference ranges. Ig levels are low in infancy and rise throughout childhood. Most isotypes reach fully adult levels between the ages of 5 and 10 years.

Antibody titers to specific antigens may be measured before or after immunization (often both). The specific antibodies most commonly assayed for protein antigens are tetanus and diphtheria toxoids. With respect to polysaccharide antigens, measurement of antibodies to pneumococcal serotypes is frequently undertaken. If a child has had a particular illness (e.g., varicella), a specific viral antibody titer may be useful. Note that the HIB vaccines and newer pneumococcal vaccines (e.g., Prevnar-13) currently in use for primary immunization in children are protein conjugates. Thus measuring a polyribosylribitol phosphate or pneumococcal serum antibody concentration in children who have received these vaccines does not necessarily reflect a "pure" polysaccharide antibody response. The adequacy of carbohydrate responses may be tested by challenge with 24-valent pneumococcal polysaccharide vaccines (e.g., Pneumovax).[438,439,467-469] This vaccine produces reliable results in normal children as young as 1 year.[470] An unconjugated meningococcal vaccine (Menomune) may also be used to assess ability to respond to polysaccharides. In children who are not fully immunized or who are too young to immunize, isohemagglutinins may be measured as an indicator of the ability to generate polysaccharide antibody.[4]

Most children have been immunized with tetanus toxoid, HIB, and Prevnar, and antibody titers may be determined at the first visit. If titers are low, response to booster immunization may be assessed.

A high index of suspicion for antibody deficiency should be maintained in patients with recurrent, refractory, or severe infections. One study of primary antibody deficiency in the United Kingdom found that the median delay in diagnosis after the onset of symptoms (infections) was 2 years (mean, 4.4 years), even after the introduction of a set of national guidelines intended to reduce this delay.[471]

IgG Replacement Therapy

The mainstay of therapy for significantly impaired ability to produce antibody is IgG replacement.[4,472] As mentioned earlier, this therapy is also important for the treatment of any combined immunodeficiency in which defective antibody production may occur. Patients with XLA were the first to receive human gamma globulin therapy. The effect of intramuscular injections of gamma globulin was prompt and dramatic, with a reduction in the occurrence of infections. With the advent of preparations suitable for intravenous administration in the 1970s, this became the most common route of administration worldwide. A retrospective study of bacterial infections in patients with XLA showed a reduction in incidence from 0.4 to 0.06 per patient per year with IVIG therapy.[473] At present, administration of IVIG provides these patients with an almost normal lifestyle.[474-479] Many immunologists also apply chronic antibiotic prophylaxis in addition to IgG for patients with XLA, but this practice is not universal. All patients with CVID and with class-switch defects should also be treated with IgG infusions.[4,472,474,477,480-483] For these patients, as with XLA, many immunologists also apply chronic antibiotic prophylaxis.

In most countries gamma globulin replacement is administered predominantly by the intravenous route every 3 to 4 weeks. However, IgG can also be administered by subcutaneous infusion.[477,480,481,484] Smaller subcutaneous doses are given weekly or twice weekly or even daily. Intravenous infusion is generally well tolerated but requires intravenous access and is associated with frequent mild (10% to 20% of patients) or moderate (5%) side effects. Subcutaneous infusions may be administered by patients themselves at their own convenience with a much lower rate of systemic effects at the cost of generally mild and transient (24-hour) local irritation. The general efficacy of intravenous versus subcutaneous administration appears to be equivalent. Subcutaneous infusion is becoming increasingly popular.

Antibiotic Prophylaxis

For milder forms of antibody deficiency such as SIGAD, IGGSD, SADNI and THI, antibiotic prophylaxis is the more common first-line approach for infection prophylaxis.[4] However, regimens are not standardized. Commonly used agents include amoxicillin and trimethoprim-sulfamethoxazole. Depending on specific circumstances, other antibiotics may be chosen, such as clarithromycin or azithromycin, amoxicillin-clavulanate, or doxycycline. Also depending on circumstances, therapeutic dose or half-dose regimens may be applied. For any of these disorders (with the possible exception of SIGAD), IgG therapy may ultimately be prescribed if the response to antibiotic prophylaxis is poor, or if intolerable side effects or hypersensitivity precludes their continued use.[424,426,477,485,486] In these circumstances, there is usually a clearly observable reduction in the frequency of infections. IgG replacement for SIGAD is especially controversial because experience has not demonstrated consistent benefit.

General Care

Patients with antibody deficiency require regular monitoring for occurrence or progression of complications and adequacy of therapy.[4] Lung function should be measured regularly along with periodic assessment of hematologic parameters, liver and kidney function, and Ig levels. Therapy for autoimmune inflammatory complications in CVID and other antibody deficiencies generally follows regimens applied in similar situations in the absence of the immunodeficiency.[365,369,371,392,480] However, patients with primary immunodeficiency who are treated with immunosuppression for autoimmune or lymphoproliferative disorders may be more susceptible to infectious complications.

COMPLEMENT DEFICIENCIES

The complement system is composed of some 20 serum proteins, 5 complement receptors with varied distribution among leukocytes, and several integral membrane regulators found on most cells of the body.[487,488] The latter group of molecules limit cell damage as a result of a low constitutive level of complement activation. There are three distinct modes of complement activation: the classical pathway, the alternative pathway, and the lectin pathway. These pathways are shown in Figure 24-10. Soluble or surface-bound antigen-antibody complexes initiate the classical pathway; the alternative pathway is activated when complexes of C3b, factor B, and properdin are deposited on bacterial surfaces or extracellular matrix components. The lectin pathway is initiated by binding of MBL to mannose-containing microbial polysaccharides. The activation of the complement system by way of any of these pathways consists of sequential proteolytic interactions having a common outcome: cleavage (activation) of the C3 complement component to yield the anaphylatoxin C3a and the C3b fragment. C3b attaches to cell membranes (e.g., bacteria) and is the principal opsonin for phagocytosis. After cleavage of the C5 component (yielding the anaphylatoxin C5a), additional reactions are nonenzymatic aggregation of C5b, C6, C7, C8, and C9 to form the macromolecular membrane attack complex (MAC). The MAC is an amphiphilic cylinder; its hydrophobic end inserts into target cell membranes and permits free flow of ions and other solutes, which leads to metabolic derangement or lysis.[489] Genetic defects of almost all complement components are known. The clinical manifestations of these defects are categorized in Table 24-9. Complement deficiencies are among the rarest of primary immunodeficiencies, accounting for fewer than 1% of all patients diagnosed.

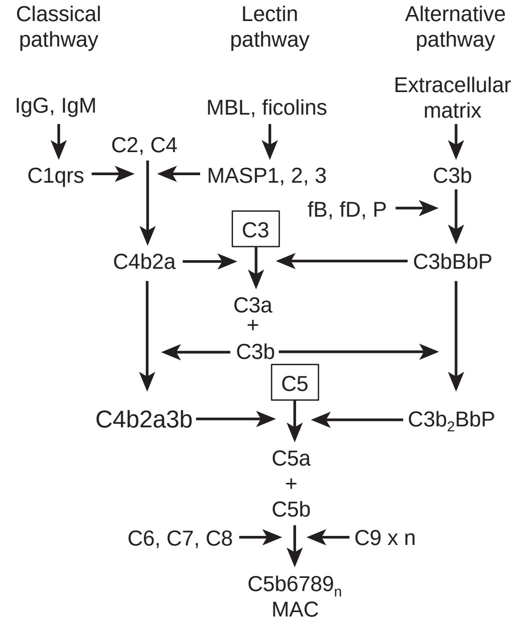

Figure 24-10 Complement activation pathways. The alternative pathway is initiated when the C3b fragment with factor B (fB) and properdin (P) bind to the extracellular matrix or some microbial polysaccharides. Factor D (fD) cleaves fB to yield the C3 convertase BbC3b. Addition of C3b creates the C5 convertase BbC3b2. The classical pathway begins when IgG or IgM bound to a surface forms a complex with C1qrs. The lectin pathway is initiated when mannose-binding lectin (MBL) or other initiators such as the ficolins and MBL-associated serine protease 2 (MASP2) bind to microbial polysaccharides. Activated C1qrs and MBL/MASP2 are both capable of cleaving C2 and C4 to yield the C3 convertase C4b2a. Addition of C3b to this complex yields the C5 convertase C4b2a3b. The C5 convertases generate C5b, the nucleus for the membrane attack complex (MAC), which is formed by the addition of one each of C6, C7, and C8 and several molecules of C9.

TABLE 24-9 Clinical Associations with Complement Deficiencies

Component	Symptoms/Syndrome Associated with Deficiency
C1q, C1r	Systemic lupus erythematosus–like syndrome
C2	Systemic lupus erythematosus–like syndrome, vasculitis, polymyositis
C4	Systemic lupus erythematosus–like syndrome
C3	Recurrent pyogenic infections
C5, C6, C7, C8, C9	Neisserial infection, systemic lupus erythematosus–like syndrome
C1-INH	Hereditary angioedema
Factor I, Factor H	Recurrent pyogenic infections, atypical hemolytic uremic syndrome
Factor D, Properdin	Neisserial infections
MBL, MASP-2	Recurrent pyogenic infections
Ficolin 3	Atopy (possibly also infection)

Deficiency of Classical Complement Pathway Components

C1 Deficiency

C1 is a large complex multimer consisting of three major subunits: C1q, C1r, and C1s. C1q is itself a complex of 18 separate polypeptides, 6 molecules each of C1qA, C1qB, and C1qC. The C1 complex is completed by the addition of two molecules each of C1r and C1s, which brings the total to 22 polypeptides. Patients with defects in any of the major subunits of C1 have a syndrome resembling systemic lupus erythematosus (SLE) and susceptibility to recurrent infections.[490-493] Deficiency of C1q may be caused by antigenically normal nonfunctional protein or complete absence of C1q. The *C1QA, C1QB,* and *C1QC* genes are closely linked on chromosome 1; defects in the *C1QB* and *C1QC* genes have been described. The genes encoding C1r and C1s are closely linked on chromosome 12, and mutations of both C1r and C1s have also been described. In addition to genetically determined or primary C1q deficiency, autoantibody-mediated depletion of C1q has been associated with SLE.[494-497]

C2 Deficiency

The monomeric C2 component of complement is the substrate for the activated C1s component of the C1q complex. Two fragments are generated in this reaction: The C2a fragment associates with C4b on a cell surface and becomes an activator for C3 (“C3 convertase”); the C2b fragment is a substrate for plasmin and releases a vasoactive peptide that may play a role in hereditary angioedema (HAE, discussed later). Approximately 50% of individuals with C2 deficiency are asymptomatic. The rest have one or more of the following: various forms of vasculitis such as discoid lupus erythematosus or SLE, chronic Henoch-Schönlein purpura, polymyositis, or recurrent pyogenic infections.[498-501] Only approximately 25% of C2-deficient patients are prone to infections. C2 deficiency is associated with low levels of IgG subclasses, and low levels of IgG4 and IgA may play a role in determining susceptibility to infection.

The gene encoding C2 resides in the class III region of the HLA complex on chromosome 6. Between 1% and 1.5% of Caucasians are heterozygous for a null *C2* allele (*C2Q0*). Thus approximately 1 in 10,000 will be homozygous for *C2Q0* and have a complete deficiency of C2.[498-501] Two forms of C2 deficiency independent of *C2Q0* have been identified. In type I, a mutation results in loss of a mRNA splice site and C2 cannot be translated. In type 2 deficiency C2 is not secreted, but the basis for this is unknown.

C4 Deficiency

The C4 component is another substrate for activated C1s. The C4a fragment is a very weak anaphylatoxin, and the C4b fragment associates with C2a on a cell membrane to constitute C3 convertase. Complete deficiency of C4 is rare. Virtually all such individuals with C4 deficiency have SLE and severe glomerulonephritis.[502-506] Renal failure and infection are the leading causes of death. The genes encoding C4 are also located in the class III region of the HLA complex. There are two copies on each chromosome, *C4A* and *C4B*. Each encodes proteins that differ in structure only minimally, although their biologic activities are distinct. Null alleles at these loci occur frequently; 35% of people have one copy of either *C4AQ0* or *C4BQ0*. Ten percent of the population have two null alleles, and 1% have three. The presence of four null alleles (complete C4 deficiency) is rare. All of these partial deficiency states are often asymptomatic. One study has found an association of *C4B* null alleles with Henoch-Schönlein purpura.[507] Older studies have found HLA haplotypes containing C4 null alleles frequently in patients with SLE.[508] However, more recent studies suggest that this association arises by linkage disequilibrium of C4 with the MHC locus rather than by the C4 genes themselves.[509] The relatively high rate of occurrence of C2 and C4 null alleles leads to combined heterozygous partial deficiencies of both in about 1 in 1000 Caucasians. Approximately one third of these patients have SLE and other autoimmune diseases.[510] It is possible that this association also may be the result of linkage disequilibrium with MHC.

C3 Deficiency

The C3 component sits at the confluence of all three pathways of complement activation. The various fragments of C3 generated through the action of C3 convertases (C4b2a and C3b2Bb), as well as other serum proteases, have diverse influences within the immune system. C3a is a potent anaphylatoxin, whereas C3b is the principal opsonin for phagocytosis and is a component of enzyme complexes in the alternative pathway of complement activation. Additional C3 fragments influence lymphocyte function. For example, C3d is an important regulator of B-cell activation.[511] Patients with C3 deficiency are susceptible to recurrent pyogenic infections affecting any organ system, and this condition is often clinically indistinguishable from hypogammaglobulinemia. These patients also may exhibit skin, kidney, and joint inflammation caused by immune complex deposition as well as a variety of autoimmune diseases.[512-514]

Deficiency of Alternative Complement Pathway Components

Properdin Deficiency

Properdin stabilizes the alternative pathway C3 convertase. The properdin gene is located on the X chromosome. Lack of properdin does not cause any changes in serum levels of complement components; activation through the alternative pathway is inefficient. Susceptibility to respiratory bacterial infections, *Neisseria* infections, and SLE has been associated with properdin deficiency.[510,515-518]

Factor D Deficiency

Factor D cleaves factor B after it associates with C3b. Factor D deficiency is associated with recurrent respiratory bacterial infections, pneumococcal sepsis, *Neisseria* infections, and glomerulonephritis.[519-522]

Deficiency of Lectin Pathway Complement Components

Mannose-Binding Lectin Deficiency

MBL is a member of the collectin protein family, which also includes lung surfactant proteins A and D.[523] MBL has a structure similar to C1q and interacts with polysaccharides containing mannose, glucose, and fucose, as well as acetylated derivatives of these sugars. Thus MBL may interact with members of virtually all classes of infectious microbes. Three proteins called MBL-associated serine proteases (MASPs) 1 to 3 are similar to C1r and C1s. MASP-2 may have the most important role in catalyzing cleavage of C2 and C4 in a manner analogous to C1s. Once this is accomplished, the classical pathway C3 convertase has been created and further reactions are identical to the classical pathway. There are polymorphisms of the gene encoding MBL that affect both structure and expression. Structural polymorphisms may impair the ability of MBL to form functional oligomers that bind effectively to carbohydrates and may also impair the association with MASP-2 and subsequent activation of the remainder of the classical pathway. Promoter polymorphisms lead to variation in blood MBL levels of greater than 1000-fold from 5 ng up to 10 μg/mL. A clinically relevant lower limit is not well established. Many studies use values of approximately 100 to 200 ng/mL, although slightly different values are occasionally used. MBL levels also vary among ethnic groups. The genotype or functional tests may be more informative than the serum level alone because it is possible to have normal serum levels of a nonfunctional protein.

The clinical significance of MBL measurement is controversial. Low blood MBL level or function does not by itself clearly predispose to disease, except possibly during infancy, when it may be associated with a higher rate of bacterial respiratory tract infections.[523] One study suggests that the (possible) increased susceptibility to pneumococcal infections in MBL deficiency may be serotype specific.[524] In other words, MBL-determined host defense is more important for some serotypes than for others. A variety of other types of conditions have been reported to be associated with MBL-deficiency, including pertussis,[525] cryptococcal meningitis,[526] cryptosporidiosis,[527] recurrent vulvovaginal candidiasis,[528] and the occurrence of septic shock in pyelonephritis as a result of *E. coli*[529] or severe course and fatality in adults with community-acquired pneumonia.[530]

Some reports also suggest that in the setting of other disease processes or stresses on the immune system, MBL deficiency predisposes to a higher rate of a variety of infectious complications. For example, one study reported a higher rate of invasive aspergillosis in MBL-deficient patients with secondary immune compromise resulting from acquired immunodeficiency virus or treatment with steroids or cancer chemotherapy.[531] Another report found a higher rate of bacterial infections in patients with low MBL and hepatic cirrhosis[532] or patients who underwent liver transplantation[533] or HSCT.[534] Another group reported a higher rate of CMV reactivation in MBL-deficient patients after lung transplantation.[535] Interestingly, overall survival was superior in those with low MBL in this study. Similarly, low blood MBL levels may also increase the severity of primary immunodeficiency, such as in CVID or with IgA/IgG subclass deficiencies or properdin deficiency.[416,536-538]

MBL levels may also impact certain inflammatory states. A low level of MBL has been associated with a lower rate of acute rejection after heart transplantation[539] and smaller infarct size and better outcome after cerebral infarction.[540] However, another study reported a higher risk for myocardial infarction in adults with MBL deficiency.[541] MBL deficiency has also been reported to predispose to or worsen autoimmune or inflammatory diseases such as rheumatoid arthritis, SLE, and celiac disease.[542]

There are also examples of studies reporting negative findings regarding disease susceptibility and MBL levels. For example, a recent study in 105 HIV patients did not find a significant association between low MBL levels and the occurrence of infection.[543] Another study did not find correlation of MBL levels with outcomes in cystic fibrosis patients[544] (whereas earlier studies had reached opposite conclusions[544-548]). The situation may be even more complex because of discrepancies between studies based on measuring MBL levels versus those based on genetics. For example, MBL genotypes generally thought to be predictive of serum level and function did not correlate with infection rates in patients with colorectal cancer, in contrast to a prior study that had found significant associations with the serum levels of MBL.[549]

In spite of the controversy surrounding the clinical significance of MBL levels, recombinant human MBL is under development as a therapeutic agent; one phase I trial has shown no adverse effects of administration to healthy MBL-deficient individuals.[550]

The ficolins 1-3 are also members of the collectin family of molecules and can interact with MASPs 1 to 3 and initiate the lectin pathway of complement activation. Deficiency of ficolin 2 (also called L-ficolin) has recently been associated with recurrent respiratory infections and atopy in children.[551] However, a more recent report from the same group suggests that the main clinical association of low ficolin levels is with atopy rather than with infection.[552] The significance of these findings requires clarification.

MASP-2 Deficiency

Abrogation of MASP-2 function is expected to have the same consequence as the absence of MBL function: failure to activate complement by way of the lectin pathway. Patients homozygous for *MASP2* mutations have been reported to have similar clinical associations as described for MBL deficiency.[524,530,549,553-555] All of the controversy regarding the clinical importance of MBL levels and genetics applies equally to a discussion of functional MASP2 polymorphisms or mutations.

Deficiency of Terminal Complement Components

Recurrent neisserial and pneumococcal infections and rheumatic disease have been reported in patients with C5,[556,557] C6,[558,559] and C7[560] deficiencies. C6 deficiency has been found in approximately 1 in 1600 African-Americans in the southeastern United States in association with a higher incidence of meningococcal meningitis.[561]

The C8 molecule is a heterotrimer of chains designated α, β, and γ. The *C8A* and *C8B* genes are on chromosome 1, whereas the *C8G* gene resides on chromosome 9. C8 deficiency is also associated with recurrent neisserial infection; patients have been identified with lesions of *C8A* or *C8B*.[562-565] Patients with lesions in the *C8A* genes also lack C8 γ protein, even though the latter genes are normal, because C8 α and γ are normally covalently linked. C9 deficiency is usually asymptomatic[566]; a few patients with recurrent neisserial infections have been described.[567,568] One patient with IgA nephropathy associated with C9 deficiency has been reported.[569] C9 deficiency has also been found in one patient with dermatomyositis.[570] Allotypy at IgG Fc receptor gene loci may have an impact on the susceptibility to infection in patients with deficiencies of terminal complement components.[571]

Deficiency of Complement Regulatory Factors

C1 Inhibitor Deficiency (Hereditary Angioedema)

HAE exhibits autosomal dominant inheritance. The symptoms of this disease are unpredictable recurrences of acute and profound subepithelial edema in a variety of anatomic locations.[572,573] Physical trauma may be a precipitating factor. The distal ends of the extremities, face, intestines, and pharynx are often affected. Extremity edema is painless but impedes function. Intestinal edema causes excruciating pain and is associated with abdominal distention, vomiting, and diarrhea. Pharyngeal edema may lead to fatal airway obstruction.[574] Attacks evolve over a period of approximately 12 hours and resolve in 24 to 48 hours. Attacks in childhood are not uncommon but occur less frequently than in adulthood. An increase in incidence and severity often occurs during puberty and is sustained thereafter. Attacks may be associated with menses in females. Patients may become symptomatic or may have more frequent symptoms when receiving estrogen replacement or contraceptive therapy.

C1 esterase inhibitor (C1-INH) is a serum serine protease inhibitor (Serpin G1) that limits activities of the complement proteases C1r and C1s, as well as those of kallikrein, coagulation factors XIa and XIIa, and plasmin.[572,573] In type I HAE (≈85% of patients), levels of C1-INH are less than 30% of normal. Patients with type II HAE have normal levels of a mutated (nonfunctional) protein. There is at least one additional form of HAE with autosomal dominant inheritance and completely normal C1-INH function. It is designated HAE type III and arises from mutations in the gene encoding coagulation factor XII.[575] The mechanism of angioedema is not known. In HAE I and II the unopposed activities of C1r, C1s, and kallikrein lead to consumption of C2, C4, and kininogen.[572,573] Diminished levels of these proteins are highly characteristic of HAE. Peptides derived from C2 and especially bradykinin cause the increase in vascular permeability and edema.

The *SERPING1* gene is located on chromosome 11. A variety of genetic lesions have been identified in patients with HAE.[575] Despite having one normal copy of the gene, patients have inadequate levels of C1-INH, well below the 50% one would expect to be due to a simple gene dosage effect. The mechanism that leads to inhibition of transcription of the normal gene is unknown. Although inheritance is autosomal dominant, as many as 10% to 25% of patients may express new mutations.

Acquired forms of HAE have also been described. In acquired angioedema (AAE) type 1, C1-INH levels decrease by an unknown mechanism, usually as a paraneoplastic process associated with lymphoma and lymphoproliferative disorders.[576-581] In AAE type 2, autoantibodies against C1-INH deplete it from serum.[582-584] These antibodies may arise sporadically, in the context of other autoimmune syndromes, or in association with lymphoproliferation.

There are three distinct problems in the management of HAE or AAE. These are the control of acute attacks when they occur and the short-term (i.e., preprocedure) and long-term prophylaxis of acute attacks.[585,586-588] For management of acute attacks in patients with HAE, medications such as epinephrine, antihistamines, and corticosteroids, used to treat angioedema subsequent to hypersensitivity reactions, are entirely useless because the mechanism of swelling in HAE is unrelated. Analgesia is indicated for painful abdominal crises. Airway protection is vital and may require a tracheostomy.

Recently, several new products have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of acute attacks of angioedema. These include the human plasma–derived purified C1 inhibitor Berinert,[589,590] a recombinant kallikrein inhibitor,[591-594] and a peptidomimetic bradykinin receptor 2 inhibitor.[595-597] All of these drugs have proved effective for the treatment of acute HAE attacks, reducing the intensity of angioedema and shortening the time to resolution. Other medications may be used if these are not available. Fresh frozen plasma has been used in attempts to replace C1-INH. There are several anecdotal reports of efficacy of this therapy.[598] However, this is not considered first-line therapy for acute attacks with the availability of newer agents. Tranexamic acid may also be effective, but this is also a second-line agent.[585-588]

Several strategies are available for short- and long-term prophylaxis of angioedema attacks. Short-term prophylaxis is indicated whenever a patient is to undergo a procedure (e.g., invasive dental work or surgery) that has the potential to precipitate an attack. Long-term prophylaxis is used to reduce the frequency of spontaneous attacks. Impeded androgens such as danazol, stanozolol, and oxandrolone are useful for short- and long-term prophylaxis.[585-588] These hormones act to increase expression of the normal *SERPING1* gene. Pediatric use is limited by the tendency of these hormones to promote epiphyseal closure; use in female subjects is limited by their undesirable virilizing effects. Tranexamic acid is also useful as long-term prophylaxis. Recently, another purified human plasma–derived C1 inhibitor product (Cinryze) has been FDA approved for prophylactic therapy.[599-601] Rituximab has been used with mixed success in a small cohort of patients with AAE resulting from autoantibodies to C1-INH.[602]

Factor I Deficiency

Also known as C3 inactivator, the disulfide-linked heterodimeric complement regulatory molecule factor I cleaves C3b. Deficiency of factor I leads to increased degradation of C3 (and factor B), principally because of the inability to control constitutive low-level activity of the alternative pathway of activation. These patients have manifestations of C3 deficiency such as recurrent infections, urticaria, and immune complex disease.[603-607] Factor I deficiency is also associated with atypical hemolytic uremic syndrome.

Factor H Deficiency

Factor H cooperates with factor I in the inactivation of C3b; its absence leads to reduced C3 and factor B and a syndrome of recurrent infections with *Neisseria meningitidis* and membranoproliferative glomerulonephritis.[608-614] Both familial and sporadic relapsing hemolytic-uremic syndromes have also been associated with mutations in factor H. Similar clinical complications result from autoantibody-mediated depletion of factor H.[615-617]

Diagnosis and Management of Complement Disorders

Clinical tests are available to assess the function of each of the three major pathways of complement activation.[4,487,488,516,618-620] The lectin pathway is not as frequently evaluated because the definition of clinically significant deficiency is controversial. Activation of the classical pathway is measured by the CH50 assay (total classical pathway hemolytic assay), which can be performed by a variety of methods. The alternative pathway is assessed by a similar test called the AH50. If the CH50 is low and the AH50 is normal, then there is likely to be a defect in C1, C2, or C4. If the AH50 is low and the CH50 is normal, there is likely to be a defect in properdin, or factor D. If both are low, it is likely that there is a defect in any of the terminal pathway components (C3-C9). Complement depletion caused by consumption in an inflammatory process will lead to depression of both CH50 and AH50. The same situation is seen in regulatory component defects (factor H and factor I) because of consumption of C3.

Antibiotic prophylaxis is the mainstay of long-term management of the infectious predisposition in complement deficiencies.[4,489,618] Patients should also maintain fully protective levels of antibodies for common encapsulated organisms such as HIB, pneumococcus, and meningococcus. The autoimmune or inflammatory complications are managed according to standard regimens as they arise. It should be noted that experience is limited with newer biologic therapies in the inflammatory states associated with complement deficiency. Eculizumab is an anti-C5 monoclonal antibody that inhibits the terminal pathway and is approved for the management of atypical hemolytic-uremic syndrome.[621]

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CHAPTER 25

Lysosomal Storage Diseases

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CHAPTER OUTLINE

THE LYSOSOMAL SYSTEM
Morphology and Physiology
Biosynthesis and Biogenesis
Pathogenesis
OVERVIEW OF LYSOSOMAL STORAGE DISEASES AND THEIR DIAGNOSES
The Sphingolipidoses
The Mucopolysaccharidoses
Multiple Sulfatase Deficiency
Mucolipidosis Type II/ III (I-Cell Disease and Pseudo-Hurler Polydystrophy)
Lysosomal Acid Lipase Deficiencies
Neuronal Ceroid Lipofuscinoses
Krabbe Disease
Metachromatic Leukodystrophy

This chapter provides an overview of the lysosomal storage diseases (LSDs) with particular reference to their specific hematologically related manifestations or treatments. Comprehensive reviews, such as *Chapters in the Metabolic and Molecular Bases of Inherited Diseases,* 9th ed., are available for molecular genetics, biochemistry, enzymology, and phenotypes, and these sources should be consulted for details.

THE LYSOSOMAL SYSTEM

The lysosome is an intracellular organelle originally encompassing two concepts: (1) an intracellular acidic vacuole for digestion of intracellular and extracellular organic materials, and (2) biogenesis mechanisms to deliver or target its constitutive proteins, lipids, and other components.[1] Inherent in these were the foundations of the pathogenesis and therapy for the LSDs (i.e., the lysosomal accumulation of specific substrates for particular digestive hydrolases and the potential to correct such abnormalities by the reconstitution of the appropriate levels of enzyme activity in the lysosome). These concepts, although still relevant, evolved to include the lysosome as an integral part of a major intracellular system for the clearance of internal and external macromolecules and organelles (i.e., the autophagy system).[2,3] In addition, the lysosome can no longer be considered as a simple digestive vacuole but rather as a complex organelle functioning as a signal system that specifies its tissue or cellular compositions, modulates major metabolic processes (e.g., cholesterol and triglyceride synthesis),[4] and initiates inflammation. This complexity and its integration with the autophagy system preclude the LSDs as a simple result of inert intracellular accumulations of macromolecules and require their reconceptualization to complex disorders with wide ramifications within specific cellular types and throughout the body. Although the specific accumulated substrates may have differing effects on and manifestations in selected tissues, the disruptions of intracellular clearance through autophagy and of dysregulated metabolism are generally unifying, albeit heterogeneous, concepts for the LSDs. Indeed, the LSD disruptions of the lysosomal/autophagy/mitophagy system should be more properly termed *the lysosomal autophagy system diseases* (LASDs) (Fig. 25-1). *LSD* will be used here in its conventional sense, but the concept of widespread disruption of cellular processing is essential to the evolving view of these diseases.[5] These disruptions lead to heterogeneous effects, including macrophage activation and cytokine or chemokine storms (e.g., Gaucher and Niemann-Pick diseases), dysmorphology and skeletal malformations (e.g., mucopolysaccharidoses), and neuroinflammation and neuronal apoptosis or necroptosis (e.g., neuronopathic Gaucher disease, neuronal ceroid lipofuscinosis, and Krabbe diseases).

Morphology and Physiology

Lysosomes are membrane-bound, specialized organelles that normally constitute approximately 5% to 10% of the intracellular volume. Lysosomes are found in all

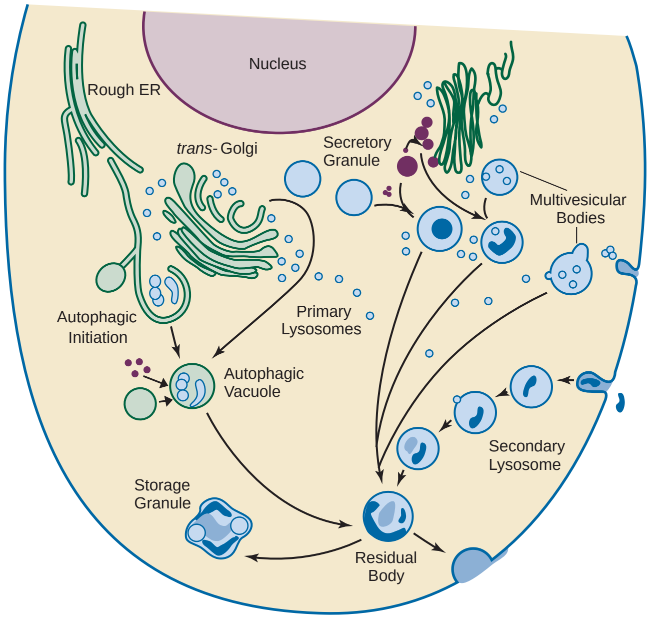

Figure 25-1 Schematic of the lysosomal/autophagy system. Primary lysosomes derive from the *trans*-Golgi as early endosomes and mature into late endosomes. These endosomes become increasingly acidic, which promotes dissociation of targeting signals from their receptors (e.g., M6P, with localization of the associated proteins to the primary lysosomes). The receptors are recycled to the Golgi and endoplasmic reticulum *(ER)* for reuse. The M6P receptor can also move to the plasma membrane for uptake of external proteins with that signal. Primary lysosomes also fuse with autophagic vacuoles for degradation of internal organelles (e.g., mitochondria) and extrusion from the cells as residual bodies. Multivesicular bodies (MBs) are formed by fusion of endosomes/secretory granules with invaginations from the plasma membranes. The internal vesicles of the MBs are inside-out plasma membranes. The MB fuse with lysosomes and provide surfaces for glycolipid degradation. In the lysosomal storage diseases, this complex machinery is disrupted by the increased physical size of the lysosomes and the biological, signaling, and toxic effects of the accumulated substrates.

nucleated cells but vary in size, morphology, and content with cell type. The characteristic azurophilic-staining granules in polymorphonuclear leukocytes and eosinophils are lysosomes. Moreover, lysosomes are abundant in cells of the macrophage/monocyte system.

Ultrastructure of the lysosome reveals a unit membrane surrounding polymorphic vacuoles, which fuse with pinocytic or phagocytic vesicles containing exogenous material (heterophagy) or with components of the autophagocytic system.[6] The undigested material remaining in secondary lysosomes becomes a residual body that can be extruded from the cell by way of an exocytosis pathway (e.g., into bile from the liver and into urine from the kidney).

Biosynthesis and Biogenesis

The lysosomal system represents the major site of intracellular compartmentalized degradation and potentially requires more than 200 different proteins for formation and modulation of its functions.[7,8] However, the protein composition of the lysosome varies considerably with the tissue or cell source. The expression of these proteins, and therefore lysosomal biogenesis, has been proposed to be controlled by the master regulator, *TFEB*, as are many of the autophagy proteins as part of the CLEAR system for intracellular degradation of macromolecules.[3] Lysosomes contain more than 70 hydrolytic enzymes, each with specificity to degrade selected proteins, glycoproteins, nucleic acids, polysaccharides, glycolipids, phospholipids, and neutral lipids. The lysosomal membrane contains many transmembrane- and membrane-associated proteins for maintenance of the acidic interior and its fusion capabilities.[7,9]

Most of the soluble lysosomal hydrolases are targeted to the lysosome by a direct route mediated by the mannose 6-phosphate receptor system.[8] In comparison, peptide sequences, rather than carbohydrate recognition markers, target most of the lysosomal transmembrane proteins indirectly, after transport to the plasma membrane and reinternalization with delivery to the lysosome.[7,10] The targeting mechanisms remain incompletely understood for several lysosomal membrane proteins, as are the roles of alternative targeting mechanisms (receptors) for some portions of soluble proteins.[8,10,11] Many soluble lysosomal enzymes are secreted from cells, and this process forms the basis of potential cross-correction of nearby cells.

All lysosomal proteins and enzymes are synthesized on the rough endoplasmic reticulum (ER) and gain access to the ER lumen by way of a leader, or signal, peptide (Fig. 25-2). The proteins are cotranslationally glycosylated on specific asparagines (*N*-linked glycosylation) as they enter the ER lumen.[12] Once glycosylated, vesicle-mediated transport delivers these proteins to the *cis*-Golgi. The oligosaccharide side chains are remodeled as they transit from the *cis*- to *trans*-Golgi and acquire specific sugars from Golgi glycosyltransferase that are resident in the *cis*, *mid*, and *trans*-Golgi.[13,14] For soluble lysosomal proteins a specific trafficking signal, mannose 6-phosphate (M6P), for lysosomal localization is added in the mid-Golgi.[8,15,16] The M6P-tagged proteins bind to the M6P receptor until they undergo an acidic pH-mediated dissociation in the late endosomes and are released into the lysosomal lumen. Small amounts of these soluble proteins are secreted from the cell and can be endocytosed by way of the M6P receptors on the plasma membrane for lysosomal delivery. This is the mechanic basis for metabolic cross-correction following hematopoietic stem cell transplantation (HSCT) in some LSDs. Membrane-bound lysosomal proteins use different receptors that recognize specific dileucine and other hydrophobic sequences for their delivery to the lysosomes.[10] The membrane-associated enzyme, acid β-glucosidase (glucocerebrosidase; i.e., the Gaucher disease enzyme) has a unique peptide sequence that binds to LIMP-2, lysosomal integral membrane protein 2, (Grabowski, unpublished), which is the specific receptor for this enzyme.[17] This enzyme and other membrane-bound lysosomal proteins do not normally secrete from cells and thus cannot cross-correct deficient cells.

Excellent recent reviews of lysosomal biogenesis, protein sorting, and molecular events of the dynamic lysosomal and autophagy systems are available, and the reader is referred to these for details.[2,18-22]

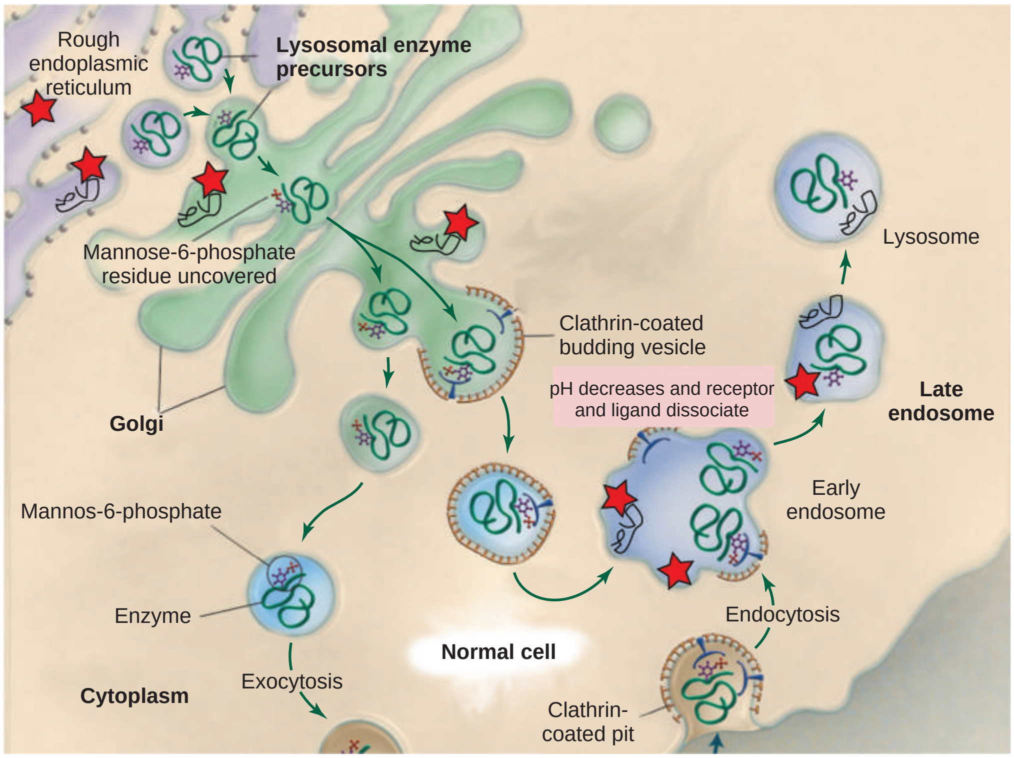
Rough endoplasmic reticulum
Lysosomal enzyme precursors
Mannose-6-phosphate residue uncovered
Golgi
Mannos-6-phosphate
Enzyme
Cytoplasm
Exocytosis
Normal cell
Clathrin-coated budding vesicle
pH decreases and receptor and ligand dissociate
Lysosome
Late endosome
Early endosome
Endocytosis
Clathrin-coated pit

Figure 25-2 Overview of targeting mechanisms for lysosomal enzymes to the lysosome. Lysosomal enzyme precursors are synthesized on the rough endoplasmic reticulum (ER) with penetration into the ER lumen by way of a leader sequence. The enzymes are cotranslationally glycosylated and transported in vesicles to the *cis*-Golgi. During transit through the Golgi, the attached oligosaccharides are remodeled with subtractions and additions of specific sugars to form *N*-linked high mannose, complex, or hybrid oligosaccharide side chains. An important modification is the addition of M6P *(blue)*, which is the targeting signal for most soluble lysosomal proteins. The M6P receptor and M6P-modified proteins dissociate, owing to a drop in pH, in the late endosomes with release of the protein into the lysosomal lumen and regeneration of the endosome. The M6P receptor is recycled to either the mid-Golgi or the plasma membrane. Alternative mechanisms exist for integral or associated lysosomal membrane proteins. The red star is the LIMP2 receptor protein that transports acid β-glucosidase, the Gaucher disease enzyme, by way of a peptide sequence to the late endosome, where they dissociate by way of an acid-mediated mechanism. LIMP2 and other lysosomal integral membrane proteins are trafficked to the lysosome by dileucine or other hydrophobic motifs.

Pathogenesis

Hers[23] conceptualized the inborn lysosomal diseases in 1965 and proposed that excessive lysosomal accumulation of substrate leads to an increased number of lysosomes within cells, disruption of normal cell functions, and possibly cell death. In general, the signs and symptoms of each LSD primarily reflect the normal pattern of distribution of the enzymes' substrates.[24,25] In Gaucher disease type 1 (discussed later), turnover of senescent erythrocyte and leukocyte membranes containing glucosylceramide leads to its accumulation in the macrophage/monocyte system. Similarly, disorders of ganglioside metabolism involve principally the central nervous system (CNS) because gangliosides normally have higher turnover in this tissue. Compounds that are normally abundant in all tissues lead to generalized organ dysfunction (e.g., mucopolysaccharidoses and glycoproteinoses). Because of the disruptions of the lysosomal and autophagy system, secondary accumulations of various compounds do occur; the mechanisms for such accumulations are poorly understood.

OVERVIEW OF LYSOSOMAL STORAGE DISEASES AND THEIR DIAGNOSES

The LSDs are caused by deficiencies of a single or multiple lysosomal proteins. This underscores the lack of redundancy for these proteins and their importance in maintaining cell homeostasis. Most LSDs result from single enzyme or protein deficiencies resulting from mutations in their respective genes. The essential need for specific enzymes implies that if a single enzyme is deficient or functionally defective, the hydrolytic pathway is halted at the level of the missing enzyme, and its specific substrate (or substrates) accumulates in the lysosome with resultant organelle, cellular, and organ dysfunction. Some LSDs have multiple enzyme or protein deficiencies that result from mutations in single genes that are necessary for posttranslational processing or assembly or stability of a multiprotein complex. Examples include mucolipidosis II and III that disrupt the M6P targeting of many soluble lysosomal proteins to the lysosome, thereby leading to intracellular deficiencies of most soluble lysosomal enzymes.[26] Multiple lysosomal hydrolase defects also result from single gene defects that alter specialized posttranslational processing or multienzyme complex formation. Multiple sulfatase deficiency, in which all, not only lysosomal, cellular sulfatase activities are deficient, results from a single gene defect that encodes an active site modification enzyme that is essential for the activity of all sulfatases.[27,28,29] Multiprotein complex formation is defective in combined β-galactosidase/neuraminidase/"protector protein" deficiency in which there is a deficiency of a "protective protein (cathepsin A)" that is essential for the formation and stability of the tri-enzyme complex.[30]

All LSDs are inherited in an autosomal recessive manner, except for Fabry disease, Hunter syndrome (MPS II), and Danon disease, which are X-linked. Extensive allelic heterogeneity exists in all the LSDs, including many single base substitutions, premature chain terminations, and insertions and deletions of part or all of the respective genes. Some of the LSDs exhibit founder effects leading to a common mutation (or mutations) in specific demographic or ethnic groups. New alleles are regularly being discovered in all of the LSDs, and databases for many LSD mutations are maintained. Polymorphic variation also occurs in some of the alleles, and these can affect the resultant phenotype of a specific disease-causing genotype. Imperfect genotype-phenotype correlations exist, but in general this information can inform the anticipated prognosis and therapeutic options (discussed later).

Because many of the enzyme-defective LSDs are very rare, enzyme assays remain the gold standard for antenatal and postnatal diagnoses. Indeed, identification of a "mutation of unknown clinical significance," which often occurs in unrelated families with an LSD, requires enzyme confirmation of the diagnosis. For known pathogenic mutations in genes encoding enzymes and proteins in specific families or ethnic groups, molecular diagnosis is highly reliable, particularly for heterozygote detection and prenatal diagnosis.

For enzyme diagnosis, serum, plasma, or mixed leukocytes provide readily available sources, and reliable assays using synthetic fluorogenic or colorimetric substrates are available. LSD enzymes require different sources for assay; for example, serum and plasma or peripheral blood leukocytes are routinely used for many of the LSD-associated enzyme assays. Nucleated cells are needed for the diagnosis of Gaucher disease because the relevant enzyme is not secreted. Recently, dried blood spots have become more generally available for screening studies, but definitive enzyme assays should be used for confirmation. Antenatal diagnosis is available for most LSDs. Such sample needs are specified on the website *Gene Tests* (ncbi.nlm.nih.gov/sites/GeneTests/).

The Sphingolipidoses

The sphingolipidoses are characterized by defective or absent lysosomal hydrolysis and accumulations of specific sphingolipids in lysosomes. Mutations leading to either defective or deficient function of sphingolipid hydrolases or their specific activator proteins (also known as *saposins*) are the bases for these diseases. The shared structures of the sphingolipids include sphingosyl and fatty acyl acid chains that are covalently linked to form the ceramide. Attachment of various head groups to the ceramide backbone produce sphingomyelin, a phospholipid, and the more than 400 glycosphingolipids that contain single to multiple glycosyl residues. The glycosyl, fatty acid acyl chain, and sphingosyl components vary in structure with tissue, cellular, and subcellular localization. The sphingolipids are crucial structural elements of cell membranes and have important roles in cell signaling and recognition. Among the sphingolipidoses, Gaucher disease; Niemann-Pick disease types A, B, and C; Farber disease; GM_1 gangliosidosis; and GM_2 gangliosidosis have significant hematologic manifestations and effects.

Gaucher Disease

Gaucher disease, one of the more common LSDs, is an autosomal recessive trait resulting from more than 350 mutations at the *GBA1* locus and the resultant deficient or defective activity of the acid β–glucosidase (glucocerebrosidase).[31] Gaucher disease has a frequency of approximately 1/57,000 in the general population, and the type 1 variant has its greatest frequency in the Ashkenazi Jewish population, with a frequency of approximately 1/855.[32]

Decreased catalytic activity or instability of acid β–glucosidase leads to accumulation of glucocerebroside (glucosylceramide) and its deacylated analog, glucosylsphingosine, in the lysosomes of visceral cells of the monocyte lineage, termed Gaucher cells.[31] Gaucher cells (Fig. 25-3) accumulate in the liver, spleen, bone marrow, lung, and lymph nodes, leading to hepatomegaly, splenomegaly, anemia, thrombocytopenia, destructive bone disease, bone marrow infiltration, pulmonary disease, and lymphadenopathy.

Three clinical variants of Gaucher disease are classically described, depending on the absence or presence and severity of primary CNS involvement. Gaucher disease type 1, the "non-neuronopathic" variant, accounts for approximately 90% of all cases in European and North

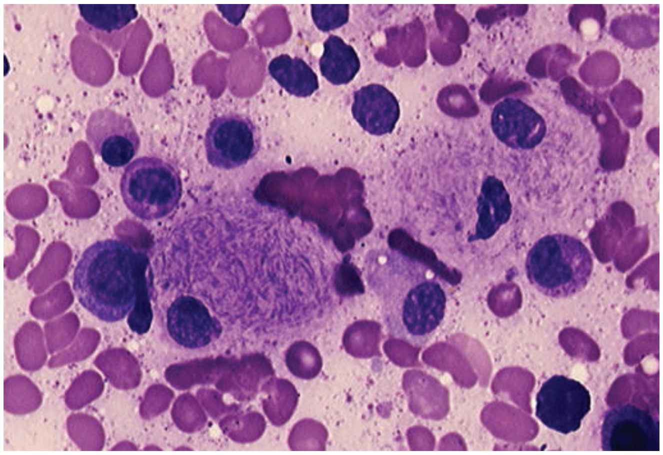

Figure 25-3 Bone marrow Gaucher cell: Light micrograph of bone marrow demonstrating characteristic enlarged, lipid-laden macrophages, "Gaucher cells" *(arrows)*, with inclusions resembling crumpled tissue paper.

American populations. Type 1 disease displays marked phenotypic variability in its age at onset, rate of progression, and degree of visceral organ involvement. Although survival may be reduced, individuals may live well into adulthood.[33] Indeed, patients have been diagnosed coincidentally in the sixth to eighth decades of life, and with particular genotypes the manifestations may be so minor that affected patients do not seek medical attention.[31]

Gaucher disease types 2 and 3 are primary neuronopathic variants distinguished by the age of onset and severity of CNS features. Although infrequent in European and North American populations, they are more commonly seen in Asia, Africa, and South and Central America. The demarcation between Gaucher disease types 2 and 3 is clinically useful but somewhat arbitrary insofar as these variants represent a continuum of disease phenotypes. Gaucher disease type 2 is a severe, early (prenatal to first 6 months of life) onset, rapidly progressive neurologic disease, particularly with brainstem abnormalities (stridor, dysphagia, aspiration) and visceral disease. Death occurs in the first 1 to 2 years of life. Gaucher disease type 3 has a more protracted course, with variable CNS and visceral organ (e.g., hepatosplenomegaly, poor weight gain) involvement. Indeed, horizontal supranuclear gaze palsy and minor cognitive deficits may be the only manifestations of neurologic disease for decades. Survival is shortened, with death usually occurring by the fourth decade of life.[31]

Although Gaucher disease type 2 demonstrates similar visceral findings to types 1 and 3, only the findings in Gaucher disease type 1 are presented here. The visceral findings in types 1 and 3 are very similar. It is important to note that types 1 and 3 can present in the first few years, and therefore the distinction between types 1 and 3 is not based on age at onset but rather by the presence of early primary CNS involvement in type 3.

Hematologic abnormalities are common in individuals with Gaucher disease type 1. Thrombocytopenia of mild to moderate severity is the most common finding in the peripheral blood and is seen in 50% to 70% of individuals at diagnosis.[31] Splenic sequestration and diminished platelet production caused by bone marrow infiltration are implicated as mechanisms. Splenectomy improves platelet counts; however, this is generally not advised because of the associated risks, as described later.

Anemia is present in approximately 20% to 30% of clinically involved individuals at diagnosis, although this varies with *GBA1* genotype.[31] The anemia is generally mild and is normocytic or microcytic and hypochromic. In developing countries severe anemia in individuals with Gaucher disease may be multifactorial, and additional contributing factors should be sought.[31] Low serum iron levels and iron-binding capacity resemble the anemia of chronic disease, but excessive iron is present in bone marrow Gaucher cells. Ferritin levels are generally elevated, likely owing to the inflammatory component of Gaucher disease. Iron therapy is generally not useful, but a short trial of iron may provide insight into its utility. Megaloblastic anemia occurs with vitamin B_{12} deficiency, which may be more frequent in this disease; evaluation for vitamin B_{12} deficiency should be considered, with appropriate treatment as indicated. Anemia requiring blood transfusions is unusual in developed countries.[31]

Coagulation factor abnormalities are common, including abnormalities of factor IX, vWF Ag, and factor VIII. These rarely require clinical intervention and may be incidental findings.[34-36] Deficiencies of factor XI are frequent, particularly in the Ashkenazi population.[31]

Leukopenia is atypical but may occur with severe splenomegaly or bone marrow failure caused by myelofibrosis.[31] Recently, the roles of excessive cytokine and chemokine have been recognized as important to the pathology of Gaucher disease. This is because of the recruitment and chronic stimulation of immune system cells, including B- and T-lymphocytes.[37] The long-term stimulation of B-cells and their precursors lead to monoclonal and oligoclonal gammaglobulinemias and an increased rate of multiple myeloma in adults. Autoimmune hemolytic anemia occurs infrequently[36]

Most patients (75% to 90%) have bone abnormalities on radiographic imaging.[31] At least half have an Erlenmeyer-flask deformity, in which expansion of the femoral midshaft leads to a tapered appearance and widening of the distal end. Destruction of the marrow cavity (resulting from marrow replacement by Gaucher cells), cortical lytic lesions (caused by infarctions), and osteopenia or osteoporosis are present in many patients. Painful bone crises, similar in clinical manifestations to bone infarcts in sickle cell disease, occur in approximately 10% of patients and are more frequent in the femoral head and shaft. These episodes can mimic osteomyelitis, with fever, local erythema, leukocytosis, and increased erythrocyte sedimentation rate. Growth retardation occurs in 35% to 40% of children, leading to a reduction in predicted adult height.[38-40]

Hepatomegaly is nearly universal in Gaucher disease types 1 and 3, although the degree of enlargement is variable, ranging from 1.25 to greater than eightfold of predicted normal volumes. The liver is usually homogenous, with evidence of fatty infiltration on magnetic resonance imaging (MRI) studies, but isolated areas of Gaucher cell accumulations do occur and may raise the suspicion of malignancy. Persistent mild elevations in serum transaminases and increased unconjugated bilirubin are not infrequent; however, liver failure is rare and is associated with other comorbidities, including hepatitis C.[31] Hepatocellular carcinomas and cholangiosarcomas have occurred, but these are unusual and confined to the adult population.

Asymptomatic splenomegaly is the most frequent presenting sign. Splenomegaly may be sufficiently pronounced to result in early satiety or to interfere mechanically with respiratory function.[31] As evaluated by MRI or ultrasound, the spleen is generally homogeneously enlarged early in life. With increasing size and age, the spleen can develop infarctions, multiple areas of scarring, and intrasplenic nodules. The scarred or damaged areas may lead to intrasplenic consumption of coagulation factors. In adults malignancy should be excluded.[41] Although infrequent, pain from splenic infarction may mimic an acute abdomen. Splenectomy rapidly improves the hematologic signs, but it is generally not recommended because it has been linked to worsening bone disease and increased frequency of pulmonary hypertension.

Several variants of pulmonary involvement occur in Gaucher disease type 1, but they are much more frequent in type 3. These include interstitial infiltrative disease, alveolar infiltration, hepatopulmonary syndrome, and pulmonary hypertension and are associated with significant morbidity and mortality risk.[31] The infiltrative diseases are more common in younger patients with type 3 disease and can be exacerbated by chronic aspiration. Pulmonary vascular disease is associated with adult female, non-Jewish patients who have undergone splenectomy. These complications can be challenging to treat and require medications for pulmonary hypertension as they respond poorly to enzyme therapy.[31,42]

Specific malignancies have a clearly increased frequency in adults with Gaucher disease type 1. These include multiple myeloma and lymphoid cancers (i.e., leukemia, Hodgkin and non-Hodgkin lymphoma).[43-46] The lifetime risk of multiple myeloma in untreated type 1 disease is estimated to be approximately 25 times that of the general population.[46] The overall cancer risk in patients with Gaucher disease is estimated at 1.8 times that of the general population.[46] However, the significance of this and the relationship to Gaucher disease is unclear. There are no data to support an increased risk of malignancy in children with types 1 and 3 diseases.

Gaucher cells are the hallmark histologic findings in all variants of Gaucher disease. These lipid-laden macrophages are approximately 20 to 100 μm in diameter, with tubular inclusions resembling crumpled tissue paper (see Fig. 25-3). This material stains positively with periodic acid–Schiff (PAS) stain. In comparison, storage cells in sphingomyelinase deficiency (discussed later) contain foamy-appearing inclusions. On electron microscopy Gaucher cells demonstrate membrane-bound inclusions containing twisted structures (Fig. 25-4). Histologic analysis of bone marrow demonstrates accumulation of Gaucher cells and fibrosis; the latter can be progressive.[31]

Clinical features suggest the diagnosis of Gaucher disease, as does the presence of Gaucher cells in tissue (i.e. bone marrow, liver, spleen) or bronchoalveolar lavage. Confirmatory laboratory diagnosis requires demonstration of decreased acid β-glucosidase activity in nucleated cells or tissues, most commonly peripheral blood leukocytes or cultured skin fibroblasts. Molecular diagnosis through the identification of pathogenic mutations in both copies of *GBA1* can provide useful prognostic information. Although genotype and phenotype correlations are not absolute, identification of known mutations may provide insight into the potential severity and risk of CNS or non-CNS disease. For example, the presence of a *GBA1* mutation encoding N370S in affected individuals is not associated with early onset progressive, primary neurologic disease. Homozygosity for N370S generally predicts a less severe disease course than other genotypes. About 50% of N370S homozygotes are predicted never to come to medical attention owing to their lack of overt signs and symptoms. For example, N370S homozygotes have an age of onset or recognition of about 32 years, whereas in individuals with the N370S/84GG (null), onset occurs by approximately 6 years. In addition, the latter genotype is associated with greater liver and spleen enlargement. Homozgyosity for the recurrent allele encoding L444P is highly associated with early onset primary CNS disease. An extensive review of these correlations from large patient populations is available.[31]

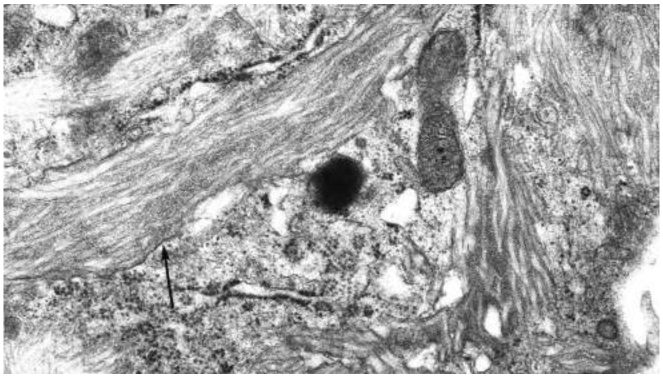

Figure 25-4 Gaucher inclusions. Electron micrograph of a Gaucher cell demonstrating characteristic membrane-bound twisted inclusions of glucosylceramide and phospholipids *(arrow)*.

Recommendations from expert panels regarding evaluation, monitoring, and treatment of patients with Gaucher disease are available and revised on a regular basis.[47-49] Gaucher disease variants are multisystem diseases that affect disease management plans. The lack of predictive biomarkers necessitates comprehensive, multidisciplinary evaluations of the major organs for associated complications. The primary focus is on the major involved organs (liver, spleen, blood, and bone) and their functions.

Although HSCT has been used to treat type 1 disease, its utility is limited by the availability of safer, specific forms of therapy. Because acid β-glucosidase is not normally secreted from cells, HSCT has not shown significant or consistent effects on the CNS manifestations in type 3 disease. Similarly, HSCT in type 2 disease has not been successful for the CNS disease.

Two treatment approaches are currently available for the treatment of Gaucher disease type 1: enzyme replacement therapy (ERT) and substrate reduction therapy (SRT). With ERT recombinant human acid β-glucosidase, a glycoprotein, is administered intravenously. For Gaucher disease the available ERT products have been modified to expose terminal α-mannosyl residues for preferential targeting to the macrophage mannose receptor. After intravenous administration such modified enzymes preferentially localize to the macrophage compartment, are internalized by receptor-mediated endocytosis, and delivered to the lysosomes where they reconstitute the enzyme activity. This leads to degradation of the stored glucocerebroside and other glucolipids. SRT is different and depends on the presence of some residual mutant enzyme activity (1% to 15%) that can degrade a lesser amount of substrate and establish a healthier steady state. The driving concept is to slow the synthesis of the offending substrates by inhibition of their synthesis. The target is the Golgi localized enzyme, glucosylceramide synthase. For either ERT or SRT, the efficacy will be limited by tissue delivery and penetration. For example, the ERT recombinant enzymes are macromolecules that do not penetrate the blood-brain barrier, or potentially other tissue barriers, in sufficient quantities to be therapeutic. The SRT small molecules may or may not be distributed to all tissue and can have off-target effects.

ERT is currently the standard of care for the treatment of Gaucher disease. Three ERT products are currently approved: Imiglucerase (Cerezyme, Genzyme, a Sanofi company, Cambridge, Mass., approved by the Food and Drug Administration [FDA] in 1991), velaglucerase alfa (VPRIV, Shire Human Genetic Therapies, Inc., Cambridge, Mass., approved by the FDA in 2010), and taliglucerase alfa (Elelyso, Pfizer, Inc., New York, N.Y., approved by the FDA in 2012). The FDA currently restricts the use of taliglucerase alfa to adults (older than 18 years). These products differ in their manufacturing processes, glycosylation patterns, and amino acid sequence. Briefly, they are all human acid β-glucosidases that are produced in large cultures of Chinese hamster ovary cells (imiglucerase), human fibrosarcoma cells (velaglucerase alfa), or carrot root cells (taliglucerase alfa). All have terminal α-mannosyl residues and target the macrophage mannose receptor; have highly similar crystal structures; and have similar, if not identical, enzyme functions. On the basis of current data, all three ERT products have similar clinical effects and safety. Direct head-to-head studies of imiglucerase and velaglucerase alfa were conducted in mouse analogs of Gaucher disease and shown to have similar biochemical, histopathologic, and antibody formation effects.[50]

The long-term (approximately 20 years) safety and efficacy of imiglucerase has been shown in Gaucher disease type 1.[51,52] In 1028 such patients, hemoglobin concentrations increased to normal or near normal within 6 to 12 months, with a sustained response through 5 years. In thrombocytopenic patients with intact spleens, improvements in platelet counts were particularly noted during the first 2 years of therapy. Further increases in platelet counts were observed during years 2 through 5; The likelihood of achieving a normal platelet count decreased with increasing severity of baseline thrombocytopenia. Similarly, significant decreases in liver (20% to 30%) and spleen (30% to 50%) volumes were noted after 2 years, with additional reductions in liver volume to near normal levels by 5 years. Further decreases in spleen volume after two years were minimal.[51] Improvement or stabilization of bone pain occurred in many patients after 2 years of therapy, with approximately a third of patients having residual bone pain reports, probably the result of chronic joint abnormalities that are not directly related to Gaucher disease. A 10-year follow-up of patients treated with imiglucerase showed sustained stability of disease.[52] The safety profile of imiglucerase has been extensively reviewed and shows that the drug is generally safe and well tolerated.[51,52] A detailed statistical study of various doses of imiglucerase shows a clear dose response of the major monitoring parameters and provides guidance for expected responses in patients initiated on any dose between 15 to 60 U/kg every two weeks.[53] Bone mineral density was also dose dependent over the long term.[54] About 13% of patients receiving ERT with imiglucerase develop immunoglobulin G (IgG) antibodies, and about half of these patients have minor adverse events or infusion-related events. These are generally easily controlled. Inhibitor antibodies have been reported in a few patients,[55,56] and immunoglobulin E (IgE) antibodies have been anecdotally reported in fewer than 10 patients. Similar long-term data are not available for velaglucerase alfa and taliglucerase alfa, but phase II and phase III studies show comparable safety and efficacy profiles to imiglucerase.[57-61]

SRT with miglustat (Zavesca, Actelion Pharmaceuticals, South San Francisco, Calif.) is licensed in several countries for use in adults with mild to moderate Gaucher disease type 1 for whom ERT is unavailable or contraindicated. Miglustat is an oral medication that produces statistically significant effects on liver and spleen volumes but only slight improvements in hematologic variables.[62-64] Miglustat is reported to positively affect bone mineral density.[65] The drug has several side effects, including diarrhea (approximately 80%) and peripheral neuropathy, which may be significant in some patients.[66]

Phase I and phase II clinical studies on a second SRT, eliglustat tartrate (Genzyme, a Sanofi company; Cambridge, Mass.), have been completed, and initial phase III results are available.[67-69] In the phase II study, 20 patients completed a total of 2 years of therapy. In these patients statistically significant improvements from baseline were observed for platelet count, hemoglobin level, spleen volume, liver volume, and bone mineral density of the lumbar spine. These findings were essentially duplicated in the initial phase III data in patients who were naïve to therapy. Also, the drug was well tolerated, without many of the side effects observed in miglustat.[68,69]

Acid Sphingomyelinase Deficiency (Niemann-Pick Disease Types A and B)

The acid sphingomyelinase (ASM) deficiencies are a group of clinically heterogeneous, autosomal recessive conditions, characterized by deficient activity of ASM resulting from mutations in *SMPD1*. Sphingomyelin, a phosphosphingolipid, accumulates in lysosomes of multiple cell types, particularly those of the macrophage or monocyte lineage, leading to characteristic lipid-laden Niemann-Pick foam cells (Fig. 25-5).[70]

Two specific variants of ASM deficiency are classically recognized. Niemann-Pick A (NPA) is an early onset neurologic disorder characterized by progressive psychomotor retardation, ocular findings (cherry-red spots [approximately 50%]), massive hepatosplenomegaly, and cachexia, leading to death in the first few years. A nonneurologic form (NPB) involves visceral disease and is compatible with survival into adulthood.[71] The NPB phenotype includes growth retardation, hepatosplenomegaly, infiltrative pulmonary disease, pancytopenia, skeletal disease, hypercholesterolemia, and lymphadenopathy. Intermediate phenotypes between NPA and NPB are

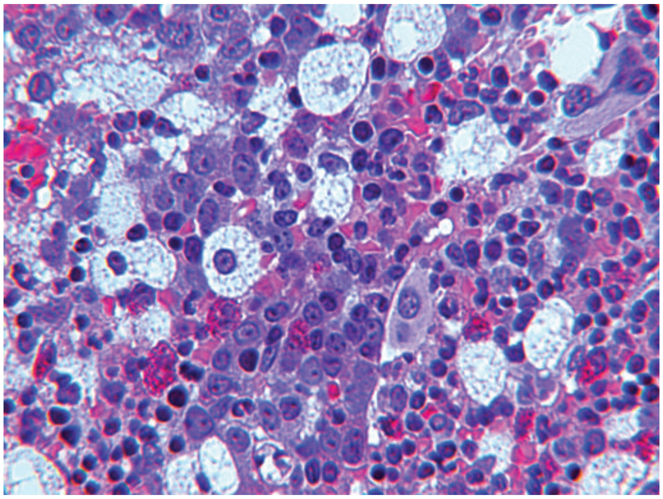

Figure 25-5 Niemann-Pick B bone marrow cells. Light micrograph of Niemann-Pick B bone marrow with characteristic lipid-laden vacuoles, which impart a foamy appearance to cells of macrophage/monocyte origin, "Niemann-Pick cells." These cells may stain a bluish color with Giemsa and are occasionally referred to as *sea-blue histiocytes*. *(Courtesy Dr. Kevin Bove.)*

frequently described. As with many LSDs, ASM deficiencies represent a disease continuum.

The incidence of the various forms of the disease varies among different populations. Based on long-term experience in Australia, the estimated combined frequencies of NPA and NPB are approximately 1 in 248,000.[32] NPA is more prevalent in the Ashkenazi Jewish population, in which approximately 1 in 80 persons are heterozygotes.[70] NPB is panethnic, with the highest incidence in individuals of Middle Eastern and North African descent. Emerging data suggest greater frequencies in Brazil, but better ascertainment is needed.

NPA presents somewhat uniformly and includes severe progressive neurovisceral disease within the first few months of life. Neonatal jaundice, hepatomegaly (usually in the absence of liver dysfunction), splenomegaly, and failure to thrive are present early. Moderate lymphadenopathy and infiltrative pulmonary disease are frequently seen. Moderate microcytic anemia, which may respond to iron supplementation, and thrombocytopenia develop progressively. During the subsequent months neurodevelopmental arrest is followed by regression, hypotonia, muscle weakness, and ultimately spasticity and rigidity as terminal stages. Ophthalmologic examination may reveal cherry-red maculae in approximately 50% of subjects. Death usually occurs by 2 to 3 years of age.[71]

NPB is a heterogeneous disease with manifestations generally restricted to the visceral organs. Most patients are diagnosed in childhood, often after hepatosplenomegaly is found on routine examination.[70] Hepatosplenomegaly is most prevalent during childhood and less so in adults. The mean liver and spleen volumes are 1.9±0.7 MN [range: 0.7-3.9 MN (MN=calculated multiples of normal)] and 11.1±5.7 MN (range 3.1-27.3 MN), respectively. About 71% of individuals had liver volumes greater than 1.5 MN, whereas 84% of individuals had spleen volumes greater than 5 MN.[72] Splenectomy has been performed in the past, particularly in those with a history of bleeding and thrombocytopenia. The removed spleens are bright orange as a result of the sphingomyelin accumulation. Splenectomy is generally discouraged because of concerns that disease severity may worsen in other organs, such as the lung, and the risk of infection related to splenectomy.

Atherogenic serum cholesterol and lipoprotein profiles include low high-density lipoprotein (HDL), and elevated total cholesterol, triglycerides, low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) levels. These are present from early childhood. Additionally, easy bruising and bleeding, infiltrative pulmonary disease, and joint or limb pain (or both) are common. Some individuals have significant bleeding (i.e., epistaxis, postsurgical bleeding) and may require medical intervention, including surgical procedures and blood transfusions. Thrombocytopenia is the most common hematologic finding, followed by anemia and leukopenia. These hematologic abnormalities are generally mild to moderate. In a cross-sectional study of patients with NPB, mean hemoglobin and hematocrit concentrations were 13.3 g/L (SD 1.5; range 9.3-16.5) and 39.1% (SD 4.5; range 27.8-48.3), respectively, whereas mean white blood cell (WBC) and platelet counts were 6.4 × 109/L (SD 2.7; range 2.1-16.2) and 158 × 109/L (SD 82; range 59-459).[72] Osteoporosis, expansion and widening of the medullary cavities, and remodeling defects can be demonstrated radiologically. Survival into adulthood is common, as is development of liver cirrhosis and fibrosis and pulmonary dysfunction.

Although NPB is not generally associated with primary neurologic involvement, a subset of such patients may have exhibit retinal abnormalities, including cherry-red maculae or a gray, granular pigmentation around the fovea. Cognitive impairment has been found with or without other neurologic findings.[72] These findings highlight the continuum of Niemann-Pick disease variants, rather than discrete subtypes.

Niemann-Pick cells, a primary histologic finding in ASM deficiencies, are approximately 25 to 75 μm in diameter. The cytoplasm is filled with lipid vacuoles, imparting a foamy appearance to the cells (Fig. 25-5).[69] The vacuoles stain positive with Sudan black B and oil red O but poorly with PAS. Within the bone marrow the intracellular material in Niemann-Pick cells may have a bluish color on Giemsa staining and are occasionally referred to as *sea-blue histiocytes*. Sphingomyelin also accumulates in hepatocytes, with inflammation and fibrosis that can lead to cirrhosis (Fig. 25-6). Alveolar histocyte-like cells filled with sphingomyelin accumulate in the lung.

ASM deficiency is suspected on the basis of the clinical features and identification of Niemann-Pick cells on histologic examination of tissues. The diagnosis is confirmed by deficient ASM activity in any nucleated cell sample or identification of pathogenic mutations in both copies of *SMPD1*. Of note, the type and severity of disease correlate poorly with the amount of in vitro residual enzyme activity.

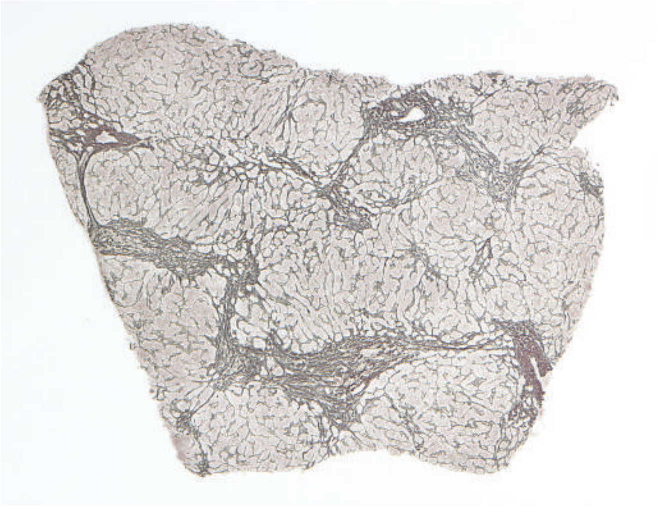

Figure 25-6 Niemann-Pick B liver. Light microscopy of a Niemann-Pick B affected liver stained for reticulin shows extensive bands of fibrous tissue, bridging fibrosis, and distortion of the normal hepatic architecture (20× magnification). *(Courtesy of Dr. Beth Thurberg.)*

No specific treatments for NPA or NPB are currently approved. Treatment is supportive. HSCT has been performed in patients with NPA and NPB. Despite engraftment, continued neurologic deterioration occurs in NPA. HSCT has been disappointing in NPB. Variable effects have been seen on the liver and lung manifestations. Two well-documented cases are instructive. Both showed improvement or resolution of pulmonary disease but no correction of hepatocellular disease despite resolution of Kupffer cell involvement. Indeed, both patients progressed to cirrhosis.[70,73-76] A phase I clinical trial evaluating the safety of ERT in NPB adults has been completed, and a phase II trial is currently being conducted.

Niemann-Pick Disease Type C

Niemann-Pick disease type C (NPC) is a clinically heterogeneous progressive neurovisceral disease resulting from mutations of *NPC1* (~95% of cases) or *NPC2* (~5% of cases) and is inherited in an autosomal recessive manner.[77] Although the major phenotypic manifestations are common to both NPC1 and NPC2, severe pulmonary disease is a unique complication and a significant source of morbidity in patients with *NPC2* mutations. NPC was originally thought to be part of the NPA and NPB family, but the identification of the molecular lesions and the role of NPC proteins in cholesterol metabolism revealed a different underlying biology. However, the nosology is prevalent in the literature and has been retained.

NPC, which is panethnic with a frequency of approximately 1 in 120,000 to 1 in 150,000,[77,78] is characterized by defective lipid transport and lysosomal accumulation of lipids (unesterified cholesterol, phospholipids, sphingomyelin, and glycolipids) in affected cells. The gene products of *NPC1* and *NPC2* are lysosomal proteins that are critical to the egress of cholesterol from lysosomes for the modulation of cellular lipid metabolism by way of the SREBP system.[79] Although partial deficiencies of ASM activity have been found in NPC patient cells, this is not the primary defect.

NPC has three variants, based on the age of onset, severity of CNS disease, and survival: neonatal/infantile, childhood, and adolescent/adult.[80] As with many LSDs, overlap exists among the various clinical forms, thereby forming a continuum of overlapping disease phenotypes.

Progressive neurologic or psychiatric manifestations and visceral (liver, spleen, and potentially lung) involvement occur in any of the variants. It is important to note that visceral findings may occur before or after the development of neurologic symptoms, and in adults visceral findings may be minimal at diagnosis. The age of onset of systemic involvement does not correlate with neurologic involvement. The age of onset of neurologic disease correlates with disease severity and survival.[81]

The neonatal and infantile variant is a rapidly progressive neurovisceral disease with onset in the first few months of life and accounts for approximately 20% to 30% of cases.[82,83] The most severe cases may present perinatally with ascites or hydrops fetalis (Table 25-1).[78] Infantile cholestatic disease or idiopathic neonatal hepatitis often develops in early infancy and is a common cause of neonatal liver disease. In approximately 10% of cases, fulminant liver disease develops and can lead to death.[84] Survivors usually experience resolution of severe liver disease by 2 to 4 months of life; however, hepatosplenomegaly often remains and becomes less conspicuous with age.[80] Failure to thrive and growth retardation are prominent. A subset of NPC2 individuals develops significant pulmonary disease, characterized by tachypnea, infiltrates on chest x-ray, and a presentation resembling alveolar proteinosis.

Those recovering from early liver disease experience progressive neurodegenerative and exhibit hypotonia, developmental delay, regression, spasticity, dystonia, dysphagia, seizures, and pyramidal signs.[77,80,82,85] Vertical supranuclear gaze palsy is a classic finding for NPC variants. Death usually occurs by 5 years.[81]

Childhood-onset NPC is the most common variant, accounting for approximately 50% to 70% of cases. These children initially appear healthy, with normal development. By early to late childhood, they exhibit neurologic deterioration manifested initially as clumsiness and gait abnormalities, behavioral problems, poor academic performance, and supranuclear gaze palsy. Gradual progression of neurodegenerative signs occurs, including psychiatric disturbances, ataxia, dystonia, seizures, cataplexy, dysarthria, and dysphagia. Individuals eventually become incapacitated. Death usually occurs in the second or third decade of life, usually from pneumonia.[80] Significant liver dysfunction is unusual in this variant.

The adolescent- or adult-onset variant accounts for approximately 5% to 10% of cases.[82,83] This variant manifests primarily as a psychiatric or neurocognitive degenerative disease in which significant visceral involvement is unusual, although hepatosplenomegaly may occur.[77] Hematologic abnormalities are not commonly present. Vertical supranuclear gaze palsy may be subtle and difficult to recognize.

TABLE 25-1 Fetal Hydrops Associated with Lysosomal Storage Diseases

Diagnosis	Fetal Features	Neonatal Findings	Pathology	Diagnostic Confirmation
Morquio IV A	Ascites, pleural effusion	May resolve by term		Reduced *N*-acetylgalactosamine-6-sulfatase activity
Mucopolysaccharidosis VII	Nuchal thickening, ascites	Coarse features	Vacuoles in lymphocytes and placenta	Reduced β-glucuronidase activity
Gaucher	Hydrops, hepatomegaly, fetal hypokinesia	Collodion-like skin, contractures, hepatosplenomegaly	Gaucher cells	Reduced acid β-glucosidase activity
Niemann-Pick A	Nuchal thickening, hydrops	Hepatomegaly, prolonged jaundice, poor feeding	Foam cells	Reduced sphingomyelinase activity
Niemann-Pick C	ascites	Prolonged jaundice, pulmonary infiltrates	Foam cells	Abnormal filipin staining, molecular testing for *NPC1* and *NPC2*
GM_1 gangliosidosis	Intrauterine growth retardation, oligohydramnios	Hypertonia, periorbital edema, cherry red spots	Placental vacuolization	Decreased β-galactosidase, normal neuraminidase activities
Galactosialidosis	Hydrops	Edema, coarse facies, hepatosplenomegaly, telangiectasias	Vacuoles in circulating cells, Kupffer cells, other organs	Decrease in *both* β-galactosidase and neuraminidase activities
Infantile sialic acid storage disease	Ascites, IUGR, nuchal thickening	Hepatomegaly, coarse facies, hypotonia, stippled epiphyses	Placental vacuolization	Increased free sialic acid in urine, amniocytes, fibroblasts
Mucolipidosis II	Short femurs	Gingival hyperplasia, coarse facies, periorbital puffiness, hepatosplenomegaly	Vacuoles in lymphocytes, not present in bone marrow	Increase in various lysosomal enzymes in plasma

Histologic analysis of bone marrow reveals characteristic foam cells containing accumulated free cholesterol, as evidenced by filipin positivity, and sphingolipids (Fig. 25-7).[77,81] Sea-blue histiocytes may be present.[81] These cells are also present in other visceral organs (hepatocytes in the liver) and CNS (neurons and glial cells). Ultrastructurally, the storage material consists of pleomorphic membrane-bound lipid inclusions with structures varying from crystalline to electron-dense laminated inclusions.[84,86,87]

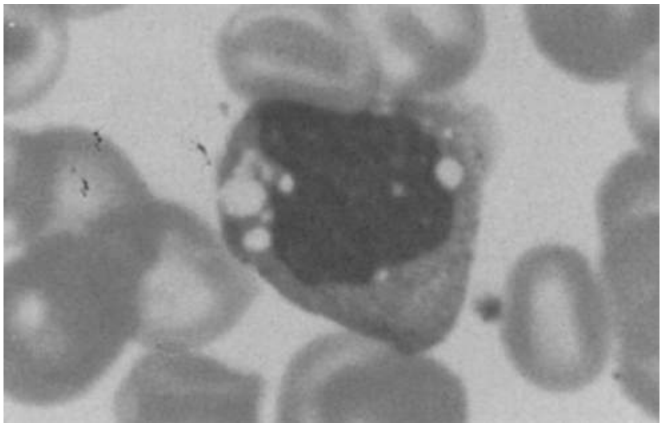

Figure 25-7 Niemann-Pick C peripheral blood leukocyte. Characteristic lipid laden "foam cell" in peripheral blood of a patient with Niemann-Pick disease type C. *(Courtesy J. Alroy, Department of Pathology, Tufts New England Medical Center.)*

Because of the significant variability of phenotypes, NPC is not always initially considered in the differential diagnosis. Indeed, the diagnosis may be delayed as a result of the absence of hepatosplenomegaly or storage cells in the bone marrow. A biochemical diagnosis is established by demonstration of impaired cholesterol esterification and positive filipin staining in cultured fibroblasts. ASM activity is normal to low. Molecular studies can provide a definitive diagnosis; Many *NPC1* or *NPC2* mutations are confined to single families.

Treatment of NPC is supportive. Bone marrow transplantation may improve visceral disease but is ineffective for CNS disease.[88] As the NPC1 protein is an integral lysosomal membrane protein with multiple transmembrane domains, it is not secreted and therefore cannot cross-correct neighboring cells. For similar reasons, liver transplantation does not benefit patients with CNS disease.

A number of pharmacologic agents have been evaluated as potential therapies for NPC, including cholesterol-lowering medications (statins), sterol-binding agents, and glucocorticoids, all without success. The safety and efficacy of miglustat (Zavesca®, Actelion Pharmaceuticals) has been evaluated in NPC1 and may stabilize oculomotor manifestations, dysphagia, and ambulation deficits. However, the effects on visceral disease manifestations in NPC were not significant.[89-92] Side effects were similar to those discussed previously and included diarrhea, flatulence, and abdominal pain. The European Medicines Agency (EMA) approved Miglustat in 2009 for the treatment of CNS manifestations in NPC. It is not approved by the FDA.

Cyclodextrin, a cholesterol-binding excipient, is being explored as a therapy for NPC. This agent is theorized to reverse the transport defect in the late endosomal or lysosomal complex, allowing sequestered free cholesterol to be transported out of the lysosome and metabolized by other cellular mechanisms.[93] Based on encouraging preclinical studies in the *Npc1-/-* mouse model, clinical trials in humans have begun.[94]

Farber Disease (Lipogranulomatosis)

Farber disease is a very rare progressive autosomal recessive disease resulting from deficient activity of acid ceramidase, which is encoded by *ASAH1*. The frequency is estimated to be less than 1 in 1,000,000.[94] Seven subtypes of Farber disease are recognized; these vary in severity and survival. Types 6 and 7 are not caused by mutations in *ASAH1* and will not be discussed here.

Classic Farber disease, the most common variant, is characterized by a triad of progressive clinical manifestations: painful and progressive deformation of joints; subcutaneous nodules, particularly near the joints and over pressure points; and progressive hoarseness resulting from laryngeal nodules. Often these findings are present within the first few weeks to months. Progressive respiratory and swallowing difficulties result in poor weight gain and impaired acquisition of development milestones; this can necessitate palliative management (i.e., tracheostomy and gastrostomy tube placement). Granulomatous lesions may also occur in the liver, spleen, lung, heart, reticuloendothelial system, bone, and CNS and contribute to the morbid manifestations. Hepatomegaly may be identified in patients. Death usually occurs within the first years of life.[94]

Intermediate and attenuated variants demonstrate similar, albeit less severe, phenotypes. Clinical manifestations begin within the first 1 to 2 years; some individuals have survived into adulthood.[94]

The neonatal-visceral variant is characterized by hepatosplenomegaly first noted in the neonatal period. In the most severe cases, patients may present with nonimmune hydrops fetalis. Infants with this severe phenotype die in early infancy, often before the onset of the more characteristic clinical features of classic Farber disease.[94]

The neurologic form is defined by progressive neurodegeneration noted within the first two years, followed by death shortly thereafter. Visceral organ involvement, including subcutaneous nodules and joint involvement, may be present but does not predominate.[94]

The principal pathologic findings are granulomatous infiltrates composed of foam cells containing a PAS-positive material that is extractable with lipid solvents. Comma-shaped curvilinear tubular, membrane-bound structures (Farber bodies) (Fig. 25-8) within vacuoles are characteristic findings on electron microscopy.

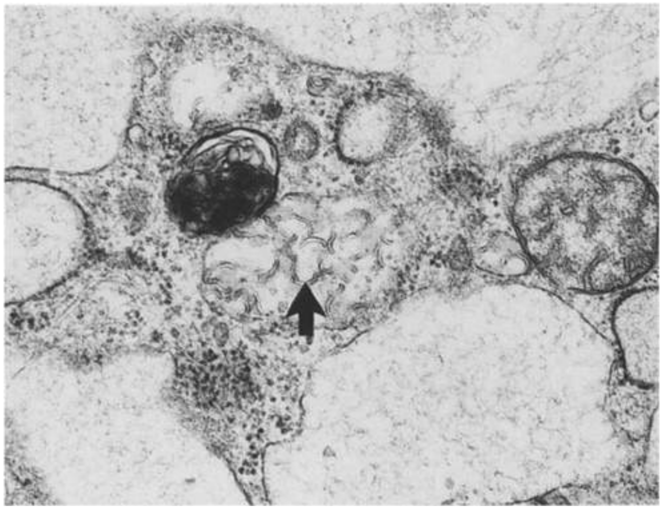

Figure 25-8 Farber cell body. Conjunctival biopsy showing a vacuole filled with tubulelike profiles of "curvilinear" bodies (Farber body). Uranyl acetate and lead citrate × 42,000. *(From Nowaczyk et al:* J Inherit Metab Dis *19:655–660, 1996.)*

The clinical features and presence of characteristic Farber bodies on histology suggest the diagnosis of Farber disease. Definitive diagnosis relies on identification of deficient acid ceramidase activity in leukocytes or cultured skin fibroblasts or by molecular analysis.

Treatment of Farber disease is supportive (i.e., tracheostomy or resection of oral granulomas for management of upper airway obstruction). HSCT in several individuals who had classic Farber disease with neurologic involvement resulted in improved visceral features, but neurologic deterioration continued, leading to death.[95,96] Several Farber disease patients without neurologic involvement have received HSCT and displayed improvement in visceral disease. These children were reported as alive and well without any evidence of neurologic deterioration.[97] Thus individuals without neurologic involvement may benefit from HSCT.

GM_1 Gangliosidosis

GM_1 gangliosidoses are heterogeneous, progressive neurovisceral disorders resulting from a deficiency of the enzyme acid β-galactosidase because of mutations in *GLB1*. There are several lysosomal β -galactosidases with different substrate specificities. In the GM_1 gangliosidoses, acid β-galatosidase deficiency leads to specific accumulation of GM_1ganglioside, asialo-GM1 ganglioside, oligosaccharides, and keratin sulfate in neurologic and visceral tissues.[98]

The GM_1 gangliosidoses are allelic to Morquio type B disease, a nonneurologic mucopolysaccharidosis primarily expressed as a skeletal dysplasia with a paucity of other visceral organ involvement. GM1 gangliosidosis has a phenotype similar to that of galactosialidosis, because acid β-galactosidase exists in multienzyme complex with N-acetylgalactosamine-6-sulfatase, neuraminidase, and protective protein cathepsin A (PPCA), and the defects in PPCA lead to deficiencies of all three enzymes.[98]

GM_1 gangiosidosis presents in three forms depending on the age of onset, severity of neurologic disease, presence or absence of visceral disease, and survival: early infantile onset (type 1), late infantile onset (type 2), and adult onset (type 3).[98] The conditions are panethnic but appear to be more common in certain populations, including those in Brazil and the Maltese Islands, and among Romani and Cypriot people.[77] The prevalence was estimated at approximately 1 in 384,000.[32]

Early infantile onset (type 1) GM_1 gangiosidosis, the most severe variant, usually manifests in the first 6 months after birth. In rare cases intrauterine growth retardation and oligohydramnios have been present. The most severe variant presents as fetal hydrops. More frequently, children exhibit feeding problems, failure to thrive, splenomegaly, hepatomegaly (with or without evidence of hepatic dysfunction), and coarsening of facial features. Generalized skeletal dysplasia may be present as joint stiffness. Developmental arrest occurs during the first few months, followed by progressive neurodegeneration. Cherry-red maculae are commonly observed and are an important clue to this diagnosis. Death usually occurs by 3 years of age, frequently as a result of pulmonary infections.[98]

Late infantile or juvenile onset (type 2) GM_1 gangliosidosis manifests from approximately 6 months to 3 years of age with developmental arrest, followed by progressive neurodegeneration. The patients may exhibit other visceral organ manifestations, including hepatosplenomegaly and skeletal involvement. Cherry-red maculae are variably present. Survival may extend into the second decade.[98]

Adult onset (type 3), or chronic, GM_1 gangliosidosis is a slowly progressive, heterogeneous CNS disease, with a variable age of onset from 3 to 30 years. Neurologic features frequently include gait disturbances, speech disturbances, dystonia, and Parkinsonism. Skeletal changes are less significant than in the more severe variants, and hepatosplenomegaly and cherry-red maculae may not be present.[98]

Blood cell counts are generally normal. The reticuloendothelial system contains foamy histiocytes filled with strongly PAS-positive granular material. Vacuolated lymphocytes can readily be recognized in peripheral blood and bone marrow, which may contain sea-blue histiocytes.[99] Additionally, lymphocytes and neutrophils may exhibit basophilic granules that stain mildly with toluidine blue.[98] Tissue macrophages with grossly distended cytoplasm filled with storage lysosomes can be identified in most organs, including the liver, kidney, spleen, lymph nodes, thymus, lung, intestine, pancreas, and skeletal muscle.[100]

GM_1 gangliosidosis is often first suspected clinically and by the detection of complex urinary oligosaccharides and glycosaminoglycans (i.e., keratin sulfate), but this is not diagnostic. Confirmation requires detection of deficient acid β-galactosidase activity in lymphocytes, fibroblasts, or plasma; however, in those with deficient acid β-galactosidase activity, subsequent neuraminidase assays are necessary to rule out galactosialidosis. Confirmation of the diagnosis may also be made by identification of a pathogenic mutation in each copy of *GLB1*.

Treatment of GM_1 gangliosidosis involves management of symptoms. HSCT was unsuccessful in stabilizing or preventing neurologic deterioration in a presymptomatic child with juvenile-onset disease, despite normalization of enzyme activity in WBCs.[101]

GM_2 Gangliosidosis, β-Hexosaminidase Deficiency Variants

The GM_2 gangliosidoses are heterogeneous neurodegenerative diseases, inherited in an autosomal recessive manner and characterized by impaired degradation of GM_2 ganglioside. This may result from deficient β-hexosaminidase activity or, more rarely, defects in the GM_2 activator protein, which is responsible for presenting the substrate to the enzyme. GM_2 ganglioside is synthesized mostly within the CNS, and consequently storage material accumulates in neuronal cells. Depending on the nature of the mutation and the biochemistry of the β-hexosaminidase enzymes, substrates can also accumulate in the visceral organs. The two isoforms of β-hexosaminidase include β-hexosaminidase A (Hex A), a heteromer consisting of α and β subunits, and β-hexosaminidase B (Hex B), a homomer of β subunits. Therefore defects in three genes, *HEXA* (encodes the α subunit), *HEXB* (encodes the β subunit), and *GM2A* (encodes the GM_2 activator) may result in impaired β-hexosaminidase activity.[102] The α-chain defects lead to isolated deficiency of Hex A and the CNS disease Tay-Sachs disease. The β-chain defects lead to deficiencies of Hex A and B, because they share the common β subunit, and the CNS and visceral disease Sandhoff disease. Hex B has non-GM_2 ganglioside substrates, oligosaccharides, and glycoproteins, which occur in the viscera, and their accumulation results in hepatomegaly and skeletal defects.

The resultant phenotypes vary in age of onset, severity of neurologic disease, presence or absence of visceral disease, and age of death.[102] Of the variants, only *Hex B* mutations are associated with visceral involvement. The reader is referred to other sources for further information regarding *Hex A* and *GM2A* deficiency disorders.[102]

Sandhoff disease is panethnic with a frequency of approximately 1 in 384,000.[32] Three variants are frequently identified, based on the age of onset, severity of disease, and survival: infantile (classic), juvenile, and adult/late onset.

Infantile onset (classic) Sandhoff disease is virtually identical to classic Tay-Sachs disease with regard to neurologic disease. Although individuals often appear normal at birth, by approximately 3 to 5 months hypotonia and exaggerated startle responses are noted. Soon afterward, a plateau in acquisition of milestones, followed by regression of development, is noted. Decreased visual attentiveness and unusual eye movements occur, and cherry-red maculae are noted on ophthalmoscopy in almost all affected individuals. After the age of 8 to 10 months, progression of the disease is rapid. Spontaneous or purposeful voluntary movements diminish, and infants becomes less responsive to their environment. Vision deteriorates rapidly, although light and dark discrimination may remain. Although not typically present at disease onset, seizures commonly develop by the age of 1 year. Progressive enlargement of the head typically begins by 18 months of age as a result of reactive cerebral gliosis and substrate deposition. In the second year decerebrate posturing, difficulties in swallowing, and increased seizures ultimately cause the affected individual to enter an unresponsive vegetative state. Death usually occurs by the age of 2 to 4 years, typically subsequent to pneumonia.[102] Storage materials (e.g., oligosaccharides and glycosaminoglycans) accumulate in the visceral organs (i.e., spleen, liver, kidneys, bone marrow, lymph nodes) and occasionally result in organomegaly; however, evidence of visceral organ dysfunction, including hematologic abnormalities, is unusual.[102]

Later onset disease variants (late infantile or juvenile onset and chronic or adult onset variants) present from early childhood to adulthood. The features are exceptionally variable among affected individuals but are typically milder and demonstrate slower rates of progression than those seen with the classic (early infantile onset) variant. These features may include muscle weakness, speech and swallowing problems, abnormal movements, gait disturbances, and seizures. Cherry-red maculae may be observed but are less common than in the classic (early infantile onset) variant.

On histologic examination, strongly PAS-positive storage material is observed in Kupffer cells in the liver, macrophages in the spleen, lymph nodes and lung, and renal tubular epithelium. These storage cells appear vacuolated in routine hematoxylin and eosin-stained paraffin sections. The storage material in these visceral organs is composed of heterogeneous lamellar structures, some of which resemble membranous cytoplasmic bodies.[102]

Sandhoff disease variants are often first suspected on the basis of the clinical features and can be distinguished from other forms of GM_2 gangliosidosis by the presence of visceral involvement, including foam cells in bone marrow. Identification of deficient total (A and B) β-hexosaminidase activity and presence of a higher than normal percentage (>70%) of the Hex-S (α-chain trimers) are strongly suggestive of the diagnosis. Confirmation is made by identification of pathogenic mutations in both copies of *HEXB*.

Currently, the treatment for hexosaminidase B deficiency is palliative. HSCT has not reversed or stabilized neurologic involvement. Miglustat treatment of affected individuals has not forestalled neurologic deterioration.[103,104]

The Mucopolysaccharidoses

The mucopolysaccharidoses are a group of inherited disorders that result from deficiency of one or more of the lysosomal enzymes required for glycosaminoglycan (GAG) or mucopolysaccharide catabolism. GAGs are long-chain complex carbohydrates that are usually linked to protein and are major constituents of connective tissue, such as cartilage and bone. Deficiencies in the specific lysosomal hydrolases needed for breakdown of chondroitin 4-sulfate, chondroitin 6-sulfate, heparan sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid result in the accumulation of incompletely degraded GAGs within the lysosome of specific cells, resulting in alterations in cell morphology and function.

Mucopolysaccharidosis Type I (Hurler Syndrome and Attenuated Mucopolysaccharidosis I)

Mucopolysaccharidosis type I (MPS I) has been divided classically into three phenotypic categories, all of which are due to deficiency in α-L-iduronidase, a soluble M6P containing enzyme. The most severe type, MPS I H, or Hurler syndrome, manifests with clinical findings during the first year but may not be recognized until the second year. Infants with MPS I H are often large for their age and may not have obvious developmental deficits until it becomes evident that language acquisition is delayed. The coarse facial features evolve over time, and review of serial photographs can be helpful in recognizing the changing phenotype. One of the earliest symptoms may be constant noisy breathing, and it is not unusual for families to complain that their child always has a cold. Lumbar prominence, termed *gibbus,* may be one of the earliest specific features. Hernias are common, and some children are recognized because of the failure of the initial surgical repair.[105,106] Increasing head circumference, sometimes the result of a communicating hydrocephalus, develops. Corneal opacities may be present during the first year but may be difficult to see with a handheld ophthalmoscope. The skeletal abnormalities that are collectively referred to as *dysostosis multiplex* include oar-shaped ribs, anterior beaking of the vertebral bodies, lumbar kyphosis, and shortening of the metacarpals and phalanges. Contractures at the hips, knees, and ankles may not be recognized until the child is standing, and it is not unusual to note limitation in shoulder movement. Evolution of symptoms during the first year is usually associated with the severe form of MPS I, which includes progressive cognitive decline.[107] The differentiation from milder forms of MPS I is crucial because of the distinct treatment options (discussed later).

The attenuated forms of MPS I, in which cognitive development is intact, have been divided historically between Scheie syndrome (onset in late childhood or adolescence) and Hurler-Scheie syndrome (onset between ages 3 and 8 years). These attenuated forms represent a continuum of severities, and referring to both as "attenuated" MPS I has become standard. It is important to remember that from the patient's point of view none of the MPS I forms are "mild." Individuals with later onset of clinical manifestations may present (1) to the orthopedist with joint stiffness, contracture, clawhand deformity, or carpal tunnel syndrome; (2) to the ophthalmologist with corneal clouding and glaucoma; (3) to the cardiologist with valve dysplasia; or (4) to the otolaryngologist with hearing loss or obstructed breathing.

Initial biochemical assessment usually involves quantitation and characterization of urine GAGs, which should reveal significant elevation (often 10- to 20-fold) of total GAG, predominantly heparan and dermatan sulfates. Iduronidase can be measured in both circulating WBCs and cultured skin fibroblasts. A pseudodeficiency state has been described,[108] which can confuse the assessment of phenotypically normal family members and interfere with prenatal diagnosis using enzyme assay. Sequencing of *IDUA* is available and provides important information for counseling of family members as well as supporting information for the prediction of severity.[109] Prenatal diagnosis is possible and should involve molecular as well as enzyme analysis. Since early diagnosis and treatment have improved the prognosis, there is interest in population based newborn screening. Pilot programs for the detection of MPS I and other treatable LSDs are beginning in several locations.

Treatment. Cross-correction of lysosomal storage by application of the appropriate enzyme was demonstrated in vitro by Neufeld and coworkers.[110] This property has been translated into treatment for MPS I using either cell-based (HSCT) or ERT therapies. HSCT from an unaffected donor has been possible for more than 20 years. Modern marrow-donor programs and transplant techniques are improving access to transplant and are fostering durable engraftment for a much larger fraction of the eligible patients.[111,112] A combined approach, using ERT during the early phase of diagnosis and preparation for HSCT, is practical, safe, and without adverse effects on graft survival.[113,114] Neurocognitive status is expected to decline for a few months after HSCT and then stabilize. For this reason earlier diagnosis and expedited referral for HSCT are important in maximizing eventual neurocognitive outcome.[115,116] The long-term benefits for children transplanted before 2 years of age include softening of coarse features, improvement in airway manifestations, and resolution of hepatosplenomegaly. However, skeletal dysplasia persists and may progress. Short stature[117,118] and the need for multiple orthopedic procedures are anticipated in transplanted individuals.[119,120,121] Odontoid dysplasia may improve after HSCT.[122] A role for additional ERT in well-engrafted individuals has not been defined.

Laronidase, recombinant α-L-iduronidase, was FDA approved in 2003 for the non-CNS manifestations of MPS I. Treated individuals, who require intravenous infusions weekly or biweekly, exhibit improvement in hepatosplenomegaly, joint range of motion, exercise tolerance, and biochemical markers (e.g., GAG excretion).[123] Intrathecal enzyme therapy has been used to a limited extent for spinal complications. A trial of intrathecal enzyme during the peritransplant period is ongoing. Although intravenous ERT may have a palliative role for individuals with MPS I H, who are not candidates for HSCT, early HSCT is superior.

Gene therapy efforts using adeno-associated virus (AAV) vectors transducing human cDNA for *IDUA* have shown restoration of enzyme activity in brain, but no human gene therapy trials have been reported.[124]

Small molecule approaches to restoration of endogenous activity have shown that GAG storage can be reduced in mice carrying a W392X mutation[57] on treatment with NB84, an aminoglycoside designed to suppress translation termination at in-frame premature termination codons. In human fibroblasts in vitro, chloramphenicol, a peptidyl transferase inhibitor, has been shown to increase α-L-iduronidase activity by 100-fold, but the mechanism appears to be different from stop codon readthrough.[125] There are currently no human trials of either of these approaches.

Mucopolysaccharidosis Type II (Hunter Syndrome)

Hunter syndrome is an X-linked recessive disorder resulting from a deficiency of iduronate-2-sulfatase. Boys with the severe variant may resemble children with the more severe variants of MPS I, although they typically present somewhat later. Many boys with Hunter disease are tall during infancy with coarse facial features, progressive evolution of developmental delay and eventual regression of milestones, stiff joints, clawhand deformity, hearing loss, and hepatosplenomegaly. Corneal clouding is absent. Behavioral difficulties, including aggression and disrupted sleep patterns, can be challenging. Diarrhea is a frequent complaint and can be difficult to manage. A specific skin feature is "peau d'orange," a white pebbly lesion often located on the back or upper arms. Boys with the "milder" form, who do not have cognitive involvement, may present with similar somatic features and progressively develop joint stiffness, carpal tunnel syndrome, and cardiac valve disease. The less severe form is compatible with survival into adulthood and with normal cognitive achievement. However, it is by no means clinically "mild."

The diagnosis may be first suspected on the basis of family history, clinical features, or abnormal radiologic findings. Quantitation and characterization of urine GAG excretion may be similar to findings in MPS I, and biochemical features do not predict clinical severity. Iduronate-2-sulfatase is present in plasma but can also be assayed in WBCs and cultured skin cells. Molecular confirmation is readily available, and hundreds of mutations of all types have been described. When clinical and biochemical evidence supports a diagnosis but sequencing fails to reveal a mutation, a deletion, which can involve contiguous genes, should be sought. In this case, an alternate diagnosis, such as multiple sulfatase (discussed later) deficiency, should be considered. Hunter syndrome is X-linked, and knowledge of the molecular lesion is important for genetic counseling in females at risk. Prenatal diagnosis is possible, and molecular analysis should be used as an adjunct to assays.

Treatment. HSCT has been disappointing in MPS II, although the reason for the difference in cognitive response to HSCT (as compared to MPS I) is unclear.[126] ERT with idursulfase was approved in 2006 to treat the non-CNS manifestations of MPS II, based on clinical endpoints of improved 6-minute walk and improved respiratory status.[127] The therapy involves weekly intravenous infusions and requires a significant family commitment to therapy. Regular infusions have been transferred to the home setting after demonstration of safety during the first several months.[128] Idursulfase does not enter the CNS, and ERT is not expected to have a significant impact on cognitive decline in severely affected boys. A clinical trial using the combination of intravenous and intrathecal enzyme is in progress. Gene therapy using AAV vectors has been studied in MPS II mice, with encouraging clearance of GAG is tissues and correction of skeletal abnormalities.[129,130] No human gene therapy trials are in progress.

Mucopolysaccharidosis Type III (Sanfilippo Syndrome)

MPS III has predominantly CNS manifestations and can be very difficult to diagnose during the toddler years. Before age 5 the mildly coarse features can be subtle, and the striking physical findings that make MPS I and II recognizable are absent (Fig. 25-9). There are four different enzymes implicated in this clinical phenotype, all of which lead to storage and urinary excretion of excessive heparan sulfate. In addition, glycosphingolipids accumulate in MPS III CNS. Phenotypic features include delayed language acquisition, hyperactivity, aggressive behavior, sleep disorders, and hirsutism. Loss of established milestones becomes evident at approximately 4 to 6 years. In general, individuals with MPS III A exhibit more rapid decline, and individuals with MPS III B have a less rapid course. The frequency of MPS III is approximately 1 in 100,000 in Western Europe, based on incidence of known cases, but many authorities suspect that MPS III may be significantly underdiagnosed.[131]

MPS III can be difficult to diagnose biochemically. Urine GAGs may be only mildly abnormal when analyzed at the quantitative level, and detection of the abnormal heparan sulfate requires characterization by electrophoresis or thin layer chromatography.[132] Only one of the enzymes, MPS III B (α-*N*-acetylglucosaminidase) can be assessed in circulating cells; the others require a skin fibroblast culture. Molecular analysis is available for all four variants and is required for accurate carrier assessment. Prenatal diagnosis is available using enzymatic analysis of cultured cells, augmented with molecular analysis.

Treatment. HSCT has been explored but does not appear to be effective, as murine models suggested.[133] Because the primary target for therapy for MPS III is the CNS, enzyme therapy trials have focused on intrathecal administration, and clinical trials are under way for MPS IIIA. Gene therapy has been attempted in MPS III B mice.[134] Genistein, which is a soy derivative widely available as a nutritional supplement, has provoked significant interest among MPS III families. Genistein decreased heparan sulfate in mouse brains[135] and decreased GAG excretion in human trials, but without a change in behavior.[136] Finally, some improvement with aspirin administration suggests that antiinflammatory drugs may play a role in altering the progressive CNS lesions of MPS III.[137]

Mucopolysaccharidosis Type IV (Morquio Syndrome)

MPS IV, a lysosomal disorder, is primarily a skeletal dysplasia without CNS disease.[138] Two different enzymes are implicated in MPS IV: MPS IV A is a deficiency of *N*-acetylgalactosemia-6-sulfatase, whereas MPS IV B is caused by deficiency of acid β-galactosidase. In both variants the degradation of keratan sulfate is impaired, and excessive keratan sulfate can be observed in urine. There is a continuum of severities, based on the age at which skeletal deformities appear. MPS IVA may present with hydrops, but hydrops and less severe presentations are found in the same family. Children with MPS IV have a short neck and trunk, prominent sternum, and kyphosis. They tend to waddle and are distinct from other individuals with MPS in that they have hypermobile joints rather than contractures. Valvular heart disease and restrictive pulmonary disease develop with age. Cognitive development is normal.

MPS IVB is biochemically interesting because it is allelic to GM_1 gangliosidosis. Some mutations affect the catabolism of both GM_1 ganglioside and keratan sulfate, resulting in a phenotype overlapping that of MPS IV B and GM_1 gangliosidosis, encompassing both neurologic and skeletal manifestations. Other mutations lead to phenocopies of MPS IVA.

Treatment. *N*-acetylgalactosamine-6-sulfatase, the MPS IV A enzyme, has been produced in therapeutic quantities in Chinese hamster ovary cells. Systemic administration to MPS IVA mice showed uptake and GAG reduction in chondrocytes.[139] Initial clinical trials in humans have shown improvement in 6-minute walk test and some pulmonary functions. A phase III trial is under way.

Mucopolysaccharidosis Type VI (Maroteaux-Lamy Syndrome)

MPS VI is an early onset pleiotropic disorder involving joint stiffness and contractures, short stature, coarse

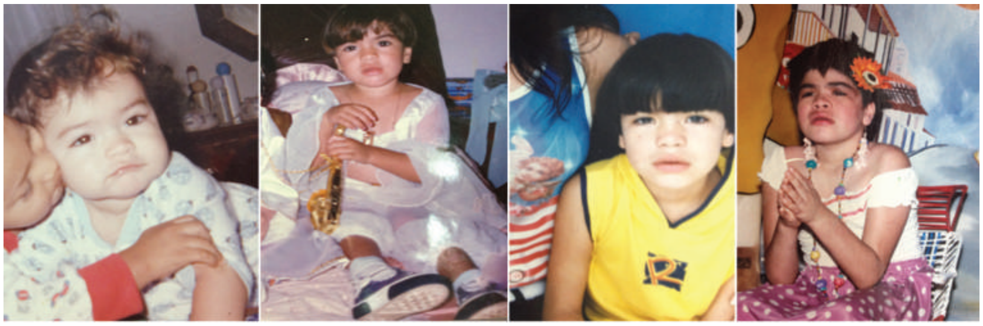

Figure 25-9 Sanfilippo disease progression of the facial phenotype. The mild coarseness of the facial dysmorphia can obscure the diagnosis early in the disease. The facial features become progressively more coarse but are still milder than in mucopolysaccharidosis I or II. *mo,* Months old; *yo,* years old.

facies, corneal clouding, and hearing loss. MPS VI can be confused with MPS I H and its attenuated variants. However, the CNS is not directly involved, and cognitive status is preserved, although some individuals require shunting because of hydrocephalus. Cardiac valve disease is common. Thickening of the dura may result in nerve impingement and myelopathy.[140] There is a spectrum of severities, and more mildly affected individuals may be underdiagnosed. MPS VI appears to be more common, albeit very rare, in South America, but overall prevalence is not known with accuracy.

Arylsulfatase B (ASB, galactosamine-4-sulfatase) is deficient in MPS VI and leads to an accumulation and excessive excretion of dermatan sulfate only, which provides a clue to the enzyme diagnosis. Measurement of ASB should be supplemented by assays of at least one other sulfatase enzyme to exclude multiple sulfatase deficiency, or molecular confirmation should be performed.[141]

Proof of the principle that MPS VI could be treated by cell transfer of enzyme was provided by earlier descriptions of improved joint mobility after HSCT.[142] ERT with galsulfatase was approved in 2006 by the FDA and EMA after clinical trials showed improved joint mobility and improved quality of life.[143]

Mucopolysaccharidosis VII (Sly Syndrome)

MPS VII, or β-glucuronidase deficiency, was initially described in 1973. Although the original patient had a rather stereotypical MPS course, with cognitive decline, organomegaly, short stature, and valvular heart disease, fetal presentation with hydrops may be the most frequent clinical variant.[144] This disorder has been studied extensively in murine models, and treatment efforts with enzyme therapy, HSCT, and gene therapy have showed variable outcomes.[145] ERT for MPS VII has been granted orphan drug status in the United States, and clinical trials are anticipated in this very rare disease.

Multiple Sulfatase Deficiency

Multiple sulfatase deficiency results from the failure of posttranslational conversion of a cysteine to 2-amino-3-oxopropionic acid (α–formylglycine), a modification required for activation of all members of the sulfatase family, which include nonlysosomal enzymes such as steroid sulfatase. The mutated gene, *SUMF-1*, encodes sulfatase-modifying factor 1. The clinical phenotype combines somatic features of all the MPS diseases owing to sulfatase deficiencies (coarse features, stiff joints, hearing loss, hepatomegaly) with those of metachromatic leukodystrophy (deficient arylsulfatase A). Ichthyosis caused by steroid sulfatase deficiency may be present. As with many lysosomal disorders, there is a spectrum of severities. Correct identification of multiple sulfatase deficiency is important to avoid inappropriate therapy with a single sulfatase replacement enzyme (idursulfase or galsulfase).[141] Development of an appropriate treatment strategy is challenging because many of the sulfatases are active in brain, are nonlysosomal, and have poorly defined functions.

Mucolipidosis Type II/ III (I-Cell Disease and Pseudo-Hurler Polydystrophy)

Mucolipidosis types II and III are allelic disorders in which internalization of lysosomal hydrolases by way of the M6P recognition marker is defective. The deficient enzyme, UDP-N-acetylglucosamine: lysosomal–enzyme N-acetylglucoseamin-1-phosphotransferase (GNPT), is encoded by *GNPTAB*. The more severe form, I-cell disease, may present with hydrops fetalis, short femurs, and intrauterine growth retardation. Coarse features may be noted in the neonatal period, and gingival hyperplasia is prominent. Growth retardation and developmental delay are usually noted earlier than in individuals with severe MPS I. The attenuated form, mucolipidosis type III, typically presents in early childhood with joint stiffness, contractures, and cardiac valve disease. Dysostosis multiplex may be noted on x-rays, but lack of excessive GAG excretion in urine provides a clue that the individual does not have MPS I, II, or VI. The assay of lysosomal enzymes in plasma show large increases in activity (e.g. 10- to 20-fold increases in β-hexosaminidases, iduronate sulfatase, and arylsulfatase A. Assays of such enzyme activities in the cell pellet of cultured cells or isolated WBCs show major deficiencies. In contrast, the plasma or cell culture media have high enzyme activities because the soluble lysosomal enzymes are secreted out of cells. Molecular confirmation through gene sequencing of *GNPTAB* is helpful for carrier determination and prenatal diagnosis. HSCT has been attempted in severe cases of I-cell disease, with some improvement in somatic features but no improvement in CNS status.[146]

Lysosomal Acid Lipase Deficiencies

Wolman disease and cholesteryl ester storage disease (CESD) are caused by defects of lysosomal acid lipase (LAL) and represent a continuum of disease. These conditions result from allelic mutations in *LIPA,* which maps to human chromosome 10q23.2–q23.3. The gene spans 45 kb and has 10 exons. LAL is essential to the degradation of cholesteryl esters and triglycerides that are delivered to the lysosome by LDL or other receptor-mediated endocytosis. LAL cleaves the fatty acids from these substrates to produce free cholesterol and fatty acids. Subsequent to LAL cleavage, cholesterol and free fatty acids exit the lysosome, enter the cytosol, and participate in the complex SREBP systems to modulate intracellular cholesterol and fatty acid synthesis.[147] In LAL deficiency, cholesteryl esters and triglycerides cannot be cleaved and their egress from the lysosome is blocked.[148] Cells sense this as cytoplasmic cholesterol and free fatty acid deficiencies with resultant upregulation of these biosynthetic pathways. LAL is ubiquitously expressed and is particularly abundant in macrophages, fibroblasts, lymphocytes, and hepatocytes. Although LAL is expressed in cells of the CNS, there seems to be no major role for this enzyme in cholesterol metabolism in the brain. A major difference between Wolman disease and CESD is the absence or presence, respectively, of small amounts of normal LAL in cells. A common *LIPA* splice junction mutation in

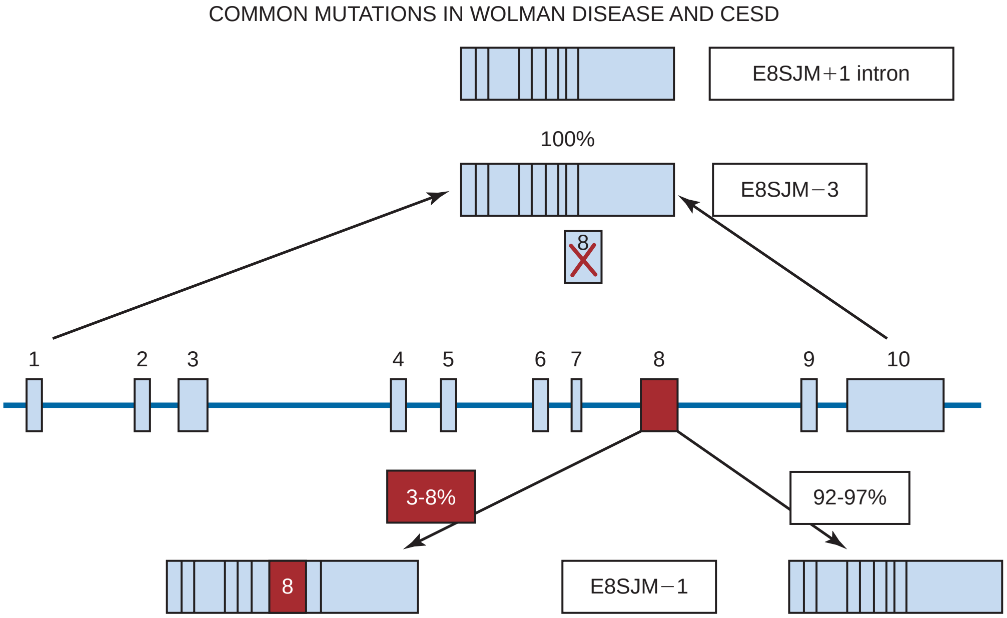

Figure 25-10 Lysosomal acid lipase (LAL) gene and splicing mutations. *LIPA* structure *(middle)* with 10 exons. Above *LIPA* are shown two different Wolman disease splice junction mutations that lead to the production of mutant LAL protein missing exon 8. Below *LIPA* is the common E8SJM-1 mutation in cholesteryl ester storage disease that leads to ~3% to 8% of normal LAL mRNA and protein being produced, whereas 92% to 97% of the LAL transcript is missing exon 8. Also, two nondeleterious mutations have been found in this same region.

CESD leads to production of about 3% to 8% of normal LAL mRNA and protein. This and the preferential cleavage of triglycerides, compared with cholesteryl esters, by LAL may account for the differences between Wolman disease and CESD (Fig. 25-10).

Wolman Disease

Wolman disease is a severe infantile disease with a mean age of death of approximately 3.5 months. It has a frequency of approximately 1 in 300,000 to 1 in 500,000 live births, but better ascertainment is needed.[149] The major clinical manifestations of severe vomiting, failure to thrive, abdominal distention, and massive hepatosplenomegaly have been reported in patients as young as 4 days of age.[150] Jaundice or persistent low-grade fever develops in a few infants. A prominent feature in about 60% of such patients is symmetrically enlarged, calcified adrenal glands.[151] Radiographic examination demonstrates extensive, stippled, or punctuate calcific deposits.

By the sixth week, progressive anemia usually develops. Peripheral blood lymphocytes contain both intracytoplasmic and intranuclear lipid vacuoles. In contrast to other diseases characterized by vacuolated cells, the vacuoles in Wolman disease are strongly sudanophilic and stain positively with oil red O, Nile red, and filipin for cholesterol. Their lysosomal origin is indicated by a strong reaction to staining for acid phosphatase activity. Lipid droplets may be present in neutrophils and monocytes. Foam cells (lipid-laden macrophages) are present in the bone marrow, the peripheral blood, and all organs.[150] Thrombocytopenia is not seen. Acanthocytosis has been reported.[152]

Vomiting, diarrhea, and inanition result from severe damage to the intestinal microvilli and the accumulation of foam cells in the intestinal mucosa. Abdominal ultrasonography may demonstrate thickened bowel walls.[153] Eventually, attempts to provide enteral nutrition may be futile, and intravenous hyperalimentation may be initiated as a palliative measure.[154] The disease progresses rapidly, and death usually occurs before 1 year.

The diagnosis is suspected by the clinical findings and deficient activity of LAL in cultured fibroblasts or circulating leukocytes. No specific blood chemical abnormalities are present in patients with Wolman disease. Hypercholesterolemia is not a feature, probably owing to malabsorption. Insufficient glucocorticoid response to adrenocorticotropic hormone (ACTH) may be observed; when present, this indicates the need for glucocorticoid supplementation.[155]

Cholesteryl Ester Storage Disease

CESD has a variable phenotype that ranges from severe progressive liver fibrosis and cirrhosis with hypercholesterolemia to a nonalcoholic fatty liver disease (NAFLD) phenotype that may remain undetected until adulthood.[156] Because of this variability the true prevalence of CESD is unknown. Based on DNA testing, approximately 1 in 40,000 and 1 in 70,000 are predicted to have CESD in Germany and Czech Republic, respectively. Among a selected group of patients with heart disease in the United States, molecular analyses indicated a prevalence of about 1 in 160,000 in Caucasians.[157] Greater suspicion in patients with NAFLD and hyperlipidemia may allow refinement of the true prevalence and phenotypic spectrum. Hepatomegaly, a prominent sign, may be evident at birth, during early childhood, or occasionally in the second decade of life. It results from the fatty infiltration of foam cells, progressive hepatocellular lipid accumulation, and ultimately hepatic fibrosis and cirrhosis. About 25% of children with CESD experience liver failure and have hepatic transplantation. Alcohol has been known to accelerate the process and therefore should be avoided. Other clinical features include recurrent abdominal pain, delayed puberty, recurrent epistaxis, jaundice, splenomegaly, esophageal varices, and decreased concentration of blood clotting factor V. Calcified adrenal glands are rarely seen. Malabsorption and malnutrition have not been described in patients with CESD. It is important to note that the CNS is not involved in CESD.

Plasma levels of cholesterol, triglyceride, and low-density lipoproteins are elevated, and the plasma high-density lipoprotein concentration is reduced. Isolated hypercholesterolemia may be the sole clinical finding in children and adults, and routine chemistry profiles may be completely normal. Lymphocytes and monocytes are the only blood cells affected in CESD. Bone marrow aspirates may demonstrate large macrophages filled with birefringent droplets. Cultured skin fibroblasts of patients with CESD demonstrate birefringence in plain polarized light. Sharply reduced lysosomal acid lipase activity in the lymphocytes or culture fibroblasts establishes the diagnosis. Liver LAL activity is reduced but detectable and is substantially greater than such activity in the liver of patients with Wolman disease.[158]

Treatment. In some patients treatment with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (lovastatin or simvastatin) and with lipophilic vitamins (A, D, E, and K) has resulted in significant reductions in plasma cholesterol, triglyceride, and low-density lipoprotein cholesterol levels, without changing the progression of hepatic disease.[159] Several patients with CESD have received liver transplants with variable results.[160-162]

A null-mutant murine model of LAL appeared normal at birth and developed normally into adulthood. However, massive accumulation of triglycerides and cholesteryl esters (30-fold increase in comparison to normal) occurred in several organs, and at 3 weeks of age the liver appeared yellow-orange and up to two times larger than normal. This murine model is a phenocopy of CESD and a biochemical and histopathologic mimic of human Wolman disease.[163] ERT with recombinant LAL resulted in nearly complete resolution of the hepatic yellow color and a decrease in hepatic weight. Histologic analysis of tissues from ERT-treated mice showed a reduction in macrophage lipid storage and a 50% to 70% decrease in triglyceride and cholesterol levels in the liver, spleen, and small intestine.[164,165] ERT with recombinant enzyme for human LAL deficiencies are currently under evaluation (http://clinicaltrials.gov).[166]

HSCT has been attempted in a few children with Wolman disease. Although long-term survival was reported in one case, generally the patients do not survive.[167] The neurologic performance of the one surviving patient, who received a transplant at 5 months of age, has been good, as would be expected insofar as LAL appears to have no significant role in CNS lipid metabolism.

Neuronal Ceroid Lipofuscinoses

Lysosomal accumulation of lipopigments is characteristic of the neuronal ceroid lipofuscinoses, a group of neurodegenerative conditions whose biochemical bases have only recently begun to be understood. The stored material consists of autofluorescent lipopigments resembling lipofuscin, the material that accumulates in neurons in normal aging. However, the storage material related to neuronal ceroid lipofuscinoses is less sharply outlined and more faintly stained by aldehyde fuchsin than the normal lipofuscin deposits.[168] The neuronal ceroid lipofuscinoses genes encode soluble and transmembrane proteins, localized to the ER or the endosomal or lysosomal organelles. The precise function of most of the neuronal ceroid lipofuscinoses proteins and their cellular dysfunction remains unclear.

The accumulations present in brain lysosomes include glycoconjugates consisting primarily of dolichol pyrophosphate-linked oligosaccharides and proteins, the major component being subunit c (unit 9) of mitochondrial adenosine triphosphate (ATP) synthase. By immunohistochemistry, this excess of subunit c can be identified in skin fibroblasts[169] and in urine.[170,171] On electron microscopy the residual bodies have a heterogeneous appearance. They are present in all tissues but are particularly abundant in the neuronal perikaryon and axon hillock, where they continue to accumulate until the nerve cell degenerates. Neuronal ceroid lipofuscinoses exert a profound effect on the CNS of affected individuals by their progressive nature, leading to blindness, neurocognitive decline, intractable seizures, and ultimately premature death.[172-174] It is not clear why these conditions involve the CNS almost exclusively because the proteins affected are also found in many extraneural tissues.[175]

The neuronal ceroid lipofuscinoses represent a broad spectrum of diseases with an overall incidence of approximately 1 in 12,500 to 25,000 births.[173] The neuronal ceroid lipofuscinoses are probably the most common group of hereditary progressive encephalopathies in children.[173] The age at onset; visual, mental, and motor signs; and types of inclusions present vary in the different forms of the disease. At least eight major forms of the disease have been identified (Table 25-2). These forms show a consistent clinical, pathologic, and genetic picture.[176] The classic forms are infantile (CLN-1), late infantile (CLN-2), and juvenile (CLN-3) neuronal ceroid lipofuscinosis. These forms are inherited in an autosomal recessive pattern and have different genetic bases. An adult onset variant without vision loss (CLN-4) also occurs. Most patients demonstrate autosomal recessive inheritance, but an autosomal dominant pattern has also been found in a few patients. Four additional variants (Finnish, or type 5; Gypsy/Indian, or type 6; Turkish, or type 7; and northern epilepsy with mental retardation, or type 8) have thus far been restricted to specific ethnic groups.[177] Their phenotypes resemble that for type 2, and their genetic bases have been identified. In neuronal ceroid lipofuscinosis

TABLE 25-2 Phenotypic/Genotypic Correlations of Neuronal Ceroid Lipofuscinoses

Form	Genetic Symbol	Onset, Range (yr)	Initial Symptoms	EM	Enzyme Activity Assay	Gene Symbol	OMIM	DNA Testing (Common Mutation)
INCL	*CLN1*	Birth-37*	Seizures, cognitive/motor decline, visual loss	GROD	PPT1	*PPT1*	256730	223A→ G451C→T
Classic late-infantile NCL	*CLN2*	2-8	Seizures, cognitive/motor decline, visual loss	CV/mixed	TPPI	*TTP1*	204500	T523-1G→ C636C→T
Juvenile NCL	*CLN3*	4-10	Visual loss, cognitive/ motor decline, seizures	FP/mixed, vacuolated lymphocytes	TM	*CLN3*	204200	1.02-kb deletion
Adult NCL	*CLN4*	15-50	A: seizures and cognitive/ motor decline; B: abnormal behavior; cognitive, motor decline	Mixed	CTSD	*CTSD, PPT, CLN4, CLN3*	204300/ 162350	NA
Finnish-variant LINCL	*CLN5*	4-7	Cognitive/motor decline, seizures, visual loss	FP/CV/RL	TM	*CLN5*	256731	Tyr392STOP
Romani/ Indian, early juvenile variant LINCL	*CLN6*	1.5-8	Cognitive/motor decline, seizures, visual loss	CV/FP/RL	NA	*CLN6*	601780	NA
Turkish-variant LINCL	*CLN7*	1-6	Seizures, motor decline, visual loss	FP/mixed	NA	MFSD8	610951	NA
Northern epilepsy	*CLN8*	5-10	Seizures and cognitive decline	CV- or GROD-like structures	TM	*CLN8*	600143	Arg24Gly

From Wisniewski KE, Zhong N, Philippart M: Pheno/genotypic correlations of neuronal ceroid lipofuscinoses. Neurology *57:576-581, 2001.*

CTSD, Cathepsin D; *CV,* curvilinear profiles; *EM,* electron microscopy; *FP,* fingerprint profiles; *GROD,* granular osmiophilic deposits; *INCL,* infantile neuronal ceroid lipofuscinosis; *LINCL,* late infantile neuronal ceroid lipofuscinosis; *NA,* not available (unknown); *NCL,* neuronal ceroid lipofuscinosis; *Northern epilepsy,* progressive epilepsy with mental retardation (website: http://www.ucl.ac.uk/ncl); *OMIM,* Online Mendelian Inheritance in Man; *PPT1,* palmitoyl protein thioesterase 1; *RL,* rectilinear complex; *TM,* transmembrane protein; *TPP1,* tripeptidyl peptidase 1.

*INCL has various phenotypes that overlap with onset of the other forms.

type 8, missense mutations have been found in most affected patients.

Children with CLN-1 are normal at birth. Symptoms usually present acutely between ages 6 and 24 months. Initial symptoms include delayed development, myoclonic jerks, seizures, and deceleration of head growth. Affected infants develop retinal blindness and seizures by 2 years of age, followed by progressive intellectual disability.[178,179] The first symptoms of CLN-2 typically appear between 2 and 4 years, usually starting with epilepsy, followed by regression of developmental milestones, dementia, ataxia, and extrapyramidal and pyramidal signs. Visual impairment typically appears at age 4 to 6 and rapidly progresses to blindness. Life expectancy ranges from 6 to older than 40 years.[172,180] The onset of CLN-3 is usually between 4 and 10 years. Rapidly progressive visual loss resulting in total blindness within 2 to 4 years is often the first clinical sign. Epilepsy with generalized tonic-clonic seizures, complex-partial seizures, or myoclonic seizures typically appears between 5 and 18 years. Life expectancy ranges from the late teens to the 30s.[181-183] Initial signs and symptoms of CLN-4 usually appear at approximately age 30, with death occurring about 10 years later. Affected individuals exhibit either progressive myoclonic epilepsy or behavior abnormalities, and all have dementia, ataxia, and late-occurring pyramidal and extrapyramidal signs.[184] Northern epilepsy, or progressive epilepsy with mental retardation (EPMR) is predominant in Finland and is characterized by tonic-clonic or complex-partial seizures, intellectual disability, and motor dysfunction. Onset occurs between 2 and 10 years.[185]

With only rare exceptions, patients who show signs before adulthood become blind, and for this reason older texts usually grouped these diseases among the familial amaurotic disorders. However, the absence of ganglioside storage and the marked brain atrophy that occur in the neuronal ceroid lipofuscinoses clearly set these diseases apart from the classic forms of gangliosidoses (i.e., Tay-Sachs disease, Sandhoff disease, and the GM$_1$ gangliosidoses). Seizures, another common sign in neuronal ceroid lipofuscinoses, may be related to selective loss of inhibitory synapses within the axon hillock and initial segment of pyramidal cells. Their presence may lead to confusion with other causes of progressive myoclonic epilepsy, including Unverricht-Lundborg disease, myoclonic epilepsy with ragged-red fibers, Lafora disease, and the sialidoses. In contrast to these other disorders, each variant of the neuronal ceroid lipofuscinoses is associated with the accumulation of autofluorescent material.

Retinal degeneration can be monitored with electroretinography. With complete degeneration of the outer retinal layer, the electroretinographic response is totally abolished. Another useful test is electroencephalography, the results of which may be abnormal even before the development of dementia. Computed tomography and magnetic resonance imaging have been useful to document loss of gray matter early in the course of infantile neuronal ceroid lipofuscinosis.[186]

Diagnosis of these relatively common disorders depends primarily on the clinical features, but examination of a skin biopsy specimen by electron microscopy also helps greatly. Lymphocyte vacuolization and azurophilic hypergranulation of neutrophils are often found in the peripheral blood smear, except in the infantile form of the disease. Electron microscopic studies of these blood elements and urinary sediment, conjunctiva, liver, skeletal muscle, and sural nerve have also aided in the diagnosis. In juvenile neuronal ceroid lipofuscinosis, fingerprint lipopigments may appear on electron micrographs of lymphocytes in the absence of vacuoles. More recently, molecular diagnosis has become clinically available for the majority of the neuronal ceroid lipofuscinoses, making the diagnosis less invasive.

Clinical, pathologic, and biochemical changes similar to those seen in humans with neuronal ceroid lipofuscinosis have been detected in several animal species.[187-190] These naturally occurring animal models provide important clues regarding the pathogenesis of neuronal ceroid lipofuscinoses and offer investigators the opportunity to study potential treatments of these disorders. HSCT and liver transplantation in English setter dogs did not lead to CNS stabilization or improvement, nor have treatments with vitamin E, selenium, levodopa, or glutathione or peroxidase enzyme. Addition of 20% corn starch to their diet did improve the quality of life but failed to alter disease progression[189]

The only treatment currently available is symptomatic. Baclofen (15 to 25 mg/day, increasing to as much as 200 mg/day) and tizanidine (1 to 9 mg/day, later 20 to 60 mg/day) have been helpful for controlling irritability, choreoathetosis, and sleep disturbances. Levomepromazine (5 to 20 mg/day) or benzodiazepines may also be beneficial.[191] Cysteamine bitartrate reduces storage material in CLN1-deficient cells. An open-label 7-year trial in CLN1-affected children failed to show overall attenuation of disease progression.[192] Moreover, HSCT is not therapeutic.

Krabbe Disease

Krabbe disease results from mutations in *GALC*, which encodes galactocerebrosidase. This enzyme uniquely degrades galactosylsphingosine, a deacylated analogue of galactosylceramide. Sulfatide and galactocerebroside are important constituents of myelin that are synthetized by oligodendrocytes and Schwann cells. Galactosylsphingosine is highly toxic to several cell types in the CNS, particularly oligodendrocytes and Schwann cells.[193] The Krabbe disease phenotype matches well with the primary involvement of these cells and secondary neuroinflammatory responses. The majority of affected individuals have infantile Krabbe disease with onset before 6 months. The later forms of Krabbe disease have a variable life expectancy and presentation but are collectively called *late onset Krabbe disease*.[194] A birth frequency of approximately 1 in 100,000 is generally given, but the results from the New York State Newborn Screening Program indicate a frequency of 1 in 275,000.[195] The carrier frequency of these panethnic diseases in individuals with no family history is approximately 1 in 150.

Infantile Krabbe Disease

Infantile Krabbe disease is divided into four stages: stage I—irritability, vomiting, and feeding problems (gastroesophageal reflux); stage II—muscular hypertonia of limbs, pyramidal signs, and difficulty feeding; stage III—hypertonia in all limbs and opisthotonic posturing followed by progressive depression of deep tendon reflexes, reflecting a polyneuropathy (optic atrophy and sluggish pupillary reactions to light are common); stage IV—"burned out stage" with immobility, either hypotonic or decerebrate, and inability to feed, jerky eye movements, seizures, unexplained fevers, and startle reflexes. These are general stages, and not all patients fit them chronologically. Malnutrition and global CNS atrophy lead to decreased head circumference. Average survival is 2 years or less.

Abnormal neurophysiologic studies are present early in infantile Krabbe disease with reduced nerve conduction studies before the appearance of specific signs.[196] Also, visual evoked potentials (VEPs) and brainstem auditory evoked responses (BAER) are abnormal early in patients with infantile Krabbe disease.[196] Peripheral neuropathy is observed in approximately 50% of patients with late onset Krabbe disease, and nerve conduction velocity (NCV) studies can be normal in some affected adults.[197] MRI signal abnormalities occur within cerebellar white matter, the deep gray nuclei, and the pyramidal tracts. Involvement of the parietooccipital white matter and corpus callosum develop rapidly and simultaneously with global atrophy.[198]

Late Onset Krabbe disease

Late onset Krabbe disease includes two forms: late infantile, with onset after 6 months to age 3, and juvenile Krabbe disease, with onset by 3 to 8 years of age. Survival correlates with age of onset, with death occurring approximately 2 years after onset for the late infantile and more than 7 years after onset for the juvenile form.[196] Late onset Krabbe disease has three main signs: motor weakness, vision loss, and intellectual regression. Progressive equinovarus, with walking difficulties, in a previously normal or mildly delayed child, may appear first. A spastic paraparesis supervenes, sometimes associated with cerebellar ataxia and dystonia. Visual failure caused by optic atrophy is almost universal with advancing age. In many cases dementia progresses after the onset of motor signs.[197] This classical progression is not present in all cases.[199] In late onset Krabbe disease, MRI reveals abnormalities in the pyramidal tracts, posterior corpus callosum, and parietooccipital white matter.[199] Hypertrophy of the optic nerves, optic chiasm, and other cranial nerves can be present, as can radial stripes inside demyelinated tracts.[200,201]

Treatment

Supportive care to control irritability and spasticity in children with infantile onset Krabbe disease is the only management available in the later stages of the disease. HSCT has been performed in patients of all ages with variable success.[202,203] HSCT in newborns with infantile Krabbe disease prolongs survival; cognitive decline is greatly slowed and peripheral nervous system function may deteriorate, but these depend on the time at which transplant was performed.[204] There are no consensus criteria for HSCT in Krabbe disease. Disease stages (I to IV) have been used to guide candidacy for HSCT.[205] Outcome analysis has demonstrated that patients with minimal to no disease progression at time of HSCT (stages I or II) have good survival after HSCT and progress in their development, whereas patients with more advanced disease (stages III or IV) have significantly higher mortality after HSCT with no developmental improvement. A multicenter study of 16 infants with Krabbe disease after HSCT in the early disease stage, who were mostly ascertained by family history, showed that all developed abnormalities of gross motor control and expressive language.[204] Receptive language and cognition were considered normal in this cohort, although assessments can be difficult in children with motor and expressive language deficits. These patients also had poor growth and developed spasticity, loss of motor milestones, and microcephaly. These studies suggest that HSCT should be performed in this age group only when symptoms are absent or mild, but despite transplantation there will be significant neurologic morbidity.[205]

Identification of Krabbe disease by newborn screening facilitates the initiation of treatment before neurologic damage ensues. The decision to treat and the time to start therapy are challenging in Krabbe disease because HSCT carries significant morbidity and mortality risks. Acute or chronic graft-versus-host disease has been reported in up to 32% of transplanted patients.[203,206] Prediction of clinical course with and without treatment is limited, as is the current understanding of genotype-phenotype correlation. The significant clinical variability in late onset forms (even those with the same genotype) makes evaluation of treatment effectiveness difficult.

Metachromatic Leukodystrophy

Metachromatic leukodystrophy (MLD) is caused by mutations in *ARSA*, which encodes arylsulfatase A (ASA). Three clinical subtypes characterize MLD: late infantile MLD (50% to 60% of cases), juvenile MLD (20% to 30% of cases), and adult MLD (15% to 20% of cases).[207] Age of onset within a family is usually consistent. The prevalence of MLD has been reported to be between 1 in 40,000 to 1 in 160,000 in different populations. The carrier frequency is approximately 1 in 100 to 1 in 200. MLD was the most common diagnosis among children in Utah with inherited leukodystrophy.[208]

MLD is suspected in individuals with progressive neurologic dysfunction and MRI evidence of a leukodystrophy. MLD is suggested by ASA enzyme activity in leukocytes that is less than 10% of normal controls; however, assay of ASA activity cannot distinguish between MLD and *ARSA* pseudodeficiency, in which ASA activity is 5% to 20% of normal and MLD fails to ensue. The diagnosis of MLD must be confirmed by molecular genetic testing and urinary excretion of sulfatides.

Late-infantile MLD is characterized by onset by 1 to 2 years in a child with previously normal development. Typical initial findings are weakness, hypotonia, frequent falls, toe walking, and slurred speech. Later signs include inability to stand, poor speech, cognitive decline, hypertonia, pain, seizures, vision and hearing impairment, and peripheral neuropathy. In the final stages, children have spasms and decerebrate posturing and are no longer aware of their surroundings.[209]

Juvenile MLD is characterized by onset between 4 years and sexual maturity. Typical presentation includes decline in school performance and behavioral problems, followed by gait abnormalities, slurred speech, and incontinence. Seizures may occur. Progression is slower than the late-infantile form. Survival for 10 to 20 or more years after the initial diagnosis is common.

Adult MLD is characterized by onset after sexual maturity until the fourth or fifth decade. Initial manifestations are problems in school or job performance, personality changes, alcohol or drug abuse, poor financial decisions, and emotional lability. In other patients neurologic symptoms predominate and include weakness and loss of coordination that progresses to spasticity and incontinence.[210] Peripheral neuropathy is common in this group. Disease course is variable and slow, with periods of stability and decline.

Treatment

MLD leads to progressive loss of function and continually changing clinical care needs. Symptomatic treatment includes management of seizures using antiepileptic medications, physical therapy to maintain joint mobility, and treatment of contractures with muscle relaxants. Aggressive therapies to preserve neuromuscular function, intellectual abilities, and mobility are a critical component of care for these patients. Family involvement is important to help anticipate decisions regarding wheelchairs, feeding tubes, suction equipment, and other care needs.

HSCT is the only potential therapy available for primary CNS manifestations in MLD.[211,212] Reduced-intensity conditioning accompanied by mesenchymal stromal cell infusion has been well tolerated in adult patients with MLD.[213] Single center experiences with HSCT have generally included a limited number of patients with MLD and with controversial outcomes. The best results are obtained when transplant is performed before signs and symptoms have developed and in the later onset forms of the disease. Poor clinical results of HSCT have been reported in symptomatic MLD patients.[214] HSCT slows CNS progression but does not prevent peripheral nervous system manifestations.[215] In an individual with juvenile MLD, there was less peripheral nervous system progression during the 2 years after HSCT, with stabilization of disease.[216] HSCT was performed in a presymptomatic neonate, and disease progression was not prevented.[217] HSCT carries a substantial risk, and long-term benefits in MLD need to be evaluated. Improvements in the effectiveness of HSCT have been attempted by incorporation of *ARSA* gene constructs into hematopoietic stem cells using lentiviral vectors.[218] After myeloablation, HSCT with the genetically modified cells led to reconstitution of the hematopoietic system and apparent nonprogression of the CNS disease.[218] This promising result likely results from repopulation of the CNS macrophages that secrete large amounts of ARSA for cross-correction of multiple affected cell types, as suggested by the high levels of CSF ARSA.

In comparison, ERT efficacy is limited in neuronopathic lysosomal diseases because of the blood-brain barrier. Phase I and phase II clinical trials of intravenous recombinant ARSA enzyme failed to show clinical improvement. Currently, ERT administered intrathecally is under evaluation (http://clinicaltrials.gov). Other research therapies include AAV vectors containing human ARSA administered into the brains of nonhuman primates. ASA was increased by 12% to 38% without adverse effects, suggesting that a similar approach could be feasible in MLD.[219]

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CHAPTER 26

Platelets and the Vessel Wall

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CHAPTER OUTLINE

PLATELET MORPHOLOGY AND SUBCELLULAR ORGANIZATION
PLATELET GENOMICS AND PROTEOMICS
ANTITHROMBOTIC COMPONENTS OF THE VESSEL WALL
REACTING TO THE BREACH: CELL SURFACE RECEPTORS THAT MEDIATE TETHERING AND ADHESION AND TRANSMIT EARLY ACTIVATION SIGNALS
PLATELET GRANULES AND THEIR ROLE IN HEMOSTASIS
FEED-FORWARD AMPLIFICATION PATHWAYS INVOLVED IN PLATELET RECRUITMENT AND THROMBUS STABILITY
ACTIVATION OF THE MAJOR PLATELET INTEGRIN, αIIbβ_3 (GPIIb-IIIa complex)—THE FINAL COMMON ENDPOINT OF PLATELET ACTIVATION
CELL SURFACE AND CYTOSOLIC PROTEINS THAT LIMIT PLATELET RESPONSIVENESS
NONHEMOSTATIC ROLES FOR PLATELETS: BLOOD VESSEL MAINTENANCE AND REPAIR, INFLAMMATION AND INFECTION, AND ATHEROTHROMBOSIS

In an 1881 communication to the Turin Royal Academy of Medicine, the Italian physician Giulio Bizzozero disclosed the presence in circulating human blood of discrete elements that he termed "*piastrine*" ("*Blutplättchen*" in a 1882 publication in a German journal and "*petites plaques*" in a communication in French).[1] Previously speculated to be merely nonphysiologic "granular aggregates," blood platelets have since become central to the understanding of thrombosis and hemostasis, and detailed understanding of their participation in cardiovascular disease, stroke, and even cancer has led to remarkable progress in the rational treatment of these disorders.

Although platelets are most often studied in the context of their ability to form a hemostatically effective plug, it is now widely recognized that their influence extends far beyond this process to all aspects of hemostasis, as well as to wound healing and vascular remodeling. For example, platelets generate or secrete biologically active mediators such as thromboxane A_2 (TXA_2) and serotonin, which not only amplify platelet activation responses but also modulate vascular tone. In addition, platelets secrete a broad array of granule constituents that stimulate vessel repair, induce megakaryocytopoiesis, promote coagulation, and limit fibrinolysis.

The same pathways that lead to platelet plug formation can also produce pathologic thrombosis, a process that has been described as hemostasis occurring at the wrong time or in the wrong place. Platelets are particularly important for hemostasis on the arterial side of the circulation, where blood flows under higher pressure and experiences greater shear force. As a result, platelet function is generally considered to be critical to the pathogenesis of arterial thrombosis and less so for venous thrombosis, and antiplatelet drugs are most widely used in the former setting. However, this distinction between the mechanisms underlying arterial and venous thrombosis is not absolute, and the spectrum of thrombotic disorders should be considered a continuum.

Arterial thrombosis is a particularly common problem in middle-aged and older adults and is a major cause of morbidity and mortality in developed countries. The thrombi that arise in atherosclerotic vessels are predominantly platelet in origin and are the proximate cause of myocardial infarction and most cerebrovascular accidents. Although arterial thrombosis is considerably less common in children than adults, it may contribute to major morbidity in patients with sickle cell disease, as well as complications of some childhood infections, Kawasaki syndrome, and various forms of arteritis, autoimmune disorders, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (see Chapter 34).

In this chapter platelet structure and function are reviewed, and special emphasis is given to the cell surface glycoproteins (GPs) that function as sentries for areas of vascular damage and the signal transduction events that both amplify and limit platelet responsiveness. The information provided should be helpful in understanding subsequent chapters that describe inherited and acquired platelet disorders (see Chapters 30 and 34) and the role of the adhesive protein von Willebrand factor (VWF) in hemostasis (see Chapter 31). Finally, there is growing appreciation of the role that platelets play in inflammation and the pathogenesis of atherothrombosis, which is briefly discussed at the end of the chapter.

PLATELET MORPHOLOGY AND SUBCELLULAR ORGANIZATION

Platelets are adhesion and signaling machines that circulate as small, disc-shaped cellular fragments in the whole blood of healthy individuals at a concentration of approximately 150,000 to 300,000 per μL. Early studies suggested that platelets might be produced via cytoplasmic fragmentation along a network of internal demarcation membranes that were observed in large, polyploid megakaryocytes.[2,3] More recent studies,[4-6] however, support the notion that proplatelets are assembled and packaged with their various constituents at the ends of long cytoplasmic extensions of differentiated megakaryocytes that have migrated from the proliferative osteoblastic niche to the capillary-rich vascular niche of the bone marrow microenvironment,[7] with the invaginated demarcation membrane system serving simply as a reservoir of internal membrane used for proplatelet extension.[8,9] Once adjacent to the adluminal face of the endothelium, proplatelets are released into the blood stream,[10] where they circulate as mature platelets for approximately 7 to 10 days before being cleared by the liver and spleen,[11] their life span being controlled at least in part by an antagonistic balance between the apoptotic proteins Bcl-xL and Bak.[12]

The size of resting platelets is somewhat variable, averaging approximately 1.5 μm in diameter and 0.5 to 1 μm in thickness. Platelet size is undoubtedly regulated by numerous factors during their biogenesis, but both the 224-kDa nonmuscle myosin heavy chain IIA (MYHIIA) and the cell-surface glycoprotein Ib complex (GPIb) appear to play critical roles. Thus mutations in the *MYH9* gene act dominantly to interfere with contractile events important for platelet formation,[13] whereas failure to express GPIb results in Bernard-Soulier syndrome[14,15] by disruption of critical associations with the cytoskeletal protein filamin,[16,17] which play an essential role both in platelet formation and in platelet compliance.[18,19] In both of these inherited platelet disorders, platelets are as large as lymphocytes (see Chapter 30). Correction of GPIb expression in GP Ib-deficient (Bernard-Soulier syndrome) mice has been shown to restore platelets to their normal size.[20]

The volume of platelets (mean platelet volume) normally ranges from 6 to 10 femtoliters. Platelet density is also variable,[21] and the issue of whether young platelets are more[22,23] or less[24] dense as they gain versus lose content during their circulating lifetime has never been satisfactorily resolved. Because platelets retain most species of messenger ribonucleic acid (mRNA) for a short period after their release from bone marrow megakaryocytes,[25] young platelets can be distinguished from older ones based on their RNA content.[26]

As shown in Figure 26-1, *A* and *C*, resting platelets are discoid in shape, largely because of the presence of a circumferential coil of microtubules,[27,28] and are packed with numerous electron-opaque α granules, a few dense (δ) granules, several mitochondria, and lysosomes.[29] Platelets also retain a few Golgi bodies, as well as occasional vestiges of rough endoplasmic reticulum—the exception being platelets from patients with rapid platelet turnover, where very young platelets containing more abundant protein synthesis machinery are readily observed in the circulation. Platelets also contain two highly specialized membrane systems not found in other cells of the body: the surface-connected open canalicular system (OCS; see Fig. 26-1, *B* and *C*), and the dense tubular system (DTS). The OCS is a series of tortuous invaginations of the plasma membrane that appear to tunnel throughout the cytoplasm of the cell[30] that serves as an internal reservoir of plasma membrane that is called upon when platelets round up, extend lamellipods and filopods (see Fig. 26-1, *B* and *D*), and spread during platelet activation—a process that can increase by more than 400% the surface area of exposed plasma membrane.[31] Because OCS channels are proximal to internal granules, they also likely function as a conduit for the rapid expulsion of α- and δ-granule contents during platelet activation.[32] The DTS, on the other hand, is a remnant of the smooth endoplasmic reticulum[33] and is found randomly dispersed throughout the cytoplasm. The DTS appears to be one of several organelles within the platelet known to harbor high concentrations of calcium[34,35] and is thought to contain a 100-kDa calcium adenosinetriphosphatase (ATPase) known as *sarcoplasmic/endoplasmic reticulum* Ca^{2+}*-ATPase 2b* (*SERCA2-b*)[36] that functions to sequester and store cytosolic calcium in resting cells. Recent evidence suggests that adenosine diphosphate (ADP) is able to induce selective release of calcium from the DTS,[37] whereas activation of the GPIb/V/IX receptor for VWF releases calcium primarily from a poorly defined acidic compartment within the cell.[38] Thrombin, a strong platelet agonist, appears to elicit calcium release from both stores upon binding to the platelet thrombin receptor PAR-1.[37]

The platelet cytoskeleton is comprised of a single, rigid but dynamic microtubule of approximately 100 μm in length coiled 8 to 12 times around the equatorial plane of the cell.[39-41] This marginal band of microtubules is largely responsible for maintaining the discoid shape of the resting cell, as illustrated by the observations that 1) incubation of platelets with colchicine, an agent that dissolves microtubules, results in their rounding[42]; 2) platelets from mice lacking β_1 tubulin remain largely spherical[43]; and 3) "platelet spherocytosis" in humans results when tubulin fails to polymerize normally into microtubules.[44] Directly underneath the plasma membrane lies an intricate, two-dimensional, tightly-woven

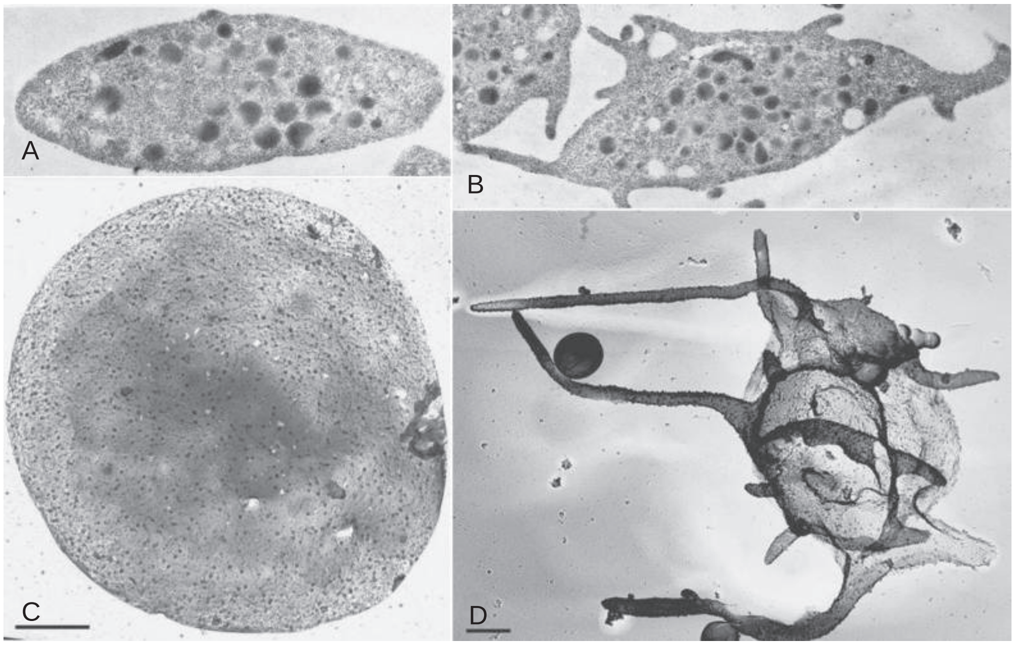

Figure 26-1 Platelet morphology. Resting platelets (**A**, thin section, and **C**, from a scanning electron micrograph of a flash-frozen, freeze-dried platelet) are shaped like a disc and contain numerous electron-opaque α granules, a few dense granules, and several mitochondria and lysosomes. A circumferential coil of microtubules is responsible for maintaining their discoid shape. Platelets also contain a number of cytoplasmic membrane systems that subserve specialized functions, including vestiges of the smooth endoplasmic reticulum that sequester calcium and tortuous invaginations of the plasma membrane that form a surface-connected open canalicular system (OCS). When platelets become activated (**B** and **D**), they rapidly round up, extend lamellipodia and filopodia, and release the contents of their granules, often into the nearby OCS. *(Courtesy of John H. Hartwig.)*

membrane skeleton[45] comprised of nonerythroid spectrin,[46,47] a network of actin filaments,[45,47] vinculin,[48] and the actin-binding protein filamin,[49] which itself is tethered to the inner face of the plasma membrane via linkages with the cytoplasmic domain of GPIb.[16,49] The membrane skeleton, because of its location, serves as a scaffold, linking elements of the plasma membrane with contractile elements of the cytoskeleton and cytosolic signaling proteins, and thereby regulates such diverse functions as receptor mobility,[50,51] receptor clustering,[52-54] and signal transduction.[55] Finally, the platelet is filled with an extensive cytoplasmic network of actin filaments[47,56] organized by the actin-binding proteins filamin[57,58] and α-actinin[59] that constitute its cytoskeleton.

When platelets become exposed to components of the extracellular matrix[60] or to soluble agonists like ADP[61] or thrombin,[62,63] they undergo dramatic changes in their morphology.[64,65] The marginal band of microtubules disappears,[56] allowing the platelet to transform from a disc to an irregular sphere. At nearly the same time, the actin filament capping protein, α-adducin, becomes phosphorylated and dissociates from existing F-actin filaments,[66] exposing the barbed end of the filament to cytosolic actin monomers and driving rapid polymerization of actin into microfilaments.[64] This has the dual effect of driving the extension of lamellipodia and filopodia, and of forcing granules towards the center of the platelet where they can fuse with membranes of the OCS and release their contents to the exterior of the cell. Phosphorylation of myosin additionally induces contractile events that facilitate centralization of the granules.[67]

PLATELET GENOMICS AND PROTEOMICS

Though anucleate, platelets contain measurable and manipulable levels of megakaryocyte-derived mRNA,[25] at least some of which is capable of being synthesized into small but detectable amounts of protein.[68,69] Both serial analysis of gene expression (SAGE) and gene microarray analyses[70-72] were employed early on to estimate the size and composition of the platelet transcriptome. Of the 20,563 genes present in the human genome, 9538 distinct transcripts (6493 in the mouse) were identified in unstimulated platelets using RNA sequencing (RNA-SEQ).[73] That is considerably fewer than normally found in a nucleated cell, but perhaps more than might have been expected from an anucleate circulating cellular fragment. One of the more surprising findings in recent years has been the identification of heavy (sometimes called *heterogeneous*) nuclear RNA (hnRNA) in the platelet cytosol, as well as all of the spliceosome components necessary to splice the hnRNA into mature message that can be thereafter translated into protein.[74] Enlisted during the activation process, signal-dependent protein translation has thus far been demonstrated for mRNAs encoding interleukin (IL) 1β,[74] tissue factor,[75] and Bcl-3,[76] the protein products of which have the potential to influence inflammation, thrombosis, and wound repair. Platelets also contain an abundance of microRNAs (miRs) that have the potential, especially in precursor megakaryocytes, to regulate transcription and translation.[77]

The platelet proteome appears to be equally complex and diverse, and unlike the transcriptome, reports both the breadth as well as the relative amounts of protein products actually present in the cell. Obtained by refined two-dimensional gel electrophoretic techniques that were originally developed in the 1970s[78,79] or by liquid chromatographic separation, proteins are fragmented and separated using a combination of proteolytic and ionization techniques and then analyzed by mass spectrometry, allowing for the identification of thousands of proteins present in complex cellular lysates or subcellular fractions. In by far the most complete and quantitative profiling analyses to date, Burkhart and colleagues have identified a core platelet proteome comprised of approximately 5000 distinct platelet proteins,[80] including an abundance of molecules involved in adhesion, signaling, cytoskeletal change, and metabolism. Others have combined prefractionation methods with suitable separation techniques to describe the inventory of platelet proteins that become phosphorylated during platelet activation,[81-85] can be palmitoylated,[86] or are enriched within various subcellular compartments such as the platelet cytoskeleton,[87] platelet α-granules,[82,88-90] the membrane fraction,[91-93] membrane rafts,[94] and microparticles.[95]

ANTITHROMBOTIC COMPONENTS OF THE VESSEL WALL

Though hundreds of thousands of platelets per microliter circulate in blood, very few, if any, interact with the intact vessel wall, because the endothelial lining of the blood vessel presents an excellent nonthrombogenic surface. In fact, this property of the vessel wall has not yet been duplicated in any prosthetic or extracorporeal device. The healthy endothelium not only provides an effective barrier between blood and the highly thrombogenic components of the subendothelium (see below), but also actively produces both membrane-bound and secretory products that limit fibrin generation and promote clot dissolution. For example, heparinlike glycosaminoglycans present on the luminal side of the endothelial cell surface recruit plasma antithrombin, effecting a conformational change that promotes binding and neutralization of thrombin and other serine proteases.[96] Thrombin, when bound to the endothelial cell surface receptor, thrombomodulin, takes on anticoagulant properties via its cleavage and activation of protein C, which in turn cleaves coagulation factors V and VIII, suppressing further thrombin generation.[97] Endothelial cells also express a specific receptor for activated protein C, which serves to concentrate the protein on the endothelial surface.[98] Finally, endothelial cells synthesize, secrete and rebind tissue plasminogen activator,[99,100] which activates plasminogen to facilitate fibrin dissolution (Fig. 26-2).

The endothelial cell also produces two important inhibitors of platelet activation: prostacyclin (PGI_2)[101-103] and nitric oxide (NO). The latter was first recognized for its role as an endothelium-derived relaxation factor.[104-106] A labile oxygenated metabolite of arachidonic acid generated by endothelial cell cyclooxygenase-2 (COX-2), PGI_2 diffuses out of the cell and binds to a platelet G_s-protein coupled receptor (GPCR) known as the isoprostenoid (IP) receptor.[107,108] Its release stimulates adenylate cyclase to increase cytosolic cyclic adenosine monophosphate (cAMP) levels, which 1) activates a pump in the DTS that decreases cytosolic calcium ions (Ca^{2+}), thus helping keep platelets quiescent, and 2) activates protein kinase A (PKA). PGI_2 also has potent vasodilatory effects, binding to IP on arterial smooth muscles cells to effect vessel relaxation.[101] PGI_2 produced by the vascular endothelium thus serves to counterbalance the proaggregatory and vasoconstrictor activities of the platelet-derived prostanoid, TXA_2, the biology of which is discussed below. In fact, upsetting the delicate balance between COX-1–derived TXA_2 and COX-2–derived PGI_2 has been shown to increase the risk of adverse cardiovascular events.[109]

Whereas PGI_2 stimulates adenylate cyclase to produce cAMP, NO, a product of L-arginine generated by endothelial nitric oxide synthase (eNOS),[110] directly activates platelet guanylate cyclase, resulting in increased levels of cytosolic cyclic guanosine monophosphate (cGMP). Although platelet responses to low levels of this cyclic nucleotide can at first be mildly stimulatory,[111] cGMP, largely via its activation of protein kinase G (PKG), has the overall effect of dampening platelet responses and inhibiting platelet adhesion,[112,113] platelet aggregation,[114-117] and platelet-mediated recruitment of leukocytes during the inflammatory response (see Fig. 26-2).[118] In addition to these two soluble metabolites, endothelial cells also express on their surface a potent ADPase known at CD39, which scavenges plasma ADP to prevent platelet aggregation.[119,120] Finally, it is important to note that inflammatory cytokines, oxidized lipids, and immune complexes can, under pathologic conditions, inhibit these protective biochemical pathways and impair the antithrombotic state of the endothelial cell. These changes permit untoward formation of platelet- and fibrin-containing thrombi, as well as thrombus formation beyond sites of vascular injury, and can thus contribute to atherothrombosis.

REACTING TO THE BREACH: CELL SURFACE RECEPTORS THAT MEDIATE TETHERING AND ADHESION AND TRANSMIT EARLY ACTIVATION SIGNALS

As antithrombotic as is the endothelial lining, the underlying extracellular matrix consists of a rich mixture of glycosaminoglycans into which are embedded an abundance of highly-concentrated prothrombotic proteins, including structural components like collagen and elastin (which comprise ≈30% of body weight) and adhesive proteins such as laminin, fibronectin, and VWF. Not surprisingly, platelets have evolved receptors for most of these proteins, and a series of rapid biochemical events is initiated both on the surface and inside the cell when exposed to them. Adhesion is an activating event.

The large number of circulating red blood cells serves to marginate platelets, and when the vessel wall is breached, either by mechanical injury or after atherosclerotic plaque rupture, the first layer of platelets to encounter exposed matrix undergoes a series of sequential events similar to that which leukocytes experience during the

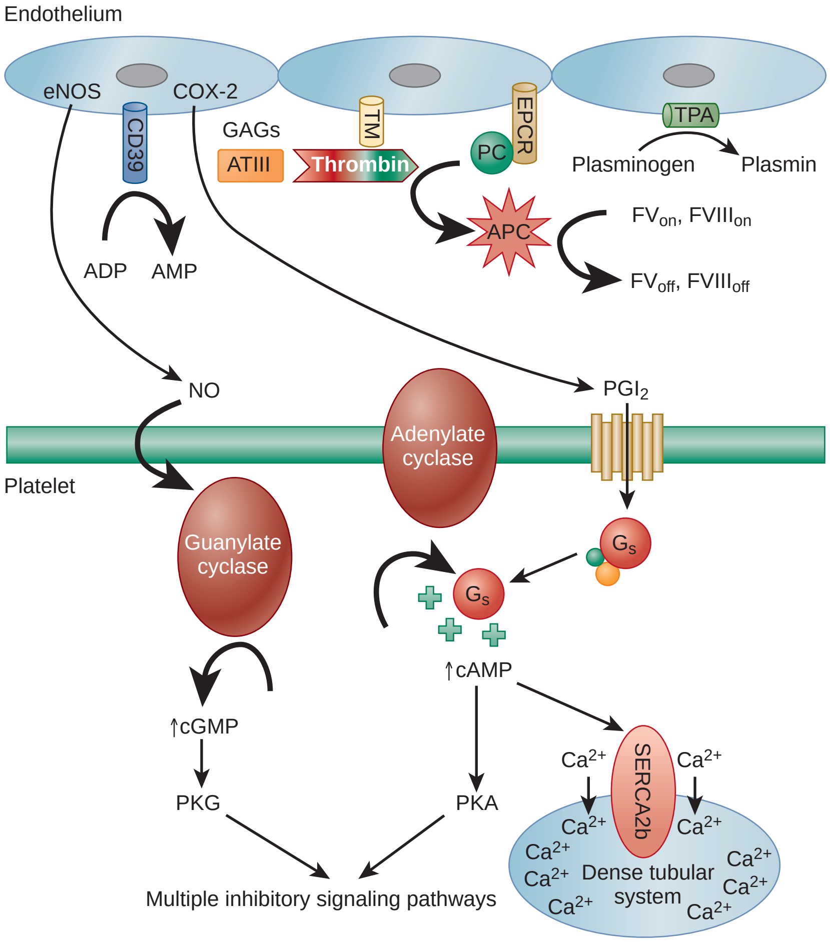

Figure 26-2 Anticoagulant and antithrombotic components of the vascular endothelium. Endothelial cells produce a number of substances, including NO and PGI$_2$ that act on platelet surface receptors to dampen platelet responsiveness. They also scavenge the platelet agonist ADP, inactivate thrombin, and activate the fibrinolytic enzyme plasmin. *ADP,* Adenosine diphosphate; *AMP,* adenosine monophosphate; *APC,* activated protein C; *Ca^{2+},* calcium ion; *cAMP,* cyclic adenosine monophosphate; *cGMP,* cyclic guanosine monophosphate; *COX-2,* cyclooxygenase-2; *GAGs,* glycosaminoglycans; *eNOS,* endothelial nitric oxide synthase; *EPCR,* endothelial cell protein C receptor; *NO,* nitric oxide; *PC,* protein C; *PGI$_2$,* prostacyclin; *PKA,* protein kinase A; *PKG,* protein kinase G; *SERCA2b,* sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; *TM,* thrombomodulin; *TPA,* tissue plasminogen activator.

inflammatory response—namely tethering, initial signaling to the cell interior, integrin-mediated adhesion, and cytoskeletally-directed cell spreading. Whereas leukocyte tethering is mediated by members of the selectin family, the first layer of platelets become tethered on VWF,[121] which is sprinkled on exposed collagen fibers via interactions with a high-affinity, platelet-specific, multi-subunit receptor known as the *GPIb/V/IX complex*.[122] This complex, which is expressed at approximately 25,000 copies/platelet,[123] binds to the A1 domain of VWF[124] with sufficiently high affinity to tether platelets even under conditions of arterial shear.[125] Loss of this receptor in both humans and mice results in a clinical condition known as *Bernard-Soulier syndrome,*[14,15] which is characterized not only by an increase in platelet size but also by prolonged bleeding caused, in large part, by the inability of platelets to adhere to the vessel wall. After engagement with its ligand, GPIb acts through membrane-proximal Src family kinases,[126] adapter molecules,[127] and to a lesser extent via its association with immunoreceptor tyrosine-based activation motif (ITAM)–bearing subunits[128,129] to transmit early activation signals.[130,131] Together the result is the recruitment and activation via tyrosine phosphorylation of phospholipase Cγ2 (PLCγ2),[132,133] a key enzymatic component of platelet amplification that is required to achieve thrombus growth and stability (Figure 26-3).[134]

Once tethered, two different platelet integrins—each of which exists in a low-affinity state on the platelet surface—begin to engage specific extracellular matrix components, and together with the small calcium transients and kinase-generated signals emanating from the GPIb complex, and from mechanical shear forces generated by the flowing blood,[135] initiate the reciprocal processes of platelet adhesion and activation. Thus the integrin $\alpha_2\beta_1$ binds to exposed collagen fibrils,[136] and the integrin receptor $\alpha_6\beta_1$ engages laminin.[137] Both of these integrins "hand off" to a member of the immunoglobulin superfamily, glycoprotein VI (GPVI),[138,139] which via its non–covalent association in the plane of the plasma membrane with the ITAM-bearing Fc-receptor γ-chain homodimer,[138,140] elicits strong PLCγ2-dependent events that 1) begin the process of cytoskeletally-directed shape change and cell spreading; 2) initiate signal transduction pathways that cause dramatic structural changes

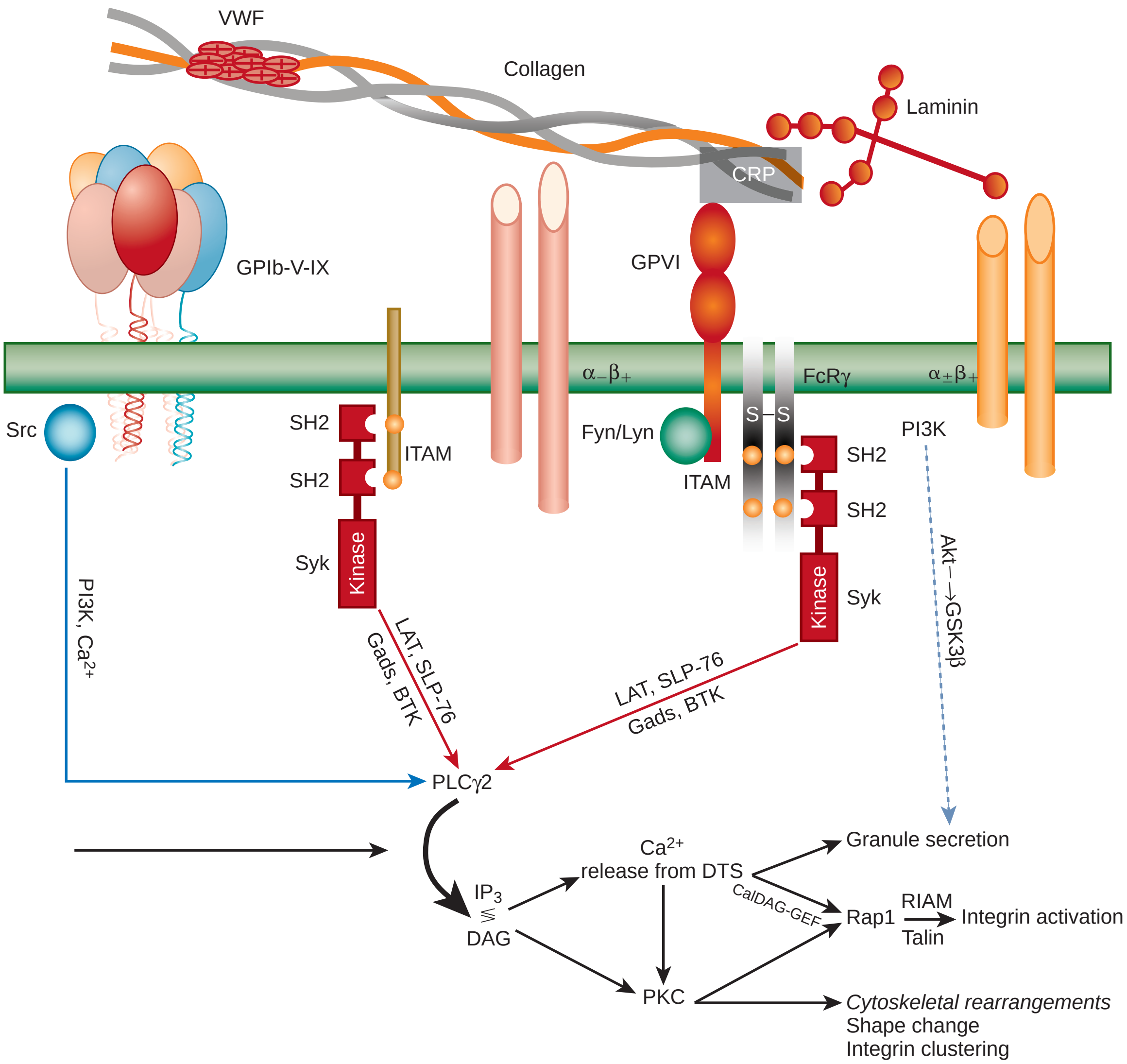
VWF
Collagen
Laminin
CRP
GPIb-V-IX
GPVI
$\alpha_{-}\beta_{+}$
FcRγ
$\alpha_{\pm}\beta_{+}$
Src
SH2
SH2
ITAM
Syk
Kinase
Fyn/Lyn
ITAM
S–S
SH2
SH2
Kinase
Syk
PI3K
PI3K, Ca^{2+}
LAT, SLP-76
Gads, BTK
LAT, SLP-76
Gads, BTK
Akt—→GSK3β
PLCγ2
IP$_3$
⩽
DAG
Ca^{2+}
release from DTS
CalDAG-GEF
Granule secretion
Rap1
RIAM
Talin
Integrin activation
PKC
Cytoskeletal rearrangements
Shape change
Integrin clustering

Figure 26-3 Platelet adhesion receptors that signal through PLCγ2. Each of the cell surface receptors shown recognizes different components of the extracellular matrix, and thus they work in coordinated fashion to send early activation signals into the cell. Binding of platelets to VWF slows platelets down so that integrins associate productively with matrix collagen and laminin. Signals emanating from each of these events are transmitted into the cell interior, in part via the action of receptor-associated Src family kinases (Src, Fyn, Lyn), which phosphorylate tyrosine residues within nearby ITAMs, thus forming a nucleation point for the assembly of miniature organelles sometime referred to as *signalosomes*. Signalosomes are themselves comprised of the adaptor proteins LAT, SLP-76 and Gads and the receptor tyrosine kinase BTK, and they function to localize, phosphorylate, and then activate PLCγ2, which coordinates all these responses by generating the classical signaling molecules IP3 and DAG. Details regarding the molecular events that take place after generation of IP3 and DAG are found in Figures 26-4 and 26-6. *CRP,* C-reactive protein; *DAG,* diacylglycerol; *DTS,* dense tubular system; *GP,* glycoprotein; *IP$_3$,* 1,4,5-inositol triphosphate; *ITAM,* immunoreceptor tyrosine-based activation motif; *PKC,* protein kinase C; *PLCγ2,* phospholipase Cγ2; *VWF,* von Willebrand factor.

in platelet integrins (see Fig. 26-3), resulting in their adopting a high-affinity, ligand-binding–competent conformation[141] (a process known as “inside-out” signal transduction); and 3) facilitate fusion of α- and δ-granules with the OCS and underlying plasma membrane. Other cell-surface receptors that participate in adhesion-initiated activation events include PEAR1,[142] FcγRIIa,[143] and the C-type lectin receptor, CLEC-2.[144]

PLATELET GRANULES AND THEIR ROLE IN HEMOSTASIS

Platelet-specific granules are synthesized, assembled, and packaged during megakaryocyte biogenesis, and at later stages of maturation they appear to come into contact with microtubules. The microtubules then transport them, via the microtubule motor protein kinesin, along the shafts of proplatelets until they reach the proplatelet tips.[145] Once inside a mature platelet, platelet granules remain relatively evenly dispersed throughout the cytoplasm, their contents awaiting threshold signals for cellular activation, at which time their membranes fuse with those of the plasma membrane, or more likely, the invaginated subdomains of the OCS.[146] The regulated secretion of granule contents ensures that hemostasis remains highly localized, an event that has been exploited to deliver non–platelet-derived procoagulant proteins like Factor VIII to sites of vascular injury.[147,148]

Box 26-1 **Platelet Granules and Their Contents**

A. α-Granules
  1. Membrane proteins enriched in the granule membrane: P-selectin, TLT-1, CD40 ligand (which is cleaved after exposure on the platelet surface to release soluble CD40L), and tissue factor.
  2. Membrane proteins present at similar concentrations as they are in the plasma membrane: GPIIb-IIIa, GPIb, PECAM-1, and perhaps many others
  3. Granule contents:
    a. Synthesized by megakaryocytes: thrombospondin, VWF, PF4, β-thromboglobulin, PDGF
    b. Endocytosed from plasma or origin not determined: albumin, fibrinogen, fibronectin, IgG, Gas6, coagulation factor V, and many chemokines and growth factors, including RANTES, bFGF, EGF, TGFβ, and VEGF
B. Dense (δ) granules: ADP, ATP, 5′-HT, Ca^{++}, polyphosphate
C. Lysosomes: acid hydrolases, elastase, cathepsins, and other degradative enzymes

5′-HT, 5′-hydroxytryptamine; *ADP,* adenosine diphosphate; *ATP,* adenosine triphosphate; *Ca^{2+},* calcium ion; *EGF,* epidermal growth factor; *FGF,* fibroblast growth factor; *IgG,* immunoglobulin G; *PDGF,* platelet-derived growth factor; *PF4,* platelet factor 4; *RANTES,* regulated on activation, normal T-cell expressed and secreted; *TGF,* transforming growth factor; *VFW,* von Willebrand factor.

Platelets harbor three distinct types of granules (Box 26-1). Two, α-granules and dense (δ) granules, are found only in platelets, whereas lysosomes are present in nearly all cell types. α-Granules are by far the most numerous, with as many as 40 to 80 per cell, and they contain a wide array of proteins and bioactive peptides. Box 26-1 classifies α-granule proteins as those that reside within the α-granule membrane (P-selectin being the most diagnostic), those pinocytosed from plasma and packaged (IgG, fibrinogen, albumin),[149] and those synthesized by megakaryocytes and stored (VWF, platelet factor 4, thrombospondin). mRNAs encoding the latter group have all been identified in the platelet cytoplasm. Upon platelet activation, granules redistribute toward the center of the cell,[146] at which time soluble *N*-ethylmaleimide–sensitive attachment protein receptor (SNARE) proteins within the α-granule membrane facilitate fusion[150,151] with members of the Rab family of low–molecular-weight GTPases playing a prominent role in vesicle docking and exocytosis.[152,153] After membrane fusion, P-selectin[154-156] and α-granule membrane-specific proteins like TLT-1[157] become expressed on the platelet surface, and the contents of the granule are released into the plasma milieu. Exposed P-selectin, diagnostic of an activated platelet,[158,159] serves to recruit leukocytes to the site of injury,[160] constituting one of a number of important links between thrombosis and inflammation.[161] Proteins secreted from the platelet include the adhesive ligands VWF and fibrinogen, which serve to support platelet–platelet interactions, growth factors and cytokines that promote cell migration,[162] wound healing,[163] maintenance of vascular integrity,[164] and autocrine factors like growth-arrest–specific gene 6 (Gas6)[165] and CD40L[166] that are released and rebind platelet receptors to help amplify platelet responsiveness. Finally, α-granules and their contents are a source of procoagulant proteins, releasing Factor V[167] and exposing tissue factor[75,168] to promote localized fibrin deposition at sites of vascular injury. Platelet α-granules, or at least their contents,[169] are severely reduced in an inherited bleeding disorder known as the *gray platelet syndrome (GPS)* (see Chapter 30).[170-173]

Dense granules (four to eight per platelet) are morphologically distinct, electron-opaque storage organelles that contain the vasoconstrictive substance serotonin,[174,175] adenine and guanine nucleotides like ADP and ATP, inorganic pyrophosphates[176] and polyphosphates,[177,178] and Ca^{2+}.[179] Complexes of the latter two are likely responsible for the dark appearance of these bodies in thin section electron microscopy.[180] Dense granule membranes contain a few components in common with lysosomal membranes, like granulophysin (CD63, lysosomal associated membrane protein [LAMP]-3)[181] and LAMP2,[182] as well as membrane proteins also present in α-granule membranes, like P-selectin,[181] suggesting a common origin during biogenesis. Like α-granule membrane proteins, these become expressed on the platelet-cell surface after granule fusion and secretion and can be used as platelet activation markers. Curiously, a number of plasma membrane GPs, including GPIb and GPIIb-IIIa, have also been reported in dense granule membrane.[183] Dense granule contents, especially ADP,[184] play a physiologically important role in hemostasis, as evidenced by characteristic platelet function defects in patients whose platelets lack dense granules or their contents,[185] collectively known as *storage pool disorders*.[186-188] Chédiak-Higashi and Hermansky-Pudlak[189] syndromes are two such examples of autosomal recessive dense granule defects that lead to platelet dysfunction and bleeding, the former being associated with immunodeficiency and the latter with albinism (see Chapter 30).

Primary lysosomes are the third organelle whose contents are secreted upon platelet activation, though only three or fewer per cell are normally identifiable.[190] Though a clear role for lysosomes in platelet function has not been identified, they do contain more than a dozen different acid hydrolases, cathepsins D and E, and other degradative enzymes that can be secreted if platelets are subjected to strong agonist stimulation. Their contents have been shown to be mildly reduced in the platelets of individuals with GPS,[191] in keeping with the notion that GPS is caused by a defect in packaging.[171-173] Membrane proteins on platelet lysosomes are typical of lysosomes in other cells, and include LAMP-1,[192] LAMP-2,[182] and LAMP-3.[181]

FEED-FORWARD AMPLIFICATION PATHWAYS INVOLVED IN PLATELET RECRUITMENT AND THROMBUS STABILITY

Although platelet adhesion, early activation signals, and granule release are necessary prerequisites for thrombus formation, efficient recruitment of additional platelets to the site of the vascular lesion to yield a stable platelet plug requires a host of additional receptor/ligand interactions, each of which results in signal transmission and therefore biochemical and cell biological changes that help sustain platelet activation. Among the most important of these is the binding of released ADP to one of its two platelet G-protein–coupled receptors, P2Y_{12}.[193,194] Like the α_2 receptor for epinephrine, P2Y_{12} is coupled to an inhibitory G protein that slows down the activity of adenylate cyclase, thus lowering cytosolic levels of cAMP (Fig. 26-4). This greatly potentiates platelet responses to other agonists, because it allows calcium ions (released from the DTS as a result of the action of phospholipase-generated 1,4,5-inositol trisphosphate [IP_3]) to remain cytosolic. "Bioavailable" calcium ions in turn support a host of additional cellular events, including more robust granule secretion, activation of metal ion-dependent proteases, and activation of cell surface integrins. The importance of ADP in amplifying platelet responses is illustrated by the clinical effectiveness of ticlopidine and clopidogrel—widely-used pharmacological agents that antagonize the activity of P2Y_{12}—in pacifying platelet reactivity and inhibiting platelet aggregation.[195]

In addition to ADP-induced, P2Y_{12}-mediated signaling, there are nearly a dozen other soluble ligands that are either generated or released at sites of vascular injury and that function in signal amplification and platelet activation. These can be broken into three classes according to the type of platelet receptor to which they bind (Fig. 26-5). The first class of ligands is comprised of ADP, thrombin, TXA_2, and serotonin (5′-hydroxytryptamine), each of which bind to a specific GPCR that is coupled to

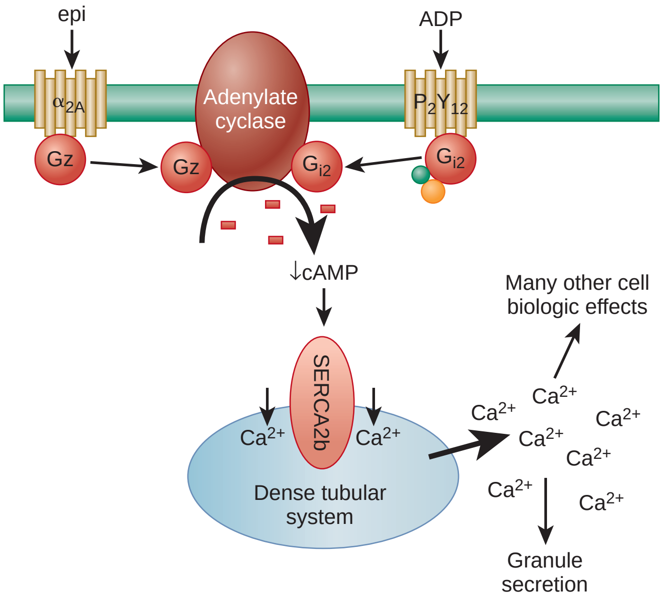
epi
ADP
α_{2A}
Adenylate cyclase
P_2Y_{12}
Gz
Gz
G_{i2}
G_{i2}
↓cAMP
Many other cell biologic effects
SERCA2b
Ca^{2+}
Ca^{2+}
Dense tubular system
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
Granule secretion

Figure 26-4 G_{α}i-protein coupled receptors on platelets inhibit adenylate cyclase and lower cAMP levels. Shown are the two major receptors that are responsible for dampening the activity of adenylate cyclase. As cAMP levels drop, the ability of the SERCA2b calcium pump to sequester cytosolic calcium ions is impaired, thereby allowing calcium-mediated activation events to occur more readily. *ADP,* Adenosine diphosphate; *Ca^{2+},* calcium ion; *epi,* epinephrine; *SERCA2b,* sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase.

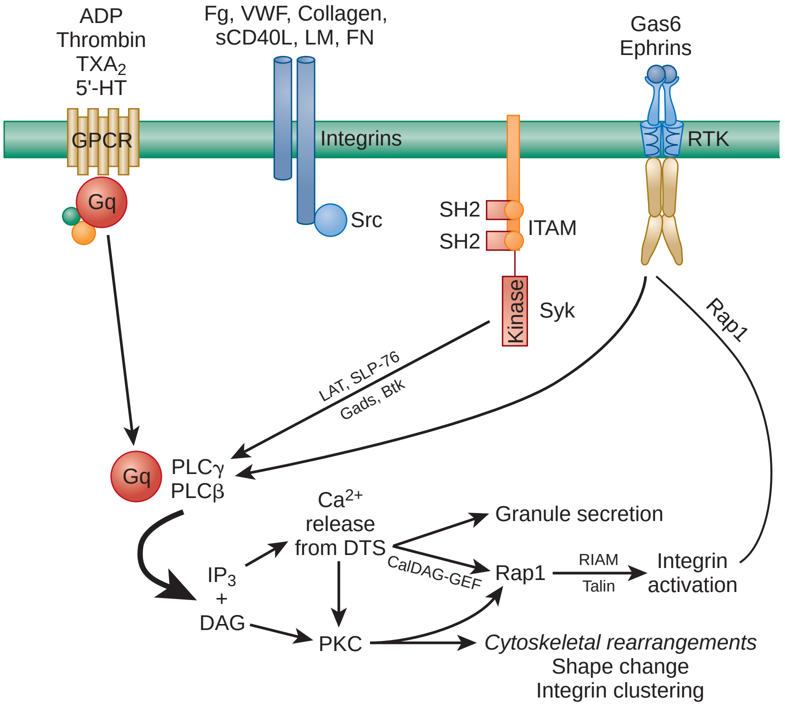
ADP
Thrombin
TXA_2
5′-HT
Fg, VWF, Collagen,
sCD40L, LM, FN
Gas6
Ephrins
GPCR
Integrins
RTK
Gq
Src
SH2
SH2
ITAM
Kinase
Syk
Rap1
LAT, SLP-76
Gads, Btk
Gq
PLCγ
PLCβ
Ca^{2+} release from DTS
Granule secretion
IP_3 + DAG
CalDAG-GEF
Rap1
RIAM
Talin
Integrin activation
PKC
Cytoskeletal rearrangements
Shape change
Integrin clustering

Figure 26-5 Agonist receptors that initiate and/or amplify platelet activation responses. Three different families of receptors are involved in signal amplification pathways: Gq-coupled GPCRs, integrins, and RTKs. Note how each activates either the γ or β isoform of PLC. The sum total of PLC-generated products serves to determine the activation state of the platelet, its ability to respond to vascular injury, and its participation in thrombus growth. *5′HT,* 5′-Hydroxytryptamine; *ADP,* adenosine diphosphate; *DAG,* diacylglycerol; *DTS,* dense tubular system; *GPCR,* G_s-protein coupled receptor; *IP_3,* 1,4,5-inositol triphosphate; *ITAM,* immunoreceptor tyrosine-based activation motif; *PLC,* phospholipase C; *RIAM,* $Rap1_{GTP}$-interacting adaptor molecule; *RTK,* receptor tyrosine kinase; *VWF,* von Willebrand factor.

the heterotrimeric a subunit, Gq. Thus ADP binds to P2Y$_1$,[196-200] thrombin to the protease activated receptors PAR1 and PAR4,[201,202] TXA$_2$ to the thromboxane receptor,[203,204] and serotonin to 5HT2A.[205] When released as a consequence of ligand binding to any of these GPCRs, the Gq subunit binds to the β isoform of phospholipase C (PLC). PLCs are lipid hydrolases that act on membrane-associated phosphatidylinositol (PI)–4,5-P$_2$ to produce the second messengers IP$_3$ and diacylglycerol (DAG). IP$_3$ binds and opens calcium channels, and DAG activates the most abundant forms of protein kinase C (PKC), initiating additional signaling cascades downstream of this serine/threonine kinase. As shown in Figure 26-5, it is the sum of these products—generated by the γ2 isoform of PLC in response to adhesion and by the β isoform of PLC in response to ligand-GPCR interactions—that the platelet integrates when deciding whether to become fully activated. This concept is important in the context of designing pharmacologic strategies to inhibit platelet function, because blocking adhesion and its consequent activation of PLCγ2 leaves PLC β-mediated platelet activation largely intact and vice versa.

The second class of signal amplifiers is the cell surface integrins themselves.[206] As illustrated in the middle section of Figure 26-5, when ligands bind to integrins, Src-family kinases (SFKs) associated with integrin cytoplasmic tails[207] trigger a series of incompletely understood SFK-mediated amplification events[208] that have been collectively termed *outside-in signaling*.[209-211] This has recently been demonstrated after interaction of the major platelet integrin αIbβ$_3$ with its ligand fibrinogen;[212] however, signals also emanate from α$_2$β$_1$[141,213,214] and α$_6$β$_1$[139] when platelets encounter collagen and laminin at sites of vascular injury. Activation signals from the latter two may be relatively weak by comparison, owing to the fact that only a few thousand of each are expressed on each platelet, compared with 50 to 80 thousand αIbβ$_3$ receptors per cell.[215,216] Protein kinases Syk and FAK have been shown to become activated downstream of αIIbβ$_3$ engagement, as has PLCγ$_2$.[217] However, the details of these events remain to be worked out. A less well-defined autocrine loop that utilizes integrin-mediated outside-in signal amplification is the cleavage and rebinding of soluble CD40L after α-granule fusion and secretion.[166]

The third class of feed-forward amplification reactions is mediated by ligand-activated plasma membrane receptor tyrosine kinases (RTKs). The first of these to be described were receptors for Gas6, a vitamin K-dependent protein related to the anticoagulant protein S. Gas6 is thought to reside in platelet α-granules,[218,219] and like other α-granule proteins, becomes secreted upon platelet activation. Interestingly, platelets have three different receptors for Gas6: Axl, Sky, and Mer, all of which have active cytoplasmic tyrosine kinase activity (see Fig. 26-5). Upon engagement, Gas6 receptors trigger tyrosine phosphorylation of the β$_3$ integrin cytoplasmic domain and thereby support outside-in integrin signaling, as well as activate PI3-kinase to further sustain granule secretion.[220] Platelets also express two members of the Eph receptor tyrosine kinase family, EphA4 and EphB1, which when in contact with their membrane-bound counter-receptor, Ephrin B1, stimulate tyrosine phosphorylation of the integrin β$_3$ tail and activate the integrin activator, Rap1b.[221] Like Gas6 signaling,[165,222,223] genetic loss or pharmacologic blockade of Ephrin/Eph kinase interactions results in decreased ability to form a stable thrombus or retract a fibrin clot.[224]

ACTIVATION OF THE MAJOR PLATELET INTEGRIN, αIIBβ$_3$ (GPIIB-IIIA COMPLEX): THE FINAL COMMON ENDPOINT OF PLATELET ACTIVATION.

Human platelets express at least five different members of the 24-member integrin family,[211,225] including three β1 integrins (α$_2$β$_1$, α$_5$β$_1$, and α$_6$β$_1$—specific for collagen, fibronectin, and laminin, respectively) and two β$_3$ integrins—avβ$_3$ and its close relative, αIIbβ$_3$ (also known as the GPIIb-IIIa complex). αIIbβ$_3$ is by far the most abundant and well-studied. This section will focus on our current understanding of how αIIbβ$_3$ becomes transformed from a resting to an active, ligand binding-competent conformation, with the understanding that the biochemical and cell biological principles described for this integrin may well apply to the others.

As shown in schematic form on the left side of Figure 26-6, αIIbβ$_3$ exists on the platelet surface in a bent-over conformation that is unable to associate effectively with its major soluble ligands, fibrinogen, VWF, and fibronectin.[226,227] Though relatively short, the cytoplasmic domains of αIIb and β$_3$ are thought to play a key role in maintaining the “off” state of this integrin complex, owing to weak charge interactions between them[228,229] that allow the hydrophobic transmembrane domain helices of each subunit to interact and maintain the integrin in a low-affinity state.[230,231] When platelets become activated—either by adhesion– or soluble-agonist–mediated events—calcium and DAG, generated as a result of the actions of PLCγ2 and PLCβ (see Figs. 26-3 and 26-5), bind to and activate PKC and the guanine exchange factor, CalDAG-GEF1. As illustrated in Figure 26-6, each of these can independently activate Rap1,[232-236] a low molecular weight GTPase that has been implicated in integrin activation.[237-239] Rap1 activates integrins via an effector molecule known as the *Rap1$_{GTP}$-interacting adaptor molecule (RIAM),* which recruits the highly abundant cytosolic protein, talin to the inner face of the plasma membrane, forming an integrin activation complex. Binding of RIAM-associated talin to the β$_3$ integrin subunit represents the final common step in integrin activation,[240,241,242] because it disrupts the weak ionic clasp between the αIIbβ$_3$ tails, allowing for tail separation[243] and a dramatic, rapid unfolding of the extracellular domain.[227] Simultaneous conformational changes in the integrin head[244,245] result in formation of an integrin receptor having high affinity for its soluble adhesive ligands. In a still-to-be-defined mechanism, the cytoskeletal protein kindlin 3 then contributes to activating and/or maintaining the activation state of αIIbβ$_3$.[246,247] As integrin-rich adhesion complexes mature, the cytoskeletal protein vinculin takes over for the nascent Rap1/RIAM complex and stabilizes adhesions.[248] Finally, integrins

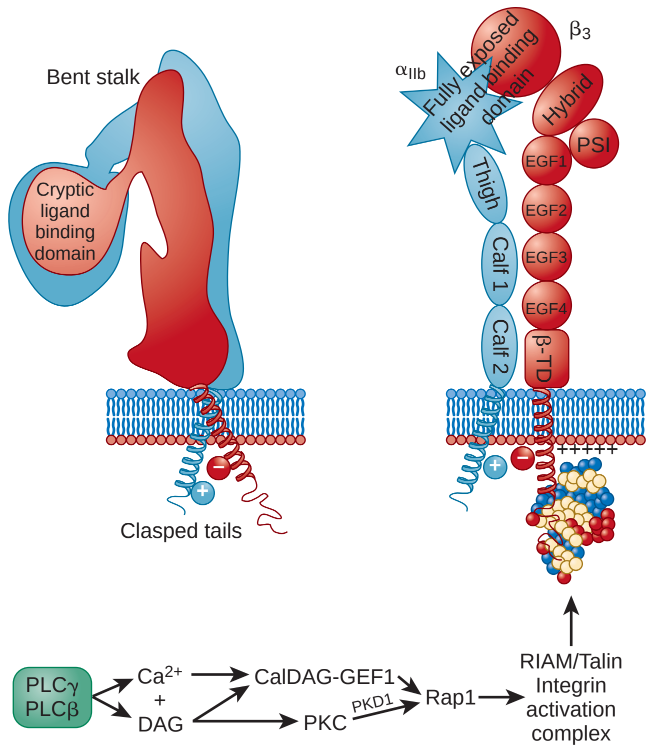

Figure 26-6 Integrin activation. Calcium and DAG, generated as a result of the combined actions of PLCγ and PLCβ, activate two proteins: PKC and the Rap guanine exchange factor, CalDAG-GEF1. Each of these is able to independently activate the small GTPase, Rap1. In its GTP-bound form, Rap1 binds to and activates one of its effector molecules, RIAM, which then binds talin, forming an integrin activation complex (IAC). When the IAC binds specific sites within the β_3 cytoplasmic domain, the clasp breaks, thereby destabilizing transmembrane domain helix associations that are thought to maintain the integrin in its low-affinity state. Breaking the hinge causes extensive conformational changes in the extracellular domain to produce a high-affinity, ligand binding-competent integrin. *DAG,* Diacylglycerol; *PKC,* protein kinase C; *PLC,* phospholipase C; *RIAM,* Rap1GTP-interacting adaptor molecule. *(Modified from Wegener, Partridge AW, Han J, et al: Structural basis of integrin activation by talin.* Cell *128:171, 2007.)*

cluster,[249] ensuring that bound ligands effectively broker with high avidity the platelet–platelet interactions that permit thrombus growth and stabilization.

CELL SURFACE AND CYTOSOLIC PROTEINS THAT LIMIT PLATELET RESPONSIVENESS

As anyone who has witnessed a myocardial infarction or thrombotic stroke will know, unrestrained thrombus growth at inappropriate sites can be as harmful as excessive bleeding, because it can result in vessel occlusion, ischemia, and tissue damage. Numerous active processes are in place to limit platelet responsiveness in healthy vessels, to keep thrombus growth localized to specific sites of vascular injury, and to facilitate dissolution of a platelet plug during recovery.

Exogenous inhibitors of platelet function produced by a healthy endothelial lining contribute to platelet passivation via continuous generation of PGI_2 and NO, which act on platelets by activating adenylate and guanylate cyclases to increase intracellular levels of cAMP and cGMP, subsequently activating PKA and PKG (see Fig. 26-2). PKG controls the threshold for platelet activation primarily by phosphorylating the IP_3 receptor-associated cGMP (IRAG) kinase substrate,[250] a protein that associates with PKG and IP_3 receptor type I to inhibit IP_3-induced calcium release from intracellular stores.[251,252] Both PKA and PKG interfere with platelet activation by phosphorylating and inactivating vasodilator-stimulated phosphoprotein (VASP),[253] a molecule with anticapping activity that is important for the processes of actin polymerization and filopod formation.[254-258] PKCδ also binds VASP and interferes with its ability to promote filopodia formation, although this pathway is unique to collagen-stimulated platelets and does not involve regulation of PKA- or PKG-mediated VASP phosphorylation.[259]

In addition to exogenous inhibitors, platelets themselves contain a plethora of inhibitory proteins designed to keep platelet activation in check, either by setting a threshold for platelet activation or by turning off activation signals once sufficient platelet responses have occurred. Regulator of G-protein signaling (RGS) proteins modulate GPCR-mediated platelet accumulation and thrombus formation at sites of vascular injury,[260,261] as do members of the β-arrestin family.[262] Platelets also express three members of the immunoglobulin gene–immunoreceptor tyrosine-based inhibitory motif (Ig-ITIM) family of inhibitory receptors, PECAM-1,[263-266] CEACAM-1,[267] and G6b-B,[92,268,269] each of which contains in its cytoplasmic domain tyrosine residues that, when phosphorylated form docking sites for phosphatases that function to shut down tyrosine kinase–dependent signaling reactions.[270-272] Interestingly, one of the five tyrosine kinases present in platelets, Lyn, actually limits platelet aggregation both in response to GPVI-specific stimuli[273] and after platelet spreading on immobilized fibrinogen,[274] perhaps via its ability to phosphorylate the ITIM tyrosines of PECAM-1.[275,276]

Several inhibitory pathways have been identified in platelets that either regulate, or are regulated by PI3 kinase, a lipid kinase that phosphorylates the 3′ position of PI 4,5-diphosphate (PIP_2) to generate PI 3,4,5-triphoshate (PIP_3), thereby creating docking sites on the inner face of the plasma membrane for pleckstrin homology (PH) domain–containing molecules.[277] The actions of PI3-kinase are opposed by the lipid phosphatase, SH2 domain-containing inositol 5′-phosphatase 1 (SHIP1), which hydrolyzes the 5′-phosphate of PIP_3.[278,279] In platelets, SHIP1 has been shown to downregulate PIP_3 generation after $\alpha IIb\beta_3$-mediated outside-in signaling,[274] and thus it may interfere with feed-forward amplification pathways that increase the efficiency with which platelets are recruited to growing thrombi. Interestingly, the 5′-inositol phosphatase activity of SHIP1 appears to be enhanced at least in part by the actions of the Src-family tyrosine kinase,

Akt (also known as *protein kinase B*) is a PH domain–containing serine/threonine kinase that is a well-characterized effector of PI3 kinase.[280,281] Akt contributes positively to platelet activation in multiple ways, one of which appears to be by inactivating the serine/threonine kinase, glycogen synthase kinase 3 (GSK3). The GSK3 family is comprised of three isoforms (α, β, β_2) that are constitutively active in resting cells but which become inactivated in activated cells by Akt-mediated phosphorylation.[282] Platelets express two isoforms of GSK3 (α and β), both of which become phosphorylated and inactivated after exposure of the platelet to multiple agonists that activate PI3 kinase and Akt.[283] Whereas initial studies reported that specific inhibitors of GSK3 activity block rather than enhance platelet responses to agonist stimulation,[283] more recent data implicate the β isoform of GSK3 as a negative regulator of platelet function both in vitro and in vivo.[284] Finally, the serine/threonine kinase PKCδ has also been shown to negatively regulate platelet activation after platelet exposure to collagen.[259,285]

NONHEMOSTATIC ROLES FOR PLATELETS: BLOOD VESSEL MAINTENANCE AND REPAIR, INFLAMMATION AND INFECTION, AND ATHEROTHROMBOSIS

In addition to being essential for primary hemostasis, activated platelets and their secreted products have the ability to influence a broad array of pathophysiologic processes. These include leukocyte trafficking and inflammation, tissue regeneration and angiogenesis, and both the beginning and end stages of atherosclerosis.

Activated platelets that become spread on components of the extracellular matrix or on each other display an altered surface phenotype, the most prominent of which is exposure of several thousand copies of the α-granule-derived membrane protein, P-selectin. P-selectin is also expressed on cytokine-activated endothelial cells. Thus after either a thrombotic or inflammatory event, P-selectin appears on the luminal face of the vessel wall, where it serves to recruit monocytes and neutrophils into the underlying tissue by binding PSGL-1, a constitutively expressed counter-receptor for P-selectin that is present on most leukocytes. In vivo, mice lacking P-selectin exhibit greatly diminished leukocyte rolling, delayed recruitment into sites of inflammation, and increased susceptibility to infection.[286,287] Whereas endothelial P-selectin no doubt has a major role in leukocyte capture, platelet P-selectin likely plays a prominent role in "secondary capture."[288,289] As in platelets, tethering also initiates activation of leukocyte integrins, which are then able to mediate cell spreading and transendothelial migration. P-selectin–PSGL-1 interactions therefore constitute an important link between thrombosis and inflammation.[161,290] Other platelet/leukocyte receptor–counter-receptor pairs have also been shown to facilitate the inflammatory response, including binding of platelet-associated fibrinogen binding to the leukocyte integrin, MAC-1[291] and platelet JAM-3 binding to MAC-1 on monocytes[292] and dendritic cells.[293]

In addition to forming a platform for leukocyte recruitment during acute inflammation, platelets also deliver to the vessel wall proinflammatory chemokines that are thought to play roles in the development of atherosclerosis by promoting further chemoattraction of leukocytes and by stimulating proliferation of vessel wall smooth muscle cells and fibroblasts. Such secreted factors include the C-X-C chemokine platelet factor 4, macrophage inflammatory protein (MIP) 1a, the C-C chemokine regulated on activation, normal T-cell expressed and secreted (RANTES), CD40 ligand, platelet-derived growth factor (PDGF), and TGFβ.[161,290,294,295] Activated platelets also synthesize *de novo* IL 1β,[74,296,297] a potent stimulator of endothelial cells and monocytes that upregulates adhesion molecule expression. Thus platelets appear to contribute in a number of ways to the development and progression of atherosclerotic lesions.

So as not to leave the reader with the impression that platelets only exacerbate chronic human disease, it should be noted that as early as 1969[164] platelets and their secreted products were shown to be able to "nurture" the vascular endothelium. They are increasingly used as a source of biological response modifiers in a plethora of conditions, including organ preservation, gum restoration after dental procedures, and tissue repair after surgery.[163] The ability of platelets to adhere to sites of vascular injury and secrete both degradative enzymes and at the same time growth-promoting factors like vascular endothelial growth factor (VEGF), PDGF, fibroblast growth factor (FGF), epidermal growth factor (EGF) and angiopoietin 1 allows them to play a uniquely supportive role in endothelial cell migration and survival during the process of wound healing and angiogenesis.[162] Platelet-rich plasma to treat bone and cartilage repair after sports injuries, though largely unsubstantiated, is also finding increasing use.[298-301] Platelet-secreted factors have also been shown to have a critical role during the time when embryonic blood vessels and lymphatic vessels separate during development[302,303] in a mechanism that involves binding of the ITAM-bearing platelet receptor CLEC-2 to the lymphatic endothelial marker podoplanin.[304] Blood flow also appears to be a critical regulator of this developmental event.[305] Platelet secreted products also play a vital role in another developmental event, namely closing of the ductus arteriosis.[306] Finally, platelets bind via their cell-surface CD36 receptor to malarial-infected red blood cells, an event that induces secretion of platelet factor 4 (PF4) from platelet α granules. PF4 in turn binds to the Duffy antigen on the red blood cells, causing lyses of the parasite digestive vacuole, ultimately killing the parasites within.[307,308]

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CHAPTER 27

Blood Coagulation

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CHAPTER OUTLINE

OVERVIEW OF COAGULATION
Initiation
Propagation
Termination
Flow Regulation
THE VITAMIN K–DEPENDENT PROTEIN FAMILY
Factor VII (Proconvertin, Convertin)
Factor IX (Plasma Thromboplastin Component, Christmas Factor, Hemophilia B Factor)
Factor X (Stuart Factor)
Factor II (Prothrombin)
Protein C (Autoprothrombin II-A)
Protein S
Protein Z
FACTOR XI, THE CONTACT PATHWAY CROSSOVER (PLASMA THROMBOPLASTIN ANTECEDENT)
COFACTOR PROTEINS
Factor V (Labile Factor)
Factor VIII (Antihemophilic Factor, Factor VIII:C)
von Willebrand Factor (Ristocetin Cofactor)
Tissue Factor (Tissue Thromboplastin CD142 and Coagulation Factor III)
Thrombomodulin
THE INTRINSIC (ACCESSORY) PATHWAY FACTORS
Factor XII (Hageman Factor, Contact Factor)
Plasma Prekallikrein (Fletcher Factor)
High–Molecular-Weight Kininogen (Fitzgerald Factor, Williams Factor)
STOICHIOMETRIC INHIBITORS
Tissue Factor Pathway Inhibitor (Extrinsic Pathway Inhibitor, Lipoprotein-Associated Coagulation Inhibitor)
Antithrombin III
Heparin Cofactor II (Leuserpin 2)
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PROTEINS OF CLOT FORMATION
Factor XIII (Fibrin-Stabilizing Factor)
Fibrinogen (Factor I) and Fibrin
CONCLUSION

OVERVIEW OF COAGULATION

Generation of the enzyme thrombin from its precursor prothrombin is the central event in the blood coagulation process, essential for hemostasis and the culprit in thrombosis. Blood loss through lack of hemorrhage control has captured the attention of individuals from many walks of life throughout history. At the end of the nineteenth century, the role of thrombin in the clotting of fibrinogen was accurately described by Schmitt.[1] Since that time hemostasis has been synonymous with blood clotting. Congenital diseases associated with absence or reduced production of thrombin (the hemophilias) represent important clinical problems (see Chapters 31 and 32). In contrast, the unregulated production of thrombin in an inappropriate location leads to another series of diseases associated with thrombotic occlusion (see Chapter 33).

The development of plasma clot–based assays in vitro has played a central role in establishing the logical relationships between the reactants and reactions of hemostasis (Fig. 27-1). The prothrombin time (PT) and the activated partial thromboplastin time (APTT) both rely on the end point of fibrin formation (clotting), which occurs with the generation of less than 5% of the thrombin ultimately produced. The primary pathway leading to hemostatic and thrombotic pathology appears to be associated with the tissue factor (TF)–initiated, “extrinsic” coagulation pathway. In contrast, components unique to the “intrinsic,” or contact, pathway (factor XII, prekallikrein, high–molecular-weight kininogen [HMWK]) do not appear to be essential to hemostasis.

The observation that tissue extracts added to plasma initiated clotting led Quick to develop the PT assay.[2] This assay is still used extensively in the management of warfarin anticoagulant therapy and establishes the continuity of components in the *extrinsic pathway*. The tissue preparations (thromboplastin) contain the protein TF and

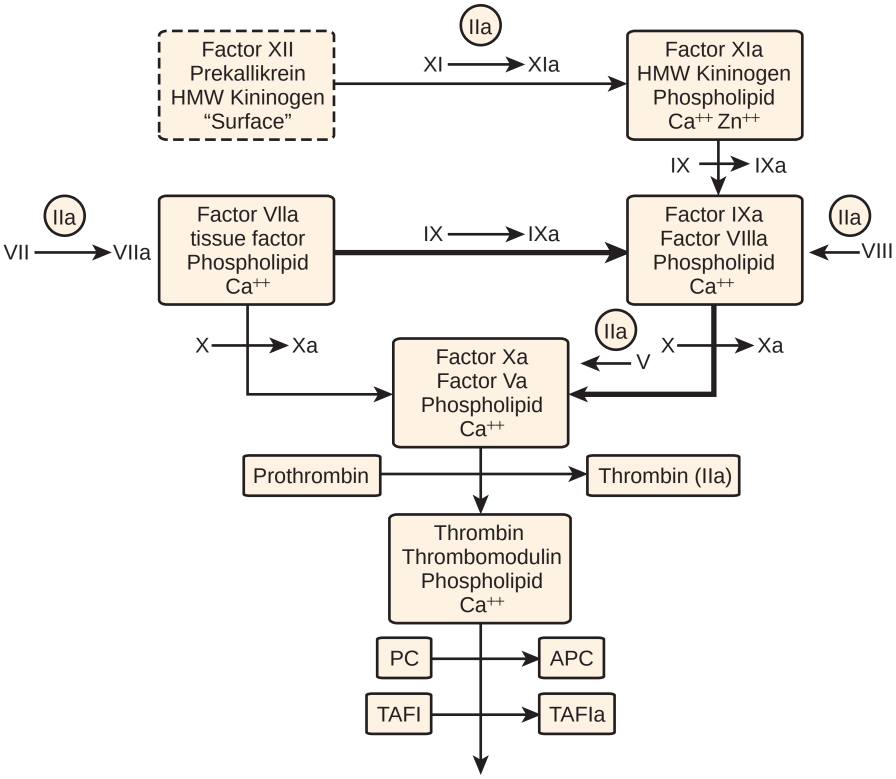

Figure 27-1 Schematic of the catalysts of the hemostatic system. The outline of the "contact catalyst" of the intrinsic pathway is dashed because of its uncertain contributions to hemostasis. The contribution of the contact catalyst to thrombosis is unresolved. The various points at which thrombin catalyzes its own generation by the conversion of zymogens and procofactors to the active species required for catalyst formation are illustrated by circles (IIa). *APC,* Activated protein C; *HMW,* high–molecular-weight; *PC,* protein C; *TAFI,* thrombin-activatable fibrinolysis inhibitor.

phospholipids, with plasma contributing factor VIIa and calcium to form the *extrinsic factor Xase*, which converts plasma factor X to factor Xa (see Fig. 27-1). Factor Xa subsequently combines with plasma factor Va, phospholipids, and calcium to activate prothrombin to thrombin, the latter serving as the catalyst that cleaves fibrinogen to produce fibrin, the clotting end point.

Langdell and colleagues[3] subsequently explored the spontaneous clotting of recalcified citrate plasma and developed the APTT, thus providing the tool that defined the *intrinsic pathway* of coagulation (see Fig. 27-1). This pathway, dependent only on the protein components present in blood plasma, was formalized by the cascade/waterfall hypotheses of MacFarlane[4] and Davie and Ratnoff.[5] The APTT assay involves the addition of an artificial surface, phospholipids, and calcium chloride to blood plasma. Initiation of this contact pathway begins with the surface-dependent activation of factor XII to factor XIIa; this leads to activation of prekallikrein to kallikrein, which, together with HMWK, provides a self-amplifying catalytic system that results in rapid activation of factor XI to factor XIa. Factor XIa, together with HMWK, phospholipid, and metal ions, catalyzes the activation of factor IX to factor IXa. Factor IXa, with factor VIIIa and a membrane surface, form the *intrinsic factor Xase*, which activates factor X to factor Xa as illustrated in Figure 27-1.

By the mid-1980s, the conclusion that TF was probably the principal initiator of the hemostatic process[6] and the absence of bleeding associated with deficiencies of the contact pathway initiators (factor XII, prekallikrein, and HMWK) presented a significant conundrum for the scientific community. This was accentuated by the absence of linkage between hemophilia A and B and the extrinsic pathway as evaluated by the PT assay. Subsequently, the intersections between intrinsic and extrinsic and the reactions linking these two "classical" pathways were identified.[7,8,9]

Of equal importance to the procoagulant process is its regulation by stoichiometric and dynamic inhibitory systems. Antithrombin (AT)[10] and tissue factor pathway inhibitor (TFPI)[11] are the primary stoichiometric inhibitors, whereas the thrombin-thrombomodulin–protein C system is dynamic in its inhibitory function. The latter catalyst activates protein C to activated protein C (APC), which proteolytically destroys the cofactors factor Va and factor VIIIa, thereby eliminating the intrinsic factor Xase and prothrombinase. TFPI blocks the factor VIIa–TF–factor Xa product complex,[12] thus effectively neutralizing the extrinsic factor Xase (Fig. 27-2). However, TFPI is present in low abundance (≈2.5 nM) in blood and can only delay the hemostatic reaction.[13] AT, normally present in plasma at twice the concentration (≈2 to 3 μM) of any potential coagulation enzyme, neutralizes all the

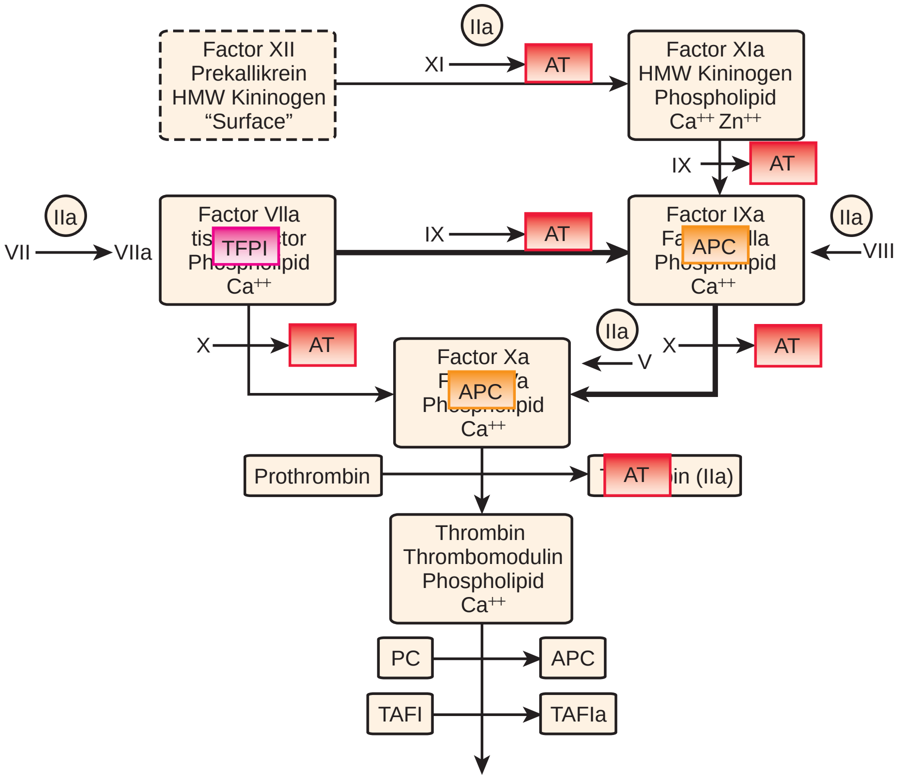
IIa
Factor XII
Prekallikrein
HMW Kininogen
"Surface"
XI
AT
Factor XIa
HMW Kininogen
Phospholipid
Ca++ Zn++
IX
AT
IIa
VII
VIIa
Factor VIIa
tissue factor
TFPI
Phospholipid
Ca++
IX
AT
Factor IXa
Factor VIIIa
APC
Phospholipid
Ca++
IIa
VIII
X
AT
IIa
X
AT
V
Factor Xa
Factor Va
APC
Phospholipid
Ca++
Prothrombin
AT
Thrombin (IIa)
Thrombin
Thrombomodulin
Phospholipid
Ca++
PC
APC
TAFI
TAFIa

Figure 27-2 Points of regulation of the catalyst of Figure 27-1 by tissue factor pathway inhibitor (TFPI), antithrombin III (AT), and activated protein C (APC) are illustrated. *HMW,* High–molecular-weight; *PC,* protein C; *TAFI,* thrombin-activatable fibrinolysis inhibitor.

procoagulant serine proteases, primarily in the uncomplexed state.[10]

The dynamic protein C system is activated by binding of thrombin to vascular thrombomodulin, with this complex (Fig. 27-3) converting protein C to its activated species APC.[14] APC proteolytically inactivates factors Va and VIIIa.[15] The protein C system, TFPI, and AT cooperate to produce steep TF concentration thresholds that act like a digital "switch" to allow or block thrombin formation.

Thus there are three membrane-associated procoagulant complexes[16] (corresponding to the proteases factor IXa, factor Xa, and factor VIIa), each interacting with a cofactor protein (factor VIIIa, factor Va, TF). An analogous anticoagulant complex is composed of thrombin-thrombomodulin (see Fig. 27-3). Each complex involves a vitamin K–dependent serine protease, phospholipid, and an associated cofactor protein assembled on a membrane surface provided by an exposed, activated or damaged cell.

The membrane-binding properties of the vitamin K–dependent proteins (VKDPs) are the consequence of posttranslational γ-carboxylation of these macromolecules. The cofactor proteins are either membrane-binding proteins (factor Va, factor VIIIa) recruited from plasma as procofactors or intrinsic membrane proteins (TF, thrombomodulin) regulated by presentation. Each complex catalyst is orders of magnitude (10^3- to 10^6-fold) more efficient than the individual serine protease acting on its substrate in solution.[17-19] When considered on a temporal scale, the amount of thrombin produced

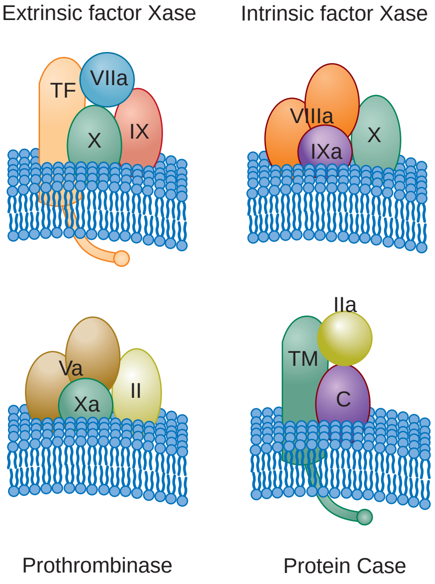
Extrinsic factor Xase
Intrinsic factor Xase
TF
VIIa
X
IX
VIIIa
IXa
X
Va
Xa
II
IIa
TM
C
Prothrombinase
Protein Case

Figure 27-3 Vitamin K–dependent complexes. Three procoagulant complexes (extrinsic factor Xase, intrinsic factor Xase, and prothrombinase) and one anticoagulant complex (protein Case) are illustrated. Each membrane complex consists of a vitamin K–dependent serine protease (factor VIIa, factor IXa, factor Xa, or thrombin [IIa]) and a soluble or cell surface–associated cofactor (tissue factor [TF] factor VIIIa, factor Va, or thrombomodulin [TM]). Each serine protease is shown in association with the appropriate cofactor protein and zymogen substrate or substrates (factors IX, X, and II and protein C) on the membrane surface. The membrane serves as a scaffold for the coagulation reactants and enhances reaction rates by 105- to 106-fold. *(Redrawn with permission from Mann KG: Dynamics of Hemostasis. Essex Junction, Vt., Haematologic Technologies, 2002.)*

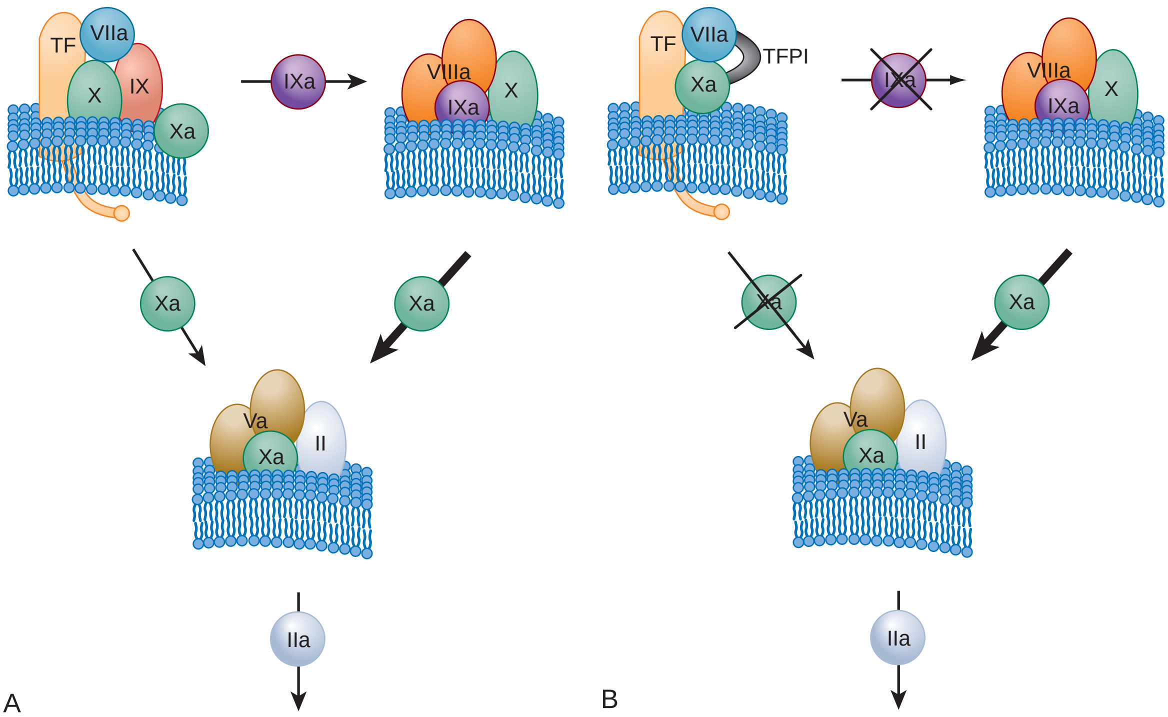

Figure 27-4 **A,** The factor Xa generated by the factor VIIa–tissue factor (TF) complex catalyzes the formation of a small amount of thrombin, which activates factors V and VIII and leads to the presentation of the intrinsic factor Xase (factor IXa-VIIIa) and prothrombinase (factor Xa-Va). At this point in the reaction, generation of factor IXa is cooperatively catalyzed by membrane-bound factor Xa and by factor VIIa–TF. The thick arrow representing generation of factor Xa by the intrinsic factor Xase illustrates the more efficient generation of factor Xa by this catalyst. **B,** The tissue factor pathway inhibitor (TFPI) interacts with the factor VIIa–TF–factor Xa product complex to block TF-initiated activation of both factor IX and X, thus leaving the factor IXaβ-VIIIa complex as the only viable catalyst for activation of factor X. *(Redrawn with permission from Mann KG: Dynamics of Hemostasis. Essex Junction, Vt., Haematologic Technologies, 2002.)*

by prothrombinase in 1 minute would be produced by factor Xa acting alone in 6 months. As a consequence, factor Xa is ineffective in hemostasis without the presentation of both factor Va and the platelet membrane binding sites on which prothrombinase is formed. The blood coagulation reaction can be described as occurring in three overlapped phases: initiation, propagation, and termination.

Initiation

The factor VIIa–TF–membrane complex (extrinsic factor Xase) (Fig. 27-4, *A*; also see Fig. 27-3) catalyzes the activation of both factor IX and factor X, the latter being the more efficient substrate.[7] Thus the initial product formed is factor Xa. Feedback cleavage of factor IX by membrane-bound factor Xa enhances the rate of generation of factor IXa in a cooperative process with the factor VIIa–TF complex.[9]

The initially formed, membrane-bound factor Xa activates small amounts of prothrombin to thrombin.[20] This initial activation of prothrombin provides the thrombin essential for acceleration of the hemostatic process by serving as the activator for platelets and the procofactors factor V[15] and factor VIII (see Fig. 27-1). Once factor VIIIa is formed, the factor IXa generated by the factor VIIa–TF complex combines with factor VIIIa on the activated platelet membrane to form the intrinsic factor Xase (see Figs. 27-3 and 27-4, *A*), which becomes the major activator of factor X. The factor IXa–factor VIIIa complex is 10^6-fold more active as a factor X activator and 50 times more efficient than the factor VIIa–TF complex in catalyzing activation of factor X (thus the bulk of factor Xa is ultimately produced by the factor IXa–factor VIIIa complex).

Propagation

As the reaction progresses, generation of factor Xa by the more active intrinsic factor Xase exceeds that of the extrinsic factor Xase complex.[21] In addition, the extrinsic factor Xase complex is subject to inhibition by TFPI[11] (Fig. 27-4, *B*). As a consequence, most (>90%) of factor Xa is ultimately produced by the factor VIIIa–factor IXa complex. In hemophilia A and hemophilia B, this amplification of factor Xa generation does not occur.[22] Factor Xa combines with factor Va on the activated platelet membrane receptors, and this factor Va–factor Xa prothrombinase catalyst (see Figs. 27-3 and 27-4) converts most prothrombin to thrombin. Prothrombinase is 300,000-fold more active than factor Xa alone in catalyzing activation of prothrombin.[17]

Termination

The same hemostatic process required for preventing leaks from the vasculature may also be life threatening when responsible for intravascular occlusion. Thus equally important to the hemostatic process are the stoichiometric and dynamic inhibitory systems, which block the presentation of thrombin. The sum of inhibitory functions is far in excess of the potential coagulant response. These inhibitory processes governed by TFPI, AT, and the protein C pathway act in synergy to provide activation thresholds, for which a minimum level of TF stimulation must be achieved before significant thrombin generation can occur.[23] The termination phase thus occurs throughout the reaction process.

Flow Regulation

Current data are consistent with the concept of a two-compartment model for regulation of the procoagulant response to a breach in a blood vessel (Fig. 27-5). Regulation of clotting in the extravascular space, apart from providing the initiating TF, is primarily passive and controlled by the availability of fresh blood, whereas regulation in the intravascular compartment is dynamic.

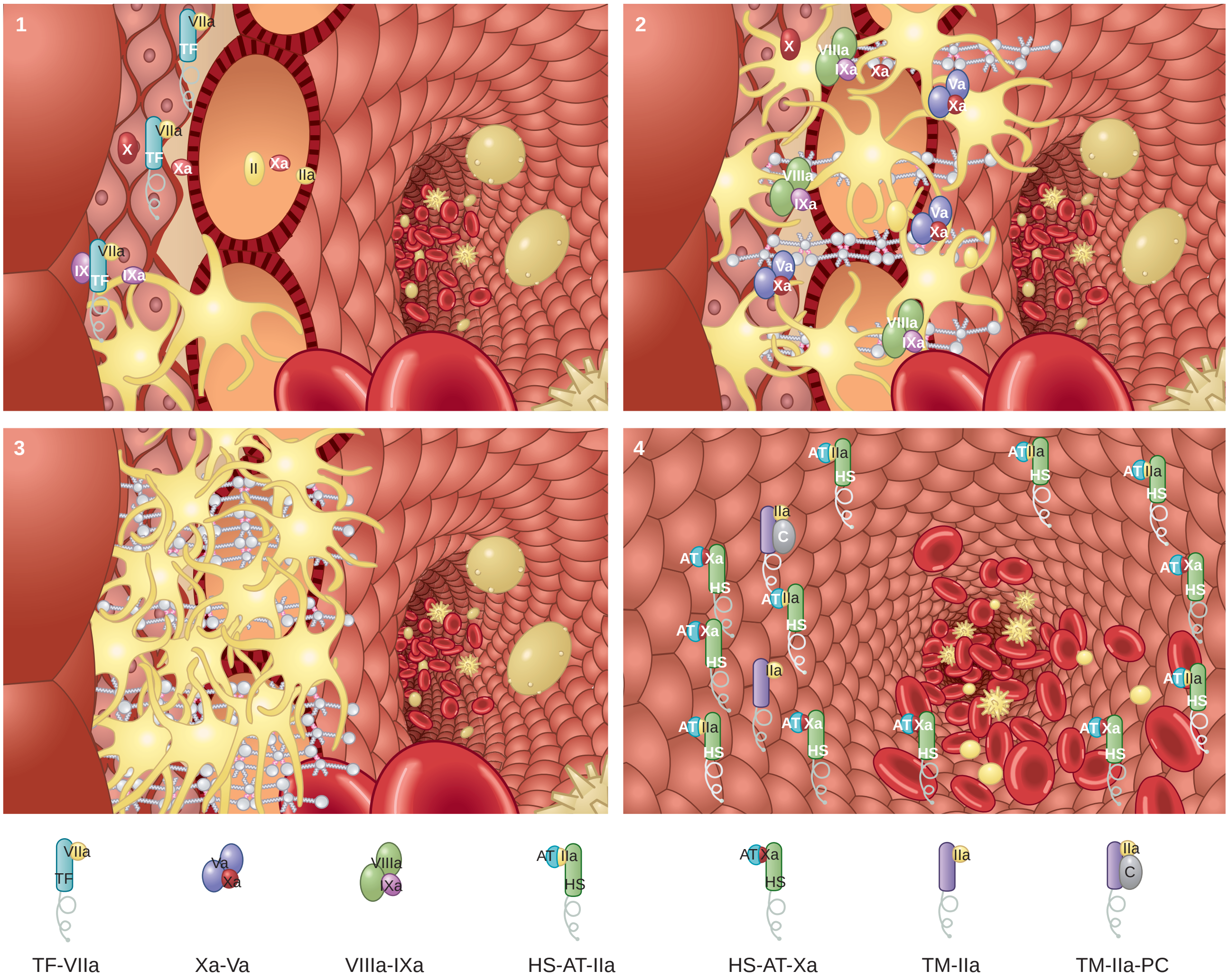

Figure 27-5 Schema of a two-compartment model of the regulation of tissue factor (TF)-initiated blood coagulation. A cross-section of a blood vessel showing the luminal space, endothelial cell layer, and extravascular region is presented at the site of a perforation. The blood coagulation process in response is depicted in four stages. TF–factor VIIa complex, TF-VIIa; prothrombinase complex, Xa-Va; intrinsic factor Xase, VIIIa-IXa; antithrombin (AT)–endothelial cell heparan sulfate (HS) proteoglycan complex bound to thrombin or factor Xa, HS-AT-(IIa or Xa); protein C (PC) bound to thrombomodulin-thrombin, TM-IIa-PC. Stage 1. Perforation results in delivery of blood and, with it, circulating factor VIIa and platelets to an extravascular space rich in membrane-bound TF. Platelets adhere to collagen and von Willebrand factor associated with the extravascular tissue, and TF binds factor VIIa, thereby initiating the process of activation of factors IX and X. Factor Xa activates small amounts of prothrombin to thrombin, which in turn activates more platelets and converts factors V and VIII to factors Va and VIIIa. Stage 2. The reaction is propagated by platelet-bound intrinsic factor Xase and prothrombinase, with the former being the principal generator of factor Xa. Initial clotting occurs and fibrin begins to fill in the void in cooperation with activated platelets. Stage 3. A barrier composed of activated platelets laden with procoagulant complexes and enmeshed in fibrin scaffolding is formed. The reaction in the now-filled perforation is terminated by consumption of reagent, which attenuates further generation of thrombin, but functional procoagulant enzyme complexes persist because they are protected from the dynamic inhibitory processes found on the intravascular face. Stage 4. View downstream of the perforation. Enzymes escaping from the plugged perforation are captured by AT-heparan complexes, and the protein C system is activated by binding of residual thrombin to endothelial cell thrombomodulin, thereby initiating the dynamic anticoagulant system. These intravascular processes work against occlusion of the vessel despite continuous resupply of reactants across the intravascular face of the thrombus. *(From Orfeo T, Butenas S, Brummel-Ziedens KE, Mann KG: The tissue factor requirement in blood coagulation.* J Biol Chem *280:42887-42896, 2005.)*

Mechanical disruption of a blood vessel results in the movement of blood to an environment where TF is exposed and platelets bind and are activated (see Fig. 27-5, *stage 1*). These events localize the initiating procoagulant reactions to blood now outside the vasculature, with adhesion and activation of platelets in this extravascular blood providing sufficient receptors and surfaces to support the propagation phase of thrombin generation (see Fig. 27-5, *stage 2*). This extravascular process rapidly becomes independent of TF as generation of thrombin becomes supplied by the intrinsic factor Xase and prothrombinase complexes. As long as new blood flows into this space, and fibrin formation and platelet aggregation have not achieved a sufficient barrier to stop flow, the system is open and the propagation phase of thrombin generation will continue. If blood ceases to move into the extravascular compartment, the system becomes closed, available prothrombin is consumed, and the complex enzymes are ultimately destroyed or sequestered into inactive complexes (see Fig. 27-5, *stage 3*). However, the stability of the prothrombin–activating potential incorporated into the platelet-rich thrombus and its localization in the extravascular space ensure that if the barrier springs a leak, robust generation of thrombin will begin immediately on contact and continue until consequent platelet activation and fibrin deposition reestablish a secure barrier.

Three mechanisms act to limit clot growth into the intravascular space (see Fig. 27-5, *stage 4*): thrombomodulin-dependent activation of protein C,[14] the presence of abundant AT, and the actively nonthrombogenic and nonadhesive surface of the vascular endothelium. Thus thrombin generation with consequent ongoing fibrin deposition and platelet activation is suppressed at the extravascular-intravascular boundary by these processes. In contrast, complexes at the extravascular side will remain relatively protected from APC and AT by the forming barrier and are therefore ready to respond to any leakage of blood.

The general properties of proteins of the blood coagulation proteome are summarized in Table 27-1. It has been stated that "a mouse is not a small furry human." A corollary is that an infant is not a tiny adult. This is especially true for the average plasma proteome concentration values for the newborn. These data are presented in parentheses in Table 27-1. Note that except for factor VIII and fibrinogen, most newborn functional protein concentrations are low relative to adult values (see Chapter 5).

THE VITAMIN K–DEPENDENT PROTEIN FAMILY

The Nobel Prize was awarded to Dam and Doisy in 1941 for their discovery of the fat-soluble vitamin K. Nearly simultaneously with the discovery of vitamin K, a naturally occurring antagonist, bishydroxycoumarin (dicumarol), was described. This antagonist was identified as a toxic agent in spoiled sweet clover that caused hemorrhage in cattle because of decreased thrombin formation. This agent was introduced as an anticoagulant in humans in 1941.[24]

Vitamin K, essential for the biosynthesis of clotting factors, contributes to the conversion of 10 to 13 glutamate residues to γ-carboxyglutamate residues (Gla). This posttranslational modification endows the vitamin K–dependent protein (VKDP) with Ca^{2+}-binding and membrane-binding qualities.[25] Gla formation is decreased or absent after treatment with coumarin derivatives.

The VKDPs (Fig. 27-6) can be divided into two classes: procoagulant (factors II, VII, IX, and X) and anticoagulant (protein C, protein S, and protein Z). The homology among these proteins is probably due to a common ancestral gene.[26] Each VKDP is composed of discreet domains characterized by highly conserved regions. The NH_2-terminal "Gla" domains (see Fig. 27-6) are followed by either kringle domains in prothrombin or epidermal growth factor (EGF)–like domains in factor VII, factor IX, factor X, protein C, protein S, and protein Z. Protein S contains four EGF domains followed by a sex hormone–binding domain (SHD). The serine protease domain for prothrombin, factor VII, factor IX, factor X, and protein C becomes functional after cleavage of specific peptide bonds. Protein S is not a serine protease precursor but is sensitive to thrombin cleavage. Protein Z contains a "pseudocatalytic domain" in the (COOH)-terminal but cannot function as a serine protease.

Synthesis of these proteins occurs primarily in the liver, although synthesis in other tissue has been reported. The plasma concentration of circulating VKDP varies 200-fold from approximately 100 μg/mL for prothrombin to 0.5 μg/mL for factor VII (see Table 27-1). Clearance rates of the VKDPs vary with their half-lives, from 6 hours (factor VII) to 2.5 days (prothrombin; see Table 27-1).

The VKDPs are synthesized as preproproteins and are modified posttranslationally at specific glutamates (γ-carboxylation) to form γ-carboxyglutamic acid (Gla).[25] Other modifications include β-hydroxylation at select aspartate and asparagine residues, tyrosine sulfation, and glycosylation. After synthesis of the single-chain precursors, the hydrophobic signal peptide directs each polypeptide to the endoplasmic reticulum. The propeptides, which play a role in docking vitamin K–dependent carboxylase,[27] are removed by an endoproteinase. For factor X and protein C, removal of an internal dipeptide or tripeptide converts them to their mature plasma two-chain zymogen forms.

The Gla domains constitute the first approximately 50 residues of the VKDPs (see Fig. 27-6). The negative charge elicited from the string of Gla residues (9 to 13) contributes to the binding of Ca^{2+} and generation of the conformation required for binding to anionic phospholipid membranes. In vivo, this surface is supplied by activated platelets or other blood cells in response to vascular damage through exposure of the phosphatidylserine-rich internal face of their cell membranes. Without vitamin K, or in the presence of vitamin K antagonists, the coagulation protein precursors are synthesized but not γ-carboxylated. In this form they are still secreted into plasma but display diminished function. The γ-carboxylation reaction is catalyzed by the enzyme γ-glutamyl carboxylase. This enzyme is located in the rough endoplasmic reticulum and requires the reduced form of vitamin K, oxygen, and

TABLE 27-1 General Properties of Blood Coagulation Proteins

		PLASMA CONCENTRATION[*]				
Protein	**Molecular Weight**	**nmol/L**	**μg/mL**	**Plasma $t_{1/2}$ (Days)**	**Chromosome[†] (Gene Location)**	**Functional Classification**
INTRINSIC PATHWAY PROTEINS						
Factor XII	80,000	500 (265)	40 (21)	2-3	Chr 5 (176 Mb)	Zymogen
Factor XI	160,000	30 (11)	4.8 (2)	2.5-3.3	Chr 4 (187 Mb)	Zymogen
Prekallikrein	85,000-88,000	486 (180)	42 (16)		Chr 4 (187 Mb)	Zymogen
HMW kininogen	120,000	670 (362)	80 (43)		Chr 3 (188 Mb)	Cofactor
LMW kininogen	66,000	1300	90		Chr 3 (188 Mb)	Cofactor
EXTRINSIC PATHWAY PROTEINS						
Prothrombin (factor II)	72,000	1400 (672)	100 (48)	2.5	Chr 11 (47 Mb)	VKD zymogen
α-Thrombin	37,000					Serine protease
Factor VII	50,000	10 (7)	0.5 (0.3)	0.25	Chr 13 (113 Mb)	VKD zymogen
Factor IX	55,000	90 (48)	5 (3)	1	X Chr (138 Mb)	VKD zymogen
Factor X	59,000	170 (68)	10 (4)	1.5	Chr 13 (113 Mb)	VKD zymogen
Protein C	62,000	65 (23)	4 (1)	0.33	Chr 2 (128 Mb)	VKD zymogen
Protein S	69,000	300 (108)	20 (7)	1.75	Chr 3 (95 Mb)	VKD protein
Protein Z	62,000	47	3	2.5	Chr 13 (113 Mb)	VKD protein
Factor V	330,000	20 (14)	6.6 (5)	0.5	1q21-q25	Procofactor
Factor VIII	285,000	1.1-1.5[‡]	0.3-0.4[‡]	0.3-0.5	X Chr (154 Mb)	Procofactor
VWF	255,000 (monomer)	Varies	10		Chr 12 (6 Mb)	Platelet adhesion, factor VIII carrier
Tissue factor	44,000	—	—	—	Chr 1 (9 Mb)	Cell-associated cofactor
Thrombomodulin	100,000	—	—	—	Chr 20 (23 Mb)	Cofactor
Fibrinogen	340,000	7400 (8380)	2500 (2830)	3-5	Chr 4 (156 Mb)	Structural protein cell adhesion
Aα	66,500					
Bβ	52,000					
γ	46,500					
Factor XIII	320,000	94 (72)	30 (23)	9-10		Transglutaminase zymogen
A chain	83,200				Chr 6 (6 Mb)	
B chain	79,700				Chr 1 (195 Mb)	
Tissue factor pathway inhibitor	40,000	1-4	0.1	6.4×10^{-4}-1.4×10^{-3}	Chr 2 (188 Mb)	Kunitz inhibitor
Antithrombin III	58,000	2400 (1510)	140 (88)	2.5-3	Chr 1 (172 Mb)	Serpin inhibitor
Heparin cofactor II	66,000	950 (408)	62 (26)	2.5	Chr 22 (19 Mb)	Serpin inhibitor
Protein C inhibitor	57,000	90	5	1	Chr 14 (94 Mb)	Serpin inhibitor

HMW, High–molecular-weight; *LMW,* low–molecular-weight; *VKD,* vitamin K–dependent; *VWF,* von Willebrand factor.

[*] Concentrations are reported as the mean of the healthy adult population, with the mean of the healthy 1-day-old infant population in parentheses. (Infant values from Andrew M, Paes B, Milner R, et al: Development of the human coagulation system in the full-term infant, *Blood* 70:165-172, 1987.)

[†] Chromosome number. Numbers in parentheses are approximate positions in megabases (from human genome build 36.2 www.ncbi.nlm.nih.gov).

[‡] Butenas S, Parhami-Seren B, Mann KG: The influence of von Willebrand factor on factor VIII activity measurements. *J Thromb Haemost* 7(1):132-137, 2009; Butenas S, Parhami-Seren B, Undas A, et al: The "normal" factor VIII concentration in plasma. *Thromb Res* 126(2);119-123, 2010.

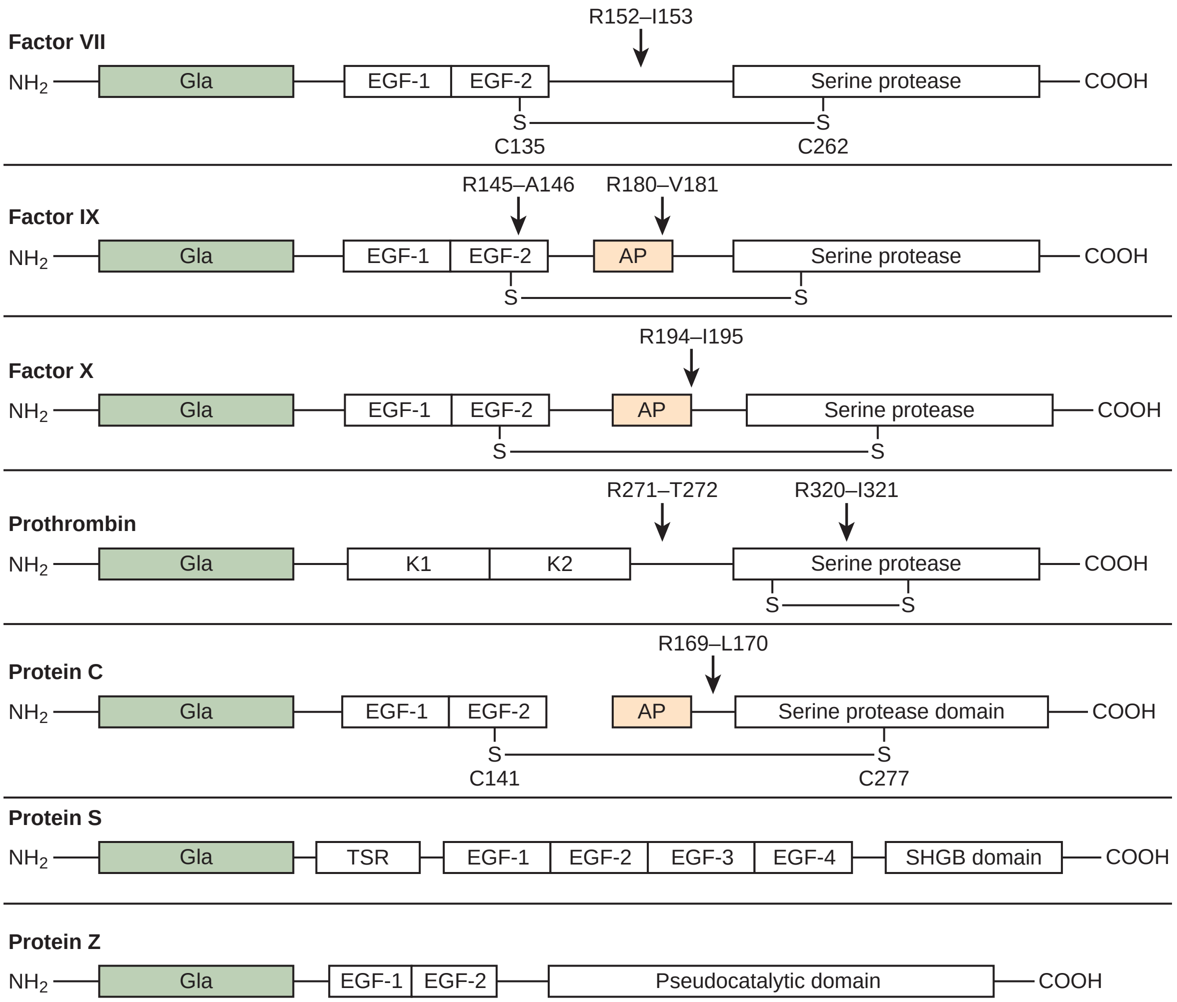
Factor VII
R152–I153
NH2
Gla
EGF-1
EGF-2
Serine protease
COOH
S
S
C135
C262
Factor IX
R145–A146
R180–V181
NH2
Gla
EGF-1
EGF-2
AP
Serine protease
COOH
S
S
Factor X
R194–I195
NH2
Gla
EGF-1
EGF-2
AP
Serine protease
COOH
S
S
Prothrombin
R271–T272
R320–I321
NH2
Gla
K1
K2
Serine protease
COOH
S
S
Protein C
R169–L170
NH2
Gla
EGF-1
EGF-2
AP
Serine protease domain
COOH
S
S
C141
C277
Protein S
NH2
Gla
TSR
EGF-1
EGF-2
EGF-3
EGF-4
SHGB domain
COOH
Protein Z
NH2
Gla
EGF-1
EGF-2
Pseudocatalytic domain
COOH

Figure 27-6 Schematic representation of the vitamin K–dependent proteins. The building blocks for these proteins are composed of Gla domains (9 to 13 Gla residues), followed by either kringle domains (K) in prothrombin or epidermal growth factor (EGF)–like domains in factors VII, IX, and X, protein C, protein S, and protein Z. Protein S contains a thrombin-sensitive region (TSR) before the EGF domain. The active site is contained within the serine protease domain for prothrombin, factors VII, IX, and X, and protein C and is functional upon cleavage at the activation site. Protein S is not a serine protease precursor and instead contains a sex hormone globulin binding (SHGB)–like domain in the COOH-terminal end. Protein Z contains a "pseudocatalytic domain" in the COOH-terminal end and does not function as a serine protease enzyme. For reference, the molecular weight for each protein is listed under its name, and disulfide bonds are illustrated as (-S-S-).

carbon dioxide[25] to convert glutamic acid residues to γ-carboxyglutamic acid.

Vitamin K_1 (phylloquinone) is primarily found in leafy green vegetables and vegetable oil. Additional K activity may be provided by vitamin K_2 (menaquinones) synthesized by intestinal gram-negative bacteria. Synthetic vitamin K_3 (menadione) has no intrinsic activity until it undergoes in vivo modification to the active menaquinone form. In vivo, vitamin K is recycled in a microsomal oxidation-reduction system for continued use in the γ-carboxylation reaction.[28,29] In order to perform the γ-carboxylation reaction, vitamin K has to be present in its reduced hydroquinone form. As the precursor proteins are carboxylated, vitamin K is oxidized to the epoxide. The epoxide in the presence of 2,3-epoxide reductase, with thiols being used as the reducing agent, yields the quinone form of vitamin K.[30] A subsequent reduced nicotinamide adenine dinucleotide phosphate (NADPH) or NADH-dependent quinone reductase reaction resynthesizes the hydroquinone form. The cycle can thus continue. Warfarin anticoagulant therapy blocks the process required for the regeneration of reduced hydroquinone vitamin K.[28,29] Because warfarin and other coumarin–derivative drugs indirectly affect carboxylation, they can be overcome with excess vitamin K.

The Gla domain is followed by two tandem EGF domains (EGF-1 and EGF-2) in factor VII, factor IX, factor X, and protein C and four EGF domains (EGF-1 to EGF-4) in protein S[31] (see Fig. 27-6). Each EGF-like domain consists of 40 to 50 amino acids, including 6 cysteine residues involved in disulfide bond formation. These domains are found widely distributed in extracellular and membrane proteins. Proteins containing these EGF-like domains are involved in blood coagulation, fibrinolysis, complement activation, connective tissue microfibril formation, and signal transduction.[32] The function of the EGF domain is still unclear. One hypothesis is that it serves as a spacer. There is a consistent elongation of the molecules of factors VII, IX, and X, protein C, and protein S. The distance between the membrane-binding Gla domain and the serine protease domain is crucial to placement of the active site in an appropriate position relative to the target peptide bond in its substrate.[33]

The serine protease domains (see Fig. 27-6) account for approximately half the mass of each protein. Peptide bond cleavage at specific sites converts the vitamin K–dependent zymogens to their active, chymotrypsin-like, serine protease forms. The mechanism of proteolysis by chymotrypsin involves a catalytic triad composed of Asp102, His57, and Ser195 (chymotrypsin numbering). The serine protease domains of all the Gla-containing factors are homologous members of the chymotrypsin family but cleave almost specifically at arginyl residues. However, unlike trypsin, which shows little specificity beyond the requirement for arginyl or lysyl residues at the cleavage site, the activated coagulation factors have extended substrate specificity pockets whereby only selected amino acid sequences are recognized by each protease. Thus despite the high degree of homology between the protease domains of protein C and factors II, VII, IX, and X, each of these factors has a highly specific function in coagulation. Some of this discrimination is mediated by surface loops, along with other domains distant from the substrate binding pocket termed exosites.

Factor VII (Proconvertin, Convertin)

The single-chain factor VII zymogen is activated to its two-chain protease form by cleavage of a single peptide bond between Arg152 and Ile153 (see Fig. 27-6). The mechanism for the initial activation of this zymogen is unclear. The resulting protease consists of an NH_2-terminal light chain linked by a single disulfide bond to a COOH-terminal heavy chain containing the catalytic domain. Factor VIIa has very poor catalytic efficiency without the presence of its cofactor TF.[34,35] Cleavage of factor VII to factor VIIa can be catalyzed by several proteases, including α-thrombin,[36] factor IXa,[37] factor Xa,[36] factor XIIa,[38] and factor VII–activating protease,[39] as well as by autoactivation by factor VIIa.[40] The active factor VIIa serine protease bound to TF forms the extrinsic factor Xase complex (see Fig. 27-3).

Factor VIIa, unlike other serine proteases of the coagulation cascade,[35] is not readily inhibited by circulating protease inhibitors, including the AT-heparin complex, unless bound to TF.[41,42] Thus regulation of TF expression is the primary means of controlling factor VIIa activity. Paradoxically, the zymogen factor VII (10 nM) is an effective competitor of factor VIIa (0.1 nM) for binding to TF. This competition downregulates the level of enzymatically active complex (factor VIIa–TF), thereby suppressing initiation of the clotting cascade. Once the extrinsic tenase complex activates factor X to factor Xa, TFPI can form a quaternary complex (factor VIIa–TF–factor Xa–TFPI) with no enzymatic activity[43] (see Fig. 27-4). Thus once assembled and functioning, the extrinsic tenase, along with its substrate factor Xa, is a target for inactivation through the action of TFPI.

Factor IX (Plasma Thromboplastin Component, Christmas Factor, Hemophilia B Factor)

Factor IX is a single-chain zymogen consisting of 415 amino acids organized with a Gla domain (12 Gla residues), two tandem EGF domains, an activation peptide region, and a serine protease domain (see Fig. 27-6). Glycosylation in the form of O-linked and N-linked oligosaccharides makes up 17% of the factor IX protein by weight.[44] Activation of factor IX is a two-stage process that requires cleavage at Arg145 and Arg180 and results in the release of a 35-residue activation peptide[45] (see Fig. 27-6). The physiologic activators of factor IX are either TF–factor VIIa (extrinsic tenase complex)[7] or factor XIa. The first cleavage at Arg145-Ala146 results in factor IXα. This cleavage has been shown to be important in its affinity for its cofactor factor VIIIa.[46] The second cleavage occurs at Arg180-Val181 and results in factor IXαβ, the active form, also referred to as *factor IX*a. Both cleavages are required for the biologic activity of factor IXa.[45] Plasma factor IXa is primarily inhibited by AT[47] (see Fig. 27-2). In vitro experiments conducted on phospholipid vesicles and cell membranes showed that protease nexin-2/amyloid β-protein precursor (PN2/APP) can also inhibit factor IXa.[48]

Factor X (Stuart Factor)

Factor X is synthesized in the liver and circulates as a two-chain molecule linked by a disulfide bond. The NH_2-terminal light chain consists of a Gla domain and two EGF-like domains (see Fig. 27-6). The COOH-terminal heavy chain consists of an activation peptide region and a catalytic (serine protease) domain. Factor X is activated by both the intrinsic and extrinsic factor Xases (see Figs. 27-1 and 27-3) by cleavage at Arg194-Ile195 (see Fig. 27-6). A 52–amino acid activation peptide is released. The resulting catalytic serine protease, factor Xa, is composed of a light chain and a disulfide-linked heavy chain. Factor X has to be bound to the membrane before activation.[19] Factor Xa participates in forming the prothrombinase complex (see Fig. 27-4).[17] The propagation phase of thrombin generation is catalyzed principally by factor Xa generated via the intrinsic tenase complex (see Fig. 27-3), which activates factor X at a 50- to 100-fold higher rate than the extrinsic tenase complex does.[9] The factor Xa active site associates with the COOH-terminus of TFPI.[49] The factor Xa–TFPI complex rapidly inactivates TF–factor VIIa to form a stable quaternary complex, TF–factor VIIa–TFPI–factor Xa.[49] Factor Xa is inhibited by AT[50] but only poorly when in the prothrombinase complex.[51]

Factor II (Prothrombin)

Prothrombin is the single-chain precursor of factor IIa (thrombin), the key enzyme in blood coagulation. The zymogen prothrombin is the most abundant of the plasma VKDPs and circulates in plasma at a mean concentration of 1.4 μM, or 100 μg/mL[52] (see Table 27-1). Prothrombin is synthesized primarily in the liver. Low levels of prothrombin expression have also been identified in other tissues, including the brain, diaphragm, stomach, kidney, spleen, intestine, uterus, placenta, and adrenal gland. Removal of the 43–amino acid propeptide generates the mature protein of 579 amino acids. An NH_2-terminal Gla domain is followed by two kringle domains and the serine protease precursor domain (see Fig. 27-6). Kringles have been identified as common motifs in many plasma proteins, including prothrombin, plasminogen, tissue plasminogen activator, urokinase, factor XII, and apolipoprotein(a).[53] The kringle 1 domain may interact with factor Va,[54] although kringle 2 appears to be the primary region mediating interaction with factor Va to form the prothrombinase complex.[55] This latter interaction may initiate conformational changes that make reaction sites accessible for enzymatic cleavage of prothrombin.[56] The NH_2-terminal Gla domain and the first kringle domain together are referred to as *prothrombin fragment 1*, with the kringle 2 domain being located in prothrombin fragment 2. The sequence of the enzyme α-thrombin is contained within prethrombin 2.

Activation of prothrombin by prothrombinase proceeds through an initial cleavage of prothrombin at Arg320-Ile321, which gives rise to meizothrombin, a two-chain disulfide-linked molecule (see Fig. 27-6). Meizothrombin expresses some of the activities of α-thrombin. However, the fibrinogen clotting ability of meizothrombin is impaired in comparison to that of α-thrombin.[57] Meizothrombin is subsequently cleaved at Arg271-Thr272 to yield the NH_2-terminal half of the molecule (prothrombin fragment 1.2) and α-thrombin. α-Thrombin consists of an NH_2-terminal 49-residue A-chain disulfide linked to a COOH-terminal 259-residue B chain containing the catalytic triad.

An alternative cleavage pathway occurs when factor Xa independently acts on prothrombin. In the presence of factor Xa and Ca^{2+}, the initial cleavage occurs at Arg271-Thr272 and gives rise to prothrombin fragment 1.2 and prethrombin 2. Subsequent cleavage at Arg320 in prethrombin 2 yields α-thrombin. Prothrombin fragment 1.2 remains noncovalently associated with α-thrombin.[58] In contrast to other VKDPs, the phospholipid-binding Gla region is not covalently attached to the serine protease domain after cleavage of prothrombin to thrombin.

α-Thrombin is the central enzyme in blood coagulation in that it contributes to reactions at all levels, thereby allowing overall maintenance of vascular integrity. Its premier role in hemostasis results in platelet-fibrin clot formation.[59] However, the events that lead up to and beyond fibrin formation also involve protein cleavage by α-thrombin, as well as activating platelets,[60,61] factor VII,[36] factor V,[15] factor VIII,[62] factor XI,[8] and factor XIII. The contributions of α-thrombin also extend to anticoagulant and fibrinolytic reactions, including activation of protein C and thrombin-activatable fibrinolysis inhibitor (TAFI).[63]

The stable form of human α-thrombin possesses at least five distinct binding sites for substrates, inhibitors, cofactors, apolar molecules, and sodium ions (Na^+).[64] Exosite I, the fibrinogen binding site, is an electropositive anion binding site distinct from, but acting in concert with, the active site of the α-thrombin molecule. In addition to fibrinogen, exosite I also recognizes the COOH-terminal domain of hirudin, the hirudin-like region of PAR-1, and the fifth and sixth EGF-like domains of thrombomodulin.[64]

Many of the effects of α-thrombin on platelets and cells are elicited through the interaction of α-thrombin with receptor molecules, with α-thrombin binding to a receptor and initiating a signal transduction mechanism. The interaction between α-thrombin and the human platelet thrombin receptor PAR-1, however, is characterized by a more unusual mechanism in which the receptor is also a substrate for α-thrombin.[65] α-Thrombin cleaves the extracellular region of the receptor and produces a "tethered ligand." The new NH_2-terminus binds back to and activates the receptor.[65,66] Two additional α-thrombin receptors homologous to PAR-1, PAR-3, and PAR-4 have been identified. α-Thrombin's role continues beyond hemostasis into the realm of tissue repair and the remodeling phase necessary to regenerate damaged vascular tissue. α-Thrombin is a potent mitogen[67] and stimulates cell division in macrophages,[68] smooth muscle cells,[69] and endothelial cells.[70]

Protein C (Autoprothrombin II-A)

Protein C circulates as a heterodimer consisting of disulfide-linked heavy and light chains[71] (see Fig. 27-6). However, approximately 5% to 10% of circulating protein C is in the single-chain form. The NH_2-terminal light chain contains the Gla domain and a hydrophobic region that connects the Gla domain to two EGF domains. The COOH-terminal heavy chain contains the serine protease domain.[72] Cleavage at Arg169-Leu170 releases the activation peptide from the heavy chain to generate the enzyme. Although the α-thrombin–thrombomodulin complex is probably the major physiologic activator of protein C,[73] other potential activators include plasmin and factor Xa.[72] The half-life of protein C (8 to 10 hours; see Table 27-1) is markedly shorter than the half-life of other members of the VKDP family, which is probably the basis of the transient hypercoagulable state that may occur subsequent to the administration of coumarin-based anticoagulants.

The anticoagulant role of the dynamic protein C inhibitory pathway is proteolytic inactivation of factor Va. APC inactivates factor Va via a sequential series of proteolytic cleavages and thus inhibits the generation of α-thrombin.[74] APC also cleaves and inactivates factor VIIIa, although the spontaneous dissociation/inactivation of factor VIIIa is probably the physiologic regulator of factor Xa generation.[75] Complete inactivation of factor Va by APC requires an anionic phospholipid surface, and rates of inactivation of factors Va and VIIIa by APC have been reported to be enhanced by protein S.[76,77] APC also has profibrinolytic effects associated with decreased activation of TAFI. TAFI is activated by the α-thrombin–thrombomodulin complex and acts to prolong clot lysis. Cleavage of factor Va by APC inhibits α-thrombin generation, thus reducing α-thrombin–thrombomodulin–mediated TAFI activation.[78]

Protein C activation and APC activity are controlled on several levels. The endothelial cell protein C receptor (EPCR) provides cell-specific binding sites for both protein C and APC.[79] Inflammatory agents such as endotoxin, interleukin-1β (IL-1β), tumor necrosis factor-β (TNF-β), and TNF-α upregulate protein C activation on endothelial cells.[80-82] TNF-α downregulates thrombomodulin on the endothelial cell surface.[82] There are multiple other factors that downregulate thrombomodulin as well. APC activity is further regulated by the protein C inhibitor, plasminogen activator inhibitor 3 (PAI-3).[83]

Protein S

Protein S is a VDKP that is not a serine protease precursor. Approximately 40% of protein S circulates in the free form, and the remaining 60% circulates as a 1 : 1 complex with C4b-binding protein (C4bBP), a regulatory protein of the complement system.[84] The precise function of protein S in the protein C inhibitory pathway is controversial. Protein S is thought to function as a cofactor for APC in the inactivation of factors Va and VIIIa.[85-87] Protein S has also been reported to bind to factor Xa,[88] factor VIII,[89] and factor Va[90] and compete for prothrombinase binding sites on the membrane surface.[91] The C4bBP–protein S complex may inhibit factor X activation as well.[92] Protein S may have other roles apart from anticoagulation.[93] α-Thrombin cleavage of protein S inhibits the ability of protein S to act as a cofactor for APC.[94] The anticoagulant activity of protein S is also neutralized when bound to C4bBP.[84,92]

Protein Z

Protein Z is an enzymatically inactive homologue of factors VII, IX, and X and protein C.[95,96] Protein Z circulates in plasma in a complex with protein Z–dependent protease inhibitor (ZPI). ZPI is a 72-kD member of the serpin superfamily. However, like protein S, protein Z does not function as a protease. Although the COOH-terminus contains a homologue to the catalytic domains present in serine protease zymogens, the catalytic triad is not present.[96] The function of protein Z in vivo is unclear. Thrombosis is observed in mice when factor V Leiden is protein Z deficient.[97]

FACTOR XI, THE CONTACT PATHWAY CROSSOVER (PLASMA THROMBOPLASTIN ANTECEDENT)

Factor XI is a substrate for thrombin (see Fig. 27-1) and, when cleaved to factor XIa, activates factor IX.[8] Factor XI circulates as a homodimer in complex with HMWK[98] (see Table 27-1) and is also found in platelets.[99] Unlike other members of the accessory or contact pathway, factor XIa has a role in coagulation as part of a positive feedback loop that enhances the generation of α-thrombin.[8] Plasma factor XI accounts for most of the factor XI activity in humans and is a disulfide-linked homodimer (see Table 27-1). The NH_2-terminus contains four tandem sequences termed Apple domains (Apple 1 to Apple 4), followed by the COOH-terminal catalytic domain. The Apple domains are homologous to the Apple domains in human plasma prekallikrein.[100] The Apple domains mediate factor XI dimerization, as well as binding to its various cofactors and substrates.[98,101]

Plasma factor XI is cleaved at an internal Arg369-Ile370 bond to yield a disulfide-linked two-chain activated serine protease. The factor XI homodimer yields two disulfide-linked heavy chains containing the Apple domains and two light chains containing the active sites.[102,103] Activation of factor XI can be accomplished by factor XIIa and α-thrombin and by autoactivation by factor XIa itself. Activation of factor XI by factor XIIa requires HMWK and an anionic surface. However, deficiencies of factor XII and HMWK do not result in bleeding diatheses, whereas factor XI deficiency is associated with hemorrhage. This suggests that factor XIIa–dependent activation of factor XI as part of the contact pathway is not likely to be the primary route of factor XIa generation in hemostasis. The physiologically relevant pathway for activation of factor XI in coagulation is believed to involve α-thrombin.[8,104] Subsequent to activation, factor XIa remains bound to the surface. Factor XIa is a trypsin-like serine protease that cleaves and activates factor IX in a Ca^{2+}-dependent fashion.[105,106] Factor IXa is the enzyme component of the intrinsic tenase complex that provides the burst of factor Xa needed for normal coagulation. As part of a positive feedback loop, α-thrombin activates factor XI. In turn, factor XIa generates factor IXa, which leads to the high levels of factor Xa that ensure efficient generation of α-thrombin.[8]

Factor XIa is regulated by four serine protease inhibitors: AT, α_1-protease inhibitor, C1 inhibitor, and α_2-antiplasmin.[107-109] Factor XIa is also reported to be inhibited by PAI-1 and protein C inhibitor.[110] In addition, platelets secrete several factor XIa inhibitors,[111] including PN2.[112]

COFACTOR PROTEINS

There are two categories of procoagulant cofactor proteins: the plasma-derived soluble procofactors factor V and factor VIII (the latter in complex with its circulating carrier von Willebrand factor [VWF]) and the cell-associated cofactors TF and thrombomodulin. Factors V and VIII are highly homologous (40% identity). These cell-associated cofactors are single-chain transmembrane proteins, each composed of extracellular, transmembrane, and cytoplasmic domains.

Factor V (Labile Factor)

The procofactors factors VIII and V are organized into discrete structural domains (Fig. 27-7). Factor V was initially recognized as an unstable plasma component necessary for the generation of α-thrombin.[113] Most factor V circulates in plasma (see Table 27-1), but 18% to 25% of the total factor V is present in platelet alpha granules.[114]

The intron-exon structure of the factor V and factor VIII genes is quite similar, and the genes probably evolved from a common ancestor. The liver appears to be the

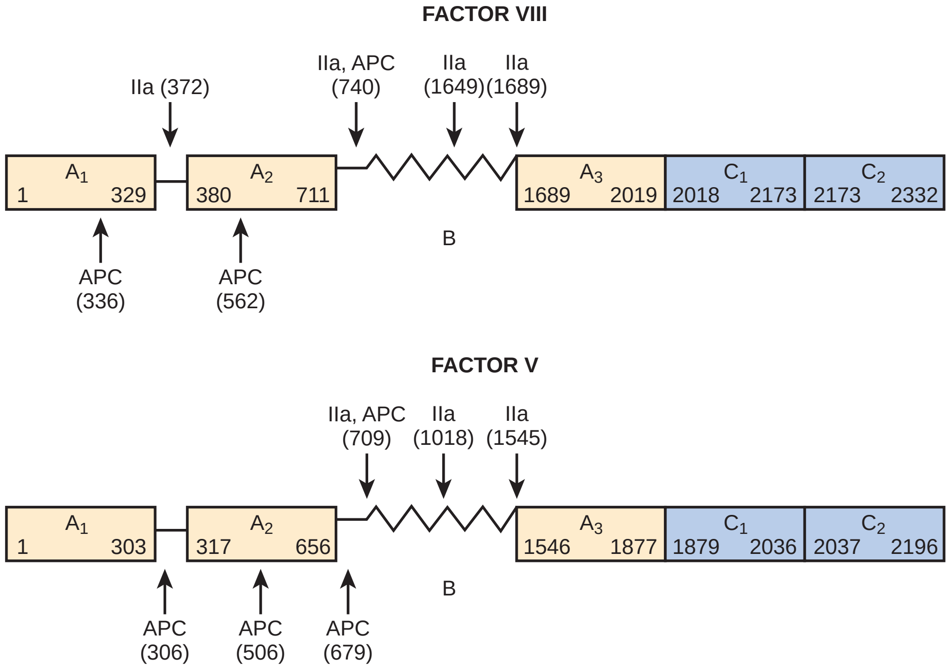

Figure 27-7 Soluble cofactor domain structures (factors VIII and V). The domain structures (A1-A2-A3-B-C1-C2) are bracketed by the beginning and ending amino acid residue. α-Thrombin (IIa) and activated protein C (APC)/factor Xa cleavage sites are shown with *vertical arrows*.

primary site of factor V biosynthesis. Although human megakaryocytes have also been reported to express factor V,[115] platelet factor V appears to be primarily derived from plasma factor V.[116] Human factor V is composed of A1-A2 domains, a central B domain, and a COOH-terminal light-chain precursor (A3-C1-C2 domains). The A domains are homologous to those found in factor VIII and ceruloplasmin; the C domains are homologous to the slime mold protein discoidin. Like factor VIII and ceruloplasmin, factor V is a copper-binding protein.[117]

Factor V undergoes extensive posttranslational modification, including phosphorylation, sulfation, and glycosylation. Phosphorylation at Ser692 affects the rate of inactivation of factor Va by APC.[118] The tyrosine sulfation status of factor V has also been related to its function.[119] Carbohydrate accounts for 13% to 25% of the mass of factor V and about 50% of the mass of the B region.[120] Differential glycosylation of Asn2182 in the C2 domain is reported to be responsible for the factor V1 and factor V2 variants observed in plasma.[121] Factors Va1 and Va2 appear to also be distinguished by functional differences.

The procofactor factor V does not bind factor Xa and is essentially completely inactive.[17] Limited proteolysis of the factor V molecule yields the active cofactor. α-Thrombin cleaves factor V at Arg709, Arg1018, and Arg1545 (see Fig. 27-7). The α-thrombin–generated form of factor Va is a heterodimer consisting of an NH_2-terminal heavy chain (A1-A2 domains) linked noncovalently to a COOH-terminal light chain (A3-C1-C2 domains).[122-125] The B domain is excised during proteolytic activation.[126] Factor Xa also cleaves factor V to produce a molecule similar to the factor Va produced by α-thrombin cleavage.[127] In addition, other enzymes may fully or partially activate factor V.[128] Factor Va functions both as a receptor for factor Xa and as a positive modulator of the catalytic potential of factor Xa in the prothrombinase complex. The cofactor forms at least part of the receptor for factor Xa on platelets and serves to anchor factor Xa to the membrane surface.[15,16,62]

Factor Va is downregulated via proteolytic inactivation by APC. APC cleaves factor Va at three sites: Arg506, Arg306, and Arg679. Initial factor Va cleavage occurs at Arg506. Subsequently, the membrane-dependent cleavage at Arg306 results in dissociation of the A2 domain as two fragments and complete loss of factor Va cofactor activity. An additional APC-mediated cleavage of factor Va fragments occurs at Arg679. The importance of this regulatory mechanism is demonstrated by the "APC resistance" syndrome associated with factor V Leiden.[129,130] The factor V Leiden Arg506→Gln mutation eliminates the first and most rapid of the sequential inactivating cleavages by APC. Factor Va Leiden thus retains cofactor activity and continues to promote α-thrombin generation for an extended period. Ultimately, inactivation of factor Va Leiden by cleavage of the Arg306 bond does occur, but at a markedly slower rate.[131]

Factor VIII (Antihemophilic Factor, Factor VIII:C)

Factor VIII circulates in plasma in complex with the large multimeric protein VWF. The ratio of factor VIII to VWF is in the range of 1 molecule of factor VIII to 50 to 100 molecules of VWF monomeric units. VWF acts to transport and stabilize plasma factor VIII. Factor VIII in complex with VWF has a plasma half-life of approximately 12 hours, whereas factor VIII alone undergoes rapid clearance and has a half-life of approximately 2 hours.[132,133] The liver and spleen are thought to be the primary sites of factor VIII biosynthesis.[134]

Though synthesized as a 2351–amino acid single-chain protein, human plasma factor VIII undergoes significant proteolytic processing that results in a heterogeneous collection of forms. The heterogeneity in molecular weight is due to proteolysis of the protein in circulation or processing before secretion (or both). The initial synthesized product is composed of three A domains, two C domains, and a connecting B domain (see Fig. 27-7). The secreted factor VIII molecule is a two-chain heterodimer as a result of intracellular proteolysis at the B-A3 junction. Additional cleavages within the B chain yield a heavy chain of A1-A2 domains linked to B fragments of variable length.[135] The B domain is highly glycosylated and contains 18 of the 25 potential N-linked glycosylation sites in factor VIII. Activation by α-thrombin involves cleavage at Arg372 (the A1-A2 junction), Arg740 (the A2-B junction), and Arg1689 in the light chain (the B-A3 junction) and excision of the remnant of the B domain. The resulting molecule contains three separate polypeptide chains: a light chain (A3-C1-C2) bound to the NH_2-derived heavy chain and the noncovalently associated A2 region of the heavy chain. Factor VIIIa is an unstable molecule, with dissociation of the A2 domain leading to spontaneous inactivation.[136] Factor VIII shares homology with the copper ion–binding protein ceruloplasmin and contains a reduced copper ion (Cu^+) binding site in the A1 domain that is important for function.[137] A specific VWF binding site has been identified between residues 1673 and 1684 of the factor VIII light chain.[138] The VWF binding site is removed from factor VIII during activation by α-thrombin cleavage at Arg1689. High-affinity factor IXa binding is observed for the factor VIIIa light-chain A3 domain.[139] Sulfation of several tyrosine residues in factor VIII is also important for function.[140]

The cofactor factor VIIIa binds factor IXa with Ca^{2+} (and membrane) to form the intrinsic factor Xase complex (see Fig. 27-3). The intrinsic factor Xase complex catalyzes the activation of factor X at a rate orders of magnitude greater than the enzyme factor IXa alone[18] (see Fig. 27-4). Factor Xa generated via the intrinsic factor Xase complex is the primary contributor of the factor Xa required for the propagation phase of thrombin generation.[21] In hemophilia A or B the intrinsic factor Xase complex cannot be formed, and the factor Xa levels required to induce the propagation phase of thrombin generation are not produced,[22] thereby resulting in lethargic thrombin formation and poor clot formation (see Fig. 27-5).

The spontaneous loss of factor VIIIa function is primarily a consequence of dissociation of the A2 subunit from the heterotrimer. Once the A2 subunit is displaced, factor VIIIa loses cofactor function. Dissociation occurs rapidly under physiologic conditions. The factor IXa–membrane complex interactions stabilize factor VIIIa and thus delay dissociation of the heterotrimer and prolong the transient activity of factor VIIIa.[141] Factor VIIIa is also regulated by limited proteolysis. Factor IXa cleaves the A1 subunit of factor VIIIa at Arg336 and eliminates factor VIIIa function.[142] APC is a key anticoagulant enzyme that likewise cleaves the A1 subunit at Arg336. APC also cleaves the A2 subunit of factor VIIIa at Arg562.[143] These APC cleavages occur sequentially, with the A1 cleavage occurring first and the A2 cleavage second.[144] Factor IXa protects factor VIIIa from APC cleavage at Arg562; however, protein S blocks the protective effect.[145] Although factor VIIIa is proteolytically inactivated by a number of enzymes, spontaneous dissociation appears to be the key regulator of cofactor function.

von Willebrand Factor (Ristocetin Cofactor)

VWF is a multifunctional protein with several key roles in coagulation. VWF circulates in plasma and is also contained in the alpha granules of human platelets.[146] In addition, it is known for its role in ristocetin-induced platelet aggregation (see Chapters 26 and 31). VWF is expressed by endothelial cells and megakaryocytes and is stored in Weibel-Palade bodies in endothelial cells and in alpha granules in platelets.[147,148] VWF is a large adhesive glycoprotein that circulates in plasma as a heterogeneous mixture of disulfide-linked multimers (see Chapter 31). The disulfide-linked multimers range in size from dimers, $M_r = 600{,}000$, to extremely large multimers consisting of more than 20 million Daltons. VWF has binding sites for factor VIII, heparin, collagen, platelet glycoprotein Ib, and platelet glycoprotein IIb-IIIa.[149] Plasma VWF transports and stabilizes factor VIII and protects it from inactivation by APC,[87] thus significantly prolonging the half-life of factor VIII in circulation.[133] VWF also acts as a bridge between platelets and promotes platelet aggregation. The primary platelet binding site for VWF is the glycoprotein Ib-IX-V receptor complex (see Chapters 26 and 31). Endothelial cells secrete VWF multimers that are larger than those found circulating in plasma. The function of these large multimeric forms of VWF is to bind to and agglutinate blood platelets under high shear rates. These large multimers of VWF are degraded by a specific metalloproteinase, ADAMTS-13.[150]

Tissue Factor (Tissue Thromboplastin Cd142 and Coagulation Factor III)

TF and thrombomodulin (Fig. 27-8) are intrinsic membrane proteins that are essential cofactors for factor VIIa and thrombin, respectively. They share no homology at the molecular level. TF is a transmembrane protein that functions as a cofactor for factor VIIa in the extrinsic factor Xase complex. In the absence of injury or stimulation, active TF is not ordinarily expressed on cellular surfaces in direct contact with circulating blood. Presentation of TF to the circulation is the event that triggers the primary procoagulant pathway of coagulation (see Fig. 27-1). Expression of TF can be induced in a number of cultured cell types. Monocytic cells and monocytes isolated from peripheral blood also express TF when stimulated by bacterial endotoxin or other proinflammatory agents. There is little or no detectable TF expression on unstimulated endothelial cells in vivo, although certain conditions such as sepsis, placental villitis, and graft rejection induce endothelial TF expression.[151] The mechanism governing TF expression under nonpathologic conditions is unknown.

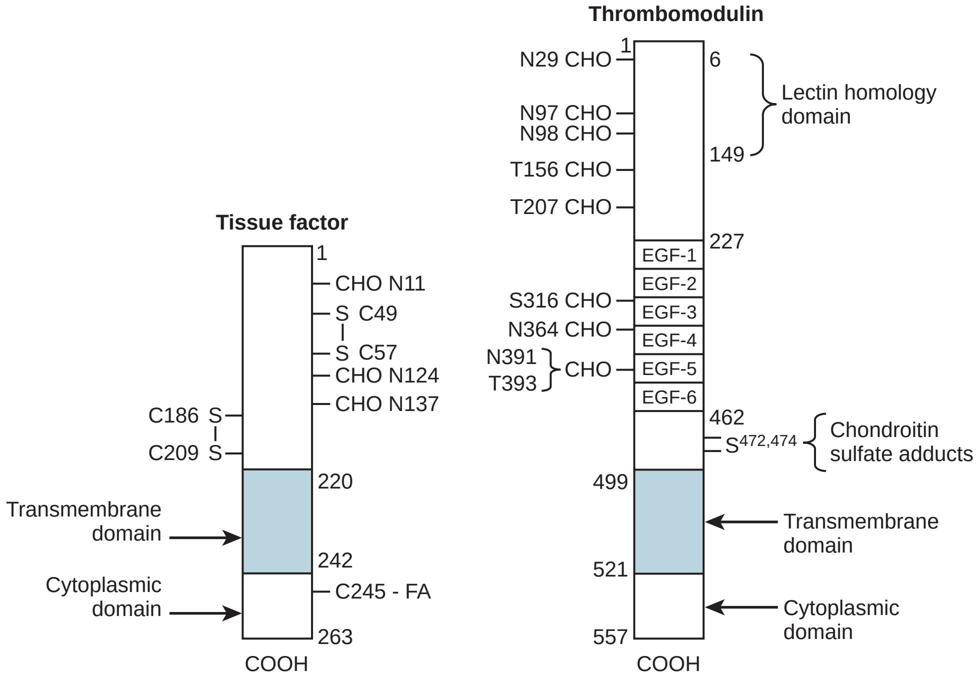

Figure 27-8 Transmembrane cofactors. Tissue factor is composed of an extracellular domain (residues 1 to 219), a transmembrane domain (residues 220 to 242), and a cytoplasmic domain (residues 243 to 263). Two disulfide bonds (S-S) and the sites of the three carbohydrate moieties (CHO) are identified by amino acid residue. One cysteine (C245) contains a thiol ester linkage to a fatty acid. Thrombomodulin is composed of five distinct domains. The domain structures are a lectin-like domain (residues 6 to 149), a domain containing six epidermal growth factor (EGF)-like regions (residues 227 to 462), a small extracellular domain rich in threonine and serine residues (two have been identified as sites of chondroitin sulfate adducts: S472 and S474), a membrane-spanning region (residues 499 to 521), and a cytoplasmic tail (residues 522 to 557). There are nine known glycosylation sites (CHO) on the thrombomodulin molecule.

TF is a member of the class 2 cytokine receptor superfamily and a type I integral membrane protein. The NH_2-terminus of TF (residues 1 to 219) is extracellular, whereas the COOH-terminus of the protein is intracellular (residues 243 to 263). TF also contains a hydrophobic membrane-spanning domain (see Fig. 27-8). This domain appears to function as an anchor for TF in the membrane.[152] The NH_2-terminal domain of TF is composed of two fibronectin type III domains and is glycosylated. The COOH-terminal cytoplasmic domain is quite short, and its function is unclear because deletion of this domain has no significant effect on TF procoagulant activity.[152]

TF can bind either factor VII or factor VIIa to form a high-affinity 1:1 complex. Once bound to TF, the zymogen factor VII may be converted to factor VIIa via limited proteolysis.[153,154] The TF–factor VIIa or extrinsic tenase complex activates both factor IX and factor X. When TF is exposed or expressed subsequent to vascular perturbation, the low levels of circulating factor VIIa bind to TF, and the extrinsic factor Xase complex triggers the procoagulant cascade (see Fig. 27-4, *A*).[9,155]

In the absence of TF, factor VIIa is relatively inert. TF alters the active site of factor VIIa, thus functioning as an allosteric activator of the enzyme.[40,155] Although the cofactor is necessary for enzymatic activity, formation of the TF–factor VIIa complex does not require an anionic phospholipid membrane surface as do the other procoagulant complexes. The membrane dependency of the extrinsic factor Xase complex function in factor X and factor IX activation arises from membrane-mediated substrate delivery. Both factors X and IX bind to the membrane surface for efficient two-dimensional transfer to the extrinsic factor Xase complex.

TF is primarily regulated through availability. In normal hemostasis, the commonly accepted source of functional TF is through exposure of the subendothelium after vascular perforation. However, controversy has arisen regarding the sources and presentation of active TF and whether functional TF circulates in blood.[156,157] Once the TF–factor VIIa complex is formed in the vicinity of an injury, the extrinsic tenase activity is modulated by TFPI and AT.

Thrombomodulin

Thrombomodulin is a type 1 transmembrane protein that is constitutively expressed on the surface of vascular endothelial cells (see Fig. 27-8). Thrombomodulin is a high-affinity receptor for α-thrombin and acts as a cofactor for the α-thrombin–dependent activation of protein C and TAFI. Thrombomodulin expression has been reported in a variety of cell types, including vascular endothelial cells.[158] The activity of thrombomodulin on the surface of endothelial cells is decreased by inflammatory cytokines, which may contribute to the hypercoagulation characteristic of inflammatory states. Homozygous thrombomodulin-deficient mice die in utero before formation of the cardiovascular system, thus suggesting a potential role for thrombomodulin in mammalian development.[159]

The domain structure of thrombomodulin is depicted in Figure 27-8. The fifth and sixth EGF domains support α-thrombin association.[160] The presence of chondroitin sulfate increases the affinity for α-thrombin 10-fold.[161] The chondroitin sulfate moiety also enhances inactivation of α-thrombin by AT[162] and modulates protein C activation.[161,162] The O-linked sugar domain of thrombomodulin is required for generation of APC on cellular surfaces. This domain is extended and rigid and probably functions to elevate α-thrombin from the membrane surface.

Production of APC by the α-thrombin–thrombomodulin complex is approximately 1000 times faster than equivalent concentrations of protein C and α-thrombin. Once bound to thrombomodulin, α-thrombin's procoagulant activities are neutralized. The high-affinity α-thrombin–thrombomodulin interaction is mediated mainly by exosite I on the α-thrombin molecule. Exosite I also binds fibrinogen, and interaction of α-thrombin with thrombomodulin therefore blocks fibrinogen binding and cleavage. In addition, thrombomodulin induces conformational changes in α-thrombin.[163,164] The changes that occur upon interaction of α-thrombin with thrombomodulin reduce the ability of α-thrombin to generate fibrin, activate factor V, or activate platelets[165] and increase the inactivation of α-thrombin by AT.[165] Thus the α-thrombin–thrombomodulin complex, or protein case (see Fig. 27-3), functions in an anticoagulant and antifibrinolytic capacity.

THE INTRINSIC (ACCESSORY) PATHWAY FACTORS

The procoagulant proteins that make up the intrinsic or accessory pathway consist of factor XII, plasma prekallikrein, HMWK, and factor XI. These proteins are responsible for the contact (intrinsic) activation of blood coagulation (see Fig. 27-1).

Factor XII (Hageman Factor, Contact Factor)

Factor XII plays a primary role in initiation of the contact or intrinsic pathway of coagulation upon binding with substances such as glass or kaolin. The contact pathway is the basis for the APTT clotting assay. Deficiency of factor XII is not associated with bleeding abnormalities, and therefore the precise role of factor XII in hemostasis is unclear, although the components of the contact pathway may provide a link between coagulation and inflammation.

The factor XII molecule is composed of two domains: a NH_2-terminal heavy chain and a COOH-terminal light chain. The heavy chain contains fibronectin type I and type II domains, two EGF-like domains, a kringle domain, and a proline-rich region. The light chain contains the serine protease catalytic domain, a region homologous to the B chain of the enzyme plasmin.

Factor XII undergoes autoactivation upon interaction with negatively charged surfaces such as glass, kaolin, dextran sulfate, ellagic acid, celite, or bismuth subgallate.[166] These are probably artifactual triggers, and the physiologic activator is unknown. Although factor XII associates with many physiologically relevant anionic surfaces, including negatively charged phospholipids, the autoactivation of factor XII induced by these surfaces in vitro does not appear to represent the mechanism of factor XII activation in vivo.[167]

Factor XIIa, prekallikrein, and HMWK form a complex on anionic phospholipids of the cell membrane, and prekallikrein is cleaved to kallikrein. Kallikrein activates factor XII by cleavage at Arg353-Val354 to generate an 80-kD two-chain enzyme, α-factor XIIa (factor XIIa). This cleavage is essential for exposure of the active site in factor XIIa.[168]

Factor XIIa activates factor XI and prekallikrein by mechanisms dependent on anionic surfaces and the cofactor HMWK. Factor XIIa also activates the C1 component of the complement system,[169] downregulates the Fc receptor on monocytes and macrophages,[170] and stimulates neutrophils.[171] Although these roles have no apparent impact on normal coagulation, factor XII/XIIa may be an important link between coagulation and inflammation. Factor XIIa also activates plasminogen to plasmin, thereby linking the contact pathway to fibrinolysis.[172]

Plasma Prekallikrein (Fletcher Factor)

Plasma prekallikrein deficiency is rare and is not associated with a hemostatic defect. Approximately 75% of plasma prekallikrein circulates in a noncovalent complex with HMWK,[173] and the remaining 25% circulates as free prekallikrein. Prekallikrein contains four tandemly repeated Apple domains in the NH_2-terminal portion of the molecule that are highly homologous to the Apple domains of factor XI. Prekallikrein is activated by factor XIIa in complex with the cofactor HMWK on an anionic surface (see Fig. 27-1). The enzyme kallikrein is a two-chain molecule composed of an NH_2-terminal heavy chain containing the four Apple domains and a COOH-terminal light chain containing the active site.[174]

Kallikrein is a member of the trypsin family of serine proteases. In the presence of an appropriate anionic surface and the cofactor HMWK, kallikrein activates factor XII to factor XIIa and proteolyses factor XIIa to β-factor XIIa. Kallikrein also undergoes autoproteolysis to yield β-kallikrein.[175] Enzyme activity is significantly reduced upon conversion of kallikrein to β-kallikrein. Kallikrein cleaves HMWK at two sites to generate the vasoactive nonapeptide bradykinin, a potent vasodilator. Kallikrein is also an activator of fibrinolytic zymogens and converts both plasminogen to plasmin and pro–urokinase plasminogen activator (pro-uPA) to uPA.[176] In addition, kallikrein has been reported to activate neutrophils and stimulate release of elastase as part of the hemostatic and inflammatory responses.[177] C1 inhibitor and α_2-macroglobulin are the major inhibitors of kallikrein.[178]

High–Molecular-Weight Kininogen (Fitzgerald Factor, Williams Factor)

HMWK acts as a cofactor for the activation of factor XII and prekallikrein and is the precursor of the vasoactive peptide bradykinin. A second form of kininogen, low–molecular-weight (LMWK), is also found in plasma. LMWK can be cleaved to yield bradykinin but has no procoagulant activity. Deficiencies of HMWK and LMWK are rare and are not associated with bleeding diatheses.[179] The major established function of the kininogens is to serve as a source of bradykinin and thereby contribute to a number of vascular events regulated by bradykinin.

The two forms of kininogen, HMWK and LMWK, are the products of a single gene and are produced by alternative splicing.[180] The NH_2-terminal heavy chains of the two forms are identical and contain potent inhibitors of cysteine proteases such as calpain.[181] The central domain of both kininogens is the bradykinin region. The two forms of kininogen have different COOH-terminal light-chain regions. The light chain of HMWK contains binding sites for prekallikrein and factor XI.[182] Kallikrein, factor XIIa, and factor XIa cleave HMWK to release bradykinin. Kallikrein will also cleave LMWK to release bradykinin.

As noted earlier, the biologic significance of the contact pathway of coagulation is not established. In hemostasis, generation of factor XIa most likely proceeds via an α-thrombin–dependent pathway, and any factor XIa generated by the contact pathway would not have a measurable impact on coagulation. Release of bradykinin as a consequence of these activation events, however, does provide a key vasoactive agent with a variety of roles and directly links the contact pathway to vascular repair processes.

STOICHIOMETRIC INHIBITORS

In vivo, the hemostatic reaction is tightly regulated to ensure that adequate but limited levels of α-thrombin are generated. Two systems, one stoichiometric and one proteolytic, regulate the generation of thrombin. In the proteolytic system (described earlier), α-thrombin participates in its downregulation by binding to thrombomodulin on the vascular cell surface and converting protein C to APC. This anticoagulant serine protease cleaves factors Va and VIIIa, thereby eliminating thrombin generation. The stoichiometric inhibitors consist of the AT-heparin complex, which inhibits virtually all the procoagulant serine proteases, and TFPI, which targets the factor VIIa–TF–factor Xa complex and principally regulates the initiation phase of the blood coagulation process (see Fig. 27-2).

Tissue Factor Pathway Inhibitor (Extrinsic Pathway Inhibitor, Lipoprotein-Associated Coagulation Inhibitor)

TFPI is a multivalent protein that modulates TF-dependent coagulation by inhibiting the extrinsic tenase complex.[183] TFPI is expressed constitutively by cultured endothelial cells and consists of an acidic NH_2-terminal region, three tandem Kunitz-like serine protease inhibitor domains (K1 to K3), and a positively charged COOH-terminal region. The tandem Kunitz domains are essential for function.[184] Two variants of TFPI (isoforms α and β) are generated by alternative splicing. A significant proportion of TFPI molecules in blood are truncated to a variable extent at the COOH-terminal end, with resultant compromised inhibitory activity. The inhibitory activity of TFPI is enhanced by heparin. Upon heparin administration, TFPI is released from endothelial cells, with a 2- to 10-fold increase in circulating levels being achieved. Release of TFPI with heparin therapy is responsible for a portion of the observed elevation in PT and raises the possibility that part of the antithrombotic effect of heparin may be mediated by release of TFPI. A minor pool of TFPI (approximately 10% of the total TFPI in blood) is located within platelets.[185] Transgenic mice with complete TFPI deficiency do not survive.[186] There are no known human TFPI-deficient individuals.

TFPI is the principal stoichiometric inhibitor of the extrinsic pathway (see Figs. 27-2 and 27-4, *B*). The TFPI mechanism allows the factor VIIa–TF complex to initiate factor Xa formation, but it suppresses the generation of high levels of factor Xa product formation by binding the enzyme-product complex (see Fig. 27-4, *B*). The inhibitor mechanism involves rapid interaction between the second Kunitz domain of TFPI with the active site of factor Xa; localization of the complex to the membrane surface is mediated by the Gla domain of factor Xa.[49] Once surface bound, the factor Xa–TFPI complex rapidly inactivates TF–factor VIIa. This complex formation depends on binding of the first Kunitz domain of TFPI to the active site of factor VIIa. These interactions together form a stable quaternary complex, TF–factor VIIa–TFPI–factor Xa. The factor IXa–TFPI complex can also inhibit factor VIIa–TF, although the physiologic relevance of this route of inhibition is unclear because high plasma concentrations of TFPI are required. At normal plasma concentrations, this multicomponent interaction of TFPI allows basal function of the factor VIIa–TF complex but inhibits it after more extensive activation takes place.

When combined with the stoichiometric inhibitor AT, a synergistic regulatory effect of blood coagulation occurs by inducing kinetic “thresholds” such that the initiating TF stimulus must achieve a significant magnitude to propel thrombin generation.[187] TF concentrations below the threshold concentration are ineffective in promoting robust generation of thrombin because of the cooperative influence of the inhibitors; concentrations in excess of the threshold yield robust and almost equivalent thrombin generation. TFPI and the dynamic protein C–thrombomodulin-thrombin system also cooperate to provide threshold-limited, synergistic inhibition of thrombin production. In this instance, TFPI slows the initiation phase while the APC system reduces the availability of the cofactors factors Va and VIIIa, thereby shutting down the propagation phase of thrombin generation.

Antithrombin

AT is a member of the serpin proteinase inhibitor family and circulates in blood as a single-chain glycoprotein. Despite its name, AT inhibits not only thrombin but also most of the proteases in the coagulation pathway. Human AT circulates as two glycoforms, the α and β variants, which contain identical polypeptide backbones but differ in both carbohydrate content and heparin affinity. AT-β binds heparin more tightly than AT-α and is observed to preferentially accumulate on the vessel wall where heparan sulfate proteoglycans are exposed.[188]

AT has a broad spectrum of inhibitory activity, with most of its target proteases participating in the coagulation cascade. It is primarily an inhibitor of thrombin, factor Xa, factor IXa, factor VIIa–TF, factor XIa, factor XIIa, and plasma kallikrein[10,189] (see Fig. 27-2). AT also displays antiproliferative and antiinflammatory properties that may derive from its ability to inhibit thrombin. Heparin and heparan sulfate potentiate these reactions and are used in the treatment of thrombosis. When AT is complexed with heparin, its rate of binding to thrombin increases 1000-fold. The general mechanism of inhibition involves reaction of the active site of the enzyme with a peptide loop structure of the serpin to form a tight, equimolar (1 : 1) complex, followed by inactivating structural rearrangements of both AT and the protease.

Heparin Cofactor II (Leuserpin 2)

Heparin cofactor II is another member of the serpin family and is uniquely stimulated by the proteoglycan dermatan sulfate. The only coagulation enzyme inhibited by heparin cofactor II appears to be thrombin.[190] However, the rate of thrombin inhibition by heparin cofactor II in the absence or presence of glycosaminoglycans is significantly slower than inhibition by AT under similar conditions. In view of the fact that the plasma concentration of heparin cofactor II is 25% to 50% that of AT and that low levels of heparin cofactor II are not strongly associated with thrombosis, the physiologic role of heparin cofactor II as a systemic thrombin inhibitor has been questioned.

Protein C Inhibitor (Plasminogen Activator Inhibitor 3)

Protein C inhibitor is a member of the serine proteinase inhibitor family. Its targets range from the procoagulant, anticoagulant, and fibrinolytic enzymes to plasma and tissue kallikreins, the sperm protease acrosin, and prostate-specific antigen. Hereditary or acquired protein C inhibitor deficiency has not been documented. Protein C inhibitor–deficient mice show impaired spermatogenesis and male infertility.[191] The importance of the regulation of APC by protein C inhibitor in humans is suggested by the use of this complex as a marker for detection of deep vein thrombosis by immunofluorometric assay measurements.[192]

PROTEINS OF CLOT FORMATION

The central event in blood coagulation is the conversion of soluble fibrinogen (factor I) to insoluble cross-linked fibrin. This conversion is accomplished when thrombin removes small polar peptides (termed fibrinopeptides A and B) from the fibrinogen molecule forming fibrin. These fibrin molecules noncovalently interact with each other to form a fibrin web. Fibrin stabilization is accomplished by the action of a second coagulation enzyme (factor XIIIa) that introduces numerous covalent cross-links between these fibrin molecules. The resulting fibrin web is able to capture platelets and red blood cells, thus effectively sealing the wound and stemming plasma loss.

Factor XIII (Fibrin-Stabilizing Factor)

Factor XIII is a protransglutaminase that when proteolytically activated, can form cross-linked amide bonds between glutamyl and lysinyl residues. The zymogen circulates in plasma as a tetramer composed of two A and two B subunits. It plays an important role in hemostasis and thrombosis, as well as participates in the physiologic processes of cell proliferation and cell migration. The product enzyme factor XIII(a) has multiple substrates, including fibrin/fibrinogen, fibronectin, α_2-plasmin inhibitor, collagen, vitronectin, VWF, actin, myosin, factor V, and thrombospondin.[193-196]

Factor XIII is distributed into plasma and platelet pools. Plasma factor XIII circulates as a 320-kD A_2B_2 heterotetramer composed of two identical A chains and two B chains associated noncovalently.[197] The A subunit is the procatalyst and contains three important functional sites: the catalytic site, a calcium binding site, and the activation peptide.[198,199] The activation peptide of one A subunit limits access to the active site cysteine of the other A subunit of the dimer. The B chain has no enzymatic activity and has been thought to function as a carrier of the A subunit.[200]

Bone marrow is the prime site of synthesis of the A chain of plasma factor XIII,[201] which is also present intracellularly as a 160-kD A_2 homodimer in platelets, megakaryocytes, and monocytes.[202,203] How the A_2 dimer is transferred out of the cell and which cell types are the primary sources of plasma A_2 are not understood. Approximately 50% of the total potential factor XIII A chain activity in human blood is found in platelets.[202] The B subunit is synthesized in hepatocytes and secreted as a monomer.[204] After being secreted, the A and B subunits associate and become an A_2B_2 tetrameric molecule in plasma.

Plasma factor XIII is activated by cleavage of thrombin at the NH_2-terminal end of the A chains, with release of a 36–amino acid activation peptide. Catalytic activity is expressed only after the A chain dimer is dissociated from the B chain. Fibrin/fibrinogen plays an important role as a cofactor in dissociation of the B chains from thrombin-cleaved factor XIII.[201] The intracellular form of factor XIII contains only the A chain and thus does not require dissociation of the B chain.

Activation of factor XIII during blood clotting is tightly correlated with fibrin formation.[205] This creates a regulated system in which cross-linked polymers occur coincident with fibrin being formed. The rate of thrombin cleavage of plasma factor XIII has been shown to be greatly accelerated by the presence of fibrin polymers.[206] This positive feedback network between thrombin, fibrin/fibrinogen, and factor XIIIa ensures that a stable clot can form rapidly to maintain hemostasis. To date, no known endogenous inhibitor has been described that regulates this important enzyme.[193-196] Fibrin is the main physiologic substrate for factor XIIIa.

Fibrinogen (Factor I) and Fibrin

Fibrinogen is composed of six polypeptide chains (two $A\alpha$ chains, two $B\beta$ chains, and two γ chains); after

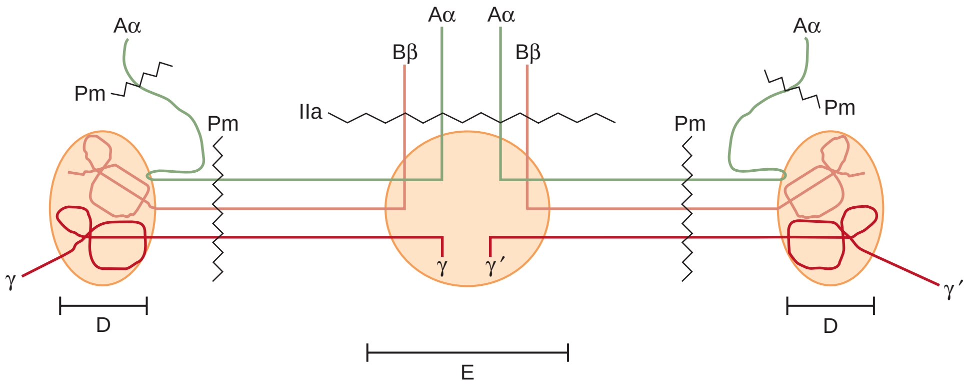

Figure 27-9 Schematic representation of fibrinogen to illustrate thrombin and plasmin cleavage sites in the intact molecule. The six polypeptide chains, Aα2Bβ2γ2 are folded into three globular structures, two "D" and one "E," which are connected by "coiled-coil" segments. The NH2-terminal regions of the Aα, Bβ chains extend from the central "E" domain and, when cleaved by thrombin, yield fibrinopeptide A and fibrinopeptide B. The macromolecular product fibrin is then referred to as $\alpha 2\beta 2\gamma 2$. A small fraction of fibrinogen contains a COOH-terminal extension of the γ chain that is referred to as γ′.

posttranslational modification, the mature protein circulates in blood with an average molecular weight of 340,000 (see Table 27-1).[207,208] The polypeptide chains are distributed into two symmetric half-molecules, each containing one Aα, one Bβ, and one γ chain, with the NH$_2$-terminal ends oriented toward each other (Fig. 27-9). The half-molecules are linked by noncovalent and disulfide bonds near their NH$_2$-terminal ends to yield a linear arrangement of three nodular structures. The outside two domains, formed by the COOH-terminal regions of the Bβ and γ chains of fibrinogen, are designated "D," whereas the central domain, which contains the NH$_2$-terminal ends of all the chains, is designated "E." Between 1.7 and 5 g of fibrinogen is synthesized per day by the liver, with approximately 75% of this fibrinogen being secreted into plasma and the remainder being distributed between the lymph and interstitial fluids.[209] Fibrinogen is an acute-phase reactant and is upregulated 2- to 10-fold in response to a variety of physiologic stresses, including trauma, pregnancy, and tissue inflammation.

The fibrinogen locus is composed of three closely linked genes (specifying the polypeptides Aα, Bβ, and γ) found as single copies in a region of approximately 50 kb. The three fibrinogen genes most likely evolved from a common ancestor through a series of duplications that began approximately 1 billion years ago.[210] Expression of the three chains is coordinately controlled and, at least for the hepatocyte, results in almost equal levels of mRNA for each chain in the cell.[211] Although most γ chains end at γ-Val411 (human numbering), approximately 10% of the time during splicing the last intron is retained, which results in a new chain, γ′, that ends at γ-Leu427.[212] A less common variant of the γ chain adds a 236–amino acid extension at the C-terminus.[213] After synthesis but before secretion, a number of posttranslational modifications occur, including glycosylation and phosphorylation. Factor XIIIa cross-linking occurs between sites on the Aα and γ chains but not on the Bβ chain.

Thrombin catalyzes the hydrolysis of Arg-Gly bonds, with small, polar NH$_2$-terminal pieces (fibrinopeptides) being removed from the α and β chains[207,208,214] (see Fig. 27-9). Cleavage of Aα-Arg16 by thrombin releases fibrinopeptide A (FPA) and forms fibrin I. The release of two FPA peptides exposes a site in the E domain that aligns with a complementary site in the D domain to form overlapping fibrils. Subsequent cleavage by thrombin at Bβ-Arg14 releases fibrinopeptide B (FPB) and leads to the formation of fibrin II, which presumably increases lateral aggregation of the protofibrils. FPA and FPB vary in length, between 13 and 21 amino acids in various mammals, and constitute less than 2% of the total mass.

Release of fibrinopeptides from fibrinogen results in the formation of an intermediate termed the fibrin monomer, which is all but indistinguishable from fibrinogen. In purified systems, removal of either FPA or FPB leads to formation of the fibrin dimer through noncovalent (i.e., charge-charge [salt links]) and hydrogen bond interactions. As fibrin monomers continue to be generated, the dimer elongates from both ends as a two-stranded molecule until it reaches approximately 30 monomers, at which point it becomes a protofibril. The second step in fibrin assembly is lateral association of the protofibrils into thicker fibrin fibers. These fibers are formed from the association of between 14 and 22 protofibrils. Because protofibrils, not dimers, are required for this step, it is believed that the forces involved in lateral association are weak and therefore become "strong" only in large numbers. Clots are known to branch, although how branching is accomplished is not clear.

The description of fibrinogen activation and fibrin assembly has been based on studies involving the use of citrated plasma or purified proteins, or both. To understand the in vivo process of fibrin formation, a system with nonanticoagulated blood has been used; in this experimental model the pattern of fibrin formation based on release of fibrinopeptide is different from systems using citrated plasma or purified fibrinogen.[205] In this study, most cleavage of FPA and subsequent clot formation take place just before the propagation phase of thrombin generation (Fig. 27-10). At the point of visual clot formation, virtually all fibrinogen (and some product

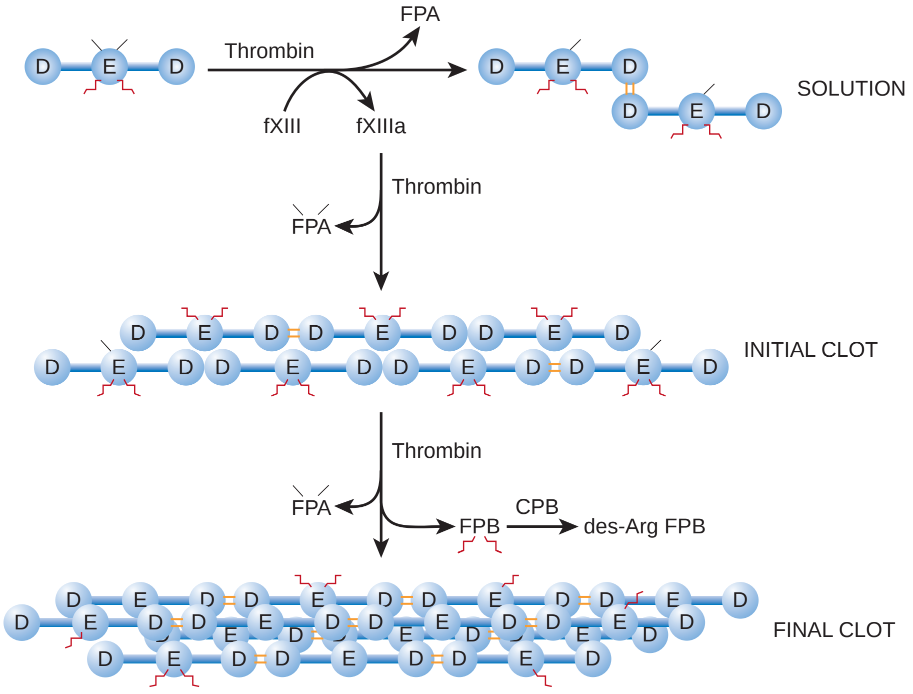

Figure 27-10 Schematic representation of whole blood fibrin formation. Thrombin at the beginning of clot formation simultaneously acts on fibrinogen (D-E-D) and factor XIII (fXIII). A portion (~40%) of fibrinopeptide A (FPA) is released from fibrinogen, and an initial clot is formed from the complementary overlap of the exposed sites between the E and D domains of adjacent molecules. Activated factor XIII (fXIIIa) simultaneously cross-links adjacent D domains (D = D). Thus the initial soluble fibrin clot is composed of fibrinogen, fibrin, and γ-γ dimers with fibrinopeptide B (FPB) still attached. The initial clot is continuously acted on by thrombin, which releases the remaining FPA and some of the FPB to yield a final clot with the majority of FPB still attached. The FPB released is selectively acted on by a carboxypeptidase B–like enzyme (CPB) to cleave the COOH-terminal arginine and produce des-Arg FPB. The significance of this cleavage is still unclear. *(Modified from Brummel KE, Butenas S, Mann KG: An integrated study of fibrinogen during blood coagulation.* J Biol Chem *1999;274: 22862-22870.)*

already cross-linked) disappears from the fluid phase of the reaction. Thus the "clot" appears to be a mixture composed of fibrin I and fibrinogen. The insoluble material present in the fibrin clot is virtually all cross-linked by factor XIIIa, whose activation occurs nearly simultaneously with removal of FPA[205] (see Fig. 27-10). It has been proposed that as many as six cross-links can form between a fibrin monomer and its neighbors and that the presence of these cross-links increases the strength, chemical resistance to urea, and lysis by plasmin.[207,208,215]

CONCLUSION

As the result of molecular investigation of blood coagulation for more than 100 years, the components of the blood coagulation system and their connections are well understood. Critical insights into this process and its regulation have often come from the study of patients with mutations in the genes encoding the relevant coagulation factors. It is only fitting that this knowledge has formed the basis for the development of reliable diagnostic tools and effective therapies for patients with both inherited and acquired disorders of hemostasis, as detailed in the ensuing chapters.

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CHAPTER 28

Molecular Basis of Fibrinolysis

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CHAPTER OUTLINE

BASIC CONCEPTS OF FIBRINOLYSIS

COMPONENTS OF THE FIBRINOLYTIC SYSTEM

Plasminogen

Plasminogen Activators

Inhibitors of Fibrinolysis

Cellular Receptors in Fibrinolysis

THE FIBRINOLYTIC ACTIONS OF PLASMIN

Fibrinogen

Fibrin

Tissue Plasminogen Activator–Mediated Plasminogen Activation

Urokinase-Mediated Plasmin Generation

PHYSIOLOGIC FUNCTIONS OF THE FIBRINOLYTIC SYSTEM

Thrombosis and Thrombolysis

Wound Healing and Tissue Fibrosis

Atherosclerosis and Cardiovascular Remodeling

Tumor Biology

Microbial Virulence and Host Defense Mechanisms

Neurologic Plasticity

NON–PLASMIN-MEDIATED FIBRINOLYSIS

Fibrinolysis is the process by which the insoluble protein fibrin is converted to a defined set of soluble degradation products. It occurs in both intravascular and extravascular locations and is essential to human health and survival. Modern molecular biologic techniques have identified the major fibrinolytic genes, the mechanisms regulating their expression, and the consequences of their deficiency or overexpression in genetically engineered mice. In many cases these experiments have yielded surprising and instructive data that have revealed physiologic and pathophysiologic roles for the fibrinolytic system that are much more complex than originally thought. Furthermore, in the current genomic age, molecular analysis of human syndromes has identified specific mutations that result in either thrombosis secondary to fibrinolytic deficiency or hemorrhage secondary to fibrinolytic excess. In this chapter the molecular basis of fibrinolysis and its physiologic roles in human health and disease are reviewed.

BASIC CONCEPTS OF FIBRINOLYSIS

In response to vascular injury and activation of the coagulation cascade, thrombin induces polymerization of the soluble plasma protein fibrinogen, thereby producing an insoluble, cross-linked end product called fibrin (see Chapter 27). Once the flow of blood has been stemmed and vascular integrity restored, fibrin, which is found in both intravascular and extravascular sites, is cleared by a process known as fibrinolysis (Fig. 28-1, *A*). Normally, the fibrinolytic process is tightly regulated by a series of activators, inhibitors, cofactors, and receptors.[1] In the presence of fibrin, which serves as a cofactor for its own destruction, tissue plasminogen activator (tPA) is released from endothelial cells and possibly other cell types and interacts with the circulating zymogen plasminogen. Plasminogen, tPA, and fibrin form a ternary complex that accelerates the catalytic efficiency of plasmin generation by approximately 500-fold. Urokinase is also an efficient plasminogen activator (uPA), but its action is only minimally enhanced by fibrin. The action of plasmin on fibrin generates soluble fibrin degradation products, many of which have their own unique biologic properties.

The dynamic regulation of plasmin generation is complex. On the surface of a fibrin-containing thrombus, tPA and plasmin are protected from their major circulating inhibitors plasminogen activator inhibitor 1 (PAI-1) and α_2-antiplasmin (α_2-AP), respectively. On release into the circulation, however, plasmin and tPA are rapidly neutralized by these inhibitors and cleared by the liver. Because uPA and the nonenzymatic plasminogen activator streptokinase do not use fibrin as a cofactor, they function well in the fluid phase. Plasminogen may also be activated, albeit rather inefficiently, by proteases of the contact system such as kallikrein, factor XIa, and factor XIIa (see Chapter 27).

Cell surfaces represent protected environments in which plasmin can be generated without the risk of

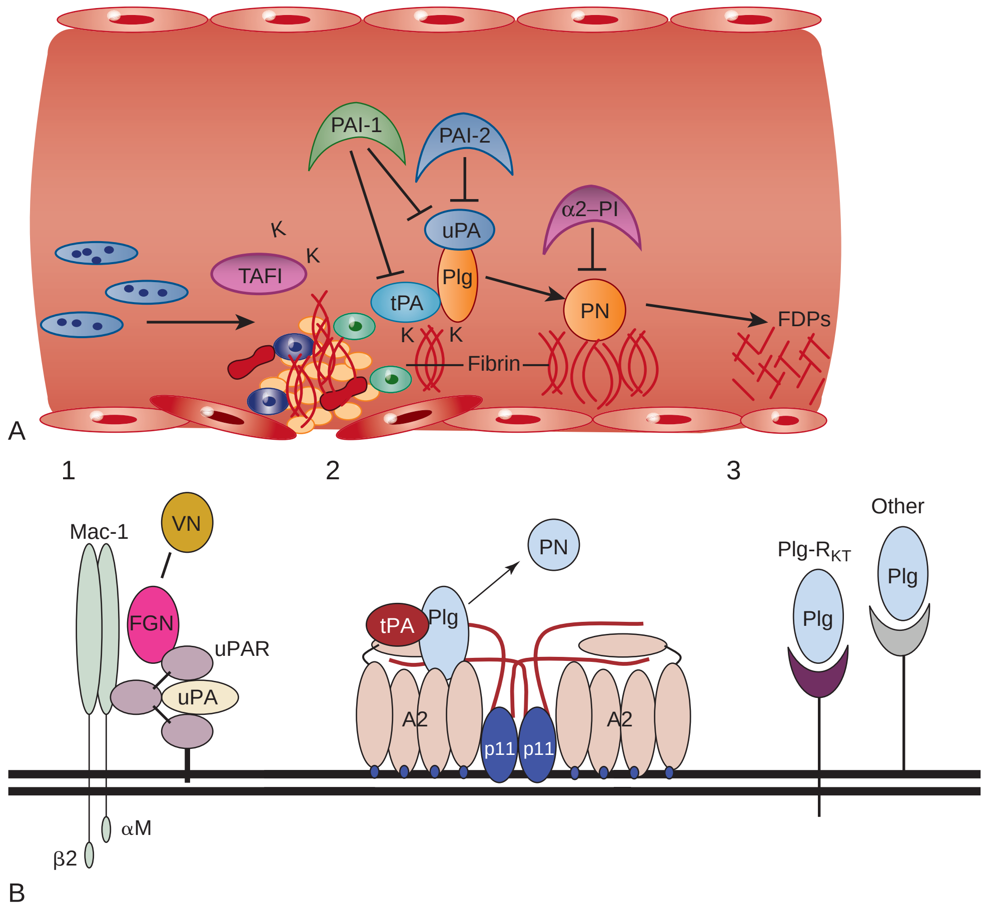

Figure 28-1 Overview of the fibrinolytic system. **A,** Fibrin-based plasminogen activation. The zymogen plasminogen (Plg) is converted to the active serine protease plasmin (PN) through the action of tissue plasminogen activator (tPA) or urokinase (uPA). The activity of tPA is greatly enhanced by its assembly with Plg through lysine residues (K) on a fibrin-containing thrombus. uPA acts independently of fibrin. Both tPA and uPA can be inhibited by plasminogen activator inhibitor 1 (PAI-1), which is released by endothelial cells, activated platelets, and other cells. PAI-2, on the other hand, neutralizes uPA more efficiently than it does tPA. By binding to fibrin, plasmin is protected from its major inhibitor α_2-antiplasmin (α_2-AP). Bound plasmin degrades cross-linked fibrin, which gives rise to soluble fibrin degradation products (FDPs). **B,** Some cell surface fibrinolytic receptors. *1,* Endothelial cells, monocytes, and macrophages express the uPA receptor (uPAR), which can interact with both uPA, thereby preserving its activity, and adhesive glycoproteins, such as fibrinogen (FGN) and vitronectin (VN). uPAR association with Mac-1 appears to facilitate adhesive interactions, whereas its interaction with uPA has a negative impact on adhesion. *2,* Endothelial cells, monocytes, and macrophages also express the annexin $(\text{A2-p11})_2$ complex, a coreceptor for tPA and Plg that augments the efficiency of plasmin generation. A2 forms a heterotetramer with protein p11, a member of the S100 family, which accentuates the efficiency of plasmin generation. *3,* Plasminogen receptors may be either transmembrane (Plg-R_{KT}) or peripheral membrane proteins (other). *(Modified from Ragno P: The urokinase receptor: a ligand or a receptor?* Cell Mol Life Sci *63:1028–1037, 2006.)*

neutralization by fluid-phase inhibitors (Fig. 28-1, *B*).[2] Endothelial cells, platelets, monocytes, macrophages, and some tumor cells all express protein receptor sites for plasminogen, tPA, or uPA.[3] The broad substrate specificity of plasmin observed in vitro may relate to its generation in nonvascular sites through fibrin-independent mechanisms. Plasmin may thus play an important role in extravascular events such as the modification of growth and differentiation factors, processing of matrix proteins, and activation of procoagulant molecules.

COMPONENTS OF THE FIBRINOLYTIC SYSTEM

Plasminogen

Synthesized primarily in the liver,[4,5] plasminogen is a single-chain proenzyme with a molecular weight (M_r) of approximately 92,000 that circulates in plasma at a concentration of approximately 1.5 μmol/L (Table 28-1).[6] The plasma half-life of plasminogen in humans is approximately 2 days.[7] Its 791 amino acids are cross-linked by 24 disulfide bridges, 16 of which yield five homologous triple-loop structures called *kringles*[8] (Fig. 28-2). The first (K1) and fourth (K4) of these 80–amino acid structures with an M_r of 10,000 impart high- and low-affinity lysine binding, respectively.[9] The lysine-binding domains of plasminogen appear to mediate its specific interactions with fibrin, cell surface receptors, and other proteins, including its circulating inhibitor α_2-AP.[10-14]

Posttranslational modification of plasminogen results in two glycosylation variants (forms 1 and 2)[15-17] (see Table 28-1). An *O*-linked oligosaccharide on Thr345 is common to both forms. Only form 2, however, contains an *N*-linked oligosaccharide on Asn288. The carbohydrate portion of plasminogen appears to regulate its

TABLE 28-1 Fibrinolytic Proteins

PROTEASES

Property	**Plasminogen**	**tPA**	**uPA**
Molecular mass (D)	92,000	72,000	54,000
Amino acids	791	527	411
Chromosome	6	8	10
Site of synthesis	Liver Neuronal cells Glial cells	Endothelium Neuronal cells Glial cells	Endothelium Kidney
Plasma concentration			
nmol/L	1500	0.075	0.150
μg/mL	140	0.005	0.008
Plasma half-life	48 hr	5 min	8 min
N-Glycosylation (%)	2	13	7
Form 1	—	N117, N184, N448	N302
Form 2	N288	N117, N448	—
O-Glycosylation			
α-Fucose	—	T61	T18
Complex	T345	—	—
Two-chain cleavage site	R560-V561	R275-I276	K158-I159
Heavy chain domains			
Finger	No	Yes	No
Growth factor	No	Yes	Yes
Kringles (no.)	5	2	1
Light-chain catalytic triad	H602, D645, S740	H322, D371, S478	H204, D255, S356

MAJOR SERPIN INHIBITORS

Property	**α_2-AP**	**PAI-1**	**PAI-2**
Molecular mass (D)	70,000	52,000	60,000 (glycosylated) 47,000 (nonglycosylated)
Amino acids	452	402	393
Chromosome	18	7	18
Sites of synthesis	Kidney, liver	ECs Monocytes/Macrophage Hepatocytes Adipocytes	Placenta Monocytes/Macrophage Tumor cells
Plasma concentration			
nmol/L	900	0.1–0.4	ND
μg/mL	50	0.02	ND
Serpin reactive site	R364-M365	R346-M347	R358-T359
Specificity	Plasmin	uPA = tPA	uPA > tPA

NONSERPIN INHIBITORS

Property	**TAFI**	**α_2-MG**
Molecular mass (D)	45,000	725,000 (monomer ~180,000)
Amino acids	423	1451
Chromosome	13	12
Sites of synthesis	Liver	Liver, Macrophage Endothelium Fibroblasts
Plasma concentration		
nmol/L	~75	~2000–5000
μg/mL	4	~1450–3625
Activators	Plasmin >> thrombin	—
Specificity	C-terminal K and R	Broad spectrum

TABLE 28-1 Fibrinolytic Proteins (Continued)

		SOME PROPOSED ACTIVATION RECEPTORS		
		Annexin A2 Complex $(A2\text{-}p11)_2$		
Property	**uPAR**	A2	S100A10 (p11)	**PlgR-$_{KT}$**
Molecular mass (D)	55-60,000	36,000	11,000	17,000
Amino acids	313	339	97	147
Chromosome	19	15	1	9
Source	ECs Monocytes Macrophage Fibroblasts Tumor cells	ECs Macrophage Early myeloid cells Tumor cells	M	Monocytes
Ligand(s)	uPA	tPA, Plg	Plg, tPA	Plg

α_2-AP, α_2-Antiplasmin; *α_2-MG,* α_2-macroglobulin; *EC,* endothelial cell; *LRP,* low-density lipoprotein receptor-like protein; *PAI,* plasminogen activator inhibitor; *ND,* not determined; *Plg,* plasminogen; *TAFI,* thrombin activatable fibrinolysis inhibitor; *tPA,* tissue plasminogen activator; *uPA,* urokinase plasminogen activator; *uPAR,* urokinase plasminogen activator receptor.

affinity for cellular receptors and may also specify its physiologic degradation pathway.

Activation of plasminogen results from cleavage of a single Arg-Val peptide bond at position 560 to 561,[18] which produces the active protease plasmin (see Table 28-1). Plasmin contains a typical serine protease catalytic triad[6] but exhibits broad substrate specificity in comparison to other proteases of this class.[19] The circulating form of plasminogen, amino-terminal glutamic acid plasminogen (Glu-Plg), is readily converted by limited proteolysis to several modified forms known collectively as lysine plasminogen (Lys-Plg).[20,21] Hydrolysis of the Lys77-Lys78

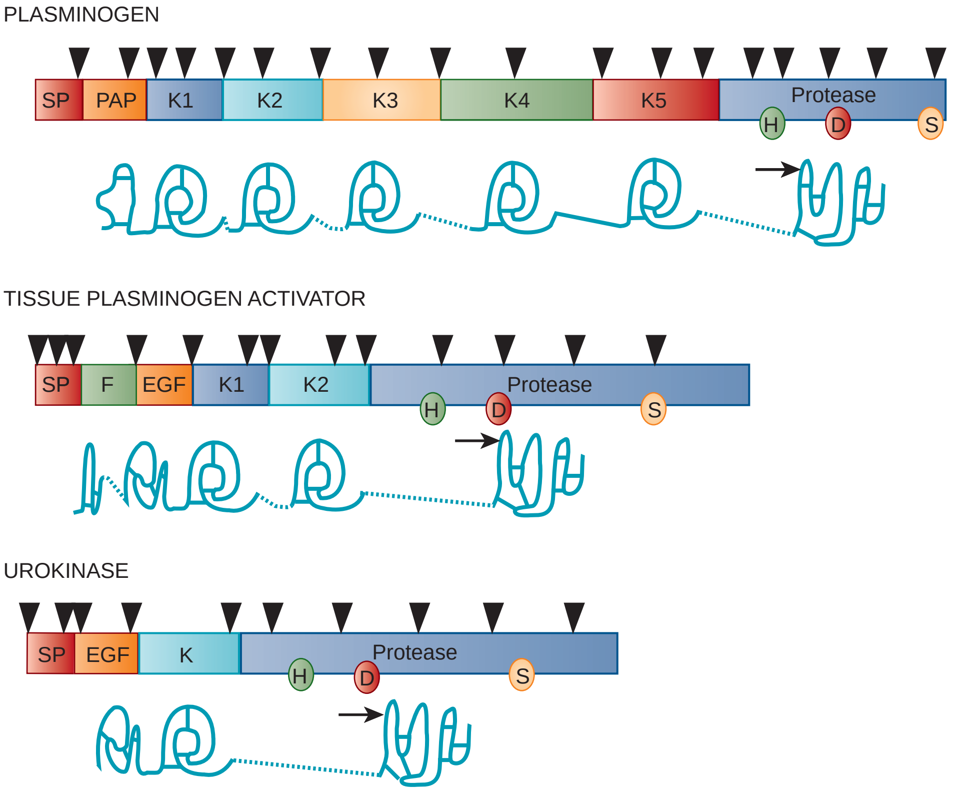

Figure 28-2 Structure-function relationships of plasminogen, tissue plasminogen activator (tPA), and urokinase (uPA) and alignment of the intron-exon structure of the plasminogen, tPA, and uPA genes with functional protein domains. Protein domains are labeled signal peptide (SP), preactivation peptide (PAP), kringle domains (K), fibronectin-like "finger" (F), epidermal growth factor–like domain (EGF), and protease. The approximate positions of the catalytic triad of amino acids histidine (H), aspartic acid (D), and serine (S) are shown within individual protease domains. The positions of individual introns relative to amino acid–encoding exons are indicated with *inverted triangles*.

peptide bond results in an altered conformation that more readily binds fibrin, displays twofold to threefold higher avidity for cellular receptors, and is activated 10 to 20 times more rapidly than Glu-Plg is.[12,18,22] Lys-Plg does not normally circulate in plasma[18] but has been identified on cell surfaces.[23]

Spanning 52.5 kilobases (kb) of DNA on chromosome 6q26-27, the plasminogen gene consists of 19 exons[24,25] and directs expression of a 2.7-kb messenger RNA (mRNA)[8] (see Fig. 28-2). Plasminogen gene activity is stimulated by the acute phase mediator interleukin-6, both in vitro and in vivo.[26] The gene is closely linked and structurally related to that of apolipoprotein(a), an apoprotein associated with the highly atherogenic low-density lipoprotein–like particle lipoprotein(a)[27] and more distantly related to other kringle-containing proteins such as tPA, uPA, hepatocyte growth factor, and macrophage-stimulating protein.[28-32] The significance of the latter two proteins to the fibrinolytic system remains to be determined.

Plasminogen Activators

Tissue Plasminogen Activator

One of the two major endogenous plasminogen activators, tPA, is a 527–amino acid glycoprotein with an M_r of approximately 72,000[33] (see Table 28-1). tPA contains five structural domains, including a fibronectin-like “finger,” an epidermal growth factor–like domain, two kringle structures homologous to those of plasminogen, and a serine protease domain (see Fig. 28-2). Cleavage of the Arg275-Ile276 peptide bond by plasmin converts tPA to a disulfide-linked, two-chain form.[33] Although single-chain tPA is less active than two-chain tPA in the fluid phase, both forms demonstrate equivalent activity when bound to fibrin.[34]

The two glycosylation forms of tPA are distinguishable by the presence (type 1) or absence (type 2) of a complex *N*-linked oligosaccharide moiety on Asn184 (see Table 28-1).[35,36] Both types, however, contain a high-mannose carbohydrate on Asn117, a complex oligosaccharide on Asn448, and an *O*-linked α-fucose residue on Thr61.[37] The carbohydrate moieties of tPA may modulate its functional activity, regulate its binding to cell surface receptors, and specify degradation pathways.

Located on chromosome 8p12-q11.2, the gene for human tPA is encoded by 14 exons spanning a total of 36.6 kb.[38-40] Most of the structural domains of tPA are encoded by one or two exons, and the organization of these exons is similar across related domains of tPA and the other fibrinolytic proteases (see Fig. 28-2). This observation suggests that the tPA gene arose by an evolutionary process called “exon shuffling,” whereby functionally related genes are generated through rearrangement of exons encoding autonomous domains. Consistent with this hypothesis, various functions of tPA can be localized to specific domains. For example, deletion of the fibronectin-like finger or kringle 2, but not kringle 1, results in a tPA resistant to the cofactor activity of fibrin, whereas catalytic activity in the absence of fibrin remains intact.[41]

The proximal promoter of the human tPA gene contains binding sequences for potentially important transcriptional factors, including AP1, NF1, SP1, and AP2,[42,43] as well as a potential cyclic adenosine monophosphate (cAMP)-responsive element.[44] In vitro, many agents have been shown to exert small effects on the expression of tPA mRNA, but relatively few enhance tPA synthesis without augmenting PAI-1 synthesis as well. Agents that regulate tPA gene expression independently of PAI-1 include arterial shear stress, thrombin, endotoxin, histamine, butyrate, retinoids, and dexamethasone.[45-50] Forskolin, which increases intracellular cAMP levels, has been reported to decrease the synthesis of both tPA and PAI-1.[43,51]

tPA is synthesized in the endothelial cell and stored within Rab3D-negative granules that are distinct from classical Weibel-Palade bodies.[52] Its release is governed by a variety of stimuli such as shear stress, butyrate, thrombin, histamine, bradykinin, epinephrine, acetylcholine, arginine vasopressin, gonadotropins, exercise, and venous occlusion.[45,47,53,54] Its circulating half-life is exceedingly short (≈5 minutes). By itself, tPA is a poor activator of plasminogen. However, in the presence of fibrin, the catalytic efficiency of tPA-dependent plasmin generation (k_{cat}/K_m) increases by at least 2 orders of magnitude[22] because of a dramatic increase in affinity (decreased Michaelis constant [K_m]) between tPA and its substrate plasminogen in the presence of fibrin. Although it is expressed by extravascular cells, tPA appears to represent the major intravascular activator of plasminogen.[19]

Urokinase

The second endogenous plasminogen activator, single-chain uPA or prourokinase, is a glycoprotein with an M_r of approximately 54,000 and consists of 411 amino acids (see Table 28-1). uPA contains an epidermal growth factor–like domain and a single plasminogen-like kringle and possesses a classic catalytic triad within its serine protease domain[55] (see Fig. 28-2). Cleavage of the Lys158-Ile159 peptide bond by plasmin or kallikrein converts single-chain uPA to a disulfide-linked two-chain derivative.[56] Located on chromosome 10, the human uPA gene is encoded by 11 exons spanning 6.4 kb and expressed by activated endothelial cells, macrophages, renal epithelial cells, and some tumor cells.[57,58] As noted earlier, its intron-exon structure is closely related to that of the tPA gene.

uPA may be induced during neoplastic transformation, possibly through a mechanism involving the transcription factors AP1 and AP2.[59] Other agents that appear to induce expression of uPA in vitro include hormones, growth factors, and cAMP.[49] Inflammatory cytokines such as interleukin-1 and lipopolysaccharide induce only small increments in uPA expression, whereas tumor necrosis factor and transforming growth factor β (TGF-β) have a more dramatic (5- to 30-fold) effect.[60-62]

Two-chain uPA occurs in both high-molecular-weight (M_r of 54,000) and low-molecular-weight (M_r of 33,000) forms that differ by the presence or absence, respectively, of a 135-residue amino-terminal fragment released by plasmin cleavage between Lys135 and Lys136.[63,64]

Although both forms are capable of activating plasminogen, only the high-molecular-weight form binds to the uPA receptor (uPAR). uPA has much lower affinity for fibrin than tPA does and is an effective plasminogen activator both in the presence and in the absence of fibrin.[65,66] The extent to which prourokinase possesses intrinsic plasminogen-activating capacity is an area of controversy.[67,68]

Accessory Plasminogen Activators

Under certain conditions, proteases traditionally classified within the intrinsic arm of the coagulation cascade (see Chapter 26) have been shown to be capable of activating plasminogen directly. Such proteases include kallikrein, factor XIa, and factor XIIa.[69-71] Normally, however, they account for no more than 15% of the total plasmin-generating activity in plasma.

Inhibitors of Fibrinolysis

Plasmin Inhibitors

The action of plasmin is negatively modulated by a family of ***ser***ine ***p***rotease ***in***hibitors called serpins[72] (see Table 28-1). All serpins share a common mechanism of action by forming an irreversible complex with the active-site serine of the target protease after proteolytic cleavage of the inhibitor by the target protease. Within such a complex, both protease and inhibitor lose their activity.

A single-chain glycoprotein with an M_r of approximately 70,000, α_2-AP circulates in plasma at relatively high concentrations (≈0.9 μmol/L) and has a plasma half-life of 2.4 days[73] (see Table 28-1). This serpin contains about 13% carbohydrate by mass and consists of 452 amino acids with two disulfide bridges.[74] In humans the gene is located on chromosome 18 and contains 10 exons distributed over 16 kb of DNA.[75] The promoter region of the α_2-AP gene contains a hepatitis B–like enhancer element that directs tissue-specific expression in the liver.[75] α_2-AP is also a constituent of platelet alpha granules.[76] Plasmin released into flowing blood or in the vicinity of a platelet-rich thrombus is immediately neutralized on forming an irreversible 1 : 1 stoichiometric, lysine binding site–dependent complex with α_2-AP. Interaction with plasmin is accompanied by cleavage of the Arg364-Met365 peptide bond, and the resulting covalent complexes are cleared in the liver.

Several other proteins inhibit the activity of fibrinolytic serine proteases (see Table 28-1). α_2-Macroglobulin (α_2-MG) is a tetrameric protein with an M_r of 725,000 that is synthesized by the liver, endothelial cells, macrophages, and fibroblasts and is found in platelet alpha granules. The gene for α_2-MG, which consists of 36 exons distributed over 48 kb of DNA on chromosome 12, directs the expression of a 1451–amino acid polypeptide.[77-79] As a generic inhibitor of all four classes of proteases (serine, cysteine, aspartyl, and metallo), α_2-MG is a nonserpin that inhibits plasmin with approximately 10% of the efficiency exhibited by α_2-AP by forming a noncovalent complex.[80] C1 esterase inhibitor can also serve as an inhibitor of tPA in plasma.[81] Protease nexin may function as a noncirculating cell surface inhibitor[82] of trypsin, thrombin, factor Xa, urokinase, or plasmin and result in protease-inhibitor complexes that are endocytosed via a specific nexin receptor.[83]

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma carboxypeptidase that acts as a potent inhibitor of fibrinolysis (see Table 28-1).[84] Identical to the previously cloned carboxypeptidase B[85] and the previously isolated carboxypeptidase U,[86] this single-chain polypeptide with an M_r of 45,000 circulates in plasma at concentrations of about 75 nmol/L and undergoes limited proteolysis in the presence of plasmin or thrombin, which leads to its activation.[87] Carboxypeptidase B–like molecules remove carboxyl-terminal lysine or arginine residues from fibrin and other proteins, thereby reducing binding of plasminogen to fibrin and cell surfaces and limiting plasmin generation.[88] The potentially antifibrinolytic effect of thrombin appears to be mediated through its ability to activate TAFI in the presence of thrombomodulin.[84,89] Anticoagulation by inhibition of factor XI also appears to have an antifibrinolytic effect in vivo by downregulation of thrombin-mediated activation of TAFI.[90] In a system of purified components, TAFI has been shown to reduce tPA-induced fibrinolysis half-maximally at approximately 1 nmol/L, which is well below its concentration in plasma.[91]

Plasminogen Activator Inhibitors

Plasminogen Activator Inhibitor 1. Of the two major plasminogen activator inhibitors[92] (see Table 28-1), PAI-1 is the most ubiquitous. This single-chain cysteine-less glycoprotein with an M_r of approximately 52,000 is released by endothelial cells, monocytes, macrophages, hepatocytes, adipocytes, and platelets.[93,94,95] Release of PAI-1 is stimulated by many cytokines, growth factors, and lipoproteins common to the global inflammatory response.[61,96-98] The PAI-1 gene consists of nine exons spanning 12.2 kb on chromosome 7q21.3-q22.[99] The serpin reactive site is located at Arg346-Met347, and activity of this labile serpin is stabilized upon complex formation with vitronectin, a component of plasma and the pericellular matrix.[100,101]

Regulation of PAI-1 gene expression is complex.[102-105] Agents that have been shown to enhance expression of PAI-1 at the message level, the protein level, or both without affecting the synthesis of tPA include the inflammatory cytokines lipopolysaccharide, interleukin-1, tumor necrosis factor α,[61,62,96,98,106-108] TGF-β and basic fibroblast growth factor,[60,96,103,105] very-low-density lipoprotein and lipoprotein(a),[109,110] angiotensin II,[111] thrombin,[107,112] and phorbol esters.[113] In addition, endothelial cell PAI-1 is downregulated by forskolin[51] and by endothelial cell growth factor in the presence of heparin.[114]

Plasminogen Activator Inhibitor 2. Originally purified from human placenta,[92,115] PAI-2 is a 393–amino acid member of the serpin family whose reactive site is the Arg358-Thr359 peptide bond[115] (see Table 28-1). The gene encoding PAI-2 is located on chromosome 18q21-23, spans 16.5 kb, and contains eight exons.[116] PAI-2 exists as both a nonglycosylated intracellular form with an M_r of 47,000 and a glycosylated form with an M_r of

60,000 secreted primarily by macrophages and keratinocytes. Functionally, PAI-2 inhibits both two-chain tPA and two-chain uPA with comparable efficiency (second-order rate constants of 10^5 mmol/L/sec). However, it is less effective in inhibiting single-chain tPA (second-order rate constant of 10^3 mol/L/sec) and does not inhibit prourokinase.

Significant levels of PAI-2 are found in human plasma only during pregnancy but can be markedly enhanced in vitro by the inflammatory mediator tumor necrosis factor.[117] Regulation of PAI-2 gene expression is modulated by a variety of factors, including a number of inflammatory mediators.[118-121] In macrophages in vitro, secretion of PAI-2 is enhanced by endotoxin and phorbol esters.[121,122]

Cellular Receptors in Fibrinolysis

Though structurally diverse, cell surface fibrinolytic receptors can be classified into two groups whose integrated actions are likely to be essential for homeostatic control of plasmin activity[3] (see Table 28-1). Activation receptors localize and potentiate plasminogen activation, whereas clearance receptors eliminate plasmin and plasminogen activators from the blood or focal microenvironments.

Activation Receptors

Plasminogen Receptors. Plasminogen receptors are a diverse group of proteins expressed on a wide array of cell types.[3] Receptors reported include α-enolase, glycoprotein IIb-IIIa complex, the Heymann nephritis antigen, amphoterin, and the annexin A2 (A2) complex (A2-p11) (see Table 28-1) and have been found to be expressed primarily on monocytoid cells,[123,124] platelets,[125] renal epithelial cells,[126] neuroblastoma cells,[127] and endothelial cells,[128-130] respectively. In addition, Plg-R_{KT}, expressed mainly on macrophages, is the first transmembrane plasminogen receptor to be identified, and it appears also to bind tPA (see Table 28-1).[131,132] All plasminogen receptors appear to interact with the kringle structures of plasminogen through carboxyl-terminal lysine residues.

The Urokinase Receptor. uPAR is expressed on monocytes, macrophages, fibroblasts, endothelial cells, and a variety of tumor cells[3] (see Table 28-1). uPAR complementary DNA (cDNA) was cloned and sequenced from a human fibroblast cDNA library[133] and encodes a protein of 313 amino acids with a 21-residue signal peptide. The gene structure consists of seven exons distributed over 23 kb of genomic DNA, which places this glycoprotein within the Ly-1/elapid venom toxin superfamily of cysteine-rich proteins.[134-136] uPAR is anchored to the plasma membrane through glycosylphosphatidylinositol linkages.[137] uPA bound to its receptor maintains its activity and susceptibility to PAI-1.[138] Formation of uPA–PAI-1 complexes appears to hasten clearance of uPA by hepatic or monocytoid cells.[139-141]

High-resolution (1.9 to 2.7 Å) analysis of the crystal structure of a complex formed by various amino-terminal uPA peptides and a soluble form of uPAR reveals that uPA engages a central cone-shaped cavity in uPAR formed by its three-finger motif. This arrangement leaves surface residues of uPAR available for binding of other proteins, such as integrins and vitronectin. It is therefore postulated that these interactions may be regulated by binding of uPA itself.[142-144]

Aside from a potential role in fibrinolysis, uPAR appears to play a pivotal role in cellular signaling and adhesion events.[145] uPAR binds to the adhesive glycoprotein vitronectin at a site distinct from the uPA-binding domain,[146,147] and uPAR-transfected renal epithelial cells acquire enhanced adhesion to vitronectin and lose their adhesion to fibronectin.[148] Furthermore, uPAR colocalizes with integrins in focal contacts and at the leading edge of migrating cells[149] and also associates with caveolin, a major component of caveolae, structures abundant in endothelial cells and thought to participate in signaling events.[150-152] Thus integrin function may be regulated by uPAR, an association signifying an integrated relationship between cellular adhesion and proteolysis.

These findings have important implications for the behavior of malignant cells, in which uPAR is frequently overexpressed.[152] High-level uPAR expression also appears to be an indicator of a poor prognosis in breast, lung, colon, esophageal, and gastric cancer.[153] uPA has been identified by protease activity profiling as being important in intravasation of fibrosarcoma,[154] and RNA interference–mediated "knockdown" of uPA and uPAR seems to block the in vivo invasiveness of highly malignant cells.[154,155]

The Annexin A2 Complex. Annexin A2 (A2) is a widely distributed, highly conserved, peripheral membrane protein with an M_r of 36,000 that is expressed abundantly on endothelial cells,[128-130] monocyte/macrophages,[159,160] myeloid cells,[161] and some tumor cells[162-164] (see Table 28-1). It belongs to a more than 60-member superfamily of calcium-dependent, phospholipid-binding proteins[165,166] that have in common a conserved carboxyl-terminal "core" region preceded by a more variable amino-terminal "tail."[167] The human *ANXA2* gene consists of 13 exons distributed over 40 kb of genomic DNA on chromosome 15 (15q21).[168]

At membrane surfaces, A2 forms a heterotetrameric complex with S100A10 (p11) [$(A2\text{-}p11)_2$]. A2 possesses the unique property of binding both plasminogen (K_d of 114 nmol/L)[129] and tPA (K_d of 30 nmol/L),[169] but not uPA,[128,170] and purified native human A2 stimulates the catalytic efficiency of tPA-dependent plasminogen activation by sixtyfold.[171] This effect is completely inhibited in the presence of lysine analogues or on treatment of A2 with carboxypeptidase B, an agent that removes basic carboxyl-terminal amino acids. Although it lacks a classic signal peptide, A2 is constitutively translocated to the endothelial cell surface within 16 hours of its biosynthesis, where it binds phospholipid via core repeat 2.[172] The $(A2\text{-}p11)_2$ complex may stimulate tPA-dependent plasmin generation even more strongly.[173]

Expression of the A2 complex on the endothelial cell surface appears to be highly regulated.[170] Translocation of A2 to the cell surface requires association with p11, tyrosine phosphorylation of A2 at tyrosine 23 by an src-like kinase, and an activating stimulus such as heat stress or thrombin stimulation.[174,175] Intracellular concentrations of p11 are regulated by A2, which masks a polyubiquitination site on p11; in the absence of sufficient A2, uncomplexed p11 is rapidly ubiquitinated and directed to the proteasome for degradation.[176] In a negative-feedback mechanism, furthermore, plasmin formed on the endothelial cell surface induces activation of PKC, which phosphorylated serines 11 and 23 on A2, dissociates the $(A2\text{-}p11)_2$ tetramer and reduces further translocation.[177]

Lipoprotein(a), an atherogenic low-density lipoprotein–like particle, competes with plasminogen for binding to A2[178,179] and reduces cell surface plasmin generation. Lipoprotein(a) contains an apoprotein called apoprotein(a) that contains kringle structures highly homologous to those of plasminogen.[27] This mechanism may contribute to atherogenesis by reducing fibrinolytic surveillance at the blood vessel wall.

Binding of tPA to A2 requires residues 8 to 13 (LCKLSL) within the amino-terminal "tail" domain of the receptor.[180] This sequence is a target for homocysteine (HC), a thiol-containing amino acid that accumulates in association with nutritional deficiencies of vitamin B_6, vitamin B_{12}, or folic acid or in inherited abnormalities of cystathionine β-synthase, methylenetetrahydrofolate reductase, or methionine synthase; it is associated with atherothrombotic disease.[181-183] In vitro, HC impairs the intrinsic fibrinolytic system of the endothelial cell by approximately 50%[184] by forming a covalent derivative with Cys9 of A2, thereby preventing its interaction with tPA.[184] The half-maximal dose of HC for inhibition of binding of tPA to A2 is approximately 11 μmol/L HC, a value close to the upper limit of normal for HC in plasma (14 μmol/L).

Studies in homozygous A2-null mice have revealed that A2 contributes to intravascular fibrin balance in vivo[185] (Table 28-2). A2 knockouts display microvascular fibrin deposition, reduced clearance of arterial thrombi, and markedly deficient microvascular endothelial cell surface plasmin generation.[186] In addition, pretreatment of the rat carotid artery with A2 prevents vessel thrombosis in response to injury.[187] A2-null mice also display defects in angiogenesis reflected in several systems, including oxygen-induced retinopathy, a model of retinopathy of prematurity, in which prevention of fibrin formation relieved the block to new blood vessel formation.[186,188] In mice with diet-induced hyperhomocysteinemia, moreover, fibrin accumulation and impaired angiogenesis were reversed on infusion of fresh, unmodified A2.[189]

In humans, moreover, overexpression of A2 has been associated with hemorrhagic disease.[190] Overexpression of A2 by acute promyelocytic leukemia (APL) cells correlates with the initial severity of hemostatic compromise at the time of diagnosis.[161,191,192] Elevated expression of p11 has also been demonstrated in an APL cell line that harbors the [t(15;17)(q22-24;q12-21)] translocation characteristic of APL.[193] It is interesting to note that diet-induced hyperhomocysteinemia has been demonstrated to reverse the hyperfibrinolytic coagulopathy in a murine model of APL.[194]

Conversely, A2 system dysfunction appears to correlate with vascular occlusive disease.[190] Patients with antiphospholipid syndrome and anti-A2 antibodies have a significantly higher risk for clinical thrombosis than do patients who lack this antibody.[195] Similarly, high titer auto-antibodies to A2 are associated with thrombosis and maternal-fetal complications in pregnancy[196] and were unusually prevalent in a cohort of patients with cerebral thrombosis.[197] In sickle cell disease, moreover, A2 polymorphisms are associated with vaso-occlusive stroke, osteonecrosis of bone, and possibly pulmonary hypertension.[198-201]

Clearance Receptors

Both uPA and tPA are cleared from the circulation via the liver.[202] In vitro, clearance of tPA–PAI-1 complexes also appears to be mediated by a large two-chain receptor called the low-density lipoprotein receptor–related protein (LRP).[202-205] This complex interaction requires both the growth factor and finger domains of tPA. An additional "receptor-associated protein" with an M_r of 39,000 copurifies with LRP and may regulate the binding and uptake of LRP ligands.[206] LRP-knockout embryos undergo developmental arrest by 13.5 days after conception, which suggests that regulation of serine protease activity may be crucial for early embryogenesis.[207,208] Although several PAI-1–independent clearance pathways for tPA involving the large LRP subunit,[209] the mannose receptor,[210] or an α-fucose–specific receptor[211] have been proposed, in vivo studies in mice suggest that LRP and the mannose receptor play a dominant role in clearance of tPA.[212]

THE FIBRINOLYTIC ACTIONS OF PLASMIN

Fibrinogen

Fibrinogen possesses distinct proteolytic cleavage sites for plasmin and thrombin (Fig. 28-3). Whereas plasmin cleaves carboxyl-terminal Aα and amino-terminal fibrinopeptide B moieties, thrombin primarily releases fibrinopeptide A, which exposes the Gly-Pro-Arg tripeptide sequence and allows fibrinogen to polymerize and form insoluble fibrin[213] (see Chapter 27). Cleavage of fibrinogen (M_r of 340,000) by plasmin initially produces carboxyl-terminal fragments from the α chain within the D domain of fibrinogen (Aα fragment).[214-217] Simultaneously, but more slowly, the amino-terminal segments of the β chains are cleaved, with release of a peptide containing fibrinopeptide B. The resulting molecule with an M_r of approximately 250,000 is termed fragment X and represents a clottable form of fibrinogen. Additional cleavage events may release the Bβ fragment from the carboxyl-terminal of the β chain, and in a series of subsequent reactions, plasmin cleaves the three polypeptide chains that connect the D and E domains to produce a free D domain (M_r of ≈100,000) plus the binodular D-E fragment known as fragment Y (M_r of ≈150,000). Finally, domains D and E are separated from each other, and some of the amino-terminal fibrinopeptide A sites on domain E are also modified. Although fragment X can be converted to fibrin by thrombin, the fragments Y, D, and

TABLE 28-2 Fibrinolytic Gene Deletion Models

	PLASMINOGEN AND FIBRIN			PLASMINOGEN ACTIVATORS			RECEPTORS			INHIBITORS			
							A2 Complex						
Genotype	**Plg$^{-/-}$**	**FgnA$^{-/-}$**	**Plg$^{-/-}$ FgnA$^{-/-}$**	**tPA$^{-/-}$**	**uPA$^{-/-}$**	**tPA$^{-/-}$ uPA$^{-/-}$**	**A2$^{-/-}$**	**p11$^{-/-}$**	**uPAR$^{-/-}$**	**PAI-1$^{-/-}$**	**PAI-2$^{-/-}$**	**α2AP$^{-/-}$**	**TAFI$^{-/-}$**
GENERAL CONSEQUENCES													
Development	nl	nl	nl	nl	nl	nl	nl	nl	nl	nl	nl	nl	nl
Fertility	♀↓	♀↓	♀↓	nl	nl	↓	nl	nl	nl	nl	nl	nl	nl
Physical growth	↓	nl	nl	nl	nl	↓	↓	nl	nl	nl	nl	nl	nl
Survival	↓	↓/nl	nl	nl	nl	↓	nl	nl	nl	nl	nl	nl	nl
HEMOSTASIS AND THROMBOSIS													
Spontaneous thrombosis	++	–	–	–	–	+	+	ND	–	–	–	–	–
Induced thrombosis	ND	–	ND	↑	↑	↑↑	↑	ND	ND	↓	nl	↓	nl
Lysis of artificial thrombi	↓	ND	ND	↓	↓	↓↓	ND	↓	nl	↑	ND	↑	ND
Fibrin deposition	++	–	–	–	+	++	++	++	–	–	–	–	–
Spontaneous hemorrhage	–	++	+	–	–	–	–	–	–	–	–	–	–

α_2AP, α_2-Antiplasmin; *A2*, annexin 2; *FgnA*, fibrinogen A; *nl*, normal; *PAI-1*, plasminogen activator inhibitor-1; *PAI-2*, plasminogen activator inhibitor-2; *PLG*, plasminogen; *TAFI*, thrombin-activatable fibrinolysis inhibitor; *uPA*, urokinase; *uPAR*, urokinase receptor.

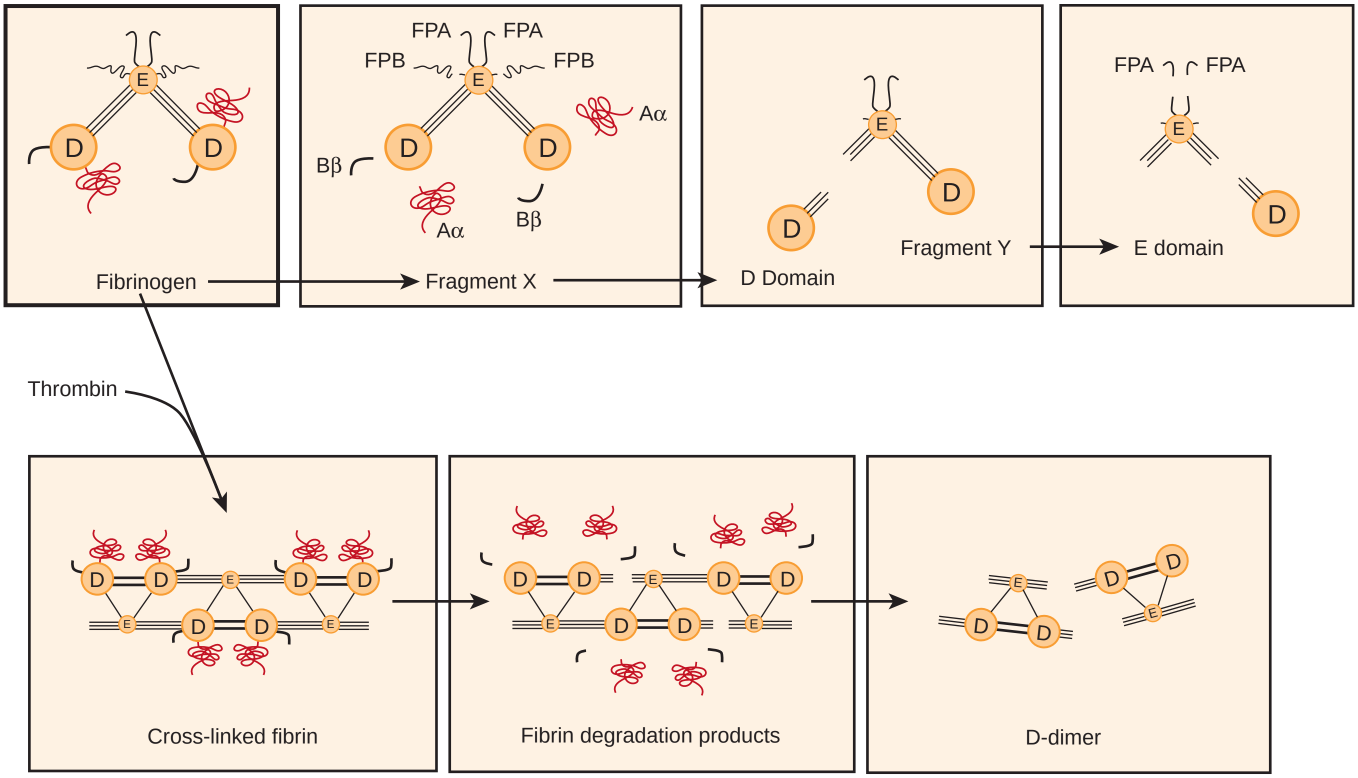

Figure 28-3 Degradation of fibrinogen and cross-linked fibrin by plasmin. *Top panel,* Plasmin initially cleaves the carboxyl-terminal regions of the α and β chains within the D domain of fibrinogen, thereby releasing the Aα and Bβ fragments. In addition, a fragment containing fibrinopeptide B (FPB) from the amino-terminal region of the β chain is released, forming the intermediate product known as fragment X. Fibrinopeptide A (FPA) remains intact. Subsequently, plasmin cleaves the three polypeptide chains connecting the D and E domains, which produces fragments D, E, and Y, and releases FPA. *Bottom panel,* Fibrinogen can also be polymerized by thrombin to form fibrin. When degrading cross-linked fibrin, plasmin initially cleaves the carboxyl-terminal region of the α and β chains within the D domain. Subsequently, some of the connecting regions between the D and E domains are severed. Fibrin is ultimately solubilized upon hydrolysis of additional peptide bonds within the central portions of the coiled-coil connectors, giving rise to fibrin degradation products such as D-dimer.

E are all nonclottable and may in fact inhibit the spontaneous polymerization of fibrinogen.[218]

Fibrin

Degradation of fibrin by plasmin leads to a distinct set of molecular products.[218] Species similar to fragments Y, D, and E but lacking fibrinopeptide sites are released from non–cross-linked fibrin. If fibrin has been extensively cross-linked by factor XIII, however, the resulting D fragments are cross-linked to an E domain fragment. An assay of cross-linked D-dimer fragments is used clinically to identify disseminated intravascular coagulation–like states associated with excessive plasmin-mediated fibrinolysis. Several biologic activities, including inhibition of platelet function,[219,220] potentiation of the hypotensive effects of bradykinin,[220] chemotaxis,[221] and immune modulation, have been ascribed to fibrin breakdown products.[222]

Tissue Plasminogen Activator–Mediated Plasminogen Activation

With or without fibrin, tPA-mediated activation of plasminogen follows Michaelis-Menten kinetics.[22] In the absence of fibrin, tPA is a weak activator of plasminogen. However, in the presence of fibrin, the catalytic efficiency (k_{cat}/K_m) of tPA-dependent plasminogen activation is enhanced by at least 2 orders of magnitude. This is the basis for its specificity as a lytic agent in the treatment of thrombosis. The affinity between tPA and plasminogen in the absence of fibrin is low (K_m of 65 μmol/L) but increases about 400-fold in its presence (K_m of 0.16 μmol/L), even though the catalytic rate constant remains essentially unchanged (k_{cat} of ≈0.05 sec^{-1}). When plasmin forms on the fibrin surface, both its lysine binding sites and its active site are occupied. Thus it is relatively protected from its physiologic inhibitor α_2-AP.[223] The interaction of tPA with fibrin is probably initiated by its finger domain. However, once fibrin is modified by plasmin, carboxyl-terminal lysine residues are generated, and these residues become binding sites for kringle 2 of tPA and kringles 1 and 4 of plasminogen.[224] Therefore fibrin accelerates its own destruction by (1) enhancing the catalytic efficiency of plasmin formation by tPA, (2) protecting plasmin from its physiologic inhibitor α_2-AP, and (3) providing new binding sites for plasminogen and tPA once its degradation has begun.

Urokinase-Mediated Plasmin Generation

For activation of Glu-Plg by uPA in a fibrin-free system, reported K_m values vary from 1.4 to 200 μmol/L, whereas

k_{cat} values range from 0.26 to 1.48 sec^{-1}. Interestingly, activation of Glu-Plg by two-chain uPA is minimally increased in the presence of fibrin, and uPA does not bind to fibrin.[225] In contrast, single-chain uPA has considerable fibrin specificity. This may reflect neutralization by fibrin of components in plasma that impair plasminogen activation.[226] It may also reflect a conformational change in plasminogen on binding to fibrin.[227] It is important to recognize, however, that the intrinsic plasminogen-activating potential of single-chain uPA is less than 1% that of two-chain uPA. Two-chain uPA has been used effectively as a thrombolytic agent for many years.[228]

PHYSIOLOGIC FUNCTIONS OF THE FIBRINOLYTIC SYSTEM

Thrombosis and Thrombolysis

Congenital plasminogen deficiency was first reported in a 31-year-old man with recurrent thrombosis.[229] In groups of patients with plasminogen deficiency, however, whether type I, in which both immunologically detectable protein and enzymatic function are decreased, or type II, in which just plasminogen function is impaired, only about 25% of patients report a history of thrombosis.[230-232] Indeed, the most common clinical manifestations of plasminogen deficiency, occurring in 80% and 34% of patients, respectively, are ligneous conjunctivitis and ligneous gingivitis, disorders characterized by the deposition of fibrin-rich pseudomembranes on mucosal surfaces.[233] These findings strongly suggest that plasminogen acts in extravascular as well as intravascular locations.[234-236]

The relative infrequency of thrombosis in patients with plasminogen deficiency may, in part, reflect the fact that in only a small fraction of patients with sonographic evidence of thrombosis does symptomatic vascular occlusion eventually develop.[237] In a study of consecutive patients with thrombophilia, the prevalence of plasminogen deficiency was only 1.9%.[238] Similarly, congenital deficiencies of plasminogen activators (tPA and uPA) have not been linked to thrombophilia in humans.[239] On the other hand, increased levels of PAI-1 were prevalent in young survivors of myocardial infarction.[240] Additionally, TAFI levels above the 90th percentile in a case-control study approximately doubled the risk for venous thrombosis.[241-243] Together these data suggest that measurement of plasma plasminogen or its activators in patients with thrombophilia is likely to be a low-yield procedure, whereas assessment of global fibrinolytic function using a phospholipid-based assay may be of value.

As one might expect, congenital deficiency of either α_2-AP or PAI-1 is generally associated with delayed rebleeding at sites of trauma or wound healing.[244,245] In patients with the Quebec platelet disorder, tandem duplication of the urokinase gene with overproduction of this plasminogen activator generally leads to a bleeding diathesis.[246] To date, no human disorders reflecting primary deficiencies of fibrinolytic receptors have been reported.

In general, analyses of genetically engineered mice support the hypothesis that the fibrinolytic system functions to regulate fibrin balance in both the intravascular and extravascular compartments (see Table 28-2). That fibrinogen is central to hemostasis is underscored by findings of postnatal bleeding at multiple sites in fibrinogen-null mice,[247] similar to that seen in human patients with afibrinogenemia. Similarly, although mice deficient in plasminogen have normal embryogenesis, survive to adulthood, and can sire offspring, they display significant runting, spontaneous thrombi develop in multiple tissues, and extravascular fibrin accumulates in the liver (see Table 28-2).[248,249] Ulcerative lesions are observed in the gastrointestinal tract, particularly the rectum, where prolapse is also seen. Plg$^{-/-}$ mice also clear induced thrombi much less readily than wild-type mice do,[250] and this defect is reversed by bolus injection of exogenous plasminogen.[251] Because the spontaneous thrombosis observed in plasminogen-deficient mice is completely reversed when fibrinogen is also deleted, it is clear that the thrombosis observed in plasminogen deficiency is related to failure of intravascular fibrin clearance.[252] Some strains of Plg-deficient mice also display ligneous conjunctivitis, a disorder similar to that observed in humans with plasminogen deficiency.[253]

Because no examples of complete deficiency of tPA or uPA have been documented in humans, the most compelling evidence for their role in fibrin balance derives from gene-knockout experiments in the mouse (see Table 28-2).[254] Both uPA- and tPA-null mice exhibit normal fertility and embryonic development. Although little spontaneous thrombosis is seen in either genotype, uPA$^{-/-}$ mice exhibit occasional fibrin deposition and tPA-deficient mice display impaired lysis of artificially induced pulmonary thrombi. In contrast, doubly deficient (tPA$^{-/-}$, uPA$^{-/-}$) mice exhibit extensive spontaneous fibrin accumulation in multiple organs, rectal prolapse, runting, and cachexia, all reminiscent of the phenotype seen in Plg$^{-/-}$ mice. These findings demonstrate that tPA and uPA are not essential for normal embryologic development, but they do play crucial roles in the activation of plasminogen, in the lysis of spontaneous and induced thrombi, and in fibrinolytic surveillance.

Although complete PAI-1 deficiency in humans is associated with a moderately severe bleeding tendency, mice deficient in PAI-1 exhibit normal development, survival, and fertility, with no evidence of spontaneous hemorrhage[255] (see Table 28-2). PAI-1–deficient mice do, however, exhibit resistance to thrombosis after injury[256] and enhanced sensitivity to thrombolytic therapy.[257] On the other hand, in transgenic mice that overexpress human PAI-1, a dramatic vascular occlusive process develops in the tail and hind limbs.[258] This finding may be attributable to unique effects of human PAI-1 in the mouse because mice overexpressing murine PAI-1 do not exhibit this abnormality.[259]

Deficiency of α_2-AP, the major serpin directly blocking plasmin activity, is a rare cause of bleeding disorder in humans.[260] Although no overt bleeding is seen in α_2-AP–deficient mice, increased lysis of artificially induced thrombi has been reported.[261] PAI-2–deficient mice exhibit no obvious phenotype even when PAI-1 is also absent in doubly deficient animals.[262] Complete TAFI deficiency in mice is fully compatible with life and does not appear to alter the response to vascular injury, artificially induced thrombosis, or endotoxin-induced disseminated intravascular coagulation.[263]

Mice transgenic for the catalytically inactive plasminogen analogue apolipoprotein(a) are subject to atherosclerosis and resistant to tPA-induced lysis of experimental pulmonary emboli.[264] The accelerated atherosclerosis observed in these mice is blunted under conditions of concomitant fibrinogen deficiency.[265] These data suggest that apolipoprotein(a)-associated atherosclerosis may be rooted in functional plasminogen deficiency and accumulation of intravascular fibrin. In addition, meta-analysis indicates a clear association between elevated plasma levels of Lp(a) and the occurrence of venous thromboembolism.[266]

Whereas complete deficiency of uPAR is associated with normal lysis of intravenously injected plasma clots in mice,[267] A2-deficient mice have impaired lysis of experimentally induced thrombi, and fibrin accumulates in both intravascular and extravascular locations.[186] In addition, A2-deficient microvascular endothelial cells lack tPA cofactor activity and migrate slowly through fibrin and collagen matrices. Interestingly, infusion of recombinant A2 amplifies the thrombolytic efficacy of tPA in a rat model of thromboembolic stroke, potentially reducing the hemorrhagic consequences of tPA-based stroke therapy.[268]

Wound Healing and Tissue Fibrosis

As discussed earlier, extravascular fibrin deposition is a hallmark of plasminogen deficiency in both mice and humans.[269,270] Plasminogen-deficient mice, as well as uPA$^{-/-}$/tPA$^{-/-}$ but not uPAR$^{-/-}$/tPA$^{-/-}$ mice, also exhibit defective wound healing characterized by impaired keratinocyte migration despite abundant neovascularization and granulation tissue within experimental wounds. Although fibrinogen-deficient mice exhibit subtle histologic defects in wound repair, the impaired healing in plasminogen-deficient mice disappears when fibrinogen is also absent.[271] Consistent with the hypothesis that plasmin is necessary for clearance of extravascular fibrin, deficiency of either PAI-1 or α_2-AP in mice is associated with accelerated cutaneous wound healing.[272,273] In addition, sciatic nerve regeneration after crush injury also appears to require tPA/plasmin-mediated clearance of fibrin to protect against axonal degeneration and demyelination.[274] Surprisingly, TAFI deficiency seems to be associated with impaired, rather than accelerated, healing of cutaneous wounds.[275]

Although extravascular fibrin commonly forms in response to injury, its exact role in tissue fibrosis is unclear. In a well-established model of bleomycin-induced pulmonary fibrosis, plasminogen$^{-/-}$, tPA$^{-/-}$, and uPA$^{-/-}$ mice, but neither uPAR$^{-/-}$ nor fibrinogen$^{-/-}$ mice, have increased deposition of both fibrin and collagen after injury.[276-278] Similarly, PAI-1–deficient mice, exhibit less bleomycin-induced pulmonary fibrosis than wild-type animals do.[278] Surprisingly, however, fibrin does not seem to be a prerequisite for the induction of pulmonary fibrosis in this model because mice deficient in fibrinogen were susceptible to fibrosis to the same degree as wild-type animals.[279] Thus the mechanism by which fibrinolytic proteins regulate fibrosis may relate to modulation of adhesive interactions that promote leukocyte adherence rather than plasmin catalytic activity.

Atherosclerosis and Cardiovascular Remodeling

Atherosclerosis is thought to represent a response to vascular injury that leads to atheromatous plaque formation and vascular remodeling.[280] In the setting of apolipoprotein E deficiency, simultaneous plasminogen deficiency accelerates vessel wall disease.[281] In this model fibrin is not strictly required for the development of atherosclerotic lesions, thus suggesting that the role of plasmin may relate to its ability to remove one or more nonfibrin substrates, liberate one or more growth factors from extracellular matrix, or activate crucial proteases.[282] Interestingly, although plasminogen deficiency accelerates hyperlipidemic atherosclerosis, the same mice are relatively resistant to transplant-associated atherosclerosis.[283]

Plasmin appears to promote the pathologic response to vascular and myocardial injury.[284] After induction of myocardial infarction in plasminogen-null mice, myocardial healing was markedly inhibited inasmuch as inflammatory cells failed to appear at the wound site, necrotic cardiomyocytes failed to disappear, and granulation and fibrous tissue failed to develop.[285] Similarly, healing of electrically injured femoral arteries, arteries treated with a flexible cuff, or arteries undergoing guidewire endothelial denudation was impaired in plasminogen-null mice, with reduced infiltration of inflammatory and smooth muscle cells and decreased neointima formation.[250,286,287] All these processes require cellular migration, which may entail plasmin-dependent matrix remodeling through activation of downstream matrix metalloproteinase 1 (MMP-1) and matrix metalloproteinase 3 (MMP-3).[288-290]

Plasmin may play a role in the activation of growth factors and in the proliferative response to blood vessel injury. Macrophages cultured on labeled matrices release both basic fibroblast growth factor and TGF-β into the medium in a plasminogen-dependent fashion.[291] TGF-β, a homodimeric polypeptide with an M_r of 25,000 whose effects on vascular cell growth and differentiation are pleiomorphic,[292] also appears to be converted from a latent to an active form by cell-associated plasmin. The latter process can be blocked by the presence of plasmin inhibitors such as aprotinin or α_2-AP and appears to involve alteration of its tertiary structure on cleavage of an amino-terminal glycopeptide.[293]

uPA but neither tPA nor uPAR appears to be required for vascular cell migration and pericellular plasmin formation in some injured vessels.[294,295] uPA appears to mediate the activation of MMPs in both atherosclerotic aortic aneurysm[296] and postinfarction cardiac rupture,[297] presumably through its activation of plasmin rather than direct cleavage of pro-MMPs. Together, these data suggest a unique role for uPA in postinjury vascular remodeling.

Tumor Biology

Tumor cells may use the fibrinolytic system for local invasion and metastasis. Although plasminogen-deficient mice display normal growth of both primary and metastatic tumors in the Lewis lung carcinoma model,[298] pulmonary metastases from a polyoma middle T antigen–induced mammary cancer were significantly reduced in plasminogen-null animals.[299] Fibrinogen-null mice exhibited normal growth of implanted Lewis lung carcinoma, whereas lymph node and hematogenous lung metastases were markedly reduced in both Lewis lung carcinoma and melanoma.[300,301] On the other hand, p11-null mice seem to exhibit reduced growth of implanted Lewis lung carcinoma in association with impaired recruitment of macrophages.[302] These data suggest that plasmin-mediated fibrin clearance may be required for tumor cell invasion, recruitment of tumor-supporting cells, or metastasis in some specific settings.

Analysis of tumor behavior in mice with genetic alterations in other components of the fibrinolytic system has produced a spectrum of results. uPA-deficient mice displayed markedly diminished murine sarcoma cell proliferation and tumor-associated angiogenesis when compared with wild-type animals.[303] These mice also failed to support local progression of cutaneous melanoma,[304] as well as polyoma middle T antigen–induced vascular tumors.[305] The absence of TAFI, on the other hand, does not appear to alter the progression of primary Lewis lung carcinoma, B16 melanoma, or T241 sarcoma tumors or their metastases.[306]

PAI-1 has a surprising stimulatory effect on the growth and invasiveness of murine tumors derived from malignant keratinocytes. In PAI-1–deficient mice, these tumors are subject to loss of local invasion and tumor vascularity,[307] thus suggesting that PAI-1 may play an adhesive rather than an anticatalytic role in this system. Similarly, murine fibrosarcoma cells implanted in PAI-1$^{-/-}$ mice display reduced tumor cell proliferation and neovascularization.[303] On the other hand, neither tumor cell burden nor overall host survival were affected by the absence of PAI-1 in a murine model of metastatic melanoma.[259] Thus the precise role of PAI-1 in tumor biology may depend critically on the specific context.

Microbial Virulence and Host Defense Mechanisms

There is increasing evidence that pathogenic bacteria can usurp elements of the host fibrinolytic system to enhance their own virulence.[308,309] In Lyme disease, for example, dissemination of *Borrelia burgdorferi* in both the tick vector and the mammalian host requires that the spirochete bind host plasminogen and use host plasminogen activator.[310] Similarly, *Borrelia* species employ host plasminogen for heart and brain invasion in relapsing fever.[311] Plasminogen-deficient mice also appear to be remarkably resistant to *Yersinia pestis*, whose own plasminogen activator (Pla) is required for the rapid progression of primary pneumonic plague.[311,312,313] Expression of human plasminogen in mice overrides their natural resistance to invasive infection with group A β-hemolytic streptococci.[314]

Components of the fibrinolytic system are required for the recruitment of host cells to sites of inflammation. In plasminogen-null mice, for example, the influx of neutrophils to the peritoneum in response to thioglycolate is normal, whereas recruitment of lymphocytes and monocytes is significantly impaired.[315] In the murine lung, similarly, recruitment of phagocytes and lymphocytes in response to *Cryptococcus neoformans*[316] and *Pneumocystis carinii*[317] requires uPA. Recruitment of neutrophils in response to *Pseudomonas aeruginosa*, on the other hand, depends on uPAR but not on uPA, thus suggesting a nonfibrinolytic, possibly adhesive role for this receptor.[318]

Neurologic Plasticity

tPA appears to play a relatively unique role in modulating plasticity and cell death within the central nervous system. tPA, which is stored within neuronal cells, is released on calcium-mediated membrane depolarization[319] and participates in the immediate early response after electrical or pharmacologic stimulation of hippocampal neurons.[319,320] tPA expression is induced in Purkinje neurons after cerebellar motor learning,[321] and excessive tPA-related proteolytic activity in granule neurons may contribute to neuronal death in the weaver mouse cerebellum.[322] tPA-deficient mice do not display discernible motor or sensory deficiencies but do exhibit a mild defect in long-term potentiation.[323,324] These animals are resistant to excitotoxic neuronal degeneration in the hippocampus, in which plasmin-catalyzed degradation of laminin is blocked both by tPA deficiency and by infusion of a plasmin inhibitor.[325] In addition, tPA-deficient mice displayed diminished central nervous system signaling and behavioral responses to cocaine,[326] and tPA-deficient mice were also protected from the NMDA-mediated toxicities of ethanol in a model of fetal alcohol syndrome.[327]

Neuronal migration within the cerebellum of the developing mouse brain is reduced by approximately 50% in tPA-deficient mice,[328] and these animals exhibit increased sensitivity to the locomotor effects of cocaine.[329] During the remodeling of neuronal connections that occurs in response to seizure activity in the hippocampus, mice lacking either tPA or plasminogen show decreased mossy fiber outgrowth in this region and accumulate an extracellular matrix component known as DSD-1-PG/phosphacan. These data suggest both plasmin-dependent and plasmin-independent roles for tPA in neuronal plasticity.[330]

In both ischemic and hemorrhagic stroke, infusion of tPA can have a major effect on clinical outcome. After ischemic stroke in mice, tPA increases infarct size in both wild-type and tPA-null animals,[331] and smaller infarcts are observed in untreated tPA-deficient animals than in uPA-deficient or wild-type mice. Interestingly, the largest infarcts in the latter study were found in PAI-1$^{-/-}$ mice.[332] In a thrombotic stroke model, on the other hand, deficiency of tPA leads to more extensive vascular fibrin deposition and cerebrovascular thrombosis and consequently a sixfold to eightfold increase in infarct volume.[333]

Engagement of LRP by the tPA–PAI-1 complex may regulate the vascular response to injury by triggering NF-κB–mediated release and activation of matrix metalloproteinase 9 (MMP-9), which may lead to weakening of the blood-brain barrier and catastrophic hemorrhage.

In a mouse model, this process was inhibited by concomitant infusion of activated protein C, which by interacting with endothelial protein C receptor or protease activated receptor 1, or both, blocked the activation of NF-κB and hence release of metalloproteinase.[334] Together these data suggest that the actions of tPA in the central nervous system extend beyond the activation of plasmin.

NON–PLASMIN-MEDIATED FIBRINOLYSIS

There is accumulating evidence that proteases other than the traditional members of the fibrinolytic family may participate in fibrinolysis. Fibrin appears to be subject to degradation by activated monocytoid cells that express intracellular cathepsins.[335] In addition, MMP-3, MMP-7, and membrane type 1 MMP may cleave fibrinogen and cross-linked fibrin into fragments similar to those produced by plasmin.[336] Matrix MMPs have been shown to act as pericellular fibrinolysins, thereby providing an alternative pathway for fibrin-based angiogenesis in a plasminogen-free milieu.[337] In particular, membrane-type matrix MMPs appear to function in this way within a three-dimensional fibrin meshwork.[338]

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CHAPTER 29

Approach to the Child with a Suspected Bleeding Disorder

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CHAPTER OUTLINE

INTRODUCTION
CLINICAL EVALUATION
Clinical Presentation of Bleeding
Relevant Clinical History
Age of Onset
Family Bleeding History
Medications
PHYSICAL EXAMINATION
LABORATORY EVALUATION
Sample Collection and Technique and Preanalytical Variables
Screening Tests
Global Tests for the Evaluation of Bleeding
BLEEDING IN THE CRITICALLY ILL CHILD

INTRODUCTION

Before initiating the laboratory workup of any patient with a suspected defect of hemostasis, the hematologist must first verify that the patient has a clinical history, signs, and symptoms compatible with a bleeding disorder. This is not an easy task because mild bleeding and bruising are common in the general population. Furthermore, bleeding, particularly in the outpatient setting, is difficult to quantify because patient reports may be biased; the definition of "excessive" bleeding or bruising is subjective and varies among individuals. Additionally, a critical consideration in children is the fact that many of them have not had specific hemostatic challenges such as surgeries or menarche that might unveil a bleeding disorder. All these factors contribute to the difficulty in the diagnosis of bleeding in the pediatric population.

Therefore, an accurate diagnosis is made primarily by obtaining a thorough personal and family history and then performing a careful physical examination, which will lead to an appropriate set of laboratory tests. Once suspicion for a bleeding disorder is confirmed, the remaining workup items are implemented in a focused manner with reference to particular differential diagnoses.

Children with suspected bleeding disorders might present for evaluation in a variety of ways: concerning signs and symptoms, abnormal presurgical screening laboratory results, or a known family history of hemostasis defects that requires consultation. The variety in severity of different bleeding disorders also results in children presenting at different ages. The goal for the consulting hematologist is to approach the child with a broad differential diagnosis in mind, narrow the options by carefully assessing the medical and family history as well as pertinent physical findings, and order the most appropriate laboratory tests to reach a diagnosis and initiate definitive therapy, if indicated.

Although the approach to the bleeding child is often similar in the outpatient and inpatient setting, the practicing hematologist is sometimes consulted because of bleeding in the critically ill patient. In such cases therapeutic measures will likely be required immediately and an accurate diagnosis made later.

CLINICAL EVALUATION

The initial clinical evaluation of a child with a suspected bleeding disorder starts with a series of questions, usually at a doctor's office or clinic, that attempt to summarize the most important aspects of the clinical presentation and history of bleeding. Although several standardized bleeding assessment tools have been used recently in research projects, their value in clinical practice is still unproven. Therefore, questions should be designed to establish certain parameters that would prompt an appropriate laboratory workup. The initial set of questions should establish the following: (1) the most common site and type of bleeding (e.g., mucocutaneous versus articular or deep muscle), (2) bleeding on hemostatic challenge such as surgeries or trauma, and (3) family history of bleeding.

TABLE 29-1 Differences in Clinical Manifestations of Primary Hemostatic Defects and Clotting Factor Deficiencies

Clinical Characteristic	Primary Hemostatic Defect	Clotting Factor Deficiency
Site of bleeding	Skin, mucous membranes	Soft tissues, muscles, joints
Bleeding after minor cuts	Yes	Rare
Petechiae	Present	Absent
Ecchymosis	Small, superficial	Large, deep, palpable
Hemarthrosis	Rare	Common
Bleeding after trauma/surgery	Immediate	Delayed

Clinical Presentation of Bleeding

Significant surgical or postoperative hemorrhage, rectal or genitourinary bleeding, and major muscle or joint bleeding are easily identified as abnormal, but they represent a minority of new cases. Most often the clinician's challenge consists in differentiating mild but common presenting symptoms of bleeding disorders, such as easy bruising and mucosal bleeding (e.g., epistaxis, menorrhagia, oropharyngeal), from those encountered in healthy children and then determining when additional laboratory evaluation is warranted. Large bruises without previous significant trauma, disseminated petechiae, intramuscular hematomas, hemarthrosis (joint effusion, warmth, and pain with passive movement) usually indicate a bleeding disorder. In young children, refusal to walk is often a sign for an extremity-related bleed and could represent the first sign of hemarthrosis in a boy with hemophilia (Table 29-1).

Susceptibility to increased bruising must first be differentiated from nonmedical causes such as abuse, a phenomenon that is, unfortunately, common enough to warrant its own set of guidelines for evaluation in recent reports from the American Academy of Pediatrics.[1,2] Inflicted trauma is most likely to manifest over the head, chest, back, and long bones (and may retain the outlines of the instrument used to inflict harm), whereas bruises associated with primary hemostasis defects are usually located over areas of typical childhood trauma, such as bony protuberances of extremities or spinous processes.[3]

Epistaxis is a frequent presenting sign in children with hemostatic disorders, but it is also a common complaint among healthy children, usually the result of local aggravating factors (dry nasal mucosa, trauma, allergic rhinitis).[4] In a recent study of 248 children referred to the otolaryngology clinic at Children's Hospital of Philadelphia for epistaxis, 11% were found to have a bleeding disorder (type 1 von Willebrand disease [VWD], platelet aggregation defect, or mild factor VII deficiency), with clinical predictive factors being younger age or previous emergency medical care for the epistaxis.[5] Other studies, however, have found an incidence as high as 25%[6] to 33%[7] for coagulopathy among children referred to pediatric hematologists for recurrent epistaxis. Therefore it is important that epistaxis be taken seriously as a presenting symptom, particularly in cases that require emergency medical care, occur in both nostrils, or appear in association with other bleeding signs or a family history of similar bleeding.

Menorrhagia is also a frequent presenting sign for mild or moderate bleeding disorders (including VWD, platelet function disorders, and other coagulopathies)[3] and can quickly lead to severe anemia and decreased quality of life. The classic definition of menorrhagia (i.e., greater than 80 mL of blood loss per cycle) is rarely used clinically[8] because most women describe limitations of their daily activities as more important than the actual volume of blood lost.[9] A more popular working definition is "excessive cyclic uterine bleeding that occurs at regular intervals over several cycles, or prolonged bleeding that lasts for more than 7 days".[10] In fact, the American College of Obstetrics and Gynecology has recommended that testing for VWD be performed in all adolescents with severe menorrhagia,[11] although a subsequent meta-analysis determined that there may not be adequate data to support this universal recommendation.[12] It is essential for practitioners to be judicious about laboratory testing in adolescents with menorrhagia. All adolescents that present with menorrhagia should be evaluated by a gynecologist; testing for a bleeding disorder is not always warranted, particularly in the absence of family and personal history or other bleeding manifestations.

Surgical (e.g., circumcision, tonsillectomy) or dental (e.g., extractions) bleeding also may be associated with disorders of hemostasis. This type usually manifests as uncontrolled bleeding during or after the procedure, bleeding that extends beyond the surgical site, unexpected need for blood transfusion, or delayed bleeding after a procedure.[3]

Hereditary hemorrhagic telangiectasia may also be manifested as mucosal bleeding, particularly epistaxis. Profuse bleeding into soft tissues or joints suggests deficiency of a coagulation factor (such as factors VIII or IX). Umbilical stump bleeding is typically seen with factor XIII deficiency, but it may also occur with deficiencies of prothrombin, factor X, and fibrinogen.[13]

After establishing the possibility of a true bleeding disorder, the clinician should next consider a broad differential diagnosis for the bleeding child and approach the history taking and physical examination with the intent to systematically remove options and pare down the list to a certain category or group of potential diseases. Briefly, the main categories to be considered should include anatomic abnormalities, quantitative and qualitative platelet defects affecting platelet plug formation (primary hemostasis), and quantitative and qualitative defects of clot propagation (secondary hemostasis). Differentiation also must be made between inherited and acquired disorders. Common diseases should be considered before rare ones, and diagnoses that are either life threatening or easy to treat should be prioritized in the evaluation process.

Finally, it is clear that the clinical assessment and the laboratory workup of the bleeding child will vary depending on the emergent nature of the clinical situation. The practicing hematologist is often consulted in the inpatient setting regarding bleeding in the intensive care unit or operating room. The approach in these situations is sometimes limited by the inability to obtain reliable baseline hemostatic laboratory values because of the presence of active disease or the fact that most patients receive hemostatic agents (including blood products) to stop the hemorrhage. Therefore it becomes difficult to distinguish congenital bleeding conditions frin acquired ones. Often the immediate need for treatment supersedes a more thorough evaluation. In these cases it is reasonable to treat the patient and then, if a baseline defect of hemostasis is suspected, perform the laboratory evaluation days or even months after the episode, when the acute process is resolved.

Relevant Clinical History

Information about the patient's previous response to hemostatic challenges (e.g., surgical procedures, invasive dental work, traumatic injuries) is an essential part of the initial evaluation. For example, a congenital hemorrhagic disorder is unlikely in a patient with a history of surgical procedures or tooth extractions without any abnormal bleeding, whereas postoperative transfusion requirements and iron-responsive anemia often signal hemostatic defects. In view of the variability of patients' perception of bleeding, as well as the lack of a uniform clinical measure of bleeding severity, the history may be most discriminatory when a standardized and validated questionnaire is used and a "bleeding score" is incorporated into the diagnosis.[14,15] Several such instruments currently exist, but consensus on a single questionnaire that can quantitate bleeding optimally is still evolving.[16-18] The International Society of Thrombosis and Hemostasis (ISTH) Joint Scientific Subcommittee (SSC) on von Willebrand factor (VWF) and Pediatric/Perinatal Bleeding Disorders recently developed a bleeding assessment tool to provide more accurate determination of bleeding phenotype in individuals with bleeding disorders and compared them to healthy subjects.[19] The main objective of all the existing bleeding assessment tools is to accurately diagnose bleeding and predict the risk of bleeding in the future. However, owing to their high negative predictive value, it is becoming increasingly clear that these new instruments are more effective at excluding the likelihood of a bleeding disorder than predicting future bleeding risk. Family history is also a key component in establishing both the likelihood of an inherited bleeding disorder and its specific nature. However, determination of which family members may have been affected by relying on testimony from one or more relatives can be difficult. Therefore the ISTH SSC on VWF has a specific definition of a positive family history for VWD.[20] In summary, a personal history of bleeding triggers clinical and laboratory evaluation, and a positive family history serves as supportive evidence for a hereditary bleeding disorder.[21]

The history of the present illness often provides useful clues to the diagnosis, including several classic types of presentation. A sick child with fever, shock, and mucocutaneous purpura may have disseminated intravascular coagulation (DIC) associated with bacteremia. Hemophilia should be considered in a male toddler who has just started crawling and exhibits subcutaneous or joint bleeding, or who bleeds after circumcision. A girl who has had severe menorrhagia since menarche may have VWD. A well-appearing child covered with petechiae likely has immune thrombocytopenia, but if the lesions are localized to the buttocks, ankles, and feet, and they present as palpable bruises, Henoch-Schönlein purpura should be considered.

When applicable, a detailed menstrual history, including age at menarche, duration of menses, interval between menses, and frequency of pad or tampon replacement necessary to control the bleeding, should be obtained. The prevalence of bleeding disorders in women with menorrhagia is as high as 20%, and menorrhagia is a common initial symptom in women with VWD (approximately 90% of female patients).[22-26]

It is also important to note whether the patient has an underlying medical disorder that may affect hemostasis, such as hepatic or renal disease, malabsorption syndrome, or Ehlers-Danlos syndrome (EDS) or another connective tissue disorder.[27] Most of the coagulation proteins are synthesized in the liver, and as a result, liver insufficiency is a common cause of prolongation of clotting times and bleeding.[28,29] Certain metabolites that accumulate in uremia can interfere with platelet function,[30,31] whereas low-molecular-weight coagulation proteins (factors IX and XI) are lost through the kidney in children with nephrotic syndrome.[32] In malabsorption syndrome, levels of the vitamin K–dependent coagulation factors (II, VII, IX, and X) may be depleted and lead to excessive bleeding.[33,34] A child with cyanotic congenital heart disease and polycythemia may have petechiae and excessive bleeding with surgery, in part as a result of thrombocytopenia, hypofibrinogenemia, or both.[35]

Age of Onset

It is also important to investigate the age of onset of clinical bleeding. Generally, early age of onset correlates with more severe bleeding and may indicate a congenital cause. Bleeding that develops later in childhood may indicate either an acquired problem or a milder congenital bleeding disorder. For example, mild hemophilia may not be diagnosed until late childhood or adolescence, when participation in contact sports presents an increased hemostatic challenge.[36,37] Postcircumcision hemorrhage, umbilical stump bleeding, cephalohematomas, and subgaleal hemorrhage are cardinal manifestations of underlying bleeding disorders and should be evaluated thoroughly. The incidence of neonatal intracranial hemorrhage among boys with hemophilia is estimated to be as high as 3% and may be as high as 25% in patients with FXIII deficiency.[3]

The neonatal period (first 4 weeks after delivery) is a time of unique transitional physiology, which includes the coagulation system. The neonate has physiologically decreased levels of most procoagulant and anticoagulant proteins (although levels of acute phase reactants, such as factor VIII and fibrinogen, are normal), and thus the hemostatic system can be easily overwhelmed, potentially during the significant head trauma associated with labor and vaginal delivery. One study reported intracranial hemorrhage (ICH) after spontaneous vaginal delivery at a rate of 1 per 1900, whereas the rate of ICH in babies delivered by vacuum-assisted delivery was 1 in 860.[38] In addition, 15% to 30% of patients with inherited bleeding disorders have bleeding manifestations in the neonatal period,[39] and certain disorders, such as neonatal alloimmune thrombocytopenia (NAIT), are unique to the neonatal period. Furthermore, the neonate is significantly affected by the state of maternal health and medications used during labor. Because of the small blood volume in a neonate, a relatively small degree of blood loss can have major hemodynamic consequences.

When evaluating bleeding in a neonate, the hematologist must first assess whether the baby is healthy or has medical conditions that may have precipitated hemorrhage. It is important to inquire about prolonged rupture of membranes, chorioamnionitis, and fetal distress during labor. Additional details about the mother's state of health, including infections, autoimmune disease, and platelet count, should be obtained. Vitamin K administration to the baby should be confirmed. The neonate should be examined, with particular attention directed to the presence of birth trauma, bruises, and petechiae and evidence of flank masses (renal vein thrombosis), which can cause thrombocytopenia. The presence of hepatosplenomegaly may suggest disseminated intrauterine infection. When blood is obtained for various coagulation tests, particular attention should be paid to the patient's hematocrit concentration and the volume of the sample. Additionally, all laboratory results should be compared with normal values for different gestational ages.[40,41]

Isolated thrombocytopenia in a healthy infant may be seen in NAIT, in maternal autoimmune thrombocytopenia, or in cases of decreased platelet production such as amegakaryocytic thrombocytopenia or the syndrome of thrombocytopenia with absent radii. Rarely, type 2B VWD may be manifested as thrombocytopenia in a well infant. Thrombocytopenia in sick neonates is often due to the underlying cause, such as infection or DIC. Isolated prolongation of the prothrombin time (PT) or activated partial thromboplastin time (APTT) in a healthy baby may be caused by a specific clotting factor deficiency in the extrinsic or intrinsic pathway, respectively, and prolongation of both the PT and APTT suggests either a common pathway clotting factor deficiency or vitamin K deficiency (hemorrhagic disease of the newborn). The so-called early vitamin K deficiency–associated bleeding usually presents in the first week of life and is associated with nutritional deficiencies or failure to administer vitamin K at birth, whereas late vitamin K deficiency bleeding occurs between 3 and 8 weeks after birth and may manifest as intracranial hemorrhage or severe gastrointestinal bleeding.[42]

Family Bleeding History

The family history is essential for assessing differential diagnoses. The hematologist should inquire about any known or suspected bleeding problems in other family members, including any specific diagnoses. If the family history is positive for bleeding, the hematologist should note the type and severity of bleeding (e.g., joint bleeding, epistaxis, menorrhagia), age at onset, and the relationship of the affected family member or members to the patient. An X-linked recessive inheritance pattern (maternal cousins, uncles, and grandfather) suggests a diagnosis of hemophilia A or B,[43] whereas an autosomal dominant pattern would be more consistent with VWD or hereditary hemorrhagic telangiectasia. Most other clinically relevant clotting factor deficiencies are inherited in an autosomal recessive manner, and the family history is frequently negative for bleeding, although consanguinity may be noted.

Approximately one third of infants and young children with newly diagnosed hemophilia have a negative family history,[3] consistent with the Haldane hypothesis for the fraction of new mutations in all lethal X-linked recessive disorders.[44,45] For VWD, considerable variation in symptoms may be noted among affected family members because of the incomplete penetrance (i.e., not every person that inherits the mutation will exhibit an abnormal phenotype) and variable expressivity (i.e., family members with the same mutation have a variable phenotype) that are hallmarks of this disease. Bleeding manifestations may be very mild in some and give the misleading impression of a negative family history. Factor XI deficiency, an autosomal trait most often (but not exclusively) seen in persons of Ashkenazi Jewish descent, may be associated with a very mild or moderate tendency for bleeding. The degree of bleeding manifestations does not correlate well with the level of factor XI or the APTT, although the patient's specific mutation may be predictive.[46,47]

Medications

A careful history of medication use should be obtained, including prescribed medications, over-the-counter drugs, recreational drugs, and herbal products.[48] A number of drugs are associated with bleeding diatheses, with mechanisms including induction of thrombocytopenia (e.g., quinine or quinidine, rifampin, trimethoprim-sulfamethoxazole, carbamazepine, cimetidine, ranitidine, valproic acid)[49-52] and platelet dysfunction (nonsteroidal antiinflammatory drugs [NSAIDs] such as ibuprofen [reversible effect] and aspirin [irreversible]).[53,54-57] In the case of medications that cause platelet dysfunction, it is important to note that they primarily exacerbate preexisting bleeding disorders rather than cause clinically relevant bleeding by themselves. In addition, prolonged antibiotic use may lead to lower vitamin K levels and induce bleeding secondary to acquired deficiency of vitamin K–dependent factors. Table 29-2 lists medications, compounds, and herbal supplements known to be associated with bleeding.

PHYSICAL EXAMINATION

A thorough physical examination must begin with an evaluation of the vital signs, with particular emphasis on

TABLE 29-2 Compounds that Decrease Platelet Number and/or Function

Effect	Compound Class	Examples	Mechanism
Platelet dysfunction	COX-1 inhibitors	Aspirin, ibuprofen, naproxen	Inhibit conversion of AA to thromboxane A2
	Thienopyridines	Clopidogrel, prasugrel, ticagrelor, cangrelor	Inhibit ADP-induced platelet activation
	Phosphodiesterase inhibitors	Dipyridamole, cilostazol	Increased cAMP decreases agonist-induced activation
	GPIIb/IIIa inhibitors	Abciximab, tirofiban, eptifibatide	Inhibit integrin activation and conformational change
	β-Lactam antibiotics	Cephalosporins, penicillins (esp. in high doses)	Inhibit epinephrine- and ADP-induced aggregation and granule release. Also, interference with platelet-VWF interaction
	SSRIs	Fluoxetine, olanzapine, citalopram, sertraline, escitalopram	Decrease platelet serotonin
	Herbs and foods	Ginkgo *(Ginkgo biloba)* Garlic *(Allium sativum)* Bilberry *(Vaccinium myrtillus)* Ginger *(Zingiber officinale)* Dong quai *(Angelica sinensis)* Feverfew *(Tanacetum parthenium)* Asian ginseng *(Panax ginseng)* American ginseng *(Panax quinquefolius)* Siberian ginseng/eleuthero *(Eleutherococcus senticosus)* Turmeric *(Curcuma longa)* Meadowsweet *(Filipendula ulmaria)* Willow *(Salix alba)* Black tree fungus *(Auricularia polytricha)*	Various
	Alcohol	Alcohol	Decrease P-selectin exposure
	Other	Amitriptyline, imipramine, chlorpromazine, cocaine, lidocaine, isoproterenol propranolol, penicillin, ampicillin, cephalothin, promethazine, diphenhydramine, carbenicillin	Interfere with platelet membrane
		Furosemide, verapamil, hydralazine, cyclosporine, hydrocortisone	Inhibit prostaglandin pathways
		Caffeine, dipyridamole, aminophylline, theophylline, vinblastine, vincristine, colchicine, papaverine	Inhibit platelet phosphodiesterase
Thrombocytopenia	Antiepileptic drugs	Phenytoin, valproate, carbamazepine	Unclear
	Glycoprotein IIB/IIIa inhibitors	Abciximab	Drug-dependent antibody
	Anticoagulants	Unfractionated heparin (more common) low-molecular-weight heparin (less common)	Drug-dependent antibody
	Foods	Quinine, white lupin, tahini, jui herbal tea	Various

AA, Arachidonic acid; *A2*, annexin 2; *ADP*, adenosine diphosphate; *cAMP*, cyclic adenosine monophosphate; COX, cyclooxygenase-1; GP, glycoprotein; SSRI, selective serotonin reuptake inhibitors; VWF, von Willebrand factor.

potential signs of severe bleeding-related anemia or intravascular volume loss, such as tachycardia (early finding) or hypotension (late finding). Next, a global overview should be undertaken to observe the pattern of bleeding stigmata. Petechiae are small capillary hemorrhages and characteristically develop in crops in areas of increased venous pressure, such as dependent parts of the body or those compressed by elastic waistbands, sock tops, or straps (e.g., from backpacks, purses, luggage). They are painless, nonpalpable, and nonblanching and must be distinguished from small telangiectases and angiomas. In general, the presence of petechiae indicates a defect in primary hemostasis (platelet number or function or vascular integrity). Ecchymoses are palpable purplish lesions induced by subcutaneous bleeding and usually indicate a defect in secondary hemostasis (clot propagation), such as deficiency of a coagulation factor. Additionally, hemarthrosis, associated with severe coagulation factor deficiency, should be evaluated for joint size, swelling, and range of motion limitations. Identification of hepatomegaly and splenic enlargement may point toward coagulopathy associated with systemic disorders such as leukemia or hepatocellular disease.

Knowledge of the bleeding manifestations of certain syndromes can help the hematologist efficiently evaluate children with known syndromes. Conversely, the hematologist's identification of a particular bleeding pattern may pave the way to an important syndrome diagnosis that can guide other system-based evaluation or interventions earlier than would otherwise be possible. For example, it is important for the hematologist to know that patients with Noonan syndrome can have thrombocytopenia, platelet function defects, and prolonged APTTs secondary to abnormalities of the intrinsic pathway (deficiencies of FVIII, FIX, FXI).[58,59] Another example is the importance of recognizing bleeding related to skin or vascular fragility as a potential manifestation of heritable connective tissue disorders such as EDS.[27] The molecular basis for bleeding in these syndromes is not well understood, but the clinical association between these syndromes and excessive hemorrhage is strong enough to warrant a careful investigation.

LABORATORY EVALUATION

Sample Collection and Technique and Preanalytical Variables

A properly drawn blood sample is crucial for adequate interpretation of coagulation test results.[60,61] Many laboratories develop independent ranges, controlling for preanalytic variables (patient posture, medical condition, lipid levels, hormones, mental and physical stress, activity before blood draw, tourniquet time, proper tube filling, specimen processing, and transport conditions).[62] Preferably, subjects should be fasting and should have refrained from smoking, caffeine, or alcohol ingestion, and they should not have taken any medications known to affect platelet function (see Table 29-2) on the day of testing; for some medications, such as aspirin and NSAIDs, the period of abstinence should be at least 2 weeks.[63] Herbal remedies, garlic, and alcohol may also cause acquired mild platelet dysfunction.[53] Blood for coagulation assays should be obtained by venipuncture performed by an experienced phlebotomist using a 19- to 21-gauge needle.[60,61] Drawing samples from an indwelling catheter often results in sample contamination with heparin or intravenous fluids and spuriously abnormal values. Improper sample collection is one of the most common reasons for falsely prolonged clotting times.[64-66] The total tourniquet time should be less than 3 minutes.[67]

The initial 1 to 2 mL of blood should be drawn into a waste tube to prevent activation of the test sample by tissue factor. Then blood is collected into citrated anticoagulant in an evacuated sample tube containing a fixed amount of buffered sodium citrate (3.2% preferred over 3.8% for coagulation assays) as anticoagulant in the ratio of one part citrate to nine parts whole blood.[63,68,69] A clinically significant difference between glass and plastic tubes has not been identified.[70] If the patient is polycythemic (hematocrit ≥55%), the amount of plasma in the tube would be reduced in proportion to the citrate, thereby leading to falsely abnormal coagulation test results. In such circumstances the amount of citrated anticoagulant should be reduced proportionate to the high hematocrit.[71,72] Conversely, anemia (hematocrit <25%) does not seem to significantly affect the accuracy of coagulation tests,[73] but it does have an effect on some global assays of hemostasis such as the Platelet Function Analyzer (PFA-100) in which hemoglobin should be greater than 10 mg/dL (and platelets should be >100,000/μL) to provide proper biorheologic effect to bring platelets in contact with the agonist-coated peripheral section of the device.[63] For infants and children, small 3-mL tubes (2.7 mL blood to 0.3 mL citrate) are available. Tubes of all sizes should be filled to at least 90% of the expected fill volume to prevent false elevation of clotting times from a disproportionately high citrate level. When tubes with various anticoagulant types are used, the citrate tubes should be collected before ethylenediaminetetraacetic acid–containing or heparin-containing tubes to avoid the potential for carryover.[69]

Once the tube is filled, the anticoagulated blood should be gently mixed by inversion three to four times, sent to a special coagulation laboratory without delay, and tested within 2 hours of collection if maintained at room temperature or within 4 hours if kept cold. Samples with visible hemolysis or clots should be discarded. Plasma samples must be frozen if they are not tested within this time frame. When they are to be analyzed, frozen samples should be rapidly thawed at 37° C and tested immediately. Normal values differ from one laboratory to another and between instruments and reagents. Thus the values obtained should be compared with the age-appropriate normal range for that particular laboratory.

Screening Tests

A focused history and physical examination may lead the physician to obtain certain assays immediately, in order to identify common disorders, life-threatening situations, or easily treated conditions. However, if the initial evaluation does not suggest a specific disorder, a panel of screening tests should be ordered, including a complete blood count with evaluation of platelet number, size, morphology, PT, APTT, and thrombin time (TT) to help in the process of differential diagnosis. Although screening tests represent a very important part of the initial laboratory evaluation, they are usually neither predictive nor confirmatory of bleeding. Therefore, if a specific bleeding disorder is suspected, the laboratory evaluation should be directed to that specific disorder. These principles are illustrated in Figure 29-1, which provides an algorithm representing the general approach to working up clinically significant mucocutaneous bleeding.

Prothrombin Time

The PT is performed by adding a thromboplastin reagent that contains calcium chloride to the citrated plasma sample. The time required for clot formation is recorded with an automated instrument that signals the end point, as defined by optical or electromechanical change. The normal (reference) range varies depending on the laboratory (its instrumentation and the lot of thromboplastin), but it is generally 10 to 11 seconds. The PT measures the activities of factors I (fibrinogen), II (prothrombin), V, VII, and X. Prolongation of the PT beyond the reference range is not generally seen until the functional level of one of these factors is less than 30% or until fibrinogen is less than 100 mg/dL. Isolated prolongation of the PT may reflect factor VII deficiency. Though rare, the PT can also be prolonged by a circulating inhibitor to one of the involved clotting factors or by the presence of abnormal fibrinogen molecules or fragments in the circulation.

The PT is also quite useful for monitoring the effect of coumarin-type anticoagulants. When the PT is used to monitor a patient taking warfarin, differences in the sensitivities of different thromboplastin preparations in different laboratories need to be taken into account. This consideration has led to the development of a

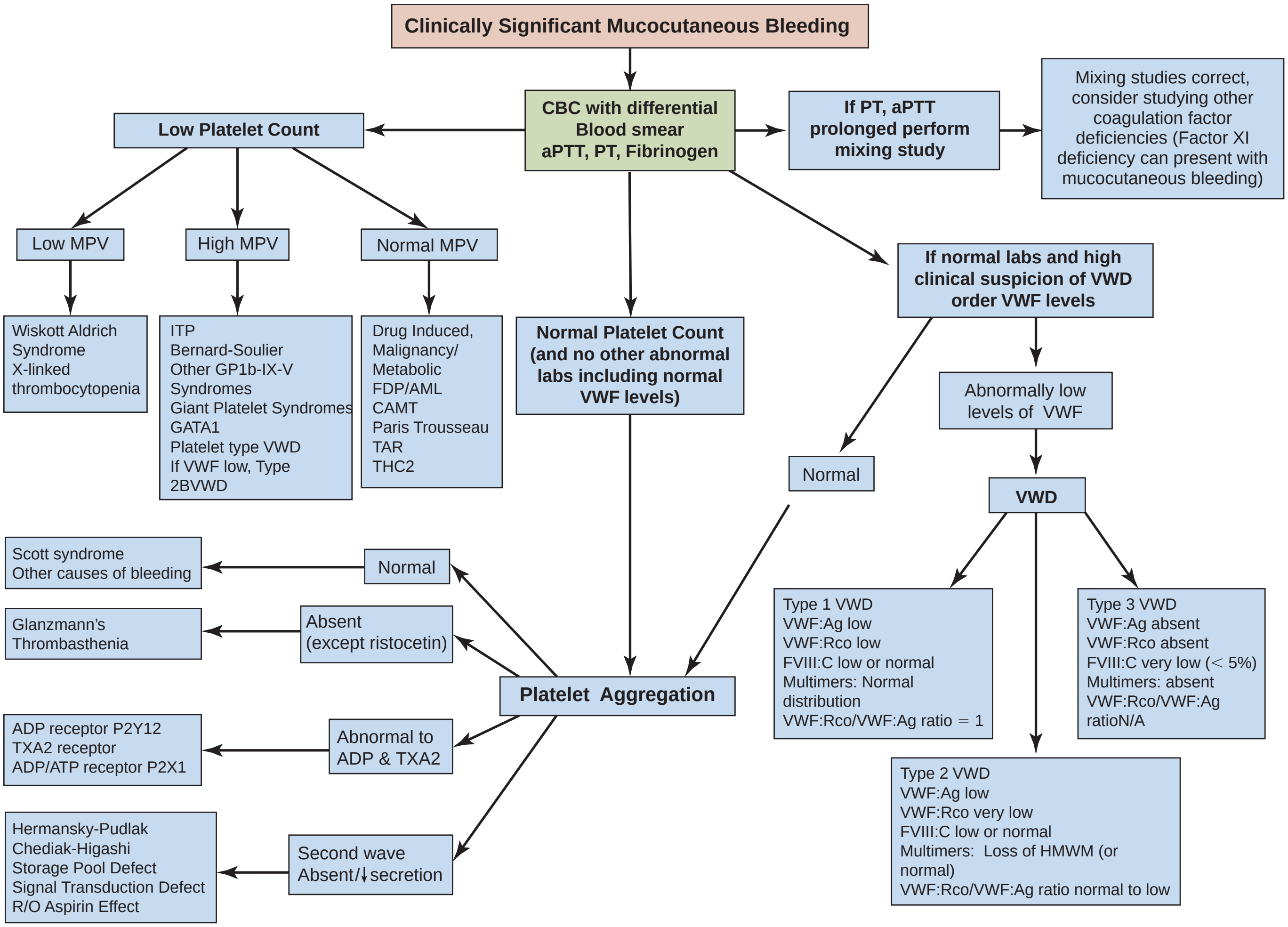
Clinically Significant Mucocutaneous Bleeding
CBC with differential
Blood smear
aPTT, PT, Fibrinogen
If PT, aPTT prolonged perform mixing study
Mixing studies correct, consider studying other coagulation factor deficiencies (Factor XI deficiency can present with mucocutaneous bleeding)
Low Platelet Count
Low MPV
High MPV
Normal MPV
Wiskott Aldrich Syndrome
X-linked thrombocytopenia
ITP
Bernard-Soulier
Other GP1b-IX-V Syndromes
Giant Platelet Syndromes
GATA1
Platelet type VWD
If VWF low, Type 2BVWD
Drug Induced,
Malignancy/ Metabolic
FDP/AML
CAMT
Paris Trousseau
TAR
THC2
Normal Platelet Count (and no other abnormal labs including normal VWF levels)
If normal labs and high clinical suspicion of VWD order VWF levels
Abnormally low levels of VWF
Normal
VWD
Type 1 VWD
VWF:Ag low
VWF:Rco low
FVIII:C low or normal
Multimers: Normal distribution
VWF:Rco/VWF:Ag ratio = 1
Type 3 VWD
VWF:Ag absent
VWF:Rco absent
FVIII:C very low (< 5%)
Multimers: absent
VWF:Rco/VWF:Ag ratioN/A
Type 2 VWD
VWF:Ag low
VWF:Rco very low
FVIII:C low or normal
Multimers: Loss of HMWM (or normal)
VWF:Rco/VWF:Ag ratio normal to low
Platelet Aggregation
Normal
Scott syndrome
Other causes of bleeding
Absent (except ristocetin)
Glanzmann's Thrombasthenia
Abnormal to ADP & TXA2
ADP receptor P2Y12
TXA2 receptor
ADP/ATP receptor P2X1
Second wave Absent/↓secretion
Hermansky-Pudlak
Chediak-Higashi
Storage Pool Defect
Signal Transduction Defect
R/O Aspirin Effect

Figure 29-1 A provider's guide to the staged workup of clinically significant mucocutaneous bleeding. *ADP,* Adenosine diphosphate; *aPTT,* activated partial thromboplastin time; *ATP,* adenosine triphosphate; *CAMT,* congenital amegakaryocytic thrombocytopenia; *CBC,* complete blood count; *FDP/AML,* familial platelet disorder with predisposition to acute myelogenous leukemia; *ITP,* immune thrombocytopenia; *MPV,* mean platelet volume; *PT,* prothrombin time; *Rco,* ristocetin cofactor test; *TAR,* thrombocytopenia with absent radii syndrome; *TXA2,* thromboxane A2; *VWD,* von Willebrand disease; *VWF,* von Willebrand factor.

standardized method of expressing the prolongation as an international normalized ratio.

Activated Partial Thromboplastin Time

The APTT is performed by adding a "partial thromboplastin" reagent, which is a source of phospholipids without tissue factor, to the patient's citrated plasma sample, plus introducing controlled activation of the contact factors (factors XI and XII, prekallikrein, and high-molecular-weight kininogen) by preincubation with a surface-activating reagent (e.g., Celite, kaolin, silica, or ellagic acid).[74] This mixture is incubated for 2 to 5 minutes before calcium chloride is added, and the time required for clot formation is recorded. As for the PT, automated instruments are generally used. The APTT measures factors I (fibrinogen), II (prothrombin), V, VIII, IX, X, XI, and XII; prekallikrein; and high-molecular-weight kininogen. Deficiency of any of the latter three factors can result in a markedly prolonged APTT in the absence of clinically significant bleeding. Isolated prolongation of the APTT in a patient with clinical bleeding is likely to result from a deficiency of factor VIII, IX, or XI. It should be noted that the sensitivity and reproducibility of the APTT are highly dependent on the specific reagents used (particularly the activator in the partial thromboplastin reagent). With most APTT reagents, the APTT will not be prolonged until the amount of factor VIII is less than 35% (0.35 U/mL). The laboratory should establish a reference range for each new lot of reagent and each new method of clot detection. The reference range will generally be approximately 26 to 35 seconds for children and adults but longer (30 to 54 seconds) in term infants (and often even longer in premature infants).

The APTT is somewhat less sensitive than the PT to deficiency of the vitamin K–dependent factors (so mild deficiencies could be missed), but it is more sensitive to the presence of circulating anticoagulants and heparin. The APTT can detect circulating anticoagulants (e.g., lupus anticoagulants) and is routinely used to monitor standard heparin therapy.[75] Among hospitalized infants or children, unintentional contamination of patient samples with heparin is a common cause of an unexpected prolongation of the APTT that does not correct on mixing.

Thrombin Time

The TT measures the thrombin-induced conversion of fibrinogen to fibrin and is performed by adding bovine thrombin to the patient's citrated plasma and recording the clotting time. The TT measures the amount and the clotting function of fibrinogen and is also prolonged in the presence of heparin or circulating fibrin degradation products (FDPs). An extremely prolonged TT usually indicates a heparin effect. Reptilase, a snake venom protease, clots fibrinogen in the presence of heparin and thus can be used to identify heparin as the cause of a prolonged TT. Thus in the presence of heparin the TT is prolonged, whereas the reptilase time is normal. Alternatively, it is possible to test for heparin activity by its anti–factor Xa activity or with the use of commercial heparinase. The sensitivity of the TT can be increased by dilution of the thrombin to give a control TT of 16 to 18 seconds.

Platelet Count

The normal platelet count (for all ages) ranges from 150,000 to 450,000 per microliter. Review of the platelets on a well-prepared stained blood smear should give a visual estimate that matches the laboratory's printed value. A spuriously low automated platelet count (pseudothrombocytopenia) may result from ethylenediaminetetraacetic acid anticoagulant plus an IgG or IgM platelet antibody, platelet cold agglutinins, unexpectedly large platelet size (e.g., as seen in idiopathic thrombocytopenia purpura) in which the cell counter is unable to distinguish the platelets from other cell types, or platelet clumping from a partially clotted sample. Electric particle counters also often provide a mean platelet volume and platelet volume (size) distribution. In the setting of thrombocytopenia, increased platelet size may suggest increased platelet turnover. Large platelets are also seen in many hereditary platelet syndromes, and very small platelets are characteristic of Wiskott-Aldrich syndrome.

Bleeding Time

The bleeding time (BT), a measurement of length of time that bleeding continues after a standardized wound is made on the forearm or ear lobe, is no longer in clinical use in the United States or United Kingdom, primarily because of its poor reliability and parental perception of the test as overly invasive. It is, however, inexpensive and uses moderately accessible materials, and therefore it is still used in developing countries as a global hemostasis assay.

PFA-100

Current platelet function tests are often viewed as inaccurate and unreliable (BT) or labor intensive and time consuming (platelet aggregation studies). Recently, a range of new instruments have been developed that attempt to simulate in vivo platelet adhesion and aggregation. The PFA-100 (Dade Behring, Marburg, Germany) is a commercially available instrument that uses test cartridges containing collagen/adenosine diphosphate- or collagen/epinephrine (CEPI)-coated membranes with a small aperture (150 μm). Citrated blood is aspirated under high shear (5000 to 6000/sec) from the sample reservoir through a capillary tube onto the membrane, and blood flow is monitored through the aperture. Platelets begin to adhere and aggregate primarily through interactions with collagen under high shear, thereby resulting in closure of the aperture. The instrument monitors the drop in flow rate with time as the aperture gradually occludes and records the final closure time (CT) with either cartridge. Although the test is reproducible between cartridge lots and instruments, each laboratory should establish normal control times. Citrate samples have been shown to be stable for up to 4 hours after sample collection, but transport through a pneumatic vacuum transport or "tube" system is not recommended.

The test is sensitive to the platelet count, hematocrit concentration, platelet function, and VWF level and function. Generally, a platelet count of less than 80 to 100 × ^{9}/L or a hematocrit concentration of less than 30% leads to a prolonged CT.[76] The PFA-100 CT is inversely proportional to the VWF levels and has been used by some as a screening test for VWD.[77] In congenital platelet disorders, PFA-100 CT depends on the severity of the disorder. Severe platelet function defects in glycoprotein Ib/V/IX (Bernard-Soulier syndrome or platelet-type VWD) and glycoprotein IIb/IIIa (Glanzmann thrombasthenia) result in a markedly prolonged CT. In platelet-dense granule deficiency or secretion defects, the prolongation in CT is variable and is often seen in the CEPI cartridge. Thus, although the PFA-100 CT is abnormal in some forms of platelet disorders, the test does not have sufficient sensitivity or specificity to be used as a screening tool for platelet disorders in general.[78] It is most consistently abnormal in severe platelet function disorders, in which clinical symptoms alone are significant enough to suggest the diagnosis. Additionally, PFA-100 CT may be altered by drugs that affect platelet function. Aspirin and other NSAIDs prolong the CEPI CT. The role of the PFA-100 in therapeutic monitoring is currently being investigated.[78] Despite its wide use, the PFA-100 has not demonstrated great overall efficacy as a screening tool for mild to moderate bleeding disorders.

Specific Tests

Specific Coagulation Factors. Each of the coagulation factors of the intrinsic pathway (prekallikrein, high-molecular-weight kininogen, and factors VIII, IX, XI, and XII) can be measured by one-stage, APTT-based methods. A specific factor assay measures the clotting time of a mixture of diluted test plasma and a specific factor-deficient substrate plasma that supplies all factors except the one being measured.[79] Though seldom performed in U.S. clinical laboratories, a chromogenic substrate assay kit for factor VIII (as well as chromogenic kits for certain other clotting factors) that is based on measuring the generation of factor Xa is also commercially available.

Each of the extrinsic and common pathway coagulation factors (V, VII, and X) can be measured by specific one-stage assays based on the PT. The patient's diluted test plasma is incubated with commercially available plasma that is deficient in either factor V, VII, or X; a mixture of tissue factor and calcium chloride is added, and the clotting time is determined.[79]

For each clotting factor assay based on the APTT or PT, the clotting times of serial dilutions of a plasma sample representing a pool obtained from a large number of normal individuals is used to construct a standard curve. The concentration of the specific factor in each of the several dilutions of patient plasma is determined from the standard curve and is used to calculate the level of that clotting factor in the patient's undiluted plasma.[79] In most laboratories, automated instruments are used to perform the assays, and many of these instruments are preprogrammed with a statistical package for plotting the data. The reference pooled normal plasma should be calibrated with a commercial or national standard that has been assayed against an international standard when available.[80]

Covalent stabilization of fibrin by factor XIIIa is essential for normal hemostasis (see Chapter 27). Factor XIIIa catalyzes the formation of covalent cross-links between the α and γ chains of fibrin, which results in increased mechanical stability of the fibrin clot and resistance to its degradation by plasmin. None of the routine screening tests (PT, APTT, or BT) detect factor XIII deficiency.[81] Screening for factor XIII deficiency can be performed with the clot solubility test: solubility of the patient's recalcified plasma clot in 5 mol/L urea or monochloroacetic acid is assessed after 30 minutes and periodically at 1, 2, 4, and 24 hours. Clots from patients with less than 1% factor XIII activity are soluble. Quantitation of factor XIII can be done with specific amine incorporation assays, although these tests are generally performed only in specialized research laboratories.[81]

Recommended tests for VWD include APTT, factor VIII assay, ristocetin cofactor assay to measure VWF activity, VWF antigen assay, and ABO blood group typing. If these tests suggest VWD, VWF multimer analysis should be performed. Treatment of VWD depends on accurate subtype diagnosis (see Chapter 31).

Fibrinogen. The concentration of fibrinogen in plasma can be measured immunologically (fibrinogen antigen) or by a chemical method in which the ability of fibrinogen to clot does not influence the assay. Normal fibrinogen antigen levels are between 200 to 400 mg/dL. Fibrinogen is an acute phase reactant, and elevated levels are commonly seen in stress or acute illness.

The level of functional fibrinogen or fibrinogen activity can also be measured. The von Clauss kinetic assay uses a dilute solution of patient plasma and an excess of thrombin, which makes fibrinogen the rate-limiting step in the clotting reaction.[82] The resulting clotting time in seconds is compared with a standard dilution curve to determine the concentration of clottable fibrinogen. Fibrinogen is decreased in congenital afibrinogenemia, hypofibrinogenemia, and consumptive states such as DIC. The assay is also sensitive to abnormalities in fibrinogen function (dysfibrinogenemia) or the presence of inhibitors of fibrin formation such as FDPs.

Euglobulin Clot Lysis Time. The euglobulin clot lysis time (ECLT) is a screening test for excessive fibrinolysis. It is performed by using the plasma euglobulin fraction prepared from fresh, citrated, platelet-poor plasma. After clot formation, lysis is measured. The normal ECLT is 60 to 300 minutes. It is shortened in conditions characterized by increased fibrinolysis (e.g. α_2-antiplasmin deficiency, plasminogen activator inhibitor 1 deficiency, or systemic fibrinolysis).

Platelet Function Tests. When ordering tests of platelet function, the physician must be aware that a wide variety of drugs can affect the platelet count and alter test results. Drugs such as aspirin irreversibly affect platelet function for the entire life span of the platelet (10 to 11 days). Thus tests of platelet function should be scheduled at a time when the individual has not taken any relevant drugs for at least 10 days.

The most common test of platelet function is platelet aggregometry. Platelet aggregation can be performed in whole blood by impedance technique or in platelet-rich plasma (PRP) by the turbidimetric technique. Whole blood platelet aggregation can be combined with release of adenosine triphosphate by using a lumi-aggregometer. A variety of platelet agonists (ristocetin, epinephrine, collagen, adenosine diphosphate (ADP), and arachidonic acid) can be added to an aliquot of the patient's sample. The rapidity and extent of platelet agglutination are detected by changes in optical density (PRP) or impedance (whole blood) and are graphically recorded for each agonist used.[83] In Glanzmann thrombasthenia, the patient's platelets will agglutinate normally with ristocetin but not at all with the addition of ADP, epinephrine, collagen, or arachidonic acid. In Bernard-Soulier syndrome, platelet agglutination will occur normally on addition of each of these agonists except ristocetin.[83]

Global Tests for the Evaluation of Bleeding

Over the last two decades, investigators and clinicians have attempted to develop global assays of hemostasis that would eventually be able to predict risk of bleeding as well as measure therapeutic efficacy of blood products and clotting factor replacement therapy. As knowledge of the coagulation system developed in the 1950s, specific assays were devised to measure the individual clotting factors. However, it soon became apparent that individual clotting factor assays were inadequate to assess the true nature of the hemostatic system. It was obvious that static end point laboratory tests such as the PT or APTT also did not accurately reflect the patient's overall hemostatic status and could not assess the dynamic clotting cascade. The common clinical observation that patients with hemophilia have great phenotypic variation in bleeding despite identical factor levels stresses this point. This has led to a revival of interest in global clotting assays. In addition, the development of technical innovations and computer-assisted methods of analysis has improved the reliability and reproducibility of global assays. Two such techniques are described here: thromboelastography (TEG) and thrombin generation assays (TGAs). TEG is usually performed on whole blood and quantitatively measures the dynamics of fibrin formation. TGAs are generally performed on plasma and measure thrombin formation.

Thromboelastography

Hartert first described TEG as a global test for blood coagulation more than 50 years ago.[84] TEG monitors the whole dynamic process of hemostasis from clot formation to its dissolution and also provides information about platelet function. TEG produces a continuous profile of the overall rheologic changes occurring during coagulation while using a small amount of whole blood. By processing the data, the viscoelastic changes occurring during whole blood clot formation can be transformed into a dynamic velocity profile that is then recorded as a tracing. Currently, reagent-modified TEG with various activators (tissue factor, kaolin) and inhibitors is used with the aim of accelerating the coagulation cascade and obtaining differential diagnostic information.[85] The two commonly used instruments are the TEG (Haemoscope Corp, Skokie, Illinois) and the rotating thromboelastometer ROTEM (Pentapharm, Munich, Germany).[86] Currently used primarily as tools to decrease transfusion requirements during cardiac[87] and hepatic surgery, these instruments are also being investigated as screening tools for bleeding disorders.[88,89]

Thrombin Generation Assays

TGAs have been used extensively in research laboratories but are cumbersome to perform.[90] A more recently developed test, calibrated automated thrombography (CAT), may be applicable to the routine clinical laboratory. CAT measures the concentration of thrombin in clotting plasma by monitoring the splitting of a fluorogenic substrate and comparing the results with known constant thrombin activity in a parallel nonclotting sample (control).[91] Application of TGAs is currently being investigated for monitoring of hemophilia treatment and anticoagulation therapy and as a means to better understand the differing clinical patterns in bleeding disorders.[92]

Overall, although significant advances have been made in the development of global assays of hemostasis, and some of them are widely used in the clinical and surgical setting, the evidence for making critical diagnostic and therapeutic decisions based on results obtained from these assays is still largely premature; caution should be exercised.

BLEEDING IN THE CRITICALLY ILL CHILD

It is not uncommon for the general pediatric hematologist to be consulted in the critical care setting for acute bleeding in the presence of abnormally prolonged clotting tests, thrombocytopenia, or suspected platelet dysfunction. Several clinical situations may lead to the presence of acquired bleeding in the inpatient setting; when the underlying cause is resolved, bleeding typically stops (Table 29-3). Perhaps the most common consult for excessive bleeding in this setting concerns consumption of clotting factors and platelets in the presence of DIC.

TABLE 29-3 Acquired Bleeding Disorders

Underlying Bleeding Disorder	Hemostatic Defect	Cause
Overwhelming sepsis	Acute DIC	Initiation of coagulation, damage to the endothelium; decrease in clotting and anticlotting factors
Liver disease	Multiple coagulation factor deficiency	Decreased hepatic synthesis Increased fibrinolysis Decreased clearance of plasminogen activators Hypercoagulable state Decreased production of natural anticoagulants Thrombocytopenia Hypersplenism
Malabsorption syndrome	Decreased production of factors II, VII, IX, and X and proteins C and S	Vitamin K deficiency
Cyanotic congenital heart disease	Mild to moderate thrombocytopenia	Shortened platelet survival Abnormal platelet function Acquired defects in platelet aggregation
Acyanotic congenital heart disease (e.g., ASD, PDA)	Decreased high-molecular-weight VWF multimers	Consumption
ECMO and CPB platelet dysfunction	Platelet activation in the oxygenator and physical damage to the platelet membrane	Coagulation factor deficiency Consumption of coagulation factors in the circuit Hyperfibrinolysis Increase in tPA and decrease in α_2-antiplasmin
Acute promyelocytic leukemia	Thrombocytopenia Decreased production in bone marrow and increased consumption	Disseminated intravascular coagulation Release of procoagulant material from the leukemic cells Hyperfibrinolysis Increased synthesis of plasminogen activators

ASD, Atrial septal defect; *CPB,* cardiopulmonary bypass; *ECMO,* extracorporeal membrane oxygenation; *PDA,* patent ductus arteriosus; *tPA,* tissue plasminogen activator; *VWF,* von Willebrand factor.

Box 29-1 **Conditions Associated with Disseminated Intravascular Coagulation**

- Sepsis (particularly gram-negative bacteremia)
- Trauma
  - Massive tissue injury
  - Head injury
- Fat embolism
- Malignancy
  - Acute promyelocytic leukemia
  - Myeloproliferative disorders
- Obstetric accidents
- Vascular disorders
  - Giant hemangiomas (Kasabach-Merritt syndrome)
  - Aortic aneurysm
- Toxins
  - Snake venom
  - Drug overdose
- Immunologic reactions
  - Severe anaphylaxis
  - Acute hemolysis
  - Acute transplant rejection

DIC is an acquired systemic disorder characterized by widespread activation of coagulation and deposition of fibrin leading to the formation of thrombi in the microcirculation accompanied by secondary activation of fibrinolyis.[72] DIC can be acute or chronic, compensated or decompensated, and caused by a wide variety of conditions (Box 29-1). DIC should be suspected in children with shock and bleeding manifestations. In DIC the PT and APTT are usually both prolonged, with decreased fibrinogen, factor VIII, and factor V. A concomitant decrease in natural anticoagulants (protein C, protein S, and antithrombin III) may also be seen. Examination of the peripheral smear usually reveals the presence of schistocytes and thrombocytopenia.[93] There is also an increase in D-dimer and FDPs secondary to the formation of plasmin. As discussed earlier in the chapter, in most cases the diagnosis of the cause of bleeding becomes complicated by the need for immediate therapeutic intervention, which usually requires replacement products.

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CHAPTER 30

Inherited Platelet Disorders

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CHAPTER OUTLINE

QUALITATIVE DISORDERS
Platelet Membrane
Glanzmann Thrombasthenia: Defective Platelet Integrin $\alpha_{IIb}\beta_3$
Other Integrins in Inherited Platelet Disorders
Bernard-Soulier Syndrome: Defective Glycoprotein Ib/IX Complex
Platelet-Type von Willebrand Disease (Pseudo–von Willibrand Disease)
Other Inherited Defects of Platelet Receptors
Defects in Signal Transduction
Platelet Storage Granule Defects
QUANTITATIVE DISORDERS
Disorders of Platelet Production

Platelets are small (1 to 4 µm in diameter) and were once thought to be fragments of other mature blood cells, dismissed as "blood dust." They are now known to be highly specialized and organized structures released (by a still partly understood process called *thrombopoiesis*) from megakaryocytes. Platelets are a critical component for the first phase of hemostasis (formation of the platelet plug), which can halt the loss of blood from vessels whose endothelial integrity has been interrupted, though it has become increasingly clear that platelets have important roles in maintaining vascular integrity and the inflammatory response. If platelets are deficient in number or defective in function, excessive bleeding may occur. The clinical manifestations of platelet-type bleeding typically involve the skin or mucous membranes and include petechiae, ecchymosis, epistaxis, menorrhagia, and gastrointestinal hemorrhage (Box 30-1). Intracranial bleeding can occur, but it is infrequent. The deep-muscle hematomas and hemarthrosis typically seen in patients with defects in the fluid-phase (plasma) hemostatic system infrequently occur in platelet disorders.

Inherited platelet disorders can involve a qualitative and/or quantitative defect and are often broadly classified according to one of these two categories. In this chapter, we have classified the platelet disorders by their predominant feature, although many involve combined defects in both platelet number and function. For example, Bernard-Soulier syndrome (BSS) results from defects in the platelet receptor glycoprotein Ibα/Ibβ/IX/V (GPIb/IX) complex, which binds von Willebrand factor (VWF) and is critical for adherence of platelets at sites of vascular injury. This receptor is anchored to the cytoskeleton and is also important in platelet formation from megakaryocytes. Thus many of these patients have macrothrombocytes and low platelet counts, as well as a defect in platelet function. Furthermore, although many inherited platelet disorders have been described, most are extremely rare. Even the well-known disorders such as BSS and Glanzmann thrombasthenia (GT) are uncommon, with a frequency in the general population of 10^{-5} to 10^{-6}, and are mostly seen in inbred populations or consanguineous relationships. In aggregate, however, these inherited disorders are not uncommon and, given the diverse nature of the underlying defects, may pose a significant diagnostic challenge.

QUALITATIVE DISORDERS

Platelet Membrane and Receptors

Sensing abnormalities in their microenvironment, adhering to damaged vascular walls, and aggregating to each other are central functions of circulating platelets. Many of the membrane receptors in these processes have been cloned, and our understanding of the interplay between membrane receptors, intracellular molecules, and the cytoskeleton continues to increase. Initially, these protein receptors were classified by their electrophoretic mobility and molecular mass and numbered sequentially. Now it is clear that many of these proteins are members of several large families of receptors, including the integrin α/β heteroduplexes, the leucine-rich receptors, the G protein–coupled receptors (GPCRs), and the immunoglobulin

Box 30-1 Clinical Features of Platelet Defects

Epistaxis, petechiae, purpura, ecchymosis
Gastrointestinal hemorrhage
Menorrhagia
Rarely, intracranial bleeding or hemarthrosis

superfamily. Such a classification takes advantage of the similar structure and function of members of these families. Frequently, family members share a common family of ligands or activate cells by a common pathway. Table 30-1 lists the members of these families of receptors that are found on platelets, as well as several receptors that do not belong to any of these families. Many of these receptors have other historical names, and these, as well as their cluster differentiation (CD) antigen nomenclature, are noted in the table. Many of the receptors involved in inherited platelet disorders are described in the following text.

Glanzmann Thrombasthenia: Defective Platelet Integrin $\alpha_{IIb}\beta_3$

In 1918, a Swiss pediatrician, Eduard Glanzmann, described a heterogeneous group of disorders that he termed *thrombasthenie* (weak platelets); these disorders were characterized by normal platelet counts but abnormal clot retraction.[1] In 1956 it was noted that these platelets failed to spread onto a surface or to stick to each other (aggregate).[2,3] Glanzmann thrombasthenia (GT; MIM 273800) is now known to be a rare, inherited, autosomal recessive bleeding disorder, the hallmark of which is failure of platelets to bind fibrinogen and aggregate after activation. The underlying defect is an abnormality in the genes encoding either chain of the integrin $\alpha_{IIb}\beta_3$ fibrinogen receptor (see Table 30-1). GT is the most common of the inherited platelet disorders associated with a severe bleeding phenotype.[4]

Biology of the $\alpha_{IIb}\beta_3$ Receptor

The α_{IIb} and β_3 subunits are encoded by separate genes (*ITGA2B* and *ITGB3*) that are closely linked on chromosome 17q21-23.[5] α_{IIb} is approximately 145 kD in size and contains 18 cysteine residues arranged into 9 disulfide bonds that are rather evenly spaced throughout its length (Fig. 30-1). The α_{IIb} prochain complexes with the β_3 subunit in the endoplasmic reticulum. During maturation in the Golgi body, the α_{IIb} prochain is cleaved into the α_{IIb} heavy fragment and the α_{IIb} light fragment, with the two fragments remaining linked by a disulfide bond. Like many other integrin α chains, α_{IIb} contains four calcium-binding domains near its N-terminal. This region in all α

TABLE 30-1 Major Platelet Membrane Receptors

Class of Receptor	Receptor	Other Names	Number of Receptors per Platelet	Ligand
Integrins	$\alpha_{IIb}\beta_3$	GPIIb/IIIa, CD41b	»80,000	Fibrinogen, VWF, fibronectin
	$\alpha_V\beta_3$		»500	Vitronectin, osteopontin
	$\alpha_2\beta_1$	CD49b	»2000	Collagen
	$\alpha_5\beta_1$	CD49e	»4000	Fibronectin
	$\alpha_6\beta_1$	CD49f		Laminin
Leucine-rich repeats receptor	GPIb-IX	CD42a, b, c	»25,000	VWF, thrombin, P-selectin
G protein–coupled receptors	PAR-1		»2000	Thrombin
	PAR-4		Low	Thrombin
	P2Y1			ADP
	P2X1			ADP
	P2Y12			ADP
	a_{2A}		»700	Epinephrine
	TP		»1000	Thromboxane
	IP			Prostaglandin I_2
	CXCR1 and CXCR2		»2000 each	Interleukin-8
	CXCR4		»2000	Stromal-derived factor 1
	CCR4		»2000	CCL22
Immunoglobulin superfamily receptors	GPVI		1000-3000	Collagen
	P-selectin	GMP-140, PADGEM, MARK		P-selectin, glycoprotein-1
	PECAM-1	CD31	»10,000	PECAM-1
	FcγRIIA	CD32	1000-5000	Immune complexes
Others	GPIV	CD36	»25,000	Collagen
	p65			Collagen

ADP, adenosine diphosphate; *GPIIb*, glycoprotein IIb; *MAPK*, mitogen-activated protein kinase; *PADGEM*, platelet activation–dependent granule–external membrane protein; *PAR*, protease-activated receptor; *PECAM-1*, platelet–endothelial cell adhesion molecule 1; *VWF*, von Willebrand factor.

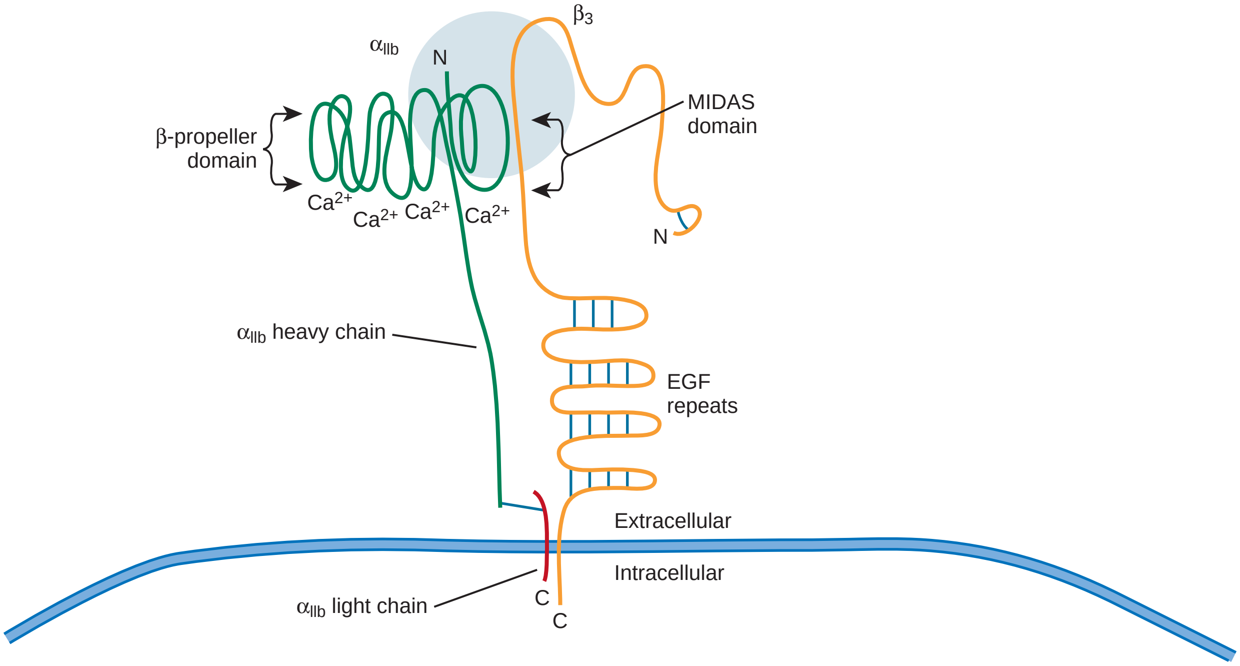

Figure 30-1 Structure of the $\alpha_{IIb}\beta_3$ receptor. Both units of the $\alpha_{IIb}\beta_3$ heteroduplex contain a transmembrane domain with short intracellular tails. The α_{IIb} extracellular domain is cleaved into two parts: the α_{IIb} heavy chain, which has the N-terminal β-propeller domain, and the α_{IIb} light chain, which spans the space from the extracellular compartment to the intracellular compartment. The β_3 chain contains multiple cysteine disulfide bonds represented by the *gray lines*. The MIDAS (metal ion–dependent adhesion site) domain is indicated. The pocket on $\alpha_{IIb}\beta_3$ that may represent the ligand-binding domain is shown as a *gray ball*. *EGF*, epidermal growth factor.

subunits contains seven homologous repeats that fold into a β-propeller structure[6]; portions of the surface loops of this structure are critical for ligand binding (in interaction with the βA domain of the β_3 subunit).[7]

Similar to other integrin β chains, β_3 is approximately 90 kD and contains 762 amino acid residues in its mature form (see Fig. 30-1).[7] β_3 contains five cysteine-rich regions for a total of 56 cysteine residues, including a large disulfide-bonded loop, termed the βA region, that extends from amino acids Cys5 to Cys435.[6] This loop participates in fibrinogen binding and contains three divalent cation-binding domains that appear to be important in ligand binding. One of these cation-binding sites (the βA MIDAS–metal ion–dependent adhesion site) interacts directly with ligand, and binding of cations to the βA domain may stabilize the ligand-occupied conformation.

There are about 8×10^4 $\alpha_{IIb}\beta_3$ receptors per platelet, making this receptor the most abundant one on platelets (see Table 30-1).[8] Most of these receptors are located on the platelet surface, although a portion are found on the inner surface of alpha granules and are involved in the localization of fibrinogen to these granules.[9] On resting platelets, $\alpha_{IIb}\beta_3$ exists in a folded, inactive state that does not interact with its ligand. However, upon platelet activation, an “inside-out” signaling event takes place in which the $\alpha_{IIb}\beta_3$ complex is activated and unfolded like a switch blade,[7] thereby resulting in binding of fibrinogen at the N-terminals of the two proteins and platelet aggregation. After ligand binding, an “outside-in” signal mediates integrin-cytoskeleton interactions and platelet spreading.[10]

Many integrin ligands contain an arginine-glycine–aspartic acid (RGD) motif that participates in integrin binding. Conversely, peptides containing RGD act as competitive inhibitors of ligand binding.[11] For example, the RGD motif located in the C1 domain of VWF appears to be necessary for binding of VWF to $\alpha_{IIb}\beta_3$. In contrast, deletion of the two RGD sequences in the fibrinogen α chain does not impair its ability to bind to $\alpha_{IIb}\beta_3$. Rather, binding of fibrinogen to $\alpha_{IIb}\beta_3$ requires a KQAGD sequence located at the carboxyl-terminal of the fibrinogen γ chain.[12]

Whereas expression of the α_{IIb} chain is restricted to megakaryocytes, the β_3 subunit is expressed more widely as a component of the $\alpha_V\beta_3$ (vitronectin) receptor. Mouse studies have shown that deletion of $\alpha_V\beta_3$ function results in placental defects and osteosclerosis,[13] although to date no differences have been noted between patients with GT caused by an α_{IIb} or β_3 defect.[4]

Clinical Features

Thrombasthenia is characterized by repeated mucocutaneous bleeding beginning at an early age (Fig. 30-2, *A*). Epistaxis and gastrointestinal bleeding are frequent issues in early childhood that often require intervention, as well as early iron supplementation. Menorrhagia is a critical problem in teenage girls.[14] The bleeding that normally accompanies pregnancy, surgical procedures, tooth extraction, or physical trauma can be excessive in GT patients. Unprovoked intracranial or gastrointestinal hemorrhage occurs and accounts for a significant portion of the observed 5% to 10% premature mortality

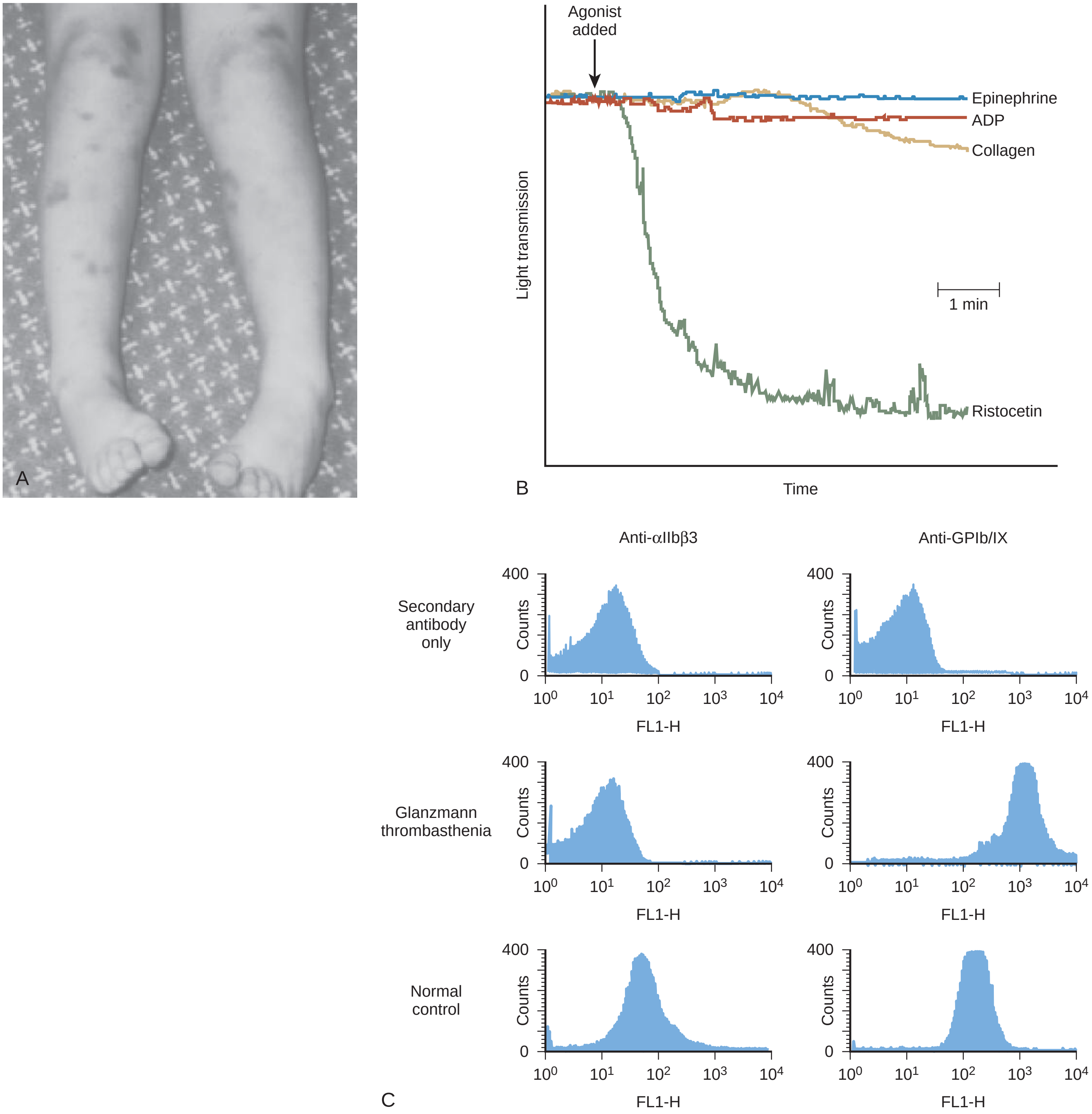

Figure 30-2 Glanzmann thrombasthenia. The patient shown has a mutation in the fourth cysteine-binding domain of α_{IIb} secondary to a Gly273Asp substitution.[183] **A**, Representative ecchymosis seen in the patient at the age of 6 years. **B**, Platelet aggregation studies show an absence of secondary aggregation to adenosine diphosphate (ADP), epinephrine, and collagen but a normal response to ristocetin. **C**, Flow cytometric platelet analysis using an anti-$\alpha_{IIb}\beta_3$ primary antibody (*left*) or an anti-GPIb/IX antibody as a positive control (*right*), followed by a fluorescein isothiocyanate–labeled secondary antibody. Shown at the *top* is the fluorescence with the secondary antibody only on normal platelets. In the *middle* is a Glanzmann thrombasthenia patient with primary antibody followed by secondary antibody, and at the *bottom* is the same with normal platelets. The patient's platelets show little $\alpha_{IIb}\beta_3$ antibody binding when compared with the control. The patient's platelets do express high levels of glycoprotein Ib/IX (GPIb/IX).

rate. In addition, some patients experience joint bleeding or muscle hematomas more characteristic of the hemophilias.[14]

Most frequent is recurrent and problematic epistaxis often complicated by prior nasal cauterizations that damage the local nasal architecture. Frequently, local compression strategies or topical applications of thrombin are useful when bleeding occurs. DDAVP (1-deamino-8-δ-arginine vasopressin) has been used as well, and oral contraceptive management of menorrhagia is generally sufficient. Platelet transfusions may be useful until resistance to platelet infusions develops, although patients do not necessarily form antibodies to the $\alpha_{IIb}\beta_3$ receptor. Antibodies may be particularly problematic because they may not only result in increased platelet clearance or platelet refractoriness but may also interfere with platelet function.[15] Case reports and small case series suggest that recombinant activated factor VII may be a useful supplement to platelet transfusions.[16,17] We have also successfully used embolization of arterioles feeding the nose and uterus in cases of recurrent, life-threatening hemorrhage.

Incidence

Although infrequent worldwide, GT is more prevalent among certain isolated populations and in the setting of consanguineous relationships, particularly among Arab populations,[17] Iraqi Jews,[18] French gypsies,[19] and individuals from southern India.[20] Heterozygotes for this disease have approximately 50% of the normal number of $\alpha_{IIb}\beta_3$ receptors, but generally no evidence of platelet dysfunction or clinically significant bleeding.[14] Although most cases are observed in specific populations or in the setting of consanguinity and are thus homozygous for a shared mutation inherited from both parents, about one third of patients with identified mutations are compound heterozygotes.[4]

Classification and Laboratory Diagnosis

In 1972, Jacques Caen classified GT according to platelet intracellular fibrinogen content and the ability of platelets to retract a fibrin clot.[21] Type I patients, representing 80% of those studied, lacked platelet fibrinogen and had an absence of clot retraction. Type II thrombasthenic platelets contained appreciable levels of platelet fibrinogen and maintained some clot retraction capability. Soon thereafter, the technique of sodium dodecyl sulfate–polyacrylamide gel electrophoresis became widespread, and it became clear that there were three different patterns seen with thrombasthenic platelet membrane glycoproteins.[4] Type I platelets lacked detectable levels of $\alpha_{IIb}\beta_3$, whereas type II platelets expressed moderate (15% to 25%) levels of these glycoproteins. Adding to the complexity of this classification was the identification of variant forms of thrombasthenia characterized by normal to nearly normal levels of a dysfunctional form of $\alpha_{IIb}\beta_3$ (type III).[4] Finally, a related integrin dysfunction syndrome was identified involving a GT-like platelet defect along with a white cell disorder termed *leukocyte adhesion deficiency type III*.[22] These patients have a deficiency of Kindlin-3 (encoded by the *FERMT3* gene on chromosome 11), an intracytoplasmic signaling molecule involved in the function of β_2- as well as β_3-based integrins. Patients have both GT-like bleeding manifestations and the infectious complications and leukocytosis of leukocyte adhesion deficiency.[15]

GT patients fail to aggregate in response to physiologic agonists such as adenosine diphosphate (ADP), thrombin, or epinephrine (see Fig. 30-2, *B*) because they all have a functional deficiency of platelet surface $\alpha_{IIb}\beta_3$ receptors. The failure to bind fibrinogen and other adhesive ligands is the reason for the inability of platelets to aggregate. No correlation exists between any of the proposed subtypes of GT and the severity of bleeding symptoms in patients.[4] Some patients with no $\alpha_{IIb}\beta_3$ have relatively mild clinical symptoms, whereas others with a full complement of $\alpha_{IIb}\beta_3$, albeit dysfunctional, can have frequent bleeding episodes requiring repeated transfusions.

Currently, the most common method used for determining levels of $\alpha_{IIb}\beta_3$ on thrombasthenic platelets involves flow cytometry and immunoblot analysis.[4] Figure 30-2, *C* illustrates flow cytometric analysis of the $\alpha_{IIb}\beta_3$ content of both a normal control and a GT patient with a severe deficiency of $\alpha_{IIb}\beta_3$ secondary to a mutation in one of the Ca^{2+}-binding domains of her α_{IIb} chain.[23] This mutation blocks export of the $\alpha_{IIb}\beta_3$ receptor out of the endoplasmic reticulum and into the Golgi body for final processing, as indicated by failure to cleave the α_{IIb} prochain into an α_{IIb} heavy chain.

Several hundred individuals with GT have been described in the literature. To date, more than four hundred different mutations have been described. As in most genetic disorders, the molecular abnormalities have been found to range from major deletions and inversions to single point mutations.[4,23a] Some of the better-characterized subgroups of mutations are described in the following text.

The earliest and largest group of thrombasthenic mutations described failed to express $\alpha_{IIb}\beta_3$ on the cell surface. Several of these patients had major deletions or inversions in their α_{IIb} or β_3 genes. In addition, a number of point mutations or small deletions in the α_{IIb} or β_3 genes have been described with no surface expression of $\alpha_{IIb}\beta_3$; both chains are formed intracellularly and may assemble the complex but fail to undergo intracellular processing or reach the cell surface. These defects result in the type 1 form of thrombasthenia. One major subgroup of these patients involves missense mutations in the N-terminal β-propeller repeats of α_{IIb} near the proposed Ca^{2+}-binding sites on its lower surface as exemplified by the patient in Figure 30-2.[23,24]

Another group of mutations occur in the same β-propeller region of α_{IIb} but take place on the upper surface and have expressed surface receptor that cannot bind its ligand. These mutations are located within three upper loops of the β-propeller, the connecting loop between the second and third blades, the loop in the middle of the third blade, and the loop connecting the third and fourth blades.[25] These mutations occur within the pocket of α_{IIb} that binds RGD ligand.[6]

Other thrombasthenic patients with significant levels of nonfunctional $\alpha_{IIb}\beta_3$ surface expression have mutations that involve the β_3 chain. These mutations result in a surface $\alpha_{IIb}\beta_3$ that is easily dissociable by chelation of external calcium ions.[26] Many of these missense mutations have been identified within the cation-binding MIDAS domain.[6] The importance of these sites was reinforced by the identification of a group of in vitro–generated mutant $\alpha_{IIb}\beta_3$ receptors expressed in Chinese hamster ovary cells, which provided independent support for the importance of the MIDAS domain in ligand binding.[27]

A second group of dysfunctional mutations with normal levels of surface $\alpha_{IIb}\beta_3$ receptors are localized to the cytoplasmic domain of β_3, thus demonstrating the importance of this domain for integrin activation and regulation of ligand binding.[4] These mutations do not affect surface expression of platelet $\alpha_{IIb}\beta_3$ complexes, but mutant receptors are unresponsive to agonist stimulation. These mutations provide compelling evidence for the role of the β_3 cytoplasmic tail in downstream platelet activation by $\alpha_{IIb}\beta_3$.

Other Integrins in Inherited Platelet Disorders

Other integrins that are infrequently associated with platelet dysfunction include the $\alpha_2\beta_1$ (glycoprotein Ia/IIa) receptor, an Mg^{2+}-dependent collagen receptor on many cells, including platelets.[28] In platelets, collagen not only serves as an adhesive substrate but it also functions as an agonist for platelet aggregation. Patients with histories of bleeding have been reported whose platelets failed to respond normally to collagen and lacked $\alpha_2\beta_1$, thus suggesting that binding of collagen to $\alpha_2\beta_1$ is a necessary component for collagen-induced signaling.[29] In addition, another report described several families with autosomal dominant thrombocytopenia and mild platelet dysfunction associated with mutations in the α_2 subunit.[30]

There is substantial variability in the density of $\alpha_2\beta_1$ on the surface of platelets from different individuals.[31] These differences in $\alpha_2\beta_1$ expression are associated with the inheritance of three different α_2 alleles. The higher the receptor density, the greater the attachment of platelets to type I collagen. Some, but not all, epidemiologic surveys suggest that increased $\alpha_2\beta_1$ density is a risk factor for cardiovascular disease.[32] An increased prevalence of the allele with lower $\alpha_2\beta_1$ density has been reported in patients with type 1 von Willebrand disease (VWD), which suggests that this allele may contribute to bleeding symptoms in these patients.[33]

Bernard-Soulier Syndrome: Defective Glycoprotein Ib/IX Complex

Bernard-Soulier syndrome (BSS: MIM 231200) was first described in 1948 in a 5-month-old infant with a prolonged bleeding time and giant platelets on blood smear and who had had a sibling who died of hemorrhage.[34] Over the following years, additional patients with the combination of mucocutaneous bleeding; enlarged platelets; normal platelet aggregation with ADP, collagen, and epinephrine with a delayed response to thrombin; and absent platelet aggregation with human VWF and ristocetin or with bovine VWF alone were described as having BSS, now the second most recognized inherited severe platelet disorder.

Biology of the Glycoprotein Ib/IX Complex

Adhesion through the GPIb/IX complex involves binding of VWF to the subendothelium. Platelet membranes contain two binding sites for VWF (see Table 30-1). One of these sites requires previous platelet activation and is located on the platelet membrane $\alpha_{IIb}\beta_3$ complex. The second binding site involves the GPIb/IX complex, and it is this membrane complex that is crucial for initial attachment and proper adhesion to the extracellular matrix of a damaged vessel wall. It should be pointed out that the GPIb/IX complex may also bind to other ligands, including P-selectin, thrombospondin-1, high-molecular-weight kininogen, and Mac-1.[35] Furthermore, GPIb/IX also binds thrombin, and its role in the activation of platelets by thrombin is discussed later.

The GPIb/IX complex is the second most abundant receptor on the platelet membrane surface, with approximately 25,000 copies per platelet.[36] This complex actually consists of four different proteins (Fig. 30-3), all of which have one or more leucine-rich repeats composed of a 24–amino acid motif with 7 conserved leucine residues. Other proteins have been described with this leucine-rich repeat, and these regions mediate ligand binding.[37]

GPIbα is the largest subunit (135 kD, 610 amino acids, chromosome 17p12) and has 7 leucine repeats. It is susceptible to cleavage by trypsin or calpain, which gives rise to a heavily glycosylated 135-kD fragment known as *glycocalicin*. In addition to containing the binding site for VWF, the glycocalicin portion of GPIbα also binds thrombin. Under normal flow in vivo, plasma VWF does not bind to the GPIb complex, but under shear stress conditions, VWF simultaneously binds to collagen and the GPIb/IX complex.[38] In clinical assays, the antibiotic ristocetin or the venom-derived protein botrocetin is used to mimic this effect by inducing conformational changes in VWF that promote binding to GPIb/IX in stirred platelet-rich plasma.[39]

GPIbα is disulfide-bonded to GPIbβ (25 kD, 181 amino acids, chromosome 22q11.2) through a single cysteine residue located in each subunit near the transmembrane domains of GPIbα and GPIbβ (see Fig. 30-3). This peptide has only one leucine repeat. The cytoplasmic tail of GPIbβ contains a filamin-binding site that links the receptor complex to F-actin below the membrane and a binding site for 14-3-3ζ. The disulfide-bound GPIbα-β is noncovalently associated with platelet GPIX and GPV. GPIX is the smallest member of the GPIb complex (22 kD, 160 amino acids, chromosome 3q29), with only one leucine repeat.[38] GPV (82 kD, 344 amino acids, chromosome 3q21) is a transmembrane protein with 15 leucine repeats.[40] This protein is a proteolytic substrate for thrombin and releases a 69-kD soluble fragment. GPV is present in only a single copy per complex, whereas there are two copies of the other three subunits in a single receptor (see Fig. 30-3). Furthermore, cleavage of the GPV extracellular domain by thrombin bound to GPIbα activates platelets.[41] Consistent with this observation, the GPV-knockout mouse has a mild prothrombotic state.[42]

Binding of activated VWF to the GPIb/IX complex activates platelets through activation of phospholipase C

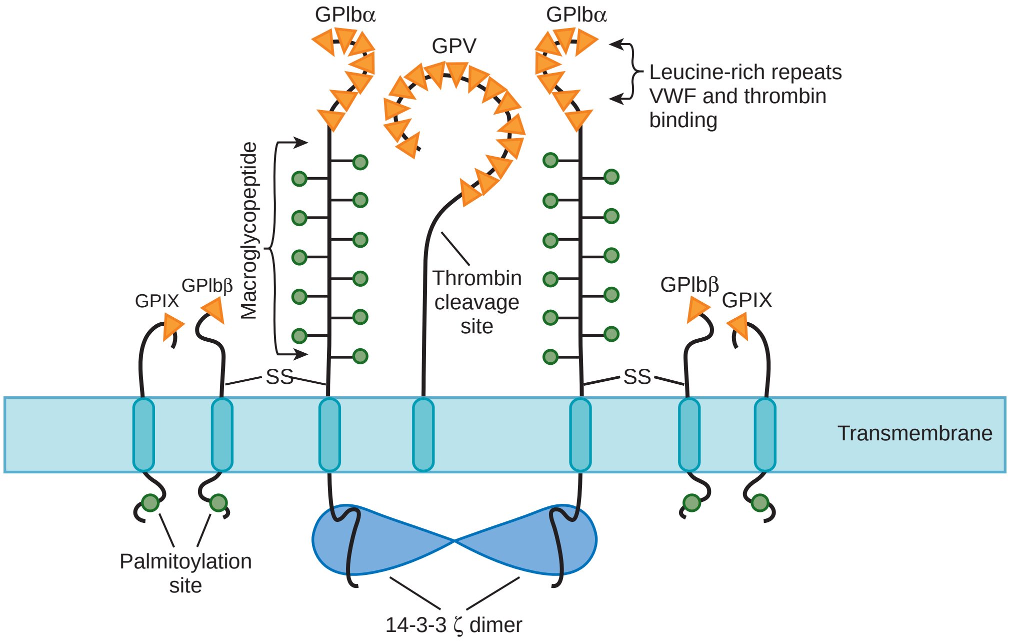

Figure 30-3 Structure of the glycoprotein Ib/IX (GPIb/IX) receptor. Each receptor consists of two GPIbα chains, each disulfide linked to a GPIbβ chain. There are two GPIX chains and one GPV chain in each complex as well. The leucine-rich repeats on all the chains are indicated, as are the sites for binding of von Willebrand factor (VWF) and thrombin. Known intracellular interactions or modification sites are also indicated. *SS*, cysteine disulfide bond.

(PLC) and mobilization of protein kinase C, which, together with increases in $[Ca^{2+}]_i$, promotes platelet secretion and potentiates platelet aggregation. The GPIb-IX complex also appears to activate the cell by binding to the cytoskeleton protein 14-3-3ζ and by interacting with the FcγRIIA receptor on platelets, where it activates an intracellular tyrosine–based activation motif receptor (see Fig. 30-3).[43]

Clinical Features

The bleeding manifestations of patients with BSS are similar to those of other patients with severe platelet dysfunction and center on mucocutaneous bleeding with purpura, epistaxis, gastrointestinal hemorrhage, and menorrhagia.[44] Alloantibodies to components of the GPIb/IX complex can develop after platelet transfusions with resulting platelet refractoriness.[44] Care of bleeding in these patients is similar to that of patients with GT discussed earlier, including the use of DDAVP.[45] Studies evaluating DDAVP have demonstrated improved bleeding times, although the improvement did not correlate with the ability of DDAVP to increase the level of circulating VWF. Recombinant activated factor VII has also been reported to be useful in patients with BSS,[46] but data are still very limited and it is probably most effective in combination with platelet transfusion.

Classification and Laboratory Diagnosis

BSS patients have a variable degree of thrombocytopenia. Most patients are thrombocytopenic to some extent, but some patients may have platelet counts as low as 20,000/µL. However, care must be taken to avoid underestimation of platelet number due to the macrothrombocytopenia.[47] The platelets have a mean diameter ranging from 3 to 20 times normal (Figs. 30-4, *B* and 30-5, *A*). Other cell types appear normal. In general, the circulating total platelet mass in people is more precisely conserved than the platelet count is, and part of the decrease in platelet count in BSS may well reflect this compensatory mechanism. Megakaryocytes in this disorder appear normal in size and appearance by light microscopy. However, on electron microscopy, a striking feature in these cells is the variable and intermittent nature of the demarcation system, which is often vacuolar.[48] Absence of GPIbα results in an abnormal demarcation membrane in a mouse model of BSS.[49] Studies have shown that GPIb/IX contributes to platelet formation in that this complex lines up with the cytoskeleton during thrombopoiesis and plays a role in cell cycle regulation.[50]

Bleeding times and platelet functional analyzer–100 measurements are prolonged in these patients,[51] but the distinctive abnormality of BSS platelets is the failure of agglutination in the presence of ristocetin, an abnormality that cannot be corrected by the addition of normal plasma. Aggregation by other agonists such as ADP, collagen, and epinephrine is normal, although the response to low-dose thrombin may be delayed.[52] Flow cytometric analysis of platelet glycoproteins shows decreased surface expression of the GPIb/IX complex and may aid in the diagnosis.[53]

Molecular Abnormalities

Molecularly, there appear to be two forms of BSS. In the biallelic form, most often homozygous mutations result in severely reduced or absent GPIb/IX expression. Heterozygous family members are usually asymptomatic with normal platelet counts. For the biallelic form, more than 50 mutations of the *GP1BA, GPIBB,* and *GP9*

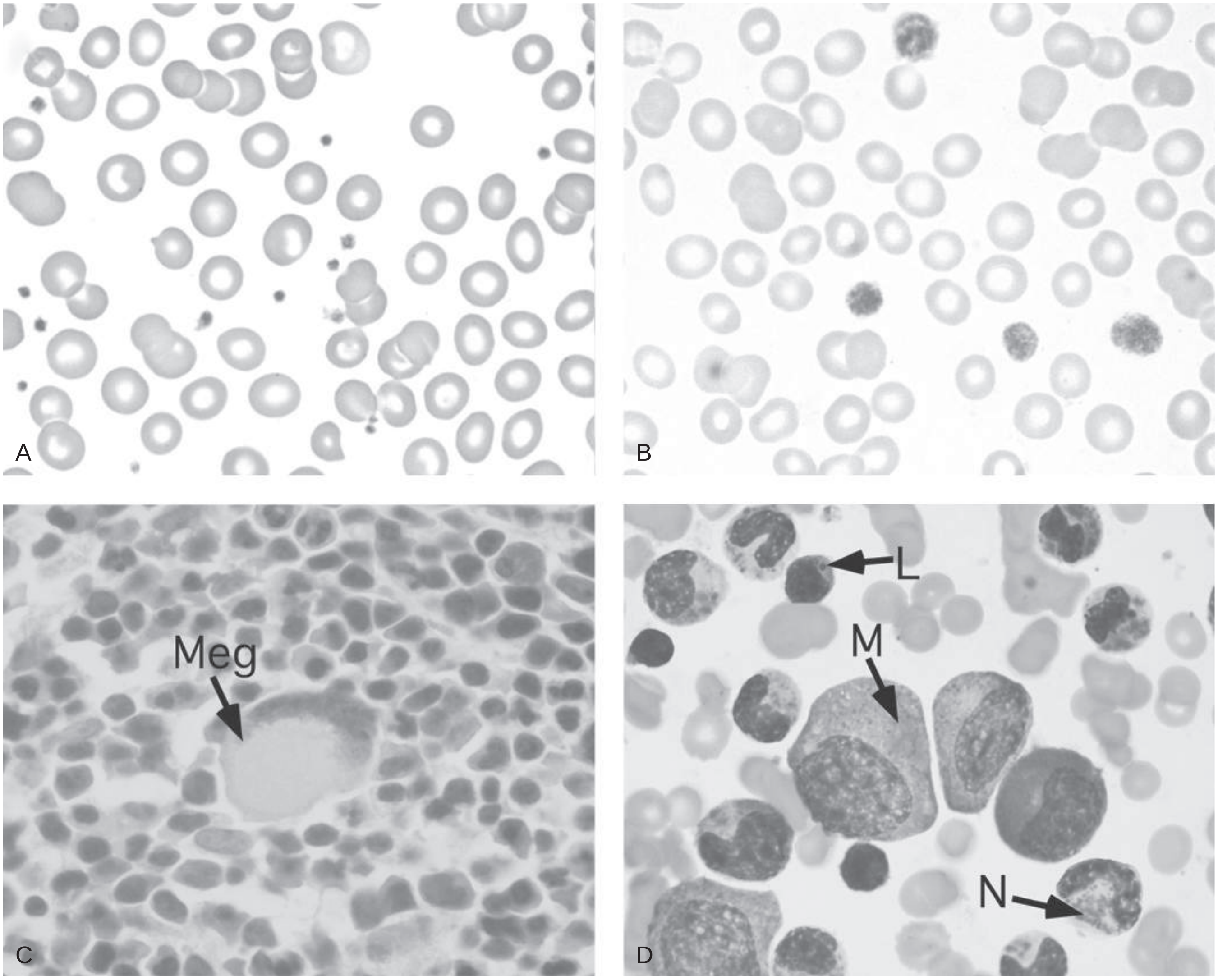

Figure 30-4 Light microscopy. **A**, Normal peripheral smear showing platelets of typical size (original magnification ×100). **B**, Peripheral smear of a patient with Bernard-Soulier syndrome showing a decreased number of enlarged platelets (original magnification ×100). **C**, Dysmorphic megakaryocyte (Meg) with the nuclei pushed to the side in a patient with a GATA1 mutation (original magnification ×50). **D**, Bone marrow in a patient with Chédiak-Higashi syndrome showing inclusion bodies in a myelocyte (M), neutrophil (N), and lymphocyte (L) (original magnification ×100). *(Courtesy of Marybeth Helfrich, The Children's Hospital of Philadelphia.)*

genes have been defined.[54] Few patients are compound heterozygotes, suggesting that this is a rare disease outside of specific populations. Most of the mutations described are missense or nonsense mutations. Deletions are uncommon but do occur, as in one patient who had partial deletion of the *GPIBB* gene on chromosome 22q11.2 in association with velocardiofacial syndrome.[55] In the monoallelic form, the platelet count is usually somewhat decreased and the bleeding tendency is often less dramatic. However, in some families,[54] there is significant thrombocytopenia and severe bleeding, in particular with the p.Ala156Val mutation in Italy, and three other missense mutations (p.Asn57His, p.Tyr70Asp, and p.Leu73Phe) in *GP1BA*. These mutations are associated with decreased expression of the GPIb/IX complex and macrothrombocytopenia. It is not clear why some mutations appear to exhibit a dominant negative effect so that heterozygous patients manifest disease whereas other heterozygous patients are asymptomatic. Indeed, patients with 22q deletion syndrome (22qDS, velocardiofacial syndrome, DiGeorge syndrome) often have deletion of one copy of *GP1BB* but may only manifest mild macrothrombocytopenia with decreased expression of the GPIb/IX complex.

Platelet-Type von Willebrand Disease (Pseudo–von Willibrand Disease)

Platelet-type von Willebrand disease (VWD), also known as *pseudo–von WIllibrand disease*, is an autosomal dominant bleeding disorder often associated with a prolonged bleeding time, mild thrombocytopenia, and decreased circulating levels of high-molecular-weight VWF multimers.[56,57] Patients with this disorder have a mild to moderate bleeding diathesis. Platelet-type VWD is very similar to type 2B VWD (see Chapter 31) in that both are characterized by platelet agglutination in the presence of low

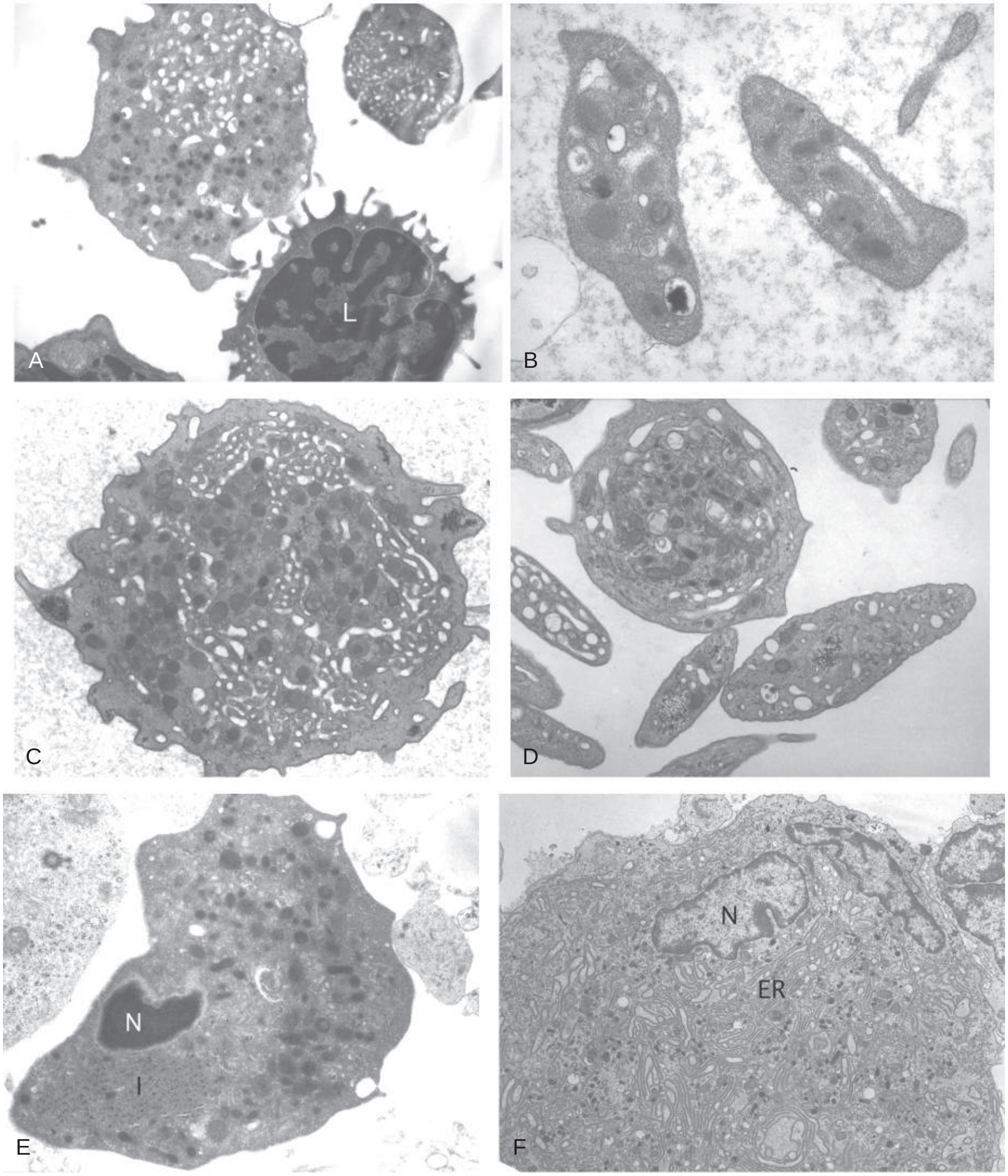

Figure 30-5 Electron microscopy. **A**, Platelet in a patient with Bernard-Soulier syndrome that is as large as a lymphocyte (L). Otherwise, platelet morphology is normal (original magnification ×18,000). **B**, Platelets in patients with Wiskott-Aldrich syndrome are half to two thirds the normal size but otherwise normal in appearance (original magnification ×38,500). **C**, Platelets in patients with Fechtner syndrome are enlarged and the canalicular system is exuberant, but otherwise the platelets look normal (original magnification ×13,000). **D**, Platelets in patients with gray platelet syndrome vary in granule content. Most of the platelets seen have an absence of alpha granules, whereas others (such as the large platelet here) may have identifiable granules (original magnification ×16,500). **E**, Neutrophil in a patient with myosin heavy–chain 9–related disorder showing an inclusion body *(I)* below the nucleus *(N)* forming longitudinally oriented filaments (original magnification ×14,000). **F**, Megakaryocyte from a patient with a GATA1 mutation showing the nucleus *(N)* pushed to the side by exuberant endoplasmic reticulum *(ER),* which fills most of the cytoplasm (original magnification ×38,000*). (All electron micrographs courtesy of Dr. James White, University of Minnesota.)*

concentrations of ristocetin and by decreased circulating levels of high-molecular-weight VWF multimers. Binding of VWF to circulating platelets results in the reduced levels of VWF seen in these patients, as well as some degree of platelet activation, mild or intermittent thrombocytopenia, and an increased risk of clotting.

Unlike type 2B VWD, where mutations in *VWF* result in increased affinity for a normal platelet GPIb/IX complex, pseudo-VWD is caused by an alteration in the platelet GPIb/IX complex that leads to increased affinity for normal VWF multimers. Platelet-type VWD can be distinguished from type 2B VWD by the addition of normal VWF to patient platelet-rich plasma, which results in spontaneous aggregation of pseudo-VWD platelets but not type 2B VWD platelets.[57] It is important to distinguish patients with platelet-type VWD from other VWD patients because care of patients with VWD often involves infusion of VWF that contains factor VIII concentrates or DDAVP. This therapy can worsen the thrombocytopenia in patients with platelet-type VWD.[57] A patient with mild thrombocytopenia and a prolonged bleeding time should be evaluated for this disorder.

The molecular basis of platelet-type VWD has been defined in multiple families.[57] Mutations, such as Gly233Val and Met239Val, occur in the region of *GP1BA* that has been previously shown to be important in VWF binding. It has been hypothesized that these mutations alter GPIbα such that it maintains an adhesive conformation much as wild-type GPIb/IX receptor becomes activated on exposure to components of the extracellular matrix at sites of vascular injury and flow.

Montreal platelet syndrome, characterized by macrothrombocytes, a prolonged bleeding time, spontaneous aggregation of platelets at pH 7.4, and a defect in platelet calpain metabolism, was thought to be a distinct macrothrombocytopenia, but in 2009 was shown to be a particular subtype of type 2B VWD, with a V1316M mutation in *VWF*.[58] This disorder demonstrates the overlap between the described phenotypes in these inherited platelet disorders and the value of molecular diagnosis.

Other Inherited Defects of Platelet Receptors

G Protein–Coupled Receptors

Many agonists that activate platelets, such as thrombin, ADP, and thromboxane A_2 (TxA_2), activate cells through a G protein–coupled receptor (GPCR) (see Table 30-1). These seven-transmembrane receptors are linked to a heterotriplex of intracellular guanosine diphosphate (GDP)/guanosine triphosphate (GTP)–binding proteins, Gα, β, and γ. The strongest platelet agonist is thrombin. As mentioned previously, one receptor for thrombin is the GPIb/IX complex. However, thrombin appears to have its greatest effect on human platelets through two GPCRs, protease-activated receptor 1 (PAR-1) and PAR-4, with PAR-1 being the major human receptor.[59] Thrombin binds to PAR-1, releases an N-terminal peptide, and leaves a new N-terminus. An oligopeptide of this N-terminal sequence, SFLLRN, can mimic the effects of thrombin and activate platelets. Surprisingly, no patients with defects in PAR-1 have been described. In mice, PAR-1 is not important for platelet biology, although its absence does have consequences because it is expressed on other tissues.[60] Instead, on murine platelets, PAR-3 and PAR-4 are biologically relevant.[61]

ADP is stored in platelet-dense granules and released upon platelet activation. When added to platelets in vitro, ADP causes TxA_2 formation; protein phosphorylation; and increase in cytosolic Ca^{2+} shape change, aggregation, and secretion. ADP also inhibits the formation of cyclic adenosine monophosphate (cAMP). Several patients with bleeding defects have been described whose platelets showed greatly diminished responsiveness to ADP and reduced numbers of binding sites for ADP analogues,[62,63] a phenotype not dissimilar from that produced by ADP antagonists such as ticlopidine or clopidogrel. ADP receptors on human platelets include P2Y1, P2Y12, and P2X1.[64] P2Y1 is a GPCR that activates PLC. P2Y12 inhibits cAMP formation by adenyl cyclase. P2X1 is a ligand-gated nonspecific cation channel that may contribute to the influx of extracellular Ca^{2+} that occurs in response to ADP. Mutations in each of these receptors have been reported in patients and have resulted in mild to moderate bleeding symptoms.[62]

In several respects, epinephrine is unique among human platelet agonists in that it causes aggregation and secretion but does not cause the cytoskeletal reorganization that underlies shape change. Activation of PLC by epinephrine appears to be dependent on TxA_2 formation and can be suppressed with aspirin. Aspirin also blocks second-wave, or secretion-dependent, platelet aggregation in response to epinephrine. This gives rise to a characteristic aggregometer tracing in which epinephrine-induced primary aggregation is followed by disaggregation of the platelet clumps. Platelet responses to epinephrine are mediated by α_2-adrenergic receptors. There are reports of families in which a mild bleeding disorder was associated with impaired epinephrine-induced aggregation and reduced numbers of α_2-adrenergic receptors.[65] However, platelets from up to 10% of apparently normal people may fail to aggregate when challenged with epinephrine, and the clinical significance of this finding is unclear.

TxA_2 is produced from arachidonate in platelets by the aspirin-sensitive cyclooxygenase pathway. When the stable thromboxane analogue U46619 is added to platelets in vitro, the platelets undergo shape change, aggregation, secretion, phosphoinositide hydrolysis, protein phosphorylation, and an increase in $[Ca^{2+}]_i$. Similar responses are seen when platelets are incubated with exogenous arachidonate. Platelets express a GPCR for TxA_2.[66] Platelets also contain a receptor for prostacyclin (PGI_2), an endothelial cell prostaglandin that is a potent platelet inhibitor. Binding of PGI_2 to this receptor stimulates adenylate cyclase activity and inhibits platelet function.[67] A few patients have been described who have a mild bleeding disorder caused by congenital absence of the TxA_2 receptor. Two Japanese patients have had their mutations defined and were found to have an R60L substitution in the first cytoplasmic loop of *TXA2R*.[68] In addition, three patients have been described who have normal TxA_2 binding but absent platelet aggregation in the presence of this agonist, thus suggesting uncoupling between the receptor and downstream signaling events.[69] There are as yet no known clinical disorders ascribed to the PGI_2 receptor, but deletion of the gene in mice increases their risk for thrombosis after arterial injury[70] and polymorphisms in the PGI_2 gene (*PTGIS*) have been reported to be risk factors for cerebrovascular disease.[71]

Immunoglobulin Superfamily Receptors

GPVI, a 62-kD protein encoded by a gene (*GP6*) on human chromosome 19q13.4,[72] is a member of the immunoglobulin superfamily of receptors and is the platelet-signaling receptor for collagen. Unlike $\alpha_2\beta_1$, GPVI's interaction with collagen initiates intracellular signaling via its surface partner, the FcRγ chain. Signaling occurs via phosphorylation of PLCγ2 and Syk.[73] Fab antibodies derived from GPVI-deficient individuals who had received platelet transfusions recognize GPVI and are capable of blocking activation of platelets by collagen, thereby resulting in thrombocytopenia and internalization and/or cleavage of surface GPVI.[74] Convulxin, a snake venom protein isolated from a South American rattlesnake, is also a potent activator of platelets via GPVI.[75]

GPVI seems to be the major collagen receptor on platelets,[73] although a few patients have been described who lack GPVI and show only a mild bleeding tendency.[76] A mutation in the *GP6* gene has been linked to absence of collagen-induced platelet activation.[77] These patients have some residual platelet aggregation to collagen, which is likely to be through the $\alpha_2\beta_1$ receptor. In addition, autoantibodies to GPVI have been described in patients with immune thrombocytopenic purpura[74] and systemic lupus erythematosus.[78] These patients lack surface GPVI on their platelets and show a mild bleeding diathesis with abnormal platelet aggregation to collagen. Interestingly, several genetic polymorphisms affecting GPVI surface expression have been described and suggested to be a risk factor for myocardial infarction[79] and may be associated with increased risk of pregnancy loss.[80] Platelets with less GPVI showed less vigorous aggregation in vitro, although further study is ongoing on the true relevance of these findings.[81]

Scott Syndrome

The platelet surface functions as one of the principal sites for plasma coagulation reactions by providing a surface on which coagulation factor complexes assemble and accelerate these important reactions several thousandfold. This property of platelet membranes has been termed *platelet factor 3 activity*. In a resting platelet, there is little anionic phospholipid on the platelet's surface. Activation by agonists such as thrombin or collagen is thought to reorient membrane phospholipids and bring anionic phospholipids, chiefly phosphatidylserine, to the outer leaflet. Activation also induces membrane vesiculation and the production of platelet microparticles with procoagulant activity.[82]

Deficiency in platelet coagulant activity is a rare clinical event that has been termed Scott syndrome, named after the propositus of a well-studied family.[83] The patient had prolonged bleeding after dental extractions, bleeding after surgical procedures, and a spontaneous retroperitoneal hematoma. The patient secreted a normal quantity of factor V, a platelet alpha-granule constituent, but had only 25% of the normal number of binding sites for factor Xa on activated platelets. When her platelets were challenged with such agonists as thrombin and collagen, aggregation and secretion were normal but fewer anionic phospholipid-binding sites were translocated to the platelet surface. In addition, the formation of microparticles is defective in patients with Scott syndrome. Standard screening tests for plasma coagulation, such as the prothrombin time and partial thromboplastin time, which use artificial lipid micelles, are normal. Recently, a splice-acceptor site mutation in the gene encoding transmembrane protein 16F (*ANO6*) was reported in the original proband, causing premature termination of a Ca^{2+}-activated chloride channel. Transfection studies provided evidence for the essential role of transmembrane protein 16F in Ca^{2+}-dependent exposure of phosphatidylserine at the platelet surface.[84]

The diagnostic abnormality is a shortened serum prothrombin time as a result of reduced consumption of prothrombin. Additionally, these patients have a prolonged dilute Russell viper venom time that corrects when the patient's plasma is added to normal platelets but not when normal plasma is added to the patient's blood.[85] This is due to the inability to generate normal platelet procoagulant activity. Patients can be readily treated with platelet transfusions, which improve hemostasis and reduce postoperative blood loss.

Defects in Signal Transduction

With multiple overlapping pathways of platelet activation, it has been difficult to define specific defects in patients with inherited disorders of platelet signal transduction. Nonetheless, a small number of patients have been described with phenotypes and abnormal platelet aggregation patterns consistent with such a diagnosis[86]; the number of these patients will likely increase as the diagnostic armamentarium improves because defects in these pathways may be quite common.

Abnormalities of the G proteins (Giα1 and Gαq) have been described in patients with defects in platelet aggregation to ADP and to multiple agonists including ADP, thrombin, and TxA_2, respectively. These patients had lifelong mucocutaneous bleeding associated with these platelet defects.[87] Both of these proteins play an important role in outside-in signaling. Mutations in Gsα (linked to receptors for such agents as PGI_2) have been associated with hypofunction and hyperfunction syndromes. Mutations in prostaglandin E_1 and pituitary adenyl cyclase–activating peptide (PACAP) lead to increased cAMP production, and patients with partial trisomy of 18p have increased PACAP, very increased cAMP, and severe neurologic abnormalities with a pronounced bleeding tendency and mild thrombocytopenia.[88] These patients (signaling defect patients) may be found to have abnormal clot retraction or abnormal spreading of platelets on surface-bound fibrinogen.

Another group of patients lack the ability to liberate arachidonic acid,[89] which may be due to deficiency of phospholipase A_2. Other patients have diminished cyclooxygenase enzyme activity that mimics an aspirin-like defect.[89,90] These patients have abnormal platelet aggregation in response to ADP, epinephrine, collagen, and arachidonic acid but respond normally to prostaglandin G_2, consistent with a defect in cyclooxygenase-1 and subsequent TxA_2 synthesis. Most patients with this disorder have normal antigen levels of a functionally abnormal prostaglandin synthetase. Other patients have been described who have decreased protein expression. Additionally, patients have been reported who have mutations in the gene coding for thromboxane synthetase that converts prostaglandin H_2 to TxA_2.[91]

Finally, pleiotropic aggregation abnormalities due to multiple pathway defects have been associated with mutations in transcription factors. Decreased platelet levels of protein kinase C and PLC-β2 have been found in patients with *RUNX1* mutations (see section on FPD-AML, below), resulting in defects in aggregation and secretion as well as plekstrin phosphorylation in response to thrombin.[91,92] In patients with *GATA1* mutations (see below), abnormal alpha-granule biogenesis, decreased collagen-induced platelet aggregation, and abnormal GPVI-dependent tyrosine phosphorylation have been reported.[93] Over the past few years, other models of platelet dysfunction secondary to a signaling defect have been recognized, including Wiskott-Aldrich syndrome (WAS), which is described later under disorders of platelet size and number. For all these signaling disorders, platelet transfusions are effective in the management of bleeding.

Platelet Storage Granule Defects

Human platelets contain several types of intracellular granules that can be distinguished by electron microscopy, including dense granules, alpha-granules, and lysosomes. Dense granules are the most rapidly secreted granules after platelet activation and contain ADP, adenosine triphosphate (ATP), serotonin, and calcium.[94] Two thirds of platelet adenine nucleotides are stored in these granules, with an ADP/ATP ratio of 3 : 2, as opposed to 1 : 8 in the platelet cytoplasm. Platelet alpha granules contribute both prothrombotic and antithrombotic proteins as well as proangiogenic and antiangiogeneic proteins.[95] After platelet activation, the alpha granules centralize in the platelet and release their contents via membrane fusion with the open canalicular system.[95] Not all proteins present in alpha granules have the same origin. Some, such as VWF, thrombospondin, and platelet factor 4 (PF4), are synthesized in and packaged by megakaryocytes,[96] whereas others, such as albumin, immunoglobulins, and fibrinogen, are endocytosed from plasma.[97]

Dense Granule Defects

Patients with defects in platelet dense granules have a form of storage pool deficiency (δ-SPD).[98] Patients with δ-SPD exhibit a mild to moderate bleeding diathesis, as well as abnormalities in platelet aggregation. The second wave of aggregation is absent in response to ADP and epinephrine and virtually absent in response to collagen. Bleeding times in these patients are often prolonged. In 1969 it was shown that these patients have a deficiency in total platelet ADP, and this deficit was subsequently localized to the ADP pool of the dense granule. The abnormalities in the ultrastructure of the dense granules are heterogeneous, but most patients show a characteristic lack of dense granules by electron microscopy (see Fig. 30-5). One study has suggested a deficiency of multidrug-resistant protein 4 may be important in two patients with δ-SPD, although the molecular basis of this defect has yet to be resolved.[99]

The two most common SPDs are *Hermansky-Pudlak syndrome* (HPS: MIM 203300)[98] and *Chédiak-Higashi syndrome* (CHS: MIM 214500).[100,101] HPS is characterized by severe tyrosinase-positive, oculocutaneous albinism in association with photophobia, rotatory nystagmus, loss of visual acuity, excessive accumulation of ceroid-like material in reticuloendothelial cells, and a mild to moderate hemorrhagic diathesis. This disease is inherited as an autosomal recessive disorder, and although it occurs in many populations, it is seen with high frequency in people from the northwest part of Puerto Rico.[102] Patients exhibit bruising and epistaxis in early childhood. Fatal bleeding is rare, but a significant proportion of patients will have received packed red cell or platelet transfusions in their lifetime. The major cause of death in some genotypes is progressive pulmonary fibrosis, which generally begins in the late 20s or early 30s and may be related to higher plasma serotonin levels but also clearly involves defects in the pulmonary alveolar epithelium.[103] This disease is also associated with a 15% incidence of granulomatous colitis. To date, mutations in nine different genes (*HPS-1* to *HPS-9*) have been described in patients with phenotypic HPS, all of which encode for proteins, which are involved in the biogenesis of lysosome-related organelles such as melanosomes in melanocytes and delta granules in platelets. All known HPS proteins are components of one of four protein complexes: biogenesis of lysosome-related organelles complex 1 (BLOC-1), BLOC-2, BLOC-3, or adaptor protein complex 3 (AP-3). Some genotype-phenotype associations have also been documented.[104] For example, pulmonary fibrosis develops in patients with mutations in *HPS-1* and *HPS-4*, whereas this life-threatening complication does not develop in patients with *HPS-3*, *HSP-5*, and *HSP-6* mutations. In addition, patients with *HPS-2* mutations have associated neutropenia and a predilection for infections, particularly in childhood.[105]

CHS (MIM 214500) is a rare autosomal disorder that has been described in approximately 200 affected individuals.[105] It is characterized by immune dysfunction, platelet storage pool defects, and partial albinism. Patients often have a white forelock or ashen hair secondary to abnormally large melanosomes. Hematologic manifestations of this disease are characterized by the presence of large intracytoplasmic granules in leukocytes, lymphocytes, monocytes, and platelets (see Fig. 30-4, *D*), with a concurrent defect in mobilization of the marrow leukocyte pool, defective chemotaxis, and decreased bactericidal activity. Many other tissues also contain abnormal granules.[106] CHS patients often die in the first 2 decades of life of either overwhelming infections or an unusual lymphoproliferative disorder called the *accelerated phase*, which involves fever, anemia, neutropenia, and, occasionally, thrombocytopenia, hepatosplenomegaly, lymphadenopathy, and jaundice. The latter disorder occurs in 85% of patients.[105] Patients who survive to adulthood have progressive neurologic dysfunction.[105] These patients are particularly susceptible to Epstein-Barr virus infections.[107] The gene involved in CHS *(LYST)* is located on chromosome 1q42.1-42.2 and encodes a 3801–amino acid protein called LYST (lysosomal trafficking regulator).[108] This complex protein is a member of the BEACH protein family and contains several ARM/HEAT domains and a BEACH motif.[109] Mutations have been defined in an increasing number of affected patients and involve truncated products, although there is no correlation between residual protein length and clinical phenotype.[110]

Finally, *Griscelli syndrome* (MIM 214450) is a rare disease in which patients have partial albinism, silver hair, and neurologic defects and/or lymphohistocytosis with associated delta-granule deficiency. Major bleeding is rare in this syndrome. Patients have mutations in myosin Va, Rab27a, or melanophilin. One patient with a mutation in *RAB27A* and *AP3B1* showed a bleeding phenotype caused by impaired secretion, dependent platelet aggregation, and abnormal delta granules.[105]

Whole-mount electron microscopy is often used for the diagnosis of these disorders but is complicated by the fact that dense granule numbers may be low in normal platelets.[111] Some laboratories use serotonin release assays and assays examining ATP : ADP ratios to aid in the diagnosis of these disorders.[112] Some studies suggest that flow cytometry on mepacrine-loaded platelets may aid in the diagnosis because mepacrine is selectively taken up by dense granules.[113]

Alpha-Granule Defects

Patients with alpha-granule deficiencies have a bleeding phenotype similar to that of patients with dense-granule deficiencies, with mild to moderate bleeding, mild thrombocytopenia, and a prolonged bleeding time. Because of the initial light microscopic observation of gray platelets in the peripheral blood smear of these patients after Romanovsky staining, this disorder was termed *gray platelet syndrome* (GPS: MIM 139090).[114] On electron microscopy, the gray platelets are most notable for the absence or marked reduction of alpha granules (see Fig. 30-5, *D*). These platelets are also deficient in alpha granule–specific proteins such as PF4, VWF, factor V, and fibronectin.[115] Gray platelet aggregation to various agonists, especially thrombin, is impaired. These platelets have a delayed and blunted Ca^{2+}-mobilization response. The vacuolar structures seen in gray platelets appear to be aborted alpha granules that contain both P-selectin and $\alpha_{IIb}\beta_3$ in their membranes. They also contain a significant amount of proteins, including immunoglobulin and albumin, which are normally endocytosed into alpha granules. Endogenously synthesized alpha granule–specific proteins such as PF4 leak out. This latter phenomenon may contribute to the observed myelofibrosis and pulmonary fibrosis associated with this disease[116] and may also contribute to thrombocytopenia by negative feedback on megakaryopoiesis.[117,118] In 2011, several groups using various approaches simultaneously reported that mutations in *NBEAL2* are responsible for GPS; the approaches included linkage analysis followed by DNA sequencing, [119] whole-exome sequencing,[120] and RNA-sequencing[121] technologies. NBEAL2 belongs to a family of proteins containing BEACH domains; the proteins are highly conserved and important for protein-protein interactions, membrane dynamics, and vesicle trafficking. These diagnostic studies demonstrate the power of these technologies to identify molecular causes of inherited disorders of platelets in rare diseases and offer the possibility that in the future, underlying defects in such affected individuals will be resolved; this would not only enhance the understanding of platelet biology but would provide knowledge about an underlying platelet defect for many individuals.

In *arthrogryposis, renal dysfunction, cholestasis syndrome* (ARC: MIM 208085, 608552, 613401), patients also have absent alpha granules as a result of a defect in granule biosynthesis due to mutations in *VPS33B* or *VPS16B* (VIPAR), a VPS33B binding protein on chromosome 14 (C14orf133).[122] VPS33B is a vesicle trafficking protein, a member of the Sec1/Munc18 family of proteins, and is predicted to function in vesicle docking and fusion during alpha-granule development. Patients present with neonatal cholestatic jaundice, renal tubular acidosis, and arthrogryposis with severe failure to thrive.[123]

Other Granular Defects

A small number of patients have been described with *combined alpha-granule and dense-granule deficiency*.[124] In these patients, the dense-granule deficiency is often much more severe than the alpha-granule deficiency. Patients also have mild to moderate bleeding histories, and laboratory results are similar to those of dense-granule–deficient patients, with a decreased platelet ADP\:/ATP ratio in the cells and low levels of serotonin. In one patient with a severe deficiency in both alpha granules and dense granules, there was a deficiency in total platelet P-selectin; this finding suggests that in this disorder, unlike in GPS, in which there may be an alpha-granule–targeting defect, platelet granule formation itself may be defective.[125]

Quebec platelet syndrome is a rare disorder that results from the degradation of alpha-granule contents within the platelet because of ectopic urokinase-type plasminogen activator expression in developing megakaryocytes and its storage in alpha granules.[126] This disorder, inherited in an autosomal dominant pattern within two large kindreds in Quebec, was previously known as *factor V Quebec syndrome* because the initial observations in these patients revealed a relative intraplatelet deficiency of factor V.[127] These patients manifest moderate to severe delayed bleeding after surgery or trauma.[128] Bleeding can be controlled by antifibrinolytic therapy with tranexamic acid, but platelet transfusions are generally ineffective. The molecular abnormality responsible for this disorder was recently found to be a direct tandem duplication of a 78-kb genomic fragment that includes *PLAU* (the gene encoding urokinase-type plasminogen activator) present in Quebec platelet syndrome patients.[129]

QUANTITATIVE DISORDERS

Megakaryocytes are large, polyploid, terminally differentiated hematopoietic cells that release platelets into the bloodstream.[130] These cells arise from self-regenerating pluripotent stem cells that also give rise to all other hematopoietic lineages. Megakaryocyte lineage commitment involves a number of precursor stages, including CFU-GEMM (colony-forming unit–granulocyte, erythrocyte, monocyte, megakaryocyte), which in culture results in a mix of granulocytes, erythrocytes, monocytes, and megakaryocytes; CFU-EMM, which gives rise to mixed colonies of erythrocytes, monocytes, and megakaryocytes; BFU-Meg (burst-forming unit–megakaryocyte), which gives rise to a burst of 10 to 20 megakaryocytes; and CFU-Meg, which gives rise to a smaller megakaryocyte colony.[131] Megakaryocyte-erythroid progenitor (M cells are proliferating cells that express CD34 and CD38low that terminally commit to the erythroid or megakaryocytic lineage.[132]

Various growth factors regulate megakaryocyte differentiation at different points of megakaryocyte development. Certain cytokines stimulate proliferation of megakaryocytic progenitors, such as interleukin-3 (IL-3), IL-6, IL-11, IL-12, granulocyte-macrophage colony-stimulating factor, and erythropoietin.[130] Additionally, PF4 may be an important negative paracrine in megakaryocytes that is especially important in regulating platelet production after injury to megakaryocytes.[118] However, all the aforementioned growth factors and cytokines have very broad effects on all hematopoietic cell lines. The discovery and cloning of thrombopoietin (TPO), the ligand for the myeloproliferative ligand receptor (Mpl), opened a new perspective in megakaryocyte differentiation study. TPO was found to be a specific factor controlling megakaryocytic cell proliferation and maturation and platelet reactivity.[133] This 353–amino acid protein is homologous at its N-terminal with erythropoietin. Either the full-length protein, a truncated N-terminal TPO, or even mimetic peptides can dimerize Mpl and stimulate megakaryocytopoiesis.[134] On immature progenitor cells, activation of Mpl stimulates proliferation, much like IL-11 or c-kit ligand. On megakaryocyte precursors and megakaryocytes it leads to increased megakaryocyte differentiation and platelet formation. Targeted disruption in murine models of either the *Mpl* or *TPO* gene results in a decrease in platelet count to approximately 20% of normal.[135,136] Thus this cytokine pathway appears to be permissive for megakaryocytopoiesis but is not absolutely required for this differentiation.

MEPs differentiate into megakaryoblasts, small lymphocyte-like cells that undergo nuclear endoduplication and cytoplasmic enlargement and accumulate a body of specific proteins to form the mature megakaryocyte with a cell volume of approximately 10^4 to 10^5 μm^3. Each of these huge cells eventually results in the formation of approximately 10^3 to 10^4 platelets. Direct visualization of thrombopoiesis in the calvarium of mice suggests that mature megakaryocytes release platelets, large cytoplasmic fragments, and whole megakaryocytes at the endothelial cell border.[137] The circulating larger fragments may subsequently release platelets within the pulmonary vascular bed.[138,139]

The mechanistic basis of thrombopoiesis is beginning to be understood, but it does not appear to involve the extensive membrane system termed the *demarcation membrane system* that may represent inverted membranes. Rather, extensive pseudopods form and re-form in a dynamic process that eventually leads to a beaded appearance that fragments to form particles with the size and ultrastructural appearance of platelets.[140] This process can be inhibited by agents that disrupt microtubules and promoted by actin filament–depolymerizing agents, thus suggesting involvement of the cytoskeleton.[141]

The normal range of platelet counts is approximately 150 to 400 × 10^3/μL. Even more constant than the circulating platelet count is the circulating thrombocrit, which is the platelet count multiplied by the mean platelet volume, so there is an inverse relationship between platelet count and platelet size.[142] Regulation of megakaryocytopoiesis by TPO is in large part regulated by the platelet mass. TPO produced by the liver is secreted at a relatively constant level. As platelet mass rises, the amount of bound TPO increases and less is available for stimulation of marrow megakaryocytes. The inverse situation would also be true: As platelet mass falls, there is more free TPO to stimulate marrow megakaryocyte development.

The molecular basis for the regulated expression of genes during megakaryocytopoiesis is only beginning to be understood. This understanding has grown with the development of targeted disruption of hematopoietic genes in mice, which has demonstrated the importance of the transcription factors GATA1, NF-E2, and FOG-1 in megakaryocyte development.[143] Both GATA1 and FOG-1 knockdowns and NF-E2 knockouts result in viable mice with large and dysmorphic megakaryocytes and very low platelet counts.[144,145]

Disorders of Platelet Production

Disorders involving platelet production include congenital amegakaryocytic thrombocytopenia (CAMT), characterized by a complete absence of megakaryocytes; thrombocytopenia with absent radii (TAR), in which there is a decrease in megakaryocytes in infancy; amegakaryocytic thrombocytopenia with radioulnar synostosis (ATRUS), characterized by skeletal anomalies and thrombocytopenia that improves with time; essential thrombocythemia (ET), resulting in increased platelet production; and disorders of hematopoietic transcription factors, often associated with an excess of dysmorphic megakaryocytes and platelets.

Disorders of the Thrombopoietin-Mpl Axis

Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare disorder associated with very low platelet counts, absence or near absence of megakaryocytes in bone marrow, and risk for the development of bone marrow failure. These children have no skeletal abnormalities, and the platelet count does not improve with age and often necessitates bone marrow transplantation because of the severe bleeding and because of the risk of development of bone marrow failure. CAMT results from mutations in the receptor for TPO (*MPL*).[146] These patients demonstrate the importance of TPO signaling in normal hematopoiesis in that patients who survive long enough exhibit pancytopenia, usually during the first decade of life.[147] In addition, no *TPO* mutations have been found in this disorder or in other disorders associated with thrombocytopenia, including TAR (see later discussion).

In contrast, excessively high platelet counts (>600 × $10^3/\mu L$) are seen in *essential thrombocythemia (ET),* an acquired myelodysplastic disorder seen primarily in adults and associated with an acquired *JAK2* mutation (V617F) in 50% to 65% of cases. Other mutations in the TPO-Mpl signaling axis have also been reported.[148] A rare congenital form of familial thrombocytosis occurs in children that can be associated with enhancing mutations in the TPO promoter[149] or activating mutations in Mpl.[150,151] Unlike the ET associated with the JAK2 V617F mutation, myelodysplastic conversion in the congenital form is rare,[152] so it is unclear whether therapeutic intervention (suppression of the platelet count with hydroxyurea or anagrelide) is necessary, although antithrombotic strategies are often still used in the management of these children.

Thrombocytopenia and Orthopedic Abnormalities

Thrombocytopenia with absent radii (TAR) is one of the more common inherited disorders of platelet number and was first described in 1951.[153] This disorder involves hypomegakaryocytic thrombocytopenia and bilateral absence of the radii. Patients are born with platelet counts in the approximate $10^4/\mu L$ range; the count improves slowly and generally reaches nearly normal levels after the first year of life. During infancy, environmental stress, such as viral illness, can precipitate episodes of severe thrombocytopenia. Several TAR patients have had an associated storage pool defect and bleeding out of proportion to their platelet count. In addition, these patients have other hematologic changes, including leukemoid reactions in the range of approximately 30,000/μL and anemia, partly as a result of blood loss. About 30% of patients have recurrent gastroenteritis and bleeding. The major skeletal manifestation is complete absence of the radii but with functional thumbs. Other orthopedic abnormalities are seen in about 50% of cases, and the more severe the upper limb deformity, the more likely the patient is to have a lower limb deformity. Approximately 30% of patients have cardiac anomalies, including tetralogy of Fallot and atrial septal defects or neural defects.[154]

Megakaryocytes are present in the bone marrow, although there may be some decrease in comparison to what one would see in thrombocytopenia secondary to increased peripheral destruction. Serum levels of TPO in patients with TAR are elevated despite normal Mpl levels on hematopoietic cells. Bone marrow cultures from these patients reveal reduced numbers of megakaryocytes, and CFU-Meg do not grow in response to TPO.[155] Together, these findings suggest a disconnection between the TPO receptor and downstream signaling events. Inherited or de novo deletion of chromosome 1q21.1 is found in the majority of affected individuals but is also seen in unaffected family members.[156] Recently, whole-exome sequencing identified a second mutation as an SNP in the first intron or the 5′ UTR for the *RBM8A* gene.[157] Subsequent analysis of other TAR syndrome patients demonstrated that reduced expression of Y14 (the protein encoded by *RBM8A*), involving a combination of deletional and hypomorphic versions of *RBM8A*, is responsible for TAR syndrome.[157] Western blots showed a significant reduction in Y14, which is one of the four components of exon junction complexes. Exon junction complexes are involved in basic cellular functions such as nuclear export and subcellular localization of specific transcripts, translational enhancement, and nonsense-mediated RNA decay.[158]

Amegakaryocytic thrombocytopenia with radioulnar synostosis (ATRUS) is autosomal dominant and characterized by amegakaryocytic thrombocytopenia, skeletal anomalies (including radioulnar synostosis, hip dysplasia, and clinodactyly or syndactyly), and sensorineural hearing loss.[159] The thrombocytopenia in these patients occurs in the neonatal period and may be severe, but often resolves in the first few years of life.[160] Mutations of *HOXA11* have been demonstrated as the molecular abnormality in most patients with ATRUS.[161] HOXA11 is a member of the homeobox group of regulatory proteins that play a role in hematopoietic proliferation and differentiation. Studies in animal models have shown the role of HOXA11 in forearm skeletal development. However, although bone marrow from HOXA11-overexpressing mice shows increased immature megakaryocyte colonies,[162] a clear role in megakaryocytopoiesis has not been defined.

Platelet Disorders Secondary to Transcription Factor Defects or Unknown Biology

X-linked anemia with severe thrombocytopenia (MIM 300367) secondary to mutations in *GATA1* has been reported.[163] There have been mutations described in different kindreds resulting in at least two distinct phenotypes with thrombocytopenia: *XLT (X-linked thrombocytopenia)* and *XLTT (X-linked thrombocytopenia with thalassemia)*. The major differentiation between the two types is the presence of dyserythropoiesis (in XLT) versus a thalassemia-like hemolytic anemia with splenomegaly and abnormal beta chain production (XLTT). The differences in phenotype may be related to whether the mutated GATA1 retains its ability to bind to FOG-1 (R216Q: XLTT) or whether the GATA1 interaction with FOG-1 is disrupted (XLT: V205M, G208R, G208S, D218G). The megakaryocytes in the marrow of these patients are similar to those described in GATA1–knockdown mice; they are numerous and large, with poorly organized granular content and nuclei pushed off to the side (see Figs. 30-4, *C* and 30-5, *F*). Some of these patients have a macrothrombocytopenia and have been described as having a form of GPS.[164] Patients present with significant bleeding early in life (especially XLT patients), with varying degrees of anemia.[163]

Familial platelet disorder/acute myelogenous leukemia (FPD/AML: 601399) is a dominantly inherited thrombocytopenia with platelet counts around 100,000/μL that is often associated with a bleeding diathesis out of proportion to the decreased platelet count. Platelet studies in some families show a classic and profound storage pool defect, but the precise defect can vary between families. There is about a 50% lifetime risk of AML developing in any affected family member, as well as an increased risk for other cancers.[165] Studies have shown that in many of these extended families, the defect resides in the *RUNX1* (previously *CBFA2* or *AML1*) gene (chromosome 21q22.1-22.2), resulting in defective RUNX1.[166] RUNX1 is a nuclear transcription factor involved in early hematopoietic differentiation from endothelial cells and is important for myeloid differentiation. Translocations involving RUNX1 result in AML or related cancers. RUNX1 is also expressed and important during megakaryopoiesis. The majority of FPD/AML mutations described are within the DNA-binding Runt domain of RUNX1 and affect heterodimerization of RUNX1 with PEBP2β/CBFβ. Haploinsufficiency of RUNX1 has been shown to affect DNA binding, heterodimerization, nuclear localization, and transactivation functions. Recently, *RUNX1* mutations have also been shown to dysregulate myosin II in megakaryopoiesis.[167] Additionally, several patients have now been described with deletions involving the *RUNX1* gene with various phenotypes that depend to some degree on the size of the deletion.[168]

Jacobsen syndrome (MIM 147791) involves a deletion of the terminal portion of chromosome 11q (23-ter), a region that includes the *ETS1* and *FLI1* genes. Ets1 and Fli1 are related zinc finger nucleases critical for megakaryopoiesis. *FLI1* is within the minimal critical region required for manifestation of the disease, and its overexpression can drive MEP cells into megakaryocyte differentiation. Over 200 cases have been reported; most cases arise from de novo deletions, which are variable in size. Almost all patients present with neonatal macrothrombocytopenia with abnormally large alpha granules of *Paris-Trousseau platelets* (MIM 188025) and increased numbers of dysmorphic megakaryocytes in the bone marrow. Additional defects in the heart, kidney, brain, and other organs are often more problematic than the platelet disorder. The thrombocytopenia may be severe at birth but tends to improve with age, although a mild to moderate bleeding diathesis persists.[169]

ANKRD26-related thrombocytopenia (ANKRD26-RT) is a recently characterized autosomal dominant thrombocytopenia with normal platelet size, which was previously mapped to chromosome 10p11.1-p12. These patients have moderate thrombocytopenia and mild bleeding. The bone marrow shows dysmegakaryopoiesis with many small dystrophic megakaryocytes with hypolobated nuclei but without clear abnormalities in platelet morphology.[54] ANKRD26 is a protein of unknown biologic function. The mechanism by which *ANKRD26* mutations result in thrombocytopenia is not known. Patients may have an increased risk of leukemia.[170]

Thrombocytopenia Associated with Macrothrombocytes

Another inherited platelet disorder involving large platelets and thrombocytopenia besides the disorders related to the GPIb/IX receptor, GPS, platelet-type VWD, and transcriptional factors is the autosomal dominant disorder known as *myosin heavy–chain 9–related disorder* (MYH9-RD). The disorder was previously called May-Hegglin anomaly (MHA: MIM 155100), Sebastian syndrome (MIM 605249), Epstein syndrome, and Fechtner syndrome (MIM 153640). In this disorder macrothrombocytes are associated with thrombocytopenia and normal coagulation studies. Leukocytes contain large spindle-shaped inclusions known as *Döhle bodies* (see Fig. 30-5, *E*). Additionally, patients may have sensorineural deafness, nephritis, and ocular abnormalities (in particular, cataracts). The original disorders were separately classified, but it is now clear that all are due to mutations in the *MYH9* gene encoding the cytoskeletal protein nonmuscle myosin heavy-chain IIA (NMMHC-IIA). Bleeding symptoms in this disorder range from mild to moderate (bruising, epistaxis, and menorrhagia), and severe bleeding episodes are rare. Platelet counts range from severe thrombocytopenia, with platelet counts of approximately 30,000/μL, to mild thrombocytopenia, with platelet counts of around 100,000/μL; giant platelets are present on peripheral blood smears. Diagnosis can be confirmed by immunofluorescence staining of peripheral blood showing aggregates of abnormal nonmuscle myosin IIA (NMM-IIA) within leukocytes and platelets. Because the bleeding issues are often limited, further evaluation of affected patients should focus on screening for hearing loss, ophthalmologic impairment, and renal dysfunction in the patient and family members.[171,172]

NMM-IIA exists as a hexamer of two heavy chains and four light chains. NMMHC contains an N-terminal motor domain (head) and a C-terminal rod domain (tail). Although genotype-phenotype correlations are not perfect, mutations within the head appear to result in more severe disease with more significant thrombocytopenia and higher risk for hearing and renal impairment. However, within families containing the same mutation there is significant heterogeneity in phenotypes.[172]

Specific mutations in *ITGA2B* and *ITGB3* have also been described to cause an autosomal dominant form of macrothrombocytopenia.[173,174] While biallelic mutations in these genes result in GT (described earlier), mutations resulting in a partially constitutively active GPIIB-IIIa complex, through disruption of the interaction of cytoplasmic tails, alter megakaryocyte: fibrinogen interactions within the bone marrow and alter platelet production.[175] These patients have thrombocytopenia with different degrees of platelet macrocytosis and some altered platelet function.

There are multiple complex disorders associated with thrombocytopenia, including congenital sitosterolemia resulting from loss of function mutations in *ABCG5* or *ABCG8*, which play a role in enterocyte and hepatocyte exclusion of plant sterols. The clinical presentation of these patients is variable, but many present with stomatocytosis, thrombocytopenia, and large platelets associated with elevated plasma concentrations of phytosterols.[176]

Another complex disorder associated with macrothrombocytopenia results from mutations in the filamin A gene *(FLNA)* located on chromosome Xq28. The most commonly described disorder associated with mutations in *FLNA* is *periventricular nodular heterotopia*. Several patients have been described with PVNH and thrombocytopenia with abnormal platelet and megakaryocyte morphology. Recently, a novel missense mutation in *FLNA* was described in a woman presenting with isolated thrombocytopenia that had been initially diagnosed as immune thrombocytopenia.[177]

Thrombocytopenia Associated with Microthrombocytes

Wiskott-Aldrich sydrome (WAS: MIM 313900) patients have microthrombocytopenia, but the phenotype involves multiple aspects of platelet and lymphocyte biology. WAS was first recognized in two case reports as a sex-linked disorder with thrombocytopenia and eczema.[178] Platelets are small, usually half-normal volume, and the platelet mass is decreased.[179] There appears to be an associated platelet storage pool defect. The thrombocytopenia seen is severe and can be less than 10,000 platelets/μL. These patients may also have a severe immune defect with an impaired ability to form antibodies to carbohydrate antigens and appear to have a defect in B and especially T lymphocytes. As in the 22q deletion syndrome, WAS can lead to an immune-based thrombocytopenia and ultimately to a leukemic- or lymphomatous-based thrombocytopenia. Splenectomy, although it increases the risk for opportunistic infection, often alleviates the thrombocytopenia and bleeding in this disorder. Immune-related complications respond variably to steroids, intravenous immunoglobulin, vincristine, or plasmapheresis.[182] Bone marrow transplantation has been efficacious.

The gene involved in WAS (MIM 301000) and a milder version termed X-linked thrombocytopenia (MIM 313900), which is not associated with the severe immunologic defects, has been localized to the short arm of the X chromosome (Xp11.22). The encoded protein WASP is 501 amino acids long, has no transmembrane domain, but has two highly charged C-terminal domains.[180] In addition, there is a potential nuclear localization signal. There is a pleckstrin homology domain and a cofilin domain by which WASP can depolymerize actin. WASP expression is limited to lymphocytes and platelets. WASP binds to a number of intracellular signaling proteins such as the Rho family member guanosine triphosphatase (GTPase) Cdc42. Tyrosine phosphorylation signaling in the cell appears to cause WASP to become bound to cytoskeletal proteins such as PSTPIP (WIN) and to be involved in megakaryocytopoiesis.[181]

Additional proof that WASP was involved in WAS came from the original description of the gene. One affected patient had a single base deletion, a second had a G→T transversion that led to an Arg86Leu substitution, and a third had a G→A transition of the same Arg leading to a His substitution.[180] Since this patient was identified, the number of defined mutations has increased (almost 300 mutations have been described), and their relationship to the genetic severity has been better defined. Patients have been described with isolated X-linked microthrombocytopenia,[182] and these patients have missense mutations in the *WAS* gene, only some of which overlap with the mutations seen in WAS. To complicate matters, the *GATA1* gene is closely linked to the *WASP* gene, and this may limit the usefulness of linkage studies for defining which of these two genes is affected in a patient with X-linked thrombocytopenia.

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CHAPTER 31

Hemophilia and von Willebrand Disease

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CHAPTER OUTLINE

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Musculoskeletal Bleeding
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Miscellaneous Hemorrhages
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VON WILLEBRAND DISEASE
Pathophysiology
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Fundamentals of Treatment

The clinical and laboratory features of the three most common hereditary bleeding disorders—hemophilia A (factor VIII deficiency), hemophilia B (factor IX deficiency), and von Willebrand disease (VWD)—are discussed in this chapter. Hemophilia A and hemophilia B are both caused by a functional deficiency of a plasma protein inherited in an X-linked manner. Because these two proteins, factor VIII and factor IX, are part of the tenase complex that subsequently activates factor X (see Chapter 27), the clinical features and many of the diagnostic considerations for hemophilia A and B are similar. Thus these two types of hemophilia are discussed together, although the treatment of each is presented separately. The last part of this chapter is devoted to VWD. Because von Willebrand factor (VWF) and factor VIII exist as a circulating noncovalent complex in plasma, historically they copurified during plasma fractionation. In fact, for many years, severe VWD and hemophilia A were treated with the same plasma-derived therapeutic products. As the products to treat hemophilia A became more pure, factor VIII was subfractionated away from its carrier protein VWF, thereby making high-purity factor VIII products unsatisfactory for the treatment of VWD. The most commonly used products to treat hemophilia A contain recombinant factor VIII and do not contain VWF. To treat severe VWD, intermediate-purity plasma-derived concentrate containing both VWF and factor VIII or, in some regions of the world, purified plasma-derived VWF concentrates are available to treat deficiency of VWF when replacement therapy is indicated. Recombinant VWF is currently being investigated in clinical studies and will likely be available to patients in the near future.

HEMOPHILIA A AND HEMOPHILIA B

Hemophilia A and hemophilia B are the most common severe inherited bleeding disorders. Historically, hemophilia has been recognized as an entity since biblical times.[1] Use of the term *hemophilia* was ascribed to Schönlein in the 1820s.[2] Progressive insight into the pathophysiologic course of the disease has led to the development of powerful tools for diagnosis and agents for improved therapy, as well as the need to minimize the primary and secondary complications of the disease and its therapy.[3] The prolonged clotting time of hemophilic blood was first noted in 1893,[4] and the associated decrease in factor VIII levels in hemophilia A was initially identified in 1947.[5,6] In 1952 levels of factor IX were found to be decreased in *Christmas disease*, which was the early term used to denote hemophilia B because it was the surname of the first patient diagnosed with the disease.[7]

Early therapy for hemophilia was primarily supportive, but after the observation in the 1930s that administration of plasma corrected the clotting time of hemophilic blood, treatment evolved to include transfusion with whole blood and plasma to achieve improved clotting function.[8,9] The modern era of treatment began in 1964 with the discovery of cryoprecipitate[9] and plasma fractions containing factor IX[10] and their use to treat bleeding episodes in hemophilia. More purified products were introduced in 1965 and were referred to as factor VIII concentrates (containing both factor VIII and VWF).[11,12] The next major improvements in replacement therapy were the result of attempts to limit transfusion-related viral infections during the 1980s, which included improved donor selection, heat treatment of the product, and further refined purification schemes.[13-15]

In 1984 and 1985 the genes for factor VIII and factor IX were cloned,[16-19] and in 1989, recombinant factor VIII was first used clinically.[20,21] In recent years more highly purified factor IX and recombinant factor IX products have been developed. With the advent of recombinant products, patients with hemophilia could more readily and safely be treated with primary[22-24,25,26] and secondary prophylactic therapy.[27] Such vigorous treatment programs markedly reduce the likelihood of spontaneous bleeding episodes and the progression of hemophilic arthropathy. Thus prophylaxis continues to be the preferred approach to patients with severe hemophilia.

The landscape of hemophilia treatment is changing significantly. New progress has been made in the development of recombinant products based on the prolongation of the half-life of factors VIII and IX. Several clinical trials in adults and children are ongoing, and it is likely that some of these new products will be available to patients shortly. Additionally, a major breakthrough in hemophilia therapy was recently made with the report of a successful gene therapy clinical trial for hemophilia B with long-lasting, sustained coagulation factor IX levels.[27a]

Pathophysiology

The structure and function of factors VIII and IX in the coagulation pathway were outlined in Chapter 27. Both factors are crucial for normal thrombin generation. The classic representation of hemostasis shows factor VII together with tissue factor activating factor X; however, subsequent studies suggested that the primary physiologic pathway of activation of factor X by tissue factor and factor VII is through the activation of factor IX.[28,29] Activated factor IX complexes with factor VIIIa, calcium, and phosphatidylserine on physiologic membranes to generate factor Xa, which subsequently participates in formation of the prothrombinase complex. Thus, physiologically, the tissue factor pathway of factor X activation requires factor VIII and factor IX for normal thrombin generation, and the absence of either protein severely impairs the ability to generate thrombin and fibrin. This absence is not identified by use of the classic prothrombin time; the prothrombin time is normal in patients with hemophilia A or hemophilia B.

After injury the initial hemostatic event is formation of a platelet plug, and the subsequent generation of fibrin is crucial to prevent continued oozing. Because of the primary clotting factor deficiency in either hemophilia A or hemophilia B, the propagation phase of coagulation is impaired, and clot formation is delayed and is not robust. Thus patients with hemophilia do not bleed more rapidly; rather, there is delayed formation of an abnormal clot.[30] Thrombin is crucial for platelet aggregation, fibrin generation, clot retraction, and activation of factor XIII. Because thrombin generation in hemophilia is markedly delayed, hemorrhage may occur after minimal or unknown trauma. Deep bleeding into joints and muscles is characteristic of hemophilia. In hemophilia the clot formed is often friable, and rebleeding is a common observation in inadequately treated patients. Medications such as aspirin and other nonsteroidal antiinflammatory drugs may alter platelet function and further impair hemostasis in affected individuals and are therefore contraindicated.[31]

Demographics and Classification

Hemophilia occurs in approximately 1 in 5000 males.[32-34] Of all patients with hemophilia, 80% to 85% have hemophilia A, and 10% to 15% have hemophilia B. Hemophilia shows no apparent racial predilection and appears in all ethnic groups.

The severity of hemophilia is classified on the basis of the patient's baseline level of factor VIII or factor IX. One unit of each factor is defined as the amount found in 1 mL of normal plasma. Factor levels are often expressed as a percentage of activity, with a 100% level (100 U/dL) being equal to the activity found in 1 mL of normal plasma. Severe hemophilia is characterized by a factor level of less than 1 U/dL (<1%). In moderate hemophilia the clotting factor level is between 1 and 5 U/dL, and in mild hemophilia factor VIII or IX levels are greater than 5 U/dL. This classification provides a rough guide for the patient's expected rate of occurrence of bleeding episodes. Although the correlation is not perfect, the clinical phenotype usually corresponds to the factor level. Patients with severe hemophilia often have bleeding episodes after minimal or unknown trauma, with children experiencing unprovoked muscle and joint bleeding between one and six times per month. With more aggressive prophylactic administration of factor VIII, instances of bleeding are markedly reduced. Patients with moderate hemophilia will have bleeding after mild to moderate injuries, whereas mild hemophilia may often be undiagnosed for many years, and these patients may have bleeding only after significant trauma or at the time of surgery.

Clinical Manifestations

Approximately 30% of male infants with hemophilia have bleeding with circumcision.[35] In some reports as many as 2% to 3% of neonates with hemophilia may experience an intracranial hemorrhage.[36,37] It is important to prevent head trauma during delivery. Forceps or vacuum extraction should be discouraged.[36,38] In the majority of patients hemophilia is diagnosed at birth because of a family history, although in approximately a third of patients the occurrence of hemophilia represents a new mutation.[39] In these latter patients the diagnosis is often made when the child begins to crawl and walk. The usual initial symptoms include easy bruising; oral bleeding, especially from a torn frenulum; hemarthrosis; and intramuscular hemorrhage. When the diagnosis of hemophilia is suspected on the basis of either clinical findings or a positive family history, the initial diagnostic studies should include determination of the prothrombin time, partial thromboplastin time (PTT), fibrinogen or thrombin time, platelet count, and studies to identify VWD (see Chapter 29). If the PTT is prolonged, assays for factor VIII and factor IX should be performed. The presence of an antibody inhibiting either factor VIII or IX can be ruled out by mixing normal plasma in equal volume with the test plasma, incubating the mixture for 30 to 60 minutes, and repeating the PTT to be sure that it has been normalized (mixing study). The specific assays for factor VIII and factor IX are not as easily affected by difficult venipuncture and are therefore more reliable. Factor VIII and VWF levels may be elevated by stress and inflammation, however, and repeated studies may be necessary to establish the diagnosis and the severity of the disorder.

Specific types of bleeding are described in the following paragraphs. In general, the clinical bleeding episodes associated with deficiency of factor VIII or factor IX are identical.

Musculoskeletal Bleeding

Although patients with hemophilia may bleed in any area of the body, the hallmark of hemophilia is deep bleeding into joints and muscles.[40] The occurrence of such hemorrhage usually corresponds to the severity of the clotting factor deficiency in patients with either hemophilia A or hemophilia B. Without prophylactic factor treatment, patients with severe hemophilia may have a bleeding episode as often as once or twice per week. Bleeding occurs most commonly into the joint space, a condition referred to as *hemarthrosis*. These joint hemorrhages usually begin when the child reaches the toddler age. As the toddler attempts to maintain an upright position, the ankle becomes particularly prone to hemorrhage. Thus in toddlers the ankle is the most common site of hemorrhage, with the knee being the second most common. As the child grows older, the most frequent bleeding sites are the knees and elbows.

Patients who sustain hemarthrosis usually first note an aura of tingling or warmth, followed shortly thereafter by increasing pain and decreased range of motion of the joint as the joint capsule becomes progressively distended.[41-43] As children get older, they are often able to tell their parents and the physician when a hemorrhage is beginning to occur, even in the absence of significant signs on physical examination. In young children who are unable to verbalize symptoms, severe pain and distention of the joint space often occur before the location of the hemorrhage can be easily determined. On examination pain and limitation of range of motion of the joint are obvious and followed by the development of warmth, swelling, and tenderness.

Prompt, early coagulation factor replacement therapy is the key to minimizing long-term complications of these hemorrhages. Patients with severe hemophilia are increasingly being treated prophylactically to prevent most bleeding episodes (discussed later). Prompt aspiration of hip hemarthrosis should be performed if joint distention is present to reduce the risk for avascular necrosis of the femoral head (a rare event). As children and their parents become familiar with the early symptoms of bleeding, aggressive early treatment can be given to reduce the degree of joint damage. If the child experiences repeated hemorrhage within a joint, chronic effusion and hyperemia often occur, and an aggressive secondary prophylactic therapy program may be needed to return the joint to a more normal state of recovery.[27] Severe chronic arthropathy may develop in older children and adults who have not received aggressive early treatment. These individuals often experience pain very early with little swelling. The joints of younger children are usually more distensible, and massive joint swelling may be observed. Splinting and application of ice packs as auxiliary measures may provide a modicum of relief but are not substitutes for early coagulation factor replacement therapy. When the physician has doubts, treatment should be initiated; it is not appropriate to watch and wait because damage to the joint can start early in life.

The clinical diagnosis of intramuscular hematoma is often elusive. Such hemorrhage occurs deep within the body of the muscle and is associated with a vague feeling of pain on motion. Because the bleeding is not in a closed space, the mass may be difficult to palpate, although the circumference of the affected limb is generally increased. Muscle hemorrhage should be considered to be as severe as hemarthrosis because it may result in severe muscular contractures as a result of fibrosis and atrophy or even pseudotumor formation. Muscle weakness also predisposes patients to joint hemorrhage. Appropriate replacement therapy is critical for reducing the size of the hematoma, and physical therapy is important to restore normal range of motion and prevent fibrosis of the muscle.

Iliopsoas bleeding is a particularly troublesome form of intramuscular hemorrhage. A patient with an iliopsoas hemorrhage is generally seen with vague symptoms of lower abdominal or upper thigh discomfort. The patient may have a characteristic gait; the hip is flexed and inwardly rotated. On examination the patient is unable to extend the hip, but usually both internal and external rotation of the hip joint is normal; the latter finding often distinguishes an iliopsoas hemorrhage from a true hemarthrosis of the hip joint. The diagnosis is made clinically by these findings and should be confirmed by either ultrasonography or computed tomography (CT).[44] Appropriate treatment is crucial; iliopsoas hemorrhages may be life threatening because large volumes of blood can be lost into the retroperitoneal space. Physical examination demonstrates loss of hyperextension of the hip. If an iliopsoas hemorrhage is identified, an aggressive infusion program should be initiated and maintained for 10 to 14 days or longer, followed by at least several months of prophylactic therapy until clinical and radiographic evidence demonstrates resolution.

Life-Threatening Hemorrhages

Bleeding episodes of the central nervous system (CNS), bleeding into and around the airway, and exsanguinating hemorrhage are the most common causes of life-threatening hemorrhage in hemophilia. The first element of treatment is prompt therapy with clotting factor concentrate to bring the plasma clotting factor level to normal. CNS hemorrhage may occur without known trauma, and early symptoms may be minimal. Thus therapy should be initiated before radiologic evaluation or lumbar puncture; when there is any doubt, it is better to treat immediately.[45-47] Rapid performance of CT is often critical in the early diagnosis of intracranial bleeding, although an astonishingly large volume of blood can be present within the cranium in infants with relatively few neurologic findings. Treatment of life-threatening hemorrhage involves replacement therapy to achieve a level of 100 U/dL for factor VIII and factor IX, maintenance of adequate hemostatic levels (>50 to 60 U/dL) for a minimum of 14 days, and a more prolonged period of prophylactic therapy for an additional 2 to 3 weeks or longer to ensure resolution of the underlying event (Table 31-1). Patients with intracranial hemorrhage should receive prophylaxis for at least 6 months after the more intense initial therapy and may benefit from lifelong prophylaxis.

Preparation for Surgery

Preparation of a hemophilic patient for surgery includes a careful history and physical examination, measurement of inhibitor titer, careful determination of the incremental recovery and half-life after infusion of the appropriate clotting factor (VIII or IX), and assurance that adequate amounts of coagulation factor replacement material are available, as well as red cells if a transfusion is needed.[48] Before surgery the replacement materials should be infused to achieve a level of 80 to 100 U/dL for factor IX and 100 to 150 U/dL for factor VIII; levels should be maintained at greater than 50 to 60 U/dL for 7 to 10 days postoperatively. Lower doses to maintain levels at greater than 20 to 30 U/dL for an additional 1 to 2 weeks may then be used and continued until healing has occurred (see Table 31-1). Because recovery of recombinant factor IX activity is less than that of therapeutic plasma-derived

TABLE 31-1 Treatment of Specific Hemorrhages in Hemophilia

Type of Hemorrhage	Hemophilia A	Hemophilia B[*]
Hemarthrosis[†]	*50 U/kg* factor VIII concentrate initially,[‡] 20 U/kg the following day; consider additional treatment every other day, depending on response	*80 U/kg* factor IX concentrate initially, 40 U/kg the following day; consider additional treatment every other day, depending on response
Muscle or significant subcutaneous hematoma	*50 U/kg* factor VIII concentrate; may need 20 U/kg every other day until well resolved	*80 U/kg* factor IX concentrate; may need 40 U/kg every other day until well resolved
Mouth, deciduous tooth, or tooth extraction	*20 U/kg* factor VIII concentrate (40 U/kg if molar extraction), antifibrinolytic therapy; remove loose deciduous tooth	*40 U/kg* factor IX concentrate (80 U/kg if molar extraction), antifibrinolytic therapy; remove loose deciduous tooth
Epistaxis	*Apply pressure* for 15-20 min, nosebleed QR, pack with petrolatum gauze, antifibrinolytic therapy; 20 U/kg factor VIII concentrate if preceding measure fails	*Apply pressure* for 15-20 min, nosebleed QR, pack with petrolatum gauze, antifibrinolytic therapy[§]; 30 U/kg factor IX concentrate if preceding measure fails
Major surgery, life-threatening hemorrhage (e.g., central nervous system, gastrointestinal, airway)	*50-75 U/kg* factor VIII concentrate, then initiate continuous infusion of 3 U/kg/hr to maintain factor VIIII > 100 U/dL for 24 hr, and then give 2-3 U/kg/hr for 5-7 days to maintain the level greater than 50 U/dL and an additional 5-7 days at a level >30 U/dL (bolus dosing to maintain these levels is acceptable); monitor factor VIII levels	*80-100 U/kg* factor IX concentrate, then 20-40 U/kg every 12-24 hr to maintain factor IX >40 U/dL for 5-7 days, and then >30 U/dL for 5-7 days[‖]; monitor factor IX levels
Iliopsoas hemorrhage	*50 U/kg* factor VIII concentrate, then 25 U/kg every 12 hr until asymptomatic, and then 20 U/kg every other day for a total of 10-14 days[¶]	*80 U/kg* factor IX concentrate, then 20-40 U/kg every 12-24 hr to maintain factor IX > 40 IU/dL until asymptomatic, and then 30 U/kg every other day for a total of 10-14 days[‖,¶]
Hematuria	*Bed rest*, 1.5 × maintenance fluids; if not controlled in 1-2 days, 20 U/kg factor VIII concentrate; if not controlled, prednisone if human immunodeficiency virus negative	*Bed rest*, 1.5 × maintenance fluids; if not controlled in 1-2 days, 30 U/kg factor IX concentrate; if not controlled, prednisone if human immunodeficiency virus negative

[*] Dosing of recombinant factor IX should be increased to 1.3 to 1.5 times the recommended doses or, preferably, be based on an individual in vivo recovery study.

[†] For hip hemarthrosis, orthopedic evaluation for possible aspiration is advisable.

[‡] For mild or moderate hemophilia, desmopressin, 0.3 μg/kg, should be used instead of factor VIII concentrate if patient is known to respond with a hemostatic level of factor VIII; if repeated doses are given, monitor factor VIII levels for tachyphylaxis.

[§] Do not give antifibrinolytic therapy until 4 to 6 hours after a dose of prothrombin complex concentrate.

[‖] If repeated doses of factor IX concentrate are required, use highly purified, specific factor IX concentrate.

[¶] Repeat radiologic assessment should be performed before discontinuation of therapy.

factor IX, 1.2 to 1.5 times the dose should be administered if using recombinant factor IX.[49,50]

Miscellaneous Hemorrhages

A common manifestation of hemophilia is oral bleeding, whether from a torn frenulum in a young child or after tooth extraction in an older patient. Replacement therapy to achieve a factor level of 30 to 40 U/dL is adequate for initial therapy. Because the mucosae of the oral cavity contains abundant fibrinolytic activity, therapy with antifibrinolytic agents (aminocaproic acid [Amicar] or tranexamic acid [Cyklokapron and Lysteda]) is useful to stabilize the clot until the wound has healed.[51-53]

Hematuria can be a particularly troublesome problem. Blood in the urine may arise spontaneously, and determination of an actual bleeding site is often difficult. Therapy is also controversial. Replacement therapy with 40 to 50 U/kg of factor VIII or IX followed by bed rest is generally advisable after other common causes of hematuria have been ruled out. Treatment with corticosteroids may be helpful.[54] Sudden abdominal or flank pain should be investigated for the possibility of ureteral obstruction and hydronephrosis secondary to bleeding. Antifibrinolytic agents are not recommended in hematuria because of the risk they present for ureteral obstruction.

Gastrointestinal bleeding is an occasional complication of hemophilia. All the common causes of gastrointestinal bleeding can occur in patients with hemophilia, as well as spontaneous hemorrhage after no known insult. An initial episode of gastrointestinal hemorrhage requires that the patient receive a thorough gastrointestinal evaluation after the acute bleeding is controlled with replacement therapy. If substantial blood loss has occurred, patients should be treated with high doses of concentrate as though they were at risk for life-threatening hemorrhage.

Long-Term Complications of Joint Bleeding

Chronic arthropathy is the major long-term disabling complication of hemophilia. The articular surface of a normal joint is lined with cartilage that is lubricated by synovial fluid produced by the synovial lining of the joint. A thick fibrous capsule protects the joint. The natural history of hemophilic arthropathy is one of a cycle of recurrent hemorrhage into a "target" joint leading to synovial thickening and friability, followed by additional hemorrhage into the joint.[41,42,55] After a joint hemorrhage, proteolytic enzymes are released by granulocytes into the joint space.[56] In addition, heme iron is ingested by macrophages and may contribute to inflammation by the release of oxidative products. The synovium of the joint, originally the width of a single cell, proliferates in response to inflammation and develops frondlike projections into the joint.

These projections are by nature friable and bleed with minimal trauma, thus setting up a vicious cycle of recurrent hemorrhage. With recurrence of hemorrhage there is further release of substances injurious to the joint cartilage, which causes it to develop a roughened, lunar landscape–like surface. As the cartilage erodes, the joint space becomes narrowed. In recent years the pathophysiology of hemophilia arthropathy has been better characterized at a molecular level; not surprisingly, it shows some similarities with other inflammatory processes that are associated with joint diseases such as rheumatoid arthritis.[57]

On examination, the clinical findings depend on the stage of arthropathy that has developed. The early stages are characterized by synovial thickening with little evidence of joint damage. With synovitis alone, the range of motion of the joint is nearly normal, although recurrent bleeding episodes usually result in weakness of the proximal muscles. With time, however, proliferative changes become associated with erosion of the cartilage. The patient at this stage may note the development of arthritic symptoms that are often difficult to distinguish from joint bleeding. Crepitus and decreased range of motion are prominent findings on physical examination. At this stage proximal muscle weakness is accentuated because of the inability of the joint to attain full range of motion. Finally, with progressive bleeding and further narrowing of the joint space, the joint fuses.

This progression of joint disease was once commonly seen in hemophilia centers throughout the United States and other countries. The most commonly affected joints were the knees, ankles, and elbows. With the development of modern concentrates for replacement therapy, home therapy, and prophylactic therapy, this cycle has now been disrupted in the vast majority of children with severe hemophilia. Primary prophylactic therapy and early diagnosis and treatment of joint hemorrhage have limited the number of "target joints" that develop. When a joint becomes a target, hemorrhage into that joint must be treated vigorously and the joint must be carefully watched for the cycle of rebleeding every 1 to 2 weeks that is characteristic of incompletely treated hemarthrosis. If evidence of early synovitis or loss of range of motion is present, a secondary prophylactic therapy program of every-other-day treatment for 30 to 90 days is often helpful for "cooling down" the affected joint and allowing healing to occur.[27]

Alternatively, a lifelong prophylactic therapy program to prevent spontaneous, severe joint bleeding may be started in young infants (discussed later), a modality that is currently widely used in the United States and other developed nations.[58] If medical therapy fails, synovectomy frequently reduces the occurrence of bleeding and may prevent progression of the joint disease. With the advent of arthroscopic means to perform synovectomy, this procedure has been more readily performed with reduced morbidity; equally important, early mobilization of the affected joint allows more rapid return of range of motion.[59,60] Another procedure in which various radioisotopes are used to reduce the mass of inflamed synovium is termed *radioactive synovectomy*.[60] This approach is recommended for patients with inhibitors. Fewer radioactive synovectomies are now performed because of the development of acute lymphoblastic leukemia in two children who underwent this procedure.[61] When aggressive medical and less invasive surgical management fails to halt progression of the joint disease, some adults have benefited from joint replacement.[62-64]

If pain and bleeding are severe in a joint with minimal range of motion, joint fusion may provide a significant decrease in pain and rebleeding. A critical element in the care of patients with chronic joint disease is the need for a team approach to the problem because the care of these children requires the combined expertise of an orthopedic surgeon, physical therapist, nurse, social worker, and hematologist to achieve optimal results.

Symptomatic Carriers

Although the usual patient with hemophilia A or hemophilia B is a male, carriers of a factor VIII or factor IX gene mutation may also be symptomatic. Known carriers should have their levels of clotting factor measured at least once to determine whether they are at increased risk for clinical bleeding episodes. There are several possible explanations for the observation of a female patient with moderate or severe deficiency of factor VIII or factor IX.[65-68] Skewed lyonization of a carrier female is the most commonly accepted explanation. As predicted by the Lyon hypothesis, the X chromosome in each female cell is randomly inactivated early in embryonic development; by chance, some carriers of hemophilia will have a high percentage of inactivated normal X chromosomes. The active abnormal X chromosome will result in deficient synthesis of factor VIII or factor IX. Other explanations for the presence of hemophilia A or B in a phenotypic female include testicular feminization of a genotypic male,[69,70] Turner syndrome with the genotype XO,[71-72] an X autosomal translocation with skewed X inactivation,[73] and a daughter of a maternal carrier and a father with hemophilia A. Factor VIII deficiency is also found in type 2N VWD as a result of mutations in the factor VIII–binding region of the VWF protein.[74]

Laboratory Evaluation

Severe hemophilia A or hemophilia B is easily identified by a markedly prolonged PTT and the absence of either factor VIII or factor IX. The diagnosis of mild hemophilia A or hemophilia B may be more difficult to make because the newborn has a slightly prolonged PTT secondary to a physiologic reduction in vitamin K–dependent factors such as factor IX. In addition, the stress of delivery and other neonatal problems may transiently elevate factor VIII levels into the normal or nearly normal range. Thus in the absence of clinical bleeding, mild deficiencies may be identified only after repeated testing in the weeks or months after birth (see Chapter 29). VWF testing must be done to distinguish mild or moderate factor VIII deficiency secondary to hemophilia from deficiencies of factor VIII secondary to VWD.

Partial Thromboplastin Time

The PTT measures all the procoagulant clotting factors except factor VII and factor XIII (see Chapters 27 and 29). In general, the PTT is most sensitive for deficiencies of the "intrinsic" pathway.[75] In general, commercial PTT reagents have good sensitivity for factor VIII deficiency, but their sensitivity for factor IX deficiency is more variable.[76-78] Use of some of these reagents will give normal PTTs even with factor IX levels of 15 to 20 U/dL. Because the hemostatic level of factor IX is approximately 30 U/dL, mild or moderate factor IX deficiency may be missed. Thus if hemophilia is suspected, a factor IX assay should be performed even if the PTT is normal. The clinical laboratory technician should study the sensitivity of each lot of PTT reagent to be able to advise the clinician about the likelihood that a normal PTT implies normal clotting factor levels. In patients with severe hemophilia A or hemophilia B, the PTT is generally prolonged to two to three times the upper limit of the normal range. The prolonged PTT will correct to within the normal range when the reagent is mixed 1 : 1 with normal plasma. Failure of the PTT to correct on 1 : 1 mixing of the reagent with normal plasma implies the presence of an anticoagulant (e.g., lupus inhibitor, antibody to factor VIII or IX, or heparin). The presence of a specific anti–factor VIII or anti–factor IX inhibitory antibody is described in more detail later.

Specific Assays for Factor VIII and Factor IX

Functional assays for factor VIII and factor IX are performed on plasma samples that contain no factor VIII or factor IX, respectively (either hemophilia A or hemophilia B plasma); alternatively, normal plasma from which the respective clotting factor is selectively removed by a monoclonal antibody can be used.[79] If adsorbed plasma is used for factor VIII–deficient plasma, the VWF content of that plasma should be normal. A standard curve is constructed with the use of normal plasma, with the 100% point being a 1 : 10 dilution of normal plasma. Serial dilutions of this normal plasma should usually be linear through a dilution of 1 : 640 or 1 : 1280 when plotted semilogarithmically versus the clotting time. Serial dilutions of the patient's plasma are compared with this normal curve and expressed in units per deciliter. One unit is the amount of factor VIII or factor IX present in 1mL of normal plasma.

Immunoassays for factor VIII and factor IX are sometimes performed to identify dysfunctional proteins, also known as cross-reacting material (CRM) positive (+) variants. The majority of patients with severe hemophilia A have undetectable levels of factor VIII protein, but some patients with mild or moderate hemophilia A possess levels of factor VIII protein that are detectable with an immunoassay.[80-82] In hemophilia B approximately 50% of patients will have detectable or even normal levels of factor IX antigen and therefore have true CRM + variants.[83,84] Immunoassays are not usually required for the clinical management of these disorders because the coagulant-based assays predict the functional concentration of these clotting factors.

Inhibitory Antibodies to Factor VIII or Factor IX

Inhibitor development is one of the most feared complications of hemophilia. The presence of a factor VIII or factor IX inhibitory antibody is usually suspected when the PTT does not correct to normal after being mixed 1 : 1 with normal plasma. Because of the kinetics of the inhibitory antibody, this mixture should be incubated 60 minutes before the PTT is performed. When a low-titer inhibitory antibody is identified, the PTT performed on a 1:1 mixture with normal plasma may initially be prolonged only by several seconds, but when the mixture is incubated for 1 hour, the PTT may be as long as or even longer than the PTT when it was originally performed on the patient's plasma.

Specific assays for factor VIII or factor IX antibodies are performed with the Bethesda assay. Dilutions of the test plasma are mixed with normal plasma and incubated for 120 minutes. A standard factor VIII or factor IX assay is then performed. One Bethesda unit is defined as the amount of antibody that will inactivate 50% of the normal factor VIII or factor IX in 2 hours when the residual factor VIII or factor IX level is between 25 and 75 U/dL.[85] The dilution of the test plasma that inhibits this amount of factor VIII is determined. If a 1:10 dilution of test plasma has this effect, the inhibitor plasma is said to contain 10 Bethesda units.

Genetic Testing

The genes encoding factors VIII and IX (*F8* and *F9*, respectively) map to the distal portion of the long arm of the X chromosome.[86,87,88,89] Therefore both hemophilia A and B are inherited as X-linked traits. Approximately two thirds of patients have a family history, while the rate of de novo mutations is approximately one in three. Genetic testing for hemophilia A and hemophilia B is now widely available.[86-88,90-92] Such testing is usually performed on the proband first, and once the mutation is identified the potential carriers of hemophilia in the family can be then tested. Prenatal testing for hemophilia is performed by amniocentesis or chorionic villus biopsy[90] and may be useful for the perinatologist and neonatologist in the management of pregnancy, delivery, and immediate neonatal care. Databases of the known mutations causing hemophilia A are available (http://hadb.org.uk). The most common genetic alteration is inversion. Gene inversion occurs in approximately 45% of patients with severe hemophilia A, with most inversions originating in male germ cells. The most common inversion occurs in intron 22 of *F8;* this inversion accounts for approximately half of the cases of severe hemophilia A.[93] In families in which the propositus has a known molecular abnormality, genetic screening and carrier detection are highly accurate. Direct DNA sequence analysis is available for families in which the results of testing for the factor VIII gene inversion are negative. Additionally, molecular genetic diagnosis can also be done by performing linkage for the affected allele if an appropriate number of family members are available. This test is clinically available (www.genetest.org). If genetic testing is uninformative or unavailable, a coagulation-based assay to detect the carrier state may also be useful[94] and is approximately 90% accurate.

In contrast to the complexity of the factor VIII gene, the factor IX gene is considerably smaller (33 versus 187 kilobases [kb]). More than 60% of factor IX gene defects are due to missense point mutations, and an identifiable defect in the gene can be found in nearly all patients[95] (http://www.factorix.org). In approximately 50% of patients with hemophilia B, a factor IX protein that is nonfunctional is produced, whereas in the other patients no factor IX protein is produced.

Fundamentals of Treatment

Knowledge of the half-life, volume of distribution, patient's inhibitor status, and appropriate replacement material is necessary to make intelligent decisions for the treatment of bleeding episodes.[96-98] Prompt and appropriate treatment of hemorrhage and prophylactic therapy are the key to excellent care of patients with hemophilia. Home therapy and prophylactic therapy have revolutionized the care of children and adults with hemophilia. Early or prophylactic treatment prevents the long-term morbidity of hemophilia. For adequate treatment of mild to moderate hemorrhage, it is necessary that factor VIII levels of 30 to 50 U/dL or factor IX levels of 30 U/dL be achieved.

For patients with mild hemophilia A who have previously shown a satisfactory response to desmopressin acetate (DDAVP, 1-deamino-8-d-arginine vasopressin), this drug is the treatment of choice for bleeding episodes of mild to moderate severity.[99-101] For life-threatening hemorrhage, the immediate aim is to correct the patient's clotting factor level to normal (100 to 150bU/dL) and to maintain a nadir level between 50 and 60 U/dL for 5 to 7 days, followed by a vigorous maintenance treatment program.

Table 31-1 summarizes the treatment of specific types of hemorrhage in hemophilia A and hemophilia B. Calculation of the dose, recommendations concerning prophylaxis, and the specific treatment products for hemophilia A and hemophilia B are reviewed later.

Hemophilia A

Calculation of Dose. Calculation of the dose required to achieve a desired hemostatic level is based on a convenient formula derived from the work of Abildgaard and colleagues[11]:

$$\text{Dose factor VIII (units)} = \text{U/dL desired rise in plasma factor VIII} \times \text{Body weight (kg)} \times 0.5$$

Thus for treatment of hemarthrosis in a 20-kg child with severe hemophilia A, to achieve a plasma level of 60 U/dL, the dose would be 60 U/dL × 20 kg × 0.5 = 600 units of factor VIII.

For patients undergoing major surgery or with serious hemorrhage, the plasma factor VIII level should be initially corrected to 100 to 150 U/dL (immediately before the procedure if given for surgery) and maintained at greater than 50 to 60 U/dL for 5 to 7 days and then at greater than 30 U/dL for an additional 5 to 7 days. This can be accomplished by infusion of intermittent doses of factor VIII based on a half-life of 12 hours (e.g., administration of 30 U/kg [60-U/dL correction] every 12 hours).

Alternatively, a level of 50 to 60 U/dL can be maintained by continuous infusion of factor VIII at a rate of 2 to 3 U/kg/hr.[102,103] The level of factor VIII should be monitored periodically to ensure that the expected levels are attained. Immediately after surgery, it is advisable to administer 25 U/kg of factor VIII concentrate to compensate for the loss of factor VIII by increased consumption and blood loss during the surgical procedure. For surgical procedures and limb- or life-threatening hemorrhage, factor VIII levels should be monitored at least every 24 hours. CNS bleeding should be treated for a 2- to 3-week course of therapy. Such patients have a risk of recurrence of CNS bleeding for approximately 6 months after an episode, and these patients often continue to receive prophylactic therapy. At the conclusion of therapy for CNS hemorrhage, CT should be performed to provide a baseline scan for comparison if any subsequent CNS symptoms appear and to demonstrate resolution of the previous hemorrhage.

For treatment of more routine bleeding episodes, such as hemarthrosis, often only one to two doses of clotting factor replacement (plasma factor VIII level of 80 to 100 U/dL), initially followed by infusions as recommended in Table 31-1, are required to produce significant clinical improvement. Nevertheless, many centers are now using a more aggressive approach to managing severe joint hemorrhage by administering follow-up infusions daily until the signs and symptoms have resolved and then every other day for 7 to 10 days to limit the potential development of chronic joint disease. For dental extractions, mouth lacerations, or recurrent epistaxis, adjuvant antifibrinolytic therapy should be used (discussed later).

Prophylactic Factor VIII Therapy. Most centers in the United States and other developed countries are now routinely giving individuals factor VIII prophylactic replacement therapy with recombinant factor VIII.[27,104,105] Most centers initiate prophylactic therapy as soon as joint bleeding occurs while others start in early life even before the first bleed occurs. This therapy is usually administered through a subcutaneous access port after the insertion of a central venous line.[106,107] Insertion of such lines is often associated with catheter infections or thrombosis (or both), so patients and family members must be aware of the signs and symptoms.[107] In general, a dose of 20 to 40 U/kg of factor VIII is administered every other day or three times a week. The dose and rate are adjusted to ensure that the nadir before the next infusion is greater than 1 U/dL. The levels attained by use of this regimen usually prevent spontaneous bleeding, although hemorrhage caused by trauma may still require additional replacement therapy. The goal is to prevent recurrent hemarthrosis and associated chronic hemophilic arthropathy and thereby promote a normal lifestyle. A number of centers throughout the world have initiated prophylactic therapy programs and have demonstrated their cost-effectiveness when productivity and lifestyle are used as indicators of success.[25,108] However, this long-term vision is not always shared by administrators of managed health care systems, and the most successful programs have been carried out in countries with nationalized health care.

Factor VIII Treatment Products. Clotting factor replacement products should be rendered as free as possible of transfusion-transmissible agents, but vigilance is still necessary. As a result, recombinant products are generally considered to be preferable to plasma-derived products. Because even recombinant products may contain human albumin added as a stabilizer, products have recently been developed that contain no human plasma–derived protein.[109,110] The question of which product to use, the rate of administration, and the potential use of prophylaxis are decisions that need to be made by the health care provider, the family, and the patient.

Recombinant Factor VIII Concentrates. The factor VIII gene has been cloned, sequenced, and used to successfully transfect mammalian cells to produce recombinant factor VIII,[111] and several recombinant factor VIII products have been licensed. Clinical trials have documented that the half-life, recovery, and efficacy of these recombinant factor VIII products are similar to those for plasma-derived factor VIII.[20,21] Advantages of these recombinant factor VIII products are their freedom from human viral contamination and their production by methods that do not require human blood or plasma donors. There was initial concern that there may be an increase in the incidence of inhibitor development in previously untreated patients, but this appears not to be warranted.[112-117] Careful study of patients receiving recombinant products identified inhibitors in 20% to 25%, as opposed to the previously reported 14% prevalence of inhibitors.[118] The difference is now thought to be due to detection of transient inhibitors that subsequently disappear and not due to differences between the immunogenicity of recombinant factor VIII and plasma-derived factor VIII. More recent studies have revived interest in this issue, and a number of studies are re-evaluating whether factor VIII in the presence of VWF might be less immunogenic.[119-122] A recombinant factor VIII product missing the B domain has been licensed, which may require a different approach to laboratory monitoring because its activity differs between one- and two-stage factor VIII assays.[123,124] More recently developed products are formulated so that other human proteins are removed.[109,110]

Plasma-Derived Factor VIII Concentrates. Several commercial plasma-derived factor VIII concentrates are available. Intermediate-purity concentrates are produced from large pools of donor plasma by a combination of cryoprecipitation or precipitation with glycine, polyethylene glycol, or ethanol.[125] These intermediate-purity products contain 2 to 5 units of factor VIII per milligram of protein. Products of higher purity have also been developed and are produced by immunopurification of factor VIII with the use of monoclonal antibodies to factor VIII[126] or VWF.[127] All these products undergo multiple purification steps that involve heat treatment, as well as other modalities, to reduce the risk of viral transmission. As a result, the risk of human immunodeficiency virus (HIV) infection; hepatitis B; hepatitis C; and, more recently, transmission of hepatitis A and parvovirus B19 has been markedly reduced or eliminated.[128-130]

With the improved purity of plasma-derived products, the incidence of complications has greatly diminished. Allergic reactions, isohemagglutinin-induced hemolytic anemia, immune complex–mediated hematuria, and granulocyte antibody–induced lung injury are rarely seen. The use of immunoaffinity-purified factor VIII concentrates and recombinant factor VIII concentrates appears to stabilize CD4$^+$ counts in HIV-infected patients with hemophilia.[131,132]

To minimize the risk for hepatitis B and hepatitis A, unimmunized patients should be immunized at the time of diagnosis.[133,134]

Cryoprecipitate. The first widely used preparation of factor VIII was made by Judith Graham Poole in 1965 by a method called cryoprecipitation.[135] Each bag of cryoprecipitate contains only 100 units of factor VIII, and thus 10 to 20 donor units are required for the treatment of joint hemorrhage in an adult. Because cryoprecipitate lacks any active method of viral inactivation, possesses significant amounts of other human proteins, and is inconvenient to administer, it is no longer considered a viable alternative to purified factor VIII products, except in areas of the world where other forms of therapy are unavailable or in short supply.

Desmopressin. Desmopressin is a synthetic vasopressin analogue that increases plasma factor VIII and VWF levels, thereby allowing successful treatment of bleeding episodes associated with dental and surgical procedures in patients with mild and moderate hemophilia A and VWD.[137] Desmopressin is now considered the treatment of choice in patients with mild and moderate hemophilia A who have shown a response to the drug during a therapeutic trial. It is not effective in the treatment of severe hemophilia A, severe VWD, or any form of hemophilia B.

The individual response to desmopressin is varied,[138] with the range of increase in factor VIII level being between twofold and fifteenfold over baseline in patients with mild or moderate hemophilia A. It is therefore recommended that patients undergo a therapeutic trial of desmopressin with laboratory measurement of response to factor VIII before it is used for the treatment of bleeding episodes or as prophylactic therapy before dental and other surgical procedures. A similar degree of response is generally seen in an individual patient with subsequent doses,[139] and thus the factor VIII level attained after a trial dose can be used to predict the response to future therapy. The level of response to factor VIII is not usually sufficient to treat life- or limb-threatening hemorrhage. Tachyphylaxis may occur with repeated doses of desmopressin, but this varies from patient to patient.[140] Consequently, if repeated doses of desmopressin are to be administered, factor VIII levels should be monitored, and if necessary, exogenous factor VIII should be administered. In general, if several days have elapsed between doses, a response similar to that for the baseline infusion can be expected. Side effects from the administration of desmopressin are minimal and include headache; flushing; a slight change in pulse rate or blood pressure; and, rarely, hyponatremia. Because hyponatremia after the administration of desmopressin may cause a seizure in rare instances, particularly in young children,[141] fluid intake should be restricted to maintenance levels for at least 24 hours. If repeated doses of the drug are given, serum sodium levels should be monitored. Because of rare reports of thrombosis after the use of desmopressin,[142] the drug should be used with caution in patients with an increased risk for thrombosis.

The recommended intravenous dose of desmopressin is 0.3 μg/kg, and it is administered in 25 to 50 mL of normal saline over a period of 20 to 30 minutes.[100] If desmopressin is being given before a procedure such as a dental extraction, it should be administered as close to the procedure as possible because the maximal response occurs 30 to 60 minutes after administration. A concentrated form of desmopressin (Stimate) is available for intranasal administration to treat bleeding disorders. This preparation should not be confused with the far less concentrated formulation (brand name DDAVP) that is used to treat enuresis; the latter formulation is ineffective for the treatment of hemophilia and VWD. The appropriate dose of intranasal Stimate is 150 μg (1 puff) for persons weighing less than 50 kg and 300 μg (1 puff in each nostril) for persons weighing more than 50 kg.[143] It is particularly important to document the response to intranasal desmopressin before it is used for the treatment of bleeding.

Antifibrinolytic Therapy. Bleeding from mucosal surfaces in patients with hemophilia is often problematic because of the increased fibrinolytic activity associated with the mucosa. Antifibrinolytic therapy has been found to be effective in controlling mucosal bleeding, especially from the oral mucosa. Although hemostasis is generally achieved with either factor VIII replacement or desmopressin, the risk of recurrent bleeding from oral mucosal surfaces is dramatically reduced with the administration of antifibrinolytic agents. Use of these agents for nasal bleeding or for bleeding from the urinary tract or gastrointestinal surfaces has not been studied extensively. The clearest demonstration of their efficacy is with dental extractions, for which transfusion therapy was previously required for 10 to 12 days. With the use of antifibrinolytic therapy, a single infusion of factor VIII and 7 to 10 days of antifibrinolytic therapy are usually sufficient to prevent recurrent hemorrhage after dental extractions.[51,144] In addition, tranexamic acid has been shown to be effective when used topically as a mouthwash.[145]

Two antifibrinolytic agents, aminocaproic acid and tranexamic acid, are available. Aminocaproic acid (Amicar) is formulated in an intravenous form, oral tablets, and an elixir.[146,147] The tablet contains 500 or 1000 mg, and the elixir contains 250 mg/mL. Because the tablets are large and the usual dose is 5 g (5 to 10 tablets), most adult patients prefer taking the elixir. The oral dose of ε-aminocaproic acid is 100 to 200 mg/kg initially (maximum dose, 10 g), followed by 50 to 100 mg/kg per dose every 6 hours (maximum dose, 5 g). The second agent, tranexamic acid, is available in 650-mg capsules (Lysteda). The dose of tranexamic acid is 25 mg/kg every 6 to 8 hours. No elixir is available for tranexamic acid. Both tranexamic acid (Cyklokapron) and aminocaproic acid are available in intravenous forms.

Treatment of Patients with Hemophilia A Who Have Inhibitory Antibodies to Factor VIII

In 14% to 25% of patients with severe hemophilia A, antibodies develop that inactivate the procoagulant activity of factor VIII; these antibodies are referred to as factor

VIII inhibitors.[112-118] The clinical hallmark of inhibitor development is failure to respond to routine replacement therapy. Patients with factor VIII inhibitors can be divided into two general categories: high responders and low responders. High responders are those in whom high-titer antibody develops with exposure to factor VIII and in whom an anamnestic response usually occurs with subsequent exposure. The titer of their antibody is in excess of 5 Bethesda units and may increase to hundreds or thousands of Bethesda units after repeat exposure. In low responders a titer of inhibitory antibody is maintained at less than 5 Bethesda units, even when they are exposed to repeated doses of factor VIII. The clinical approach is different for these two groups.

Low-Responding Factor VIII Inhibitors. Patients with inhibitor titers of less than 5 Bethesda units[148] can generally be treated with factor VIII concentrates at higher doses. Because the effect of hemophilic factor VIII inhibitor is usually time dependent, the Bethesda titer in plasma is determined after a 2-hour incubation period. As a result of this time delay, continuous administration of factor VIII is usually found to be effective.[149] For a serious limb- or life-threatening bleeding episode, a bolus infusion of 100 U/kg of factor VIII is administered, and the level is maintained by treatment at a rate of 20 U/kg/hr. An assay for factor VIII should be performed 1 hour after the bolus infusion and at least daily thereafter. As the antibody titer drops (caused by the formation of an immune complex with the high-dose factor VIII), the daily level of factor VIII may rise and thus require downward adjustment of the continuous infusion rate. For routine joint and muscle hemorrhage, patients can usually be managed[144] with infusions at twice the usual dosage. A survival study can be performed to determine the peak and half-life of the infused factor VIII. Routine inhibitor assays should be performed after exposure to factor VIII to determine whether an anamnestic response has occurred. Whether the patient remains a low responder or becomes a high responder will determine how the patient is treated for future bleeding episodes.

High-Responding Factor VIII Inhibitors. Treatment of patients with high-responding factor VIII inhibitors requires a much more aggressive approach with one of several alternative therapies. Such treatment is usually performed in sophisticated tertiary care centers by experts in the management of hemophilia.

Continuous Factor VIII Infusion. Most clinicians caring for patients with limb- or life-threatening bleeding episodes prefer to use products for which therapeutic levels can be monitored. As described earlier, continuous administration of factor VIII is often effective because of the time delay in inhibition by the antibody. An initial dose of 100 to 200 U/kg can be administered, and factor VIII levels can be determined 1 hour after initiation of continuous infusion at a rate of 20 to 40 U/kg/hr. If a factor VIII level cannot be obtained (i.e., patients with Bethesda titers greater than 5 to 10 Bethesda units), alternative approaches are usually indicated.

Porcine Factor VIII Concentrate. Because of the high degree of functional homology with human factor VIII, porcine factor VIII represents an attractive alternative for patients with hemophilia and high-responding inhibitors. Plasma-derived porcine factor VIII was a highly purified material produced by polyelectrolyte ion exchange chromatography.[150,151] In patients whose Bethesda titer was less than 50 Bethesda units, a therapeutic factor VIII level was usually attained by using this porcine factor VIII concentrate. Because of contamination with parvovirus, this plasma-derived porcine factor VIII is no longer available. Recombinant porcine factor VIII is being explored as a new therapeutic option for factor VIII inhibitor patients, but it is not yet licensed.[152]

Recombinant Factor VIIa. A recombinant factor VIIa (activated factor VII) concentrate has been developed and demonstrated to produce hemostasis in patients with high-responding inhibitors.[153,154] It is thought to interact with tissue factor and platelets at the site of vascular injury and thereby activate factor X and promote thrombin generation in a manner that bypasses the factor VIII–dependent steps.[155]

The initial dose is 90 to 120 μg/kg of factor VIIa, with additional doses administered every 2 hours. The subsequent rate of infusion and duration of therapy must then be individualized on the basis of the clinical response and severity of bleeding. Recently, the use of higher bolus doses of 180 to 270 μg/kg has been advocated to presumably establish a higher "thrombin burst" at the site of vascular injury.[156]

Prothrombin Complex Concentrate. Prothrombin complex concentrates may sometimes be used to treat routine joint and muscle hemorrhage in patients with hemophilia A and inhibitors. The exact mechanism by which the beneficial effects of these concentrates are produced is still not fully understood. A multicenter controlled study of hemarthrosis demonstrated that these concentrates promoted hemostasis in 50% of bleeding episodes. The initial dose is 75 U/kg of factor IX. If there is no response after two or three infusions given 12 hours apart, it is unlikely that additional treatments will be successful. If repeated doses of these concentrates are administered, consideration should be given to the addition of heparin owing to the potential increased risk for thrombosis.

Activated Prothrombin Complex Concentrates. There is currently one commercially available "activated" prothrombin complex concentrate, FEIBA (factor eight inhibitor–bypassing activity). This product has been purposefully activated during the fractionation process to achieve increased amounts of activated factor VII, factor X, or thrombin. Double-blind controlled trials have demonstrated that this product has small but significant advantages over standard prothrombin complex concentrates.[157] In contrast to porcine factor VIII or continuous-infusion factor VIII, these products are effective even in patients with high-titer inhibitors. The initial dose of 75 U/kg can be repeated in 6 to 12 hours. If multiple doses are administered, the patient should be monitored for the development of disseminated intravascular coagulation or even myocardial infarction, which has been reported with the use of these concentrates.[158] The simultaneous use of antifibrinolytic therapy should be avoided in these patients. If this treatment is not successful, recombinant factor VIIa treatment is usually considered.

Immune Tolerance to Factor VIII. Immune tolerance was first attempted in the 1970s and was achieved by the administration of high doses of factor VIII (100 to 150 U/kg) twice daily and the use of activated prothrombin complex concentrates for acute bleeding episodes.[159] This strategy attempts to eliminate inhibitors through administration of repetitive doses of factor VIII with or without immunosuppressive therapy. After a period of months or even years, the titer of factor VIII antibodies was markedly reduced, and the dose could be decreased to a smaller daily dose or even doses given every other day. A less aggressive approach using lower doses of factor VIII has generally been demonstrated to be less effective.[160] Proposed immune tolerance induction mechanisms include clonal deletion of antibody-producing B cells, induction of suppressor T cells, and synthesis of anti-idiotype antibodies. This approach is effective in as many as 70% of patients with hemophilia A, but much less so in hemophilia B. If bleeding occurs during immune tolerance induction, a bypass agent is used. Either activated prothrombin complexes or activated recombinant factor VIIa has been shown to be efficacious in this setting. Various reports have also described the use of intravenous gamma globulin, immunosuppressive therapy, removal of the antibody by extracorporeal immunoadsorption on *Staphylococcus* protein A columns, or a combination of these modalities.[161] Immune tolerance in four of five patients treated with porcine factor VIII on a home-based infusion program has also been reported.[162] Reports suggest that early treatment of patients with inhibitors to induce immune tolerance is more successful and that higher doses of factor VIII may allow hemophiliacs to become tolerized more rapidly.[163-165]

Hemophilia B

Calculation of Dose. Because factor IX has a volume of distribution different from that of factor VIII, the amount of plasma-derived factor IX that is required to achieve a similar plasma level is approximately twice that for factor VIII:

$$\text{Dose of factor IX (units)} = \text{U/dL (percent) desired rise} \times \text{Body weight (kg)}$$

Thus for the treatment of hemarthrosis in a 20-kg child in whom a level of 80 U/dL is desired, the dose would be 80 U/dL × 20 kg = 1600 units of factor IX.

If recombinant factor IX concentrate is used, the dose must be increased 1.2- to 1.5-fold because of the poorer recovery of recombinant factor IX.[166] Thus in the example just presented, the dose of recombinant factor IX would be 1920 to 2400 units. It is recommended that the recovery of factor IX after an infusion be established for each patient to accurately determine subsequent therapeutic doses. The half-life of factor IX (18 to 24 hours) is much longer than that of factor VIII (10 to 12 hours), and therefore factor IX does not need to be administered as often. Whereas the minimal hemostatic level of factor VIII is 30 to 40 U/dL, that of factor IX appears to be 25 to 30 U/dL.[167]

Factor IX Concentrates

Plasma-Derived Factor IX Concentrates. Hemophilia B can now be treated with highly purified factor IX products derived from plasma through purification or monoclonal immunoaffinity.[168] These highly purified factor IX concentrates are preferred over prothrombin complex concentrates for the treatment of major bleeding episodes, especially in patients with impaired liver function, massive crush injury, or a history of thrombosis. As discussed later, prothrombin complex concentrates are much more likely to be thrombogenic because of the presence of activated vitamin K–dependent clotting factors. Anaphylaxis in association with inhibitor development has been reported in patients receiving high-purity factor IX concentrates.[169,170] It is therefore usually recommended that administration of the first several doses of factor IX be observed in an emergency department where anaphylaxis can be treated if necessary.

Recombinant Factor IX Concentrates. Recombinant factor IX is considered the preferred product for treating patients with hemophilia B. As mentioned earlier, there is reduced recovery of recombinant factor IX, so 20% to 50% more factor IX must be used to achieve the same plasma concentration as plasma-derived factor IX.[171,172]

Prothrombin Complex Concentrates. The vitamin K–dependent clotting factors are of similar size and have similar biochemical and physical chemical properties. Thus fractionation procedures such as barium sulfate precipitation and column chromatography with various adsorbents purify many of the vitamin K–dependent clotting factors together.[173,174] This material is therefore called prothrombin complex concentrate. These concentrates have been found on rare occasion to cause life-threatening thrombotic complications, including deep venous thrombosis, myocardial infarction, pulmonary emboli, and disseminated intravascular coagulation.[175,176] The risk of thrombosis is increased in patients receiving high or repetitive doses, in patients with impaired fibrinolysis (liver disease or after treatment with antifibrinolytic agents), and in those with impaired ability to clear activated clotting factors (i.e., patients with liver disease). When prothrombin complex concentrates are used repetitively, especially in high doses, the International Society of Thrombosis and Hemostasis (ISTH) has recommended the addition of 100 units of heparin to each 500 units of factor IX concentrate.[177] When factor IX is replaced, this level of heparin is not a problem.

Rather than adding heparin to the prothrombin complex concentrates, most physicians currently prefer to use the more purified factor IX products or recombinant factor IX to treat patients with factor IX deficiency when high or repetitive doses are required. Factor IX concentrates and prothrombin complex concentrates have undergone dry heating or vapor heating at high temperatures to reduce the risk of HIV infection and hepatitis C.[178]

Fresh Frozen Plasma. Although fresh frozen plasma was historically used to treat hemophilia B, its use today is limited to areas of the world where factor IX concentrates are unavailable or in emergency situations when factor IX concentrates are not available. If large amounts of fresh frozen plasma are used, the plasma should be ABO blood type compatible to prevent the complication of hemolytic anemia caused by the infusion of isohemagglutinins. Patients with a normal cardiovascular system can safely tolerate a dose of 15 to 20 mL/kg.[179]

Treatment of Hemophilia B Patients with Factor IX Inhibitors

The incidence of inhibitory antibodies to factor IX is much lower than that seen with factor VIII, but these antibodies are commonly associated with the development of anaphylaxis. When such antibodies are identified, activated prothrombin complex concentrates and recombinant factor VIIa have been found to be the most successful therapies. However, the use of activated prothrombin concentrates is contraindicated in patients in whom anaphylaxis has developed along with the inhibitor because they too contain factor IX. Recombinant factor VIIa does not contain any factor IX and is therefore the preferred therapeutic approach. The induction of immune tolerance has been disappointing in patients with factor IX inhibitors. In several patients, nephrotic syndrome has developed when factor IX was given in high doses for induction of tolerance.[180,181]

Complications of Transfusion Therapy for Hemophilia A and Hemophilia B

Transfusion-transmitted infectious diseases are a complication of transfusion therapy with plasma-derived therapeutic products in patients with hemophilia A and hemophilia B. Such diseases include HIV infection, hepatitis B, hepatitis C, and hepatitis A. With the use of recombinant factor VIII and factor IX and the newer methods of viral attenuation, many of the secondary problems associated with these complications can be avoided or minimized. As discussed earlier, hepatitis B and hepatitis A vaccines should be administered to patients before or concomitant with initial therapy.

Comprehensive Care and Home Therapy

Care of patients with hemophilia requires a comprehensive team of health care providers that includes a hemophilia specialist, nurse coordinator, social worker, psychologist, physical therapist, dentist, orthopedic surgeon, financial counselor, primary care physician, and sometimes an infectious disease specialist.[182-184] This approach is considered to be the standard of care and is provided primarily through comprehensive hemophilia treatment centers. The occurrence of primary and secondary complications of hemophilia is reduced, and patient performance both at school and in the workplace is improved.[182] Additionally, overall survival appears to be significantly greater among patients with hemophilia who receive care at hemophilia treatment centers.[184]

A revolution occurred in hemophilia care with the development of factor concentrates and the potential for treatment of bleeding episodes at home. This has become the standard venue for the administration of replacement therapy for the majority of hemorrhages in patients with hemophilia. With home therapy, treatment can be administered sooner, which can reduce the long-term morbidity of hemophilia and thereby reduce the total cost of the disease. Home care programs should be monitored closely by the comprehensive hemophilia team and requires the physician, nurse coordinator, patient, and family to have a close working relationship.[185] Today many patients receive routine primary prophylactic therapy at home administered through a subcutaneous port and a central venous access line.

Future Gene Therapy for Hemophilia A and Hemophilia B

Because of the potential for a complete cure, gene therapy for hemophilia is still being actively pursued on an investigational basis. Animal models of hemophilia A and hemophilia B have been developed or maintained in dogs, mice, and sheep. Early success with gene therapy in these animal models has been achieved, but long-term maintenance of a satisfactory plasma level of clotting factor continues to be an issue. Human gene therapy trials have been carried out for both hemophilia A[187-189] and hemophilia B.[190-192] Although extensive preclinical studies have demonstrated the potential for gene therapy in hemophilia; those clinical trials were somewhat disappointing. More recently a successful trial was published in which the investigators obtained phenotypic correction with persistent expression of greater than 1% factor IX activity. The trial used an adeno-associated virus vector engineered for factor IX expression specific to the liver, resulting in factor IX expression in each of six individuals treated.[27a] New approaches include the use of gain of function factor IX variants that may increase the efficiency of the vector. Because inhibitory antibodies will develop in more than 25% of patients treated with clotting factor concentrates, it is not yet clear whether gene therapy will have a similar incidence or whether continuous release by the transgene will provide tolerance in these patients. One recent preclinical approach consists of directing factor VIII expression to platelets where the factor VIII is stored and can be released locally by platelets.[194-196,197] One group has demonstrated that this approach appears to be effective even in the presence of high-titer inhibitory antibodies.[196] Gene therapy has made significant gains in the last 10 years, but many problems remain, including selection of both appropriate vectors and the appropriate cell in which to express the gene. Control of potential deleterious side effects must still be solved.[186]

VON WILLEBRAND DISEASE

VWD is a disorder of VWF, a large multimeric glycoprotein that: (1) contributes to the adherence of platelets to damaged endothelium and (2) serves as a carrier protein for plasma factor VIII. Deficiency of VWF results in mucocutaneous and posttraumatic or postsurgical bleeding, but if VWF is absent or markedly reduced, the secondary reduction in plasma factor VIII may also result in muscle or joint bleeding similar to that seen in hemophilia A. Erik von Willebrand first described this disorder in 1925 in persons living on the Åland Islands off the coast of Finland.[198-200] VWD is a very common but heterogeneous disorder, resulting from a variety of genetic defects. These include congenital absence of VWF, mutations in which the structure or function of the molecule is markedly abnormal, and mutations associated with mild decreases in expression. Some patients have low VWF levels without a defined genetic defect. It is inherited autosomally; thus in contrast to hemophilia A and hemophilia B, the incidence of VWD in men and women is comparable, although more women may receive the diagnosis because of menorrhagia.

Pathophysiology

VWF is a large multimeric glycoprotein that is synthesized in megakaryocytes and endothelial cells and stored in specific cellular storage granules: the Weibel-Palade body in endothelial cells and the alpha granule in platelets.[201,202-204] Figure 31-1 depicts the role of VWF in localizing and facilitating the hemostatic process. After an injury to the endothelial cell wall of the blood vessel, the local concentration of VWF is increased by the interaction of VWF with components of the subendothelial matrix, such as collagen.[205] When VWF binds collagen, it changes conformation, allowing it to bind to the platelet glycoprotein Ib-IX-V complex (GPIb-IX-V) receptor, thereby resulting in adherence of circulating platelets to the site of damaged endothelium.[206,207] VWF is also capable of interacting with the $\alpha_{IIb}\beta_{III}$ (GPIIbIIIa) receptor on platelets (Chapter 26), and this action may be promoted by shear stress.[208-210] Once VWF causes the platelet to adhere by way of the GPIb-IX-V receptor complex, the platelet is activated, causing recruitment of additional platelets.[211] This is termed *platelet aggregation* and results in formation of a platelet plug (see Fig. 31-1). Formation of the platelet plug also depends on the presence of plasma fibrinogen, the primary ligand for platelet aggregation. Thus in the absence of VWF (or in the presence of VWF with abnormal structure), platelets are poorly targeted to the site of endothelial cell damage, resulting in less than optimal hemostasis.

VWF also serves as a carrier protein for plasma factor VIII and is necessary for normal recovery and survival of factor VIII.[212-214] In the absence of VWF, lack of the carrier molecule function results in a secondary deficiency of plasma factor VIII as the result of accelerated clearance.[74,215] Recombinant factor VIII is produced more efficiently within cells transfected with genes for both VWF and factor VIII.[216,217] Thus patients with severe VWD (type 3) usually have a prolonged bleeding time and prolonged PTT caused by a decrease in both VWF and factor VIII. Milder forms of this disorder can differ and are described in more detail later.

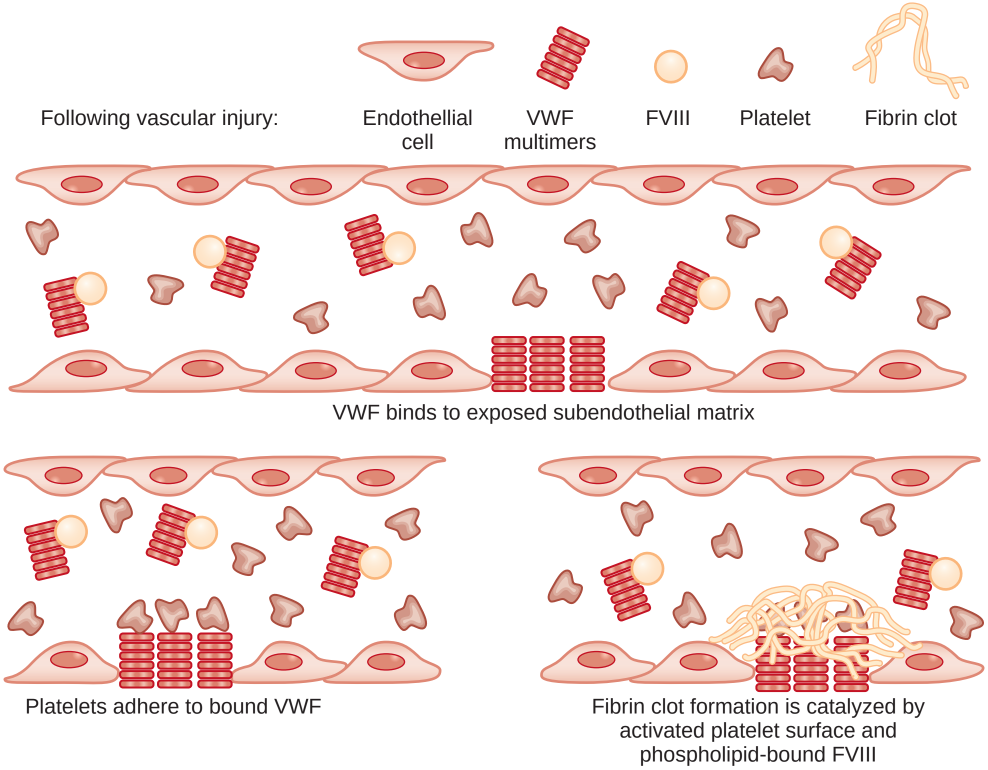

Following vascular injury:
Endothellial cell
VWF multimers
FVIII
Platelet
Fibrin clot
VWF binds to exposed subendothelial matrix
Platelets adhere to bound VWF
Fibrin clot formation is catalyzed by activated platelet surface and phospholipid-bound FVIII

Figure 31-1 After vascular injury, plasma von Willebrand factor (VWF) binds to the subendothelial matrix. Under shear stress VWF changes conformation, which enables the adherence of circulating platelets (platelet adhesion) through the glycoprotein receptor GPIb. Platelets are activated and recruit other platelets through platelet aggregation (platelet cohesion and aggregation). These activated platelets alter their surface phospholipids so that factor VIII (FVIII) binds, interacts with FIXa to activate the X-ase complex, and facilitates formation of the fibrin clot (coagulation).

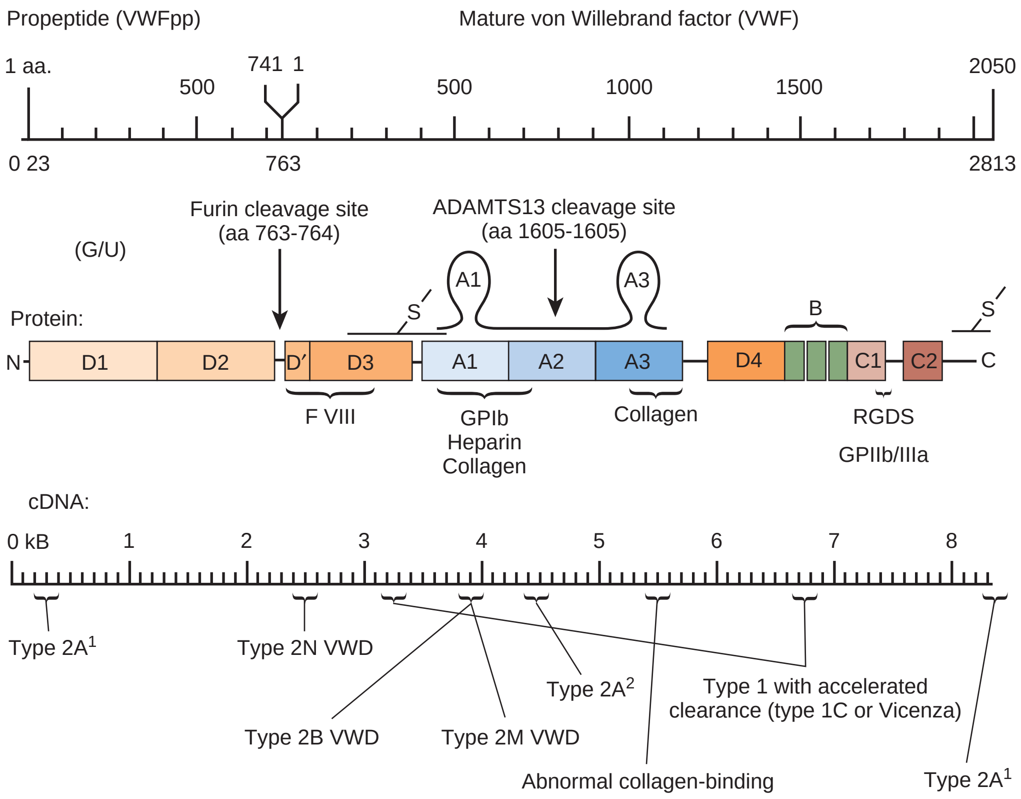

Propeptide (VWFpp)
Mature von Willebrand factor (VWF)
1 aa.
500
741 1
500
1000
1500
2050
0 23
763
2813
Furin cleavage site (aa 763-764)
ADAMTS13 cleavage site (aa 1605-1605)
(G/U)
A1
A3
S
S
Protein:
N
D1
D2
D′
D3
A1
A2
A3
D4
B
C1
C2
C
F VIII
GPIb Heparin Collagen
Collagen
RGDS
GPIIb/IIIa
cDNA:
0 kB
1
2
3
4
5
6
7
8
Type 2A^1
Type 2N VWD
Type 2A^2
Type 1 with accelerated clearance (type 1C or Vicenza)
Type 2B VWD
Type 2M VWD
Abnormal collagen-binding
Type 2A^1

Figure 31-2 Protein structure and its relationship to von Willebrand disease (VWD) variant subtype mutation location. Von Willebrand factor (VWF) contains specific domains that have been shown to have discrete functions, including (1) the interaction with factor VIII (FVIII; D' and D3), glycoprotein Ib (GPIb; A1), collagen (A1 and A3), ADAMTS-13 (A2), platelet GPIIb/IIIa (C1), and furin (D2-D'). The lower panel localizes hot spots for DNA mutations that cause VWF variants that tend to correlate with the functional domains. The variants include type 2A^1 (2A variants that fail to multimerize properly), type 2N (decreased interaction with FVIII), type 2B (increased binding to platelet GPIb), type 2M (decreased binding to GPIb but with normal VWF multimers), type 2A^2 (2A variants caused by increased spontaneous proteolysis of VWF by ADAMTS-13), abnormal binding to collagen, and type 1C (type 1 VWD variant with accelerated VWF clearance). aa, amino acid. *(Courtesy R. R. Montgomery.)*

Variant forms of VWF have been characterized in patients with type 2 VWD (discussed later) that cause reduced binding to factor VIII, reduced binding to platelets or collagen, and reduced size of VWF multimers. Each of these forms can therefore lead to abnormal hemostasis and clinical VWD.

Genetics

VWD is an autosomal disorder, but some patients exhibit a classic recessive inheritance (type 3 and type 2N VWD), whereas others exhibit dominant inheritance (type 2A and type 2B VWD).[218-220] The most common form, type 1 VWD, has variable penetrance and symptoms even within the same family.[218,221] Studies have shown that VWD may occur in as many as 1% to 2% of the general population,[222,223] but this high frequency likely does not represent true VWD (discussed later).[224-226] VWF gene defects are rare in individuals with VWF antigen (VWF:Ag) levels just below the normal range.[218,221,227,228,229]

The *VWF* gene spans 178 kb and contains 52 exons.[230,231,232] It encodes an 8.5 kb message that directs the synthesis of a 22 amino acid signal peptide and a 2791 amino acid pro-VWF monomer that is subsequently cleaved into a 741 amino acid propeptide and a 2050 amino acid mature VWF monomer. Figure 31-2 illustrates the relationship of the complementary DNA (8.5 kb) to the structural and functional regions of the VWF protein. The areas of this gene that have been found to contain clusters of genetic defects that cause similar phenotypes are illustrated and include type 2A,[235,236] type 2B,[237,238] type 2N,[74,239,240] hereditary persistence of pro-VWF,[241] and a type 1 variant of VWD with accelerated clearance of VWF (Vicenza variant or type 1C VWD).[242,243,244] These genetic defects can be identified either by sequencing segments of the gene following amplification by polymerase chain reaction (PCR) or by reverse transcription of messenger RNA from circulating platelets and sequencing the derived complementary DNA after PCR amplification.[236,239,240] Confirmation that any sequence variations found are responsible for the disease requires expression of the abnormal protein after transfection in vitro.[240]

Many laboratories are now carrying out such DNA analysis on unusual variants. Three studies of relatively large cohorts of families have identified a number of VWF mutations associated with the most common type of VWD, type 1.[218,228,245-248] A database of the mutations and polymorphisms found in the *VWF* gene has been established by the VWF Subcommittee of the International Society on Thrombosis and Haemostasis (https://grenada.lumc.nl/LOVD2/VWF/home.php), and a summary of the variant types of VWF has been published.[220] Care must be taken, however, in interpreting DNA sequencing results because polymorphisms are frequent in certain ethnic populations and may mistakenly be labeled as disease-causing mutations.[224] In the mouse extragenic causes of low VWF have been found and are the result of accelerated clearance of VWF owing to abnormal glycosylation.[251] In humans the major modifier of plasma VWF levels is the ABO blood group, because of glycosylation differences in the ABH antigens.[252,253] Additional modifiers of VWF levels or of clinical bleeding symptoms are being sought.

Low von Willebrand Factor Versus Type 1 von Willebrand Disease

Historically, a cutoff level below 2 standard deviations (SD) of normal VWF has been used to define a diagnosis of VWD. However, a 2 SD normal range leaves 2.5% of the population above that range and 2.5% below that range. Some studies suggest that VWD is present in 1% to 2% of the general population.[222,223] The diagnosis of mild type 1 VWD has been challenged on the basis of the argument that the plasma level of VWF is a continuous variable and may be a risk factor for bleeding rather than a defined disease state.[224,225,254] Guidelines have been published by the National Institutes of Health (www.nhlbi.nih.gov/guidelines/vwd) in which the suggested level for the designation of type 1 VWD was a VWF:Ag or VWF:RCo level of less than 30 IU/dL. Patients with bleeding and VWF levels below the normal range may still benefit from therapy such as desmopressin to raise the level of plasma VWF, even if their level is greater than 30 IU/dL. Controversy remains surrounding this issue, and many clinicians still use the term “mild type 1 VWD” to refer to individuals with VWF:Ag levels between 30 and 50 IU/dL. Low VWF or even clinical bleeding symptoms are not always associated with a clearly identifiable VWF gene mutation. Conversely, not all patients inheriting a VWF gene mutation will manifest bleeding symptoms (incomplete penetrance),[255,256] and among those who do bleed, severity can vary widely (variable expressivity).[218,246,257] Alternatively, low VWF or clinical bleeding symptoms in the absence of a VWF gene mutation may result from the effects of mutations in another gene or genes (locus heterogeneity). Research is still necessary to characterize these complex genetic factors in greater detail.

Demographics and Classification

In theory, males and females should have a similar prevalence of VWD, but diagnosis is more common in teenage and adult female subjects because of the frequent complication of menorrhagia. VWD occurs in all racial groups, although localized pockets with a very high incidence of VWF do exist, such as those in the Åland Islands of Finland and areas of Sweden, Israel, and Iran.[258-260] African-Americans have been shown to have higher levels of plasma VWF:Ag, but their VWF activity is not similarly increased.[261,262]

VWD has numerous subtypes that can be defined by structural, functional, and molecular genetic techniques. It is often categorized by quantitative or qualitative defects. Type 1 is characterized by partial deficiency of VWF and type 3 by complete absence of VWF. Type 2 variants are defined by qualitative defects. Of patients with VWD 65% to 80% have type 1 VWD, characterized by a parallel reduction in both VWF antigen and VWF activity as measured by the ristocetin cofactor activity assay (VWF:RCo).[263-265] The second most common variant is type 2A WD, in which the multimeric structure of VWF is altered such that only the smallest VWF multimers are present in plasma. This results in a marked reduction in VWF:RCo with a somewhat milder reduction in VWF:Ag.[266,267] Figure 31-3 summarizes the more common variants of VWD and their relative prevalence. Descriptions of the other VWF variants are provided in subsequent sections. Figures 31-4 and 31-5 depict the mechanisms for disease for the different types of VWD and their correlation with laboratory assays.

Clinical Screening Using Bleeding Assessment Tools

Several recent studies have focused on the clinical symptoms associated with VWD and have attempted to quantify these symptoms in the hopes of improving the specificity of the diagnosis. These scoring systems evaluated epistaxis, skin bleeding, oral or dental bleeding, gastrointestinal bleeding, muscle or joint bleeding, and CNS bleeding.[268,269] The original scoring system was modified to give weight to surgical, dental, or delivery procedures that were not treated and did not result in excessive bleeding. Modified versions have been developed for use in children, but concerns remain given that children have had less time to develop symptoms and fewer exposures to hemostatic challenges than adults.[270]

Laboratory Evaluation

Laboratory evaluation of VWD often requires multiple assays to quantitate VWD and characterize its function and structure. VWD cannot be ruled out on the basis of a normal PTT, a normal bleeding time, or a platelet function analyzer such as the PFA-100.[271,272] Therefore specific assays for VWF are required for diagnosis of VWD in patients with a history of significant bleeding.

von Willebrand Factor Antigen

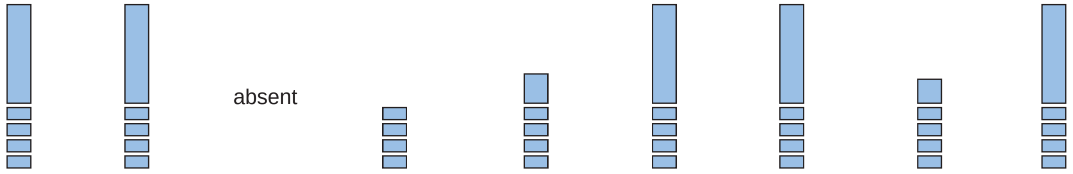
Presence of the VWF protein (VWF:Ag) can be measured by a variety of quantitative immunoassays. The most widely used is the enzyme-linked immunosorbent assay (ELISA).[273-275] The World Health Organization (WHO) and ISTH have established standards for these assays and recommend reporting of results in international units standardized by the WHO.[277]

Ristocetin Cofactor Activity Assay

The activity of VWF is most commonly measured in vitro with the antibiotic ristocetin (VWF:RCo).[264,265] Ristocetin induces binding of VWF to the GPIb receptor on platelets.[208] Large multimers of VWF have more VWF:RCo activity than do the intermediate and small VWF multimers. Some laboratories perform ristocetin-induced platelet aggregation, which is a measure of both binding of VWF to the GPIb receptor and the ability of this binding to induce aggregation. This test is not nearly as sensitive as

VON WILLEBRAND DISEASE VARIANTS AND PLATELET DISORDERS AFFECTING VWF BINDING

	Normal	Type 1	Type 3	Type 2A	Type 2B	Type 2N	Type 2M	PT-VWD*	BSS*
VWF:Ag	N	↓	absent	↓	↓	N or ↓	↓ or N	↓	N
VWF:RCo	N	↓	absent	↓ ↓ ↓	↓ ↓	N or ↓	↓ ↓	↓ ↓	N
FVIII	N	↓ or N	1-6%	N or ↓	N or ↓	↓ ↓	N	N or ↓	N
RIPA	N	often N	absent	↓	often N	N	N or ↓	often N	absent
LD-RIPA	absent	absent	absent	absent	↑↑↑	absent	absent	↑↑↑	absent
PFA	N	N or ↑	↑↑↑	↑	↑	N	↑	↑	↑ ↑ ↑
BT	N	N or ↑	↑↑↑	↑	↑	N	↑	↑	↑↑↑
Platelet count	N	N	N	N	↓ (rarely N)	N	N	↓	↓
Usual Tx		DDAVP VWF conc	VWF conc	VWF conc (DDAVP)	VWF conc	VWF conc (DDAVP)	VWF conc	platelets	platelets
Response to DDAVP		good	none	poor	decreases platelets	poor	poor	decreases platelets	none or modest
Response to VWF Conc		good	good	good	good	good	good	decreases platelets	no response
Frequency in general population		reported as 1–2%	very rare 1:250,000	rare	rare	rare	rare	rare	rare
VWF multimers	N	N or ↓	absent	abnormal	abnormal	N but ↓	N but ↓	abnormal	normal



* Platelet disorders that affect VWF interaction with platelets.

Figure 31-3 Summary of types of von Willebrand disease (VWD types 1, 3, 2A, 2B, 2M, 2N) and absent or defective platelet glycoprotein Ib receptor for von Willebrand factor (VWF) (platelet-type VWD [PT-VWF]) or Bernard-Soulier syndrome (BSS). The *lower portion* illustrates the VWF multimers that are identified by sodium dodecyl sulfate–agarose gel electrophoresis of VWF for each of these variants. *BT,* Bleeding time; *Conc,* concentrate; *DDAVP,* desmopressin (1-deamino-8-d-arginine vasopressin); *LD-RIPA,* ristocetin-induced platelet aggregation to low-dose ristocetin; *N,* normal; *PFA,* platelet function analyzer; *Tx,* treatment; *VWF:Ag,* von Willebrand factor antigen; *VWF:RCo,* VWF activity by ristocetin cofactor assay.

the ristocetin cofactor assay and should not be used to rule out the diagnosis of VWD. Various methods have been developed to automate the VWF:RCo.[278-280] Because ristocetin is a nonphysiologic agonist, a number of alternative assays attempt to use shear as the stimulus for platelet binding, but to date VWF:RCo remains the most widely used assay for VWF function. Newer assays for ristocetin cofactor are being developed that use bound GPIb in an ELISA format either with or without ristocetin.[281-284] The VWF:RCo is also affected by *VWF* sequence variations such as the A1 domain polymorphism D1472H that may affect VWF binding to ristocetin without decreasing in vivo function.[285]

Low-Dose Ristocetin-Induced Platelet Aggregation

One specific use for the low-dose ristocetin-induced platelet aggregation (LD-RIPA) assay is to diagnose the hyperresponsiveness of VWF with platelets in either type 2B

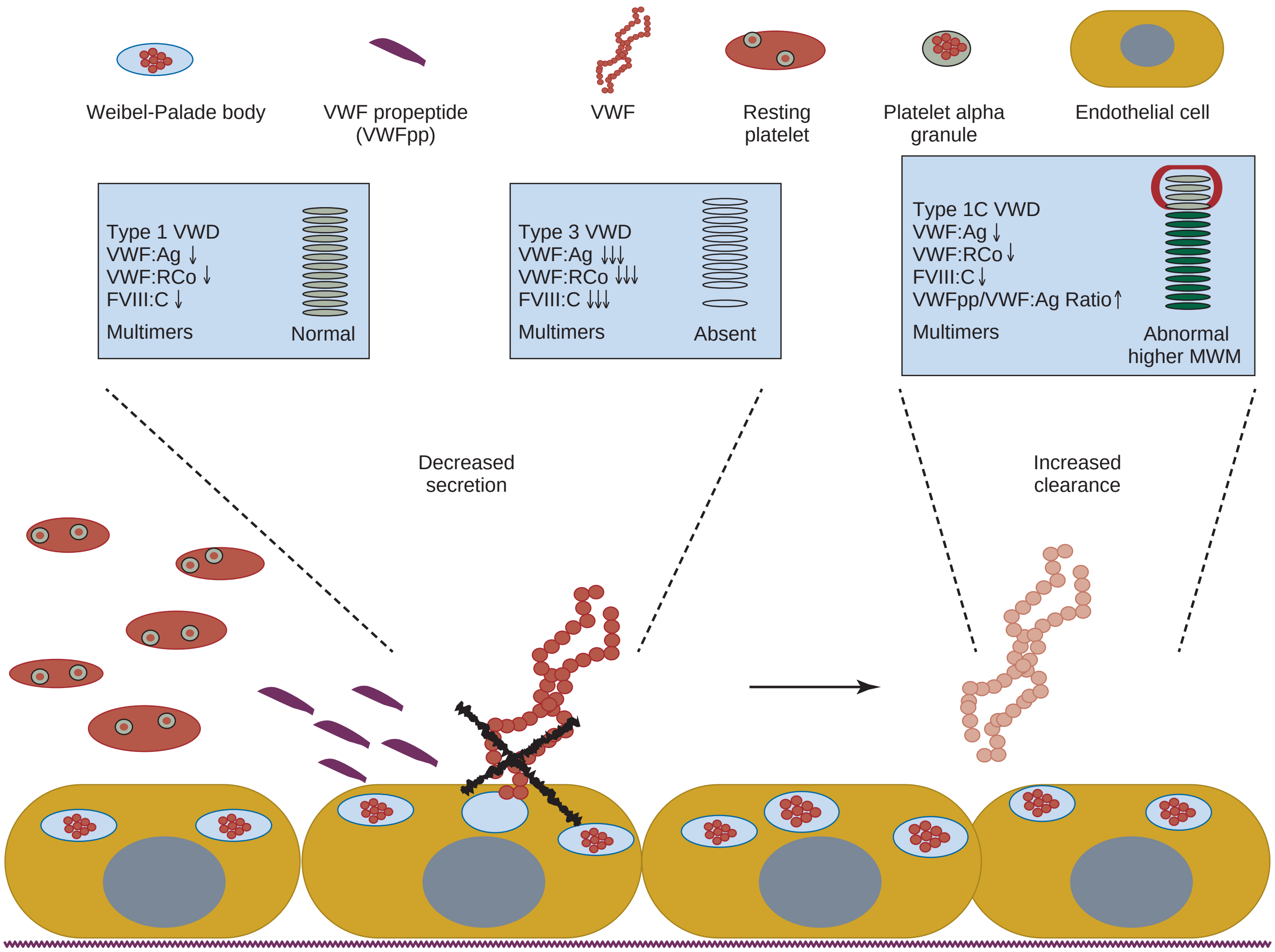

Weibel-Palade body
VWF propeptide (VWFpp)
VWF
Resting platelet
Platelet alpha granule
Endothelial cell
Type 1 VWD
VWF:Ag ↓
VWF:RCo ↓
FVIII:C ↓
Multimers
Normal
Type 3 VWD
VWF:Ag ↓↓↓
VWF:RCo ↓↓↓
FVIII:C ↓↓↓
Multimers
Absent
Type 1C VWD
VWF:Ag ↓
VWF:RCo ↓
FVIII:C ↓
VWFpp/VWF:Ag Ratio ↑
Multimers
Abnormal higher MWM
Decreased secretion
Increased clearance

Figure 31-4 Mechanism of disease for type 1 and 3 VWD and the effect on diagnostic clinical laboratory results.

VWD or platelet-type, pseudo-VWD (see Fig. 31-3).[286] These variants are important to differentiate appropriately because their response to the administration of desmopressin may be suboptimal. Each variant requires specific treatment to correct the qualitative abnormality, with VWF concentrates usually used for type 2B VWD and platelet transfusion for platelet-type, pseudo-VWD.

Collagen-Binding Assay

Assays have been developed to measure the binding of plasma VWF to collagen. In general, large multimers of VWF bind preferentially, although the type of collagen used may affect this specificity. Some have advocated replacement of the VWF:RCo assay with a collagen-binding assay (VWF:CB),[287] but the two assays measure different activities—one with platelets and the other with subendothelial matrix collagen. A few families have been identified who lack binding of VWF to collagen, with mutations located in the VWF A3 domain.[288-290] Some patients with type 2M VWD will show low binding in ristocetin cofactor assays but normal results with collagen-binding assays.[291,292] Most collagen-binding assays use type 1 collagen or type 3 collagen, although a combination of the two may provide the most sensitive results.[293] Recently, collagen binding with type 6 collagen has been described and may identify different defects than assays that use type 1 or type 3 collagen.[294]

von Willebrand Factor Multimers

VWF is present in plasma in multimers ranging from dimers of approximately 600,000 daltons to very large multimers of up to 20 million daltons.[267] The full range of VWF multimers is best demonstrated through the use of low concentrations of agarose (0.65%) and staining the multimers with radiolabeled antibody to VWF. The multimers are visualized by luminography.[266,293] Quantitative assays for multimer distribution are under development.[296] Figure 31-6 demonstrates the multimers in type 1 VWD, as well as in type 2A, type 2B, and type 3 VWD. Acquired alteration of VWF multimers may also occur in various clinical disorders, with the loss of high-molecular-weight multimers seen in patients with disseminated intravascular coagulation or ventricular septal defects.[297] Larger than normal multimers can be identified in patients with recurrent thrombotic thrombocytopenic purpura (Chapter 34).[298] Because multimer assays are technically

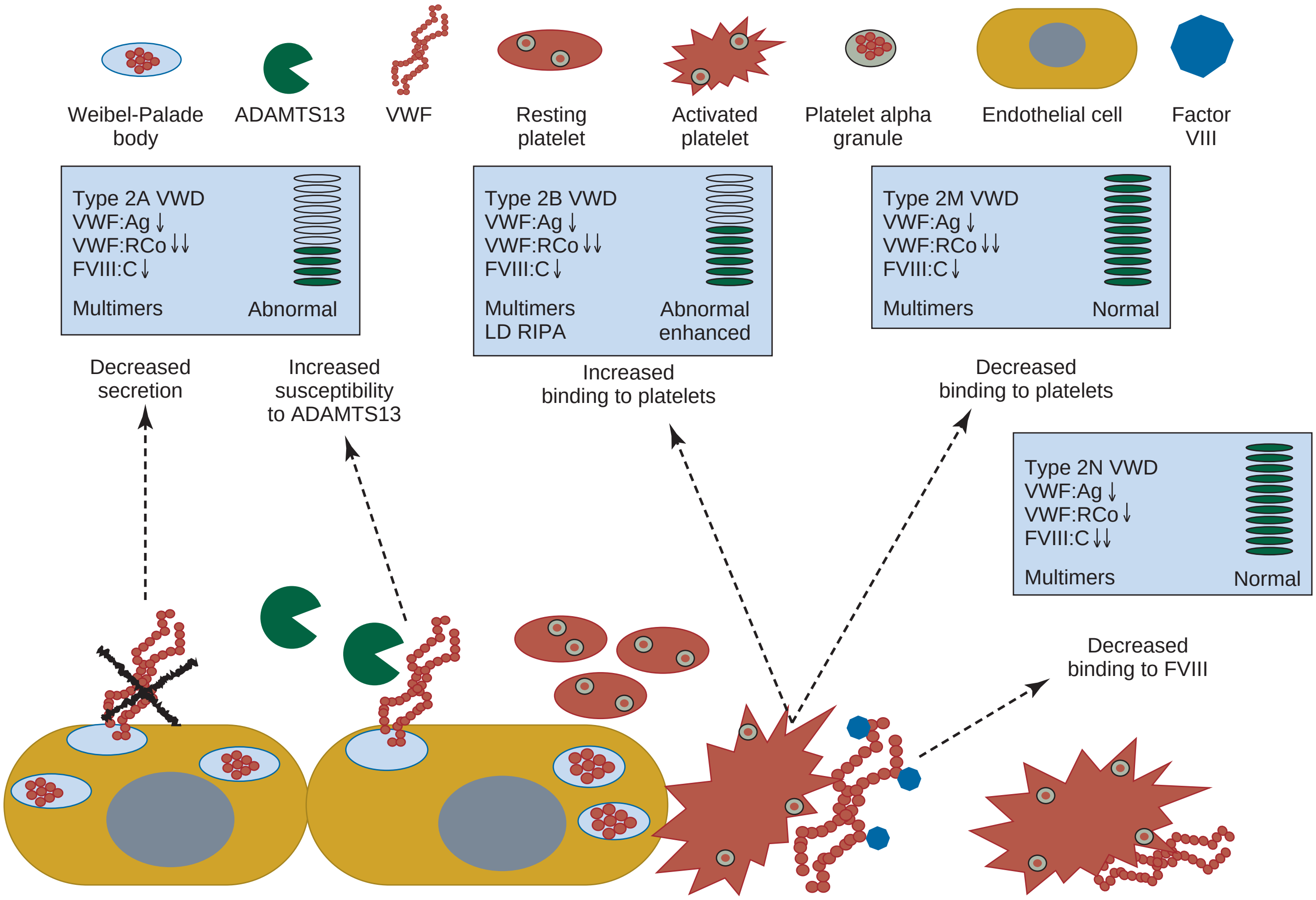

Weibel-Palade body
ADAMTS13
VWF
Resting platelet
Activated platelet
Platelet alpha granule
Endothelial cell
Factor VIII
Type 2A VWD
VWF:Ag ↓
VWF:RCo ↓↓
FVIII:C ↓
Multimers
Abnormal
Type 2B VWD
VWF:Ag ↓
VWF:RCo ↓↓
FVIII:C ↓
Multimers
LD RIPA
Abnormal
enhanced
Type 2M VWD
VWF:Ag ↓
VWF:RCo ↓↓
FVIII:C ↓
Multimers
Normal
Decreased secretion
Increased susceptibility to ADAMTS13
Increased binding to platelets
Decreased binding to platelets
Type 2N VWD
VWF:Ag ↓
VWF:RCo ↓
FVIII:C ↓↓
Multimers
Normal
Decreased binding to FVIII

Figure 31-5 Mechanism of disease for the qualitative type 2 variants of VWD and the effect on diagnostic clinical laboratory results.

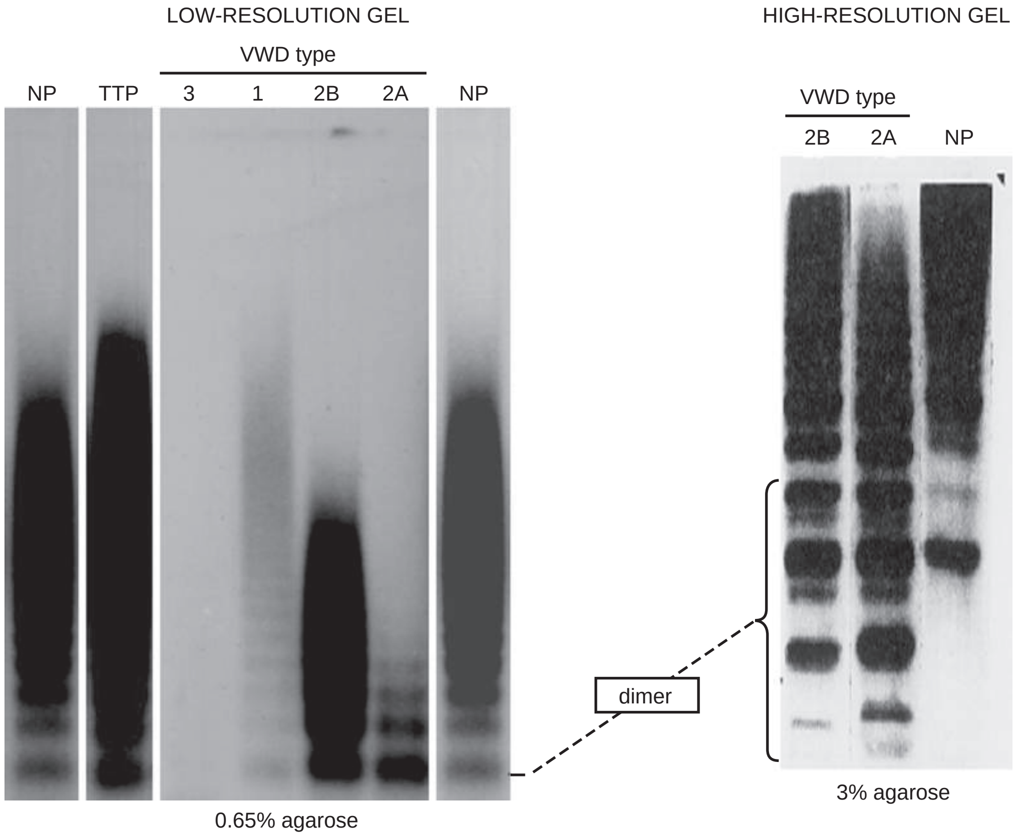

LOW-RESOLUTION GEL
VWD type
NP
TTP
3
1
2B
2A
NP
0.65% agarose
HIGH-RESOLUTION GEL
VWD type
2B
2A
NP
dimer
3% agarose

Figure 31-6 Multimeric analysis of von Willebrand factor (VWF) with low (0.65%) and high (3%) concentrations of agarose. Representative samples are compared and demonstrate that the low-agarose gels are preferable for detecting the loss of high-molecular-weight multimers, as seen in some VWF variants, and the increased size of VWF multimers seen in patients with thrombotic thrombocytopenic purpura (TTP). The gels with higher agarose concentration *(left)* demonstrate that single multimer bands *(left)* can be resolved into multiple satellite bands that may be selectively increased in plasma from patients with variant forms of VWD. *NP,* Normal plasma.

difficult, they are usually performed only at specialized centers.

von Willebrand Factor Propeptide

A number of laboratories now offer quantitation of the VWF propeptide (VWFpp). Because this 741-amino acid propeptide of VWF is synthesized on a 1 : 1 basis with VWF monomer, quantitation of this protein is useful in characterizing clinical states in which VWF has accelerated clearance by identifying individuals whose plasma VWFpp/VWF:Ag ratio is markedly elevated.[235,299] This is possible because the accelerated clearance affects only the VWF:Ag level and not the VWFpp level. This ratio is also markedly elevated in acquired VWD associated with an autoantibody that promotes rapid clearance of VWF.[300] Quantitation of VWFpp and VWF:Ag has also been suggested as a marker to differentiate acute and chronic endothelial perturbation.[301]

VWF Gene Sequencing

Identification of mutations or sequence variations in *VWF* is now possible with gene sequencing, either of the whole *VWF* coding sequence or select regions, such as exon 28. The diagnosis of VWD may be confirmed when well-characterized mutations are found. However, the high frequency of benign sequence variations, particularly in certain ethnic groups, makes the routine use of sequence data more challenging.[224]

Other Assays to Define Variants of von Willebrand Disease

Several additional assays are available at specialized centers to help classify variant forms of VWD. In type 2B VWD, the VWF spontaneously binds to platelets in the presence of low-dose ristocetin. Measurement of this interaction is performed with the type 2B platelet-binding assay.[239,302] In type 2N VWD, VWF has reduced affinity for factor VIII, which is best studied with a type 2N VWF factor VIII-binding assay.[240,303] The VWF:RCo/VWF:Ag ratio has been studied and helps identify patients with type 2M VWD.[291] These assays are depicted in Figure 31-7.

Variables That Affect Laboratory Assays for von Willebrand Factor

A large number of preinstrument variables may affect the determination of VWF. Standardization of assays for VWF has only recently been initiated, and a number of clinical reference plasma samples may have altered ratios of VWF antigen to activity, as well as loss of the high-molecular-weight multimers. Therefore it is imperative that each laboratory establishes its own normal range and performs careful quality control. Alternatively, samples can be sent to laboratories that specialize in VWF testing. When frozen samples are shipped, meticulous attention must be paid to the presence of contaminating platelets in the frozen plasma. If plasma is centrifuged only at 1500*g* for 5 to 10 minutes, the residual platelet count may be as high as 30,000 to 40,000/mL. When this plasma containing platelets is frozen, platelet proteases are released that alter the multimeric structure of VWF and result in an increase in the apparent amount of VWF antigen and a decrease in the VWF activity.[304] Furthermore, refrigeration of samples of whole blood before separation of plasma at a central reference laboratory may result in reducing the amount of VWF in the plasma to be tested.[305]

Clinical Disorders That Affect Assays for von Willebrand Factor

A variety of clinical disorders may affect the level of VWF. VWF behaves as an acute phase reactant protein.[306-308] Its level is markedly elevated in collagen vascular disorders, after surgery, in pregnancy, and in conditions associated with marked endothelial stimulation, such as intravascular clotting or liver disease. The VWF level is reduced in hypothyroidism and increased in hyperthyroidism.[309-312] In hypothyroidism VWF levels as low as 15 to 20 IU/dL have been observed and therefore may be associated with clinical bleeding. The multimeric structure of plasma VWF from patients with dissenminated intravascular coagulation, thrombotic thrombocytopenic purpura, hemolytic-uremic syndrome, or ventricular septal defects may be altered with the loss or gain of high-molecular-weight multimers.

Pregnancy and even the clinical stress of a difficult phlebotomy, especially in anxious young children, may double or triple the level of VWF, thus making it impossible to rule out VWD without repeated testing. The stress of normal vaginal delivery has a marked stimulatory effect on levels of VWF in newborns,[313,314] hence making it difficult to rule out VWD in the neonatal period.

Clinical Manifestations and Medical History

The clinical manifestations of VWD are primarily epistaxis, ecchymoses, menorrhagia, and postoperative or postpartum bleeding. Because this disorder is often mild, the initial manifestation may be bleeding after surgery such as tonsillectomy and adenoidectomy. It is rare to find hemarthrosis in VWD except in patients with severe (type 3) disease. In eliciting the patient's clinical history, it is important to determine whether the size and distribution of ecchymoses are proportional to the severity of the initiating trauma. Because both bruising and epistaxis are moderately common complaints in children and are usually a result of normal childhood trauma, identification of either severe recurrent epistaxis or bruising in unusual places (back and shoulders, upper part of the arms, and thighs) may be more suggestive of a hereditary bleeding disorder. In a normal active child, minor trauma to the anterior tibial region or the forearm may result in small ecchymoses. Recurrent epistaxis is a common complaint in childhood, but in an older child or adult it is an unusual symptom that may require further evaluation. Occasionally patients with VWD have petechiae, although petechiae are more commonly associated with mild platelet function defects.

A careful family history may identify symptoms in the parents or a sibling that are similar to the patient's. The presence of recurrent epistaxis and excessive bruising in an adult is more often abnormal and suggestive of a familial bleeding disorder. The clinician should determine whether either of the parents has experienced

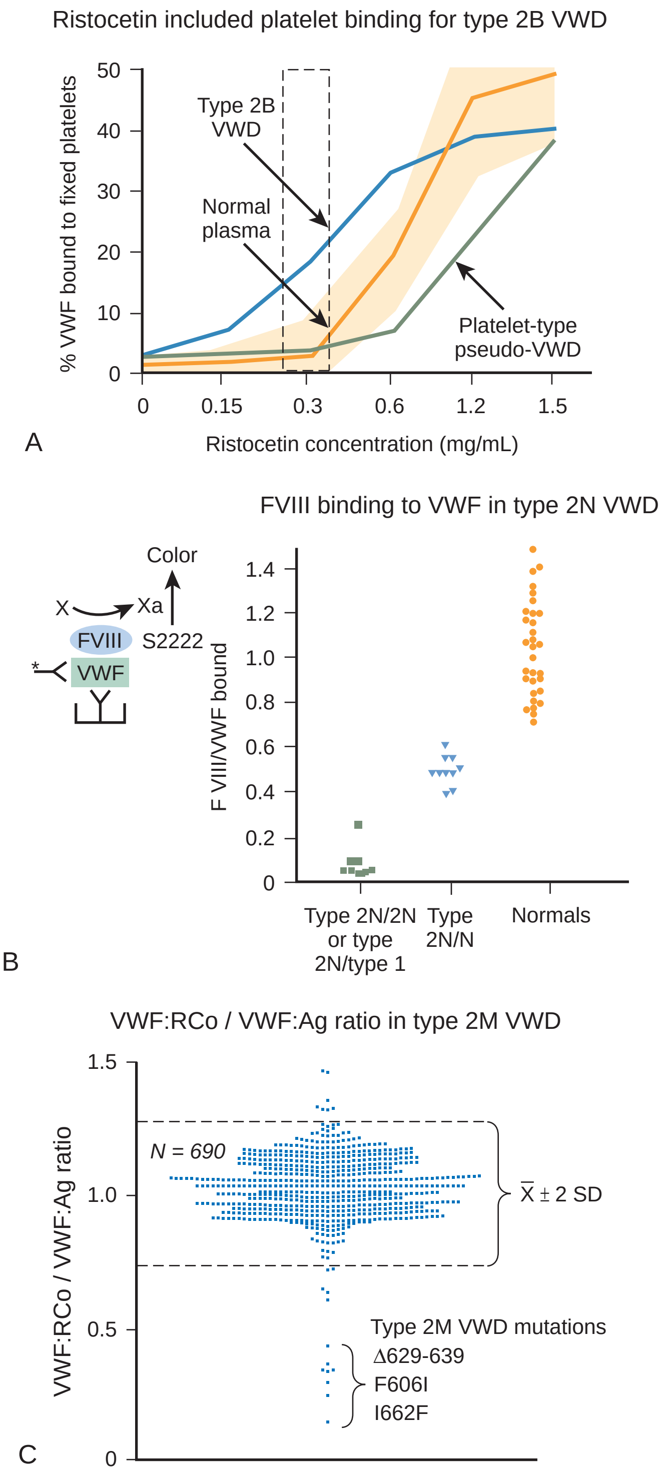


Figure 31-7 Variant forms of von Willebrand disease (VWD) can be identified by specialized phenotypic assays of patient plasma. **A,** A noninhibitory, radiolabeled antibody is added to patient plasma, and binding to normal formalin-fixed platelets is determined with various concentrations of ristocetin. At a low concentration of ristocetin (0.3 mg/mL), normal von Willebrand factor (VWF) does not bind, but type 2B VWF exhibits increased binding that is due to a shift of the dose-response curve to the left. In contrast, plasma from a patient with platelet-type pseudo-VWD shifts to the right. Thus if platelet aggregation with platelet-rich plasma (PRP) exhibits increased aggregation to low-dose ristocetin, type 2B VWD can be clearly differentiated from platelet-type pseudo-VWD with this dose-response curve. **B,** Patient VWF is bound to a microtiter well with a monoclonal antibody to VWF after patient factor VIII (FVIII) is removed. Recombinant FVIII is added and the activity of the bound FVIII determined by chromogenic (S2222) assay. The ratio of bound FVIII to captured VWF:Ag is determined and identifies heterozygous type 2N VWF (usually asymptomatic) and type 2N patients with absent FVIII binding (homozygous or compound heterozygous with a 2N allele and a "VWF-null" mutation allele). **C,** Ristocetin cofactor–antigen (VWF:RCo/VWF:Ag) ratio in a large group of patients evaluated with low-normal or abnormally low VWF levels. The ratios of eight patients with type 2M VWD are clearly below 2 standard deviations of normal, and each of these patients has an identified point mutation (F1379I or I1425F) or a deletion (?1392-1402) in the A1 domain of VWF. Patients with ratios below 0.7 and normal VWF multimers are considered to have type 2M VWD.

postoperative bleeding after major surgery, particularly tonsillectomy. Because VWD is often mild and some families learn to accept increased bruising as the norm in their family, they may not seek medical attention until bleeding occurs in the postoperative state after routine surgery or dental extraction. In teenagers epistaxis or menorrhagia, when excessive, will sometimes lead to iron-deficiency anemia; therefore, iron deficiency, particularly in menstruating teenage girls, may trigger evaluation for the presence of VWD. In a patient with severe (type 3) VWD, profuse epistaxis may occur and even result in shock,[315] although replacement therapy is generally needed less often in patients with type 3 VWD than in patients with hemophilia. In rare instances, VWD has been reported in association with congenital hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome); telangiectases should be sought in a patient with recurrent epistaxis or significant gastrointestinal bleeding.[237,316,317] Gastrointestinal blood loss in VWD may result from either occult or overt bleeding. Although bleeding may be associated with telangiectasia, usually no such anatomic cause can be determined. Such gastrointestinal bleeding may be more common in elderly patients with VWD than in children.

In patients with mild VWD, the stress associated with certain operations (e.g., ruptured appendix) may minimize clinical bleeding symptoms, whereas relatively minor surgery, such as cosmetic or mucosal surgery, may induce a major hemorrhage in a nonstressed individual.

Von Willebrand Disease Classification

Type 1 von Willebrand Disease. Type 1 VWD is the most common variant (70% to 80% of all occurrences of VWD). Type 1 VWD is generally caused by the heterozygous deficiency of VWF. The VWF produced is structurally normal, but its concentration is reduced. Most patients have levels of VWF antigen and ristocetin cofactor activity between 20 and 40 IU/dL. Patients with mild deficiencies of VWF generally do not experience spontaneous hemorrhage unless it is provoked by a major injury, surgical procedure, or dental extraction. Patients with VWF:Ag less than 20 to 30 IU/dL are more likely to have a genetic mutation in VWF than those with higher VWF:Ag levels.[218,228] Intracellular retention and increased clearance have been demonstrated as the mechanism causing low VWF levels for a select group of type 1 VWD mutations.[318,319] A gene deletion involving exons 4-5 has also been demonstrated in type 1 and type 3 VWD.[320] Type 1 VWD is characterized by a proportional decrease in VWF antigen and activity.

Type 1C or the Vicenza Variant of von Willebrand disease. Within the type 1 VWD group are individuals with accelerated clearance of their VWF who usually have plasma VWF:Ag and VWF:RCo levels below 12 to 20 IU/L and factor VIII below 30 IU/dL. The initial prototype for this disorder was termed the *Vicenza variant* because of a number of families who were identified in Italy and subsequently shown to have a common mutation (R1205H in the VWF D3 domain).[244,321,322] These patients have also been shown to have larger than normal VWF multimers and normal levels of platelet VWF. Recently, four additional families were identified with this phenotype and different mutations in the D3 and D4 domains (W1144G and S2179F).[235] This group is now referred to collectively as *type 1C* because its members have type 1 laboratory results secondary to increased clearance of VWF.[235,299] VWF synthesis in type 1C is normal. When desmopressin is administered, patients exhibit hyperresponsiveness, and their plasma VWF will increase fivefold to tenfold, but the half-life is 2 to 3 hours rather than the normal 10 to 14 hours.[235,323] Furthermore, the half-life of factor VIII released by desmopressin is similarly accelerated.[235] Infused VWF and factor VIIII have a normal half-life. Thus treatment with desmopressin will normalize the plasma concentration of VWF and factor VIII, but not for the normal time period. Minor bleeding can be treated with desmopressin, but major bleeding or surgery may require VWF concentrates to maintain the level of VWF activity and FVIII in the normal range. In this group assays of VWFpp versus VWF:Ag are diagnostically elevated in patient plasma, reflecting the accelerated clearance of VWF:Ag, but not VWFpp.[235] Type 1C VWD is characterized by an elevated VWF propeptide to antigen ratio.

Type 3 von Willebrand Disease. Type 3 VWD is usually due to autosomal recessive or compound (double) heterozygous inheritance of a null VWF allele. Thus the level of plasma VWF is usually undetectable, and the factor VIII level is generally less than 6 IU/dL. This plasma level of factor VIII is the steady-state level in the absence of its carrier protein VWF. Patients with type 3 VWD may have spontaneous bleeding, including profound epistaxis; subcutaneous hemorrhage; or, rarely, joint bleeding, as seen in patients with hemophilia.[324,325]

Type 2 Variant Types of von Willebrand Disease

Type 2A von Willebrand Disease. In type 2A VWD, there is a loss of intermediate- and high-molecular-weight multimers as a result of two different mechanisms. These were defined in the first molecular report of VWF mutations in this variant form of VWD.[356] One group is characterized by failure to synthesize intermediate- and high-molecular weight VWF multimers (referred to as type 2A[1] in Fig. 31-2); in the other group, full-length VWF is synthesized, stored, and released, but the VWF undergoes rapid proteolysis by ADAMTS-13 (referred to as type 2A[2] in Fig. 31-2).[326] Bleeding in type 2A VWD is typically more severe than that seen in either type 1 or type 2M VWD, with an observed increase in gastrointestinal bleeding as well as other mucosal bleeding.[327] Type 2A VWD is characterized by low VWF activity and a VWF:RCo/VWF:Ag ratio less than 0.7 along with loss of high molecular weight multimers.

Type 2B von Willebrand Disease. Type 2B VWD is due to a mutant VWF molecule that binds sponatenously to platelets under physiologic shear. This results in clearance of the highest-molecular-weight multimers and usually mild thrombocytopenia.[238,239,328-330] The thrombocytopenia is caused by spontaneous agglutination and clearance of platelets as a consequence of the abnormal VWF and results in loss of the high-molecular-weight multimers. Stress, pregnancy, or surgery can trigger increased levels of VWF with resultant increased agglutination of platelets and enhanced thrombocytopenia.[331,332] Thrombocytopenia is not always present, necessitating careful consideration of the diagnosis.[333] Type 2B VWD is characterized by low VWF activity and a VWF:RCo/VWF:Ag ratio less than 0.7 along with loss of high molecular weight multimers and spontaneous binding to platelets by either direct binding or increased aggregation with low-dose ristocetin, as detailed later.

Type 2N von Willebrand Disease. Type 2N VWD is the result of an abnormal VWF molecular with reduced binding of factor VIII.[74,334,335] This leads to accelerated clearance of the unbound factor VIII and a resultant reduction in plasma factor VIII as compared with the level of VWF.[336] In fact, some type 2N VWD patients may be misdiagnosed as having mild hemophilia A. It appears that the phenotype is clinically expressed only in the presence of a second allele carrying a *null* mutation or a second type 2N VWD allele (see Fig. 31-5). Misdiagnosis of type 2N VWD as mild factor VIII deficiency has been reported and will result in improper treatment of bleeding episodes.[337] Therefore if the plasma VWF level is reduced modestly and the factor VIII level is even more reduced, or if a patient with mild hemophilia A is given factor VIII and the half-life is markedly shortened, type 2N VWD should be considered. For diagnosis of type 2N VWD, a specific assay has been developed in which the patient's VWF is captured with a monoclonal antibody in a microtiter well, factor VIIII is removed by changes in pH or salt concentration or the addition of thrombin, and binding of recombinant factor VIII is determined. Type 2N VWD is characterized by low factor VIII activity and abnormal binding of VWF to factor VIII.

Type 2M von Willebrand Disease. Type 2M VWD is the result of a mutation in VWF that decreases the interaction of VWF with the platelet GPIb receptor, the converse of the effect of type 2B VWD, and also resulting in reduced VWF:RCo.[290,291,338,339] Similar to type 2A VWD, plasma VWF exhibits discordance between VWF:Ag and VWF:RCo (See Fig. 31-3), but patients with type 2M VWD have normal VWF multimers, whereas those with type 2A VWD have abnormal multimers. If the VWF:Ag and VWF:RCo assays are carefully standardized, the ratio of VWF:RCo to VWF:Ag may be used to diagnose type 2M VWD. In this variant the VWF protein can be released by desmopressin, but the released VWF exhibits abnormal interaction with platelets. Thus for major bleeding episodes or in preparation for surgery, replacement therapy with VWF concentrates should be given. Studies of type 1 VWD in two large cohorts identified some patients with mild type 2M VWD,[218,227,340] but whether clinical treatment is different in these patients will require additional study. Type 2M VWD is characterized by low VWF activity and a VWF:RCo/VWF:Ag ratio less than 0.7 with a normal multimer distribution. Mutations in the VWF gene are much more likely in patients with a VWF:RCo/VWF:Ag ratio less than 0.4.[341] Patients with mutations that affect collagen bindings are currently also classified as type 2M VWD.

Figure 31-8 depicts a simplified algorithm for the stepwise diagnosis of the different types of VWD based on laboratory data, in patients in whom there is a high index of suspicion based on personal history of mucocutaneous bleeding.

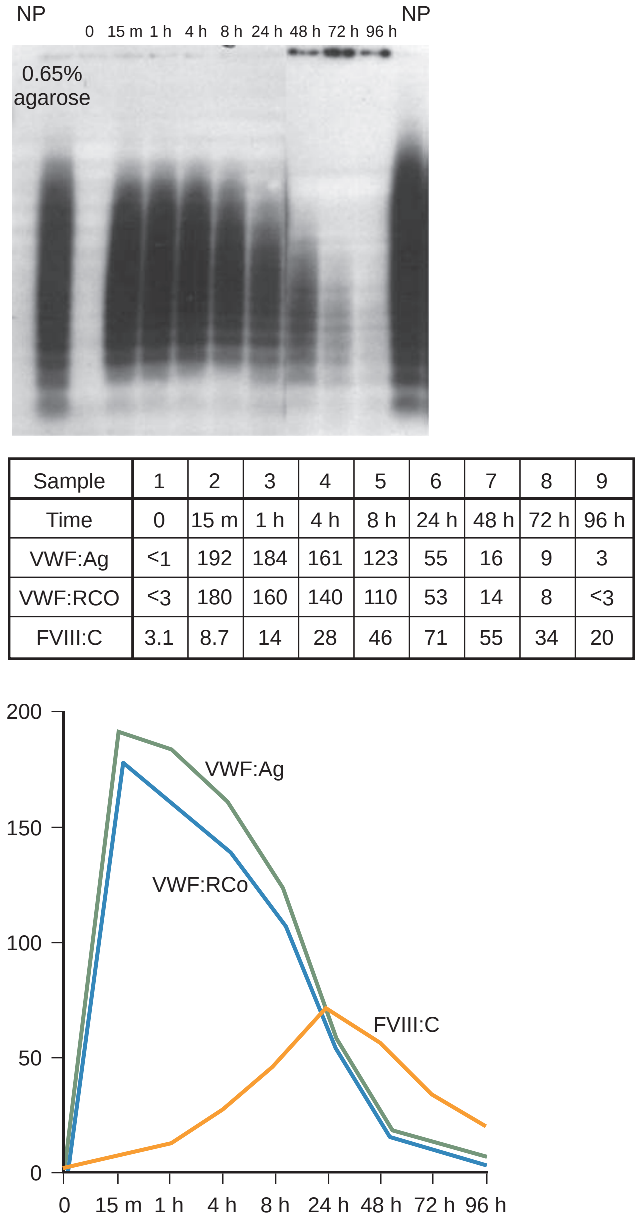
Fundamentals of Treatment

Appropriate treatment of VWD requires a specific diagnostic laboratory workup and correct classification of the type of VWF deficiency. If the patient produces normal VWF but its concentration is reduced, the plasma level can usually be increased by administering desmopressin, which induces release of VWF from endothelial storage sites into plasma.[136] If the protein that is present is abnormal, mild bleeding episodes might still be resolved with increased concentrations of the "abnormal" VWF (induced by desmopressin), but severe bleeding episodes may require the administration of normal VWF from plasma-derived products. Cryoprecipitate contains substantial levels of VWF but is not used regularly because of the lack of viral attenuation treatment. The use of cryoprecipitate is reserved for areas of the world where VWF-containing concentrates are not available.

Desmopressin

The dose of desmopressin is 0.3 µg/kg of body weight administered intravenously over a period of 20 to 30 minutes. This represents the most effective route of administration. Alternatively, subcutaneous injections of desmopressin at approximately the same dose have been used effectively.[342] Stimate (1.5 mg/mL desmopressin) is a concentrated form of desmopressin that has been shown to be effective intranasally.[343,344] The dose in teenagers and adults is 2 sprays, given as 1 spray (150 µg) in each nostril for a total dose of 300 µg. Children weighing less than 50 kg should receive 1 spray (150 µg). For the drug to be effective, the child should be old enough to cooperate with administration. The clinician should be aware that a diluted intranasal solution of desmopressin (100 µg/mL) is available for patients with diabetes insipidus and enuresis. This latter dilute form is ineffective in patients with bleeding disorders. Clinicians should also be aware of the risk of hyponatremia and seizures in patients receiving desmopressin and consider monitoring sodium levels, particularly in high-risk patients.

Although one or two doses of desmopressin combined with either aminocaproic acid or tranexamic acid (see earlier discussion under hemophilia A) is generally effective for dental extractions, major surgery usually requires maintenance of increased levels of VWF and factor VIII until healing has occurred. For most patients repeated infusions of desmopressin at 12- to 24-hour intervals are effective, but tachyphylaxis may develop.[140,345] Thus preinfusion and 30- to 60-minute postinfusion measurements of either VWF:RCo or VWF:Ag should be performed to establish the continued clinical responsiveness of the patient to desmopressin if used repeatedly. If a patient with type 1 VWD has a very low level of VWF (<20 IU/dL) and is to undergo major surgery, the levels achieved after desmopressin may be insufficient even though a twofold increase is achieved. Such patients, like those who either show no response to desmopressin or



Sample	1	2	3	4	5	6	7	8	9
Time	0	15 m	1 h	4 h	8 h	24 h	48 h	72 h	96 h
VWF:Ag	<1	192	184	161	123	55	16	9	3
VWF:RCO	<3	180	160	140	110	53	14	8	<3
FVIII:C	3.1	8.7	14	28	46	71	55	34	20

Figure 31-8 Simplified algorithm for the laboratory evaluation of a patient with possible diagnosis of von Willebrand disease.

have tachyphylaxis, must be treated with plasma-derived VWF/factor VIII.

Von Willebrand Factor Concentrates

The current Food and Drug Administration (FDA)-licensed products that contain both VWF and factor VIII for the treatment of VWD are Humate-P, Alphanate, and Wilate. Clinical trials have demonstrated their efficacy in the cessation of bleeding in patients with VWD.[346-349] Studies have compared these and other products for VWF and factor VIII content, as well as for VWF multimer content.[219] In these comparative studies Humate-P usually has the larger forms of VWF multimers, similar to normal plasma. The products also differ in their relative concentration of factor VIII, with Humate-P containing approximately 0.5 IU factor VIII per IU VWF:RCo and Alphanate containing 2.0 IU factor VIII per IU VWF:RCo. Currently Humate-P is approved by the FDA for surgery and urgent bleeding in patients with types 1, 2, and 3 VWD, and Alphanate is approved for surgery in patients with types 1 and 2 VWD. Wilate is the most recent VWF concentrate to obtain FDA approval for treatment of bleeding in all types of VWD. It contains a 1 : 1 ratio of FVIII and VWF.[350] Other intermediate purity factor VIII and VWF concentrates are sometimes used off-label, but such use is not currently recommended.

The dose of VWF concentrate is calculated as follows:

$$\text{Dose of VWF : RCo} = \text{IU/dL (percent) desired rise in plasma VWF} \times \text{body weight (kg)} \times 0.75.$$

One unit per kg of body weight of VWF will increase the plasma VWF level by 1.5 IU/dL. In preparing a patient for surgery, the clinician usually tries to correct the plasma level to approximately 100 IU/dL; plasma levels should be maintained at greater than 50 to 60 IU/dL for 3 to 7 days after major surgery. For dental extractions or minor surgery, such as repair of a laceration, the patient can generally be treated with one or two infusions of VWF concentrate along with antifibrinolytic therapy.

In Europe a concentrate containing very little factor VIII is approved, but these products are not available in the United States.[351-355] Because synthesis of factor VIII is normal in a type 3 VWD patient, providing VWF alone prevents the problems of elevated factor VIII in patients treated with factor VIII–containing VWF concentrates.[356,357] Figure 31-9 illustrates the delayed rise in endogenous factor VIII when a patient with severe type 3 VWD is administered a concentrate primilary containing VWF.

Type-Specific Treatment of von Willebrand Disease

For type 1 VWD patients, the safest therapy is to augment the patient's plasma VWF concentration by stimulating the endogenous release of VWF with desmopressin, as described earlier.[136] More than 90% of patients with type 1 VWD have a twofold to fourfold increase in their plasma levels of VWF (Fig. 31-10) and factor VIII within 15 to 30 minutes of infusion, with the same half-life as transfused VWF or factor VIII (approximately 12 hours). Occasionally, patients are encountered who do not mount an adequate response to the infusion of desmopressin. This is particularly true for children younger than 1 to 1½ years of age, who may exhibit a diminished response or symptoms of hyponatremia, or both.[141] It is usually recommended that patients in whom VWD is diagnosed be given a therapeutic trial of desmopressin to determine their responsiveness to the drug so that when future therapy is required, the clinician knows that this form of treatment will be effective.

For type 3 VWD patients, administration of desmopressin is not effective because their cells do not contain endogenous VWF and the desmopressin-induced release of factor VIII is dependent on endogenous synthesis of VWD.[215] Sometimes a therapeutic trial of desmopressin in these patients is worthwhile because it might identify patients with type 1C who have VWF:Ag levels below the detection limit in some laboratories.[323] In patients with undetectable VWF or an inadequate response to desmopressin, bleeding symptoms must be managed with plasma-derived VWF or factor VIII/VWF concentrates. Management is similar to that for patients with severe hemophilia and consists of factor VIII concentrates that contain normal or nearly normal VWF. Survival of the transfused VWF is virtually identical to that for factor VIII (discussed earlier). Levels similar to those used for treatment of hemophilia, both preoperatively and postoperatively, should be maintained for adequate hemostasis. Because the licensed concentrates have different ratios of factor VIII to VWF, care must be exercised to ensure adequate factor VIII levels with the first infusion and to avoid excessively high factor VIII levels after several days of intensive or postsurgical treatment.[358]

A number of centers are now using prophylaxis to prevent bleeding in type 3 VWD patients if clinically indicated because of the amount or severity of recurrent bleeding.[359-362] Since the half-lives of VWF and factor VIIII are comparable, the approach is similar to that used in hemophilia A (factor VIII deficiency). In patients with type 3 VWD and large gene deletions, there is an increased likelihood of antibody development.[363,364] Recombinant factor VIIa has been reported to be effective in some patients with severe VWD and alloantibodies.[365-367]

For type 2A VWD patients, if desmopressin is administered, the correction of VWF multimers is transient, and the bleeding time may or may not shorten.[368] Therefore patients with type 2A VWD may require treatment with plasma-derived VWF/factor VIII concentrate containing normal VWF multimers (see earlier). It may be possible to treat minor bleeding episodes and bleeding after dental extractions with desmopressin, which gives a brief increase in plasma levels of VWF. A trial of desmopressin and measurement of in vitro survival of VWF:RCo are required to determine potential efficacy.

For type 2B VWD patients, although one might assume that desmopressin would not be efficacious, studies have demonstrated its effectiveness in some patients.[369,370] Therefore for mild bleeding a trial of desmopressin can be initiated and the platelet count and bleeding monitored. For internal bleeding or major surgery, administration of VWF/factor VIII concentrate is generally required.

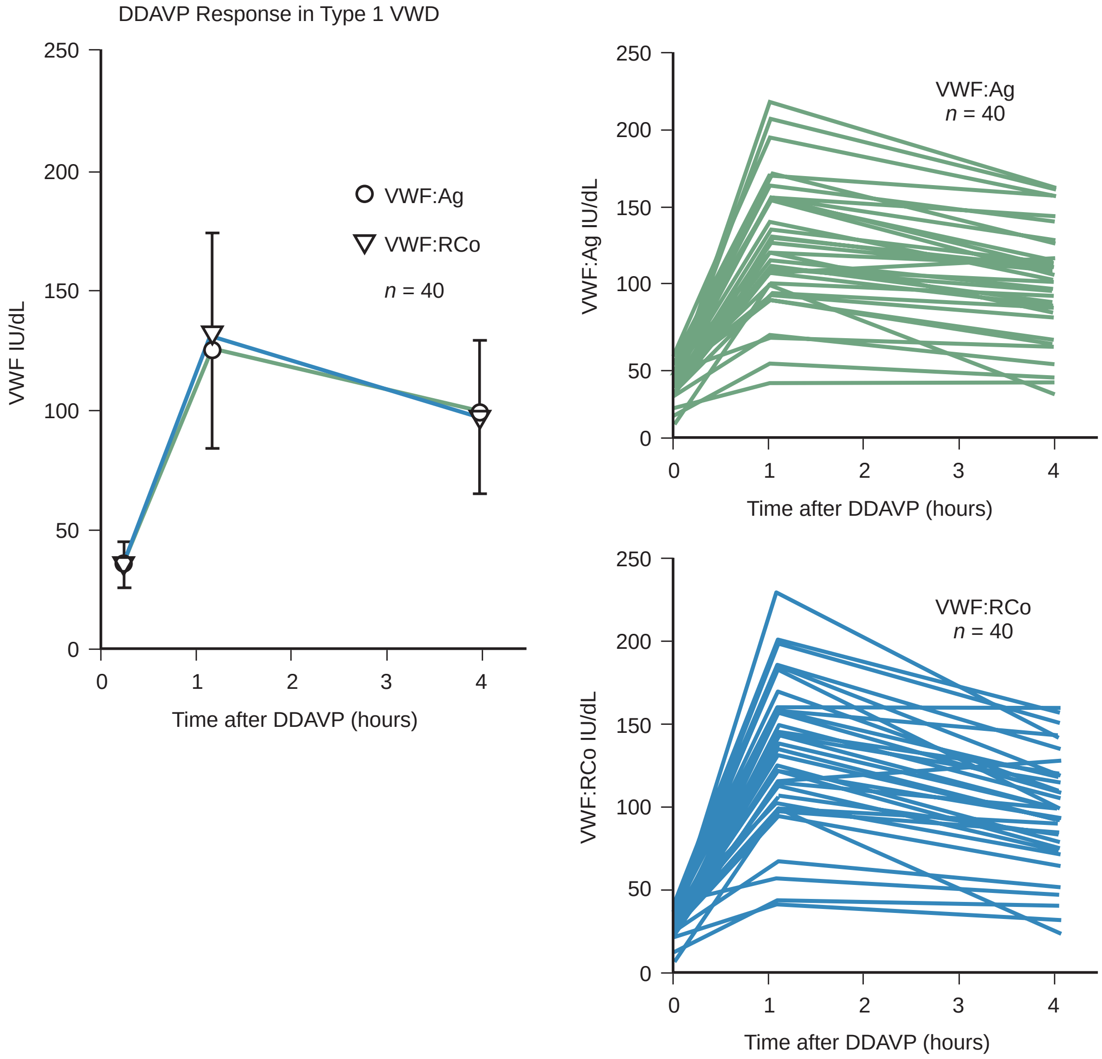


Figure 31-9 A patient with severe type 3 von Willebrand disease was infused with a von Willebrand factor (VWF) concentrate containing minimal amounts of factor VIII (FVIII). Within 15 minutes after infusion, VWF antigen (VWF:Ag) and ristocetin cofactor (VWF:RCo) are markedly increased. At 24 hours the factor VIII level in the patient has increased to 71 U/dL because of the endogenous production of factor VIII. *NP,* Normal plasma.

Although thrombocytopenia is usually present, therapy should be focused on correcting the plasma VWF level. The dose is identical to that used for the treatment of severe type 1 or type 3 VWD. If profound thrombocytopenia is present, concomitant administration of platelets may be necessary. Administration of platelets alone without replacement of VWF is not usually effective. Sometimes, pseudothrombocytopenia may be present and cause the platelet count to appear reduced as a result of platelet clumping.[369,371,372]

Although *platelet-type pseudo-VWD* is caused by a defect in the platelet GPIb receptor[373,374] (see Chapter 30), it is considered in the differential diagnosis of type 2B VWD because of the loss of high-molecular-weight multimers and reduced levels of VWF. The results of plasma VWF assays are identical to those for patients with type 2B VWD, but these disorders must be distinguished because of the markedly different approaches to therapy (see Fig. 31-3). Platelet-type pseudo-VWD should be treated with platelets.[375] Although the plasma level of VWF is reduced and the distribution of multimers is abnormal, these findings are secondary to the abnormal GPIb receptor on platelets. Thus as platelets are appropriately used to treat bleeding, the secondary VWF abnormalities will actually lessen. Standard doses of platelets are used to treat hemorrhage. If the hemorrhage is severe or if bleeding persists, VWF/factor VIII concentrates may be needed to correct the secondary deficiency of VWF. Similar to the normal platelet counts found in patients with intrinsic platelet disorders, the increment in platelets is not necessarily a good measure of the response to therapy because a nearly normal number of abnormal platelets will remain in the circulation. Improvement in the hemostatic abnormality may not become evident until a large proportion of the platelets are replaced by normal platelets from transfusion.

For patients with type 2N VWD, administration of desmopressin or recombinant factor VIII might have only a transient effect on factor VIII levels.[336] For a major bleeding episode or in preparation for surgery, the

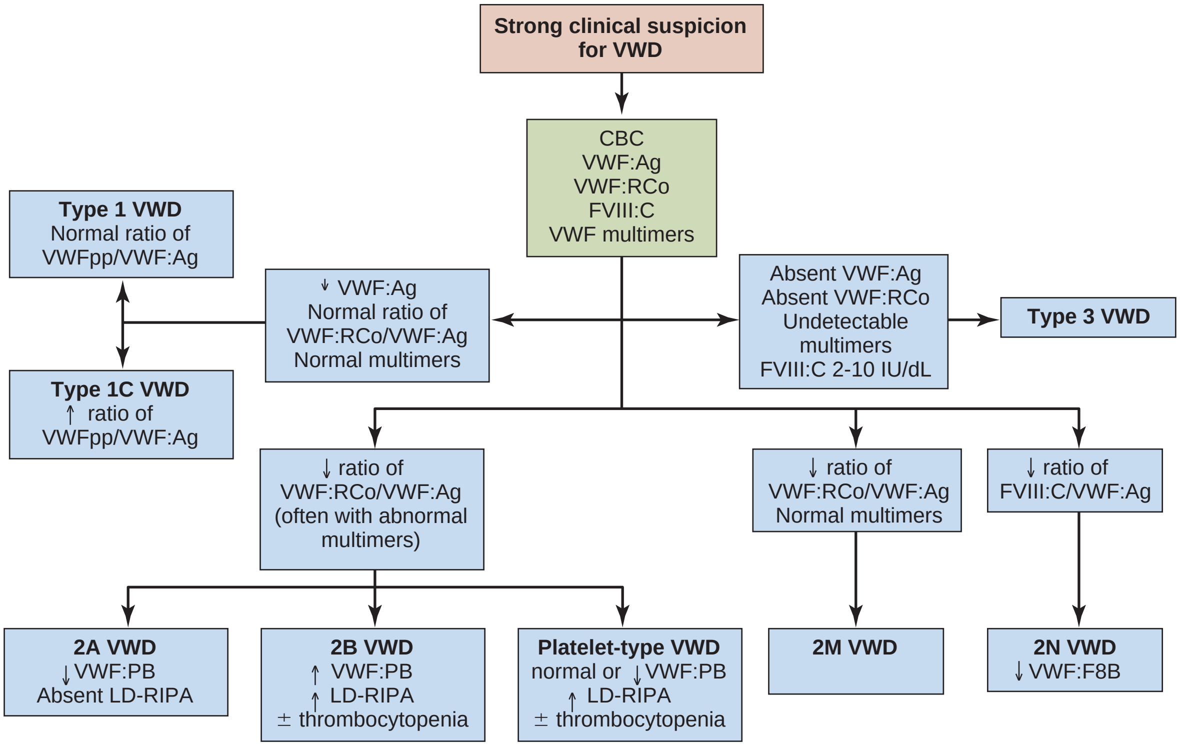

Strong clinical suspicion for VWD
CBC
VWF:Ag
VWF:RCo
FVIII:C
VWF multimers
Type 1 VWD
Normal ratio of VWFpp/VWF:Ag
Type 1C VWD
↑ ratio of VWFpp/VWF:Ag
↓ VWF:Ag
Normal ratio of VWF:RCo/VWF:Ag
Normal multimers
Absent VWF:Ag
Absent VWF:RCo
Undetectable multimers
FVIII:C 2-10 IU/dL
Type 3 VWD
↓ ratio of VWF:RCo/VWF:Ag (often with abnormal multimers)
↓ ratio of VWF:RCo/VWF:Ag
Normal multimers
↓ ratio of FVIII:C/VWF:Ag
2A VWD
↓ VWF:PB
Absent LD-RIPA
2B VWD
↑ VWF:PB
↑ LD-RIPA
± thrombocytopenia
Platelet-type VWD
normal or ↓ VWF:PB
↑ LD-RIPA
± thrombocytopenia
2M VWD
2N VWD
↓ VWF:F8B

Figure 31-10 Desmopressin (*DDAVP*, 0.3 μg/kg intravenously) was administered to 40 patients with type 1 von Willebrand disease (VWD). Plasma samples were obtained before infusion and at 1 and 4 hours after infusion. The *lefthand* panel illustrates the mean and 1-SD range for these 40 individuals. The *righthand* panels illustrate the consistency of the response in these 40 individuals for both von Willebrand factor antigen (VWF:Ag) and ristocetin cofactor (VWF:RCo) activity. *(Courtesy J. P. Scott and R. R. Montgomery.)*

preferred treatment is plasma-derived VWF/factor VIII concentrate.

Acquired von Willebrand Disease

Acquired VWD, also known as acquired von Willebrand syndrome, may be caused by at least three pathophysiologic mechanisms: (1) an acquired clinical state such as hypothyroidism resulting in decreased VWF synthesis, (2) an acquired autoimmune state in which an autoantibody develops against VWF and results in accelerated clearance of VWF (and usually factor VIII), or (3) a clinical disease develops in which VWF structure and function may be altered (Fig. 31-11).

Congenital or acquired hypothyroidism can result in reduced VWF and subsequent bleeding symptoms.[318,376-379] Treating these patients to make them euthyroid results in normalization of the VWF level. If acute bleeding is present, the patient can generally be managed as would a patient with type 1 VWD, by administration of desmopressin or VWF concentrate. In hypothyroid patients the VWFpp level is usually reduced in parallel with the VWF level and suggests normal clearance of VWF.

Acquired VWD can also be the result of an isolated acquired autoantibody to VWF, autoantibody to VWF in autoimmune diseases (e.g., systemic lupus erythematosus), or monoclonal gammopathies.[380-382] Assays for an inhibitor of VWF:RCo activity are generally negative because the autoantibody is directed against other regions of VWF and promotes only clearance. An abnormal VWFpp/VWF:Ag ratio can be used to demonstrate increased clearance of VWF.[235,299,383] In monoclonal gammopathies, VWF multimers may demonstrate loss of high-molecular-weight multimers. Either plasmapheresis or a trial of intravenous immunoglobulin, or both, may sometimes be beneficial.[384,385] Desmopressin may also induce a transient rise in the VWF level, but its survival may be markedly reduced. Some success has been reported with recombinant factor VIIa.[386-388]

Wilms tumor has been reported to cause acquired VWD. In at least one case, synthesis of VWF was still present in endothelial cells in the removed kidney, and in another study the elevated hyaluronidase resulted in a reduced amount of VWF by Laurell quantitative immunoelectrophoresis.[389-392] Acquired loss of high-molecular-weight multimers has been identified in children with ventricular septal defects[294] and pulmonary hypertension[393,394] as well as in patients with aortic stenosis.[395]

von Willebrand Disease Without Known Subtyping

Occasionally, a clinician is called on to treat VWD when the appropriate subtype is not known and the patient's acute condition does not permit taking the time to collect appropriate samples to determine the subtype. In such instances a blood sample should be obtained for appropriate subtyping of VWD, followed by a carefully monitored therapeutic trial of desmopressin, VWF concentrates, or both. The response to such treatment should

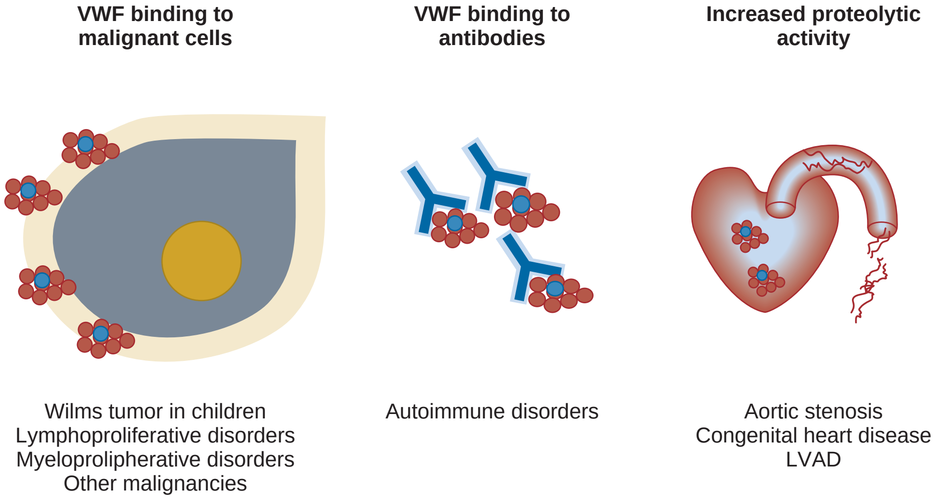

VWF binding to malignant cells
VWF binding to antibodies
Increased proteolytic activity
Wilms tumor in children
Lymphoproliferative disorders
Myeloproliphertive disorders
Other malignancies
Autoimmune disorders
Aortic stenosis
Congenital heart disease
LVAD

Figure 31-11 Mechanisms that account for acquired von Willebrand disease and the conditions and diseases associated with it.

be monitored by close observation of the platelet count and the response of VWF activity and its survival. In a patient with known but untyped VWD and major hemorrhage, VWF/factor VIII concentrates are probably the preferred treatment. This therapeutic approach will appropriately treat all forms of VWD, with the exception of platelet-type, pseudo-VWD. The use of desmopressin may be either ineffective or exacerbate thrombocytopenia in patients with type 2A or type 2B VWD, respectively.

Thus therapy for VWD must be closely integrated with the correct laboratory diagnosis and often requires intensive laboratory monitoring through one episode of treatment. However, once an appropriate response is obtained with a given therapy, such monitoring can be greatly curtailed.

General Comments on the Treatment of von Willebrand Disease

Although the previous section presents the current clinical approach to VWD, many unanswered questions remain. Our current clinical approach may overdiagnose type 1 VWD because of the presence of low plasma VWF levels (see the earlier section on low VWF versus type 1 VWD). Because these patients in general have a preexisting history of excessive bleeding, we do not always know the contribution of their low VWF levels to these symptoms. We therefore generally treat them as having VWD. However, in postsurgical states in normal individuals, it is not uncommon to have VWF and factor VIII levels in excess of 200 to 300 IU/dL, yet we usually focus our therapy only to achieve a hemostatic level above 60 to 100 IU/dL. Whether the normal postsurgical elevation is a pathologic state brought on by surgical stress or a physiologic response to adapt to the surgical stress remains unknown. Normally, VWF is present in plasma, endothelial cells, platelets, and subendothelial matrix. When we treat a patient with type 3 VWD, we normalize only plasma VWF; endothelial cells, platelets, and subendothelium remain abnormal, and their relative contribution to hemostasis is not fully defined. If clinical bleeding continues in the absence of adequate plasma VWF and factor VIII, consideration should be given to platelet transfusion as a means of providing normal platelet VWF.[396]

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CHAPTER 32

Rare Hereditary Coagulation Factor Abnormalities

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CHAPTER OUTLINE

FIBRINOGEN DISORDERS
Hypofibrinogenemia and Afibrinogenemia
Dysfibrinogenemia
PROTHROMBIN DISORDERS
Factor V Deficiency
Combined Deficiency of Factor V and Factor VIII
Factor X Deficiency
Factor VII Deficiency
Factor XI Deficiency
Factor XIII Deficiency
Contact Factor Deficiencies
ANTIPLASMIN DEFICIENCY
Plasminogen Activator Inhibitor 1 Deficiency
Antitrypsin Pittsburgh
MULTIPLE FACTOR DEFICIENCIES

Rare bleeding disorders consist of inherited quantitative or functional deficiencies of factors I (fibrinogen), II, V, VII, X, XI, and XIII; combined factor deficiencies, particularly of factors V and VIII; inherited quantitative or functional deficiencies of vitamin K–dependent factors; and disorders of excessive fibrinolysis. Rare bleeding disorders constitute about 3% to 5% of all inherited bleeding disorders.[1] Many of these disorders, which are variable in their bleeding phenotype, may be encountered in the neonatal period and early childhood, whereas others present later in life. Each of these disorders is reviewed in this chapter; however, the reader is referred to reviews or hemostasis texts in the references for a more complete description of these abnormalities.

FIBRINOGEN DISORDERS

Fibrinogen is the most abundant clotting factor in human plasma, with normal concentrations ranging between 200 to 450 mg/dL. The hereditary fibrinogen deficiency states (afibrinogenemia and hypofibrinogenemia) and functional abnormalities (dysfibrinogenemias), which account for only 7% of the rare bleeding disorders in the most recent World Federation of Hemophilia annual survey, are among the rarest of the rare bleeding disorders.[1] These disorders should be considered when a prolonged thrombin time (TT) is found in a patient with a history of bleeding who is otherwise essentially healthy. Prolongations of the prothrombin time (PT) and activated partial thromboplastin time (APTT) are found in patients with afibrinogenemia and variably in patients with severe hypofibrinogenemia and some types of dysfibrinogenemia, with PT being more sensitive to a moderate decrement in plasma fibrinogen levels.

Hypofibrinogenemia and Afibrinogenemia

Genetic abnormalities that cause decreased fibrinogen synthesis may occur either as a heterozygous deficiency (hypofibrinogenemia) or as a homozygous deficiency (afibrinogenemia).[2] Clinical findings include ecchymoses, subcutaneous hematomas, hemarthroses, mucosal bleeding, menorrhagia, and postoperative bleeding. Because bleeding severity is strongly associated with fibrinogen activity, patients with fibrinogen activity levels of greater than 100 mg/dL seem to be completely protected from bleeding symptoms, whereas patients with activity levels greater than 70 mg/dL are protected from spontaneous bleeding.[3] Afibrinogenemia presents early in life, frequently in the neonatal period, with gastrointestinal bleeding, hemorrhage from delivery-associated trauma, and bleeding with circumcision. In contrast, patients with hypofibrinogenemia may remain undiagnosed until later in life; their condition may be detected as a result of preoperative screening or after posttraumatic bleeding, except in females, for whom menorrhagia may be the only symptom. Pregnancy complications, particularly spontaneous abortions and placental abruption, have also been reported and are known to occur in laboratory animals that have severe hypofibrinogenemia.[4,5] Surprisingly, symptoms in patients with afibrinogenemia are not as severe as those seen in persons with the classic hemophilic disorders.

Laboratory evaluation of a patient with afibrinogenemia reveals prolongations of the TT, PT, and APTT (see Chapter 29). The best screening tests to detect fibrinogen deficiency are the TT and reptilase time, which measure the time required for conversion of fibrinogen in plasma to a fibrin clot.[6] Reptilase is a thrombinlike enzyme obtained from snake venom. Unlike the TT, the reptilase time is unaffected by heparin treatment. Thus if one encounters a very prolonged TT, the possibility of heparinization must be considered, but marked prolongation of the reptilase time is suggestive of fibrinogen deficiency. Fibrinogen may be detected by functional, precipitation, or immunologic assays. The amount of fibrin clot needed for clot detection is greater with automated clotting instruments than with manual methods. Consequently, these tests are frequently prolonged to the limits of the instrument even though some fibrinogen may be present and a clot may be detected with use of manual methods. However, patients with afibrinogenemia generally have undetectable levels of fibrinogen. Although more than 80 distinct mutations in genes encoding fibrinogen have been identified, genetic testing of patients with afibrinogenemia and hypofibrinogenemia is of no direct value in guiding clinical management.[7]

Because fibrinogen is the ligand for the glycoprotein IIb/IIIa receptor that enables platelet aggregation, both the bleeding time and the results of platelet aggregation tests are usually abnormal. Interestingly, in the absence of fibrinogen, von Willebrand factor is able to bind to the glycoprotein IIb/IIIa complex on platelets and provides a backup mechanism for platelet aggregation,[8,9] which may result in a reduction in symptoms associated with this disorder. In many patients a small amount of platelet fibrinogen might facilitate some of these interactions, although this fibrinogen is not believed to be derived from synthesis by megakaryocytes; rather, it is believed to be acquired from plasma.

Clinical bleeding episodes in patients with afibrinogenemia, similar to those observed in patients with hemophilia, require episodic replacement therapy. Because of the long plasma half-life of fibrinogen (about 4 days), prophylactic infusions of fibrinogen should be considered in patients with severe symptoms. With the availability of fibrinogen concentrates, fibrinogen replacement therapy has become simpler compared with cryoprecipitate and fresh-frozen plasma (FFP). RiaSTAP is the only fibrinogen concentrate available in the United States.[10] Because it is derived from pooled human plasma, the risk of transmission of bloodborne pathogens is always a possibility, although such transmission is exceedingly rare. Compared with cryoprecipitate, which has a fibrinogen content of about 10 mg/mL, the fibrinogen content of RiaSTAP is more than 20 mg/mL. Hemorrhagic symptoms are usually controlled by initially achieving plasma levels of 80 to 100 mg/dL and achieving maintenance at a level greater than 50 to 60 mg/dL until the bleeding subsides. To raise the fibrinogen level to 100 mg/dL in an afibrinogenemic patient, approximately 0.17 unit/kg of cryoprecipitate and 60 mg/kg of RiaSTAP must be infused.[11] Because of the prolonged half-life of fibrinogen, replacement therapy can be provided at intervals of 3 to 4 days.

Dysfibrinogenemia

Numerous dysfibrinogenemias, which are named after the city in which they were originally identified, have been reported.[7] Most dysfibrinogenemias are associated either with no clinical symptoms or with hemorrhage, whereas others, interestingly, are associated with a predisposition to venous or arterial thrombosis (see Chapter 33).[12] In one series more than half of patients experienced no clinical complications, whereas bleeding occurred in 25% and thrombosis occurred in 20%.[13] Functional abnormalities of fibrinogen are generally inherited in an autosomal-dominant manner. Occasionally, however, index cases are associated with consanguinity and major hemorrhagic or thrombotic symptoms. Hereditary dysfibrinogenemia that is associated with hemorrhage usually results in a prolonged TT. Laboratory evaluation for dysfibrinogenemia includes functional and immunologic assays for fibrinogen, which should show a normal level of fibrinogen antigen in the presence of reduced functional fibrinogen. The ratio of functional to antigenic fibrinogen is usually less than 0.5. The reptilase time may also be prolonged. An occasional person with dysfibrinogenemia may have a prolonged PT or APTT. The inability of some abnormal fibrinogen to clot completely in vitro can result in false-positive results in fibrin(ogen) degradation product tests. TT and reptilase time are often prolonged in persons who have dysfibrinogenemia associated with thrombosis, though the TT is substantially shortened in some variants.[14] Molecular testing may be of clinical significance in persons with dysfibrinogenemia because some mutations may be predictive of a bleeding phenotype and other mutations may be predictive of thrombosis, but currently molecular testing is only available through research laboratories.[15] Infusion of fibrinogen in the form of cryoprecipitate may be indicated in patients who have clinical bleeding, with a normal rate of survival of this fibrinogen to be expected. Fibrinogen concentrates are not approved for use in persons with dysfibrinogenemia but may be used off-label in patients with a severe bleeding diathesis.

Severe liver disease is occasionally associated with acquired dysfunction of fibrinogen.[16] Studies usually demonstrate that this disease is not hereditary in nature, and the liver disease is generally overt.

PROTHROMBIN DISORDERS

Prothrombin, or factor II, is a vitamin K–dependent glycoprotein with a plasma concentration of approximately 100 μg/mL. The hemostatic level of prothrombin is around 20% to 40%.[17] Prothrombin deficiency is exceedingly rare, with a prevalence of about 1 in 1 to 2 million people, but in a few North American series it has been noted to disproportionately affect Latinos.[18] Similar to congenital abnormalities of fibrinogen, defects in prothrombin present either as quantitative (hypoprothrombinemia, or type 1 disorders) or functional deficiency (dysprothrombinemia, or type 2 disorders).[19] Aprothrombinemia is incompatible with life. Hypoprothrombinemia is inherited as autosomal-recessive defects in the prothrombin gene, leading to decreased production of protein. The mutations resulting in dysprothrombinemia either cause defective activation of the prothrombin molecule by factor Xa or result in its altered interaction with fibrinogen. Occasionally prothrombin deficiency can occur in association with combined deficiency of other vitamin K–dependent factors because of mutations in either the γ-glutamyl carboxylase gene or the vitamin K epoxide reductase gene.[20]

Clinical presentation is strongly associated with the level of functional protein. Heterozygotes are usually asymptomatic, although postoperative bleeding has been reported. Clinical bleeding in homozygotes ranges from easy bruising and mucosal bleeding to severe posttraumatic or postoperative bleeding. Hematomas, umbilical cord bleeding, intracranial bleeding, and major hematomas after vaccination have been reported in the neonatal period.[18] Although the APTT may be slightly prolonged, the hallmark of disorders of prothrombin is usually a prolonged PT and a normal TT. In some cases of dysprothrombinemia, such as prothrombin Cardeza, the PT may be nearly normal even though prothrombin activity is markedly reduced,[21] and thus specific prothrombin assays comparing immunologic levels with functional levels are required to diagnose dysprothrombinemias. The lack of deficiencies of other vitamin K–dependent factors (i.e., factors VII, IX, and X) and liver-dependent factors (i.e., factor V and fibrinogen) usually directs attention to the diagnosis of a hereditary deficiency of prothrombin. Immunoassays and assays of electrophoretic mobility help identify dysprothrombinemia. Molecular testing is only available in the research setting and does not provide much clinical utility.

Treatments currently available for prothrombin deficiency include FFP and prothrombin complex concentrates (PCCs).[10,22] Acute bleeding episodes can be easily treated with FFP. A dose of 15 to 20 mL/kg will usually raise the factor level by about 25%. Given the long half-life of prothrombin (approximately 60 hours), transfusion volume is usually not an issue. Three-factor PCCs contain factors IX, X, and prothrombin and a small amount of factor VII; the amounts vary from product to product and from lot to lot. Four-factor prothrombin factor concentrates contain all four vitamin K–dependent procoagulant factors and the antithrombotic proteins C and S; a description of the actual units of each factor in each vial accompany the product. Four-factor PCCs are indicated for the urgent reversal of acquired coagulation factor deficiency induced by vitamin K antagonist (e.g., warfarin) therapy. These products are labeled and dosed on the basis of factor IX units. These products are not approved for prothrombin deficiency, but a dose of 20 to 30 units/kg will usually be hemostatic. Thrombotic complications resulting from supratherapeutic levels of other factors remain a potential problem. Antifibrinolytic agents are a useful adjunct to factor replacement but are best avoided in combination with PCCs.

Factor V Deficiency

Factor V deficiency, which was first reported by Owren in 1947, has also been termed *parahemophilia*.[23] Its prevalence has been estimated to be 1 in 1 million people with no ethnic predisposition.[1] It is transmitted autosomally and is generally symptomatic only in the homozygous or compound heterozygous state.[24] Factor V activity has limited correlation with bleeding severity. Mucocutaneous bleeding and hematomas are the most common symptoms, but hemarthroses, gastrointestinal tract bleeding, and central nervous system bleeding are rarely encountered. Menarche is frequently associated with severe menorrhagia. Both the PT and the APTT are prolonged, which usually prompts specific factor assays. Immunoassays for factor V are not readily available, and thus most cases are defined by identification of functional deficiencies of factor V with the use of clotting assays. Liver disease, disseminated intravascular coagulopathy, a factor V inhibitor, and combined factor V and VIII deficiency states must be ruled out before diagnosing factor V deficiency.

Clinical bleeding can be controlled with FFP, which is the only currently available source of factor V. Normal hemostasis is achieved with levels greater than 20%. In a patient with severe factor V deficiency, hemostasis can be achieved with a loading dose of 15 to 20 mL of plasma per kilogram of body weight. The half-life of factor V is 12 to 36 hours, and hence daily infusions of plasma are sufficient. Factor V is more labile in FFP compared with other hemostatic factors, and thus it is important to use FFP that is less than 1 to 2 months old. Mild mucocutaneous bleeding can be treated with antifibrinolytic agents.[25] In rare instances, alloantibodies to factor V can develop in patients with congenital factor V deficiency who are, of course, quite symptomatic, but their condition can generally be managed by infusing fresh platelets that contain normal platelet factor V and account for about 20% of circulating factor V.

Occasionally one may encounter a patient who has an acquired inhibitor to factor V. These patients have usually undergone surgical procedures in which topical bovine thrombin has been used.[26] A mixing study can be performed to confirm the presence of an inhibitor. Even though the PT is markedly prolonged, some of these patients do not have major hemorrhagic symptoms.

Combined Deficiency of Factor V and Factor VIII

More than 50 pedigrees featuring an autosomal-recessive bleeding disorder characterized by a combined deficiency of both factor V and factor VIII have been reported.[27,28] In some cases the mutations responsible for this disorder are in the “lectin mannose-binding 1” *(LMAN1)* gene (also known as endoplasmic reticulum–Golgi intermediate compartment protein, or ERGIC-53), which functions as a molecular chaperone from the endoplasmic reticulum to the Golgi apparatus in the biosynthesis of both factor V and factor VIII.[29,30] In other kindreds, this disorder is associated with mutations in the multiple coagulation factor deficiency 2 *(MCFD2)* gene.[31] *MCFD2* encodes a protein that forms a calcium-dependent stoichiometric complex with LMAN1 and acts as a cofactor in the intracellular trafficking of factors V and VIII. The disease is associated with a moderate bleeding tendency and plasma factor V and factor VIII levels of 5% to 30% of normal. Combined factor V and factor VIII deficiency is usually accompanied by fewer symptoms compared with hemophilia A, but when hemostasis is required, it generally can be achieved with FFP.

Factor X Deficiency

Factor X deficiency is a rare autosomal disorder estimated to have a worldwide incidence of about 1 in 1 million people; it is more prevalent in populations that practice consanguineous marriage, such as in Iran.[32,33] Factor X is a vitamin K–dependent factor produced by the liver, and activation of factor X by the "tenase" complexes is considered the first step in the common pathway of coagulation. Similar to other coagulation factors, deficiencies of factor X can also be classified as type 1, in which both antigenic and functional activity are proportionally decreased, and type 2, characterized by near-normal antigenic levels but reduced functional activity.[34] A reduced factor X level is identified by a factor X clotting assay. In patients with dysfunctional factor X, the factor X clotting assay is prolonged, but results of the immunoassay are normal.[35,36] Mucocutaneous bleeding together with posttraumatic bleeding are the most frequent bleeding symptoms. Hemarthrosis and intracranial bleeding are reported in persons with functional levels of less than 1%.[37] The diagnosis is usually suspected when both PT and APTT are prolonged. A factor X functional assay and immunologic assays to measure factor X antigen confirm the diagnosis. Additional assays include the Russell viper venom (RVV) time. RVV is a metalloproteinase that directly activates factor X, and the RVV time will be abnormal in persons with factor X deficiency, although this assay may be normal in persons with some type 2 factor X deficiencies. Factor X levels are low at birth and reach adult levels by about 6 months of age. Factor X levels may also be low in persons with liver disease, accompanied by low levels of factors II, V, VII, and IX.

The biologic half-life of factor X is about 40 hours. Factor X levels of 20 to 30 units/dL are generally associated with the cessation of hemorrhagic symptoms. Infusion of approximately 20 mL of FFP per kilogram of body weight followed by 6 mL/kg every 12 hours increases the level of factor X sufficiently to achieve hemostasis for minor bleeding episodes. Although not approved for the treatment of factor X deficiency, PCC can also be used to treat major hemorrhage (see the previous discussion regarding use of PCCs in persons with prothrombin deficiency); however, their use can potentially be thrombogenic. Although factor X is a vitamin K–dependent factor, vitamin K has no therapeutic effect on factor X in patients with a congenital deficiency. Antifibrinolytic agents can also be adjunctive but are best avoided in combination with PCCs.[34]

Although it is not usually a pediatric problem, amyloidosis may be associated with factor X deficiency because of adsorption of factor X to the amyloid material.[38,39] Therapeutic infusions are not generally efficacious in patients with acquired deficiency of factor X as a result of amyloidosis.

Factor VII Deficiency

Factor VII deficiency has an estimated incidence of 1 per 500,000 in the general population worldwide.[40] Inherited as an autosomal-recessive disorder, the hemorrhagic predisposition in affected patients is highly variable and correlates poorly with plasma factor VII activity levels.[41,42] Heterozygous deficiency is not usually associated with hemorrhagic symptoms. Patients with levels that are less than 1% of normal can experience severe bleeding episodes, including hemarthroses and crippling arthropathy, comparable with patients who have the classic hemophilias. Neonates who have factor VII deficiency are at increased risk for the development of intracranial hemorrhage, which is attributable to trauma at the time of delivery.[42]

Hemostasis screening tests in patients with factor VII deficiency reveal a prolonged PT with a normal PTT and a normal TT. Heterozygotes typically have normal PTs and are identified only after specific factor VII assays are performed in family members of patients who have homozygous or compound heterozygous factor VII deficiency. Laboratory evaluation of factor VII deficiency relies on specific assay of plasma factor VII coagulant activity with the use of animal or human thromboplastin, as well as immunologic quantitation of factor VII antigen.[43,44] Patients have been categorized by their plasma level of factor VII antigen or cross-reacting material (CRM; CRM–, low or absent antigen; CRM^R, reduced antigen; CRM+, normal antigen). CRM– defects result from defective factor VII biosynthesis or accelerated clearance in vivo, CRM+ defects are structurally abnormal factor VII molecules, and CRM^R defects may result from a combination of these mechanisms. Some CRM^R or CRM+ factor VII molecules give variable plasma factor VII coagulant activity results when assayed with tissue factor from different animal species (e.g., rabbit brain, ox brain, simian brain, or human brain).[45-47] As anticipated, the molecular basis of factor VII deficiency is heterogeneous, and several different types of mutations have been described in patients with factor VII deficiency.[48] Most mutations have been missense mutations, but nonsense mutations, small deletions, splice site abnormalities, and promoter mutations have also been identified.

Factor VII is a vitamin K–dependent factor, but vitamin K administration is of no therapeutic benefit in the treatment of hereditary deficiencies. Although commercial PCCs may contain some factor VII, the amount is highly variable among the different commercial preparations. The factor VII content also varies between different lots produced by a single manufacturer. Because the plasma half-life of factor VII is short (approximately 3 to 6 hours), plasma infusions may not achieve adequate levels for normal hemostasis. Factor VII concentrates have been available for patients with factor VII deficiency in clinical trials or on a compassionate-use basis.[49]

The use of activated recombinant factor VII, which has a half-life similar to that of factor VII, has also been shown to provide effective hemostasis in patients with factor VII deficiency[50] and has been approved for this indication in many countries, including the United States. Anecdotally, the therapeutic effect of factor VII infusions appears to be longer than the time during which measurable factor VII levels can be identified in plasma. In one patient treated with PCCs on a regular basis, mucocutaneous bleeding was controlled for 3 to 4 days after a single infusion, even though factor VII was not measurable after 24 hours. It was not clear whether this control of mucocutaneous bleeding resulted from surface binding of factor VII to endothelial cells or platelets, removal of it from plasma, or some nonspecific effect from the concentrate infusion.

Factor XI Deficiency

Factor XI deficiency is usually inherited as an autosomal-recessive disorder. The APTT is prolonged in factor XI deficiency, whereas the PT is normal. A specific assay for factor XI coagulant activity is necessary to make the diagnosis. Factor XI assays are best performed on fresh plasma because the apparent factor XI level may be increased after freezing plasma.[51]

Although factor XI deficiency is most frequently encountered in Jewish persons of Ashkenazi descent, it is occasionally found in other ethnic groups, and the mutations have been characterized.[52,53,54] In Israel, 1 to 3 per 1000 Ashkenazi Jews are homozygous or doubly heterozygous for this deficiency, which calculates to a gene frequency of 4.3%.[55] Sephardic Jews, on the other hand, are rarely affected by factor XI deficiency. Three different point mutations in the factor XI gene account for nearly all cases of factor XI deficiency in Ashkenazi Jews: (1) a splice site mutation at the last intron that disrupts normal messenger ribonucleic acid processing (type I), (2) a nonsense mutation in exon 5 resulting in a premature stop codon (type II), and (3) a missense mutation in exon 9 resulting in substitution of Leu for Phe at 238-amino acid.[56] Although the genotype correlates well with plasma factor XI levels, there is not good correlation with the hemorrhagic tendency.[57] In Ashkenazi Jewish patients who are homozygous or doubly heterozygous for factor XI deficiency, the APTT is often longer than it is in patients with either severe factor VIII or factor IX deficiency.

Although factor XI deficiency has been termed *hemophilia C*, the bleeding tendency is highly variable, and bleeding almost always occurs in association with surgery or trauma. Epistaxis, soft tissue hemorrhage, and bleeding after dental extractions may occur, but hemarthroses and concomitant arthropathy are not seen. Affected women may experience menorrhagia. Even though low factor XI levels are not always associated with bleeding, patients with a significant personal or familial history of bleeding should probably receive FFP before major surgery. In the absence of such a history, prophylaxis with FFP should probably be administered for ear, nose, and throat (e.g., tonsillectomy), neurosurgical, cardiac, or urologic procedures. Patients who have previously undergone major surgery without bleeding or who are having minor procedures that can be controlled easily by local pressure (e.g., dental extraction) may be monitored closely, with initiation of treatment only if hemorrhage occurs. Heterozygous patients with factor XI levels greater than 20% of normal also may occasionally experience bleeding.[58]

Plasma infusions of 1 unit/kg body weight can increase the circulating factor XI concentration by about 1.5 unit/dL. A loading dose of 15 to 20 mL of plasma per kilogram will result in plasma levels of 20 to 30 units/dL, a level that is usually sufficient to control moderate hemorrhage. Additional plasma infusions of 7.5 to 10 mL/kg can be administered at 12- to 24-hour intervals until hemostasis is achieved. The half-life of factor XI is in excess of 48 hours. In factor XI–deficient patients who are deemed to be at risk for bleeding, antifibrinolytic agents such as ε-aminocaproic acid or tranexamic acid can be used to prevent hemorrhage after minor surgical procedures (e.g., dental extractions). A plasma-derived factor XI concentrate is available for use in Israel but not in most other countries.[10] The development of inhibitors to factor XI has been reported in patients with severe factor XI deficiency who have received prior treatments with plasma.[59]

Factor XIII Deficiency

Factor XIII (fibrin-stabilizing factor) is responsible for clot stabilization and cross-linking of the fibrin polymer, protecting it from fibrinolysis,[60] and hence deficiency of factor XIII is characterized by delayed bleeding. In a typical patient who sustains trauma on one day, a bruise or hematoma will develop on the following day. Other symptoms of factor XIII deficiency include delayed umbilical cord hemorrhage, delayed separation of the umbilical stump in the neonatal period (beyond 3.5 to 4 weeks), intracranial hemorrhage with little or no trauma, poor wound healing, and recurrent spontaneous abortions in women.[61] Although factor XIII is a heterodimer of two enzymatic A subunits and two carrier B subunits, most known cases are due to mutations in the A subunit.[62] The deficiency states of factor XIII are also classified as type 1, or antigenic deficiency, and type 2, or dysfunctional protein, with the latter being exceedingly rare.

Laboratory evaluation reveals normal hemostasis screening test results, including bleeding time, APTT, PT, and TT. For a patient with normal screening test results but clinical symptoms of a bleeding disorder, one must perform a factor XIII assay to avoid a misdiagnosis. Screening tests such as clot solubility tests have poor sensitivity but may be the only option in a resource-poor setting.

Recently a heat-treated, lyophilized, plasma-derived factor XIII concentrate, Corifact, has become available.[63,64] Because factor XIII has a very long half-life of 5 to 7 days and a level of 2 to 3 units/dL is hemostatic, the recommended dose of Corifact is 40 international units/kg every 28 days. When needed, an infusion of 5 to 10 mL/kg of FFP is also effective. Cryoprecipitate may also be used. One bag of cryoprecipitate contains 75 units of factor XIII.

Rare cases of acquired factor XIII deficiency as a result of autoantibody against factor XIII-A has been reported.[65] This deficiency is usually associated with autoimmune conditions, and neutralizing antibodies can be detected with mixing studies. Treatment is usually immunosuppression.

Contact Factor Deficiencies

Although factor XI deficiency may be associated with bleeding symptoms, deficiency in any of the other contact factors (i.e., factor XII, prekallikrein, and high-molecular-weight kininogen) is not associated with bleeding symptoms,[66] and thus these factors do not appear to play a major physiologic role in normal hemostasis (see Chapter 27). Because these contact factors function at the initiation step of the intrinsic system, the APTT in severely deficient individuals (i.e., persons with contact factor activity less than 1% of normal) frequently demonstrates prolongations beyond those seen with hemophilia A and hemophilia B. As a result, one encounters the paradoxic situation in which an extremely prolonged PTT is not associated with a bleeding disorder.

Clinicians usually encounter contact factor deficiency when they are asked to evaluate a patient without bleeding symptoms who is found to have a markedly prolonged APTT on a preoperative screening test. It is frequently advisable to carry out these same screening tests for other family members once the specific contact factor deficiency is determined to identify affected family members who could have future surgery inappropriately delayed because of the need for such clotting studies.

Paradoxically, in a number of case reports, venous thromboembolism or myocardial infarction has been noted in factor XII–deficient patients,[67] including the initial patient described with the abnormality. This thrombophilic tendency has been attributed to reduced plasma fibrinolytic activity.[67] In a literature review of 121 patients with factor XII deficiency, an 8% incidence of thromboembolism was found, including several myocardial infarctions in relatively young individuals.[68] Interpretation of such data is difficult because unlike in asymptomatic patients with factor XII deficiency, these complications are likely to be reported in the literature. This situation led some groups to perform cross-sectional analyses of thromboembolic events in larger numbers of unselected families with factor XII deficiency. In Swiss families with factor XII deficiency, 2 of 18 homozygous or doubly heterozygous patients had experienced deep venous thrombosis, although each episode occurred at a time when other predisposing thrombotic risk factors were present.[69] Among heterozygotes with factor XII deficiency, only 1 in 45 had a possible history of venous thrombosis. These investigators concluded that heterozygous factor XII deficiency does not constitute a major thrombotic risk factor, although a severe deficiency may predispose some affected persons to venous thrombosis. Other groups have found a 10% to 20% incidence of thrombotic episodes in heterozygotes.[70,71] Thus it remains uncertain whether an increased thrombotic risk is associated with factor XII deficiency.

ANTIPLASMIN DEFICIENCY

Antiplasmin is a major regulatory protein that controls the activity of plasmin, the pivotal enzyme in the fibrinolytic system that cleaves fibrin. In familial α_2-antiplasmin deficiency, the plasmin activity generated under physiologic conditions is poorly regulated and results in a bleeding disorder.[72] These patients have mucocutaneous bleeding and joint hemorrhage and must be differentiated from persons with mild hemophilia. The usual hemostatic screening test results are normal, including the PT, APTT, and TT. The euglobulin clot lysis time, a measure of fibrinolytic activity, is generally shortened. Although FFP contains α_2-antiplasmin, this clinical disorder is most commonly treated with the intermittent administration of antifibrinolytic agents, including ε-aminocaproic acid and tranexamic acid.

Plasminogen Activator Inhibitor 1 Deficiency

Deficiency of plasminogen activator inhibitor 1 (PAI-1) has been shown to cause a hereditary bleeding disorder.[73,74] In one homozygous case, the genetic defect was shown to be a frameshift mutation.[75] Affected patients can experience recurrent bleeding, especially after surgery or trauma. The PT, APTT, and TT are normal in such patients. The euglobulin clot lysis time can be abnormally short. Specific assays for PAI-1 antigen and activity are required to make a diagnosis of PAI-1 deficiency. Treatment with antifibrinolytic agents, such as tranexamic acid and ε-aminocaproic acid, is effective in treating bleeding complications.

Antitrypsin Pittsburgh

Antitrypsin is not normally a significant regulatory protein of hemostasis. However, several families with an alteration in the α_1-antitrypsin molecule (Met358Arg) have been described, conferring spontaneous antithrombin activity.[76] Continuous inactivation of thrombin results in delayed clot formation and a hemorrhagic syndrome that, like α_2-antiplasmin deficiency, resembles mild hemophilia. Therapy for a disorder featuring excessive antithrombin activity is more difficult than simple replacement in a patient with deficiency of a plasma protein.

MULTIPLE FACTOR DEFICIENCIES

Hereditary deficiencies of multiple factors are rare clinical disorders that are associated with a bleeding diathesis of variable severity. Combined deficiency of the vitamin K–dependent factors (i.e., factors II, VII, IX, and X) can result from deficient vitamin K–dependent γ-carboxylase activity,[77] which mediates posttranslational modifications of these proteins, or defective vitamin K metabolism as a result of mutations in vitamin K 2,3-epoxide reductase.[20,78] Vitamin K 2,3-epoxide reductase is the target of the oral anticoagulant warfarin. As mentioned earlier, factor V and factor VIII deficiency has been reported in more than 50 families. In general, when a patient with multiple factor deficiencies is identified, it is imperative to rule out acquired causes such as liver disease or vitamin K deficiency and to perform a family study before one assumes that such a defect is hereditary.

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CHAPTER 33

Inherited Disorders of Thrombosis and Fibrinolysis

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CHAPTER OUTLINE

HEREDITARY THROMBOTIC DISORDERS
- Antithrombin III Deficiency
- Protein C Deficiency
- Protein S Deficiency
- Resistance to Activated Protein C and the Factor V Leiden Mutation
- Prothrombin G20210A Mutation

HYPERHOMOCYSTEINEMIA

DYSFIBRINOGENEMIAS

INHERITED ABNORMALITIES OF DIMINISHED FIBRINOLYSIS

MANAGEMENT OF PATIENTS WITH INHERITED THROMBOTIC DISORDERS
- Acute Thrombosis and Long-Term Management
- Antithrombin III Deficiency
- Coumarin-Induced Skin Necrosis
- Homozygous or Doubly Heterozygous Protein C Deficiency and Neonatal Purpura Fulminans
- Evaluation of Relatives of Patients with Hereditary Thrombophilia

For many years, researchers suspected that hereditary coagulation defects underlie a large percentage of venous thromboembolic events that could not be attributed to identifiable acquired risk factors. Acquired risk factors for venous thrombosis consist of a heterogeneous group of conditions and clinical disorders with diverse and poorly understood prothrombotic mechanisms. With expanding scientific knowledge of the hemostatic mechanism and the regulatory role of natural anticoagulants, major advances have been made in identifying hereditary defects in the heparan sulfate–antithrombin and protein C pathways during the past three decades. To date, whole genome screening and sequencing efforts have identified several additional genes of interest, although each individually makes a relatively small contribution to thrombotic risk.[1] In this chapter we review the hereditary disorders of thrombosis and fibrinolysis associated with an increased risk for thrombosis. Issues relating to diagnosis and treatment of the hereditary thrombophilias are also discussed.

HEREDITARY THROMBOTIC DISORDERS

Before 1980, the medical literature included only isolated reports of thrombosis in the pediatric population. Although, when compared with adults, thromboembolic events are uncommon in young patients, the incidence is increasing rapidly.[2] This increase is particularly true of children who experience stays in the hospital and is attributed to advances in critical care medicine, improved survival of high-risk patients (e.g., neonates and children with severe congenital heart disease), and increased awareness. The paucity of thrombotic events in children may be related more to vascular/endothelial factors than to hematologic factors, although some of the physiologic inhibitors do not attain full normal adult levels until mid childhood (e.g., protein C and protein S; see Chapter 5). Even patients with hereditary deficiencies of antithrombin (AT), protein C, or protein S, in association with moderate reductions in the levels of these natural physiologic anticoagulants, have few thrombotic symptoms during early childhood. During the past 30 years, however, more vigorous supportive and specific treatment measures for sick preterm infants have resulted in a markedly improved survival rate of even very preterm infants. Because these infants are quite prone to thrombotic events, the physiologic "hypercoagulability" of the developing hemostatic system has received increased attention and is discussed elsewhere (see Chapter 5). Thus the newborn infant can be expected to be at greater risk for thrombosis if he or she also has an underlying hereditary deficiency of an anticoagulant protein. Inherited alterations of coagulation proteins that have been clearly associated with a prethrombotic state are listed in Box 33-1.

Box 33-1 Inherited Alterations of Coagulation Proteins That Have Been Clearly Associated with a Prethrombotic State

- Antithrombin deficiency
- Protein C deficiency
- Protein S deficiency
- Resistance to activated protein C because of the factor V Leiden mutation (factor V Arg506Gln)
- Prothrombin G20210A mutation
- Dysfibrinogenemias (rare)

Antithrombin Deficiency

AT is a plasma protein synthesized by the liver that binds and neutralizes the serine proteases generated by the coagulation cascade, including thrombin, factor Xa, and factor IXa (see Chapter 27). Inhibition of these coagulation serine enzymes by AT is relatively slow but is markedly enhanced by binding to heparin. AT has two major active functional sites: the reactive center toward the carboxyl end and the heparin-binding site located at the amino terminus of the molecule. Thrombin cleaves the reactive site followed by the formation of an inactive complex, which is rapidly cleared from the circulation.

AT deficiency was the first hereditary hypercoagulable disorder identified. In 1965, Egeberg investigated a Norwegian family with a strong history of thrombosis and found that affected persons had plasma AT concentrations that were 40% to 50% that of normal concentrations.[3] Subsequent studies described families with similar clinical and laboratory abnormalities.[4,5] Heterozygosity for AT deficiency can be found in approximately 4% of families with inherited thrombophilia and in 1% of consecutive patients with a first episode of deep venous thrombosis.[6]

The thrombotic risk associated with AT deficiency depends on the population selected. In older reviews of AT deficiency that included families with a high penetrance of thrombosis, more than 50% of affected patients experienced venous thrombotic episodes.[7,8] The initial clinical manifestations occur spontaneously in about 42% of subjects but are related to pregnancy, parturition, hormonal contraceptives, surgery, or trauma in 58% of patients.[7] In children, the odds ratio (OR) of thrombosis with AT deficiency is estimated to be 9.44 (95% confidence interval [CI], 3.34 to 26.66).[9] The most common sites of disease are the deep veins of the leg and the mesenteric veins. Recurrent thrombotic episodes occur in approximately 60% of affected persons, and clinical signs of pulmonary embolism are evident in 40% of affected persons.[7] The literature includes case reports of neonatal and childhood arterial ischemic stroke in association with AT deficiency, but a causal relationship remains to be proven. Although cases have been reported in which AT-deficient infants sustained cerebral venous thrombosis,[7-9] thrombotic episodes are relatively uncommon in affected children before puberty, after which the risk of thrombosis increases substantially with advancing age.[7] First-degree relatives of symptomatic persons with AT deficiency have an eightfold to tenfold increased risk for thrombosis compared with the risk in noncarriers.[10,11] In the Leiden Thrombophilia Study—a case-control study of 474 consecutive patients after an initial episode of deep venous thrombosis[12]—the prevalence of AT deficiency was just 1.1%, and the OR for thrombosis was 5.0.[13]

AT deficiency is inherited in an autosomal-dominant fashion and thus affects both sexes equally. Two major types of inherited AT deficiency have been delineated (Table 33-1). The type I deficiency state is a result of reduced synthesis of biologically normal protease inhibitor molecules.[14] In these cases the antigenic and functional activities of AT in blood are reduced in parallel. The molecular basis of this disorder is either deletion of a major segment of the AT gene or, more commonly, the occurrence of small deletions/insertions or single base substitutions. These mutations introduce a frameshift, a direct termination codon, a change in messenger ribonucleic acid (mRNA) processing, or unstable translation products. The AT mutation database includes more than 100 distinct mutations in patients with type I deficiency.[15]

The second type of AT deficiency is produced by a discrete molecular defect within the protease inhibitor (type II). Plasma levels of AT are greatly reduced as judged by functional activity, whereas AT antigen is essentially normal. The first family with type II AT deficiency was described in 1974.[16] As additional families with this type II AT deficiency have been reported, they have been further subcategorized on the basis of two different functional assays of AT activity. The first is the antithrombin-heparin cofactor assay, which measures the ability of heparin to bind to lysyl residues on the inhibitor and catalyze the neutralization of coagulation enzymes such as thrombin and factor Xa. The second test is the progressive AT activity assay, which quantitates the capacity of this inhibitor to neutralize the enzymatic activity of thrombin in the absence of heparin.

Heterozygous type II patients with both diminished heparin cofactor activity and progressive AT activity have mutations near the thrombin-binding site at the carboxyl terminus of the molecule; patients with reductions only in heparin cofactor activity have mutations in the heparin-binding domain at the amino terminus of the molecule. The distinction between type II defects is clinically relevant because variants with heparin-binding site defects are infrequently associated with thrombotic events, except when they are present in the homozygous state.[17] Patients with homozygous mutations in the heparin binding site leading to AT deficiency were young children in whom severe venous or arterial thrombosis (or both) developed along with plasma antithrombin-heparin cofactor levels measuring less than 10%.[18,19] In each

TABLE 33-1 Assay Measurements in Heterozygous Antithrombin Deficiency

Type	Antigen	Activity	Heparin Cofactor	Progressive Antithrombin
I		Low	Low	Low
II	Active-site defect	Normal	Low	Low
	Heparin-binding site defect	Normal	Low	Normal

instance, a history of parental consanguinity was noted, and the parents had type II AT deficiency with heparin-binding site defects.

The prevalence of type I AT deficiency in the adult population is approximately 1 in 2000.[20,21] Studies of healthy blood donors with functional assays that measure heparin cofactor activity have found a prevalence of AT deficiency in the general population of 1 in 250 to 1 in 500[22,23]; a substantial number, however, have a type II defect with mutations at the heparin-binding site.[21] The best single screening test for the disorder is the antithrombin-heparin cofactor assay, which measures inhibition of factor Xa; it is the functional AT assay that is most widely used today.

The mean concentration of AT in normal pooled plasma is approximately 140 µg/mL. Most laboratories report a normal range between 75% and 120% of normal pooled plasma for antithrombin-heparin cofactor determinations and a somewhat wider range for immunoassay results. A variety of pathophysiologic conditions can reduce the concentration of AT in blood. AT levels can drop substantially in patients with disseminated intravascular coagulation (DIC), sepsis, burns, and severe trauma but less commonly in the setting of acute thrombosis.[24] Reduced levels can also be seen in persons with liver disease as a result of decreased synthesis or in persons with nephrotic syndrome as a result of urinary protein loss.[25,26] Modest reductions are also found in users of oral contraceptives or estrogens.[27,28] In addition, the administration of heparin decreases plasma AT levels, presumably by accelerated clearance of the heparin-AT complex.[29] Thus evaluation of plasma samples from persons during a period of heparinization can potentially lead to an erroneous diagnosis of AT deficiency.

Healthy newborns have about half the normal adult concentration of AT[30,31] and gradually reach the adult level by 6 months of age.[32] Levels may be considerably lower in infants born at 30 to 36 weeks of gestation[32] and are even further reduced in infants with respiratory distress, necrotizing enterocolitis, sepsis, or DIC.[33] In the absence of heparin, AT contributes approximately 80% of the thrombin-neutralizing capacity of normal adult plasma.[34,35] The levels of a second thrombin inhibitor, α_2-macroglobulin, are higher during the first two decades of life than in adults, and this factor may lessen the risk for thromboembolic complications in AT-deficient patients during childhood.[36]

A number of clinical disorders can be associated with reductions in the plasma concentration of AT, and thus making a definitive diagnosis of the hereditary state is often difficult. Although an AT level in the normal range is usually sufficient to exclude the disorder, low levels should be confirmed at a later date. This determination is ideally performed when the person is no longer receiving warfarin because plasma AT concentrations can occasionally be elevated into the normal range in persons with the deficiency state. In such situations, clinical assessment of the person's risk for recurrent thrombosis will determine whether discontinuation of warfarin is feasible. In most AT-deficient subjects, however, this effect of oral anticoagulants is not of sufficient magnitude to obscure the diagnosis.[37] Confirmation of the hereditary nature of the disorder often leads to the investigation of other family members. Diagnosis of other affected family members allows appropriate counseling regarding the need for prophylaxis for venous thrombosis and avoidance of prothrombotic risk factors.

Protein C Deficiency

Protein C is a vitamin K–dependent protein synthesized by the liver that circulates as a zymogen. It exerts its anticoagulant function after cleavage to the serine protease activated protein C (APC). This activation process can be mediated by thrombin alone but occurs much more efficiently when thrombin is bound to thrombomodulin on endothelial cells (see Chapter 27).

In 1981, Griffin and colleagues[38] described low levels of protein C in a family with recurrent thrombotic events. Subsequently, reports were made of many other families with heterozygous protein C deficiency.[39-41] Heterozygous protein C deficiency is inherited in an autosomal-dominant fashion; a more severe form of protein C deficiency is an autosomal-recessive disorder. The phenotype of patients with heterozygous protein C deficiency is similar to that of persons with hereditary AT deficiency. In severely affected families, approximately 75% of protein C–deficient persons experienced one or more thrombotic events. The initial episode occurs spontaneously in approximately 70% of persons experiencing such events. The remaining 30% have the usual associated risk factors (e.g., pregnancy, parturition, hormonal contraceptives, surgery, or trauma) at the time that the acute thrombotic events take place. Symptoms occur infrequently until affected persons are in their early twenties, with increasing numbers of persons experiencing thrombotic events as they reach the age of 50 years.

The most common sites of disease are the deep veins of the legs, the iliofemoral veins, and the mesenteric veins. Approximately 63% of affected patients experience recurrent venous thrombosis, and approximately 40% exhibit signs of pulmonary embolism.[42] Superficial thrombophlebitis of the leg veins, as well as cerebral venous thrombosis, can occur in protein C–deficient patients.[39] The literature includes reports of nonhemorrhagic arterial stroke in children and young adults with hereditary protein C deficiency, but a causal relationship is unproven.

The prevalence of protein C deficiency in outpatients who have experienced an initial episode of venous thromboembolism (VTE) ranges from 0.5% to 4%.[6,13] In earlier reports of more select patient populations, protein C deficiency was more frequently identified and occurred in 2% to 9% of patients. Initial estimates placed the prevalence of protein C deficiency between 1 in 16,000 and 1 in 32,000 within the general population based on the assumptions that protein C was an autosomal-dominant disorder with high penetrance and that at least half the persons with the deficiency would demonstrate symptomatic thrombosis. However, it was difficult to reconcile this figure with the infrequent history of thrombosis in the parents of infants with purpura fulminans, which is due to the homozygous or doubly heterozygous form of protein C deficiency. This disparity led to studies of healthy blood donors in which a much higher prevalence of heterozygosity for protein C deficiency was found, ranging from 1 in 200 to 1 in 500.[43,44]

The risk of thrombosis initially attributed to protein C deficiency was subject to selection bias in that it was overestimated from familial reports. Data from the Leiden Thrombophilia Study indicate that heterozygous protein C deficiency is associated with about a sevenfold increased risk for an initial episode of deep venous thrombosis compared with persons who have normal protein C activity.[13] Among Italian patients, protein C deficiency is associated with a similar sevenfold increase in venous thrombotic risk.[10] In asymptomatic carriers of protein C deficiency, the incidence of thrombosis is fairly low, at 0.4% to 1.0% annually.[45] Similar to other inherited thrombophilias, this variability in phenotypic expression of protein C deficiency is not explained by differences in the particular genetic defect alone and probably represents a complex interaction with other modulating factors. In pediatric patients, the odds ratio of thrombosis with protein C deficiency is estimated at 7.72 (95% CI, 4.44 to 13.4).[9]

Two major subtypes of heterozygous protein C deficiency have been identified with the use of immunologic and functional assays (Table 33-2). The type I deficiency state is the most common form and is characterized by a similar degree of reduction in both the immunologic and biologic activity of protein C. More than 195 different mutations have been identified, most commonly missense or nonsense mutations.[46] The functional or type II deficiency state is characterized by normal synthesis of an abnormal protein. The functional capacity of protein C is most often assessed by using a snake venom protease (Protac)-based assay to directly activate the protein. After activation, an amidolytic assay can assess the functionality of the catalytic site of the protein. A few persons with normal levels of protein C antigen and amidolytic activity but with substantial reductions in protein C anticoagulant activity have been described.[47] In such cases, protein C anticoagulant activity can be measured with a clotting assay (based on prolongation of the activated partial thromboplastin time [APTT]), and abnormalities presumably reflect a reduced ability of activated protein C to interact with the platelet membrane or its substrates such as factor Va and factor VIIIa. The coagulant assay thus has the highest sensitivity in screening patients for hereditary protein C deficiency.

Levels of protein C antigen in the heterozygous deficiency state overlap with those in the normal population, thus making it difficult to define the diagnosis in some patients. In general, antigen levels of 60% to 70% of normal represent borderline values and warrant repeat testing. Protein C antigen levels can also vary with age. Protein C levels in newborns are 20% to 40% that of normal adult levels,[48] and preterm infants have even lower levels.[49] Neonates with significant perinatal thrombosis can have levels suggestive of homozygous deficiency (<10%).[50] Protein C levels often reach adult levels by 1 year of age (see Chapter 5), although further increases are reported into adolescence. In adults, protein C levels typically increase 4% per decade.[43]

TABLE 33-2 Assay Measurements in Heterozygous Protein C Deficiency

		Activity	
Type	**Antigen**	**Amidolytic**	**Coagulant**
I	Low	Low	Low
II	Normal Normal	Low Normal	Low Low

Acquired protein C deficiency is found in numerous disease states, including liver disease, DIC, sepsis, and acute thrombosis. A particularly severe form of acquired protein C deficiency has been reported in association with purpura fulminans and DIC in persons with acute meningococcal infections.[50,51] In contrast to AT, antigenic concentrations of vitamin K–dependent plasma proteins, including protein C, are often elevated in persons with nephrotic syndrome.[52] Most persons with uremia have low levels of protein C anticoagulant activity but normal levels of protein C amidolytic activity and antigen.[53] This situation is attributable to a dialyzable moiety in uremic plasma that interferes with most clotting assays for protein C activity.[54]

As for other γ-carboxylase coagulation proteins (see Chapter 27), warfarin therapy reduces functional and, to a lesser extent, immunologic measurements of protein C, thus complicating diagnosis of the deficiency state.[47] Several researchers have proposed that the diagnosis can still be made in this setting on the basis of the ratios of protein C antigen to factor II or factor X antigen. However, this method will not detect patients with type II deficiency, and it can be used only in subjects in a stable phase of oral anticoagulation.[55] Other groups have used protein C activity assays in conjunction with functional measurements of factor VII, a vitamin K–dependent zymogen with a similar plasma half-life.[56] In practice it is preferable to investigate persons suspected of having the deficiency state either before the initiation of oral vitamin K antagonists or after these medications have been discontinued for at least 1 week, and family studies should also be performed. In patients in whom discontinuation of warfarin is impossible because of the severity of the thrombotic diathesis, studies can be performed while they are undergoing therapy with heparin, which does not alter plasma protein C levels.

Warfarin-induced skin necrosis has been associated with the presence of heterozygous protein C deficiency.[57-59] This syndrome typically manifests during the first several days of warfarin therapy, often in association with the administration of large loading doses of the medication. Skin lesions occur on the extremities, breasts, trunk, and penis and marginate over a period of hours from an initial central erythematous macule. If a product that contains protein C is not administered rapidly, the affected cutaneous areas become edematous, central purpuric zones develop, and the lesions ultimately become necrotic. Biopsies demonstrate fibrin thrombi within cutaneous vessels with interstitial hemorrhage. The dermal manifestations of warfarin-induced skin necrosis are clinically and pathologically similar to those seen in infants who have purpura fulminans resulting from severe protein C deficiency (discussed later).

The pathogenesis of warfarin-induced skin necrosis is attributable to the emergence of a transient hypercoagulable state. Initiation of the drug at standard doses leads to a decrease in protein C anticoagulant activity to approximately 50% of normal within 1 day.[47] Although factor VII activity follows a pattern similar to that of protein C, levels of the other vitamin K–dependent factors decline at slower rates, consistent with their longer half-lives. Increased thrombin generation has been documented in patients during this early phase of warfarin therapy.[60] During this period the drug's suppressive effect on protein C has a greater influence on the hemostatic mechanism than does its reduction of factor VII. Only about a third of patients with warfarin-induced skin necrosis have an inherited deficiency of protein C,[61] and this complication is a rather infrequently reported event in persons with the heterozygous deficiency state. This syndrome has been reported in association with an acquired functional deficiency of protein C.[62] Reports also have been made of hereditary protein S deficiency and the factor V Leiden mutation in association with warfarin-induced skin necrosis.[63-66]

The long-established syndrome of purpura fulminans is classically described as a complication of meningococcal infection in children. However, newborns rarely exhibit a syndrome of purpura fulminans, which is associated with laboratory evidence of DIC and protein C antigen levels that are less than 1% of normal.[67-76] In some instances a history of consanguinity exists in the family, leading to homozygous deficiency of affected infants.[68,71,74] Affected newborns can also be double heterozygotes, as was demonstrated in a Chinese patient[72] who had a five-nucleotide deletion in one protein C allele and a missense mutation in the other.[77] The heterozygous parents of infants with purpura fulminans in association with severe protein C deficiency only infrequently have thromboses, in contrast to patients with thrombotic histories and a hereditary partial deficiency of protein C. However, numerous reports have been made of older patients with homozygous or doubly heterozygous protein C deficiency in whom purpura fulminans was not present. These persons generally had protein C levels that were less than 20% of normal in the absence of oral anticoagulant therapy, and their clinical manifestations were similar to those of severely affected subjects from thrombophilic kindreds with the heterozygous deficiency state.[78,79]

Protein S Deficiency

Protein S is a vitamin K–dependent protein that enhances the anticoagulant effect of APC. Primarily synthesized by hepatocytes but also by endothelial cells, megakaryocytes, and brain cells, protein S serves as a cofactor for APC, which then inactivates factors Va and VIIIa. Factor Va inactivation occurs by an ordered series of peptide bond scissions in the molecule's heavy chain: first, rapid cleavage at Arg506 takes place, followed by slower cleavage at Arg306 and then Arg679 (see Chapter 27).[80] Interaction of protein S with APC results in both an increased affinity for negatively charged phospholipids and a twentyfold enhancement of the slower phase of factor Va inactivation. In plasma, only 40% of protein S is available in the "free" form, whereas the remainder is bound to C4b-binding protein and cannot interact with APC.

Protein S deficiency is an autosomal-dominant disorder that was originally described in 1984 in several kindreds with low levels of protein S and a striking history of recurrent thrombosis.[81,82] A frequency of thrombosis of approximately 10% has been reported in families with inherited thrombophilia[10,83]; however, the prevalence is lower (between 1% and 7%) in consecutive outpatients with a first episode of deep venous thrombosis.[6,13] Protein S deficiency is generally considered to confer a risk of thrombosis similar to that in persons with protein C deficiency, although the association has been complicated by considerable phenotypic variability.

The clinical findings in patients with heterozygous protein S deficiency are similar to those outlined for deficiencies of AT and protein C. Among 71 protein S–deficient members from 12 Dutch pedigrees,[84] 74%, 72%, and 38% of the individuals sustained deep venous thrombosis, superficial thrombophlebitis, and pulmonary emboli, respectively. The mean age at the first thrombotic event was 28 years, with a range of 15 to 68; 56% of the episodes were apparently spontaneous, and the remainder were precipitated by an identifiable factor. Thrombosis has also been reported in the axillary, mesenteric, and cerebral veins. In pediatric patients with VTE, the OR for thrombosis attributed to protein S deficiency is estimated to be 5.77 (95% CI, 3.03 to 11.0).[9] Although the literature includes case reports of young patients with arterial thrombosis (including arterial ischemic stroke) who were found to have protein S deficiency, current data have not established causation between hereditary protein S deficiency and an increased risk for arterial thrombosis.[85]

Three types of protein S deficiency states can be identified on the basis of measurements of total and free antigen, as well as functional activity (Table 33-3). The type I deficiency state is associated with approximately 50% of the normal total protein S antigen level[82] and greater reductions in free protein S antigen and protein S functional activity.[86] This defect is most often a result of missense mutations and base pair insertions or deletions. The type II qualitative deficiency state, characterized by normal total and free protein S levels but abnormal functional activity, has been identified infrequently, thus

TABLE 33-3 Assay Measurements in Heterozygous Protein S Deficiency

Type	Protein S Antigen	PROTEIN S ACTIVITY	
		Total	Free
I	Low	Low	Low
II	Normal	Normal	Low
III	Normal	Low	Low

suggesting that current functional assays may not screen for all such defects. In persons with type III deficiency, total protein S antigen levels are normal, with disproportionately decreased free protein S and functional activity. Thus initial evaluation for protein S deficiency should test for protein S functional activity.

The biologic basis of the type III protein S deficiency state is uncertain. Furthermore, coexistence of type I and type III deficiency has been reported in some protein S–deficient families, which has led to the proposal that the two types of protein S deficiency are phenotypic variants of the same genotype and are not the product of distinct genetic mutations.[87] In a follow-up analysis of one large family that had a high prevalence of both type I and type III deficiency, total protein S antigen levels—but not free protein S antigen levels—were shown to directly correlate with age.[88] These findings were independent of gender and were also seen in nondeficient family members. The researchers concluded that a single point mutation in the protein S gene was responsible for the quantitative type I deficiency but that the type III phenotype was actually due to an age-dependent free protein S deficiency; with increasing age, the relative concentrations of free protein S to total protein S decreased. However, in a Dutch cohort study of first-degree family members with protein S deficiency it was found that affected relatives of probands with type I deficiency were at increased risk for VTE, whereas affected relatives of probands with type III deficiency were not at increased risk for VTE.[85]

Acquired protein S deficiency has multiple causes, including pregnancy and oral contraceptive use. Protein S levels are commonly low in inflammatory states, including DIC and acute thrombosis, largely because of protein S interaction with C4b-binding protein. C4b-binding protein is an acute phase reactant and shifts protein S to the complexed, inactive form, which leads to decreased protein S activity.[89] Levels of total and free protein S are significantly reduced in men infected with human immunodeficiency virus.[90] Although total protein S antigen measurements are generally increased in persons with nephrotic syndrome, functional assays are often reduced[52] because of loss of free protein S in urine and elevated C4b-binding protein levels.

The lower limit of normal total and free protein S in plasma is approximately 65%; however, considerable overlap exists between the heterozygous deficiency state and levels at the low end of the normal range. Such overlap is largely due to the influence of age, which leads to an increase in protein S levels, and the influence of gender, with females having a lower normal limit for plasma protein S levels compared with males. Thus it is difficult to diagnose heterozygous protein S deficiency by performing a single assay; repeat sampling and family studies are usually required to make the diagnosis.

An epidemiologic study demonstrated that adults with low protein S levels and a negative family history of venous thrombosis have minimal, if any, increase in risk of a first episode of VTE.[91] Among 5317 patients and control subjects evaluated, a free protein S level below the 2.5th percentile (i.e., <53 units/dL) did not confer an increased risk of thrombosis compared with a higher free protein S level (OR, 0.82; 95% CI, 0.56 to 1.21). A free protein S level below the 0.10th percentile (i.e., <33 units/dL) was associated with an increase in risk of VTE that was not statistically significant (OR, 5.4; 95% CI, 0.61 to 48.8). Total protein S levels did not correlate with thrombosis, even below the 0.10th percentile. The results of this study imply that the increased risk of VTE is minimal in persons with protein S deficiency in the absence of a family history.

Young patients with recurrent VTE disease associated with doubly heterozygous or homozygous protein S deficiency have been identified.[81] The parents of these patients were asymptomatic and had laboratory studies consistent with type I protein S deficiency. Neonatal purpura fulminans has been described in association with homozygous protein S deficiency.[92,93]

Resistance to Activated Protein C and the Factor V Leiden Mutation

Before 1993, hereditary deficiencies were infrequently identified in patients with familial thromboembolic disease. Dahlbäck and colleagues[94] identified several probands with venous thrombosis that appeared to be resistant to APC in an APTT-based clotting assay. When compared with control subjects, many family members of the probands also had a blunted anticoagulant response to APC. The following year, Bertina and colleagues[95] identified the genotype underlying most cases of APC resistance as a single point mutation in the factor V gene, leading to substitution of Arg506 by glutamine. This mutation at an APC cleavage site renders factor Va relatively resistant to inactivation by APC.[80,95]

After the description of APC resistance by Dahlbäck and colleagues,[94] several studies revealed that this defect was relatively common in persons with venous thrombosis. In 1994 Svensson and Dahlbäck[96] screened 104 consecutive Swedish persons referred for evaluation of venous thrombosis and found that 33% of the subjects demonstrated APC resistance. In a U.S. referral population of persons younger than 50 years who had unexplained VTE disease, Griffin and colleagues[97] found that approximately 50% exhibited APC resistance.

Dutch investigators had previously initiated the Leiden Thrombophilia Study to identify risk factors for a first episode of venous thrombosis. Excluding patients with known malignancies, 345 consecutive outpatients younger than 70 years who had confirmed deep venous thrombosis were initially reported.[98] With use of a screening APTT-based assay, APC resistance was identified in 21% of persons with thrombosis and in 5% of age- and sex-matched healthy control subjects. The factor V Arg506Gln (or factor V Leiden) mutation was found in more than 80% of the patients who had been found to be APC resistant.[95] The lower frequency of APC resistance identified in the Leiden Thrombophilia Study compared with previous studies is attributable to differences in selection criteria and referral cohorts.

The U.S. Physicians' Health Study has also provided valuable data regarding factor V Leiden as a risk factor for venous and arterial thrombosis. In a retrospective case-control study of 14,916 healthy men older than 40 years with a mean follow-up period of 8.6 years, heterozygosity for the factor V Leiden mutation was identified in 12% of patients (14/121) who had a first episode of deep venous thrombosis or pulmonary embolism and in 6% of control subjects.[99] The relative risk for VTE was increased 3.5-fold in persons with no other concomitant risk factors but was reduced to 1.7-fold in persons who had preexistent cancer or had recently undergone surgery. This study also showed that elderly patients with venous thrombosis frequently have the mutation.[99] Among men older than 60 years who had initial episodes of venous thrombosis and no identifiable triggering factors, 26% (8/31) were heterozygotes for factor V Leiden mutation.

A cohort study of more than 9000 randomly selected adults in Denmark found that the simultaneous presence of smoking, obesity (body mass index >30 kg/m^2), older age (>60 years), and the factor V Leiden mutation resulted in absolute 10-year VTE rates of 10% in heterozygotes and 51% in homozygotes.[100]

A systematic review and meta-analysis of pediatric studies found that factor V Leiden heterozygosity conferred an odds ratio of 3.77 (95% CI, 2.98 to 4.77) for a first episode of VTE and 0.64 (95% CI, 0.35 to 1.18) for recurrent VTE in children.[9] In a cohort of children who had spontaneous VTE, factor V Leiden still did not increase the risk of recurrent thrombosis (OR, 1.35; 95% CI, 0.91 to 1.98). A systematic review and meta-analysis of observational studies, including pediatric patients with cerebral sinovenous thrombosis, reported an OR for thrombosis of 2.74 (95% CI, 1.73 to 4.34) in factor V Leiden mutation carriers.[101]

A prospective cohort study determined the incidence of VTE in asymptomatic carriers of the factor V Leiden mutation who were identified through family studies of symptomatic probands. Nine events occurred in 1564 observation-years, for an annual incidence of 0.58%. It was concluded that the absolute annual incidence of VTE in asymptomatic carriers of the mutation is low.[102]

A number of studies have examined whether factor V Leiden mutation, a prevalent abnormality in white populations, leads to an increased risk for arterial thrombotic events. No convincing data are available to indicate that other thrombophilic states such as deficiencies of AT, protein C, and protein S confer an increased risk for arterial thrombosis, but evaluation of these associations is complicated by the relative infrequency of these defects. In a cohort of men older than 40 years who had a low prevalence of smoking in the U.S. Physicians' Health Study, an association was not found between the factor V Leiden mutation and myocardial infarction or stroke.[99] In a younger cohort of Italian patients who experienced myocardial infarction before 45 years of age, an increased incidence of the factor V Leiden mutation relative to that in control subjects also was not found.[103] Like other hereditary thrombophilic states, factor V Leiden has been associated with pediatric arterial ischemic stroke,[101] although data are lacking to prove causation.

Because of the frequency of heterozygosity for the factor V Leiden mutation in white persons, homozygosity is not a particularly rare occurrence, and its presence is compatible with normal gestational survival. However, homozygotes for factor V Leiden are at significantly higher risk for venous thrombosis than are heterozygotes[104,105] and demonstrate heightened APC resistance in APTT assays.[95,104,106] Patients with heterozygosity for the factor V Leiden mutation along with deficiencies of protein C, AT, or protein S[107-111] are also at significantly greater risk for VTE than are persons who have only a single genetic defect. The genes for factor V and AT are both located on the long arm of chromosome 1, thereby allowing coinheritance of the factor V Leiden mutation and an AT mutation within all affected members of a family. This situation is expected to lead to an even more severe thrombotic diathesis.[109]

Obstetric complications such as severe preeclampsia, abruptio placentae, fetal growth retardation, and stillbirth are associated with intervillous or spiral artery thrombosis and consequent placental insufficiency. Factor V Leiden, as well as other hereditary thrombophilias, is associated with an approximate tripling of the risk for late fetal loss.[112,113] In contrast, first-trimester loss does not correlate with factor V Leiden or other hereditary thrombophilias. An increased incidence of factor V Leiden, as well as other thrombophilias, was also reported in women in association with other obstetric complications[114]; however, associations with preeclampsia and intrauterine growth restriction/retardation have not been corroborated.[115,116]

The prevalence of heterozygosity for the factor V Leiden mutation in white persons, including European, Jewish, Israeli Arab, and Indian populations, ranges between 1% and 8.5%. The mutation apparently is rare (<1%) in African blacks and in Chinese, Japanese, or Native American populations.[117] In Europe, the mutation has been found to be more prevalent in northern countries such as Sweden than in southern countries such as Spain and Italy. By using dimorphic sites in the factor V gene to perform haplotype analysis, data have been provided that support the existence of a single founder allele among whites of differing ethnic backgrounds.[118] It was also estimated that the mutation originated approximately 30,000 years ago, after the evolutionary divergence of white, African, and Asian populations.

Two mutations at the Arg306 residue in factor V, the second APC cleavage site in the activated cofactor, have been described in patients who have a history of thrombosis. These mutations are replacement of Arg306 with threonine (factor V Cambridge)[119] and with glycine (in Chinese persons from Hong Kong).[120] The latter mutation, however, evidently has no clinical relevance because it is not associated with APC resistance and the mutation is as common in healthy Chinese blood donors as it is in patients with thrombosis (4.5% and 4.7%, respectively).[121] The factor V Cambridge mutation is extremely rare among white persons with VTE disease.

Co-segregation of heterozygous APC resistance as a result of the factor V Leiden mutation and type I factor V deficiency has been reported in some patients.[122-124] The plasma of these persons manifests severe APC resistance in partial thromboplastin time (PTT) assays, as found in homozygous factor V Leiden patients (i.e., the patients with such co-segregation are pseudohomozygous). These patients were seemingly more prone to thrombosis than were heterozygous relatives with factor V Leiden alone, thus suggesting that their clinical phenotype is similar to that of homozygous factor V Leiden patients.

Several polymorphisms are present in the factor V gene.[125,126] An extended factor V gene haplotype (HR2) containing the R2 polymorphism (His1299Arg) is associated with mild APC resistance and occurs with increased frequency in heterozygous patients with the factor V Leiden mutation and the lowest APC resistance ratios.[126] Although one case-control study found that the R2 allele was a risk factor for VTE with an OR of 2.0 after excluding subjects with genetic defects such as factor V Leiden,[127] another case-control study found no significant increase in risk.[128]

The initial observations of Dahlbäck and colleagues[94] facilitated the development of a PTT-based assay that serves as a screening test for APC resistance. The PTT assay is performed in the presence or absence of a standardized amount of APC, and the two clotting times are converted to an APC ratio. Results can be interpreted by comparing the ratio with the normal range or by normalizing it to the APC resistance ratio obtained with normal pooled plasma. Although this first-generation APC resistance assay was conceptually quite simple and easy to perform in a coagulation laboratory, it required careful standardization and determination of the normal range in at least 50 control subjects. The level of APC, the PTT reagent, and the instrumentation used for clot detection affected the performance characteristics of the assay. Some assays using this format therefore had inadequate sensitivity and specificity for the factor V Leiden mutation. Moreover, patients who were receiving anticoagulants or had an abnormal PTT because of other coagulation defects could not be evaluated with this assay, and the test was not validated in patients with acute thrombosis or in pregnant women.

The discovery that factor V Leiden is the dominant genetic defect responsible for APC resistance facilitated the development of second-generation coagulation tests that, with proper standardization, can provide nearly 100% sensitivity and 100% specificity for detection of the mutation. For these tests, patient plasma is diluted in a sufficient volume of factor V–deficient plasma, and a PTT-based assay is then performed. This modification also permits evaluation of the plasma of patients who are receiving anticoagulants or have abnormal PTT results because of coagulation factor deficiencies other than factor V.

Because the dominant mutation underlying APC resistance is factor V Leiden, diagnosing this defect by analyzing genomic deoxyribonucleic acid (DNA) in peripheral blood mononuclear cells is an attractive option. This analysis can be readily accomplished by amplifying a DNA fragment containing the factor V mutation site with polymerase chain reaction.

Prothrombin G20210A Mutation

In 1996, investigators from Leiden reported that a G-to-A substitution at nucleotide 20210 in the 3′-untranslated region of the prothrombin gene is associated with a 30% elevation of plasma prothrombin levels and an increased risk for venous thrombosis.[129] This mutation, which was discovered by directly sequencing the prothrombin gene of selected patients with venous thrombosis, is located in the 3′-untranslated region at the cleavage site for polyadenylation of prothrombin mRNA. The prothrombin G20210A mutation changes the position of the 3′-cleavage/polyadenylation reaction in prothrombin mRNA, thereby leading to increased prothrombin biosynthesis by the liver[130]; this finding contrasts with previous data indicating that the mutation changes mRNA stability by increasing mRNA 3′-end formation.[131]

Investigation of a referral population with a personal and family history of venous thrombosis demonstrated that 18% had the mutation in the 3′-untranslated region of the prothrombin gene, whereas it was present in only 1 of 100 healthy control subjects.[129] Among these patients with thrombosis, 40% also carried the factor V Leiden mutation, thus emphasizing the current view of venous thrombosis as a multigene disorder.

In the Leiden Thrombophilia Study, 6.2% of patients with venous thrombosis and 2.3% of healthy matched control subjects had the prothrombin gene mutation.[129] This mutation independently confers a 2.8-fold increased risk for venous thrombosis, and the effect is operative in both sexes and all age groups. Among heterozygotes with the prothrombin gene mutation, 87% of patients with thrombosis and control subjects in the study had prothrombin activity levels that were greater than 1.15 units/mL, whereas only 23% of those with a normal prothrombin genotype had levels elevated to this degree. A systematic review and meta-analysis of pediatric studies found that prothrombin gene mutation G20210A heterozygosity conferred an OR of 2.64 (95% CI, 1.60 to 4.41) for a first episode of venous thromboembolism and an OR of 1.88 (95% CI, 1.01 to 3.49) for recurrent VTE.[9] A similar analysis of cases of cerebral sinovenous thrombosis in children found that prothrombin mutation carriers had an OR for thrombosis of 1.95 (95% CI, 0.93 to 4.07).[101] An association with arterial ischemic stroke was also observed.[101]

In numerous other studies the prothrombin G20210A mutation has been documented to have a significantly higher frequency in patients with venous thrombosis than in healthy control subjects.[132-134] Large population studies have shown an overall prevalence of about 2% in the general white population, with significant geographic variation. In southern Europeans the prevalence is about 3%, but it was only very rarely identified in persons of Asian or African descent.

Polymerase chain reaction methods are used to detect the prothrombin G20210A mutation in genomic DNA.[129] Although plasma prothrombin activity and antigen levels are significantly higher in persons with the prothrombin G20210A mutation, prothrombin concentrations cannot be used to screen for the defect because of significant overlap with the normal population.[129,135]

HYPERHOMOCYSTEINEMIA

Homocysteine is a sulfur-containing amino acid involved in metabolic pathways that lead to the formation of other amino acids; methionine is generated through the remethylation of homocysteine or metabolized to cysteine via transsulfuration. The normal plasma homocysteine concentration is 5 to 16 µmol/L. Mild (16 to 24 µmol/L) or moderate (25 to 100 µmol/L) hyperhomocysteinemia has been identified as a seemingly independent risk factor for venous and arterial thrombotic events.[136-138] Hyperhomocysteinemia results from genetic and acquired abnormalities. The most common causes of acquired hyperhomocysteinemia are deficiencies of vitamin B_{12}, folate, or vitamin B_6, which are cofactors in homocysteine metabolism. A strong inverse correlation exists between hyperhomocysteinemia and folate levels and, to a lesser extent, with vitamin B_{12} and B_6 concentrations. Elderly patients frequently have elevated plasma homocysteine concentrations, even in the absence of vitamin deficiencies, as do patients with renal failure. Cigarette smoking is also associated with acquired hyperhomocysteinemia. The prevalence of hyperhomocysteinemia in the U.S. population has declined since the wheat supply was fortified with folic acid in the late 1990s. Patients with gluten sensitivity (as a result of celiac disease or other causes) may have mild hyperhomocysteinemia as a result of folate deficiency.

Homocystinuria is a rare autosomal-recessive inborn error of metabolism that presents in childhood. The most frequent cause is homozygous cystathionine β-synthase deficiency, which has a frequency in the general population of approximately 1 in 250,000. As a result of impaired intracellular metabolism of homocysteine, increased amounts of the amino acid (often >100 µmol/L) accumulate in blood and are excreted in urine. Children with homocystinuria exhibit premature atherosclerosis and arterial and venous thromboembolism, along with mental retardation, ectopic lenses, and skeletal abnormalities. The mechanisms by which hyperhomocysteinemia acts as an atherogenic and thrombogenic risk factor have been only partially elucidated.[139,140] Heterozygous cystathionine β-synthase deficiency is found in approximately 0.3% of the general population. Persons with heterozygous defects in this gene may have slightly elevated or even normal fasting plasma homocysteine levels. Testing 4 hours after an oral methionine load increases the sensitivity of this test.

In contrast to homocystinuria, a variant of methyltetrahydrofolate reductase (MTHFR) with an alanine-to-valine substitution at amino acid 222 (due to a C to T polymorphism at nucleotide 677) is common[141] and can be present in at least one copy in up to 61% of the population, depending on their origin, and homozygous in 1.4% to 15%.[138] This mutation causes thermolability of MTHFR and a 50% reduction in its specific activity. Defects in the remethylation pathway as a result of *MTHFR* gene defects or inadequate folate or vitamin B_{12} levels tend to cause elevated homocysteine levels under fasting conditions. The prevalence of hyperhomocysteinemia is almost twice as high when it is based on homocysteine measurements performed after methionine loading than when it is based on fasting levels. Genetic testing for the common C677T polymorphism in *MTHFR* is also possible. Because current data indicate that persons with mild hyperhomocysteinemia and a history of venous or arterial thrombosis should not be managed any differently from persons with normal homocysteine levels, justification no longer exists for routinely determining homocysteine levels or testing for *MTHFR* polymorphisms in such patients. In addition, results from the Leiden MEGA (Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis) Study indicate that the presence of homozygosity for the *MTHFR* C677T polymorphism, which mildly increases homocysteine levels, is not associated with an increased risk for a first episode of venous thrombosis.[142] Although it was reported that hyperhomocysteinemia significantly increased the risk for a first venous thrombotic event in patients with the factor V Leiden mutation,[143] a meta-analysis of published studies found no additive or multiplicative interaction between these abnormalities for the risk of venous thrombosis.[144]

Because folic acid, vitamin B_6, and vitamin B_{12} supplementation can decrease plasma homocysteine levels, it was hypothesized that treatment with these supplements would reduce the risk for arterial and venous thrombosis. However, results of large randomized placebo-controlled clinical trials showed that clinical outcomes are not improved by providing supplements of these vitamins to patients with histories of ischemic stroke,[145] myocardial infarction,[146] vascular disease,[147] or venous thrombosis.[148]

DYSFIBRINOGENEMIAS

The dysfibrinogenemias are a heterogeneous group of disorders that may be asymptomatic or may be manifested as a bleeding diathesis or as recurrent venous or arterial thromboembolism. Qualitative fibrinogen abnormalities are inherited in an autosomal-dominant manner. A small number of variant fibrinogens have been reported to be associated with thrombotic complications. These defects can be detected with thrombin and reptilase times, which are often prolonged. Functional fibrinogen measurements are usually substantially lower than antigenic measurements in the plasma of affected patients. An occasional patient with dysfibrinogenemia may have a prolonged prothrombin time or PTT, and the inability of some abnormal fibrinogens to clot completely in vitro can result in false-positive results in fibrin(ogen) degradation product tests.

The functional and biochemical defects of a number of abnormal fibrinogens associated with thromboembolic disease have been characterized.[149,150] The conversion of fibrinogen to fibrin by thrombin results in the proteolytic cleavage of fibrinopeptides A and B from the molecule. Defects in the release of these two peptides[151-154] or abnormalities in fibrin polymerization[155-158] have been reported, although the link between these functional defects and thrombosis risk is unclear. Abnormalities in the binding of thrombin to fibrin have also been found in some dysfibrinogenemias.[159-162] In one of these kindreds, three homozygous siblings with a β-chain substitution of Ala by Thr at position 68 had a severe clinical phenotype and sustained both arterial and venous thrombosis at a young age.[161,163] It has been suggested that decreased binding of thrombin by this mutant fibrinogen may lead to the presence of excessive thrombin in the circulation and the occurrence of thrombosis.[163] Other fibrinogen mutants have been shown to cause abnormal fibrin polymerization.[155-157,160] Some abnormal fibrinogens have been evaluated for their ability to resist or promote fibrinolysis upon incorporation into a fibrin clot. The fibrin formed from fibrinogen “Chapel Hill III” has been demonstrated to be abnormally resistant to lysis by plasmin.[155] Activation of plasminogen is decreased in the presence of the fibrin formed from fibrinogen “Dusart” despite normal binding of tissue plasminogen activator to the substrate.[164-168] These abnormalities clearly have the potential for decreasing fibrinolytic activity in vivo, which results in a familial thrombotic diathesis in biochemically affected persons. The coexistence of factor V Leiden may predispose persons with certain dysfibrinogenemias to thromboembolism.[169]

INHERITED ABNORMALITIES OF DIMINISHED FIBRINOLYSIS

In contrast to the relationship between hyperfibrinolytic states and delayed, postprocedural bleeding, it is logical that diminished fibrinolytic activity may explain some cases of hereditary thrombophilia. Disorders of plasminogen activity or elevated plasminogen activator inhibitor–1 (PAI-1) function have been linked to thrombosis, whereas α_2-antiplasmin disorders have not been described in patients with thrombosis. Elevations in PAI-1 have been associated with arterial and atherothrombotic events in adults.[170] The 4G allele of the 4G/5G polymorphism in the promoter of the PAI-1 gene is associated with increased PAI-1 activity and was reported to be associated with a modest increase in thrombotic risk[171]; however, this association has not been confirmed.[172] The association between elevated PAI-1 activity and thrombosis have not been identified in pediatric patients.

Although investigators have identified a few persons with inherited abnormalities of the fibrinolytic mechanism and recurrent VTE, the clinical association is considerably less striking than that in many kindreds with deficiencies of AT, protein C, or protein S or with prothrombin gene mutation or APC resistance as a result of factor V Leiden mutation.

Dysplasminogenemia or hypoplasminogenemia has been reported in a number of persons with thromboembolic disease; the first case of an abnormal plasminogen was identified in Japan.[173] The propositus had a history of recurrent thrombosis, and family studies demonstrated that the biochemical abnormality followed an autosomal-dominant inheritance pattern. Despite the hereditary nature of the defect, none of the other biochemically affected members of the kindred had experienced a thrombotic event. Other Japanese pedigrees without thrombosis have since been described with the same biochemical defect; the gene frequency of this variant in the Japanese population was 0.018.[174] Population studies in the United States have not uncovered any cases of this dysplasminogenemia. The non-Japanese cases of dysplasminogenemia and hypoplasminogenemia have also been characterized by the absence of thrombotic episodes in biochemically affected family members other than the propositi.

Severe type 1 plasminogen deficiency is associated with a rare chronic inflammatory disease of the mucous membranes. Although this disorder can occasionally be life threatening, venous thrombosis was not observed in these patients. The disorder is characterized by the presence of fibrin-rich pseudomembranous lesions, and the most common clinical manifestations are ligneous conjunctivitis (80%) and ligneous gingivitis (34%); less common sites of involvement are the vagina, respiratory tract, ears, and gastrointestinal tract.[175] Patients with this disorder have been found to be homozygous or compound heterozygous for a number of different mutations in the plasminogen gene.[175] A severely affected infant with ligneous conjunctivitis has been treated successfully with a purified plasminogen concentrate,[176] but this product is not generally commercially available. The absence of thromboembolic events in persons with severe plasminogen deficiency and in most persons with hypoplasminogenemia and dysplasminogenemia supports the argument that diminished plasminogen function does not pose a significant thrombotic risk.

Some reports have documented the existence of thrombophilic families with impaired fibrinolysis.[177,178] Reevaluation of several of these families demonstrated the presence of hereditary protein S deficiency and no association between elevations in PAI-1 activity and a history of thrombosis.[179,180]

MANAGEMENT OF PATIENTS WITH INHERITED THROMBOTIC DISORDERS

Acute Thrombosis and Long-Term Management

When a patient with one of the inherited thrombotic disorders is discovered, family studies should be considered because approximately half the members of a given kindred may be affected. Institution of long-term anticoagulation is not generally recommended for persons who have not yet sustained thrombotic episodes, especially children. However, affected persons should receive counseling regarding the implications of the diagnosis and advice regarding the symptoms that require immediate medical attention. In women of childbearing age who have known hereditary thrombophilia, hormonal contraceptives are generally contraindicated in view of the increased thrombotic risk associated with the use of these medications. All thrombophilia carriers should be carefully evaluated before surgical, medical, or obstetric procedures that carry an increased thrombotic risk, and use of appropriate prophylactic anticoagulation regimens should be considered. In addition to prophylactic anticoagulation, nonpharmacologic preventive strategies should be discussed, along with relevant replacement of specific deficiencies with recombinant or concentrated products to raise plasma levels of the protein to the normal range during the perioperative period. In general, women with previous thrombotic episodes receive prophylactic heparin throughout pregnancy and asymptomatic women with thrombophilia are treated with either observation or heparin during the third trimester and in the postpartum period.

Management of an acute thrombotic event in patients with a hereditary thrombotic disorder is generally similar to that for patients who do not have an identifiable risk factor. Anticoagulant therapy generally consists of 3 to 6 months of heparin, low molecular weight heparin, or an oral vitamin K antagonist (e.g., warfarin). Warfarin is used less frequently in young children because of challenges in maintaining an international normalized ratio in the therapeutic range as a result of variable nutritional intake or other issues (e.g., compliance and malabsorption). Newer oral anticoagulants including dabigatran, rivaroxaban, and apixaban have not been adequately studied in children, although off-label use has begun following regulatory agency approval for the treatment and prevention of venous thromboembolism without the need for laboratory monitoring. The indications for thrombolytic therapy in patients with these disorders are similar to those in other patients who have had acute deep venous thrombosis or pulmonary embolism.

In adult patients with a first episode of symptomatic VTE, Prandoni and colleagues[181] found that the cumulative incidence of recurrent venous thrombosis after the cessation of anticoagulant therapy was 24.8% at 5 years and 30.3% at 8 years. Other investigators have confirmed that this risk is about 5% to 15% per year for the first several years after a first or even a second episode of unprovoked venous thrombosis. In children, first-year recurrence rates for VTE are reported to be 8%, with this rate rising to 18% in subsequent years.[182,183,184] Inherited thrombophilia can increase recurrence rates further in children, with the exception of the factor V Leiden and prothrombin gene mutations (discussed later). Recurrences are less common when the initial event is associated with a transient risk factor (e.g., surgery, trauma, or pregnancy). Despite the relatively high recurrence risk in patients with a first episode of unprovoked venous thrombosis, anticoagulation has not proved to have sufficient benefit in excess of known bleeding risk to support long-term prophylaxis for all patients who are at substantial risk for recurrence. For example, one controlled trial evaluated the efficacy of long-term warfarin therapy (international normalized ratio of 2 to 2.85) for 6 months or indefinitely in 227 patients who had a second venous thrombotic episode, but who had not specifically inherited thrombophilia.[185] Long-term use of warfarin was highly effective in preventing recurrences when compared with 6 months of therapy (2.6% vs. 21% over a period of 4 years); this benefit was partially counterbalanced by a trend toward an increased incidence of major hemorrhage (8.6% vs. 2.3%), and no difference in mortality rate was found between the two groups. Persistent elevation of factor VIII and/or D-dimer 1 month after cessation of anticoagulation is a risk factor for thrombosis recurrence in pediatric patients, whether familial or acquired.[182]

Because of the relatively high frequency of the factor V Leiden mutation in adult patients who have a first episode of VTE, a substantial amount of data is available on the risk of recurrence. Although two groups of investigators initially reported that patients with the factor V Leiden mutation who had a first venous thrombotic event were more than twice as likely to have a recurrent episode compared with persons who did not have the mutation,[143,186] several other groups have not found that heterozygosity for this defect or the prothrombin G20210A mutation confers a higher risk for recurrence.[187-191] Thus the consensus view is that neither of these defects alone is predictive of recurrence, and authors of several studies have argued that long-term warfarin therapy should not be used after a first thromboembolic episode in these patient populations. No increase in mortality has been observed among patients with the factor V Leiden mutation.[192] The recurrence risk appears to be significantly higher in the small subset of patients who are heterozygous for two hereditary prothrombotic defects or homozygous for factor V Leiden.[107-110,190,193]

As a result of the relatively low frequency of AT, protein C, or protein S deficiency in unselected cohorts who have an initial episode of VTE, randomized clinical trials have included too few patients with these deficiencies to draw firm conclusions. A literature review and retrospective cohort study suggested that they have a high annual incidence of recurrent VTE during the years immediately after a first episode and that this incidence declines thereafter.[194] Retrospective studies are unable to demonstrate an increase in mortality in patients with AT[195] or protein C deficiency.

For the individual adult patient, the decision to continue anticoagulation indefinitely requires estimation of the quantitative risk over time for recurrent thrombosis (including fatal pulmonary embolism) and major bleeding (including fatal bleeding). The approach to pediatric patients involves additional considerations. Pediatric patients have lower rates of thrombosis and recurrent thrombosis than do adults. Although children may be less predisposed to bleeding events from anatomic lesions (e.g., ulcers and diverticulosis) than are older adults, adolescents often display risk-taking behaviors and are prone to accidental trauma, increasing their risk of bleeding while they take anticoagulation agents. Patient compliance has a major impact on the success of therapy, and patient preferences and behaviors must be factored into the decision. Because future events in a patient with only one previous thrombotic episode cannot currently be accurately predicted and there is a finite risk of bleeding associated with indefinite anticoagulation therapy, recommendations regarding long-term anticoagulation are individualized. The following clinical features should be considered when making this decision:

1. **The number, sites, and severity of thrombosis.** A patient who previously sustained a massive pulmonary embolism is more likely to receive long-term anticoagulation than is a subject in whom deep venous thrombosis developed in a calf vein unless the patient has symptoms or signs of a significant postphlebitic syndrome.
2. **Whether the thrombotic episodes were spontaneous or whether precipitating factors were present.** If a

precipitating event such as a major abdominal operation was a factor when the initial venous thrombotic event occurred, the risk of recurrence is substantially lower than if the event was spontaneous, and the patient does not require long-term anticoagulation after the initial episode is adequately treated and the precipitating factor is removed.

3. **The patient's risk of bleeding while receiving anticoagulation.** A history of bleeding or risk-taking behaviors or difficulty maintaining therapeutic anticoagulation because of altered gastrointestinal function will lead to an increased risk for bleeding.
4. **The lifestyle of the individual.** Situations that may influence the decision to recommend long-term anticoagulation include occupations that entail prolonged periods of immobilization and therefore might be associated with an increased risk for thromboembolism, jobs with a higher than average chance of trauma that might lead to thrombotic or bleeding complications, or both. Gender may influence the choice of anticoagulant, because warfarin is generally contraindicated in pregnancy.
5. **A history of thromboembolism in other biochemically affected members of the family with an inherited thrombotic disorder.** Although marked intrafamilial and interfamilial heterogeneity has been observed in the phenotypic expression of inherited thrombotic disorders, it is reasonable for biochemically affected patients from severely affected kindreds with a single thrombotic episode to consider indefinite anticoagulation therapy.

In pediatric patients, "indefinite" anticoagulation therapy is often discussed and reevaluated annually. Given the cumulative bleeding risk through the remainder of childhood, adolescence, young adulthood, and beyond, indefinite anticoagulation therapy is not commonly used in pediatric patients. In their 2012 guidelines, the American College of Chest Physicians suggest consideration of ongoing anticoagulation in pediatric patients who have recurrent idiopathic thrombosis or thrombosis with an ongoing risk factor.[196] The impact of thrombophilia on this discussion in pediatrics is unclear and considered individually.

Antithrombin Deficiency

Non–heparin-based anticoagulants are often used for long-term anticoagulation for patients who have AT deficiency. However, patients with AT deficiency can usually be treated successfully with intravenously administered heparin,[197] although in some situations unusually high doses of the drug are required to achieve adequate anticoagulation. In patients with AT deficiency who are receiving heparin for the treatment of acute thrombosis, the adjunctive role of AT supplementation is not clearly defined because controlled trials have not been performed.[197] Two AT products are available: a concentrate purified from human plasma and a recombinant human AT purified from the milk of transgenic goats.[198] AT replacement should be considered in patients who have difficulty achieving adequate heparinization or if recurrent thrombosis is observed despite adequate anticoagulation. It is also reasonable to treat subjects who are deficient in AT with concentrate before major surgery or in obstetric situations in which the risks of bleeding from anticoagulation are unacceptable. The manufacturing processes used to prepare AT concentrate result in a product that is greater than 95% pure; they also inactivate hepatitis B virus and human immunodeficiency virus type 1.[199,200] Hence it is preferable to administer AT concentrate rather than fresh-frozen plasma.

The biologic half-life of AT is approximately 48 hours.[14] Infusion of 50 units of plasma-derived AT concentrate per kilogram of body weight (1 unit is defined as the amount of AT in 1 mL of pooled normal human plasma) will usually raise the plasma AT level to approximately 120% in a congenitally deficient person with a baseline level of 50%.[200-204] Plasma levels should be monitored to ensure that they remain higher than 80%; administration of 60% of the initial dose at 24-hour intervals is recommended to maintain inhibitor levels in the normal range.[204] Recombinant AT is administered as an infusion after a loading dose to target plasma AT levels between 80% to 120%.

Coumarin-Induced Skin Necrosis

Because coumarin-induced skin necrosis is a rare complication, therapy has been guided primarily by knowledge regarding its pathogenesis. The diagnosis should be suspected in patients with painful, red skin lesions that develop within a few days after initiation of the drug, and immediate intervention is required to prevent rapid progression and reduce complications. Therapy should consist of immediate discontinuation of warfarin, administration of vitamin K, and infusion of heparin at therapeutic doses. However, lesions have been reported to progress despite adequate anticoagulation with heparin. In patients with hereditary protein C deficiency, administration of a source of protein C should be given serious consideration, and it may also be appropriate in other patients with coumarin-induced skin necrosis because they invariably have reduced plasma levels of functional protein C when the skin lesions first appear. Fresh-frozen plasma has been used, but improved results can be expected after the administration of a highly purified protein C concentrate, which facilitates rapid and complete normalization of plasma protein C levels.[205]

Because of the infrequent occurrence of coumarin-induced skin necrosis, it may be advisable to take special precautions when initiating warfarin in a patient who is previously known or likely to have protein C deficiency. Warfarin should be started only when the patient is fully heparinized, and the dose of the drug should be increased gradually, starting at a relatively low level. Patients with heterozygous protein C deficiency and a history of warfarin-induced skin necrosis have been successfully retreated with warfarin, although the emergence of alternative anticoagulants may reduce reexposure to warfarin. Protein C administration either in the form of fresh-frozen plasma or protein C concentrate provides protection against the development of recurrent skin necrosis until a stable level of anticoagulation is achieved.[58,206]

Homozygous or Doubly Heterozygous Protein C Deficiency and Neonatal Purpura Fulminans

Management of neonatal purpura fulminans in association with homozygous or doubly heterozygous protein C deficiency is complicated, and neither heparin therapy nor antiplatelet agents have been shown to be effective.[67,69-73] Administration of a protein C source appears to be critical in the initial treatment of these patients. Fresh-frozen plasma has been used successfully to treat these infants. However, the half-life of protein C in the circulation is only about 6 to 16 hours,[47,207] and frequent administration of plasma is limited by the development of hyperproteinemia or hypertension, loss of venous access, and the potential for exposure to infectious viral agents. A highly purified concentrate of protein C has been developed and is efficacious in treating neonatal purpura fulminans.[208] Warfarin has been administered to these infants without the redevelopment of skin necrosis during the phased withdrawal of fresh-frozen plasma infusions,[67,72,74,209,210] and this medication has been used on a long-term basis to control the thrombotic diathesis. A 20-month-old child with liver failure and homozygous protein C deficiency successfully underwent liver transplantation, which normalized his protein C levels and resolved the thrombotic diathesis.[211]

Evaluation of Relatives of Patients with Hereditary Thrombophilia

Family history is an essential component of the evaluation of patients with thrombotic complications and often influences the decision to test for hereditary risk factors. Pediatric hematologists/oncologists are frequently asked whether to screen a pediatric patient because of identification of a family member with thrombophilia or whether to screen other family members after thrombophilia is identified in a pediatric patient. Appropriate testing of the patient with thrombosis is important, but screening asymptomatic children who have not experienced thrombophilic events is generally not recommended. Equipoise exists with regard to whether to screen family members of probands who have a history of thrombosis unless there is a very strong history of VTE. In a study of 192 kindreds with a member homozygous for either the factor V Leiden or prothrombin gene mutation, the incidence of VTE was higher in affected relatives when the symptomatic proband was heterozygous for either mutation rather than being homozygous.[212] A study of 533 family members of 206 children with VTE identified a ninefold higher risk of thrombosis in family members with hereditary thrombophilia compared with probands who did not have a defect; the risk was highest in patients with deficiencies of AT, protein C, or protein S.[213] The type of thrombophilia and family history should therefore guide counseling for individual families. However, caution should be exercised in providing false reassurance to family members who test negative and in providing overly aggressively anticoagulation for asymptomatic persons who test positive.

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CHAPTER 34

Acquired Platelet Defects

David B. Wilson

CHAPTER OUTLINE

QUANTITATIVE PLATELET ABNORMALITIES
Thrombocytopenia
Thrombocytosis

ACQUIRED DEFECTS IN PLATELET FUNCTION
Drug-Induced Platelet Dysfunction
Platelet Abnormalities Associated with Systemic Diseases

Platelets play a critical role in hemostasis. When the vascular endothelium is disrupted, platelets adhere to the subendothelium and initiate primary hemostasis. The details of normal platelet physiology and function are presented in Chapter 26. Excessive bleeding occurs if primary hemostasis is abnormal because platelets are either deficient in number or defective in function. Acquired platelet defects, both quantitative and qualitative, are discussed in this chapter, and the corresponding inherited disorders are discussed in Chapter 30.

The normal circulating platelet count for all ages ranges from 150,000 to 400,000/μL. Circulating platelets constitute two thirds of total body platelets; the remaining platelets are located within the spleen.[1] Platelets exhibit marked heterogeneity in size. Two factors have been proposed to account for this heterogeneity.[2] First, as platelets age, they may become smaller as a result of fragmentation or loss of granule contents or membrane proteins. Second, megakaryocytes produce platelets of varying size. In thrombolytic states megakaryocytes preferentially produce large platelets, a phenomenon analogous to stress erythropoiesis.[2] The average life span of platelets is 7 to 10 days, although survival of transfused platelets in a thrombocytopenic recipient is reduced proportionately to the severity of the thrombocytopenia.[3] These findings suggest either that there is increased platelet utilization in thrombocytopenic states or that a fixed number of platelets are removed from the circulation each day, irrespective of the platelet count.[4]

QUANTITATIVE PLATELET ABNORMALITIES

Thrombocytopenia

The clinical manifestations of thrombocytopenia typically involve the skin or mucous membranes and include petechiae, ecchymoses, prolonged bleeding at incision or venipuncture sites, epistaxis, gastrointestinal hemorrhage, hematuria, and menorrhagia. Intracranial hemorrhage (ICH) may occur but is rare. As discussed in Chapter 29, the deep muscle hematomas and hemarthroses typically seen in individuals with deficiencies of factor VIII or factor IX generally do not occur with platelet disorders. Causes of thrombocytopenia fall into three broad categories: platelet sequestration (usually in the spleen), increased platelet destruction, and decreased platelet production. The differential diagnosis of thrombocytopenia in children is outlined in Box 34-1. Discriminating among these diagnoses is important because the cause of thrombocytopenia affects the choice of therapy.

When a patient with thrombocytopenia is assessed, the risk of bleeding episodes should be estimated. If the risk is significant, treatment is warranted. Unfortunately, there is a lack of direct correlation between the platelet count and the risk of bleeding episodes, which confounds treatment decisions. The risk of hemorrhage is affected by many factors, such as coexisting coagulation defects, trauma, and surgery. In older children and adults, serious spontaneous bleeding does not occur until the platelet count is less than 20,000/μL. Many physicians use a platelet count of 10,000 to 20,000/μL as the threshold for intervention. This threshold was derived from a study of children with leukemia and may not be relevant for all cases of thrombocytopenia. For example, because of the increased risk for ICH in neonates, a threshold of 20,000 to 50,000/μL is often used. In addition, patients with a defect in production are more likely to have serious bleeding than those with a destructive platelet problem because in the latter patients, platelets tend to be larger and more functional.

Box 34-1 **Differential Diagnosis of Thrombocytopenia in Children and Adolescents**

DESTRUCTIVE THROMBOCYTOPENIAS

PRIMARY PLATELET CONSUMPTION SYNDROMES

- Immune thrombocytopenias
  - Acute and chronic ITP
  - Autoimmune diseases with chronic ITP as a manifestation
    - Cyclic thrombocytopenia
    - ALPS and its variants
    - SLE
    - Evans syndrome
    - Antiphospholipid antibody syndrome
    - Neoplasia-associated immune thrombocytopenia
  - Thrombocytopenia associated with HIV
  - Neonatal immune thrombocytopenia
    - Alloimmune
    - Autoimmune (e.g., maternal ITP)
  - Drug-induced (including HIT)
  - Posttransfusion purpura
  - Allergy and anaphylaxis
- Nonimmune thrombocytopenias
  - Thrombocytopenia of infection
    - Bacteremia or fungemia
    - Viral infection
    - Protozoan infection
  - Thrombotic microangiopathic disorders
    - TTP
    - HUS
    - BMT–associated microangiopathy
    - Drug-induced
  - Platelets in contact with foreign material
  - Congenital heart disease
  - Drug-induced via direct platelet effects (ristocetin, protamine)
  - Type 2b VWD or platelet-type VWD

COMBINED PLATELET AND FIBRINOGEN CONSUMPTION SYNDROMES

- DIC
- Kasabach-Merritt syndrome
- HLH

IMPAIRED PLATELET PRODUCTION

- Hereditary disorders (see Chapter 30)
- Acquired disorders
  - Aplastic anemia
  - MDS
  - Marrow infiltrative process
  - Nutritional deficiency states (Fe, folate, vitamin B_{12}, anorexia nervosa)
  - Drug- or radiation-induced thrombocytopenia
  - Neonatal hypoxia or placental insufficiency

SEQUESTRATION

- Hypersplenism
- Hypothermia
- Burns

ALPS, Autoimmune lymphoproliferative syndrome; *BMT,* bone marrow transplant; *DIC,* disseminated intravascular coagulation; *HLH,* hemophagocytic lymphohistiocytosis; *HIT,* heparin-induced thrombocytopenia; *HIV,* human immunodeficiency virus; *HUS,* hemolytic-uremic syndrome; *ITP,* immune thrombocytopenic purpura; *MDS,* myelodysplastic syndrome; *SLE,* systemic lupus erythematosus; *TTP,* thrombotic thrombocytopenic purpura; *VWD,* von Willebrand disease.

Thrombocytopenia Reflecting Laboratory Artifact or Sequestration

Spurious Thrombocytopenia. Thrombocytopenia may be an incidental finding. If the history and physical examination do not suggest a defect in primary hemostasis, a low platelet count may represent a laboratory artifact. Potential causes of a falsely low platelet count include platelet activation during blood collection, undercounting of megathrombocytes,[5] or pseudothrombocytopenia as a result of in vitro agglutination by ethylenediaminetetraacetic acid (EDTA)-dependent antibodies.[6] In a patient with artifactual thrombocytopenia, large clumps of agglutinated platelets may be found at the periphery of the blood film, or platelets may be adherent to leukocytes and form platelet "satellites" (Fig. 34-1).[7]

Pseudothrombocytopenia secondary to EDTA-dependent antibodies can be confirmed by repeating the platelet count with another anticoagulant (e.g., citrate, oxalate, heparin) or by preparing a blood film directly from a finger- or heel-puncture sample. The cause of EDTA-associated pseudothrombocytopenia is an immunoglobulin G (IgG) or immunoglobulin M (IgM) directed against a cryptic platelet antigen exposed only in the presence of this anticoagulant.[6] The phenomenon is not associated with any particular pathology and may be observed in both healthy subjects and patients with diseases. In some individuals the antibodies responsible persist indefinitely, whereas in others the antibodies are transient. No abnormalities in hemostasis or thrombosis have been reported in any of these patients.

Other causes of pseudothrombocytopenia include drugs[8] and EDTA-independent cold agglutinins.[9] The overall incidence of pseudothrombocytopenia in hospitalized adult patients is approximately 1%, but it is less common in pediatric patients.[6] No further evaluation or treatment is indicated for a patient documented as having a spuriously low platelet count.

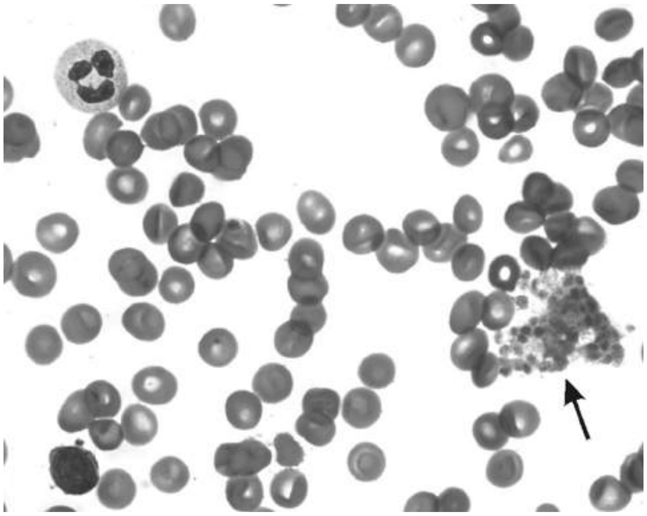

Figure 34-1 Pseudothrombocytopenia: photomicrograph of EDTA–dependent platelet clumping *(arrow)*.

Apparent Thrombocytopenia Caused by Hypersplenism. The spleen normally retains about a third of the body's platelets in an exchangeable pool.[1] The fraction of platelets sequestered in the spleen increases in proportion to spleen size. Thus an apparent thrombocytopenia can result from increased pooling in an enlarged spleen—a condition referred to as *hypersplenism*. In patients with hypersplenism, recovery of transfused autologous platelets is only 10% to 30%, whereas in normal individuals it is 60% to 80% and in asplenic patients it is 90% to 100%. Splenic blood flow is the major determinant of the size of the exchangeable splenic platelet pool in splenomegalic states.[10] Administration of intravenous epinephrine causes constriction of the splenic artery and results in passive emptying of platelets into the circulation. This increase in platelet count is proportionately greater in patients with splenomegaly than in normal subjects.

In general, the apparent thrombocytopenia that results from pooling in an enlarged spleen is mild (50,000 to 150,000/μL). Platelet counts of less than 50,000/μL should not be attributed to splenomegaly alone without further investigation. The degree of hypersplenism is proportional to spleen weight, whether the splenomegaly is due to congestion (e.g., cirrhosis with portal hypertension), hemolytic anemia (e.g., hemoglobin SC disease), or other causes. Because thrombocytopenia secondary to splenic pooling is not usually clinically important, no treatment is warranted, although splenectomy may be indicated for patients with severe thrombocytopenia, as can occur in Gaucher disease or other storage diseases.

Apparent Thrombocytopenia Caused by Hypothermia. Platelets are transiently sequestered in the spleen, liver, and other organs of experimental animals subjected to hypothermia. On rewarming of the animal, these platelets return to the circulation. A similar phenomenon has been observed in hypothermic patients. Transient thrombocytopenia (platelet counts of 7000 to 62,000/μL) has been reported in hypothermic patients of various ages.[11,12] Less significant thrombocytopenia has been observed in patients undergoing cardiac surgery with hypothermic perfusion. Treatment of the thrombocytopenia associated with hypothermia consists of rewarming and documentation of return of the platelet count to the normal range, which usually occurs in 4 to 10 days.

Thrombocytopenia Caused by Increased Platelet Destruction

A major etiologic classification of acquired thrombocytopenia of childhood is increased platelet destruction. Clinical conditions associated with increased platelet destruction are listed in Box 34-1. These conditions can be further subgrouped into immune and nonimmune causes.

Immune Thrombocytopenias. Autoantibodies, alloantibodies, or drug-dependent antibodies may associate with platelet membranes and target the cells for accelerated destruction by phagocytes of the reticuloendothelial system. An antibody mediating immune destruction of platelets may be directed against a platelet membrane antigen, or it may be part of an immune complex that binds Fc receptors on platelets.

Platelet antigens fall into two general classes. Glycoproteins that occur predominantly on platelets, such as the glycoprotein IIb/IIIa (GPIIb/IIIa) or GPIb/IX/V complexes, are often termed *platelet-specific antigens*. The glycoproteins (e.g., human leukocyte antigen [HLA] class I) and glycolipids (e.g., blood group ABH antigens) expressed on platelets, leukocytes, and other cell types are termed *platelet-nonspecific antigens*. Antibodies against platelet-specific and platelet-nonspecific antigens are responsible for a number of clinical syndromes, including autoimmune thrombocytopenia, neonatal alloimmune thrombocytopenia (NAIT), posttransfusion purpura (PTP), and platelet transfusion refractoriness. Antibodies directed against platelet integrins are common in immune thrombocytopenias. For example, autoantibodies directed against GPIIb/IIIa are seen in immune thrombocytopenic purpura (ITP)[13,14] and in drug-induced thrombocytopenia.[15,16] Several clinically significant alloantigens are located on GPIIb/IIIa, including HPA-1a, the human platelet antigen most frequently implicated in NAIT (discussed later). Autoantibodies against GPIb or GPIX also have been reported in patients with ITP[13,14] and drug-induced thrombocytopenia.[15,17]

HLA class I antigens are expressed on a wide range of cells, including platelets, and are important for the recognition of self by cytotoxic T cells. HLA-A and HLA-B antigens are strongly expressed on platelets.[18] Alloantibodies against HLA-A or HLA-B antigens frequently form in multiparous women and multiply transfused patients, and these alloantibodies contribute to platelet transfusion refractoriness. HLA-C antigens are weakly expressed on platelets and are far less likely to induce alloantibodies, thus making it less necessary to match the HLA-C locus for donor-recipient compatibility in platelet transfusions. HLA class II antigens, which function in antigen presentation by macrophages and B lymphocytes, are not expressed on platelets.

ABH blood group antigens are carried on a number of platelet glycoproteins, including the GPIIb/IIIa and GPIb/IX/V complexes.[19] ABH antigens are also expressed on glycolipids.[19] In rare instances these antigens have been implicated as the cause of immune-mediated platelet destruction, but in general these platelet-nonspecific antigens play only a minor role in immune thrombocytopenias.

Platelet Antibody Testing. A large number of assays have been developed to detect antibodies directed against or associated with platelet membrane antigens.[20] These assays can be categorized into direct (detecting antibody associated with the patient's platelets) or indirect (detecting antibody in the patient's serum that binds to control platelets). Indirect tests may detect, in addition to autoantibodies, alloantibodies (e.g., HLA related), particularly in multiparous women and individuals who have received multiple transfusions, and thus give false-positive results. Autoantibodies tend to be associated with the patient's platelets and are usually present in low concentration in serum. Therefore a direct test is the preferred test for autoimmune thrombocytopenias such as ITP.[20]

A number of direct assays measure immunoglobulin on platelets, regardless of whether the immunoglobulin

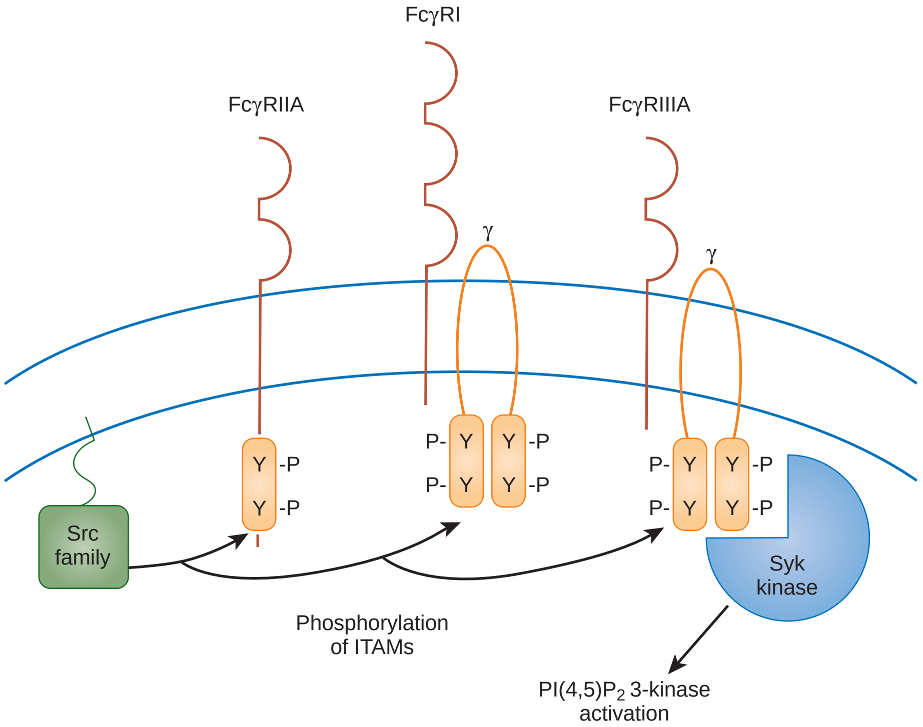

Figure 34-2 Activating Fcγ receptors on macrophages. Fcγ receptors signal phagocytosis via their phosphorylated ITAM (immunoglobulin gene–related tyrosine activation motif) domains. Receptor cross-linking stimulates src family kinases to phosphorylate tyrosine (Y) residues within the ITAM domain of FcγRIIA or within the dimerized γ subunits of FcγRI or FcγRIIIA. The tyrosine kinase syk is then recruited to the phosphorylated ITAM domains and activated. Syc mediates phagocytosis by activating phosphatidylinositol 3-kinase and phospholipase C. PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate. *(Modified from Aderem A, Underhill DM: Mechanisms of phagocytosis in macrophages.* Annu Rev Immunol *17:595, 1999.)*

is specifically or nonspecifically bound to the platelet surface. These assays generally detect platelet-associated IgG (PAIgG), but they can also be engineered to detect IgM or IgA. PAIgG is increased in immune disorders such as ITP and in nonimmune thrombocytopenic disorders such as leukemia and myelodysplastic syndrome. Thus the specificity of these tests is limited. Moreover, the sensitivity of these tests is limited because autoantibodies, such as those responsible for ITP, represent only a small fraction of the total PAIgG. Megakaryocytes nonspecifically take up plasma proteins, including IgG and albumin, and incorporate these proteins into the alpha granules of platelets, especially in disease states associated with increased thrombopoiesis.[21] Therefore increased PAIgG can result from elevated antibody production for any reason.

More recent assays use antigen capture techniques to detect platelet glycoprotein–specific antibodies, such as those that recognize GPIIb/IIIa and GPIa/IX/V, and have high specificity. Unfortunately, the sensitivity is generally too low for these assays to be used for the routine serologic diagnosis of most immune thrombocytopenias.[20] A newer method is based on flow cytometric detection of autoantibodies reacting with specific platelet proteins immobilized on microbeads.[22]

Macrophage and Platelet Fcγ Receptors. Receptors for the Fc domain of IgG (Fcγ receptors) are found on a variety of cell types, including macrophages and platelets. These receptors have been shown to play a critical role in immune complex–mediated platelet destruction. Fcγ receptors are diverse in structure and function and fall into two major classes: those that activate effector functions, such as phagocytosis or platelet activation, and those that inhibit effector functions.[23]

Activating Fcγ receptors on macrophages include the low-affinity receptors FcγRIIA and FcγRIIIA and the high-affinity receptor FcγRI (Fig. 34-2).[23] As discussed later, cross-linking of the activating receptor FcγRIII promotes phagocytosis of antibody-coated platelets in certain disease states, including ITP. The only Fcγ receptor expressed on platelets is the activating receptor FcγRIIA. Binding of IgG complexes to this receptor results in receptor cross-linking, which in turn initiates platelet aggregation and activation. This process is involved in heparin-induced thrombocytopenia (HIT), the most common drug-induced thrombocytopenia.

Immune Thrombocytopenic Purpura. ITP is a disorder characterized by accelerated destruction of antibody-sensitized platelets by phagocytic cells, especially those of the spleen. In many affected individuals, inhibition of megakaryopoiesis also contributes to the degree of thrombocytopenia. ITP is the most common autoimmune disorder affecting a blood element. The annual incidence is about 1 in 10,000 children. Two major forms are seen: acute ITP and chronic ITP. Acute ITP is usually a benign, self-limited condition that occurs in young children, typically those younger than 10 years. Often a viral infection or vaccination precedes the onset of acute ITP. In most of these patients, the thrombocytopenia resolves within weeks or a few months of the original manifestation. Chronic ITP is defined arbitrarily as persistence of thrombocytopenia (platelet count <150,000/μL) for longer than 6 months after the initial manifestation, although some hematologists advocate a later cutoff point (Box 34-2).

Box 34-2 **Controversial Issues Involving Immune Thrombocytopenic Purpura**

CONTROVERSIAL ISSUE 1

What is the definition of chronic ITP? Traditionally, chronic ITP has been defined as thrombocytopenia lasting longer than 6 months. Many pediatric hematologists take exception to this definition because a significant fraction of children with ITP recover in 6 to 12 months. The Intercontinental Childhood ITP Study Group has recommended that a 12-month rather than a 6-month cutoff point be used to define chronicity.[24]

CONTROVERSIAL ISSUE 2

Is it necessary to perform bone marrow aspiration in patients with suspected ITP before starting glucocorticoid therapy? Although the diagnosis of leukemia is extremely unlikely when the clinical history, physical examination, and peripheral smear are consistent with ITP, many hematologists routinely perform bone marrow aspiration before initiating glucocorticoid therapy. Retrospective studies[25-27] suggest that this may not be necessary. Bone marrow aspiration or biopsy is warranted for children with atypical laboratory features and for those in whom initial therapy fails.

CONTROVERSIAL ISSUE 3

Where does splenectomy belong in the therapeutic decision tree for chronic ITP? Experts generally agree that splenectomy should be deferred as long as possible and be reserved for patients with severe, symptomatic thrombocytopenia. The favorable safety profile of rituximab and the possibility of a sustained response could justify its use instead of splenectomy in the decision tree, but this has yet to be demonstrated in a prospective clinical trial. Despite the invasive and irreversible nature of splenectomy, some pediatric hematologists advocate the use of rituximab only after splenectomy has failed because splenectomy has a superior 5-year response rate compared with rituximab (71% versus 26%).

ITP, Immune thrombocytopenic purpura.

Children in whom ITP is diagnosed have an excellent chance of spontaneous recovery, irrespective of therapy. The platelet count returns to normal in 4 to 8 weeks in approximately half of the patients and by 3 months after diagnosis in two thirds of children.[28] In a review[29] of 12 publications involving more than 1500 children with ITP, 76% achieved complete remission within 6 months of initial evaluation. Spontaneous recovery was documented in 37% of the remaining patients with thrombocytopenia persisting longer than 6 months. These findings have been confirmed in other large studies.[24,30] Factors associated with the development of chronic ITP include age older than 10 years, insidious onset, and female gender.[30]

Pathogenesis. ITP is caused by autoantibodies that interact with membrane glycoproteins on the surface of platelets and megakaryocytes. These antibodies result in accelerated platelet destruction and may also impair thrombopoiesis. More than a third of adults with ITP have inadequate platelet production despite increased numbers of megakaryocytes in their bone marrow.[31] Certain antiplatelet antibodies have been shown to inhibit megakaryocytopoiesis or egress of platelets from the marrow space.[32,33] The GPIIb/IIIa complex is the autoantigen implicated most often as the cause of childhood and adult ITP.[14] Autoantibodies directed against the GPIb/IX/V and GPIa/IIa complexes have also been reported.[34] In rare instances platelet glycolipids have been implicated as autoantigen targets in chronic ITP.[35] Autoantibodies diminish or disappear when platelet levels are restored to normal.[36,37]

Factors that trigger platelet autoantibody formation in acute ITP are not well understood. Although various mechanisms by which viruses may induce autoimmune disease have been suggested, the link between the immune response initiated by infection (or vaccination) and the subsequent production of platelet autoantibodies has not been established. Proposed mechanisms include adsorption of virus to platelets, deposition of virus-containing immune complexes onto platelet membranes, or exposure of cryptic neoantigens on the platelet surface.[38] There are data to both support[39] and refute[40] the hypothesis that acute ITP is triggered by antiviral antibodies that cross-react with platelet antigens.

Some evidence suggests that T lymphocytes also play a role in the pathogenesis of ITP. Dysregulated helper T cells can promote the expansion of antiplatelet antibody–producing B-cell clones.[41] Cytotoxic T cells from some ITP patients have the capacity to destroy platelets ex vivo.[42] CD3+ T lymphocytes from ITP patients are resistant to glucocorticoid-induced apoptosis, thus suggesting that disturbed apoptosis may contribute to defective clearance of autoreactive T lymphocytes.[43]

Genetic factors have been proposed to influence the development of ITP. Studies have failed to detect linkage of the disease to particular HLA genotypes. Polymorphisms in genes encoding phagocyte Fcγ receptors or proinflammatory cytokines may influence the development of ITP.[44] Immunodeficiency states that have been associated with chronic ITP are discussed later.

Clinical and Laboratory Features. The typical manifestation of acute ITP is the abrupt onset of bruising and bleeding in an otherwise healthy child. Frequently, there is a history of a viral illness in the weeks preceding the onset of bruising. Seasonal fluctuation in the diagonsis of ITP has been noted, with a peak during spring and a nadir in the autumn.[45] Petechiae and ecchymoses are evident in most patients. Epistaxis and oral mucosal bleeding are seen in fewer than a third of patients. Hematuria, hematochezia, or melena is evident in fewer than 10%. Menorrhagia may be observed in adolescent women with ITP. Although children with ITP may have extremely low platelet counts, bleeding episodes are less severe in these patients than in those with hypoproductive thrombocytopenia. This finding has been attributed to enhanced platelet production and young, large, hemostatically effective circulating platelets.[46] A palpable spleen is present in about 10% of reported cases of childhood ITP; this finding alone should not justify performing imaging studies on the affected child's abdomen.[47] Malaise, bone pain, and adenopathy are uncommon and should raise concern for another cause, such as acute leukemia.

TABLE 34-1 Characteristics of Childhood Versus Adult Immune Thrombocytopenic Purpura

Childhood ITP	Adult ITP
Females = males	Females > males (2 : 1)
Abrupt onset	Insidious onset
Infectious prodrome common	Infectious prodrome uncommon
<20% chronic	>50% chronic

ITP, Immune thrombocytopenic purpura.

The peak age at diagnosis is 2 to 6 years. Although acute ITP may be diagnosed in children of any age, adolescents and infants are more likely to have chronic ITP develop in combination with some other immune disorder. In children ITP is seen equally in males and females, whereas in adults ITP is seen predominantly in females by a 2-to-1 ratio (Table 34-1).

Roughly 80% of children have platelet counts below 20,000/μL, often less than 10,000/μL.[24,30] Leukocyte and red cell counts are usually normal, although anemia may be seen in as many as 15% of these children, especially those with a significant history of epistaxis, hematuria, or gastrointestinal bleeding. A review of the peripheral blood film is mandatory in every child suspected of having ITP; features inconsistent with a diagnosis of ITP should prompt further investigation. Bone marrow aspiration or biopsy reveals normal or increased numbers of megakaryocytes. Increased numbers of eosinophils and their precursors may be noted, although this is not predictive of outcome.

The need for bone marrow examination in children with typical features of acute ITP is a subject of debate. There is a consensus that bone marrow aspiration is not necessary if the initial management is observation alone or administration of intravenous immunoglobulin (IVIG) or anti-D. Controversy exists regarding whether bone marrow aspiration should be performed in all children before glucocorticoid therapy is started to exclude the possibility of acute leukemia (see Box 34-2).

Additional tests that may be considered in the initial evaluation and management of suspected ITP include blood group and Coombs test (required if anti-D therapy is contemplated), quantitative immunoglobulin levels (before IVIG therapy), and antinuclear antibody test. Retrospective studies have shown that the antinuclear antibody test is positive in approximately 30% of pediatric patients with otherwise uncomplicated ITP and may predict a subset of patients at risk for the development of further autoimmune symptoms.[48] Tests not considered useful unless specific reasons are identified in the patient's history and physical examination include screening coagulation tests, liver and renal function tests, serum complement levels, thyroid function tests, and testing for human immunodeficiency virus (HIV) or *Helicobacter pylori* infection.[47]

Options for the Initial Management of Immune Thrombocytopenic Purpura. Childhood acute ITP is usually a benign, self-limited disorder that requires minimal or no therapy in the majority of cases.[24,29,49-52] There is no convincing evidence that medical therapy alters the natural history of the disease. Indications for treatment vary among practitioners and are a source of debate.[29,49,53] One set of practice guidelines put forth by the American Society of Hematology (ASH)[31] recommends that children with ITP and platelet counts less than 20,000/μL plus significant mucosal membrane bleeding or those with platelet counts less than 10,000/μL and minor purpura be treated with IVIG or a glucocorticoid. However, many pediatric hematologists take exception to this recommendation.[50,52] In treatment guidelines put forth by other expert panels, a child's condition, rather than the platelet count, steers management; children with bruising but without mucosal or more severe hemorrhage may be treated by observation alone, irrespective of the platelet count.[49,53]

At the heart of the treatment debate is the perceived risk for ICH, a rare but potentially life-threatening complication in children with ITP. In a review of 12 case series involving 1293 children, the incidence of ICH was 0.9%.[29] Other surveys[30,54-57] suggest that this may be an overestimate and that the true incidence of ICH in children with ITP is between 0.1% and 0.5%. The subgroup of children with ITP who appear to be at greatest risk for ICH are those with platelet counts less than 20,000/μL and additional risk factors such as a history of head trauma, aspirin use, or arteriovenous malformation.[29] However, the platelet count alone has never been shown to predict the severity of bleeding symptoms.[28]

There is no evidence that medical therapy, such as administration of a glucocorticoid or IVIG, reduces the incidence of ICH. Indeed, retrospective studies[56-58] have shown that ICH may occur despite previous or concomitant therapy with IVIG or a glucocorticoid. The low incidence of ICH in children with ITP precludes a randomized clinical trial to determine whether treatment reduces the risk.

The results of randomized trials for various treatment approaches have been summarized previously.[29] Regardless of whether pharmacologic therapy is used, detailed education and careful follow-up should be provided to the patient and family. The child's activities should be limited, and aspirin-containing medications should be avoided. Although hospitalization is appropriate for the treatment of a child with a severe bleeding episode, there is no evidence to support routine hospitalization of patients with otherwise uncomplicated ITP.

OBSERVATION ONLY. For a patient with ITP and only minor purpura, medical therapy may not be necessary. There is uniform consensus that patients with platelet counts greater than 20,000/μL and only minor purpura do not require therapy. As noted earlier, management of patients with platelet counts lower than 20,000/μL and minor bleeding is controversial.

FIRST-LINE MEDICAL THERAPIES. Glucocorticoids: Glucocorticoids are presumed to act through several mechanisms, including inhibition of both phagocytosis and antibody synthesis, improved platelet production, and increased microvascular endothelial stability.[59] The latter effect may explain why symptomatic bleeding episodes often subside before the platelet count increases.

In randomized trials prednisone therapy has been shown to induce normalization of the platelet count more promptly than placebo does.[60-62] Although various doses have been used, the traditional glucocorticoid regimen is prednisone at 2 mg/kg/day (maximum of 60 to 80 mg) for approximately 21 days.[60] A regimen of 4 mg/kg/day for 7 days and then tapered to day 21 is equally effective and appears to cause fewer side effects.[62] Prednisone at a dose of 4 mg/kg/day orally for 4 days with no tapering is also effective.[63] An alternative to these regimens is megadose pulse therapy (methylprednisolone, 30 mg/kg/day intravenously or orally for 3 days).[64] Side effects of glucocorticoid therapy include cushingoid facies, weight gain, fluid retention, acne, hyperglycemia, hypertension, moodiness, pseudotumor cerebri, cataracts, growth retardation, avascular necrosis, and osteoporosis.

IVIG: Imbach and colleagues[65] first reported the successful use of IVIG for the treatment of acute ITP in a small series of children. This was followed by many reports that documented the ability of IVIG to cause a rapid increase in the platelet count in patients with acute and chronic ITP.[66-68]

IVIG slows the clearance of antibody-coated blood cells from the circulation by inhibiting the phagocytic activity of cells of the reticuloendothelial system.[69] Studies in the early 1980s suggested that this effect is mediated primarily through the Fc portion of IgG. Specially treated preparations of intravenous IgG lacking the Fc portion of the molecule were inferior to unmodified IgG at increasing the platelet count in ITP patients. The proposed mechanism for this effect was Fc receptor blockade.[69] Subsequent studies of FcγRIIB-deficient mice[70] suggested that IVIG elicits its effect by activating these inhibitory receptors and not simply by Fc receptor blockade (Fig. 34-3). However, recent publications cast doubt on the conclusion that IVIG acts through activation of FcγRIIB.[71,72] Although some data support the notion that anti-idiotypic antibodies may also be present in commercial preparations and contribute to the action of IVIG,[73]

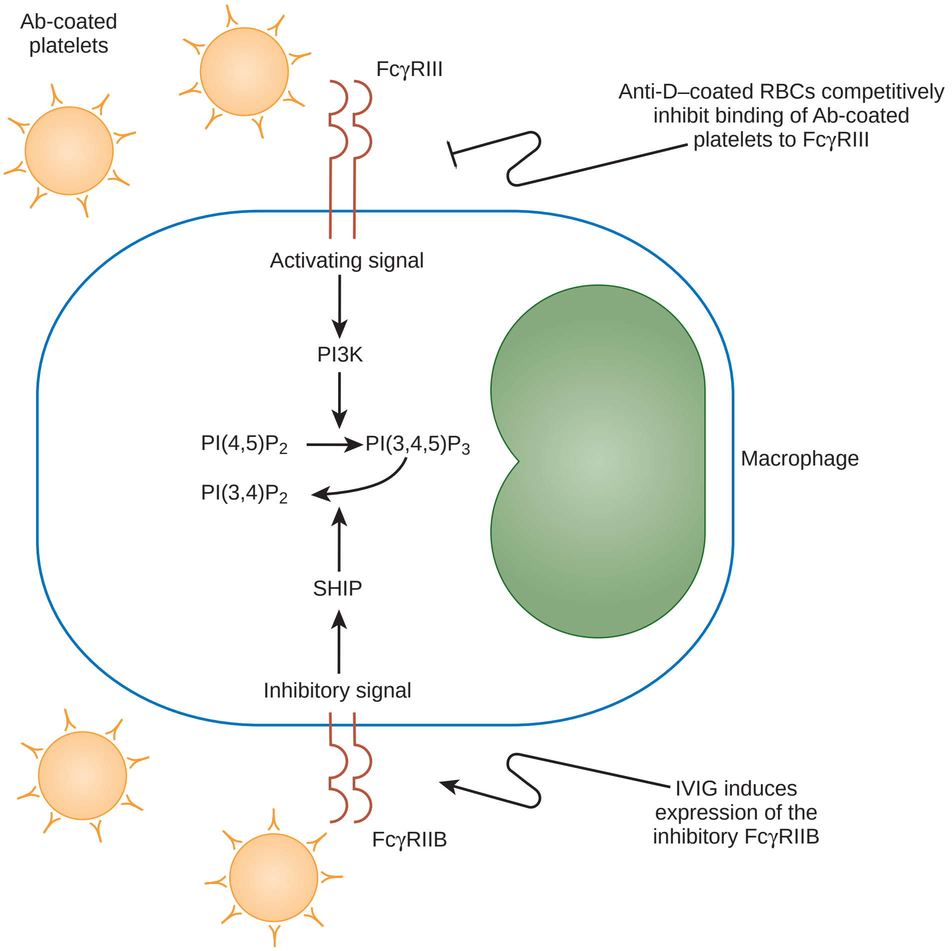
Ab-coated platelets
FcγRIII
Anti-D–coated RBCs competitively inhibit binding of Ab-coated platelets to FcγRIII
Activating signal
PI3K
PI(4,5)P$_2$
PI(3,4,5)P$_3$
PI(3,4)P$_2$
SHIP
Inhibitory signal
Macrophage
FcγRIIB
IVIG induces expression of the inhibitory FcγRIIB

Figure 34-3 Mechanism of action of intravenous IgG (IVIG) in immune thrombocytopenia. Binding of antibody-coated platelets to activating Fcγ receptors (FcγRIII) on macrophages results in the production of phosphatidylinositol 3,4,5-triphosphate (PI[3,4,5]P$_3$) via the action of phosphatidylinositol 3′-kinase (PI3K). These activated macrophages phagocytose the platelet-antibody complexes. IVIG induces expression of the inhibitory Fc receptor (FcγRIIB) on macrophages. Stimulation of the inhibitory receptor results in recruitment of SHIP, a 5-phosphatase that degrades PI(3,4,5) P$_3$ into phosphatidylinositol 3,4-bisphosphate PI(3,4)P$_2$. *(Modified from Lin SY, Kinet JP: Immunology: giving inhibitory receptors a boost.* Science *291:446, 2001.)*

the clinical importance of this mechanism remains unproved.

The traditional dose of IVIG is 2 g/kg divided over a period of 2 to 5 days. However, several studies suggest that lower doses are effective. In a randomized trial,[67] pediatric patients who received 0.8 g/kg IVIG had at least as rapid a response rate as those who received 2 g/kg over a 2-day period. In more recent randomized trials,[68,74] favorable results have been seen with even lower doses of IVIG (250 mg/kg/day for 2 days). Among pediatric patients in whom a response is seen, IVIG produces a more rapid increase in platelet count than do traditional doses of a glucocorticoid (2 mg/kg/day of prednisone), as documented in controlled trials.[62,75]

Several other general conclusions have emerged from studies on IVIG in pediatric patients.[76] First, both splenectomized and nonsplenectomized patients may respond to IVIG. Second, responses to IVIG are generally reproducible. Third, the duration of the response is brief, approximately 2 to 4 weeks.

Enthusiasm for the use of IVIG is offset by cost considerations. IVIG is considerably more expensive than glucocorticoids or anti-D.[77] One study concluded that IVIG may be cost-effective if it reduces hospital stay or if it prevents the need for a splenectomy.[66]

Complications associated with IVIG are common and occur in 15% to 75% of patients.[29] Frequent side effects include flulike symptoms such as headache, nausea, lightheadedness, and fever. Some of these adverse effects can be alleviated by pretreatment with analgesics or antihistamines. Approximately 10% of patients treated with IVIG (2 g/kg) experience aseptic meningitis manifested as a severe, protracted headache and photophobia.[78] These symptoms, which can cause considerable anxiety in patients, parents, and physicians, may spur additional diagnostic studies (e.g., computed tomography) or result in prolonged hospitalization. A rare but serious side effect of IVIG infusion is anaphylaxis, which can occur in patients who are totally deficient in IgA.[79] Most preparations of IVIG contain small amounts of IgA, and IgE antibodies responsible for anaphylaxis form after an initial exposure to IgA in these preparations.

Anti-D: Anti-D is a plasma-derived immunoglobulin prepared from donors selected for a high titer of anti-Rh_0(D) antibody. Anti-D elicits a rise in platelet count, along with mild to moderate anemia, in most patients with ITP.[67,77,80-83] A role for anti-D in the treatment of chronic ITP is now well established. Its utility in acute ITP has been studied less extensively, but it is also effective in this clinical setting.[81,83,84]

Anti-D can be used to treat only Rh_0(D)-positive patients with ITP because Rh_0(D)-negative patients do not show a response. The presumed mechanism of action is phagocytic cell blockade. Patients with intact spleens are more likely to respond to anti-D than splenectomized patients are.

The generally recommended dose is 50 to 75 µg/kg as a short intravenous infusion[81] or subcutaneous injection.[85] Uncontrolled studies have demonstrated that anti-D treatment increases the platelet count in approximately 80% of Rh_0(D)-positive children.[80,82] The therapeutic effect of anti-D lasts for 1 to 5 weeks. A controlled trial showed that anti-D therapy was less effective than IVIG or a glucocorticoid in terms of days required to attain a platelet count of 20,000/µL.[67] Anti-D is less expensive than IVIG, with an estimated cost savings of 35% per episode of ITP.[77] The shorter administration time required for anti-D therapy also makes the medication more convenient to use than IVIG.

Adverse reactions of headache, nausea, chills, dizziness, and fever have been reported in 3% of infusions, and these adverse reactions were classified as severe in only a minority of cases.[78,86] Some degree of hemolysis, the main adverse reaction with anti-D, is inevitable because of binding of anti-D antibody to Rh_0(D)-positive erythrocytes. There is laboratory evidence of hemolysis in most patients. The average decline in hemoglobin ranges from 0.5 to 1 g/dL,[81-83] and most cases of hemolysis do not require medical intervention. In a small subset of patients, more significant hemolysis has been observed, which has tempered enthusiasm for use of the drug. Postmarketing surveillance by the U.S. Food and Drug Administration[87] documented 15 cases of hemoglobinemia or hemoglobinuria after anti-D therapy. Of these patients six required transfusion, eight experienced an onset or exacerbation of renal insufficiency, and two underwent dialysis. The incidence of intravascular hemolysis was estimated to range from 0.1% to 1.5%. Why certain patients experience severe intravascular hemolysis after anti-D therapy is unclear. Subcutaneous delivery of anti-D may be associated with a lower incidence of severe hemolytic reactions.[85]

Relapses or Treatment Failures. Many patients treated with a glucocorticoid, IVIG, or anti-D will become thrombocytopenic again after a few weeks. These patients are likely to respond again to the therapy used initially. Patients who require therapy and whose thrombocytopenia does not respond to initial treatment with a glucocorticoid, IVIG, or anti-D are usually treated with one of the alternative frontline therapies. No clinical studies document the response rate in these instances. Patients with persistent mild hemorrhage may benefit from low-dose glucocorticoid therapy.

Management of Chronic Immune Thrombocytopenic Purpura. In approximately 20% of children in whom ITP is diagnosed, thrombocytopenia persists for longer than 6 months. Chronic ITP is more common in female adolescents and adults than in their male counterparts. Chronic ITP sometimes occurs in association with other autoimmune diseases or in conjunction with an underlying condition known to predispose to disorders of autoimmunity, such as lymphoma (Box 34-3).

In up to a third of children with chronic ITP, spontaneous remission will occur months or years later. It is unclear why the condition resolves in these individuals. An estimated 5% of children with chronic ITP have recurrent ITP characterized by intermittent episodes of thrombocytopenia followed by lengthy periods of remission. This is presumed to reflect a chronic compensated state of ITP. During periods of remission, increased platelet production balances the increased rate of platelet destruction. During exacerbations, platelet production by

Box 34-3 **Conditions Associated with Chronic Immune Thrombocytopenic Purpura of Childhood**

- Immunodeficiency
  - Hypogammaglobulinemia
  - Common variable immunodeficiency
- Lymphoproliferative disorders
  - Autoimmune lymphoproliferative syndrome
  - Hodgkin lymphoma
- Collagen vascular disorders
  - Systemic lupus erythematosus
- Infection
  - Human immunodeficiency virus

the marrow is suppressed by viral infections or other factors and is unable to offset the rate of destruction.

Evaluation of patients with chronic ITP should include bone marrow aspiration, if not already done; screening tests for immunodeficiency (e.g., quantitative immunoglobulin levels/subsets, specific antibody titers, lymphocyte subsets), systemic lupus erythematosus (SLE), and other autoimmune diseases (antinuclear antibody tests, direct Coombs test, antiphospholipid antibody tests, thyroid function tests); and serologic testing for HIV. Obtaining blood counts and peripheral blood smears from the parents may help exclude familial disorders.

The primary goal of treatment in patients with chronic ITP is to prevent bleeding, not cure the disease. As with acute ITP, therapy decisions should be based more on symptoms than on platelet count. In formulating a treatment plan, the physician must balance the potential side effects of treatment with the risk of bleeding episodes caused by the disease. Because the goal of treatment is a safe platelet count, not necessarily a normal platelet count, observation alone is an appropriate approach for many patients, especially those with minimal symptoms.[88]

SPLENECTOMY. Splenectomy is thought to be effective because in most patients the spleen is the major site of both platelet destruction and autoantibody production. Platelet survival studies indicate improved platelet life span after splenectomy.[57]

Splenectomy remains the most predictable intervention to achieve long-term remission in children with chronic ITP.[47] Splenectomy should be considered in a pediatric patient with chronic ITP whose risk for hemorrhage precludes the use of observation alone. However, there is debate about where splenectomy belongs in the therapeutic decision tree for chronic ITP (see Box 34-2). Because remissions are often delayed in children and the risk of postsplenectomy sepsis is significant, especially in children younger than 5 years, splenectomy is deferred longer in children with ITP than in adults. ASH practice guidelines[29,52] recommend that splenectomy be considered for children in whom ITP has persisted for at least 1 year and who have bleeding symptoms and a platelet count of less than 10,000/µL (ages 3 to 12) or 10,000 to 30,000/µL (ages 8 to 12 years). Practice guidelines in the United Kingdom are similar.[55]

Laparoscopic splenectomy is preferred over open splenectomy in children with chronic ITP.[89,90] Advantages of the laparoscopic procedure include less postoperative pain, earlier return of gastrointestinal function, shorter hospitalization, more rapid resumption of normal activities, and smaller incisions. To ensure adequate hemostasis, the platelet count can be raised preoperatively with a glucocorticoid, IVIG, or anti-D. Despite these interventions, many patients will still have thrombocytopenia at the time of splenectomy; however, most will not exhibit excess bleeding. Accordingly, prophylactic platelet transfusions are not warranted. Instead, platelet transfusions should be reserved for patients with intraoperative bleeding. Most experienced surgeons have become comfortable performing laparoscopic splenectomy on patients with platelet counts in the range of 50,000 to 100,000/µL. Overall perioperative mortality is less than 1%.

The majority of children with chronic ITP respond to splenectomy. In a review[29] of 16 case series involving 271 children undergoing elective splenectomy for chronic ITP, a complete remission rate of 72% was reported. The platelet count usually rises immediately after splenectomy and reaches a maximum 1 to 2 weeks postoperatively. If the peak platelet count achieved after splenectomy is greater than 500,000/µL, permanent remission is likely.[91] Certain patient characteristics are associated with a higher probability of response to splenectomy, including a previous response to glucocorticoid therapy.[92-94] However, there is no single factor or combination of factors that will predict the response to splenectomy in all cases.[95]

Relapse after an immediate response to splenectomy may result from transient viral suppression of thrombopoiesis. If the thrombocytopenia persists, an accessory spleen should be considered. Approximately 40% of patients with persistent thrombocytopenia will have a residual accessory spleen that can be seen with sensitive imaging techniques such as ^{99m}Tc sulfur colloid scans (Fig. 34-4) or ^{111}In platelet localization studies.[96] Many of these patients will achieve complete remission after removal of the accessory spleen.[96] The presence of Howell-Jolly bodies on a peripheral blood smear does not rule out an accessory spleen.

The major risk after splenectomy is fatal sepsis caused by encapsulated organisms. The risk of septicemia in splenectomized patients is estimated to be 1 per 300 to 1000 patient-years.[97] To lessen the risk of future sepsis, patients should receive vaccinations for pneumococcus, *Haemophilus influenzae* type b, and meningococcus at least 2 weeks before surgery.[98] In addition, penicillin prophylaxis is indicated for splenectomized patients younger than 5 years. The benefit of penicillin prophylaxis in older asplenic children is controversial.[99]

PHAGOCYTE BLOCKADE. Intermittent maintenance therapy with a glucocorticoid, IVIG, or anti-D can be used to delay splenectomy. These medications may also be useful in the management of patients with chronic ITP who experience symptomatic thrombocytopenia after splenectomy.

In some patients a safe platelet count can be maintained with low doses of prednisone. However, even low doses of prednisone, comparable to physiologic cortisol secretion, can cause osteoporosis.[100]

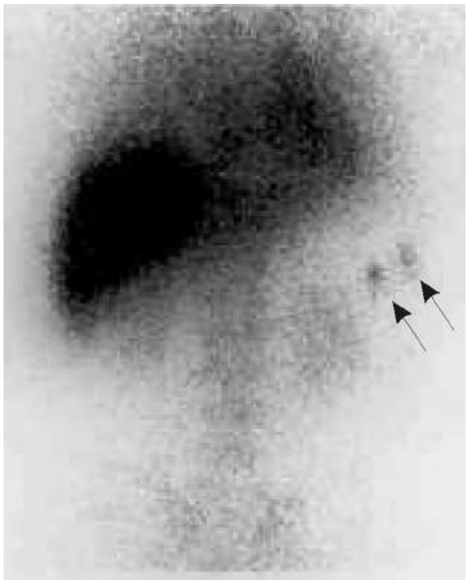

Figure 34-4 Detection of accessory spleens after splenectomy. A sulfur colloid scan shows accessory spleens *(arrows)* in a teenage girl with chronic immune thrombocytopenic purpura and persistent thrombocytopenia after splenectomy. *(Courtesy Dr. Robert Minkes, Louisiana State University.)*

IVIG (1 to 2 g/kg total dose administered) elevates the platelet count in most children with chronic ITP.[61,62] Periodic doses of IVIG may be used as maintenance therapy to defer splenectomy in young children with chronic ITP.[66] This approach is cost-effective in young children.[101] In approximately 25% of children, ITP will become refractory to maintenance therapy with IVIG.[102] Low-dose alternate-day prednisone therapy can be used as an adjunct to maintenance IVIG therapy. Although repeated infusions of IVIG induce short-term platelet responses in most patients with chronic ITP, there is no evidence that such therapy will affect the natural history of the disease and induce a long-lasting remission.

Anti-D administration raises the platelet count in most Rh_0(D)-positive children with chronic ITP. However, the benefit is usually transient, with a median duration of 3 to 5 weeks.[103] Anti-D can be effective as maintenance therapy in patients who are not appropriate candidates for splenectomy or other therapies because of young age or other factors.[103,104] In Rh_0(D)-positive patients with chronic ITP, anti-D is preferred over IVIG because of ease of administration, comparable efficacy, and lower cost.

MONOCLONAL ANTIBODIES THAT TARGET B CELLS OR T CELLS. Rituximab: Rituximab, a humanized monoclonal antibody directed against CD20, is now commonly used to treat chronic ITP despite limited clinical trial data documenting its efficacy in children with this condition (see Box 34-2).[105] Rituximab is generally administered in four weekly intravenous infusions of 375 mg/m^2 each, but there is no standard dose for rituximab in children.[106] There are reports of children with ITP who responded to lower doses of rituximab.[106] This agent causes rapid depletion of pre-B cells and B cells that lasts for 6 to 12 months. Pre-B cells efficiently present antigen to T cells, and disruption of this interaction may attenuate the autoimmune response. Approximately 30% to 60% of patients with chronic ITP respond to rituximab.[107-113] The initial response may be immediate (within 1 week) or delayed (up to 3 months).[107-113] Relapses are common but often respond to retreatment. The estimated 5-year sustained response rate in children is 26%.[113] Side effects of rituximab include fever, urticaria, pruritus, throat tightness, and serum sickness.[110] Another serious complication associated with rituximab is progressive multifocal leukoencephalopathy (PML).[112] More than 50 cases of PML have been reported in patients treated with rituximab, including a patient with ITP.[114]

Alemtuzumab: Alemtuzumab (Campath 1H) is a humanized monoclonal antibody that targets CD52, a protein on both B and T cells. There are anecdotal reports of its use in patients with chronic ITP.[115] Part of the effect of alemtuzumab can be attributed to B-cell depletion, analogous to rituximab, and part to depletion of T cells. Unlike rituximab, alemtuzumab induces profound generalized immunosuppression associated with opportunistic infections.[105]

OTHER IMMUNOSUPPRESSIVE AGENTS. A large number of other immunosuppressive medications have been used to treat refractory ITP, as reviewed elsewhere.[48,116,117] No randomized studies have evaluated the effectiveness of these various therapies. The choice of therapy must be individualized to the patient.

Azathioprine: Azathioprine acts preferentially on lymphocytes and was one of the first drugs reported to be effective in the treatment of chronic refractory ITP.[118,119] Recommended doses range from 50 to 200 mg/m^2/day orally. Continuous therapy for 4 to 6 months is required before a patient is considered unresponsive. Once a response is obtained, the dose should be tapered to the lowest level that results in a hemostatic platelet count. In adults with refractory ITP treated with azathioprine, approximately 20% achieve a complete response and 45% exhibit a partial response. Relapse often occurs when the therapy is stopped. The combination of azathioprine and oral steroids may be synergistic, and azathioprine therapy may allow a reduction in the dose of glucocorticoids. Toxicities include dose-related leukopenia, opportunistic infection, and an increased risk for malignancy.

Cyclophosphamide: The alkylating agent cyclophosphamide has also been used to treat chronic refractory ITP, with a response rate similar to that with azathioprine.[119] Greater risks are associated with the use of cyclophosphamide than with azathioprine. Side effects include myelosuppression, alopecia, nausea, infertility, teratogenicity, hemorrhagic cystitis, and an increased risk for malignancy.

Cyclosporine: The potent immunosuppressive agent cyclosporine disrupts T-cell function through calcineurin inhibition. There are case reports of patients with refractory ITP who had increases in platelet counts after treatment with cyclosporine.[120] Response rates vary. A starting dose of 5 mg/kg/day given in divided oral doses is recommended, with the aim of maintaining a serum cyclosporine level of 200 to 400 ng/mL. The drug should be discontinued after 4 weeks if there is no response. Hypertension, renal insufficiency, hirsutism, and liver dysfunction are common side effects.

Tacrolimus: Though not structurally related to cyclosporine, tacrolimus has a similar mechanism of action and has been used anectodally to treat chronic ITP.[41] Initial dosing recommendations for tacrolimus in children are 0.15 to 0.3 mg/kg/day given in divided oral doses. Doses should be adjusted to maintain trough tacrolimus concentrations of 5 to 20 ng/mL in whole blood. Common adverse effects associated with tacrolimus include hypertension, tremor, headache, and hyperglycemia.

Mycophenolate mofetil: There are reports showing the effectiveness of another immunomodulatory drug, mycophenolate mofetil (MMF), in patients with steroid-resistant chronic ITP.[121,122] MMF is a prodrug of mycophenolic acid, a noncompetitive inhibitor of inosine-5α-monophosphate dehydrogenase, a key enzyme in the purine biosynthetic pathway. MMF inhibits proliferation of both T cells and B cells. In one series[122] a sustained platelet increase to a level greater than 50,000/μL was seen in 7 of 18 patients with refractory ITP. For adults the usual starting dose is 250 mg orally twice daily, with an increase to 1 g twice daily by 3 weeks. MMF has few side effects and is generally well tolerated.

Vinca alkaloids: The vinca alkaloids vincristine and vinblastine may induce a response in some patients with chronic refractory ITP.[123] These drugs bind tubulin and inhibit microtubule polymerization, which is presumed to disrupt phagocytosis. Either vincristine or vinblastine may be used; the efficacy of the two drugs is comparable. The usual dose of vincristine is 1.5 mg/m^2 (maximum of 2 mg) intravenously, whereas the dose of vinblastine is 6 mg/m^2 (maximum of 10 mg) intravenously. These doses are repeated weekly for a month. If no response is seen, additional doses are unwarranted because subsequent improvement is unlikely. Patients who show a response often require repeated doses at 2- to 3-week intervals to maintain a safe platelet count. In adults the response rate is approximately 12%, and the partial response rate is 35%. The response rate in children is not established and varies considerably in the reports available. Adverse effects include peripheral neuropathy, constipation, and alopecia. Vinblastine causes dose-related myelosuppression.

Danazol: Danazol, an attenuated androgen with mild virilizing effects, has been shown to increase the platelet count in patients with refractory ITP, although the mechanism of action is uncertain. Approximately 40% of adult patients respond to danazol with an increase in platelet count to greater than 50,000/μL.[124] A typical dose is 300 to 400 mg/m^2/day orally, although smaller doses (50 mg/m^2) have been reported to be effective.[125] In general, 2 months of therapy is required before a response is seen. Often the medication must be continued to maintain the platelet count, although effort should be made to minimize the dose. Danazol may be especially useful for the treatment of older adolescent or adult women with chronic, refractory ITP and uncontrollable menorrhagia. Side effects of danazol include acne, fluid retention, hirsutism, deepening of the voice in women, headache, nausea, rash, breast tenderness, and oligomenorrhea or amenorrhea. Patients should be monitored for the development of liver toxicity.

Interferon alfa: Although the mechanism is unknown, 25% of adults with chronic ITP treated with interferon alfa display a significant, albeit transient, increase in platelet count.[126] Interferon-alfa has also been used to treat children with chronic ITP.[127] The recommended dose is 3 million U/m^2 subcutaneously three times per week for 4 weeks. Adverse effects of interferon alfa include flulike symptoms and neutropenia.

Combination chemotherapy: Combination chemotherapy with or without autologous stem cell transplantation has been used successfully to treat some adults with chronic, refractory ITP, with an overall sustained remission rate of approximately 40%.[128,129]

THROMBOPOIESIS-STIMULATING AGENTS. In ITP the rate of platelet production is inadequate to offset the increased rate of platelet destruction.[31] Two newer drugs for the treatment of chronic ITP act by stimulating thrombopoiesis. Romiplostim is a recombinant protein that contains two domains: a peptide that binds to the thrombopoietin (TPO) receptor c-Mpl and an antibody Fc domain that increases the half-life in circulation.[130] In a placebo-controlled, phase II study this agent was efficacious when administered subcutaneously on a weekly basis (1 to 3 μg/kg) to adults with chronic ITP.[131] Toxicity was minimal. In children there has been one randomized clinical trial of romiplostim[132] and several case series.[106,133,134] Romiplostim increased the platelet count in 88% of the children in this study, and quality-of-life measurements documented reduced parental burden.[135] Another small-molecule c-Mpl agonist for chronic ITP is eltrombopag,[130] which binds to the transmembrane region of c-Mpl rather than the TPO binding site. This agent is given orally once daily. A phase II trial on adults with chronic ITP showed that eltrombopag at doses of 50 and 75 mg was significantly better than placebo at increasing platelet counts to greater than 50,000/μL. The drug appears to have few side effects, one of which is reversible liver toxicity.[136] Romiplostim and eltrombopag are licensed for use in adults with chronic, severe ITP but not yet for use in children.

One concern is that myelofibrosis may develop after prolonged treatment with TPO receptor agonists.[106] A slight but significant increase in reticulin fibrosis has been demonstrated in the bone marrow of adult patients receiving TPO receptor agonists.[137] Until additional safety data are collected, monitoring for marrow changes with an annual bone marrow biopsy seems prudent. Thromboembolic events have also been reported in patients treated with romiplostim and eltrombopag.[106] Other limitations for the use of TPO receptor agonists include cost, the indefinite duration of therapy required, and the risk of severe thrombocytopenia in patients who interrupt therapy.[106,138]

Management of Life-Threatening Hemorrhage in Immune Thrombocytopenic Purpura. ICH or another life-threatening form of bleeding mandates intervention with multiple therapeutic modalities, including IVIG (1 g/kg), anti-D (50 to 75 μg/kg), high-dose glucocorticoids (e.g., methylprednisolone, 30 mg/kg intravenously), platelet transfusions, or a combination of these interventions. Because IVIG and anti-D act to inhibit phagocytosis by distinct and potentially synergistic mechanisms (see Fig. 34-3), these two agents may be used together for instances of severe bleeding. TPO receptor agonists have a limited role in this setting because it takes an average of 2 weeks to see a response.[106] Recombinant factor VIIa has also been used in the management of ICH in patients with thrombocytopenia refractory to platelet-enhancing agents.[139] If a craniotomy is required, consideration should be given to splenic artery embolization[140] or emergency splenectomy,[141] although most hematologists reserve splenectomy for patients in whom ITP does not respond rapidly to therapy with glucocorticoids, IVIG, anti-D, and platelet transfusions.[29,142] Optimal therapy for ICH also includes supportive care with mechanical ventilation and mannitol.

Thrombocytopenia Associated with Lymphoproliferative and Autoimmune Disorders. Chronic ITP may be a manifestation of an associated autoimmune disease or immunodeficiency state. This is especially true in young children in whom chronic, refractory ITP develops or in adolescent girls with systemic symptoms.

Autoimmune lymphoproliferative syndrome (ALPS) is a rare syndrome that occurs in early childhood and is characterized by massive, nonmalignant lymphadenopathy, splenomegaly, hypergammaglobulinemia, and autoimmune manifestations, including immune thrombocytopenia and hemolytic anemia (see Chapter 13).[143] ALPS has been linked to inherited defects in *FAS* and other genes that regulate lymphocyte apoptosis. An abnormal accumulation of lymphocytes results in lymphadenopathy, hepatosplenomegaly, and hypersplenism. In young children the lymphadenopathy may be pronounced and then wane later in life. Impaired elimination of autoreactive lymphocytes also causes autoimmune manifestations, including autoantibody formation. Patients with ALPS commonly exhibit episodic autoimmune cytopenia, including thrombocytopenia, hemolytic anemia, neutropenia, or combined cytopenias.

A significant proportion of young children with chronic ITP may have a variant of ALPS.[144-146] For example, in one survey[145] of 20 pediatric patients with chronic cytopenia, 25% were found to have profound defects in Fas-mediated lymphocyte death. Two of these patients, ages 4 and 9 years, had chronic refractory ITP without other clinical manifestations of ALPS. Neither patient had an identifiable mutation in *FAS* or caspase-10, and it is presumed that they may have defects in one of the molecules involved in Fas signaling. Similar results have been reported in other studies,[144,146] thus suggesting that altered Fas signaling, independent of *FAS* mutations, is associated with and may precipitate chronic hematologic autoimmunity, including ITP.

The lymphadenopathy and cytopenias associated with ALPS generally respond to glucocorticoid therapy. Mycophenolate mofetil and sirolimus are also highly effective in the treatment of ALPS.[147] Because rituximab may lead to prolonged hypogammaglobulinemia when used in ALPS, alternative immunosuppressants should be tried before using this agent.[147] Patients with ALPS have a very high risk of postsplenectomy sepsis, so splenectomy is not recommended.[147]

Thrombocytopenia occurs at some time in approximately 25% of patients with SLE and may be the first manifestation of autoimmunity.[148] Both autoantibodies and complement-fixing immune complexes have been implicated in the pathogenesis of thrombocytopenia in SLE.[149] In addition, antiphospholipid antibodies are frequently observed in the sera of patients with SLE.[150] Antibodies against antigens nonspecifically adsorbed to the surface of platelets, such as DNA, have also been reported.[151]

The initial therapy for thrombocytopenia in patients with SLE is administration of a glucocorticoid, but there is a high incidence of recurrent thrombocytopenia when the steroid dose is tapered. Long-term use of low doses of glucocorticoids may be justified, especially if these drugs also control the other symptoms of SLE. Splenectomy can be used in the management of patients with SLE and chronic, symptomatic thrombocytopenia. In some case series[152] splenectomy for SLE has been shown to yield response rates comparable to those for ITP, whereas in other series[153] the response rate has been lower.

Thrombocytopenia may be associated with an antiphospholipid antibody syndrome, whether the syndrome occurs as a primary disorder or in association with SLE or another autoimmune disorder. Patients with antiphospholipid antibody syndrome often experience recurrent venous or arterial thrombotic complications rather than bleeding (see Chapter 35). Autoantibodies against GPIIb/IIIa, GPIa/IIa, and GPIb/IX have been documented in this syndrome.[154] Enhancement of platelet activation by antiphospholipid antibodies may contribute to the prothrombotic state in these patients.[155] In general, the degree of thrombocytopenia is moderate. A variety of therapies have been used to treat antiphospholipid antibody syndrome and the accompanying thrombocytopenia, including glucocorticoids and other immunosuppressive agents.

Evans syndrome is the coexistence of autoimmune hemolytic anemia and thrombocytopenia. Autoimmune neutropenia also develops in many of these patients (see Chapter 13).[156] Frequently, patients with Evans syndrome have associated disorders, such as dysgammaglobulinemia. Distinct antiplatelet and antierythrocyte antibodies rather than cross-reactive antibodies are responsible for the association of autoimmune hemolytic anemia and thrombocytopenia. In many patients the course of the disorder is marked by exacerbations of thrombocytopenia, anemia, or neutropenia. Unlike the majority of patients with ITP or isolated autoimmune hemolytic anemia, therapy with glucocorticoids, IVIG, or splenectomy often fails in patients with Evans syndrome.[156] Patients with Evans syndrome have been treated with rituximab, which induces remission in the majority, although relapses tend to occur within 12 months.[156] Other options for treatment include mycophenolate mofetil and cyclosporine.[156] For severe and refractory cases, hematopoietic stem cell transplantation is a consideration.

Immune thrombocytopenia can complicate other autoimmune diseases, including dermatomyositis, juvenile rheumatoid arthritis, Hashimoto thyroiditis, Graves disease, myasthenia gravis, inflammatory bowel disease, sarcoidosis, and protein-losing enteropathy.[157-160] Immune thrombocytopenia can also precede, accompany, or follow neoplastic disease, especially lymphoproliferative disorders such as Hodgkin and non-Hodgkin lymphoma.[161] Immune thrombocytopenia has also been seen in association with childhood acute lymphoblastic leukemia.[162] In some patients the thrombocytopenia of neoplastic disease resolves in response to antineoplastic therapy, whereas in others the thrombocytopenia requires specific treatment.

Thrombocytopenia occurs in 5% to 10% of patients with HIV infection. Immune-mediated platelet destruction is a major contributor to the thrombocytopenia seen in patients with HIV. The antibodies in HIV-associated thrombocytopenia are directed against the same target epitopes commonly seen in idiopathic ITP—namely, epitopes on GPIIb/IIIa.[163] Immune complexes composed of anti-idiotype antibodies and antibodies directed against the HIV antigen gp160/120 may also induce platelet destruction.[163] Whereas half of patients with ITP have normal or increased platelet production, most patients with HIV-associated thrombocytopenia have decreased platelet production.[164] Infection of megakaryocytes or bone marrow stromal cells may account for the reduced platelet production noted in HIV-associated thrombocytopenia.

Thrombocytopenia may occur early in the course of HIV infection and is often seen within the first 2 years.[165] Platelet counts are rarely lower than 50,000/μL, and symptomatic bleeding is uncommon. However, in HIV-infected patients with hemophilia, the bleeding can be more severe, even life threatening. Therapy is similar to that for ITP, except that HIV-associated thrombocytopenia may respond to antiviral therapy. Patients with severe or symptomatic thrombocytopenia that persists despite antiviral therapy should be managed in the same way as patients with severe ITP.

Neonatal Alloimmune Thrombocytopenia. NAIT, also termed fetomaternal alloimmune thrombocytopenia, is a syndrome characterized by transient, isolated, severe thrombocytopenia. The syndrome results from placental transfer of maternal alloantibodies directed against paternally inherited antigens present on fetal platelets but absent from maternal platelets. Therefore this condition is the platelet counterpart of hemolytic disease of the newborn.

Alloantigens. Platelet alloantigens and the platelet membrane glycoproteins on which they reside are listed in Table 34-2. Most of the antigens were originally named on the basis of the patient's surname. In many cases duplicate names were assigned to the same alloantigens (e.g., Pl^{A1} = Zw^{a}, Pen^{a} = Yuk^{b}). The HPA, or human platelet antigen, nomenclature was proposed in the 1990s to systemize platelet alloantigens.[166-168] Each HPA system represents a biallelic polymorphism caused by a single amino acid substitution (see Table 33-2).

In white populations the most frequently implicated alloantigen in NAIT is HPA-1a (Pl^{A1}), which accounts for 78% of serologically confirmed cases.[169] Incompatibility for the HPA-5b alloantigen is the next most common cause. Approximately 98% of white individuals express the HPA-1a antigen on their platelets; most of the remaining 2% are homozygous for an alternative allele that encodes the HPA-1b (Pl^{A2}) antigen. The protein products of these two alleles differ only by a single amino acid (Leu33 versus Pro33). The incidence of NAIT is approximately 1 in 2000 births,[170] which is much lower than the predicted incidence based on distribution of the HPA-1a and HPA-1b alleles in the population. Other immune

TABLE 34-2 Platelet Alloantigen Systems*

Current Designation	Original Designation	Platelet Localization	Polymorphism	PHENOTYPE FREQUENCY	
				Whites	Japanese
HPA-1a -1b	Pl^{A1}, Zw^{a} Pl^{A2}, Zw^{b}	GPIIIa	Leu33 Pro33	0.98 0.027	0.99 0.037
HPA-2a -2b	Ko^{b} Ko^{a}, Sib^{a}	GPIb$_{\alpha}$	Thr145 Met145	0.99 0.017	ND 0.25
HPA-3a -3b	Bak^{a}, Lek^{a} Bak^{b}, Lek^{b}	GPIIb$_{\alpha}$	Ile843 Ser843	0.85 0.66	0.79 0.71
HPA-4a -4b	Pen^{a}, Yuk^{b} Pen^{b}, Yuk^{a}	GPIIIa	Arg143 Gln143	>0.999 <0.0001	>0.999 0.017
HPA-5a -5b	Br^{b}, Zav^{b} Br^{a}, Zav^{a}	GPIa	Glu505 Lys505	0.99 0.021	0.99 0.087
HPA-6b	Tu^{a}, Cu	GPIIIa	Gln489	<0.01	ND
HPA-7b	Mo^{a}	GPIIIa	Pro407	<0.01	ND
HPA-8b	Sr^{a}	GPIIIa	Cys636	<0.01	ND

GPIbα, Glycoprotein Ibα; *GPIa,* glycoprotein Ia; *GPIIIa,* glycoprotein IIIa; *HPA,* human platelet antigen; *ND,* not determined.

*Other low-frequency human platelet alloantigens exist but are not listed here. Comprehensive reviews are available.[166,167]

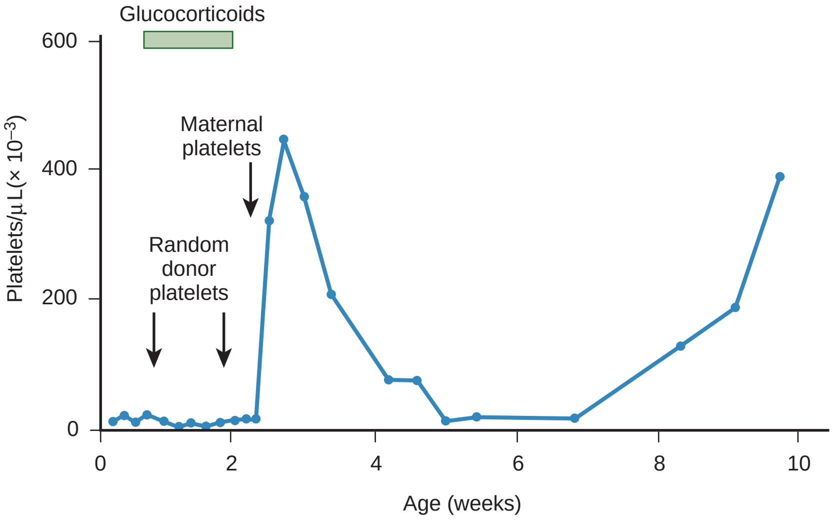

Figure 34-5 Treatment of neonatal alloimmune thrombocytopenia with washed, irradiated maternal platelets. *(Courtesy Dr. Lori Luchtman-Jones, Washington University.)*

response genes appear to regulate HPA-1a alloantibody formation. Women with the HLA-DR3 antigen have about a twentyfold relative risk of forming HPA-1a alloantibodies. In Asian populations incompatibility of the HPA-4 system is the most common cause of NAIT (80%), followed by incompatibility of the HPA-3 system.[171] HLA class I antigens are expressed on platelets, and maternal sensitization to HLA has been implicated as a cause of NAIT in a few patients.[172] HLA alloantibodies are present in 10% to 30% of pregnant women, especially multiparous women. The relative rarity of NAIT secondary to HLA alloantibodies may result from binding of these antibodies to the placenta or other tissues. Blood group ABH alloantibodies have been found in a small number of patients with NAIT.[173]

Clinical and Laboratory Features. The degree of thrombocytopenia in NAIT can be severe, with platelet counts of less than 10,000/μL on the first day of life.[173] Common hemorrhagic manifestations include petechiae (90%), hematomas (66%), and gastrointestinal bleeding (30%). The maternal platelet count is normal, an important laboratory value for distinguishing this condition from neonatal thrombocytopenia secondary to maternal ITP[174] (discussed later). Unlike Rh disease of the newborn, NAIT can occur in both the first and subsequent pregnancies. A history of a previously affected infant provides strong supportive evidence for a diagnosis of NAIT.

Patients with NAIT are at increased risk for ICH, both prenatally and postnatally.[170,173] About 15% of affected neonates have ICH, and 50% of these cases occur antenatally. In utero bleeding can result in hydrocephalus, porencephaly, seizures, or fetal loss.[175] The pronounced bleeding diathesis associated with anti–HPA-1a alloimmune thrombocytopenia may reflect the combination of severe thrombocytopenia and antibody-mediated interference with the function of the GPIIb/IIIa complex on the few remaining platelets.

Treatment. The diagnosis of NAIT is confirmed by serologic or genotypic testing, including immunophenotyping of maternal, paternal, and occasionally neonatal platelets; maternal or fetal serum also can be examined for the presence of antiplatelet antibody. Several sensitive assays have been developed for platelet antigen typing and detection of alloantibody.[176] Despite the availability of sensitive tests, serologic evidence of alloantibodies is lacking in a least a third of cases identified by clinical criteria; therefore NAIT is a clinical diagnosis. Because delays may be associated with serologic testing, therapy should be initiated as soon as the diagnosis is suspected.

The treatment of choice for severe NAIT (platelet count <30,000/μL or clinically significant bleeding) is transfusion of washed, irradiated maternal platelets (Fig. 34-5).[168] Irradiation prevents transfusion-associated graft-versus-host disease. In the interest of time, normal donor screening procedures may have to be abridged. There are often medical or practical reasons that maternal platelets cannot be collected in a timely manner. Random-donor platelet infusions can be used in the treatment of NAIT if maternal platelets are not immediately available.[177] However, only a modest and short-lived increase in the platelet count may be observed after the infusion of random-donor platelets. IVIG (1 g/kg daily for 2 days) or a glucocorticoid (methylprednisolone, 2 mg/kg/day) can also be used as a temporary measure, although these interventions may be of limited benefit. If the offending alloantigen is known, platelets from an antigen-negative unrelated donor may be used. In white populations the use of HPA-1a– and HPA-5b–negative donors has been advocated because these two antigens are responsible for more than 95% of cases of NAIT. To provide transfusion support for fetuses and neonates with NAIT, the National Blood Service of England has established an accredited platelet donor panel of HPA-1a–negative individuals with no antibodies to HLA, HPA, or red cell antigens.[178] The majority of these donors are HPA-5b negative.

Platelets produced by neonates with alloimmune thrombocytopenia may exhibit accelerated clearance for weeks, until antiplatelet antibody is removed from the circulation (see Fig. 34-5). Consequently, it is not unusual for additional transfusions of maternal (or an antigen-compatible unrelated donor) platelets to be required after the first few weeks of life.

Management of Future Pregnancies. The likelihood is high that subsequent infants born to mothers of infants with NAIT will be affected. Moreover, the severity of antenatal and postnatal hemorrhage tends to increase in later pregnancies.[179] Therefore detailed counseling on the risk of recurrence should be provided to the family at the time that thrombocytopenia is diagnosed in the first infant. Although the neonate's thrombocytopenia may have resolved before completion of the full diagnostic laboratory evaluation, it is important that the diagnosis and presumed antigen incompatibility be confirmed by formal serologic and genotypic testing to guide the management of future pregnancies. Antibody titers begin to decline after delivery; thus the evaluation should ideally be initiated in the postpartum period. Because the likelihood of thrombocytopenia in subsequent infants depends on whether the father is HPA-1a homozygous or HPA-1a/1b heterozygous, performance of DNA-based genotyping is desirable.

Fetuses at risk for ICH should be monitored with serial ultrasound examinations, although fetal ICH may not always be detected by this method. In some centers prenatal diagnosis of the platelet alloantigen genotype has been performed with DNA obtained by chorionic villus sampling or amniocentesis. More commonly, percutaneous umbilical blood sampling is performed at 20 to 22 weeks of gestation to obtain a fetal platelet count and perform platelet antigen typing. However, this procedure may be associated with fetal loss because of exsanguination; thus compatible antigen-negative (e.g., maternal) platelets should be available when percutaneous umbilical blood sampling is performed and should be infused if the fetus is severely thrombocytopenic.[170]

When the diagnosis of alloimmune thrombocytopenia is confirmed in the fetus, two antenatal treatment approaches are available. In most North American centers, the favored approach is weekly maternal infusion of IVIG.[180,181] In some European centers the preferred approach for severe cases of alloimmune thrombocytopenia is repeated in utero platelet transfusions; administration of IVIG and a glucocorticoid is reserved for mildly affected fetuses.[181]

Neonatal Autoimmune Thrombocytopenia. This condition can occur in infants of mothers with immune thrombocytopenia as a result of ITP, SLE, or other autoimmune disorders and is caused by placental transfer of maternal autoantibodies.[174] Antigens implicated as causes of neonatal autoimmune thrombocytopenia mirror those seen in ITP. In general, this condition is less serious than NAIT.

This diagnosis should be considered when both the neonate and the mother exhibit signs of thrombocytopenia or in infants of mothers with a previous history of immune thrombocytopenia. Neonatal thrombocytopenia has been observed in infants of mothers rendered asymptomatic for chronic ITP by splenectomy. Maternal ITP must be distinguished from the mild decrease in platelets that frequently accompanies pregnancy at term, presumably because of plasma volume expansion.[182]

The thrombocytopenia associated with maternal immune thrombocytopenia is generally milder than NAIT. The incidence of cord platelet counts lower than 50,000/μL is only about 3%,[183] and the incidence of ICH is only about 1%.[174] Although clinically significant bleeding episodes are rare, newborns with this condition should be monitored closely because platelet counts often decrease in the days after birth. The threshold for medical intervention in neonates with thrombocytopenia differs from that in older children with ITP because of concern about ICH in the newborn. A platelet count of less than 40,000 to 50,000/μL in a neonate is considered by many to be a useful indicator for starting therapy. IVIG (1 g/kg daily for 2 days) elevates the platelet count in most patients.[184] A glucocorticoid (methylprednisolone, 2 mg/kg/day) may be used in addition to or in lieu of IVIG therapy.

Perinatal management of mothers with immune thrombocytopenia, including the mode of delivery of a thrombocytopenic fetus, is controversial. Because the cord platelet count rarely is below 50,000/μL, perinatal ICH is uncommon and not necessarily related to delivery. The degree of maternal thrombocytopenia is a poor predictor of the degree of neonatal thrombocytopenia, although the clinical course of thrombocytopenia in the first sibling does predict that of the next sibling.[174] Otherwise, there are no reliable predictors of severe thrombocytopenia in the newborn. Direct determination of the fetal platelet count by percutaneous umbilical venous sampling can be performed, but this procedure is rarely justified for this condition.

Posttransfusion Purpura. PTP is a very rare syndrome in childhood. Typically, this syndrome affects a multiparious woman with HPA-1a–negative platelets who is reimmunized with that alloantigen through blood transfusion.[185] Severe, acute thrombocytopenia ensues 1 week later, presumably reflecting an anamnestic response to the platelet alloantigen. PTP has been reported to develop in males and in nulligravida females as a result of initial exposure to the alloantigen through a previous blood transfusion. HPA-1a is the alloantigen found in 90% of occurrences of PTP.

Alloantibodies cause PTP, but the mechanism of platelet destruction is unclear. Several hypotheses[185] have been put forward to explain how alloantibody promotes destruction of autologous platelets, including immune complex–mediated platelet destruction; adsorption of allogeneic platelet antigens onto autologous platelets, which renders them susceptible to alloimmune destruction; and transient induction of autoantibodies. Because on average 2% of transfusion recipients have an HPA-1a incompatibility and PTP is very rare, other factors probably influence the development of this syndrome. Thrombocytopenia is usually severe (platelet count <10,000/μL), and hemorrhage is common. A high-titer alloantibody is often detectable. Patients generally recover in a few weeks. However, life-threatening bleeding, including

ICH, may occur. Prednisone and plasma exchange have been used, but IVIG is probably the treatment of choice for severe occurrences.[186] Random-donor platelet infusions are ineffective and should not be administered. The efficacy of compatible, alloantigen-negative platelet transfusions is controversial.

Severe, transient thrombocytopenia has been associated with passive transfusion of plasma-containing products that have high-titer alloantibodies against HPA-1a or HPA-5b.[187] This condition has been reported in neonates.[188] The thrombocytopenia occurs within hours of transfusion. Most patients recover spontaneously, but treatment with IVIG or a glucocorticoid should be considered for severely affected patients.[188]

Drug-Induced Immune Thrombocyopenia. Numerous drugs have been implicated as causes of antibody-mediated thrombocytopenia.[189,190,191] In general, the risk associated with any drug is low, but some drugs cause immune thrombocytopenia more commonly than others do. The thrombocytopenia generally resolves when use of the drug is discontinued, but the severity of bleeding symptoms and the duration of thrombocytopenia vary widely among patients. In some instances the sensitizing drug induces a second, drug-independent antibody (autoantibody) in addition to a drug-dependent one.[192] Usually, these drug-induced autoantibodies are transient and disappear when treatment with the drug is discontinued. Occasionally, however, autoantibody production may be sustained and lead to a clinical picture indistinguishable from that of ITP.[192] Once established, sensitivity to a drug generally persists indefinitely.[189]

Pathogenesis. Several mechanisms have been implicated in drug-induced immune thrombocytopenia.[189]

HAPTEN FORMATION. Small compounds can become covalently linked to blood cell membrane proteins and stimulate the production of antibodies specific for the hapten-protein complex. This mechanism is responsible for the hemolytic anemia seen in some patients treated with penicillin (see Chapter 13). Similarly, antibodies directed against hapten-protein complexes appear to be responsible for cases of penicillin-induced thrombocytopenia.[193]

COMPOUND EPITOPE FORMATION. In patients with quinine-induced thrombocytopenia, antibodies that bind to normal platelets can be detected only when quinine is present in the soluble phase. These antibodies are usually specific for GPIIb/IIIa[194,195] or GPIb/IX.[196] The mechanism by which quinine promotes binding of antibody to platelets is not well understood. It is postulated that this agent binds noncovalently to one or more platelet membrane proteins and creates a “compound epitope” recognized by an antibody. Alternatively, the drug may interact with the target protein and cause a conformational change elsewhere in the molecule for which the antibody is specific.[197] In addition to quinine, sulfonamide antibiotics and certain other drugs have been shown to induce antibodies that bind to platelets only when the sensitizing drug or one of its metabolites is present in the soluble state.[189] Among children sulfamethoxazole is one of the more common causes of drug-induced immune thrombocytopenia.

DRUG-INDUCED AUTOANTIBODIES. Certain drugs, such as gold salts, procainamide, levodopa, and interferon alfa, induce true autoantibodies that bind to platelet membrane targets in the absence of the drug.[189,198] The mechanisms by which these drugs induce autoantibodies is poorly understood.[192]

Clinical Features. Severe thrombocytopenia generally develops within 1 day of ingestion of quinine in a previously sensitized individual. A minimum of 1 week is required for initiation of the primary immune response in individuals taking this drug for the first time. Prodromal symptoms such as fever and chills may be observed. Bleeding may be severe, and its onset is abrupt. Hemorrhagic vesicles are common in the oral mucosa. The presence of a platelet antibody may be verified by a variety of techniques. Readministration of a suspected drug in an attempt to confirm an etiologic relationship is not recommended as a routine diagnostic measure.

Treatment. Discontinuation of treatment with the offending drug is the main therapy. With quinine the thrombocytopenia usually begins to abate in 1 day if no additional drug is taken, and a normal platelet count is restored within 1 week. In patients with severe drug-induced thrombocytopenia, IVIG or a glucocorticoid has been used to ameliorate bleeding.[199] However, there is little evidence that these medications are effective in this clinical setting. For life-threatening hemorrhage, platelet transfusion may be used, although this may be of limited value because of the persistence of slowly dissociating drug-antibody complexes. Because a sensitized individual is presumed to remain at lifelong risk for additional episodes of drug-induced thrombocytopenia, further use of the offending drug is contraindicated.

Heparin-Induced Thrombocytopenia and Thrombosis. HIT is a complex clinical syndrome with an overall estimated incidence of 1% to 5% in adult patients exposed to heparin; the incidence in children is probably less, and it is virtually nonexistent in neonates.[200-202] The thrombocytopenia usually begins 5 to 10 days after heparin therapy is started, but it can occur within hours if the patient previously received heparin.[201] The risk of HIT is higher for therapeutic than for low drug doses, for bovine than for porcine heparin, and for unfractionated than for low-molecular-weight heparin (LMWH).[203] In contrast to drug-induced thrombocytopenia caused by quinine or sulfonamides, severe thrombocytopenia (platelet count <10,000/μL) is rare in HIT. Typically, platelet counts range from 20,000 to 150,000/μL, with a median of 50,000/μL.[189,204] The thrombocytopenia itself is rarely severe enough to cause bleeding symptoms and resolves after heparin therapy is discontinued. However, as many as 30% of patients with HIT experience arterial or venous thromboses. The development of thrombosis in patients with HIT is known as thrombocytopenia with thrombosis syndrome (HITTS), and this condition is associated with significant morbidity and mortality.

Pathogenesis. The antibodies responsible for HIT recognize the complex of heparin and platelet factor 4 (PF4), an alpha granule constituent.[205] After treatment with heparin, PF4 is released into the circulation and binds with heparin to form a reactive antigen on the platelet surface.[204] The heparin-PF4 complex is immunogenic in some patients. IgG against this complex triggers platelet activation by binding to FcγRIIA receptors on the surface membrane. The activated platelets release substances that stimulate other platelets and result in thrombin generation.[206] PF4 bound to glycosaminoglycans on the surface of endothelial cells may also serve as a target for antibody binding and lead to antibody-mediated injury to the endothelium and a predisposition to thrombosis.[207]

Approximately 50% of patients exposed to heparin during cardiac catheterization and subsequently during cardiac surgery form antibodies against heparin-PF4 complexes, but HITTS develops in only a small percentage.[208] It is unclear why heparin-PF4 complexes, which are composed of two substances normally present in the body, are so immunogenic. Moreover, it is not known why thrombocytopenia or thrombosis develops in some patients and not in others.

Diagnosis. The diagnosis of HIT is based on the interpretation of clinical findings together with laboratory confirmation of heparin-PF4 antibodies. A scoring system has been proposed to assess the pretest probability of HIT,[209] although this system has not been validated for pediatric patients. The gold standard laboratory test for diagnosing HIT is a functional test, the platelet [^{14}C] serotonin release assay. Unfortunately, the complexity and availability of this assay limit its usefulness. An enzyme-linked immunosorbent assay (ELISA) for detection of antibodies against heparin-PF4 complexes is available, and results obtained with the serotonin release assay and ELISA are generally in agreement in patients with a clinical diagnosis of HIT.[203,210] However, ELISA has a more limited specificity and therefore produces false-positive results. A negative ELISA result generally excludes HIT. If the ELISA result is positive, a serotonin release assay may be performed to confirm the diagnosis or to invalidate a false-positive ELISA result.[211]

Treatment. It is standard practice to discontinue heparin in patients in whom HIT is suspected, but many of these patients will have thrombotic complications over the subsequent days. Therefore ongoing antithrombotic therapy is indicated. Patients with HIT should not be given warfarin as a substitute for heparin during the acute episode because this can cause an abrupt decrease in the level of protein C and put the patient at greater risk for thromboembolic complications such as warfarin-induced limb gangrene.[196,212] LMWH is not recommended for the treatment of HIT because unfractionated heparin and LMWH preparations display significant platelet aggregation cross-reactivity in vitro and in vivo.[203,213] Hirudin, a compound originally purified from the leech salivary gland, inactivates thrombin by forming a 1:1 complex. A recombinant hirudin, lepirudin, is effective in the treatment of HIT or HITTS.[214] In children lepirudin should be started as a continuous infusion at 0.1 mg/kg/h without a preceding bolus, with a target activated partial thromboplastin time (APTT) of 1.5 to 2.5 times the patient's baseline value (first assessed 4 hours after infusion).[200] Other agents that are effective in this clinical setting are the synthetic antithrombin argatroban[215] and the synthetic pentasaccharide fondaparinux.[216] Danaparoid, a low-molecular-weight heparinoid consisting of a mixture of heparan sulfate, dermatan sulfate, and small amounts of chondroitin sulfate, has been used for more than a decade to treat patients with HIT.[217] Danaparoid was voluntarily withdrawn from the United States market with the introduction of fondaparinux but is still available in other countries.

Box 34-4 **Causes of Schistiocytic Hemolysis and Thrombocytopenia**

- Thrombotic thrombocytopenic purpura
  - Acquired
  - Hereditary
- Hemolytic-uremic syndrome
  - Typical
  - Atypical
    - Familial
    - Sporadic
- Disseminated intravascular coagulation
- Vasculitis, including systemic lupus erythematosus and sclerodermal kidney
- Drug-induced (e.g., cyclosporine, tacrolimus)
- Dysfunctional prosthetic heart valves
- Endocarditis
- Bone marrow transplant–associated microangiopathy

Thrombotic Microangiopathic Disorders. The thrombotic microangiopathic spectrum of disorders is characterized by destructive thrombocytopenia, disseminated capillary thrombosis, and microangiopathic hemolytic anemia (Box 34-4). Often organ dysfunction occurs because of microvascular thrombosis and ischemic necrosis. Thrombotic microangiopathic disorders may be primary or secondary. There are both acute and chronic (familial) relapsing forms.

The two major thrombotic microangiopathic disorders affecting children and adolescents are thrombotic thrombocytopenic purpura (TTP), a classic disease of hematology, and hemolytic-uremic syndrome (HUS), a classic disease of nephrology. Patients with TTP always have thrombocytopenia and schistiocytic hemolysis, frequently with central nervous system and renal impairment. In HUS the microangiopathic process is usually but not always limited to the kidneys. However, the boundary between these two clinical syndromes may be nebulous. Renal impairment can be prominent in TTP, and neurologic injury can occur in HUS. In adults these two microangiopathic syndromes are often viewed as a joint entity termed *TTP-HUS*.[218] In pediatrics, however, the two syndromes are generally considered to be distinct, albeit overlapping, entities. In addition to TTP and HUS, other conditions can produce schistiocytic hemolysis and thrombocytopenia, including bone marrow transplant–associated microangiopathy (see Box 34-4).

Thrombotic Thrombocytopenic Purpura. TTP is a syndrome characterized by reversible aggregation of platelets in the microvasculature that results in waxing and waning ischemia in various organs.[219-221] The defining clinical features of TTP are listed in Table 34-3. The pentad of thrombocytopenia, microangiopathic

TABLE 34-3 Clinical Manifestations of Thrombotic Thrombocytopenic Purpura

Symptoms	Cases	%
Fever	237/243	98
Pallor or anemia	246/256	96
Hemorrhage	241/251	96
Neurologic abnormalities	250/271	92
Renal abnormalities	191/217	88
Jaundice	113/271	42
Fatigue, malaise	92/271	34
Nausea and vomiting	69/271	25
Abdominal pain	36/271	13
Chest pain or arrhythmias	21/271	8
Arthralgia or myalgia	18/271	7

Modified from Amorosi EL, Ultman JE: Thrombotic thrombocytopenic purpura. Medicine (Baltimore) *25:139, 1966.*

hemolytic anemia, neurologic abnormalities, fever, and renal abnormalities is present in many patients. Before the development of an effective therapy, the risk of mortality associated with the disease was greater than 90%. With the advent of plasma infusion/exchange therapy, TTP has become a curable disease. There are two major forms of TTP: an acute, acquired form and a rare, inherited, chronic relapsing form. Acute TTP can be further subcategorized into primary (idiopathic) and secondary forms. Drugs, infections, and other factors have been associated with secondary TTP.

PATHOPHYSIOLOGY. The characteristic pathologic lesion of TTP is a hyaline thrombus composed of aggregated platelets in the absence of a perivascular inflammatory response. The thrombotic lesions of TTP typically involve terminal arterioles and capillaries and are most commonly seen in the brain, abdominal viscera, and heart, but not the lungs. Red blood cells are damaged as a result of interactions with microthrombi in the small vessels.

Immunohistochemistry of early lesions of TTP reveals an abundance of von Willebrand factor (VWF) but little fibrin.[222,223] VWF within these thrombi is thought to be critical in pathogenesis. In 1982 unusually large multimers of VWF (UL-VWF) were observed in the plasma of patients with chronic relapsing TTP (Fig. 34-6).[224,225] UL-VWF multimers are most prominent in plasma samples taken from these patients during remission and decrease at the time of relapse. These UL-VWF multimers, which are synthesized by endothelial cells, are composed of increased numbers of mature VWF subunits. Under conditions of shear stress, the UL-VWF forms are more efficient than other VWF multimers at binding to the GPIb/IX/V and GPIIb/IIIa complexes on platelets and inducing aggregation.[224,226,227] These findings, coupled with the observation that VWF was abundant in the early thrombotic lesions of TTP, led to the hypothesis that intravascular platelet aggregation in TTP was due to either excessive release of UL-VWF multimers into plasma or impaired degradation of these UL-VWF multimers into smaller, nonaggregating forms.

Under ordinary circumstances, UL-VWF multimers are processed into multimers of normal size through the action of ADAMTS13, a VWF-cleaving metalloproteinease in normal plasma.[228,229] This protease cleaves a specific Tyr-Met bond in the second of the three A domains in VWF subunits (Fig. 34-7).[230] Physiologically, UL-VWF multimers are thought to be cleaved by ADAMTS13 under conditions of high shear stress, which partially unfolds the molecules. In vitro this partial denaturation can be mimicked by guanidinium or urea treatment.

Acquired or constitutional deficiency of the VWF-cleaving protease predisposes individuals to TTP. Chronic relapsing TTP, which usually begins in early childhood, is caused by loss-of-function mutations in the ADAMTS13 gene.[231-234,235] The acute TTP more commonly seen in adults is usually caused by an acquired deficiency in

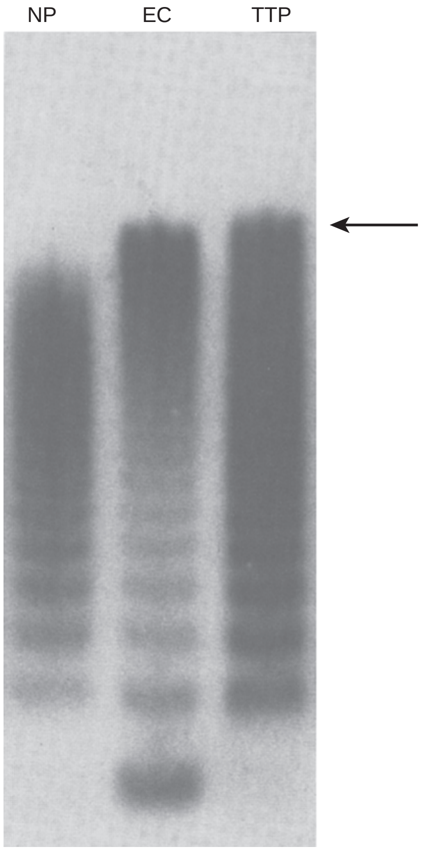

Figure 34-6 Autoradiogram demonstrating unusually large multimers of von Willebrand factor (VWF) near the top of the gel *(arrow)* in the plasma of a patient (thrombotic thrombocytopenic purpura [TTP]) obtained early during a single episode of TTP. Normal pooled plasma *(NP)* and the culture supernatant from human umbilical vein endothelial cells *(EC)* are shown for comparison. EDTA-anticoagulated samples were subjected to agarose gel electrophoresis in sodium dodecyl sulfate–urea-EDTA. VWF multimers were visualized by incubation with ^{125}I-labeled rabbit antihuman VWF antibody followed by autoradiography. *(From Moake JL, Chow TW: Thrombotic thrombocytopenic purpura: understanding a disease no longer rare.* Am J Med Sci *316:108, 1998.)*

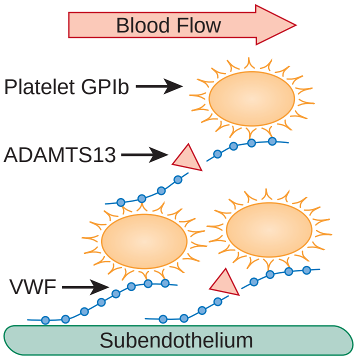

Figure 34-7 Pathogenesis of thrombotic thrombocytopenic purpura (TTP). The von Willebrand factor (VWF) multimers facilitate platelet adhesion to the subendothelium by binding to exposed connective tissue and then to platelet glycoprotein Ib (GPIb). In flowing blood shear stress unfolds ultralarge VWF multimers in the platelet-rich thrombus and enables ADAMTS13 to cleave a specific Tyr-Met bond in the second of the three A domains in VWF subunits. Cleavage reduces VWF multimer size and limits thrombus growth. In the absence of ADAMTS13, VWF-dependent platelet accumulation continues and eventually results in microvascular thrombosis and TTP. *(Courtesy Dr. Evan Sadler, Washington University.)*

VWF-cleaving protease activity resulting from an IgG autoantibody against ADAMTS13.[236,237] The reasons for this transient immune dysregulation and for the selective antigenic targeting of ADAMTS13 are unknown. Fresh frozen plasma, cryoprecipitate-poor plasma (cryosupernatant), and plasma treated with solvent or detergent all contain the VWF-cleaving protease activity, which presumably accounts for the efficacy of plasma exchange therapy (discussed later).

CLINICAL FEATURES. TTP is a relatively rare disorder with an estimated annual incidence of 1 to 4 per million population.[238,239] TTP is more common in women than in men (ratio of 3:2) and has a peak incidence in persons between the ages of 30 and 40 years. The condition has been reported in adolescents; indeed, the first instance reported in the literature was a teenage female.[220] The incidence of the disease is increasing, in part because of increased awareness of the condition and the availability of effective therapy.[218,240]

TTP usually develops in previously healthy individuals (idiopathic TTP). In approximately 15% of patients, the syndrome is associated with an underlying condition (secondary TTP), such as bacterial or viral infection,[241,242] pregnancy,[243] collagen vascular disorders,[244] pancreatitis,[245] and malignancy.[246] Several drugs[230] have been associated with the development of secondary TTP, including ticlopidine, clopidogrel, lopidogrel, quinine, mitomycin C, cyclosporine, and tacrolimus. The mechanisms by which these drugs promote TTP are not well understood. Inhibitory antibodies against ADAMTS13 have been observed in ticlopidine- and clopidogrel-associated TTP.[247,248]

In the usual occurrence of TTP, the onset of symptoms is abrupt. The most common initial manifestations are bleeding and neurologic abnormalities. The neurologic symptoms can progress rapidly and may include headache, confusion, stupor, coma, hemiparesis, cranial nerve palsies, and seizures. Fever is uncommon initially but invariably develops during the course of the disease. TTP is a clinical diagnosis that rests on the signs and symptoms presented in Table 34-3. The primary diagnostic criteria are thrombocytopenia and microangiopathic hemolytic anemia. Some patients may not be anemic initially, although their hematocrit levels may fall sharply afterward, and in some patients red cell fragmentation may not be apparent.[249] Therefore the diagnosis of TTP initially is often provisional. Serum lactate dehydrogenase (LDH) levels are increased, frequently to very high levels, because of not only hemolysis but also diffuse tissue ischemia. If considerable tissue ischemia and necrosis are present, disseminated intravascular coagulation (DIC) may ensue.

The diagnosis of TTP can be confirmed by measuring ADAMTS13 activity and by screening for inhibitory antibodies against this enzyme.[235] These tests may also provide prognostic information in cases of idopathic TTP. In one prospective study[250] 6 of 14 patients (43%) with severe ADAMTS13 deficiency later relapsed, whereas only 2 of 25 patients (8%) without severe ADAMTS13 deficiency relapsed. In two other series[251,252] patients with detectable anti-ADAMTS13 antibodies had a higher mortality rate than patients without detectable antibodies did. More recently, the presence of high-titer anti-ADAMTS13 IgA has been associated with a higher mortality rate.[253]

Though less common than acquired TTP secondary to autoantibodies, TTP caused by mutations in the ADAMTS13 gene is an autosomal recessive disease (also referred to as Upshaw-Schulman syndrome) that can be manifested at birth or in early childhood, with rare patients remaining asymptomatic until adulthood. The age at onset and severity can be highly variable, even among patients in the same family (carrying the same ADAMTS13 mutation).[230-235] Congenital TTP occurring in early childhood may be misdiagnosed as chronic ITP or Evans syndrome.[235]

TREATMENT. Congenital TTP typically exhibits a chronic relapsing course that usually requires long-term treatment with fresh frozen plasma to replace the deficient ADAMTS13 protein. The regimen is determined empirically, and stable remission can generally be maintained with plasma infusions as infrequently as once every few weeks to months.[224,225,231-233]

Plasma exchange is the mainstay of therapy for acquired TTP (secondary to anti-ADAMTS13 autoantibodies). At least 50% to 80% of patients recover with this therapy. A randomized trial demonstrated the superiority of plasma exchange over plasma infusion.[254] This finding has been attributed to removal of antibody against VWF-cleaving protease. Another randomized study showed that fresh frozen plasma and cryoprecipitate-poor plasma were equally effective in the initial treatment of adults with TTP.[255] The risks of plasma exchange are substantial and include hemorrhage and catheter-related complications such as pneumothorax and infection.[246] Glucocorticoid therapy is often used as an adjunct to plasma exchange therapy, but there is no direct evidence that the addition of glucocorticoids produces results that are superior to those of plasma therapy alone.[218]

Although TTP in most patients responds to plasma exchange therapy, the response is varied. Mental status changes may resolve immediately. Typically, the initial recovery from thrombocytopenia requires several days. Serum LDH levels may improve promptly, but patients often remain dependent on red cell transfusion for days. Recovery from renal failure is generally slow. Chronic renal insufficiency, defined as creatinine clearance of less than 40 mL/min/1.73 m^2 at 1 year after diagnosis, occurs in about 25% of patients.

Preliminary studies[256-258] suggest that rituximab can induce a complete response in patients with idiopathic TPP who are refractory to plasma exchange. This agent may also be beneficial as prophylatic therapy in patients with idiopathic TTP who are at high risk for relapse.[256-258]

Patients with secondary TTP caused by dose-related drug toxicity (e.g., mitomycin C) or allogeneic marrow transplantation may not benefit from plasma exchange treatment. Likewise, the course in a patient who has a clinically apparent additional condition, such as SLE, is unpredictable. Management of plasma exchange treatment is often dictated by the activity of the associated condition.

Hemolytic-Uremic Syndrome. HUS is another microangiopathic hemolytic anemia characterized by capillary thrombosis and ischemic necrosis. The kidneys are the end organs most severely affected, but ischemic injury of the intestines, central nervous system, or other organs may occur. HUS typically follows a diarrheal illness (mainly *Escherichia coli* O157:H7 infection) but also occurs in nonenteropathic settings. HUS occurs most often in infants and young children and is the most common cause of acute renal failure in this age group. The annual incidence is approximately 1 in 100,000 and may be increasing.[259] HUS can also occur in adults, particularly in the elderly, which suggests that susceptibility to HUS is greatest at the extremes of age.

ENTEROPATHIC HEMOLYTIC-UREMIC SYNDROME. Pathogenesis: Certain strains of enteric bacteria produce a toxin that is cytopathic to Vero cells, a monkey kidney cell line. In the early 1980s Karmali and associates[260] reported that 75% of children with HUS had evidence of gastrointestinal infection by verotoxin-producing *E. coli*. Subsequent studies showed that strains of other enteric pathogens, including *Shigella, Salmonella,* and *Campylobacter,* produce verotoxin and can cause HUS.

The term *verotoxin* has been replaced by the term *Shiga toxin* or *Shigalike toxin* (Stx). This toxin is the key factor that differentiates infection with enterohemorrhagic *E. coli* (EHEC), which is associated with watery diarrhea, hemorrhagic colitis, and HUS, from enteropathogenic *E. coli* (EPEC), which is associated only with watery diarrhea and vomiting. Stx1 and Stx2, the two most prevalent forms of the toxin found in EHEC strains, are encoded by bacteriophages. Stx1 is identical to Shiga toxin produced by *Shigella dysenteriae* type I. Stx2 is immunologically distinct from Stx1.[261]

Stx is structurally related to ricin and cholera toxin. Each of these toxins is composed of a single A subunit noncovalently associated with a pentamer of identical B subunits.[261] The enzymatic activity of the toxin resides in the A subunit, whereas the B subunits are required for cellular uptake. Stx is taken up via endocytosis and transported to the endoplasmic reticulum via retrograde trafficking through the secretory pathway.[262] The A subunit enters the cytosol and targets 28S ribosomal RNA, which is depurinated by the toxin at a specific adenine residue and causes protein synthesis to cease and the infected cells to die by apoptosis. Renal microvascular endothelial cells appear to be particularly sensitive to Stx.[263,264] Hemorrhagic enterocolitis has been ascribed to the action of Stx on endothelial cells in vessels of the intestinal submucosa. The toxin also inhibits protein synthesis in certain nonendothelial cells, including renal mesangial and cortical tubular cells.[265,266] Because EHEC is not invasive, it has long been assumed that HUS-related tissue damage results from the spread of bacterial products from the intestine to the kidney and other target organs. It is thought that Stx enters the systemic circulation after translocation across the intestinal epithelium.[267]

Clinical and laboratory features: Approximately 50% of cases of acquired HUS appear after gastrointestinal infection with *E. coli* O157:H7. For unclear reasons, HUS develops in only 9% to 30% of persons with *E. coli* O157:H7 colitis. Elevated leukocyte counts and C-reactive protein levels have been identified as risk factors for progression to HUS.[268,269] Other risk factors are the extremes of age, the presence of bloody diarrhea, and the use of antimotility drugs.[270]

Gastrointestinal symptoms can range from mild to severe. The colonic mucosa is hemorrhagic and edematous; ischemic infarcts can occur in the colon, and perforations have been reported. Stool cultures may be positive for the pathogenic organism.[271] Other diagnostic techniques include demonstration of Stx or specific bacterial DNA in stool samples or a rise in the titer of specific antibodies.

The anemia and thrombocytopenia associated with HUS tend to be less severe than those associated with TTP. The platelet count is greater than 100,000/μL in half of the patients, and bleeding is uncommon. Laboratory evidence of DIC is not usually present, but it can occur, especially in HUS associated with *Shigella*. The serum LDH level is less elevated than in TTP. A study of 16 children in whom HUS developed after *E. coli* O157:H7 infection showed normal levels of VWF-cleaving protease activity in plasma.[270] This finding has been confirmed by other investigators.[272]

Acute renal failure with oliguria, hypertension, and proteinuria usually develops in affected patients. Renal histopathologic examination reveals a preglomerular and glomerular thrombotic microangiopathy.[273] Mental status changes attributable to uremia are common. More rarely, focal neurologic signs are evident.

Treatment: Supportive care is the mainstay of therapy. Parenteral volume expansion with isotonic fluid during acute *E. coli* O157:H7 enteritis may attenuate the renal injury.[274-275] Dialysis and renal transplantation are performed when necessary. Independent trials have shown that antimotility agents increase the risk for HUS after *E. coli* O157:H7 infection.[253]

There is no compelling evidence that antibiotics are beneficial in the treatment of HUS, and there is some indirect evidence that the use of antimicrobials may be dangerous. Antibiotics increase the amount of Stx released from EHEC in vitro, so antibiotic therapy might paradoxically increase the risk for HUS in vivo. A prospective controlled study[276] evaluated the effect of trimethoprim-sulfamethoxazole in children with *E. coli* O157:H7 enteritis. There was no statistically significant effect of treatment on progression of symptoms, fecal pathogen excretion, or the incidence of HUS. A prospective study of 71 children with diarrhea caused by *E. coli* O157:H7 suggested that antibiotic therapy was associated with an increased risk for HUS.[269] It is now generally accepted that antibiotics should not be administered in an effort to decrease the severity or duration of gastrointestinal symptoms or to prevent HUS in patients with *E. coli* O157:H7 enteritis.[277] There might be a role for azithromycin in elimination of Stx-producing *E. coli* from postsymptomatic carriers,[278] although this remains controversial.[277]

Other treatments that have been tried without obvious benefit include infusion of fresh frozen plasma, glucocorticoids, heparin, thrombolytic agents, and prostacyclin.[279,280] Although plasma infusion or exchange has no proven role in the management of diarrhea-associated HUS in children, this therapy may be beneficial in adults with HUS or children with nonenteropathic HUS (discussed later).[202] Despite treatment, approximately 5% of children with HUS die of the disease and 10% have severe, chronic renal impairment.[281,282]

ATYPICAL HEMOLYTIC-UREMIC SYNDROME. Approximately 10% of pediatric patients seen with an HUS-like illness do not have antecedent bloody diarrhea and are classified as having atypical HUS (aHUS). There are both familial and sporadic forms of aHUS. The associated mortality rate is higher than that of enteropathic HUS. About half of survivors have a relapse, and a third require chronic dialysis.[283]

Pathogenesis: Deficiency of complement regulators is a major risk factor for the development of aHUS. More than 50% of patients with aHUS have a mutation in one of three complement control proteins: factor H (fH), membrane cofactor protein (MCP; CD46), or factor I (fI).[284-286] Additionally, autoantibodies against fI have been described in patients with sporadic aHUS.[285] MCP and fH serve as cofactors for fI, a serine protease that inactivates C3b (and C4b) through limited proteolytic cleavage (Fig. 34-8). Deficiency of fH, MCP, or fI results in excessive complement deposition, which promotes the development of microthrombi in the kidneys and other tissues.[287]

Invasive infection with certain strains of pneumococcus can also cause aHUS.[288] These strains produce neuraminidase, which exposes the normally hidden Thomsen-Freidenreich antigen on erythrocytes, platelets, and renal glomerular cells. Naturally occuring IgM antibodies in human plasma bind the exposed antigen; promote complement fixation; and trigger anemia, thrombocytopenia, and uremia.[288] Other nondiarrheal infections have also been linked to aHUS.[289]

Treatment: Although plasma therapy is often used in the management of aHUS secondary to complement

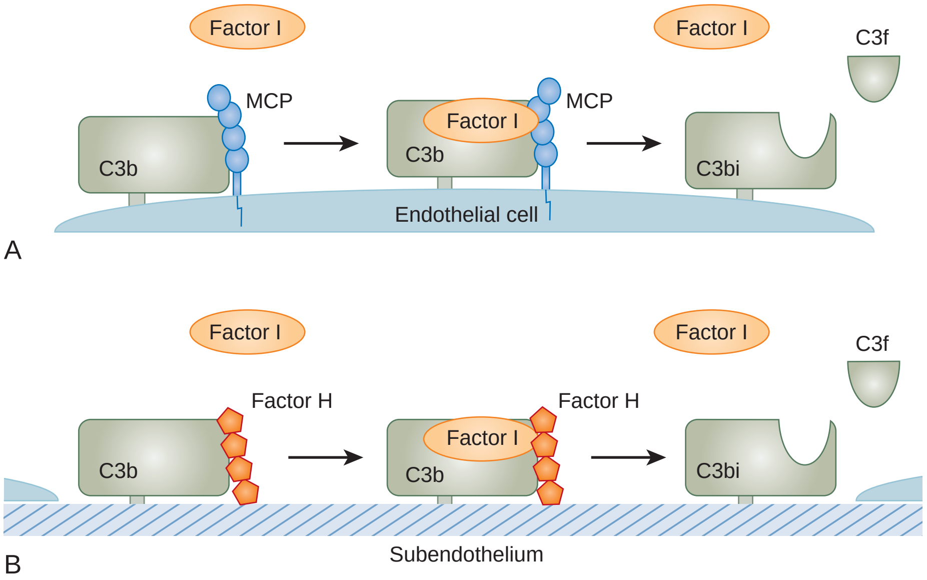

Figure 34-8 Role of complement regulatory proteins in the pathogenesis of atypical hemolytic-uremic syndrome. **A,** Membrane cofactor protein (MCP), a complement regulatory protein, is expressed on the surface of endothelial cells and other cell types and binds to C3b that has been deposited on the cell surface either spontaneously or in response to injury. In cooperation with MCP, the plasma serine protease factor I then cleaves C3b into its inactive form C3bi, thereby preventing further complement activation via the alternative pathway. In patients with MCP or factor I deficiency, excessive complement deposition occurs and leads to further cellular injury, endothelial cell necrosis, and ultimately thrombosis. **B,** Endothelial cell necrosis exposes basement membranes that are devoid of complement regulators. Factor H, a plasma-derived complement regulatory protein, binds to exposed basement membranes and, in cooperation with factor I, inactivates C3b that is deposited at these sites. Factor H deficiency leads to excess complement deposition on basement membranes. *(Courtesy Dr. J. P. Atkinson, Washington University.)*

regulator deficiency, its benefit in preventing progression to end-stage renal disease or relapse is questionable.[290-292] Patients with an MCP mutation have a satisfactory outcome after renal transplantation because MCP is a transmembrane protein and the allografts are protected by wild-type MCP.[284] In contrast, recurrent aHUS develops in patients with a deficiency of fH or fI after renal transplantation because the allograft cannot compensate for deficiency of these plasma proteins, which are produced primarily in the liver. Liver transplantation is an option for some of these patients.[286] Eculizumab, a humanized monoclonal antibody that inhibits cleavage of C5, preventing the generation of the proinflammatory peptide C5a and the membrane attack complex C5b-9, is licensed for treatment of aHUS, particularly for patients nonresponsive to plasma therapy.[287,293]

Patients with aHUS caused by neuraminidase-producing pneumococcus should receive only washed red blood cells or platelets if transfusion is required.[288] Plasma products are not recommended for use in these patients.

Bone Marrow Transplant–Associated Thrombotic Microangiopathy. Thrombotic microangiopathy has emerged as one of the major complications of bone marrow transplantation (BMT).[294] The incidence ranges from 6% to 21%.[295] It is seen after allogeneic and less commonly after autologous BMT. The diagnosis may be difficult to make because of the presence of coexisting complications of BMT, such as graft-versus-host disease, cytomegalovirus infection, and hepatic veno-occlusive disease. Although BMT-associated microangiopathy (BMT-MA) shares features with other microangiopathies such as TTP and HUS, it has a distinctive clinical course and response to therapy. The pathogenesis of BMT-MA is unknown but appears to be distinct from that of primary TTP.

The condition ranges in severity from self-limited microangiopathic hemolytic anemia to a fulminant, usually fatal disorder. The fulminant form of the disease is more common in adults than children. In one adult series[296] the incidence of BMT-MA was 2% with matched sibling donor transplants and 6% with unrelated donor transplants. Risk factors for the development of BMT-MA in adults include the use of unrelated or mismatched donors, grade II through IV acute graft-versus-host disease, and systemic bacterial or fungal infection.[295] In contrast to primary TTP, plasma exchange therapy appears to be of limited benefit in BMT-MA.[295] Most pediatric patients primarily exhibit signs of renal insufficiency. The mainstay of management is supportive care and, if feasible, a reduction of the dose of cyclosporine or tacrolimus. If the patient's clinical condition deteriorates (e.g., worsening hemolysis, progressive renal insufficiency, neurologic symptoms), plasma exchange should be considered.[294]

Other Causes of Increased Platelet Destruction. Systemic bacterial or fungal infections are also commonly associated with thrombocytopenia. The degree of thrombocytopenia may range from moderate to severe. Thrombocytopenia associated with systemic bacterial or fungal infection is usually caused by increased platelet destruction. A variety of mechanisms contribute to this destruction, including immune-mediated factors, direct effects of bacterial toxins, DIC, and hypersplenism.

Acute viral infections in children are commonly complicated by thrombocytopenia. For example, thrombocytopenia develops in about 75% of patients with infectious mononucleosis caused by Epstein-Barr virus.[297] Viral-induced thrombocytopenia may be immediate or delayed, and the decrease in platelet count may range from mild to severe. Thrombocytopenia can follow immunization with live virus vaccines. Viruses may cause thrombocytopenia through both nonimmune and immune mechanisms, including cytotoxic effects on megakaryocytes, direct injury to platelets, immune complex–mediated destruction (see earlier discussion of ITP and HIV), and hyperspenism. Thrombocytopenia is a manifestation of hemophagocytic lymphohistiocytosis. Thrombocytopenia caused by increased platelet destruction is a uniformly seen complication of malaria and African trypanosomiasis.[298]

Thrombocytopenia commonly accompanies extracorporeal membranous oxygenation, cardiopulmonary bypass, hemodialysis, and apheresis.[299] There are many causes, including adherence of platelets to synthetic materials in the perfusion device, hemodilution, hemorrhage, and drug effects (e.g., heparin). The thrombocytopenia associated with hemodialysis or apheresis is generally mild, but more severe thrombocytopenia may be seen in association with extracorporeal membranous oxygenation.

A healthy fetus has a platelet count of greater than 150,000/μL by the second trimester of pregnancy, and only 2% of term infants are thrombocytopenic at birth.[300] Severe thrombocytopenia, defined as a platelet count lower than 50,000/μL, is evident in fewer than 3 of 1000 term infants, the most important cause being NAIT. In contrast, thrombocytopenia is common among patients in neonatal intensive care units. Thrombocytopenia is also common in patients with intrauterine growth retardation and has been ascribed to placental insufficiency or chronic hypoxia. Thrombocytopenia is seen in preterm neonates with respiratory distress syndrome. Neonatal viral infections, including rubella, herpes, cytomegalovirus, and enterovirus, have also been associated with thrombocytopenia. Acute thrombocytopenia commonly accompanies necrotizing enterocolitis and is often an early sign of the disease. Thrombocytopenia is commonly seen in neonates with polycythemia, especially if they have associated cyanotic heart disease, although this phenomenon is probably multifactorial.[301] Infants with severe Rh hemolytic disease of the newborn may exhibit thrombocytopenia along with neutropenia.[302]

Kasabach-Merritt Syndrome. Kasabach-Merritt syndrome is the association between thrombocytopenia and a giant hemangioma of infancy. Most patients are seen in the first few weeks of life, although rare occurrences have been described in later childhood.[303] The vascular lesions are usually solitary and involve the extremities, neck, or trunk (Fig. 34-9). Sometimes the lesions are located in the viscera, including the retroperitoneum. The possibility of an occult hemangioma should be considered in a neonate

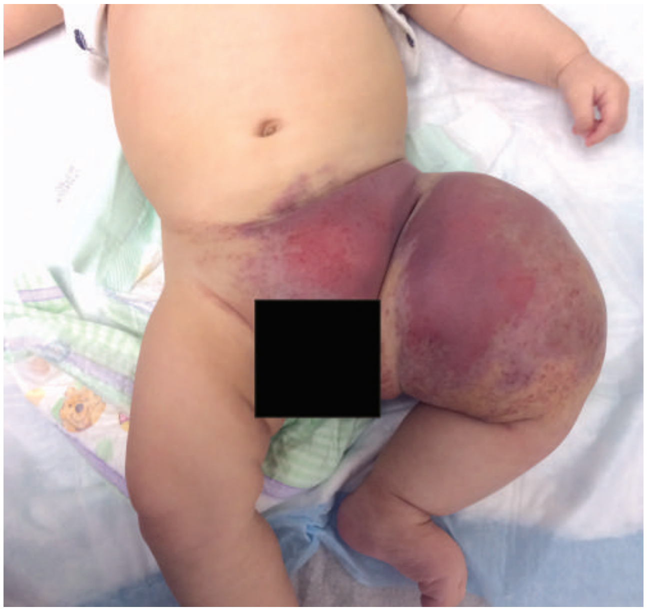

Figure 34-9 Infant with Kasabach-Merritt syndrome. Histopathology showed a kaposiform hemangioendothelioma. *(Courtesy Dr. Adam Vogel, Washington University.)*

with unexplained thrombocytopenia. Detection may require radiographic imaging of the brain, liver, spleen, or gastrointestinal tract.

The hemangiomas associated with Kasabach-Merritt syndrome have a natural history of rapid growth for several months, followed by spontaneous regression. The thrombocytopenia subsides as the hemangioma regresses. Kasabach-Merritt syndrome, which is marked by severe thrombocytopenia and spontaneous regression in the first few years of life, is distinct from the mild, chronic thrombocytopenia associated with venous malformations, which are lifelong lesions (Box 34-5).

The pathogenesis is poorly understood, but platelets are thought to be trapped by abnormal endothelium within the hemangioma. Usually, the platelet count is less than 50,000/μL, and schistocytes are frequently present on the peripheral blood film. In a majority of cases there is laboratory evidence of DIC. These tumors grow rapidly to a large size and are often characterized by cutaneous purpura, edema, and an advancing ecchymotic margin. Imaging studies are essential in delineating the extent of the lesion and determining whether it is surgically resectable. In contrast to common hemangiomas, magnetic resonance imaging shows diffuse enhancement with ill-defined margins, cutaneous thickening, stranding of subcutaneous fat, hemosiderin deposits, and small feeding and draining vessels.[303]

The mortality rate is as high as 40%,[308] and the development of a life-threatening consumptive coagulopathy warrants aggressive treatment. Surgical removal may be effective, but most lesions cannot be resected. Radiation therapy can be effective but carries serious long-term risks for a young infant[309] and is not appropriate for most patients. Vascular ligation or embolization has been used to promote regression of a large hemangioma, especially in patients in whom a solitary, major feeding vessel can be obliterated.[303] Glucocorticoid therapy may be beneficial in some patients.[303] Interferon alfa has been shown to be effective if no response to glucocorticoid therapy is seen.[310] Vincristine is another treatment option in the management of patients with Kasabach-Merritt syndrome; response rates of greater than 80% have been reported with vincristine, although complete resolution is rare and the use of vincritistine is limited by neurotoxicity and other side effects.[311,312] Propranolol, an agent highly effective at treating common infantile hemangiomas, appears to beneficial in only a minority of patients with Kasabach-Merritt syndrome.[312]

Thrombocytopenia Caused by Decreased Platelet Production

The second major etiologic classification of acquired thrombocytopenia of childhood is impaired platelet production, with the associated conditions listed in Box 34-1. Patients with thrombocytopenia caused by a production defect are more likely to have serious bleeding than patients with destructive thrombocytopenia are.

A common cause of thrombocytopenia in patients with cancer is chemotherapy-induced bone marrow suppression. This is an expected complication of therapy and rarely poses a diagnostic problem. Traditional management is straightforward and relies on transfusion of platelet concentrates and adjustment of chemotherapy doses. A recent clinical trial demonstrated that bleeding risks are higher for children versus adults given prophylactic platelet transfusions for treatment-induced hypoproliferative thrombocytopenia.[313]

Valproic acid is commonly used in the treatment of childhood epilepsy, bipolar disorder, and other conditions. Hematologic toxicity, including thrombocytopenia, is often seen in patients treated with valproate.[291] Valproate causes direct bone marrow suppression that may vary in onset and severity and is more common in patients with serum valproate levels greater than 100 μg/mL.[314] In addition to bone marrow suppression, valproate may have other effects on platelets, including immune-mediated destruction and impaired function.[314]

Box 34-5 Clinical Pearls

Hemangiomas and Kasabach-Merritt syndrome. Thrombocytopenia is evident in only a small proportion of infants with hemangiomas (0.3%).[303,304] Kasabach-Merritt syndrome is not associated with the common infantile capillary/cavernous hemangioma, which is seen in 5% to 10% of children. Rather, Kasabach-Merritt syndrome appears to be a complication of two rare, aggressive histopathologic subtypes: kaposiform hemangioendothelioma and tufted hemangioma.[305-307] The histologic features of kaposiform hemangioendothelioma and tufted hemangioma are abnormal when compared with the relatively well-organized vessels evident in capillary hemangiomas. These abnormal histologic features may contribute to platelet trapping and activation.

Thrombocytopenia can result from replacement of normal marrow elements by abnormal cells, as seen in leukemia, solid tumors (neuroblastoma, rhabdomyosarcoma, Ewing sarcoma), storage diseases, and disseminated Langerhans cell histiocytosis. Other acquired disorders of hematopoietic cells that are associated with thrombocytopenia include myelodysplastic syndrome, aplastic anemia, paroxysmal nocturnal hemoglobinuria, and osteopetrosis. In osteopetrosis the bone marrow space is obliterated by new bone formation. This condition can be diagnosed by x-ray studies but may not be clinically obvious in a neonate with thrombocytopenia. Peripheral smear findings include teardrop cells and nucleated erythrocytes.

Although iron deficiency is usually associated with thrombocytosis, thrombocytopenia may accompany severe iron deficiency in children and adults[315] (see Chapter 11). Anemia caused by folate or vitamin B_{12} deficiency (see Chapter 10) is also often associated with thrombocytopenia. Severe thrombocytopenia secondary to impaired hematopoiesis has been reported in association with anorexia nervosa.[316] The thrombocytopenia reverses with improved nutrition.

Thrombocytosis

Thrombocytosis is defined as a platelet count greater than 2 SD above the mean. Conditions associated with thrombocytosis in childhood are listed in Box 34-6.

When the platelet count is elevated, the clinician must determine whether the patient has a true increase in total body platelet mass. Under normal circumstances a third of the body's platelets are sequestered in the spleen. Individuals with surgical or functional asplenia who have a normal total body platelet mass will have an elevated platelet count. This situation is normal for an asplenic individual, and no therapy is warranted. Spuriously elevated automated platelet counts may be caused by the presence of microspherocytes, Pappenheimer bodies, red cell and leukocyte fragments, or bacteria.

Causes of true thrombocytosis fall into two categories: autonomous (or primary) thrombocytosis and reactive (or secondary) thrombocytosis. In primary thrombocytosis platelet production is unresponsive to normal regulatory processes.[317] The decrease in megakaryocyte size that normally results from an increase in platelet mass is not seen. In childhood most occurrences of thrombocytosis are secondary. In general, occurrences of reactive thrombocytosis are moderate in degree and asymptomatic and respond with treatment of the underlying disorder. Reactive thrombocytosis is often seen in association with inflammatory disorders (see Box 34-6). In these situations the degree of thrombocytosis usually parallels the activity of the underlying condition. Therapy to decrease the platelet count is not indicated. Iron deficiency is often associated with an increase in the platelet count (see Chapter 11). Moderate thrombocytosis that persists for 1 to 2 weeks occurs commonly after major trauma or surgery.[318]

Rarely, a child with thrombocytosis will have a primary myeloproliferative disorder such as essential thrombocythemia or chronic myelogenous leukemia. These patients may be predisposed to thrombosis or hemorrhage.

Box 34-6 Causes of Thrombocytosis in Childhood

PRIMARY OR AUTONOMOUS THROMBOCYTOSIS

- Myeloproliferative disorders
  - Essential thrombocythemia
    - Nonfamilial
    - Familial
  - Polycythemia vera
  - Chronic myelogenous leukemia
  - Agnogenic myeloid metaplasia
  - Acute megakaryoblastic leukemia
  - 5q–syndrome

SECONDARY OR REACTIVE THROMBOCYTOSIS

- Inflammatory diseases
  - Infection
    - Acute
    - Chronic (tuberculosis, hepatitis, osteomyelitis)
  - Rheumatoid arthritis
  - Inflammatory bowel disease
  - Ankylosing spondylitis
  - Sarcoidosis
  - Acute rheumatic fever
  - Kawasaki syndrome
- Hematologic disorders
  - Iron deficiency
  - Chronic hemolytic anemias
  - Vitamin E deficiency
  - Acute hemorrhage
  - "Rebound" following thrombocytopenia
- Drug-induced
  - Vinca alkaloids
  - Corticosteroids
- Neoplastic diseases
  - Lymphoma
  - Neuroblastoma
  - Other childhood solid tumors
- Miscellaneous
  - After exercise
  - Following surgery
  - Caffey disease

RELATIVE THROMBOCYTOSIS DUE TO DECREASED SPLENIC POOLING

- Asplenia
  - Surgical
  - Congenital
  - Afunctional
- Drug-induced
  - Epinepherine

SPURIOUS THROMBOCYTOSIS

- Microspherocytes
- Pappenheimer bodies
- Red cell and leukocyte fragments
- Bacteria

ACQUIRED DEFECTS IN PLATELET FUNCTION

Drug-Induced Platelet Dysfunction

Many substances have been shown to inhibit platelet function, including both prescription drugs and medicinals such as garlic and various Chinese herbs.[319,320] In otherwise normal individuals the impairment in platelet function produced by these substances is of no clinical significance. On the other hand, these agents may cause serious hemorrhage in patients with underlying coagulopathies, such as uremia, hemophilia, or thrombocytopenia, or in patients taking anticoagulants such as heparin or warfarin.[321]

Oxygenated metabolites of arachidonic acid, collectively termed eicosanoids, are involved in a variety of processes, including thrombosis, inflammation, and allergy. Eicosanoids include prostaglandins, thromboxanes, and other compounds. Interference with eicosanoid synthesis is the basis for the action of many therapeutic agents, including analgesics, antiinflammatory agents, and antithrombotic drugs. The first enzyme in the synthetic pathway of prostaglandins and thromboxanes is cyclooxygenase (COX). There are two isoforms of his enzyme, COX-1 and COX-2. The former is constitutively expressed in most cells, including platelets. The latter is not normally present but may be induced in certain cells after stimulation with cytokines or other factors. In platelets thromboxane X_2 (TxA_2) is the major product of COX. TxA_2 induces platelet aggregation and vasoconstriction. Aspirin blocks production of TxA_2 by covalently acetylating a serine residue near the active site of COX.[322] Because platelets do not synthesize new proteins, the action of aspirin is permanent and lasts the life of the platelet. Complete inactivation of platelet COX-1 is achieved when adults take 160 mg of aspirin daily. In pregnant women aspirin may cross the placenta and impair platelet function in the fetus.[323,324] Aspirin prolongs the bleeding time but seldom produces values above the normal range.

Most other antiinflammatory drugs act on platelets in a manner similar to that of aspirin, although the majority are less potent. Such agents include ibuprofen, naproxen, phenylbutazone, indomethacin, sodium salicylate, and related drugs. In contrast to those of aspirin, the effects of these medications are reversible.

Dipyridamole is a vasodilator that interferes with platelet function by increasing the intracellular concentration of cyclic adenosine monophophate (cAMP). This effect is mediated by inhibition of cyclic nucleotide phosphodiesterase.[325] In combination with warfarin, dipyridamole is used to inhibit embolization from prosthetic heart valves. Other drugs that raise cAMP levels by inhibiting phosphodiesterase, such as methylxanthines, also inhibit platelet function.

The thienopyridine derivative class of drug, including ticlopidine, inhibits platelet activation[326] and prolongs the bleeding time by blocking signaling through the $P2Y_{12}$ platelet adenosine diphosphate (ADP) receptor. Abnormal platelet function continues for several days after use of the drug is discontinued. A newer member of this class, clopidogrel, has supplanted ticlopidine because of its efficacy and better safety profile.[327-329]

Though used sparingly in pediatrics, GPIIb/IIIa antagonists are now widely used as anticoagulants in adult patients with heart disease.[330,331] These agents inhibit the final common pathway of platelet aggregation mediated by fibrinogen or VWF binding to GPIIb/IIIa. Therefore GPIIb/IIIa antagonists more completely inhibit platelet aggregation than aspirin or clopidogrel does, each of which inhibits only one initiating pathway of platelet activation (COX and ADP receptors, respectively). Several GPIIb/IIIa inhibitors are commercially available for use in coronary intervention procedures or unstable angina.[330,331]

A number of antibiotics inhibit platelet aggregation and the release reaction,[332] although the mechanisms are not well understood. Penicillin causes platelet dysfunction when administered at high doses, but this effect is usually clinically insignificant. However, certain semisynthetic penicillins, notably carbenicillin, may cause clinically significant bleeding when administered at the usual therapeutic doses.[333-336] Bleeding times are prolonged in patients receiving carbenicillin therapy, and serious episodes of hemorrhage have been reported.

Calcium channel blockers, such as verapamil and nifedipine, interfere with both adrenergic receptors and TxA_2 receptors in the platelet membrane[337] but do not prolong the bleeding time. Some antidepressants, including amitriptyline, imipramine, and chlorpromazine, interfere with platelet function through ill-defined effects on platelet membranes.[338] Propranolol and other β-adrenergic receptor antagonists mildly inhibit platelet function through effects on the cell membrane and serotonin transport.[339] Sulfonylureas used in the management of hyperglycemia have been associated with mild qualitative platelet defects.[340] Valproic acid, in addition to causing thrombocytopenia, can directly inhibit platelet function.[314]

Platelet Abnormalities Associated with Systemic Diseases

Uremia

Platelet dysfunction is a well-recognized and potentially serious complication of uremia. Patients with severe renal insufficiency may experience petechiae, purpura, gastrointestinal hemorrhage, and other forms of bleeding. Dialysis can correct the platelet dysfunction associated with uremia.[341] The precise cause of the platelet defect in uremia is unknown. A dialyzable substance in plasma causes platelet dysfunction, but the identity of this factor (or factors) remains controversial.

Bleeding may be severe, although the incidence of serious hemorrhage has declined in recent years because of more intensive dialysis and avoidance of drugs that further impair platelet function. If there is symptomatic bleeding or surgery is planned, patients should be treated with dialysis or desmopressin. Typically, only partial correction is achieved. The anemia of chronic renal insufficiency should be treated with erythropoietin because this has been shown to reduce the risk of serious bleeding.[341-346] If these approaches are not effective, cryoprecipitate may be added.

Liver Disease

Patients with liver disease have a multifactorial bleeding disorder. A reduction in plasma coagulation factors synthesized by the liver contributes to the increased risk of bleeding in these patients. Moderate thrombocytopenia is a common finding in liver disease and is well tolerated in most instances. Thrombocytopenia in cirrhotic liver disease has long been ascribed to portal hypertension and concomitant hypersplenism. Recent studies suggest that altered thrombopoietin production may contribute to the thrombocytopenia in liver disease. Qualitative platelet defects have also been reported to occur in adult patients with liver failure,[347-350] although the mechanisms responsible are not well understood.

Box 34-7 Vascular Purpuras of Childhood

- Vasculitis
  - Henoch-Schönlein purpura
  - Kawasaki syndrome
  - Polyarteritis nodosa
- Purpura fulminans
- Connective tissue diseases
  - Ehlers-Danlos syndrome
  - Marfan syndrome
  - Osteogenesis imperfecta
  - Minkes disease
  - Pseudoxanthoma elasticum
- Hereditary hemorrhagic telangiectasia

Vascular Purpuras

The vascular purpuras, also termed the *nonthrombocytopenic purpuras,* are mentioned in this chapter because these diseases share features with ITP. These conditions often follow acute infection. A list of childhood vascular purpuras is presented in Box 34-7.

Henoch-Schönlein Purpura. Henoch-Schönlein purpura (HSP), also known as allergic purpura or anaphylactoid purpura, is a vasculitis affecting capillaries of the skin, synovial membranes, renal mesangium, and small intestine. The purpuric manifestations of the disease are usually self-limited.

HSP is presumably the result of an autoimmune process. The basic lesion is a microvasculitis with endothelial cell injury. Deposits of IgA are evident on capillary endothelial cells of the skin, kidney, and gastrointestinal tract. The renal manifestations of HSP are indistinguishable from those of so-called IgA nephropathy.[351]

The median age at diagnosis is 5 years (range, 0.4 to 15 years). Males predominate at a ratio of 3 : 2. An acute febrile illness precedes the syndrome in a majority of cases. In about 50% of patients, raised purpuric papules (palpable purpura) are evident over the lower extremities and buttocks, whereas in the remainder of patients the purpuric rash is more extensive.[352] Bullae, urticaria, diffuse erythema, and ulceration may also be seen. Joint pain or swelling is evident in about 80% of cases. Two thirds of patients exhibit abdominal signs or symptoms, including pain, hematemesis, or melena. Intussusception occurs in about 4% of patients with HSP. Edema, including scrotal swelling, is common. Renal involvement is evident in 40% of patients, many of whom exhibit severe proteinuria. The manifestations of this syndrome resolve within 1 to 6 weeks in the vast majority of patients. In a small subset of patients, permanent renal or joint damage may ensue.[353]

Glucocorticoid therapy can reduce the skin, joint, and gastrointestinal manifestations of the disease but should be reserved for those with severe symptoms. There is little evidence that glucocorticoids prevent renal involvement[354,355] or intussusception.

Purpura Fulminans. Purpura fulminans is a potentially fatal disorder that follows infection with meningococcus, streptococcus, varicella, and rubella. Thrombosis of small arterioles leads to infarction and hemorrhage of skin, subcutaneous tissues, and muscle. The disease occurs suddenly 2 to 4 weeks after a relatively mild infection such as varicella or scarlet fever. It begins with purpuric lesions on the skin that coalesce and then become necrotic. Patients become extremely ill with fever, hypotension, and laboratory evidence of DIC. The disease resembles pupura fulminans neonatalis, a disorder associated with homozygous protein C or protein S deficiency (see Chapter 33).

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CHAPTER 35

Acquired Disorders of Hemostasis

Steven Pipe and Neil Goldenberg

CHAPTER OUTLINE

ACQUIRED HEMORRHAGIC DISORDERS
Drug-Induced Bleeding
Disseminated Intravascular Coagulation
Liver Disease
Vitamin K Deficiency
Massive Transfusion Coagulopathy
Acquired Inhibitors of Coagulation Proteins
Acquired Inhibitors to Prothrombin—Hemorrhagic Lupus Anticoagulant Syndrome
ACQUIRED THROMBOEMBOLIC DISEASE
Characterization of Thromboembolism in Children
Venous Thromboembolism
Ischemic Arterial Stroke

ACQUIRED HEMORRHAGIC DISORDERS

Chapter 29 describes how the application of clinical skills forms the cornerstone for the assessment of a potential bleeding disorder. After confirming that a bleeding disorder is present through a comprehensive medical history and physical examination, the clinician should next address whether the condition is likely familial or acquired. This is sometimes difficult because some patients with mild underlying heritable hemostatic defects (e.g., mild von Willebrand disease [VWD] or platelet function defects) may not demonstrate suspicious clinical symptoms until faced with a traumatic injury or a surgical challenge at a later age. Nevertheless, acquired bleeding disorders remain a frequent clinical scenario, with presentations ranging from acute unexpected bleeding during or immediately after surgery to unusual or excessive clinical manifestations of bruising, petechiae, epistaxis, gum bleeding, or hematoma formation that occur over weeks to months. Distinguishing whether the bleeding most likely represents an abnormality in primary hemostasis, fibrin formation, or fibrinolysis provides a framework for the generation of a differential diagnosis (See Chapter 29). For example, petechiae are almost exclusively seen with defects in platelet number or function, whereas deep tissue hematomas are more likely associated with defects in fibrin formation, as would occur with clotting factor deficiencies. Standard screening laboratory tests such as the prothrombin time (PT) and activated partial thromboplastin time (aPTT) are most often used in the initial evaluation but are insensitive to mild but clinically significant reductions in hemostatic capacity.[1] In addition, a platelet count does not provide any assessment of platelet function. Therefore the clinician must rely on the history and physical findings to guide the extent of additional coagulation studies. The most common causes of acquired hemorrhagic disorders include drug-induced bleeding (e.g., anticoagulants); disseminated intravascular coagulation; liver disease; vitamin K (VK) deficiency; massive transfusion; renal disease; and, rarely, acquired inhibitors to coagulation proteins.

Drug-Induced Bleeding

Many drugs have been associated with abnormal platelet function, although not all result in bleeding manifestations. The major classes include antiinflammatory agents, antibiotics, cardiovascular drugs, psychotropic drugs, anticonvulsants, and anticoagulants. Aspirin is the commonest example, acting by irreversibly acetylating a serine residue at the active site of cyclooxygenase,[2] whereas other nonsteroidal antiinflammatories (e.g., ibuprofen, naproxen) are reversible inhibitors.[3] β-Lactam antibiotics can interfere with platelet function through binding to the platelet membrane.[4,5] Calcium channel blockers (e.g., nifedipine)[6] and tricyclic antidepressants, such as amitriptyline,[7] can result in decreased platelet aggregation responses but are unlikely to result in clinical bleeding. Valproic acid has become one of the most commonly used anticonvulsants in children. Interestingly, several mechanisms of relevant valproate-induced coagulopathies have been described, including thrombocytopenia and platelet dysfunction, acquired VWD, decreased VK-dependent clotting factors, hypofibrinogenemia, and decreased factor XIII levels.[8] Therapeutic anticoagulants (heparin, low-molecular-weight heparin, warfarin, and direct inhibitors of factor Xa and thrombin) should be the most readily apparent cause of drug-induced bleeding and in most patients can be correlated with laboratory monitoring. Platelet receptor antagonists (IIb/IIIa inhibitors; e.g., abciximab, eptifibatide, tirofiban, and adenosine diphosphate receptor antagonists; e.g., clopidogrel) are in increasing use in patients with congenital heart disease and patients undergoing interventional studies.[9]

In most cases discontinuation of the offending drug should resolve the hemorrhagic manifestations, but this will depend on the reversibility and half-life of the drug. VK administration will in most cases rapidly reverse warfarin-induced bleeding. Alternatively, when more rapid replacement is necessary to correct low levels of factors II, VII, IX, and X, infusion with a prothrombin complex concentrate (PCC) or fresh frozen plasma (FFP) can be considered. PCCs are formulated with either three factors (II, IX, and X) or four factors (II, VII, IX, and X) and have some advantages over FFP. PCCs can be rapidly reconstituted in small infusion volumes, have a fast onset of action, do not require identification of the patient's blood group, have an exceptional safety profile with respect to risk of viral transmission owing to pathogen reduction and inactivation steps incorporated into their manufacturing, and reduced risk of adverse reactions such as transfusion-associated circulatory overload or transfusion-associated acute lung injury.[10] A four-factor PCC, Kcentra, was recently approved by the U.S. Food and Drug Administration (FDA) for the urgent reversal of acquired coagulation factor deficiency induced by VK antagonism therapy in adults with acute major bleeding. Transfusions of fresh platelets are indicated in those patients with life-threatening or unremitting bleeding caused by drug-induced platelet dysfunction. In cases of minor bleeding in which the drug cannot be readily discontinued (e.g. anticonvulsants), desmopressin has been used with some success.[11]

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation (DIC) is a pathologic syndrome in which the normal physiology of coagulation is disturbed by the simultaneous action of four mechanisms: increased thrombin generation, suppressed physiologic anticoagulant pathways, activation and subsequent impairment of fibrinolysis, and activation of the inflammatory pathway.[12] This leads to widespread intravascular deposition of fibrin with resultant thrombotic end-organ complications and consumption of platelets and coagulation proteins, resulting in severe bleeding. Associated damage to the microvasculature can contribute to organ dysfunction, capillary leak, and shock.

Etiology

DIC is always a secondary phenomenon and not a disease entity in its own right. Thus its recognition should prompt the clinician to identify and treat the underlying cause rather than merely react to the bleeding manifestations. It most frequently occurs in the settings of sepsis, trauma, and systemic inflammatory syndrome, with an approximate frequency in hospitalized children of 0.4% to 1%, with sepsis accounting for approximately 95% of cases.[13,14] It is primarily a clinical diagnosis based on the evaluation of laboratory results in patients with a clinical condition known to be associated with DIC (Box 35-1).

Box 35-1 Causes of Disseminated Intravascular Coagulation

INFECTIOUS

Meningococcemia (purpura fulminans)
Other gram-negative bacteria (Haemophilus, Salmonella)
Gram-positive bacteria (group B Streptococcus)
Rickettsia (Rocky Mountain spotted fever)
Viruses
Malaria
Fungus

TISSUE INJURY

Central nervous system trauma (massive head injury)
Multiple fractures with fat emboli
Crush injury
Profound shock or asphyxia
Hypothermia or hyperthermia
Massive burns

MALIGNANCY

Acute promyelocytic leukemia
Acute monoblastic or myelocytic leukemia
Widespread malignancies (neuroblastoma)

VENOM OR TOXIN

Snake bites
Insect bites

MICROANGIOPATHIC DISORDERS

"Severe" thrombotic thrombocytopenic purpura
Hemolytic-uremic syndrome
Giant hemangioma (Kasabach-Merritt syndrome)

GASTROINTESTINAL DISORDERS

Fulminant hepatitis
Severe inflammatory bowel disease
Reye syndrome

HEREDITARY THROMBOTIC DISORDERS

Homozygous protein C deficiency

MISCELLANEOUS

Severe graft rejection
Acute hemolytic transfusion reaction
Severe collagen-vascular disease
Kawasaki disease
Heparin-induced thrombosis
Infusion of "activated" prothrombin complex concentrates
Hyperpyrexia/encephalopathy, hemorrhagic shock syndrome

From Colman R, Marder V: Disseminated intravascular coagulation (DIC): pathogenesis, pathophysiology, and laboratory abnormalities. In Colman R, Marder V, Salzman E, editors: Hemostasis and thrombosis: basic principles and clinical practice. *Philadelphia, 1982, JB Lippincott, p. 654.*

Clinical Presentation

The clinical manifestations of DIC include bleeding, thrombosis, or both, however typically bleeding predominates. Early indicators are bleeding resulting from venipunctures, intravascular accesses, and surgical wounds. Mucocutaneous bleeding may manifest as bruising, petechiae, epistaxis, gum bleeding, blood from tracheal aspirates, gastrointestinal bleeding, and hematuria. In fulminant cases it may lead to bleeding into vital organs. However, with increasingly sensitive diagnostic tests that can detect endogenous activation of the hemostatic process, the clinical spectrum of DIC is broad. Nonovert DIC describes a stressed but compensated hemostatic system, in which the laboratory tests are abnormal but there are no clinical manifestations.[15] Overt DIC is described as a stressed and decompensated hemostatic system, in which the laboratory tests are abnormal and clinical bleeding or microvascular thrombosis and organ dysfunction exists. Overt DIC may be controlled or uncontrolled depending on whether the process will resolve when the underlying stimulus is removed.

Diagnosis

The clinical presentation of a profoundly ill child with bleeding believed to be secondary to DIC can be supported by laboratory tests showing evidence of a consumptive coagulopathy with activation of the fibrinolytic cascade. Moderate to severe thrombocytopenia with or without anemia will be evident from the complete blood count. Thrombocytopenia is present in approximately 50% of patients and suggests consumption of platelets.[16] Anemia could be caused by bleeding or, when accompanied by schistocytes on blood smear, evidence of microangiopathic hemolytic anemia. The PT and aPTT are prolonged in 50% to 60% of patients,[17] reflecting consumption of many coagulation proteins, including prothrombin; factors V, VII, and VIII; and fibrinogen. Fibrinogen or fibrin degradation products (FDPs) and D-dimers are both increased in concentration in most patients with DIC, suggesting activation of the fibrinolytic process.[18] Marked reductions in plasma anticoagulants including proteins C and S and antithrombin (AT) have also been described.[12,19] The most sensitive tests for diagnosis of DIC are markers of endogenous thrombin generation: prothrombin fragment 1.2 and thrombin-AT (TAT) complexes.[20] Prothrombin fragment 1.2 is released when thrombin is generated from prothrombin. TAT complexes are generated by binding of thrombin with its inhibitor AT. The standard assays (PT, aPTT, platelet count, and D-dimers) are relatively rapid and simple to perform. However, changes in these test results do not always occur at the same time, and laboratory values change rapidly depending on the patient's clinical status. This may create confusion in patient management and make the diagnosis of DIC at an early stage particularly difficult. The International Society on Thrombosis and Haemostasis (ISTH) diagnostic scoring system for overt DIC has been widely used in intensive care units, and several publications have validated the score against morbidity scores.[15,21,22] The five-step algorithm assigns a score based on the severity of abnormality for each of the following: platelet count (>100 × 109/L = 0; <100 × 109/L = 1; <50 × 109/L = 2), elevated fibrin-related markers (no increase = 0; moderate increase = 2; strong increase = 3), prolonged prothrombin time (<3 seconds = 0; <3 seconds but <6 seconds = 1; >6 seconds = 2), and fibrinogen level (>1 g/L =0; <1 g/L = 1). A total score of 5 or more is considered compatible with overt DIC. The sensitivity and specificity of this scoring system are greater than 90%. However, the algorithm should be applied only in the presence of an underlying disorder known to be associated with DIC. Despite its use in many pediatric intensive care units in the United States, there remains a paucity of clinical research studies examing the utility of the ISTH scoring system in children. Investigators at Texas Children's Hospital have compared their institutional modified DIC criteria with the ISTH diagnostic score against a gold-standard diagnostic method of confirmation of DIC in a subset of children who died.[23] Their modified criteria is not a scoring system but a sequential analysis of coagulation assays (PT, platelet count, fibrinogen, and D-dimer) as evaluated by transfusion medicine specialists along with the patient's clinical condition. Such an approach yielded a higher sensitivity, owing to the inclusion of sequential testing, to recognize a trend in the evolution from an early-phase DIC to overt DIC.

Treatment

The fundamental principal of DIC treatment is the specific and vigorous treatment of the underlying disorder. In some cases DIC will completely resolve within hours after resolution of the underlying condition (e.g., appropriate control of sepsis with antimicrobials). However, in other cases supportive measures are required to control the DIC until the underlying condition is resolved (e.g., the use of all-*trans*-retinoic acid and chemotherapy for the treatment of acute promyelocytic leukemia and DIC.[24]) Therapeutic interventions remain controversial and must be individualized according to the underlying basis for the DIC and severity of the clinical symptoms. For example, in nonovert DIC, children do not usually require therapy for the DIC itself. However, in the presence of uncontrolled overt DIC, therapeutic intervention including blood replacement products may be indicated to improve hemostasis while waiting for effective therapy for the underlying condition. Treatment modalities investigated include blood component therapy, anticoagulants, restoration of natural anticoagulant pathways, and other agents.

Blood Component Therapy. In general, the more severe the laboratory abnormalities, in particular the degree of thrombocytopenia and coagulation factor depletion, the greater the risk of bleeding complications with DIC. Hence treatment with FFP, fibrinogen, cryoprecipitate, and platelets appears to be a rational therapy in bleeding patients or patients who are at risk for bleeding with a significant depletion of these hemostatic factors. However, blood component therapy should not be instituted on the basis of laboratory results alone; it is indicated only in patients with active bleeding, those who require an invasive procedure, or those who are otherwise at risk for bleeding complications.[25] Large volumes of plasma (15 to 30 mL/kg) may be necessary to correct the coagulation defect. PCC may be considered in actively bleeding patients if FFP transfusion is not possible. However, these products lack certain essential coagulation factors, such as factor V. The efficacy and safety of recombinant factor VIIa in DIC with life-threatening bleeding are unknown.

Reasonable goals are to maintain platelet counts above 50×10^9/L, fibrinogen concentrations above 1.5 g/L, and PT values less than double the normal range.

Anticoagulant Therapy. Considering the central role played by thrombin in DIC, the use of heparin or other anticoagulants to inhibit thrombin generation appears reasonable. Heparin can at least partly inhibit the activation of coagulation in sepsis and other causes of DIC.[26] However, a beneficial effect of heparin on clinically important outcome events in patients with DIC has never been demonstrated in controlled clinical trials and is not a standard of care in overt cases of DIC. However, therapeutic doses of heparin may be indicated in patients with clinically overt thromboembolism, chronic DIC, or extensive fibrin deposition such as seen in purpura fulminans or acral ischemia.[27]

Replacement of Natural Anticoagulant. Depleted levels of AT, protein C, and protein S cannot be effectively replaced with FFP alone because of the short plasma half-life of these proteins. AT and protein C concentrates have been extensively evaluated in patients with DIC.[28-32] Trials of AT therapy have not yet provided conclusive evidence sufficient to make treatment recommendations.[33] The double-blind, placebo-controlled, phase III trial of recombinant human activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) demonstrated a significant decrease in mortality for the protein C–treated group compared with placebo.[31] However, these findings were not replicated in later trials, treatment was associated with a higher risk of bleeding, and recombinant human protein C was ultimately withdrawn in 2011.[34] Soluble recombinant human thrombomodulin acts by reducing thrombin-mediated clotting, enhancing protein C activation at the site of clotting, as well as demonstrating some antiinflammatory properties. However, a large randomized controlled trial failed to demonstrate a benefit in 28-day mortality rates.[35]

Other Agents. Recently, recombinant factor VIIa (rVIIa) has become an attractive strategy to control bleeding in various scenarios.[36] In situations where volume overload is an issue or bleeding persists despite adequate blood component support, use of rVIIa has been shown to be effective.[37] However, these data have mostly been generated from anecdotal reports, and given the potential adverse thrombotic complications with this agent, controlled randomized trials are required to address its safety and efficacy in these patients. Antifibrinolytic agents can be effective in bleeding patients, but the use of these agents in patients with bleeding associated with DIC is generally not recommended. They could be considered in cases when hyperfibrinolysis is suspected, such as may occur with acute promyelocytic leukemia or trauma.[38]

Liver Disease

Pathophysiology

The liver is the main site of synthesis for most hemostatic components. However, the hemostatic impairment in liver disease involves a variety of mechanisms, including impaired hepatic synthesis, activation of the coagulation and fibrinolytic systems, poor clearance of activated hemostatic components, loss of hemostatic proteins into ascitic fluid, concurrent VK deficiency, thrombocytopenia, and impaired platelet function.[39,40] When both procoagulants and anticoagulants are reduced, hemostatic dysregulation results in both a propensity to hemorrhage and a propensity to thrombose. Along with reduced synthesis there may be secretion of abnormal forms of the hemostatic proteins that may function as inhibitors to coagulation. For example, abnormal fibrinogens (dysfibrinogenemias) are very common in liver disease.[41] Similarly, VK-dependent proteins (factors II, VII, IX, X, protein C, protein S, and protein Z) decrease in liver disease. However, they may also be secreted in forms with abnormal gamma-carboxylation reactions of the glutamic acid residues on the amino-terminal portion of the protein leading to reduced functional activity.[42] Hypofibrinogenemia may be related to decreased synthesis, or increased consumption (e.g., DIC). However, in patients with acute liver failure, plasminogen activator inhibitor (PAI-1) is increased, shifting the balance toward hypofibrinolysis. This occurs despite elevated levels of tissue plasminogen activator (tPA) that may be secondary to increased release from the activated endothelium or reduced clearance by the diseased liver (or both). The other proteins involved in fibrinolysis (plasminogen, α2-antiplasmin, thrombin activatable fibrinolysis inhibitor, and factor XIII) are typically reduced because of decreased liver synthesis.[43] In addition, several structural manifestations of liver disease can contribute to the bleeding in these patients, including portal hypertension, varices, gastritis, and hemorrhoids.

Clinical Presentation

Clinical symptoms are variable and dependent, to some extent, on the etiology of the liver failure and associated invasive procedures. Symptoms include ecchymosis and petechiae, mucous membrane bleeding, hemorrhage from gastrointestinal varices, and hemorrhage into the abdomen or central nervous system (CNS). However, clinically significant bleeding occurs rarely in acute liver failure (approximately 5% to 10% of cases).[43] Coagulation screening tests (aPTT, PT, thrombin clotting time) are usually prolonged, platelet counts are reduced, and bleeding time is prolonged. Plasma concentrations of FVII; FV; and, less commonly, fibrinogen are decreased.[39] Levels of FVIII may be normal or elevated, possibly reflecting reduced clearance via low-density-lipoprotein receptor-related protein,[44] and can be helpful in distinguishing severe liver disease from DIC. However, both conditions may occur simultaneously.[45] FDPs or D-dimer levels are often increased in hepatic failure and contribute to prolongation of the thrombin clotting time and impaired platelet function.[46] Dysfibrinogenemia will contribute to prolongations of the PT and aPTT, but the thrombin time and reptilase assays are more sensitive. Dysfibrinogenemia may be confirmed by demonstrating an abnormal ratio of clottable fibrinogen (functional assay) to fibrinogen antigen (immunoassay).

Treatment

Treatment for the hemostatic manifestations of severe hepatic failure is difficult. Enhanced hemostasis after replacement therapy is usually transient because adequate volumes of FFP cannot be infused, owing to associated hypervolemia. In addition, many coagulation proteins are rapidly cleared, thereby limiting any beneficial effect. Usually, therapy specifically directed at the coagulopathy is reserved for active bleeding or to facilitate invasive procedures. A common indication for FFP is an international normalized ratio (INR) greater than 2 or a PT prolongation greater than 4 seconds.[47] However, there are no firm guidelines, and some interventions may safely be performed with INR in the 2 to 2.5 range.[48] Although desmopressin can induce the release of factor VIII and von Willebrand factor (VWF) with a shortening of the bleeding time, in randomized trials desmopressin failed to control bleeding from acute variceal hemorrhage[49] or decrease blood loss with hepatectomy.[50] Cryoprecipitate can be administered to increase plasma fibrinogen concentrations if necessary. Secondary VK deficiency owing to impaired VK utilization or impaired VK absorption owing to cholestasis from intrahepatic and extrahepatic biliary atresia requires treatment with VK.[51] Isovolumetric plasmapheresis may aid in restoration of hemostasis by replacing coagulation factors while removing activated clotting factors, tPA, fibrin, and FDPs.[52] Recombinant VIIa has been shown to correct the prolonged PT in nonbleeding cirrhotic patients and has been used to avoid volume overload before invasive procedures likely to cause bleeding. However, randomized trials in the setting of liver biopsy and variceal bleeding have shown either no difference in hemostasis or only early, modest reductions in rebleeding rate but no difference in overall bleeding or transfusion requirements.[42] Larger studies are still required to evaluate the efficacy and safety of this agent in liver disease.

Patients requiring liver transplantation invariably have severe, end-stage hepatic failure with its associated hemorrhagic complications. Additional risk factors that contribute to bleeding in children during transplantation include extensive scar tissue from previous surgery, prolonged duration of the surgery, the need to reduce the size of the donor organ to fit the small patient, dilutional coagulopathy, and excessive activation of the fibrinolytic system during the anhepatic phase.[53-56] Although treatment with antifibrinolytic agents is an attractive option, this approach should be used with caution because children who undergo liver transplant may later develop either hemorrhagic or thrombotic complications. The prophylactic use of recombinant VIIa to reduce blood loss and transfusion requirements in patients undergoing liver transplantation has been studied in two multicenter randomized controlled trials.[57,58] These trials failed to demonstrate a benefit to support its use as a universal preemptive therapy for liver transplantation.[59] Recombinant VIIa could be considered in individual cases as “rescue therapy” for patients with life-threatening coagulopathy and bleeding during transplantation or in situations where blood products are unavailable or unacceptable (e.g., the Jehovah witnesses).[60]

Vitamin K Deficiency

The role of VK in coagulation is detailed in Chapter 27, and VK deficiency bleeding in newborns is discussed in detail in Chapter 29.

Clinical Presentation

Clinical symptoms of VK deficiency include mild to moderate bleeding; ecchymoses; oozing from intravenous (IV) puncture sites; and, rarely, internal bleeding. The primary disorder is usually identified on the basis of the history and physical examination.

Primary VK deficiency in healthy children is uncommon because of a relatively low VK requirement and the widespread distribution of VK in plant and animal tissues. In plants the only important molecular form is phylloquinone (VK1). Bacteria synthesize a family of vitamin K compounds called menaquinones (VK2). The contribution of the microbiologic flora of the gut to VK dietary intake is unknown but may be as high as 50%.[61] Although broad-spectrum antibiotics may reduce VK2 production by intestinal bacteria, VK deficiency is rare in the presence of a normal diet.

Groups of children at high risk for VK deficiency include breastfed newborns, chronically ill children with an inadequate dietary intake of VK, children with disorders that interfere with the absorption of VK (e.g., diarrhea, cystic fibrosis, cholestatic liver disease, celiac disease), and children with poor nutrition who are receiving broad-spectrum antibiotics.[62-65] Cystic fibrosis, biliary atresia, and obstructive jaundice interfere with the absorption of fat-soluble vitamins and interfere with transport to the liver, with resultant VK deficiency. Therapeutic drugs can interfere with VK metabolism (e.g., phenytoin, warfarin). However, at least in the case of warfarin administration, this is an expected side effect. Accidental exposure to supratherapeutic doses may occur in small children with access to warfarin in the home. Anticoagulant rodenticides can be another source of accidental exposure. Owing to the emergence of warfarin-resistant strains of rats, newer “long-acting” second-generation compounds have been utilized. These so-called “superwarfarins” have higher affinity for vitamin K1-2,3 epoxide reductase, are able to disrupt the vitamin K1-epoxide cycle at more than one point, exhibit hepatic accumulation, and have unusually long biological half-lives as a result of their high lipid solubility and enterohepatic circulation. After accidental ingestion these compounds can cause anticoagulation for several days or even weeks. However, in most cases involving small children, the exposure is low and bleeding manifestations are rare.[66,67] VK deficiency has been described in bone marrow transplant and oncology patients in whom the etiology is multifactorial as a result of drug antagonism, liver dysfunction, fat malabsorption, anorexia, or inadequate dietary intake. A study of children evaluated before undergoing bone marrow transplantation identified 31% (8 of 26) with evidence of VK deficiency (as evidenced by circulating PIVKA-II), although only one patient had a prolonged PT, highlighting that the PT alone is an insensitive marker of VK deficiency. Of the eight affected patients, six exhibited bleeding manifestations peri-transplant.[68]

Laboratory Diagnosis

The PT and aPTT may both be abnormal in VK deficiency but should correct in a 1:1 mix with normal plasma. Other acquired or congenital coagulopathies can give similar screening test abnormalities. Therefore specific coagulation factor assays must be performed. The finding of more than one VK-dependent factor deficiency should increase the suspicion of an acquired deficiency. A normal factor V in addition to normal liver function studies would suggest that there is no significant liver disease underlying the presentation. A normal FVIII and fibrinogen level and absence of elevated D-dimers would make DIC unlikely. However, VK deficiency can complicate other coagulopathies, such as DIC or liver disease. If there is any doubt about the diagnosis, the patient should receive VK therapy in conjunction with other supportive care.

Treatment

The route and specific type of therapy are dictated by the urgency of the clinical situation and potential side effects of therapy. For example, severe anaphylactoid reactions, although rare, have complicated IV VK administration, even when the solution is diluted and infused slowly. Therefore IV administration of VK, at a rate no faster than 1 mg per minute, should be restricted to those situations in which other routes are not feasible and the risk is justified. Intramuscular VK is not recommended because it can result in pain, swelling, and hematoma formation. The preferred systemic route is subcutaneous because it is safe and effective. Oral VK is effective if absorption is unimpaired. Although oral administration is generally thought to produce a slower correction of the PT (6 to 8 hours) compared with parenteral VK (2 to 6 hours), a study in adults suggested that the oral route may actually be faster, at least in reversing the effect of warfarin.[69]

Asymptomatic patients with mildly abnormal coagulation results that are presumed secondary to VK deficiency should be given VK subcutaneously (1 to 5 mg depending on size). This approach is both therapeutic and diagnostic, with coagulation abnormality correction within 2 to 6 hours. A patient bleeding because of VK deficiency should receive 2 to 10 mg of VK subcutaneously. FFP (10 to 20 mL/kg) is particularly useful when the precise nature of the coagulopathy is unknown and bleeding is severe. However, the amount of plasma needed for total correction of a severe VK deficiency is so great that it may result in volume overload. A patient with a life-threatening hemorrhage or an intracranial hemorrhage (ICH) would likely benefit from prothrombin complex concentrates at a minimum dose of 50 units/kg, in addition to systemic VK (5 to 20 mg). Prothrombin complex concentrates contain relatively uniform amounts of factors II, IX, and X, with amounts of factor VII/VIIa varying.[70] Recombinant VIIa has been used anecdotally.[71,72]

Prevention

Prophylactic VK should be given to patients at risk, such as patients with inadequate nutrition and those who are receiving broad-spectrum antibiotics. Most patients requiring total parenteral nutrition (TPN) are supplemented with VK. There has been concern in the past that maternal anticonvulsant therapy may be associated with increased risk of neonatal bleeding linked to enzymatic degradation of VK.[73] This has prompted recommendations for VK prophylaxis to pregnant mothers on anticonvulsants. However, recent studies have demonstrated that the incidence of bleeding in infants exposed to these drugs antenatally is not higher than controls[74] and have shown no benefit to routine VK prophylaxis.[75]

Massive Transfusion Coagulopathy

Uncontrolled bleeding can be a clinical problem associated with the management of trauma and surgical patients, often necessitating the transfusion of large amounts of blood and blood derivatives. Massive transfusion has been commonly defined as replacing at least one blood volume in 24 hours or 50% of one blood volume in 3 hours.[76,77] Criteria used by the University of Michigan Hospitals for adults is 4 U of red blood cells utilized in less than 4 hours and ongoing uncontrolled bleeding, and for children 30 mL/kg of red blood cells utilized and ongoing uncontrolled bleeding. Patients with this degree of bleeding will develop defective hemostasis related to the transfusions in addition to the underlying precipitating trauma or surgical insult. The resulting bleeding diathesis may be complex and may include evidence of DIC; depletion of hemostatic factors through blood loss, tissue injury, and consumption of factors; dilutional coagulopathy owing to aggressive blood component resuscitation; hypothermia; platelet dysfunction; and excessive fibrinolysis.[76] Hemostatic resuscitation advocates for the transfusion of red blood cells, plasma, and platelets in a 1:1:1 ratio in order to address the underlying dilutional coagulopathy. Massive transfusion protocols are now utilized worldwide for patients with severe life-threatening bleeding and are intended to standardize the approach to these patients and provide structure and organization to what is usually a chaotic situation. These protocols standardize the ratio for blood products that are empirically delivered; initiate unique communication processes among the blood bank, the clinical laboratories, and the clinician; and call attention to appropriate management of acidosis, hypothermia, and hypocalcemia that may be aggravating the coagulopathy. Clinical studies with massive transfusion protocols have demonstrated decreased crystalloid use, improved survival rates, decreased chance of organ failure, and decreased risk of early death resulting from hemorrhage. Fibrinogen concentrates for acquired fibrinogen deficiency have been evaluated by postmarketing surveillance studies and retrospective analyses and show results consistent with in vitro studies demonstrating that fibrinogen replacement can improve clot firmness and improve survival of severely injured massively bleeding patients. Recombinant factor VIIa has been evaluated in several randomized clinical trials for bleeding in the context of trauma and surgery but has failed to improve outcomes.[78,79]

Acquired Inhibitors of Coagulation Proteins

Inhibitors directed against coagulation factors are circulating antibodies that specifically neutralize the procoagulant activity or increase the clearance of those coagulation factors leading to a plasma deficiency and are often accompanied by bleeding manifestations.[80,81] They may be either alloantibodies or autoantibodies. Examples of alloantibodies include those directed against infused factor VIII and are observed in 15% to 25% of patients with hemophilia A (discussed in Chapter 31). Autoantibodies have also been observed directed against endogenous factor VIII in nonhemophiliacs and occur typically in postpartum women, elderly individuals, or people who have autoimmune disorders. However, autoantibodies have also been ascribed to other coagulation proteins, including fibrinogen; factors II, V, VII, IX, X, XI, and XIII; andVWF. Antiphospholipid antibodies (APLAs), or lupus anticoagulant (LA), are a heterogeneous group of antibodies that react with proteins bound to phospholipids. Although these primarily function as inhibitors to in vitro phospholipid-dependent clotting assays, they paradoxically predispose to thrombosis rather than hemorrhage. However, a unique LA coagulopathy has been observed with a hemorrhagic syndrome associated with hypoprothrombinemia (discussed later).

Acquired Inhibitors to Factor VIII

Anti–factor VIII autoantibodies are rare in children but can result in severe bleeding and significant morbidity. They are associated with underlying medical conditions in half of those affected, including malignancy, autoimmune disease, lymphoproliferative disorders, and drugs (e.g., penicillin).[82] Patients can present with bleeding symptoms ranging from easy bruising to intracranial hemorrhage. In one patient the clinical course was also complicated by nephrotic syndrome.[83] Laboratory screening studies will show a prolonged aPTT that does not fully correct on mixing with normal plasma in a 1 : 1 mix ("Inhibitor Screen"). In addition, this can be distinguished from an LA by performing the aPTT 1 : 1 mix again with an incubation phase (typically at least 30 minutes at 37° C) in which the aPTT will show further prolongation indicating progressive neutralization of factor VIII provided by the normal plasma mixing. There will be no significant additional prolongation seen on incubation in the presence of an LA. In contrast to congenital hemophilia with inhibitors, these antibodies typically demonstrate type II (nonlinear) inactivation kinetics. Thus the baseline factor VIII levels may be higher than 1% despite high titer inhibitory antibodies.[84]

Treatment is two-pronged, directed at stopping the bleeding and eradicating the inhibitor. Hemostasis can be achieved with high doses of factor VIII concentrates, activated prothrombin complex concentrates (APCC), and recombinant VIIa. Immunosuppression may be accomplished with steroids alone or in combination with mycophenolate-mofetil, rituximab, γ-globulin, or cytotoxic agents such as cyclophosphamide.[85,86] Inhibitors have also been eradicated through extracorporeal immunoadsorption and immune tolerance with regular exposure to high-dose factor VIII infusions. Review of the available literature suggests that, in contrast to adults, anti–factor VIII autoantibodies in children may resolve more quickly and easily and do not necessarily require steroids and cytotoxic agents.[82,86,87] Recombinant factor VIII should be considered in the initial treatment with additional hemostatic support from APCCs and recombinant VIIa as needed. Withdrawal of instigating drugs such as penicillin may also facilitate resolution.[88]

Acquired Inhibitors to Factor IX

Few cases of spontaneous factor IX inhibitors have been described in nonhemophiliac patients, and most adult cases have been related to underlying systemic disorders such as systemic lupus erythematosus, hepatitis, multiple sclerosis, rheumatic fever, collagen vascular disease, postpartum status, and postprostatectomy status. Reports in children are limited to a few case reports.[86,89-91] Bleeding manifestations included cutaneous ecchymoses and soft tissue hematomas. Diagnostic studies are similar to those with anti–factor VIII inhibitors. Treatments have included corticosteroids, γ-globulin, and cyclophosphamide, with rapid resolution of the inhibitor. One child had a spontaneous resolution.[90] Recombinant factor IX and recombinant VIIa can be considered for acute management of bleeding.

Acquired Inhibitors to Factor V

Most cases of inhibitors directed against factor V develop as a result of risk factors such as surgical procedures, antibiotic administration (typically the β-lactam group), blood transfusions, cancers, and autoimmune disorders. However, the majority of cases arise after exposure to bovine thrombin.[81] Topical preparations of bovine thrombin are widely used for surgical hemostasis and contain fibrinogen as well as small amounts of factor V and other proteins. Acquired inhibitors to coagulation factors, as a consequence of exposure to this highly immunogenic agent, is a frequent occurrence, albeit often unrecognized. In this context antibodies directed against factor V are most frequent, although anti-prothrombin and anti–factor X antibodies have also been described.[92,93] The incidence of symptomatic inhibitors is not known. In a study of 151 adult patients exposed to bovine thrombin during cardiac surgery, 56% of the patients developed antibodies directed against human coagulation proteins.[92] However, adverse clinical outcomes did not correlate with antibody formation. A literature review identified 12 cases of inhibitors in pediatric patients after thrombin exposure.[93] In eight cases the antibodies were directed against human coagulation proteins; five of these patients had hemorrhagic complications. Bleeding manifestations included cutaneous, gastrointestinal, pulmonary, and cerebral hemorrhages. Patients exhibited both PT and aPTT prolongations and mixing studies consistent with an inhibitor. Specific factor activity assays revealed the target for the inhibitor. However, there was no correlation between laboratory studies and clinical bleeding. Corticosteroids and γ-globulin have been used with resolution of the inhibitor over days to weeks. Platelet transfusions are another option, insofar as approximately 20% of factor V activity is present in platelet granules that are shielded from circulating antibodies.[94] Coagulation factor inhibitors should be suspected in any postoperative patient exposed to topical bovine thrombin presenting with a prolonged PT and aPTT.

Acquired Inhibitors to Other Coagulation Factors

Antifibrinogen antibodies are extremely rare and may or may not be associated with clinical bleeding. An increased rate of fibrinogen antibodies has been described in pregnant and peripartum women.[95] Acquired inhibitors directed against coagulation factors VII and X have been observed with malignancies, autoimmune disorders, and drug exposure. In addition, acquired inhibitors against factor XI have been rarely reported, including in patients with underlying SLE.[96]

Acquired von Willebrand Syndrome

Acquired von Willebrand syndrome (AVWS) is a rare bleeding disorder with clinical and laboratory findings similar to those in inheritedVWD. Since its original description in 1968 in a patient with systemic lupus erythematosus, fewer than 700 cases have been reported.[97] AVWS usually occurs in individuals with no personal or family history of VWD and is accompanied by bleeding symptoms in about three quarters of patients, whereas others are diagnosed on the basis of abnormalities found during routine hemostasis screening tests.[98] As would be expected, mucocutaneous bleeding symptoms predominate. Underlying disorders are identified frequently and fall into the following main categories: lymphoproliferative and myeloproliferative disorders; solid tumors; immunologic and cardiovascular disorders; and other miscellaneous conditions, including drug associations. It is typically a syndrome of advanced age, although it has also been reported in children, and is primarily associated with congenital heart disease, collagen vascular diseases, Wilms tumor, hypothyroidism, and use of certain drugs. Drug-associated AVWS has been described in up to 20% of pediatric patients who received valproic acid, although bleeding manifestations were mild and did not require discontinuation of the anticonvulsant.[99]

Four main pathogenic mechanisms can lead to AVWS: (1) reduced VWF synthesis (as seen in severe hypothyroidism or certain drugs (e.g., valproic acid); (2) inhibition or clearance by paraproteins or autoimmune inhibitors (this has been observed in association with B-cell lymphomas, monoclonal gammopathies, multiple myeloma, and autoimmune disorders); (3) absorption of VWF high-molecular-weight multimers onto malignant cell clones or platelets; (4) increased shear stress and proteolysis, as may occur with aortic stenosis, artificial heart valves, left ventricular assist devices, or other cardiac defects with disturbed flow.[97] Laboratory studies rely on detecting abnormally reduced functional VWF studies (ristocetin cofactor activity, collagen-binding assay) in comparison to VWF antigen and a selective deficiency of the high-molecular-weight multimers. A search for evidence of an inhibitor should be performed by measuring VWF activity after mixing experiments involving patient plasma and normal plasma incubated at 37° C.

The three goals of treatment should be control of active bleeding, prevention of bleeding with necessary invasive procedures, and treatment of the underlying disease. Desmopressin and factor VIII/VWF concentrates have provided only short-term control of bleeding in the presence of autoantibodies.[100] In those cases γ-globulin, corticosteroids, or other immunosuppressive agents have been administered. Recombinant VIIa may also be effective with this presentation.[101] In noninhibitor-associated AVWS, factor VIII/VWF concentrates are effective in most patients, although the half-life may be shorter.[97] Desmopressin may be used to control bleeding, although there is a relatively lower rate of success among patients with cardiovascular or myeloproliferative disorders.[102] Ultimately, normalization of hemostasis may not be seen until the underlying precipitating condition is treated effectively (e.g., with surgery or chemotherapy for Wilms tumor).

Acquired Inhibitors to Prothrombin—Hemorrhagic Lupus Anticoagulant Syndrome

LA represent a diverse group of antibodies directed against proteins bound to phospholipids. They may be found in patients with systemic lupus erythematosus and other autoimmune disorders but also in otherwise healthy individuals and have been implicated in thrombotic complications. However, in children they are most often identified after investigation of a prolonged aPTT seen as part of pre-surgical evaluation or associated with a history of infection.[103] In the vast majority of these cases, they are asymptomatic (including no bleeding manifestations), transient, usually resolving over days to weeks, and do not preclude proceeding with invasive procedures. However, a rare hemorrhagic LA syndrome (ecchymoses, epistaxis, gastrointestinal bleeding, hematomas and even hemarthroses) has been observed with associated low prothrombin levels.[104-109] This would be manifest by a concurrent prolongation of the PT (unusual for an uncomplicated LA) and pathogenically is believed to be caused by antiprothrombin antibodies. These are not typically neutralizing and, as a result, mixing studies may not indicate the presence of an inhibitor. Rather, the formation of immune complexes leads to increased plasma clearance.[110] Antibodies have been characterized directed against the carboxy terminal portion of the prothrombin molecule.[111] Bleeding symptoms usually resolve without therapy, although patients with significant bleeding symptoms may be treated with prothrombin complex concentrates. Corticosteroids and other immune suppressants have been used in some cases.

ACQUIRED THROMBOEMBOLIC DISEASE

Thromboembolism (TE) in children has become increasingly diagnosed in recent years thanks to enhanced survival rates associated with serious underlying illnesses, heightened use of invasive vascular procedures and devices, and a growing (albeit still suboptimal) awareness that TE does indeed occur in children. The subsequent portion of this chapter summarizes the clinical spectrum and characterization of pediatric TE, key considerations for diagnostic evaluation and antithrombotic management, and long-term outcomes. Emphasis is also placed on unresolved and emerging clinical and investigative issues in the field.

Characterization of Thromboembolism in Children

TE is anatomically classified by vascular type (i.e., venous versus arterial), distribution (e.g., distal lower extremity versus proximal lower extremity versus central; superficial versus deep vasculature), and organ system affected, if applicable (e.g., renal vein thrombosis, cerebral sinovenous thrombosis, pulmonary embolism [PE]). TE is also distinguished by the additional clinically relevant factors of first episode versus recurrent, symptomatic versus asymptomatic, acute versus chronic (a sometimes-difficult distinction), veno-occlusive versus nonocclusive, and idiopathic versus risk associated. This last category includes both clinical prothrombotic risk factors (e.g., exogenous estrogen administration, indwelling central venous catheter, reduced mobility) and blood-based thrombophilic conditions (e.g., transient or persistent antiphospholipid antibodies, acquired or congenital anticoagulant deficiencies, the factor V Leiden or prothrombin 20210 mutations), as discussed in greater detail later in the section "Etiology." In light of the frequency of indwelling central venous catheters as a clinical risk factor for TE in children, provoked TE is often also classified as catheter related versus non–catheter related.

Venous Thromboembolism

Epidemiology

Several national or international registries in the past two decades have evaluated the occurrence of venous thromboembolism (VTE) in children. From these data a cumulative incidence of 0.07 per 10,000 (5.3 per 10,000 hospitalizations) was estimated for extremity deep venous thrombosis (DVT) and PE among non-neonatal Canadian children,[112] and an incidence rate of 0.14 per 10,000 children per year was reported for all VTE in the Netherlands.[113] In 2004 evaluation of the National Hospital Discharge Survey and census data for all VTE in the United States disclosed an overall incidence rate of 0.49 per 10,000 per year.[114] More recently, an analysis of the Pediatric Health Information System database revealed a nearly 70% increase in the diagnosis of VTE among hospitalized children, as defined by International Classification of Disease-9 diagnostic codes, over a 7-year period from 2001 to 2007.[115]

Closer examination of epidemiologic data reveals that the age distribution of the incidence rate for VTE in children is bimodal, with peak rates in the neonatal period and adolescence. With regard to the newborn period, the cumulative incidence of venous or arterial TE was reported to be 0.51 per 10,000 births in Germany[116] and 24 per 10,000 admissions to neonatal intensive care units in Southern Ontario.[117] The Dutch registry indicated a VTE-specific incidence rate of 14.5 per 10,000 per year in the neonatal period, approximately 100 times greater than the overall rate in childhood.[113] Among adolescents 15 to 17 years of age, the VTE-specific incidence rate in the United States was determined to be 1.1 per 10,000 per year, a rate nearly threefold that observed overall in childhood.[114]

Although differing selection criteria have contributed to the considerable variability in occurrence estimates in the preceding example, it is clear that the incidence of VTE in children is lower than that among middle-aged and elderly adults. Nevertheless, as discussed later in this chapter, the sequelae of VTE in children, particularly with regard to the post-thrombotic syndrome (PTS), appear to be at least as frequent and severe as those among adults.

Etiology

The pathogenesis of VTE can be readily appreciated by considering Virchow's triad, consisting of venous stasis, endothelial damage, and the hypercoagulable state. In children, greater than 90% of VTE are risk-associated[113,118,119] (compared with approximately 60% in adults), with risk factors often disclosed from more than one component of this triad. Specific examples of VTE risk factors in children are shown in Figure 35-1. Among the most common of clinical prothrombotic risk factors in childhood is an indwelling central venous catheter. Over 50% of cases of DVT in children and over 80% of cases in newborns occur in association with central venous catheters.[112,117] The reported cumulative incidence or prevalence of catheter-related thrombosis (CRT) in children receiving home TPN ranges widely, from 1% to 80%[120-125]; this broad variation is largely influenced by differing study designs, selection criteria, and diagnostic imaging modalities. The presence of an indwelling central venous catheter, the underlying malignancy or disorder for which bone marrow transplantation was undertaken, and congenital cardiac disease and its corrective surgery were all highly prevalent in the Canadian pediatric thrombosis registry,[119] whereas underlying infectious illness and the presence of an indwelling central venous catheter were identified as pervasive clinical risk factors in a recent cohort study analysis from the United States.[118] It is likely that differences in the composition of referral populations strongly contribute to differences in the composition of VTE etiologies across major pediatric thrombosis centers.

With regard to blood-based risk factors for VTE, thrombophilic conditions (e.g., severe anticoagulant deficiencies , antiphospholipid antibodies [APAs]) in children are frequently acquired, and severe inherited thrombophilias are rare. By contrast, mild congenital thrombophilic traits (e.g., the factor V Leiden and prothrombin 20210 mutations) are common in Caucasian children, as in adults (see Chapter 33). Thrombophilia is potentially caused by any alteration in hemostatic balance that increases thrombin production, enhances platelet activation or aggregation, mediates endothelial activation or damage, or inhibits fibrinolysis. Common examples of acquired thrombophilia in children include increased factor VIII activity with significant infection and inflammatory states, anticoagulant deficiencies resulting from consumption in bacterial sepsis and DIC, the production

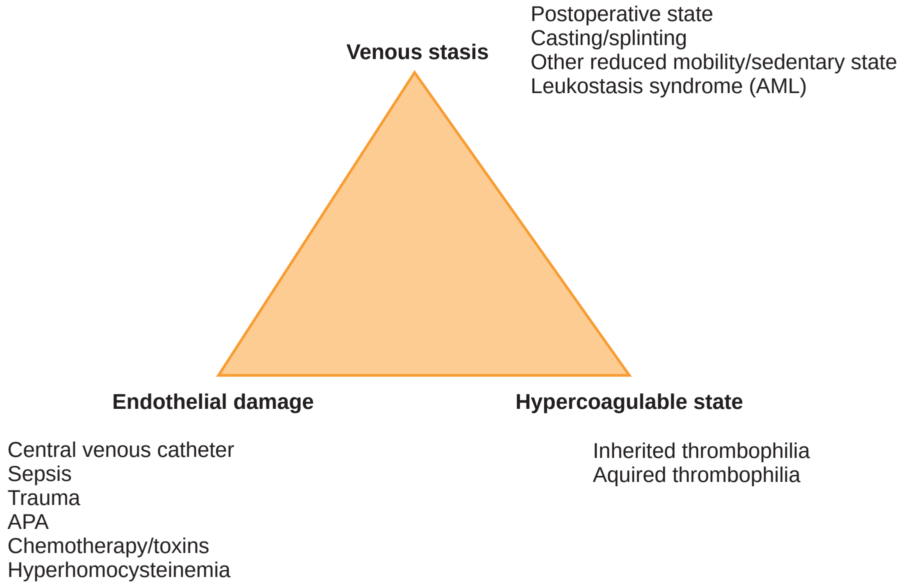

Figure 35-1 Clinical prothrombotic risk factors: Virchow's triad applied to venous thromboembolism in children.

of inhibitory antibodies in acute viral infection, and parainfectious development of APA. To provide an appreciation of the magnitude of VTE risk increase associated with several congenital or genetically influenced thrombophilia traits, population-based VTE risk estimates derived from the adult literature are shown in Table 35-1. For example, the addition of standard-dose estrogen oral contraceptive pill to underlying heterozygous factor V Leiden substantially increases the risk for VTE from a baseline risk of 15 per 10,000 U.S. females aged 15 to 17 years per year[114] to a risk of more than 500 per 10,000 (or 5%) per year.

Clinical Presentation

The degree of clinical suspicion for acute VTE in children should be principally influenced by clinical prothrombotic risk factors and family history of early VTE or other vascular disease elicited on thorough interview, known thrombophilia traits and risk factors, and clinical signs and symptoms. The signs and symptoms of VTE depend on anatomic location and organ systems affected and are influenced by characteristics of veno-occlusiveness and chronicity. The classic manifestation of acute extremity DVT is painful unilateral limb swelling. The lack of other physical examination findings (e.g., Homans sign, the presence of a palpable cord in the popliteal fossa in the clinical evaluation for lower extremity DVT) should not reduce the clinical index of suspicion of DVT. In upper extremity DVT with extension into, and occlusion of, the superior vena cava (SVC), signs and symptoms may include swelling of the neck and face, bilateral periorbital edema, and headache. PE is classically manifested by sudden-onset, unexplained shortness of breath with pleuritic chest pain. When PE is proximal or extensive bilaterally in the distal pulmonary arterial tree, hypoxemia is often demonstrated. Associated right-sided heart failure may manifest with hepatomegaly and peripheral edema. Proximal PE and especially saddle embolus can present with cyanosis or sudden collapse. However, in many cases PE may be asymptomatic or produce only subtle symptoms in children,[126-129] especially when involving limited segmental branches of the pulmonary arteries and not eliciting a significant pleural reaction. In one retrospective series 14% of affected children had no clinical symptoms attributable to PE.[127] Acute cerebral sinovenous thrombosis (CSVT) may present with unusually severe and persistent headache, blurred vision, neurologic signs (e.g., cranial nerve palsy, papilledema), or seizures. The classic findings in renal vein thrombosis (RVT) are hematuria and thrombocytopenia, sometimes associated with uremia (especially when bilateral). Presenting signs include oliguria (especially when bilateral), and in the neonatal period (the time at which RVT is most common during childhood) a flank mass is often palpable on examination.[130] RVT in older children is often associated with nephrotic syndrome (a risk factor for VTE in general owing to the loss of anticoagulant proteins) and hence may present with associated stigmata of peripheral and periorbital edema when diagnosed at presentation of nephrosis.[128] Thrombocytopenia may be a presenting manifestation

TABLE 35-1 Venous Thromboembolism Risk Estimates for Selected Thrombophilia Traits and Conditions

Trait/Condition	VTE Risk Estimate
Hyperhomocysteinemia	2.5×
Prothrombin 20210 mutation, heterozygous	3×
OCP (standard dose estrogen)	4×
Factor V Leiden mutation, heterozygous	2-7×
Factor V Leiden mutation, heterozygous, + OCP	35×
Factor V Leiden mutation, homozygous	80×

OCP, Oral contraceptive pill; *VTE,* venous thromboembolism.

not only of RVT but also of intracardiac (e.g., right atrial) thrombosis, especially as a CRT associated with sepsis.[131] Isolated intracardiac thrombosis in association with cardiac surgery or central venous catheter placement is most often asymptomatic.[132] Portal vein thrombosis is one of the most common causes of portal hypertension in children, characteristically presenting with splenomegaly, and is associated with findings of hypersplenism (anemia, neutropenia, and thrombocytopenia); gastrointestinal bleeding at presentation typically signals the presence of gastroesophageal varices.[133,134] Internal jugular vein thrombosis may manifest with neck pain or swelling, and when part of Lemierre syndrome is classically also associated with fever, trismus, pain on lateral neck rotation, and a palpable mass in the lateral triangle of the neck.[135]

Chronic VTE may be diagnosed incidentally without signs or symptoms (as sometimes occurs for CSVT during unrelated brain imaging), or it may present with signs and symptoms of chronic venous obstruction or PTS, including pain and edema of dependent extremities, dilated superficial collateral veins, venous stasis dermatitis, and frank ulceration of the skin.

Diagnosis

Whereas venography has historically been the gold standard for diagnosis of venous thrombosis, this modality is invasive and has become less prevalent in recent years with the development of effective noninvasive or minimally invasive radiologic imaging technologies. Radiologic imaging is used not only to confirm the clinical diagnosis of VTE but also to define both the extent and occlusiveness of thrombosis. For suspected DVT of the distal or proximal lower extremity, compression ultrasound with Doppler is typically employed for objective confirmation. When the thrombus may affect or extend into deep pelvic or abdominal veins, computed tomography (CT) is often necessary. For circumstances in which radiation exposure is a significant concern or alternative diagnoses (e.g., myositis) are being evaluated, magnetic resonance imaging (MRI) may alternatively be employed. Magnetic resonance venography may be useful in documenting May-Thurner syndrome, an uncommon cause of all lower extremity DVT (but seemingly a common cause of spontaneous or minimally provoked left-sided iliofemoral DVT) in which the iliac vein is compressed by an overriding right common iliac artery.[136] In suspected DVT of the upper extremity, compression ultrasound with Doppler effectively evaluates the limb, but echocardiography or CT is required to disclose involvement of more central vasculature (e.g., right atrial thrombosis). In the case of asymptomatic nonocclusive extremity DVT, venography may be used as an alternative to CT. It is important to recognize that venography (in which radiographic contrast is administered into a vein in the limb) should not be confused with "line-o-grams" (in which radiographic contrast is instilled into a central venous catheter). The latter studies are useful for delineating catheter tip thrombi but cannot reliably detect DVT along the intravascular length of a central venous catheter.[137] Magnetic resonance venography has replaced conventional venography in most centers. However, dynamic conventional venography may be required to evaluate for thoracic outlet syndrome (mechanical compression and entrapment of the subclavian vessels in the region of the thoracic outlet, whether owing to a congenitally narrow subclavian passage between the clavicle, first rib, and a hypertrophied subclavius muscle in athletes [Paget-Schroetter syndrome] or to an accessory rib).[138] To establish a diagnosis of DVT of the jugular venous system, compression ultrasound with Doppler is typically sufficient.

PE in children is commonly diagnosed by spiral CT or, alternatively, by ventilation-perfusion scan, the latter generally being suboptimal in cases wherein other lung pathology exists and at centers wherein availability of, and expertise with, this modality are limited. CSVT is typically diagnosed by standard CT or CT venography, and alternatively by MRI or magnetic resonance venography. The diagnosis of RVT is most often made clinically in neonates and is supported by Doppler ultrasound findings of intrarenal vascular resistive indices; however, in some cases a discrete thrombus may be suggested by Doppler ultrasound, especially when extending into the inferior vena cava. When RVT occurs in older children, Doppler ultrasound or CT is often diagnostic. Similarly, portal vein thrombosis is typically visualized by Doppler ultrasound or CT.

When new-onset venous thrombosis is being evaluated in patients in areas of anatomic abnormality of the venous system (e.g., extensive collateral venous circulation caused by a prior VTE episode, May-Thurner anomaly, atretic inferior vena cava with azygous continuation), more sensitive methods such as CT or magnetic resonance venography are often required to adequately delineate the vascular anatomy as well as the presence, extent, and occlusiveness of thrombosis.

Diagnostic laboratory evaluation for pediatric acute VTE includes a complete blood count, evaluation for thrombophilia (see "Etiology"), and beta-human chorionic gonadotropin in postmenarchal women. Additional laboratory studies may be warranted depending on associated medical conditions and VTE involvement of specific organ systems.

Treatment

A summary of conventional antithrombotic agents and corresponding target anticoagulant levels, based on recent pediatric recommendations,[139] is provided in Table 35-2 for both initial (i.e., acute phase) and "extended" (i.e., subacute phase) treatment. Conventional anticoagulants attenuate hypercoagulability, decreasing the risk of thrombus progression and embolism, and rely on intrinsic fibrinolytic mechanisms to break up the thrombus over time. The most commonly employed conventional anticoagulants in children include heparins and warfarin. Heparins, including unfractionated heparin and low-molecular-weight heparin (LMWH), enhance the activity of AT, an intrinsic anticoagulant that serves as a key inhibitor of thrombin. Warfarin acts through antagonism of VK, thereby interfering with γ-carboxylation of the VK-dependent procoagulant factors II, VII, IX, and X.

TABLE 35-2 Recommended Intensities and Durations of Conventional Antithrombotic Therapies in Children, by Etiology and Treatment Agent

	Agents and Target Anticoagulant Activities		
Episode	Initial Treatment	Extended Treatment	**Duration of Therapy, by Etiology**
First	UFH 0.3-0.7 anti-Xa U/mL LMWH 0.5-1.0 anti-Xa U/mL	Warfarin INR 2.0-3.0 LMWH 0.5-1.0 anti-Xa U/mL	Resolved risk factor: 3 months No known clinical risk factor: 6-12 months Chronic clinical risk factor: 12 months Potent congenital thrombophilia: indefinite
Recurrent	UFH 0.3-0.7 anti-Xa U/mL LMWH 0.5-1.0 anti-Xa U/mL	Warfarin INR 2.0-3.0 LMWH 0.5-1.0 anti-Xa U/mL	Resolved risk factor: 6-12 months No known clinical risk factor: 12 months Chronic clinical risk factor: indefinite Potent congenital thrombophilia: indefinite

INR, International normalized ratio; *LMWH,* low-molecular-weight heparin; *UFH,* unfractionated heparin.

Initial Anticoagulation. Initial anticoagulant therapy (i.e., acute phase) for VTE in children generally uses unfractionated or LMWH. LMWH has become increasingly employed as a first-line agent for initial anticoagulant therapy in children, given the relative ease of subcutaneous over intravenous administration, the decreased need for blood monitoring of anticoagulant efficacy, and a decreased risk of the development of heparin-induced thrombocytopenia (HIT). Unfractionated heparin is typically preferred in circumstances of heightened bleeding risk or labile acute clinical status, given the rapid extinction of anticoagulant effect following cessation of the drug. In addition, unfractionated heparin is used for acute VTE therapy in renal insufficiency, given the renal excretion of LMWH. Common initial maintenance dosing for unfractionated heparin in nonneonatal children ranges from 15 to 25 U/kg/h preceded by a loading dose of 50 to 75 U/kg. In full-term neonates a maintenance dose of up to 50 U/kg/h may be required, especially if the clinical condition is complicated by AT consumption. The starting dose for the LMWH enoxaparin in nonneonatal children commonly ranges between 1 and 1.25 mg/kg; no bolus dose is given. In full-term neonates an enoxaparin starting dose of 1.5 mg/kg is typically necessary.[140] For the LMWH dalteparin, initial maintenance dosing of 100 to 150 anti–factor Xa U/kg) appears appropriate based on available pediatric data.[141] Heparin therapy is monitored most accurately by anti–factor Xa activity. For unfractionated heparin the therapeutic range is 0.3 to 0.7 anti–factor Xa activity U/mL, whereas for LMWH the therapeutic range is 0.5 to 1 U/mL. When anti–factor Xa assay is not available, the activated partial thromboplastin time (aPTT) may be used, but it is suboptimal in the pediatric age group, in which antiphospholipid antibodies may interfere with or artifactually prolong the clotting endpoint; indeed, one study of pediatric heparin monitoring demonstrated inaccuracy of aPTT approximately 30% of the time.[142] When dosed by weight in childhood, LMWH does not require frequent monitoring, but anti–factor Xa activity should be evaluated with changes in renal function. In addition, in cases of acute VTE in which acquired AT deficiency is related to consumption in acute infection or inflammation, anti–factor Xa activity may rise as AT levels normalize with resolution of the acute illness; in this circumstance follow-up evaluation of anti–factor Xa activity is warranted in the subacute period. The recommended duration of initial heparin therapy for acute VTE, 5 to 10 days, has been extrapolated from adult data.[143] Unfractionated heparin treatment is rarely maintained beyond the acute period, given the risk of osteoporosis with extended administration[139] and the inconvenience of continuous intravenous administration. Although adult data suggest efficacy of subcutaneous administration of unfractionated heparin for acute VTE,[144] this has been evaluated only for the acute therapy period before extended therapy with warfarin, and the appropriateness of such an approach in children has not been established.

Extended Anticoagulation. "Extended" anticoagulant therapy (i.e., subacute phase) for VTE in children may employ LMWH or warfarin. Warfarin may be started during the acute phase; however, because severe congenital deficiencies involving the protein C pathway can present as VTE in early childhood and are associated with warfarin skin necrosis,[145] warfarinization should ideally be initiated only after therapeutic anticoagulation is achieved with heparin-based treatment. Warfarin is monitored by INR, derived from the measured PT. The therapeutic INR range for warfarin anticoagulation in VTE is 2 to 3.[139] Historical evidence for maintaining a higher INR (2.5 to 3.5) in the presence of APA is not supported by data from more recent studies[146,147]; however, maintaining an INR of 2.5 to 3, when feasible, satisfies both older and more recent literature criteria with regard to appropriate safety and efficacy in this subset of patients.

Duration of Therapy. Pediatric recommendations for the duration of antithrombotic therapy in acute VTE[139] are largely derived from evidence from adult trials. For a provoked first episode of VTE in children, the recommended duration of anticoagulant therapy is 3 months, as long as the provoking risk factor does not persist beyond 3 months (e.g., postoperative VTE), and—in the authors' view—absent a potent chronic thrombophilia state (e.g., APA syndrome, homozygous anticoagulant deficiency). The recommended duration is 6 to 12 months when spontaneous and 12 months to lifelong when a chronic risk factor persists (e.g., systemic lupus erythematosus). Recurrent VTE is treated for 6 to 12 months in the presence of an underlying reversible risk factor, 12 months to lifelong when spontaneous, and lifelong when a chronic risk factor persists. Children with SLE and persistence of an LA have a 16- to 25-fold greater risk of TEs than children with SLE and no LA.[148] However, in children with primary (i.e., idiopathic) or secondary (i.e., associated with SLE or other underlying chronic inflammatory condition) VTE, it is possible that the autoimmune disease will become quiescent in later years, such that the benefit of continued VTE prophylaxis may be reevaluated. Some experts have recommended consideration of low-dose anticoagulation as secondary VTE prophylaxis after a conventional 3- to 6-month course of therapeutic anticoagulation for VTE in children with SLE who have APA syndrome.[149] However, further study to optimize the intensity and duration of therapy or secondary prophylaxis for VTE in children with APA syndrome is urgently needed, especially in light of evidence in adult provoked VTE that secondary prophylaxis with low-dose warfarin not only may offer little risk reduction beyond no anticoagulation but also is associated with bleeding complications despite a reduced warfarin dose.[150,151]

Thrombolytics. Whereas the aforementioned anticoagulant therapies are conventional in pediatric acute VTE, thrombolytic approaches are gaining increased attention and use during acute VTE therapy in children with hemodynamically significant PE or extensive limb-threatening VTE. Unlike conventional anticoagulants, thrombolytics directly promote fibrinolysis. Tissue-type plasminogen activator is an intrinsic activator of the fibrinolytic system and has been administered exogenously as a systemic bolus or short-duration infusion, a systemic low-dose continuous infusion, or a local catheter-directed infusion with or without interventional mechanical thrombectomy or thrombolysis. A few small cohort study analyses of children with acute proximal limb DVT who had an a priori high risk of poor post-thrombotic syndrome by virtue of completely veno-occlusive thrombus with plasma FVIII activity greater than 150 U/dL or D-dimer concentration greater than 500 ng/mL have revealed that a thrombolytic regimen followed by standard anticoagulation offers potential reduction in the risk of post-thrombotic syndrome when compared (either concomitantly or historically) to standard anticoagulation alone.[152,153] A National Institutes of Health–sponsored planning grant was begun in 2012 for a future randomized controlled trial on the efficacy and safety of thrombolysis versus standard anticoagulation for acute completely occlusive proximal lower-extremity DVT in children (Goldenberg and Manco-Johnson, personal communication).

Other Anticoagulants. Other antithrombotic agents include fondaparinux, intravenous and oral direct thrombin inhibitors, and oral direct factor Xa inhibitors. Fondaparinux is a parenteral selective factor Xa inhibitor. It is a synthetic pentasaccharide that rapidly binds to AT in the blood, potentiating the natural inhibitory effect of AT against factor Xa, thus indirectly inhibiting thrombin. Fondaparinux is typically administered once daily as a subcutaneous injection. Dosing in children has been informed by a pediatric pharmokinetic and safety study.[154] Direct thrombin inhibitors, by contrast, inhibit thrombin directly by binding to exosite I or the active site of thrombin (or both).[155] These drugs include hirudin and recombinant hirudins such as lepirudin. Argatroban is a synthetic small molecule derived from L-arginine and functions as a reversible direct thrombin inhibitor. Intravenous direct thrombin inhibitors are routinely monitored by aPTT, with the therapeutic goal ranging from a 1.5- to 3.0-fold aPTT prolongation. At present, intravenous direct thrombin inhibitors are indicated for the treatment of HIT, particularly when associated with acute thrombosis (HITT). Indeed, this is the setting in which pharmacokinetic studies of argatroban have been conducted.[156] However, pediatric use of intravenous direct thrombin inhibitors has also been reported in settings of catastrophic APA syndrome and thrombotic storm.[157]

Oral direct thrombin inhibitors and oral direct factor Xa inhibitors have been developed as a possible alternative to warfarin. They have the advantage of oral bioavailability, low intersubject variation, no metabolism by the hepatic cytochrome P450 system, a low incidence of drug-drug interactions, and no dietary influence. Early-phase pediatric clinical trials in pediatric VTE prevention or treatment with the oral direct thrombin inhibitor dabigatran and the oral direct factor Xa inhibitors apixaban and rivaroxaban were all initiated between 2011 and 2012, after regulatory agency approvals for the corresponding adult indications.[158]

Other Therapeutic Agents. Other products may have antithrombotic roles in selected circumstances but lack definitive demonstration of efficacy and safety in clinical trials. For example, plasma replacement with protein C concentrate is a useful adjunctive therapy for VTE or purpura fulminans owing to microvascular thrombosis in severe congenital protein C deficiency[159-161] and may play a beneficial role in the treatment of purpura fulminans owing to microvascular thrombosis in children with sepsis, particularly in meningococcemia.[162-164] In addition, case series have suggested a role for AT replacement in prevention of VTE in children and young adults with congenital severe AT deficiency,[165] for the prevention of L-asparaginase–associated VTE in pediatric acute lymphoblastic leukemia (ALL),[166,167] and as combination therapy with defibrotide in the prevention and treatment of hepatic sinusoidal obstruction syndrome (formerly termed *veno-occlusive disease*) in children undergoing hematopoietic stem cell transplanation.[168] The potential benefit toward VTE risk reduction of a regimen of AT replacement combined with daily prophylactic LMWH during induction and consolidation phases of therapy in ALL has also been suggested by a historically controlled cohort study of the BFM 2000 protocol experience in Europe.[169] AT replacement may also be worthy of consideration in patients with acute VTE undergoing heparinization in whom significant AT deficiency prevents the achievement of therapeutic anti–factor Xa levels (i.e., heparin "resistance"). This may be seen in neonates with clinical conditions complicated by AT consumption superimposed on the physiologic relative deficiency of this key intrinsic thrombin inhibitor in the newborn period. However, AT replacement in preterm neonates has been investigated to improve the outcomes of respiratory distress syndrome, intracranial hemorrhage, and sepsis but has failed to demonstrate benefit and may contribute to adverse events.[170]

Vena Cava Filters. In certain circumstances vena cava filters may serve an adjunctive role in antithrombotic management in children. The use of vena cava filters should be considered in children of appropriate size in whom contraindications to anticoagulation exist or in whom recurrent VTE (especially PE) has occurred despite therapeutic anticoagulation and in the absence of a reversible risk factor. In addition, "temporary" or retrievable vena cava filters may be considered during times of especially heightened risk for PE or at times when therapeutic anticoagulation is transiently considered to engender a heightened risk of hemorrhagic complications (e.g., recent neurosurgery). The presence of the filter alone is not an indication for long-term anticoagulation because the risk for vena caval thrombosis related to the device is low. Long-term outcomes in adult series have shown a failure rate (clinically relevant, symptomatic PE) of 3.3% to 5.6%.[171-173] Major complications were infrequent (<1%) and include rare cases of caudal migration, filter fractures, or caval wall perforation.[173] A retrospective review of 15 children 18 years of age or younger suggested efficacy of long-standing vena cava filters with regard to primary and secondary prevention of PE.[174] Follow-up was a mean of 9.2 years (range 19 months to 16 years), and there were no significant complications, complementing similar findings in other small case series.[175,176] However, until the impact of such nonretrievable devices on the vena cava of the developing child has been better studied, their use in preadolescents should be undertaken with caution. Experience with surgical removal of retrievable vena cava filters is quite limited in children with unknown risks and costs of extraction. Surgical removal of permanent vena cava filters should be strongly discouraged because this often requires vena caval ligation.[177]

Outcomes

Complications of VTEs can occur both acutely and over the long term and are germane to both CRT and non-CRT. Short-term adverse outcomes include major hemorrhagic complications of antithrombotic interventions, as well as of the thrombotic event itself (e.g., post-thrombotic hemorrhage in the brain or adrenal gland); thrombus progression (involvement of previously unaffected contiguous venous segments, or change from nonocclusive to occlusive); early recurrent VTE (including DVT and PE[178]); SVC syndrome in DVT of the upper venous system[178,179]; acute renal insufficiency in RVT[180]; catheter-related sepsis,[181] PE,[182,183] and catheter malfunction (sometimes necessitating surgical replacement) in CRT; severe acute venous insufficiency leading to venous infarction with limb gangrene in rare cases of occlusive DVT involving the extremities; and death from hemodynamic instability in extensive intracardiac thrombosis or proximal PE.[112] Long-term adverse outcomes in pediatric VTE have been previously reviewed[184] and include recurrent VTE; chronic hypertension and renal insufficiency in RVT; variceal hemorrhage in portal vein thrombosis[112]; chronic SVC syndrome in CRT involving SVC occlusion; loss of availability for venous access in recurrent or extensive CRT of the upper venous system; and development of PTS, a condition of chronic venous insufficiency following DVT. The manifestations of PTS include (in order of increasing severity) edema, visibly dilated superficial collateral veins, venous stasis dermatitis, and venous stasis ulcers (Fig. 35-2). Given the long-term and sometimes lifelong risks of disease sequelae as in RVT and functional impairment as in PTS, venous thrombosis is best considered a chronic disorder in children.

Long-Term Outcomes. The long-term outcomes of VTE in children, specifically with regard to recurrent VTE, PTS, major hemorrhage, and death, are summarized in Table 35-3. Registry[112,119,185] and cohort study[118] data in pediatric VTE of all types indicate that children appear

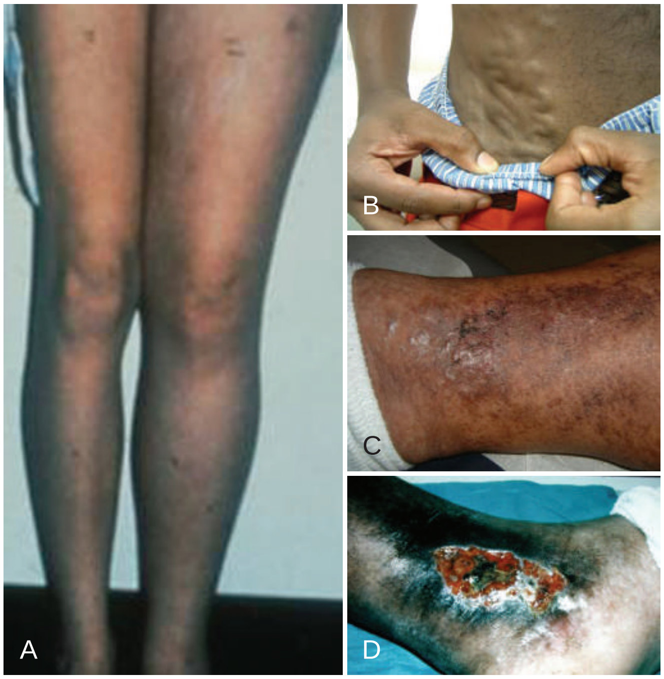

Figure 35-2 Manifestations of post-thrombotic syndrome in children. **A,** Stage 1: chronic edema with iliofemoral deep venous thrombosis *(DVT)* at 16 years of age. The patient was heterozygous for factor V Leiden and was taking oral contraceptive pills. **B,** Stage 2: dilated superficial venous collateral circulatioin in a 14-year-old adolescent with inferior vena cava *(IVC)* DVT as a result of antiphospolipid antibody *(APA)* and prolonged travel. **C,** Stage 3: chronic venous stasis dermatitis in a 13-year-old with IVC DVT who was heterozygous for factor V Leiden and exhibited APA. **D,** Stage 4: chronic ulceration in a 10-year-old with femoral popliteal DVT, homozygous protein C deficiency, and a tibial fracture.

TABLE 35-3 Outcomes of Venous Thromboembolism in Children

Author	Year of Publication	Country	# VTE Cases	Mean/Median Duration of Follow-up	Major Bleeding	Recurrent VTE	PTS	VTE-Related Mortality
Monagle et al.[119]	2000	Canada	405	3 years	N/A	8%	12%	2%
Nowak-Göttl et al.[188]	2001	Germany	301	7 years	N/A	21%	N/A	0%
Massicotte et al.[189]	2003	Canada	76	6 months	9%	9%	N/A	0%
Van Ommen et al.[185]	2003	Netherlands	100	4 years	N/A	9% (3 years)	70%	1%
Ören et al.[190]	2004	Turkey	186	N/A	N/A	9%	N/A	2%
Goldenberg et al.[118]	2004	U.S.A.	74	1 year	0%	6%	33%	0%

N/A, Not available; *PTS,* post-thrombotic syndrome; *VTE,* venous thromboembolism.

to have a lower risk of recurrent TE than adults (cumulative incidences at 1 to 2 years of 6% to 11% versus 12% to 22%,[186,187] respectively). However, the risk of PTS in children with DVT of the limbs appears to be at least as great as that in adults (cumulative incidences at 1 to 2 years of 33% to 70%[113,118] versus 29%, respectively[187]). In addition, in a German cohort study of children with spontaneous VTE[188] (i.e., VTE in the absence of identified clinical risk factors), the cumulative incidence of VTE at a median follow-up time of 7 years was 21%, suggesting that, in this subgroup of pediatric VTE, the risk for recurrent VTE is long-lived. Although VTE-specific mortality in children is quite low, ranging from 0% to 2% in these and other[189,190] studies, a considerably higher all-cause mortality indicates the severity of underlying conditions (e.g., sepsis, cancer, congenital cardiac disease) in pediatric VTE. Neonate-specific outcome data in pediatric non-RVT VTE reflect an all-cause mortality of 12% to 18%,[116,117,191] including one series of premature infants with CRT treated with enoxaparin.[191] Bleeding (including all severities) occurs in approximately 20% of children treated with a therapeutic course of oral VK antagonists and 17% receiving LMWH; so-called "major" hemorrhage occurs in 2% to 4%.[192]

Outcomes by Anatomic Site. Outcomes of VTE in children may differ among specific anatomic sites and organ systems affected, as well as in relation to the presence or absence of a central venous catheter. Analyses devoted specifically to CRT in children are limited; however, given the high prevalence of CRT in registries and cohort studies of pediatric VTE, outcomes of CRT are likely to considerably influence the data discussed previously for VTE in general. In a Canadian study of CRT in children from 1990 to 1996,[183] VTE-specific mortality was 4%—and 20% among PE cases. No major bleeding episodes were observed. At a median follow-up duration of 2 years, the cumulative incidence of symptomatic recurrent VTE was 6.5%, and PTS developed in 9% of children. In published series of RVT[130,180,193-195] (primarily among neonates), VTE-related death has been very rare, and the cumulative incidence of recurrent VTE has ranged from 0% to 4%, albeit with wide variation in duration of follow-up across studies. The cumulative incidence of chronic hypertension in RVT in these studies was reported at 22% to 33%. The pediatric literature devoted to CSVT[196,197,198,199] is somewhat smaller than that for RVT and suggests a broad range of VTE-specific mortality of 4% to 20%, with a cumulative incidence of recurrent VTE of 8% for neonatal CSVT cases and 17% for CSVT occurring in older children.[198] Long-term neurologic sequelae were noted in 17% to 26% of CSVT cases diagnosed in the neonatal period and 8% to 47% of those occurring later in childhood. It is worth noting that in the aforementioned pediatric series of RVT and CSVT, both the proportion of children who received anticoagulation and the duration of the anticoagulation course varied considerably across studies. Few pediatric series reporting outcomes of portal vein thrombosis have been published; however, it appears that the risk of developing recurrent gastrovariceal bleeding in this population is substantial, often despite surgical interventions to reduce portal hypertension.[200] With regard to long-term outcomes of PE in childhood, in a recent single-institutional cohort study[201] of 58 patients with acute PE treated with conventional anticoagulation (nearly half of whom had hypoxemia at presentation, and in which signs of right ventricular dysfunction of triscuspid regurgitant velocity >3 m/s, septal flattening, and right ventricular dilation were each present acutely on echocardiogram in 25%), there were no PE-related deaths and the cumulative incidence of recurrent VTE in long-term follow-up was 9%. The prevalence of persistent PE at 6 months follow-up was 18%, and on follow-up echocardiogram at a median time of approximately 1.5 years, signs of right ventricular dysfunction of triscupid regurgitant velocity greater than 3 m/s, septal flattening, and right ventricular dilation were observed in 0%, 13%, and 13%, respectively. It is noteworthy that 26% of the cohort had received acute thrombolytic therapy with tPA.

An additional outcome of interest to patients and families of children with VTE is persistence versus resolution of thrombosis. Data to date, largely from adult VTE populations, suggest that the persistence of thrombosis after a therapeutic course of anticoagulation of appropriate duration does not appreciably increase the risk for recurrent VTE, including PE. However, some evidence[202] indicates that persistent thrombosis is associated with the development of venous valvular insufficiency, an important risk factor for the development (albeit an imperfect correlate[203]) of PTS. The prevalence of thrombus persistence despite adequate anticoagulation in neonatal VTE has ranged from 12% in premature newborns with CRT[183] (representing one of eight patients with VTE, in whom thrombus extension led to progressive SVC syndrome and death) to 62% in full-term neonatal VTE survivors.[119] Among primarily older children, the prevalences of persistent thrombosis have ranged broadly from 37% to 68% in the few longitudinal studies to date that have systematically evaluated thrombus evolution radiologically.[118,141]

Prognostic Indicators. The ability to predict clinically relevant long-term outcomes of VTE at diagnosis and during the acute phase of treatment is essential to a future risk-stratified approach to antithrombotic management in children. Historically, few prognostic indicators have been defined in pediatric VTE. The presence of inherited anticoagulant deficiencies in children with incident VTE has been associated with an increased risk of recurrent VTE.[204] The presence of multiple thrombophilia traits in children with unprovoked (i.e., spontaneous) VTE has been associated with an increased risk of recurrent VTE.[188] In addition, the radiologic finding of complete veno-occlusion at diagnosis of DVT has been associated with an increased risk of persistent thrombosis[205] (which in turn has been associated with the development of venous valvular insufficiency,[203] as noted previously). Plasma FVIII activity greater than 150 U/dL and D-dimer concentration greater than 500 ng/mL at the time of diagnosis of VTE in children, as well as after 3 to 6 months of standard anticoagulation, have been defined as markers of a composite adverse thrombotic outcome, characterized by persistent thrombosis, recurrent VTE, and the development of PTS[118]; similar prognostic findings have been observed in adult VTE.[206-208] Other factors suggested to be associated or possibly associated with development of post-thrombotic syndrome, using a standardized and validated pediatric instrument in recent studies, include an acutely positive dilute Russell viper venom test for the LA in patients treated with conventional anticoagulation,[209] and in mechanically thrombolysed patients have included the diagnosis of May-Thurner or Paget-Schroetter syndrome and a lag time from symptom onset to lytic intervention exceeding 10 days.[153]

Arterial Ischemic Stroke

Epidemiology

The incidence of perinatal and neonatal arterial ischemic stroke (AIS) has been estimated from the U.S. National Hospital Discharge Survey at approximately 18 per 100,000 per year, or 1 in 5000 live births annually.[210] The rate of stroke in later childhood is approximately one tenth of that for the neonatal period, at approximately 2 in 100,000 children per year in the U.S.[211] Similar estimates have been provided by the Canadian and Swiss pediatric stroke registries.[212,213] However, just as for VTE in children, these epidemiologic data for pediatric AIS likely represent underestimates because of the relatively low index of suspicion for cardiovascular and cerebrovascular events in children compared with adults, as well as because of inaccuracies engendered by International Classification of Diseases, 9th revision (ICD-9) coding.[214]

Etiology

The causes of AIS in children are less well characterized than in adults, largely because of the challenges posed by the lower incidence of the disorder in the pediatric population. Whereas hypertension, diabetes, smoking, and hypercholesterolemia are prominent cerebrovascular disease risk factors in adults, in children sickle cell disease, infection, cerebral arteriopathy,[215,216,217] head and neck trauma (with or without identified arterial dissection),[218-220] congenital cardiac disease, previous transient ischemic attack,[221] and thrombophilia[221-225] appear to be the most prevalent identified risk factors for AIS. Whether anemia and polycythemia are independent risk factors for pediatric AIS, or instead constitute epiphenomena of underlying acute and chronic diseases, remains unclear. The subsequent discussion focuses principally on AIS in children without sickle cell disease; for a detailed discussion of AIS in children with sickle cell disease, see Chapter 20.

Arteriopathy in pediatric AIS may be characterized by arterial dissection, narrowing, thrombosis, and irregular arterial contour (suggestive of possible arteritis). Overall, arteriopathy may be identified in as many as one half of non-neonatal AIS cases.[226] Dissection has been demonstrated in 7% to 20% of children with acute AIS.[221,226] The distinction between dissective and nondissective causes of arterial narrowing and thrombosis may be difficult, with variables including the imaging technology employed (e.g., CT with angiography, magnetic resonance angiography, cerebral angiography), the timing in relation to the event, and the experience of the neuroradiologist. Dissection may be caused by head or neck trauma or may be spontaneous; limited data suggest that the prevalence of underlying connective tissue disorders may be increased among AIS cases that are associated with spontaneous dissection, although further controlled studies are needed.[227] Although some studies have indicated that antecedent head trauma appears to be prevalent among pediatric AIS cases, minor head trauma is not infrequent in active children, and there is also considerable potential for recall bias in such studies. A minority of cases of childhood AIS with arteriopathy represent moyamoya syndrome (defined as stenosis in the terminal portion of the internal carotid arteries bilaterally with the formation of collateral arteries, producing the classic angiographic “puff of smoke”) or possible moyamoya syndrome (characterized as either unilateral stenosis in the terminal segment of an internal carotid artery with collaterals or the presence of bilateral stenosis of the terminal portion of the internal carotid arteries without collaterals[217]). Other causes of nondissective cerebral arteriopathy in children include a history of recent varicella zoster infection within the previous year,[198] neurofibromatosis type 1, and cranial irradiation for malignancy.[217,228] Despite the high prevalence of arteriopathy in AIS, its etiology in most cases remains unclear and not attributable to moyamoya syndrome, dissection, or defined infections such as varicella. Efforts to further define the etiologies and natural history of arteriopathic AIS are ongoing through the International Pediatric Stroke Study (IPSS) group.

Infection as an underlying cause for pediatric AIS has been classically associated with tuberculous meningitis, human immunodeficiency virus infection, and primary herpes simplex and varicella virus infection as well as secondary herpes zoster and varicella zoster infections. Local bacterial and fungal infections of the head and neck may also contribute to the etiology of AIS in some pediatric patients. The pathophysiologic mechanisms of infection-associated AIS in children may include direct angioinvasion by the pathogen, local arteritis without direct angioinvasion, and systemic inflammatory and hypercoagulative responses. It is likely that in many instances more than one process contributes to the genesis of AIS. Based on the challenges of etiologic classification in childhood-onset AIS, Bernard and colleagues in the IPSS group have proposed an anatomic-based classification system, the CASCASE criteria, which largely reflect the presence or absence of cardiac abnormalities on echocardiography or ischemic involvement of multiple arterial territories on neuroimaging in support of cardioembolism, as well as the distribution of any aortic, cervical, or cerebral arterial abnormalities in support of dissection, moyamoya syndrome, and the unilateral focal cerebral arteriopathy so frequently observed in childhood-onset AIS.[229]

Thrombophilia Risk Factors

Thrombophilia may contribute to AIS risk via arterial thrombosis or by cerebral embolism of a venous thrombus by way of a cardiac lesion with right-to-left shunt. Thrombophilia risk factors in pediatric AIS include anticoagulant deficiencies (particularly deficiency of protein C[222]), elevated plasma levels of lipoprotein(a),[230] and hyperhomocysteinemia.[231,232] In some cases of AIS following acute varicella infection, antibody-mediated acquired protein S deficiency (i.e., anti–protein S antiphospholipid antibody) has been demonstrated.[233] It is likely that, as is the case for VTE in children, anticoagulant deficiencies are most commonly acquired in the setting of pediatric AIS and are most often parainfectious. However, as in VTE, severe congenital anticoagulant deficiencies associated with AIS may be most likely to present at an early age. Markedly elevated levels of homocysteine are classically associated with metabolic disorders such as homocysteinuria (caused by cystathionine β-synthase deficiency), and appears to be rare among individuals possessing the thermolabile variant of methylenetetrahydrofolate reductase (MTHFR C677T) in countries in which routine folate supplementation of the diet is undertaken. Therefore most evidence suggests that this highly prevalent polymorphism is not an independent risk factor for AIS. Although a single-institutional U.S. study[234] may contradict this evidence, no contemporaneous control group was employed, and further study is necessary. It remains possible that an alternative mechanism to hyperhomocysteinemia may mediate the AIS risk, if substantiated. With regard to lipoprotein(a), a recent U.S. study failed to substantiate the meta-analysis findings that were largely derived from German cohorts but did find elevated lipoprotein(a) as well as small apo(a) isoform size to be risk factors for recurrence of childhood-onset AIS.[235]

Clinical Presentation

The clinical presentation of AIS in neonates and older children largely depends on the territory and extent of the CNS affected by ischemia or infarct. The most common presentation for perinatal and neonatal AIS is seizure, sometimes associated with subtle hemiparesis when AIS involves a unilateral middle cerebral artery (MCA) distribution. Apnea and poor feeding in a term infant should also raise concern for possible perinatal or neonatal AIS. Prenatal MCA-distribution AIS, by contrast, is frequently asymptomatic until noted by parents as one-sided weakness, spasticity, early handedness, or disuse as development of motor activity progresses, frequently with asymmetric gross motor delay between 4 and 12 months of age.

The presentations of acute AIS occurring after the neonatal period (i.e., childhood-onset AIS) include acute- or subacute-onset focal neurologic deficit (e.g., hemiparesis, cranial nerve palsy, dysphasia, incoordination), severe headache, or unexplained new-onset seizure, sometimes accompanied by altered mental status. In cases of dissection-associated AIS in children, a recent history of head or neck trauma is common (see also "Etiology," in this section). Interestingly, a single-center retrospective series of AIS in non-neonatal children found that a "non-abrupt" pattern of neurologic symptoms and signs (including those in whom the maximum severity of symptoms and signs in acute AIS developed more than 30 minutes from the time of symptom onset, the presentation of symptoms and signs was waxing and waning or was preceded by recurrent transient symptoms and signs with intercurrent resolution) was significantly associated with findings of arteriopathy on diagnostic neuroimaging.[236] Ischemic stroke that is not clearly arterial in distribution may result from global hypoxic-ischemic injury (e.g., with watershed distribution), a sequela of CSVT (e.g., symmetrical parasaggital infarction), or metabolic in etiology. A classic example of ischemic stroke of metabolic etiology occurs in mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes (MELAS). MELAS is caused by mitochondrial DNA defects encoding tRNA involved in the generation of ATP via the electron transport chain in the mitochondrion. The clinical presentation of MELAS is ischemic stroke, often recurrent in nature, in the preschool or school-aged child. Imaging most often reveals persistent ischemia or infarct in the posterior cerebral hemispheres, with relative sparing of deep white matter; the affected territory often results in hemianopia.[237] Unlike the cerebral lesions in acute AIS, acute abnormalities at the time of stroke in MELAS are bright on apparent diffusion coefficient (ADC) maps of diffusion-weighted MRI.[238] Associated symptoms and signs in MELAS, which are of varying severity and penetrance in affected individuals owing to threshold effect and heteroplasmy in mitochondrial DNA disorders,[239] include short stature, hearing loss, recurrent seizure, proximal muscle weakness, and optic atrophy.[237]

Diagnosis

Imaging Studies. The diagnostic evaluation for suspected acute AIS in children involves either urgent CT of the head with CT angiogram (CTA) of the head and neck or, alternatively, MRI of the brain with diffusion-weighted imaging and magnetic resonance angiography (MRA) of the head and neck. Whereas routine CT and MRI evaluates for ischemia, hemorrhage, mass/mass effect, and other non-AIS pathologies, the addition of angiography permits evaluation for arteriopathy, including dissection, stenosis, irregular contour, or intraarterial thrombosis. CT is often more readily available on an urgent basis than MRI; however, CT engenders greater radiation exposure than MRI (especially if angiography is performed) and is less sensitive for the diagnosis of AIS, especially in the acute phase of ischemia. For the latter reason, if CT is performed initially and is unremarkable in the setting of an acute focal neurologic deficit of unclear etiology, this imaging should be followed by MRI of the brain with diffusion-weighted imaging and MRA of the head and neck. In circumstances in which the presence or extent of arteriopathy is unclear by MRA and/or CTA, or in cases of moyamoya syndrome, the more invasive approach of cerebral angiography is warranted to establish a definitive diagnosis and guide subsequent management.

The pattern of ischemia and infarction may be informative with respect to etiology and consequent management of AIS. For example, multifocal infarction may suggest a thromboembolic etiology of AIS. Subcortical distribution of ischemia and infarct may suggest small arterial disease. Watershed distribution of ischemia and infarct may suggest an alternative diagnosis of hypoxic-ischemic injury related to hypotension and other global causes of poor perfusion.

An additional important component to diagnostic imaging in pediatric AIS is echocardiography (either transthoracic with peripheral intravenous saline injection or transesophageal) to evaluate for right-to-left shunting cardiac lesion, with potential implications for management.

Laboratory Studies. Diagnostic laboratory evaluation in pediatric acute AIS involves a complete blood count, toxicology screen, complete metabolic panel, erythrocyte sedimentation rate/C-reactive protein, β–human chorionic gonadotropin testing in post-menarchal females, fasting lipid profile, and thrombophilia assessment (see the section on VTE diagnosis in this chapter). When APA results are positive in the neonate with AIS, it is informative to evaluate for APA in the mother to establish a possible etiology of vertical (i.e., transplacental) transmission of immunoglobulin G APA. In childhood-onset AIS with the common finding of unilateral focal cerebral arteriopathy, consideration should be given to infectious disease investigation, including serology for such viral infections as varicella-zoster virus, herpes simplex virus, Epstein-Barr virus, parvovirus, and enterovirus; a thorough workup may also include enteroviral cultures of the mouth and rectum and blood testing for *Helicobacter pylori*. If there is associated meningitis or in the presence of relevant clinical risk factors, tuberculosis testing should also be undertaken. In the setting of arteritis, rheumatologic evaluation should be considered and should include testing of antinuclear antibodies and other markers as warranted. In certain circumstances additional studies may be warranted, including evaluation for metabolic (including mitochondrial DNA-based) disorders, particularly in AIS with encephalopathy of unclear etiology, nonarterial distribution, lactic acidosis, or other multisystem disorders of unclear etiology (e.g., hearing loss, myalgia, endocrinopathy).

The role of lumbar puncture is controversial, especially when weighed against the need to withhold anticoagulation periprocedurally. Unfortunately, the circumstances in which risk for AIS progression or early recurrence is among the greatest (e.g., acute or subacute period of AIS with vasculopathy), and therefore in which maintenance of effective anticoagulation may be most critical, is also the same circumstances in which lumbar puncture for infectious disease investigation would be among the most informative (yet requires suspension of anticoagulation).

Treatment

Initial management of AIS in children must emphasize appropriate control of blood pressure, temperature, glycemia, oxygenation, and hydration. Unfortunately, there is no evidence from clinical trials on which to base specific interventions for acute AIS in children who do not have sickle cell disease. Nevertheless, pediatric AIS treatment recommendations[139,240] have been developed on the basis of registry and cohort study data in neonates and older children, as well as to some extent on evidence extrapolated from studies in adult AIS. Acute antithrombotic therapy use and regimens in childhood-onset AIS are highly variable worldwide and within the United States.[241]

In the absence of suspected cardioembolism or potent thrombophilia, antithrombotic therapy is rarely employed in perinatal and neonatal AIS, as appropriate to the low risk (cumulative incidence of approximately 3% at a median follow-up duration of 3.5 years[242]) of recurrent AIS or VTE. Therefore for most cases of perinatal AIS advancing the knowledge of acute and subacute neuroprotective interventions is likely to be more beneficial than employing therapeutic strategies aimed at risk reduction of recurrent AIS.

In contrast to neonates, antithrombotic intervention is generally recommended as secondary AIS prophylaxis in older children with acute AIS, given a relatively high recurrence rate (cumulative incidence of approximately 40% at 5 years[221]). A nonrandomized prospective cohort-based study of LMWH versus aspirin as secondary AIS prophylaxis detected no significant difference in the cumulative incidence of recurrent AIS between these two therapies.[243] The optimal antithrombotic regimen and duration for a given CASCADE subtype of childhood-onset AIS remain unclear in the absence of evidence from randomized controlled trials. Antiplatelet therapy with daily aspirin is widely used in childhood AIS, with variable intensity (e.g., 1 to 5 mg/kg per dose) and duration (3 months, 1 year, indefinitely). Patients who experience recurrent AIS while on antiplatelet therapy should be considered for therapeutic anticoagulation.

Pediatric AIS with moyamoya syndrome presents a particular challenge to optimal management. Anticoagulation and antiplatelet agents have been considered as possible therapies. However, given heightened risks for both intracerebral hemorrhage and recurrent AIS, neurosurgical approaches at revascularization (including indirect means, such as encephaloduroarteriomyosyangiosis [EDAMS],[244] and direct means, such as superficial temporal artery branch-to-middle cerebral artery branch [STA-MCA] and anterior cerebral artery branch [ACA] bypass[245]) are generally preferred, and anticoagulation or antiplatelet therapy is perhaps best reserved for children with appropriately defined "possible moyamoya" in the absence of recurrent symptoms or recurrent AIS- or transient ischemic attack–related events.

Thrombolytic approaches have been employed in isolated cases of pediatric AIS.[246] Because only a minute proportion of cases of acute AIS in children are diagnosed within the first few hours of symptomatic onset, a multicenter collaborative clinical trial approach with stringent uniform exclusion criteria will be required to address whether adult evidence for a beneficial role of systemic tPA administration in the immediate period after onset of AIS may also apply to children. An early-phase dose-finding trial is currently underway through the IPSS group.[247] Other thrombolytic approaches include interventional pharmacomechanical thrombolysis and thrombectomy for acute AIS associated with arterial thrombosis. However, technical limitations (i.e., size constraints) are posed by small-caliber pediatric cerebral vasculature, and pediatric recommendations reflect the similar cautions found in adult recommendations.[192]

In the subacute period after AIS in children and over the long term, treatment to optimize outcomes includes rehabilitation services (e.g., physical, occupational, and speech therapy) and neuropsychological interventions (e.g., educational, vocational, and mental health services). The need for these services should be assessed on a case-by-case basis and provided in accordance with the findings of comprehensive multidisciplinary assessments involving the expertise of pediatric neurology, rehabilitation medicine, and neuropsychology staff members. Furthermore, the impact of rehabilitative and neuropsychological interventions on the progress and future needs of the pediatric AIS patient should be regularly reassessed in extended follow-up.

Outcomes

Short-term adverse outcomes of AIS include death caused by uncontrolled increased intracranial pressure with uncal herniation, status epilepticus, or hemodynamic instability in extensive intracardiac thrombosis associated with cardioembolic AIS; major hemorrhagic complications, including those related to antithrombotic interventions as well as postischemic CNS hemorrhage; acute neurologic deficits; and early recurrence of AIS. Findings from the IPSS indicate that neurologic deficits are present at hospital discharge in approximately 70% of cases of childhood-onset AIS.[241]

Long-term outcomes of AIS in children include residual neurologic deficits, cognitive impairments, and recurrent AIS. These outcomes, stratified by diagnosis in the neonatal period versus older childhood, are summarized in Tables 35-4 and 35-5 based on data from selected published studies.[198,212,221,226,242,248,249,250,251,252] In brief, death caused by perinatal or neonatal AIS is quite rare, and for childhood AIS the case-fatality rate (i.e., cumulative incidence) ranges between 2% and 16% in various analyses, although the period over which death was attributable to AIS has not been well delineated in most studies. The risk (i.e., cumulative incidence) of recurrent AIS in perinatal or neonatal AIS is approximately 3% at a median follow-up duration of 3.5 years,[242] whereas the risk for recurrence after non-neonatal AIS varies widely between 19% and 40% at a fixed follow-up duration of 5 years. With regard to neurologic and rehabilitative (e.g., neuromotor, speech) outcomes, the prevalence of neuromotor deficits after perinatal or neonatal AIS varies widely between 36% at a median follow-up of 19 months and approximately 60% at average follow-up times 2 to 6 years across studies. Furthermore, the prevalence of either hemiplegia or asymmetric tone without hemiplegia has been shown in one study to be 30% at approximately 5 to 7 years of age.[252] By comparison, the prevalence of persistent neuromotor deficits for AIS diagnosed in

TABLE 35-4 Long-Term Outcomes of Neonatal Arterial Ischemic Stroke*

Author	Year of Publication	Country	# Cases	Average Duration of Follow-Up	Recurrent AIS	Persistent Neuromotor Deficits	Persistent Cognitive Deficits	AIS-Related Mortality
de Veber et al.[197]	2000	Canada	33	2 years	N/A	64%	3%	0%
Kurnik et al.[242]	2003	Germany	215	3.5 years	2%	N/A	N/A	N/A
Mercuri et al.[252]	2004	UK	22	~6 years	N/A	59%	14%	2%
Kirton et al.[251]	2007	Canada	14	19 months	N/A	36%	N/A	N/A
Fullerton et al.[250]	2007	U.S.A.	84	6 years	0%	N/A	N/A	2%

AIS, Arterial ischemic stroke; *N/A,* not available.

*Expressive speech deficits were evaluated in one study,[62] with a defined prevalence of 12%.

TABLE 35-5 Long-term Outcomes of Childhood-Onset* Arterial Ischemic Stroke†

Author	Year of Publication	Country	# Cases	Average Duration of Follow-Up	Recurrent AIS	Persistent Neuromotor Deficits	Persistent Cognitive Deficits	AIS-Related Mortality
de Veber et al.[197]	2000	Canada	90	2 years	11%	67%	17%	0%
Lanthier et al.[249]	2000	Canada	46	N/A	17%	41%	N/A	N/A
Chabrier et al.[226]	2000	France	59	2.5 years	22%	73%	N/A	N/A
Sträter et al.[248]	2002	Germany	301	3.7 years	7%	N/A	N/A	2%
Steinlin et al.[212]	2005	Switzerland	38	6 months	N/A	74%	N/A	3%
Ganesan et al.[221]	2006	UK	212	2.2 years	41% (5 yrs)	N/A	N/A	2%
Fullerton et al.[250]	2007	U.S.A.	92	5 years	19% (5 yrs)	N/A	N/A	4%

AIS, Arterial ischemic stroke.
*Non-neonatal.
†Expressive speech deficits were evaluated in one study,[62] with a defined prevalence of 18%.

later childhood is approximately 70% at average follow-up durations ranging from 6 months to 2.5 years across studies. Expressive speech impairments have been noted in 12% of perinatal or neonatal and 18% of non-neonatal AIS cases in one study.[198] As for neuropsychologic outcomes, cognitive or behavioral deficits have been discerned in a few of the aforementioned studies in 3% to 14% of children with perinatal or neonatal AIS at 2 to 6 years average follow-up and in 17% of children at 2 years follow-up in whom AIS occurred in later childhood.

Prognostic Indicators. As in pediatric VTE, the ability to predict clinically relevant acute and long-term outcomes of AIS at diagnosis and during the acute phase of treatment is essential to the development of appropriate management paradigms in children. However, such efforts have been particularly challenged by the relative infrequency of pediatric AIS cases and the heterogeneity in etiologies and AIS subtypes in this complex disorder. In perinatal or neonatal ischemic stroke, a cohort study[253] of 24 neonates with ischemic stroke, including mostly MCA-territory AIS cases but some with primarily watershed area involvement, suggested that an abnormal background on early neonatal encephalography (as opposed to focal epileptiform discharges) and a distribution that includes a cerebral hemisphere along with both the internal capsule and basal ganglia (as opposed to only one or two of these regions) serve as factors that are each associated with the development of a persistent neurologic deficit. The deficits consisted of either hemiplegia or asymmetry of tone without hemiplegia. These data were subsequently substantiated at later follow-up in early childhood.[252] In a smaller series the presence of signal abnormalities in the posterior limb of the internal capsule or the cerebral peduncles on magnetic resonance with diffusion-weighted imaging and apparent diffusion coefficient mapping in acute perinatal or neonatal AIS was recently found to be associated with the development of either hemiplegia or asymmetry of tone without hemiplegia at a median follow-up of greater than 18 months[254] and are supported by similar findings in a separate cohort at a median follow-up of 5.5 years. More recently, the use of computer-assisted quantitation of degree of descending corticospinal tract signal abnormality on magnetic resonance with diffusion-weighted imaging in a series of 14 neonates with AIS demonstrated that the percentage of cerebral peduncle affected and the total length of descending corticospinal tract involved correlates with the development of hemiparesis.[251] Although correlates of neuromotor outcome in perinatal and neonatal AIS are becoming better defined, predictors of recurrent stroke in this group have been elusive, particularly given the infrequency of recurrent AIS after perinatal or neonatal AIS. In childhood-onset AIS, an IPSS analysis in 2009 identified arteriopathy, bilateral ischemia, and decreased consciousness at stroke presentation or diagnosis to each be independently prognostic of a composite acute adverse outcome of death or neurologic deficit at hospital discharge.[241] With regard to AIS recurrence, a single-center retrospective study in the 1990s demonstrated a statistically significant increase in the odds of recurrent AIS among patients with more than one AIS risk factor (e.g., arteriopathy, thrombophilia traits, cardiac disorders, infection) identified at the time of the acute event compared with children who had one or no risk factors.[252] In addition, patients with moyamoya syndrome are at greatly increased risk for recurrent AIS and persistent neurologic deficits.[221,226,255] In one large single-center cohort study of mixed prospective-retrospective design,[221] moyamoya was one of only two independent risk factors for recurrent ischemic events (consisting of AIS, transient ischemic attack, or death with reinfarction). The association of arteriopathy with increased risk of recurrent AIS in children has been demonstrated in a U.S. population-based cohort,[250] confirming prior findings from a large multicenter German prospective cohort study of childhood AIS.[242] In the German study elevated serum levels of lipoprotein(a), congenital protein C deficiency, and arteriopathy were each found to be independent risk factors for recurrent AIS. Although APAs were not evaluated in the multivariate model of the German cohort study, a two-center British-Canadian cohort study of non-neonatal AIS, including 185 children, reported that no increased risk for recurrent AIS appears to exist with respect to persistently positive immunoglobulin G–type anticardiolipin antibody titers.[256] Although the statistically insignificant unadjusted difference in 3-year recurrence rates between anticardiolipin immunoglobulin G–positive and –negative groups at 38% versus 26% might nevertheless be considered clinically meaningful, the difference in proportional hazard functions for AIS recurrence between the two groups was also not statistically significant after adjustment for covariates of AIS etiologies and treatment types. However, few cases of high-titer antibodies were represented in the cohort. Clearly, further basic and translational research is needed to discern which subtypes of anticardiolipin antibodies may be most arteriopathic and thrombogenic and will likely involve secondary characterization of APAs using functional assays. With regard to lipoprotein(a), as noted earlier, its importance as a prognostic factor for recurrent childhood-onset AIS from the German cohort was recently substantiated in a U.S. cohort, which also identified small apo(a) isoform size as a prognostic factor for recurrence.[241]

Future studies should seek to validate the predictive capacity of these potentially quite meaningful associations in pediatric AIS cohorts distinct from those in which these associations have been derived. Collaborative multicenter cohort studies and trials groups, such as the IPSS group, offer optimism for the establishment of more robust evidence of predictors of outcome in neonatal and childhood AIS according to common etiologies and vascular pathologies; these data will help clinicians optimize risk-stratified approaches to the management of pediatric AIS in future years.

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CHAPTER 36

Transfusion Medicine*

Steven R. Sloan

CHAPTER OUTLINE

RED BLOOD CELL TRANSFUSIONS
- Indications for Transfusion
- Packed Red Blood Cells
- Leukoreduction of Red Blood Cells
- Irradiation of Red Blood Cells
- Immunologic Considerations
- Compatibility Procedures
- Selection of Blood for Transfusion to Patients with Blood Group Alloantibodies
- Selection of Blood for Transfusion to Patients with Blood Group Autoantibodies
- Adverse Effects of Transfusion

PLATELET TRANSFUSIONS
- Indications for Transfusion
- Platelet Units
- Platelet Dosing
- Platelet Refractory State
- Preventing Platelet Alloantibodies

GRANULOCYTE TRANSFUSIONS

PRINCIPLES OF HEMATOPOIETIC CELLULAR THERAPY
- Hematopoietic Cell Sources
- Infusion-Related Toxicities
- Component Assessment
- Processing and Storage of Hematopoietic Progenitor Cell Components
- Future Directions

APHERESIS
- Donor Apheresis
- Therapeutic Apheresis
- Manual Apheresis
- Automated Apheresis
- Unique Aspects of Apheresis in Pediatrics
- Indications

ERYTHROCYTAPHERESIS (RED BLOOD CELL EXCHANGE) FOR SICKLE CELL DISEASE
- Acute Chest Syndrome
- Stroke
- Priapism
- Red Blood Cell Exchange for Chronic Transfusion
- Normovolemic Red Blood Cell Transfusion

LEUKAPHERESIS

PERIPHERAL BLOOD PROGENITOR CELLS

EXTRACORPOREAL PHOTOPHERESIS

PLASMA EXCHANGE
- Autoimmune Disorders
- Other Coagulopathies
- Renal Disease
- Hypercholesterolemia
- Transplantation
- Disseminated Intravascular Coagulation
- Special Applications and Selective Adsorptions

CONCLUSION

Transfusion medicine focuses on the administration of blood, blood components, and purified blood proteins to patients for therapeutic purposes. Red blood cells (RBCs) are the most frequently used blood component. In this chapter RBC transfusions are discussed first, and issues that pertain to all transfusions, such as transfusion reactions and transfusion-transmitted diseases, are discussed in this section. Platelets are often transfused to some patients, such as those receiving oncologic treatment, and are discussed in the next section of this chapter. While not a traditional blood component prepared by blood banks, hematopoietic progenitor cells are a blood derived cellular component that are being infused with increasing frequency over the past two decades. Although these cells are often said to be transplanted rather than transfused, they are also discussed in this chapter. Finally, granulocyte components collected by apheresis for transfusion and other apheresis procedures that allow for the simultaneous removal and transfusion of blood components are discussed at the end of this chapter.

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RED BLOOD CELL TRANSFUSIONS

Indications For Transfusion

The primary function of RBCs is to bind oxygen as the RBCs circulate through the pulmonary bed and release oxygen in the capillaries at a sufficiently high pressure for tissue diffusion. The configuration of the oxyhemoglobin dissociation curve provides for proper diffusion as long as only part of the oxygen is released during capillary transit. The oxygen tension of fully oxygenated blood in the arteries is approximately 100 mm Hg; a release of 20% of the oxygen in the capillaries maintains a pressure of 40 mm Hg, which is sufficient to propel oxygen to all cells within a truncated cone segment around the capillary.[1] RBCs compensate for a loss in oxygen-carrying capacity relatively rapidly by a shift in the oxyhemoglobin dissociation curve to the right, which decreases oxygen's affinity for hemoglobin (Hb). In addition, cardiac output increases. On a more chronic basis, blood volume, the size of the vascular bed, and the rate of production of RBCs increases. These compensatory mechanisms permit states of mild to moderate anemia without significant symptoms.

For most patients the only indication for RBC transfusion is to provide a patient with sufficient RBCs to prevent or reverse tissue hypoxia caused by insufficient compensation. One large multicenter randomized controlled study of adult critically ill patients is probably applicable to some pediatric patients, especially adolescents.[2] That trial compared a liberal transfusion strategy in which patients received RBC transfusions when their Hb level was below 10 g/dL to a restrictive transfusion strategy in which patients were transfused when their Hb level was below 7 g/dL. Among patients younger than 55 years old, those who were transfused according to the more restrictive strategy had a significantly lower 30-day mortality rate. A similar study of stable critically ill pediatric patients in intensive care units found similar clinical outcomes but fewer RBC transfusions in patients transfused at an Hb concentration at or below 7 g/dL compared with patients transfused at an Hb concentration at or below 9.5 g/dL.[3] In that study patients could be transfused for active bleeding or hemodynamic instability even if their blood Hb concentration had not reached the assigned trigger.

Two prospective randomized controlled studies have compared transfusion strategies for low-weight premature infants. The triggers used for transfusion depended on the oxygen support requirements in both studies[4,5] and also on the baby's age in one of the studies.[5] Because neonates often receive multiple aliquots from the same RBC unit, there was no statistical difference in donor exposure between the liberal and restrictive transfusion groups in either study. One study by Bell and colleagues found that neonates on the liberal transfusion strategy were less likely to develop intraventricular hemorrhage or periventricular leukomalacia.[4] However, a follow-up study of these patients when they were between 8 and 15 years old found better neurocognitive development in the children transfused more conservatively.[6] In contrast, the other larger multicenter study by Kirpalani and coworkers found no differences in short-term outcomes between patients assigned to liberal and restrictive groups, but a follow-up study of the patients when they were 18 to 21 months age suggested cognitive delay in those transfused more conservatively.[5,7] Taken together, these studies suggest that a more liberal transfusion strategy might be advisable. A liberal RBC transfusion protocol might protect against obvious brain injury without significant risk in the neonatal period. Additionally, the larger of the two randomized studies suggested better neurocognitive outcomes with a more liberal use of RBC transfusions.

Children who are chronically anemic as a result of thalassemia may also benefit from a more liberal transfusion policy. These patients benefit from a hypertransfusion program that maintains their Hb concentration above 9 to 10 g/dL. Hypertransfusion of these patients allows them to attain normal height and weight and reduces the risk of hepatosplenomegaly, osteoporosis, and cardiac dilation.[8-12] Transfusions benefit these patients by increasing tissue oxygenation and particularly by suppressing endogenous erythropoiesis.

Acute Blood Loss

RBC transfusions are frequently used to replace blood loss either in the operating room or after trauma. In practice, diagnosis of the presence and degree of blood loss is quite difficult, especially in a young otherwise healthy child. In fact, a child may sustain a relatively large hemorrhage with few external signs of distress. Signs of impending shock, such as pallor, anxiety, and tachypnea, are frequently subtle and easily overlooked or attributed to other causes. By the time signs of cardiovascular compromise become evident (i.e., stupor, tachycardia or bradycardia, hypotension, coolness of the extremities, weak peripheral pulses, and decreased capillary filling), the patient has most likely lost at least 25% of the total blood volume. Hypotension is one useful clinical sign of moderate to severe blood loss. Patients who have lost more than 25% of their blood volume frequently manifest age-related systolic hypotension: less than 65 mm Hg (younger than 4 years of age); less than 75 mm Hg (5 to 8 years of age); less than 85 mm Hg (9 to 12 years of age); and less than 95 mm Hg (adolescents and adults).

Chronic Transfusion Therapy

In chronic anemia the body is deficient in red cells, whereas the plasma volume is normal or increased so that the total blood volume is normal. Box 36-1 lists the childhood conditions that are likely to require intermittent or long-term transfusion therapy.

The following guidelines apply when planning for chronic transfusions (months to years):

1. If possible, transfusion should be avoided or minimized by aggressive treatment of the underlying disorder (e.g., recombinant human erythropoietin [r-HuEPO] administration for patients on dialysis or with chronic renal failure).
2. Leukocyte-reduced RBCs should be used to avert or delay sensitization to leukocyte alloantigens, the

Box 36-1 Diseases Requiring Intermittent or Chronic Red Cell Transfusion in Childhood

- Chronic renal failure
- Thalassemia
- Sickle cell disease
- Aplastic anemia
  - Constitutional
  - Acquired
  - Oncologic (leukemia, solid tumors)
  - Diamond-Blackfan anemia
  - Transient erythroblastopenia

most common cause of febrile nonhemolytic transfusion reactions.

3. RBCs should be administered in an amount sufficient to prevent symptomatic anemia and allow for normal growth.
4. The patient's iron status should be monitored through determination of serum ferritin, iron, iron-binding capacity, and liver iron stores (see Chapter 21 for more information).

Packed Red Blood Cells

A packed cell product is the component of choice for replacement during surgery, RBC loss, and chronic transfusion therapy. Most RBC units contain an additive nutrient solution such as Adsol or Nutricel and have a hematocrit concentration of 50% to 60%, whereas RBC units collected and stored in citratephosphate-dextrose-adenosine (CPDA-1) have an average hematocrit concentration of approximately 75%.[13] Additives vary in their composition and contain some combination compounds, including dextrose, citrate, mannitol, and adenine. Although no systemic pediatric risk has been demonstrated for additives, some institutions avoid using them or remove them before transfusing in large quantities to neonates.[14]

Blood Storage

During storage RBC metabolism continues, and some leakage through RBC membranes occurs. This results in decreasing plasma dextrose and increasing plasma potassium. These changes are acceptable for most transfusions, but in some situations the increased potassium in solution may cause problems. In these cases fresh blood (less than 7 to 10 days old) may be beneficial. This is indicated when small patients receive large amounts of blood, such as neonates receiving exchange transfusions or infants receiving large transfusions rapidly during surgery. However, neonates can tolerate 5 to 20 mL/kg transfusions of older units of RBCs, and some blood banks dedicate a unit of blood for repeated transfusions to one infant to minimize the number of donors to which each infant is exposed.[14] This protocol can lead to transfusion of older RBC units, which are safe for infants, including premature infants.[15]

Leukoreduction of Red Blood Cells

Transfusion of allogeneic cellular blood products is associated with deleterious effects caused by the presence of residual leukocytes. These side effects include alloimmunization to histocompatibility antigens in patients with leukemia, transmission of some viruses, febrile reactions, and graft-versus-host disease (GVHD).[16,17] Filters are used to reduce the number of leukocytes from whole blood, RBCs, and platelets.[18,19] Routine leukoreduction of red cells to less than 5×10^6 leukocytes per unit reduces the frequency of febrile nonhemolytic transfusion reactions in patients who are transfusion dependent.[20,21] The removal of leukocytes from blood also reduces the risk of leukocyte-associated virus transmission, such as cytomegalovirus (CMV). Leukoreduction of RBCs and platelets to a level of less than 5×10^6 has been demonstrated to substantially reduce CMV transmission in neonates and transplant patients.[22,23] However, leukoreduction is not sufficient to prevent transfusion-associated GVHD.

Irradiation of Red Blood Cells

Transfusion-associated GVHD is a rare complication after transfusion of cellular products. Patients who are the most susceptible include premature neonates, some patients with congenital immunodeficiency syndromes, immunosuppressed oncology patients being treated with chemotherapy or irradiation (or both), and patients receiving certain immunosuppressive drugs after solid organ transplantation and certain other indications.[24-26] In addition, normal recipients who are heterozygous for human leukocyte antigens (HLAs) do not reject lymphocytes that are transfused from a donor who is homozygous for one of the recipient's haplotypes.[27] This is not merely theoretic. Transfusion-associated GVHD has developed in normal recipients undergoing surgery who received unirradiated directed donor blood from a first-order relative.[28,29] Because of the wide variety of patients at risk for GVHD, some transfusion services have chosen to irradiate all cellular blood products to ensure that no patient develops this life-threatening complication.

Immunologic Considerations

Multiple antigens that are expressed on erythrocyte cell surfaces can be recognized by the immune system of a transfusion recipient. Before an RBC transfusion, erythrocytes that are immunologically compatible with the patient must be identified. Erythrocyte antigens are polymorphic inherited structural characteristics located on proteins, glycoproteins, or glycolipids on the outside surface of the RBC membrane. Erythrocyte antigens are clinically important in the immune destruction of RBCs in allogeneic blood transfusions, maternal-fetal blood group incompatibility, autoimmune hemolytic anemia, and organ transplantation. The ability to detect and identify antibodies to erythrocyte antigens and identify compatible donor RBCs lacking the specific antigens has contributed significantly to the safe supportive blood transfusion practices used today.

The ABO and H System

The H Antigen. A and B antigens on RBCs are defined by a terminal sugar attached to a carbohydrate chain on membrane glycosphingolipids. The H antigen is the precursor molecule on which A and B antigens are built. The H antigen on RBCs is defined by fucose at the terminus

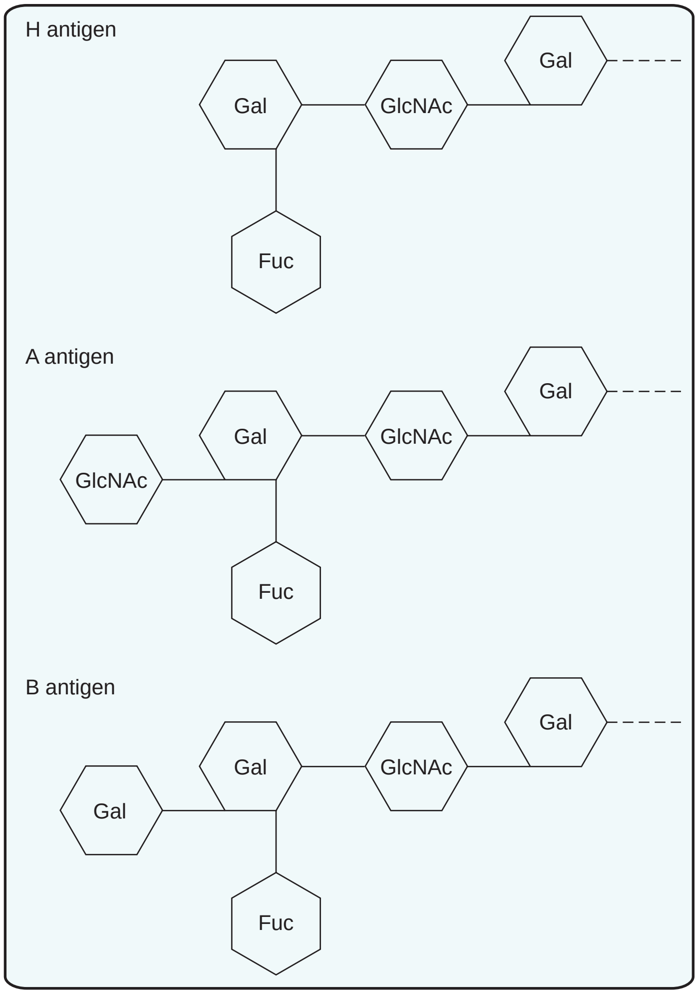

Figure 36-1 Structure of the precursor H structure and the A and B antigens. *GlcNAc, N*-acetylglucosamine.

of carbohydrate chains.[30] Expression of the H antigen is determined by a fucosyltransferase that transfers a fucose to an oligosaccharide side chain that is attached to integral membrane proteins on RBCs. The fucosyltransferase (and thus the H antigen) is present in all persons except those with the rare Bombay (Oh) phenotype.[31]

The A and B Antigens. Transferases responsible for the A or B antigens add *N*-acetyl galactosamine or galactose, respectively, to the precursor chain carrying the H antigen (Fig. 36-1).[30,32-36] The genes for the A and B blood group antigens are codominant because people with both transferases will transfer galactosamine and galactose and thus express A and B antigens. Group O is the amorph of the ABO blood group system. RBCs from group O persons have H antigens but lack A and B antigens.[37]

Although ABO antigens are detectable on the RBCs of 5- to 6-week-old embryos, these antigens are not fully developed until 2 to 4 years of age.[38-41] RBCs from infants carry A, B, and H antigens on linear oligosaccharide chains, which have only one terminus to which the A, B, and H sugars can be added.[30,32,34,42-44] The fact that the A and B antigens are not fully developed at birth may be one reason that ABO hemolytic disease of the newborn (HDN) is usually a mild disease.[44,45] As the infants grow older, the oligosaccharides branch, creating additional termini to which A, B, and H sugars can be added.[35] After the children reach 2 to 4 years of age, expression of A and B antigens remains fairly constant throughout life.

Antibodies to A and B antigens, also known as isohemagglutinins, develop as a result of environmental stimulants, such as bacteria, that carry similar antigens.[46] Because isohemagglutinins are naturally occurring, it is essential to select ABO-compatible blood for transfusion.[47] Indeed, ABO incompatibility is the most frequent cause of fatal hemolytic transfusion reactions.

Isohemagglutinin production begins several months after birth, reaching a peak at 5 to 10 years of age and declining with increasing age.[48] Thus infants younger than 4 months of age who have isohemagglutinins will have acquired them through placental transfer of maternal immunoglobulin G (IgG) anti-A, anti-B, or anti-A,B.[44]

Isohemagglutinins from group A and B individuals are predominantly immunoglobulin M (IgM) that do not usually cross the placenta and cause HDN.[49] However, as group O serum contains IgG isohemagglutins, ABO HDN is most frequently seen in non–group O infants of group O mothers.

The Lewis System

The Lewis antigens, Le^a and Le^b, are not intrinsic to RBCs but are instead carried on plasma glycosphingolipids that are adsorbed onto the RBC membrane.[50] These antigens are structurally related, with the Le^b antigen containing an additional fucose that is not present on the Le^a antigen. Furthermore, the one additional fucose present on the Le^b antigen is not very different from the Le^a antigen. Thus Le^a individuals rarely make anti-Le^b. Hence in most cases Lewis antibodies are made only in individuals who are Le(a–b–).[44]

In most cases Lewis antibodies do not cause hemolysis, and donor RBCs lacking the Lewis antigens do not usually need to be selected.[44] However, rare hemolytic transfusion reactions have been reported when Le^a+ RBCs were transfused to recipients with anti-Le^a.[51] This may occur when the antibody reacts at 37° C. In these cases it is not difficult to identify Le^a– donor units because the RBCs in almost 80% of donors are Le^a–.[52] Anti-Le^a and anti-Le^b are not known to cause HDN. They are usually IgM, and fetal RBCs type Le(a–b–).[44] Interestingly, Lewis antigens are also expressed on *Helicobacter pylori,* a causative agent of gastric ulcers, and play a role in adhesion and colonization of the bacteria in the gastric mucosa.[53,54]

The Ii Collection

The I and i antigens reside in the subterminal portions of carbohydrate chains on glycolipids or glycoproteins of the RBC membrane. The i antigen on fetal RBCs appears when disaccharide units are linked in a straight chain.[55] In the first 2 years of life, many of these linear chains are modified into branched chains.[35] I specificity results at the expense of i antigens when the branched structures appear. Adults have RBCs with predominantly I antigens and little or no i antigen, whereas newborns have RBCs with strong expression of i antigens and small amounts of I antigen.[56]

Anti-I is a common autoantibody that is reactive at room temperature and below and usually produces no hemolysis.[57,58] In cold agglutinin disease, anti-I causes in vivo hemolysis, and such pathologic autoantibodies often react at 30° C in albumin.[59] Adult I-negative RBCs are not readily available for transfusion, and patients with cold agglutinin disease caused by anti-I are transfused with I-positive RBCs through a blood warmer.[44] The titer and thermal range of anti-I is often increased after infection with *Mycoplasma pneumoniae*.[44] The presence of a cold agglutinin caused by an anti-I antibody frequently occurs in *M. pneumoniae* infection, but this is neither a specific nor a sensitive test for this infection.[60] Analogously, cold agglutinins resulting from anti-i antibodies are sometimes present in Epstein-Barr virus infection, but this is neither a sensitive nor a specific test for this infection.[61]

Levels of the i antigen can aid in differentiating Diamond-Blackfan anemia from transient erythroblastopenia of childhood. The i antigen is enhanced on RBCs from patients with Diamond-Blackfan anemia and is absent or of reduced strength on RBCs from children with transient erythroblastopenia of childhood.[62-64] However, the enhanced i antigen associated with Diamond-Blackfan anemia reflects stress hematopoiesis[65] and is not a specific aspect of the disease. In transient erythroblastopenia of childhood, the reduced levels of i antigen reflect the selective suppression of erythropoiesis secondary to infection; however, during recovery from this disorder the patient's marrow is active and the i antigen may be enhanced as a result of hematopoietic stress.[64]

The P System

Like the A, B, and H antigens, the P blood group antigens are defined by sugars added to precursor glycosphingolipids. Antigens in this group include P1, P, and P^K. Among white people, 21% have the P1-negative phenotype, and they commonly produce anti-P1. This cold reactive IgM antibody does not cross the placenta and has rarely been reported to cause hemolysis in vivo.[66] An autoantibody, autoanti-P, is found in patients with paroxysmal cold hemoglobinuria and in some patients with acquired immune hemolytic anemia. It is called the Donath-Landsteiner antibody and is most frequently seen in children.[67] This IgG autoantibody is a biphasic hemolysin that binds to RBCs in the cold and then hemolyzes them when warmed. The patient's serum causes hemolysis of RBCs that have been incubated first in melting ice and then incubated at 37° C.[68] This antibody should be considered when the patient has hemoglobinuria or anemia (or both) and C3 alone is present on the RBCs.

The P antigen serves as a receptor for parvovirus B19[69] and pyelonephritic *Escherichia coli*.[70,71] Persons with the very rare p phenotype lack the antigens P1, P, and P^k and are not susceptible to infection by parvovirus B19.

People with the rare p phenotype may produce anti-P1+P+P^k, a potent hemolytic antibody that can cause immediate hemolytic transfusion reactions.[44,72] This antibody has also been associated with HDN, fetal death, and miscarriages during early pregnancy in women whose RBCs have the p phenotype.[73]

The Rh System

The Rh blood group system includes D, C, c, E, e, and 40 other antigens.[74] The terms *Rh positive* and *Rh negative* refer to the presence and absence of the D antigen, respectively. The D antigen is, after A and B, the most important RBC antigen in transfusion practice. Persons who lack the D antigen do not have anti-D unless they are exposed to D antigen by pregnancy or transfusion. More than 80% of persons who lack the D antigen can produce anti-D after receiving a unit of D-positive donor blood, but only approximately 22% of D-negative patients transfused with D-positive RBCs make an anti-D antibody.[75-77] Although these statistics probably do not apply to infants younger than 4 months of age, some infants probably can respond to the Rh(D) antigen.[78]

The RBCs of 0.2% to 1% of white individuals and a greater percentage of African Americans carry reduced expression of the D antigen known as weak D. Some RBCs expressing weak D may not be detected by routine anti-D reagents, but almost all are detected by a more sensitive test, the indirect antiglobulin test. D and weak D have the same RH (D) protein complex on their surface but weak D RBCs have fewer RH (D) proteins.[79] That is usually due to point mutations in the gene encoding the Rh(D) antigen, the *RHD* gene.[80,81] Most patients of the weak D phenotype can safely receive blood transfusions from individuals who are Rh(D) positive. However, some individuals express a truncated form of the D protein, known as a partial D, and can make an anti-D antibody. Erythrocytes from such an individual may type as D-positive or weak D. Often only reference labs can determine the specific type of weak D that is present, sometimes using molecular techniques.

All D-negative donor blood is tested for weak D by the antiglobulin test to ensure that D-negative patients are not transfused with weak D RBCs that can induce an anti-D antibody that can hemolyze weak D RBCs.[47] Indeed, there are rare reports of fetuses with weak D having HDN when born to a mother with anti-D antibody in her plasma.[44] Weak D donor RBCs are labeled as Rh(D) positive to ensure that Rh(D)-negative individuals do not receive weak D RBCs. Analogously, at birth an infant is designated as Rh(D) negative only when negative results are obtained for routine anti-D and weak D.[47] This ensures that Rh(D)-negative mothers receive postpartum doses of Rh immune globulin if their baby is Rh(D) positive or weak D positive.

Rh antigens are carried on Rh proteins that pass through the RBC membrane 12 times and are encoded by at least two genes on chromosome 1.[82-85] These Rh proteins associate with the RHAG protein that is required for cell surface expression of the Rh antigens. D, C, c, E, and e antigens are associated with specific amino acid substitutions on the Rh proteins.[84,85] Patients with erythrocytes deficient in Rh proteins owing to complex mutations in the Rh locus have a mild spherocytosis and a mildly hemolytic state known as Rh-deficiency syndrome, or Rh null.[86,87] Mutations of the *RHAG* gene result in regulatory-type Rh deficiency (Rh null or Rh mod) associated with mild hemolytic anemia with overhydrated hereditary stomatocytosis showing increased permeabiltity to cations.[88] Although the function of the Rh proteins is unknown, they may play a role in ammonium transport.[89,90]

The MNS System

The M, N, S, s, and U antigens are carried on sialoglycoproteins on the RBC membrane. M and N are encoded by paired allelic forms of the glycophorin A gene *(GPA)* and are the result of amino acid substitutions at residues 1 and 5 of GPA.[91-93] Human RBCs type as M+N–, M–N+, or M+N+, representing homozygosity for M, homozygosity for N, and heterozygosity for both genes, respectively. S and s antigens are encoded by alternative forms of the glycophorin B gene *(GPB)*. GPA and GPB are encoded by homologous genes on chromosome 4q28-q31.[94] GPA binds *E. coli*[95] and plays a role in malarial invasion of RBCs. RBCs lacking GPA, GPB, or GPC are resistant to invasion by *Plasmodium falciparum* to varying degrees.[96]

Anti-M and anti-N can be IgG, IgM, or a mixture of both. These antibodies are usually clinically insignificant and are often present in persons who have not been previously transfused or pregnant. Those antibodies that react at 37° C may cause hemolysis of transfused cells. Antibodies to S, s, and U usually occur after stimulation and are capable of causing hemolytic transfusion reactions and HDN.[44]

The Kell System

The Kell system antigens reside on two transmembrane proteins, the Kell protein and the XK protein, that are linked by a single disulfide bond. The Kell protein is a 93-kd glycoprotein that carries more than 20 antigens, and the XK protein is a 440–amino acid protein that carries just one antigen known as Kx.

The major antigen of the Kell blood group system resides on the Kell protein. This antigen is the K antigen, which is present in 9% of white donors.[72] Anti-K is the most common immune RBC antibody, after those in the ABO and Rh systems. However, because more than 90% of donors are K–, it is not difficult to find compatible blood for patients with anti-K antibodies. The molecular basis of antithetical K and k antigens is an amino acid substitution of methionine to threonine at residue 193 of the Kell protein.[97] This allows for prenatal testing for K and k antigens of amniocytes from a mother whose fetus is at risk for HDN owing to anti-K or anti-k. Antibodies to other antigens on the Kell protein, such as Kp^a, Kp^b, Js^a, and Js^b, are less common but are also clinically significant.[72,98] The Kell protein is a member of a large family of zinc-dependent endopeptidases.[99]

The other protein of the Kell system, the XK protein, carries one antigen, the Kx antigen.[100] The XK protein is encoded by a gene on the X chromosome and is a 444–amino acid integral membrane protein that has some features of a membrane transporter.[101] The absence of membrane XK protein, known as the McLeod phenotype, results in diminished expression of the Kell protein on the membrane and reduced expression of all antigens in the Kell blood group system. The blood of patients with the McLeod phenotype often has a variety of abnormalities, including acanthocytotic erythrocytes, hemolytic anemia, some teardrop erythrocytes, and bizarre poikilocytes.[102] Most patients with McLeod syndrome develop a subclinical myopathy associated with an elevated creatine phosphokinase level.[103] The neuropathy may first manifest as areflexia and is later characterized by basal ganglia atrophy causing dystonic or choreiform movements, psychiatric symptoms, and cognitive changes.[104,105] Most individuals with McLeod syndrome also develop cardiac symptoms, such as dilated cardiomyopathy and arrhythmias.

The *XK* gene is less than 500 kb beyond the chronic granulomatous disease (CGD) locus on the short arm of the X chromosome (p21.1).[106] Consequently, males with deletions encompassing both loci have both CGD and McLeod syndrome.[98] Patients with CGD and McLeod syndrome usually make antibodies to the XK and Kell proteins if they are transfused with RBCs containing the XK and Kell proteins. This can lead to major problems in transfusing these patients.[107] To avoid this problem, McLeod patients should be transfused with blood of the McLeod phenotype. Ko blood that lacks the Kell protein is not appropriate because such RBCs have increased expression of the Kx antigen that is expressed on the XK protein. However, male subjects with McLeod phenotype without concomitant CGD (non-CGD McLeod) do not make anti-Kx and can be transfused with McLeod or Ko type blood.[98,108]

The Duffy System

The Duffy antigens are epitopes present on a cell surface glycoprotein. The Duffy glycoprotein is a chemokine receptor that is expressed on many cell types and can bind a variety of chemokines.[109] Although the Duffy chemokine receptor can bind chemokines, its function is unknown and people lacking the receptor on their RBCs or on all cells are totally healthy.[110]

The two most common alleles in people of European or Asian descent are Fy^a and Fy^b, which differ by a single amino acid substitution. Anti-Fy^a and -Fy^b have been implicated in transfusion reactions.[44] Anti-Fy^a has caused mild HDN, but anti-Fy^b has not been implicated.[44] Anti-Fy^a is commonly encountered, but because one third of donors are Fy(a-), compatible blood is not difficult to find.

The RBCs of many people of African descent lack the Fya and Fyb antigens (Fy[a–b–]) on their erythrocytes. However, the Fy^b allele is usually expressed in lung, colon, and spleen of these individuals.[111] Thus these individuals cannot mount an immune response to the Fy^b antigen but can produce anti-Fy^a.[44,72] In West Africa most black people are Fy(a-b-) as a result of natural selection; these RBCs are resistant to *Plasmodium vivax* malaria because the Duffy protein is a receptor for *P. vivax*.[109,111,112,113]

The Kidd System

The Kidd gene encodes two antigens, Jk^a and Jk^b, which represent a single amino acid substitution (Asp280Asn) in the Kidd glycoprotein.[114] These are usually expressed in one of three phenotypes: Jk(a+b–), Jk(a–b+), and Jk(a+b+). The fourth phenotype Jk(a–b–) is exceedingly rare.[72] Antibodies to both Kidd antigens can cause delayed hemolytic transfusion reactions. Because these antibodies are often weak and may become undetectable over time, they may escape detection in the sensitized patient's serum before transfusion. If the patient is then transfused with antigen-positive RBCs, the anamnestic response causes an increase in the titer of the antibody and hemolysis of the transfused antigen-positive RBCs.[44] Once the antibody is identified, compatible blood is not difficult to find because one fourth of donors are negative for each antigen.

The Kidd glycoprotein is a urea transporter that prevents RBCs from shrinking and swelling as they pass into and out of the high urea concentration of the renal medulla.[115-117] The Kidd urea transporter also prevents RBCs from carrying urea away from the renal medulla and decreasing the urea-concentrating efficiency of the kidney.[117] Individuals with RBCs with the Jk(a–b–) phenotype are unable to concentrate urine as efficiently as people who have the Kidd glycoprotein.[118-120]

The Lutheran System

The Lutheran blood group antigens are carried on a cell surface glycoprotein. The Lu^a antigen is present on the RBCs of 8% of people, whereas the antithetical antigen, Lu^b, is present in more than 99% of random blood samples. Antibodies in this system are variable and are rarely encountered.[44] The glycoprotein carrying these antigens has five extracellular immunoglobulin-superfamily domains that serve as laminin-binding receptors.[121,122] Lu glycoproteins may be involved in the pathogenesis of sickle cell vaso-occlusive crises and may also be involved in the metastasis of certain types of malignancy.[123,124]

About 1 person in 5000 inherits a dominantly acting inhibitor, called In(Lu),[125,125a] which partially suppresses expression of Lu^a and Lu^b such that they are undetectable by standard agglu-tination tests. The Lu(a-b-) phenotype of the dominant type is associated with acanthocytosis but no hemolysis.[125] Patients with the In(Lu) Lu(a-b-) phenotype have abnormally shaped red cells but no hemolysis.[125] The Lu antigens directly interact with spectrin, independently of protein 4.1, which may partially explain the abnormal RBC morphology in some patients with the In(Lu) phenotype.[126] Osmotic fragility of fresh In(Lu) Lu(a–b–) red cells is normal, but during in vitro incubation the cells lose K^+ and become osmotically resistant.

Other Systems

Other systems are included in Table 36-1.[74] Antibodies to antigens in these systems are less common than those described previously, and information regarding their clinical significance is summarized in Table 36-2.

TABLE 36-1 Blood Group Systems with Associated Gene Product

Blood Group System	Gene Product
Carbohydrate Antigens	
ABO	Glycosyltransferase
P	Glycosyltransferase
Lewis	Glycosyltransferase
Hh	Glycosyltransferase
Protein Antigens	
MNS	Glycophorin A, glycophorin B
Rh	D polypeptide *RHCE* polypeptide CcEe polypeptide
Lutheran	Lutheran glycoprotein
Kell	Kell glycoprotein
Kx	Xk glycoprotein
Duffy	Fy glycoprotein
Kidd	Jk glycoprotein
Diego	Band 3 (AE1)
Yt	Acetylcholinesterase
Xg	Xg^a glycoprotein
Scianna	Sc glycoprotein
Dombrock	Glycoprotein (possibly adenosine 5′-diphosphate[ADP]-ribosyltransferase)
Colton	Channel-forming integral protein
LW	Glycoprotein
Chido/Rodgers	C' component 4 (C4)
Gerbich	Glycophorin C, glycophorin D
Cromer	CD55 (DAF)
Knops	CD35 (CRI)
Indian	CD44

Maturation of Blood Group Antigens

Several blood group antigens are not expressed or are only weakly expressed on cord RBCs and usually reach adult levels by 2 years of age. Antibodies to these antigens are unlikely to cause HDN. Cord RBCs do not express Le^a, Sd^a, Ch, Rg, or AnWj antigens. Cord RBCs express the following antigens more weakly than RBCs from adults: A, B, H, I, Le^b, P_1, Lu^a (but not Lu^b), Yt^a, Vel, Do^a, Do^b, Gy^a, Hy, Jo^a, Xg^a, and Bg.[44,72,127]

Clinical Significance of Blood Group Antibodies

A blood group antigen has no immediate untoward effect on blood transfusion. It is the corresponding antibody that has clinical relevance and dictates the need for tests in a transfusion service to detect blood group antibodies.

Antibodies recognizing antigens in the ABO blood group system are by far the most clinically significant.

TABLE 36-2 Clinical Significance of Selected Alloantibodies to Antigens in Blood Group Systems

Antibody	IgG	IgM	Complement	Transfusion Reaction Binding	HDN*
ABO	Some	Most	Common	Mild to severe; immediate	Moderate
MNS					
MN	Some	Some	Rare	Extremely rare	Extremely rare
SsU	Most	Some	Rare	Immediate or delayed	Rare
P1	Rare	Most	Rare	None	None
RH	Most	Some	No	Immediate or delayed	Mild/severe
Lutheran					
Lu^a	Most	Some	No	None	None
Lu^b	Most	Rare	No	Mild; delayed	Mild/rare
Kell	Most	Some	Some	Immediate or delayed	Mild/severe
Lewis					
Le^a	Some	Most	Some	Rare; immediate	None
Le^b	Rare	Most		None	None
Duffy					
Fy^a	Most	Rare	Rare	Delayed	Mild
Fy^b	Most		No	Mild; delayed	None
Kidd	Most	Few	Common	Immediate or delayed	Mild/rare
Diego	Most			Delayed	Mild/severe
Yt	Most			Some; mild	None
Xg	Most		Some	None	None
Scianna	Most			None	None
Dombrock	Most			Delayed	Mild
Colton	Most			Mild; delayed	Mild/rare
LW	Most			Mild; delayed	Mild
Ch/RG	Most			None	None
H	Rare	Most		None (except in O$_h$)	None
XK	Most		None known	Delayed	None
Gerbich	Most	Some	Some	Immediate or delayed	None
Cromer	Most			Delayed	Mild
Knops	Most			None	None
Indian	Most			None	None

*The most severe form of hemolytic disease of the newborn (HDN) that has been reported is indicated. Remember that exceptions exist.

With antibodies in the other blood group systems, as a general rule, the more immunogenic an antigen, the more clinically significant its corresponding alloantibody. Other clinically significant antibodies occur in the following order, from most commonly to least commonly encountered in transfusion practice: anti-D, anti-K, anti-E, anti-c, anti-Fy^a, anti-C, anti-Jk^a, anti-S, anti-Jk^b.[128,129] All other clinically significant antibodies occur with an incidence of less than 1% of serum containing antibodies. Anti-P$_1$, anti-M, anti-N, anti-Lu^a (Lutheran), anti-Le^a, anti-Le^b, and anti-Sd^a are all considered clinically insignificant unless the alloantibodies are reactive in tests performed strictly at 37° C.[44,128] Clinically insignificant antibodies that react at 37°C by indirect antiglobulin test are those of the Knops and Ch/Rg systems and anti-JMH.[44,128] Other alloantibodies are clinically significant in some patients but not in others; examples are anti-Vel, anti-Ge (Gerbich), anti-Yt^a, and anti-Co^a (Colton).[44] Table 36-2 summarizes the clinical significance of antibodies to RBC blood group antigens.

The clinical significance of an antibody to a low-incidence antigen depends on whether it was found during crossmatching or prenatal testing. The specificity of a low-incidence antibody detected during compatibility testing need not be identified to locate antigen-negative blood with which to transfuse that patient because the full crossmatch will be positive in the rare event that a unit of donor blood from the stock supply carries the low-incidence antigen. In contrast, if an antibody to an uncommon antigen is detected in the serum of a pregnant woman, identification of the antibody, or determination of the antigen carried by the fetal or paternal RBCs, can be used as a predictor for the likelihood and severity of HDN. Blood for exchange transfusion will not be difficult to find.[44]

If a patient's serum contains alloantibodies to a high-incidence antigen, blood may be difficult to find. Examples are anti-U, anti-Kp^b, anti-Js^b, anti-Lu^b, and all antigens in the high series recognized by the ISBT Working Party.[74] Whether the investigation is for transfusion purposes or prediction of HDN, the antibody should be identified. This will aid in both the assessment of its clinical significance and the location of appropriate blood for transfusion.

Blood group antibodies in donor plasma present little hazard to an antigen-positive recipient or RBC units that have low plasma volumes. However, components that have significant plasma, such as fresh frozen plasma (FFP) or platelets, should generally be selected to lack ABO antibodies against recipient antigens. If platelets lacking ABO antibodies against the patient are unavailable, there is a small risk of a potentially severe reaction, and some blood banks have implemented procedures to minimize transfusion of incompatible isohemagglutinins. Donor blood is always tested for the presence of antibodies to antigens other than ABO and blood components containing such antibodies are generally not transfused to patients expressing the corresponding antigen.

Sera from some patients contain autoantibodies that recognize antigenic determinants on RBCs from the majority of individuals as well as their own. In such cases it may be impossible to locate compatible blood and only incompatible blood will be available. This may arise in such cases as autoimmune hemolytic anemia, cold agglutinin disease, and paroxysmal cold hemoglobinuria; these are discussed later in this chapter.[44]

Testing Donor Blood

Donor blood is routinely tested for its ABO and Rh blood group status. More extensive typings for other antigens are performed either when antigen-negative RBCs are required for a recipient whose serum contains clinically significant antibodies or when building an inventory of donors with certain antigenic profiles for future use, such as in the transfusion management of sickle cell disease (discussed later). The more alloantibodies are present, especially to high-frequency antigens, the longer the time needed to locate compatible units.

Compatibility Procedures

The routine approaches used involve testing a blood sample from a prospective recipient for ABO and Rh blood groups and for detection of blood group antibodies.[47] In most cases no unexpected antibodies are detected and donor RBCs of the appropriate ABO and Rh blood type will be selected for transfusion. A sample of this blood may be crossmatched with the patient's serum by an immediate spin procedure or an "electronic crossmatch" in which a validated computer system verifies that the patient is receiving an RBC unit that is ABO and Rh(D) compatible with the patient's blood group.

When an antibody is detected, an attempt should be made to identify its specificity. Knowing the specificity of an antibody allows the clinician to establish whether it is likely to be clinically significant and the approach that will be necessary to provide an adequate supply of compatible RBCs.

Compatibility in ABO and Rh

Determination of the ABO and Rh(D) type of the patient is the first step in compatibility testing. In neonates the ABO group is determined by testing the neonate's RBCs only because the plasma may contain maternal antibodies and young neonates' immune systems do not make isohemagglutinins. In patients older than 4 months, the ABO group is determined by testing both the patient's RBCs (forward group) and the plasma or serum (reverse group). The forward and reverse groups should correspond, except that infants younger than 9 months old may not yet make isohemagglutinins. In all other patients, until any discrepancy is resolved, only group O RBCs and AB plasma should be given.

Blood and blood components must be selected to be ABO and Rh compatible (Table 36-3). In general, for patients older than 4 months of age, type-specific RBCs and whole blood are selected, which means that donor and recipient have the same ABO and Rh type; it is acceptable to transfuse ABO and Rh compatible but not identical RBCs. For transfusions of a fetus, group O Rh(D−), irradiated, CMV antibody–negative or leukoreduced RBCs (or both) are usually given regardless of the ABO group of the recipient. For neonates some centers transfuse type-specific RBCs, whereas other centers transfuse group O RBCs to all infants. If the RBCs selected for transfusion are not group O, the neonate's serum or plasma should be tested for maternal anti-A or anti-B IgG antibodies in addition to IgM isohemagglutinins.[47] If anti-A or anti-B is detected, RBCs that are ABO-compatible with the antibodies should be transfused.[47]

Compatibility in Other Blood Groups

Compatibility testing for blood groups other than ABO is achieved by the antibody screen and crossmatch (Figs. 36-2 and 36-3). These tests detect unexpected RBC antibodies in the patient's serum. The patient's serum or plasma—or in the case of a neonate, maternal serum or plasma—may be used for the antibody screen.[47] In the antibody screen the patient's serum is mixed with group O reagent RBCs, which have most of the important blood group antigens. Because neonates are unlikely to produce new antibodies, the antibody screen must be performed only once per admission if the initial antibody screen is negative.[47] In the crossmatch the patient's serum is mixed with donor RBCs or "electronically" crossmatched using a validated computer system.[47] In patients older than 4

TABLE 36-3 Compatible Donors for Red Blood Cell Transfusion

Patients ABO/D	O Positive	O Negative	A Positive	A Negative	B Positive	B Negative	AB Positive	AB Negative
O positive	+	+						
O negative		+						
A positive	+	+	+	+				
A negative		+		+				
B positive	+	+			+	+		
B negative		+				+		
AB positive	+	+	+	+	+	+	+	+
AB negative		+		+		+		+

months of age, the crossmatch is performed for every unit of RBCs or whole blood transfused. In neonates if the initial antibody screen is negative, it is unnecessary to crossmatch donor RBCs.[47]

If a blood group alloantibody is detected in the recipient's serum, every attempt should be made to identify the antibody. The clinical significance of an antibody is based on knowledge of the specificity of the alloantibody and the reported experience in other patients whose serum contains the same antibody specificity (see Table 36-2).[44] If the antibody has potential clinical significance, antigen-negative blood should be selected and crossmatched by a procedure that includes the antiglobulin phase.[37,47]

Selection of Blood for Transfusion to Patients with Blood Group Alloantibodies

Many alloantibodies detected in vitro in human sera do not have the potential to induce in vivo destruction of incompatible RBCs. No technical procedure is able to differentiate, with certainty, between clinically benign and clinically significant antibodies. Although the in vitro characteristics of an antibody may not reflect its behavior in vivo, the following features can be used to help establish its potential significance in a patient. In general, antibodies of the IgG class are more clinically significant than those of the IgM class.[44] Also, the higher the titer of an antibody, the more likely it is to destroy antigen-positive RBCs in vivo. An antibody that causes in vitro hemolysis owing to complement activation (see Table 36-2) of antigen-positive RBCs is likely to be clinically significant. Antibodies that react at 37° C are more likely to be clinically significant than those that do not.[44] For cold-reacting IgM antibodies, the wider the thermal range of reactivity in vitro, the more likely it is to be clinically significant. Alloantibodies that react in the cold and are not usually clinically significant include antibodies to M, N, P_1, Le^a, and Le^b antigens.[128] In difficult cases a few reference laboratories can also perform a monocyte monolayer assay that has some value in predicting whether an antibody will cause a hemolytic transfusion reaction.[130]

Once a patient is actively immunized to an RBC antigen and produces a clinically significant alloantibody, the patient is considered immunized for life and must be transfused with RBCs lacking the corresponding antigen for life. Neonates with passively acquired maternal

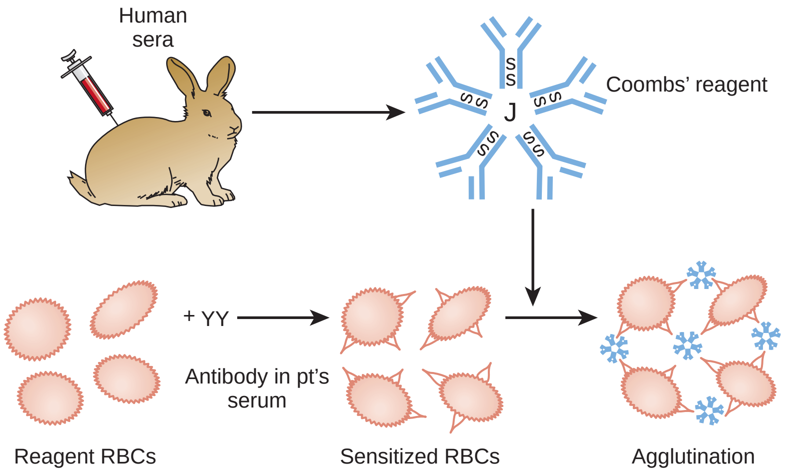

Figure 36-2 Antibody screen. Reagent red blood cells *(RBCs)* of known antigenic phenotypes are mixed with a patient's serum and Coombs' reagent. If the patient's cells agglutinate, the patient has an antibody to an antigen present on the reagent cells.

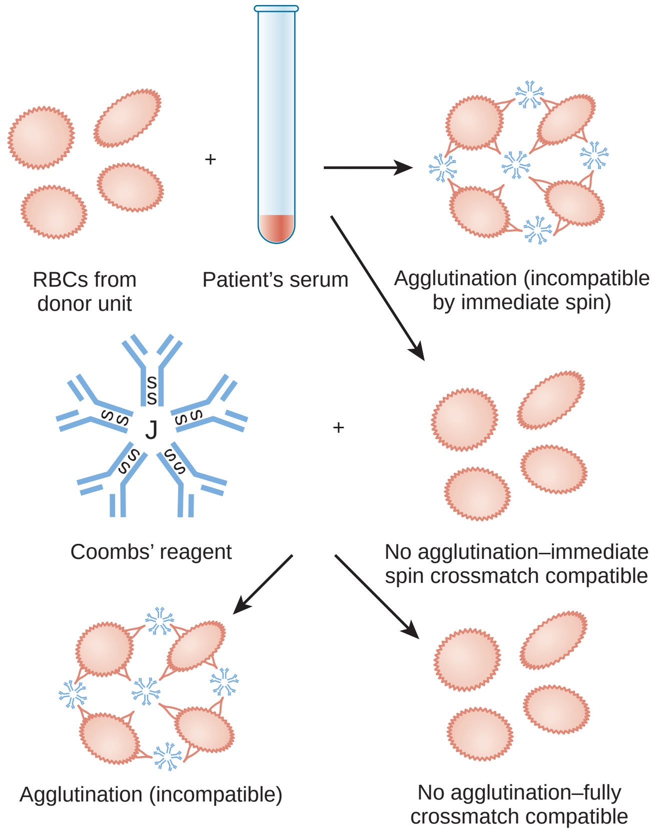
RBCs from donor unit
+
Patient's serum
Agglutination (incompatible by immediate spin)
S S
J
+
Coombs' reagent
No agglutination–immediate spin crossmatch compatible
Agglutination (incompatible)
No agglutination–fully crossmatch compatible

Figure 36-3 Compatibility procedures. Red blood cells *(RBCs)* from a donor unit are mixed with a patient's serum. If agglutination of the cells occurs, the cells are incompatible by the immediate spin technique. If a full crossmatch is performed, Coombs' reagent is added to the cells and serum and the cells are examined for agglutination.

antibody are not actively immunized and thus need to be transfused with antigen-negative RBCs only while the antibody is still present.

Emergency Need

When RBC transfusion is urgently needed in a rapidly bleeding patient, compatibility testing should be abbreviated so as not to delay transfusion. The clinician should indicate the need for emergency release of blood and send a pretransfusion patient blood sample to the blood bank. The blood bank can issue group O Rh(D–) RBCs and AB plasma or, if time permits, ABO type-specific RBCs. In some cases of inventory shortages, these patients, especially male ones, may receive Rh(D+) RBCs. In cases of emergency transfusion, the antibody screen and crossmatch may be performed after the blood has been transfused, and the clinician is notified of an incompatible crossmatch.[131]

Autologous Blood

Autologous blood transfusion is possible for some children who undergo elective surgery for which transfusion is anticipated. Transfusion of such blood reduces the risk of transfusion-transmitted viral diseases, but no blood transfusion is without risk. For example, autologous blood transfusions can be contaminated with bacteria or can cause volume overload, and there is always the risk of transfusing the incorrect unit. Intraoperative RBC salvage can be used if tumor cells or bacteria are not expected to contaminate the surgical field.

To donate autologous blood before surgery, a child must meet size or age requirements of the blood donor center, have adequate venous access, and have an Hb concentration of 11 g/dl or greater and a hematocrit of 33% or greater.[131] Some blood donor centers will collect small units that are proportional to the child's size. Donation should begin 4 to 5 weeks before surgery to allow time for in vivo RBC regeneration. Oral iron supplementation is often given. Recombinant erythropoietin is not routinely recommended for autologous blood donors and has not been licensed by the Food and Drug Administration for autologous donation, but it may be useful in certain circumstances.[132-135]

Directed Donations

Some institutions allow family and friends to direct their blood donations to be used for a child or infant. This is no safer than random banked blood and adds additional administrative expenses. GVHD can occur when the patient shares an HLA haplotype with an HLA homozygous blood donor, and this is more likely to occur if a patient receives blood from a relative.[28,29,136] Hence directed donations from blood relatives should be irradiated, and most institutions irradiate all blood donated by directed donors. Theoretically, blood from some female relatives has a higher chance of containing anti-HLA or antineutrophil antibodies that can contribute to transfusion-related acute lung injury (TRALI). Additionally, transfusion from a relative may induce antibodies to that relative's HLA antigens, complicating future donations of other tissues or organs from that relative.

Choice of Red Blood Cell Units for the Fetus

For intrauterine transfusions, group O Rh(D–) (and antigen-negative if mother has an alloantibody) RBCs should be used. To prevent graft-versus-fetus disease, blood is irradiated. To prevent CMV infection, CMV antibody–negative RBCs or leukocyte-reduced RBCs (or both) should be used.

Choice of Blood for the Neonate

Cellular components should be selected from CMV antibody–negative donors for neonate recipients weighing less than 1200 g at birth if their mother's are CMV antibody–negative.[137] Alternatively, leukocyte-reduced blood can also be used to prevent CMV infection if the blood product has less than 5×10^6 donor leukocytes.[138] Cellular blood components should be irradiated to prevent GVHD in these low-weight premature infants.[139]

In exchange transfusions for the neonate with HDN, RBCs used must be negative for the implicated antibody (e.g., Rh[D–] RBCs when hemolysis is caused by anti-Rh[D]). In terms of selection of ABO group, group O RBCs and plasma of the infant's ABO group are often used. Group-compatible RBCs and plasma can also be used if the RBCs are also compatible with any maternal antibodies that are present in the infant's circulation.[44]

TABLE 36-4 Approaches to Antigen Typing of Blood for Patients with Sickle Cell Disease

Approach	Advantages	Disadvantages
Treat like other patients (i.e., avoid antigens to which the patient has made antibodies)	Simple; avoids unnecessary work	Some patients will make antibodies that can result in serious transfusion reactions that may be especially dangerous in these patients Patients are probably at increased risk for the development of autoantibodies
Provide fully antigen-matched blood before the patient makes antibodies (i.e., matched for D, C, E, c, e, K, Fy^a, Fy^b, Jk^a, and Jk^b antigens). Note that antigen matching for Fy^b is not needed for patients of African descent who are Fy(a– b–) because they have the *FYB* gene, which is expressed in the lung and colon, and do not mount an immune response to the Fy^b antigen on transfusion of Fy(b+) RBCs	Reduces the risk of alloantibody and autoantibody formation and the risks associated with these antibodies	Significant work to reduce the risk for an antibody that may never develop; may be difficult to identify units with extended phenotype matches
Provide antigen-matched blood after the first antibody is made	Reduces the chance of multiple antibodies with less work than extended phenotype matching for all patients	Some patients will make multiple antibodies quickly
Provide partially antigen-matched blood (i.e., matched for D, C, E, and K antigens)	Reduces the chance of multiple antibodies with less work than extended phenotype matching that includes antigens that are not very immunogenic	Some patients will make antibodies against unmatched antigens

RBCs, Red blood cells.

Choice of Blood for Patients with Sickle Cell Anemia

Two important principles must be followed in the transfusion of patients with sickle cell anemia: (1) avoidance of excessive viscosity and (2) prevention of sensitization to RBC antigens.

Sickle cells are intrinsically less deformable than normal cells; thus elevating the hematocrit concentration without significantly reducing the proportion of sickle cells may raise the blood viscosity to dangerous levels.[140] Simple transfusion must be used with caution, particularly in children with hematocrit concentrations greater than 30%.

The question of what to use to transfuse patients with sickle cell anemia has been the subject of many heated debates.[141-143] There is still no consensus as to the best and most practical approach. Patients with sickle cell anemia, like all patients, are at risk for developing alloantibodies, and alloantibodies may pose specific hazards to patients with sickle cell disease. Patients with sickle cell disease usually need many transfusions throughout their lives, and an antibody to a blood group alloantigen that develops in childhood may be undetectable later in their lives. If they are then transfused with blood expressing that antigen, they are likely to develop an anamnestic response to the antigen and develop a delayed hemolytic transfusion reaction. Patients with sickle cell anemia have been reported to develop painful crises secondary to delayed hemolytic transfusion reactions. Additionally, a patient with sickle cell anemia who develops RBC alloantibodies may be at increased risk for the development of RBC autoantibodies that can significantly complicate the patient's disease.[143] For these reasons some transfusion services provide phenotype-matched blood for patients with sickle cell disease. Approaches that are used include those described in Table 36-4. Note that none of these approaches will prevent the production of antibodies to high-incidence antigens, such as U, Js^b, Cr^a, hr^S, and hr^B. African Americans tend to be null for antigens commonly expressed in the general donor population, which is mostly Caucasian in the United States. For this reason some blood donor facilities have programs to recruit African Americans to donate blood to support the transfusion needs of patients with sickle cell anemia. One potential risk of this approach is that patients are then likely to be exposed to many more Rh variant genes that are prevalent in the African-American population.

Thalassemia

In thalassemia the goals of transfusion therapy are the prevention of anemia and the maintenance of a circulating Hb level that is sufficient to suppress endogenous erythropoiesis. For most patients maintenance of a pretransfusion Hb level of 9 to 10 g/100 mL is sufficient.[144] RBC alloantibodies are not a significant problem in most patients with thalassemia, possibly because their transfusions are usually initiated at an early age when tolerance may develop or because they don't generally develop crises associated with hemolysis, as occurs in sickle cell disease. Therefore most institutions in the United States do not provide RBCs that are phenotypically matched to the patient's red cells.[145,146]

Choice of Blood for Allogeneic Hematopoietic Progenitor Cell Transplants

Because an HLA-identical donor is usually required for allogeneic hematopoietic progenitor cell (HPC) transplantation, the donor may not have the same ABO and

TABLE 36-5 Choice of Blood for Bone Marrow Transplant Patients

Recipient	Donor	Red Cells
A	O	O
A	B	O
A	AB	A or O
B	O	O
B	A	O
B	AB	B or O
AB	O	O
AB	B	B or O
AB	A	A or O
O	A	O
O	B	O
O	AB	O

Rh type as the patient.[37] In major ABO incompatibility, the recipient has antibodies against donor RBCs (e.g., recipient is group O and has anti-A and anti-B; donor is group A). In minor ABO incompatibility, the donor has antibodies against recipient RBCs (e.g., donor is group O and has anti-A and anti-B; recipient is group A). These incompatibilities may produce hemolysis,[44] but hemolysis is often minimized by proper selection of blood products for transfusion.

In major ABO incompatibility (e.g., donor is group A, recipient is group O), host-versus-graft reactions can cause delayed erythropoiesis and even RBC aplasia.[147] To prevent recipient destruction of donor RBCs at the time of HPC infusion, donor RBCs are removed from the HPC before infusion. The group O recipient may continue to produce anti-A and anti-B after transplantation and cause hemolysis of the newly produced donor group A RBCs. If transfusions are necessary during this period, group O RBCs can be given (Table 36-5). Before transplantation the group O recipient has anti-A and anti-B. After engraftment of group A donor marrow, the anti-B will persist but the anti-A will become undetectable. Donor group A RBCs are given only after the recipient stops producing anti-A—that is, when the recipient's group O blood type changes to the donor group A and anti-A is not detected in the patient's serum.

In minor ABO incompatibility, hemolysis of recipient RBCs by donor antibody is usually preventable. To prevent hemolysis of recipient RBCs by passively transfused donor antibody during HPC infusion, plasma is removed from the HPC preparation before infusion. In addition, passenger B lymphocytes in the donor HPC may produce antibodies that may destroy recipient RBCs. To prevent hemolysis of transfused RBCs, group O RBCs are used for transfusions during the posttransplant period if the antibody is detected. If the donor or recipient has a clinically significant antibody to an erythrocyte antigen, blood lacking the antigen should be given after transplantation.[47]

Choice of Blood for Organ Transplantation

The ABO antigens are strong histocompatibility antigens and thus are of major importance in organ transplantation. Because the ABO antigens are expressed on the vascular endothelium, the recipient can reject and damage vascular endothelium of a donor organ that is a major ABO mismatch. For this reason, major ABO-incompatible vascular organ (liver, kidney, heart, lungs, pancreas) transplants are usually not performed. However, ABO-incompatible heart transplants may be performed in infants and young children who have not yet produced isohemagglutinins or who have produced only low levels of isohemagglutinins.[148]

When organ transplants are performed in the presence of minor ABO incompatibility, the same hemolytic problems as in HPC transplant may occur.[149] Passenger B lymphocytes may produce antibodies against recipient RBCs. Hemolysis of transfused RBCs can usually be prevented when an antibody is detected, by transfusion of group O RBCs for ABO incompatibility and transfusion of antigen-negative RBCs for incompatibility owing to other blood groups.

Selection of Blood for Transfusion to Patients with Blood Group Autoantibodies

Autoimmune hemolytic anemia is described in detail in Chapter 13. In hemolytic anemia resulting from warm-reactive autoantibodies, no compatible blood can usually be found. The direct antibody test ([DAT]; Fig. 36-4) will almost always be positive in autoimmune hemolytic anemia. Blood bank serologic testing is aimed at determining whether underlying clinically significant alloantibodies are present and identifying the specificity of any such antibodies that exist. This can be determined, with time, at a hospital transfusion service, which may require that the testing be performed at an immunohematology reference laboratory. Patients without a history of transfusion or pregnancy are unlikely to have underlying alloantibodies. Transfused RBC units should be compatible with any underlying clinically significant alloantibodies.

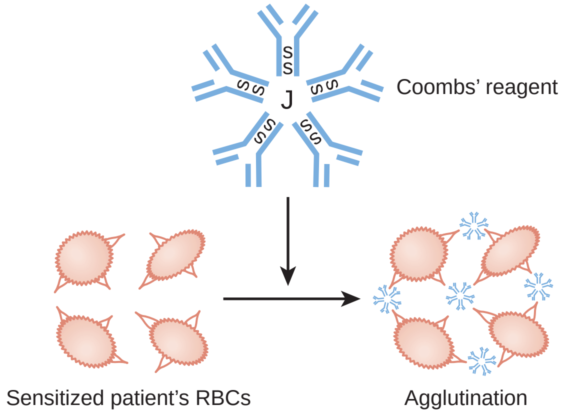

Figure 36-4 Direct antiglobulin test. Red blood cells *(RBCs)* are incubated with Coombs' reagent; if the cells agglutinate after incubation, the direct antiglobulin test is positive.

However, the blood is still usually incompatible with the patient's serum, and the clinically necessary amount of RBCs should be transfused with careful monitoring but without delay. In some patients the warm autoantibody has a clear-cut specificity for an antigen, and antigen-negative RBCs may be compatible in the crossmatch test.[150]

In paroxysmal cold hemoglobinuria associated with the Donath-Landsteiner antibody, transfusion is not usually necessary. The antibody binds RBCs only at cold temperatures (<20° C). In theory, it is reasonable to transfuse blood through a blood warmer; however, in practice crossmatch-compatible, unwarmed random RBCs have been given without problem.[151] Only rarely is transfusion of RBCs of the rare phenotype p needed.[152,153] Most cases of paroxysmal cold hemoglobinuria in children are transient and occur after viral infections.

In cold agglutinin disease, a rare condition in children, RBCs should be transfused through a blood warmer. As described previously, the same issue of testing for underlying alloantibodies pertains here.

Adverse Effects of Transfusion

All reactions other than mild allergic (urticarial) reactions require the immediate cessation of the transfusion and sending of a fresh blood sample from the patient to the blood bank for serologic evaluation.

Allergic Reactions

The typical urticarial reaction is characterized by itching and hives. Localized urticaria simply requires interrupting the transfusion and administering an antihistamine. If the symptoms resolve within 30 minutes the transfusion can be continued. Recurrent allergic reactions may require pretreatment with antihistamine.

Some children exhibit more severe allergic reactions, such as laryngospasm and bronchospasm. These patients may require steroids, epinephrine, or both (Table 36-6). The same unit of blood should not be restarted, even after symptoms have responded to treatment. Saline-washed blood products may be helpful in patients with severe or recurrent allergic reactions. Anaphylactic reactions may occur if the patient is IgA deficient and has formed an

TABLE 36-6 Guidelines for the Management of Transfusion Reactions

Agent	Use	Dose	Comment
Diphenhydramine	Treating pruritus and rash (hives)	6.25 to 25 mg IV over period of 10 to 20 min	Diphenhydramine cannot prevent and has no place in the treatment of severe transfusion reactions
Epinephrine	Severe reactions characterized by bronchospasm, hypotension, and shock	0.1-0.4 mg SC (or IV if the patient is hypotensive)	For patients in shock not responding to epinephrine, treatment with an initial fluid bolus should be initiated; as in patients with shock from other causes, fluids, vasopressors, airway protection, and oxygenation should also be used
1:20 Aqueous suspension of epinephrine	After stabilization with epinephrine	0.05-0.2 mg SC	
Fluids			For hypotensive patients, a bolus of 20 mL/kg of normal saline should be administered simultaneously with epinephrine and steroids
Narcotics	Specific and effective treatment of rigors	0.1 mg/kg of morphine IV (or equivalent dose of meperidine hydrochloride)	
Acetaminophen (Tylenol)	Prevention or reversal of elevations in temperature in mild to moderate febrile reactions	Appropriate for age	
Steroids	In moderate to severe reactions; may occasionally be required for severe urticaria; indicated in all reactions characterized by fever, shaking chills, diaphoresis, and pallor	1 to 2 mg/kg of methylprednisolone (or equivalent dose of dexamethasone or hydrocortisone by intravenous push)	

IV, Intravenously; *SC,* subcutaneously.

IgE anti-IgA antibody. In these cases, either washed blood products or products drawn from rare IgA-deficient donors are indicated.

Febrile Nonhemolytic Reactions

The occurrence of fever, chills, or diaphoresis usually results from cytokines and other pyrogens released from leukocytes and platelets.[154] Cytokines can be released from leukocytes during storage or on infusion to a patient who has antibodies that react with antigens on foreign white blood cells. Febrile nonhemolytic transfusion reactions are not associated with serious sequelae and may be treated with antipyretics. Of note, fever during a transfusion may represent another, more serious types of transfusion reaction: acute hemolysis, sepsis from a bacterially contaminated unit, or TRALI.

Acute Hemolytic Transfusion Reactions

Acute hemolytic transfusion reactions are most often the result of donor-recipient ABO incompatibility. These reactions are generally caused by managerial errors, such as the misidentification of a transfusion recipient.[155,156] The clinical presentation of acute hemolytic transfusion reactions varies. Signs and symptoms may include any or all of the following: fever, chills, dyspnea, tachycardia, hypotension, flank pain, abdominal pain, pain at the infusion site, apprehension, nausea, vomiting, and hemoglobinuria. Other serious sequelae may include acute renal failure, shock, and disseminated intravascular coagulation (DIC), which may present as bleeding. Some acute hemolytic reactions are characterized by only minor initial symptoms. In anesthetized recipients the only manifestations may be diffuse bleeding (owing to DIC) or red urine (hemoglobinuria). When an acute hemolytic transfusion reaction is suspected, the transfusion should be stopped immediately and an investigation should be initiated. The identity of the patient and unit should be reconfirmed. The remainder of the unit and a fresh blood sample should be sent to the blood bank, where the required workup includes the following: (1) a clerical check, (2) visual inspection of the recipient's plasma for hemolysis, and (3) a DAT. If there is no hemolysis in the patient's plasma or urine and the DAT result is negative, immune-mediated hemolysis can be excluded.

Therapy of an acute hemolytic reaction is supportive. Depending on the size of the child, a urine output of 40 to 100 mL/h should be maintained. Early consultation with nephrologists is warranted. Intravenous administration of furosemide or mannitol, which increases renal blood flow, may be required.

Delayed Hemolytic Transfusion Reactions

Delayed hemolytic transfusion reactions are of two different types: primary immunization and anamnestic response. Primary immunization is mild and occurs weeks after transfusion. It rarely causes significant hemolysis and may be suspected after an unexplained decrease in Hb level 2 to 3 weeks after transfusion.

Secondary immune responses occur in patients who have been sensitized to one or more non-ABO blood group antigens during a prior transfusion or pregnancy. When a patient is reexposed to the antigen, an anamnestic response occurs, resulting in a rapid rise in antibody level and accelerated clearance of the transfused antigen-positive RBCs. These patients experience some of the symptoms of a hemolytic reaction 3 to 10 days after transfusion. The clinical syndrome is usually mild, but delayed transfusion reactions can result in profound anemia. These reactions should be suspected in patients who have received multiple transfusions and develop unexplained anemia or bilirubinemia (or both) 3 to 10 days after transfusion. The diagnosis is confirmed by a positive DAT result and identification of RBC alloantibodies in the patient's serum or on the patient's RBCs (or both).

Transfusion-Associated Acute Lung Injury

TRALI presents with some combination of fever, chills, dyspnea, cyanosis, hypotension, and bilateral pulmonary infiltrates.[157,158] The reported incidence is 1 in 5000 transfusions but may be lower in pediatric patients.[159] Many cases appear to result at least in part from antibodies in donor plasma that react with patient HLA or neutrophil antigens. Blood banks minimized the use of plasma from female subjects that is most likely to contain such antibodies, resulting in a significant reduction in TRALI incidence. TRALI is often self-limited, with resolution in 48 to 96 hours in most patients who receive supportive care such as supplemental oxygen and often mechanical ventilation. However, in approximately 20% of patients TRALI can be severe and recovery prolonged; some cases, in fact, are fatal.

Transfusion-Associated Circulatory Overload

Because blood components have high oncotic pressure, transfusion-associated circulatory overload (TACO) may occur, resulting in pulmonary edema. This is mostly seen in adults, although very young children may also be at risk. TACO is treated with diuretics, phlebotomy, and supplemental oxygen.[160]

Transfusion-Associated Gut Injury

Several case series reports have found an association between RBC transfusions and the development of necrotizing enterocolitis (NEC) in premature neonates.[161,162] Although these studies suggest an association, they do not prove that NEC is caused by transfusion and not by some other factors that occur around the time of RBC transfusions.[163]

Transfusion-Transmitted Diseases

Because of improvements in both donor screening and product testing, blood products are currently extremely safe. But even though the risks of transfusion-transmitted disease have been greatly reduced, they have not been eliminated entirely. Currently, all blood donations in the United States are tested for the presence of human immunodeficiency virus 1 and 2 (HIV-1 and HIV-2), hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell lymphotropic virus 1 and 2 (HTLV-1 and HTLV-2), West Nile virus (WNV), and syphilis, and all blood donors are tested for Chagas disease at least once. The battery of tests performed includes a combination of serologic tests and nucleic acid tests (NATs) that taken together are exquisitely sensitive. However, very recent infections may be missed (so-called window-period donations). The risks listed in the subsequent sections and summarized in Table 36-7 are based on mathematical models estimating the number of infectious donors whose blood tests negative for a disease because they are in the window period.

TABLE 36-7 Estimated Risk of Transfusion-Transmitted Diseases

Agent	Number of Infections/Million Units
HIV	0.5
HCV	0.5
HBV	2 to 5
HTLV	0.5 to 4

HBV, Hepatitis B virus; *HCV,* hepatitis C virus; *HIV,* human immunodeficiency virus; *HTLV,* human T-cell lymphotropic virus.

Posttransfusion Hepatitis

Hepatitis B Virus. The estimated risk of posttransfusion HBV infection is presently 1 in 200,000 to 1 in 500,000 units transfused.[164] Acute HBV infection is symptomatic in approximately 50% of adults, and 5% to 10% of those infected become chronic carriers. In contrast, HBV infection acquired in early childhood is usually asymptomatic and leads to a chronic-carrier state in more than 70% of those infected.[165]

Hepatitis C Virus. With the routine use of NAT screening, the per-unit risk of HCV is currently estimated to be about 1 in 2,000,000.[164] Children who receive HCV-infected blood products have a 30% to 50% chance of spontaneously clearing their infection.[166] Most others become chronically infected. Approximately 20% of children who have been infected with HCV develop signs of cirrhosis after 18 years of age.[167,168]

Human Immunodeficiency Virus. All units are tested for HIV-1 and HIV-2 by immunoassay as well as by NAT. With NAT the window period for HIV has been reduced to approximately 10 days. The per-unit risk of HIV infection is currently estimated to be 1 in 2,000,000.[164]

Human T-Cell Lymphotrophic Virus. HTLV is the causative agent for both adult T-cell leukemia and endemic spastic myelopathy.[169,170] Most cases have been reported in residents of or emigrants from Japan, Africa, and the Caribbean region. The most comprehensive report of transfusion-transmitted HTLV indicated that 65% of patients who received whole blood or cellular products from HTLV antibody–positive donors seroconverted. Blood from all donations is tested for HTLV-1 and HTLV-2; the current per-unit risk is estimated to be 1 in 641,000.[171]

West Nile Virus. WNV is a flavivirus common in Africa, West Asia, and the Middle East. Approximately 20% of infected individuals will develop a febrile illness. Rarely, WNV infection causes an encephalitis syndrome; elderly and immunocompromised individuals are those primarily at risk. WNV has recently become established in North America. The vast majority of cases are transmitted by mosquito bites, but transfusion transmission does occur in rare cases. As with HIV and HCV, NAT has almost completely eliminated WNV from the U.S. blood supply.

Cytomegalovirus. CMV is carried in and transmitted by lymphocytes. In most patients transfusion-associated CMV infection causes no symptoms, although it may cause a mild mononucleosis-type illness 3 to 4 weeks after transfusion. Transfusion of CMV-containing blood products into an immunocompromised host with no serologic evidence of prior exposure to CMV can cause a lethal systemic infection. Patients at risk for severe CMV infection include bone marrow transplant patients, organ transplant candidates and recipients, and high-risk neonates. Therefore patients who have never been infected with CMV and have an inherited or acquired immunodeficiency state should receive CMV-negative or leukoreduced RBCs.[172]

Babesia. *Babesia* spp., especially *Babesia microti,* is responsible for more transfusion-transmitted infectious diseases than any other pathogen in the United States. Many infections are asymptomatic, but disease can occur, especially in immunocompromised patients such as premature infants and patients without functional spleens. Symptoms generally occur 1 to 9 weeks after infection and can include fever, headache, chills, sweats, myalgia, malaise, and hemolytic anemia; symptoms have progressed to death in at least 12 cases of transfusion-transmitted babesiosis in the USA.[173] Although transfusion-transmitted babesiosis predominantly occurs in the endemic areas of the northeast and upper midwest United States, it is not restricted to those regions because both blood donors and units of blood travel.

Bacteria (Platelet Units). Platelets are associated with the same range of infectious pathogens as any other blood component, but septic transfusion reactions, caused by bacterially contaminated units, comprise a unique risk of platelet transfusion. In contrast to all other blood components, which are stored either at 4° C or frozen, platelets are stored at room temperature. The reason is that if platelets are chilled before transfusion, they are cleared rapidly from the recipient's circulation.[174] Although room temperature storage dramatically improves platelet survival in vivo, it also tends to promote bacterial growth. Currently, bacteria can be cultured from approximately 1 in 5000 apheresis platelet units.[175] In the United States blood donor centers generally use culture methods to screen for bacteria in all platelet products, although alternative rapid testing methods are also available. Using current screening methods, the per-unit risk of a clinically apparent septic transfusion reaction is estimated to be 1 in 75,000 platelet transfusions.[164]

PLATELET TRANSFUSIONS

Indications for Transfusion

Bone Marrow Failure

Bone marrow failure resulting in thrombocytopenia is the major reason for patients to receive platelet transfusions. Iatrogenic induction of bone marrow failure, secondary to chemotherapy or irradiation, is the most common cause of bone marrow failure in all age groups. Patients with malignancies involving the bone marrow (e.g., leukemia, lymphoma, neuroblastoma, metastatic disease from solid tumors, myelofibrosis) may also require platelet support. The decreased marrow production in these diseases is caused by displacement of megakaryocytes with malignant elements. In some cases the tumor cells produce substances (e.g., tumor necrosis factor) that inhibit the growth of normal marrow cells. If there is no evidence of platelet dysfunction, patients usually do not experience active bleeding if the platelet count is greater than 10,000/mm^3 and have minimal risk for spontaneous life-threatening hemorrhage until the count is less than or equal to 5000/mm^3.[176-180] Although the bleeding rate is higher in patients undergoing pediatric oncology and bone marrow transplant procedures, the bleeding risk is not closely associated with the platelet count, and transfusion triggers of 10,000/mm^3 are still recommended for pediatric patients.[181] However, additional coagulation abnormalities, such as acquired or congenital platelet dysfunction, clotting protein deficiencies, and vascular or tissue defects, require more aggressive platelet support to decrease the risk of hemorrhage.

Patients with primary bone marrow failure caused by a defect such as Fanconi anemia or aplastic anemia will also be thrombocytopenic. However, platelets are usually withheld from these patients unless they are actively bleeding because they have a high rate of alloimmunization to HLA antigens, which in turn will significantly reduce the success of future transfusions and complicate bone marrow transplants. Studies conducted before the introduction of modern leukoreduction filters found that exposure of patients with aplastic anemia to more than five platelet donors results in a significant reduction in successful transplantation owing to rejection of the donor bone marrow.[182] Although it is unclear whether this finding applies to current leukoreduced platelets, if these patients do require treatment for bleeding, it is best to use irradiated, single-donor, leukoreduced platelets in an attempt to minimize sensitization. The decision to transfuse a patient who has been newly diagnosed with aplastic anemia should not be taken lightly because even a single exposure to transfused platelets may adversely affect long-term survival if bone marrow transplantation fails. Once the patient has become refractory to single-donor and HLA-matched platelets, family donors might also undergo plateletpheresis if the patient is *not* a candidate for bone marrow transplantation.

Platelet Destruction

Shortened platelet survival may result from immune-mediated destruction or nonimmune mechanisms, leading to accelerated platelet consumption (Table 36-8). In general, prophylactic platelet transfusions are contraindicated in these patients because the infused platelets are rapidly destroyed and thus a “safe” platelet count cannot be maintained. Therefore platelet transfusions are reserved for episodes of active bleeding or in preparation for surgical procedures.

TABLE 36-8 Platelet Alloantigen Phenotype and Frequency

Alloantigen System	Glycoprotein Location	Allelic Forms	Phenotypic Frequency
HPA-1 (P1^{A}, Zw)	GPIIIa Leu–Pro33	HPA-1a (P1^{A1})/ HPA-1b (P1^{A2})	72% a/a 26% a/b 2% b/b
HPA-2 (Ko, Sib)	GP1b Thr–Met145	HPA-2a (Ko^{b})/ HPA-2b (Ko^{a})	85% a/a 14% a/b 1% b/b
HPA-3 (Bak, Lek)	GPIIb Ile–Ser843	HPA-3a (Bak^{a})/ HPA-3b(Bak^{b})	37% a/a 48% b/b 15% b/b
HPA-4 (Pen, Yuk)	GPIIIa Arg–Gln143	HPA-4a (Pen^{a})/ HPA-4b(Pen^{b})	99% a/a <0.1% a/b <0.1% b/b
HPA-5 (Br, Hc, Zav)	GPIa Glu–Lys505	HPA-5a (Br^{b})/ HPA-5b (Br^{a})	80% a/a 19% a/b 1% b/b
HPA-6 (Ca, Tu)	GPIIIa Arg–Gln489	HPA-6a (Ca^{b})/ HPA-6b (Ca^{a})	99% a/a <1% a/b <1% b/b
HPA-7 (Mo)	GPIIIa Pro–Ala407	HPA-7a (Mo^{b})/ HPA-7b (Mo^{a})	99% a/a <1% a/b <1% b/b
HPA-8 (Sr^{a})	GPIIIa Arg–Cys636	HPA-8a (Sr^{b})/ HPA-8b (Sr^{a})	99% a/a <1% a/b <1% b/b

Nonimmune Mechanisms. Nonimmune mechanisms include the utilization of platelets in areas of endothelial damage, such as hemolytic-uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP), or NEC. DIC results in decreased platelet life span owing to consumption of platelets and activated clotting factors, such as fibrinogen in the formation of microthrombi in association with sepsis, shock, acidosis, or severe head trauma. In these syndromes it is essential to assess the risk of bleeding in determining the need for transfusion. Infants with NEC are often transfused more aggressively because many demonstrate marked gastrointestinal blood loss and may have a preexisting central nervous system hemorrhage associated with prematurity. Similarly, patients with DIC are often given platelets even though their counts may not be less than 10,000/mm^3, owing to consumption of clotting factors and evidence of ongoing bleeding. Successful outcome for these patients depends on elimination of the underlying disease—for example, treating sepsis with antibiotics, restoration of organ perfusion in states of shock, or removal of necrotic tissue. Patients with large cavernous sinus hemangiomas

(Kasabach-Merritt syndrome) have localized consumption of platelets and clotting factors, resulting in a laboratory picture of DIC.[183-185] Until the lesion is removed or begins to involute, the platelet and clotting factor consumption will continue. These patients usually do not require transfusion for thrombocytopenia because the high rate of platelet turnover results in younger and more hemostatically effective platelets in circulation. Finally, in TTP and HUS, platelet infusions are withheld as long as possible because it is thought that the infused platelets would lead to further hemolysis and organ damage by contributing to the formation of microthrombi in areas of endothelial damage.[186]

Hypersplenism may result in poor platelet survival owing to trapping of platelets in the expanded splenic bed. A more complex clinical situation arises with acute or chronic liver disease.[187] Splenic pooling, DIC, and poor platelet production may all contribute to the thrombocytopenia of hepatic disease. The presence of portal hypertension and esophageal varices from cirrhosis may be an indication to consider platelet transfusion in the presence of gastrointestinal bleeding or when the platelet count falls below 20,000/mm^3.[188,189] Acquired platelet dysfunction is associated with both acute and chronic liver disease owing to decreased hepatic clearance of circulating fibrin degradation products. When platelet transfusion is required, splenomegaly may affect initial platelet recovery measured in the first hour after transfusion, but not platelet survival as determined by the platelet count 24 hours later.[188-191]

Immune-Mediated Thrombocytopenia. In immune thrombocytopenia antibodies bind to epitopes on platelet membranes resulting in rapid removal of the platelets from the circulation. These antibodies are either "self-reactive" autoantibodies or specific for alloantigens, polymorphic epitopes expressed on platelet membrane receptors. Alloantibodies cause thrombocytopenia in neonatal alloimmune thrombocytopenia and posttransfusion purpura (PTP), both of which are discussed later in this chapter. Alloantibodies responsible for poor response to platelet transfusion are discussed in the section on platelet refractory state.

Autoantibody-Mediated Thrombocytopenia. Autoantibody to platelets may be present in association with other diseases, such as systemic lupus erythematosus, Epstein-Barr virus infection, CMV infection, varicella virus infection, multiple sclerosis, diabetes, thyroid disease, and drug-induced autoantibody as seen with valproic acid. It may also be present without other disease associations, as in idiopathic thrombocytopenic purpura. In either setting there is rapid removal of antibody-coated platelets in the spleen and, to a lesser extent, in the liver. Immune complexes may nonspecifically adhere or bind by means of Fc receptors on the platelet surface. A particularly interesting example of the latter is the entity heparin-induced thrombocytopenia (HIT).[192] In this case patients exposed to heparin therapy produce antibodies directed to platelet factor 4 (PF4) bound to heparin. Immune complexes of PF4-heparin and antibody bind via Fc receptors to platelets, causing platelet activation and a prothrombotic state in which platelets are consumed. This may be a devastating complication of heparin therapy that may be suspected in patients who show falling platelet counts a few days after initiating heparin therapy. Platelet transfusions are generally contraindicated insofar as they may exacerbate the prothrombotic state.

In states that increase platelet turnover, the bone marrow responds by increasing megakaryocyte number, size, and ploidy. This, in turn, results in fresher platelets that are slightly larger in size and represent a younger population than normal owing to the rapid turnover of thrombocytes. Thus the bleeding time in patients with immune-mediated thrombocytopenia is shortened, relative to the absolute number of platelets, and such patients rarely experience major bleeding even though the platelet count may fall below 10,000/mm^3.[193] Autoantibodies that interfere with functional epitopes on platelet glycoprotein receptors, such as in acquired Glanzmann thrombasthenia, are a notable exception to this rule. In this setting patients present with bleeding even with platelet counts greater than 20,000/mm^3.

In patients with rapid consumption of platelets by autoantibody, platelet transfusions are generally not indicated unless there is evidence of bleeding, regardless of the platelet count. Central nervous system and gastrointestinal bleeding may be the presenting features of autoantibody-mediated thrombocytopenia and require immediate aggressive therapy, including platelet transfusion. Although the platelets are rapidly consumed, active bleeding may stop or decrease significantly. Platelet transfusions should be used judiciously in combination with therapy aimed at reducing autoantibody production and blocking reticuloendothelial cell clearance of antibody-coated cells. The therapy for immune-mediated thrombocytopenia is discussed elsewhere.

Alloantibody-Mediated Thrombocytopenia—Neonatal Alloimmune Thrombocytopenic Purpura. In neonatal alloimmune thrombocytopenic purpura (NATP), maternal antiplatelet alloantibody has crossed the placenta, resulting in thrombocytopenia in the fetus and neonate. Harrington and Sprague first reported the observation that pregnant women may become alloimmunized to antigens expressed on fetal platelets.[194] Even during the first pregnancy, maternal antiplatelet alloantibodies can cross the placenta and cause profound thrombocytopenia in the fetus and newborn. In North America the alloantibodies identified are most commonly directed against human platelet antigen 1a (HPA-1a, also known as PLA-1). A complete list of platelet alloantigens and their frequency in those populations that have been studied thus far are listed in Table 36-8. The presentation of thrombocytopenia in a healthy infant whose mother has a normal platelet count is often the result of maternal alloantibodies.

The diagnosis of NATP is made with the demonstration of antiplatelet antibody in maternal plasma, reactive with *paternal* platelets. Maternal plasma is also screened to rule out the presence of autoantibody. More than 50% of the platelet-specific alloantibodies in NATP are reactive with the HPA-1a epitope on glycoprotein IIIa.[195] Healthy infants may not bleed or develop petechiae until the platelet count drops below 20,000/mm^3. Reports suggest that immune response genes may also play a role in platelet alloimmunization.[196-198] On the basis of these studies, there appears to be an increased incidence of alloimmunization against HPA-1a in mothers positive for HLA-B8 and HLA-DR3.

The diagnosis of maternal alloimmunization is usually made after the birth of a mother's first thrombocytopenic infant. Women who are then identified as sensitized to a platelet-specific alloantigen should be monitored very closely throughout subsequent pregnancies. Intermittent screening of maternal serum should be performed similar to prenatal Coombs testing in women who are Rh negative. All women in the mother's family should also be evaluated before or during their pregnancies to determine whether they might also be at risk of alloimmunization. If a HPA-1a–negative woman is also HLA-DR3 (DRw52) positive, she has a greatly increased risk of alloimmunization even during her first pregnancy.[198-200]

To determine whether the infant is at risk for thrombocytopenia secondary to maternal alloantibody, it is advantageous to establish the platelet phenotype of the fetus early in pregnancy. As early as the eighth week of gestation, DNA from nucleated cells obtained from chorionic villus sampling can be amplified through the use of the polymerase chain reaction. DNA typing is then determined with glycoprotein-specific oligonucleotide primers and probes unique for each alloantigen sequence or phenotype.[200-202] Results from a prospective study described a 100% correlation between DNA typing and subsequent serologic response in mothers with alloantibodies to PlA1 or Bak.[203] This technique is useful only where the nucleotide sequences that define the polymorphisms are known (see Table 36-8).[201]

Because of the high frequency of these alloantigens, random donor platelets are usually ineffective in treating alloimmune thrombocytopenia in the neonate. When platelets are given, it is best to use platelets lacking the implicated antigen. If the antigen is unknown or antigen-negative platelets are unavailable, then washed, irradiated *maternal* apheresis platelets can be used because they lack the alloantigen. Washing is required to remove maternal plasma that contains the alloantibody. *Irradiation is routinely performed on all blood products from first-degree relatives where there is an increased risk of GVHD, particularly in neonates*. When severe thrombocytopenia in the newborn is anticipated, antigen-negative platelets may be procured before elective delivery and made available for transfusion shortly after birth for infants with NATP diagnosed prenatally. Intravenous immunoglobulin has also been used successfully in the newborn.[204-208] In families with no history of prenatal intracranial hemorrhage, fetal platelet count should be closely monitored and ultrasonography performed to rule out bleeding before delivery.[209]

Platelet Transfusions for Surgery

Retrospective data suggest that a platelet count above 50,000/mm^3 is acceptable for a patient to undergo major surgery.[210] Certain circumstances may warrant more aggressive correction of thrombocytopenia before or after surgery. In areas where even a small amount of bleeding might cause permanent deficits, such as the eye or central nervous system, it is traditional to aim for a platelet count higher than 100,000/mm^3. During prolonged procedures where the patient will be on cardiopulmonary bypass or extracorporeal membrane oxygenation (ECMO), platelets may be dysfunctional owing to activation after passage through extracorporeal perfusion equipment. In general, there is no need to administer platelets to patients after extracorporeal circulation if there is no evidence of bleeding and the platelet count is at least 50,000/mm^3.[211-213] When bleeding is present, it is more commonly caused by heparin, inadequate protamine correction, fibrinolysis, or problems with surgical closure.

Minor procedures such as liver biopsies have been performed safely with platelet counts greater than 25,000/mm^3.[188] Similarly, lumbar puncture has been shown to be safe in the setting of thrombocytopenia. Howard and colleagues[214] reported a series of 956 consecutive pediatric patients with newly diagnosed acute lymphoblastic leukemia who underwent lumbar puncture. No serious bleeding complications occurred in 5223 lumbar punctures, including 170 procedures performed when the platelet count was 11,000 to 20,000/μL. The authors concluded that prophylactic platelet transfusion was unnecessary in patients with platelet counts above 10,000/μL. These procedures were performed in only 29 patients with platelet counts below 10,000/μL, so it was not possible to assess with confidence the risk of bleeding in patients with extremely low platelet counts.

Platelet Transfusions for Patients with Dysfunctional Platelets

Patients with congenital platelet dysfunction, such as Glanzmann thrombocytopathy or Bernard-Soulier syndrome, may experience episodes of profuse bleeding. In this setting platelets may be administered to control massive hemorrhage. Because normal platelets express proteins that these individuals lack, some patients develop isoantibodies to these receptors and eventually become refractory to all platelet transfusions. Efforts to minimize menstrual bleeding using hormonal suppression may decrease the number of platelet infusions in women. Bleeding in patients with congenital platelet dysfunction caused by abnormal platelet secretion may respond to desmopressin, avoiding exposure to blood.[215,216]

Acquired platelet dysfunction in children is most commonly caused by drugs, chronic renal failure, or antiplatelet antibodies. Rarely, children present with platelet dysfunction as a manifestation of myelodysplastic syndrome or chronic leukemia. Platelet transfusions may be used if the patient is actively bleeding, but eventually the transfused platelets will be affected by exposure to drug, uremia, or antiplatelet antibodies, which limit their survival and function. For this reason platelets are not given prophylactically in these patients. Every effort should be made to ameliorate the platelet dysfunction so that exposure to blood product is avoided. Patients with uremia may respond to infusions of desmopressin, and those with antiplatelet autoantibodies may respond to immunoglobulin infusions or corticosteroid therapy. When possible, alternative medications should replace those known to cause platelet dysfunction.

Platelet Units

Platelets are prepared for transfusion from a single unit of freshly collected whole blood (whole blood–derived [WBD] platelets) or from apheresis by means of continuous-flow centrifugation (apheresis platelets). Regardless of the technique used, platelets must always be collected and stored at 20 to 24° C and in anticoagulant-containing citrate-phosphate-dextrose (CPD) or CPDA-1 or an additive solution that may contain some combination of acetate, magnesium, potassium, or gluconate. Citrate anticoagulates the blood by binding plasma calcium, whereas dextrose is a nutrient source for actively metabolizing platelets.

Whole Blood–Derived Platelets

In the United States WBD platelets are prepared from whole blood by the platelet-rich plasma (PRP) method. PRP is separated from packed RBCs by centrifugation of whole blood ("soft spin"). The PRP is then centrifuged again ("hard spin"). All but 50 to 70 mL of the platelet-poor plasma (PPP) is transferred to an adjoining bag to be used for the production of fresh frozen plasma or other blood components, such as cryoprecipitate or albumin. When optimal centrifugation techniques are used, approximately 85% of the platelets (5 to 7 × 10^{10} platelets) are recovered from a unit of whole blood.[217-219] In Canada and Europe the alternate "buffy coat" method is used to generate platelet concentrates from whole blood.

Apheresis Platelets

Apheresis platelets from one individual should yield more than 3 × 10^{11} platelets in 200 to 300 mL of plasma. Modern apheresis equipment is often able to yield more than 6.5 × 10^{11} platelets per procedure and typically incorporates a leukoreduction step that leaves fewer than 1 × 10^{6} leukocytes per product. This allows units to be split into two or even three apheresis platelet units, each with five to six WBD platelet equivalents.[220,221]

Once they are processed, platelet units are stored on elliptical, circular, or flat-bed agitators.[222,223] This constant mild agitation prevents platelet clumping or activation as platelets settle to the bottom of the bag.[224,225] Constant agitation also facilitates gas exchange, which is important for maintaining an optimal pH range for platelet metabolism and function at 20°C.[226] Because of the risk of bacterial growth associated with room temperature storage, platelet units are usually stored for no more than 5 days.

Platelet Dosing

Guidelines for determining platelet dose have been published in a number of reviews.[227-229] In general, one unit of random donor platelets (5 to 7 × 10^{10} platelets) per 12 kg of body weight should increase the platelet count by 40,000 to 50,000/mm^3 within 1 hour after the infusion. In the average-size adolescent or adult, 6 units of platelet concentrate, or 1 single-donor apheresis unit (3 to 5 × 10^{11}), will increase the platelet count by 50,000/mm^3 or greater. This dose or a lower dose are equivalent in bleeding outcomes, whereas a higher dose increases the number of platelets transfused without any benefit in bleeding outcomes.[181,230] Concentrating or washing the platelets can result in variable platelet loss and decrease the platelet count increments after transfusion.

To determine the effectiveness of platelet transfusion, a platelet count should be obtained before infusion, at 1 hour, and at 24 hours after infusion. There is no significant difference in the posttransfusion platelet count when measured at 10 minutes versus 60 minutes after transfusion.[231]

Platelet Refractory State

When a patient no longer achieves the expected rise in platelet count after a platelet transfusion, the clinician must first assess whether the shortened platelet recovery and survival result from immune or nonimmune mechanisms. Examples of nonimmune mechanisms are listed in Table 36-8 and are responsible for the majority of cases in which posttransfusion count increments are lower than expected. The most notable of these include splenomegaly, DIC, and fever.

Normally, one third of the platelets released into circulation from the bone marrow are stored in the spleen. Hypersplenism causes a larger percentage of transfused platelets to be sequestered in the spleen. Hypersplenism results in a decrease in the platelet recovery immediately after platelet infusion but does not have an effect on platelet survival once a steady state has been achieved.[191]

Fever may reduce platelet recovery by 20% to 40%.[232,233] In one study decreased platelet recovery was documented in patients who underwent bone marrow transplant with fever and negative blood cultures. Although occult infection cannot be ruled out, in these patients fever alone was the only change associated with poor platelet increment.[233]

Consumptive coagulopathy (DIC) refers to the excessive activation of thrombin resulting in an accelerated conversion of fibrinogen to fibrin. This process may be acute and accompanied by rapid consumption of procoagulant factors and platelets in association with extensive bleeding, as in sepsis, shock, or certain forms of malignancy.[187,234] A chronic form of DIC may occur if the procoagulant consumption is slow enough for the liver to compensate for a limited consumption, as found in hemangiomas and necrotic lesion in the lung or brain, for example. In either form of DIC, recovery and survival of transfused platelets is shortened.[183-185] Diseases associated with primary endothelial cell damage, such as HUS and TTP, are also associated with a decreased platelet recovery owing to the formation of microaggregates in the areas of vascular damage.[186] Additional bleeding may be seen with these syndromes because of the acquired platelet dysfunction associated with renal failure and uremia.[190,228]

Older platelets may not remain in circulation as long as freshly collected platelet concentrates because of excessive activation during storage or metabolic effects of acidosis.[226] Platelets also express small amounts of ABO antigens, making ABO-incompatible transfusions slightly less effective than ABO-matched transfusions. Finally, analysis of multiply transfused patients show that there is a tendency to decreased average count increments with successive transfusions. A patient's first platelet transfusion may result in a 30% higher count increment than their fifteenth, despite equal numbers of platelets being transfused and in the absence of other factors. No definitive cause of this decrease has been discerned, other than the hypothesis that patients subjected to multiple rounds of chemotherapy may sustain subtle endothelial damage that consumes transfused platelets.[235]

To compare the effectiveness of platelet transfusion on different occasions or with different donors and recipients, the best ratio to use is the corrected count increment (CCI):

$$\mathrm{CCI} = \frac{\begin{array}{c}(\text{Posttransfusion count} - \text{Pretransfusion count})\\ (\text{body surface area m}^2)\end{array}}{\text{Number of platelets given } (\times 10^{11})}$$

Using this equation, the clinician can correct for the variations in number of platelets infused and for differences in patient size.[228,229] For example, if the platelet count is increased from 5000 to 65,000/mm^3 after a transfusion of 3×10^{11} platelets in a child with a body surface area of 1.2 m^2,

$$\mathrm{CCI} = 60{,}000 \times 1.2 / 3 = 24{,}000$$

This formula is most commonly used to define platelet refractoriness (CCI <5000 at 1 hour), but it has limited use to compare single tranfusions directly, insofar as it does not factor in the many other factors that may affect platelet count increments, as outlined in the preceding sections (Table 36-9).

Alloimmune Mechanisms

Alloimmunization is characterized by increasingly poor recovery and survival of platelets in the multiply transfused patient. The majority of alloantibodies react to determinants on HLA class I molecules and platelet-specific antigens expressed primarily on membrane glycoproteins.[236] The production of antiplatelet alloantibodies is weakly associated with the number of exposures or transfusions. However, there is an association with underlying disease state. For example, only 30% to 60% of patients with leukemia become alloimmunized, in contrast to 80% to 90% of aplastic anemia patients.[237,238]

Unlike the process for RBCs, it is not practical or efficient to crossmatch platelet donors and recipients in most blood centers or hospitals. The antigens known to elicit an immune response on platelets are allelic molecules. These include a variety of class I HLA alloantigens, platelet-specific alloepitopes such as HPA-1a, and blood group antigens. It is not reasonable to match all donors and recipients for HLA or platelet-specific alloantigen antigens. As seen in Table 36-9, the platelet-specific alloepitopes are diallelic with a high frequency of expression of one allele in the general population. Therefore the incidence of alloimmunization to platelet-specific antigens after a single transfusion may be a serious problem for only a very small percentage of the population depending on which epitope is involved.[229,238] An algorithm for dealing with patients who appear to be refractory to platelet transfusions is shown in Figure 36-5.

TABLE 36-9 Variables Resulting in Decreased Post-Transfusion Platelet Counts

Platelet Component	Clinical Condition
Donor platelet count	Fever
Technique of platelet collection	Sepsis
Temperature of storage	Amphotericin B therapy
Platelet component age	DIC
Storage conditions	Hepatosplenomegaly
Infusion time	Alloantibodies
Blood filter type	Autoantibodies
Leukocyte removal	Massive blood loss
Reducing platelet plasma volume	HUS
Washing platelets	TTP
ABO match	Necrotizing enterocolitis Multiple previous platelet transfusions

DIC, Disseminated intravascular coagulation; *HUS*, hemolytic-uremic syndrome; *TTP*, thrombotic thrombocytopenic purpura.

The majority of alloantibodies that mediate the rapid clearance of transfused platelets in the refractory patient bind to epitopes on class I HLA molecules. Platelets express the class I (HLA-A, -B, and -C) gene products,[239] but the class II molecules (HLA-DR, -DP, -DQ) have been shown only on platelets in a high turnover state, such as immune thrombocytopenic purpura, in which the majority of circulating platelets have just been released from the bone marrow.[240] As with other precursor cells, early megakaryoblasts express HLA-DR in normal individuals[241,242]; however, this antigen is generally not detectable on circulating platelets.

Initially, class I HLA alloantigens were defined serologically—that is, with plasma from patients or postpartum women with high-titer alloantibodies. Subsequently, molecular typing and monoclonal antibodies helped clarify the number and types of epitopes associated with the HLA-A, HLA-B, and HLA-C loci. Interestingly, it is the serologic definitions that are important in platelet refractoriness. In categorizing the alloantigens serologically, it became clear that two categories of epitopes were expressed. The first group of alloantibodies recognizes private epitopes, those antigens that are uniquely associated with only one HLA gene product. The second category contains alloantibodies that recognize public epitopes, which are expressed on more than one class I HLA gene product. The broad reactivity associated with the platelet refractory state is most commonly caused by alloantibodies that recognize *public* epitopes

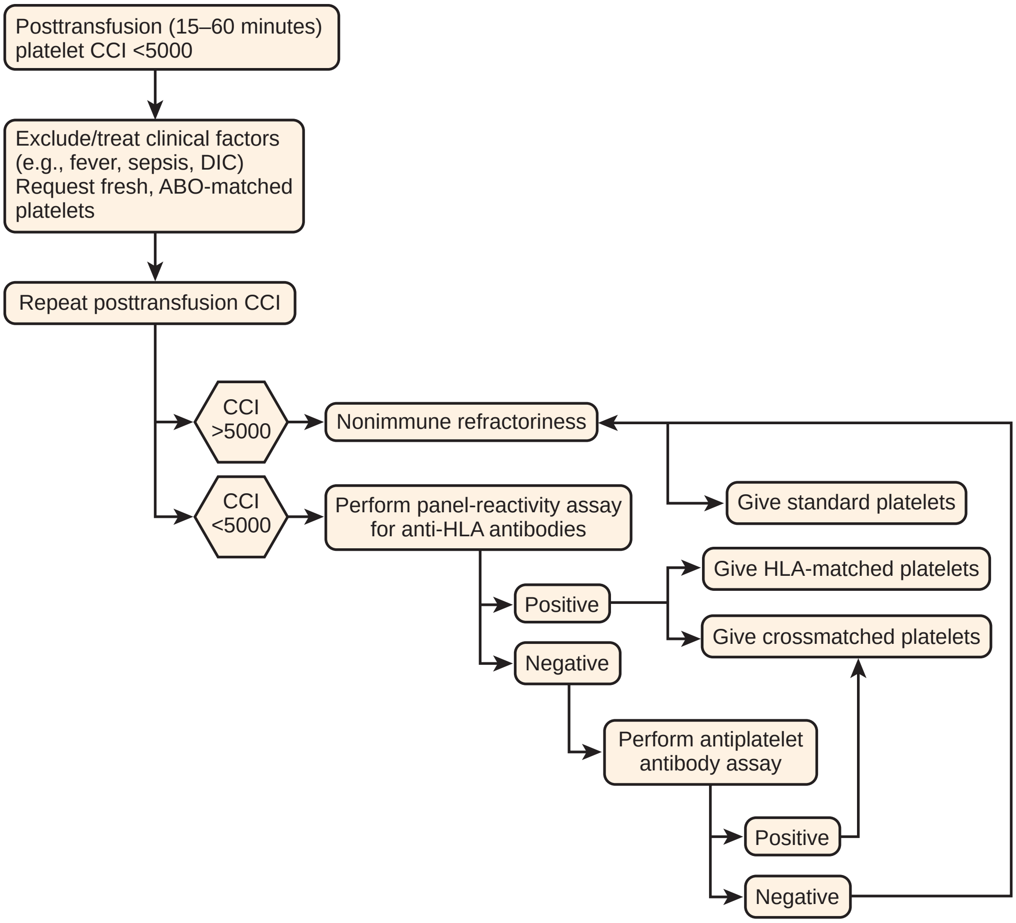
Posttransfusion (15–60 minutes) platelet CCI <5000
Exclude/treat clinical factors (e.g., fever, sepsis, DIC) Request fresh, ABO-matched platelets
Repeat posttransfusion CCI
CCI >5000
Nonimmune refractoriness
CCI <5000
Perform panel-reactivity assay for anti-HLA antibodies
Give standard platelets
Give HLA-matched platelets
Positive
Give crossmatched platelets
Negative
Perform antiplatelet antibody assay
Positive
Negative

Figure 36-5 Algorithm for the management of patients refractory to platelet transfusion. *CCI,* Corrected count increment; *DIC,* disseminated intravascular coagulation.

shared by many HLA gene products.[243,244] By categorizing HLA-A and HLA-B loci according to these public epitopes or cross-reactive groups (CREGs), blood banks may be able to provide single-donor platelets based on reactivity to CREG subsets. Blood banks take advantage of this observation to provide "HLA-compatible" platelets (type B matches) when HLA-identical donors are limited in number.[245] Although the platelets may not be identical at the HLA-A or HLA-B loci, if the CREGs shared by a subset of class I allotypes can be avoided, then non-HLA identical product can still be used, often with adequate platelet recovery and survival. Using this system, blood centers can expand their pool of volunteers providing single-donor platelets for multiply transfused patients; however, it remains difficult to provide fully HLA-matched platelets consistently. Because of this, the overall success rate for attaining suitable posttransfusion count increments with HLA-matched platelets is only approximately 50% in alloimmunized patients, even in major centers.[245]

Preventing Platelet Alloantibodies

Leukoreduction of platelet and RBC units for patients who are expected to require multiple platelet transfusions significantly reduces the rate of development of platelet alloantibodies. Although platelets express class I gene products, it is the class II molecules expressed on contaminating leukocytes that are primarily responsible for the induction of alloantibodies.[246,247] With the use of washed platelets devoid of white cells, multiply transfused animals failed to develop lymphocytotoxic antibodies.[248,249] These studies were eventually repeated in patients comparing pooled random-donor platelets with or without leukocyte depletion. At least 90% of the contaminating leukocytes had to be removed (less than 10^6 remaining), but there was a striking decrease in the rate of alloimmunization from 93% to 24%.[250-254] Prospective studies in humans suggest that the leukocyte threshold for alloimmunization is less than or equal to 5×10^6 cells per infusion. As outlined previously, the TRAP study[255] has conclusively demonstrated in a randomized, prospective study that the use of leukocyte-reduced products can effectively reduce, but not eliminate, the risk of alloimmunization. Most centers recommend leukoreduction in the blood center during collection,[256] which may also decrease the accumulation of cytokines produced by white cells during storage.[257,258]

PTP occurs 7 to 10 days after blood product infusion in a patient who has been previously transfused or pregnant.[259] Paradoxically, alloantibodies are produced that destroy not only any transfused platelets but also the patient's own platelets. Although extremely rare, PTP occurs most commonly in the less than 2% of the population who are HPA-1a negative.[195] The posttransfusion plasma usually has high-titer anti-HPA1a activity, but the mechanism of autologous platelet destruction is unclear. Hypotheses suggesting that there is a cross-reactive alloantibody that binds to autoepitopes in close proximity to the HPA-1a site have yet to be proved. There are individual cases reported of PTP secondary to alloantibody to Bak^a, Pen^a, and two cases of anti–HPA-1b antibodies.[260-262] Of the approximately 100 cases reported since its description in 1959, only four men have been described as having PTP.[195] A series of PTP patients with anti–HPA-1a alloantibodies were HLA typed and more than 90% were HLA-DR3 positive, with the supertype HLA-DRw52 predominating in these individuals.[197,198] Patients are usually in their late forties or older, but PTP has been reported in a 16-year-old girl not previously pregnant.[262]

Platelet transfusions are contraindicated in PTP patients in whom the treatment of choice is intravenous immunoglobulin, plasmapheresis, or both.[263] The frequency of transfusion reaction in these patients is quite high, with 46% experiencing chills and fever during blood product infusion. Therefore RBCs should be washed and filtered whenever possible. If platelets must be given for life-threatening hemorrhage, they should be HPA-1a negative, washed, and leukoreduced. Further discussion of therapy and possible mechanisms of autologous platelet destruction in PTP are more thoroughly addressed in two extensive reviews of this disorder.[201,259]

GRANULOCYTE TRANSFUSIONS

Granulocytes are available from some blood banks and blood centers. Three groups of patients may benefit from granulocyte transfusions. One group consists of neutropenic patients with a bacterial or fungal infection whose bone marrow is likely to recover. This group consists predominantly of patients who have undergone bone marrow transplant and patients who have received chemotherapy. The other groups that may benefit from granulocyte transfusions include neonates with sepsis and septic patients with dysfunctional granulocytes, such as patients with CGD. If there is a benefit, it is likely only for patients with a high risk of mortality receiving a relatively high dose of granulocytes.[264,265] Additionally, granulocyte transfusions are associated with adverse effects, including fevers and pulmonary complications, and high rates of alloimmunization to HLA antigens, a potentially serious complication for patients who may be candidates for bone marrow transplantion.[266] Granulocyte transfusions to patients at risk of CMV disease should be collected only from CMV-seronegative donors because granulocytes from CMV-seropositive donors contain the virus. Granulocyte transfusions should be closely coordinated with the blood bank and should be transfused as soon as possible after collection and no more than 24 hours after collection.

PRINCIPLES OF HEMATOPOIETIC CELLULAR THERAPY

With the advancement of molecular technology, a better understanding of human immunology, and the increased interest in regenerative medicine, the use of cellular therapy is no longer limited to hematopoietic stem cell transplantation (HSCT). Current applications have expanded to include the use of cells as adoptive immunotherapy and cancer vaccines. Future applications may also include the use of cells as a drug-delivery vehicle or as a source of tissue engineering for regenerative medicine.[267] However, HSCT is still the best-established type of hematopoietic-based cellular therapy. In this section the sources and processing of hematopoietic cell-based therapy and complications associated with its use will be reviewed.

Hematopoietic Cell Sources

The donors of therapeutic hematopoietic cells can be autologous or allogeneic. Autologous donors are usually patients who will be undergoing HSCT. These donors have their own cells collected and cryopreserved and have their own cells infused during transplantation. Because the donor and recipient are the same, complications caused by major histocompatibility complex (MHC) or HLA disparities are negligible for autologous cell infusions. The MHC/HLA molecules are displayed on cell surfaces and responsible for lymphocyte recognition and "antigen presentation." They control the immune responses through recognition of "self" and "nonself" and consequently serve as targets in transplantation. However, during allogeneic transplantation, the degree of HLA disparity is extremely important for donor selection because large numbers of lymphocytes are transfused. These donor lymphocytes can recognize recipient's tissue as foreign or "nonself" and mediate GVHD. HLA molecules are located on chromosome 6. There are two classes of HLA antigens, class I and class II. Class I antigens such as HLA-A and -B and class II antigen HLA-DR are the three most important molecules contributing to GVHD. Given that each person has two alleles of chromosomes, a minimum of six out of six HLA antigens matching is often required for transplantation. The donors for allogeneic hematopoietic stem cells are often siblings of the recipients and referred to as *related donors*. For patients who do not have related donors, cells from HLA-matched unrelated donors are frequently used. These donors are obtained from international donor registries such as National Marrow Donor Program (NMDP), and the amount of time spent searching for appropriate and available donors can be unpredictable. Because only 50% to 60% of patients have an HLA-matched sibling or an unrelated donor, not all patients who may benefit from HSCT can proceed to transplant. Timely search for an appropriate donor is essential for successful allogeneic HSCT.[268] A prolonged donor-search period can lead to death, increased risk, or ineligibility of the patients. The incidence of GVHD is also significantly increased when HSCT is performed with unrelated donors.[269,270]

TABLE 36-10 Differences in Transplanted Cell Dose and Hematopoietic Recovery Between Cell Sources

	Total Cell Dose Transplanted		Days to Hematopoietic Recovery	
Hematopoietic Stem Cell Source	Mononuclear Cells/kg ($\times 10^8$)	CD34$^+$ Cells/kg ($\times 10^6$)	Neutrophil	Platelets
G-CSF–stimulated peripheral blood	9.6 ± 6.1	9.3 ± 7.0	13 ± 4	17 ± 15
Bone marrow	2.6 ± 1.5	1.8 ± 1.2	19 ± 5	23 ± 12
UCB	0.44 ± 0.12	0.23 ± 0.14	29 ± 16	52 ± 23

From Dana-Farber Cancer Institute adult hematopoietic stem cell data, 2005.
G-CSF, Granulocyte colony-stimulating factor; *UCB,* umbilical cord blood.

Bone marrow, stimulated peripheral blood, and umbilical cord blood (UCB) are the sources of hematopoietic stem cells for transplantations. Unstimulated peripheral blood cells are used either in an unmanipulated state or can be further processed to be used as adoptive immunotherapy. Characteristic differences between various hematopoietic cell sources will be discussed individually.

HSCT can be performed with unmanipulated bone marrow, peripheral blood, or cord blood. The original HSCTs were performed using bone marrow. In recent years investigators have routinely used mobilized peripheral blood for transplantation[271-273] because of the higher number of CD34+ hematopoietic stem cell content and faster posttransplant hematopoietic recovery (Table 36-10). CD34+ cells are typically used as a marker for hematopoietic stem cells, and although CD34+ cell numbers are a good marker to predict the success of engraftment, some hematopoietic stem cells are actually CD34–.[273a] Depending on the source of cellular products used, they can be contaminated with different degrees of RBCs and leukocytes. Compared to peripheral blood–derived hematopoietic stem cells, bone marrow products have higher RBC contamination and lower numbers of T cells and CD34+ cells. Overall clinical outcomes of transplants from the two sources are equivalent.[274]

Mobilized peripheral blood collected by leukapheresis has become the most commonly used hematopoietic progenitor cell component for both autologous and allogeneic HSCT because of the ease of collection and ample number of CD34+ stem cells.[275] Peripheral-blood donors recover more quickly from pain related to the collection than do bone marrow donors.[276] Additionally, compared with autologous bone marrow transplants, autologous peripheral blood transplants result in more rapid engraftment, decreased need for transfusions, and decreased incidence of infection and antibiotic usage, as well as shorter hospital stay.[277] To mobilize stem cells into the peripheral blood, patients are treated with cytokines such as granulocyte-colony stimulating factor (G-CSF), possibly in combination with plerixafor, an inhibitor of the interaction between CXCR4 and CXCL12 that helps maintain stem cells in the hematopoietic microenvironment. Autologous donors are often mobilized after receiving chemotherapy that also contributes to the mobilization. The cells are usually collected in the outpatient setting. After chemotherapy the patient will undergo leukapheresis at the time his or her cell counts recover from the nadir. Circulating peripheral blood CD34+ cell levels may be used to predict yield.[278,279] If cytokines are used without prior chemotherapy, collection is begun on a predetermined day during the course of administration. Approximately 10% of autologous peripheral blood stem cell donors cannot be mobilized successfully with G-CSF alone, but approximately three fourths of these patients can be successfully mobilized with the addition of plerixafor.[280,281] The risk factors for poor mobilization include the patient's disease status and marrow environment related to history of heavy chemotherapy and radiation therapy.[282-285] Patients who fail mobilization with any agent are at increased risk of delayed engraftment, and non-transplant treatments or transplants of hematopoietic progenitor cells directly collected from the bone marrow are often considered.

If peripheral blood collections are used for allogeneic transplantation, collection is begun on the fourth or fifth day of G-CSF administration. As in the autologous setting, engraftment may be more rapid with allogeneic peripheral blood compared with bone marrow. However, peripheral blood components typically contain at least one log more T cells than bone marrow components, potentially increasing the risk for GVHD. Nevertheless, the incidence of acute GVHD is only moderately elevated, and the overall survival rate is equivalent.[286] This may in part result from effects of the G-CSF used for mobilization on the T cells. However, reports have suggested that chronic GVHD may be more frequent in recipients of peripheral blood components.[287] But peripheral blood transplants have less graft failure and equivalent overall survival rates compared with bone marrow transplants.[274]

The biology of CD34+ stem cell mobilization is now better understood. The bone marrow environment heavily regulates the movements of these CD34+ hematopoietic progenitor stem cells. Cytokines such as G-CSF stimulate the generation of granulocytes in both bone marrow and peripheral blood.[288,289] These granulocytes release serum proteases such as cathepsin G and neutrophil elastase. According to some reports, the accumulation of these serum proteases coincides with the increased inhibition of adhesion molecule interaction between the very late antigen 4 (VLA-4) on the CD34+ hematopoietic stem cells and the vascular cell adhesion molecule 1 (VCAM-1) on the surface of bone marrow stromal cells.[290] This adhesion molecule disruption allows the CD34+ hematopoietic progenitor cells to be free floating within the bone marrow environment, and their movement can then be influenced by chemokines. Interestingly, proteolysis of chemokine receptor CXCR4 on CD34+ cell surface and the chemokine stromal cell–derived factor 1, also known as CXCL12, is also observed to coincide with serum proteases during G-CSF and chemotherapy, such as cyclophosphamide in human bone marrow.[291] With decreased chemotaxis signals within the marrow, the free-floating CD34+ hematopoietic stem cells can traffic toward the peripheral blood compartment.[292] With G-CSF, chemotherapy, and possibly plerixafor mobilization, peripheral blood circulating CD34+ hematopoietic stem cells can be increased to approximately tenfold to a hundredfold.

Many centers are also evaluating the use of UCB as a source of allogeneic hematopoietic stem cells. The use of UCB as an alternate source of allogeneic hematopoietic stem cells was first reported by Gluckman and colleagues in 1989.[293] Since that time, hundreds of UCB transplants have been performed in North America and Europe.[294] Because of the success of UCB transplantation in both related and unrelated donor settings, many cord blood banks (CBBs) have been established worldwide. UCB is collected from umbilical cord or placental veins after delivery of the infant and therefore is a readily available component. Parents have the option of banking the cells for their own use in a private CBB or donating them to a public CBB. If the UCB samples are donated to public CBBs, these cells undergo stringent quality-control evaluation; only qualified products are processed and cryopreserved for use. The UCB products stored in public CBBs are available for public use when requested and prescribed by appropriate transplantation physicians. The number of CBBs and the size of the inventory within the CBBs have been increasing.[295] There are at least 15 public CBBs in the United States. UCB transplantations offer many advantages: Various reports claim that UCBs are less immunogenic, and transplants may be performed across HLA barriers with less risk of GVHD than would be anticipated if a bone marrow donor with the same HLA type were used.[296] Therefore UCB transplant is an attractive alternative for patients requiring allogeneic transplantation who do not have an HLA-matched donor. In contrast to mobilized peripheral blood stem cells and bone marrow harvests, the collection of UCB after birth carries no risk for the donor. The collected UCB is HLA typed, tested for infectious agents, and banked for use—and therefore available immediately on request to be shipped to any transplant center in the world. Despite the ease of availability and safety of the donation procedure, UCB transplants have limitations. Studies continue to show that appropriate stem cell dose is essential for speedy hematopoietic cell engraftment and successful transplantation.[297-299] The limited cell dose in the UCB products poses problems especially for large adolescent and adult recipients. In the case of disease progression or relapse, it is also impossible to go back to the original UCB donor for lymphocyte or additional stem cell collections. Research working on the expansion of cord blood stem cells is ongoing.[300,301] Double UCB transplantation in adult patients is being attempted to overcome the dosing limitation issues.[302]

Infusion-Related Toxicities

Infusion of hematopoietic stem cell products can be associated with either immediate reactions or long-term side effects. The immediate infusion toxicities are similar to blood transfusion reactions. They include febrile reactions resulting from infusion of large number of leukocytes and related cytokines, allergic reactions secondary to plasma protein content, and occasional hemolytic reactions caused by donor-recipient erythrocyte ABO incompatibility. To reduce the occurrence of febrile and allergic reactions, patients are always premedicated with antipyretic medication and antihistamines before hematopoietic stem cell infusion. Special manipulation to reduce the erythrocyte contents can be performed to prevent hemolytic reactions. For hematopoietic stem cell products or lymphocytes that are cryopreserved in dimethyl sulfoxide (DMSO) for storage in liquid nitrogen, the infusion of thawed products can lead to frequent DMSO-related toxicities. Common symptoms attributed to DMSO include nausea, vomiting, flushing, and headaches. Anaphylactoid symptoms caused by the release of histamine are also common. Embolic events associated with infusion of bone marrow and apheresed hematopoietic stem cell products have been reported. Most of the described cases involved infusing of products with particulate matter, owing to cell clumps or bone fragments, in recipients with congenital cardiac septal defect.[303,304]

GVHD is the most common long-term toxicity associated with allogeneic HSCT. Approximately 30% to 70% of patients undergoing allogeneic HSCT will develop GVHD.[305] GVHD is mediated primarily through donor graft lymphocytes (T-cell population). Graft lymphocytes target tissues with alloantigens and cause alloimmune reactions in recipient organs.[306,307] Severe GVHD is observed when donors are not fully identical at MHC antigens (HLA-A, B, DR) with the recipient. However, most allogeneic HSCTs are conducted with donors and recipients who are identical at MHC antigens. In these recipients minor histocompatibility antigens (mHAs) are thought to be the primary targets of donor alloimmunity, and severe GVHD can occur.[308-310] Studies have shown that mHAs can be expressed on hematopoietic tumor cells. When donor T cells target leukemia mHAs, alloimmunity results in graft-versus-leukemia (GVL) and can contribute to eradication of leukemia cells.[311,312] The use of donor lymphocytes as adoptive immunotherapy for patients with relapse of chronic myelogenic leukemia has shown to be successful in patients in cytogenetic relapse after allogeneic HSCT.[313-315] Conversely, depletion of T-cell content in the graft has shown to reduce the incidence of GVHD but at the expense of losing GVL effect.[307]

Component Assessment

For customized cell therapy such as HSCT, each product is donor-patient specific. The quality of the product varies for different donors and even changes daily for a product that came from the same donor. As mentioned previously, the content of the product can lead to various types of infusion reactions, immunologic effects, and time to hematopoietic cell engraftment. Most important, as with any blood product, infusion of products with microbial or viral contamination can lead to transmission of infectious diseases.

Achieving rapid hematopoietic cell engraftment is essential for successful HSCT. Insufficient infusion of hematopoietic stem cells is linked to prolonged hematopoietic cell recovery. Patients who experienced delayed hematopoietic engraftment require long-term blood component support and are more likely to contract severe bacterial, fungal, and other opportunistic infectious disease as a result of prolonged neutropenia and lack of proper immune function. Functional assays such as granulocyte-macrophage colony-forming unit (CFU-GM), granulocyte-erythroid-macrophage-megakaryocyte colony-forming unit (CFU-GEMM), and long-term culture–initiating cell (LTC-IC) content have been described as measures of hematopoietic stem cell quality and content. The results of these assays correlate well with posttransplantation hematopoietic recovery. However, these culture-based assays have long turnaround times. Enumeration of CD34+ cells using flow cytometry analysis is now commonly performed as a surrogate for hematopoietic stem cell content because of its fast turn-around-time and proven correlation with clinical engraftment data. As with other assays, CD34+ cell enumeration methods vary among centers, making the characterization of a hematopoietic stem cell component that is adequate to achieve hematologic engraftment more difficult.[316,317] Standardized protocols for CD34+ cell enumeration have been proposed in an attempt to limit variability and permit comparison of hematopoietic stem cell content of components collected and infused at different centers.[318] Methodologies to determine absolute cell concentrations instead of percentages may further improve the accuracy of the CD34+ cell enumeration procedure.[319] Analysis of graft T-cell content using flow cytometry in hematopoietic stem cell or donor lymphocyte products is also used to correlate with GVHD outcome.

In addition to quantifying hematopoietic stem cell content, components must be tested for contaminants. Contamination rate increases with component manipulation, such as ex vivo cell isolation or depletion and long-term cell culture, and may be associated with adverse clinical sequelae. This emphasizes the need to include bacterial cultures as a component of quality control of hematopoietic cell collection, processing, cryopreservation, thawing, and re-infusion procedures.[320]

Lastly, critical evaluation of the donor of the product is also essential in assessing the quality of the product. All allogeneic and autologous products should be tested for infectious disease markers, just like those from routine blood donors. Donors who are positive for certain viral markers can still donate their cells for transplantation if the transplantation was deemed as the only life-saving option and the patient was informed of the risk by the transplant physicians. However, the practice of using high-risk allogeneic donors for transplantation is discouraged.

Processing and Storage of Hematopoietic Progenitor Cell Components

Autologous and, occasionally, allogeneic hematopoietic progenitor cell components are collected before the patient receives myeloablative therapy and are cryopreserved. Cells are suspended in a mixture of an electrolyte solution, a protein source and a cryoprotectant such as DMSO or hydroxyethyl starch.[321] Cryopreserved cells are infused rapidly when thawed, and the freezing solution can be infused along with the cells. Premedication is required to minimize toxicities.[322-324]

In the allogeneic setting, processing may be performed to prevent acute hemolysis associated with infusion of an ABO-incompatible component.[325] If there is a major ABO incompatibility, which occurs when the recipient's plasma contains antibodies against donor RBCs, many centers will remove RBCs from the donor component. If there is a minor ABO incompatibility, wherein the donor plasma contains antibodies against the recipient's RBCs, the component will be washed or volume reduced to remove plasma. Alternatives to these procedures include plasma or RBC exchange in the recipient as well as the infusion of sources of donor blood group antigens to absorb recipient antibodies.[326,327]

Laboratories may perform more extensive procedures in an attempt to deplete unwanted cell populations from the hematopoietic stem cell component. Tumor cells are removed from autologous components in an attempt to decrease the risk for relapse, whereas T lymphocytes are removed from allogeneic components to abrogate acute GVHD. Most of these procedures are performed using either negative- or positive-selection methods.

Negative selection targets the cells to be removed from the component while preserving other cell populations.[328] The methods for negative selection that have been used include attempting to separate cells on the basis of physical characteristics, in vitro sensitivity to chemotherapeutic agents, and monoclonal antibody (MoAb)–mediated separation or damage. Density gradient centrifugation and counterflow centrifugal elutriation have been used to separate cells according to size. Trials have used 4-hydroxycyclophosphamide or mafosfamide to kill tumor cells. Cells can bind to MoAbs conjugated to magnetic beads, dense particles, or surfaces and be physically separated from the other cell populations within a component. In addition, MoAbs may be used to destroy tumor cells or T cells by way of conjugation with toxins or complement-mediated lysis.[329]

In contrast, positive selection enriches the cell population to be retained while nonspecifically depleting all other cell populations. The systems currently available for positive selection use MoAbs directed against various hematopoietic cell surface antigens. Cells are incubated with the MoAbs, and separation is achieved by biotin-avidin, magnetic bead, or high-speed cell-sorting methods.[330-333] Most of these cell-manipulation techniques are initially designed to reduce the incidence of GVHD or disease relapse associated with HSCT. Recently, some of these techniques have also been applied to the development of cell-based immunotherapy. For example, monocytes or

CD34+ cells from peripheral blood can be isolated using selection techniques and treated in vitro so that the cells differentiate into potent antigen-presenting dendritic cells. Animal models have shown that dendritic cells can act as tumor vaccines to stimulate and enhance cell-mediated immunity in patients who may have defective tumor immunity.[334-336] Specialized lymphocytes can also be isolated and expanded with appropriate growth factors and antigen stimulation to target specific tumor cells or infectious agents. Most of the cell-based immunotherapies are still under investigation and therefore considered experimental. Encouragingly, early clinical data using dendritic cell therapy in melanoma-, prostate cancer–, and Epstein-Barr virus (EBV)–specific T cells targeting posttransplant lymphoproliferative diseases have shown immunologic responses with potential clinical effects.[337-340]

Future Directions

Several areas of active investigation involve the use of hematopoietic stem cells. First, definitive trials are necessary to clarify whether there is any clinical benefit associated with CD34 positive cell selection, tumor cell, or T-cell depletion. Second, building on platforms where cells have been expanded but differentiated,[341] as new cytokines are identified and new systems developed, it may be possible to expand primitive hematopoietic stem cells. Expanded populations of primitive cells would be useful for infusion to patients whose autologous graft contains insufficient numbers for transplantation. In addition, the ability to expand hematopoietic stem cells could make UCB components available to larger recipients with less perceived risk. In addition, expanded cells may be useful for gene therapy approaches.

Much effort has taken place in recent years to use hematopoietic stem cells as a strategy for clinical gene therapy or to use other cells therapeutically. Stable expression of a transgene in a primitive hematopoietic stem cell would, in theory, be an ideal method to replace missing gene products in a patient. However, some retroviral vectors induce leukemia owing to insertional mutagenesis of CD34+ cells.[342] Current studies are using vectors designed to minimize the risk of oncogenesis and are aimed at treating severe congenital diseases involving the hematopoietic system.[343]

APHERESIS

Apheresis (from the Greek *aphairesis*, meaning "removal") refers to a technique of drawing peripheral blood, separating it, and selectively removing one or more of the components while returning the remainder. Apheresis techniques may be manual or automated, and the phases of drawing, separating, removing, and returning blood and components may be continuous or discontinuous. The technique of apheresis may be used for collecting transfusable blood components from donors, and it may be used to treat patients with conditions for which removal or replacement of a portion of the blood may be therapeutic. The terms *cytapheresis, erythrocytapheresis, leukapheresis, plateletpheresis,* and *plasmapheresis* refer to the use of apheresis to remove, respectively, cells, RBCs, white cells, platelets, or plasma. The terms *plasma exchange* and *RBC exchange* imply that the component removed is replaced in like amount with normal plasma or RBCs. In practice, nearly all donor and therapeutic apheresis is performed using automated techniques with specialized programmable separation devices designed for this purpose.

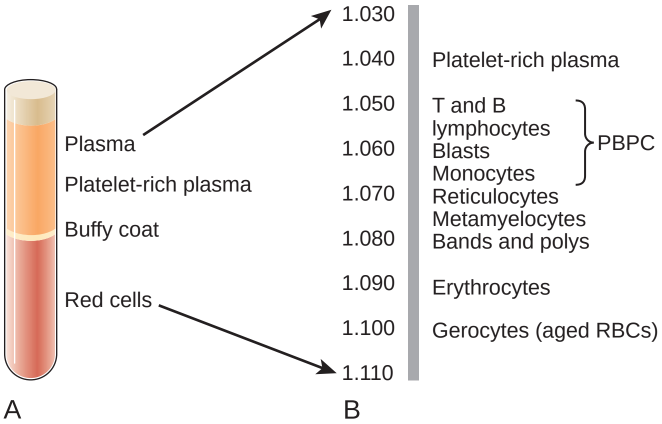

Figure 36-6. **A**, Separation of anticoagulated whole blood. **B**, Specific gravity of blood components. *PBPC,* Peripheral blood progenitor cells; *Polys,* polymorphonuclear leukocytes; *RBCs,* red blood cells.

The principle by which most apheresis instruments fractionate the components of whole blood is simple density centrifugation. The instrument contains a sterile, closed, and disposable centrifugation chamber in which whole blood is separated into RBC, plasma, and buffy-coat layers. The densest component, the RBCs, is segregated to the bottom of the centrifugal field, the least dense component (plasma) is pushed to the top, and the other cellular components layer in between, as shown in Figure 36-6. The efficiency of separation depends not only on the density of the components but also on the geometry and rotational speed of the centrifugal chamber and on the specific technique for isolating the separated layers. Pure separation of plasma from RBCs and concentrated suspensions of platelets nearly devoid of RBCs can be achieved reliably. Because the white cell layer is much smaller than either the RBC or plasma layers and close in density to the RBC layer, both the RBC and the platelet components will contain leukocytes. For the same reason, whereas it is possible to use apheresis to isolate leukocyte fractions that are enriched in granulocytes, or in mononuclear cells, these fractions will also contain other types of white cells as well as RBCs and platelets.

Other apheresis instruments separate blood by filtration rather than centrifugation. Because this technique cannot separate cellular elements, it is used primarily in donor applications for collection of plasma.

Donor Apheresis

Overall, the most common medical use of automated apheresis technology is for donor procedures, in which platelet or plasma is collected from individual volunteer donors. The principle reason to use apheresis techniques to collect these products, rather than deriving them from whole blood donations, is that the yield per donation is much higher. Six to 20 times the number of platelets can be harvested in one apheresis donation than can be isolated from one unit of whole blood. Automated systems can be programmed to collect two or even three separate blood components from one donor during a single apheresis procedure—that is, units of packed cells, plasma, or single-donor platelets. The same advantage applies to these apheresis collection techniques. A much larger yield is obtained per donation, with little or no compromise in donor safety. The blood products derived from these donor procedures have slightly different quality-control characteristics from WBD components, but in clinical use they are as efficacious as those derived from whole-blood donations.

Granulocyte concentrates drawn by apheresis technology to treat refractory fungal or bacterial infections in patients with profound neutropenia are once again being evaluated.[344] Studies in the 1970s showed that granulocyte transfusions had limited efficacy and considerable toxicity. However, the advent of a granulocyte-specific hematopoietic cytokine (G-CSF) in concert with apheresis technology has made it possible to collect up to tenfold more granulocytes for transfusion than were available for the older studies.[345] Unlike products from unstimulated donors, granulocyte concentrates collected with cytokine stimulation of the donor can yield substantial increments in peripheral granulocyte count in the recipient.[346,347] Whether these larger increments correspond to greater efficacy and tolerable toxicity has yet to be determined.[348-351]

Apheresis technology can also facilitate obtaining multiple units of plasma from the same donor to provide single-donor plasma support to a recipient with a congenital coagulation factor deficiency treatable with plasma, such as factor X or XIII deficiency.

Although adult-donor apheresis, especially for platelet collection, has a large impact on the blood banks that supply blood products for pediatric patients, child-donor apheresis is rarely used. The one exception is donor apheresis collections of peripheral blood progenitor cells, described later.

Therapeutic Apheresis

A broad spectrum of indications exists for automated apheresis used for therapeutic effects in children. For some indications the mechanism of the therapeutic benefit is readily apparent and the efficacy of the treatment well substantiated. For many other indications the rationale for use of apheresis to treat a disease condition is less clear and is based on extrapolation from studies in adults or on anecdotal evidence. It is important to remember that the therapeutic value may be derived from the removal of a portion of the patient's blood, from the administration of a fluid to replace what was removed, or from both. In some cases the therapeutic value of an apheresis procedure is derived from the ability to control the patient's blood volume independent of the rate and total volume of blood product administered, as with complete exchanges of the RBC mass or the plasma volume.

Manual Apheresis

Although it is theoretically possible to carry out most apheresis procedures by entirely manual techniques, with steps analogous to those used to make blood components from donated whole blood, in practice, manual methods are usually reserved for situations in which the automatic equipment is not available or in which the volume of blood to be processed is too small to warrant use of the automated cell separator. For example, for a newborn with isoimmune thrombocytopenia, it might be possible to obtain an adequate dose of platelets by drawing one unit of whole blood from the mother, isolating the platelets manually, and transfusing the residual RBCs and plasma back to the mother, rather than subjecting the mother to an automated plateletpheresis procedure. Another manual apheresis procedure is the whole blood exchange for neonatal hyperbilirubinemia, typically performed in a discontinuous fashion with reconstituted whole blood, an empty blood-collection bag, a three-way stopcock, and a syringe to remove and replace blood in 10-mL cycles. Occasionally a manual exchange of RBCs or plasma may be the only alternative in a patient whose venous access is inadequate for an automated exchange and for whom a simple transfusion is not an alternative.

Automated Apheresis

Modern apheresis machines offer the advantages of a sterile, closed, single-use system; automated control of volume balance; traps to prevent air embolism; and other safety features. Each machine can perform several types of apheresis procedures, with highly automatic modes as well as the capacity to modify procedure parameters for individual patient needs. By comparison, manual exchanges are more awkward to perform, usually use open systems, involve manual tracking of intake and output, and are therefore potentially less safe. Apheresis devices may be either continuous or discontinuous. A discontinuous device withdraws a volume of blood, separates it, removes the selected component, and returns the remainder, all in serial steps. The sole advantage of a discontinuous device is that it requires only a single site of vascular access. In the donor setting this is often reason enough to use a discontinuous device. However, compared with a continuous device, discontinuous apheresis technique requires longer processing times and involves larger volume shifts in the donor or patient. The latter consideration may be critically important in pediatric patients and imposes a practical limitation on the use of these devices in children. In contrast, continuous devices withdraw, separate, and return blood simultaneously, permitting apheresis procedures that affect the patient's intravascular volume only minimally.

For any apheresis procedure, there is a fixed extracorporeal blood volume and extracorporeal RBC mass that must be taken into account in planning the procedure. The extracorporeal blood volume depends on the specific make and model as well as the type of procedure planned and fall into a range from 100 to 400 mL.

Unique Aspects of Apheresis in Pediatrics

Performing apheresis procedures in pediatric patients is often more challenging than performing analogous procedures in adults. The specific challenges include vascular access, anticoagulation, volume shifts, hypothermia, and patient cooperation.

Vascular Access

The vessel used for the apheresis draw line, through which whole blood is drawn into the extracorporeal system, must be large and resilient enough to accommodate a large catheter and to sustain the required flow rate without collapsing. When peripheral veins are used, a rigid needle, 18 gauge or larger, is used for drawing. In adults and older children, it is usually possible to find veins in the antecubital fossae large enough to accommodate this needle, but children under the age of 7 years often do not have adequate access. The access requirements for returning blood are less demanding because a smaller gauge (20 to 22 g) flexible catheter may be used. Although every child undergoing apheresis should be evaluated first for peripheral access, it is often necessary to obtain access to a larger central vessel. This may be accomplished in several ways: a single-lumen central vein catheter or an arterial line to draw, and a peripheral vein to return, or a double-lumen venous catheter. The catheters, often referred to as “dialysis catheters” because the same access issues apply in hemodialysis, may be designed either for temporary placement (usually stiff, straight catheters intended for bedside percutaneous placement) or permanent (softer, tunneled catheters with cuffs that require placement in the operating room).[352] Temporary polyurethane catheters are available as small as 7 French for patients as small as 5 to 10 kg. Permanent catheters designed for apheresis are available as small as 8 French, for children as small as 10 to 12 kg.[353] It is important to realize that most of the double- and triple-lumen catheters available for general purpose central access in children may fail under the draw pressure of an apheresis procedure because the lumen is too small or the catheter wall too soft. Some multiple-lumen catheters also have inadequate separation of the openings of the lumens, permitting recirculation and decreasing the efficiency of apheresis.[353] Hence it is imperative to consider the possibility that apheresis will be required before placement of a central catheter. Finally, there is growing experience with successful use of implanted access devices (ports) to support chronic apheresis therapy in children.[354,355]

Anticoagulation

Because the blood is exposed to artificial surfaces, apheresis procedures usually require an anticoagulant to prevent activation of platelets and the coagulation cascade and the formation of clot within the extracorporeal circuit. The standard anticoagulants used in apheresis are sodium citrate, heparin, or combinations of those two. The anticoagulant is added to the circuit at a controlled rate just at the point where the blood is drawn, and a portion of the anticoagulant is eventually returned to the patient. The amount of anticoagulant that the patient receives depends on the type of procedure, the replacement fluid, and the rates of blood drawing and replacement.

Citrate chelates the available calcium, a necessary cofactor for the clotting cascade, and also prevents platelet aggregation. The infusion of citrated plasma or blood does not cause systemic anticoagulation in the donor or patient, but it may reduce the ionized calcium level in the plasma. Pediatric patients are particularly subject to citrate toxicity, in part because the flow rates used during apheresis procedures, and therefore the citrate doses, are often higher on a per-kilogram basis than for adults. Citrate toxicity first manifests as tingling around the mouth and fingers and sometimes nausea and abdominal discomfort. More serious citrate toxicity includes hypotension and cardiac rhythm disturbances.[356] Because a young or critically ill child may be unable to inform the operator about the earlier warning signs of citrate toxicity, use of citrate as an anticoagulant requires careful attention to ionized calcium levels, calcium replacement by mouth or by intravenous infusion,[91] and monitoring of blood pressure and heart rate.

Alternatively, citrate may either be replaced or supplemented with heparin anticoagulation, which may be monitored by activated clotting time, a crude measure of whole-blood clotting time.[357,358] Heparin anticoagulation may be achieved with a bolus, with or without subsequent continuous infusion. The dose of heparin used for an apheresis procedure is similar to doses used for therapeutic anticoagulation, and the patient may be expected to have a systemic anticoagulation effect lasting 4 to 6 hours after the end of the procedure, a side effect that may be significant in a patient who has had recent surgery or is otherwise at risk for bleeding. Citrate confers minimal bleeding risks, and citrate without heparin is generally used to anticoagulate patients with bleeding risks.

Volume Considerations

The extracorporeal volume for an apheresis procedure, including the centrifugal chamber, tubing, filters, air traps, and possibly a blood warmer, may exceed 400 mL, depending on the machine and tubing configuration. Because the majority of this extracorporeal volume will consist of whole blood or packed cells within the centrifugal chamber, there is also an obligate extracorporeal RBC mass. Discontinuous machines carry out stepwise cycles of removal and return of the extracorporeal volume. In a continuous apheresis machine, the extracorporeal volume is filled at the start by drawing blood from the patient and remains fixed through the duration of the procedure. An older child or an adult with a blood volume of approximately 5000 mL can usually tolerate the temporary loss of 300 to 400 mL of blood in either a discontinuous or a continuous technique. At the other extreme, for a child of 12 kg with a blood volume of about 800 mL, the extracorporeal volume represents roughly half the patient’s blood. Thus for younger children discontinuous apheresis technique is not feasible, and continuous apheresis procedures may require priming with exogenous RBCs, reconstituted whole blood, or albumin to compensate for the extracorporeal volume and RBC mass.

Many apheresis devices have automated modes that at completion of the procedure return the RBCs in the circuit to the patient by flushing with saline. This return cycle results in rapid reinfusion of up to 450 mL over several minutes. An older child or adult, who was able to tolerate the temporary loss of volume and RBC mass incurred during a discontinuous or unprimed continuous apheresis procedure, will usually tolerate the rapid return of the volume. However, a younger patient may not tolerate the return cycle, which means that some or all of the obligate extracorporeal RBC mass must be left in the machine. This loss of RBC mass must be compensated for by using packed RBCs as the priming fluid at the start of the procedure.

Beyond the principles that have been described here, there are also strategies to avoid fluid overload without the use of RBC priming, to administer or remove intravascular fluid, and to administer blood products during the course of an apheresis procedure. The apheresis physician must take into consideration the patient's size, the extracorporeal volume of the apparatus in use, the patient's RBC mass and volume status, and disease factors that may affect the patient's ability to tolerate rapid changes in blood volume and RBC mass. The management of volume and RBC balance during apheresis procedures in children requires careful, individualized planning and a thorough understanding of the operation and programming of the automated cell separator.

Hypothermia

Because a portion of the patient's blood volume circulates outside the body during apheresis, considerable heat loss to the environment can occur. Adult platelet donors may lose sufficient heat to require blankets by the end of a 90-minute collection. For children undergoing apheresis, the physiologic impact of this heat loss is much greater, and the use of a blood warmer may be required to prevent life-threatening hypothermia. The amount of heat loss is a function of: (1) the ratio of extracorporeal volume to patient's blood volume, (2) the rate of blood removal or replacement, and (3) the thermal properties of the system. A blood warmer reduces this heat loss at the expense of increasing the extracorporeal volume, albeit modestly. Only approved devices should be used to warm the blood.

Other hazards of therapeutic apheresis are rare. These include some severe complications, but they appear to be largely related to the underlying disorder as opposed to the apheresis itself.[358,359] This is consistent with the rarity of serious complications in apheresis platelet donors. Complications have also been reported in pediatric series. However, more were related to the need for vascular access than to the procedure itself.[357,360]

Cooperation

The staff of a pediatric apheresis unit must be proficient in age-appropriate communication and psychosocial support. The aspects of the apheresis procedure that are most upsetting to children are the needle sticks, the need to remain seated, the immobilization of one or both arms, the frequent squeezing of the blood-pressure cuff, and boredom with a prolonged procedure. Expertise in phlebotomy and placement of intravenous lines is a basic requirement for the staff. The other issues can usually be managed with age-appropriate explanations, parental involvement, and distracting entertainment. Sedation is almost never necessary, although extremely uncooperative patients, such as those with a significant psychiatric diagnosis or movement disorder, might require sedation unless central access is used.

Indications

A consensus committee for the American Society for Apheresis has periodically reviewed the apheresis literature supporting and refuting the use of apheresis-based therapies for a variety of disorders in which it has been tried (Table 36-11).[361] For each disorder the strength of the evidence supporting the efficacy of apheresis was evaluated and the indication placed into one of five categories. Category I means that efficacy is well documented and use of therapeutic apheresis is "standard and acceptable"; category II, that apheresis is probably efficacious and its use is "generally accepted" but as an adjunct to primary therapy; category III, that efficacy is debatable and evidence is mostly anecdotal or conflicting; category IV, that apheresis has been shown to be ineffective and use of apheresis should be "discouraged"; and category P (for Pending), wherein an apheresis device is not approved for use in the United States, although trials may be under way. Table 36-11 is excerpted from the consensus review, listing only those diagnoses usually encountered in the practice of pediatric apheresis.[362]

ERYTHROCYTAPHERESIS (RED BLOOD CELL EXCHANGE) FOR SICKLE CELL DISEASE

In sickle cell disease small-vessel occlusion occurs when RBCs deformed (sickled) by polymerized Hb S obstruct blood flow in capillaries. If the percentage of cells that can be deformed in this way is reduced below 30% to 50% by transfusion with normal RBCs, many of the clinical manifestations of sickling substantially decrease. This reduction can be accomplished either by serial simple transfusion or by erythrocytapheresis. When a rapid reduction in the percentage of Hb S–containing cells is desirable, such as in the acute management of stroke or chest syndrome or preparation for emergency surgery, erythrocytapheresis is the method of choice. A series of two to four simple transfusions given over 2 to 3 weeks can also reduce the percentage of Hb S–containing cells to less than 30%, an approach suitable for preparation for elective surgery.

Acute Chest Syndrome

Acute chest syndrome is a leading cause of premature death among patients with sickle cell disease. Transfusion with RBCs is commonly used to treat acute chest syndrome, both to increase the oxygen-carrying capacity of the blood and to reduce the percentage of Hb S. Although controversy exists regarding the advantages

TABLE 36-11 Indications for Therapeutic Apheresis in Pediatrics

Disorders	Therapeutic Procedure	Category*
Autoimmune		
Catastrophic antiphospholipid syndrome	Plasma exchange	III
Systemic lupus erythematosus, nephritis	Plasma exchange	IV
Systemic lupus erythematosus, other	Plasma exchange	III
Polymyositis-dermatomyositis	Plasma exchange	IV
Rheumatoid arthritis	Lymphocytapheresis Immunoadsorption	II II
Hematology and Oncology		
ABO-incompatible stem cell transplant	Plasma exchange (recipient)	II
Aplastic anemia/red cell aplasia	Plasma exchange	III
Autoimmune hemolytic anemia	Plasma exchange	III
Babesiosis, severe	Erythrocytapheresis	II
Coagulation factor inhibitors	Plasma exchange Immunoadsorption	III III
Erythrocytosis/polycythemia vera	Erythrocytapheresis	II
GVHD, skin	Extracorporeal photopheresis	II
GVHD, nonskin	Extracorporeal photopheresis	III
Hyperleukocytosis, leukostasis	Leukapheresis	I
Hyperleukocytosis, prophylaxis	Leukapheresis	III
Idiopathic thrombocytopenic purpura, refractory	Immunoadsorption	II
Idiopathic thrombocytopenic purpura, any	Plasma exchange	IV
Malaria, severe	Erythrocytapheresis	II
Sickle cell disease, life or organ threatening	Erythrocytapheresis	I
Sickle cell disease, stroke prophylaxis	Erythrocytapheresis	II
Sickle cell disease, prevention of iron overload	Erythrocytapheresis	II
Thrombocytosis, symptomatic	Thrombocytapheresis	II
Thrombocytosis, prophylactic or secondary	Thrombocytapheresis	III
TTP	Plasma exchange	I
Platelet refractoriness	Plasma exchange Staphylococcal protein A adsorption	III III
Metabolic		
Acute liver failure	Plasma exchange	III
Familial hypercholesterolemia, homozygotes	Selective removal	I
Familial hypercholesterolemia, heterozygotes	Plasma exchange, selective removal	II
Hypertriglyceridemic pancreatitis	Plasma exchange	III
Mushroom poisoning	Plasma exchange	II
Other poisoning and overdoses	Plasma exchange	III
Phytanic acid storage disease (Refsum)	Plasma exchange	II
Sepsis	Plasma exchange	III
Thyrotoxicosis	Plasma exchange	III

Continued on following page

TABLE 36-11 Indications for Therapeutic Apheresis in Pediatrics (Continued)

Disorders	Therapeutic Procedure	Category*
Miscellaneous		
Dilated cardiomyopathy	Immunoadsorption	P
Inflammatory bowel disease	Adoptive cytapheresis	P
Neurology		
Acute disseminated encephalomyelitis	Plasma exchange	III
Acute GBS	Plasma exchange	I
Chronic inflammatory demyelinating polyneuropathy	Plasma exchange	I
Lambert-Eaton myasthenic syndrome	Plasma exchange	II
Multiple sclerosis, Devic syndrome	Plasma exchange	III
Myasthenia gravis	Plasma exchange	I
PANDAS, severe	Plasma exchange	I
Sydenham chorea, severe	Plasma exchange	I
Rasmussen encephalitis	Plasma exchange	II
Renal		
ANCA-associated RPGN (Wegener)	Plasma exchange	II
Focal segmental glomerulosclerosis	Plasma exchange	III
Goodpasture's disease (anti-GBM)	Plasma exchange	I
HUS, diarrhea associated	Plasma exchange	IV
HUS, other	Plasma exchange	III
Rapidly progressive nephritis (without anti-GBM)	Plasma exchange	III
Renal allograft rejection, antibody mediated	Plasma exchange	II
Renal allograft HLA desensitization	Plasma exchange	II
Rheumatic		
Scleroderma	Plasma exchange	III
Scleroderma	Extracorporeal photopheresis	IV
Transplantation		
ABO-incompatible solid organ transplant		
Kidney	Plasma exchange	II
Heart (infants)	Plasma exchange	II
Liver	Plasma exchange	III
Heart allograft rejection, prophylaxis	Extracorporeal photopheresis	I
Heart allograft rejection, treatment	Extracorporeal photopheresis	II
Heart allograft rejection, treatment	Plasma exchange	III
Lung transplant	Extracorporeal photopheresis	III

From Shaz BH, Linenberger ML, Bandarenko N, et al: Category IV indications for therapeutic apheresis: ASFA fourth special issue. J Clin Apher *22:176-180, 2007.*

ANCA, Antineutrophil cytoplasmic antibody; *GBM,* glomerular basement membrane; *GBS,* Guillain-Barré syndrome; *GVHD,* graft-versus-host disease; *HUS,* hemolytic-uremic syndrome; *NS,* not specified; *P,* pending; *PANDAS,* pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections; *RPGN,* rapidly progressive glomerulonephritis; *TTP,* thrombotic thrombocytopenic purpura.

*I: Therapeutic apheresis is standard and acceptable, either as primary therapy or as a valuable first-line adjunct to other initial therapies. Efficacy is documented by controlled clinical trials and published experience.

II: Therapeutic apheresis is generally accepted as supportive to other more definitive treatments; there is some literature support of its efficacy.

III: Anecdotal reports may suggest the use of therapeutic apheresis; however, no controlled trials have documented its efficacy. No consensus can be reached regarding its risk-to-benefit ratio.

IV: Controlled trials show lack of efficacy of therapeutic apheresis. Anecdotal studies suggesting efficacy have not been reproduced.

of exchange transfusion over simple transfusion (see Chapter 20), the efficacy of both methods in acute chest syndrome is probably similar.[363] In practice, exchange transfusion by automated apheresis is often performed in severe acute chest syndrome to reduce the Hb S percentage in a shorter time, and without the accompanying fluid loading and increases in hematocrit concentration, than could be achieved by simple transfusion or manual exchange.[141]

Stroke

The observed incidence of stroke in patients with sickle cell disease varies from 6% to 34%. Although transfusion therapy is not thought to affect the neurologic outcome of completed strokes, it remains the mainstay of acute management as a measure to halt progression of cerebral vasculopathy. Considerable evidence suggests that long-term transfusion after a central nervous system event is effective prophylaxis against progression of central nervous system disease and against recurrence of clinical stroke (secondary stroke prevention).[364] Transfusion has also been shown to be effective in primary prevention of stroke in patients with sickle cell disease who have been identified as high risk on the basis of transcranial Doppler ultrasound studies of blood flow velocity in the circle of Willis.[365] For secondary stroke prevention, some neurologically stable patients may be maintained safely at a target Hb S concentration of less than 50%, which is usually easily achievable on a program of chronic erythrocytapheresis.[366] For primary stroke prevention, the standard approach is to maintain the hemoglobin S percentage at less than 30%, which is also achievable for most patients using erythrocytapheresis.

Priapism

Although prepubescent priapism has a good prognosis for eventual potency, multiple episodes of postpubescent priapism are associated with eventual impotence. In addition, there is some correlation of the duration of the episode of priapism with loss of erectile function. Whether this is because of efficacious therapeutic intervention or simply because the worst cases last longer and are more likely to cause irreversible damage remains unclear. Some authors advocate RBC exchange for severe cases,[367] but the efficacy of exchange transfusion for priapism in sickle cell disease is unclear.

Red Blood Cell Exchange for Chronic Transfusion

Erythrocytapheresis has an important role to play in chronic transfusion therapy in sickle cell disease. Children with sickle cell disease who develop complications for which chronic, lifelong RBC transfusion is indicated are at risk to develop transfusional iron overload. Although this can often be successfully managed with chelation therapy, patient responses are variable and chelation therapy does not always succeed in achieving and maintaining optimal iron concentrations. The iron overloading of long-term simple transfusion therapy can be substantially reduced or eliminated by a program of erythrocytapheresis.[368] This approach involves removal and replacement of approximately half of the patient's RBC mass every 3 to 4 weeks. Transfusion by erythrocytapheresis will generally require 23% to 73% more donor units and exposures than simple transfusion. Because the general risks of infectious disease transmission and transfusion reactions are low, the additional risk of these donor exposures may be outweighed by the benefit of reducing or avoiding iron overload and chelation therapy. Of greater concern is the relatively high risk for RBC alloantibody formation in patients with sickle cell disease. It is not clear whether patients chronically transfused by erythrocytapheresis have a greater risk of alloimmunization than those treated by simple transfusion because both populations will eventually receive large numbers of donor exposures.

Normovolemic Red Blood Cell Transfusion

RBC transfusion in hemodynamically unstable patients may be accomplished in a normovolemic fashion using apheresis technology. For example, thalassemia patients with end-stage cardiac failure secondary to transfusional iron overload may better tolerate transfusion to raise the Hb if the blood volume is not increased rapidly. Exchange transfusion in the setting of overwhelming hemolysis has also been advocated when transfusion requirements would exceed the patient's ability to handle the volume to be transfused. This may occur in autoimmune hemolytic processes (especially IgM-mediated ones) as well as in infectious hemolytic processes, including malaria and babesiosis.[369,370]

Erythrocytapheresis may also be used to remove RBCs from a patient or donor. Patients with polycythemia secondary to chronic cyanotic congenital heart disease, complicated by Eisenmenger's syndrome, may have improved hemodynamic function and symptomatic relief after phlebotomy, which is well tolerated using apheresis technology.[371] Finally, more than one unit of RBCs at a time can be drawn safely from a donor if normovolemia is maintained.[372] This technique is being used increasingly in routine red cell collection,[373,374] in autologous red cell collection,[175] and is especially useful when obtaining blood from rare donors or from a single donor to support a specific recipient.

LEUKAPHERESIS

Leukocytosis is a negative prognostic indicator in childhood leukemia, especially when the presenting white blood cell count exceeds 200,000/μL. In many centers it is standard practice to perform leukapheresis or whole blood exchange for patients presenting with extremely high counts, with the goal of treating or preventing symptoms related to hyperviscosity and tumor lysis syndrome.[375] Although the leukocyte count can be reduced rapidly, typically dropping by 50% to 75% immediately after the procedure, a long-term survival advantage for patients treated by leukapheresis for hyperleukocytosis has not been demonstrated. The value of this intervention may in part depend on the presenting immunophenotype. Specifically, complications of hyperleukocytosis, such as cerebral thrombosis and hemorrhage, are more common in acute myeloblastic leukemia than in acute lymphoblastic leukemia.[376] Furthermore, in one retrospective study electrolyte abnormalities during induction chemotherapy were reduced in the group of patients with acute lymphoblastic leukemia presenting with hyperleukocytosis who received leukapheresis or exchange transfusion, suggesting that leukapheresis may reduce the severity of tumor lysis syndrome.[377] Some authors propose that all patients with acute myeloblastic leukemia or acute undifferentiated leukemia with presenting white blood cell counts in excess of 200,000/mm^3 should undergo exchange or leukapheresis, whereas only selective patients with acute lymphoblastic leukemia require this intervention.[378] Technically, leukapheresis may be performed either by using specific tubing sets designed to remove the buffy-coat layer or by using the tubing set for plasmapheresis and collecting down into the RBC interface. It is often desirable to maintain a relatively low hematocrit concentration during therapeutic leukapheresis because the RBC mass contributes to the viscosity of the blood.

PERIPHERAL BLOOD PROGENITOR CELLS

Peripheral blood progenitor cells or stem cells are hematopoietic progenitors with the potential for self-renewal that circulate in the blood. At steady state they are present in very low numbers, but during the early phase of recovery from chemotherapy-induced myelosuppression or as a result of exogenous hematopoietic cytokine mobilization (e.g., G-CSF), their relative numbers may increase tenfold to a hundredfold. This observation has made it possible to collect sufficient numbers of progenitor cells by apheresis to allow autologous hematopoietic rescue after high-dose chemotherapy and to serve as the sole source of stem cells, in lieu of bone marrow, for allogeneic transplantation.[379-382] Collection of peripheral progenitor cells for autologous transplantation in children was reported in 1989 by several centers.[379-382] Early engraftment of granulocyte and platelets after high-dose chemotherapy was observed, similar to the findings in adult trials.[383] (See Chapter 8 for a discussion of the role of HSCT and evaluation of progenitor harvests.)

Because almost all pediatric autologous peripheral blood progenitor protocols are directed toward treatment of aggressive cancers with high risks of relapse (e.g., relapsed lymphoma, high-risk brain tumors), the protocols have most commonly timed the collection of cells to occur during the initial rise in white blood cell count after chemotherapy-induced myelosuppression. Most protocols also use mobilization with a hematopoietic cytokine (G-CSF).[384-388] The general considerations for access for progenitor cell collections are the same as have been described previously. Because many of the candidates for peripheral blood progenitor cell collections will have central lines for their chemotherapy and supportive care placed at the time of diagnosis or relapse, it is important to select a catheter stiff enough to use in leukapheresis. As with large-volume leukapheresis collections in adults, four to seven blood volumes may be processed by the cell separator in a single 4-hour apheresis collection. More prolonged collections are technically feasible and do increase the net yield but may tax the patience of the child and apheresis staff. The adequacy and quality of collections are usually monitored with a complete blood cell count and a measure of progenitor content, using either tissue culture assays for CFU-GM or, more commonly, counts of CD34+ cells by fluorescence-activated cell-sorter analysis, or both. Whereas a typical yield from a single collection in adults might be 2.5×10^6 CD34+ cells per kg of recipient body weight, yields from pediatric collections are often much higher. Actual yields vary widely, depending on factors such as history of previous chemotherapy, timing of collections, intercurrent illness, and precollection counts.[385-388] Cytopenias, especially thrombocytopenia, have been reported after progenitor cell collections,[389] but catheter-related problems such as mechanical obstruction, thrombosis, and infection are the most common difficulties.[390]

EXTRACORPOREAL PHOTOPHERESIS

Extracorporeal photopheresis is a therapeutic use of apheresis technology that takes advantage of the conversion by ultraviolet A light (UVA, spectrum 320 to 400 nm) of 8-methoxypsoralen from an inactive molecule to an agent that can induce irreversible lesions in the DNA of leukocytes. This induces apoptosis of re-infused leukocytes, which in turn can induce regulatory T cells and stimulate interleukin-10 production.[391,392] Disorders mediated by excessive autoreactivity or alloreactivity might theoretically be treated by this approach. Some photopheresis protocols involve performing leukapheresis to collect mononuclear cells, ex vivo psoralen treatment and UV irradiation of the cells, and re-infusion of the treated cells. In other protocols the patient is treated with the psoralen systemically before leukapheresis and UV irradiation of the product. The best-documented therapeutic effect of photopheresis is in the treatment of cutaneous T-cell lymphomas (Sézary syndrome found exclusively in adults), in which response rates greater than 60% and cure rates of 10% to 15% have been achieved.[393]

More recently it has been applied to treat rejection of solid organ grafts in which immunosuppressive interventions have failed and to treat chronic GVHD, especially skin GVHD, after bone marrow transplantation, including in children.[394-399] According to some anecdotal reports, striking responses have been observed in patients with cardiac rejection refractory to standard treatment, and a randomized pilot study demonstrated efficacy of photopheresis in prevention of cardiac allograft rejection.[400] Controlled trials are needed before the efficacy of this application can be evaluated.[401] Additional reports have been made describing the control of rejection of other solid organ transplants (including lung) by extracorporeal photopheresis.[402,403]

PLASMA EXCHANGE

Plasma exchange is performed either to remove harmful constituents of the plasma (e.g., autoantibodies, excess cholesterol) or to replace missing plasma proteins, such as coagulation factors in liver failure. The extent of removal of a particular molecule or compound by plasmapheresis is a function of the amount of plasma removed and of the distribution of the molecule in the body. The most efficient clearance is achieved for substances (e.g., IgM) that are limited to the intravascular compartment. If the molecule is distributed within the compartment defined by the extracellular water (e.g., IgG), then the efficiency of removal is also affected by the rate of reequilibration between the extravascular and the intravascular space. Plasmapheresis is least efficient when the molecule targeted for removal is distributed over the entire body (as with many drugs and toxins). Similarly, the efficiency of replacement of missing plasma factors is a function of the factors' distribution and ongoing rate of consumption.[357]

For some indications the underlying mechanism of an observed therapeutic effect of plasmapheresis may be unknown. For example, in TTP, controlled clinical trials[393] documented the superiority of plasma exchange over simple plasma transfusion well before the pathogenesis of the disease was understood. This result was commonly extrapolated to the treatment of HUS because it shares certain clinical features with TTP, even though the empiric evidence supporting the use of plasma exchange in adult HUS[404] and pediatric HUS[357,405,406] was not well founded. In light of the demonstration in patients with TTP (but not HUS) of an autoantibody that removes the activity of a serum metalloproteinase responsible for cleaving von Willebrand factor,[407-409] it appears that plasma exchange treats TTP by removal of the autoantibody as well as by replacement of the serum metalloproteinase and normal von Willebrand factor. Although this discovery has provided a sound explanation of how plasma exchange helps in TTP, it has invalidated the prior assumption that HUS and TTP represent a spectrum of a single disease process that will respond to plasma exchange.

The role of plasma exchange in liver failure is controversial. In acute severe liver failure, metabolic toxins (the so-called middle molecules such as ammonia that are not effectively removed by conventional dialysis) accumulate and lead to hepatic encephalopathy, coma, and death. Furthermore, failure of production of intravascular proteins, including albumin and coagulation factors, leads to ascites, edema, and bleeding. Plasma exchange can offer temporizing supportive care by providing the missing plasma components in larger doses than could be delivered by simple transfusion. However, plasmapheresis is inefficient for removal of the toxins that accumulate in liver failure and has not been shown to prevent or forestall the development of hepatic encephalopathy. The use of plasma exchange to provide a bridge to support patients awaiting liver transplantation or to normalize coagulation parameters immediately before transplant surgery is well documented.[410]

Autoimmune Disorders

Guillain-Barré Syndrome

Several controlled studies comparing plasmapheresis to conventional therapy for Guillain-Barré syndrome (GBS) have documented a therapeutic benefit from plasmapheresis, especially when initiated early in the course of the illness.[411] Lack of uniform response to plasmapheresis had led some researchers to speculate whether the syndrome may be a common outcome of diverse causes.[412] Because children with GBS have a somewhat better prognosis than adults, plasmapheresis is generally reserved for those who deteriorate rapidly or who require ventilator assistance.[413] High-dose intravenous immunoglobulin (IVIG) has also been shown to have efficacy in treatment of GBS, and a randomized trial in adults demonstrated that IVIG may be slightly superior to plasma exchange in GBS.[414] This result has led neurologists to treat with IVIG first and reserve plasmapheresis for refractory or especially severe cases. Both IVIG and plasmapheresis have efficacy in childhood GBS but have not been compared in a randomized trial[415,416] Patients with chronic inflammatory demyelinating polyradiculopathy, an insidious peripheral neuropathy with a waxing and waning course and some similarities to GBS, may also benefit from plasmapheresis.[416-418]

Myasthenia Gravis

Myasthenia gravis is a neuromuscular disorder characterized by progressive proximal muscle weakness. It follows damage to motor end plates owing to an autoantibody directed against postsynaptic acetylcholine receptors. Plasmapheresis has been shown to provide acute temporary relief of myasthenic crises and to be a useful adjunct along with immunosuppressive therapies for longer-term treatment.[419,420]

Systemic Lupus Erythematosus

Systemic lupus erythematosus is an autoimmune disorder with protean manifestations that may affect many organ systems, especially the skin, kidney, central nervous system, and blood. Controlled trials of plasmapheresis in systemic lupus erythematosus have generally failed to show long-term benefit.[421] Nonetheless, some authors advocate the use of plasmapheresis to treat certain acute flares of the disease, especially when they are severe or threaten the function of vital organs (e.g., kidney, central nervous system). Another proposed appropriate indication for apheresis is a lupus flare during pregnancy when other more toxic treatments might be contraindicated.[422]

Immune Hemolysis

Cold agglutinin disease, a form of autoimmune hemolytic anemia mediated by an RBC antibody of the IgM isotype, may be effectively treated in the acute phase by plasmapheresis, because IgM antibodies are efficiently removed by plasmapheresis.[423] Plasmapheresis has also been used in life-threatening IgG-mediated autoimmune hemolysis, even though the removal of IgG is much less efficient. The reduction in hemolysis is transient in either case, and in general, apheresis is warranted only to buy time until other treatments take effect or to treat disease refractory to other therapies.[424]

Other Autoimmune Disorders

Rasmussen encephalitis, a rare disease resulting in an intractable seizure disorder previously treated by hemispherectomy, has been shown to result from autoantibodies directed against the glutamate receptor (GluR3) in the brain.[425] The antibodies reportedly act as constitutive agonists, resulting in excessive neuronal activation and subsequent seizures.[426,427] Case reports suggest that plasmapheresis has a role in acute treatment. A syndrome called pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) consists of a movement disorder with involuntary tics and a behavioral disorder with obsessive-compulsive features.[428] The pathophysiology is thought to involve group A β-hemolytic streptococcus triggering antibodies that cross-react with the basal ganglia of genetically susceptible individuals, a mechanism parallel to the pathophysiology of Sydenham chorea.[429] One randomized trial has demonstrated that both IVIG and plasma exchange have efficacy in treating the symptoms of PANDAS,[430] and another trial suggests that plasmapheresis and IVIG may have efficacy in Sydenham chorea.[431]

Hyper-IgE syndrome (Job syndrome) is a rare autosomal recessive disorder characterized by extremely high IgE levels, recurrent infections (typically with *Staphylococcus* organisms), and chronic dermatitis. Plasmapheresis results in a reduction of IgE levels and correspondingly decreases inflammatory lesions. It is usually reserved for patients who have failed to respond to other therapies, including antibiotics and IVIG.[432] An unusual complication of hyper-IgE syndrome is keratoconjunctivitis, which may threaten vision; it has reportedly been improved using apheresis.[433]

Other Coagulopathies

A preexisting coagulopathy has historically been a contraindication to putting a patient on ECMO, owing to the additional anticoagulation required to prevent clotting in the membrane oxygenation circuit and the high risk of intracranial hemorrhage. Several centers have observed that it is possible to perform plasmapheresis using the ECMO circuit itself, thereby replacing the missing coagulation factors with the hope of decreasing the risk of hemorrhage.[434,435]

Renal Disease

Cryoglobulinemia, a disorder rare in pediatrics, involves the pathologic production of proteins that reversibly precipitate or gel on exposure to cold. In general, they are antibodies, although they are not necessarily limited to the IgM class. These immune complexes may deposit in the kidney, resulting in end organ damage. Because they are largely intravascular, they are efficiently cleared by apheresis.[436] Apheresis has also been advocated in Goodpasture syndrome, an autoimmune disorder caused by antibodies against glomerular basement membranes. Controlled trials to measure the efficacy of plasmapheresis, often as an adjunct to other immunosuppressive agents, have yielded conflicting results. Some appear to show clear benefit,[437] whereas benefit in other studies appears marginal.[438]

Whereas plasmapheresis has not been shown to prevent renal allograft rejection, it may prove useful as an adjunctive therapy in preventing recurrence of corticosteroid-resistant nephrotic syndrome with focal sclerosing glomerulosclerosis (FSGS) in the transplanted kidney when used in conjunction with cyclophosphamide and pulse corticosteroids.[439,440] The mechanism of this therapeutic effect is thought to be the removal of a protein permeability factor in the plasma that mediates the renal injury in FSGS.

Hypercholesterolemia

Genetic absence of the low-density lipoprotein receptor results in extreme hypercholesterolemia and development of severe atherosclerotic complications within the first several decades. Cholesterol-lowering drugs are largely ineffective in patients with completely absent or nonfunctional receptors. Hence plasmapheresis is the treatment of first choice. It may be performed by simply removing plasma and replacing it with albumin or by using selective lipid absorption columns. The reported advantages of the latter treatment are the greater volumes that may be processed with less depletion of coagulation factors, immunoglobulins, and high-density lipoproteins. Columns come in many varieties; the most commonly used are disposable dextran sulfate columns. A dual column method has been developed that cannot be saturated; collected lipid is eluted from one column while the second is collecting. This system has been shown to be effective in pediatric patients.[441] Disadvantages of using columns include additional extracorporeal volume (150 to 400 mL) added to the system and some reports of adverse side effects.

Transplantation

ABO-incompatible bone marrow or solid organ transplant is most problematic when the recipient has IgM antibodies against the donor ABO type.[442] Apheresis can be used to deplete isohemagglutinins (naturally occurring anti-A, anti-B antibodies).[443] Although this approach is effective in reducing IgM titers, it often requires several daily procedures, resulting in large numbers of donor exposures.[444] Most bone marrow transplant programs have switched to depleting the donor marrow of RBCs, a one-time procedure that has not been shown to compromise engraftment. For ABO-incompatible cardiac transplantation in neonates, however, plasmapheresis, either as a separate procedure or incorporated into cardiopulmonary bypass, may be used immediately before or after transplant surgery to remove isohemagglutinins.[148] Plasmapheresis has also been used to reduce HLA antibodies in patients who are candidates for renal transplantation but who have high degrees of HLA sensitization that restrict compatible organ availability and increase the risk of hyperacute rejection.[445]

Disseminated Intravascular Coagulation

Purpura Fulminans

Plasmapheresis may be used in desperate situations. Specifically, the dramatic coagulopathy of purpura fulminans associated with meningococcal sepsis can be treated with plasmapheresis. In trials using historical controls, improved survival and correction of coagulopathy have been documented, but because the trials have no concurrent controls, it is impossible to be sure that plasmapheresis contributed to the improvement.[446]

Special Applications and Selective Adsorptions

Plasmapheresis allows the selective removal of components in plasma by additional processing of the separated plasma. The columns currently available in the United States are the previously described lipid adsorption columns. Other columns not available for clinical use in the United States use beads coated with antigen to remove a specific antibody.

Another selective-removal apheresis methodology has been developed to target the removal of some populations of granulocytes and monocytes based on their adherence to cellulose acetate beads. According to several reports from Japan, this form of granulocytapheresis is efficacious and safe in the treatment of ulcerative colitis, but the device is also not currently available in the United States.[447-449]

CONCLUSION

The addition of mechanical apheresis devices to the therapeutic armamentarium of transfusion medicine specialists has allowed for dramatic interventions in both acute and chronic disorders. The complexity of the clinical settings in which apheresis is sometimes used often makes it difficult to assess the value of apheresis-based therapies. As economic constraints continue to encroach on the application of medical technologies, it is imperative to design well-controlled studies to document the efficacy of the intervention. Consensus statements provide an opportunity to pose testable questions for interventions in multicenter trials. The involvement of pediatric transfusion medicine specialists is warranted early in the design of such trials owing to the technical complexity of the procedures and the unique requirements of pediatric patients. Producers of apheresis devices must also be mindful of these unique requirements because protocols designed for adults are not always applicable to children. Reduction in extracorporeal volume, for example, may be beneficial to adults as well as pediatric patients.

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CHAPTER 37

Hematologic Manifestations of Systemic Diseases*

Rachael F. Grace

CHAPTER OUTLINE

COLLAGEN DISORDERS
Collagen Vascular Disease
Vasculitis
Connective Tissue Disorders
INFECTIOUS DISEASES
General Hematologic Signs of Infection
Hematologic Aspects of Specific Infections
CARDIAC DISEASE
Hemolysis
Acquired Coagulation Abnormalities
Platelet Abnormalities
Miscellaneous Hematologic Manifestations of Heart Disease
PULMONARY DISEASE
Cystic Fibrosis
Idiopathic Pulmonary Hemosiderosis
Pulmonary Hemosiderosis from Other Causes
Hematologic Findings in Other Pulmonary Disorders
RENAL DISEASE
The Red Blood Cell
The White Blood Cell
Coagulation Abnormalities
Platelet Abnormalities
GASTROINTESTINAL DISEASE
Diseases of the Gastrointestinal Tract
Disease of the Pancreas
Diseases of the Liver
ENDOCRINE DISORDERS
Thyroid Gland
Adrenal Gland
Pituitary Gland
Other Endocrine Disorders
Diabetes Mellitus
METABOLIC DISEASES
Disorders in Lipid Metabolism
Other Metabolic Disorders
NEUROLOGIC AND PSYCHIATRIC DISORDERS
Neuroacanthocytoses
Anorexia Nervosa
CUTANEOUS DISEASES
Eczema and Psoriasis
Hereditary Hemorrhagic Telangiectasia
Mast Cell Disease
PREGNANCY
Anemia of Pregnancy
Thrombocytopenia and Pregnancy
Disseminated Intravascular Coagulation and Pregnancy
Sickle Cell Disease and Pregnancy
Inherited Bleeding Disorders and Pregnancy
Pregnancy and Thrombosis
Inherited Hypercoagulable States and Pregnancy
Antiphospholipid Antibody Syndrome and Pregnancy
TOXINS AND OTHER INSULTS
Alcohol and Hematopoiesis
Thermal Injury
Venoms

Evaluation of almost any patient who seeks medical attention, particularly in a hospital setting, includes the obligatory complete blood cell count. The vast majority of these patients do not have a primary hematologic problem. However, there are many pathophysiologic paradigms that have secondary effects on hematopoiesis and/or the coagulation system. One of the most common examples of these effects includes the anemia of chronic disease or inflammation, which is associated with a wide variety of acute and chronic illnesses, including cancer, collagen vascular disease, severe tissue injuries, renal failure, and infectious processes.[1] A detailed discussion of the anemia of chronic disease can be found in Chapter 11.

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In this chapter, both an organ system and disease-specific approach is used. The goal of this chapter on the hematologic manifestations of systemic diseases is to focus on information that is not included in other sections of this book. For overlapping topics, the reader will be guided to other relevant chapters throughout the book.

COLLAGEN DISORDERS

Collagen Vascular Disease

Many of the hematologic manifestations of the various collagen vascular diseases are similar. However, the characteristics that are unique to each syndrome are emphasized in this section.

Systemic Lupus Erythematosus

Anemia. Anemia is the most common hematologic abnormality in systemic lupus erythematosus (SLE).[2,3] There are multiple causes of anemia in this disease, although the anemia of chronic disease is probably the most common (see Chapter 11).

Acquired autoimmune hemolytic anemia (AIHA) may precede the onset of active SLE. It is typically characterized by a warm-type AIHA with a serologic pattern of immunoglobulin (Ig) G plus complement on the red blood cell surface.[4] Although positive results on an antiglobulin test are common, true hemolysis is seen in less than 10% of patients.[5] Chapter 13 details the pathophysiology and management of AIHA.

Mechanical hemolytic anemia may be seen in SLE-associated thrombotic thrombocytopenic purpura (TTP). Myelofibrosis has also been described in several patients with SLE; immune complexes have been suggested but not proven to be the cause of this rare complication of SLE.[6] Aplastic anemia has been reported in association with SLE.[7]

Leukopenia. A reduction in the total white blood cell count is seen in most patients but is more common in adults than children. The leukopenia is usually caused by a combination of decreased numbers of granulocytes and lymphocytes.[8] Lymphopenia may be severe in SLE[9] and is sometimes due to IgG or IgM antilymphocyte antibodies.[10-12] The magnitude of the reduction in T cells parallels disease activity and is associated with defects in cellular immunity, as measured by skin tests of delayed hypersensitivity, blast transformation in response to mitogen (phytohemagglutinin and concanavalin A), and macrophage-inhibiting factor production.[9,13] Reduced numbers of regulatory T cells contribute to the loss of T- and B-cell tolerance in SLE.[14] Unlike T-cell function, B-cell function is increased despite the decrease in the absolute number of B cells with emergence of autoreactive B cells.[15,16] There is actually an increase in IgG-synthesizing peripheral blood lymphocytes, as well as elevated numbers of cells capable of binding native DNA and elevated numbers of IgM- and IgG-producing cells with antibody specificity against DNA antigen.[17,18] These changes in peripheral blood lymphocytes are associated with certain morphologic alterations. A strongly basophilic cytoplasm and a high nuclear-to-cytoplasmic ratio are noted in many lymphocytes believed to be “immunoblasts.”[19] On electron microscopic examination, inclusions are identified within lymphocytes that appear as undulating tubules characteristically associated with the endoplasmic reticulum.

Antibodies to granulocytes have been proposed as one mechanism for the granulocytopenia. Granulocyte antibodies and peripheral granulocyte destruction have been observed.[20] Bone marrow depression of granulocyte formation may also occur. Sera from patients with SLE can inhibit mouse bone marrow colony-forming units.[21] Qualitative abnormalities in granulocyte function have also been noted. Although several investigators have found no abnormalities in chemotaxis,[22,23] others have observed abnormal migration by the skin window technique and diminished in vitro phagocytosis.[24,25] These defects parallel the observed depressions in complement. The qualitative defects in granulocyte function may be the result of altered humoral factors rather than defects in the phagocytic cells themselves.

Thrombocytopenia. Thrombocytopenia secondary to platelet immune sensitization is the most common immune-mediated hematologic manifestation of SLE (see Chapter 34). Thrombocytopenia may be an initial manifestation of SLE, and consideration of SLE should be part of the evaluation of immune thrombocytopenia (ITP) in adults and adolescent females. Occasionally, ITP may manifest years before serologic evidence or clinical manifestations of SLE are detectable.[26] Similar to primary ITP, platelet survival is shortened and there are typically increased numbers of bone marrow megakaryocytes. The platelet antibody is commonly an IgG with a molecular mass of 150,000 to 330,000 daltons, and it binds complement.[27] ITP in patients with SLE has the same therapeutic implication as primary ITP, that is, corticosteroids or intravenous immune globulin as primary intervention, with other treatments, including rituximab and splenectomy, reserved for patients who fail these approaches.

Rarely, a qualitative defect in platelet function may also be present with an antibody that interferes with platelet aggregation.[28,29] Thrombocytopenia may be seen in association with SLE and TTP.[30]

Lupus Anticoagulant and Antiphospholipid Syndrome. Patients with SLE may also have a circulating anticoagulant in their plasma.[31,32] The antibody-anticoagulant is directed against various phospholipids involved in phospholipid-dependent anticoagulation assays.[33] Most investigators have reported that lupus anticoagulant is an immunoglobulin that may be of the IgG, IgM, or mixed IgG-IgM class.[34-37] The pathologic significance of the lupus anticoagulant and other antiphospholipid antibodies is their association with thrombosis, mainly venous, as well as miscarriages and spontaneous abortions (see Chapter 35). Among adult SLE patients, 12% to 30% have anticardiolipin antibodies and 15% to 34% have lupus anticoagulant antibodies.[38-41] The prevalence of thrombosis in pediatric SLE patients with lupus anticoagulant is not known. In adults, the prevalence of thrombosis is about 30%, with the predominant site being the legs in 66%, followed by the peripheral arteries in 10% and the cerebral arteries in 2%.[42]

The partial thromboplastin time is invariably prolonged in patients with the lupus anticoagulant and is considered to be the most sensitive screening test.[43] The inhibitor is suspected when the addition of normal plasma to the patient's plasma fails to correct the defect.[34] The prothrombin time in patients with the lupus anticoagulant is often normal or minimally prolonged because of the influence of the inhibitor. True prothrombin deficiency may occur in patients with the inhibitor, but it is rare.[44,45] Prolonged thrombin times in the absence of elevated levels of fibrin split products may occasionally be seen.[46] The exact significance of this finding is unclear.

A minority of patients have a catastrophic form of the disease.[47,48] This often fatal syndrome is defined by the clinical involvement of at least three different organs over a period of days to weeks, with pathologic evidence of multiple occlusions of large or small vessels.[49] In general, involvement of small vessels is more common in these patients, who tend to present with an acute thrombotic microangiopathy involving the kidney, lungs, central nervous system, heart, or skin, along with disseminated intravascular coagulation (DIC). The mortality rate is 30% to 50%.[48] Aggressive therapy with combinations of anticoagulants, corticosteroids, intravenous immune globulin, and plasmapheresis have been tried in anecdotal reports with varying success.

In children, the lupus anticoagulant is usually found coincidentally on routine preoperative coagulation screening.[50] In the majority of cases, this lupus anticoagulant is not associated with SLE or bleeding symptoms. In these cases, the lupus anticoagulant typically resolves spontaneously within weeks to months.[51]

Juvenile Idiopathic Arthritis (Rheumatoid Arthritis)

Anemia in rheumatoid arthritis is caused typically by both anemia of chronic disease and iron-deficiency anemia.[52] A high incidence of iron deficiency has historically been seen in children with juvenile idiopathic arthritis (JIA), because of gastrointestinal blood loss secondary to long-standing use of aspirin and nonsteroidal analgesics in the symptomatic treatment of this disease.[53] The severe inflammatory nature of this disorder makes it a model for anemia of chronic disease, a sideropenic anemia with reticuloendothelial iron overload. Iron is not only loaded inappropriately into the reticuloendothelial system but also accumulates in the involved joint, possibly playing a role in joint destruction.[54]

Microcytosis is very common in juvenile chronic forms of arthritis, with a rate of occurrence of about 40%. The degree of microcytosis relates directly to disease activity.[55] Occasionally, macrocytic anemia develops in patients with JIA.[56] Although abnormal vitamin B_{12} metabolism has been suggested as a cause, this is not seen in children. Folate metabolism may, however, be abnormal. Some children with rheumatoid arthritis have diminished plasma and red blood cell folate levels.[57] Increased folic acid plasma clearance and reduced protein binding have also been observed.[58]

A few patients have had mild shortening of red blood cell survival as a result of an extracorpuscular defect.[59] More severe hemolytic anemia is much less common than in other collagen vascular diseases. Erythroid aplasia responsive to corticosteroid therapy has been reported in a child with juvenile rheumatoid arthritis.[60] Circulating inhibitors of erythropoiesis have also been noted.[61]

Leukocytosis and neutrophilia are common during acute flare-ups of JIA; however, this is uncommon in the adult form of the disorder. Neutrophil chemotaxis may be mildly diminished.[22] Phagocytosis is normal or mildly impaired.[62] Nitroblue tetrazolium dye reduction may be increased during active phases of this disease.[63] These minimal alterations in white blood cell function do not produce any clinical disturbances. Wound healing, for example, is normal even after surgery in patients who have not been receiving high-dose corticosteroid therapy.[64] A peripheral blood eosinophil count greater than 5% has been noted in slightly more than 50% of children with JIA.[65] Some will also demonstrate basophilia and plasmacytoid lymphocytes.[66,67]

Secondary thrombocytosis is common in JIA, as part of the inflammatory response. A consumptive coagulopathy may be seen in association with systemic JIA.[68-70] Acquired circulating inhibitor to factor VIII has been described in JIA and may cause bleeding.[69] However, clinical problems related to coagulation abnormalities in this disorder are rare.[70]

Felty Syndrome

Felty[71] originally described the triad of rheumatoid arthritis, splenomegaly, and neutropenia in 1924. Whether Felty syndrome occurs with JIA is unclear. Splenomegaly is seen in about 20% of all children with JIA, especially in those with the acute extraarticular exacerbations of this disease. However, splenomegaly alone does not suggest Felty syndrome.

The etiology for the neutropenia of Felty syndrome is multifactorial. The neutropenia is not always a consequence of hypersplenism per se because only 60% of adults who undergo splenectomy experience resolution of their neutropenia.[72] In 50% of patients, a maturation arrest bone marrow pattern is demonstrated, possibly from immune humoral or cellular mechanisms. Neutropenia in association with cytotoxic lymphocytes directed against granulocyte progenitors has also been reported,[73] as have lower than normal levels of granulocyte colony-stimulating factor (G-CSF) activity.[74] The neutropenia of Felty syndrome may also be immunologic in origin, because some patients have IgG antibodies directed against neutrophils.[75,76]

The neutropenia may be associated with serious infections. Recombinant human G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF) both effectively raise the neutrophil count in essentially all patients with Felty syndrome.[77,78] The neutrophil count decreases again once the growth factor treatment is stopped, but often it stabilizes at a level higher than it was before treatment.[77,79] The drug can be used long term and may be cost-effective in selected patients with a high incidence of infections and repeated hospitalizations.[80,81]

Methotrexate treatment in Felty syndrome can be effective in correcting the neutropenia.[82] Splenectomy may be indicated in those in whom G-CSF or GM-CSF is ineffective or rapid blood cell improvement is desired. A rise in the neutrophil count after splenectomy is experienced by 50% to 80% of patients.[77]

Vasculitis

Polyarteritis Nodosa

Microangiopathic hemolytic anemia can be associated with renal disease or hypertensive crises in polyarteritis nodosa. The hemolytic process in these circumstances parallels disease activity. Anemia of chronic disease is common in polyarteritis nodosa.

Neutropenia occurs and is associated with the presence of antineutrophil cytoplasmic antibodies.[83] Neutrophilia and eosinophilia are also common. The eosinophilia may be marked, particularly in those patients with clinically apparent pulmonary involvement.

Wegener Granulomatosis

Wegener granulomatosis, characterized by necrotizing vasculitis (particularly in the lungs and kidneys), is rare but can affect children.[84,85] The disease can also occur in the neonatal period.[86] The course of the disease is marked by fever, cough, hemoptysis, epistaxis, nasal discharge, obliteration of the nasal sinuses, and nodular pulmonary infiltrates. Renal failure may occur. A microangiopathic hemolytic anemia can develop.[87] An associated anemia of chronic disease is common.[87,88]

The white blood cell count is elevated, often coupled with an eosinophilia.[89] The disease is characterized by marked thrombocytosis.[87] Autoantibodies directed against cytoplasmic components of neutrophil granulocytes and monocytes have been described as a disease-specific marker for Wegener granulomatosis.[90,91]

Connective Tissue Disorders

Bleeding and bruising are common manifestations of connective tissue disorders related to fragility of capillaries and perivascular connective tissue rather than disorders of clotting or platelet function.[92] Primary hemostasis depends on the interaction of the vessel wall, platelets, and the clotting cascade. In connective tissue disorders, the connective tissues of the skin, subcutaneous tissue, and blood vessel wall have increased fragility, which portends increased bruising and bleeding. Laboratory evaluation for disorders of platelet aggregation and clotting factors is typically normal. Genetic counseling is recommended when a connective tissue disorder is suspected.

Ehlers-Danlos Syndrome

Most Ehlers-Danlos subtypes are caused by mutations in fibrillar collagens. The elastic tissue defects result in an increased bleeding tendency. Different forms of Ehlers-Danlos have been associated with platelet and coagulation defects, including disorders of platelet aggregation and factor deficiencies.[93-97] It is not clear whether these hematologic defects are sporadic associations in individual patients or true associations with the subtypes of Ehlers-Danlos.

In Ehlers-Danlos syndrome, the bleeding symptoms are typically easy bruising, gingival bleeding, prolonged bleeding with surgical or dental procedures, and menorrhagia. Other common clinical findings include skin hyperextensibility, “cigarette paper–like” scars, and joint hypermobility. The vascular Ehlers-Danlos syndrome, type IV, is an autosomal dominant disorder caused by mutations in the collagen type III alpha 1 *(COL3A1)* gene. Type IV Ehlers-Danlos is associated with small and large vessel bleeding, including arterial bleeding, and spontaneous ruptures of the bowel, uterus, and lungs. Nonemergent surgical interventions are to be avoided in these individuals owing to the high risk for perisurgical bleeding and complications. With serious bleeding in Ehlers-Danlos syndrome patients, efficacy of desmopressin and of factor VIIa has been reported.[98-100]

Other Inherited Connective Tissue Disorders

Marfan syndrome is not associated with fragility of capillaries or small to medium-sized arteries or veins. However, many Marfan syndrome patients report easy bruising, which has not been explained. A defective fibronectin has been proposed as the cause of the hypermobility and platelet dysfunction in some patients.[101] Easy bruising has also been reported in individuals with osteogenesis imperfecta.

Scurvy

Ascorbic acid functions as a cofactor, enzyme complement, cosubstrate, and antioxidant for a wide variety of metabolic processes.[102] Vitamin C is involved in reactions involving iron, copper, vitamin E, and folic acid. Deficient intake of ascorbic acid results in impaired collagen synthesis, mitochondrial fatty acid transport, and synthesis of neurotransmitters.[102-104] Although scurvy is uncommon in developed countries, certain patient subpopulations remain at risk for this type of nutritional deficiency, such as those with neurologic impairment, psychiatric disease, alcoholism, small bowel disease, and iron-overload disorders associated with vitamin C renal wasting.[105-108] Scurvy occurs when vitamin C has been eliminated from the diet for at least 3 months. Hematologic symptoms of scurvy arise mainly from defective collagen synthesis and include gingival bleeding, subperiosteal hemorrhage with painful joint bleeding, and organ hemorrhage. Scurvy is associated with a multifactorial anemia related to bleeding, dietary deficiencies, oxidative hemolysis, and altered metabolism of iron and folate.[109,110] Other manifestations include poor wound healing, defective dentine formation, tooth and hair loss, and bony changes. The symptoms of scurvy, including the bleeding manifestations, rapidly respond to enteral vitamin C supplementation.

INFECTIOUS DISEASES

General Hematologic Signs of Infection

Red Blood Cell Disturbances

The anemia of chronic disease is common to all infections. Even common childhood infections, especially those associated with inflammation, will cause a decline in hemoglobin concentration. During active inflammation, the hemoglobin concentration declines about 13%, usually within 1 week, followed by an increase of nearly 25% during resolution of the active inflammation.[111] The actions of hepcidin probably deprive microorganisms of iron, which is an essential nutrient for microbial replication, and may be a component of host immunity against infection.[112] Serum levels of iron decrease after a bacterial challenge, and the lactoferrin released from stimulated granulocytes is a conduit for this iron to return to reticuloendothelial cells. Many examples of bacterial pathogenicity being dependent on iron availability (e.g., *Neisseria gonorrhoeae, Neisseria meningitidis*) support the hypothesis that the anemia of infection is the body's compromise in an attempt to deprive invading microorganisms of iron.[113,114]

Although some infections, particularly viral ones, such as parvovirus, cause transient bone marrow aplasia or selective erythroid aplasia, anemia from this cause is rare because of the long life span of red blood cells. In contrast, patients with chronic hemolytic anemias may experience a rapid decrease in hemoglobin concentration or an "aplastic" crisis during viral and some bacterial infections.

Severe hemolytic anemia may be observed in certain types of infections. Clostridial infections may result in a high titer of hemolysins and cause severe anemia. A similar severe anemia may result from sepsis related to other bacterial organisms, including staphylococci, streptococci, pneumococci, and *Haemophilus influenzae*.[115] Immune hemolytic anemia mediated by a cold agglutinin may be observed with *Listeria* and *Mycoplasma* infections[116] and occasionally with infections by other organisms such as Epstein-Barr virus.

Many viral illnesses may be associated with what appears to be a mild hemolytic anemia for which no pathologic mechanism has been defined. The most common morphologic finding in these circumstances is poikilocytosis. Certain viruses, such as most strains of influenza virus, contain neuraminidase activity, which is, at least theoretically, capable of affecting the sialic acid content of the red blood cell membrane. Whether this plays any significant role in the hemolysis associated with some viral diseases is not known.

Many congenital infections, including cytomegalovirus, herpes simplex, rubella, toxoplasmosis, and syphilis, produce profound hemolytic anemia in the neonatal period, even though these same agents may not significantly alter red blood cell survival at other times of life.

White Blood Cell Disturbances

The white blood cell count may be normal, low, or high with infection. Viral illnesses may be associated with leukocyte counts lower than 5000 cells/mm^3, although bacterial diseases of certain types or overwhelming sepsis of any type may also cause leukopenia. The most common viral illnesses associated with leukopenia are infectious hepatitis, infectious mononucleosis, rubella, measles, and, occasionally, influenza. Of the bacterial infections, shigellosis may produce leukopenia with a marked increase in band cell forms. Sepsis caused by meningococci, pneumococci, staphylococci, and a few other bacterial pathogens may also cause leukopenia.

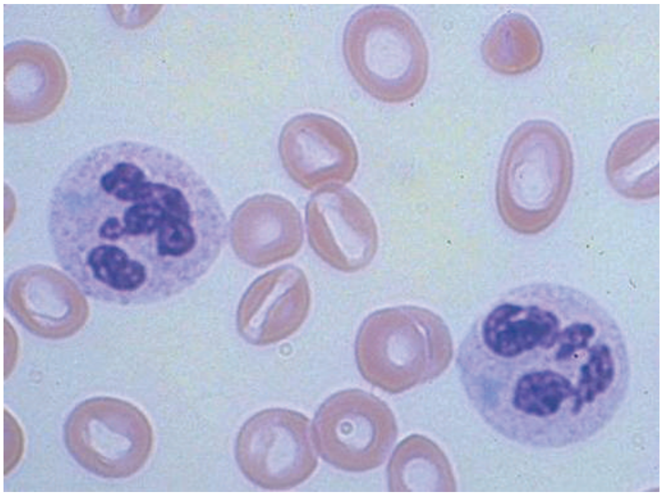

Figure 37-1 Döhle bodies.

Neutrophilia, with or without an increase in band cell count, is a common result of bacterial infection. White blood cell and neutrophil counts do not differ between children of different races with bacteremia.[117] Occasionally, viral illness also initially will be manifested as neutrophilia. A variety of morphologic changes may appear in the neutrophils of patients with infection. Döhle bodies (Fig. 37-1), which are pale blue cystlike inclusion bodies usually located in the periphery of the cytoplasm of neutrophils, may appear in bacterial infections.[118] They are occasionally associated with viral illness but are also commonly seen in patients with burns, massive trauma, and cancer, as well as in pregnancy and after the use of cyclophosphamide.[118] In addition, Döhle bodies are seen in the May-Hegglin anomaly.[119] Increased size of neutrophil granules ("toxic granulation") may be found in both bacterial and viral illnesses, as well as in many of the other disorders associated with the presence of Döhle bodies. Vacuolization of the cytoplasm of neutrophils is the next most common morphologic abnormality of neutrophils in patients with significant bacteremia. In overwhelming sepsis, the organisms are frequently evident in the vacuoles of the polymorphonuclear cells. In a study of the neutrophils of patients with bacteremia, Zipursky and associates[120] found toxic granulation, Döhle bodies, and vacuolization in 75%, 29%, and 24%, respectively, of the patients studied.

Increased neutrophil alkaline phosphatase activity and nitroblue tetrazolium dye reduction may also occur,[121,122] but neither of these characteristics is specific for bacterial infection. Infections may be associated with development of the Pelger-Huët anomaly (Fig. 37-2), in which granulocytes and eosinophils have one or two lobes per nucleus and assume a round, dumbbell, or peanut shape.[123]

In neonates, especially those born prematurely, an increase in total white blood cell or mature neutrophil counts may not be seen in the presence of infection. In fact, a decrease in the neutrophil count often occurs. The

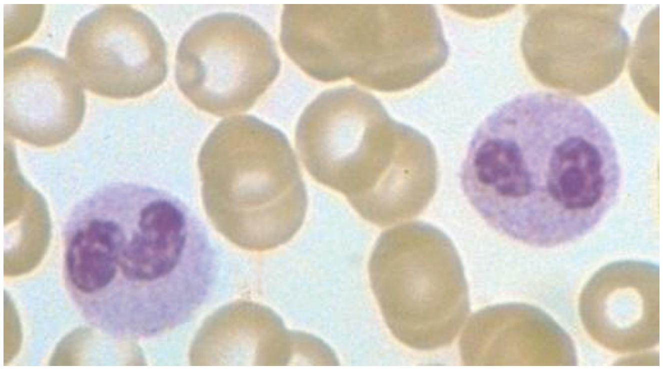

Figure 37-2 Pelger-Huët anomaly.

most helpful signs of septicemia in this age group are an increase in the band cell count and the presence of toxic granulations and Döhle bodies.[120,124]

Leukocytosis may result from lymphocytosis. The most common infections producing the greatest increases in lymphocyte counts are infectious mononucleosis, cat-scratch disease, and pertussis. Many other viral illnesses, such as cytomegalovirus, rubella, mumps, and hepatitis, may also cause an increase in the lymphocyte count. Lymphopenia of T lymphocytes is a common finding after measles infection.[125]

Eosinophilia may reflect the presence of parasitic infections. In the United States, the most common cause of marked elevations in eosinophil counts is *Toxocara* infestation, which is often accompanied by high titers of isohemagglutinins. Other parasites commonly causing eosinophilia include organisms belonging to *Trichinella, Echinococcus, Filaria, Strongyloides, Schistosoma, Enterobius,* and *Ancylostoma* and tapeworms other than *Echinococcus.* Allergic sensitization to mites may cause eosinophilia, as well as fungal infections, especially aspergillosis.[126] Eosinophilia is not specific for infestation. Marked degrees of eosinophilia may occur in association with prematurity. An absolute eosinophilia may be expected in about 75% of low-birth-weight infants.[127] In some, the eosinophilia is marked (3000 cells/mm^3) and the maximal increase seems to occur at about the time that birth weight is regained, although this is not true in all infants.[128]

Monocytosis is occasionally reported with specific infections, especially tuberculosis, syphilis, and subacute bacterial endocarditis. Monocytosis is often noted early in the course of many infections and again on recovery, particularly if it is associated with granulocytopenia.

Basophilia is rarely seen in infection but has been reported with tuberculosis, influenza, and hookworm infestation.[129]

Clotting Abnormalities and Thrombocytopenia

DIC may be triggered by infectious processes. Of the infectious causes, gram-negative septicemia is probably most common. *Meningococcus, Escherichia coli, Proteus, Pseudomonas, Aerobacter,* and *Klebsiella* are among the most common etiologic agents recovered from the bloodstream.[130,131] Gram-positive septicemia can cause a similar picture. The most common offender is *Streptococcus pneumoniae,* especially in asplenic individuals. Other gram-positive agents causing DIC include *Staphylococcus aureus,* other streptococcal species, and *Clostridium.* A wide range of viral infections may cause a consumptive coagulopathy that often leads to purpura fulminans. Among the most common agents are those that cause infectious hepatitis, measles, rubella, varicella, and infectious mononucleosis. Less common causes of DIC are severe mycoplasmal, rickettsial, and malarial infections.

Thrombocytopenia occurring separately from a true disseminated consumptive process is quite common in many infectious processes, especially in association with infectious mononucleosis, cytomegalovirus, rubella, measles, gram-negative bacteremia, and rickettsial diseases. Congenital viral infections and congenital syphilis and toxoplasmosis, if clinically apparent, are almost invariably associated with increased platelet turnover with or without thrombocytopenia. Pediatric immune thrombocytopenia is commonly associated with viral infections.[132] Immune thrombocytopenia may also occur after immunization with the measles, mumps, and rubella vaccine.[133] Corrigan found that thrombocytopenia without consumptive coagulopathy is an extremely common finding in infants and children with septicemia.[134] In contrast, thrombocytosis is often present during the active phases of infectious processes. The platelet distribution width and mean platelet volume may be helpful in predicting whether the thrombocytopenia is due to infection. In the late neonatal period, thrombocytopenia associated with an infection dramatically increases the mean platelet volume and platelet distribution width as determined by electronic counting equipment.[135]

Bone Marrow Abnormalities

The bone marrow frequently contains clues as to the source of infectious diseases. Histoplasmosis (Fig. 37-3), tuberculosis, kala-azar, *Salmonella typhi,* and *Candida* (Fig. 37-4) all have been identified in marrow macrophages. Marrow granulomas may be a marker of disseminated tuberculosis or histoplasmosis (Fig. 37-5).

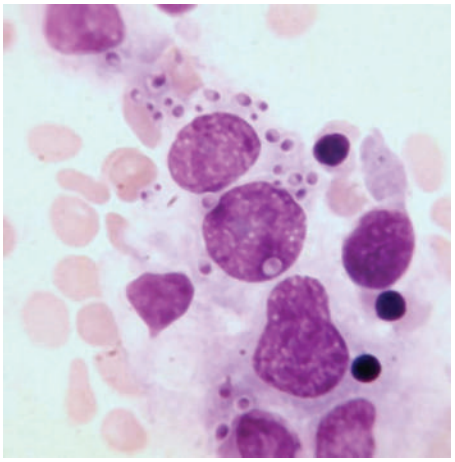

Figure 37-3 Histoplasmosis in the bone marrow.

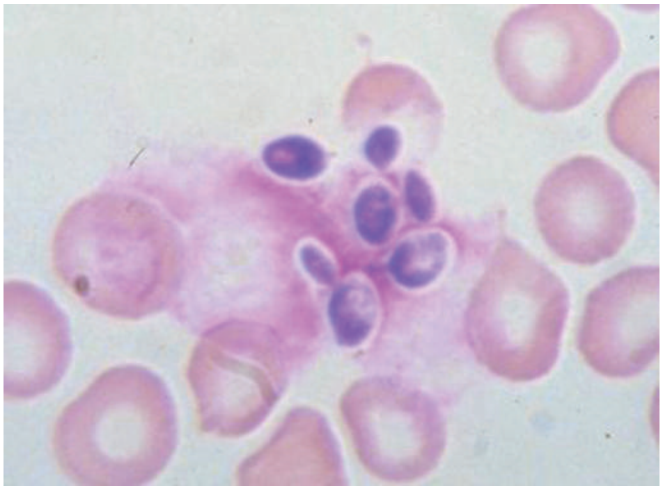

Figure 37-4 *Candida albicans.*

Histoplasmosis, tuberculosis, brucellosis, and *Salmonella* can be successfully cultured from the marrow aspirate.

Hematologic Aspects of Specific Infections

Bacterial Infections

Clostridial Sepsis. *Clostridium perfringens* septicemia is seen in a variety of clinical situations but must particularly be considered in patients with penetrating wounds, septic abortions, peritonitis after a perforated viscus, or cholecystitis or cholangitis; in immunosuppressed patients with gastrointestinal or hematologic malignancies; and in neonates with necrotizing enterocolitis.[135-151] In patients with clostridial sepsis, severe, rapidly progressive intravascular hemolysis and microspherocytosis may occur (Fig. 37-6).[135,137,143,150,151] Hemolysis of the entire red blood cell mass has been reported.[135,142,145,152-154]

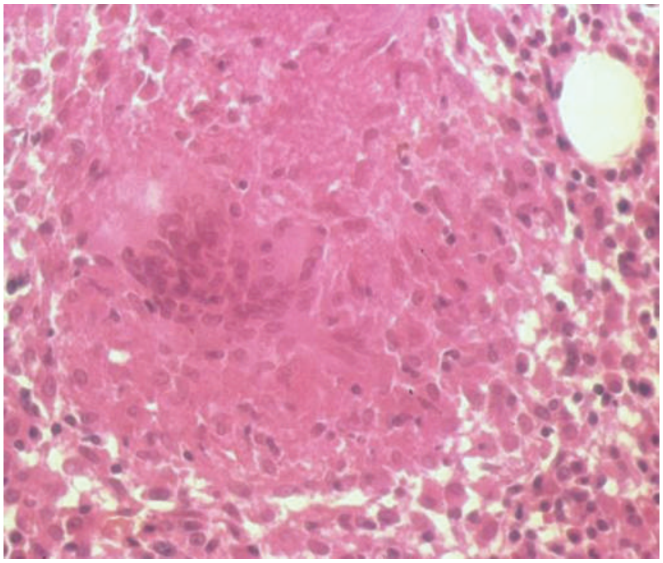

Figure 37-5 Bone marrow granuloma.

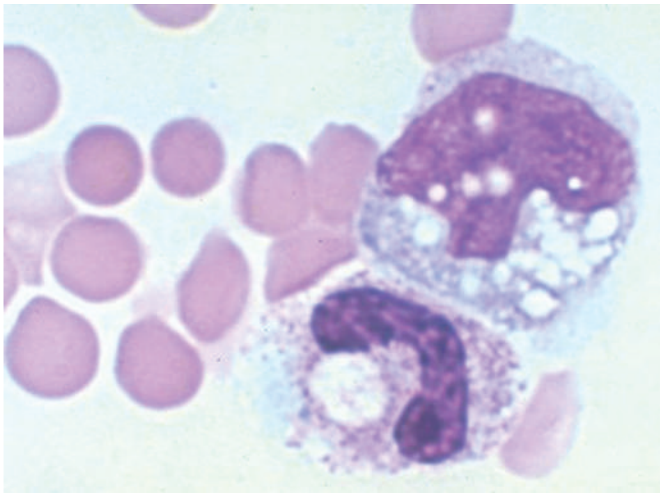

Figure 37-6 Peripheral blood in *Clostridium perfringens* septicemia demonstrating microspherocytes, neutrophils with toxic granulation, vacuolization, and Döhle bodies and erythrophagocytosis.

Complications include shock, acute renal failure, and death. Transfusion therapy may be ineffective. Antibiotics and hyperbaric oxygen occasionally have been used successfully to treat clostridial infections.[139,144,145]

The mechanism of red blood cell damage is uncertain and may vary between patients. The bacteria produce several hemolytic toxins including α-toxin, a 43-kD protein that contains an NH_2-terminal phospholipase domain and a COOH-terminal domain required for hemolysis, and θ-toxin, a 54-kD cholesterol-binding protein that aggregates and forms membrane pores leading to colloid osmotic hemolysis.[144,155-160] α-Toxin appears to induce hemolysis through the activation of sphingomyelin metabolism.[160-162] *C. perfringens* contains a neuraminidase that cleaves terminal sialic acids from red blood cell glycoproteins in some patients.[163] The underlying galactose residues form the Thomsen-Friedenreich cryptoantigen (or T antigen). Anti-T antibodies are present in almost all adult plasma; thus T-antigen activation can lead to significant hemolysis.[135,136,138,164] In infected infants and children who lack T antibodies, transfusion may lead to massive hemolysis and death. Rarely, T-antigen activation may precede the intravascular hemolysis, leading to early detection of clostridial sepsis and lifesaving therapeutic intervention.[135]

Pertussis. Pertussis may cause a marked increase in the white blood cell count, with elevations to 40,000 cells/mm^3 or higher, most of which are due to an increase in the lymphocyte count. Marked leukocytosis has been associated with a more severe course.[165] Hyperleukocytosis with white blood cell counts greater than 100,000 cells/mm^3 has been associated with the presence of immature leukocytes and obstruction in the pulmonary microcirculation. Successful leukoreduction has led to clinical improvement in a number of young infants with severe pertussis.[166,167]

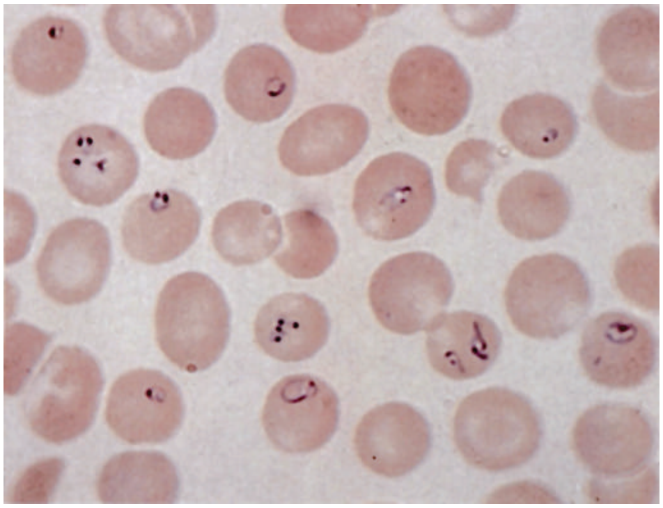

Figure 37-7 Babesiosis in the peripheral red blood cells.

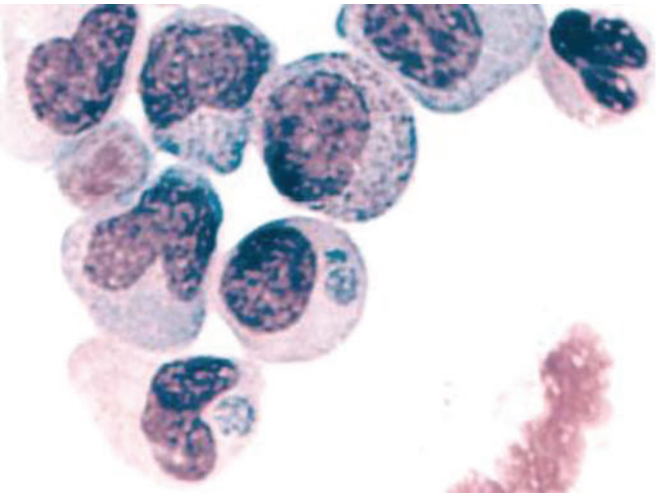

Figure 37-8 Intramonocytic morula in human monocytic ehrlichiosis.

Babesiosis. In healthy individuals, a mild hemolytic anemia is caused by *Babesia microti* and *Babesia divergens* microorganisms.[168,169] Occasional cases of combined *B. microti* babesiosis and Lyme disease are described, attributable to the shared geographic locale of the tick vectors for these diseases.[170] The disease can also be acquired by transfusion.[171] Babesiosis is not life threatening except in asplenic subjects in whom the parasitized red blood cells are not contained.[168,169,172] Then, the infection can be rapidly progressive and life threatening. In extreme cases, nearly all the red blood cells may be parasitized (Fig. 37-7).[173] Patients may have malaise, headache, fever, shaking chills, profuse sweating, jaundice, and dark urine. There may be intravascular hemolysis and, occasionally, pancytopenia and hemophagocytosis.[174,175] Diagnosis is made by finding the parasites in blood smears or by serologic tests or amplification of parasitic DNA using the polymerase chain reaction (PCR) assay. Current treatment of symptomatic cases is quinine plus clindamycin, but treatment failures have been reported in asplenic patients. Exchange transfusion may reduce the parasite load and be beneficial.[173]

Anaplasmosis (Ehrlichiosis). A variety of hematologic manifestations are found in infections with *Ehrlichia chaffeensis,* the causative bacteria of human monocytic ehrlichiosis (HME, Fig. 37-8), and *Anaplasma phagocytophilum,* the causative bacteria of human granulocytic anaplasmosis (HGA, Fig. 37-9)).[176] The disease is transmitted through tick bites, typically through the Lone Star tick or *Ixodes scapularis,* the shared tick vector of Lyme disease and babesiosis.[177] Transmission has also been reported through blood transfusions.[178] Symptoms are variable but are most severe in immunocompromised individuals. The obligate intracellular bacteria grow within membrane-bound vacuoles in leukocytes. General symptoms can include fever, headache, rash, and neurologic symptoms.[179,180] Thrombocytopenia and leukopenia are common, often with both lymphopenia and neutropenia.[181] Lymphopenia can be followed by an atypical lymphocytosis in the later stages of disease. Intraleukocytic morulae detected on review of the peripheral blood smear or buffy coat can be seen in up to 50% to 70% of infected individuals.[181] Diagnosis can also be made through serologic tests or PCR assay. Doxycycline is the treatment of choice; however, rifampin has been reported to be effective in young patients.[182]

Mycobacteria. Tuberculosis produces a variety of hematologic abnormalities. Leukemoid reactions mimicking myeloproliferative disorders are common.[183] Bone marrow involvement in miliary tuberculosis may result in a leukoerythroblastic pattern with teardrop-shaped red blood cells, nucleated red blood cells, and myeloblasts apparent on the peripheral blood smear.[184] Bone marrow biopsy may show evidence of granulomas. Monocytosis is common, and thrombocytopenia and pancytopenia have been reported.[185]

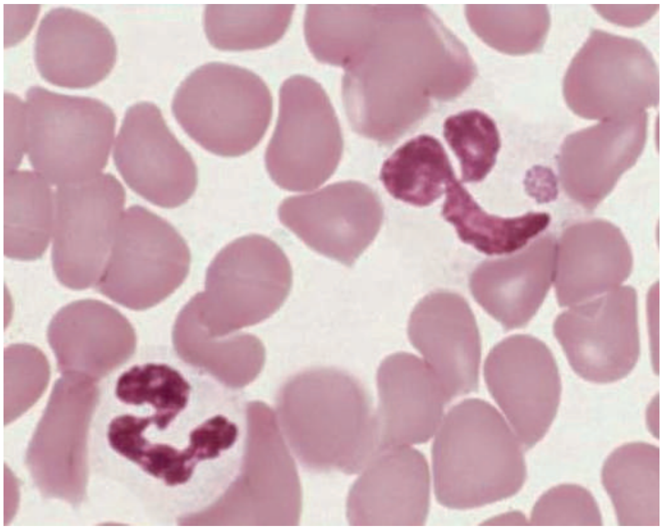

Figure 37-9 Morula-infected neutrophil in human granulocytic anaplasmosis.

Viral Infections

Parvovirus B19. Parvovirus B19, the cause of fifth disease (erythema infectiosum) in children, infects and kills early erythroid progenitors bearing the P blood group antigen.[186] This infection, which is discussed in Chapter 6, is not clinically significant unless the infection occurs in the setting of chronic hemolytic processes such as sickle cell disease or hereditary spherocytosis, or in patients with compromised immunity.[187] In these situations, life-threatening red blood cell aplasia or even pancytopenia may be caused by this virus. The infection typically presents as fever, vomiting, and abdominal pain, along with pallor, fatigue, and other symptoms of anemia.[188] Sometimes multiple family members are affected simultaneously. Parvovirus B19 infections are often associated with mild neutropenia or thrombocytopenia, and isolated cases of transient pancytopenia, hemophagocytosis, and transient myelodysplasia are reported.[189-192] The virus is a particular danger to pregnant women because it can cause fetal death due to anemia and nonimmune hydrops fetalis.[193]

Infectious Mononucleosis. Infectious mononucleosis is caused by the Epstein-Barr virus (EBV) and historically associated with the triad of (1) the classic clinical picture, (2) atypical lymphocytosis, and (3) positive results on a heterophil antibody test.[194] Serologic testing for EBV is now often used in addition or instead of the heterophil antibody test to confirm acute infection.

Epidemiology. EBV infection is acquired at an early age in lower socioeconomic groups. In economically privileged children, infection is often delayed until adolescence and young adulthood. About 40% of American children are seropositive for EBV by 5 years of age.[195-197] In a report from the U.S. Military Academy at West Point, 63.5% of entering cadets had EBV antibody, indicative of previous infection.[198] During the college years, the infection rate among susceptible individuals is 12% to 15% per year.

The pattern of infection in different groups of susceptible persons is probably best explained by transmission of this virus in throat secretions. EBV is present in the saliva of most patients with infectious mononucleosis, in up to 20% of healthy persons with EBV antibodies, and in 50% or more of seropositive patients receiving immunosuppressive drugs. The ease with which EBV is recovered from the oral secretions of persons with primary or reactivated EBV infection suggests that a cell type that freely permits EBV replication exists in the oropharynx. The oropharyngeal epithelial cell may be the target cell type that is productively infected in infectious mononucleosis.[199] Transmission requires intimate contact. Intrafamilial spread, however, does occur frequently, and, in this setting, an incubation period of 4 to 6 weeks has been demonstrated.[200] Another possible route of transmission of EBV is transfusion through white blood cells.

Clinical Manifestations. The classic clinical manifestations of infectious mononucleosis are generally seen only in adolescents and young adults. Younger children rarely exhibit the typical findings of this disease, and most generally have only a mild viral respiratory illness.[195-197]

The onset of typical illness is usually a subtle prodrome consisting of fatigue, malaise, sweating, feverishness, and anorexia. Headache, nausea, and vomiting are seen frequently. The most common symptom during this period is a sore throat, which begins slowly and increases in intensity over a 1-week period. The usual findings of infectious mononucleosis then follow (Table 37-1). The

TABLE 37-1 Approximate Rate of Occurrence of Various Signs and Symptoms of Infectious Mononucleosis in Young Adults

Symptom or Sign	%
Adenopathy	100
Malaise and fatigue	90-100
Fever	80-95
Sweats	80-95
Sore throat, dysphagia	80-95
Pharyngitis	65-85
Anorexia	50-80
Nausea	50-70
Splenomegaly	50-60
Headache	40-70
Chills	40-60
Bradycardia	35-50
Cough	30-50
Periorbital edema	25-40
Palatal enanthema	25-35
Liver or splenic tenderness	15-30
Myalgia	12-30
Hepatomegaly	15-25
Rhinitis	10-25
Ocular muscle pain	10-20
Chest pain	5-20
Jaundice	5-10
Arthralgia	5-10
Diarrhea or soft stools	5-10
Photophobia	5-10
Rash	3-6
Conjunctivitis	5
Abdominal pain	5
Gingivitis	3
Pneumonitis	3
Epistaxis	3

From Finch SC: Clinical symptoms and signs of infectious mononucleosis. In Carter RL, Penman HG, editors: Infectious mononucleosis, *Oxford, 1969, Blackwell Scientific, p. 35.*

course of fever often follows a specific pattern, with no temperature elevation in the morning but daily afternoon or evening peaks of 38.3° C to 39.4° C (101° F to 103° F). Occasionally, higher temperatures are observed. The fever usually lasts about 2 weeks.

Lymphadenopathy is seen in all patients. Symmetric, moderately enlarged, discrete, slightly tender nodes, especially in the posterior cervical region, are most characteristic. Adenopathy is common in the axillary, epitrochlear, and inguinal areas. The nodes are not matted and do not show signs of heat, redness, or fluctuation. Rarely, enlargement of the mediastinal glands constitutes the only evidence of adenopathy and may be confused with a lymphomatous process.

Splenomegaly occurs in more than 50% of patients with infectious mononucleosis. The spleen is usually just barely palpable but on rare occasion may be quite large. It is smooth, soft to firm, and sometimes slightly tender. In some patients, splenomegaly persists for months, but the most common situation is resolution by 2 to 3 weeks after onset of the illness.

Hepatomegaly occurs in about 20% of patients, and clinical jaundice is seen in 10%. The jaundice is invariably mild. Despite the low rate of occurrence of apparent hepatic dysfunction, virtually all patients with infectious mononucleosis demonstrate abnormal liver enzyme levels. Except in rare patients with acute liver failure, the hepatitis associated with this illness is self-limited. There is no evidence that chronic liver disease or cirrhosis results from infectious mononucleosis.

Rashes occur occasionally, and their manifestation follows no particular pattern. The rash may be a diffuse, faint, erythematous or maculopapular eruption, or it may be urticarial, scarlatiniform, petechial, or herpetiform. In general, the rashes of infectious mononucleosis have no unique features and are of little or no help for diagnosis. Circulating immune complexes and complement sequence activation occur only when rashes are present.[201] If ampicillin is administered to patients with infectious mononucleosis, a rash will develop in 69% to 100%.[202] Other penicillins may cause this response but less often. This rash is not an allergic reaction, and these antibiotics may be administered subsequently without ill effect.

Only a few patients with infectious mononucleosis have no pharyngitis. Almost all demonstrate hyperplasia of the pharyngeal lymphoid follicles. The presence of an exudate is common, and membrane formation occurs frequently. The inflammation may be severe enough to cause respiratory obstruction. Peritonsillar abscesses may complicate the course of the illness.[203] A palatal enanthema is seen in about a third of patients and consists of crops of sharply circumscribed petechiae, symmetrically distributed at the junction of the soft palate. Unfortunately, such petechiae are not specific for infectious mononucleosis and have been described in rubella and other viral disorders. In fact, the entire pharyngeal manifestation of infectious mononucleosis is clinically indistinguishable from that of streptococcal disease.

Periorbital edema is not rare and occasionally leads to the erroneous suspicion that renal disease or hypoproteinemia is present. The edema is self-limited and lasts only a few days.

TABLE 37-2 Reported Complications of Infectious Mononucleosis

Type of Complication	Diagnosis or Description of Abnormalities
Neurologic	Bell palsy, cerebellar syndrome, encephalitis, encephalomyelitis, encephalomyelopathy, Guillain-Barré syndrome, meningitis, meningoencephalitis, myelitis, optic neuritis, peripheral neuritis, psychosis, radiculoneuritis, ataxia, positive Babinski sign, coma, convulsions, diplopia, extraocular palsy, facial diplegia, hemiplegia, hyperesthesia, meningismus, mental confusion, nystagmus, papilledema, psychotic reaction, ptosis, respiratory paralysis, positive Romberg sign, seizure, status epilepticus, scotomas
Cardiac	Electrocardiographic changes, myocarditis, pericarditis
Ocular	Conjunctivitis, diplopia, cyclic edema, hemianopia, lacrimal pericyclitis, nystagmus, optic neuritis, ptosis, retinal edema, retinal hemorrhage, retroorbital pain, scotomas, uveitis
Respiratory	Laryngeal obstruction, peritonsillar abscess, pharyngeal edema, pleural effusion, pleuritis, pneumonitis
Hematologic	Acquired hemolytic anemia, agranulocytosis, eosinophilia, fibrinolysis, pancytopenia, splenic rupture, thrombocytopenia
Digestive	Esophageal varices, gingivitis, hepatic dysfunction, hepatic necrosis, jaundice, melena
Renal	Hematuria, hemoglobinuria, nephritis, nephrotic syndrome, porphyrinuria, proteinuria
Other	Bullous myringitis, endocervicitis, orchitis, otitis media, pancreatitis, porphyria, rashes

From Finch SC: Clinical symptoms and signs of infectious mononucleosis. In Carter RL, Penman HG, editors: Infectious mononucleosis, *Oxford, 1969, Blackwell Scientific, p. 35.*

Complications. Much attention is paid to the complications associated with infectious mononucleosis, although their overall occurrence is low (Table 37-2). Hematologic complications of infectious mononucleosis include disturbances resulting in anemia, granulocytopenia, thrombocytopenia, and occasional coagulation defects. The rate of occurrence of these hematologic abnormalities is shown in Table 37-3.

AIHA can occur with EBV infection and developed in approximately 3% of West Point cadets with infectious mononucleosis.[198] When hemolysis does occur, it usually begins 1 to 2 weeks into the course of the illness. The majority of occurrences terminate in less than 1 month, and chronic hemolysis is rare. Although usually mild, hemolysis occasionally occurs rapidly and can then result in severe anemia. Jenkins reported the first instance of

TABLE 37-3 Infectious Mononucleosis—Laboratory Features

Findings	% Positive
Lymphocytosis, relative and absolute	100
Atypical lymphocytes, definite*	100
Epstein-Barr virus antibody in serum	100
Heterophil antibody	80-100
Liver enzyme abnormalities	80-100
Leukocytosis	60-80
Neutropenia	60-80
Hyperbilirubinemia	30-50
Bone marrow granulomas	50
Slight thrombocytopenia	25-50
Increased cold agglutinins	10-50
Occult hemolysis	20-40
Hyperuricemia	15-20
Leukopenia	10-20
Severe thrombocytopenia with bleeding	Rare
Positive direct Coombs test results	Rare
Significant anemia (usually caused by hemolysis)	Rare

*Twenty percent or more of white blood cells in peripheral blood.

hemolytic anemia in infectious mononucleosis that was mediated by the temporary induction of a high-thermal amplitude cold agglutinin of anti-i specificity.[204,205] Since then, several series have verified the high incidence of anti-i antibody as the cause of immune-mediated hemolysis in infectious mononucleosis. It should be noted that although hemolysis is not common in infectious mononucleosis, the presence of anti-i is seen in as many as 50% of all patients. Not all instances of immune hemolysis in infectious mononucleosis are caused by anti-i antibodies. Anti-N antibodies have also been reported, and in some patients the nature of the antibody has not been identified.[206]

Aplastic anemia has been reported to follow the onset of infectious mononucleosis.[207] Aplastic anemia caused by EBV infection has also been reported after bone marrow transplantation.[208] Localization of EBV in the bone marrow of some patients with aplastic anemia supports a causative role of the virus in these aplastic anemia patients.[209]

Granulocytopenia is common during the acute phase of infectious mononucleosis. It is rarely severe but, on occasion, is associated with a secondary bacterial infection.[210] Bone marrow myeloid hyperplasia with myeloid arrest is the most typical finding.[211] Spontaneous resolution is the rule.

Immune thrombocytopenia occurs rarely in infectious mononucleosis.[212] Most occurrences of thrombocytopenia are mild.[213] The signs and symptoms of severe occurrences are similar to those of primary ITP. Severe hemorrhagic complications are rare.[214] Treatment of the thrombocytopenia of infectious mononucleosis in children is guided by bleeding symptoms. Treatment approaches are similar to primary ITP with first-line therapies with corticosteroids and/or intravenous immune globulin.[215]

The incidence of the neurologic complications reported varies from 0.37% to 7.3%, depending on the series.[216] A rare occurrence in adolescence is the "Alice in Wonderland" phenomenon in which objects are visualized in a very distorted fashion, with exaggerations in size, being either too large or too small.[217,218] A transverse myelopathy characterized by a sudden onset of profound weakness of the lower extremities and urinary retention may complicate the clinical course of infectious mononucleosis.[219]

Cardiac complications are rare and occur in 1% to 6% of reported series. They usually consist of only nonspecific T-wave changes or minor conduction abnormalities. Myocarditis and pericarditis are rare. Liver function abnormalities are rarely severe, although enzyme changes are common. Primary EBV infection has been associated with a Reye syndrome–like illness.[220] Severe liver dysfunction causing death is a common complication of EBV infection in the X-linked lymphoproliferative syndrome. Hepatic dysfunction is uniformly present with this disorder at the time of death and is the cause of death in about a third of such patients.[221] An unusual manifestation of infectious mononucleosis is intense jaundice. It generally results from a combination of hemolysis and mild hepatitis.[222] Spontaneous rupture of the spleen may occur.[223]

Respiratory difficulties usually consist of upper airway obstruction. Transient interstitial infiltrations, some with effusions, have been recorded. Infectious mononucleosis should be considered in the differential diagnosis of any child with pleural effusions.[224] Renal complications of infectious mononucleosis generally consist of hematuria associated with a mild nephritis. Not all occurrences have been clearly separated from poststreptococcal glomerulonephritis. Severe rhabdomyolysis can be associated on rare occasion with EBV infection.[225] Eye findings in infectious mononucleosis are unusual but may be significant when they do occur. Severe retinochoroiditis is such an ocular complication.[226] In some patients with infectious mononucleosis, lethargy, particularly daytime lethargy, is seen for prolonged periods, often longer than a year.[227-229]

X-linked lymphoproliferative syndrome is associated with fatal or severe infectious mononucleosis, acquired hypogammaglobulinemia, and malignant lymphoma. Details about X-linked lymphoproliferative syndrome can be found in Chapter 24.

A variety of malignancies have been linked to EBV, including clonal T-cell proliferations.[230] In patients who undergo transplantation, a spectrum of lymphoproliferative diseases may occur as a result of activation of EBV. These diseases may vary from an infectious mononucleosis–like polyclonal B-cell proliferation to a monoclonal B-cell lymphoma. EBV infection can also result in a hemophagocytic syndrome.[231] The total spectrum of the rare complications and unusual syndromes associated

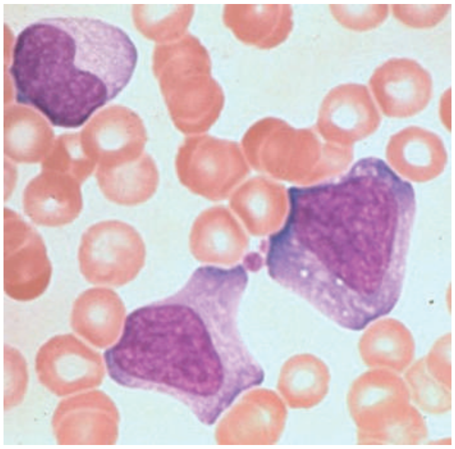

Figure 37-10 Atypical lymphocytes (Downey cells) in infectious mononucleosis.

with EBV infection have been reviewed by Timár and colleagues.[232]

Laboratory Diagnosis. Atypical lymphocytosis is the hallmark of infectious mononucleosis (Fig. 37-10). Several attempts have been made to classify these abnormal cells on morphologic grounds, the best known method being that of Downey and McKinlay.[233] However, it is clear that atypical lymphocytes cannot be easily classified into separate categories and that a spectrum of cell types exists. In general, the atypical lymphocytes of infectious mononucleosis are large, but they vary in size considerably. Their outlines are irregular, and many cells show a characteristic tendency to flow around adjacent erythrocytes. Nuclei are large and usually eccentrically located and pleomorphic, with abundant coarse chromatin and occasional nucleoli. The cytoplasm is generally abundant and typically basophilic. Cytoplasmic vacuoles may be seen. These types of cells are not morphologically specific for infectious mononucleosis and can be seen in other viral infections and after administration of a number of medications.[234]

The vast majority of atypical lymphocytes from patients with infectious mononucleosis are thymus derived.[235,236] These atypical cells possess human T-lymphocyte–specific antigens, as well as sheep erythrocyte receptors.[235] T lymphocytes appear to lack receptors for EBV, and it would appear that only B lymphocytes are infected by this virus.[236,237] A possible unifying interpretation of the role of T lymphocytes is that these cells represent an immune reaction that protects against this potentially oncogenic virus. Increased numbers of B cells are found during the first week of illness and decline to normal levels in 3 weeks. T lymphocytes reach their peak later, usually 10 to 14 days after the onset of symptoms, and remain elevated for 5 weeks.[238] There may be an early reversal of the ratio of T to B lymphocytes, with a subsequent increase in the percentage of T cells during the second through fifth weeks of illness. It is possible that both T and B cells may be "transformed" into atypical lymphocytes—B cells by infection with EBV and T cells by an immunologic response to viral antigen itself—or the B cells may respond to altered antigens on their surface. EBV-infected B lymphocytes account for only a minority of the atypical lymphocytes found in peripheral blood. In the very early stages of symptomatic illness, however, nearly 20% of all B cells in the circulation may be infected with the virus. The majority of atypical lymphocytes are T lymphocytes. Natural killer (NK) cell activity has been shown to be present during the acute phase of infectious mononucleosis. Interferons, which are inducers of NK cell activity, may have an inhibitory effect on the outgrowth of EBV-infected B lymphocytes in vitro. Significant anergy and diminished lymphocyte responsiveness in vitro to mitogens and antigens exist during the first week of illness. These lymphocyte changes are reflected in a great increase in uric acid turnover, with 55% of infected patients having serum uric acid levels of 8 mg/dL or higher.[239]

Patients with infectious mononucleosis have lymph node pathologic findings that are easily confused with lymphoma. The nodal architecture is distorted by large, dark lymphoid cells, and the capsule may be infiltrated. Reed-Sternberg cells have been reported on several occasions.[240,241] Pathologic changes are not confined to lymphoid tissue, however. Perivascular cuffing of the brain vasculature, inflammation of the liver, and inflammatory infiltration of the kidney and bone marrow have been repeatedly observed.

The heterophil antibody is so named because the antigen to which the antibody reacts is found in more than one species. The antibody, like anti-i, is an IgM macroglobulin. It agglutinates sheep red blood cells and can be removed completely from serum by preincubation with beef red blood cells but not with guinea pig kidney. Heterophil antibody titers usually increase after the third day of illness, peak at 2 weeks, and may remain positive for several months before ultimately becoming negative (unlike the antibody specific for EBV).[242] This traditional Paul-Bunnell serologic agglutination method has been largely replaced for screening purposes by the spot test, in which finely ground guinea pig kidney or beef red blood cell stroma is added to serum on a slide, followed by a drop of horse cells.[243] Results of the test are considered positive if agglutination occurs in the presence of guinea pig kidney (which absorbs out Forssman antibody but not heterophil antibody) but are negative with beef red blood cell stroma. The spot test requires only 2 minutes and is 96% to 99% accurate. Results of both the Paul-Bunnell test and the spot test are usually negative in preschool-aged children, in whom heterophil antibody production is limited.[244] This age group does produce diagnostic levels of EBV-specific antibody.

EBV is a herpeslike DNA virus. It is a relatively complex virus, and a variety of virus-associated antigens have been described.[245] Antibodies to viral capsid antigen (VCA) and early antigen (EA) are detected early after the onset of EBV-associated infectious mononucleosis.[246] Levels of antibodies to VCA reach their peak at about 3 weeks after the onset of clinical illness. Their levels decline somewhat thereafter, but they remain for life.[247] Antibodies to EA usually last 2 to 4 months. EA has two components that are differentiated by their immunofluorescent staining: "D" for diffuse and "R" for restricted

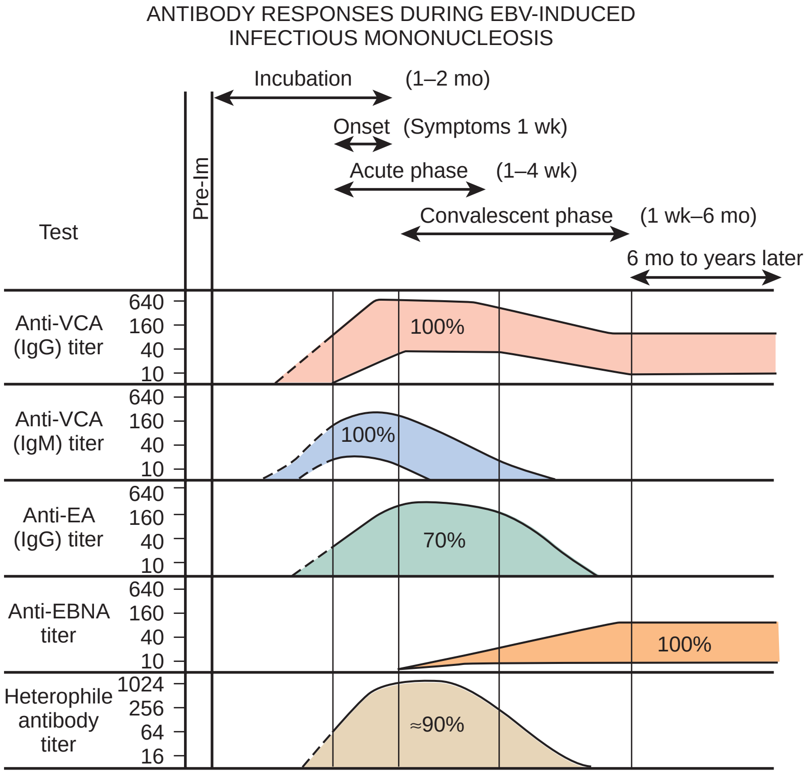

Figure 37-11 Characteristic Epstein-Barr virus–specific antibody response observed in young adults with acute infectious mononucleosis. *(Modified from Henle W, Henle GE, Horwitz CA: Epstein-Barr virus specific diagnostic tests in infectious mononucleosis.* Hum Pathol *5:551–565, 1974; and Sullivan JL: Epstein-Barr virus and the X-linked lymphoproliferative syndrome.* Adv Pediatr *30:365–399, 1984.)*

staining. A technique for determining EBV-specific IgM has been described.[248] EBV-specific IgM almost always occurs in the acute phase of infectious mononucleosis. It rarely persists more than 2 to 3 months. During this period, virus shedding from the oropharynx is easily demonstrable.[249]

Figure 37-11 shows the characteristic antibody patterns observed in young adults with EBV-induced infectious mononucleosis.[250] Before infection, no antibodies are present. During the acute phase of illness, high titers of IgM and IgG antibodies to VCA are seen. IgM antibodies are transient and disappear after 1 to 2 months. Antibodies appearing against EA disappear after a few weeks to months. Antibodies against Epstein-Barr nuclear antigen are the last to appear and are seen 1 to 2 months after the illness. In young infants, VCA IgM is found in only 60% of patients and EA antibody is identified in only 50%. The only persistent antibody response should be VCA IgG and antibodies to Epstein-Barr nuclear antigen.

Treatment. There is no evidence that bed rest or rest in general shortens the clinical course of infectious mononucleosis. Patients will determine their own level of activity. The most significant risk during acute illness is splenic rupture, but its incidence is extremely low.

Corticosteroid therapy is often used for infectious mononucleosis. Although these agents may produce improvement of symptoms, enhancement of general well-being, and reduction of fever, their use for these purposes should be restricted.[251] Less controversial is the use of corticosteroid therapy for patients with airway obstruction secondary to tonsillar hypertrophy, severe hemolytic anemia, or thrombocytopenia and bleeding.[252]

Human Immunodeficiency Virus

Epidemiology. In the early 1980s, a newly described constellation of symptoms that resulted from immune compromise and led to death from opportunistic infections and unusual malignancies was recognized.[253-255] These early descriptions of what is now known as acquired immunodeficiency syndrome (AIDS) heralded the beginning of what has become one of the major medical, public health, and social issues of our time. Identification of the human immunodeficiency virus (HIV), a retrovirus, as the etiologic agent that transmits the disease[256,257] has led to major advances in understanding the pathogenesis of AIDS. At least two types of HIV, HIV-1[256,257] and HIV-2,[258] exist; both are considered lentiviruses based on similarities in genetic composition, mechanisms of replication, and interaction with their hosts.[259] A characteristic of lentivirus infections is that they cause slowly progressive disease with an incubation period of months to years before clinical symptoms appear. The worldwide spread of AIDS has led to a plethora of information about the biology of the virus and its epidemiology and incidence projections in different countries, as well as a concerted search for effective means of prevention and treatment.

The first occurrences of AIDS in the pediatric population in the United States took place in children born to mothers infected with HIV or in children, especially neonates, who had received blood products contaminated with HIV.[260] The AIDS pandemic in Africa and, in particular, southern Africa has reached catastrophic proportions. The incidence of the disease in eastern Europe, India, and Thailand and throughout most of the Third World poses many political, social, and medical challenges, a discussion of which that is beyond the scope of this chapter.

Transmitted primarily through contact with infected lymphocytes and monocytes, HIV may be found in blood, semen, and vaginal secretions.[261,262] Isolation of the virus from 13- and 15-week abortuses, placenta, and cord blood provides strong support for intrauterine infection.[260,263-265] Maternal-fetal transfusion at delivery is another possible route of infection.[266] Postpartum transmission via breastmilk, although possible, is rare. Accordingly, in countries in which safe feeding substitutes are not readily available, breastfeeding should not be curtailed; however, in developed countries where alternatives exist, breastfeeding by HIV-infected women is discouraged.

The rate of transmission from an untreated mother to child is estimated to be about 25% based on several studies from the United States and Europe.[267-270] Transmission from mother to child can be significantly reduced by the use of antiretroviral therapy during pregnancy, labor, and the neonatal period.

Major advances in screening and testing of the blood supply in the United States after 1985 led to a reduction in the risk of receiving a contaminated single transfusion in 1988 to 1 in 250,000.[271] The first screening test is a questionnaire to exclude people who engage in high-risk activities. The next step is an enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to HIV antigens. In rare individuals who are infected but seronegative, HIV can escape detection, but sensitive screening tests detect the viral genome by PCR assay.[272] Tragically, individuals with bleeding disorders such as hemophilia A and B who received clotting factors from a large donor pool for a single infusion had an increased likelihood of acquiring AIDS before modern blood screening practice and factor preparation.

Clinical Manifestations. Infection with HIV results in a spectrum of disease from an asymptomatic state to severe immunodeficiency involving multiple organ systems. The heterogeneity of symptoms is explained by the underlying pathophysiologic course in that (1) the incubation period may be weeks, months, or years; (2) the direct cytopathic effect on target cells, predominantly CD4+ lymphocytes, monocytes, and accessory cells, results in dysregulation of the immune system; and (3) the viral elimination phase that is accompanied by a host response to infection varies with the immune competence of the host. Because many children are infected congenitally, they show signs early in life and often have constitutional symptoms such as unexplained diarrhea, fever, night sweats, generalized lymphadenopathy, and hepatosplenomegaly. Failure to thrive and developmental delay are often prominent early manifestations of HIV infection in children. Susceptibility to recurrent infections by common bacterial and viral pathogens, as well as to opportunistic infections, increases the index of suspicion that the underlying disorder is AIDS. These and other manifestations of HIV infection are summarized in Box 37-1.

The hematopoietic dysfunction that is invariably associated with HIV infection results from global dysregulation of the physiologic cascades of antibody formation, coagulation and complement, and direct infection of key regulatory cells. Acute and chronic infections and therapies for the underlying disorder also affect hematopoiesis. Although for the purposes of discussion it is simpler to view these changes according to cell lineage, it should be stressed that these events rarely occur in isolation. Examination of the peripheral blood smear from the majority of AIDS patients reveals anemia and granulocytopenia and, in a third of patients, associated thrombocytopenia.[273]

Bone Marrow Findings. In several studies, the bone marrow morphologic changes in patients with AIDS were reported.[274] The most common findings were hypercellularity, lymphoid aggregates, plasmacytosis, and dysplasia. When thrombocytopenia is present, the marrow often contains adequate megakaryocytes, thus suggesting an immune mechanism of platelet destruction. The anemia and granulocytopenia appear to be related to the dysplastic bone marrow and result from ineffective hematopoiesis. Increases in reticulum and fibrosis have been reported in association with *Mycobacterium avium* infection.

Thrombocytopenia. The underlying mechanism of thrombocytopenia, which can be the initial finding of HIV infection in children, appears to be immune destruction of platelets. Several studies have identified a high incidence of cytophilic antibodies[275,276] and have highlighted concomitant complement deposition on the platelet.[277] In addition, a 25-kD platelet-associated antigen has been detected in thrombocytopenic adults and children with AIDS[278]; however, the presence of the antibody in serum does not invariably lead to thrombocytopenia. Spontaneous recovery from thrombocytopenia does occur; for instance, in one study, 8 of 25 patients with thrombocytopenia recovered.[279]

Adequate treatment of HIV infection with highly active antiretroviral therapy is the first-line approach for HIV-associated ITP and typically improves the thrombocytopenia.[280] ITP-specific treatment is considered when the platelet count decreases to less than 30,000/mL or a clinical bleeding episode is encountered. After HIV-directed treatment, first-line treatment of HIV-associated ITP includes the standard options of prednisone, intravenous immune globulin, and anti-D globulin.[281] Second-line ITP-directed treatment options can be considered with failure of the first-line agents and include splenectomy and thrombopoietin-receptor agonists.[277,282]

Anemia and Granulocytopenia. Anemia and granulocytopenia are found in most patients with AIDS and reflect, in large part, ineffective hematopoiesis. The anemia is typically normochromic and normocytic, with a low reticulocyte count and mild to severe anisocytosis and poikilocytosis. Up to 40% of patients with AIDS may

Box 37-1 **Clinical Manifestations of HIV Infection**

PRIMARY MANIFESTATIONS

HEMATOLOGIC AND IMMUNE ABNORMALITIES

Hypergammaglobulinemia
Lymphopenia (CD4+ cells decrease)
Decreased CD4/CD8 ratio
Thrombocytopenia
Anemia
Neutropenia
Drug allergies (e.g., trimethoprim in 40% to 60% of patients)

NONSPECIFIC FINDINGS (includes children with two or more unexplained findings for more than 2 months)

Failure to thrive
Hepatosplenomegaly
Generalized lymphadenopathy
Parotitis
Diarrhea (three or more loose stools per day)

NEUROLOGIC DISEASE

Loss of intellectual ability or developmental milestones
Impaired brain growth (acquired microcephaly, brain atrophy, or both)
Progressive systemic motor defects
Paresis
Abnormal tone
Pathologic reflexes
Ataxia or gait disturbance

CARDIOVASCULAR DISEASE

Cardiomyopathy
Arrhythmias

OTHER DISEASES

Hepatitis
Nephropathy (sclerosing glomerulonephritis)
Dermatologic diseases (most commonly seborrheic dermatitis)

SECONDARY MANIFESTATIONS

SECONDARY INFECTIONS

Pneumocystis carinii (now *Pneumocystis jirovecii*) pneumonia
Chronic cryptosporidiosis
Disseminated toxoplasmosis (onset after 1 month of age)
Extraintestinal strongyloidiasis
Chronic isosporiasis
Candidiasis (esophageal, bronchial, and pulmonary)
Extrapulmonary cryptococcosis
Disseminated histoplasmosis
Mycobacterial infection
Cytomegalovirus infection (onset after 1 month of age)
Coccidioidomycosis
Nocardiosis
Progressive multifocal leukoencephalopathy
Lymphocytic interstitial pneumonitis

SECONDARY CANCERS

Kaposi sarcoma
B-cell non-Hodgkin lymphoma
Primary lymphoma of the brain

From Centers for Disease Control and Prevention: Classification systems for human immunodeficiency virus (HIV) in children under 13 years of age. MMWR Morb Mortal Wkly Rep *36:225–230, 235–236, 1987.*

have positive findings on a direct Coombs test as a result of absorbed immunoglobulin. Although antibodies have been detected on the surface of granulocytes,[283,284] the mechanism for granulocytopenia appears to be suppression of bone marrow progenitors. Bone marrow from patients with HIV infection exhibits colony formation (GM-CSF and burst-forming units–erythrocyte) similar to that in HIV-seronegative control subjects when cultivated in serum from an HIV-seronegative donor. However, there is selective suppression of colony formation in bone marrow derived from a patient with HIV infection versus bone marrow from seronegative donors when cultivated in seropositive sera. This suggests that progenitor cells and progeny are able to be infected with HIV and express HIV antigens on their surface that are recognized by HIV antibodies, thus accounting for the suppression. Further support for this hypothesis comes from the demonstration of provirus in megakaryocytes from the bone marrow of patients with AIDS.[285,286]

Alloantibodies are detected in 30% to 60% of patients with AIDS in different series, with anti-i and anti-I being the most common.[283] Anti-i has also been associated with EBV and cytomegalovirus infections, both of which often coexist with HIV infection and may account for the presence of specific red blood cell antibodies. Antibodies to Le, PL, E, K, Lu, and Sd all have been found, although their relationship to the pathophysiologic course of the anemia in HIV, which in large part does not appear to be hemolytic, remains an open question.

Antiretroviral agents often causes anemia and neutropenia. Macrocytosis with 25- to 40-unit increases in mean corpuscular volume is observed in 74% of patients receiving zidovudine.[287] The macrocytosis is not usually accompanied by anemia, but when it does occur the anemia is usually mild and dose related. In a subgroup of patients, anemia caused by bone marrow suppression is observed. Neutropenia (absolute neutrophil count of 750 to 1000 cells/μL) is seen early in antiretroviral treatment and can be dose limiting.

Laboratory Diagnosis. Detection of antibodies to HIV antigens is a standard approach to the diagnosis of HIV infection. The first screen is an ELISA; positive results may be confirmed by a specific immunoblot. However, the presence of anti-HIV immunoglobulin of the IgG subclass in an infant does not necessarily indicate that the infant is infected with HIV because maternal IgG crosses the placenta and has a half-life of 28 days. In fact, persistence of maternal IgG has been detected beyond 15 months of age.[268] In contrast, several seronegative HIV-infected children have been reported.[288-290] Although IgM or IgA anti-HIV antibodies are better indicators of active infection in children because these subclasses of antibody do not cross the placenta, the sensitivity and specificity of the ELISAs for these antibody isotypes are much lower than those for IgG anti-HIV. Definitive proof of HIV infection rests with viral culture and demonstration of the provirus via the very sensitive PCR assay.[290]

Treatment. Current U.S. treatment guidelines for pediatric HIV infection advocate aggressive therapy with combination antiretroviral regimens. The goal is durable suppression of viral replication with preservation of immune function.[291] The expense and difficulty of adhering to this regimen limit access to the vast majority of infected children in the world. Prevention and education remain the most effective short-term modes of limiting the spread of AIDS.[292]

Infections in the Developing World

A detailed review of the prevalent infections in developing countries, including malaria, visceral leishmaniasis (kala-azar), schistosomiasis, trypanosomiasis, hookworm, bartonellosis, and dengue can be found in Chapter 38.

CARDIAC DISEASE

The focus of this section is on the three major hematologic complications of cardiac disease: hemolytic anemia, coagulopathy, and increased platelet turnover.

Hemolysis

A number of instances of continuing hemolysis and progressive anemia have been reported after the insertion of prosthetic valves, particularly in the aortic area.[293,294] These conditions may also occur postoperatively when intracardiac patches have been placed (the "Waring blender" syndrome). They are also being recognized as an increasing problem after endoluminal closure of the ductus arteriosus[295,296] and occasionally after repair of a ventriculoseptal defect.[297] The mechanism of such erythrocyte destruction has been related to failure of endothelialization of patches, thrombosis or perforation of prosthetic valves, and improper placement of prosthetic valves, especially when insufficiency develops at the suture lines. Erythrocyte destruction and ensuing hemolysis, however, have been described in the absence of these complications and been attributed to red blood cell mechanical trauma associated with apparently normal function of the prosthetic valve.[298,299]

Red blood cell survival studies have clearly shown that prosthetic valve and patch hemolysis is due to an extracorpuscular defect.[300] In general, hemolytic anemia results from fragmentation of the red blood cells as they are mechanically "battered" against a distorted vascular surface (Fig. 37-12).[301] In some instances, it has been postulated that the red blood cell fragmentation may be caused by contact with fibrin deposited in small blood vessels as a result of localized intravascular coagulation.[302] Most times, however, hemolysis is the result of direct mechanical trauma. Nevaril and associates[301] demonstrated that a shearing stress of 300 dynes/cm^2 causes hemolysis in vitro whereas less stress may result in deformed red blood cells morphologically similar in appearance to cells in cardiac hemolytic anemia and microangiopathic hemolytic anemia.[301]

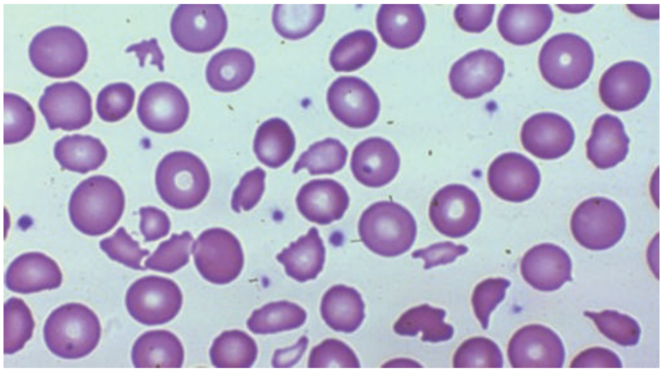

Figure 37-12 Red blood cell fragmentation due to a prosthetic heart valve.

The consequence of the process is hemolytic anemia of the intravascular type associated with hemoglobinemia and hemoglobinuria. Iron deficiency develops quickly in patients with this disorder as a result of increased loss of body iron in the form of hemosiderin, which is shed within the renal tubular cells into urine.[303,304] The onset of iron deficiency may be of clinical importance. The microcytic hypochromic cell in iron deficiency is more rigid, thereby leading to an accelerated rate of hemolysis from mechanical shearing in the microvasculature.[305] Plasma haptoglobin levels fall. Large quantities of red blood cell lactate dehydrogenase are released into serum, and a close correlation exists between the logarithm of serum lactate dehydrogenase and the half-life of chromium-labeled erythrocytes.[306] The rate of hemolysis may or may not result in anemia. If surgical correction of the defect causing hemolysis is not possible, the patient should be treated with both iron and folate, as well as red blood cell transfusion. The latter is intended to correct anemia, reduce stroke volume, and presumably reduce shear force. Sears and Crosby[307] observed that the severity of hemolysis is directly related to physical activity. This finding has been used as a test to determine whether the hemolysis is of cardiac origin because, if it is, rest also diminishes the rate of hemolysis. If the source of the problem cannot be corrected surgically, propranolol, which reduces the shearing stress between red blood cells and the vascular wall by slowing the velocity of the circulation, may be given.[308]

Mechanical injury to red blood cells may result in loss of pieces of cell membrane, with or without loss of hemoglobin. Such loss will lead to the formation of spherocytes. Consequently, in many patients with heart valve hemolysis, results of the red blood cell osmotic fragility test may be abnormal.[308] Mechanical destruction of red blood cells on abnormal surfaces of the vasculature can result in hyperkalemia. This has been reported to cause ventricular arrhythmias.[309]

Occasionally, AIHA is observed after cardiac surgery involving the placement of foreign material within the vascular system.[310] Children with 22q11.2 deletion syndrome, often with underlying congenital heart disease, are at higher risk for AIHA.[311]

Erythrocyte disturbances in children with cardiac disease must be understood in the context of compensatory states or the context of iron deficiency as a complication. For example, infants with cyanotic congenital heart disease have erythropoietin-induced compensatory polycythemia. Aortic oxygen saturation higher than 80% is usually associated with low erythropoietin titers and hemoglobin levels that will not cause hyperviscosity. Even with moderate degrees of hypoxemia, elevated erythropoietin levels are not seen; presumably, the modest elevation in hemoglobin levels provides adequate tissue oxygenation.[312-314] Infants with cyanotic congenital heart disease have higher iron requirements because of the greater hemoglobin mass. Diminished iron stores in patients with cyanotic congenital heart disease are associated with a more right-shifted oxyhemoglobin dissociation curve.[315] Most children with cyanotic congenital heart disease have evidence of mild macrocytosis. A mean corpuscular volume greater than the 90th percentile for age and sex nearly eliminates the possibility of iron deficiency.[315]

Acquired Coagulation Abnormalities

Many investigations have suggested that a coagulopathy exists in some patients with cyanotic congenital heart disease. Thrombocytopenia, low plasma fibrinogen levels, defective clot retraction, hypoprothrombinemia, factor V and VIII deficiency, and evidence of fibrin degradation products in serum have been reported.[316-320] The presence of coagulation abnormalities may correlate with the extent of polycythemia.[321] Marked derangements in coagulation often accompany surgery involving cardiopulmonary bypass.[322] Conflicting data suggest that the coagulation defects associated with cyanotic congenital heart disease must be multifactorial in origin and may be associated with both a bleeding and prothrombotic predisposition.[323] Therefore, each child should be studied individually.

Acquired von Willebrand disease is well recognized in children with congenital heart disease as well as children with aortic stenosis, with ventricular-assist devices, and on extracorporeal life support.[324-326] Mechanical destruction of high-molecular-weight multimers occurs owing to increased shear force within the abnormal cardiac anatomy. Acquired von Willebrand disease causes a mucocutaneous and/or gastrointestinal bleeding predisposition.[327] Laboratory results will demonstrate decreased von Willebrand factor collagen binding and decreased large von Willebrand factor multimers. Treatment includes supportive care with von Willebrand factor/factor VIII concentrate and/or desmopressin as a bridge to correcting the underlying contributing cardiac defect.

Repair of congenital heart disease can also be associated with coagulopathy.[328] The Fontan operation is the final surgery in the staged palliation of different forms of congenital heart disease with a univentricular heart. After the Fontan procedure, there is often increased systemic venous pressure that causes hepatic dysfunction and/or protein-losing enteropathy, both of which are associated with abnormalities of coagulation.[329]

Management of the hemostatic defects is not settled. It is agreed that they may predispose patients to postoperative hemorrhage, but they are rarely associated with preoperative clinical bleeding tendencies. Suggested management procedures have included the use of heparin[330] to lower viscosity, erythrocytapheresis with plasma exchange,[331,332] and aminocaproic acid to inhibit fibrinolysis.[333] Whether any of these modalities are indicated and, indeed, whether they might be uniformly effective is speculative. If erythrocytapheresis is chosen, the procedure should be done with great care in cyanotic patients. Withdrawal of red blood cells must be accompanied by infusion of an equal volume of fresh-frozen plasma. Simple removal of red blood cells without volume replacement in polycythemic individuals may cause an acute increase in viscosity, vascular collapse, seizures, and even stroke.[334]

Because of the high incidence of cerebrovascular accidents in children with cyanotic congenital heart disease, some have believed that a hypercoagulable state exists. Hyperviscosity alone may account for some ischemic infarctions. The clinician should be alert to the possibility of iron deficiency predisposing patients to stroke in the presence of polycythemia. Several children in whom neurologic deficits developed after the onset of iron deficiency in the presence of polycythemia have been described.[335,336] Card and Weintraub[337] showed that red blood cells from animals with iron deficiency have decreased deformability. Altered deformability in the presence of increased blood viscosity could theoretically result in vascular ischemia.

Platelet Abnormalities

Quantitative and qualitative platelet abnormalities are commonly associated with cardiac disease. Certain syndromes, such as DiGeorge syndrome, Noonan syndrome, and Jacobsen syndrome, are associated with both congenital heart disease and platelet abnormalities.[338] However, even in those without a diagnosed genetic syndrome, platelet abnormalities are commonly found.

In one series, the mean platelet count in cyanotic patients with an arterial oxygen saturation of less than 60% was 185,000 cells/mm^3, as compared with 315,000 cells/mm^3 in patients with an arterial oxygen saturation greater than 60%.[339,340] These abnormalities are readily noted on preoperative evaluation of the coagulation status of individuals about to undergo surgery.[341] Examination of bone marrow has failed to demonstrate any quantitative changes in megakaryocytes to account for these platelet differences.[339] This, together with the finding of shortened platelet survival in many patients, has suggested that the mechanism of the thrombocytopenia is destructive. Shortening of platelet survival with or without thrombocytopenia has been observed in both children and adults with prosthetic heart valves.[342] The increased platelet turnover may result from a combination of mechanical damage to the platelet and adhesion to the foreign material. Some patients, especially those with minimal cyanosis, may have elevated platelet counts. Although iron deficiency is commonly associated with cyanotic congenital heart disease, the quantitative platelet abnormalities are not related to iron status.

Qualitative platelet defects associated with cyanotic congenital heart disease may include abnormal aggregation in response to adenosine diphosphate, epinephrine, and collagen.[343] In as many as 70% of patients, either a delayed or no second wave of platelet aggregation is observed.[344] These platelet functional abnormalities appear to be due to a defective platelet release mechanism because diminished release of carbon-14–labeled serotonin occurs in response to adenosine diphosphate whereas uptake of it is normal.[345] Platelet release abnormalities are more common in patients older than 4 years, in those with a hematocrit greater than 60%, and in those with platelet counts less than 175,000 cells/mm^3.

Miscellaneous Hematologic Manifestations of Heart Disease

Subacute bacterial endocarditis may be associated with a variety of hematologic manifestations. Anemia is often present and is usually the result of chronic infection. The white blood cell count is generally normal, although marked leukocytosis or leukopenia may occur. A 25-year review of the evolving pattern of pediatric endocarditis shows that anemia is present in 40% of children with endocarditis and leukocytosis in 30% of such patients.[346] Pancytopenia has been reported,[347] and thrombocytopenia has been noted in a few patients.[112,113]

Congestive heart failure may result in sufficient hypoxia to cause nucleated red blood cells to appear in the peripheral blood in association with mild reticulocytosis.[114] Thrombocytopenia may be present but is almost exclusively the result of hypersplenism.[348]

Cyanotic heart disease may result in poor perfusion of the spleen and subsequent functional hyposplenism manifested by Howell-Jolly bodies in peripheral blood.[349] This finding is likely to lead to the mistaken conclusion that a child may have the asplenia syndrome (absence of the spleen, cardiovascular malformations, abdominal situs inversus, and other anatomic malformations), which is a separate and distinct diagnosis.

PULMONARY DISEASE

Hypoxia may be caused by a wide variety of pulmonary disorders and, in turn, results in a form of secondary polycythemia and compensatory shifts in the hemoglobin-oxygen dissociation curve that can be attributed to an increase in the red blood cell content of 2,3-diphosphoglycerate (2,3-DPG). Rarely, the polycythemia results in a state of extreme hyperviscosity and decreased tissue blood flow. Other than polycythemia, the hematologic findings of specific pulmonary disorders tend to be unique to those disorders.

Cystic Fibrosis

Patients with cystic fibrosis demonstrate a significant impairment in erythropoietic response to hypoxemia.[350] There is neither an appropriate increase in hemoglobin level nor an adequate shift in the red blood cell–oxygen affinity curve. Failure of the hemoglobin-oxygen dissociation curve to compensate means that such patients may be more symptomatic at any given hemoglobin level than patients who have anemia and other disorders. Disturbances in erythropoietin regulation, as seen in the anemia of chronic disorders and iron deficiency, appear to be the principal cause of the relative anemia in children with cystic fibrosis.[351,352] Deficiencies in vitamin E and iron are also common.[353] If a severe hemolytic anemia is observed in a subject with cystic fibrosis, it may be related to vitamin E deficiency.[354]

Idiopathic Pulmonary Hemosiderosis

Idiopathic pulmonary hemosiderosis (IPH) is an uncommon chronic disease that usually affects children and young adults. However, the age at onset may be as early as the neonatal period.[355] IPH is characterized by recurrent intrapulmonary hemorrhage and may result in hemoptysis and pulmonary insufficiency. The hematologic manifestation of this disorder is iron-deficiency anemia.

The etiology of IPH is not known. A hereditary or familial tendency has been suggested. An immunologic cause seems likely because other occasional findings include positive Coombs test results, the presence of cold agglutinins, and an increased number of mast and plasma cells in the lungs. Autoimmunity is also suggested by frequent association with celiac disease, vasculitis, and collagen vascular disease, as well as by response to treatment with immune suppression. Historically, an association with hypersensitivity to milk has been suggested as well as a structural defect in the alveolar capillaries.[356-358]

The most helpful clinical signs for identifying IPH are iron-deficiency anemia and recurrent or chronic cough, hemoptysis, dyspnea, wheezing, and, frequently, cyanosis. Any single feature may be present without the others. For example, occasionally the only clinical sign is iron-deficiency anemia.[359] Pulmonary symptoms may be present without radiologic findings and vice versa. When pulmonary symptoms are predominant, there may be associated fever, tachycardia, tachypnea, leukocytosis, an elevated sedimentation rate, and, occasionally, abdominal pain.[360] Radiographic abnormalities vary from minimal transient infiltrates to massive parenchymal involvement with atelectasis, emphysema, and hilar adenopathy.[361,362]

The anemia reflects an iron-deficiency state resulting from excessive accumulation of iron in the lungs. This iron is usually sequestered in alveolar macrophages and is largely unavailable for new red blood cell formation. With time, however, the iron is eventually lost from the lungs.[363] In some patients, iron administration fails to correct the anemia. This is usually a reflection of inadequate heme synthesis resulting from the anemia of chronic disease. AIHA may occur. Eosinophilia is found in 15% to 20% of children with IPH.

A diagnosis of IPH may be made by the finding of siderophages in the gastric aspirate. Siderophages will stain positive with Prussian blue dye. Sputum evaluation and bronchoalveolar lavage may reveal hemosiderin-laden alveolar macrophages. If the diagnosis of IPH is seriously being considered, it may be necessary to perform a lung biopsy. Typical findings include alveolar epithelial hyperplasia, degeneration with excessive shedding of cells, large numbers of siderocytes, varying amounts of interstitial fibrosis and mast cell accumulation, elastic fiber degeneration, and sclerotic vascular changes.[364] Electron microscopic examination shows no evidence of subendothelial deposits or basement membrane lesions.[365] No evidence for localization of IgG, IgM, C1q, or fibrinogen has been found.[363] The results of needle aspiration or needle biopsy may also provide a diagnosis.[366]

Treatment of IPH continues to be a controversial subject due to its rarity. In those patients with concomitant celiac disease, a gluten-free diet is recommended. Given the historic link with hypersensitivity to cow's milk, a trial of removal of cow's milk from the diet is appropriate in some instances.[367] Corticosteroid therapy will sometimes produce a remission of disease activity.[368,369] Immunosuppressive therapy may be useful, but the results of such treatment are unpredictable.[370,371]

Pulmonary Hemosiderosis from Other Causes

Clinical findings of hypochromic and microcytic anemia and pulmonary infiltrates may be seen in association with glomerulonephritis (Goodpasture syndrome), collagen vascular disease, Wegener granulomatosis, and, occasionally, SLE.[372-375] Episodes of pulmonary hemosiderosis have also been described in patients with cystic fibrosis and celiac disease.[376,377]

Hematologic Findings in Other Pulmonary Disorders

Sarcoidosis does not usually cause specific hematologic disturbances, although anemia is present in some patients. Sarcoidosis can be associated with splenic enlargement in up to 25% of patients, often with signs of hypersplenism with anemia, leukopenia, and thrombocytopenia. A higher than average incidence of blood group antigen A has been reported in this disorder.[378,379] Bone marrow granulomas are noted occasionally.[380] The significance of this finding is not known, but the altered immune response of these patients[381] and an association of sarcoidosis with hemolytic anemia[382] and immune thrombocytopenia[383-386] suggest that sarcoidosis represents a generalized autoimmune disorder.

Eosinophilia may be observed in a variety of pulmonary disorders, including asthma, Löffler syndrome, tropical pulmonary eosinophilia, polyarteritis nodosa, and sarcoidosis.

RENAL DISEASE

Renal disease may produce disturbances in red blood cells, white blood cells, platelets, and coagulation factors. In many instances, the abnormalities that are found do not parallel the status of renal function but rather reflect the activity of the disease process that results in renal dysfunction. The hematologic aspects of renal insufficiency have been reviewed in detail.[387]

The Red Blood Cell

Anemia

Anemia is a common finding in acute and chronic renal disease and is often so severe in these conditions that it constitutes the major morbidity of renal disease.[388] Although the anemia is often multifactorial, the main cause is erythropoietin deficiency.[389] Progressive renal disease is accompanied by an erythropoietin decline that results in a hypoproliferative, usually normochromic, normocytic anemia. However, other factors can intervene to impair erythropoietin responsiveness and aggravate already low levels of the hormone. These include infection and inflammation (anemia of chronic disease), inadequate dialysis, and secondary hyperparathyroidism.[390,391] In patients treated with erythropoietin, deficiencies of cobalamin (vitamin B_{12}), folic acid and, especially, iron may limit response. Transfusion support frequently becomes a necessary intervention, although elevated 2,3-DPG levels and a right-shifted oxygen dissociation curve make the red blood cells of uremic subjects more effective in the delivery of oxygen than normal red blood cells.[392] The anemia usually develops when the creatinine clearance decreases below 35 to 45 mL/min, although no direct correlation is found between the severity of the anemia and the degree of renal impairment. The hemoglobin level is generally in the 7- to 8-g/dL range in uremic states.

Decreased red blood cell production is the major cause of the anemia of chronic renal failure. Although deficiency in erythropoietin production is the primary cause of decreased red blood cell production in renal failure,[393,394] suppression of hematopoiesis by serum inhibitors may be a contributing factor.[395,396] In patients with acute or chronic renal failure as a result of nephrectomy, normal or even increased erythropoietin production can be maintained.[397] Several series have documented improved hematocrits and erythrocyte volumes proportional to the intensity of dialysis treatments, which may remove the inhibitors.

In addition, excess parathyroid hormone is also an inhibitor of erythropoiesis in uremia, a defect that renal failure states share with primary hyperparathyroidism.[398] The mechanism of this contribution to anemia[399] is likely due to the replacement of the marrow cavity by fibrous tissue in hyperparathyroid states. Removal of hypertrophied parathyroid glands improves the osteitis fibrosa of involved marrows and the anemia that accompanies this abnormality.[400,401] Treatment with 1,25-dihydroxycalciferol also decreases marrow fibrosis and diminishes the anemia of renal failure.[402,403] The role of parathyroid hormone in these anemias is in remodeling the environment in which hematopoiesis takes place; the more advanced states of bone disease seen in patients with uremia may even lead to splenomegaly secondary to extramedullary hematopoiesis.[404]

In renal disease, unlike many other chronic disease states, close correlation exists between erythrocyte mass and erythrocyte survival.[405] This implies that shortening of red blood cell survival has an important role in the development of anemia in these patients. About 70% of patients with renal disease have shortened red blood cell survival related to a host of potential alterations. These cells demonstrate increased mechanical fragility, autohemolysis, diminished deformability, and a variety of metabolic defects.[406,407]

A circulating inhibitor of red blood cell metabolism accumulates in some but not all uremic patients. This inhibitor diminishes recycling of glucose through the hexose monophosphate shunt,[406] a defect that may be clinically important. There are several reports of severe hemolytic disease in uremic patients given sulfa drugs. Because of this problem, caution in the use of oxidant drugs is advised in those with azotemia.[408] The decreased shunt activity may be secondary to a metabolic defect within the shunt itself or due to a block within the main glycolytic pathway. Other incidental causes of hemolysis may be observed during hemodialysis. Contaminants such as copper, nitrate, and chloramines in hemodialysis baths may cause varying degrees of hemolysis.[409-411]

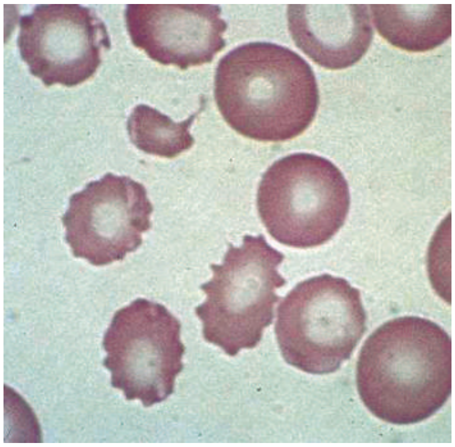

Figure 37-13 Burr cells caused by uremia.

The erythrocytes of patients with renal disease are generally normochromic and normocytic without any distinguishing morphologic characteristics. Occasionally, scalloped or burr cells are observed, usually in association with uremia secondary to specific causes or related to specific syndrome complexes such as hemolytic uremic syndrome (Fig. 37-13).[412] In diseases associated with microangiopathic hemolytic anemia, red blood cell fragmentation is common. It may be observed in patients with malignant hypertension, renal cortical necrosis, polyarteritis nodosa, hemolytic uremic syndrome, SLE, and TTP.

Anemia associated with megaloblastic changes in bone marrow is not uncommon in chronic renal failure. This anemia is usually due to folate deficiency[413] caused by poor dietary intake, hemodialysis, peritoneal dialysis, or the effects of immunosuppressive drugs. In addition, defective protein-mediated folate transport may be present.[414] Serum from uremic patients may contain an increased concentration of folic acid–binding protein.[414] Folate deficiency is usually suspected when large numbers of macroovalocytes are seen in association with hypersegmentation of polymorphonuclear leukocytes on the peripheral blood smear. The anemia of renal disease can also be worsened in dialyzed patients by superimposition of iron deficiency due to blood loss during hemodialysis.

The primary role of erythropoietin deficiency in the anemia of renal failure has been incontrovertibly confirmed with the successful trials of recombinant human erythropoietin in both dialyzed and nondialyzed renal failure patients (see Chapter 1).[415,416] Administration of the hormone has eliminated the need for red blood cell transfusions or androgens, previously the mainstays in treating the anemia of renal failure.[417,418] The therapeutic success of erythropoietin-stimulating agents (ESAs) has brought to a close any doubts concerning the major cause of the anemia of renal failure.[419,420] The administration of ESAs may unmask iron and folate deficiency, their presence being signaled by an apparent refractoriness to erythropoietin administration.[421] Controversy exists about the goal hemoglobin level in patients with renal insufficiency treated with ESAs, although most data support a lower hemoglobin level of 10 to 11 g/dL.[422,423] Administering ESAs to reach higher target hemoglobin levels greater than 13 g/dL is associated with fewer red blood cell transfusions; however, higher hemoglobin levels are also associated with an increased risk for stroke, hypertension, and thrombosis.[424,425] It remains unclear whether the higher hemoglobin is linked to these adverse outcomes or high doses of ESAs.

Polycythemia

Secondary erythrocytosis is a well-recognized complication of renal disorders. The increase in red blood cell mass is due to release of erythropoietin directly from renal tumor cells, renal ischemia, and hydronephrosis and in association with chronic glomerulonephritis, pyelonephritis, nephrosclerosis, and nephrotic syndrome in adults.[426,427] Erythrocytosis is also a common finding after renal transplantation.[428]

The White Blood Cell

Azotemia and uremia per se appear to have relatively little effect on white blood cell function. Granulocytopenia is common during hemodialysis[429] and appears to be due to transient sequestration of granulocytes in the pulmonary vascular bed followed by later release back into the circulation. After hemodialysis, a transient augmentation in granulocyte adherence coincides with the time of sequestration of the pulmonary vascular bed.[430] The adherence-augmenting factor in the plasma of patients undergoing dialysis is heat stable.

Neutropenia as a result of folate deficiency may be present in patients with renal failure. The cause of the neutrophil nuclear hypersegmentation associated with uremia is unknown and should not necessarily be attributed to folate deficiency.

Vascular occlusion of the kidney may result in eosinophilia. Approximately 80% of adult subjects with embolic renal disease have eosinophil counts ranging from 540 to 2000 cells/mm^3.[431]

Coagulation Abnormalities

Patients with uremia may have varied changes in some coagulation factors. Among the most common abnormalities observed are mild to moderate depressions in factors V, VII, IX, and X.[432,433] These abnormalities are related to the high incidence of hepatic dysfunction in patients with uremia or vitamin K deficiency.[343] The level of fibrinogen is regularly elevated because it is an acute phase reactant.[434-436] Increased fibrinogen turnover has been demonstrated in experimental models,[437] and excessive fibrin degradation products have been found in the urine and serum of uremic patients.[438] When a decrease in one or more consumable clotting factors (factors I, II, V, or VIII) is also present, it may signify the existence of consumptive coagulopathy.[432,433,439] Because fibrin deposition may occur in the absence of such a coagulopathy, it is often helpful to determine whether high-molecular-weight fibrin degradation products are present in the urine and serum. If found only in the urine, such products are probably derived from the dissolution of fibrin deposits in the kidneys. If demonstrable simultaneously in urine and serum, their presence indicates renal injury because such degradation products cannot be demonstrated in the urine of patients with streptokinase-induced fibrinolysis who have no renal disease.[440,441] Fibrin degradation products in the urine and serum usually indicate active renal disease, although other reasons for this finding may be seen in patients with renal disease. Fibrin degradation products may also result from concomitant liver disease or from an external shunt used for hemodialysis.[337] Urinary fibrinolytic activity is either diminished or absent in renal failure, and inhibitors of urokinase-induced plasminogen activation are demonstrable.[440]

Nephrotic syndrome is often accompanied by changes in a number of coagulation factors.[442] Increased levels of fibrinogen, factor VIII, and the factor VII-X complex are commonly present.[443] A prolonged partial thromboplastin time is often observed and is usually due to a decrease in the plasma level of factor IX.[444] Factor IX is consistently found in urine containing more than 10 g of protein per 24 hours in patients with nephrotic syndrome.[445] Plasma levels of factor IX rarely decrease to less than 10%, and clinical bleeding episodes are unusual. With resolution of the proteinuria in patients receiving corticosteroid therapy, levels of factor IX increase. Factors II and VII have also been identified in considerable quantity in the urine of patients with nephrotic syndrome despite normal levels of these factors in plasma.[446] This finding suggests that loss of some coagulation factors into urine can be fully compensated for by an increased rate of synthesis. A prolonged partial thromboplastin time may also be due to a low level of Hageman factor (factor XII).[447] Hageman factor has a molecular mass of approximately 75,000 kD, similar to that of albumin, and losses in urine are to be expected.[448] Although factor XII levels may decrease to less than 10%, as with congenital deficiency of this factor, bleeding is not encountered. Patients with nephrotic syndrome appear to have a substantially higher risk for thromboembolic complications.[440] The coagulation defects associated with nephrotic syndrome have been reviewed by Kaufmann and colleagues.[449]

The hypercoagulable state seen in many forms of renal disease may be compounded by the occurrence of lupus-like anticoagulants. This is common in a variety of end-stage renal diseases, with a rate of occurrence as high as 22%.[450] Whether therapy focused on prevention of fibrin deposition within the kidney is of benefit to patients with certain forms of glomerulonephritis remains uncertain. Kincaid-Smith[451,452] used a combination of an anticoagulant, dipyridamole, and cyclophosphamide in a series of patients with membranoproliferative glomerulonephritis. A similar approach was reported by Robson and associates,[453] but with azathioprine substituted for cyclophosphamide. In both studies, it was suggested that these therapies produced improvement in the patients' clinical course. The concept of anticoagulation therapy for these disorders has been reviewed.[454]

Platelet Abnormalities

Mild thrombocytopenia occurs in approximately 25% of patients with acute renal failure and 10% of those with chronic renal disease.[455] The thrombocytopenia is most likely due to impaired production, because platelet life span in uremia is generally normal.[435]

Platelet function is abnormal in patients with renal failure. The standardized Ivy bleeding time is usually prolonged and generally correlates with the clinical tendency for bleeding in these patients.[456] Platelet adhesion and platelet factor 3 availability are diminished in acute and chronic uremia.[457] Impaired aggregation of platelet-rich plasma in the presence of adenosine diphosphate,[458] epinephrine,[459] thrombin,[459] and collagen[459] has been observed. Clinical bleeding episodes related to these platelet abnormalities can usually be managed with desmopressin given intravenously.[460] Long-term administration of recombinant erythropoietin also improves the biochemical and functional changes in the platelets of uremic patients.[461] Rarely, enhanced platelet function characterized by hyperaggregability has also been observed in uremia.[462]

Platelet functional abnormalities are not due to an intrinsic platelet defect because dialysis quickly reverses these changes and incubation of normal platelets in plasma from uremic patients promptly induces functional aberrations. Rather, platelet dysfunction appears to be due to the interaction of intrinsically normal platelets with various abnormal dialyzable metabolites found in the plasma of uremic patients.[463] If urea and creatinine levels are elevated, some of these findings can be reproduced.[463] Phenols[434] and guanidinosuccinic acid,[464] which accumulate in the plasma of uremic patients, can also account for platelet functional abnormalities. These findings are discussed in detail in Chapter 34.

Several hereditary disorders have been reported in which quantitative or qualitative platelet abnormalities occur in association with nephritis. MYH9 disorders are caused by defects in the myosin heavy chain IIa and associated with macrothrombocytopenia and a bleeding tendency. Because myosin IIA is a major component of the podocyte foot process and maintains capillary wall integrity, many MYH9 disorders, including Epstein syndrome, Fechtner syndrome, and Sebastian platelet syndrome, are associated with kidney dysfunction in addition to hearing loss and cataracts.[465,466] In these disorders it is hypothesized that disordered early platelet release contributes to the thrombocytopenia. The inherited platelet disorders are discussed in Chapter 30.

The pathophysiology and management of hemolytic uremic syndrome are discussed in Chapter 34.

GASTROINTESTINAL DISEASE

Hematologic complications of gastrointestinal disease appear in many disorders. This section does not include the wide range of situations in which blood loss from the gastrointestinal tract occurs but rather focuses on specific diseases and their hematologic complications.

Diseases of the Gastrointestinal Tract

Esophagus

Plummer-Vinson syndrome (dysphagia, postcricoid webs, and iron deficiency)[467] occurs in older individuals and is discussed in Chapter 11. Iron-deficiency anemia may be the only manifestation of gastroesophageal reflux; thus endoscopy is important in the evaluation of unexplained iron-deficiency anemia.[468] Chronic gastroesophageal reflux may cause Barrett esophagus with its attendant risk for the development of adenocarcinoma.[469]

Stomach

The gastric mucosa is important in both vitamin B_{12} and iron absorption, and disorders of the gastric mucosa may cause defective absorption of either of these nutrients. The gastrointestinal causes of iron and vitamin B_{12} deficiency are reviewed in Chapters 10 and 11.

Gastric resection may result in iron or vitamin B_{12} deficiency, the former from bleeding at sites of anastomosis and the latter, years later, from lack of intrinsic factor. Vitamin B_{12} deficiency may develop in infants breastfed by women who have undergone gastric or intestinal bypass procedures.[470] Macrocytic, megaloblastic anemia resulting from vitamin B_{12} deficiency has been reported in association with gastric trichobezoars.[471] This is presumed to be due to bacterial overgrowth of the upper gastrointestinal tract. In congenital intrinsic factor deficiency, gastric biopsy results are normal and no antibodies to parietal cells or intrinsic factor are present.[472]

Small Intestine

Celiac disease and inflammatory bowel disease are both frequently associated with anemia and are covered in detail in Chapter 11.

Crohn disease has also been associated with coagulopathy, particularly with acute flares. Low factor XIII levels have been observed in active disease associated with regional enteritis.[473] An abnormality in arachidonic acid metabolism may occur in patients with chronic inflammatory bowel disease, including regional enteritis. This may result in alterations in platelet function.[474] Diminished neutrophil function may be seen in both regional enteritis and other forms of inflammatory bowel disease. The condition may be identified by decreased oxidative metabolism and low superoxide dismutase content of neutrophils.[475] Perinuclear antineutrophil cytoplasmic antibodies are commonly found in patients with inflammatory bowel disease.[476,477] Inflammatory bowel disease, particularly during acute flares, is also associated with an increased risk for thrombosis.

Disorders such as tropical sprue, celiac disease, bowel lymphoma, amyloidosis, and connective tissue disturbances (including Ehlers-Danlos syndrome and pseudoxanthoma elasticum) may produce panselective or selective malabsorption. Certain disorders such as intestinal lymphangiectasia may cause protein loss of a magnitude sufficient to impair globin chain synthesis. Protein loss may also be associated with selective loss of T lymphocytes into the bowel lumen, which causes lymphopenia and altered delayed-type hypersensitivity. This lymphopenia may be associated with hypogammaglobulinemia.[478]

Eosinophilic gastroenteritis is a disorder most often characterized by recurrent bouts of abdominal pain, nausea, vomiting, diarrhea, and an elevated peripheral blood eosinophil count for which there is no other explanation.[479] The gut wall may be infiltrated by eosinophils.[479]

Common diarrheal illnesses during infancy may result in life-threatening methemoglobinemia, especially if the diet has been high in nitrates.[480-483]

Large Intestine

Ulcerative colitis is often associated with iron-deficiency anemia as a result of blood loss. AIHA identified by positive results of a Coombs test and immune thrombocytopenia are relatively rare complications of ulcerative colitis, however, and develop in less than 1% of patients.[484] Occasionally, vitamin B_{12} deficiency occurs after many years if a "backwash" ileitis is present. Polymorphonuclear leukocytosis is common. Occasionally, an increase in plasma fibrinolytic activity will be observed. A significant decrease in antithrombin III levels may be found.[485] Some clinicians have treated this complication with ε-aminocaproic acid.[486] Platelet functional abnormalities may exist and are discussed in Chapter 34. Alterations in T-cell subsets may also occur in children with inflammatory bowel disease.[487]

Constipation has been reported to cause an increase in sulfmethemoglobin levels.[488] It has been suggested that excess nitrite absorption from the gut may be present in some patients as a result of abnormal bowel function.[489]

Symptoms of Peutz-Jeghers syndrome (gastrointestinal polyposis and mucocutaneous pigmentation) may occur in early childhood and are associated with an increased risk for intestinal cancer.[490,491]

Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu disease) may result in iron deficiency from gastrointestinal bleeding. Alterations in hemostasis (DIC, platelet dysfunction, and factor XI deficiency) have been reported.[492] An association between this disorder and von Willebrand disease may exist.[493]

Disease of the Pancreas

Acute hemorrhagic pancreatitis may cause acute anemia during the first week of the illness as a result of hemodilution, intravascular coagulation, and blood loss.[494] Leukocytosis with neutrophilia is a usual finding. Markedly elevated levels of fibrin split products usually suggest that a consumptive coagulopathy is present. A concomitant decrease in platelet count is also common.[495] High levels of methemalbumin are common in the ascitic fluid of individuals with hemorrhagic pancreatitis[496] and can be used to distinguish this disorder from nonhemorrhagic pancreatitis. Patients with ascitic fluid are the ones most likely to have evidence of consumptive coagulopathy. For example, fibrin degradation products are seen in 40% of subjects with fulminant pancreatitis without ascites but in 100% of patients with clinically apparent peritoneal fluid.[497]

Shwachman-Diamond syndrome and Pearson syndrome with associated congenital exocrine pancreatic insufficiency and bone marrow failure are discussed in Chapter 7.

Cystic fibrosis may cause malabsorption, which produces the expected hematologic abnormalities. A specific pattern of anemia in association with edema and hypoproteinemia may be observed in some children with cystic fibrosis. It is found most commonly in children who have low dietary intake of nitrogen, such as those who receive their protein primarily from breastmilk or soybean formula.[498] The edema is caused by hypoalbuminemia. The anemia responds to adequate dietary protein enrichment and pancreatic enzyme supplementation but not to iron administration. Cystic fibrosis may also be associated with malabsorption of fat-soluble vitamins, and the consequent coagulopathy is related to vitamin K deficiency. Vitamin E deficiency may also occur and is manifested by mild hemolytic anemia and abnormal red blood cell peroxide hemolysis, as well as by hyperaggregation of platelets.[499]

Diseases of the Liver

Red Blood Cell Disturbances

Anemia is common in acute and chronic liver disease and has a diverse etiology.[500] Liver disease is often accompanied by increased portal pressures, with the resultant congestive splenomegaly potentially creating any combination of anemia, leukopenia, or thrombocytopenia. The marrow's compensation for such hemolytic insults is frequently dampened or absent in the setting of liver disease because of concomitant vitamin nutritional deficiencies. In addition, portal hypertension and liver disease are associated with increased risk for bleeding and concomitant iron deficiency. Shortened red blood cell survival may be observed in some patients with no evidence of blood loss.[501,502] The exact mechanism by which red blood cell survival is shortened is unclear.

Erythrocytes from patients with active liver disease have an increased tendency to form Heinz bodies after incubation with an oxidant chemical such as acetylphenylhydrazine or sodium ascorbate. This increase in Heinz body formation is associated with increased instability of red blood cell reduced glutathione, decreased hexose monophosphate shunt activity, and reduced glucose recycling through the hexose monophosphate shunt.[503] Activation of superoxide dismutase and glutathione reductase may be observed in the red blood cells of patients with profound liver disease. This may partially explain the alterations in the antioxidant system noted with these disorders.[504] The hemolytic anemia associated with liver disease secondary to Wilson disease is especially severe.[505] The same is true of the hemolytic anemia related to protoporphyria.[506]

The exact significance of these metabolic alterations has not yet been determined. Red blood cell metabolism appears to return to normal within a few weeks after an insult to the liver. All of the metabolic consequences of liver disease can be reproduced in normal intact red blood cells by prolonged incubation in plasma from patients with active liver disease. In patients with active liver disease, it may be wise to withhold any drug that has the potential to present an oxidant challenge to the red blood cell. Similar acquired abnormalities in hexose monophosphate shunt activity have been associated with drug-induced hemolysis in patients with uremia.[507]

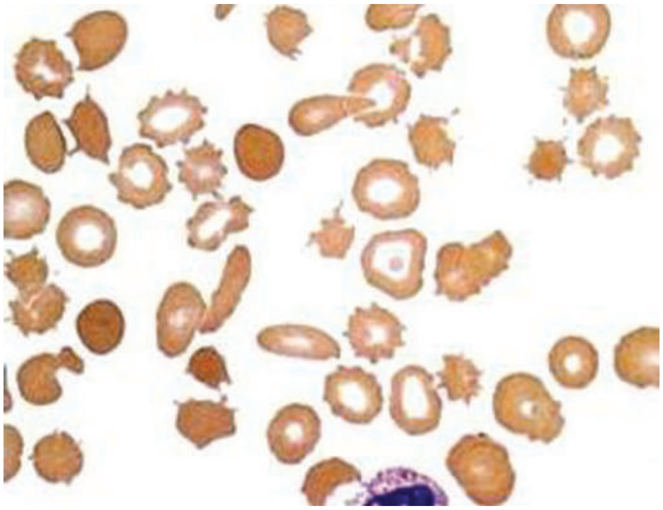

Figure 37-14 Acanthocytes and target cells due to liver disease.

The red blood cells in liver disease are often macrocytic, with mean corpuscular volumes in the range of 100 to 110 fL. In patients with biliary obstruction, the red blood cell surface lipids increase and cause target cells. With severe hepatocellular damage, acanthocytes or spur cells are commonly observed (Fig. 37-14).[508,509] These morphologic abnormalities appear to be in direct proportion to the increase in red blood cell membrane cholesterol that accompanies hepatocellular liver disease.[510-512] Increased osmotic resistance is a consequence of the increased red blood cell surface area. Stomatocytosis has been reported in alcoholic liver disease.[513]

Coagulation Abnormalities

Because the liver is involved to some extent in the synthesis of most of the coagulation factors, it is not unexpected that liver dysfunction would be associated with the presence of abnormal clotting studies and bleeding. The coagulation abnormalities common in patients with fulminant hepatic failure have been reviewed in detail.[514,515]

Patients with liver disease commonly manifest a spectrum of coagulation abnormalities that are highly suggestive of DIC.[516,517] Findings consistent with DIC include hypofibrinogenemia, thrombocytopenia, increased fibrinogen catabolism, increased levels of fibrin degradation products, and depressed levels of other coagulation factors. This pattern of abnormalities is not specific for DIC and may simply reflect the severity of the hepatocellular disease process. For example, fibrinogen levels may be depressed on a synthetic basis alone. Increased catabolism of fibrinogen may reflect distribution in extravascular spaces, such as the formation of ascitic fluid or proteolysis by enzymes other than thrombin. Thrombocytopenia may occur for a variety of reasons and frequently is not present when other signs of DIC are seen. Changes in levels of factors V and VIII are varied and nonspecific. Because fibrin degradation products are cleared by the liver, severe liver dysfunction itself may cause elevations of these products without implicating DIC.[515]

TABLE 37-4 Hematologic Effects of Thyroid Hormone

↑ Thyroid Hormone	↓ Thyroid Hormone
↑ PRBC volume	↓ PRBC volume
Variable effect on RBCs	↑ Or normal MCV; spiculated RBCs
↑ Glucose utilization and hexose monophosphate shunt activity	↑ O_2 affinity
↓ RBC glutathione, reduced	↓ 2,3-Diphosphoglycerate
↑ Red blood cell 2,3-diphosphoglycerate	↓ RBC Na^{+}
↓ O_2 affinity	↓ Platelet adhesion
↑ Diphosphoglycerate mutase activity	↓ (Slight) platelet aggregability
↑ Glyceraldehyde-3-phosphate dehydrogenase	↓ Factor VIII activity
↑ Glucose 6-phosphate activity	↑ Fibrinolytic activity
↓ RBC carbonic anhydrase	↑ Plasminogen
↓ RBC zinc	↑ Plasminogen activation
↑ RBC Na^{2+}	
↑ (Slight) platelet turnover	↓ Capillary fragility
↑ Platelet adhesion	↑ Factors VIII, VII, IX, XI activity
↑ Platelet aggregability	
↑ Factor VIII activity	
↓ Plasminogen activation	
↑ Capillary fragility	

MCV, Mean corpuscular volume; *PRBC,* packed red blood cell; *RBC,* red blood cell.

The acquired coagulation abnormalities of liver disease are discussed in detail in Chapter 35.

ENDOCRINE DISORDERS

Disturbances in endocrine balance tend to produce relatively mild hematologic abnormalities. The most thoroughly evaluated of these abnormalities are those caused by thyroid disorders.

Thyroid Gland

Thyroid hormone appears to have many hematologic effects (Table 37-4), with the most impressive being its effect on erythropoiesis. Individuals with hyperthyroidism sometimes have an elevated red blood cell mass secondary to the effects of excessive thyroid hormone and have an increased requirement for folic acid to accommodate the increased red blood cell synthesis.[518] The red blood cells of patients with hyperthyroidism may have increased osmotic resistance.[519] The red blood cell sodium concentration is significantly increased in hyperthyroidism, and decreased sodium pump activity may be present.[520] A macrocytic anemia may complicate hyperthyroidism in the setting of Graves disease because pernicious anemia and other immune disorders are linked to Graves disease.[521,522] Neutropenia may occur in approximately 5% of children with hyperthyroidism.[523] The mechanism of this abnormality is unclear, although some patients will have antineutrophil antibodies.[524]

The anemia of hypothyroidism is caused by a reduced red blood cell mass secondary to the decreased oxygen requirements of the hypothyroid state.[525,526] The anemia is usually normochromic and normocytic and occasionally mildly macrocytic.[527] Patients with hypothyroidism often (20% to 65%) have a small number (0.5% to 2%) of acanthocytes on peripheral blood smears.[528] Given the high incidence of hypothyroidism relative to other disorders that cause acanthocytosis, it has been suggested that the presence of acanthocytes should prompt physicians to consider thyroid testing, especially in adults. This approach has led to the diagnosis of previously unsuspected hypothyroidism. Red blood cell survival is normal, and ferrokinetic studies demonstrate a decreased rate of iron clearance. In adults with hypothyroidism, an unusually high incidence of iron deficiency, often linked to menorrhagia, and pernicious anemia is found.[529,530]

Thyroid hormone may play a role in the regulation of red blood cell metabolism. Thyroxine has been reported to increase red blood cell glycolysis.[531] It helps regulate red blood cell carbonic anhydrase I concentrations.[532] Hexose monophosphate shunt activity may increase in the presence of triiodothyronine.[533] The activity of glucose-6-phosphate dehydrogenase is consistently elevated in thyrotoxicosis, whereas normal levels are found in most patients with mild hyperthyroidism.[534] Glutathione levels are usually depressed in hyperthyroidism. Thyroid hormone may affect the activities of several other red blood cell enzymes. This hormone stimulates diphosphoglycerate mutase and glyceraldehyde-3-phosphate dehydrogenase.[535] These effects may be the cause of the elevated levels of red blood cell 2,3-DPG that are seen in patients with hyperthyroidism and that result in decreased whole blood oxygen affinity and a shift to the right in the hemoglobin-oxygen dissociation curve.[536] Opposite effects are seen in hypothyroidism. The carbonic anhydrase activity of erythrocytes is diminished in hyperthyroidism and increased in hypothyroidism. Because red blood cell zinc is almost exclusively contained in carbonic anhydrase, hyperthyroidism will result in a decrease in total red blood cell zinc levels.[537]

Multiple effects on platelets and blood coagulation factors may occur in thyroid disease states. Marked hyperthyroidism may reduce platelet survival.[538] Egeberg[539] reported on a patient with hypothyroidism who had a prolonged bleeding time and low factor VIII activity, similar to findings in von Willebrand disease.

Simone and associates[540] reported on patients with hypothyroidism who had multiple coagulation factor deficiencies. Diminished platelet responsiveness to epinephrine and low platelet adhesiveness may be present as well.[541] Impressively low levels of factor VIII occur occasionally, with milder depression of factors VII, IX, and XI being found either alone or in combination.[542] In hypothyroidism, increased plasma fibrinolytic activity may result from an elevation in plasma plasminogen and a decrease in an inhibitor of plasminogen activation.[542,543] This may account for the mild to moderate increase in levels of fibrinogen and fibrin split products occasionally found in patients with hypothyroidism.[542]

Hypothyroidism is also associated with acquired type I von Willebrand disease in 2% to 5% of cases.[544] The low von Willebrand factor antigen is due to a reduction in its synthesis and/or secretion.[544] Similar to inherited type I von Willebrand disease, acquired von Willebrand disease in hypothyroidism is associated with mucocutaneous bleeding and responds to desmopressin and antifibrinolytic agents.[545,546] Acquired von Willebrand disease typically reverses with correction to a euthyroid state.

Adrenal Gland

Adrenal corticosteroids appear to have a stimulatory effect on erythropoiesis. In Addison disease, the adrenal insufficiency may cause mild to moderate anemia, probably as a result of reduced basal metabolism. In most patients, a decrease in plasma volume masks the decreased red blood cell mass. After treatment of adrenal insufficiency, the plasma volume corrects quickly, whereas the red blood cell mass responds over a period of several weeks. The usual effect of treatment is therefore a prompt decrease in hemoglobin concentration followed by a gradual increase.[547] In addition to anemia in Addison disease, neutropenia, eosinophilia, and lymphocytosis are common.[548]

Erythrocytosis may occur in Cushing syndrome, but in most patients the reported increase has been slight. Cushing syndrome is also associated with coagulation abnormalities, suggesting a hypercoagulable state.[549]

A variety of effects on leukocytes occur after corticosteroid administration or endogenous overproduction of a corticosteroid, including granulocytosis, a reduced lymphocyte count, involution of lymphatic tissue, and a decrease in peripheral blood eosinophils and monocytes. The mechanism by which endogenous or exogenous corticosteroids lower the number of circulating eosinophils is unknown.

Pituitary Gland

The hematologic effects of pituitary disease are usually a consequence of the action of trophic hormone on target endocrine organ function. For example, anemia is common in patients with hypopituitarism.[550] This normochromic, normocytic anemia is associated with findings of lymphocytosis and eosinophilia, which indicate that this effect of hypopituitarism results from adrenal insufficiency. Erythropoietin production may be diminished as a consequence of a lower metabolic rate.

Growth hormone may play an important role in erythropoiesis.[551] This hormone stimulates erythropoiesis directly and indirectly by increased production of erythropoietin. The red blood cell count may decrease with isolated growth hormone deficiency.[552] Erythrocyte glucose-6-phosphate dehydrogenase activity is low in hypopituitarism and increases with growth hormone administration.[553] Growth hormone depresses erythrocyte glycolysis.[554]

Other Endocrine Disorders

Hematologic abnormalities are rare in association with gonadal dysfunction. Androgens stimulate erythropoiesis, whereas estrogens, in general, depress red blood cell production. Castration of the adult male results in a definite decrease in red blood cell mass.[555] In disorders associated with androgen excess, such as Cushing syndrome and congenital adrenal hyperplasia, the hemoglobin concentration may exceed normal values. Exogenous androgen administration may cause polycythemia and may also increase red blood cell 2,3-DPG.[556] Androgens appear to stimulate erythropoiesis by increasing erythropoietin production, as well as by having a direct effect on bone marrow stem cells.

Diabetes Mellitus

It has long been recognized that diabetic patients are prone to anemia,[557] infection,[558] and thrombotic episodes with macrovascular and microvascular sequelae.[559]

Red Blood Cells

An unusual hemoglobin component in hemolysates prepared from the blood of some diabetic patients was first noted by Rahbar.[560] A component that migrated near the position of fetal hemoglobin was described on agar gel electrophoresis at pH 6.2 in citrate buffers. This hemoglobin is present in normal individuals; it constitutes 5% to 7% of total hemoglobin[561] and is called hemoglobin A_{1c}. Rahbar's group observed a twofold increase in this hemoglobin fraction in some diabetic patients.[562] Structurally, hemoglobin A_{1c} is a condensation product, by means of a Schiff base, between one molecule of hemoglobin A and one molecule of an aldehyde or ketone, linked at the amino-terminals. The aldehyde or ketone group appears to be one or more hexoses, thus making hemoglobin A_{1c} a glycohemoglobin. Bunn and Briehl[563] have shown that the oxygen affinity of hemoglobin A_{1c} is little affected by the addition of 2,3-DPG, which leads to decreased oxygen affinity when added to hemoglobin A. Studies have shown highly significant relationships between the percentage of hemoglobin A_{1c} and the response to an oral glucose tolerance test and overall diabetic control, as reflected in quantitative urinary glucose determinations.[564,565] In this sense, measurement of the glycohemoglobin provides an insight into diabetic control over many days.

Red blood cell survival, as measured by chromium (^{51}Cr) labeling, may be mildly impaired during periods of poor diabetic control (mean erythrocyte half-life of 27 days). With improvement of diabetic control, red blood cell survival will increase (mean half-life of 31 days).[566] During episodes of ketoacidosis, rapid shifts in the oxygen affinity of hemoglobin may also occur. A decrease in pH causes a shift to the right with an increase in oxygen unloading. Very rapid correction of pH may impede oxygen delivery. A prompt decrease in red blood cell 2,3-DPG levels occurs at these times.[567] This decrease is probably compensatory—an attempt to correct the oxygen affinity disturbances caused by the acidosis. A decrease in red blood cell 2,3-DPG levels may also result from hypophosphatemia, if it occurs during insulin treatment.

Other red blood cell abnormalities have been found in diabetic subjects. The red blood cells of such individuals show evidence of lipid peroxidation and antiperoxidative enzyme changes when compared with those of healthy subjects.[568] An increase in the glycosylated form of erythrocyte superoxide dismutase has been found in diabetic subjects and is related to nonenzymatic glycosylation of this enzyme.[569] Erythrocytes from diabetic subjects also show sodium influx.[570] Red blood cell sorbitol concentrations will increase in subjects with poorly controlled diabetes; the amount of red blood cell sorbitol is related to the degree of diabetic control.[571] Finally, if electronic counting equipment is used to determine the mean corpuscular volume of red blood cells, artifactual elevations in mean corpuscular volume may be seen in subjects with hyperglycemia.[572]

Thiamine-responsive megaloblastic anemia, diabetes mellitus, and sensorineural deafness is a rare autosomal recessive disorder[573,574] that begins in childhood and is caused by defects in the high-affinity thiamine transporter-1 *(SLC19A2)*, on chromosome 1q23.3.[575-577] The anemia and diabetes respond to high-dose thiamine treatment, although the diabetes can relapse years later, despite continued thiamine treatment.[578]

White Blood Cells

Polymorphonuclear cell function may be disturbed in diabetic patients. Leukocyte adherence is diminished in individuals with poor control of diabetes, as is phagocytic capacity.[579] Chemotaxis may also be abnormal, but this does not correlate with the status of diabetic control.[580] Impaired cellular metabolism and DNA synthesis have been reported in the lymphocytes of some patients with diabetes.[581] Increased superoxide production by mononuclear cells is observed in patients with diabetes and hypertriglyceridemia.[582]

Platelets

Many studies have emphasized the abnormalities in platelet adhesion and aggregation in patients with diabetes. The finding that enhanced platelet aggregation occurs before clinical evidence of diabetic vascular disease suggested that this defect may be acquired early in the natural history of diabetes and could underlie the vascular disease.[583] Platelet aggregation in diabetic subjects is characterized by a shortened adenosine diphosphate–induced aggregation time.[584] Increased platelet adhesiveness and platelet factor 3 and 4 activity also occur.[566,585] Normal platelets incubated in the plasma of diabetic patients will become abnormal. This aggregation-enhancing activity is present in both plasma and serum and is nondialyzable and heat resistant.[584] In patients with this plasma factor, von Willebrand factor activity is also increased, thus suggesting that the two are related.[586] The enhanced in vitro responsiveness of platelets from diabetic patients can be decreased by prostaglandin synthetase inhibitors.[583,587] Platelets from diabetic patients demonstrate increased activity of the prostaglandin synthetase system, which results in increased synthesis of prostaglandin endoperoxides and, therefore, of prostaglandin E_2.[588] When induced to aggregate with arachidonic acid, the platelets of diabetic patients with vascular disease tend to produce more thromboxane A_2 than those of normal subjects do.[589] A lower prevalence and severity of diabetic retinopathy have been observed in a group of diabetic patients with concurrent rheumatoid arthritis who were taking high doses of aspirin. This finding suggests that inhibition of platelet aggregation and prostaglandin synthesis may be desirable in the management of diabetic patients.[590] The increased glycosylation of connective tissue proteins in diabetic patients has been suggested to increase their aggregation potency.[591]

Other Hematologic Findings in Diabetes Mellitus

A state of hypercoagulability associated with changes in clotting factors and platelet function has been postulated to be important in the increased thrombotic complications in diabetic patients. Approximately a third of patients with diabetes will show abnormally increased factor VIII coagulant activity and diminished antithrombin III levels. Abnormalities in fibrinolysis do not appear to be common.[592] In addition to this, factor XI and factor XII levels are higher in some diabetic patients with evidence that the kallikrein-prekallikrein system has been activated.[593] Ketoacidosis may be associated with DIC.[594] Altered levels of factors V and VIII and diminished fibrinolytic activity have been reported in a few patients.[595]

The hematologic consequences of diabetes also extend to infants born of diabetic mothers. An increased incidence of thrombosis or thromboembolic phenomena is well recognized in these infants.[596] Complications such as renal vein thrombosis, peripheral gangrene secondary to vascular occlusion, and cerebral thrombosis may occur.[597]

METABOLIC DISEASES

Discussion in this section is restricted mainly to the hematologic consequences of the lipid disorders of metabolic origin. The hematologic findings of the other storage disorders and metabolic diseases are covered in Chapter 25 and throughout the book.

Disorders in Lipid Metabolism

Tangier Disease

Tangier disease, caused by mutations in the gene encoding ATP-binding cassette 1 *(ABCA1)*, is a rare autosomal recessive condition in which homozygotes have splenomegaly, absent or very low levels of high-density lipoproteins (HDLs), reduced levels of low-density lipoproteins (LDLs) (typically 40% of normal), hypertriglyceridemia, mild corneal clouding, peripheral neuropathy, bone marrow foam cells (70% of patients), and characteristic orange cholesterol ester–laden tonsils.[598] *ABCA1* is a major determinant of plasma HDL cholesterol and is critically involved in cellular trafficking of cholesterol and choline phospholipids. *ABCA1* mediates the first step in reverse cholesterol transport, the transfer of cholesterol and phospholipids to lipid-poor apoproteins.[599,600] Cholesterol ester levels are greatly elevated in tissue macrophages in Tangier disease owing to the lack of HDLs, which normally return cholesterol esters to the liver. The HDL deficiency is caused by hypercatabolism of the lipoprotein. Normally, HDLs bind to specific receptors on macrophages, are internalized (presumably to load up on cholesterol esters), and then recycle to the membrane surface. In Tangier disease, HDLs are misrouted to macrophage lysosomes and destroyed.

Hematologic examination of one patient with Tangier disease demonstrated stomatocytosis, hemolysis (hemoglobin concentration of 8.5 g/dL; reticulocyte count of 6 to 9%), and osmotically sensitive red blood cells.[601] Because hematologic data have rarely been reported in patients with Tangier disease, it is difficult to be certain that this patient's data are not exceptional. However, previous reports of unexplained hemolysis in three patients suggest that they are not.[602-604]

Familial Lecithin-Cholesterol Acyltransferase Deficiency

Familial lecithin-cholesterol acyltransferase (LCAT) deficiency is a rare autosomal recessive disorder characterized by anemia, corneal opacities, hyperlipemia, proteinuria, chronic nephritis, and premature atherosclerosis.[605,606] LCAT deficiency is relatively asymptomatic during childhood. Proteinuria and corneal opacities are among the earliest manifestations. The latter consist of minute grayish dots that concentrate near the edge of the cornea, resembling arcus senilis. Almost all of the reported patients have had a moderate normochromic anemia (hemoglobin level of 8 to 11 g/dL) characterized by prominent target cell formation and decreased osmotic fragility.[607,608,609]

Hematologic studies in LCAT deficiency suggest that the anemia is due to a combination of moderate hemolysis and decreased erythropoietic compensation, but interpretation of the evidence is complicated by the coexisting renal disease. Other studies have shown that the red blood cells are abnormally susceptible to peroxidative threat and have mechanically fragile membranes.[608] There is no obvious explanation for these changes, but it is possible that they contribute to the shortened red blood cell life span. LCAT deficiency is caused by a variety of mutations in the *LCAT* gene; affected individuals are either homozygotes or compound heterozygotes for these mutations.[609,610]

Modeling of the mutations has provided insight into *LCAT* structure and function.[611] As expected from the absence of LCAT activity, there is a pronounced decrease in cholesteryl esters and an increase in free cholesterol and phosphatidylcholine in plasma lipoproteins. Because LCAT is required for normal lipoprotein formation and catabolism, nascent lipoproteins and abnormal lipoprotein remnants accumulate in the plasma. One of the latter is LP-X, which is believed to be responsible for target cell formation in patients with the acquired LCAT deficiency of obstructive liver disease.[612] Free cholesterol and phosphatidylcholine levels are markedly elevated in the red blood cell membranes of patients with LCAT deficiency; however, total red blood cell phospholipid levels are normal because the increase in phosphatidylcholine is balanced by a decrease in phosphatidylethanolamine and sphingomyelin.[605] As with liver disease, red blood cell targeting and lipid abnormalities can be induced by incubation of normal red blood cells in LCAT-deficient lipoproteins and reversed by incubation in normal serum.[613] Other plasma membranes are also probably affected by the abnormal LCAT-deficient lipoproteins. For example, accumulations of lipid in endothelial membranes may underlie the atherosclerotic and nephritic complications seen in some patients. Phagocytosis of abnormal lipoproteins by reticuloendothelial cells leads to the appearance of foam cells and sea-blue histiocytes in the bone marrow, spleen, and other organs.[607,614] Serum and red blood cell lipids are improved in vivo when LCAT is supplied by infusions of normal plasma; however, the short half-life of this protein, 4 to 5 days, and the large amounts of plasma required make chronic replacement therapy impractical.

Fish-Eye Disease

Fish-eye disease is caused by partial deficiency of LCAT and is also associated with mutations in the *LCAT* gene.[605,609] It is characterized by corneal opacities (which give the disease its name), hypertriglyceridemia, and very low levels of HDLs. One report noted increased target cells and decreased osmotic fragility.[615]

Other Metabolic Disorders

Cystinosis is a rare lysosomal storage disorder associated with poor growth, proximal tubular renal Fanconi syndrome with associated electrolyte and protein loss, and complications from cystine accumulation in various organs, including the reticuloendothelial organs and corneal tissues.[616] All forms of cystinosis are caused by mutations in the *CTNS* (cystinosin, lysosomal cystine transporter) gene, with more than 80 mutations reported. Diagnosis can be established by demonstrating cystine crystals in macrophages of the bone marrow (Fig. 37-15). In rare cases, cystine can accumulate to cause bone marrow failure with pancytopenia.

Primary oxaluria, caused by overproduction of oxalate, is an autosomal recessive disorder associated with failure

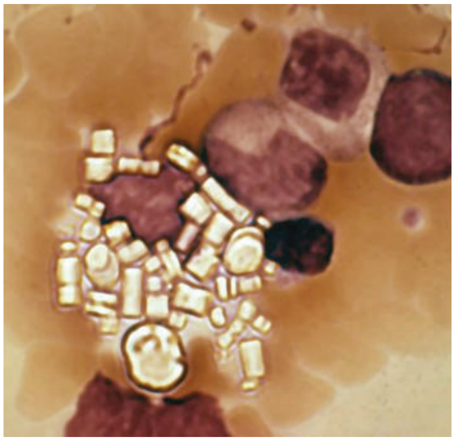

Figure 37-15 Cystine crystals in a macrophage of the bone marrow in a patient with cystinosis.

to thrive, urolithiasis, renal failure, and complications from oxalate accumulation in the joints, retina, skin, heart, and bone marrow.[617] Diagnosis can be made by measuring urinary oxalate, genetic testing, and/or bone marrow biopsy.

Osteopetrosis is a heterogenous group of disorders caused by defective osteoclastic resorption of bone with associated increased bone density. Childhood forms are typically more severe and often linked to autosomal recessive mutations in the *TNFSF11* (tumor necrosis factor [ligand] superfamily, member 11; formerly *RANKL*) gene.[618] Symptoms include fractures, short stature, pancytopenia due to bony expansion, and resultant hepatosplenomegaly from extramedullary hematopoiesis.

Essential fatty acid deficiency will result in a variety of characteristic changes in plasma lipids. Decreased prostaglandin formation in in vitro platelet studies involving animals with essential fatty acid deficiency has been reported.[619] This results in impaired platelet aggregation and may reflect a deficiency of arachidonic acid necessary for the formation of thromboxane A_2.[619] Clinically, essential fatty acid deficiency is being recognized more often as a result of prolonged fat-free parenteral nutrition.[620] Several infants have been described with hemorrhagic complications from this disorder.[619]

A child has been described with altered vitamin B_{12} metabolism in association with megaloblastic anemia and homocystinuria secondary to a defect in methionine biosynthesis.[621] Homocystinuria and megaloblastic anemia responsive to vitamin B_{12} therapy have also been reported as an inborn error of metabolism caused by a defect in cobalamin metabolism.[622] Infants and children with orotic aciduria, hyperglycinemia, and hyperglycinuria may have neutropenia.[623] Patients with orotic aciduria may also exhibit megaloblastic changes in bone marrow and have macrocytic indices.

NEUROLOGIC AND PSYCHIATRIC DISORDERS

Discussion in this section relates to the hematologic findings in the neuroacanthocytoses and anorexia nervosa. Neuroacanthocytosis refers to a group of diseases in which neurologic changes are found in the setting of acanthocytes in the peripheral blood smear.[624,625] In some cases of neuroacanthocytosis, acanthocytes are not detected on the peripheral smear. However, because routine genetic testing and classic laboratory findings are not available for most of these disorders, the detection of acanthocytes is one of the hallmarks of clinical diagnosis. The most sensitive and specific method for measuring acanthocytes is using isotonically diluted blood combined with unfixed wet blood preparation with the normal range of less than 6.3% acanthocytes of the total erythrocytes.[626] The mechanism of acanthocyte formation in the individual subsets of the neuroacanthocytoses is related to specific membrane protein defects, including band 3 and the XK protein of the 4.1 multiprotein complex.[627,628,628a] The subsets of neuroacanthocytoses are abetalipoproteinemia, McLeod syndrome (reviewed in Chapter 36), chorea-acanthocytosis, Huntington disease–like 2, and pantothenate kinase–associated neurodegeneration.

Neuroacanthocytoses

Abetalipoproteinemia

Abetalipoproteinemia (Bassen-Kornzweig syndrome) is an autosomal recessive disorder that causes a failure to synthesize or secrete serum apolipoprotein B–containing lipoproteins. With the lack of apolipoprotein B there is defective intestinal absorption of lipids and extremely low serum cholesterol and triglyceride levels.[629] Owing to deficient absorption of fat-soluble vitamin E, individuals with abetalipoproteinemia develop severe, but often reversible, neurologic problems, such as spinocerebellar degenerative ataxic disorder with peripheral neuropathy and retinitis pigmentosa leading to blindness.[630,631] These findings are also associated with the presence of acanthocytic red blood cells. The cholesterol content of the red blood cell is normal or slightly increased, whereas the phospholipid content reflects that of serum.[629] Autohemolysis[632] and peroxidative hemolysis[633] may be increased, but osmotic fragility[632] and rates of glycolysis[632] are normal. Anemia, if present, is mild.

This rare disease is caused by various mutations in the microsomal triglyceride transfer protein gene *(MTTP)*, which catalyzes the transport of triglyceride, cholesterol ester, and phospholipid from phospholipid surfaces.[634,635] MTTP forms a heterodimer with a multifunctional protein called disulfide isomerase, which is located in the lumen of hepatic microsomes and intestinal epithelia, the sites of lipoprotein synthesis.[636-638] Disulfide isomerase is the only tissue-specific component, other than apolipoprotein B, required for secretion of apolipoprotein B–containing lipoproteins.[639]

Related entities (hypobetalipoproteinemia, normotriglyceridemic abetalipoproteinemia, and chylomicron retention disease) exist that are associated with partial production of apolipoprotein B–containing lipoproteins or with secretion of lipoproteins containing truncated forms of apolipoprotein B. Patients with these diseases may also manifest acanthocytosis and neurologic disease, depending on the severity of the lipoprotein defect. The majority of patients with heterozygous hypobetalipoproteinemia do not have acanthocytosis.

Characteristically, 50% to 90% of the red blood cells are acanthocytes (Fig. 37-16).[640] The shape defect is not evident in nucleated red blood cells or reticulocytes and worsens as the erythrocytes age. Membrane protein composition is normal, but membrane lipid composition is not. Phosphatidylcholine concentration is decreased by about 20%, and the amount of sphingomyelin is correspondingly increased. The cholesterol-to-phospholipid ratio is normal to mildly elevated.[641-643] These changes reflect abnormalities in the distribution of plasma phospholipids and a decrease in LCAT activity.[644] The relative increases in cholesterol and sphingomyelin concentration both decrease lipid fluidity, particularly in the outer one half of the bilayer, and presumably contribute to the acanthocytic shape.[642]

Because of fat malabsorption and the absence of LDLs, which transport vitamin E, the red blood cells of these patients are markedly deficient in vitamin E.[645,646] Oxidant sensitivity can be prevented by treatment with a

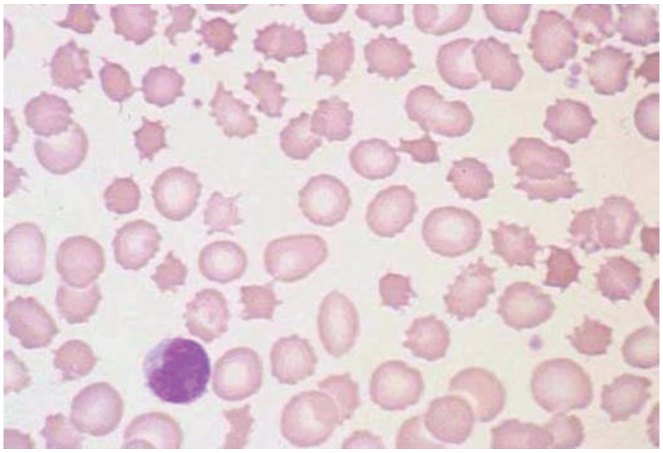

Figure 37-16 Acanthocytosis in abetalipoproteinemia.

water-soluble form of vitamin E (e.g., D-α-tocopherol polyethylene glycol succinate).[646] Despite increased lipid viscosity and vitamin E deficiency, the hemolysis experienced by these patients is mild.[641]

Patients with familial hyperbetalipoproteinemia (type II hyperlipoproteinemia) have abnormal platelet function[647] characterized by increased sensitivity to aggregating agents and release of increased amounts of nucleotides in response to aggregating agents. These changes are associated with platelet plasma membrane ultrastructural changes, particularly in type IIa hypercholesterolemia.[648] Such subjects may truly demonstrate a "hypercoagulable state." The latter has been demonstrated in severely hypertriglyceridemic conditions.[649] These findings suggest the possibility that platelet function may be involved in the thrombotic complications of familial hyperbetalipoproteinemia.

Chorea-Acanthocytosis

This syndrome was first described by Estes and colleagues.[650,651] Since then, this autosomal recessive disorder has been well characterized as a progressive movement disorder with acanthocytosis, myopathy, and cognitive and behavior changes beginning in adolescence or adult life (8 to 62 years).[652] The movement disorder is most typically characterized by limb chorea, but also can present as parkinsonism, orofacial dyskinesia, lip and tongue biting, involuntary vocalizations leading to mutism, seizures, and decreased or absent tendon reflexes.[653,654] The lingual-buccal-facial dyskinesia and oral self-mutilation are most characteristic of chorea-acanthocytosis. The myopathy causes distal muscle wasting and hypotonia, as well as increased levels of creatine phosphokinase. The extent of mental deterioration varies but often resembles that of the frontal lobe syndrome.[655]

The mutant protein in chorea-acanthocytosis, chorein, encoded by the vacuolar protein sorting 13 homolog A gene *(VPS13A)* on chromosome 9q21, has been identified and over 70 different mutations have been reported.[656-659] The function of chorein is unknown, but *VPS13A* is a member of a conserved gene family involved in membrane protein trafficking. The loss of chorein may be diagnostic for this disorder, because it is not seen in similar disorders, such as Huntington disease or McLeod syndrome.[658,660] Genetic testing is commercially available but is difficult owing to the large gene size and the heterogeneity of mutations. The diagnosis of this disorder is still often made through radiologic and clinical findings, as well as evidence of muscle disease. Magnetic resonance imaging (MRI) and pathologic examinations show atrophy of the basal ganglia, particularly the caudate and putamen. In some patients, little or no hemolysis occurs; in others, acanthocytosis precedes the onset of neurologic symptoms.[655] In others, acanthocytes do not appear until late in the disease course.[661] In some patients, splenomegaly is noted, likely owing to hemolysis.[661] Multiple red cell alterations have been described in the acanthocytes of patients with Choreacanthocytosis.[628a] On electronic microscopy, these red cells have a heterogeneous distribution of membrane skeleton. An increased amount of gamma-glutamyl lysine isopeptide is associated with the membrane in some patients. Choreacanthocytic red cells have a perturbation of the skeletal network, which may lead to the instability in bridging band 3 protein complexes in the absence of chorein. These red cells have a reduced response to drugs, inducing endovesicles.[628b]

Of note, one family has been described with a defect near the COOH-terminal end of band 3, Pro868Leu.[662] Hematologically, affected individuals of this kindred have 20% to 25% acanthocytes and mild anemia with reticulocytosis. However, this family's defect seems to be a distinct disorder separate from the more typically described chorea-acanthocytosis.

Huntington Disease–like 2

In 2001, Margolis and associates described a pedigree with an autosomal dominant, Huntington-like disease due to CAG/CTG repeats in a novel site.[663] Huntington disease–like 2 has since been characterized as a hyperkinetic disorder similar to Huntington disease that mostly or exclusively affects patients of African ancestry. This neuroacanthocytosis typically is manifested in the third decade by weight loss, dystonia, rigidity, chorea, behavioral changes, and acanthocytosis.[664,665] Acanthocytosis in this disorder may be related to involvement of small G proteins in membrane skeletal rearrangement.[628a] Brain imaging demonstrates marked striatal atrophy and moderate cortical atrophy, similar to Huntington disease. The CTG/CAG trinucleotide repeat expansion has been found within a variably spliced exon on the junctophilin 3 gene *(JPH3)* on chromosome 16q24.3.[666]

Pantothenate Kinase–Associated Neurodegeneration

This autosomal recessive progressive movement disorder is caused by a mutation in the pantothenate kinase 2 gene *(PANK2)* on chromosome 20p13.[667,668] In the classic form of this neuroacanthocytosis, patients present within the first decade of life with a rapidly progressive motor disease leading to loss of ambulation. The atypical and intermediate forms present later in life and progress more gradually. Individuals with an earlier onset of symptoms typically develop pigmented retinopathy. Those with a later onset tend to also develop psychiatric and speech disorders. The motor disease is characterized by rigidity in the lower and, later, the upper extremities. Choreic type movements often coincide with the rigidity. Owing to the effects on voluntary movement and muscular tone, patients also develop difficulty chewing and swallowing.[669] Ten percent of patients have acanthocytes on the peripheral smear.

The diagnosis of this disorder is made clinically and radiologically. Brain MRI pallidal abnormalities, described as the "eye of the tiger" sign, consist of decreased signal intensity in T2-weighted images, compatible with iron deposits, and of a small area of hyperintensity in the internal segment.[670] All patients with this disorder have the "eye of the tiger" sign on brain MRI.[671,672] However, this sign is not specific only to pantothenate kinase–associated neurodegeneration.[671,673] Historically, this disease was referred to as Hallervorden-Spatz disease, named after Julius Hallervorden and Hugo Spatz who originally described this disorder. In 2001, Zhou and colleagues suggested this disorder be referred to as pantothenase kinase–associated neurogeneration to avoid the objection to its eponym, because Hallervorden and Spatz were able to make their original descriptions of this disorder by actively participating in the euthanasia program in Germany during World War II.[668]

Anorexia Nervosa

Patients with anorexia nervosa may demonstrate many of the hematologic manifestations of severe malnutrition with concomitant vitamin B_{12}, folate, and iron deficiency as well as more rare nutritional deficiencies. Normochromic, normocytic anemia will be present in 20% to 30% of patients with anorexia.[674] Acanthocytosis is common in patients with severe anorexia nervosa (Fig. 37-17).[674,675] The cause of the acanthocytosis is unknown. Plasma lipid and LDL levels are typically normal or slightly decreased. However, even in those patients with low levels of LDLs, acanthocytosis may not be due solely to the lipoprotein deficiency, because patients with much lower concentrations of LDLs (e.g., those with hypobetalipoproteinemia) usually have normal red blood cell morphology.[676] Red blood cell life span is normal or only slightly shortened. Severe starvation or malnutrition due to causes other than anorexia nervosa can also produce acanthocytosis or target cells and hypobetalipoproteinemia.[677]

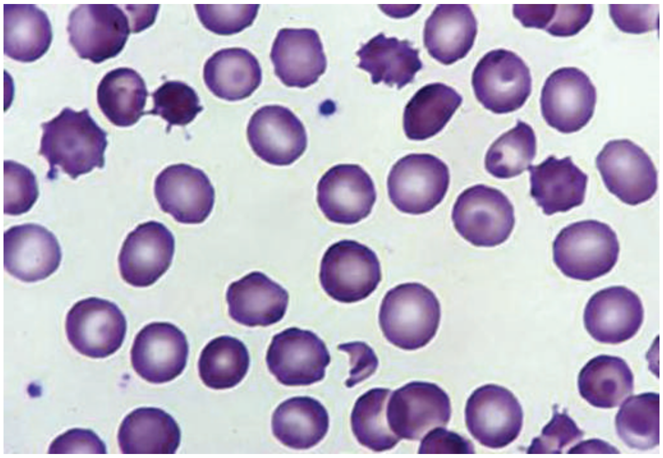

Figure 37-17 Acanthocytosis in anorexia nervosa.

Leukopenia and neutropenia are common, and this may be associated with an increased risk for infection.[678] Leukocyte response to bacterial infection may be suboptimal. Mild thrombocytopenia is often seen.[674,675,679-681] A marked increase in platelet hyperaggregability may be found.[682] In addition to specific nutrient deficiencies, cytopenias can also be due to a hypoplastic bone marrow filled with gelatinous material.[683,684] Several months may be needed for the blood cell abnormalities in anorexia nervosa to resolve after adequate caloric intake.

CUTANEOUS DISEASES

Skin diseases are only rarely a direct cause of hematologic disturbances. More often than not, the hematologic alterations reflect a simultaneous disturbance in more than one developmental system, such as the skin and blood. Certain disorders that affect the hematologic system and the skin, such as Wiskott-Aldrich syndrome, are described in Chapter 24.

Eczema and Psoriasis

Patients with extensive eczema or psoriasis commonly have a mild anemia.[685] The anemia is usually normochromic and normocytic, although microcytosis may occur. Typically, this anemia is associated with a low serum iron level and normal or decreased iron-binding capacity and best classified as an anemia of chronic disease.[685] A defect in red blood cell deformability in eczematous patients has been reported.[686] Some individuals with extensive rash will have greatly expanded whole blood and plasma volumes, which may result in a dilutional anemia.[687,688] In rare patients with eczema or psoriasis, long-standing steatorrhea may develop and cause folate malabsorption and macrocytic anemia.[689] Psoriasis may be a widespread membrane disorder. Alterations in red blood cell sodium and potassium fluxes are seen, as well as alterations in membrane lipid composition (an increase in arachidonic acid and unsaturated fatty acid content).[690,691] The increased erythrocyte membrane arachidonate and platelet malondialdehyde production noted in patients with psoriasis may normalize after treatment with fish oil.[692]

Hereditary Hemorrhagic Telangiectasia

This inherited structural abnormality of the vasculature is characterized by mucocutaneous telangiectases caused by localized dilation and convolution of venules and capillaries. It is an autosomal dominant disorder that results in a bleeding tendency because of the friable blood vessels. Telangiectases predominate on the lips, buccal mucosa, gingivae, palate, tongue, and skin of the face and upper parts of the body and may be present in visceral organs as well. Pulmonary arteriovenous fistulas are not rare but tend to appear later in life. Easy bruisability, epistaxis, and respiratory and gastrointestinal bleeding may be caused by these telangiectases.

Mast Cell Disease

In mast cell disease, large numbers of mast cells are found, often diffusely located in the skin or the gastrointestinal tract.[693] These cells periodically release histamine and heparin-like substances.[693,694] Trauma to involved areas may trigger release of these substances and may also cause urticaria and blistering of affected parts of the body. A bleeding diathesis can be caused by mast cell disease due to an increased systemic heparin concentration and/or an increased fibrinolysis by tissue plasminogen activator release.[695] Because fatal hemorrhage may result from this excessive anticoagulant effect, treatment with protamine sulfate may be necessary.

Systemic mast cell activation can occur with systemic mastocytosis, in which clonal CD25 positive mast cells typically carry an activating *KIT* point mutation D816V.[696] In systemic disease, mast cells may infiltrate the bone marrow and cause cytopenias. *KIT* D816V can also be expressed in mast cells in patient with cutaneous mastocytosis. Serum tryptase is often elevated in these conditions.

Urticaria pigmentosa is one form of mast cell disease. On rare occasion, mast cell disease and urticaria pigmentosa precede the onset of mast cell leukemia.[697]

PREGNANCY

Pregnancy, with a reorientation of the mother's physiology to support the expanding growth of the fetus, is frequently complicated by hematologic disorders. Some of these arise from the progressive hypercoagulable state of pregnancy,[698] in preparation for placental separation, whereas other disorders result from pathologic initiation of the clotting cascade secondary to the release of placental, amniotic, or fetal elements.[699] Antecedent hematologic disorders are compounded by the demands of pregnancy and may be exaggerated in severity and impact because of their dual effect on mother and fetus. Management of these conditions must also take into account the effect that any hematologic defects or medications used to reverse these defects may have on the fetus. A detailed review of the hematologic manifestations of pregnancy is beyond the scope of this pediatric textbook, but a broad overview of the types of hematologic findings is provided in this section.

Anemia of Pregnancy

During the first and second trimesters of pregnancy, a physiologic expansion of the mother's plasma volume occurs, which outdistances a concomitant increase in the red blood cell mass.[699] The plasma volume starts to increase at about 6 weeks and reaches about 1250 mL at term, an increase of about 50% above the nonpregnant state.[700] The red blood cell mass increases about 20%, or 250 mL, without iron supplementation and may reach 400 mL with iron supplements. This increase in plasma volume results in a reduction in the normal woman's hemoglobin and hematocrit. The hemoglobin nadir is 11.4 ± 0.8 at 25 to 26 weeks of gestation, which makes the minimum value (–2 SD) 9.8 g/dL.[701] In general, the minimum hemoglobin value is 11 g/dL in the first trimester and 10 g/dL in the next two trimesters.[702,703]

A number of studies show a relation between prematurity, low birth weight, and maternal anemia.[704-709] One large epidemiologic study found that the risk for premature delivery increased by 20% in pregnancies in which the hemoglobin value is between 10 and 11 g/dL and by 60% when it was in the 9- to 10-g/dL range.[707] Below 9 g/dL the risk doubled and tripled for each additional decrease of 1 g/dL. Another study found that perinatal mortality tripled when the mother's hemoglobin level decreased below 8 g/dL.[709]

On the other hand, there is evidence that the normal expansion of maternal plasma volume is needed for an optimal pregnancy outcome.[710] Several studies demonstrate a positive correlation between the weight of the neonate and the increase in plasma volume within the normal range.[710-714] Whether the increase is needed for optimal fetal oxygenation and growth or whether normal fetal growth somehow triggers the increase is not known. However, high hemoglobin levels portend pregnancy complications, such as fetal growth restriction, premature birth, pregnancy-induced hypertension, and perinatal fetal distress or death.[715-718] For example, the frequency of hypertension in primiparas with hemoglobin levels less than 10.5 g/dL is only 7% but rises to 42% when the hemoglobin concentration is more than 14.5 g/dL.[710]

The surge in red blood cell production during pregnancy, even though less than the plasma volume, often outstrips the marginal iron and folate stores of the expectant mother. Iron deficiency accounts for about 75% of the anemias in women who do not take iron supplements. It is estimated that about 80% of pregnant women who do not take iron supplements will have hemoglobin values less than 11 g/dL at term.[719] The consequences to the fetus are less certain. Some investigators observe a direct relationship between maternal and fetal iron levels; others find that the level of iron in the mother has little effect on that in the neonate.[720,721] The fetal demands seem to dominate. Mild to moderate iron-deficiency anemia in the mother does not cause significant anemia in the fetus.[720]

Folic acid deficiency is estimated to exist at term in 60% to 95% of pregnant women who have not taken folate supplementation,[719] although frank megaloblastic anemia is uncommon. Folate deficiency predisposes to devastating defects in closure of the neural tube and thus in spinal cord development,[722-724] but folate supplementation to prevent this malformation must be given before conception because neural tube closure occurs during the fourth week of embryogenesis. The risk for neural tube defects is higher in mothers with the common thermolabile variant of 5,10-methlyenetetrahydrofolate reductase,[725] which impairs enzyme activity and raises blood levels of homocysteine.

Low values of serum iron (<30 μg/dL) and ferritin (normal values in pregnancy 55 to 70 μg/L) are reliable indicators of iron deficiency.[702] Reliance on a low mean corpuscular volume (MCV) measurement may be misleading during pregnancy because a combined deficiency may cancel out the extremes of iron (low MCV) and folate (high MCV) deficiency.[726] To avoid the development of anemia secondary to these deficiencies, it is standard practice to supplement all pregnant women with prenatal vitamins with iron and folate. For those women who cannot tolerate iron, monitoring of serum ferritin permits assessment of iron stores and the specific need for iron supplementation.[727]

Other causes of anemia during pregnancy are much rarer. An aplastic anemia that resolves with abortion of the fetus and recurs with subsequent pregnancies is presumed to have an immunologic basis when it occurs during pregnancy.[728] The devastating anemia secondary to intravascular hemolysis that is associated with clostridial sepsis may be a lethal complication of septic abortions.[729]

Thrombocytopenia of Pregnancy

With its potential to exaggerate bleeding during the birth process, thrombocytopenia needs careful assessment and management in the pregnant woman.[730] Thrombocytopenia, with a platelet count less than 100 kcells/μL is observed in 1% of pregnant women.[731] The incidence of all of the classic thromboconsumptive processes of ITP, TTP, and DIC is increased during pregnancy and is joined by platelet abnormalities as a component of eclampsia of pregnancy.[732] The diagnosis of ITP during pregnancy requires the same constellation of findings as that in the nonpregnant state.[731,733] Because the antibodies in ITP cross the placenta and may create thrombocytopenia in the infant, this condition can create problems for the mother and fetus.[734]

The mechanism of thrombocytopenia in eclampsia is not certain.[735] The fact that the low-dose aspirin prevents the perinatal consequences of preeclampsia in high-risk mothers[736,737] suggests a platelet-endothelial defect may underlie the disease. The spectrum of toxemia has been extended to include the HELLP syndrome (*h*emolysis, *e*levated *l*iver enzymes, and *l*ow *p*latelets), which may or may not be accompanied by the more classic evidence of toxemia.[738-740] The disease occurs in 0.2% to 0.9% of pregnancies, typically at between 32 and 34 weeks of gestation, although it occurs after delivery in 30%.[740] The cardinal clinical symptom is right upper quadrant or epigastric pain with nausea, vomiting, or malaise. The disease often progresses to a life-threatening state. Management of the hematologic problems of toxemia and HELLP syndrome entails management of the toxemia state, which frequently requires delivery of the infant if the maturity of the fetus allows.

Disseminated Intravascular Coagulation and Pregnancy

Because the products of conception can initiate the coagulation cascade when released into the maternal circulation, the course of pregnancy and delivery are not infrequently accompanied by DIC. DIC is covered in detail in Chapter 35. Fetal death in utero,[741] abruptio placentae,[742,743] and amniotic fluid embolism[744] all are often signaled by bleeding or coagulation abnormalities. Management of these abnormalities requires delivery of the fetus while providing blood component therapy with platelets, fresh-frozen plasma, and cryoprecipitate to replace the consumed elements. Amniotic fluid embolism has the especially threatening complication of cardiopulmonary collapse, which requires cardiopulmonary support and corticosteroids in addition to blood product administration.[745] DIC may also accompany the severe hemolytic anemia that occurs when clostridial sepsis follows an abortion.

Sickle Cell Disease and Pregnancy

The management of sickle cell during pregnancy is covered in Chapter 20.

Inherited Bleeding Disorders and Pregnancy

Neither of the classic hemophilic syndromes complicates pregnancy because of their X-linked mode of inheritance. von Willebrand disease does require recognition, although the incidence of bleeding secondary to this problem in pregnancy is mitigated by the physiologic increase in factor VIII and von Willebrand factor during pregnancy. Treatment of bleeding due to von Willebrand disease consists in the use of von Willebrand factor concentrate or, for short-term control of bleeding, infusions of desmopressin.[746] The latter may normalize von Willebrand factor levels in the various subgroups of von Willebrand disease, although its use in type IIB disease may lead to thrombocytopenia owing to release of abnormal von Willebrand factor in this group.[747] Dysfibrinogenemias and factor XIII deficiency are both associated with spontaneous abortions and abnormal bleeding; plasma infusions throughout pregnancy may be successful in averting complications in these groups.[748]

Pregnancy and Thrombosis

Pregnancy represents a progressive hypercoagulable state during which a marked increase in the incidence of venous thrombosis is seen.[749,750] Venous thromboembolism is the leading cause of maternal mortality during pregnancy in Western countries. Low-molecular-weight heparin is used to prevent and/or treat thrombosis in pregnancy.[751,752] The drug is discontinued at the time of delivery but reinitiated 24 hours after delivery because of the ongoing risk for thrombosis that persists for 8 to 12 weeks after delivery. Warfarin (Coumadin) is avoided during pregnancy, especially during the first trimester, because of the risk for an embryopathy. Heparin escapes this complication because it does not cross the placenta, although it is associated with the threat of bleeding and fetal loss. Either can be used in the postpartum period because neither drug creates problems for the nursing mother or infant.[753,754]

Inherited Hypercoagulable States and Pregnancy

The inherited thrombophilic disorders are covered in Chapter 33. Women who inherit defects in these proteins are more likely to experience fetal loss, particularly stillbirths, than controls.[755] The risks are greatest in antithrombin III deficiency and in women with more than one inherited risk factor. These defects also appear to play a role in other gestational vascular complications, including fetal growth restriction, preeclampsia, and placental abruption.[755,756] Many women with a personal history of venous thrombosis, three unexplained miscarriages, abruptio placentae, stillbirths, or recurrent fetal growth restriction are screened for inherited thrombophilias. Current guidelines suggest that women with recurrent early pregnancy loss should be screened for antiphospholipid antibodies, but women with a history of other pregnancy complications but no history of thrombosis should not be screened for an inherited thrombophilia.[752]

Antiphospholipid Antibody Syndrome and Pregnancy

Antiphospholipid antibodies and the antiphospholipid antibody syndrome are discussed in Chapters 29 and 35. In addition to the risk for venous and arterial thrombosis, pregnant women with antiphospholipid antibodies have an increased risk for fetal loss, premature delivery, preeclampsia, and uteroplacental insufficiency.[757] The risk to the pregnancy involves all periods: preembryonic (<6 weeks), embryonic (6 to 9 weeks), and fetal (≥10 weeks). The mechanisms are not well defined but may include antiphospholipid antibodies interfering with binding of the protective anticoagulant annexin V to phospholipid surfaces on endothelial cells and trophoblasts.[758]

Prospective trials show that heparin plus low-dose aspirin is more effective than aspirin alone in achieving live births in pregnant women with antiphospholipid antibodies and predominantly preembryonic and embryonic loss.[752,759] Doses of heparin sufficient to produce full anticoagulation are recommended for all women with antiphospholipid antibodies and prior thromboembolism.

TOXINS AND OTHER INSULTS

Toxins, venoms, and thermal injury can cause marked disturbances in hematopoiesis. The most common and extensively described toxin is alcohol. The hematologic complications of snake bites are covered in Chapter 38.

Alcohol and Hematopoiesis

Significant hematologic damage is incurred by alcoholics because of the combined direct toxicity of alcohol on the marrow and the multiple associated abnormalities in the nutritional and gastrointestinal systems that exist in the alcoholic.[760] Alcohol supplies empty calories for nutrition and is also frequently accompanied by multivitamin deficiencies; alcohol-induced gastritis is threatened with a double jeopardy for bleeding imposed by the combination of thrombocytopenia and a qualitative platelet dysfunction created by the toxin.[761] Congestive splenomegaly, as an accompaniment to both acute alcoholic fatty liver and chronic cirrhosis, may further deplete the red blood cell pool. These myriad potential pathologic contributions of ethanol to hematopoiesis and its environment readily explain the frequent occurrence of anemia in about 50% of patients hospitalized with acute or chronic alcoholism.[762]

Alcohol and the Red Blood Cell

Heavy alcohol ingestion of over 1 week's duration produces a characteristic vacuolization of the red blood cell precursors in the marrow, a finding shared with chloramphenicol toxicity, phenylalanine deficiency, and hyperosmolar states (Fig. 37-18).[763] More chronic ingestion of lesser amounts of alcohol results in a mild macrocytosis unaccompanied by any megaloblastic features in the marrow.[764]

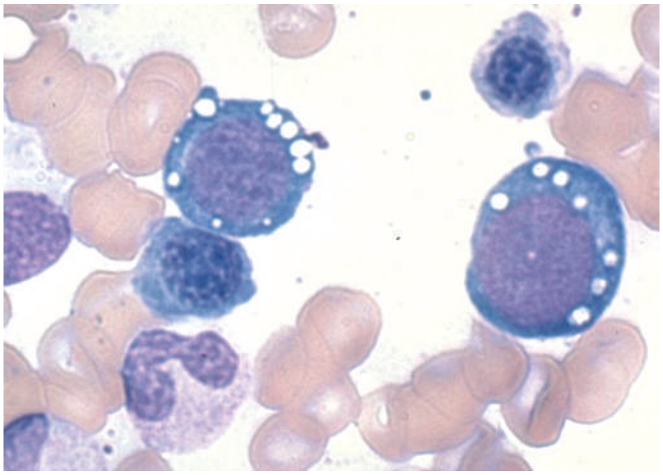

Figure 37-18 Vacuolated normoblasts in the bone marrow of a patient with alcoholism.

Evaluations of adults with macrocytosis (MCV >100 fL) show that alcohol is one of the most common causes, along with drugs and liver disease, especially alcoholic liver disease.[765,766] Surprisingly, only about 10% of patients with macrocytosis have megaloblastic anemias, although the fraction increases greatly at higher MCVs (>120 fL). In fact, a high or high normal MCV (>94 fL) is one of the best screening tests for identifying hazardous drinkers in general practices.[767]

The vacuolization of normoblasts is short-lived (3 to 5 days) after ethanol withdrawal, whereas the macrocytosis of alcoholism persists for 1 to 2 weeks after abstinence from the toxin.[768] The mechanism for the production of these abnormalities is not well established. Vacuolization may just be a nonspecific sign of injury. It occurs with chloramphenicol toxicity and in diseases associated with erythroblast damage, such as parvovirus B19 infection.

Macrocytosis can also be due to folic acid deficiency, which is common in alcoholics, but macrocytosis may also be a consequence of bone marrow toxicity by alcohol, because it occurs in response to any reversible bone marrow injury. During recovery from such damage, the bone marrow releases "stress" erythrocytes, which have some of the characteristics of fetal red blood cells, including large size, increased fetal hemoglobin, and the i antigen. Finally, hemolysis and bleeding must always be considered in the evaluation of macrocytosis, because reticulocytes have a much higher MCV than mature erythrocytes.

Acquired stomatocytosis is another red blood cell abnormality that is common in alcoholics, particularly in those with acute alcoholism, and it may be associated with moderate hemolysis.[769] In one study, 15% of alcoholic patients had marked stomatocytosis (≥10% stomatocytes) and 29% of the patients had 3% to 9% stomatocytes.[770] The MCV was significantly higher in these patients.

Ethanol is also a direct toxin to hematopoietic cells with evidence that ethanol and its breakdown product, acetaldehyde, both inhibit erythroid marrow colony growth in vitro.[771] A further insult to erythropoiesis is produced by a direct antifolate effect of alcohol on the marrow; this compounds an already reduced availability of folate due to alcohol's interference with the normal enterohepatic recirculation of folate and decreased amounts of the active congener of folate, methyl tetrahydrofolate.[772,773] The sum of these insults to folate metabolism is escalated further by the simultaneous presence of folate deficiency as a result of a poor diet, producing megaloblastic anemia as the most common form of anemia in alcoholics.[774]

The megaloblastic transformation of the marrow due to folate deficiency may not be accompanied by the anticipated striking macrocytosis in the peripheral blood because the condition is often compounded by iron deficiency. This frequently produces a dimorphic red blood cell picture in which there exists competing contributions of both deficiencies to red blood cell size. Hypersegmentation of the polymorphonuclear cells still remains as a marker of megaloblastic hematopoiesis, an important clue when macrocytosis has been blunted by iron deficiency.[775] Measurement of red blood cell folate confirms the deficiency, one that may occur in the course of only a few weeks of a folate-deficient diet.

Megaloblastic transformation of the marrow is frequently further complicated by ringed sideroblast formation in which iron accumulates in and around altered mitochondria in red blood cell precursors.[776] This iron fails to be normally incorporated into heme, and the red blood cell products of this pathway are hypochromic in the presence of iron-overloaded mitochondria. The defect originates in alcohol's direct interference in protoporphyrin ring synthesis or in a toxic effect of alcohol on the mitochondria. These insults to normal hemoglobin production and the ineffective hematopoiesis characteristic of megaloblastic lesions alter iron kinetics within the body.

After alcohol intake has been discontinued and nutritional supplements provided, the megaloblastic component of the anemia is rapidly reversed. Serum iron levels plummet as normal erythropoiesis supervenes. Sideroblastic features persist for 7 to 10 days, a more prolonged aftermath of alcohol toxicity. The marrow, freed from alcohol's suppressive effect, responds with a reticulocytosis that has frequently been misinterpreted as evidence for hemolysis in the jaundiced, anemic drinker. The finding of reticulocytosis in alcoholics with fatty liver and hypertriglyceridemia became the triad of Zieve syndrome.[777] The major component of the reticulocytosis represents the return of a marrow formerly suffering the dual effects of alcohol suppression and vitamin deficiencies.

Alcohol and the White Blood Cell

Leukopenia may be caused by an ethanol effect on the marrow independent of any megaloblastic interference with hematopoiesis. Direct marrow suppression of granulopoiesis by alcohol is accompanied by inhibition of release of colony-stimulating factors by T lymphocytes.[778,779] This may contribute to the high morbidity and mortality rates associated with pneumococcal sepsis in the alcoholic with leukopenia.[780]

The alcoholic is further handicapped as an infected host because of diminished adherence, chemotaxis, and motility of granulocytes in response to infection.[781] Macrophage function is impaired with decreased motility and phagocytic performance.[782,783] Immune defenses are also diminished. Both lymphopenia and inhibition of lymphocyte transformation are common in alcoholism.[784] The ability to respond with delayed hypersensitivity to new antigens is also compromised.[785] The mechanism of alcohol-related functional defects of white blood cells is not established, although a raised intracellular concentration of cyclic adenosine monophosphate in the presence of ethanol has been suggested as the cause.[786]

Alcohol and Platelets

Both platelet number and function are adversely affected by alcohol in the absence of any supplementary contribution of vitamin deficiency or hypersplenism. The thrombocytopenia originates in a modest shortening of platelet survival combined with a more significant impairment of megakaryocytopoiesis. In vivo and in vitro experiments confirm that the number and proliferation of megakaryocytes are not altered in the presence of ethanol.[787] The lesion created by the agent appears at the later stages of the megakaryocyte maturation, with inhibition of platelet production. Withdrawal of ethanol is followed within 3 to 5 days by a release of platelets, with levels rebounding to thrombocythemic levels before returning to normal levels in 1 to 3 weeks.[788] Alcohol suppression of thrombopoiesis may be severe enough to result in bleeding, especially when other defects such as gastritis already exist in the patient.

Qualitative platelet abnormalities are also induced by ethanol. With moderate doses (more than 50 g) of ethanol, no direct effect is seen on bleeding time, although this amount of ethanol significantly potentiates prolongation of the bleeding time induced by aspirin and nonsteroidal analgesics. Larger ethanol ingestion results in prolongation of the bleeding time even in the absence of any thrombocytopenia.[789] Defects in platelet aggregation, thromboxane release, and platelet factor 3 availability occur on exposure of platelets to alcohol. These qualitative defects may be secondary to direct toxic effects of ethanol on the platelet membrane in addition to any contribution from an increased plasma osmolality in the presence of ethanol.

Thermal Injury

More than 140 years ago Schultze observed that red blood cells heated to temperatures approaching 50° C for short periods developed membrane budding, fragmentation, and microspherocytosis.[790] This phenomenon was further defined by the careful studies of Ham and colleagues and others and is now believed to be due to heat denaturation of spectrin or other membrane proteins. Similar changes are observed acutely in patients with major cutaneous burns, presumably because of heat exposure of red blood cells in the skin and subcutaneous

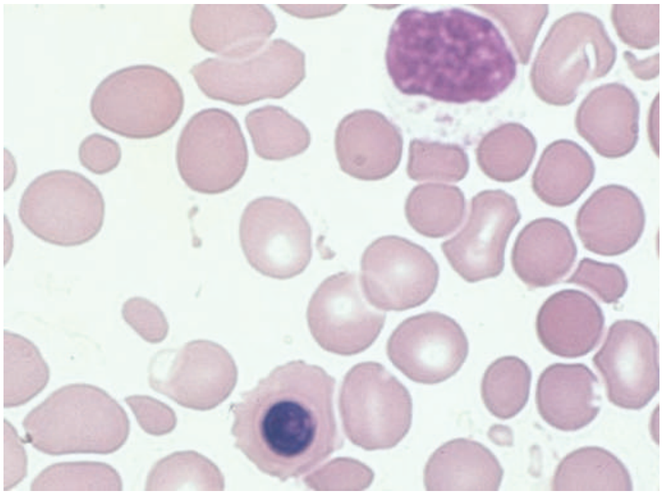

Figure 37-19 Spherocytes, red blood cell fragmentation, and a nucleated red blood cell in a patient with burn-associated hemolysis.

tissues. During the first 24 to 48 hours after the burn, intravascular hemolysis develops, which is associated with red blood cell fragmentation and spherocytosis (Fig. 37-19).[791,792] The severity of the reaction is related to the extent and degree of the burn. Generally, hemolysis is evident in patients with third-degree burns involving 15% to 20% or more of the body surface area.[791,793] In severely burned patients, up to 30% of the red blood cell mass may be destroyed. This acute hemolytic process is usually complete by the third day after the burn and is followed by a chronic anemia resembling that seen in various chronic disorders.[794,795] An unusual variant of this disorder has been reported after infusion of red blood cells that were inadvertently overheated in a blood warmer.[796,797]

Venoms

The venoms of cobras and certain vipers and rattlesnakes may produce severe hemolysis and spherocytosis.[798-802] Spherocytic hemolytic anemia may also be seen in patients bitten by the common brown spiders (*Loxosceles reclusus* and *Loxosceles lata*) of South America and the central and southern sections of the United States[803,804] and in individuals with massive numbers of stings of honeybees, wasps, or yellow jackets.[804-807] Hemolysis in a child stung by a Portuguese man-of-war jellyfish has also been reported.[808] A detailed review of the hematologic manifestations after snake bites can be found in Chapter 38.

A full description of the numerous toxins in these venoms and their mechanisms of action is beyond the scope of this chapter. In general, the mechanisms of hemolysis after envenomation are not fully understood. The venoms of snakes and insects often contain phospholipases (particularly phospholipase A_2) and protein toxins that enhance their action.[809] Examples of toxins include the mastoparans of wasp and hornet venoms and melittin, a polypeptide contained in honeybee *(Apis mellifera)* venom that immobilizes and clusters erythrocyte band 3, producing protein-free areas of the lipid bilayer that are susceptible to phospholipase action.[810-816]

Hemolysis after brown recluse spider bites is characteristically delayed from 1 to 5 days and is caused by a different mechanism.[817] Affected patients develop a transient, spherocytic, hemolytic anemia that produces positive Coombs test results with erythrophagocytosis and IgG and C3 deposition on the red blood cell surface.[818-821] Venom from the brown recluse spider induces activation of an endogenous metalloproteinase, resulting in glycophorin cleavage from the erythrocyte membrane that facilitates the complement-mediated hemolysis.[822-824] The hemolysis usually subsides within a week, but it may be fatal. Complement appears to play a critical role because complement-deficient guinea pigs are resistant to the venom.[825] In vitro models demonstrate that the complement-mediated hemolysis, after exposure to this venom, may be partially inhibited by treatment with complement inhibitor therapy.[825a] The glycoprotein, CD59, may play a previously unrecognized role in membrane protection from venom-induced injury.[826] The venom also contains a phospholipase D that may contribute to the hemolysis.[827]

Except for spider bites, which may be clinically deceptive initially, the potential for a hemolytic catastrophe should be obvious in patients who have been bitten or stung.[817] An early sign of impending hemolysis may be a rapid rise in the serum potassium level caused by the prelytic leak of this ion from the red blood cells. Once hemolysis is established, therapy other than transfusions is usually of little use. In patients with snake bites, localization or drainage of the venom and prompt administration of antivenom can be lifesaving.

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CHAPTER 38

Hematologic Manifestations of Systemic Diseases in Children of the Developing World

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CHAPTER OUTLINE

PREVALENCE AND MULTIPLE CAUSES OF ANEMIA IN THE THIRD WORLD
HEMATOLOGIC CHANGES ASSOCIATED WITH SPECIFIC INFECTIONS IN THE TROPICS
Malaria
Visceral Leishmaniasis (Kala-azar)
Schistosomiasis
Trypanosomiasis
Hookworm
Bartonellosis
Complex Hematology of Childhood Human Immunodeficiency Virus Infection in Developing Countries
TROPICAL EOSINOPHILIA
HEMATOLOGIC ASPECTS OF MALABSORPTION IN THE TROPICS
HEMOSTATIC FAILURE AS A MANIFESTATION OF SYSTEMIC DISEASE IN THE TROPICS
Dengue
Other Viral Hemorrhagic Fevers
Snake Bite
GEOGRAPHIC VARIABILITY IN THE DISTRIBUTION AND EXPRESSION OF HEMATOLOGIC DISEASE
Lymphoma and Leukemia
Hereditary Blood Disorders
Aplastic Anemia
Hematologic Disorders Unique to Certain Countries
ASPECTS OF CLINICAL MANAGEMENT
Treatment of Severe Anemia in the Tropics
Problems with Blood-Borne Infection and Blood Transfusion
Management of Imported Malaria
Leishmaniasis

Diagnosis and management of hematologic disorders in children in developing countries pose a number of problems that are not encountered in advanced Western societies.[1,2] Although all the conditions that have been described in this book are seen in these populations, their clinical features may be modified to a varying degree by the coexistence of malnutrition, chronic bacterial infection, or parasitic illness. Furthermore, many of the common killers, particularly in tropical climates, produce their own complicated hematologic manifestations.

It therefore follows that the study of hematologic disease in these populations presents a particular challenge to hematologists. It is often very difficult to define the clinical and pathophysiologic features of a single hematologic disorder in this setting. Sickle cell anemia is a good example. This disorder, as described in Chapter 19, usually produces a well-defined clinical and hematologic picture in North American or European children. However, the disease may be different in the rural populations of Africa, where its course is generally complicated by diarrheal illness, malnutrition, endemic malaria, and as yet ill-defined climatic effects on the clinical phenotype. It is very difficult to separate the environmental and genetic factors that must underlie the extraordinarily varied severity of this condition in different parts of the world.

The same problems are encountered when attempts are made to define the hematologic manifestations of disorders that are peculiar to tropical climates. For example, despite many years of intensive investigation, it is still not possible to provide a clear picture of the pathophysiologic cause of the anemia of malaria caused by *Plasmodium falciparum* infection. Although investigators have been unable to provide a clear picture for many reasons, the major difficulty for persons who wish to study this important problem is finding patients with malaria who do not have other diseases that may cloud the clinical picture, such as chronic bacterial infection, human immunodeficiency virus (HIV) infection, malnutrition, hemoglobinopathies, or red blood cell glucose-6-phosphate dehydrogenase (G6PD) deficiency (see Chapter 17).

Despite these difficulties, in recent years it has been possible to start to understand the pathogenesis of some of the hematologic manifestations of systemic disease in children in the Third World. In this chapter some of the progress that has been made in this extremely important area of childhood hematology is reviewed.

PREVALENCE AND MULTIPLE CAUSES OF ANEMIA IN THE THIRD WORLD

Numerous surveys have been conducted to determine the prevalence of anemia in tropical populations.[3-6] However, because of differences in methodology and demographic design, it is very difficult to interpret the results and compare one study with another. Representative data from several countries, using the World Health Organization definition of anemia (Table 38-1),[7] are shown in Table 38-2. It is clear that in many populations the prevalence of anemia in preschool children is extremely high, and in some locations almost 100% of the population is affected. Although the data shown in Table 38-2 were compiled more than 20 years ago, the situation remains serious. Although the 2010 Global Burden of Disease study investigators described a modest decrease in the global prevalence rates of anemia, they also reported that the burden of anemia, as measured in years lived with disability (YLDs), had increased from 65.8 to 68.3 million YLDs, or 8.8% of the global total of YLDs from all conditions.[8]

It is equally difficult to determine the relative importance of different causes of anemia in the tropics. Most surveys have concentrated on only one mechanism, such as iron or folate deficiency. However, to get a true picture of the cause of anemia in a particular population, it is necessary to obtain consecutive data over a substantial period. For example, studies in Gambia have shown that mean hemoglobin levels in children vary significantly at different times of the year; anemia is much more common in the wet season when malaria transmission is at its highest (Fig. 38-1).[5] The wet season is also the time when diarrhea and malnutrition are most common because heavy rains after many dry months have profound effects on the community, sanitation measures are disrupted, and food stores are at a low point in the annual cycle. Although these observations emphasize the multifactorial cause of anemia, it is clear that iron deficiency, which affects at least 20% of the world's population, is the major factor and that the numerous other diseases that may exacerbate anemia are often operating in the setting of low stores of iron in the body.

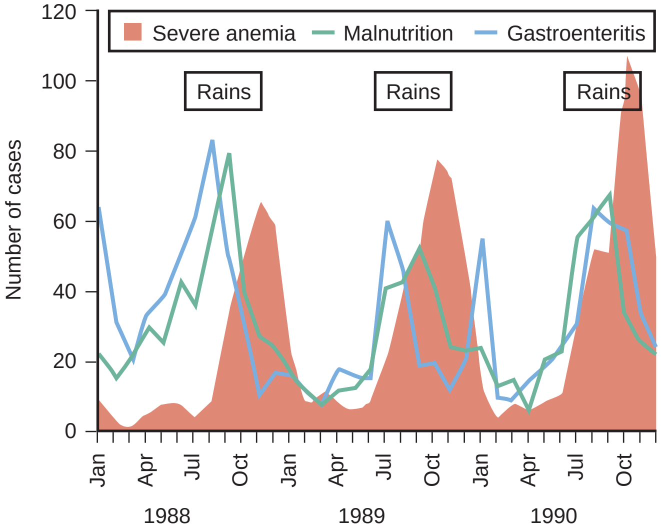

Figure 38-1 Admissions to the children's ward of a hospital in Gambia, where malaria transmission is confined to the rainy season. The incidence of severe malarial anemia corresponds to seasonal epidemics of malaria fever and cerebral malaria. Gastroenteritis and malnutrition also reach a peak incidence in the early part of the rainy season and thus contribute to the multifactorial cause of the anemia. *(Data from Brewster DR, Greenwood BM: Seasonal variation of paediatric diseases in The Gambia, West Africa.* Ann Trop Paediatr *13:133–146, 1993.)*

The body's response to infection may also reduce iron stores and iron utilization. The level of hepcidin, the body's main regulator of iron absorption,[9] is modified by proinflammatory mediators such as tumor necrosis factor

TABLE 38-1 World Health Organization Criteria for Hemoglobin Concentrations below Which Anemia Is Considered to Be Present in Populations at Sea Level

Age	Hemoglobin Concentration (g/dL)
Children, 6 mo–6 yr	11
Children, 6-14 yr	12
Adult males	13
Adult females (nonpregnant)	12
Adult females (pregnant)	11

From World Health Organization: Nutritional anemias. Report of a WHO group of experts. World Health Organ Tech Rep *Ser 503:1–29, 1972.*

TABLE 38-2 Prevalence (%) of Anemia (World Health Organization Criteria) in Different Populations

Geographic Area	Preschool Children	Nonpregnant Women	Pregnant Women	Adult Males
Latin America (7 countries)	–	17	24	4
Chile	35	–	–	–
Nigeria	63	46	52	36
Northern India	90	84	80	48
Southern India	76	81	88	56
Burma	3-27	5-15	82	1-5
Philippines	42	37	72	7

From Weatherall DJ, Ledingham JGG, editors: The Oxford textbook of medicine, *Oxford, 1987, Oxford University Press.*

(TNF) and interleukin (IL)-6, which are elevated in a wide variety of infections. High hepcidin levels stimulated by malaria infection or bacterial infections reduce absorption of iron from the gut and also reduce incorporation of iron into red blood cells. Iron may act as a growth factor for malaria parasites, and thus a raised hepcidin level and a reduction in available iron may form part of a protective innate response to malaria infection. However, this protective response may contribute to functional iron deficiency and anemia in endemic areas.

Iron deficiency and anemia are less common in groups that have persisted as hunter-gatherers, such as the Hadza in Tanzania and the pastoralists who drink blood and eat meat—for example, the Masai in Kenya.[2] On the other hand, absorption of nonheme iron, except from breastmilk, is comparatively restricted, and the content of iron in breastmilk is very low. Therefore iron deficiency is common in communities whose food is predominantly of vegetable origin.[10] The three great staples in these populations are rice, wheat, and maize. Sorghum and millet are also important in some areas of Africa and Asia. Soy and similar legumes are major sources of protein in many countries. The iron content of these diets is generally low and absorption is inhibited by fiber, phytates, phosphates, and polyphenols, which are all found at high levels in these largely vegetarian diets.[10] Loss of iron from chronic hemorrhage resulting from hookworm infection or schistosomiasis further contributes to the high incidence of iron deficiency anemia in the developing world.

The infants of mothers with iron deficiency have low iron stores, their folate status at birth reflects that of their mother, and the folate content of breastmilk is diminished by maternal deficiency and maternal malaria.[11-13]

In many populations, anemia may be associated with folate deficiency.[3,4,13] Again, the reasons for the deficiency are complex and multifactorial. Although intake of folate varies widely among different populations, depending on the way in which food is prepared and the temperature at which it is cooked, it is clear that low intake is not necessarily the result of lack of folate in the diet. Work in Africa suggests that the continuous anorexia associated with recurrent infections such as malaria or tuberculosis is the most important cause of folate deficiency in children.[2] As will be discussed later, postinfective malabsorption is a particularly common cause of folate deficiency, especially in the Indian subcontinent. Folate deficiency may be exacerbated by erythroid hyperplasia associated with chronic malarial infection or hemoglobinopathy.[14]

Although nutritional vitamin B_{12} deficiency is uncommon, Indian infants born to mothers with sprue (discussed later) who are fed breastmilk or goat milk containing insufficient vitamin B_{12} may be susceptible to megaloblastic anemia with locomotor complications during the early months of life.[2] This syndrome, which is often fatal, appears to be complicated by a marked predisposition to infection.

Although many of the population surveys on the prevalence of anemia in tropical countries have concentrated on one particular cause and intercurrent illness has not been assessed, studies in Africa—in which attempts have been made to assess body iron stores, folate levels, and the presence of intercurrent infection—indicate that chronic recurrent malaria, without other important complicating factors, is the major cause of anemia in these populations.

A major issue is whether iron supplementation is the most effective way to prevent anemia in regions where malaria is highly endemic. For example, a study in Tanzania showed that either iron supplementation or malaria chemoprophylaxis could be used to reduce the incidence of anemia in the first year of life but that the benefits of malaria prophylaxis were outweighed by increased risk for both malaria and anemia after the prophylaxis was stopped, thus suggesting that iron supplementation is the better option.[15] Then again, a reduction in iron levels is part of the natural host defense against infection, and a long-standing debate has ensued about whether supplementation has adverse consequences, particularly on mortality from infectious diseases.

Studies with a large sample size are needed to address this issue, and results of two such studies have recently been published. On Pemba Island, Zanzibar, where malaria is endemic, an initial study found that iron supplementation improved motor and language development,[16] but a subsequent larger study involving 24,000 children found that deaths and hospital admissions were significantly higher in children who received iron and folate supplementation compared with children who did not receive such supplementation.[17] Although the cause of increased deaths in the children receiving supplementation was not precisely defined, malaria appeared to play a major role. In contrast, authors of a study of 25,000 preschool children in southern Nepal found that children who received iron and folic acid had no significant difference in mortality compared with children who did not receive the supplements, although modest protective effects against diarrhea, dysentery, and acute respiratory illness were suggested.[18]

Iannotti and colleagues[19] reviewed 26 randomized controlled trials of preventive oral iron supplementation in children younger than 5 years. Their general conclusion was that iron-deficient children may need to be identified to ensure that iron supplementation programs are effectively targeted to improve hemoglobin levels and cognitive and motor development. Although no effect on morbidity was found in most studies, few had an adequate sample size or study design to resolve this issue. The outcome of a supplementation program may depend greatly on the pattern of endemic disease in the population.

HEMATOLOGIC CHANGES ASSOCIATED WITH SPECIFIC INFECTIONS IN THE TROPICS

Malaria

Malaria is the most important parasitic illness that affects humans.[20] The total burden of disease has recently been estimated to be 515 million episodes annually, and malaria is responsible for 18% of all childhood deaths in sub-Saharan Africa (equivalent to 800,000 deaths each year).[21,22] With increasing drug resistance of the malarial parasite, the problems of both treatment and control are becoming more complex, and this disease remains one of the major unsolved world health problems, although the widespread use of insecticide and bed nets appears to be reducing the incidence of severe malaria in many parts of Africa.

Life Cycle and Species

Because of its peculiar life cycle (Fig. 38-2), the malarial parasite is particularly prone to cause hematologic manifestations. During a blood meal, female anopheline mosquitos inject sporozoites that disappear from the circulation after about 1 hour and enter liver parenchymal cells, where they proliferate into thousands of merozoites (Fig. 38-3). The period of development in liver cells varies between species. Merozoites rupture from liver cells, pour into the bloodstream, and invade erythrocytes. Further development of the intraerythrocytic parasite follows one of two pathways: asexual differentiation or differentiation into sexual parasites called gametocytes (Fig. 38-4). The latter continue their development within anopheline mosquitos. Asexual parasites develop from young ring forms through trophozoites to dividing forms called schizonts (Fig. 38-5). Upon rupture of infected erythrocytes, forms called merozoites are released, invade other erythrocytes, and thus continue the erythrocyte cycle. When billions of schizonts rupture simultaneously and release cytokine-inducing toxins, they cause paroxysms of malarial fever.

When a child is infected with malaria for the first time, the result is usually an intermittent febrile illness lasting a few weeks, but complete eradication of malarial parasites from the bloodstream may take months or years. In the phase between cessation of the fever and final resolution of the infection, the child may appear well, but the destruction of red blood cells continues. From a hematologic viewpoint, the major question is how soon will the child be reinfected, because in some communities reinfection occurs almost every day, and immunity to malaria is slowly acquired and never complete. In regions of high transmission, children eventually acquire the ability to maintain a parasite density below the level that causes fever, but chronic or repeated infections cause a state of chronic anemia.

Four species of *Plasmodium* infect humans—*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*—and a fifth species, *P. knowlesi*, which is normally restricted to macaque monkeys, has recently been discovered in the human population in Borneo.[23] Each has particular morphologic and biologic properties and clinical manifestations. *P. falciparum* is the predominant cause of clinical malaria in Africa and much of Southeast Asia, whereas *P. vivax* tends to predominate in Central America and the Indian subcontinent, but their distribution overlaps considerably and multiple infections are not uncommon. *P. vivax* is essentially absent in populations of Central and West Africa because their erythrocytes fail to express the Duffy antigen or IL-8 receptor, to which the merozoites of this species attach during invasion.[24,25]

Another peculiarity of *P. vivax*, also shared with *P. ovale*, is the ability to form hypnozoites, which can

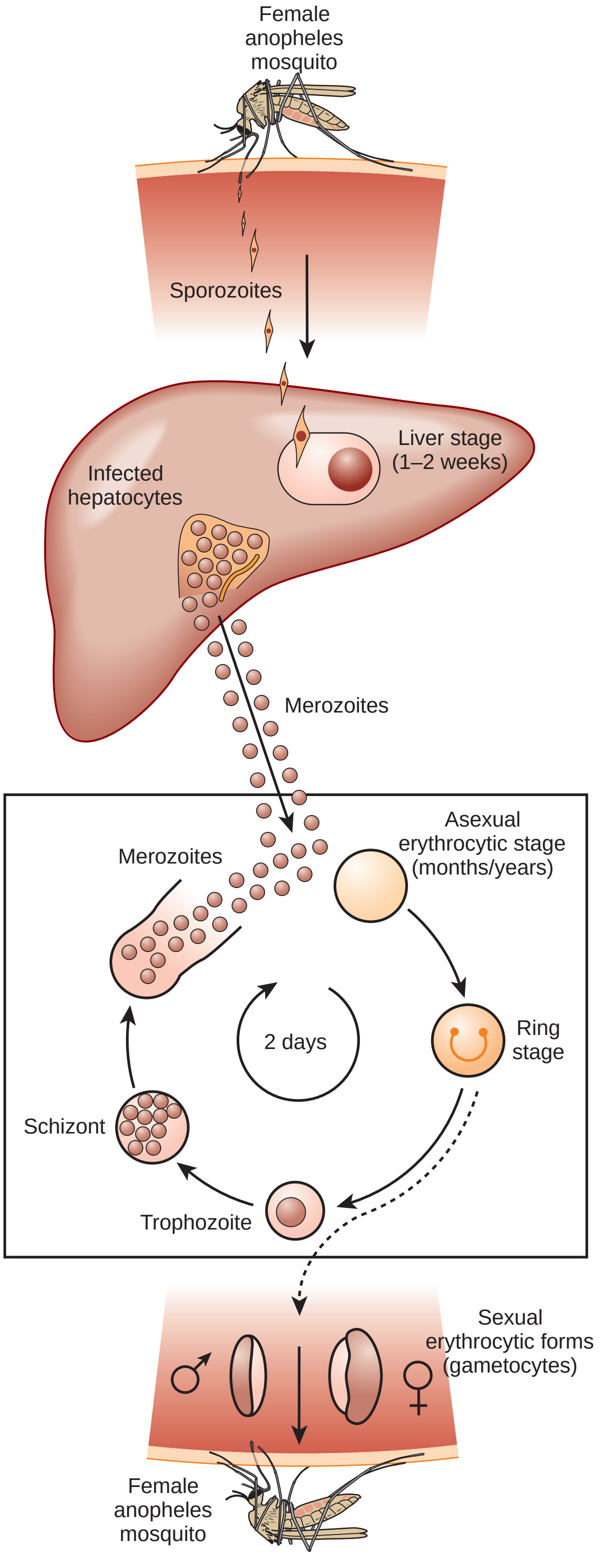

Figure 38-2 The life cycle of malarial parasites. The duration of the erythrocyte cycle is 48 hours for all human malaria parasites except *Plasmodium malariae*, which has a 72-hour cycle.

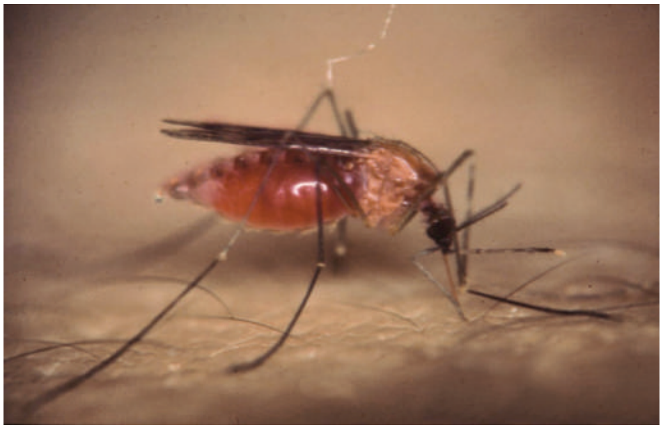

Figure 38-3 An anopheline mosquito after a blood meal. *(Courtesy Professor Herbert Gilles, Liverpool School of Tropical Medicine.)*

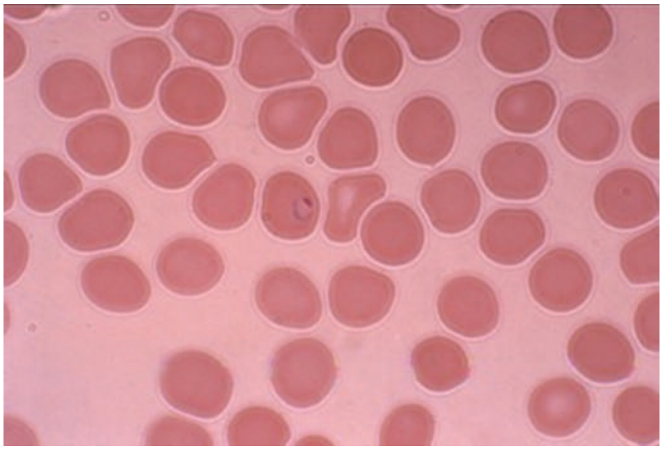

Figure 38-5 A *Plasmodium falciparum* malarial ring form.

remain dormant in cells for months or years. This ability results in relapsing infections that are often associated with mild chronic anemia. However, profound anemia and other grave complications are almost always seen with *P. falciparum* malaria, and the following sections apply mainly to this particular species.

Severe Malaria

Life-threatening complications are estimated to occur in about 1% of episodes of *P. falciparum* infection in African children, and it has been estimated that a child in rural Africa has a 15% lifetime risk of a malaria infection that requires hospital admission as a result of complications.[26] Such complications include profound anemia, cerebral malaria (a syndrome of unarousable coma that is often accompanied by severe convulsions), hypoglycemia, jaundice, renal failure, pulmonary edema, and coagulation abnormalities. Important and as yet largely unexplained differences are seen in the clinical spectrum of severe *P. falciparum* malaria in different parts of the world.[27] In Southeast Asia, all the aforementioned complications are commonly seen, whereas in Africa, where cerebral malaria and severe malarial anemia together account for about 1 million deaths per year and hypoglycemia is also common, the other complications of *P. falciparum* malaria are surprisingly rare. Malarial illness affects mostly children in Africa, whereas adults are more commonly affected in some other parts of the world; it has been suggested that much of the variation in clinical symptomatology is related to age. However, this theory does not provide a full explanation. Even among African children regional differences exist, and the impression is growing that higher rates of malarial transmission lead to a greater prevalence of anemia but, paradoxically, a low incidence of cerebral malaria.[28]

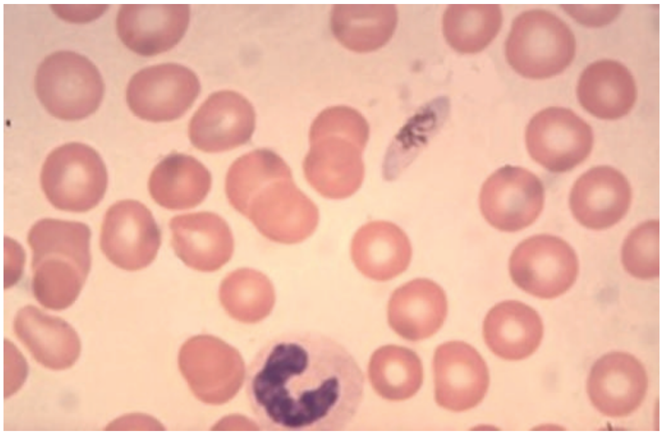

Figure 38-4 A *Plasmodium falciparum* malarial gametocyte in the blood.

The lethality of *P. falciparum* compared with other species of *Plasmodium* probably stems from two biologic properties. First, the parasite density that it achieves is typically a hundred times higher than that of other species before its growth is curtailed by host defense mechanisms.[29] Second, mature intraerythrocytic forms of *P. falciparum* adhere to the endothelium of postcapillary venules and thus sequester in tissues. Because these properties are common to all strains of *P. falciparum*, the greater puzzle is why lethal complications arise in only a minority of infected persons. Some important clues have emerged recently. Parasite sequestration is mediated by a parasite-derived molecule known as PfEMP1 that is expressed on the surface of a mature infected erythrocyte and binds to a variety of endothelial adhesion molecules, including CD36, intracellular adhesion molecule 1, E-selectin, and vascular cell adhesion molecule.[30] Whether a parasite will bind to a specific endothelial adhesion molecule is determined by its PfEMP1 type, which shows an extraordinary degree of phenotypic variability.[31] Expression of endothelial adhesion molecules varies between organs and is regulated by cytokines released by the host in response to the infection, such as TNF.[32] Another potentially important pathophysiologic mechanism is vascular sludging, which results from the clumping of parasitized erythrocytes, either with platelets ("autoagglutination" or "clumps") or with unparasitized erythrocytes ("rosettes"). Both phenomena are strain specific and have been associated with the severity of malaria.

As usual, the parasite uses diverse adhesive mechanisms; complement receptor–1 is a key binding target for some rosetting parasites,[33] whereas the platelet glycoprotein CD36 and other as yet unknown proteins are used in the formation of autoagglutinates or clumps.[34] Rosetting has been found to be reduced in persons with blood group O[35] who are known to be protected against severe malaria.[36]

Recognition is growing that a significant proportion of cases of severe malaria in the tropics are bacteremic.[37] Sickle cell trait has been found to be protective against bacteremia, which has led to the discovery—using an epidemiologic method known as mendelian randomization—that *P. falciparum* infection is a strong predisposing factor for bacteremia in Kenyan children.[38] An interesting relationship exists between salmonellosis, malaria, and malarial anemia.[39,40] It remains open to speculation whether *Salmonella* infection aggravates malarial anemia, whether anemia is a marker of chronic malarial infections that favor salmonellosis, or whether some other explanation exists for this curious and striking relationship.[41]

Malarial Anemia

The pattern of hematologic changes in malaria varies considerably, depending on the type of patient. During an acute attack of *P. falciparum* malaria in a nonimmune person, the hematocrit starts to decrease after 1 or 2 days and continues to decrease for about 1 week after antimalarial treatment.[42,43] Subsequently, a steady increase in the hematocrit usually occurs, although several weeks may pass before the hematologic picture is back to normal. The anemia, which is not usually life threatening, is characterized by both hemolysis and an ineffective marrow response,[42,43] features that have also been documented in persons with acute *P. vivax* infection. When profound anemia occurs with acute *P. falciparum* malaria, it is often associated with multiorgan failure, although it can also occur as an isolated complication.

From the perspective of the health of children who live in the tropics, the most important hematologic problem is seen in a child chronically infected with *P. falciparum*, whose anemia is debilitating and sometimes fatal. Although this problem most commonly occurs in areas of high malarial transmission, it is a growing problem in regions of lower transmission because of the increase in resistance to antimalarial drugs, which has served to prolong the average duration of infection. Attempts to investigate the problem of chronic malarial anemia are often hampered by the coexistence of iron or folate deficiency and hemoglobinopathies. In a study in which patients with these conditions were carefully excluded, the most striking pathophysiologic findings were hemolysis, hypersplenism, and a suboptimal bone marrow response.[43] In the sections that follow, some of the mechanisms for these components of the anemia of malaria are discussed in more detail.

Role of Hemolysis. Normal children living in rural Africa typically have extremely low levels of haptoglobin that increase significantly after institution of malaria prevention programs,[43,44] thus providing some indication of the burden of chronic hemolysis that malaria causes in many tropical communities. The mechanisms of red blood cell destruction in persons with *P. falciparum* malaria are complex and not fully understood.[45] Clearly, erythrocytes are destroyed when schizonts rupture and release their progeny, and depending on the state of immunity, a proportion of the parasitized erythrocytes are destroyed by the host before schizont rupture can take place. Both mechanisms can have devastating consequences if the patient is hyperparasitemic (sometimes more than 50% of erythrocytes are infected), but such patients are rare. In most infections, fewer than 1% of erythrocytes are infected, a loss that is important in the context of chronic infection but would not account for the severity of anemia that is commonly observed. Both mathematical modeling and clinical observation suggest that 10 times as many uninfected erythrocytes are removed from the circulation for each infected erythrocyte.[46] Indeed, cross-transfusion experiments clearly indicate that erythrocyte survival is shortened both in the acute phase of malaria and during convalescence.[47,48]

Role of the Spleen. Some degree of splenomegaly is a normal feature of malarial infection, and the prevalence of splenomegaly in regions of malarial transmission is used as a major indicator of the level of malarial endemicity. The importance of the spleen in host defense against malaria has been demonstrated in experimental systems, and persons whose spleens have been surgically removed are thought to be more susceptible to severe infection (discussed later). The phenomenon of parasitic sequestration, discussed earlier, is thought to have evolved primarily as an immune evasion strategy whereby the mature parasite can avoid passing through the spleen.[49]

Several studies have attempted to define the pathophysiologic changes that occur in the spleen during the acute phase of malaria. In animal models, malaria is accompanied by increased intravascular clearance of infected or rigid heat-treated cells by the spleen,[50] alterations in the splenic microcirculation,[51] and recruitment of myelomonocytes to the spleen and liver.[52] In studies of human malaria it has been found that increased splenic clearance of heated red blood cells occurs during acute attacks.[53] Reduced deformability of uninfected red blood cells is observed in *P. falciparum* malaria and is a significant predictor of the severity of anemia, consistent with the notion that these cells are being removed by the spleen[54] (discussed later). It has also been found that immunoglobulin G (IgG)-sensitized red blood cells are rapidly removed from the circulation by the spleen and that unusually rapid clearance persists well into the convalescent phase.[55] Undoubtedly, the activity and number of macrophages are increased during human malarial infection, which may therefore contribute to the increased removal of uninfected cells.[56-58]

In rodent malaria, a model of chronic anemia has been developed in which *Plasmodium berghei*–infected semi-immune mice show a fivefold to tenfold increase in the clearance of uninfected erythrocytes, although the extent of parasitemia remains below 1%.[59] In this study, clearance of uninfected red blood cells was delayed in mice depleted of macrophages, thus supporting the view that removal of uninfected erythrocytes is an important factor in persons with anemia.

All the available evidence therefore points to increased reticuloendothelial clearance in *P. falciparum* malaria that persists long after recovery. These changes are presumably a host defense mechanism to maximize the clearance of parasitized erythrocytes. Clinical studies have also uncovered a curious phenomenon whereby some erythrocytes appear to be returned to the circulation after having had their parasites removed,[60] but killing parasites usually means destroying erythrocytes, and it appears that the survival of uninfected red blood cells is reduced in the process.

These observations in patients are consistent with findings at the cellular level. Active erythrophagocytosis is a conspicuous feature within the bone marrow of persons with *P. vivax* and *P. falciparum* malaria,[61,62] and it is highly probable that it also occurs within the spleen. Children with acute *P. falciparum* malaria have high circulating levels of interferon-γ and TNF,[63] a synergistic combination of cytokines that activate macrophages.[64] Transgenic mice that constitutively overexpress human TNF are anemic and show enhanced clearance of autologous erythrocytes, which is presumed (though not proved) to be due to erythrophagocytosis.[65] When infected with *Plasmodium yoelii* or *P. berghei*, transgenic mice suppress parasite density much more effectively than their littermates do. This finding supports the notion that with relatively nonspecific effector mechanisms such as erythrophagocytosis within the spleen, anemia is part of the price that the host has to pay for protection against overwhelming parasitemia.

Massive Intravascular Hemolysis. A peculiar example of malaria-associated hemolysis is blackwater fever.[45,66] The classic form of this syndrome, often reported in colonial times among Europeans living in Africa, was characterized by fever and massive intravascular hemolysis associated with low or no parasitemia; it led to renal failure and was associated with high mortality. It was suspected that intermittent quinine ingestion might have led to a drug-induced immune hemolysis, although this mechanism was never proved and recent reports have implicated newer antimalarial drugs such as halofantrine and mefloquine/artemisinin combinations.[67,68] Classic blackwater fever is now much less common, but it is important to remember that massive intravascular hemolysis with hemoglobinuria remains an important complication of *P. falciparum* malaria in Southeast Asia in both children and adults.[42] Blackwater fever is sometimes associated with high parasitemia or destruction of G6PD-deficient red blood cells by oxidant antimalarial drugs. A survey of cases in Vietnam found considerable overlap of quinine ingestion, G6PD deficiency, and concurrent malaria, suggesting that these different factors may interact, but they certainly do not all have to be present for blackwater fever to occur.[69] It is conceivable that hemolysis is caused by the rupture of a large number of sequestered parasites, but the possibility of another hemolytic process has yet to be ruled out.

Decreased Erythrocyte Deformability. The increased clearance of uninfected erythrocytes is due not only to the activation of splenic macrophages but also to extrinsic and intrinsic factors that enhance their recognition and phagocytosis. Uninfected erythrocytes have reduced deformability, which leads to enhanced clearance in the spleen. The mechanism responsible for the loss of deformability is not completely understood, however. Increased oxidation of membranes in uninfected erythrocytes has been demonstrated in children with severe *P. falciparum* malaria, and the ongoing inflammatory insults associated with acute malaria (proinflammatory cytokines) or the direct effects of parasite products have been shown to cause loss of red blood cell deformability.[57,70,71] Intriguingly, a severe reduction in red blood cell deformability measured on admission is also a strong predictor not just for anemia but also for mortality, both in adults and children with severe malaria.[54,72]

Immune Complex–Mediated Hemolysis. It is also possible that at least part of the hemolysis in *P. falciparum* malaria might have an immune basis. A positive direct Coombs antiglobulin test (DAT) is seen in some patients with malaria.[43,73-79] As with all studies of malaria, considerable differences in the incidence of positive DAT results in malarial infection seem to exist in different populations and at different ages. For example, in Thai children or adults during their first attack or with subsequent attacks in persons who live in areas where a degree of immunity has not developed, DAT results are invariably negative.[78] Furthermore, studies using labeled immunoglobulin have shown that the number of antibodies bound to the red blood cells of patients with malaria of this type is no different from that in uninfected control subjects of the same age and in the same location.[78]

The position is different in African children who have experienced repeated attacks of malaria. Positive DAT results have been found in up to 50% of these children, and the incidence is significantly higher in children with active malarial infection than in those who are not infected.[74-77,79] The phenomenon is age related and is more common in young children. DAT results may remain positive for several weeks after an acute infection. Use of specific antisera has revealed that the most common type of red blood cell sensitization occurs with C3; other specificities include IgG alone, IgG plus C3, IgG plus C3 and C4, and various other combinations of IgG with complement components.[74-78] The IgG that can be eluted from these cells has specific activity against *P. falciparum* schizont antigen,[74] which suggests that the erythrocyte coating results from passive attachment of circulating complement-fixing malarial antigen-antibody complexes. It is therefore likely that the development of positive DAT results is part of the immune response to *P. falciparum*.

Other studies have suggested that the *P. falciparum* ring surface protein 2 (RSP-2) may be one component of the immunoglobulin-antigen complexes deposited on uninfected erythrocytes. This protein is expressed on infected erythrocytes shortly after merozoite invasion and may mediate some adhesion of infected erythrocytes to endothelial cells. RSP-2 is also deposited on uninfected

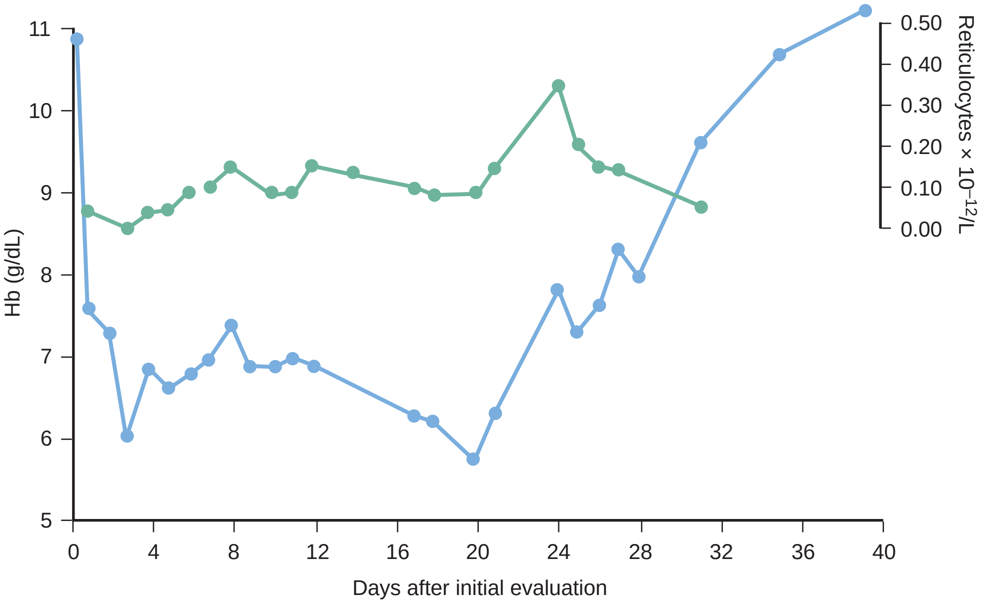

Figure 38-6 Hemoglobin (Hb) level and reticulocyte response in a nonimmune patient with *Plasmodium falciparum* malaria.

erythrocytes and forms immune complexes that contribute to the phagocytosis of uninfected erythrocytes. Limited clinical studies have shown that high levels of anti–RSP-2 antibodies are found in the sera of immune adults and children with severe anemia.[80] This antigen is also present on the surface of erythroblasts in the bone marrow of patients infected with *P. falciparum*, thus indicating that clearance or damage to developing erythroid cells by RSP-2 and anti–RSP-2 could contribute to the development of anemia.

However, it is far from clear whether the presence of positive DAT results indicates that immune destruction of red blood cells occurs in African children with malaria. Several studies have shown that no correlation exists between the degree of anemia and positive DAT results,[78,79] and other measured parameters of hemolysis have not correlated with coating of red blood cells. Nonetheless, immune destruction may occasionally be of importance. A small number of patients with severe anemia and positive DAT results have been described who had active C3 components on their red blood cells.[75] In these patients, striking monocyte erythrophagocytosis of nonparasitized erythrocytes was seen.

In some patients with *P. falciparum* infection, IgM antibodies have been reported to develop against triosephosphate isomerase, and disappearance of the antibodies coincides with disappearance of the hemolysis. Because these antibodies can induce erythrocyte lysis and complement activation in vitro, it has been postulated that they may contribute to a hemolytic process in vivo.[81]

In short, little evidence exists for immune destruction of red blood cells in *P. falciparum* malarial infections in nonimmune adults. Although a varying proportion of children with chronic malaria in Africa have positive DAT results, only occasionally is evidence of genuine immune destruction of red blood cells seen. Of course, it is possible that a sensitized red blood cell population is very rapidly destroyed and that subsequent serologic studies have missed this event. However, the persistence of shortened red blood cell survival of nonparasitized cells in the absence of any consistent serologic abnormalities suggests that another factor must be involved in the shortened red blood cell survival in persons with malaria. This factor appears to be nonspecific “overactivity” of the monocyte/macrophage populations of the spleen and liver, as previously discussed.

Defective Marrow Response. In addition to the hemolytic components of the anemia of *P. falciparum* malaria, an inappropriate marrow response undoubtedly occurs.[42,43,45,82,83] For many years it has been noted that the reticulocytosis in response to the decrease in hematocrit is often inappropriately low and delayed (Fig. 38-6). It remains unclear whether defective erythropoietin production is a significant cause, with some studies suggesting an appropriate rise[84] or even enhanced response[83] and other investigators observing a blunted response,[85,86] but there is no question that significant dysfunction of marrow takes place and appears to have a complex multifactorial basis.

It is now clear that disturbances in iron metabolism are mediated by hepcidin.[9] Hepcidin is regulated by proinflammatory mediators such as TNF and IL-6, which are elevated in both murine infections and in patients presenting with severe falciparum malaria, although other parasite-derived and host factors may be involved in stimulating hepcidin production. Hepcidin levels decrease in the most severely ill children because hypoxia inhibits hepcidin production.[87] High hepcidin levels stimulated by blood stage malaria infection are associated with inhibition of liver stage malaria infection in mice[88] and reduced incorporation of iron into red blood cells in humans.[89] Evidence from murine malaria suggests that a low level of serum iron caused by an elevated level of hepcidin

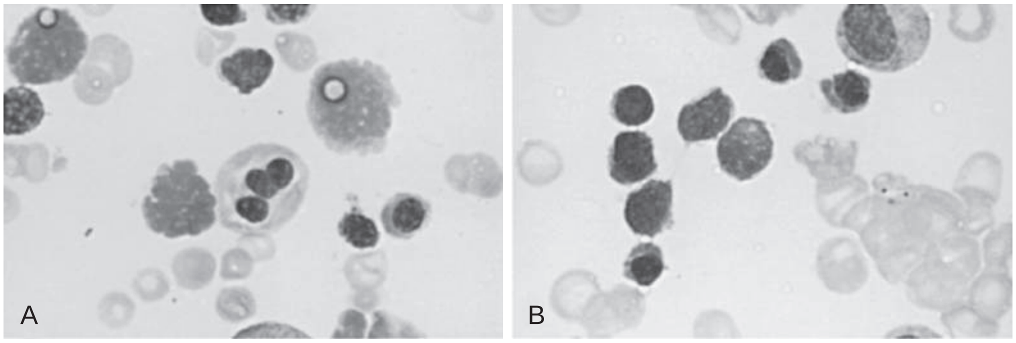

Figure 38-7 Bone marrow from a person with *Plasmodium falciparum* malaria. **A**, Multinucleate red blood cell precursors. **B**, Intercytoplasmic bridging (Leishman stain, magnification ×500).

reduces parasite growth and progression to severe disease.[90] These experimental and clinical findings are consistent with a role for iron as a growth factor for malaria parasites, as well as a role for an increased level of hepcidin and a reduction in available iron as a protective innate response to malaria infection.

Some of the features of iron metabolism and bone marrow structure and function in persons with acute malaria resemble those of other acute infections; thus the serum iron level rapidly decreases and iron appears to be sequestrated into the storage compartments of the marrow.[42,43] Serum ferritin levels are extremely high during the acute phase, although studies of isoferritins suggest a complex source. In many instances it appears that much of the ferritin is released as a consequence of liver damage.[42]

During acute malarial infections, marked dyserythropoietic changes in the bone marrow may occur.[42,43] Although changes are most marked in African children with chronic relapsing malaria,[43] they have also been observed in nonimmune Thai people[42] and in travelers returning to the United Kingdom.[45] These morphologic abnormalities, which have been studied by both light and electron microscopy, consist of erythroblast multinuclearity, karyorrhexis, incomplete and unequal amitotic nuclear divisions, and cytoplasmic bridging (Fig. 38-7).[43] Upon electron microscopic examination, binucleate or multinucleate erythroblasts, bizarre myelination with loss of parts of the nuclear membrane, and widening of the space between the two layers of the nuclear membrane have been observed.[43,62] Some degree of iron loading of the mitochondria and a reduction in electron density of the cytoplasmic matrix appear to occur, together with a paucity of ribosomes. In addition, widespread sequestration of erythrocytes in the sinuses of the bone marrow occurs in persons with an acute infection.[62]

With use of [^{3}H]thymidine autoradiography and Feulgen microspectrophotometry, it is clear that a significant abnormality of red blood cell proliferation in the bone marrow occurs in persons with acute malaria. Changes include an increased proportion of red blood cell precursors in the G_2 phase and arrest in progress of cells in the S phase. These findings are nonspecific and have been observed in other conditions associated with ineffective erythropoiesis.[82]

In addition to these dyserythropoietic changes, erythrophagocytosis is particularly common in the bone marrow in persons with *P. falciparum* malaria.[43] This phenomenon is not restricted to the marrow and may be seen in the spleen and other organs and, as mentioned earlier, may play a role in the hemolytic component of the disease.

Although the mechanism of these dyserythropoietic changes is unknown, it is possible that they are an exaggerated example of the bone marrow suppression that occurs in other situations of chronic infection. An important factor may be the high levels of TNF production that occur during malarial infection because this cytokine has been strongly implicated in the anemia of chronic infection and severe malarial anemia has been associated with certain TNF promoter polymorphisms.[91] TNF suppresses the proliferation of erythroid progenitor cells in human marrow culture, although the effect declines as the cells differentiate.[92] On the other hand, TNF stimulates fibroblasts to secrete growth factors for colony-forming unit–granulocyte-erythrocyte-monocyte-macrophage and burst-forming unit–erythrocyte.[93] In nude mice implanted with transfected Chinese hamster ovary cells that can constitutively express the human TNF gene, erythropoiesis is preferentially suppressed, with a marked reduction of colony-forming unit–erythrocyte and burst-forming unit–erythrocyte in the marrow and spleen.[94] Chronic malaria has specific features that might augment these effects because huge numbers of pigment particles are ingested by the resident macrophages of the spleen and marrow, thereby providing a sustained stimulus for TNF production at the site of erythropoiesis.[95] Furthermore, experimental findings in mice are consistent with a role for TNF in the dyserythropoietic changes of malaria.[96]

Other cytokines have also been implicated. IL-10 is an antiinflammatory cytokine that inhibits TNF, and two clinical studies in African children with severe malarial anemia have found a low ratio of IL-10 to TNF in plasma, thus leading the investigators to propose that defective IL-10 production may pave the way to marrow suppression by TNF.[97,98] IL-12 protects against severe anemia in experimental murine malaria,[99] which might reflect both its antiparasitic actions and effects on erythropoiesis.[100]

Several studies have suggested that a parasite byproduct of hemoglobin digestion, hemozoin, may have an indirect or direct role in impaired erythroid development.[83] Hemozoin stimulates the secretion of biologically active endoperoxides from monocytes, such as 15(*S*)-hydroxyeicosatetraenoic acid and hydroxynonenal, via oxidation of membrane lipids,[101,102] which may affect erythroid growth.[103]

Hemozoin and TNF-α also have additive effects on erythropoiesis in vitro, and in a clinical study, hemozoin-containing macrophages and plasma hemozoin were associated with anemia and reticulocyte suppression.[83] Moreover, bone marrow sections from children who died with severe malaria show a significant association between the quantity of hemozoin (located in erythroid precursors and macrophages) and the proportion of erythroid cells that were abnormal. These findings are consistent with a direct inhibitory effect of hemozoin on erythropoiesis.

Coagulation Abnormalities

Modest thrombocytopenia is not uncommon during acute *P. falciparum* attacks, and, occasionally, the platelet count decreases to as low as 10,000 to 20,000/mL.[42] The mechanisms have not been determined but probably involve platelet activation and adhesion of infected erythrocytes to platelets, and experimental studies in mice suggest that platelets sequester in venules during malarial infection.[104] Adherence of platelets to epithelium appears to be mediated through a specific receptor-ligand interaction involving lymphocyte function antigen–1. Evidence indicating reduced thrombopoiesis during acute infection also exists.

In some patients with severe *P. falciparum* malaria, a severe bleeding diathesis may develop during the acute phase of the illness. Although it has been suggested that the diathesis results from disseminated intravascular coagulation, studies in Thailand have not substantiated this supposition.[42] Rather, the bleeding appears to reflect gross thrombocytopenia together with liver damage. However, it is becoming apparent that approximately 10% of African children with severe malaria also have bacteremia,[39] and thus when disseminated intravascular coagulation occurs, the cause may be complex.

Malaria in Pregnancy

In endemic regions, it is extremely common for women to have malaria while they are pregnant. The placenta provides a favorable environment for parasite replication, and specific pathways of adhesion of infected erythrocytes to the surface of syncytiotrophoblasts have now been unraveled. A specific variant antigen, PfEMP1, which is expressed on the surface of infected erythrocytes (varCSA-2), mediates adhesion of infected erythrocytes to chondroitin sulfate. Primigravidae are particularly susceptible to malarial infection and may become systemically unwell, but immunity to this variant antigen modulates the duration and severity of infection in subsequent pregnancies. These observations suggest that varCSA-2 may be an excellent candidate antigen for a pregnancy-specific malaria vaccine. Apart from the effects on the mother, malaria is an important cause of low birth weight and increased perinatal mortality. Studies of cord hemoglobin levels indicate that it is also a significant cause of fetal anemia. Because the severity of the fetal anemia is out of proportion to the degree of maternal anemia, it has been suggested that intrauterine hemolysis may be involved.[105] Malaria during pregnancy is a preventable illness, although it has become more difficult to control with the rise of resistance to antimalarial drugs. Studies in different parts of Africa have found placental malaria to be an important risk factor for anemia in the first 6 months of life.[106,107]

Given the high prevalence of malaria in pregnancy and the strong association of placental malaria with infantile anemia, it is surprising how few infants have overt symptoms of congenital malaria in the tropics. On the rare occasions when it does occur, it can result in a perplexing hematologic disorder. The manifestations of malaria may not be seen until several months after birth, presumably because of passive immunization from the mother and possibly the fact that *P. falciparum* tends to grow less effectively in cells that contain relatively large amounts of fetal hemoglobin. The disease is characterized by a febrile illness associated with anemia that may often be profound.[105] As in conventionally acquired malaria, the pathophysiologic course of the anemia is a complex combination of hemolysis and marrow suppression. Thus it is important to obtain a careful travel history from the parents of any infant with unexplained hemolysis. This condition is commonly misdiagnosed because it is not considered as a possibility.

Malaria in Splenectomized Patients

Although it is generally believed that malaria may be associated with particularly severe infections in persons who have had a splenectomy, no clear evidence was found in a review[108] of this important question. Until this question is resolved, it is wise to advise visitors to the tropics who have had their spleens removed and splenectomized patients who live in regions where malaria is endemic to be particularly careful about continuing to maintain malaria prophylaxis.

Hyperreactive Malarial Splenomegaly Syndrome

Although splenomegaly is a feature of many tropical disorders, increasing evidence (particularly from work in East and West Africa and Papua New Guinea) indicates that a specific entity, formerly known as tropical splenomegaly syndrome, occurs widely throughout Africa, India, and Southeast Asia.[1,109,110] This condition is characterized by gross splenomegaly, high malarial antibody titer, and serum IgM levels at least two standard deviations above the local mean. The classic syndrome regresses after prolonged antimalarial prophylaxis with proguanil, a dihydrofolate reductase inhibitor marketed outside the United States as Paludrine.

Etiology and Pathophysiology. Evidence that malaria plays an important role in hyperreactive malarial splenomegaly (HMS) syndrome is increasing. Epidemiologic studies indicate that its prevalence is related to patterns of malarial transmission and that affected persons have a higher malarial antibody titer than do those who live in the same environment and are unaffected; this process seems to involve IgM rather than IgG. These observations, together with the partial clinical response to treatment with antimalarial drugs and the fact that persons with sickle cell trait seem to be partially protected, suggest that the condition probably results from an unusual form of immune response to chronic malarial infection. For this reason it is now commonly referred to as HMS.

With progressive splenomegaly, pooling of the formed elements of blood occurs, and in patients with particularly large spleens, a third to half of the total circulating red blood cell mass may be sequestered. Such sequestration produces a dilutional anemia that is made worse by an absolute increase in plasma volume. Pooling of neutrophils and platelets also occurs, and thus the hematologic picture is characterized by pancytopenia. No characteristic changes in bone marrow are seen.

Some evidence of a genetic predisposition to HMS exists.[111] A recent study in Ghana found that splenomegaly, high IgM levels, and anemia were more common in relatives of patients with HMS than in the general population, but no clear pattern of mendelian segregation was observed, thus suggesting that the cause in this population is likely to be complex and involve multiple genetic and environmental factors.[112] For some years researchers have debated about whether HMS is premalignant because it sometimes becomes refractory to antimalarial treatment, and it can also be associated with a lymphocytosis resembling chronic lymphocytic leukemia. Clonal rearrangements of the J_H region of the immunoglobulin gene have been noted in some patients whose clinical syndrome appears to be intermediate between HMS and chronic lymphocytic leukemia, thus suggesting that HMS has premalignant potential.[113]

Clinical Features. The clinical picture of HMS is typical and consists of massive splenomegaly, weight loss, and a variable degree of anemia.[109,110] Serum IgM levels are high, and a relative increase in T lymphocytes in peripheral blood may be seen. Serum may also show increased cold agglutinin titers and an increase in rheumatoid factor, antibodies to thyroglobulin and antinuclear factor, and circulating immune complexes.

The prognosis for patients with this condition is remarkably poor in parts of Africa, with up to 50% mortality in some populations. It is managed by long-term antimalarial prophylaxis, most commonly with proguanil.

Visceral Leishmaniasis (Kala-azar)

Leishmaniasis is an infection caused by intracellular protozoan parasites transmitted by various species of sandflies.[114,115] Human infections can result in three main forms of disease: cutaneous, mucocutaneous, and visceral (kala-azar). The important hematologic manifestations of leishmanial infection are found in the visceral forms.

The generalized form of leishmanial disease involves the liver, spleen, bone marrow, and lymph nodes and is caused by organisms belonging to the *Leishmania donovani* complex. The parent species, *L. donovani*, is found throughout Asia and Africa and can affect persons of all ages. However, the parasite that causes kala-azar in countries bordering the Mediterranean, in southern Europe, and in North Africa affects primarily young children and infants. It differs from *L. donovani* to such an extent that it warrants the designation of a special species and is called *Leishmania infantum*. Similarly, in the Western hemisphere, kala-azar is also a disease of very young children; the causative organism is *Leishmania chagasi*.

Although visceral leishmaniasis is primarily a disease of indigenous populations, it may be contracted on short-term visits. For example, in the United Kingdom it is being recognized increasingly after a return from Mediterranean holidays, and particularly in young children, it can cause considerable diagnostic difficulty; occurrences have been mislabeled as leukemia when the diagnosis of leishmaniasis was not considered. The risk for leishmaniasis is greatly increased in persons infected with HIV.[116]

Although the incubation period is usually 1 to 3 months, it can be as short as a few weeks. The onset is usually insidious and consists of fever, sweating, malaise, and anorexia, although a much more acute onset may be seen. As the disease progresses, the acute symptoms abate, but gradual enlargement of the spleen and liver ensues for several months. By this time the patient may have marked splenomegaly, cachexia, and anorexia. Generalized lymphadenopathy is also present.

Hematologic findings in later stages of the illness include anemia, neutropenia, and thrombocytopenia, which are manifestations characteristic of hypersplenism. The bone marrow is hyperplastic with dyserythropoietic changes, and the diagnosis can usually be made by finding Leishman-Donovan bodies—that is, macrophages containing intracellular organisms with characteristic staining properties (Fig. 38-8). The diagnosis can also be made by splenic or lymph node puncture.

Red blood cell survival studies and ferrokinetic analyses have suggested that hemolysis is the major cause of anemia in persons with leishmaniasis[117,118,119] but that plasma volume expansion also may be associated with the massively enlarged spleen.[119] Surprisingly, ferrokinetic

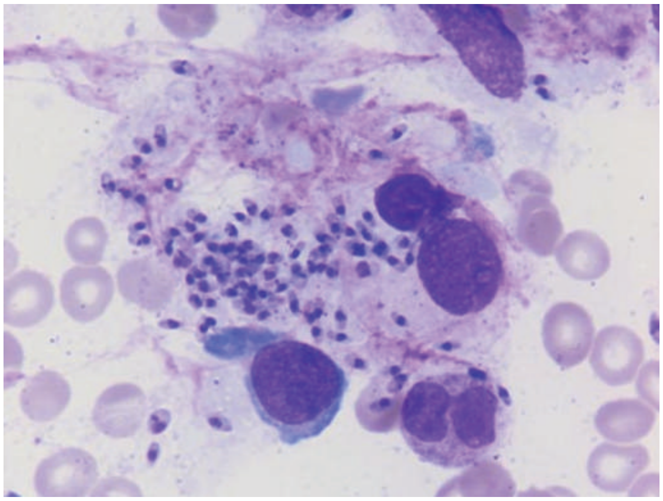

Figure 38-8 Leishman-Donovan bodies in the bone marrow of a 2-year-old child.

studies have shown very little evidence of ineffective erythropoiesis, but a reduced plasma iron level in the presence of greatly increased iron stores suggests that the reticuloendothelial hyperplasia is accompanied by abnormal iron retention by macrophages, typical of the anemia of chronic disorders.[119] This condition may limit the marrow response to hemolysis.

In babies or young children with acute visceral leishmaniasis, the clinical and hematologic findings may differ from those described earlier. For example, in Mediterranean populations a very rapid onset of anemia with severe hemolysis is commonly observed.[120] Occasionally both IgG and complement components are found on the red blood cells, but this finding is not consistent, and its significance remains to be determined. In most instances no evidence of immune hemolysis is found, and it appears that nonsensitized red blood cells are destroyed by macrophages recruited to the spleen and liver as part of the inflammatory response to the parasite. Severe neutropenia may also occur in young children, and this condition, together with dyserythropoietic changes in the marrow, marked erythrophagocytosis, and bizarre mononuclear infiltrates, may cause confusion with leukemia.

Schistosomiasis

The schistosomes are a group of trematodes that cause major health problems in many parts of the developing world.[121] The major human parasites include *Schistosoma haematobium*, *Schistosoma mansoni*, and *Schistosoma japonicum*. Schistosomiasis affects mainly children and young adults. Two hundred million people worldwide are estimated to have schistosomiasis, with 80% of them in sub-Saharan Africa.[122]

The location of adult worms in the human host varies with the parasite species: *S. hematobium* in the veins of the bladder, *S. mansoni* in the superior mesenteric veins, and *S. japonicum* in the inferior mesenteric veins. Sexual maturity of female schistosomes depends on their interaction with living male worms. Egg deposition occurs in the small venules of the bladder or portal venous system. When freshly deposited, the ova are partially developed. Hatching of the eggs leads to the liberation of miracidia. Further development occurs when miracidia penetrate a snail intermediate host. After two generations, cercariae are released that are capable of penetrating intact skin and migrating through tissues to reach their final home.

S. haematobium infections occur in Africa and the Middle East, whereas *S. mansoni* is found in Africa, the Middle East, and parts of South America. *S. japonicum* occurs in Japan and the Philippines, and together with the related species *Schistosoma mekongi*, it occurs in mainland Indochina.

The most consistent hematologic changes are found in association with *S. mansoni* infection. The acute phase is characterized by fever, myalgia, and progressive hepatosplenomegaly. At this time the most common finding in peripheral blood is eosinophilia. As the disease progresses, the patient may have massive hepatosplenomegaly associated with anemia, neutropenia, and thrombocytopenia.[123] Few good descriptions of the appearance of bone marrow in this condition are available, and the limited data suggest that the anemia is predominantly due to hypersplenism and hemodilution from the massive splenomegaly.

S. haematobium infection leads to urinary tract symptoms—in particular, hematuria and dysuria. The hematuria may be gross and persistent and may lead to iron deficiency anemia. In addition, affected children have typical features of the anemia of chronic disorders.

A major advance in the control of schistosomiasis is the availability of a highly effective drug, praziquantel, which is safe in both children and pregnant women. The cost of control programs may be reduced by combination therapy for schistosomiasis, gastrointestinal helminths, and filariasis. Major efforts to provide mass drug administration in high-burden areas have been undertaken[122] and have led to reductions in the intensity and prevalence of infection, but questions remain about the best strategy for long-term control, particularly in view of concerns about possible emerging resistance.[124]

Trypanosomiasis

Trypanosomal infections are a major cause of ill health throughout the world. The disease syndromes produced by these infections vary widely, but in general, they are divided into the American and African forms.[125-127]

American Trypanosomiasis (Chagas Disease)

American trypanosomiasis is a zoonosis caused by *Trypanosoma cruzi*, which is transmitted to humans by blood-sucking insects. It was first described by Carlos Chagas in 1909.

In its invertebrate host, *T. cruzi* grows extracellularly. Epimastigotes divide in the insect gut and differentiate into metacyclic trypomastigotes, which are the infective form for mammalian hosts. They are released close to the site of a bite and enter the host via the skin or mucous membranes. After invasion they enter cells, where they replicate as rounded amastigote forms that differentiate into trypomastigotes, which are released into the circulation and tissues. A variety of organs are involved, but the most important pathophysiologic result is a chronic cardiomyopathy.

Characteristic clinical findings are noted at each of these different stages of infection. After penetration of the skin, a local cutaneous lesion called an inoculation chagoma develops and is accompanied by swelling of the local lymph glands. Occasionally the route of inoculation is through the eye, where the initial lesion is characterized by unilateral conjunctivitis with edema of the eyelids. Invasion may or may not be followed by an acute phase consisting of fever, generalized lymphadenopathy, hepatosplenomegaly, and acute myocarditis.

The chronic phase of Chagas disease takes several forms. If cardiac involvement is predominant, a slowly progressive cardiomyopathy with heart failure, arrhythmias, and embolic complications is seen. In other patients, gastrointestinal symptoms predominate as a result of denervation and destruction of the myenteric plexus, which may give rise to megaesophagus or megacolon.

A congenital form of Chagas disease may occur. The organism can traverse the placental villi and from there be released into the fetal circulation. The most common

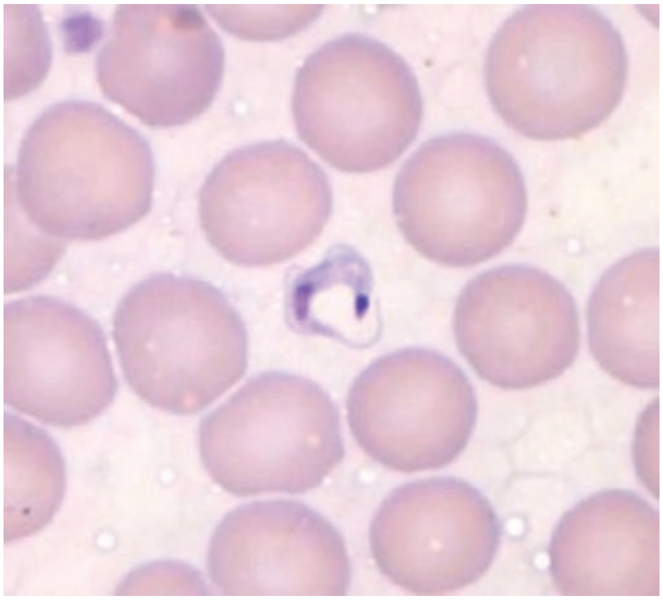

Figure 38-9 *Trypanosoma cruzi* parasite in the blood.

findings include growth retardation; hepatosplenomegaly; acute myocarditis; neurologic lesions, including a meningoencephalitic picture with tremors and convulsions; and, occasionally, a generalized hemorrhagic tendency with purpura or more widespread bleeding. The prognosis for patients with congenital Chagas disease is poor, and most infants die during the first week of life.

The hematologic changes in Chagas disease are nonspecific. In the acute phase, mild anemia and lymphocytosis may occur. At this stage the parasite can be found in the blood or in leukocyte concentrates (Fig. 38-9). Parasites may be concentrated by sedimentation of heparinized blood cells with the addition of phytohemagglutinin. Similarly, it is possible to find amastigote forms of *T. cruzi* in muscle or lymph node biopsy samples. The results of serologic tests for anti–*T. cruzi* IgM antibodies typically become positive about 20 to 40 days after the onset of symptoms.

African Trypanosomiasis

In Africa trypanosomiasis, conditions are caused by subspecies of the hemoflagellate *Trypanosoma brucei* known as *T. brucei gambiense*, which causes a chronic illness called sleeping sickness, and *T. brucei rhodesiense*, which is associated with a more acute illness. *T. brucei* is transmitted by the tsetse fly *Glossina*, in which it undergoes a variety of developmental changes.

A small nodule called a trypanosomal chancre forms at the site of the tsetse bite. Organisms may be isolated from this nodule. After this self-limited lesion subsides, the parasite invades and multiplies extracellularly in the blood, which leads to the systemic phase of the illness characterized by fever, lymphadenopathy, and splenomegaly. In the later phase of African trypanosomiasis, the central nervous system is invaded, which results in meningoencephalitis.

During the acute phase, normochromic normocytic anemia associated with hypoalbuminemia may be present. Occasionally patients have hemorrhagic manifestations and evidence of disseminated intravascular coagulation. The total white blood cell count is either normal or elevated with relative lymphocytosis. The sedimentation rate is markedly elevated, and circulating immune complexes may be present together with rheumatoid factor.

The parasite can often be demonstrated in thin or thick blood films stained with Giemsa or Field stain. Several concentration techniques have been developed to increase the likelihood of finding the parasite. A variety of immunodiagnostic tests are available, notably indirect immunofluorescence and enzyme-linked immunosorbent assay, but visualization of the parasite is necessary for a confident diagnosis. In the more chronic stage, it is important to examine cerebrospinal fluid, which often shows lymphocytosis, an elevated protein level, and the presence of morular cells of Mott or motile trypanosomes. High levels of IgM in cerebrospinal fluid may suggest the diagnosis in advanced disease if trypanosomes have not been found.

A novel test for African trypanosomiasis involves analysis of serum samples by mass spectrometry to detect distinct proteomic signatures; this test appears to provide more sensitive and specific detection than other diagnostic tests.[128]

Hookworm

It has been estimated that more than 900 million people are infected with hookworms.[129] The two main species that infect humans are *Ancylostoma duodenale* and *Necator americanus*, which are sometimes called the Old World and the New World hookworms, respectively. Both species are widely distributed in tropical and subtropical Asia and Africa. The prevalence of infection ranges from 80% to 90% in rural areas in the moist parts of the tropics, such as west Bengal, to 10% to 20% in relatively dry countries, such as those of the Middle East and Pakistan.

Both species of hookworm produce enormous numbers of eggs—approximately 20,000 per day per female. The eggs are discharged into the intestinal lumen, where they undergo a number of cell divisions before being passed in stool. Given appropriate conditions, the eggs develop further and hatch, with liberation of larvae. These free-living stages go through a series of developments designated L1 to L3, after which they penetrate the skin and the infective filarial larval forms migrate to the intestinal tract via the circulation, lungs, and respiratory tract. The final larval stage, L4, and the adult worms are found in the small intestine.

Hookworms attach to the mucosa of the upper intestine by their buccal capsules. Although they are found mainly in the jejunum, in persons with very heavy infections they may be distributed as low as the ileum. Attachment of the worm results in bleeding into the gastrointestinal tract.

The worms change their attachment site every few hours. Continuous or intermittent suction causes tissue and blood to be drawn into the worm's intestinal tract.[130] Only about 50% of the iron lost as hemoglobin into the gut may be reabsorbed, resulting in a marked increase in fecal iron concentration that is related directly to the worm load, which can be assessed by the fecal egg count. Direct recordings of blood loss have indicated values on the order of 0.03 mL/day per worm for *N. americanus* and up to 0.26 mL/day per worm for *A. duodenale*,[130] with the latter species tending to cause more profound levels of iron deficiency in the community.[131]

The hematologic findings in children or adults with heavy hookworm infections are characteristic. Because the condition is usually chronic, anemia is the major clinical feature. In severe cases, hemoglobin values of 2 to 5 g/dL are common.[132] The blood film shows the typical changes of iron deficiency anemia, such as microcytosis, hypochromia, and a normal reticulocyte count. The serum iron level is low, and iron is absent from bone marrow stores. Because of associated protein loss, affected children often have hypoalbuminemia.[132-135] Mild eosinophilia may be present[132] in patients with established illness, although very high eosinophil counts may occur during the phase of the illness in which the worms are migrating from the skin to the gastrointestinal tract (discussed later).

The diagnosis is made by analysis of stool for eggs. In persons with heavy infections, a fecal film can be examined directly after the sample is mounted in saline or iodine solution. In persons with lighter infections, concentration by zinc sulfate flotation is required.

Affected children require oral iron treatment to restore the iron stores, as well as treatment with anthelmintic drugs. Although many of these children are severely anemic, they usually have had chronic anemia for many years, and rapid restoration of the hemoglobin level is generally not required. However, in profoundly anemic children with high-output failure and associated hypoalbuminemia, it may be necessary to administer red blood cells slowly, together with appropriate diuretics. These children may have greatly increased blood volume, and it is easy to precipitate circulatory overload and cardiac failure. For patients with severe heart failure, an exchange transfusion consisting of removal of blood from one arm while infusing red blood cells into the other arm may be helpful. Whatever method is chosen, monitoring of the patient's central venous pressure is essential.

In recent years a growing number of tropical countries have made concerted efforts to reduce the effects of hookworm infection through use of anthelmintic strategies in the community. For example, mass administration of mebendazole to 30,000 school children in Zanzibar was estimated to prevent 1260 cases of moderate to severe anemia and 276 cases of severe anemia in 1 year, although it appeared that at least two doses per year would be necessary to maintain this level of protection, thus raising the issue of long-term sustainability.[136]

Bartonellosis

Bartonellosis (also known as Carrión disease and Oroya fever) is an infectious disease that is endemic in the western Andes of Peru and is seen occasionally in Colombia and Ecuador.[137] It results from infection by an aerobic, gram-negative bacillus, *Bartonella bacilliformis*. It is usually transmitted through a sandfly bite. After inoculation, the organism multiplies in endothelial and red blood cells. In severe cases of the disease, almost 100% of red blood cells are infected with numerous bacteria (Fig. 38-10). Hence the major feature of the disease is profound anemia resulting from the infection and subsequent phagocytosis of red blood cells. Although the precise mechanisms of the anemia are not fully understood, it seems to have many of the features of anemia of acute malarial infection. Survival of infected and noninfected red blood cells is shortened, and a limited response to the hemolysis associated with dyserythropoietic changes in the bone marrow is seen.

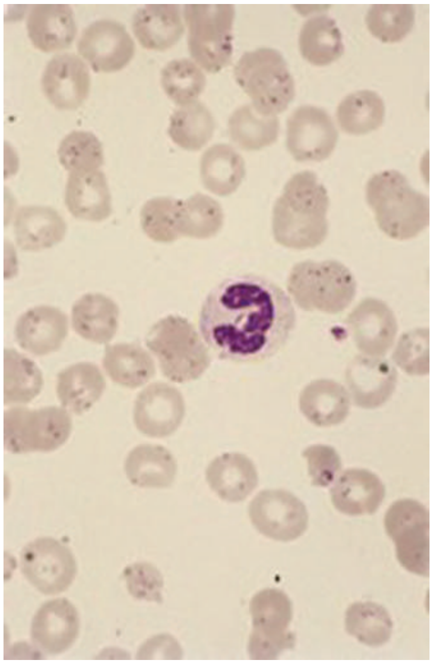

Figure 38-10 Bartonellosis in the peripheral blood.

The disease has two stages, anemic and eruptive, with an asymptomatic period between the stages. After an incubation period of approximately 60 days, the onset of the condition is acute and consists of malaise, chills, fever, and headache. The clinical picture is then dominated by severe hemolytic anemia associated with hepatosplenomegaly, lymphadenopathy, and, occasionally, a fine petechial rash. In the later eruptive stage, nodule lesions of varying size appear on the face, trunk, and limbs. Arthralgia and fever may also be present. The lesions, which are widespread on the arms and legs, may be confused with leprosy, yaws, or even Kaposi sarcoma. Although the infection is often present for a prolonged period, the eruptive phase tends to regress spontaneously.

During the anemic stage, moderate to severe hemolytic anemia is seen with reticulocyte counts in the 5% to 10% range. The organisms can be seen on thick and thin blood films stained with Giemsa, Wright, or related stains. The bone marrow is hyperactive with dyserythropoietic changes. The white blood cell count is not usually elevated, but thrombocytopenia is common.

The principal complication of this disease, apart from profound anemia, is superinfection with other organisms, particularly *Salmonella typhi* and *Mycobacterium tuberculosis*. Fortunately, *B. bacilliformis* is extremely sensitive to chloramphenicol, penicillin, and tetracyclines. Supportive treatment includes regular transfusion through the anemic phase of the illness.

Complex Hematology of Childhood HIV Infection in Developing Countries

The devastating impact of perinatally acquired HIV infection in an increasing number of developing countries is mirrored by the complexity of the hematologic complications of this disease in these populations. In some central African countries, seroprevalence rates in pregnant mothers in 1990 ranged from 20% to 30%, and in several other countries they exceeded 10%.[138] In India and Southeast Asia, the figures are presently much lower than this but are rapidly rising. Perinatal transmission rates have been difficult to establish with certainty but seem to be considerably higher than in the West. In 1989, an estimated figure in Zaire was 39%.[139] Although some children are infected through breastfeeding, the current consensus is that the benefits of breastfeeding probably outweigh the risk in countries where infectious diseases are the major cause of infant mortality.

The clinical picture of childhood HIV infection in the tropics does not differ greatly from that seen elsewhere, except that protein-energy malnutrition occurs much earlier. Tuberculosis and measles, both major causes of mortality by themselves, are more severe in HIV-infected children, but surprisingly, the severity of malaria does not seem to be affected. In Malawi, it is estimated that approximately half of perinatally infected infants die in the first year of life,[140] a considerably earlier mortality than that seen in the West.[141]

HIV infection provides yet another example of the complex pathogenesis of hematologic problems in the tropics. The virus can infect hematopoietic precursors,[142,143] as well as bone marrow macrophages and stromal fibroblasts, which may explain the marked dyserythropoiesis that is commonly observed in the bone marrow of affected children; however, these changes may also be complicated by opportunistic infection, malnutrition, and drug toxicity. Anemia with a low reticulocyte count and a microcytic hypochromic blood picture is common and may reflect both coexistent iron deficiency and the anemia of chronic disorders.[141,144,145] Although HIV may cause immune thrombocytopenia early in the course of the disease, it is not regarded as an adverse prognostic feature. Thrombocytopenia may also be accompanied by microangiopathic hemolytic anemia as part of thrombotic thrombocytopenic purpura, which, in the context of HIV infection, responds well to plasma infusion. Later, in addition to the progressive lymphopenia that characterizes acquired immunodeficiency syndrome, both reduced production and increased cellular clearance may contribute to neutropenia and thrombocytopenia.[145,146] Finally, advancing immunodeficiency contributes to a greatly increased risk for lymphoid neoplasms, particularly lymphoma.

The wide spectrum of hematologic complications associated with HIV and the high prevalence of infection make HIV testing an essential part of the investigation of cytopenia in most tropical areas. One of the most difficult dilemmas that HIV infection poses for pediatricians in the tropics is management of a child with life-threatening anemia. The conventional approach would be blood transfusion, but in many regions blood transfusion is now a major risk factor for HIV infection that is not adequately prevented by the available screening programs.[147,148] This problem is discussed further in a later section of this chapter.

TROPICAL EOSINOPHILIA

The structure and function of eosinophils are described in Chapter 21. Here the importance of eosinophilia in the tropics is discussed.

After anemia, eosinophilia is probably the next most common hematologic abnormality in children in the tropics.[149] An increase in eosinophils in bone marrow and peripheral blood, as well as in tissues, is a major feature of infections caused by worms that migrate through extraintestinal organs. A strong association exists between eosinophilia and infection with tissue nematodes, trematodes, or cestodes. In contrast, however, there is no well-documented evidence that worms that do not invade tissues or protozoal infections are associated with elevated eosinophil counts.

Some common causes of eosinophilia in the tropics are summarized in Box 38-1. A functional role for eosinophils in host protection against some invasive helminths has been demonstrated. For example, incubation of human eosinophils with the larval forms of *S. mansoni* or *Trichinella spiralis*, together with specific antibodies or complement, causes the destruction of these organisms. These in vitro studies have been confirmed by in vivo experiments in mice depleted of eosinophils; animals treated in this manner lose resistance to these helminths. It appears that killing of these organisms is initiated by attachment of eosinophils to the parasite surface, followed by extracellular degranulation and deposition of electron-dense material.

Box 38-1 **Causes of Eosinophilia in Tropical Populations**

TISSUE NEMATODES

- *Wuchereria bancrofti*
- *Loa loa*
- *Onchocerca volvulus*
- *Ankylostoma braziliense*
- *Strongyloides*
- *Ascaris lumbricoides*
- *Necator americanus*
- *Toxocara*
- *Trichinella spiralis*

TISSUE TREMATODES

- *Schistosoma*
- *Fasciola hepaticum*

TISSUE CESTODES

- *Echinococcus granulosus*

An absolute elevation in the peripheral blood reticulocyte count may occur in any of the conditions listed in Box 38-1. However, the term *tropical eosinophilic syndrome* was first used in the 1940s to describe patients with a paroxysmal cough and wheezing, particularly at night, scanty sputum production, weight loss, low-grade fever, lymphadenopathy, and extreme blood eosinophilia (>3000/μL). Later this syndrome was discovered to be due to filarial infection.

Hypereosinophilic syndrome is seen in children or young adults and is found most commonly in India, Indonesia, Sri Lanka, Pakistan, and Southeast Asia, although it may occur in any area where human filariasis is common.[150-153] In addition to typical clinical and hematologic findings, these patients have very high levels of serum IgE together with increased levels of specific antifilarial antibodies. Although it is usually easy to make this diagnosis in a tropical setting, if patients with these symptoms return to nontropical countries, other conditions that cause eosinophilia must be considered, including chronic eosinophilic pneumonia, allergic aspergillosis, vasculitic syndromes, and idiopathic hypereosinophilic syndrome. The major distinguishing features of tropical hypereosinophilic syndrome are a marked elevation in antifilarial antibody and very high IgE levels. It may be possible to identify filaria on blood films. Traditionally, better results have been obtained with films prepared from blood samples taken at night, although some studies suggest that this step may not be necessary.[153]

What is the best approach to determine a diagnosis in a child who has spent time in the tropics and who has persistent eosinophilia? It is important to determine which parts of the tropics have been visited. A careful respiratory history should be obtained. Detailed studies of stool for cysts and ova should be performed several times by an experienced observer. Serologic studies for *Filaria*, *Toxocara*, and *Trichinella* infection should be carried out, and immunoglobulin levels should be determined. If a diagnosis has not been determined after these investigations, it is important to exclude nontropical causes of eosinophilia, including collagen vascular disease, allergy, drug reactions, lymphoma, and leukemia associated with an eosinophil response. It may be necessary to initiate a trial of the antifilarial drug diethylcarbamazine under careful medical supervision as part of the diagnostic workup.

HEMATOLOGIC ASPECTS OF MALABSORPTION IN THE TROPICS

One of the major difficulties in discussing the problem of malabsorption in the tropics is definition.[154] A large proportion of people in tropical climates, both indigenous populations and expatriates who have lived and worked in rural areas, have mild abnormalities of the intestinal mucosa that are often associated with impairment of absorption. These structural and functional alterations of the gut have been called *tropical enteropathy*.[155-157] It is likely that they reflect an adaptation to life in the contaminated environment of the tropics with its recurrent enteric infections and peculiarities of diet. Interestingly, similar morphologic lesions have been demonstrated in the colon of otherwise healthy residents of southern India. After expatriates return to temperate climates, these changes in the gut revert to normal.[158]

The more severe malabsorption syndromes, sprue and postinfective malabsorption, are associated with chronic diarrhea, wasting, and a variety of hematologic changes.

The term *sprue* was first coined by the English physician Manson while working in China; it is an Anglicization of the Dutch term *indische sprouw*. During the nineteenth and twentieth centuries, tropical sprue was thought to be a disease of expatriates, but during World War II it became apparent that similar syndromes commonly occur in indigenous populations. The occurrence of severe malabsorption syndromes is not distributed evenly in the tropical world.[158-160] They are particularly common in the Indian subcontinent, Burma, Malaysia, Vietnam, Borneo, Indonesia, and the Philippines. They are also seen in the West Indies, in parts of Central America (particularly Puerto Rico, Cuba, and the Dominican Republic), and in northern parts of South America. A few reports have come from the Middle East and temperate areas. Tropical Africa seems to be spared.

These syndromes are believed to originate from an infection.[161,162] They usually start as an acute attack of diarrhea, which then becomes chronic. Many well-documented epidemics of sprue have been reported both in southern India and in the Philippines among American military personnel. However, despite a vast amount of work and the establishment of units specifically assigned to study the problem, no organism has been isolated that could even approach meeting Koch's postulates. It may be that these disorders can follow infection with a variety of agents, but the reason for the peculiar geographic distribution remains unexplained. Remarkably, genetic factors have been almost totally ignored in the investigation of this disease.

Tropical malabsorption syndromes can occur in persons of any age. They are characterized by intermittent diarrhea, weight loss, and anemia. Varying degrees of mucosal damage are found upon biopsy of the small intestine, although an absolutely flat mucosa, as seen in gluten-induced enteropathy, is rare. Hematologic findings in patients with tropical malabsorption syndromes vary considerably. In persons with more advanced disease, megaloblastic anemia is common; it is usually caused by folate deficiency but may also be complicated by vitamin B_{12} deficiency. Frequently, bizarre mixed pictures of iron and folic acid deficiency are found.

Interestingly, although much of the data have been derived from uncontrolled studies, many of the symptoms and hematologic changes associated with these syndromes can be reversed by a course of oral tetracycline. However, recovery is much more rapid if folate or vitamin B_{12} treatment is provided as well.

In a tropical setting, malabsorption can also result from colonization of the small bowel by specific parasites, including *Giardia lamblia*, *Strongyloides stercoralis*, *Cryptosporidium*, and others. Furthermore, abdominal tuberculosis with malabsorption is particularly common, and in Africa, HIV infection may be an important cause of malabsorption.[163,164] The local name "slim disease" acknowledges the severe wasting that can occur with HIV infection.[163]

Hematologists without experience in the tropics should be aware that the clinical and hematologic findings of tropical malabsorption syndromes can be extreme. In young children in particular a good history of diarrheal illness may not always be available, and in many tropical populations, recurrent diarrheal illness is the norm anyway. In addition to producing anemia, folate deficiency may give rise to severe neutropenia and thrombocytopenia with associated infection or bleeding. If intercurrent infection is present, as is often seen in severely affected children, the bone marrow appearance may be deceiving, although some megaloblastic change nearly always occurs even if overall the bone marrow is much less hyperplastic than is usually observed with folate or B_{12} deficiency in Western settings.

A major diagnostic problem that is often encountered in children who return from the tropics with persistent diarrhea and malabsorption is determining whether they have postinfective malabsorption or celiac disease. If the symptoms do not resolve, it may be necessary to initiate a trial of a gluten-free diet and then reintroduce gluten at a later date while monitoring progress with repeated small bowel biopsies.

HEMOSTATIC FAILURE AS A MANIFESTATION OF SYSTEMIC DISEASE IN THE TROPICS

Many tropical infections or other hazards produce serious bleeding disorders. Malaria was considered in an earlier section. A few other causes of bleeding disorders that are seen in the tropics are discussed in this section.

Dengue

The dengue viruses are four antigenically related but distinct organisms that are transmitted to humans by mosquitos of the species *Aedes aegypti*.[165] The clinical manifestations of this very common infection vary in different parts of the world. In American, African, and Indian populations the disease is characterized by classic dengue fever, but in Southeast Asia a much more serious condition, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), develops in many children. The latter condition develops in infants born to dengue-immune mothers during initial infections or in children older than 1 year during secondary infections. It appears that this curious paradox reflects enhancement of dengue viral infection in mononuclear phagocytes mediated by subneutralizing concentrations of dengue antibody.

The pathophysiologic course of DHF/DSS is quite remarkable.[165,166] Dengue viruses appear to be parasites of mononuclear phagocytes. Furthermore, they use antibody as a specific receptor for gaining entry to these cells. Such antibodies are known as infection-enhancing antibodies. Epidemiologic studies have shown that children 1 year or older always have detectable dengue antibody before acquiring a subsequent infection that results in DHF/DSS. Infants who acquire dengue antibody passively from their mothers are also at risk for development of the syndrome. DHF/DSS is characterized by simultaneous activation of the complement and hemostatic systems, together with a marked increase in vascular permeability.[166-168] It appears that complement activation follows both the classical and alternative pathways. The hemostatic abnormalities include gross thrombocytopenia, prolonged bleeding time, elevated prothrombin time, and a reduction in the levels of factors II, V, VII, and IX, together with marked hypofibrinogenemia and an increase in fibrin degradation products. The abnormalities in vascular permeability are characterized by an elevated hematocrit, normal or low serum protein levels because of selective loss of albumin, and variable serous effusions.

Precisely what triggers these remarkable events is not clear. However, it seems likely that the mediators are the product of dengue virus–infected mononuclear phagocytes. Studies in Thailand have suggested that low levels of heterotypic neutralizing antibody from a single previous infection prevent the illness in persons who acquire dengue grade II infections.[165,168] On the other hand, children who have circulating dengue-enhancing antibodies but no neutralizing antibodies are at high risk.

Once DHF has been triggered, the immune disturbance is not confined to antibodies. Both the $CD4^+$ and $CD8^+$ subpopulations of T lymphocytes are more intensively activated in persons with DHF than in persons with uncomplicated dengue fever.[169] High levels of TNF and other procoagulant cytokines may also contribute to the pathologic changes.[170] A further factor might be antibodies to plasminogen, which are found in a significant proportion of dengue infections, particularly if hemorrhage is present.[171] These antibodies are thought to arise because of structural homology between plasminogen and the dengue envelope glycoprotein.[172] Findings in Vietnamese children with DSS suggest that rather than causing true disseminated intravascular coagulation, the virus may act to directly degrade fibrinogen and thereby result in secondary activation of procoagulant homeostatic mechanisms.[173]

The clinical findings of DHF/DSS consist of fever, malaise, and anorexia, and about 2 to 5 days later a second phase occurs in which widespread hemorrhagic phenomena are observed, including purpura, large spontaneous ecchymoses, and bleeding from previous venipuncture sites. These children then enter a phase of profound shock. The hematologic findings are characterized by a normal or even high hematocrit associated with gross thrombocytopenia and evidence of disseminated intravascular coagulation.

The fact that the most serious pathophysiologic mechanism in this disease is fluid loss rather than hemorrhage has focused attention on the development of treatment regimens similar to those used for the management of severe diarrheal illnesses in children in the tropics. A study in Vietnamese children concluded that initial resuscitation with lactated Ringer solution was appropriate for children with moderately severe DSS, whereas hydroxyethyl starch resulted in fewer adverse reactions than did dextran for colloid treatment of severe DSS.[174] Clinical trials of heparin treatment to counteract disseminated intravascular coagulation have not produced impressive results.

Other Viral Hemorrhagic Fevers

Severe hemorrhagic fevers caused by viruses occur throughout the tropical world and are encountered on every continent except Australia and North America.[175] These fevers are the result of ribonucleic acid viruses of four distinct families, each of which is a zoonosis with typical epidemiologic features. These viruses are transmitted by a variety of agents, including tics and mosquitos. In many instances the reservoirs and vectors are unknown. These diseases are associated with specific clinical entities in different parts of the world, such as Rift Valley fever, Lassa fever, Argentine hemorrhagic fever, Bolivian hemorrhagic fever, and others.

Many of these conditions are accompanied by hemorrhagic diatheses of varying severity ranging from mild purpura to severe hemostatic failure.[176] As with dengue, shock is a major feature and is often associated with an elevated hematocrit. The bleeding manifestations reflect both increased vascular permeability and consumptive coagulopathy. Few extensive coagulation studies have been performed in patients with these disorders, although disseminated intravascular coagulation has been implicated as the basis for the hemostatic failure.

Snake Bite

Snake bite is an important and neglected problem in the rural tropics; its incidence is underestimated because of the lack of reliable epidemiologic data.[177] In northern Ghana, the carpet viper (*Echis* complex of species) is responsible for some 86 bites per 100,000 population per year, with 28% mortality. This particular snake, whose geographic range extends throughout the northern half of Africa and the Middle East to India, probably bites and kills more people than any other species. In the Indian subcontinent, the most important species are the cobra (*Naja naja*), the common krait (*Bungarus caeruleus*), Russell's viper (*Vipera russelli*), and *Echis* species. In Sri Lanka about 60,000 bites and 900 deaths (6 per 10,000 population) occur each year. In Southeast Asia, the Malayan pit viper (*Calloselasma rhodostoma*), *Daboia russelli*, and green pit vipers cause most bites and deaths. In Burma, Russell's viper bite has been the fifth major cause of death, with an annual mortality of about 15 per 100,000 population. Snake bite is also a common public health problem in Central and South America.

The hematologic complications of snake bite are a reflection of the complex chemistry of venoms.[178-180] Although much work remains to be done, at least some components of snake venom have been well defined. Venom may contain 20 or more different components. More than 90% of the dry weight of venom is protein in the form of enzymes, nonenzymatic polypeptide toxins, and nontoxic proteins. The mechanisms of envenomation have been most clearly defined for some of the venom procoagulants. For example, *V. russelli* venom contains a glycoprotein that activates factor X and an arginine ester hydrolase that activates factor V. *Echis* venom contains a zinc metalloprotein called ecarin that activates prothrombin. Many crotaline venoms contain proteases that cleave fibrinogen (e.g., arvin and reptilase). Some venoms contain phospholipase A_2 (lecithinase), which probably contributes to their neurotoxicity, cardiotoxicity, and hemolysis and is also responsible for increasing vascular permeability.

After a snake bite, swelling and bruising of the limb result from increased permeability induced by proteases, phospholipase, polypeptide toxins, and hyaluronidase, as well as by endogenous activators released by the venom such as histamine, 5-hydroxytryptamine, and kinins. The acute hemostatic failure that may follow envenomation is mediated in several ways.[181,182] Bites from snakes that produce procoagulants that activate factors V or X or prothrombin are associated with a rapid consumptive coagulopathy. In other instances rapid defibrinogenation may occur that is due to either direct attack on fibrinogen or activation of the endogenous fibrinolytic system. Thrombocytopenia results from either aggregation or consumption. The bleeding is potentiated by damage to vascular endothelium. The combination of defibrination, thrombocytopenia, and increased vessel wall permeability leads to massive bleeding, which is a common cause of death.

Although, as mentioned earlier, many venoms are hemolytic in vitro, massive intravascular hemolysis is seen rarely and usually in association with bites by *V. russelli*.

Management of snake bite includes supportive treatment, careful correction of the coagulation abnormalities, appropriate respiratory support if the venom is associated with neurotoxins, and judicious use of appropriate antivenin. Hemostatic disturbances usually respond well to antivenin treatment, and replacement therapy with fresh whole blood, fresh frozen plasma, specific factors, or platelet concentrates is not generally required. Even though a theoretical case can be made for the use of heparin in patients bitten by Russell's viper, particularly if there is definite evidence of disseminated intravascular coagulation, thus far clinical trials have not shown any benefit.

Although the long-term prognosis for persons who recover from a snake bite is good, work in Burma has shown that chronic pituitary failure and renal failure may develop later. These conditions are believed to be caused by infarction of the pituitary gland during the phase of intravascular coagulation, which is similar in pathophysiology to that observed after childbirth and in this setting is called Sheehan syndrome.[183] It may be accompanied by a mild normochromic anemia.

GEOGRAPHIC VARIABILITY IN THE DISTRIBUTION AND EXPRESSION OF HEMATOLOGIC DISEASE

In addition to the unique hematologic problems caused by disorders that are specific to the tropics or other parts of the developing world, the pattern of distribution of primary disorders of the blood varies considerably among different populations. Furthermore, some hematologic syndromes, as yet ill defined, are unique to certain parts of the developing world.

Lymphoma and Leukemia

The pathophysiologic and clinical features of the lymphomas and childhood leukemias[184,185] are described elsewhere in this book. Burkitt lymphoma is endemic throughout large regions of sub-Saharan Africa and in Papua New Guinea. In Burkitt's classic descriptions of the tumor, he pointed out that its occurrence is related closely to altitude, temperature, and rainfall, features that are similar in sub-Saharan Africa and Papua New Guinea. Although this distribution—together with work relating this B-cell tumor to infection with Epstein-Barr virus and evidence for loss of immunologic control of Epstein-Barr virus during acute attacks of malaria[176,186]—relates the prevalence of the tumor to malaria, this is certainly not the whole story. For example, Burkitt lymphoma is not found in Zanzibar, although it is very common in the coastal regions of neighboring Tanzania.

The T-cell leukemia/lymphoma syndrome, first recognized in southern Japan and now known to be due to infection with human T-cell leukemia/lymphoma virus type 1 (HTLV-1), has a very restricted distribution in southern Japan and the Caribbean.[185] It is not yet clear which particular lymphocyte populations are involved in the very common histiocytic lymphomas that occur throughout the Middle East and across parts of Central and South America.

The curious syndrome of primary upper intestinal lymphoma (Mediterranean lymphoma) and immunoproliferative disease of the small intestine must be distinguished from the rare primary intestinal lymphomas that occur sporadically throughout the world, usually in elderly patients.[187,188] In endemic regions, primary upper intestinal lymphoma and immunoproliferative disease of the small intestine occur in much younger age groups, usually of poor socioeconomic background. The pathologic lesions are found predominantly in the upper part of the small intestine and are associated with a clinical picture characterized by malabsorption, which often precedes development of the tumor.

Although this condition is still known as Mediterranean lymphoma, it was first described in Peru. However, the largest epidemic area is the region of the Mediterranean and the Middle East, and many cases have now been reported from Israel, Lebanon, Iraq, Syria, and Iran, as well as Greece.

Studies of the population distribution of classic Hodgkin disease have also shown some discrepancies.[184,189] High rates are found in most affluent westernized countries, and lower rates are found in poorer countries and the tropics; this finding does not seem to be attributed entirely to ascertainment bias. Interestingly, particularly low rates are seen in Japan, as well as in China, India, and Malaysia. In addition, considerable variations in age distribution and histologic subtype exist in different parts of the world. In poorer or lesser developed countries, Hodgkin disease occurs at a younger age and is usually the histologic type with a poorer prognosis.

Some interesting differences in rate of occurrence and type of acute leukemia throughout the world are also seen.[184,190] In North America and Europe and in all other westernized populations, acute lymphoblastic leukemia (ALL) accounts for a high proportion of cases of acute leukemia in children between the ages of 2 and 5 years. In contrast, ALL is uncommon in the tropics, particularly in sub-Saharan Africa, where Burkitt lymphoma is the predominant childhood tumor. On the other hand, in older children the incidence of acute myeloid leukemia and various subtypes of ALL is similar to that in Western societies.

Hereditary Blood Disorders

The distribution of hemoglobin disorders in the world is described elsewhere in this book. The reasons for their remarkably high frequency include heterozygote protection against malaria, a high frequency of consanguinity in many of the high-frequency regions, and the epidemiologic transition toward a reduction in childhood mortality as a result of malnutrition and infection, allowing children with these conditions who previously would have died early in life to survive long enough to be evaluated for treatment.[191] A remarkable heterogeneity in the frequency of these disorders has recently been shown in many high-frequency populations, even within short geographical distances,[192] which may reflect variation in malaria transmission in these populations. It has also been found recently that strong epistatic interactions occur between protective polymorphisms to malaria. For example, whereas persons with the sickle cell trait or α thalassemia trait are protected against malaria, persons who inherit both traits lose this protection.[193] By modeling these interactions, it is becoming possible to provide further information about the mechanism for the heterogeneous distribution of these disorders.[194] For economic, environmental, and cultural reasons, the presentation, diagnosis, and management of these conditions raise problems in many developing countries that are completely different from the problems encountered in developed countries.[191]

Remarkable differences have been found between high-frequency regions with regard to other common hematologic disorders that have reached a high frequency as a result of malaria resistance. For example, although G6PD deficiency is always associated with drug-induced hemolysis and favism, it is also associated with a high frequency of neonatal jaundice in some, but not all, populations; the reason for this discrepancy is still unknown.[195] Another example is the extremely high frequency in Melanesia of ovalocytosis, a condition that is relatively rare in Western populations. This condition is asymptomatic in heterozygotes, although the absence of reported homozygotes suggests that it may have a much more severe phenotype. Evidence clearly shows that it has reached its high frequency in Melanesia by providing protection against malaria, particularly cerebral malaria.[196]

Aplastic Anemia

Although more data are needed for confirmation, evidence is growing that aplastic anemia, particularly in childhood, is much more common in parts of Southeast Asia and South America than in developed Western countries.[197] The ease of availability of potentially toxic agents such as chloramphenicol may be reflected by this finding but is probably not the whole story. The possibility exists that that aplastic anemia reflects the action of an unidentified infectious agent. This challenging question requires further detailed prospective studies because a full hematologic workup, including a bone marrow trephine biopsy, is rarely available in many parts of the world, even at tertiary referral hospitals.

Hematologic Disorders Unique to Certain Countries

A number of ill-defined hematologic disorders appear to be common to particular countries. For example, a well-recognized disorder called acquired prothrombin complex deficiency occurs in Thailand and is seen mainly in infants who have been breastfed.[198] Despite a great deal of investigation, the cause is not yet known, although it is suspected that mothers may have ingested substances antagonistic to prothrombin.

A very curious condition called "acquired platelet dysfunction with eosinophilia," which is also seen in Thailand,[199] is a pediatric disorder characterized by bleeding and ecchymoses. The disorder is associated with eosinophilia and abnormal platelet function test results. The cause is unknown, with current efforts focused on attempts to define a parasitic cause.

In India an increase in hemolytic disease of the newborn that appears to be due to fetomaternal ABO incompatibility has been noted.[200] Evidence is increasing that this condition may be related to the administration of tetanus toxoids to pregnant women to prevent the major problem of tetanus neonatorum in that population because a marked increase occurs in the maternal titer of anti-A/anti-B antibodies after injection of the tetanus toxoid.

ASPECTS OF CLINICAL MANAGEMENT

Treatment of Severe Anemia in the Tropics

The definition of severe anemia depends on the clinical context, and the normal level of hemoglobin in the community must be considered. In an African child with malaria, a hemoglobin level of approximately 5 g/dL is commonly used as the criterion for transfusion, although a transfusion would normally be given sooner if the parasitemia were extremely high. The fatality rate for severe malarial anemia varies widely between clinics, but a typical figure is approximately 7%.[201] Until recently, most tropical pediatricians have endeavored to improve survival by improving the availability of transfusions.

The situation has altered dramatically as a result of the significant risk of HIV infection that now accompanies blood transfusion in many tropical regions.[147,202] Apart from the logistic and financial difficulties of screening blood donations, these screening procedures are an unreliable means of preventing transfusion-associated HIV infection in the developing world[148] (discussed later). Other problems with blood transfusion also exist that tend to be ignored. Crossmatching can be erratic in a clinic with inadequate resources, and the infusion is often unsupervised. Occasionally the consciousness level of a child with malaria deteriorates dramatically after the transfusion. Such occurrences are rare, but there are theoretical reasons why transfusion might worsen sequestration, such as the blood group favoring the formation of erythrocyte rosettes.[203]

The need to define the clinical criteria that make transfusion absolutely essential is urgent. Decision trees have been devised,[204] but the underlying risk-benefit equation may vary from place to place, and a meta-analysis found no clear evidence of benefit for transfusion in anemic children whose conditions are clinically stable.[205] One issue that has become clear is the prognostic importance of respiratory distress. For example, in Kenyan children, malarial anemia rarely causes death unless respiratory distress is present,[206] and the only clear evidence that blood transfusion is beneficial is seen in the group of children who have both anemia and respiratory distress.[207]

Investigations into the clinical physiologic course of severe malarial anemia in African children have yielded important insight into the management of this condition. Lactic acidosis appears to be the main cause of the relationship between anemia and respiratory distress,[208] and a substantial oxygen debt is present.[209] Rapid transfusion may be lifesaving, not only because it increases hemoglobin but also because volume repletion reduces acidosis.[208] A carefully conducted study in Kenya showed no evidence that rapid transfusion was likely to cause fluid overload, although in different parts of the world, different pathophysiologic factors may be operative.[210] However, it is now clear that bolus fluid supplementation of severely ill children on admission may result in higher mortality (discussed later). Therefore ill children should be carefully assessed clinically and appropriate fluid or blood transfusion should be administered to correct hypovolemia or severe anemia.

With the steady increase in levels of resistance to antimalarial drugs worldwide, the incidence of severe childhood anemia in the tropics is expected to increase. As the criteria for transfusion become stricter, the need exists for more data on optimal hematinic therapy for children with malarial anemia. The practices of pediatricians vary, but many pediatricians prescribe both iron and folic acid in addition to antimalarial therapy. One concern has been that iron deficiency may help suppress parasite growth, and in some studies[211,212] (but not others[15]), iron repletion has been found to increase the risk for subsequent infection. Another concern has been that folic acid supplements might reduce the efficacy of the antifolate drugs that are increasingly being used to treat chloroquine-resistant malaria in the community.[213] These issues were addressed in a study of moderately anemic Gambian children with *P. falciparum* infection.[214] Supplementary iron therapy was found to result in a small but significant improvement in hemoglobin levels after 1 month and did not increase the risk for reinfection within this period. Folic acid supplements failed to improve hemoglobin levels and increased the risk for parasite recrudescence if sulfadoxine-pyrimethamine was used to treat the infection. However, it cannot be assumed that these findings would necessarily apply to regions with different rates of malarial transmission and different nutritional problems, and the need for locally based research into this type of problem cannot be overemphasized.

Problems with Blood-Borne Infection and Blood Transfusion

The risk of contracting an infection after blood transfusion is increasing and is becoming a major economic burden for developing countries. Whereas the most important infections are those resulting from blood-borne HIV and hepatitis B and C viruses, the incidence of other infections is increasing. As previously mentioned, although donor screening has had a major effect in reducing the occurrence of HIV infection in patients who are receiving regular blood transfusions, such as patients with thalassemia, even if blood is screened adequately, the risk for infection remains. Hepatitis C is becoming a major problem throughout the southern Mediterranean region, the Indian subcontinent, and Southeast Asia, whereas hepatitis B remains a problem in many of the poorer countries. With the reappearance of malaria as a major problem in parts of the Indian subcontinent and Southeast Asia, posttransfusion malarial infection is increasing. Indeed, in many clinics it is becoming standard practice to treat recipients of transfusions with antimalarial drugs prophylactically.

In addition to these common viral and parasitic infections, the risk of blood-borne disease caused by other bacteria and parasites must always be considered, particularly in places where these infectious diseases are endemic. For example, Chagas disease is often transmitted through blood transfusion, although remarkably, pretreatment of blood with gentian violet seems to result in a major reduction of this complication. In parts of South America, transfusion-borne infections with *Bartonella* may occur. Diseases associated with HTLV-1 and HTLV-2, notably tropical spastic paraparesis and adult T-cell leukemia, which seem to be restricted mainly to the Caribbean and certain Japanese populations, can also be transmitted by blood transfusion.

Clearly, screening programs must be tailored to the particular infections that are common in individual communities, and blood should be administered only when absolutely necessary in these populations.

Management of Imported Malaria

The most important part of the management of malaria is to be constantly aware of the possibility of the diagnosis.[215] Obtaining an accurate travel history is critical. Children who have flown from one nonmalarious region to another may have had a brief stopover during which they could have been infected. It is vital to obtain a detailed account of all countries visited and the use of malaria prophylaxis. Clinical symptoms are usually seen within a month of infection, but they can occasionally appear after several months if the infection has been partially suppressed by a prophylactic drug with a long half-life, such as mefloquine. In a child who has previously been infected with *P. vivax* or *P. malariae* and in whom parasites in the dormant stage in the liver have not been eradicated, symptoms may recur years after the initial infection. If malaria is suspected, it is important that the diagnosis not be discarded if parasites are not found at first; thick blood film stains may need to be repeated several times, ideally during episodes of fever. Results with thin films are unreliable for diagnostic screening, but they can be useful for determining the species of parasite when infection has been confirmed. Dipstick assays based on detection of *P. falciparum* antigens in serum are now available. The most commonly used is based on *P. falciparum* lactate dehydrogenase, which is present in blood at the time of infection but is cleared rapidly.[216] An alternative is based on *P. falciparum* histidine-rich protein 2, which persists for some time after infection.[217] A positive result with this test may be helpful in a medical emergency, but the thick blood film remains the diagnostic "gold standard."

A child with malaria may lack the classic symptoms of paroxysmal fever and body pain. The presence of vomiting, diarrhea, cough, or tachypnea can be particularly misleading. Febrile convulsions are common and need to be distinguished from the seizures that accompany cerebral malaria, the defining feature of which is coma due to *P. falciparum* from which patients cannot be aroused. Hypoglycemia sometimes contributes to the coma and must be immediately excluded. Other potential problems include severe anemia, jaundice, circulatory collapse, pulmonary edema, renal failure, and coagulation abnormalities. Vigilance is needed because, although these potentially lethal complications occur in only a minority of infections, they can arise after treatment has commenced.

The choice of antimalarial treatment depends on the species of parasite, the geographic source of infection, the age of child, and the severity of the infection. Here it is only possible to outline the general principles involved; specific recommendations should be sought from an up-to-date formulary or reference center. Oral chloroquine is generally the drug of choice for all species other than *P. falciparum*, although it is important to be aware of the increasing prevalence of chloroquine-resistant *P. vivax* in Papua New Guinea and Indonesia.[218] With *P. vivax* or *P. ovale* infection, it is also necessary to destroy the dormant liver forms with primaquine. The child's G6PD activity should be checked before administering this drug; if it is deficient, a lower dose of primaquine may be administered over a longer period. If the infective species is not known, treatment should initially be the same as for falciparum malaria because this form is the most dangerous.

Chloroquine-resistant forms of *P. falciparum* are now so widespread that chloroquine can no longer be recommended for imported infections with this species. For uncomplicated infection, several alternative treatments are available. The most convenient are combination therapies, such as proguanil with atovaquone (Malarone) or artemether with lumefantrine (Riamet). The alternative and classic method is a course of oral quinine, but this treatment needs to be followed by a course of pyrimethamine-sulfadoxine (Fansidar) if the patient is from an area where the parasites are likely to be sensitive, a course of doxycycline if the patient is older than 12 years, or a course of clindamycin.

For patients with severe malaria, the standard therapy is quinine or quinidine administered by carefully controlled intravenous infusion. The use of intravenous infusion is impractical for many pediatric wards in the tropics because of nursing shortages and inadequate infusion equipment. In this situation, quinine is commonly given intramuscularly (after being diluted to reduce the likelihood of abscess formation).[219] Artemisinin (qinghaosu) derivatives are increasingly being used because of their rapid antimalarial action.[220,221]

In areas in which malaria is endemic, the recommended treatment of uncomplicated falciparum malaria is artemisinin-based combination therapy.[222] A large multicenter study of African children with severe malaria found that those treated with parenteral artesunate had significantly lower mortality than did those treated with parenteral quinine.[223] It is therefore a matter of grave concern that *P. falciparum* is becoming increasingly resistant to artemisinin in parts of Southeast Asia, because this situation has the makings of a global health disaster if the problem spreads to Africa.[224]

The principles of intensive management for complications of *P. falciparum* infection are the same as those for any other serious systemic infection. Dextrose infusion is often required to maintain blood glucose levels, which may be suppressed by quinine or by the infection.[225] Renal function and hemoglobin levels need to be closely monitored. In a child with cerebral malaria, overt seizures are usually controlled by diazepam and phenobarbital, but subclinical seizures are common and may be highly refractory to treatment. If high doses of an anticonvulsant are necessary, it is important to recognize that the child's system is often trying to compensate for metabolic acidosis and that respiratory arrest is the most common mode of death[226]; indeed, high doses of phenobarbital have been shown to increase mortality.[227]

Fluid management of a child with severe malaria has been the focus of considerable debate recently. Studies in Kenyan children with severe malaria have concluded that overall volume status is generally low and that relatively aggressive fluid replacement may be beneficial.[208] In contrast, investigators studying Gabonese children with severe malaria found no evidence of significant hypovolemia,[228] and these authors recommend that fluid replacement regimens be relatively conservative because intracranial pressure tends to be moderately elevated.[229] A major new randomized controlled trial by Maitland and colleagues[230] showed that bolus fluid supplementation in acutely ill children resulted in an unexpected increase in 48-hour mortality in the children who were given a fluid bolus upon admission.[230] Fluids and blood transfusion should therefore be given only after appropriate and careful clinical assessment. Use of steroids does not improve the outcome for adult cerebral malaria; whether this is true for children remains unknown. No conclusive evidence indicates that any other form of adjunctive therapy is beneficial for children with cerebral malaria. Exchange transfusion is sometimes used in patients with very high parasitemia, although its efficacy has not been evaluated in a controlled trial. More effective forms of treatment of severe malaria are badly needed.

Leishmaniasis

Leishmaniasis may be contracted on short visits to countries where the condition is endemic. It is a disease that may cause considerable diagnostic difficulty when seen in the setting of a hospital in the developed world. In children with this disorder, a primary hematologic disorder such as leukemia may be misdiagnosed if the diagnosis of visceral leishmaniasis is not considered. It may be manifested as an acute disorder with fever, hepatosplenomegaly, and hemolysis or as a more chronic condition characterized by increasing hepatosplenomegaly, lymphadenopathy, and pancytopenia. The hematologic and diagnostic features were mentioned earlier in this chapter.

Treatment remains difficult, with several different drugs to chose from; all have potential toxic effects, and thus expert advice should be sought.[231-233] For many years pentavalent antimony has been the drug of choice. The preparation that is available in the United States, stibogluconate (Pentostam), is classified as an investigational drug and is obtained from the Centers for Disease Control and Prevention in Atlanta, Georgia, which provides advice on dosage schedules for particular preparations. However, significant resistance is emerging, particularly in patients with kala-azar in India, and relapses may occur even in patients who show a good response to treatment. Pentamidine has long been used as alternative for antimonial-resistant visceral leishmaniasis, but it is relatively toxic.

During the past decade we have seen the introduction of new therapies, including liposomal amphotericin, oral miltefosine, and paromomycin.[233] Although these advances are significant, they are not ideal drugs, and cheaper and less toxic therapies are still needed. Liposomal amphotericin has been shown to be at least as effective and less toxic than compounds that contain antimony for the treatment of visceral leishmaniasis, and it is now licensed in the United States for that purpose, although its high cost is a significant impediment to use in the developing world. Liposomal amphotericin has been used successfully for the treatment of visceral leishmaniasis in children.[234] Miltefosine, an alkyl phospholipid developed for use in cancer treatment, has been demonstrated to be an effective oral treatment of visceral leishmaniasis, including antimony-resistant infection.[235] A recent comparison of the different regimens for visceral leishmaniasis concluded that sodium stibogluconate is the most cost-effective option in areas with high response rates to antimonial agents; in other areas, miltefosine is most cost-effective, but its use as a first-line drug is limited by teratogenicity and the rapid development of resistance, and liposomal amphotericin remains an expensive option for poor countries where the disease is endemic.[236]

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CHAPTER 39

Origins and Evolution of Pediatric Cancers

Mel Greaves

CHAPTER OUTLINE

PEDIATRIC VERSUS ADULT CANCERS
CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA AS A MODEL OF CLONAL EVOLUTION
EVOLUTIONARY PENETRANCE OF DISEASE
EVOLUTIONARY DYNAMICS AND CLINICAL OUTCOME

"Nothing in biology makes sense except in the light of evolution" was Dobzhansky's bold but persuasive claim, and nothing is as natural in biology as natural selection. It has been occurring for approximately 3 billion years whenever heritable traits vary in replicating populations, affecting the survival and reproductive fitness of molecules, unicellular or multicellular organisms, each in the face of environmental selective pressures.

When complex multicellular organisms evolved some 600 million years ago,[1] the potential for individual cells to be selectable, or to clone, was suppressed or rendered subservient to the fitness of the entire individual.[1] Two classic exceptions to this rule exist, however: the immune system and cancer. In the former, lymphoid cells genetically diversify their immunoglobulin heavy chain in B cells or T-cell receptor in T cells antigen recognition gene segments by recombinase-mediated rearrangement[2] to produce more than 10^{11} potentially selectable variants, collectively covering the recognition of essentially any infectious antigenic epitope. This naturally selected or adaptive system is under tight network control, although collapse of regulation can result in autoimmune disease or lymphoid cell cancers.

All pediatric and adult cancers are nonphysiologic, nonbeneficial clonal evolutions that follow the same rules of engagement as speciation in ecosystems (Fig. 39-1).[3] They are, in essence, breaking a 600-million-year-old covenant. It has been known for some time that cancer is a paradigmatic Darwinian, adaptive system.[4] The implications for cancer risk, progression of disease, prognosis, drug resistance, and clinical response have only more recently been appreciated.[3,5,6] Genomics has contributed significantly to this sharpness of focus,[7] just as it has reinforced the underlying principles of evolutionary phylogeny and natural selection of species and variants.[8,9] As in speciation, what emerges in cancer in terms of functionally important mutations (the so-called *drivers*) or subclones is the consequence of selective pressures exercised via the tissue habitat or ecosystem (Fig. 39-2), with outcome being heavily modulated both by variation in inherited susceptibility and by chance. Inherited susceptibility is a marked feature of pediatric cancers, both in terms of familial syndromes and in sporadic cases with little or no family history, as revealed by genomewide association studies.[10-13] The element of chance applies in both cancer and evolution in general because mutations arise throughout the genome in a stochastic manner and independently of the functions encoded by particular genes.[14] For mutations to have a functional impact in cancer requires that they occur in a manner that alters protein function (and consequently cell fitness) and in the appropriate context with respect to cell type and the presence (or absence) of other mutations with which they may be epistatic or interactive. The vast majority of mutations are neutral passengers of no consequence. The mutations we see recurrently are the result of natural or therapeutic selection.

As clones evolve over time (within a few years for most pediatric cancers compared with decades for adult carcinomas) they also move in space or throughout tissue ecosystems, both within the primary site and to secondary sites.[15,16] This characteristic has important implications for biopsy-based sampling, because anything other than the complete tumor will be a biased selection of subclones.[3] This phenomenon has been well documented for adult cancers, and we assume that the same phenomenon must apply to most if not all pediatric solid tumors.

In evolutionary or Darwinian selection, it is the phenotype that is the focus of selectability—for example, a predator with sharp teeth, a thermoresistant bacteria, or a drug-resistant bacterium or cell. In addition, stable phenotypes are initiated and sustained by genotypic

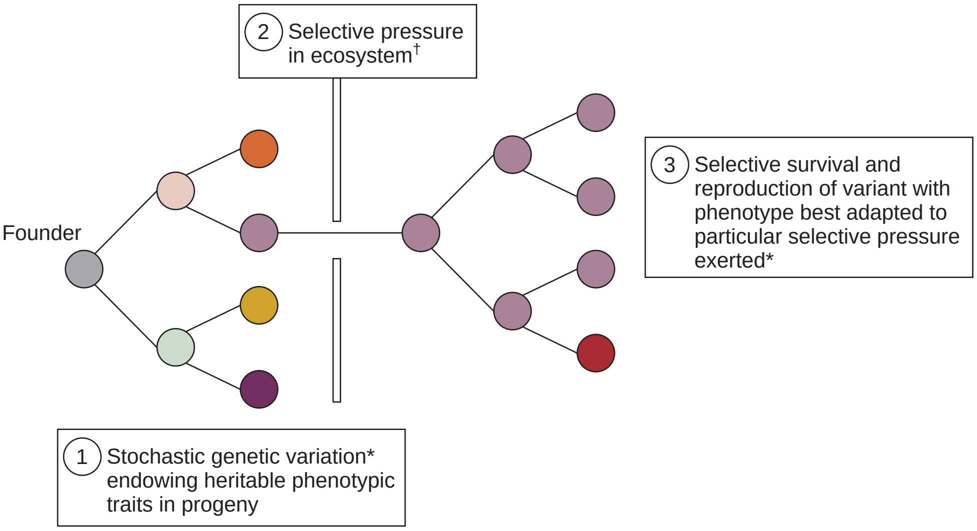

2 Selective pressure in ecosystem†
Founder
3 Selective survival and reproduction of variant with phenotype best adapted to particular selective pressure exerted*
1 Stochastic genetic variation* endowing heritable phenotypic traits in progeny

Figure 39-1 Principles of evolution by natural selection. *Different colored circles* represent genetic variants. If selection is stringent, there may be a selective sweep (winner takes all), as shown in the figure, with one or a few individuals surviving. A selective sweep may occur in key junctures in the evolution of cancer—for example, with in situ carcinoma to invasive cancer transition in adults, with metastases (which are usually monoclonal), and with the emergence of drug-resistant mutants in postchemotherapy relapse or recurrence. However, note that winners can become losers if the selective conditions change; as George Williams noted, "Evolution has no eyes to the future." *Must be present in germ cells for sexually reproducing multicellular organism selection, or, in cancer, in self-renewing stem cells.[22] †A wide variety of selective pressures exist in a diverse ecosystem, including interspecies competition for limited resources (food or mates), predation, and climatic factors.

alterations that are either mutational or epigenetic. However, any evolutionary system must have a "unit of selection," which is defined classically as any unit that replicates and in which a stable phenotype is linked to survival and/or reproductive success.[17] For bacteria and protists, this unit is the single cell. For multicellular organisms, it is the entire individual.[1] For cancer it will be somatic cells but probably not every cancer cell. The key attribute of any unit of selection is extensive replicative potential plus expression of the selectable phenotype. Extensive replication capacity, or self-renewal, is the key and exclusive feature of cancer stem cells.[18] The numbers of stem cells vary greatly in individual cancers and are generally increased in frequency with progression of disease and clinical malignancy.[19] In addition, the stem cell state may not be entirely stable, and under some circumstances (microenvironmental or mutational), non–stem cell progenitors can acquire or reacquire stem cell status.[20,21] Despite these variables, it is likely that cancer stem cells are the units of selection that drive and sustain the evolution of cancer clones and are the repositories of drug resistance.[22]

PEDIATRIC VERSUS ADULT CANCERS

All cancers are consequences of stable variation in somatic cells that facilitate clonal escape.[23] However, fundamental differences exist between pediatric and adult cancers

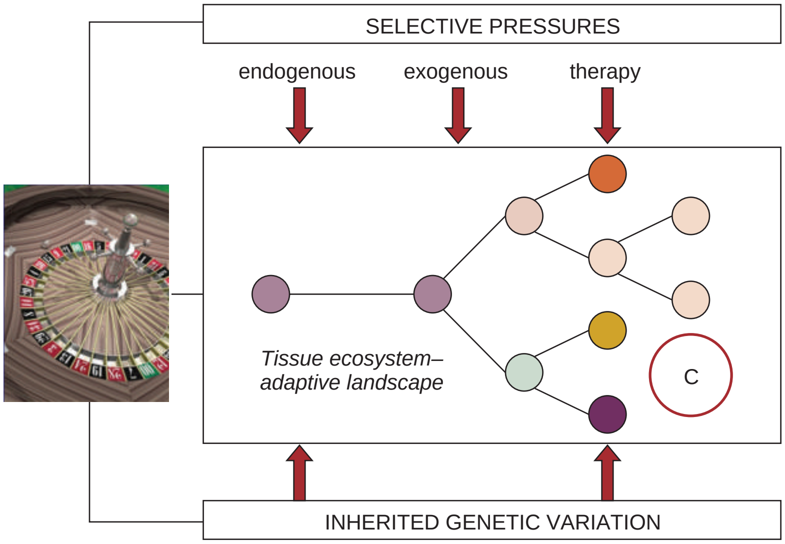

SELECTIVE PRESSURES
endogenous
exogenous
therapy
Tissue ecosystem–adaptive landscape
C
INHERITED GENETIC VARIATION

Figure 39-2 Determinants of clonal selection in cancer. The *roulette wheel* represents chance. *Different colored* circles represent genetically distinct cancer cells. *C,* Cancer.

TABLE 39-1 Differences Between Pediatric and Adult Cancers

Variable	Pediatric	Adult
Predominant lineage origins	Transient/ developmental tissue progenitors in embryo and fetus Fetal blood	Epithelia Adult blood
Time frame of natural history	Months to 15 yr	2-3 decades
Number of "driver" mutations required	2 (?)	5-8 (?)
Genetic instability	Rare	Common
Major risk factors	Endogenous proliferative stress (?)	Genotoxic exposures, persistent proliferative stress (hormonal), or infection
Cumulative risk	~1 in 800 (0-15 yr)	~1 in 3 (16-90 yr)
Age-associated incidence distribution	Defined, age-linked peak incidence in infancy or childhood	Increases as power of age*

*Some exceptions are testicular cancer and Hodgkin lymphoma in young adults and choriocarcinoma.

(Table 39-1). The most striking of these differences is the context of tissue or cell type involved, which in turn can be rationalized in terms of underlying risk factors. Most adult cancers are the consequences of protracted environmental or lifestyle exposures to epithelia.[24] In contrast, most pediatric cancers can best be viewed as developmental accidents arising during very restricted windows of tissue morphogenesis in the embryo or fetus when particular stem or progenitor cells are proliferating.[25-27] This view is supported by the finding that many of the inherited gene variants that contribute to the risk of pediatric cancer[11,13] and many of the somatic mutations that are restricted to the cancer cells themselves[28] are encoded by genes that are critical for the normal developmental biology and differentiation of the cells involved. Very few pediatric cancers are attributable to any known in utero external exposure (i.e., via the pregnant mother), despite an epidemiologic quest to find them lasting decades. This is not to say that pediatric cancers are not subject to variable risk factors. Aside from inherited variation in susceptibility, the numbers of cells at risk and proliferative stress may play a part. Higher birth weight is consistently associated with excess risk of pediatric cancer[29] and may operate by simply increasing the number of proliferating "target" cells at risk. Patterns or timing of infection (especially in affluent, "hygienic" societies) may promote or trigger the clinical development of childhood B-cell precursor acute lymphoblastic leukemia (ALL).[30] This mechanism is likely to operate via proliferative stress on preleukemic clones that are generated in utero (rather than via specific viral transformation).[30] Similarly, the striking peak of incidence of long bone osteosarcoma in adolescents might reflect the clinical emergence of a cancer initiated long before (prenatally) but promoted by the proliferative stress of the long bone growth spurt associated with puberty.[31]

A few types of cancer occur in both children and ageing adults, with acute leukemias and gliomas being clear examples. However, it is interesting that the genetics or mutational spectrum of these cancers,[32] and probably their etiology, is very different according to age. Two nonexclusive "seed or soil" interpretations of this distinction have been proposed. One interpretation is that the mutations are different because the microenvironments and selective pressures are different, resulting in different mutant genes providing clonal advantage. A second interpretation is that the mutations are cell context–sensitive and that the cell type of origin is different and developmentally constrained. Evidence for the latter interpretation came from mouse modeling of the impact of *GATA1* mutations, which revealed that the cellular targets that, with this mutation, lead to acute megakaryocytic leukemia in infants with Down syndrome, are developmentally restricted to the yolk sac and fetal liver.[33]

The tissue distribution, composite phenotypes (or cell type), and gene expression profiles of pediatric cancers provide a strong indication of the possible lineage and cell type or origin,[34-38] and this indication is endorsed by animal models (Table 39-2).[39-41] Subtypes of some cancers, such as medulloblastoma, may have distinct developmental origins.[42] No unambiguous way exists to identify precisely when and where any childhood cancer is initiated by somatic mutations. Most mutations are assumed to originate prenatally either in the embryo or the developing fetus, possibly when the cell lineage stem or progenitor cell "targets" are under maximum risk from proliferation.[43]

CHILDHOOD ALL AS A MODEL OF CLONAL EVOLUTION

Childhood leukemias lend themselves to interrogation of their natural history more than any other pediatric cancer type. This situation occurs because, although it is initiated in tissue sites such as fetal liver, bone marrow, or thymus, the clone disseminates very readily into the accessible blood, including in the early stages of clinical covert natural history, such as at the birth of the child. Figure 39-3 illustrates a minimal two-stage model proposed for the development of the common form of B-cell precursor ALL in children.[30,44] The challenge was to backtrack clonal history from the point of diagnosis when the genetic features of the clone were definable. For this task, chromosome translocation–generated chimeric gene fusion sequences are the ideal markers for clonal tracking, as illustrated in Figure 39-4 for the most frequent of such fusion genes, ETV6-RUNX1 (formerly TEL-AML1).[45,46] Three tactics have been successfully adopted for backtracking clonal history in ALL (Fig. 39-5),[44,47] and collectively they have provided persuasive evidence for the in utero origin of disease and the two-step model that has been proposed. Formal proof of a prenatal origin of other

TABLE 39-2 Developmental, Lineage Origins of Pediatric Cancers

1. Central nervous system	Gliomas	Glial precursor (different brain regions)
	Ependymoma	Radial glial cells
	Medulloblastoma*	
	(i)	Cerebellum (granule neuron precursor cells)
	(ii)	Dorsal brain stem
2. Neuroblastoma		Embryonic neural crest cells
3. Rhabdomyosarcoma		Muscle stem/progenitor cells (Satellite cells?)
4. Hepatoblastoma		Embryonic liver precursor cells (?)
5. Osteosarcoma		Bone progenitor cells
6. Retinoblastoma		Embryonal retinoblasts
7. Ewing sarcoma		Mesenchymal stem cells (?)
8. Wilms tumor		Embryonic urogenital ridge (mesenchymal) progenitor/stem cells
9. Acute leukemias		Fetal, hematopoietic stem/progenitor cells
	(i) Childhood ALL	B (or T) progenitor cells (fetal liver or bone marrow)
	(ii) Infant ALL	Fetal B/monocytic progenitor?
	(iii) AML	Myeloid restricted stem/progenitor cells
	(iv) Down syndrome AMKL	Fetal liver megakaryocytic erythroid progenitors

ALL, Acute lymphoblastic leukemia; *AMKL,* acute megakaryoblastic leukemia; *AML,* acute myelogenous leukemia.
*More distinctive subtypes in terms of cellular and anatomic origin probably exist than are listed here.

pediatric cancers is not available, although the very young age of many patients at diagnosis and the embryonal phenotypes of some make a prenatal origin very likely.[43] The oldest age at diagnosis of ALL to date with backtracking evidence for an in utero origin is 14 years.[48] However, it is possible that some leukemias and other tumors in older children and even young adults are initiated prenatally with a protracted clinically silent latent period—for example, osteosarcoma in teenagers and testicular cancer in adults in their twenties and thirties.[37] This scenario has yet to be formally assessed, however.

Monozygotic twins with concordant or discordant clinical ALL have been especially informative in defining the sequence of prenatal and later secondary but essential genetic hits (Fig. 39-6, *A* and *B*).[47,49,50,51] These data strongly suggest that fusion genes (or hyperdiploidy) are common initiating, prenatal genetic events in childhood ALL. This suggestion is supported by modeling experiments with murine and human blood cells, which indicate that *ETV6-RUNX1* can initiate a preleukemic phenotype but that additional genetic changes are required for progression of disease and overt clinical ALL.[52,53] When coupled with in-depth second-generation, whole-genome sequencing, it becomes possible to define the genetic landscape of ALL as assembled via the two independent prenatal and postnatal steps (Fig. 39-7).

This explanation still leaves unclear how the "set" of secondary abnormalities (copy number alterations and single nucleotide variants) are accrued; that is, are they sequentially acquired, and if so, does this acquisition occur in any preferential order or as some type of bolus? This question has been resolved by single-cell genetic analysis and inferred clonal phylogeny (Fig. 39-8).[54,55] The striking—although, from an evolutionary perspective, not

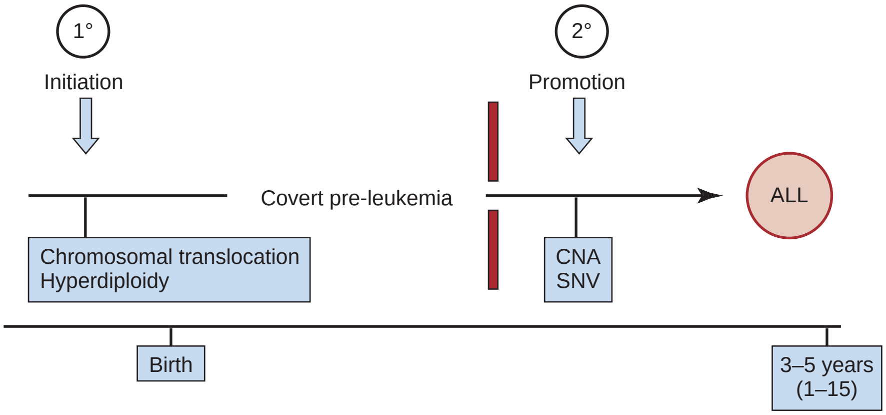


Figure 39-3 A two-step model for childhood acute lymphoblastic leukemia (ALL). *CNA,* Copy number (gene/locus/chromosome) alteration (i.e., gain or loss from diploid normal), identified by single nucleotide polymorphism arrays; *SNV,* single nucleotide variant (or mutation), identified by exome sequencing; *3-5 years,* peak age incidence of diagnosis; *1-15 years,* age range of diagnoses in B-cell precursor ALL (hyperdiploid or *ETV6-RUNX1*⁺). *(Modified from Greaves M: Infection, immune responses and the aetiology of childhood leukaemia.* Nat Rev Cancer *6:193–203, 2006.)*

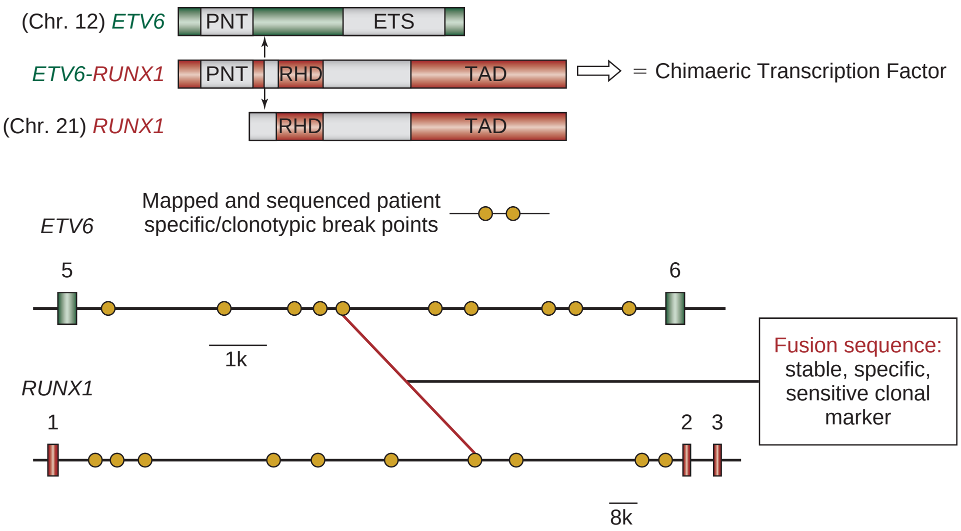

(Chr. 12) ETV6
PNT
ETS
ETV6-RUNX1
PNT
RHD
TAD
= Chimaeric Transcription Factor
(Chr. 21) RUNX1
RHD
TAD
Mapped and sequenced patient specific/clonotypic break points
ETV6
5
6
1k
Fusion sequence: stable, specific, sensitive clonal marker
RUNX1
1
2
3
8k

Figure 39-4 The t(12;21) *ETV6-RUNX1* gene fusion in acute lymphoblastic leukemia. *Circles* represent individual, patient-specific (clonotypic) break points determined by long-range polymerase chain reaction amplification of genomic deoxyribonucleic acid, sequencing, and mapping. The diagram is for the purpose of illustration. Actual sequencing mapping data is provided in references 80 and 81. *Chr,* Chromosome; *ETS,* Ets domain (DNA binding); *PNT,* pointed domain (dimerisation); *RHD,* runt homology domain (DNA binding and interaction with CBFb); *TAD,* transactivation domain.

surprising—result is the branching clonal architecture, which is now seen as a hallmark feature of cancer,[3,7] just as it is seen as a hallmark feature of biological speciation and evolution in general (Fig. 39-9). Relapse can originate from within either major or minor subclones that are present at diagnosis[54,56] and can involve positive selection of prior, very rare subclones with mutations that render these cells resistant to particular drug therapies.[57] An implication of these data is that a patient with ALL (and other pediatric cancers) has multiple subclones with malignant potential. This implication in turn implies that distinct subclones should be generated and sustained by genetically distinct (and diverse) self-renewing stem cells. This situation has been shown to be the case for ALL by comparing single-cell genetics and clonal phylogenies of diagnostic samples with those generated by serial transplantation in NOD/SCID-IL2R$\gamma^{-/-}$ (NSG) mice.[54] Stem cells are the "units of selection" in the evolutionary development of cancer,[22] and as such their genetic diversity and plasticity of phenotype (including quiescence option) is a key or even possibly the key parameter in clinical outcome.

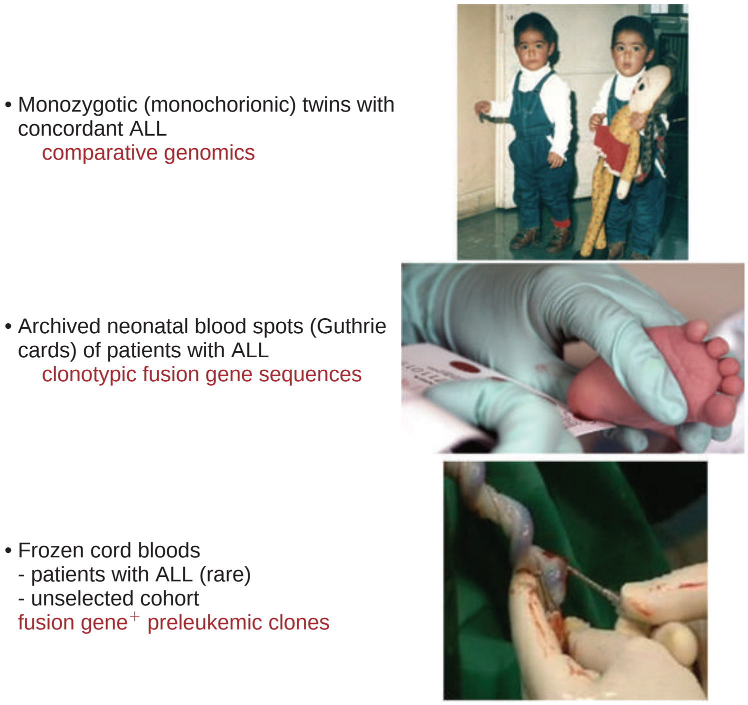

• Monozygotic (monochorionic) twins with concordant ALL
comparative genomics
• Archived neonatal blood spots (Guthrie cards) of patients with ALL
clonotypic fusion gene sequences
• Frozen cord bloods
- patients with ALL (rare)
- unselected cohort
fusion gene+ preleukemic clones

Figure 39-5 Three strategies for backtracking the prenatal origins of childhood leukemia. *ALL,* Acute lymphoblastic leukemia. *(Reviewed in references 44 and 47. The photograph of the twins is used with the permission of the parents.)*

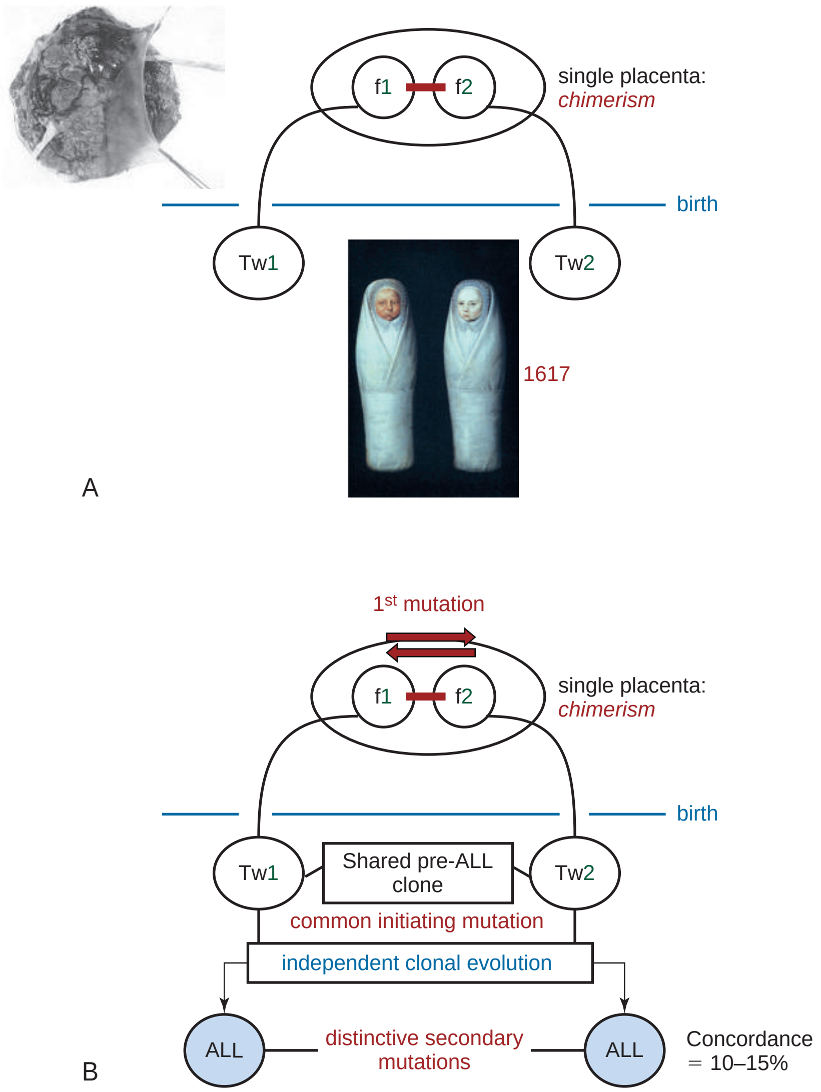

f1
f2
single placenta:
chimerism
birth
Tw1
Tw2
1617
A
1st mutation
f1
f2
single placenta:
chimerism
birth
Tw1
Shared pre-ALL clone
Tw2
common initiating mutation
independent clonal evolution
ALL
distinctive secondary mutations
ALL
Concordance = 10–15%
B

Figure 39-6 Leukemia in identical twins. **A,** Blood cell chimerism in monozygotic, monochorionic twins. The photograph of the twins is from a 1617 painting of Dutch twins who likely had twin-twin transfusion syndrome (the twin on the right is anemic and the one on the left is polycythemic, which is a common morbidity of twinning). The upper left photograph is of a single (monochorionic) placenta. Two cords are visible at 2 and 7 o'clock. **B,** Concordance of leukemia and divergent clonal evolution in monozygotic, monochorionic twins. *ALL,* Acute lymphoblastic leukemia; *f1, f2,* two fetuses with shared blood circulation (via vascular anastomoses); *Tw1, Tw2,* two twins after birth. *(**A,** Photograph of painting of twins from Berger HM, de Waard F, Molenaar Y: A case of twin-to-twin transfusion in 1617.* Lancet *356:847–848, 2000.)*

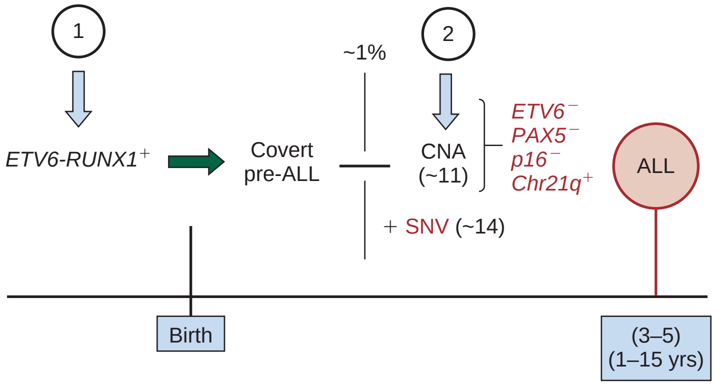

1
2
~1%
ETV6-RUNX1+
Covert pre-ALL
CNA (~11)
ETV6−
PAX5−
p16−
Chr21q+
ALL
+ SNV (~14)
Birth
(3–5) (1–15 yrs)

Figure 39-7 Sequential evolution in B-cell precursor acute lymphoblastic leukemia (ALL). *Chr21q+,* Extra copy of most of long arm of chromosome 21; *CNA,* copy number alterations (~7): average number per patient sample; *ETV6*$^{-}$, *PAX5*$^{-}$, one copy/heterozygous loss of *ETV6; p16*$^{-}$, loss of one copy of *p16/CDKN2A*; *SNV,* single nucleotide variants, nonsynonymous (~7): average number per patient sample; *3-5 years,* peak age incidence of diagnosis; *1-15 years,* age range of diagnosis of *ETV6-RUNX1*$^{+}$ ALL; *~1%,* probability of transition between covert preleukemia and overt, clinically silent diagnosed ALL. *(From Mori H, Colman SM, Xiao Z, et al: Chromosome translocations and covert leukemic clones are generated during normal fetal development.* Proc Natl Acad Sci U S A *99:8242–8247, 2002.)*

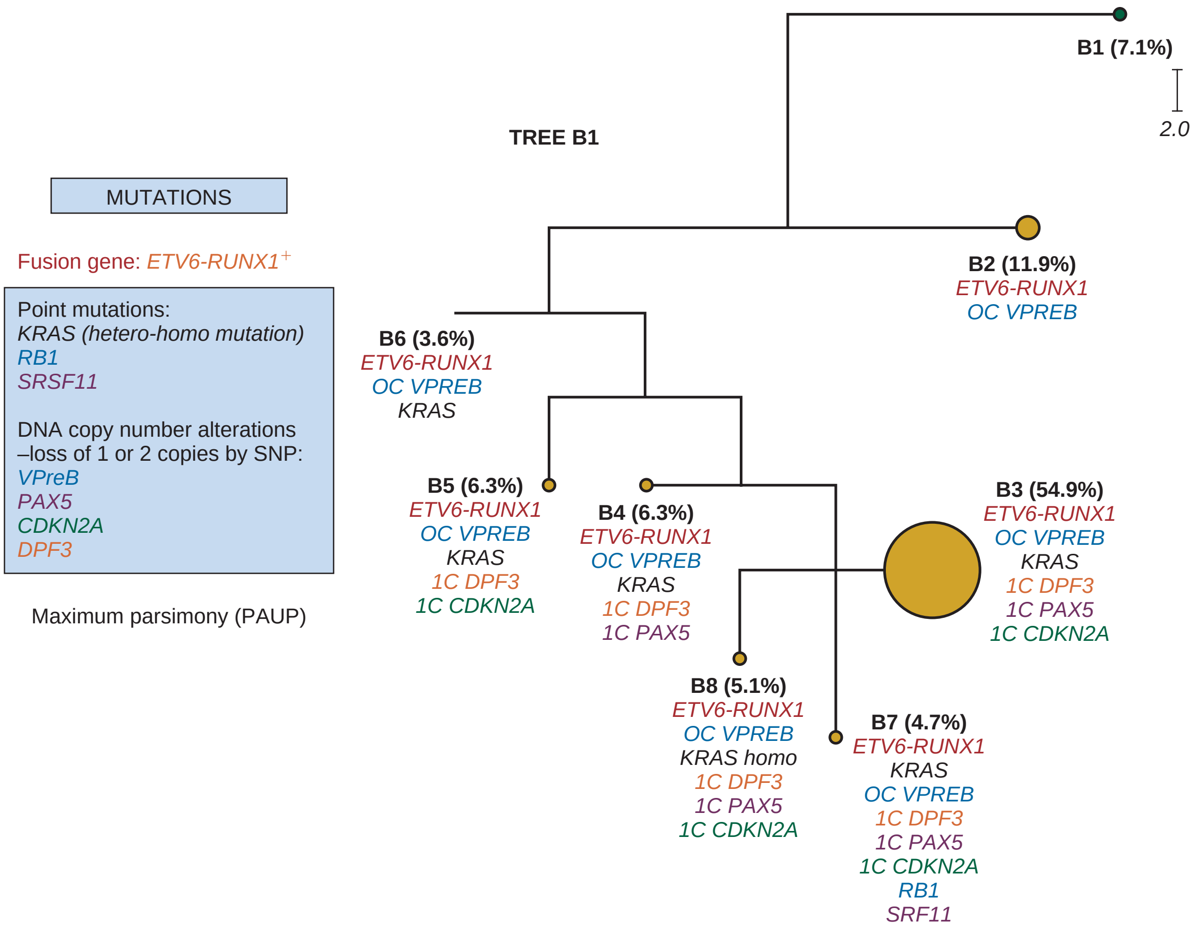

TREE B1
B1 (7.1%)
2.0
MUTATIONS
Fusion gene: ETV6-RUNX1+
Point mutations:
KRAS (hetero-homo mutation)
RB1
SRSF11
DNA copy number alterations –loss of 1 or 2 copies by SNP:
VPreB
PAX5
CDKN2A
DPF3
Maximum parsimony (PAUP)
B2 (11.9%) ETV6-RUNX1 OC VPREB
B6 (3.6%) ETV6-RUNX1 OC VPREB KRAS
B5 (6.3%) ETV6-RUNX1 OC VPREB KRAS 1C DPF3 1C CDKN2A
B4 (6.3%) ETV6-RUNX1 OC VPREB KRAS 1C DPF3 1C PAX5
B3 (54.9%) ETV6-RUNX1 OC VPREB KRAS 1C DPF3 1C PAX5 1C CDKN2A
B8 (5.1%) ETV6-RUNX1 OC VPREB KRAS homo 1C DPF3 1C PAX5 1C CDKN2A
B7 (4.7%) ETV6-RUNX1 KRAS OC VPREB 1C DPF3 1C PAX5 1C CDKN2A RB1 SRF11

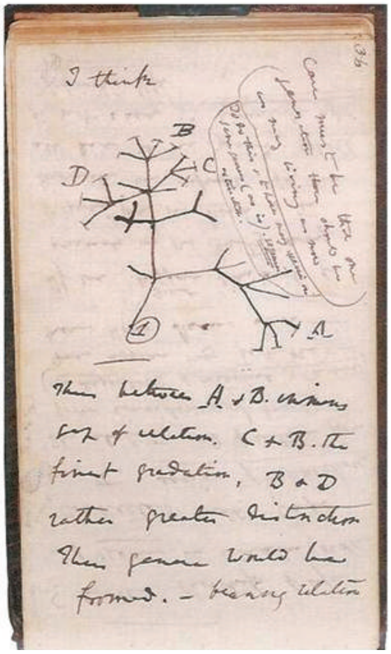
Figure 39-8 Inferred clonal architecture and phylogenetic tree in acute lymphoblastic leukemia (ALL). B1, Normal cells; B2-7, ALL subclones with individual genotypes listed alongside with the percentage representation in the entire population. B3 at 54.0% is the dominant subclone. Mutations listed were determined by whole exome sequencing (depth 100×). Deoxyribonucleic acid copy number alterations were determined by single nucleotide polymorphism array.[55] Single cells were flow sorted and genotyped by multiplex quantitative polymerase chain reaction using the Fluidigm BioMark microfluidic platform (Fluidigm, South San Francisco, Calif.). Maximum parsimony (PAUP) was the method used for assembling the phylogenic tree and the software that was used. *(From Potter N, Ermini L, Papaemmanuil E, et al: Single cell mutational profiling and clonal phylogeny in cancer.* Genome Res *23:2115–2125, 2013.)*

Another marked feature of ALL clonal architecture and its genetic variegation is the finding that the same gene is frequently altered, or mutated, independently in distinct subclones (or phylogenetic branches) of ALL in individual patients.[54,58] Reiterated gene mutation is seen in some other leukemias and solid tumors[16] and presumably reflects strong selection for particular gene alterations that have an impact on cell fitness. Additionally, however, some mutations may not be entirely stochastic or random. Bioinformatic scrutiny of *ETV6-RUNX1*–positive ALL genomes have revealed that half or more of all recurrent copy number alterations, mostly deletions, have lymphoid recombinase, *RAG1/RAG2* recognition motif heptamer nonamer sequences close to the break points.[59] Recombination-activating genes are expressed constitutively in normal B- and T-cell precursors as part of the physiologic program of immunoglobulin heavy chain and T-cell receptor gene rearrangement.[60] These enzymes continue to be expressed in ALL, where they may saturate their physiologic targets and mutate other substrates.

EVOLUTIONARY PENETRANCE OF DISEASE

Pediatric cancers are rare in human and animal species. Were they not, their highly deleterious impact on reproductive fitness (prior to the advent of successful therapy) would have led, by natural selection, to more potent suppressive mechanisms. The evolution of complex multicellular organisms required the elaboration of suppressor gene functions[31,61] to ensure cohesion and integration of diverse cell functions. That this mechanism so clearly fails in ageing adult humans is, we assume, a reflection of the weak impact of natural selection postreproductively and the chronic exposure patterns associated with human societies that can overwhelm suppression.[14]

It might then be assumed that the 1 in 800 risk of childhood cancer is a compound of the various components of risk (Fig. 39-2). However, it is well recognized that the evolutionary progression of adult cancers is both sluggish and inefficient because substantially more covert, early or premalignant lesions (e.g., carcinomas in situ) exist than ever emerge as a clinical malignancy.[62] This situation



36

I think

Thus between A & B. immens
gap of relation. C & B. the
finest gradation, B & D
rather greater distinction
Thus genera would be
formed. — bearing relation

Figure 39-9 Branching phylogeny in speciation as depicted by Charles Darwin in his 1837 notebook. Founder individual 1 gives rise via a branching architecture to descendents A, B, C, and D.

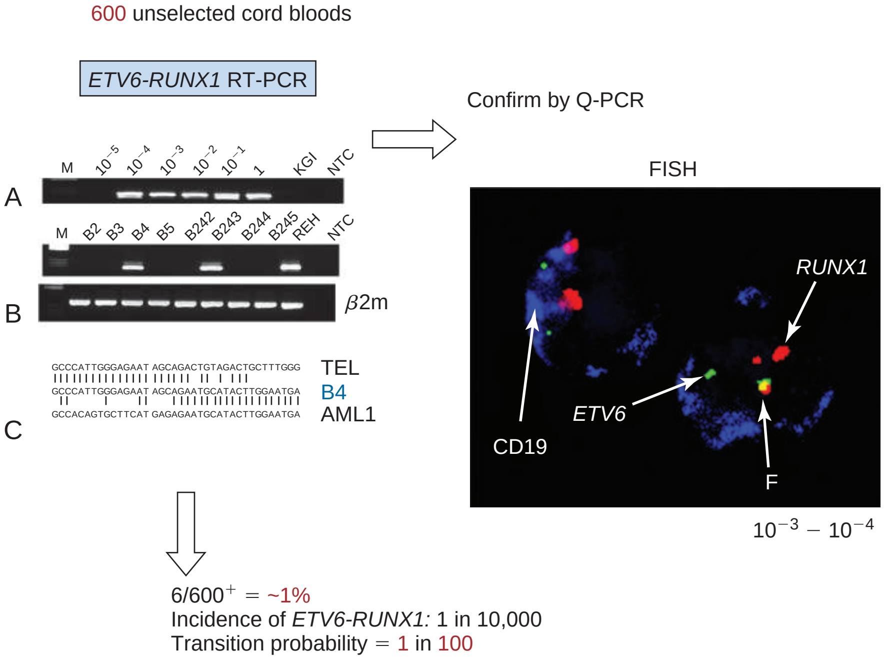
raises an interesting question with regard to pediatric cancer: how often is the disease initiated prenatally but then stalls or regresses and fails to evolve to overt cancer? Presumably more frequently than 1 in 800 instances? An answer to this question has been determined for ALL using a tactic available for bloodborne premalignant cells. Careful molecular screening of a large series (approximately 600) of unrelated cord blood samples revealed that expanded clones of *ETV6-RUNX1*–positive B lineage cells were present at some 100 times the rate of clinical ALL with the same fusion gene (Fig. 39-10).[63]

Is this result peculiar to childhood leukemia? Probably not. Autopsy histopathologic data suggest a similar approximately 100-fold incidence of the premalignant lesions for neuroblastoma[64] and Wilms tumor[65] (resulting in approximately a 1% incidence in newborns). This finding presumably reflects a low probability of the acquisition of necessary secondary genetic events. This low penetrance could be due to a limited life span of the premalignant clone, an absence of the required promotional impact of postnatal proliferative stress, altered microenvironmental selective pressures, or an influence of inherent genetic susceptibility.

EVOLUTIONARY DYNAMICS AND CLINICAL OUTCOME

The clonal dynamics of cancer are extremely variable, but the time frame to overt malignancy for most pediatric cancers is measured in a few years versus a few decades in adults. As in ecologic speciation,[66] the pace of evolution is not constant or steady; periods of stasis or slow, parallel clonal evolution and codominance (or even regression) are occasionally punctuated by major selective sweeps where, in cancer, one subclone emerges to dominance. Genetic analysis of both adult[67] and pediatric cancers[68] suggests that these key transition times in clonal evolution occur when in situ tumors become diffuse, with metastases and posttherapeutic recurrence or relapse. These changes reflect the imposition of stringent selective pressures.

In contrast with the common adult carcinomas, a striking feature of pediatric cancers is the low mutational burden.[69] Most pediatric cancers have around 10 nonsynonymous exomic nucleotide alterations compared with



600 unselected cord bloods
ETV6-RUNX1 RT-PCR
Confirm by Q-PCR
A
M
10⁻⁵
10⁻⁴
10⁻³
10⁻²
10⁻¹
1
KGI
NTC
M
B2
B3
B4
B5
B242
B243
B244
B245
REH
NTC
B
β2m
C
GCCCATTGGGAGAAT AGCAGACTGTAGACTGCTTTGGG
GCCCATTGGGAGAAT AGCAGAATGCATACTTGGAATGA
GCCACAGTGCTTCAT GAGAGAATGCATACTTGGAATGA
TEL
B4
AML1
FISH
RUNX1
ETV6
CD19
F
10⁻³ – 10⁻⁴
6/600⁺ = ~1%
Incidence of ETV6-RUNX1: 1 in 10,000
Transition probability = 1 in 100

Figure 39-10 Determining the transition probability for acute lymphoblastic leukemia (ALL). Multicolor fluorescence in situ hybridization of interphase nuclei (of cord blood cells). The cell on the left has two normal signals of *RUNX1 (red)* and *ETV6 (green)* and is normal. The cell on the right has fused *(yellow) ETV6-RUNX1* signal, plus loss of one *ETV6 (green)* signal with one normal *RUNX1 (red)* signal and one remnant *RUNX1 (small red)* signal resulting from translocation. 10^{-3}-10^{-4}: Frequency of positive cells within the CD19+ *(blue)* population. Reverse transcription polymerase chain reaction *(RT-PCR)* for *ETV6-RUNX1* (ribonucleic acid). **A,** Dilution series using positive cell line (REH) versus negative cell line (KG1) and no template control (NTC). **B,** Results with eight cord blood samples. B4 and B243 give positive results. **C,** Sequencing of amplicons from sample B4 showing a bona fide, in frame *ETV6-RUNX1* sequence. *(Data from Mori H, Colman SM, Xiao Z, et al: Chromosome translocations and covert leukemic clones are generated during normal fetal development.* Proc Natl Acad Sci U S A *99:8242–8247, 2002.)*

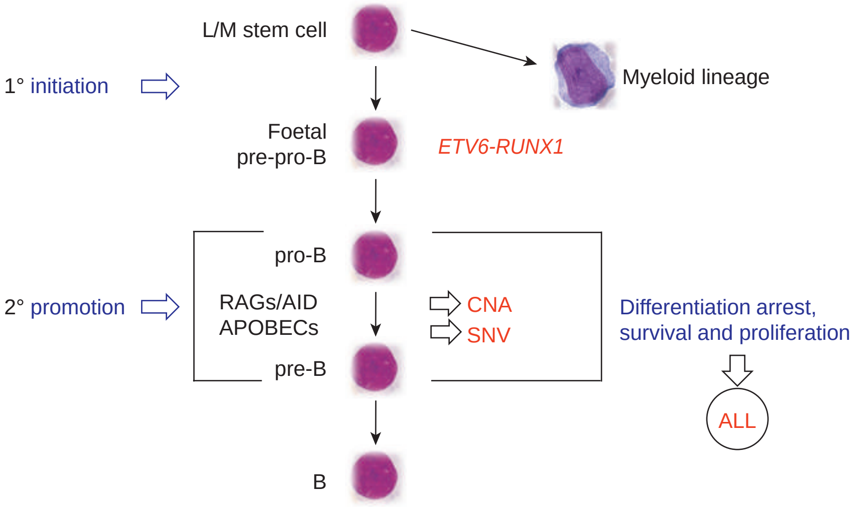


Figure 39-11 Developmental model for childhood B-cell precursor acute lymphoblastic leukemia (ALL). Lymphomyeloid (L/M) stem/progenitor cells. The model shows preleukemic ALL clones initiated (by gene fusion or hyperdiploidy) in the fetal, early pre-pro-B stage. An overt ALL clone with secondary genetic changes (copy number alterations [CNA]/single nucleotide variants [SNV]) becomes trapped in differentiation arrest. Off-target RAG1/RAG2 recombinase activity (R) generates reiterated and highly recurrent CNA. APOBEC enzymes induce SNV.[59]

50 to 70 for many adult cancers and several thousand for the adult cancers associated with known genotoxic exposures (e.g., lung cancer and melanoma).[69] Part of this distinction can be attributed to the frequent presence of genetic instability in many adult cancers, which itself is a possible adaptive cellular response to genotoxic exposure.[70] However, it is also possible that within the architectural constraints of a structured epithelium, more restraints on cancerous growth must be abrogated by mutations. The minimal number of mutations necessary for the evolution of malignant pediatric cancer is difficult to determine, but it could be as few as two or even one for rare pediatric cancers in which the initiating genetic lesion is a major chromatin modifier with pleiotropic impact on multiple pathways, such as *MLL-AF4* fusion in infant ALL[71] and *SMARCB1* in rhabdoid cancer.[72] Two mutations might be sufficient for most pediatric cancers if these mutations effectively uncoupled proliferation from differentiation and if, in the developmental context, this uncoupling caused relatively rapid onset of a diagnosable disease, leading to relatively early detection of these cancers in their evolutionary trajectory.

These considerations may be relevant to the success in treatment of pediatric cancer. Prior to the advent of combination chemotherapy, all cases of childhood ALL were universally fatal within around 12 months.[73] The current long-term remission and possible cure rate for *ETV6-RUNX1*+ ALL is close to 100%.[74] Other rarer subtypes of ALL are considerably more intransigent.[75] What is it about the evolutionary biology of the major, or common, subtypes of pediatric ALL that render them curable with use of relatively generic cytotoxics? In adult cancers, evidence indicates that the extent of clonal, genetic diversity is a strong predictor of progression of disease[76] or eventual clinical outcome.[77] One interpretation of this situation is that genetic diversity is fueled by genetic instability and high diversity, which substantially increases the likelihood of acquisition of drug-resistant mutations. Minimal genetic diversity arising at the time of "early" diagnosis may therefore be critical to the curability of *ETV6-RUNX1*+ ALL. However, other factors probably also contribute, including an origin from a progenitor (B) population that is intrinsically very sensitive to deoxyribonucleic acid (DNA) damage and apoptosis.[78] The same dynamic may apply to embryonic progenitors that give rise to other pediatric cancers that are intrinsically sensitive to radiation and chemotherapy.[25] In addition to mutation rate or load, the particular mutations selected may also be crucial. Adult cancers with genetic instability have a very high frequency of *TP53* mutations or deletions,[79] which may reflect an adaptive cellular response to DNA damage; the downside is resistance to chemotherapy. *TP53* mutations are rarer in pediatric cancer and absent from *ETV6-RUNX1*$^+$ ALL.[59] Also missing from curable *ETV6-RUNX1*$^+$ ALL are kinase mutations[59] that are a hallmark feature of high-risk childhood ALL.[75] Although deletions of the cell cycle inhibitor *CDKN2A* (*p16*) are very common in all ALL (and other pediatric cancers), the lack of proliferative "driver" oncogene mutations (i.e., activated kinases) is striking. This finding supports the notion that the common variants of childhood ALL and possibly several other types of pediatric cancer, including Wilms and germ cell tumors, are in effect minimally modified, albeit expanded, clones in differentiation arrest and that this status renders them chemosensitive (Fig. 39-11).

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CHAPTER 40

Epidemiology of Leukemia in Childhood

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CHAPTER OUTLINE

DESCRIPTIVE EPIDEMIOLOGY
International Patterns
Age-Specific Incidence and Sex Ratio
Ethnic Origin
Socioeconomic Status
Time Trends
Clustering of Leukemia in Space and Time
GENETIC FACTORS AND FAMILIAL AGGREGATION
Concordance of Childhood Leukemia in Twins
Leukemia and Cancer in the Families of Children with Leukemia
Cancer in Offspring of Patients Treated for Childhood Leukemia
Other Conditions in Relatives of Patients with Leukemia
Human Leukocyte Antigen–DR and Susceptibility to Childhood ALL
Susceptibility to Childhood ALL: Genetic Polymorphisms
PRENATAL ORIGIN OF CHILDHOOD ALL
INFECTION
The Kinlen Hypothesis: Residence in Areas with High Population Mixing
Infections during the Index Pregnancy
Antibiotic Use and Immunizations during the Index Pregnancy
Association of Childhood Leukemia with Factors Related to the Immune System
Breastfeeding
Immunizations
Sibship Size
Early Child Care and Preschool Experiences
Infection and Related Exposures during the Lifetime of the Index Child
Chloramphenicol
Index Child's Contact with Animals
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Leukemias of childhood are a highly heterogeneous group of diseases. In reviewing the descriptive and analytic epidemiology of these malignancies, we have, when possible, emphasized specific subgroups as defined by morphologic, cytogenetic, or molecular features. In selected instances, evidence indicates that specific subgroups of leukemia may have distinct causes and that molecular abnormalities associated with particular subgroups may be linked with specific causal mechanisms. In assessing risk factors, studies of the childhood leukemias present several methodologic advantages compared with those addressing adult leukemias. For example, because the interval between exposure to putative risk factors and the onset of leukemia is shorter, recall of exposures is likely to be more accurate and intervening factors are less likely to be of importance than are those associated with adult leukemias. These characteristics of childhood leukemia better lend themselves to an approach that includes both population studies and molecular epidemiologic techniques, permitting the design of studies to assess interactions between genes and the environment. However, in striking contrast to the impressive advances in the treatment and biology of childhood leukemia, remarkably little has been achieved regarding our understanding of the cause of leukemia, the most common form of childhood malignancy.

DESCRIPTIVE EPIDEMIOLOGY

Leukemias are the most common cancers affecting children, accounting for 32% of all occurrences of cancer among children younger than 15 years and 27% of occurrences of cancer among children younger than 20 years.[1] Each year in the United States, leukemia is diagnosed in approximately 3540 children who are younger than 20 years. Of these occurrences, acute lymphoblastic leukemia (ALL) accounts for 73%, acute myeloid leukemia (AML) accounts for approximately 18%, and chronic myeloid leukemia (CML) is rarely seen, accounting for less than 4%.

International Patterns

A wide variation exists in the incidence of childhood leukemia by geographic location. The highest annual incidence rates are reported in Costa Rica, Ecuador, Hong Kong, Denmark, and Singapore (57.9 to 51.0 per million population), whereas some of the lowest rates are found in Zimbabwe, India, Israel, and Algeria (23.1 to 26.0 per million).[1] The annual incidence of childhood leukemia for many regions, including North America, Australia, Northern and Western Europe, China, Japan, the Philippines, and Singapore, ranges between 35 and 50 per million (Table 40-1). When evaluating geographic variation in disease incidence, concerns always exist regarding the quality and completeness of reporting. Ecologic studies of childhood leukemia incidence according to annual per-capita gross national income demonstrates substantial variation within low-income countries and a much narrower range in middle- and high-income countries.[2] In addition, a significant correlation exists between the incidence of childhood leukemia and population mortality rates for children younger than 5 years in low- and middle-income countries. Some of the lowest reported childhood leukemia rates are within countries with high mortality rates for children younger than 5 years, suggesting that children with undiagnosed leukemia may die in some low-income countries as a result of anemia or fever that is attributed to infectious disease.

Age-Specific Incidence and Sex Ratio

The incidence rate for all leukemias is highest among children younger than 5 years and decreases with age.[3] The age-adjusted incidence for boys exceeds that for girls, with a sex ratio typically between 1.1:1 and 1.4:1.[1]

TABLE 40-1 Incidence of all Childhood Leukemias, ALL, and AML in Selected International Sites

	ANNUAL INCIDENCE RATE (PER MILLION POPULATION)*		
Country	**Leukemia**	**ALL**	**AML**
Costa Rica	57.9	46.3	8.9
Ecuador	56.3	39.6	9.1
Hong Kong	53.9	40.6	3.9
Denmark	53.0	42.8	8.4
Singapore	51.0	39.5	6.9
Canada	50.8	41.0	6.3
Finland	49.9	41.9	5.4
Australia	49.9	39.9	8.0
Italy	49.1	37.9	7.9
Sweden	48.7	40.1	6.7
Norway	48.6	38.3	8.0
Philippines	48.1	25.2	8.6
United States (white)	46.9	38.0	6.0
Germany	46.6	39.0	6.7
Uruguay	44.0	29.3	7.4
Colombia	42.8	31.5	6.4
United Kingdom	40.8	32.8	6.3
France	40.5	31.7	6.1
China	40.3	17.4	6.7
Netherlands	40.1	30.9	5.8
Japan	38.5	22.6	7.2
Korea	38.1	20.2	8.7
Cuba	37.7	25.4	5.7
Czech Republic	37.1	28.4	5.1
India (Delhi)	36.0	23.1	6.1
Peru	35.9	25.4	6.9
Brazil	33.7	21.9	5.3
Thailand	29.8	19.8	5.8
United States (black)	29.4	20.8	6.2
Algeria	26.0	14.3	5.7
Israel	25.7	18.6	5.3
India (Bombay)	25.4	16.0	4.8
Zimbabwe	23.1	11.6	11.0

Data from Parkin DM, Kramárová PE, Draper GJ, et al, editors: International incidence of childhood cancer, *vol II (IARC Scientific Publications No. 144). Lyon, France, 1998, International Agency for Research on Cancer.*

ALL, Acute lymphocytic leukemia; *AML,* acute myeloid leukemia.

*Children 0 to 14 years of age.

Acute Lymphoblastic Leukemia

In developed countries, the age-incidence curve for ALL is characterized by a peak between the ages of 1 and 4 years (Fig. 40-1).[4] Thus a sharp peak in ALL incidence is seen among 2- to 3-year-olds (>100 per million), which decreases to a rate of 20 per million for 8- to 10-year-olds. The incidence of ALL among 2- to 3-year-olds is approximately fourfold greater than that for infants and is nearly tenfold greater than that for 19-year-olds. Although this age distribution is well recognized and attributable to common ALL (cALL),[5] it has not always been present. It was first observed in England and Wales in the 1920s,[6] among American whites in the 1940s,[6] among African Americans[7] and in Japan in the 1960s,[8,9] and among Jews and non-Jews in Israel in the 1970s.[10] In Kuwait, where the incidence was low during the 1970s, the age peak has recently been observed, whereas in the African series, in which the incidence is also low, the peak has not yet been reported.[11]

An exaggerated peak incidence among 2- to 3-year-olds, with a very low incidence of ALL in older children, was observed in certain areas of England and Wales and in the rural north of Scotland and coincided with a high socioeconomic status. These observations have led to a hypothesis that an elevated risk of ALL is influenced by community characteristics such as isolation, high socioeconomic status, and population mixing,[12] which in turn are related to immunologic isolation in infancy and influence patterns of exposure to common infectious agents in the early years of life before the appearance of leukemia.[13] These hypotheses are discussed in detail later. The ratio of incidence rates of ALL for boys and girls ranges between 1.1 : 1 and 1.3 : 1.[11]

Acute Myeloid Leukemia

Among children, the incidence rates for AML are highest in infancy and are fairly uniform in older children (see Fig. 40-1).[4] In the United States, during 1986 to 1995, the incidence in the 0- to 4-year-old age group was 10.3 per million, and in the 5- to 9-year-old and 10- to 14-year-old groups it was 5.0 per million and 6.2 per million, respectively. Comparable rates have been reported elsewhere in Europe and in England. The male-to-female ratio is close to unity for AML.[1]

Chronic Myeloid Leukemia

The incidence rates of CML in one of the largest series, originating from the United Kingdom, were 1.2 per million for infants and 1.6, 0.5, and 0.7 for children aged 1 to 4 years, 5 to 9 years, and 10 to 14 years, respectively,[14] which are comparable to rates observed in the United States for these age ranges.[4] The rate for CML increases during the 15- to 19-year age interval. The male-to-female sex ratio is 1.6 : 1.[1]

Myelodysplastic Syndrome and Other Myeloproliferative Diseases

The International Classification of Childhood Cancer[15] includes a classification for myelodysplastic syndrome (MDS) and other myeloproliferative diseases. In the 0- to 19-year age group, rates for MDS are very similar to those for CML; however, in contrast, the incidence of MDS is highest among infants and drops essentially to 0 by age 8 years.[4] The incidence is almost twice as high among males.

Ethnic Origin

In the United States, substantial variation is seen in the incidence rates of ALL by ethnicity. The highest rates are reported among Hispanics, Filipinos, and Chinese, and the lowest rates are reported among African Americans.[4] The incidence rates among whites are moderate to high by international standards, whereas those for American Indians are somewhat lower.

On the other hand, two studies have investigated the incidence of childhood cancer by ethnicity in England, but results were negative. The lack of variation in the incidence of ALL among ethnic groups in England, in conjunction with the markedly lower incidence of ALL in the Indian subcontinent and Africa than in England, suggests that the incidence of ALL depends to some extent on environmental factors in association with geographic location. The contrast between the ethnic variations in incidence could be a reflection of the socioeconomic differences by ethnicity (or the lack thereof) in the two countries.[14] A recent report from the Malaysia-Singapore Leukemia Study Group describes differences in prognostically important chromosomal abnormalities among an unselected multiethnic Asian population.[16] Ethnic groups differed significantly with regard to the proportion of patients with ALL who had T-cell ALL (the incidence is higher in Chinese and Indians), *ETV6-RUNX1* (with a lower incidence in Chinese persons), and *BCR-ABL* (with a lower incidence in Indian persons). Beyond ethnic differences in the occurrence of childhood ALL, it has been well documented that race/ethnicity is associated with prognosis.[17] The black-to-white ratio of the incidence of AML in the United States is 0.85. The rates are highest among Hispanics.[1,18] This higher rate of AML contributed to the higher incidence of acute promyelocytic leukemia seen among Hispanics than among non-Hispanics, thus raising the question of genetic predisposition to acute promyelocytic leukemia or exposure to distinct environmental factors.[19] As with ALL, race/ethnicity also has prognostic importance.[20]

Socioeconomic Status

Socioeconomic factors have been proposed as an explanation for the age peak in childhood leukemia.[21] Thus it has been hypothesized that with economic development, poorer communities move from a situation in which leukemia is rare (T-cell ALL is the predominant type of ALL) through an intermediate stage in which cALL begins to appear to a state of high socioeconomic status that is associated with a high incidence of ALL and cALL. Numerous descriptive studies have investigated the relationship between leukemia and socioeconomic status.[22-28] In the vast majority of these studies, the area of residence of the patients (or those who died) was used as a measure of socioeconomic status. Other measures used included household income and years of schooling. In most of

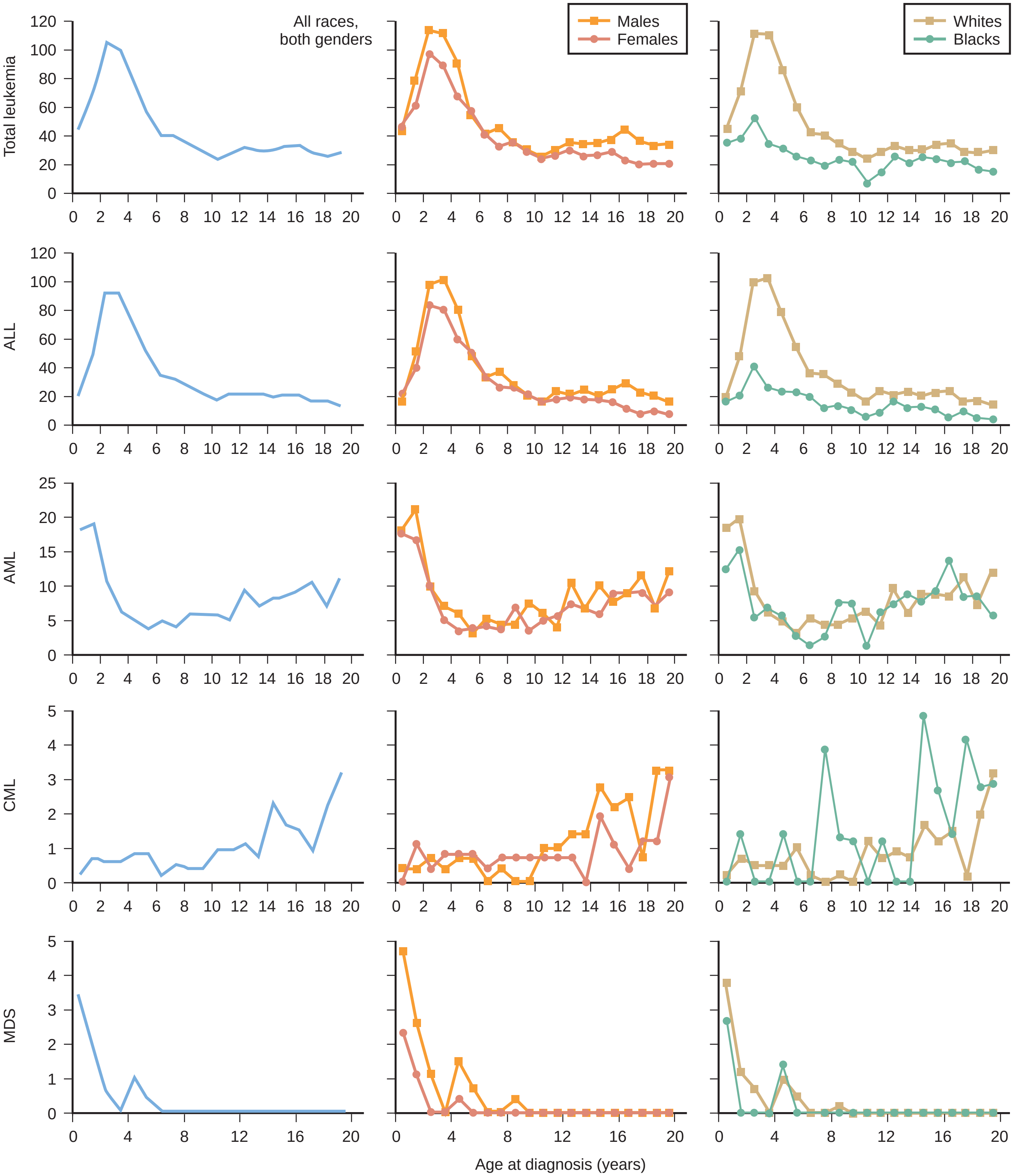

Figure 40-1 Incidence of leukemia (per million children) by age at diagnosis. *ALL,* Acute lymphoblastic leukemia; *AML,* acute myeloid leukemia; *CML,* chronic myeloid leukemia; *MDS,* myelodysplastic syndrome.

these studies, a weak positive association between leukemia and high socioeconomic status was observed, with a few exceptions.[24,27,29-31] In contrast, several investigators have examined the relationship between AML and socioeconomic status and have failed to show an association.[23]

Time Trends

Several investigators have examined temporal trends for leukemia, but the findings are difficult to interpret,[32-37] primarily because of the varying periods covered and different methods of analysis used. Some investigators analyzed the 0- to 14-year-old age group as a whole, whereas others examined trends in specific age groups. Some investigators reported trends for each sex, whereas others combined the two sexes in their analyses. In some analyses all leukemias were grouped together, whereas others conducted their analyses by the leukemia subtype. In addition, diagnostic improvements have led to more accurate and precise classification of leukemias over time, which could contribute to the observed temporal trends in incidence.

In the studies reporting an increase in the incidence of ALL, the increase is fairly modest and is confined to the 0- to 4-year-old age group. Moreover, in some reports, the increase in incidence of ALL appeared to be accompanied by a decrease in the incidence of AML or other leukemias, which could reflect diagnostic shifts and more accurate and precise classification.[38-40] A temporal increase in the incidence of childhood ALL has been reported in England[41] and is attributed to an increase in the precursor B-cell subtype.[42] Between 1974 and 2000, the average annual increase in childhood ALL was 0.7% overall but 1.4% for cALL. Similarly, analyses of childhood ALL time trends in Italy demonstrated increases, some of which were related to maternal time variables that included maternal age and maternal birth cohort, which were considered to be consistent with an infectious cause.[43,44] On the other hand, in another study, Linet and associates[45] report no substantial change in the incidence of childhood leukemia diagnosed in the United States between 1975 and 1995. The Czech national registries reported a 1.5-fold increase in childhood ALL incidence between the ages of 1 to 4 years during the period of substantial socioeconomic transition in the European postcommunist countries.[46]

Clustering of Leukemia in Space and Time

Spatial clustering of childhood leukemia has been observed to varying degrees in England,[47,48] France,[49] and Greece[50] but not in the United States.[51-55] Where spacial clustering was identified, it was strongest for leukemias diagnosed in younger children. Although spatial clustering was confined to sparsely populated areas in England, such clustering was found only in urban areas in Greece. Birch and colleagues[56] addressed the issue of space-time clustering by using the Manchester Children's Tumor Registry. All methods showed highly significant evidence of space-time clustering on the basis of place of birth and time of diagnosis of childhood leukemia. The authors concluded that the results were consistent with an infection hypothesis (discussed later). Furthermore, the investigators found an excess of instances in males versus females in space-time pairs, thus suggesting gender-specific susceptibility to infections. More recently they have reported statistically significant cross-clustering among childhood leukemia and central nervous system (CNS) tumors and between ALL and astrocytoma, suggesting possible common causes, possibly of an infectious nature.[57] The correlation between childhood leukemias and CNS tumors is of interest, given the recently reported correlation in international rates for these two pediatric cancers.[58]

Historically, clusters of childhood leukemia have been observed in which the number of cases reported exceed those expected within a given time and geographic location. Investigation of these clusters has typically not identified any causal agent. A recent leukemia cluster in the United States gained substantial public and scientific attention because of the marked excess observed within a small population.[59] Comprehensive investigation including biologic samples from persons within the population that were analyzed for chemicals, viral markers, and genetic polymorphisms, in conjunction with air, water, soil, and dust sampling, failed to identify any leukemia risk factor.[60]

GENETIC FACTORS AND FAMILIAL AGGREGATION

Certain genetic syndromes are associated with an increased susceptibility to leukemia.[61] These syndromes include Down syndrome,[62] neurofibromatosis type 1,[63] and chromosome breakage syndromes, such as ataxia telangiectasia,[64] Bloom syndrome,[65] Shwachman syndrome,[66,67] Fanconi anemia, and Langerhans cell histiocytosis.[68] Shaw and associates[69] reported an association between Klinefelter syndrome and childhood leukemia on the basis of an observation of two children with ALL who had a 47XXY karyotype. However, Hasle and colleagues[70] subsequently reported no increase in the incidence of childhood leukemia in a cohort of 696 men with Klinefelter syndrome. In a recent study of malformation syndromes among children with cancer, an association was reported between Bardet-Biedl syndrome and ALL.[71]

Although specific associations have been described mainly as case reports, data on the proportion of instances of leukemia that are known to have a genetic etiology or are associated with specific genetic syndromes are limited. In a study from England, 2.6% of children with leukemia were reported to have a recognized genetic condition, and this percentage was almost entirely accounted for by Down syndrome (2.3% of all leukemias).[72] This proportion is similar to that found in studies from the United States and Nordic countries.[73-75]

Population-based studies using record-linkage approaches have documented that approximately 97% of cancers occurring among children with Down syndrome are leukemia.[76] Persons with Down syndrome have an estimated 56-fold increase in leukemia between the ages of 0 to 4 years, which declines to tenfold for leukemia risk between the ages of 5 to 29 years. More recently, interest has been increasing in the identification of environmental factors that may contribute to the development of leukemia among patients with Down syndrome.[77] Case-control investigations comparing persons with Down syndrome who have leukemia with persons with Down syndrome who do not have leukemia have found that reduced risk of leukemia is associated with maternal vitamin supplementation in the periconceptional period and reported history of infection in the first 2 years of life.[78,79]

Concordance of Childhood Leukemia in Twins

Assessment of concordance for childhood leukemia in twins may provide etiologic clues. If childhood leukemia were to have a predominantly genetic cause, monozygotic twins would be expected to be concordant for leukemia more often than dizygotic twins because of their genetic similarity. If childhood leukemia were caused by an abnormal intrauterine environment, it would be expected that if one member of a dizygotic twin pair were affected, the other twin would be affected more often than a nontwin sibling because of the shared environment. Recently, in a study of leukemia in a pooled series from the United States, Canada, and England, only three concordant pairs (1.5%) were found among 197 pairs in which one or both twins had leukemia, with the concordance rate for monozygotic twins reported to be 3.9% (95% confidence interval [CI], 0.8 to 11.1).[80] Although the concordance rate in twins of like sex (probably monozygotic twins) is higher than the zero concordance rate reported for twins of unlike sex (dizygotic twins), the concordance rate in twins of like sex is quite variable.[80,81] These findings suggest that inherited genetic factors most likely play a relatively small part in the cause of childhood leukemia.

The occurrence of concordant leukemic pairs has been hypothesized to be due to parallel expansions of clones descended from a single, ancestral cell transformed in utero, with a malignant clone arising in one monozygotic twin and entering the circulation of the co-twin through anatomizing placental vessels. This mechanism would account for the early and similar age of onset of leukemia and the similar cytogenetic findings.[82,83] Support for this hypothesis is provided by the recent observation of shared clonal but nonconstitutional mixed lineage leukemia gene (*MLL)* rearrangements in the leukemic cells of three pairs of monozygotic twins concordant for infant acute leukemia.[84] The rearrangements were not observed in nonleukemic cells of the twins and were not present in the maternal or paternal blood of two of the three twins.

Leukemia and Cancer in the Families of Children with Leukemia

In studies of leukemias of all types combined, no excess of cancer was observed among siblings, parents, or offspring.[85-91] In studies focusing on acute leukemia, an excess of acute leukemia was observed among siblings, in whom the expected rates were extremely low.[92-94] Limitations of these studies include the inclusion of distant relatives, in whom verification of cancer becomes problematic, incomplete follow up, and, finally, the inclusion of families with a history of consanguinity.

Cancer in Offspring of Patients Treated for Childhood Leukemia

Few studies have reported the rate of occurrence of cancer in the offspring of patients treated for childhood leukemia. Only recently have enough patients survived that the number of their offspring permitted estimates of their risk of developing malignant neoplasms. After performing a review of the literature, Hawkins and associates[89] estimated that the proportion of heritable cases among survivors is unlikely to exceed 5%, assuming that the age of onset of all heritable cases is 15 years of age or younger and that the diseases had a penetrance of 70% or more. Chromosomal instability was examined in 20 apparently healthy children of survivors of childhood malignancy. Compared with control subjects, no increases in spontaneous or bleomycin-induced aberrations were found in these “index children.” The results suggest that the offspring of subjects who previously received chemotherapy, radiotherapy, or both for childhood malignancy probably have no increased risk of latent chromosomal instability.[95]

Other Conditions in Relatives of Patients with Leukemia

Savitz and Ananth[96] reported an excess of major birth defects among the siblings of patients with ALL (relative risk [RR], 3.2; 95% CI, 1.3 to 7.7; adjusted for age at diagnosis, sex, and year of diagnosis). Mann and colleagues[97] reported an excess of congenital defects among parents, uncles, aunts, and other distant relatives of patients with ALL compared with community control subjects. Buckley and associates[98] reported an excess of a number of conditions in siblings, parents, and grandparents of index patients with various types of ALL. These conditions included musculoskeletal disorders among relatives of patients with the common cell type; gastrointestinal, hematologic, and musculoskeletal disorders and allergies for those with the pre–B-cell type; an excess of gastrointestinal disorders for those with the T-cell type; and an excess of congenital heart and lung disease for those with the null cell type. The offspring of adult survivors of childhood ALL were not found to have an increased risk of occurrence of congenital anomalies compared with the offspring of sibling control subjects.[98] Similarly, Kenney and colleagues[99] reported no excess of congenital anomalies among the offspring of adult survivors of childhood cancer compared with the offspring of sibling control subjects.

Human Leukocyte Antigen–DR and Susceptibility to Childhood ALL

Both genetic and environmental factors could possibly play an interactive role in the development of childhood ALL. Because of the demonstration of the influence of major histocompatibility complex on mouse leukemia, a human leukocyte antigen (HLA) association has been considered as a possible genetic risk factor. Dorak and associates[100] demonstrated a moderate association with the most common allele in the HLA-DR53 group, HLA-DRB1*04, which was stronger in males. In addition, homozygosity for HLA-DRB4*01, encoding the HLA-DR53 specificity, was increased among patients. Confounding these associations was a male-specific increase in homozygosity for HLADRB4*01. The cross-reactivity between HLA-DR53 and H-2Ek, extensive mimicry of the immunodominant epitope of HLA-DR53 by several carcinogenic viruses, and the extra amount of deoxyribonucleic acid (DNA) in the vicinity of the *HLA-DRB4* gene provide arguments for the case that HLA-DRB4*01 may be one of the genetic risk factors for childhood ALL. Comparison of DQA1 and DQB1 alleles in 60 children with ALL and 78 newborn infants (control subjects) revealed that male but not female patients had a higher rate of occurrence of DQA1 *0101/*0104 and DQB1 *0501 than did appropriate control subjects.[101] The authors concluded that this finding represented a male-associated susceptibility haplotype in ALL, supporting an infectious cause. Additional investigations from the United Kingdom have suggested that cALL in children may be associated with HLA-DPB1 through a mechanism that involves the presentation of specific antigenic peptides, possibly derived from infectious agents, which leads to activation of helper T cells that mediate proliferative stress on preleukemic cells.[102]

Susceptibility to Childhood ALL: Genetic Polymorphisms

In the search for gene-environment interactions in the cause of childhood leukemia, investigations have focused on the genetic variability in xenobiotic metabolism, DNA repair pathways, and cell-cycle checkpoint functions that might interact with environmental factors to influence leukemia risk. Although much of the research in this area has been limited by small sample size, data suggest a potential role for polymorphisms in genes encoding cytochrome P450, glutathione S-transferase, reduced nicotinamide adenine dinucleotide phosphate quinone oxidoreductase, methylenetetrahydrofolate reductase (MTHFR), cycle-cycle inhibitors, and DNA-repair polymorphisms.[103-111]

In general, few reports have addressed the potential role of these polymorphic genes in childhood leukemia, and in many instances they provide contradictory findings. This situation is particularly true with regard to studies of GSTM1 and GSTT1 and CYP-P450 enzymes.[112,113] Low folate intake or alterations in folate metabolism as a result of polymorphisms in the enzyme MTHFR have been associated with neural tube defects and some cancers. Polymorphic variants of MTHFR lead to enhanced thymidine pools and better quality DNA synthesis that could afford some protection from the development of leukemias, particularly those with translocations. Wiemels and colleagues[103] and Smith and colleagues[111] reported an association of MTHFR polymorphisms in infant leukemias with *MLL* gene rearrangements and childhood leukemia with either *ETV6-RUNX1* fusions or hyperdiploid karyotypes. These studies provide evidence that molecularly defined subgroups of childhood leukemias may have different causes and also suggest a role for folate in the development of childhood leukemia. In a case-control study, MTHFR genotypes TT677/AA1298 and CC677/CCC1298 were associated with a reduced risk of childhood ALL.[114] Moreover, the findings of these studies suggested a gene-environment interaction based on timing of implementation of folic acid supplementation in the Canadian population. To date, no direct gene-environment associations have been established convincingly. When considering the potential importance of the prenatal origin of childhood leukemia, it becomes important to consider the genotypic profile of the mother with respect to metabolizing enzymes. Investigation of maternally mediated genetic effects through reduced nicotinamide adenine dinucleotide phosphate quinone oxidoreductase did not affect risk for childhood ALL.[115]

PRENATAL ORIGIN OF CHILDHOOD ALL

As mentioned earlier, molecular studies on several pairs of identical twins, aged between 2 months and 14 years at diagnosis, first provided strong evidence that concordant ALL arises in monozygotic twins after mutation and clonal expansion of one cell in one fetus in utero.[84,116,117,118,119] Because the disease in the twins is not clinically or biologically different from that in singletons, Gale and associates[119] hypothesized that some of the singletons are likely to have a prenatal initiation of their leukemia. They further hypothesized that an additional event or exposure postnatally results in the clinically overt leukemia at a variable time after birth. To test these hypotheses, they used the neonatal blood spots or Guthrie cards[119] to identify the presence of clonotypic or patient-specific leukemia fusion-gene sequences *(ETV6-RUNX1)*.[120] The association between the t(12;21) and the deletion of the nontranslocated allele of *TEL* is among the most frequent abnormalities observed in B-lineage ALLs.[121] Neonatal blood samples are routinely used to screen for inherited metabolic disorders. By reverse-transcriptase polymerase chain reaction screening of blood or bone marrow, the investigators identified *ETV6-RUNX1* fusion in 12 children and a pair of identical twins, aged 2 to 5 years, with newly diagnosed ALL. They identified *ETV6-RUNX1* fusion sequences in blood spots from the identical twins and in six of the nine studied ETV6-RUNX1–positive patients. Gale and colleagues concluded that childhood ALL is often initiated by a chromosome translocation event in utero. This important observation has been more extensively investigated and characterized.[122,123] Screening of neonatal cord/blood samples has revealed a putative leukemia clone with the *ETV6-RUNX1* fusion gene in 1% of newborn babies,[124] which represents a frequency 100 times greater than the prevalence of ALL defined by this fusion gene later in childhood. In children with cALL who have a *ETV6-RUNX1* fusion gene, deletion of the unrearranged or normal *TEL* allele is a common secondary occurrence. The causal events that lead to the initial *ETV6-RUNX1* fusion in utero and any postnatal secondary mutations are not known.[125] Some epidemiologic data provide support for the idea that an abnormal immunologic response under particular social conditions could account for certain critical postnatal events.[13,126] Further epidemiologic studies need to focus on in utero exposures that could result in the initial *ETV6-RUNX1* fusion and postnatal exposures or events that could explain the secondary mutations.

INFECTION

Considerable interest and research has focused on the potential role of infection in the cause of childhood ALL through a mechanism of stimulation of an inappropriate immune response or via a direct transformation.[127] This avenue of research, proposed and spearheaded by British researchers, reflects two different but conceptually related hypothesized mechanisms.

The Kinlen Hypothesis: Residence in Areas with High Population Mixing

In 1988 Kinlen[128] hypothesized that the high incidence of leukemia in children in the vicinity of the Sellafield and Dounreay nuclear reprocessing plants in England could be due to a rare response to some unidentified mild and subclinical infection, the transmission of which is facilitated by contacts between large numbers of people. An influx of people of diverse origin into a previously isolated area would particularly facilitate transmission. Thus a high incidence of leukemia in childhood would be expected in isolated areas into which a significant influx of people had occurred. The hypothesis was first tested in an area in Scotland. Glenrothes New Town was identified a priori as an area where, after a period of relative isolation, a sudden burst of in-migration occurred in 1964 because of the opening of the Forth Road bridge.[128] A significant number of persons younger than 25 years who died of leukemia were identified during the period from 1951 to 1967. The incidence of leukemia was not excessive in the period from 1968 to 1985, when the community became much less isolated. The hypothesis has been tested in a number of other situations in England, France, and other parts of Europe and Hong Kong.* A 3.6-fold increase in childhood leukemia was observed in the wartime cohort compared with national Scottish rates in Orkney and Shetland (England's northernmost islands) during World War II, when the local people were outnumbered by servicemen stationed there. The rates normalized to the national rates in the postwar period.[141] A recent analysis of the U.S. Surveillance Epidemiology and End Results data also provided confirmatory evidence for the population mixing hypothesis, with findings that changes in rural county population sizes from 1980 to 1989 were associated with increased incidence rates for childhood ALL.[142]

Infections during the Index Pregnancy

An alternative model for the cause of childhood ALL places the critical infectious event during pregnancy rather than early childhood. In this model, the etiologic agent causing the primary infection in the mother is transmitted to the fetus, and as a consequence of this in utero infection, the child is at increased risk for the development of ALL before the age of 5 years. The characteristics that the causative infectious agent should possess include: (1) the ability to induce genomic instability, (2) specific effects on B lymphocytes and not on T lymphocytes, (3) higher rates of infection in regions with lower socioeconomic status, (4) limited general oncogenic potential, (5) minimal symptoms associated with the primary infection, and (6) the ability to cross the placenta and infect the fetus but not result in severe fetal abnormalities. A virus that meets several of these criteria is the JC virus, a member of the polyomavirus family.[143]

The possibility that maternal infection during pregnancy could potentially be leukemogenic is supported by the observation that leukemia in cats is caused by a feline virus transmitted from the mother to the fetus.[144] Three cohort studies of maternal infection during pregnancy and subsequent childhood cancer revealed an excess of cancer in these children compared with the age- and sex-matched general population.[145-147] In case-control studies, no association between maternal infection with influenza or varicella and subsequent childhood cancer was seen.[98,148-153] With regard to maternal infection in general, a twofold excess of childhood cancer has been reported. However, the studies are small or the reported prevalence of exposure is low and may be attributed to recall bias. Neonatal blood spots have been used to identify evidence of selected in utero viral infections.[154,155] Investigation of cytomegalovirus and human parvovirus B19 infection failed to identify DNA in the small groups of childhood ALL cases compared with healthy control subjects. First-trimester serum samples from mothers of children diagnosed with ALL derived from large maternal cohorts in Finland and Iceland have been tested for antibodies to *Chlamydia trachomatis*, *Helicobacter pylori,* and *Mycoplasma pneumonae*.[156] Comparison of ALL cases to control subjects identified an increased risk associated with *M. pneumoniae* immunoglobulin M and, in Iceland, *H. pylori* immunoglobulin G. Within the same maternal cohort, evidence was found for activation of maternal Epstein-Barr virus infection in both childhood ALL and non-AML cases.[157]

Smith and colleagues[158] sought to assess the relationship of childhood ALL with hygiene conditions, an aspect of socioeconomic development affecting rates of exposure to infectious agents. The data suggested that improved public hygiene conditions measured by decreased prevalence of hepatitis A virus infection (an agent with a fecal-oral route of transmission) were associated with high childhood ALL incidence rates. The model presented in this study supports the plausibility of the hypothesis that decreased childhood exposure to a leukemia-inducing agent associated with hygiene conditions leads to higher rates of ALL in children by increasing the incidence of in utero transmission caused by primary infection during pregnancy (or by increasing the number of persons infected in early infancy because of a lack of protective antibodies).

Antibiotic Use and Immunizations during the Index Pregnancy

No association between antibiotic use during pregnancy and leukemia has been identified.[148,150,152,159-161] No consistent association has been reported between vaccination during pregnancy and leukemia. Salonen and Saxen[162]

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*References 6, 12, 25, 49, 129-134, **135**, 136-140.

found a positive association between leukemia and maternal polio vaccination during pregnancy. Similarly, Gilman and associates[160] reported a positive association between total childhood deaths and vaccination during pregnancy that was largely accounted for by neoplasms of the reticuloendothelial system. However, results of a population-based study in the Netherlands showed no association between vaccination during pregnancy and ALL.[150]

Association of Childhood Leukemia with Factors Related to the Immune System

It has been proposed that some instances of pediatric ALL arise as a rare response to a common childhood infection and that the leukemia-inducing potential of the agent is related to the timing of infection, with a greater leukemogenic effect for later infections compared with those occurring during infancy.[163] In this context, factors related to the child's immune system are of special interest. Several investigators have sought to identify factors such as birth order and breastfeeding that can potentially be used as markers of infections or confer protection against infection before the diagnosis of leukemia.[164]

Breastfeeding

Breastfeeding is well known to have a protective effect against infection in infants.[165] Data regarding the association between breastfeeding and subsequent childhood leukemia are important because of the proposed hypothesis about the etiologic role of infections, taking into consideration the fact that breastfeeding protects against infections. This association was examined in several studies with mixed results. Whereas some researchers failed to show any association between breastfeeding and childhood leukemia,[152,166-172] other researchers do show that a protective effect is offered by breastfeeding.[173] A report of a large North American case-control study revealed that breastfeeding was associated with a reduced risk of childhood acute leukemia.[173] The inverse association was stronger with a longer duration of breastfeeding for patients with ALL and AML. A recent meta-analysis suggested that breastfeeding of any duration was associated with a 9% lower risk of ALL, but little evidence was found of an association for risk of childhood AML.[174]

Immunizations

Previous studies suggested that vaccinations during infancy may reduce the risk of subsequent childhood leukemia. No consistent association between leukemia and immunization has been identified, although two recent studies suggested that early immunization against *Haemophilus influenzae* type b may reduce the incidence of childhood leukemia.[175,176] Auvinen and associates[176] compared the vaccination histories of 439 children in whom ALL was diagnosed and 439 community control subjects matched to patients with regard to age, race, and telephone exchange. No association was demonstrated between most infant vaccinations such as oral poliovirus, diphtheria-pertussis-tetanus, and measles-mumps-rubella. However, infants receiving the conjugate *H. influenzae* type b vaccine had a reduced risk for subsequent childhood ALL. Confirmatory studies are needed.

Sibship Size

Sibship size might be an indirect indicator of exposure to common childhood infections. However, although no consistent association between ALL and sibship size has been identified,[177] authors of a study from Canada reported that when all variables defining family structure are included in a model, having older siblings at the time of diagnosis was a risk factor among children in whom ALL was diagnosed before 4 years of age, whereas having older siblings in the first year of life was a protective factor among children in whom ALL was diagnosed at 4 years or later.[171] More recently, analysis of the Swedish Family-Cancer Database demonstrated that having three or more older siblings is associated with a significantly reduced risk of childhood AML and ALL.[178]

Early Child Care and Preschool Experiences

Day care of the index child and of siblings has been used as a proxy measure of exposure to infections. The findings of case-control studies have been inconsistent regarding association between attendance at day care centers and risk of leukemia.[177,179-185] A recent study conducted by the United Kingdom Childhood Cancer Study (UKCCS) investigators was specifically designed to test the early infection hypothesis, including the role of day care. These investigators found that increasing levels of social activity were associated with reductions in the risk of ALL in a dose-response manner.[185]

Infection and Related Exposures during the Lifetime of the Index Child

Studies of an association between childhood cancer and postnatal infection of the index child are problematic because of the difficulty in assessing whether the episodes of infectious disease were a possible cause of the childhood cancer or represented a prediagnostic sign of the disease. Some investigators have attempted to overcome this concern by restricting attention to infection during the first year or first 6 months of life or by considering infections reported only during a specified period before diagnosis in patients and during a reference time in control subjects. Specifically, investigators explored the role of parvovirus B19 with its hematotropic effects and the potential to precipitate varying forms of cytopenia in patients before or after the diagnosis of ALL,[186] but they failed to find an association between parvovirus B19 and childhood ALL. Other epidemiologic studies have provided inconsistent findings for an association between leukemia and recorded or reported infections in early life.[98,149,153,167,186-191] In the UKCCS investigation, which utilized general practitioner records for visits during the first year of life, it was found that children with ALL who were diagnosed between the ages of 2 to 5 years had significantly more clinically diagnosed infectious episodes in infancy.[191] Another case-control study from the UKCCS group explored the relationship between childhood leukemia, infant infection, and three markers of infection exposure: birth order, infant day care attendance, and area-based deprivation. The study findings indicated that immune dysregulation among children who are diagnosed with ALL is detectable from an early age.[192] Moreover, recent epidemiologic data indicate that the risk of cALL is increased by higher socioeconomic status, isolation, and other community characteristics suggestive of abnormal patterns of infection during infancy,[13] and population-based incidence patterns have identified small peaks in the incidence of childhood ALL that coincide with years immediately following influenza epidemics.[42]

Chloramphenicol

Chloramphenicol can cause bone marrow suppression and could potentially play an etiologic role in childhood leukemia. Shu and colleagues[193] reported a positive association with the use of chloramphenicol from a study conducted in Shanghai. The risk of leukemia associated with chloramphenicol use increased with an increasing total number of reported days of use. The association was stronger for AML than for ALL. A major limitation of the study was the long interval between diagnosis (1974 to 1986) and interview (1985 to 1986) for the patients, whereas the control subjects were recruited in the 2-year period of 1985 to 1986.

Index Child's Contact with Animals

Although considerable interest has been generated in a possible role of feline leukemia virus because of similarities between human and feline ALL in clinical findings, laboratory data, and response to treatment,[194] evidence is lacking for a biologic association.[195]

Bovine leukemia virus has also been studied for a possible role in the cause of human leukemia. A case-control study of 131 children with leukemia and 136 regional population control subjects was conducted to investigate this association. However, none of the DNA samples from patients or control subjects hybridized with a bovine leukemia virus DNA probe, providing strong evidence that genomic integration of bovine leukemia virus is not a factor in childhood ALL.[196]

Buckley and associates[98] reported a positive association with exposure of the mother, father, and index child to farm animals, compared with control subjects who had other types of cancer or community control subjects. However, no overall association with cat ownership was identified. A recent large case-control study failed to show any association between pet ownership and childhood acute leukemia.[197]

Leukemia and Bacillus Calmette-Guérin Vaccination

Laboratory evidence that tubercle bacilli of the Calmette-Guérin strain (BCG) can prevent or suppress challenge from leukemia or tumor grafts in laboratory animals[199] led investigators to explore whether BCG vaccination might reduce the incidence of cancer in humans and of leukemia in particular. However, no consistent association between BCG vaccination and the subsequent risk of leukemia was found.[198-201]

Seasonal Variations in the Onset of Childhood Leukemia

Seasonal variations in onset of disease could provide supportive evidence of an infectious cause. Westerbeek and colleagues[202] demonstrated significant seasonal variation in the date of the first symptom of childhood ALL, with peaks occurring in November. However, a larger study of 15,835 patients with childhood leukemia failed to reveal any evidence of seasonality in either month of birth or month of diagnosis overall or in any subgroups by age, sex, histologic analysis, or immunophenotype.[203] However, this study did find a statistically significant February peak in month of birth for patients born before 1960 and an August peak in month of diagnosis for patients in whom leukemia was diagnosed before 1962. Although these findings may be due to chance, they are also consistent with changes over time in the seasonality of exposure or immunologic response to a relevant infection. Changes in the seasonal variation in the fatality rate of a preleukemic illness, such as pneumonia, could be another explanation. Ross and associates[204] examined data from the Children's Cancer Group and the Pediatric Oncology Group for seasonal variation patterns. A statistically significant seasonal variation for ALL was found, with a peak in the summer. Biologic mechanisms underlying these seasonal patterns are probably multifactorial and need to be investigated.

ENVIRONMENTAL EXPOSURES

Although a large body of literature describes the associations between a variety of environmental exposures and childhood leukemia, insufficient evidence exists for a causal relationship in most exposure studies, with the exception of those for ionizing radiation. Retrospective studies are limited because of the reliance placed upon parental self-report of the exposure in the context of a case-control study design, which can be hampered by recall bias. In addition, the nonspecific nature of the exposure being assessed makes it difficult to explore causal mechanisms involved with the associations. Moreover, a majority of the exposure assessment has focused on paternal exposures, with little emphasis placed on maternal exposures. To more clearly evaluate the importance of these exposures in future investigations, improvement is needed in the following areas: (1) importance given to maternal exposures; (2) sophisticated exposure techniques used; (3) attention paid to the mechanism, timing, and route of exposure; and (4) the provision of evidence that the exposure is actually transferred to the child's environment.

Radiation Exposure

In utero exposure to radiation has been reported to be associated with a small yet statistically significant increased risk of development of childhood leukemia in numerous case-control studies.[†] Reported estimated risks generally ranged from 1.1 to 2.0, with most of the risk ratios being equal to or lower than 1.6. It is reasonable to expect that in utero levels of exposure to radiation would have declined substantially over time given the knowledge of health-related risks associated with radiation exposure and the reduced level of radiation required for diagnostic procedures. Studies from Sweden, England,

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[†] References 166, 205-208, **209**, 210-213.

and the United States have demonstrated higher risk estimates among children born in earlier eras (e.g., the 1930s to 1950s) compared with more recent periods.[213-216] Nonetheless, the potential role of in utero exposure to radiation in childhood leukemia has remained controversial. Much of the debate has focused on the lack of an observed association among offspring of mothers who were exposed while pregnant at the time of the Hiroshima and Nagasaki atomic bombs[217,218] coupled with concerns regarding the lack of good exposure assessment and the potential for recall bias in case-control studies of the topic. Results from a large case-control study in North America did not identify an overall increased risk associated with in utero pelvimetric diagnostic x-rays or associations within subgroups defined by immunophenotype.[219] It is likely that the reduced rate and dose of exposure in North America has resulted in an inability to detect the small risk that may exist.

In contrast to the in utero data, postnatal exposure to radiation has not been consistently associated with the risk of childhood leukemia.[166,206,212] Regardless, even if leukemia risk is increased, the potential number of instances of leukemia that currently might be attributed to in utero or postnatal exposure to radiation would probably be very small when one considers the modest magnitude of risk and the limited level of exposure. Results of a case-controlled study of 302 cases of infant leukemia suggested that paternal low-level radiation exposure before conception is associated with an increased risk of infant leukemia, although the nature of this association needs to be further evaluated.[220]

A number of investigations of environmental exposures of children to sources of radiation from nuclear detonations and power plants were conducted to determine the potential impact on leukemia risk. In studies of children who survived the atomic bombs of Hiroshima and Nagasaki, an increased occurrence of leukemia 5 to 15 years later was seen.[221] Leukemia risk was positively correlated with the dose of radiation among the atomic bomb survivors. Reports relating to follow-up of nuclear accidents at Three Mile Island and early reports from Chernobyl did not provide strong evidence of an increased risk of childhood leukemia. Analyses that included residents in the area surrounding the Three Mile Island facility did not reveal an increase in the ratio of observed to expected incidence of leukemia.[222] Similarly, investigation of specific regions of the former Soviet Union did not reveal increased rates of childhood leukemia within the first 5 years after the Chernobyl accident.[223] Because of the widespread fallout from Chernobyl, a series of investigations were undertaken in affected countries, including Sweden,[224] Finland,[225] England,[226] Scotland,[227] Germany,[228] and Greece.[229] With the exception of higher rates in Scotland and an observed excess of infant leukemia (i.e., in children younger than 1 year) in Greece,[230] results were generally negative for an observed increase after the accident. The most recent investigation of the acute and long-term risks for childhood leukemia resulting from radiation exposure (a median estimated dose of <10 mGy) after the Chernobyl power station accident identified a significant increase in leukemia risk with increasing radiation dose to the bone marrow.[231] However, the potential overestimate of childhood leukemia risk within one region raised serious concerns about conclusions that could be drawn with regard to the level of risk resulting from the Chernobyl accident.

During the 1950s, testing of nuclear weapons took place in Nevada, with nuclear fallout affecting regions of Nevada and Utah. Studies were carried out in Utah to determine the potential impact on childhood leukemia, using geographic region–specific radiation dose estimates to the bone marrow. No significant trend between estimated dose and risk of leukemia mortality was found.[232] However, the risk of death from leukemia was found to be higher in those exposed at the highest dose level (6 to 30 mGy) compared with those in the lowest dose group (0 to 2.9 mGy).

During the past several decades, the potential impact of nuclear facilities, either nuclear energy plants or nuclear reprocessing plants, has generated considerable interest with regard to cancer risk among surrounding residents. Studies addressing childhood leukemia have been conducted in England,[233,234] France,[235,236] the United States,[237,238] Germany,[239] and Canada.[240] In aggregate, these investigations have not provided strong evidence of an increased occurrence of childhood leukemia, although several have reported increases in incidence and mortality.

Although the biologic plausibility of indoor radon exposure and the cause of childhood leukemia are speculative[241] and debatable,[242] studies have been conducted to address the topic. Although ecologic investigations have provided some evidence for a correlation,[241-243] case-control studies incorporating measurement of indoor radon levels have not found higher levels within homes of children with ALL[244] or AML.[245]

Parental Occupational Exposures

Occupational exposure of parents and its association with childhood leukemia have been the subject of several investigations.[246-249] Occupations and exposures of fathers were investigated more often than were those of the mother. Detailed assessment with use of a job exposure matrix to classify occupational exposures identified an increased risk for childhood AML in association with maternal exposure to 1,1,1-tricholorethane, toluene, mineral spirits, alkanes, and mononuclear aromatic hydrocarbons, although dose-response associations were not present.[249] Exposure in the home was not associated with increased risk. Investigation within the UKCCS identified small but statistically significant increased risks among children with ALL whose fathers reported their occupation to involve motor vehicle driving and were exposed to exhaust fumes and/or inhaled particulate hydrocarbons.[248] The studies have several limitations related to the quality of exposure assessment, small numbers of exposed children, multiple comparisons, and possible bias toward the reporting of positive results. Although some evidence exists for an association between childhood leukemia and paternal exposure to solvents, paint, and employment in motor vehicle–related occupations, clearly established associations between parental occupation and childhood leukemia are lacking.

Exposure to Chemicals and Dust

Pesticides

The association between pesticides—including insecticides, fungicides, and herbicides—and childhood leukemia has generated considerable interest, primarily because of the observed higher incidence of leukemia in rural areas and because of the biologic activity of these chemicals. Leiss and Savitz[250] reported a positive association between the use of pest strips and childhood leukemia. The insecticide used in pest strips was dichlorvos, an organophosphate that has been associated with adult-onset leukemia in men.[251] Authors of another study reported an association between the use of pesticides in gardens and farms and childhood leukemia,[252] but no association of leukemia with reported home pest extermination was found in this study. A positive association between maternal exposure during pregnancy and lactation and paternal exposure to household pesticides and garden pesticides or herbicides during the time the mother was pregnant was reported by Lowengart and associates.[253] A recent study provides further evidence for an association between an increased risk of leukemia and rural residence and use of household pesticides.[254] Authors of several other studies report an association between pesticide exposure and childhood leukemia.[255,256]

In a multicenter study of 404 patients who had ALL and individually matched community control subjects, Buckley and associates[98] reported a positive association between ALL and both paternal exposure (RR, 2.8; $P < .001$) and exposure of the index child (RR, 5.0; $P < .001$) to insecticides. The strongest associations for both paternal exposure and exposure of the index child were apparent for T-cell and common cell ALL.

In another case-control study consisting of 204 patients with AML and matched community control subjects, Buckley and associates[257] reported a positive association with maternal occupational exposure to pesticides before, during, and after pregnancy and paternal occupational exposure during the same period. In addition, an independent association of maternal exposure to household fly sprays, pesticides, and garden and agricultural sprays and treatment of the house by insect exterminators in the month before the last menstrual cycle and during the index pregnancy was found, along with direct exposure of the index child to household and garden insecticides. These associations were independent of parental occupational exposure.

Thus, although a large body of literature exists regarding the association between pesticide exposure and childhood leukemia,[255,256] the studies are limited by the nonspecific nature of the exposure, the reliance of these studies on parental self-report, and the lack of sufficient evidence for a causal relationship. Moreover, use of pesticides may be an indicator of rural isolation, and thus there is a possible confounding effect because of the patterns of exposure to infection that may be associated with population mixing.

Agent Orange. Wen and colleagues[258] reported a small increase in the risk of the diagnosis of AML before the age 2 years among offspring of veterans who had served in Cambodia or Vietnam.[258] The etiologic importance of these observations remains to be determined. Exposure to Agent Orange was evaluated in this study, but no association was identified. Other studies also concluded that exposure to the herbicides in Agent Orange was not associated with an increased risk of childhood leukemia among the children born to veterans who reported the exposure.[259]

Hydrocarbons and Solvents

Parental exposure to hydrocarbons at work has been investigated as a potential risk factor for childhood leukemia, with no consistent association between paternal or maternal occupational exposure to hydrocarbons and leukemia (all types and specifically ALL).[260-264] Similarly, no association between paternal exposure to benzene and leukemia has been found.[253,257,262-265] Maternal occupational exposure has been less well studied, but in two of the three studies, a positive association with maternal occupational exposure to hydrocarbons was found that is compatible with the observation that benzene is associated with AML in adults.[162,193,257] Two studies indicated that maternal exposures to hydrocarbons during the preconception period, during pregnancy, and during the postnatal period were related to an increased risk of ALL.[266] A positive association between ALL and paternal exposure to hydrocarbons during the preconception period was also found.

No clear association with residential proximity to industrial sources of hydrocarbons has been observed,[267,268] although authors of one study reported an increased risk of childhood leukemia, and AML in particular, associated with living near petrol stations and/or repair garages.[264] An increased risk of childhood leukemia associated with paternal occupational exposure to chlorinated solvents was reported by several investigators.[253,257,263] The risk has been assessed for exposure during the periods of 1 year before pregnancy, during pregnancy, and after delivery.[257] Several investigators reported a positive association with postnatal exposure of the index child to solvents.[98,153] No consistent association with maternal exposure to solvents is apparent.[269] A recent study revealed a significant association between substantial participation by household members in some common household activities involving organic solvents and childhood leukemia,[270] but further substantiation of these findings is necessary.

Metal Dusts and Fumes

A positive association between the total reported duration of paternal occupational exposure to lead and AML has been reported by Buckley and associates.[257] In the same study, these investigators reported an association between maternal occupational exposure to "metal dusts and fumes." Similar findings were reported by Shu and colleagues[193]; in their study in Shanghai, maternal occupational exposure to lead was associated with an increased risk of acute leukemia. A positive association between maternal exposure to molten metal and ALL (null cell and T cell) and a positive association between paternal exposure to metals and T-cell ALL have been reported.[98] However, ecologic studies have failed to show an association between leukemia and proximity to industrial facilities with an increased exposure to metal or metal fumes.[271,272]

Wood Dust

An association between maternal occupational exposure to wood dust before conception of the index child and childhood leukemia and non-Hodgkin lymphoma has been reported. A few women were exposed during or after pregnancy.[263] Significantly elevated RRs have been reported for paternal occupational exposure to wood dust before conception, during the periconceptional and gestational periods, and postnatally.[246] The association of exposure to wood dust and leukemia was studied by Buckley and associates.[98,257] They found that the risk was elevated compared with that for other cancers and also compared with that for community control subjects.

Traffic

Occupational exposure to elevated concentrations of benzene is a known cause of leukemia in adults. Concentrations of benzene from motor vehicle exhaust fumes could be elevated along highly trafficked streets. The hypothesis that exposure to traffic-related air pollution increases the risk of development of childhood cancer was examined by several investigators.[273-275] Results indicate that an association might exist between residence on a busy street and childhood leukemia. However, these results need to be viewed with caution, because most of the results are based on ecologic studies, with an imperfect assessment of individual exposure.

Electromagnetic and Radiofrequency Field Exposure

An initial report in 1979 suggested an association between childhood leukemia and residential proximity to sources of electric and magnetic fields, as assessed by wiring configurations.[276] Subsequently, a large number of investigations have been conducted to further evaluate the hypothesis that exposure to extremely low-level electric and magnetic fields may influence the risk of childhood leukemia and other pediatric malignancies.[‡] These studies, although they provide some consistent findings, are difficult to interpret given the inconsistencies observed in associations between inferred exposure (i.e., wire configuration) compared with measured fields.

Moreover, many of these studies are limited by small sample size and the inability to adequately consider potential confounding factors within the analysis. Three of the more recently reported investigations from the United States,[287] Canada,[288] England,[289,290] and Japan[291] provide rather convincing evidence that electric and magnetic field exposure is not associated with a significantly increased risk of childhood ALL. Nonetheless, results of meta-analyses[292,293] have been interpreted to suggest that risk may be increased at the highest exposure levels (i.e., >0.4 μT). However, it is important to note that even if an increased risk does exist at this highest exposure level, the proportion of the population exposed is extremely small and thus the attributable risk would be negligible. Beyond investigation of risk for leukemia, reports have been made of possible associations between electric and magnetic field exposure and prognosis.[294,295]

Although not as extensively studied as electric and magnetic fields, radiofrequency exposure has been suggested as a possible cancer-related risk factor.[296] A recent report investigating radiofrequency exposure in 1928 patients with childhood leukemia observed a twofold risk associated with living within 2 km of an amplitude modulation radio transmitter, which was statistically significant in a dose-dependent fashion for childhood ALL.[297]

LIFESTYLE

Maternal Diet and Vitamin Supplement Use during Pregnancy

Diet and use of vitamin supplements have been shown to be associated with the risk of several cancers among adults. In pediatric malignancies, research has focused primarily on use of vitamin supplements during pregnancy, with little attention given to diet.[159,298] Investigation of maternal diet during pregnancy of children with ALL between the ages of 1 to 5 years found that ALL risk was statistically significantly lower with increased consumption of fruits, vegetables, and fish/seafood, with a significantly higher risk related to increased maternal intake of sugar/syrups and meat products, but these findings will require confirmation in other populations.[299]

Among the available studies of childhood leukemia and maternal consumption of vitamin supplements during pregnancy, no association was found,[159,162,298] with the exception of one study reporting a decrease in risk of ALL among mothers who took iron supplements during the index pregnancy.[300]

Nearly 80% of infants with leukemia have a specific genetic abnormality in their leukemic blast cells involving the *MLL* gene on chromosome band 11q23.[301] Abnormalities involving 11q23 have also been reported in persons with secondary AML after exposure to topoisomerase inhibitors. Therefore Ross and colleagues[301] hypothesized that infant leukemias involving 11q23 may result from exposure to naturally occurring topoisomerase inhibitors. These agents include caffeine and a variety of fruit and vegetables. A subset of mothers of infants with leukemia diagnosed at 1 year of age or less and control children selected by random digit dialing included in three multicenter case-control studies of childhood leukemia in the United States[257,298] was reapproached to obtain additional information on maternal diet during the index pregnancy.[302] On the basis of data from a food-frequency questionnaire assessing consumption of 26 food items during the index pregnancy, no association was found between total estimated dietary intake of topoisomerase II inhibitors and leukemia of all types combined or ALL. However, a statistically significant positive association for AML was noted with increasing consumption of dietary topoisomerase II inhibitors. A study conducted in 2000 identified bioflavonoids, which are natural substances in food and in dietary supplements that cause site-specific DNA cleavage in the *MLL* breakpoint cluster region in vivo.[303] These results suggest that maternal ingestion of bioflavonoids may induce MLL breaks and potentially induce translocations in utero, leading to infant leukemia. In utero exposures and their association with infant acute leukemia were assessed by Alexander and associates.[304] Use of cigarettes and alcohol, the ingestion of certain herbal medicines and drugs classified as "DNA damaging," and exposure to pesticides were associated with an increased risk of leukemias associated with alterations in *MLL*.

[‡] References 228, 277, 278, 279-286, 287-289.

Postnatal Diet of the Index Child and Use of Vitamin Supplements

The role of diet of the index child in the development of childhood leukemia has not been the subject of intensive investigation. Results of investigations exploring the association between the intake of certain food items thought to be precursors or inhibitors of N-nitroso compounds have been controversial. Some investigators report a positive association with hot dogs,[253] whereas others have failed to show any association with consumption of hot dogs or other cured meats.[298] No associations have been reported with postnatal use of vitamins by the index child[98] or consumption of fish, dried milk, fruit juice, or canned foods.[207]

Tobacco Smoking by Parents

Parental smoking and its association with childhood leukemia has been the focus of several investigations. However, issues such as publication bias (with a greater likelihood of positive associations being published) limit the quality of the data. In summary, the literature shows no consistent association between childhood leukemia and parental exposure to tobacco.

Furthermore, no consistent association between maternal smoking during pregnancy and leukemia[305-308] or between paternal smoking and all types of leukemia combined or AML has been found. In most studies, the period of exposure considered has been up to the birth of the index child. Although a positive association between paternal smoking and ALL has been reported by several investigators,[309-312] other investigators have failed to show any association.[305-308,313,314] The largest investigation of maternal smoking and risk of childhood leukemia, which was conducted in Sweden, included more than 1.4 million children born between 1983 and 1997.[315] In this study it was found that maternal smoking was associated with a lower risk of ALL but a higher risk of AML, particularly among heavy smokers. Sandler and associates[316] evaluated the cancer risk from cumulative household exposure to cigarette smoke in a case-control study. Cancer risk was greater for persons who were exposed during both childhood and adulthood than for persons who were exposed during only one period. Some evidence indicates that the effect of parental smoking could be modified to some extent by variant alleles in the *CYP1A1* gene.[306]

An earlier study reported an association between AML (type M4 or M5) and reported use of mind-altering drugs (primarily marijuana) by the mother in the year before or during the index pregnancy.[159] However, a subsequent investigation by the same investigators that was designed to test the hypothesis of maternal marijuana exposure failed to reproduce the association.[317] Thus, overall, maternal smoking during pregnancy or the exposure of infants to cigarette smoke shortly after birth is unlikely to contribute substantially to the risk of childhood leukemia. Moreover, it seems unlikely that passive smoke inhalation during childhood strongly affects the risk of development of childhood leukemia. However, the demonstration of genotoxic effects on the fetus of exposure to metabolites of tobacco smoke should lead to strong recommendations aimed at fully protecting fetuses, newborns, and infants from tobacco smoke.

Alcohol

The majority of studies pertain solely to alcohol consumption by the mother and only to consumption during the pregnancy leading to the birth of the index child. No consistent associations have been found between maternal alcohol consumption before or during the index pregnancy and leukemia of all types combined or ALL.[149,161,193,310] A recent report indicates that maternal alcohol consumption before (adds ratio [OR], 1.37; 95% CI, 0.99-1.9) or during pregnancy (OR, 1.39; 95% CI, 1.01-1.93) may contribute to an increased risk of childhood leukemia.[318] However, some evidence from a number of studies shows an association between maternal alcohol consumption and AML, primarily among persons in whom leukemia is diagnosed early in life. Severson and colleagues[305] reported an increased risk of AML among children in whom leukemia was diagnosed at or before 2 years of age and whose mothers reported consuming alcohol during their pregnancies; the association was particularly pronounced among patients with monocytic and myelomonocytic leukemia. However, the authors advised cautious interpretation of the data because of the small number of subjects included in the subgroup analysis. A positive association between alcohol consumption and AML has also been reported by others.[161,310] The risk appears to be increased for alcohol consumption during each of the three trimesters of pregnancy and for each type of beverage. A dose-response relationship is also suggested.[305,310] No association between paternal alcohol consumption and childhood leukemia has been found.[193,305,310]

MATERNAL REPRODUCTIVE HISTORY

Maternal Age and Birth Order

Accumulation of chromosomal aberrations and mutations during the maturation of germ cells are mechanisms hypothesized for the association between increasing maternal age and cancer in offspring. Most studies have failed to show an association between leukemia and maternal age.[193,319-322] However, a report from 1999 using the Swedish Family-Cancer Database revealed a maternal age effect for childhood leukemia (of about 50% greater in persons older than 35 years).[323]

Most studies based on index cases do not show a positive association with first birth. However, one of the largest of these studies revealed a decreasing trend with increasing birth order for children in whom ALL was diagnosed between the ages of 0 and 4 years.[322] This study also showed a positive association between AML and birth order, adjusted for age, sex, calendar period, and maternal age at the birth of the child. This association was also reported from a combined analysis of data for children who had AML that was diagnosed before 1 year of age in the United States.[319]

Prior Fetal Loss

The association between childhood leukemia and prior fetal loss has been examined by many investigators, with the reported RRs ranging from 0.3 to 1.8.[153,319,320] Data from a Children's Cancer Group case-control study were analyzed to test the hypothesis that this association depended on the number of previous fetal losses and age at leukemia diagnosis. Overall, a modestly increased risk of leukemia was found to be associated with a history of fetal loss. Stratification by age at diagnosis of leukemia showed that this association was significant only for patients in whom ALL and AML was diagnosed before 4 years of age and was most significant in patients in whom leukemia was diagnosed before 2 years of age.[324] Ross and associates[319] also reported a positive association between infantile AML and prior fetal loss. These reports suggested that childhood acute leukemia occurring at younger ages may be associated with an underlying genetic abnormality or chronic environmental exposure, which can be either lethal to the developing fetus or mutagenic and result in the development of acute leukemia.[319]

Oral Contraceptives

McKinney and associates[152] reported no association between leukemia and an interval of less than 3 months between when a woman stopped taking oral contraceptives and conception. Van Steensel-Moll and colleagues[150] reported no association between childhood leukemia and any use of oral contraceptives.

Maternal Illness and Use of Medications during the Index Pregnancy

Ever since the use of diethylstilbestrol to treat women during pregnancy was causally linked with clear-cell adenocarcinoma of the vagina and cervix, several investigators have explored a possible association between use of medications during pregnancy and childhood cancer.

Van Steensel-Moll and colleagues[150] reported a significant positive association with "threatened miscarriage" and "drugs to maintain pregnancy." Another study reported a positive association with hormonal treatment for infertility in the preconceptual period and reported infertility investigations.[148] However, in 1991 Kobayashi and associates[325] from Japan showed that no instance of maternal ovulation induction was recorded for the mothers of 2301 children with leukemia. The Northern California Childhood Leukemia Study investigators observed a significantly increased risk of ALL among mothers reporting influenza/pneumonia during the index pregnancy.[300] Lastly, in the Children's Oncology Group study of 1840 childhood ALL cases, it was reported that significant associations existed with parental use of amphetamines or diet pills and mind-altering drugs before and during the index pregnancy.[326]

Ultrasound Examinations

No association has been found between routine ultrasound exposure in pregnancy and childhood leukemia.[219,327-329]

Anesthesia during Labor

No consistent association has been found between childhood leukemia and cesarean section, which is an indication for anesthesia during labor.[148,190,321] McKinney and associates[152] found a positive association between leukemia and use of narcotic and opioid analgesics, mainly dextropropoxyphene hydrochloride, and between leukemia and the use of barbiturates in labor, but other investigators failed to show any association.[148]

MEDICAL HISTORY OF THE INDEX CHILD

Wen and colleagues[330] investigated the association between childhood ALL and allergic disorders (e.g., asthma, hay fever, food or drug allergies, eczema, and hives) using a case-control study design. The results of this study were in agreement with those of most previous studies of adult cancer, suggesting that allergic disorders may be associated with reduced risk of childhood ALL.

Congenital Anomalies

The presence of cancer and congenital anomalies in the same child may sometimes be explained by an underlying genetic abnormality. A study of these associations might provide clues to the identification of genes that are important to the cause of childhood leukemia. The increased risk of leukemia associated with congenital anomalies appears to be accounted for largely by Down syndrome.[331] Children with Down syndrome have a thirtyfold increased risk of developing childhood leukemia, with the proportion of leukemia attributable to Down syndrome by age 14 years estimated to be 2.7%.[332] To evaluate the risk of leukemia associated with congenital anomalies, a series of matched case-control studies was carried out by the Children's Cancer Group.[333] A total of 2117 children in whom ALL was diagnosed and 605 children in whom AML was diagnosed were compared with matched regional population control subjects. Data on congenital anomalies in the index child were collected by telephone interview with the biologic mother. More congenital anomalies were reported in index case patients who had ALL than in control subjects and included statistically significant increases in multiple birthmarks, Down syndrome, congenital heart defects, and gastrointestinal anomalies. Similarly, birth defects were reported more often among index case patients with AML than among control subjects and included a significant increase in multiple birthmarks, Down syndrome, mental retardation, and congenital heart defects. Exclusion of patients with Down syndrome from the analysis did not change the statistically significant excess of gastrointestinal anomalies in patients with ALL or the excess of birthmarks in patients with AML. However, the majority of the observed associations with congenital anomalies occurred in children with Down syndrome.

In a study of 20,029 children with cancer in England between 1971 and 1986, the occurrence of congenital anomalies was significantly lower in children with leukemia or lymphoma (2.6%) than in children with solid cancers (4.4%).[334] This finding is compatible with the hypothesis that mutations leading to the development of leukemias and lymphomas occur at a much later stage in development in the cells committed to hematopoiesis.

Birth Weight and Length at Birth

Birth weight might serve as an indirect indicator of the intrauterine stresses to the fetus and therefore has been studied by numerous investigators for its association with leukemia.[§] The accumulating literature on high birth weight and risk of childhood ALL, particularly at younger ages, is becoming increasingly convincing as demonstrated in a meta-analysis.[340] High levels of insulin-like growth factor–1, which might both produce a larger baby and contribute to leukemogenesis, has been postulated as an explanation for this association.[341] No consistent association has been reported between length of child at birth and leukemia.[152,321]

Vitamin K

Several studies have explored the association between vitamin K administered in the neonatal period and leukemia, with inconsistent results.[148,342-347] The initial studies by Golding and associates[342,343] from England showed an association between intramuscularly (but not orally) administered vitamin K and childhood leukemia. The biologic basis for the association between vitamin K and childhood leukemia was the observation that concentrations of vitamin K in the infants 12 to 24 hours after injection have been shown to increase sister chromatid exchanges in human placental lymphocytes in vitro and sheep fetal lymphocytes in vitro.[348] However, in vivo studies in human neonates did not substantiate this observation.[349] Moreover, the positive association between leukemia of all types and vitamin K reported by Golding and associates has not been confirmed by analytic studies in England,[346] Germany,[345] Denmark,[350] Sweden[344] and the United States.[351] Parker and associates[352] reported a positive association between parenterally administered vitamin K and childhood ALL diagnosed between the ages of 1 and 6 years, although no association was seen in a cohort-based study. The authors stated that a definite conclusion regarding the relationship between vitamin K and childhood leukemia has not yet been reached. Thus the relationship between vitamin K and childhood leukemia is tenuous at best and the attributable risk is extremely small, with some as yet unidentifiable biologic predisposition placing the patients at increased risk of the development of childhood leukemia.[353]

FUTURE DIRECTIONS

The occurrence of cancer during childhood remains one of the leading causes of childhood mortality, even with the marked improvements in the treatment and cure of pediatric malignancies during the past three to four decades. Although the volume of epidemiologic research addressing the cause of childhood cancer is considerable, relatively little has been achieved regarding our understanding of the cause of childhood leukemia, which would have direct impact on potential future prevention strategies. The absence of progress is not the result of a lack of research in populations around the world directed toward investigation of environmental and genetic factors. This body of research includes large case-control epidemiologic studies including thousands of pediatric leukemia cases, with many incorporating state-of-the-art clinical and biological characterization of cases along with environmental sampling designed to enhance exposure assessment. Future research in this, the most common malignancy of childhood, will probably need to focus on patients with a common primary genetic lesion, such as those with BRC-ABL, MML-AF4, or ETV6-RUNX1 fusion, or hyperdiploidy. By investigating within such well-defined groups, the potential exists to identify common environmental and genetic factors that result in the specific genetic lesion.

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    The infection hypothesis was tested by examining the impact of major rural-urban migration on mortality from childhood leukemia in the 1950s and 1960s—an era that preceded the decline in mortality brought about by effective chemotherapy. Greece and Italy, the two countries with the most striking levels of rural migration, also had the highest mortality rates from childhood leukemia, suggesting that marked rural population mixing in Greece and Italy may have contributed to their high mortality rates from childhood leukemia in the 1950s and 1960s.

13. Greaves MF, Alexander FE: An infectious etiology for common acute lymphoblastic leukemia in childhood? *Leukemia* 7:349–360, 1993.

    Epidemiologic data, reviewed in this article, indicate that risk of B-cell precursor, a form of ALL (cALL), is increased among persons with higher socioeconomic status, isolation, and other characteristics suggestive of abnormal patterns of infection during infancy. The authors conclude that these data may be compatible with the concept that cALL may be a rare response to common infection(s).

17. Bhatia S, Sather HN, Heerema NA, et al: Racial and ethnic differences in survival of children with acute lymphoblastic leukemia. *Blood* 100:1957–1964, 2002.

    African-American and Hispanic children with ALL who were treated with Children's Cancer Group protocols had worse outcomes and Asian children had better outcomes when compared with non-Hispanic white children. The poorer outcomes among black children were most apparent among patients with standard-risk features, whereas poorer outcomes in Hispanic children were most evident among patients with high-risk features. Asian children had better outcomes than all racial and ethnic groups among high-risk patients, particularly in the recent era.

61. Shannon K: Genetic predispositions and childhood cancer. *Environ Health Perspect* 106(Suppl 3):801–806, 1998.

    The role of genetic susceptibility in childhood cancer is discussed, with a particular emphasis on problems with ascertaining inherited cancer risk and the role of tumor-suppressor gene mutations in cancer predispositions. The association between

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[§] References 98, 319, 322, 324, 335, 336-339.

neurofibromatosis type 1 and childhood leukemia is used to illustrate issues related to identifying and analyzing at-risk persons.

62. Zipursky A, Poon A, Doyle J: Leukemia in Down syndrome: a review. *Pediatr Hematol Oncol* 9:139–149, 1992.

The authors use observations from their own experience, reports in the literature, and data accumulated in the Canadian Down Syndrome Leukemia Registry to describe the epidemiologic and clinical characteristics of leukemia in children with Down syndrome.

71. Merks JH, Caron HN, Hennekam RC: High incidence of malformation syndromes in a series of 1,073 children with cancer. *Am J Med Genet A* 134A:132–143, 2005.

The authors describe the prevalence and nature of malformation syndromes associated with childhood cancer in 1073 children with cancer. They observe that the prevalence of patients with a proven or suspected syndrome is high, suggesting a possible association.

72. Narod SA, Stiller C, Lenoir GM: An estimate of the heritable fraction of childhood cancer. *Br J Cancer* 63:993–999, 1991.

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This report describes three pairs of infant twins with concordant leukemia who each share unique but nonconstitutive HRX rearrangements in their leukemic cells, demonstrating that the leukemogenic event originates in utero and provides support for intraplacental "metastasis" for leukemia concordance in twins.

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*This study demonstrates an association between childhood cALL and an allele at the HLA-DPB1 locus, DPB1*0201, pointing to a mechanism of cALL susceptibility that involves the presentation of specific antigenic peptides, possibly derived from infectious agents, by DPbeta1*0201–related allotypic proteins and leading to the activation of helper T cells, mediating proliferative stress on preleukemic cells.*

103. Wiemels JL, Smith RN, Taylor GM, et al: Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and risk of molecularly defined subtypes of childhood acute leukemia. *Proc Natl Acad Sci U S A* 98:4004–4009, 2001.

This study examines the role for alterations in folate metabolism as a result of polymorphisms in the enzyme methylenetetrahydrofolate reductase (MTHFR) in the risk of three subgroups of pediatric leukemias: infant leukemias with MLL *rearrangements and childhood lymphoblastic leukemias with either ETV6-RUNX1 fusions or hyperdiploid karyotypes. Carriers of C677T demonstrated protection from leukemias with* MLL *translocations. A1298C homozygotes and C677T homozygotes offer protection from hyperdiploid leukemia, providing preliminary evidence for a role of folate in the development of childhood leukemia.*

117. Ford AM, Bennett CA, Price CM, et al: Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia. *Proc Natl Acad Sci U S A* 95:4584–4588, 1998.

The ETV6-RUNX1 genomic sequence has been characterized in a pair of monozygotic twins with common ALL diagnosed at ages 3 years, 6 months and 4 years, 10 months. The twin leukemic DNA shared the same ETV6-RUNX1 fusion sequence, suggesting a single cell origin of the TEL-AML fusion in one fetus in utero, probably as a leukemia-initiating mutation, followed by intraplacental metastasis of clonal progeny to the other twin.

118. Wiemels JL, Ford AM, Van Wering ER, et al: Protracted and variable latency of acute lymphoblastic leukemia after TEL-AML1 gene fusion in utero. *Blood* 94:1057–1062, 1999.

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CHAPTER 41

Informatics

Samuel L. Volchenboum

CHAPTER OUTLINE

DNA-BASED METHODS
Comparative Genomic Hybridization
Genotyping
Capillary (Sanger) Sequencing
Pyrosequencing
DNA Microarrays
Single Nucleotide Polymorphism Arrays
Chromatin Immunoprecipitation, ChIP-Chip, ChIP-Seq
Genome-Wide Association Studies
Exome Sequencing/Targeted Sequencing
Whole Genome Sequencing
RNA-BASED METHODS
Whole Transcriptome Shotgun Sequencing (RNA-Seq)
Expression Profiling
PROTEIN-BASED METHODS
Protein Microarrays
Mass Spectrometry
INFORMATICS
False-Positive Rate, Sample Size, and Validation
Clustering and Classification
Gene Set Enrichment Analysis
CONCLUSIONS

The first genome to be sequenced came at a cost of $100 million. Only 13 years later, the price for sequencing an entire genome is nearing $1000 (Fig. 41-1). Rapid increases in the speed and complexity of genomic sequencing technologies, coupled with dramatically decreasing costs, have created an overwhelming array of platforms, methods, and informatics analysis algorithms. In many publications the authors assume the reader is facile with the platform used in the study described, yet most clinicians and researchers have a limited or narrow knowledge of the wide range of methods capable of describing the genomic or proteomic content of a biologic sample. This chapter focuses on describing the basic framework with which to understand most of the common platforms in use today.

Genomic platforms can be classified in many ways, and one can consider the starting material (e.g., deoxyribonucleic acid [DNA], ribonucleic acid [RNA], proteins, and small molecules) or the questions being asked (e.g., copy number, mutations, loss of heterozygosity [LOH], transcript expression, and methylation). The classification scheme shown in Table 41-1[1,2] classifies each platform according to the underlying structure, and the remainder of this chapter is organized in this fashion.

DNA-BASED METHODS

Most of the time the goal of studying DNA is to learn about the genes encoded, including mutations, rearrangements, and changes in copy number. Low-resolution methods prevailed for years as the standard way of understanding copy number variations and rearrangements (e.g., karyotyping and fluorescent in situ hybridization [FISH]), and low-throughput techniques were required to evaluate point mutations and pathogenic sequences (e.g., capillary sequencing, pyrosequencing, polymerase chain reaction [PCR], and microbial arrays). The emergence of high-throughput, high-resolution techniques has revolutionized the study of the genome.

Comparative Genomic Hybridization

Until the development of comparative genomic hybridization (CGH), chromosomal or subchromosomal gains and losses were discovered though Giemsa banding ("karyotyping") or FISH. Although similar in resolution to these techniques (5 to 10 megabases[3]), CGH can be used in an unbiased and agnostic way to detect unbalanced chromosomal abnormalities by competitive FISH using different fluorophores for two different isolates of DNA (e.g., test and control).[4-6] The technique was also

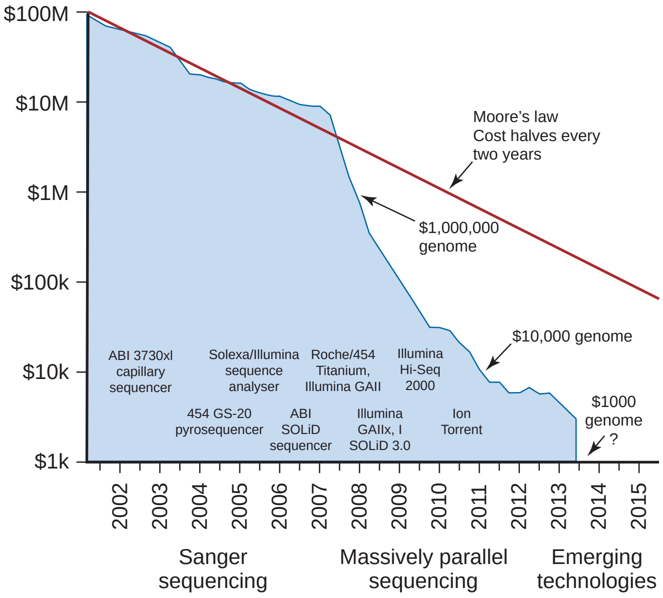

Figure 41-1 The decreasing cost of genome sequencing. Note that the price has been falling faster than Moore's law would predict. *(Modified from MacConaill LE: Existing and emerging technologies for tumor genomic profiling.* J Clin Oncol *31:1815–1824, and Mardis ER: Next-generation DNA sequencing methods.* Annu Rev Genomics Hum Genet *9:387–402, 2008.)*

novel because it did not require cells to be undergoing active cellular division.[7] With the popularization of DNA microarrays, a higher resolution technique was developed: array CGH (aCGH).[8-10] As is common to all microarray techniques, probes (oligonucleotides) are deposited onto a solid support (glass slide), with the resolution dependent on the size of the probes and the genomic distance separating them.[11] DNA from a test sample and control are extracted, labeled with different fluorescent dyes, and applied to the probes (Fig. 41-2). Complementary strands will bind and can be visualized with use of a digital imaging system to quantify the relative amounts of each target bound. The ratio of test to control for each DNA region can then be used to determine copy number variation throughout the genome. Compared with prior methods, aCGH can detect copy number changes at any locus, as long as it is represented in the array. The technique has been productively applied to the study of thousands of diseases and complex traits, and despite the emergence of newer high-resolution methods, aCGH continues to be a significant platform for the study of copy numbers.

Genotyping

Genotyping is simply determining the genetic variation in an individual. Many methods may be used to perform genotyping with varying degrees of throughput. Restriction fragment length polymorphism analysis was productively applied to describe human leukocyte antigen polymorphisms, followed by PCR-based methods using membrane- or bead-bound sequence-specific oligonucleotide probes and sequence-specific priming (reviewed in references 12 and 13).[12,13] For the specific application of identifying a point mutation or allelic variant, genotyping via PCR followed by restriction digestion is a simple approach that has been applied to many diseases and conditions, including fatty acid binding protein mutations in insulin resistance[14,15] and identification of polymorphisms in thiopurine methyltransferase in leukemia[16] and methylenetetrahydrofolate reductase in childhood acute leukemia.[16]

Capillary (Sanger) Sequencing

Sanger sequencing, which was first developed in 1977, has been routinely used for more than 25 years for genomic studies.[17,18] In the classic method (Fig. 41-3), chain-terminating nucleotides are incorporated by DNA polymerase during DNA replication with a synthesized primer specific to the region of interest. Compared with normal deoxynucleoside triphosphates, terminating dideoxynucleoside triphosphates lack a 3′-hydroxyl group, and DNA polymerase is unable to create the phosphodiester bond between two nucleotides, thus halting transcription. Four DNA replication samples are prepared, each with a different radiolabeled or fluorescently tagged dideoxynucleoside triphosphate, and the resulting DNA products are separated by electrophoresis and visualized by autoradiography or ultraviolet light. Current instruments use dye terminators instead of fluorescent labels, resulting in faster and more accurate readings.[19,20] The relatively high cost for reagents and the nonautomated nature of traditional Sanger sequencing have been largely obviated by the development of microfluidic technology.[21-24] All of the steps for Sanger sequencing are carried out on a small chip using nanoliter volumes. This "lab-on-a-chip" or "sequencing-on-a-chip" technique increases speed and accuracy while decreasing costs. Despite limited resolving power for the first 25 to 50 bases and read lengths fewer than 1000 bases, Sanger sequencing is still being used for smaller scale projects or when a long contiguous read is desired.

Pyrosequencing

In pyrosequencing, liberated pyrophosphates from nucleotide incorporation are detected and the DNA sequence is determined by light emitted upon nucleotide incorporation.[25-28] Because only one nucleotide at a time is presented to the DNA template and DNA polymerase, the base responsible for the emitted light is the one incorporated into the growing strand. The pyrophosphate release during the reaction is converted to adenosine triphosphate, fueling a luciferase-based fluorescent reaction. Although the reads are shorter (300 to 500 base pair [bp]), multiple reactions can be detected simultaneously, thereby increasing throughput.

DNA Microarrays

A microarray is simply a small piece of DNA (the "probe") that is attached to a solid support, which is usually a glass, plastic, or silicon chip (e.g., an Affymetrix [Santa Clara, CA] genome chip, gene array, or DNA chip) or polystyrene beads (Illumina, San Diego, CA). The process of "printing" the array refers to the deposition of the DNA onto the solid support, either through "spotting"

TABLE 41-1 Types of "-omics" Data

Underlying Structure	"-omics"	Alterations in Disease	Technologies
DNA	Genome	Point mutations	Capillary (Sanger) sequencing Pyrosequencing Genotyping Targeted-sequencing/WES RNA-seq
		Copy number gains or losses	FISH Array CGH SNP array Targeted-sequencing/WES WGS
		Rearrangements/fusion genes	Karyotyping FISH WGS RNA-seq
		Pathogenic sequences	PCR Microbial arrays WGS RNA-seq
	Epigenome	DNA methylation Histone modifications	Bisulfite sequencing Methyl-specific PCR ChIP-seq
mRNA	Transcriptome	Altered transcript expression Altered allele-specific expression Differential alternative splicing	Microarrays RNA-seq
microRNA	Epigenome	Altered transcriptional control	Microarrays
Proteins	Proteome	Mutated or deleted proteins Altered posttranslational modification Increased or decreased regulation	Microarrays Mass spectrometry
Small molecules	Metabolome	Modulations in small molecules	Mass spectrometry NMR spectroscopy

Modified from Chadeau-Hyam M, Campanella G, Jombart T, et al: Deciphering the complex: methodological overview of statistical models to derive OMICS-based biomarkers. Environ Mol Mutagen *54:542–557, 2013, and MacConaill LE: Existing and emerging technologies for tumor genomic profiling.* J Clin Oncol *31:1815–1824, 2013.*

CGH, Comparative genomic hybridization; *ChIP-seq,* chromatin immunoprecipitation followed by massively parallel sequencing; *DNA,* deoxyribonucleic acid; *FISH,* fluorescent in situ hybridization; *mRNA,* messenger ribonucleic acid; *NMR,* nuclear magnetic resonance; *PCR,* polymerase chain reaction; *RNA,* ribonucleic acid; *RNA-seq,* ribonucleic acid sequencing (transcriptome sequencing); *SNP,* single nucleotide polymorphism; *WES,* whole exome sequencing; *WGS,* whole genome sequencing.

(complementary DNA [cDNA] microarray[29]) or by synthesizing the oligonucleotides directly onto the array surface.[30-32] For a spotted array, the probes are produced beforehand, either through the production of a cDNA library, by PCR, or by the generation of oligonucleotides that are then "spotted" directly onto the support surface, often by a robot.[33,34,35]

Single Nucleotide Polymorphism Arrays

A single nucleotide polymorphism (SNP) is any sequence variation in the genome for which both alleles occur at a relatively high frequency. The vast majority do not appear to confer functional consequences.[36] Although they are located most often in noncoding regions, SNPs can occur

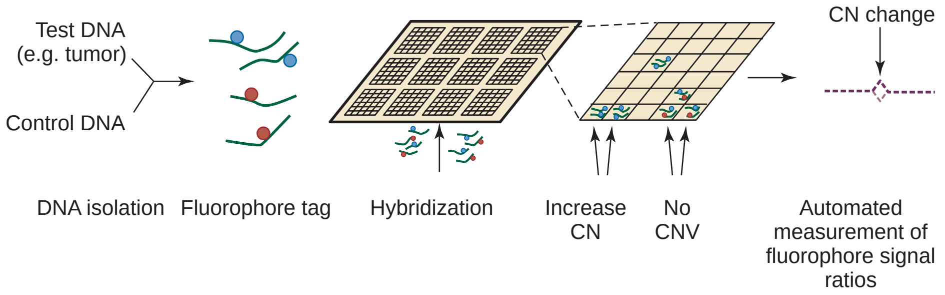

Figure 41-2 Array comparative genomic hybridization workflow. Deoxyribonucleic acid (DNA) is isolated from patient/tumor and control, differentially labeled with fluorophores, hybridized to oligonucleotides on a solid support, and analyzed for differences in ratios of fluorescence. *CN,* Copy number; *CNV,* copy number variation.

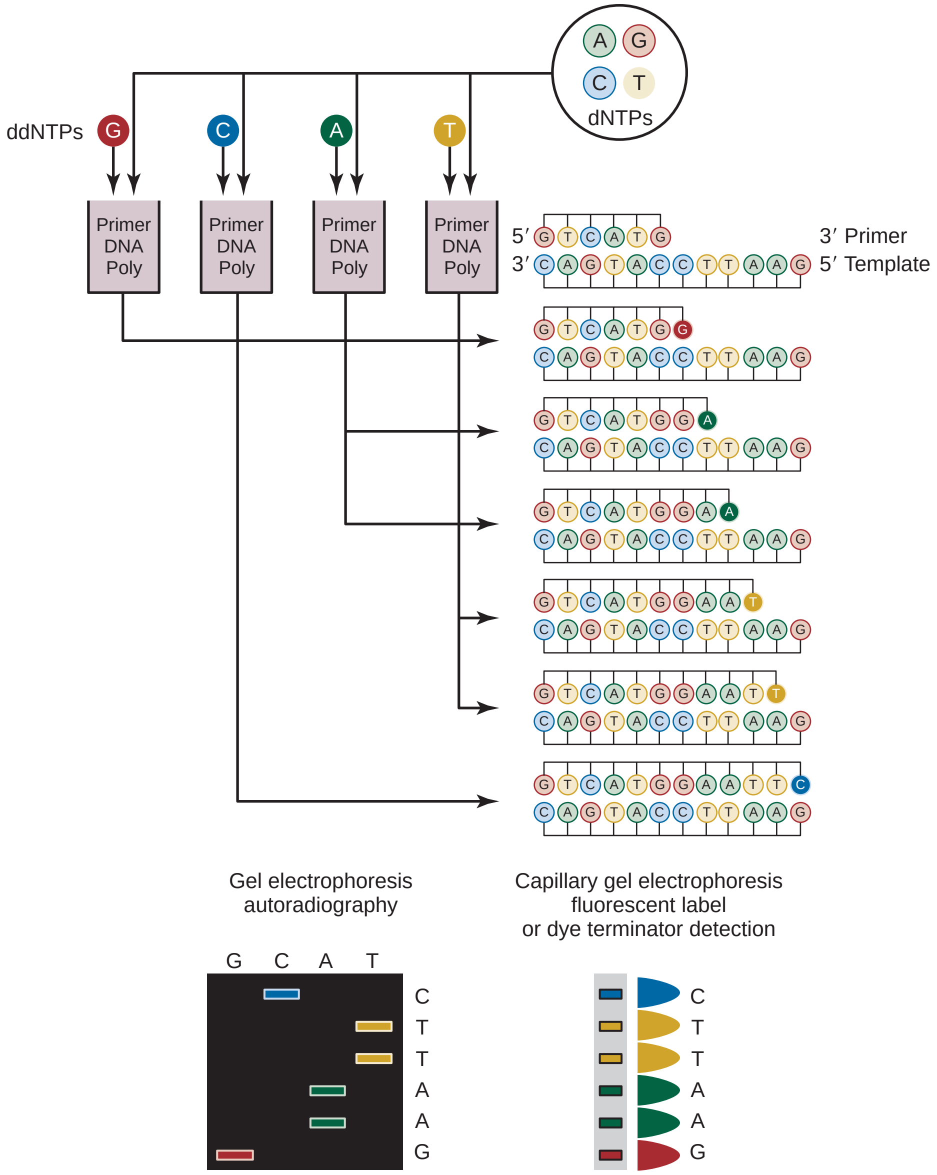
dNTPs
A
G
C
T
ddNTPs
G
C
A
T
Primer DNA Poly
Primer DNA Poly
Primer DNA Poly
Primer DNA Poly
5′ 3′ Primer
3′ 5′ Template
Gel electrophoresis autoradiography
G C A T
C T T A A G
Capillary gel electrophoresis fluorescent label or dye terminator detection
C T T A A G

Figure 41-3 Capillary (Sanger) sequencing. The dideoxynucleoside triphosphates *(ddNTPs; black)* terminate the elongation reaction by deoxyribonucleic acid *(DNA)* polymerase. Results are visualized by autoradiography or fluorescence detection. *dNTPs,* Deoxynucleoside triphosphates.

anywhere throughout the genome. More than 50 million SNPs have been cataloged in the SNP database.[37] High-density SNP arrays can be used for genetic linkage studies to map disease loci and complex traits.[38,39] Because SNP arrays can detect slight differences between individual genomes, the polymorphisms detected can be used to characterize disease susceptibility or drug effectiveness.[40,41] A comparison of intensities of DNA-bound SNP probes can be used to determine the relative DNA copy number for a given locus based on the SNP map.[42] A specific application of SNP arrays is in detection of LOH in tumors and other malignancies (Fig. 41-4).[43-46] When examining paired blood and tumor samples, SNPs detected as heterozygous in blood and homozygous in tumor (LOH) may be part of a region where a normal copy of a tumor suppressor gene was lost. A special case of LOH called uniparental disomy is a copy-neutral gene conversion.[47-49] Instead of a deletion leading to the loss of the normal allele, a nondisjunction event results in LOH without a change in copy number. Although undetectable by FISH or karyotyping, this important type of LOH can be inferred from an SNP virtual karyotype.

Chromatin Immunoprecipitation, ChIP-Chip, ChIP-Seq

Chromatin immunoprecipitation (ChIP) is used to investigate the interaction between proteins and DNA. In addition to defining the locations of transcription factor binding, ChIP can also aid in determining the location of histone modifications.[50-52] The basic concepts for all ChIP studies are similar (Fig. 41-5). Proteins are cross-linked to DNA, and the cell is lysed. The protein-bound DNA is sheared, and immunoprecipitation is used to “pull down” the protein-bound DNA regions of interest.[53] The DNA is then isolated and purified, and different

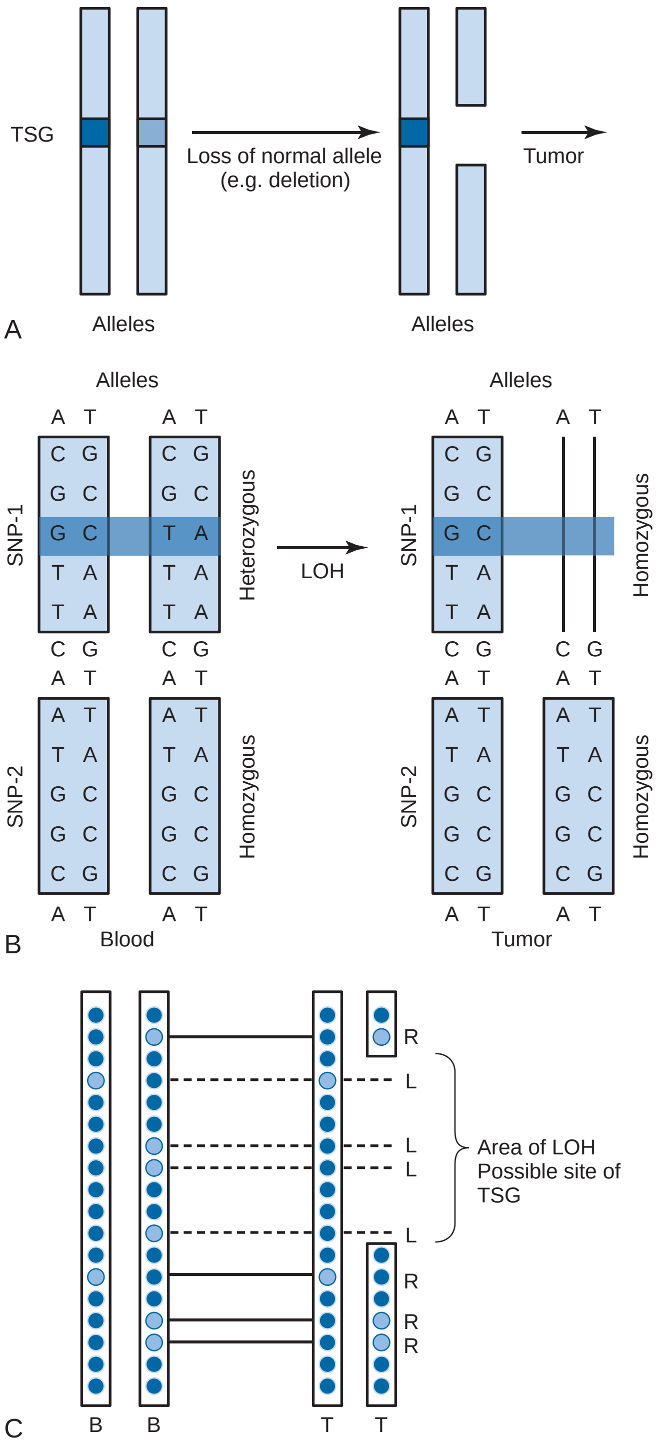

Figure 41-4 Using single nucleotide polymorphism *(SNP)* array technology to detect loss of heterozygosity (LOH) with detection of a possible tumor suppressor gene *(TSG)*. **A,** Part of a chromosome with a mutated *(black)* and normal *(gray)* copy of a TSG. Loss of the normal TSG leads to tumor formation, because the remaining TSG is not functional. **B,** Region of chromosome from blood and tumor showing two SNPs. The subject is homozygous for SNP-2 in the blood but heterozygous for SNP-1. In the tumor sample from the same patient, SNP-1 is detected as homozygous, because one of the alleles has been lost. This "loss of heterozygosity" can be a marker for chromosomal deletion. **C,** An expanded view of both alleles showing many SNPs. In the tumor, several of the heterozygous SNPs have been lost, indicating the site of a possible TSG. Homozygous SNPs are noninformative, because the loss of an allele will not be detected.

techniques are used to determine the sequence and region of DNA bound by the protein. In standard ChIP, low-resolution, traditional sequencing methods[54] or PCR are used, followed by genome mapping. ChIP has been productively applied to study histone binding,[55,56] histone acetylation,[57] and cell differentiation in myeloid leukemia cells.[58] To increase the coverage and depth of DNA binding site discovery, the analysis of protein-bound DNA was extended to DNA microarrays ("ChIP-chip" or "ChIP-on-chip").[59-61] This technique has been applied to describe histone binding patterns,[62,63] identify therapeutic targets in persons with acute myelogenous leukemia,[64] and describe and map transcriptional networks and histone modifications in leukemia.[65,66] With the development and popularization of next-generation sequencing (NGS) techniques, the products of ChIP can be subjected to high-resolution sequencing ("ChIP-seq"). This technique has been used to describe a genome-wide map of chromatin binding in stem cells,[67] delineate histone phosphorylation,[68] identify transcription factor binding sites,[69] demonstrate the requirement of histone ligase in mixed-lineage leukemia–rearranged leukemia,[70] describe transcriptional networks,[65] and provide overall maps of chromatin binding and histone modifications in leukemia.[66]

Genome-Wide Association Studies

The purpose of a genome-wide association study (GWAS) is typically to compare two groups (e.g., cases/control) to find common genetic variants associated with either group.[71-73] As a by-product of sequencing the human genome, the discovery of large numbers of SNP variants facilitated an unbiased interrogation of the genome. Useful SNPs must be selectively neutral and have relatively high frequencies (generally >0.05). More than 1 million SNPs were collected for the HapMap project, capturing most of the genomic variation in a select number of human populations.[74] GWA studies are only dependent on linkage disequilibrium between the genotyped SNP and the nongenotyped causal variants, with the strength of the association depending on their allele frequencies (Fig. 41-6). Hundreds of loci have been discovered for many complex diseases and traits such as Crohn disease,[75] ulcerative colitis,[76] and short stature.[77] Metabolic traits, autoimmune diseases, cancer, and many complex traits have been studied extensively and with large sample sizes by GWA studies.[77a] Data from a GWA study are typically visualized with use of a Manhattan plot (Fig. 41-7).[78] Much of the statistical analysis of GWA study data focuses on determining the significance threshold given the sample size and the large number of features (multiple testing).[79]

Exome Sequencing/Targeted Sequencing

Although GWA studies have identified many loci contributing to diseases and complex traits, most findings account for only a small proportion of the heritability.[80,81] Many mendelian disorders have been attributed to underlying genetic changes through linkage analysis and resequencing. Nevertheless, the causative mutations for more than half of the ~6000 known mendelian disorders remain

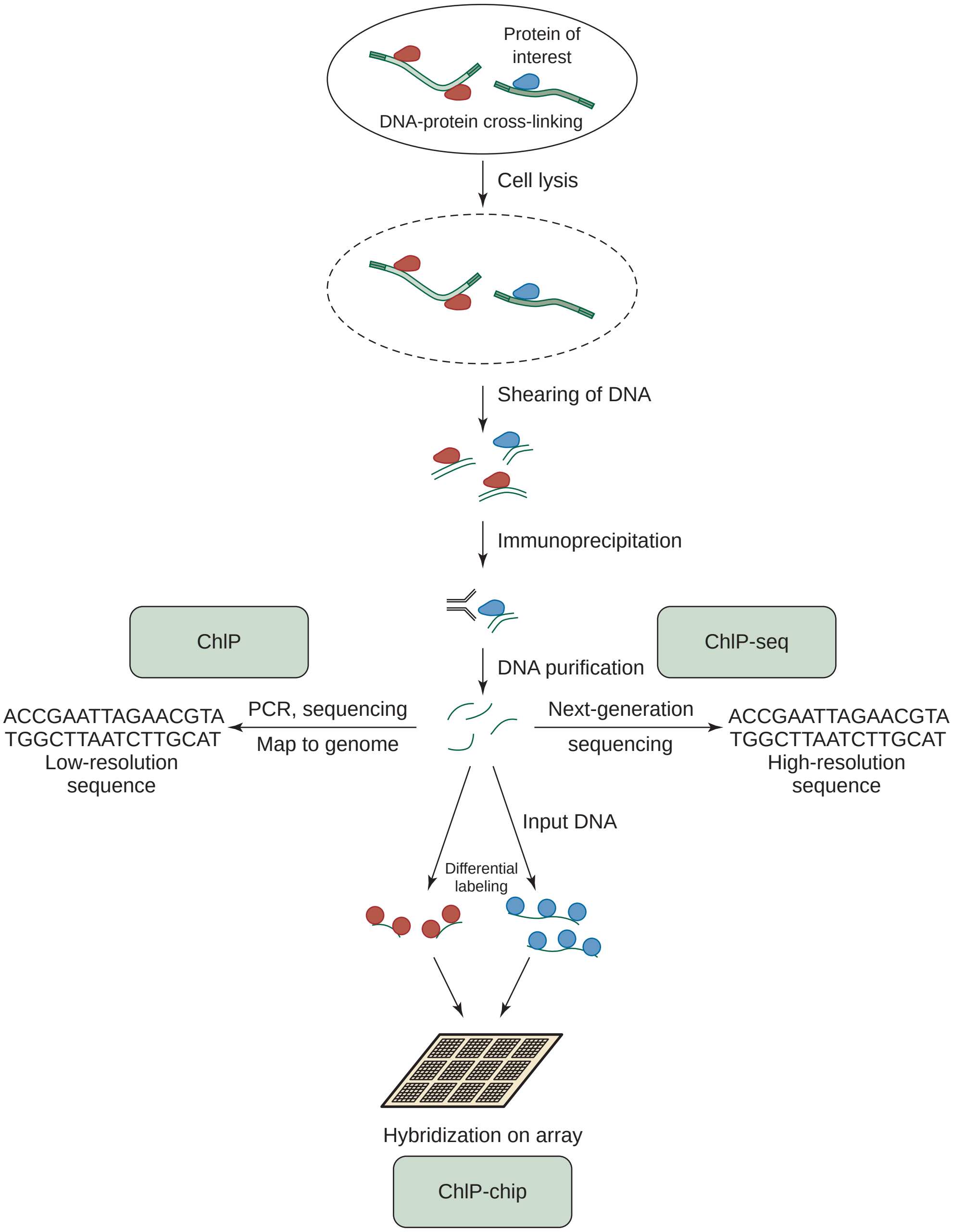
Protein of interest
DNA-protein cross-linking
Cell lysis
Shearing of DNA
Immunoprecipitation
ChIP
ChIP-seq
DNA purification
ACCGAATTAGAACGTA
TGGCTTAATCTTGCAT
Low-resolution sequence
PCR, sequencing
Map to genome
Next-generation
sequencing
ACCGAATTAGAACGTA
TGGCTTAATCTTGCAT
High-resolution sequence
Input DNA
Differential labeling
Hybridization on array
ChIP-chip

Figure 41-5 Chromatin immunoprecipitation *(ChIP)* is used to determine the deoxyribonucleic acid *(DNA)* sequences bound to proteins of interest. In traditional ChIP *(left)*, proteins are cross-linked to DNA, the cells are lysed, and the DNA is sheared. Immunoprecipitation is used to pull down the protein-DNA complexes. Purified DNA is then subjected to sequencing through traditional methods with mapping to the genome. In ChIP-chip (ChIP-on-chip), the DNA is not sequenced but is instead labeled and applied to a DNA array. By applying differentially labeled input DNA, the regions to which the proteins were bound can be ascertained. Most recently, high-resolution sequencing (ChIP-seq) techniques have emerged in which the isolated and purified DNA is subjected to high-resolution next-generation sequencing. *PCR,* Polymerase chain reaction.

unknown, mostly because of the paucity of cases and low penetrance.[82-84] Therefore an abundance of low-penetrant common variants are now described that reportedly contribute to the inheritance of common diseases (Fig. 41-8).[85] Highly penetrant but rare mutations that contribute to diseases with mendelian inheritance are being identified through NGS high-throughput methods that parallelize the sequencing process, producing millions of concurrent sequences.[86-89,90-92] To better focus on these potential causes of disease, target enrichment strategies were developed to facilitate a focused sequencing to determine the coding variation in the entire genome (Fig. 41-9).[93,94] The exome represents the 1% of the genome (30 megabases) that is translated into proteins.[95] Although the untranslated regions are part of the exon, exome sequencing studies usually do not include these regions. The general workflow for whole exome sequencing (WES) involves DNA extraction, fragmentation, exome segment isolation, and sequencing (Fig. 41-10).[96] For many diseases for which conventional approaches have failed,

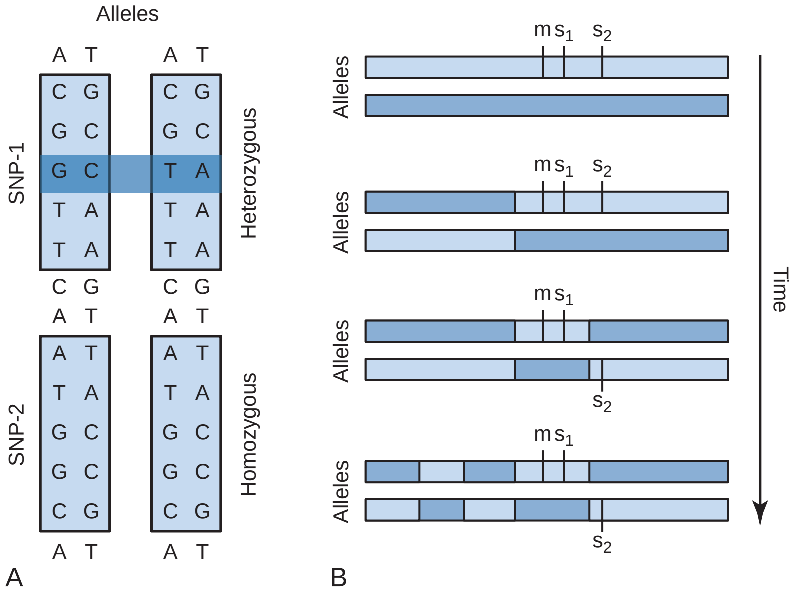

Figure 41-6 **A,** Two single nucleotide polymorphisms *(SNPs)* are shown, both on the same chromosome. This individual is heterozygous for SNP-1 and homozygous for SNP-2. **B,** A gene mutation is shown, along with two SNPs. The first SNP is closely associated with the mutation, whereas the second is in strong linage disequilibrium. Note how during recombination, the haplotype block containing the mutation and SNP-1 remains intact, whereas SNP-2 does not remain associated. By comparing large numbers of SNPs in persons with a disease to persons without a disease, one can infer the location of mutations associated with the condition.

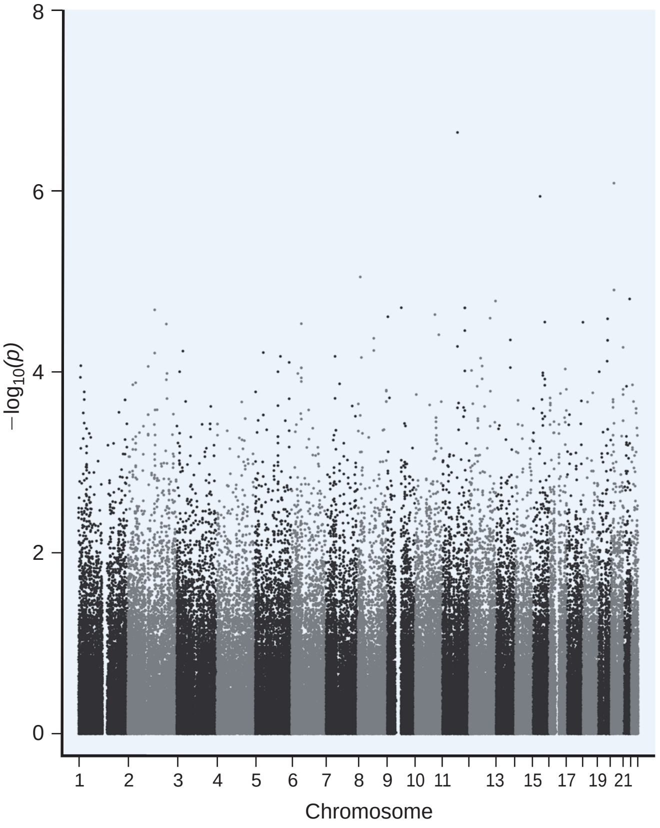

Figure 41-7 A typical Manhattan plot showing the significance of genetic variants (single nucleotide polymorphisms) along each chromosome. The highest ones are most likely to be statistically significant.

exome sequencing has led to candidate gene identification.[97,98,99,100] Targeted exome sequencing has also provided evidence for alternative transcriptional start sites in colorectal carcinoma,[101] RET gene fusion in lung cancer in nonsmokers,[102] and stage-specific alternative splicing in neuroblastoma.[103] Of course, identifying the disease-related genes among all of the sequencing errors and non–disease-causing polymorphisms remains a considerable challenge. The application of exome sequencing typically results in identification of more than 20,000 single nucleotide variants, and although most are known polymorphisms, other details are necessary, such as the pedigree, mode of inheritance, and sample size. The informatics techniques for identifying causal and alternatively spliced variants are well established.[97,104-106] Although the costs of whole genome sequencing (WGS) are decreasing, WES remains a mainstay for identifying causal variants of genetic diseases.

Whole Genome Sequencing

NGS methodologies can be applied to determine the entire sequence of an organism's genome. A current role for WGS and WES is to study whether common complex diseases are driven by rare variants ("rare variant hypothesis")[107-109] or by many common variants with small effects on risk ("common disease–common variant").[110-112] Methods for WGS are similar to exome and whole transcriptome sequencing (Fig. 41-10). Isolated and fragmented DNA is subjected to repetitive and overlapping sequencing to achieve the most robust coverage. Although the cost of WGS remains relatively high, identification of rare disease-causing variants is often achieved by family studies in which sequencing efforts are focused on individuals at the extremes of the trait's

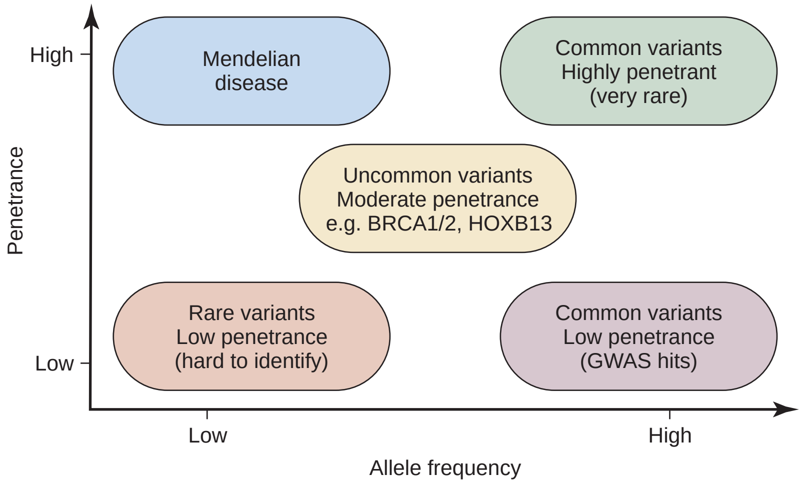
High
Low
Penetrance
Mendelian disease
Common variants Highly penetrant (very rare)
Uncommon variants Moderate penetrance e.g. BRCA1/2, HOXB13
Rare variants Low penetrance (hard to identify)
Common variants Low penetrance (GWAS hits)
Low
High
Allele frequency

Figure 41-8 Genetic architecture showing common variants with low penetrance identified by genome-wide association studies (GWAS). Conversely, mendelian diseases are characterized by being rare but highly penetrant. Many interesting findings will fall in the middle range with modest variant frequency with moderate penetrance. *(Modified from McCarthy MI, Abecasis GR, Cardon LR, et al: Genome-wide association studies for complex traits: consensus, uncertainty and challenges.* Nat Rev Genet *9:356–369, 2008, and Eeles R, Goh C, Castro E, et al: The genetic epidemiology of prostate cancer and its clinical implications.* Nat Rev Urol *11:18–31, 2014.)*

distribution or by studying the most distant coaffected subjects.[109] As might be expected, considerable informatics challenges exist for determining significant rare variants.[113]

RNA-BASED METHODS

Whole Transcriptome Shotgun Sequencing (RNA-Seq)

Although hybridization-based (e.g., microarray) studies target the millions of common alleles (SNPs) in the genome, such libraries are limited in their ability to detect rare variants that cause disease. NGS methodologies facilitate the unbiased sequencing of all RNA transcripts in a cell (RNA-seq[114,115]; Fig. 41-9), providing a means to study gene fusions,[116] mutations, alternative splicing,[117-119] and modulation in gene expression levels.[120-124] Subpopulations of RNA such as microRNA (miRNA), transfer RNA, and ribosomes can be characterized as well.[125-129] Sequencing of the transcriptome has been applied to many malignancies, including prostate cancer,[130] mesothelioma,[131] squamous cell lung cancer,[132] Fanconi anemia,[133] and breast cancer.[134] The general workflow for transcriptome sequencing is similar to WGS and WES, except that isolated RNA must first be converted into a cDNA library (Fig. 41-10). Once sequencing data have been collected, alignment and contig generation depend on the presence of a scaffold for a known genome or de novo methods for a novel genome.[114,135-137]

Expression Profiling

By measuring the activity of thousands of genes simultaneously, it is possible to make broad global assessments of cellular activity or compare expression levels of genes between two different states or conditions.[35,138] The development and commercialization of DNA microarrays have commoditized the analysis of gene expression, making it a common method of study of biologic systems. The general process is similar to other microarray-based workflows (Fig. 41-11). Commercial systems (e.g., Affymetrix GeneChip and Illumina BeadArray) utilize oligonucleotide arrays of varying density, depending on the application. The target sample is prepared by extracting miRNA, creating cDNA using reverse transcriptase,

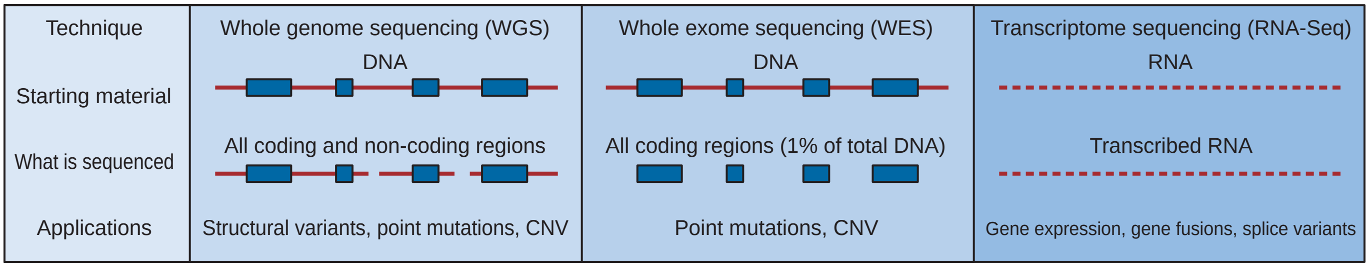
Technique
Starting material
What is sequenced
Applications
Whole genome sequencing (WGS)
DNA
All coding and non-coding regions
Structural variants, point mutations, CNV
Whole exome sequencing (WES)
DNA
All coding regions (1% of total DNA)
Point mutations, CNV
Transcriptome sequencing (RNA-Seq)
RNA
Transcribed RNA
Gene expression, gene fusions, splice variants

Figure 41-9 Strategies for next-generation sequencing *(NGS)*. Whole genome sequencing determines the deoxyribonucleic acid *(DNA)* sequence for all DNA, both introns and exons, whereas whole exome sequencing only describes the coding portions. In transcriptomics, the transcribed ribonucleic acid *(RNA)* is sequenced, leading to information about gene expression, gene fusions, and splice variants. *CNV,* Copy number variation. *(Modified from Simon R, Roychowdhury S: Implementing personalized cancer genomics in clinical trials.* Nat Rev Drug Discov *12:358–369, 2013.)*

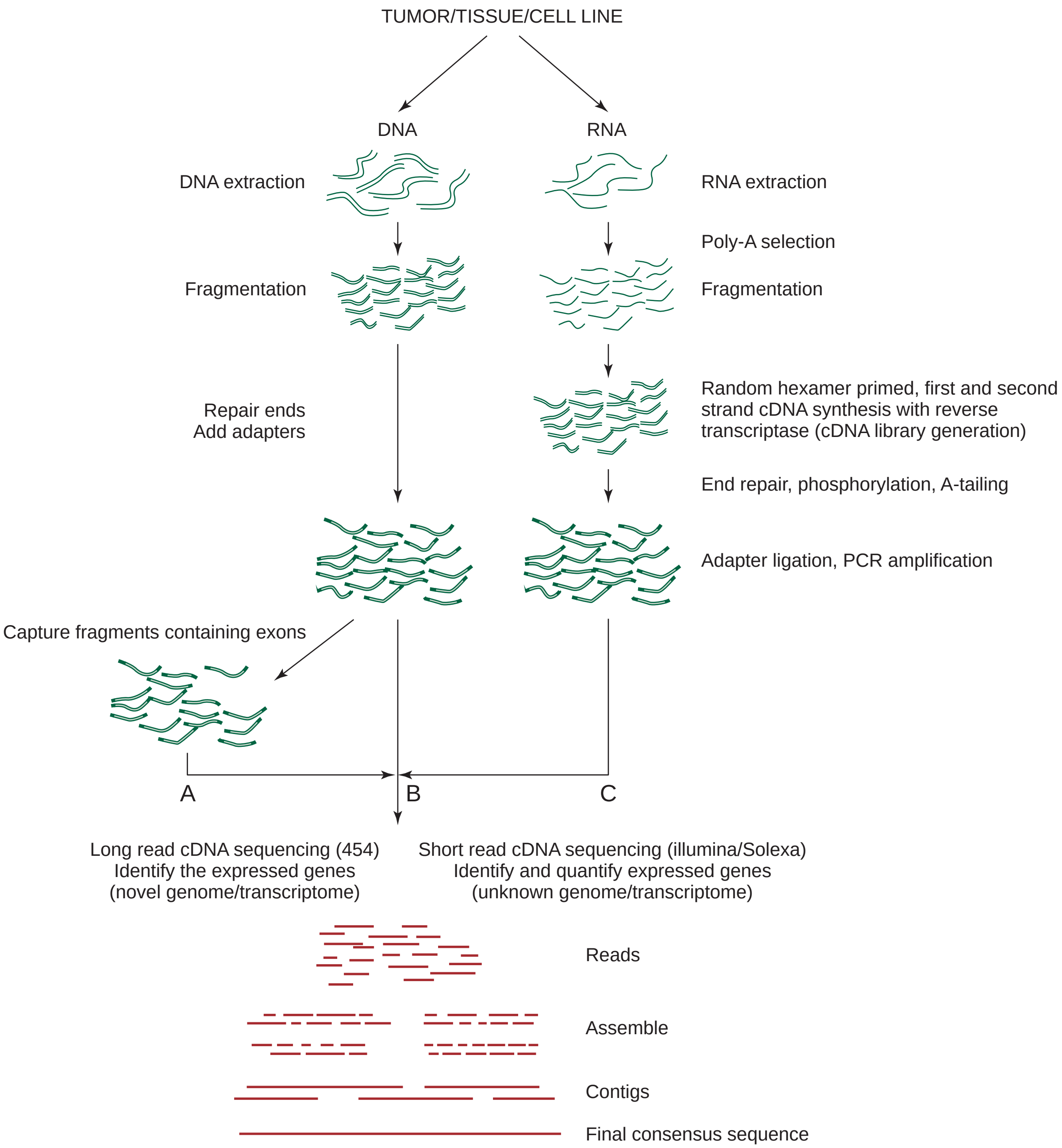
TUMOR/TISSUE/CELL LINE
DNA
RNA
DNA extraction
RNA extraction
Poly-A selection
Fragmentation
Fragmentation
Repair ends
Add adapters
Random hexamer primed, first and second strand cDNA synthesis with reverse transcriptase (cDNA library generation)
End repair, phosphorylation, A-tailing
Adapter ligation, PCR amplification
Capture fragments containing exons
A
B
C
Long read cDNA sequencing (454)
Identify the expressed genes
(novel genome/transcriptome)
Short read cDNA sequencing (illumina/Solexa)
Identify and quantify expressed genes
(unknown genome/transcriptome)
Reads
Assemble
Contigs
Final consensus sequence

Figure 41-10 Workflow for whole exome sequencing *(A)*, whole genome sequencing *(B)*, and ribonucleic acid *(RNA)*-seq/whole transcriptome sequencing *(C)*. *DNA*, Deoxyribonucleic acid; *cDNA*, complementary DNA; *PCR*, polymerase chain reaction. *(Partially modified from Bras J, Guerreiro R, Hardy J: Use of next-generation sequencing and other whole-genome strategies to dissect neurological disease.* Nat Rev Neurosci *13:453–464, 2012.)*

and then labeling the DNA. Even though each chip characterizes a single sample, the high reproducibility of the system facilitates comparison of expression levels between more than one condition. To compare two conditions directly, they are first differentially labeled (e.g., green and red), combined, and then applied to a cDNA array with subsequent measurement of the intensity ratios.[139] Three applications for gene expression profiling are: (1) class comparison, (2) class prediction, and (3) class discovery.[138] For class comparison, the objective is to understand which genes are differentially expressed between two known disease subtypes or conditions (Fig. 41-12).[140] The goal of class prediction is to define a classifier that can facilitate class membership for a sample based on its gene expression profile.[141-143,144-146] Class discovery is useful when trying to define clinically relevant molecular subtypes.[140,146-150] MiRNA functions as a regulator of gene expression both during and after transcription.[151] The expression of miRNA can be monitored with use of microarrays, as described earlier.[152,153] Such methods were used to show a novel miRNA profile for chronic lymphocytic leukemia.[154-156]

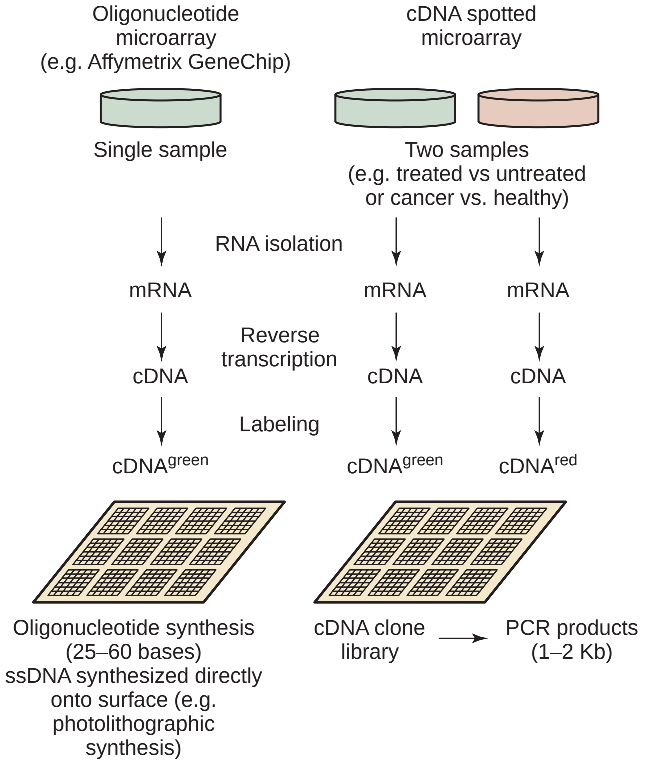
Oligonucleotide microarray (e.g. Affymetrix GeneChip)
cDNA spotted microarray
Single sample
Two samples (e.g. treated vs untreated or cancer vs. healthy)
RNA isolation
mRNA
mRNA
mRNA
Reverse transcription
cDNA
cDNA
cDNA
Labeling
cDNA^green
cDNA^green
cDNA^red
Oligonucleotide synthesis (25–60 bases) ssDNA synthesized directly onto surface (e.g. photolithographic synthesis)
cDNA clone library
PCR products (1–2 Kb)

Figure 41-11 Oligonucleotide arrays *(left)* require synthesis of the deoxyribonucleic acid *(DNA)* sequence directly onto the solid support, whereas complementary DNA *(cDNA)* spotted arrays *(right)* are produced by the deposition of DNA onto the slide. Because of the high reproducibility of the oligonucleotide arrays, comparisons between chip analyses can be performed to determine differential levels of gene expression. To compare two conditions using cDNA microarrays, a single experiment using two different color fluorophores is required. *mRNA,* Messenger ribonucleic acid; *PCR,* polymerase chain reaction; *ssDNA,* single-stranded DNA.

PROTEIN-BASED METHODS

Protein Microarrays

The large-scale monitoring of protein expression, function determination, and interaction can be assessed through the use of protein microarrays. Similar to DNA microarrays in concept, proteins are immobilized on the surface of a solid support, such as a glass slide.[157,158] Protein probes labeled with fluorescent dye are applied to the array and the interactions are monitored by laser scanning. Protein microarrays have been used to construct interactome maps,[159] monitor protein levels in patients with chronic graft-versus-host disease,[160] and facilitate biomarker identification for cancer and other diseases.[161-164]

Mass Spectrometry

Proteomic analysis by mass spectrometry (MS) remains the mainstay of comprehensive protein analysis in cells and other complex systems. In general, most MS-based studies follow a paradigm of protein isolation, enzymatic digestion, separation, MS, and informatic analysis (Fig. 41-13).[165] For quantitative studies,[166] cells can be grown in differential isotopic media (with stable isotope labeling by amino acids in culture) or labeled during or after enzymatic digestion (oxygen-18 [18O],[167] isobaric tag for relative and absolute quantitation [iTRAQ][168]) so that amounts of nonlabeled (light) and labeled (heavy) peptides can be compared. Traditional proteomic approaches involving data-dependent acquisition are based on selecting the top most abundant precursor ions for fragmentation. As a result, protein identifications are disproportionate, with the abundant proteins in a sample

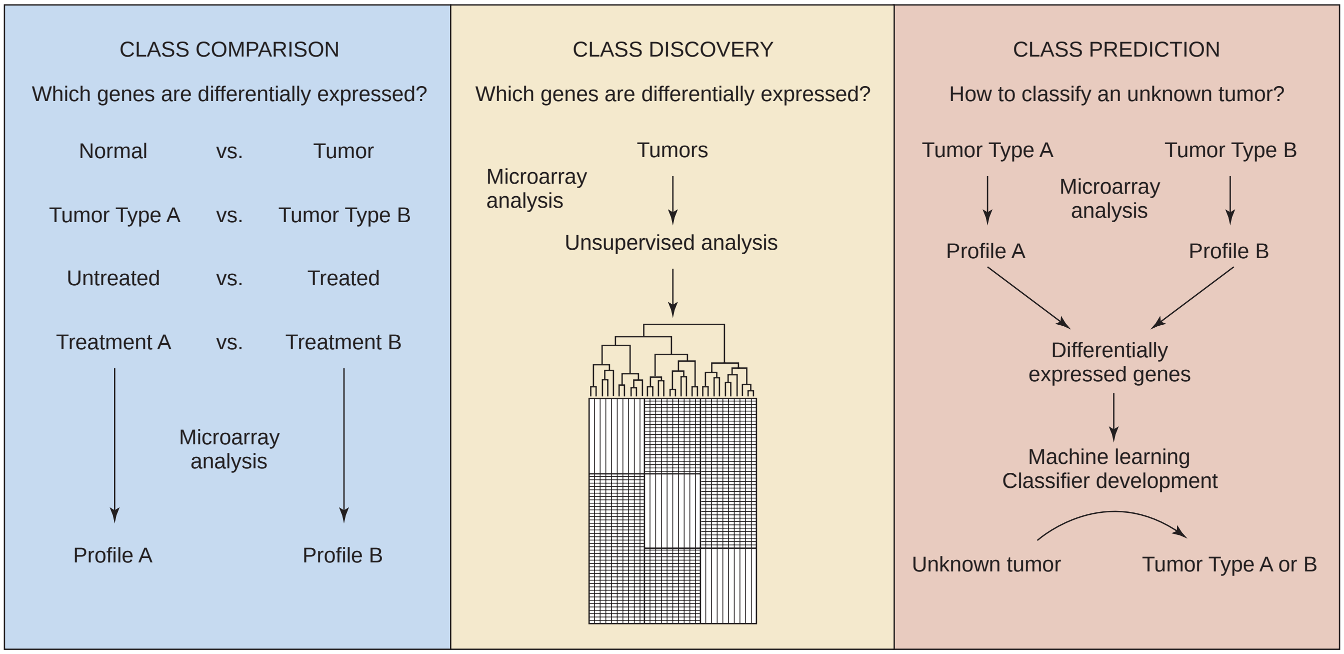
CLASS COMPARISON
Which genes are differentially expressed?
Normal vs. Tumor
Tumor Type A vs. Tumor Type B
Untreated vs. Treated
Treatment A vs. Treatment B
Microarray analysis
Profile A
Profile B
CLASS DISCOVERY
Which genes are differentially expressed?
Tumors
Microarray analysis
Unsupervised analysis
CLASS PREDICTION
How to classify an unknown tumor?
Tumor Type A
Tumor Type B
Microarray analysis
Profile A
Profile B
Differentially expressed genes
Machine learning Classifier development
Unknown tumor
Tumor Type A or B

Figure 41-12 Applications for gene profiling include class comparison *(left)* in which two different conditions or tumors are compared to determine which genes are differentially expressed. Class discovery *(middle)* does not require prior knowledge about the tumor types but instead looks for subgroups of differentially expressed genes. Class prediction *(right)* results in the discovery of a gene classifier that can predict the class of an unknown sample.

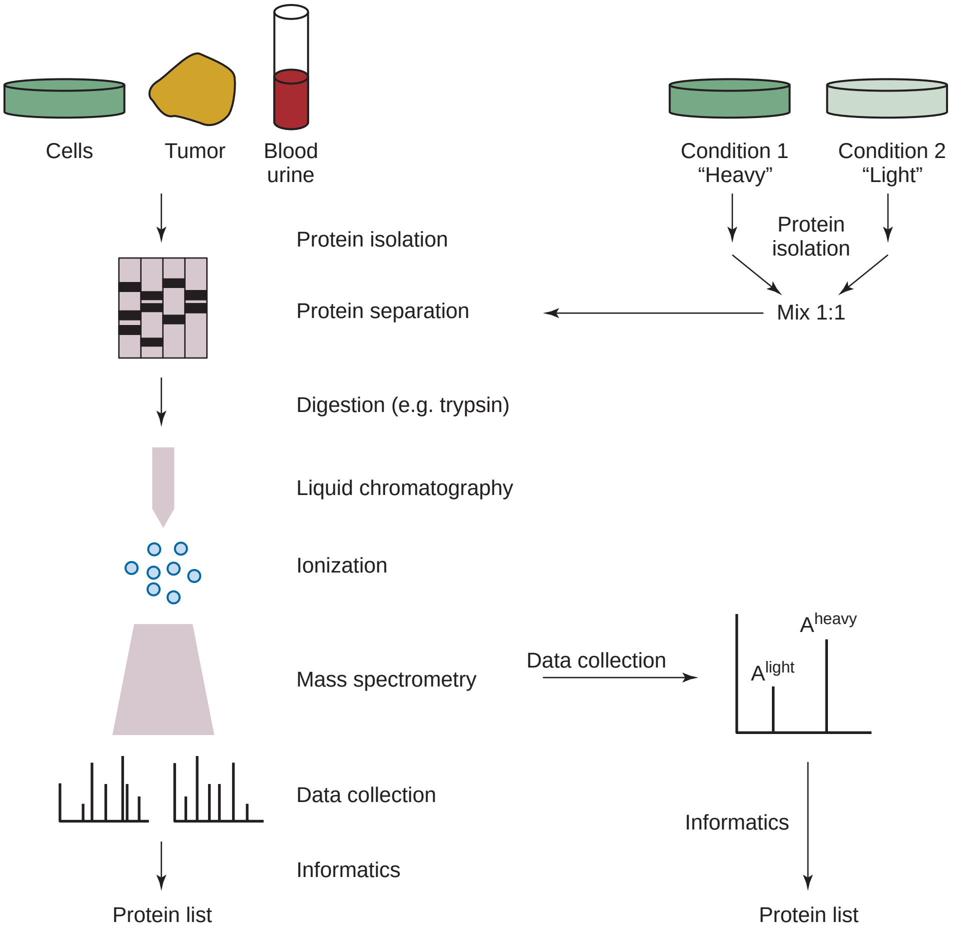

Figure 41-13 The standard workflow for mass spectrometry–based proteomics *(left)* includes isolation of proteins from a complex sample followed by separation, usually by gel electrophoresis. The proteins are then subjected to trypsin digestion and then separated by liquid chromatography prior to ionization and injection onto the mass spectrometer. All data are collected and then analyzed offline to match spectra to peptides and to create the most parsimonious protein list. A quantitative proteomics experiment *(right)* often involves growing cells in a "light" (normal) and "heavy" isotope-labeled media. The proteins are then mixed in equal amounts and subjected to a similar workflow. Peptides from each condition can be identified by the characteristic mass shift, and their relative peak heights correspond to quantitative differences.

getting more coverage and low-abundance (and often more interesting) proteins being missed entirely. The downside of narrow dynamic range is evident in MS analysis of complex samples such as human serum, wherein the most abundant proteins account for more than 85% to 90% of total protein content,[169,170] whereas the more informative proteins remain in the low molecular weight serum proteome.[171-173] To circumvent these issues, researchers typically incorporate the use of labeled cell lines, enrichment strategies, fractionation techniques, and protein depletion methods (e.g., Agilent [Santa Clara, Calif.] Multiple Affinity Removal System column) into experimental protocols.[171,174] On the analysis front, inclusion lists and single/multiple reaction monitoring assays for targeted proteomics are used and have been shown to be sensitive[175,176] even in the context of biomarker validation in human plasma.[177,178] However, these methods still have a fundamental flaw in that the candidates for inclusion are chosen without regard for the overlap between the proteins of interest and the dominant background. Thus, in the context of analyzing important cancer protein markers, the complexity of the human proteome makes it difficult for the MS platform to characterize a few select proteins effectively. Informatics challenges present a second major obstacle to the effective use of proteomics in a clinical setting. Providing researchers and clinicians with access to powerful computing resources and data management tools that are capable of rapidly processing the hundreds of samples in large-scale clinical settings remains a challenge. Existing proteomics pipelines such as the Trans-Proteomic Pipeline,[179] OpenMS,[180] and ProteoWizard[181,182] run on workstations but do not work effectively at these scales. On-demand cloud computing services, such as that operated by Amazon Web Services, provide an intriguing alternative,[183] and cloud-based options for data processing are available.[184-186] Galaxy-P (https://usegalaxyp.org/), developed at the University of Minnesota, is a framework for performing computation-intensive proteomic data processing on their computer cluster. Inspired by the public Galaxy server[187,188] that is used for genomics at the University of Pennsylvania, Galaxy-P offers key proteomics software applications on a freely available platform.

INFORMATICS

A full summary of the informatics tools and techniques across the broad spectrum of platforms and methods discussed is beyond the scope of this review. Nevertheless, it is important to understand a few fundamental concepts when evaluating genomic data. With the popularization and wide availability of tools for genomic data analysis, it is common for research groups to perform their own analysis despite little or no experience in bioinformatics and statistics. However, the interpretation of complex and dense data is subject to many caveats and pitfalls. The algorithms for class comparison, discovery, and prediction all require specialized knowledge for proper application. The general procedure for analysis of microarray data includes background correction, normalization, logarithmic transformation, batch effect correction, outlier removal, and probe filtering.[189-193] Each of these steps has many variations, and understanding the indications and caveats for each is an important part of data analysis.

False-Positive Rate, Sample Size, and Validation

With a large number of features, the number of false-positive results becomes significant. For instance, when testing 20,000 genes for differential expression, even if none is truly significant (i.e., no true-positives), a traditional false-positive rate of 5% would indicate that 1000 genes will be falsely classified as differentially expressed. One possible approach is simply to increase the stringency by ranking the genes by *P* value and testing the value for significance based on 5% multiplied by the rank of the *P* value divided by the number of features, thus limiting the false-discovery rate to less than 5%. A simpler method is the Bonferroni correction, in which the *P* value is divided by the number of tests.[194] The most important factors influencing the *P* value are the absolute proportion of true-positive differentially expressed genes, the distribution of the differences, the variability in expression, and the sample size.[195] Of course, overly aggressive methods for controlling for the false-discovery rate will raise the false-negative rate. Increasing the sample size can help alleviate this problem.[195,196] It is critical that differentially expressed genes be validated, either in another experiment using a different sample set or by using an alternative method such as reverse transcriptase–PCR.[196]

Clustering and Classification

Classification is the process of predicting membership in predetermined groups and requires training data with known class assignments for each point.[197] For example, with a set of gene expression data on patients with leukemia of known risk group status, one could use classification techniques to predict which features (genes) could be used to classify an unknown patient. Classification is a form of supervised learning and requires knowledge of not only the feature values (gene expression) but also the class of each sample. Supervised learning methods are usually neighbor based, such as k-nearest neighbors, or based on discriminating hyperplanes, such as linear discriminant analysis, neural networks, and support vector machines.[198,199] In clustering analysis, no predefined classification is required. Instead, the classification is to be learned from the data only, because the algorithms divide the data into natural groups based on intrinsic properties only. A common unsupervised method is principal component analysis, which is particularly useful for microarray expression data where many intracorrelations exist between a large number of the variables.[200,201] Transforming the original high-dimensional data into a lower dimensional space retains the intrinsic critical information while still facilitating a meaningful separation of the samples into relevant groups. Hierarchical clustering is another form of unsupervised analysis, which is commonly visualized through heat maps.[197]

Gene Set Enrichment Analysis

After using statistical analyses with gene expression data, it may still be difficult to extract useful meaning from the data. For instance, after correction for multiple hypotheses testing, few or no genes may be found to be differentially expressed. Alternatively, the list of differentially expressed genes may appear to have no underlying biologic theme. Additionally, the overall effect of pathway interactions may be masked as a result of the measurement of one gene at a time. One way to compensate for these shortcomings is to apply gene set enrichment analysis to the data.[202] The strategy in gene set enrichment analysis is to use predefined knowledge of gene pathways to evaluate microarray data at the level of the gene set. This process is a means of identifying functionally related groups of genes.[203]

CONCLUSIONS

It remains challenging to keep pace with the rapid advancements in systems biology technology. The development and widespread adoption of NGS technologies have resulted in torrents of data. The informatics challenges are considerable and include issues of storage, access, and analysis. These problems will only continue to grow because the rate of data accumulation is likely to increase. As more institutions adopt genomic testing platforms, informaticists, geneticists, pathologists, and other clinicians will need to work together to apply these tools appropriately.[204] With the concurrent popularization and widespread availability of genomics analysis tools, it is increasingly important to retain expertise in the acquisition and analysis of high-density genomic data. Many successful research institutions have established bioinformatics core service teams that serve as collaborators throughout the experimental process, from planning to data collection to analysis and interpretation. Bioinformatics experts are most likely to have access to the most current and advanced tools and will understand the subtleties and nuances inherent to complex data analysis and interpretation. For the nonexpert, it is most important to understand the context in which the technology or platform is being used. As old methods are supplanted by new ones, the most well-informed scientist, researcher, or clinician will recognize the progression and adoption of newer technologies and appreciate any positive and negative implications. We have never experienced a more exciting time in medicine. In the next 10 years, enormous growth will occur in our knowledge about complex biological processes, resulting in new methods of disease prevention and therapy.

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CHAPTER 42

Genetic Predisposition to Cancer

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CHAPTER OUTLINE

PRINCIPLES OF CANCER PREDISPOSITION
IDENTIFICATION OF A CHILD WITH A CONDITION PREDISPOSING TO CANCER
- Features of Family History Suggestive of a Heritable Predisposition to Cancer
- Tumor Features Suggestive of a Heritable Predisposition to Cancer
- Other Clinical Features Suggestive of a Heritable Predisposition to Cancer
- Incidental Identification of a Child with a Condition Predisposing to Cancer
- Genetic Testing for a Heritable Predisposition to Cancer
- Management of Children with a Heritable Predisposition to Cancer

SPECIFIC SYNDROMES PREDISPOSING TO CANCER
- Hereditary Retinoblastoma
- Li-Fraumeni Syndrome
- *WT1*-Related Wilms Tumor Predisposition Syndromes
- Wilms Tumor, Aniridia, Genital Anomalies, Retardation Syndrome
- Denys-Drash Syndrome
- Frasier Syndrome
- Hereditary Paraganglioma/Pheochromocytoma Syndrome
- Neurofibromatosis Type 1
- Von Hippel–Lindau Syndrome
- Beckwith-Wiedemann Syndrome and Isolated Hemihyperplasia
- Rhabdoid Tumor Predisposition Syndrome
- *DICER1*-Related Tumor Predisposition Syndrome
- Constitutional Mismatch Repair–Deficiency Syndrome

CONCLUSIONS, CHALLENGES, AND LOOKING TO THE FUTURE

It is currently estimated that 1% to 10% of children with cancer develop the disease due to an underlying genetic predisposition.[1-4] Over the last 3 decades, there has been an explosion in knowledge regarding the heritable causes of cancer, including the discovery of many genes that when mutated in the germline significantly increase the risk for tumor formation in children. This knowledge has allowed for a better understanding of the mechanisms leading to tumor formation and provided insights into the development of novel cancer therapies targeting defective genetic pathways. Through presymptomatic genetic testing, it is also now possible to identify children who are at increased risk for tumor formation and to initiate surveillance protocols with the intent to improve overall outcomes through the detection of early-stage tumors that are more readily cured with less invasive and/or toxic therapies. In this chapter, we review the principles of cancer predisposition, steps involved in the recognition and testing of children for heritable cancer syndromes, and concepts underlying tumor surveillance. We also describe the clinical and genetic features typifying specific syndromes and review current recommendations regarding management. As there are too many conditions to discuss in this chapter, we focus on several syndromes that are associated primarily with the development of solid tumors. For a review of additional childhood cancer syndromes, including those that increase the risk for hematologic malignant tumors, we refer the reader to several excellent recent reviews.[5-8]

PRINCIPLES OF CANCER PREDISPOSITION

Seminal insights into cancer predisposition were gained in 1971 when Alfred Knudson developed the *two-hit hypothesis* to explain the epidemiology of retinoblastoma (RB), the most common pediatric eye tumor.[9] Children with RB show two patterns of tumor formation, including: (1) early-onset disease characterized by bilateral eye involvement and autosomal dominant inheritance; or (2) later-onset disease typified by unilateral eye involvement with no family history of RB. According to the two-hit hypothesis, children with bilateral RB are at increased risk for tumor formation because they carry an altered copy of a growth regulatory gene within the cells of the body; this constitutional (germline) mutation is known as the first hit. Knudson proposed that if the second gene copy underwent an inactivating mutation within a developing retinal cell (i.e., the second hit), that cell would then become susceptible to tumor formation. Because every cell in a child with heritable RB carries the first hit, children with this form are likely to develop more than one tumor (multifocal and/or bilateral RB) at a very young age. In contrast, for those with nonheritable RB, both hits would need to occur within a single retinal cell. As these mutational events are exceedingly rare, it is expected that the process would take longer and be less likely to occur. Thus, children with nonheritable RB would be older at presentation and only develop unilateral tumors.

In 1986, Stephen Friend confirmed Knudson's prediction by demonstrating that patients with bilateral RB harbor inactivating germline mutations in the RB susceptibility gene *RB1*, the first tumor suppressor gene to be identified.[10] Since this initial observation, many additional tumor suppressor genes have been cloned, and it is now clear that inactivating tumor suppressor gene mutations account for the majority of heritable cancers in humans. Other genetic mechanisms that contribute to heritable cancers include the presence of constitutional activating or "gain-of-function" mutations involving one copy of a growth-promoting oncogene, inactivating mutations in one or both alleles of an X-linked or autosomal recessive gene, and larger structural chromosomal abnormalities such as translocations, deletions, and duplications that disrupt or enhance the expression of a tumor suppressor gene or oncogene, respectively.

IDENTIFICATION OF A CHILD WITH A CONDITION PREDISPOSING TO CANCER

Pediatric oncologists play an important role in recognizing hereditary cancer syndromes in children and their families. Toward this end, there are several possible clues that point to the existence of a condition that predisposes a child to cancer, including: (1) a positive family history of cancer; (2) the diagnosis of specific tumor types in the child; (3) tumors that involve paired organs; and (4) the presence of associated physical or syndromic features. Pediatric oncologists should consider each of these factors when evaluating a patient and refer the child to a geneticist or genetic counselor when there is a suspicion of an underlying cancer predisposition.

Features of Family History Suggestive of a Heritable Predisposition to Cancer

Features of the family history that are suggestive of a genetic predisposition to cancer include the presence in the child or his or her close relatives of one or more cancers presenting at a younger than expected age, involving both of paired organs (e.g., eyes, kidneys, or adrenal glands) or exhibiting multifocality (more than one discrete tumor within an organ). Other suspicious features include the existence of multiple individuals on the same side of the family (maternal or paternal lineage) with the same type of cancer or cancers known to cluster together in certain syndromes (e.g., the clustering of soft tissue and bone sarcomas, early-onset breast cancer, brain tumors, adrenocortical cancers, and leukemia in Li-Fraumeni syndrome [LFS]).

Pediatricians and pediatric oncologists should take a careful family history and generate a pedigree as a routine part of each patient's initial clinic visit. Ideally, the pedigree should include information about the child being seen (the proband), as well as his or her first-degree relatives (parents and siblings), second-degree relatives (aunts, uncles, and grandparents), and third-degree relatives (first cousins). For any individuals who have developed a cancer, its type, site of origin, stage (laterality, focality), and the age at which it was diagnosed should be determined. It is important to note whether individuals have had more than one cancer, and if so, to distinguish whether multiple cancers represent recurrence of the initial tumor or development of a second primary malignant tumor. Finally, it is important to gather information about the ethnic background of the family, as some genetic syndromes manifest more commonly in individuals from specific geographic regions (as is the case with hereditary breast and ovarian cancer, which is more common in Ashkenazi Jewish individuals with ancestors from Eastern Europe). Information about the biologic relationship between parents can provide insights into the mode of inheritance. For example, if parents are closely related or both come from a similar geographic region, there is a greater chance for consanguinity and the presence of an autosomal recessive condition.

The importance of collecting a family history cannot be underestimated; however, this procedure can have its limitations. Some hereditary syndromes exhibit incomplete penetrance where not everyone with the inherited mutation will develop a cancer. Variable expressivity can be a complicating factor, with affected individuals manifesting in different ways within a given family. One must recognize that family histories evolve over time. A child may have an affected parent; however, this parent might be young or not yet have developed a cancer or other features typical of the condition. In some cases, affected family members may die of other causes before they develop a cancer. Finally, the family history may be unrevealing when there is a de novo germline mutation in an affected child, the presence of autosomal recessive or X-linked recessive conditions (where carriers are clinically unaffected), nonpaternity, or situations in which a child is adopted and there is little knowledge of the biologic parents' family history. Due to these factors, it may be difficult to ascertain from a pedigree whether a specific syndrome is present. Nonetheless, the family history remains an important tool in recognizing conditions predisposing a child to cancer, and one should be obtained, regularly updated, and reviewed for the presence of a possible predisposition.

Tumor Features Suggestive of a Heritable Predisposition to Cancer

In addition to whether the tumor is unilateral or bilateral, unifocal or multifocal, specific tumor types should raise the suspicion of a syndrome predisposing to cancer, even in the absence of a positive family cancer history (Table 42-1). RB is an excellent example as there is a hereditable

TABLE 42-1 Pediatric Tumor Types and Underlying Syndromes Predisposing to Cancer

Tumor Type	Predisposition Syndrome	Gene(s)
Adrenocortical carcinoma*	Li-Fraumeni syndrome (LFS)	*TP53*
Astrocytoma	LFS Tuberous sclerosis	*TP53* *TSC1, TSC2*
Atypical teratoid or rhabdoid tumor*	Rhabdoid tumor syndrome	*SMARCB1/INI1*
Basal cell carcinoma	Nevoid basal cell carcinoma syndrome (NBCCS)	*PTCH1*
Choroid plexus carcinoma*	LFS	*TP53*
Cystic nephroma* Bilateral or multifocal	Pleuropulmonary blastoma (PPB) family tumor and dysplasia syndrome/*DICER1* syndrome	*DICER1*
Endolymphatic sac tumors	von Hippel–Lindau syndrome (VHL)	*VHL*
Fibroma	Familial ademonatous polyposis (FAP)	*APC*
Gardner fibroma (desmoid fibroma)		
Cardiac fibroma	NBCCS	*PTCH1*
Hemangioblastoma	VHL	*VHL*
Glioblastoma	LFS Constitutional mismatch repair–deficiency syndrome (CMMR-D)	*TP53* *MLH1, MSH2, MSH6, PMS2*
Hepatoblastoma	Beckwith-Wiedemann syndrome (BWS) FAP	11p15, *CDKN1C* *APC*
Leukemia		
Pre-B acute lymphoblastic (low-hypodiploid)* leukemia	*PAX5*-associated familial leukemia LFS	*PAX5* *TP53*
Other types†	CMMR-D Neurofibromatosis type 1 (NF1)	*MLH1, MSH2, MSH6, PMS2* *NF1*
Lipoma	PTEN hamartoma tumor syndrome (PHTS)	*PTEN*
Malignant peripheral nerve sheath tumor	NF1	*NF1*
Medulloblastoma	FAP NBCCS	*APC* *PTCH1*
Neuroblastoma* Bilateral or multifocal	Hereditary neuroblastoma	*ALK* *PHOX2B*
Optic pathway glioma	NF1	*NF1*
Ovarian sex-cord stromal tumors	PPB family tumor and dysplasia syndrome/*DICER1* syndrome Peutz-Jeghers syndrome (PJS)	*DICER1* *STK11/LKB1*
Papillary cystadenoma of epididymis or broad ligament	VHL	*VHL*
Paraganglioma (PGL)/ pheochromocytoma (PCC)*	Hereditary PGL/PCC syndrome Multiple endocrine neoplasia type 2 (MEN2) NF1 VHL	*SDHA, SDHB, SDHC, SDHD, SDHAF2, MAX, TMEM127* *RET* *NF1* *VHL*
Pineoblastoma	Hereditary retinoblastoma (RB)	*RB1*
PPB*	PPB family tumor and dysplasia syndrome/*DICER1* syndrome	*DICER1*
Retinoblastoma*	Hereditary RB	*RB1*
Sarcoma		
Liposarcoma	LFS	*TP53*
Rhabdomyosarcoma	LFS PPB family tumor and dysplasia syndrome/*DICER1* syndrome Costello syndrome	*TP53* *DICER1* *HRAS*

TABLE 42-1 Pediatric Tumor Types and Underlying Syndromes Predisposing to Cancer (Continued)

Tumor Type	Predisposition Syndrome	Gene(s)
Osteosarcoma	Hereditary RB LFS	*RB1* *TP53*
Schwannoma		
Acoustic neuroma*	Neurofibromatosis type 2 (NF2)	*NF2*
Schwanommatosis	Rhabdoid tumor syndrome	*SMARCB1/INI1*
Thyroid		
Medullary*	MEN2	*RET*
Nonmedullary	PHTS	*PTEN*
Wilms tumor	BWS	11p15, *CDKN1C*
Bilateral or multifocal*	*WT1*-related syndromes	*WT1*
Or with associated GU defects in males (hypospadias, undescended testicles)		

Seif AE: Pediatric leukemia predisposition syndromes: clues to understanding leukemogenesis. Cancer Genet *204(5):227-244, 2011; and Stieglitz E, Loh ML: Genetic predispositions to childhood leukemia.* Ther Adv Hematol *4(4):270-290, 2013.*

*Tumor types that warrant genetic testing even if there is no other personal or family history of cancer (where noted, only if tumor is bilateral or multifocal).

†Several other leukemia predisposition syndromes exist that are not discussed here.

component in 40% of patients.[11,12] Additional tumor types associated with a high likelihood of an underlying genetic predisposition include: (1) Wilms tumor (WT), where features such as bilaterality, early age of onset, and genitourinary anomalies in boys indicate a higher likelihood of a germline *WT1* mutation[13,14]; (2) pheochromocytoma (PCC) and paraganglioma (PGL), where more than 70% carry a germline mutation in *SDHB* or other associated PCC/PGL genes[15,16]; (3) atypical teratoid/rhabdoid tumor, where 35% carry a mutation in the *INI1/SMARCB1* gene[17]; (4) pleuropulmonary blastoma (PPB), where more than 70% of cases in one study carried a germline *DICER1* mutation[18]; (5) hepatoblastoma (HB), where up to 10% carry a mutation in the *APC* gene that is causative of familial adenomatous polyposis (FAP)[19]; (6) medullary thyroid cancer (MTC), where 20% to 25% carry a mutation in the *RET* protooncogene[20]; (7) medulloblastoma, where onset at an age younger than 5 years and desmoplastic histology are indicative of possible nevoid basal cell carcinoma syndrome (NBCCS, Gorlin syndrome)[21,22], where patients harbor germline mutations of *PTCH1*; and (8) adrenocortical carcinoma (ACC), choroid plexus carcinoma (CPC), and rhabdomyosarcoma presenting under the age of 3 years, where 50% to 80%, 35% to 100%, and 20% of children, respectively, carry germline mutations in the *TP53* gene.[23-27] The diagnosis of an adult-onset tumor in a child should also raise suspicion for a predisposing condition. For example, the presence of colorectal cancer could indicate a constitutional mismatch repair–deficiency (CMMR-D).[28-30]

Other Clinical Features Suggestive of a Heritable Predisposition to Cancer

Specific clinical features can provide clues to the diagnosis of an underlying cancer predisposition. These features include physical findings, cognitive or developmental disabilities, and the presence of benign tumors (Table 42-2). Primary physicians and pediatric oncologists should be alert to the presence of these manifestations in their patients. If suspicion of a hereditary cancer syndrome is raised, the child should be referred to a geneticist or genetic counselor, as well as appropriate specialist(s) for evaluation, genetic testing, and management.

Dermatologic Findings

Benign or malignant skin findings are a common feature of several cancer predisposition syndromes, and evaluation by a dermatologist may be helpful in characterizing these findings and facilitating the diagnosis. Café-au-lait macules and axillary and inguinal freckling are a well-known example and are crucial in establishing a clinical diagnosis of neurofibromatosis type 1 (NF1)[15,31] (Fig. 42-1). Café-au-lait macules are often the earliest clinical manifestation of NF1, as they are present in 99% of affected children by 1 year of age.[32] Café-au-lait macules are also a cardinal feature of CMMR-D.[33,34] Skin pigmentation differences may also provide a clue to the diagnosis of PTEN hamartoma tumor syndrome (PHTS). For example, boys with PHTS may exhibit pigmented macules of the glans penis, which should raise the suspicion of the diagnosis, especially if features such as macrocephaly, developmental delay, or autism are present. Other skin findings are also found in PHTS, including trichilemmomas and papillomatous papules; however, these may not be diagnosed until later in life.[35] Individuals with Peutz-Jeghers syndrome (PJS) commonly exhibit mucocutaneous pigmentation resembling “freckling” around the mouth, eyes, or nostrils, or on the buccal mucosa or perianal area. Skin cancers in children may also indicate the presence of a syndrome predisposing to cancer. NBCCS predisposes individuals to basal cell carcinomas, which generally appear in the late teens or early adult years, but can occasionally develop in young children. Benign skin findings such as palmar and plantar pits and facial milia may be present in individuals with NBCCS.[36]

TABLE 42-2 Clinical Manifestations of Syndromes Predisposing to Cancer

Manifestation	Predisposition Syndrome	Gene(s)
Cutaneous		
Café-au-lait macules	Neurofibromatosis type 1 (NF1)	*NF1*
	Constitutional mismatch repair–deficiency syndrome (CMMR-D)	*MLH1, MSH2, MSH6, PMS2*
Axillary or inguinal freckling	NF1	*NF1*
Penile freckling	PTEN hamartoma tumor syndrome (PHTS)	*PTEN*
Mucosal pigmentation	Peutz-Jeghers syndrome (PJS)	*STK11*
Plantar or palmar pits	Nevoid basal cell carcinoma syndrome (NBCCS)	*PTCH1*
Pilomatrixomas	Familial adenomatous polyposis (FAP)	*APC*
Dental anomalies		
Extra or missing teeth	FAP	*APC*
Jaw osteomas	FAP	*APC*
Jaw keratocysts	NBCCS	*PTCH1*
Ear anomalies		
Earlobe creases or pits	Beckwith-Wiedemann syndrome (BWS)	11p15 abnormalities, *CDKN1C*
Eye abnormalities		
Aniridia	Wilms tumor (WT), aniridia, genitourinary malformations, retardation syndrome (WAGR)	*WT1*
Cataracts	Neurofibromatosis type 2 (NF2)	*PTCH1*
	NBCCS	*NF1*
Lisch nodules	NF1	*NF2*
Congenital hypertrophy of retinal pigmented epithelium (CHRPE)	FAP	*APC*
Strabismus or leukocoria	Hereditary retinoblastoma (RB)	*RB1*
Genitourinary anomalies	*WT1*-related WT syndromes	*WT1*
Hypospadias, undescended testes, undermasculinized male external genitals, streak gonads		
Kidney abnormalities	*WT1*-related WT syndromes	*WT1*
Horseshoe kidney		
Nephroblastomatosis		
Overgrowth		
Macrosomia	BWS	11p15 abnormalities, *CDKN1C*
	Simpson Golabi Behmel syndrome	*GPC3, CXORF5*
	NBCCS	*PTCH1*
Macrocephaly	PHTS	*PTEN*
Macroglossia	BWS	11p15 abnormalities, *CDKN1C*
Hemihypertrophy	BWS	11p15 abnormalities, *CDKN1C*
Marfanoid habitus	Multiple endocrine neoplasia type 2B	*RET*
Neurologic issues		
Ataxia	Ataxia-telangiectasia	*ATM*
Autism-spectrum disorders	PHTS	*PTEN*
Cognitive delays	WAGR	*WT1*
	NF1	*NF1*
	NF2	*NF2*
Seizures	NF1	*NF1*
	NF2	*NF2*

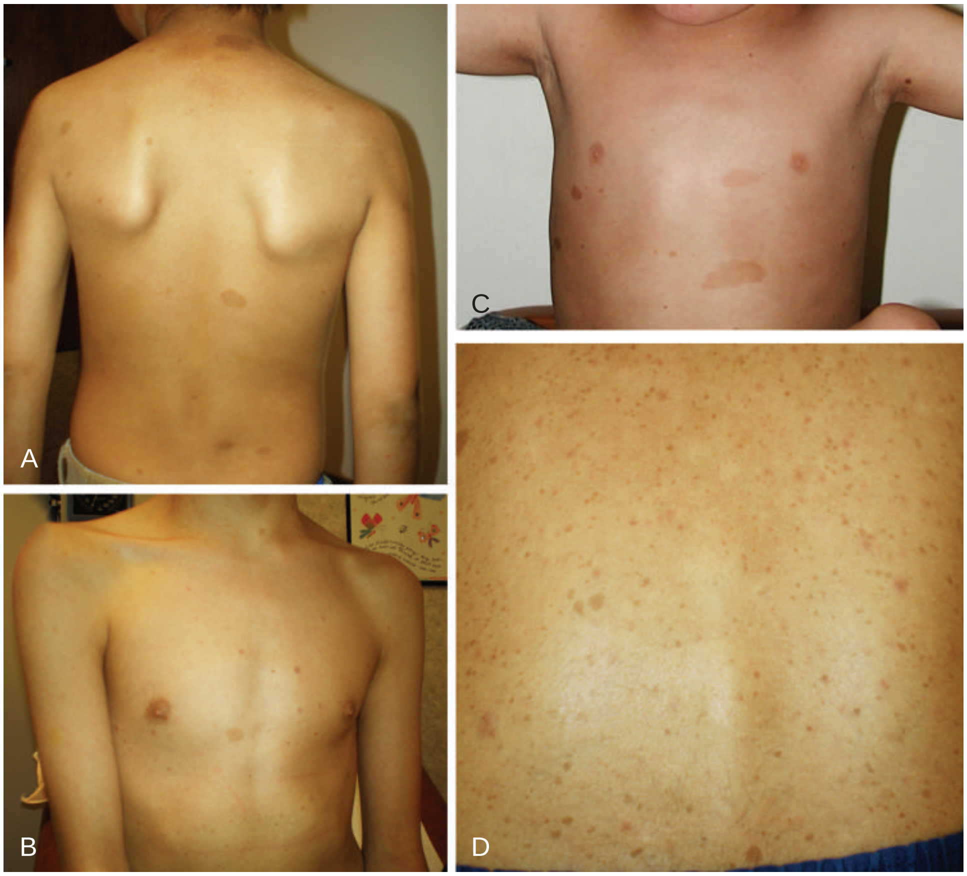

Figure 42-1 Café-au-lait spots in neurofibromatosis type 1 (NF1). (**A** to **D**). Photographs depicting the appearance of typical café-au-lait spots, as seen in NF1. Café-au-lait spots exhibit distinct edges and are slightly darker than the surrounding skin. Café-au-lait spots can increase in size, number, and darkness throughout childhood. Multiple café-au-lait spots alone are not a definitive indication for NF1; they must be present with other established criteria in order to make the diagnosis. In (**C**), there is also evidence of bilateral axillary freckling.

Developmental or Cognitive Abnormalities

Developmental or cognitive disabilities, including delays in achieving motor milestones, intellectual disabilities, and autism-spectrum disorders are commonly seen in cancer predisposition syndromes, including PHTS; WT, aniridia, genitourinary malformations, and retardation (WAGR) syndrome; tuberous sclerosis; 13q deletion syndrome (which predisposes to RB); and NF1. Seizures may also be a feature, where they are often seen in NF1 and tuberous sclerosis, and may indicate the presence of an underlying tumor. Any child with cancer who has a history of developmental or intellectual disabilities, autism-spectrum disorders, seizures, and/or other neurologic features should be considered a candidate for referral for a possible cancer genetics evaluation.

Overgrowth

Generalized or focal overgrowth of specific parts of the body can be a feature of a condition predisposing to cancer, such as Beckwith-Wiedemann syndrome (BWS), PHTS, and Simpson Golabi Behmel syndrome. In BWS overgrowth may manifest with generalized macrosomia at birth, and some patients may also exhibit macroglossia and/or asymmetric overgrowth of one side of the body (hemihypertrophy). Hemihypertrophy may involve the extremities or external physical features (face, labia, buttocks), as well as internal organs (where, for example, one kidney may be larger than the other). Macrosomia is also a feature of Simpson Golabi Behmel syndrome and Proteus syndrome (PS), while macrocephaly can be a manifestation of PHTS and NBCCS.

Specific Congenital Anomalies

In some cases, the presence of specific congenital anomalies can serve as an early indicator of a condition predisposing to cancer. For example, an omphalocele or umbilical hernia, especially in a child with macrosomia, macroglossia, ear pits or creases, or neonatal hypoglycemia, can point to the diagnosis of BWS. Another good example is WAGR syndrome, where affected children have aniridia, often in combination with genitourinary abnormalities such as hypospadias or cryptorchidism.

Other Physical Features

Other physical manifestations may also suggest an underlying hereditary cancer syndrome. Examples include individuals with multiple endocrine neoplasia type 2B (MEN2B), who often present with mucosal neuromas of the lips and tongue, ganglioneuromas, and marfanoid habitus, and those with FAP, who may present with dental abnormalities (missing or extra teeth), epidermal cysts or pilomatrixomas, Gardner fibromas (also known as desmoid tumors), osteomas (usually involving the jaws), and congenital hypertrophy of the retinal pigmented epithelium. Generally these manifestations present prior to the development of colon polyps.

Incidental Identification of a Child with a Condition Predisposing to Cancer

Until recently, genetic testing for hereditary cancer syndromes was only offered when an individual presented with a specific cancer type, physical or developmental features consistent with a known syndrome predisposing to cancer, or a suggestive family history. Due to the increasing use of genome-wide approaches to establish a genetic diagnosis, including single-nucleotide polymorphism arrays, multigene panels, and whole-exome sequencing, the "incidental" discovery of a gene mutation that predisposes to cancer is becoming more and more common. This information can lead to the unintended diagnosis of a predisposing syndrome prior to its clinical manifestation in the child and/or his or her family. Toward this end, several recent studies of genome-wide microarrays have reported the identification of copy number variations involving genes predisposing to cancer in individuals presenting with a noncancer phenotype.[37-39] In these studies, the frequency of incidental findings involving genes predisposing to cancer was estimated to be between 0.18% and 0.60%.[40-42]

The testing of tumor samples for somatic genetic mutations is also being pursued with the intent of facilitating "personalized" cancer care by identifying and therapeutically targeting mutated genetic pathways in tumors. When evaluating tumors, it is often necessary to compare tumor-derived sequences to those obtained using normal tissue from the same individual. As a result, it is possible to identify incidental constitutional mutations that are indicative of an underlying cancer predisposition or other nononcologic condition.[43,44] In one recent study, most patients undergoing tumor genetic testing expressed interest in receiving incidental germline genetic test results, as most thought this information would be valuable to family members. However, some perceived it to be an additional burden on top of their cancer diagnosis.[45] Many laboratories have specific policies regarding the sequencing of normal tissue and return of incidental results. Therefore, prior to ordering tumor testing, pediatric oncologists should be familiar with the performing laboratory's policy, discuss the possibility of obtaining incidental genetic findings with the patient and his or her parents, and determine whether or not they would like to receive these results as part of the informed-consent process.

Genetic Testing for a Heritable Predisposition to Cancer

Complexities of Childhood Cancer Genetic Testing

Genetic testing for cancer predisposition is a complex process that should be explored with the patient and family, preferably by a genetic counselor or trained genetics professional, prior to performing the testing. Counseling should include information about the natural history of the condition under consideration, methods of testing and its possible outcomes, as well as the potential risks, benefits, and limitations of testing. Patients may have concerns about privacy, confidentiality, insurance coverage, and genetic discrimination, all of which must be addressed. For the oncologist, it is important to recognize that genetic test results have implications not only for the individual being tested but also for his or her family members. This factor necessitates counseling of the whole family and discussion of how the results will be communicated between various members. When the individual under consideration is a minor (defined here as a person <18 years), the importance of these issues is further magnified by the fact that children generally do not have the capacity to fully understand the risks, benefits, and limitations of genetic testing. Therefore, they are not able to make their own informed decisions about whether or not to be tested. Instead, this decision falls to the child's parent(s) or guardian(s), and strips the child of the opportunity to make an autonomous decision about genetic testing later in life. Testing in childhood and disclosure of results to parents, guardians, and/or other family members may violate the child's privacy, confidentiality, and right not to know.[46,47,48] Many also fear that performing genetic testing on a child may have adverse psychological consequences, such as depression, anxiety, fear of the future, altered self-image, limited horizons (i.e., impact on thoughts about future education and career choices), and altered family relationships.[49] However, others have argued for the possible benefits of genetic testing in childhood, such as relief if test results are negative, providing certainty about genetic risk, and allowing the child to integrate genetic information into the self-concept at a younger age, when it is less disruptive to the sense of self. Until recently, the concerns about testing children have more or less superseded the possible benefits of such testing.

Despite much debate, available evidence suggests that the negative consequences of cancer genetic testing may be fewer and less severe than anticipated. Studies of the psychosocial consequences of genetic testing in adults have generally shown minimal or no negative consequences and suggested that emotional outcomes may be more dependent on factors such as pretest emotional state and social support than on the actual genetic test result itself.[50,51] Several studies report that adults undergoing genetic testing for LFS consider obtaining certainty about cancer risk a psychological benefit of testing, even if adults are found to be affected.[51,52] Data on the outcomes of presymptomatic cancer genetic testing of children are more limited. Much of the data comes from testing for FAP, where affected individuals are prone to develop multiple gastrointestinal polyps, which if left untreated invariably lead to early-onset colorectal cancer. In FAP, polyps generally begin to form during adolescence. Consequently, it is recommended that children begin surveillance with colonoscopy around the age of 10 to 12 years. Based on these factors, it is deemed appropriate to offer

FAP genetic testing to minors. Published reports describing the psychosocial consequences of pediatric FAP genetic testing reveal that children by and large do not experience significant distress related to this testing.[53,54,55] One report describing the psychological outcomes of genetic testing of minors for LFS did not note any significant negative outcomes after up to 12 years of follow-up.[56] However, in this study, the participation rate in testing for minors was low, with only 4 of 26 parents who were offered testing for their children actually following through with the test. Therefore, it remains to be determined whether and how genetic testing for these or other conditions predisposing to cancer impacts the well-being of children and their families.

Guidelines for Testing Children for a Heritable Predisposition to Cancer

The controversies surrounding genetic testing of children have prompted several groups to issue guidelines and recommendations. Most recommendations indicate that the primary justification for presymptomatic (i.e., predictive) genetic testing is medical benefit for the child. Therefore, testing should be offered to minors only if the condition is known to manifest in childhood and there are effective preventive or therapeutic interventions available. Conversely, if cancer risk is not increased in childhood or if there are not effective surveillance or intervention measures, it is recommended that testing be delayed until adulthood, when a child can then independently decide whether or not to pursue the testing. Families must be counseled about the benefits, risks, and limitations of genetic testing in order to give informed consent for genetic testing on behalf of their child. As with other medical interventions, it is the role of the health care provider to advocate for the best interests of the child when discussing the option of genetic testing. If possible, minors should be encouraged to participate in decision making about genetic testing; however, the capacity of children to understand genetic information is variable and depends upon their age, maturity, education, and cognitive abilities. Disclosure of results to tested minors is also encouraged once the minor reaches the legal age of majority, or possibly sooner if it is felt that the minor is capable of understanding and coping with the results.[49,56-61,62]

Management of Children with a Heritable Predisposition to Cancer

The identification of a presymptomatic child with a heritable predisposition to cancer has the potential to improve survival and minimize morbidity by allowing prospective monitoring for tumor formation and often life-saving prophylactic surgeries or other preventive measures. This information can also benefit children who already have cancer, as it can influence choice of the most appropriate or least toxic cancer-directed therapy. For example, one should avoid the use of radiation therapy in children with hereditary RB or LFS, as these children are at increased risk of developing secondary irradiation-induced malignant disease due to their constitutional genetic mutations. Similarly, in a child with a WT predisposition syndrome, it is recommended that a renal tumor be removed using nephron-sparing surgery and not total nephrectomy. Because the remaining kidney tissue remains at risk for tumor formation and could thus become the target of future surgical procedures, it is deemed advisable to leave as much healthy kidney in place as possible. Below, we review some of the salient principles of tumor surveillance and cancer risk reduction for children with heritable predisposition to cancer.

Principles of Tumor Surveillance

Cancer surveillance guidelines exist or are being developed for several conditions predisposing to childhood cancer. The primary goal of surveillance is to detect cancers at the earliest and most curable stage. For this reason, cancer surveillance protocols are best suited for solid tumors, where survival is often linked to the size and extent of spread of the tumor at diagnosis. Tumors identified in individuals undergoing regular monitoring may be smaller. As a result, surveillance allows for less invasive surgical procedures and can also reduce or eliminate the need for additional chemotherapy and/or irradiation. However, surveillance can also be complicated by the possibility of false-positive results, which often lead to worry about cancer, excess rates of follow-up imaging and/or biopsy, and possibly increased costs of care.

Several factors must be taken into account in the development and implementation of a cancer surveillance protocol. First, there must be a benefit for early cancer detection in an individual undergoing monitoring (i.e., there should be an effective treatment available and preferably also demonstration that early detection improves outcome). Second, the age-specific tumor risks for syndromes predisposing to cancer must be known and deemed high enough to warrant surveillance (generally, a tumor incidence of ≥5% is considered sufficiently high to initiate monitoring). This information helps to identify candidates for surveillance, when it should be initiated, and how long it should be continued. The surveillance method(s) and interval between specific tests must also be carefully considered in light of the specific tumor risks and growth rate associated with a given syndrome. Ideally, surveillance methods should be readily available, safe, and have high sensitivity and specificity. Whenever possible, screening tools that involve no or minimal irradiation should be used, as patients with hereditary cancer syndromes may be at increased risk to develop irradiation-induced cancers (as is the case for hereditary RB, LFS, ataxia telangiectasia, and other genetic syndromes associated with defects in DNA repair).

Cancer Prevention Strategies

For some syndromes, early identification of at-risk children allows for the elimination or dramatic reduction of cancer risk through prophylactic surgery. MEN2 and FAP are excellent examples, where removal of the at-risk organ may be considered early in a patient's lifetime to prevent the development of thyroid or colorectal cancer, respectively. In MEN2, there is a nearly 100% lifetime risk for MTC, an aggressive type of thyroid cancer with a high metastatic potential. As a result, it is recommended that individuals with MEN2 undergo prophylactic thyroidectomy, a procedure that has greatly reduced the likelihood of developing MTC.[63] It should be noted that while all individuals with MEN2 are at increased risk for MTC, there are significant genotype-phenotype correlations that predict the age of thyroid cancer onset, and this information can dictate the appropriate timing for prophylactic removal of the thyroid.[20] In FAP, individuals have a similarly high risk of early-onset colon cancer that nears 100% if colonic polyps are not removed. To prevent colon cancer, affected individuals should undergo colectomy when polyps become too numerous to clinically follow or remove, generally in the late teens to early 20s. Studies of the efficacy of colectomy reveal a significant reduction in colon cancer risk.[6] Due to the morbidities associated with colectomy, alternatives to surgery are being sought. Nonsteroidal antiinflammatory medications such as sulindac and Cox-2 inhibitors reduce polyp formation in adults with FAP[64-66]; however, these medications are currently under examination for use in children with the condition.[67]

SPECIFIC SYNDROMES PREDISPOSING TO CANCER

There currently exist more than 30 genetic conditions that predispose to cancer in which children are at risk for tumor formation (several examples are summarized in Table 42-3). Each of these conditions is typified by distinct clinical and genetic manifestations. For some of these conditions, recommendations regarding genetic testing, surveillance, and management are well established. However, for others these issues are only recently emerging. Here we focus on some of these conditions, including the clinical and molecular features and current recommendations regarding management.

Hereditary Retinoblastoma

Incidence

Hereditary RB is an example of a classic syndrome predisposing to cancer and the first for which the underlying genetic mechanism was recognized. RB is a malignant tumor of the embryonic neural retina that has an

TABLE 42-3 Clinical and Genetic Features of Selected Childhood Syndromes Predisposing to Cancer

Syndrome	Gene(s)	Inheritance	Cancers or Tumors	Other Features
Beckwith-Wiedemann syndrome	Chromosome 11p15, *CDKN1C*	Imprinting defects Autosomal dominant (AD)	Hepatoblastoma (HB) Wilms tumor (WT) Others (rare): Adrenocortical cancer Neuroblastoma Pheochromocytoma (PCC) Rhabdomyosarcoma Rhabdoid tumor Gonadoblastoma	Macrosomia Macroglossia Omphalocele Umbilical hernia Hypoglycemia Hemihypertrophy Ear pits or creases
Constitutional mismatch repair–deficiency syndrome	*MLH1* *MSH2* *MSH6* *PMS2*	Autosomal recessive (AR)	Gastrointestinal polyps (adenomas) Colon cancer Brain cancer Leukemia or lymphoma Endometrial cancer	Café-au-lait spots Axillary or inguinal freckling Lisch nodules
DICER1 syndrome	*DICER1*	AD	Pleuropulmonary blastoma Cystic nephroma Sertoli–Leydig cell tumor Thyroid goiter WT Rhabdomyosarcoma Colon polyps (hamartomas)	Pulmonary cysts
Familial adenomatous polyposis	*APC*	AD	Gastrointestinal polyps Colorectal cancer Small bowel cancer Papillary thyroid cancer HB Medulloblastoma Soft tissue tumors Gardner fibroma Desmoid tumor Osteomas	Epidermoid cysts Pilomatrixomas Supernumerary or missing teeth Congenital hypertrophy of retinal pigmented epithelium (CHRPE)
Hereditary paraganglioma (PGL)-PCC syndrome	*SDHA/B/C/D/AF2* *MAX* *TMEM127*	AD	PCC PGL Papillary thyroid cancer Renal cancer Gastrointestinal stromal tumor (GIST)	

TABLE 42-3 Clinical and Genetic Features of Selected Childhood Syndromes Predisposing to Cancer (Continued)

Syndrome	Gene(s)	Inheritance	Cancers or Tumors	Other Features
Hereditary neuroblastoma	*ALK* *PHOX2B*	AD	Neuroblastoma	Hirschsprung disease (*PHOX2B*) Congenital central hypoventilation (*PHOX2B*)
Hereditary retinoblastoma	*RB1*	AD	Retinoblastoma Pineoblastoma Soft tissue or bone sarcoma Melanoma	Multiple congenital anomalies (13q syndrome)
Juvenile polyposis syndrome	*BMPR1A* *SMAD4*	AD	Colon cancer Gastric cancer Pancreatic cancer Gastrointestinal polyps Juvenile type	Arteriovenous malformations (*SMAD4*)
Li-Fraumeni syndrome	*TP53*	AD	Adrenocortical carcinoma Choroid plexus carcinoma Bone or soft tissue sarcoma Breast cancer Brain cancer Leukemia Colon cancer Many other cancers	None
Multiple endocrine neoplasia type 1	*MEN1*	AD	Parathyroid adenoma Gastrinoma-insulinoma Anterior pituitary adenoma Carcinoid tumor Angiofibroma Collagenoma Lipoma	None
Multiple endocrine neoplasia type 2	*RET*	AD	Medullary thyroid cancer PCC Parathyroid adenoma (MEN2A) Mucosal neuroma (MEN2B) Ganglioneuroma (MEN2B)	Hyperparathyroid (due to parathyroid adenomas) (MEN2A) Marfanoid habitus (MEN2B) Mucosal neuromas (MEN2B)
Neurofibromatosis type 1	*NF1*	AD	Neurofibromas Optic pathway gliomas Astrocytomas Schwannomas Juvenile myelomonocytic leukemia Acute myeloid leukemia PCCs	Café-au-lait macules Axillary or inguinal freckling Lisch nodules Tibial bowing Macrocephaly Developmental delay, intellectual disability, or autism
Neurofibromatosis type 2	*NF2*	AD	Vestibular schwannomas Meningiomas Astrocytomas Schwannomas Gliomas Neurofibromas	Posterior subcapsular lenticular opacities Hearing loss Peripheral neuropathy Seizures
Nevoid basal cell carcinoma syndrome (Gorlin syndrome)	*PTCH1*	AD	Basal cell carcinoma Medulloblastoma Cardiac and ovarian fibromas	Macrocephaly Palmar or plantar pits Jaw keratocysts Bifid ribs Calcificiation of falx Developmental delay, intellectual disability, autism

Continued on following page

TABLE 42-3 Clinical and Genetic Features of Selected Childhood Syndromes Predisposing to Cancer (Continued)

Syndrome	Gene(s)	Inheritance	Cancers or Tumors	Other Features
Peutz-Jeghers syndrome	*STK11/LKB1*	AD	Gastrointestinal polyps (Peutz-Jeghers type) Colon cancer Gastric cancer Breast cancer Lung cancer Pancreatic cancer Ovarian cancer Cervical cancer	Mucocutaneous hyperpigmentation
PTEN hamartoma tumor syndrome	*PTEN*	AD	Breast cancer Thyroid cancer (nonmedullary) Endometrial cancer Renal cancer Lipomas Thyroid nodules or goiter Gastrointestinal polyps (hamartomas)	Macrocephaly Arteriovascular malformations Trichilemmomas Papillomatous papules Developmental delay, intellectual disability, or autism
Rhabdoid tumor syndrome	*SMARCB1/INI1*	AD	Atypical teratoid/rhabdoid tumors (CNS) Malignant rhabdoid tumors (renal or extrarenal) Schwannomatosis	None
Tuberous sclerosis	*TSC1* *TSC2*	AD	Astrocytoma Cortical tuber Subependymal nodules Cardiac rhabdomyoma Angiomyolipomas Renal cell carcinoma (RCC) Colon polyps (hamartomas)	Facial angiofibroma Hypopigmented macules (ash leaf macules) Ungual fibromas Shagreen patch Confetti skin lesions Dental pits Gingival fibromas Developmental delay, intellectual disability, or autism Seizures or infantile spasms
Von–Hippel Lindau syndrome	*VHL*	AD	Hemangioblastoma (retinal or cerebellar) RCC Pancreatic neuroendocrine tumors PCCs Endolymphatic sac tumors Papillary cystadenomas Epididymis (males) Broad ligament (females)	Renal cysts Pancreatic cysts
WT1-related syndromes	*WT1*	AD	WT Gonadoblastoma	Aniridia (WAGR syndrome) Genitourinary abnormalities Developmental delay, intellectual disability, or autism

WAGR, Wilms tumor, aniridia, genital anomalies, and retardation.

incidence of two to five cases per million children per year and accounts for 3% to 4% of all pediatric cancers.[4] Nonhereditary RB accounts for 60% of cases and is associated with development of unilateral eye tumors at a median age of 22 months.[68,69] The remaining 40% of patients have hereditary RB, which involves both eyes in more than 80% of cases and presents at a median age of 11 months.[68,69] Heritable RB is highly penetrant, with more than 90% of patients developing RB and 0.5% to 15% also developing intracranial tumors of the pineal gland.[70,71] Over the course of their lives, survivors of hereditary RB remain at increased risk to develop second malignant neoplasms, including bone and soft tissue sarcomas, which commonly occur in previously irradiated sites, as well as melanomas and cancer of the lungs, gastrointestinal tract, and bladder.[72]

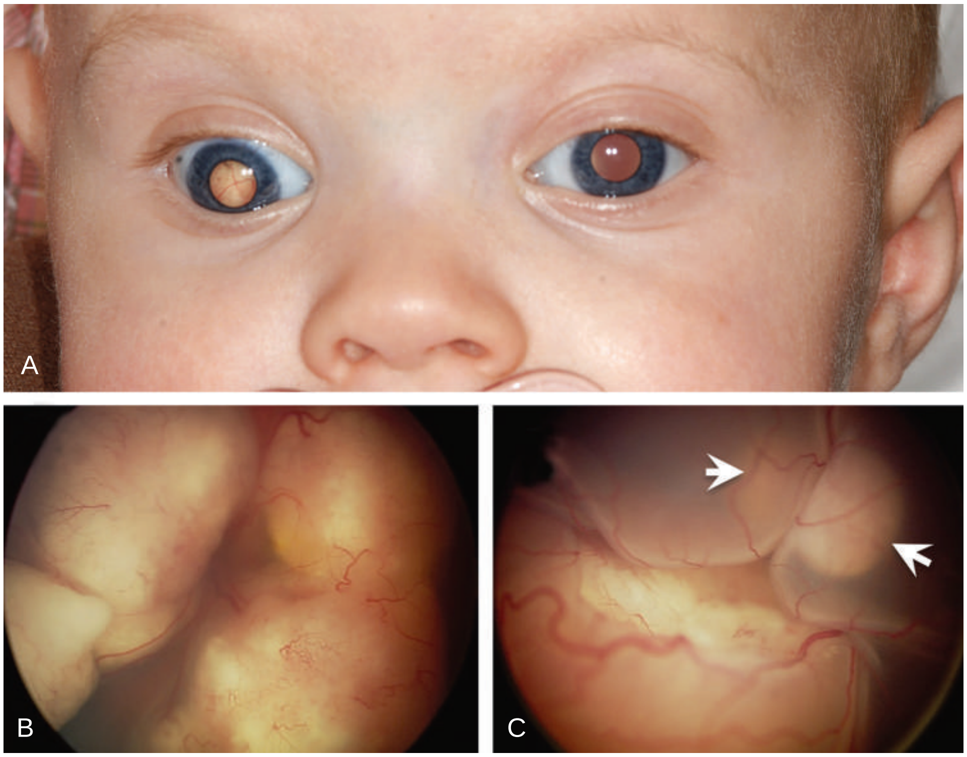

Figure 42-2 A child with hereditary retinoblastoma (RB) with bilateral eye involvement. **A**, The face of an infant with bilateral RB demonstrating bilateral leukocoria, one of the most common presentations for this eye tumor. **B** and **C**, Retinal photographs of this child's ocular tumors, which appear as large white masses. In **B**, the right eye has an advanced-stage (group E) tumor. In **C**, the left eye has a large (group D) tumor with evidence for subretinal seeding (*arrows*).

Genetics

RB is caused by mutations in *RB1*, which resides at chromosomal locus 13q14.[10] *RB1* encodes the RB protein (pRB), a potent tumor suppressor that regulates cell growth by inhibiting the function of the E2F family of transcription factors and preventing inappropriate S-phase entry. pRB also regulates cellular glucose tolerance, mitogenesis, glutathione synthesis, and the expression of genes involved in carbon metabolism.[73] When both RB1 alleles are mutated within a developing retinal cell, pRB expression and/or function are diminished, cell division proceeds in a dysregulated manner, and, ultimately, RB tumors form.

In patients with nonhereditary RB, both RB1 gene copies are mutated within a single postzygotic retinal cell. In contrast, patients with hereditary RB harbor one mutated RB1 allele within the germline. This mutation is inherited from a similarly affected parent in 10% to 20% of cases.[69] In the remaining cases, the RB1 mutation arises de novo within one of the parent's gametes[74] or a cell of the developing embryo. Mutation of the remaining RB1 allele occurs somatically. Genetic testing for germline RB1 gene mutations can identify mutations in more than 90% of individuals with hereditary disease.[75,76] While most patients can be identified clinically due to the presence of multifocal or bilateral eye tumors and/or a positive family history, 10% to 15% of patients have single eye tumors and no family history of RB. These latter factors make them indistinguishable from those with the sporadic form of disease. The identification of RB1 mutations in unilateral patients enables appropriate primary eye tumor treatment as well as initiation of surveillance for involvement of the pineal gland and later second cancers. Identification of a germline RB1 mutation in any child with RB also allows for presymptomatic testing of other family members, including siblings who may not yet have developed RB but carry a mutated RB1 gene copy, as well as children of RB survivors.

Tumor Risks and Surveillance

Individuals harboring germline RB1 mutations are at greatly increased risk to develop bilateral RB tumors, generally during the first 5 years of life (Fig. 42-2). During this time period patients are also at increased risk to develop tumors involving the pineal gland (so-called trilateral RB). Later in life, individuals with hereditary RB can develop second malignant neoplasms. While the risk for these cancers is greatest in those treated with external-beam radiation therapy, nonirradiated long-term survivors of hereditary RB have an approximately 10% to 15% risk of developing one or more second malignant neoplasms[77]

The early detection of eye tumors has been shown to significantly increase ocular retention and preserve vision.[78] Therefore, it is recommended that children at risk for RB be examined by fundoscopy beginning 1 to 2 weeks after birth and then every 2 weeks until the age of 3 months.[78] Subsequently, examinations should be performed under general anesthesia until the age of 4 to 5 years.[79] The frequency of examination depends upon the age of the patient, with examinations recommended every 2 months during the first year of life and then gradually spaced to every 6 months during the fourth to fifth years of life.[79] A study by Abramson and colleagues[80] showed that patients with a positive family history who received ophthalmologic examinations from birth had an overall survival rate of 93.2% and an ocular retention rate of 67.7%. This is in contrast to patients who did not receive screening, whose survival rate measured 87.4% and ocular retention rate only 38.2%. In addition to increased ocular retention, early screening and close follow-up results in a long-term visual acuity measuring between 20/20 and 20/40 for approximately 90% of patients with hereditary disease.[78] To screen for pineal tumors, it is recommended that children undergo magnetic resonance imaging (MRI) examination of the brain every 6 months until the age of 5 years.[71] Screening in this manner successfully identifies tumors when they are smaller and has been reported to increase the 5-year survival rate from 0% to 27%.[71] While the basis for ocular and pineal gland surveillance is well established, it remains unclear whether monitoring for second malignant neoplasms is of benefit. As a result, monitoring for these tumors is not yet recommended and remains the subject of investigation.

Li-Fraumeni Syndrome

Incidence

LFS is a highly penetrant cancer syndrome first described in 1969 in four families with soft tissue sarcoma and early-onset breast cancer.[81] Since this initial observation, more than 500 families have been reported with complete or partial LFS phenotypes, and it is possible that many more families exist that have not yet been identified. In 1990, it was determined that germline *TP53* mutations were responsible for most cases of LFS.[82] The *TP53* mutation carrier rate has been reported to be about 1 in 5000 births,[23] although the true incidence and prevalence of germline *TP53* mutations are unknown. *TP53* germline mutations are more common in southern Brazil, due to a founder mutation with a high population prevalence of nearly 0.3%.[83-85,86]

Genetics

TP53 encodes p53, which is often referred to as the “guardian of the genome” because it is a critical transcription factor that mediates cellular stress responses and initiates DNA repair, cell-cycle arrest, senescence, and apoptosis. *TP53* somatic alterations have been detected in nearly every type of human cancer, and patients with LFS who harbor a mutated copy of this important cancer-preventing gene are much closer to the threshold of developing cancer in any of their body tissues. Although there are no strong genotype-phenotype correlations, early tumor onset has been reported to be more common in those with nonsense, frameshift, or splice site mutations.[87] Modifiers of tumor risk have included increased burden of copy number variants,[88] shorter telomeres,[89] a single-nucleotide polymorphism at codon 72 (R72) of *TP53*,[90,91] and a single-nucleotide polymorphism (rs2279744) affecting the p53 inhibitor MDM2.[91] As more LFS families are studied, these associations and the genotype-phenotype correlations will be more clearly elucidated.

Over the years, several classification systems have been developed to help identify possible LFS families and determine those to whom *TP53* genetic testing should be offered (Table 42-4). Currently, *TP53* mutations are

TABLE 42-4 Clinical Classification Schemes for Li-Fraumeni Syndrome

Classification Criteria	Description
Classic Li-Fraumeni syndrome (LFS)	Proband with sarcoma diagnosed before 45 years of age and First-degree relative with cancer diagnosed before 45 years of age and First- or second-degree relative on same side of family with cancer diagnosed before 45 years of age or sarcoma at any age
Chompret classification	Proband with LFS spectrum cancer diagnosed before 36 years of age and At least one first- or second-degree relative on same side of family with cancer (other than breast cancer if proband has breast cancer) diagnosed before 56 years of age or A relative with multiple primary cancers at any age A proband with cancer with a relative with cancer or multiple primaries, two of which are sarcoma, brain tumor, breast cancer, and/or adrenocortical carcinoma, with the initial cancer occurring before 46 years of age, regardless of family history A proband with adrenocortical carcinoma or choroid plexus carcinoma at any age, regardless of family history
Birch classification	Proband with any childhood cancer or sarcoma, brain tumor, or adrenocortical carcinoma diagnosed before 45 years of age and First- or second-degree relative with typical LFS cancer (sarcoma, breast cancer, brain tumor, leukemia, or adrenocortical carcinoma) diagnosed at any age and First- or second-degree relative on same side of family with any cancer diagnosed before 60 years of age
Eeles classification	Two first- or second-degree relatives with LFS-related malignant disease (sarcoma, breast cancer, brain tumor, leukemia, adrenocortical carcinoma, melanoma, prostate cancer, or pancreatic cancer) at any age

identified in 70% or more of patients who meet the strict diagnostic criteria for LFS. The mutation detection rate drops to 20% in families classified as "Li-Fraumeni–like," who exhibit some but not all of the features associated with LFS (such as very early onset or multiple primaries).[92] Even without an identified *TP53* mutation, cancer-prone families with an LFS pedigree are considered to be at high risk for an inherited cancer syndrome, and it is recommended that members of these families be monitored carefully for early cancer development using modalities currently in place for those with genetically verified LFS.

Tumor Risks and Surveillance

Patients with classic LFS are at risk for nearly every type of cancer, and the lifetime cancer risk is extraordinarily high, with a 68% risk in men and 93% risk in women.[93] Patients with LFS most commonly present with breast cancer (nearly 30% of patients), soft tissue sarcomas (<20%), brain tumors (<14%), osteosarcomas (12% to 14%), and ACCs (6.5% to 10%).[23,85,87,93] In children, the tumors most strongly associated with LFS include ACC, CPC, rhabdomyosarcoma presenting before 3 years of age, and osteosarcoma presenting before 5 years of age.[23,27,87] Recently, *TP53* germline mutations have been described in nearly half of patients with hypodiploid pre-B acute lymphoblastic leukemia,[94] most notably those children whose leukemia cells are considered "low hypodiploid" and contain a median of 32 to 39 chromosomes. A germline *TP53* mutation was also described in an additional kindred with familial transmission of acute lymphoblastic leukemia.[95] Nearly half of cancers in LFS present before 30 years of age.[93] The phenomenon of genetic anticipation has been observed in LFS, with a decrease in age and increase in cancer onset in successive generations.[96] Subsequent tumors also are an issue in LFS, with more than an 80-fold relative risk for a second malignant neoplasm in patients with their first cancer under the age of 20, a 10-fold relative risk if diagnosed between 20 and 44 years, and a 1.5-fold relative risk if diagnosed at over 45 years.[97]

The recommendations for cancer surveillance in patients with LFS are emerging, although no formal guidelines for affected children are in place at this time. For *TP53* mutation carriers, a healthy lifestyle with avoidance of ionizing radiation and DNA-damaging agents is encouraged. Annual dermatology visits are recommended, along with avoidance of unprotected sun exposure. Annual physical examinations are also recommended, with careful attention to the thyroid and abdomen for masses and a neurologic examination for indication of brain tumors. For adults, colonoscopy is recommended beginning at age 25 years; if there is a family history of colorectal cancer, the patient can consider screening at an age 5 to 10 years younger than the earliest age at which colorectal cancer has been diagnosed in other family members. For women, annual mammograms and breast MRI are recommended beginning at age 25 years, with these procedures alternated so that the breasts are screened every 6 months. The cost versus benefit of mammography in this high-risk population is still being explored, and these recommendations may change in the future.[98]

Currently, studies are ongoing to determine whether newer radiologic approaches, alone or in combination with other screening measures, are effective in detecting tumors in patients with LFS. One study of rapid whole-body MRI combined with other imaging and laboratory measures has reported increased survival rates in patients with LFS,[99] and this now can be considered an option for surveillance (Fig. 42-3). Additional measures being offered include abdominal ultrasound (to screen for ACC or other intraabdominal tumors), assessments of complete blood count (to detect leukemia), and measurements of adrenal hormones (to screen for ACC). Although it has not yet been definitively proven that surveillance is beneficial, many practitioners will test for *TP53* mutations in order to begin a tumor surveillance program in children who are determined to be at increased cancer risk. Cancers identified in LFS patients can be treated according to standard protocols, although the avoidance of agents that might damage DNA (e.g., exposure to ionizing radiation) is recommended due to the increased risk for second malignant tumors in this population.

WT1-Related Wilms Tumor Predisposition Syndromes

Incidence

Wilms tumor (WT, also known as nephroblastoma) is the most common renal tumor of childhood, accounting for 8% of all pediatric cancers[100] and 95% of pediatric renal cancers.[101] As many as 10% of WTs develop in the setting of a hereditary predisposition, where it is estimated that up to 5% of tumors are caused by germline mutations in the *WT1* tumor suppressor gene.[102] As with other predisposition syndromes, children with germline *WT1* mutations are more likely to present with bilateral kidney involvement[103] and at a younger-than-expected age.[100] Patients may also exhibit a variety of nononcologic clinical manifestations.[13]

Genetics

The *WT1* gene, located at chromosomal locus 11p13, encodes a zinc finger transcription factor that regulates mesenchymal to epithelial transition in embryonic renal and genitourinary development[104] and thus functions as a tumor suppressor gene.[105] The *WT1* gene consists of 10 exons, which encode an N-terminal proline and glutamine-rich transactivation domain, and a C-terminal zinc-finger domain involved in DNA binding.[106,107] Loss of heterozygosity at 11p13 in WT samples suggests a key role as a tumor suppressor,[108] and numerous constitutional mutations in *WT1* have been described in association with familial WT.[100] In cases of *WT1*-associated WT, a second mutation in either CTNNB1 or *IGF2* is often also required for oncogenesis.[101]

Tumor Risks and Surveillance

Patients harboring a germline *WT1* mutation develop WT in the context of several distinct clinical syndromes (Table 42-5), including WT, aniridia, genital anomalies, and retardation (WAGR), Denys-Drash syndrome (DDS),

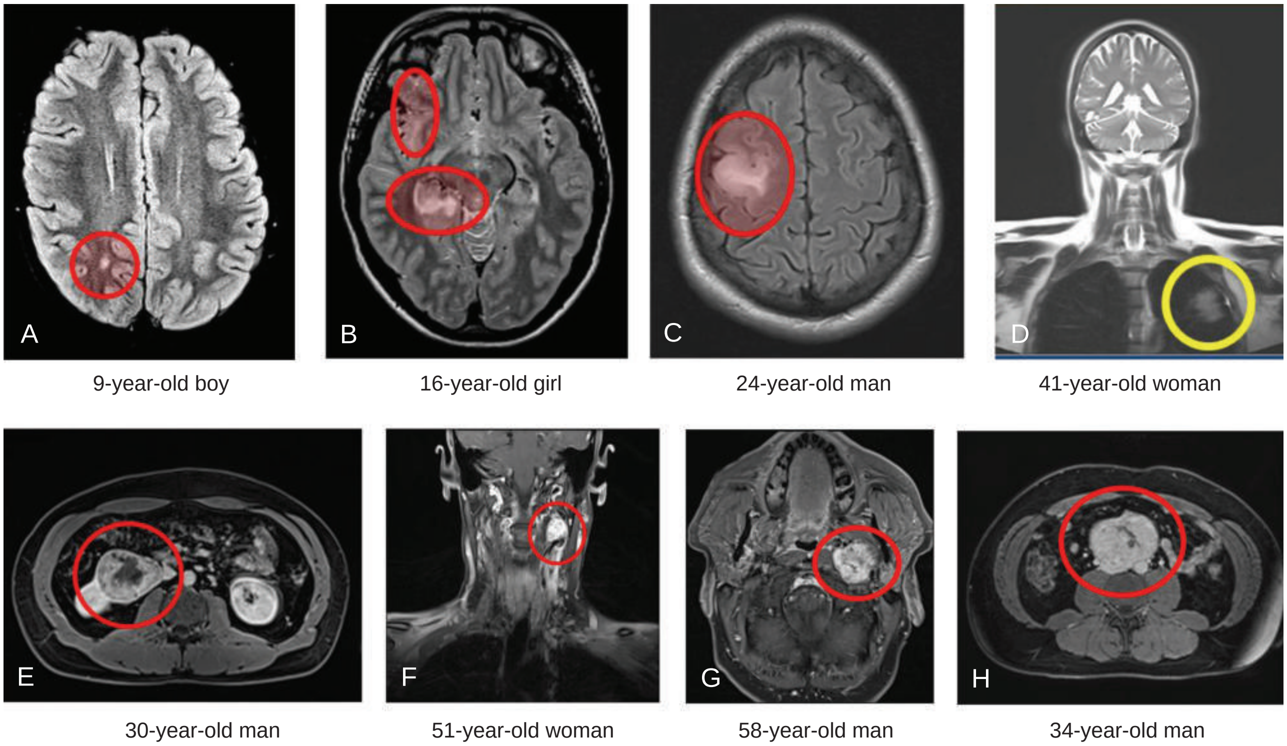

Figure 42-3 Tumors identified by magnetic resonance imaging (MRI)-based surveillance of asymptomatic TP53 and SDHB mutation carriers. **A** to **D**, Examples of lesions in *TP53* mutation–positive patients with Li-Fraumeni syndrome. **A**, Unknown lesion, followed regularly on brain MRI. **B**, Diffuse astrocytoma (World Health Organization [WHO] grade II), resected. **C**, Diffuse fibrillary astrocytoma (WHO grade II), resected. **D**, Lung adenocarcinoma, resected. **E** to **H**, Examples of lesions found in patients with hereditary paraganglioma/pheochromocytoma (PCC), who carried mutations in *SDHB*. **E**, Renal cell carcinoma, removed by nephrectomy. **F**, Carotid body tumor, resected. **G**, Carotid body tumor, resected. **H**, PCC in the organ of Zuckerkandl, resected.

and Frasier syndrome (FS). In a large study of WT patients, only 20% with a germline *WT1* mutation did not have associated congenital abnormalities.[103]

Wilms Tumor, Aniridia, Genital Anomalies, Retardation Syndrome

WAGR syndrome is caused by a contiguous gene deletion at 11p13, which leads to the loss of a single copy of *WT1* as well as neighboring genes, including *PAX6*, a transcription factor critical for development of the eye and other neural organs. Haploinsufficiency for WT1 is believed to result in genitourinary abnormalities, which are most notable in males and include hypospadias and undescended testes,[101] as well as an almost 50% risk of developing WT.[109] Haploinsufficiency for PAX6 contributes to the development of complete or partial aniridia. The etiology of the cognitive delay in WAGR syndrome has not been elucidated, though it is likely that haploinsufficiency for genes adjacent to *WT1* are responsible for this finding.

TABLE 42-5 Clinical and Genetic Features of *WT1*-Related Syndromes Predisposing to Wilms Tumor

Syndrome	Genetic Alteration	Molecular Pathogenesis	Wilms Tumor Risk	Clinical Phenotype
Familial Wilms tumor (WT)	*WT1* mutation or deletion	Altered *WT1* function	Variable risk of WT	Nonsyndromic, hypospadias or undescended testes in males
WAGR syndrome	*WT1* deletion	*WT1* haploinsufficiency	~50% risk of developing WT	Aniridia, genital abnormalities, developmental delays, possibility for streak gonads in girls
Denys-Drash syndrome	*WT1* missense mutation	Dominant negative mutation	>90% risk of WT	Development of diffuse mesangial sclerosis, undermasculinized genitalia in 46 XY males
Frasier syndrome	*WT1* splice site mutation	Altered ratio of *WT1* splice forms	5% to 10% risk of WT	Development of focal segmental glomerulosclerosis, gonadoblastoma, undermasculinized genitalia in 46 XY males

Denys-Drash Syndrome

DDS is characterized by the development of WT, renal diffuse mesangial sclerosis, and undermasculinized external genitalia in 46 XY males. It is caused by missense mutations affecting exon 8 or 9 of *WT1*,[103] which lead to the generation of a WT1 protein with impaired DNA-binding capacity. These WT1 mutants function in a dominant negative fashion, and as a result patients with DDS exhibit a very high incidence (>90%) of WT.[13] Furthermore, approximately 74% of patients develop end-stage renal disease within 20 years of diagnosis.[110] While the molecular pathogenesis of the diffuse mesangial sclerosis remains unclear, murine studies have implicated *WT1* in the regulation of podocyte formation,[111] suggesting a central role for *WT1* in lifelong renal homeostasis. Genitourinary abnormalities of varying severity are common in males with DDS, and there is also an increased risk for gonadoblastoma. In contrast, most females with DDS have normal genitourinary development and no risk for gonadal tumors.[112]

Frasier Syndrome

FS is typified by the development of renal focal segmental glomerulosclerosis, gonadoblastoma, and undermasculinized external genitalia in 46 XY males. FS is caused by point mutations in the WT1 intron 9 donor splice site.[113] Normally, distinct messenger RNA (mRNA) isoforms are produced by WT1 as the result of alternative splicing. The intron 9 alternative splice donor site mutation seen in FS leads to loss of three amino acids (KTS, or lysine, threonine, and serine), thus disrupting the normal ratio of WT1 isoforms critical for proper gonadal and renal development. The altered ratio of WT1 isoforms is associated with decreased expression of *SRY* and *SOX9*, which both encode transcription factors required for normal testicular development.[114] Interestingly, most patients with FS do not develop WT; however, a few cases have been reported,[115] with overall estimates for WT of 5% to 10%.[116] Most males with FS, however, have severe genitourinary abnormalities, resulting in a high risk for gonadoblastoma.[112]

The management of children with WT1-associated WT predisposition syndromes involves genetic testing, tumor surveillance, and organ-sparing therapies. It is recommended that children presenting with WT, genitourinary abnormalities, and/or early-onset renal failure be tested for germline *WT1* mutations.[103] Testing should also be considered for patients with bilateral WT without associated abnormalities, although the yield of testing in this instance is lower.[103] Generally, it is not recommended that children with unilateral WT and no associated abnormalities be tested, as the prevalence of *WT1* mutations in these patients is only 1.4%.[100] All children with aniridia should be screened by fluorescence in situ hybridization studies or single-nucleotide polymorphism array, as up to one third of patients will exhibit loss of one copy of *WT1*[117] and thus be at increased risk for WT formation.

As the treatment strategies for WT have become more successful, screening programs are unlikely to affect these outcomes. However, the goal of screening is to promote earlier detection and, thus, easier surgical management, less intensive chemotherapy, and possible avoidance of radiotherapy. Ninety-eight percent of children with *WT1*-associated WT are diagnosed with their tumors by 7 years of age.[118] As a result, it is generally recommended that patients with germline *WT1* mutations undergo kidney surveillance with renal ultrasound examinations every 3 months through the first 7 years of life.[116] Given the increased risk for multifocal and bilateral disease, it is recommended that for those who do develop WT, nephron-sparing surgery be used whenever possible. Newer areas of investigation focus on the use of neoadjuvant chemotherapy to eradicate renal tumors and thereby avoid the use of surgery. Patients with a germline *WT1* mutation are particularly at risk for this end-stage renal disease. Therefore patients should undergo lifelong monitoring of blood pressure and renal function, with early treatment of hypertension and avoidance of nephrotoxic medications.[110]

Hereditary Paraganglioma/Pheochromocytoma Syndrome

Incidence

PGLs and PCCs are neuroendocrine tumors that generally involve the parasympathetic and sympathetic chains or adrenal glands. PGLs are rare in the general population, occurring at an estimated incidence of 1 : 30,000 to 1 : 1 million.[119-121] However, in the presence of an underlying predisposing condition, the tumor rate can be extraordinarily high.[122,123] Toward this end, a recent study demonstrated that 30% and 81% of children with nonmetastatic and metastatic PGLs/PCCs, respectively, carried germline mutations in genes encoding subunits of the succinate dehydrogenase (SDH) complex.[124] In addition to *SDH* genes, germline mutations in *NF1*, *VHL*, *RET*, *TMEM127*, and *MAX* also predispose to PGL/PCC. Therefore, the true incidence of germline mutations in children with these tumors may be even higher than currently estimated.[125]

Genetics

Hereditary PGL/PCC is caused by germline mutations in the *SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* genes (collectively *SDHx*), which encode components of *SDH*, a mitochondrial enzyme complex responsible for converting succinate to fumarate as part of the tricarboxylic acid cycle.[126] *SDHA*, *SDHB*, *SDHC*, and *SDHD* encode for proteins that make up SDH, while *SDHAF2* encodes for SDH assembly factor 2, a protein needed to assemble the SDH proteins into a fully functioning complex.[16] Mutations in the *SDHx* genes lead to a faulty tricarboxylic acid cycle with increased levels of succinate and subsequent increases in hypoxia-inducible factor (HIF) signaling and histone deregulation. It was recently reported that *SDHx* mutations are associated with a hypermethylator phenotype, which may also contribute to tumorigenesis.[127,128] PGLs are usually benign tumors, but when caused by an *SDHx* mutation, particularly *SDHB*, they exhibit an aggressive metastatic phenotype. The biology behind this clinical difference is not yet known. Hereditary PGL/PCC is inherited in an autosomal dominant fashion; however, *SDHD* and *SDHAF2* are maternally imprinted, with only the children of fathers, not mothers, developing tumors. Nevertheless, mothers can still pass along *SDHD* and *SDHAF2* mutations to their children, who will not develop PGL/PCC but remain at 50% risk to transmit the disease to future generations.

Tumor Risks and Surveillance

The SDHx-related tumor spectrum is broad and includes PGL (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*), PCC (*SDHA*, *SDHB*, *SDHC*, *SDHD*), gastrointestinal stromal tumors (GISTs) (*SDHA*, *SDHB*, *SDHC*, *SDHD*), papillary thyroid cancer (*SDHB*, *SDHD*), renal cell carcinoma (RCC) and oncocytoma (*SDHB*, *SDHC*), neuroblastoma (*SDHD*), and, most recently, pituitary adenoma (*SDHA*).[93,126,129,130] Patients who develop PGLs as well as GISTs have a condition known as Carney-Stratakis syndrome.[131] In rare instances, PGLs, GISTs, and pulmonary chondromas occur together, in which case they are referred to as the Carney triad. Traditionally, tumors occurring in the context of Carney triad have not been associated with underlying SDHx mutations.[131,132]

Hereditary PGL/PCC has no defining clinical criteria other than a possible family history of *SDHx*-related tumors. Therefore, any child diagnosed with a PGL, PCC, or GIST should undergo genetic testing due to the strong association between these tumors and underlying *SDHx* mutations. Immunohistochemical staining of tumor tissue for *SDHB* protein expression can identify patients at risk for underlying germline *SDHx* mutations.[133,134] Specifically, absent *SDHB* staining indicates that the SDH protein complex is not intact, and this finding may be caused by a mutation in any of the *SDH*-related genes. Clinical laboratories have started to offer *SDHB* immunohistochemical staining to facilitate genetic diagnosis of hereditary PGL/PCC, although the sensitivity and specificity of this test needs to be determined.

Due to the aggressive nature of *SDH*-related tumors, early identification of small tumors in asymptomatic patients may reduce morbidity and mortality. No consensus screening guidelines exist for tumor surveillance, but many clinicians will perform annual physical examinations, blood pressure checks (to detect hypertension due to increased secretion of catecholamines), and blood work for serum metabolites (fractionated catecholamines, including epinephrine, norepinephrine, dopamine; fractionated free metanephrines, including metanephrine and normetanephrine; and chromogranin A).[135,136] Regular imaging can also identify PGL/PCC, and this is especially important for those with non–catecholamine-secreting tumors.[137-140] Common imaging approaches include computed tomography, ^{18}F-fluorodeoxyglucose positron-emission tomography, and [^{123}I]-metaiodobenzylguanidine scintigraphy. Some centers are beginning to use rapid-sequence whole-body MRI, with or without dedicated neck MRI, as a screening measure for individuals with germline *SDHx* mutations (see Fig. 42-3). Recently, the University of Utah published findings showing that this approach has a sensitivity of 88% and specificity of 95% for detecting new tumors compared with isolated biochemical testing, which exhibited a sensitivity of 38% and specificity of 95%.[141] As *SDHx* mutations are increasingly recognized and studied, we will learn more about the genetic, epigenetic, and metabolic alterations linked to cancer and discover novel approaches to tumor prevention and treatment.

Neurofibromatosis Type 1

Incidence

Neurofibromatosis type 1 (NF1) is considered one of the most common inherited cancer predisposition syndromes, affecting 1 in 3000 children.[142,143] NF1 is caused by germline heterozygous inactivating mutations in the *NF1* gene, which is located on chromosome 17 and encodes the protein neurofibromin.[142-144] In addition to cancer, patients with NF1 are at increased risk to develop benign tumors such as neurofibromas and Lisch nodules (iris hamartomas), as well as nononcologic manifestations, including café-au-lait macules and intertriginous freckles (see Fig. 42-1), bone deformities (long bone pseudarthrosis, skeletal dysplasias, scoliosis, osteoporosis), vascular abnormalities, short stature, and neurocognitive delay.[145-148]

Genetics

The *NF1* gene encodes a cytoplasmic protein, neurofibromin, which is mainly expressed in neurons, Schwann cells, oligodendrocytes, and leukocytes. Neurofibromin functions as a tumor suppressor by restricting cell proliferation through its effects on the RAS signaling pathway. Interestingly, there is wide variation in the phenotypic presentation of NF1, even among family members with the identical *NF1* mutation. Szudek and associates[149] examined familial aggregation of NF1 phenotypes in 904 affected individuals from 373 families with NF1 and concluded that unlinked modifying genes are likely to contribute to the distinct presentations.[149] Most recently, Sabbagh and colleagues[150] evaluated phenotypic correlations between affected relatives in 750 patients with NF1 in 275 multiplex families; they also concluded that genetic modifiers unlinked to the *NF1* locus contribute to the variable phenotypic severity observed in NF1. Thus, similar to other hereditary cancer disorders, loss of *NF1* serves as the first hit but the presence of other modifying genes and/or accumulation of tissue-specific second hits may ultimately dictate the phenotype.

Tumor Risks and Surveillance

The tumors that develop in children with NF1 include malignant peripheral nerve sheath tumors (MPNSTs) and astrocytomas (including optic gliomas [OPGs]), and, less commonly, rhabdomyosarcomas and neuroblastomas. Adults with NF1 remain at increased risk for MPNSTs and gliomas, as well as GISTs, somatostatinomas, and PCCs.[151] Half of patients with NF1 develop plexiform neurofibromas, the majority of which grow slowly but can cause significant disfigurement and pain. A small proportion transform to MPNST, which can be aggressive and resistant to therapy.[152-155] OPGs occur in up to 25% of patients and may lead to blindness if left untreated.

OPGs usually present before 6 years of age, although they may not become problematic until later in life as they encroach along the optic pathway.[156-159]

The diagnosis of NF1 is often clinical and based on a patient having at least two or more of the following criteria: (1) six or more café-au-lait macules measuring more than 5 mm in greatest diameter in prepubertal individuals and more than 15 mm in postpubertal individuals; (2) two or more neurofibromas of any type or one plexiform neurofibroma; (3) freckling in the axillary or inguinal regions; (4) an OPG; (5) two or more Lisch nodules (iris hamartomas); (6) osseous lesions such as sphenoid dysplasia or tibial pseudarthrosis; or (7) a first-degree relative (parent, sibling, offspring) with NF1 as defined by the above criteria. The clinical features of NF1 increase with age. Therefore, many children meet the criteria for diagnosis by 8 years.[32,156] Young children who manifest only with café-au-lait macules should be followed closely for development of other manifestations and considered to have NF1 unless proven otherwise.[160]

Surveillance for early cancers remains controversial in NF1.[58,145,161-163] Upon diagnosis, patients should undergo a thorough physical examination with a complete ophthalmologic evaluation (to assess for compromised vision, which could indicate an underlying OPG) and developmental assessment. Currently, some institutions order baseline and annual head MRIs to screen for OPGs. Other institutions order MRIs only if patients are symptomatic or if visual or central nervous system abnormalities are detected. Avoidance of radiation therapy is encouraged in patients with NF1 as this treatment may be associated with a higher risk for developing subsequent MPNSTs.[153,164]

Von Hippel–Lindau Syndrome

Incidence

Von Hippel–Lindau syndrome (VHL) is an autosomal dominant disorder caused by mutation of the *VHL* gene, which resides at chromosomal locus 3p25.5. VHL is a rare disease, with a prevalence of 1:36,000 live births and an estimated de novo mutation rate of 1% to 3%.[165,166] VHL is primarily considered to be a genitourinary cancer syndrome because of its strong association with RCC, but as discussed below, several different tumor types are associated with germline VHL mutations.

Genetics

VHL encodes a protein with diverse functions, including activity as an ubiquitin ligase as well as a tumor suppressor involved in transcriptional regulation, posttranscriptional gene expression, apoptosis, and extracellular matrix formation.[166,167] The VHL protein functions as part of a complex that targets other proteins for degradation, including HIF1α-.[168] The fact that it participates in the same pathway that is affected by *SDHx* mutations could explain the clinical similarities between VHL and hereditary PGL/PCC; both are characterized by the development of PGL, PCC, and RCC. It is also proposed that VHL's involvement in HIF1α signaling might explain the tendency of patients to form highly vascularized tumors such as hemangioblastomas.[166]

Tumor Risks and Surveillance

VHL is associated with an increased risk of developing benign as well as malignant tumors, including retinal angiomas; central nervous system hemangioblastomas; clear cell RCC; pancreatic islet cell tumors; endolymphatic sac tumors of the inner ear; renal, pancreatic, and epididymal cysts; PGLs; and PCCs. Of these tumors, RCC is one of the most common and develops in nearly 70% of VHL patients by the age of 60 years. Although RCC is common, it is rarely the first tumor to present.[169] In fact, over a third of patients initially present with cerebellar hemangioblastomas decades prior to RCC.[170] Unfortunately, most VHL patients succumb to RCC, the leading cause of mortality in this disorder.[165,169] Somatic *VHL* mutations are found in inherited and sporadic RCC, and are thus recurring drivers of RCC formation.[171]

Any patient who presents with a VHL-related tumor becomes a candidate for *VHL* genetic testing. Asymptomatic children born to a parent with VHL should be tested within the first year of life, given the 50% risk for inheriting the syndrome and the importance of early intervention. *VHL* germline mutations are typically identified in close to 100% of affected individuals, making the genetic test a very reliable one.[172] VHL is typified by strong genotype-phenotype correlations, which can guide the approach to genetic testing and/or screening.[167,173] For example, the VHL "type 1" phenotype lacks PCCs and is associated with *VHL* deletions, insertions, and truncations. The VHL "type 2" phenotype presents with PCCs and is associated with *VHL* missense mutations. "Type 2A" lacks RCC and pancreatic cysts and is associated with specific missense alterations, including the pathogenic *VHL* Y98H, Y112H, and V116F variants. "Type 2B" patients develop RCC and pancreatic cysts, and harbor R167Q and R167W variants. Finally, VHL "type 2C" is associated with PCC only, and is associated with the V155L, R238W, and L188V variants of *VHL*.

Although no evidence-based guidelines exist, many clinicians follow a similar protocol for tumor surveillance. In affected individuals, ophthalmologic examinations should be initiated by 5 years of age or earlier to examine for retinal angiomas. In those at risk for PGL or PCC, plasma catecholamines and metanephrines should be measured annually, along with regular blood pressure monitoring and abdominal ultrasound examinations starting before the age of 10 years. Rapid whole-body MRI may be useful as a means of locating various tumors; however, this approach has not yet been systematically evaluated in VHL. Audiology assessments are suggested to occur every 2 to 3 years starting at age 5 to assess for the presence of endolymphatic sac tumors. In those with hearing loss, tinnitus, or vertigo, hearing evaluation and/or MRI scans should be completed annually. MRI of the brain and spine is recommended to commence around midadolescence to assess for the presence of central nervous system hemangioblastomas, and close neurologic examinations starting at birth and continuing throughout life are also recommended. Collectively, these approaches have proven to be effective in VHL, where they have greatly decreased morbidity and increased life expectancy for affected children and adults.[174]

Beckwith-Wiedemann Syndrome and Isolated Hemihyperplasia

Incidence

BWS is an overgrowth syndrome characterized by macrosomia, neonatal hypoglycemia, abdominal wall defects (omphalocoele, umbilical hernia), hemihyperplasia, macroglossia, ear anomalies, and increased risk for tumor formation.[175] BWS is estimated to affect 1 in 13,700 live births, with 85% of cases being sporadic and 15% familial.[175] Approximately 7.5% to 10% of BWS patients develop malignancy, most commonly hepatoblastoma (HB) or WT.[175,176] ACC, neuroblastoma, and rhabdomyosarcoma are also rarely encountered.[3,33,63] Atypical teratoid/rhabdoid tumor and PCC have been identified in single case reports but may be coincidental findings.[177-179] Isolated hemihyperplasia (IHH) is defined by asymmetric overgrowth of one or more body parts in the absence of the other clinical findings associated with BWS. IHH may represent part of the clinical spectrum of BWS as, in many cases, it has a similar genetic etiology and profile of cancer predisposition.[180]

Genetics

Multiple genetic and epigenetic abnormalities are observed in patients with BWS or IHH, all of which affect chromosomal locus 11p15.5. Among the genes included this region are *IGF2*, which encodes insulin-like growth factor 2, and *CDKN1C*, which encodes cyclin-dependent kinase inhibitor 1C. These and surrounding genes are "imprinted," or differentially methylated, depending on whether the gene copy is inherited from the mother or the father. As a result, only the maternal or paternal copy is expressed at any given time. The epigenetic aberrations in BWS/IHH lead to hypomethylation, and thus overexpression, of *IGF2*, as well as hypermethylation and, thus, reduced expression of *CDKN1C*.[175,181] The overexpression of growth-promoting genes and inhibition of growth-controlling genes may contribute to the overgrowth and tumor predisposition that occur in BWS/IHH.

Fifty-five percent to 65% of BWS patients have a defect at one of two imprinting control centers that determine methylation of the genes in the region. Paternal uniparental disomy at chromosome 11p15.5, in which a child has two copies of the chromosome or chromosomal region from the father and none from the mother, is the genetic abnormality identified in 20% to 25% of patients with BWS.[175,182] About 5% of patients with sporadic BWS harbor germline mutations in *CDKN1C*, a tumor suppressor gene encoding a cell cycle inhibitor, whereas approximately 40% of familial cases display this defect.[175,182] In total, about 75% to 80% of patients with clinical BWS have identifiable constitutional genetic abnormalities. The remaining patients may harbor mutations in novel genes, or be genetic mosaics for alterations in known genes linked to BWS. In mosaicism, the genetic or epigenetic alteration occurs during embryogenesis, with the result that only a subpopulation of cells displays the genetic abnormality. When mosaicism is present, the disease-associated genetic abnormality may be difficult to detect if only a small percentage of cells is affected, or if it is isolated to tissues that are not as easily sampled as the blood.

Tumor Risks and Surveillance

Cancer screening recommendations for patients with BWS or IHH are aimed primarily at detecting HB and WT. Although the incidence of neuroblastoma is higher in patients with BWS than in the general population, it is not considered high enough to warrant screening. Screening should be used for all patients with a clinical diagnosis of BWS or IHH, even if no underlying molecular abnormality is identified. Screening for HB begins when the diagnosis of BWS is made or suspected. It is recommended that patients undergo abdominal ultrasound examinations every 3 months, as well as serial evaluations of serum alpha-fetoprotein (AFP), as AFP is typically increased in patients with HB and thus serves as a very sensitive tumor marker. Although the timing of when to obtain AFP levels remains a subject of debate, it is generally recommended that levels be measured at a minimum of every 3 months.[183] AFP levels tend to be higher in patients with BWS, even in the absence of HB. In addition, levels tend to decrease more slowly over time.[183] Therefore, if the initial AFP level is elevated but there is no abnormality on ultrasound or other imaging, the test may be repeated in 4 to 6 weeks. If the levels are falling, they may be rechecked every 4 to 6 weeks until they return to normal. If the initial elevation is accompanied by a radiologic abnormality, if it fails to fall, or if it exponentially increases, the patient should be referred to an oncologist for further evaluation. Screening for HB can be discontinued at age 4 years as the majority of these tumors develop before this time.

Screening for WT consists of serial ultrasound examinations every 3 months from the time of diagnosis until the age of 8 years. In the first 4 years, the abdominal ultrasound performed to screen for HB is also used to evaluate for renal masses.[184] After age 4 years, imaging may be limited to renal ultrasound, which is faster and less expensive than abdominal ultrasound and does not require that the child fast for the examination. For HB and WT screening, ultrasound is favored over MRI or computed tomography as it is widely available, is noninvasive, leads to no radiation exposure, only rarely requires sedation, and is generally faster and less expensive.

There is increasing evidence to suggest that within the spectrum of BWS and/or IHH, distinct molecular abnormalities confer different risks for tumor development. For example, paternal uniparental disomy and abnormalities at the imprinting control center 1 have the highest risk of tumor development, whereas mutations of *CDKN1C* may not increase a child's risk for malignancy.[62,182,185,186] However, these genetic findings have not yet been used to direct decisions in most screening programs. It is likely, however, that future screening programs will be more precisely designed to take into account the particular risks related to the underlying genetic abnormality.

Data pointing to the benefits of screening in BWS are sparse but encouraging. Choyke and associates[187] report that patients with BWS or IHH who did not undergo screening had an increased rate of high-stage WT (stage III or IV) compared with patients who underwent screening. A report from the National Wilms Tumor Study Group examining 4669 children between 1980 and 1995 found that 1.1% of children with WT had BWS.[188] Interestingly, the children with BWS were diagnosed at younger ages (mean 28 months) than children without BWS (mean 44 months) and with lower-stage WT (71.7% of BWS patients with stage I or II tumors vs. 55% of non-BWS patients). Furthermore, none of the BWS patients presented with metastatic disease, compared with 12% of the non-BWS children. It is not possible to discern how much these differences were affected by screening. Nonetheless, it remains feasible that some of them were due to the positive effects of the monitoring for tumors in this population. Few studies exist evaluating the efficacy of screening for HB, although case reports document presymptomatic detection of tumors in some BWS patients.[189]

Rhabdoid Tumor Predisposition Syndrome

Incidence

Rhabdoid tumors are rare, highly aggressive malignant growths that generally present in infancy or early childhood[190] and are most commonly found in the kidney or central nervous system.[191] The annual incidence of rhabdoid tumors in children under 15 years of age in the United States is 0.19 per million for renal tumors, 0.89 per million for central nervous system tumors, and 0.32 per million for tumors involving other sites (SEER data, 2011). The characteristic somatic genetic abnormality and the primary initiating event includes mutation of the *INI1/SMARCB1* gene located on chromosome 22q11.2.[192]

Genetics

SMARCB1 is part of a chromatin-remodeling complex involved in regulating gene expression, including genes involved in cell cycle control, DNA repair, and differentiation.[193] Both alleles must be inactivated in order for a tumor to arise, consistent with its function as a tumor suppressor gene. Approximately 35% of rhabdoid tumor patients have germline *INI1/SMARCB1* alterations.[194] While the majority of germline mutations are de novo, families with rhabdoid tumor predisposition have been reported in which multiple siblings have been affected. In rare cases, a parent is an unaffected carrier, and can have multiple children with rhabdoid tumor before the condition comes to the attention of a clinical geneticist. Additionally, there have been reports of families in which some members were diagnosed with schwannomatosis, a disorder in which an individual develops two or more benign tumors of the nerve sheath, commonly referred to as schwannomas, while others developed rhabdoid tumors.[194] Most cases of familial schwannomatosis are associated with germline *INI1/SMARCB1* mutations.[10,27,195,196] The penetrance and expressivity of *INI1/SMARCB1* abnormalities are under investigation, and may vary with the specific genetic mutation.[194]

Tumor Risks and Surveillance

The major tumor risk in rhabdoid tumor predisposition syndrome (RTPS) is for the development of renal or extrarenal rhabdoid tumors, which generally have a very aggressive phenotype. In National Wilms Tumor Study Group trials, patients with stage I or II renal rhabdoid tumors had an overall survival rate of 42% at 4 years; those with stage III, IV, or V disease fared less well, with a survival rate of about 16% when evaluated as a group.[190] For those with central nervous system disease, the prognosis is comparably dismal, with a registry review identifying a median overall survival time of 16.75 months.[197] Event-free survival appears to be better in patients with gross total resection compared with those who had subtotal resections of their central nervous system tumors.[197] Thus, if central nervous system tumors were detected earlier, they might be more easily resected and the outcome might be improved. Individuals with RTPS have also been reported to develop multiple schwannomas and meningiomas later in life.[194]

Currently, there are no standard recommendations for surveillance in patients with RTPS. Experts in the field have suggested that patients have thorough physical and neurologic examinations as well as head ultrasound imaging studies monthly during the first year of life to detect the presence of central nervous system tumors. It is also suggested that patients undergo abdominal ultrasound imaging every 1 to 3 months to assess for the presence of renal lesions. After age 4 years, the risk of developing a rhabdoid tumor rapidly declines, and at this point one could consider periodic screening using whole-body MRI. Although there are as yet no studies to confirm the benefit of screening patients with germline *INI1/SMARCB1* mutations, the aggressive natural history of the disease, apparently high penetrance, and well-defined age of onset for central nervous system rhabdoid tumors suggest that screening could prove beneficial. Given the potential survival benefit of surgically resectable disease, it is reasonable to postulate that early detection might improve overall survival rates.

DICER1-Related Tumor Predisposition Syndrome

Incidence

PPB is a rare cancer of embryonal lung tissue, which presents in infancy or early childhood. The first known case was reported in 1945, and the term PPB was proposed in 1988 to describe a range of rare pulmonary and pleural tumors made up primarily of fetal tissues.[198] Over 50 cases have been described, with a median age of 34 months at diagnosis.[199] Hereditary PPB has been observed in a small number of families worldwide, and in 2009 germline *DICER1* mutations were identified as the cause of hereditary PPB.[200] Since this discovery, germline mutations in *DICER1* have been implicated in up to 60% to 70% of PPB cases. Furthermore, careful review of *DICER1*-mutation–positive individuals and families reveals that there are many additional tumors associated with germline *DICER1* mutations, including cystic nephroma, ovarian Sertoli-Leydig cell tumor, embryonal rhabdomyosarcoma, intraocular medulloepithelioma, and supratentorial primitive neuroectodermal tumor.[201]

Genetics

The *DICER1* gene, located at 14q32.13, encodes a cytoplasmic endoribonuclease, which cleaves precursor molecules into microRNAs (miRNAs) that subsequently modulate cellular mRNA expression.[201] Impaired miRNA processing decreases the posttranslational regulation of mRNAs, and germline *DICER1* mutations have recently been shown to promote tumorigenesis via this mechanism.[202] Interestingly, all individuals with familial PPB show haploinsufficiency at 14q32.13 due to germline mutations affecting a single *DICER1* allele.[201] Studies of zebrafish[203] and mice[204] suggest that *DICER1* haploinsufficiency with retention of a single wild-type allele is sufficient for cancers to develop. Perturbations in miRNA concentrations have been implicated in studies of many human cancers, including lung, breast, ovarian, and skin cancers, with diverse and unclear associations between defects in miRNA processing and tumor phenotype depending on the tissue and mutation studied.[205] Consistent with this observation, several recent studies have implicated somatic mutations in *DICER1* in a variety of adult solid tumors.[206,207] Further translational studies will be necessary to explain the exact cause of miRNA-mediated oncogenesis, but it seems likely that miRNA dysregulation represents a new and important pathway in tumor formation.

Tumor Risks and Surveillance

DICER1 syndrome should be considered and clinical gene testing pursued in patients who present with PPB, cystic nephroma, ovarian Sertoli-Leydig cell tumors, embryonal rhabdomyosarcoma, intraocular medulloepithelioma, supratentorial primitive neuroectodermal tumor, or multinodular goiter. In a large retrospective cohort, 11 of 14 (79%) PPB cases, 2 of 3 (67%) cystic nephromas, and 3 of 6 (50%) cases of ovarian Sertoli-Leydig cell tumor were associated with germline *DICER1* mutations.[18] *DICER1* mutations are inherited in an autosomal dominant manner with variable expressivity and incomplete penetrance.[18] A negative family history does not exclude the diagnosis, especially in patients with typical tumors due to de novo germline *DICER1* mutations.

Since *DICER1* syndrome has been only recently described, the understanding of its phenotype and associated tumor risks is evolving. As a result, there are not yet consensus guidelines for surveillance among individuals harboring *DICER1* mutations. In cross-sectional studies, many individuals harboring a *DICER1* mutation do not develop cancer.[18] Given the rare and varied nature of malignant disease associated with *DICER1* germline mutations, individual practitioners will need to account for penetrance, age of tumor onset, clinical impact of early diagnosis, and risk of false-positive screening tests when weighing the risks and benefits of surveillance. Once PPB develops, complete surgical resection (often combined with adjuvant chemotherapy) is indicated and can lead to cure.[199] The overall 5-year survival rate in a cohort of 50 PPB patients was 45%,[13] although there was significant biologic variability within the cohort. A recent European review of 65 cases of PPB reveals that stage, grade, and degree of resection were highly correlated with survival,[208] suggesting that screening and early detection may indeed improve patient outcomes. Patients with *DICER1* mutations warrant close follow-up, and patients and their physicians should remain vigilant for changes in respiratory function, alterations in menses, and development of goiter, as each of these manifestations can herald the development of malignancy in this high-risk patient population.

Constitutional Mismatch Repair–Deficiency Syndrome

Incidence

CMMR-D (also known as CoLoN [Colon tumors and/or Leukemia/Lymphoma and/or Neurofibromatosis features],[210] childhood cancer syndrome,[211] and Lynch III syndrome[212]) is a recently described predisposition syndrome associated with the development of several different types of cancer, and sometimes multiple cancers, in children.[213] CMMR-D is caused by homozygous or compound heterozygous germline mutations in the genes that function in DNA mismatch repair (MMR), including *MLH1*, *MSH2*, *MSH6*, and *PMS2*. Individuals who carry a heterozygous mutation in one of these genes have a condition known as hereditary nonpolyposis colon cancer, or Lynch syndrome [LS]), which predisposes to many cancer types, including early-onset colorectal cancer, endometrial cancer, and ovarian cancer, with an average age of onset of cancer in the 40s or 50s. CMMR-D was first described in two consanguineous LS families in which the children were noted to have features of NF1 and early-onset hematologic and gastrointestinal malignancies.[214,215] To date, about 100 cases have been reported[216,217]; however, the exact incidence of CMMR-D is not known. In addition to cancer, children with CMMR-D develop café-au-lait macules, axillary and/or inguinal freckling, and Lisch nodules. Due to its similarity to NF1, children may be misdiagnosed as having neurofibromatosis.

Genetics

The normal role of the MMR proteins is to identify and repair small errors, such as base pair mismatches and small insertions and deletions, which occur during DNA replication. Additionally, the DNA MMR system has a role in apoptosis in response to DNA damage. Defective MMR leads to the accumulation of additional mutations, which eventually lead to tumor development. MMR-deficient tumors are often characterized by instability in microsatellites, short repeated sequences of DNA that are highly susceptible to errors during DNA replication. In LS, the second copy of an affected MMR gene is lost during tumor development. Therefore, LS-associated tumors are MMR-deficient and high in microsatellite instability (MSI).

Some genotype-phenotype correlations have been reported in CMMR-D. Individuals with biallelic mutations in *MLH1* or *MSH2* are more frequently diagnosed at younger ages and with hematologic malignant disease compared with individuals who carry mutations affecting *MSH6* or *PMS2*. In contrast, brain and other LS-associated tumors are more common in individuals with *MSH6* or *PMS2* mutations compared with *MLH1* or *MSH2*.[213] There is some evidence that individuals with more severe mutations (deletions or truncating mutations resulting in no functional protein) exhibit a more severe phenotype, with hematologic and brain cancers developing in the first decade of life, compared with individuals with less damaging mutations. These latter individuals are more likely to develop hematologic, brain, and gastrointestinal malignant disease in the second to fourth decades of life.[212,213]

Establishing a molecular diagnosis of CMMR-D is similar to making the diagnosis of LS, which can be accomplished in a stepwise manner beginning with testing for MSI and/or loss of expression of an MMR protein, followed by mutational analysis of the appropriate MMR genes. Because patients with CMMR-D have constitutional MMR deficiency, MSI and lack of MMR protein expression should theoretically be detectable in tumor as well as normal tissue. Interestingly, immunohistochemical staining of tumor was recently shown to be 100% sensitive and specific in diagnosing MMR deficiency, whereas MSI was neither sensitive nor specific and thus did not help in making the diagnosis.[209] If one or more of the familial MMR mutations are already known, it may be possible to proceed directly to genetic testing in the individual in question. It is important to note that diagnostic and genetic testing for this disorder is challenging, and it should be coordinated by professionals with experience in the diagnosis of LS and/or CMMR-D.[212,213,218]

Tumor Risks and Surveillance

The cancers most commonly associated with CMMR-D include hematologic malignant tumors, brain tumors, and LS-associated cancers (most notably, very early-onset gastrointestinal cancer).[209] Less common cancers include neuroblastoma and rhabdomyosarcoma.[212,213,217] In recent reviews, LS-associated cancers (mostly gastrointestinal) accounted for 32% to 37% of all cancers, brain tumors for 32% to 48%, and hematologic cancers for 15% to 24%; 5% to 7% were other types of cancer.[209,213] Multiple primary tumors have been reported in 20% to 40% of patients.[216,217] Age of onset varies by tumor type, with hematologic malignant tumors presenting in early childhood (mean age of onset, 5.5 years), brain tumors presenting in slightly older children (mean age of onset, 8 years), and LS-associated tumors in adolescence and young adulthood (mean age of onset, 15 to 16 years for gastrointestinal and genitourinary tract cancers and 24 years for endometrial cancer). LS-associated tumors may present as a second or third malignancy in patients who survive their initial cancer diagnosis.[213] In one study, gastrointestinal polyps or cancers were the first manifestation of CMMR-D in up to one third of patients.[217]

CMMR-D should be considered for young children who present with hematologic malignant disease, brain tumors, and/or LS-related cancers, in conjunction with: (1) café-au-lait macules and/or other signs of NF1 and/or hypopigmented skin lesions; (2) consanguineous parents; (3) a family history of LS-related cancers; (4) a second malignant tumor; and (5) siblings with childhood cancer. The lack of a family history of LS-related cancers should not rule out the possibility of CMMR-D, as some families could carry less penetrant mutations and thus not meet clinical criteria for a diagnosis of LS.[212,216,219] Because CMMR-D shares clinical features with NF-1, FAP, and LFS, it is important to keep this diagnosis in mind when evaluating children who present with one or more of these features.[213,216,217,220]

Development of a surveillance protocol has been challenging in CMMR-D because of the variety of associated cancers and the possibility of multiple primary cancers. There are currently no standard cancer screening guidelines, although some groups have suggested protocols including surveillance for colon and other gastrointestinal, hematologic, endometrial and ovarian, and other cancers.[217,221] Recently, it was reported that a surveillance protocol including semiannual brain MRI, annual gastrointestinal endoscopy, and blood work every 4 months successfully identified 39 asymptomatic tumors in a cohort of 23 children with CMMR-D, including 2 malignant gliomas, 2 gastrointestinal carcinomas, and 6 cases of polyposis.[209] There are currently very few data regarding the optimal treatment of cancers in patients with CMMR-D. However, clinicians should be aware of the possibility of increased toxicity and reduced efficacy of chemotherapy, as well as increased risk of second primary malignant tumors.[219,222]

CONCLUSIONS, CHALLENGES, AND LOOKING TO THE FUTURE

Since the isolation of *RB1* as the first gene predisposing to cancer, we have experienced tremendous advances in cancer genetics, including sequencing of the human genome and elucidation of DNA, RNA, and the epigenetic landscape of several different cancers. These new data have led to a profound increase in our knowledge of genes that predispose to cancer and the heritable basis of cancer. The characterization of these genes has highlighted critical roles for the encoded proteins in processes ranging from cell cycle control, DNA repair, apoptosis, signaling, miRNA processing, and metabolism. Because many of these predisposing genes are also mutated on a somatic level in a wide array of sporadic cancers, the study of rare cancer syndromes— many of which affect children—has provided invaluable insights into the processes underlying tumor formation.

Along with these advances have come many challenges. For example, we continue to know little about the psychosocial, developmental, and behavioral issues related to the genetic testing of children or adults for heritable predisposition to cancer. We do not yet understand which factors influence the pursuit and uptake of genetic testing by families, whether patients truly understand genetic test results and their implications, how individuals choose to communicate results within their immediate and/or extended families, and how genetic information influences the health-promoting and risk-taking behaviors of children and other family members. For many conditions predisposing to cancer, it is impossible to know when and what types of cancer will occur. This uncertainty makes it difficult to establish effective surveillance protocols. Assessment of the costs and benefits of these protocols is further compounded by the rarity of specific syndromes.

These challenges are only likely to exponentially increase as genetic technologies evolve and are increasingly applied to the clinical setting. Although these technologies will further our knowledge of the pathogenic mutations linked to cancer, they will also reveal many less-damaging variants. In many cases, it may not be readily evident whether these variants influence the expression or function of an encoded protein. Therefore, it will be difficult to know how to react to these results. Will these variants influence disease susceptibility, response to treatment, and/or therapy-associated toxicities? How do these variants cooperate with one another or with environmental factors during the process of tumor formation? As more information is gained and functional assays are developed, variants may eventually be reclassified as benign polymorphisms or disease-causing mutations. Who will be responsible for reanalyzing these data, reporting the updated results, and explaining their implications to clinicians and patients? Finally, how will we translate the increasing body of genetic information to the clinical setting to predict cancer risk, direct tumor screening, and prevent tumors from occurring? Although it is expected that genetic discoveries will enable development of new and more effective therapies or preventive measures, unfortunately we are yet to realize these benefits for the majority of human cancers. Ultimately, the challenge lies in establishing national and international collaborations, where information on these rare, yet informative, syndromes predisposing to cancer can be gathered, integrated, and analyzed to address these important and as yet unanswered questions.

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CHAPTER 43

Genetics and Epigenetics of Childhood Cancers

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CHAPTER OUTLINE

FUNCTIONAL GENETICS OF CHILDHOOD CANCER PREDISPOSITION SYNDROMES
DYSREGULATION OF CELL DIVISION AND GROWTH
DNA REPAIR AND GENOME INSTABILITY
ABERRANT GENE EXPRESSION
SOMATICALLY ACQUIRED GENE MUTATIONS AND CANCER GENOMICS
GENOMIC REARRANGEMENTS AND CHROMOTHRIPSIS
DNA METHYLATION, CHROMATIN, AND THE EPIGENETICS OF CHILDHOOD CANCER
DNA METHYLATION
CHROMATIN-MODIFYING ENZYMES
SPLICING FACTORS
METABOLIC ENZYMES
CONCLUSIONS AND FUTURE OUTLOOK

The first scientific reports of childhood cancers such as Virchow glioma (neuroblastoma), Wilms tumor (nephroblastoma), and Ewing sarcoma provided a classification system based on organ involvement but shed little light on the natural causes of these diseases. Subsequent study of cancer cells and animal tumors, and in particular Boveri's analysis of cell mitoses and Rous' study of oncogenic viruses, revealed that cancer is at least in part a disease of genes and their aberrations.[1,2]

With the advent of molecular biology, we learned that animal oncogenic viruses contain mutant versions of cellular genes, termed *oncogenes*, as well as gene products that negatively regulate endogenous tumor suppressor genes.[3] Expressed in normal cells, these tumorigenic viral genes can cause unrestrained proliferation and, ultimately, immortalization and malignant transformation, leading to the concept that human cancer is caused by the dysregulation of endogenous protooncogenes and tumor suppressor genes.[4-6]

In particular, the idea of endogenous tumor suppressor gene inactivation in human cancers is strongly supported by (1) the analysis of the age dependence of retinoblastoma incidence and (2) the finding that in children who inherit one mutant allele, bilateral tumors develop at an earlier age than in persons who have tumors that develop as a result of spontaneous biallelic inactivation.[6] Although this "two-hit" model provides a powerful explanation of the incidence of retinoblastomas, similar analyses of other human cancers led to the conclusion that at least six different genetic mutations are needed to explain the apparent age-dependent incidence of some human tumors.[7,8] Indeed, a large majority of human cancers contain many gene mutations in a complex clonal architecture, as revealed by recent analysis of tumor genomes by next-generation sequencing. Some tumors, however—particularly those in children—can have surprisingly low mutation rates. Finally, even cancers with extremely low numbers of gene mutations contain mutations outside of genes that could contribute to tumorigenesis. Consequently, the extent to which alterations in noncoding genetic elements and epigenetic changes have the potential to cooperate with or substitute for genetic mutations has become an active area of investigation.

In addition to the polygenic nature of human cancers, many features of tumor cells are not directly explained by the mutation of cancer-causing genes but instead involve complex cell and tissue biologic systems. These "hallmarks of cancer," which include limitless cell replication, self-sufficiency in growth signaling, circumvention of cell death, immune system evasion, and the ability to metastasize, provide an expedient phenomenologic model to understand cancer cell behavior.[9,10]

Recently, contributions from changes in the chemical modifications of deoxyribonucleic acid (DNA) and chromatin have been increasingly implicated in cancer pathogenesis. These changes are often referred to as "epigenetic" because they may have the potential to result in somatically heritable changes in gene expression and phenotype that do not result directly from changes in DNA composition. Normal development provides a vivid illustration of the power of epigenetic changes, because virtually every cell in the body contains mostly identical coding DNA sequences and yet cells display markedly different behaviors and fates. For example, mature neurons have a distinct differentiated appearance and never divide, whereas hematopoietic stem cells divide throughout a person's lifetime. Further, these cellular fates remain stable over years of somatic cell division. Given the role of epigenetic regulation in the control of cell fate, perhaps it is not surprising that epigenetic perturbations may also contribute to oncogenesis. Although they are conceptually distinct, genetic and epigenetic dysregulation can be closely associated in cancer cells, while genetic mutations of epigenetic regulators are increasingly recognized, particularly in childhood tumors.

Whereas somatic cancer mutations are relatively easy to identify by comparing the sequence of DNA between tumor and nontumor cells, epigenetic causes of cancer are much more difficult to discern because the normal epigenetic state varies with cell type, which makes defining aberrant features a challenge. With respect to DNA methylation, it has become evident that cancer genomes overall tend to be hypomethylated compared with normal tissues and that they have focal areas of hypermethylation in the promoters of specific genes associated with silencing of gene expression. Similarly, the recent discovery of mutations in genes that regulate chromatin structure suggests that aberrant chromatin modifications may contribute to tumorigenesis via epigenetic dysregulation rather than through the induction of additional genetic mutations, although in most cases this conjecture has yet to be proven.

Arguably, our ability to decipher the biologic basis of human cancer will depend on understanding the relationship between molecular aberrations and dysregulated properties of cancer cells. Awareness of the importance of this problem is increasing in the field of pediatric oncology, leading to the recognition that although some childhood tumors can have a remarkably low number of recurrently mutated genes, others can contain thousands of DNA substitutions and complex chromosomal rearrangements. Pediatric cancers may be conceptually distinct from those that occur in adults, because in the absence of a syndromic predisposition, children otherwise have relatively low rates of somatic genetic alterations and toxin exposures due to aging. Thus childhood cancers may involve unique mechanisms of tumorigenesis.

In this chapter we review the mechanisms of molecular pathogenesis of childhood cancers. Our goal is not to provide a comprehensive encyclopedia (electronic publishing and online databases* now enable ready access to this information) but to develop a systematic classification of the molecular lesions responsible for childhood tumors, with a focus on biologic mechanisms and functional principles. Instead of organizing this chapter on the basis of cancer types, which are covered individually in other chapters of this volume, we have attempted to develop a functional framework that we hope will provide helpful insights into the relationship between molecular aberrations and behaviors of cancer cells *sui generis*. Such an approach should inform the development of improved clinical therapies, particularly if cancer treatment is to become more precise through the use of rationally targeted agents.

FUNCTIONAL GENETICS OF CHILDHOOD CANCER PREDISPOSITION SYNDROMES

The study of childhood cancer predisposition syndromes is an important source of information about the genetic basis of human cancers. Indeed, the retinoblastoma 1 *(RB1)* gene, which is mutated in familial retinoblastomas, was the first human tumor suppressor gene identified.[6,11,12] What follows is an overview of known genes mutated in childhood cancer predisposition syndromes based on the major cell biologic pathways thought to be perturbed by the inherited gene mutations.

DYSREGULATION OF CELL DIVISION AND GROWTH

A large number of childhood cancer predisposition syndromes involve mutations of genes that regulate cell growth and division. One of the first to be described, the *RB1* gene, is mutated in children who are predisposed to the development of retinoblastoma, pinealoblastoma, and osteosarcoma. The *RB1* gene is part of the G1 cell cycle checkpoint, where its product directly binds and regulates the activity of the E2F family of transcription factors and the DREAM chromatin remodeling complex in response to stress.[13] Cancer-causing deletions and nonsense mutations of *RB1* can cause entry into the cell cycle by otherwise quiescent cells and mitigation of senescence that limits cellular life span.[14] Whereas loss of *RB1* in cellular and mouse models causes defects in mitotic chromosome segregation that lead to aneuploidy,[15,16] most human retinoblastomas are largely diploid,[17] suggesting that the canonic chromosome segregation effects of *RB1* inactivation in human retinoblasts are functionally compensated. Human retinoblastomas invariably exhibit biallelic inactivation of *RB1*, but otherwise often have a comparatively low mutation rate. One study found that *RB1* was the only known cancer gene mutated in these tumors.[17] However, additional subclonal DNA mutations were observed, with some mutations located outside of coding regions, which may cooperate with *RB1* loss to drive retinoblastoma formation. In addition, epigenetic alterations have also been implicated in cooperating with *RB1* mutation.[17]

The adenomatous polyposis coli *(APC)* gene, which is mutated in children with familial adenomatous polyposis who are predisposed to hepatoblastoma and colon carcinoma, also controls the G1 cell cycle checkpoint.[18] APC serves as a ubiquitin ligase that regulates the protein stability of β-catenin, which controls transcription of cyclins, including cyclin D, which in turn associates with cyclin-dependent kinase (CDK)4/6 to negatively regulate *RB1* activity.[19] Disease-associated dominant mutations of *APC* tend to occur in distinct regions of the gene, leading to the generation of truncated protein products, consistent with the requirement of residual or neomorphic activity of the mutant proteins.[20] Because *APC* regulates the stability of many proteins—and because, in addition to the cell cycle progression, β-catenin also regulates cell adhesion and differentiation—the mechanism of tumorigenesis induced by *APC* mutations is thought to be pleiotropic.

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*At the time of this writing, the following publicly accessible databases of cancer-associated mutations and structural aberrations are available: http://target.cancer.gov; http://pediatriccancergenomeproject.org; http://www.sanger.ac.uk/genetics/CGP; and http://cansar.icr.ac.uk.

In addition to mutations of genes whose products regulate cell division, cancer-causing mutations also directly dysregulate mitogenic signaling and cell growth. The most common cancer predisposition syndrome, neurofibromatosis, is predominantly caused by autosomal-dominant inheritance of inactivating mutations of the neurofibromin 1 *(NF1)* gene, with the less frequent form caused by mutations of neurofibromin 2 *(NF2)* or *merlin*. Children with mutations of *NF1* are predisposed to the development of benign and malignant tumors, including gliomas, peripheral nerve sheath tumors, pheochromocytomas, and leukemias.[21] NF1 is a large protein that is not completely understood but is known to regulate the activity of rat sarcoma (Ras).[22,23] In turn, *NF1* mutant tumors exhibit increased Ras guanosine triphosphate hydrolase activity, which stimulates the mitogen-activated protein and AKT kinase cascades that, among other effects, result in the transcription of genes involved in cell growth and stimulation of protein synthesis.[24-26] Although most patients with germline *NF1* mutations experience the development of neurofibromas, which harbor biallelic inactivation of the gene, only a minority of these tumors evolve to become malignant. This finding indicates that disease-associated *NF1* mutations have limited transforming ability, and that additional lesions are required for carcinogenesis.

The complex relationship between cancer-causing gene mutations and their effects on tumorigenesis is also evident from mutations of *ras* genes themselves. Genes related to *ras*, including Kirsten rat sarcoma *(KRAS)*, neuroblastoma rat sarcoma (*NRAS)*, and Harvey rat sarcoma *(HRAS)*, are some of the first human protooncogenes identified, based on the involvement of the corresponding viral oncogenes in the pathogenesis of rat sarcomas.[27] However, whereas *NF1* mutations stimulate Ras activity and cause neurofibromatosis, patients with inherited mutations of the *ras* genes instead experience the development of cardiofaciocutaneous, Noonan, LEOPARD, and Costello syndromes. Inherited mutations of the *ras* genes are missense activating mutations, analogous to the oncogenic viral *ras* mutations, yet the spectrum of tumor phenotypes in these patients is distinct. In addition to gliomas these phenotypes also include leukemias, neuroblastomas, and rhabdomyosarcomas.[28,29] These differences between *NF1*- and *ras*-mutant cancer predisposition syndromes are not simply due to differences in tissue expression of the two genes, because both appear to be expressed in neuronal, mesenchymal, and hematopoietic tissues. Rather, they may reflect the developmental consequences of Ras activation as a result of the inactivation of an endogenous negative regulator as opposed to direct mutational activation of an oncogenic signaling pathway, a property that may inform the development of strategies to target these lesions therapeutically.

DNA REPAIR AND GENOME INSTABILITY

The prevention of de novo cancer-causing mutations ultimately depends on efficient DNA repair and maintenance of genome stability. Indeed, several cancer predisposition syndromes involve defective DNA repair. For example, inherited mutations of the tumor protein 53 (*TP53)* tumor suppressor gene cause most cases of Li-Fraumeni syndrome, which is associated with the predisposition to sarcomas, leukemias, and breast carcinomas.[30] The genotype-phenotype relationship of *TP53* cancer-causing mutations is complex, with some mutations having apparent loss-of-function, dominant-negative, or gain-of-function properties in experimental models.[31-34] p53 regulates the expression of genes involved in cell division, apoptosis, and DNA repair, and its mutation in cells leads to chromosomal instability.[35,36] This process is mediated partly by direct effects of p53 on the transcription and translation of target genes, as well as via feedback interaction with its ubiquitin ligase murine double minute 2 (MDM2), which also has oncogenic effects independent of its negative regulation of p53.[37] As a result, the dysregulation of cellular DNA damage and stress response by *TP53* mutations is profoundly tumorigenic, contributing to the somatic pathogenesis of most human cancers. The p53-MDM2 feedback regulation also complicates therapeutic targeting, because inhibition of MDM2 aimed at restoring p53 tumor suppression may in effect enhance the deleterious activity of gain-of-function p53 mutant proteins.[38]

Defective DNA damage response is also a hallmark of Fanconi anemia (FA), a cancer predisposition syndrome characterized by bone marrow failure and development of myelodysplasia, leukemia, and carcinomas.[39,40] Although mutations of more than 15 different genes can cause FA, most of the known FA-mutant gene products physically interact with the components of a conserved DNA damage response mechanism, breast cancer 1 (BRCA1), ataxia telangiectasia mutated (ATM), Nijmegen breakage syndrome 1 (NBS1), and radiation-sensitive 51 (RAD51), and are required for the repair of chromosome defects that occur during homologous recombination.[39] FA pathway–deficient cells are hypersensitive to DNA-damaging agents, and over time they accumulate deleterious gene mutations (usually segmental deletions).[41]

Several other chromosome-breakage syndromes with cancer predisposition involve defects in closely related biochemical pathways of DNA repair of homologous recombination errors, such as ataxia telangiectasia (due to mutations of *ATM*), Nijmegen breakage syndrome (due to mutations of *NBS1*), and Bloom syndrome (due to mutations of *BLM*). Genes involved in nucleotide excision or transcription-coupled DNA repair can also be mutated and cause the cancer predisposition syndromes xeroderma pigmentosum and Cockayne syndrome, usually due to accumulation of ultraviolet radiation–induced mutations (typically nucleotide substitutions).[42]

ABERRANT GENE EXPRESSION

Another hallmark of cancer cells is expression of genes in inappropriate developmental states, often driven by mutations of gene products that regulate gene expression. For example, patients with inherited mutations of the Wilms tumor 1 *(WT1)* gene experience development of the Denys-Drash syndrome (due to loss-of-function missense mutations) or the Wilms tumor, aniridia, genitourinary anomalies, and mental retardation (WAGR) syndrome (due to gene deletions), both with predisposition to the development of bilateral Wilms tumors.[43] In spite of years of study, the function of the WT1 protein is incompletely understood. WT1 is a key regulator of mesenchymal cell development, including kidney mesenchyme, in part through its functions as a transcriptional repressor.[44] However, WT1 also interacts with messenger RNA (mRNA) and splicing factors, consistent with possible posttranscriptional functions in regulation of gene expression.[45] In addition, although recessive mutations of *WT1* appear consistent with its tumor suppressive functions in Wilms tumorigenesis, in other developmental and tissue contexts, particularly myeloid leukemias, WT1 can enhance cell survival and proliferation and as a result can act as an oncogene or haploinsufficient tumor suppressor gene.[46] Such tissue-dependent phenotypes are characteristic of cancer-associated genes that affect gene expression and cell fate determination, insofar as the functional consequences of mutations depend on the spectrum of expressed genes themselves and differentiation state in general.

Tumorigenesis due to aberrant regulation of gene expression can also occur posttranscriptionally, as in the case of inherited mutations of the *DICER1* gene, which predispose to the development of pleuropulmonary blastoma, cystic nephroma, and rhabdomyosarcoma.[47] *DICER1* is a ribonuclease that is required for the generation of endogenous microRNAs (miRNAs), which negatively regulate gene expression by inducing degradation and translational blockade of specific mRNAs.[48] Cancer-associated mutations affect conserved, enzymatically required residues in *DICER1* and cause defective miRNA processing, leading to increased expression of target genes.[49] The reason inherited *DICER1* mutations predispose specifically to the development of pleuropulmonary blastoma, cystic nephroma, and rhabdomyosarcoma is not understood, but it is reasonable to hypothesize that this predisposition is due to the specific requirements for miRNA-mediated gene expression in these tissues or expression of specific target protooncogenes.

SOMATICALLY ACQUIRED GENE MUTATIONS AND CANCER GENOMICS

Many cancer-causing gene mutations that are inherited as part of familial cancer predisposition syndromes can also be acquired somatically in tumor cells. These mutations include most of the genes described in the preceding sections, but with notable differences in pathogenic mechanisms.

First, in many instances of somatic mutations of tumor suppressor genes, only one of the alleles is mutated, leaving the other wild-type allele intact and expressed.[50] Such haploinsufficient mutations, as opposed to the classic "two-hit" tumor suppressor gene mutations, are thought to exert their effects through reduced tumor suppressive activities as part of tumor initiation. In addition, the functional contributions of the wild-type allele may be required for tumor maintenance in some cases. This distinction may be crucial for the development of therapeutic strategies to target haploinsufficient tumor suppressors, because restoration of tumor suppressor gene functions may be not be useful in tumors in which residual wild-type activity is required for tumor maintenance.[51]

Second, the tumor spectrum and phenotype, and therefore the exact mechanism of tumorigenesis, are often different for the same mutation when it is developed somatically as opposed to inherited in the germline. For example, inherited mutations of *WT1* cause Denys-Drash syndrome and predisposition to Wilms tumors but never leukemias, whereas somatic mutations of *WT1* occur in 10% of cases of acute myeloid and T-cell acute lymphoblastic leukemias (ALLs).[52] This difference may be due to the differences in gene expression and differentiation state of cells that are transformed by these tumorigenic mutations. The same principle is likely responsible for the nonequivalence of many other somatically acquired tumor mutations that have counterparts that occur as part of inherited developmental syndromes, such as the loss-of-function mutations of BCL6 corepressor *(BCOR)* that occur somatically in medulloblastomas and myeloid leukemias but cause oculofaciocardiodental syndrome without cancer predisposition when inherited in the germline.[53-55]

Finally, recent whole-genome surveys have revealed that recurrence rates of somatically acquired gene mutations may be rare when considered individually but can involve coherent biologic pathways that are mutated frequently as a functional group. This functional co-occurrence is often suggested by the mutual exclusivity of the individual mutations. We review the mechanisms of tumorigenesis by these mutations, as organized below by shared biochemical activities or biologic pathways.

GENOMIC REARRANGEMENTS AND CHROMOTHRIPSIS

Structural genomic rearrangements and copy number changes are particularly prevalent in adult cancers, both at the level of whole chromosomes and chromosomal segments, particularly in advanced or histologically anaplastic diseases, but they are generally somewhat less prominent in pediatric cancers. The mechanisms of their generation and tumorigenic sequelae at least in part appear to be related to the dysfunction of DNA repair and maintenance of genome stability. In particular, whole chromosome aneuploidy, often as a result of chromosomal instability due to defects in mitosis such as centrosome amplification or failure of spindle assembly, is a prevalent feature of anaplastic tumors.[56] In some cases, such as acute myeloid leukemias, mutations of the components of the mitotic cohesin complex, including stromal antigen 2 (STAG2), structural maintenance of chromosomes 3 and 1A (SMC3 and SMC1A), and radiation-sensitive 21 (RAD21), may lead to chromatin cohesion defects and aneuploidy.[57] However, identification of such cohesin complex mutations in karyotypically normal acute myeloid leukemia (AML) raises the possibility that their tumorigenic effects rely on nonmitotic functions of the cohesin complex components.[58]

In model systems, aneuploid cells appear to have increased rates of mutation and susceptibility to DNA damage.[59,60] Noncancerous aneuploid human cells tend to be nonviable, with the exception of cells with trisomies of chromosomes 13, 18, and 21 and certain ring chromosomes.[61] However, somatically acquired chromosomal aneuploidy is far more prevalent in tumors than in constitutional syndromes, suggesting that additional molecular changes that enable tumor cells to tolerate aneuploidy must exist. Indeed, tumors with characteristic aneuploidies often have specific additional mutations—for example, overexpression and mutational activation of cytokine receptor-like factor 2 (CRLF2) signaling in B-cell ALL (B-ALL) with trisomy of chromosome 21.[62-64] Defining the molecular basis of these interactions may reveal potential opportunities to therapeutically target mechanisms that permit tolerance of aneuploidy in cancer cells.[65]

One of the best-studied genomic aberrations in cancer cells is recurrent chromosomal translocations. These chromosomal rearrangements generally lead either to the aberrant expression of protooncogenes as a result of juxtaposition of regulatory transcriptional elements or to the generation of chimeric fusion gene products that have gain-of-function tumorigenic effects.[66] Chromosomal translocations are frequently observed in hematopoietic cancer cells, where they lead to increased survival, growth, and malignant transformation, such as MYC-overexpressing t(8;14) translocation in Burkitt lymphomas and t(9;22) translocation generating the BCR-ABL1 fusion proteins in B-ALL and chronic myeloid leukemia. In lymphoid cells, these translocations are generated at least in part by aberrant V(D)J recombination and activation-induced deaminase activities that occur at the immunoglobulin and T-cell receptor loci undergoing somatic hypermutation. In addition, recombination of endogenous repetitive elements and DNA topoisomerase II failures also appear to cause chromosomal translocations, such as those generating mixed-lineage leukemia (MLL) fusion proteins.[67,68]

The chimeric fusion proteins generated by these translocations have been found to disrupt cell differentiation and specification, along with enhanced cell survival and growth.[69] For example, the TEL-AML1 fusion protein leads to abnormal self-renewal of hematopoietic stem cells, making them susceptible to additional mutations and ultimate transformation to B-ALL.[70,71] The EWS-FLI1 fusion protein in Ewing sarcoma disrupts specification of mesenchymal progenitors, blocking cell differentiation.[72-74]

By virtue of being tumor-specific, fusion gene products generated by chromosomal translocations represent attractive targets for rational therapy. Indeed, introduction of all-*trans*-retinoic acid and arsenic trioxide to induce the degradation of the chimeric PML-RARA fusion protein caused by the t(15;17) chromosomal translocation in the majority of cases of acute promyelocytic leukemia has revolutionized the treatment of this disease by eliminating the need for chemotherapy.[75]

In addition to the gains and losses of whole chromosomes and their segments, cancer cells can exhibit other distinct and complex types of genomic rearrangements. In particular, some tumors such as neuroblastomas and gliomas, for example, have been found to contain short episomal DNA fragments, termed *double minutes,* that lack centromeres and telomeres.[76,77] In neuroblastomas, these double minutes contain multiple copies of the *MYCN* protooncogene, leading to its high-copy amplification and overexpression. The mechanisms generating these episomal DNA fragments are not well defined but may involve recombination or transposition events involving endogenous *Alu, LINE*, and *MER* elements, or microhomology-based aberrant nonhomologous end-joining reactions.[77]

Recently a highly complex form of genomic rearrangements, termed *chromothripsis*, has been described in a variety of tumors, including neuroblastomas, medulloblastomas, and osteosarcomas.[78-80] This phenomenon is defined by extensive intrachromosomal rearrangements, often numbering in the thousands, of a single chromosome.[81] It has been proposed to be caused by aberrant DNA replication and microhomology-based nonhomologous end-joining repair, as well as by damage of chromosomes trapped in micronuclei as a result of mitotic errors.[80,82,83] The mechanisms of the generation of these complex rearrangements and their functional consequences remain to be defined but promise to reveal important insights into the mechanisms of human tumorigenesis, given their marked prevalence in distinct tumor types.

DNA METHYLATION, CHROMATIN, AND THE EPIGENETICS OF CHILDHOOD CANCER

Structural abnormalities in the genomes of cancer cells seem essential for tumorigenesis, but increasingly, the contribution of aberrant epigenetic regulation to the development of human cancers in general and pediatric cancers in particular is also becoming recognized. Methylation of cytosines in DNA can cause profound changes in transcription, and methylation of CpG islands in the promoters of genes can lead to transcriptional silencing.[84,85] In addition, nuclear DNA is tightly bound to histones as part of chromatin, and sequence-specific posttranslational modification of histones, including acetylation, methylation, and ubiquitination, have all been shown to modulate DNA accessibility and transcription.[86,87] Consequently, the transcription of individual genes depends both on the degree of DNA compaction and accessibility, as well as on the individual chemical modifications of histones surrounding specific genes.

DNA METHYLATION

Methylation of cytosines in DNA is a key regulator of mammalian cell development, and changes in DNA methylation have long been known to be associated with human cancer. For example, loss of normal DNA methylcytosine imprinting patterns on chromosome 11 causes Beckwith-Wiedemann syndrome, a developmental disorder that predisposes to the formation of hepatoblastomas and Wilms tumors.[88] Indeed, abnormal patterns of cytosine methylation are frequently observed in tumor cells, and recently mutations of enzymes that control DNA methylation have been found in AML and myelodysplastic syndromes.

The de novo DNA methyltransferase 3A (DNMT3A) is recurrently mutated in a large fraction of cases of AML.[89,90] The mutations tend to be missense and heterozygous, involving an evolutionarily conserved DNA-binding interface of DNMT3A, consistent with a possible gain-of-function effect that presumably alters locus-specific DNA methylation. Loss-of-function deletions and nonsense mutations of methylcytosine dioxygenase 2 *(TET2)* are also observed in AML and myelodysplastic syndromes.[91-93] TET2 functions by hydroxylating methylcytosine, and these mutations are thought to lead to the loss of DNA hydroxymethylcytosine, although whether this is carcinogenic in itself or transforms cells indirectly via its effects on histone modifications and DNA demethylation remains to be determined.[94,95] Consistent with this notion, the CpG island methylator phenotype, which is a key factor in the tumorigenesis of adult colorectal carcinomas, is yet to be definitively established in tumors with defects in DNA methylation.[96]

CHROMATIN-MODIFYING ENZYMES

Whereas perturbation of nuclear appearance in cancer cells was long ago realized to represent a change in chromatin structure, the molecular underpinnings of this phenomenon have begun to be elucidated only recently. Perhaps the strongest evidence implicating perturbation of chromatin structure in carcinogenesis is the finding that chromatin-remodeling complexes are mutated at a high frequency in a wide variety of cancers. Some of these include genes that encode proteins that contribute to the methylation and acetylation of histone lysines. Others include those that encode subunits of complexes that mobilize histones and remodel chromatin structure. Numerous other modifications of histones also occur, such as arginylation, succinylation, and glycosylation, but their significance is only beginning to be assessed.

One of the earliest genes involved in chromatin modification that was identified as being mutated in pediatric cancers is the *MLLT1* gene, which contains the SET lysine N-methyltransferase domain that is characteristically lost as a result of chromosomal translocations that generate chimeric MLL fusion proteins in infant and topoisomerase inhibitor–induced leukemias.[97,98] Wild-type MLL and mutant MLL fusion proteins physically interact with a large supramolecular complex involved in regulation of gene expression by chromatin modification.[99] MLL fusion–induced leukemias are characterized by unique patterns of gene expression associated with a distinct spectrum of chromatin modifications, particularly those involving methylation of lysine 79 of histone 3 (H3K79), which in turn is associated with high levels of gene expression.[100] Notably, these activities require the preferential recruitment of the DOT1L lysine methyltransferase by the MLL fusion proteins compared with wild-type MLL, and DOT1L activity is required for leukemogenesis in preclinical models, potentially offering a therapeutic strategy.[101,102] Aberrant methylation of H3K79 at target genes of the MLL fusion protein therefore likely contributes to *MLLT1* translocation-induced leukemogenesis.

Whereas increased target gene H3K79 methylation by aberrant recruitment of a lysine methyltransferase represents a gain-of-function of tumorigenic methyltranferase activity, loss-of-function mutations of lysine methyltransferases have also been observed in tumors. In particular, loss-of-function frameshift and nonsense mutations of the suppressor of zeste 12 homologue *(SUZ12)*, embryonic ectoderm development *(EED)*, and enhancer of zeste homologue 2 *(EZH2)* genes are frequently found in T-cell ALL (T-ALL).[103,104] Similar mutations have also been observed in cases of myelodysplastic syndromes.[105,106] These gene products are physical components of the Polycomb repressive complex (PRC) 2, which methylates lysine 27 of histone 3 (H3K27), a modification associated with silencing of target gene expression.[107] It is important to note that whereas *EZH2* undergoes loss-of-function mutations in T-ALL, gain-of-function missense mutations of *EZH2*, which are associated with neomorphic enzymatic activity of the mutant protein, are observed in cases of diffuse large B-cell lymphoma.[108] The complexity of the tumorigenic mechanism of these mutations is further emphasized by the recent finding of loss-of-function mutations of the H3K27 demethylase *UTX*, which, when mutated, ought to potentiate H3K27 methylation in some cases of B-ALL.[109] The importance of regulation of H3K27 modifications was recently further highlighted by the finding that H3K27 itself is frequently mutated in pediatric diffuse pontine gliomas, in which lysine to methionine mutations occur in histone 3.3 or 3.1[110,111] and result in inhibition of PRC2.[112] Collectively, these findings indicate that the developmental and epigenetic state of the cell likely makes key contributions to tumorigenesis by the mutant chromatin-modifying enzymes by providing specific substrates for their altered enzymatic activities.

The recognized role of chromatin modifiers has continued to expand via cancer genome sequencing efforts and has led to identification of the switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complex as the most frequently mutated chromatin regulatory complex. Genes that encode subunits of this complex are mutant in 20% of all human cancers.[113] This 12- to 15-protein complex serves important roles in transcriptional regulation by utilizing adenosine triphosphate hydrolysis to modulate chromatin structure at the promoters and enhancers of its target genes. Identification of biallelic inactivating mutations of *SNF5/INI1/SMARCB1*, a core SWI/SNF subunit, in nearly all cases of malignant rhabdoid tumors represented the first identified link between an adenosine triphosphate–dependent chromatin remodeler and cancer.[114,115] Heterozygous *SMARCB1* mutation was also identified as the basis of a familial cancer predisposition syndrome.[114] Other SWI/SNF subunits have also recently been found to be recurrently mutated in pediatric malignancies, including *ARID1A* in Burkitt lymphoma, *ARID1A* and *ARID1B* in neuroblastoma, and *ARID1A* and *SMARCA4* in medulloblastoma. Although these findings broadly implicate the SWI/SNF complex in tumor suppression, the mechanisms by which these mutations drive cancer formation remain unclear.

The complex serves roles in transcriptional regulation by modulating the chromatin landscape at promoters and enhancers of its target genes; in addition, the complex has also been implicated in DNA repair.[116-121] However, recent sequencing of rhabdoid tumor genomes indicates that these cancers contain a remarkably simple protein coding genome, with mutations of *SMARCB1* essentially the sole recurrent genetic mutation; in some cases it is sole coding sequence mutation identified.[122-124] These findings have been interpreted as suggesting that oncogenesis caused by the mutation of the SWI/SNF complex occurs not via induction of genomic instability but rather via epigenetic dysfunction.[125] Perhaps providing mechanistic insight, mutation of *SMARCB1* was reported to result in unbalanced epigenetic antagonism between the SWI/SNF and PRC2 complexes, generating a unique dependence upon EZH2.[126] Preclinical small molecule inhibition of EZH2 has been reported to be effective against rhabdoid tumors in preclinical models but has yet to be tested in humans.[127]

Cancer genome sequencing has also identified other chromatin remodelers mutated in cancer. For instance, alpha-thalassemia mental retardation syndrome, X-linked *(ATRX)* and death-domain associated protein *(DAXX)*, which encode subunits of a chromatin-remodeling complex required for replication-independent deposition of histone H3.3, were identified in glioblastoma and were present in 100% of tumors that also contained G34R or G34V mutations in H3.3 itself, raising the possibility of mutations with coordinate effects upon chromatin structure.[110] Mutations in *ATRX* also occur in older patients with neuroblastoma.[78] Collectively, chromatin modifiers have emerged as a major new class of tumor suppressors.

SPLICING FACTORS

In addition to mutations affecting factors that regulate chromatin, recent studies have also identified recurrent mutations of genes encoding components of the spliceosome, including mutations of the *U2AF35*, *ZRSR2*, *SRSF2*, and *SF3B1* genes in myelodysplastic syndromes, AML, and juvenile myelomonocytic leukemia.[128,129] The mutations tend to be missense and mutually exclusive, and they cluster in distinct conserved parts of the genes, suggesting gain-of-function phenotypes. All of these genes encode components of the spliceosome that are involved in splicing of pre-mRNAs, suggesting that their tumorigenic mechanism may involve dysfunctional control of gene expression arising from aberrant mRNA splicing. Indeed, mutated splicing factor gene products participate in the 3′ splice site selection during pre-mRNA processing, inducing abnormal splicing in reporter assays.[128] However, SF3B1 can also interact with components of the Polycomb complex involved in regulation of chromatin structure and gene transcription, raising the possibility that its mutation and tumorigenesis may involve mechanisms other than splicing.[130] Because transcription and splicing are often coregulated in normal cells and mutations of splicing factor genes often co-occur with mutations affecting regulators of gene expression in tumor cells, a definition of their tumorigenic mechanisms and phenotypic consequences may require a comprehensive analysis of the potential interactions of the co-occurring mutations.

METABOLIC ENZYMES

Another unanticipated finding from the analysis of tumor genomes has been the discovery of mutations in metabolic enzymes. Initially observed in gliomas, missense mutations of the isocitrate dehydrogenase 1 and 2 *(IDH1* and *IDH2)* genes that encode isocitrate dehydrogenase enzymes, which are components of the mitochondrial citric acid cycle, have also been found in a large fraction of AMLs.[131,132] The majority of mutations involve conserved arginine residues in the enzymatic active sites, which aberrantly generate 2-hydroxyglutarate that accumulates to high concentrations in tumor cells, tissues, and even circulating blood.[133,134] Because 2-oxoglutarate, the normal metabolite of *IDH1* and *IDH2*, is an essential cofactor of many cellular enzymes, the effects of oncogenic *IDH1* and *IDH2* mutations and excess production of 2-hydroxyglutarate may be pleiotropic.[135] Indeed, *IDH1* and *IDH2* mutations can lead to the inhibition of enzymatic activity of *TET2* that hydroxylates 5-methylcytosine in DNA, as well as associated changes in DNA methylation and gene expression in leukemia cells.[136] Mutations of IDH and production of 2-hydroxyglutarate have also been found to inhibit H3K9 demethylase KDM4C in glioma cells.[137] In addition, in glioma cells, generation of 2-hydroxyglutarate by mutant IDH enzymes also inhibits EGLN prolyl hydroxylases, leading to diminished hypoxia-inducible factor levels and enhanced cell growth.[138]

The stereospecific nature of the effects of 2-hydroxyglutarate—wherein the (R)- but not the (S)-enantiomer appears to be oncogenic yet both metabolites can inhibit 2-oxoglutarate–dependent cellular enzymes—may lead to the definition of specific mechanisms and their ultimate therapeutic blockade.[139] Mutations of other metabolic enzymes, such as succinate dehydrogenase and fumarate hydratase, can also be tumorigenic, causing gastrointestinal stromal tumors, paragangliomas, and renal cell carcinomas,[140] which suggests that aberrant metabolic properties of cancer cells may be not only adaptive but in and of themselves tumorigenic.

CONCLUSIONS AND FUTURE OUTLOOK

The study of the genetic basis of pediatric cancers has revealed important principles of human carcinogenesis that help explain the diverse traits of cancer cells. In particular, the study of human cancer predisposition syndromes, many of which clinically present in childhood, has established the prevalence of mutations of tumor suppressor genes and oncogenes that cause the dysregulation of cell growth, DNA repair, and gene expression in human cancers. These studies have also established the essential contributions of the cellular state on the effects of tumorigenic mutations, with the same mutations having distinct functional consequences and phenotypic effects that depend on the cancer cell of origin. Likewise, the increasing recognition of epigenetic alterations in human cancers may reveal fundamental developmental pathways that are required for normal and cancerous cell growth. Finally, because tumorigenesis is inherently a multistep process, the accumulation of genetic lesions that ultimately lead to symptomatic disease is nonrandom, establishing discrete combinations of molecular lesions that functionally cooperate to transform cells. As a result, the clonal structure of tumors is organized hierarchically, and its future study will reveal important principles of biologic cell function and dysfunction. This notion is further bolstered by the frequent observation of somatically acquired gene mutations that are rare when considered individually but involve coherent biologic pathways that are mutated frequently as a functional group. Consequently, future studies of the biologic basis of human cancer that are faithful and therapeutically relevant may necessitate the generation of compendia of molecular lesions and cancer models based on such ontogenies.

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CHAPTER 44

Targeted Therapies in Oncology

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CHAPTER OUTLINE

TRADITIONAL CHEMOTHERAPY
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Lessons Learned from Cytotoxic Chemotherapy
Shortcomings of Cytotoxic Chemotherapy
INTRODUCTION TO TARGETED THERAPY
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Advantages of Targeted Therapy
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Classes of Targeted Therapies
Targeted Therapy: Lessons Learned from Adult Oncology
NEW AND EMERGING OPPORTUNITIES IN PEDIATRIC CANCER
Special Considerations in Targeted Therapy Development for Children
Emerging Genetic Targets in Pediatric Cancer
Emerging Lineage-Dependent Targets in Pediatric Cancer
Emerging Synthetic Lethal or Empiric Track: Targets in Pediatric Cancer
CONCLUSIONS

With the discovery of nitrogen mustard as an anticancer agent in the 1940s, medicinal and hence systemic cancer therapy became a reality. The identification and application of cytotoxic agents dominated the next 60 years of cancer treatment. The success of imatinib for *BCR-ABL* rearranged chronic myelogenous leukemia (CML), the dramatic responses seen in patients with *BRAF*-mutated melanoma and *ALK* rearranged non–small cell lung cancer to targeted inhibitors, and the unparalleled ability to systematically characterize cancer genomes have catapulted the community into a new era of targeted cancer therapy. Nonetheless, there is much to learn from the 60-year experience in empiric-based therapy that should be directly applicable to the development of molecularly targeted therapy. Cytotoxic chemotherapy, although often regarded as largely unsuccessful, has led to substantial cure rates in a number of well-defined malignancies, particularly pediatric. In this chapter we will discuss the fundamental concepts, advantages, and disadvantages of cytotoxic cancer therapy and their relevance to targeted drug therapy for malignancy. We will define targeted therapy, its advantages and disadvantages, discuss the credentialing of targets, and provide a system for categorizing classes of targets and classes of therapies. We will then focus on particular lessons learned in the early development and testing of new targeted therapies for adult malignancies, with examples from each of the target classes. Our intent is to highlight illustrative examples rather than to provide an exhaustive list of all targeted therapies in development. We will conclude with a discussion of the challenges facing us in the development of targeted therapies for children and highlight emerging pediatric cancer-based targets.

TRADITIONAL CHEMOTHERAPY

Conventional Chemotherapy: Not Entirely Nonspecific

Although conventional chemotherapy agents are often seen as nonspecific cytotoxic drugs, over the years more information has come to light about their mechanisms of action in killing tumor cells. In general, these agents were designed to interfere with molecular pathways required for nucleotide synthesis, DNA replication, and the mechanics of cell division. Thus these agents have significant but often predictable, or at least stereotypical, morbidity related to target-mediated suppression of hematopoiesis or of the cell-division process required for homeostasis of other organ systems. These agents are discussed in greater detail in Chapter 45.

Lessons Learned from Cytotoxic Chemotherapy

The use of cytotoxic chemotherapy agents is an imperfect science at best, but researchers have learned many lessons over the years about the medicinal treatment of cancer,

Box 44-1 Lessons Learned from Cytotoxic Chemotherapy

1. Combination therapy is required for cure.
2. Appropriate dose intensity is required for improved outcome.
3. Toxicity is acceptable when therapy is curative.
4. Establish curative regimens, and then attempt to reduce toxicity.

lessons to strongly consider in the development of targeted therapy (Box 44-1).

Combination Therapy Is Required for Cure

The goals of combination therapy are numerous. First, because it is difficult to predict which single agent will be most effective against an individual patient's tumor, the use of multiple drugs increases the potential for an initial complete response. Second, combination therapy is likely critical for preventing the development of therapeutic resistance. Third, drug combinations may have additive or synergistic interactions, increasing the total activity of the drugs and potentially enabling lower dosing and reduced toxicity. One example is the successful dose reduction of cytotoxic chemotherapy with the addition of all-*trans*-retinoic acid (ATRA) for the treatment of patients with acute promyelocytic leukemia (APL).[1,2] With few exceptions, curative systemic therapy has required the combination of highly active agents rather than combinations of active and inactive agents.

The need for multiagent therapy was first appreciated in the treatment of acute lymphoblastic leukemia (ALL). At best, 60% of patients are estimated to achieve complete remission with single-agent chemotherapy. Despite continued use, almost all patients will relapse within 6 to 9 months with single-agent therapy. However, complete remission rates exceed 95% in patients treated with combination chemotherapy, and cure rates today for children with ALL exceed 80% with multiagent treatment regimens.[3,4] Similarly, patients with APL had very poor remission and cure rates until the discovery of ATRA differentiation therapy for this disease. ATRA achieved a high complete remission rate in use as a single agent, but virtually all patients ultimately relapsed.[5,6] It was not until the combination of cytotoxic chemotherapy with ATRA and the recent combination of ATRA with arsenic trioxide that these patients obtained the best long-term survival rates of any acute myeloblastic leukemia (AML) subtype.[1,7]

Appropriate Dose Intensity Is Required for Improved Outcome

A guiding principle in the delivery of cytotoxic chemotherapeutic agents is the concept of the maximum tolerated dose (MTD): the simple idea that more is better. Drugs are delivered at the maximum dose possible, a dose greater than which unacceptable toxicity is encountered. Here, without a clear cellular biomarker of cytotoxic activity, it is not possible to relate the maximum molecular effect to maximum achievable efficacy. Thus cytotoxic drugs are first evaluated in phase I studies with the primary study goal of establishing the MTD. Subsequent phase II and III trials are then based on this dosing schedule. MTD assumes that some toxicity is acceptable and, in fact, expected in the pursuit of cure. The most extreme example of this concept has been the use of autologous stem cell transplantation to deliver what otherwise would be lethal doses of chemotherapy. In this case the MTD is surpassed by replacing the dose-limiting factor, the hematopoietic system, with stem cells harvested from the patient before delivery of the conditioning chemotherapy. While clearly a brute force approach, the MTD paradigm also established an important corollary: significant side effects can be tolerated when therapy is administered with curative intent (discussed later).

Many retrospective studies of chemotherapy dose intensity and outcome support this premise, particularly in the pediatric setting. In high-stage neuroblastoma the addition of dose intensification with stem cell transplantation has improved the outcome for patients with metastatic disease.[8-11] In pediatric ALL several studies suggest that dose intensification is important for long-term outcome, including one randomized study comparing standard versus dose-reduced methotrexate with mercaptopurine.[12-15] Moreover, in Ewing sarcoma chemotherapy intensification through interval compression in a randomized trial was also associated with an improved outcome in patients with localized disease.[16] However, fewer prospective studies than retrospective studies have been performed to address the issue of dose intensity and outcome for pediatric tumors. Significantly, while these studies begin to emerge, some previous assumptions about MTD have come into question. For example, retrospective studies support the notion that doxorubicin or methotrexate dose intensity is important for outcome in patients with localized high-grade osteosarcoma.[17,18] However, prospective studies involving large patient numbers have called into question the role of further dose intensification for this disease.[19,20] Furthermore, randomized trials for malignancies such as breast cancer have demonstrated that massively increasing dose with stem cell transplantion does not always improve long-term survival.[21,22] Well-controlled prospective trials are critical to address the issue of dose intensification, even for cytotoxic agents.

Toxicity Is Acceptable When Therapy Is Curative

The oncology community has accepted toxicity as inherent in the mission of cure. With the implementation of dose-intensive regimens, however, comes additional toxicity. Because of the relatively nonspecific nature of traditional chemotherapy, morbidity from these treatment regimens is significant. Acute toxicity takes the form of infection, transfusion dependence, renal injury, and hepatic injury. There are also potential long-term consequences such as cardiac injury, infertility, chronic renal insufficiency, hearing loss, skeletal injury, and secondary malignancies.[23] Ongoing efforts are focused on decreasing these short- and long-term side effects. For example, to minimize acute toxicity, chemotherapy dosing is usually individualized on the basis of body surface area (mg/m^2). For some cytotoxic agents, such as methotrexate and busulfan, drug levels can be monitored to minimize toxicity. Dose intensity can also be achieved with regional tumor delivery, growth factor support, or rescue with stem cells. Furthermore, better supportive care, such as infection prophylaxis, has enabled tolerance of prolonged immunosuppression. To decrease long-term morbidity, "safe" maximum cumulative doses are being established, such as maximum cumulative doxorubicin to minimize late cardiac effects. Although toxicity can be tolerated, it is essential that the level of toxicity be commensurate with the level of benefit. Marginally effective therapeutic regimens that result in substantial toxicity should not become accepted standards.

Establish Curative Regimens and Attempt To Reduce Toxicity

Treatment strategies for most of the pediatric malignancies, including ALL and the solid tumors, have used disease stratification to decrease dose in treatment regimens. A dose-intensive regimen is initially used to establish efficacy. Then, patients with a good prognosis are treated with less intensive regimens, with the goal of comparable disease-free outcomes but decreased treatment-related morbidity. This is particularly important for pediatric disease, in which the long-term toxicity of treatment is experienced over a lifetime. Historically, these stratifications were based on factors such as age, stage, and histology, but now they include molecular genetic markers, such as *MYCN* amplification in neuroblastoma, and *MLL* rearrangement in pediatric ALL. For example, the National Wilms'Tumor Study evaluated a half-dose chemotherapy regimen for infants with low-risk disease in an attempt to decrease the incidence of toxic deaths without compromising long-term survival. These infants had acceptable toxicity with no toxic deaths, and long-term survival was not compromised.[24] A similar approach has been taken with infants and young children with low-stage neuroblastoma,[25,26] and therapy for children with ALL has been dose-reduced based on rapid clearance of minimal residual disease.[27]

While our understanding of the molecular basis of malignancy improves, so will our ability to develop fine-grained prognostic categories and patient-tailored treatment regimens, perhaps even for traditional chemotherapy agents. While we implement targeted therapy, it is expected that disease stratification and patient selection will be based on molecular genetic findings related to the credentialed target. In the clinical development of targeted therapies, we must continue to consider these key concepts: combination therapy, appropriate dose intensification, tolerance of toxicity for substantial efficacy and cure, and treatment adjustment within established favorable prognostic groups.

Shortcomings of Cytotoxic Chemotherapy

Although clinicians have made great progress with the cytotoxic armamentarium, further strides in the treatment of cancer are limited with this approach alone. Several shortcomings of this approach are intimately related to the lack of specific target knowledge (Box 44-2).

1. Toxicity is significant.
2. Optimization of anticancer effect versus side effects is difficult.
3. Mechanistic understanding of nonresponding and resistant disease is lacking.
4. Predictors of patient response to a specific drug are poor.
5. To date cytotoxics have explored a narrow range of cellular and molecular mechanisms.

Box 44-2 Shortcomings of Cytotoxic Chemotherapy

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3. Mechanistic understanding of nonresponding and resistant disease is lacking.
4. Predictors of patient response to a specific drug are poor.
5. Cytotoxics have explored a narrow range of cellular and molecular mechanisms.

One major shortcoming to cytotoxic therapy is morbidity. Because these agents are relatively nonspecific, there is significant toxicity to normal host cells. Patients can expect to experience short- and long-term morbidity from these drugs and even incur the risk of therapy-related fatality. Furthermore, because the most relevant targets of these drugs are poorly understood, it is difficult to optimize the anticancer effect versus the toxicity, and it is difficult to separate on-target from off-target toxicities. Often, there is no relationship between the administered dose (pharmacokinetics) or the MTD and inhibition of the putative target (pharmacodynamics). Developing a strong correlation between the pharmacokinetics of a drug and its pharmacodynamic effect is obviously impossible when the target of a drug is unknown. Similarly, in the absence of target knowledge, our understanding of resistant or refractory disease is poor at best, and it is very difficult to predict responders a priori. This has necessitated long clinical development programs with large trials to identify responsive subpopulations of patients and then follow-up trials to more thoroughly investigate response in this patient subset. Targeted therapy has the potential to address these shortcomings, to enable more rational tumor-specific therapy with less morbidity risk, and to explore previously unexploited cancer-related molecular mechanisms. With the sequencing of the human genome, the development of high-throughput genomic technologies, and an ever-increasing understanding of the molecular pathogenesis of cancer, this promise is likely to be realized.

INTRODUCTION TO TARGETED THERAPY

What Is Targeted Therapy?

Although we have made great strides with the cytotoxic armamentarium, further strides in the treatment of cancer are limited with this approach alone. We still fail to cure many patients with this approach, and several shortcomings of this approach are closely related to the lack of specific target knowledge (see Box 44-2). Targeted therapy, defined as a therapeutic procedure whose preclinical development focused on modulation of a single molecular target (typically inhibition but occasionally activation of the molecular entity), has the potential to address these shortcomings. Targeted therapy relies on the concept of cancer cell dependence—that is, the malignant cell or tumor as a whole depends for its survival or proliferation on the particular gene product or pathway. The discovery of targeted therapy differs significantly from that of cytotoxics. Historically, cytotoxic therapy discovery relied on simple, phenotypic cell death assays in which small-molecule libraries were screened in vitro against a panel of cancer cell lines. Because cytotoxic compounds are believed to cause nonspecific cell death, effective compounds should, in principle, have efficacy for all cancer types. In practice, however, this has not proved to be the case. Thus although phenotypic screens may be of significant value in identifying molecules with complex mechanisms of action, the direct prediction of clinical efficacy from limited in vitro testing remains problematic. This is in sharp contrast to the concept of targeted therapy.

There are three critical elements to the implementation of targeted therapy. First, on the basis of available data, a hypothesis is generated suggesting a causal or pathogenic link between a specific protein or gene (the potential drug target) and cancer. Experiments are then performed and data generated to test the validity of this hypothesis. Is this candidate target relevant to oncogenesis or the maintenance of the malignant phenotype? Second, there must be a valid therapeutic approach to interfering with the target activity through small molecules, antibodies, or protein- or cell-based biologics. Third, the clinician must estimate the likely impact of target manipulation in a relevant clinical application. For example, a targeted therapeutic may have limited clinical application if the disease state is already readily cured. The process of evaluating potential targets that emerge is target "validation."

Advantages of Targeted Therapy

Targeted therapy offers several potential advantages over nonspecific cytotoxic treatments (Box 44-3).

1. Improved patient selection is enabled.
2. Drug efficacy and target inhibition are linked, enabling rational dosing.
3. Side effects are more predictable.
4. Understanding of target and pathway increases options for the subsequent development of therapeutics with improved efficacy.
5. The mechanisms of resistance may, in some cases, be more readily discovered and interdicted with second-generation therapeutics or specific combinations.
6. Rational pathway-based combination regimens can be tested.

By way of example, hormonal therapy has become standard of care for patients with estrogen receptor (ER)–positive breast cancer. In this case there is a lineage-dependent target in the malignant cell, the ER, and antiestrogen agents, such as tamoxifen, have been developed to inhibit competitive binding of estrogen to the ER. Numerous studies exploring estrogen antagonist therapy with tamoxifen for patients with ER-positive versus ER-negative disease demonstrate benefit only in the ER-positive patients.[28-31] In conjunction with diagnostic tools to identify hormonal receptor status, patients are now rationally chosen for therapy, thus eliminating exposure to drug toxicity for patients not likely to respond.

A second advantage of targeted therapy is the strong link between the target inhibition and efficacy. The dosing goal is achievement of complete target inhibition, not MTD. Thus there is no need to suffer undue toxicity in the achievement of an MTD. In fact, studies have shown that tamoxifen treatment responses do not correlate with serum levels and that doses greater than 20 mg daily do not increase efficacy compared with that of this dose.[32] A related third advantage of targeted therapy is that the side effects can generally be anticipated and serve as a marker for on-target inhibition. For example, the side effects of tamoxifen are predominantly related to the antiestrogen effects of this drug: vaginal dryness, hot flashes, and irregular menses. Because this drug also has proestrogen effects, thromboses and endometrial cancer can be seen.

A fourth advantage of targeted therapy is that understanding the target and the pathway increases the possibilities for therapeutic design. In the case of modifying ER signaling, there are several methods to accomplish this other than direct ER antagonism. One method is to decrease ovarian production of estrogen with luteinizing hormone–releasing factor agonists, which induce a medical ovarian ablation. A second method of interfering with ER signaling is to decrease estrogen production through the inhibition of aromatase activity. Aromatase is the final enzyme in estrogen synthesis converting the androgens androstenedione and testosterone to the estrogens estrone and estradiol. Aromatase inhibitors work by decreasing this conversion and thus decreasing circulating estrogens. Aromatase inhibitors have improved efficacy over tamoxifen and have the advantage of fewer side effects in terms of hot flashes, endometrial disease, and ischemic cerebrovascular effects.[33,34] Because of the clear dependence of some breast cancers on ER and detailed knowledge regarding estrogen biosynthesis and signaling, multiple agents could be developed to inhibit this pathway.

The fifth advantage of targeted therapeutics is the ability to more readily understand the molecular mechanisms associated with resistance. The success of targeted inhibition of BCR-ABL in CML and of epidermal growth factor receptor (EGFR) in lung cancer was rapidly followed by the specific elucidation of direct resistance

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mutations in the target leading to decreased activity of the relevant small-molecule inhibitors, imatinib and erlotinib or gefitinib, respectively.[35,36] In the case of CML, new agents were developed in short order to treat resistance in patients by more efficient inhibition of the mutant BCR-ABL proteins.[37]

The final advantage of targeted therapies may be the ability to generate rational and novel combination regimens. Currently, the vast majority of combination strategies derive from combining a novel agent with components of existing standards of care without regard to mechanism. This strategy is largely based on clinical expedience, existing clinical efficacy (for the standard of care) and tolerability. The difficulty of empirically discovering new synergistic combinations stems from the large number of permutations required to test all possible combinations against multiple in vitro and in vivo models or multiple cancer types. Targeted therapeutics are often related to one another in the context of a pathway. This allows testing of specific pathway-related hypotheses. For example, combinations might target upstream and downstream components of the same pathway to defeat feedback mechanisms, target components of parallel pathways to defeat pathway redundancy, or target distinct antiproliferative and prosurvival pathways.

Classes of Targets

Much of current cancer therapy development focuses on specific changes that alter the malignant cell, setting it apart from its normal counterpart and creating distinct molecular or pathway dependence for cell survival and replication. These may be genetic mutations, gene rearrangements, epigenetic modifications such as altered patterns of methylation or acetylation, lineage legacies, or other metabolic liabilities. Genes whose expression and activation have been increased, resulting in a cancer-dependent state, are prime targets for therapy development. On the basis of cancer dependencies, cancer targets can be categorized in four classes: genetic, lineage, host, and synthetic lethal or empiric (Box 44-4).[38] Although some targets may cross over from one class to another, this classification may be useful in the design of clinical trials and elucidation of the parallel biomarker strategy.

Genetics Track

The genetics track relies on the idea that a cancer develops from a finite number of genetic alterations, that these alterations give the malignant cell a selective advantage, and that these genetic changes are selected for during malignant cell replication. Hence the malignant cell becomes dependent on a set of these changes for continued survival, a process sometimes referred to as *oncogene addiction*.[39,40] This cancer cell dependence or addiction should provide a window for drug specificity for the malignant cell compared with normal cells. Targeted therapeutics taking advantage of genetic abnormalities should, in theory, be more injurious to the malignant cell than to the normal cellular counterpart. Advances in cytogenetic studies, high-throughput gene sequencing, detection of copy number alteration, whole genome expression profiling, and proteomic approaches have revolutionized candidate genetic target discovery. Now, tens of thousands of genes in hundreds of tumor samples can be analyzed simultaneously. These candidate targets must then be carefully credentialed for their suitability as a drug target. Is there cancer cell dependence on the genetic abnormality, and can the target be pharmacologically manipulated? Imatinib is one of a number of examples of clinically validated targeted therapeutics exploiting a genetic dependence, in this case the dependence of CML on the activity of the Abelson kinase (ABL). The very existence of the cancer-dependent state is dramatically illustrated by the emergence of resistance mechanisms that largely appear to restore Abelson activity (see further discussion later in this chapter).

Box 44-4 Classes of Targets

1. Genetics track
2. Lineage track
3. Host track
4. Synthetic lethal or empiric track

Lineage Track

Cancer cells often maintain developmental features of the lineage from which they were derived. This is well supported with gene expression studies that report tumor cells more closely resembling the normal lineage counterpart than tumors of a different cell type. The notion that lineage or "legacy" features of a tumor cell may be exploited with targeted therapy is known as *lineage addiction*.[41] One of the earliest examples of the success of this approach has been the antagonism of ER and its signaling pathway in patients with ER-positive breast cancer. Similarly, the vast majority of patients with prostate cancer derive significant benefit from antagonism of the androgen receptor (AR). In both ER-positive breast cancer and in prostate cancer (virtually all forms of which express AR), the emergence of hormone-refractory disease largely appears to rely on mechanisms that restore steroid receptor signaling.[42] Thus as in the case of imatinib resistance, the dependent state is illustrated by these resistance mechanisms. In many cases lineage dependence may also derive from the normal hierarchy of differentiation beginning with the stem cell.

Host Track

The importance of tumor cell environment to the development and maintenance of the malignant state has become increasingly recognized and represents another potential inroad to targeted therapy. It is possible that tumors, by evolving in a particular environmental context, may become dependent on certain growth factors or neighboring cells for survival in that niche. Preclinical evidence suggests that many tumors require de novo blood vessel development for their growth and maintenance. The identification of these angiogenesis factors has enabled the development of modifiers of the angiogenesis process, leading to the successful clinical application of bevacizumab, an anti–vascular endothelial growth factor

(VEGF) antibody, in patients with colorectal cancer and the approval of kinase insert dependent receptor inhibitors (sunitinib and sorafenib) in renal cell carcinoma (see further discussion later in this chapter). The interaction of tumors with the bone microenvironment has also been targeted using inhibitors of osteoclastogenesis. Here, multiple bisphosphonate agents are approved for the prevention of cancer-related fractures.[43]

Synthetic Lethal or Empiric Track

A number of highly efficacious anticancer agents do not conveniently map to the first three categories. Admittedly, we can say that the understanding of the mechanisms of efficacy of such agents remains opaque. Nonetheless, it is quite likely that such agents, including many of the cytotoxic agents described previously, take advantage of aspects of cancer cell biology that remain unknown but may be broadly termed as *synthetic lethal interactions*. Synthetic lethal genetic interactions are defined as mutations that are lethal in combination but that do not produce a lethal phenotype as a single event. In a similar way, gain-of-fitness alterations in a malignant cell, which give the cell a survival advantage, can sensitize the cell to other stresses that have no consequence in a normal state but may be lethal with the malignancy promoting alteration. Thus cancer-promoting mutations give the malignant cell a survival advantage, but they may also incur a distinct liability that can be exploited therapeutically. Rather than two synthetic lethal genetic events, the clinician can also apply such synthetic lethal concepts to a preceding cancer-related molecular alteration followed by the application of a therapeutic. For example, a striking synthetic lethal relationship was discovered between loss of BRCA1 or BRCA2 function and inhibition of the poly(ADP-ribose) polymerase I enzyme (PARP). Here, loss of PARP1 was found to induce double-strand DNA breaks that required the recruitment of the RAD51 homologous recombination repair complex to the site of these breaks for effective repair. Because it was known that BRCA1 and 2 were required for RAD51 recruitment to such complexes, it was hypothesized that loss of BRCA1 or BRCA2 would create sensitivity to PARP inhibition. This was demonstrated both with siRNA and small-molecule inhibitors of PARP.[44] Moreover, clinical activity of PARP inhibitors has now been seen in patients with *BRCA1* and *BRCA2* mutations with ovarian or breast cancer.[45-48]

Credentialing Targets

By the strictest of measures, a drug target is not validated until a selective inhibitor of the target of interest has proven clinical activity *and* when resistance emerges through specific alterations in the direct target. Although very few cancer targets have achieved this degree of validation, we can envision preclinical experimental methods that will allow the researcher to credential a target for further drug discovery efforts. In this effort, the goals are to build data sets that either prove or disprove certain target-based hypotheses and to use such data sets to prioritize target selection for future drug discovery efforts.

Box 44-5 **Credentialing Targets**

Target epidemiology: study of a target in human cancers
  Target expression
  Genetic alteration
    - Gain-of-function
      - Translocation
      - Activating point mutations
      - Copy number gain
    - Loss-of-function
      - Translocation
      - Inactivating point mutations
      - Deletion
        a. Homozygous
        b. Heterozygous
        c. Loss of heterozygosity
  Protein Epidemiology
Functional validation: study of a target in a model system of tumor biology
  Gain-of-function: address sufficiency of target
  Loss-of-function: address necessity of target

We can consider three key questions regarding the relevance of any given target to anticancer therapy:

1. Is the activity of the target required for the development and maintenance of the cancer-dependent state?
2. Is it possible to identify a strategy for developing a therapeutic for the target of interest?
3. Is there a clinical application for the eventual therapeutic?

In the subsequent sections we will focus on the approaches relevant to addressing the role of a given target in cancer dependence. In this specific regard there are two general sources of information: the study of a target in human cancers (target epidemiology) and the functional study of a target in model systems of tumor biology (functional validation) (Box 44-5).

Target Epidemiology

Preclinical models of cancer are limited in both number and in their qualitative relationship to human cancers. Comprehensive collection of human tumor specimens could, in theory, be used to study a given target in a large array of samples broadly covering tissue distribution and stage distribution (primary versus metastatic sites). In practice, however, there are limited sets of comprehensive tumor collections, and generally the largest tumor-type specific collections are limited to fixed, paraffin-embedded specimens. The availability of tissue samples paired with robust clinical data remains limited. Furthermore, the vast majority of human cancer collections come from surgical resection samples, which do not necessarily reflect the disease state in which a therapeutic will be initially tested. Despite these limitations, molecular epidemiology studies in tissue samples, enabled by tissue microarrays, single nucleotide polymorphism (SNP) arrays, expression microarrays, reverse protein arrays, and next-generation sequencing technologies remain critical to building a case for the cancer relevance of a given target.

Target Expression. The desire to discover genes "selectively" expressed in cancer led first to the development of differential expression methods, including the subtractive hybridization cDNA libraries, differential display, and serial analysis of gene expression (SAGE) as examples.[49,50] Following the elucidation of the human genome sequence and the development of DNA-based microarrays, comparative expression profiling largely replaced difference methods, and more recently, RNA sequencing has become a commonly used tool. In all cases the basic premise has been to identify genes overexpressed in cancer with the assumption that overexpression will equate with increased sensitivity to target inhibition and hence serve to improve the therapeutic index. This basic idea, however, must be challenged and carefully considered for each target, for the simple reason that resistance to therapeutics can likewise be engendered through overexpression. Indeed, on the basis of standard biochemical principles, increased target expression should necessitate a higher drug concentration to achieve target inhibition. This is clearly the case in the amplification of dihydrofolate reductase (DHFR) in generating resistance to methotrexate.[51] Likewise, data suggest that increased expression of AR can lead to resistance to AR blockade.[42] Thus caution is required before proceeding with target selection based solely on expression data in the absence of a demonstrated causal or functional link to the maintenance of a cancer state.

Genetic Alterations. As illustrated by the success of ATRA, trastuzumab, and imatinib, direct genetic alteration leading to gene activation of a given drug target remains perhaps the best evidence for the causal role of a given gene in carcinogenesis. Genetic alterations that are highly frequent in a given tumor type and are present in the majority of cells are a strong indicator of causality. In the era of large-scale resequencing projects, it is critical that one distinguishes "driver" mutations (causal mutations) from so-called passenger mutations.[52] In addition, driver mutations that arise late in the course of disease, are present in only a minor fraction of the tumors, or are present in only a fraction of tumor cells in a given single tumor must be given less weight. These findings would suggest a reduced likelihood of this lesion possessing a strong causal link to the initiation of the disease state, although they may pose an eventual mechanism of drug resistance. The types of genetic alterations (e.g., point mutations, focal amplifications, broad amplifications, and translocations) are also distinguished with respect to the extent to which they can be used to establish causality or to infer dependence.

Gain-of-Function Genetic Alterations. Gain-of-function genetic alterations include translocations, activating point mutations, and increases in gene copy number. Translocations were among the first discovered genetic alterations initially described using chromosome spreads. Approaches to the discovery of chromosomal rearrangements include exon microarrays, which can potentially detect imbalanced or amplified translocations, cancer outlier profile analysis (COPA), paired-end resequencing of BAC clones from a genomic library (giving a structural map of a cancer genome),[53] and newer methods of single-molecule sequencing (discussed later). Translocations are highly informative with respect to causality because they typically alter only two genes, and because background or random translocation events are relatively rare, with chromothripsis (massive DNA rearrangements affecting one or a few chromosomes that occur as a result of one catastrophic event) as a notable exception.[54]

Amplifications are now routinely detected by methods of DNA copy number analyses: comparative genomic hybridization (CGH) array, high-density SNP arrays, and whole exome or whole genome sequencing. There are two general types of increases in gene copy: focal amplifications and broad copy number increases covering an entire chromosome or chromosome arm. With sufficient sample numbers it is possible to discern one to several genes contained within a focal amplicon. Typically, the minimal region of amplification is a guide. However, methods for detecting copy number alterations (e.g., genomic identification of significant targets in cancer [GISTIC]) also use the amplitude of amplification as an important parameter.[55] Although it is not definitive proof, amplification lends evidence to a causal relationship between a given gene within an amplicon and a cancer state. In some amplicons, multiple genes remain strong candidates for oncogenes targeted by the amplification. One notable example is the 11q13 amplification with genes including *Cyclin D1*, *FGF3*, and *FGF4*.[56]

Larger-scale chromosomal alterations leading to trisomy or gain of an entire chromosome arm are frequent yet are far more difficult to study. As such, identifying single genes (or drug targets) within such broad regions remains a challenge. In this case the number of genes implicated in such large-scale alterations is so large that almost no importance can be ascribed to the cancer relevance of a gene contained within such broad chromosomal-level alterations. Outlier analysis may begin to allow the elucidation of multigene targeting by such broad regions and is illustrated by the analysis of trisomy 7 in glioma revealing a dependence on cMET and its ligand HGF, both located on chromosome 7.[57]

Subtle nucleic acid level alterations, including point mutations and small insertions and deletions, are an important mechanism for the activation of oncogenes. Point mutations leading to the constitutive guanosine triphosphate (GTP)–bound and hence activated form of N-RAS and K-RAS,[58] as well as genetic events leading to activation of the BRAF kinase in melanoma, thyroid cancer, colorectal cancer,[59] pediatric pilocytic astrocytoma,[60-62] and Langerhans cell histiocytosis[63] are exemplars of this mechanism. Large-scale sequencing projects that unveiled new mutations activating oncogenes such as *EGFR*[36] and *PIK3CA*[64] have led to national and international cancer genome sequencing projects. From these studies it has become apparent that the detection of passenger mutations, constituting the background rate of mutation, can pose a significant challenge. These passenger mutations must be separated from the more prevalent and recurrent driver mutations.[52] Such passenger mutations may be exceedingly high in certain types of tumors, particularly those bearing mutations in mismatch repair genes. Indeed, in one case inactivating mutations in the mismatch repair gene *MSH6* were discovered in glioma tumors recurring after treatment with temozolomide. Here the sequencing of *MSH6* was triggered by the very high rate of observed passenger mutations in treated versus untreated tumors.[65,66] These data suggest that cautious interpretation of low-frequency events is warranted when considering mutation analysis from larger-scale modalities. A framework for context-specific mutation analysis has been provided by a systematic study of mutation data across many cancer types in The Cancer Genome Atlas (TCGA) project and others, and these studies are revealing remarkably simple genomes in many pediatric cancers (Fig. 44-1).[67]

Loss-of-Function Genetic Alterations. Loss-of-function alterations do not typically directly indicate or point to a specific gene as a candidate drug target. However, such alterations may predict sensitivity to agents acting against protein targets contained within downstream or parallel pathways. For example, clinical trials are under way testing inhibitors of mammalian target of rapamycin (mTOR) function against sporadic tumors or hereditable cancer predisposition syndromes resulting from loss of the tumor suppressors PTEN and TSC1 or TSC2. In both cases loss-of-function mutations in these tumor-suppressor genes are thought to lead to constitutive activation and hence constitutive dependence on mTOR (discussed later in this chapter).

Similarly, in the hedgehog pathway, a key development pathway regulating cell patterning, deletions or mutations in the gene encoding the Patched receptor (PTCH) are associated with hereditary and sporadic basal cell carcinomas and sporadic medulloblastoma.[68-70] Loss of PTCH, a receptor for the ligands in the hedgehog family, leads to constitutive activation of the atypical G protein–coupled receptor (GPCR)–like protein Smoothened (Smo).[71] Cyclopamine, a naturally derived inhibitor of Smo, and other, newer small-molecule inhibitors, have had significant activity in preclinical models lacking *PTCH* gene function[72] and in clinical trials.[73,74] These examples illustrate that identification of loss-of-function mutations can reveal downstream target molecules in critical cancer pathways.

Translocations are typically thought of as gain-of-function genetic events in that they act dominantly. However, they can functionally inactivate endogenous genes through the creation of dominant-negative protein fusions. For example, the PML-RARα fusion protein is believed to act, at least in part, as a dominant-negative inhibitor of endogenous RARα function.[75] This type of molecular action can be difficult to elucidate in functional experiments. Nonetheless, it is worth remembering this possibility when evaluating proteins or genes as drug targets when they are involved in new or existing translocations.

Chromosomal-level alterations leading to loss-of-function events include homozygous deletions, heterozygous deletions, and loss of heterozygosity. Homozygous deletions are typically highly focal in their nature. When the boundaries are defined with large sample sets, in

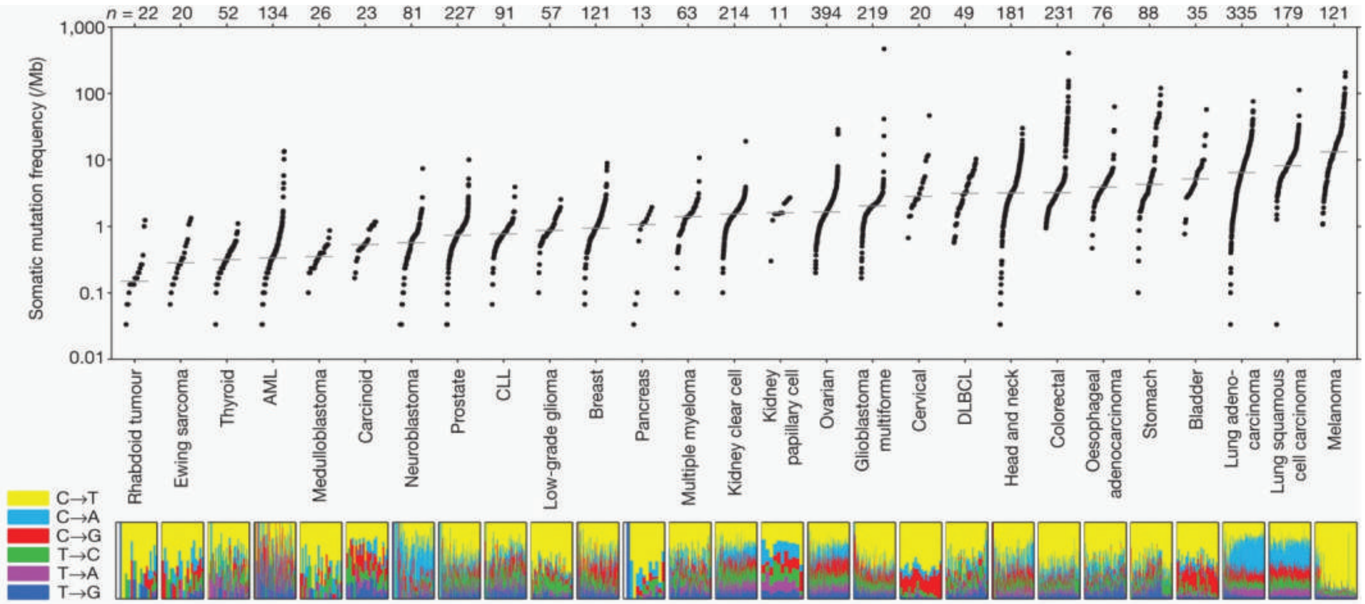

Figure 44-1 Somatic mutation frequencies observed in exomes from 3083 tumor-normal pairs. Each *dot* corresponds to a tumor-normal pair, with vertical position indicating the total frequency of somatic mutations in the exome. Tumor types are ordered by their median somatic mutation frequency, with the lowest frequencies *(left)* found in pediatric tumors and hematologic malignancies, and the highest *(right)* in tumors induced by carcinogens such as tobacco smoke and ultraviolet light. Mutation frequencies vary more than 1000-fold between lowest and highest across different cancers and also within several tumor types. The *bottom panel* shows the relative proportions of the six different possible base-pair substitutions, as indicated in the label on the left. *AML,* Acute myeloblastic leukemia; *CLL,* chronic lymphocytic leukemia; *DLBCL,* diffuse large B-cell lymphoma. *(Modified from Lawrence, et al: Mutational heterogeneity in cancer and the search for new cancer-associated genes.* Nature *499:214–218, 2013.)*

many cases, only a few genes are found in the overlapping region. For example, in the case of focal deletions on 9p, the typical minimal deleted regions in tumors such as melanoma contain only *CDKN2A* and *CDKN2B*.[76] Heterozygous deletions are typically larger and usually involve entire chromosomes or chromosome arms. As in the case of amplifications, such large-scale broad genetic alterations make it difficult to ascertain the cancer relevance of any single gene contained within a larger region. Loss of heterozygosity (LOH) can occur as an event associated with heterozygous deletion leaving only one parental chromosome behind, which is thus homozygous. LOH can also occur when duplication of the remaining parental chromosome creates a situation known as *uniparental disomy.* This is significant because it may indicate preservation of a mutated allele, leading to two mutant alleles of a given gene. In the case of the tumor suppressor gene *p53*, copy-neutral LOH is common.

Inactivating point mutations are a common means of gene inactivation. In this case stop codons are the most efficient mechanism for disrupting gene function. As a result, tumor suppressor gene mutations often are enriched for stop or truncating mutations. This creates certain technical and cost difficulties in sequencing genes for inactivating mutations because it is necessary to sequence all coding exons, consider sequencing of the regulatory elements governing splicing, and consider the detection of promoter mutations. As a result of these difficulties, precise clinical analysis of tumor suppressor gene mutations remains challenging even with the application of next-generation sequencing technologies.

Protein Molecular Epidemiology by Immunohistochemistry. It is important to understand the relationship between the expression or activity of a gene product and patient outcome or other clinicopathologic data. Although establishing a relationship between protein expression and clinical data (e.g., survival) does not provide a causal link to cancer, such linkage to patient outcome likely indicates a nonrandom association with cancer behavior. Here the clinician should be careful to consider the relationship between protein expression and the state of differentiation of the cancer. Because the degree of cancer differentiation state (usually referred to as *grade*) is typically already linked to patient prognosis, candidate drug targets whose protein levels vary with differentiation state may be secondarily linked to patient outcome. Thus an independent association between a protein's expression and patient outcome should be sought in multivariate analysis. In the case of the targeted disruption of HER2 in patients with breast cancer, the association of *HER2* amplification with poor outcome provided a significant motivation for the development of trastuzumab, an anti-HER2 monoclonal antibody.[77]

The ability to perform larger-scale epidemiology analyses typically relies on archived tissue for which longer-term clinical follow-up is available. This largely dictates that target epidemiology be carried out in paraffin-embedded, fixed tissue. Here RNA and DNA can be examined by in situ hybridization (ISH), fluorescence in situ hybridization (FISH), or next-generation sequencing approaches. Historically, the most common method for interrogating human tissue samples is protein-based immunohistochemistry (IHC). These studies have been made higher throughput by the advent of tissue microarrays. Such arrays align hundreds of tumor samples on a single slide, facilitating the experimental procedures. IHC is primarily limited by the quality of the antibody reagents that are used and the care with which such reagents are subject to rigorous validation. Too often, poorly described or unvalidated antibodies are used in IHC studies after a quality assessment based solely on the observed staining pattern in tissue sections. In all cases where IHC is used, the controls should include experimental, fixed cell blocks derived from negative control cells or tissues known to lack the antigen of interest and positive control cells or tissue known to harbor the antigen of interest. These controls can typically be obtained from blocks created from cell lines where the negative controls can be generated by using short hairpin RNA (shRNA). With the development of protocols for next-generation sequencing from paraffin-embedded tissues, the use of such approaches has become more commonplace for the interrogation of archived primary patient tissues.[78,79]

Functional Studies to Address Cancer Dependence

Ideally, the functional relevance of a putative cancer target should be elucidated before embarking on the lengthy process of drug discovery. The concept is simple: It is preferable to "fail" or invalidate a putative target in preclinical studies than to fail later in the clinic. Although there are multiple reasons for the failure of a given therapeutic in clinical development, ideally failure should be avoided because the drug is made against an irrelevant target. Hence one should develop a robust understanding of the science surrounding a given drug target and tie this scientific understanding to cancer-relevant processes. It is useful to consider two questions in the context of designing experiments for functional validation. First, is the putative cancer target *necessary* for the maintenance of a cancer-related phenotype? Second, is the putative cancer target *sufficient* for inducing a cancer or transformation-related phenotype? These questions are generally answered by experiments directed at inducing loss of function of the target (addressing necessity) and by experiments directed at inducing gain of function (addressing sufficiency).

Gain-of-Function Experiments. Gain of function of a given protein typically involves overexpression of the cDNA or of an activated allele generated by mutation. The functional consequences of gene activation are assayed first in in vitro growth assays, in transformation assays, and in cotransformation experiments. Typically, gain of function in transformation assays would be assessed in murine embryonic fibroblasts, in immortalized NIH3T3 cells, Ba/F3 cells, or in Rat1a fibroblasts. In addition, the ability to transform human primary epithelial cells has been made possible through the use of telomerase and has provided a set of assays that can be used to measure the ability of a given gene to induce transformation in human cells.[80]

Short-term validation experiments using in vivo systems have become more attractive with the advent of tissue reconstitution murine models, enabling rapid genetic manipulation. Examples of this approach include the use of murine hematopoietic cells or murine fetal hepatoblast cells. In each case retroviruses directing the expression of the relevant candidate oncogene can be introduced into the respective primary cells that are then re-introduced into host animals. As an example, mutations in *JAK2* discovered in myeloproliferative syndromes recapitulate the disease entity when re-introduced in retroviral vectors into the hematopoietic reconstitution system.[81-83] Similarly, the use of hepatoblasts has been useful in demonstrating that certain gene activation events can induce hepatocellular carcinoma.[84,85] These systems provide some of the speed and flexibility of in vitro systems but maintain the more authentic relationship between stroma and the generated tumor cells.

Fully genetically engineered models, including transgenic mice, can be useful in studying gene activation in the specific tissue of origin depending on the extent to which promoter regulation can be used to direct gene expression to the indicated cell. Knock-in animals have also been generated in which mutant *RAS* alleles are re-introduced into the endogenous murine *RAS* gene locus.[86] Such animals have been used to demonstrate sufficiency of *K-RAS* for the induction of lung adenocarcinomas,[86] carcinomas of the pancreas[87] and myeloproliferative disease.[88] These engineered models also provide a stable resource for testing candidate drug compounds in highly defined mechanistically driven models.

Loss-of-Function Experiments. For many years there were no feasible methods for readily inducing loss-of-function alterations in mammalian systems. Consequently, the elaboration of a cell-penetrant small-molecule inhibitor or tool compound was one of the only ways to achieve the desired inhibitory effect. The development of robust small interfering RNA (siRNA) methods, taking advantage of the endogenous siRNA processing systems, now allows for transient, stable, or regulated knockdown of nearly any mammalian gene product.[89,90] Genome-editing technologies, such as transcription activator-like effector nucleases (TALEN) and the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas system have also been developed to enable targeted gene-disruption events.[91,92] These powerful new tools are complemented by existing methods for loss-of-function experiments, including the use of inhibitory antibodies for extracellular targets, soluble protein traps for extracellular ligands and receptors, dominant-negative proteins expressed from cDNAs, murine germline knockouts, and inducible knockouts. Generally, loss-of-function experiments would be attempted first in human cancer cell line models. Increasingly, cell line models are being characterized in greater genetic detail, enabling more rational selection of models for functional inactivation experiments by either small molecules or genetic perturbations.[93,94,95] Additionally, shRNAs have been merged with the bone marrow transplantation and the hepatoblast tissue reconstruction models described previously (Fig. 44-2).[96] Finally, regulated germline shRNAs can now be engineered in the mouse as a rapid method for generating tissue-specific and temporally regulated gene knockdowns.[97]

Genetic Studies in Lower Organisms

The topic of genetic studies in lower organisms is too broad for a complete discussion in this chapter. However, it is worth noting the utility of defining genetic dependence in the context of highly conserved pathways that are shared between mammals, flies *(Drosophila melanogaster)* and worms *(Caenorhabditis elegans)*. In some cases lower organisms have a reduced set of homologues for any given pathway member, and thus the necessity of a single gene can be tested directly. Although establishing the relationship between a gene and a cancer phenotype in these organisms is not usually possible, the genetic dependence or epistatic relationships elucidated in such model systems have proved highly informative in humans. A notable example is the discovery of the relationship between loss-of-function mutations in the tuberous sclerosis genes and activation of the mTOR and S6K (ribosomal S6 kinase) pathway (discussed later).

Target Credentialing Summary

The credentialing of drug targets ultimately must be conceived as a set of hypotheses to be tested experimentally. In each case these hypotheses must be built around the relevant biology. It is important to distinguish targets with cell-autonomous effects versus those in which the interaction between cancer cell and host factors is critical. In the case of drug targets that are encoded by bona fide human oncogenes with definitive genetic alterations, one can have a high degree of confidence in the likely therapeutic effect of inhibitory molecules. Unfortunately, this group is the minority of the drug targets for consideration. Thus the intent of credentialing is to enable a rational stratification of potential drug targets and a rational application of resources to specific drug discovery efforts.

Classes of Targeted Therapies

Targeted therapies can be divided into two main classes: drugs (small molecules, natural products, antisense, and siRNA) and biologics (antibody, protein/peptide, and cell-based) (Box 44-6). Biologics, as defined by the U.S. Food and Drug Administration (FDA), include a wide range of products such as vaccines, blood and blood components, somatic cells, gene therapy, tissues, and recombinant therapeutic proteins. They can be composed of sugars, proteins, or nucleic acids or complex combinations of these substances, or they may be living entities such as cells and tissues. Biologics are isolated from a variety of natural sources—human, animal, or microorganism—and may be produced by biotechnology methods. From a practical point of view, drugs entering the market require the filing of a New Drug Application (NDA), whereas biologics require the filing of a Biologic License Application (BLA).

Drugs

Small molecules are defined as carbon-containing compounds that usually have a molecular weight of less than

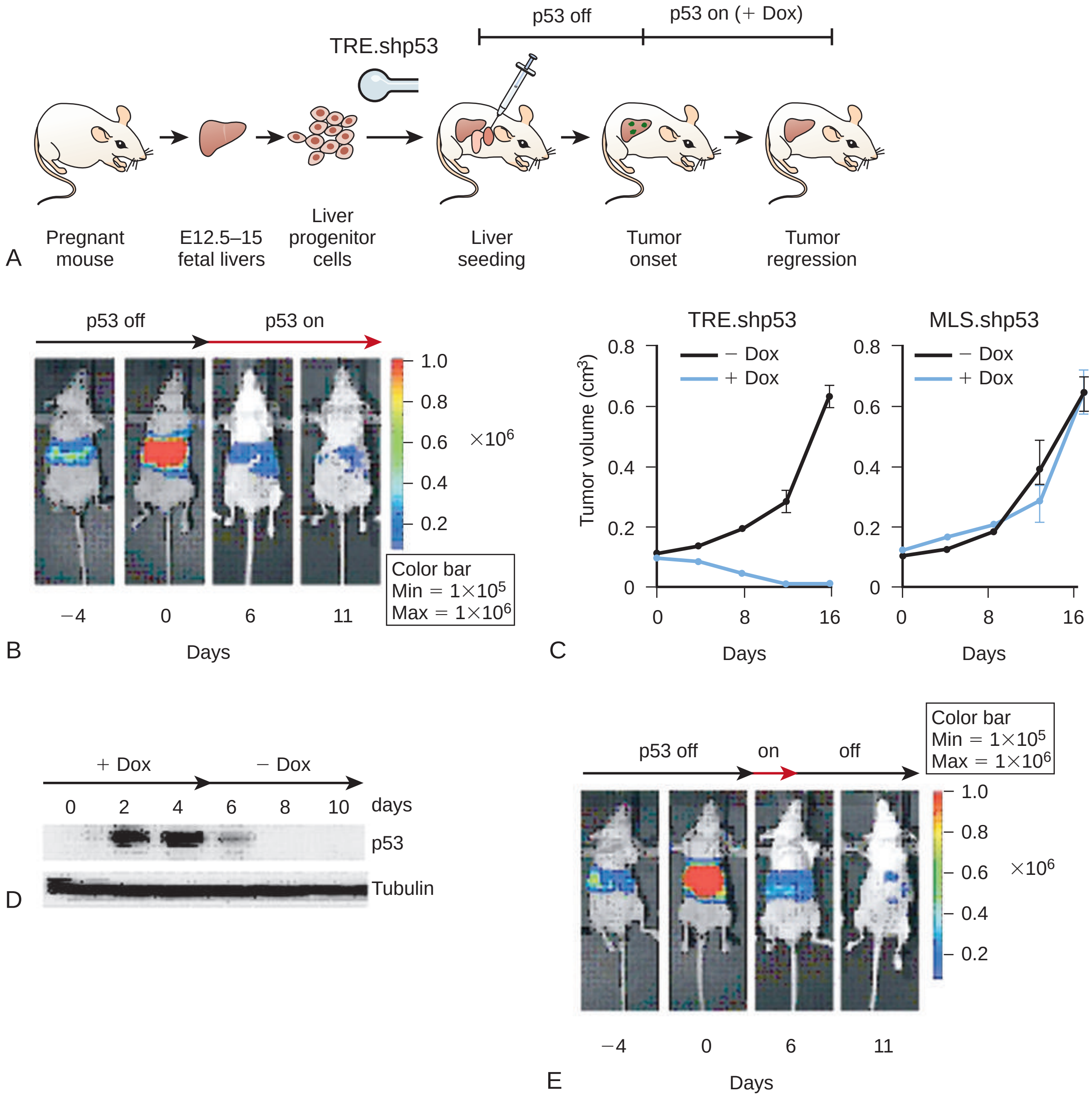

Figure 44-2 Reactivation of p53 results in liver tumor regression. **A,** Embryonic liver progenitor cells were transduced with a tetracycline-regulatable p53 shRNA (TRE.shp53), tTA, and H-rasV12. After onset of liver tumors, p53 expression could be restored by doxycycline (Dox) treatment. **B,** Reactivation of p53 leads to rapid tumor regression. Tumor-bearing mice were treated with Dox starting at day 0 and imaged at the indicated time points ($n = 9$). **C,** Subcutaneous tumors derived from ras-transformed liver progenitor cells with tet-off shRNA (TRE.shp53) or a nonregulatable shRNA (MLS.shp53) were grown in nude mice. Values represent mean s.d. ($n = 4$). **D,** p53 reactivation is reversed by Dox withdrawal. Protein lysates from liver progenitor cells pulse-treated with Dox for 4 days were immunoblotted for p53. **E,** Representative mice ($n = 6$) as in **B** were pulse-treated with Dox for 4 days and imaged at the indicated time. *(Modified from Xue, et al: Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas.* Nature *445:656–60, 2007.)*

2000 g/mol. These small organic compounds have the advantage of purity, high permeability into target cells, ease of manufacturing, and stability, and they remain the primary class amenable to oral delivery. However, disadvantages include insufficient selectivity resulting in off-target toxicity, challenges with poor bioavailability, and challenges with metabolism of the molecule. Over the last decade synthetic methods have been developed for producing a diversity of chemical structures, either by using directed medicinal chemistry approaches or by using combinatorial chemistry, the synthesis of numerous organic compounds by combining variations of each of the building blocks that make up the compounds.[98] Similarly, there has been the elaboration of a diverse set of methods for identifying the initial small-molecule hits against a desired target. High-throughput screening involves the in vitro assay of target activity against chemical libraries. In silico screening can be used to dock millions of compounds into known or modeled three-dimensional structures of a given target. Fragment-based screening uses either

Box 44-6 Classes of Therapies

- DRUGS
  - Small molecules
  - Natural products
  - Antisense oligonucleotides
  - RNA interference
- BIOLOGICALS
  - Antibody
  - Protein
    - Whole protein
    - Peptides
    - Soluble receptors
- CELLULAR

radiographic or nuclear magnetic resonance–based structural approaches to identify very-low-molecular-weight fragments that can interrogate the binding surface of a new target.[99] Finally, target-specific cell-based screens can be used to identify cell-penetrant small molecules perturbing a target-specific reporter or phenotype.

Natural products have been important in drug development and have played a dominant role in cancer therapy.[100] Approximately three fourths of the current anticancer drugs are natural products or derived from natural products.[101] The chemical diversity of natural products complements that of synthetic libraries. As a result of a long evolutionary process, natural products tend to be sterically complex with greater diversity of ring systems compared with synthetic and combinatorial libraries.

Natural products are classified according to shared scaffolding elements and can be divided into several structural classes, such as terpenes, alkaloids, polyketides, and nonribosomal peptides. The building blocks for the natural products are most often monomeric components of primary metabolic pathways that are then shunted into secondary metabolic pathways. Plants and soil microbes are traditional sources of natural products with new sources, such as fungi and marine life (e.g., sponges and algae), under active exploration.[102]

Drug development with natural products may have specific advantages to that of synthetic molecules. The complexity of natural products may lead to greater specificity. In addition, unanticipated activities may be discovered that would be difficult to engineer into a small molecule. For example, rapamycin (derived from a soil fungus from Easter Island [Rapa Nui]), creates a novel protein-drug-protein interaction between the protein FKBP12, rapamycin, and the mTOR kinase, resulting in an exquisitely selective inhibition of mTOR.[103,104] However, although the natural products have been developed into very successful drugs, there are several disadvantages to these libraries. First, natural product extracts are generally impure, and hence identifying the active compound can be difficult. Second, the synthesis of these compounds in production level quantities for medicinal purposes can be challenging. Third, subsequent optimization of the lead natural product can be difficult in the absence of a complete in vitro synthesis of the relevant natural product. New high-throughput strategies are under development to tackle roadblocks to the use of natural products for medicinal purposes.

Antisense oligonucleotides are single strands of short deoxynucleotide sequences (18-21 oligomers) that bind to target mRNA sequence by Watson-Crick hybridization and induce transcript destruction. They interact with the target transcript with sequence specificity and inhibit production of the target protein by several potential mechanisms. First, they activate endogenous nucleases, such as RNAse H, which then cleave the RNA strand of an RNA-DNA heteroduplex. A second potential mechanism of antisense activity is a noncatalytic effect with steric inhibition of the translational machinery with induction of translational arrest and inhibition of protein synthesis. A third mechanism is interference with normal RNA splicing, resulting in the inhibition of specific splice variants. Antisense therapeutics held the promise of pharmacologically inhibiting targets that had been intractable with small molecule approaches. In theory, virtually any gene can be targeted with antisense approaches with an expectation of high target selectivity. However, the development of antisense therapy has faced many challenges, including poor solubility, limited intracellular uptake, and rapid degradation. To overcome these issues, subsequent antisense therapy focused on chemical modification of the backbone to which the nucleoside bases are attached.

Despite these efforts, the utility of antisense therapy for cancer has been disappointing. However, there have been some clinical responses seen in trials. Oblimersen (Genasense), a phosphorothioate antisense therapy targeting BCL-2, has shown some activity in patients with heavily pretreated chronic lymphocytic leukemia (CLL).[105] Furthermore, recent data from a randomized phase III trial for patients with relapsed or refractory CLL comparing fludarabine plus cyclophosphamide with or without oblimersen demonstrated an increase in complete response and nodular partial response in patients in the oblimersen arm, particularly for those patients with fludarabine-sensitive disease.[106,107]

RNA interference (RNAi) is a more recent approach to nucleic acid–based therapy. Similar to antisense therapy, RNAi may have therapeutic application, particularly for targets that have been considered intractable by traditional drug discovery approaches. RNAi is a double-stranded RNA (dsRNA)–induced mechanism of gene silencing naturally occurring in plants and animals. Over the last few years, it has become possible to target virtually any gene in the genome for knockdown with small, dsRNA gene-silencing by using siRNAs or expressed shRNAs. The RNAi pathway is hypothesized to have evolved early in eukaryotes as a protection against viral and genetic pathogens. Double-stranded RNA viruses and genetic elements are subject to RNAi-dependent gene silencing as a means of cell-based immunity. Furthermore, endogenously expressed shRNAs may regulate gene expression during development through the RNAi pathway.[89,90] If exploited as a potential cancer therapy, RNAi has the potential advantages of (1) relative specificity for a particular gene target, (2) efficiency with the potential to achieve more than 90% knockdown, and

(3) widespread applicability with the potential of targeting any gene in the human genome. Unlike antisense oligonucleotides, RNAi takes advantage of a naturally occurring cellular mechanism for gene silencing.

As in the case of oligonucleotides, there are impasses to the development of RNAi-based therapies, including the difficulty of delivering large nucleic acid molecules to intracellular targets in humans. Unmodified siRNAs have a short half-life in human plasma and are rapidly excreted by the kidneys. Furthermore, to have activity the siRNA or shRNA must reach the cytoplasm of the targeted cell, and naked siRNAs are not efficiently taken up by mammalian cells unaided. One method of delivering shRNA that is commonly used in the laboratory to develop stable knockdown is the use of viral vectors. However, with viral vector delivery, there remains significant concern regarding insertional mutagenesis, carcinogenesis, direct cellular toxicity and short-lived on-target effects.[108-111] Other nonviral methods of delivery are actively under exploration. One possibility is to chemically modify the siRNA to increase its stability with modifications such as locked nucleic acid (LNA) residues[112,113] or phosphorothioates.[114] A second approach to increase the stability of the siRNA is to package it in liposomes[115] or other nanoparticles such as the cationic polymer polyethylenimine[116] or to use cholesterol modifications.[117] These packaging systems can also be modified to contain tissue-specific homing signals, such as the attachment of specific antibodies. Despite the problems with delivery and stability of RNAi, novel therapeutics using RNAi have entered clinical trials for multiple diseases.[118-120]

Biologicals

Antibody therapy with monoclonal antibodies (Mabs) is an increasingly important mode of targeted therapy for cancer. Early attempts at antibody-based therapy with murine Mabs were limited by immunogenicity, short half-life in humans, and poor ability to activate human immune effector mechanisms. With the evolution of recombinant DNA technology, it is now routine to create humanized Mabs. Recombinant antibodies with human crystallizable fragments (Fc) regions are much less immunogenic in humans than murine Mabs and can facilitate activation of immune effector mechanisms. Thus recombinant Mabs may have a dual mechanism of anticancer activity secondary to the specificity of epitope targeting with the antigen-binding fragment (Fab) and the recruitment of immune effectors with the Fc region. As a direct effect of the Mab variable domain binding to the target, they can have antagonist effects on receptor-ligand interactions and receptor-receptor interactions or agonistic effects on the target's biological activity. Second, if engineered to be of the immunoglobulin G1 (IgG1) subclass and to contain an Fc region, they induce immune effector function against I the target cell after their interactions with complement or with receptors for the Fc region, such as FcγR, a class of surface glycoproteins expressed predominantly on white blood cells. The Fc region interactions with FcγR can induce antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, endocytosis of immune complexes followed by antigen presentation, and release of inflammatory mediators.[121] Antibodies can be linked to chemical moieties with additional antitumor activity, such as in gemtuzumab ozogamicin, where an antibody to CD33 was conjugated to the antitumor antibiotic calicheamicin.

Box 44-7 **Advantages and Disadvantages of Antibody-Based Therapy**

ADVANTAGES

1. High-degree of specificity results in reduced off-target effects.
2. Pharmacokinetic properties are similar across different antibodies.
3. Spectrum of targets amenable to antibody-based therapy differs from that of small molecules.

DISADVANTAGES

1. Intravenous administration is necessary.
2. Preclinical in vivo studies are difficult to perform.
3. Neutralizing antibodies can develop.
4. Antibodies cannot access intracellular antigens.
5. Volume of distribution is limited by large molecular weight of antibody.
6. The cost of production is higher.

Bispecific antibodies: Whereas MAbs that are clinically effective usually recruit immune cells expressing an Fc receptor, T cells are notably FcR negative. To engage T cells in the antitumor response, bispecific antibodies (bsAbs) can be deployed. These are artificially designed molecules that bind simultaneously to two different antigens, one on the tumor cell, the other one on an immune cell, such as T cells, cross-linking tumor cells and T cells. For example, blinatumomab, a bsAb that directs T cells to CD19-expressing tumor cells, such as B-ALL and non-Hodgkin lymphoma (NHL) cells, has been demonstrated to have compelling activity in clinical trials, including complete responses.[122-124]

Antibody-based therapy has several advantages to small molecule–based therapy (Box 44-7). First, there is a high degree of specificity for the target of interest, resulting in reduced off-target effects. Second, pharmacokinetic properties are very similar from one antibody product to another, with the half-life of an infused antibody generally lasting 2 weeks. Unlike the case of small molecules, there is a consistent path for metabolism, thus eliminating a highly variable aspect of low-molecular-weight drug development. Third, the spectrum of targets amenable to antibody-based therapeutics is largely distinct from small molecules and includes large extracellular domains and secreted ligands that are otherwise "undruggable."

On the other hand, there are several disadvantages of antibody-based therapy compared with small-molecule therapy. Currently antibody-based therapies are generally delivered intravenously. A second disadvantage is that preclinical in vivo studies can be difficult to conduct.[125] The researcher must address whether the antibody in development will recognize the appropriate antigen in the animal model being tested. In some cases only primates conserve the relevant human epitope, limiting the selection of species available for toxicology studies. Efficacy studies, in particular against stromal or host factors, may require generation of a "murine equivalent" antibody, although equivalence is difficult to quantitate. The robust evaluation of host effector function elicited by an antibody and the role of this function in antitumor activity is also difficult to assess in the typical human tumor xenotransplant study in rodents. A third challenge with antibody-based therapy is that the patient can develop neutralizing antibodies to the therapy. This was particularly problematic with murine-based antibody therapy. Fourth, antibodies cannot easily gain access to intracellular antigens. Fifth, the volume of distribution of antibodies is limited by the large molecular weight, and tumor penetration is therefore a concern, at least theoretically. Finally, antibody-based treatment is generally more costly to produce compared with small-molecule therapy.

Cellular Adoptive Therapy

Most recently, cell-based biological products have also seen dramatic clinical responses. The transfer of autologous T cells modified to express chimeric antigen receptors (CARs) specific for the B-cell antigen CD19 has shown antitumor activity in low-grade B-cell malignancies and in refractory adult and pediatric B-cell ALL.[126,127] These exciting advances are discussed in more detail in Chapter 46.

Targeted Therapy: Lessons Learned from Adult Oncology

With rare exceptions, most drugs are first tested in adults before evaluation in children. This has been true for targeted agents as well. In the following section several examples of successful targeted therapies for the treatment of adult patients with cancer are briefly discussed, with an emphasis on particular lessons learned in the development and testing of these early agents.

Genetics Examples of Targeted Therapy

Treatment of Acute Promyelocytic Leukemia with All-*Trans*-Retinoic Acid. The successful treatment of patients with M3-AML (acute promyelocytic leukemia, or APL) with ATRA is one of the earliest examples of targeted therapy in the genetic class. This therapeutic discovery was serendipitous. The observation that myeloid blasts differentiate in response to retinoic acid derivatives preceded the discovery of the involvement of the retinoic acid receptor in the pathogenesis of APL.[128,129] ATRA treatment is one example of how discovery of a compound inducing a phenotypic alteration can lead to greater understanding of the mechanisms underlying the state change. By morphologic examination APL is characterized by a predominance of malignant hypergranular cells blocked at the promyelocyte stage of differentiation. At the molecular level a reciprocal translocation involving the *retinoic acid receptor α (RARα)* gene on chromosome 17q21 is invariably present. This translocation most commonly fuses *RARα* to the *PML* gene on chromosome 15q22. RARα is a hormone-dependent, DNA-binding, nuclear receptor transcription factor that can act as a transcriptional activator or inhibitor.[75,130,131] In the presence of physiologic amounts of retinoic acid, it normally functions as a transcriptional activator. Abnormal RARα fusion proteins function as transcriptional repressors, enhancing interactions with the corepressor complex.[132,133] Pharmacologic doses of ATRA appear to overcome the PML-RARα–induced transcriptional repression by dissociating the corepressors from PML-RARα and restoring normal ATRA-mediated myeloid differentiation.[134] Furthermore, the PML-RARα protein undergoes proteolytic cleavage in APL cells after ATRA treatment.[135] ATRA treatment increases the fraction of differentiated cells with functional characteristics of neutrophils, induces a mature membrane phenotype, inhibits leukemia cell proliferation, and ultimately induces apoptosis. Historically, APL was among the most fatal subtypes of AML. However, with the addition of ATRA to APL therapy regimens, the overall 2-year survival rate is more than 75%. APL now has the highest cure rate of the AML subtypes.[1,5,136]

The success of ATRA in the treatment of PML-RARα rearranged AML illustrates several important issues regarding targeted therapy. First, specific somatic rearrangements, such as PML-RARα, can be exploited with targeted therapy, and the specific underlying genetic event predicts response, not the phenotype. For example, other RARα rearrangements, such as PLZF- RARα, are associated with an APL phenotype. However, the PLZF-RARα fusion protein is associated with nuclear corepressor interactions that are resistant to ATRA therapy. Patients with the *PLZF-RARα* rearrangement are resistant to ATRA therapy.[137] A second important concept that emerges from the success of ATRA is that differentiation therapy is feasible for some malignancies. In general, terminally differentiated cells lose the capacity to divide and ultimately undergo apoptosis. This is indeed the case for APL cells treated with ATRA, which raises the possibility that such approaches may be more broadly applicable. A third important lesson from this example is that modulation of transcription factors is therapeutically feasible. Rearrangements involving transcription factors are a common event in the acute leukemias and in the pediatric solid tumors. Although well-characterized contributors to the malignant state, these transcription factor abnormalities have been considered "undruggable" or pharmacologically intractable because there is no obvious approach to inhibiting their function. However, ATRA therapy for the genetic lesion PML-RARα lends credence to the idea that these lesions can be targeted and that their modulation can have therapeutic efficacy. Finally, this example reinforces the importance of combination therapy not only for cytotoxic agents but also for targeted therapy. Although the majority of patients with APL in early trials achieved a complete remission with ATRA as a single agent, it was only in combination with conventional chemotherapy or arsenic trioxide that long-term cure rates were achieved.[6,136,138,7]

Imatinib in Chronic Myelogenous Leukemia. The development of imatinib for the treatment of CML stands out as the first example of a designed small-molecule inhibitor developed for the purpose of targeting an activated oncogene. Moreover, its dramatic single-agent activity in its initial phase I and II trials, along with the rapid path to registration, has provided the impetus for attempting to identify highly responsive patient populations during preclinical and early clinical development. In 1960 Nowell and Hungerford first detected the presence of the then-named *Philadelphia chromosome* in nearly all patients with CML.[139] This aberrant chromosome, resulting from the fusion of chromosomes 9 and 22, was subsequently shown to encode a fusion protein linking a gene of unknown function (*BCR,* or *breakpoint cluster region*) to the product of the *Abelson* gene—the cellular homologue of an oncoprotein activated by the Abelson leukemia virus. The discovery that the *Abelson* gene product (ABL) could function as a protein tyrosine kinase and that the normal autoregulatory domain of ABL was replaced with coding sequences from BCR led to the idea that constitutive activation of ABL kinase activity was likely a causal genetic event in CML.[140-142] In animal models production of BCR-ABL was sufficient to induce both acute and chronic leukemia.[143] These data, along with the presence of a catalytic domain, made BCR-ABL kinase an attractive drug target. Based on these elements, a drug-discovery program was launched, culminating in the discovery and clinical testing of imatinib (Gleevec), a small molecule inhibitor of BCR-ABL.

In the initial phase I trial by Druker and colleagues, 83 patients with interferon-refractory CML were enrolled and treated with doses of imatinib ranging from 25 to 1000 mg per day. No MTD was reached, and nausea, myalgias, edema, and diarrhea were the most frequent side effects. Remarkably, at a dose of 140 mg or higher, all patients had a hematologic response. At doses of 300 mg per day or greater, 53 of 54 patients had complete hematologic responses, 17 had major cytogenetic responses, and 7 had complete cytogenetic remissions.[144] Numerous subsequent trials demonstrated remarkable activity in CML and CML in accelerated phase, lymphoid or myeloid blast crisis or with Ph+ ALL in adults, including complete molecular responses.[144-148]

On the basis of these data, imatinib became the gold standard for first-line therapy of CML. Nonetheless, resistance to imatinib does occur. In vitro experiments conducted by using randomly mutagenized *ABL* cDNA libraries inserted into sensitive CML cells revealed that a defined spectrum of mutations in the *ABL* gene itself could preserve kinase activity but could disrupt binding to imatinib.[149] More important, sequencing of the *ABL* kinase domain in patients relapsing on imatinib revealed a similar spectrum of mutations in patient samples.[150,151] When these mutations were isolated and placed into ABL kinase constructs and re-inserted into sensitive CML cells in vitro, these mutant ABL constructs also rescued lethality induced by imatinib inhibition. These data, although initially concerning because of the potential for long-term control of the CML disease state, definitively demonstrated in humans the absolute requirement for ABL kinase activity in CML and closed the loop on the notion of a fully validated drug target. Moreover, the identification of such mutants provided the impetus for testing second-generation inhibitors that might overcome these resistance alleles. Here, two separate approaches were considered. First, although imatinib is a fairly selective inhibitor, it is not a particularly potent inhibitor. Because many mutations shifted the IC_{50} of the kinase activity, it remained possible that a more potent inhibitor might be able to overcome or prevent the emergence of these resistance alleles. Nilotinib is a second-generation inhibitor designed to target BCR-ABL and preserve the interaction with the inhibited form of the ABL kinase, the type II binding mode.[152] Nilotinib is more potent in biochemical and cellular assays of ABL activity and in phase I and phase II trials had significant activity in imatinib-resistant CML.[153] Nilotinib was later demonstrated to be superior to imatinib in patients with newly diagnosed chronic phase CML.[154] Dasatinib, initially developed as a src kinase inhibitor, binds to the "active" form of its kinase targets. Src kinases are highly related to ABL kinase activity, and in vitro testing showed that dasatinib had activity against imatinib-resistance alleles.[155] In phase I and II trials dasatanib has shown significant activity in imatinib-resistant patients.[156] Both dasatinib and nilotinib have gained FDA approval in imatinib-resistant patients with CML and in CML-accelerated phase and blast crisis. Ultimately, both drugs were approved for upfront therapy of patients with CML.[157]

Unfortunately, both nilotinib and dasatinib lack activity against a common but difficult to target *ABL* mutation, the T315I or "gatekeeper" mutation. This mutation creates a steric problem in the adenosine triphosphate–binding domain. This particular kinase domain conformation creates challenges in the drug design process. Notably, it has been difficult to make potent *and* selective inhibitors of this conformation inhibition. Recently, a drug with activity against the T3151 mutation, ponatinib, was granted accelerated approval by the FDA for the treatment of adult patients with chronic, accelerated, or blast-phase CML that is resistant or intolerant to prior tyrosine kinase inhibitor therapy or BCR-ABL–positive ALL that is resistant or intolerant to prior tyrosine kinase inhibitor therapy (Fig. 44-3).[158] However, the safety of this drug was later called into question secondary to concerns about drug-related arterial thrombotic events.[159,160]

Imatinib and Other Malignancies. Imatinib, in addition to inhibiting ABL kinase activity, also inhibits c-KIT and platelet-derived growth factor receptor (PDGFR). In preclinical cell line models of gastrointestinal stromal tumors (GIST), a tumor defined by frequent mutations in the *c-KIT* gene, efficacy of imatinib both in vitro and in murine xenograft models was demonstrated.[161] Based on these early preclinical findings, a patient with inoperable, recurrent, and treatment-refractory *c-KIT*-mutated GIST was treated with imatinib. After 4 weeks of therapy, the fludeoxyglucose-positron emission tomography scan showed a complete resolution of tracer uptake compared with the pretreatment scan, and over the ensuing 8 months the patient had a progressive diminution in the tumor volume.[162] Multiple subsequent studies demonstrated response to tyrosine kinase inhibitors in GIST,[163-166] and in 2008 the FDA granted accelerated approved for imatinib in the adjuvant setting for completely resected primary GISTs 3 cm and larger.

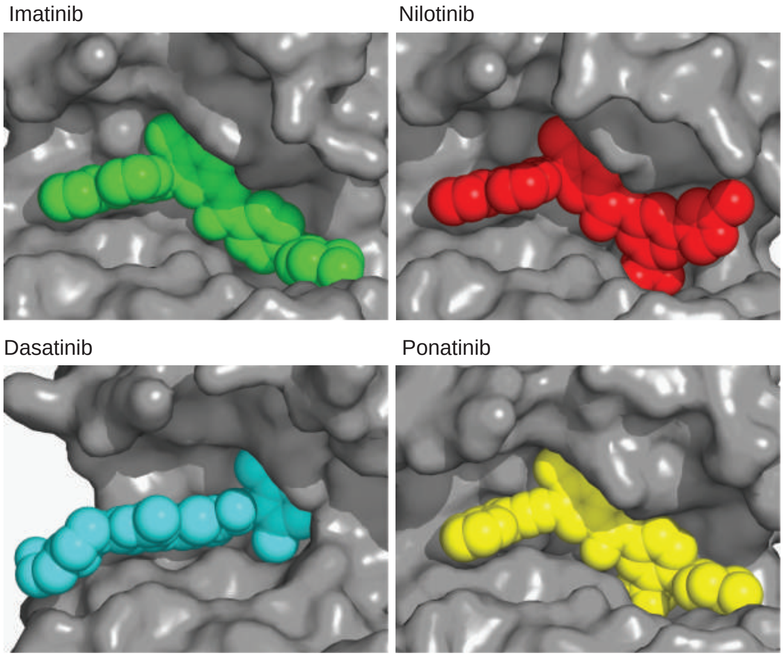

Figure 44-3 Abl inhibitors. Shown are representative structural images of the indicated small molecule inhibitors *(colored)* bound to the ABL kinase domain *(gray)*. Imatinib, nilotinib, and ponatinib are type II inhibitors and contain molecular components that occupy the hydrophobic pocket that is exposed when the kinase is in an "inactive" conformation. Dasatinib is a type I inhibitor and does not have extended interactions with the hydrophobic pocket and instead binds to the "active" conformation of the kinase. *(Courtesy Sandra Jacobson, Novartis Institutes for BioMedical Research, Emeryville, Calif.)*

The activity of imatinib against PDGFR or KIT has enabled clinical development in other diseases characterized as harboring specific genetic alterations activating these receptors. In the case of dermatofibrosarcoma (DFS) the majority of patient lesions harbor translocations between chromosomes 17 and 22 fusing the *PDGFB* ligand gene downstream of the *Col1a* promoter.[167] This leads to constitutive ligand production and receptor activation and sensitivity to imatinib in in vitro models, transplantable primary tumors from patients, and in patients treated with the drug.[168-170] In hypereosinophilic syndrome (HES) Schaller and Burkland report that empiric imatinib treatment of a patient with HES induced a complete and rapid remission of the disease.[171] Similarly, other studies revealed patients with HES responding to imatinib.[172] Gleich and colleagues reported the empiric treatment of five HES patients, four of whom had a prompt and sustained response to imatinib with a resolution of the eosinophilia.[173] The basis for these dramatic observations was uncovered in 2003, when Cools and coworkers reported the discovery of an intrachromosomal deletion that placed *PDGFRA* downstream of the promoter of the *FIP1L1* gene, resulting in constitutive upregulation of the receptor.[174]

The remarkable finding in the examples of the FDA-approval of imatinib for CML, GIST, dermatofibrosarcoma, and HES is that in each case a specific genetic event leads to constitutive activation of a molecular target of imatinib, and in each case a high degree of efficacy is achieved. Moreover, we have learned that efficacy is identified rapidly in selected patient populations, with dramatic responses even in phase I. We have learned that MTD does not necessarily set the appropriate effective dose, and, indeed, MTD end points were not reached in a number of the early phase I trials.

Dramatic Responses in Select Genotypes. With the success of imatinib for the treatment of patients with CML, a high bar was set for subsequent small-molecule kinase inhibitors. The possibilities that this targeted approach held for the poorly treated adult epithelial malignancies generated great excitement. The EGFR pathway is believed to be important in the development, maintenance, and proliferation of many malignancies, and EGFR is frequently overexpressed in epithelial solid tumors. For these reasons EGFR was an attractive target for small-molecule inhibition. Gefitinib (Iressa) and erlotinib (Tarceva), both orally administered tyrosine kinase inhibitors, were developed to bind competitively at the adenosine triphosphate pocket of EGFR, inhibiting autophosphorylation, and suppressing downstream EGFR signaling. Surprisingly, these EGFR inhibitors have been largely ineffective in the treatment of solid tumors with one exception: the treatment of a subset of patients with non–small cell lung cancer (NSCLC).[175-179]

After these initial clinical studies, numerous reports characterized the clinical predictors of tumor response (as opposed to survival) in patients. It soon became apparent that these patients frequently shared similar demographic features: a nonsmoking history, a well-differentiated adenocarcinoma histology, female sex, and Asian origin, although the underlying genetic reason for these response differences was not known.[178-180] One hypothesis was that expression levels of EGFR correlated with these clinical characteristics and hence response. However, these initial clinical trials failed to identify a correlation between receptor expression and clinical outcome.

Later, in 2004, two research groups independently identified a correlation between mutations in the tyrosine kinase domain of the EGFR and patient responders.[36,181] These *EGFR* mutations enhance ligand-dependent EGFR activation and sensitivity to gefitinib/erlotinib and are more common in nonsmokers, women, Asians, and patients with adenocarcinoma. Approximately 90% of these mutations occur in a few amino acids. Between 45% and 50% of mutations are in-frame deletions of exon 19 (codons 746 to 750), and 35% to 45% of mutations are missense mutation leucine to arginine at codon 858 (L858R) in exon 21.[182-185] Several studies have also reported that patients with EGFR mutant tumors have a longer survival when treated with EGFR small- molecule inhibitors compared with patients with wild-type EGFR tumors.[184,186-188]

This work highlights the importance of developing a molecular understanding of disease and response. The molecular characterization of EGFR abnormalities in NSCLC and their relationship to response to tyrosine kinase inhibitors have been illuminating for the clinical application of targeted therapy. More recently, the dramatic responses seen to B-RAF inhibition in patients with V600E *B-RAF*-mutant melanoma and to ALK inhibition in patients with *EML4-ALK* rearranged lung cancer underscores the critical role of matching tumor genotype with targeted drug therapy (Fig. 44-4).[189,190-192,193]

Lineage-Dependence Examples of Targeted Therapy

Several successful targeted cancer therapies capitalize on the lineage dependence of the tumor as exemplified by antibody-based therapy directed against cancers with hematologic lineage markers such as rituximab (Rituxan). As discussed in the subsequent sections, these therapies have the advantage of target specificity, with predictability of side effects based on the expression pattern of the lineage marker.

Rituximab Therapy for Non-Hodgkin Lymphoma. Rituximab (Rituxan) in 1997 became the first monoclonal antibody approved by the FDA for the treatment of malignancy (NHL). It is a chimeric, anti-CD20 monoclonal antibody composed of murine variable regions fused with human IgG1 heavy and κ light chains. CD20 is a transmembrane protein expressed on normal and malignant mature B lymphocytes[194] thought to be important in calcium transportation and to be involved in B-cell activation, differentiation, and growth regulation.[195] Rituximab binds to CD20 and depletes CD20-positive B cells in the circulation and in lymph nodes.

The first trials evaluating rituximab were in patients with indolent lymphoma, which has no curative therapy. The pivotal multicenter trial in patients with relapsed low-grade or follicular lymphoma demonstrated an overall response rate of 50% and a complete remission rate of 6%. These results were comparable with the best single-agent cytotoxic therapy for relapsed follicular lymphoma.[196] Rituximab has also been evaluated in combination with cytotoxic chemotherapy for patients with indolent B-cell lymphomas. One of the earliest studies, published in 1999, evaluated 40 patients with low-grade or follicular lymphoma treated with six cycles of CHOP (**c**yclophosphamide, **h**ydroxydaunorubicin, **o**ncovin, and **p**rednisone or **p**rednisolone) and rituximab (R-CHOP). The initial report suggested an additive benefit of

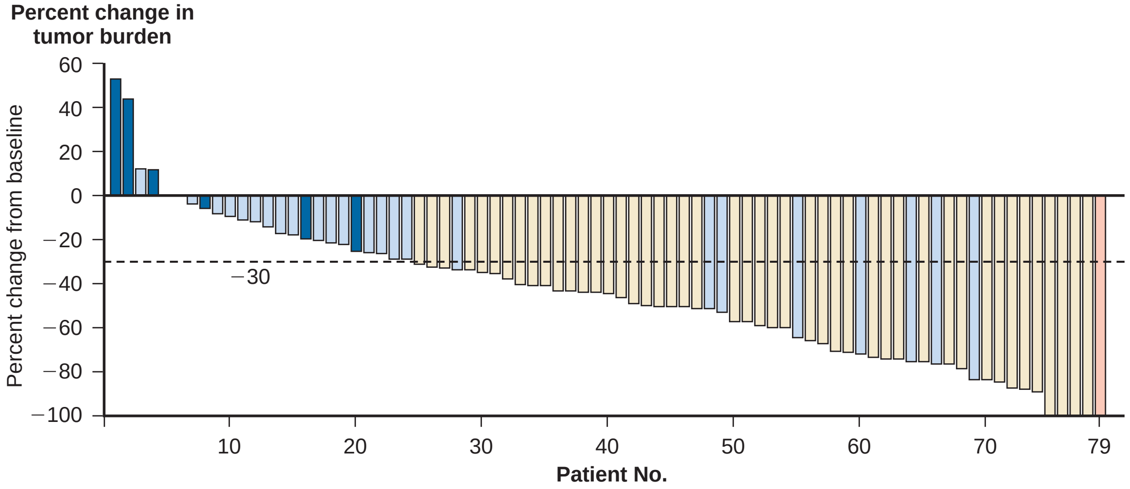

Figure 44-4 Response to ALK inhibition in patients with ALK-positive non–small cell lung cancer. Shown is the best response of patients with *ALK*-positive tumors who were treated with crizotinib, compared with pretreatment baseline. Numbers along the *x* axis indicate arbitrarily assigned patient numbers from 1 to 79. The *bars* indicate the percentage of change in tumor burden from baseline. *Black bars* indicate disease progression, *blue* stable disease, *yellow* partial response, and *orange* complete response. Four patients had complete resolution of their target lesions but were classified as having had a partial response on the basis of stability in nontarget lesions. Eight patients had tumor shrinkage of more than 30% but were classified as having stable disease either because confirmatory scans were not available by the data-cutoff point or early restaging was performed at 6 weeks after crizotinib initiation. The *dashed line* indicates a tumor reduction of 30% from baseline, the minimal percentage of decrease that constitutes a partial response, according to Response Evaluation Criteria in Solid Tumors. *(Modified from Kwak, et al: Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer.* N Engl J Med *363:1693–703, 2010.)*

rituximab with no additional toxicity when these two therapies are used in combination.[197] The overall response rate was 95%, and the complete response rate was 55% in the intent-to-treat group. One of the larger clinical trials of 428 patients with previously untreated, advanced-stage follicular lymphoma assigned to CHOP or R-CHOP demonstrated an overall response rate of 90% with CHOP and 96% with R-CHOP and estimated survival rate at 2 years of 90% and 95%, respectively.[198]

The combination of rituximab with cytotoxic chemotherapy has also been evaluated for aggressive NHL. In a randomized phase III study comparing CHOP with R-CHOP in elderly patients with diffuse large B-cell lymphoma, the rate of complete response was higher in the group receiving R-CHOP (76% versus 63%, $P = 0.005$). Furthermore, at 2 years both event-free and overall survival rates were higher in the R-CHOP arm, with no significant additional toxicity.[199] A randomized study of CHOP-like chemotherapy alone or in combination with rituximab in adult patients ages 18 to 60 years with good-prognosis diffuse large B-cell lymphoma demonstrated a 3-year event-free survival rate of 59% with chemotherapy alone versus 79% with the addition of rituximab and at 6 years of 56% and 74%, respectively.[200,201]

Thus R-CHOP is now considered the standard of care for the first-line treatment of CD20-positive diffuse large B-cell lymphoma and for CD20-positive follicular B-cell NHL in combination with chemotherapy. Rituximab is also indicated for the treatment of patients with relapsed or refractory CD20-positive low-grade or follicular NHL.

Host Track Example of Targeted Therapy

Bevacizumab and Colorectal Cancer. Angiogenesis plays an important role in tumor formation. Continued tumor growth is dependent on new blood vessels for the supply of oxygen and critical nutrients. Moreover, certain somatic and germline alterations, including those disrupting the function of the *von Hipple-Lindau (VHL)* tumor-suppressor gene, appear to act largely by triggering dysregulated hypoxic and angiogenic responses.[202-205] Over the last several years, numerous angiogenic growth factors have been characterized, including vascular endothelial growth factor (VEGF). VEGF is a member of a large family of dimeric glycoproteins acting as growth factors. It is essential for the normal development of blood vessels and is a growth factor for vascular endothelium.[206,207] VEGF binds to the transmembrane tyrosine kinase receptors VEGFR1, VEGFR2, and VEGFR3. In the malignant state VEGF secreted by the tumor or surrounding stroma leads to endothelial cell proliferation and migration. Because VEGF and its receptors play a critical role in tumor formation, progression, and maintenance, VEGF or VEGFR inhibition became a focus of targeted antiangiogenesis therapy.[208] Several approaches are in development, but the two with the most success thus far are (1) neutralizing antibodies that inhibit the binding of VEGF to its receptors and (2) small-molecule tyrosine kinase inhibitors that block downstream signaling from the VEGFR.

Bevacizumab (Avastin) is a recombinant, humanized monoclonal antibody that binds to VEGF-A, preventing its interaction with the VEGF receptors. It is believed that bevacizumab affects tumor vasculature by several mechanisms: (1) inducing regression of tumor vasculature, (2) normalizing the tumor vasculature, (3) inhibiting new blood vessel formation, and (4) preventing recruitment of progenitor cells from the marrow. It may also improve chemotherapy delivery by altering tumor vasculature and decreasing interstitial pressure.[209] Bevacizumab has been approved by the FDA for first- (2004) and second- (2006) line treatment of patients with metastatic colorectal cancer and the treatment of nonsquamous metastatic lung cancer, although it extends survival by less than 6 months.[210-214]

As with most of the targeted therapies, many of the side effects of bevacizumab were predictable. Some of the reported side effects include hypertension, proteinuria, arterial thromboembolic events, bleeding, poor wound healing, gastrointestinal perforation, and reversible posterior leukoencephalopathy syndrome (RPLS). Most of these side effects are predictable from the known activity of VEGF. For example, VEGF is a homeostatic factor for regulating blood pressure. Blocking of VEGF or VEGFR leads to increased vascular tension and thus problems with hypertension and RPLS. It is interesting to note that the side effects of VEGF inhibition are similar to the signs of preeclampsia and eclampsia (hypertension, proteinuria, and mental status changes), a disease in which soluble VEGFR-1, which antagonizes VEGF functions, has been implicated.[215,216]

Other VEGFR pathway inhibitor development has focused on inhibition of the kinase activity of the VEGF receptor. In December of 2005 the FDA approved the drug sorafenib (Nexavar), a small-molecule VEGFR inhibitor, for the treatment of patients with advanced renal cell carcinoma, and in January of 2006 the FDA approved the multitargeted inhibitor (including VEGFR and KIT) sunitinib (Sutent) for the treatment of patients with both advanced renal cell carcinoma and for patients with GIST who are intolerant of, or who have had disease progression with, imatinib treatment.

The "success" of bevacizumab for colorectal cancer is a humbling one for drug development. In reality, there is a long way to go when the successful drug extends overall survival by less than 6 months. In pediatric cancer care this would hardly be deemed a victory. However, many lessons have been learned from this experience. One is that antiangiogenesis-directed therapy can play a role in some disease treatments. A second is the continued importance of combination therapy, even for targeted therapy. Bevacizumab alone had very little activity. It was in combination with standard cytotoxic agents that a clinical benefit was demonstrated. Third, careful evaluation of targeted therapy benefits and toxicities may provide new insight into human disease.

Empiric or Synthetic Lethal

As described previously, classical chemotherapy is likely to exploit cancer-dependent states that are not fully understood. For example, microtubule inhibitors, such as the taxanes, specifically interact with protein components of microtubules and are efficacious in a number of cancers, although there is no current scientific rationale for their selective efficacy. A number of emerging targeted therapeutics target highly selective protein targets involved in diverse basic cellular processes and were elaborated according to specific synthetic lethal hypotheses.

Histone Deacetylase Inhibitors. Histone deacetylase (HDAC) inhibitors are an example of an empirically acting targeted agent. Transcriptional repression and transcriptional activation, at least in part, reflect the balance of histone modification of chromatin structures surrounding gene regulatory regions. Histone acetylation is of particular importance. In many cases transcriptional activators recruit histone acetylase activity to gene regulatory regions, whereas transcriptional repression complexes typically recruit HDAC to the same regions. In keeping with the notion that repression might underlie the function of oncogenic transcription factor translocations, HDAC enzymes are recruited to the critical promoters by various translocation partners, including RARα and ETO. These data provide a compelling rationale for the development and testing of HDAC inhibitors. In keeping with the underlying concept of synthetic lethality, it is possible to hypothesize an enhanced dependence on, or constitutive requirement for, HDAC enzymes by the aforementioned repressive oncogenes. Although the original therapeutic concept evolved from a link between leukemogenesis and transcriptional repression, a large array of proteins are now known to be modulated through acetylation at the hands of 11 related HDACs.[217] This will invariably create difficulty in elucidating the underlying therapeutic mechanisms linked to the anticancer activity now observed in the clinic with relatively broad-acting HDAC inhibitors.

An array of HDAC inhibitors targeting the major classes of HDAC enzymes are now in the clinic. The majority of these inhibitors have a core hydroxamate structure that sits deep in the enzymatic pocket, making contacts with a key coordinated zinc atom in the enzyme. The most advanced of these compounds, vorinostat (suberoylanilide hydroxamic acid [SAHA]), was approved for the treatment of cutaneous T-cell lymphoma (CTCL).[218] In initial phase I studies testing vorinostat, diarrhea, anorexia, dehydration, and fatigue were found to be the limiting side effects. Thrombocytopenia was also frequent.[219] Based on early evidence of efficacy in patients with CTCL, a phase II trial of vorinostat was conducted exploring three dose levels.[218] Eight of 33 patients achieved a partial response. A phase IIB multicenter trial was subsequently conducted. In this trial of 74 patients who had all progressed or relapsed on at least two prior treatment regimens, 30% achieved an objective response and 77% of patients had some measure of disease improvement. One patient achieved a complete response.[220] These data provided the basis for the FDA approval of vorinostat for CTCL in 2006.

Romidepsin (depsipeptide, FR901228, FK228), a natural product bicyclic depsipeptide originally isolated from a broth culture of *Chromobacterium violaceum,* was discovered to have potent transcriptional activation properties and to be a potent inhibitor of HDAC enzymatic activity.[221] In the early clinical experiences with romidepsin, in addition to the observed toxicities with HDAC inhibitors discussed previously, cardiac abnormalities were also reported.[222,223] In a phase I trial conducted in pediatric patients taking romidepsin, the dose-limiting toxicities were T-wave inversions and transient sick sinus syndrome, which were asymptomatic.[224] In phase II trials romidepsin was tested in patients with neuroendocrine tumors and in patients with metastatic renal cell carcinoma. The neuroendocrine study was terminated because of serious cardiac adverse events, including ventricular arrythmias and prolonged QTc.[225] In the renal cancer trial, prolonged QTc interval (two), grade 3 atrial fibrillation and tachycardia (one), and sudden death (one) were observed.[226] There was insufficient antitumor activity to warrant further development in these indications. A phase II trial of romidepsin in patients with CTCL reported an overall response rate of 38%.[227] In 2009 the FDA approved romidepsin for CTCL in patients who have received at least one prior systemic therapy. Similarly, panobinostat (LBH589), a synthetic hydroxamic acid–based small-molecule inhibitor that was specifically designed as part of a drug-discovery effort directed at producing a pan-HDAC inhibitor, has been demonstrated to have activity in patients with CTCL but also to have the potential liability of cardiac toxicity.[228]

It is clear from the summation of these emerging data that HDAC inhibition results in significant activity in CTCL, although the reason for this empiric observation remains obscure. HDAC inhibitors are now being tested more widely in other solid tumor and hematologic malignancies. The key to these studies will be the elucidation of effective yet well-tolerated dosing regimens.

NEW AND EMERGING OPPORTUNITIES IN PEDIATRIC CANCER

Targeted therapies have been introduced into the pediatric cancer therapeutic arsenal.[229] Thus far, nearly all of these agents have already been tested in the adult community. Several have had success in the pediatric setting where the potential target is shared by the phenotypically identical adult and pediatric tumor, such as ATRA for children with APL,[230,231] imatinib for BCR-ABL–rearranged CML[232] and ALL,[233] and rituximab for lymphoma.[234,235] There are many targets—those shared by both adult and pediatric malignancies and targets specific to pediatric cancers—not yet thoroughly explored with targeted approaches. In the following sections we will discuss these new and emerging targets and the special considerations in bringing targeted therapy to the pediatric setting (Box 44-8).

Special Considerations in Targeted Therapy Development for Children

Majority of Children with Cancer Are Cured

There are numerous special considerations in the development of targeted therapy for pediatric cancer. In contrast to adults with cancer, the majority of children with cancer will be cured of the malignancy, making the selection of pediatric patients for testing of new targeted

Box 44-8 **Special Considerations in Pediatric Targeted Therapy Development**

1. Most children with cancer are cured.
2. Pediatric cancer is rare.
   a. Market incentive is reduced.
   b. Clinical trial design and accrual are difficult.
   c. Limited number of agents can be reasonably tested
3. Toxicity may be specific to developmental stage.
4. Ethical considerations are more difficult.
   a. Informed consent for experimental therapy must be obtained.
   b. Informed consent for participation in biological study must be obtained.

therapies more challenging and raising the required level of activity necessary for "success." For example, in de novo pediatric pre–B-cell ALL, with cure rates greater than 80%, it is difficult to introduce a new targeted agent for fear of detracting from an already excellent cure rate by diluting curative agents. Although there are many interesting new targets emerging, such as *PAX5* and other genes important in B-cell developmental pathways,[236] it would be difficult to justify the inclusion of upfront novel targeted therapy in patients with this excellent outcome. Thus it is likely in the relapsed setting, or in de novo disease with poor outcome, that novel targeted therapies will undergo initial testing. If efficacy and safety are demonstrated, researchers may then consider incorporation into upfront clinical trials.

Pediatric Cancer Is Rare

A total of 1,660,290 new diagnoses of cancer and 580,350 cancer deaths are projected to occur in the United States in 2013.[237] In contrast, it is estimated that only 14,023 children in the United States younger than age 20 years were diagnosed with cancer in 2009, and 1964 died of cancer in 2009.[238] Hence pediatric cancers are quite rare compared with adult malignancies, and there is a reduced market incentive for pediatric-specific targeted drug development by the pharmaceutical industry. Furthermore, researchers have been understandably hesitant to test new drugs in children. For these reasons, there are virtually no drugs that have been developed specifically for pediatric malignancies. Even when a common target has been identified in adult and pediatric cancer, it may be years before a drug reaches a pediatric trial. Because pediatric cancers are rare, even with a drug in hand to test, clinical trials are difficult. A rare disease incidence means slow accrual of patients and necessitates large cooperative groups to power the trial with appropriate patient numbers. The limited number of patients also precludes testing of all possible new agents. New methods are needed to more rationally choose novel targeted agents for testing in children.

One national attempt to prioritize the selection of new agents for testing in children with cancer was the development of the Pediatric Preclinical Testing Program (PPTP) by the National Cancer Institute. This group is prospectively testing new agents against a panel of xenografts and genetic models of pediatric cancer, such as neuroblastoma, rhabdomyosarcoma, osteosarcoma, and ALL. The PPTP builds on earlier work demonstrating the ability of preclinical testing of new agents in rhabdomyosarcoma and neuroblastoma xenografts to predict in vivo activity in children with these malignancies.[239,240] The development of new genetically engineered models of pediatric cancer should also facilitate preclinical evaluation of targeted therapies and better prioritization of new agents for clinical trial, particularly for diseases such as Ewing sarcoma, for which no such model yet exists.

Developmental Considerations in Targeted Therapy for Children

Children may tolerate targeted therapies differently from adults secondary to age-specific differences in physiology that result in altered pharmacokinetics or to drug interference with critical developmental pathways. In normal development, vascularization of cartilage is prominent during embryogenesis and during periods of growth when neoangiogenesis occurs at growth plates.[241,242] Inhibition of VEGF signaling has been reported in animal models to result in thickening of the epiphyseal growth plates secondary to an expansion of the hypertrophic chondrocyte zone.[243,244] These findings have been attributed to a delayed vascular invasion of the epiphyseal growth plate with a subsequent reduced rate of chondrocyte apoptosis. Inhibition of VEGF signaling has also been reported to impair trabecular bone formation, induce dental dysplasia, and cause ovarian atrophy.[245] Although the animal data are concerning, the actual long-term effects of this class of drug on human children is unknown.

Ethical Considerations in Targeted Therapy for Children

The testing of drugs in children raises issues universal to cytotoxic and targeted therapies, as well as challenges more specific to targeted treatments. Adults can make informed decisions about the risk and potential benefit of experimental therapies and biological studies and actively participate in treatment decisions. Particularly for young children, however, true informed consent from the patients for experimental therapies cannot be obtained. Here it is the parent or legal guardian making decisions about participation in a trial. In light of this potential conflict of interest, special regulations have been instituted to afford additional protection to children. These include the Code of Federal Regulations, Part 46; Protection of Human Subjects, Subpart D; and the related FDA regulation, Part 50, Protection of Human Subjects, Subpart D. These regulations outline allowable research in children based on the risk to the child, potential benefit to the child or other children, and alternative therapies. In the testing of targeted therapies, pharmacodynamic studies with sampling of tumor tissue before and after therapy initiation become more critical. For obvious reasons these studies can be difficult to perform with the current regulations, particularly for solid tumors or brain tumors. In general, tissue collection in children for the purpose of biological studies must be considered no greater than a minor increase in minimal risk. Innovative methods are needed to evaluate pharmacodynamic response, such as new imaging technologies, and better methods are needed to assess surrogate tissues.

Emerging Genetic Targets in Pediatric Cancer

Significant progress has been made in our understanding of the pediatric leukemias and solid tumors. As such, numerous potential genetic targets have emerged over the past 10 years. In light of the excellent cure rates for some pediatric malignancies, it might be difficult to incorporate target-directed therapies associated with good outcome genetic lesions, such as *TEL-AML1* in pediatric ALL. In these early stages of targeted therapy for pediatric malignancies, the focus will likely be on lesions associated with poor outcome. In the following section we will discuss some of these genetic lesions with potential for therapeutic targeting.

FLT3 and the Acute Leukemias

Although much progress has been made in the treatment of childhood leukemias, patients with AML with mutations in the Fms-like tyrosine kinase 3 *(FLT3)* gene remain a high-risk group. FLT3 is a class III receptor tyrosine kinase that plays a critical role in normal hematopoiesis and shares structural homology with PDGFR, c-KIT, and CSF1R. Upon the binding of FLT3 ligand, wild-type receptors dimerize and become activated by phosphorylation, leading to activation of downstream signal transduction pathways. Mutations in *FLT3* can be identified in blasts from 30% of adult patients with AML with constitutive activation of the kinase resulting from internal tandem duplications (ITD) in the juxtamembrane domain or point mutations in the activation loop.[246] The *FLT3* ITD is present in 15% and *FLT3* point mutations in 7% of pediatric AML, and children with FLT3 ITD have a particularly poor prognosis.[247,248]

FLT3 has also been implicated as a target in pediatric patients with *MLL*-rearranged ALL. Gene-expression profiling studies comparing *MLL*-rearranged ALL with all other ALL and AML have demonstrated a unique expression profile for *MLL*-rearranged leukemia. *FLT3* is the gene most frequently overexpressed in *MLL*-rearranged leukemia compared with the other acute leukemias.[249,250] An initial study reported mutations in *FLT3* in approximately 15% of samples of *MLL*-rearranged ALL. All of these were point mutations in the activation loop, including mutations not previously reported in patients with AML (deletion of isoleucine 836). These mutations lead to constitutive activation of the kinase and increased sensitivity to small-molecule inhibitors of FLT3. Furthermore, *MLL*-rearranged lymphoblasts with high levels of wild-type FLT3 were sensitive to the FLT3 inhibitor PKC412.[251] Subsequent studies have reported an incidence of *FLT3* mutations in infant MLL samples ranging from 3% to 18%. These studies also confirmed increased expression of FLT3 in *MLL*-rearranged infant ALL compared with both infant and noninfant patients with germline *MLL*. Even in the absence of documented mutation, a high level of FLT3 expression was associated with FLT3 ligand independence and with increased sensitivity to the FLT3 inhibitor PKC412.[252,253] In addition to its role as a target in *MLL*-rearranged ALL, FLT3 is a potential target in children with T-cell ALL (T-ALL), particularly with early precursor T-ALL, and in children with hyperdiploid ALL, wherein *FLT3* mutations have been reported.[253-257]

Several FLT3 inhibitors have been tested in clinical trials for adults with AML.[258-263] Most of the early trials testing these compounds have had some transient responders with decreased peripheral blood or marrow blasts, and the compounds have generally been well tolerated. However, very few patients enter a complete remission, and the majority of patients eventually progress. The high rate of initial response failure, as well as the development of progressive disease despite an initial clinical response, has tempered enthusiasm for FLT3 inhibitors in AML.[264] There are several possible explanations for the lack of dramatic clinical responses, including issues related to pharmacology and the failure to achieve sustained, complete inhibition of FLT3.[265,266] Another explanation is the acquisition of resistance mutations in *FLT3* itself. Indeed, the newer drug quizartinib (AC220), with greater potency and sustained inhibition of FLT3, has had promising initial results in clinical trials.[267] Moreover, point mutations within the kinase domain of *FLT3*-ITD, conferring quizartinib resistance, were recently reported.[268] This finding is similar to the emergence of *BCR-ABL*–resistance mutations in patients with CML treated with imatinib. Significantly, this study provided gold-standard validation that FLT3-ITD is an oncogenic driver of AML.

The clinical study of FLT3 inhibitors in children with leukemia is ongoing. A phase I trial testing the multikinase inhibitor sorafenib, with activity against FLT3, in children with refractory solid tumors and leukemia, was reported by the Children's Oncology Group (COG).[269] This trial established a recommended phase II dose of sorafenib for children with leukemias. Rash, diarrhea, and liver enzyme elevation were the most common sorafenib-related toxicities. Two patients with AML with the FLT3-ITD achieved less than 5% bone marrow blasts with 4 or 5 cycles of sorafenib. A second phase I trial evaluated the effects of sorafenib in combination with clofarabine and cytarabine in children with relapsed or refractory leukemia.[270] On day 8 of this trial, 10 of 12 patients demonstrated a decrease in blast percentage after sorafenib alone. After combination chemotherapy, six patients (three of these with FLT3-ITD) achieved a complete remission, two with FLT3-ITD had a complete remission with incomplete count recovery, and one patient with FLT3 wild-type had a partial remission. This trial also demonstrated downregulation of AKT, RPS6, and 4E-BP1 phosphorylation in leukemic blasts. Finally, there was another report of three patients with relapsed FLT3-ITD AML who achieved a sustained remission with sorafenib in combination with other cytotoxic chemotherapy.[271] Based on these encouraging initial results, there are now multiple ongoing trials to test the efficacy of FLT3 inhibitors in pediatric patients with leukemia.

JAK Mutations in Pediatric Leukemia

With the marked success of imatinib in the treatment of *BCR-ABL*-rearranged ALL in children (Fig. 44-5), there has been interest in the identification of other kinase abnormalities in childhood cancers, including leukemia.[233] Recurrent mutations in Janus kinases (JAKs) have

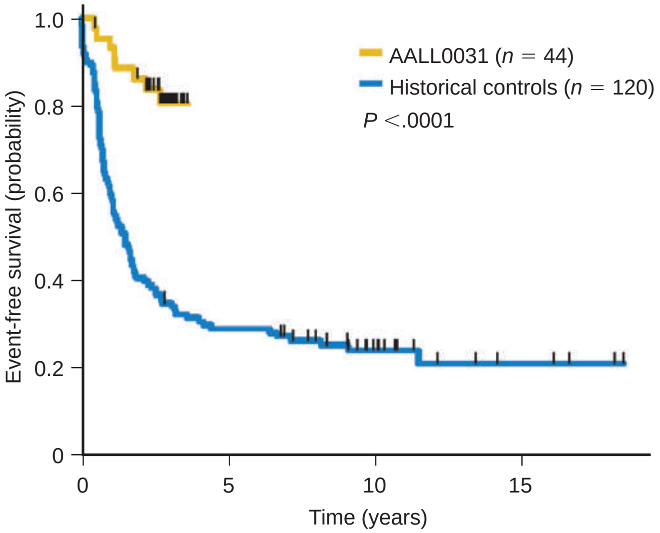

Figure 44-5 Early event-free survival in patients with Philadelphia chromosome–positive acute lymphoblastic leukemia treated with imatinib. Treated patients in cohort 5 ($n = 44$) were compared with patients previously treated on Pediatric Oncology Group protocols AL inC 14, 15, and 16 from January 1986 through November 1999 (n = 120). *(Modified from Schultz, et al: Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study.* J Clin Oncol *27:5175–5181, 2009.)*

emerged as immediately targetable in a subset of pediatric patients with leukemia. JAKs phosphorylate substrates, including STAT proteins, on ligand binding to a type I cytokine receptor, leading to altered transcription of growth-enhancing and anti-apoptotic genes. Interest in targeting JAK2 was sparked by the finding of recurrent *JAK2* mutations (most frequently involving valine 617) in patients with myeloproliferative disorders.[81-83,272] Subsequently, activating mutations in *JAK2* and *JAK3* were reported in patients with AML and Down syndrome–associated acute megakaryoblastic leukemia[273,274] and in 18% to 28% of patients with Down syndrome–associated ALL.[275-278] In the case of Down syndrome–associated ALL, the mutations affect a highly conserved arginine residue (R683) in the pseudokinase domain of JAK2 and have been demonstrated to induce JAK/STAT activation and promote growth factor independence in BaF3 cells and response to JAK inhibitors.

In a subgroup of pediatric patients with poor-outcome ALL, a gene expression signature similar to that observed in patients with *BCR-ABL*–rearranged ALL was identified by genome-wide expression profiling in patients lacking the *BCR-ABL* rearrangement.[279] It was hypothesized that these *BCR-ABL*–negative cases might harbor other kinase abnormalities. Indeed, recurrent mutations in *JAK1*, *JAK2*, and *JAK3* were identified in approximately 11% of patients with high-risk B-precursor ALL (B-ALL), with mutations in *JAK2* being the most common.[280,281] As was seen in Down syndrome, the mutations in *JAK2* frequently involved the R683 residue. Mutations in *JAK2* are also frequently associated with overexpression of CRLF2 (cytokine receptor–like factor 2), a type I cytokine receptor subunit also referred to as *thymic stromal lymphopoietic receptor*.[282,283] Overexpression of CRLF2 has been linked to genomic rearrangement of *CRLF2* by way of a translocation of the immunoglobulin heavy chain (*IGH)* gene and by way of an interstitial deletion centromeric of *CRLF2* that results in a P2RY8-CRLF2 fusion.

There has been some controversy regarding the prognostic significance of *CRLF2* overexpression/genomic lesions and *JAK* mutations. In a study of 1061 pediatric patients with B-precursor ALL (both high risk and standard risk) treated on COG trials, it was found that only 50% of patients with high CRLF2 expression had *CRLF2* genomic lesions; the reason for high CRLF2 in the other 50% remains unclear. All *CRLF2* genomic lesions and nearly all *JAK* mutations were observed in samples with high CRLF2 expression. In this study high CRLF2 expression was associated with a very poor outcome in the high-risk patients with ALL but not in the standard-risk patients.[284] Thus targeting of JAKs, particularly JAK2, is of interest given its association with CRLF2 overexpression and poor outcome and the preclinical data supporting activity of JAK inhibitors in *JAK*-mutated ALL.[285] Clinical trials testing JAK inhibitors in pediatric patients are now ongoing.

ALK in Anaplastic Large Cell Lymphoma and Neuroblastoma

The discovery of the recurrent translocation t(2;5)(p23;q35) in anaplastic large cell lymphoma led to the cloning of *ALK*, a novel tyrosine kinase, as the key oncogene dysregulated by the gene fusion. Subsequently, *ALK* was found to be the transforming partner fused to the *EML4* promoter in approximately 4% of patients with adenocarcinoma of the lung[189] and to be activated by point mutations in neuroblastoma[286] and in inflammatory myoblastic tumors.[287,288] These data, in particular the findings in lung adenocarcinoma, triggered attempts to exploit this genetic information for therapeutic gain. Cells driven by the EML4-ALK fusion were shown to be sensitive to ALK inhibition in the original work describing the fusion event,[189] and it was known that crizotinib, originally developed as a MNNG HOS transforming gene (MET) inhibitor, was also a potent ALK inhibitor. Indeed, crizotinib showed significant preclinical efficacy in models of anaplastic lymphoma.[289] These data led to the rapid clinical testing of crizotinib in patients with lung adenocarcinoma tumors bearing ALK fusions. In the phase I testing of crizotinib, there was an overall response rate of 57%, including one complete response, and an additional 33% of patients achieved disease stabilization.[190] These data led to a randomized phase III trial comparing crizotinib with chemotherapy in patients with tumors harboring *ALK* translocations. In this trial the response rate to crizotinib was 65% compared with 20% in the chemotherapy arm (P >.001) and progression-free survival was 7.7 months in the crizotinib-treated patients compared with 3 months in the chemotherapy group.[290] Crizotinib received FDA market approval for the treatment of ALK-positive lung adenocarcinoma in August of 2011.

Crizotinib has recently been tested in pediatric patients with solid tumors enriched for those with ALK alterations.[291] The investigators enrolled 79 patients with a diversity of tumor types in children with an average age of 10.1 years. Crizotinib was well tolerated in children. Efficacy was marked in patients with tumor types known to harbor genetic alterations in *ALK*. In nine patients with anaplastic lymphoma, there were eight responders, including seven who responded completely and one whose disease remained stable. In addition, three of seven patients with inflammatory myofibroblastic tumors also responded to crizotinib, and the remainder all had stable disease. One patient (out of 11) with neuroblastoma also had a response. In summary, crizotinib is a promising agent in pediatric patients with these diseases.

In adenocarcinoma of the lung, treatment with crizotinib, although achieving marked efficacy, is eventually associated with the development of therapeutic resistance. In some cases this is accompanied by point mutations in the kinase domain creating specific resistance to the binding of the drug. Significantly, the most common de novo mutation in *ALK* observed in children with neuroblastoma is the *ALK* F1174L, a mutation reported to engender crizotinib resistance. Novel ALK inhibitors are now entering clinical trials in the hopes of both overcoming resistance and improving on the upfront therapy of tumors bearing genetic alterations in *ALK*. NVP-LDK378 is a selective ALK inhibitor that is more potent than crizotinib and has activity against some of the resistance mutations seen with crizotinib.[292] In a phase I trial of NVP-LDK378 in adults, both crizotinib-naïve and crizotinib-refractory patients were enrolled on ascending doses. Among 88 patients receiving doses greater than 400 mg daily, the overall response rate was 70% with a response rate of 73% in the crizotinib-refractory setting. NVP-LDK378 was well tolerated in this trial. On the basis of these data, the FDA granted Breakthrough designation to LDK387 in March 2013. Early clinical data for AP26113, a second-generation ALK inhibitor that also inhibits EGFR, have been reported. Here, among 18 evaluable ALK-positive patients there were 10 responses. In the 12 patients previously treated with an ALK inhibitor, eight patients responded (67%).[293] Finally, the ALK inhibitor CH5424802 showed a remarkable 93.5% response rate in Japanese patients with ALK-positive lung cancer not previously treated with crizotinib.[294] The evolution of this field has been rapid and promises to bring highly active ALK inhibitors to pediatric patients with *ALK*-mutant cancers, including anaplastic lymphoma, neuroblastoma, and inflammatory myoblastic tumors.

Mammalian Target of Rapamycin in Tuberous Sclerosis

Tuberous sclerosis is a rare disorder characterized by skin and mucosal manifestations, including angiofibromas and subungual fibromas and by central nervous system manifestations, including cortical tubers and a high incidence of seizures. Two notable tumors in this disorder are renal angiomyolipomas and subependymal giant cell astrocytoma (SEGA). Tuberous sclerosis arises from inactivating germline mutations in the tumor-suppressor genes *TSC1* or *TSC2*. The protein products of these genes, known as hamartin and tuberin, are negative regulators of the kinase known as mTOR, a critical regulator of cell growth and cell size. These cellular control mechanisms are primarily linked to the role of this protein complex in controlling protein translation. The regulatory interaction between the TSC genes and the mTOR pathway was first discovered in *Drosophila* organisms, wherein genetic interaction studies revealed that cells lacking tuberous sclerosis function were dependent on mTOR activity and the activity of the downstream kinase S6K for their aberrant growth characteristics. These studies suggested that rapamycin and other inhibitors of mTOR might have therapeutic benefit in patients with tuberous sclerosis.[295,296]

Rapamycin (sirolimus) is a natural product that acts as an allosteric inhibitor of mTOR. It does so by binding to FKBP12 and recruiting FKBP12 to the mTOR complex, creating an inactivated complex. Sirolimus was tested in a small study of patients with renal angiomyolipomas and pulmonary lymphangioleiomyomatosis, a disorder also caused by mutations in *TSC* genes. In this study five patients with renal angiomyolipomas had a tumor response over a 24-month period.[297] Everolimus is a derivative of rapamycin that has improved oral availability and demonstrated efficacy and market approval in a number of adult malignancies, including renal cell carcinoma, neuroendocrine tumors, and breast cancer. Everolimus has been tested in patients with tuberous sclerosis who have either angiomyolipomas or SEGA tumors. In a phase III randomized trial 35% of patients with SEGA (EXIST-1) responded to everolimus compared with 0% in the placebo control arm. Significantly, no patients in the treated arm progressed during the trial.[298] Similarly, in a phase III trial (EXIST-2) patients with angiomyolipoma had a response rate of 42% in the everolimus-treated arm compared with 0% in the placebo control arm.[299] On the basis of these data, everolimus was approved for use in these indications.

In earlier uncontrolled studies there was evidence of a reduction in seizure frequency that appeared independent of the effect on SEGA tumor volume reduction. This raised the possibility that ongoing dysregulation of mTOR signaling in the central nervous system could contribute directly to seizure frequency. To further understand these findings, a phase II study examining seizure frequency was conducted. Here, everolimus reduced the seizure frequency by 50% or more in 12 of 20 patients, and in total 17 of the 20 patients experienced seizure reduction with an overall median decrease of 73% ($P < .001$).[300] Together these data support the notion that everolimus is a highly effective agent in the treatment of SEGA and angiomyolipomas in children with tuberous sclerosis disease. In addition, everolimus might be effective in seizure control, although additional randomized data will be required.

RAS Pathway Activation and Inhibition in Childhood Cancers

Genetic alterations leading to activation of the RAS-MAPK pathway are frequent in pediatric cancers and precancer syndromes. Germline mutations in RAS pathway components, including *NF1, KRAS, NRAS, SHP2 (PTPN11), MEK2, SHOC2,* and *CBL,* result in a set of related disorders known collectively as “RASopathies.” These disorders include neurofibromatosis, Noonan syndrome, LEOPARD syndrome, Costello syndrome, cardiofaciocutaneous syndrome, and Legius syndrome.[301] Moreover, somatic activating mutations in *KRAS* and *NRAS* are also common in a diversity of pediatric tumors. Juvenile myelomonocytic leukemia (JMML), in both syndromic and nonsyndromic forms, appears to be a disease wherein RAS is activated in nearly all settings. In this disorder loss-of-function mutations in *NF1,* gain-of-function mutations in *SHP2,* and activating mutations in *RAS* genes all lead to constitutive RAS pathway activation.[302] In B-ALL, mutations in *NRAS* and *KRAS* are among the most common mutations found.[281,303] Similarly, *NRAS, KRAS, BRAF, SHP2,* and *NF1* are, in aggregate, frequently mutated in pediatric T-ALL.[257] In pediatric AML activating mutations in *NRAS* are seen in 10% of patients.[304]

Outside of the hematologic malignancies, recent discoveries highlight the role of alterations in BRAF, a key immediate downstream mediator of RAS signaling. In pilocytic astrocytoma duplications of the *BRAF* locus occurred in 28 of 53 patients (53%).[62] This genomic rearrangement results in a *KIAA1549-BRAF* fusion gene and constitutive activation of BRAF. Another surprising discovery was the finding of the oncogenic mutation *BRAFV600E,* most commonly seen in melanoma, in 57% of patients with Langerhans cell histiocytocis[63] and 50% of patients with the related disorder Erdheim-Chester disease.[305] In aggregate, both germline and somatic genetic alterations in RAS pathway components frequently occur in pediatric cancers, and these observations strongly support the rationale for testing RAS pathway inhibitors in these pediatric indications.

Emerging data from the targeted treatment of adult patients with melanoma suggest that we divide the therapeutic approach to the RAS pathway into two distinct categories: *BRAF* mutant tumors and those tumors with direct *RAS* mutation or with upstream genetic events (e.g., *NF1*) that lead to RAS activation. BRAF inhibitors have had dramatic efficacy in patients with melanoma bearing the V600E *BRAF* mutation.[306,307] These data have led to the market approval of vemurafenib and dabrafenib. In the NRAS or KRAS setting, however, BRAF inhibitors are not effective. In addition, the current generation of BRAF inhibitors all share the property of *activating* rather than inhibiting other RAF family members (CRAF or ARAF), especially in the setting of *KRAS* mutation. Hence although in the setting of *BRAF* mutation these drugs indeed turn off pathway signaling and lead to tumor regression, in settings of the wild-type RAS pathway or in the setting of *KRAS* mutation the drugs lead to pathway activation. Indeed, this so-called paradoxical activation results in a plethora of vemurafinib-mediated dermatologic side effects, including the induction of keratoacanthomas. In addition, at least one patient was found to have a *KRAS* mutant leukemia while on therapy for metastatic melanoma. Withdrawal of the vemurafenib led to partial regression of the leukemia,[308] suggesting that the drug was inducing tumor growth. These findings imply that the use of BRAF inhibitors is likely to be restricted to indications in which *BRAF* itself is mutated.

Studies attempting to elucidate the mechanisms of resistance to BRAF inhibition, both preclinical and clinical, have identified a number of alterations leading to therapeutic resistance primarily linked to activation of downstream signaling by MEK or ERK. These observations have led to combination clinical trials in which MEK inhibition has been combined with BRAF inhibition. Although many such trials are under way, the most advanced is the trial of dabrafenib and the MEK inhibitor trametinib. A phase II randomized study comparing this combination with dabrafenib alone found that median progression-free survival rate was increased from 5.8 months to 9.4 months. In addition, the response rate increased from 54% to 76%. Notably, the incidence of skin manifestations caused by "paradoxical activation" was significantly reduced, and hence the combination was well-tolerated.[309] These data suggest that the BRAF/MEK inhibitor combination will become a standard of care for *BRAF*-mutant melanoma.

Do these findings apply to pediatric patients? At the time of this review, phase I trials of dabrafenib and vemurafenib were ongoing in pediatric patients. Although results in pilocytic astrocytoma were not yet reported, Haroche and coworkers reported treating three patients with Erdheim-Chester disease with vemurafenib. For all three patients, treatment led to substantial and rapid clinical improvement, with tumor responses confirmed by imaging studies.[310] These exciting results support the notion that BRAF inhibition will find significant clinical utility in these disorders.

Therapeutic intervention in tumors driven by RAS or upstream activation event has proved more challenging. It has not been possible to develop direct inhibitors of RAS. Therefore current efforts are focused on interrupting the outputs of oncogenic RAS, including downstream effectors in the MAP kinase pathway, such as MEK, or downstream effectors in the PI3K pathway, such as PI3K or mTOR. In adult *NRAS*-mutant melanoma there is evidence for activity of MEK inhibitors, notably MEK162 which had a 20% response rate among *NRAS*-mutant patients in a phase II trial in melanoma.[311] In pediatric patients MEK inhibitor phase I trials are under way primarily in glioma, but on the basis of preclinical data there is also strong support for the clinical testing of MEK inhibitors in JMML.[312] As mentioned previously, mutations in the *NF1* gene result in activation of RAS. To date, most therapeutic interventions have focused on inhibiting mTOR downstream of RAS in patients with type 1 neurofibromatosis. Although clinical trials are progressing, no results have been reported. As in the case of *BRAF*-mutant tumors, it is likely that combination therapy will be required. In this case there is a rationale for testing the combinations of mTOR or PI3K pathway inhibitors and MEK inhibitors in pediatric disorders resulting from RAS activation.

Notch1 in T-ALL

With intensive chemotherapy the majority of children with T-ALL will be cured. However, ALL is still a leading cause of death from cancer in children, and intensive chemotherapy is not without toxicity. One important

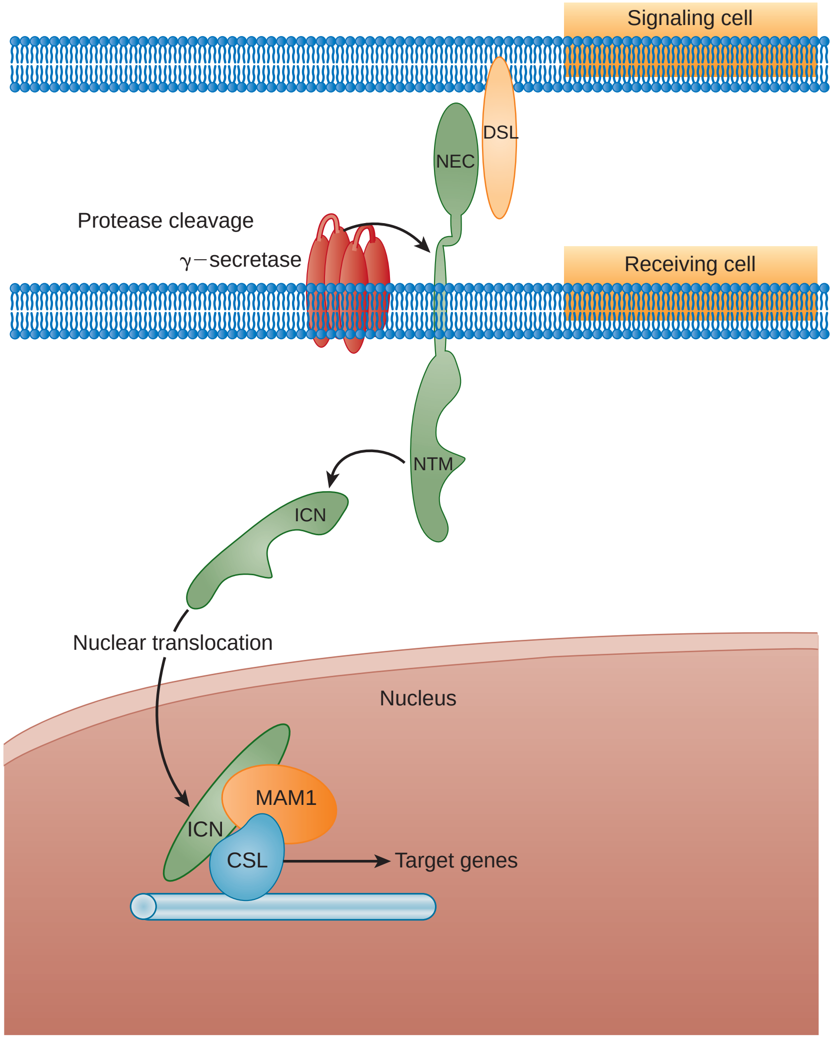

Figure 44-6 Notch1 signaling. Notch is activated by the binding of the ligands of the Delta-Serrate-Lag2 (DSL) family, which trigger a series of proteolytic cleavages. Notch receptors are cleaved by ADAM metalloproteases producing two noncovalently associated subunits, NEC and NTM. Next, cleavage by γ-secretase releases the intracellular domain of Notch (ICN), which translocates to the nucleus and forms a transcriptional activation complex with CSL and Mastermind-like (MAM1) cofactors. *CSL,* CBF1, Suppressor of Hairless, Lag-1; *NEC,* notch extracellular subunit; *NTM,* notch transmembrane subunit.

target in T-ALL is Notch1. *NOTCH1* encodes a transmembrane receptor required for T-cell differentiation and for the assembly of pre–T-cell receptor complexes in immature thymocytes (Fig. 44-6). Notch is activated by the binding of ligands of the Delta-Serrate-Lag2 (DSL) family that triggers a series of proteolytic cleavages, with the last step catalyzed by the γ-secretase complex, releasing the intracellular domain of Notch (ICN) into the cytoplasm, for subsequent translocation to the nucleus. Once in the nucleus, Notch acts as a transcriptional activator complexed with the DNA-binding protein CSL (CBF1, Suppressor of Hairless, Lag-1) and the transcriptional co-activators of the Mastermind-like family (MAML1).[313,314]

The oncogenic role of *NOTCH1* was first identified through its involvement in a rare (7;9) translocation in T-ALL.[315] Later, mutations in *NOTCH1* were discovered in 50% to 60% of T-ALL samples,[316,317] and *NOTCH1* mutations have also been reported in numerous murine models of T-ALL.[318-320] Enforced Notch signaling also induces T-ALL in mice.[321] In humans these mutations are activating mutations that involve the heterodimerization domain or the PEST domain of *NOTCH1* (or both), leading to constitutive activation or increased stability of the protein. Inhibition of γ-secretase is one possible approach to inhibiting Notch signaling. Initially, γ-secretase inhibitors (GSIs) were developed because of their potential therapeutic role in Alzheimer disease in cleaving amyloid precursor protein.[322] However, they were tested in Notch-dependent cancers in an attempt to block the production of the processed, nuclear form of Notch.[323] Many human T-ALL cell lines with Notch mutation showed a G0/G1 cell-cycle arrest in response to a GSI.[316] A Notch pathway inhibitor clinical trial with the GSI MK-0752 was opened in 2005 for patients with refractory T-ALL.[324] The drug was tolerated in a limited number of patients at doses below 300 mg/m^2. However, further dose escalation by continuous infusion was discontinued secondary to diarrhea. This gastrointestinal toxicity is thought to result from blockade of Notch1 and Notch2 in the gut, leading to intestinal secretory metaplasia, an increased number of goblet cells, and arrested proliferation in the crypts of the small intestine.[325] Because of the limited antileukemic activity in this trial and the severe gastrointestinal toxicity, studies were conducted to identify combination therapies with gamma secretase inhibitors (GSIs). One promising combination identified is GSI with glucocorticoids (GCs). This combination has been demonstrated to alleviate the gut toxicity by induction of cyclin D2 (CCND2), a cyclin associated with cell-cycle progression, and by the downregulation of Krüppel-like factor 4 (KLF4), a negative regulator of the cell cycle that is required for goblet cell differentiation.[326] It also potentiates the antileukemia effect of either drug given alone.[327,328] Alteration in the schedule of GSI delivery has also been evaluated, and a 3 day on, 4 day off dosing schedule was better tolerated.[329] Other approaches to Notch inhibition are being explored, including antibody-based targeting of the Notch1 receptor,[330,331] targeting of Notch ligands,[332] direct inhibition of the Notch1 complex,[333] and selective targeting of the maturation of the mutant receptor.[334]

Although the genetic and functional data to support targeting Notch1 in hematologic malignancy are compelling, and multiple approaches to targeting Notch may be feasible, there are still numerous challenges that lie ahead. One difficulty is the on-target side effects of inhibiting Notch, as discussed previously. Another concerning toxicity, reported in a recent trial performed in patients with Alzheimer disease, is the development of secondary malignancies because Notch pathway loss-of-function mutations have been reported in squamous cell carcinomas of the head and neck,[335,336] skin, and lung.[337] Here the hope is that intermittent dosing of the Notch inhibitor will mitigate this risk of secondary malignancy. A second concern in targeting Notch1 has been the largely cytostatic effect of Notch1 inhibition with GSIs and antibody-based therapy in preclinical testing. As in the case of cytotoxic therapy for cancer, targeted therapies will likely need to be used in combination with other drugs to achieve maximal clinical efficacy. In addition to the combination of GSI with GCs discussed previously, several studies support the combination of Notch1 inhibitors with inhibitors of the PI3K-AKT-mTOR pathway for T-ALL.[338,339] Notch pathway inhibitors have yet to be brought to pediatric testing.

Sonic Hedgehog Signaling and Medulloblastoma

Medulloblastoma is the most common malignant pediatric brain tumor. The sonic hedgehog signaling pathway, implicated in the pathogenesis of this disease, is a potential pathway for targeted therapy. Aberrant sonic hedgehog signaling was first implicated in the pathogenesis of medulloblastoma with the discovery of germline mutations in the sonic hedgehog (Shh) receptor gene *PTCH1* in patients with Gorlin syndrome (basal cell nevus syndrome) who have an underlying predisposition to develop medulloblastoma.[68,69] Heterozygous loss of *PTCH1* occurs in approximately 10% of sporadic medulloblastoma. Mutations in the downstream members of the hedgehog pathway, *Smo*, and *suppressor of fused (SUFU)*, have also been reported.[340-345] Furthermore, the hedgehog pathway is activated in approximately 30% of human medulloblastoma based on gene expression studies.[346]

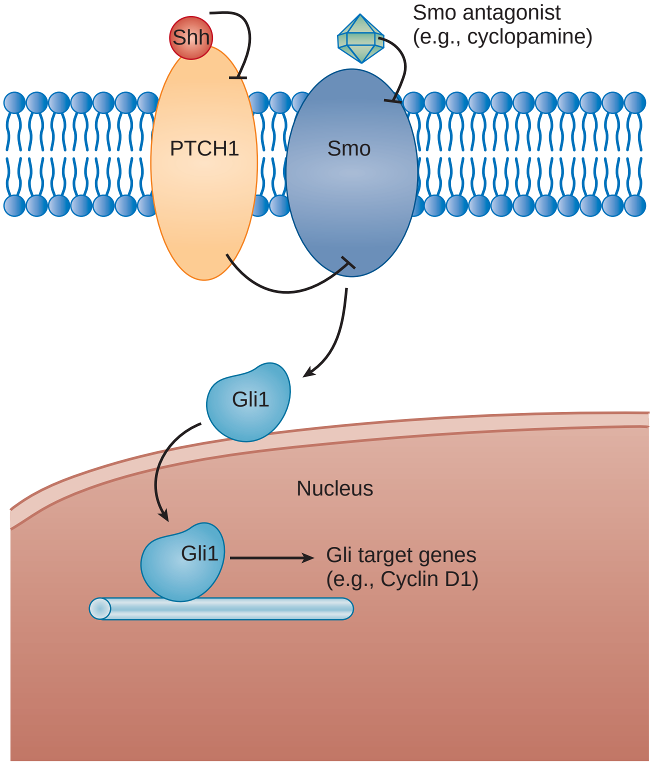

Figure 44-7 Hedgehog signaling. Patched (Ptch1) is a transmembrane receptor for the ligand sonic hedgehog (Shh). In the absence of Shh, Ptch1 inhibits Smo, a transmembrane protein with homology to G protein–coupled receptors. Upon Shh binding to Smo, this repression is relieved and signal is transduced through Smo to the nucleus, upregulating Gli1 transcription factors, which turn on a host of other transcriptional targets, including cyclin D1. *(Courtesy of Curtis Glavin and William Sellers.)*

PTCH1 is a transmembrane receptor for the ligand Shh (Fig. 44-7). In the absence of Shh, PTCH1 inhibits Smo, a transmembrane protein with homology to G-protein–coupled receptors. Upon Shh (ligand) binding to PTCH1, the repression is relieved and signal is transduced through Smo to the nucleus. This activity upregulates the Gli1 transcription factors, which in turn induces a set of transcriptional targets, including cyclin D1, PTCH1, and Gli1 themselves. SUFU appears to have a role in the repression of hedgehog signaling by interacting with GLI proteins in the cytoplasm and the nucleus.[347-349] The hedgehog pathway is critical for the development of the cerebellum and regulates the proliferation of granule neuron precursor cells in the cerebellum. Medulloblastoma is thought to originate from these immature granule cells.[343]

The goal of identifying inhibitors of the pathway began with the identification of cyclopamine, a plant-derived alkaloid from *Veratrum calcifornicum* initially described because of its causal role in the development of holoprosencephaly in livestock ingesting the plant.[350,351] Cyclopamine inhibits hedgehog signaling by binding to and inhibiting Smo, and this observation led to the therapeutic hypothesis that inhibition of Smo would reverse the transformed phenotype of Ptch-deficient medulloblastoma cells.[72] Indeed, cyclopamine inhibits the growth of medulloblastoma in vitro and in xenograft models of medulloblastoma.[72,352,353] Genetically engineered mouse models of medulloblastoma have been created with mice heterozygous for the *PCTH1* gene and null for *p53*. These animals develop medulloblastoma as early as week 2 and die from the disease by week 16.[354] In this animal model of medulloblastoma, a benzimidazole derivative, Hh-Antag691, with hedgehog-inhibitory activity via binding to Smo, led to a dose-dependent downregulation of hedgehog pathway members, decreased tumor burden, and increased disease-free survival rates in treated mice.[355]

These data led to the development and early clinical testing of Smo inhibitors in patients with basal cell carcinoma and medulloblastoma. Among the Smo inhibitors that have been in clinical testing are vismodegib (GDC-0449), sonidegib (NVP-LDE225), BMS-833923, PF04449913, LY2940680, saridegib (IPI-962) and TAK-441. Clinical trials of the latter two molecules have been discontinued. Vismodegib is the first Smo inhibitor and is currently approved in basal cell carcinoma on the basis of the tumor response rates in metastatic and locally advanced basal cell carcinoma (30% and 43%, respectively).[356,357] In the first report of its activity in medulloblastoma, a heavily pretreated patient with medulloblastoma had short-term tumor regression followed by progression at 3 months.[74] Analysis of this patient's tumor revealed an acquired point mutation in the Smo receptor likely mediating therapeutic resistance.[358] These data in aggregate suggested that medulloblastomas bearing a mutationally activated hedgehog pathway would be responsive to Smo inhibitors while also pointing out the potential problems of emergent resistance. Phase I testing of vismodegib led to a recommended phase II dose from this trial of 150 mg by mouth based on a maximal plasma exposure and pharmacodynamics reponse.[359] The activity of vismodegib in medulloblastoma is being evaluated in an ongoing phase II trial. Early results from the first 32 patients with refractory or relapsed medulloblastoma treated daily with 150 mg of vismodegib have been presented in preliminary form. Among 20 patients with sonic hedgehog–positive tumors, there were three with sustained response compared with no response among the patients with either no evidence of Shh activity or indeterminate tumors, and six patients remained on trial for longer than 6 months.[360]

Sonidegib (NVP-LDE225) is a potent Smo inhibitor with reasonable blood-brain barrier penetration as a feature of the molecule.[292] In phase I testing sonidegib was escalated from 100 to 3000 mg daily and also tried at 400 and 750 mg twice daily. Sonidegib was tolerated with doses as high as 800 mg daily. The MTD was defined by exposure-related creatine kinase elevations thought to be related to target-mediated effects on muscle. Gli1 mRNA, a marker of hedgehog pathway activity, was reduced by as much as 95% and 99% in skin and tumor biopsies. Preliminary evidence of activity was noted with one partial remission and one metabolic partial remission in medulloblastoma and one complete remission and four partial remissions in advanced basal cell carcinoma. Based on these data a phase II trial is being conducted in pediatric and adult patients with medulloblastoma in which post hoc evaluation of hedgehog activity is being conducted by using a five-gene hedgehog pathway mRNA signature. Preliminary results have been presented from 37 patients with medulloblastoma treated with sonidegib. In this cohort six patients responded (two complete remissions and four partial remissions) to sonidegib, and all responders were predicted to have hedgehog pathway–activated tumors. Of the remaining 31 patients, 29 were predicted to have hedgehog-inactive tumors and two were predictive to be hedgehog active. These latter two patients had stable disease. Based on the high concordance between the five-gene signature and patient response, this signature is now being used for patient selection to a randomized phase III trial comparing sonidegib with temozolamide in refractory or relapsed adult and pediatric medulloblastoma.[361] Taken together the aforementioned clinical data for vismodegib and sonidegib strongly support the notion that such inhibitors have significant clinical activity in hedgehog-activated medulloblastoma.

The hedgehog pathway is also thought to play a role in the maintenance of cancer stem cells. In particular, the preclinical data suggests that leukemia stem cells may be particularly dependent on hedgehog-pathway signaling.[362-364] To test this hypothesis the Smo inhibitor PF-0449913 has been tested clinically and found to have preliminary evidence of efficacy in AML; a complete remission with incomplete blood count recovery was noted in one patient, and five patients with AML had 50% or greater reduction in bone marrow blast counts. In addition, a major cytogenetic response with loss of the *T315I* mutation was observed in one patient with CML. These data suggest the Smo inhibitors may also find a role outside of tumor types driven by genetic mutations in the hedgehog pathway.

Transcription Factors and Pediatric Cancer

Abnormalities of transcription factors are a common genetic theme in the development of pediatric solid tumors, as well as leukemia. Often, the transcription factor is involved in a translocation rendering a new protein with aberrant transcriptional activity. Some examples of common transcription factor rearrangements are as follows: Ewing sarcoma *(EWS-FLI*, *EWS-ERG)*, alveolar rhabdomyosarcoma *(PAX3-FKHR, PAX7-FKHR)*, infantile fibrosarcoma *(TEL-NTRK3)*, ALL *(TEL-AML1, MLL* rearrangements, *E2A-PBX)*, and AML *(PML-RARα, AML1-ETO, CBFβ-MYH11, MLL* rearrangements). A second mechanism of transcription factor aberrancy is upregulation by amplification, such as amplification of *MYCN* in neuroblastoma and medulloblastoma. The high frequency of these abnormalities and the tumor cell specificity make these genetic lesions attractive targets. However, transcription factors have been considered "undruggable." Unlike enzymatic targets, such as kinases, transcription factors are not easily amenable to high-throughput screening assays. With the exception of the treatment of *PML-RARα*–rearranged APL, no current therapy directly targets these transcription factor abnormalities. Alternative approaches to small molecule discovery are needed. Technologies, such as RNAi, may facilitate therapeutic targeting of oncogenic transcription factors as better delivery methods are developed. Furthermore, these evolving technologies have enabled new methods for drug screening.

One approach to address the challenges to transcription-factor targeting exploits gene expression signatures. With this method gene expression signatures, defined by using genome-wide expression profiling, are used to identify different biological states. Then a small molecule library is screened with a low-cost, higher-throughput assay to identify compounds that induce the change in gene expression signature from "state A" to "state B." The gene expression signature definition, amplification, and detection are generic. Furthermore, a priori knowledge of a target is not needed. The gene expression signature serves as a surrogate for the biological state in question. The method is thus well suited for discovery of modulators of oncogenic transcription factors in which the mutant transcription factor's transforming mechanism is not known. One method developed, gene expression–based high-throughput screening (GE-HTS), used Affymetrix microarrays to define expression signatures of the two biological states of interest. From these high-complexity arrays, as many as 100 genes are selected as marker genes for each state. These signature genes are then amplified and detected with a high-throughput assay.[365,366] This approach was successfully used in a proof-of-concept experiment to identify inducers of AML differentiation. It was then extended to the modulation of transcription factors in an effort to identify small-molecule modulators of EWS-FLI in Ewing sarcoma, AML1-ETO in AML, and Notch1 in T-ALL.[334,367,368] GE-HTS provides an avenue for identifying tool compounds for the study of these genetic lesions and for identifying therapeutic leads. In fact, there are industry-based efforts incorporating gene expression fingerprints for drug discovery.

A second signature-based approach to address this problem uses a reference collection of gene expression profiles from cells treated with bioactive small molecules. This approach was initially demonstrated to be feasible in yeast with a pivotal study confirming that gene expression data could be used to functionally annotate small molecules and genes.[369] A pilot study next demonstrated the feasibility of this approach in human cell lines with the expression profiling of 164 distinct small molecule perturbagens, including many FDA-approved drugs and known bioactive compounds. Here the goal was to create an in silico tool (the Connectivity Map, or C-Map) for discovering the relationships among diseases, physiologic processes, and small molecule interventions.[370,371] One example pertinent to pediatric leukemia identified rapamycin as a small molecule candidate to reverse dexamethasone resistance in ALL.[372] In this example the C-Map was screened for molecules whose profile overlapped with a gene expression signature of GC sensitivity or resistance in ALL cells. The screen indicated that the mTOR inhibitor rapamycin profile matched the signature of GC sensitivity. Rapamycin was confirmed to sensitize ALL to GC-induced apoptosis, suggesting that the combination of rapamycin and GCs has potential utility in lymphoid malignancies. A clinical trial testing this hypothesis for pediatric patients with relapsed ALL is ongoing.

One approach to target the interface between proteins involved in transcriptional regulation uses peptides. One example is the use of specific peptide interference with BCL6 (B-cell lymphoma 6) transcriptional mechanisms in B-cell lymphoma.[373] Regulatory elements of the *BCL6* gene are frequently mutated in human diffuse large B-cell lymphoma, leading to sustained expression of BCL6.[374,375] This is thought to enhance B-cell survival, proliferation, and block differentiation. BCL6 possesses an N-terminal BTB/POZ (Bric-a-brac, Tramtrack, Broad complex/Poxvirus zinc finger) domain, and the BTB domain recruits the corepressors SMRT (silencing mediator of retinoid and thyroid receptors), NCoR (nuclear receptor corepressor) and BCoR (BCL6 corepressor).[376] The interface through which BCL6 recruits corepressor proteins was characterized with x-ray crystallography, and based on this structure a peptide inhibitor blocking the interaction of these proteins was designed.[377] This peptide disrupted BCL6-mediated repression and reactivated the expression of BCL6 target genes. Furthermore, in B-cell lymphoma cells positive for BCL6, the peptide caused apoptosis and cell cycle arrest. With improved methods of delivering peptides to cells, peptide therapy has become more feasible. One method fuses the peptide with protein transduction domains, which mediate receptor-independent cell penetration by binding the peptide to lipid rafts and triggering macropinocytosis.[378]

A second potential peptide-based approach to achieving this goal might use a reported stapled peptide technology. This approach has been applied to the activation of apoptosis by using a hydrocarbon-stapled BH3 peptide.[379,380] In contrast to the use of helical peptides to interfere with protein-protein interactions, which has been limited because of the loss of secondary structure in solution, susceptibility to degradation, and poor penetration of intact cells, the hydrocoarbon-stapled peptides proved to be helical, protease-resistant, and cell-permeable molecules that bound with increased affinity to target pockets. Hydrocarbon stapling of native peptides may provide a useful strategy for experimental and therapeutic modulation of protein-protein interactions, especially in disrupting transcription factor homodimers and heterodimers requiring extended helical interaction surfaces, as is the case for the MYC family.

Finally, multiple recent studies have used high-throughput in vitro screening of cancer cell lines to define genetic-, lineage-, and gene expression–based predictors of drug response. These studies focused primarily on adult cancers and involved the testing of a limited number of small molecules. Nevertheless, two of the most remarkable insights to emerge from those studies (discussed in greater detail later) were the discovery of marked sensitivity of Ewing sarcoma cell lines with EWS-ETS rearrangements to PARP inhibitors and the marked sensitivity of

MYCN-amplified neuroblastoma cell lines to BET bromodomain inhibitors.[381] These discoveries illustrate the potential power of comprehensive, pediatric cancer–focused screening to identify either compounds that directly interfere with aberrant transcription factors or synthetic lethal relationships in the presence of a transcription factor abnormality. Moreover, the potential of small molecule inhibitors of epigenetic regulators to alter aberrant transcriptional programs is revealed in the preclinical efficacy of BET bromodomain inhibitors in *MYCN*-amplified neuroblastoma tumors.

Emerging Lineage-Dependent Targets in Pediatric Cancer

Anti-GD2 and Neuroblastoma

Cure rates for children with high-stage neuroblastoma have improved with dose intensity of chemotherapy. However, even with double autologous stem cell transplantation, most children with stage 4 disease will relapse. For long-term survivors, therapy-related morbidity is high. Alternative approaches to cytotoxic therapy are clearly needed. One approach to targeted therapy for this disease has exploited the high-density expression of the neuronal lineage marker disialoganglioside, GD2, on neuroblastoma cells.[382] Several anti-GD2 monoclonal antibodies have been developed and tested in clinical trials.

One of the first was a murine IgG3 monoclonal antibody 3F8 directed at GD2. According to reports, 3F8 mediates neuroblastoma cell death by human complement activation and by activation of lymphocytes, monocytes, and neutrophils.[383-385] Initial studies testing 3F8 showed evidence of activity, particularly in children with minimal disease at the time of immunotherapy initiation.[386] Subsequent studies evaluated whether the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) to 3F8 would increase response by increasing the number of granulocytes and by priming effector cells for greater cytotoxicity. There were 45 patients with high-stage disease not in remission enrolled in the study.[387] Of the 15 patients with refractory bone marrow disease, 80% had a complete remission in bone marrow. Eleven patients were treated for recurrent neuroblastoma that was refractory to salvage therapy. Five of ten with marrow disease had complete remission in the marrow, but the one patient with soft tissue disease progressed. Additionally, the 15 patients treated for progressive disease did quite poorly, with all continuing to progress. The combination of GM-CSF and 3F8 was well tolerated, with the principal side effects of pain and rash. The authors concluded that the combination of 3F8 and GM-CSF was well tolerated and shows promise for treatment of minimal residual neuroblastoma in the bone marrow.

Another anti-GD2 antibody, 14.G2a, a murine monoclonal IgG2a antibody, has been tested in a clinical trial.[388,389] In an attempt to improve this antibody, a chimeric human/murine anti-GD2 monoclonal antibody (ch14.18) was developed and tested in phase I clinical trials, with some encouraging results.[390,391] This construct uses the 14.G2a variable region's heavy and light chains and human constant region genes for IgG1 heavy chain and kappa light chain. However, a large retrospective study of 334 assessable children older than 1 year of age (166 received ch14.18 consolidation treatment, 99 received 12-month low-dose maintenance chemotherapy, and 69 received no additional treatment) failed to demonstrate an advantage to ch14.18 in event-free survival or overall survival with multivariate analysis.[392] Similarly, an analysis of infants younger than 1 year of age failed to show a difference in outcome with ch14.18 consolidation therapy.[393]

Ch14.18 was also tested in combination with GM-CSF in a phase I study with manageable toxicities, including pain, urticaria, and emesis.[394] Testing was performed with humanized ch14.18 linked to IL-2 (hu14.18-IL2) with the hypothesis that IL-2 would augment activation of preexisting antigen specific T cells, enhancing destruction of tumor cells and activation of natural killer cells. A phase I trial demonstrated manageable side effects: hypotension, allergic reaction, fever, pain, marrow suppression, and blurred vision. Three patients out of 27 experienced some antitumor activity, with decreased tumor burden in the bone marrow.[395]

A landmark phase III trial evaluated ch14.18 administered in combination with interleukin-2 (IL-2) and GM-CSF and isotretinoin versus isotretinoin alone for patients after stem cell transplantation with a total of 226 eligible patients randomly assigned to each treatment group.[396] With a median duration of follow-up of 2.1 years, this immunotherapy was superior to standard therapy with event-free survival of 66 ± 5% versus 46 ± 5% and overall survival of 86 ± 4% versus 75 ±– 5%, and the trial was stopped early owing to efficacy based on event-free survival. This study reported that 52% of patients experienced grade 3, 4, or 5 pain; 23% capillary leak syndrome; and 25% hypersensitivity reactions, expected side effects attributable to antibody binding to GD2 on normal nerves, cytokine-mediated leak, and hypersensitivity to either antibody or cytokines, respectively. Immunotherapy was also more effective in patients with minimal, rather than high, levels of residual disease. Patients who had biopsy-proven persistent disease before entering the study were nonrandomly assigned to immunotherapy, and event-free survival for these patients was only 36% ± 10% at 2 years. Nevertheless, this study suggests the more routine use of lineage-directed immunotherapy for patients with neuroblastoma with minimal residual disease after stem cell transplantation.

Emerging Synthetic Lethal or Empiric Track: Targets in Pediatric Cancer

Insulin-like Growth Factor Receptor Signaling and Pediatric Solid Tumors

More than 10 years ago, the insulin-like growth factor-1 receptor (IGF-1R) signaling pathway was implicated in Ewing sarcoma. It has also been implicated in other pediatric and adult tumors.[397] With the evolution of small molecule inhibitors of receptor tyrosine kinases and the development of antibody-based therapy, therapeutic inhibition of this pathway has become a reality. IGF-1R is a heterodimeric tyrosine kinase receptor. IGF-1 and IGF-2, and to a lesser extent insulin, bind to IGF-1R, leading to receptor activation, autophosphorylation, and phosphorylation of downstream targets. The receptor is important for development, cell growth, transformation, and the prevention of apoptosis.

One early study demonstrated that IGF-1 mRNA was expressed in 90% of Ewing tumor samples positive for the EWS-FLI translocation. Furthermore, this work demonstrated that blockade of the IGF-1R with a monoclonal antibody inhibited serum free growth in a Ewing cell line.[398] Next, it was demonstrated that IGF-1R is essential for EWS-FLI transformation of fibroblasts and that expression of the EWS-FLI fusion may alter the IGF-1R signaling pathway.[399] In addition, the expression of insulin-like growth factor binding protein (IGFBP-3) is strongly induced with siRNA against EWS-FLI, and EWS-FLI can bind the IGFBP-3 promoter and repress its activity, suggesting that dysregulation of IGFBP-3 contributes to the development of Ewing sarcoma.[400,401] With the advent of inhibitors of IGF-1R, it was next demonstrated that small molecule inhibitors of IGF-1R, such as NVP-AEW541, a pyrrolopyrimidine derivative with selectivity for IGF-1R versus insulin receptor, had activity in vitro and in vivo models of Ewing sarcoma.[402,403] Furthermore, a dominant negative mutant IGF-1R inhibited tumorigenesis and induced apoptosis in Ewing sarcoma cells.[404]

In addition to Ewing sarcoma, IGF-1R has been implicated in numerous other pediatric solid tumors. One study reported inhibition of proliferation in several pediatric cancer–related cell lines (neuroblastoma, rhabdomyosarcoma, and osteosarcoma) with an antagonistic, monoclonal antibody (EM164) directed against IGF-1R.[405] Other evidence implicating IGF-related signaling in rhabdomyosarcoma, particularly for alveolar, includes upregulation of IGF-2 in cell lines, cooperation of IGF-2, and PAX3-FKHR, and inhibition of rhabdomyosarcoma growth with overexpression of IGFBP-6, an inhibitor of IGF-2.[406-408] In osteosarcoma, expression of IGF-1R has been reported in both canine and human cells.[409] In neuroblastoma the small molecule inhibitor NVP-AEW541 has had in vitro and in vivo activity,[410] and data suggest that IGF-1R expression may regulate metastases to bone.[411]

Initial phase I trials testing monoclonal antibodies against IGF-1R demonstrated tolerability of this approach in patients with sarcoma, with excitement generated about responses seen specifically in patients with Ewing sarcoma.[412-414] However, this initial excitement was tempered in subsequent follow-up trials wherein moderate activity has been observed. A phase I/II study of figitumumab, a fully human IgG2 monoclonal antibody against the IGF-1R, enrolled 106 evaluable patients with Ewing sarcoma in the phase II component of the trial.[415] Of these, 15 had a partial response (overall response rate = 14.2%) and 25 had stable disease. However, the median duration of response was only 4.7 months, and median overall survival was only 8.9 months. Another phase II study testing R1507, the fully human IgG1 monoclonal antibody against the IGF-1R, enrolled 115 patients with Ewing sarcoma.[416] For all patients the overall response rate was 10% with a 29-week median duration of response and a median overall survival of 7.6 months. This study identified patients with bone primaries as more likely to respond to antibody therapy compared with patients with extraskeletal sites. A third phase II study tested the fully human monoclonal antibody antagonist of IGF-1R, ganitumab, in patients with Ewing sarcoma and desmoplastic small round blue cell tumors (DSRCT).[417] In this trial, of the 35 evaluable patients, only two had an objective response for an overall response rate of 6%, although 49% of patients had stable disease.

Although the data suggest modest activity of anti–IGF-1R antibody therapy in a subset of patients with Ewing sarcoma, and possibly DSRCT, biomarkers of sensitivity have yet to be identified. The *IGF1R* gene is not commonly mutated in Ewing sarcoma, and although some studies have correlated IGF-1 serum levels with a longer median overall survival, neither serum IGF-1 nor tumor IGF-1R have been demonstrated to be associated with response to IGF-1R antibody therapy.[415,417-419] Identification of biomarkers of response and mechanisms of rapid resistance to IGF-1R therapy are critical next steps in determining the role of IGF-1R–directed therapy for patients with Ewing sarcoma. Moreover, the identification of highly efficacious combination therapies with IGF-1R antibodies is another active avenue of pursuit.[420]

Poly(ADP-ribose) Polymerase Inhibitors in Ewing Sarcoma

In an effort to uncover new biomarkers of drug sensitivity and resistance, several groups have screened large collections of genetically annotated cancer cell lines against hundreds of drugs.[93,94] Although well-known connections between genotype and chemosensitivity have been validated (e.g., BCR-ABL–positive cell lines are sensitive to multiple ABL inhibitors), new tumor vulnerabilities are emerging. For example, in a study conducted by Garnett and colleagues, 639 human cancer cell lines were screened with 130 drugs under clinical preclinical testing. Ewing sarcoma cell lines expressing EWS-FLI fusions were identified as highly sensitive to multiple structurally distinct PARP inhibitors, undergoing apoptosis in vitro.[94] Tumors harboring either *BRCA1* or *BRCA2* mutations have been demonstrated to have increased sensitivity to PARP inhibitors owing to defects in homologous recombination and reliance on alternative pathways to repair DNA damage that are targeted by these inhibitors. Ewing sarcoma tumors, however, have not been demonstrated to have genomic events involving *BRCA1* or *BRCA2*. Rather, these investigators confirmed that the presence of the EWS-FLI fusion engenders sensitivity to PARP inhibitors by demonstrating that mouse mesenchymal cells transformed with EWS-FLI were more sensitive to the PARP inhibitor olaparib than when they were transformed with FUS-CHOP. Expression of EWS-FLI in NIH3T3 cells also conferred greater sensitivity to this compound class compared with a control.

Although the precise mechanism of Ewing sarcoma cell line sensitivity to PARP inhibitors is still under consideration, a study by Brenner and colleagues suggests that EWS-FLI or EWS-ERG fusions themselves promote DNA damage.[421] In this study the authors report that Ewing sarcoma cell lines have DNA double-strand breaks induced by the expression of the EWS-ETS fusion and that treatment with PARP inhibitors potentiates this DNA damage. Moreover, they found that EWS-FLI enhances the expression of PARP in a positive feedback loop, and a synergistic response was seen when PARP inhibitors were combined with the DNA-damaging agent temozolomide in preclinical models of Ewing sarcoma. These initial preclinical observations were rapidly translated to phase I/II trials for patients with Ewing sarcoma, with results of these trials still pending.

BRD4 in Pediatric Cancers

Another example of the power of high-throughput in vitro screening of cancer cell lines to define genetic, lineage, and gene expression–based predictors of drug response is provided by the identification of the sensitivity of *MYCN*-amplified neuroblastoma cells to BET bromodomain inhibitors.[381] The BET family is composed of BRD2, BRD3, BRD4, and BRDT. All are defined by two N-terminal bromodomains sharing high levels of sequence conservation. BET family members are epigenetic "readers," which recognize covalent modifications of histone proteins or DNA.[422] One important modification associated with open chromatin, and thus transcriptional activation, is the ε-N-acetylation of lysine residues on histone tails.[423] Context-specific molecular recognition of acetyl-lysine residues is mediated by 61 diverse bromodomains, comprising 46 human proteins.[424]

The BET family member BRD4 has been reported to be a critical mediator of cell cycle progression and transcriptional elongation, functioning to recruit the positive transcription elongation factor b (P-TEFb).[425-427] BRD4 has been identified as a component of a recurrent t(15;19) chromosomal translocation in NUT midline carcinoma (NMC), resulting in the expression of the twin N-terminal bromodomains of BRD4 as an in-frame chimera with the NUT protein.[428] Functional studies in patient-derived NMC cell lines have validated the essential role of the BRD4-NUT oncoprotein in maintaining a proliferation advantage and differentiation block.[429]

Recently, multiple BET bromodomain small molecule inhibitors have been developed that displace BET bromodomains from chromatin by competitively binding to the acetyl-lysine recognition pocket. Not only have these molecules had remarkable preclinical activity in NMC, but they have also been shown to modulate the transcriptional function of MYC in several diseases, including AML, T-ALL, and multiple myeloma.[430-434] BET proteins bind to acetylated histone lysines and modulate transcription by recruiting transcriptional activators; MYC-regulated transcription is associated with increases in histone lysine acetylation. Unexpectedly, however, it was discovered that MYC itself is transcriptionally regulated by BET bromodomains.[431,432,434] In multiple myeloma cells JQ1 treatment leads to depletion of the MYC oncoprotein and downregulation of the coordinated MYC transcriptional program, leading to cell cycle arrest and senescence. This was associated with significant therapeutic responses in orthotopic xenograft studies as well as genetically engineered mouse models of multiple myeloma. Moreover, in AML, JQ1 treatment inhibited MYC transcription and promoted AML differentiation and response in a mouse model of MLL-rearranged leukemia,[432] and in ALL, BET inhibition targeted IL7R with prolonged survival in mice xenografted with primary human *CRLF2*-rearranged B-ALL treated with JQ1.[435]

An unbiased screen of a collection of 673 genetically characterized cancer cell lines was conducted to understand response and resistance to BET inhibition and to find new opportunities for therapeutic development. Neuroblastoma was identified as one of the most JQ1-sensitive diseases and *MYCN* amplification as the most predictive marker of sensitivity.[381] Subsequent studies revealed a strong correlation between *MYCN* amplification and sensitivity to bromodomain inhibition. Bromodomain-mediated inhibition of MYCN impaired growth and induced apoptosis in neuroblastoma and suppressed the MYCN pattern of gene expression. BRD4 knockdown phenocopied these effects, establishing BET bromodomains as transcriptional regulators of *MYCN*. BET inhibition conferred a significant survival advantage in three in vivo neuroblastoma models, including a primary human orthotopic xenograft and a MYCN GEMM, and this efficacy correlated with blockade of both MYCN and of MYCN target genes, providing a strong rationale for developing BET bromodomain inhibitors in patients with neuroblastoma. The preclinical evaluation of BET bromodomain inhibitors in other MYCN-driven malignancies, such as medulloblastoma, is ongoing.

BET bromodomain inhibitors have entered the clinic, with trials now ongoing in adult diseases. Indeed, investigators reported the safety of chronic administration of the BET inhibitor RVX-208 from a clinical trial to treat atherosclerosis.[436] Future trials are under development for pediatric malignancies.

EZH2 in Pediatric Cancer

Whereas some targets have emerged from high-throughput screening efforts, other pediatric cancer vulnerabilities have been uncovered through the detailed study of genetic models of specific diseases. One recent example is the discovery of epigenetic antagonism between the polycomb family member EZH2 and SWI/SNF complexes in malignant rhabdoid tumors. Polycomb group proteins contribute to epigenetic-driven gene silencing during development, and increasing evidence supports a critical role for this protein class in oncogenesis. EZH2 is the core catalytic protein in the polycomb repressor complex 2 (PRC2) and catalyzes the trimethylation of histone 3 lysine 27 (H3K27), leading to the repression of hundreds of genes in embryonic stem cells.[437] EZH2 forms a complex with other PRC2 components, including the cofactors SUZ12 and EED and the protein Jarid2, which recruit PRC2 to promoters. EZH2 has been implicated in numerous malignancies. It is highly expressed in many tumors, including those of the breast, prostate, colon, and pancreas, as well as lymphomas and sarcomas.[438] In 2010 the discovery was made of recurrent Tyr 641 mutations in the catalytic SET domain of *EZH2* in diffuse large

B-cell lymphomas and in follicular lymphomas, which increase H3K27me3 in an interaction with the remaining wild-type *EZH2* allele.[439-441] More recently, a tumor-suppressor function of EZH2 in some malignancies has been described. Homozygous deletions or inactivating mutations have been reported in approximately 6% of myelodysplastic syndrome and in certain subsets of myeloproliferative disease.[442-445] Moreover, loss of *EZH2* is reported to cause aggressive T-ALL in mice, and loss-of-function mutations in multiple members of the PRC2 complex, including *EZH2*, *SUZ12*, and *EED*, have been reported in adult and early precursor T-ALL.[257,446,447]

Recent studies support the role for EZH2 dependency in malignant rhabdoid tumor. The first line of evidence that SWI/SNF complexes oppose epigenetic silencing by polycomb group proteins was supported by genetic studies in *Drosophila* species in which defects in body segment identity induced by mutations in polycomb proteins were suppressed by mutations in the SWI/SNF complex.[448] It was later discovered that polycomb proteins can oppose the enzymatic activity of the SWI/SNF complex.[449,450] Malignant rhabdoid tumors, a highly aggressive pediatric malignancy, arise from the biallelic inactivation of *SNF5*, a SWI/SNF complex member.[451,452] It was reported that EZH2 expression and H3K27me3 levels at lineage-specific target genes are upregulated in these SNF5-deficient tumors, with SNF5 directly repressing EZH2 expression.[453] Reduction of EZH2 levels in primary cells lacking SNF5 expression led to downregulation of stem cell signatures. Moreover, it was determined that EZH2 is required for the development and growth of tumor cells with biallelic inactivation of *SNF5*. Although these studies were performed with genetic manipulation, it has only recently become possible to selectively inhibit the enzymatic activity of EZH2 with a small molecule inhibitor with the development of EZH2 inhibitors by multiple pharmaceutical companies.

Small molecule inhibitors of EZH2 were initially described and reported in *EZH2*-mutated lymphoma, in which as many as 22% of germinal center diffuse large B-cell lymphoma and follicular lymphomas harbor mutations in the Y641, located in the catalytic SET domain of *EZH2*. GSK126, a selective S-adenosyl-methionine competitive small molecule inhibitor of EZH2 methyltransferase activity, was reported to decrease global H3K27me3 levels, activate the expression of genes suppressed by PRC2, and inhibit *EZH2*-mutated lymphoma in vitro and in xenograft mouse models of the disease.[454] Similarly, the compound EPZ005687 was reported to reduce H3K27 methylation in lymphoma cells, resulting in apoptosis of lymphoma cells with heterozygous mutations in Y671 or A677, but with little effect on lymphoma cells with wild-type EZH2.[455] A second-generation EZH2 inhibitor, EPZ-6438, with improved potency and pharmacokinetics from EPZ005687, was developed and tested in malignant rhaboid tumor.[456] This compound had remarkable activity, with treatment leading to a dose-dependent regression of malignant rhabdoid tumors in mouse models of disease, a decrease in intratumoral levels of H3K27me3, and even prevention of regrowth after treatment was discontinued. This study demonstrated the dependency of rhabdoid tumors on not only the EZH2 protein but specifically the enzymatic activity of this molecule.

EZH2 has been implicated in other pediatric malignancies. For example, in Ewing sarcoma EWS-FLI has been reported to directly upregulate the expression of EZH2.[457,458] In this study EZH2 was reported to regulate the expression of genes related to stemness and to genes involved in neuroectodermal and endothelial differentiation. Furthermore, in this study the downregulation of EZH2 by shRNA suppressed oncogenic transformation in Ewing sarcoma cells by inhibiting in vitro clonogenicity. Whether Ewing cells depend on the enzymatic activity of EZH2 or another function of the EZH2 protein (as has been implicated in prostate cancer)[459] has yet to be determined. Finally, a potential role for EZH2 has been implicated in neuroblastoma. Here, EZH2 has been found to be physically associated with MYCN in a complex and shRNA directed against EZH2 to alter growth of neuroblastoma cells.[460,461] However, as in Ewing sarcoma, the therapeutic role of EZH2 inhibition in neuroblastoma has not been fully explored. As in the examples discussed previously for other epigenetic modifying drugs, EZH2 inhibitors are now entering clinical trials for adults with cancer.

DOT1L in Mixed-Lineage Leukemia-Rearranged Leukemia

As discussed before, epigenetic modifiers have come to the forefront as therapeutic targets in multiple pediatric cancers. Mixed-lineage leukemia (MLL)-rearranged leukemia represents another illustrative example of a defined genetic subtype of childhood leukemia in which multiple epigenetic vulnerabilities have been identified.[462,463] There are two primary genetic aberrations of *MLL* in leukemia. The most common event is a balanced translocation; more than 70 different fusion partners with *MLL* have been described. A second class of aberration of the *MLL* gene creates a repeat within the N-terminal of MLL resulting in an internal partial tandem duplication (PTD). As noted above, BRD4 has emerged as one potential target in this genetic subtype of leukemia. Another potential target is the H3K79 methyltransferase DOT1L (Fig. 44-8). Whereas MLL-fusion proteins have lost the histone H3K4 methyltransferase activity of wild-type MLL, many of these fusion proteins have been shown to copurify with protein complexes normally involved in transcriptional elongation and activation, such as polymerase-associated factor complex (PAFc), P-TEFb, and DOT1L.[464] It has thus been hypothesized that MLL-fusion proteins promote leukemia through enhanced elongation or activation of leukemia-promoting genes.

The association of MLL fusions with DOT1L precipitated the study of H3K79 methylation levels in *MLL*-rearranged leukemia. Numerous studies demonstrate enhanced H3K79 methylation associated with genes overexpressed in *MLL*-rearranged leukemia cells.[465-467] As in the studies of EZH2 and malignant rhaboid tumor, a therapeutic effect of targeting DOT1L in the context of certain MLL fusions has been demonstrated with both genetic and chemical approaches. In one study abnormal H3K79me2 profiles were identified on MLL-AF9 fusion

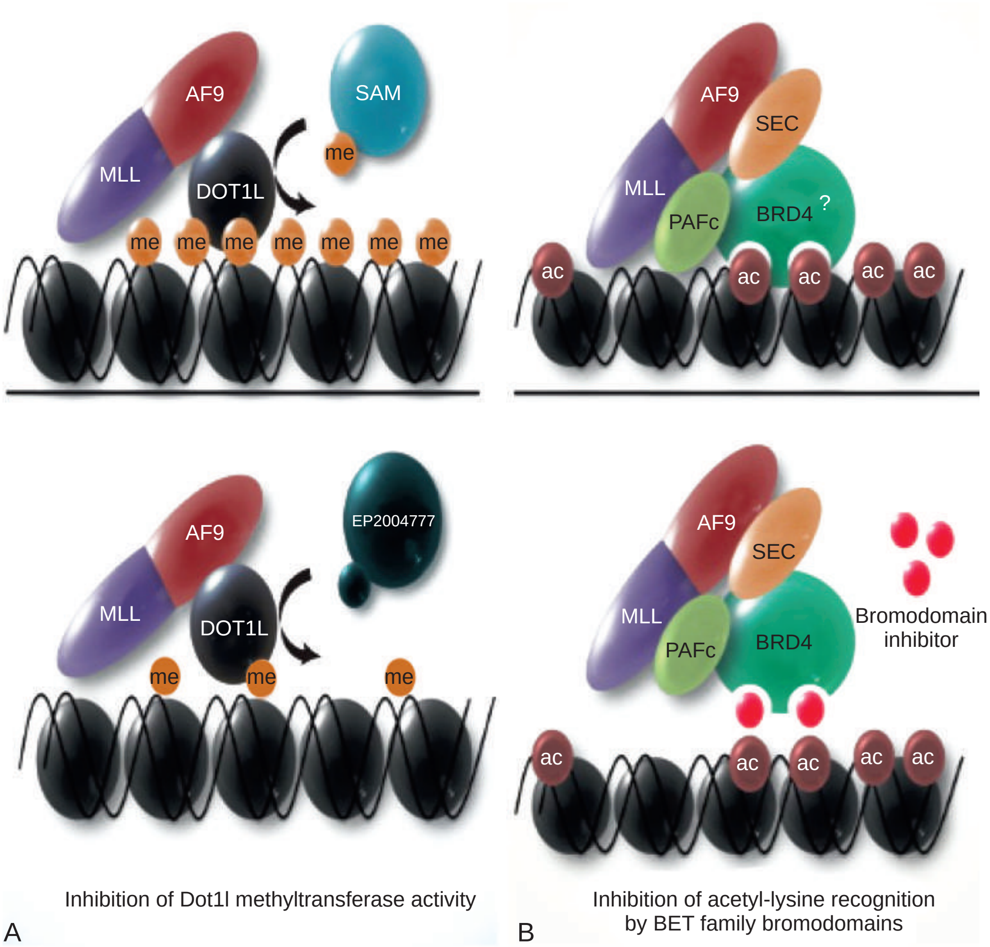

Figure 44-8 Pharmacologic targeting of chromatin regulator pathways in mixed-lineage leukemia (MLL)-rearranged leukemias. **A,** Depiction of the association of DOT1L with the AF9 component of the MLL-AF9 fusion. DOT1L methylates histone 3 at lysine residue 79 by using S-(5′-adenosyl)-1-methionine (SAM) as a donor. The use of a SAM mimetic (e.g., EPZ004777), as shown in the lower panel, can potentially inhibit DOT1L activity and target oncogenic H3K79 hypermethylation. **B,** MLL fusions recruit polymerase-associated factor complex (PAFc) through the N-terminal portion of MLL. A number of nuclear fusion partners, such as AF9 and ENL, are members of the super elongation complex (SEC), which includes positive transcription elongation factor b (P-TEFB). Both PAFc and P-TEFb have been implicated in MLL-fusion positive leukemias. The BET family member BRD4 was shown to co-purify with several components of the PAFc and SEC. Small molecule inhibitors of BRD4, which inhibit its recognition of acetyl lysine, impair the targeting of the PAFc and SEC components to chromatin, leading to altered gene expression. BRD4 inhibitors might therefore inhibit MLL-mediated leukemogenesis. *(Modified from Deshpande, et al: Chromatin modifications as therapeutic targets in MLL-rearranged leukemia.* Trends Immunol *33:563–570, 2012.)*

target loci in leukemic stem cells and inactivation of Dot1l led to the downregulation of MLL-AF9 target genes.[468] Moreover, in this study, by using an experimentally derived mouse model of MLL-AF9 leukemia, it was determined that Dot1l is required in vivo for both leukemic transformation and maintenance. Similar findings were observed in an independent study by using a tamoxifen-inducible Cre-mediated loss-of-function mouse model in which it was demonstrated that Dot1l is required for the initiation and maintenance of MLL-AF9–induced leukemogenesis in vitro and in vivo.[469] The critical relevance of the enzymatic activity of DOT1L in *MLL*-rearranged leukemia was determined with the testing of EPZ004777, a selective small molecule inhibitor of DOT1L. Treatment of MLL cells with the compound inhibited H3K79 methylation, blocked expression of leukemogenic genes, resulted in the selective killing of *MLL*-rearranged leukemia cells, and led to a modest extension of survival in a mouse MLL-leukemia xenograft model.[470] Subsequently, it was demonstrated that other MLL fusions, including MLL-AF6, MLL-AF10, and CALM-AF10, require the H3K79 methyltranasferase activity of DOT1L.[471,472] As in the case of BET bromodomain and EZH2 inhibitors, DOT1L inhibitors are just beginning to enter clinical trials.

CONCLUSIONS

Tremendous progress has been made in the treatment of pediatric cancer over the last century. The empiric combination of cytotoxic agents has proved curative for many patients, but at the expense of toxicity. Over the last decade, heralded by the success of targeting BCR-ABL in CML, we have entered a new era of drug discovery with an emphasis on target-based treatment with the promise of improved cure rates and decreased toxicity. New and developing high-throughput genomic and proteomic technologies are dramatically altering the landscape of research, enabling the identification and credentialing of new targets with a speed previously unfathomable. In this chapter we have provided a framework for classifying and credentialing new targets. We have highlighted examples of successful targeted approaches from the treatment of adults with cancer and have discussed emerging targets for pediatric cancer. Although it is an exciting time in cancer therapy, there are many sobering challenges that lie ahead in the development of targeted therapy, particularly for pediatric cancer. Continued advancement will require innovative approaches to intractable targets (e.g., transcription factors), rational approaches to combination therapy, and collaborative efforts between industry and academic centers to identify new pediatric targets along with target-specific drugs. For maximal progress in the treatment of children with cancer, we will need to collectively solve the issues that confront the clinical development of novel therapeutics. Trials that allow for biopsies, genetic profiling of tumors, and "window" treatment periods may be necessary to bring targeted therapies into the pediatric realm.

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CHAPTER 45

Cytogenetic and Molecular Pathology of Pediatric Cancer

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CHAPTER OUTLINE

CYTOGENETIC AND MOLECULAR METHODOLOGIES
- Karyotyping
- Molecular Cytogenetics
- Polymerase Chain Reaction–Based Molecular Assays
- Array Comparative Genomic Hybridization and Single Nucleotide Polymorphism Profiling Assays
- Next-Generation Sequencing and Other Techniques

GENOMIC MECHANISMS IN PEDIATRIC TUMORS
- Causes and Predisposing Factors for Chromosomal Translocations

BIOLOGIC CONSEQUENCES OF CHROMOSOMAL TRANSLOCATION
- Chromosomal Rearrangements Involving Transcription Factor Genes
- Chromosomal Rearrangements Involving Protein Tyrosine Kinase Genes
- Chromosomal Rearrangements Associated with Transcriptional Upregulation of Nonfusion Oncogenes

BIOLOGIC BASIS FOR SPECIFICITY OF BALANCED CHROMOSOMAL REARRANGEMENTS: DIAGNOSTIC RELEVANCE

CYTOGENETIC AND MOLECULAR PATHOLOGY: MAJOR TUMOR TYPES
- Mesenchymal Neoplasms: Soft Tissue and Bone Tumors
- Nonmesenchymal Solid Tumors
- Blastomas
- Central Nervous System Tumors
- Germ Cell Tumors
- Hematologic Neoplasms

Cytogenetic and molecular analyses have provided pivotal biologic and clinical insights into pediatric neoplasia. It is increasingly evident that genetic assays of various types can provide essential diagnostic or prognostic information about pediatric solid tumors and hematologic malignancies (Tables 45-1 to 45-3). In this chapter we offer an overview of the methods in use for the analysis of cytogenetic and molecular aberrations (Table 45-4) in pediatric cancers together with their relative attributes, discuss the causes of these genetic aberrations, and summarize their diagnostic and predictive relevance in clinical practice.

CYTOGENETIC AND MOLECULAR METHODOLOGIES

The pathognomonic genetic aberrations in pediatric cancer can be evaluated by various methods. Because each of these methods has a different profile of substrate requirements, sensitivity, and specificity (see Table 45-4), the optimal approach must be tailored individually in regard to both the nature and quantity of material available and to the precise information being sought.

Karyotyping

The traditional karyotype provides a low-resolution snapshot of the entire genome. Cells are cultured, arrested in metaphase, and then subjected to staining of the chromosomes to produce characteristic banding patterns,[1-4] which are described using standardized nomenclature (Box 45-1). Probably the major advantage of karyotypic analysis is the provision of a global overview of the chromosomal composition of a cultured cellular population without any requirement for preknowledge, especially enabling the identification of completely novel aberrations (Fig. 45-1). However, karyotyping has distinct limitations, including the requirement for fresh material in sufficient amounts and slow turnaround because of time required for tumor cell growth.

Need for Sufficient Viable Tumor Cells

Solid tumors may comprise substantial nonviable or hypocellular regions containing few neoplastic cells. Such regions may be extensively necrotic because the tumor cells have died, having outstripped their blood supply.

Text continued on p. 1345

TABLE 45-1 Typical Genetic Aberrations in Soft Tissue and Bone Tumors

Histologic Findings	Cytogenetic Events	Molecular Events	Frequency (%)	Clinical Relevance
Alveolar soft part sarcoma	t(X;17)(p11;q25)	*ASPSCR1 (ASPL)-TFE3* fusion	>90	
Aneurysmal bone cyst (extraosseous)	16q22 and 17p13 rearrangements	*USP6* fusion genes	>50	
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11)	*FUS-ATF1* fusion	10	
		EWSR1 (EWS)-GREB1 fusion	80	
Chondromyxoid fibroma	Deletion of 6q		>75	
Chondrosarcoma				
Skeletal	Complex*		>75	
Extraskeletal myxoid	t(9;22)(q22;q12)	*EWS-NR4A3* fusion	>75	
	t(9;17)(q22;q11)	*TAF15-NR4A3* fusion	<10	
	t(9;15)(q22;q21)	*TCF12-NR4A3* fusion	<10	
Clear cell sarcoma—melanoma of soft parts	t(12;22)(q13;q12)	*EWS-ATF1* fusion	>	
Dermatofibrosarcoma protuberans	Ring form of chromosomes 17 and 22	*COL1A1-PDGFB* fusion	>75	PDGFRB therapeutic inhibition
	t(17;22)(q21;q13)	*COL1A1-PDGFB* fusion	10	PDGFRB therapeutic inhibition
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWS-WT1* fusion	>75	
Endometrial stromal tumor	t(7;17)(p15;q21)	*JAZF1-SUZ12*	30	
Ewing sarcoma	t(11;22)(q24;q12)	*EWS-FLI1* fusion	>80	
	t(21;22)(q12;q12)	*EWS-ERG* fusion	5-10	
	t(2;22)(q33;q12)	*EWS-FEV* fusion	<5	
	t(7;22)(p22;q12)	*EWS-ETV1* fusion	<5	
	t(17;22)(q12;q12)	*EWS-ETV4* fusion	<5	
	t(16;21)(p11;q12)	*FUS-ERG* fusion	<5	
	t(2;16)(q33;p11)	*FUS-FEV* fusion	<5	
	inv(22)(q12q12)	*EWS-PATZ1 (ZSG)* fusion	<5	
Fibromatosis (desmoid)	Trisomies 8 and 20		30	
	Deletion of 5q	*APC* inactivation	10	Association with germline *APC* mutation and familial adenomatous polyposis
		CTNNB1 mutation	70	
Fibromyxoid sarcoma, low-grade	t(7;16)(q33;p11)	*FUS-CREB3L2 (BBF2H7)* fusion	50	
Fibrosarcoma, infantile	t(12;15)(p13;q26)	*ETV6 (TEL)-NTRK3* fusion	>75	NTRK3 therapeutic inhibition
	Trisomies 8, 11, 17, and 20		>75	
Gastrointestinal stromal tumor	Monosomies 14 and 22		>75	
	Deletion of 1p		>25	
		KIT or *PDGFRA* mutation	>90	KIT-PDGFRA therapeutic inhibition
Giant cell tumor				
Bone	Telomeric associations		>50	
Tenosynovial	Trisomies 5 and 7		>25	
	t(1;2)(p13;q35)	*CSF1-COL6A3* fusion	25	CSF1R therapeutic inhibition
Hibernoma	11q13 rearrangement		>50	

TABLE 45-1 Typical Genetic Aberrations in Soft Tissue and Bone Tumors (Continued)

Histologic Findings	Cytogenetic Events	Molecular Events	Frequency (%)	Clinical Relevance
Inflammatory myofibroblastic tumor	2p23 rearrangement	*ALK* fusion genes	50	ALK therapeutic inhibition
Leiomyoma				
Uterine	t(12;14)(q15;q24) or deletion of 7q	*LHFP (HMGIC)* rearrangement	40	
Extrauterine	Deletion of 1p		?	
Leiomyosarcoma	Deletion of 1p		>50	
Lipoblastoma	8q12 rearrangement or polysomy 8	*PLAG1* oncogenes	>80	
Lipoma				
Typical	12q15 rearrangement	*HMGIC* rearrangement	60	
Spindle cell or pleomorphic	Deletion of 13q or 16q		>75	
Chondroid	t(11;16)(q13;p12-13)		?	
Liposarcoma				
Well-differentiated	Ring form of chromosome 12		>75	
Myxoid, round cell	t(12;16)(q13;p11)	*FUS-DDIT3 (CHOP)* fusion	>75	Trabectedin therapeutic response
	t(12;22)(q13;q12)	*EWS-CHOP* fusion	<5	
Pleomorphic	Complex*		90	
Malignant fibrous histiocytoma				
Myxoid	Ring form of chromosome 12		?	
High-grade	Complex*		>90	
Myxofibrosarcoma	(see "Malignant fibrous histiocytoma")			
Malignant peripheral nerve sheath tumor	(see "Schwannoma")			
Mesothelioma	Deletion of 1p	*?BCL10* inactivation	>50	
	Deletion of 9p	*p15*, *p16*, and *p19* inactivation	>75	
	Deletion of 22q	*NF2* inactivation	>50	
	Deletions of 3p and 6q		>50	
Neuroblastoma				
Good prognosis	Hyperdiploid, no 1p deletion		40	
Poor prognosis	1p deletion		40	
	Double-minute chromosomes	*MYCN* amplification	>25	
Oligodendroglioma	Deletion of 1p and 19q			Therapeutic response to CDDP
Osteochondroma	Deletion of 8q	*EXT1* inactivation	>25	
Osteosarcoma				
Low-grade	Ring chromosomes		>50	
High-grade	Complex*	*RB1* and *TP53* inactivation	>80	
Pericytoma	t(7;12)(p22;q13-15)	*GLI-ACTB* fusion	?	
Pigmented villonodular synovitis	(see "Giant cell tumor: Tenosynovial")			
Primitive neuroectodermal tumor	(see "Ewing sarcoma")			
Rhabdoid tumor	Deletion of 22q	*SMARCB1 (INI1)* inactivation	>90	

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TABLE 45-1 Typical Genetic Aberrations in Soft Tissue and Bone Tumors (Continued)

Histologic Findings	Cytogenetic Events	Molecular Events	Frequency (%)	Clinical Relevance
Rhabdomyosarcoma				
Alveolar	t(2;13)(q35;q14)	*PAX3-FOXO1* fusion	60	Prognosis
	t(1;13)(p36;q14), double minutes	*PAX7-FOXO1* fusion	10-20	Prognosis
Embryonal	Trisomies 2q, 8 and 20		>75	
		Loss of heterozygosity at 11p15	>75	
Schwannoma				
Benign	Deletion of 22q	*NF2* inactivation	>80	
Malignant, low-grade	None			
Malignant, high-grade	Complex*		>90	
Synovial sarcoma				
Monophasic	t(X;18)(p11;q11)	*SS18 (SYT)-SSX1* or *SYT-SSX2* fusion	>90	Prognosis
Biphasic	t(X;18)(p11;q11)	*SYT-SSX1* fusion	>90	
Wilms tumor	Deletion 11p13	*WT1* inactivation		Association with syndromic Wilms tumor
	1p deletion			Adverse prognosis in low-stage FH
	16q deletion			Adverse prognosis in low-stage FH
	1q gain			Increased relapse risk

CDDP, *cis*-Diamminedichloroplatinum; *FH*, favorable histology; *PDGFRA*, platelet-derived growth factor receptor alpha; *PDGFRB*, platelet-derived growth factor receptor beta.

*Indicates presence of complicated numeric and structural chromosomal aberrations.

TABLE 45-2 Typical Genetic Aberrations in Pediatric Lymphomas

Histologic Findings	Cytogenetic Events	Molecular Events	Frequency (%)
Hodgkin, classic	Complex*	Clonal Ig rearrangements	95
		Clonal *IGH* translocations	17
		Clonal *IGK* translocations	1
		Clonal *IGL* translocations	3
		Clonal TCR rearrangements	1-2
Hodgkin, nodular lymphocyte predominance	t(3;14)(q27;q32)	*BCL6* rearrangement	50
Burkitt	t(8;14)(q24;q32)	*IGH-MYC* rearrangement	80
	t(2;8)(p11;q24)	*IGK-MYC* rearrangement	5
	t(8;22)(q24;q11)	*IGL-MYC* rearrangement	15
Lymphoblastic (see Table 45-4) ALL			
Anaplastic large cell	t(2;5)(p23;q35)	*NpM1 (NPM)-ALK* fusion	80
	Various inversions and translocations	*ALK* fusions with *TPM3, ATIC, CLTC* and others	10-20
Diffuse large B-cell	Complex*		
Subcutaneous panniculitic T-cell		Clonal T-cell receptor rearrangements	
Hepatosplenic T-cell	Isochromosome 7q; trisomy 8	Clonal T-cell receptor rearrangements	>75

*Indicates presence of complicated numeric and structural chromosomal aberrations.

TABLE 45-3 Typical Genetic Aberrations in Pediatric Leukemias and Myeloproliferative Disorders

Histologic Findings	Cytogenetic Events	Molecular Events	Frequency	Clinical Relevance
Early T-cell precursor ALL	*MEF2C* rearrangements (e.g., *MEF2C-PITX2*) or related rearrangements *(NKX2-5/BCL11B, SPI1(PU.1)/BCL11B, ETV6/NCOA2)* *RUNX1-AFF3* rearrangement	AML-like aberrations: Cytokine receptor and RAS signaling mutations (*NRAS, KRAS, NF1, FLT3, IL7R, JAK3, JAK1, SH2B, BRAF;* 67%) Inactivating hematopoietic transcription factor mutations (*GATA3, ETV6, RUNX1, IKZF1;* 58%) Inactivating histone modifier mutations (*EZH2, EED, SUZ12, SETD2; EP300;* 48%) Novel and other mutations *(WT-1, DNM2, ECT2L, RELN)*	Accounts for 5-16% pediatric T-ALL	Associated with poor prognosis, early T-cell differentiation arrest (CD1a–, CD8–, CD5weak, stem-cell or myeloid marker+, biallelic *TRG* deletions absent)
T-cell ALL	t(1;14)(p34;q11)	*SCL(TAL1)*-TCR alpha-delta locus fusions *or SIL-SCL* rearrangements	30% T-cell ALL	
	t(1;3)(p34;p21)	*SCL-TCTA* fusion	Rare	
	Chromosome band 11p13 translocations	*LMO2*-TCR loci fusions	10%-20% T-cell ALL	
	Chromosome band 11p15, 19p13.2, or 10q24 translocations	*LMO1, LYL1, TLX1 (HOX11)-TCR* loci fusions	?	
	t(7;9)(q34;q34)	*TRB (TCRB)-NOTCH1* fusion, activating *NOTCH1* mutations	<1% T-cell ALL	Gamma secretase inhibitor sensitivity
	t(6;7)(q23;q34)	*TCRB-MYB (CMYB)* fusion		Associated with young age
		Activating *NOTCH1* mutations	50% T-cell ALL	
		PTEN gene mutation	8% T-cell ALL	Associated with disease progression
	t(10;14)(q24;q11)	*HOX11(TLX1)* overexpression, *HOX11(TLX1)-TCRD* fusion		Favorable prognosis
	Chromosome band 9p21 deletion	*CDKN2A* ($p16^{INK4A}$-$p14^{ARF}$) deletion; variable *CDKN2B* ($p15^{INK4B}$) deletion		Associated with relapse, poor prognosis
Cortical T-cell ALL	*NKX2-1* or *NKX2-2* rearrangement *TLX1* rearrangement *MYB* rearrangement			CD1+ immunophenotype, associated with favorable prognosis
T-cell ALL, precursor B-cell ALL	Translocations, inversions involving chromosome bands 14q32, 14q11, 7q34, 7p14	Hybrid antigen receptor gene rearrangements	?	
	Chromosome band 9p21 deletion	*CDKN2A* ($p16^{INK4A}$)/$p14^{ARF}$) deletion/ variable *CDKN2B* ($p15^{INK4B}$) deletion	60% T-cell ALL, 20% childhood precursor B-cell ALL	

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TABLE 45-3 Typical Genetic Aberrations in Pediatric Leukemias and Myeloproliferative Disorders (Continued)

Histologic Findings	Cytogenetic Events	Molecular Events	Frequency	Clinical Relevance
Precursor B-cell ALL	Chromosome band 11q23 translocations	*MLL (KMT2A)* gene rearrangements	80% of infant ALL	Poor prognosis especially in ALL and therapy-related cases, associated with FAB M4, FAB M5, other AML morphologies, leukemia cutis, extramedullary involvement, and topoisomerase II (TOP2) poison exposure
	Hyperdiploidy		30% precursor B-cell ALL	Favorable prognosis if >50 chromosomes and specific trisomies
	Hypodiploidy		6%-8% precursor B-cell ALL	Unfavorable prognosis if <44 chromosomes, −7, dicentric chromosome
	t(12;21)(p13;q21) (cryptic; detectable by FISH only)	*TEL-AML1* fusion	25% of “common” ALL	Favorable prognosis
	t(9;22)(q34;q11)	*BCR-ABL1* fusion	5% precursor B-cell ALL	Poor prognosis, associated with older age, imatinib mesylate sensitivity
	t(1;19)(q23;p13.3)	*E2A-PBX1* fusion	~4% Precursor B-cell ALL; 25% cIg+ cases; <1% cIg– cases	Poor prognosis in cIg+ cases
	t(17;19)(q22;p13.3)	*E2A-HLF* fusion	1% precursor B-cell ALL	Coagulopathy, poor prognosis
	t(5;14)(q31;q32)	*IGH-IL3* fusion	<1% precursor B-cell ALL	Peripheral eosinophilia
	t(6;14)(p22.3;q32)	*IGH-ID4* fusion	?	Favorable prognosis
	t(1;19)(q23;p13.3)	*MEF2D-DAZAP1* fusion	?	
		FLT3 point mutation	5% childhood precursor B-cell ALL, 16% infant ALL, ~21%-28% high hyperdiploid ALL, 18% *MLL*-rearranged ALL	Poor prognosis, increased FLT3-ITD allelic ratio associated with AML relapse, sensitivity to FLT3 tyrosine kinase inhibition
		*FLT3*ITD	2% precursor B-cell ALL	Poor prognosis, increased FLT3-ITD allelic ratio associated with AML relapse, sensitivity to FLT3 tyrosine kinase inhibition
		IKZF1 mutations	10%-15% of precursor B-cell ALL	Poor prognosis
	iAMP21	Accompanying *IKZF1, CDKN2A/B, PAX5, ETV6,* and *RB1* alterations	~2% of B-cell precursor ALL in older children	Poor outcome with standard regimens
Precursor B-cell ALL,	Chromosome band 9p21 deletion	*CDKN2A (p16*^{INK4A}-*p14*^{ARF} deletion; variable *CDKN2B* (*p15*^{INK4B}) deletion		Associated with relapse, poor prognosis
Precursor B-cell ALL “Ph-like ALL”	Cryptic rearrangements *EBF1-PDGFRB, BCR-JAK2, STRN3-JAK2, PAX5-JAK2, NUP214-ABL1, ETV6-ABL1, RANBP2-ABL1, RCSD1-ABL1, IGH-EPOR* *SH2B3* deletion	May also have *IL7R* mutation. Accompanying *IKZF1* deletions (40%) Accompanying *JAK* mutations	10% of precursor B-cell ALL, ~15% of cases of high-risk precursor B-cell ALL	Poor prognosis, preclinically responsive to TKI and JAK inhibition

TABLE 45-3 Typical Genetic Aberrations in Pediatric Leukemias and Myeloproliferative Disorders (Continued)

Histologic Findings	Cytogenetic Events	Molecular Events	Frequency	Clinical Relevance
Precursor B-cell ALL and precursor B-cell ALL in DS	*CRLF2* rearrangements (Xp22.3/Yp11.3) with IGH (14q32) *CRLF2* interstitial deletion (*P2RY8-CRLF2* rearrangement)	*CFLF2* lesion may be F232C point mutation Accompanying *JAK1* or *JAK2* mutations in 50% Accompanying *IKZF1* mutation/deletion Accompanying *IL7R*	Ph-like ALL, 15% of high-risk precursor B-cell ALL, 60% Down syndrome ALL, in non-DS high-risk ALL	Associated with poor prognosis high relapse rate, high MRD, associated with Hispanic ethnicity; preclinically responsive to signal transduction inhibitors (JAK, PI3K/mTOR)
B-cell ALL	t(8;14)(q24;q32)	*IGH-MYC* fusion	Rare	Poor prognosis
	t(2;8)(p12;q24)	*IGK-MYC* fusion	Rare	
	t(8;22)(q24;q11)	*IGL-MYC* fusion	Rare	
AML	t(8;21)(q22;q22)	*AML1-ETO* fusion	~12% pediatric AML	Associated with FAB M2 morphology, granulocytic sarcoma presentation, favorable prognosis
	Chromosome band 11q23 translocations	*MLL* gene rearrangements	80% myelomonocytic-monoblastic AML in infants and young children; ~7%-18% pediatric AML	Poor prognosis especially in ALL and therapy-related cases, associated with FAB M4, FAB M5, other AML morphologies, leukemia cutis, extramedullary involvement, TOP2 poison exposure
	inv(16)(p13q22) or t(16;16)(p13;q22)	*CBF-MYH11* fusion	~7% pediatric AML	Associated with abnormal eosinophils (adults), FAB M2 or M4 AML without eosinophilia (pediatrics), favorable prognosis
	t(8;16)(p11;p13)	*MOZ-CBP* fusion	Rare	Unfavorable prognosis, erythrophagocytosis, coagulopathy, associated with leukemia cutis and spontaneous remission in neonates
	t(6;9)(p23;q34)	*DEK-NUP214 (CAN)* fusion	Rare	Unfavorable prognosis
	May be associated with t(8;21) or inv(16)	*KIT* mutation	37% CBF AML	Sensitivity to imatinib, sensitivity to FLT3 tyrosine kinase inhibition
		NPM mutation	~7%-8% pediatric AML	Favorable prognosis in absence of *FLT3* ITD
		FLT3 point mutation	7%-9% pediatric AML	Poor prognosis, increased FLT3-ITD allelic ratio associated with AML relapse, sensitivity to FLT3 tyrosine kinase inhibition
		FLT3-ITD	~12%-15% pediatric AML	Poor prognosis, increased FLT3-ITD allelic ratio associated with AML relapse, sensitivity to FLT3 tyrosine kinase inhibition
APL	t(11;17)(q23;q21)	*PLZF-RARA* fusion	Rare	Insensitive to ATRA
		FLT3-ITD	~30% pediatric APL	
Therapy-related APL	t(15;17)(q22;q21)	*PML-RARA* fusion		Favorable prognosis, ATRA sensitivity, arsenic trioxide sensitivity
Therapy-related MDS/AML, de novo MDS/AML	Monosomy 7/del(7q)		~40% pediatric MDS, 49% RC, 4%-5% pediatric AML	Alkylating agent exposure, associated with disease progression, poor prognosis, can be familial
De novo or therapy-related AML, ALL, MDS	Chromosome band 11p15 translocations	*NUP98* gene rearrangements	?	

Continued on following page

TABLE 45-3 Typical Genetic Aberrations in Pediatric Leukemias and Myeloproliferative Disorders (Continued)

Histologic Findings	Cytogenetic Events	Molecular Events	Frequency	Clinical Relevance
Therapy-related MDS/AML	Monosomy 5, del(5q)			Alkylating agent exposure
	May be associated with segmental jumping translocations	*TP53* mutation	? (more common in therapy-related than de novo MDS, AML)	Alkylating agent exposure, associated with AML in Li-Fraumeni syndrome
Therapy related ALL and AML after exposure to TOP2 poisons	Chromosome band 11q23 translocations	*MLL* gene rearrangements		Poor prognosis especially in ALL and therapy-related cases, associated with FAB M4, FAB M5, other AML morphologies, leukemia cutis, extramedullary involvement, TOP2 poison exposure
5q– syndrome, refractory anemia	del(5q)	*RPS14* deletion	?—not described in children	Lenalidomide sensitivity
JMML, AML, MDS, precursor B-cell ALL		*PTPN11* mutation	30%-35% of JMML, 4% of AML, 18% of FAB M5 AML	Associated with Noonan syndrome
JMML	May be associated with monosomy 7	*NF1* mutation	~30% of JMML, ~50% of JMML in constitutional NF1 syndrome	Associated with constitutional NF1 syndrome
JMML, AML, MDS	May be associated with monosomy 7 in JMML/MDS or with t(8;21) or inv(16) in AML	*KRAS* or *NRAS* mutation	20%-30% JMML, ~20%-30% pediatric AML, 30% monosomy 7 MDS	
CMML	t(5;12)(q33;p13)	*TEL-PDGFRB* fusion	Rare	Associated with eosinophilia, progression to AML
TAM, AMKL	May be associated with +8 if progression to AMKL	*GATA1* mutations		Associated with Down syndrome
Non–Down syndrome AMKL	t(1;22)(p13;q13)	*OTT-MAL* fusion	Rare	Found in neonates
Refractory cytopenia (RC)	Trisomy 8		9% of RC	

ALL, Acute lymphocytic leukemia; *AMKL,* acute megakaryoblastic leukemia; *AML,* acute myeloid leukemia; *APL,* acute promyelocytic leukemia; *CBF,* core-binding factor; *CMML,* chronic myelomonocytic leukemia; *FAB,* French-American-British classification; *FISH,* fluorescence in situ hybridization; *ITD,* internal tandem duplication; *JMML,* juvenile myelomonocytic leukemia; *MDS,* myelodysplastic syndrome; *MRD,* minimal residual disease; *NF1,* neurofibromatosis type 1; *TAM,* transient abnormal myelopoiesis.

TABLE 45-4 Complementary Nature of Cytogenetic and Molecular Assays

	METHOD				
Assay Consideration	Karyotyping	FISH	CGH	SNP	PCR Sequencing
Cost of assay	High	Low	High	High	Low
What types of tumor material can be used?	Fresh	Any	Fresh, frozen*	Fresh, frozen*	Any
Does assay detect translocations?	Yes	Yes	No	No	Yes
Does assay detect point mutations?	No	No	No	No	Yes
Does assay detect deletions?	Yes	Yes	Yes	Yes	Yes, if qPCR
Does assay detect amplifications?	Yes	Yes	Yes	Yes	Yes, if qPCR
Does assay detect low-frequency mutations?	No	Yes; 1 in 100	No	No	Yes; 1 in 10,000
Does assay permit genome-wide evaluation?	Yes	No	Yes	Yes	No

CGH, Comparative genomic hybridization; *FISH,* fluorescence in situ hybridization; *PCR,* polymerase chain reaction; *qPCR,* quantitative PCR; *SNP,* single-nucleotide polymorphism analysis.

*Can be performed using DNA extracted from paraffin materials, but with some loss of resolution.

Other regions of a tumor mass may be composed largely of blood (hemorrhage) or scar tissue (fibrosis). Therefore in the case of solid tumor karyotyping, it is crucial that the pathologist select a maximally viable region for analysis.

Unpredictable Tumor Cell Growth in Culture

Even when tumor tissue has been selected from an optimal region, the unpredictable growth of neoplastic cells in culture remains a major consideration. Benign tumors often contain only few mitotic cells and so one generally has to wait several days before such specimens proliferate actively in culture. In the meantime, the culture may become overgrown by nonneoplastic cells, such as fibroblasts. Perhaps surprisingly, even highly malignant solid tumors may grow poorly in tissue culture, despite the fact that they grew well in the patient. Such tumor cultures may occasionally be stimulated by the use of specialized culture media or growth factors,[5] but it is impractical in most clinical cytogenetic laboratories to troubleshoot tissue cultures to optimize the growth of each tumor type. Therefore in practice it may be challenging to culture and

Box 45-1 **Cytogenetic Abbreviations and Definitions**

cDNA: complementary DNA
cen: centromere
CGH: comparative genomic hybridization
CISH: chromogenic in situ hybridization
del: deletion
dmin; double minute chromosome (extrachromosomal amplicon)
FISH: fluorescence in situ hybridization
hsr: homogeneously staining region (intrachromosomal amplicon)
ins: insertion within chromosome
inv: inversion of chromosome segment
ISH: in situ hybridization
mar: marker chromosome (aberrant chromosome whose origin cannot be ascertained)
p: chromosome short arm
q: chromosome long arm
r: ring chromosome
SKY: spectral karyotyping
t: translocation
tel: telomere

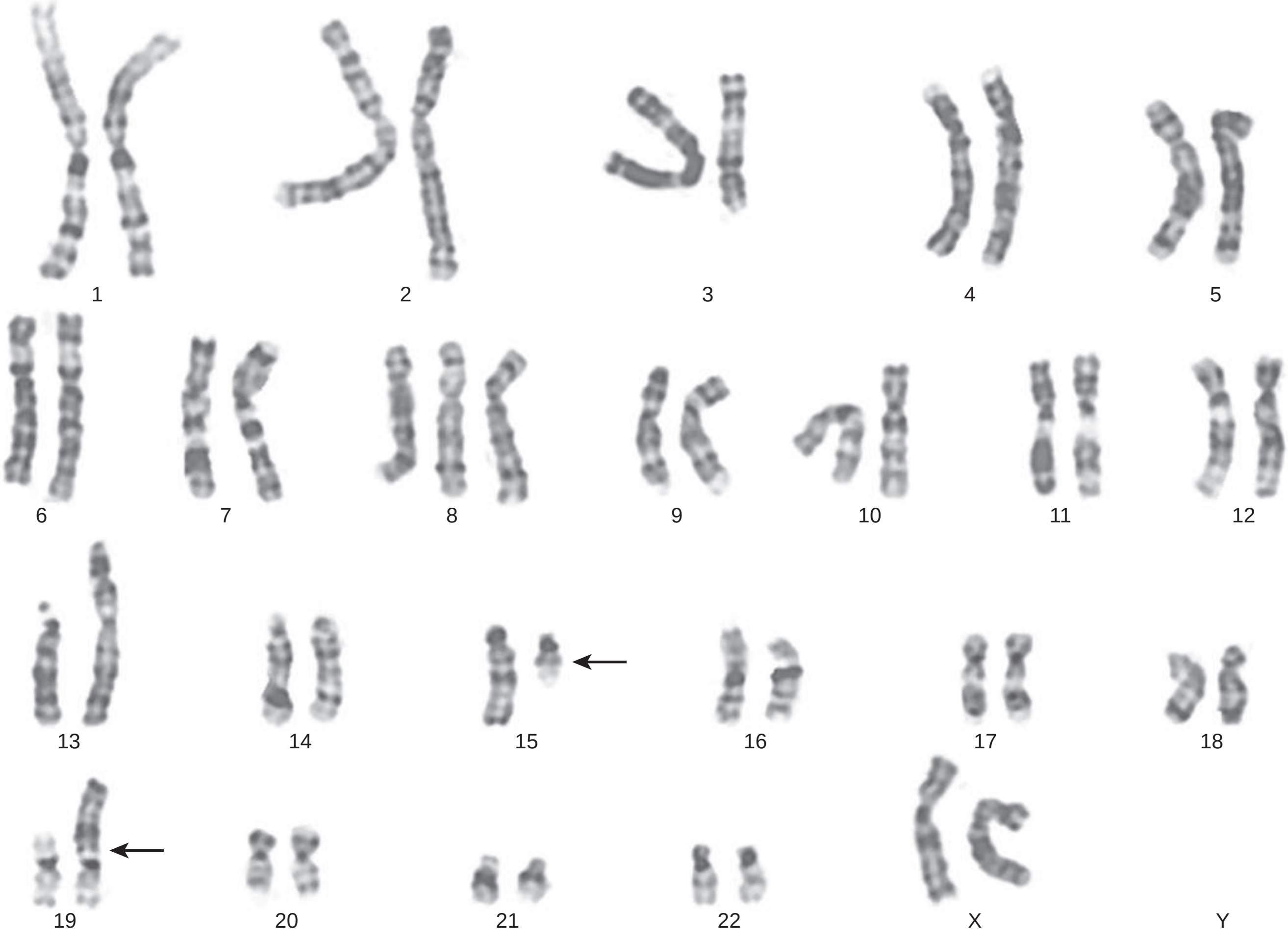

Figure 45-1 Giemsa-banded karyotype of highly lethal NUT midline carcinoma, demonstrating balanced t(15;19) with *arrows* indicating the translocation breakpoints. Other cytogenetic findings, including chromosome 3 and 13 rearrangements and trisomy 8, are secondary aberrations.

karyotype certain types of pediatric tumors in the clinical laboratory. In the case of bone marrow cytogenetics, it is well known that posttherapeutic specimens can be difficult to analyze, being hypocellular and containing cells—reactive and/or neoplastic—that have been temporarily growth-arrested by therapy and therefore fail to give rise to metaphases in culture.

Nonrepresentative Cell Culture

It is also important to recognize that only a subpopulation of cells within a given sample might be capable of growing under a particular set of culture conditions. Therefore one cannot necessarily assume that a clonally abnormal karyotype is representative of the overall neoplastic process. For example, in a given tumor, the final karyotype might be representative of the components of the tumor that were more or less clinically aggressive, depending on which component was best suited for growth under the particular culture conditions used at that time.

Culture Overgrowth by Nonneoplastic Cells

All tumor samples contain mixtures of neoplastic and nonneoplastic cells. The nonneoplastic elements may include hematopoietic cells, fibroblasts, normal epithelial cells, endothelial cells, or glial cells, depending on the type and location of the tumor. Any of these reactive or support cell types might proliferate more successfully than the neoplastic cells in culture. Invariably, when a pediatric cancer submitted for cytogenetic analysis is reported to have a normal karyotype, the normal karyotype is a false-negative result, only signifying that the culture was overgrown by normal cells. Therefore the cytogenetic analysis must always be timed carefully, so that metaphases are analyzed at a point at which the neoplastic population is actively dividing. This technical challenge can, in the case of solid tumors, be met if the cytogeneticist develops familiarity with the distinctive morphologies of the various neoplastic and reactive cell types and then inspects the tissue cultures daily to determine when the neoplastic cells have the proliferative advantage.[5]

Complex Karyotype

Beyond the initial challenges incurred at the culture stage, various additional considerations influence the success of cytogenetic evaluation in different types of pediatric cancer. For example, conventional karyotyping can be challenging in many high-grade solid tumors, in which the karyotypes are often complex compared with those seen in leukemias and lymphomas. A single metaphase cell from such cancers can contain dozens of clonal and nonclonal chromosomal aberrations, making it impractical to characterize the exact mechanisms of rearrangement responsible for each chromosomal aberration or to estimate the relative significance of any of these abnormalities.

Technical Limitations in Detecting Aberrations

There are certain aberrations that cannot be detected by karyotyping, because the resolution is too low. These include smaller deletions or amplifications and point mutations, but also cryptic or masked aberrations, which include, for example, the chromosomal translocation t(12;15) characteristic of congenital or infantile fibrosarcoma, congenital cellular mesoblastic nephroma, secretory breast carcinomas, and a subset of acute myelogenous leukemias (Fig. 45-2).[6,7] Another translocation that cannot

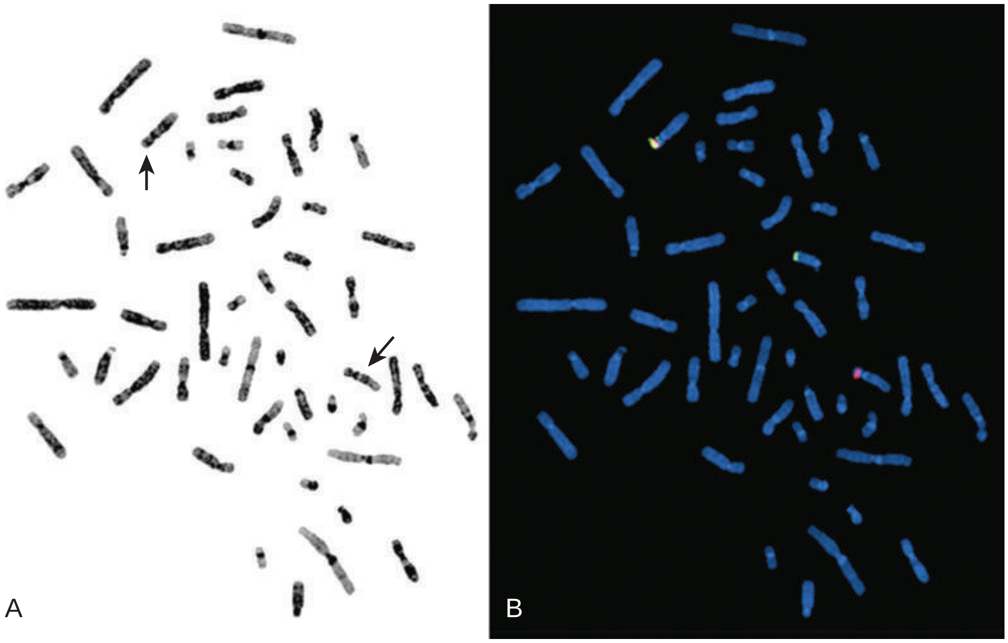

Figure 45-2 **A,** Giemsa banding studies in infantile fibrosarcoma do not reveal the characteristic, but cytogenetically cryptic t(12;15), associated with *TEL-NTRK3* oncogenic fusion. *Black arrows* indicate the normal *(top)* and rearranged *(middle)* copies of chromosome 12, as demonstrated by *TEL* break-apart fluorescence in situ hybridization (FISH). **B,** The FISH studies reveal yellow probe fusion signal on the normal chromosome 12, whereas the t(12;15) results in split red and green FISH signals on rearranged chromosomes 12 and 15, respectively.

be resolved by conventional karyotype analysis is the t(12;21), which is the most common translocation in pediatric leukemia. It is found in a large fraction of common acute lymphoblastic leukemia (ALL) in children and fuses the *ETV6 (TEL)* and *RUNX1 (AML1)* genes.

In essence, then, the major advantage of karyotypic evaluation of solid tumors is the breadth of the data provided. Notable challenges include the successful culture of representative tumor cells, particularly because this is essentially a one-shot approach, with no opportunity to return to the specimen, because the window of opportunity for culture is confined to the short period of viability of fresh cells after biopsy. Even when culture is successful, meaningful interpretation of the findings on karyotype may represent a further challenge, depending on the number and nature of the aberrations.

Although solid tumors are less readily accessible for biopsy compared with hematologic neoplasms, minimally invasive sampling by a percutaneous approach is becoming more common. Needle biopsy is often performed under ultrasound or computed tomographic (CT) guidance and can involve fine-needle aspiration or needle core biopsy of the tumor. Solid tumor samples obtained by these methods can be karyotyped successfully,[8-11] but the small amount of starting material is a constraint in that fewer cultures can be established. Because the successful cytogenetic evaluation of tumors in standard practice is limited by these factors, alternative, more robust methods are needed for the successful routine demonstration of genetic aberrations. Traditionally, these have included fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR) techniques, which have the distinct advantage of applicability to fixed material and therefore also to large cohorts of archival cases. Both FISH and molecular analyses can be straightforward also in fine-needle specimens.[12,13]

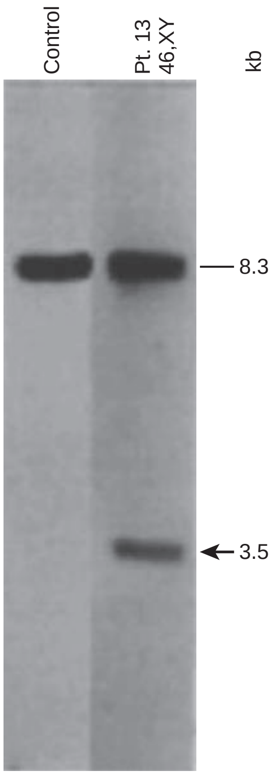

Figure 45-3 Detection of cryptic *MLL* bcr rearrangement by Southern blot analysis in treatment-associated acute myelogenous leukemia (AML). The karyotype was normal, but the French-American-British (FAB) M4 morphology and clinical history of dactinomycin exposure informed further analysis of *MLL*. The *dash* indicates the germline pattern, and the *arrow* shows a rearrangement, which panhandle polymerase chain reaction molecular cloning revealed to be an *MLL* internal tandem duplication. *(Modified from Megonigal MD, Rappaport EF, Jones DH, et al: Panhandle PCR strategy to amplify* MLL *genomic breakpoints in treatment-related leukemias.* Proc Natl Acad Sci U S A *94:11583–11588, 1997.)*

Molecular Cytogenetics

Recombinant DNA Technologies

Southern Blot Analysis. In pediatric solid tumors, leukemias, and lymphomas, recombinant DNA technologies, including Southern blot analyses of candidate genes relevant to particular disease states, can overcome limitations of conventional karyotype analysis for the detection of chromosomal aberrations (Fig. 45-3). Although no longer used extensively in most clinical diagnostic laboratories, Southern blotting has served an important role in gene discovery. That is, genomic Southern blot analyses, coupled with PCR-based molecular cloning and gene sequencing strategies, have identified unknown partner genes fused to various known genes in chromosomal translocations. For example, the demonstration of immunoglobulin *(IG)* and T-cell receptor *(TCR) loci* rearrangements in leukemias and lymphomas, as evidenced by altered size of restriction enzyme fragments on genomic Southern blot analyses, has been essential in highlighting crucial regions that could be studied by molecular cloning strategies and that characterize the genomic translocation breakpoint junction sequences that define these tumors (Fig. 45-4). The molecular principles that provide the basis for current clinical molecular cytogenetic diagnostic strategies (e.g., FISH) in pediatric leukemias and solid tumors are not substantially different from the recombinant DNA concepts used in Southern blotting. Some seminal examples of gene rearrangements characterized originally by Southern blotting and now detected routinely by FISH include *MYC* gene rearrangements, involving the *IGH* or *IGL* chain loci in Burkitt lymphoma,[14] and *MLL (KMT2A)* rearrangements, of which the first characterized type was associated with t(4;11), resulting in *MLL-AFF1 (AF4)* fusion.[15]

Another application of Southern blot analysis has been in discerning gene copy number variation. One example is *MLL* gene amplification (see Fig. 45-4), involving an interlocus DNA rearrangement known as segmental jumping translocation in which the unrearranged *MLL* DNA segment is amplified and translocated to various regions of the genome.[16]

Fluorescence and Chromogenic in Situ Hybridization. Whereas conventional cytogenetic analyses are performed using staining techniques that highlight chromosomal banding patterns (1;2), the various molecular cytogenetic

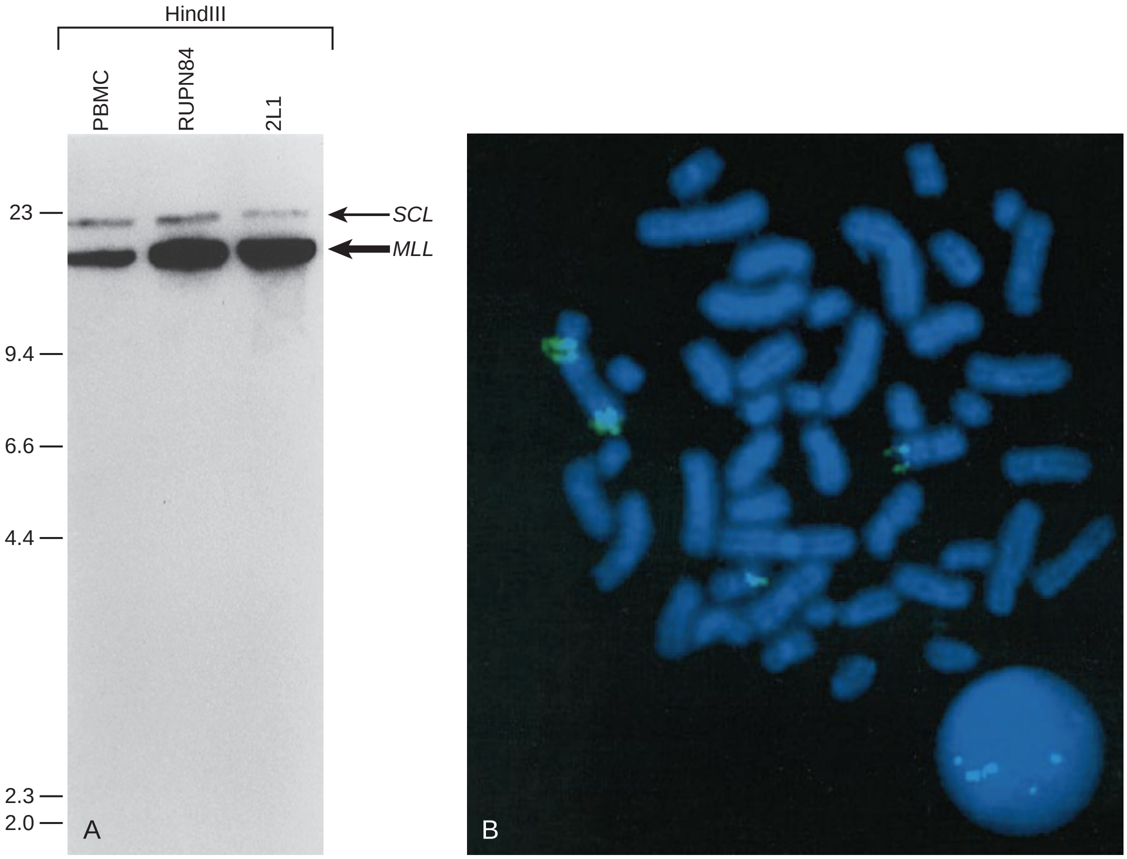

Figure 45-4 **A,** Southern blot analysis indicating *MLL* gene amplification in treatment-related acute myelogenous leukemia (AML). Southern blot analysis of *HindIII*-digested genomic DNA from French-American-British M4 AML cells (RUPN84) of a patient whose AML karyotype showed no evidence of chromosome band 11q23 rearrangement that would suggest *MLL* gene rearrangement and a cell line established from the same AML (2L1). Cohybridization with a *MLL* bcr-region probe and a loading control *SCL* probe demonstrates unrearranged but amplified *MLL* with signal intensity of 4.3 : 1 in the AML compared with normal peripheral blood mononuclear cell (PBMC) control DNA. **B,** Fluorescence in situ hybridization confirms *MLL* genomic amplification in RUPN84. *(Modified from Felix CA, Megonigal MD, Chervinsky DS, et al: Association of germline* p53 *mutation with* MLL *segmental jumping translocation in treatment-related leukemia.* Blood *91:4451–4456, 1998.)*

methods interrogate chromosomal regions of interest by using nucleic acid probes.[17,18] Most molecular cytogenetic methods use some variant of in situ hybridization (ISH), in which DNA probes are hybridized and evaluated in the cellular context. These probes are usually cosmids, containing an approximately 40-kb sequence of interest or bacterial artificial chromosomes, containing hundreds to a few thousand kilobases of sequence. ISH assays can be performed with fluorescence or enzymatic detection, referred to as FISH and CISH (chromogenic in situ hybridization), respectively (Figs. 45-5 and 45-6).[18]

Flexibility of in Situ Hybridization: Substrate Options. FISH and CISH analyses are performed routinely in cytogenetic preparations (metaphase spreads) and in paraffin sections and other archival pathologic preparations. Substantial advantages of using paraffin sections include the following: (1) the abundance of source material for each case permits retrospective and repeat analyses; and (2) the well-preserved cell morphology can guide evaluation of the chromosomal events in the relevant cell populations. However, a drawback in the use of paraffin sections is that the nuclei are generally incomplete, having been sliced through during preparation of the sections by microtomy.[19] This limitation can be addressed by performing the ISH assays with nuclei disaggregated from thick (50- to 60-μμ) paraffin sections[20] or from thin cores of the paraffin block.[21]

Resolution. Most ISH studies have a resolution of one to several megabases on metaphase spreads. Notwithstanding the aforementioned problems of interphase FISH on fixed material, the technique offers the advantage of higher resolution, given the less compact arrangement of DNA in interphase nuclei. Higher-resolution FISH techniques have been developed in which the DNA is essentially stretched out over a slide, as in direct visual hybridization (DIRVISH), resulting in a resolution of between 700 and 5 kb,[22] or fiber FISH, in which the resolution is between 500 kb and just a few kilobases.[23]

Highly Combinatorial Modifications of FISH. Various molecular cytogenetic methods have expanded the capabilities of FISH, enabling evaluation of the entire genome or karyotype. Examples include comparative genomic hybridization (CGH)[24,25] and spectral karyotyping (SKY).[26] CGH is performed by extracting total genomic DNA from a tumor of interest and from a nonneoplastic control cell population. These DNAs are differentially

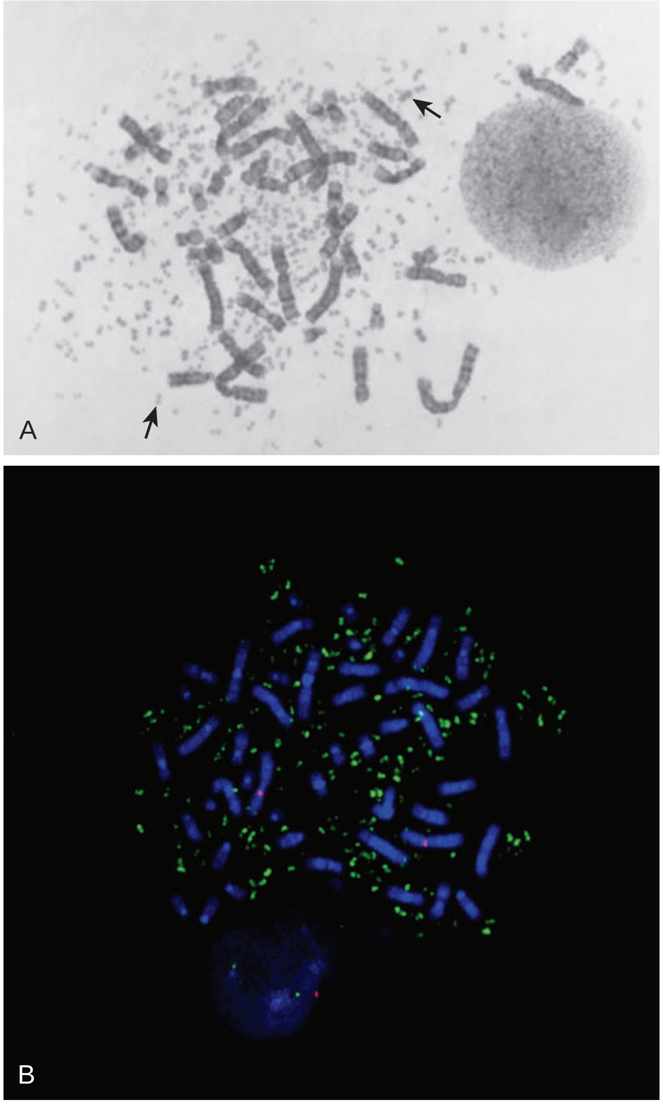

Figure 45-5 **A,** Giemsa-stained neuroblastoma metaphase cell (and with nucleus from a different cell on right) showing hundreds of variably sized double-minute chromosomes, as indicated by *arrows*. **B,** *MYCN* fluorescence in situ hybridization (FISH) evaluation of neuroblastoma metaphase cell, showing numerous extrachromosomal double minutes containing *MYNC (green)* whereas the reference chromosome 2 pericentromeric FISH probe *(red)* shows two copies.

labeled (e.g., tumor DNA with fluorescein and control DNA with rhodamine) and in the earliest applications were cohybridized against normal metaphase cells (metaphase CGH), although these methods have been rapidly supplanted by higher-resolution assays in which the sample DNAs are hybridized against arrayed clones (array CGH) or interrogated for presence of informative single nucleotide polymorphisms (SNPs) as an indicator of copy number and heterozygosity across the genome (discussed later).

An advantage of CGH, compared with conventional karyotyping, is that the tumor DNA can be isolated from frozen or even paraffin specimens, without any need for cell culture, thereby avoiding the selection pressures imposed by culture and instead capturing an overview of the in vivo situation, although diluted by the admixture of support stromal DNA. In addition, the array CGH methods (discussed later) can detect very small deletions, which would be overlooked by traditional cytogenetic banding assays and might even be missed by FISH. However, CGH does not detect the balanced chromosomal rearrangements, particularly the translocations that are the genetic hallmarks of many pediatric cancers. Furthermore, while detecting chromosomal amplifications and deletions reliably, the technique does not provide a functional assay in terms of expression level changes.

Genome-wide molecular cytogenetics can be performed using spectral karyotyping or M-FISH, in which entire panels of DNA probes are simultaneously hybridized against tumor metaphase cells.[26] Whereas conventional FISH techniques involve hybridization of one or two fluorescence-tagged probes, the spectral karyotyping and M-FISH methods use probes for each chromosome or chromosome arm (24 or more probes). Each probe is then detected combinatorially, using different ratios of fluorescence markers, such as fluorescein and rhodamine. By varying the ratio of fluorescence tags, each chromosome can be visualized with a unique color (Fig. 45-7). Thus spectral karyotyping enables a comprehensive ISH screen of the entire tumor cell karyotype. Spectral karyotyping and M-FISH are powerful research tools in tumor cytogenetics and have been useful in identifying rearrangements that are cryptic by conventional cytogenetic banding methods or that are highly complex.[27,28] However, these techniques have generally been supplanted by array CGH and SNP profiling methods (see later).

Routine Applications of FISH. Most ISH studies performed in clinical laboratories are focused on investigating deletions, rearrangements, or amplifications of particular gene loci or chromosomes. The probes used for

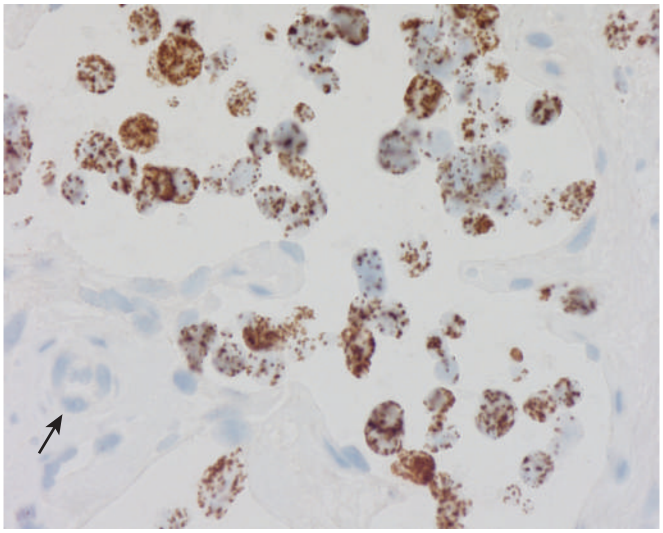

Figure 45-6 *MYCN* chromogenic in situ hybridization in a neuroblastoma paraffin section. Peroxidase-DAB detection *(brown)* of *MYCN* fluorescence in situ hybridization probe demonstrates high-level *MYCN* amplification in neuroblastoma cells but not in fibrovascular nonneoplastic cells *(arrow)*.

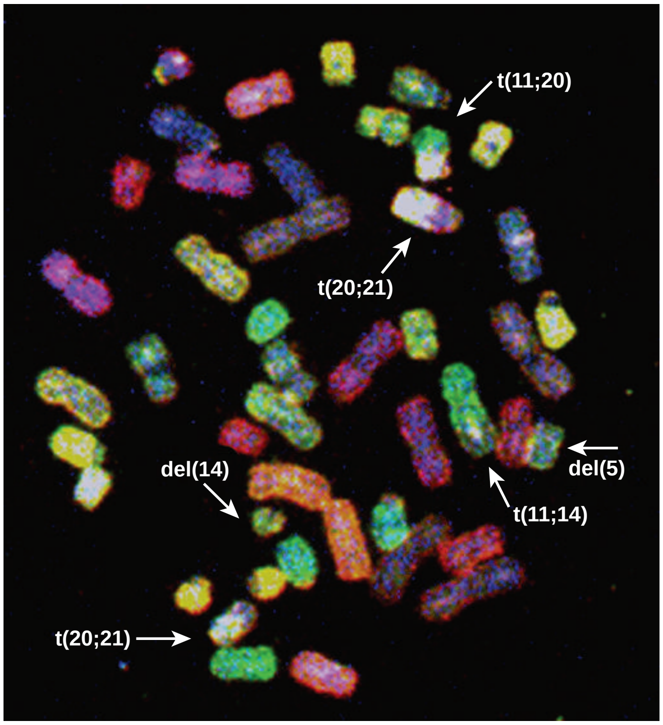

Figure 45-7 Metaphase spectral karyotype in treatment-associated acute myelogenous leukemia, showing typical 5q deletion, among other clonal aberrations. *(Courtesy Hesed, National Institutes of Health, Bethesda, Md.)*

FISH analysis of chromosomal translocations (and other balanced cytogenetic rearrangements) generally fall into two categories. First, there are those that hybridize to a region spanning a breakpoint on one involved chromosome, producing a split signal in the presence of chromosomal rearrangement (see Fig. 45-2), Second, there are probes designed to come together in the presence of a fusion, in which a probe labeled with one fluorochrome hybridizes to one partner gene and the other, differentially labeled, to its fusion partner, with these producing a fused fluorescent signal only in the presence of a gene rearrangement.

Maximizing Data Yield from Individual FISH Probes for Rearrangements. Judicious selection of break-apart FISH probes can allow applicability to a wide variety of tumors, in which the probe interrogates a frequently rearranged gene such as *EWSR1* in various solid tumors or *MLL* in leukemias. The *EWS* gene undergoes rearrangement with various ETS family genes in Ewing sarcoma and *EWSR1* is rearranged in pediatric desmoplastic round cell tumors and clear cell sarcomas of soft parts, among others. Each of these diagnostically useful *EWSR1* rearrangements can be demonstrated using a single *EWSR1* break-apart FISH probe (Fig. 45-8), with ultimate diagnosis requiring interpretation of the molecular findings in the context of the histology.

Additional gross genetic aberrations routinely accompanying chromosomal translocation may be exploited to refine interpretation of FISH-based analysis in certain contexts. For example, *FOXO1* gene FISH can distinguish the *PAX7-FOXO1* and *PAX3-FOXO1* oncogenic fusions in alveolar rhabdomyosarcoma, given that the *PAX7-FOXO1* (but not the *PAX3-FOXO1*) is invariably highly amplified in these tumors. This distinction is warranted because of its prognostic significance. In the typical scenario, in which routine pathologic evaluation by light microscopy of hematoxylin and eosin (H&E)-stained sections with accompanying immunohistochemistry has generated a reasonably narrow differential diagnosis, genetic testing by this type of assay is highly informative and increasingly being used. Generally, this somewhat looser FISH-based assay may be regarded as having optimal attributes, providing greater sensitivity than karyotypic analysis and being capable of detecting cryptic or masked translocations[29] while not being burdened by the restrictive specificity of PCR-based assays, a feature that is especially advantageous for detection of variant translocations.

FISH in the Analysis of Gains and Losses. Although quantitative assessment of gene amplifications and deletions can be obtained by quantitative PCR (qPCR) or FISH, in practice such assays are more often conducted in clinical laboratories by FISH-based methods. In the example of *MYCN* amplification in neuroblastoma, FISH detection is performed routinely (see Figs. 45-5 and 45-6). Assessment of *MYCN* amplification may alternatively be done by semiquantitative PCR, using a standard PCR assay with normal (unamplified) and known amplified controls and multiplexing the reaction to include a housekeeping gene along with the gene of interest. However, such assessments can be confounded by intratumoral

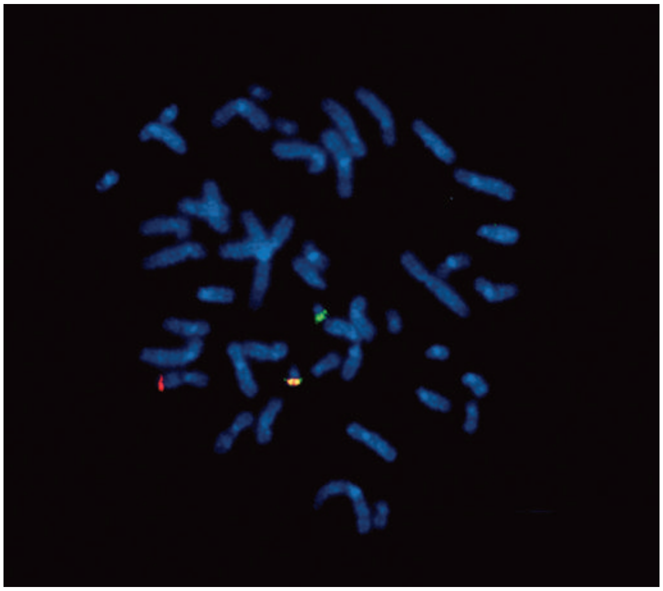

Figure 45-8 *EWS* break-apart fluorescence in situ hybridization probe, hybridized to Ewing sarcoma metaphase cell, in which probe components centromeric and telomeric to *EWSR1* are detected with fluorescein isothiocyanate *(green)* and rhodamine *(red)*, respectively. *EWSR1* rearrangement is indicated by the breaking apart of the normal yellow (*green-red fusion)* signal into the red and green components, which localize to different chromosomes as a result of translocation.

heterogeneity. In neuroblastoma, this heterogeneity may encompass substantial variations in cellular composition and maturation, and thus careful selection of tumor tissue for analysis is essential. The analysis of poorly selected tissue for *MYCN* evaluation (e.g., submission of schwannian stroma) will otherwise yield a false-negative result.[30] Moreover, contributions of different components of the neuroblastic tumor in the final DNA isolate can dilute out regional (neuroblast-specific) *MYCN* amplification, again yielding false-negative results. In this context, there is a distinct advantage to the FISH assays for *MYCN* amplification, because such methods permit correlation with cytology or morphology for the detection of regional heterogeneity of *MYCN* amplification, particularly when performed in tissue sections or multifocal touch imprints.

Detection of deletions by FISH is a more complex matter and particularly challenging when using fixed tissue, in which the assay is for an absence of signal and one is dealing with interphase nuclei, by definition incomplete (sliced through by microtomy), with an admixture of normal stromal cells further confounding the picture. Where cytogenetically abnormal metaphase spreads are available, these problems are largely circumvented, but there are other potentially more suitable technical strategies that might be used, including PCR-based assays—again with the caveat of normal cell admixture—to establish loss of heterozygosity (LOH). The immunohistochemical stain may provide a ready assay for such detection, as in the case of the *SMARCB1* gene on chromosome 22q, commonly deleted in malignant rhabdoid tumors (Fig. 45-9), and atypical teratoid rhabdoid tumors.[31]

Polymerase Chain Reaction–Based Molecular Assays

General Applications of PCR-Based Methods

Since the initial description of the basic PCR assay,[32] critical modifications of the technique have led to massive expansion of the applicability of this technology. The initial methodology involved the use of a DNA template from which a sequence of interest could selectively be amplified. This methodology provides the basis for testing for LOH, which relies on the natural allelic heterogeneity of repeat sequences in the vicinity of a gene of interest.[33] Where a genomic region is deleted, the concomitant loss of an adjacent allelic repeat sequence results in PCR amplification of only one version of the repeat sequence, thus producing hemizygosity (seeming homozygosity) at that locus.

Reverse-Transcriptase PCR Assay. When a particular question revolves around the presence of a specific transcript, the starting material is RNA, which is subjected to reverse-transcriptase PCR (RT-PCR), a highly sensitive technique.[34] The principle is that total RNA extracted from fresh, flash-frozen, or fixed material is reverse-transcribed into first-strand complementary DNA (cDNA). This provides the template[34,35] for testing for the presence of fusion oncogene transcripts using primers complementary to the involved partner genes flanking the fusion point in the chimeric transcript and which cannot therefore generate a product from unrearranged normal transcript. The technique is extraordinarily sensitive, able to detect as few as 1 in 10,000 cells bearing a fusion transcript.[36] The presence of just a small neoplastic component within a tissue sample for genetic testing is therefore sufficient for detection. Thus this PCR-based technology is useful for the detection of minimal residual disease[35] and well suited for the surveillance of hematologic malignancy, in which serial peripheral blood or bone marrow specimens are obtained for routine follow-up of patients. However, this same sensitivity of PCR-based methods carries with it the risk for false-positive results, from cross-contamination of minute amounts of fusion gene templates within assays or detection of very-low-level biologically insignificant fusion transcripts within specimens. Although moot in the context of demonstrating uniquely tumor-associated aberrations (e.g., fusion products), ideally DNAse I treatment of the RNA substrate should be performed to eliminate the possibility of inadvertent genomic amplification during RT-PCR.

Specificity of RT-PCR Reaction. Breakpoint variability within the genes involved in translocation-generated fusion oncogenes has prognostic implications in some cases. This is reported for Ewing sarcoma and synovial

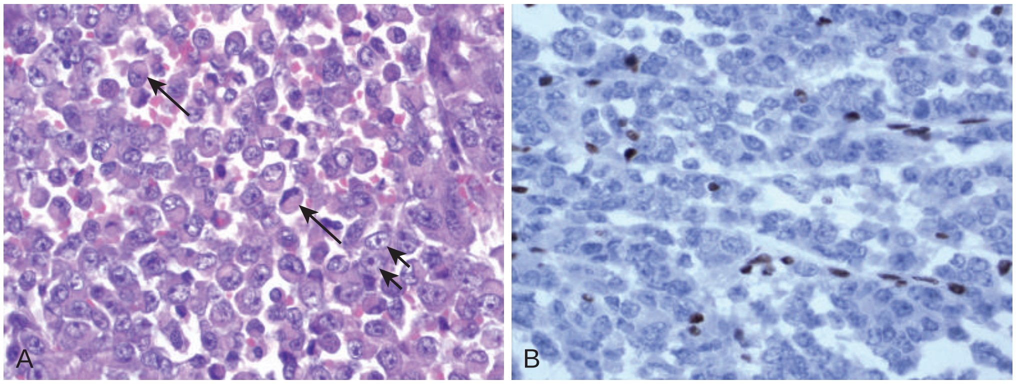

Figure 45-9 **A,** Rhabdoid tumor showing loosely cohesive cells, many containing intracytoplasmic inclusions *(arrows)*. Nuclei are vesicular and contain prominent nucleoli *(short arrows)* (H&E stain). **B,** INI1 immunohistochemistry, showing entirely negative reaction in the neoplastic cells, reflecting loss of 22q, and with only the endothelial and occasional admixed inflammatory cells staining positively.

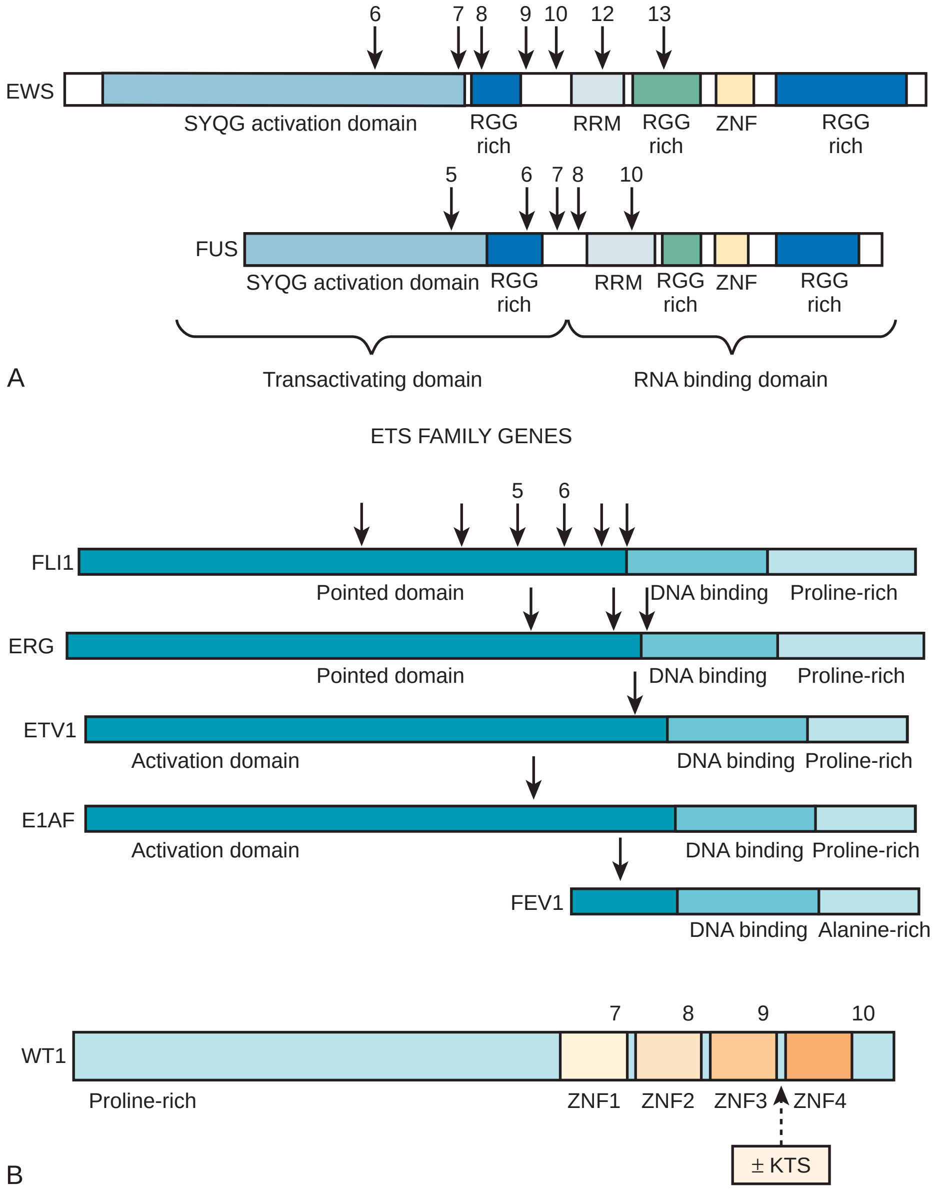
6
7
8
9
10
12
13
EWS
SYQG activation domain
RGG rich
RRM
RGG rich
ZNF
RGG rich
5
6
7
8
10
FUS
SYQG activation domain
RGG rich
RRM
RGG rich
ZNF
RGG rich
Transactivating domain
RNA binding domain
A
ETS FAMILY GENES
5
6
FLI1
Pointed domain
DNA binding
Proline-rich
ERG
Pointed domain
DNA binding
Proline-rich
ETV1
Activation domain
DNA binding
Proline-rich
E1AF
Activation domain
DNA binding
Proline-rich
FEV1
DNA binding
Alanine-rich
7
8
9
10
WT1
Proline-rich
ZNF1
ZNF2
ZNF3
ZNF4
± KTS
B

Figure 45-10 **A,** *EWS* and *FUS* genes encode an N-terminal transcriptional activation domain, RGG-rich regions, RNA recognition motif, and a zinc finger. *Arrows* indicate various breakpoints reported in *EWS* or *FUS* oncogenes. **B** *(top)*, Domains encoded by the various ETS-family genes fused with *EWS* or *FUS* in Ewing sarcoma. *Arrows* indicate various breakpoints that have been reported in fusion rearrangements. The most common oncogenic rearrangements in Ewing sarcoma involve the fusion of the *EWS* exon 7 to *FLI1* exon 6 (type 1 transcript) or exon 5 (type 2 transcript). **B** *(bottom)*, Domains encoded by the *WT1* gene, which is fused with *EWS* in desmoplastic small round cell tumor. Two WT1 isoforms are produced by an alternative spicing event (±KTS) between zinc fingers 3 and 4.

sarcoma, in which a favorable prognosis is associated with a type I fusion of *EWS* exon 7 to *FLI1* exon 6 compared with all other combinatorial variations in Ewing sarcoma (Fig. 45-10).[37-39] *SYT-SSX2* fusion in synovial sarcoma is similarly reportedly associated with better patient outcome.[40,41] Assays may be designed to address breakpoint variability through choice of primers, selectively amplifying only one variant product, or by choosing consensus primers that will amplify several transcripts[42,43] and then sequencing the product to establish the precise nature of the transcript. An indication of transcript type might already have been obtained from gel electrophoresis demonstrating the product size (Fig. 45-11; see Fig. 45-10). PCR-based assays are best suited to the detection of such fine genetic detail, which cannot be detected by karyotypic analysis and only by specifically designed FISH assays. Currently, distinction among variant transcripts generally remains of academic interest because it provides no basis for therapeutic stratification.

In the case of *MLL* translocations, not only can one of more than 50 different partner genes be fused to *MLL* but it has also been suggested that the various partner genes of *MLL* affect the prognosis.[44] In this case, PCR

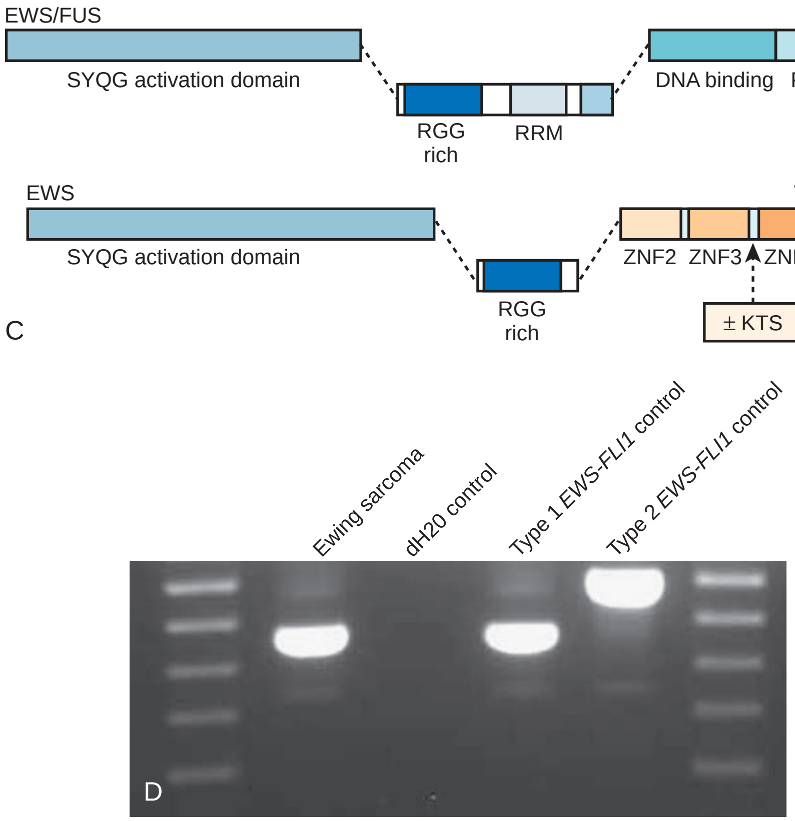

Figure 45-10, cont'd **C** *(top)*, Domains encoded by oncogenic fusions between *EWS* or *FUS* with an ETS family member in Ewing sarcoma. *Dotted lines* indicate regions that are variably included, depending on the breakpoint locations. **C** *(bottom)*, Domains encoded by the *EWS-WT1* fusion oncogene in desmoplastic small round cell tumor. *Dotted lines* indicate encoded sequences that are variably included, depending on the breakpoint locations (RGG-rich region) and alternative splicing events (KTS). **D**, Reverse-transcriptase polymerase chain reaction demonstration of type 1 *EWS-FLI1* transcript in Ewing sarcoma (lane 1). Positive controls for types 1 and 2 transcripts are in lanes 3 and 4, respectively.

with gene-specific primers has been one approach to identify fusion transcripts involving some of the more common partner genes of *MLL*, and primer combinations for different *MLL* transcripts can be combined in a multiplex PCR reaction. This is most feasible in ALL, where the *MLL* partner genes are less heterogeneous than in acute myeloid leukemia (AML). Alternatively, approaches such as cDNA panhandle PCR can detect the fusion transcript produced by the 5′-*MLL* partner gene 3′ rearrangement using primer sequences derived from MLL. This is accomplished by the generation of a stem loop template in which a known *MLL* sequence has been attached to the unknown partner sequence by reverse transcribing the first-strand cDNA from total RNA using a 5′-*MLL*-random hexamer adapter-3′ primer for the primer extension.[45]

Additionally, genomic panhandle PCR approaches[46-48] and long distance inverse PCR approaches[49,50] are alternatives for characterizing translocations in which the sequence of only one of the involved genes is known. And increasingly, intron-capture methods, coupled with sequencing, are used in high-throughput sequencing screens to identify oncogene fusion partners.

One clinical scenario that requires the ability to detect a broad range of chromosomal translocations, including unknown or rare fusion variants, is in surveillance screening for evidence of a preleukemic state in chemotherapy-exposed patients, before these patients manifest frank secondary leukemia.[51] Advances in sensitive molecular PCR-based screening methods and molecular cytogenetic screening methods such as FISH will better enable the prospective detection of chromosomal translocations, which is crucial in such applications.

Quantitative PCR Assay. Simultaneous PCR of a constitutively expressed housekeeping gene provides an internal control. A standard positive control may be used as a comparator across a series of cases to rank these in a semiquantitative approach.[52] Use of qPCR methods can provide precise information about the actual copy number of oncogene transcripts (gene expression level) by qRT-PCR or gene copy number when genomic DNA is used as template material. qPCR has advantages of increased reliability and reproducibility over conventional PCR. Because it provides data accrued during the entire cycle, rather than just end-point analysis, as in standard PCR, it will discriminate between different assays quantitatively rather than based on product size differences. Additionally, the graphic readout of accumulating fluorescence generated within each tube or well during the reaction is instantaneous (real time) and therefore no delaying additional step for product analysis is

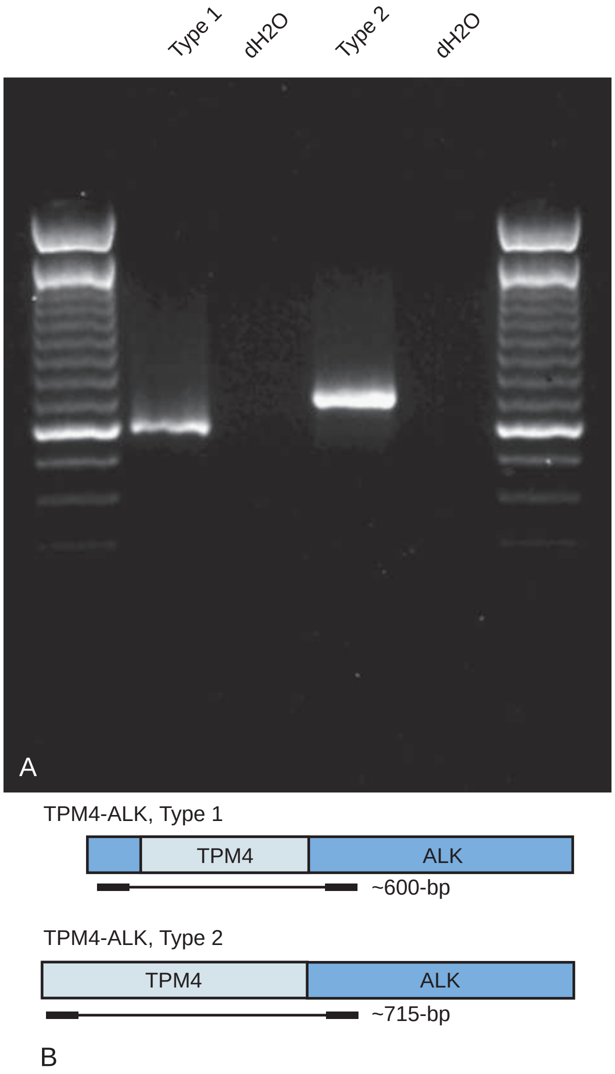

Figure 45-11 Reverse-transcriptase polymerase chain reaction (RT-PCR) demonstration of various *TPM4-ALK* fusion oncogene transcripts in inflammatory myofibroblastic tumors, as distinguished by gel electrophoresis of PCR products generated with the same primers (**A**), with primer hybridization sites as indicated (**B**).

required. In conventional PCR, the amplification products must typically be evaluated by gel electrophoresis. Therefore the assay turnaround time is minimized in qPCR, so that a complete analysis can be performed in only a few hours. qPCR is more sensitive, with an ability to detect twofold changes in expression level as opposed to a tenfold cutoff for standard PCR assays. If desired, the products can still be subjected to gel electrophoresis (e.g., to evaluate product size). Various forms of qPCR are available, including (1) an SYBR green-based method, detecting double-stranded DNA product accumulation by binding to the minor groove of double-stranded DNA nonspecifically and thus being more readily subject to false positivity because of contamination or detection of primer-dimer; and (2) TaqMan methodology, based on highly specific primer-probe combinations. In TaqMan qPCR, a probe labeled with a fluorescent dye at one end (5′) and a quencher at the other (3′) is designed to bind within the sequence amplified by qPCR. In the unbound state, the proximity of quencher to the fluorescent dye inhibits detection of the fluorescence through Foerster resonance energy transfer (FRET). Once the probe has bound within the amplified sequence, 5′ exonuclease activity of the polymerase enzyme leads to probe cleavage and dissociation of fluorescent dye and quencher, with ensuing accumulation of fluorescence proportional to product amplification (strictly probe cleavage). Multiplexing of reactions is possible with TaqMan but not SYBR green technology.

Array Comparative Genomic Hybridization and Single Nucleotide Polymorphism Profiling Assays

Conventional metaphase CGH, which was summarized previously, was made obsolete by the advent of array CGH, in which the tumor DNA is hybridized instead to high-density arrays of probes covering the entire genome.[53] Array CGH provides a much higher-resolution genome-wide definition of genetic gains and losses. However, conventional and array-based CGH do not detect balanced chromosome translocations, a hallmark of many pediatric cancers. Genome-wide SNP assays (Fig. 45-12), like array CGH, are used increasingly as a higher-resolution alternative to conventional karyotyping for genome-wide assessments of gene copy number alterations, and, unlike array CGH, the SNP methods have the advantage of demonstrating LOH, even in situations in which gene copy number is normal (copy number neutral LOH). However, SNP profiling generally provides less quantitative assessment of copy number gains compared with array CGH.

Next-Generation Sequencing and Other Techniques

In light of the ever-increasing number of gene fusions identified in pediatric sarcomas and hematologic neoplasms, the need for more efficient, multiplexed methods for their detection is becoming more pressing. Increasingly, molecular diagnostic laboratories are implementing variations of next-generation sequencing toward these ends. Whereas few laboratories now employ comprehensive genome sequencing (exome, whole genome, or whole transcriptome) in routine diagnostics, more targeted high-throughput sequencing panels have become commonplace. Typically, these panels focus on gene perturbations with known prognostic, predictive, or diagnostic relevance in cancer, detecting hundreds of clinically relevant mutations in a single panel. Although the translation of these new approaches based on next-generation sequencing into routine diagnostic assays is moving quickly,[54] there remain challenges to routine implementation for diagnosis, including the need for relatively large amounts of high-quality nucleic acids. In addition to next-generation sequencing, there are various other novel and promising methods for detecting the ever-expanding variety of diagnostically relevant gene rearrangements in cancer. These include the NanoString nCounter system, which detects individual messenger RNA (mRNA) molecules without PCR amplification in a quantitative and highly multiplexed fashion[55] and antibody detection of translocations assays.[56]

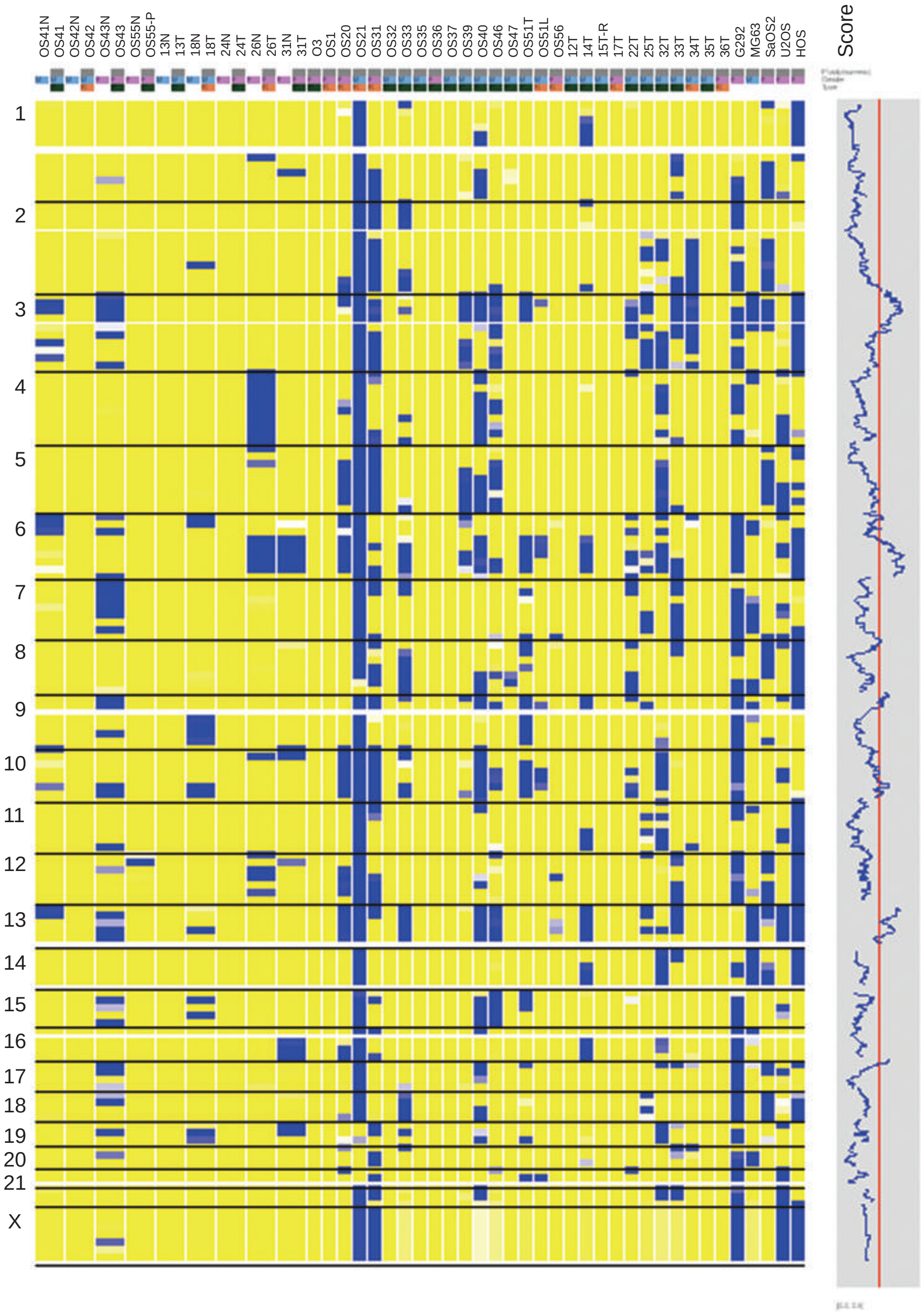

Figure 45-12 Genome-wide single-nucleotide polymorphism (SNP) analysis of osteosarcomas, with each column showing data from an individual case and the rows showing SNP data for each chromosome. Most osteosarcomas have numerous regions of SNP loss of heterozygosity, which are indicated in *blue,* whereas admixed regions of retained heterozygosity are in *yellow.*

It might be surmised that the optimal setup of a tumor genetics laboratory in the pediatric context would strive to culture cells from all newly diagnosed tumors. It would largely rely on FISH-based assays to screen for rearrangements or amplifications of commonly involved genes such as *EWSR1*, *FOXO1*, *MYCN*, *MLL*, and *ALK* but retain expertise in PCR for detection of gene rearrangements and minimal residual disease and array-based or sequencing screens for more comprehensive evaluations of actionable mutations across the broad spectrum of pediatric hematologic neoplasms and solid tumors.

GENOMIC MECHANISMS IN PEDIATRIC TUMORS

The cytogenetic aberrations in pediatric tumors can be extremely simple, involving loss or rearrangement of only a single chromosome, or can be highly complex, as manifested by a karyotype showing dozens of abnormal chromosomes. Complexly abnormal pediatric cancer karyotypes, containing 10 or more clonal rearrangements, are most often found in highly malignant solid tumors, such as osteosarcoma. On the other hand, a subset of highly malignant solid tumors, such as Ewing sarcoma and synovial sarcoma, has noncomplex karyotypes. Therefore the absence of cytogenetic complexity is not, in itself, a reassuring finding.

Clinically relevant chromosomal aberrations in pediatric tumors result in amplification, translocation, or deletion of various target genes. Amplifications, manifest as intrachromosomal homogeneously staining regions or extrachromosomal double minutes (see Fig. 45-5), are of greatest clinical relevance in neuroblastoma. They are also seen frequently in subsets of rhabdomyosarcoma, osteosarcoma, and central nervous system (CNS) tumors[57,58] and in leukemia, in which DNA segments encompassing various amplified oncogenes can be translocated to different regions of the genome. This phenomenon is termed *segmental jumping translocation.*[16,59] Deletions are generally believed to target tumor suppressor gene loci. Although of limited ancillary diagnostic value, detection of deletions may have prognostic implications (see Tables 45-1 and 45-3). Chromosomal translocations are particularly frequent in leukemias, lymphomas, and sarcomas, where they typically create fusions of regulatory or coding sequences in genes located at the breakpoints of the participant chromosomes.[60,61] Translocations result from double-strand DNA breaks, which are believed to occur more frequently in cancer cells than in normal cells, presumably as a manifestation of the genetic instability that is characteristic of many cancers. Most random translocations arising in cancer cells do not create functionally significant oncogenes and are therefore not selected for and retained by most of the cells in that cancer. The cytogenetic evidence suggests that at most a limited variety of translocations serve an oncogenic role in any given type of pediatric cancer. Many such recurring translocations have clinical use as diagnostic biomarkers (see Tables 45-1, 45-2, and 45-3) or for identifying tumor-specific therapeutic targets (see Tables 45-1 and 45-3).

Some translocation breakpoints directly interrupt the coding sequences of the target genes, leading to the creation of a fusion oncogene. Other breakpoints are outside of the gene coding sequences but nonetheless alter transcriptional regulation of the target gene. In the case of coding sequence fusions, important functional domains from the two component genes are generally brought together by the chromosomal rearrangement. For example, fusion kinase oncogenes often result from apposition of the kinase gene catalytic domain with a translocation partner's protein-protein association domain (oligomerization domain). Such fusions lead to constitutive activation of the kinase. In other translocations, the intact coding sequence of a particular gene, usually one expressed at weak to undetectable levels in the nonneoplastic progenitor cell, is overexpressed by translocational juxtaposition to highly active promoter and enhancer sequences. One well-known example occurs in Burkitt lymphomas, in which *MYC* oncogene expression is increased by translocation into various transcriptionally active *IG* gene regions. The following sections review considerations of causative factors for translocation-associated oncogenes and provide key examples of their structural and functional roles in pediatric cancers.

Causes and Predisposing Factors for Chromosomal Translocations

The underlying mechanisms responsible for the genesis of chromosomal translocations are still poorly understood, but DNA double-strand breakage is a key step in this process. This may be induced by intracellular (endogenous) or extracellular (exogenous) agents. Some endogenous processes associated with double-strand breakage include V(D)J recombinase-mediated intrachromosomal rearrangements at *IG* or *TCR* loci, meiotic recombination between homologous chromatids, topoisomerase II (TOP2)–mediated changes in DNA topology that are required for DNA strand passage during mitosis and for the relaxation of supercoiled DNA for RNA transcription, and production of DNA-damaging agents (e.g., oxygen free radicals) from spontaneous hydrolysis, which themselves can alter DNA topology. Among the many exogenous insults causing double-stranded DNA breakage, ionizing radiation is the most extensively studied and has been shown to disrupt hydrogen bonds and sugar-phosphate backbones, damage purine and pyrimidine bases, and induce cross-links between DNA strands. Such damage can lead to single- or double-strand DNA breakage. Notably, the locations of chromosomal breakage induced by ionizing radiation are not entirely random. Rather, smaller chromosomes appear to be disproportionately affected, as are regions rich in GC repeats. Other inducers of chromosomal breakage include ultraviolet A–activated psoralens, chemotherapeutic agents, and DNA endonucleases.

One class of chemotherapeutic agents that can induce chromosomal breakage is the alkylating agents, such as nitrogen mustards, mitomycin C, nitrosoureas, and platinum compounds, which form DNA adducts and cause intrastrand and interstrand DNA cross-linkage. DNA damage caused by treatment with these agents is associated with secondary myelodysplastic syndrome (MDS) and secondary leukemias, whose salient molecular cytogenetic features include complex numerical and structural abnormalities that often involve loss of chromosome 5, 5q, 7, or 7q. Noteworthy also is that germline and somatic mutations in the TP53 tumor suppressor protein, which is critical for DNA damage recognition, are frequent in secondary leukemias with chromosome 5q abnormalities and a complex karyotype, suggesting that genomic instability caused by loss of TP53 predisposes to this treatment complication.[16,62] The second class of chemotherapeutic agents that can induce chromosomal breakage are the TOP2 poisons, which target the nuclear enzyme topoisomerase II (e.g., the epipodophyllotoxins etoposide and teniposide; the anthracyclines doxorubicin, daunorubicin, and idarubicin; the anthracenedione mitoxantrone; and the combined topoisomerase I/topoisomerase II poison dactinomycin).[63]

Studies on genomic instability mechanisms in the yeast *Saccharomyces cerevisiae* have implicated defects in double-strand DNA breakage repair as causative factors in chromosomal translocations.[64] These repair defects can involve homologous recombination repair or nonhomologous end joining. Homologous recombination repair necessitates guidance of the DNA repair mechanism by two homologous sister chromatids and is therefore more active in the G2 and S phases of the cell cycle. Nonhomologous end joining is intrinsically mutagenic and does not require extensive homology (usually <10 base pairs [bp]) between the two sequences that are joined. Nonhomologous end joining does not require the presence of guiding sister chromatids and is therefore most active during the G1 phase of the cell cycle. Malfunction of these DNA repair systems is responsible for the chromosomal instability in some cancer and premature aging syndromes such as Bloom syndrome, an autosomal recessive disorder caused by inactivating mutations of the *BLM* gene. *BLM* encodes a nuclear protein related to the RecQ family of helicases, which are DNA-unwinding proteins involved in homologous recombination. Bloom syndrome patients show increased rates of chromosomal breakage and exhibit sister chromatid exchanges in the form of quadriradials, which are four-armed structures composed of two chromosomes intersecting at regions of chromatin homology. Bloom syndrome patients develop various benign and malignant tumors, generally occurring at an earlier age than in the normal population.

Other chromosomal instability syndromes result from defects in RecQ helicase proteins. For example, both Werner and Rothmund-Thompson syndromes are autosomal recessive disorders with germline mutations of *RECQL2 (WRN2)* and *RECQL4 (WRN4)*, respectively. Affected individuals develop various tumors, with osteosarcomas being particularly characteristic of patients with the Rothmund-Thompson syndrome. Ataxia-telangiectasia is an autosomal recessive disorder conferring exquisite sensitivity to ionizing radiation.[65] Ataxia-telangiectasia generally results from mutations in the *ATM* gene, which encodes a protein kinase that participates in surveillance for double-strand DNA breakage. Lymphocytes from ataxia-telangiectasia patients show increased levels of chromosomal rearrangement.

Nijmegen breakage syndrome shares several features with ataxia-telangiectasia but is caused by mutations of the *NBN (NBS1)* gene. The NBN (NBS1) protein participates in a complex with apparent roles in homologous recombination repair and nonhomologous end joining. Patients with ataxia-telangiectasia and Nijmegen breakage syndromes are particularly susceptible to the development of leukemias and lymphomas.

Fanconi anemia is a heterogeneous group of autosomal recessive disorders with predisposition to cancer, particularly leukemias and squamous cell carcinomas. At least eight distinct genes involved in DNA double-strand breakage repair have been implicated in the genesis of Fanconi anemia.[66] The chromosomes of patients with Fanconi anemia exhibit increased sensitivity to DNA cross-linking agents, such as diepoxybutane, and this feature can be useful in diagnosis of the disease. Fanconi anemia DNA repair defects can also result from germline mutations of the *BRCA2* gene.

The Li-Fraumeni syndrome is a familial cancer predisposition syndrome that manifests as early onset of a constellation of tumors (rhabdomyosarcoma, breast carcinoma, osteogenic sarcoma, astrocytic brain tumors, adrenal cortical carcinoma, acute myelogenous leukemia) caused by germline mutation in the TP53 tumor suppressor protein.[67]

Certain DNA sequences may be targeted for chromosomal rearrangements. For example, certain sequences within the *MLL* transcription factor gene might comprise TOP2 DNA-binding sites that promote leukemia-associated rearrangements, and this has been studied in detail.[68] Notably, *MLL* is typically rearranged in secondary leukemias after exposure to topoisomerase II poisons, including epipodophyllotoxins, and in leukemia in infants in whom maternal-fetal exposures to dietary or environmental topoisomerase II poisons have been implicated.[63] Even though there is not a clear consensus TOP2 DNA-binding sequence in *MLL* and the preferred sites of enzyme recognition are modified by specific chemotherapeutic agents, it has been shown that *MLL* translocation breakpoints occur near functional sites of chemotherapy-enhanced TOP2 cleavage in in vitro assays.[63] TOP2 is an essential cellular enzyme that relaxes supercoiled DNA by transiently cleaving and religating both strands of the double helix. TOP2 catalyzes the sequential reactions of double-strand DNA cleavage, DNA strand passage, and DNA strand rejoining (religation). The DNA cleavage reaction occurs when each subunit of the TOP2 enzyme homodimer covalently attaches to and introduces staggered nicks in the DNA, with the nicked DNA remaining tethered to the enzyme subunits, forming a fleeting TOP2/DNA intermediate called the cleavage complex. Particular anticancer drugs such as etoposide alter the cleavage-religation equilibrium by decreasing the rate of religation, which damages the DNA by increasing cleavage complexes. Chemotherapeutic agents that interact with TOP2 in this manner are termed *topoisomerase II poisons* because the enzyme is converted to a cellular toxin that promotes strand breakage. Although sometimes called TOP2 inhibitors when used clinically, these agents are distinct in their activity from catalytic inhibitors of this enzyme. The association of various chemotherapeutic TOP2 poisons used for anticancer treatment with secondary leukemias characterized by balanced chromosomal translocations has suggested that TOP2 has a role in other balanced translocations, such as t(8;21), t(15;17), and inv(16) in addition to those involving *MLL*.

Other examples of recombination-promoting sequences are the heptamer-nonamer sequences adjacent to translocation breakpoints in many B-cell lymphomas. These sequences serve as sites of V(D)J recombination and are, therefore, recombination signals in the B-cell context. Similarly, the sites of V(D)J recombination also promote translocations of *TCR* genes with heterologous gene loci in T-cell ALL and T-cell lymphomas.

Physical proximity of genes in interphase nuclei has also been implicated in their incorporation into fusion genes. For example, radiation-induced thyroid carcinomas frequently exhibit rearrangements of the protein tyrosine kinase gene *RET* on chromosome 10q11, which is most often fused with the *H4* locus, telomeric (band 10q21) to *RET* on the same chromosome arm.[69,70] *RET* and *H4* are physically juxtaposed in 35% of normal human thyroid cells, providing a hypothesis as to why these two particular genes are disproportionately likely to be fused after radiation-induced double-strand DNA breakage.[71] Indeed, the three-dimensional topography of the genome explains why certain rearrangements, particularly between neighboring regions on a given chromosome, are commonplace both in cancer and nonneoplastic cells.[72] Accordingly, whole transcriptome evaluations in various cancers have revealed hundreds of apparent intrachromosomal rearrangements in a given tumor, many of which involve closely neighboring genome regions and most of which are unlikely to create functionally important oncogenes. The constraints of genome organization and nuclear proximity likely determine that certain genes are the favored partners in cancer-associated rearrangements, even in situations in which other members of the same gene family would appear to be equally suitable, biologically, in the oncogenic roles. As one example, *MYC,* rather than its biologically similar family member, *MYCN,* is rearranged with an *IG* locus in Burkitt lymphoma; however, *MYCN* if moved to the *MYC* genomic locus effectively undergoes oncogenic rearrangement with *IGH.*[73]

One intriguing aspect of cancer chromosomal translocations is that they are sometimes amplified, providing additional copies of the associated fusion oncogenes. After their formation, the translocation breakpoint regions can be amplified as tandem repeats within the original chromosome or can be amplified as extrachromosomal structures, such as double-minute chromosomes. One example of intrachromosomal amplification occurs with the typically low-level amplification of *PDGFB* fusion genes in the spindle cell sarcoma dermatofibrosarcoma protuberans (Fig. 45-13, *A*).[74,75] An example of extrachromosomal amplification is found in a subset of alveolar rhabdomyosarcomas, in which double-minute chromosomes contain numerous copies of the *PAX7-FOXO1* fusion oncogene (see Fig. 45-13, *B*).[76] In either of these situations, the amplification event suggests that multiple copies of the fusion oncogenes are required to accomplish cellular transformation.

The *EWSR1* gene is rearranged in a striking number of pediatric sarcomas including Ewing sarcoma and desmoplastic small round cell tumor. There are, however, striking racial discrepancies with respect to Ewing sarcoma incidence, such that children of European ancestry are affected with a higher frequency than African-American, Asian, or Native American children. A recent study has shown, however, that the incidence of EWSR1-rearranged sarcoma is not uniformly higher in European descendants, because desmoplastic small round cell tumor (DSRCT), for example, occurs with higher frequency in African-American children.[77]

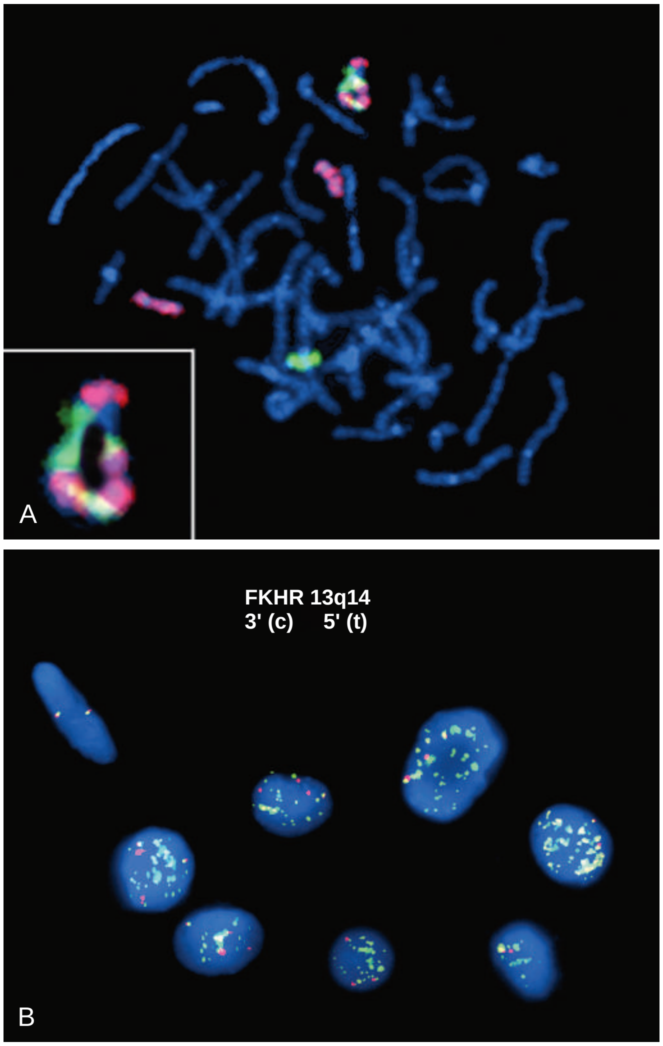

Figure 45-13 **A,** Fluorescence in situ hybridization (FISH), with whole chromosome "painting" probes to chromosomes 17 *(pink)* and 22 *(green),* demonstrates alternating segments of chromosomes 17 and 22 in a dermatofibrosarcoma protuberans ring chromosome *(magnified in inset),* resulting in threefold amplification of the characteristic *COL1A1-PDGFB* fusion oncogene. **B,** FISH demonstration of highly amplified and rearranged *FOXO1* gene *(green)* in an alveolar rhabdomyosarcoma with *PAX7-FOXO1* oncogenic fusion. Break-apart probe components 5′ and 3′ to the *FOXO1* gene are *red* and *green,* respectively. Normal fibroblast nucleus at upper left contains two normal fusion probe signals.

BIOLOGIC CONSEQUENCES OF CHROMOSOMAL TRANSLOCATION

Chromosomal Rearrangements Involving Transcription Factor Genes

Transcription factors are a heterogeneous group of DNA-binding proteins, most of which have domains involved in DNA binding, protein dimerization (for interaction with homologous proteins), and gene transactivation (for activation of gene transcription) or transcriptional repression. There are several classes of transcription factors, primarily relating to the structure of their DNA-binding domains. Some of the major categories include homeodomain proteins, zinc finger proteins, leucine zipper proteins, forkhead proteins, and helix-loop-helix proteins. Transcription factor genes are disrupted by many chromosomal translocations, resulting in their aberrant expression and function. Often, these transcription factor genes control lineage-specific developmental pathways and their abnormal activation can induce ectopic or asynchronous expression of corresponding lineage-specific antigenic markers, which then become a defining aspect of the transformed phenotype.

Many chromosomal translocations involving transcription factors have been described in leukemias and lymphomas. Chromosomal translocations in leukemias often interfere with the normal differentiation program of myeloid and lymphoid lineages,[78] although the presence of a chromosomal translocation per se does not prevent subclone evolution via further lineage-associated differentiation (e.g., progression from *IGH* gene rearrangement only to *IGH* plus *IGL*-chain rearrangement in precursor B-cell ALL). In acute leukemias, the targets for various chromosomal translocations are genes encoding hematopoiesis-related transcription factors, including AML1 (also known as RUNX1) and CBFB (see Fig. 45-12).

AML1 is a DNA-binding protein with significant homology to the *Drosophila melanogaster* developmental protein Runt. AML1 binds to the DNA enhancer sequence TGTGGT; the DNA-binding capabilities of AML1 are enhanced through interactions with the non–DNA-binding protein subunit of CBF (core-binding factor) called CBFB, thereby regulating expression of several genes involved in hematopoiesis. One of the most common chromosomal translocations observed in AMLs (approximately 12% of cases) is the t(8;21)(q22;q22), which fuses *AML1* on chromosome 21q to *RUNX1T1 (ETO)* on chromosome 8.[79] *ETO* encodes a nuclear phosphoprotein expressed in the nervous system and in CD34+ hematopoietic progenitor cells. ETO is a transforming protein that normally participates in a multiprotein complex involved in chromatin remodeling and transcriptional repression. The fusion protein AML1-ETO retains the Runt domain of AML1 and almost the entire sequence of ETO.[80] It appears that the ETO domains incorporated into the fusion oncoprotein can dominantly repress transcription of certain genes, the expression of which is normally activated by AML1. As an example, the tumor suppressor protein p14^{ARF} normally is transcriptionally activated by AML1, but p14^{ARF} is transcriptionally repressed by AML1-ETO.[81,82] *AML1* is also involved in several other chromosomal translocations in leukemias and myeloproliferative disorders, including *AML1-MDS1* in myelodysplastic (preleukemic) syndromes, *AML1-EVI1* in chronic myelogenous leukemia in blast crisis, and *TEL (ETV6)-AML1* in pre-B ALL. All these fusion genes retain the AML1 Runt domain.[83]

Another example of a fusion gene affecting the normal function of the AML1-CBFB DNA-binding transcription factor protein complex is *CBFB-SMMHC,* which results from rearrangement of chromosome 16 in the French-American-British (FAB) M4Eo subtype of AML.[84] *CBFB-SMMHC* can result from translocation or pericentric inversion of chromosome 16, thereby fusing sequences from the 5′ end of *CBFB* to the 3′ end of *SMMHC.* The resultant fusion oncoprotein contains the AML1-binding heterodimerization domain of CBFB juxtaposed to the coiled-coil domains of the MYH11 (SMMHC) protein. The fusion protein CBFB-SMMHC binds to the AML1 Runt domain more effectively than the normal CBFB and has a dominant negative effect on the AML1-CBFB complex.[85]

MLL is a transcription factor protein in which the amino and carboxyl aspects contribute transcriptional repression and activation activities, respectively. MLL undergoes posttranslational processing so that the transcriptional repression and activation regions are separated by cleavage, but they then reassociate with one another in a large multiprotein chromatin remodeling complex. MLL maintains but does not initiate expression of its target genes via histone methyltransferase activity, which is provided by a SET domain within the MLL carboxyl terminus.[86] A wide range of MLL fusion oncoproteins resulting from translocations of the *MLL* gene at chromosome band 11q23 contain the amino terminus of the MLL transcription factor and the carboxyl terminus of partner proteins that are themselves involved in transcriptional regulation. However, there is substantial diversity in MLL partner protein function and not all MLL partner proteins are transcription factors.[86]

Chimeric (fusion) transcription factor oncogenes are also featured in many soft tissue tumors. A much-studied example is the *EWS-FLI1* fusion oncogene resulting from the t(11;22)(q24;q12) translocation[87] in Ewing sarcoma. This translocation fuses *EWS* 5′ sequences to *FLI1* 3′ sequences. *EWS* encodes a ubiquitously expressed protein involved in DNA transcription, and *FLI1* is a member of a large family of DNA transcription factors that contain the highly conserved ETS domain. The *EWS-FLI1* fusion oncogene structure varies, depending on which *EWS* and *FLI1* exons are retained in the chimeric gene (see Fig. 45-10). *EWS* can be fused with other ETS family members to provide the oncogenic impulse in Ewing sarcoma, and, alternatively, *FUS* (an EWS family member) can instead be fused with ETS family members in Ewing sarcomas. EWSR1-ETS fusion proteins are necessary but insufficient for cellular transformation,[88,89] a process for which IGF1R is vital.[90] Some progress has been made in recent years in understanding the oncogenic mode of action of the EWSR1-ETS fusion protein, with a rather complex and multifaceted picture emerging whereby gene targets may be direct or indirect and include transcriptional regulators such as EZH2,[89,91] NROB1,[92] and NKX2.2,[93] as well as genes involved in signal transduction or genes involved in cell cycle control. EWS-FLI1 can directly bind promoters of target genes and modulate gene expression[94,95] by upregulating (e.g., IGF1, CCND1, hTERT[96]) or repressing targets (e.g., cyclin-dependent kinase inhibitors and IGFBP3, the latter potentially allowing increased IGF1 signaling). Reduced target expression may result from decreased transcription through EWS-FLI1 reducing Pol II at the target gene promoter or through posttranscriptional reduction of transcript half-life.[97] Any additional posttranscriptional role, for example, of microRNAs putatively targeted by the fusion oncogene remains to be elucidated fully,[98] and there is emerging evidence that posttranslational protein modification is a further important function of *EWS-ETS* fusions. The direct binding of EWSR1-ETS to the *EZH2* promoter specifically with upregulation of EZH2 is interesting in relation to the ongoing debate regarding the cell of origin of Ewing sarcoma. EZH2 is a subunit of the Polycomb repressor complex, PRC2, and its expression holds cells through reversible epigenetic means, in an undifferentiated state of "stemness."[99]

Similarly, *EWS* or *FUS* is fused with other genes (not belonging to the ETS family) in various Ewinglike and frankly non-Ewing mesenchymal small round blue cell neoplasms, always resulting in fusion oncoproteins with EWS or FUS at the amino-terminal end of the fusion protein. DSRCT, a clearly defined tumor that is considered absolutely distinct from Ewing sarcoma, is an exceptionally malignant cancer composed of nests of small round tumor cells sharply delineated by a reactive fibroblastic proliferation. It is cytogenetically characterized by t(11;22)(p13;q12), which fuses *EWS* to the *WT1* gene.[100] The EWS-WT1 fusion oncoprotein retains the EWS transactivation domain and the WT1 DNA-binding domain. *WT1,* located on chromosome 11p13 encodes a zinc finger transcription factor with crucial roles in genitourinary tract development. Germline *WT1* mutations cause Wilms tumor syndromes, including Denys-Drash and WAGR (*W*ilms-*A*niridia-*G*enitourinary abnormalities and mental *R*etardation) syndromes. Further examples of fusion genes involving *EWSR1* or *FUS* and non-ETS genes include isolated cases or small cohorts of small round blue cell tumors, which to a variable degree share histologic features with Ewing sarcoma. The partner genes may again be transcription factors, as seen with POU5F1 or NFATc2,[101,102] or in the case of SMARCA5,[103] a chromatin remodeler, whereas single reports each of a small round blue cell tumor with EWS-SP3[104] and of EWS-ZSG[105] represent fusions of EWS with zinc finger proteins. The latter case particularly has more similarity with DSRCT, insofar as it failed to react with CD99, a classic marker of Ewing sarcoma, but did express desmin. Indeed with respect to both these cases, fusion with a zinc finger gene resonates with the situation in DSRCT. The rarity of such cases precludes major comment on their likely biologic relationship with Ewing sarcoma versus DSRCT.

A recently described undifferentiated small round cell tumor is characterized by recurrent chromosomal translocations t(4;19)(q35;q13.1) or t(10;19)(q35;q26) fusing the high mobility group box [HMG] transcription factor *CIC* with one or other of the *DUX4* double homeobox transcription factor genes located within macrosatellite repeats on 4q and 10q[106]; thus these tumors are known as CIC-DUX4 tumors. An isolated report documenting t(18;19)(q23;q13.2) in a soft tissue tumor resembling Ewing sarcoma most likely represents a variant also involving *CIC.*[107]

Tumors of epithelial differentiation (carcinomas) often have complex karyotypes, which can obfuscate recurrent translocations. However, translocations involving transcriptional regulatory genes have been identified in several pediatric carcinomas, including follicular thyroid carcinomas, renal cell carcinomas, and NUT midline carcinoma.[108,109,110] Follicular thyroid carcinomas can feature *PAX8-PPARγ* fusion transcription factor genes resulting from t(2;3)(q13;p15)[108] (Fig. 45-14), whereas in the highly lethal NUT midline carcinomas, the translocation t(15;19)(q13;p13) (Fig. 45-15) results in oncogenic fusion of the bromodomain transcriptional regulator *BRD4* with a novel testis-restricted gene, *NUT.*[110] Although exhibiting epithelial immunophenotypic features, NUT midline carcinomas typically have a primitive round cell morphology (see Fig. 45-15).

Chromosomal Rearrangements Involving Protein Tyrosine Kinase Genes

Chromosomal translocations in various neoplasms produce protein tyrosine kinase fusion genes, and such

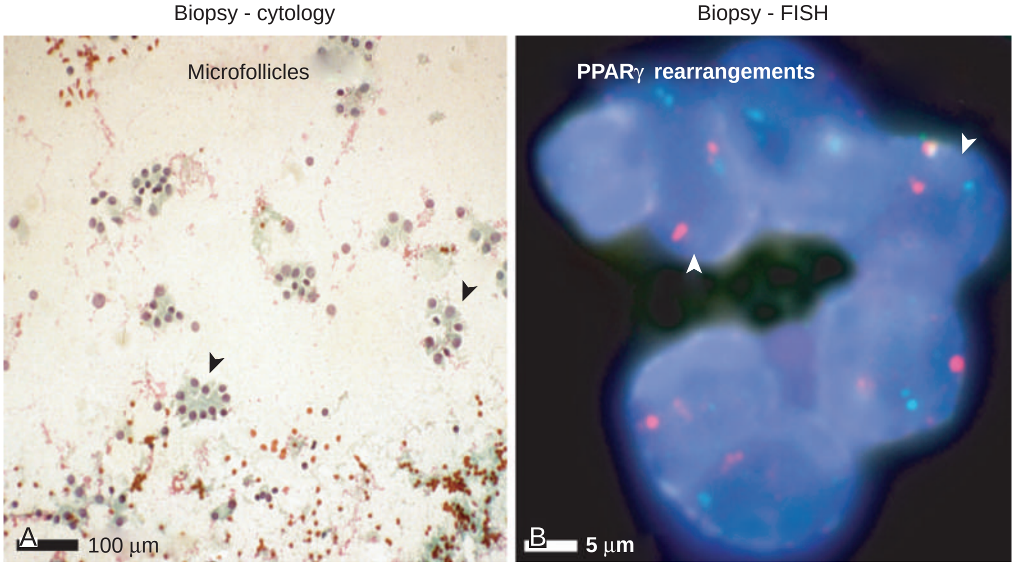

Figure 45-14 **A,** Cytology and **B,** fluorescence in situ hybridization (FISH) of follicular thyroid carcinoma, demonstrating rearrangement of the *PPARγ* gene, using break-apart probe, in microfollicle cells. *(Courtesy Dr. Todd Kroll.)*

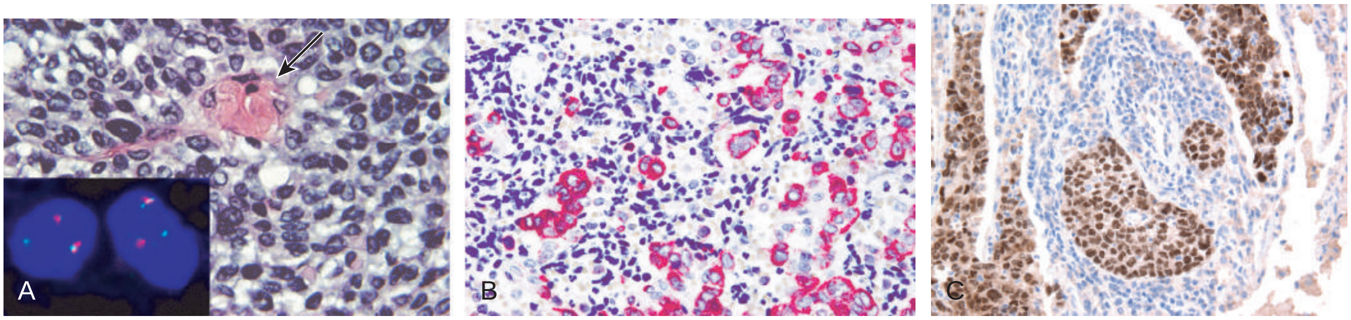

Figure 45-15 **A,** NUT midline carcinoma (sinonasal primary) composed of undifferentiated rounded cells with focal keratinization *(arrow)*. *Inset*, *BRD4* break-apart fluorescence in situ hybridization reveals rearrangement. Immunohistochemical study demonstrates reactivity for pan-keratin (**B**), indicating epithelial differentiation (alkaline phosphatase red). The NUT protein (**C**) (peroxidase DAB) is expressed in tumor cells but not in admixed histiocytes and lymphocytes.

oncogenes are of considerable clinical importance. Protein tyrosine kinases comprise a large family of proteins, which are primarily involved in signal transduction. All tyrosine kinase proteins contain a highly conserved kinase (catalytic) domain, which mediates the phosphorylation of tyrosine residues in protein substrates. Phosphorylated tyrosines serve to stabilize various protein-protein interactions and to enhance kinase activity in those substrates with intrinsic kinase function. Tyrosine kinase fusion oncoproteins resulting from chromosomal translocations in cancer have a consistent structure. The carboxyl-terminal end of these fusion oncoproteins typically contains the entire kinase domain from the tyrosine kinase protein, whereas the amino-terminal end contains an oligomerization domain from the other fusion partner. The oligomerization domain facilitates spontaneous interactions between kinase fusion oncoproteins, and the complexed oncoproteins can then phosphorylate each other, resulting in further upregulation of kinase activity.

The Philadelphia (Ph) chromosome is the cytogenetic hallmark of chronic myelogenous leukemia (CML) and was the first diagnostic translocation identified in a human cancer, also providing the best known example of a fusion tyrosine kinase oncogene.[111] The Ph chromosome results from chromosomal translocation between chromosomes 9 and 22, in which the *BCR* gene on chromosome 22 is fused with the *ABL1 (ABL)* kinase gene on chromosome 9. This translocation is found in most CMLs, 25% of ALLs in adults, 5% of ALLs in children and, less often, in AML. *ABL* encodes a 145-kD nonreceptor protein tyrosine kinase that shuttles between the nucleus and cytoplasm and is involved in various cellular processes, such as cell cycle regulation and apoptosis. *BCR* encodes a 160-kD protein with dimerization, serine-threonine kinase, Rho-GEF (guanine nucleotide exchange factor), and Rac-guanosine triphosphatase (GTPase) domains. The BCR-ABL fusion oncoprotein is expressed as three structural variants, depending on the location of the breakpoint in the *BCR* gene. The BCR-ABL fusion transcript types segregate with specific neoplasms so that the 190-, 210-, and 230-kD BCR-ABL proteins are typically expressed in ALL, CML, and chronic neutrophilic leukemia, respectively. In AML, BCR-ABL fusion oncoproteins can be the P210 or P190 form.[112] The BCR-ABL fusion oncoproteins feature constitutive activation of the ABL kinase domain, resulting in autophosphorylation and tyrosine phosphorylation of various substrates. The phosphotyrosine residues serve as binding sites for various signaling proteins, resulting in activation of signaling pathways, such as Ras/MAPK (implicated in cell proliferation) and PI3K/AKT (implicated in cell survival).

Translocation-associated fusion kinase oncogenes are also found in various soft tissue tumors, including congenital fibrosarcoma, a pediatric spindle cell neoplasm with an excellent prognosis. Cytogenetically, congenital fibrosarcoma is characterized by the balanced translocation t(12;15)(p13;q25), which fuses the 5′ end of the transcription factor gene *TEL* to the 3′ end of the *NTRK3* protein tyrosine kinase gene (see Fig. 45-2).[113] The TEL-NTRK3 fusion oncoprotein retains the TEL helix-loop-helix dimerization domain and the NTRK3 kinase domain. *TEL* is also rearranged with other genes, including protein tyrosine kinase genes in various leukemias. The *TEL-NTRK3* fusion gene is seen in pediatric renal tumors known as congenital cellular mesoblastic nephromas, which are histologically similar to congenital fibrosarcoma (Fig. 45-16).[7] This finding suggests that congenital fibrosarcoma and cellular mesoblastic nephroma belong to the same spectrum of tumors. TEL-NTRK3 is also found occasionally in AML[6] and is one of a few fusion oncogenes known to play transforming roles not only in soft tissue and hematopoietic neoplasms[114] but also in epithelial neoplasia (secretory breast carcinoma).

Inflammatory myofibroblastic tumor (IMT) is an unusual entity that arises predominantly in the abdominal cavity/peritoneum of young patients. Similar to congenital fibrosarcoma, IMT is usually associated with an excellent prognosis and metastases are rare. However, a subset of patients with IMT do develop disseminated disease and it is notable that many IMTs have chromosomal translocations that create activated fusion forms of the *ALK* receptor tyrosine kinase gene detectable by immunohistochemical means (Fig. 45-17).[114] *ALK* oncogenic fusion genes were initially described as a consequence of the chromosomal translocation t(2;5)(p23;q35), which occurs in a subset of anaplastic large cell lymphomas (ALCLs).[115] At least nine *ALK* fusion genes have now been described in IMT and ALCL; in all of them,

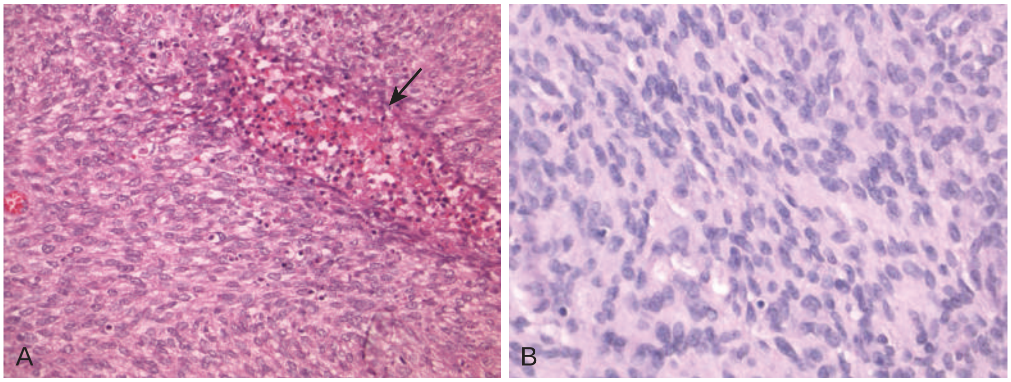

Figure 45-16 **A,** Congenital mesoblastic nephroma and **B,** infantile fibrosarcoma are histologically indistinguishable tumors composed of mitotically active plump spindle cells, with varying extent of fascicular arrangement and showing islands of spontaneous necrosis as a characteristic feature *(arrow)*.

the resultant fusion oncoproteins contain the ALK kinase domain fused to an oligomerization domain of another protein. Two of the *ALK* fusion genes, *TPM3-ALK* and *CLTC-ALK*, have been found in IMT and ALCL, indicating that an identical oncogenic mechanism can contribute to these different tumors.[114,116] Most recently, *ALK* kinase domain mutations and gene amplification have been demonstrated in hereditary and sporadic neuroblastoma.[117,118,119] Other receptor tyrosine kinase translocations in pediatric cancer include those in papillary thyroid cancers, which can target the *RET* or *NTRK1* genes.[120,121] In these fusion oncoproteins, the RET or NTRK1 kinase domains are activated by fusion with various oligomerization-inducing domains.

A further mechanism of protein tyrosine kinase activation by chromosomal translocation is seen in dermatofibrosarcoma protuberans (DFSP).[122] DFSP is a subcutaneous plump spindle cell sarcoma that exhibits high local recurrence rates but rarely metastasizes. Cytogenetically, this tumor is characterized by the chromosomal translocation t(17;22)(q21;q13), often amplified within a circular or ring chromosome (see Fig. 45-13A). The t(17;22) creates a *COL1A1-PDGFB* fusion gene, in which the entire *PDGFB* coding sequence is placed under the transcriptional control of the highly active *COL1A1* promoter in an event referred to as promoter swapping. This mechanism results in *PDGFB* overexpression, with resultant autocrine activation of the PDGFB receptor.[122] The same genetic event underlies the development of giant cell fibroblastoma, which is a pediatric tumor believed to be closely related to DFSP.[74,123]

Chromosomal Rearrangements Associated with Transcriptional Upregulation of Nonfusion Oncogenes

Many pediatric lymphomas feature juxtaposition of intact protooncogenes to transcriptionally active loci (e.g., the *IG* locus in B-cell lymphomas or the *TCR* locus in T-cell lymphomas). V(D)J recombination and class switch recombination, which normally occur within the *IG* or *TCR* loci in lymphocytes, have been implicated in the genesis of these nonfusion genes.[124] V(D)J recombination and class switch recombination mechanisms are exemplified by the nonfusion genes targeting *MYC*, a regulator of cell growth and survival, in many Burkitt lymphomas. V(D)J recombination facilitates the chromosomal translocation t(8;14)(q24;q32) in endemic Burkitt lymphomas found in patients from equatorial Africa. This translocation juxtaposes *MYC* to the intronic *IG* heavy chain gene enhancer, leading to MYC overexpression. In contrast, most nonendemic Burkitt

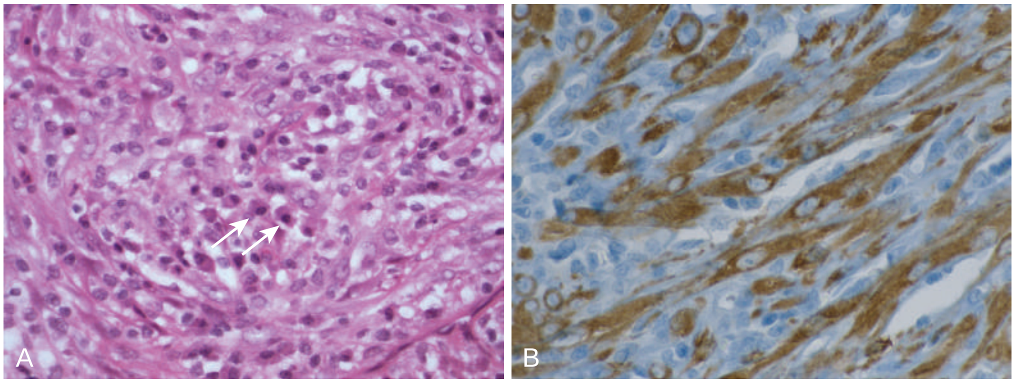

Figure 45-17 **A,** Inflammatory myofibroblastic tumor composed of plump neoplastic spindle cells with a reactive infiltrate featuring abundant plasma cells *(arrows)*. **B,** Immunohistochemistry in a case with *TPM4-ALK* fusion, showing diffuse cytoplasmic staining with nuclear sparing in spindle cell (myofibroblastic) component and absence of immunoreactivity in the inflammatory cell infiltrate.

lymphomas have translocation breakpoints downstream elsewhere in the *IG* heavy chain gene locus, juxtaposing *MYC* to other enhancers. Alternatively, *MYC* overexpression can result from juxtaposition to the *IG* κ or λ light chain genes resulting from t(2;8) or t(8;22).

Like Burkitt lymphomas, follicular cell lymphomas of B-cell origin also feature rearrangements of *IG* loci, but with *BCL2* or *BCL6,* rather than *MYC,* typically overexpressed by translocation into the region of an *IG* gene promoter. *BCL2* alterations, conferring well-described antiapoptotic effects, often result from *IG* heavy chain V(D)J recombination, with a breakpoint in exon 3 of *BCL2* becoming fused to the 5′ end of J_H or D-J_H heavy chain segments. These mechanisms result in *BCL2* transcriptional upregulation, mediated by the *IG* heavy chain gene enhancer. Although deregulated BCL2 expression provides a survival advantage in such lymphomas, additional gene mutations are needed for full neoplastic transformation.[125] However, in the pediatric population, follicular lymphomas are uncommon and the vast majority of cases feature elevated BCL6 expression. Cases with t(14;18) fusing the *IGH* chain locus with the BCL2 gene comprise only a small subset of the cases, which is in contrast to adults, but increased BCL2 expression is associated with advanced disease and a poor prognosis in pediatric lymphoma.[126]

TCR locus rearrangements are demonstrable in many T-cell leukemias and lymphomas, resulting in transcriptional upregulation of various translocated genes, similar to the mechanisms involving *IG* gene regions in various B-cell malignancies. The T-cell leukemia and lymphoma cytogenetic rearrangements dysregulate expression of transcription factor genes, such as *MYC, HOX11, TLX3 (HOX11L2), TAL-SCL, LYL1, LMO1,* and *LMO2,* by placing these genes under the control of highly active *cis*-acting transcriptional regulatory *TCR* elements, although dysregulation of these T-cell oncogenes can also occur in the absence of cytogenetic *TCR* rearrangements.[127,128]

Translocations resulting in nonfusion oncogenes can be found in pediatric solid tumors; one example is the *PDGFB* fusion gene in DFSP (see earlier). Other examples include translocations of chromosome 8, targeting the *PLAG1* gene, a zinc finger transcription factor, in salivary gland pleomorphic adenoma and the primitive adipose tumor lipoblastoma. In both tumor types, the chromosomal breakpoints occur in the 5′ noncoding regions of the involved genes, resulting in promoter swapping, in which the transcriptionally inactive *PLAG1* promoter is replaced by the highly active promoter of the translocation partner gene, leading to *PLAG1* overexpression.[129-131]

BIOLOGIC BASIS FOR SPECIFICITY OF BALANCED CHROMOSOMAL REARRANGEMENTS: DIAGNOSTIC RELEVANCE

Recurrent chromosomal rearrangements involving specific breakpoint regions are increasingly being used as diagnostic markers in various pediatric cancers. Translocations are the most extensively studied of these rearrangements, but other mechanisms include chromosomal insertions, inversions, and interstitial deletions. Although diagnostic rearrangements, such as those involving chromosome 22 in Ewing sarcoma, have become useful biomarkers, it is unclear whether most such characteristic rearrangements are initiating tumorigenic events or later events responsible for tumor progression. However, their oncogenic nature is generally convincing in that they can transform cells, as can be demonstrated by expressing the oncogenes in nonneoplastic cells in vitro or in mice. Although *EWS* and its family member *TLS-FUS* are frequently rearranged in chromosomal translocation in sarcomas, the partner gene varies and appears occasionally to dictate the tumor phenotype (see Table 45-1). For example, *EWS-WT1* transcripts are found in DSRCT but not Ewing sarcoma, whereas *EWS-ATF1* fusion occurs in soft tissue clear cell sarcoma but not other sarcomas. It has not yet been clearly established whether it is the partner gene that determines lineage in these tumors or whether only certain cells, already lineage committed, might tolerate the presence of these oncogenic transcripts.

Translocations of the *MLL* gene at chromosome band 11q23 are examples of leukemia-associated chromosomal translocations that create potent fusion oncoproteins. *MLL* translocations are believed to be the events that initiate these leukemias.[132] Analogous to *EWS* variant rearrangements in pediatric solid tumors, particular *MLL* partner genes are more likely to occur in ALL than in AML, even though there is some overlap, and leukemias with *MLL* translocations can also exhibit dual lineage or actually switch lineage.[133]

There is substantial evidence that single genetic aberrations are insufficient to produce cancer. Rather, tumor development requires many gene mutations, perturbing different aspects of the cell biology (including apoptosis, proliferation, differentiation, and adhesion), which collectively results in the neoplastic phenotype. Even with *MLL* translocations in which there is a short latency from the in utero occurrence of the translocation to manifestation of disease, which is often in the young infant (<3 months old) or even in the neonate or the fetus,[134] there are characteristic secondary alterations in addition to the translocations.[135]

Some of the same characteristic genetic alterations, including chromosomal translocations, observed in human cancers have been detected at very low levels (e.g., 1 in 10,000 cells) in nonneoplastic cell populations.[136,137] The rare cells containing these alterations are individually at low risk for progression to malignancy, presumably because it is unlikely that they will acquire the other mutations required to result in clinically evident cancer. However, this likely is more pertinent to solid tumors, in which a greater number of events generally is believed to be required for full oncogenesis than in leukemias. Similarly, it is likely that various diagnostic genetic alterations can be detected at very low levels in cancer types in which they are not characteristically encountered and where they likely are not serving an important transforming role for the vast majority of cells in that cancer. Therefore when using genetic alterations as diagnostic markers, it is important to have some sense that the alteration is found in most cells in the cancer, rather than being a rare and perhaps trivial event. It is similarly crucial to correlate the genetic findings with the histology, thereby confirming that the molecular diagnosis is credible.

The recurring and relatively specific association of chromosomal translocations with one or a few types of cancer has enabled the development of assays in which the translocations serve as ancillary diagnostic markers. There is a biologic basis to such tumor specificity because the neoplastic phenotype is essentially a symbiotic interaction between cell environment and translocation product, and certain genetic alterations, particularly those resulting from translocations, appear oncogenic in only a limited number of cancer cell environments.[138] Support for this concept is provided by B-cell lymphomas, in which diagnostic translocations often juxtapose oncogenes to the *IG* loci. The *IG* gene promoters are extremely active in the B-cell context, and ectopic placement of oncogenes near these promoters results in striking overexpression of the oncogenes, provided that the chromosomal rearrangement occurs in the B-cell context. By contrast, this same chromosomal rearrangement would presumably be irrelevant in other cell types, in which the *IG* genes are transcriptionally silent. Translocations that occur in B-cell lymphoma have not been found in other varieties of human cancer. Another factor accounting for tumor specificity is the restriction of transforming properties of translocation-associated oncoproteins to certain cell lineages. Fusion oncoproteins always require interactions with other cellular proteins that facilitate and support the effect of that oncoprotein. Therefore cell lineages lacking the relevant interacting proteins will not be transformed by a given fusion oncoprotein. These concepts are underscored by the observation that the BCR-ABL fusion oncoprotein, which is highly transforming in hematopoietic progenitor cells, fails to transform fibroblast cell lines.[139]

In pediatric tumors, genetic aberrations useful for testing as ancillary diagnostic findings are especially frequent in sarcomas, leukemias, and lymphomas. Interestingly, in contrast to adult tumors, diagnostic alterations are also found in many pediatric carcinomas, including papillary and follicular thyroid carcinomas, renal cell carcinomas, and lethal midline carcinoma. Although cytogenetic and molecular profiles are diagnostic in some pediatric cancers, there are many others that lack specific genetic or cytogenetic aberrations or have extremely complex karyotypes in which the specific aberrations, if present at all, are difficult to identify.

CYTOGENETIC AND MOLECULAR PATHOLOGY: MAJOR TUMOR TYPES

Cytogenetic and molecular analyses have provided extraordinary insights into the biology and pathogenesis of pediatric cancer and, in some cases, these insights have then formed the basis for more accurate assessment of diagnosis and prognosis. However, neither cytogenetic nor molecular methods are required routinely in the clinical setting. In some pediatric solid tumors, there is not yet a basis for genetic testing because recurrent genetic aberrations are unknown. In others, particularly those that are benign clinically, the histopathology diagnosis and prognosis are generally straightforward and genetic adjuncts are therefore irrelevant. Some pediatric solid tumors, such as high-grade osteosarcoma, have extremely complex karyotypes, so that to date, there is little clinical advantage in genetic analysis, given the formidable task of describing the many genetic aberrations in a given tumor and the questionable prognostic relevance of the individual genetic perturbations. Widespread application of genetic assays in such tumors awaits the identification of key genetic predictors, which might then be determined in tumor interphase cells by molecular cytogenetic assays.

The following sections will highlight applications of cytogenetic and molecular assays in subsets of pediatric tumors, in which the technical challenges of genetic assays are manageable, and in which genetic findings are acknowledged to provide important diagnostic or prognostic information.

Mesenchymal Neoplasms: Soft Tissue and Bone Tumors

Ewing Sarcoma

Ewing sarcomas are highly aggressive tumors, characteristically occurring in bone and soft tissue, but also, less commonly, in viscera, and in which the neoplastic cells are generally of the small, round, blue cell type. Ewing sarcoma and peripheral primitive neuroectodermal tumor (pPNET), although historically considered distinct entities, share molecular and morphologic features and are now regarded as essentially the same entity (herein referred to simply as Ewing sarcoma). Ewing sarcomas typically contain chromosomal translocations involving the Ewing sarcoma gene (*EWS* or *EWSR1*), which is located on the long arm of chromosome 22. These translocations involve a number of partner genes (see Table 45-1), with the most common translocation being t(11;22)(q24;q12), resulting in oncogenic fusion of 3′ sequences of the *FLI1* gene on chromosome 11 with 5′ sequences of *EWS*.[140-144] *FLI1* belongs to the winged helix-loop-helix transcription factor family that share a conserved 85-amino acid ETS domain that is present in all EWS-ETS fusions. The oncogenic *EWSR1-FLI1* fusion gene encodes an activated version of transcription factor in which the DNA binding domain of *FLI1* is fused to the more potent *trans*-activating domain (TAD) of *EWSR1*, replacing the TAD of *FLI1*. In variant translocations, *EWS* is fused with alternative ETS transcription factor family members (see Fig. 45-10 and Table 45-1)[145-149] and, rarely, the *EWS*-related gene, *FUS*, can also be fused with an alternative ETS family member in Ewing sarcomas (Fig. 45-18; see Table 45-1).[150] The *EWS* family and ETS family gene translocations are apparently essential for oncogenesis, being found in almost all Ewing sarcomas.

These translocations are readily detected by conventional cytogenetic methods, even using needle biopsy material, because Ewing sarcoma cells grow well in tissue culture.[9] The translocations can also be detected by FISH,

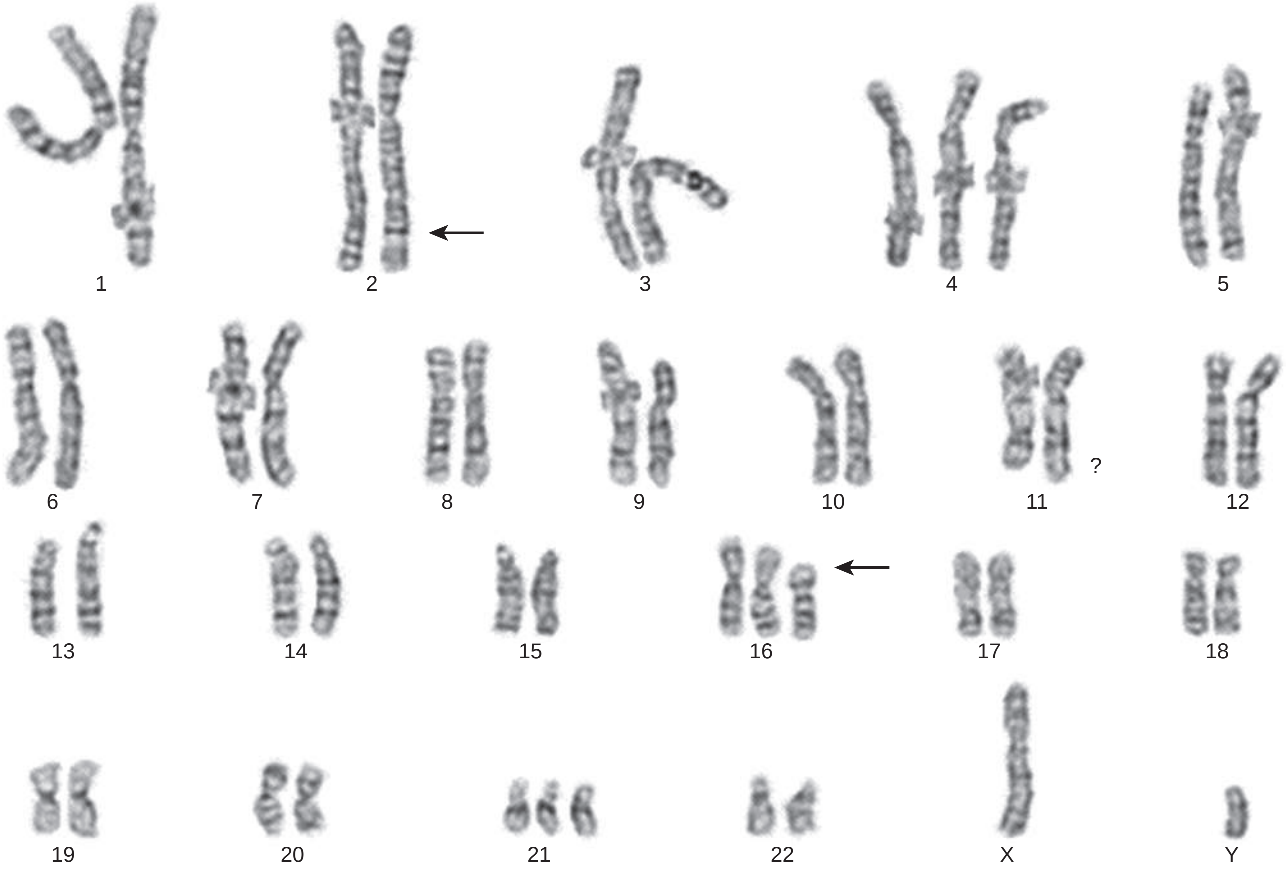

Figure 45-18 Ewing sarcoma with t(2;16)(q35;p11), resulting in oncogenic fusion of the *FEV1* and *FUS* genes (*arrows* indicate translocation breakpoints).

typically using dual-color probes flanking the *EWSR1* locus (see Fig. 45-8).[151] Although this does not identify the partner gene fused to *EWSR1*, that information is generally unnecessary for diagnostic purposes. Another method for detecting these translocations is RT-PCR.[152] Advantages of PCR include superior sensitivity, identification of the *EWS* fusion partner gene, and identification of the breakpoint locations within the genes. The most common fusion is between *EWS* exon 7 and *FLI1* exon 6, known as type I fusion, whereas type II fusion involves fusion of *EWSR1* exon 7 with *FLI1* exon 5, and many further variants have been described (see Fig. 45-10). Although several studies have indicated that *EWSR1-FLI1* breakpoint locations might be prognostic in Ewing sarcoma,[37,39,153] with tumors bearing the type I transcript doing better than all others, more recent studies do not confirm this, and such information is not used routinely to guide therapeutic decisions.

Although histomorphologic and immunohistochemical features are often sufficient to establish the diagnosis of Ewing sarcoma, genetic corroboration of the diagnosis is highly reassuring in distinguishing Ewing sarcoma from other small round blue cell tumors, particularly in situations in which tumor arises at an unusual site, or outside the typical age range, in the context of smaller biopsies, or if tumor cell preservation is compromised and/or immunohistochemistry is suboptimal. Although the cells are essentially undifferentiated, and superficially similar or identical to those in many other small round cell tumors, there are nonetheless characteristic findings, such as subtle cytologic features and crisp cytoplasmic membrane–associated expression of the CD99 (O13-HBA71, pseudoautosomal gene-encoded membrane glycoprotein [Fig. 45-19], or specifically for *Erg*-rearranged Ewing sarcoma, anti-Erg monoclonal antibody[154]) that aid the diagnosis.

Thus the combined traditional histologic evaluation of H&E-stained sections, together with immunohistochemical profiling, enables distinction of Ewing sarcoma from, for example, a reasonable mimic in alveolar rhabdomyosarcoma by the demonstration of CD99 expression in Ewing sarcoma versus expression of myogenic markers such as myogenin, MyoD1, and desmin in rhabdomyosarcoma (Fig. 45-20). The characteristic oncogene fusions in Ewing sarcoma *(EWS-FLI1)* and alveolar rhabdomyosarcoma *(PAX-FOXO1)* might contribute to the distinct cell differentiation profiles that separate these two round cell tumors immunophenotypically and morphologically. Evidence to this effect has been obtained from in vitro studies in which *EWS-ETS* oncogenes can induce neuroectodermal differentiation,[155] whereas *PAX-FOXO1* induces a myogenic program.[156,157] Secondary

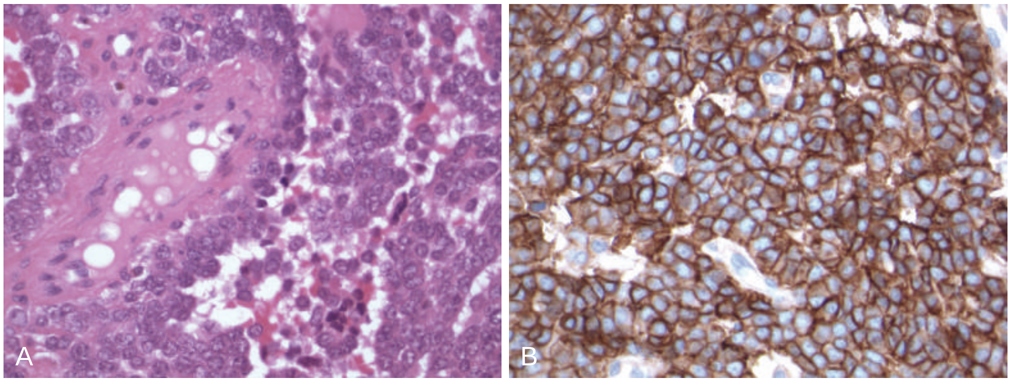

Figure 45-19 Ewing sarcoma composed of undifferentiated round cells (**A**) arranged around thick fibrovascular cores and demonstrating strong and crisp CD99 expression confined to the cell surface (**B**), producing a mosaic-like effect.

genetic aberrations in Ewing sarcoma include *TP53* mutation and *CDKN2A* deletion, which are each found in approximately 25% of cases and are more frequent in cases that are clinically aggressive and poorly chemoresponsive.[158]

Similarly, gain of 1q was strongly associated with relapse and poor outcome, and this is apparently due to overexpression of *DTL (CDT2)*, a ubiquitin ligase gene located on chromosome 1q.[159,160] Independent loss of 16q was also predictive of worse overall and event-free survival regardless of stage at presentation.[159] In that study, gain of chromosome 12 was, at least in cases of localized disease at presentation, associated with worse event-free survival whereas deletion of 6p has been associated with worse outcome also.[161] Genomic instability portends poorly insofar as tumors with more than three copy number alterations reportedly did worse than those with three of fewer.[162,163] However, currently these secondary genomic findings are not used in therapeutic decisions.

Rhabdomyosarcoma

Rhabdomyosarcomas are malignant tumors featuring skeletal muscle differentiation, with several histologic subtypes (see Fig. 45-20). The major varieties in

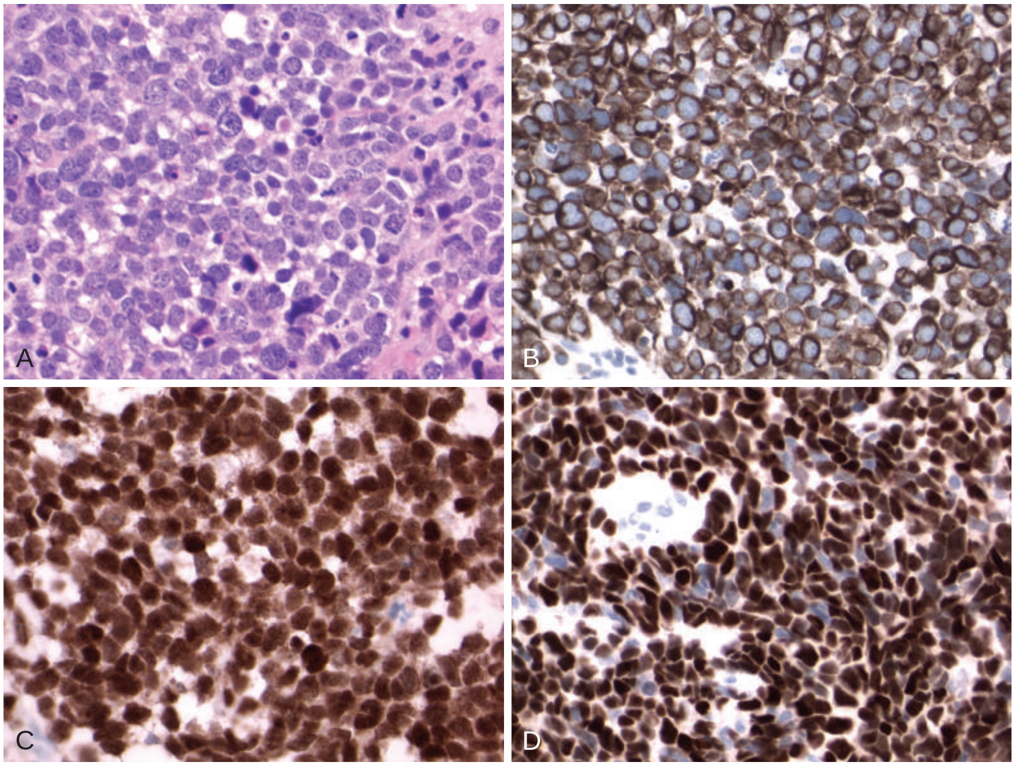

Figure 45-20 Solid variant of alveolar rhabdomyosarcoma composed of undifferentiated, moderately pleomorphic round cells histologically (**A**), but with convincing evidence of myogenic differentiation, as demonstrated by strong and diffuse cytoplasmic expression of desmin (**B**) and nuclear reactivity for MyoD1 (**C**) and myogenin (**D**). Of note, the percentage of nuclei reacting for both myogenin and MyoD1 is very high, as is typical of the alveolar variant of rhabdomyosarcoma.

childhood are the embryonal and alveolar subtypes and, although there are classic morphologies of alveolar and embryonal rhabdomyosarcoma, the distinction is not always clear-cut, because a solid variant alveolar rhabdomyosarcoma exists and composite cases also occur. Therefore especially with decreasing biopsy size, traditional morphologic distinction between embryonal and alveolar subtypes of rhabdomyosarcoma is not always a reliable means of subclassifying the tumors but is nonetheless essential for appropriate therapeutic stratification of patients with these two biologically different tumors. Alveolar rhabdomyosarcoma exhibits a higher proportional nuclear reactivity for both myogenin and MyoD1 than embryonal rhabdomyosarcoma,[164] but even this feature may be unconvincing in small biopsies and thus be of little value in firmly establishing the diagnosis. The biologically distinct nature of embryonal and alveolar subtypes is confirmed by genetic studies, which show translocations targeting the chromosome 13 *FOXO1A* (forkhead transcription factor) gene in most alveolar rhabdomyosarcomas, and nontranslocation cytogenetic alterations, including 11p deletion, trisomy 8, and trisomy 20, in embryonal rhabdomyosarcomas. In 70% to 80% of alveolar rhabdomyosarcomas there is fusion of the *FOXO1* gene with the *PAX3* gene on chromosome 2,[165-167] and approximately 10% have fusions of *FOXO1* with the *PAX7* gene on chromosome 1.[168] The *PAX7-FOXO1* fusion is often highly amplified in the form of double-minute chromosomes (see Fig. 45-13B), whereas the *PAX3-FOXO1* fusions are not usually amplified. This difference appears to reflect the lower intrinsic expression of *PAX7-FOXO1,* relative to that of *PAX3-FOXO1,* with genomic amplification therefore required to provide a comparable and sufficient level of oncogenic transcript.[169] The *FOXO1, PAX3,* and *PAX7* genes encode transcription factors, and the *PAX3-FOXO1* and *PAX7-FOXO1* fusion oncogenes encode activated forms of those transcription factors[170,171] that function to induce myogenic differentiation programs.[156,157,172] Notably, alveolar rhabdomyosarcomas with *PAX7-FOXO1* rearrangements were reported in one study to have better prognoses than those with *PAX3-FOXO1* when comparing patients presenting with metastatic disease.[173] Furthermore, an argument has been made for movement toward designation of rhabdomyosarcomas based on translocation status rather than on morphology; and in that vein, cases of morphologic alveolar rhabdomyosarcoma have been termed *fusion-positive* versus *fusion-negative*. The observation that gene expression profiles of fusion-negative alveolar rhabdomyosarcomas are indistinguishable from those of embryonal rhabdomyosarcomas[174-177] might lend considerable support to such subclassification. Whether molecular categorization however translates well to clinical prognosis and treatment stratification remains unresolved,[176,178,179] and a combined morphomolecular diagnosis is currently considered best practice.[180]

A proportion of cases of rhabdomyosarcoma lacking the canonical *PAX-FOXO1* fusions show t(2;2)(p23;q35) or t(2;8)(q35;q13) in which *PAX3* is fused with either *NCOA1* or *NCOA2,* which are nuclear receptor transcriptional coactivators,[181] whereas isolated individual cases of rhabdomyosarcoma were reported to show t(2;6)(p23;p21.1),[182] t(4;22)(q35;q12) fusing *EWSR1* with *DUX4,*[183] and a complex t(8;13;9)(p11.2;q14;q32) apparently resulting in amplification of *FGFR1-FOXO1* fusion.[184]

Synovial Sarcoma

Synovial sarcomas may be monophasic (Fig. 45-21), in which the tumor is predominantly composed of spindle cells, or biphasic, in which the tumor contains both spindle cell and epithelioid elements, with the latter exhibiting more convincing epithelial immunophenotypic characteristics or, uncommonly, poorly differentiated, with a primitive round cell morphology reminiscent of Ewing sarcoma. Apart from the epithelial markers epithelial membrane antigen (EMA) and cytokeratins, transducer-like enhancer protein 1 (TLE1) also has been validated from gene expression profiles as a reliable marker for synovial sarcoma.[185-187] Synovial sarcomas feature translocation of chromosomes X and 18, t(X;18)(p11;q11).[188,189] t(X;18) is found in more than 90% of synovial sarcomas but not generally in histologic mimics such as hemangiopericytoma, mesothelioma, leiomyosarcoma, or malignant peripheral nerve sheath tumor. The molecular underpinnings of t(X;18) are complex in that the oncogene on chromosome 18 (*SYT* or *SS18*) can be

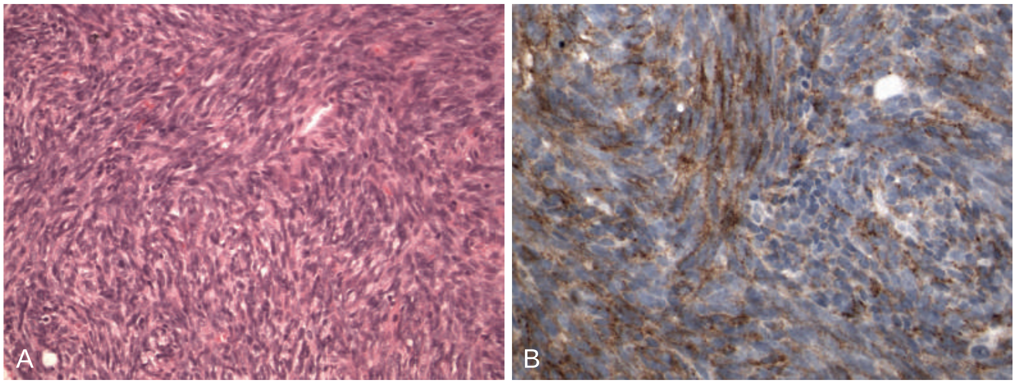

Figure 45-21 **A,** Monophasic synovial sarcoma, with interdigitating fascicular arrangement of fusiform spindle cells. **B,** Weak, patchy immunohistochemical staining for epithelial membrane antigen (EMA) in the spindle cells.

fused with one of several almost identical genes (generally *SSX1* or *SSX2* and, rarely, *SSX4*) on chromosome X.[190,191] *SSX1* and *SSX2* are adjacent genes and, given their close proximity, it is impossible to distinguish *SS18-SSX1* and *SS18-SSX2* translocations using conventional chromosomal banding methods. However, the alternative *SSX* fusions can be demonstrated by FISH or RT-PCR using specific probes or primers, respectively.[192,193] One study has suggested that synovial sarcomas with the *SS18-SSX1* fusion are typically biphasic, whereas those with *SS18-SSX2* can be biphasic or monophasic and have better metastasis-free survival compared with those bearing *SS18-SSX1* fusions.[194] This morphologic-genotypic correlate is substantiated by studies of SS18-SSX1 and SS18-SSX2 interactions with SNAIL and SLUG, both transcriptional repressors of E-cadherin, which mediates mesenchymal to epithelial transition.[195]

The SS18-SYT fusion protein is both necessary and sufficient for cellular transformation.[196] SS18 and SSX contribute, respectively, transcriptional coactivator and repressor domains to the SS18-SSX fusion proteins, and, despite the lack of any DNA binding domain, exert an effect on gene transcriptional dysregulation apparently through association with chromatin remodeling proteins Trithorax (TRX) and the Polycomb repressor complex (PRC2).[197-200] Mass spectrometry has revealed that SS18-SSX fusion proteins bind TLE1 and ATF2 to form a complex necessary for tumor cell survival. ATF2 is the DNA binding partner of SS18-SSX, and the epigenetic repression of ATF2 is dependent on TLE1, the recruitment of which can be abrogated by HDAC inhibitors, which have already shown considerable promise in preclinical trials.[201] In addition, SS18-SSX competes physically with wild-type SS18, forming an altered complex lacking SMARCB1. This altered complex transactivates *SOX2*, which is a diagnostic marker that is universally overexpressed in synovial sarcoma and essential for synovial sarcoma proliferation.[202]

Adipose Tumors

Most subtypes of adipose tumors, whether benign or malignant, contain distinctive genetic aberrations (see Table 45-1). Useful diagnostic markers include 8q rearrangement in lipoblastoma, 12q rearrangement in lipoma, ring chromosomes in well-differentiated and dedifferentiated liposarcoma, and t(12;16) in myxoid or round cell liposarcoma.

Lipoblastomas are pediatric adipose tumors containing variable numbers of primitive cells (lipoblasts) and generally bearing translocations of the chromosome 8 long arm at bands 8q11-12, resulting in rearrangement of the *PLAG1* zinc finger oncogene.[130] Reports of individual cases each of lipoblastoma[203] and lipoma[204] bearing translocations involving 8q21.1 region in each case as well as with a t(9;12)(p22;q14) in a pediatric intramuscular lipoma[205] add to the variability in observations in these benign fatty neoplasms. Hibernomas, which contain adipose cells with a brown fat phenotype, generally have rearrangements of the chromosome 11 long arm,[206] although the gene target of that translocation has not been identified.

Another diagnostically useful aberration is the translocation of chromosomes 12 and 16 found in myxoid liposarcomas.[207-209] This translocation is also found in round cell liposarcomas and is retained in myxoid liposarcomas that acquire round cell features and thereby undergo transition to higher histologic grade.[210,211] t(12;16) results in fusion of the *DDIT3 (CHOP)* transcription factor gene on chromosome 12 to the *TLS* gene on chromosome 16,[212,213] and the resultant fusion oncoprotein is an activated transcription factor. t(12;16) has not been found in other subtypes of liposarcoma or in other types of myxoid soft tissue tumors.[214,215] It appears to be relevant therapeutically, in that patients with myxoid or round cell liposarcoma show impressive responses to trabectedin chemotherapy.[216] However, liposarcoma is rare in childhood and virtually unheard of in the first decade of life.

Clear Cell Sarcoma: Malignant Melanoma of Soft Parts

Clear cell sarcomas of the soft tissues resemble cutaneous melanomas histologically, and therefore have been referred to as melanoma of soft parts. Immunohistochemically, the tumors are consistently positive for S-100 protein, usually at least focally for HMB45, microphthalmia transcription factor (MITF), and Melan A.[217] Despite their histologic and immunohistochemical overlaps, clear cell sarcoma and true melanoma are different clinically. Whereas most melanomas are of cutaneous origin, clear cell sarcomas generally present as isolated masses in deep soft tissues, without apparent origin from, or involvement of, skin. Less commonly they arise in the gastrointestinal tract. Most clear cell sarcomas contain a chromosomal translocation, t(12;22)(q13;q12), while those arising in the gastrointestinal tract will more commonly bear the variant t(2;22)(q34;q12).[218] The latter has, however, also been described in a minor subset of soft tissue–based clear cell sarcoma.[154,219] These translocations have never been reported in cutaneous melanoma and therefore serve as a reliable markers in distinguishing these two tumor types (Fig. 45-22). t(12;22) fuses the *ATF1* gene on chromosome 12 with the *EWS* gene on chromosome 22.[219,220] *ATF1* encodes a transcription factor, and the biologic implications of the translocation are probably similar to those in Ewing sarcoma translocations (see earlier). t(2;22) fuses the *CREB1* gene on chromosome 2, a *CREB* gene family member closely related to *ATF1,* with the *EWSR1* gene.

Desmoplastic Small Round Cell Tumor

DRSCTs are aggressive and chemotherapy-resistant neoplasms that arise most commonly from the peritoneum or intraabdominal soft tissues, particularly in adolescent males.[221] They are composed of undifferentiated malignant small round cells in a striking desmoplastic reaction, ensnaring sharply demarcated islands of tumor cells (Fig. 45-23).[222] Almost all cases express an *EWSR1-WT1* fusion oncogene[222,223] resulting from translocation between the chromosome 11 short arm and chromosome 22 long arm.[224,225] The EWS-WT1 oncoproteins are expressed as several isoforms (see Fig. 45-10), each of which is composed of the EWSR1 amino-terminal

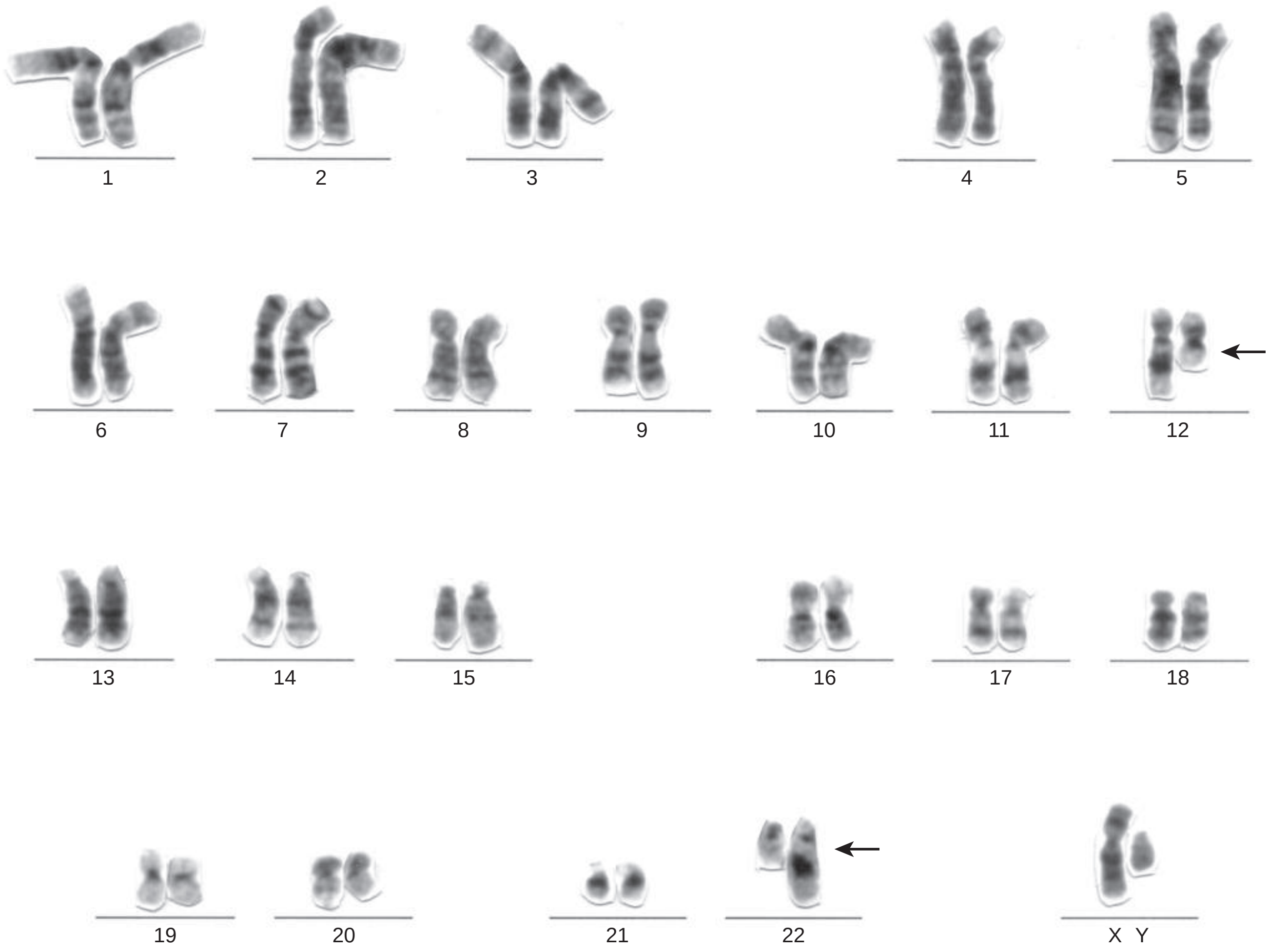

Figure 45-22 Clear cell sarcoma karyotype showing balanced translocation, t(12;22), as the only cytogenetic aberration. *Arrows* indicate translocation breakpoints.

transactivation region fused to the last three of four zinc fingers at the WT1 carboxyl-terminal end.[226,227] These oncoproteins result from fusion of *EWSR1* exon 7, 8, or 9 to *WT1* exon 8. The *EWSR1-WT1* fusion typically results in expression of the carboxyl-terminal but not amino-terminal aspect of WT1 in DSRCT, which may be detected by immunohistochemistry using appropriate antibodies (see Fig. 45-23). Quantitatively minor secondary transcripts lacking exon 6 of *EWSR1* and/or exon 9 of *WT1* are also expressed, although the functional relevance of these transcripts is unknown.[226] However, it is worth noting that the usual immunostaining pattern was not observed in an isolated reported case with atypical transcript that lacked *WT1* exons 9 and 10.[228] The EWSR1-WT1 oncoprotein retains an alternative splicing site (±KTS) between the third and fourth WT1 zinc fingers (see Fig. 45-10). The KTS− isoform regulates genes that are believed to have important biologic roles in DSRCTs, including platelet-derived growth factor-α (PDGFA) and insulin-like growth factor receptor I, whereas the KTS+ isoform regulates a different set of genes.[229] Notably, only the KTS− isoform has shown transforming activity when evaluated in vitro.[230] PDGFA is known to activate potent receptor (platelet-derived growth factor receptor [PDGFR])-mediated mitogenic signaling pathways in fibroblasts,[231] and EWSR1-WT1 might therefore induce desmoplastic proliferation via PDGFA transcriptional upregulation.[227]

Dermatofibrosarcoma Protuberans

DFSP is a low-grade spindle cell tumor, which can occasionally progress to a more aggressive fibrosarcomatous phase. Most DFSPs contain ring chromosomes composed of sequences from chromosomes 17 and 22 (see Fig. 45-13A).[232,233] The ring chromosomes contain multiple copies of a fusion gene, *COL1A1-PDGFB*, in which *COL1A1* (a collagen gene) is contributed from chromosome 17 and *PDGFB* (platelet-derived growth factor-β gene) from chromosome 22.[75,122] Alternatively, DFSP, particularly in pediatric patients, occasionally has t(17;22) translocations, resulting in a single copy of the *COL1A1-PDGFB* fusion gene.[234] The *COL1A1-PDGFB* oncogene

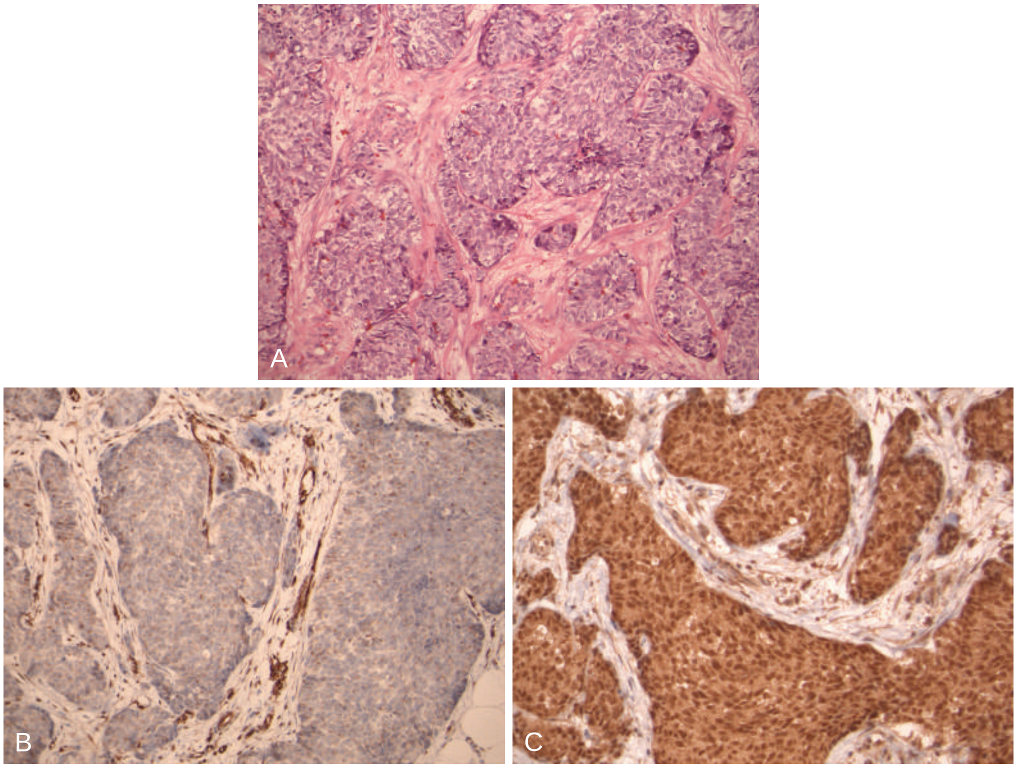

Figure 45-23 **A,** Desmoplastic small round cell tumor with sharply demarcated islands of blue tumor cells separated by pink densely fibrotic "desmoplastic" stroma. Immunoreactivities with antibodies to N-terminal (**B**) and C-terminal (**C**) regions of WT1 are negative and strongly positive, respectively, in the tumor cells, in keeping with EWS-WT1 fusion oncoprotein expression.

results in overexpression of PDGFB, which is a growth factor that activates platelet-derived growth factor receptor-β (PDGFRB) and PDGFRA. This cytogenetic observation has suggested that patients with inoperable DFSP might benefit from treatment with PDGFR inhibitors such as imatinib, and this hypothesis has been confirmed by impressive clinical responses to PDGFRB therapeutic inhibition with imatinib.[235,236,237,238] *COL1A1-PDGFB* oncogenic fusion is also seen in giant cell fibroblastoma (GCF), a pediatric neoplasm closely related to DFSP.[123,239] Studies of GCF and composite GCF/DFSP cases have shown the presence of a single translocation t(17;22) and the resultant *COL1A1-PDGFB* fusion, in the GCF component, with acquisition of additional copies of the oncogene being associated with progression to DFSP.[74]

Desmoid Tumors

Desmoid tumors, also known as deep fibromatoses, contain various genetic aberrations, including the *APC* (adenomatous polyposis coli) gene or *CTNNB1* mutations,[240,241] and trisomies for chromosomes 8 or 20.[242] Cytogenetic deletions of the chromosome 5 long arm are seen in occasional desmoids, resulting in loss of the *APC* tumor suppressor gene.[243,244] However, the most common mutations, particularly in nonfamilial desmoid tumors, are activating *CTNNB1* mutations, which are found in at least 50% of cases.[240,241] Both *APC* inactivating and β-catenin activating mutations result in stabilization and resultant overexpression, of the β-catenin protein with roles in cell proliferation, motility, and differentiation. This overexpression of β-catenin can be detected immunohistochemically in a nuclear distribution.[245] In a retrospective analysis of primary desmoid tumor, the precise β-catenin mutation type was strongly predictive of recurrence, with S45F being associated in multivariate analysis with decreased time to recurrence and overall percentage recurrence.[246] The cytogenetic aberrations in most desmoid tumors, particularly the trisomies 8 and 20, are mechanisms of progression and are acquired subsequent to the *APC* or *CTNNB1* mutations. Interestingly, an as yet unexplained association of desmoidal fibromatosis in patients with gastrointestinal stromal tumor (GIST) has been reported.[247]

Infantile Fibrosarcoma

Infantile (also congenital/infantile) fibrosarcomas characteristically present within the first year of life. These are tumors composed of plump fibroblast-like cells (see Fig. 45-16), which often show high mitotic activity, and feature trisomies of chromosomes 8, 11, 17, and 20.[248] This same group of trisomies occur in the histologically identical pediatric renal tumor, congenital cellular mesoblastic nephroma (see Fig. 45-18).[249] Most infantile fibrosarcomas and congenital cellular mesoblastic nephromas also contain a diagnostic chromosomal translocation, t(12;15)(p13;q26), which is cryptic when studied by traditional cytogenetic banding methods (see Fig. 45-2).[7,113,250] A complex three-way t(12;15;19) translocation has also been reported in infantile fibrosarcoma.[251] t(12;15) (p13;q26) results in fusion of the *TEL* gene on chromosome 12 to the *NTRK3* receptor tyrosine kinase gene on chromosome 15, producing constitutive tyrosine kinase activation. Although challenging to detect by banding methods, the t(12;15) translocation is demonstrated readily by FISH with a dual-color break-apart TEL probe (see Fig. 45-2) or RT-PCR assay.[7,113,250]

Inflammatory Myofibroblastic Tumor

Inflammatory myofibroblastic tumors, belonging to the group of inflammatory pseudotumors, are relatively rare tumors occurring primarily in children and young adults. They are composed of myofibroblastic cells admixed with an infiltrate of lymphocytes and plasma cells (see Fig. 45-17). The inflammatory component of these tumors is nonneoplastic and therefore lacks cytogenetic aberrations, whereas the myofibroblastic cells often contain clonal chromosomal aberrations[252-254] involving rearrangement and oncogenic activation of the *ALK* receptor tyrosine kinase gene at chromosome band 2p23.[114] The *ALK* oncogenes cause constitutive activation of the ALK kinase by enhanced oligomerization resulting from fusion of the ALK kinase domain to the oligomerization domain of the partner gene.[114] *ALK* oncogenic activation is associated with strong, immunohistochemically detectable ALK expression in the inflammatory myofibroblastic tumor spindle cells in about 50% of cases (see Fig. 45-17).[114] The ALK immunohistochemical staining pattern reflects the fusion type, insofar as fusion of ALK with most of the various partners (TPM3, TPM4, CARS, CLTC, SEC31A (SEC31L1),[255] PPFIBP1,[256] ALO17) produces cytoplasmic staining, whereas ALK-RANBP2 fusion produces nuclear membrane–associated reactivity.[257,258] These immunohistochemical findings can distinguish inflammatory myofibroblastic tumor from its histologic mimics, but, given the considerable rate of nonimmunoreactivity, demonstration of *ALK* rearrangement is especially helpful in confirming the diagnosis. An aggressive form of IMT characterized by round cell or epithelioid morphology is described that typically arises intraabdominally in male patients and shows in virtually all cases ALK immunoreactivity in a nuclear, or less commonly, cytoplasmic and perinuclear distribution.[259]

ALK-rearranged inflammatory myofibroblastic tumors are effectively treated with ALK inhibitor crizotinib; however, the emergence of secondary resistance can limit efficacy.[260,261]

Gastrointestinal Stromal Tumor

Most adult GISTs contain activating mutations of the *KIT* or *PDGFRA* oncogenes[262-264] or rarely *BRAF*.[265] These mutations have been targeted with considerable success using the KIT and PDGFRA inhibitor imatinib (Gleevec)[266,267] and second-line tyrosine kinase inhibitors in the context of resistant disease.[268] In addition, germline (inherited) *KIT* mutations are responsible for rare syndromes of familial, multifocal GISTs.[269] However, because most pediatric GISTs do not contain *KIT* or *PDGFRA* mutations they have been termed *wild-type GISTs,* and these predominate as gastric tumors in prepubescent girls.[270-272] In keeping with this, children with metastatic GISTs show only very limited benefit from the KIT-PDGFRA kinase inhibitor therapies. Moreover, pediatric GISTs lack the typical sequential cytogenetic deletions that seem to drive genetic and clinical progression in adult GISTs.[273] Largely through an appreciation of the occurrence of GIST as a component of Carney-Stratakis syndrome/dyad, which is the dominantly inherited predisposition to paraganglioma and GIST,[274,275] underpinned by germline inactivation of subunits B, C or D of succinate dehydrogenase (SDH), it has become evident that deficiency in cellular respiration in fact appears to provide the pathologic basis to all pediatric/wild-type GISTs, despite the low incidence of SDH mutation in such tumors.[276] Germline *SDHA* mutations have been described in isolated cases of paraganglioma and adult wild-type GIST.[277,278] Inactivation of any of the four subunits A, B, C, or D of SDH leads to enzyme complex destabilization and inactivation. Immunohistochemical staining for the B subunit of succinate dehydrogenase (SDHB) provides a surrogate marker for SDH function and is characteristically negative in all pediatric/wild-type GISTs. On this basis, a proposal has been made that GISTs no longer be classed as "mutant" and "wild-type" based on receptor tyrosine kinase mutation but rather be designated type 1 and type 2 as SDHB-positive and SDHB-negative, respectively.[279] Although a strong association with neurofibromatosis type 1 (NF1) exists, in this particular context GISTs usually arise only in adulthood.[280]

Malignant Peripheral Nerve Sheath Tumors

Benign peripheral nerve sheath tumors and malignant peripheral nerve sheath tumors (MPNSTs) are frequent complications in patients with hereditary neurofibromatosis syndromes. These syndromes are the most common tumor predisposition syndromes, affecting 1 in 3500 individuals worldwide. Neurofibromas and malignant peripheral nerve sheath tumors are common in those NF1, whereas benign schwannomas are common in neurofibromatosis type 2 (NF2, central neurofibromatosis). Characterization of the neurofibromatosis syndrome genes has shed substantial light on the pathogenesis of peripheral nerve sheath tumors. The *NF1* and *NF2* genes are located on chromosomes 17 and 22, respectively, and both these genes encode tumor suppressor proteins that normally constrain cellular proliferation.[281-286] NF1 protein tumor suppressor mechanisms involve regulation of RAS GTPase activity,[282,283] with *NF1* gene deletion therefore resulting in constitutive RAS activation, suggesting that therapeutics targeting RAS (or downstream signaling intermediates such as RAF and MEK-MAPK) might be useful for clinical management of MPNSTs and other NF1-associated cancers.[287] MPNSTs often have *NF1* gene deletions, which can be demonstrated by FISH assay. The *NF1* gene aberrations are accompanied by a generally complex karyotype, suggesting that genetic instability plays a prominent role in the development of MPNSTs. Notably, *NF1* gene deletions can be shown in only the Schwann cell component of neurofibromas.[288] This observation supports the view that neurofibromas are clonal Schwann cell neoplasms, whereas the other admixed cell lineages—including fibroblasts, mast cells, and perineural cells—are reactive.

Recent studies demonstrated that the evolution of benign peripheral nerve sheath tumors to MPNST is accompanied predominantly by *loss* of expression of a large number of genes, including, in a vast majority of cases, inactivation of TP53[289] with associated downregulation of the pro-apoptotic miR-34a. MiR-10b, which specifically targets NF1, is upregulated in NF1-associated MPNSTs.[290] Homozygous loss of CDKN2A is also associated with malignant transformation,[291,292] whereas high-resolution copy number profiling has shown, among others, recurrent gains of *PDGFRA, MET, HGF, TP73, EGFR, TERT, MYC,* and *IGF1R* and losses of *NF1, p16^{INK4A}/CDKN2A, RB1,* and *TP53,*[293,294] providing potential basis for novel therapeutic agents[294,295] in this therapeutically challenging sarcoma.

Neuroblastoma

Most neuroblastomas have distinctive cytogenetic features that correlate with their clinical behavior. Favorable prognosis neuroblastomas, which respond well to chemotherapy and can even undergo spontaneous regression, generally feature near-triploid karyotypes without 1p deletion or *MYCN* amplification.[296] Unfavorable prognosis neuroblastomas (Fig. 45-24), by contrast, feature near-diploid or near-tetraploid karyotypes, often accompanied by 1p deletion, 17q amplification, and/or *MYCN* amplification,[297] whereas deletions of 3p and 11q are reportedly predictive of metastasis.[298] In essence, gains of whole chromosomes are more typical of low- or intermediate-risk neuroblastoma whereas segmental chromosomal aberrations are more commonly seen in high-risk cases. *MYCN* amplification, typically manifested as double-minute chromosomes (see Figs. 45-5 and 45-6), is arguably the most ominous of the adverse genetic markers. *MYCN*-amplified neuroblastomas are rarely curable, although complete remission can be achieved through intensive myeloablative chemotherapy. Therefore genetic parameters can be useful adjuncts for determining appropriate intensity of therapy, particularly for those children whose prognoses are not clear based on clinical parameters alone.[297] Cytogenetic analysis of neuroblastoma has been difficult, however, because the tumor cells from most favorable prognosis neuroblastomas fail to divide in culture.[297] *MYCN* amplification and chromosome 1p or 11q deletion and 17q gain can be demonstrated in interphase cells by FISH or CISH (see Fig. 45-6), obviating the need for tumor cell culture.[299,300] It remains unclear whether one or more conventional tumor suppressor genes are targeted by the 1p deletions found in most poor-prognosis neuroblastomas. However, several microRNAs in the critical 1p deletion region function to maintain neuroblastoma survival and activate *MYCN* transcription.[301,302] *ALK* kinase domain mutations and gene amplifications have been demonstrated in hereditary neuroblastoma that may otherwise be underscored by *PHOX2B* germline mutation.[117-119,303,304] About 15% of sporadic neuroblastomas also show aberrations of ALK,[117-119,305] suggesting that ALK inhibitors might be useful therapeutically in neuroblastoma. SNP variations at 6p22.3,[306] 2q35 (the *BARD1* gene),[307] and copy number aberration at 1q21 (the *NBPF23* gene)[308] are associated with increased risk for development of neuroblastoma but without further elucidation of the likely mechanisms involved biologically.

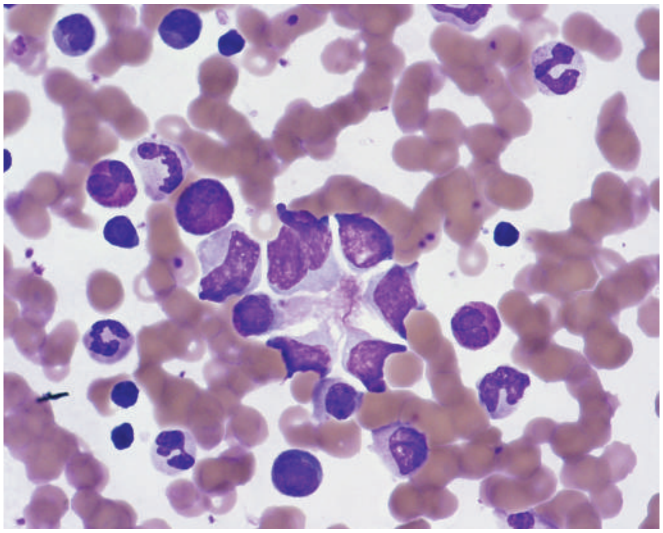

Figure 45-24 Bone marrow involvement by disseminated neuroblastoma, showing a classic rosette of tumor cells, with bluish cytoplasm and neuropil. *(Courtesy Marybeth Helfrich, Children's Hospital of Philadelphia.)*

Rhabdoid Tumor

Malignant rhabdoid tumors generally have dismal outcomes, particularly those presenting in infancy, and most cases, whether arising in soft tissues, kidney, or CNS (where they are known as atypical teratoid/rhabdoid tumors), have deletions of the chromosome 22 long arm, targeting the *SMARCB1* (*INI1)* tumor suppressor gene. Biallelic *SMARCB1* mutation is present in the vast majority of rhabdoid tumors, with frequent truncating mutations occurring. In a small number of cases, instead of *SMARCB1,* the *SMARCA4 (BRG1)* gene is inactivated.[309] *SMARCB1* encodes a member of the SWI/SNF protein complex involved in chromatin remodeling.[310,311] Chromosome 22 deletion is often the only detectable cytogenetic aberration, suggesting that *SMARCB1* inactivation is an early oncogenic event in these remarkably aggressive tumors,[312,313] which are classically diploid and genomically stable.[314,315] Indeed, sequencing studies have revealed a remarkably simple tumor genome otherwise with virtually no oncogenic mutations identifed.[315,317] Additional evidence of an essential tumorigenic role includes the finding of germline *INI1* mutations in some individuals with rhabdoid tumors[311,318,319] and the development of rhabdoid tumor models in mice with inactivating *SMARCB1* mutations.[320] Notably, germline *SMARCB1* mutations are not restricted to rhabdoid tumors but have been implicated also in familial schwannomatosis[321] and in nonrhabdoid CNS tumors, including medulloblastoma.[318] and multiple meningiomas[322] SMARCB1 inhibits cyclin D1 expression by recruiting a histone deacetylase 1 complex to the *CCND1* (cyclin D1) promoter, resulting in G1 cell cycle arrest.[323] The relevance of cyclin D1 in *SMARCB1*-mediated tumorigenesis is underscored by the observation that rhabdoid tumors develop in SMARCB1+/– mice expressing cyclin D1 whereas SMARCB1+/– mice with cyclin D1 deficiency (cyclin D1–/–) do not develop rhabdoid tumors.[324] These observations suggest that drugs targeting cyclin D1 might be relevant for rhabdoid tumors,[325] and clinical trials testing this are planned.

Recent work has unraveled an epigenetic antagonism between SMARCB1 and Polycombs of the PRC2 complex.[326] The Polycomb proteins are transcriptional repressors that modulate chromatin structure to silence gene expression, and they are key regulators of cell fate transitions. Their function frequently becomes deregulated in cancer.[327] The Polycombs have been shown to be required for the repression of differentiation genes in stem cells[328,329] so that this repression of differentiation might be facilitated by loss of the PRC2 antagonist SMARCB1.

Rhabdoid tumors are characteristically composed of cells with vesicular nuclei and prominent nucleoli, with the nucleus often being pushed to an eccentric location within the cell by a dense intracytoplasmic inclusion (see Fig. 45-9), which, as seen by electron microscopy, consists of a tightly arranged whorl of intermediate filaments. These filaments stain nonspecifically for various markers, giving the tumor a polyphenotypic appearance. Absence of SMARCB1 expression in the tumor cells but not the normal stromal support nuclei, as is readily demonstrable by immunohistochemistry, is useful for diagnosing rhabdoid tumor (see Fig. 45-9).[31,330]

Alveolar Soft Part Sarcoma

Alveolar soft part sarcoma is a rare tumor, occurring mostly in adolescents and young adults, with a predilection for involvement of soft tissues in the head and neck region. However, other soft tissue and even hollow viscera may be involved. Characterized by a distinctive morphology, with nested or alveolar-like growth, the tumor cells are epithelioid, with abundant clear to eosinophilic cytoplasm, sharply defined cellular borders, and diastase-resistant, periodic acid–Schiff (PAS)-positive intracytoplasmic crystals (Fig. 45-25), which on electron microscopic examination are rhomboidal. Alveolar soft part sarcoma generally exhibits indolent behavior but nonetheless has a high risk for late metastases and indeed represents the sarcoma most likely to metastasize to the CNS.[331] There are no histologic features to predict clinical behavior. The classic cytogenetic feature of ASPS is t(X;17)(p11;q25), producing fusion of the *ASPSCR1* and *TFE3* genes.[332] This same translocation is seen in a subset of pediatric renal cell carcinomas, with the distinct difference that the *ASPS*-related translocation is *un*balanced and involves gain of Xp telomeric to *TFE3* and loss of 17q sequence telomeric to q25[333] while the renal carcinoma–associated translocation is balanced. The ASPSCR1-TFE3 fusion protein induces strong expression of MET tyrosine kinase in tumor cells.[334] Antibody raised to the carboxyl terminus of TFE3 produces strong nuclear reactivity in *ASPSCR1-TFE3* fusion-positive tumors.[335]

Nonmesenchymal Solid Tumors

Renal Tumors

Most pediatric renal tumors contain characteristic cytogenetic aberrations. Deletion of 11p is a well-known aberration in Wilms tumor, but several other aberrations, including extra copies of chromosome 12, are more frequent and have apparent prognostic relevance, as does LOH of 1p and 16q.[336,337] Another pediatric renal tumor with consistent cytogenetic aberrations is congenital cellular mesoblastic nephroma, in which various chromosomal trisomies and oncogenic fusion of the TEL and NTRK3 proteins are observed reliably.[7,250] More recently, it has emerged that translocation-associated carcinomas make up a majority of pediatric renal carcinomas.[338,339] In addition, neuroblastoma patients are at hugely increased risk (329-fold)[340] of developing oncocytoid renal carcinoma[341]; however, pediatric renal carcinoma may also follow certain sarcomas.[342,343]

Pediatric Renal Cell Carcinomas with Xp11 or 6p21 Translocations. Pediatric renal cell carcinomas are uncommon and they generally have a papillary morphology, accompanied by translocations involving chromosomes X or 6.[344] By comparison, adult papillary renal cancers typically have a distinctive profile of chromosomal trisomies, although a rare few contain the same translocations seen in pediatric renal cancers. The

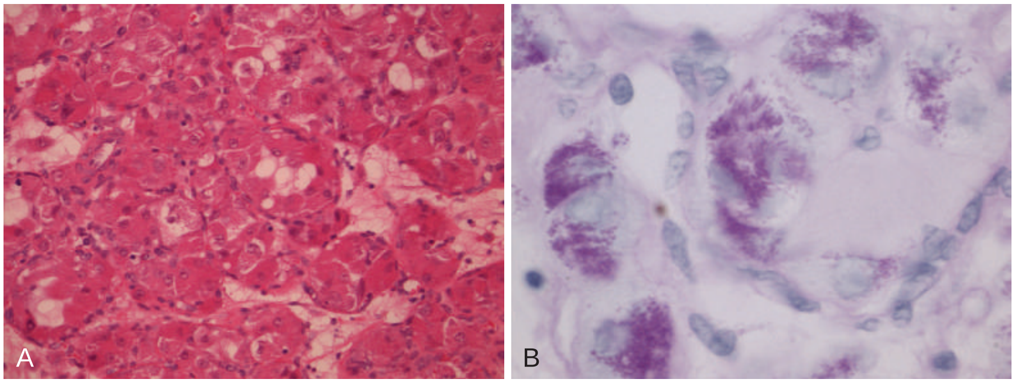

Figure 45-25 Characteristic histologic features of alveolar soft part sarcoma include an alveolar-like arrangement of cells with abundant deeply eosinophilic cytoplasm (**A**) and diastase-resistant, periodic acid–Schiff-positive intracytoplasmic crystals (**B**).

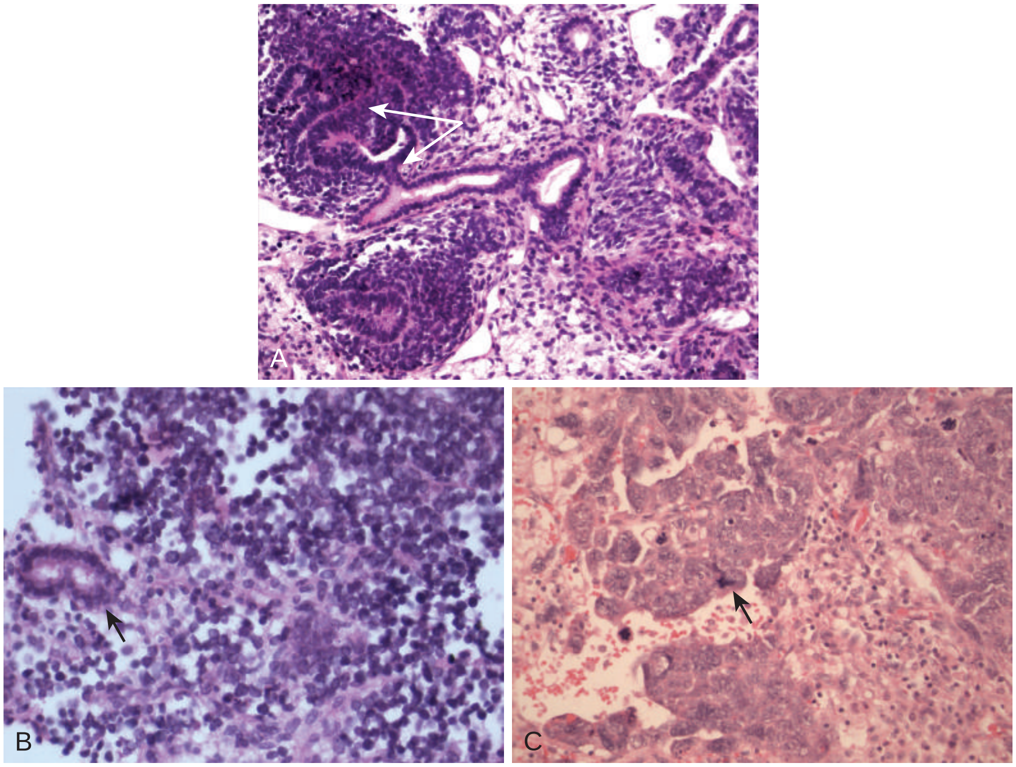

Figure 45-26 **A,** Wilms tumor with triphasic histology. The tumor recapitulates nephrogenesis with condensation of mesenchyme at tips of tubular branch-points. Here, the blastema is condensed around a focus of tubular differentiation *(arrows)*. **B,** High-power image showing undifferentiated blastema, which in the absence of obvious tubular differentiation *(arrow)* might be difficult to distinguish from other undifferentiated small round cell tumors. **C,** Anaplastic Wilms tumor with bizarre mitoses and massively enlarged hyperchromatic nuclei *(arrow)*.

unifying theme for the pediatric carcinoma translocations is that they target various members of the MiT family of transcription factors. These include t(X;1)(p11;q21), resulting in fusion of *PRCC* (at chromosome band 1q21) and the MiT family member *TFE3* (at chromosome band Xp11). There are variants of this translocation, including t(X;17)(p11;q25), in which *TFE3* is fused with the *ASPSCR1* gene at chromosome band 17q25.[345] Also involving an MiT family member is t(6;11)(p21;q13), which fuses *TFEB* (at chromosome band 6p21) with the *Alpha* gene (at chromosome band 11q13).[346] The *TFEB*-rearranged tumors more consistently express melanocytic markers HMB45 and Melan A than do *TFE3*-translocated cases.[347] TFE3 and TFEB immunostains, respectively, show specific reactivity in a nuclear distribution in the correspondingly rearranged cases but may rarely be negative in the presence of molecular proof of a translocation, and a robust FISH assay has also been developed to confirm rearrangement of *TFEB*.[347] Intriguingly, however, whereas ASPS cases with ASPSCR1-TFE3 fusion were found to uniformly express cathepsin K, a target of MiTF, and the majority of PRCC-TFE3 rearranged carcinomas did similarly, the ASPSCR1-TFE3 fusion-positive carcinomas were all negative for cathepsin K,[348] a feature that might be exploited in certain diagnostic settings.

Wilms Tumor. Wilms tumors are the most common type of renal cancer in children. They typically contain primitive blastemal cells that differentiate into tubular epithelial, glomerular, and/or mesenchymal populations. The classic triphasic Wilms tumor contains an admixture of blastemal, epithelial, and mesenchymal components (Fig. 45-26), and all three cell types may contain the same clonal chromosomal aberrations.[349] Various genetic alterations are found in subsets of Wilms tumors, including trisomies 6, 8, 12, and 18, and deletions of 11p13 (see later). Deletions of 11p15, 1p, and 16q bode ill in patients with low-stage favorable histology tumors and 1q gains are associated with relapse. These can all readily be tested on interphase nuclei by FISH assay (see Table 45-1).[337,350-352]

Deletion 11p13 is the most extensively characterized cytogenetic aberration in Wilms tumors. This aberration became the focus of many studies after reports that individuals with the WAGR syndrome (***W***ilms, ***A***niridia, ***G***enitourinary abnormalities, and mental ***R***etardation) often had constitutional deletions at chromosome band 11p13, harboring the Wilms tumor suppressor gene *(WT1)*.[244,245] WAGR phenotype, however, results from deletions of several genes, with the *WT1* gene deletion responsible for predisposition to Wilms tumors and with deletion of a neighboring gene, *PAX6,* responsible for aniridia.[246] Although most or all WAGR-associated Wilms tumors have complete inactivation of *WT1,* such inactivation is found in fewer than 20% of sporadic Wilms tumors, and here again it appears to function as a tumor suppressor gene, the homozygous deletion of which is essential for Wilms tumorigenesis.[353] Familial Wilms tumor predisposition is rare and primarily linked to loci on 17q or 19q and only uncommonly to *WT1*.[354,355] Approximately 7% to 30% of Wilms tumors have deletion of the *WTX* gene, which notably is the first putative tumor suppressor gene identified on chromosome X.[247-249] *WTX* loss, invariably a somatic event in Wilms tumor, results from truncating mutations or more commonly from deletion of the corresponding region on the single copy of chromosome X in males, although there is controversy as to whether the deletion in females invariably involves the active copy of chromosome X.[250,251] Interestingly, although germline mutation of WTX is associated with osteopathia striata congenita with cranial sclerosis, these patients have no increased risk for developing Wilms tumor.[356] The WTX protein promotes β-catenin ubiquitination and degradation,[254] and it is therefore expected that *WTX* gene deletions in Wilms tumors would hyperactivate WNT–β-catenin signaling pathways. This is in keeping with the finding of virtually mutual exclusivity of β-catenin mutations (found in about 15% Wilms tumors) and *WTX* mutations.[255-258] On the other hand, CTNNB1 (protein)-stabilizing mutations largely overlap with inactivating mutations of *WT1*[357,358] but occur later in oncogenesis. The suggestion has been made that WTX inactivation, although frequent in Wilms tumor, is a late event and of unclear clinical significance.[359]

Loss of heterozygosity (LOH) or loss of imprinting (LOI) of 11p15 is seen in approximately 70% of Wilms tumors and leads to upregulation of IGF2,[359-361] which occurs through biallelic expression of IGF2 owing to germline mutation of imprinting in Beckwith-Wiedemann syndrome.[362,363]

Nephrogenic rests—persistent islands of uncompleted organogenesis believed to represent precursor lesions—are found in surrounding kidney in approximately 30% of sporadic Wilms tumors and almost all bilateral or multifocal Wilms tumors.[364,365] These may take the form of intralobar or perilobar nephrogenic rests (ILNR and PLNR, respectively), each being associated with particular histologic phenotype[364,366] and genetic mutations, such that PLNRs are more commonly blastema rich, associated with overgrowth syndromes and show aberrations in the 11p15 imprinted region, whereas ILNRs typically are rich in stroma, including skeletal muscle or adipose elements, are associated with Denys-Drash and WAGR syndromes, and have *WT1* or β-catenin mutations.[367,368]

Various cytogenetic findings are associated with anaplastic histology in Wilms tumor. Complex karyotypes with chromosome counts in the triploid to tetraploid range are generally found in tumors with diffuse or focal anaplasia.[259] Similarly, Wilms tumors with *TP53* tumor suppressor gene mutations generally show anaplasia (see Fig. 45-26),[260] a finding linked more with therapy resistance than to a more biologically aggressive phenotype per se.

Clear Cell Sarcoma of the Kidney. Also formerly known as bone-metastasizing renal tumor, clear cell sarcoma of the kidney lacks any specific immunomarker and may show very variable histological features, making diagnosis a potential challenge. This tumor has a propensity to occur in boys younger than age 5 years.[369,370] Until recently, virtually nothing was known about the biology of this tumor of unfavorable histology, apart from individual case reports of a t(10;17)(q22;p13) recently characterized to show a fusion of the gene encoding the 14-3-3 ε family member of ubiquitously expressed modulators of phosphoserine-containing proteins (YWHAE) with *NUTM2* genes on 10q22, which encode proteins that are related to the NUT protein (see later).[371] This transcript was found to be expressed in 12% of clear cell sarcomas of the kidney and is similar to the translocation underpinning high-grade endometrial stromal sarcoma.[372] Although the full functional consequences of this translocation remain to be elucidated, upregulation of CCND1 is a feature in high-grade endometrial stromal sarcoma bearing YWHAE-FAM rearrangements.[372]

Mesoblastic Nephroma. Mesoblastic nephromas are the most common renal tumors diagnosed in infancy and may rarely also be encountered in older children. Histologically, mesoblastic nephroma falls into two categories: those tumors composed of bland, benign-appearing spindle cells interdigitating with the surrounding tissues, essentially recapitulating the morphology of fibromatosis, are known as classic mesoblastic nephroma,[261] whereas those with greater cellularity, sometimes in a fascicular arrangement, that are well delineated with a pushing border and composed of cells with plump nuclei, less cytoplasm, more mitoses, blood lakes, and often zonal necrosis, are known as cellular mesoblastic nephroma (Fig. 45-27; see Fig. 45-16).[262] Trisomy 11 is a consistent cytogenetic aberration in mesoblastic nephromas but is found only in the cellular group.[187,263] Approximately 70% of cellular mesoblastic nephromas contain trisomy 11, often accompanied by trisomies for chromosomes 8 and 17.[187] By contrast, clonal chromosomal aberrations have not been identified in classic mesoblastic nephroma. These findings suggest that trisomy 11, with or without other clonal chromosomal aberrations, is associated with

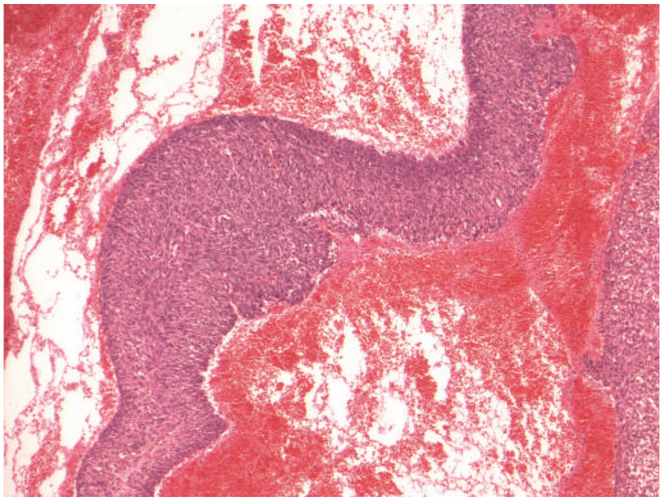

Figure 45-27 Cellular congenital mesoblastic nephroma with prominent blood lakes.

progression from classic to cellular histology in mesoblastic nephroma.[187] Mesoblastic nephromas with trisomy 11 generally also contain a balanced translocation t(12;15)(p13;q26), resulting in oncogenic fusion of the *TEL* and *NTRK3* genes. The t(12;15)(p13;q26) is cryptic by cytogenetic evaluation using chromosomal banding but is readily demonstrable by FISH or RT-PCR (see Fig. 45-2). These cytogenetic associations have been useful diagnostically, because Wilms tumors (the entity most likely confused with mesoblastic nephroma on small biopsy when blastema only has been sampled) rarely have trisomy 11 and have not been found to bear t(12;15).[241,242]

NUT Midline Carcinomas

Based on initial case reports, NUT midline carcinoma was considered a malignancy of children and adolescents arising in midline structures, especially upper aerodigestive tract and thymus.[110,373,374] Midline origin below the diaphragm is now well recognized, and the entity may also present in adulthood.[375,376] With the exception of an isolated atypical case,[377] all reported patients have died of disease or treatment complications within months of diagnosis. The histologic appearance is variable, typically including undifferentiated cells but often accompanied by immunohistochemical evidence of epithelial differentiation and, in some cases, with histologically convincing squamous differentiation (see Fig. 45-15). This entity was initially defined by the midline location, undifferentiated carcinoma–like histology, highly lethal course, and characteristic cytogenetic feature of t(15;19) (see Fig. 45-1). The t(15;19) results in oncogenic fusion of the *BRD4* (bromodomain-containing) and *NUT* genes on chromosomes 19 and 15, respectively, encoding a BRD4-NUT fusion oncoprotein. A subset of NUT midline carcinomas features similar oncogenic mechanisms in which *NUT* is fused with *BRD3*, or with yet unidentified translocation partners.[375] Diagnosis can be confirmed by karyotypic demonstration of t(15;19), molecular cytogenetic demonstration of *NUT* gene rearrangement, or RT-PCR demonstration of *BRD4-NUT* or *BRD3-NUT* fusion oncogenes.[375,376,378] The oncogenic function of BRD3-4-NUT oncoproteins is not yet well understood and the normal function of NUT is unknown. Interestingly, however, the NUT fusion oncoproteins do appear to block squamous differentiation in epithelial cell precursors.[378]

Thyroid Carcinomas

Thyroid malignancies include medullary carcinoma, which may be sporadic, or familial in the setting of multiple endocrine neoplasia type IIA (MEN-IIA) and MEN-IIB with germline *RET* tyrosine kinase gene mutations. Papillary thyroid carcinomas may also be sporadic, but are encountered with greatly increased frequency in individuals exposed to radiation, including adults after external-beam irradiation and children exposed to radioactive fallout, as from the Chernobyl disaster. Regardless of the patient's age or exposure to radiation, papillary carcinomas can feature chromosomal rearrangements resulting in oncogenic *RET* fusions.[70,379] *BRAF* mutations, on the other hand, although found in many adult papillary thyroid carcinomas, are uncommon in childhood thyroid carcinomas.[380,381] Follicular thyroid carcinomas are uncommon but are associated with a diagnostic translocation t(2;3), resulting in oncogenic fusion of the *PAX8* and *PPAR*γ genes (see Fig. 45-14).[108]

Blastomas

The central tumorigenic role of RB1 inactivation in retinoblastoma has served as a paradigm for tumor suppressor genes in oncology, but little is known of the other genetic mechanisms responsible for tumor progression in this disease. Recently, specificity of the cone-rod homeobox-containing gene *(CRX)* as an immunohistochemical marker for metastatic retinoblastoma versus other extracranial small round blue cell tumors was described.[383] Similarly, the molecular underpinnings of hepatoblastoma, pancreatoblastoma, and pleuropulmonary blastoma are only poorly understood. Trisomies of chromosomes 2 and 8 are found in many hepatoblastomas and pleuropulmonary blastomas,[384,385] but the genes targeted by these trisomies have not been identified. The identification in patients with familial pleuropulmonary blastoma of germline inactivating mutations of *DICER1*, an RNAse endonuclease essential in the processing of microRNAs,[386,387] has led to the recognition of a "DICER1 syndrome," predisposing patients to a wide variety of tumors, notably pleuropulmonary blastoma, cystic nephroma, and ovarian Sertoli-Leydig tumors.[388] The risk for tumors, however, is low, because most mutation carriers do not develop tumors. In contrast to the classic tumor suppressor model, monoallelic loss of DICER1 predisposes to tumor development through haploinsufficiency whereas loss of the second allele is inhibitory.[389,390]

Central Nervous System Tumors

Although heritable syndromes, including neurofibromatosis 1 and 2, von Hippel-Lindau syndrome, tuberous sclerosis, Li-Fraumeni syndrome, Gorlin syndrome, and Turcot syndrome account for a minor proportion of childhood CNS tumors, they have provided considerable pathogenetic insights including the important roles of Wnt, SHH, and TP53 pathways in medulloblastoma, for example.[391] Medulloblastomas are now, on a molecular basis, categorized into four groups based on alterations of SHH and Wnt pathways.[392-396] Dysregulation of Notch and HGF/MET pathways are also important in medulloblastoma. It has long been known that isochromosome 17 i(17)q was a common aberration in medulloblastoma and correlates with poor clinical outcome.[397,398] Further, cytogenetic aberrations also occur in medulloblastoma and many of these can be established by FISH assay, which has been used to generate a molecular stratification system from worst to best, including *MYC/MYCN* amplification, 6q gain, 17q gain, 6q/17q balance, and 6q loss.[397] Catastrophic DNA rearrangements were reported in pediatric medulloblastoma in the context of TP53-associated chromothripsis.[399] Meanwhile, high-resolution SNP arrays have identified recurrent copy number alterations indicative of epigenetic modification contributing to tumorigenesis[400] and evidence is emerging of a role for microRNA dysregulation also contributing.[400,401] Recently, somatic copy number aberration analysis has shown novel potentially targetable alterations in medulloblastoma including transforming growth factor β (TGF-β) and nuclear factor κB (NF-κB) signaling pathways.[402] In supratentorial PNET, deletion of *CDKN2A* was associated with metastatic progression[403] and amplification of *KIT* and *PDGFRA* have been described in some cases also,[404] potentially providing therapeutic targets. Recently, considerable further progress has been made in understanding supratentorial PNET (also known as CNS PNET).[405] Copy number and gene expression analyses detected three molecular subgroups of sPNET: primitive neural, oligoneural, and mesenchymal lineage based on differential expression of cell lineage markers Lin28 and OLIG2 with distinct survival and metastatic characteristics. These markers can be evaluated by immunohistochemistry.

Many genetic alterations have been identified in ependymoma without further pointers as to candidate genes until recent studies,[406-409] which have helped generate three subsets of ependymoma. Until very recently little if anything was known about the biology of diffuse pontine gliomas, because these are rarely sampled. Oligo array CGH conducted on archival postmortem cases has now provided initial insights into this tumor.[410] Response predictors identified in oligodendroglioma include amplification of *EGFR* (epidermal growth factor receptor) at 7p12 and homozygous deletion of *CDKN2A* at 9p21, which correlate with poor response to chemotherapy and reduced survival.[411] *EGFR* amplification in high-grade gliomas and amplification of *PDGFRA* in 15% malignant pediatric gliomas similarly call for investigation of its role as a molecular therapeutic target in that tumor.[412,413] More than half of pilocytic astrocytomas bear tandem duplications, translocations, or activating mutations of *BRAF*, which are associated with improved prognosis.[414-417]

Germ Cell Tumors

Many germ cell tumors contain a diagnostic cytogenetic marker, isochromosome 12p, often found in the context of a moderately complex karyotype, among other clonal chromosomal aberrations (Fig. 45-28).[418] Isochromosome 12p results from duplication of the chromosome 12 short arm on either side of the centromere and is uncommon in carcinomas and sarcomas.[419] Therefore demonstration of isochromosome 12p, particularly in any poorly differentiated cancer, should provoke strong suspicion of a germ cell origin.

Hematologic Neoplasms

Hodgkin Lymphoma

Hodgkin disease (HD), first described by Thomas Hodgkin in 1832, remained a poorly understood disease for over a century and a half, largely because of the sparse nature of neoplastic cells (typically <1%) within the tumor mass, even raising occasional doubt as to

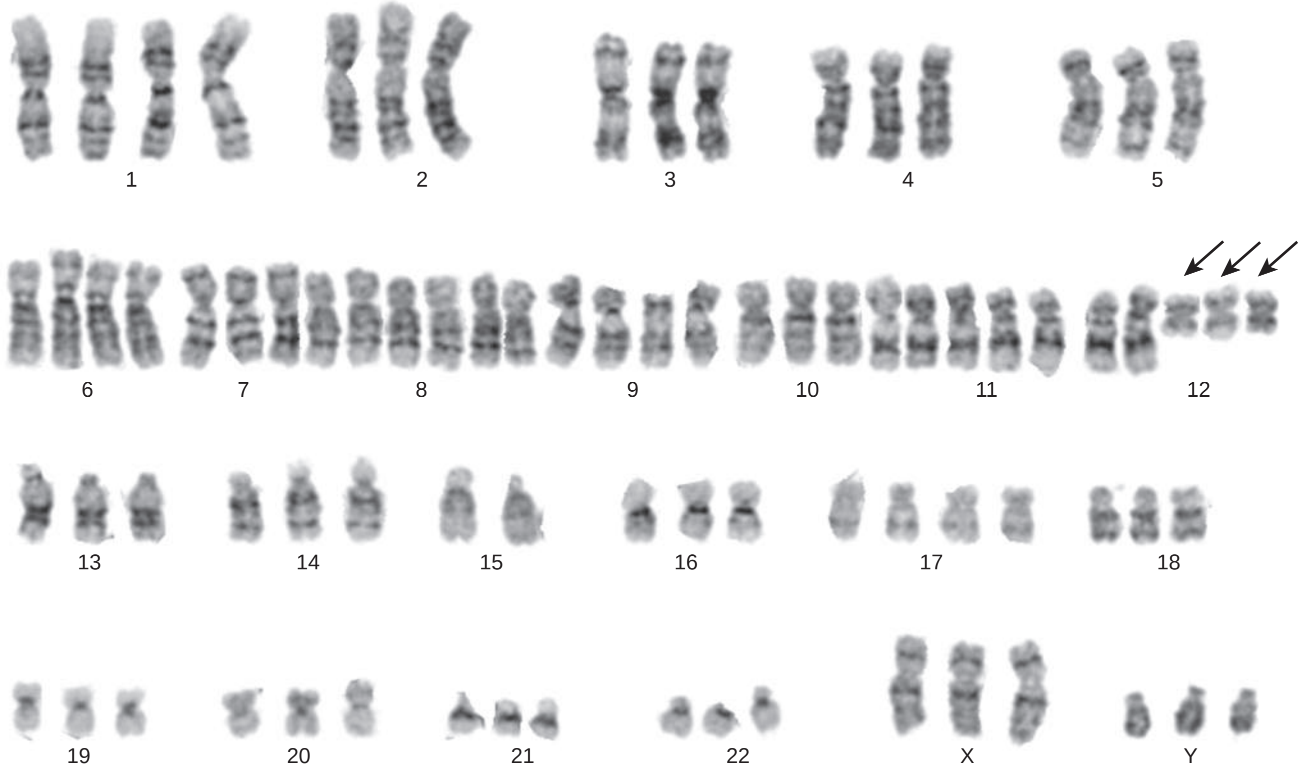

Figure 45-28 Malignant germ cell tumor karyotype showing three copies of the pathognomonic isochromosome 12p *(arrows)*.

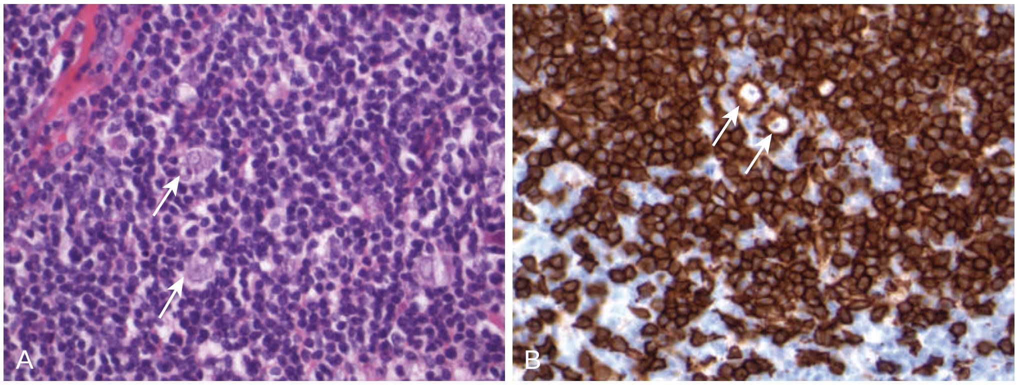

Figure 45-29 Nodular lymphocyte-predominance Hodgkin lymphoma featuring lymphocyte and histiocyte popcorn neoplastic cells (**A**, *arrows*) among small reactive lymphocytic infiltrate, and with immunohistochemical reactivity for the CD20 pan-B marker in both reactive and neoplastic cells (**B**, *arrows*).

the neoplastic nature of the entity. This sparseness of malignant cells, combined with their poor proliferative index, confounded attempts to investigate the molecular basis of HD. Karyotypic evaluations have been challenging, but nonetheless yielded early insights into the nature of HD. The capacity to grow Hodgkin cells continuously in vitro and the fact that injection of these cells into athymic nude mice produced tumors supported a neoplastic nature,[420] as did the aneuploid karyotypes demonstrated in Hodgkin lymphoma (HL) cell cultures.[421]

Light microscopic examination has permitted morphologic assignment of HL into classic and nodular lymphocyte-predominance subtypes,[422-424] a categorization recognized by the World Health Organization (WHO); this provided an attempt at cell of origin classification. The nodular lymphocyte-predominant subtype features a lymphocyte and histiocyte (L&H) neoplastic cell population, also known as popcorn cells (Fig. 45-29), with B-cell immunophenotype—expressing CD45, CD20, and BCL6 but lacking CD15 and CD30—whereas classic Hodgkin-Reed-Sternberg (H-RS) cells show only equivocal expression of the pan–B-cell marker CD20 and lack CD45 reactivity but express CD15 and CD30 in a membrane and Golgi staining pattern (Fig. 45-30).[425,426] Classic HL is subclassified into nodular sclerosis, mixed cellularity, lymphocyte-depleted, and lymphocyte-rich variants, depending on the composition of the background nonneoplastic elements in the tumor. The lack of any obvious

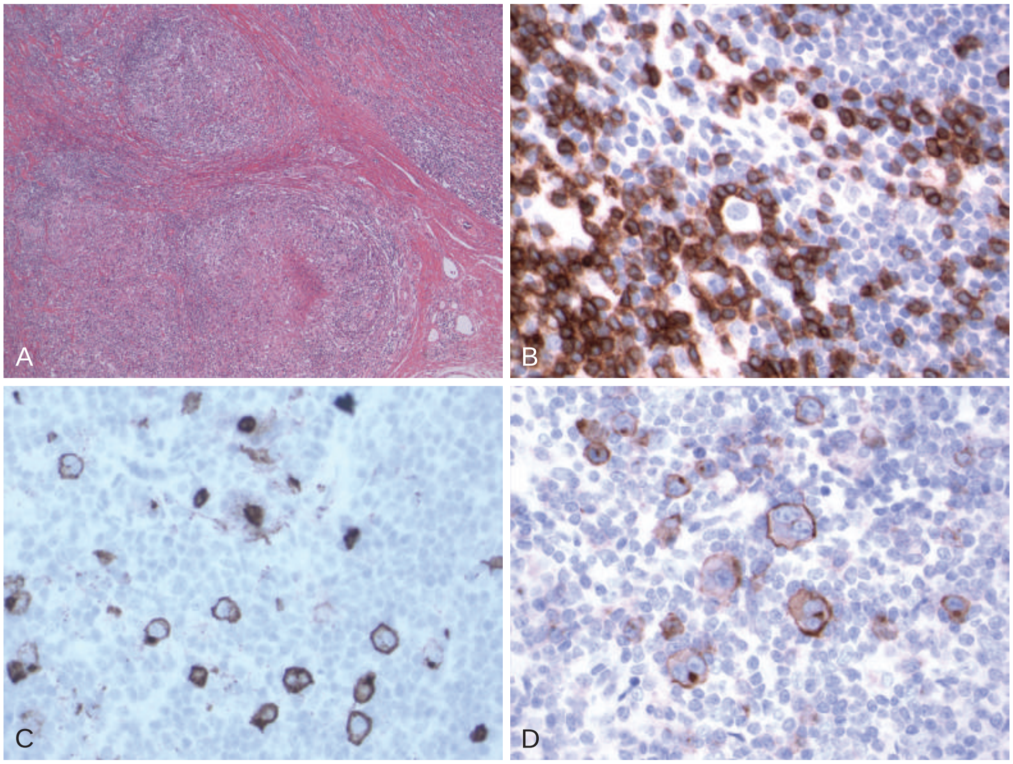

Figure 45-30 Nodular sclerosis Hodgkin lymphoma showing prominent fibrotic bands (**A**) and rimming of characteristic Reed-Sternberg cells by CD57+ lymphocytes (**B**). The Reed-Sternberg elements show cell membrane and Golgi expression of CD15 (**C**) and CD30 (**D**).

physiologic counterpart, together with its otherwise noncommittal immunophenotype, initiated a search to reveal the true nature of the H-RS cell, and molecular studies have assisted substantially in these efforts (see later).

Substantial insights into HL pathogenesis have come from single-cell studies showing that L&H and the vast majority of H-RS cells are B cells and clonal in nature.[427] These neoplastic H-RS cells seem to be of germinal center B-cell derivation,[428] with 96% featuring clonal *IG* rearrangements but lacking *IG* gene transcripts because of defects in *IG* gene regulatory elements. These findings apply in both Epstein-Barr virus (EBV)-associated and EBV-unassociated HL.[429] L&H cells express BCL6, in keeping with a germinal center differentiation stage, whereas H-RS cells express CD138 (syndecan), more in keeping with a postgerminal center differentiation stage.[430] Single-cell studies of H-RS cells have shown clonal *IG* gene rearrangements supporting a B-cell phenotype in 95% of cases. Even the 15% of HL with H-RS cells expressing T-cell markers generally contain clonal *IG* gene rearrangements and, overall, only 1% to 2% of classic HL cases have clonal *TCR* gene rearrangements.[431-434] Thus T-cell marker expression is generally considered aberrant in H-RS cells.

Both classic and nodular lymphocyte-predominance HL (NLPHL) are characterized by chromosomal instability, which is manifest by extremely complex cytogenetic aberrations in the neoplastic cells.[435] Molecular cytogenetic studies typically demonstrate chromosomal aberrations in the hyperdiploid range.[436]

Nodular Lymphocyte-Predominance Hodgkin Lymphoma. The observation that NLPHL corresponds to germinal center stage B-cell differentiation is in keeping with the finding of recurrent *IGH-BCL6* fusions, resulting from t(3;14)(q27;q32).[437] HL karyotyping evaluations have not revealed IGH region 14q32 rearrangements in NLPHL; these aberrations are undoubtedly being masked by the overall complexity of the HL karyotypes, with the recurring t(3;14) being cytogenetically cryptic.[438] However, molecular cytogenetic studies have demonstrated *BCL6* rearrangements in almost 50% of NLPHL.[437,439-441] CGH analyses in NLPHL have underscored the genomic complexity of these tumors, with frequent copy number gains involving chromosomes 1, 2q, 3, 4q, 5q, 6, 8q, 11q, 12q, and X and copy number losses for chromosome 17.[442] The copy number gains involving 2q, 4q, 5q, 6, and 11q are otherwise uncommon in hematologic neoplasia, and therefore might signify genomic aberrations of unique importance in the pathogenesis of NLPHL.

Classic Hodgkin Lymphoma. In classic HL, gains of 2p and 9p are identified in 30% to 50% of cases and 12q aberrations are also noted.[443] FISH studies have confirmed that the aberrations involve the locus of the *JAK2* gene at 9p23-24[444] and *MDM2* at 12q14-15.[445,446] The 2p copy number gains, which involve the *REL* gene (encoding one of the five subunits of NF-κB), might contribute to constitutive NF-κB activation, which is a hallmark of HL.[447]

Evaluations of *IG* gene aberrations in classic HL have demonstrated *IGH, IGL,* and *IGK* rearrangements in 17%, 3%, and 1% of cases, respectively.[448] These rearrangements juxtaposed the *IG* loci with genes at 2p16 *(REL),* 3q27 *(BCL6),* 8q24 *(MYC),* 14q24, 16p13.1, 17q12, and 19q13.2 *(BCL3-RELB)*. *IGH-BCL6* fusions were found in 4 of 70 cases, although a previous study had not shown such events in 40 cases of nodular sclerosis and mixed cellularity classic HL.[441] t(14;18), resulting in the typical *IGH-BCL2* fusion, has been reported in one case of follicular lymphoma evolving into Hodgkin lymphoma.[449]

Non-Hodgkin Lymphoma

Non-Hodgkin lymphomas (NHLs) account for 7% to 10% of all pediatric malignancies. Cytogenetic and molecular genetic aberrations have been studied extensively in adult NHL, but there have been few comparable studies in pediatric NHL, with the exception of well-characterized *MYC* gene rearrangements in Burkitt lymphomas. Most pediatric NHLs belong to one of four overall categories: Burkitt (40%), lymphoblastic (30%), large B-cell (20%), and anaplastic large cell (10%). Cytogenetic hallmarks include *MYC* rearrangements in Burkitt lymphomas, 14q11 rearrangements in T-cell lymphoblastic leukemia and lymphomas, and *ALK* rearrangements in anaplastic large cell lymphomas.[450] The proposed WHO classification of lymphoid neoplasms incorporates the Revised European American Lymphoma (REAL) classification, which takes into account morphologic, immunophenotype, genetic, and clinical features. Notably, there can be marked differences in molecular mechanisms and clinical response when comparing pediatric with adult forms of the same NHL subtype. For example, adult large B-cell lymphomas are heterogeneous entities, many of which have a phenotype suggesting follicular center cell origin and containing *IGH* region translocations that upregulate the *BCL2* or *BCL6* genes. Pediatric large B-cell lymphomas, by contrast, generally have gene expression and immunophenotypic features of germinal center cell origin and lack *IGH-BCL2* rearrangements but have a better prognosis than adult cases.[451] Lymphoblastic lymphomas are on a biologic continuum with ALL, and the biology of this subtype is discussed in the later section on ALL.

Burkitt Lymphoma. The cytogenetic underpinnings of Burkitt lymphoma were first reported in 1972, when Manolov and Manolova described aberrations of the chromosome 14 long arm[452]; these were later shown to represent t(8;14)(q24;q32), resulting in rearrangement of the *IGH* gene with the chromosome 8q *MYC* oncogene region.[14,453] *IGH-MYC* rearrangements are found in approximately 80% of Burkitt lymphomas, irrespective of whether the tumors are EBV positive or EBV negative and whether they are sporadic or arise in an endemic region.[454] However, *IGH-MYC* rearrangement can be found rarely in diffuse large cell lymphomas, so this cytogenetic aberration is not diagnostic of Burkitt lymphoma. Alternative cytogenetic mechanisms dysregulate *MYC* transcription by juxtaposing *MYC* with the κ or λ *IG*

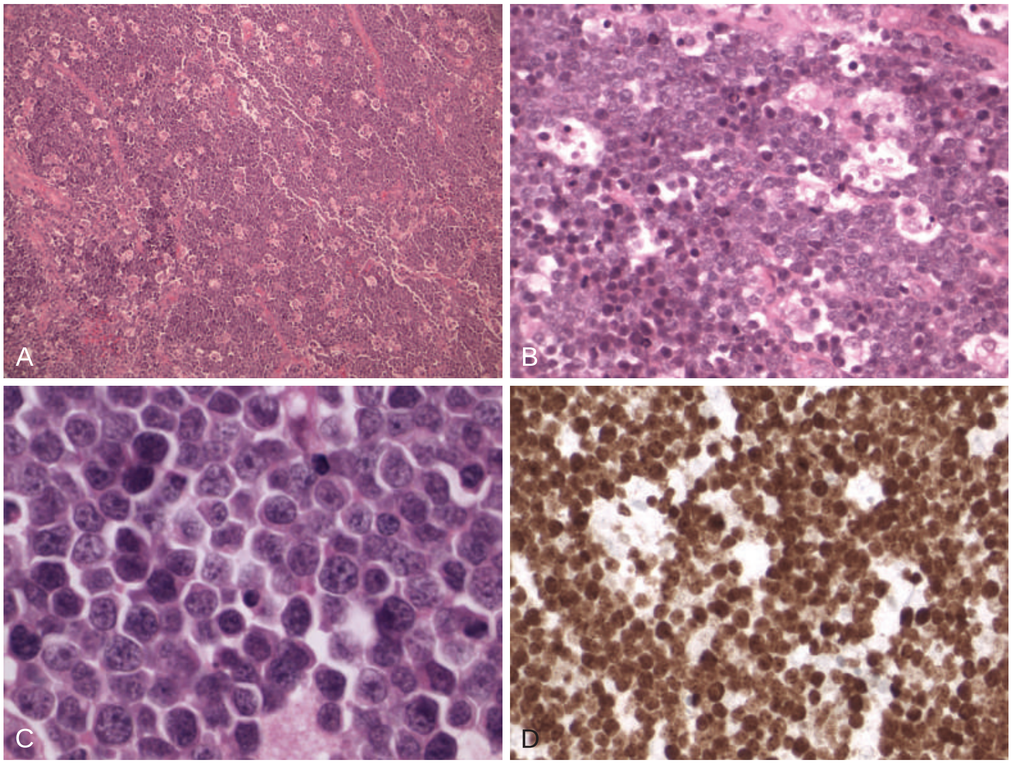

Figure 45-31 Burkitt lymphoma low-power (**A**) and intermediate-power (**B**) views show starry sky appearance resulting from engulfment of cellular apoptotic debris by interspersed macrophages; higher-power view shows typical squaring off of cells from one another (**C**). The Ki-67 proliferation marker is expressed strongly by almost 100% of the lymphoma cells (**D**).

gene loci.[455] The κ and λ *IG* rearrangements result from t(2;8) and t(8;22), which are found in 5% and 15% of Burkitt lymphomas, respectively.

Burkitt lymphoma has a characteristic cytomorphologic appearance in which the cells are squared off from each other. A starry sky appearance results from engulfment of cellular apoptotic debris by interspersed macrophages, whereas the actual neoplastic cells have relatively uniform intermediate-sized nuclei and exhibit at least a 99% proliferative index, as demonstrated by immunostaining for Ki-67 (Fig. 45-31). *MYC* region genomic breakpoints vary between the endemic and sporadic types of Burkitt lymphoma. The breakpoints in endemic Burkitt lymphomas can be as much as 300 kb upstream or downstream of the *MYC* coding sequences, whereas those in sporadic Burkitt lymphomas typically involve the first exon or first intron of *MYC*.[455] Despite this considerable variability in genomic breakpoint location, all the rearrangements serve the purpose of dysregulating *MYC* expression and can be detected by a simple dual-color *MYC* break-apart FISH assay, in which the probe components are more than 300 kb upstream and downstream of *MYC*, thereby flanking most *MYC* region translocation breakpoints (Fig. 45-32). Secondary cytogenetic aberrations occur in conjunction with *MYC* translocations in some Burkitt lymphomas. Several of these, particularly 1q duplication and 13q deletion, may be associated with worse prognosis, although the numbers of patients studied have been few.[456]

Follicular Lymphoma. Follicular lymphoma is uncommon in childhood. When diagnosed, it more commonly affects boys and presents as localized disease in lymph nodes or tonsils. Such cases generally lack *BCL2* rearrangement and BCL2 expression.[457]

Diffuse Large B-Cell Lymphoma. Diffuse large B-cell lymphomas may be categorized into germinal center, activated B-cell–like, or primary mediastinal types. In children, diffuse large B-cell lymphomas are frequently of the germinal center type and associated with an overall better prognosis than for adults. Despite the germinal center phenotype, however, diffuse large B-cell lymphomas in children generally lack t(14;18), which is seen in a proportion of adult cases of this subtype.[451]

Anaplastic Large Cell Lymphoma. ALCLs are predominantly mature T-cell disorders with excellent prognosis in the pediatric population.[458] ALCL is a diagnostic pitfall for the pathologist, although the morphology is uniquely different from all other hematopoietic malignancies. The cells often appear relatively cohesive and have abundant cytoplasm, so they may mimic an epithelial neoplasm more so than suggesting a lymphoid one; ALCLs, furthermore, classically express epithelial membrane antigen (Fig. 45-33). The cells typically react for CD30 and often also express the anaplastic ALK receptor tyrosine kinase protein (see Fig. 45-33), which is constitutively activated by oncogenic fusions to various partners containing

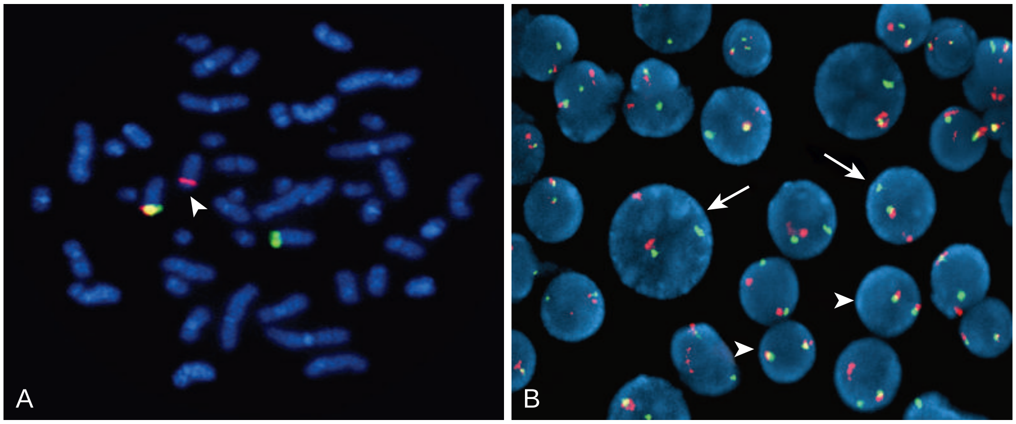

Figure 45-32 Burkitt lymphoma fluorescence in situ hybridization (FISH) using *MYC* break-apart probe in metaphase cell (**A**) and interphase cells (**B**) from malignant pleural effusion in a 5-year-old child. The metaphase cell shows a t(8;14), indicative of *IGH-MYC* fusion, in which the centromeric *(red)* and telomeric *(green)* FISH probe components localize to chromosomes 8 *(arrowhead)* and 14, respectively. The interphase cells include *MYC*-rearranged Burkitt lymphoma *(arrows)* and reactive nonneoplastic lymphocytes *(arrowheads)*.

dimerization domains. The most common of these fusions, found in 80% of ALCLs, results from t(2;5) fusing the 3′ end of the *ALK* gene to the 5′ end of *NPM*,[459] and serving as a useful diagnostic marker of ALCL. Less common *ALK* fusions involve *ATIC, TFG, CLTC, TPM3, TPM4,* and *MSN.*[116,460-462] ALCL occurs in a cutaneous form, which is usually ALK-negative, whereas nodal and extranodal systemic forms express ALK in about 95% of pediatric cases. ALK encodes a receptor tyrosine kinase that is normally expressed in neural cells of the intestine, testis, and brain but not in nonneoplastic lymphoid cells. A smaller number of pediatric ALCLs are B-cell disorders, and these cases often feature oncogenic fusions of the *ALK* and clathrin genes.[458] The pattern of ALK immunostaining in association with *ALK-NPM1 (NPM)* fusion resulting from t(2;5) is one of nuclear and cytoplasmic staining, with distinctive nucleolar accentuation (see Fig. 45-33). Similarly, other ALK oncoproteins are associated with distinctive immunostaining patterns, including the granular cytoplasmic staining with clathrin-*ALK*

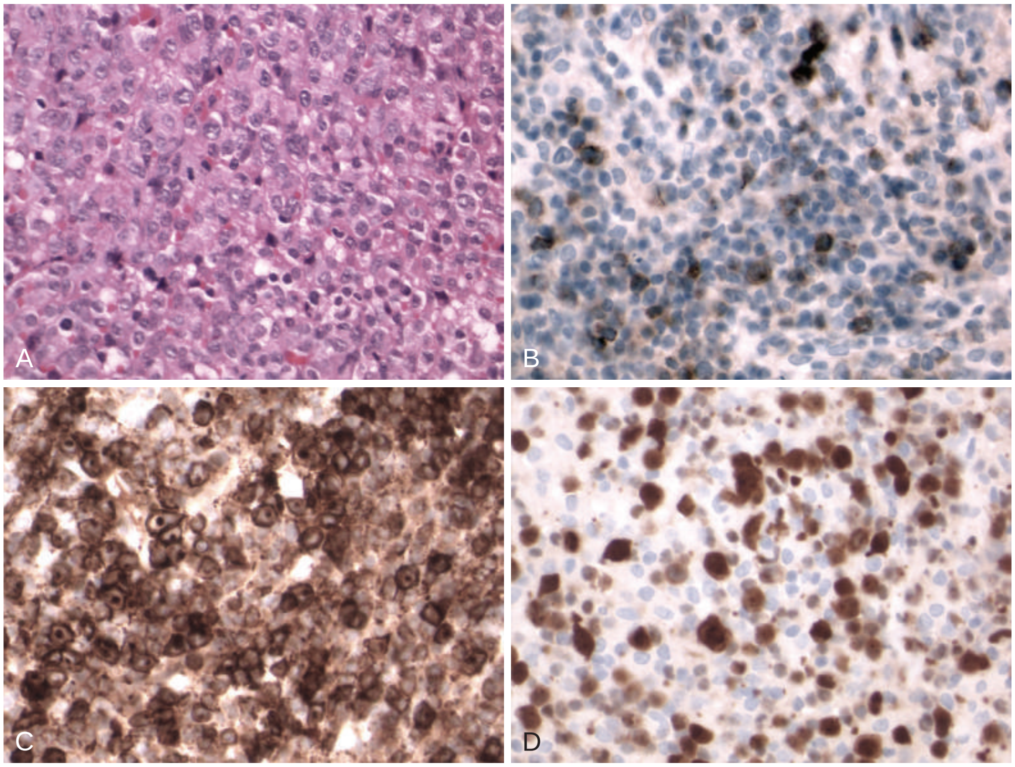

Figure 45-33 In anaplastic large cell lymphoma, histologic features include cohesive cells with abundant cytoplasm, imparting an epithelioid appearance (**A**). Immunohistochemical features include patchy expression of epithelial membrane antigen (**B**), strong membrane and Golgi reactivity for CD30 (**C**), and anaplastic lymphoma kinase immunostain (**D**), which in this t(2;5)-positive case shows reactivity in both nuclear and cytoplasmic compartments.

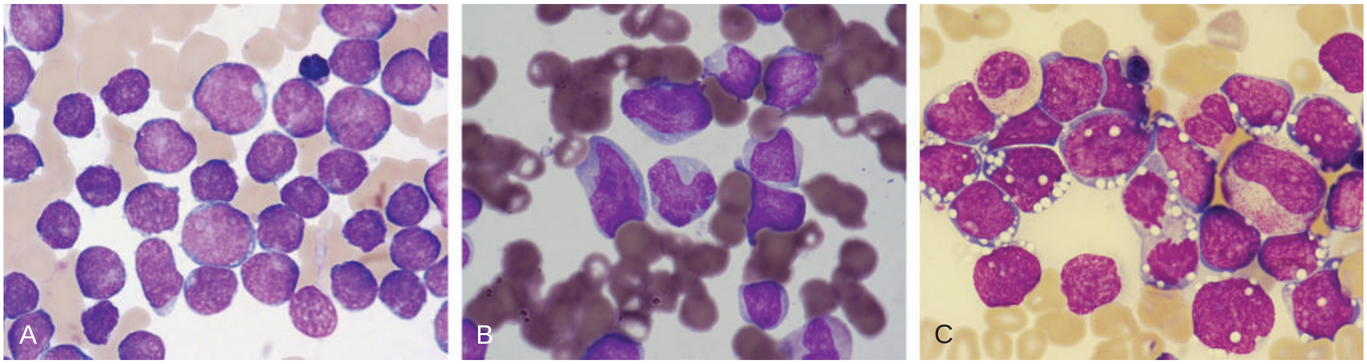

Figure 45-34 Acute lymphoblastic leukemia historical French-American-British subtypes. **A,** L1, with smaller, more uniform cells and a high nuclear-to-cytoplasmic ratio. **B,** L2, with somewhat larger cells, and a more varied nuclear-to-cytoplasmic ratio. **C,** L3 (Burkitt), with larger cells and numerous vacuoles. *(Courtesy Marybeth Helfrich, Children's Hospital of Philadelphia.)*

rearrangement, diffuse cytoplasmic staining in the case of *TFG-ALK* and *ATIC-ALK* rearrangements, diffuse membranous and cytoplasmic staining with *TPM3-ALK,* and nuclear membrane staining with *RANBP2-ALK* rearrangement. However, *ALK* staining is not specific for ALCL. As noted earlier (see "Mesenchymal Neoplasms: Soft Tissue and Bone Tumors"), *ALK* oncoprotein expression is also characteristic of inflammatory myofibroblastic tumors. In the hematopoietic group of neoplasms, *ALK* reactivity as an indicator of clathrin-*ALK* fusion oncoproteins also has been described for large B-cell neoplasms.[463]

Subcutaneous Panniculitic T-Cell Lymphoma. This lymphoma subtype produces firm nodular infiltrates of skin, which occasionally progress to ulceration. These infiltrates are generally confined to subcutaneous fat, with sparing of the overlying dermis and epidermis. Other helpful histologic features include evidence of cellular atypia and characteristic rimming of adipocytes by the neoplastic cells. In the presence of such histologic features, demonstration of *TCR* gene rearrangement confirms the diagnosis.

Hepatosplenic T-Cell Lymphoma. This is an extranodal lymphoma of γδ T-cells. Clinical presentation is generally that of hepatosplenomegaly without accompanying lymphadenopathy, although other anatomic locations, typically also extranodal, may be involved. The histologic findings on a diagnostic liver biopsy may mimic those of hepatitis. The neoplastic infiltrate is sinusoidal in distribution, sparing the portal tracts. Expansion of the sinusoids by a monotonous lymphoid population should prompt consideration of this diagnosis. Demonstration of *TCRγ* gene rearrangement is confirmatory, and another helpful genetic feature, seen in many cases, is the cytogenetic finding of isochromosome 7q,[464,465] often accompanied by trisomy 8.[466] Development of hemophagocytic syndrome may ensue and heralds poor outcome.

Acute Lymphoblastic Leukemia

ALL, the most common childhood cancer, comprises a heterogeneous spectrum of diseases characterized by clonal proliferations of lymphoid precursor cells at varying stages of development but traditionally assigned to one of three FAB subtypes (Fig. 45-34). Classic teaching has been that the ALL cell of origin is a committed lymphoid progenitor, with the particular stage of lymphoid differentiation during which the transforming event occurs being highly variable. However, although controversial, studies have suggested that leukemic transformation in ALL may occur in a more primitive stem cell.[467] Irrespective of whether the nonneoplastic cell that is targeted for transformation is a committed lymphoid cell, the major demonstrable immunophenotypic ALL subsets are B- or, less often, T-cell precursors. About 85% of cases are B-cell precursor ALL, whereas 10% to 15% are T-cell precursors. Expression of the CD10 B-cell differentiation antigen characterizes common ALL, which is the most frequent form of childhood ALL (Fig. 45-35). By contrast, the vast majority of cases of ALL in infants (defined as <1 year old) are negative for CD10 cell surface antigen expression. Burkitt leukemias, with their characteristic phenotype of mature B-cell surface IG expression, account for only approximately 0.5% of cases of childhood ALL.[2] Heterogeneous primary molecular genetic aberrations cause leukemic transformation and are associated with distinctive biologic properties and clinical features in the different major subtypes of childhood ALL.

Intralocus *IG* and *TCR* Gene Rearrangements. Historically, the patterns of *IG* and *TCR* gene rearrangements in pediatric ALL have provided insights into the ordered hierarchy of rearrangements of *IG* and *TCR* genes in lymphoid maturation.[468,469] The diversity of rearrangements of variable (V), diversity (D), and joining (J) segments generated within these loci in the human immune response affords a signature of unique markers of lineage, clonality, and maturation stage in each ALL. This is particularly true because *TCR* gene rearrangements can be identified even in precursor B-cell ALL and, conversely, *IG* gene rearrangements can occur in T-cell ALL. Furthermore, *IG* and *TCR* gene rearrangements are not static but can evolve after leukemic transformation via subclone progression (e.g., from D-J to complete V[D]J assembly or from *IGH* to *IGK* light-chain gene

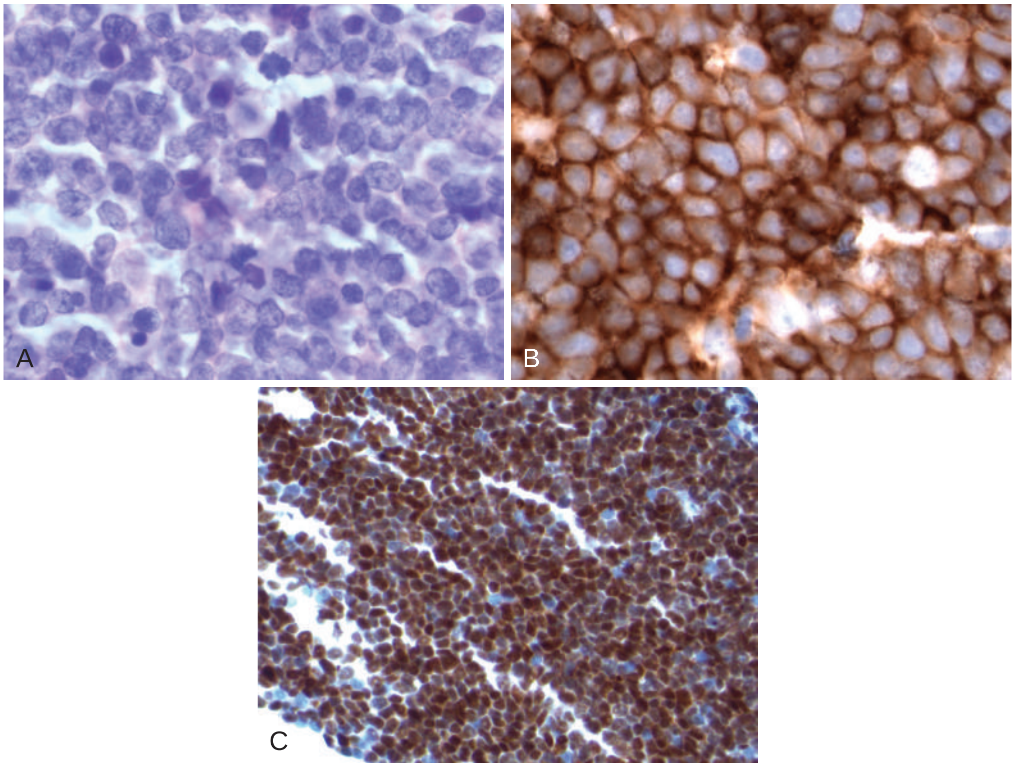

Figure 45-35 **A,** Lymph node infiltration by pre–B-cell acute lymphoblastic leukemia lacking any appreciable cytoplasm and with slightly irregular nuclear contours. The nuclear chromatin is finely dispersed. This case shows typical strong immunohistochemical staining for CD10 (common ALL antigen) (**B**) and nuclear TdT expression (**C**).

rearrangement).[470] However, because of the related nature of the ALL subclones in any given patient, *IG* and *TCR* gene rearrangements can serve as sensitive markers for detecting minimal residual disease and predicting relapse.[471,472] Comparable sensitivity in detecting minimal residual disease can also be achieved by multiparameter flow cytometric analysis of various antigens, expression of which is restricted to leukemic cells.[473]

One study has suggested that about 25% of cases of B-cell precursor ALL undergo transformation at the pro–B- to pre–B-cell stage, characterized by cytoplasmic μ expression and assembly of the pre–B-cell receptor complex. The pre–B-cell differentiation stage is a critical cell survival checkpoint because most normal B cells cannot form intact immunoglobulin and instead undergo apoptosis.[474] The subset of ALL transitioning from pro-B to pre-B cells exhibits steroid sensitivity.[474]

Interlocus *IG* and *TCR* Gene Rearrangements and Translocations. Not only do *IG* and *TCR* genes undergo intralocus rearrangements, but their aberrant recombination also causes interlocus chromosomal translocations and inversions. These chromosomal rearrangements can create hybrid antigen receptor genes when two different antigen receptor loci recombine with each other. A small fraction of pediatric ALLs of B- or T-cell lineage harbor these hybrid antigen receptor gene rearrangements.[475] Alternatively, chromosomal rearrangements can juxtapose transformation-inducing non-*IG*/non-*TCR* genes with the regulatory enhancer and promoter elements of *IG* or *TCR* genes as a second type of interlocus rearrangement. V(D)J recombination errors of this type,[476] causing transcriptional upregulation of the juxtaposed non-*IG*/non-*TCR* genes, occur in subsets of pediatric ALL, especially in T-cell ALL.

SIL-SCL rearrangements and *SCL* translocations disrupting *TCR* genes are present in approximately 30% of cases of T-cell ALL overall. The *SCL (TAL1)* gene at chromosome band 1p34, which encodes a basic helix-loop-helix (bHLH) transcription factor, was first discovered at the t(1;14)(p34;q11) breakpoint junction involving the *TCRαδ* locus in a stem cell leukemia.[477] Other T-cell ALL cases harbor a different type of *SCL* rearrangement, in which illegitimate V(D)J recombinase activity at recombinase-like signal sequences in *SCL* and *SIL* (SCL-interrupting locus) creates an interstitial deletion on chromosome 1p. The *SIL-SCL* rearrangement causes aberrant regulation of *SCL* expression by the *SIL* promoter.[478,479] The t(1;3)(p34;p21) translocation, fusing *SCL* and the nonantigen receptor gene *TCTA,* is mediated by a similar mechanism of illegitimate V(D)J recombinase activity.[480]

In other recurrent translocations in T-cell ALL, various oncogenic transcription factor genes such as *LMO1, LMO2, LYL1,* and *HOX11 (TLX1)* are fused to different *TCR* loci, leading to unscheduled expression of the relevant transcription factor protein.[78] *LMO2* translocations occur in 10% to 20% of T-cell ALLs. Microarray experiments have shown that oncogenic transcription factor genes can be overexpressed in T-cell ALL subsets at distinct maturation stages and are prognostic markers, even when conventional karyotyping studies do not reveal such translocations.[481] For example, dual-color FISH analysis and a ligation-mediated PCR assay have revealed cryptic translocations of the *HOX11 (TLX1)* gene with the *TCRD* locus in most T-cell ALLs in which there was high *HOX11* expression.[482] This finding is particularly intriguing because high-level *HOX11* expression in T-cell ALL correlates with a favorable prognosis.[482] In addition, the CD34–, CD1a+, CD4/CD8 double-positive cortical immunophenotype has suggested that the immature β pre-αβ stage of maturation arrest is associated with high *HOX11* expression.[482] These results have demonstrated that molecular cytogenetics can provide more sensitive and consistent evaluations of ALL oncogenic mechanisms compared with conventional karyotyping.

The most recently identified example of a recurrent *TCR* gene translocation in T-cell ALL is t(6;7)(q23;q34), which juxtaposes the *MYB* gene at chromosome band 6q23 with the *TRB* locus.[483] Although the subtelomeric locations of *TRB* and *MYB* precluded recognition of this rearrangement by conventional karyotyping, the rearrangement is readily detectable with methods such as FISH, Southern blotting, or PCR assay.[483] Molecular cloning has revealed a heptamer-like sequence adjacent to the translocation breakpoint in the *MYB* gene, consistent with illegitimate V(D)J recombinase activity as the mechanism of this translocation. In this translocation, placement of *MYB* in close proximity with the *TRB* enhancer regulatory element results in aberrant expression of the translocated *MYB* allele and a gene expression signature consistent with upregulation of various proliferation and mitosis genes.[483] t(6;7) is of clinical interest because the median age of the patients is only 2.2 years old,[483] which is unusual because T-cell ALL is otherwise infrequent in young children. This translocation exemplifies the importance of cooperating mutations in pediatric ALL pathogenesis, because many cases of T-cell ALL with t(6;7) also contain *NOTCH1* mutations and *CDNK2A (p16^{ARF})* deletions.[483] Dysregulated *MYB* expression in T-cell ALL of the very young can also result from gene duplications, as demonstrated by molecular and fiber FISH analyses of *MYB* gene copy number.[483] *MYB* is expressed at high levels in the thymus, and it has been suggested that *MYB* deregulation results from sustained rather than ectopic expression[483]; in that sense, it differs from several other transcription factor oncogenes disrupted in T-cell ALL.

Another T-cell ALL translocation, t(7;9)(q34;q34.3), fuses *TCRB* to the gene encoding the NOTCH1 transmembrane receptor protein, which regulates T-cell maturation.[484] This is an extremely uncommon translocation, occurring in less than 1% of cases of T-cell ALL; however, it was more recently shown that at least 50% of T-cell ALL cases have activating intragenic *NOTCH1* mutations, including cases with other major translocations.[485] Activating *NOTCH1* mutations affecting the extracellular heterodimerization domain result in ligand-independent activation, whereas those affecting the intracellular PEST (proline [P], glutamic acid [E], serine [S], and threonine [T]) domain cause increased stability of the protein. The mutant NOTCH1 protein is a potential therapeutic target of gamma secretase inhibitors, which can block NOTCH1-mediated signaling in T-cell ALL.[485] Inhibition of NOTCH1 signaling by gamma secretase inhibitors is associated with upregulation of the phosphatase and tensin homologue (PTEN) tumor suppressor protein.[486] Although a phase I/II clinical trial of γ secretase inhibition did not show efficacy in T-cell ALL, the observed drug resistance could be attributed in part to *PTEN* gene deletion. PTEN inhibits the PI3K/AKT pathway, and loss of PTEN function is associated with AKT activation and a switch in oncogene addiction from the NOTCH1 to the AKT signaling pathway. *PTEN* mutational analyses have indicated that a subset of T-cell ALL (8%) harbors mutations of this gene at diagnosis, independently or together with *NOTCH1* mutations, or, alternatively, the *PTEN* mutations can be secondary alterations associated with disease progression.

Analogous to the interlocus *TCR* gene rearrangements in T-cell ALL, molecular approaches have also been revealing in precursor B-cell ALL, and recurrent novel translocations that juxtapose *IG* genes with oncogenic transcription factor genes have been identified and characterized. One recent example is the t(6;14)(p22.3;q32.22) that fuses the joining region of the *IGH* gene with the gene encoding *ID4*.[487] The translocation causes overexpression of the ID4 bHLH transcription factor and is associated with a CD19+, CD10+, HLADR+, TdT+ common precursor B-cell immunophenotype, low-risk clinical features, and favorable prognosis.[487] This translocation is often accompanied by deletion of the chromosome 9p genes *CDKNA* and *PAX5,* suggesting cooperativity of these alterations in the pathogenesis of this disease.[487] Another example of *IGH* gene rearrangement is seen with t(5;14), which occurs in less than 1% of cases of precursor B-cell ALL. t(5;14) places the *IL3* gene under transcriptional regulatory control of the *IGH* locus, resulting in the hallmark clinical feature of peripheral eosinophilia associated with this translocation.[78]

In the rare entity of Burkitt leukemia, expression of *MYC* is altered by translocation into the *IGH* or, less often, the *IGK* or *IGL* light chain locus, but oncogenic transformation at a more mature B-cell developmental stage is reflected by IgM expression on the leukemia cell surface.

Another area of interest has been in the application of molecular approaches to determine the temporal origins of ALL-specific *IG, TCR,* or transcription factor gene rearrangements. Studies of ALL in monozygous twins and studies of ALL molecular cytogenetic aberrations in neonatal Guthrie blood spots have been useful in indicating whether various ALL subtypes arise before or after birth. In the case of T-cell ALL there is recent evidence that the disease is not initiated in utero or that the clonal aberration in most cases is present below the detection sensitivity of PCR at the time of birth.[488] However, these findings are in contrast to PCR-based findings on *TCR* gene rearrangements in two other studies, which were consistent with a prenatal origin of T-cell ALL.[489,490] In one of three cases of T-cell ALL with *NOTCH1* mutation, the *NOTCH1* mutation was detectable in neonatal

Guthrie blood spot DNA, whereas the *SIL-SCL* rearrangement in the same case occurred later as a postnatal event.[491] Thus ALL-specific *IG* and *TCR* gene rearrangements and/or leukemia-associated transcription factor alterations in at least some T-cell ALLs are in utero events, even though the latency to clinically evident disease can be protracted. Most subtypes of precursor B-cell ALL also initiate in utero (see later).

Chromosomal Translocations Not Involving *IG* or *TCR* Genes

TEL-AML1 ***Translocations.*** Several of the major recurrent chromosomal translocations in pediatric ALL do not involve *IG, TCR,* or other genes with obvious V(D)J recombination signal sequences. The most frequent chromosomal translocation in all pediatric cancers[492] is t(12;21), which is present in 25% of cases of CD10+ B-cell lineage common ALL. This translocation is cryptic by conventional Giemsa-banded karyotyping and must be detected by FISH or molecular analyses. The leukemogenic fusion of the *TEL (ETV6)* gene from chromosome band 12p13 to the *RUNX1 (AML1)* gene from chromosome band 21q21 is associated with recruitment of complexes containing histone deacetylases to AML1 target genes, causing aberrant transcriptional repression.[493] There is evidence that t(12;21) occurs as an in utero event[493,494] in cells that are at a stem or B-cell progenitor developmental stage, before *IG* and *TCR* gene rearrangement. Rearrangement of the immune receptor loci occurs after the initiating *TEL-AML1* translocation.[495] *TEL-AML1* translocations occur in cord blood specimens of healthy neonates at 100 times the incidence of *TEL-AML1* translocations in leukemia, indicating that progression to overt ALL is rare in *TEL-AML1*–positive cells. Similarly, the low concordance (5% to 10%) of *TEL-AML1* leukemia in monozygous twins has suggested that postnatal mutations are required for transformation of *TEL-AML1*–positive progenitor cells into frank ALL.[496] These additional mutations often include loss of the normal *TEL* allele.[492] The *TEL-AML1* translocation is associated with a favorable outcome.[492]

TCF3 (E2A) ***Gene Translocations.*** t(1;19) occurs in approximately 5% of cases of pediatric ALL, fusing the *E2A* gene on chromosome band 19p13.3 to the *PBX1* gene on chromosome band 1q23. This translocation is most often found in cIg+ (pre-B) rather than cIg– B-cell precursor ALL (25% vs. <1% of cases).[497] t(1;19)(q23;p13.3) is associated with a poor prognosis in the cIg+ (pre-B) cases. *E2A* is a critical B-cell developmental gene that encodes an IG enhancer binding protein. The *E2A* gene encodes three different bHLH transcription factors via alternative splicing of the *E2A* transcript. The *PBX1* gene product is a homeobox transcription factor, and t(1;19) results in the production of a fusion oncoprotein from the der(19) chromosome, in which the DNA-binding domain of PBX1 replaces the carboxyl DNA-binding bHLH domain of *E2A*. t(1;19)(q23;p13.3) is detectable in neonatal Guthrie cards in only about 10% of cases, suggesting a postnatal origin, and is different in that sense from the other major subtypes of pediatric ALL.[496,498] Split-signal FISH using different color probes for the *E2A* and *PBX1* genes is a useful tool for the definitive identification of this translocation,[499] which is otherwise indistinguishable karyotypically from a different translocation sharing breakpoints in the same chromosome bands. The alternate form of t(1;19)(q23;p13.3) in ALL does not disrupt the *TCF3 (E2A)* and *PBX1* genes but rather fuses the *DAZAP1* RNA-binding protein gene on chromosome band 19p13.3 to the *MEF2D* DNA-binding protein gene on chromosome band 1q23.[500]

A rare t(17;19) *E2A* variant occurs in 1% of cases of childhood B-cell precursor ALL, which creates a *E2A-HLF* (hepatic leukemia factor) fusion gene. t(17;19) is associated with coagulation abnormalities and has a poor prognosis.[497]

BCR-ABL1 ***Translocations.*** About 5% of cases of B-cell precursor ALL harbor t(9;22)(q34;q11), fusing *BCR* with the *ABL1* tyrosine kinase gene. This translocation is associated with the clinically high-risk features of older age and high white blood cell count and an especially grim prognosis.[78] However, emerging data have indicated that the molecularly targeted ABL kinase inhibitor imatinib mesylate has clinical activity against BCR-ABL+ ALL in the pediatric population as well as in adults.[501]

MLL (KMT2A) ***Translocations.*** Leukemia is the second most common malignancy during infancy. The infant cases comprise 2.5% to 5% of ALLs and 6% to 14% of AMLs in the pediatric population overall. The annual incidence of infant ALL and AML is 19 and 10/million, respectively.[133,502] Infant leukemia is a special subtype of pediatric leukemia characterized by chromosomal translocations that generate fusion oncoproteins, and 75% to 80% of cases of infant ALL and myelomonocytic-monoblastic AMLs feature balanced chromosomal translocations of the *MLL* gene at chromosome band 11q23.[503] Cases of ALL with *MLL* translocations often coexpress myeloid-associated surface antigens, especially CD15. Additionally, *MLL* translocations can be associated with true bilineal acute leukemias, in which there are separate lymphoid and myeloid cell populations (Fig. 45-36), and acute leukemias with *MLL* translocations can undergo lineage switch. *MLL* translocations are also found in secondary leukemias in patients treated with chemotherapeutic TOP2 poisons.[63] The *MLL* gene encodes a large complex oncoprotein that regulates transcription[15,504-507] and has regional amino acid similarity to that of *Drosophila* Trithorax (TRX).[132,505,506] Whereas TRX is involved in the maintenance of expression of homeotic gene complexes during embryonic development,[508] of relevance to leukemia, MLL maintains *HOX* gene expression during hematopoiesis.[509] *MLL* translocations in various types of pediatric leukemia disrupt an 8.3-kb breakpoint cluster region and fuse the 5′ portion of *MLL* with one of more than 80 different partner genes, generating diverse leukemogenic fusion oncoproteins containing the amino-terminus of MLL and the carboxyl-terminus of the partner protein.[49,68,86,510]

The native MLL protein has complex transcriptional regulatory functions. MLL undergoes proteolytic cleavage into amino- and carboxyl-segments that reassociate with one another in a multiprotein complex,[511-513]

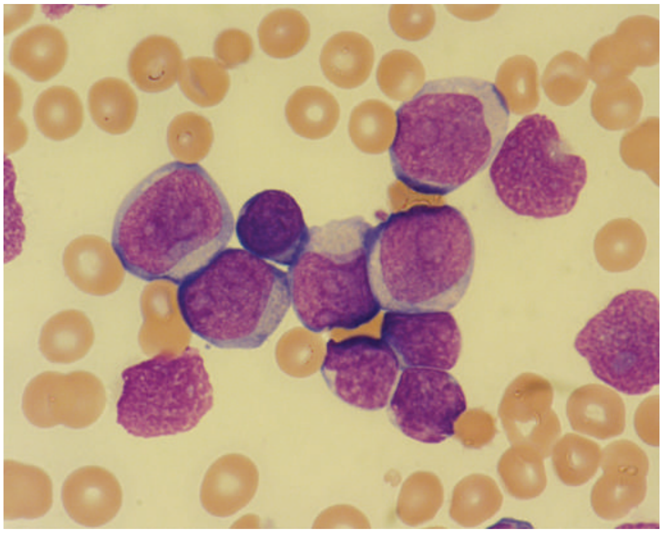

Figure 45-36 Infant bilineal acute leukemia with *MLL* translocation showing lymphoid and myeloid blast cell populations. *(Courtesy Marybeth Helfrich, Children's Hospital of Philadelphia.)*

regulating *HOX* gene expression and epigenetic modifications of nucleosomes and histones.[514] The MLL chimeric oncoproteins resulting from the various *MLL* partner gene rearrangements lack the MLL proteolytic cleavage site and affect leukemogenic transformation through transcriptional dysregulation of MLL target genes.[515] Although many *MLL* partner genes themselves encode important proteins in transcriptional regulation or cell signaling pathways,[132,515] it is unclear how the partner gene disruption contributes to leukemogenesis mediated by *MLL* translocations.

Of the approximately 75% of infant ALL cases with *MLL* translocations, 70% and 13% of cases, respectively, involve the *AF4* (chromosome band 4q21) or *MLLT1 (ENL)* (chromosome band 19p13) transcription factor partner genes.[516] In AML, the partner genes of *MLL* are much more heterogeneous, including not only genes that encode nuclear transcription factors and transcriptional regulatory proteins but also cytoplasmic proteins and proteins in various cellular organelles.[49]

MLL translocations in infant leukemias exemplify another principle in the molecular cytogenetics of pediatric leukemias, namely, the association of host risk factors and gene-environment interactions with specific aberrations. The *MLL* translocation in infant leukemia is an in utero event, the origin of which has been established by the detection of identical nonconstitutional *MLL* rearrangements in leukemias of monozygous twins,[517,518] as well as the detection of *MLL* translocations in neonatal Guthrie blood spot DNA in nontwin cases.[519] Maternal-fetal exposure from consumption of food containing TOP2–interacting substances during pregnancy has been implicated as a risk factor in *MLL*-rearranged infant AML.[520] Moreover, an inactivating polymorphism in the gene encoding reduced nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidoreductase 1 (NQO1) increases the risk for all *MLL*-rearranged infant acute leukemias, especially cases with t(4;11) fusing *MLL* to *AF4*, which are primarily ALL.[521,522] The inactivating NQO1 polymorphism is a plausible host predisposition factor because the *p*-benzoquinone detoxified by NQO1 is a TOP2 poison[523] and four-base 5′ overhangs from TOP2 cleavage lend readily to DNA damage resolution by nonhomologous end joining (NHEJ) repair, features of which usually are evident at *MLL* translocation breakpoint junctions.[47,524] Infant leukemia risk is also modulated by a genetic polymorphism in the methylene tetrahydrofolate reductase *(MTHFR)* gene; it has been suggested that the resultant increased thymidine synthesis, which enhances DNA repair, protects against *MLL* translocations.[525]

Infant ALLs with *MLL* translocations are composed of CD10– early B progenitor cells, but they may have monocytic features and they typically express the myeloid-associated CD15 cell surface antigen.[133] Clinically, *MLL* translocations are important because of their unfavorable prognosis.[516] The *MLL* translocation in infant ALL is associated with a high leukemia burden manifest as marked hyperleukocytosis, massive hepatosplenomegaly, and meningeal involvement.[133] Neonatal leukemia (leukemia in the first month of life) has an annual incidence of 4.7/million live births[526] and comprises an important fraction of leukemia in infants. The clinical manifestations of neonatal leukemia include stillbirth, signs of leukemia at birth, or hematologic abnormalities preceding the leukemia diagnosis within a few weeks.[134] Clinical hallmarks include hepatosplenomegaly, hydrops, and polyhydramnios detected antenatally, and the archetypal marked hyperleukocytosis, hepatosplenomegaly, leukemia cutis, and CNS involvement detected after birth.[134] *MLL* translocations in infant and neonatal leukemia have significant adverse effects on response to treatment, especially in cases of early B-cell lineage ALL.[44,134,516,527] The substantial adverse impact of *MLL* translocations on prognosis places infant ALL at the extreme of pediatric ALL in which outcome is the poorest.[44,134,516,527] However, in AML, in which outcome is less favorable overall than in ALL, *MLL* translocations are not independent prognostic factors. Overexpression of *FLT3* mRNA is a common secondary alteration in infant ALL with *MLL* translocations.[528] In these cases, the *FLT3* gene may be mutated or upregulated and the FLT3 protein is highly expressed.[135,529,530]

There is limited copy number variation[531] and few additional mutations have been detected, (e.g., in *NRAS, KRAS, FLT3,* and *IKZF1*[532-534]) in *MLL*-R infant ALL beyond the translocations. In contrast, altered methylation, especially hypermethylation, is a very characteristic epigenetic aberration of *MLL*-rearranged(R) ALL and the altered methylation within different subsets of *MLL*-R ALL is similar.[535]

In a microarray analysis of an infant ALL cohort (n = 97) from the COG P9407 trial representing the largest group of infants to be studied in this manner, the most significant determinant of gene expression was infant age at diagnosis.[536] Remarkably, there was a very distinct change in the gene expression pattern at approximately 90 days of age, which also corresponds with the cutoff for worst outcome. Interestingly, whereas the gene expression signature of older infants was that of a more differentiated B-cell lineage precursor, in infants younger than 90 days of age, interleukin-related genes, heat-shock proteins, and human leukocyte antigen genes linked through IL13 and IL1A were more prominent, suggesting different pathways in leukemogenesis. In addition, outcome predictors beyond age and *MLL* rearrangement status were discovered that were highly predictive of event-free survival.

Molecular Aberrations in Subsets of Precursor B-Cell ALL Identified by Integration of Gene Expression Profiling, Analysis of Copy Number Alterations, and Genomic Sequencing. The subtypes of precursor B-cell ALL that are distinguishable by distinctive cytogenetic aberrations also are able to be distinguished by their gene expression profiles. Conversely, application of integrated genomic approaches including gene expression profiling, analysis of copy number alterations, and, later, next-generation sequencing has led to the identification of clusters of precursor B-cell ALL otherwise classified as "high-risk" by conventional definition (age, white blood cells, cytogenetics) encompassing subsets with well-recognized molecular cytogenetic aberrations, such as *KMT2A* gene rearrangements and t(1;19), but also subsets lacking molecular cytogenetic markers and subsets with particularly favorable or poor outcome. For example, a cluster with 94% 4-year relapse-free survival defined by high *AGAP1, CCNJ, CHST2/7, CLEC12A/B,* and *PTPRM* expression and intragenic *ERG* deletions was identified as well as a cluster with 9.4% 4-year relapse-free survival with high *BMPR1B, CRLF2, GPR110, GPR171, IGJ, LDB3,* and *MUC4* expression and *EBF1, NUP160-PTPRJ, IL3RA-CSF2RA, SLX4IP, ADD3, IKAROS/IKZF1,* and *VPREB1* deletions.[537] The genomic basis for the differences in gene expression were subsequently elucidated and continue to be elucidated by next-generation sequencing initiatives.

Through the application of sophisticated bioinformatics algorithms, the patterns of gene expression not only have been correlated with molecular cytogenetic subtypes, but they also have been used to develop classifiers that are predictive of minimal residual disease at the end of induction and the risk for relapse within cases characterized by the presence of, for example, *JAK* mutations, kinase signatures, *IKAROS/IKZF1* deletions, or t(1;19). Whereas high expression of *RGS2, NFKBIB, NR4A3, DDX21,* and *BTG3* was associated with 87% relapse-free survival, high expression of genes including *BMPR1B, CTGF (CCN2), TTYH2, IGJ, PON2, CD73, CDC42EP3, TSPAN7,* and *SEMA6A* was associated with relapse-free survival of only 29%.[538] Additionally a 21-gene classifier in the diagnostic marrow was discerned that was able to predict the minimal residual disease burden at the end of induction.[538] Such gene expression signatures underscore the heterogeneity in gene expression within ALL in general and high-risk ALL in particular, and they are significant because they are possibly expected to have prospective therapeutic utility in the near future.

IKZF1 ***Deletions in B-Cell Precursor ALL.*** Deletion of the *IKZF1* tumor suppressor gene is present in 10% to 15% of B-cell precursor ALL. Findings from a large international study of 1128 cases of childhood B-cell precursor ALL have suggested that *IKZF1* gene deletion, as detected by approaches including PCR assay, array CGH, and Sanger sequencing, is an independent poor prognostic feature.[539]

"BCR-ABL1–*like*" *B-Cell Precursor ALL.* *IKZF1* deletions are also a frequent finding marking approximately 40% of cases of "*BCR-ABL1*-like" B-cell precursor ALL,[539] a molecular subtype of high-risk precursor B-cell ALL without *BCR-ABL1* rearrangement but with a "*BCR-ABL1*–like" gene expression signature.[540] The "*BCR-ABL1*–like" gene expression signature is estimated to be present in approximately 10% of cases of B-cell precursor ALL and approximately 15% of cases of high-risk precursor B-cell ALL and has been associated with a poor prognosis.[539-541] By mRNA-seq analysis, the "*BCR-ABL1*–like" cases were found to be characterized by cryptic chromosomal rearrangements such as *EBF1-PDGFRB, BCR-JAK2, STRN3-JAK2, PAX5-JAK2, NUP214-ABL1, ETV6-ABL1, RANBP2-ABL1,* and *RCSD1-ABL1; IGH-EPOR* fusions; *IL7R* mutations; or *SH2B3* deletion.[541] *JAK1, JAK2,* and *JAK3* mutations also have been linked to the "*BCR-ABL1*–like" gene expression signature.[542] Of therapeutic interest, consistent with the ABL1 and/or JAK/STAT signaling activation in these cases, in preclinical experiments the "*BCR-ABL1*–like" leukemias are sensitive to tyrosine kinase inhibitors (e.g., imatinib, dasatinib) as well as to the JAK2 inhibitor roxolitinib.[541]

CRLF2 ***Aberrations in Precursor B-Cell ALL.*** The "*BCR-ABL1*–like" precursor B-cell ALLs also encompass cases with *CRLF2* aberrations.[540,541] This subset of high-risk precursor B-cell ALL has surfaced through cytogenetic and FISH analyses, as well as advanced genomics studies, including microarray analysis, analysis of copy number alterations, and next-generation sequencing, and is characterized by rearrangement or mutation of the chromosome pseudoautosomal region 1 Xp22.3/Yp11.3 gene *CRLF2* (cytokine receptor-like factor 2), which encodes the TSLP (thymic stromal lymphopoietin) receptor.[543,544] *CRLF2* rearrangements are present in approximately 60% of ALL occurring in patients with Down syndrome.[545] *CRLF2* aberrations also account for approximately 8% and 15%, respectively, of pediatric precursor B-cell ALL and high-risk precursor B-cell ALL.[546-548] The *CRLF2* gene can be disrupted by rearrangements with the *IGH* locus at chromosome band 14q32, by *CRLF2* interstitial deletion resulting in *P2RY8-CRLF2* rearrangement or, alternatively, by F232C point mutation.[543,547,549] The *CRLF2* aberrations are frequently associated with *JAK1/JAK2* mutations, *IKZF1* mutations, and, less often, *IL7R* mutations. Clinically, the *CRLF2* aberrations are important because they have been correlated with a poor prognosis.[543,547] Within high-risk ALL, the poor outcome has been further linked to high minimal residual disease burden and high *CRLF2* expression.[547] They also have been linked to Hispanic/Latino ethnicity.[550] In contrast, in a recent large international study of Down syndrome ALL[545] and a number of smaller studies of Down syndrome ALL,[544] the *CRLF2* aberrations did not affect prognosis. The *CRLF2* aberrations may have further significance for therapeutic targeting because they result in sensitivity to JAK and PI3K/AKT/mTOR (mammalian target of rapamycin) signaling inhibition in preclinical models.[551,552]

iAMP21 (Intrachromosomal Amplification of Chromosome 21) in Precursor B-Cell ALL. iAMP21 is a cytogenetic abnormality that occurs in approximately 2% of B-cell precursor ALL in older children, which has a known association with inferior outcome in standard treatment protocols and for which the molecular basis has been poorly understood. In a recent analysis of leukemia cells from 94 patients with this abnormality, the common region of amplification was narrowed to a 5.1-Mb region of chromosome 21 that encompasses *RUNX1,* miR-802, and genes in the Down syndrome critical region.[553] Genomic abnormalities affecting *IKZF1, CDKN2A/CDKN2B, PAX5, ETV6,* and *RB1* also were frequently detected in these cases in addition to iAMP21.[553] Interestingly, a very detailed analysis of the heterogeneous leukemic subclones that were present and their relationships to the iAMP21 suggests that iAMP21 and not these other abnormalities was the initiating alteration and that other aberrations such as *P2RY8-CRLF2* were secondary alterations to iAMP and were acquired later in the subclones.[553] However, the specific chromosome 21 gene within the 5.1-Mb regions that is most relevant to leukemogenesis remains to be determined.

Hereditary Susceptibility to B-Cell Precursor ALL. Until recent genome-wide association studies, few heritable factors influencing B-cell precursor ALL susceptibility had been identified by candidate gene approaches (e.g., *ABCB1; MTHFR; GSTM1, GSTT1;* SNPs in *CDKN2A, CDKN2B, CDKN1A, CDKN1B* promoters; *CYP1A1*). However, a number of genetic variants modulating disease susceptibility with predilection to influence the development of particular subsets of this disease have recently been discovered. One genome-wide association study conducted on a large number of cases and controls implicated variants in three different genes as risk loci.[554] The cases studied were predominantly B-cell precursor (824 B-cell ALL, 83 T-cell ALL). The most significant variant was rs4132601 in the *IKZF1* transcription factor gene, which encodes a master regulator of lymphoid development. The rs7089424 polymorphism in the gene encoding ARID5B (AT-rich interactive domain 5B), a member of the AT-rich interaction domain family of transcription factors involved on B-cell development, was a particularly strong risk variant. A variant (rs2239633) in the gene *CEBPE,* encoding CCAAT/enhancer binding protein, epsilon, was also associated with increased risk.[554] Interestingly, the *ARID5B* variant is particularly selective for hyperdiploid ALL,[554] an association validated by a second study.[555] The latter study determined that 18 different SNPs varied in allele frequencies between cases and controls and also found that an OR2C3 variant is particularly linked to *ETV6-RUNX1* leukemia.[555] Most recently, two different polymorphisms (rs3824662 and rs3781093) in the hematopoietic transcription factor *GATA3* gene were found to significantly increase the risk for "BCR-ABL1–like" disease both relative to nonleukemia controls and relative to non–"BCR-ABL1–like" leukemia cases.[556]

Molecular Alterations in Early T-Cell Precursor Leukemia. Early T-cell precursor (ETP) ALL is a recently recognized form of T-cell ALL characterized by a CD1a−, CD8−, CD5$^{\text{weak}}$, CD34+ stem cell and CD33 and/or CD13 myeloid marker+ immunophenotype and absence of biallelic *TCRG* deletions consistent with a differentiation arrest at an immature T-cell developmental stage.[557] Whole-genome sequencing in 12 pediatric cases has shown that ETP leukemia is characterized by AML-like aberrations.[558] These include cytokine receptor and *RAS* signaling mutations affecting *NRAS, KRAS, NF1, FLT3, IL7R, JAK3, JAK1, SH2B3,* or *BRAF* in 67% of cases; inactivating mutations in hematopoietic transcription factor genes *(GATA3, ETV6, RUNX1, IKZF1)* in 58% of cases; and inactivating mutations in genes encoding histone modifying factors *(EZH2, EED, SUZ12, SETD2, EP300)* in 48% of cases, as well as other novel types of mutations not otherwise seen in T-cell ALL (e.g., *WT1, DNM2, ECT2L, RELN*).[558] Interestingly, adult ETP ALL has been shown by whole-exome sequencing to manifest mutations in *ETV6, NOTCH1, JAK1,* and *NF1;* frequent recurrent novel mutations involving *FAT1* (25%), *FAT3* (20%), and *DNM2* (35%); as well as other mutations in the *MLL2, BMI1,* and *DNMT3A* genes encoding epigenetic regulators and at least a single mutation in *DNMT3A, FLT3,* or *NOTCH1* in 60% of cases.[559] Moreover, based on gene expression profiling and cluster analysis of pediatric T-cell ALL, ETP ALL comprises a unique cluster and it has been suggested that the driving aberration in ETP leukemia involves upregulation of expression of the *MEFC2* transcription factor gene on chromosome 5q14, which is a member of the MADS-box family of transcription factors, as a consequence either of novel *MEFC2* gene rearrangements (e.g., *MEF2C-PITX2*) or of rearrangements of related factors, examples of which include *NKX2-5/BCL11B, SPI1(PU.1)/BCL11B,* and *ETV6/NCOA2* translocations.[560] The NKX2-5 regulatory protein directly binds the *MEF2C* promoter.[560] The PU.1 transcription factor controls *MEF2C* expression during lymphoid development.[560] NCOA2 is also a *MEF2C* coregulator. The ETP leukemias highly express *HHEX, LYL1,* and *LMO2,* which are MEF2C-regulated targets.[560] Interestingly, *MEF2C* has been implicated as a target gene of HOXA9 and is also highly expressed in *MLL*-rearranged AML. Yet another case was characterized by a variant reciprocal *RUNX1-AFF3* translocation with upregulated *MEF2C* expression by an unknown mechanism. ETP leukemia is clinically important because of its poor outcome.

Alterations Associated with T-Cell ALL with Cortical Arrest and a Proliferative Gene Expression Profile. The same study that identified *MEFC2* and related aberrations in ETP leukemia also has identified a novel proliferative cluster of T-cell ALL characterized by a CD1+ immunophenotype consistent with a cortical arrest, expression of cell cycle genes, and ectopic *NKX2-1* or *NKX2-2* expression, also resulting from a number of novel *NKX2-1* and *NKX2-2* gene rearrangements or *TLX1* rearrangements.[560] These include *NKX2-1* inversions to the *TCRA* or *IGH* genes, a translocation with

TCRB [t(7;14)(q34;q13)] and a *TCRD/NKX2-2* gene rearrangement. In one case, array-CGH suggested *NKX2-1* amplification due to duplication or insertion into another chromosome. The subset of cases in the proliferative cluster marked not by *NKX2-1* or *NKX2-2* rearrangement but by *TLX1* gene rearrangements are also characterized by *NKX2-1* expression. Less often there are *MYB* gene translocations in the proliferative cluster of T-ALL. This subset of leukemia is clinically important because of its more favorable prognosis.[560]

Other Clinically Important Genomic Aberrations in Acute Lymphoblastic Leukemia

FLT3 ***Mutations.*** Aberrations of the FMS-like tyrosine kinase 3 (FLT3) receptor tyrosine kinase protein are crucial oncogenic events that contribute to cellular proliferation and survival and arrested differentiation in a subset of ALL.[561,562] *FLT3* mutations have been proposed to function as secondary oncogenic events in ALL, in which there is already a proliferation-inducing primary oncogenic mutation involving another gene.[532] *FLT3* molecular aberration types include in-frame deletions and internal tandem duplications (ITDs) in the juxtamembrane region and point mutations in the activation loop of the kinase domain.[135,532,563] All of these are associated with FLT3 protein overexpression and constitutive activation of FLT3 signaling pathways through STAT5, MAP kinase, and AKT.[532,564] *FLT3*-ITD mutations are found in approximately 2% of childhood ALL,[565] and *FLT3* point mutations are found in approximately 5% and 16% of childhood B-cell lineage ALL and infant ALL, respectively.[532,565] *FLT3* mutations have a predilection for particular molecular/cytogenetic ALL subtypes, being found in 21% to 28% of cases of high hyperdiploid ALL[135,530,532] and in 18% of ALL with *MLL* gene translocations, which consequently also express high levels of constitutively active FLT3 protein.[529,530] Overexpression of the wild-type FLT3 protein is another mechanism of FLT3 activation in leukemias with *MLL* translocations,[135] and microarray analyses have demonstrated increased *FLT3* mRNA expression as a distinguishing feature of ALL cases with *MLL* translocations.[528,566] Alternatively, the expression of FLT3 ligand by leukemia cells can cause autocrine activation of FLT3 signaling by increasing constitutive FLT3 phosphorylation.[135,530,567] *FLT3* mutations are clinically important in infant ALL because they are associated with a poor prognosis.[532]

Alterations in* BCL2 *Expression. In addition to classic oncogenes and tumor suppressor genes, genes that control cell death and survival decisions are clinically important, not only in pediatric ALL but in all types of pediatric cancer because they have an impact on chemosensitivity and resistance. The intrinsic (mitochondrial) cell death pathway is regulated by the formation of homotypic and heterotypic dimers involving BCL2 and many other BCL2 family protein members with opposing pro-apoptotic or anti-apoptotic actions that collectively determine the apoptosis threshold.[568-572] Almost all cases of pediatric ALL express detectable BCL2 protein.[573] BCL2 mRNA and protein are particularly abundant in infant and pediatric leukemias with t(4;11) or other *MLL* translocations compared with ALL and AML cases without *MLL* translocations,[574] but *BCL2* expression is not exclusively a feature of *MLL* disease.[573,575,576]

CDKN2 ***Tumor Suppressor Gene Deletions.*** Chromosome band 9p21 is a site of recurring deletions in pediatric ALL. The critical deleted region at chromosome band 9p21 contains the *CDKN2A* gene, which encodes two different tumor suppressor proteins p16^{INK4A} and p14^{ARF}. The adjacent gene *CDKN2B* encodes p15^{INK4b} and is often codeleted with the *CDKN2A* gene.[577] p16^{INK4A} and p15^{INK4b} inhibit the cyclin D–dependent kinases CDK4 and CDK6.[578] Deletions of the *CDKN2B* and *CDKN2A* tumor suppressor genes at the *CDKN2* loci are present in 60% and 20% of cases, respectively, of T-cell and B-cell precursor ALL, and the p15 promoter is inactivated by hypermethylation in additional cases. *CDKN2* alterations have been implicated in relapse and poor response to treatment,[577-582] and homozygous loss of p16^{INK4A} is more frequent in pediatric ALL at relapse than at diagnosis, indicating that this aberration portends disease progression.[583] Notably, hemizygous *CDKN2A* deletion may be constitutional and confer susceptibility to leukemia and other forms of cancer.[584]

SMAD3 ***Alterations.*** Loss of *SMAD3* expression is important in the pathogenesis of pediatric T-cell ALL.[585] TGF-β is a cytokine family composed of three isoforms that have a role in tumor suppression. The *SMAD3* component protein in the TGF-β signaling pathway was discovered to be absent or barely detectable in each of 10 cases of T-cell ALL, but sequencing of the corresponding *MADH3* gene from the leukemia cells of affected patients did not reveal mutations and the mechanism of loss of *SMAD3* expression is unknown. Nonetheless, the loss of *SMAD3* impairs the growth-suppressive effects of TGF-β on T cells. *SMAD3* loss is apparently restricted to T-cell ALL and is not observed in precursor B-cell ALL or AML.[585] Deletions resulting in haploinsufficiency of the cyclin-dependent kinase inhibitor *CDKN1B (p27^{Kip1})* at chromosome band 12p12 are also recurring abnormalities in T-cell ALL,[586] and *SMAD3* loss has been demonstrated to cooperate *CDKN1B* loss in a murine leukemogenesis model.[585]

Numerical Chromosomal Abnormalities. The largest cytogenetic subset of childhood precursor B-cell ALL (approximately 30%) is characterized by a hyperdiploid karyotype, which results when nondisjunction of chromosomes causes chromosomal gains. Hyperdiploidy can be of prenatal origin, as evidenced by its presence in cord blood and neonatal Guthrie blood spots, suggesting that hyperdiploidy itself may be the initiating event in this form of ALL.[587,588] High hyperdiploid ALL (>50 chromosomes) has a favorable prognosis, especially when there are specific chromosomal trisomies, such as +4, +10, +18[589] or +10, and +17.[78,590] About 25% of cases of high hyperdiploid ALL have *FLT3* mutations, whereas other cases exhibit overexpression of the FLT3 protein through autocrine activation.[135,530]

In contrast, hypodiploidy occurs in only 6% to 8% of childhood ALL, is frequently associated with chromosomal translocations, and is a poor prognostic factor.[590,591] In one study, the adverse prognosis was especially pronounced in cases with near-haploid karyotypes containing 24 to 28 chromosomes.[590] In a more recent study there was no difference in outcome when 24 to 29, 33 to 39, or 40 to 43 chromosomes were present.[592] Nonetheless, hypodiploidy with less than 44 chromosomes is generally regarded as a marker of poor prognosis. Patients with ALL containing 44 or 45 chromosomes have a better outcome unless there is monosomy 7 or a dicentric chromosome.[592]

Importantly, the major pediatric ALL leukemia subtypes recognizable at the cytogenetic and molecular levels can now be distinguished based on their unique gene expression profiles. These broad gene expression profile differences provide new insights into ALL biology and predictive markers and might be useful in identifying novel, molecularly targeted treatments.[593]

MicroRNA Aberrations. A burgeoning class of abnormalities in cancer cells involves changes in gene expression mediated by the aberrant activity of microRNAs, which affect the translation of coding mRNAs into their respective proteins.[594,595] MicroRNAs, which are short noncoding RNAs, originate via the excision of 19 to 25 nucleotide (nt) segments after formation of a hairpin pre-microRNA loop from a larger primary pri-microRNA transcript. The base pairing interactions of microRNAs with protein coding mRNAs[595] result in microRNA-mediated translational repression. Using microRNA profiling arrays, it recently has been possible to discern unique microRNA signatures and microRNA mutations in particular leukemia subtypes.[596] For example, ALL and AML can be distinguished by differential expression of microRNAs. The microRNAs miR-128a and miR-128b are overexpressed, and the microRNAs let-7b and miR-223 are underexpressed in ALL compared with AML.[597] MicroRNAs can regulate oncoprotein expression, as shown by mir-203 regulation of BCR-ABL, as relevant in CML and BCR-ABL+ ALL.[598] It has also been suggested that microRNA signatures have prognostic relevance in leukemias in adults.[599,600] Therefore the further characterization of microRNA mechanisms has important implications for the development of new antileukemia targeted therapeutics.

Aberrant DNA Methylation Patterns in B- and T-Lineage ALL. In addition to the granularity with which recurring structural aberrations and mutations have been characterized with new developments and applications of next-generation sequencing and other genomics methods, genome-wide analysis of cytosine methylation profiles has demonstrated an overarching signature of methylation across all subtypes of B-cell lineage ALL relative to normal B cells regardless of genetic subset, but also that the different ALL genetic subtypes, whether of B- or T-cell lineage, are characterized by distinct methylation patterns.[535] The common methylation signature in B-cell lineage ALL affects cell signaling genes *(TIE1, MOS, CAMLG, GPRC5C),* cell cycle regulatory genes *(MCTS1, DGKG),* RNA metabolism genes *(PABPN1, PABPC5),* transcription factor and transcriptional regulatory genes *(PROP1, TAF3, H2AFY2, ELF5, ZBTB16, CNOT1, TADA2A),* and *HOX* genes *(HOXA5, HOXA6).* Interestingly, however, different and robust ALL clusters defined by hypomethylation and hypermethylation signatures were associated with the different genetic subtypes. At one extreme, high hyperdiploid B-ALL was the most hypomethylated, B-ALL characterized by ERG alterations was more balanced in regard to hypermethylated versus hypomethylated loci, and *CRLF2*-rearranged cases exhibited few differentially methylated regions. Moreover, the subtype-specific methylation signatures also were well correlated with gene expression patterns and there was differential methylation of genes associated with T-cell versus B-cell differentiation that were lineage dependent.[535]

However, the molecular complexity of ALL extends beyond the primary aberration, acquisition of secondary alterations, and epigenetic signatures. In a study of the molecular complexity of subclone evolution that occurs within *ETV6-RUNX1* ALL, Anderson and associates used multiplex FISH to determine in unprecedented detail the relationships of cells within leukemia subclones to one another to understand their “clonal architecture.”[601] The results suggested a tremendous amount of genetic variegation that includes continued diversification in propagating cells relative to ancestral cells in the development of subclones.[601] This genetic diversification has substantial implications for the understanding of the results of genome-wide analyses that are now more commonly being undertaken.[601]

Acute Myeloid Leukemia

Pediatric AMLs exhibit heterogeneous but characteristic clinical and biologic features, which reflect underlying molecular and cytogenetic abnormalities. Many of the characteristic AML cytogenetic aberrations are similar to those in adult AML, although the relative frequencies of the aberrations differ between pediatric and adult AML. The major FAB morphologic subtypes of AML (Fig. 45-37) are, in general, associated with typical balanced chromosomal translocations.[602] The WHO has reclassified AML based on molecular and cytogenetic aberrations, which underlie unique biologic subgroups, have clinical importance, and form the basis for assignment to appropriate treatment regimens.[603] The current WHO classification system incorporates genetic, immunophenotypic, clinical, and biologic features for diagnosis of the myeloid neoplasms.[603]

Major molecular cytogenetic aberrations in AML recognized in the WHO classification system are the following: AML with t(8;21)(q22;q22), *(AML1-ETO);* AML with abnormal bone marrow eosinophils and inv(16)(p13q22) or t(16;16)(p13;q22), *(CBFB-MYH11);* acute promyelocytic leukemia (APL) with t(15;17)(q22;q21), *(PML-RARA),* and variants; and AML with 11q23 *(MLL)* abnormalities. It has been estimated that the t(8;21), t(15;17), inv(16), and *MLL* translocations are present in 12%, 7%, 12%, and 7% of cases of pediatric AML, respectively.[602,604] However, these frequencies are inexact, with another study using standard cytogenetic analyses finding *MLL* translocations in approximately 18% of cases.[605] Furthermore, other characteristic recurrent molecular cytogenetic aberrations are observed.

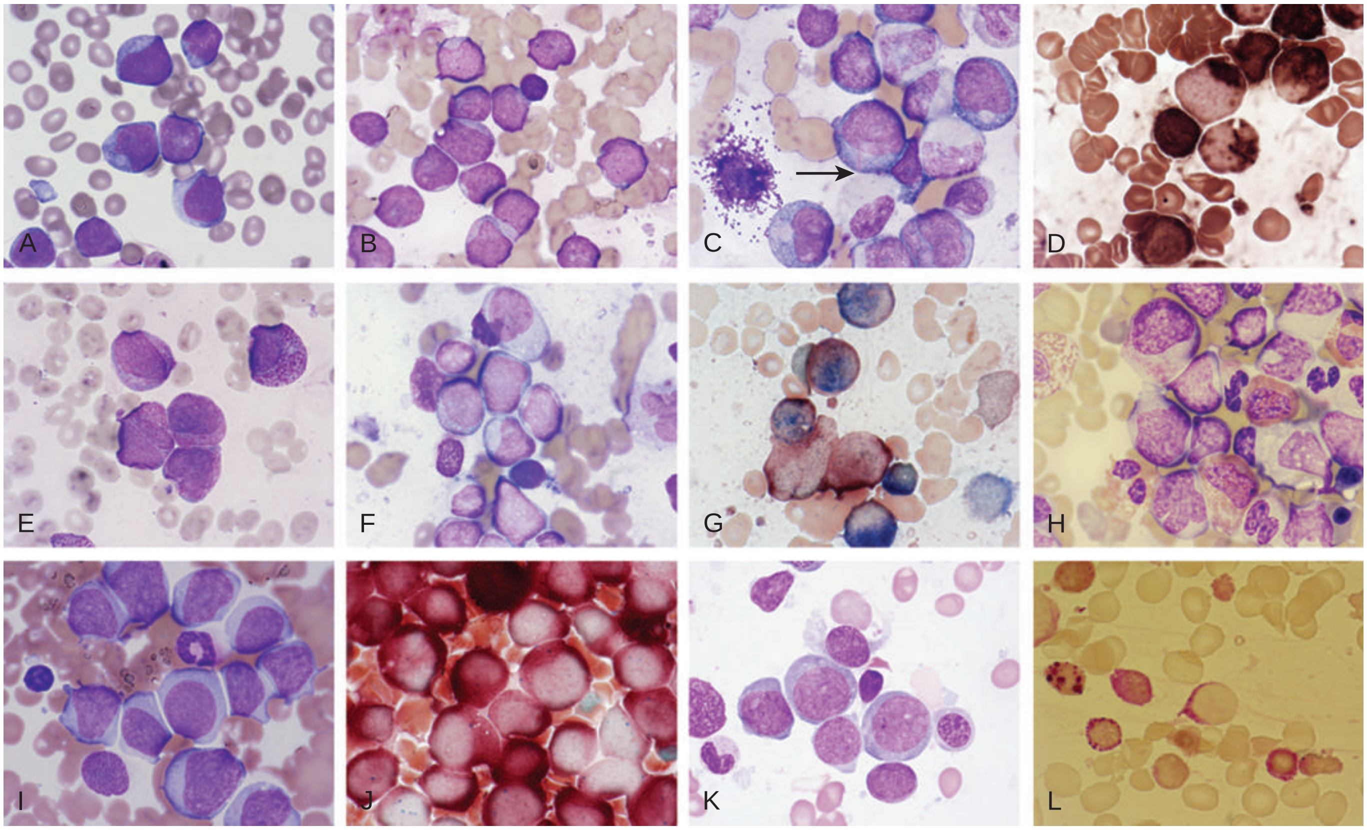

Figure 45-37 Major acute myeloid leukemia subtypes. M0 (**A**) and M1 (**B**) have minimal differentiation. **C,** M2 shows greater myeloid differentiation and may feature Auer rods *(arrow)* along with strong myeloperoxidase staining (**D**). **E,** M3 promyelocytic leukemia with typical coarse cytoplasmic granules. **F,** M4 myelomonocytic leukemia with staining for chloroacetate esterase *(blue)* in the myeloblasts, and nonspecific esterase *(brownish red)* in the monoblasts (**G**). **H,** M4eo with pronounced eosinophilic features. **I,** M5 monoblastic leukemia with staining for nonspecific esterase (**J**). **K,** M6 erythroleukemia with staining for periodic acid–Schiff (**L**). *(Courtesy Marybeth Helfrich, Children's Hospital of Philadelphia.)*

Although morphology no longer serves as the diagnostic gold standard for AML, the observation of a dyshesive, monotonous, immature cell population should trigger and guide further investigations by histochemical, immunophenotypic (including flow cytometric), and molecular cytogenetic methods. AML may exhibit morphologic features of any of the various hematopoietic progenitors or even of several simultaneously. The cytologic features, cytochemical staining pattern, and immunotype all assist in the subclassification of AML morphology by the FAB system, which recognizes the eight different subcategories (see Fig. 45-37) described here.

In FAB M0 AML there are more than 30% blasts with no or minimal features of myeloid differentiation. FAB M0 AML nuclei are generally large, with remarkably fine nuclear chromatin, prominent nucleoli, and high nuclear-to-cytoplasmic ratio.[606] The cytoplasm may exhibit focal granularity, and Sudan black reactivity is typical. Myeloperoxidase expression is inapparent by light microscopy but can be demonstrated by electron microscopy.

In FAB M1 AML, like in FAB M0, there are more than 30% blasts with minimal differentiation but, unlike in FAB M0, myeloperoxidase expression is readily demonstrable and accompanies Sudan black and chloracetate esterase positivity.

The diagnosis of FAB M2 AML is based on the observation of myeloblasts with some maturation, generally requiring more than 30% total blasts, with more than 10% of the blasts showing granulocytic differentiation. FAB M2 AML blasts also exhibit myeloperoxidase and chloracetate esterase staining. FAB M2 morphology is typical in AML with t(8;21).[607] Although the typical morphology of AML with inv(16)(p13;q22) is FAB M4eo, in one series a substantial fraction of pediatric AML with inv(16)(p13;q22) showed FAB M2 morphology.[608]

The classic FAB M3 APL has coarse cytoplasmic granules, the nuclear chromatin is less fine than in myeloblasts, and the nucleus often is eccentric. FAB M3v is an APL microgranular variant in which the cytoplasmic granules are finer than in classic FAB M3 APL, and the nuclei may have irregular contours, appearing reniform or folded. The genetic underpinnings, typically associated with t(15;17), are similar to FAB M3, but FAB M3v APL does not carry the risk for massive hemorrhage during chemotherapy induction that is associated with classic FAB M3 APL.

FAB M4 AML is acute myelomonocytic leukemia, which is composed of myeloblasts, monoblasts, and promonocytes. The monoblast has a substantially lower nuclear-to-cytoplasmic ratio than the myeloblast or promyelocyte; its nucleoli are more prominent and its cytoplasm is more abundant, with fine granularity and occasional vacuolation. Although the promonocyte has similar features, it shows a different cytoplasmic hue. Both monoblasts and promonocytes, whether in FAB M4 or FAB M5 AML, are nonspecific esterase positive. FAB M4 and FAB M5 AMLs also express chloracetate esterase, myeloperoxidase, and α-naphthol acetate, and some stain with PAS. FAB M4 and FAB M5 AMLs are typically, although not exclusively, associated with *MLL* gene translocations. FAB M4 AML with abnormal bone marrow eosinophils (FAB M4eo) is associated with inv(16)(p13;q22).

FAB M5 acute monocytic leukemias encompass two subcategories; one subcategory with no differentiation is FAB M5a (monoblastic leukemia), whereas the other with differentiation is FAB M5b. Monoblasts predominate in FAB M5a AML, but promonocytes predominate in the FAB M5b morphologic subtype.

FAB M6 AML is acute erythroid leukemia, which generally exhibits a predominance of erythroblasts but often also has a substantial myeloblast component. The erythroblast shows a high nuclear-to-cytoplasmic ratio and a condensed rounded nucleus. The cytoplasm is deeply basophilic and may contain vacuoles. FAB M6 cases express glycophorin and stain for PAS but not other cytochemical markers.

The FAB M7 category is acute megakaryoblastic leukemia (AMKL). FAB M7 AML may be associated with prominent marrow fibrosis, making aspiration difficult. The cells are pleomorphic (see Fig. 45-39) and the megakaryocytic nature may not be obvious by morphology alone. FAB M7 AMKL cells may express α-naphthol acetate. Expression of glycoprotein Ia, IIb, or IIIa or factor VIII can aid in the diagnosis of FAB M7 AMKL. As described in more detail later, AML in Down syndrome is AMKL; however, the t(1;22) is found in some non–Down syndrome cases.[609]

Major Chromosomal Translocations

t(8;21). The archetypal chromosomal translocation in FAB M2 AML is the t(8;21), which fuses the 5′ terminus of the *AML1 (RUNX1, CBFA2)* gene at chromosome band 21q22, with almost the entire *ETO* gene from chromosome band 8q22.[79] In one series, 82% of pediatric AML with this translocation had FAB M2 morphology.[607] These morphologic-cytogenetic correlates, although compelling, are imperfect. Therefore some classifications have taken account of cytogenetic abnormalities, thereby highlighting crucial biologic pathways and, in some cases, therapeutic targets.[603] The t(8;21) also is strongly associated with extramedullary granulocytic sarcoma presentations.[607,610] Leukemias with this translocation, which disrupt the *AML1* gene encoding the DNA-binding subunit of core binding factor, comprise one of the two subtypes of core binding factor leukemias. The AML1 fusion oncoprotein binds the non–DNA-binding subunit of core binding factor, CBFB, as does native AML1, but the fusion oncoprotein interacts with nuclear corepressors that inhibit AML1-mediated transcriptional activation through the deregulation of histone deacetylation.[79] The t(8:21) has been associated with a favorable prognosis in studies conducted by the Pediatric Oncology Group[611] and the Children's Cancer Group.[612] The t(8;21) has been associated with a mixed outcome in the experience of St. Jude Children's Research Hospital, but the treatment regimens did not specify repetitive high-dose cytarabine as did other regimens in which the t(8;21) was associated with a favorable prognosis.[607,612,613] Variant *AML1 (CBFA2)* translocations involving other partner genes have heterogeneous morphologic MDS and AML presentations, but these translocations occur primarily in adult leukemias and tend to be treatment related rather than to occur de novo.[614]

inv(16) and t(16;16). The second subtype of core binding factor AML has inversions or, less often, translocations fusing the *CBFB* gene encoding the non–DNA-binding subunit of CBF at chromosome band 16p13 to the *MYH11* gene at chromosome band 16q22.[615] The classic morphologic presentation of AMLs with inv(16) and t(16;16) in adults is myelomonocytic leukemia with eosinophilia (FAB M4eo)[616] but many pediatric cases present as FAB M2 or M4 AML, without eosinophilia.[608,617] RT-PCR analysis and FISH are particularly useful and important for the detection of this rearrangement.[618-621] Additionally, the leukemic blast cells may coexpress the CD2 T-cell surface antigen.[622] The inv(16) is leukemogenic via a mechanism of dominant transcriptional repression of AML1-regulated genes, so that the pathways involved and the biologic consequences are similar to those resulting from the t(8;21).[79] The outcome associated with this aberration is also favorable in several pediatric series, similar to the t(8;21).[623] In one large study of adults and children, the outcomes were comparable in patients undergoing allotransplantation and receiving high-dose or low-dose cytarabine, and the outcome was better in the younger patients.[624] Pediatric studies have found that current regimens incorporating intensive high-dose cytarabine into postremission treatment are associated with event-free survival rates of approximately 70% or higher.[605,612]

t(15;17). The t(15;17) fusing the *PML* and *RARA* genes at chromosome bands 15q22 and 17q21, respectively, is associated with FAB M3 APL.[625,626] The t(15;17) and variant translocations associated with APL disrupt the function of the retinoic acid receptor α (RARα), also called the RARA protein, which is a ligand-dependent transcription factor important in myeloid differentiation. The RARα protein forms a heterodimer with another ligand-dependent protein, retinoic X receptor protein (RXR). The natural ligand of RARα is all-*trans*-retinoic acid (ATRA), whereas RXR is responsive to ATRA or 9-*cis*-retinoic acid.[625] In the presence of ligand, the heterodimeric complex interacts with transcriptional co-activators to induce myeloid differentiation. In contrast, the molecular pathogenesis of APL involves the aberrant recruitment of transcriptional co-repressors and the histone deacetylase complex to RARα target genes. Notably, the first molecularly targeted agent ever to be implemented for antileukemia treatment was ATRA. Variant translocations fuse *RARA* with partner genes other than *PML,* including *NPM, NUMA1 (NuMA), STAT5b, PRKAR1A,* and *ZBTB16 (PLZF).*[625,627-630] The

PRKAR1A-RARA fusion was first identified by *RARA* FISH analysis in an ATRA-sensitive adult with APL who had normal cytogenetics by conventional karyotype analysis.[630] *PRKAR1A* encodes the regulatory subunit type 1α of cyclic adenosine monophosphate–dependent protein kinase,[630] and the karyotypically cryptic nature of the translocation highlights the importance of molecular cytogenetic testing. APLs with *PLZF-RARA* translocation are insensitive to ATRA, whereas those with the other *RARA* fusion types are ATRA responsive.[625]

Mixed-Lineage Leukemia* (MLL) *Gene Translocations. In contrast to ALL, in which *MLL* partner genes are limited and *AF4* is the most common, the partner genes are much more diverse in AML.[631] Nonetheless, *MLL* partner genes in ALL and AML, and in de novo and treatment-related leukemias, are at least partially overlapping. Many MLL partner proteins have structural motifs of nuclear transcription factors,[15,132,506] transcriptional regulatory proteins,[632] or other nuclear proteins.[633-635] Others are cytoplasmic proteins or cell membrane proteins.[132,518,636-642] *MLL* also undergoes self-fusion in a type of rearrangement termed *partial tandem duplication;* therefore in AML, MLL itself is an MLL partner protein.[45,643,644] As in ALL, cDNA panhandle PCR analysis has been applied to detect novel and rare partner genes of *MLL* and covert *MLL* rearrangements in AML.[645-647] There is no obvious functional relationship between the many partner genes of *MLL* but some are members of the same gene families.[518,638-640,648,649] It has been suggested that the nuclear partner proteins mediate transcriptional activation[132,649,650] and that cytoplasmic partner proteins cause forced MLL dimerization or oligomerization.[651] Several MLL partner proteins also interact with one another.[652] Fusion proteins from the der(11) chromosome, which retain the AT hook, SNL, and MT domains of MLL but replace the MLL PHD, transactivation, and SET domains with the carboxyl partner protein, transform hematopoietic progenitors and cause leukemia in mice.[653,654] In murine models, MLL fusion proteins constitutively activate *Hoxa9,* and *Hoxa9* activation is essential for leukemogenesis with some MLL fusion proteins (e.g., MLL-ENL).[515] However, altered *Hox* expression is not essential for leukemogenesis with other MLL fusion proteins, including MLL-AF9 and MLL-GAS7.[654,655]

As described earlier, balanced *MLL* gene translocations are the primary molecular aberrations underlying most cases of acute leukemia in infants.[503] AML is more common than ALL in neonates, unlike during the rest of infancy and childhood.[134,656] It has been estimated that *MLL* translocations are present in half of all ALLs and AMLs diagnosed during the neonatal period.[134] *MLL* translocations also comprise 5% to 10% of acquired chromosomal rearrangements in childhood AML.[657] The particular partner gene fused to *MLL* affects cell lineage and differentiation features, which are reflected in the immunophenotype. *MLL* translocations in AML are often associated with myelomonocytic and monoblastic features.[133] Leukemias with *MLL* translocations can also present as treatment-associated myelodysplastic syndrome or AML[658,659]; this is particularly characteristic of cases with t(11;16) fusing *MLL* with *CBP* (CREB binding protein).[632,660-662] Unlike in ALL, the presence of an *MLL* translocation is not an independent prognostic factor in pediatric AML with current treatment. However, in some series, the *MLL-MLLT3 (AF9)* fusion resulting from t(9;11) has been associated with a more favorable outcome than other *MLL* translocations[617] (e.g., *MLL-MLLT10 [ALF10]*).[663]

Certain clinical and laboratory features are associated with the archetypal neonatal FAB M4 and FAB M5 leukemias with *MLL* translocations. These features include cutaneous nodules (leukemia cutis), petechiae, and ecchymoses, in addition to the hematologic abnormalities (hyperleukocytosis, anemia, thrombocytopenia) and CNS involvement.[134,656,664] Curiously, congenital *MLL*-rearranged AML may be present predominantly in the skin and not in the marrow (aleukemic leukemia cutis) and, on occasion, can undergo spontaneous remission.[664,665] In these situations, molecular analysis of bone marrow and skin biopsy material is warranted because of the propensity for progressive disease when a clonal *MLL* rearrangement is identified.[664,665]

As discussed earlier for infant ALL, the *MLL* translocations in many infant AMLs are in utero events.[518] In some cases, however, an in utero origin cannot be demonstrated, possibly because the clonotypic sequences of the translocations are present below the detection limit of the PCR assay.[666] The occasional stillbirth presentations[134] and the presentation of monoblastic AML with an *MLL-ELL* rearrangement as a granulocytic sarcoma diagnosed by prenatal ultrasonography[667] further substantiate that *MLL* translocations arise in utero in infant AML. However, latency to leukemia underscores that additional secondary alterations are important, and perhaps required, for the development of leukemia with an *MLL* translocation.[132]

t(8;16). The chromosome 16 rearrangement in t(8;16)(p11;p13) is in the same band (16p13) disrupted by inversions and translocations of the *MYH11* gene in M4Eo AML.[615,668,669] However, the 16p13.3 breakpoints in t(8;16) target a different gene, encoding the transcriptional coactivator CREB binding protein (CBP; CREBBP) at chromosome band 16p13.3, which is fused with the *MOZ (MYST3)* gene on chromosome 8.[670] The CBP protein contains RARA and CREB binding domains, a bromodomain, and E1A and transcription factor IIB (TFIIB) binding regions, whereas MOZ contains zinc finger motifs and a MYST acetyltransferase domain.[670-674] The *CBP-MOZ* fusion transcript encoded by the der(16) chromosome is not in-frame, whereas the *MOZ-CBP* transcript is believed to have transforming properties and clinical relevance.[670,675,676] MOZ-CBP tumorigenic mechanisms have been proposed to involve inhibition of AML1-mediated transcriptional activation.[677] The *MOZ-CBP* rearrangement is associated with a gene expression signature that includes *HOXA9, MEIS1,* and *FLT3* overexpression, similar to the gene expression profiles in AML with *MLL* translocations.[678] Notably, *CBP* is also an occasional fusion partner of *MLL* in MDS.[632,660-662] *CBP* oncogenic aberrations can be constitutional, as evidenced by the germline *CBP* point mutations, microdeletions, and translocations that underlie the Rubinstein-Taybi syndrome.[679-681]

The t(8;16) is associated with a prognostically unfavorable subset of FAB M4 and FAB M5 AMLs, other features of which include erythrophagocytosis and coagulopathy.[682,683] Therefore recognition of this AML subset is clinically important and can be accomplished by molecular cytogenetic detection of the rearrangement. Although a rare translocation, accounting for only approximately 2% of pediatric AML,[684] most AMLs with this translocation have occurred in infants and children.[670,683] The t(8;16) in neonatal AML, like translocations in several other AML subtypes, can be associated with leukemia cutis and spontaneous remission.[685,686]

A variant t(10;16) translocation fuses the *KAT6B (MORF)* gene at chromosome band 10q22 to *CBP* and was discovered in a case of childhood FAB M5a AML.[687] The MORF protein contains zinc fingers, nuclear localization signals, and a histone acetyltransfersase domain[687] and, similar to MOZ, contributes to chromatin remodeling.[674] Additional variant translocations result in fusion of *MOZ* with the *NCOA2 (TIF2)* or *EP300 (p300)* genes[688-690]; p300 is related structurally to CBP, and the MOZ-TIF2 oncoprotein recruits CBP. Therefore CBP-mediated oncogenic transcriptional regulatory functions appear crucial in the several translocation variants that compose this AML molecular cytogenetic subset.

t(6;9). The t(6;9) (p23;q34) translocation is a rare recurrent translocation in AML, primarily seen in adults, the clinical and molecular features of which include myelodysplasia, basophilia, a CD34– immunophenotype and *FLT3* mutations.[691-693] AML with t(6;9) in children and adolescents has had a poor prognosis.[684,695-696] The translocation occurs in various morphologic AML subtypes, including FAB M1, M2, and M4, and fuses the 5′ end of the *DEK* gene (chromosome 6) to the 3′ end of the *NUP214 (CAN)* gene (chromosome 9), creating a *DEK-CAN* oncogene.[697-699] The *DEK* gene product is a multimeric chromatin protein, regulated by CK2 kinase, which modifies DNA topology by the introduction of supercoils into closed circular DNA.[700,701] The *CAN* gene at chromosome band 9q34 (also called *CAIN,* because of its chromosomal proximity to *ABL*), encodes the nucleoporin protein NUP214 of the nuclear pore complex. DEK-CAN oncogenic roles in leukemogenesis involve a myeloid lineage-specific increase in protein translation, which occurs in conjunction with increased eIF4E translation initiation factor phosphorylation.[702] Leukemias with the *DEK-CAN* fusion may have oncogenic mechanisms in common with other AML subtypes, as is suggested by the observation that nucleoporin-specific FG repeats in the fusion oncoprotein can induce transcriptional activation by recruitment of the transcriptional coactivators CBP and p300. Reverse-transcriptase and real-time PCR methods have been helpful for AML monitoring in patients with this rearrangement, because the persistence of the fusion transcript identifies patients at risk for relapse.[703-705] A translocation variant resulting in the *SET-CAN* oncogenic fusion gene has been observed in AML of the FAB M0 subtype.[697]

NUP98 *Translocations.* The *NUP98* gene at chromosome band 11p15 encodes a nucleoporin protein that serves as a structural component of nuclear pore complex. The NUP98 protein is located on the nucleoplasmic side of the nuclear pore and provides a docking site for the export of mRNA between the nucleus and cytoplasm.[706,707] Various chromosomal translocations produce *NUP98* oncogenic fusions and, depending on exactly which partner gene is fused with *NUP98*, these translocations can be associated with de novo or treatment-related AML, ALL, or MDS.[706-726] Many of the *NUP98* partner genes encode homeobox transcription factors, but some of the partner genes, including *TOP1* and *TOP2B,* have more diverse functions.[708,717,723] Several *NUP98* fusions, such as those involving *NSD1*, *HOXD13,* and *PSIP1 (LEDGF),* are found in infant and pediatric de novo AML.[718,724,725] t(7;11)(p15;p15), which fuses *NUP98* with *HOXA9,* and t(10;11)(q23;p15), which fuses *NUP98* with *HHEX,* have been shown to shift the cellular distribution of NUP98 away from the nuclear pore complex and into intranuclear aggregation bodies.[711,719] Because of the substantial variety of *NUP98* partner genes, it is most feasible to detect these rearrangements using a general *NUP98* break-apart FISH strategy.[723,727] cDNA panhandle PCR assay has also been applied as a gene discovery tool to identify new partner genes in leukemias with *NUP98* translocations.[728]

Therapy-Related Acute Myeloid Leukemias and Myelodysplastic Syndromes

Therapy-related AMLs and MDS comprise a separate WHO AML subgroup[603] relevant to pediatrics; two major categories have been recognized. Therapy-related leukemias are of increasing concern in children, in whom survivorship—as a percentage of patients diagnosed with cancer—is greater than in the adult cancer population.[729] Therapy-related leukemia or MDS is also well-recognized as a long-term complication after autologous hematopoietic stem cell transplantation.[730-732] The two major categories of chemotherapies associated with leukemia and MDS are alkylating agents and TOP2 poisons. The forms of leukemia associated with these agents have distinctive molecular cytogenetic features, which can also be found in subsets of leukemia arising de novo.

All alkylating agents engender some risk for leukemia as a treatment complication, but the risk is variable, depending on the particular agent(s), cumulative dose, patient age, and superimposed effects of radiation therapy.[733-736] The latency period is variable, ranging from 2 to 12 years after exposure, but the peak incidence occurs at approximately 6 years.[737] The archetypal cytogenetic features in alkylating agent–related leukemias are complete or partial deletions of chromosomes 5 and 7 and complex, unbalanced, numeric and structural cytogenetic abnormalities.[738] The chromosome 7 aberrations are the most common cytogenetic events in leukemias after alkylating agent treatment.[739,740] Most cases with chromosome 7q deletions exhibit allelic loss of the chromosomal segment between bands 7q22 and 7q31 but, despite identification of various candidate genes in this genomic region, the exact tumor suppressor gene(s) have not been convincingly identified.[741-745] In treatment-associated MDS and AML lacking chromosome 5 aberrations, the monosomy 7 or del(7q) may be acquired by subclone evolution and accompanied by other key mutational aberrations, including t(3;21), *RAS* mutation, and CDKN2B promoter hypermethylation.[740]

Monosomy 5 and del(5q) are the next most common aberrations in alkylating agent–related MDS and AML. FISH analyses have revealed that the chromosome 5q abnormalities frequently are covert unbalanced translocations.[740] The chromosome 5q abnormalities arise as primary aberrations and not by subclone evolution, and additional aberrations in therapy-related MDS and AML with monosomy 5 or del(5q) can include monosomy 7 or del(7q), *TP53* mutations, and gene duplications, such as *MLL* segmental jumping translocations.[16,740,746] The critical genes targeted by 5q deletion have been difficult to pinpoint because of variable involvement of a large genomic region that encompasses chromosome bands 5q13 to 5q33.[739,747-753] Two recently implicated chromosome 5q31 candidate genes are *CTNNA1* encoding the α-catenin protein[754] and the *EGR1* gene,[755] although there have been several others.[739,747-753] Epigenetic dysregulation may also contribute to the pathogenesis, with transcriptional silencing in the retained nondeleted *CTNNA1* allele resulting from promoter methylation and histone deacetylation.[754]

A tumor suppressor gene, *RPS14,* has been identified as the target of 5q– in the syndrome of refractory anemia, in which there is preservation of megakaryopoiesis and myelopoiesis,[756] but the role of *RPS14* in therapy-related MDS and AML remains to be determined. This gene encodes a ribosomal protein required for processing of 18S pre-rRNA.[756] The strategy of screening normal human CD34+ cells with short hairpin RNAs (shRNAs) against various genes in the minimal 5q– deletion region has revealed that *RPS14* silencing causes erythroid maturation block and increased apoptosis in differentiating erythroid cells, similar to the increased apoptosis observed in MDS.[756] Furthermore, lentiviral RPS14 expression restored erythroid differentiation in bone marrow cells from patients with 5q– refractory anemia syndrome.[756] These insights underscore the role of ribosomal genes in anemia, given that other ribosomal genes, *RPS19* and *RPS24,* contribute to Diamond-Blackfan anemia.

Most AMLs developing after autologous hematopoietic stem cell transplantation have complex numeric and structural karyotypic aberrations, including losses of chromosomes 5 and 7; these are also features of AML arising after exposure to alkylating agents.[732,757-759] The secondary AMLs can occur early after transplantation, and the cytogenetic aberrations in these treatment-associated leukemias are often demonstrable in pretransplantation specimens, suggesting that the genotoxic damage results from the prior therapy.[757,760] On the other hand, such cytogenetic aberrations are not demonstrable in the peripheral blood stem cell autografts.[761]

Therapy-related leukemias can be caused by exposure to various chemotherapeutic TOP2 poisons, including epipodophyllotoxins and anthracyclines, the anthracenedione mitoxantrone, and the TOP1/TOP2 interacting agent dactinomycin.[63] Balanced translocations, often involving the *MLL* gene,[562] are the most typical molecular alterations in leukemias related to TOP2 poisons. The typical latency is 2 to 2½ years after chemotherapy exposure,[658,762] although more protracted latencies of up to 10 years have been described.[645,763] A particularly high risk has been reported in association with high cumulative doses of etoposide and anthracyclines,[764] but other studies have not found a correlation between etoposide cumulative dose and secondary leukemia risk.[765] Intermittent weekly or twice-weekly etoposide dose schedules, and semicontinuous schedules, have also been associated with increased risk for secondary AML.[764,765] The *MLL* translocations in therapy-related AML (as is the case in de novo AML) are often associated with myelomonocytic and monoblastic morphology.[766,767] Other translocations in therapy-related MDS and AML include t(8;21) and its variants,[614,768-777] t(15;17),[778] inv(16) and t(16;16),[762,779-782] t(8;16),[783,784] t(9;22),[783] and various translocations involving the *NUP98* gene at chromosome band 11p15.[708,709,711,785-788]

Treatment-related MDS/AML can present as bizarre and highly abnormal morphology in some cases (Fig. 45-38) but, in others, can be insidious and challenging to diagnose, particularly in patients actively receiving treatment, in whom the hematologic features of cytopenia and monocytosis can be indistinguishable from ordinary side effects of chemotherapy and recovery of the marrow. Additionally, some patients with treatment-related MDS and AML, particularly in the early stages, have no obvious peripheral blood cell abnormalities at all.[51,637] Therefore one front-line pediatric neuroblastoma treatment protocol has specified routine bone marrow surveillance every 3 to 6 months, not only for neuroblastoma relapse but also for leukemia-associated aberrations.[51] In addition to the insidious nature of the clinical presentations, the spectrum of known molecular cytogenetic abnormalities associated with therapy-related MDS and AML is constantly increasing and is already very large, creating the yet-unmet need for broad molecular cytogenetic screening

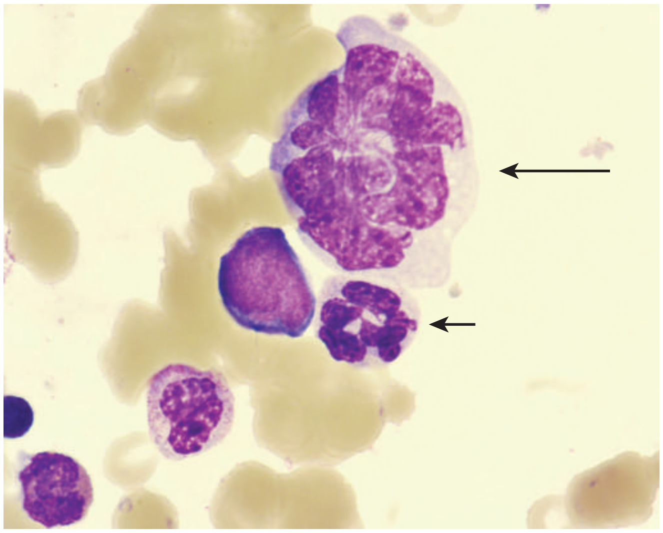

Figure 45-38 Therapy-related MDS showing hypersegmented granulocyte *(short arrow)* below an extremely dysplastic hypersegmented neutrophil *(arrow)*. *(Courtesy Marybeth Helfrich, Children's Hospital of Philadelphia.)*

methods with which to monitor high-risk patients. For example, even though there are few data on patients observed in this manner, it seems that most of the protean *MLL* translocations emerging during treatment, which involve many different partner genes, are harbingers of MDS or AML.[51,637,789] An *MLL-FRYL* translocation that was traced prospectively by molecular cytogenetic testing (FISH, panhandle PCR assay) caused clonal replacement of the bone marrow without affecting differentiation, only after a long latency culminated in clear-cut therapy-related MDS.[51] However, clinical progression is apparently not inevitable because, in one patient, the morphologically normal clone harboring an *MLL-ARHGEF17* translocation gradually regressed and neither leukemia nor MDS ensued.[790]

Additional challenges for molecular characterization result from the variable locations of *MLL* translocation breakpoints, which can be within or outside a breakpoint hot spot region.[45,47,51,63,121,134,139,155,463,465-470,632,647,791,792] Secondary myeloid disorders are particularly challenging to diagnose in patients previously diagnosed with AML, for example, the *MOZ* oncogenic rearrangement resulting from a novel t(2;8)(p23;p11.2) translocation in treatment-related MDS after AML in a pediatric patient.[793]

Clinically, the molecular cytogenetic features of the therapy-related leukemias are important prognostic factors. Most cases, except those with favorable translocations—t(8;21), inv(16), t(15;17)—are resistant to current treatment strategies, but intensive regimens, including hematopoietic stem cell transplantation, have been more successful. Prognosis is affected further by the reduced tolerance to additional intensive antileukemia therapy after primary cancer treatment.[794] Nonetheless, just as in the de novo leukemias, additional targeted therapy options are emerging from an understanding of the molecular cytogenetic aberrations in these challenging disorders.

Myelodysplasia and Myeloproliferative Diseases

Myelodysplasia and myeloproliferative diseases comprise a very small fraction (<10%) of all hematologic malignancies in children. Incomplete coverage of various unique features of pediatric MDS and myeloproliferative diseases in the current WHO classification system has led to the development of diagnostic guidelines for MDS and myeloproliferative categories applicable specifically to children.[795] To fulfill minimal diagnostic criteria for MDS, at least two of the following four features are required: (1) sustained unexplained cytopenia; (2) bi-lineage morphologic myelodysplasia; (3) acquired clonal hematopoietic cell cytogenetic abnormality; and (4) 5% or more blasts in the marrow.[795] In contrast to AML, which is defined by a bone marrow blast threshold of 20% and aberrant differentiation, and to MDS, myeloproliferative disorders are characterized by expansion of one or more hematopoietic lineages but retained differentiation.[795,796] Three major categories of MDS and myeloproliferative diseases are applicable to children: (1) a group of myelodysplastic-myeloproliferative disorders comprising primarily juvenile myelomonocytic leukemia (JMML); (2) myeloid leukemia of Down syndrome; and (3) MDS.[795]

Juvenile Myelomonocytic Leukemia. The constellation of hepatosplenomegaly, lymphadenopathy, pallor, and rash, especially in children younger than 3 years old, comprises the clinical features of JMML, the diagnostic criteria for which include peripheral monocytosis, absence of the *BCR-ABL* rearrangement, less than 20% blasts in the bone marrow, and at least two of the following: increased fetal hemoglobin, circulating myeloid precursors (Fig. 45-39), a white blood cell count more than 10 × 10^9/L, presence of a clonal cytogenetic abnormality, and in vitro hypersensitivity of myeloid precursors to granulocyte-macrophage colony-stimulating factor (GM-CSF).[796] Children with neurofibromatosis type 1 (NF1) or Noonan syndrome are predisposed to JMML. Notably, several of the key molecular abnormalities contributing to JMML pathogenesis result in RAS signaling pathway hyperactivation.

Many hematopoietic growth factors transduce signals from the cell surface to the nucleus through RAS family proteins, which regulate cellular proliferation and differentiation by cycling between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate

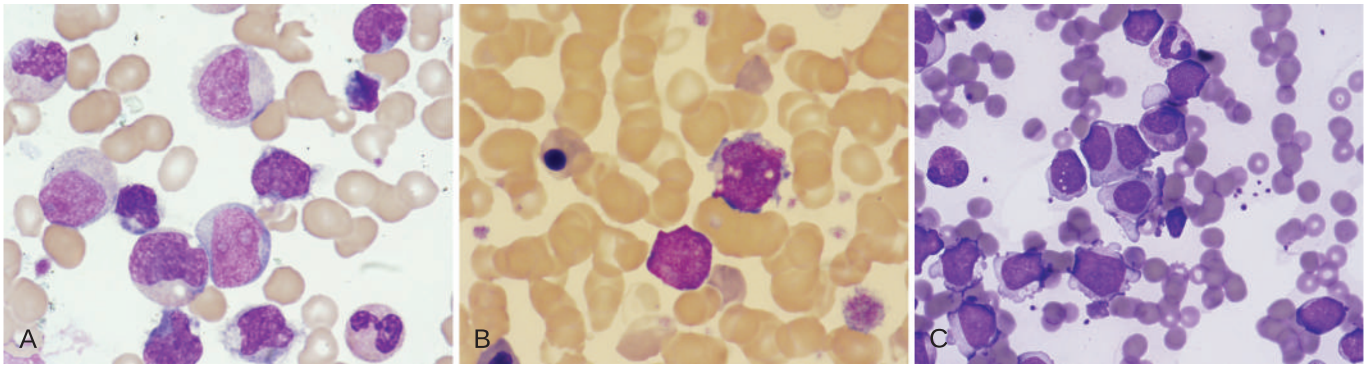

Figure 45-39 **A,** Juvenile myelomonocytic leukemia peripheral blood showing leukocytosis, monocytosis, myeloid left shift, and myelodysplasia. **B,** Down syndrome transient abnormal myelopoiesis (TAM). This peripheral blood specimen shows a megakaryoblast, nucleated red blood cell, and giant platelet. **C,** Acute megakaryoblastic leukemia (FAB M7) featuring pleomorphic blasts and cytoplasmic blebbing. *(Courtesy Marybeth Helfrich, Children's Hospital of Philadelphia.)*

(GDP)-bound states.[797,798] Somatic mutations in *RAS* oncogenes are one mechanism of RAS pathway activation and are found in 20% to 30% of JMMLs,[798,799] often involving *KRAS* or *NRAS* at codons 12, 13, or 61. Some cases of *RAS*-mutant JMML undergo spontaneous regression,[800] whereas *NRAS*-mutant JMML can progress with loss of the remaining normal *NRAS* allele.[801] Monosomy 7 is also found in some cases of JMML with *RAS* oncogene mutations.[802]

The *NF1* tumor suppressor gene product, neurofibromin, is a GTPase activating protein that accelerates GTP hydrolysis on RAS proteins.[803] Germline mutations in the *NF1* gene, often with accompanying loss of the normal *NF1* allele, result in elevated levels of GTP-bound RAS. Thus germline *NF1* mutations comprise an alternative mechanism for hyperactivating the RAS signaling pathway, predisposing to JMML.[803,804] LOH at the *NF1* locus is observed in approximately 50% of JMML arising in patients with NF1.[803] In NF1 kindreds, JMML is seen particularly in boys with maternal transmission of the mutant *NF1* allele.[805] Additional mutations in the *NF1* gene, with or without LOH, occur in sporadic JMML that is not associated with the NF1 syndrome.[806] In some of these cases, the *NF1* mutations are germline, with JMML being the presenting feature of the NF1 syndrome. Overall, it is estimated that *NF1* mutations are present in approximately 30% of all JMMLs.[806] Monosomy 7 occurs in addition to *NF1* mutation in some JMMLs.[803] However, *NF1* and *RAS* mutations in JMMLs are mutually exclusive.[806]

Noonan syndrome is an autosomal dominant disorder associated with germline mutations in the *PTPN11 (SHP2)* gene in approximately 50% of cases and is characterized by JMML, facial dysmorphology, short stature, and cardiac defects.[807] The *PTPN11* gene product, SHP2 phosphatase, regulates RAS-MAPK pathway signaling but is inactive in its baseline state.[808,809] *PTPN11* mutations in Noonan syndrome result in SHP2 activation and, consequently, hyperactivation of RAS-MAPK signaling.[808] In pediatric patients without Noonan syndrome, *PTPN11* somatic mutations can be found in sporadic cases of JMML, MDS, AML (particularly the FAB M5 subtype), and B-cell lineage ALL.[810-814] Somatically acquired *PTPN11* mutations are present in 30% to 35% of de novo JMML.[811,814] Generally, the types of *PTPN11* mutations occurring as somatic events in nonsyndromic cases are more strongly activating than the germline *PTPN11* mutations in patients with Noonan syndrome.[796,815] The fact that *PTPN11* mutations are less strongly activating in patients with Noonan syndrome might explain why syndromic JMML is associated with less severe hematologic abnormalities and can resolve spontaneously.[796,811] Thus dysregulation of the RAS signaling pathway by *RAS, NF1,* or *PTPN11* mutations is instrumental to the aberrant cell growth and differentiation in JMML, and these three mechanisms of RAS dysregulation are usually mutually exclusive.[814]

The myeloproliferative disorder known as monosomy 7 syndrome has clinical overlap with JMML and is characterized by myelodysplasia, thrombocytopenia, anemia, and hepatosplenomegaly.[816] However, this disorder is not included in the more recent classification system for pediatric myelodysplastic and myeloproliferative diseases.[795]

Chronic Myelomonocytic Leukemia. Chronic myelomonocytic leukemia (CMML) is a clonal bone marrow stem cell disorder characterized by monocytosis, myelodysplasia, and absence of *BCR-ABL* fusion.[41,603] t(5;12)(q33;p13), which generates the *TEL-PDGFRB* fusion product, is a recurring but rare rearrangement in CMML.[603] The case in which the fusion transcript was first described occurred in an adolescent patient.[817] t(5;12) is associated with eosinophilia and progression to AML on acquisition of additional cytogenetic changes.[817] However, only secondary and not de novo CMML is included as a diagnostic category in the pediatric adaptation of the WHO classification of myelodysplastic and myeloproliferative diseases.[795]

Transient Abnormal Myelopoiesis and Acute Megakaryoblastic Leukemia of Down Syndrome. Ten to 20 percent of neonates with Down syndrome are affected with transient abnormal myelopoiesis (TAM), and 20% to 30% of TAM will progress to AMKL by 4 years of age (see Fig. 45-39).[818-820] Several laboratories have identified truncating *GATA1* point mutations as in utero mutational oncogenic events that initiate both TAM and AMKL.[818,821] GATA1 is a hematopoietic transcription factor that, via interactions with the FOG1 cofactor and GATA2, regulates erythroid and megakaryocyte maturation.[818] However, whereas the *GATA1* mutation is sufficient for TAM, postnatal secondary changes such as *TP53* mutation and/or trisomy 8 are required for subsequent clonal expansion, a prerequisite for disease progression to AMKL.[822] In many cases of TAM, however, there is no disease progression because the clone with *GATA1* mutation is ultimately extinguished in the absence of requisite secondary alterations.[818] The TAM and AMKL in Down syndrome have distinct gene expression profiles.[819]

Several chromosome 21 genes, including *CBS* (cystathionine beta-synthase), at band 21q22 may contribute to the unique forms of leukemogenesis in Down syndrome.[823] The *CBS* gene is overexpressed, above and beyond the levels predicted based on the trisomy 21 increased gene dosage. This upregulation seems to result from mechanisms involving transcriptional upregulation through the *CBS* promoter.[823] Consequences of *CBS* overexpression include reduced levels of plasma homocysteine, methionine, *S*-adenosylmethionine and *S*-adenosylhomocysteine, resulting in altered CpG methylation and consequent dysregulation of gene expression. Other effects include perturbed folate metabolism and genetic instability (increased DNA mutations), which may predispose to accumulation of the secondary genetic mutations in AMKL.[823] Another consequence of *CBS* overexpression is increased cytarabine sensitivity,[823] which contributes to increased treatment-related toxicities and to the more favorable outcome associated with myeloid leukemia of Down syndrome.[824]

t(1;22)(p13;q13), which fuses *RBM15 (OTT)* and *MLK1 (MAL),* is associated with non–Down syndrome

AMKL, another distinct form of AMKL peculiar to neonates.[134,503,825]

Myelodysplastic Syndrome. The three MDS morphologic subtypes of greatest relevance in pediatrics are refractory cytopenia, refractory anemia with excess blasts (RAEB), and RAEB in transformation (RAEB-T).[796] Monosomy 7 or del(7q) occurs in approximately 40% of cases and is the most commonly acquired cytogenetic aberration in pediatric MDS.[826] In cases of refractory cytopenia, monosomy 7 is associated with disease progression and poor outcome.[795,827] Similarly, monosomy 7, with or without other cytogenetic abnormalities, is a frequent finding in RAEB-T.[826] Despite the generally poor prognosis of −7/del(7q) in MDS, spontaneous and durable hematologic remissions have been described in some children diagnosed with MDS characterized by this cytogenetic feature.[828] Rarely, MDS with monosomy 7 is familial and observed in siblings.[805,829] In one study of refractory anemia of childhood, which is a form of refractory cytopenia, cytogenetic analyses have demonstrated monosomy 7 in 49% of cases, whereas trisomy 8 was the next most common abnormality, found in 9% of cases.[827] The 5q− syndrome associated with an indolent refractory anemia but with preservation of megakaryopoiesis and myelopoiesis[756] has not been described in children.[795] FISH analysis with chromosome-specific probes in combination with immunophenotyping has enabled characterization of the cell lineages affected by the clonal aberrations in MDS[830] as well as evaluation of the lineages affected by MDS clonal expansion.[831] MDS secondary to prior therapy has been discussed earlier. As is true for adults,[734] pediatric patients with MDS secondary to cytotoxic therapies (see earlier) often have chromosome 5 and 7 abnormalities.[832] Constitutional disorders associated with congenital bone marrow failure syndromes and acquired aplastic anemia are included in the differential diagnosis of MDS in children.[795]

Other Clinically Important Genomic Aberrations in Myeloid Disorders

It has been proposed that in addition to the major chromosomal aberrations that underlie the various AML morphologic subtypes, cooperating mutations affecting proliferation and differentiation are essential in AML pathogenesis[503] and may affect prognosis and treatment. The balanced chromosomal translocations that disrupt major hematopoietic transcription factor genes are generally regarded as the primary molecular alterations in the associated AML morphologic subsets. Translocations that disrupt major hematopoietic transcription factor genes generally alter differentiation,[503] whereas additional aberrations, including kinase or RAS-RAF pathway gene mutations, often contribute to hyperproliferative behavior.

***FLT3* Alterations.** Although less common than in adult AML, *FLT3* mutations are nonetheless found in a considerable minority of pediatric AML cases, with juxtamembrane region ITD and activation loop point mutations identified in 12% to 15% and 7% to 9% of pediatric cases, respectively.[565,833,834] Indeed, *FLT3* mutations might represent the most frequently occurring molecular abnormalities in AML. *FLT3* mutations are common in pediatric AML with *MLL* translocations; 23% of *MLL*-rearranged AML in one large series harbored either *FLT3*-ITD or kinase domain mutations.[565] Also, *FLT3* mutations are particularly common in AMLs with normal karyotypes, in which they are demonstrable in up to 60% of cases.[565] Another subset of AML features autocrine FLT3 activation caused by the expression of FLT3 ligand, resulting in constitutive FLT3 phosphorylation and signaling.[834] *FLT3*-ITD mutations in pediatric AML are associated with poor outcomes, similar to those in adult cases.[834-836] An increased *FLT3*-ITD allelic ratio is associated with relapse, and molecular diagnostic tests have been developed to detect *FLT3* mutations and quantify the *FLT3*-ITD allelic ratio.[837,838] *FLT3*-ITD mutations occur in approximately 30% of childhood APL.[837] *FLT3*-ITD mutations, although common in de novo AML, are infrequent in treatment-related cases, as reported in an adult series.[839]

***KIT* Mutations.** Mutations in the *KIT* receptor tyrosine kinase gene are also common in pediatric AML and occur most often in cases with t(8;21) or inv(16), which disrupt DNA or non–DNA-binding subunits of CBF.[840] The *KIT* mutations are associated with constitutive, ligand-independent activation of the KIT protein. One recent study has estimated that 37% of all AML in children with either t(8;21) or inv(16) harbor *KIT* mutations. *KIT* mutations do not affect prognosis but are nonetheless significant because preclinical studies have indicated that AML with *KIT* mutations are sensitive to imatinib mesylate and FLT3 inhibition.[840]

***RAS* Mutations.** It has long been recognized that the *RAS* oncogene family members can be activated by mutations in myeloid neoplasms,[841] although these mutations do not usually have prognostic relevance.[840] Mutations of *NRAS* or *KRAS* codons 12, 13, or 16 have been described in approximately 20% of pediatric AMLs with *MLL* translocation[842] and in approximately 30% of pediatric AMLs with t(8;21) or inv(16).[840] *RAS* mutations are also found in about one third of MDS with monosomy 7.[843] In secondary AML, *RAS* mutations are less common than in de novo disease[844,845] but are seen more often in alkylating agent–related cases, especially cases with monosomy 7, than in AML arising after exposure to TOP2 poisons. Additional evidence of RAS pathway oncogenic roles is seen in the germline *RAF1* mutations in adult therapy-related AML.[846] RAF1 is activated by RAS binding and participates in RAS signaling.

***PTPN11* Mutations.** The RAS/RAF/MEK/ERK signaling pathway can also be activated by mutations in the SHP2 phosphatase gene, *PTPN11*. Although somatic *PTPN11* mutations are uncommon, and occur in only approximately 4% of childhood AMLs overall, *PTPN11* mutations have a predilection for the FAB M5 morphologic subtype, in which they are found in approximately 18% of cases.[813]

PI3K/AKT/mTOR Pathway Aberrations. The PI3K/AKT/mTOR pathway is a signaling pathway that promotes cell proliferation and survival in leukemia. In adults, constitutive AKT activation, leading to downstream mTOR activation, contributes to the progression of MDS to AML.[847,848]

PTEN is a tumor suppressor protein that inhibits the PI3K/AKT/mTOR signaling pathway. Although *PTEN* gene mutations and LOH are uncommon in AML, aberrant *PTEN* mRNA splicing has been demonstrated, primarily in adult AML.[849] Constitutive PTEN phosphorylation, which results in PTEN inactivation, may contribute to AML,[850,851] whereas reduced PTEN expression may be important in MDS progression.[847] These oncogenic mechanisms may impede normal PTEN functions, which include not only attenuation of PI3K survival signaling but also promotion of hematopoietic stem cell quiescence and preservation of the self-renewal capacity of the hematopoietic stem cell compartment.[852]

***TP53* Mutations.** TP53 is a crucial cell cycle regulatory tumor suppressor protein that senses and responds to DNA damage by regulation of the G1-S cell cycle checkpoint. *TP53* mutations are features of alkylating agent–related leukemias in adults and children.[16,746,845,853] In contrast to *RAS* mutations, *TP53* mutations are more common in therapy-related than de novo cases.[845] Segmental jumping translocations are chromosomal abnormalities in which multiple copies of various oncogenes are dispersed extrachromosomally and throughout the genome.[16] Gene amplification accompanies loss of wild-type TP53 after exposure to genotoxic agents, and *TP53* mutations and *MLL* segmental jumping translocations are strongly associated, with both occurring after alkylating agent treatment.[746] The *TP53* mutations may be of germline origin.[16,854] Similarly, *TP53* gene mutations are associated with *AML1* gene amplification or duplication in MDS and AML after alkylating agent treatment.[855] *TP53* mutations occur in de novo AML in patients affected with the Li-Fraumeni syndrome.[67,856]

***NPM1* (Nucleophosmin) Mutations.** The *NPM1* gene encodes a nucleolar shuttle protein with multiple functions in protein trafficking, preribosomal assembly, and ARF-TP53 pathway regulation.[857,858] t(5;17), a variant translocation in APL, involves fusion of the *NPM1* gene to *RARA*,[628] and *NPM1* is fused to *ALK* in a subset of anaplastic large cell lymphomas.[859] Identification of *NPM1* point mutations in adult AML without karyotypic abnormalities has led to the recent analysis and discovery of *NPM1* mutations in childhood AML. The mutations cause loss of nucleolar localization with accumulation of nucleophosmin in the cytoplasm. Leukemic cells with *NPM1* mutations have a particular gene expression signature of *HOXB2, HOXB3, HOXB6,* and *HOXD4* upregulation.[860] In contrast to adult AML, in which *NPM1* mutations are present in 25% to 35% of cases, and even in up to 60% of cases with a normal karyotype, the frequency of *NPM1* mutations in pediatric AML is only 7% to 8%. Similar to adult AML, however, *NPM1* mutations are concentrated in patients with normal cytogenetics and, in the absence of *FLT3*-ITD mutations, *NPM1* mutations are associated with an especially good prognosis.[857,858] *NPM1* mutations in AML are more common in older than younger children and in girls than boys.[857] In addition, *NPM1* mutations are significant because they may facilitate risk stratification and allow for minimal residual disease detection in cases in which no other molecular cytogenetic markers are available.[858]

Monosomy 7 and del(7q). Monosomy 7 or del(7q) occurs with or without other cytogenetic aberrations in only 4% to 5% of pediatric AMLs but in 40% of pediatric MDSs.[826] Because of the infrequency of these aberrations, an international retrospective study was conducted to characterize −7 and del(7q) in pediatric AML and MDS. del(7q) is more common than −7 in AML cases with favorable cytogenetic features, such as t(8;21), inv(16), t(15;17), or t(9;11), whereas −7 is more common in cases with inv(3), t(9;22), i(17q), or +21. Further subgroup analysis, which was enabled by the size of the study, has demonstrated that −7 is associated with inferior survival compared with del(7q) and that survival is worse in the del(7q) subgroup without other favorable cytogenetic features compared with the del(7q) group having other favorable cytogenetic features.[826]

***IDH1* and *IDH2* Mutations.** A novel type of mutations in AML affects the isocitrate dehydrogenase 1 *(IDH1)* and isocitrate dehydrogenase 2 *(IDH2)* genes, which encode the metabolic enzymes responsible for conversion of isocitrate to α-ketoglutarate.[861] These mutations result in the production of an aberrant oncometabolite 2-hydroxyglutarate. They are associated with a global pattern of DNA hypermethylation because of the dependence of *TET2* on α-ketoglutarate and the resultant impaired oxidation of 5mC by the TET2 enzyme.[862] Somatic *IDH1* and *IDH2* mutations characterize15% to 23% of cases of adult AML, and they occur in AML cases in which there are few other molecular cytogenetic abnormalities.[861,863] A genomic classifier in adult AML has suggested that the co-occurrence of *IDH1* or *IDH2* mutation with *NPM* mutation in absence of the *FLT3*-ITD predicts a favorable prognosis.[863] However, in pediatric AML, *IDH1* and *IDH2* mutations appear to be much less common. In studies conducted by the Children's Oncology Group, there were no *IDH1* mutations[864] and *IDH2* mutations were detected in 2.2% of cases, in which accompanying t(8;21), MLL translocation, or *NPM* mutation was detected.[865] In another study, two *IDH1* and one *IDH2* mutations were detected in 206 cases of pediatric AML. Interestingly, both *IDH1* mutations occurred in cases with MLL–partial tandem duplication of FAB M0 morphology.[866]

***TET2* Mutations.** The TET2 DNA methylase enzyme catalyzes the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) in a reaction that is dependent on α-ketoglutarate.[862] Circos maps have suggested that *IDH1* or *IDH2* mutations and *TET2* mutations in adult AML are mutually exclusive.[862] Both *IDH1* and *IDH2* mutations as well as *TET2* mutations are associated with impaired hematopoietic differentiation programs.[862] *TET2* mutations have been reported in 6% of pediatric AML, a frequency more similar to that in adult AML.[863,865] In a second pediatric study, their frequency was only 1.7% and they were found with *FLT3*-TKD, *FLT3*-ITD plus *CEBPα*, and *KRAS* plus *WT1* mutations.[866] *TET2* mutations in adult AML are most often associated with an intermediate prognosis.[862] The results of the latter pediatric study, which also examined the mutation frequency in *ASXL1* (1.1%) and *DNMT3* (0), altogether indicate that the occurrence of mutations in genes whose products regulate DNA methylation and histone modification is less in pediatric AML than in AML in adults, but also that, when present, there are more accompanying molecular cytogenetic aberrations in the pediatric cases.[866]

MicroRNA Abnormalities

Levels of a particular microRNA, miR-223, are low in AML with the t(8;21) translocation.[597] The underlying mechanism was shown to involve epigenetic transcriptional silencing of miR-223 by the AML1-ETO fusion oncoprotein through the recruitment of several chromatin-remodeling enzymes, including DNA methyltransferases and HDAC1 to the pre–miR-223 gene.[867] The ages of the patients studied were not specified (they were presumably adults), but t(8;21) is a feature of AML in adult and pediatric patients.

CONCLUSION

It is now possible to characterize and understand the cytogenetic and molecular pathobiology of the many forms of pediatric cancer in unprecedented detail using a panoply of approaches starting with decades-old conventional cytogenetic analysis of larger chromosomal aberrations that have withstood the test of time, advanced genomic methods reliant on the many derivatives of recombinant DNA technologies that followed later on and, now, next-generation sequencing of the whole transcriptome, genome, and epigenome, not only globally but within single cells. The ascertainment of cancer gene alterations and the cytogenetic and molecular subclassification of the many pediatric cancers outlined in this chapter is significant because the disruption of particular oncogenic pathways has prognostic and therapeutic relevance and is leading to the discovery of therapeutic targets that provide opportunities for new directed treatments.

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CHAPTER 46

Cancer Chemotherapy for Pediatric Patients

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CHAPTER OUTLINE

CHEMOTHERAPEUTIC AGENTS
CLINICAL APPLICATION OF CHEMOTHERAPY
CHEMOTHERAPY IN CHILDREN
PHARMACOKINETIC CHANGES DURING CHILDHOOD
Absorption
Volume of Distribution
Hepatic Metabolism
Renal and Hepatic Excretion
Influence of Underlying Disease on Metabolism
Unique Drug Effects in Pediatric Patients
DRUG INTERACTIONS
Combination Chemotherapy
Coadministration of Non-Anticancer Drugs
Coadministration of Complementary and Alternative Medicine
PHARMACOGENETICS AND PHARMACOGENOMICS OF CHEMOTHERAPEUTIC AGENTS IN CHILDREN
Thiopurines
Methotrexate
Glucocorticoids
Asparaginase
Vincristine
Anthracyclines
Cytarabine
Imatinib Mesylate
Etoposide
Cyclophosphamide
Platinum Compounds
Irinotecan
Temozolomide
Pharmacogenetics, Pharmacogenomics, and the Future
NEW DRUG DEVELOPMENT
Preclinical Development
Choice of Starting Dose for Clinical Studies
Dose Escalation Schemes
Parameter Estimates
STRATEGIES TO IMPROVE THE THERAPEUTIC INDEX
Dosage Based on Body Surface Area
Pathophysiologic Status
Dose Adaptation Using Pharmacokinetic-Pharmacodynamic-Pharmacogenetic Principles
CONCLUSION AND PERSPECTIVES

In the past two decades we have seen substantial advances in the number of available chemotherapeutic agents to treat a variety of neoplastic diseases, as well as in the discovery and development of new anticancer drugs, while our understanding of the biology and genetics of cancer continues to grow.[1,2,3,4] The tools at our disposal now include classes of molecules with highly distinct structures and innovative mechanisms of action and an impressive array of new techniques and novel uses for existing agents. In this chapter we will review the principles of chemotherapy in children, discuss the factors that influence individual responses to chemotherapy, and describe how pharmacokinetic, pharmacodynamic, and/or pharmacogenetic principles are used when conducting clinical trials that provide therapy tailored to individual patients.

CHEMOTHERAPEUTIC AGENTS

Chemotherapeutic agents used to treat pediatric cancer are generally similar to those used with adults, including conventional cytotoxic drugs and molecularly targeted agents. The former class can be subdivided into two large groups based on the dependence of their mechanism of action on the cell cycle. Cell cycle–nonspecific agents, which include alkylating agents, platinum agents, and most antitumor antibiotics, kill both quiescent and cycling tumor cells. Cell cycle–specific agents include antimetabolites and agents that interact with tubulin and topoisomerases. Conventional cytotoxic agents can target both malignant and normal cells and often cause adverse effects. During the past decade, the development of molecularly targeted chemotherapy has been revolutionized by a greater understanding of the biologic and genetic features of malignant disease.[2-4] Table 46-1 provides an overview of the drugs used to treat childhood cancers and their key pharmacologic features.

CLINICAL APPLICATION OF CHEMOTHERAPY

Chemotherapy has four major roles in the management of cancer: (1) primary cytoreduction therapy, (2) adjuvant therapy, (3) neoadjuvant therapy, and (4) site-directed therapy. Primary cytoreduction therapy is used when no superior alternative treatment is available (such as surgery and radiation therapy). Hematologic malignancies and some solid tumors of childhood are significantly chemosensitive and can be cured by serial courses of chemotherapy (e.g., induction, consolidation, and continuation therapy for acute lymphoblastic leukemia [ALL]). The chemosensitive malignancies also include acute myeloid leukemia (AML), lymphomas (both non-Hodgkin and Hodgkin), germ cell tumors, Wilms tumor, and embryonal rhabdomyosarcoma. Chemotherapy can also be used as an adjuvant therapy, wherein systemic treatment is applied to enhance local tumor control and to eradicate occult disseminated disease after surgery and/or radiation therapy. Occult residual disease at original sites and micrometastases are frequently present even after

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TABLE 46-1 Key Features of Drugs Used in Pediatric Oncology

Drug (Trade Name; Chemical Class)	Mechanism of Action	Antitumor Activity	Route of Administration	Primary Pathway of Elimination	Enzymes and Transporters	Principal Adverse Effects
Alkylating Agents						
Mechlorethamine (Mustargen; nitrogen mustard, mustine, HN2)	Alkylation; DNA cross-linking	Hodgkin lymphoma (MOPP)	IV injection	Hydrolysis; liver	*N*-demethylation (CYP)	A, M, N&V, Mu, HU, Derm, V, Phleb, G, SM
Melphalan (Alkeran; mechlorethamine analogue)	Alkylation; DNA cross-linking	HDCT	IV injection or infusion; oral	Hydrolysis; renal	GSTP1, LAT1, ABCB1	A, M, N&V, D, Mu, Derm, Vas, Phleb, SIADH, G, SM
Chlorambucil (Leukeran; mechlorethamine analogue)	Alkylation; DNA cross-linking	Hodgkin lymphoma (ChlVPP)	Oral	Hydrolysis	GSTP1, ABCC2	A, M, N&V, HU, P, S, G, SM
Cyclophosphamide (Cytoxan; mechlorethamine analogue)	Alkylation (prodrug); DNA cross-linking	Leukemias, lymphomas, neuroblastoma, sarcoma	IV infusion; oral	Hydrolysis; liver	CYP2B6, CYP2C9, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1, ALDH3A1	A, M, N&V, Cy, SIADH, C, P, G, SM
Ifosfamide (Ifex; mechlorethamine analogue)	Alkylation (prodrug); DNA cross-linking	Sarcomas, germ cell	IV infusion	Hydrolysis; liver	CYP2A6, CYP2B6, CYP3A4, CYP2C19, OCT2	A, M, N&V, Met, Cy, SIADH, NT, C, P, R, G, SM
Bendamustine (Treanda)	Alkylation; DNA cross-linking; blocks purine synthesis	Hodgkin lymphoma, lymphoma, leukemia	IV infusion	Liver	CYP1A2	M, N&V, H, P, PE, NT
Thiotepa (Thioplex; aziridine)	Alkylation; DNA cross-linking	HDCT	IV injection or infusion; intrathecal, intravesical	Liver	NA	A, M, N&V, HU, Derm, Cy, G, SM
Busulfan (Myleran; alkyl alkane sulfonate)	Alkylation; DNA cross-linking	HDCT	IV infusion; oral	Conjugation with glutathione; liver	CYP3A4, GSTA1, GSTP1	A, M, N&V, Mu, Derm, HU, AI, NT, VOD, P, S, G, SM
Carmustine (BiCNU; BCNU, nitrosourea)	Alkylation; DNA cross-linking; carbamoylation	Brain tumors, Hodgkin lymphoma, lymphoma	IV infusion; wafer implant (glioblastoma)	Chemical hydrolysis; liver	CYP	M, N&V, Phleb, S (wafer), C, H, P, R, G, SM

TABLE 46-1 Key Features of Drugs Used in Pediatric Oncology (Continued)

Drug (Trade Name; Chemical Class)	Mechanism of Action	Antitumor Activity	Route of Administration	Primary Pathway of Elimination	Enzymes and Transporters	Principal Adverse Effects
Lomustine (CeeNU; CCNU, nitrosourea)	Alkylation; DNA cross-linking; carbamoylation	Brain tumors, Hodgkin lymphoma, lymphoma	Oral	Chemical hydrolysis; liver	CYP2D6	M, N&V, NT, C, H, P, R, G, SM
Dacarbazine (DTIC, DTIC-Dome; imidazole-carboxamide)	Methylation inhibition (prodrug)	Hodgkin lymphoma, neuroblastoma, sarcomas	IV infusion	Chemical hydrolysis; liver, renal	N-demethylation (CYP); CYP1A2, CYP2E1	M, N&V, Flu, H
Procarbazine (Mutulane; imidazole-carboxamide)	Methylation inhibition (prodrug)	Brain tumors; Hodgkin lymphoma (ChlVPP)	Oral	Chemical hydrolysis; liver, renal	CYP, MAO	M, N&V, Mu, Flu, NT, Derm, G, SM
Temozolomide (Temodar; imidazole-carboxamide)	Methylation inhibition (prodrug)	Brain tumors	Oral	Chemical hydrolysis	CYP (minor)	M, N&V, NT, PE, H
Antimetabolites						
Cytarabine (Cytosar; Ara-C, cytosine arabinoside; cytidine analogue)	Incorporated into DNA; inhibits DNA polymerase; terminates DNA chain elongation	Leukemias	IV injection or infusion; intrathecal; SC	Activated to triphosphate; deamination	Cytidine deaminase, CNT1, ENT1, ENT2, ABCC4, ABCC11	M, N&V, Mu, GI, HU, Derm, HFS, PE, Conj, NT, H, PE
Azacytidine (Vidaza; cytidine analogue)	Hypomethylation of DNA (low dose)	MDS, AML	SC	Activated to triphosphate; chemical degradation; deamination	Cytidine deaminase	M, N&V, F, Mu, D, Inj, Derm
Decitabine (Dacogen; cytidine analogue)	Hypomethylation of DNA (low dose)	MDS, AML	IV infusion	Activated to triphosphate; chemical degradation; deamination	Cytidine deaminase	M, N&V, F, Mu, D, Derm
Gemcitabine (Gemzar; cytidine analogue)	Incorporated into DNA; inhibits DNA polymerase; terminates DNA chain elongation; inhibits ribonucleotide reductase (prodrug)	Solid tumors, lymphoma	IV infusion	Activated to triphosphate; deamination	Cytidine deaminase, CNT1, ENT1	M, N&V, Mu, D, F, Flu, Derm, NT, PE, H, R
Cladribine (Leustatin; adenosine analogue)	Incorporated into DNA; inhibits DNA polymerase; terminates DNA chain elongation; inhibits ribonucleotide reductase (prodrug)	Leukemias	IV infusion	Activated to triphosphate; renal; liver	Oxidative cleavage, oxidation, conjugation, CNT1, CNT2, CNT3, ENT1, ABCG2	M, N&V, D, Derm, NT

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TABLE 46-1 Key Features of Drugs Used in Pediatric Oncology (Continued)

Drug (Trade Name; Chemical Class)	Mechanism of Action	Antitumor Activity	Route of Administration	Primary Pathway of Elimination	Enzymes and Transporters	Principal Adverse Effects
Clofarabine (Clolar; adenosine analogue)	Incorporated into DNA; inhibits DNA polymerase; terminates DNA chain elongation; inhibits ribonucleotide reductase (prodrug)	Leukemias	IV infusion	Activated to triphosphate; extrahepatic	NA	M, N&V, D, C, H, R
Fludarabine (Fludara; adenosine analogue)	Incorporated into DNA; inhibits DNA polymerase; terminates DNA chain elongation; inhibits ribonucleotide reductase (prodrug)	AML, CML, indolent lymphomas	IV infusion	Activated to triphosphate; renal	NA	M, HA, Derm, fever, H, NT, TLS
Nelarabine (Arranon)	Incorporated into DNA; inhibits DNA synthesis	ALL	IV infusion	Activated to ara-guanine triphosphate; liver, renal	Adenosine deaminase	M, N&V, NT, P, PE
6-Mercaptopurine (Purinethol; hypoxanthine analogue)	Incorporated into DNA, RNA; blocks purine synthesis (prodrug)	ALL	Oral	Liver, renal (high dose)	TPMT, HGPRT, ENT2, OAT2, OAT3, ABCC4	M, H, Mu, N&V, HU, crystalluria, GI
6-Thioguanine (guanine analogue)	Incorporated into DNA, RNA; blocks purine synthesis (prodrug)	ALL, AML	Oral	Liver	TPMT, HGPRT, CNT3, ENT2, OAT3, ABCC5	M, H, Mu, N&V, HU, crystalluria
Hydroxyurea (Hydrea, Droxia)	Interferes with synthesis of DNA synthesis; inhibits ribonucleoside diphosphate reductase	ALL, AML	Oral	Liver, renal	OATP1B3, UT-A, UT-B1	M, H, Mu, N&V
5-Fluorouracil (fluoropyrimidine)	Incorporated into RNA, DNA; inhibits thymidine synthase (prodrug of 5′-DFUR)	Carcinomas, hepatic tumors	IV injection or infusion	Liver	DPD, GSTP1, hNT1, OAT2,	M, Mu, N&V, HFS, D, Derm, NT, C
Capecitabine (Xeloda; fluoropyrimidine)	Incorporated into RNA, DNA; inhibits thymidine synthase (prodrug of 5′-DFUR)	Colorectal cancer (in development)	Oral	Urine	CES2, CES1A1, cytidine deaminase, thymidine phosphorylase, DPD, CNT1, ABCA1	M, Mu, N&V, HFS, D, Derm, NT, C

TABLE 46-1 Key Features of Drugs Used in Pediatric Oncology (Continued)

Drug (Trade Name; Chemical Class)	**Mechanism of Action**	**Antitumor Activity**	**Route of Administration**	**Primary Pathway of Elimination**	**Enzymes and Transporters**	**Principal Adverse Effects**
Methotrexate (Trexall; antifolate)	Interferes with folate metabolism	Leukemias, lymphomas, osteosarcoma	Oral; IM; SC; IV injection; intrathecal	Renal	FPGS, GGH, OAT1, OAT2, OAT3, OAT4, OATP1A2, OATP1B1, OATP1B3, OATP4C1, RFC, PCFT, ABCB1, ABCC1, ABCC2, ABCC3, ABCC4, ABCG2	M, Mu, Derm, H, R, NT
Pemetrexed (Alimta; antifolate)	Interferes with folate metabolism	Various (in development)	IV infusion	Renal	NA	M, fatigue, Mu, HFS, Derm
Antitumor Antibiotics						
Bleomycin (Blenoxane)	Free radical–mediated DNA strand breaks	Lymphomas, germ cell tumor	IV injection; IM, SC	Renal	OCT6	P, Derm, fever, Mu, A, HSR, N&V
Dactinomycin (Cosmegen)	DNA cross-linking	Solid tumors	IV injection	Renal, liver	NA	M, Mu, N&V, D, A, H, V
Mitomycin C (Neulasta)	DNA cross-linking	Carcinomas	IV injection or infusion; intravesical	Liver	ABCB1	M, N&V, Mu, HU, anemia, VOD
Daunorubicin (Daunomycin; anthracycline)	DNA strand breaks (topo II)	ALL, AML, lymphomas	IV injection or infusion	Renal, liver	OCTN1, ABCB1, aldoketoreductase	M, Mu, N&V, D, A, C, V
Doxorubicin (Adriamycin; anthracycline)	DNA strand breaks (topo II)	ALL, AML, lymphomas, solid tumors	IV infusion	Liver	CYP2D6, CYP3A4, GSTP1, OCT6, OCTN1, ABCB1, ABCB5, ABCG2, aldoketoreductase	M, Mu, N&V, A, D, C, V
Epirubicin (Ellence; anthracycline)	DNA strand breaks (topo II)	Solid tumors, leukemia (in development)	IV infusion	Renal, liver	ABCB1, ABCC1, ABCG2, aldoketoreductase	M, Mu, N&V, A, C, V
Idarubicin (Idamycin; anthracycline)	DNA strand breaks (topo II)	ALL, AML, lymphomas	IV injection or infusion; oral	Renal, liver	ABCB1, aldoketoreductase	M, Mu, N&V, D, A, C, V
Mitoxantrone (Novantrone; anthracenedione)	DNA strand breaks (topo II)	ALL, AML, lymphomas	IV infusion; IP	Renal, liver	OCTN1, ABCB1, ABCG2	M, Mu, N&V, A, urine, veins and nail discoloration
Asparaginases						
l-Asparaginase (Elspar, Kidrolase; native asparaginase from *Escherichia coli*)	Depletion of plasma asparagine	ALL, lymphomas	IV infusion (>30 min), IM	Nonrenal	NA	HSR, thrombosis, GI, glucose intolerance, coagulopathy, hyperglycemia, H
Crisantaspase (Erwinase; Erwinia l-asparaginase, native asparaginase from *Erwinia chrysanthemi*)	Depletion of plasma asparagine	ALL, lymphomas	IV infusion (>30 min), IM	Nonrenal	NA	HSR, thrombosis, GI, glucose intolerance, coagulopathy, hyperglycemia, H

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TABLE 46-1 Key Features of Drugs Used in Pediatric Oncology (Continued)

Drug (Trade Name; Chemical Class)	Mechanism of Action	Antitumor Activity	Route of Administration	Primary Pathway of Elimination	Enzymes and Transporters	Principal Adverse Effects
Pegasparaginase (Oncaspar; PEG-asparaginase, from *E. coli*)	Depletion of plasma asparagine	ALL, lymphomas	IV infusion (1-2 hours)	Nonrenal	NA	HSR, thrombosis, GI, glucose intolerance, coagulopathy, hyperglycemia, H
Corticosteroids						
Dexamethasone (Decadron)	Receptor-mediated lympholysis	Leukemias, lymphomas	Oral, IV injection; IM	Liver	CYP3A4, ABCB1	Muscle weakness, osteoporosis, Derm, hypertension, N&V, headache
Prednisolone (Orapred OTD, Pediapred)	Receptor-mediated lympholysis	Leukemias, lymphomas	Oral; IV injection	Renal, liver	CYP3A4, sulfation, glucuronidation	Muscle weakness, osteoporosis, Derm, hypertension, N&V, headache
Prednisone (Deltasone)	Receptor-mediated lympholysis (prodrug)	Leukemias, lymphomas	Oral	Renal, liver	CYP3A4, sulfation, glucuronidation	Muscle weakness, osteoporosis, Derm, hypertension, N&V, headache
Platinum Compounds						
Carboplatin (Paraplatin)	Platination; DNA cross-linking	Brain tumors, neuroblastoma, sarcomas	IV infusion	Renal	CTR1, ATP7A	M, N&V, NT, EA, HSR
Cisplatin (Platinol)	Platination; DNA cross-linking	Testicular, brain tumors, osteosarcoma, neuroblastoma	IV infusion	Renal	GSTP1, OCT1, OCT2, CTR1, ABCC2, ABCC4, ATP7B, MATE1	N&V, R, NT, M, EA, O, HSR
Oxaliplatin (Eloxatin)	Platination; DNA cross-linking	Colorectal cancer, lymphoma (in development)	IV infusion	Renal	GSTP1, OCT1, OCT2, OCT3, OCTN1, OCTN2, MATE1, MATE2-K	NT, N&V, D, M, R, HSR
Retinoids						
All-*trans*-retinoic acid (ATRA; Vesanoid; tretinoin; retinol derivative)	Differentiation agent	Acute promyelocytic leukemia	Oral	Liver	CYP2C8, CYP2A6, CYP2B6, CYP2C9	Retinoic acid syndrome, pseudotumor cerebri, cheilitis
13-*cis*-retinoic acid (Accutane; retinol derivative)	Differentiation agent	Neuroblastoma	Oral	Liver	CYP2B6, CYP2C8, CYP2C9, CYP3A4/5, CYP2A6, CYP2D6	Cheilitis, conjunctivitis, dry mouth, xerosis, pruritus, headache

TABLE 46-1 Key Features of Drugs Used in Pediatric Oncology (Continued)

Drug (Trade Name; Chemical Class)	Mechanism of Action	Antitumor Activity	Route of Administration	Primary Pathway of Elimination	Enzymes and Transporters	Principal Adverse Effects
Topoisomerase-Interactive Agents						
Etoposide (VePesid; VP-16)	DNA strand breaks (topo II)	ALL, AML, lymphomas, neuroblastoma, sarcomas, brain tumors	IV infusion	Renal, liver	CYP3A4, CYP3A5, CYP1A2, CYP2E1, UGT1A1, OCTN2, OATP1B1, ABCB1, ABCC1, ABCC2	M, N&V, hypotension, HSR, SM
Teniposide (Vumon; VM-26)	DNA strand breaks (topo II)	ALL	IV infusion; oral	Liver	CYP3A4, ABCB1	M, N&V, HSR, hypotension, SM
Irinotecan (Camptosar; CPT-11; camptothecin analogue)	DNA strand breaks (topo I; prodrug of SN-38)	Rhabdomyosarcoma, solid tumors	IV infusion	Renal, liver	CYP3A4, CYP3A5, CYP2B6, CES2, UGT1A1, OATP1B1, ABCB1, ABCC2, ABCC4, ABCG2,	M, D, N&V, A, H
Topotecan (Hycamptin; camptothecin analogue)	DNA strand breaks (topo I)	Neuroblastoma; rhabdomyosarcoma	IV infusion	Renal	OAT3, ABCB1, ABCC4, ABCG2	M, Mu, D, N&V, A, Derm, H
Tubulin-Interactive Agents						
Docetaxel (Taxotere; taxanes)	Microtubule depolarization inhibitor	Solid tumors	IV infusion	Liver	CYP3A4/5, CYP2C8, OAT2, OATP1B3, ABCB1, ABCC2, ABCC10	M, HSR, A, NT, Derm, Mu
Paclitaxel (Taxol; Abraxane; taxane)	Microtubule depolarization inhibitor	Solid tumors	IV infusion	Liver	CYP2C8, CYP2C9, CYP3A4/5, OAT2, OATP1B1, OATP1B3, ABCB1, ABCC2, ABCC10	M, HSR, A, NT, M, C
Vinblastine (Velban; *Vinca* alkaloid)	Microtubule polarization inhibitor	Histiocytosis, Hodgkin lymphoma, testicular cancer	IV push or infusion	Liver	CYP3A4, CYP2D6, ABCB1, ABCC1, ABCC2, ABCC10, ABCG2	M, A, Mu, NT, V
Vincristine (Oncovin; *Vinca* alkaloid)	Microtubule polarization inhibitor	ALL, lymphomas, solid tumors	IV injection	Liver	CYP3A5, CYP3A4, ABCB1, ABCC1, ABCC2, ABCC10	NT, A, SIADH hypotension, V
Vinorelbine (Navelbine; *Vinca* alkaloid)	Microtubule polarization inhibitor	Solid tumor, Hodgkin lymphoma (in development)	IV injection of infusion	Liver	CYP3A4, CYP2D6	M, NT, A, V
Tyrosine Kinase Inhibitors						
Imatinib (Gleevec; STI-571, benzamide analogue)	Abl inhibitor	Ph+ CML, ALL	Oral	Liver	CYP3A4, CYP3A5, CYP2D6, CYP2C9, CYP2C19, OATP1A2, OATP1B3, OCT1, ABCB1, ABCC1, ABCC4, ABCG2	N&V, F, D, H, M

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TABLE 46-1 Key Features of Drugs Used in Pediatric Oncology (Continued)

Drug (Trade Name; Chemical Class)	Mechanism of Action	Antitumor Activity	Route of Administration	Primary Pathway of Elimination	Enzymes and Transporters	Principal Adverse Effects
Dasatinib (Sprycel; carboxamide analogue)	Src-Abl inhibitor	Ph+ CML, ALL	Oral	Liver	CYP3A4, FMO3, OATP1B3, ABCB1, ABCC4, ABCG2	M, D, N&V, Derm, fatigue, P, headache
Nilotinib (Tasigna)	Abl inhibitor	Ph+ CML, ALL	Oral	Liver	CYP3A4, OATP1B1, OATP1B3, ABCB1, ABCG2	M, N&V, D, C, Derm, MS, P
Ponatinib (Iclusig)	Src-Abl inhibitor	Ph+ CML, ALL (in development)	Oral	Liver	CYP3A4, CYP2C8, CYP2D6, CYP3A5	M, N&V, D, C, Derm, H, GI, MS
Sunitinib (Sutent; carboxamide analogue)	VEGFR, PDGFR inhibitor	In development	Oral	Liver	CYP3A4, CYP1A2, OATP1B1, ABCB1, ABCG2	M, D, Derm, anorexia, hypertension, fatigue, headache
Sorafenib (Nexavar; carboxamide analogue)	Raf and FLT3 inhibitor	In development	Oral	Liver	CYP3A4, UGT1A9, OATP1B1, OATP1B3, ABCB1, ABCG2	M, D, Derm, anorexia, hypertension, fatigue, headache
Erlotinib (Tarceva; OSI-774, quinazolinamine analogue)	EGFR inhibitor	Solid tumors (in development)	Oral	Liver	CYP3A4/5, CYP1A2, ABCG2	Derm, D, anorexia, fatigue, N&V, dyspnea, fatigue
Gefitinib (Iressa; ZD1839, anilinoquinazoline analogue)	EGFR inhibitor	Solid tumors (in development)	Oral	Liver	CYP3A4/5, CYP2D6, OATP1B3, ABCB1, ABCG2	D, Derm, N&V
Crizotinib (Xalkori)	ALK inhibitor	ALCL, non–small cell lung cancer, neuroblastoma (in development)	Oral	Liver, Renal	CYP3A4/5, OATP1B1, OATP1B3, ABCB1	Ocular, N&V, Edema, H, NT
Pazopanib (Votrient)	Vascular endothelial growth factor inhibitor	Solid tumors (in development)	Oral	Liver	CYP3A4, CYP1A2, CYP2C8, OATP1B1, OATP1B3, ABCB1, ABCG2	D, N&V, M, H, C, MS, P
Vemurafenib (Zelboraf)	BRAF inhibitor	Melanoma	Oral	Liver	CYP3A4, OATP1B3, ABCB1	Derm, N&V, MS, PE
Proteasome Inhibitors						
Bortezomib (Velcade)	Proteasome inhibitor	Leukemia, lymphoma (in development)	IV injection, SC	Liver	CYP2C19, CYP3A4, CYP1A2	M, N&V, NT, D, P
Histone Deacetylase Inhibitors						
Vorinostat (Zolinza)	Histone deacetylase inhibitor	Leukemia, lymphoma (in development)	Oral	Liver, Renal	NA	D, N&V, PE, A, M, NT, R

TABLE 46-1 Key Features of Drugs Used in Pediatric Oncology (Continued)

Drug (Trade Name; Chemical Class)	Mechanism of Action	Antitumor Activity	Route of Administration	Primary Pathway of Elimination	Enzymes and Transporters	Principal Adverse Effects
Other Molecular Targeting Agents						
Arsenic trioxide (Trisenox)	Degrades PML-RARA and induces apoptosis	Acute promyelocytic leukemia, MDS	IV infusion	Hydrolysis	NA	C, NT, Derm, N&V, D, M, H, P
Everolimus (Afinitor)	mTOR kinase inhibitor	Solid tumor, leukemia, subependymal giant cell astrocytoma (in development)	Oral	Liver	CYP3A4, ABCB1	N&V, D, HR, P, PE, Derm, M, Mu
Temsirolimus (Torisel)	mTOR kinase inhibitor	Solid tumor, leukemia (in development)	IV infusion	Liver	CYP3A4, ABCB1	N&V, D, HR, P, PE, Derm, M, Mu
Ruxolitinib (Jakavi)	Janus kinase inhibitor	In development	Oral	Liver	CYP3A4	M, D, N&V, H, P, PE
Vismodegib (Erivedge)	Hedgehog inhibitor	In development	Oral	Liver	CYP2C9, CYP3A4, ABCB1	M, A, MS
Antibodies						
Rituximab (Rituxan)	CD20 antibody	Leukemia, lymphoma	IV infusion	NA	NA	M, N&V, F, PE, NT
Gemtuzumab ozogamicin	CD33 antibody, DNA double-strand break	AML	IV infusion	NA	NA	M, H, C, F, N&V, R
Brentuximab vedotin (Adcetris)	CD30 antibody, disrupt microtubule network	Hodgkin lymphoma, ALCL	IV infusion	Liver	CYP3A4	NT, F, PE, Derm, N&V, D, M, P
Alemtuzumab (Campath)	CD52 antibody	Leukemia, lymphoma, HSCT	IV infusion, SC	NA	NA	F, M, MS, C
Bevacizumab (Avastin)	Vascular endothelial growth factor antibody	Solid tumors (in development)	IV infusion	NA	NA	M, C, D, D, N&V, MS, P, R
Biological Response Modulator						
Aldesleukin (Proleukin)	Interleukin-2	Melanoma, leukemia, cell therapy	IV infusion, SC	Renal	NA	D, F, Flu, C, PE, Derm, N&V, M, R, P

A, Alopecia; *ABC*, adenosine triphosphate–binding cassette transporter; *AI*, adrenal insufficiency; *ALCL*, anaplastic large cell lymphoma; *ALDH*, aldehyde dehydrogenase; *ALK*, anaplastic lymphoma kinase; *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *ANC*, absolute neutrophil count; *AS*, addisonian syndrome; *BRAF*, v-raf murine sarcoma viral oncogene homolog B; *C*, cardiac toxicity; *CES*, carboxylesterase; *ChlVPP*, chlorambucil, vinblastine, procarbazine, and prednisolone; *CML*, chronic myelogenous leukemia; *CNT*, concentrative nucleoside transporter; *Conj*, conjunctivitis; *CTR*, copper transporter; *Cy*, cystitis; *CYP*, cytochrome P-450 enzyme; *D*, diarrhea; *Derm*, dermatologic toxicity (skin rash and/or nail changes); *5′-DFUR*, 5′-deoxy-5-fluorouridine; *DNA*, deoxyribonucleic acid; *DPD*, dihydropyrimidine dehydrogenase; *EA*, electrolyte abnormalities; *EGFR*, epidermal growth factor receptor; *ENT*, equilibrative nucleoside transporter; *F*, fever; *FLT3*, fms-related tyrosine kinase 3; *Flu*, flu-like syndrome; *FMO3*, flavin-containing monooxygenase 3; *G*, gonadal toxicity; *GGH*, γ-glutamyl hydrolase; *GI*, gastrointestinal (ulcers, pancreatitis); *GST*, glutathione-*S*-transferase; *H*, hepatic; *HA*, hemolytic syndrome; *HDCT*, high-dose chemotherapy; *HFS*, hand-foot syndrome; *hNT1*, human nucleoside transporter 1; *HSR*, hypersensitivity reactions; *HU*, hyperuricemia; *IM*, intramuscular injection; *Inj*, injection site reaction or irritation; *IP*, intraperitoneal injection; *IV*, intravenous; *LAT*, L-type amino acid transporter; *M*, myelosuppression; *MAO*, monoamine oxidase; *MATE*, multidrug and toxin extrusion transporter; *MDS*, myelodysplastic syndrome; *Met*, metabolic acidosis; *MOPP*, mechlorethamine, vincristine, prednisone, and procarbazine; *MS*, muscular skeletal; *mTOR*, mammalian target of rapamycin; *Mu*, mucositis; *NA*, information not available; *N&V*, nausea and vomiting; *NT*, neurotoxicity; *O*, ototoxicity; *OAT*, organic anion transporter; *OATP*, organic anion-transporting polypeptide; *OCT*, organic cation transporter; *P*, pulmonary toxicity; *PCFT*, proton-coupled folate transporter; *PDGFR;* platelet-derived growth factor receptor; *PE*, peripheral edema; *Ph+*, Philadelphia chromosome–positive; *Phleb*, phlebitis; *Plt*, platelets; *PML-RARA*, promyelocytic leukemia-retinoic acid receptor, alpha; *R*, renal toxicity; *RFC*, reduced folate carrier; *RNA*, ribonucleic acid; *S*, seizures; *SC*, subcutaneous injection; *SIADH*, syndrome of inappropriate antidiuretic hormone; *SM*, secondary malignancy; *TLS*, tumor lysis syndrome; *UGT*, uridine diphosphate glucuronosyltransferase; *UT*, urea transporter; *V*, vesicant; *Vas*, vasculitis; *VEGFR*, vascular endothelial growth factor receptor; *VOD*, veno-occlusive disease.

macroscopic resolution of local disease. Before the routine use of adjuvant chemotherapy, 60% to 95% of children with localized solid tumors experienced relapse at primary or metastatic sites after receiving local control therapy alone. Thus the goal of adjuvant chemotherapy is to reduce the risk of local and systemic recurrence by eradicating microscopic residual disease or micrometastases in lungs, bone, bone marrow, lymph nodes, or other sites. The efficacy of adjuvant chemotherapy for Wilms tumor, Ewing sarcoma, rhabdomyosarcoma, osteosarcoma, and brain tumors (e.g., medulloblastoma) is well established. Neoadjuvant therapy may be used to reduce the size of primary and metastatic tumors, allow definitive surgical resection, and eradicate possible micrometastases when localized cancers cannot otherwise be optimally managed. This approach is often used with childhood solid tumors, including osteosarcomas, neuroblastomas, hepatoblastomas, and Wilms tumors. Examples of site-directed therapy include instillation of chemotherapy agents into sanctuary sites, such as intrathecal chemotherapy for leukemia and site-directed perfusion therapy (e.g., injection of agents into the feeding artery of tumors such as sarcomas in the extremities, liver tumors, and retinoblastoma).

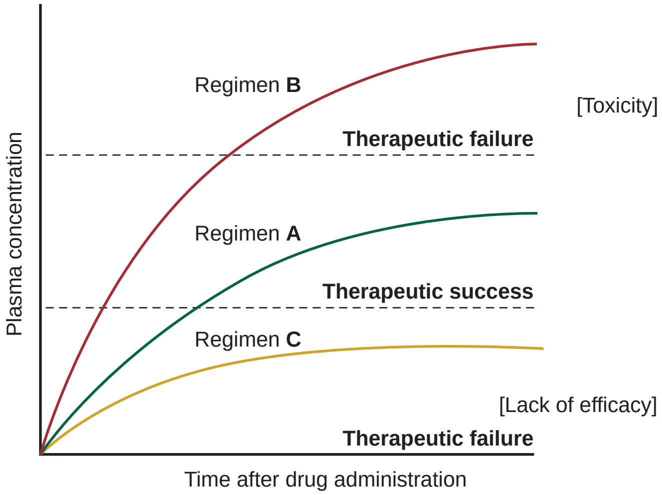


Figure 46-1 Narrow therapeutic index for chemotherapy administration. When a drug is given at a fixed dose and at fixed time intervals, it accumulates within the body until a plateau is reached. With regimen A, therapeutic success is achieved after a period of time. With regimen B, the therapeutic plasma concentration is achieved more quickly, but the plasma concentration ultimately becomes too high and causes toxicity. Regimen C does not reach therapeutic plasma concentration.

CHEMOTHERAPY IN CHILDREN

The current strategy is to administer chemotherapeutic agents at the maximum tolerated dose—that is, the dose just below that which causes unacceptable toxicity. This approach is based on a series of prospective and retrospective analyses showing that the greater the dose intensity (i.e., the dose delivered over a standard time interval), the better the outcome.[5,6] However, the narrow therapeutic index (i.e., the ratio of the theoretical minimum effective dose to the maximum tolerated dose) of most anticancer drugs demands a rigorous effort to optimize these regimens (Fig. 46-1). Effective systemic treatment requires judicious application of cytotoxic drugs that differ only slightly in their toxicity to normal cells and their therapeutic lethality to malignant cells. Pediatric oncologists and pharmacologists have studied multiple approaches in search of the best way to exploit this narrow therapeutic margin, including the use of drug combinations with different dose-limiting toxicities, the rescue or reconstitution of normal tissues (e.g., administration of leucovorin after methotrexate and use of bone marrow transplantation after high-dose chemotherapy), and the use of biologic modifiers to counteract specific mechanisms of resistance.

The therapeutic approaches used in adults cannot be directly transferred to growing children because certain agents have an extremely narrow therapeutic range in pediatric patients, and in most cases little or no information is available about the intrinsic sensitivity of a tumor to a particular agent and the tolerability of a given dose. Physiologic differences between children and adults, such as body composition, hepatic metabolism, and renal function, may affect the pharmacokinetics of a drug (i.e., drug disposition involving its absorption, distribution, metabolism, and excretion and the plasma concentration–time profile of the drug in the body) and thus the pharmacodynamics of a drug (i.e., drug action; the quantitative study of the effects of a drug, including efficacy and toxicity).[7,8] Children may also be more susceptible than adults to the toxicity of agents even when the pharmacokinetics are similar, as in the case of all-*trans*-retinoic acid for pseudotumor cerebri.[9] Further, pharmacokinetic studies have shown substantial interpatient variability in drug disposition and systemic drug exposure in children; these findings may underlie the observed variability in toxicity and response. The pharmacokinetics and pharmacodynamics of a drug, and thus its anticancer effects, are also influenced by physiologic factors that differ in children and adults, by drug-drug interactions, and by genetic influences (pharmacogenomics). Although pediatric cancer is rare compared with adult cancer, optimizing treatment in a patient group with a higher cure rate and a longer expected survival becomes critical to minimize the incidence of preventable acute and late complications in children while maintaining efficacy.

PHARMACOKINETIC CHANGES DURING CHILDHOOD

Pharmacokinetic parameters include half-life ($T_{1/2}$), area under the plasma concentration versus time curve (AUC), bioavailability, volume of distribution (Vd), steady state concentration, and clearance (Table 46-2). Body composition and organ function at the extremes of age can affect drug disposition and effect.[10,11] Changes as a child develops and matures may alter absorption, distribution, metabolism, and excretion of chemotherapeutic agents (Fig. 46-2).

The importance of understanding the influence of age on the pharmacokinetics and pharmacodynamics of

TABLE 46-2 Pharmacokinetic Parameters

Parameter	Units	Definition
Half-life ($T_{1/2}$)	Time (h)	The time necessary to halve the plasma concentration. Useful for determination of frequency of drug administration to obtain the desired plasma concentration. The half-life of a particular drug is usually independent of the dose administered. However, it may change according to the saturation of a mechanism (e.g., elimination, catabolism, or binding to plasma proteins).
Area under the curve (AUC)	Concentration × time (μM × h)	Calculated as *f* ([C] × Dt); [C] is measured concentration and Dt is the interval of time between two measurements. The integral of the plasma concentration versus an interval of definite time. It represents an important quantitative measure of total systemic drug exposure. Also, it is required for the calculation of other pharmacokinetic parameters such as clearance and bioavailability.
Bioavailability (F)	Fraction (%)	The percentage of the administered drug concentration that is achieved in the central compartment. Generally measured by comparing the AUCs obtained after intravenous administration with those obtained after oral administration. For example, the AUC obtained after intravenous administration usually corresponds to bioavailability (i.e., 100%). After oral administration, the AUC corresponds at best to an identical bioavailability; it is generally lower.
Volume of distribution (Vd)	Volume (L)	The fictitious volume in which the drug would have been distributed by supposing that its concentration is homogeneous (i.e., the average tissue concentration is identical to that of plasma). Expressed as Vd = dose/C0 (initial concentration). For example, after intravenous injection of 100 mg of a drug whose initial concentration, C0, in plasma is 10 mg/L, the volume of distribution is 10 L. For a given drug, the knowledge of its desired concentration in blood and of its volume of distribution allows evaluation of the dose to administer.
Steady state concentration (Css)	Concentration (μM)	The state of equilibrium obtained at the end of a certain number of administrations. To obtain an increase in the plasma concentration with repeated administrations, it is necessary that a residual concentration persists at the time of the following administration. At the steady state, if the dose and the frequency of administration remain constant, the concentration obtained will also be constant. The steady state is usually obtained at the end of approximately five half-lives.
Clearance (Cl)	Volume/Time (mL/min)	Defined as the theoretic volume from which drug is completely removed per unit time. Plasma clearance is the apparent volume of plasma from which drug is cleared per unit of time. Total clearance is the fraction of the volume of distribution, Vd, which is completely cleared per unit of time. The total clearance depends on the constant of elimination and thus on $T_{1/2}$ and on Vd, which is considered to be sum of metabolic and spontaneous chemical degradation, as well as biliary/fecal and renal elimination.

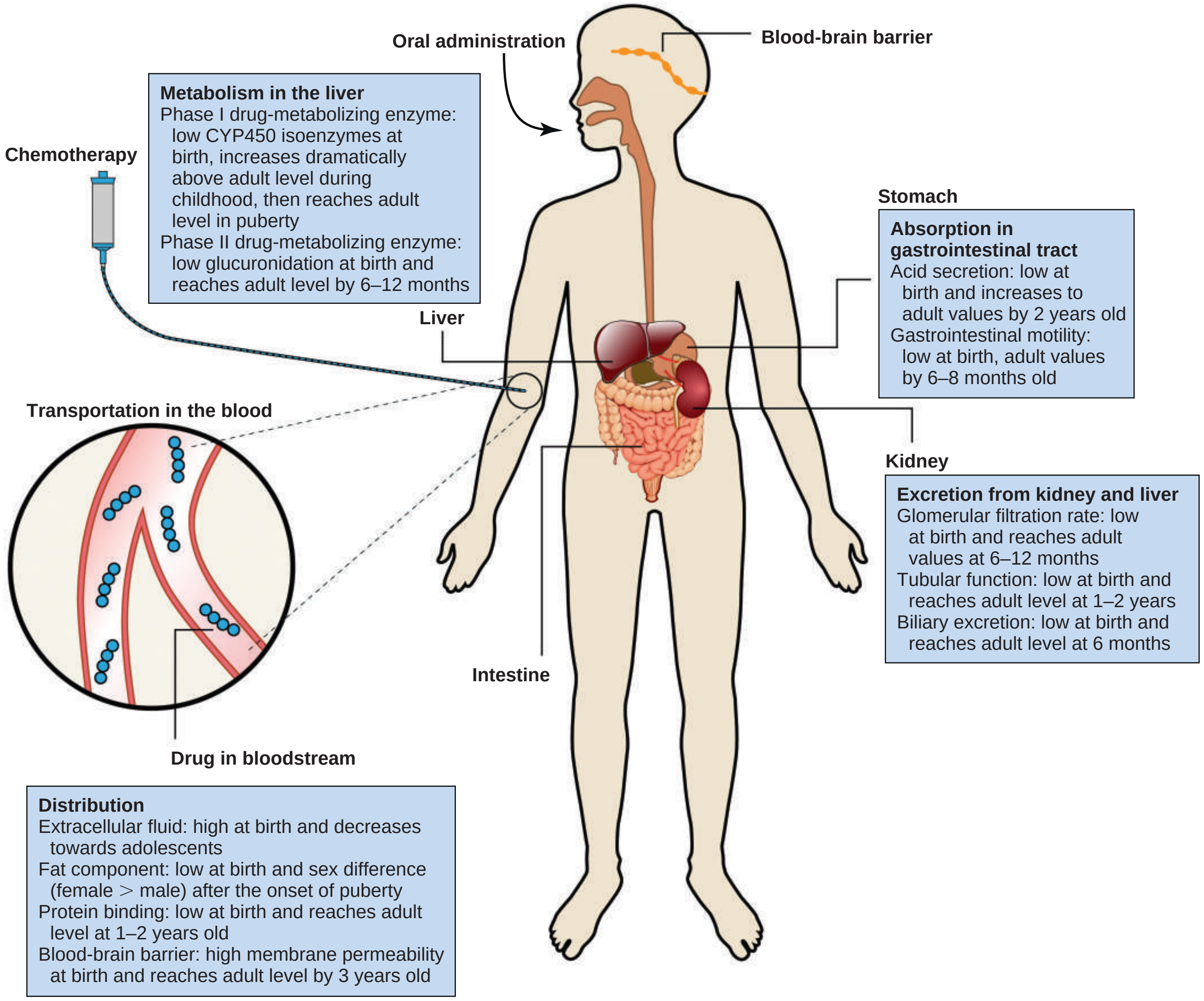
individual anticancer agents has increased steadily with advances in the treatment of infant and childhood malignancies. Although the influence of age has been formally evaluated for a limited number of cytotoxic drugs, further efforts to improve the individualization of drug therapy on the basis of maturation and development are necessary to enhance the odds of therapeutic success.

Absorption

Medications are typically administered via the gastrointestinal tract (mainly orally), intravenously (IV), intramuscularly, and via the skin (subcutaneous or transdermal). Orally administered agents must surmount multiple barriers to reach the systemic circulation (Figs. 46-2 and 46-3). Gastric pH, gastrointestinal motility, transporter-mediated uptake and efflux, and metabolism before a drug enters the systemic circulation play important roles in the absorption of many drugs. Although chemotherapy is rarely administered intramuscularly or transdermally, the lack of muscle and fatty tissue in newborns and infants and their thin skin would cause erratic absorption and increase the risk of toxicity.

Gastric pH is neutral during the first few weeks of life and does not approximate adult values until the age of 2 years, which may affect the bioavailability of compounds.[7,8] Higher pH delays the absorption of weak acids and increases the absorption of weak bases. Methotrexate absorption, for example, is significantly reduced by coadministration with milk, which may further increase gastric pH.[12] The solubility of tyrosine kinase inhibitors (e.g., dasatinib and nilotinib) is also dependent on pH.[13] Further, pH-dependent drug degradation in the stomach affects the quantity of intact drug that reaches the small intestine. Absorption of lipophilic drugs requires their micellar solubility, which is dependent on biliary acids, whose secretion in turn is affected by food intake. Bile acid secretion is low at birth and increases to the adult level at approximately 6 months.

Gastric emptying time varies with gestational age and is typically longer in premature infants and neonates than in older children.[14] Low gastrointestinal motility persists until 6 to 8 months of age and may cause delayed absorption or increased absorption because of greater contact time. In addition, drugs may be administered in altered



Metabolism in the liver
Phase I drug-metabolizing enzyme: low CYP450 isoenzymes at birth, increases dramatically above adult level during childhood, then reaches adult level in puberty
Phase II drug-metabolizing enzyme: low glucuronidation at birth and reaches adult level by 6–12 months

Absorption in gastrointestinal tract
Acid secretion: low at birth and increases to adult values by 2 years old
Gastrointestinal motility: low at birth, adult values by 6–8 months old

Excretion from kidney and liver
Glomerular filtration rate: low at birth and reaches adult values at 6–12 months
Tubular function: low at birth and reaches adult level at 1–2 years
Biliary excretion: low at birth and reaches adult level at 6 months

Distribution
Extracellular fluid: high at birth and decreases towards adolescents
Fat component: low at birth and sex difference (female > male) after the onset of puberty
Protein binding: low at birth and reaches adult level at 1–2 years old
Blood-brain barrier: high membrane permeability at birth and reaches adult level by 3 years old

Figure 46-2 Pharmacokinetic changes during childhood development. Changes in body composition and organ function during childhood development can affect drug disposition and effects by altering absorption, distribution, metabolism, and excretion of chemotherapeutic agents. *CYP450,* Cytochrome P450.

forms to small children. For example, tablets may be crushed and added to food or slurries/suspensions and sometimes may be given via nasogastric tube, which may alter their rate and extent of absorption.

Transporter proteins are involved in drug uptake and movement across the intestinal epithelium (Fig. 46-3). Intestinal epithelial cells contain several uptake transporters, including one or more members of the organic anion-transporting polypeptide (OATP) family, peptide transporter (PEPT)–1 (PEPT1; solute carrier [SLC]–15A1 [SLC15A1]), ileal apical sodium/bile acid cotransporter (SLC10A2), and monocarboxylic acid transporter 1 (SLC16A1) in their apical (luminal) membranes (Fig. 46-4, *A*). Adenosine triphosphate–binding cassette (ABC) transporters such as ABCB1 (P-glycoprotein, multidrug resistance protein [MDR]-1), ABCC1 (multidrug resistance-associated protein [MRP]–1), ABCC2 (MRP2), and ABCG2 (breast cancer resistance protein [BCRP]) are located on the apical membranes of intestinal epithelial cells, where they secrete substrates back into the intestinal lumen, thereby limiting intestinal absorption and decreasing bioavailability.[15,16] The basolateral membranes of intestinal epithelial cells contain organic cation transporter–1 (OCT1; SLC22A1), heteromeric organic solute transporter α-β, and ABCC3 (MRP3). Some drugs are eliminated by hepatocytes into the bile before they are metabolized (Figs. 46-3 and 46-4, *B*). Phase I (e.g., cytochrome P [CYP]–450) and phase II (e.g., glutathione *S*-transferase) drug-metabolizing enzymes in the gastrointestinal tract can metabolize drugs before they enter the systemic circulation (see Fig. 46-3). Although the data are limited, some studies have shown that developmental changes in at least some of these enzyme activities and transporter proteins can affect drug absorption.[17-19]

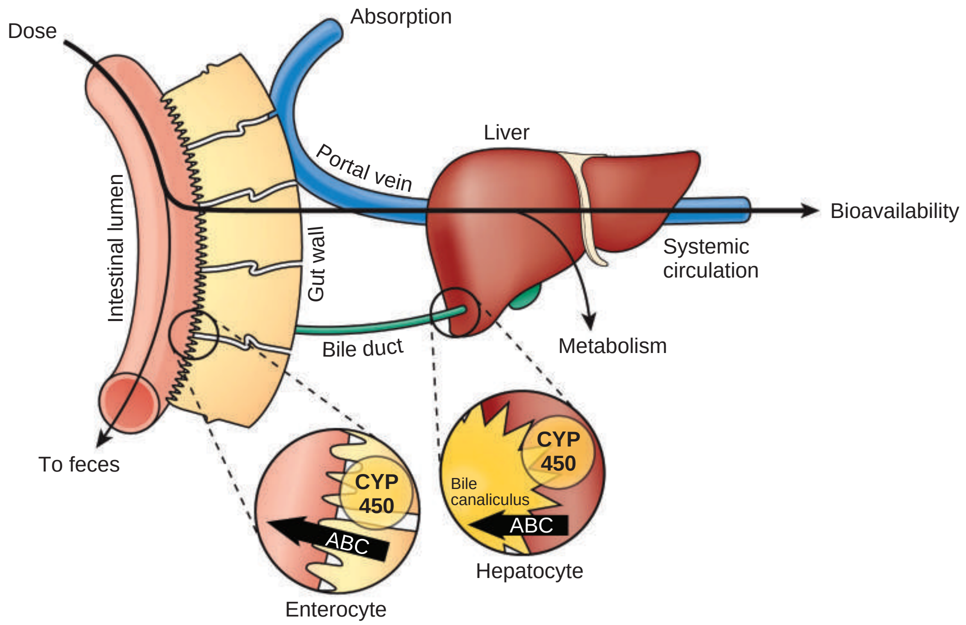


Figure 46-3 Absorption of orally administered medications and their bioavailability and metabolism. The role of active transport toward the gut lumen and bile by adenosine triphosphate–binding cassette *(ABC)* transporters expressed in the intestinal epithelium and liver, respectively, after oral administration of chemotherapeutic agents. Cytochrome P450 *(CYP450)* isoenzymes can metabolize drugs before they reach systemic circulation.

Volume of Distribution

The volume of distribution (Vd) of a systemically absorbed drug is the extent to which the drug penetrates extravascular tissues (see Fig. 46-2 and Table 46-2). Vd is affected by body composition (water and fat) and plasma proteins. Changes in body composition between birth and adolescence can alter pharmacokinetic parameters. The proportions of body water compartments, particularly extracellular fluid volume, change dramatically between birth and adulthood; extracellular fluid volume represents 50% of body weight in premature infants, 35% in infants 4 to 6 months old, and 20% in adolescents and adults.[20] Thus polar drugs, which are distributed primarily in body water, will have a larger volume of distribution in infants than in older children and adults. The net result of an isolated increase in Vd is a lower peak concentration and prolonged terminal half-life, assuming that drug clearance remains unchanged.

Newborns have less skeletal muscle mass and subcutaneous fat than do older infants. Fat components gradually increase after birth, but they differ in boys and girls after the onset of puberty. During adolescence, boys actually lose body fat (reaching a mean of 12% of body weight), whereas girls gain body fat (reaching a mean of 25% of body weight). Therefore gender-related differences in Vd and clearance of lipophilic drugs may be more prominent during adolescence than in preadolescent children or older adults.

Drug distribution may also be affected by plasma proteins. Theoretically, drug displacement from blood components by lower protein binding increases the apparent distribution volume. Although the resulting increase in the free drug fraction may make the drug more available to its target, it also enhances metabolic and renal elimination. Protein binding may be reduced because of persistence of fetal albumin or by low plasma protein content, particularly albumin, α1-acid glycoprotein, and gamma globulin.[21,22] Although the protein binding of acidic drugs may reach adult levels between 1 and 2 years of age, adult gamma-globulin levels are not reached until age 7 to 12 years.

One unique aspect of infancy is the immaturity of specific organs. For example, the myelin content of the brain is lower in newborns. Because of incomplete maturation of the blood-brain barrier (which reaches adult level by age 3 years), membrane permeability is greater in the infant brain, and the brain-to-plasma ratio of some drugs has been shown to vary with age.[23,24]

Hepatic Metabolism

The liver plays an important role in drug metabolism (see Fig. 46-2). Drug-metabolizing enzymes are divided into two classes: phase I (oxidation, reduction, hydrolysis, or demethylation) and phase II (conjugation or coupling of endogenous molecules, including glucuronide, sulfate, acetate, amino acid, methyl, and glutathione moieties, to the parent drug or a phase I metabolite; Table 46-3). Immature organ systems in infants and young children can alter the disposition of many classes of drugs, including antibiotics, anticonvulsants, and antineoplastic agents.[7,8,10] Phase I enzymes (e.g., CYP450 microsomal enzymes) are highly expressed in the liver. They can diminish the pharmacologic activity of a drug but can also convert a prodrug to its biologically active form (e.g., cyclophosphamide and codeine). CYP3A4 is the phase I

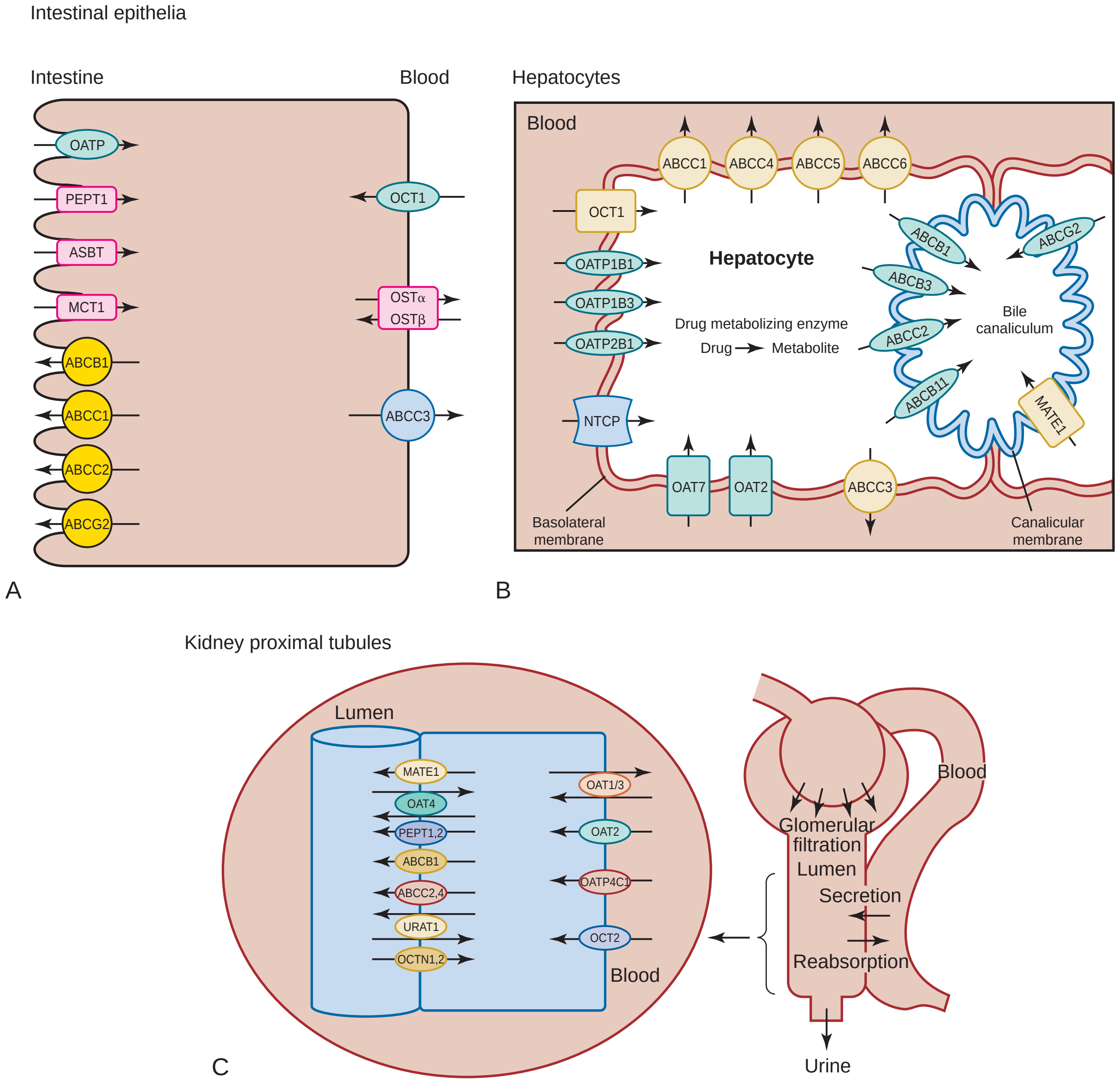

Intestinal epithelia
Intestine
Blood
OATP
PEPT1
ASBT
MCT1
ABCB1
ABCC1
ABCC2
ABCG2
OCT1
OSTα
OSTβ
ABCC3
A
Hepatocytes
Blood
ABCC1
ABCC4
ABCC5
ABCC6
OCT1
OATP1B1
OATP1B3
OATP2B1
NTCP
Hepatocyte
Drug metabolizing enzyme
Drug → Metabolite
ABCB1
ABCG2
ABCB3
ABCC2
ABCB11
MATE1
Bile canaliculum
OAT7
OAT2
ABCC3
Basolateral membrane
Canalicular membrane
B
Kidney proximal tubules
Lumen
MATE1
OAT4
PEPT1,2
ABCB1
ABCC2,4
URAT1
OCTN1,2
OAT1/3
OAT2
OATP4C1
OCT2
Blood
Glomerular filtration
Lumen
Secretion
Reabsorption
Blood
Urine
C

Figure 46-4 Common mechanisms involved in anticancer drug uptake and elimination through the gastrointestinal tract (**A**), liver (**B**), and kidney (**C**). *ABC,* Adenosine triphosphate–binding cassette transporter; *ASBT,* ileal apical sodium/bile acid cotransporter; *MATE,* multidrug and toxic extrusion transporter; *MCT,* monocarboxylic acid transporter; *NTCP,* (Na$^+$)-taurocholate cotransporting polypeptide; *OAT,* organic anion transporter; *OATP,* organic anion-transporting polypeptide; *OCT,* organic cation transporter; *OCTN,* novel organic cation transporter; *OST,* organic solute transporter; *PEPT,* peptide transporter; *URAT,* urate transporter.

enzyme involved in the metabolism of the largest number of drugs, followed in approximate order by CYP2D6, CYP2C8/9/19, CYP1A2, CYP2B6, CYP2A6, and CYP2E1.[7] CYP3A7 is highly expressed in the fetus and then decreases rapidly after birth.[25] The metabolic activity of other CYP450 enzymes (e.g., CYP3A4, CYP2D6, and CYP2C9) is low in neonates but increases dramatically between age 2 weeks and 3 years. Clearance rates by CYP450 enzymes rise from 20% of that in adults to two to six times that in adults between infancy and adolescence, and then they decline gradually to adult values during puberty.[26] Low hydrolysis rates have been reported for drugs such as procaine in premature infants and neonates, but normal activity is measurable by 12 months. Phase II glucuronidation is almost exclusively a detoxification mechanism that inactivates a compound and facilitates its excretion by enhancing its hydrophilicity and its recognition by biliary canicular efflux proteins. The activity glucuronidation enzyme rises to 30% of adult activity by 3 months of age and then reaches adult levels between 6 and 12 months of age.[27] Esterase activity, which is important for the metabolisms of agents such as irinotecan, gradually increases during the first year of life.[28]

Renal and Hepatic Excretion

The kidney is predominantly responsible for excretion of drugs and their metabolites (see Fig. 46-2). The immature kidney has a low glomerular filtration rate (GFR). In full-term infants, the GFR is 40 mL/min/1.73 m^2 and varies

TABLE 46-3 Drug Metabolizing Enzymes

Enzymes	Reactions
Phase I Enzymes	
Cytochrome P450s	C and O oxidation, dealkylation, others
Flavin-containing monooxygenases	N, S, and P oxidation
Epoxide hydrolases	Hydrolysis of epoxides
Phase II Enzymes	
UDP-glucuronosyltransferases	Addition of glucuronic acid
Glutathione-*S*-transferases	Addition of glutathione
Sulfotransferases	Addition of sulfate
N-acetyltransferases	Addition of acetyl group
Methyltransferases	Addition of methyl group
Other Enzymes	
Alcohol dehydrogenases	Reduction of alcohols
Aldehyde dehydrogenases	Reduction of aldehydes
NADPH-quinone oxidoreductase	Reduction of quinones

C, Carbon; *N,* nitrogen; *NADPH,* nicotinamide adenine dinucleotide phosphate; *O,* oxygen; *P,* phosphorus; *S,* sulfur; *UDP,* uridine diphosphate.

substantially among individuals.[29,30] The GFR gradually increases, reaching adult values between age 6 and 12 months. Tubular function and passive resorption may also be significantly lower in the neonate. Because tubular resorption matures more slowly than does glomerular filtration, toddlers may have remarkably high clearance rates for compounds that undergo tubular resorption in older children.

GFR, as assessed by technetium-99m diethylenetriamine pentaacetic acid filtration, was related to body surface area (BSA) in children 2 months to 17 years of age, but GFR was not related to age when normalized to BSA.[29] Premature and term infants require dose adjustments based on GFR measurement or estimation for drugs eliminated primarily by glomerular filtration, such as carboplatin. Formulas are available for estimation of creatinine clearance in infants and older children on the basis of serum creatinine and height[30,31] or on another endogenous surrogate marker of GFR, such as cystatin C.[32]

Many drugs are excreted in bile as the parent compound or as a metabolite. Biliary excretion favors compounds that have a molecular weight greater than 300 and contain both polar and lipophilic groups. Conjugation, particularly with glucuronate, increases biliary excretion. After the bile empties into the intestine, a fraction of the drug may be reabsorbed and eventually return to the liver (enterohepatic cycling). The remainder is excreted into the feces. Biliary excretion is low at birth and reaches adult levels at approximately 6 months of age.

Many SLC family transporters and ABC family transporters have been identified; these transporters mainly facilitate cellular drug uptake and elimination in kidney and liver (see Fig. 46-4, *B* and *C*).[33] The apical (luminal) membranes of renal proximal tubules contain organic anion transporter (OAT)–4 (OAT4, SLC22A11), urate transporter–1 (SCL22A12), PEPT1 (SLC15A1) and PEPT2 (SLC15A2), ABCC2 and ABCC4 (MRP4), multidrug and toxin extrusion (MATE) protein–1 (MATE1, SLC47A1), ABCB1, organic cation/ergothioneine transporter (OCTN1; SLC22A4), and organic cation/carnitine transporter (OCTN2; SLC22A5). The basolateral membranes of the proximal tubules contain the uptake transporters OATP4C1 (SLCO4C1); OCT2; and OAT1, OAT2 (SLC22A7), and OAT3 (SLC22A8).

Uptake transporters in the hepatocyte basolateral (sinusoidal) membrane include the sodium/taurocholate cotransporting peptide (SLC10A1), three members of the OATP family (OATP1B1 [SLCO1B1], OATP1B3 [SLCO1B3], and OATP2B1 [SLCO2B1]), OAT2 and OAT7 (SLC22A9), and OCT1. Efflux pumps in the hepatocyte basolateral membrane include ABCC1 (MRP1), ABCC3, ABCC4, ABCC5 (MRP5), and ABCC6 (MRP6). Apical (canalicular) efflux pumps of the hepatocyte comprise ABCB1, ABCC2, ABCB3 (transporter associated with antigen processing 2 [TAP2]), ABCB11 (bile salt export pump [BSEP] or sister of P-glycoprotein [SPGP]), ABCG2, and MATE1.

ABCB1 activity in lymphocytes peaks at birth, decreases between ages 0 and 6 months, and stabilizes at the adult level between the ages of 6 months and 2 years.[34] Developmental changes in the functions of other transporters have not been fully defined, but many are under investigation.

Influence of Underlying Disease on Metabolism

Pathophysiologic changes associated with specific pediatric malignancies may alter drug disposition. For example, the clearance of antipyrine and lorazepam was observed to increase between diagnosis and the end of remission induction therapy in children with ALL.[35] The clearance of unbound teniposide is lower in children with relapsed ALL than during first remission.[36] Because leukemic infiltration of the liver is common at the time of diagnosis of ALL, drugs metabolized by the liver may have reduced clearance, as has been documented in preclinical models.[37]

In mouse models, certain tumors elicited an acute-phase response that coincided with downregulation of human hepatic CYP3A4 and of the mouse orthologue Cyp3a11.[38] The reduction of murine hepatic *Cyp3a* gene expression in tumor-bearing mice resulted in decreased Cyp3a protein expression and consequently a significant reduction of Cyp3a-mediated metabolism of midazolam. These findings support the possibility that tumor-derived inflammation may alter the pharmacokinetic and pharmacodynamic properties of CYP3A4 substrates, leading to reduced drug metabolism in humans.

Unique Drug Effects in Pediatric Patients

Experience has shown that children often tolerate the conventional toxicities of chemotherapy better than adults, and that children (with the exception of neonates) experience very low regimen-related mortality. This finding may be partly explained by relatively low drug exposure caused by higher drug clearance rather than by altered pharmacodynamics. Lower exposure may explain briefer periods of neutropenia, less mucositis, and rare hepatic veno-occlusive disease. Children's greater ability to tolerate intensive therapy may partly explain why children with ALL have a higher cure rate compared with adults.[39,40]

However, the immature organ systems of the very young may be more susceptible to certain aspects of toxicity, including late cardiomyopathy, neuron developmental disorders, delayed puberty, growth plate arrest, and infertility. Children appear to be more sensitive to the cardiotoxic effects of anthracyclines (e.g., doxorubicin, daunorubicin, idarubicin, and mitoxantrone).[41,42] Follow-up studies have shown that months or years after intrathecal therapy for central nervous system (CNS) leukemia, with or without cranial irradiation, children treated for ALL may experience a chronic demyelinating encephalopathy and/or cognitive late effects.[43,44] Further, the latent effects of chemotherapy on fertility appear to vary with the developmental stage of the patient at the time of treatment. Treatment of leukemia and lymphoma in prepubertal boys, for example, does not appear to produce sustained testicular damage unless large cumulative doses of alkylating agents (e.g., cyclophosphamide >6 g/m^2) are used.[45] Treatment during puberty is more likely to permanently damage the germinal epithelium, although sexual maturation may proceed on a normal schedule.[46] Most prepubertal girls treated for leukemia with antimetabolites achieve menarche and progress through puberty.[47] Ovarian failure is associated mainly with alkylating agents and gonadal radiotherapy and is directly related to cumulative dose and age at exposure.[48] Nearly 30% of survivors treated with alkylating agents and abdominal-pelvic irradiation experienced nonsurgical primary ovarian failure (defined as cessation of menses before age 40 years).[49] Procarbazine exposure at any age, cyclophosphamide exposure from age 13 to 20 years, and ovarian radiation doses greater than 10 Gy were independent risk factors for acute ovarian failure (defined as loss of ovarian function within 5 years after diagnosis).[50] Most girls treated for brain tumors with procarbazine and/or nitrosourea and with neuraxis radiation showed evidence of ovarian hormonal failure.[51]

DRUG INTERACTIONS

Combination Chemotherapy

The vast majority of pharmacologic studies of anticancer agents have modeled the effects of a single drug. However, as a consequence of somatic mutations and/or selection of genetically resistant clones, tumor cytotoxicity tends to diminish over subsequent courses of single-agent treatment. Therefore cancer chemotherapy is most frequently given as a combination of drugs that individually have shown activity against the specific cancer to be treated but that have different pharmacologic mechanisms of action. The selection of agents that exert maximal anticancer effects and minimal adverse effects requires a clear understanding of the biochemical, molecular, and pharmacokinetic mechanisms of action and of individual and overlapping toxicities of the agents. The treatment for ALL provides an example of excellence.[3] Most of the drugs used were developed before 1970. However, their dosage and schedule of administration have been optimized on the basis of leukemic-cell biologic features, response to therapy (e.g., minimal residual disease), and patient pharmacodynamic and pharmacogenomic findings. The 5-year survival rate for pediatric ALL has been approximately 90% in recent trials.[52]

Optimal combination chemotherapy theoretically should meet the following criteria:

- Drugs that show single-agent activity should be selected. Because of primary resistance, rates of complete response to a single agent rarely exceed 20%.
- Drugs with different mechanisms of action should be combined. The various classes of anticancer agents have different cellular targets. The use of multiple agents with different mechanisms of action enables independent killing of cells by each agent. Cells resistant to one agent may be sensitive to another drug or drugs in the regimen. Known patterns of cross-resistance must be taken into consideration.
- Drugs with different mechanisms of resistance should be combined. Resistance to many agents may be the result of mutations selected by those agents. However, a single mutational change can also cause resistance to multiple drugs. The number of known mechanisms of resistance is continuously increasing and is partly drug dependent. Because drug-resistant mutants may be present at the time of clinical diagnosis, the earliest possible use of non–cross-resistant drugs is recommended to avoid the selection of double mutants. Adequate cytotoxic doses of drugs must be administered as frequently as possible to achieve maximal killing of sensitive and moderately resistant cells.
- If possible, drugs with different dose-limiting toxicities should be combined, because it is more likely that drugs with nonoverlapping toxicities can be used at the full dosage, optimizing their potential effectiveness.

As noted, multidrug therapy can give rise to clinically important drug-drug interactions, which typically occur when the pharmacokinetic behavior of one drug is altered by the other (Fig. 46-5 and Table 46-1). These interactions are important in the design of drug combinations, because diminished therapeutic efficacy or increased toxicity of one or more of the administered agents can occur. Combinations of drugs may also show pharmacodynamic interactions that cannot be explained by altered pharmacokinetic profiles. Some of these interactions are at the cellular level or are related to the cell cycle. Interactions can be classified as synergistic (i.e., the effect of the combination is greater than the sum of the individual effects), additive (the effect of two drugs is the sum of the effects of each), or antagonistic (the effect of two drugs is less than the sum of their individual effects). Provided that the drugs used are active against a particular disease,

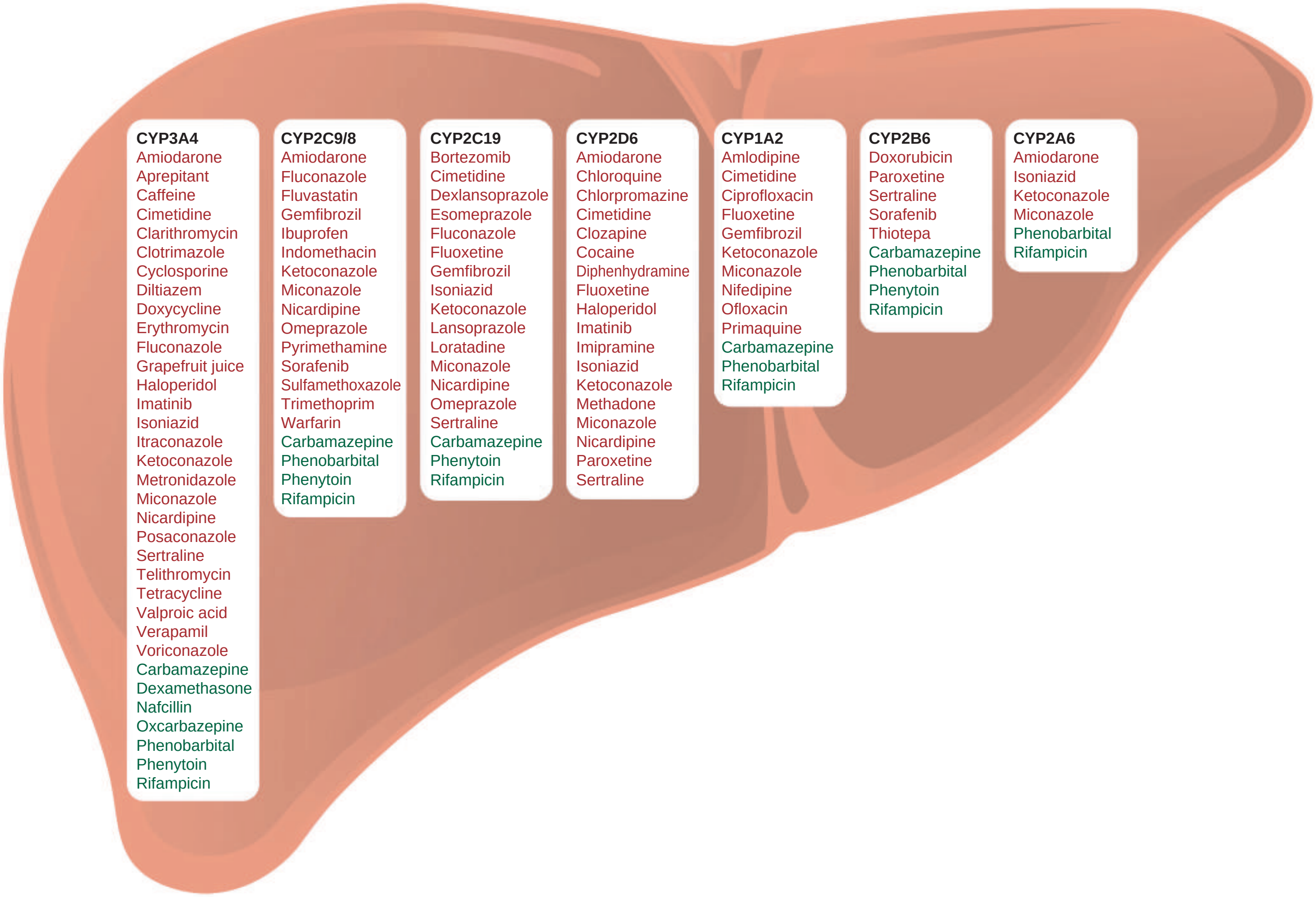

CYP3A4
Amiodarone
Aprepitant
Caffeine
Cimetidine
Clarithromycin
Clotrimazole
Cyclosporine
Diltiazem
Doxycycline
Erythromycin
Fluconazole
Grapefruit juice
Haloperidol
Imatinib
Isoniazid
Itraconazole
Ketoconazole
Metronidazole
Miconazole
Nicardipine
Posaconazole
Sertraline
Telithromycin
Tetracycline
Valproic acid
Verapamil
Voriconazole
Carbamazepine
Dexamethasone
Nafcillin
Oxcarbazepine
Phenobarbital
Phenytoin
Rifampicin
CYP2C9/8
Amiodarone
Fluconazole
Fluvastatin
Gemfibrozil
Ibuprofen
Indomethacin
Ketoconazole
Miconazole
Nicardipine
Omeprazole
Pyrimethamine
Sorafenib
Sulfamethoxazole
Trimethoprim
Warfarin
Carbamazepine
Phenobarbital
Phenytoin
Rifampicin
CYP2C19
Bortezomib
Cimetidine
Dexlansoprazole
Esomeprazole
Fluconazole
Fluoxetine
Gemfibrozil
Isoniazid
Ketoconazole
Lansoprazole
Loratadine
Miconazole
Nicardipine
Omeprazole
Sertraline
Carbamazepine
Phenytoin
Rifampicin
CYP2D6
Amiodarone
Chloroquine
Chlorpromazine
Cimetidine
Clozapine
Cocaine
Diphenhydramine
Fluoxetine
Haloperidol
Imatinib
Imipramine
Isoniazid
Ketoconazole
Methadone
Miconazole
Nicardipine
Paroxetine
Sertraline
CYP1A2
Amlodipine
Cimetidine
Ciprofloxacin
Fluoxetine
Gemfibrozil
Ketoconazole
Miconazole
Nifedipine
Ofloxacin
Primaquine
Carbamazepine
Phenobarbital
Rifampicin
CYP2B6
Doxorubicin
Paroxetine
Sertraline
Sorafenib
Thiotepa
Carbamazepine
Phenobarbital
Phenytoin
Rifampicin
CYP2A6
Amiodarone
Isoniazid
Ketoconazole
Miconazole
Phenobarbital
Rifampicin

Figure 46-5 The main cytochrome P450 isoenzyme–inducing and P450 isoenzyme–inhibiting agents used in pediatric oncology. Drugs listed in *red* are inhibitors and those listed in *green* are inducers of the cytochrome P450 enzyme system.

knowledge of cellular kinetics can be used to consider the use of non–cell cycle phase-specific agents (e.g., alkylating agents, platinum agents, and anthracyclines), first to reduce tumor bulk and second to recruit slowly dividing cells into active deoxyribonucleic acid (DNA) synthesis. After the latter is achieved, treatment can be continued with cell-cycle phase-specific agents (e.g., methotrexate or fluoropyrimidines), which affect cells mainly during DNA synthesis. Similarly, repeated courses of S-phase–specific drugs, such as cytarabine and methotrexate, are most effective if administered during the rapid rebound recovery of DNA synthesis that follows suppression of DNA synthesis in the previous course.[53] If pharmacokinetic and pharmacodynamic interactions exist, the drug doses and sequence of administration that allow safe administration of combination chemotherapy are typically defined during early clinical trials (i.e., phase I and phase II studies).

Coadministration of Non-Anticancer Drugs

Many prescription and over-the-counter medications are used during chemotherapy to improve quality of life or to treat other diseases, but they may interact with anticancer agents, thus altering their pharmacokinetic characteristics, clinical effectiveness, and/or toxicities.[54,55,56] More than 100,000 deaths per year in the United States alone are attributed to drug-drug interactions, placing these interactions between the fourth and sixth leading cause of death.[57] Clearly, all aspects of pharmacokinetics (i.e., absorption, distribution, metabolism, and excretion) may be affected when a drug is given in combination with another drug. The generally narrow therapeutic index of cytotoxic chemotherapy means that drug-drug interactions would predispose some persons to excessive toxicity or inadequate efficacy.

Pharmacokinetic Interactions Affecting Drug Absorption

Because many chemotherapeutic agents are given via an IV line, factors that affect absorption have little effect on their pharmacokinetics. However, absorption plays a crucial role in the bioavailability of orally administered drugs; absorption is contingent on adequate intestinal uptake and circumvention of intestinal and subsequent hepatic metabolism of the drug (see Figs. 46-2 and 46-3).[13,58,59] Food intake can delay gastric emptying, raise intestinal pH, increase hepatic blood flow, and slow gastrointestinal transit, which may significantly affect the pharmacokinetic profile of orally administered medications.[55] Mercaptopurine is metabolized by xanthine oxidase into inactive metabolites. Ingestion of foods that contain xanthine oxidase, such as dairy products, can reduce the effectiveness of mercaptopurine.[58] Therefore patients are instructed to take mercaptopurine on an empty stomach, preferably at night (2 hours after food intake).[56] Proton-pump inhibitors and antacids increase gastric pH and decrease the solubility and absorption of tyrosine kinase inhibitors (with the exception of imatinib).[60]

Another important mechanism of interaction with orally administered anticancer agents is their affinity for ABC transporters (e.g., ABCB1, ABCC1, ABCC2, and ABCG2), which are located on the apical membranes of intestinal epithelial cells and secrete substrates from the epithelial cell back into the intestinal lumen, thereby limiting intestinal absorption and bioavailability (see Figs. 46-3 and 46-4, *A*).[15,16] Some chemotherapeutic agents are substrates of these transporters, the activity of which may be inhibited by other medications, foods, and herbal products. In addition, extensive metabolism of anticancer drugs in the gut wall and/or the liver (e.g., by CYP450 enzymes) during the first pass can keep them out of the systemic circulation and is another potential drug-drug interaction mechanism (see Fig. 46-3). An ideal chemotherapeutic drug would have an adequate absolute bioavailability, as shown by little drug-drug interaction or interpatient/intrapatient variability in absorption. However, the most commonly used oral agents, including mercaptopurine, methotrexate, etoposide, and cyclophosphamide, do not meet these criteria.

Pharmacokinetic Interactions Affecting Drug Distribution

Chemotherapeutic agents can bind to several blood components, including albumin, lipoproteins, immunoglobulins, and α1-acid glycoprotein, and the increased free-drug fraction caused via displacement by another drug may make the displaced drug more available to the target and enhance metabolism and elimination.[55] Highly protein-bound drugs, such as warfarin, may interact with protein-bound cytotoxic drugs such as etoposide and paclitaxel. The hematologic toxicity of etoposide is more closely related to systemic exposure to the unbound drug than to the total drug.[61] However, the therapeutic implications of displacement of many anticancer drugs from their protein-bound state are as yet undefined.

Pharmacokinetic Interactions Affecting Drug Metabolism

Most important drug-drug interactions involve altered liver metabolism—for example, altered expression or functionality of CYP450 isoenzymes, such as CYP3A4, 2C9/8, 2D6, and 2C19 isoforms, which oxidize anticancer drugs to more polar and usually inactive metabolites (see Table 46-1).[62] Induction of CYP450 activity, which increases a drug's metabolic rate, may reduce plasma drug concentration and thus reduce the therapeutic effect. For example, anticonvulsant drugs (such as phenytoin, phenobarbital, and carbamazepine) induce drug-metabolizing CYP450 enzymes and thereby increase the clearance of various anticancer agents (e.g., anthracyclines, epipodophyllotoxins, and vinca alkaloids) in children (see Table 46-1 and Fig. 46-5).[56,63] Enzyme-inducing anticonvulsants have been shown to be associated with lower event-free survival, hematologic relapse, and CNS relapse in persons with ALL.[63] Conversely, suppression (inhibition) of CYP450 activity may increase plasma drug concentration, causing greater toxicity commensurate with overdose. Valproic acid, azole antifungals (e.g., fluconazole, voriconazole, and posaconazole), the antiemetic aprepitant, and macrolide antibiotics (e.g., clarithromycin, erythromycin, and azithromycin) should be avoided or prescribed with caution when CYP450-metabolized chemotherapeutic agents are given concomitantly.[56] Although drugs are typically metabolized to their inactive forms, prodrugs such as cyclophosphamide are metabolized to their active forms by CYP450 enzymes and have the opposite consequences of metabolic inhibition or activation.

Pharmacokinetic Interactions Affecting Drug Excretion

ABC transporters such as ABCB1, ABCC1/2, and ABCG2 are involved in drug elimination.[33] ABCB1 in the brush-border membrane of the proximal renal tubule plays an important role in renal elimination of chemotherapeutic agents by active secretion into the urine (see Fig. 46-4, *C*). Inhibition of ABCB1 can result in increased systemic exposure and tissue distribution of drugs that are ABCB1 substrates (see Table 46-1), whereas induction of ABCB1 leads to decreased systemic exposure. ABCB1 is also expressed in canalicular membranes of the liver; it has a role in elimination of drugs into bile, from which they can be reabsorbed in the intestine or eliminated in the feces (see Fig. 46-4, *B*). Excretion of irinotecan and its metabolites is mediated by several ABC transporters (e.g., ABCB1 and ABCG2).[64] Substrates and/or inhibitors of these transporters (e.g., cyclosporine and gefitinib) may interfere with renal and/or biliary excretion of irinotecan and its metabolite, SN-38, and increase the plasma concentration and toxicity. Organic anion transporters (e.g., OAT1, OAT3, and OAT4) are highly expressed on the renal proximal tubules and are involved in the renal elimination of methotrexate.[65] Nonsteroidal antiinflammatory drugs (e.g., salicylate, ibuprofen, and indomethacin), probenecid, and penicillin can inhibit these OATs and delay renal excretion of methotrexate, leading to severe and even life-threatening drug interactions, including bone-marrow suppression and acute renal failure.[66,67]

Coadministration of Complementary and Alternative Medicine

In recent years, interest in complementary and alternative medicine (CAM) has grown rapidly in the industrialized world.[68,69] No compelling clinical trial data support the use of CAM in children with cancer, and the potential interaction of CAM agents with conventional, possibly curative, treatment may be harmful. Families are more likely to use CAM for their child if a parent uses CAM, if the child has a poor prognosis, or if the parents are older and better educated.[68,69] Surveys within the past decade have estimated that CAM is used in 31% to 84% of pediatric oncology cases, and in many cases the treating clinician is unaware of the patient's use of CAM.[68,69] In addition to nonherbal therapies (e.g., hypnosis, guided imaginary, massage, and acupuncture), many children use herbal treatments, such as St. John's wort (*Hypericum perforatum*), garlic, and ginseng (*Panax ginseng*), in combination with allopathic therapies.[68,69] The risk of herb-drug interactions is a growing concern, and the need is increasing to understand possible adverse interactions. This concern is of particular relevance to pediatric oncology, because CAM use is more common in this group than in the general population.

During the past decade, a wealth of in vitro and in vivo evidence has shown that many herbal preparations interact extensively with drug-metabolizing enzymes and drug transporters. A number of clinically important interactions are now recognized, although causal relationships have not consistently been established and confirmatory studies in children are lacking. Most of the observed interactions suggest that the herbs inhibit or induce several isoforms of the CYP family, and concurrent use of some herbs with chemotherapy is likely to have serious clinical and toxicologic implications. An additional consideration for cancer chemotherapy is that herb-mediated induction or inhibition of phase I and II metabolic enzymes and ABC/SLC transporters may alter response. Therefore rigorous testing is urgently required to identify the pharmacokinetic interactions of anticancer drugs with widely used herbs. Drug-drug interaction potential has been investigated for only a limited number of herbs. For example, St. John's wort, melatonin, and whey protein are CYP450 inducers, and curcumin, goldenseal *(Hydrastis canadensis)*, and grapefruit juice *(Citrus paradise)* are strong inhibitors.[68,69] Milk thistle *(Silybum marianum)*, *Echinacea (Echinacea purpurea)*, saw palmetto, *Ginkgo biloba*, valerian, and green tea have showed no significant interactions. Because of the widespread use of herbal medicines in the United States, physicians should include it in their routine drug histories and review potential hazards with individual patients. Detailed information can be obtained from the following websites[68,69]:

- National Center for Complementary and Alternative Medicine, National Institutes of Health: *http://nccam.nih.gov*
- Office of Cancer Complementary and Alternative Medicine, National Cancer Institute, National Institutes of Health: *http://cam.cancer.gov/*
- PDQ Cancer Information Summaries: Complementary and Alternative Medicine, National Cancer Institute, National Institutes of Health: *http://www.cancer.gov/cancertopics/pdq/cam*
- Integrative Therapies Program for Children with Cancer, Columbia University Medical Center: *http://integrativetherapies.columbia.edu/*
- About Herbs, Botanicals and Other Products, Memorial Sloan Kettering Cancer Center: *http://www.mskcc.org/mskcc/html/11570.cfm*
- Complementary/Integrative Medicine, MD Anderson Cancer Center: *http://www.mdanderson.org/departments/cimer/*
- Natural Standard: *http://www.naturalstandard.com/*

PHARMACOGENETICS AND PHARMACOGENOMICS OF CHEMOTHERAPEUTIC AGENTS IN CHILDREN

Pharmacogenetics/pharmacogenomics investigates the influence of human genomic variation in drug response. Inherited (germline) or acquired (somatic) variation in genes encoding drug-metabolizing enzymes, drug receptors, drug transporters, and drug targets have all been shown to influence anticancer drug response in humans and they may also influence drug toxicity.[70]

A single nucleotide polymorphism (SNP) is defined as a single base change in a DNA sequence.[71] Unless it occurs in at least 1% of the population, it is considered a variant or a mutation. Accumulation of mutations during evolution, in concert with random selection, has made SNPs the most common form (about 90%) of genetic variation in the human genome.

The advent of pharmacogenomics was characterized by the passage from a candidate gene approach to a genome-wide approach. This advance was initially made possible by the international HapMap project, which included 270 samples from three geographic areas of genetic descent—African descent (Yoruban population); European descent (Center d'Etude du Polymorphisme Humain population), and Asian descent (Japanese and Han Chinese population)—and more than 2 million publicly available SNPs.[72] Subsequently, the "1000 Genomes Project" has provided the most detailed catalogue of human genetic variation. The recent development of next-generation sequencing technologies has allowed better coverage of all genetic variants.[73]

Advances in treatment have greatly improved the survival rates of children with cancer. However, the efficacy of therapy, as well as its short- and long-term adverse effects, can vary among patients, even when their chemotherapeutic or radiologic treatment is identical. Thus it is very important to identify inherited (germline) genomic variations that can alter the absorption, distribution, metabolism, and/or excretion of chemotherapeutic agents, thereby altering their effects and toxicities (Table 46-4).

Thiopurines

Thiopurines frequently used for the treatment of childhood ALL are 6-mercaptopurine and 6-thioguanine (see Table 46-1). As prodrugs, they require conversion to thioguanine nucleotides (TGNs) via the purine-salvage pathway, and 6-thioguanosine 5′-triphosphate (TGTP) and deoxy-TGTP are then incorporated into ribonucleic acid (RNA) or DNA, respectively (Fig. 46-6). The active DNA mismatch repair system identifies these base-pair mismatches and induces cell cycle arrest and apoptosis.[74,75] An alternative protein complex that includes high mobility group box 1 and 2 proteins, heat shock cognate protein 70 (HSC70), protein disulfide isomerase (ERp60), and glyceraldehyde 3-phosphate dehydrogenase trigger apoptosis when it detects changes in DNA structure caused by incorporation of nonnatural nucleosides.[76] Thiopurines are inactivated by methylation catalyzed by thiopurine methyltransferase (TPMT). The relation between *TPMT* polymorphisms and thiopurine efficacy and toxicity provides a well-established example of the

TABLE 46-4 Pharmacogenomics of Chemotherapeutic Agents Used for Pediatric Cancer

Agent	Genes Involved	Effects
Thiopurines	*TPMT*3A* (460 G>A and 719 A>G), *TPMT*3C* (719 A>G), and *TPMT*2* (238G>C)	Higher incidence of hematopoietic cell toxicities, secondary malignancies in ALL
6-mercaptopurine	*PACSIN2* (rs2413739, T allele)	Lower TPMT activity and higher incidence of severe gastrointestinal toxicity in ALL
6-thioguanine	*ITPA* (rs41320251, C>A)	Higher methylmercaptopurine nucleotides and higher incidence of febrile neutropenia when mercaptopurine dose was adjusted with *TPMT* genotype in ALL
Methotrexate	*SLC19A1* (G80A, AA or AG vs. GG)	Worse EFS and higher gastrointestinal toxicities in ALL
	SLC19A1 AA80 and positive *GSTM1*	Higher incidence of hepatotoxicity in ALL
	DHFR (G308A, AA or AG vs. GG) and *DHFR* *1b haplotype	Worse EFS in ALL
	TYMS (3/3 vs. 2/3 or 2/2)	Increased risk of hematologic relapse in ALL
	MTHFR (C677T, TT vs. CC)	Increased risk of relapse in ALL
	MTHFR (T677A1298 haplotype)	Lower EFS in ALL, especially in the presence of *TYMS* (3/3)
	MTHFD1 (A1958 variant, AA or GA vs. GG)	Lower EFS in ALL, especially in the presence of *TYMS* (3/3)
	SLCO1B1 (rs11045879 [T allele], rs4149081 [G allele], and rs4149056 [TT])	Higher methotrexate clearance and higher incidence of gastrointestinal toxicity in ALL
Glucocorticoids Prednisone Prednisolone	*NR3C1* (-627 AA vs. AG or GG, intron 2+646 CG or GG vs. CC, and 9b TT vs. CC or TC)	Worse EFS in ALL
Dexamethasone	*NR3C1* (1088 AG vs. AA)	Increased risk of hematologic relapse in ALL patients with *GSTM1* null genotype
	GSTT1 (*0/0 vs. *A/A)	Better early response to prednisone in ALL
	GSTP1 (codon105 Val/Val or Val/Ile vs. Ile/Ile, codon 114 Ala/Ala vs. Ala/Val or Val/Val)	Increased central nervous system relapse in ALL
	GSTM1 (null vs. normal)	Severe infection in ALL
	SNPs in *CNTNAP2, LEPR, CRHR1, NTAN1, SLC12A3, ALPL, BGLAP,* and *APOB*	Association with hypertension during remission-induction therapy in ALL
	ACP1 (rs 12714403 AA or AG vs. GG)	Higher incidence of symptomatic osteonecrosis in ALL
	TYMS (2/2 vs 3/3 or 2/3) and *VDR* (rs2228570, CC vs. TT or CT)	Higher incidence of osteonecrosis in ALL
	PAI-1 (rs6092, AA or GA vs. GG)	Higher incidence of osteonecrosis in ALL
Asparaginase		
Escherichia coli asparaginase	*ATF5* T1562C (TT or CT vs. CC)	Inferior EFS when treated with *Escherichia coli* asparaginase in ALL
PEG-asparaginase	SNPs in *ADSL, DARS, ASS1, DARS2, NARS2, ADSSL1*	Association with in vitro asparaginase sensitivity in ALL
Erwinia asparaginase	*GRIA1* (rs4958381, A allele vs. G allele)	Higher incidence of asparaginase hypersensitivity in ALL
Vincristine	*VDR* intron 8 (AA or AG vs. GG), *CYP3A5*3* (AA or AG vs. GG)	Increased peripheral neuropathy in ALL
	PMP22 duplication (Charcot-Marie-Tooth disease)	Increased peripheral neuropathy in ALL
Anthracycline		
Daunorubicin	*CBR3 V244M* (GG vs. GA or AA)	Increased cardiomyopathy, especially with anthracycline dose at 1-250 mg/m^2
Doxorubicin	*SLC28A3* (rs7853758, L461L)	Decreased risk of cardiotoxicity
Mitoxantrone	*ABCC1* (rs3743527TT, combination of rs3743527TT-rs246221TC/TT)	Increased cardiotoxicity in ALL
Idarubicin	*CAT* (rs10836235; c.66 + 78C > T, CC vs. TC)	Increased cardiotoxicity in ALL
Cytarabine	*SLC29A1* haplotype with −1345C>G, −1050G>A, or −706G>C	Associated with high hENT1 expression
	DCK −360G allele (GG or CG vs. CC)	Increased risk of mucositis after low-dose cytarabine in ALL
	CDA A79C (CC vs. AA or AC)	Higher postinduction treatment-related mortality in AML
	CDA G208A (GA or AA vs. GG)	Lower activity of CDA and increased sensitivity to cytarabine in ALL, AML, and NHL

TABLE 46-4 Pharmacogenomics of Chemotherapeutic Agents Used for Pediatric Cancer (Continued)

Agent	Genes Involved	Effects
Etoposide	*ABCB1* exon 26 (3435C>T, CC or CT or TT)	Higher clearance in patients with ALL who have prednisone pretreatment
	*CYP3A5*3* (AA vs. AG or GG) and *GSTP1* (313 A>G, AA vs. AG or GG)	Lower clearance in black patients with ALL who have prednisone pretreatment
	VDR intron 8 (GG vs. AG) and *VDR* Fok 1 (CC vs. CT or TT)	Higher clearance in black patients with ALL who do not have prednisone pretreatment
	UGT1A1 (7/7 vs. 6/7 or 6/6)	Lower clearance in black patients with ALL who do not have prednisone pretreatment
	SNPs in *AGPAT2, IL1B,* and *WNT5B*	Higher etoposide catechol AUC in black and white patients with ALL
	SNPs in *UVRAG, SEMA5A, SLC7A6,* and *PRMT7*	Association with etoposide cytotoxicity
		Association with etoposide cytotoxicity
	MDM2 309T>G	Decreased sensitivity to etoposide
Cyclophosphamide	*CYP2B6*4* (AG or GG vs. AA), recipient	Higher incidence of oral mucositis in patients with leukemia who received MSD HSCT
	*CYP2B6*2* (CT or TT vs. CC), recipient	Higher incidence of hemorrhagic cystitis in patients with leukemia who received MSD HSCT
	*CYP2B6*6* (GG vs. GT or TT), donor	Higher incidence of SOS in patients with leukemia who received MSD HSCT
	CYP2B6 rs3211371, R487C (Arginine), recipient	Reduced toxicity <100 days after allogeneic HSCT
	SOD2 rs4880, V16A (Valine), recipient	Higher overall toxicity after allogeneic HSCT
Platinum compounds Cisplatin Carboplatin Oxaliplatin	*ERCC2* Lys751Gln G allele (TG or GG vs. TT)	Poor tumor response and shorter EFS in patients with osteosarcoma treated with cisplatin
	GSTM3 (*B allele vs. *A allele)	Protective effect for cisplatin-induced ototoxicity in solid tumor and CNS malignancies
	GSTP1 (^{105}Val/^{105}Val vs. ^{105}Ile/^{105}Ile or ^{105}Ile/^{105}Val)	Protective effects in long-term cisplatin-induced hearing impairment in testicular cancer
	GSTM1 and *GSTT1* polymorphisms (≥1 null type vs. non-null type)	More cognitive impairment after therapy in children with medulloblastoma
	TPMT (rs12201199, A allele) and *COMT* (rs9332377, A allele)	Increased cisplatin-induced hearing loss in patients with solid tumor and CNS malignancies
	Megalin (rs2075252, AG or AA vs. GG)	Increased cisplatin-induced hearing loss in patients with solid tumor and CNS malignancies
Irinotecan	*UGT1A1*28* (7/7 vs. 6/7 or 6/6)	No association with severe toxicity when treated with low dose and protracted schedule

ALL, Acute lymphoblastic leukemia; *AML,* acute myeloid leukemia; *AUC,* area under the plasma concentration versus time curve; *CDA,* cytidine deaminase; *CNS,* central nervous system; *EFS,* event-free survival; *hENT1,* human equilibrative nucleoside transporter; *HSCT,* hematopoietic stem cell transplantation; *MSD,* matched sibling donor; *NHL,* non-Hodgkin lymphoma; *SNP,* single nucleotide polymorphism; *SOS,* sinusoidal obstruction syndrome; *TPMT,* thiopurine methyltransferase.

potential clinical importance of pharmacogenomics (Fig. 46-7 and Table 46-4).[77-79] SNPs in the *TPMT* gene are associated with different inherited levels of TPMT enzyme activity.[80-83] One in approximately 400 white persons has profound TPMT deficiency resulting from inheritance of two nonfunctional variants of *TPMT*, 6% to 11% have intermediate activity by having heterozygous variants of *TPMT*, and 89% to 94% have normal activity (homozygous wild-type *TPMT*).[74,84,85] Ethnic differences exist in the frequency of occurrence of these variant alleles and thus in the frequency of TPMT deficiency. To date more than 20 *TPMT* variants have been identified, but three variants—*TPMT*3A* (460 G>A and 719 A>G), *TPMT*3C* (719 A>G), and *TPMT*2* (238 G>C)—account for more than 90% of inactivating alleles.[86,87]

The specific activity of TPMT determines the efficacy of thiopurine treatment and serves as an indirect indicator of hematologic toxicity.[88] At conventional thiopurine doses, patients with inherited TPMT deficiency accumulate excess intracellular concentrations of thioguanine nucleotides (TGN) especially in hematopoietic cells, predisposing them to moderate to severe hematologic toxicity depending on their deficiency status (i.e., heterozygous vs. homozygous).[77,89] In addition, several studies have found a relationship between low TPMT activity and secondary neoplasms, especially at high doses (e.g., mercaptopurine, 75 mg/m^2/day).[90,91] Patients with homozygous and heterozygous TPMT deficiency can be treated safely with appropriate adjustment of the thiopurine dosage.[92,93]

Because some patients with wild-type *TPMT* experience toxicities during mercaptopurine treatment, a genome-wide association study was performed to identify additional genetic determinants of thiopurine toxicity (see Table 46-4). In an analysis of a panel of human HapMap cell lines followed by validation in patients with ALL, the protein kinase C and casein kinase substrate in neurons–2 *(PACSIN2)* gene was among the top genes

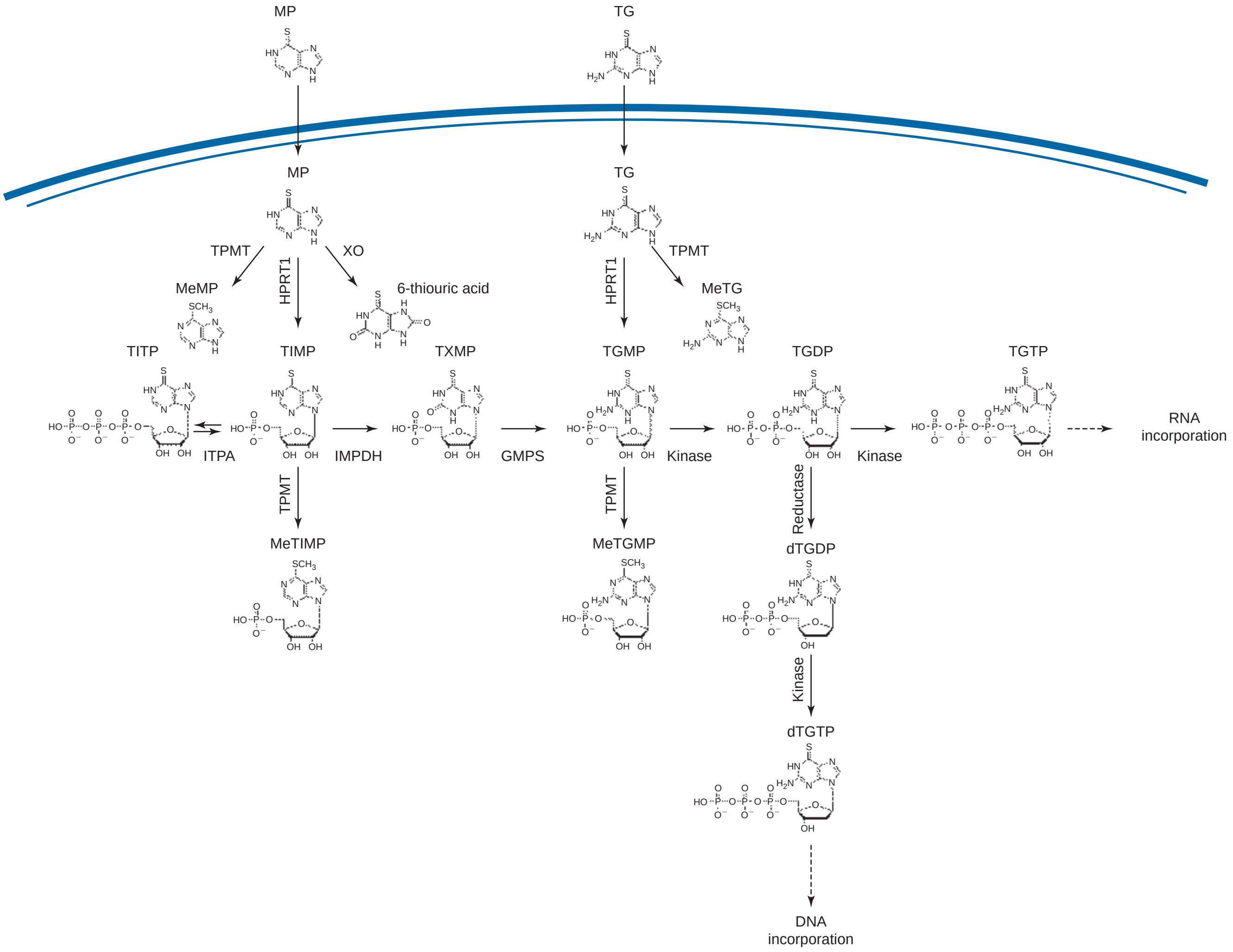


Figure 46-6 Metabolic pathways of 6-mercaptopurine and 6-thioguanine. 6-Mercaptopurine *(MP)* and 6-thioguanine *(TG)* are prodrugs that require conversion to thioguanine nucleotides (TGNs) via the purine-salvage pathway. The TGNs are incorporated into deoxyribonucleic acid *(DNA)* or ribonucleic acid *(RNA)*. Thiopurines are inactivated by thiopurine methyltransferase *(TPMT)* through catalytic methylation and by xanthine oxidase *(XO)*. Inosine triphosphate pyrophosphatase *(ITPA)* catalyzes the hydrolysis of thioinosine triphosphate *(TITP)* to 6-thioinosine 5′-monophosphate *(TIMP)*. *dTGDP,* Deoxy-6-thioguanosine 5′-diphosphate; *dTGTP,* deoxy-6-thioguanosine 5′-triphosphate; *GMPS,* guanosine monophosphate synthetase; *HPRT1,* hypoxanthine phosphoribosyltransferase 1; *IMPDH,* inosine-5′-monophosphate dehydrogenase; *Me,* S-methyl; *TGDP,* 6-thioguanosine 5′-diphosphate; *TGMP,* 6-thioguanosine 5′-monophosphate; *TGTP,* 6-thioguanosine 5′-triphosphate; *TXMP,* 6-thioxanthosine 5′-monophosphate.

identified, and the T allele of the *PACSIN2* SNP rs2413739 was significantly associated with lower TPMT activity and a higher incidence of severe gastrointestinal toxicity during consolidation therapy for ALL.[94] PACSIN2, also called syndapin II, has a role in various biologic processes, including endocytosis, cell-cycle control, and autophagy. PACSIN2 may influence TPMT activity by influencing *TPMT* messenger RNA levels and/or TPMT protein degradation. Furthermore an SNP (rs41320251, C>A) in the inosine triphosphate pyrophosphatase *(ITPA)* gene was associated with significantly higher accumulation of methylmercaptopurine nucleotides and a higher incidence of febrile neutropenia during ALL therapy in patients whose mercaptopurine doses had been individualized to the *TPMT* genotype.[95] These findings illustrate that even when treatment has been adjusted for the strongest genetic determinant of drug response, additional genetic variants may emerge as determinants of a drug's effects.

Methotrexate

Methotrexate is a folate analogue commonly used in the treatment of certain pediatric malignancies, including ALL and osteosarcoma (Fig. 46-8 and Table 46-1).[96] Cellular uptake is mediated mainly by the reduced folate carrier RFC1 (also known as SLC19A1).[97-99] In cells, cytosolic folylpolyglutamyl synthase converts methotrexate to methotrexate polyglutamates (MTXPGs) by adding as many as five glutamate residues. Methotrexate and MTXPG inhibit dihydrofolate reductase (DHFR), the enzyme responsible for conversion of dihydrofolate to its active form, tetrahydrofolate. In addition, MTXPG

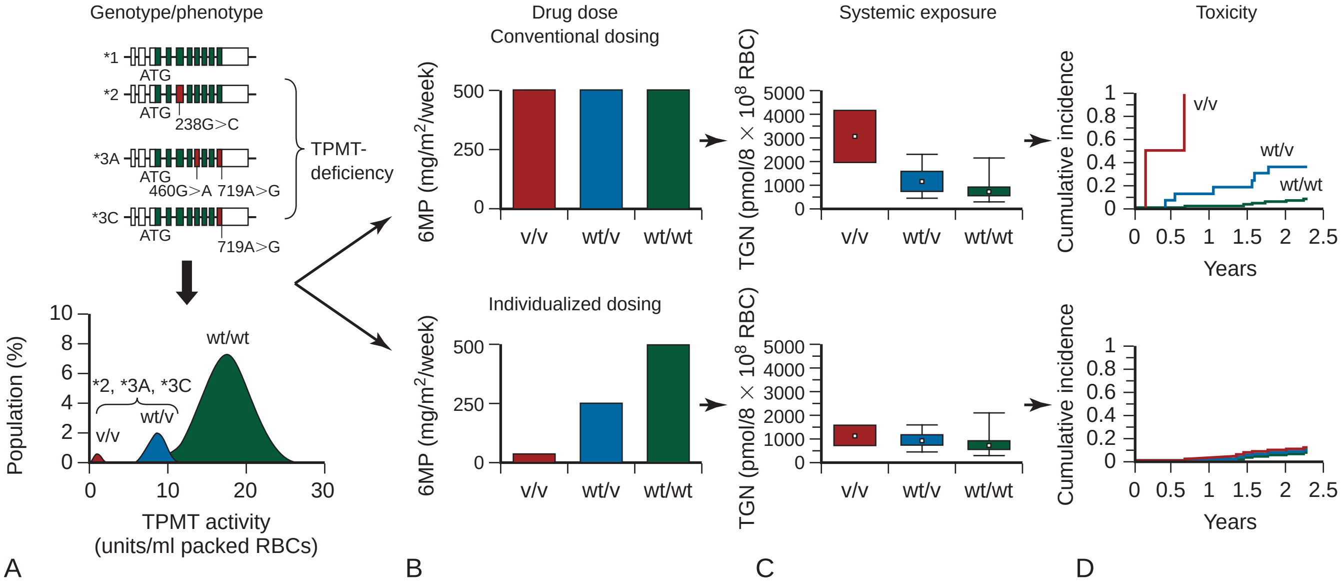


Figure 46-7 Polymorphisms in thiopurine methyltransferase and their effects on the pharmacogenetics of mercaptopurine toxicity. Wild-type thiopurine methyltransferase (*TPMT*1*) and the three predominant *TPMT* variant alleles (*TPMT*2*, *TPMT*3A*, and *TPMT*3C*) are shown (**A**). These changes cause autosomal codominant inheritance of TPMT activity in humans. The wild-type *(wt/wt)* enzyme is most active, homozygous variants *(v/v)* are least active, and the heterozygous wild-type/variant enzyme *(wt/v)* has intermediate activity. When mercaptopurine *(MP)* treatment is given to a cohort of patients, dosing can be conventional or individualized according the patient's genotype (**B**). When conventional doses of mercaptopurine are given, TPMT-deficient patients *(v/v)* have a systemic exposure to active thioguanine nucleotides *(TGN)* 10 times that of wild-type patients *(wt/wt)*, and heterozygous patients have TGN concentrations about two times that of wild-type patients (**C**). The higher concentrations of TGN cause a significantly higher frequency of hematopoietic toxicity (**D**). When doses specifically adjusted to the genotype are given in an individualized therapeutic regimen, similar cellular TGN concentrations are achieved in all patients, and all three TPMT phenotypes can be treated without acute toxicity. *RBC,* Red blood cell. *(Modified from Cheok MH, Evans WE: Acute lymphoblastic leukaemia: a model for the pharmacogenomics of cancer therapy.* Nat Rev Cancer *6:117–129, 2006.)*

inhibits thymidylate synthetase (TYMS), thereby blocking purine and thymidine synthesis, as well as DNA and RNA synthesis. MTXPG is not readily effluxed from cells, making its formation advantageous for anticancer effects of methotrexate. Other enzymes such as 5,10-methylenetetrahydrofolate reductase (MTHFR) and methylenetetrahydrofolate dehydrogenase–1 (MTHFD1) are also key elements in folate metabolism and in methotrexate effects.[100,101] MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate of homocysteine remethylation to methionine, and MTHFD1 catalyzes interconversion of 1-carbon derivatives of tetrahydrofolate, which are substrates for methionine, thymidylate, and de novo purine synthesis. MTXPG can be degraded via cleavage by lysosomal γ-glutamyl hydrolase. Several transporters (e.g., ABCG2, ABCC2, OATP1B1 [SLCO1B1], OAT1, OAT3, and OAT4) also participate in methotrexate clearance.

Interpatient variability in the efficacy and toxicity of methotrexate treatment is associated with genetic variations in methotrexate metabolic pathways (see Table 46-4). The G80A polymorphism of *SLC19A1* replaces Arg with His at position 27 of the major methotrexate transporter RFC1 (encoded by *SLC19A1*). ALL patients with the A80 variant had lower event-free survival estimates than did those with the GG genotype.[102,103] However, this association has not been widely replicated, and patients homozygous for A80 had higher plasma levels of methotrexate than the other genotype groups; the mechanism by which this SNP might confer a worse prognosis is not clear.[103] In other studies, the G80A *SLC19A1* genotype was associated with a substantially higher incidence of gastrointestinal toxicity,[104] and patients with AA80 and positive *GSTM1* (glutathione S-transferase mu–1) had a higher incidence of hepatotoxicity.[105] Patients with Down syndrome experience greater methotrexate toxicity, requiring dose reduction and early leucovorin rescue.[106] This toxicity may partly reflect enhanced intracellular uptake of methotrexate, because *SLC19A1* is located on chromosome 21 and thus three copies are present in all cells of persons with Down syndrome, although other mechanisms, such as enhanced methotrexate polyglutamination and reduced folate depletion, may also be involved.

Altered levels of DHFR expression have been found in patients with relapsed ALL and in ALL cells with a methotrexate-resistant phenotype.[107,108] Genetic variations in both a major promoter and a noncoding minor transcript region of *DHFR* have been identified.[109] Patients with ALL who had an A allele of the G308A polymorphism and the *1b haplotype of *DHFR* had a significantly poorer outcome because of increased DHFR expression.

The promoter region of the *TYMS* gene exhibits a genetic polymorphism, having either two or three 28-base-pair tandem repeat sequences.[110] *TYMS* (3/3) was associated with greater TYMS expression and a

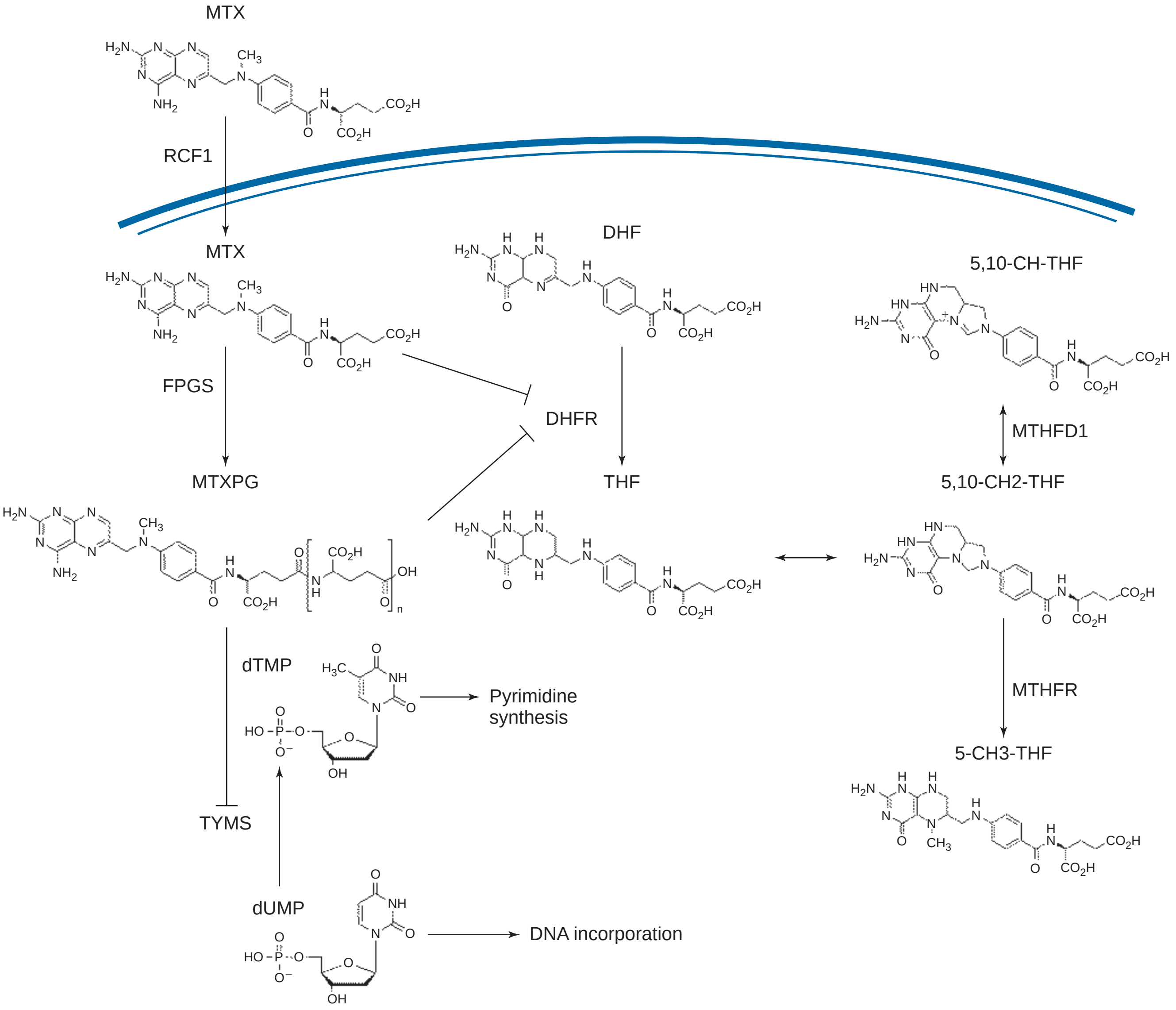


Figure 46-8 Metabolic pathways of methotrexate. Cellular uptake is mediated mainly by reduced folate carrier–1 *(RFC1)*. Within cells, cytosolic folylpolyglutamyl synthase *(FPGS)* converts methotrexate to methotrexate polyglutamates *(MTXPGs)* by adding glutamate residues. Methotrexate *(MTX)* and MTXPGs inhibit dihydrofolate reductase *(DHFR)*, the enzyme responsible for conversion of dihydrofolate *(DHF)*, to its active form, tetrahydrofolate *(THF)*. In addition, MTXPGs inhibit thymidylate synthetase *(TYMS)*, thereby blocking purine and thymidine synthesis, as well as deoxyribonucleic acid *(DNA)* and ribonucleic acid synthesis. 5,10-methylenetetrahydrofolate reductase *(MTHFR)* and methylenetetrahydrofolate dehydrogenase 1 *(MTHFD1)* are also key elements in folate metabolism and in methotrexate effects. *5,10-CH-THF,* 5,10-methenyltetrahydrofolate; *5,10-CH2-THF,* 5,10-methylenetetrahydrofolate; *5-CH3-THF,* 5-methyltetrahydrofolate; *dTMP,* deoxythymidine monophosphate; *dUMP,* deoxyuridine monophosphate.

greater risk of hematologic relapse of ALL than was observed in children with a 2/2 or 2/3 genotype, possibly implicating resistance to methotrexate, which targets this enzyme.[111,112]

Polymorphisms in the *MTHFR* gene, such as C677T and A1298C, which result in reduced enzymatic activity, have been implicated in the response to methotrexate treatment.[113-115] The *MTHFR* C677T polymorphism is a common genetic variant, and enzyme activity is reduced by 70% in patients with the homozygous TT polymorphism, conferring a greater risk of relapse in children with ALL.[116,117] Patients with either the *MTHFR* T677A1298 haplotype or the *MTHFD1* A1958 variant also had a lower rate of event-free survival, which became more evident in the presence of *TYMS* (3/3).[100]

A genome-wide analysis has identified common inherited variations in *SLCO1B1,* encoding OATP1B1, as important determinants of methotrexate pharmacokinetics and clinical effects.[118] Two SNPs, rs11045879 and rs4149081, were in linkage disequilibrium with each other and with the functional polymorphism T521C (rs4149056). These SNPs were associated with methotrexate clearance and gastrointestinal toxicity. The association of *SLCO1B1* SNP rs4149056 with methotrexate clearance was further validated in five different treatment regimens.[119] Of note, rare genetic variants of the

SLCO1B1 gene have been shown to have a greater impact on methotrexate clearance than common variants and to contribute to the inherited basis of methotrexate clearance and toxicity.[120]

Glucocorticoids

Glucocorticoids (prednisone or prednisolone and dexamethasone) are important components of ALL treatment (see Table 46-1). Glucocorticoids diffuse into the cell and bind to the glucocorticoid receptor, leading to its translocation into the nucleus.[121-123] In the nucleus, the glucocorticoid receptor transactivates or transrepresses glucocorticoid-responsive genes,[124] leading to inhibition of cytokine production, alteration of oncogene expression, cell-cycle arrest, and apoptosis.[125] Glucocorticoids can be metabolized by CYP450 enzymes (mainly CYP3A4) and glutathione-*S*-transferases (GSTs) and are transported by ABCB1.[126-129]

Sensitivity to glucocorticoid therapy is variable among patients, both in efficacy and the prevalence and severity of adverse effects. Numerous polymorphic sites in the glucocorticoid receptor gene (*NR3C1*) are described as being associated with prognosis (see Table 46-4).[130] Polymorphisms −627A/G, intron 2 +646C/G, and 9bT/C of *NR3C1* have each been associated with event-free survival in childhood ALL.[131] The *NR3C1* AG genotype (1088A>G) showed a greater association with hematologic relapse than did the *NR3C1* AA genotype among children with the *GSTM1*-null genotype.[111] Association between polymorphisms of the phase II enzyme GST and prognosis has also been reported. A deletion polymorphism in glutathione-*S*-transferase theta–1 *(GSTT1)* modulates the early response to glucocorticoids in childhood ALL; patients with *GSTT1**0/0 had a better early response to prednisone than did patients with *GSTT1**A/A.[132] Polymorphisms in glutathione-*S*-transferase pi–1 *(GSTP1)* codon 105 (Ile/Val) and codon 114 (Ala/Val) were associated predominantly with CNS relapse.[133]

The acute and late adverse effects associated with glucocorticoids include hypertension, infection, and osteonecrosis. Among 203 candidate polymorphisms, those in eight genes (*CNTNAP2* [contactin-associated protein-like–2], *LEPR* [leptin receptor], *CRHR1* [corticotropin releasing hormone receptor–1], *NTAN1* [N-terminal asparagine amidase], *SLC12A3*, *ALPL* [alkaline phosphatase, liver/bone/kidney], *BGLAP* [bone gamma-carboxyglutamate (gla) protein], and *APOB* [apolipoprotein B]) were associated with hypertension during remission-induction therapy (prednisone, 40 mg/m^2/day for 28 days) for childhood ALL.[134] In another candidate gene study that included *NR3C1*, *ABCB1*, *GSTM1*, *GSTP1*, *GSTT1*, and interleukin 10 *(IL10)* genes, the *GSTM1*-null genotype was associated with severe infections.[135] Glucocorticoid-induced osteonecrosis causes severe pain, limited range of motion of joints, and eventual joint destruction and mechanical failure because of compromised bone vascularization, demineralization, and trabecular thinning.[136] Clinically, older age, greater treatment intensity, lower serum albumin levels, higher cholesterol, and low dexamethasone clearance rates were important prognostic factors for osteonecrosis.[137,138,139,140,141,142] Polymorphisms (AA/AG) in acid phosphatase–1, soluble *(ACP1;* e.g., rs12714403), which regulates lipid levels and osteoblast differentiation, were associated with a greater risk of osteonecrosis and with lower albumin and higher cholesterol concentrations compared with those in patients with the GG genotype.[141] In addition, variants in *TYMS* (enhancer 2-repeat allele), vitamin D (1,25-dihydroxyvitamin D3) receptor *(VDR)* Fok I (rs2228570), and plasminogen activator inhibitor–1 *(PAI1;* rs6092) have been found to be associated with a higher incidence of osteonecrosis.[137,143]

Asparaginase

Asparaginase catalyzes the hydrolysis of asparagine to aspartic acid and ammonia (see Table 46-1). ALL cells have a low level of asparagine synthetase (ASNS) and are therefore dependent on external sources of asparagine.[144,145] Depletion of circulating endogenous asparagine by asparaginase therapy selectively kills leukemic cells by decreasing their protein synthesis. Three different formulations of asparaginase are available: native asparaginase derived from *Escherichia coli* (*E. coli* asparaginase); a pegylated form of the native *E. coli* asparaginase (polyethylene glycol [PEG] asparaginase); and asparaginase isolated from *Erwinia chrysanthemi* (*Erwinia* asparaginase).[146,147] The different asparaginase preparations have different pharmacokinetic properties, and thus the dosing schedule of each preparation is adjusted based on the half-life of the preparation; serum asparaginase concentrations at or above 0.1 international units/mL are believed to deplete asparagine levels to below the limit of detection.[148]

Asparaginase resistance appears to be caused by various mechanisms, and ASNS plays a variable functional role (see Table 46-4).[149-152] An analysis of 14 polymorphisms in the regulatory and coding regions of *ASNS*, activating transcription factor–5 *(ATF5)*, and argininosuccinate synthase–1 *(ASS1)* genes showed that *ATF5* T1562C was associated with lower event-free survival rates in patients with ALL who were treated with *E. coli* native asparaginase.[153] T1562 was associated with greater *ATF5* promoter activity, which may increase ASNS expression and lead to worse outcome. In a genome-wide study seeking to identify determinants of asparaginase sensitivity in lymphoblastoid HapMap cell lines and patient-derived ALL cells, the aspartate metabolic pathway was the most overrepresented.[154] SNPs primarily involved in this pathway included those in adenylosuccinate lyase *(ADSL)*, aspartyl-tRNA synthetase *(DARS)*, *ASS1*, *DARS2*, asparaginyl-tRNA synthetase–2 *(NARS2)*, and adenylosuccinate synthase-like–1 *(ADSSL1)*.

Asparaginase may be associated with adverse effects, including hypersensitivity, pancreatitis, hyperglycemia, and thrombosis.[155-158] Asparaginase hypersensitivity can lead to antibody formation, which is more commonly observed with native *E. coli* asparaginase than with PEG asparaginase.[159,160] Antibody formation was related to low systemic exposure to asparaginase and even to higher systemic clearance of dexamethasone.[161,162] A genome-wide association approach identified variations in the glutamate receptor, ionotropic, AMPA1 *(GRIA1)* gene, especially SNP rs4958381, as being associated with the risk of hypersensitivity to asparaginase among children with ALL.[163]

Vincristine

Vincristine is a natural alkaloid isolated from *Catharanthus roseus* (the Madagascar periwinkle) and is widely used in the treatment of both hematologic and solid malignancies (see Table 46-1).[164] Vincristine interferes with microtubule formation and mitotic spindle dynamics by interacting specifically with β-tubulin, causing mitotic arrest and cell death.[165] Vincristine is metabolized by CYP3A4 and CYP3A5 in the liver, mainly forming a secondary amine, M1 metabolite.[166-168] Vincristine appears to be exported from cells by ABCB1, ABCC1, ABCC2, and ABCC10.[169-173]

Vincristine is associated with a dose-limiting peripheral neurotoxicity, and several factors may influence its incidence and severity (Table 46-4). For example, underlying neuropathy (e.g., Charcot-Marie-Tooth disease [especially with duplication of peripheral myelin protein–22 *(PMP22)*] and Guillain-Barré syndrome) or myopathy (e.g., muscular dystrophy) and malnutrition have been reported to be associated with greater vincristine neuropathy.[165] The mechanism of vincristine-induced peripheral neuropathy has not been fully elucidated. *CYP3A5* is highly polymorphic, with variants including *CYP3A5*3*, *CYP3A5*6*, and *CYP3A5*7*.[168,174,175] The association of genetic polymorphisms with vincristine-induced neurotoxicity appears to be inconsistent and may be influenced by the treatment context (e.g., disease and concomitant therapy). VDR regulates CYP3A4, CYP3A5, and ABCB1.[176] Variants in *VDR* intron 8 (AA or AG) and *CYP3A5*3* (AA or AG) were associated with a higher incidence of peripheral neurotoxicity during continuation therapy in children with ALL,[104] and children with ALL who express a functional CYP3A5 experienced less vincristine-induced peripheral neuropathy.[177] In another study, however, patients with the *CYP3A4*1B* and *CYP3A5*3* variant genotypes had a decreased risk of peripheral neuropathy that was statistically significant in a univariate analysis but not in a multivariate analysis.[178] Expression of the efflux transporters ABCB1 and ABCC1 in cancer cells has been reported to be associated with reduced accumulation of, and in vitro resistance to, vincristine.[179] However, a retrospective analysis of children with ALL showed no association between the *ABCB1* SNPs C3435T and G2677T and vincristine pharmacokinetics or toxicity.[180] Thus additional studies are needed to identify genomic variations associated with vincristine neurotoxicity and/or cancer cell resistance.

Anthracyclines

The anthracyclines (e.g., daunorubicin, doxorubicin, mitoxantrone, and idarubicin) are antibiotic chemotherapeutic agents used for both hematologic and solid malignancies (Table 46-1).[181,182] The antitumor activity of anthracyclines is attributed to their ability to intercalate between base pairs of DNA and inhibit the topoisomerase II enzyme, preventing the relaxing of supercoiled DNA and thus blocking transcription and replication.[183-185] Daunorubicin differs from doxorubicin by the substitution of a hydroxyl group for a hydrogen atom in the acetyl radical.[186] Although anthracyclines are considered to enter the cell via passive diffusion, transport proteins are also involved.[187,188] Within cells, the anthracyclines are substrates for the ABC efflux transporters. The mechanism of resistance to anthracyclines can involve increased efflux via ABCB1, ABCC1, and/or ABCG2.[189-191] To overcome this resistance, functional modulation of the ABC drug transporters has been investigated, mainly in adults.[192,193]

The cumulative dose of anthracyclines is limited by their selective toxicity to cardiomyocytes, which are vulnerable to free radical damage due to low activity of antioxidant enzymes. Free oxygen radicals cause DNA damage and mitochondrial dysfunction.[194] Daunorubicin and doxorubicin are metabolized to the more cardiotoxic hydroxyl derivatives, daunorubicinol and doxorubicinol, respectively, by the myocardial cytosolic carbonyl reductases (CBRs) and aldo-keto reductases. In humans, two monomeric CBRs (CBR1 and CBR3) are encoded by genes located on chromosome 21.[195,196] The *CBR3* V244M polymorphism showed a trend toward association with the risk of congestive heart failure (see Table 46-4).[197] Although the risk of cardiomyopathy generally increases with cumulative doses of anthracycline greater than 250 mg/m^2, a homozygous G allele in *CBR3* V244M increases the risk of cardiomyopathy even at low to moderate cumulative doses (1 to 250 mg/m^2), suggesting that no safe dosage exists for patients with this SNP (Fig. 46-9).[198] In a study of 2977 SNPs in 220 key drug biotransformation genes, it was found that the synonymous coding variant rs7853758 (L461L) within the *SLC28A3* gene had a significantly protective effect

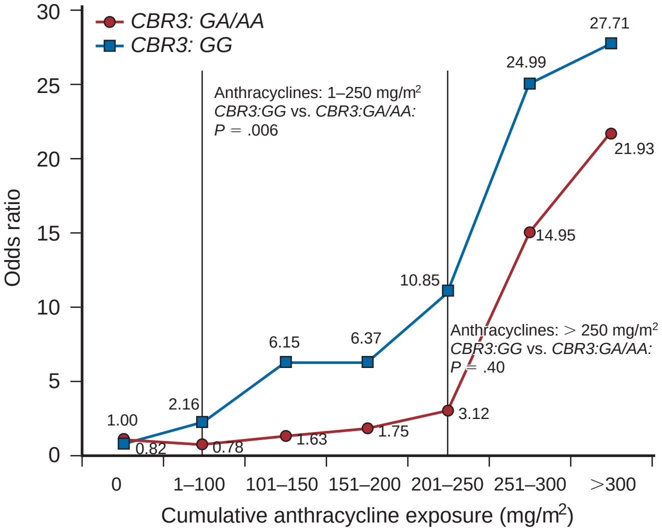


Figure 46-9 Dose-response relationship between cumulative anthracycline exposure and risk of cardiomyopathy, stratified by patients' carbonyl reductase–3 *(CBR3)* genotype (*CBR3*:GG or *CBR3*:GA/AA). Patients with the *CBR3*:GA/AA genotype who were not exposed to anthracyclines served as the reference group. Magnitude of risk is expressed as odds ratio. *(From Blanco JG, Sun CL, Landier W, et al: Anthracycline-related cardiomyopathy after childhood cancer: role of polymorphisms in carbonyl reductase genes—a report from the Children's Oncology Group.* J Clin Oncol *30:1415–1421, 2012.)*

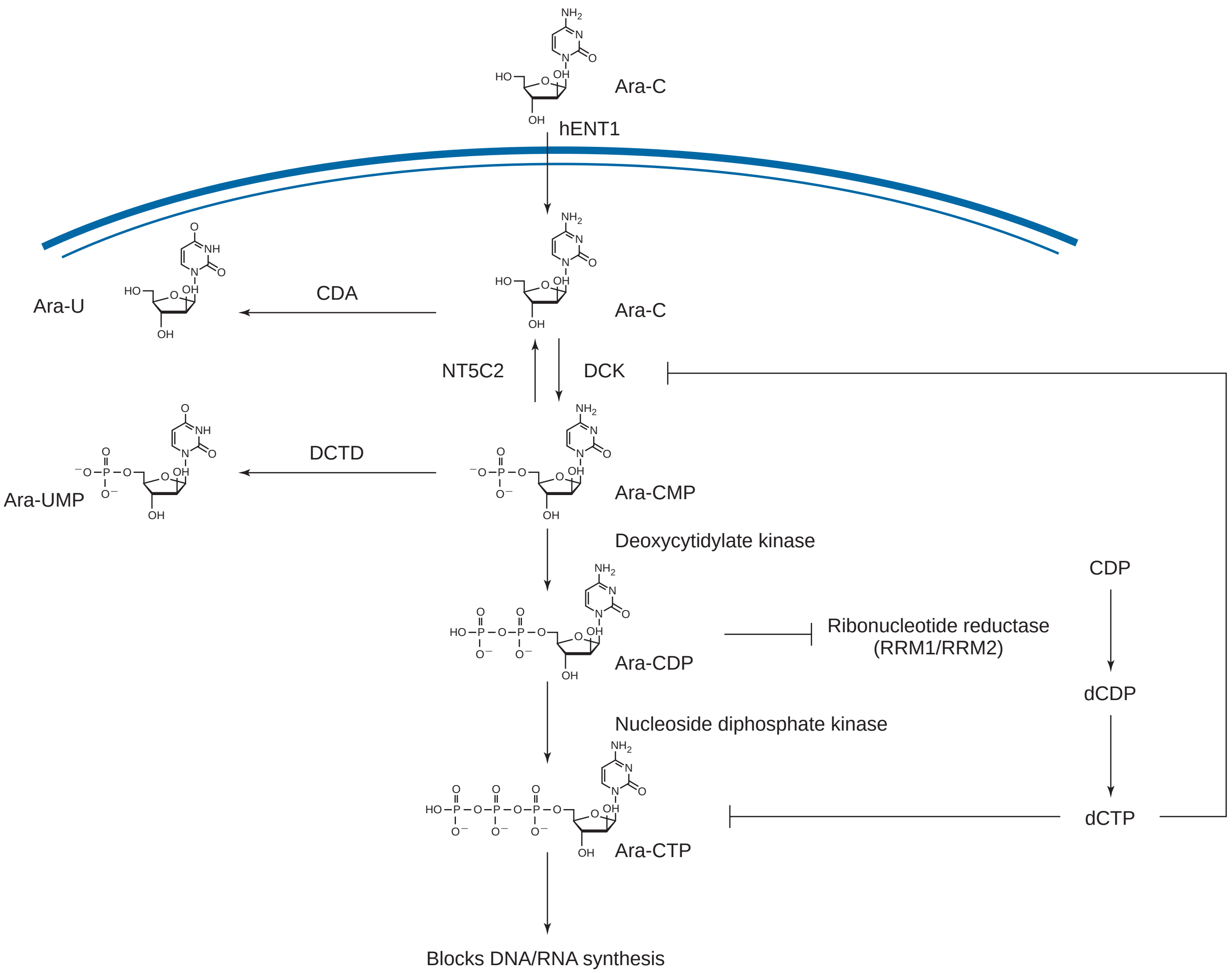


Figure 46-10 Metabolic pathways of cytarabine. Cytarabine *(Ara-C)* is transported through the cell membrane by the human equilibrative nucleoside transporter *(hENT1)*. It is first phosphorylated by deoxycytidine kinase *(DCK)* to form arabinosylcytosine 5′-monophosphate *(ara-CMP)*, which is then phosphorylated by deoxycytidylate kinase to arabinosylcytosine 5′-diphosphate *(ara-CDP)* and converted by nucleoside diphosphate kinase to the active arabinosylcytosine 5′-triphosphate *(ara-CTP)*. Ara-CMP can be dephosphorylated back to ara-C by 5′-nucleotidase *(NT5C2)*. Both ara-C and ara-CMP can be converted to inactive forms (uracil arabinoside *[ara-U]* and uracil arabinoside monophosphate *[ara-UMP]*) by cytidine deaminase *(CDA)* and deoxycytidylate deaminase *(DCTD)*, respectively. Larger intracellular pools of deoxycytidine triphosphate *(dCTP)*, which is regulated by ribonucleotide reductase, can compete with deoxyribonucleic acid *(DNA)* incorporation of ara-CTP and inhibit DCK activity. *CDP,* Cytidine diphosphate; *dCDP,* deoxycytidine diphosphate; *RNA,* ribonucleic acid; *RRM,* ribonucleotide reductase M.

against anthracycline-induced cardiotoxicity.[199] Risk and protective variants in other genes included *SLC28A1* and several ABC transporters (*ABCB1*, *ABCB4*, and *ABCC1*). Combination of these multiple variants into a single prediction model that included clinical risk factors was able to identify patients at high risk of anthracycline cardiotoxicity. Other studies found cardiotoxicity to be associated with the *ABCC1* rs3743527TT genotype and the rs3743527TT-rs246221TC/TT genotype combination[200] and with CC homozygosity at rs10836235 of the catalase gene (*CAT* c.66 + 78C > T) in childhood ALL.[201]

Cytarabine

Cytarabine (cytosine arabinoside, ara-C, 1-β-arabinofura nosylcytosine), is one of the most effective chemotherapeutic agents for treatment of AML and ALL (see Table 46-1). Ara-C is a deoxynucleoside analogue and is transported through the cell membrane by the human equilibrative nucleoside transporter (hENT1/SLC29A1) (Fig. 46-10). Within the cell, ara-C undergoes a series of phosphorylations. It is first phosphorylated by deoxycytidine kinase (DCK) to form arabinosylcytosine 5′-monophosphate (ara-CMP), which is then phosphorylated by pyrimidine kinases to the active 5′-triphosphate derivative, arabinosylcytosine 5′-triphosphate (ara-CTP).[202-204] Ara-CTP is incorporated into DNA and causes chain termination, blocking DNA and RNA synthesis.[205,206] Ara-CMP can be dephosphorylated back to ara-C by 5′-nucleotidase (NT5C2). Both ara-C and ara-CMP can be converted into inactive forms (uracil arabinoside [ara-U] and uracil arabinoside monophosphate [ara-UMP]) by cytidine deaminase (CDA) and deoxycytidylate deaminase, respectively.[207] Insufficient cellular uptake of ara-C caused by a low level or activity of hENT1 reduce levels of activating enzymes (primarily DCK), and increased levels of inactivating enzymes (e.g., NT5C2 and CDA) reduce the level of ara-CTP.[208,209] Further, larger intracellular pools of deoxycytidine triphosphate (dCTP), which is regulated by ribonucleotide reductase, can compete with DNA incorporation of ara-CTP and inhibit DCK activity.

The expression of hENT1 appeared to be related to the cytarabine sensitivity of childhood AML cells in vitro,[210] and haplotypes including −1345C>G, −1050G>A, and −706G>C of the *SLC29A1* promoter region were related to higher hENT1 expression, which may alter ara-C chemosensitivity (see Table 46-4).[211]

DCK activity and expression vary widely in normal and malignant cells but may be related to ara-C sensitivity and patient survival.[212] Polymorphisms in the *DCK* gene differ between races; SNPs in the 5′ regulatory region (−201C>T and −360C>G) are common in the Chinese population and less common in Caucasians.[213] Adult Chinese patients with AML who have the −360CG/−201CT and −360GG/−201TT compound genotype had higher levels of DCK mRNA and a greater response to chemotherapy than patients with the −360CC/−201CC compound genotype.[214] The *DCK* −360G allele was found to increase the risk of mucositis after exposure to low-dose cytarabine in Asian children with ALL.[215]

Analysis of functional SNPs in human *CDA* led to the identification of three polymorphisms (A79C, G208A, and T435C) in the coding region. Analysis of the impact of the *CDA* A79C genotype on therapy outcomes in children with AML showed that postinduction treatment-related mortality was significantly greater in patients with the CC genotype, which is associated with low CDA activity.[216] The G208A polymorphism results in an alanine to threonine substitution (A70T) within the conserved catalytic domain, and the 208A genotype was associated with lower CDA activity and greater sensitivity to ara-C in pediatric leukemia cells.[217] Another polymorphism in the *CDA* promoter, SNP C-451T, reduces the expression level and enzymatic activity of CDA.[218] *CDA* SNP C-451T was found to be a significant, independent predictor of survival in adult AML, in which the TT genotype predicted higher risk.

Imatinib Mesylate

Imatinib mesylate (Gleevec) or 4-[(4-methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl] benzamide methanesulfonate is a breakpoint cluster region-Abelson (BCR-ABL) tyrosine kinase inhibitor commonly used for the treatment of chronic myeloid leukemia and Philadelphia chromosome–positive ALL (see Table 46-1).[219-222] Imatinib is metabolized to the active metabolite N-desmethyl-imatinib by CYP3A4; CYP3A5 and other CYP enzymes (e.g., CYP2D6, CYP2C9, and CYP2C19) play minor roles.[223] Imatinib is a substrate of the ABC transporters ABCB1 and ABCG2. It is bound to plasma proteins, predominantly albumin and α1-acid glycoprotein.[224] Pharmacogenetic studies of imatinib, conducted mainly in adults, implicated SNPs in *ABCB1* (positions 1236, 2677, and 3435) and *ABCG2* (rs2231142) as affecting drug response.[225-228] Authors of a study in children with solid tumors and adults with gastrointestinal stromal tumors investigated the effect of several demographic, biologic, and pharmacogenetic covariates on the disposition of imatinib.[229] Morphologic and biologic characteristics (i.e., body weight, albumin, and plasma α1-acid glycoprotein levels) influenced imatinib pharmacokinetics more strongly than pharmacogenetics. Somatic DNA changes or selection of subclones with preexisting mutations in the tyrosine kinases targeted by imatinib are the predominant genetic mechanisms underlying imatinib resistance.[230]

Etoposide

Etoposide (VP-16), 4′-demethylepipodophyllotoxin-9-(4,6-O-ethylidene)-β-D-glucopyranoside, is a semisynthetic derivative of podophyllotoxin that inhibits DNA topoisomerase II and is used for the treatment of several malignancies, including AML and solid tumors (see Table 46-1).[231] Etoposide is metabolized to a catechol etoposide through an O-demethylation reaction catalyzed mainly by CYP3A4 and CYP3A5 and to a minor degree by CYP1A2 and CYP2E1.[232,233] CYP3A4 and CYP3A5 expression is regulated in part by the VDR.[176] Etoposide catechol metabolite binds covalently to DNA and cellular proteins and displays intrinsic cytotoxic properties.[234] Etoposide metabolites can be conjugated to glutathione by GSTs and can be glucuronidated by the uridine diphosphate–glucuronosyltransferase 1A1 *(UGT1A1)* gene.[235] Etoposide and its metabolites are substrates for the efflux transporters ABCB1, ABCC1, and ABCC2 and for the influx transporter OCTN2.[236,237] In addition, the pharmacokinetics of etoposide in children are reported to be influenced by other factors, including body weight and excessive urinary carnitine loss.[237,238]

Genetic variants that contribute to etoposide metabolism and etoposide-induced cytotoxicity have been identified (see Table 46-4). A study in children with ALL showed that the pharmacokinetics of etoposide and its catechol metabolite are related to polymorphisms in *CYP3A5*, *ABCB1*, *GSTP1*, *UGT1A1*, and *VDR* in a manner influenced by race and by prednisone use.[239] Etoposide clearance rates were significantly higher in patients who had recently received prednisone, which can modulate expressions of CYP3A and ABCB1. The *ABCB1* exon 26 (3435C>T) CC genotype was associated with higher etoposide clearance for patients on day 29 of remission-induction therapy, after 28 days of prednisone treatment, and the *CYP3A5*3* AA and *GSTP1* (313 A>G) AA genotypes predicted lower clearance in black patients. At week 54 of continuation treatment without preceding prednisone treatment, the *VDR* intron 8 GG and *VDR* Fok 1 CC genotypes predicted higher etoposide clearance, and *UGT1A1* 7/7 predicted lower clearance, in black patients. The *UGT1A1 7/7* genotype predicted higher etoposide catechol AUC in both black and white patients.

A genome-wide study using the HapMap human lymphoblastoid cell lines identified 63 genetic variants that contribute to interindividual variation in etoposide cytotoxicity.[240] These variants included genes previously reported to be associated with carcinogenesis and sensitivity to etoposide (e.g., 1-acylglycerol-3-phosphate O-acyltransferase–2 *[AGPAT2]*, interleukin-1 beta *[IL1B]*, and wingless-type MMTV integration site family, member 5B *[WNT5B]*) and genes not yet known to be associated with sensitivity to etoposide. The influence of heritable genetic variation on etoposide cytotoxicity was confirmed by the identification of additional genomic regions and SNPs in *UVRAG* (UV radiation resistance–associated), *SEMA5A* (sema domain, seven thrombospondin repeats [type 1 and type 1-like], transmembrane domain [TM] and short cytoplasmic domain, [semaphorin] 5A), *SLC7A6*, and *PRMT7* (protein arginine methyltransferase–7), which may also be associated with adverse effects, including myelosuppression, therapy-related leukemia, and neurotoxicity.[241] Cell lines homozygous for the *MDM2* (murine double minute 2) SNP309 T to G change in the promoter region showed a lower response to etoposide that was attributable, at least in part, to the lower expression of topoisomerase II.[242]

Cyclophosphamide

Cyclophosphamide is an alkylating agent widely used in the treatment of childhood cancers, including leukemia, lymphoma, neuroblastoma, rhabdomyosarcoma, and brain tumor (Table 46-1). Cyclophosphamide is a prodrug first metabolized into 4-hydroxycyclophosphamide (4-OHCP) in the liver, which is mediated by CYP2B6, CYP2C9, and CYP3A4.[243,244] 4-OHCP exists in equilibrium with the aldophosphamide, which spontaneously degrades to the active DNA-alkylating agent phosphoramide mustard and to acrolein. Cyclophosphamide and 4-OHCP can be transformed to the inactive metabolites dechloroethylcyclophosphamide and 4 keto-cyclophosphamide, respectively, by CYP3A4 and CYP3A5.[243,245] Cyclophosphamide metabolites are detoxified by aldehyde dehydrogenase (ALDH) enzymes (ALDH1A1 and ALDH3A1) and the glutathione-*S*-transferase enzyme system (GSTP1 and glutathione-S-transferase alpha–1 [GSTA1]).[246]

The adverse effects of cyclophosphamide include cardiotoxicity, nephrotoxicity, and hemorrhagic cystitis, but these effects vary among patients (see Table 46-4). In children and adults with leukemia who received human leukocyte antigen–identical sibling-donor hematopoietic stem cell transplants, recipient CYP2B6*4 (AG or GG), recipient CYP2B6*2 (CT or TT), and donor CYP2B6*6 (GG) were associated with oral mucositis, hemorrhagic cystitis, and hepatic sinusoidal obstruction syndrome, respectively.[247] Another study showed that CYP2B6 rs3211371 R487C, which inhibits the activation of cyclophosphamide, was significantly associated with reduced toxicity less than 100 days after transplantation.[248] Superoxide dismutase–2 (SOD2) is a mitochondrial enzyme that converts endogenous toxic superoxides to hydrogen peroxide, and the valine variant has lower enzymatic activity. In *SOD2* rs4880 V16A polymorphism, the valine allele was associated with significantly greater overall toxicity.

In adult women with breast cancer who were treated with cyclophosphamide, women with at least 1 *GSTP1* (rs1695) variant G allele had a lower risk of neutropenia and leukopenia than those with the AA genotype,[249] and women with *GSTA1**B/*B, which has reduced enzyme activity, had a higher survival rate than those with *GSTA1**A/*A and *GSTA1**A/*B.[250]

Platinum Compounds

Platinum-based drugs (cisplatin, carboplatin, and oxaliplatin) are widely used for the treatment of childhood solid tumors, including osteosarcoma, neuroblastoma, and hepatoblastoma (see Table 46-1).[251] Cisplatin (cis-diamminedichloroplatinum[II]) was first approved by the Food and Drug Administration in 1978, followed by carboplatin (cis-diammine[cyclobutane-1,1-dicarboxylate-O,O′] platinum[II]) in 1989 and oxaliplatin ([1*R*,2*R*]-cyclohexane-1,2-diamine[ethanedioato-O,O′] platinum[II]) in 2002. Although passive diffusion through the cellular lipid bilayer was hypothesized,[252-254] recent studies have shown the significant role of a variety of ion pumps and transporters.[255,256] The copper transporter protein copper transporter–1 (SLC31A1), which is involved in copper homeostasis, appears to be a major influx transporter of the platinum compounds.[257,258] ABCC2, ABCC4, copper-transporting P-type adenosine triphosphatases (ATP7B), MATE1, OCT1, and OCT2 are suggested to have a role in the cellular accumulation of platinum compounds.[259-263] GST catalyzes the conjugation of cisplatin with glutathione, which leads to its inactivation and excretion. Within cells, the platinum compounds are aquated and are then more reactive with the drug's cellular targets.[264-267] They bind to DNA and induce lesions that are recognized by multiple repair pathways, mainly the nucleotide excision repair system.[268-272] However, the mismatch repair system is also implicated in the recognition and repair of these lesions.[273,274] When the lesions cannot be repaired, apoptosis is induced through different signaling cascades.[275,276]

Previous studies using the lymphoblastoid cell lines have identified genetic variants associated with cisplatin-induced cytotoxicity (see Table 46-4).[277,278] *ERCC2* (excision repair cross-complementation group–2) is one of the genes involved in the nucleotide excision repair pathway; common variations in the *ERCC2* Lys751Gln G allele (TG or GG) were associated with poorer tumor response and shorter event-free survival than the TT allele in pediatric and young adult patients with osteosarcoma who were treated with cisplatin, suggesting that polymorphic variants in DNA repair genes could be useful predictors of response.[279]

Platinum compounds can cause adverse effects (e.g., ototoxicity and nephrotoxicity) in children, and cisplatin, carboplatin, and oxaliplatin have different toxicity profiles.[280] Cisplatin-induced hearing loss is usually bilateral and irreversible, affecting 41% to 61% of children treated, and several candidate genes may be associated with ototoxicity.[281] The *GSTM3**B allele was found to provide a protective effect against cisplatin-induced ototoxicity, compared with the *GSTM3**A allele.[282] GST genes were also associated with cisplatin-induced long-term hearing impairment in testicular cancer survivors; the genotype ^{105}Val/^{105}Val-*GSTP1* appeared to be protective in comparison with ^{105}Ile/^{105}Ile- or ^{105}Ile/^{105}Val-*GSTP1*.[283] The role of GST genes in other cisplatin-induced toxicities was confirmed by the discovery that *GSTM1* and *GSTT1* polymorphisms may affect cognitive impairment in children after treatment for medulloblastoma; patients with at least one null genotype were at greater risk than those without a null genotype.[284] Authors of another genotyping study of 1949 SNPs in 220 drug-metabolism genes in children identified genetic variants in *TPMT* (rs12201199, A allele) and catechol-O-methyltransferase *(COMT;* rs9332377, A allele) that were associated with cisplatin-induced hearing loss.[285] TPMT and COMT are methyltransferases dependent on the *S*-adenosylmethionine methyl donor substrate in the methionine pathway. The authors of this study proposed that higher levels of this substrate could provide a putative mechanism for cisplatin ototoxicity mediated by reduced TPMT and COMT activity. They suggested that decreased TPMT enzyme activity may also reduce the inactivation of cisplatin-purine compounds, thereby increasing cisplatin cross-linking efficiency and cisplatin toxicity, but no mechanistic data were provided. Megalin is a member of the low-density lipoprotein receptor family and is highly expressed in marginal cells of the *stria vascularis* of the inner ear, which accumulate high levels of platinum DNA adducts. The nonsynonymous SNP rs2075252 in *megalin* was found to be associated with cisplatin-induced ototoxicity.[286]

Irinotecan

Irinotecan, known as 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin or CPT-11, is a semisynthetic analogue of the natural alkaloid camptothecin (Fig. 46-11 and Table 46-1). It is a prodrug converted to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), by carboxylesterases in the liver and gastrointestinal tract.[287,288] SN-38 is a potent topoisomerase I inhibitor and has shown promising activity against pediatric solid tumors alone or in combination with other chemotherapeutic agents.[289-291] Irinotecan is inactivated after oxidation by CYP3A4 to 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin and 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin.[292,293] The majority of irinotecan and its metabolites are excreted in bile; bile excretion is enhanced by the formation of SN-38 glucuronide (SN-38G), catalyzed primarily by UGT1A1.[294]

The *UGT1A1*28* polymorphism reflects a change in the number of TA repeats in the TATA box of the *UGT1A1* promoter from the wild-type 6 repeats to the variant 7 repeats (Table 46-4).[295] Approximately 7% to 19% of the white population is homozygous (7/7) for the variant allele, as seen in Gilbert syndrome, which leads to reduced enzyme expression. Several studies in adults found that patients with the *UGT1A1*28* 7/7 genotype had lower SN-38G/SN-38 AUC ratios and greater toxicity after treatment with a high dosage of irinotecan (e.g., 350 mg/m^2, once a month) than did those with the 6/6 or 6/7 genotype.[296,297] Children with the *UGT1A1*28* 7/7 genotype who received low-dose irinotecan (15 to 75 mg/m^2) on a protracted schedule (5 days weekly for 2 consecutive weeks) tended to have higher SN-38 AUC values and lower SN-38G/SN-38 AUC ratios.[298] However, severe toxicity was not increased in these patients, suggesting that *UGT1A1* genotyping is not a useful predictor of severe toxicity for patients treated with this irinotecan dosage and schedule.

Temozolomide

Temozolomide is one of the imidazotetrazinone derivatives discovered in the 1980s and is used to treat CNS malignancies (see Table 46-1).[299] Temozolomide is a prodrug that undergoes spontaneous conversion to the reactive metabolite 5-(3-methyltriazen-1-yl-) imidazole-4-carboxamide (MTIC) under physiologic conditions.[300,301] MTIC is degraded to the methyldiazonium cation, which transfers its methyl group to DNA and to the final degradation product, 4-amino-5-imidazole carboxamide. The most common methylation site in DNA is the N^7 position of guanine, followed by the N^3 position of adenine and the O^6 position of guanine.[300,302] N^7-methylguanine and N^3-methyladenine, which constitute nearly 90% of the total methylation events, are efficiently repaired by the base excision repair pathway and have low cytotoxicity potential. O^6-methylguanine represents only 5% to 10% of methylation events, but these adducts trigger the DNA mismatch repair pathway and are highly cytotoxic. O^6-methylguanine methyltransferase (MGMT) is a cellular DNA repair protein that rapidly reverses alkylation (including methylation) at the O^6 position of guanine, thereby neutralizing cytotoxic effects.[303] MGMT can be silenced by abnormal methylation of CpG islands in its promoter region, which prevents DNA damage repair.[304,305] In several studies, adult patients with glioblastoma who had a methylated MGMT promoter had longer survival after temozolomide treatment than did patients without methylation.[306-308] Thus the methylation status of the MGMT gene promoter may provide a marker of temozolomide sensitivity. Results in the pediatric population are inconsistent, and more studies in larger cohorts are needed.[115,117,309]

Pharmacogenetics, Pharmacogenomics, and the Future

Many studies are underway to relate interindividual variability in pharmacokinetics to drug efficacy and toxicity and their implications for improving the therapeutic index of anticancer agents in children. Evaluation of the whole genome in a large series of patients will deepen our understanding of how pharmacogenetics and pharmacogenomics can be used to improve patient outcomes. This knowledge can be used to develop individualized therapy for patients. However, thus far the clinical application of pharmacogenomics research findings has been slow. The cost of genotyping will diminish over time and with advances in genomic technology, but the consistent interpretation of results and their efficient communication to the clinician are essential. Key components to successful clinical implementation of pharmacogenomics will include not only clinician understanding of pharmacogenomic test results but also availability of clinical prescription guidelines based on the test results,

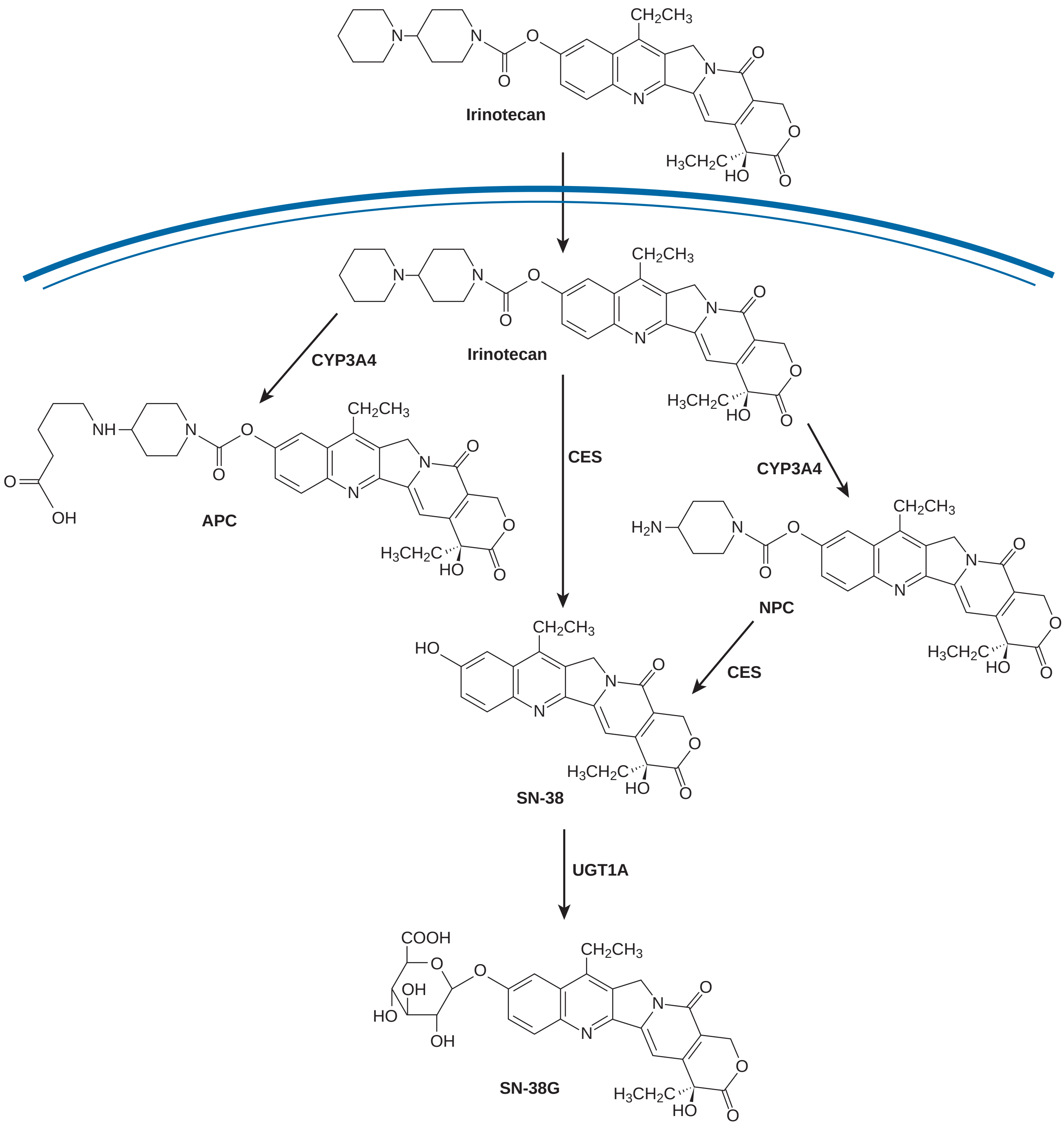


Figure 46-11 Metabolic pathways of irinotecan. Irinotecan is a prodrug converted to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), by carboxylesterases *(CES)* in the liver and gastrointestinal tract. Irinotecan is inactivated after oxidation by CYP3A4 to 7-ethyl-10-94-N-[5-aminopentanoic acid]-1-piperidino) carbonyloxycamptothecin *(APC)* and 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin *(NPC)*. The majority of irinotecan and its metabolites are excreted in bile; bile excretion is enhanced by the formation of SN-38 glucuronide *(SN-38G)*, catalyzed primarily by uridine diphosphate *(UDP)*–glucuronosyltransferase 1A1 *(UGT1A1)*.

knowledge/evidence-based decision support systems, and appropriate sharing of this sensitive information with patients. Pharmacogenomics data cannot substitute for close clinical monitoring of patients, but they can be used to improve outcomes through informed treatment planning and anticipation of toxicities.

NEW DRUG DEVELOPMENT

The era of molecular-targeted therapy arrived with the approval of the *BCR-ABL1* inhibitor imatinib for treatment of chronic myeloid leukemia.[219] Numbers of new agents have been tested in clinical trials along with further

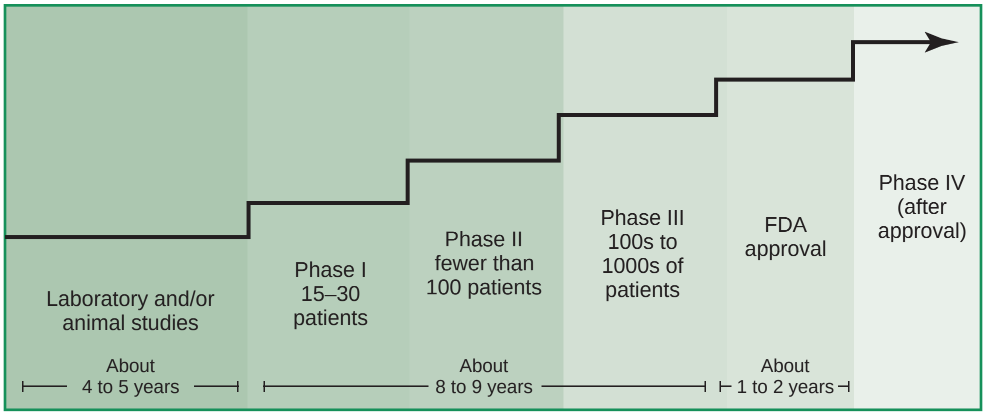

Laboratory and/or animal studies
Phase I 15–30 patients
Phase II fewer than 100 patients
Phase III 100s to 1000s of patients
FDA approval
Phase IV (after approval)
About 4 to 5 years
About 8 to 9 years
About 1 to 2 years

Figure 46-12 Clinical trials process. Clinical trials are conducted after preclinical development in laboratory and/or animal studies and encompass four phases. *FDA,* Food and Drug Administration.

understanding of molecular alterations and signal transduction pathways in pediatric cancer (see Table 46-1).

Whether for a single agent or for use in combination chemotherapy, determination of appropriate dosing through preclinical evaluation and clinical trials is crucial for appropriate use of new drugs. After preclinical development in cellular or animal models, clinical trials progress through four phases (Fig. 46-12).[310,311] The entire process can take more than 10 years. A phase I trial is performed to evaluate the safety of a new agent and the tolerable dosage. The study determines the maximum dose that can be given (maximum tolerated dose, MTD) without causing harmful side effects. Phase I trials enroll a small number of patients (15 to 30 patients) who have advanced cancers that cannot be treated effectively with standard regimens or for which no standard treatment exists. Although phase I trials do not focus on effectiveness, responses are recorded. The most important issue in pediatric phase I trials is selection of the appropriate investigational agent, because the number of pediatric participants is remarkably smaller than that of adult participants and therefore the number of agents that can be investigated is limited. Also, the new agent must be continuously available to progress through the stages of drug development, but it may be withdrawn by pharmaceutical firms if it lacks efficacy in adult trials.

A phase II trial evaluates the effectiveness of the new drug in patients with a specific type of cancer or a group of related cancers, typically by using the MTD determined in the phase I trial. Safety is also monitored. These studies usually enroll fewer than 100 patients but may include as many as 300, depending on the breadth of cancer diagnoses evaluated. The patients who participate in phase II trials may or may not have been treated previously with standard therapy for their type of cancer. Although efficacy in a specific population is the main question of phase II studies, these studies are almost never designed to compare the efficacy of an intervention with that of standard therapy. This comparison is done in phase III trials, which usually enroll large groups of patients (100 to several thousand). Participants are randomly assigned to groups that are typically stratified according to patient characteristics such as age, sex, and extent of disease to ensure that the groups have similar characteristics. This balance and patient number are important in confirming that the treatment is responsible for any significant differences between the two groups in responses or adverse effects. Randomization is usually accomplished by a computer-based algorithm, and trial participants or researchers cannot influence assignment to specific groups. Neither the participants nor their doctors may know what treatment the participants are receiving (double-blind study). Phase IV trials take place after Food and Drug Administration approval to evaluate the effectiveness and long-term safety of drugs in larger populations (several hundred to several thousand patients).

Preclinical Development

In general, preclinical data come from cytotoxic effects in vitro in various animal and human cancer cell lines and in vivo antitumor activity in mouse tumor models or in human tumors xenografted into immunodeficient mice.[312,313] In addition, toxicology studies are conducted in various species to determine toxicity end points, such as the lethal dose for 10% of the animals. These evaluations in vitro and in mouse models clearly have value in defining the target plasma drug concentrations, although direct extrapolation to humans is often difficult, because these models are oversimplified and can be influenced by species differences in metabolic transformation or any other form of drug elimination. Nonetheless, these studies may guide the choice of starting dosage and schedules of administration for phase I clinical trials. For example, if drug cytotoxic activity is shown to be S-phase–specific, prolonged exposure may be required, suggesting the use of a prolonged IV infusion if the agent has a relatively short terminal half-life. Preclinical data can also be valuable in defining the therapeutic window. Theoretically, it is possible to define a two-dimensional plasma concentration-versus-time window, the lower limit of which is the plasma concentration at which antitumor activity begins and whose upper limit is the plasma concentration at which unacceptable toxicity begins (see Fig. 46-1).[314] This approach is used to define the plasma concentration parameters for therapeutic drug monitoring and is widely used for cancer drugs and for numerous noncancer drugs, including antibiotics and antiretroviral agents.[315] In the United States, the Pediatric Preclinical Testing Program of the National Cancer Institute evaluates the therapeutic potential of new agents against molecular characteristics of pediatric cancers identified by the work of the St. Jude Children's Research Hospital and Washington University Pediatric Cancer Genome Project and others.[316]

Choice of Starting Dose for Clinical Studies

Historically, the starting dose of anticancer agents for early clinical trials has been selected empirically on the basis of toxicologic studies in rodents (mice and rats) and dogs; 10% of the murine lethal dose for 10% of the animals is often selected as the starting dose for safety studies in patients, because intolerable toxicity is unlikely at this dose.[312,313,317] Theoretical considerations and an extensive review of preclinical toxicologic studies support the appropriateness and safety of using toxicologic models based on mouse and rat data for anticancer drug development.[318,319] Importantly, however, much higher plasma concentrations can generally be achieved in animals than in humans, and this difference is not always explained by a difference in drug clearance. Therefore, in extrapolating the results of efficacy studies in tumor-bearing animals to clinical use, it is important to remember that these tumors have been exposed to drug levels that in most cases cannot be achieved in patients. This factor is particularly relevant when the relationship between plasma levels and antitumor effects is poorly understood.

Drug development for pediatric cancer has had to evolve while survival rates have improved, making fewer patients eligible for investigational drug studies.[311,313] Agents developed for use in adults may readily be investigated for potential pediatric use. However, new agents targeted to specific molecular abnormalities in adult cancers may have little value in treating pediatric cancers, which typically do not have the same underlying molecular abnormalities.

In a retrospective comparison of phase I studies performed between 1999 and 2004, the pediatric MTD was strongly correlated with the adult MTD. Adult data, such as MTD, pharmacokinetics, toxicity profile, and efficacy findings, are available for many candidate agents. Dose-finding pediatric phase I studies often start at approximately 80% of the adult MTD,[313] even though the MTD in children is often higher when adjusted to BSA. Pediatric formulations (e.g., liquid formulation or crushed form of tablets) are often necessary for younger children who cannot swallow capsules or tablets. However, pharmaceutical companies have little financial incentive to invest in the development of pediatric formulations, which have a very small market. It should also be recognized that the "starting dosage" is typically a dosage that has been tolerated by essentially everyone and thus will be lower than the MTD for patients with rapid drug clearance, and perhaps intolerable for a small subset of patient with very slow drug clearance (possibly because of an inherited defect in drug metabolism, as exemplified by TPMT and mercaptopurine, in which 1 in ~400 children can tolerate only 10% of the standard dosage).

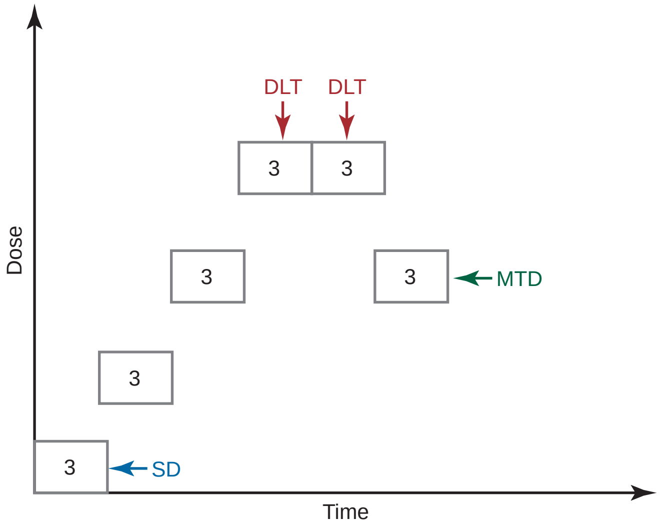


Figure 46-13 Graphic depiction of dose escalation methods using the traditional 3+3 design in phase I cancer clinical trials. Each box represents a cohort consisting of the indicated number of patients treated at a given dose level. After three patients have undergone treatment without experiencing dose-limiting toxicity *(DLT)*, the dose is escalated for the next set of three patients. *MTD*, Maximum tolerated dose; *SD*, starting dose.

Dose Escalation Schemes

Most phase I oncology trials use rule-based designs, which include the traditional 3+3 design or its variations.[312] This model implements any prior assumptions of the dose-toxicity curve. Doses are escalated or de-escalated by using the "up-and-down" methods created by Dixon and Mood in 1948.[320] In the 3+3 model, a minimum of three participants are evaluated at each dose level (Fig. 46-13). If none of the three experiences a dose-limiting toxicity (DLT), an additional three participants are enrolled at the next higher dose level. If one of the three experiences a DLT, an additional three participants are evaluated at the same dose level. Dose escalation continues in this manner until at least two participants in a cohort of three or six experience DLT (i.e., >33% of participants have a DLT at this dose level). Because the MTD is exceeded at this level, an additional three participants (as high as a total of six) are treated at the next lower dose level, which is defined as the MTD if no more than one of six participants (0% to 17%) experience DLT.

Dose escalation is usually based on the modified Fibonacci sequence, in which steps escalate in decreasing increments (e.g., 100%, 67%, 50%, 40%, and 30% to 35% of preceding doses). Because good correlation between adult and pediatric MTDs has been shown, little value exists in testing more than four dose levels or testing dose levels greater than 1.6 times the adult MTD.[321] Limiting pediatric phase I trials to a maximum of four dose levels increasing in 30% increments (e.g., 0.7, 1.0, 1.3, and 1.6 times the adult MTD) would significantly shorten the time required for study completion and can expect therapeutic activity, although it is not the major purpose. Historically, children participating in phase I studies have been treated with therapeutic intent, although legal guardians and/or patients should be fully informed that the focus of a phase I study is safety rather than efficacy.[322]

Alternative rules other than the 3+3 design have been proposed to shorten the study time line.[312] For example, accrual is suspended after enrollment of the third patient in each cohort. Further, when one of the three patients in a cohort cannot be evaluated for toxicity (usually because of disease progression), the cohort would not be completed until one additional patient completes the evaluation. The "rolling 6" design[323] has been proposed to shorten the time frame by allowing concurrent enrollment of two to six patients at a dose level. The decision about the dose level at which to enroll upcoming patients is based on the number of the patients currently enrolled and evaluable, the number of patients experiencing DLT, and the number of patients still at risk of DLT. For example, when three participants are enrolled at a dose level, if toxicity data are available for all three and no DLTs have been observed, the fourth participant is enrolled at the next higher dose level. If toxicity data are not yet available for all three participants but no DLT has been observed, or if one patient has experienced DLT, the new participant is enrolled at the same dose level. If two or more of the three participants have experienced DLT, the dose level is de-escalated. This process is repeated for the fifth and sixth participants. Accrual is suspended only when a cohort of six is filled. A discrete-event simulation study showed that the rolling 6 model significantly shortened the duration of pediatric phase I trials without increasing the risk of toxicity; this strategy has been incorporated into pediatric phase I studies.[324]

Parameter Estimates

The pharmacokinetic parameters of the investigational agent, including peak concentration, drug clearance, half-life, Vd, AUC, and metabolic profile, should be defined at different dose levels to be used clinically.[312,313] Because the effect of a therapeutic agent in the body is generally a function of its concentration at the (molecular) site of action, a description of the spatial-temporal behavior of the drug in the body is helpful, if not essential, for understanding and predicting normal tissue toxicity and optimizing tumor response.[312,313] Measures of drug exposure (e.g., AUC) are clearly more informative than the absolute dose. Ideally, drug exposure should be measured at the molecular locus of action or at least at the tissue or tumor level, but with the exception of leukemia, drug concentrations are usually measured only in plasma, which is the only readily accessible surrogate. The detection of metabolites and definition of their pharmacokinetic behavior and pharmacologic activity are also extremely important, particularly in the case of agents that require metabolic activation, such as cyclophosphamide and irinotecan.

Data from early clinical studies can be used to define the therapeutic window of the agent and design more rational schedules of administration and combination therapies; it may also provide clues about potential cumulative toxicity or organ-specific toxicity. The measured parameters can be compared with the observed clinical outcome, particularly in terms of toxicity, to help define pharmacokinetic-pharmacodynamic relationships that may be of use in further clinical testing of the agent. In addition, dose-effect relationships, at least in terms of toxicity, can best be assessed at this early stage of clinical development, when a wide range of doses is being administered to patients.

After a dose is selected, the same dose is usually maintained throughout treatment unless serious toxicity occurs, in which case the dose is empirically decreased during subsequent treatment courses. In contrast, the dose of chemotherapy is rarely increased in the absence of toxicity, despite the possibility of undertreatment. Although wide interpatient variability has been demonstrated in all aspects of anticancer drug pharmacokinetics, several studies have observed reasonably predictive relationships between a measure of drug exposure and toxicity or antitumor efficacy in pediatric patients (Table 46-5). These findings would add to the argument to

TABLE 46-5 Pharmacokinetic-Pharmacodynamic Relationships of Chemotherapeutic Agents

Drug	Toxicity/Efficacy (Disease)	Pharmacokinetic Parameter
Busulfan	Hepatotoxicity	AUC
Carboplatin	Thrombocytopenia	AUC
Cisplatin	Nephrotoxicity Neurotoxicity	C_{max} (unbound drug) C_{max} (unbound drug)
Cyclophosphamide	Cardiotoxicity	AUC
Docetaxel	Neutropenia	AUC
Doxorubicin	AML Leukocytopenia Thrombocytopenia	$C_{3\ hr}$ AUC Doxorubicinol AUC
5-Fluorouracil	Mucositis Leukocytopenia	AUC >30 µg × h/mL AUC and C_{ss}
Irinotecan	Neutropenia Diarrhea	AUC Biliary index
6-Mercaptopurine	ALL	AUC; RBC 6-TGN levels
Methotrexate	Mucositis, myelosuppression	$C_{48\ hr}$ >0.9 µM
Paclitaxel	Neutropenia	Time above 0.05 µM
Teniposide	Lymphoma, leukemia	C_{ss} and CL
Topotecan	Myelosuppression	Lactone AUC
Vinblastine	Neutropenia	C_{ss}
Vincristine	Neurotoxicity	AUC

ALL, Acute lymphoblastic leukemia; *AML,* acute myeloid leukemia; *AUC,* area under the plasma concentration vs. time curve; $C_{3\text{-}hr}$, plasma concentration 3 h after intravenous bolus administration; $C_{48\ hr}$, plasma concentration at 48 h; C_{max}, peak plasma concentration; C_{ss}, plasma concentration at steady state; *CL,* total plasma clearance; *RBC,* red blood cell; *6-TGN,* 6-thioguanine nucleotide (intracellular active metabolite of 6-mercaptopurine).

increase the dose in cases of limited toxicity, although this question remains unresolved.

Various mathematical models have been used to analyze the relation between drug concentration in biologic fluids (e.g., plasma) and the pharmacologic effects of anticancer agents. One such model is the modified Hill equation or the E_{max} model—that is, the drug concentration producing the maximum effect. The Hill equation reflects the sigmoidal relationship of pharmacologic response to AUC, steady state exposure, and threshold concentration, respectively; the response is usually measured as hematologic toxicity, expressed as a percentage. A number of commonly used anticancer drugs, including carboplatin, doxorubicin, etoposide, teniposide, mercaptopurine, 5-fluorouracil, paclitaxel, and vinblastine, have demonstrated this type of relationship.[325] When nonhematologic toxicities are dose limiting, they can be more difficult to model because of the subjective nature of grading them, in contrast to hematologic toxicity, which is a more quantitative continuous variable. However, efforts to model these adverse effects have yielded useful correlations between nephrotoxicity and cisplatin AUC and between gastrointestinal toxicity and glucuronidation rates of the irinotecan metabolite, SN-38.[326]

Clinical trials in childhood ALL that revealed a relation between drug clearance or drug exposure (AUC or steady state plasma concentration) and treatment efficacy (event-free survival) also have been conducted[327,328]; therefore treatment outcome may be improved by randomized clinical trials that adjust doses on the basis of drug clearance.[5]

STRATEGIES TO IMPROVE THE THERAPEUTIC INDEX

Dosage Based on Body Surface Area

The traditional method of individualizing anticancer drug dosage in children is by using body surface area (BSA) (Table 46-6).[329,330] BSA has become the standard variable for determining the dose of anticancer agents in pediatric patients for several reasons. First, an established correlation exists between BSA and some relevant individual patient characteristics, such as GFR, blood volume, and basal metabolic rate.[331] Second, the starting dose of agents in phase I studies is based on data derived from animal models, in which the drug dose is calculated on the basis of weight (mg/kg) or BSA (mg/m²). Third, in the 1950s, when attempts were first made to define a more accurate method for administration of cytotoxic drugs to children, several studies suggested that BSA be used in dose calculation. In 1958, Pinkel[332] reported a retrospective analysis that used a BSA-based formula for adults and children and Meeh's formula for animals to determine the pediatric and adult doses of five cytotoxic drugs (mercaptopurine, methotrexate, mechlorethamine, triethylene thiophosphoramide, and actinomycin). When the conventional doses were calculated according to unit surface area, similar values were obtained for all agents tested in children and adults except for mercaptopurine; therefore, the author recommended that doses of cytotoxic agents be normalized by using BSA.

TABLE 46-6 Strategies to Improve Therapeutic Index of Anticancer Drugs in Children

Dose Adjustment Method	**Example**
Based on Patient Characteristics	
Body surface area*	All drugs
Based on Pharmacokinetic-Pharmacodynamic Principles	
Therapeutic drug monitoring	Methotrexate, 5-fluorouracil, busulfan, etoposide, cisplatin
Feedback-controlled dosing	Methotrexate
Circadian rhythm–based dosing	6-Mercaptopurine, busulfan, carboplatin, docetaxel, 5-fluorouracil, oxaliplatin
Biomodulation	
Pharmacokinetic modulation	CYP3A4 inhibitor (erythromycin, quinidine, ketoconazole, cyclosporin A) ABCB1 inhibitor (cyclosporin A)
Pharmacodynamic modulation	Leucovorin for methotrexate and 5-fluorouracil Amifostine for cisplatin Dexrazoxane for anthracycline Cefixime for irinotecan
Drug scheduling	Etoposide, methotrexate, dexamethasone
Drug administration sequence	Methotrexate–5-fluorouracil Lestaurtinib–chemotherapeutic agents in acute lymphoblastic leukemia Deoxyadenosine analogues (fludarabine, cladribine, clofarabine)–cytarabine
Based on Pharmacogenetic Principles	
Pharmacogenetics-based dosing	6-Mercaptopurine (*TPMT*)

ABC, Adenosine triphosphate–binding cassette transporter; *CYP,* cytochrome P-450 enzyme; *TPMT,* thiopurine methyltransferase.
*Weight (mg/kg) is used in children who weigh less than 10 kg.

BSA is commonly estimated by using a formula derived primarily to determine basal metabolism from weight and height alone; more recently, the original formula has been confirmed as surprisingly accurate, considering the small sample size used in deriving it.[330] The formulas by Mosteller

$$BSA = \sqrt{\frac{W \times H}{3600}} = 0.016667 \times W^{0.5} \times H^{0.5}$$

and Du Bois

$$(\mathrm{BSA} = 4.688 \cdot \mathrm{W}^{(0.8168 - 0.0154 \cdot \log \mathrm{W})})$$

are most commonly used.[333] Because of the difficulty of measuring height in infants and their physiologic differences from older children (e.g., a higher proportion of body water and a lower proportion of body fat, immature enzyme systems, and lower renal clearance rates), the majority of pediatric protocols apply a 10-kg cutoff for surface area–based dosing.[333] Below this weight, the dosing of chemotherapeutic drugs is specified on a mg/kg basis, usually based on the assumptions that an individual with BSA of 1-m^2 weighs 30 kg and that there is a linear relationship between weight and BSA. This method may result in significantly lower calculated doses for children whose body weight is less than 10 kg. This lower dose may avert excessive toxicity in infants and younger children, who have immature hepatic and renal function. However, some patients may receive subtherapeutic dosing, and a significant step-up in dosage can occur as children cross the 10 kg boundary by moving from mg/kg to mg/m^2 dosing during their treatment.

The usefulness of normalizing anticancer drug dose to BSA in adults has recently been questioned, because BSA does not vary as widely in adults as in children and is not related to the clearance of most anticancer drugs in adults.[334] BSA is considered more important for drug dose calculation in pediatric patients, whose BSA values vary over a greater range.[335]

Pathophysiologic Status

Most pharmacologic studies of anticancer agents have shown important interindividual and intraindividual variability in pharmacokinetic behavior. In some cases, this variation is caused by cancer-induced changes in physiologic status or dysfunction of specific organs involved in drug elimination (Table 46-7). For example, if urinary excretion is an important elimination route for a given drug, any decrement in renal function could decrease drug clearance and result in drug accumulation and toxicity. It would therefore be logical to reduce the drug dose relative to the degree of impaired renal function to maintain plasma concentration within a targeted therapeutic window, as has been well documented for bleomycin, carboplatin, cisplatin, cyclophosphamide, etoposide, methotrexate, and topotecan.[336] The best known example of this a priori dose adjustment of an anticancer agent is carboplatin, which is excreted renally, almost entirely by glomerular filtration. Various strategies have been developed to estimate carboplatin dose on the basis of renal function of patients, either by using creatinine clearance[337] or radioisotopically measured GFR.[338] Use of these methods has substantially reduced pharmacokinetic variability, such that carboplatin is one of the few drugs routinely administered to achieve a targeted exposure rather than on a mg/m^2 or mg/kg basis.

TABLE 46-7 Characteristics That Affect the Pharmacokinetics of Chemotherapeutic Agents in Children

Parameter	Anticancer Drug
Renal function	Bleomycin, carboplatin, cisplatin, cyclophosphamide, etoposide, methotrexate, topotecan
Hepatic function	Anthracyclines, vinblastine, vincristine
Serum albumin	Etoposide, dexamethasone
Third spaces	Methotrexate

Although a decline in renal clearance of drugs can be predicted when glomerular filtration is impaired, it is less easy to predict the effect of impaired liver function on drug clearance. The main problem is that hepatic enzymes are typically poor indicators of metabolizing activity or anticancer drug pharmacokinetics. A proposed alternative dynamic measure of liver function is based on the sum of serum bilirubin, alkaline phosphatase, and either alanine aminotransferase or aspartate aminotransferase values (each scored according to the World Health Organization grading system) to provide a hepatic dysfunction score.[339] Guidelines based on carefully conducted pharmacokinetic studies in patients with normal and impaired hepatic function have been proposed for adjustment of the doses of several agents, including anthracyclines (doxorubicin and daunorubicin), etoposide, and vinca alkaloids (vinblastine and vincristine), for patients with liver dysfunction.[340]

The binding of drugs to plasma proteins, particularly when drugs are highly bound, may also have significant clinical implications for therapeutic outcome.[341] Although protein binding is a major determinant of drug action, it is clearly only one of a myriad of factors that influence drug disposition. The extent of protein binding is a function of drug and protein concentrations, the affinity constants for the drug-protein interaction, and the number of protein-binding sites per class of binding site. Because only unbound (or free) drug in plasma is available for diffusion from the vascular compartment to the tumor interstitium, the therapeutic response will be related to free drug concentration rather than total drug concentration. Several clinical factors, including liver and renal disease, can significantly decrease the extent of plasma binding and may increase free drug concentration and the risk of unexpected toxicity, despite unaltered total (free plus bound forms) plasma drug concentration. High systemic exposure to asparaginase induces hypoalbuminemia and is associated with lower apparent clearance of dexamethasone in patients with ALL.[342] Hepatic synthesis of proteins involved in dexamethasone clearance, rather than decreased plasma protein binding, is thought to be implicated in such cases. Third-space fluid collection, such as pleural effusion and ascites, alters the pharmacokinetics of chemotherapeutic agents (e.g., methotrexate), and dose modification and strict monitoring are required.

Adjustment of the dosage a posteriori on the basis of a patient's tolerance is also widely practiced, although it may result in dose reduction, which can compromise dose intensity and lead to treatment failure. Clearly, the seriousness of the first adverse effect is the major determinant of whether it can be used as an end point. Examples of this type of dose adjustment are the platelet nadir-directed individualized approach to carboplatin dosage[343] and leukopenia-targeted dosing of methotrexate and mercaptopurine in children with ALL.[344] However, this approach can be untenable when, as often occurs, treatment consists of multiple anticancer agents with overlapping toxicities (especially hematologic toxicity).

Dose Adaptation Using Pharmacokinetic-Pharmacodynamic-Pharmacogenetic Principles

Therapeutic Drug Monitoring

Prolonged infusion schedules offer a very convenient setting for anticancer drug dose adaptation in individual patients. When steady state concentration is achieved, it is possible to modify the infusion rate for the remainder of the treatment course if a relationship is known between this steady state concentration and a desired pharmacodynamic end point (see Table 46-6). This method has been successfully used to adapt the dose during continuous infusions of methotrexate, 5-fluorouracil, busulfan, and etoposide, and for repeated oral administration of etoposide or repeated IV administration of cisplatin.[340] Methotrexate plasma concentration is routinely monitored after administration of high-dose methotrexate to identify patients at high risk of toxicity and to adjust leucovorin rescue and hydration in patients with delayed excretion. In some cases, ongoing methotrexate infusion can be slowed or discontinued if the concentration is considered to be critically high. Such monitoring has significantly reduced the incidence of serious toxicity (including toxic death) and has in fact improved outcome by eliminating unacceptably low systemic exposure.[5]

Feedback-Controlled Dosing

It remains to be seen how information about variability can eventually be used to devise an optimal dosage regimen of a drug for the treatment of a given disease in an individual patient. Obviously, the desired objective would be most efficiently achieved if the individual's dosage requirements could be calculated before the drug is administered. Although this ideal cannot be completely met in clinical practice, some success may be achieved by adopting feedback-controlled dosing, as in the case of methotrexate (see Table 46-6).[5,345] In adaptive dosage with feedback control, population-based predictive models are initially used but allow the possibility of dosage alteration based on feedback revision. In this approach, patients are first treated with the standard dose, and then during treatment pharmacokinetic information is estimated by a limited-sampling strategy, and compared with that predicted from the population model with which dosage was initiated. More patient-specific pharmacokinetic parameters are calculated on the basis of this comparison, and dosage is adjusted accordingly to maintain the target exposure that produces the desired pharmacodynamic effect for subsequent courses. It has been proposed that, despite its mathematical complexity, this approach may be the only way to achieve the desired precise exposure of an anticancer agent. The use of population pharmacokinetic models is increasingly studied in adults in an attempt to accommodate as much pharmacokinetic variability as possible in terms of measurable characteristics, and mathematical equations based on BSA, gender, and protein levels are provided, for example, to predict drug clearance with an acceptable degree of precision.

Circadian Rhythm–Based Dosing

Administration at certain times of the day may improve the therapeutic index of several anticancer drugs (see Table 46-6).[346,347] For example, significant improvement in survival has been demonstrated for children with ALL who received 6-mercaptopurine in the evening compared with the morning.[348] Chronotherapy schedules based on this concept showed improved tolerability in several clinical trials using other anticancer drugs, including busulfan, carboplatin, docetaxel, 5-fluorouracil, and oxaliplatin.[349-351]

Although the precise mechanisms are still unknown, such findings have led to the assumption that tolerability rhythms are coupled with the rest-activity cycle in patients with cancer. For example, one of the major limiting factors for 6-mercaptopurine is bone marrow toxicity. Normal bone-marrow cells are thought to have their nadir of DNA synthesis activity at night.[352] Thus administration of 6-mercaptopurine in the evening could provide normal cells an advantage over leukemic cells, which have minimal and variable diurnal changes in DNA synthesis. It remains unclear whether the potential benefits will justify the major inconvenience of drug administration in the middle of the night.

Pharmacogenetics-Based Dosing

The *TPMT* genetic polymorphism probably best illustrates the potential impact of pharmacogenetics on pediatric oncology (Figs. 46-6 and 46-7; see also Table 46-6).[87] TPMT plays an important role in inactivation of thiopurines (6-mercaptopurine, 6-thioguanine, and azathioprine) by catalyzing the transfer of the methyl group from *S*-adenosyl methionine to the sulfur atoms of thiopurines. TPMT-deficient patients accumulate excessive concentrations of thioguanine nucleotides after conventional doses of thiopurines, leading to severe and potentially fatal hematologic toxicity.[353,354] Heterozygous patients have an intermediate risk of toxicity and typically require only a modest dose reduction (as much as 50%), whereas patients who have inherited two nonfunctional alleles require treatment with only 5% to 10% of the standard dose to avoid toxicity without compromising efficacy.[92,355,356] Pretreatment knowledge of a patient's TPMT status is now used for dose optimization to reduce the likelihood of toxicity.[356] *TPMT* genotyping (e.g., use of restriction fragment length polymorphism or high-throughput DNA arrays such as the Affymetrix DMET chip [Affymetrix, Santa Clara, Calif.]) is highly efficient in identifying patients at risk of thiopurine-related toxicity. TPMT enzymatic activity in erythrocytes can also be directly measured by radiochemical assay, but heterologous red blood cell transfusions within the past ~30 to 60 days can cause spurious results.

Mercaptopurine, thioguanine, and their metabolites (e.g., thioguanine nucleotides) can be quantified by reverse-phase high-performance liquid chromatography assay.[357] A significant inverse relationship between TGN and TPMT activity in erythrocytes has been observed in several studies, and acute hematologic toxicity (e.g., neutropenia) has been clearly related to erythrocyte TGN concentration; unacceptable toxicity is reported when doses of 50 to 75 mg/m^2 per day are given to children with *TPMT* deficiency.[353,358] When thiopurines are administered according to genotype, comparable cellular thioguanine nucleotide concentrations are achieved; therefore all patients, regardless of *TPMT* phenotypes, can be treated without adverse effects and without compromising efficacy. It is unclear whether adjustment of thiopurine doses on the basis of TGN levels improves treatment efficacy, but TGN measurement is helpful for detecting noncompliance and explaining hematopoietic toxicity.[358,359]

Intentional Biomodulation

Pharmacokinetic Alterations. Intestinal metabolic systems and drug efflux pumps located in the intestinal mucosa are another limitation of the bioavailability of orally administered drugs.[360] Several enzymes located in enterocytes, such as CYP3A4, are involved in the presystemic metabolism of many cytotoxic agents, including cyclophosphamide and etoposide, thereby limiting the oral absorption of these drugs.[361] The bioavailability of these drugs might be substantially enhanced by pharmacologic modulation of enteric CYP3A4 activity (see Table 46-6). Several investigators confirmed that coadministration of specific inhibitors of CYP3A4 activity, such as erythromycin, quinidine, ketoconazole, and cyclosporin A, increase the oral bioavailability of various anticancer agents such as etoposide while also diminishing variability in absorption.[362] Similarly, ABCB1, which is abundantly present in the gastrointestinal tract, has been shown to limit intestinal absorption of numerous (anticancer) agents.[363] Combined inhibition of intestinal ABCB1 and CYP3A4 by cyclosporin A was shown to substantially increase systemic exposure to oral paclitaxel in adult patients with cancer,[364] suggesting that simultaneous modulation of transporter and enzyme could be considered when anticancer agents that have poor bioavailability are given orally.

Pharmacodynamic Alterations. The coadministration of specific agents with anticancer drugs to increase the therapeutic index has made a substantial impact in certain diseases (see Table 46-6). One of the most widely used biomodulating agents is leucovorin, a reduced folate used in combination with both methotrexate and 5-fluorouracil. This agent has been shown to decrease methotrexate-induced toxicity in various normal tissues and to enhance 5-fluorouracil–mediated cytotoxicity to tumor cells by inhibition of thymidylate synthase, thereby allowing escalation of the anticancer drug dose without affecting its disposition profile.[365] Other examples of agents that interfere with anticancer drug pharmacodynamics to reduce toxicity include (1) the use of amifostine to reduce cisplatin-associated myelosuppression, nephrotoxicity, and neurotoxicity,[366] (2) dexrazoxane to decrease anthracycline-induced cardiotoxicity,[367] and (3) coadministration of cefixime to ameliorate irinotecan-induced diarrhea.[290]

Pharmacodynamic biomodulation with ABCB1-blocking agents has also been extensively studied in adults in an attempt to improve the therapeutic index of anticancer agents.[368] This multidrug-resistant drug-efflux transporter encoded by the *ABCB1* gene is abundant in various types of human cancer. Studies performed during the past several years have shown that intrinsic and acquired expression of ABCB1 may play a role in clinical drug resistance in specific hematologic malignancies. Consequently, in clinical trials performed worldwide, ABCB1 blockers such as verapamil and cyclosporin A have been administered with anticancer drugs with the intent of attenuating carrier-mediated tumor-cell drug resistance. Unfortunately, decreased systemic clearance of the anticancer drugs became a serious problem in these trials and necessitated substantial dose reductions, thereby precluding the potential benefits of increased antitumor activity.[368] In most cases, pharmacokinetic interference appears to be the result of competition for enzymes (mainly CYP3A4) involved in drug metabolism.[369]

Although this modulation can be beneficial in pediatric cancer treatment, prospective pharmacokinetic and pharmacodynamic studies must be conducted to determine at which level (i.e., kinetics or dynamics) the interaction takes place and whether biomodulation ultimately improves the therapeutic index of anticancer agents.

Drug Scheduling and Sequence of Administration

The antitumor activity of certain chemotherapeutic agents is highly schedule dependent (see Table 46-6). For these drugs, the antitumor response or toxicity profile of a dose fractionated over several days can differ from those of the same dose given over a shorter period. For example, markedly greater efficacy in adult patients with small-cell lung cancer was documented when an identical total dose of etoposide was administered on a 5-day divided-dose schedule rather than as a 24-hour infusion.[370] Pharmacokinetic analysis showed that both schedules produced very similar overall systemic drug exposure (as measured by AUC) but that the divided-dose schedule produced twice the duration of exposure to an etoposide plasma concentration >1µg/mL. This observation was consistent with preclinical data, and the authors speculated that the duration of exposure to this threshold concentration was important in achieving clinical efficacy. This finding has led to the use of prolonged oral administration of etoposide to treat pediatric leukemia and solid tumors.[371,372] Similar schedule dependency has been demonstrated for a number of other anticancer agents. In children with ALL, 24-hour infusion of high-dose methotrexate (1 g/m^2) produced significantly greater accumulation of MTXPG$_{1-7}$ in leukemia cells than did 4-hour infusion.[373] The 24-hour infusion increased the duration of methotrexate exposure greater than 1 µmol/L by a factor of 2.5, producing greater inhibition of de novo purine synthesis in bone-marrow ALL cells and greater reduction of circulating leukemia cells. Furthermore, alternate-week (10 mg/m^2 per day on days 0 to 6 and 14 to 20) rather than continuous (10 mg/m^2 per day on days 0 to 20) administration of dexamethasone in childhood ALL significantly reduced osteonecrosis despite delivering a higher cumulative dose.[142]

Combinations of anticancer drugs can also exhibit schedule-dependent toxicity or antitumor activity,

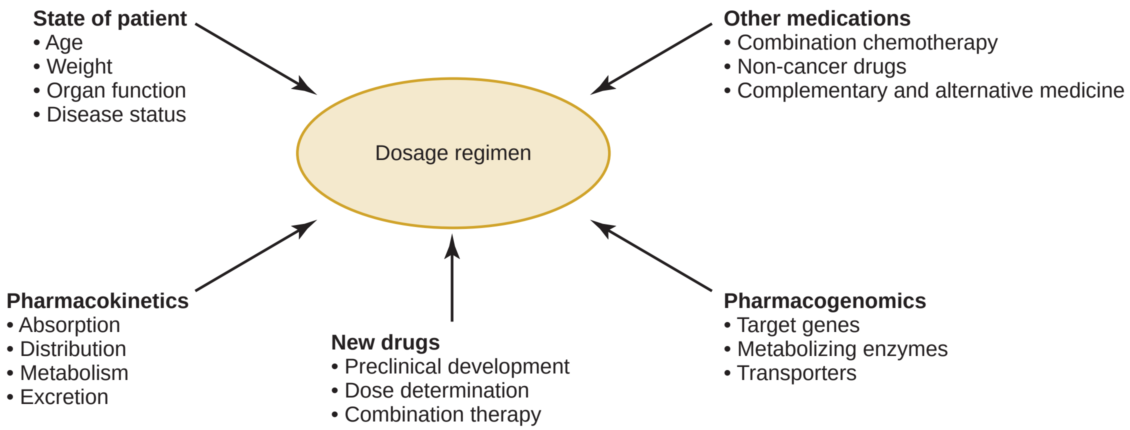


Figure 46-14 Determinants of the dosage regimen for an anticancer drug.

reflecting either pharmacokinetic or pharmacodynamic modulation. For example, the sequence of administration of 5-fluorouracil and methotrexate seems to determine the tumor cytotoxicity of this combination. In vitro, if methotrexate precedes 5-fluorouracil by at least 1 hour, synergistic results are obtained,[374] which may be due to increased activation of 5-fluorouracil to its nucleotide form. The opposite sequence of administration leads to drug antagonism due to blockage of the thymidylate synthase pathway by 5-fluorouracil. This blockage preserves the intracellular folates in their active tetrahydrofolate form, which diminishes the inhibition of dihydrofolate reductase by methotrexate. Similarly, a sequence-dependent interaction was observed in the treatment of *MLL*–rearranged ALL cells with the fms-related tyrosine kinase–3 (FLT3) inhibitor lestaurtinib and six chemotherapeutic agents (dexamethasone, vincristine, L-asparaginase, daunorubicin, cytarabine, and etoposide).[375] When chemotherapy was given before lestaurtinib, the combination was markedly synergistic, whereas the opposite sequence was antagonistic. This sequence dependence was caused by cell cycle suppression via FLT3 inhibition. Deoxyadenosine analogues such as fludarabine, cladribine, and clofarabine have been used in patients with leukemia.[376-378] They inhibit ribonucleotide reductase and decrease de novo synthesis of deoxynucleotide, lessening the feedback inhibition of deoxycytidine kinase. When combined with cytarabine in patients with AML, deoxyadenosine analogues are given first to increase the intracellular ara-CTP accumulation in leukemia cells.

In vitro and in vivo studies and phase I and II trials of combination chemotherapy typically reveal relevant pharmacokinetic and pharmacodynamic interactions and define drug doses and sequences of administration that can be safely administered.

CONCLUSION AND PERSPECTIVES

Substantial progress has been made in the optimization of cancer chemotherapy in recent years with the contributions of pharmacokinetics, pharmacodynamics, and pharmacogenetics and our increasing knowledge about physiologic changes associated with growth and development, the role of disease status, and drug-drug interactions (Fig. 46-14). Pharmacogenomics studies now include the application of next-generation sequencing technologies to cover all genetic variants,[284,285] and the inherited nature of differences in drug disposition and pharmacodynamic effects is being elucidated to provide a stronger scientific basis for optimization of drug therapy on the basis of an individual patient's genome.[30,286] The development of technologies such as cancer genome analysis, high-throughput drug screening, and combinatorial chemistry is likely to exponentially increase the number of drug targets and candidate compounds, and a deeper understanding of each compound's pharmacologic properties is expected to emerge during the exploratory stages of drug development.[287] In addition, the enormous quantity of patient-oriented information will require the use of models and computer simulation to guide clinical decisions for individualized therapy (i.e., systems biology).[288,289] In the next several years we should see the growth of targeted anticancer chemotherapy designed to exploit genetic abnormalities in individual tumors, with the reduction of toxicity and the enhancement of efficacy through judicious analysis of constitutional and genomic factors that modulate the pharmacokinetics (e.g., absorption, distribution, metabolism, and excretion) and pharmacodynamics (e.g., sensitivity and resistance) of chemotherapeutic agents.

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CHAPTER 47

Immunotherapy of Cancer

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CHAPTER OUTLINE

KEY COMPONENTS OF THE IMMUNE SYSTEM AND THEIR RELEVANCE IN CANCER IMMUNOTHERAPY
Humoral Arm of the Immune System
Cellular Arm of the Immune System
TARGET ANTIGENS IN CANCER IMMUNOTHERAPY
Antigens Arising from Genetic Alterations
Oncoviral Proteins
Clonally Rearranged Immunoglobulin Genes
Altered Glycolipid and Glycoprotein Antigens
Oncofetal Antigens
Products of Genes That Are Selectively Expressed in Immune-Privileged Sites
Histocompatibility Antigens
Molecules That Activate Recognition of the Innate Immune System
Normal Proteins Overexpressed in Tumor Tissue
Lineage-Specific Tumor Antigens
TUMOR VACCINES
Dendritic-Cell Vaccines
Peptide-Based Vaccines
Whole-Cell Vaccines
Idiotype Vaccines
DNA Vaccines
CYTOKINES
Interferons
Interleukin-2
Interleukin-7
Interleukin-15
ANTIBODY-BASED IMMUNOTHERAPY
Unconjugated Antibodies
Bispecific Antibodies
Immunoconjugates
Antibodies in the Modulation of Regulatory Pathways
ADOPTIVE CELLULAR IMMUNOTHERAPY
GVL Effect in HSCT and Donor Lymphocyte Infusions
Adoptive Therapies Exploiting NK-Cell Alloreactivity
Adoptive Therapy Using Cytokine-Induced Killer Cells
Adoptive T-Cell Therapy
Allogeneic Adoptive T-Cell Therapy for the Control of Viral Infections After HSCT
Mesenchymal Stem Cells
IMMUNE RESPONSE CRITERIA
CONCLUSION AND FUTURE DIRECTIONS

The role of the immune system in controlling cancer was subject to debate for decades, reflecting the limited understanding of the immune system at the time and the complexity of tumor immunology. However, over time a wealth of evidence from murine and human studies[1-3] has demonstrated that the immune system can control cancer. Numerous animal models have demonstrated the ability of T cells to reject tumors, revealed the importance of cytokines for antitumor immunity, and showed the susceptibility of immunodeficient mice to tumor formation. Compelling evidence of tumor immunosurveillance in humans is provided by reports of rare spontaneous tumor regressions in persons with melanoma and renal cell carcinoma,[4] particularly after infection,[5] paraneoplastic diseases (which are autoimmune neurologic manifestations of an antitumor response), and the higher incidence of melanoma in patients undergoing chronic pharmacologic immunosuppression after organ transplantation.[6] Pediatric patients with immunodeficiencies are at risk for lymphoma, and posttransplant lymphoproliferative disease (PTLD) is a well-known entity in iatrogenically immunosuppressed patients. The immune system can prevent virus-induced malignancies, eliminate tumor cells via recognition of tumor antigens that distinguish them from “self,” and promptly terminate inflammation to avoid tumorigenesis. However, when the latter process is in disarray, such as in chronic inflammatory conditions and autoimmunity, the immune system is implicated in tumorigenesis. The control of tumor growth by the immune system also applies selection pressure, which may ultimately promote tumor progression. In this process, termed "cancer immunoediting,"[7] cancer cells develop immune escape mechanisms and establish a tumor microenvironment that interferes with antigen presentation, T-cell activation, and differentiation, thereby leading to a gradual loss of control by T cells (Fig. 47-1).[8] An expanding body of preclinical and clinical experiences has established that the immune system can be manipulated in increasingly sophisticated ways to prevent, control, or aid in the control of established cancers. Careful selection of the appropriate target antigen(s) and consideration of preexisting immunologic memory are key features in the pursuit of successful immunotherapy. Antigens that are unique to the tumor and absent on healthy tissues often require priming of the immune system but possess the advantage that the induced antitumor response is unlikely to have autoimmune adverse effects. In the case of antigens that are present or overexpressed on tumor cells but are also detectable in some healthy tissues, depletion of T-cell clones with high avidity to the antigen may have polarized existing memory T cells toward tolerance. In this scenario, effective

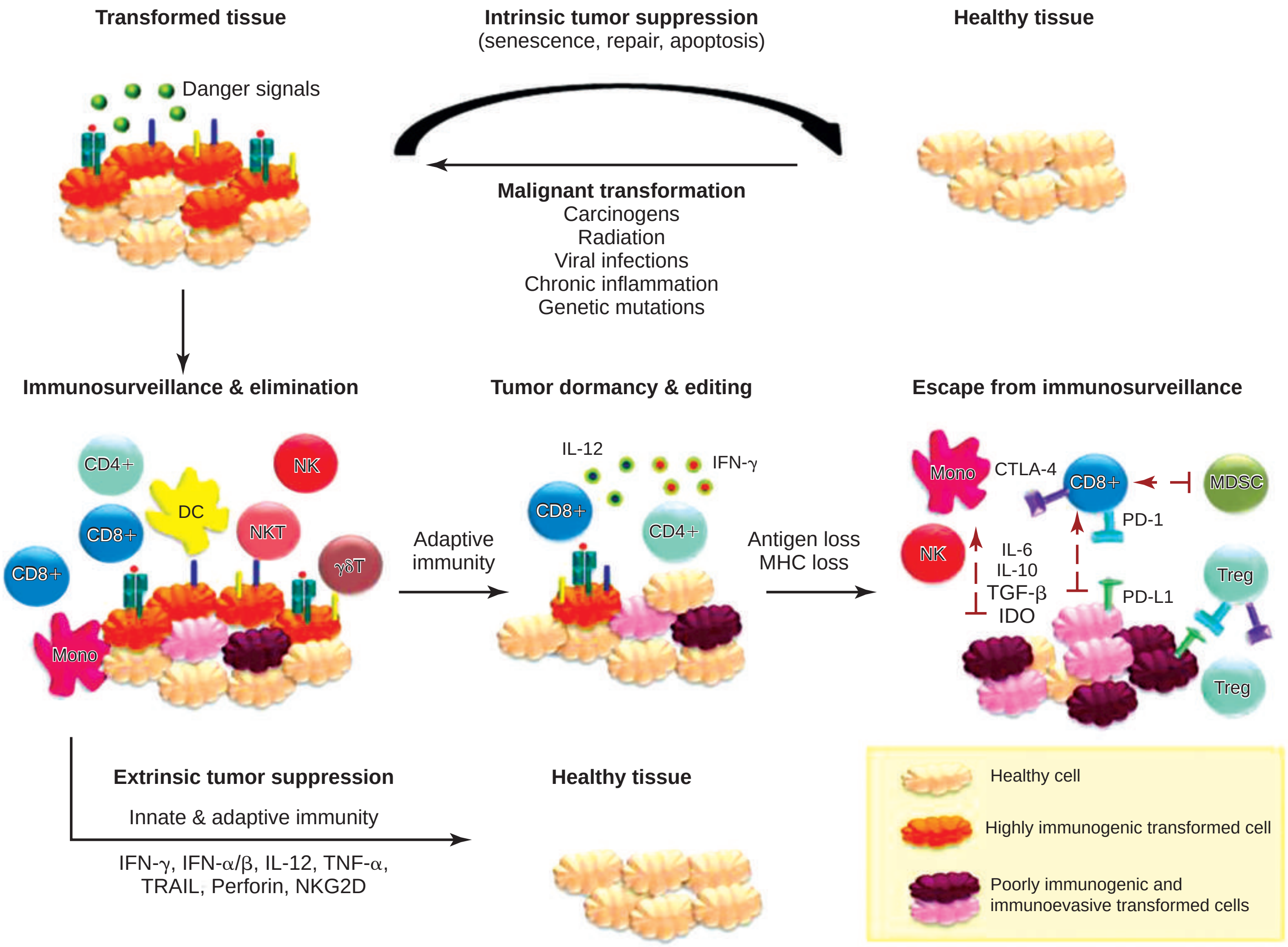

Transformed tissue
Intrinsic tumor suppression
(senescence, repair, apoptosis)
Healthy tissue
Danger signals
Malignant transformation
Carcinogens
Radiation
Viral infections
Chronic inflammation
Genetic mutations
Immunosurveillance & elimination
Tumor dormancy & editing
Escape from immunosurveillance
CD4+
NK
DC
CD8+
NKT
CD8+
γδT
Mono
Adaptive immunity
IL-12
IFN-γ
CD8+
CD4+
Antigen loss
MHC loss
Mono
CTLA-4
CD8+
MDSC
PD-1
NK
IL-6
IL-10
TGF-β
IDO
PD-L1
Treg
Treg
Extrinsic tumor suppression
Innate & adaptive immunity
IFN-γ, IFN-α/β, IL-12, TNF-α,
TRAIL, Perforin, NKG2D
Healthy tissue
Healthy cell
Highly immunogenic transformed cell
Poorly immunogenic and immunoevasive transformed cells

Figure 47-1 The concept of cancer immunoediting. Malignant transformation can be triggered by multiple insults, including inherited or acquired genetic mutations, exposure to radiation, carcinogens, infections, and chronic inflammation. Intrinsic tumor suppression mechanisms such as deoxyribonucleic acid repair, apoptosis, and senescence are in place to eliminate transformed cells. The extrinsic tumor suppressor mechanism of cancer immunoediting involves both the innate and adaptive arms of immunity and contributes to the control of a developing tumor by recognition of danger signals from immunogenic malignant cells (such as expression of tumor antigens and stress ligands specific for receptors on innate immune cells). If complete elimination of transformed cells is achieved, the person remains free of cancer. However, if rare tumor cell variants or poorly immunogenic cells survive elimination, they may enter a phase of equilibrium during which adaptive immune mechanisms control tumor outgrowth but do not achieve complete elimination. The immune selection pressure may lead to editing of tumor immunogenicity and emergence of tumor escape mechanisms. These mechanisms include (1) loss of antigen or stress ligand expression and major histocompatibility complex–I downregulation to escape recognition by adaptive immunity, (2) desensitization to immune effector mechanisms, and (3) induction of an immunosuppressive tumor microenvironment. If tumor outgrowth is no longer blocked by immune mechanisms, tumor cells emerge to cause clinically apparent disease. *DC,* Dendritic cell; *IDO,* indolamine 2,3-dioxygenase; *IFN,* interferon; *IL,* interleukin; *MDSC,* myeloid-derived supressor cell; *NK,* natural killer cell; *NKT,* natural killer T cell; *PD-1,* programmed cell death–1; *PD-L1,* programmed cell death–1 ligand; *TGF-β,* transforming growth factor β; *TNF-α,* tumor necrosis factor-α; *TRAIL,* TNF-related apoptosis-inducing ligand; *Treg,* regulatory T cell. *(Modified from Schreiber RD, Old LJ, Smyth MJ: Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion.* Science *331:1565–1570, 2011.)*

immunotherapy has to break tolerance and is more likely to induce undesired autoimmunity. The clinical use of immunotherapeutic modalities such as monoclonal antibodies (mABs), vaccines against oncogenic viruses, cytokines, and hematopoietic stem cell transplantation (HSCT) has been the standard of care in the prevention, treatment, and supportive care of cancer for many years. More recently, an exciting renaissance in cancer immunotherapy occurred as a result of the success of proof-of-concept clinical trials for a variety of novel immune-modulating strategies. As will be discussed further, these strategies include cellular therapies such as chimeric antigen receptor (CAR) T-cells (CARTs) and the first Food and Drug Administration (FDA)–approved cancer vaccine (sipuleucel-T); blockade of regulatory pathways with anti–cytotoxic T-lymphocyte antigen 4 (anti-CTLA-4) mABs; and antibody-targeted delivery of drugs via immunoconjugates such as brentuximab vedotin and trastuzumab emtansine. Many early-phase immunotherapy trials are being conducted for adult malignancies first and will be discussed when they are applicable. While promising results and toxicity data emerge from adult trials, the benefits of immunotherapy will also increasingly become available to pediatric patients with cancer.

KEY COMPONENTS OF THE IMMUNE SYSTEM AND THEIR RELEVANCE IN CANCER IMMUNOTHERAPY

The immune response can be divided into innate and adaptive immunity to differentiate between an early, generalized response against evolutionarily conserved molecular patterns and a highly specific response that forms fully as a result of exposure to specific antigens. One can also distinguishe a humoral from a cellular arm of the immune system. The humoral arm consists of soluble factors that are secreted by immune cells and includes antibodies and cytokines. The cellular arm encompasses antigen-presenting cells (APCs), phagocytes, lymphocytes, and innate immune cells.

Humoral Arm of the Immune System

Antibodies

B cells have the ability to differentiate into plasma cells and secrete antibodies. B-cell receptors, which represent surface-expressed antibodies against a vast variety of potential antigens, are created by somatic V(D)J-recombination. Subsequent antigen exposure leads to clonal expansion of B cells with the cognate B-cell receptor and facilitates large-scale antibody production. Antibodies consist of two heavy chains (γ, δ, α, μ, or ε) and two light chains (λ or κ). The composition of the heavy chains determines the immunoglobulin (Ig) subclass, which can be IgM, IgG, IgA, IgE, or IgD. These immunoglobulin subclasses have different binding valencies and functions; for example, IgM is produced early in the immune response followed by a class switch to IgG, whereas IgA is predominantly involved in mucosal immunity. Antibodies function by binding to the antigen of their specificity via the antigen-binding fragment (Fab; consisting of one constant and one variable domain from each of the heavy and light chains), thereby marking their target for destruction in a process termed "opsonization." Cellular destruction is mediated by the crystallizable fragment (Fc) component, which is composed of two heavy chains (Fig. 47-2). Depending on the effector cell and type of Fc receptor (FcR), the immune response can be modulated in multiple ways. Activating FcRs contain a cytoplasmic immunoreceptor tyrosine activation motif, whereas inhibitory FcRs signal via an immunoreceptor tyrosine inhibition motif. FcR binding can lead to antibody-dependent phagocytosis (ADP) by macrophages or APCs or cell destruction by natural killer (NK) cells via antibody-dependent cellular cytotoxicity (ADCC). The Fc component can also fix complement, leading to complement-dependent cytotoxicity (CDC). Antibodies can further kill tumor cells by direct induction of apoptosis and blockade of growth factors or other stimulatory signals (Fig. 47-3).

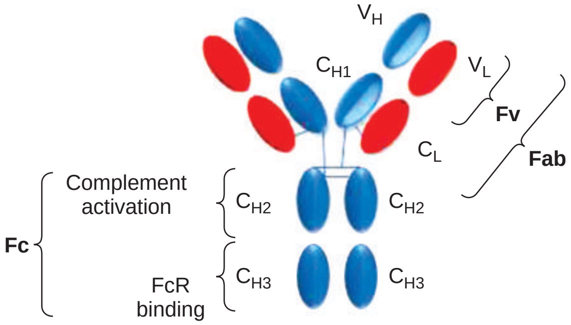


Figure 47-2 Structure of an antibody molecule. Antigen-binding fragment *(Fab)* and crystallizable fragment *(Fc)* regions are distinguished on the basis of proteolytic cleavage by papain. Functionally, the variable region *(Fv)* represents the antibody binding site, whereas the Fc region mediates complement activation and binding to Fc receptors *(FcRs)*. Structurally, one further differentiates heavy chains (*blue*; γ, δ, α, μ, or ε) from light chains (*red*; λ or κ).

Cytokines

Cytokines are soluble proteins that are predominantly produced by leukocytes and carry out intercellular signaling function. Based on their varied functions, they can be classified into interleukins (ILs), interferons (IFNs), chemokines, and hematopoietic growth factors. Several cytokines have been used in cancer immunotherapy either as a single agent or to enhance the effect of other therapeutic agents. Growth factors such as granulocyte-colony stimulating factor (G-CSF) also play an important role in facilitating the neutrophil recovery necessary for compressed chemotherapy regimens, which have led to improved survival for children with multiple types of pediatric solid tumors. Lastly, analysis of the cytokine response triggered by immune therapies is informative in elucidating the mechanism of action and associated toxicities and can guide lifesaving therapeutic interventions, as demonstrated by the successful use of an IL-6 receptor (IL-6R) antagonist in the treatment of cytokine

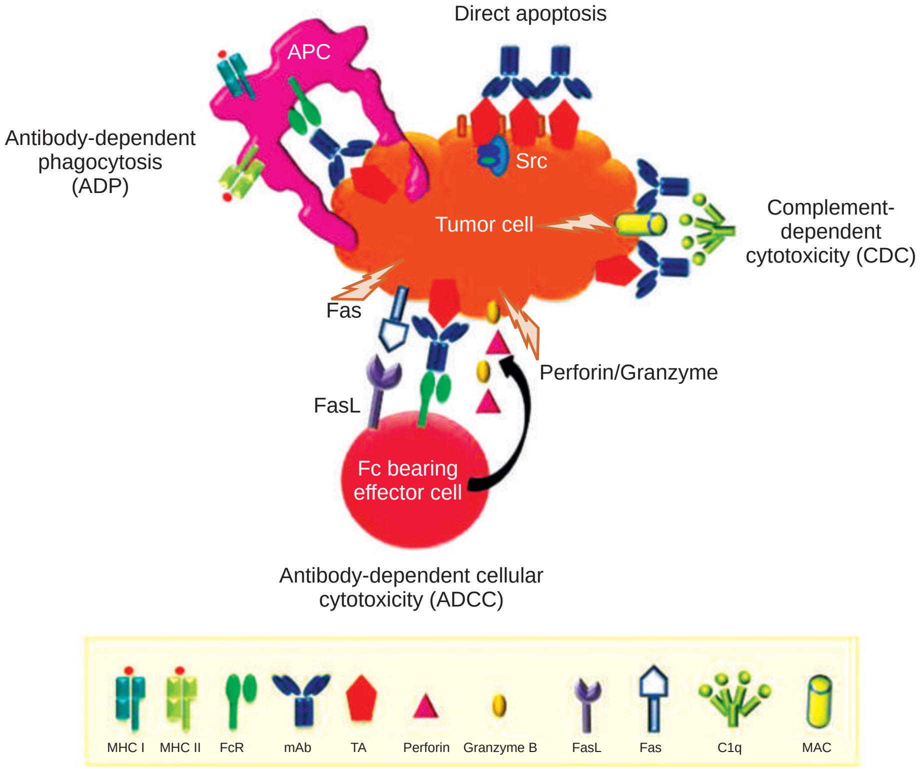


Figure 47-3 Antibody-mediated mechanisms of tumor cell destruction. Antibody-dependent phagocytosis *(ADP)*: Via the antibody-dependent phagocytosis pathway, antibodies entice crystallizable fragment receptor *(FcR)*–bearing phagocytes to take up tumor cells, process a variety of different tumor antigens, and present them in major histocompatibility complex *(MHC)*–restricted fashion to cluster of differentiation (CD)4+ and CD8+ cells. This process can lead to the vaccinal effect of monoclonal antibody *(mAb)* therapy. Antibody-dependent cellular toxicity *(ADCC)*: FcR-bearing effector cells such as natural killer cells can mediate antibody-dependent cellular cytotoxicity via perforin/granzyme B or induce apoptosis via Fas ligand *(FASL)*/Fas after recognition of the crystallizable fragment *(Fc)* portion of the bound antibody. Complement-dependent cytotoxicity *(CDC)*: The C1q protein of the complement system binds to antigen : antibody complexes on the tumor cell surface and causes activation of the complement cascade, resulting in formation of the membrane attack complex *(MAC)* and tumor cell lysis. Direct apoptosis: Cross-linking of antibody molecules and tumor antigens *(TAs)* in the lipid raft can activate signaling pathways involving Src kinases that mediate direct apoptosis. *APC,* Antigen-presenting cell.

release syndrome associated with cluster of differentiation (CD)19-CAR therapy.

Cellular Arm of the Immune System

T Cells

T cells are a major component of the adaptive immune system (Fig. 47-4). They are derived from bone marrow, but in contrast to B cells, they travel to the thymus to undergo V(D)J-recombination of their T-cell receptors (TCRs), editing, and maturation. Somatic recombination of the α- and β-chains of the TCR results in the incredibly diverse T-cell repertoire from which clonal expansion can occur upon encounter of a specific antigen. To prevent autoimmunity, TCRs specific for “self” undergo negative selection in the thymus but also are subject to regulatory mechanisms in the periphery. Generation of an adaptive antitumor response begins with capture of tumor antigens by dendritic cells (DCs) in the peripheral tissues. DCs then travel to the draining lymph nodes, where they interact with T cells. Successful activation of a T-cell response (T-cell priming) requires presentation of the antigen on major histocompatibility complex class I (MHC-I) or class II (MHC-II) molecules for TCR-mediated recognition (signal 1), as well as a costimulatory signal (signal 2). Classically, the costimulatory signal is delivered to CD28 on T cells via the B7 family (CD80 and CD86) on APCs, but many other costimulatory molecules have been identified. The absence of costimulation leads to anergy. T cells may also receive inhibitory signals via CTLA-4, programmed death 1 (PD-1), and various other receptors, which play a physiologic role in terminating an immune response. The tumor microenvironment exploits some of these mechanisms to escape immune surveillance by MHC downregulation, as well as expression of inhibitory ligands such as PD-1 ligand (PD-L1). The discovery and characterization of other modulatory signaling pathways is ongoing and will continue to be a platform for therapeutic intervention.

T cells express CD3 and are divided in CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ helper T cells. DCs can process and present antigens via MHC-I and MHC-II pathways. CD8+ CTLs are generally activated via MHC-I. This pathway predominantly processes antigens derived from the cell itself (such as viral antigens), but it can also

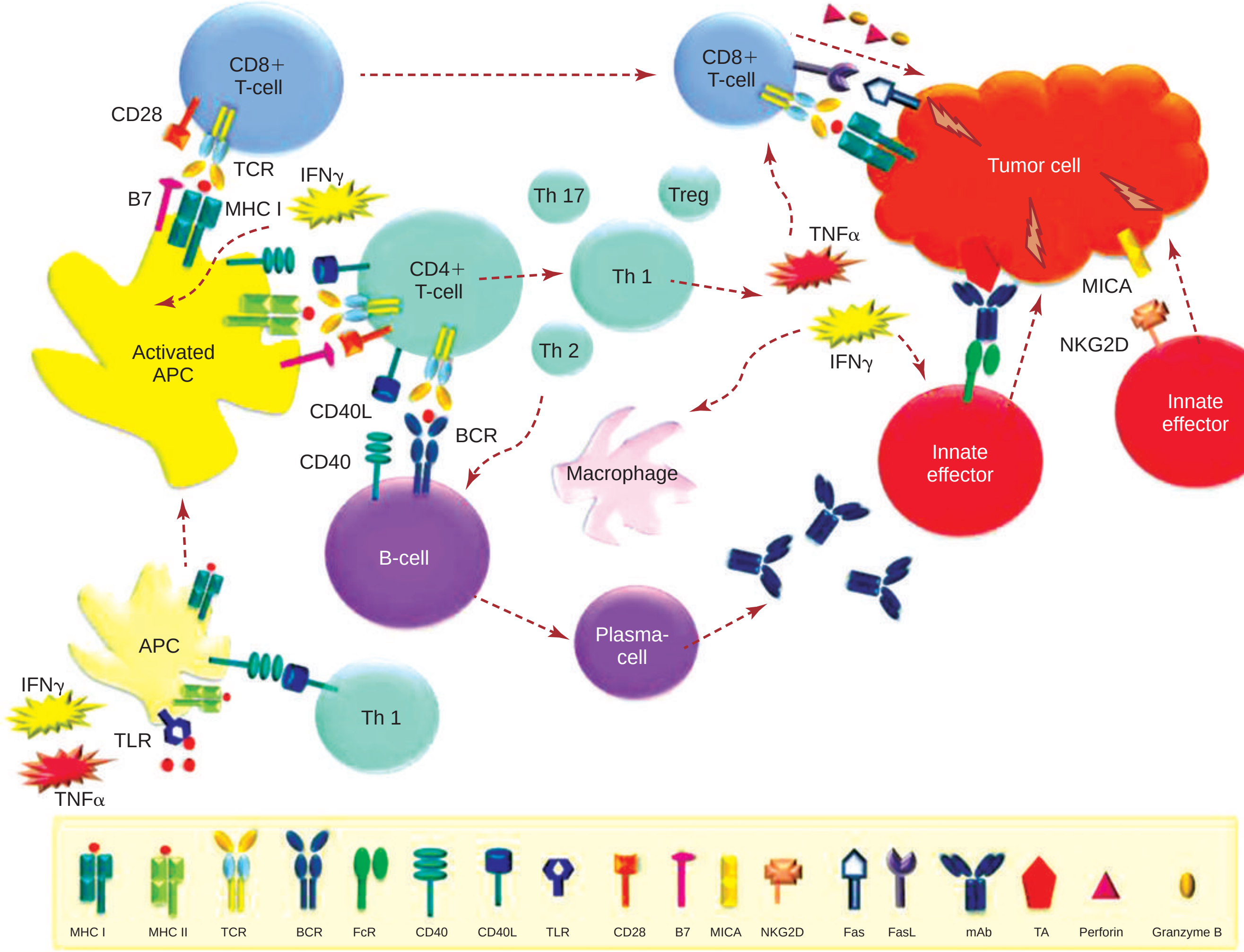


Figure 47-4 Antitumor effects of the immune system. Innate effector cells (such as natural killer cells) are regulated by a system of activating and inhibitory receptors and often recognize major histocompatibility complex *(MHC)*-I or MHC-I–related molecules that are upregulated by cells under the stress of malignant transformation. They are capable of rapid killing of tumor cells or mediation of apoptosis upon recognition of such ligands. They also mediate antibody-dependent cellular toxicity via interaction of their crystallizable fragment receptor *(FcR)* with the Fc portion of antibodies directed against tumor antigens *(TAs)*. Initiation of the adaptive immune response to tumors relies on TA uptake by an antigen-presenting cell (APC) in the context of appropriate maturation signals such as Toll-like receptor *(TLR)* signaling, cluster of differentiation (CD)40/CD40L interaction with CD4+ T cells, and cytokines. In its activated state the APC presents TAs on MHC molecules and provides the necessary costimulatory signals (e.g., CD80/CD86, also referred to as B7) to activate a T-cell response in lymph nodes. Via the MHC-I pathway, APCs prime CD8+ cytotoxic T lymphocytes (CTLs), which then clonally expand, recognize the TA on tumor cells via their cognate T-cell receptor *(TCR)*, and mediate tumor cell lysis. APCs present antigens to CD4+ helper cells via MHC-II molecules. CD4+ T cells do not directly partake in tumor cell destruction but provide help to other immune cells. They are polarized toward functionally distinct subtypes. T helper *(Th)*1 cells mediate tumor immunity through production of cytokines such as interferon *(IFN)*-γ and tumor necrosis factor *(TNF)*-α, which enhance macrophage and CTL functions, and provision of CD40L signals that enhance APC maturation. Th2 cells are crucial for the induction of B-cell responses and antibody production against TAs. Regulatory T cells *(Tregs)* are immunosuppressive, and their presence in the tumor microenvironment dampens a vigorous antitumor immune response. *BCR,* B-cell receptor; *FasL,* Fas ligand; *mAb,* monoclonal antibody; *MICA,* MHC class I–related chain A.

present antigens taken up by the cell through a mechanism referred to as cross-presentation. MHC-I is present on all nucleated cells, and upon recognition of the MHC-restricted cognate antigen on a tumor cell, primed CD8+ CTLs mediate tumor cell death by virtue of releasing perforin/granzyme B or by inducing apoptosis through interactions of death receptors such as Fas/Fas ligand. MHC-II expression is limited to APCs such as DCs, macrophages, and B cells, which process phagocytosed antigens and activate CD4+ T cells via the MHC-II pathway. CD4+ T cells do not generally directly mediate tumor cell lysis, but they critically support and influence the immune response by producing cytokines and providing “help” to CD8+ T cells and B cells via CD40 signaling. Depending on the polarization of their cytokine profile and function, CD4+ helper T cells are divided into Th1, Th2, Th17, and follicular helper T-cell subtypes and regulatory T cells (Tregs). Th1 responses are characterized by proinflammatory cytokines such as IFN-γ and tumor necrosis factor (TNF)-α, which enhance macrophage and CTL function and promote secretion of immunostimulatory cytokines, such as IL-12 by APCs. Th2 responses are essential for antibody production and class switching of B cells, are involved in allergic responses, and produce an array of cytokines such as IL-4, IL-9, IL-10, and IL-13 that contribute to eosinophil and mast cell activation. The Th17 subset was only described relatively recently. Although it is involved in the pathogenesis of autoimmune and inflammatory diseases, it may also play an important role in tumor immunity.[9] Lastly, Tregs express CD25 and the forkhead box (FOX)P3 transcription factor in addition to CD4+. They serve to inhibit immune activation, induce self-tolerance under physiologic conditions, and notably expand in response to IL-2. Treg blockade or elimination is an important factor in facilitating an effective antitumor immune response, and therapeutic expansion of Tregs may play a role in controlling graft-versus-host disease (GVHD).

γδ T Cells

γδ T cells represent a small subgroup of circulating T cells that function at the interface of innate and adaptive immunity. Their TCR also undergoes somatic recombination and editing but consists of a γδ heterodimer. This subset may recognize specific antigenic determinants via the TCR or by a TCR independent pathway similar to that of NK cells.

Natural Killer T Cells

Natural killer T cells (NKTs) are a unique subset of T cells with a highly restricted TCR repertoire, allowing recognition of certain glycolipid antigens, such as GD2, in conjunction with the nonclassical MHC-I molecule CD1d. They contribute to antitumor immunity as an early source of cytokine production, resulting in activation of other effector cells. Higher numbers of tumor-infiltrating NKTs have been associated with improved long-term disease-free survival (DFS) in children with stage IV neuroblastoma.[10] NKTs are thought to inhibit tumor growth by targeting tumor-associated macrophages (TAMs) that have been shown to have clinical significance in neuroblastoma,[11] Hodgkin lymphoma (HL),[12] and other malignancies. NKTs are attracted by the chemokine (C-C motif) ligand 2 (CCL2). *MYCN*, the hallmark of aggressive neuroblastoma, mediates CCL2 repression and therefore reduces NKT infiltration.[13] Via another escape mechanism, TAMs in the neuroblastoma microenvironment produce a different chemokine, CCL20, in response to hypoxia, thereby attracting NKTs into hypoxic traps. IL-15 was shown to protect NKTs from the inhibitory effects of hypoxia and enhances antimetastatic activity of NKTs.[14]

NK Cells

NK cells play a key role in the innate immune response, in an early defense against infection or malignant transformation. NK cells can be phenotypically distinguished from other peripheral blood mononuclear cells because they are CD56+ and lack B-cell (CD19), T-cell (CD3), and monocytic (CD14) markers. Subsets of NK cells also express the FcRs CD16 and CD11b that mediate ADCC and participate in CDC. Contrary to T and B cells, NK-cell receptors do not undergo somatic recombination and do not require recognition of a cognate antigen to mediate cytotoxicity. Traditionally it has been thought that they do not develop immunologic memory, although this paradigm may be changing.[15] Their function is tightly regulated by a complex system of activating and inhibitory receptors, which often recognize MHC-I or MHC-I–related molecules. These interactions provide the basis for the therapeutic exploitation of NK-cell alloreactivity. The key regulators of NK cells are activating and inhibitory killer cell immunoglobulin-like receptors (KIRs), which activate natural cytotoxicity receptors including NKp30, NKp46, and NKp44 and the activation receptor NKG2D. NK cells are capable of rapid killing via degranulation and release of toxic granules, primarily through a perforin and granzyme B–dependent pathway. They can also mediate apoptosis by utilizing Fas ligand or TNF-related apoptosis-inducing ligand (TRAIL) and execute ADCC via their FcRIII (CD16) receptor. NK cells further secrete chemokines such as CCL2 through CCL5, XCL1, and CXCL8. Their clinical significance in pediatrics is underscored by the susceptibility of infants born with NK-cell deficiencies to fulminant viral infections and the NK-cell defects underlying primary hemophagocytic lymphohistiocytosis. NK cells facilitate stem cell engraftment, fight infection, and control cancer, yet they do not cause GVHD. These characteristics make them attractive therapeutic candidates for adoptive NK-cell therapies.

Dendritic Cells

Dendritic cells (DCs) represent a small subset of peripheral blood mononuclear cells (1% to 2%). They are mobile sentinels that collect antigen from peripheral tissues and carry it to the secondary lymphoid organs to activate T cells. DCs are termed “professional APCs” based on their ability to process and present antigenic peptides on MHC-I or MHC-II molecules. In steady state, they reside in the periphery in an immature state and play an important role in maintaining tolerance by taking up self-antigens. Successful T-cell activation requires DC activation. Upon activation, DCs upregulate MHC molecules, costimulatory molecules (which provide the second signal for T-cell activation), and chemokine receptors (which enhance homing to lymphoid organs) and secrete inflammatory cytokines such as IL-12.[16] DC activation can occur in response to a variety of signals, such as the receipt of exogenous and endogenous danger signals via Toll-like receptors (TLRs), which recognize evolutionarily conserved molecular patterns from products of damaged or dying cells and from pathogens.[17] DCs are also matured by proinflammatory cytokines secreted by other cells (e.g., innate effectors) or by CD40 ligation. In addition, DCs are important in launching humoral immunity, partly because of their capacity to directly activate B cells.[18]

Myeloid DCs and plasmacytoid DCs are the two main subsets among various other DCs. They express high levels of human leukocyte antigen (HLA)-DR (MHC-II), but they differ in their expression of CD11c (myeloid DCs), CD123 (plasmacytoid DCs), and TLRs, among other markers. DCs are critical regulators of adaptive T- and B-cell responses and are central to the development of successful tumor vaccines. Because of their low abundance in the peripheral blood, sufficient numbers of DCs can be generated in vitro either by expansion of isolated DCs or by generation from monocytes using cytokine cocktails. DCs further play a role in the pathogenesis of GVHD and are required for full graft-versus-leukemia (GVL) effects, and thus their therapeutic manipulation is of relevance in HSCT.

Neutrophils

Neutrophils are crucial to the rapid destruction of pathogens, particularly bacterial, fungal, and certain parasitic organisms, as evidenced by the high risk of sepsis during periods of neutropenia in the oncology population. However, neutrophils also contribute to antitumor activities of the immune system. Through their FcRs, they can be involved in ADCC-mediated tumor destruction. In addition, neutrophil activation at sites of the tumor via administration of cytokines such as GM-CSF can play a role in tumor cell degradation and enhance antigen presentation.[19]

Monocytes/Macrophages

Monocytes are myeloid-derived phagocytic cells that differentiate into resident macrophages upon recruitment into tissues. Macrophages are able to ingest apoptotic cells and opsonized cells via their FcRs. Aside from their role in clearing cellular debris, they are able to present antigenic peptides of the digested cells in an immunogenic way. Activated macrophages prominently secrete cytokines such as IL-12, which may be critical in activating local immune responses by NK cells and T cells. Macrophages were originally classified into M1 (proinflammatory) and M2 (antiinflammatory) macrophages according to their polarization status. Although this classification remains conceptually helpful, it is now clear that tissue macrophages display a high degree of functional and phenotypic plasticity in response to changing microenvironmental stimuli and that M1 and M2 polarization represent extremes of a continuum of activation states. Increasing evidence indicates that TAMs are implicated in protumor effects and can play a role in tumor initiation, progression, and distant metastasis.[20,21] Limited evidence suggests that myeloid-derived suppressor cells develop from a common myeloid progenitor without a monocytic intermediate and have predefined immunosuppressive properties, whereas monocytes lack suppressive activity in steady state and acquire this function in response to certain stimuli. In persons with cancer, the majority of studies support an association between high TAM density and poor clinical outcome, as has been demonstrated in several malignancies, including neuroblastoma.[11,12] However, TAM function in tumor immunity appears to be dynamic and heterogeneous depending on the tumor type, tumor progression, location within the tumor, and perhaps most importantly, polarization of the TAM. In this regard, increased density of M2-like TAMs is a marker of poor prognosis, whereas the presence of M1-like subsets is advantageous. Based on these findings, subpopulations of TAMs are now being explored as new therapeutic targets.[20]

TARGET ANTIGENS IN CANCER IMMUNOTHERAPY

Tumor antigens (TAs) are molecules that enable the recognition of tumors by components of the immune system. A wide array of target antigens have been identified and may be classified into various subgroups according to their origins. Tumor-specific antigens are epitopes that are found exclusively on malignant cells. This group includes proteins arising from mutated oncogenes, clonally rearranged immunoglobulin genes, and oncoviral gene products. Tumor-associated antigens are expressed on some healthy and malignant tissues alike. Tumor-associated antigens may arise from overexpressed normal proteins, oncofetal proteins, lineage-restricted markers, histocompatibility molecules, and proteins that are normally expressed in immune-privileged sites.

Antigens Arising from Genetic Alterations

A number of malignancies are associated with specific genetic mutations or translocations, resulting in the synthesis of novel proteins that are foreign to the host's immune system, thereby offering the potential of immune recognition while sparing healthy tissues. Commonly they are drivers of or contribute to tumorigenesis, as is the case for oncogenes or mutated tumor suppressor genes, but they may also arise from mutations not directly involved in the neoplastic process. A variety of malignancies express gene products derived from mutated tumor suppressor genes, such as *p53* or *ras*. Examples of antigens derived from mutated oncogenes include the EWS-FLI fusion protein resulting from the t(11:22) translocation characteristic of Ewing sarcoma and the p210 and p190 products of the bcr/abl translocation in chronic myelogenous leukemia (CML) and Philadelphia chromosome–positive acute lymphoblastic leukemia (ALL), respectively.

Oncoviral Proteins

Viral infections play a causal role in several malignancies and give rise to immunogenic viral proteins that can be recognized and eliminated by the immune system. This process is underscored by the fact that immunosuppressed persons, particularly those with impairment of T-cell immunity, have a higher propensity to develop Epstein-Barr virus (EBV)–associated lymphomas and human papillomavirus (HPV)–associated skin and cervical cancers. Examples of oncoviral proteins are the expression of the EBV antigens EBNA-1, EBNA-3, LMP-1, and LMP-2 in PTLD and some lymphomas and the papillomavirus E6 and E7 proteins in cervical carcinomas.

Clonally Rearranged Immunoglobulin Genes

Surface immunoglobulin is a marker of B-cell maturity and is present in mature B-cell non-Hodgkin lymphomas (NHLs) and multiple myeloma. Because surface immunoglobulin expression results from separate somatic recombination events in each B cell, all malignant cells in a monoclonal B-cell malignancy express the clonally unique immunoglobulin variable region, or idiotype. Immunoglobulins are proteins and can therefore be immunogenic and lead to the development of idiotype vaccines. Another approach to targeting this group of TAs includes the generation of anti-idiotype mABs in mice, which in turn are immunogenic and amplify the immune response upon administration to the patient as a type of vaccine.

Altered Glycolipid and Glycoprotein Antigens

Most human tumors express higher levels or abnormal forms of surface glycoproteins and glycolipids, which serve as targets for immunotherapy. Some aspects of the malignant phenotype of tumors, including tissue invasion and metastatic behavior, may reflect altered cell surface properties as a result of the abnormal glycolipid and glycoprotein synthesis. Most of these epitopes are not exclusively expressed on tumors but are present at higher levels on cancer cells compared with healthy tissues. Examples of such glycolipids are GD2, which is expressed in neuroblastoma and some osteosarcomas, as well as GM2, GD2, and GD3 in melanomas. These molecules are actively being explored in vaccination strategies, and mABs against the carbohydrate groups or peptide cores of such molecules have been generated and clinically used with success.

Oncofetal Antigens

Oncofetal antigens are proteins that are expressed at high levels in cancer cells and during normal fetal development, but not in adult tissues. Alpha-fetoprotein expression in hepatoblastomas and germ cell tumors and carcinoembryonic antigen in colorectal cancer are examples of aberrant expression of fetal antigens. Although they are largely utilized as biomarkers, they are also being explored as targets of CART therapy.

Products of Genes That Are Selectively Expressed in Immune-Privileged Sites

An interesting group of antigens are the so-called "cancer-testes antigens." These nonmutated proteins arise from germline-associated genes that are preferentially expressed in immune-privileged sites such as the testes and placenta. These antigens include NY-ESO, MAGE, BAGE, and RAGE and are expressed in melanomas and many carcinomas. They are promising targets given their relatively tumor-specific distribution and have been explored in the context of tumor vaccination.

Histocompatibility Antigens

Histocompatibility antigens are of importance in the allogeneic HSCT setting. Because HLAs are widely expressed in normal tissues and are immunologic targets for T-cell responses, donor selection intends to maximize major histocompatibility matches between the donor and recipient to minimize GVHD. However, minor histocompatibility antigens (mHAs) may generate a GVL response both in matched sibling and in unrelated donor (URD) transplantation. mHAs may have tissue restricted expression, such as HA-1 and HA-2, which are expressed only on hematopoietic cells. Such mHAs are particularly appealing immunologic targets because the induced immunologic response will not cause GVHD in nonexpressing tissues.

Molecules That Activate Recognition of the Innate Immune System

Cells of the innate immune system do not require presentation of antigens via MHC molecules but instead have the ability to respond to MHC-I–related ligands, which tissues may express inducibly in association with cell stress, infection, or malignant transformation, thus allowing for a system in which the body alerts the immune system by creating internal danger signals. Such molecules may not be recognized by endogenous T and B cells but can induce potent responses by innate effector cells. The advantage of such danger signals is that they are absent or expressed at very low levels in steady state. In healthy humans, MICA and MICB, which are ligands for the NKG2D receptor on NK cells and CD8+ T cells, are expressed only by intestinal epithelial cells at low levels, likely as a consequence of stimulation by the local bacterial flora. However, these molecules are significantly upregulated in a variety of tumor types[22] and are thus promising targets.

Normal Proteins Overexpressed in Tumor Tissue

Increased gene transcription or amplification of normal genes in tumor cells may result in aberrantly high expression of proteins. Examples include MYCN amplification in high-risk neuroblastoma, overexpression of human epidermal growth factor receptor 2 (Her2/neu) in a subset of breast and ovarian cancers, as well as some sarcomas, and gp100 and MART in melanomas. Because these proteins are also found at lower levels in some healthy tissues, immune tolerance to such antigens may have developed and needs to be overcome by effective immunotherapy.

Lineage-Specific Tumor Antigens

Many tumors express differentiation antigens that are not restricted to the malignant cell but rather to their lineage of origin. Examples include CD19 and CD22 in pre–B-cell ALL (pre–B-ALL), CD33 in acute myeloid leukemia (AML), and prostate-specific antigen (PSA) in prostate cancer. Similar to overexpressed proteins, the utility of these antigens as targets for tumor immunotherapy is highly dependent on the expression pattern on healthy tissues. As such, the B-cell aplasia accompanying CD19-targeted therapies may be transient and can be managed with exogenous administration of immunoglobulin. Similarly, targeting of PSA may be associated with immune destruction of normal prostate cells, yet this destruction is pathophysiologically tolerable. In contrast, lineage-specific antigen expression on vital organs would be prohibitive to this approach.

TUMOR VACCINES

One of medicine's great successes was the introduction of vaccines for the prevention of infectious diseases.

Vaccines that provide protection against oncogenic viruses effectively prevent certain cancers as well. Based on Center for Disease Control and Prevention estimates, approximately 26,000 new cervical cancers each year are attributable to HPV.[23] Therefore the quadrivalent HPV vaccine, which was implemented in 2007 and is recommended for female and male adolescents, has tremendous potential to prevent cervical cancer.[24,25,26] Another example is the prevention of hepatocellular carcinoma by way of hepatitis B virus vaccination.[27] The generation of therapeutic cancer vaccines is much more challenging, given that such vaccines must overcome preestablished tumor tolerance. However, the discovery that patients can harbor CD8+ and CD4+ T cells specific for antigens expressed in their tumors[28] gave rise to the idea that therapeutic cancer vaccines could activate and amplify this preexisting reaction and possibly induce new immune responses. Adjuvants such as keyhole limpet hemocyanin (KLH) or Montanide are important components of cancer vaccines because they act to enhance the immunogenicity of the antigen, activate DCs, and help overcome preexisting tolerance.

Dendritic-Cell Vaccines

Because of the central role of DCs in the orchestration of adaptive immune responses, DCs have been a special focus of tumor vaccination strategies. Immature DCs take up antigen and rely on appropriate maturation signals to effectively prime T cells, whereas antigen presentation by immature DCs leads to immune tolerance. It is essential to consider the complexity of DC biology in the context of DC vaccine design.[29] Delivery of the TA to DCs and provision of maturation signals can be provided ex vivo or in vivo. Because of the low frequency of DCs in the peripheral blood, approaches have been developed to generate inflammatory DCs from monocytes under culture conditions with cytokine combinations such as GM-CSF and IL-4[30] and then pulse them with the desired antigen ex vivo. Such vaccines have been clinically tested for more than a decade,[31] and it has been concluded that they are safe and can induce expansion of circulating tumor-specific CD4+ and CD8+ T cells, with objective and potentially long-lasting responses in some patients.[32,33] Sipuleucel-T, a vaccine based on enriched blood APCs that are briefly cultured with a fusion protein of prostatic acid phosphatase and GM-CSF, was the first tumor vaccine to be approved by the FDA after it demonstrated a prolonged median survival of patients with metastatic prostate cancer in a randomized phase I trial.[34] In pediatrics, the experience has been limited to a few early nonrandomized trials. Administration of tumor lysate/KLH pulsed autologous DCs to children with relapsed solid tumors was well tolerated and demonstrated induction of specific T-cell responses. Significant regression of metastatic sites was observed in one patient, with several other patients having sustained stable disease.[35] In a related approach, patients with metastatic or relapsed sarcomas or neuroblastomas are receiving Treg-depleted autologous lymphocytes in combination with tumor lysate/KLH pulsed DCs ± IL-7 (trial NCT00923351). An initial study demonstrated that consolidative immunotherapy consisting of adoptive T-cell transfer with peptide-pulsed DCs can improve survival compared with historic control subjects when given in a minimal residual disease (MRD) setting of high-risk solid tumors.[36] An ongoing trial of decitabine followed by vaccination with autologous DCs pulsed with overlapping peptides derived from full-length MAGE-A1, MAGE-A3, and NY-ESO-1 antigens may hold promise in the induction of remission of relapsed neuroblastoma[37] or sarcoma (trial NCT01241162).

DCs can also be targeted in vivo by fusing the selected antigen to an antibody directed against a DC surface receptor. This technique has been shown to elicit potent antigen-specific CD4+ and CD8+ T-cell responses.[38] The choice of a DC receptor that is targeted is an important consideration because distinct DC subsets induce different types of T-cell polarization, which determine in part the elicited immune response.[39] The engagement of DC receptors by such targeting antibodies also provides activation signals, which are crucial to avoid the induction of T-cell anergy. In fact, it is now accepted that the adjuvant components of vaccines (even non–DC-based vaccines) primarily act by triggering DC maturation.

The delivery of antigens and adjuvants into the same intracellular DC compartment has been shown to be crucial.[40] Nanovaccines are a promising preclinical technology to achieve synchronized delivery of antigen and adjuvant cargo to DCs. Nanoscale complexes such as polymer-based or lipid-based nanoparticles provide protection against unwanted antigen degradation or systemic immune activation by soluble adjuvants. Upon injection into the skin, nanovaccines are transported to the lymph nodes by cell-based drainage or free interstitial lymphatic flow (depending on particle size), where they are internalized by lymph node resident DCs and generally directed to the MHC-II pathway.[41] Another bioengineering-based approach is now entering clinical trials and involves implantation of absorbable polymer scaffolds, pulsed with tumor lysate and adjuvants, to facilitate DC-based T-cell priming in situ.[42]

Peptide-Based Vaccines

The immunogenicity of peptide epitopes depends on their associations with specific polymorphic variants of MHC molecules, such that a given peptide might only elicit a response in persons with a certain HLA allele. Studies with HLA-restricted peptides thus must be carried out in patients with unique HLA alleles. Given that the population of pediatric patients is small a priori, the exploration of peptide vaccines has been much more feasible in adults who have common malignancies. Largely as a result of a poor understanding of the mechanisms of immunization and the role of DCs, in many early trials patients were unsuccessfully treated with short peptide-based vaccines in the absence of effective adjuvants.[43] Short peptides have pharmacokinetic properties that make them susceptible to rapid clearance in the absence of an adjuvant that can effectively trigger DC maturation and may promote tolerance rather than immunity. A notable exception is the administration of a short-peptide vaccine derived from gp100 in conjunction with

IL-2, which augmented tumor responses and prolonged progression-free survival (PFS) compared with IL-2 alone in persons with advanced melanoma.[44] Longer peptides (~20-mer) require further processing by DCs but have the potential to prime against a greater variety of antigens and capture CD8+ and CD4+ lymphocyte responses more efficiently compared with peptides (10- to 12-mer) that fit the MHC-I antigen-binding groove unedited. A long-peptide vaccine against HPV-16 oncoproteins administered in incomplete Freund adjuvant to patients with vulvar intraepithelial neoplasia proved to have good efficacy, including complete responses in a phase II trial.[45] These favorable results may be based on the selection of viral gene products for vaccination that are more readily recognized as foreign. In contrast, a long-peptide vaccine derived from p53 (a tumor suppressor protein mutated in many cancers), which was delivered in the emulsion-adjuvant Montanide, induced no tumor regressions in patients with advanced ovarian cancer, emphasizing preexisting tolerance and the need to optimize these formulations.[46]

Full-length protein vaccines harbor a wider profile of epitopes, and a diverse set of targets—including two cancer testes antigens, MAGE-A3 and NY-ESO-1—have been explored.[47,48] Phase I and II trials have been conducted with adjuvant-mixed, recombinant MAGE-A3 vaccines in melanoma and non–small cell lung cancer, demonstrating potent antitumor B- and T-cell responses, tumor-specific cell persistence for years after vaccination, and encouraging clinical effects.[49,50] "MAGRIT"—an ongoing large, randomized phase III trial accruing more than 2500 HLA-A2–positive patients with non–small cell lung cancer—is exploring a recombinant MAGE-A3 fusion protein in combination with an adjuvant consisting of a saponin/lipid-A emulsion and TLR4 and TLR9 agonists.[51]

Whole-Cell Vaccines

Whole-cell vaccines contain the broadest range of TAs, including mutated proteins. A meta-analysis of 173 immunotherapy trials involving a variety of solid tumors revealed that patients vaccinated with whole-cell vaccines had low objective response rates that were nonetheless significantly higher than those of patients treated with molecularly defined antigen vaccines.[52] GVAX, a vaccine strategy in which autologous or allogeneic whole tumor cells are engineered to secrete GM-CSF (to activate APCs at the site of vaccination) and are irradiated prior to injection, reached the furthest in clinical development. Phase I trials of GVAX were conducted in adults who had a variety of malignancies.[53] These early-stage trials were not powered to assess clinical endpoints, yet they consistently demonstrated immune cell infiltration at the sites of vaccination, as well as extensive tumor necrosis mediated by dense infiltrates of intratumoral CD4+ and CD8+ lymphocytes and plasma cells. Although some patients had promising results after receiving GVAX in preceding trials, a phase III trial in persons with prostate cancer that used allogeneic tumor cell lines did not demonstrate clinical efficacy.[54] This results indicate that this strategy may be most effective when autologous tumor cells are used or when it is combined with other immune-modulating therapies. Administration of anti-CTLA4-mAb to patients previously immunized with GVAX evoked objective responses of metastatic melanoma with minimal toxicities.[55] Furthermore, administration of GVAX early after allogeneic HSCT for adult AML or myelodysplastic syndrome was shown to be safe and immunogenic,[56] with encouraging responses that have led to further exploration in a randomized phase II study (trial NCT01773395). In a similar approach, a pediatric phase I trial with IL-2-transduced autologous neuroblastoma cells in 10 patients resulted in the generation of antitumor antibodies and increased antitumor cytotoxic activity, which correlated well with clinical responses. This study observed one complete response, one partial response, and three patients with stable disease, of whom one patient entered complete remission (CR) after oral etoposide therapy.[57] In a subsequent study of 21 patients treated with an allogeneic neuroblastoma vaccine engineered to secrete lymphotactin and IL-2, two complete responses and one partial response were induced.[58]

Idiotype Vaccines

The clonal immunoglobulin idiotype, which is displayed on the surface of most malignant B cells, is a patient- and tumor-specific antigen that can be used for therapeutic vaccination. An idiotype vaccine conjugated with KLH and administered with GM-CSF indicated prolonged PFS in one phase III trial, whereas several other trials failed to demonstrate a therapeutic benefit.[59]

DNA Vaccines

Deoxyribonucleic acid (DNA) vaccines consist of viral vectors that encode TAs and are based on the idea that strong immune responses against the viral vaccine components enhance the reactivity to the TA. A phase II trial, PROSTVAC, utilized a recombinant vaccinia virus that encoded PSA along with costimulatory and adhesion molecules with the intention of rendering infected cells into surrogate APCs, followed by administration of a similarly configured fowlpox vector in a prime-boost strategy. For additional immune stimulation, GM-CSF was administered with the vectors. This trial demonstrated a median overall survival (OS) benefit of 25.1 months in the treatment group versus 16.6 in the control group[60] and prompted initiation of a phase III trial.

CYTOKINES

Interferons

IFNs are a class of cytokines that were initially investigated for their ability to interfere with viral infections, and they play an important role in the propagation of an immune response. Clinically meaningful activity of IFN-α has been shown against CML,[61] although its clinical use in CML has largely been abandoned with the advent of tyrosine kinase inhibitors. IFN-α remains a very relevant agent in the adjuvant therapy of high-risk operable melanoma, and high-dose IFN-α has demonstrated a consistent and significant improvement in DFS and OS.[62]

Interleukin-2

IL-2 plays a crucial role in T-cell growth and proliferation. It is primarily produced by CD4+ T cells after antigen stimulation, and to a lesser extent by CD8+ cells, NKT cells, activated DCs, and mast cells. IL-2 transcription is mediated by multiple transcription factors, including the nuclear factor of activated T cells (NF-AT) family proteins, AP-1 and NF-κB. The immunosuppressive drugs cyclosporine and tacrolimus act by blocking NF-AT translocation to the nucleus. The IL-2 receptor (IL-2R) is composed of an α, β, and γ chain. The IL-2R β chain is shared with IL-15, and the common γ chain receptor (γcR) is shared with six other cytokines. The crucial role of IL-2 in T-cell growth is evidenced by the severe combined immunodeficiency phenotype of patients with γcR defects. IL-2 preferentially promotes differentiation of Th1 and Th2 cells while inhibiting Th17 and follicular helper T-cell development, yet it can promote Th17 expansion once polarization has occurred. Importantly, IL-2 also drives the development of Tregs, which promote tolerance, and is essential for activation-induced cell death, an important regulator of T-cell homeostasis. Besides its potent effect on T cells, IL-2 induces proliferation and cytolytic activity of NK cells and promotes B-cell proliferation and antibody production.[63]

Clinical administration of IL-2 in an effort to activate immune cells has been in use for many years, with FDA approval in 1992 for metastatic renal cell carcinoma and in 1998 for metastatic melanoma.[64] In a small subset of 5% to 10% of patients with these malignancies, IL-2 treatment has resulted in long-term CRs, and the addition of adoptive therapy approaches have improved cure rates for metastatic melanoma to a range of 20% to 40%.[65] A limitation of IL-2 is its toxicity, including the propensity for severe capillary leak syndrome.

In pediatrics, a randomized children's oncology group trial of 289 children with AML in CR1 who received either no further therapy or two short courses of IL-2 after intensive chemotherapy did not show improvement in DFS or OS.[66] However, IL-2 administration plays a role in the treatment of high-risk neuroblastoma, where it is used in conjunction with anti-GD2 mAb therapy, GM-CSF, and isotretinoin, an approach that has led to significant improvements in outcome.[67] The rationale underlying coadministration of IL-2 is the promotion of ADCC via FcRs of IL-2–activated NK cells and macrophages, which demonstrated an enhanced antitumor effect in murine models compared with IL-2 or mAb alone.[68]

As the effects of IL-2 on Tregs have become more clearly delineated, exogenous administration of IL-2 and therapeutic interventions that may prompt high levels of endogenous IL-2 production are being considered carefully. In fact, the tolerance-inducing aspect of IL-2 administration is now being explored for the treatment of GVHD. It has been shown that daily low-dose IL-2 can be safely administered in patients who have chronic GVHD, leading to a preferential and sustained Treg expansion in vivo and ameliorating chronic GVHD symptoms in a substantial portion of patients.[69,70] Furthermore, IL-2 remains an invaluable tool for the in vitro expansion and preparation of adoptive T-cell therapeutics.

Interleukin-7

IL-7 is produced by nonhematopoietic stromal cells and in small amounts by DCs but is not produced by T cells, B cells, or NK cells. It signals through the IL-7 receptor (IL-7R), a heterodimer consisting of IL-7Rα and the γcR, and mediates antiapoptotic and costimulatory proliferative signals through activation of phosphatidylinositol 3-kinase (P13K) and Janus kinase (JAK) signal transducers and activators of transcription (STAT), as well as antiapoptotic pathways such as B-cell lymphoma–2 (B-CL2). IL-7 is required for normal human T-cell development as demonstrated by the absence of T cells in patients with severe combined immunodeficiency as a result of mutations in the IL-7Rα or γcR, and it may play a role in normal B-cell development. Further, IL-7–mediated signaling in DCs has been implicated as a regulator of peripheral CD4+ T-cell homeostasis. Contrary to typical activation cytokines that do not act on resting T cells, IL-7 primarily affects naive T cells, which then downregulate IL-7R upon T-cell activation. An exception to this rule occurs during primary immune responses when IL-7R expression is maintained on a small subset of T cells destined to enter the central memory pool. Whereas IL-7 is continuously available in secondary lymphoid organs, plasma IL-7 is a limited resource and increases in lymphopenic states as a result of decreased use, thereby enhancing T-cell proliferation.[71] In preclinical models, IL-7 therapy augments antigen-specific T-cell responses after vaccination and adoptive cell therapy.[72,73] It also facilitates immune reconstitution in lymphopenic settings, largely via thymus-independent homeostatic expansion of peripheral T-cell populations,[74,75] and has been implicated in antagonizing inhibitory networks.[76]

In light of the preclinical promise, several clinical trials are using recombinant human IL-7 (rhIL-7) to treat patients with malignancies, chronic viral infections, and idiopathic CD4+ lymphopenia, as well as after HSCT. In the first adult human trial of patients with refractory malignancies, rhIL-7 was well tolerated apart from mild toxicities. After an initial decline in the numbers of circulating lymphocytes, IL-7 showed consistent biologic activity with substantial increases of CD8+ and CD4+ cells in the peripheral blood, as well as in spleen and lymph nodes, whereas proliferation rates of Tregs inversely correlated with IL-7 treatment. Notably, rhIL-7 preferentially induced the nonclonal proliferation of naive T cells, resulting in broadening of the TCR repertoire diversity without increasing thymopoiesis. However, in this subset of highly refractory patients, no significant antitumor effects were observed.[77] The first trial that used rhIL-7 after allogeneic HSCT was performed in recipients of T-cell–depleted grafts, given that IL-7 had boosted alloreactive T cells and GVHD in murine models.[78] IL-7 was well tolerated, with only one case of acute GVHD of the skin. In contrast to other clinical scenarios, IL-7 in the posttransplant setting preferentially expanded effector memory T cells rather than naive T cells but increased

TCR diversity and functional viral-specific T cells.[79] Further studies are needed to determine whether these effects translate clinically into reduced morbidity and mortality and protection from infection. In pediatric sarcomas, IL-7 therapy is currently being explored in the context of DC vaccination (trial NCT00923351).

Interleukin-15

IL-15 was discovered in 1994 and has pleiotropic immune-enhancing properties. It is thought to be critical for the stimulation and differentiation of NK cells, NKTs, and memory CD8+ T cells. It also promotes proliferation and differentiation of CD4+ T cells, nonmemory CD8+ T cells, and B cells; it further enhances cytolytic activity of CD8+ T cells and induces DC maturation and immunoglobulin synthesis by B cells. Although IL-15 messenger ribonucleic acid (mRNA) is expressed by a large number of tissues, the detection of IL-15 protein is largely limited to DCs and monocytes/macrophages. Free soluble IL-15 is limited, indicating tight regulation of this proinflammatory cytokine. The composition of the heterotrimeric IL-15 receptor (IL-15R) and the main signaling mechanism of IL-15 via presentation in trans are unique. IL-15 shares the β chain (IL-2Rβ/IL-15Rβ) and γcR chain with the IL-2R and signals through these domains via the JAK1/JAK3/STAT5 signaling pathway. Contrary to IL-2 signaling, where the IL-2Rα domain assembles with the IL-2Rβ and γc subunits on the recipient cell, the IL-15Rα domain is located on APCs, binds IL-15 with high affinity, and then presents IL-15Rα–bound IL-15 to the IL-15Rβ/γcR heterodimer on NK or T cells in trans. IL-15 is also thought to have minor signaling pathways that involve direct binding to the IL-15Rβ/γcR as well as binding to IL-15Rα on the target cell and presentation in cis to the IL-15Rβ/γcR on the same target cell.[80] Unlike IL-2, IL-15 does not stimulate immunosuppressive Tregs and has an antiapoptotic effect on T cells. Preclinical and clinical data suggest a dual role for IL-15. Through the link between inflammation and cancer, it has been suggested that IL-15 plays a proleukemic role in hematologic malignancies. In adults with pre–B-ALL, IL-15 expression was associated with an inferior 5-year relapse-free survival (RFS), as well as mediastinal and lymph node involvement.[81] In children without central nervous system involvement at diagnosis, high IL-15 expression levels were associated with a higher risk of central nervous system relapse.[82] A phase I clinical trial blocking IL-15 presentation in trans for patients with T-cell large granular leukemia was well tolerated but showed no responses.[83] On the other hand, evidence for a protective role of IL-15 through enhanced tumor immunosurveillance is provided with the antitumor effects of IL-15 in several preclinical tumor models.[84,85] Ongoing phase I trials for adults with advanced melanoma are investigating the administration of rhIL-15, either alone or after transfer of tumor-infiltrating lymphocytes (TILs; trials NCT01021059 and NCT01369888). IL-15 is also used for ex vivo preparation of cellular immunotherapies such as in a pediatric trial of haploidentical HSCT and IL-15–stimulated NK cells for refractory solid tumors (trial NCT01337544).

ANTIBODY-BASED IMMUNOTHERAPY

The specific recognition of surface antigens on pathogens or tumor cells was first conceptualized by Paul Ehrlich more than a century ago. In contrast to cellular immunotherapy, which often requires costly and individualized preparation, antibody-based immunotherapy is feasible for off-the-shelf use and thus is more cost effective. Antibody-based therapy may rely on recognition of TAs or interfere with tumor cell growth by disrupting negative regulatory and survival pathways as well as ligand receptor–dependent cell growth. Unconjugated antibodies predominantly function by recruiting other immune effector cells and initiating pathways such as ADCC, CDC, ADP, and direct apoptosis. Via the ADP pathway, antibodies entice APCs to take up not only the targeted antigen but also a variety of tumor contents that are processed to induce T-cell responses in MHC-restricted fashion.[86] This effect is termed the "vaccinal effect" of antibody therapy because it promotes not only immediate tumor cell death, but also vaccinelike antitumor effects with ensuing humoral and cellular immunologic memory. Clinically this effect has been shown to be relevant in solid tumors and lymphomas.[87] Although conventional antibodies activate innate effector mechanisms, they do not trigger T-cell immunity directly. The development of bispecific antibodies represents a promising approach to recruit and activate T cells directly at the site of the tumor cell by providing specificity not only to the TA but also to CD3 (Fig. 47-5).

Unconjugated Antibodies

Rituximab, a genetically engineered chimeric mouse antihuman CD20 mAb, was the first mAb to be approved by the FDA for the treatment of cancer in 1997. The murine Fab domain of rituximab binds CD20 on mature B-cell malignancies and healthy B cells, and the humanized Fc domain mediates effector functions. The mechanism of cytotoxicity is primarily via ADCC and CDC, although rituximab has also been shown to sensitize resistant lymphoma cells to chemotherapy and Fas ligand–induced apoptosis,[88] which led to its synergistic use with chemotherapy regimens in clinical practice. It may also have a vaccinal effect, leading to T-cell priming and lasting antilymphoma immunity. Rituximab is now a well-established component of therapeutic regimens for adult CD20+ NHL[89,90] and chronic lymphocytic leukemia (CLL) in the United States and Europe.[91] CD20 is expressed by almost all cases of pediatric B-cell NHL,[92] yet clinical experience with rituximab in pediatric lymphomas is still limited and in evolution. Pediatric randomized controlled trials of antibody therapy for B-cell lymphoma have not been published, but a limited number of phase I and II studies suggest a low incidence of adverse effects and encouraging response rates.[93] In a study of 38 children with stage III/IV B-cell NHL who were treated with a combination of FAB/LMB96 chemotherapy and rituximab, the 3-year event-free survival (EFS) and OS was 95%.[94] As rituximab is studied further in the pediatric population, it may improve outcomes

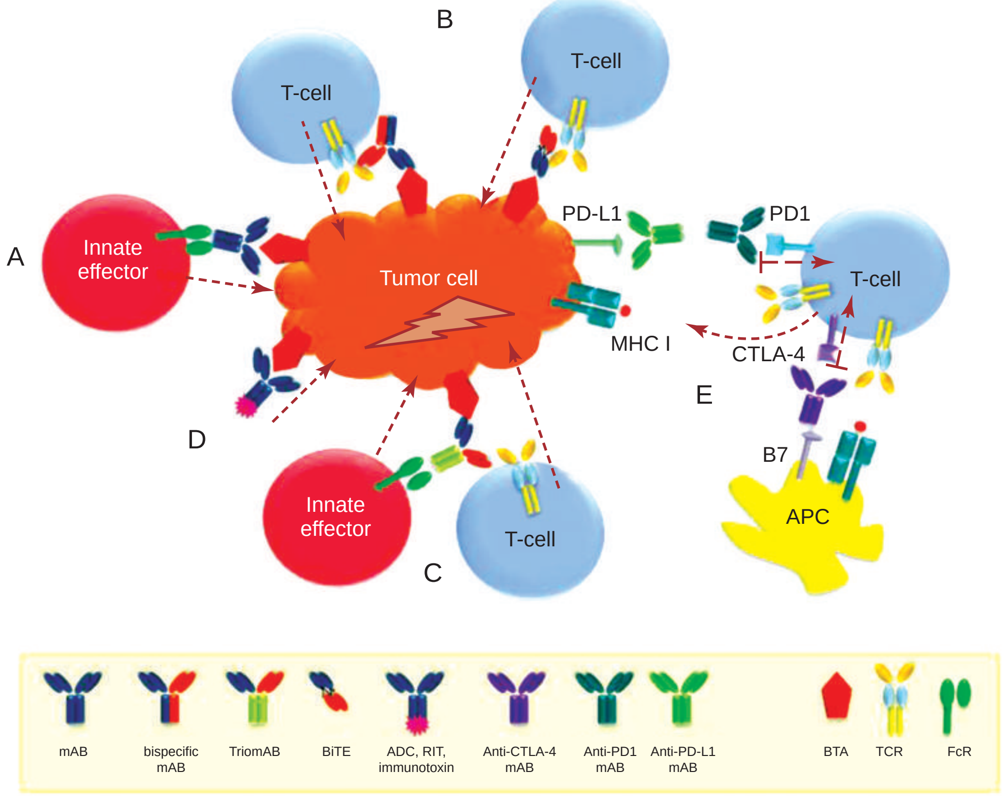


Figure 47-5 Mechanisms of antibody-based immunotherapy approaches. Various iterations of monoclonal antibodies *(mABs)* have been developed for the treatment of cancer. **A,** Unconjugated mABs bind to their target and engage effector cells (see Fig. 47-4). **B,** Bispecific mABs and bispecific T-cell engagers *(BiTEs)* are engineered to bind to the tumor antigen *(TA)* and engage cluster of differentiation (CD)3 on cytotoxic T lymphocytes (CTLs), thereby activating CTLs against tumor cells, independent of their T-cell receptor *(TCR)* specificity. **C,** Triomab antibodies *(TriomABs)* bind to TA and CD3 but also recruit natural killer cells and macrophages via their crystallizable fragment receptor *(FcR)*. **D,** Immunoconjugates deliver cytotoxic agents directly to the target cell. **E,** Antagonistic mABs to cytotoxic T-lymphocyte antigen 4 *(CTLA-4)*, programmed death–1 *(PD-1)*, programmed death–1 ligand *(PD-L1)*, or programmed death–2 ligand block ligand-receptor interactions that otherwise deliver inhibitory signals to the T cell and unleash their antitumor potential. *ADC,* Antibody drug conjugate; *APC,* antigen-presenting cell; *MHC,* major histocompatibility complex; *RIT,* radioimmunotherapy. *(Modified from Baumeister SH, Dranoff G: Principles of targeted immunotherapy. Translational cancer research: molecular therapeutics. In Kurzrock R, editor:* Targeted therapy in cancer, *Wiley Publications, Hoboken, N.J., in press.)*

both for newly diagnosed patients and patients who have had a relapse or have refractory disease, and facilitate chemotherapy regimens with reduced toxicity. Rituximab further finds application in the treatment of PTLD, either as a single agent or in combination with low-dose chemotherapy.[95] Isolated reports also indicate its utility in the treatment of pediatric paraneoplastic syndromes.[96] It should be emphasized that rituximab temporarily depletes healthy B cells, and thus patients may require immunoglobulin replacement.

Specific to the treatment of pediatric high-risk neuroblastoma is a highly effective unconjugated mAb against the disialoganglioside GD2. When immunotherapy (consisting of ch14.18 anti-GD2 mAb in conjunction with IL-2 and GM-CSF) was administered with isotretinoin after standard intensive multimodal therapy in a phase III trial, 2-year EFS increased dramatically to 66% versus 46% and OS increased to 86% versus 75%.[67]

Also noteworthy is trastuzumab, an antibody against HER2, which is approved by the FDA for the treatment of HER2-overexpressing breast cancer and gastric adenocarcinoma.

Bispecific Antibodies

Staerz and colleagues[97] first introduced the concept of bispecific antibodies to engage CTLs for cancer cell lysis. Such antibodies have dual specificity for both CD3 and the TA of interest, thereby bringing CTLs in close proximity to the tumor cell and facilitating the killing of tumor cells, regardless of the T-cell's TCR specificity. Increasingly sophisticated constructs have been developed with time. Bispecific T-cell engagers (BiTEs) can be distinguished from other bispecific constructs by their high potency at picomolar range and support of serial lysis at low effector-to-target ratios. BiTEs against multiple different target antigens including CD19, GD2, epithelial cell adhesion molecule (EpCAM), Her2/neu, epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA), CD33, ephrin type A receptor 2 (EphA2), and melanoma-associated chondroitin sulfate proteoglycan (MCSP) have been constructed. Conventional EGFR antibodies such as cetuximab can mediate an ADCC effect via FcRs but appear to act predominantly by blocking EGFR, based on analyses showing that patients with downstream mutations in *KRAS* and *BRAF* genes do not have an overall survival advantage.[98] EGFR-BiTE antibodies, however, can activate T cells via CD3 when engaging a tumor cell and have been shown to lyse even KRAS-mutated colorectal cancer cell lines.[99] EpCAM is frequently expressed on human adenocarcinomas and some squamous cell carcinomas, but also on cancer stem cells.[100,101] MT110,[102] an EpCAM BiTE, is being tested in a phase I trial for lung, gastrointestinal (GI), breast, ovarian, and prostate cancers (trial NCT00635596). Blinatumomab, a CD19 BiTE that targets B-cell malignancies, first demonstrated efficacy in persons with advanced NHL[103] and has shown promising phase II results as a single agent in adult patients with ALL who have persistence or recurrence of MRD, leading to a 80% response rate with elimination of MRD[104] and a 61% hematologic RFS at a median follow-up of 33 months.[105] Of note, some but not all of the patients who experienced a relapse had CD19-negative relapses. As expected, blinatumomab treatment resulted in B-cell depletion, but interestingly, it also resulted in a transient decline followed by a significant expansion of T cells bearing the early activation marker CD69.[106] CD4+ T cells expanded more profoundly than did CD8+ T cells, but Treg numbers generally remained low. A single-arm phase II multicenter international trial is currently evaluating blinatumomab in pediatric and adolescent patients with relapsed or refractory ALL (trial NCT01471782). In the first reported pediatric experience of compassionate blinatumomab use in patients with refractory ALL who had experienced multiple relapses after HSCT, all three patients were able to reach MRD-negative CRs, although only one patient who was able to undergo subsequent HSCT had a lasting remission.[107] From a practical standpoint, blinatumomab is administered as a continuous 24-hour infusion for 4 weeks per 6-week cycle via a portable mini pump. The most prominent adverse effects of blinatumomab include lymphopenia, pyrexia, hypogammaglobulinemia, and hypokalemia, but reversible neurologic toxicities such as seizures and ataxia have been reported. Other BiTEs such as a CD33/CD3 BiTE for AML[108] and BiTEs engaging NK cells via CD16[109] are still in preclinical development.

In yet another iteration, Triomab antibodies are specific for the TA and engage T cells via CD3, but they also recruit NK cells and macrophages via their FcR. Catumaxomab, a Triomab antibody against EpCAM, was approved in Europe in 2009 for the treatment of malignant ascites in a variety of EpCAM+ abdominal tumors, following its success in a phase II/III clinical trial.[110] Other Triomab antibodies are in phase I/II adult clinical trials, such as a CD20 Triomab antibody in the therapy of B-cell lymphoma in conjunction with donor lymphocyte infusion (DLI) after allogeneic HSCT (trial NCT01138579) and ertumaxomab, a Triomab antibody against HER2/neu+ advanced solid tumors (trial NCT01569412). An anti-GD2 Triomab antibody has thus far been tested in animal models of neuroblastoma.[111]

Immunoconjugates

Various immunoconjugates have emerged from the idea that mABs against TAs could be exploited to deliver cytotoxic agents directly to the tumor cell site and minimize systemic adverse effects. In this approach, mABs are linked to radionuclides (radioimmunotherapies), drugs (antibody-drug conjugates, or ADCs), toxins (immunotoxins), enzymes (antibody-directed enzyme prodrug therapy), and cytokines (immunocytokines) to boost the cytotoxic mAb effects on target cells. Two radioimmunotherapies targeting CD20 have been approved by the FDA for adults. Yttrium-90 ibritumomab tiuxetan, approved in 2002, is used in the therapy of follicular NHL or relapsed/refractory low-grade NHL. Iodine-131 tositumomab was approved by the FDA in 2003 and is now indicated in the treatment of CD20+ relapsed or refractory follicular or transformed NHL.[112,113]

A very promising recent ADC, brentuximab vedotin, carries the antimitotic drug monomethyl auristatin E and targets CD30, leading to tumor cell apoptosis. It has shown remarkable efficacy in adults with relapsed or refractory HL or anaplastic large-cell lymphoma (ALCL).[114-116] These results led to accelerated FDA approval for brentuximab in 2011 for the treatment of HL and ALCL; it was the first new drug to be approved for HL in more than 30 years. Brentuximab has also been used successfully prior to allogeneic HSCT, as well as for relapse after allogeneic HSCT.[117,118] Its efficacy in other CD30+ adult malignancies is being tested in the phase II trials NCT01461538 and NCT01421667, and several clinical trials are being conducted to evaluate its safety and efficacy in conjunction with combination chemotherapy as a frontline agent (e.g., trial NCT01060904). In the pediatric population, brentuximab is currently being explored in a phase I/II trial for relapsed or refractory ALCL and HL (trial NCT01492088), but successful single-agent remission induction in a pediatric patient with ALCL who has experienced multiple relapses has been reported.[119] The Children's Oncology Group is planning to evaluate inclusion of brentuximab in the upfront therapy of newly diagnosed patients with classic HL across all risk strata in an effort to improve initial response and minimize radiotherapy, as well as to evaluate administration of brentuximab in conjunction with gemcitabine for relapsed/refractory HL.[120] Cooperative groups are also pursuing evaluation of brentuximab vedotin in combination with standard chemotherapy in children with ALCL.[121] Based on CD30 expression in patients with GVHD,[122] brentuximab is also being investigated as an agent to treat and prevent GVHD (trials NCT01596218 and NCT 01700751).

Trastuzumab emtansine most recently gained FDA approval in 2013 for the treatment of Her2+ metastatic breast cancer. This ADC combines the Her2-targeting antitumor properties of the mAb trastuzumab with the cytotoxicity of the microtubule inhibitory agent DM1, a derivative of maytansine. In a randomized international phase III trial it showed significantly prolonged PFS with less toxicity compared with a standard chemotherapeutic regimen[123] and further compared favorably to trastuzumab and docetaxel in a phase II trial of patients with metastatic breast cancer who had progressed after receiving Her2-targeted therapy with trastuzumab.[124] This drug has not been evaluated in children but may hold promise, given reports of Her2 expression in subgroups of patients with osteosarcoma,[125] Ewing family tumors,[126] neuroblastic tumors, and some Wilms tumors.

Gemtuzumab ozogamicin (GO) is a humanized anti-CD33 mAb conjugated with the anthracycline calicheamicin. It targets CD33, which is expressed in more than 80% of patients with AML. In phase II results of adults older than 60 years who had their first relapse, GO as a single agent was associated with a 30% overall response rate,[127] leading to accelerated FDA approval in 2000. Other randomized phase II trials comparing GO with induction chemotherapy and chemotherapy alone confirmed these results.[128,129] However, a large phase III study evaluating the effects of GO in combination with chemotherapy versus standard induction therapy in adults younger than 60 years with newly diagnosed AML failed to show superiority of the GO arm with respect to overall efficacy, RFS, and OS and demonstrated increased induction mortality in the GO arm.[130] Based on results from this study and concerns about high frequencies of hematologic and liver toxicity, approval for GO was voluntarily withdrawn in 2010. Since then, resurrection of GO has been widely discussed, citing the exceptionally low induction mortality of 1% in the control arm of the aforementioned study and typical induction mortality of 5% in the GO arm, as well as a lower dose of daunorubicin in the GO arm, complicating direct comparison of both groups.[131] Several other large randomized trials in adults with newly diagnosed AML have shown no difference in remission rates but a significantly higher EFS and OS in patients receiving GO in conjunction with chemotherapy, with particular benefit for younger adults who have favorable cytogenetics.[132,133] Furthermore, trials using lower dose GO either at single or fractionated dosing no longer showed an increase in hepatotoxicity or veno-occlusive disease.[133] In pediatric patients, an increased incidence of veno-occlusive disease was also limited to high-dose GO, and a phase I study of GO in combination with busulfan and cyclophosphamide conditioning for 12 children with CD33+ AML showed no GO-associated dose-limiting toxicities.[134] CD33 expression in childhood AML is heterogeneous, but high CD33 expression is associated with adverse disease characteristics such as fms-related tyrosine kinase 3 internal tandem duplication (FLT3-ITD) and independently predicts poor outcome.[135] Initial phase I and compassionate use data suggested efficacy of GO monotherapy in some patients,[136,137] and subsequent studies established that combination of GO with chemotherapy was well tolerated in the pediatric population.[138,139] In a study that administered chemotherapy in combination with GO to patients with poor end-induction MRD responses, 27 of 29 patients had a reduction in MRD and 13 of 29 patients became MRD negative, including 4 of 8 patients who had MRD levels greater than 25%.[140] However, no published pediatric trials have compared GO with chemotherapy versus chemotherapy alone in a randomized fashion.

Inotuzumab ozogamicin is a calicheamicin-linked mAb directed against CD22, a marker that is highly expressed in ALL. It has been evaluated in a phase II trial for relapsed or refractory adult ALL with promising responses and in a phase I/II trial for adults with relapsed NHL, where it showed high response rates and PFS in combination with rituximab.[141] It is currently undergoing further evaluation in several adult trials. No pediatric clinical trials have been conducted thus far, although preclinical studies suggest sensitivity of pediatric B-ALL blasts to inotuzumab ozogamicin in vitro.[142]

Within the group of immunotoxins, BL22 is an antibody that is bound to a modified Pseudomonas exotoxin and directed against CD22. A phase I trial of BL22 in pediatric patients with relapsed or refractory CD22+ ALL or NHL had an acceptable safety profile but did not show any responses, leading to an ongoing phase I trial of a second-generation antibody, CAT-8015, with improved binding affinity and in vitro cytotoxicity (trial NCT00659425). BL22 has shown promise in phase II trials for the treatment of hairy cell leukemia in adults,[143] and CAT-8015 is also being investigated in a phase I trial. Neutralizing antibody responses to BL22 have been documented in some patients and present an obstacle to retreatment.

Antibodies in the Modulation of Regulatory Pathways

During a physiologic T-cell response, the balance between costimulatory and inhibitory signals (so-called immune checkpoints) is critical to allow for efficient T-cell activation while limiting the duration and amplitude of T-cell responses, thereby protecting against chronic inflammation and autoimmunity.[144] CTLA-4 and PD-1 are examples of inhibitory receptors that are upregulated by T cells during the course of an immune response and inhibit T cells upon binding to their respective ligands. In the process of immunoediting, tumor cells or nontransformed cells in the tumor microenvironment express inhibitory ligands to escape immune surveillance.[145] However, given that many of these immune checkpoints are mediated by ligand-receptor interactions, they lend themselves to therapeutic blockade with mABs. In contrast to TA-specific mABs, such mABs do not target the tumor but rather target lymphocyte receptors or their ligands to unleash intrinsic antitumor activity.

CTLA-4 was the first inhibitory receptor to be clinically targeted. CTLA-4 is upregulated on activated T cells, where it dampens T-cell activation upon engagement with the B7 (CD80/CD86) receptors on DCs. Although expressed by CD8+ T cells, its major impact is the inhibition of CD4+ helper T cells and enhancement of the activity of Tregs, which express CTLA-4 constitutively.[146] Although the exact mechanism by which CTLA-4 enhances Treg function is not known, numerous studies have documented that the ratio of effector T cells to Tregs in the tumor microenvironment plays a crucial role in the control of tumors and correlates with outcome.[147,148] After demonstration of significant tumor regressions by single-agent anti-CTLA-4 mAb in conjunction with GVAX by Leach et al.[149] and van Elsas et al.,[150] two fully humanized anti-CTLA-4 mABs, ipilimumab and tremelimumab, entered phase I clinical trials in 2000. These mABs demonstrated ~10% objective response rates in patients with refractory metastatic melanoma who received either GVAX[151] or gp100 peptide vaccines.[152] The initial phase III trial with tremelimumab plus dacarbazine compared with dacarbazine alone did not show benefit at the dose used,[153] but a later trial comparing ipilimumab plus dacarbazine versus dacarbazine plus placebo showed improved outcomes with ipilimumab.[154] After a phase III trial with ipilimumab alone or combined with a gp100 peptide vaccine versus gp100 alone, which demonstrated a survival benefit in both ipilimumab groups, ipilimumab was approved in 2011 by the FDA for use in persons with advanced melanoma.[155] Up to 30% of patients may experience severe immune-related adverse effects (the most common being colitis) with ipilimumab, which modestly correlate with response. Steroids and TNF blockade have been used in clinical management; some adverse effects such as hypophysitis may be long lasting or irreversible, although they are readily managed with hormone replacement therapy. Trials in several adult malignancies are assessing Ipilimumab alone or in combination regimens such as with chemotherapy (trials NCT01331525 and NCT01473940), in combination with GM-CSF (trial NCT 01530984), or with androgen ablation in persons with prostate cancer (trial NCT 01377389). The necessity for rigorous evaluation of novel combination regimens is highlighted by significant hepatotoxicity observed during concurrent administration of ipilimumab and the BRAF inhibitor vemurafenib.[156] In the pediatric population, a first phase I trial is underway to evaluate ipilimumab in the treatment of refractory solid tumors and lymphoma (trial NCT01445379).

The PD-1/PD-L1 axis has been another focus of clinical investigation. PD-1 is expressed by activated T cells, B cells, and NK cells. PD-1 binding to its ligands PD-L1 or PD-L2 (expressed on DCs and in peripheral tissues) inhibits kinases that are involved in T-cell activation. Furthermore, PD-1 is expressed on Tregs, where it enhances proliferation upon PD-1 engagement with its ligand.[157] Whereas CTLA-4 regulates the immune response at the time of T-cell activation in the lymphoid organs, PD-1 primarily limits the activity of T cells in peripheral tissues. PD-1 ligands are upregulated on the tumor cell surface and on myeloid cells in the tumor microenvironment of a variety of human malignancies, where they mediate immune resistance.[145,158,159] Studies in mouse models demonstrate enhanced antitumor immunity through blockade of PD-1 or its ligands, while suggesting a favorable safety profile compared with CTLA-4 based on a comparatively mild phenotype of PD-1, PD-L1, and PD-L2 knockout mice. The first phase I trial with a single-dose fully human anti–PD-1 mAb yielded promising results, including one complete response.[160] A subsequent phase I trial evaluating sequential treatment with an anti–PD-1 mAb in adults with a variety of malignancies demonstrated quite durable objective response rates of 36% in patients with PD-L1+ tumors and a safety profile comparable with or better than that of ipilimumab.[161] Investigators of a concurrent phase I trial exploring blockade of PD-L1 rather than PD-1 reported an objective response rate of 6% to 17% depending on the underlying malignancy but did not consider PD-L1 expression in the tumor.[162] Ongoing phase I/II trials are investigating blockade of PD-1/PD-L1 in various malignancies either alone (trial NCT01354431); with ipilimumab (trial NCT01024231); with a gp100, MART-1, and NY-ESO-1 peptide vaccine (trial NCT01176461); or in conjunction with chemotherapeutic agents (trial NCT01313416). Several other immune-checkpoint molecules are emerging as candidates for therapeutic blockade, such as lymphocyte-activation gene 3 (*LAG3*), 2B4, B- and T-lymphocyte attenuator (BTLA), T-cell immunoglobulin and mucin domain 3 (TIM3), A2aR, and KIR. Anti-KIR mABs aim to interfere with the interaction of inhibitory KIR receptors on NK cells and their ligands to create a missing-ligand situation and induce NK-cell–mediated tumor killing. Results from two phase I trials in adults with multiple myeloma and AML have determined safe and tolerable doses at which inhibitory KIR saturation can be achieved, and RFS in patients with AML compared favorably with that in historic control subjects.[163,164]

The stimulation of activating costimulatory pathways such as 4-1BB and inducible costimulator (ICOS) by way of therapeutic mAb administration is another strategy undergoing evaluation in early trials.[165]

ADOPTIVE CELLULAR IMMUNOTHERAPY

GVL Effect in HSCT and Donor Lymphocyte Infusions

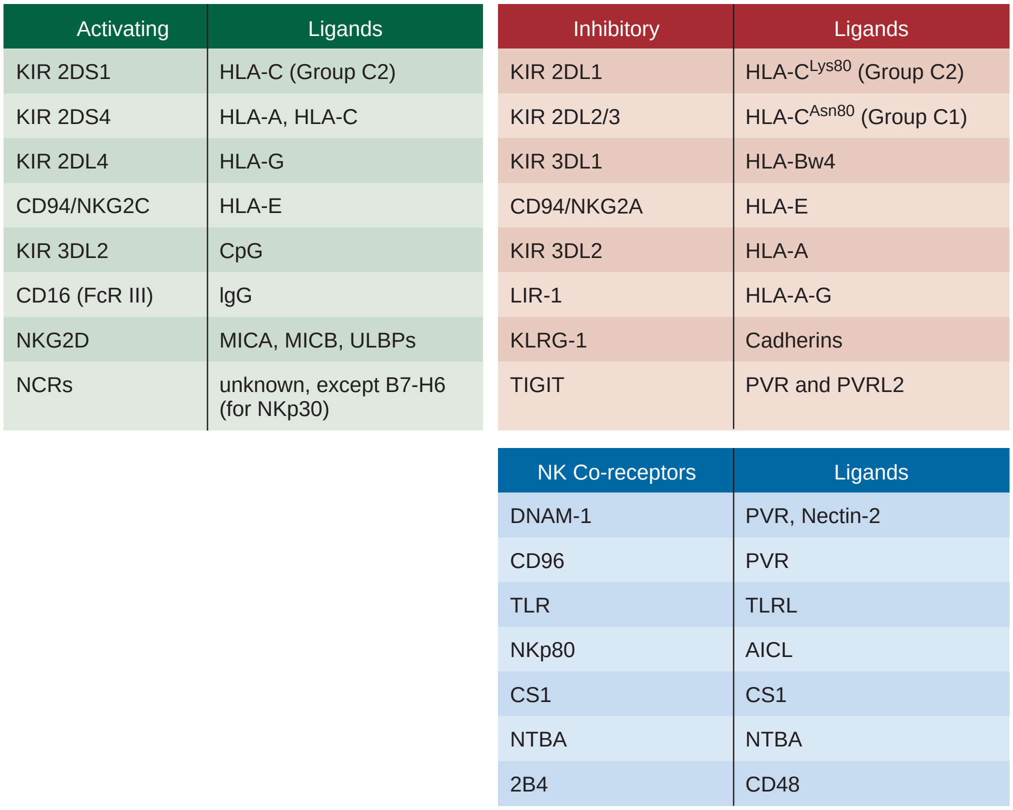
Allogeneic HSCT is a well-established curative treatment, particularly for hematologic malignancies, and immune-mediated antileukemia effects are increasingly recognized as central to its therapeutic benefit. Myeloablative preparative chemotherapy regimens with or without total body irradiation are aimed at eradicating residual tumor cells and suppressing host immunity to prevent rejection of donor HSCs. However, patients can also be cured with reduced-intensity conditioning regimens, which facilitate engraftment but may not lead to complete eradication of leukemia cells. This outcome attests to the importance of immune recognition and eradication of residual tumor cells by donor cells in a process termed GVL, which is critical for long-term cure. Evidence of the GVL effect was provided by polymerase chain reaction–based evaluation of *BCR/ABL* transcripts in 92 patients with CML, of whom 80% to 83% still had detectable *BCR/ABL* transcripts within 6 months after transplant. Six to 24 months after transplant, 80% to 88% of patients who had received a T-cell–depleted transplant remained PCR positive compared with 26% to 30% who had received unmodified marrow.[166] Although a patient's own immune cells may have been rendered tolerant to their cancer, donor cells can recognize tumor cells through expression of TAs and mHAs. Donor and recipient are maximally matched for the HLA loci on chromosome 6 to minimize GVHD, but immune recognition of mHAs encoded by genetic polymorphisms throughout the human genome can occur.[167] Initial evidence for GVL was provided by reports that recipients of syngeneic stem cells were more likely to relapse than were HLA-matched allogeneic recipients, in whom GVHD developed.[168] Subsequently a sizable body of clinical literature established that patients experiencing GVHD were less likely to relapse, that leukemia remissions could occur in association with worsening GVHD[169] and after stopping immunosuppressive GVHD prophylaxis,[170] and that T-cell depletion of grafts was associated with higher relapse rates in persons with myeloid leukemias.[171]

Kolb and colleagues.[172] first showed that DLIs could mediate a GVL effect without additional chemotherapy or radiation, and that they induced disease remissions in patients with relapsed CML after allogeneic HSCT. The use of DLI has been less effective for patients with acute leukemias after HSCT, with CR rates ranging from 5% to 18% and 15% to 29% for ALL and AML, respectively, compared with 60% to 80% for CML.[173,174] This finding can be partially explained by higher disease burden and rate of leukemic growth, but for reasons that are not well understood, myeloid leukemias overall appear more amenable to DLI. Limited data on the efficacy of DLI are available in the pediatric population, but responses have been reported in patients with pediatric AML[175] and juvenile myelomonocytic leukemia.[176] Importantly, toxicities from DLI are not trivial and include GVHD and marrow aplasia, with a treatment-related mortality rate estimated in the 12% to 22% range.[177-179] Unfortunately, the most efficient GVL responses often arise in the setting of GVHD. Therefore efforts have focused on dissecting the immunologic mechanisms of GVL and exploiting the GVL effect while preventing GVHD. Identification of tissue-restricted expression of antigens targeted by GVL versus GVHD has been key in this endeavor. As shown in a phase I trial, adoptive transfer of T cells specific for mHas is feasible, but restricted expression of the mHA on tumor or hematopoietic cells is crucial for a favorable toxicity profile.[180,181] Ongoing trials are exploring the infusion of donor lymphocytes specific for Wilms tumor–1 protein,[182,183] a transcription factor that is overexpressed in leukemias, as a directed DLI strategy (trials NCT00620633 and NCT00608166). Genomic approaches are likely to identify previously unknown mHAs that could be targeted.[184] Another area of interest concerns the role of alloreactive NK cells, which mediate a GVL effect and are intriguing because they do not cause GVHD.

Adoptive Therapies Exploiting NK-Cell Alloreactivity

NK cells are innate effector cells capable of rapid killing via degranulation and release of toxic granules in an early defense against malignant transformation. NK cells facilitate stem cell engraftment, fight infection, and control cancer, yet they do not cause GVHD. NK-cell alloreactivity is an important concept in haploidentical HSCT and allogeneic NK-cell therapies.[185] Human NK-cell activity is regulated by a complex system of inhibitory and activating receptors. One of the key regulators is the polymorphic KIR gene family. KIRs can be activating or inhibitory in nature and are named on the basis of their structure. This naming system distinguishes between the number of immunoglobulin-like domains (e.g., KIR*2D* vs. KIR*3D*), as well as *l*ong and *s*hort tails (KIR2D*L* vs. KIR2D*S*), and it numbers the different receptors within the structural group in order of discovery (KIR2DL*1* vs. KIR2DL*2*). With the exception of KIR2DL4, KIRs with long tails are generally inhibitory, whereas KIRs with short tails are activating in nature (Fig. 47-6). Three levels of KIR diversity exist: gene content, allele, and expression. A unique feature of the human KIR system is the existence of two distinctive groups of gene-content haplotypes that were identified in population studies. Group A haplotypes have a simple and constant gene content dominated by inhibitory KIRs, whereas group B haplotypes are characterized by a higher content of activating KIRs and greater gene content and variability. KIR distribution along the centromeric and telomeric motifs of the haplotype further plays a role in the ability to regulate NK-cell activity (Fig. 47-7).[186] Substantial allelic polymorphism occurs within the haplotypes, and KIR genotypic alleles are numbered analogously to HLA alleles (e.g., KIR2DL1*001 vs. KIR2DL1*002). Lastly, the expression level of each of the inherited KIR genes may vary greatly, because KIRs are clonally distributed on NK cells and individual cells express different sets of inhibitory or activating KIRs.[187] This situation illustrates why both genotyping and cell surface phenotyping are needed to accurately predict NK-cell alloreactivity between donor and recipient. Eight of the KIR family members are known to recognize MHC-I molecules, including HLA-A, HLA-B, HLA-C, and HLA-G.[188] NK cells are further regulated by a variety of other receptors, many of which recognize stress ligands that are upregulated by tissues as a signal for inflammation, infection, DNA damage, and malignant transformation (see Fig. 47-6).

An important concept of NK-cell biology is the "licensing" of NK cells. Although the exact mechanisms of licensing are yet to be fully understood, licensing provides a scientifically based concept of how NK cells maintain the balance between self-tolerance and the ability to respond to infection and malignant transformation.[189] Licensed NK cells have a matching HLA ligand for their inhibitory KIR receptor(s), which "educates" them with respect to ligand expression on healthy tissues. In steady state, ligation of the inhibitory KIR prevents them from attacking healthy tissues. However, "understanding" what normal ligand expression looks like, it renders them functional for attack in a missing ligand situation (either due to HLA class I downregulation in the tumor microenvironment or in an allogeneic setting where the recipient does not express the corresponding HLA ligand) or when tissues upregulate stress ligands that trigger activating NK-cell receptors. Unlicensed NK cells express a certain inhibitory KIR in the absence of its cognate ligand, which is possible because KIR and HLA genes are located on separate chromosomes and segregate independently. Because the ligand is lacking, unlicensed NK cells are "uneducated" and functionally are considerably less responsive in steady state.[190] However, when stress ligands are upregulated and deliver a signal to activating NK-cell receptors, unlicensed NK cells more readily mediate cytotoxicity than licensed NK cells because they do



Activating	Ligands
KIR 2DS1	HLA-C (Group C2)
KIR 2DS4	HLA-A, HLA-C
KIR 2DL4	HLA-G
CD94/NKG2C	HLA-E
KIR 3DL2	CpG
CD16 (FcR III)	IgG
NKG2D	MICA, MICB, ULBPs
NCRs	unknown, except B7-H6 (for NKp30)

Inhibitory	Ligands
KIR 2DL1	HLA-C$^{\mathrm{Lys80}}$ (Group C2)
KIR 2DL2/3	HLA-C$^{\mathrm{Asn80}}$ (Group C1)
KIR 3DL1	HLA-Bw4
CD94/NKG2A	HLA-E
KIR 3DL2	HLA-A
LIR-1	HLA-A-G
KLRG-1	Cadherins
TIGIT	PVR and PVRL2

NK Co-receptors	Ligands
DNAM-1	PVR, Nectin-2
CD96	PVR
TLR	TLRL
NKp80	AICL
CS1	CS1
NTBA	NTBA
2B4	CD48

Figure 47-6 Overview of the receptors governing natural killer-cell activity and their ligands. Natural killer *(NK)* cell surface receptors are classified based on their function as inhibitory receptors, activating receptors, or activating coreceptors. Several other receptors are involved in the regulation of NK-cell activity but are not depicted here. *AICL,* Activation-induced C-type lectin; *CD,* cluster of differentiation; *CpG,* CpG oligodeoxynucleotide; *HLA,* human leukocyte antigen; *IgG,* immunoglobulin G; *KIR,* killer cell immunoglobulin-like receptor; *KLRG-1,* killer cell lectin-like receptor subfamily G, member 1; *LIR,* inhibitory leukocyte immunoglobulin-like receptor 1; *MICA,* MHC class I–related chain A; *NCR,* natural cytotoxicity receptors; *NK,* natural killer; *NTBA,* NK-T-B antigen; *PVR,* poliovirus receptor; *TLR,* toll-like receptor; *TIGIT,* T cell immunoreceptor with Ig and ITIM domains.

not have to overcome inhibition via inhibitory KIRs (Fig. 47-8).[191]

Approximately 60% of persons have inhibitory KIRs for which they lack the cognate HLA class I ligands, and they may possess substantial numbers of unlicensed NK cells. This situation has been associated with autoimmune disease, protection from infection and cancer, and pregnancy outcome, but it may also predict the immunologic response to therapy. In an analysis of KIR-HLA immunogenetics in patients with high-risk neuroblastoma who were undergoing multimodal therapy, including autologous stem cell transplant (ASCT) and anti-GD2 mAb therapy, the presence of unlicensed NK cells was associated with significantly improved OS and reduced cumulative incidence of progression.[192] The mechanism of this benefit appeared to be due to enhanced ADCC by unlicensed NK cells during the anti-GD2 mAb therapy rather than ASCT.[193]

The importance of human NK cells in cancer surveillance was documented in an epidemiologic study by Imai and colleagues,[194] and the initial model of NK-cell alloreactivity in the HSCT setting was proposed by Valiante and Parham[195] in 1997. Ruggeri et al.[196] first reported that the KIR ligand was an important factor in determining AML relapse risk after haploidentical HSCT. These investigators demonstrated that the probability of relapse at 5 years was 0% in 20 patients with a KIR-ligand incompatible donor, compared with 75% in 37 patients with a KIR-ligand compatible donor,[196] and later consolidated similar results in a larger cohort.[197] A plethora of clinical studies have since been conducted in the sibling donor, URD, and cord blood (CB) setting with conflicting results. As succinctly reviewed by Leung,[188] several studies showed no survival benefit or even inferior survival benefit in patients transplanted with a mismatch in the KIR/KIR-ligand system. This finding may in part be related to differences in T-cell alloreactivity and the definition and use of “KIR-mismatch” models, which varies widely in different studies.[188] The ligand-ligand mismatch model is based on the “missing-self” hypothesis, in which presence of the corresponding HLA ligand prevents NK-cell alloreactivity, whereas absence of the ligand in the HSCT recipient triggers attack by the NK cell. The receptor-ligand mismatch model takes into account that a “missing-self” ligand may go unnoticed if donor NK cells do not express the cognate KIR for this ligand and thus assesses not only the HLA-ligand repertoire in the recipient but also the donor KIR genotype and phenotype. The KIR-haplotype model is based on the presence or absence of a B-KIR haplotype in the donor. Likely as

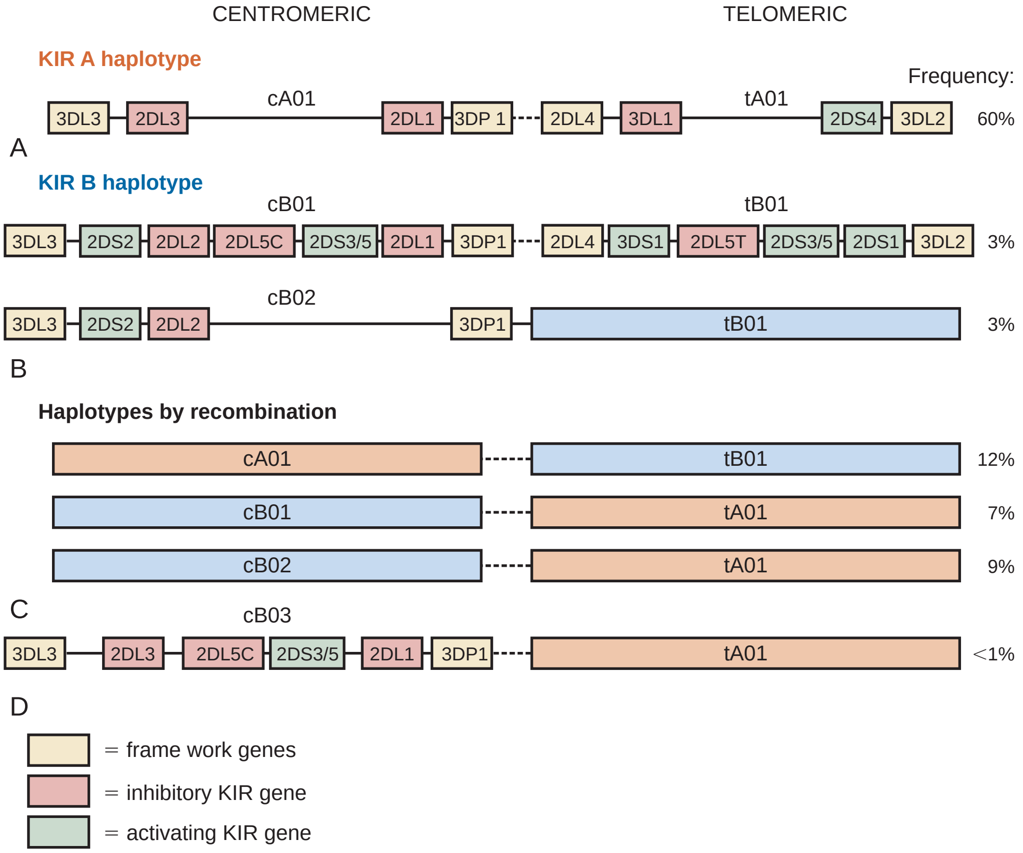


Figure 47-7 Simplified genomic map of killer cell immunoglobulin-like receptor. The human killer cell immunoglobulin-like receptor (KIR) locus maps to chromosome 19 and contains a family of highly polymorphic genes. Genetic diversity in the human KIR gene family arises from two factors: variability in KIR gene content and allelic polymorphism. Based on the combination of both, most unrelated individuals differ in KIR genotypes. A unique feature of the human KIR system is the existence of two distinctive groups of haplotypes (A and B). **A,** Approximately 60% of persons have "A" haplotype (cA01/tA01), with a simple and constant gene content, dominated by genes encoding inhibitory receptors. **B,** Group "B" haplotypes have variable and greater gene content involving genes with distinctive inhibitory receptors and a higher content of activating KIRs. The KIR haplotype model is founded on the notion that a higher number of activating KIR genes (B haplotype) in the donor increases the potential for alloreactivity and prevents relapse. **C,** Through recombination of a centromeric and telomeric cluster, various other haplotypes with "B" content have evolved, and their relative frequencies in a reference population are indicated. **D,** cB03 is an unusual B haplotype that has only been found in individuals of African descent. It contains all "A" haplotype genes but also a "B" fragment. *(Modified from Leung W: Use of NK cell activity in cure by transplant.* Br J Haematol *155:14–29, 2011.)*

a result of the fact that the B haplotype carries more activating genes than does the A haplotype, it was shown that transplants from donors with the B haplotype (and specifically centromeric motifs) reduced the risk of leukemia relapse (Fig. 47-9).[198-200]

Given that ligand-ligand mismatch always equates to the presence of a major HLA class I mismatch at the antigen level, it is not surprising that such mismatch leads to significant T-cell alloreactivity and poor survival unless it is minimized by T-cell depletion (which is standard in haploidentical SCT) or in single-unit cord blood transplantation (CBT), where up to two HLA class I mismatches are acceptable and GVHD is generally lower. Presence of T cells in the graft also alters NK-cell reconstitution, leading to lower KIR expression.[201] Differences in T-cell depletion, conditioning regimens, the source and number of stem cells, the type of GVHD prophylaxis, and the clinical status of the patient population may further explain the discrepancies between different studies. Additional factors add to the complexity of the system: Activating KIRs and specifically KIR2DS1 play a substantial role in mediating alloreactivity,[202] and in adults with AML undergoing URD HSCT, those with donors who were positive for KIR2DS1 had lower rates of relapse.[203] Killing of target cells also depends on the density of KIR-independent activating receptors on NK cells and the expression of their ligands on malignant cells. Lastly, haploidentical grafts from the mother rather than the father have been shown to confer a survival advantage, whereas donor sex did not matter in haploidentical sibling HSCT.[204] Careful consideration of all factors and a more unified approach toward prospective donor selection is poised to shed light on this complicated and fascinating field. Donor selection may include consideration of the degree of receptor-ligand KIR mismatch, origin from the mother versus father, B-haplotype content, expression of specific donor-activating KIRs such as KIR2DS1, size of the alloreactive donor NK-cell pool, and in vitro cytolytic activity.

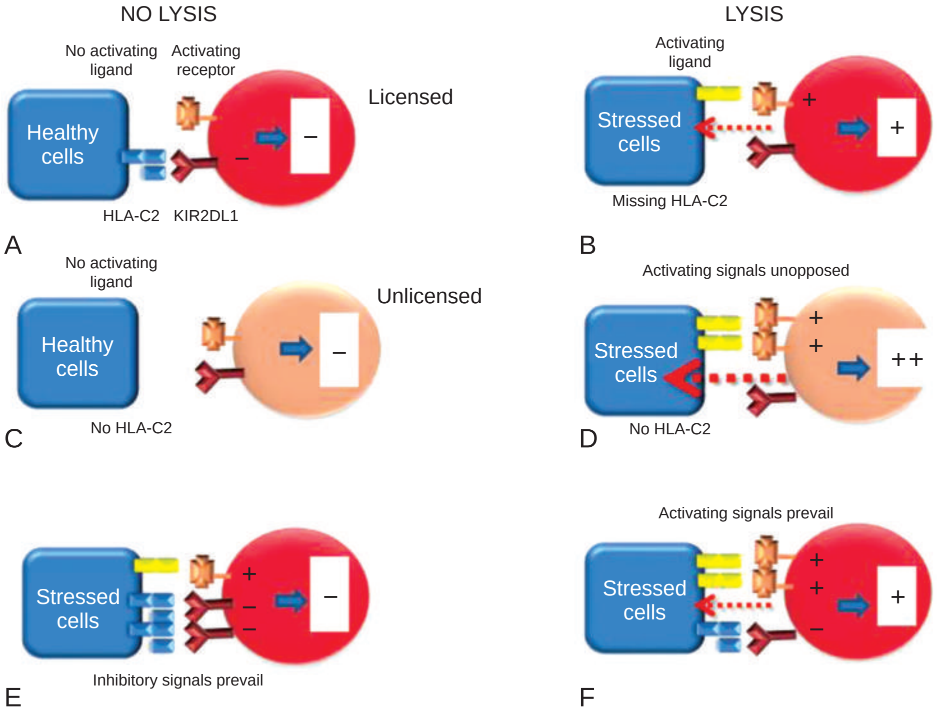
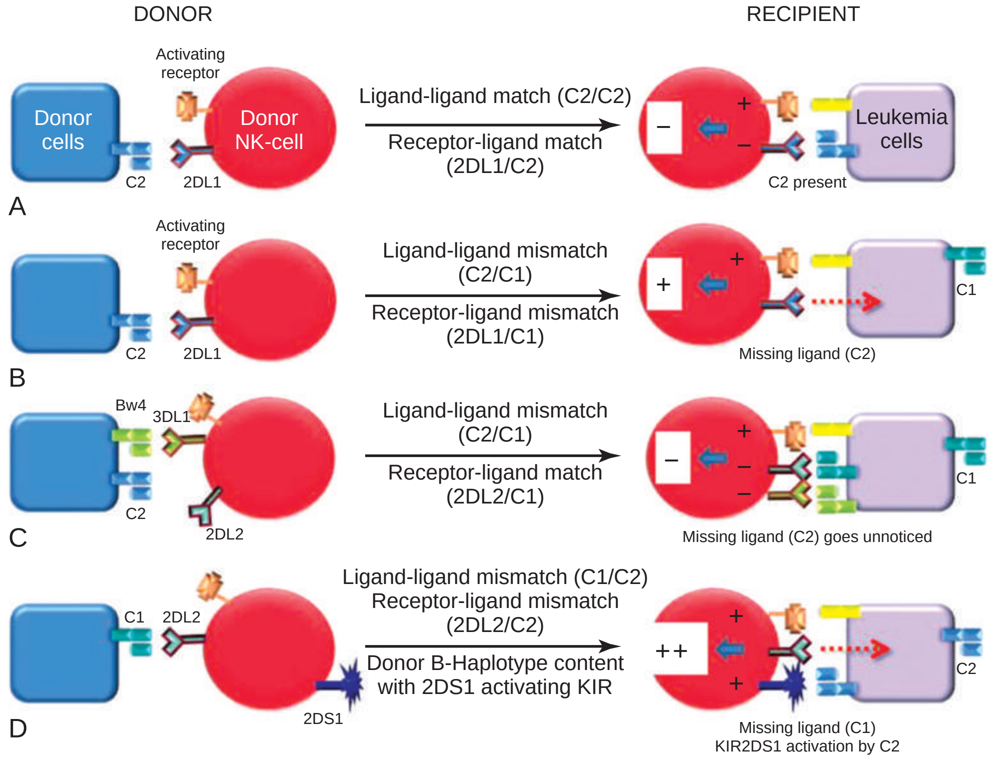


Figure 47-8 Natural killer cell reactivity is regulated by the balance of inhibitory and activating natural killer cell receptors. **A,** The presence of the appropriate ligand for an inhibitory killer cell immunoglobulin-like receptor (KIR) in an individual "licenses" the natural killer (NK) cell but delivers an inhibitory signal to the cell. **B,** If the cognate ligand is downregulated (such as in the tumor microenvironment of the same individual) or absent (in a ligand-negative allogeneic recipient), but activating ligands are expressed, NK-cell mediated killing takes place. **C,** Because of independent segregation of human leukocyte antigen (HLA; chromosome 6) and KIR (chromosome 19), an individual may lack the cognate ligand for its inhibitory receptor (autologous KIR-HLA mismatch). This scenario results in unlicensed NK cells that are hyporesponsive at baseline. **D,** However, in the presence of activating ligands, unlicensed NK cells are able to mediate killing much more readily than licensed NK cells because they do not have to overcome inhibitory signals. **E,** In contrast, licensed NK cells have to overcome inhibitory signals and will only mediate killing if activating signals prevail (**F**).

NK-cell alloreactivity is actively explored in pediatric trials by various strategies. Haploidentical HSCT involves a myeloablative or reduced-intensity conditioning regimen followed by a T-cell–depleted hematopoietic stem cell (HSC) graft from a haploidentical donor. Advantages of this approach include optimization of NK-cell alloreactive effects, immediate donor availability if no matched sibling is identified, and availability of the donor for post-transplant cellular therapies. With advances in graft manipulation and cell dose, increasingly promising results have been reported in children with malignant and benign hematologic disorders[205-208] and have led to its exploration as a treatment strategy for refractory solid tumors.[209] Novel approaches, such as use of αβ-TCR negative selection aimed at facilitating rapid engraftment and immunity by maintaining NK cells and γδ–T cells in the graft are currently being tested (trial NCT01810120). Haploidentical NK-cell transplantation as a consolidation strategy in pediatric AML (trial NCT00187096) and in relapsed or refractory neuroblastoma (trial NCT00698009) is to be distinguished by administration of a nonmyeloablative immunosuppressive regimen followed by haploidentical KIR-mismatched NK-cell infusion devoid of HSCs.[210] Yet another approach is the infusion of haploidentical KIR-mismatched NK cells without a preparative regimen after ASCT for hematologic malignancies in an effort to minimize relapse risk (trial NCT00660166).

Adoptive Therapy Using Cytokine-Induced Killer Cells

Cytokine-induced killer cells (CIKs) are ex vivo–activated cytotoxic lymphocytes characterized by a CD3+ CD56+ phenotype, acquisition of NK-cell properties, and marked upregulation of NKG2D. They can be generated from bone marrow, CB, or peripheral blood mononuclear cells by sequential addition of IFN-γ, OKT-3 (an anti-CD3 agonistic antibody), and IL-2 or IL-15.[211] Although CIKs are capable of specific MHC-restricted recognition, they can lyse a broad array of tumor targets in a non–MHC-restricted, NKG2D-dependent fashion without displaying significant alloreactive potential that could lead to GVHD.[212] Clinical experience with CIKs is still limited. Several small phase I trials demonstrated feasibility and a relatively low incidence of GVHD when donor-derived CIKs were administered to relapsed patients after allogeneic HSCT, although modest clinical benefit was limited to a few patients.[213-215] A study of CIKs in the


DONOR
RECIPIENT
Activating receptor
Donor cells
Donor NK-cell
C2
2DL1
Ligand-ligand match (C2/C2)
Receptor-ligand match (2DL1/C2)
−
+
−
Leukemia cells
C2 present
A
Activating receptor
C2
2DL1
Ligand-ligand mismatch (C2/C1)
Receptor-ligand mismatch (2DL1/C1)
+
+
C1
Missing ligand (C2)
B
Bw4
3DL1
C2
2DL2
Ligand-ligand mismatch (C2/C1)
Receptor-ligand match (2DL2/C1)
−
+
−
−
C1
Missing ligand (C2) goes unnoticed
C
C1
2DL2
2DS1
Ligand-ligand mismatch (C1/C2)
Receptor-ligand mismatch (2DL2/C2)
Donor B-Haplotype content with 2DS1 activating KIR
++
+
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C2
Missing ligand (C1)
KIR2DS1 activation by C2
D

Figure 47-9 Natural killer cell alloreactivity. **A,** If donor and recipient have matching killer cell immunoglobulin-like receptor *(KIR)* ligands (C2/C2), no natural killer (NK) cell alloreactivity is expected. **B,** If the donor has a KIR ligand (C2) that is absent in the recipient (no C2; recipient has C1), lysis of recipient cells occurs, which is the basis for the ligand-ligand mismatch model. **C,** However, depending on the donor's KIR genotype and/or phenotype, the KIR for the recipient's "missing ligand" may have no or very low expression on donor NK cells. In such event, the absence of C2 in the recipient may not be noticed. This scenario forms the basis of the receptor-ligand model, which considers KIR receptors on the donor's NK cells and KIR ligands on the recipient cells, rather than respective ligands alone. **D,** Example of a scenario with optimal donor NK-cell reactivity: The donor has a strong inhibitory KIR allele (KIR2DL2) and the NK cell is licensed by recognition of KIR2DL2's cognate ligand C1 in the donor. C1 is absent in the recipient, and this missing-ligand situation is recognized by the licensed NK cell. The donor carries a B haplotype containing the strong activating KIR2DS1. KIR2DS1 may also activate the NK cell by engaging its ligand C2 on the recipient leukemia cells.

treatment of relapsed adult HL and NHL after ASCT demonstrated partial responses in select patients.[216] Pediatric patients with relapsed AML or sarcomas after haploidentical HSCT have also been reported to have received CIKs safely, but with limited efficacy in these heavily pretreated patients.[211] Novel preclinical approaches that may improve efficacy include introduction of CARs into CIKs as a mode of redirection against surface markers such as CD33 and CD123 for the treatment of AML.[217]

Adoptive T-Cell Therapy

The potential therapeutic role of lymphocytes was first revealed by studies of melanoma showing that the isolation, expansion, and adoptive transfer of TILs back into the patient[218] could result in significant clinical response rates.[219,220] Adoptive transfer of autologous TILs also showed promise in pilot clinical trials for ovarian cancer.[221,222] However, the isolation of TILs is labor intensive and technically difficult, and TILs cannot be obtained in all patients. Morgan et al.[222] developed a strategy to overcome this problem by cloning the α and β chains of a TCR recognizing the MART-1 TA from a patient with metastatic melanoma who demonstrated near-complete regression after adoptive transfer of TILs. This strategy permitted adoptive T-cell therapy of HLA-matched patients with melanoma after their autologous T cells had been engineered to express the anti-MART1 TCR via retroviral gene transfer. With this method, select patients who sustained high levels of circulating engineered T cells achieved objective regressions of metastatic melanoma lesions.[223] However, this approach involved MHC-restricted recognition of the TA, thereby limiting availability to HLA-matched patients and remaining vulnerable to MHC downregulation in the tumor microenvironment.

CARs have since emerged as a powerful alternative tool to redirect the specificity and function of T cells and other immune cells. Gross and colleagues[224,225] pioneered the generation of TA-specific T cells by genetically modifying non–tumor-reactive T cells to express a CAR that specifically binds to TA in an MHC-unrestricted manner. CARs are hybrid receptors consisting of a single-chain variable fragment (scFv) antibody or another extracellular domain recognizing the TA of interest linked to intracellular signaling modules that mediate T-cell activation upon ligation of the CAR's extracellular domain (Fig. 47-10, *A*). Gene transfer of the CAR into T cells

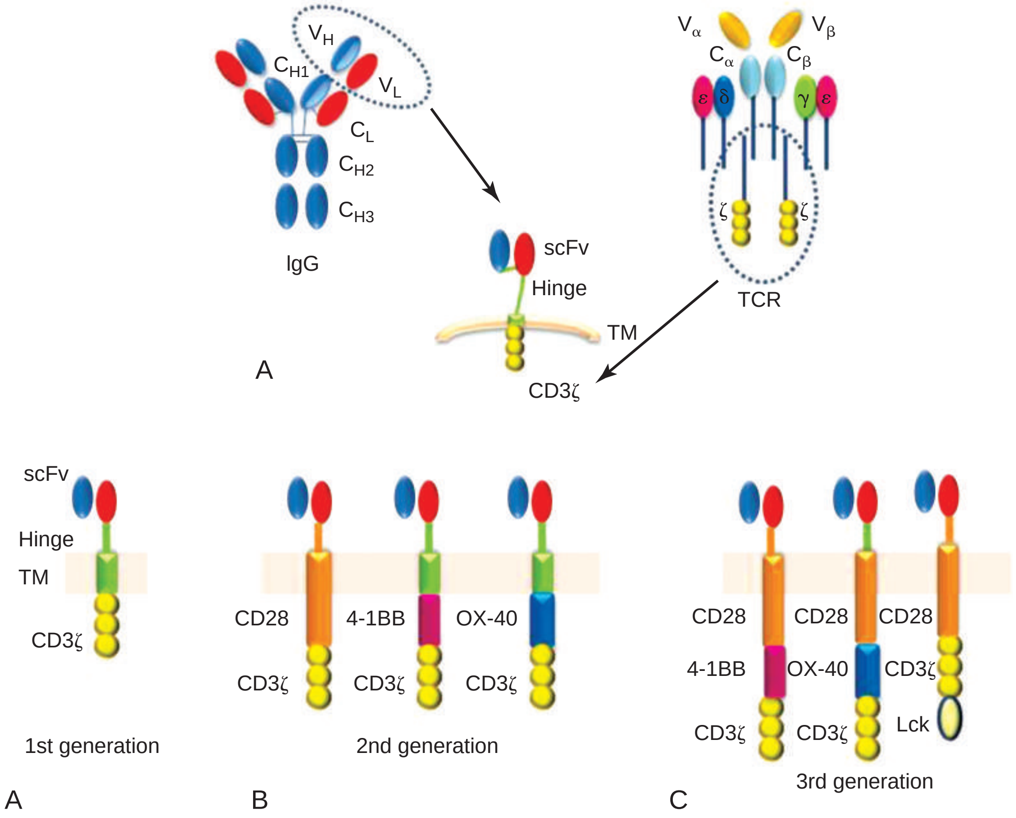

Figure 47-10 Chimeric antigen-receptor T-cells. **A,** T cells can be transduced to express chimeric antigen-receptor cells (CARs) that endow them with specificity to a particular tumor antigen (TA; signal 1), regardless of their cognate T-cell receptor *(TCR)*. Although many other iterations have been developed, the CAR commonly consists of the single-chain variable fragment *(scFv)* derived from a monoclonal antibody against a TA that is linked to an immunoreceptor tyrosine activation motif such as the CD3ζ chain. **B,** Second-generation CARs also include a costimulatory signaling domain (signal 2) in the same CAR to improve T-cell activation, expansion, and persistence. **C,** Third-generation CARs include two costimulatory signaling domains (to enhance signal 2), although it is not clear whether this is beneficial. *IgG,* Immunoglobulin G; *TM,* transmembrane region. *(Modified from Baumeister SH, Dranoff G: Principles of targeted immunotherapy. Translational cancer research: molecular therapeutics. In Kurzrock R, editor:* Targeted therapy in cancer, *Wiley Publications, Hoboken, N.J., in press.)*

can be achieved with use of γ-retroviral vectors, lentiviral vectors, or electroporation.[226] The transduced T cell acquires specificity for the targeted TA while retaining its endogenous TCR and can be endowed with supraphysiologic properties to carry out immediate and long-term effector functions. The ability to recognize TAs in HLA-independent fashion allows CARTs to attack tumor cells in the tumor microenvironment, where MHC downregulation is a major immune evasion mechanism. Furthermore, a single CAR construct can be used for the transduction of patient T cells regardless of HLA type and allows for large-scale production of the viral vector for clinical use. However, CARTs still have to be manufactured individually, either from a patient's autologous T cells or from matched allogeneic donor-derived T cells after HSCT. In contrast to native TCRs, which cannot recognize certain targets such as glycolipids, CARTs can be targeted to any cell surface antigen to which an mAb can be generated. Humanized scFvs have been developed from murine mABs[227] and are preferentially used to avoid CART elimination by way of an immune response against the murine antibody fraction.[228] With emerging clinical experience and scientific insights, CAR design has become considerably more sophisticated and continues to evolve. First-generation CARs typically consist of an extracellular binding moiety such as the scFv of an mAb, linked via a hinge and transmembrane region to an intracellular immunoreceptor tyrosine activation motif such as the CD3ζ-chain or, less commonly, the FcεRIγ (see Fig. 47-10, *A*). Such CARs deliver "signal 1," resulting in modest T-cell activation, target cell lysis, IL-2 secretion, and in vivo antitumor function (Fig. 47-11, *A*). However, a costimulatory "signal 2" is required for full T-cell activation, and the lack thereof may result in T-cell anergy. To mimic physiologic T-cell activation, second-generation CARs were designed to include a costimulatory domain such as CD28, ICOS, or TNF receptor family members (e.g., 4-1BB or OX40; Fig. 47-10, *B*). Third-generation CARs include two or more costimulatory domains but may not necessarily be superior (Fig. 47-10, *C*). Costimulatory support can be provided to CARTs by different approaches. Frequently, the costimulatory

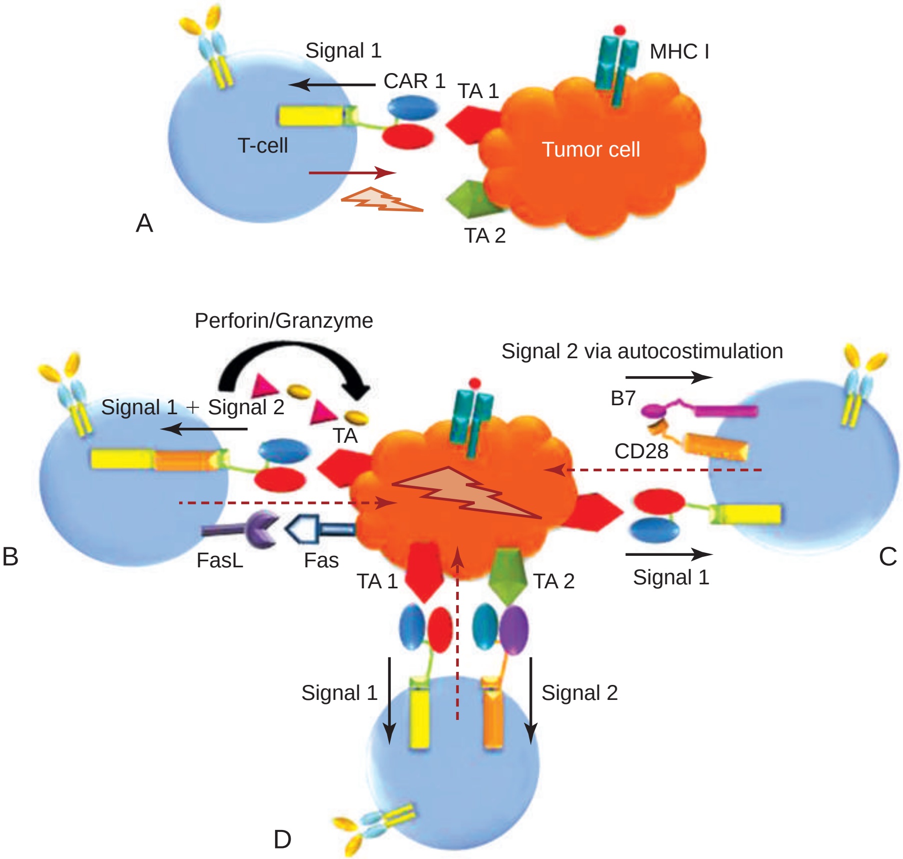

Figure 47-11 Tumor-cell elimination by chimeric antigen-receptor T cells. **A,** T cells expressing the chimeric antigen receptor (CAR) construct retain their cognate T-cell receptor but are newly endowed with specificity for the tumor antigen *(TA)*. CAR T cells can recognize the TA in major histocompatibility complex *(MHC)*–unrestricted fashion and become activated upon binding to the TA. In the absence of costimulatory signals, first-generation CARs are unable to achieve full antitumor potential and have demonstrated poor persistence in clinical phase I studies. Therefore different strategies to provide costimulatory support to CARTs were developed and significantly enhance CART-mediated killing of tumor cells and CART persistence in the patient. **B,** Costimulatory signaling domains can be embedded into the CAR construct. **C,** T cells can be engineered to express the ligand(s) for their costimulatory receptor(s) independent of the CAR, facilitating auto-costimulation. **D,** A transsignaling strategy whereby the T-cell activation signal and costimulatory signal are dissociated in two CARs with differing TA specificity not only provides a costimulatory signal but can also minimize toxicity against healthy tissues expressing one but not both of the TAs, because full CART activation is dependent on recognition of both TAs. *(Modified from Baumeister SH, Dranoff G: Principles of targeted immunotherapy. Translational cancer research: molecular therapeutics. In Kurzrock R, editor:* Targeted therapy in cancer, *Wiley Publications, Hoboken, N.J., in press.)*

domain is directly embedded into the CAR (Fig. 47-11, *B*). Alternatively, the CART can be transduced to express the ligand(s) for its costimulatory receptor(s) to enable auto-costimulation (Fig. 47-11, *C*).[229] A sophisticated strategy that also improves safety by rendering T cells specific for a tumor in the absence of a tumor-restricted antigen involves combinatorial antigen recognition and balanced, dissociated signaling; T cells are transduced both with a first-generation CAR that provides suboptimal activation upon binding of one TA and a chimeric costimulatory receptor recognizing a second TA. In this scenario, full T-cell activation is only achieved if both CARs bind to their respective antigen and tissues expressing only one of the TAs are spared from CART attack (Fig. 47-11, *D*).[230] The optimal number and configuration of costimulatory signals within a CART may vary depending on the clinical scenario and is the subject of ongoing investigation. Unifying features of second-generation CARs include enhanced cytokine production, expression of antiapoptotic proteins in resting T cells, and proliferation in response to antigen when compared with first-generation CARs.[231-236] Compared with CARs that contain TNF receptor, CD28 costimulation leads to higher levels of IL-2 and IFN-γ secretion.[237] However, IL-2 can lead to Treg-mediated CART suppression,[238] and lower cytokine levels may be beneficial because they carry a reduced risk for cytokine release syndrome and may lead to sustained clinical activity. 4-1BB appeared superior in a xenograft model of ALL, but in a mesothelioma tumor model, CD28 and 4-1BB containing CARs were equivalent.[237,239] Stringent comparative studies to identify optimal CAR design would be prudent but are complicated by other variables such as use of different scFvs, the nature and length of extracellular spacer moieties,[240] methods of transduction, tumor models, and culture conditions.

CARs against a broad range of targets have been developed, and the identification of novel targets is ongoing. Early proof-of-principle trials were directed against carbonic-anhydrase-IX for the treatment of renal cell carcinoma,[241] folate receptor for metastatic ovarian cancer,[242] CD171 for neuroblastoma,[243] and CD20 for NHL.[244] Although these trials had a reasonable toxicity profile, T-cell persistence and efficacy were poor. Increasing evidence indicates that the degree of expansion and persistence of CARTs correlates with clinical efficacy but may also increase associated toxicity.[245,246] In an effort to improve persistence through ongoing antigenic TCR stimulation and to avoid theoretic off-target toxicity against unknown antigens via the endogenous TCR, the concept of selectively transducing EBV+ or cytomegalovirus (CMV)+ T cells has been explored. An anti-GD2-CAR trial in children with neuroblastoma utilized this approach by coinfusing EBV-specific and polyclonal anti-GD2-CARTs. Although this trial showed encouraging clinical results in several patients, CART persistence was concordant with the percentage of CD4+ T cells and central memory T cells, rather than EBV+ CARTs.[245] Consideration of the phenotypic and functional heterogeneity of the T-cell pool is key because naive T cells, effector T cells, effector memory T cells, and central memory T cells differ substantially in their readiness to deliver effector function, proliferate, and persist.[247] Depending on the therapeutic goal of achieving rapid effector function versus long-term persistence, certain T-cell subtypes may be preferentially selected for CART therapy. Furthermore, some studies identified an inverse correlation between disease burden and persistence of CARTs, suggesting that conditioning chemotherapy may be beneficial.[243,248]

Many adult and ongoing pediatric CART clinical trials have focused on CD19, which is expressed by B-cell malignancies and healthy B cells but is absent on hematopoietic stem cells, early B-precursor cells, and other tissues. Undesired on-target toxicity is thus limited to B-cell aplasia. Although transient B-cell aplasia can be supported with immunoglobulin replacement, opportunistic infections are of concern, especially if CARTs and associated B-cell aplasia might persist over the life span of a young patient. Several phase I/II clinical trials have evaluated the safety and efficacy of CD19 CARs in patients with CLL, ALL, and NHL. Although the CD19-CAR constructs used in the respective trials differ in the scFvs against CD19, the type of costimulatory domain(s) used, and lentiviral versus γ-retroviral transduction regimens, the therapeutic promise of CD19 CARTs has been demonstrated by several groups.[248,249] Brentjens et al.[250] demonstrated induction of molecular remissions with a CD19-28-ζ-CAR in five adults with MRD+ ALL. Porter et al.[246] reported on three patients with CLL who were treated with chemotherapy followed by infusion of a CD19-4-1BB-ζ-CAR, whereby two patients achieved a CR and one patient achieved a partial remission. Whereas in vivo persistence of CARTs beyond a few weeks was problematic in many of the early CART trials, this group demonstrated not only persistence of CARTs in the blood and bone marrow exceeding 6 months, but also in vivo expansion of infused CARTs by greater than three log steps. Although multiple pediatric centers are now conducting CD19-CAR trials, Grupp et al.[251] reported first results utilizing a CD19-4-1BB-ζ-CAR construct in two pediatric patients with refractory pre–B-cell ALL who had experienced multiple relapses. Both patients achieved CRs, with one ongoing at 11 months after treatment. The second patient experienced a relapse 2 months after therapy with emergence of a CD19-negative escape variant. Similar to the adult trial, significant CART expansion and persistence occurred. Additionally, CARTs were detected in the cerebrospinal fluid. However, the remarkable therapeutic potential of CD19 CARTs is also associated with considerable toxicity. Adult and pediatric patients have been described as developing variable degrees of a febrile, potentially life-threatening cytokine-release syndrome that has been managed with Etanercept (a TNF-α blockade) and most effectively by Tocilizumab (an IL-6R blockade).[251] Transient neurologic toxicities such as mental status changes have also been observed and could be related to the presence of CD19 CARTs in the cerebrospinal fluid. In addition to CD19 CARTs, several pediatric trials are underway to evaluate CARTs directed against CD30+ lymphomas (trialsNCT01316146 and NCT01192464), Her2+ malignancies including osteosarcoma and glioblastoma multiforme (trials NCT00902044 and NCT 01109095), and GD2 in neuroblastoma (trials NCT00085930 and NCT01822652). As CARTs against new targets such as CD22+ B-precursor ALL[252] enter clinical trials, they may provide tools to prevent or treat CD19-negative escape variants. Allogeneic CART therapy after stem cell transplantation is another area of active investigation (trials NCT01087294 and NCT01475058).

From a safety perspective, one adverse event was reported for an adult with metastatic colon cancer who received a large dose of third-generation Her2-28-4-1BB-ζ-CARTs and developed an acute respiratory distress syndrome–like clinical picture followed by multiorgan failure, likely as a result of low level Her2 expression in healthy lung tissue.[253] A patient with CLL who was treated with CD19-28-ζ-CARTs after chemotherapy died within days of the T-cell infusion, but this death was ultimately attributed to an infectious cause.[254] To date, CARTs have otherwise been infused safely and with manageable and largely transient toxicity profiles. However, the relative toxicity or safety of a given target may only be evident when CARTs represent a significant amount of circulating lymphocytes, and trials with poor T-cell expansion may not be conclusive in this regard. Several strategies are being investigated to improve the safety and specificity of CARTs. These strategies include the integration of inducible suicide genes such as herpes simplex virus thymidine kinase (HSV-tk) or iCaspase-9 that can be pharmacologically activated and have been explored in the context of GVHD,[255-257] pharmacologically activated riboswitches that can regulate CAR expression and T-cell proliferation,[258] and inclusion of extracellular moieties such as human EGFR, which could allow targeted destruction via anti–human EGFR mAbs.[259] Contrary to gene transfer into HSCs, transduction of differentiated T cells has not been associated with insertional mutagenesis or leukemogenesis. More than 540 patient-years of follow-up from three clinical trials evaluating a CD4-ζ-CAR for human immunodeficiency virus infection showed no evidence of persistent clonal expansion or enrichment for integration sites near genes implicated in growth control or transformation.[260]

Several interesting CAR constructs have been explored in preclinical models and hold promise for clinical application. Examples include a NKG2D-receptor-CAR targeting the natural ligands of NKG2D that are overexpressed on a multitude of tumors,[261] a CAR directed against vascular endothelial growth factor receptor 2,[262] IL-12 secreting CARTs,[263] and an anti–fluorescein isothiocyanate CAR that could be used more universally to eradicate cells marked by a fluorescein isothiocyanate–labeled antibody against various TAs.[264] Other strategies of interest include expression of chemokine receptors such as CXCR2 and CCR4 to enhance trafficking of CARTs to the site(s) of tumor[265,266] and coupling of negative T-cell regulators such as PD-1 to an activating intracellular signal, allowing the T cell to prevail over PD-L1 signals in the tumor microenvironment.[267]

CAR technology is poised to include new targets and optimize extracellular binding moieties, costimulatory domain constellations, selection of T-cell subsets, transduction regimens, safety mechanisms, and other variables. The growing body of clinical experiences should further aid in the optimal incorporation of CART therapy into existing treatment regimens and inform the prevention and management of clinical toxicities.

Allogeneic Adoptive T-Cell Therapy for the Control of Viral Infections after HSCT

Viral infections after HSCT are among the most common causes of death as a result of prolonged and profound immunosuppression of the host. This scenario is especially true after CBT or in T-cell–depleted grafts, where the graft does not contain appreciable numbers of virus-experienced T cells to confer protection. Pharmacologic therapy is available but differs in efficacy depending on the virus and may not be sufficient to eradicate infection in the absence of an intact immune system. Walter et al.[268] first established in 1995 that transfer of CMV-specific CD8+ T-cell clones derived from donor bone marrow safely and effectively boosted cellular immunity against CMV after allogeneic HSCT, whereas virus-specific CD4+ helper cells are required for persistence of the transferred CD8+ CTLs. This approach was subsequently expanded to the treatment of EBV, EBV-associated PTLD, and adenovirus and further evolved toward the generation of multivirus-specific CTLs.[269-271] However, the laborious manufacturing procedure and necessary availability of the donor with respective viral immunity presented significant challenges for a broader implementation. To overcome this barrier, third-party virus-specific CTLs were introduced.[272] Establishment of a cell bank with virus-specific lines from third-party persons possessing common HLA polymorphisms and immunity to EBV, CMV, or adenovirus permitted off-the-shelf use of these CTLs. In vitro studies predicted a high degree of alloreactivity mediated by HLA-mismatched virus-specific CTLs; however, a recent multicenter study in which 50 patients with severe, refractory viral infections after HSCT received banked third-party virus-specific CTLs reported a low incidence of de-novo GVHD in two patients with a cumulative rate of complete or partial responses of 74% in the entire group.[273]

Mesenchymal Stem Cells

Multipotent mesenchymal stem cells (MSCs) are characterized by their capacity to give rise to colony-forming–unit fibroblasts and to differentiate into tissues of mesenchymal origin in vivo and in vitro. They were first isolated from bone marrow and later from other tissues such as adipose tissue and umbilical CB. Their phenotypic definition requires expression of CD73, CD90, and CD105 in the absence of CD11b, CD14, CD34, CD45, CD19, CD79a, and HLA-DR. MSC interactions with the immune system are complex, but MSCs support hematopoiesis and co-localize with HSCs during ontogeny.[274] These characteristics generated interest in using MSCs for the prevention of graft failure and promotion of hematopoietic recovery after HSCT.[275,276] A role for MSCs in bone marrow failure syndromes has also been suggested.[277] A pediatric trial of 13 patients who received parental-derived MSC coinfusions at the time of their CBTs failed to demonstrate improved hematologic recovery or reduction in rejection, but a significantly lower risk of grade 3/4 acute GVHD was observed in the patients treated with MSC compared with historic control subjects.[278] Based on their non–HLA-restricted immunoregulatory and regenerative properties, MSCs are now being actively explored as a cellular therapy for GVHD and autoimmune/inflammatory conditions. MSCs in vivo are rare, and although they can easily be obtained by simple bone marrow aspiration, they require in vitro expansion to obtain sufficient numbers. In a multicenter phase II trial, 55 patients who had severe acute GVHD refractory to first-line steroid therapy were treated with one or more infusions of non–HLA-matched bone marrow–derived MSCs. This study reported no acute or long-term adverse effects and demonstrated 30 complete and 9 partial responses. It included 25 pediatric patients, of whom 17 achieved a complete response and four achieved a partial best response.[279] A subsequent multicenter compassionate use study evaluated a premanufactured, universal donor formulation of MSCs in 12 children who had treatment-resistant grade 3/4 acute GVHD and demonstrated an overall complete response in 58%, a partial response in 17%, and a mixed response in 25%. MSC treatment was most efficacious for GI acute GVHD, with resolution of GI symptoms in 75% of patients; however, two patients experienced recurrence of GVHD with only a partial response to retreatment.[280] Adult studies have reported a mixture of complete responses, partial responses, and nonresponses in the use of MSCs for the treatment of chronic GVHD.[281] Thus far, limited studies of MSCs in pediatric patients appear to have a very favorable toxicity profile and may offer a therapeutic option for patients with treatment-refractory GVHD.

IMMUNE RESPONSE CRITERIA

An important element of cancer immunotherapy is the accurate assessment of clinical efficacy. As has been reviewed in this chapter, immunotherapy promotes the interaction between tumor and immune cells and triggers inflammatory reactions, which often correspond with an increase in tumor size during the early phases of treatment. The Response Evaluation Criteria in Solid Tumors (RECIST) or modified World Health Organization criteria that have been used to assess chemotherapy-induced solid tumor regression may therefore not provide an adequate assessment in the context of immunotherapy. In fact, false-negative interpretation as progression could preclude the most promising immunotherapy regimens. A randomized phase III trial of ipilumimab in persons with stage IV melanoma, which showed a twofold improved OS for recipients of anti-CTLA-4 mAb, most clearly illustrated that lesions might initially increase in size, and that immunotherapy responses develop more slowly than do chemotherapy responses, reflecting the gradual buildup of a lasting immune response.[155] Clearly a need exists to develop validated biomarkers to monitor the development of an immune response after therapy and identify those that best correlate with and predict clinical efficacy.[282] Careful immune analysis will also provide further mechanistic insights and translate into improved regimens. Collaborative efforts to standardize laboratory protocols for biomarkers and to implement new immune-related response criteria are underway[283,284] and will undoubtedly benefit pediatric patients as well.

CONCLUSION AND FUTURE DIRECTIONS

Critical advances have been made in appreciating the complexity of tumor immunology and dissecting the regulatory and effector components of immunity in the tumor microenvironment and beyond. As a result, a multitude of strategies capitalizing on the immune system's ability to control cancer have emerged, both with experimental therapies and increasingly with approved clinical therapies. Ongoing preclinical discoveries and important insights from clinical trials critically inform the development of novel and optimized therapies. Although some immunotherapeutic agents have shown impressive efficacy, the overall median survival advantage may not always be overwhelming. This outcome is not surprising because phase I, II, and III studies are often performed in patients with progressive disease for whom all established therapies have failed, and the studies often use immunotherapy alone. Success in cancer therapy in general has only been achieved by using multiagent or multimodality treatments, which is likely to be the case with cancer immunotherapy as well. Immunotherapy may be most beneficial when used in upfront therapeutic regimens or in MRD states, where it can aid in the complete eradication of tumor cells and prevent relapse by establishing immunologic memory. Although lymphodepleting chemotherapeutic regimens may be important to create "space" for cellular therapies, tumor vaccines or antibody-based strategies rely on a functional immune system to carry out effector functions, and such capacity may be affected in heavily pretreated patients. Several studies suggest that the immune system has the ability to increase the efficacy of existing therapeutic modalities in cancer, and the prospect of a synergistic effect of immunotherapy is highly encouraging. Although small molecule kinase inhibitors can effectuate impressive tumor responses, these drugs are frequently limited by the emergence of drug resistance. The full effect of immunotherapy is not immediate, but once developed, it can offer a sustained response that is unparalleled by other therapeutic modalities. Tumor-specific memory T cells or persistence of infused cellular products can offer long-term control, whereas other therapies are short-lived. Upon successful initiation of antitumor immunity, the immune response can be broad and reach beyond the initially targeted antigen, thus minimizing escape mutations. The combination of targeted molecular therapy with targeted immunotherapy may thus prove to be particularly efficacious and well tolerated.[285] In pediatric oncology, effective immunotherapeutic combination regimens hold particular promise for improving survival and quality of life by sparing patients from treatment with chemotherapeutic and radiotherapeutic agents that have long-term toxicity and the propensity for secondary malignancies.

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CHAPTER 48

Pediatric Radiation Oncology

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CHAPTER OUTLINE

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Osteosarcoma
Ewing Sarcoma
Retinoblastoma
SEQUELAE OF TREATMENT
CONCLUSION

PRINCIPLES OF RADIATION ONCOLOGY

Radiation oncology is an effective and often critical component of multidisciplinary cancer care for many childhood malignancies. The delivery of radiation for the pediatric population is highly complex because of the diversity of patients, tumor location, and biology. Additional concerns regarding the use of radiation for this patient population include a high likelihood of cure, coupled with the well-known and often lifelong complications from oncologic treatment. As survival from childhood cancer has improved, there is better recognition of the impact that long-term toxicities can have on the quality of life for patients. Practitioners have also learned a great deal over the past few decades regarding the required dose of radiation to provide cure, as well as the dose that specific healthy organs can tolerate. For each radiation plan it is now easier to deliver the desired target dose while minimizing the dose to healthy organs, allowing for disease control with decreased morbidity. Still, radiation is not without side effects and for each patient the risks and benefits must be considered to determine an acceptable treatment plan. Multimodality treatment is required for nearly all pediatric cancer patients, and physicians from different subspecialties must work closely together to determine the best management for these children. The desires of children and their parents must be taken into consideration to determine what life-long side effects are considered acceptable in order to achieve the best outcome.

THE PHYSICAL BASIS OF RADIOTHERAPY

Radiation is by no means a novel phenomenon; background radiation is ever-present. The understanding and use of radiation, however, is a relatively recent development in human history. The discoveries of x-rays in 1895 by the German physicist Wilhelm Konrad Röntgen and radioactivity in 1898 by Henri Becquerel led to the recognition of biologic effects of radiation.[1] By the early part of the twentieth century, ionizing radiation had rapidly come into use to treat malignant and benign conditions. At the 1922 International Congress of Oncology in Paris, Coutard presented the first evidence of the use of fractionated radiotherapy to cure advanced laryngeal cancer without disastrous sequelae.[2] This event marked the beginning of the field of radiation oncology.

Ionizing radiation, which includes both particulate radiation and electromagnetic waves, produces ionizations and excitations during the absorption of energy in

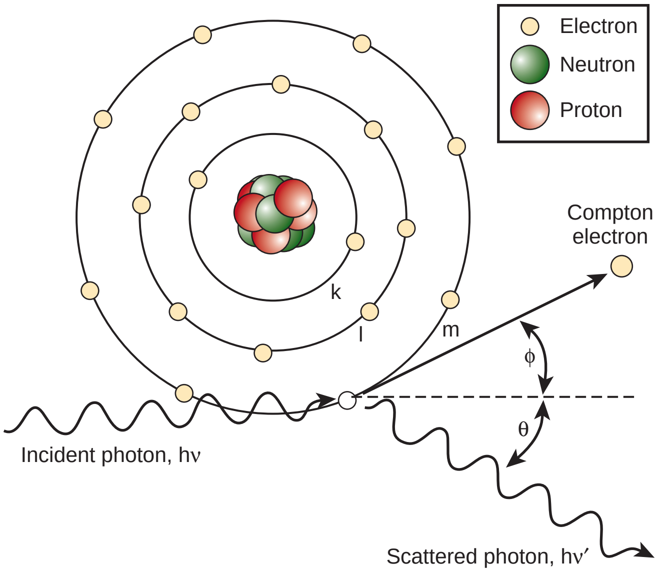

Figure 48-1 The Compton effect.

exposed tissue. Electromagnetic waves are part of a broad spectrum that includes radio waves, microwaves, visible light, x-rays, and gamma rays. In therapeutic radiation oncology, x-rays, gamma rays, and particulate radiation are used. Gamma rays and x-rays share similar general properties, differing in the source and energies. X-rays are produced when charged particles, generally electrons, are accelerated and bombard a high-density target. The target, most commonly tungsten, emits photons of varying energies up to the peak energy of the accelerated electrons. The energy (E) of the photons is determined using the relationship $E = h\nu$, where h is the constant of proportionality known as the *Planck constant*, and ν is the frequency of the wave. Substituting for the frequency, the equation becomes $E = hc/\lambda$, where c is the speed of light and λ is the wavelength. In contrast, gamma rays are emitted by a source; the most commonly used source for gamma rays used in therapeutic radiation is cobalt.

Radiation may be directly ionizing or indirectly ionizing. Direct ionization results in a direct disruption of the atomic or molecular structure of tissue through which the beam of radiation passes. This disruption causes biochemical and molecular damage. Particle beams are directly ionizing. Electromagnetic waves and neutrons are indirectly ionizing; when absorbed in tissue they give up their energy by producing fast-moving charged particles. These charged particles directly damage tissue. Photons in tissue interact in several different ways, including producing the Compton effect or the photoelectric effect, coherent scattering, pair production, and photodisintegration. The dominant reaction, the Compton effect, is governed by the energy of the photons. This process, shown in Figure 48-1, involves the interaction of the photon with a loosely bound orbital electron. Part of the energy of the incident photon is transferred to the electron as kinetic energy; this Compton electron may then interact with electrons in the surrounding tissue. The remaining energy is carried away by another photon that is less energetic than the original photon. The probability of Compton interactions is independent of the atomic number of the target tissue.

Whereas photons interact mainly with orbital electrons, protons, neutrons, and other heavy particles interact with the nuclei of atoms. Heavy particles also cause showers of lower energy and densely ionizing protons, neutrons, and other particles, depositing large amounts of energy over a very short distance. This is referred to as *linear energy transfer (LET)* and depends on the type of radiation used. Photons and electrons both have low rates of energy transfer. Heavy particles tend to deposit their energy in a track over a relatively short distance, and these particles are considered high-LET radiation.

External-beam radiation treatment systems produce ionizing radiation by radioactive decay of a nuclide, most commonly cobalt-60, or electronically through the acceleration of electrons or other charged particles such as protons. In a linear accelerator, electrons are accelerated down a waveguide by the use of alternating microwave fields. In a cobalt-60 unit the source is always on, because radioactive decay occurs continuously. The source is moved to an unshielded position to initiate a treatment. In a linear accelerator, no source is present until the unit is energized; there is an *on* switch and an *off* switch. The energy spectra are different for the two types of treatment units. Cobalt-60 units emit two monoenergetic gamma rays with each decay, producing a discrete spectrum with peaks at 1.17 and 1.33 MeV. A linear accelerator produces a continuous x-ray spectrum with a maximum energy of E_{max} (the accelerating potential) and all other photon energies down to zero. An average x-ray produces the energy of approximately one-third E_{max}. A linear accelerator can produce a treatment beam of electrons as well as photons. The accelerated electron beam exits the treatment unit under controlled conditions of scatter.

The basic components of the treatment machine include a radiation source, a collimating system to form and direct the beam, inherent shielding for protection, a light field to delineate visibly the area being treated, a control system to turn the beam on and off, a system to rotate the beam, and a support apparatus for the patient. Most treatment machines are assembled with isocentric geometry. The isocenter is a point in space at which all of the treatment machine's rotational axes intersect. Any mechanical rotation occurs around an axis that passes through the isocenter.

The linear accelerator has many degrees of geometric movement and can produce a treatment field in almost any orientation relative to the patient. Figure 48-2 shows a photon linear accelerator. The beam source is mounted on a gantry that rotates fully about the patient. The patient is on a treatment couch that can pivot with respect to the plane of gantry rotation. This degree of movement allows multiple fields to approach the target volume. In addition, the collimation assembly mounted on the gantry allows the radiation treatment field to be geometrically conformed to the target volume intersection. The delivery of proton therapy has evolved to utilize a gantry system adapted from linear accelerators and cobalt delivery systems.

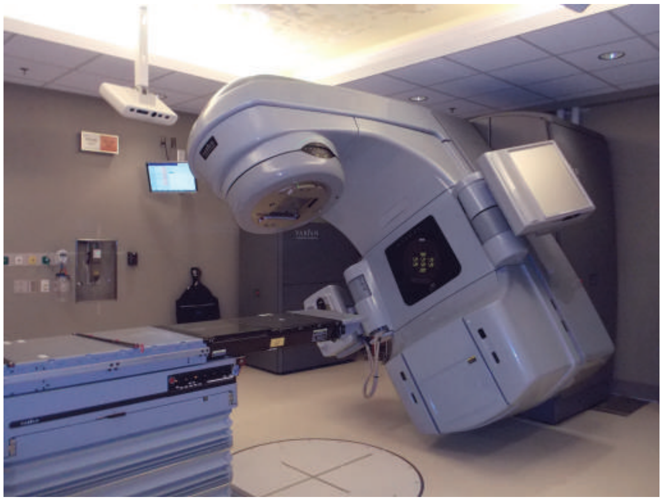

Figure 48-2 Photon linear accelerator. The patient is positioned on a couch *(black table)* that can pivot for patient positioning. The gantry can rotate fully around the patient. The radiation beam can be delivered at almost any orientation desired for treatment.

The amount of energy deposited per unit mass represents the absorbed dose of radiation. The official unit of dose is now the gray, in honor of L.H. Gray, the noted British radiobiologist who discovered the oxygen effect; it replaces the older unit, *rad*. One joule per kilogram is called a *gray (Gy)* and 1 gray equals 100 cGy. Clinical radiotherapy uses external radiation beam delivery, most often x-rays (photons) or electrons. Linear accelerators accelerate electrons to a given energy, generally 4 to 25 MeV; the electrons bombard a target, usually tungsten, resulting in the formation of an x-ray beam. The x-ray beams contain photons of varying energies up to the peak of the accelerated electrons.

In addition to external-beam radiotherapy, other radiotherapy delivery modalities are used in specific circumstances. Implanting radioactive sources directly into a tumor is known as *brachytherapy,* from the Greek *brachys*, meaning *short*. Brachytherapy can involve the placement of encapsulated sources into a body cavity (intracavitary), directly into the tumor or adjacent tissue (interstitial implants), or on the surface adjacent to the tumor (plaque therapy). Permanent implants in the form of radioactive seeds are placed under the guidance of ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI). Temporary implants are performed by using hollow catheters or loading devices inserted at the time of surgery and then subsequently loaded with radioactive sources. The chief advantage of this technique is to deliver a high dose to the target tissue while sparing the nearby normal tissue. Iridium-192 and iodine-125 are the most commonly used radionuclides. Radionuclides are embedded in seeds and sealed in thin plastic strands that can be inserted into afterloading catheters. Iodine-125, with a relatively short half-life of 57 days, is often used in permanent implants. The other feature of iodine-125 that makes it appealing for use in pediatrics is the lower energy of the photons emitted, which simplify the radiation safety concerns compared with iridium-192. There are radiobiological differences between brachytherapy and external-beam radiotherapy. Implants deliver continuous doses of 30 to 100 cGy per hour to the target tissue over the duration of the implant. This is in contrast to external-beam treatment in which the target tissue receives a short pulse of approximately 200 cGy daily, at a dose rate of 100 cGy per minute, over many weeks. Decreasing the dose rate may reduce the cell kill rate of a given dose, because repair of sublethal damage continues throughout the protracted exposure. High-dose-rate brachytherapy techniques are becoming more widely used, although late complications increase with higher dose rates.[3] For dose rates exceeding 1 Gy per hour, a reduction in the total dose is considered. In addition, fractionation of high dose rates can help to diminish the late complications of high-dose-rate delivery.

RADIOBIOLOGY

Mechanisms of Radiation Damage

The biologic effects of ionizing radiation result primarily from the formation of double-strand breaks in cellular deoxyribonucleic acid (DNA). Although most single-strand breaks in DNA caused by radiation are repaired, double-strand breaks can result in irreparable damage that leads to mitotic cell death. Photon radiation (x-rays or gamma rays) can cause damage via direct interaction with the DNA molecule or via the formation of free radicals that subsequently damage the DNA (indirect damage). Charged particles such as helium, carbon, and neon cause damage predominantly through direct interactions. High-energy neutrons interact with the nucleus of an atom, resulting in the creation of densely ionizing recoil protons, alpha particles, and nuclear fragments. LET measures the average energy deposited in tissue per unit distance traveled by a particle or photon. Conventional radiation is sparsely ionizing (low LET), whereas fast neutrons and heavy particles are more densely ionizing (high LET).[4]

Radiation causes complex cascades of molecular events that affect cell cycle checkpoints, apoptosis, DNA damage response, and DNA repair. These effects offer many potential approaches to enhancing radiation damage of tumor cells and to protecting normal tissues. These techniques are discussed in this chapter.

Clonogenic Survival Curves

A typical clonogenic survival curve is shown in Figure 48-3. The surviving fraction is plotted on a logarithmic scale, and the dose of radiation is drawn on a linear scale. The resulting cell survival curve has two distinct regions. In the initial “shoulder” portion of the curve, the fraction of cells killed increases slightly with increasing radiation doses. In the latter part of the curve, killing becomes exponential, which means that a given dose increment kills a constant fraction of cells. The slope of the exponential portion of the survival curve is called the D_0. This term is related to the inherent radiosensitivity of the cell. The smaller the D_0, the more sensitive the cells are to radiation. This is in contrast to radio responsiveness, which refers only to the rate of

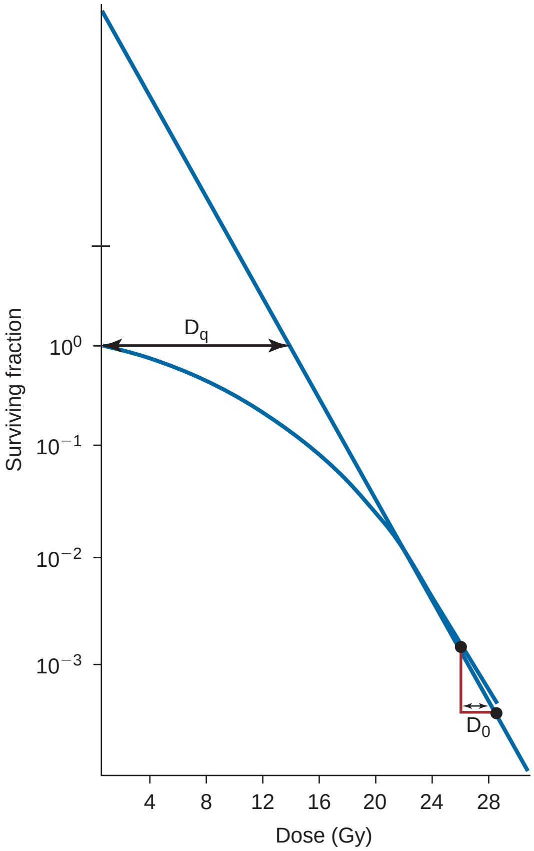

Figure 48-3 Idealized radiation cell survival curve. The fraction of surviving cells is plotted on a logarithmic scale against dose on a linear scale. The D_0 is the dose necessary to reduce the surviving fraction to 0.38 of e^{-1}. The smaller the D_0, the more sensitive the cells are to radiation. The D_q is the quasi-threshold dose and is a quantitative measure of the sublethal repair capability. *(Modified from Hall EJ:* Radiobiology for the radiologist*, ed 2, Hagerstown, Md., 1978, Harper Row.)*

disappearance of a tumor after irradiation and has no correlation with the innate sensitivity of the cells. The D_0 for mammalian cells in vitro generally falls between 1 and 2 Gy. The relatively narrow range of values for widely varying types of cells is striking. The initial nonexponential portion of the curve is believed to result from the capacity of most mammalian cells to repair nonlethal radiation injury. Saturation of the repair capability is thought to occur at the point where killing cells becomes exponential.[4]

Additional mathematical models have been used to characterize the cellular response to ionizing radiation. One of the most commonly used models, the linear-quadratic survival model, is routinely used to investigate how different tumor repopulation kinetics between radiation treatments influence the scheduling of radiation treatment. As various fractionation schemes have been examined clinically, including hyperfractionation, accelerated fractionation, and combined modality therapy (chemoradiation), the linear-quadratic model has been used to calculate the tolerability of specific tissues to various radiation schedules.[5] The concept of the biologically effective dose, a tool offered by the linear-quadratic model, allows quantification of the effects of radiation schedules on tumors and on normal structures.[6] Although this model has been helpful when applying alternative fractionation schemes to patient treatment, caution must be exercised in its application, because features such as faster tumor growth rate and higher repair capacity result in greater variations in the outcome of the survival fraction. Gaps in treatment, planned or unplanned, also accentuate the differences in the survival fraction under varying growth dynamics.[7] These limitations notwithstanding, mathematical modeling of the cellular response to radiation has contributed greatly to the clinical application of radiation to combating malignancies.

The Oxygen Effect

Tissue oxygenation influences the response of tumors and normal tissues to ionizing radiation. Poorly oxygenated tissues are two to three times more resistant to radiation than are normally oxygenated tissues. Oxygen is thought to mediate indirect damage, combining with free radicals to make DNA damage irreversible. The effect of oxygenation is measured by the oxygen-enhancement ratio. This is the ratio of doses under hypoxic versus aerated conditions required to produce a given level of cell kill. As LET increases, the effect of oxygen enhancement decreases.[4]

Radiation Interactions with Chemotherapeutic Agents

A number of chemical substances may alter the radiation response of a cell.[8] The effects may be additive, such as those of most alkylating agents and antimetabolites, or they may be synergistic, such as those of the antibiotic dactinomycin.[9] Doxorubicin and dactinomycin markedly reduce the shoulder region of the radiation cell–survival curve.[9] Dactinomycin also steepens the slope of the exponential portion of the cell-survival curve and potentiates the radiation effect; thus it is thought to be a true radiation sensitizer.

Combining radiation and chemotherapy can achieve better clinical outcomes than either modality alone but can also potentiate toxicities to normal tissues. Interactions between chemotherapy and radiation can increase the risk for toxicity in normal tissues, even when the two modalities are temporally spaced. In 1959 Dr. G.J. D'Angio described an interesting yet enigmatic phenomenon termed *radiation recall*. Radiation recall describes the remembering, or reemergence, of an inflammatory reaction that occurs within the radiation field and is prompted by the administration of particular agents days to years after initial radiation exposure. In the original case described by D'Angio, dermatitis emerged within the radiation field after application of actinomycin D to the skin.[9] Although radiation recall is most commonly documented as skin reactions, other tissues and organs are also susceptible to this phenomenon, including oral and intestinal mucosa, larynx, lungs, muscles, and the central nervous system (CNS).[10] The cause of radiation recall remains poorly understood. Although timing and dosing factors clearly impact the risk and severity of the recall reaction, there is no clear radiation dose threshold.

TECHNOLOGICAL ADVANCES IN RADIATION ONCOLOGY

All technological advances in radiation oncology allow for improved dose delivery to the target or tumor and/or better avoidance of healthy tissues outside of the area at risk of disease recurrence. The field of radiation has benefited from many major developments over the past several decades in the fields of diagnostic radiation and physics, allowing for markedly improved radiation delivery to pediatric cancer patients.

The advent of three-dimensional (3D) imaging with the invention of the CT scanner in 1971 opened a new world for not only diagnostic imaging but also for radiation treatment planning.[11] The ability to define structures and tumors in 3D and to place radiation fields and beam angles with this enhanced information marked a major advancement for the field. Additional imaging developments including MRI and positron emission tomography (PET) further enhanced capabilities to define the area at risk for tumor recurrence. Most radiation centers obtain CT scans in the radiation oncology department in the desired treatment position and employ fusion software to fuse MRI and PET scans to assist in target delineation. Figures 48-4 and 48-5 show fusions used for radiation planning. By defining this region more accurately, practitioners not only improve disease control but can also decrease the volume of healthy tissue receiving unnecessary radiation. Before the use of CT, radiation oncologists used x-rays to plan fields, and beam arrangements were less well guided by anatomic detail. Because of the inability to define tumors as precisely, fields of radiation were large for fear of local recurrence. In addition, the inability to evaluate radiation plans in 3D made the details of dose deposition less clear. Without 3D imaging radiation oncologists would not have been capable of developing the many advanced treatment delivery techniques such as intensity modulated radiotherapy (IMRT) and stereotactic radiotherapy (SRT) that are widely available today.

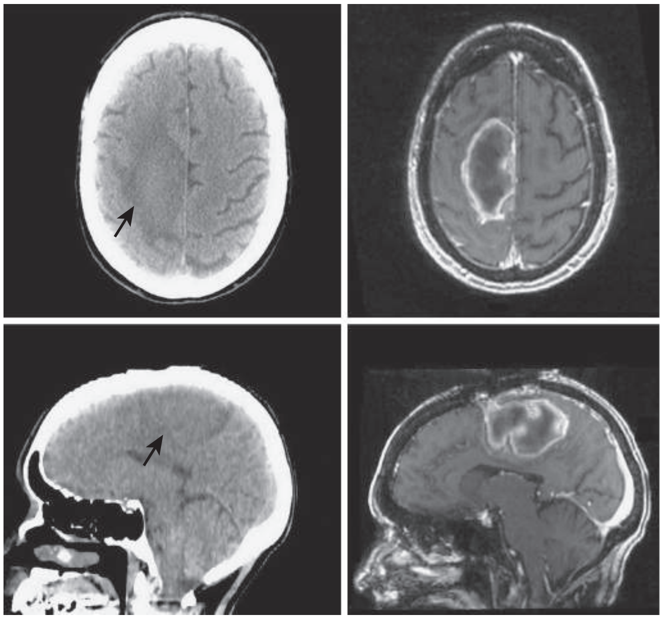

Figure 48-4 CT axial and sagittal images (*left upper* and *lower panels*) and MRI T1 post-gadolinium axial and sagittal images *(right)* showing brain tumor *(arrows)* that is much more easily visualized on MRI sequences. Fusion software enables more accurate delineation of tumor volumes by registering images together.

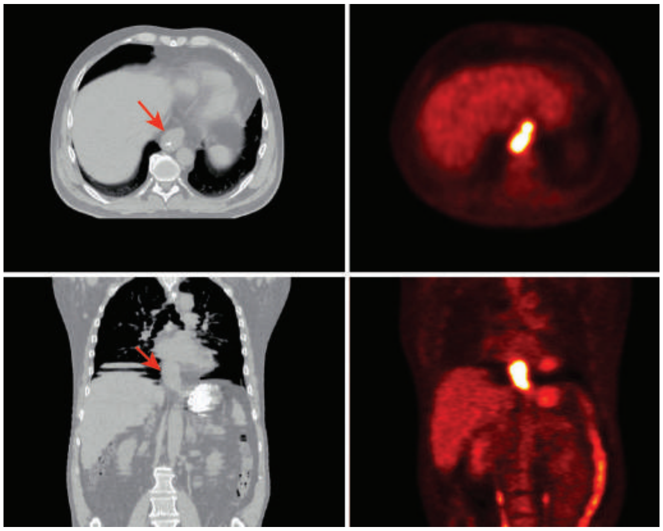

Figure 48-5 CT axial *(upper)* and coronal *(lower)* images *(left)* and PET images *(right)*. Fusion software allows for registration of PET images to planning CT so that PET avid disease *(arrow)* can be easily defined on planning CT images. Due to respiratory motion, accurate fusion can be more challenging in the chest and abdominal region.

Conformal radiotherapy describes treatment that delivers a high-dose volume that is shaped to conform to the target volume while minimizing the dose to critical normal tissues in the adjacent area. Because conformal radiotherapy attempts to conform the dose to the target, careful and accurate delineation of the target is critical. Patient immobilization for setup accuracy and to limit patient motion is imperative. The International Commission on Radiological Units and Measurements-50 defined target volumes that are used in treatment planning.[12] The gross tumor volume (GTV) is the volume of macroscopic tumor that is visualized on imaging studies. The clinical target volume (CTV) is the volume that should be treated to high dose, typically incorporating both the GTV and the surrounding tissues assumed to be at risk because of microscopic spread of the disease. The planning target volume (PTV) is the volume that should be treated to ensure that the CTV is appropriately covered; this takes into account systematic and random setup errors between treatments and during treatment (Fig. 48-6). The treated volume is the volume of tissue enclosed by a specific isodose line. The treated volume is greater than the PTV.

Intensity-Modulated Radiotherapy

Sophisticated planning techniques have significantly improved the ability to tailor the radiation field to the desired treatment region. IMRT is one such novel technique that allows the radiation oncologist to modulate the intensities of individual beamlets within each beam. IMRT requires highly sophisticated software that allows physicians to indicate the desired doses to tumor volumes and organs. This software then runs multiple iterations

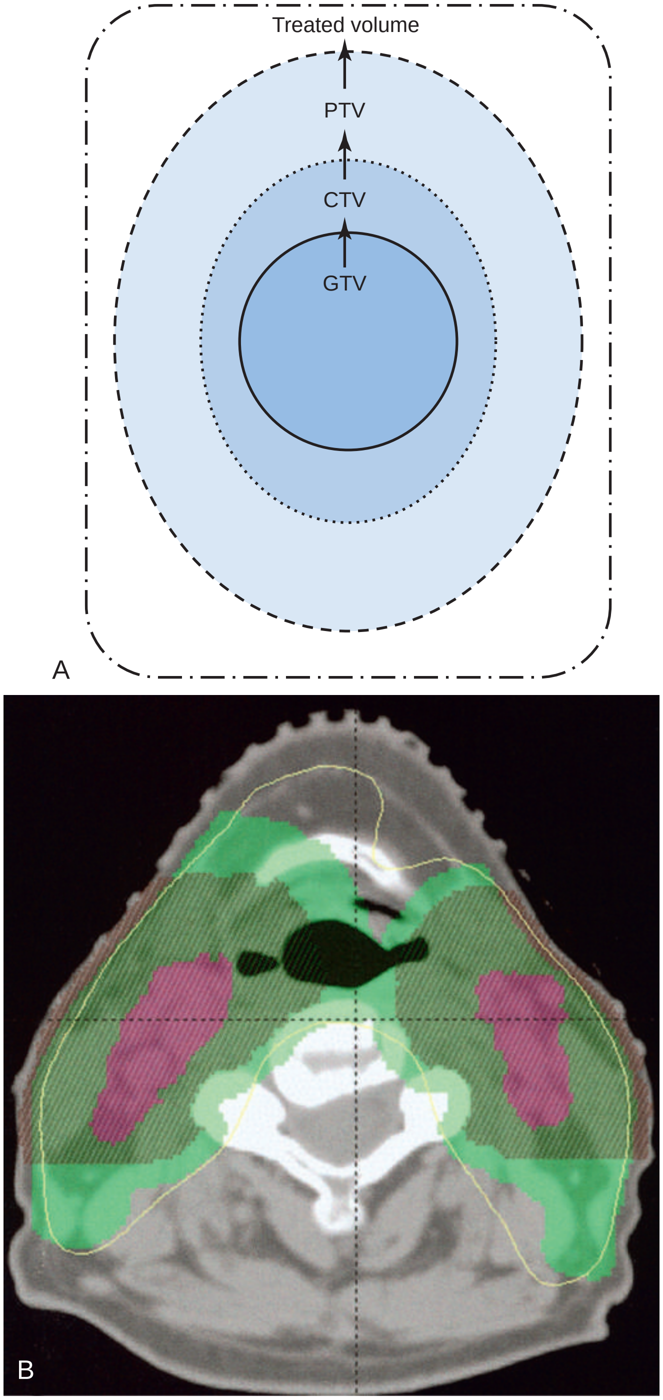

Figure 48-6 **A**, Target volumes for treatment planning. *CTV,* Clinical target volume; *GTV,* gross tumor volume; *PTV,* planning tumor volume. **B**, Axial CT image showing contoured target structures. Gross target volume shown in *pink*; CTV *hatched*; PTV in *green*.

of treatment plans involving numerous small fields and several beam angles. The physician either accepts the optimized plan or provides new parameters until the desired dose distribution has been met. IMRT accomplishes excellent conformity to target volumes while decreasing the high dose to nearby critical structures. Figure 48-7 demonstrates an IMRT field arrangement and plan for a young child with a localized ependymoma.

Stereotactic Radiotherapy

Stereotactic radiosurgery (SRS) is delivery of radiation to a target in a single treatment. This is generally reserved for smaller brain tumors, although stereotactic body radiosurgery has been developed in recent years. SRS is accomplished by delivering multiple small beams of radiation in order to deliver the prescription dose to the target but a much lower dose to the surrounding tissues. Precision is of paramount importance for SRS, and typically rigid frames and fiducial markers are used. Several delivery systems exist for photon-based SRS (e.g., Gamma-Knife, X-knife, Cyber-Knife). Protons can also be used to deliver SRS. Stereotactic treatments may also be delivered over several fractions; this is generally referred to as *SRT* (stereotactic radiation treatment). Figure 48-8 shows an SRS plan.

Proton-Beam Radiation

Proton-beam radiation is a form of particle radiotherapy that allows for deposition of maximum dose at a great range of depths (up to 32 cm) and complete sparing of tissues beyond the area of maximum dose deposition.[13] This is in contrast to photons that are limited in the depth at which they deliver maximum dose (less than 3 cm) and

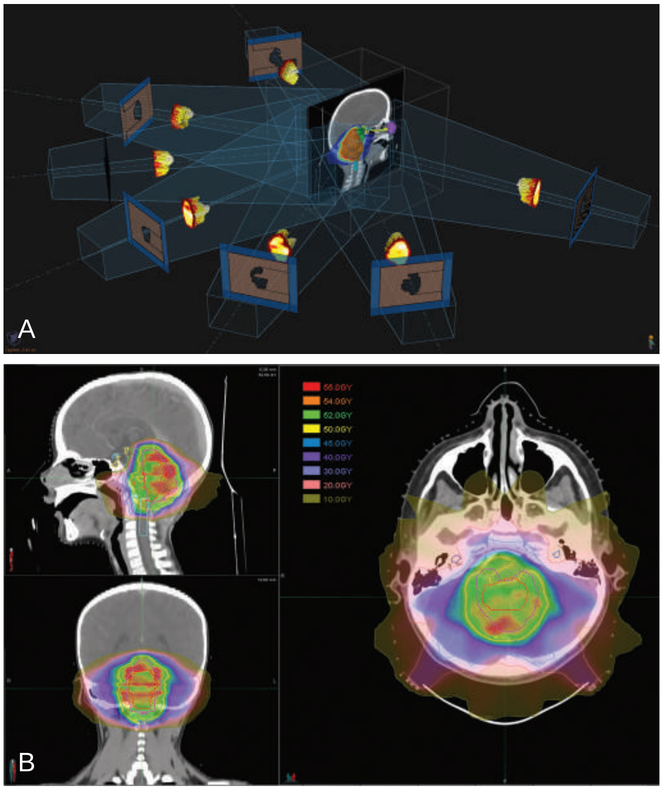

Figure 48-7 **A**, Multiple beam angles each deliver many small rays or beamlets of radiation that are used to conform the high dose to the target volume and minimize the high dose to avoid structures for this IMRT plan for a localized infratentorial ependymoma. **B**, Treatment plan showing dose distribution for the aforementioned beam arrangement demonstrating the high-dose region conforming very tightly to the target volume and reduced dose to critical structures such as the cochlea. This dose distribution would not be achievable with standard photon therapy.

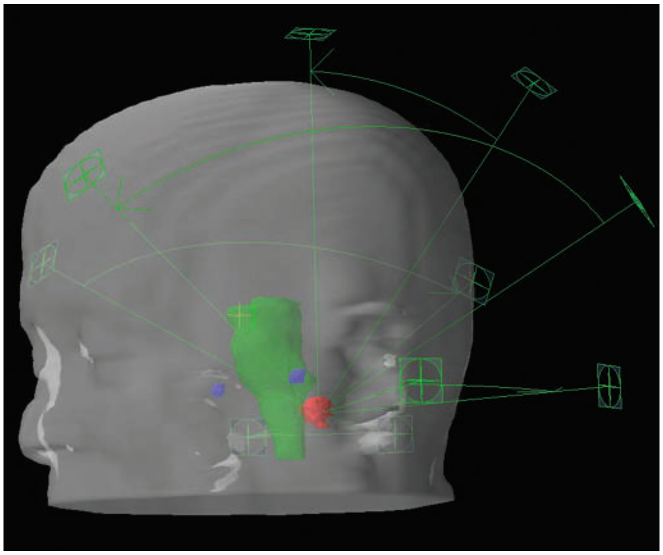

Figure 48-8 Multiple small beams of radiation deliver radiation to a small brain tumor while delivering a low dose to the surrounding healthy brain, often allowing for delivery of radiation in a single fraction.

continue to deposit the dose until passing through and exiting the body. A single proton beam delivers a small and relatively constant dose until near the end of its range, when the majority of the dose is delivered in the Bragg peak. Beyond this point, no dose is delivered.[14] Because a single Bragg peak covers only 0.5 to 1 cm, multiple Bragg peaks are required to treat a tumor.[14] CT planning is required for proton planning, because the impact of tissue inhomogeneity is greater and the density of the tissue must be taken into account by converting Hounsfield units to proton stopping power. For clinical treatment, proton beams are either combined to form a spread out Bragg peak (SOBP; 3D conformal/scattered proton treatments), or multiple proton beams are scanned across the target volume (scanning beam technique). 3D conformal radiotherapy (CRT) with protons requires patient-specific hardware to shape, or conform, the beam to the target. Lucite compensators shape the distal edge of the beam to the target volume and correct for changes in tissue density, and brass apertures or multileaf collimators shape fields to the target volume laterally. Figure 48-9 shows the delivery of 3D CRT proton treatment for a child using brass apertures and Lucite compensators. When multiple inhomogeneous scanning beam fields are used to treat a target volume, the term *intensity-modulated proton therapy (IMPT)* is used. Figure 48-10 demonstrates the use of multiple inhomogeneous beams for an IMPT plan.[15,16] Proton radiation is prescribed in gray radiobiological equivalents (Gy [RBE]) as opposed to Gy, which is used for prescribing photon radiation.[17,18] The Gy (RBE) takes into account a slightly higher average relative biologic effectiveness. Therefore the prescribed dose and biologic effects of protons are predicted to be equivalent to photons. The difference between protons and photons is the physical dose distribution. These physical properties of protons allow for a reduction in both high and low doses of radiation exposure.

In recent years, proton therapy has received much attention as a therapy that has the potential to drastically reduce late morbidity for children. Proton therapy is, however, much more capital-intensive and operationally expensive than traditional photon-based therapy. The high costs of a proton facility can be prohibitive, and for this reason proton centers are available at only a limited number of facilities. Although there has been a recent growth in the use of proton radiation both in the United States and abroad, this form of radiation is still capacity-constrained and physicians must select patients that will benefit most from this form of radiation. Children who are thought to benefit most from the tissue-sparing characteristics of proton therapy include very young children with curable tumors. Locations in the body that may benefit more from this highly conformal modality include the brain and the head and neck region. Additional malignancies and anatomic sites may also receive benefit from this form of radiation.

Image-Guided Radiotherapy

To address patient immobilization and daily positioning, some centers are using image-guided techniques. Image-guided radiotherapy (IGRT) allows the radiation oncologist

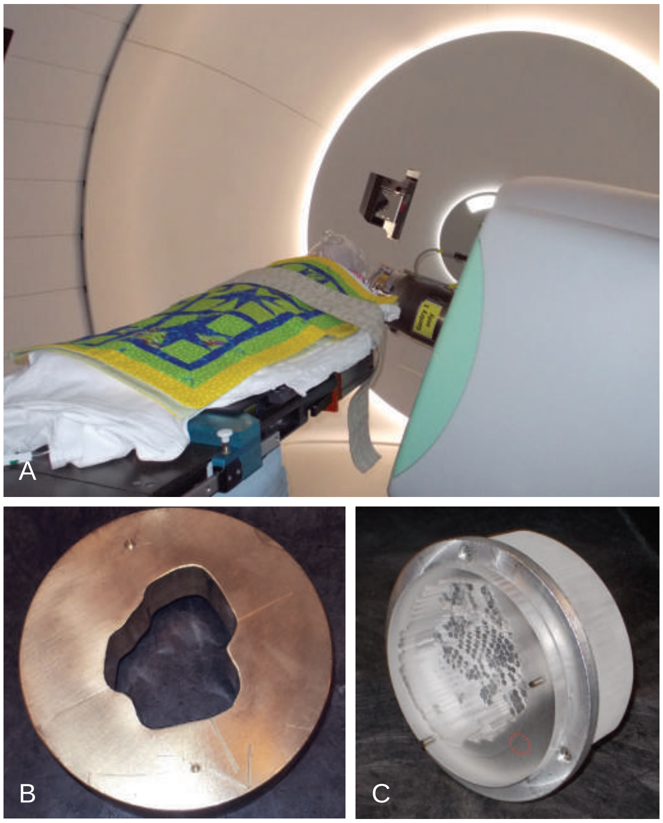

Figure 48-9 **A**, An anesthetized child receiving 3D conformal proton radiation for a brain tumor. For each field a custom brass aperture and Lucite compensator is mounted to the end of the gantry (snout). Anesthesia is required for young children to ensure that there is no movement during treatment. **B**, Brass aperture custom made to shape the field to the tumor and block healthy tissues outside of the tumor. **C**, Lucite compensator custom made to correct for tissue inhomogeneity and to shape the distal edge of the proton SOBP to the tumor.

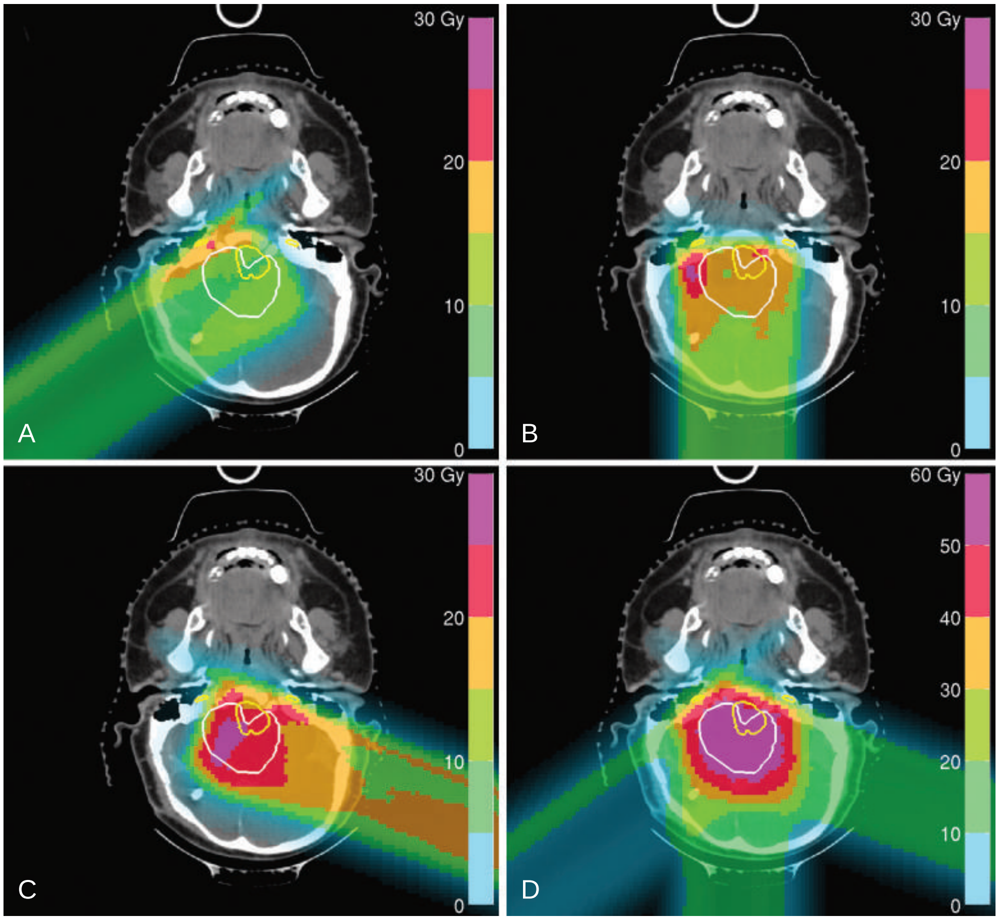

Figure 48-10 Three inhomogeneous fields composed of thousands of individual Bragg peaks are used to create a homogenous dose distribution for the composite IMPT plan. **A**, Right posterior field. **B**, Posteroanterior field. **C**, Left posterior field. **D**, Composite dose distribution of the three fields. The color scales for images **A**, **B**, and **C** are identical.

to confirm the tumor location every day. One technique to minimize tumor and patient movement is the use of a CT image of the patient using the "cone beam" technique that allows the generation of an image of the tumor and all surrounding normal structures using the same linear accelerator with which the patient is being treated. Appropriate adjustments can then be made daily to assure that the tumor is receiving the appropriate dose of radiation and normal tissues are receiving doses within their tolerance range. Gold seeds can be implanted into or near a tumor. The seeds can be tracked during the treatment. The technique of tracking the tumor during respiration and treating it at a particular phase of breathing is called *gated,* or *breath-synchronized radiotherapy* (BSRT) IGRT. This is also known as *four-dimensional (4D) IGRT.* (The fourth dimension is time.)

CLINICAL TREATMENT WITH RADIATION

Radiation is used for a wide variety of indications and disease sites. The decision to include radiotherapy in the management of a particular childhood malignancy, as well as the radiation dose used and volume treated is individualized for each patient and is dependent on many patient-specific and tumor-related characteristics. Primary among these are age, biology, stage of the cancer, and tolerance of surrounding normal tissues. Radiation delivery may also depend on surgical and other adjuvant treatments. The full management of each malignancy is beyond the scope of this chapter and the reader is referred to the chapters dedicated to the specific disease sites for a full description of disease manifestations, work up, staging, and details of therapy other than radiation.

Central Nervous System Malignancies

Tumors of the CNS are the most common pediatric malignancy requiring radiotherapy. The use of radiation dose and volume is highly dependent on pathology, extent of resection, and dissemination of disease. Given the numerous critical structures in close proximity to brain tumors including the developing brain, hypothalamus, pituitary gland, visual, and auditory structures, it is highly preferable to choose a conformal modality for children who have a good prospect of long-term survival. The use of MRI to delineate target areas is crucial for this location. The use of 3D conformal planning has markedly increased capabilities for the treatment of CNS malignancies. Advanced radiation modalities including IMRT, Tomotherapy, SRS/SRT, Gamma-Knife, and proton therapy are often employed for treatment of these malignancies.

Medulloblastoma Supratentorial Primitive Neuroectodermal Tumors

Medulloblastoma, an embryonal tumor located in the cerebellum, is a common pediatric brain tumor for which radiation is an important component of curative treatment.[19] Disease extent is categorized as *standard-risk* or *high-risk,* with standard risk defined as nonmetastatic disease in children over the age of 3 with 1.5 cm^2 or less of residual disease after surgery; all others are considered to have high-risk disease. Although not included in the stratification, anaplastic histology has been shown to carry an inferior prognosis compared with classic medulloblastoma. Molecular subtypes will likely be incorporated into future stratification, which will impact radiation dose as research indicates that prognosis is contingent on molecular characteristics.[20,21] Supratentorial primitive neuroectodermal tumors (SPNETs) are embryonal tumors located in the cerebrum. The treatment for SPNETs is the same or similar to that for high-risk medulloblastoma. Patients with medulloblastoma or SPNET have a high risk of relapse in the cerebrospinal axis even if gross disease is not detected on imaging or in spinal fluid, and patients over the age of 3 years should receive craniospinal irradiation (CSI) as a component of treatment. For patients with standard-risk disease, 23.4 Gy CSI and a boost to the area of primary disease or posterior fossa to a dose of 54 Gy is recommended. Cure rates for these patients exceed 80%.[22] Outcomes are less favorable for patients with high-risk medulloblastoma and SPNET, with cure rates on the order of 60% to 70%. These patients should receive 36 Gy CSI with a boost to the primary tumor bed or posterior fossa to 54 Gy.[23-25] For medulloblastoma, the standard volume to boost is the whole posterior fossa. Although the involved field boost has become customary at some institutions, it is not yet considered to be standard of care at the time of publication, as results from the Children's Oncology Group (COG) study, *ACNS0331,* were not available.[26] This study randomized all children enrolled to whole posterior fossa radiation versus involved field radiation. This protocol also randomized standard-risk patients under the age of 8 to reduced-dose CSI (18 Gy) versus standard dose radiation (23.4 Gy). For children with high-risk medulloblastoma or SPNET, the COG protocol *ACNS0332* is evaluating the addition of daily carboplatin as a radio sensitizer concurrent with radiation and posttreatment isotretinoin.[26] Modern techniques including IMRT, Tomotherapy, and proton radiation can decrease the dose and/or volume of uninvolved tissues receiving radiation. Figure 48-11 demonstrates the ability of protons to eliminate exit dose to organs anterior to the vertebral bodies for children receiving CSI.

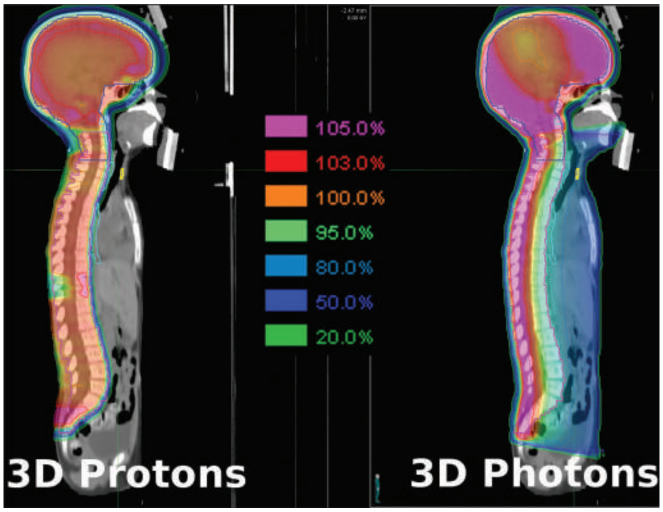

Figure 48-11 CSI delivered with protons versus photons. Protons deliver the dose to a chosen depth with no dose delivered beyond this point. This allows for sparing of all organs anterior to the vertebral bodies. Note that the entire vertebral body is treated to an even dose to prevent asymmetric growth for children who have not yet reached full height. The photon plan delivers unnecessary dose to the thyroid, heart, lung, gastrointestinal tract, and ovaries.

For children under the age of 3 years, CSI is considered to lead to unacceptable neurocognitive outcomes, and for this reason treatment of these children generally consists of chemotherapy first to delay radiotherapy, and the subsequent radiation volume is often limited to the involved field or sometimes even omitted if the child is under age 3 years at the time of radiation and did not have metastatic disease.[27,28] Outcomes are inferior, perhaps to a large extent because of the omission, deferral, or alteration of radiotherapy. The COG study *ACNS0334* delivers intensive chemotherapy, including high dose chemotherapy and stem-cell rescue. Radiation is optional on this trial, and when used it calls for delivery of radiation to the involved field to a dose of 50.4 to 54 Gy. Again, highly conformal techniques are recommended to achieve sparing of CNS structures from unnecessary radiation.

Low-Grade Glioma

Astrocytomas are the most common pediatric CNS tumor. Complete surgical resection can be achieved in many cases. With cure rates exceeding 90% for complete surgical resection, radiation is not required. Low-grade gliomas of the thalamus, hypothalamus, optic chiasm, tectum, or other region that is not amenable to complete resection may require radiation. Although observation may be appropriate if the child is asymptomatic, progression of the tumor will likely occur and treatment will be required at some point. Chemotherapy is also considered, although for the majority of patients this is only a temporizing measure. In general, chemotherapy is used for young patients, whereas radiation is the first choice for older patients, but there is no clear specific age to determine the appropriate treatment modality. Treatment decisions for these patients are best made in a multidisciplinary setting. Chemotherapy may allow for neurocognitive development and delay neuroendocrine or other radiation-related side effects, but there is a high risk of tumor progression that may lead to irreversible loss of function. Radiation, although more definitive, carries neurocognitive, neuroendocrine, and other risks. When radiation is used, a dose of 50.4 to 54 Gy is recommended. The treatment volume includes all gross tumor and the tumor bed as seen on MRI. Because many low-grade gliomas either do not enhance at all or have a nonenhancing component,

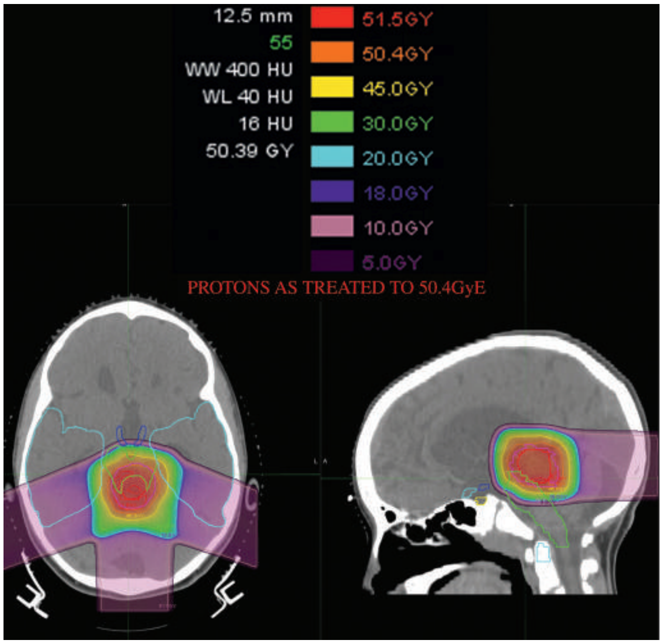

Figure 48-12 3D Conformal proton plan for a tectal glioma showing sparing of the temporal lobes and hypothalamus and pituitary gland. Protons reduce low- and high-dose radiation delivered to healthy structures of the brain.

care should be taken to include all areas of T2 abnormality that are suspicious for tumor. T2 fluid-attenuated inversion recovery (FLAIR) sequences are often useful. A margin of 5 mm to account for areas of potential microscopic disease is recommended for well-demarcated tumors. Excellent long-term outcomes have been documented after CRT.[29] Figure 48-12 shows a proton plan for a tectal glioma.

Anaplastic Astrocytoma/Glioblastoma Multiforme/Diffusely Infiltrating Pontine Glioma

Anaplastic astrocytoma and glioblastoma multiforme (GBM) carry a poor prognosis compared with other pediatric brain tumors, although long-term survival may be achieved for some patients.[30] Surgery followed by radiation with or without chemotherapy is standard. Initial treatment volumes typically include T2 FLAIR changes and enhancing tumor plus a margin for radiographically occult tumor. This volume is treated to a dose of 45 Gy. The tumor volume is then reduced to the gross tumor with a margin to deliver a total dose of 54 Gy to 59.4 Gy to this region, depending on age and adjacent critical structures.

Another highly malignant tumor is a diffusely infiltrating pontine glioma (DIPG). These tumors arise in the pons and have a classic appearance on MRI of a nonenhancing T2 bright lesion that expands the pons. Radiation is indicated for treatment of this disease, and response is typically prompt. Many patients experience a reversal of observed neurological symptoms when treated to a dose of 54 Gy. Unfortunately, response is not durable and symptoms typically recur several months after radiation. The median survival for these children is 1 year, and cure is not achievable with current available treatment.

Craniopharyngioma

Craniopharyngiomas are histologically benign tumors that can be locally destructive, causing neuroendocrine and visual disturbances. When resection is incomplete, most of these tumors recur, so radiotherapy is generally recommended. Limited surgery and radiation leads to excellent rates of control in excess of 90%. Target volume includes residual solid and cystic components and the postoperative bed with a margin of approximately 5 mm for microscopic disease. A dose of 50.4 to 54 Gy is recommended. These tumors typically have a cystic component that may change in volume during radiation, and a quick T2 MRI (Half-Fourier Acquisition Single-Shot Turbo Spin-Echo [HASTE] sequence) or CT during radiation treatment is advised so that the radiation plan can be adjusted to ensure that the cystic component remains in the treatment field with maximal sparing of uninvolved tissue. Highly conformal techniques are recommended to provide better sparing of the surrounding brain. Given neuroendocrine structures are involved by tumor, it is rarely possible to provide sparing of these structures even with the most conformal of modalities.

Ependymoma

Intracranial ependymoma represents 5% to 8% of pediatric brain tumors. The majority of these tumors arise in the fourth ventricle with fewer occurring in the supratentorial brain. Standard treatment for nondisseminated disease consists of maximal surgical resection followed by involved field radiotherapy even for children under the age of 3 years. With surgery and CRT, long-term local control rates of approximately 80% have been achieved.[31,32] Trials attempting to use chemotherapy and omit radiation have led to inferior outcomes, although chemotherapy may be recommended after a subtotal resection in attempt to allow for a second surgery and complete resection. Radiotherapy is prescribed to the tumor bed plus a margin of approximately 5 to 10 mm. Care must be taken to carefully delineate the tumor bed and areas that the tumor was in contact with before surgery as well as any areas of residual gross disease. The prescribed dose is 54 Gy to 59.4 Gy in 30 to 33 fractions to this volume. The COG protocols (*ACN0121* and *ACNS0831,* respectively) prescribe a total dose to 59.4 Gy, but *ACNS0831* requires that critical structures outside of the area of highest risk be blocked after 54 Gy.[26] Given the young age of these patients, it is important to use a very conformal technique to avoid the normal developing brain, cochlea, and neuroendocrine structures. The use of 3D CRT, IMRT, and protons has been shown to provide superior sparing of nearby critical structures while maintaining excellent outcomes.[31,32] Whereas local recurrence accounted for the majority of recurrences in historical series, recent patterns of relapse show a trend towards a more even ratio of distal and local recurrences but no absolute increase in distal recurrences; this is a reflection of the improved local control achieved as a result of advances in surgery and radiation.[31,32a]

Germ Cell Tumors

CNS germ cell tumors (GCTs) are midline CNS tumors that typically arise within the infundibulum or pineal gland but can occur elsewhere in the brain.[33] GCTs are divided into two main histologic subgroups that are highly prognostic; pure germinomas and GCTs with nongerminomatous components (NGGCTs). Pure germinoma is the most common type of GCT and typically holds the most favorable prognosis. Excellent outcomes can be achieved with radiation alone or a combination of radiation and chemotherapy. Whole-ventricle radiotherapy to a dose of 24 Gy followed by a boost to the tumor bed to total 45 Gy to 50 Gy is considered a standard treatment for pure germinoma. With the use of chemotherapy before radiotherapy, the radiation dose may be reduced, but attempts to reduce volumes to the primary tumor bed have led to an increased rate of in the ventricles. After a complete to near-complete response to chemotherapy, whole-ventricle radiation to a dose of 21 Gy followed by a dose to the tumor bed to 30 to 36 Gy is most standard. The COG protocol *ACNS1123* is investigating the use of chemotherapy followed by radiotherapy to a dose of 18 Gy to the whole ventricles, which is then followed by a boost to a total of 30 Gy for localized pure germinomas showing a good response to chemotherapy. For patients with disseminated pure germinoma, the prognosis is still excellent with radiation alone or chemotherapy followed by radiation. Doses are generally the same as stated above; however, the volume at risk for microscopic disease is the entire craniospinal axis rather than the whole ventricle. NGGCT carries a less favorable prognosis, and combined modality treatment is standard. The COG study *ACNS0122* delivered 6 cycles of induction chemotherapy (carboplatin, ifosfamide, and etoposide) followed by CSI to 36 Gy and an involved field boost to 54 Gy. Early reports suggest excellent outcomes. Reducing the volume and dose of radiation for patients who have NGGCT with localized disease and an excellent response to chemotherapy in attempt to decrease toxicity is the goal of the COG trial *ACNS1123,* which delivers the same chemotherapy as *ACNS0122* followed 30.6 Gy to the whole ventricles and 54 Gy to the tumor bed. Patients with disseminated disease still receive CSI and are not eligible for this study. A whole ventricular atlas is available on the Quality Assurance Review Center (QARC) website to help delineate this structure consistently for *ACNS1123.* Figure 48-13 shows images from this atlas.

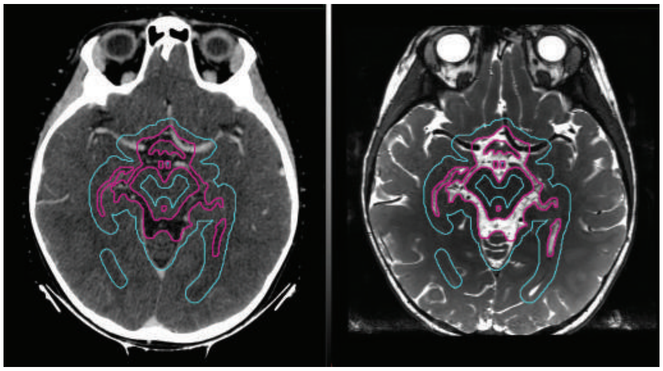

Figure 48-13 CT image *(left)* and fused T2 MRI image *(right)* showing contours for the ventricle volume. Ventricle volume is in *magenta* and PTV is in *cyan.*

Atypical Teratoid/Rhabdoid Tumors

Atypical teratoid/rhabdoid tumor (AT/RT) is a highly malignant, small, round, blue cell tumor that has only recently been recognized as a distinct entity since discovery of the tumor suppressor gene, *INI1.* Initial reports suggested a dismal prognosis for children with AT/RT, but studies incorporating high-dose chemotherapy have shown markedly improved outcomes compared with historical series.[34,35] Maximum surgical resection, high-dose chemotherapy with stem-cell rescue, and early radiation provide the best chance of cure.[35] Children diagnosed with AT/RT are generally very young, and children under the age of 3 years should receive involved field radiation to a dose of 50.4 to 54 Gy with highly conformal techniques.[36] Early radiation and CSI for children older than age 3 years seem to lead to improved outcomes and are generally recommended.[37]

Spinal Tumors

Primary spinal cord neoplasms are rare in children, accounting for approximately 5% of CNS malignancies. Most tumors are either low-grade astrocytomas or myxopapillary ependymomas. Surgical resection is the primary treatment, and radiation is reserved for situations when either complete resection is not possible or in the setting of tumor recurrence. The prescribed dose is typically 45 to 54 Gy based primarily on spinal cord tolerance, risk of relapse, and patient age.

Hematologic Malignancies

Advancements in cancer therapy have led to extraordinary improvements in outcomes for children with hematologic malignancies. Management has also greatly changed over the past few decades. Survival is excellent for children with Hodgkin disease (HD), non-Hodgkin lymphoma (NHL), and acute lymphoblastic leukemia (ALL).[38] Contemporary therapy places an emphasis on risk adaptation and avoidance of treatment-related complications while maintaining high rates of cure.[38,39] Children with hematologic malignancies are currently treated with combinations of cytotoxic chemotherapy and sometimes radiation. Advances in chemotherapy over the past several decades has allowed for a reduction in radiation dose and volume for those patients requiring it.

Hodgkin Disease

HD is a highly curable tumor with radiation alone or combined modality therapy. Contemporary treatment utilizes risk-adapted treatment with chemotherapy followed by response-based volume and dose-reduced radiotherapy. Radiotherapy alone was the first curative therapy established for HD, but the high doses used and large volumes treated with radiation led to substantial late morbidity including musculoskeletal developmental abnormalities, cardiac morbidity, and second malignancies. Many pediatric trials related to HD focus on tailoring chemotherapy and radiation to provide adequate disease control while minimizing late effects of treatment.[38,40,41] Most patients are treated with multiagent chemotherapy followed by involved field radiation to 15 to 25.5 Gy. Many studies are investigating response-adapted radiation or the omission of radiation for patients with disease showing an early favorable response to chemotherapy. Radiation volumes have been successfully reduced to the involved field, defined as a region of lymph nodes including and in close proximity to the disease as defined by diagnostic imaging. Standard involved field guidelines defining radiation field borders by anatomic landmarks are available.[42] Involved nodal radiation is being used on several trials.[43] This treatment involved contouring the areas that contained abnormal or PET-positive lymph nodes and adding a margin to these nodes to define the target for treatment. The EORTC-GELA group has established guidelines for this treatment.[43]

Leukemia

Leukemia, most often ALL, is the most common pediatric malignancy. Despite the commonality of this disease, radiation oncologists, because the development of more effective chemotherapeutic regimens, see children with leukemia less often. At present, radiation is used only in the setting of high-risk disease, refractory disease, or relapse. For patients with B-cell ALL, current guidelines indicate radiation for patients with the following high-risk features; WBC > 100,00, CNS3 disease (>5 WBC/micro-liter or blasts in the cerebrospinal fluid [CSF]), Philadelphia translocation t(9;22) ALL, T-cell phenotype, or other high-risk disease with poor response to induction chemotherapy.[44] Cranial radiation to a dose of 12 to 18 Gy is recommended. The other site for which radiation may be necessary is the testicle, but only when there is disease involvement.[45] The recommended dose for testicular radiation for leukemia is 20 to 24 Gy.

Neuroblastoma

Neuroblastoma is the most common solid non-CNS malignancy and is notable for its diverse behavior, with some cases showing spontaneous regression and others demonstrating dismal outcomes. Radiotherapy is recommended for patients with high-risk disease or sometimes for children with intermediate risk disease progressive after chemotherapy or surgery. The standard dose of radiotherapy recommended to the postchemotherapy presurgical volume is 21.6 Gy.[46,47] For children with residual disease after surgery, a total dose of 36 Gy is recommended to areas of gross disease. Radiotherapy to a dose of 21.6 Gy is also recommended to any metastatic sites that persist after induction chemotherapy. In the setting of multiple metastatic sites still metaiodobenzylguanidine (MIBG) avid after induction chemotherapy, consideration must be given to the amount of tissue to be irradiated. In this situation children are reevaluated after further chemotherapy, and an attempt is made to limit the number of metastatic sites receiving radiation to three. Urgent radiation is sometimes required for respiratory distress as a result of liver metastases in infants. If necessary, a dose of 1.5 Gy for 3 fractions is recommended. Although the radiation dose required to control neuroblastoma is low, these children are typically very young and treatment volumes may be large. Advanced radiation techniques such as IMRT, intraoperative radiation, and proton radiation should be considered.[48-50]

Wilms Tumor and other Renal Malignancies

The North American standard established by the National Wilms Tumor Study (NWTS) and COG cooperative group studies begins with up front surgery followed by radiation if indicated.[51,52] The rationale is to obtain pathology and to surgically define the extent of disease to best determine the appropriate therapy. The International Society of Paediatric Oncology (SIOP) group advises delivering chemotherapy first in attempt to decrease intraoperative tumor spillage. COG radiotherapy guidelines are based on both stage and pathology.[53] Flank radiation is recommended for patients with Stage I to III unfavorable histologies (e.g., diffuse anaplasia, focal anaplasia, clear cell sarcoma of the kidney [CCSK], and CCSK-stages II and III) and for Stage III favorable histology (FH). The standard dose is 10.8 Gy in fractions of 1.8 Gy, but higher doses are recommended for recurrent disease and stage III Diffuse Anaplasia (DA). An additional 10.8 Gy is recommended for any gross residual disease present after resection.[54] Whole abdominal radiotherapy (WART) to a dose of 10.5 Gy at 1.5 Gy per fraction is recommended for positive cytology, diffuse spillage, previous biopsy, or peritoneal seeding. COG guidelines recommend that radiotherapy commence by postoperative day 9 if feasible and no later than postoperative day 14. Whole lung irradiation (WLI) to 12 Gy is recommended for patients with anaplastic disease who have pulmonary metastases at diagnosis or children with favorable histology such as Wilms and pulmonary metastases that fail to respond to initial chemotherapy.[55] Brain, liver, and bone irradiation is indicated when metastases are present.

Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of childhood and may occur in any location of the body. The COG studies stratify patients into three risk groups (low, intermediate, and high) on the basis of site, stage, histology, and group. Risk stratification is highly complex, but in general risk groups include: 1) low risk: embryonal nonmetastatic favorable sites and embryonal group 1 or 2 unfavorable sites; 2) intermediate risk: embryonal group III unfavorable sites and nonmetastatic alveolar RMS at any site; 3) high-risk: metastatic disease. See Chapter 59 for more details on prognostic factors and risk stratification of RMS.

Combined modality therapy is always used for children with RMS, and this represents another disease site that requires multidisciplinary discussion regarding best management. Radiation treatment depends on several prognostic factors and feasibility of surgery with organ preservation. Surgery is performed up front for resectable tumors and may be attempted after chemotherapy if tumors become resectable, usually with the goal of decreasing radiation dose and sometimes volume.

Contemporary treatment and the existing COG RMS trials require 3D treatment planning to allow full visualization of areas at risk and target volumes. Radiation is omitted only for patients with completely resected favorable histology disease. Thirty-six Gy is recommended for microscopic disease after surgical resection, and nodal disease is treated to a dose of 41.4 Gy. Patients with gross disease receive a dose of 50.4 Gy. Target volumes delineate prechemotherapy volume as the GTV. A margin of 1 cm around this volume to respect anatomic boundaries is recommended to encompass microscopic disease.

Osteosarcoma

Osteosarcoma is the most common primary pediatric bone tumor, but one for which radiation is less often utilized. The use of multiagent chemotherapy has led to a marked improvement in the cure of this disease. Local control is generally achieved with surgery when feasible with reasonable functional outcome. For primary tumors of the base of skull, pelvis, and spine, surgery is not possible and radiation is administered in attempt to achieve local control. Small studies indicate that high radiation doses (50 to 76 Gy) are necessary to provide a reasonable chance of durable local control.[56-60] Proton radiation or brachytherapy may be required to deliver high doses of radiation while limiting the high dose to normal surrounding structures.[56]

Ewing Sarcoma

Ewing sarcoma is the second most common pediatric sarcoma, and multimodality treatment is required. Surgery is again the preferred method of local control if feasible. Primary radiation is indicated for inoperable tumors for which surgical resection cannot be achieved or would lead to major functional impairment.[61,62] Radiotherapy is also indicated for either partially or suboptimally resected tumors. Several studies have helped define recommendations for a radiation dose in the range of 50 to 55.8 Gy to the involved area of bone with a margin for microscopic disease. Most protocols advise that definitive radiation be initiated relatively early, usually by week 12 to 14 of treatment. The initial tumor volume should be treated to a dose of approximately 45 Gy and include all areas of tumor involvement at the time of diagnosis (with adjustments for pushing tumors that have regressed with chemotherapy) and a margin for microscopic disease of approximately 1.5 cm. Gross disease at the time of treatment along with initially involved bone with a margin of approximately 1.5 cm should receive an additional dose of 10.8 Gy to total 55.8 Gy with the exception of tumors located in the vertebral body (total 45 Gy because of spinal cord tolerance).

Retinoblastoma

Retinoblastoma is a rare disease of young children who are almost all under the age of 3 years. Many patients have the hereditary form of retinoblastoma with a high propensity for bilateral disease and subsequent malignancies.[63] Treatment depends on extent of involvement, whether disease is unilateral or bilateral, hereditary status, and sometimes response to treatment and parental preference.[64] Radiation is generally recommended for disease refractory to chemotherapy and local therapies (cryotherapy and laser therapy) in an eye with useful vision, whereas enucleation is advised if it is thought that there is no meaningful visual potential. The standard dose is 45 Gy. Postoperative radiation is delivered for advanced disease after enucleation. A dose of 45 Gy to the orbit and up to the optic chiasm is necessary in cases of frank orbital disease and high-risk pathology features (transscleral extension or tumor involvement of the cut end of the optic nerve). Plaque brachytherapy may be an option for unifocal tumors that are located in the peripheral retina.[65] Radiation does provide an excellent chance at durable control of disease, but carries risks of facial hypoplasia and radiation-induced malignancies.[66] Most data for these toxicities, however, comes from old radiation series with dated techniques as far back as 1914.[67] Modern radiation treatments should maintain disease outcomes while reducing many of these risks. Figure 48-14 depicts a photon radiation plan from 1985 compared to a proton radiation plan from 2012. Given the young age of these children and the propensity of children with hereditary retinoblastoma for second malignancy, proton radiation, which provides the best sparing of involved structures from both high and low doses of radiation, may be the best radiation option for this particular group of patients.

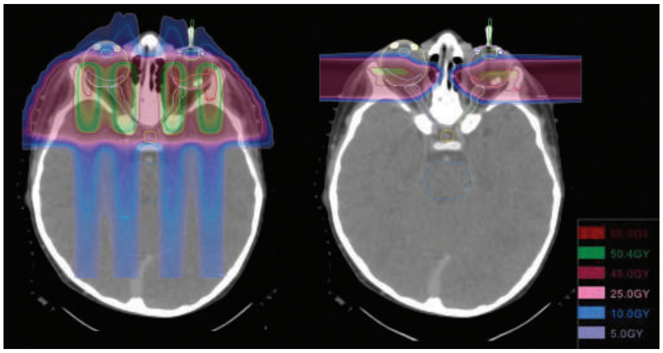

Figure 48-14 Radiation for bilateral retinoblastoma. This figure shows a radiation treatment plan with a 3D conformal photon technique from 1985 *(left)* compared with a modern proton plan from 2012 *(right)*. Protons spare tissues outside of the target volume from low and high doses of radiation. Given that these children are highly vulnerable to second malignancies, avoidance of radiation of any dose is highly desirable.

SEQUELAE OF TREATMENT

Although the goal of therapeutic radiation is to eradicate tumors, tumors are not isolated in petri dishes but occur in the human body in the midst of normal tissues that are susceptible to damage from irradiation. This normal tissue damage occurs during the course of treatment or long after the treatment has been given. The term *acute effects* refers to reactions during or very soon after treatment; reactions occurring more than 3 months after treatment are termed *late effects*. Normal tissue tolerance to fractionated treatment is a function of the volume of the organ or tissue, the total dose, and the time course over which treatment is given. When chemotherapy is given concurrently with radiotherapy, some acute and late toxicities may be enhanced. Acute and late effects are generally restricted to tissues within the radiation field or at least to tissues that received some dose of radiotherapy. The one exception to this is the systemic fatigue, which is experienced by some patients undergoing treatment.

The most typical acute effects are those on skin, on the mucosa of the aerodigestive tract, and on bone marrow. Most acute effects require supportive care, such as topical creams for skin reactions and ongoing hydration, topical anesthetics and in severe cases, narcotics for mucositis. Abdominal irradiation can cause nausea, vomiting, and diarrhea. These toxicities are managed with antiemetics, hydration, and antidiarrheal agents if symptoms are severe. Acute toxicities can be severe and occasionally require treatment breaks. However, prolonged breaks are undesirable, because the overall treatment time is an important factor in tumor control.

The mechanisms of late complications resulting from radiotherapy, though not precisely known, are speculated to be caused by vascular endothelial damage, damage to parenchymal stem cells, or damage to DNA. The normal tissue tolerance doses for the various bodily organs have been well studied.[4] The tolerance doses for adult normal tissue are applied in children with some exceptions, as discussed later. Late effects are dependent on the total dose and fraction size.[68] The potential for late toxicity is one of the most important factors in the decisions regarding dose and volume, and it is a major concern in the treatment of children with cancer.

The late toxicities of radiotherapy are dependent upon both patient factors and treatment factors. The age of the patient, the tolerance of the normal tissue treated, and the dose of the radiotherapy delivered determine the late effects and their severity. Systems most severely affected include the musculoskeletal, endocrine, and reproductive systems, and the CNS.

In children, treatment before full development has been reached can cause abnormal or arrested development. The severity of the effect on musculoskeletal development depends on the dose of irradiation, the area treated, and the age of the patient. Doses of irradiation to the growth plate that are above 20 Gy are likely to have significant effects.[69,70] The age of the patient influences the severity of the damage. Younger children with greater growth potential are more profoundly affected. Irradiation of the pituitary gland and hypothalamus can also lead to short stature by decreasing the production of growth hormone.[71,72] Spinal irradiation in the treatment of medulloblastoma in prepubertal children causes a shortened spine or decreased sitting height.[73,74]

Neurocognitive sequelae from brain irradiation are among the most serious late effects when radiotherapy is used in children.[75,76] The age of the patient, the dose, and the volume of brain treated all influence the severity of these toxicities.[75,77,78] Irradiation of the cochlea can lead to sensory peripheral neuropathy manifested as high-frequency hearing loss.[79] Many children receive ototoxic chemotherapy such as cisplatin, which itself causes hearing loss.[80] When cochlear irradiation is combined with cisplatin, profound hearing loss or sometimes deafness can result.[80]

Germ cell survival in testes and ovaries can be impacted by radiotherapy.[81] Males treated with whole abdominal irradiation may develop gonadal dysfunction. Temporary decreases in sperm count occur in the weeks after low doses of radiotherapy (0.2 to 3 Gy). Recovery occurs in 1 to 3 years, depending on the dose and the age of the patient. Direct testicular irradiation with doses of 24 Gy as used to treat testicular relapses of ALL cause sterility and Leydig cell dysfunction.[82] Total body irradiation combined with cyclophosphamide for stem-cell transplant results in azoospermia for almost all males.[83,84]

Abdominopelvic irradiation in females damages the ovaries resulting in ovarian failure.[85] The incidence of ovarian failure depends upon the age at irradiation and the dose of radiotherapy to the ovaries.[86] When prepubertal girls were treated with WART with 20 to 30 Gy, 71% of girls did not enter puberty and 26% had premature menopause.[86a] CSI with photons can have similar results when treating prepubertal girls, depending upon the dose received by the ovaries.[87]

In addition to organ and system damage, one of the most devastating late effects of radiotherapy is the development of subsequent neoplasms. Survivors of childhood cancer have an increased risk of developing subsequent neoplasms that can be benign or malignant.[88] They can be solid tumors or myelodysplasia/acute myeloid leukemia. Treatment with radiotherapy is associated with the development of subsequent solid tumors.[88] Radiation-associated second tumors occur within the prior radiotherapy field, are of a different histology from the primary tumor, and appear after a latency period of generally at least several years. Several factors are involved in the development of second tumors, including the patient's underlying genetic predisposition, the dose of radiation, and the age and gender of the patient, as well as the specific tissue irradiated.[89] The risk of subsequent radiation-related subsequent neoplasms continues to increase with increased follow-up.[90] This is in contrast to the risk of therapy-related myelodysplasia or acute myeloid leukemia, which plateau after 10 to 15 years.

CONCLUSION

Tremendous improvements have been made in the field of pediatric radiation oncology over the past few decades. Although many children require radiation for curative treatment of their malignancy, treatment can be delivered with fewer side effects. Technological advances allow for more precise delivery of radiation and much better avoidance of healthy tissues outside of the region requiring treatment. Thanks in large part to many cooperative group studies, there is better knowledge of the dose of radiation required to control various tumors. Chemotherapy has allowed for dose reduction for many tumor sites as well as improved outcomes. Pediatric oncologists are beginning to better understand the molecular characteristics and biology of tumors. In the future, this should lead to improved risk stratification, as well as targeted agents to work synergistically with radiation and further improve outcomes for these children.

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CHAPTER 49

Pediatric Surgical Oncology

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CHAPTER OUTLINE

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Successful treatment of pediatric solid tumors requires collaborative management by the disciplines of surgery, oncology, and radiotherapy. Multimodal therapy achieves the highest rate of cure in many solid tumors and often minimizes long-term morbidity. Smooth collaboration provides optimal care. The role of the surgeon varies greatly depending on the primary tumor, its site, and the presence or absence of metastatic disease. Surgeons should be involved in decisions regarding primary resection versus biopsy with neoadjuvant chemotherapy and delayed resection. The surgeon's knowledge of the risks of primary resection is critical; the method of biopsy is also very important. Inappropriate initial excisional biopsies with positive pathologic margins will require more extensive subsequent resection than if an initial incisional biopsy were performed. Similarly, inappropriate biopsies such as thoracoscopic biopsy of a chest wall Ewing sarcoma or a percutaneous or open biopsy of a Wilms tumor needlessly contaminate the thoracic and abdominal cavities, respectively, and require more intensive therapies, particularly the use of radiation therapy. A growing emphasis on minimizing the morbidity of therapy is required, because the survival for infants and children with many solid tumors is quite high. It is important, therefore, to avoid any diagnostic or therapeutic missteps that require more intensive therapy.

Surgery is critical in the staging of pediatric solid tumors. This is well established in Wilms tumor. In a classic study by Beimann Othersen and the National Wilms Tumor Study Group (NWTSG), surgeons had a 31.3% false-negative rate in their clinical assessment of lymph-node involvement with tumor by gross inspection and a false-positive rate of 18.1%.[1] It should be stressed that in Wilms tumor the protocols of the NWTSG and the Children's Oncology Group (COG) determine the intensity of chemotherapy as well as whether or not abdominal radiotherapy is utilized. Patients with pulmonary metastases (stage IV) with local stage I or stage II tumors receive intensified chemotherapy and pulmonary radiation but no abdominal radiation.

The vital importance of adequate lymph-node biopsy has been demonstrated in several studies. An increased incidence of local relapse occurred in children enrolled in the NWTSG-4 study if no biopsy was performed of the abdominal lymph nodes, particularly in stage-I cases.[2] This finding suggests that inadequate treatment of local disease in children without lymph-node biopsy results in an increased rate of local relapse. Although lymph-node biopsy is essential in children with Wilms tumor, an extensive retroperitoneal lymph-node resection has not been demonstrated to improve local control.[3]

Lymph-node biopsy in patients with rhabdomyosarcoma is critical as well. The incidence of lymph-node involvement in rhabdomyosarcoma is determined by the primary site. Children without evidence of distant metastases have an overall 10% incidence of lymph-node involvement. It is most common in tumors arising in the prostate (41%), paratesticular (26%), and genitourinary sites (24%).[4] Lesions in the extremities have an intermediate rate of 12%, whereas the orbit (0%), truncal sites (3%), and nonorbital head and neck (7%) have the lowest rate of lymphatic dissemination. In the extremity and genitourinary sites, assessment of lymph-node involvement is essential to ensure that radiation fields are appropriately designed and sufficiently inclusive. Whether the sentinel lymph-node biopsy technique optimizes the identification of nodal spread in rhabdomyosarcoma has not been established, but it has been reported to be feasible in pediatric patients with minimal morbidity.[5] Although lymph-node biopsy is critical to identify the extent of spread, a lymph-node dissection should generally not be performed, because it may produce lymphedema that complicates radiotherapy and subsequent surgical resection of the primary tumor.

Radiographic assessment is inadequate for staging rhabdomyosarcoma as it is in Wilms tumor. In the Intergroup Rhabdomyosarcoma Study III (IRS-III), 121 boys with paratesticular rhabdomyosarcoma had a retroperitoneal lymph-node dissection to evaluate nodal status.[6] The lymph nodes were assessed to be clinically negative based on computed tomography (CT) scan in 18% of the boys, 14% of whom had positive nodes when biopsy or retroperitoneal lymph-node dissection was performed. In the boys with clinically positive lymph nodes based on the CT scans, 94% were confirmed to be positive pathologically. Retroperitoneal relapse occurred in only two of the 121 boys, one of whom had pathologically negative lymph nodes and did not receive radiotherapy. Thus CT was very accurate if lymph-node abnormalities were identified, but it was not extremely sensitive in identifying nodal involvement. In a subsequent study, Weiner and colleagues reported an increased incidence of retroperitoneal relapse in children treated during IRS-IV. The use of abdominal radiation in this study was based on thin-cut CT scans in 98% of cases as compared with children treated during the preceding IRS-III, in which 94% had retroperitoneal biopsy or lymph-node dissection. In the subsequent study, IRS-IV, a decrease in stage-2 disease (positive lymph nodes) from 35% to 17% occurred when the staging was based on radiographic findings. This resulted in a decrease in the use of abdominal radiation in this cohort. The result was a fourfold increase in retroperitoneal lymph-node relapse. Another example of how critical adequate staging is to avoid local failure.[7]

This chapter discusses some of the critical surgical issues in the treatment of infants, children, and adolescents with solid tumors. The use of anesthesia in patients with an anterior mediastinal mass, appropriate methods of biopsy, the role of neoadjuvant chemotherapy, limb salvage procedures for osseous tumors, and the management of pulmonary metastases are addressed.

ANESTHESIA IN PATIENTS WITH AN ANTERIOR MEDIASTINAL MASS

Patients presenting with an anterior mediastinal mass pose a therapeutic challenge to the anesthesiologist and the surgeon. Correct diagnosis of the tumor provides the greatest chance of cure. There are, however, a plethora of reports in the anesthesia and surgical literature of patients with an anterior mediastinal mass suffering respiratory collapse upon the induction of general anesthesia. This occurrence has led to an understandable reluctance among anesthesiologists to use general anesthesia in this setting. Respiratory symptoms are a poor index of the risk of respiratory collapse, with the clear exception of orthopnea, which suggests a high risk that respiratory collapse will occur. An initial attempt to establish the radiographic parameters that would correlate with respiratory collapse upon induction of general anesthesia involved a retrospective evaluation of 74 adults with Hodgkin disease.[8] In this study, the authors used the ratio of the transverse diameter of the mediastinal mass and the transverse diameter of the chest as the critical parameter. They found the incidence of respiratory collapse was 2.1% with a mediastinal mass less than 31% of the transverse diameter, compared with 10.5% for a mass with a ratio of 32% to 44% and 33.3% for a mass with a ratio greater than 45%. Similar results were reported by Turoff and associates.[9]

Thorne Griscom and colleagues demonstrated that CT scan can accurately determine the tracheal dimensions in children and adolescents, and he established the normative values in children.[10-12] Azizkhan first used this methodology in reviewing 50 children with anterior mediastinal masses. He found that 15 patients in his cohort had "marked" tracheal compression that was defined as a tracheal cross-sectional area less than 66% of predicted. When general anesthesia was induced in eight of these 13 patients, total airway obstruction occurred in five, all of whom had 50% or less of the predicted tracheal area. It was suggested, based on these findings, that general anesthesia should be avoided in children with less than 66% of the predicted tracheal area.

In a retrospective study at Boston Children's Hospital, 42 patients were identified who had an anterior mediastinal mass and a CT scan and underwent a surgical procedure. These patients were divided into those with tracheal area greater than 75% of expected (19 cases), those with 50% to 75% predicted area (16 cases), those with 25% to 50% (5 cases), and those with less than 25% (2 cases).[13] One clear finding of that study was that the presence or absence of respiratory symptoms did not correlate well with the degree of tracheal narrowing shown by CT scan, except for orthopnea (Fig. 49-1). General anesthesia with spontaneous ventilation was performed in four patients (with tracheal areas 33%, 73%, 76%, and 98% of predicted). General endotracheal anesthesia with paralysis was used in the remaining 32 patients, only three of whom had tracheal area of less than 50% of

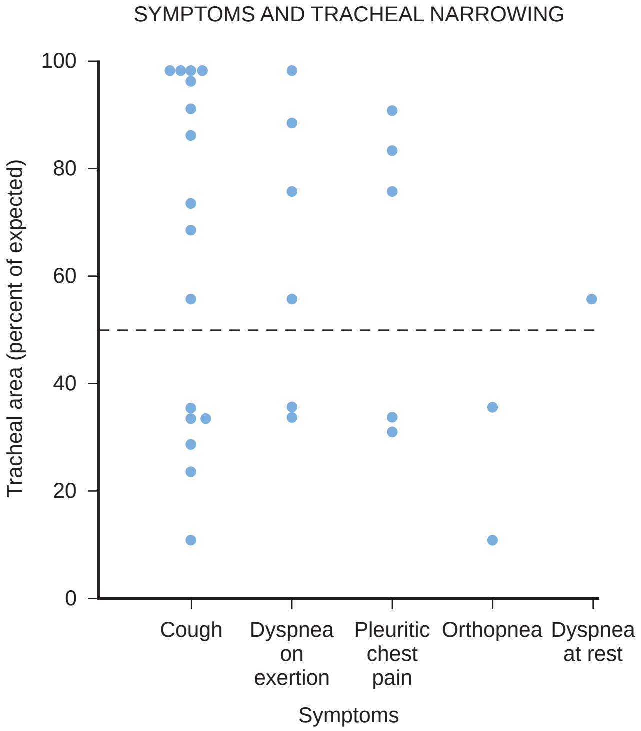

Figure 49-1 Correlation of symptoms with tracheal areas is shown for a cohort of 42 children and adolescents with an anterior mediastinal mass. *(Modified from Shamberger RC, Holtzman RS, Griscom NT et al: CT quantitation of tracheal cross sectional areas as a guide to the surgical anesthetic management of children with anterior mediastinal mass.* J Pediatr Surg *26:138–142, 1991.)*

predicted (30%, 26%, and 24%). One patient received preoperative radiation therapy (26%). None of these 32 patients had symptoms of orthopnea or dyspnea at rest, and only one had dyspnea on exertion. All tolerated anesthesia without difficulty, and no patients suffered respiratory or cardiovascular collapse. Adequate material for pathologic diagnosis was obtained in all cases. It was concluded in this study that general anesthesia was safe in patients with tracheal areas greater than or equal to 50% of predicted.

In a subsequent prospective study, 31 children with a mediastinal mass were evaluated with both CT scan and pulmonary function tests.[14] Although the use of pulmonary function tests had been proposed by multiple investigators as a method to assess anesthetic risk, little evaluation of this modality had been performed. Miller and Hyatt evaluated the flow volume loop curves in a series of patients with intrathoracic and extrathoracic obstruction resulting from strictures of the trachea, malignant tumors, or bilateral vocal cord paralysis.[15] The predominant distortion of the flow loops for intrathoracic obstruction was a marked reduction in the maximum expiratory flow rate. Hence in this prospective study, the peak expiratory flow rate was used as a critical factor. Patients with either a peak expiratory flow rate of less than 50% of predicted or a tracheal area of less than 50% of predicted had local anesthesia for their procedures. General endotracheal anesthesia was used only in patients with greater than 50% of predicted for both parameters. All patients in this study did well when general anesthesia was applied (Fig. 49-2). Further analysis of this cohort revealed that patients with a tracheal area greater than 50% but a peak expiratory flow rate (PEFR) less than 50% had one of two problems: either a very low total lung capacity (38% and 55% of predicted) resulting from the massive size of the tumor or moderate to severe bronchial narrowing by qualitative estimation (four of these five patients) (Fig. 49-3).

In a subsequent study by King and coauthors, 51 children with Hodgkin lymphoma and non-Hodgkin lymphoma (NHL) were evaluated. Respiratory symptoms were present in 49% of the children in this series and were more prevalent in children with non-Hodgkin lymphoma (76%) than in those with Hodgkin lymphoma (30%). The extent of aberration in the pulmonary function test was also worse in those with non-Hodgkin lymphoma. The first-second forced expiratory volume (FEV$_1$) was more affected in the group with NHL (mean value of 63 +/– 27% of predicted) compared with the children with Hodgkin lymphoma (mean value of 87 +/– 16% of predicted).

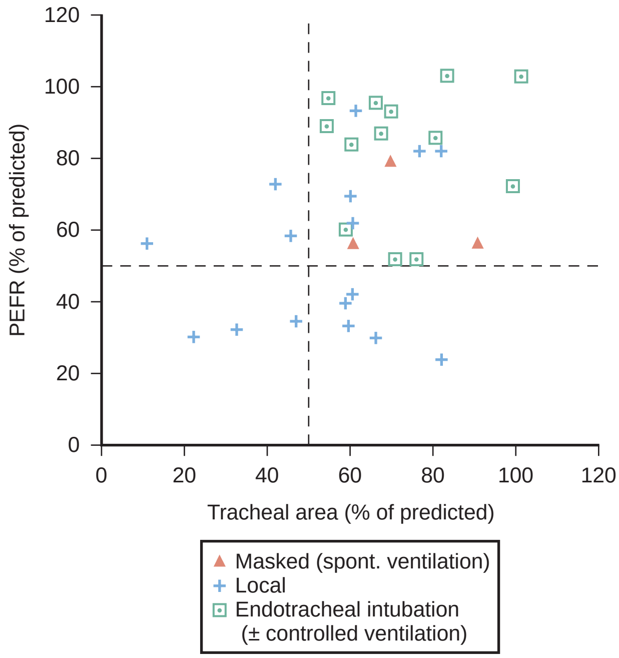

Figure 49-2 This figure shows the relationship between peak expiratory flow rate (PEFR) and tracheal area in a cohort of 31 children prospectively evaluated with an anterior mediastinal mass. Children with PEFR and/or tracheal area less than 50% of predicted (below or to the left of the *dotted lines*) all received local anesthetic and did well. Those children with PEFR and tracheal area greater than 50% of predicted received predominantly general anesthesia and did well *(right upper quadrant)*. The five children with tracheal areas greater than 50% of predicted but with peak PEFR less than 50% of predicted (in *lower right box*) might have been considered for general anesthesia if the tracheal area was the only parameter considered in assessing risk. Although the study could not demonstrate that these children would have had anesthetic problems, it did confirm that the parameters of greater than 50% PEFR and tracheal areas greater than 50% of predicted were safe for the administration of general anesthesia. *(Modified from Shamberger RC, Holzman RS, Griscom NT et al: Prospective evaluation of computed tomography and pulmonary function tests of children with mediastinal masses.* Surgery *118:468–471, 1995.)*

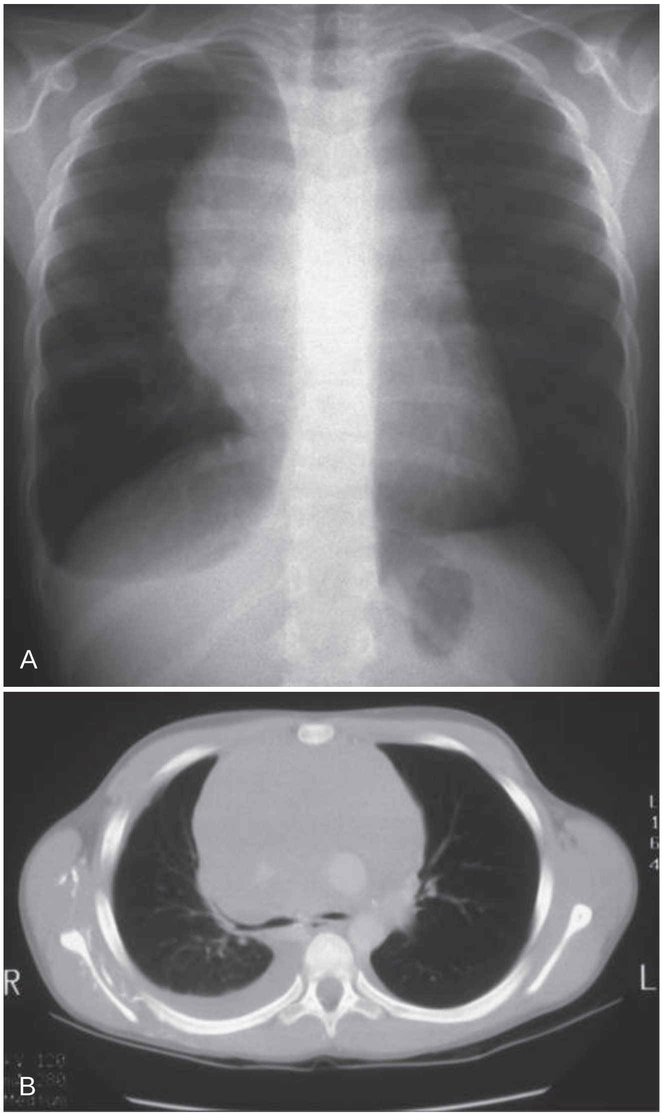

Figure 49-3 Chest imaging of an 11-year-old female who had a several-week history of a cough and dyspnea on exertion, as well as puffy eyes and orthopnea. The radiograph (**A**) reveals an anterior mediastinal mass, and a CT scan (**B**) shows a tracheal area that was 82% of predicted but revealed marked narrowing of the bronchi. The patient's peak expiratory flow rate was only 42% of predicted while sitting and 24% of predicted while supine. Aspiration of her pleural effusion confirmed the diagnosis of lymphoblastic lymphoma. *(From Shamberger RC: Preanesthetic evaluation of children with anterior mediastinal masses.* Semin Pediatr Surg *8:61–68, 1999.)*

Patients with severe obstruction have one of three therapeutic options available. Patients can be treated in a preliminary fashion with either radiation therapy or steroids/chemotherapy for the most likely diagnosis based on the clinical findings (Fig. 49-4). In a series of young adults who underwent a postradiation biopsy of an anterior mediastinal mass, a histologic diagnosis could not be obtained in eight of 10 patients because of extensive necrosis.[16] In some cases, one area of tumor can be shielded for subsequent biopsy while the bulk of the tumor receives radiation. Non-Hodgkin lymphoma (NHL) is also extremely responsive to steroid therapy. Pretreatment with steroids also often obscures the histologic diagnosis.[17,18]

The second option is aspiration of a pleural effusion if present. Patients with lymphoblastic lymphoma have a higher incidence of an associated pleural effusion (71%) than children with Hodgkin lymphoma (11.4%).[19] Aspiration of the effusion in patients with lymphoblastic lymphoma is often diagnostic. Cells in the effusion can be assayed by immunocytochemical studies as well

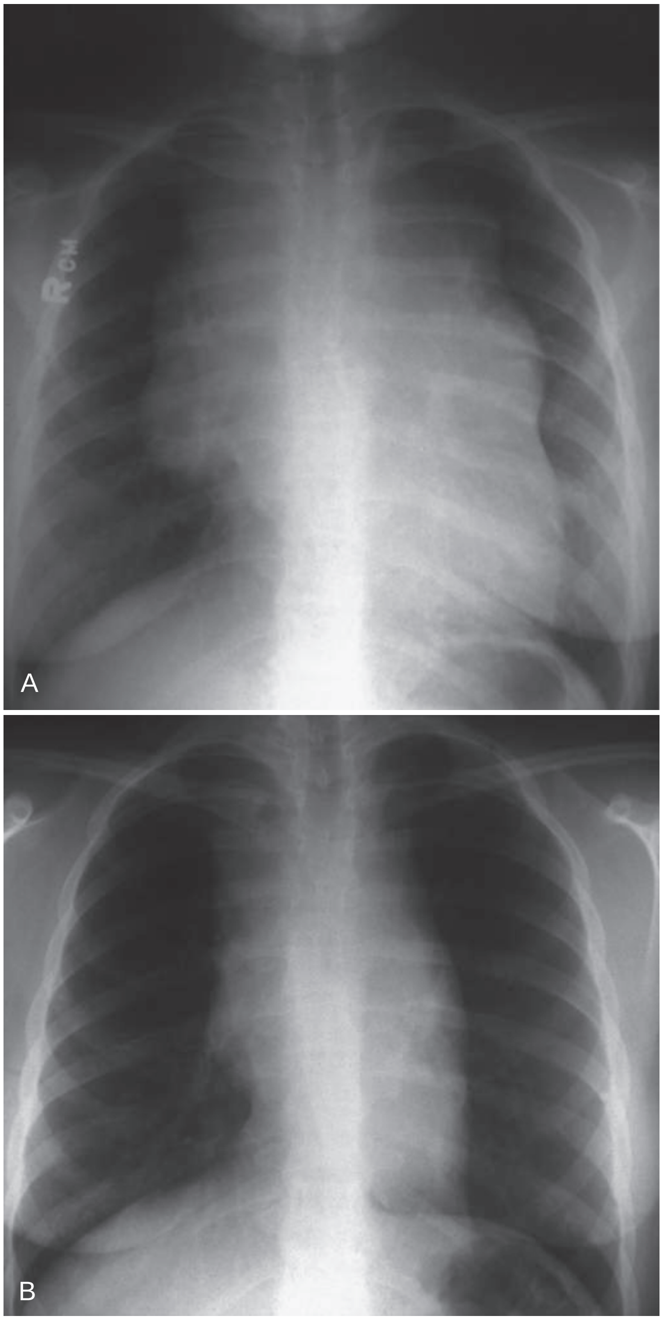

Figure 49-4 A 14-year-old girl with a nonproductive cough, retrosternal pain, 19-pound weight loss, and orthopnea. **A**, The chest radiograph showed a large anterior mediastinal mass. A biopsy was obtained in the suprasternal notch revealing lymphoblastic lymphoma. She received two courses of radiotherapy (250 cGy/per dose) over 2 days. Her chest radiograph obtained 7 days later (**B**) showed a remarkable response of the tumor to the radiation. *(From Shamberger RC: Preanesthetic evaluation of children with anterior mediastinal masses.* Semin Pediatr Surg *8:61–68, 1999.)*

as by cytogenetic evaluation, immunophenotyping, and cytology.

Third, in children with significant respiratory compromise and no pleural effusion and no lymph nodes palpable outside the chest, either a percutaneous needle biopsy under radiographic guidance or an open anterior thoracotomy (Chamberlin procedure) can be performed successfully when the child is under local anesthesia. Children should be seated in a semiupright position and spontaneously ventilating to maximize their pulmonary function (Fig. 49-5). This position also decreases venous congestion if it is present. Spontaneous ventilation minimizes collapse of the trachea by the negative pressure exerted by the chest wall. It has also been shown that in children, image-guided percutaneous needle biopsy can in most instances provide a diagnosis.[20] The major challenges have been in children with Hodgkin lymphoma, where initial biopsy was able to provide a conclusive diagnosis in only 50% of the children (six of 12). An algorithm for safe management of these children and adolescents is shown in Figure 49-6.[21] Following these

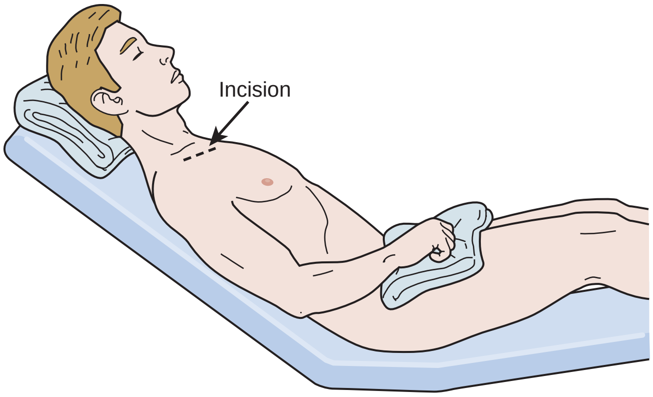

Figure 49-5 Proper position for patient with airway compromise to obtain a biopsy of an anterior mediastinal mass. The upright position optimizes pulmonary function and facilitates ventilation as well as venous drainage from potentially engorged vessels. *(From Shamberger RC: Preanesthetic evaluation of children with anterior mediastinal masses.* Semin Pediatr Surg *8:61–68, 1999.)*

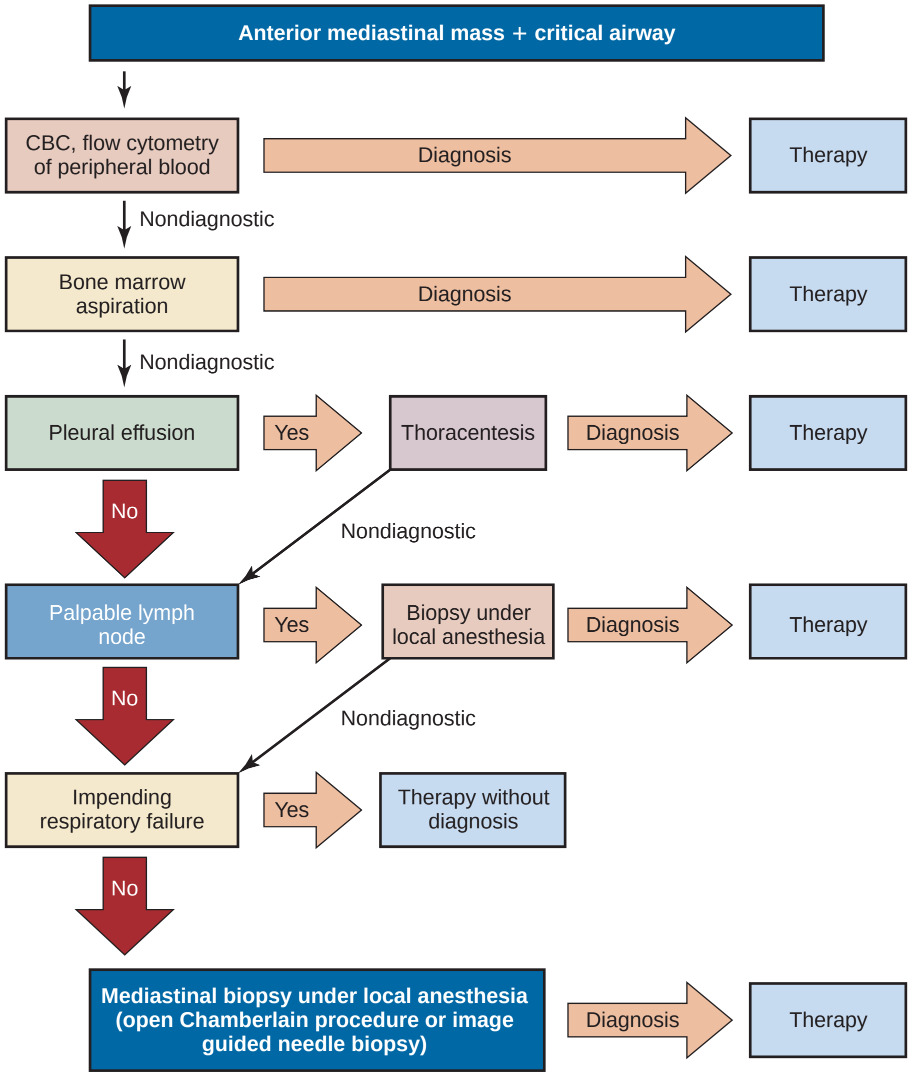
Anterior mediastinal mass + critical airway
CBC, flow cytometry of peripheral blood
Diagnosis
Therapy
Nondiagnostic
Bone marrow aspiration
Diagnosis
Therapy
Nondiagnostic
Pleural effusion
Yes
Thoracentesis
Diagnosis
Therapy
No
Nondiagnostic
Palpable lymph node
Yes
Biopsy under local anesthesia
Diagnosis
Therapy
No
Nondiagnostic
Impending respiratory failure
Yes
Therapy without diagnosis
No
Mediastinal biopsy under local anesthesia (open Chamberlain procedure or image guided needle biopsy)
Diagnosis
Therapy

Figure 49-6 Algorithm for workup of patients with a critical airway resulting from compression by an anterior mediastinal mass. *CBC,* Complete blood count.

guidelines for the use of general and local anesthesia and the biopsy techniques discussed, a biopsy can be obtained safely in essentially all children and adolescents with an anterior mediastinal mass.

METHODS OF BIOPSY

A biopsy serves as the basis of diagnosis and treatment of a tumor. It will establish the prognosis and define the multimodality therapy required for cure. Beyond establishing the diagnosis, a biopsy may be required for several other important purposes in the delivery of modern oncological care: to confirm the presence of metastatic disease; to determine the response to treatment; and to document the presence of complications from treatment, especially suspicious lesions that may be infectious in nature and pose a significant risk to a patient with immunosuppression.

The Five Principles

Five principles must be followed when considering a biopsy. First, to minimize the psychological and physical stress for the patient and family, the need for a biopsy, how it will be performed, and the possible complications must be discussed with the patient and family.

Second, a preprocedure conference between the oncologist, pathologist, radiologist, and surgeon must ensue to determine the best biopsy technique to obtain the required amount of tissue and that the tissue is processed appropriately (light microscopy, electron microscopy, cytogenetic and other biogenic marker evaluation, and immunohistochemical analysis) to provide the maximum information.

Third, the precise biopsy route and site must be selected to facilitate future therapy. The wrong incision or biopsy site can greatly complicate the subsequent “local” therapy, whether it be resection or radiotherapy. Factors that must be considered are local tissue trauma, risk of tumor spillage or hemorrhage, and minimal violation of anatomic planes.

Fourth, the patient’s general state of health, past medical history, and immune status must also be considered to fully assess the physiologic impact of anesthesia and biopsy, including risk of hemorrhagic and respiratory collapse.

Lastly, the possibility of combining several invasive procedures into one anesthetic should be considered. For example, a patient that requires a biopsy of a lesion may also need central venous access or a bone marrow biopsy and aspirate, and these procedures could all be combined. Although the grouping of procedures is not always possible, it is an option that the oncologic team needs to discuss in advance and then present a uniform plan to the patient and parents.

Method of Biopsy Overview

The type of biopsy a patient requires varies greatly and is dependent upon the site of the mass, the characteristics of the lesion itself (cystic or solid), the condition of the patient, and the diagnostic possibilities. Once the decision has been made to proceed with a biopsy, the first technical question that must be answered is the amount of tissue required and what biopsy technique will avoid increasing the stage of the patient or complicate future therapy. The fundamental separation of techniques is into small- or large-specimen biopsies.

Small-Specimen Biopsies

Small-specimen biopsies are those that are acquired through the use of hollow needles that sample an aspirate (fine needle aspiration [FNA]) or core (core needle biopsy [CNB]) of the tissue. They are often obtained with imaging assistance (ultrasound, fluoroscopy, or CT). The advantages of small-specimen biopsies are that they can often be performed with minimal or no anesthesia and are less invasive than an open procedure. Cost is also generally less for this approach, and recovery time for the patient is quicker. The disadvantages to this approach, however, are that the amount of tissue returned is quite small, which may create challenges in establishing a diagnosis or may not provide adequate tissue to complete the necessary secondary tests to adequately define the lesion. Furthermore, if imaging modalities are used to assist in this form of biopsy, then radiation exposure will occur if CT or fluoroscopy is used as opposed to magnetic resonance imaging (MRI) or ultrasound. This issue of radiation exposure is of extreme importance in the pediatric population, because there is an increased risk of developing a malignancy from radiation exposure.[22,23] In addition, there is the possibility of tumor spread in needle tracts or hemorrhage.[24-26] The risk of sampling error in which the specimen harvested may not adequately represent the entirety of the mass is always a concern with biopsies, and the smaller the sample, the larger the concern. Despite these limitations, small-specimen biopsies are an accepted standard of practice in pediatric oncology, and their use will be described in detail in the following section.

Fine Needle Aspiration and Core Needle Biopsy. FNA specimens are acquired by using a 20- to 25-gauge needle connected to a standard syringe with the plunge partially withdrawn (Fig. 49-7). The needle is inserted directly into the lesion guided by palpation or with image guidance (Fig. 49-8). Multiple passes are taken from the lesion as suction is applied to the syringe. Upon removing the needle, the contents of the syringe and needle are placed directly onto slides for cytological analysis. Multiple biopsies (3 on average) can be taken with this approach from the same lesion at the same time, and the cytological

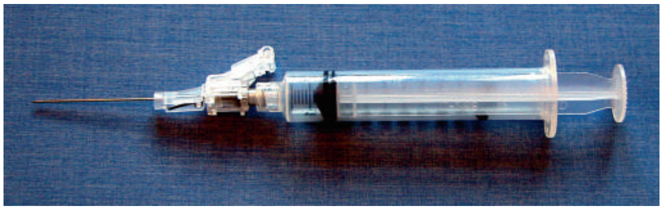

Figure 49-7 This figure shows a standard fine needle aspiration device with a 22-gauge needle attached to a 10-cc syringe that has the plunger partially withdrawn to facilitate the removal of the aspirate.

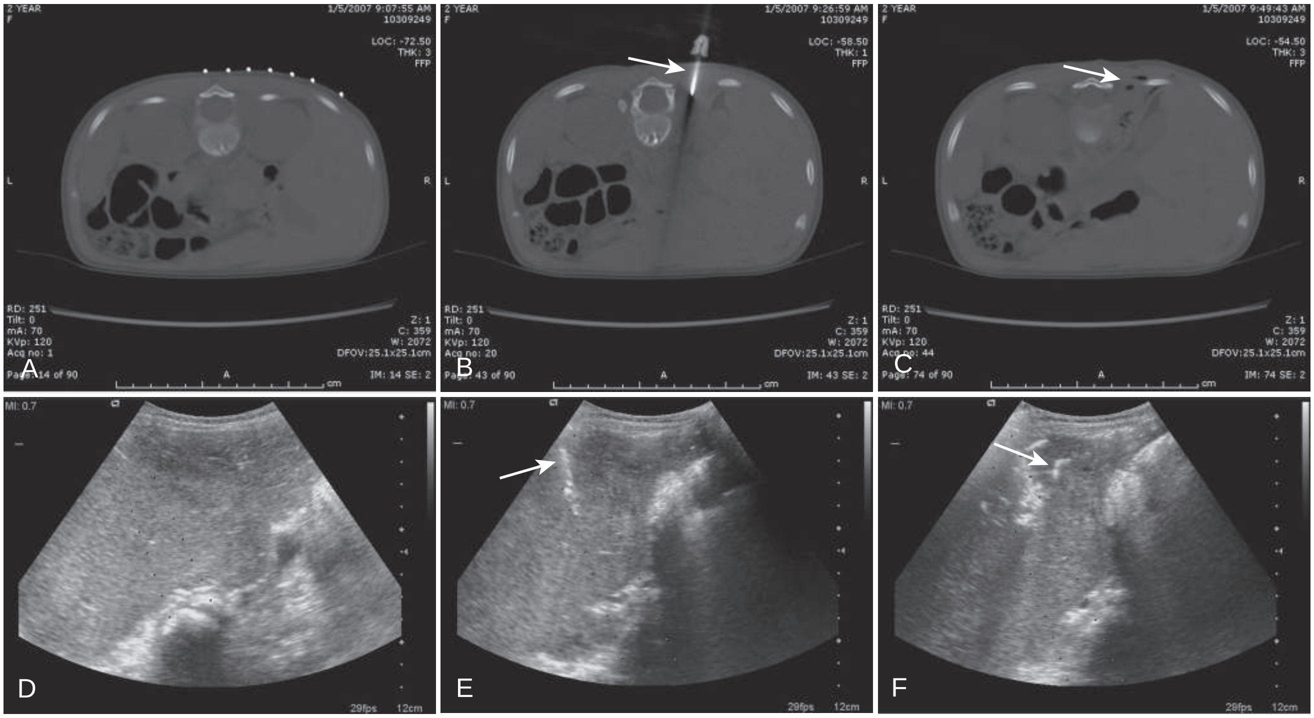

Figure 49-8 **A**, Prebiopsy computed tomography (CT) of a retroperitoneal mass with external grid for biopsy marking. **B**, The same patient with the needle in the retroperitoneal mass *(arrow)*. **C**, Postbiopsy CT with air in the mass and soft tissues *(arrow)* from the biopsy procedure. **D**, Prebiopsy ultrasound of a liver mass. **E**, Ultrasound-guided biopsy of a liver mass *(arrow)*. **F**, Postbiopsy hemorrhage in the mass is depicted *(arrow)*.

results are available for almost immediate review. However, no histologic assessment is possible using this technique, thus causing difficulty in diagnosing certain tumors with cytologic appearances that are quite similar, such as the small, round, blue cell tumors.

Histopathologic analysis is possible with a CNB. CNB specimens are retrieved using larger, hollow-bore needles (13- to 20-gauge) attached to spring-loaded devices that allow for one-step tissue harvest and extraction (Fig. 49-9). Image guidance is often used during this procedure, just as it is for the acquisition of FNA samples. The CNB device is often inserted through a coaxial sheath or cylinder that allows the operator to instill a coagulant into the tissue defects made by the CNB device to reduce the incidence of hemorrhage and tumor spillage (Fig. 49-10). Generally multiple specimens (10 or more) from different areas of the lesion are gathered with this technique to minimize sampling error and to provide more tissue for complete pathological analysis.[27-29] A representative example of the microscopic appearance of the biopsy specimens for FNA and CNB is shown in Fig. 49-11. Imaging techniques can often distinguish areas with viable versus necrotic tissue to help identify the area best to biopsy.

The results and outcomes of small-specimen biopsies from almost any organ in the body have been reported extensively in adult patients with excellent diagnostic accuracy and minimal procedural morbidity. However, small-specimen biopsy techniques and comparison of these techniques in pediatric oncology patients, especially with abdominal lesions, have only recently gained widespread acceptance and usage.[29-35]

Hugosson and colleagues in 1999 reviewed their series of image-guided percutaneous small-specimen biopsies in children from 1992 through 1996.[31] A total of 90 biopsies (53 FNA, 37 CNB) were taken in 75 patients (mean age of 6.9 years). They compared the use of FNA with CNB (1.2 mm core specimens) under ultrasound guidance. Their cohort had many different malignant

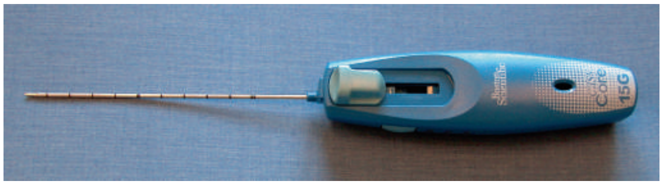

Figure 49-9 An automated 15-gauge core needle biopsy device. *(Courtesy of Easy Core Biopsy System, Boston Scientific, Natick, Mass.)*

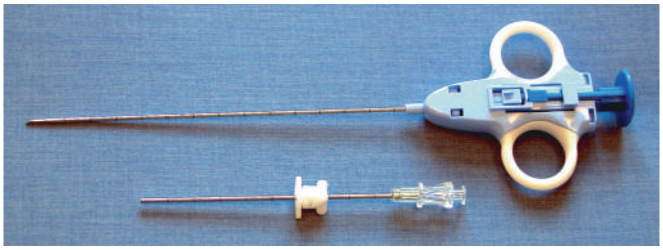

Figure 49-10 An automated 16-gauge core needle biopsy device with a 15-gauge coaxial sheath. *(Courtesy of Coaxial Temno Evolution, Allegiance Healthcare Corporation, McGraw Park, Ill.)*

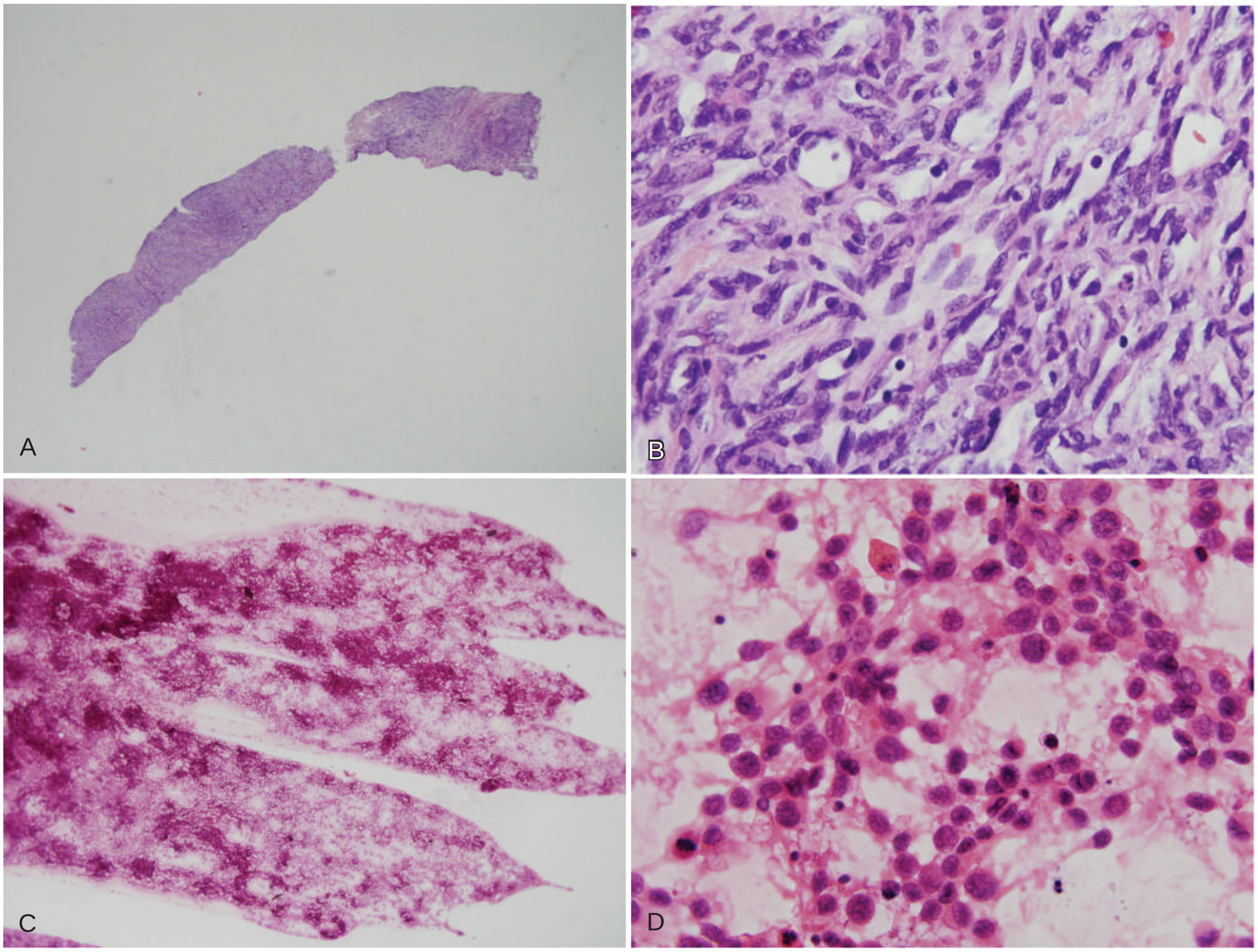

Figure 49-11 **A**, Core needle biopsy specimen from a liver mass at ×2 (angiosarcoma). **B**, CNB at ×600 with sheets of moderately pleomorphic spindled cells and lumina formation. **C**, Fine needle aspiration specimen from a submandibular lymph node at ×2 (nasopharyngeal alveolar rhabdomyosarcoma). **D**, Specimen at ×600 revealing round and elongated pleomorphic tumor cells with marked apoptosis.

processes, and they obtained biopsies from the liver, kidney, adrenal gland, spleen, pelvic masses, and enlarged abdominal lymph nodes. Their FNA diagnostic accuracy was 77% (73% for malignant lesions and 83% for benign lesions) and 95% for CNB (93% malignant lesions and 100% for benign lesions). The complication rate was 8%. They concluded that CNB is a more accurate technique with a similar complication rate to FNA. These results were echoed by Hussain and colleagues, who examined their institutional experience on image-guided percutaneous biopsies (using CNB) in children (mean age, 4 years-old).[32] Ninety-four percent of their cohort had abdominal or pelvic masses. Their reported diagnostic accuracy was 88%, and the initial diagnosis was subsequently confirmed by whole-specimen pathologic analysis. Their complication rate was approximately 8%, and they also concluded that CNB was generally successful in diagnosing tumors in children.

Sklair-Levy and colleagues published their institution's 10-year experience in small-specimen biopsies on pediatric patients.[33] They performed 69 (62 CNB) biopsies in 57 patients using image guidance. Their diagnostic accuracy was 88.7% (55 of 62 patients). For malignant lesions, CNB had an accuracy of 98% as an isolated diagnostic technique in solid malignant tumors, although seven CNBs had to be repeated because the initial results were nondiagnostic. All seven repeated CNBs were correct on subsequent evaluation. They performed seven FNAs with a diagnostic accuracy of only 28%, and these patients then underwent surgical excision of the lesion in question. Although this accuracy rate is far lower than previously reported,[31] none of the lesions in question were abdominal or pelvic masses, and the majority of these lesions were enlarged lymph nodes. Therefore direct comparison of the utility and diagnostic accuracy of FNA in this situation is not possible. Their accuracy with primary, metastatic, or recurrent lesions was in excess of 94% for all tumor types except lymphomas, for which they were correct only 60% of the time. Interestingly, they did not have a single complication.

A more comprehensive study was published by Skoldenberg and colleagues. They examined their institution's results with 147 CNBs in 110 children (mean age 4 years) from all anatomic sites (61 abdominal and pelvic lesions of the total 110 children).[34] An overall accuracy of 89% was achieved for the CNBs performed. Twenty-four children required 37 additional biopsies for a nondiagnostic first specimen (12), a question of recurrence (22), and an insufficient quantity of tissue for all the diagnostic studies needed (3). They reported a sensitivity of 82% and a positive predictive value of 98.8%. A complication rate of 7% (10 of 141 cases) was seen, of which half were hemorrhagic in nature. Fourteen of the patients required narcotics for pain management postprocedure. A statistically significant difference in the occurrence of postprocedure pain was noted in older children. The authors strongly encourage the use of CNB in the diagnosis of pediatric malignancy, and they tout its superiority to FNA.

St. Jude's Children's Research Hospital published their experience of CNB in pediatric solid tumors over 5 years.[30] Two hundred and two CNBs were performed using image guidance and anesthesia. Ultrasound was the predominant mode of imaging assistance (124 of 202), and the biopsies were performed to evaluate both primary and metastatic sites of disease. One hundred and three primary CNBs were performed with a 96.9% sensitivity, a 100% specificity, and an accuracy of 98%. For metastatic or recurrent lesions, they measured a sensitivity of 83.1%, a specificity of 100%, and an accuracy of 87.9%. There were no false positive results. In the cohort of patients with abdominal and pelvic tumors, the sensitivity and accuracy of CNB was 100%. They had a complication rate of 13.4%, with hemorrhage being the most common. Their results and experience across all anatomic regions and pathologic lesions demonstrate that small-specimen biopsies should be considered the initial diagnostic procedure of choice. Therefore solid masses in the pediatric population should be approached where indicated with image-guided small-specimen biopsies, especially using the CNB technique.

Finally, Sebire and Roebuck[36] reported the results from a systematic literature review they conducted to determine the utility, success, and complications from image-guided CNBs. Echoing similar studies, they report that 94% of all procedures obtained adequate tissue for evaluation, and of that group an accurate diagnosis by histopathologic analysis was achieved in 94% with only a 1% complication rate.

Large-Specimen Biopsies

Large-specimen tissue biopsies involve the removal of all (excisional) or part (incisional) of a lesion using an open or minimally invasive surgery (MIS) technique. This method of biopsy should not be confused with a formal extirpative procedure, because the surgeon does not remove either part or all of the entire mass with concerns about adequate margins of resection. The goal of these procedures is to remove a piece or all of the mass in question to provide tissue for a diagnosis, not for local control of the primary site. Large-specimen biopsies are also performed in anatomic areas or on lesions where image-guided small-specimen biopsies are more difficult because of proximity to major vessels or nerves, the lesions are very vascular, or the lesions cannot be reached without damaging overlying organs and structures (i.e., retroperitoneal lymph nodes). Large-specimen biopsies may be performed sequentially with a small-specimen biopsy using the same anesthetic if there is any doubt regarding the adequacy for a histopathologic diagnosis of the FNA or CNB.

Several disadvantages of large-specimen biopsies are a longer procedural and anesthetic time, recovery time, and need for hospitalization. They can delay the initiation of adjuvant therapies and are more costly. Assuming there are no complications, the vast majority of pediatric patients who have a small-specimen biopsy performed on a solid abdominal mass do not stay more than 8 hours postprocedure, as evidenced in recent large studies.[30,32-35,37] The same cannot be said for large-specimen biopsies. Finally, operative exploration for large-specimen sampling can complicate definitive local-control therapies, especially from an operative standpoint. Violating the thoracic or peritoneal cavities induces adhesion formation that can complicate the subsequent resection.

Although significant disadvantages to large specimen biopsies exist, there are marked advantages. First, large biopsy samples are obtained, and multiple areas are sampled within the lesion, decreasing the likelihood of sampling error. Second, a detailed macroscopic evaluation of the lesion in question and all surrounding structures can be performed to search for extension or metastasis from the tumor. Third, radiation exposure is avoided for the patient and the healthcare team. Finally, hemostasis and tumor sampling is controlled and confirmed directly before closing the incision to minimize the complications of tumor spillage and hemorrhage.

In the last decade, several groups have reported success using a MIS approach for either incisional or excisional large specimen biopsies. The MIS approach relies on the same basic tenets of surgical discipline as does an open procedure, but it uses smaller incisions and advanced technology to achieve these goals. An operating telescope capable of excellent image resolution and magnification is used to display a picture on closed-circuit monitors. Dissection and biopsy is performed with long, fine instruments introduced through a percutaneously placed 2- to 12-mm canula. When operating in the peritoneal cavity, carbon dioxide gas (CO_2) is introduced by an insufflation apparatus that distends the abdominal wall to provide adequate space to assess the abdominal structures. The pressure is kept as low as possible, consistent with adequate exposure. An important point that must be discussed with the anesthesiologist is the degree of respiratory impairment that CO_2 insufflation may induce, especially in pediatric patients with extremely compliant diaphragms. If at any point the child becomes physiologically unstable, the CO_2 gas is released and the procedure is terminated. Advantages of the MIS approach are decreased postoperative pain from the small incisions, a shorter postoperative stay, improved cosmetic results, and an earlier return of bowel function.[35,38] A complication rate in the pediatric population of 4% or less has been reported in a recent large series, but this rate is clearly related to the experience of the surgeon.[38]

In 1973 Gans and Berci described their successful experiences with MIS in pediatric patients, but their procedures were not related to tumors.[39] In fact, it was not until Holcomb and colleagues' report from the Surgical Discipline Committee of the Children's Cancer Group (CCG) in 1995 that the MIS approach was formally reviewed for its efficacy in oncology.[40] In this study from 15 participating centers, 25 abdominal and 63 thoracic explorations were undertaken for the purposes of diagnosis (primary, relapsed, and metastatic lesions), staging, assessment of resectability, and the evaluation of treatment complications. All abdominal lesions (16), and 50 of 51 thoracic lesions that required biopsy were successfully diagnosed. There were no perioperative mortalities, and only seven complications—all in the thoracic cohort.

A prospective trial sponsored by the National Institutes of Health (NIH) concerning the use of MIS in pediatric oncology patients was begun by the combined Surgical Discipline Committees of the CCG and the Pediatric Oncology Group (POG) in 1996.[40] The study failed for lack of accrual, and it was closed after 2 years. A questionnaire soliciting reasons for the failure of this study documented that lack of familiarity with the MIS technique by both the surgeon and the institution, surgeon and pediatric oncologist bias against the MIS technique, and the failure to submit the necessary paperwork to local institutional review boards for study approval were statistically significant factors in the study's failure. Despite these results, MIS is gaining acceptance in pediatric oncology, especially for the diagnosis of lesions.

Spurbeck and colleagues at the St. Jude's Children's Research Hospital recently published their experience with MIS in pediatric oncology patients from 1995 through 2000.[41] One hundred and one patients underwent 113 operations (64 abdominal and 49 thoracic procedures). Abdominal biopsies were attempted in 25 patients and were successful in 23 (92%). Evaluation of resectability was determined successfully in two other cases, and seven extirpative procedures were performed. There were three complications in the abdominal group, and four MIS procedures were converted to open procedures. There were three complications reported in the abdominal cohort. The thoracoscopic procedures had similar rates of success, conversion, and complications. They reported no port-site recurrences or metastases in either the abdominal or thoracic cohorts of their study. Although a feared complication, the incidence of port-site recurrence is rare in thoracic cases (a single report by Sartorelli and colleagues).[42] However, abdominal port-site recurrence rates are higher but depend on the tumor involved.[43-46] Needless to say, these are retrospective data spanning many institutions, surgeons, tumors, and techniques. The conclusions reached by Spurbeck and colleagues attest to the safety and efficacy of MIS techniques in the successful diagnosis of pediatric tumors, but its use in the formal extirpation of tumors remains to be established.

A recent analysis by de Lijster and colleagues[47] attempted to define the role for and success of MIS techniques versus open procedures in pediatric abdominal and thoracic tumors, but they discovered that there were no randomized controlled or controlled clinical trials on this topic in the literature. Hence no formal recommendation could be made as to the effectiveness, superiority or equivalency of MIS techniques versus open procedures in childhood tumors. Obviously this topic deserves greater scrutiny and investigation through the development and completion of a rigorous controlled trial as has been done in adults.

Extremity Tumors

Biopsy of a soft-tissue or bone tumor in an extremity should be considered the first interventional procedure in limb salvage. Although the technical aspects are quite simple, the decision-making process is not. At risk are not only the patient's limb, but also overall disease control and outcome.

As noted, biopsy is truly a multidisciplinary process involving medical oncologists, surgeons, radiologists, pathologists, and radiation oncologists. Accuracy and outcome of biopsy procedures are improved when each of these specialists has a knowledge of musculoskeletal tumors and limb salvage principles. This is often best accomplished at tertiary centers with experience in the management of sarcomas.[48,49]

The first step in planning a biopsy is performing a complete work-up. Based on history and physical examination findings, suspicion is raised about a tumor. Plain radiographs are often diagnostic of a bone malignancy and are crucial. MRI is also essential for both bone and soft-tissue tumors to define the extent of the tumor itself and involvement of neighboring compartments and neurovascular structures. It should be performed before biopsy to avoid artifact signal. Ultrasound is helpful to determine whether a soft-tissue mass is cystic or solid, but MRI is the gold standard for the evaluation of a suspicious mass.

Based on the findings from the work-up, a differential diagnosis can be established through collaboration at a multidisciplinary conference. All the specialists involved should be aware of the potential of a malignancy so that proper techniques are used for the procedure itself and for handling of the tissue. The biopsy approach is selected by the surgeon based on knowledge of limb-salvage surgery. The biopsy tract should be along the planned incision for limb salvage so that the entire tract can be excised later with the resection of the tumor. This is critical, because errors in placement of the biopsy incision can contaminate neurovascular and other important structures, making limb salvage impossible or necessitating large soft-tissue flaps for coverage.[48,49] The tumor should be approached in the most direct manner and through a muscle, not in between muscles, to decrease contamination of normal tissue.

Biopsy of an extremity tumor can be performed by open or closed (CNB) techniques (Table 49-1). Open biopsy offers the advantage of a larger tissue sample so that adequate pathologic evaluation can be performed. However, the major disadvantages are a higher risk of contamination of critical structures and complications such as bleeding, infection, and pathologic fracture. It is also more expensive and requires more time. Incisional

TABLE 49-1 Comparison of Open versus Needle Biopsy

	Open	Needle
Sample size	Larger	Smaller
Accuracy	Higher or equivalent	Lower or equivalent
Morbidity	Higher	Lower
Risk of contamination	Higher	Lower
Complications	Higher	Lower

biopsy is performed by entering into the tumor itself and removing a small section of it. The pseudocapsule is repaired, and careful hemostasis is obtained to try to decrease tumor-cell spillage or creation of a hematoma. Excisional biopsy involves removing the whole tumor in one piece. This is rarely indicated for extremity tumors, because resection surgery for malignant tumors is so vastly different from that for benign tumors. Also, most musculoskeletal tumors are treated with neoadjuvant therapy before excision. This approach is used for two reasons. First, it will increase the ability to perform limb-salvage surgery. Second, it provides an in vivo assay of response to standard chemotherapy (see Neoadjuvant Therapy for Solid Tumors).

Needle biopsy on the other hand is associated with less morbidity for the patient. It carries a lower risk of complications and contamination and is often simpler to perform. However, the smaller tissue sample size is a disadvantage and decreases the accuracy rate of the diagnosis. Use of a large-bore needle to remove a core of the tumor is thus usually more advantageous than FNA, as discussed. Sampling error is another disadvantage of CNB. Image-guidance with CT (Fig. 49-12) or ultrasound improves accuracy and decreases sampling error, but it is imperative that the surgeon discuss the approach with the radiologist performing the procedure. There is a risk of local recurrence if the needle biopsy tract is not excised with the tumor, but the risk is probably less than with open biopsy.

Pathologic analysis also requires specific knowledge of musculoskeletal tumors, especially with smaller sample sizes. The specialist obtaining the biopsy should discuss the case with the pathologist. A frozen section at the time of biopsy is essential, not for definitive diagnosis, but to ensure sufficient neoplastic tissue is available to the pathologist to make a diagnosis. Proper tissue handling is also crucial for the pathologist to be able to arrive at the correct diagnosis. Hematoxylin and eosin staining as well as most immunohistochemistry can be performed on tissue placed in standard formaldehyde. However, electron microscopy, cytogenetic investigation, tissue culture, flow cytometry, and immunofluorescence all require special handling and often fresh tissue. The discovery of specific cytogenetic abnormalities in certain tumors (e.g.,, t[11;22] in Ewing sarcoma [EWS] and t[2;13] in alveolar rhabdomyosarcoma) has increased the ability of pathologists to make the diagnosis with small tissue samples, but they must be processed in the correct manner.

Combined discussion of the pathology completes the biopsy process. The multidisciplinary team caring for the patient can then review all of the staging studies with the known diagnosis in mind and arrive at a recommendation for treatment.

NEOADJUVANT THERAPY FOR SOLID TUMORS

Wilms Tumor

Cooperative group trials by the NWTSG in North America, the International Society of Paediatric Oncology (SIOP) in Europe, and the United Kingdom Children's Cancer Group (UKCCG) have provided a great wealth of information regarding the optimal management of Wilms tumor. A major difference in approach exists between SIOP and NWTSG. In North America, primary resection followed by chemotherapy and radiotherapy is favored. In the SIOP studies, children generally first receive either chemotherapy or radiotherapy followed by resection. This approach has been driven by a high incidence of operative tumor rupture in their early series. In SIOP 1 the use of preoperative radiation therapy (20 Gy) was randomized versus primary resection. The rate of intraoperative rupture decreased from 33% to 4% with the use of radiation.[50] Survival was not affected, and the incidence of local recurrence was not reported. The frequency of operative rupture that occurred in NWTS 1 and 2 were 22% and 12%, respectively.[51,52] In a subsequent SIOP study, preoperative therapy was randomized between abdominal radiation (20 Gy) and actinomycin D compared with those receiving vincristine and

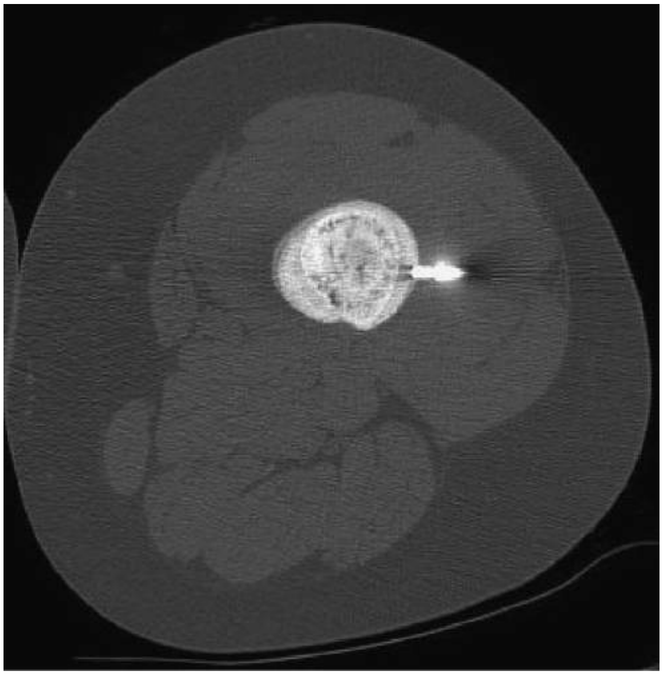

Figure 49-12 Core needle biopsy of an osteosarcoma of the femur under guidance with computed tomography.

actinomycin D. The frequency of rupture was 9% and 6%, respectively.[53]

In SIOP 6, which started in 1980, all patients received initial preoperative chemotherapy (vincristine and actinomycin D). Radiotherapy was given after resection to children with stage II N1 and stage III disease. Children with stage II N0 disease were randomized to receive either 20 Gy abdominal radiotherapy or no radiation. All children received vincristine and actinomycin D for 38 weeks. The radiotherapy randomization was halted after 123 children were randomized, 64 to radiotherapy and 59 to chemotherapy alone. Six of the 59 children who did not receive radiotherapy had local recurrences, whereas no recurrences occurred in the group who did. These findings suggest that prenephrectomy treatment altered the pathologic findings that would have led to a diagnosis of stage II N1 or stage III disease (i.e., lymph-node involvement or capsular penetration), resulting in the standard administration of local radiation. No statistical difference in survival has been seen in these children after extended follow-up observation, because those who relapsed had treatment alternatives.[54,55]

In SIOP 9, which began in 1987, preoperative therapy was randomized between 4 and 8 weeks' duration. The goal was to produce a larger proportion of stage I tumors.[56] In this study, postoperative radiotherapy for children whose disease was stage II N0 was replaced with an anthracycline (epirubicin or doxorubicin). Preoperative treatment for patients without distant metastases consisted of four weekly courses of vincristine and three 2-day courses of actinomycin D every 2 weeks versus 8 weeks of the identical therapy. There was no advantage seen from the extended therapy in terms of staging at resection (stage I, 64% versus 62%) or in intraoperative tumor rupture (1% versus 3%). Therapy after resection was based on the pathologic findings. In SIOP 9, the surgery-related complications were reported to be 8%.[57]

In both the NWTSG protocols and those of the COG, primary surgical resection is generally recommended. There are certain generally accepted situations where preoperative therapy is appropriate. These include the occurrence of Wilms tumor in a solitary kidney, bilateral renal tumors, and respiratory distress from extensive metastatic disease in the lungs. In these cases pretreatment biopsy should be obtained, and percutaneous biopsies are often used. Although needle tract seeding has been reported, this is quite rare in Wilms tumor. The aim of neoadjuvant treatment in the bilateral tumors and solitary kidney is to preserve the maximum amount of renal parenchyma and function. Current COG protocols for bilateral renal tumors have intensified the neoadjuvant therapy to include three agents (vincristine, actinomycin D, and doxorubicin) with each cycle of therapy. The duration of therapy has also been limited to avoid incremental increases in the intensity of therapy when further regression in the size of the tumor is unlikely.

The efficacy of preoperative treatment to achieve partial nephrectomy in patients with unilateral Wilms tumors has been evaluated by several centers. McLorie and associates in Toronto obtained percutaneous biopsy in 31 children with Wilms tumors. Multiagent chemotherapy was then administered for 4 to 6 weeks. Partial nephrectomy was performed in nine children (four with unilateral tumors and five with bilateral tumors).[58] Two of these children suffered an intraabdominal relapse, and only four of the 30 unilateral tumors (13.3%) were amenable to partial nephrectomy. Hence in unilateral cases not identified by prospective radiographic imaging in patients with syndromes, a partial nephrectomy is rarely possible even with preoperative therapy. Another assessment of the feasibility of partial nephrectomies was performed at St. Jude Children's Research Hospital.[59] The preoperative CT scans of 43 children with nonmetastatic unilateral Wilms tumor were reviewed. The criteria used to define those tumors suitable for partial nephrectomy were involvement by the tumor of one pole and less than one third of the kidney, a functioning kidney, no involvement of the collecting system or renal vein, and clear margins between the tumor and surrounding structures. Only two of 43 scans (4.7%) met the criteria, suggesting that a partial nephrectomy was feasible. The primary concerns regarding the administration of preoperative chemotherapy (as in the studies of Kozzi and associates and Moorman-Voestermans and co-workers) to create "resectable" small tumors is that these children may be curable by surgical resection alone without subjecting them to the toxicity of additional treatments.[60,61]

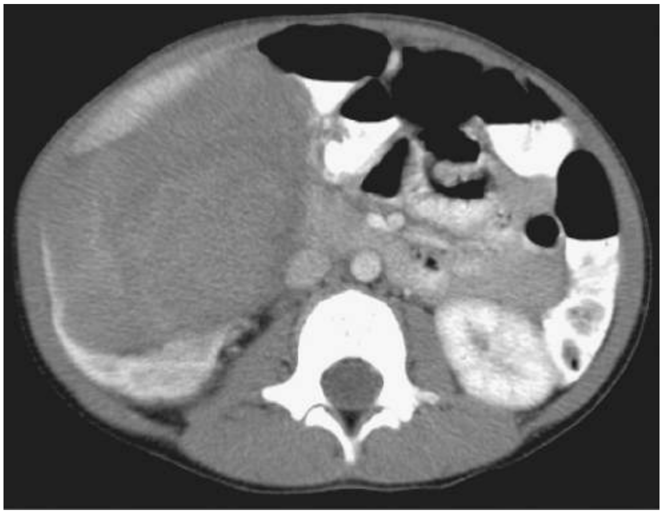

Figure 49-13 Computed tomography scan in a 4-year, 10-month-old girl who had a palpable abdominal mass. The study revealed a large tumor with a very thin renal capsule. A primary resection was performed, and the tumor was stage II with favorable histology. She went on to receive double agent therapy with vincristine and actinomycin D.

The surgical resectability of Wilms tumors has been poorly correlated with radiographic studies. In fact, extremely large tumors can be stage II lesions and be fairly safely resected (Fig. 49-13). The surgical recommendations in the NWTSG studies have been that if resection of the tumor would involve resection of adjacent organs (i.e., liver, pancreas, or spleen) because of adherence, biopsies should be obtained and subsequent chemotherapy applied before resection, because such resections are associated with an increased rate of complication (Fig. 49-14). Complications in the NWTSG-4

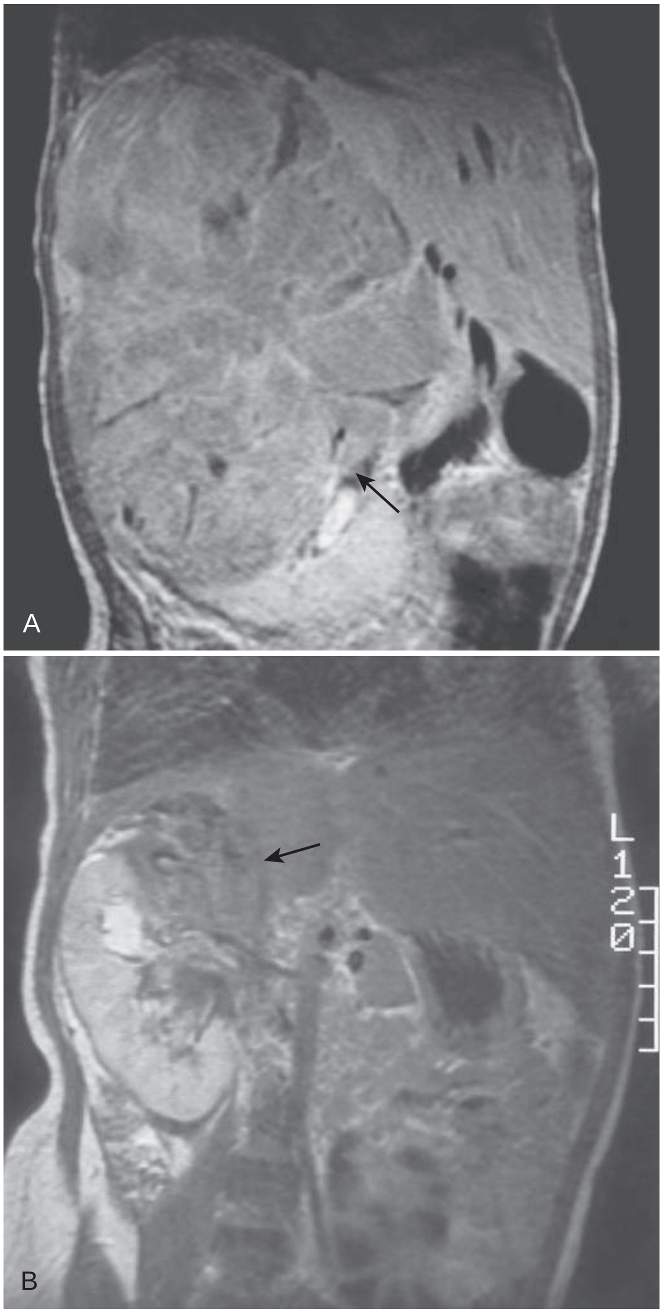

Figure 49-14 **A**, Magnetic resonance imaging (MRI) scan of a 2 and a half-year-old girl first seen with a massive right-sided abdominal mass *(arrow)* that appeared to arise from the kidney. Chest radiograph revealed pulmonary nodules as well as stage-IV disease. Because of the size of the primary tumor and the presence of metastatic disease, she received neoadjuvant chemotherapy after needle biopsy demonstrated a standard-risk tumor (diffuse blastemal subtype). She first received three courses of actinomycin D, vincristine, and cyclophosphamide, which achieved some shrinkage of the tumor. At attempted resection, the extensive tumor was densely adherent to the posterior aspect of the liver and the vena cava. The attempted resection was halted at that time, because it was clear that a portion of the liver would have to be resected with the nephrectomy. Repeat biopsies again demonstrated a standard histology Wilms tumor with tumor invasion into the liver. She then received more intensive therapy involving cisplatin and VP-16, as well as abdominal and thoracic radiation. **B**, MRI after additional therapy showed remarkable shrinkage of the tumor, allowing a less extensive and safer resection. A plane could be developed between the liver and kidney at this point, and hepatectomy was not required.

study have been assessed.[62] Complications occurred in 12.7% of a random sample of 534 of the 3335 patients enrolled on the study. Intestinal obstruction was the most common complication (5.1%), followed by extensive hemorrhage (1.9%), wound infection (1.9%), and vascular injury (1.5%). Factors associated with an increased incidence of complication included intravascular extension into the inferior vena cava or atrium and nephrectomy performed through a flank or paramedian incision. Tumor diameter greater than or equal to 10 cm was also associated with increased complications. In SIOP 9, complications occurred in 8% of 598 patients. These patients were pretreated with vincristine, actinomycin D, and epirubicin or doxorubicin before nephrectomy.[57] Their complications included small bowel obstruction in 3.7% and tumor rupture in 2.8%. The latter is not reported as a complication in the NWTSG reviews.

Neuroblastoma

Surgery plays a primary role in the treatment of low-stage neuroblastoma. Tumors that the surgeon deems readily resectable should generally have a primary resection for staging and to establish both the diagnosis and the biologic risk factors of the tumor. In many children with small tumors and favorable biologic factors, surgery is the only therapy required. Resection is generally accepted before administration of chemotherapy for most thoracic, pelvic, and cervical primaries and limited abdominal lesions that do not extend across the midline. This approach is used to avoid the toxicity of chemotherapy, and in many patients with low-stage favorable biology tumors, resection alone is curative. In those situations where resection would entail removal of vital organs or where there is extensive lymph-node extension, preliminary treatment with adjuvant chemotherapy, particularly in the biologically adverse tumors, would be most appropriate. In patients with favorable biologic features, even subtotal resection may be the only therapy required.[63]

Of note, however, is the report by Nuchtern and colleagues[64] documenting the results of the COG trial on expectant management in infants (younger than age 6 months) with small adrenal masses. Eighty-seven cases were enrolled, and four patients were elected for upfront surgery. Of the remaining cohort, only 16 other patients had their adrenal lesions resected, with neuroblastoma diagnosed in 10 children, low-grade adrenocortical tumors in two, adrenal hemorrhage in two, and extrapulmonary pulmonary sequestrations in two. The overall, 3-year survival rate in the entire cohort was 100%, with a neuroblastoma event-free survival (EFS) of almost 98%. These results are quite encouraging in defining a cohort of children who can be spared any treatment for small adrenal-based neuroblastomas, but the suitable application of these data to nonadrenal primary sites, larger tumors, and in older children remains to be determined.

Regrettably, the majority of infants and children have metastatic disease at diagnosis. In these cases, the diagnosis and biologic features must be established. Tissue may be obtained by either laparotomy or thoracotomy, as well as by laparoscopy and thoracoscopy. In older patients with metastatic disease where Shimada staging is not required to determine the patient's treatment, needle biopsy from either bone marrow or the primary metastatic tumor provides adequate material for both pathology and biologic grading. For patients in whom the Shimada criteria are critical to determine their therapeutic treatment, open or minimal access surgery is required to obtain adequate tissue.

In patients with extensive abdominal primary tumors, adjuvant therapy has been useful. It is well recognized by surgeons that preoperative chemotherapy decreases the vascularity and friability of the tumor and facilitates resection, particularly in developing the dissection plane between the tumor and the great vessels.[65] Lymph-node involvement surrounding the aorta, vena cava, and renal vessels is often seen in children with advanced stage abdominal adrenal or paravertebral primaries (Fig. 49-15).

Although two studies have shown no difference in the surgical complication rate between initial and postinduction resection, others have demonstrated a higher incidence of complications, including nephrectomy in the group undergoing primary resection.[66,67,68-71] Furthermore in patients with high-risk tumors who often require autologous bone-marrow transplant, preservation of the ipsilateral kidney during the resection is critical.[72] A neoadjuvant approach has contributed to decreased operative complications requiring nephrectomy.[73]

A multiinstitutional review of children treated over an 11-year interval demonstrated a 14.9% incidence of nephrectomy or renal infarction as a result of surgery for local control (52 of 349 children). There was a 25% incidence among those with initial resection (29 children) and a 9.9% incidence in the postchemotherapy resection (23 children). Children with initial resection had more than a twofold increase in their risk of nephrectomy. Hence the approach for neuroblastoma regarding primary lesions is quite different from that in Wilms tumor. The other determining factor is that the pathologic staging information is not as critical for determining local control in neuroblastoma as in Wilms tumor, and essentially all of these children with large primary lesions require adjuvant chemotherapy and radiation. Hence administration preoperatively in no way increases the chance that chemotherapy will be required and is generally recommended in most current protocols.

Of note, however, is the report from Simon and colleagues[74] reporting the results of the German cooperative trial NB97 that evaluated the role of surgery (upfront and postneoadjuvant therapy) in patients older than 18 months with stage IV disease. In this study, 278 patients were evaluated, and their data report that neither upfront surgery nor operations performed postneoadjuvant chemotherapy positively impacted EFS, local progression-free survival (LPFS), or overall survival (OS). They concluded their manuscript by calling into question the role and utility of surgery in this patient cohort secondary to the lack of compelling data supporting effective local control or survival rates. Obviously, more data are required to definitively answer these questions.

Hepatoblastoma

Surgery is the cornerstone in the treatment of hepatoblastoma, and children are rarely cured without resection. Neoadjuvant and adjuvant chemotherapy facilitates its feasibility and success. Historically, resection with complete removal of all radiographically identifiable disease cured only 25% of children; 50% of children who presented with hepatoblastoma had resectable lesions, and of those only 50% survived.[75,76] Failure was attributed to occult metastatic disease not identified radiographically,[77] and Weinblatt and colleagues[78] reported the early success of neoadjuvant chemotherapy to convert unresectable liver tumors to resectable lesions with more than 75% of the lesions becoming resectable in both series (Fig. 49-16).

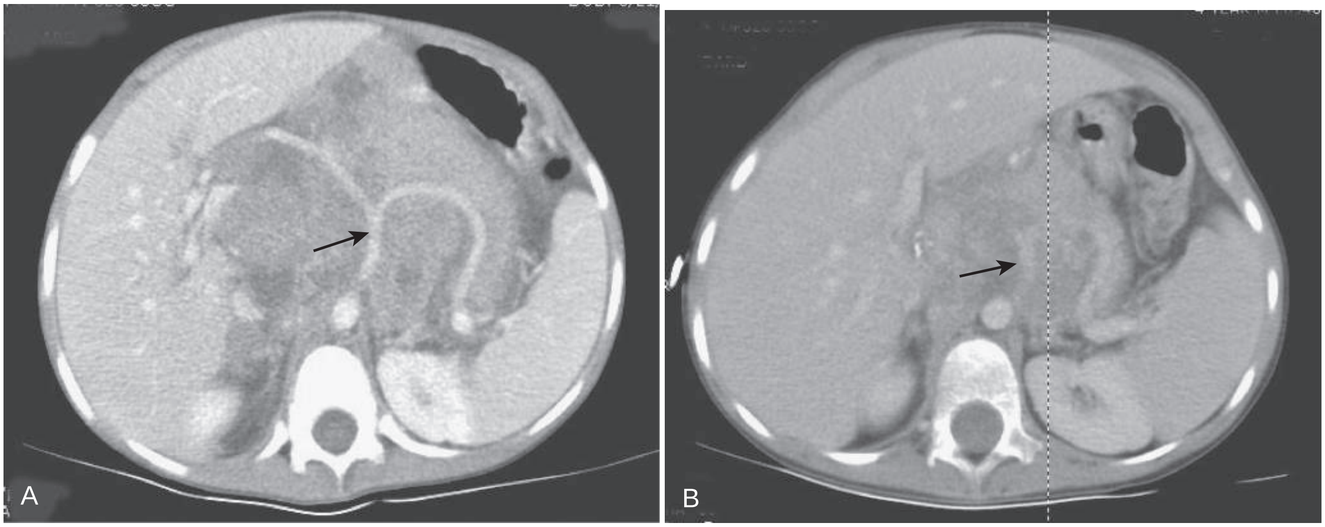

Figure 49-15 Four-year-old with bone pain and palpable abdominal mass. Computed tomography (CT) showed an extensive mass arising from the adrenal gland (**A**). This scan shows extensive involvement of the lymph nodes around the aorta with the celiac axis projecting anteriorly surrounded by tumor. In this section, the splenic artery as well as the hepatic artery are demonstrated surrounded by tumor. Subsequent CT (**B**) after four courses of multiagent chemotherapy revealed marked resolution in the size of the mass, although it still surrounded the celiac axis *(arrow)*.

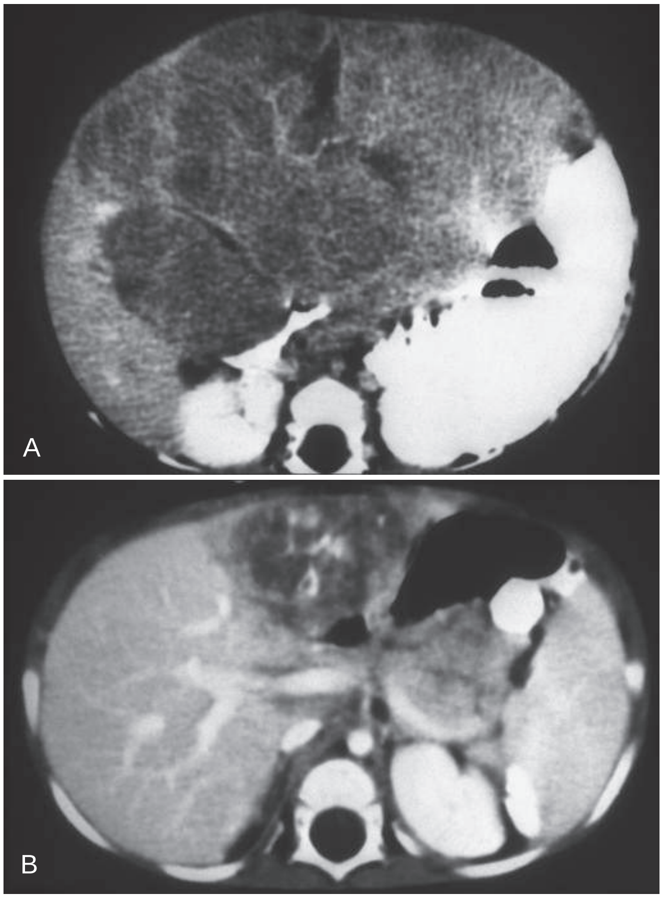

Figure 49-16 A 9-month old female with a palpable abdominal mass extending to below her umbilicus. **A**, Computed tomography (CT) scan revealed an extensive mass in her liver. **B**, CT scan after four courses of chemotherapy showed remarkable resolution of the mass that was subsequently resected by a left trisegmentectomy.

Only children with tumors with pure fetal histology do not require adjuvant therapy.[79] Subsequent reports document the utility of various chemotherapeutic regimens in hepatoblastoma to treat unresectable lesions or residual disease.[80-83,84-86] These reports establish the efficacy of doxorubicin, cisplatin, vincristine, and 5-fluorouracil alone or in various combinations. Cisplatin has been shown to be as efficacious as cisplatin with doxorubicin for standard-risk hepatoblastoma.[87] Furthermore both adjuvant and neoadjuvant regimens in combination with surgery achieved survival rates greater than 65% and tumor response rates greater than 90%. Although the regimens varied, most patients received at least two cycles before definitive surgery. The extent of tumor necrosis has also been shown to predict survival, as does negative surgical margins.[88] The critical need for resection of the primary tumor before the development of chemoresistance was demonstrated by von Schweinitz and colleagues.[84] They documented tumor regrowth as early as after the fourth course of therapy in 6 of 11 patients. Five-year survival rates for all patients with hepatoblastoma increased in just 2 decades to over 85% with the use of neoadjuvant and adjuvant chemotherapy.

Since 2000, a defined treatment protocol consisting of neoadjuvant chemotherapy and surgical resection or early orthotopic transplant evaluation for those patients with tumors that are too extensive to resect has been developed and reported by the SIOP Liver Tumor Group (SIOPEL).[89,90] This regimen was termed *PLADO,* and it consisted of a pretreatment imaging-defined staging protocol (PRETEXT), pretreatment diagnostic biopsy (core needle or incisional biopsy), neoadjuvant chemotherapy of continuous infusion cisplatin (day 1) followed by continuous infusion of doxorubicin (days 2 and 3), surgical resection after 4 to 6 cycles 12 to 20 weeks from diagnosis, and orthotopic liver transplant (OLTX) evaluation and referral for tumors confined to the liver that remained unresectable despite chemotherapy. The only tumors resected primarily were those deemed to be confined to a single section of the liver (i.e., right anterior, right posterior, left medial, left lateral; PRETEXT stage I). The overall 5-year survival rate was 75% for the entire cohort and 85% for those following the complete protocol, including neoadjuvant chemotherapy regardless of PRETEXT stage. PRETEXT stages I through IV had OS rates of 100%, 91%, 58%, and 57%, respectively, which compares favorably with survival rates achieved by Ortega and colleagues[91] through a CCG/POG cooperative trial comparing multimodality treatment protocols utilizing different chemotherapeutic regimens. Surgical mortality was 6%, and overall mortality was 24%. Twenty percent of patients had pulmonary metastases. Ninety-two percent of patients had a complete resection with this protocol, and there were 10 patients who relapsed: five with local recurrences and five with pulmonary metastases. SIOPEL's use of OLTX as primary treatment for local control was published by Otte and colleagues.[92] Those patients whose tumors involved all sectors of the liver or were intimately involved with either the hepatic or portal veins but had no evidence of metastatic disease and demonstrated some response to neoadjuvant chemotherapy were considered for a primary OLTX. Another cohort of patients who had intrahepatic recurrences of disease after resection or incomplete resections was eligible for rescue OLTX. Ten-year survival rates for the primary and rescue OLTX groups were 85% and 40%, respectively. The authors advocated, based on their results, for the following: 1) total resection of all gross disease including the retrohepatic vena cavae if needed; 2) the use of preoperative chemotherapy to control extrahepatic micrometastases and promote primary tumor regression; 3) transplantation for patients with synchronous pulmonary metastases if they had responded to neoadjuvant therapy; 4) no transplantation for those patients with evidence of metastatic disease present at surgery; 5) limiting the interval between diagnosis and OLTX to account for the rise of chemoresistance and the availability of donors (cadaveric or living); and 5) questioning the need of post-OLTX chemotherapy in the setting of a patient with immunosuppression. Although their cohort was small (12), they reviewed the world's experience with OLTX for hepatoblastoma in the same report, and the results were similar. As a corollary to this report, Austin and colleagues[93] reported on the experience of the United Network of Organ Sharing (UNOS) for 152 OLTXs performed in 135 patients in the setting of hepatoblastoma. Their actuarial 1-, 5-, and 10-year survival rates were 79%, 69%, and 66%, respectively. More than 54% of patients died of recurrent disease, and the only statistically significant predictors of favorable outcome were the preoperative condition of the patient (intensive care unit [ICU] hospitalization versus non-ICU hospitalization versus no hospitalization) and the era when the patient received the transplant (before or after December 31, 1994). These reports document not only the feasibility but also the success of OLTX as a component in the multimodality treatment of hepatoblastoma.

Rhabdomyosarcoma

Except for Wilms tumor, no other pediatric solid tumor has benefited so greatly from the combined efforts of oncologists of all disciplines in forming multimodality protocols designed to adequately diagnose, stage, and treat a lesion as has rhabdomyosarcoma. The 5-year survival rate of children with this disease was less than 25% before 1972 when the IRS study group was formed.[93,94] During the next 3 decades, this figure has increased almost threefold to 70%.[95-97] Because rhabdomyosarcoma is a tumor of mesenchymal origin, it may appear almost anywhere in the body and from almost any organ. Treatment protocols and outcomes vary greatly depending on the site and stage of the tumor. Initial pretreatment evaluations document the characteristics of the primary lesions and the regional lymph-node basins. If an extremity lesion is excised with positive margins and proves to be a rhabdomyosarcoma, it has been shown that pretreatment reexcision with generous margins (0.5 cm or greater) of uninvolved tissues and local lymph node sampling results in improved survival.[98] Debilitating resections producing loss of function are not currently warranted, except in cases with residual disease after adjuvant radiotherapy and chemotherapy. Regional and distant lymphadenopathy diagnosed clinically or radiographically must be biopsied to establish the presence or absence of tumor that will impact treatment strategies and prognosis.[99] Aggressive lymphadenectomies are not warranted, but a stepwise progression of draining lymph-node basins should be sampled to determine the lymph-node status of these contiguous basins and hence the extent of regional radiotherapy. Finally, so called "second-look" operations performed on the primary site in IRS III proved useful in the excision of residual tumor, the confirmation of the effectiveness of adjuvant therapies, and the improved prognosis of all tumors except for pelvic sites that had a pathologically confirmed complete response to therapy.[100] These principles of complete resection of the primary tumor and lymph-node biopsy determine postoperative clinical groupings that determine adjuvant treatment therapies and prognosis.

Radiotherapy is a core modality of treatment for local control, and its field is based on the size of the mass before resection. Patients receive a radiation field with at least a 2-cm margin around the primary mass. Therapy is generally begun 8 to 12 weeks after initiation of chemotherapy and continues for 5 to 6 weeks. All histopathologic subtypes except group I embryonal disease receive radiation therapy.[97] All other groups and rhabdomyosarcoma variants receive at least 4000 rads for microscopic residual disease and as much as 5000 rads for macroscopic residual disease,[97,101,102] providing a strong basis for primary resection if safely feasible.

Adjuvant chemotherapy is critical, and its use is based on a risk stratification schema that incorporates tumor site of origin, tumor histopathology, tumor-nodes-metastasis (TNM) staging, and the postoperative clinical grouping. Patients are stratified into low-, intermediate- or high-risk categories. Patients with low risk typically receive a three-drug regimen of vincristine, dactinomycin, and cyclophosphamide. Patients with intermediate risk generally receive the same three-drug regimen, but those with tumors from favorable sites and those with completely resected tumors from unfavorable sites receive dose-intensified cyclophosphamide.[102] Patients at high risk, however, should receive a two-drug regimen of ifosfamide with etoposide or doxorubicin.[103,104] Patients with metastatic disease or alveolar subtypes should also receive topotecan or irinotecan in addition to standard therapy.[105,106] Ideally, neoadjuvant chemotherapy can reduce tumor burden and convert unresectable lesions to resectable ones with decreased patient morbidity (Fig. 49-17).

Osteosarcoma, Ewing Sarcoma

The single most important factor in improved survival for patients with osteosarcoma (OSA) or Ewing sarcoma (EWS) has been the development of effective chemotherapy. Before 1970, patients with OSA had an OS of around 10%. Before the 1960s, survival was even less than 10% for patients with EWS. Ablative surgery and/or radiation were the only modalities available at that time. Local control, however, was not the issue. Patients succumbed primarily from metastatic disease that was believed to be present at diagnosis, although it could not be demonstrated radiographically. Systemic treatment was necessary to achieve improved survival and cure. Medications were identified and successfully applied in cooperative multiinstitutional trials. With modern chemotherapy protocols, OS for OSA is now 60% or better and approaches 60% for EWS.

Current protocols for OSA involve neoadjuvant chemotherapy, surgery, and then further adjuvant chemotherapy. Protocols are similar for EWS except that radiation can be used in combination with surgery or alone as local treatment. The advantages of this approach are that tumor cells in the primary tumor and in micrometastatic form are treated immediately and that histologic response to the chemotherapy can be assessed. The potential advantage of making limb salvage surgery safer is debatable, but certainly, any reduction in size of the primary tumor improves the feasibility of surgical resection (Fig. 49-18). The disadvantages are that in patients who do not respond to the usual drugs, their disease may progress, and drug resistance may develop. Because response to chemotherapy has been identified as one of the more important prognostic factors with potential

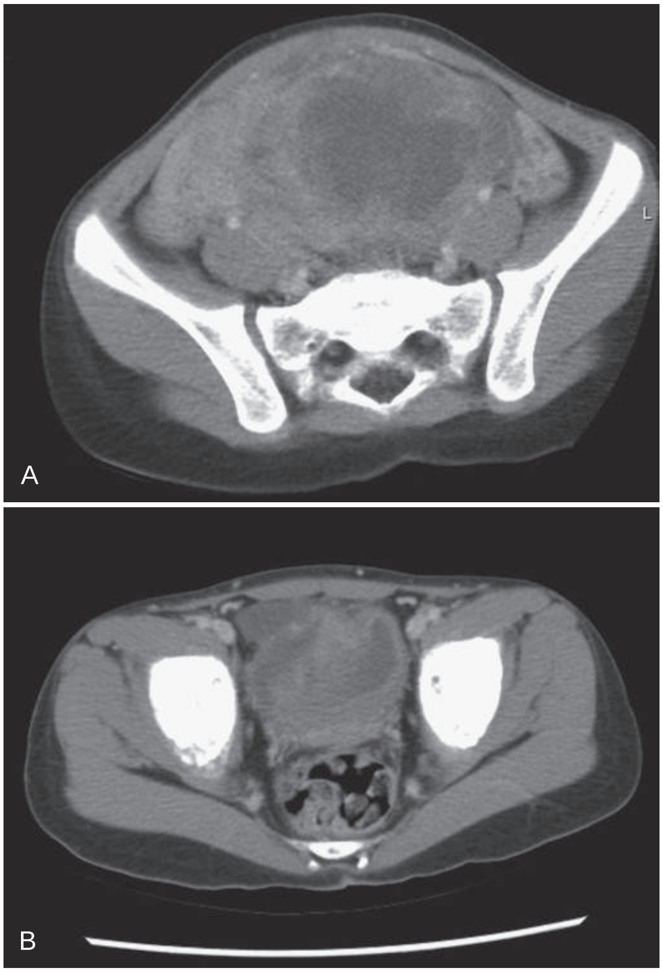

Figure 49-17 **A**, A 7-year-old with a large bladder rhabdomyosarcoma prechemotherapy. **B**, A significant decrease in the size of the tumor.

implications for changes in therapy, this overall approach is used in the majority of trials. The reality, though, is that no new drugs or changes in therapy have been shown to improve survival to any great extent in poor responders. Efforts to improve survival for poor responders and for patients who relapse are the focus of current protocols and research.

LIMB SALVAGE PROCEDURES

Surgical Principles

The surgical treatment of bone sarcomas has evolved from the prechemotherapy era where all patients had amputations, radiation therapy, or both, to the current practice where 80% of patients or more receive a limb-salvage resection.[107] The resection can be thought of in two phases: wide resection of the tumor and involved bone and the reconstruction, for which there are multiple alternatives. A “safe” resection requires complete excision of the involved bone and soft tissues with a cuff of normal tissue surrounding the entire specimen, a so-called *wide margin*. The necessary thickness of this cuff of tissue and the length of normal marrow away from the tumor is not precisely known,[108-112] but at a minimum there should not be “ink on tumor.” While experience with these operations grows, most surgeons now accept soft-tissue margins of 2 to 5 mm of normal soft tissue and 1 to 2 cm of marrow margin, but there is no evidence-based minimum margin that has been documented to be safe with respect to local recurrence. Neoadjuvant chemotherapy probably lessens the likelihood of a local recurrence.[113-116] Recent data would suggest that a wide margin in a patient with a good response to chemotherapy has a low likelihood of local recurrence.[117,118,119] Resections with a narrower margin (marginal or intralesional) and/or those with a poor response to chemotherapy have a higher risk of local recurrence. It is clear that a local recurrence is associated with a poor prognosis for survival.[120-123]

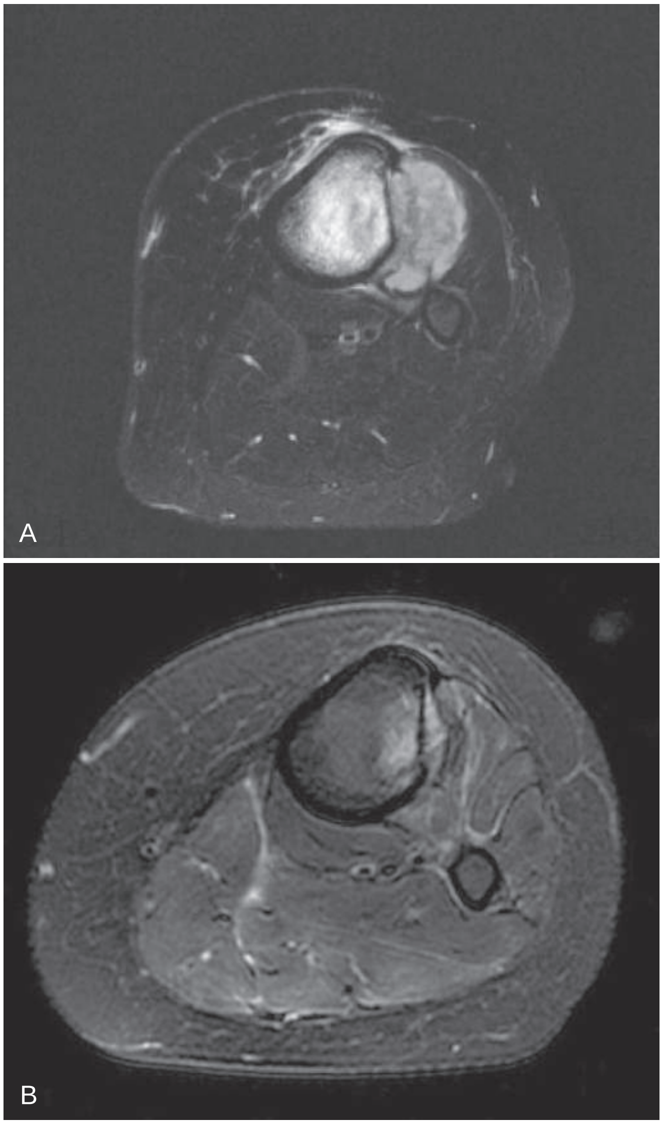

Figure 49-18 Ewing sarcoma of the proximal tibia: T2-weighted sequence with fat saturation, axial magnetic resonance imaging. **A**, At initial diagnosis; note the large anterolateral soft-tissue extension outside of the bone. **B**, After neoadjuvant chemotherapy, the soft-tissue mass has noticeably decreased in size, making surgery, especially dissection around the arterial trifurcation, less difficult.

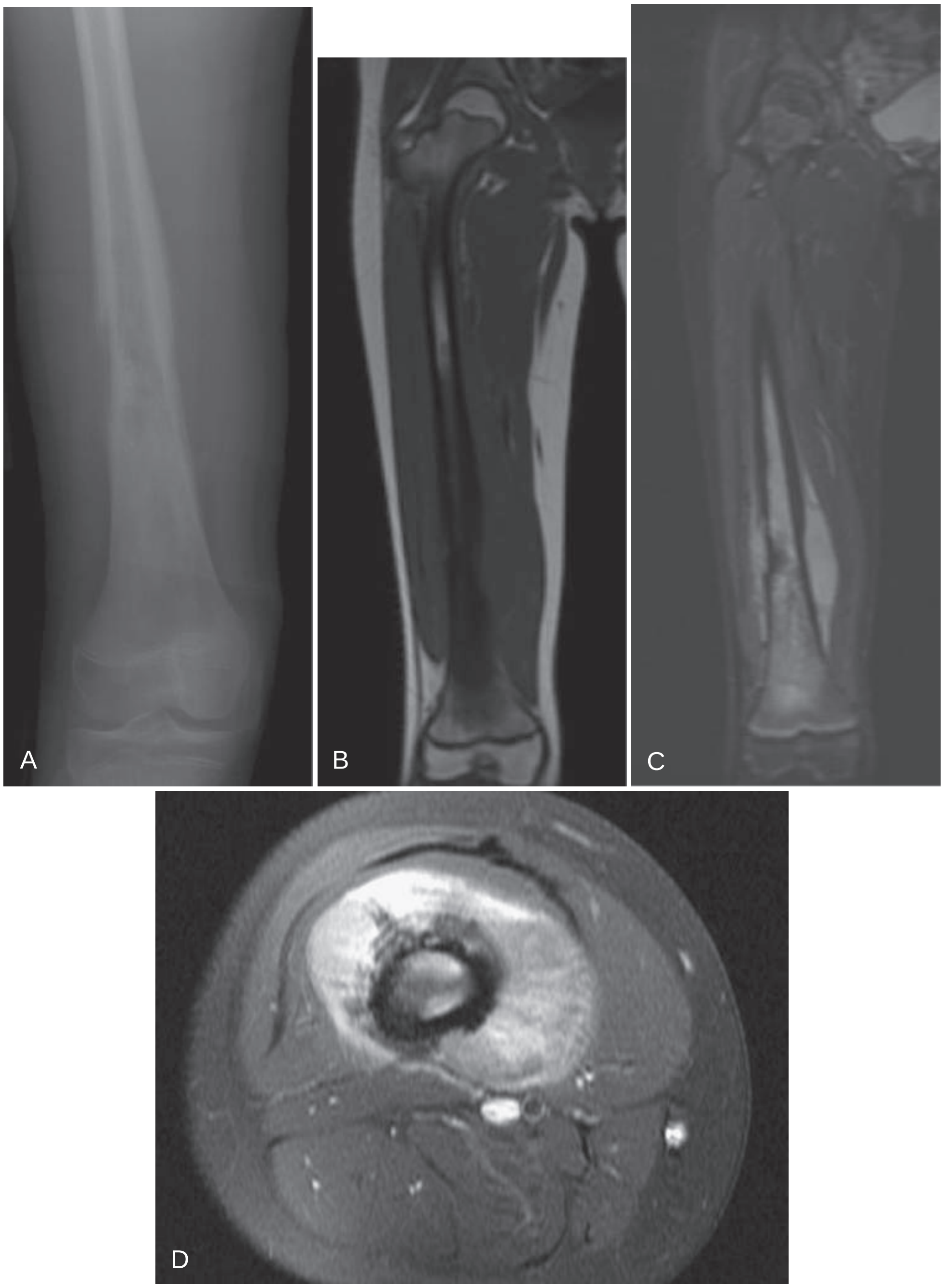

Figure 49-19 **A**, Osteosarcoma of the femur showing ill-defined bone destruction in the metaphysis and soft tissue mass. **B**, Coronal T1-weighted images show the extent of the tumor in the medullary canal. **C** and **D**, Coronal and axial images show the extent of the soft-tissue mass and the relationship to the femoral artery and vein.

The decision of resection versus amputation and the type of reconstruction requires careful evaluation of the imaging studies with a multidisciplinary team that includes an experienced musculoskeletal radiologist and a detailed discussion with the patient and family regarding the alternatives. Although most patients would prefer to save their limb if possible, it should be understood that current reconstruction options cannot restore a normal limb and most are not meant to return the patient to athletic activities. Patients with lower-extremity primary tumors who want to continue contact and running sports are better served with an amputation or rotationplasty.

The resection requires a careful analysis of the radiograph and MRI. The entire bone should be imaged to exclude the possibility of skip metastases. MRI is the best study to assess the extent of the tumor within the bone and soft tissue (Fig. 49-19).[124-128] OSAs may cross growth plates to involve the epiphysis of the bone, but they rarely cross the articular cartilage unless there has been a fracture.[124,126] Adjacent joint involvement is rare, but if present it requires an extraarticular resection. Tumors may enter the joint by extending along the joint capsule (e.g., in the proximal humerus the metaphysis is partially intraarticular) or cruciate ligaments (knee) or ligamentum teres (hip). The major nerves and vessels must be uninvolved or resectable to achieve the required margin. Finally, there must be sufficient muscle remaining after the resection to allow soft-tissue coverage of the reconstruction and to power the limb. At times rotational flaps or free-tissue transfers may be indicated to obtain soft-tissue coverage and lessen the chance of a wound complication.[129,130] For all resection types, infection is the worst immediate complication and its incidence can be lessened with adequate soft-tissue closure.

Once the resection has been completed, the pathologist should examine the specimen grossly to ensure complete resection. The use of frozen section analysis of the marrow margins is a routine and accepted practice but is of uncertain value.[131] Bisecting the specimen with a band saw and visualizing it will reassure the surgeon that an adequate resection has been accomplished.

Types of Reconstruction in Children

Reconstruction options include the use of metallic endoprostheses, bone allografts, and rotationplasty. Some sites require no bony reconstruction. These "expendable" bones include the fibula, clavicle, rib, and iliac wing (if the acetabulum can be preserved). Most OSAs are located about the knee, hip, and shoulder and involve resection of the distal femoral, proximal tibial, proximal femoral, or proximal humeral metaphysis and epiphysis. In addition to the bony defect, a major growth center is included that in the lower extremity adds complexity to the reconstruction not encountered in adults. For patients close to skeletal maturity (10 to 12 years old in girls and 14 to 16 years in boys) this is seldom a major issue and can be addressed by reconstructing with a slightly longer implant than the length resected. Limb-length discrepancies of 2 cm or less are seldom a clinical problem and can be treated with a simple shoe lift. For tumors around the knee in younger patients, limb length must be addressed by the reconstruction chosen. The alternatives include the use of an expandable prosthesis, bone allografts, and rotationplasty. Each has advantages and disadvantages. An expandable prosthesis in theory allows "growth" of the prosthesis over time but will need to be revised to an adult prosthesis at skeletal maturity.[132-136] Some of these prostheses require several operations to achieve lengthening,[136] whereas others have nonoperative mechanisms of expansion.[132] They are expensive, complex prostheses with many potential complications. Children younger than 8 years of age have difficulty cooperating with physical therapy, so joint contractures are a problem. In addition, mechanical failure of the prosthesis, metal debris, loosening, and infection can be significant problems.[137] Bone allografts have the advantage of preserving the adjacent bone and its growth plate (i.e., the proximal tibia can be preserved after distal femoral resection) but obviously have no capacity to grow. There are also issues of finding an allograft of appropriate size and shape, but if a suitable bone is available, length equalization can be addressed by inserting a graft 1 to 2 cm longer than the length resected and addressing limb length subsequently with standard pediatric orthopaedic techniques of contralateral epiphyseodesis. At skeletal maturity, closed femoral shortening and limb-lengthening techniques are also available. Rotationplasty addresses this issue because the prosthesis can be adjusted to the desired length while the child grows.

Bone Allografts

Bone allografts are used to reconstruct defects primarily at the distal femur, proximal tibia, proximal femur, and proximal humerus.[138-142] The obvious concern is transmission of bacterial and viral disease, but bone banks are now accredited by the American Association of Tissue Banks, and the safety record of these grafts is quite good since better testing for human immunodeficiency virus (HIV) and hepatitis became available.[143-145] The advantages in young patients are that they are intended to restore bone stock and joint structures (articular cartilage, ligaments, and joint capsule) and delay the need for metallic prostheses. In theory they restore bone stock, but experience has shown that the grafts are only partially replaced by host bone, and it is likely that most patients who receive osteoarticular grafts will require a joint arthroplasty at some point (5 to 20 years from the index procedure). Delaying artificial joints until adulthood is probably advantageous for long-term salvage of the limb; however, these secondary operations are challenging as well.[146] In a young child a major advantage of osteoarticular allografts is the avoidance of resection or placing an implant across the adjacent growth plate. These are complex operations, and they require the availability of a tissue bank and surgical expertise with the use of allografts. The procedure is associated with a significant complication rate. Infection, reported in the 0% to 20% range,[138-142] is the worst complication and usually requires removal of the graft. Nonunion of the allograft–host junction (reported to occur overall in 17%) is higher (27%) in patients receiving chemotherapy[147] but can usually be addressed by revision of the fixation and bone grafting of the osteosynthesis site. Fracture is a later complication and occurs in about 15% of patients.[138,140-142,147-149] Occasionally the fracture heals with conservative means, but more often revision to another allograft, augmentation with a vascularized fibular graft,[150] or conversion to an endoprostheses is required.[151] Postoperative management includes closed suction drains for 24 to 48 hours, preoperative and postoperative antibiotic coverage (there are no data regarding the necessary length of antibiotic coverage), venous thrombosis prophylaxis, and initial immobilization. Physical therapy to start joint motion is begun after 6 weeks, and the limbs are protected with crutches and bracing until the osteosynthesis site heals (approximately 6 to 9 months in patients getting chemotherapy).

Endoprostheses

Metallic prostheses were initially custom made to fit the expected bone resection defect. There are a number of available modular systems that allow the surgeon to

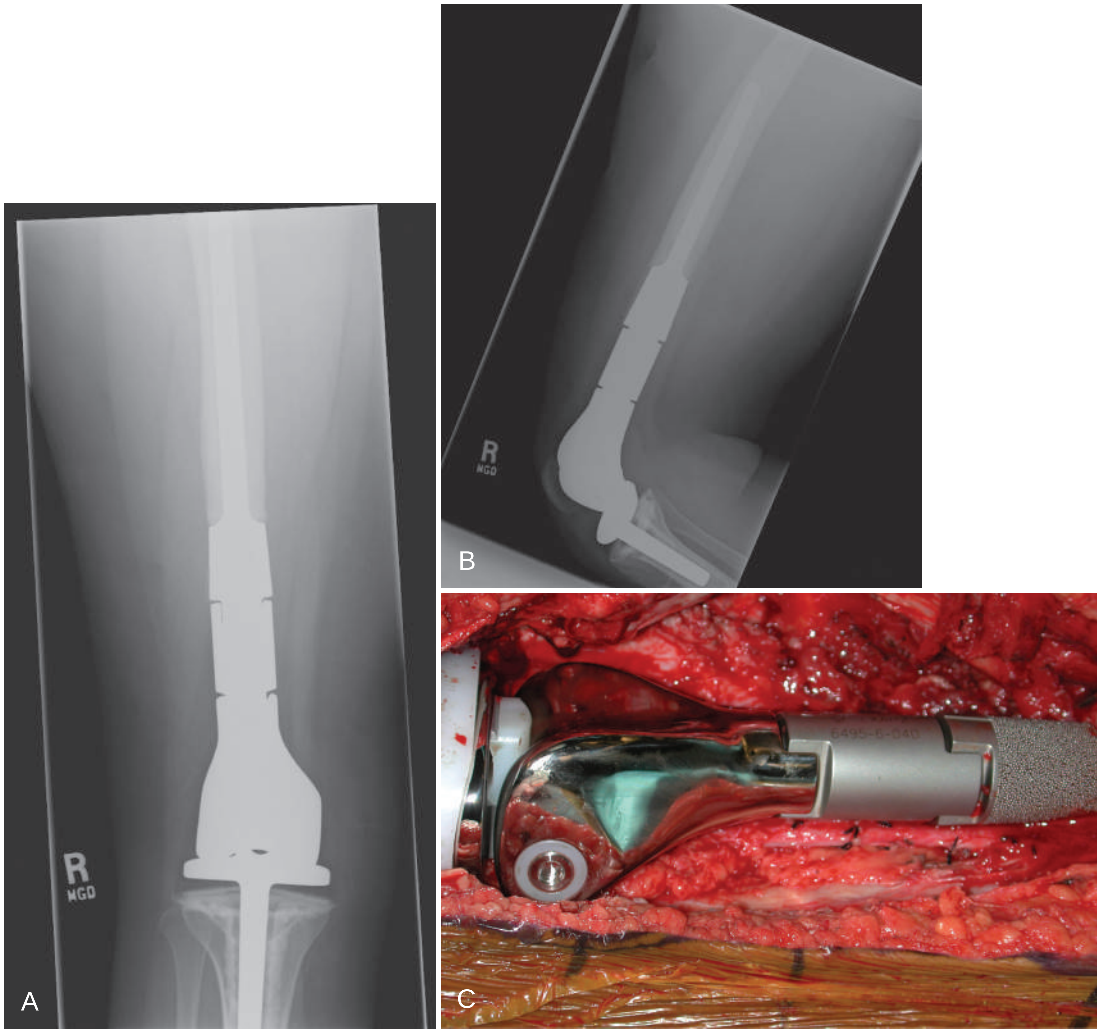

Figure 49-20 **A** and **B**, Anteroposterior and lateral views of a modular tumor prosthesis used to reconstruct after a resection for distal femoral osteosarcoma. A rotating hinge design is used, and the modular nature of the prosthesis can be appreciated. **C**, The prosthesis after insertion during the operation.

reconstruct the defect at the operating table.[152-159] In older children close to skeletal maturity, adult prostheses can be used. Most reconstructions around the knee use a rotating hinge construct that reduces the forces that are transmitted to the host-prosthesis interface, and this design is believed to increase the longevity of the implants because of delayed loosening of the stems. Modern stem designs include the option of cemented or uncemented stems, and in the femur there are curved and straight stem designs. In theory, uncemented stems offer the advantage of avoiding osteolysis and loosening, but whether bony ingrowth is reduced in patients receiving adjuvant therapies is uncertain at this time.[154,155,160,161] The main advantage to endoprostheses is that they are as stable and functional as they will ever be as soon as the wound heals, so return to function is more rapid than with allografts. Patients do not need to wait for an osteosynthesis to heal, and early weight bearing and range-of-motion exercises are permitted. The disadvantages include infection (although the reported rates are lower than with allografts), loosening or mechanical failure of the implants, metal debris, and decreased longevity.[159,160,162-165] Because 70% to 80% of children with bone sarcomas now survive, it is unlikely that these implants will last the lifetime of the patient. Revision is possible, but multiple revisions over time may limit the options and lead to amputation. The longevity (not function) of the implants is about 60% to 80% at 5 years and 50% to 80% at 10 years for lower extremity implants depending on the specific series.* The expandable endoprostheses are more complex and likely to have a higher complication rate.[132-137,170-172]

Examples by Anatomic Site

Distal Femur and Proximal Tibia

Tumors around the knee can be reconstructed by any of these methods. A few examples will be provided. Figure 49-20 shows examples of an endoprosthesis of the distal femur. The implant is a modular prosthesis that is cemented into the remaining femur and proximal tibia after an intraarticular resection. Initial function of these constructs is quite good, but it is expected that loosening will occur sometime in the future. It is known from adult arthroplasty and the early experience using custom and modular prostheses that the interface between the cement and the bone will fail over time in part because of particulate debris and the biologic response to it. Design improvements such as ingrowth surfaces near the stem–implant junction that allow bony- or soft-tissue ingrowth may delay or prevent loosening.[166] Uncemented stems may also offer longevity advantages. A further concern is stress shielding. The stem takes the mechanical load from the adjacent bone, and the host responds by resorbing the cortical bone over time. The concern is that in future revisions, insufficient bone stock will remain. There are newer stem devices that load the bone to avoid stress shielding and offer the hope of more longevity. The early data from these stems is encouraging.[173] Unfortunately, there are no direct comparisons between osteoarticular allografts (Fig. 49-21) and endoprostheses at this site, but the current literature would suggest that for the distal femur, an endoprostheses is superior.[169] Some surgeons believe that in the skeletally immature, it is still advantageous to use allografts at this site for the reasons stated.

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*References 136, 155, 157-163, 166-169.

In the proximal tibia, an osteoarticular allograft offers the advantage of providing a site for attachment of the patellar tendon. This is much more difficult to achieve in an endoprostheses. As shown in Figure 49-22, an intraarticular proximal tibial resection is achieved including the host menisci (although they can be preserved depending on the extent of the tumor). The reconstruction allows repair of the cruciate ligaments, joint capsule, and patellar tendon. A gastrocnemius flap is sometimes required for soft-tissue coverage and is always required to augment soft-tissue attachment of the patellar tendon if an endoprostheses is used. Fixation is achieved with standard plates and screws used to treat fractures. Plates that span the entire length of the allograft or intramedullary fixation are desirable to lessen the fracture rate. Intramedullary rods require supplemental plate fixation to control rotation at the osteosynthesis site. Newer locking-plate designs may lessen the incidence of nonunion and fracture.[174] A variation of this construct is to combine an allograft and standard total knee prosthesis (allograft-prosthetic composite), usually a rotating hinge design for stability (Fig. 49-23). This approach allows a stable knee construct initially and a site for reattachment of the patella tendon. It has the disadvantage in a skeletally immature patient of placement of an implant across the adjacent (distal femoral) growth plate, creating a greater potential limb-length discrepancy.

A further option at the knee is an expandable prosthesis (Figs. 49-24 and 49-25). These can be used for the distal femur and proximal tibia. Many parents are now aware of these devices and are requesting them, but their long-term outcome is still uncertain. One could argue that an attempt at this is reasonable if the alternative is amputation or rotationplasty, planning to deal with complications later if the patient survives. The downside is that it restricts young children from normal activities including sports; if they pursue them, the likelihood of failure is higher.

Rotationplasty

For large tumors of the distal femur or very young patients with distal femoral sarcomas, rotationplasty is an excellent alternative to amputation.[175-177] A rotationplasty preserves the distal leg, foot, and ankle, and places it at the level of the contralateral knee (Fig. 49-26). The tibia replaces the femoral shaft, and by rotating the limb 180 degrees, the ankle and foot act like a knee, making the construct function like a below-knee amputation rather than a high-thigh amputation. A modification of this procedure can be performed for proximal tibial and proximal femoral sarcomas.[178-180] The margin achieved is wider than a standard resection, because with the exception of the sciatic nerve, no other structures need to be saved. The vessels can be dissected and coiled like the nerve at closure or resected and anastomosed after the osteosynthesis has been achieved. This procedure may be more suitable for patients with pathologic fracture because of the wider margin it achieves, but if the patient has a widely displaced fracture and a large sarcoma at diagnosis and requires spica immobilization during preoperative chemotherapy, there is a higher risk of the anastomosis failing because of venous congestion in the leg (personal observation). Rotationplasty has the advantage of avoiding phantom pain, because the sciatic nerve and its branches are preserved and growth is not an issue because the prosthesis can be adjusted as the patient grows. It usually requires a single operation with a low complication rate and allows the child to have full activities. The functional results are rewarding. The main drawback is the cosmetic appearance. Patients must be carefully selected, and it is essential that the child and parents meet a patient who has had a rotationplasty and work with an experienced therapist preoperatively so that they fully understand the operation. Many patients, parents, and surgeons refuse this option because it is distasteful, but those who accept it do extremely well functionally and emotionally. This has been documented in long-term studies.[175,181,182,183-187]

Proximal Femur

Resection of the proximal femur requires careful assessment of the hip joint (Figs. 49-27 and Fig. 49-28). If an intraarticular reconstruction can be achieved, the reconstruction options include either a metallic prosthesis or an allograft.[154,158,188,189] The likelihood that an allograft will fit perfectly into the acetabulum is low, so most allografts are used as allograft-prosthetic composites at this site. If the acetabulum requires resection, the reconstruction is much more complex. An allograft offers the theoretical advantage of a site for reattachment of the abductor muscles and lessening the abductor lurch during gait, but attaching the abductors to a metallic prosthesis or the iliotibial band has been shown to be quite successful and probably equivalent. The decision of whether to do a total hip prosthesis or a hemiarthroplasty is more difficult. Although the hemiarthroplasty is easier and

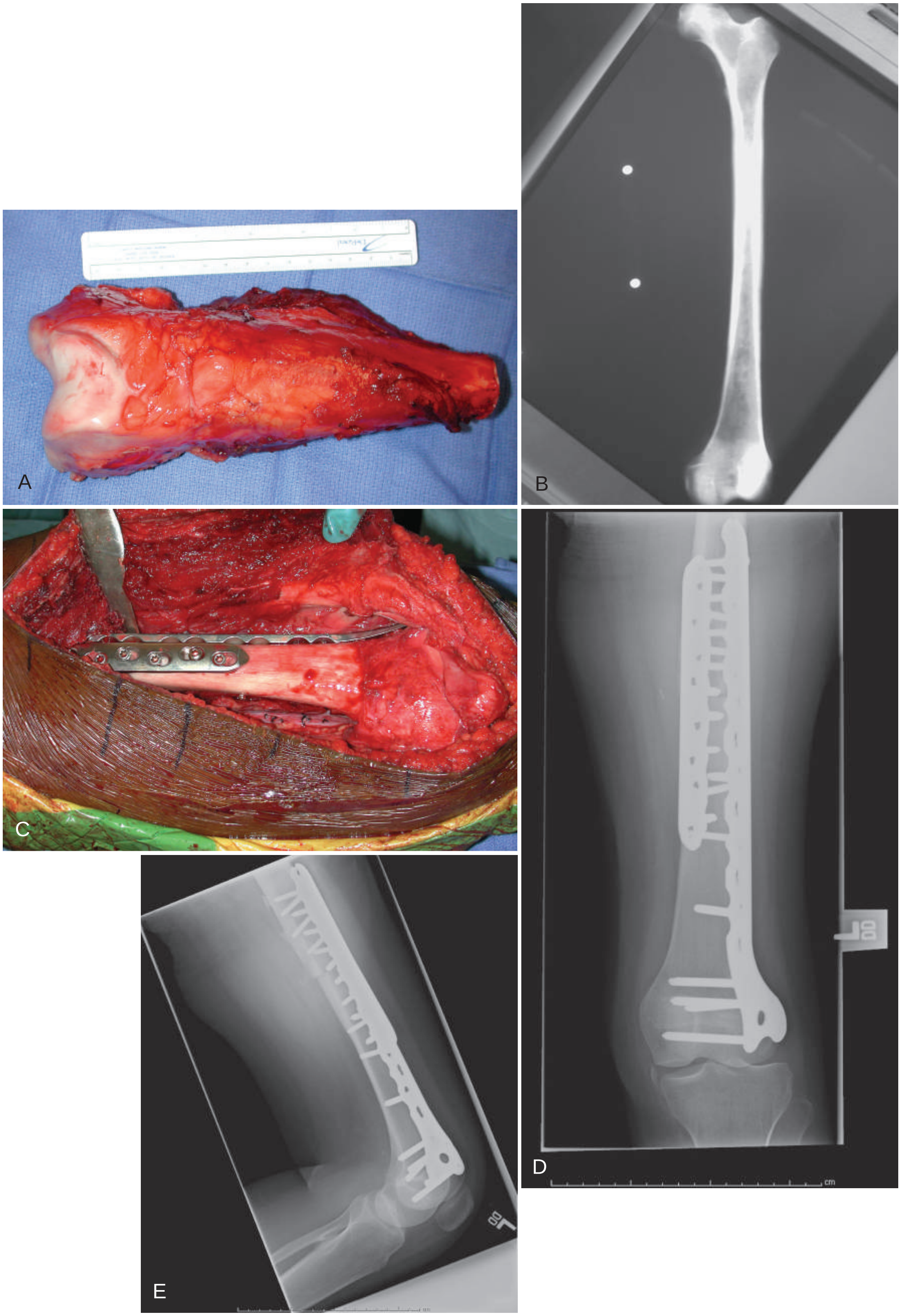

Figure 49-21 **A,** A resection specimen after intraarticular resection of the distal femur for osteosarcoma showing a margin of muscle surrounding the tumor. **B,** An osteoarticular allograft from a bone bank is thawed and then cut to fit the defect created. **C,** An intraoperative photograph shows the graft held in place with dynamic compression plates after ligament reconstruction. **D** and **E,** The postoperative appearance is shown on radiographs.

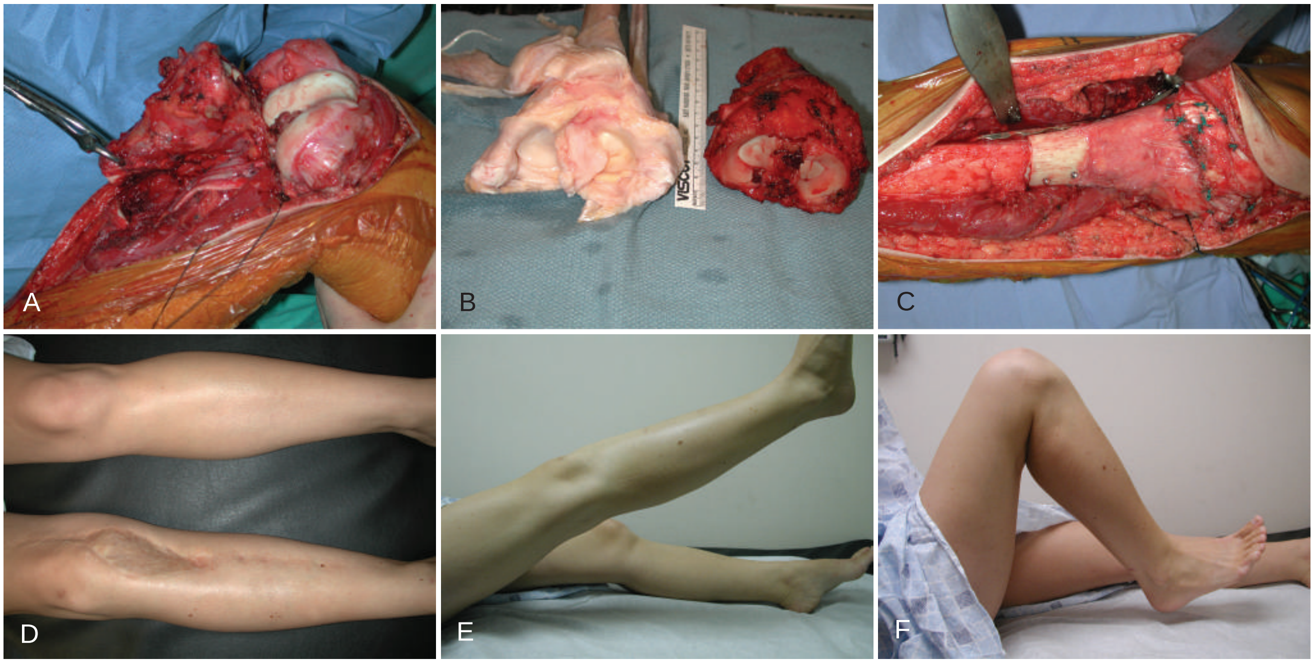

Figure 49-22 **A**, Resection of a proximal tibial osteosarcoma is shown with preservation of the tibial vessels and sacrifice of the menisci. **B**, Comparison of the resected specimen with menisci *(right)* to the allograft *(left)*. **C**, The allograft is in place after plate fixation and ligament reconstruction. The sutures in the patellar tendon are evident. **D**, The postoperative photograph shows a rotational gastrocnemius flap and skin graft. Function of a patient is demonstrated by full extension and straight leg raising (**E**) and the flexion arc (**F**).

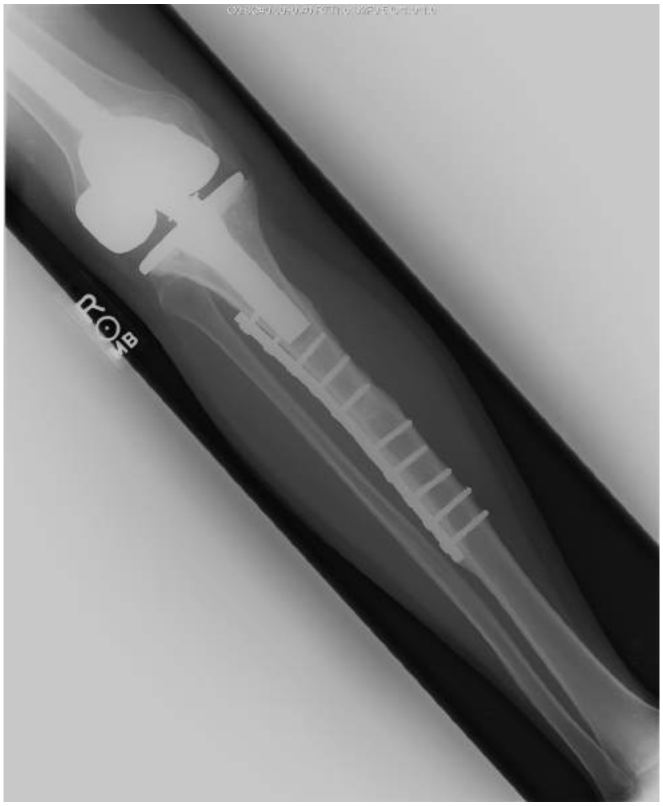

Figure 49-23 The radiographic appearance of an allograft prosthetic composite of the proximal tibia is shown.

more stable than a total hip arthroplasty, recent data in children suggest that these may subluxate as the child grows and the acetabulum develops, necessitating revision to a total-joint arthroplasty.[190]

Pelvis

In general, patients with sarcomas of the pelvis have a worse prognosis than those of the extremities.[191-196] Assessment of response to chemotherapy is critical. For EWS, the alternative of irradiation for local control is available, but for OSA surgical resection is required even if this means hemipelvectomy if the goal is cure. Resections are difficult and often require the expertise of the orthopaedic oncologist, a surgical oncologist, and at times a vascular surgeon or urologist. The site of the tumor is critical. Tumors of the iliac wing can be resected without much functional loss if the acetabulum can be preserved (Fig. 49-29). Bone grafts are sometimes used to restore the pelvic ring. Similarly tumors involving the pubic rami can be resected and require little reconstruction other than synthetic mesh to prevent herniation of the abdominal contents (Fig. 49-30). The difficult area is the acetabulum. Resection of the acetabulum or other areas of the pelvis that include the acetabulum result in complex reconstruction challenges. Often the best alternative is to leave the hip flail (Fig. 49-31). Significant shortening will occur, but function is better than an amputation even if a major nerve is sacrificed. Attempts at arthrodesis of the proximal femur to the remaining

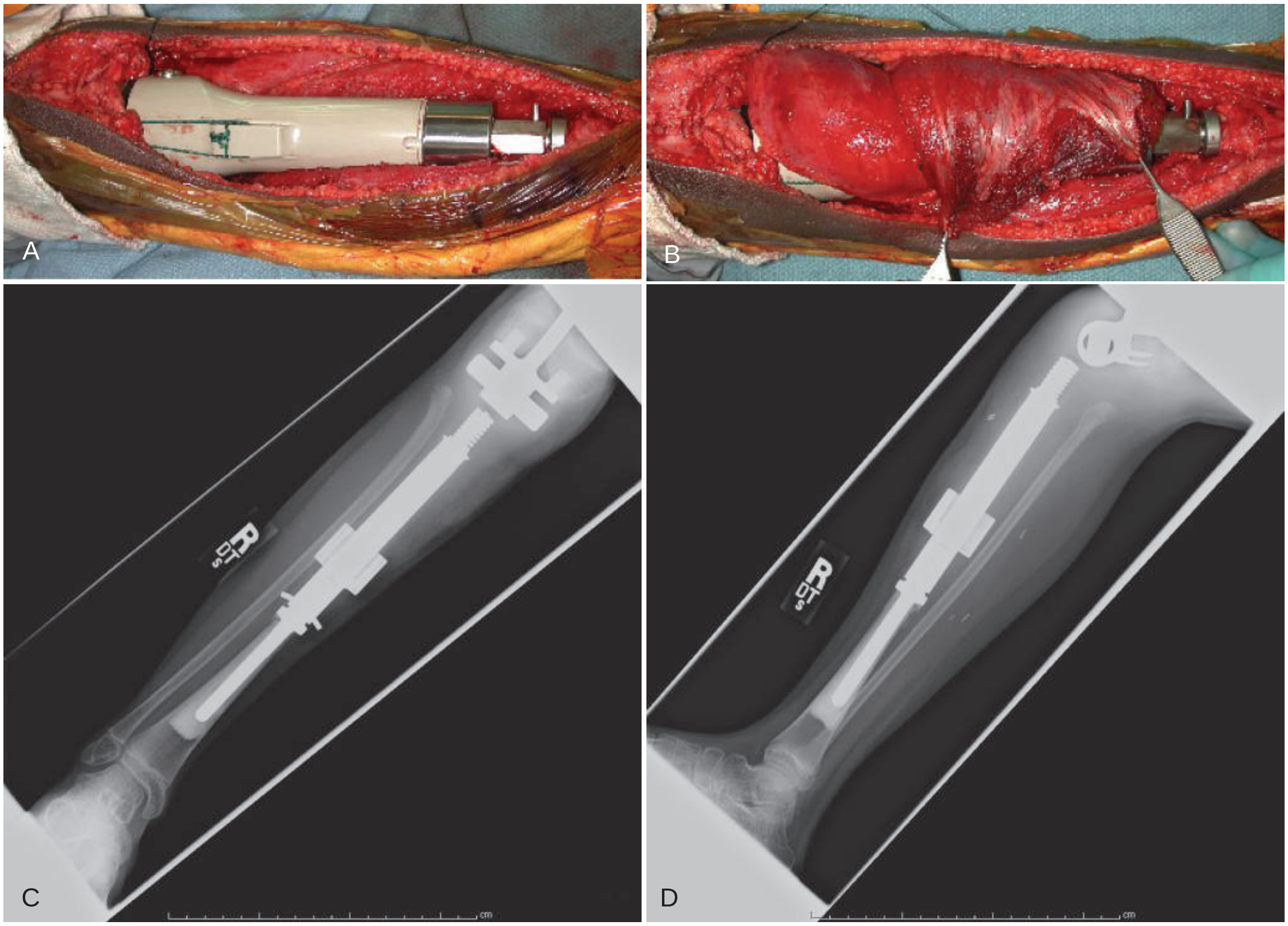

Figure 49-24 **A**, This is an example of an expandable prosthesis for the proximal tibia. The sutures for attachment of the patellar tendon are seen. **B**, The implant has been covered with a gastrocnemius and soleus flap that aid in anchoring of the patellar tendon and wound healing. This particular prosthesis allows lengthening without an operation. **C** and **D**, Anteroposterior and lateral views show the radiographic appearance of the prosthesis in place. The spring mechanism allows for the expansion after an electromagnetic field transiently melts a restraint to expansion.

pelvis can be considered but are very difficult to achieve. Allografts have been used, but are reported to have significant morbidity including a high infection rate and long-term mechanical problems such as fracture.[197-200] Custom metallic replacements have also been used,[201] but they require careful planning and have a high failure rate. All of these considerations are amplified if the adjacent sacrum requires resection as well. The multidisciplinary team and the family must have a careful discussion of the goals, resection options, reconstruction alternatives, and other treatment strategies before these pelvic resections are undertaken. Surgical expertise with the intraoperative and postoperative management of these patients is essential for success.

Proximal Humerus

All attempts should be made to avoid amputation in patients with upper extremity tumors. Unlike the lower extremity, there are no prostheses that replicate the function of the hand. Limb length in the upper extremity is usually not a major concern. A resection of the proximal humerus and scapula en bloc (Tikhoff-Linberg resection) is preferable to a forequarter amputation and is usually possible.[202-207]

For those patients with proximal humeral sarcomas that are amenable to intraarticular resections, reconstruction can be accomplished with metallic prostheses (Fig. 49-32),[158,163,208-211] osteoarticular allografts (Fig. 49-33), or allograft-prosthetic composites.[138,212-214] Function is more a result of whether the deltoid, axillary nerve, and rotator cuff can be preserved. A flail shoulder is superior to an amputation if all these muscles must be resected. For high-grade sarcomas, the deltoid is often resected, but some active motion can be preserved if all or part of the rotator cuff can be preserved. Allografts provide sites for attachment of these motors, but metallic prostheses combined with synthetic materials around the prosthesis and synthetic suture materials can result in active motion as well. Even tumors that require resection of the radial nerve can be reconstructed and the resultant nerve deficit treated either by nerve graft or tendon transfers to restore radial nerve function in the hand. If the joint is involved, an extraarticular resection including the glenoid can be performed. The decision is between the above constructs

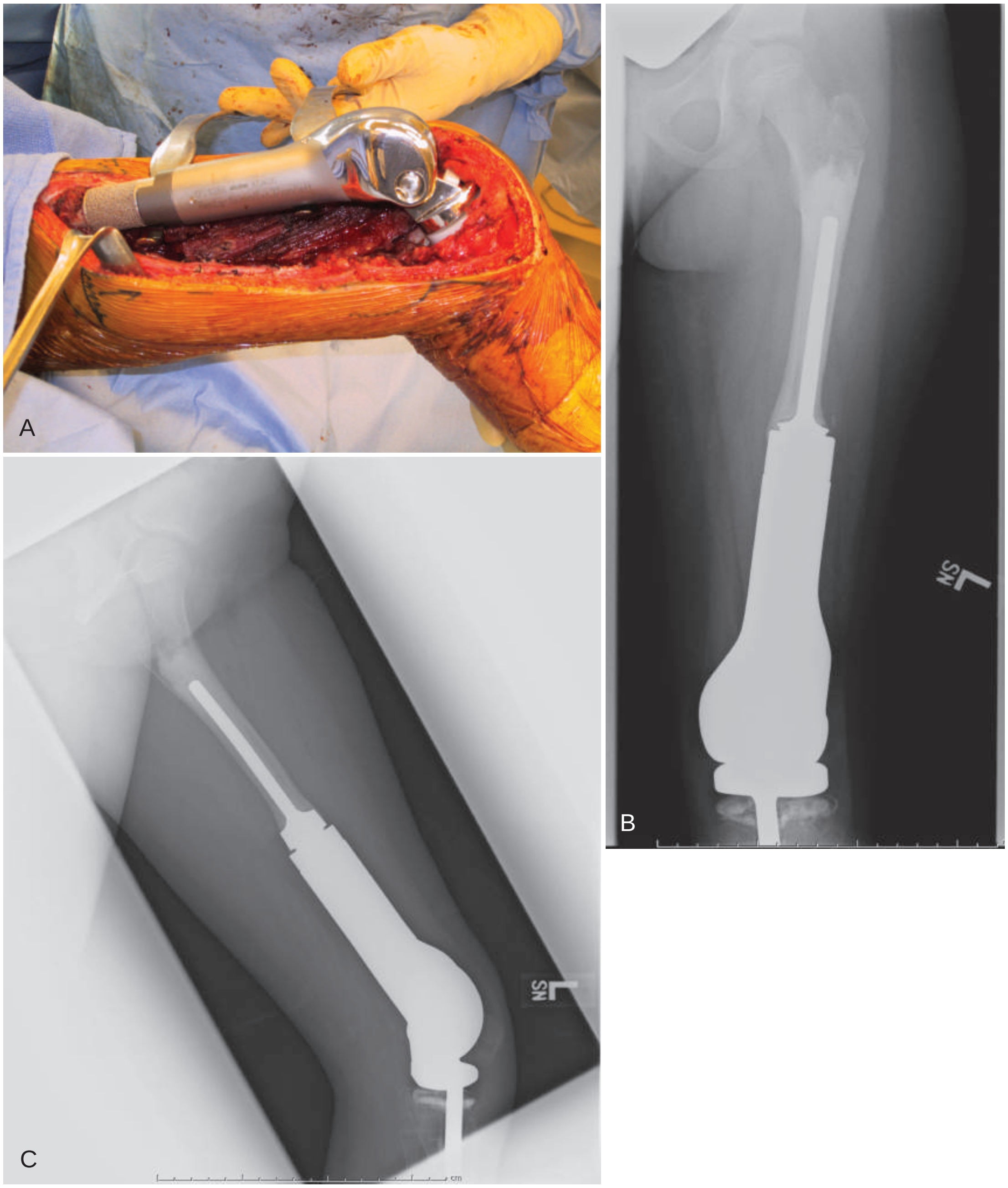

Figure 49-25 **A**, This figure shows the intraoperative appearance of another expandable prosthesis of the distal femur. This prosthesis requires an open procedure to lengthen it. **B** and **C**, The radiographic appearance of the prosthesis cemented in place. An uncemented smooth stem placed across the tibial physis allows that physis to grow.

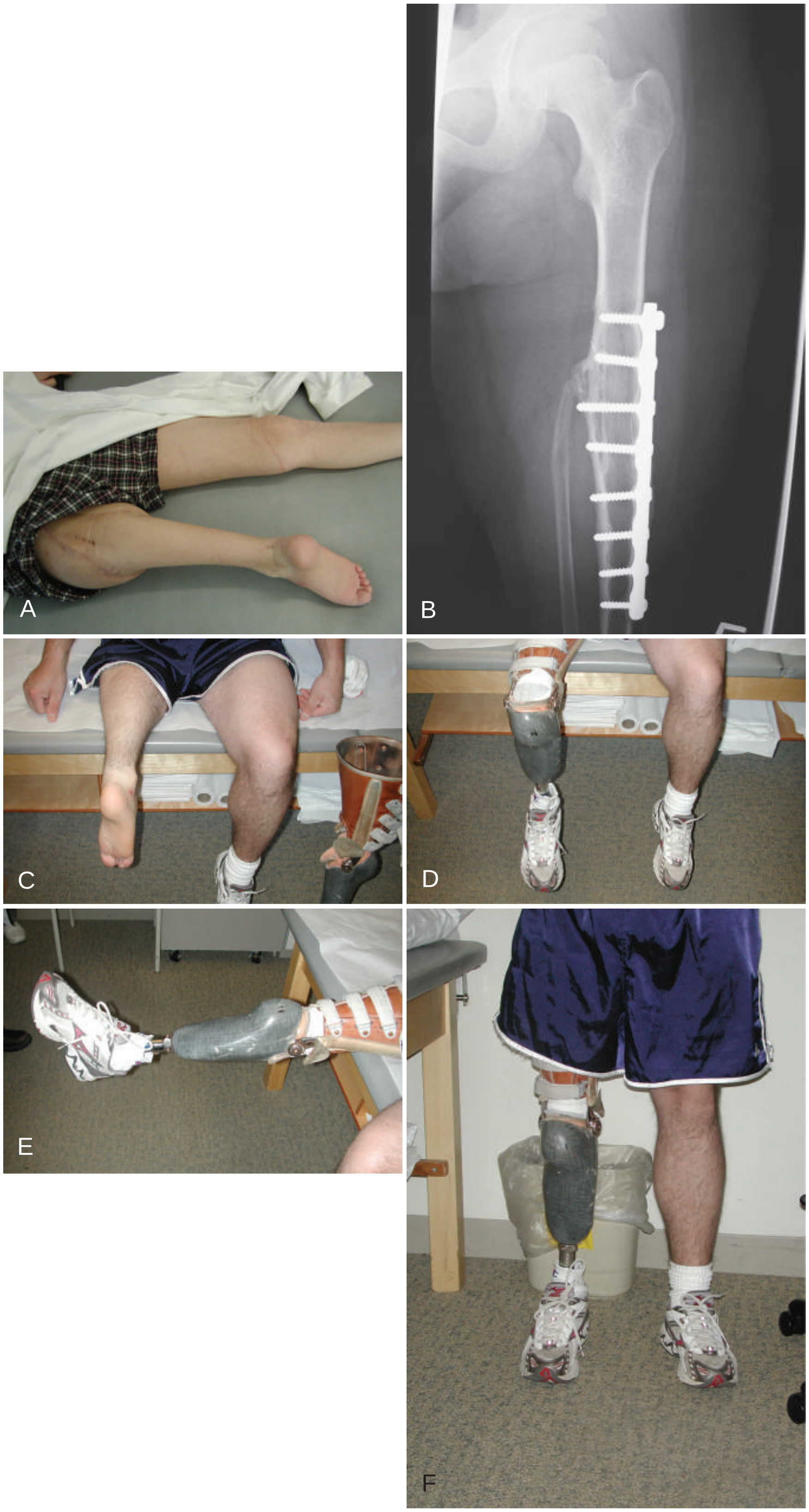

Figure 49-26 **A**, Patient with a rotationplasty for a distal femoral osteosarcoma. **B**, The radiographic appearance reveals the osteosynthesis of the tibia to the femur. **C** and **D**, These pictures show the appearance of the patient with the prosthesis off (**C**) and on (**D**). **E**, Patient has the ability to actively extend the knee against gravity. **F**, The appearance in stance.

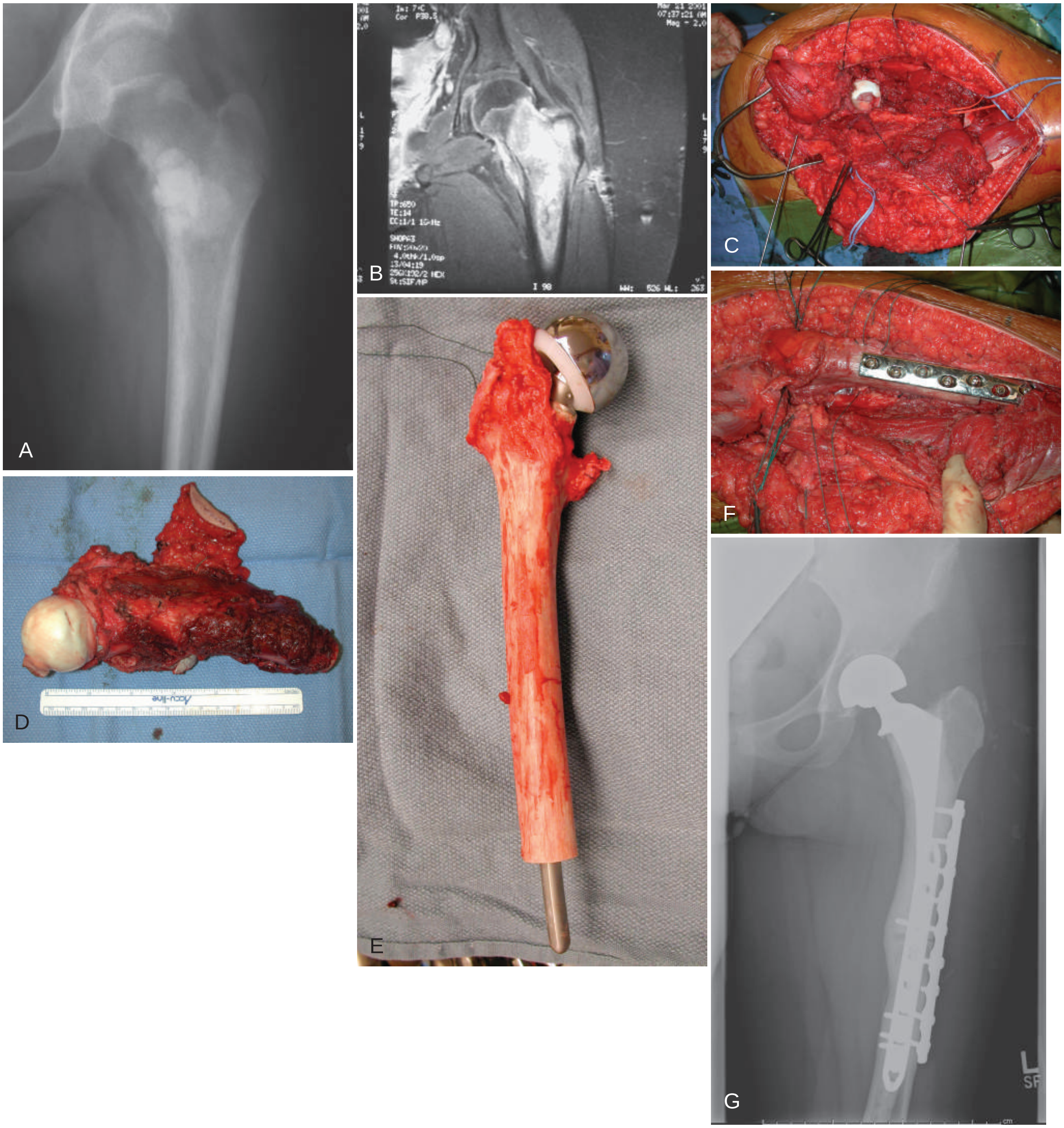

Figure 49-27 **A**, Radiograph of an osteosarcoma of the proximal femur. **B**, Magnetic resonance imaging shows that the hip joint is not involved. **C**, Intraoperative findings after resection. The acetabulum can be seen, and sutures are retracting the gluteus medius and minimus. **D**, The resected specimen is shown with the biopsy tract included. **E**, The allograft after a bipolar long-stem prosthesis has been cemented into it. **F**, Intraoperative photograph after the allograft has been fixed to the host bone. A unicortical plate is used for rotational control. The hip abductors have been sutured to the corresponding sites on the allograft. **G**, Postoperative appearance.

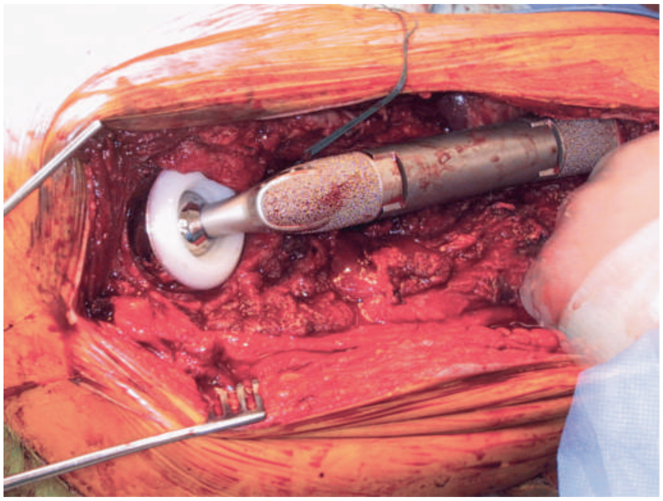

Figure 49-28 The intraoperative appearance of a modular metallic bipolar prosthesis with sites for sutures to secure the abductors. The rough surface is designed to allow tissue ingrowth with time. Distally, a similar surface allows placement of bone graft to seal the bone-cement interface and retard loosening.

and an arthrodesis using allografts with or without vascularized fibular grafts.[142,212,213,215] The latter allows for a stable shoulder, which is useful in a laborer but is difficult to achieve.

Outcomes

Although the goal of preserving a limb is laudable, and with experience the success rate of restoring functional limbs is improving, limb-sparing procedures are very complex operations. The short- and long-term complications are not trivial. It should also be remembered that young patients with amputations of the lower extremity do quite well with modern prostheses. We are only beginning to assess the quality of life and functional outcomes after these reconstructions, and the tools we have to assess these parameters are limited. The data that exists suggests that the energy cost of walking is better with a metallic prosthesis than an above-knee amputation.[216] A rotationplasty is similar to a below-knee amputation in that regard.[217] Most of the outcome studies report function using the Musculoskeletal Tumor Society rating system that has not been validated and is based on a surgeon assessment. Other outcome tools like the Short Form Health Survey 36 (SF-36) or the Toronto Extremity Salvage Score (TESS) are patient driven and not specific to sarcomas or a rare measure of disability.[218] The data

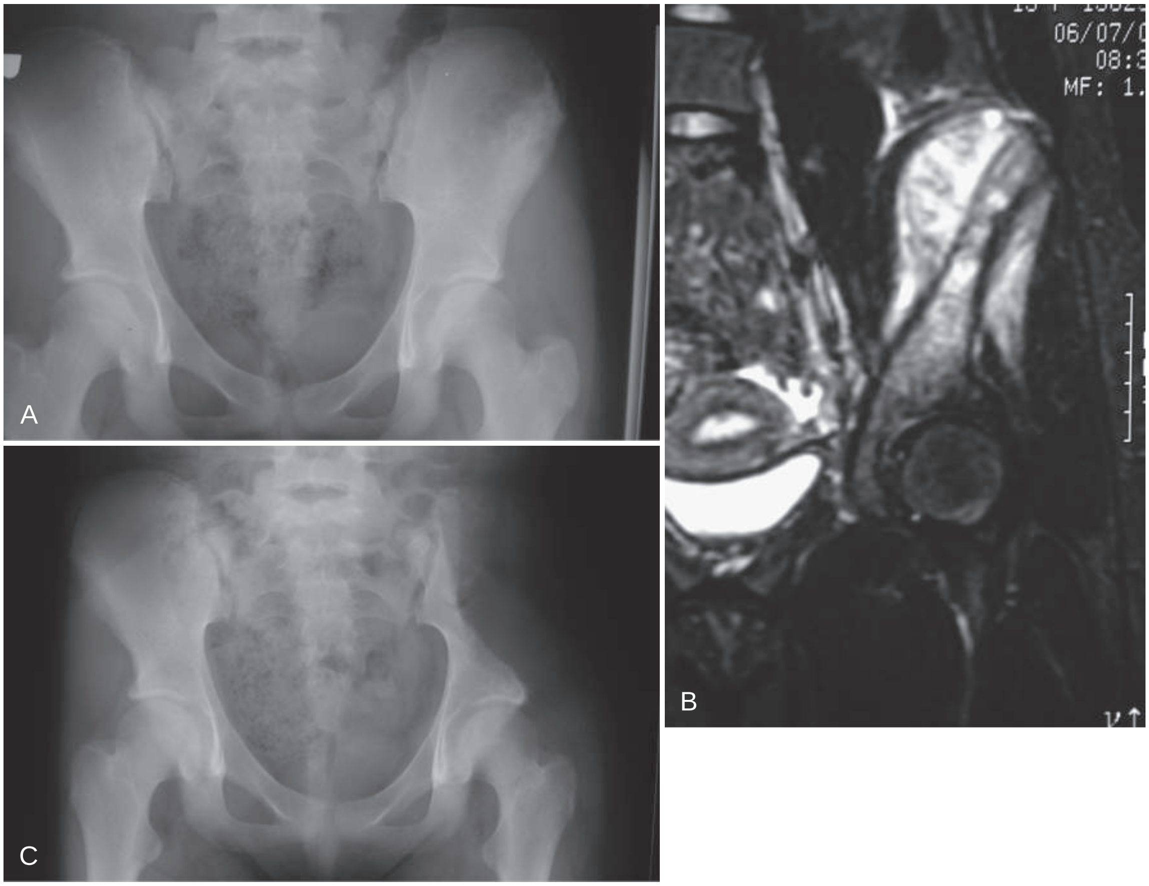

Figure 49-29 **A**, Radiograph of a Ewing sarcoma of the iliac wing. **B**, The magnetic resonance imaging reveals the extent of the soft-tissue mass. **C**, After chemotherapy, the soft-tissue mass was smaller, and resection was possible. The acetabulum was preserved, allowing reasonable function with a mild abductor lurch.

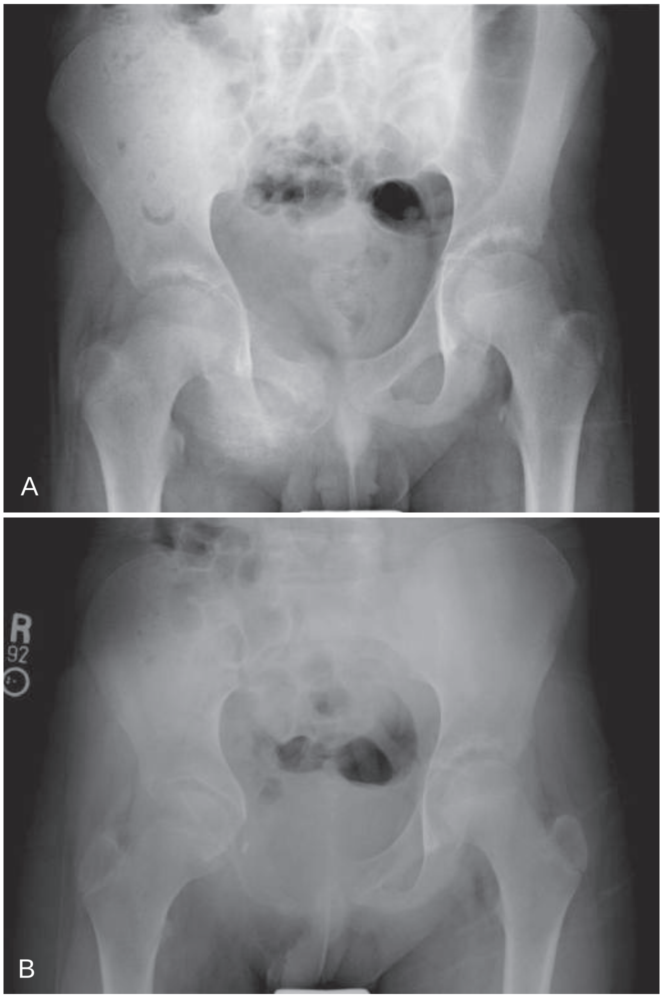

Figure 49-30 **A**, Preoperative radiograph of an osteosarcoma of the pubic rami. **B**, The appearance after resection showing preservation of the acetabulum is shown. Function in these situations is nearly normal, although hernia formation may be a problem.

suggest that there is little difference in outcomes and quality of life between amputees and limb-salvage patients and even less between the various types of reconstruction. However, the data are meager, and no controlled studies have been performed. This is an area for further study and development while the reconstruction options and prosthesis continue to evolve. Much progress has been made in the ability to treat pediatric bone sarcomas and to preserve limbs, but better reconstruction options are needed, including future developments in tissue engineering.

MANAGEMENT OF PULMONARY METASTASIS IN PEDIATRIC SOLID TUMORS

The value of the pediatric surgical oncologist in the locoregional control of malignant lesions in children was established well before the advent of chemotherapy or radiotherapy. However, the same cannot be said for the treatment of tumor metastases, especially in the lung. Two excellent reviews of the evolution and disease-specific implementation of pediatric pulmonary metastasectomy have been published.[219,220] The last five decades have produced a rapid expansion in the use of pulmonary metastasectomy for pediatric cancers, but evidence-based proof of its efficacy is limited. This is the result of a general lack of tumor-specific, prospective, randomized clinical trials critically evaluating this therapeutic intervention. Reported studies are primarily from a single institution, retrospective in nature, and often span years to decades and multiple treatment protocols. The studies also generally clump multiple tumors, thereby weakening the strength of any recommendations. Despite these limitations, some general principles and lesion specific recommendations can be made.

The Four Principles

When considering the management of a suspected metastatic pulmonary lesion, four principles must be addressed for each patient. First, the primary tumor diagnosis must

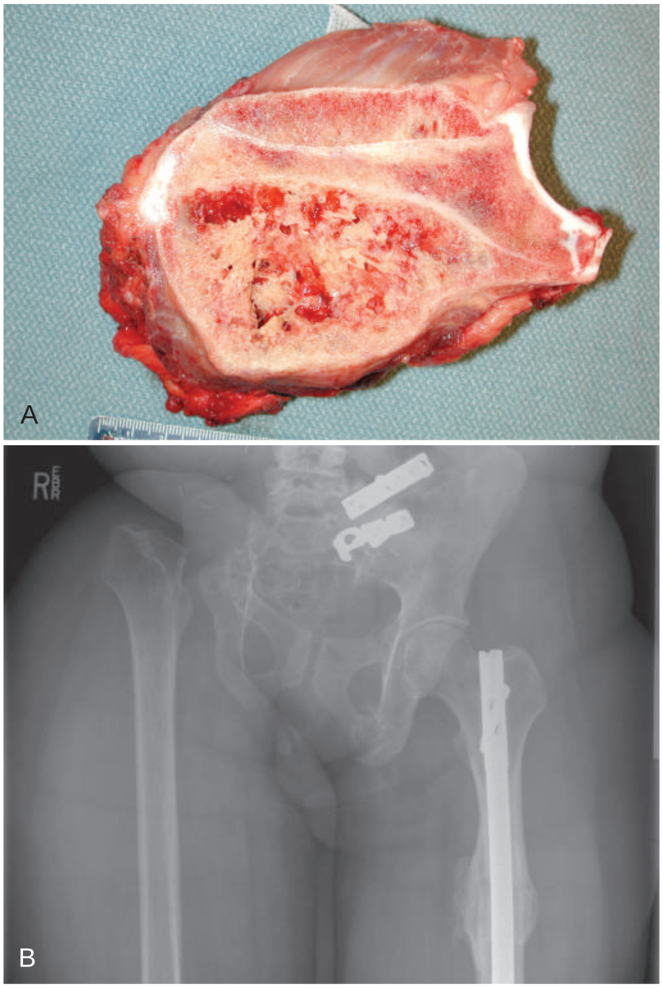

Figure 49-31 **A**, A specimen after resection of the iliac crest including the acetabulum for osteosarcoma (OSA). Function after these procedures is poor but better than an external hemipelvectomy. **B**, The radiograph of another patient more than 20 years after resection of her right ilium and acetabulum for OSA. She has had a closed femoral shortening on the left side to partially balance limb lengths. Despite her disabilities, she works full time and has a family.

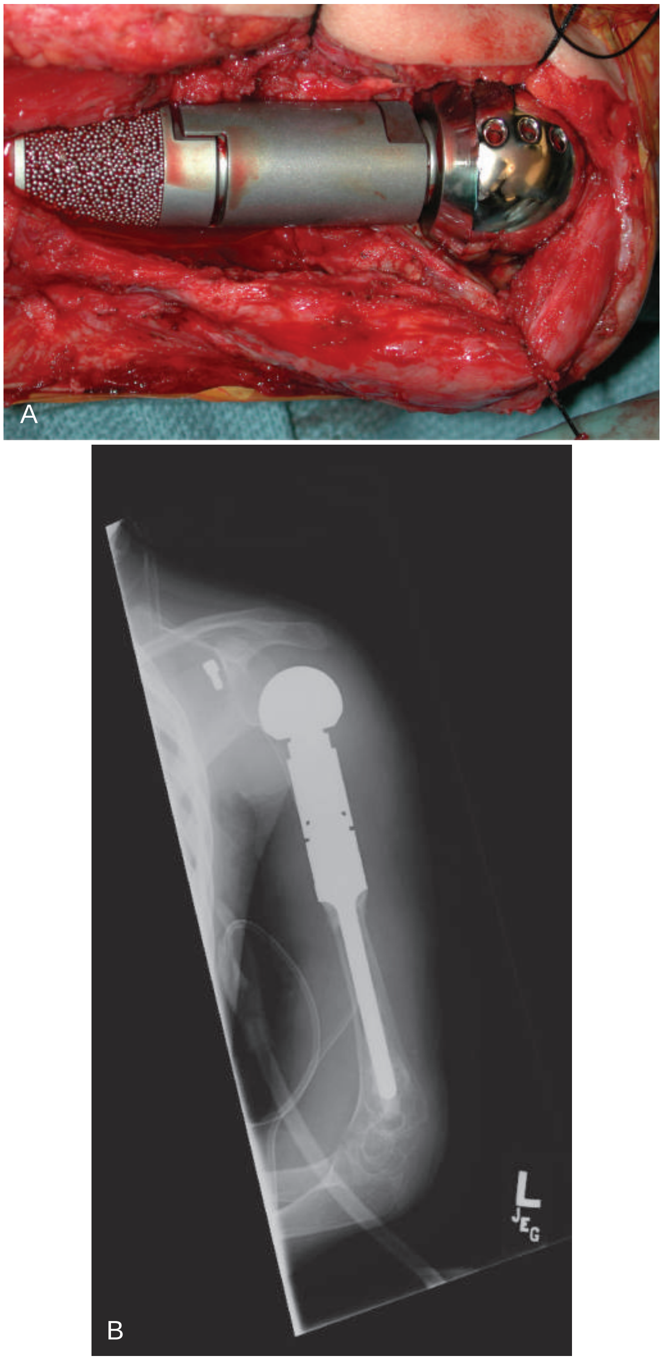

Figure 49-32 **A**, Intraoperative photograph of a metallic prosthesis used to reconstruct a patient with Ewing sarcoma who had a local recurrence after intercalary resection and allograft reconstruction. **B**, A postoperative radiograph of the prosthesis is shown. Active abduction is limited, but she has normal hand and elbow function.

be established. Response of pulmonary lesions to chemotherapy and radiotherapy is linked not only to the primary diagnosis but to the biologic subtype involved (i.e., favorable versus anaplastic Wilms tumor).

Second, the primary tumor must be controlled. A surgeon should not consider pulmonary metastasectomy before achieving local control of the primary lesion. However, biopsy to establish the presence of metastasis is required in some settings, because it determines the intensity of therapy.

Third, the therapeutic benefit to surgical resection must be assessed, and this is clearly dependent on the specific tumor. Ideally, the cancer should be chemoresponsive so as to control residual micrometastases. The lack of effective adjuvant therapy may temper the enthusiasm for pulmonary metastasectomy.

Finally, the extent of the pulmonary resection required to remove all metastatic disease should be assessed. The extent of the resection must be balanced with the therapeutic benefit achieved. An aggressive resection creating pulmonary impairment is difficult to support in the presence of chemotherapy-resistant disease, particularly in the presence of multiple lesions. Some cancers (e.g., OSA, adrenocorticocarcinoma) require an aggressive operative approach to remove all gross evidence of disease because of limited chemotherapy response in an attempt to provide an improved overall and disease-free survival.

Metastasis Localization

Depending on the cancer involved, a combination of radiographic studies and nuclear imaging modalities are used to identify metastases, especially in the lungs. Multidetector CT (MDCT) is the mainstay of identifying pulmonary lesions, and nuclear imaging is used for osseous lesions. More than 80% of lesions greater than or equal to 3 mm can be readily identified with this imaging modality,[221,222] but increasing sensitivity comes with decreasing specificity. Many lesions identified on MDCT may not be

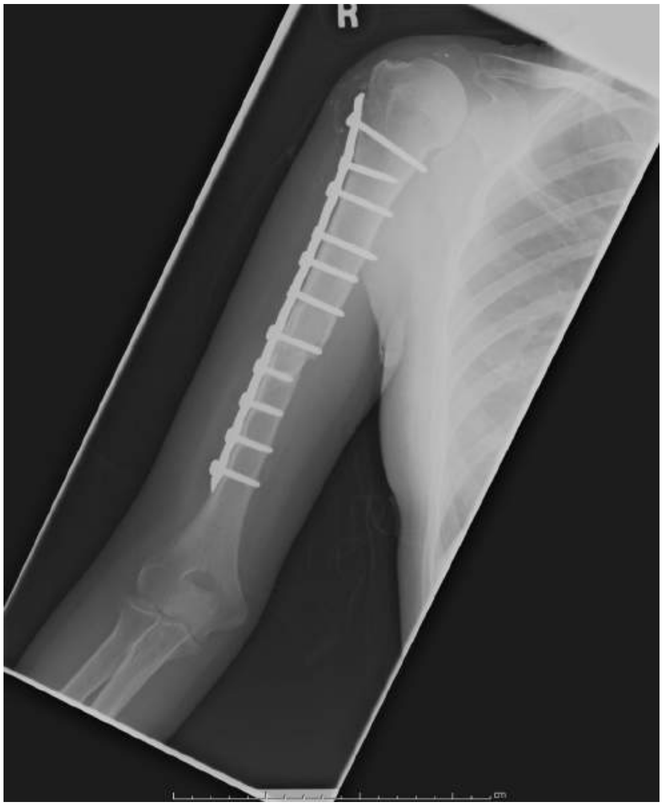

Figure 49-33 A proximal humeral osteoarticular allograft was used to reconstruct an intraarticular resection of the proximal humerus.

malignant. Although the appearance and location of the lesion may help, peripheral lesions that are smooth and well circumscribed are more likely to be malignant; biopsy is required for small lesions. Multiple lesions are also more likely to be malignant than isolated lesions.[223] Beyond this modality, nuclear medicine studies have been used for specific tumors with some success. Nuclear medicine studies exploit the biologic pathways of substrate molecules unique to specific tumors to identify these lesions as "hot spots" on subsequent whole-body scanning. These studies seldom stand alone as the only means to identify metastases, and they routinely act as corollary or confirmatory tests for lesions identified on MDCT. For example, a bone scintigraphy has been used to identify lesions in OSA,[220] but it is only taken up by large and biologically active lesions. Iodine[131] total body scans are routinely used in the evaluation and identification of pulmonary or osseous metastases in differentiated thyroid cancer, and it appears to be very sensitive and specific.[224-231] Finally, whole-body positron emission scanning utilizing fluorodeoxyglucose (18F-FDG PET) has been reported to have a sensitivity of 87% to 89% and a specificity of 100% in sarcomatous[232,233] and carcinomatous[233] pulmonary metastases, but this technique has limited assessment in pediatric primary malignancies, let alone metastases.

Biomarkers may be confirmatory tests for suspicious lesions found on MDCT or nuclear scintigraphy. Examples of tumor types with such markers that are routinely studied are adrenocortical carcinoma, differentiated thyroid carcinoma, germ cell carcinoma, and hepatoblastoma.

Technical Considerations

Pulmonary metastasectomy can be performed safely with minimal morbidity and a mortality rate of less than 1% documented in the International Registry of Lung Metastases.[233] The type of pulmonary resection can be either anatomic or nonanatomic ("wedge") despite early reports to the contrary,[234] but the vast majority of pulmonary metastasectomies performed are wedge resections that remove the lesion with a small rim of normal lung parenchyma (5 to 10 mm). Collective studies document the success of this procedure without the need for a formal lung resection along anatomic boundaries.[223,233, 235,236-238]

The use of MIS approaches for the resection of pulmonary lesions has been described in several studies.[239-244] Additionally, a recent report by Zaman and colleagues[245] analyzed various techniques—finger palpation, intraoperative ultrasound, hook-wire techniques, spiral-wire techniques, and fluoroscopic and/or radio guided detection—that can be used to assist in the localization of subcentimeter pulmonary nodules in conjunction with an MIS approach. Their conclusion was that radio guided techniques are superior to all others, especially finger palpation, which they recommended should be abandoned. MIS alone or in conjunction with other intraoperative localizing procedures has become an established technique except in the case of OSA, in which MDCT scanning has been shown to underestimate the number of lesions and formal thoracotomy has been recommended.[246] A cohort of patients with OSA and unilateral, metachronous pulmonary metastases confirmed by MDCT within 2 years of the primary lesion were found to have occult metastases on the contralateral side in 78% of cases.[247] Therefore staged or sequential bilateral thoracotomies are recommended in patients with OSA by some authors, although this approach has not been shown in a prospective study to prolong either EFS or OS.

Select Pediatric Malignancies

Adrenocortical Carcinoma

Pulmonary metastases from adrenocortical carcinoma should be considered for complete surgical removal, because effective chemotherapy is not available. A recent review of the International Pediatric Adrenocortical Tumor Registry revealed that the mean age of patients at presentation was 3.2 years, and more than 90% of the lesions were functional tumors (84% virilizing).[248] The 5-year EFS was 54%, and the incidence of pulmonary metastases was 7% at presentation. Prognosis was improved for younger patients with completely resected, small (<200 g), early-stage tumors with virilizing symptoms. Separate analysis of the patients with lung metastases could not be performed because of the small number of patients. Isolated case reports in the literature document long-term survival in patients after pulmonary metastasectomy for adrenocortical carcinoma.[249,250] If one examines the adult studies addressing this question, the role for complete tumor excision becomes readily apparent. Kwauk and colleagues examined their series of 24 primarily adult patients with adrenocortical carcinoma and pulmonary metastases.[251] They noted that complete surgical excision of all metastatic disease achieved a 5-year survival rate of 71% versus 0% for those with unresected disease. Though their patient cohort was small and not randomized, they concluded that in the light of ineffective chemotherapy, complete pulmonary metastasectomy was not only indicated but mandatory to achieve long-term survival.

Schulick and colleagues echoed these results and sentiments when analyzing their institution's results with metastatic adrenocortical carcinoma in predominantly adult patients.[252] In fact, not only do they advocate for an initial pulmonary metastasectomy but for repeated resections as well. Some patients in their series had as many as seven resections for recurrent disease. Although this may seem overly aggressive, they note that for every reoperation, the median survival was longer for complete versus incomplete resection groups, which is probably a function of the biology of the tumor involved and anatomic limits of resection as opposed to the operation performed (Fig. 49-34). Furthermore the majority of metastatic resections performed were for pulmonary disease. Thus the data confirm the important role for surgery in the treatment of adrenocortical carcinoma metastatic to the lungs with resectable disease where increased survival and cure can be achieved.

Differentiated Thyroid Cancer

Pulmonary metastases from differentiated thyroid cancer should not be treated by surgical excision except in cases

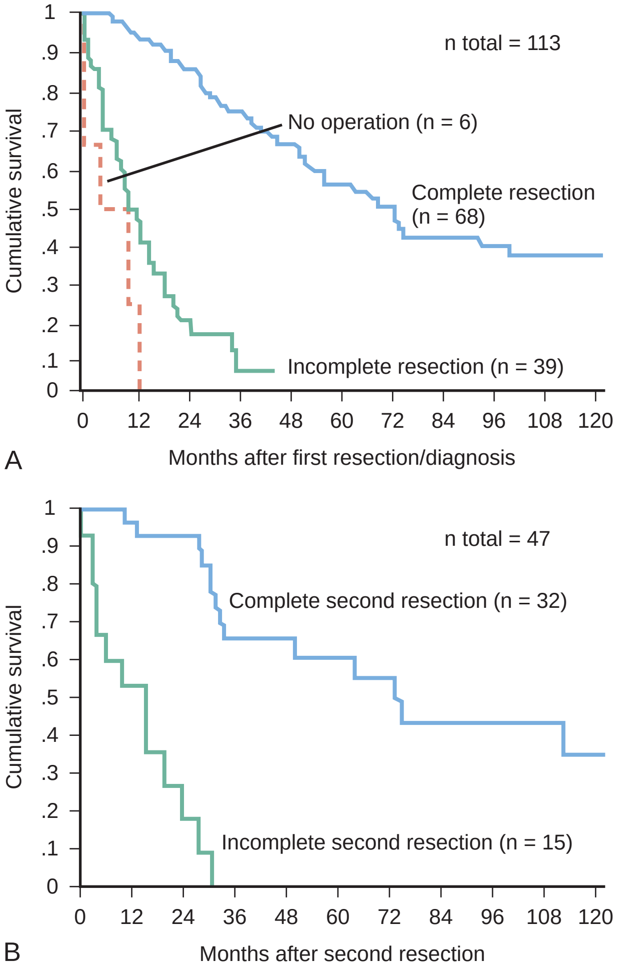

Figure 49-34 **A** and **B**, Disease-specific survival rates for patients undergoing complete versus incomplete primary resections for adrenocortical carcinoma. *(Modified from Schulick RD, Brennan MF: Long-term survival after complete resection and repeat resection in patients with adrenocortical carcinoma.* Ann Surg Oncol *6(8):719–726, 1999.)*

in which there is indecision about the nature of the diagnosis, especially after treatment. Despite the presence of disseminated disease to the lungs, prognosis is still quite good. Unlike skeletal and pleural involvement, patients with pulmonary metastases have mortality rates from 0% to 6%.[226,227,229-231,252] Studies have reported the incidence of pulmonary metastases of 6% to 25%.[227,231,228,229-231] Interestingly, these studies have also documented that pulmonary metastases are very responsive to radioactive iodine treatment. They are seen more often in younger patients and can first appear more than 10 years from the initial diagnosis.

A recent comprehensive review of the treatment of thyroid cancer in children exposed to radiation from the Chernobyl nuclear power station comprises the largest series of pediatric thyroid carcinoma.[231] Seven hundred and forty cases of thyroid cancer occurred in 681 children with direct exposure to the accident. One hundred thirty-one cases (17.7 %) of pulmonary disease were diagnosed by radionuclide scan with a mean presentation of 19 months after the primary diagnosis. Only 2.3% of patients diagnosed had pulmonary metastases that were found on chest radiograph; hence the authors recommended the use of radioactive iodine scanning as the procedure of choice because of its increased sensitivity and specificity. The risk factors for metastatic disease were female gender, a younger age at diagnosis, and the presence of clinical symptoms. Patients were treated with radioactive iodine for their initial pulmonary metastasis. With therapeutic radioactive iodine administration for pulmonary metastases, 28.9% had a complete response (negative nuclear imaging and thyroglobulin <1 ng/mL), 47.7% had a partial but stable response (negative nuclear imaging and thyroglobulin 1 to 10 ng/mL), and 23.4% had a partial response. The 5- and 10-year survival rates were 99.5% and 98.8%, respectively, with only 5 children dying of distant metastases (3 medullary, 1 follicular, and 1 papillary) with a mean follow-up period of 115 months. This study and others document the success of medical therapy. Surgery is required only for diagnosis if the lesions fail to take up iodine.

Germ Cell Tumors (Gonadal)

Pulmonary metastases from gonadal germ cell tumors should be biopsied only if their origin is in question. This tumor is very responsive to chemotherapy, so resection is performed only after treatment if radiographic abnormalities persist. Germ cell tumors comprise a broad range of tumors, 20% of which are malignant.[253] The malignant lesions are chemosensitive, and with the advent of cisplatin-based regimens long-term survival has increased to 90%.[254] However, residual disease after adjuvant therapies may harbor viable tumor,[254-258] with the lung being the most common site of distant metastasis.[255] Synchronous pulmonary metastases are found in 50% of patients with retroperitoneal lymphadenopathy and in 10% of patients without adenopathy.[254] Therefore the role of pulmonary metastasectomy is primarily to define the presence of viable tumor in residual lesions after chemotherapy. This would establish a basis for further therapy. Several groups have examined their data concerning the role and utility of pulmonary metastasectomy.

Cagini and colleagues examined their cohort of 141 patients over a 17-year period with both pulmonary and mediastinal involvement; 51% of patients had isolated pulmonary disease, 24% had pulmonary and mediastinal disease, and 24% had mediastinal disease alone.[254] Roughly, one third of patients had a single lesion, one third had 2 or 3 lesions, and one third had more than 3 lesions. Eighty-seven percent had a complete resection, and histological analysis revealed that 23% of the lesions had only necrosis or fibrosis, 45% had mature teratoma, and 32% were malignant. Biomarker analysis in these patients was not helpful, because 23% of patients with malignant disease had negative biomarkers. A second recurrence occurred in 47% of patients. The overall mortality rate was 19% for the entire group. Actuarial survival after pulmonary metastasectomy was 78% at 5 years, 76% at 10 years, and 66% at 15 years posttreatment. Statistically significant prognostic factors were the pathology of the specimen (complete necrosis or fibrosis of the specimen as opposed to the presence of viable tumor), presence of residual disease (mediastinal disease alone had a better outcome than pulmonary disease with or without mediastinal disease), and the number of resected lesions.

These results were echoed by Liu and colleagues who examined their cohort of 157 patients with isolated pulmonary metastases after chemotherapy over a 28-year period.[254] The majority of patients had multiple lesions (72%), and 89% of patients underwent nonanatomic resections. The mortality rate was 0.6%, and the morbidity rate was 6%. Pathologic analysis revealed viable tumor in 44% of the cohort, 37% of the cohort had a subsequent recurrence of disease in their chest, and 56% of these died. The overall mortality rate was 32%. Statistically significant negative prognostic factors were age older than 25 years, no cisplatin-based chemotherapy, the presence of viable tumor when the lesions were resected posttherapy, and the existence of metastatic disease outside of the thorax or retroperitoneum. The authors conclude that surgery plays a critical role in determining those patients who would benefit from further therapy. These results are also confirmed by other groups,[256-258] but patient selection is an important criteria. Ultimately, surgical salvage using pulmonary metastasectomy is a viable alternative to patients with residual, radiographically identifiable lesions to determine the chemoresponsiveness of these tumors and to provide for an increased long-term survival with complete surgical resection.

Hepatoblastoma

Pulmonary metastases from hepatoblastoma should be treated first with adjuvant chemotherapy, and only then any residual lesions should be resected. Data to support this concept have appeared over the last several decades with an initial report by Black and colleagues.[259] They reported four patients who were long-term survivors, and they stressed the need for complete local control and adjuvant chemotherapy. Furthermore they correlated the need to perform the pulmonary metastasectomy with the patient's α-fetoprotein levels, especially advocating early surgery for those patients with rising titers on appropriate therapy.

Feusner and colleagues[260] representing the CCG published their results, and they confirmed the premises outlined by Black and colleagues. Of their cohort of six patients with pulmonary recurrence, four had a complete remission and three had a survival of more than 5 years. Their approach was to combine adjuvant chemotherapy with aggressive pulmonary resections. Some caution is appropriate, however, when it is recognized that the OS or EFS of patients who have stage-IV disease in hepatoblastoma is 16%, and patients with relapse generally do worse having already received the primary therapies. Beyond these North American results, two reports from the SIOPEL have documented their results with hepatoblastoma and pulmonary metastases. The first report by Perilongo and colleagues[261] in 2000 documented the results of SIOPEL 1: 20% of patients with hepatoblastoma had pulmonary metastases, and they achieved a 5-year EFS rate of only 28%. A sustained remission of 26% of the cohort with metastatic pulmonary lesions was achieved from chemotherapy alone. Schnater and colleagues[90] analyzed the surgical approach in SIOPEL 1 and documented that 41% of patients who had pulmonary metastases were long-term survivors, but the four patients who underwent surgery and chemotherapy to treat their pulmonary metastases were alive at data analysis (minimum of 2.5 years posttreatment). In a nonrandomized study, the apparent difference in outcome may result from selection factors. Their data also suggest that recurrent pulmonary disease could be cured with surgery and chemotherapy as long as there was local control of the tumor. Thus these studies cumulatively support a possible role for pulmonary metastasectomy for hepatoblastoma in selected residual lesions.

Neuroblastoma

Pulmonary metastases from neuroblastoma are rare, and they should not be treated by surgical excision except in cases in which there is indecision about the nature of the lesion, especially after or during treatment. The incidence of pulmonary metastases from neuroblastoma has been reported to occur in fewer than 1% to 23% of cases[262-266]; however, most large studies report rates of 5% or less.[264-266] They can be synchronous or metachronous, and they are usually multiple, bilateral, subcentimeter, and peripheral. Patients with pulmonary metastases from neuroblastoma have a poor prognosis, a high association of unfavorable Shimada histology, *MYCN* amplification, and a lower EFS rate.[265,266] Hence the utility of performing resections except to confirm the diagnosis is not established.

Wilms Tumor

Pulmonary metastases from nephroblastoma should generally not be resected, and suspected lesions should only be biopsied, although some authors[236,267] have advocated for primary pulmonary metastasectomy to spare the effects of radiotherapy on patients.[268] North American centers under the protocols established by the NWTSG have demonstrated the efficacy of chemotherapy and radiotherapy in the treatment of pulmonary metastases over chemotherapy and surgical excision, regardless of pathologic subtype.[269-271] Specifically, Green and colleagues in 1991 examined patients enrolled in NWTSG studies 1 and 3 with pulmonary recurrences. Of the patients meeting study criteria, the resection of pulmonary metastases within 30 days of diagnosis did not affect 4-year survival outcomes in favorable or unfavorable histopathologic cohorts. Furthermore, Green and colleagues[271] reported results from NWTSG-4, in which pulmonary metastasectomy had almost no role in the treatment of patients with stage-IV disease patients. It was performed for residual lesions after chemotherapy and radiotherapy, but it did not affect the OS rates achieved of greater than 75% to 90%. The importance of biopsy for suspicious lesions, especially small lesions (<1 cm) found only on CT was confirmed recently by Erhlich and colleagues,[272] who reviewed the cases of those children enrolled in NWTSG-5. Thirty-three percent of those who received radiotherapy had a prior biopsy that was negative for tumor, and hence they were overtreated. These results were for patients with both isolated and multiple lesions. These studies collectively confirm the general success of combined chemotherapy and radiotherapy in the treatment of pulmonary metastases in nephroblastoma. Whether in fact those children with metastatic disease seen only on MDCT and resolved with chemotherapy need pulmonary radiotherapy remains an unanswered question. Finally, surgical intervention for pulmonary lesions in nephroblastoma should only be utilized to confirm the existence of malignancy in MDCT-defined masses before administering therapy.

Sarcomas

Pulmonary metastases from sarcomatous pediatric tumors should be biopsied at a minimum, and complete resections should be performed depending on the precise histologic subtype. The most comprehensive reports concerning this topic are by Temeck and colleagues from the National Cancer Institute, who published two reports in the 1990s that documented their results with initial[273] and recurrent pulmonary metastasectomies.[274] Their initial report examined 152 patients who underwent 258 thoracotomies over 18 years. The lesions included OSA (50%), nonrhabdomyosarcoma soft-tissue sarcomas (27%), EWS (18%), and rhabdomyosarcoma (3%). Eighty percent of patients had a complete resection, and the cohort had morbidity and mortality rates of approximately 6% and 1.3%, respectively. The highest median survival occurred in the OSA group (3.1 years), and the lowest was in patients with rhabdomyosarcomas (0.4 years). Statistically significant poor prognostic factors included the presence of three or more metastases, a diagnosis other than OSA, and an incomplete resection. Their follow-up study on recurrent lesions further documented the importance of histological subtypes, because no patients with rhabdomyosarcoma were eligible for evaluation. They also documented that CT missed 39% of lesions, 24% of which were found to be malignant, supporting the need for thorough evaluation of the thorax through an open approach. Furthermore they documented a complete resection rate of 77% with 5.6% morbidity rate and a 1% mortality rate. Finally they documented a statistically significant improved survival rate in those patients who underwent complete resections, regardless of the number of recurrences. In fact this survival advantage was eight to 20 times greater depending on the number of recurrences. These data, though encouraging for sarcomas as a whole, must be considered by individual histologic variants. The one sarcomatous variant that may benefit from pulmonary metastasectomy is OSA.

The utility of complete, recurrent pulmonary metastasectomy in OSA was ushered in with the report by Martini and colleagues describing their experience in 1971.[275] They reported a 45% survival rate in patients who had complete pulmonary metastasectomies, which is four times greater than that of chemotherapy alone. Reports from the last decade[†] have not only confirmed these results but clarified the therapeutic approach to pulmonary metastases in OSA. First, OSA should be treated with a multiagent regimen that addresses synchronous pulmonary lesions at the time of local control. Second, pulmonary lesions should undergo repeated complete resections, because this is the best chance for long-term disease-free survival and OS. Third, extirpation of extrapulmonary metastases does not confer the same survival benefits as does pulmonary metastasectomy and is often less feasible, because many of these osseous. Fourth, CT imaging does not identify all pulmonary lesions, and most lesions are not calcified. Therefore staged bilateral thoracotomies are suggested by some for complete evaluation and resection of all pulmonary disease. Fifth, once metachronous lesions have been identified and treated surgically, a majority of patients will have at least one pulmonary recurrence, and most recurrences are within 12 months of the first resection. Therefore continued surveillance and frequent repeat imaging are essential. Finally, though tumor burden was and still is an important prognostic factor in the determination of outcome, several biologic features (e.g., disease-free interval and percentage of tumor necrosis on histopathologic evaluation) have become even more significant in recent reviews. Hence aggressive, repeated complete resection of pulmonary metastases is critical to the successful treatment of patients with OSA.

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CHAPTER 50

Acute Lymphoblastic Leukemia

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CHAPTER OUTLINE

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FUTURE DIRECTIONS

The acute leukemias of childhood are rare diseases that collectively represent about 30% of malignancies in children younger than age 15 years.[1] Approximately 3000 new cases of childhood leukemia occur annually in the United States, 80% of which are acute lymphoblastic leukemia (ALL). Between the 1960s and 1990s, the prognosis for children with ALL improved dramatically. With current multiagent chemotherapy regimens, 80% to 90% of children with ALL are long-term relapse-free survivors.[2,3,4] In this chapter, childhood ALL is reviewed with respect to classification, molecular pathogenesis, clinical presentation, laboratory findings, differential diagnosis, treatment strategies, prognosis, and late effects of therapy. Causative and epidemiologic considerations, including the incidence, prevalence, and life span of afflicted individuals, are discussed in Chapter 40. Discussion of the cytogenetic aspects of the leukemias appears in Chapter 45, and the molecular basis of human malignancy is further discussed in Chapter 33. Details concerning the various chemotherapeutic agents are provided in Chapter 46, and myelogenous leukemias and myeloproliferative disorders are discussed in Chapter 51.

CLASSIFICATION

The childhood leukemias can be classified as acute or chronic. *Acute leukemia* is characterized by clonal expansion of immature hematopoietic or lymphoid precursors, whereas *chronic leukemia* refers to conditions characterized by the expansion of mature marrow elements. *Congenital leukemia* refers to leukemias diagnosed within the first 4 weeks of life.

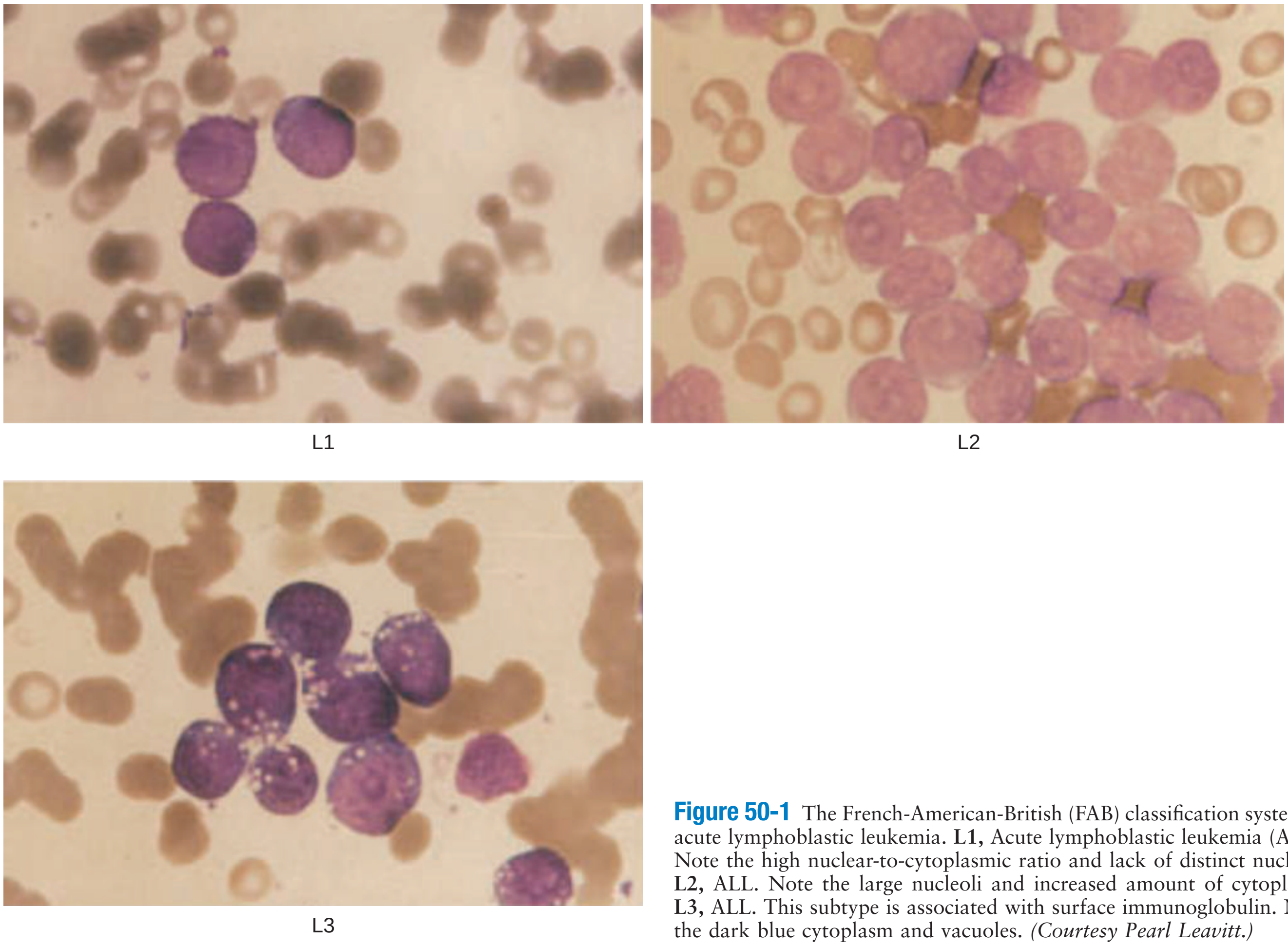
Acute leukemia is characterized by replacement of normal marrow elements with malignant blast cells, relatively undifferentiated cells with diffusely distributed nuclear chromatin, one or more nucleoli, and basophilic cytoplasm. A number of methods exist for characterizing malignant blast cells, including morphology, cytochemistry, and immunophenotype, as well as chromosomal and molecular genetic aberrations. Approximately 80% of cases of childhood acute leukemia are lymphoblastic.

Morphologic and Cytochemical Classification

The production of blast forms is part of the normal maturational sequence of hematopoietic and lymphoid elements. Blast cells are primitive precursors, lacking many of the features of differentiation. Under normal conditions, blast forms constitute fewer than 5% of the nucleated cells of the bone marrow. Blast cells are usually not observed in the peripheral blood, except during periods of profound overproduction of blood cells in response to infection or bleeding or from bone marrow invasion by granulomas, fibrosis, or tumor cells (leukoerythroblastosis). Leukemic blast cells can be difficult to distinguish morphologically from normal, nonmalignant blasts, although the finding of more than 5% blast forms in the marrow, or the presence of blast cells in the peripheral blood, should raise the suspicion of leukemia. In acute leukemia, patients often present with a marrow that is almost fully replaced by blast forms.

Once the diagnosis of leukemia is made, it is sometimes difficult to differentiate a lymphoid from a myeloid blast. The morphologic characteristics of lymphoblasts are shown in Figure 50-1. Wright-Giemsa–stained lymphoblasts have smooth, homogeneous nuclear material with indistinct nucleoli and only a small rim of light blue–staining cytoplasm, generally without granules. In contrast, myeloblasts differ from lymphoblasts in that the former have a lower nuclear-to-cytoplasmic ratio, more finely developed nuclear chromatin, and more distinct punched-out nucleoli. Cytoplasmic granules are often present in myeloblasts, and the detection of eosinophilic Auer rods is pathognomonic. However, small myeloblasts may be confused with lymphoblasts morphologically, although they can usually be distinguished by flow cytometric or cytochemical studies.

A standardized morphologic classification system devised by the French-American-British (FAB) Cooperative Working Group is generally used to categorize the appearance of leukemic blasts.[5] In this system, ALL is subdivided into three morphologic categories: L1, L2, and L3 (see Fig. 50-1). L1 is the most common subtype, observed in about 90% of cases of childhood ALL. L1 lymphoblasts are small cells characterized by a high nuclear-to-cytoplasmic ratio. The pale blue cytoplasm is scanty and limited to a small portion of the perimeter of the cell. The cells have indistinct nucleoli and nuclear membranes that vary from round to clefted. Cells in the L2 category, found in 5% to 15% of pediatric cases, are larger than those classified as L1, show marked variability in size, and have prominent nucleoli and more abundant cytoplasm. They may be difficult to distinguish morphologically from the M1 variant of myeloid leukemia, and thus flow cytometric analysis of cell surface markers is essential to differentiate ALL from acute myelogenous leukemia (AML). L1 and L2 lymphoblasts do not differ significantly in terms of cell surface markers, genetic abnormalities, or prognosis.[6,7] Only 1% to 2% of



L1

L2

L3

Figure 50-1 The French-American-British (FAB) classification system of acute lymphoblastic leukemia. **L1,** Acute lymphoblastic leukemia (ALL). Note the high nuclear-to-cytoplasmic ratio and lack of distinct nucleoli. **L2,** ALL. Note the large nucleoli and increased amount of cytoplasm. **L3,** ALL. This subtype is associated with surface immunoglobulin. Note the dark blue cytoplasm and vacuoles. *(Courtesy Pearl Leavitt.)*

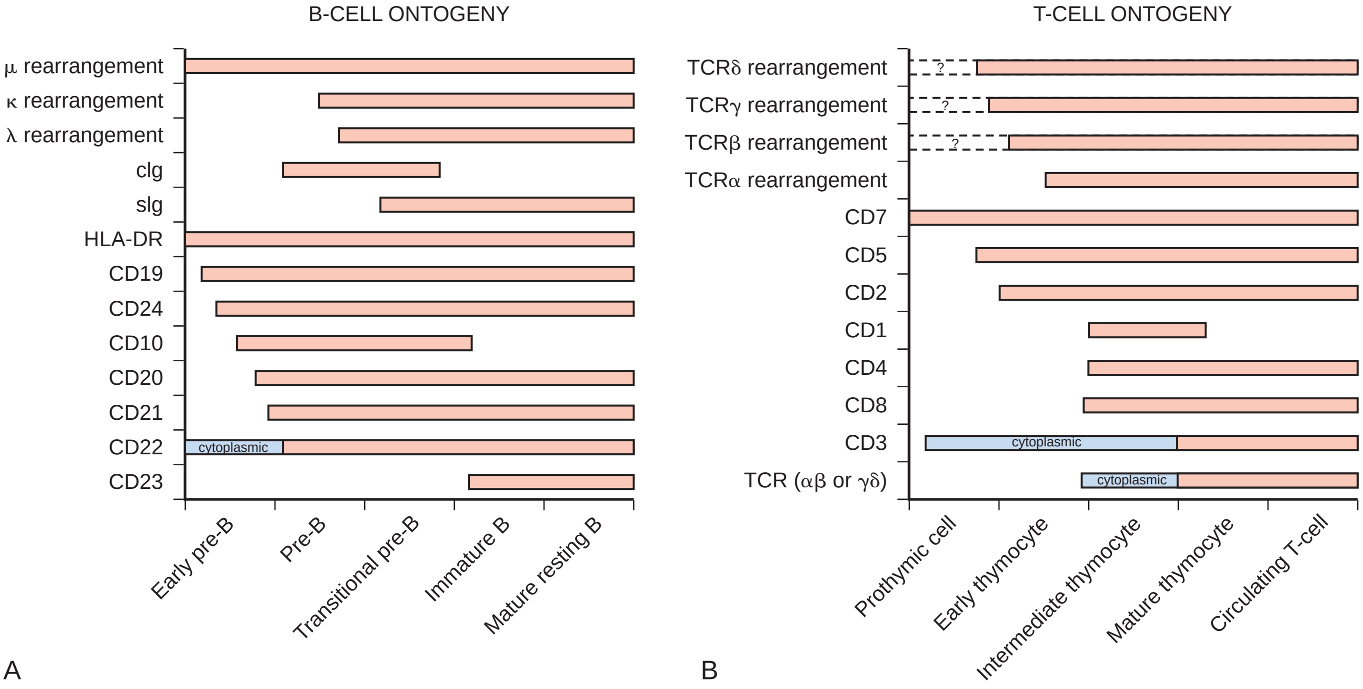


Figure 50-2 Schematic representation of human lymphoid differentiation. **A,** Hypothetical schema of marker expression and gene rearrangement during normal B-cell ontogeny. **B,** Hypothetical schema of marker expression and gene rearrangement during normal T-cell ontogeny. *(Modified from Pui CH, Behm FG, Crist WM: Clinical and biologic relevance of immunologic marker studies in childhood acute lymphoblastic leukemia.* Blood *82:343–362, 1993.)*

ALL pediatric patients have L3 lymphoblasts, which appear identical to Burkitt lymphoma cells, with deeply basophilic cytoplasm and prominent cytoplasmic vacuolization. L3 morphology is almost always associated with a mature B-cell phenotype (including expression of surface immunoglobulin) and translocations involving the *MYC* gene on chromosome 8; patients with these characteristics should be treated with regimens for advanced-stage Burkitt lymphoma rather than ALL.[8,9] Other morphologic variants of ALL, including ALL with hand mirror cells or granules, have been described but do not appear to have prognostic significance.[7]

Various cytochemical stains can distinguish ALL from AML. In approximately 80% of cases of ALL, lymphoblasts react positively with periodic acid–Schiff (PAS), which stains cytoplasmic glycogen.[10] Myeloid-specific stains, such as myeloperoxidase, specific and nonspecific esterase, and Sudan black (a cytoplasmic lipid stain), are usually negative in ALL. In contrast, myeloblasts are myeloperoxidase positive in 75% of patients. Terminal deoxynucleotidyl transferase (TdT), an enzyme that catalyzes the polymerization of deoxynucleoside monophosphates into a single-strand DNA primer, can be demonstrated in most cases of ALL but is rarely present in cases of myeloid leukemia.

Immunophenotype

ALL is subcategorized based on the expression of lineage- and maturation-specific antigens present on the cell surface and in the cytoplasm of lymphoblasts. In addition, immunophenotypic studies of lymphoblasts have provided important insights into the differentiation and maturation of normal B and T lymphocytes. Because the leukemic blast represents the neoplastic expansion of a lymphoid progenitor cell, many phenotypic and genotypic features of the lymphoblast mimic those of the normal lymphoid cell counterpart of specific lineage and maturational stage.[11] Figure 50-2 depicts a schematic overview of lymphoid cell differentiation.[12]

Based on reactivity with a panel of lineage-associated antibodies, ALL has been subclassified into three broad categories: B-precursor cell, mature B cell, and T cell. The immunophenotypic subsets are associated with distinctive clinical features (Table 50-1).[13]

B-Precursor Cell ALL

The B-precursor cell phenotype is present in 80% to 85% of children with ALL. These leukemia cells are characterized by reactivity with monoclonal antibodies specific for B-cell–associated antigens (e.g., CD9, CD19, CD22, and CD79a) and are distinguished from mature B-cell ALL by the absence of surface immunoglobulin. In the vast majority (80% to 90%) of B-precursor cell ALL, the cells express CD10, also known as the common ALL antigen (CALLA).

Over 90% of cases of B-precursor cell ALL have evidence of immunoglobulin gene rearrangements, predominantly involving the immunoglobulin heavy chain (IgH).[14] Of note, T-cell receptor (TCR) gene rearrangements also may be observed in B-precursor cell ALL and IgH gene rearrangements have been observed in T-cell ALL.[15] These leukemia-specific clonal IgH and TCR gene

TABLE 50-1 Correlation of Immunophenotype with Clinical Characteristics

Parameter	Early Pro–B Cell, CD10–	Early B-Precursor Cell, CD10+	Pre–B Cell (cIgM+)	T Cell
No. of patients	52	635	156	124
Gender (% male)	39%	53%	50%	75%
Age (yr)				
<1	33%	1%	6%	1%
1 to <10	50%	82%	80%	62%
≥10	17%	17%	14%	37%
Leukocyte count ($\times 10^9$/L)				
≤20	38%	75%	53%	23%
>50	44%	11%	21%	57%
Platelet count ≤100 $\times 10^9$/L	77%	75%	81%	56%
Hemoglobin ≤8 g/dL	58%	40%	60%	15%
Splenomegaly[*]	50%	34%	46%	57%
Hepatomegaly[*]	56%	46%	48%	61%
Mediastinal mass	0%	0%	1%	72%
Lymphadenopathy	35%	36%	41%	78%
CNS-positive (CNS-3)[†]	10%	1%	1%	11%

From Reiter A, Schrappe M, Ludwig WD, et al: Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients: results and conclusions of the multicenter trial ALL-BFM 86. Blood *84:3122–3133, 1994; and H. Riehm, personal communication, 1996.*

cIgM+, Intracytoplasmic immunoglobulin M positive; *CNS*, central nervous system.

[*] >4 cm below the costal margin.

[†] CNS-3, diagnostic cerebrospinal fluid specimen with five or more white blood cells/high-power field and lymphoblasts seen on cytospin preparation.

rearrangements have been used to quantify submicroscopic levels of residual leukemia in patients using polymerase chain reaction (PCR)-based assays.[15]

The presence of intracytoplasmic immunoglobulin (cIg) and various cell surface markers has been used to distinguish different subsets of B-precursor cell ALL based on their level of differentiation. These subsets include pro-B ALL (3% to 4% of pediatric patients), early pre-B ALL (60% to 70% of pediatric patients), and pre-B ALL (20% to 30% of pediatric patients):[16]

- Pro–B-cell ALL, believed to be derived from a very immature B-cell precursor, is characterized by CD10-negative immunophenotype and the absence of cIg. It is most frequently observed in infants with ALL, especially those with abnormalities involving chromosome 11q23 (*MLL* [also known as *KMT2A*] gene locus).[17,18]
- Early pre–B-cell ALL is the most common subtype of B-precursor cell ALL in children. It is believed to be derived from a more mature B-cell precursor than pro-B ALL. Early pre–B cells frequently express CD10 but still lack cIg. CD10 (CALLA)-positive leukemias without cytoplasmic immunoglobulin are also referred to as common ALL (c-ALL).[19]
- Pre–B-cell ALL is characterized by the presence of cIg.[20] It is believed to derive from an intermediate B-precursor cell, more mature than those lacking cIg (early pre–B cells) but not as mature as those with surface immunoglobulin (mature B cells). Like early pre–B cells, pre–B lymphoblasts typically express CD10 and human leukocyte antigen (HLA)-DR. Approximately 25% of cases of pre–B-cell (cIg+) ALL have the chromosomal translocation t(1;19)(q23;p13), which fuses the *TFPT* (formerly *E2A*) gene on chromosome 19 with the *PBX1* gene on chromosome 1.[20] This translocation is observed in only 1% of cases of early pre-B (or common) ALL.

Mature B-Cell ALL

Mature B-cell ALL accounts for 1% to 2% of childhood ALL cases.[21] It is characterized by the presence of surface immunoglobulin, most often IgM, which is monoclonal for κ or λ light chains. The cells generally express other B-cell antigens, including CD19, CD20, and HLA-DR. Morphologically, the blasts tend to be classified as FAB L3, with intensely staining cytoplasmic basophilia similar to that of erythroblasts. Cases of mature B-cell ALL without L3 morphologic features have been reported but are rare.[22] Almost all cases of mature B-cell ALL are associated with one of three nonrandom chromosomal translocations—t(8;14)(q24;q32) or, less commonly, t(2;8)(p12;q24) or t(8;22)(q24;q11).[23] The chromosomal breakpoints involve the *MYC* oncogene on chromosome 8 and the genes for the IgH (chromosome 14), κ light chain (chromosome 2), or λ light chain (chromosome 22). Mature B-cell ALL is clinically indistinguishable from disseminated Burkitt lymphoma and is much more successfully treated with regimens used for that disease than when treated with conventional childhood ALL therapy.[8,9]

T-Cell ALL

The T-cell immunophenotype is present in 10% to 15% of children with ALL.[24] Compared with B-precursor cell ALL, T-cell ALL is more frequently associated with older age at diagnosis, higher presenting leukocyte counts, and bulky extramedullary disease (including lymphadenopathy, hepatosplenomegaly, overt central nervous system [CNS] leukemia, and an anterior mediastinal thymic mass).[16] Historically, patients with T-cell ALL had an inferior outcome compared with those with B-precursor cell ALL, although this prognostic difference is not observed when more intensive regimens are used.[25] However, the time period in which relapses occur differs between the two immunophenotypic subtypes, with earlier relapses observed more frequently in T-cell ALL.[25]

T-cell ALL can be subclassified using monoclonal antibodies that recognize surface antigens expressed during discrete stages of normal T-cell development.[26] Most of the phenotypically defined subsets of T-cell ALL have limited clinical relevance.[27,28] However, gene expression profile studies have identified a biologically distinctive subset of T-cell ALL that, in retrospective analysis, was associated with a high risk for treatment failure.[29] These patients were noted to have a distinctive “early T-cell precursor” phenotype consisting of weak expression of CD5 and coexpression of stem cell or myeloid markers.[29] Similarly, another retrospective study using array comparative genomic hybridization (CGH) and quantitative DNA-PCR techniques identified the absence of V(D)J recombination at the TCRγ locus, a feature that is characteristic of early T-cell precursor cells, as a predictor of early treatment failure in patients with T-cell ALL.[30] Thus, T-cell ALL patients with features of development arrest at the earliest stages of T-cell development appear to have an inferior outcome.

Myeloid Antigen Coexpression

In some instances, individual leukemic cells simultaneously express lymphoid and myeloid surface antigens. Up to 20% of cases of ALL (i.e., with lymphoid surface antigen predominance) also coexpress myeloid antigens.[31,32] Myeloid antigen coexpression is associated with young age at diagnosis (<12 months), pro–B-cell (CD10−) immunophenotype, and certain chromosomal abnormalities, such as rearrangements of chromosome 11q23 (frequently observed in infants with ALL), the Philadelphia (Ph) chromosome [t(9;22)], and the *ETV6-RUNX1* [t(12;21)] fusion.[33-35] Older studies suggested that patients with biphenotypic ALL had a worse prognosis,[36] but more recent reports have indicated that myeloid antigen coexpression lacks independent prognostic significance.[31-33] Cases of ALL with myeloid antigen coexpression should be distinguished from leukemia of ambiguous lineage, an uncommon entity in which the predominant lineage (lymphoid or myeloid) cannot be determined by immunophenotype and histochemistry. The World Health Organization classification system requires that cells express myeloperoxidase or evidence of monocytic differentiation, along with either T-cell antigen CD3 (cytoplasmic or surface) or B-cell surface antigens (CD19, CD79a, CD22, and CD10).[37]

MOLECULAR PATHOGENESIS

Lymphocytes arise from hematopoietic stem cells through a series of proliferation and differentiation steps that result in the formation of mature B or T lymphocytes, each expressing a specific immunoglobulin or TCR capable of responding to a narrow range of foreign antigens. The development of a fully competent adaptive immune system is dependent on the generation of a population of mature B and T lymphocytes with extraordinary diversity in antigen specificity, which collectively provide the ability to mount an effective immune response against a broad range of pathogens. This diversity in antigen receptor specificity is achieved through an elaborate process of somatic mutagenesis, including V(D)J recombination and somatic hypermutation, designed to introduce unique variations in the immunoglobulin or TCR gene structure of each individual developing lymphocyte. Despite their carefully regulated nature, these programmed mechanisms of somatic mutagenesis can go awry and introduce somatic mutations at other genomic loci. Mutations in oncogenes or tumor suppressors acquired during lymphocyte development can result in the aberrant proliferation and differentiation arrest that are characteristic of ALL.

Although a comprehensive review of the molecular pathogenesis of ALL is beyond the scope of this chapter, we highlight here select molecular lesions that have proven particularly informative to our understanding of the pathobiology of ALL and review in more detail the molecular pathogenesis of three particularly high-risk subtypes of ALL: BCR-ABL, hypodiploid, and early T-cell progenitor ALL.

Transcriptional Regulators

ETV6-RUNX1 Fusion Genes in B-Precursor Cell ALL

In 20% to 25% of childhood ALL cases a cryptic t(12;21) translocation results in expression of a fusion *ETV6-RUNX1* (also known as *TEL-AML1*) translocation.[38] *ETV6* and *RUNX1* are transcription factors that are each required for normal hematopoiesis, and both are involved in other leukemia-related translocations. The t(12;21) translocation results in the fusion of the HLH domain of *ETV6* to almost all of *RUNX1*. The mechanisms through which *ETV6-RUNX1* fusions drive leukemogenesis remain incompletely understood, but recent work has shown that expression of this fusion oncoprotein in hematopoietic stem cells markedly increases their numbers and promotes their transformation after chemical mutagenesis.[39] Interestingly, *ETV6-RUNX1* translocations can often be detected in prenatal blood specimens collected from children who went on to develop *ETV6-RUNX1*–rearranged ALL many years later, indicating that it is likely the initiating mutation in ALL.[40] However, this translocation is not sufficient for leukemogenesis because the incidence of detectable *ETV6-RUNX1* fusions in normal neonatal blood is 100-fold greater than the incidence of leukemia with this translocation.[41] Several studies have shown that the presence of an *ETV6-RUNX1* translocation is associated with a favorable prognosis in childhood ALL.[42,43] Indeed, *ETV6-RUNX1* expression is associated with decreased expression of the *ABCB1* (also known as *MDR1*) multidrug resistance gene and of genes involved in purine metabolism, which may explain the particular sensitivity of these cases to mercaptopurine and methotrexate.[44,45]

MLL Fusion Genes in B-Precursor Cell ALL

Chromosomal translocations resulting in the fusion of the amino-terminus (N-terminus) of the *MLL* gene on 11q23 to the carboxy-terminus (C-terminus) of one of more than 60 different translocations partners occur in 70% of infant ALL, in 10% of AML, and in the majority of secondary AML cases that are induced by prior treatment with topoisomerase II inhibitors.[46] The presence of *MLL* translocations is associated with inferior outcomes despite aggressive chemotherapy in ALL, as discussed later in this chapter. *MLL* is the human ortholog of *Drosophila trithorax*, which positively regulates expression of multiple genes of the *HOXA* cluster and acts antagonistically to the polycomb group of proteins. The breakpoints of *MLL* translocations cluster between exons 5 and 11 of *MLL*, resulting in expression of fusion oncoproteins that retain the N-terminal domains of the protein that bind DNA in a sequence-nonspecific manner, whereas the C-terminal domains of MLL that mediate association of the wild-type protein with its endogenous chromatin modification complex and its Histone H3 lysine-4–specific *N*-methyltransferase activity are invariably lost.[47] Instead, the C-terminus of MLL fusion oncoproteins is provided by one of more than 60 different translocation partners, with common translocations such as the t(4;11), t(9;11), and t(11;19)(q23;p13.3) resulting in the in-frame fusion of *MLL* to the C-terminal domains of *AF4* (also known as *AFF1*), *AF9* (also known as *MLLT3*), and *ENL* (also known as *MLLT1*), respectively.[48,49] Expression of MLL fusion oncoproteins in murine hematopoietic precursors induces leukemia,[50-54] an effect that is dependent on MLL fusion oncoprotein–driven overexpression of several genes of the *HOXA* cluster.[55,56]

Until recently, the mechanism through which the C-terminal fusion partners of MLL contributed to leukemogenesis was unclear, because there were no apparent sequence similarities between the fusion partners involved. However, recent work has shown that several MLL translocation partners are functionally linked through their association in protein complexes that regulate transcriptional elongation.[47] MLL fusion oncoproteins bind at least three distinct complexes, including the polymerase-associated factor complex, the positive transcription elongator factor (pTEFb) complex, and the DOT1L histone methyltransferase complex.[57] The DOT1L methyltransferase is required for the maintenance of MLL-driven leukemias but appears to be less important for normal hematopoiesis, thus suggesting a therapeutic window for targeting DOT1L in MLL-rearranged ALL.[58-62] Indeed, a small molecule DOT1L inhibitor has recently been developed and demonstrates promising preclinical activity in murine models of MLL-rearranged leukemia, raising the possibility that such a therapeutic strategy could improve the prognosis for patients with MLL-rearranged ALL.[63]

MYC Translocations in Mature B-Cell ALL

Chromosome translocations that place the coding region of the *MYC* protooncogene under the control of gene regulatory elements of one of the immunoglobulin receptor genes, such as the t(8;14), t(2;8) or t(8;22), lead to aberrant overexpression of the *MYC* oncogenic transcription factor. *MYC* overexpression is potently oncogenic in a wide range of experimental systems. Although MYC has long been believed to be a traditional transcription factor that regulates a specific set of target genes, recent work has revealed that MYC accumulates at the promoters of essentially all transcriptionally active genes and acts as a nonlinear amplifier of existing gene expression programs.[64,65] Thus, rather than inducing expression of a specific gene expression program, these findings suggest that MYC instead acts to lock a cell's gene expression program into its existing form, which presumably can be oncogenic when it occurs in a cell that is in a highly proliferative state that normally should be transient. *MYC* translocations are characteristically found in mature B-cell leukemia, which is best considered a disseminated form of Burkitt lymphoma that responds poorly to standard ALL therapy. Outcomes for these patients are much improved with treatment regimens specifically designed for high-grade B-cell non-Hodgkin lymphomas.[8,9]

Activating *NOTCH1* Mutations in T-Cell ALL

NOTCH1 encodes an unusual cell surface receptor that, on activation by ligand binding to its extracellular domain, undergoes two distinct proteolytic cleavage events, culminating in release of the intracellular domain of NOTCH1 (ICN) into the cytoplasm. The ICN protein subsequently translocates to the nucleus, where it functions as a transcription factor that generally activates expression of its targets.[66] NOTCH1 is required at multiple stages of T-cell development, and its ectopic activation in murine T-cell progenitors is highly leukemogenic.[66-69] Although NOTCH1 can be activated by chromosomal translocations in very rare cases of T-cell ALL,[70] more than 50% of T-cell ALL cases harbor activating *NOTCH1* mutations, and these occur in all of the molecular subtypes of T-cell ALL.[71] Activating *NOTCH1* mutations typically occur either as missense mutations in the heterodimerization domain, where they promote ligand-independent proteolytic activation of the ICN domain, or as truncating or frameshift mutations in the C-terminal PEST domain of ICN, which lead to ICN protein accumulation as a result of impaired proteasomal degradation.[71,72] The pathogenic role of *NOTCH1* in T-cell ALL is mediated largely by its role as a transcriptional activator of two key downstream targets, *MYC* and *HES1*.[66,73-75]

One of the key proteolytic cleavage events required for NOTCH1 activation is mediated by the γ-secretase complex. Treatment of NOTCH1-mutant T-cell ALL cell lines with γ-secretase inhibitors leads to modest reductions in cell proliferation in a subset of cell lines,[71,76] but a number of NOTCH1-mutant T-cell ALL cell lines fail to respond to these inhibitors. The mechanisms of resistance to NOTCH1 inhibition have been an area of intense investigation, and recent work has revealed that inactivation of the *FBXW7* tumor suppressor, an E3 ubiquitin ligase that targets both ICN and *MYC* for proteasomal degradation, is one mechanism of resistance to γ-secretase inhibitors.[77,78] Additionally, resistance to inhibition of NOTCH1 or its downstream target *MYC* can be mediated by PTEN inactivation and resultant AKT activation in T-cell ALL, thus suggesting the potential therapeutic utility of AKT pathway inhibitors as a strategy to reverse resistance to γ-secretase inhibitor.[79,80] Moreover, glucocorticoid resistance in T-cell ALL has been shown to be reversible by γ-secretase inhibitors, and it appears that glucocorticoid therapy can ameliorate the intestinal toxicity of γ-secretase inhibitors.[81] Taken together, these findings provide a compelling rationale for clinical trials combining glucocorticoids with small molecule inhibitors of NOTCH1 and AKT.

Growth Factor Signaling

Cytokine Receptor Signaling

Cytokine receptor signaling plays key roles in normal lymphocyte development, with inactivating mutations in key cytokine receptor subunits (e.g., interleukin-2 receptor γ [*IL2RG*] and interleukin-7 receptor [*IL7R*]) or their downstream effector kinases (e.g., Janus kinase-3 [*JAK3*]), resulting in severe lymphopenia and the lack of a functional adaptive immune system characteristic of severe combined immunodeficiency. Conversely, aberrant activation of cytokine receptor signaling has recently been implicated in the molecular pathogenesis of ALL.

Cytokine receptor–like factor 2 *(CRLF2)* encodes a cytokine receptor subunit that heterodimerizes with IL7R to form the receptor for the cytokine TSLP (thymic stromal lymphopoietin), which is a proinflammatory cytokine secreted by endothelial cells that drives lymphocyte proliferation.[82] *CRLF2* rearrangements leading to aberrant overexpression have recently been described in B-precursor cell ALL, typically as a result of an intrachromosomal deletion leading to a *P2RY8-CRLF2* fusion, or translocations of the IgH locus to *CRLF2*.[83-85] *CRLF2* rearrangements are found in 5% to 10% of B-precursor cell ALL cases but in more than 50% of patients with Down syndrome–associated ALL, strongly suggesting that these collaborate with trisomy 21 in B-lymphoblast transformation. *CRLF2* rearrangements are often associated with activating mutations of Janus kinase 2 *(JAK2)*, and CRLF2 overexpression and mutated JAK2 cooperate to induce cytokine-independent growth to Ba/F3 pro-B cells.

IL7R encodes a cytokine receptor subunit that can heterodimerize with the IL2Rγ (the common γc chain) to form IL7R, or with CRLF2 to form the TSLP receptor. Recent work has identified activating IL7Rα point mutations in 9% of cases of T-cell ALL, as well as in the "BCR-ABL–like" subset of B-precursor cell ALL (discussed in more detail later).[86,87] Interestingly, most of these mutations result in the introduction of a novel cysteine residue into the extracellular domain of IL7Rα, and aberrant signaling results because disulfide bond formation between these mutant cysteines can allow mutant IL7Rα proteins to aberrantly dimerize, leading to activation of downstream signaling pathways independent of ligand or of the common γc chain.[86,87] The potential therapeutic utility of targeting aberrant oncogenic signaling downstream of mutationally activated cytokine receptors is an area of active investigation.

PTEN and the *PI3K-AKT* Axis

The *PI3K-AKT* oncogenic pathway, which is negatively regulated by the *PTEN* tumor suppressor, mediates prosurvival and proliferative signaling downstream of activated growth factor receptors and is aberrantly activated in a broad range of human cancer subtypes.[88] Recent work has revealed that nearly half of T-cell ALL cases harbor mutational activation of oncogenic signaling through this pathway, most often as a result of truncating mutations or deletions of the *PTEN* tumor suppressor, but activating mutations of *PI3K* and *AKT* genes also occur.[79,89-91] *PTEN* deletions are a strong predictor of early treatment failure in T-ALL cases[86,89] and *PTEN* inactivation has been shown to induce resistance to *NOTCH1* or *MYC* inhibition in zebrafish and murine models of T-cell ALL.[79,80] These findings highlight the potential application of small molecule inhibitors of the *PI3K-AKT* pathway for high-risk T-cell ALL.

Molecular Pathogenesis of Select High-Risk Subtypes

BCR-ABL and *BCR-ABL*–like ALL

The discovery of the Ph chromosome, which arises from the t(9;22)(q34;q11) translocation resulting in expression of a BCR-ABL fusion oncoprotein, was a major milestone in molecular oncogenesis that provided compelling support for the hypothesis, first put forth by Boveri in 1914, that cancer arises due to a genetic lesion in a single cell, resulting in outgrowth of a clonal population.[92,93] *BCR-ABL* translocations are pathognomonic of chronic myeloid leukemia and are also found in 4% to 5% of childhood and 25% of adult cases of B-precursor cell ALL.[38] The translocation underlying formation of the Ph chromosome results in expression of a BCR-ABL fusion oncoprotein with constitutively active tyrosine kinase activity, which transforms cells at least in part due to activation of the RAS-MAPK pathway, PI3K, and JUN-kinase pathways.[94] Although historically associated with an extremely poor prognosis, hematopoietic stem cell transplantation (HSCT) proved to be the first highly effective therapeutic modality for this high-risk subtype of ALL.[95] Additionally, the development of imatinib mesylate, a small molecule inhibitor of oncogenic signaling by the BCR-ABL kinase, has opened novel therapeutic opportunities for the management of Ph chromosome–positive ALL. Although single-agent therapy with imatinib leads to rapid development of resistance in Ph chromosome–positive ALL, pilot trials of the combination of conventional chemotherapy with imatinib or other tyrosine kinase inhibitors have been promising.[96,97]

Although the poor prognosis of BCR-ABL ALL was traditionally believed to be due to expression of the BCR-ABL oncoprotein, recent work has shown that this subtype of ALL is also characterized by inactivation of the DNA-binding protein Ikaros, which is encoded by the *IKZF1* gene.[98] *IKZF1* deletions have been identified in up to 15% of BCR-ABL–negative pediatric ALL cases and have been shown to be an independent predictor of adverse outcome.[99,100] These cases have been classified as "BCR-ABL–like" ALL on the basis of similarities in gene expression profiles with Ph chromosome–positive ALL.[99,100] Recent work has revealed that most of these "BCR-ABL–like" cases harboring Ikaros alterations, but lacking BCR-ABL translocations, commonly harbor mutational activation of tyrosine kinase signaling as a result of translocations or activating point mutations of receptor or cytoplasmic kinases, such as PDGFRB, CRLF2, EPOR, IL7R, FLT3, JAK2, and ABL1. Importantly, preclinical studies have demonstrated the potential therapeutic utility of targeting the aberrantly activated kinase involved for at least some of these cases.[101]

Hypodiploid ALL

The presence of hypodiploidy (<44 to 45 chromosomes) portends a poor prognosis in B-precursor cell ALL, with event-free survival (EFS) rates less than 40%.[102] Alterations in whole-chromosome number (aneuploidy) are very common in human cancer[103] and can be oncogenic.[104] However, the mechanisms through which hypodiploidy contributes to leukemogenesis in B-precursor cell ALL remain largely unknown.

A recent genomic study has shed key insights into the molecular pathogenesis of hypodiploid ALL.[105] Both copies of chromosome 21 were always retained in each of the 124 cases of hypodiploid ALL cases analyzed, which, given that trisomy 21 is one of the most common genetic alterations in B-precursor cell ALL, highlights the pathogenic role of additional copies of genes on chromosome 21 in B-precursor cell ALL.

Rather than representing a single subtype of ALL, hypodiploid ALL instead harbors distinct biologic subsets, including low hypodiploidy (32-39 chromosomes) and near haploidy (24-31 chromosomes), which differ strikingly on a molecular basis.

- *Near-haploid ALL (24 to 31 chromosomes):* Seventy percent of these cases are characterized by genetic lesions leading to activation of growth factor signaling pathways, including NRAS, KRAS, NF1, MAPK, FLT3, and PTPN11. Notably, deletions of tumor suppressors such as *NF1* and *PTPN11* were typically homozygous as a result of hypodiploidy, and loss of heterozygosity of tumor suppressors provides one mechanism through which hypodiploidy can contribute to oncogenesis. These data support the potential therapeutic utility of targeting these kinases, or their key downstream effectors, although such interventions await further preclinical investigation.
- *Low-hypodiploid ALL (32 to 39 chromosomes):* In marked contrast to near-haploid ALL, almost all cases of low-hypodiploid ALL harbored mutations of the *TP53* tumor suppressor, whereas mutations of growth factor signaling pathways were rare in this subset. Mutations of *TP53* were present in remission bone marrow specimens in almost half of pediatric cases, strongly suggesting a germline origin consistent with the Li-Fraumeni syndrome; indeed, germline transmission was confirmed in one kindred.[105] Given that the induction of aneuploidy triggers *TP53*-dependent cell cycle arrest,[106] these data support a model in which *TP53* inactivation occurs early in the pathogenesis of low-hypodiploid ALL, thus allowing the subsequent acquisition of hypodiploidy to be tolerated by these cells. However, it remains entirely unclear why *TP53* mutations should be so rare in other ALL subtypes with striking degrees of aneuploidy, including near-haploid and hyperdiploid ALL. Low-hypodiploid cases were also associated with a high frequency of inactivating mutations of the *RB1* tumor suppressor, as well as a very high frequency of mutations involving Ikaros family members, particularly *IKZF2* (encoding Helios), which was mutated in more than 50% of cases.

A subset of cases with an apparently hyperdiploid chromosome complement was found to in fact represent "masked" hypodiploidy in which the hypodiploid genome has undergone reduplication, based on loss of heterozygosity apparent on the single-nucleotide polymorphism arrays performed. Although these cases might be classified clinically as hyperdiploid ALL (which has a favorable prognosis), masked hypodiploid ALL cases are indistinguishable from the respective hypodiploid ALL subtype (low-hypodiploid or near-haploid) based on gene expression profiling and pattern of oncogenic lesions, thus suggesting the need for clinical tests to distinguish this subtype from cases of "typical" hyperdiploidy, which has a favorable prognosis. Given that chromosomes 4 and 17 are lost in almost all cases of hypodiploid ALL, the presence of these trisomies may help to distinguish cases of true hyperdiploidy from masked hypodiploidy.[107]

Early T-Cell Progenitor T-Cell ALL

Recent work has revealed that differentiation arrest at the earliest stages of T-cell development, defined either by absence of biallelic TCRγ deletion (ABD; indicating failure to complete V(D)J recombination) or by an early T-cell progenitor gene expression profile pattern, identifies an overlapping subset of T-cell ALL cases at very high risk for treatment failure.[29,30] These cases are associated with aberrant oncogenic signaling through the RAS-MAPK, PI3K-AKT, and JAK-STAT pathways, typically owing to associated mutations in *RAS*, *FLT3*, *IL7R*, *BRAF*, and *JAK* kinases, as well as deletions of the *PTEN* tumor suppressor.[30,86] These cases are defined as T-cell ALL based on expression of cytoplasmic CD3 and harbor mutations in defining T-cell ALL oncogenes such as *NOTCH1*.[29,30] However, these cases also harbor additional mutations that are typically seen in AML, myelodysplastic, and myeloproliferative syndromes, such as *RUNX1*, *ETV6*, *EED*, *EZH2*, *SUZ12*, *DNMT3A*, *IDH1*, and *IDH2*.[29,108,109] Moreover, gene expression analyses have shown that early T-precursor T-cell ALL harbors similarities to normal hematopoietic stem cells and AML leukemic stem cells. The identification of effective targeted therapies is a subject of active investigation.

CLINICAL MANIFESTATIONS

ALL may present insidiously or acutely, as an incidental finding on a routine blood cell count of an asymptomatic child, or as a life-threatening hemorrhage, infection, or episode of respiratory distress. Common presenting symptoms include fever, pallor, bruising, petechiae, bone pain (presumably secondary to stretching of the periosteum or joint capsule by leukemic infiltration), and limp.

Although ALL is primarily a disease of the bone marrow and peripheral blood, any organ or tissue may be infiltrated by the abnormal cells. Such infiltration may be clinically apparent by physical examination. At initial diagnosis, 30% to 50% of children have enlargement of the liver or spleen, with organs palpable more than 4 cm below the costal margins (see Table 50-1). Lymphadenopathy caused by leukemic infiltration is an equally frequent presenting sign. Generally, the degree of organ infiltration correlates with peripheral blood blast count, thus reflecting the total leukemic mass. Leukemic invasion of tissues may, however, be occult and detectable only by histologic sampling.

Peripheral Blood

Clinical laboratory data often provide a broad spectrum of abnormal findings at the time of diagnosis of leukemia. Anemia, abnormal leukocyte counts and differential, and thrombocytopenia are common (see Table 50-1). However, some children with ALL may have normal peripheral blood cell counts at the time of diagnosis, even when the bone marrow is replaced by leukemic cells.

The red blood cells are usually normochromic and normocytic. Failure of erythroid production is manifested by a low reticulocyte count. Peripheral blood smear may reveal teardrop forms and nucleated red cells, consistent with marrow invasion.

Platelet counts may vary from normal to extremely low. Most children have fewer than 100,000 cells/mm^3 at presentation, and the platelets are usually of normal size. In contrast to idiopathic thrombocytopenic purpura (ITP), thrombocytopenia at the time of ALL diagnosis is usually accompanied by other hematologic or physical manifestations of leukemia.[110]

There is a wide range of leukocyte counts observed at the time of diagnosis, from extremely low to more than 1 million cells/mm^3. Approximately 20% of children with ALL present with leukocyte counts of more than 50,000 cells/mm^3.[111] Even with high presenting leukocyte counts, absolute neutropenia is common. Blast forms may or may not be present on routine smears of peripheral blood. Even when present, peripheral blast cells may be misleading, and the definitive diagnosis of leukemia cannot be made from the morphologic examination of the peripheral blood. For example, myeloblasts may be detected in the peripheral blood when the marrow has been infiltrated by various disorders, including osteopetrosis, myelofibrosis, granulomatous infections, sarcoidosis, metastatic tumor, and even ALL. Additionally, in patients with leukemia, the morphologic appearance of leukemic blast cells in the peripheral blood may differ from that of the marrow. Peripheral blood flow cytometry may be useful in distinguishing malignant blasts from "normal" myeloblasts, and differentiating ALL from AML, in patients with high presenting leukocyte counts.

Hypereosinophilia has been described in association with ALL.[112] The eosinophilia may precede the diagnosis of ALL, appear at presentation, and then disappear with successful remission induction.[112,113] Eosinophilia in B-precursor cell ALL has been associated with the t(5;14)(q31;q32) translocation, involving the *IL3* gene on chromosome 5 and the IgH gene *(IGH)* on chromosome 14.[114,115]

In regard to hyperleukocytosis, approximately 10% of children with ALL present with extremely high leukocyte counts (>100,000 cells/mm^3) (Table 50-2).[2,3,116] In patients with markedly elevated leukocyte counts, blood flow in the microcirculation can be impeded by intravascular clumping of the poorly deformable blasts. This may result in local hypoxemia, endothelial damage, hemorrhage, and infarction, especially in the CNS and lung. Clinically significant leukostasis, resulting in intracerebral hemorrhage or respiratory failure, is more common with hyperleukocytosis in AML than in ALL.[117] In one series, neurologic or pulmonary symptoms or both were observed in 5% to 10% of children with ALL and a

TABLE 50-2 Presenting Features by Age in 5181 Children and Adolescents with Acute Lymphoblastic Leukemia (ALL) Treated on ALL-BFM Trials (1986-1999)

	Age (yr)		
Presenting Feature	<1 (2.5% of Patients)	1 to <10 (79% of Patients)	10-18 (18.5% of Patients)
Gender			
Male	52%	56%	61%
Female	48%	44%	39%
WBC count ≥100 × 10^9/L	59%	10%	15%
Phenotype			
B-precursor cell	96%	90%	73%
T-cell	4%	10%	27%
CNS-positive*	21%	2.3%	3.9%
High hyperdiploidy†	1.5%	27%	15%
ETV6-RUNX1 fusion†	4.5%	27%	11%
t(9;22) *BCR-ABL*†	0%	2.2%	5.1%

Modified from Moricke A, Zimmermann M, Reiter A, et al: Prognostic impact of age in children and adolescents with acute lymphoblastic leukemia: data from the trials ALL-BFM 86, 90, and 95. Klin Padiatr *217:310–320, 2005.*

CNS, Central nervous system; *WBC*, white blood cell.

*CNS-3, diagnostic cerebrospinal fluid specimen with five or more WBCs/high-power field and lymphoblasts seen on cytopsin preparation.

†Results for B-precursor cases only.

presenting leukocyte count higher than 200,000 cells/mm^3 and CNS hemorrhage was diagnosed in 2% of those patients (all of whom had leukocyte counts higher than 400×10^9/L).[117] Treatment of hyperleukocytosis consists of vigorous intravenous hydration. Red blood cell transfusions should be administered cautiously to avoid increasing whole blood viscosity and worsening symptoms. For patients with very high presenting leukocyte counts and symptomatic leukostasis, leukapheresis or exchange transfusion may prevent hyperviscosity and leukemia cell lysis–related problems.

Disseminated intravascular coagulation is infrequently observed in patients with ALL at diagnosis. It is more common in T-cell ALL as a result of thromboplastic substances released from T lymphoblasts.[118] It also has been reported in patients with the uncommon t(17;19) translocation, associated with the B-precursor cell immunophenotype.[119] Disseminated intravascular coagulation should be treated promptly with infusions of fresh-frozen plasma and cryoprecipitate.

Bone Marrow

Evaluation of the bone marrow by aspiration and biopsy is essential for making the diagnosis of leukemia. The marrow specimen is usually hypercellular and characterized by a homogeneous population of cells. In most cases of ALL, the marrow generally has more than 50% blasts.

A bone marrow aspirate may be difficult to obtain at the time of diagnosis. This is usually caused by the density of blast forms in the marrow, but it may be caused by marrow fibrosis or necrosis.[120,121] In such cases, a diagnosis can be made by bone marrow biopsy. "Touch preps" of the biopsy specimen can be helpful in elucidating morphology when aspiration is not successful.

Differences may occur in leukemic involvement found in marrow aspirates derived from widely separated sites. Patchy marrow involvement of leukemic infiltration has been reported at diagnosis and relapse.[122] If clinical findings suggest leukemia, but a single-site bone marrow specimen is nondiagnostic, the marrow should be sampled at additional sites.

The distinction between ALL with lymph node involvement and non-Hodgkin lymphoblastic lymphoma with bone marrow invasion (stage IV) is arbitrary. Commonly the disease is classified as ALL when there are more than 25% lymphoblasts in the marrow and as stage IV non-Hodgkin lymphoblastic lymphoma when there are fewer than 25% lymphoblasts in the marrow. Although the lymphoblasts of ALL and lymphoblastic lymphoma appear morphologically and immunophenotypically indistinguishable, gene expression profiling studies have suggested that there may be differences in underlying biology between these two diagnoses.[123] However, the distinction may not be clinically important because advanced-stage lymphoblastic lymphoma and ALL respond similarly to intensive ALL-type therapy.[124,125]

Extramedullary Leukemia

Although marrow replacement is the major cause of symptoms of leukemia, extramedulllary involvement may also contribute to presenting signs and symptoms.

CNS Manifestations

Up to 20% of children will have blast cells visible on a cytocentrifuged cerebrospinal fluid (CSF) specimen at diagnosis.[126,127] CSF lymphoblasts can usually be identified by the use of cytocentrifugation and Wright-Giemsa staining. Such morphologic evaluation is necessary to distinguish the pleocytosis of leukemic meningitis from that induced as a result of intrathecal chemotherapeutic agents (arachnoiditis) or from CNS infections.[128]

The vast majority of children with detectable CSF lymphoblasts at initial diagnosis do not have any symptoms related to CNS involvement. Symptomatic patients may present with diffuse or focal neurologic signs and symptoms, including manifestations of increased intracranial pressure (vomiting, headache, papilledema, and lethargy), seizures, and nuchal rigidity. Cranial nerve palsies can occur, with the facial nerve being the most frequently involved.[129,130]

Intracranial hemorrhage in patients with high presenting leukocyte counts is uncommon in ALL but can lead to severe and devastating neurologic consequences.[131] Clinically significant spinal cord involvement has been reported in ALL, manifesting as a localized epidural leukemic infiltrate compressing the cord.[132]

Anterior Mediastinal Mass

Leukemic infiltration of the thymus appears as an anterior mediastinal mass on a chest radiograph. It is observed in about 10% of newly diagnosed patients and is almost always associated with T-cell immunophenotype (see Table 50-1). Leukemic infiltration of the mediastinal structures may cause life-threatening tracheobronchial or cardiovascular compression. Pleural effusion may also be associated with thymic enlargement, which can exacerbate respiratory distress. Prompt initiation of systemic chemotherapy (e.g., corticosteroids) is necessary to handle such emergencies and, rarely, emergent radiation may be indicated.

Genitourinary Tract Manifestations

Testicular Enlargement. The clinical presentation of testicular ALL is a painless enlargement of one or both testes. Clinically detectable testicular leukemia is uncommon at diagnosis, occurring in 1% to 2% of boys, and does not appear to have prognostic significance.[133,134] Occult testicular involvement at diagnosis is more common, especially in the presence of a high tumor burden. In a study in which testicular biopsies were performed in boys with newly diagnosed ALL, approximately 20% had microscopic leukemic involvement.[135] However, this finding does not appear to have any prognostic significance, and routine testicular biopsies at the time of diagnosis to document occult disease are not recommended.

Other Genitourinary Tract Sites of Involvement. Ultrasonographically enlarged kidneys have been observed in children with ALL at the time of diagnosis and are believed to be primarily caused by leukemic infiltration.[136] Renal enlargement in acute leukemia also may be related to hyperuricemia, hemorrhage, and pyelonephritis. Hypertension is more commonly associated with the treatment of leukemia, especially with the prolonged use of corticosteroids, than with renal involvement. Occasionally, urolithiasis is observed at presentation or during therapy for ALL. Most renal stones in ALL patients are calcium-based in composition and occur most frequently during phases of therapy that include corticosteroids.[137]

Priapism is rare and usually associated with an elevated white blood cell count.[138] The pathogenesis may be caused by involvement of sacral nerve roots, or it may be related to mechanical obstruction of the corpora cavernosa and dorsal veins by leukemic infiltration or leukostasis.

Bone and Joint Manifestations

Bone pain, joint pain, and limp are common presenting symptoms in childhood ALL.[139-141] Bone pain may be the result of direct leukemic infiltration of the periosteum, periosteal elevation of underlying cortical disease, bone infarction, or expansion of the marrow cavity by the leukemic cells. Pain and swelling of the joint are less frequent but may be presenting manifestations of disease and can initially cause confusion in the diagnosis.[142] Migratory joint pain accompanied by swelling and tenderness can be misdiagnosed as juvenile rheumatoid arthritis (JRA) or rheumatic fever.[140,142]

Up to 25% of children with ALL have characteristic radiographic changes, such as osteopenia and fracture at diagnosis, including vertebral compression fractures.[140,141,143] Some patients have radiographic changes in the absence of bone pain, whereas others have bone pain unaccompanied by radiographic changes. Radiographic changes are most often seen in the long bones, especially around the areas of rapid growth (e.g., the knees, wrists, and ankles), and include subperiosteal new bone formation, transverse metaphyseal radiolucent bands, osteolytic lesions involving the medullary cavity and cortex, diffuse demineralization, and transverse metaphyseal lines of increased density (growth arrest lines).[144,145] The latter probably represent regions of growth arrest during active phases of the disease rather than direct infiltration by leukemic cells.

Gastrointestinal Manifestations

Hepatosplenomegaly is a common feature at the time of diagnosis. Severe hepatic dysfunction, manifested as hyperbilirubinemia, presumably from leukemic infiltration, has been observed at diagnosis in some children with ALL and can complicate initial therapy, because many induction agents are hepatically metabolized or hepatotoxic.[146,147] A short course of corticosteroid monotherapy before the initiation of multiagent induction chemotherapy may improve hyperbilirubinemia.[147]

Ocular Manifestations

Ophthalmic manifestations of leukemia can be observed in more than a third of all newly diagnosed patients.[148,149] Leukemia can involve almost all ocular structures. Retinal hemorrhages, the most frequent ocular abnormality, are presumably caused by thrombocytopenia or anemia.[150] However, local infiltration of the capillary vessel walls with subsequent rupture and hemorrhage also may occur, especially in patients with very high leukocyte counts. Ocular motor palsies and papilledema are indicative of meningeal leukemia. Occasionally the optic nerve may be directly involved by leukemic infiltration.[151] In such situations visual acuity may be markedly affected and patients may present with monocular blindness. Prompt radiation therapy may be necessary to salvage useful vision. Leukemic infiltration of the anterior chamber with hypopyon and iritis has also been reported and can be the first manifestation of relapse.[152] Symptoms include conjunctival injection, photophobia, pain, blurring, and decreased vision.

Pulmonary Manifestations

Pulmonary leukostasis, which can lead to respiratory failure, can be seen at diagnosis, especially in patients with high presenting leukocyte counts.[117] This complication is more common in AML than in ALL. Radiographic distinction among infection, leukemic infiltration, and hemorrhage may be difficult.

Dermatologic Manifestations

Skin infiltration is uncommon in childhood leukemia, with the exception of congenital leukemia (both AML and ALL).[153] Leukemia cutis typically manifests as red or violaceous papules, nodules, or plaques.

DIFFERENTIAL DIAGNOSIS AND PROGNOSIS

A careful history and physical examination, together with an examination of the peripheral blood and bone marrow, result in a straightforward diagnosis of leukemia in the vast majority of cases. However, at times, ALL may present as the signs and symptoms of other conditions. These include ITP, aplastic anemia, JRA, infectious mononucleosis and other infections, and metastatic solid tumors.

Differential Diagnosis

Idiopathic Thrombocytopenic Purpura

ITP is the most common cause of the acute onset of petechiae and purpura in children. Patients with ITP usually present with isolated thrombocytopenia, often with large platelets seen on a blood smear, contrasting sharply to the more generalized blood cell abnormalities and small platelets typically observed in patients with ALL.[110] Physical findings in ITP are usually limited to bruising or bleeding associated with thrombocytopenia. An occasional patient develops modest splenomegaly, probably caused by a recent viral infection. In most cases, the leukocyte level and differential count are unremarkable and there is no anemia unless bleeding has been substantial or an unrelated anemia is present. Bone marrow examination is rarely necessary in uncomplicated cases of pediatric ITP (i.e., children with otherwise normal blood cell counts, consistent peripheral blood smear, and absence of adenopathy, hepatosplenomegaly, or other concerning findings on physical examination). However, if the diagnosis of ITP is in question or any of these concerning findings are present, clinicians should consider marrow evaluation to rule out leukemia before starting corticosteroids to avoid partially treating occult ALL.[154] The bone marrow aspirate is usually diagnostic because the marrow elements in ITP appear normal or show increased megakaryocytes.

Aplastic Anemia, Myelodysplasia, and Myeloproliferative Disorders

Like patients with leukemia, those with aplastic anemia, myelodysplasia, and myeloproliferative disease may present with pancytopenia and have fevers or infections associated with granulocytopenia. Lymphadenopathy and hepatosplenomegaly are unusual in aplastic anemia. Also, the radiographic skeletal changes sometimes seen in leukemia do not occur in aplastic anemia. To differentiate acute leukemia from these other disorders, marrow biopsy is mandatory and usually revealing.

Rarely, patients with ALL present with a transient pancytopenia preceding the leukemic phase.[155] The pancytopenic period may persist for a few days or weeks and be accompanied by high fevers. Spontaneous recovery with a period of normal blood cell counts often occurs before the onset of ALL.[155] In those cases, ALL typically becomes manifest several months after the period of aplasia. However, ALL may also develop after a period of aplasia, without any recovery of blood cell counts.[156] Distinguishing pre-ALL pancytopenia from aplastic anemia may be difficult. Repeated bone marrow aspirates or biopsies ultimately establish the correct diagnosis by demonstrating areas of marrow that are infiltrated with lymphoblasts. The pathogenesis of the preleukemic pancytopenia prodrome is unclear; in one case report, leukemia-associated genetic lesions were identified in the aplastic marrow when it was retrospectively analyzed.[157]

Juvenile Rheumatoid Arthritis and Connective Tissue Disease

Because ALL often presents primarily as joint or extremity complaints (especially limp, arthritis, or arthralgia), it may be confused with JRA or other autoimmune disorders.[142] Children with JRA can manifest fever, pallor, splenomegaly, and anemia, and patients with ALL can present with a positive test for antinuclear antibody, highlighting how difficult it may be to distinguish these conditions from each other.[142] Because of this difficulty, bone marrow aspiration should be considered before initiating corticosteroid therapy in children with presumed JRA.

Infectious Mononucleosis and Other Viral Infections

Childhood infectious mononucleosis and other viral illnesses can masquerade as leukemia. Patients may have generalized lymphadenopathy, splenomegaly, rash, fevers, and peripheral blood lymphocytosis. The atypical lymphocytes observed with acute Epstein-Barr virus (EBV) and other viral infections can sometimes be confused with peripheral leukemic blasts because they are larger than normal lymphocytes. Usually, viral infections can be differentiated from leukemia without bone marrow aspiration, but the procedure is sometimes necessary for accurate diagnosis. The detection of acute EBV infection in the blood by serology or PCR testing may be helpful in establishing the correct diagnosis.

Metastatic Solid Tumors

ALL and neuroblastoma may have similar presenting signs and symptoms, including fever, bone pain, and pancytopenia. Children with neuroblastoma frequently have malignant involvement of liver, lymph nodes, bone, or bone marrow; the marrow involvement may be extensive. In the bone marrow, neuroblasts tend to cluster and form pseudorosettes, in contrast to the diffuse involvement generally observed in ALL. Other solid tumors also rarely present as extensive marrow involvement, including rhabdomyosarcoma and Ewing sarcoma, and can initially be confused with leukemia. If questions remain after the examination of marrow morphology, other studies, such as immunophenotype and cytogenetics, may be helpful in distinguishing leukemia from a metastatic solid tumor.

Prognostic Factors

An array of clinical and biologic features have been identified as prognostically significant in childhood ALL, including age, presenting leukocyte count, immunophenotype, chromosomal abnormalities (ploidy, translocations), the presence of overt CNS leukemia at diagnosis, and the rapidity with which patients respond to initial induction chemotherapy.[111] Ultimately the prognostic significance of any factor is treatment dependent and the importance of a particular presenting feature in predicting outcome may vary, depending on the therapy delivered to that patient.

Age

The age of patients with ALL significantly correlates with clinical outcome. In childhood ALL, infants and adolescents have a worse prognosis than patients in the intermediate age group (aged 1 to 10 years).[116] ALL in infancy (<1 year at diagnosis) is associated with high presenting leukocyte counts, increased frequency of CNS leukemia at presentation, and a high incidence of rearrangements of the *MLL* gene on chromosome 11q23 (see Table 50-2).[17,18,116] Even when treated with intensified regimens, infants with *MLL* gene rearrangements have a dismal prognosis, with long-term EFS rates ranging from 10% to 40%.[116,158-161]

Adolescents (10 to 21 years of age) with ALL also have a less favorable outcome than children aged 1 to 10 years, although it is not as poor as that in infants. Adolescents with ALL more frequently present with "high risk" features at diagnosis, including T-cell immunophenotype, higher presenting leukocyte counts, and a lower incidence of cytogenetic abnormalities associated with a favorable prognosis, including high hyperdiploidy and the *ETV6-RUNX1* gene fusion (see Table 50-2).[116,162] Multiple retrospective studies have shown that adolescents fare better with pediatric ALL regimens than on treatments designed for adults with ALL.[162,163-165]

Leukocyte Count

The initial peripheral blood leukocyte count is a significant predictor of treatment outcome, with worsening outcomes as the leukocyte count increases.[111] Since 1996, based on the recommendation of the Cancer Therapy Evaluation Program of the National Cancer Institute (NCI), many investigators have considered a leukocyte count of 50,000 cells/mm^3 as the level separating patients with a higher risk for relapse from those with a more favorable prognosis.[111]

Immunophenotype

Historically, immunophenotype was considered an important prognostic factor, with inferior outcomes observed in patients with mature B-cell and T-cell disease. However, if they are treated with more intensive regimens, children with T-cell phenotype fare as well as those with B-precursor cell disease.[25] As noted earlier, mature B-cell ALL is more effectively treated with the same therapy used for advanced-stage Burkitt lymphoma.[8,9]

Some investigators have reported prognostic differences within subsets of patients with B-precursor cell ALL. Initial studies have suggested that patients with pre–B-cell ALL (cIg+) have a worse outcome than those with early pre–B-cell ALL (cIg–).[166] Subsequent reports indicated that the adverse outcome of patients with pre–B-cell ALL was caused by the subset with the t(1;19) translocation.[167] However, with greater treatment intensity, even patients with this cytogenetic abnormality do not appear to have an adverse prognosis.[168]

An early T-precursor phenotype is observed in 10% to 15% of cases of childhood T-cell ALL. This subset was initially indentified by gene expression profile analyses and is characterized by a distinctive immunophenotype (CD1a and CD8 negativity, with weak expression of CD5 and coexpression of stem cell or myeloid markers).[29] Retrospective analyses suggested that patients with early T-precursor cell ALL have a poorer prognosis.[29,169] In another retrospective study, patients with T-cell ALL with early T-precursor cell features were identified by ABD, indicating failure to complete V(D)J recombination at this locus.[30] Like early T-precursor cell ALL patients defined by immunophenotype, T-cell ALL patients with ABD were also found to have an inferior prognosis.

In 15% to 30% of patients with newly diagnosed ALL, flow cytometry reveals coexpression of at least one myeloid antigen on the cell surface of the lymphoblasts.[32,33,36] Myeloid antigen coexpression has been associated with several genetic abnormalities, including the *ETV6-RUNX1* fusion [t(12;21)], *MLL* gene (11q23) rearrangements, and the Ph chromosome (*BCR-ABL* rearrangement), but is almost never observed in high hyperdiploid ALL (51 to 65 chromosomes).[33,35,170] Myeloid antigen coexpression was previously believed to be associated with an inferior outcome, but several more recent reports have indicated that it is not an independent prognostic factor.[33,171]

Chromosomal Abnormalities

Several recurrent chromosomal abnormalities are important prognostic factors in childhood ALL. Two abnormalities, high hyperdiploidy (51 to 65 chromosomes or a DNA index greater than or equal to 1.16) and the cryptic t(12;21) (*ETV6-RUNX1* fusion), have been associated with a favorable prognosis.[43] High hyperdiploidy is observed in 25% to 30% of cases of childhood ALL and is more common in younger (noninfant) children with B-precursor cell phenotype and low leukocyte counts. [172] The most favorable outcomes in high hyperdiploid ALL patients have been associated with the presence of trisomies of chromosomes 4, 10, and 17.[107,173,174]

The *ETV6-RUNX1* fusion, which is rarely detected by karyotypic analysis, has been identified in approximately 20% of children with ALL using other more sensitive techniques, such as fluorescence in situ hybridization and the reverse-transcriptase PCR assay.[175,176] Like high hyperdiploidy, the *ETV6-RUNX1* fusion is more common in younger (noninfant) patients with low leukocyte counts and is observed almost exclusively in patients with B-precursor cell phenotype.[42,176] Up to 80% of children with B-precursor cell ALL diagnosed between the ages of 2 and 7 years have either high hyperdiploidy or the *ETV6-RUNX1* fusion,[172] although almost never both; these two chromosomal abnormalities are almost always mutually exclusive.[172,177] Several studies have indicated that children with *ETV6-RUNX1*–positive ALL have favorable EFS rates.[42,43,176] When relapses are observed in children with *ETV6-RUNX1*–positive ALL, they tend to occur relatively late and may be more responsive to postrelapse salvage therapy.[176,178-180]

Chromosomal abnormalities associated with an adverse prognosis include hypodiploidy (fewer than 44 or 45 chromosomes),[102,181,182] rearrangements of the *MLL* gene on chromosome 11q23,[158,181,183] and the Ph chromosome [t(9;22)].[181,184-187] *MLL* gene rearrangements are more frequent in infants than in older children with ALL.[183] The incidence of Ph chromosome–positive ALL increases with age at diagnosis and is more common in adolescents and young adults.[184,186] Patients with Ph chromosome–positive ALL appear to fare better if treated with allogeneic hematopoietic stem cell transplantation (HSCT) in first remission[187]; recent work has suggested that favorable outcomes may also be achieved with the combination of tyrosine kinase inhibitors and intensive chemotherapy without transplant.[96,97]

Intrachromosomal amplification of the *AML1* gene on chromosome 21 (iAMP21), detected in 1% to 2% of children with ALL, may also be associated with an inferior outcome.[188] Patients with this abnormality appear to fare better with more intensified "high risk" treatment than with less intensive regimens intended for patients with "standard risk" disease.[189,190] The t(1;19) translocation, observed in approximately 5% of cases of childhood ALL, had been previously associated with inferior EFS rates, but more recently conducted clinical trials have not found that this translocation is associated with an adverse prognosis. Some investigators have reported that patients with the t(1;19) translocation are at higher risk for CNS relapses,[191] but this finding has not been confirmed by others.[43,192,193] Chromosomal abnormalities in childhood ALL are reviewed in depth in Chapter 44.

Overt CNS Disease at Diagnosis

Lymphoblasts in the CSF can be detected in 15% to 20% of children who present with ALL.[126,127] Some children, such as those diagnosed within the first 12 months of life and those with T-cell ALL, have a higher incidence of CNS leukemia at diagnosis.[116]

Most investigators consider the presence of overt CNS disease at diagnosis to be an adverse prognostic indicator, and these patients are usually treated with more aggressive therapy. CNS status at presentation is usually classified as CNS-1 (no blast cells), CNS-2 (fewer than five leukocytes/μL with blast cells), and CNS-3 (five or more leukocytes/μL with blast cells or cranial nerve palsy).[111] Historically, CNS leukemia was defined as CNS-3 status (observed in approximately 5% of patients at diagnosis).[126,127] CNS-3 status at diagnosis is associated with a lower probability of EFS.[127] Several investigators have reported that patients with CNS-2 status also have a higher risk for relapse, although it does not appear to be as high as in patients with CNS-3 status at diagnosis.[126,127] Intensification of CNS-directed therapy (e.g., increasing the frequency of intrathecal chemotherapeutic treatments) may abrogate the adverse prognostic significance of CNS-2 status.[194] Thus patients with CNS-2 status at diagnosis usually receive extra doses of intrathecal chemotherapy without other changes in treatment whereas those with CNS-3 status are typically classified as higher risk and receive more intensive systemic and CNS-directed therapies.

Traumatic lumbar punctures with lymphoblasts on cytospin have also been associated with an adverse prognosis.[127,195] Like patients with CNS-2 status, those with traumatic lumbar punctures with lymphoblasts at diagnosis may also benefit from additional doses of intrathecal chemotherapy.[127]

Early Response to Induction Chemotherapy

The rapidity with which a patient responds to initial chemotherapy is a significant predictor of long-term outcome. Early response to therapy has been evaluated using morphologic measures (residual microscopic leukemia) and more sensitive techniques, such as PCR assay and flow cytometry.

Morphologic Response to Therapy. Patients who require two or more cycles of induction chemotherapy to achieve complete remission have a much worse prognosis than those who achieve complete remission within 1 month of diagnosis.[196-198] The Berlin-Frankfurt-Munster (BFM) group treats patients with 1 week of corticosteroid monotherapy (and one dose of intrathecal methotrexate) before beginning multiagent induction chemotherapy and has reported that poor peripheral blood response at the end of that week (defined as an absolute blast count of 1000/mm^3) is an independent predictor of adverse outcome.[3] Others have reported that the rate of clearance of blasts from the peripheral blood after multiagent chemotherapy is also an independent predictor of relapse.[199-201] Similarly, the persistence of leukemia in bone marrow specimens obtained 7 or 14 days after beginning multiagent chemotherapy strongly correlates with poor outcome,[202] although intensification of therapy can abrogate the adverse prognostic significance of slow early response.[203]

Minimal Residual Disease. Minimal residual disease (MRD) evaluation involves the measurement of very low levels of leukemia using sensitive techniques, such as PCR assay or specialized multiparameter flow cytometry. Leukemic cells are identified using targets identified at diagnosis, including leukemia-specific immunophenotypes (for flow cytometry–based assays), chromosomal translocations, or lymphoblast-specific immunoglobulin or T-cell antigen receptor gene rearrangements (for PCR-based assays). Using these techniques, leukemia cells have been identified at levels as low as 1 in 1,000 to 1 in 100,000 cells.[204-207]

Many studies have demonstrated that MRD levels early in therapy are a significant and independent predictor of long-term outcome.[204,208-212] For patients achieving a morphologic remission at the end of induction therapy, those with higher levels of marrow MRD at that time point have a higher risk for relapse than those with lower or undetectable MRD.[204,208-210,212-214,215] The risk for relapse directly correlates with MRD level, that is, those with the highest MRD levels at the end of remission induction have the worst prognosis. High levels of MRD detected soon after beginning with postinduction therapy (weeks 12 to 14 of treatment) have also been associated with an increased risk for relapse,[215,216] as have high levels measured in the peripheral blood as early as day 8 and in the marrow at day 15 of induction therapy.[214,217]

Other Prognostic Factors

Gender and Race. Some investigators have reported that male patients fare worse than female patients.[218,219] This observation had been attributed, in part, to the risk for testicular relapse,[218] although higher relapse rates in males have been observed even when testicular relapse rates were low.[219] Race may also influence outcome, with lower EFS rates reported for African-American, Hispanic, and Native American patients, even after adjustment for differences in prognostically significant presenting features. [220-222] The reasons why patients of different race have varying responses to therapy on certain regimens have not been fully elucidated but may in part be related to differences in the biologic subtypes of ALL observed in different groups. For example, African-American children have a higher relative incidence of T-cell ALL and lower rates of high hyperdiploidy, a favorable genetic subtype of precursor B-cell ALL.[223] Hispanic children also have a lower frequency of *ETV6-RUNX1*–rearranged ALL[224] and, in one study, were noted to have a higher incidence of ALL with rearrangements of CRLF2, a subtype associated with an inferior prognosis.[225]

Pharmacogenomics. Several studies have suggested that outcome may be affected by how rapidly and effectively an individual patient metabolizes certain chemotherapeutic agents. Polymorphisms in genes involved in chemotherapy drug metabolism have been associated with risk for relapse.[226] More favorable long-term outcomes have been reported in patients with mutant thiopurine methyltransferase (TPMT) phenotypes involved in the metabolism of thioguanines, such as 6-mercaptopurine,[227,228] although such patients may also be at higher risk for developing significant treatment-related toxicities, including low blood cell counts, infection, liver dysfunction, and second malignancies.[229,230] Investigations of other genes, such as the glutathione S-transferase genes (encoding enzymes involved in the intracellular detoxification of various compounds)[231,232] and the folate metabolism genes (e.g., thymidylate synthase, methylenetetrahydrofolate reductase, and methylenetetrahydrofolate dehydrogenase)[233-235] have led to conflicting results, without clear consensus regarding the prognostic significance of various polymorphisms.

Treatment Adherence. Compliance with taking oral chemotherapy during the maintenance phase has been shown to impact outcome. In one study of pediatric ALL patients, nonadherence to 6-mercaptopurine (as assessed by electronic monitoring device that measured cap opening of the medication bottle) was associated with an inferior outcome. Adolescents and Hispanic patients were at higher risk for noncompliance. After adjusting for sex, NCI risk group, cytogenetics, ethnicity, and other factors, 6-mercaptopurine compliance was an independent prognostic factor, with a progressive increase in risk for relapse with decreasing levels of adherence.[236]

THERAPY

Historical Background

Over the past 50 years, there has been a dramatic improvement in the prognosis of children with ALL (Fig. 50-3). Prior to 1947, when the first complete remission in childhood ALL was attained by Farber and colleagues,[237] the median duration of survival from the time of diagnosis was 2 months.[238] During the 1950s, drugs such as 6-mercaptopurine, methotrexate, and corticosteroids were found to be active in leukemia-bearing mice and subsequently in human leukemias.[239,240] The first controlled clinical trials were conducted by Frei and associates,[241] who ushered in the era of single-agent (and soon thereafter combination-agent) antileukemic chemotherapy trials.[241,242] Active drugs introduced in the 1960s and 1970s included the anthracyclines (doxorubicin and daunorubicin), L-asparaginase, and the epipodophyllotoxins (etoposide and teniposide).[243-245]

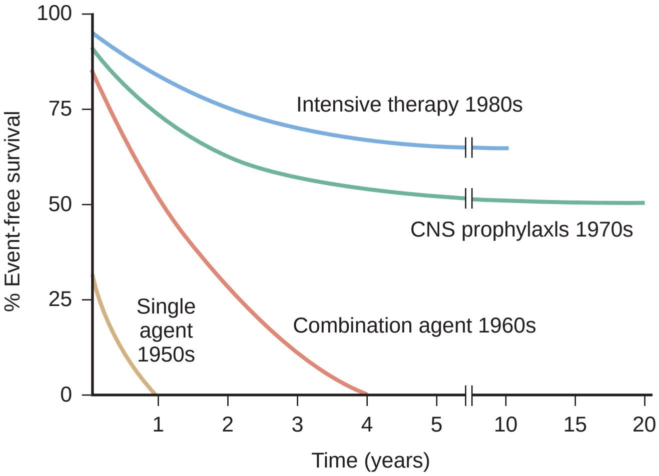


Figure 50-3 Historical perspective of the treatment of childhood acute lymphoblastic leukemia. The single-agent era resulted in few complete remissions and no cures. The combination-agent era without adequate central nervous system (CNS) treatment resulted in high complete remission rates but almost uniform mortality. Between the mid-1960s and 1970s, combination chemotherapy and CNS treatment resulted in prolonged, disease-free survival for approximately 50% of children. Intensive therapy in the 1980s and 1990s resulted in an event-free survival of 75% to 80% of children.

TABLE 50-3 Outcome by Presenting Features of 491 Children and Adolescents (Ages 0 to 18 Years) Treated on Dana-Farber Cancer Institute ALL Consortium Protocol 95-01 (1996-2000)

Presenting Feature	No. of Patients	10-Year EFS (±SE)	10-Year OS (±SE)
Overall	491	79 ± 2	89 ± 2
Age at diagnosis (yr)			
<1	14	42 ± 13	42 ± 13
1-9	385	82 ± 2	93 ± 1
≥10	92	75 ± 5	78 ± 4
WBC (× 10^9/L)			
<10	239	83 ± 3	93 ± 2
10-49	155	80 ± 3	89 ± 3
50-99	43	70 ± 10	88 ± 5
≥100	54	66 ± 7	73 ± 6
Immunophenotype			
B-precursor cell	438	78 ± 2	89 ± 5
T-cell	52	85 ± 5	90 ± 4
Gender			
Male	274	78 ± 3	88 ± 2
Female	217	81 ± 3	91 ± 2
B-Precursor cell National Cancer Institute (NCI) risk group			
Standard	303	83 ± 2	94 ± 2
High	121	72 ± 5	82 ± 4

Modified from Silverman LB, Stevenson KE, O'Brien JE, et al: Long-term results of Dana-Farber Cancer Institute ALL Consortium protocols for children with newly diagnosed acute lymphoblastic leukemia (1985-2000). Leukemia *24:320–334, 2010.*
EFS, Event-free survival; *OS*, overall survival; *SE*, standard error; *WBC*, white blood cell.

With current regimens for the treatment of childhood ALL, more than 95% of patients achieve complete remission and approximately 80% are long-term event-free survivors (Table 50-3 and Fig. 50-4).[24,246,247] Overall survival, which includes patients who are salvaged after relapse, is now approximately 90%.[248] Improvement in outcome over the past 30 to 40 years occurred despite the fact that, with few exceptions, the drugs used for the treatment of ALL today were all available by the late 1960s and early 1970s. Improvement in cure rates can be attributed to many factors, including the development of complex chemotherapeutic regimens designed to achieve clonal eradication, improvements in

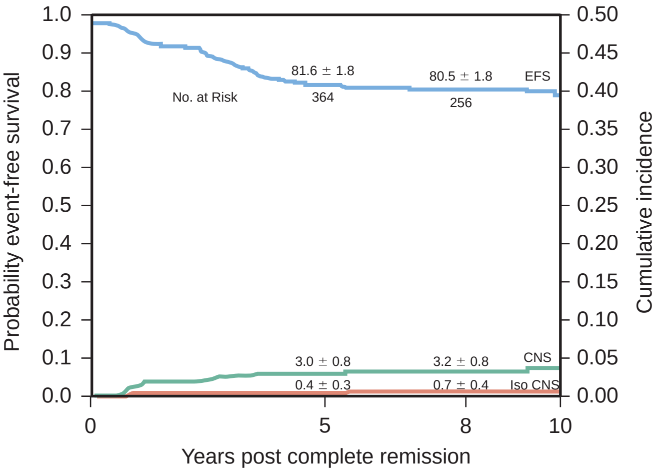


Figure 50-4 Event-free survival *(EFS)* and cumulative incidence of isolated or any central nervous system *(CNS)* relapse for 491 patients treated on Dana-Farber Cancer Institute ALL Consortium Protocol 95-01 (1996-2000). Median follow-up was 8.6 years. *(Modified from Silverman LB, Stevenson KE, O'Brien JE, et al: Long-term results of Dana-Farber Cancer Institute ALL Consortium protocols for children with newly diagnosed acute lymphoblastic leukemia [1985-2000].* Leukemia *24:320–334, 2010.)*

supportive care, recognition of the CNS as a sanctuary site, and application of risk-directed therapy.

Risk-Adapted Therapy

After the addition of CNS-directed therapy improved cure rates to approximately 50% in the 1970s, investigators compared presenting features in patients who were cured and those who relapsed to establish clinically relevant prognostic factors. Subsequent clinical trials used these prognostic factors to stratify therapy. More intensive therapy was administered to those patients considered to be at the highest risk for relapse. In contrast, some of the more morbid components of therapy were modified or eliminated for those children considered to have the best prognosis. The goal of risk-adapted therapy is to treat away adverse presenting features so that high-risk and low-risk patients have similar cure rates. For example, on Dana-Farber Cancer Institute (DFCI) ALL Consortium protocols, children with T-cell ALL (historically, a subgroup with an inferior prognosis) are treated as high-risk patients (receiving higher cumulative dosages of anthracycline and corticosteroid than lower-risk patients); with that therapy, their outcomes are as favorable as those with B-precursor cell disease.[25]

For many years, pediatric cooperative groups and institutions applied prognostic factors differently when defining risk categories for clinical trials. A more uniform approach to risk classification was proposed and agreed on at an NCI-sponsored workshop held in 1993.[111] For patients with B-precursor cell ALL, the standard-risk category was defined as age between 12 months and younger than 10 years and initial leukocyte count lower than 50,000/mm^3. The remaining patients were considered to have high-risk ALL. Other characteristics used by the various cooperative groups to classify patients as high risk include T-cell phenotype, CNS-3 status at diagnosis, and slow early response to initial therapy, including a high peripheral blood absolute blast count at the end of a 7-day prednisone prophase.[111] These various prognostic factors are discussed earlier.

Contemporary regimens have incorporated cytogenetic abnormalities and MRD levels into risk group stratification, and in some cases, only these factors are considered (and not more standard factors, such as age and leukocyte count) when assigning risk groups.[215] Patients with certain chromosomal abnormalities (e.g., *MLL* gene rearrangements and low hypodiploidy) are treated as high risk, regardless of other presenting features. Similarly, end-induction MRD is used by almost all groups as a factor determining the intensity of postinduction treatment, with patients found to have higher levels allocated to more intensive therapies.[215,249] On some protocols, MRD levels at a later time point (week 12) are also considered when risk-stratifying patients, because these have been shown to be prognostically important, especially in patients with T-cell ALL.[216]

MRD measurements, in conjunction with cytogenetics, have also been used to identify subsets of patients with an extremely low risk for relapse who may be successfully treated with less intensive therapies. The Children's Oncology Group reported a very favorable prognosis for patients with the B-precursor cell phenotype, NCI standard risk age/leukocyte count, CNS-1 status, and favorable cytogenetic abnormalities (either high hyperdiploidy with favorable trisomies or the *ETV6-RUNX1* fusion) who had low peripheral blood MRD levels at day 8 and low marrow MRD at end of induction.[214]

Ultimately, treatment is the most important prognostic factor, and those factors used to risk-stratify patients on one regimen may not be useful for patients treated on a different regimen.

Phases of Therapy

In general, treatment regimens for children with newly diagnosed ALL include three phases: remission induction, consolidation (or intensification), and continuation (or maintenance). The remission induction phase of therapy is designed to destroy measurable leukemia cells rapidly and minimize residual leukemia burden (i.e., the total number of leukemic cells in the body). The consolidation or intensification phase(s) is designed to reduce the total-body leukemia cell burden further and address issues of antileukemic drug resistance. The continuation or maintenance phase, consisting of low-dose chemotherapy, is designed to eradicate the residual leukemia cell burden. Throughout all phases, CNS-directed treatments (e.g., intrathecal chemotherapy) are an essential component because many systemically administered agents do not adequately penetrate the brain and spinal cord.

Remission Induction

After the diagnosis of ALL has been established and any urgent medical issues, such as metabolic imbalances, have been addressed, antileukemic chemotherapy should be initiated without delay. The initial phase of treatment, remission induction, is designed to reduce the leukemic cell burden to a clinically and hematologically undetectable level. *Hematologic remission* is defined as attainment of a normocellular bone marrow with 5% or fewer blasts and peripheral blood without lymphoblasts, with return of "normal" peripheral blood counts, that is, a granulocyte count exceeding 500 to 1000/mm^3 and a platelet count exceeding 100,000/mm^3. *Complete remission* is defined as achievement of these criteria along with the absence of any signs and symptoms of extramedullary leukemia. A complete remission must be induced before the next component of therapy is begun. The duration of most induction regimens is 4 to 6 weeks.

Clinical trials in the 1960s demonstrated that combinations of two agents were consistently superior to single agents for inducing complete remission in children with ALL. Vincristine and prednisone produced complete remission in approximately 90% of pediatric patients with ALL.[250,251] Addition of a third drug increased both the number of patients who achieved complete remission and the long-term relapse-free survival.[250,252] Current induction regimens, therefore, consist of at least three agents, most often L-asparaginase or an anthracycline, in addition to vincristine and prednisone.

Many groups have further intensified induction regimens to include four or more agents. In theory, these more intensive induction regimens may prevent the emergence of drug-resistant leukemic clones by an initial leukemic cell lysis of greater rapidity and magnitude.[253] The benefit in terms of long-term EFS of using four drugs during induction therapy (vincristine, prednisone, L-asparaginase, and an anthracycline) is widely accepted in higher-risk patients[254] but less clear in lower-risk patients.[255] Intensive induction regimens may enhance long-term EFS but can result in increased short-term morbidity, especially from infections during periods of granulocytopenia. However, even with four-drug induction regimens, few toxic deaths are reported (i.e., 1% to 2% of patients).[24,246,256]

The initiation of CNS treatment is an integral component of induction therapy. After the diagnosis of ALL has been established by bone marrow examination, a lumbar puncture is performed for diagnostic and therapeutic purposes. It is generally recommended that intrathecal chemotherapy be given with the first diagnostic lumbar puncture to reduce the theoretical risk for inadvertently seeding the meninges with peripheral blood lymphoblasts.[195] In addition to the diagnostic lumbar puncture, intrathecal chemotherapy is usually administered at least one more time during the first month of treatment. Patients with lymphoblasts present on their initial CSF specimen (CNS-2 or CNS-3 status, or traumatic lumbar puncture with blasts) typically receive more doses of intrathecal chemotherapy during the induction phase than those without any detectable CSF lymphoblasts (CNS-1 status).[127]

Failure to achieve remission after 1 month of therapy (induction failure) is uncommon, occurring in fewer than 5% of patients.[196,197] Induction failure is observed more commonly in patients with high presenting leukocyte counts or T-cell phenotype.[196,197] Successful treatment of refractory ALL has been reported with the use of drugs such as cytarabine, epipodophyllotoxins (e.g., etoposide, teniposide), and anthracyclines (e.g., idarubicin).[257-259] Nelarabine has been shown to induce remissions in patients with refractory T-cell ALL.[260,261] Ultimately, 80% to 90% of children with initially refractory disease are able to achieve a complete remission.[196,197] Despite this relatively high complete remission rate, overall survival for patients with a history of initial induction failure is poor (long-term overall survival rates of 20% to 30%).[196-198] Allogeneic HSCT in first remission may improve the outcome for patients with initial induction failure.[262] In a large retrospective series, a trend for superior outcome with allogeneic HSCT compared with chemotherapy alone was observed in patients with initial induction failure and either the T-cell phenotype (any age) or B-precursor cell phenotype and age younger than 6 years.[198] In that study, B-precursor cell ALL patients who were aged 1 to 5 years at diagnosis and did not have any adverse cytogenetic abnormalities (*MLL* translocation, *BCR-ABL*) had a relatively favorable prognosis, without any advantage in outcome with the utilization of HSCT compared with chemotherapy alone.

Consolidation (Intensification) Therapy

The goals of consolidation therapy are to reduce the disease burden further and to adjust the intensity of treatment based on the risk for subsequent relapse. A wide variety of agents and schedules have been used during postinduction consolidation in pediatric trials. In general, there has been an attempt in lower-risk patients to limit exposure to agents associated with significant acute and late toxicities while reserving the use of more intensive therapies for patients at higher risk for relapse.

A commonly used postinduction consolidation regimen was first introduced by the German BFM study group.[3] This consolidation scheme includes (1) a 4-week chemotherapy course (sometimes referred to as "consolidation" or "induction IB") immediately after the initial induction phase consisting of cyclophosphamide, low-dose cytarabine, and a thiopurine (mercaptopurine or thioguanine), followed by (2) an interim maintenance phase, which includes multiple doses of either high-dose or escalating doses of methotrexate with or without leucovorin rescue, and then (3) a reinduction course (or delayed intensification), which typically includes the same agents used during the initial induction/consolidation cycles. Augmentation of this regimen, including the use of two delayed intensification cycles instead of one, has led to improved outcomes for higher-risk patients, including those with a slow early morphologic response to initial induction therapy.[203,263-265] The DFCI ALL Consortium uses an alternative postinduction consolidation regimen that includes 20 to 30 weeks of L-asparaginase and, for higher-risk patients, additional doses of doxorubicin.[2]

Continuation Therapy

Almost all current treatment regimens for ALL include a phase of continuation or maintenance therapy, in which patients are treated with less-intensive chemotherapy to complete at least 2 years of therapy. Most continuation regimens consist of weekly low-dose methotrexate and daily oral 6-mercaptopurine. Some groups have added regular pulses of vincristine and corticosteroid to this regimen, although their benefit remains controversial.[266-268]

Several pharmacologic studies have documented highly variable bioavailability after oral administration of 6-mercaptopurine and methotrexate during the continuation phase.[269-273] This may have important prognostic implications, because lower intracellular levels of thioguanine nucleotides and methotrexate polyglutamates, the major metabolites of 6-mercaptopurine and methotrexate, have been correlated with a higher risk for relapse.[270,273,274] Variable bioavailability may be the result of various factors, including concurrent food, which may interfere with drug absorption.[272] Bioavailability of oral mercaptopurine appears to be improved if it is given in the evening, without food or milk.[272,275,276] Polymorphisms within genes involved in drug metabolism also affect rates of drug activation and clearance.[229] For example, patients with deficiency in the activity of TPMT, an enzyme that inactivates mercaptopurine, have higher rates of toxicity during continuation therapy (but also may have better survival) than patients with normal TPMT activity.[227,229] Most physicians attempt to compensate for the interindividual variations of bioavailability and metabolism by tailoring the dose of both drugs to the leukocyte count. Administration of maximally tolerated doses of methotrexate and 6-mercaptopurine during continuation therapy may improve outcome, as suggested by studies demonstrating that patients with lower leukocyte or neutrophil counts during continuation chemotherapy have lower relapse rates.[277,278] Lack of patient compliance with oral medications, as well as physician compliance with protocol dosages, may adversely affect the efficacy of continuation chemotherapy.[236,279,280]

6-Thioguanine is an alternative thiopurine that, unlike 6-mercaptopurine, does not rely on TPMT for its metabolism, resulting in higher intracellular levels of thioguanine nucleotides.[281] 6-Thioguanine also has been shown to be more potent than 6-mercaptopurine in inducing cell death of lymphoblasts in vitro.[282] The results of one randomized clinical trial comparing the two thioguanines indicated that 6-thioguanine was associated with a superior EFS (but not overall survival) in boys, primarily owing to a reduction in the frequency of CNS relapses.[283] However, other randomized trials comparing the two thiopurines did not confirm an EFS advantage for 6-thioguranine.[284,285] Additionally, the use of 6-thioguanine during the maintenance phase has been associated with significant hepatotoxicity, including veno-occlusive disease and cirrhosis, as well as higher remission death rates, primarily caused by infection.[283,284] Thus 6-mercaptopurine remains the thiopurine of choice during the continuation phase.

Several investigators have studied the relative efficacy and toxicity of two corticosteroids used during postinduction therapy: prednisone and dexamethasone. Interest in substituting dexamethasone for prednisone stems from studies suggesting that dexamethasone has more potent in vitro antileukemic activity, higher free plasma levels, and enhanced CSF penetration.[286-289] In several clinical trials conducted in the 1990s, the nonrandomized substitution of dexamethasone for prednisone appeared to affect outcome favorably.[290,291] This finding was confirmed in several randomized clinical trials that indicated that dexamethasone was associated with a superior 5-year EFS.[292-294] However, dexamethasone may also be associated with increased corticosteroid-related toxicities, including higher rates of fracture, osteonecrosis, and infectious complications, especially in older children and adolescents.[292-296] Thus the optimal corticosteroid preparation and dosing have yet to be determined and may differ based on presenting features (e.g., age) and risk for relapse.

Attempts to intensify continuation therapy by administering rotating pairs of non–cross-resistant drugs, including many agents not traditionally incorporated during the continuation phase, such as cyclophosphamide, epipodophyllotoxins, and cytarabine, have led to mixed results. In studies conducted by St. Jude Children's Research Hospital, this strategy resulted in improved outcomes for higher-risk but not lower-risk patients[297] but also was associated with high rates of toxicity, including secondary AML.[298] Based on this risk, these agents (especially epipodophyllotoxins) are, in general, no longer administered to most patients during the continuation phase.

CNS-Directed Therapies

In the 1960s, as systemic chemotherapy became more effective in prolonging the duration of hematologic remission in patients with ALL, the incidence of CNS leukemia as an initial site of relapse became progressively more common.[299,300] It was hypothesized that leukemia cells, even if subclinical, were present in the CNS in all patients and that these cells were protected by the blood-brain barrier from systemically administered chemotherapy. Thus, the concept of the CNS as a "pharmacologic sanctuary" (i.e., an anatomic space that is poorly penetrated by systemically administered chemotherapeutic agents) emerged. The introduction of radiation therapy to treat subclinical CNS leukemia in the 1970s was a pivotal step in boosting long-term disease-free survival rates in childhood ALL to 50%.[301] Although most pediatric patients are now treated without prophylactic cranial radiation, the importance of effectively treating subclinical CNS leukemia is undisputed for childhood ALL.

All successful treatment regimens for ALL include therapy directed at treating CNS leukemia. Options for CNS-directed therapies include cranial irradiation, intrathecal chemotherapy, and CNS penetrant systemic chemotherapy.

Radiation therapy was the first modality successfully used to prevent CNS relapses. The use of radiation for CNS treatment was based on experiments demonstrating that L1210 murine leukemia could be cured when cranial irradiation was added to systemic treatment with cyclophosphamide.[302] In the 1960s and 1970s, studies performed at St. Jude Children's Research Hospital documented the effectiveness of CNS radiation as preventive therapy in children with ALL.[303] In one study, patients were randomized to receive 2400 cGy of craniospinal radiation or no radiation. The difference in CNS relapse rates was striking: only 2 of 45 irradiated patients (4%) had their initial complete remissions ended by meningeal relapse, compared with 33 of 49 nonirradiated patients (67%).[304] Moreover, 31 of the patients who received prophylactic radiation remained alive after 18 to 20 years, compared with only 10 patients in the nonirradiated group. Subsequent studies demonstrated that 2400-cGy cranial irradiation with intrathecal methotrexate was as effective in preventing CNS relapse as 2400-cGy craniospinal irradiation without intrathecal chemotherapy.[305,306] Because craniospinal radiation was associated with increased toxicity, including excessive myelosuppression and spinal growth retardation, radiation directed only to the cranium administered with intrathecal chemotherapy became the standard form of CNS treatment in the 1970s.

Although 2400-cGy cranial irradiation (with intrathecal chemotherapy) effectively prevents CNS relapses in children with ALL, it is also associated with subsequent learning disabilities, growth and neuroendocrinologic abnormalities, and an increased risk for second malignant neoplasms. Thus many investigators have studied alternative CNS treatments, including lower doses of cranial radiation (1200 to 1800 cGy), systemically administered CNS-penetrant chemotherapy (i.e., agents known to achieve therapeutic levels in CSF), or intrathecal chemotherapy. Current regimens for childhood ALL include some or all of these CNS-directed therapies.

The proportion of patients receiving cranial radiation has decreased significantly over the past several decades; on current regimens, most pediatric patients are treated without radiation. When it is administered, cranial radiation is generally restricted to patients with a higher risk for relapse involving the CNS, especially those with T-cell disease, high presenting leukocyte counts, or CNS-3 status at diagnosis.[307] Doses lower than 2400 cGy have been shown to be effective, and therefore are typically used when cranial radiation is administered. For instance, CNS and marrow relapse rates were not significantly different when the dose of cranial radiation was lowered from 2400 to 1800 cGy in consecutive Children's Cancer Study Group trials.[308] The BFM and DFCI groups have successfully used 1200-cGy irradiation in the context of an intensive systemic regimen that included high-dose methotrexate (known to penetrate the CNS effectively).[294,309,310] Irradiated patients also typically receive intermittent intrathecal chemotherapy in addition to cranial irradiation as CNS preventive therapy.

Systemic therapy plays an important role in the prevention of CNS leukemia. Penetration of the CSF by drugs has been clearly demonstrated with the use of glucocorticoids[286] and high doses of methotrexate and cytarabine.[311,312] It has also been shown that systemically administered asparaginase, whose efficacy is a function of asparagine depletion, effectively lowers CSF asparagine levels.[313,314]

Intrathecal chemotherapy, used in conjunction with intensive systemic therapy and/or cranial irradiation, is an important component of CNS treatment. Many investigators have replaced cranial irradiation with frequent dosing of intrathecal chemotherapy, especially in lower-risk patients. The success of these efforts appears to depend on the frequency with which intrathecal chemotherapy is delivered, as well as the intensity and CNS penetration of the systemic chemotherapy administered. Investigators from the Children's Cancer Group demonstrated that administering intrathecal methotrexate throughout all phases of therapy was as effective in preventing CNS relapses in lower-risk patients as 1800-cGy cranial irradiation combined with only 6 months of intrathecal methotrexate, but only in the context of an intensified systemic regimen.[315] When a less-intensive systemic regimen was used, more CNS relapses were observed in nonirradiated patients. Similarly, in a DFCI ALL Consortium study, excessive CNS relapses were observed in standard-risk males when cranial irradiation was eliminated without increasing the frequency of intrathecal chemotherapy or the intensity of systemic chemotherapy.[316] However, in a subsequent study conducted by the same group, standard-risk patients who received more frequent doses of intrathecal chemotherapy had as low a CNS relapse rate as irradiated patients.[246] Some controversy exists regarding the relative efficacy of intrathecal methotrexate alone and "triple intrathecal chemotherapy" (intrathecal methotrexate, cytarabine, and hydrocortisone). In one randomized study in standard-risk patients, triple intrathecal chemotherapy was associated with a lower rate of isolated CNS relapse but there was no difference in overall EFS.[317] Randomized studies have also demonstrated that the use of dexamethasone instead of prednisone, which is believed to be less CNS penetrant, is associated with a lower rate of isolated CNS relapses in nonirradiated patients.[292,293] Thus those regimens that have successfully eliminated cranial radiation include extended and/or frequent dosing of intrathecal chemotherapy in conjunction with intensified, more CNS penetrant systemic therapy, including dexamethasone and/or high- or intermediate-dose methotrexate.[194,246,290,318-320]

Several clinical trials have been conducted on which cranial radiation was omitted for all patients, including high-risk subsets.[321-323] Most of these trials include multiple doses of either high-dose methotrexate or high-dose cytarabine and increased frequency of intrathecal chemotherapy. While the overall EFS on these trials was similar to that of contemporaneously conducted trials on which some patients received prophylactic radiation, some nonirradiated patient subsets had a significantly high risk for CNS relapse, including patients with T-cell phenotype or the presence of blasts in CSF at diagnosis. Additionally, some patients with high-risk features on these trials were treated with allogeneic SCT in first complete remission (which included total-body irradiation).[321]

The goal of eliminating cranial radiation in pediatric patients is to minimize long-term sequelae (see later). Although several studies have indicated that intrathecal chemotherapy and intensive systemic therapy can be as effective as cranial irradiation in preventing CNS relapses, the relative late neurocognitive toxicities of these CNS treatment strategies remains unsettled. Moreover, higher rates of acute neurotoxicity, including seizures, have been observed with regimens that included multiple courses of high-dose methotrexate and frequent doses of intrathecal chemotherapy.[324]

Cessation of Treatment

The optimal duration of therapy remains unknown. Most investigators continue to treat patients for 2 to 3 years, based on results of older studies, in which patients received therapy that was less intensive than in current regimens.[325] Randomized studies conducted by the Children's Cancer Group in the 1970s indicated that there was no significant difference in outcome when comparing 5 versus 3 years of therapy.[326,327] In the Childhood Leukaemia Trial UKALL VIII, a randomized study of 2 versus 3 years of continuation therapy conducted in the 1980s, the researchers observed no significant survival advantage with longer therapy. Patients receiving the shorter duration of therapy had a higher relapse rate, but this was counterbalanced by a higher remission death rate in those receiving 3 years of treatment.[328] Some early studies suggested that the optimal duration of therapy may be different for boys and girls, with boys benefiting from a more prolonged continuation phase,[329] although this finding may be less relevant with current regimens.

Even with more intensive regimens, attempts to shorten therapy duration from 2 years have not been successful. The BFM group randomized patients to receive 18 or 24 months of treatment and observed a higher relapse rate with patients who received the shorter course of treatment.[310] Similarly, high relapse rates were observed in a nonrandomized study conducted by the Tokyo Children's Cancer Study Group, in which patients received intensified therapy for only 12 months, suggesting that truncated therapy, even if intensive, is inadequate for most children with ALL.[330]

Treatment of Special Patient Subsets

Philadelphia Chromosome–Positive ALL Patients

The Ph chromosome, t(9;22)(q34;q11), which leads to fusion of the *BCR* and *ABL* genes, is detectable in approximately 5% of children and 20% of adults with ALL.[331] In pediatric ALL, it is more frequent in adolescents than in younger children.[116] When treated on regimens with conventional chemotherapeutic agents, these patients have a dismal prognosis, with long-term EFS rates ranging from 0% to 25%.[95,184-186] Allogeneic SCT in first remission has been shown to lead to more favorable outcomes and, prior to the availability of tyrosine kinase inhibitors, was considered the treatment of choice for children with Ph chromosome–positive ALL.[332] In one study, which reviewed the outcome of 610 children with Ph chromosome–positive ALL treated by 10 study groups in the pre–tyrosine kinase inhibitor era between 1995 and 2005, transplantation in first remission from an HLA-matched related or unrelated donor was associated with a higher likelihood of disease-free survival compared with chemotherapy alone.[187]

Imatinib mesylate is a selective inhibitor of the *BCR-ABL* tyrosine kinase. Phase I and II studies of single-agent imatinib in children and adults with relapsed or refractory Ph chromosome–positive ALL have demonstrated relatively high response rates, although these responses tended to be of short duration.[333,334] Results from pediatric trials have suggested that the administration of imatinib in combination with multiagent chemotherapy is feasible, without excessive toxicity.[96,97] In a pilot trial conducted by the Children's Oncology Group, patients treated with the combination of chemotherapy and imatinib (but without transplant) appeared to do at least as well as other patients on the same trial who underwent SCT in first remission.[96]

Infants

Patients diagnosed during the first year of life represent 2% to 4% of cases of childhood ALL.[335] The outcome of infants with ALL is significantly worse than that for older children, with reported long-term EFS rates of 20% to 40%.[160,336,337] The major cause of treatment failure is relapse, which tends to occur early, often in the first 1 to 2 years after diagnosis.[159,160,338-341] In vitro chemosensitivity assays have indicated that lymphoblasts from infants with ALL are relatively resistant to agents commonly used in childhood ALL regimens, such as corticosteroids and L-asparaginase.[342] Infants also appear to be more vulnerable to the toxic effects of therapy, especially severe infections.[160,339,343]

The poor outcome of infants appears to be related to the biologic distinctiveness of the disease observed in this age group. Up to 80% of infants with ALL present with molecularly detectable rearrangements of the *MLL* gene on chromosome 11q23.[17,18,160,161] The presence of a *MLL* gene rearrangement is an independent predictor of poor outcome in infant ALL, with the worst outcomes observed in *MLL*-rearranged patients who present at a very young age (<6 months at diagnosis) and with high presenting leukocyte counts.[160,161]

Because of their poor prognosis, infants with ALL are usually treated on separate protocols with intensified therapy. In vitro drug sensitivity testing and small, nonrandomized clinical trials have suggested that high-dose cytarabine, an agent more often used in AML, might benefit infants with ALL.[342-344] In the Interfant-99 trial, the largest clinical trial conducted for infants with ALL, a cytarabine-intensive regimen resulted in a 4-year EFS of 47%.[161]

The role of allogeneic SCT in first complete remission in infants with ALL remains controversial.[345,346] On the Interfant-99 trial, a subset analysis suggested that SCT in first remission may be beneficial in infants with high-risk features (*MLL* gene rearrangement, age younger than 6 months at diagnosis, and presenting leukocyte count at or above 300,000/µL); an EFS advantage for transplant was not demonstrated in other *MLL*-rearranged infants.[347]

Adolescents

Older children and adolescents (aged 10 to 18 years at diagnosis) have a less favorable outcome than children aged 1 to 10 years at diagnosis, and more aggressive treatments are generally administered to these patients. Adolescents present more frequently with adverse prognostic features, such as a high leukocyte count, T-cell immunophenotype, and the *BCR-ABL* translocation, and a lower frequency of favorable features, such as high hyperdiploidy and the *ETV6-RUNX1* fusion gene.[116,162,348] Adolescents also appear to be at higher risk than younger children for developing treatment-related complications, including osteonecrosis, pancreatitis, and thrombosis.[162,295,349] Several investigators have compared outcomes of older adolescents with ALL (aged 15 to 20 years) treated in pediatric or adult clinical trials, and all have found that these patients fare better with a pediatric regimen.[163-165,350] The superior outcome of older adolescents treated with pediatric ALL regimens may be related, in part, to the components of therapy; pediatric regimens tend to include higher cumulative dosages of vincristine, corticosteroid, and L-asparaginase than treatments designed for adults with ALL.[163]

Children with Down Syndrome

Children with Down syndrome have increased risk for developing both ALL and AML. Approximately one half to two thirds of cases of acute leukemia in children with Down syndrome are ALL. Although the vast majority of cases of AML in children with Down syndrome occur before the age of 4 years, ALL in children with Down syndrome has an age distribution similar to that of ALL in non–Down syndrome children.[351,352] Patients with ALL and Down syndrome almost always present with B-precursor cell phenotype and have a lower incidence of favorable and unfavorable cytogenetic abnormalities.[353-356] They appear to be at higher risk for developing treatment-related complications, especially those related to methotrexate, such as mucositis and myelosuppression.[357,358] Outcomes for children with Down syndrome and ALL have generally been reported to be less favorable than those of non–Down syndrome patients, which may be related to a higher incidence of treatment-related mortality because of infection and a lower frequency of favorable biologic features, such as high hyperdiploidy and the *ETV6-RUNX1* fusion.[352-356]

High expression of *CRLF2* occurs in 50% to 60% of cases of ALL in children with Down syndrome, compared with 5% to 10% of non–Down syndrome B-precursor cell ALL patients.[83,84,359] A higher frequency of activating mutations in *JAK2* has also been observed in Down syndrome ALL (~20% of cases)[360,361]; almost all patients with *JAK2* mutations also have *CRLF2* overexpression.[83,84,360] It does not appear that *CRLF2* genomic aberrations or *JAK2* mutations have independent prognostic relevance in Down syndrome ALL.[360] However, in one study, deletions of the *IKZF1* gene, observed in 35% of Down syndrome ALL cases (and frequently associated with *CRLF2* overexpression), was found to be a strong independent predictor of inferior EFS.[360]

COMPLICATIONS OF TREATMENT

At Diagnosis and during Treatment

At the time of diagnosis, the major clinical problems are the results of metabolic disturbances secondary to leukemia cell lysis, disrupted hematopoiesis leading to abnormal peripheral blood counts, and leukemic infiltration of nonhematopoietic organs. Initiation of chemotherapy may exacerbate these issues and lead to additional problems, including myelosuppression, bacterial and fungal infections, and mucosal toxicity.

Metabolic Complications

Rapid turnover of leukemia cells before and immediately after the initiation of chemotherapy leads to the release of intracellular contents, which can overwhelm the body's normal excretory mechanisms, especially in patients with high presenting leukocyte counts. The resultant metabolic disturbances include hyperkalemia, hyperuricemia, hyperphosphatemia, and hypocalcemia.[362,363] Patients with high levels of uric acid are at risk for the development of acute renal failure secondary to uric acid deposition in the kidney. To ensure that uric acid remains in solution in the renal tubule for optimal excretion, intravenous hydration should be instituted immediately after diagnosis to maintain dilute urine. In addition, administration of a xanthine oxidase inhibitor such as allopurinol, which prevents the formation of uric acid during cell lysis, should be started before the institution of antileukemic drugs. Recombinant urate oxidase, which catalyzes the breakdown of uric acid to the more soluble allantoin, may be used instead of allopurinol to decrease levels of uric acid rapidly, especially in patients presenting with high uric acid levels, high presenting leukocyte counts, or impaired renal function or in whom aggressive hydration is contraindicated.[364,365]

Hypercalcemia, although uncommon, has been reported in patients with ALL at diagnosis and appears to be related in many cases to elevated levels of parathyroid hormone–related peptide.[366] Hypercalcemia has also been associated with the t(17,19) translocation (*E2A-HLF* fusion), an uncommon chromosomal abnormality that also leads to disseminated intravascular coagulation.[366]

Infection

Life-threatening infection, observed at the time of diagnosis and during therapy, is one of the most frequent complications experienced by patients with ALL. Infections, most often bacterial and often associated with granulocytopenia, are the primary cause of treatment-related mortality in children receiving therapy for ALL.[256] Documented episodes of bacteremia have been reported in 15% to 30% of children receiving chemotherapy for ALL, and deaths caused by serious infection have been reported in up to 3% of patients.[3,256] Any febrile patient receiving therapy for ALL must be considered potentially bacteremic. Cultures should be obtained promptly, and the patient should be started immediately on broad-spectrum antibiotics with activity against bowel and respiratory organisms while awaiting culture results.[367]

As chemotherapy regimens have become more intensive, fungal disease, including invasive infections of *Candida* and *Aspergillus* species, have also been increasingly observed during the initial treatment of leukemia.[368,369] Viral infections can also cause significant morbidity in children with ALL. Varicella complicated by pneumonitis, hepatitis, or cerebritis can be particularly devastating.[370] Postexposure prophylaxis (within 72 hours) with varicella-zoster immunoglobulin and treatment with acyclovir once symptoms develop have reduced the frequency of severe disease.[371-373] However, even with these interventions, deaths from varicella have been reported in children undergoing therapy for ALL.[374] Rare cases of measles in children with ALL have been accompanied by a high incidence of morbidity and mortality.[375,376]

Pneumocystis jirovecii can cause a severe, often fatal interstitial pneumonitis in children receiving multiagent chemotherapy. The incidence of *P. jirovecii* increases with the degree and duration of immunosuppression and thus is usually observed during the later phases of ALL therapy, but it has been observed in the earlier phases as well.[377] Prophylaxis with trimethoprim-sulfamethoxazole markedly reduces the incidence of infection with *P. jirovecii* and is recommended for all children receiving therapy for ALL.[378]

The use of hematologic growth factors, such as filgrastim (granulocyte colony-stimulating factor), remains a matter of controversy. In prospective randomized studies, the use of filgrastim in children and adults receiving treatment for ALL was associated with a shorter duration of neutropenia and a tighter adherence to the planned treatment schedule.[379-382] However, although some investigators have reported a significantly decreased incidence in severe infections in patients treated with filgrastim during ALL therapy,[381,382] others have not confirmed this finding.[379,380] Also, filgrastim has not been shown to prolong survival or reduce the cost of supportive care for children with ALL.[380]

Typhlitis

A specific syndrome of right lower quadrant pain with rebound tenderness, abdominal distention, vomiting, and sepsis, referred to as typhlitis or necrotizing enterocolitis, can develop as a consequence of intensive chemotherapy, especially during the remission induction phase.[383,384] Findings on plain abdominal radiographs vary, but frequently show a paucity of air in the right lower quadrant.[383] Ultrasonography or computed tomography may reveal a characteristic thickening of the colonic mucosa.[384-386] The pathogenesis of typhlitis is most likely related to mucosal and bowel wall damage secondary to chemotherapy in the setting of prolonged neutropenia. It has rarely been observed before the institution of chemotherapy.[387,388] Management includes bowel rest, intravenous fluids, and broad-spectrum antibiotics. Surgery is usually not indicated, except in the rare case of perforation or uncontrolled bleeding.[389,390]

Acute Neurologic Toxicity

Intrathecal chemotherapy, high doses of systemic methotrexate, and cranial radiation, either alone or in combination, have been associated with acute neurotoxicity, including seizures.[324,391,392] Some of the highest rates of acute neurotoxicity have been observed in clinical trials that have used frequent dosing of intravenous and intrathecal methotrexate as a substitute for cranial radiation. For example, in a randomized trial conducted by the Pediatric Oncology Group in children with lower-risk B-precursor cell ALL, the incidence of acute neurotoxicity was approximately 10% in patients receiving repeated courses of intermediate-dose intravenous methotrexate compared with 4% in those receiving low-dose oral methotrexate.[324]

A transient episode of somnolence, anorexia, lethargy, and fever often can be observed 6 to 8 weeks after cranial radiation.[393,394] This “somnolence syndrome” often is accompanied by electroencephalographic changes, CSF pleocytosis, and fever.[395] Most children recover uneventfully, and it is unclear whether there are any long-term neurologic implications.[395]

Peripheral and autonomic neuropathy secondary to vincristine is frequently seen in children receiving ALL therapy. Signs and symptoms vary in severity and include constipation, absent deep tendon reflexes, distal muscle weakness, gait abnormalities, parasthesias, and cranial nerve palsy.[396] Rarely, postural hypotension, bladder atony, and the syndrome of inappropriate secretion of antidiuretic hormone can occur.[396,397] Vincristine-associated neurotoxicity is usually reversible after therapy is completed, and long-term sequelae are rare.[396,398]

Other Acute Toxicities

Therapy with L-asparaginase is associated with pancreatitis in 5% to 10% of patients, and allergic reaction occurs in up to one third of children.[246,291] L-Asparaginase can also induce coagulopathy by inhibition of protein synthesis of clot-forming and clot-inhibitory proteins, including antithrombin III, plasminogen, and fibrinogen.[399-403] CNS and peripheral thromboses have been observed in 2% to 4% of patients receiving L-asparaginase for ALL.[246,291,404] The use of this agent has also been associated with transient hyperlipidemia.[405] Complications from corticosteroids include hypertension, hyperglycemia, myopathy, mood and behavioral problems, and an increased incidence of bone fractures.[293,295,406-408]

Late Effects of Treatment

With prolonged survival of children with ALL, late effects related to the disease and its treatment have become increasingly evident. The clinical magnitude of late effects is a function of treatment and host-related factors, such as age at diagnosis. Late effects include neurocognitive deficits, short stature, obesity, bony morbidity, cardiac dysfunction, and second malignant neoplasms.

CNS Sequelae

Historically, low and low-average intelligence quotients were frequent findings in survivors of ALL.[409-411] These learning disabilities were related to a slow speed of processing information, distractibility, and difficulty in dealing with complex or conceptually demanding material.[412,413] With contemporary therapy, neurocognitive late effects appear to be less severe and are related to treatment and patient characteristics. For example, younger children (<3 to 5 years old at diagnosis) are more vulnerable than older children[409,411,414] and, in some studies, girls are more vulnerable than boys.[412,415-417] Children with meningeal relapse who receive a second course of CNS treatment are at particularly high risk for significant and progressive intellectual loss.[418-420]

Most long-term neuropsychological effects have been attributed to cranial radiation.[409,410,413,417,421] The most severely impaired long-term survivors are those who received cranial radiation doses (24 to 28 Gy) that are higher than doses used in current treatment regimens (12 to 18 Gy).[422-424] Long-term survivors treated with 18 Gy of radiation (especially those who were 3 years or older at diagnosis) appear to fare relatively well, with less severe neurocognitive sequelae than children who had received higher doses of radiation. In some studies, this group of survivors does not demonstrate any significant cognitive deficits, although subtle effects are observed with detailed neuropsychological testing.[414,425]

The contribution of systemic and intrathecal chemotherapy to the development of neurocognitive sequelae has not been fully elucidated. There is evidence that cognitive deficits are present in long-term survivors treated without cranial radiation,[424-427] but these deficits do not tend to be severe, and overall neurocognitive function is generally within the normal range.[417,428] In most studies of long-term survivors, nonirradiated patients have fewer and less severe impairments than those who received cranial radiation,[411,421] although with current chemotherapy regimens and lower doses of radiation, the differences between the two groups may be subtle.[425] Systemic chemotherapy, including high-dose methotrexate and dexamethasone, may also contribute to neurocognitive late effects.[424,429,430]

Neuropathologic changes in the CNS have been identified in survivors of childhood ALL. Leukoencephalopathy, a relatively rare phenomenon, is characterized by multifocal demyelination visible on radiographic imaging (computed tomography or magnetic resonance imaging).[431] Patients may present with a wide range of neurologic and behavioral problems, including poor school performance, confusion, memory problems, personality changes, and, in its most severe form, progressive dementia and coma.[431] Risk factors for the development of leukoencephalopathy include large doses of cranial radiation (2400 cGy or higher) or high cumulative doses of systemic and intrathecal methotrexate.[378] In one study, the development of leukoencephalopathy was associated with escalating doses of weekly systemic methotrexate beyond 50 mg/m^2 in previously irradiated children.[432] Microcephaly has also been reported as a late effect of CNS treatment and was found to be dependent on the radiation dose.[433] Radiation-induced cavernous hemangiomas and other vasculopathies, sometimes leading to cerebral hemorrhage, have been reported in survivors of childhood ALL.[434,435]

Growth

In several studies, survivors of ALL are shorter than expected for their age.[436-440] Although all survivors of childhood ALL appear to be at increased risk for adult short stature, the greatest impact on final height has been noted in patients treated with 2400-cGy cranial irradiation, with less severe growth failure noted in patients whose CNS treatment included 1800-cGy or no cranial irradiation.[437,440,441] Young age and female gender are associated with more severe growth impairment.[437,439,440] Short stature in survivors of childhood ALL is occasionally associated with growth hormone deficiency,[442-444] suggesting that growth hormone replacement may have a potential therapeutic role in some patients.

Obesity is also prevalent among survivors of childhood ALL and, like short stature, is more frequently observed in girls and in those treated at a young age.[439,445-448] The cause of obesity is unclear; some investigators have reported an increased risk for obesity in survivors who received cranial radiation, especially at higher doses (>20 Gy),[448,449] whereas others have not observed any relationship between body mass index and prior radiation.[439,447,450] Corticosteroids may also play a role in the development of obesity, with increased rates of obesity associated with higher cumulative dosages.[447]

Bony Morbidity

Bony morbidity, including osteopenia, fractures, and osteonecrosis, has been reported to occur in up to 30% of survivors of childhood ALL and is primarily secondary to corticosteroids.[295] Several investigators have demonstrated that children with ALL have reduced bone mineral density during therapy,[451,452] resulting in an increased risk for fractures during and immediately after treatment.[407,453] However, it appears that bone mineral density improves once therapy is completed, although some degree of residual osteopenia may persist.[454-457] The clinical implications, if any, related to persistent osteopenia in long-term survivors have not yet been fully elucidated.

Osteonecrosis, also known as avascular necrosis, is a disabling bony toxicity, frequently involving multiple joints. Osteonecrosis can lead to significant pain and loss of function, sometimes necessitating total joint replacement.[458-460] Symptomatic osteonecrosis has been observed in 2% to 9% of children treated for ALL, with higher rates associated with higher cumulative dosages of corticosteroids.[295,349,461] Osteonecrosis occurs much more frequently in adolescents than in younger children.[294,349,461-463] Female sex has also been identified as a risk factor for the development of this complication.[349,462,463] In some studies, dexamethasone (when used instead of prednisone) has been associated with a higher risk for osteonecrosis.[294,462]

Cardiac Sequelae

Echocardiographic abnormalities, particularly increased afterload and decreased contractility, are well-characterized late effects of anthracycline therapy.[464,465] The mechanism of this toxicity is impairment of myocardial growth. The severity of cardiac dysfunction is correlated with higher cumulative doses of anthracycline and higher dose rates.[464-467] Patients treated at a young age, females, and those with Down syndrome appear to be more vulnerable to anthracycline-associated cardiac toxicity.[466,467] Over the past two decades, as patients have received lower cumulative dosages of anthracyclines, symptomatic congestive heart failure has become increasingly uncommon and now only rarely occurs in long-term survivors of childhood ALL.[465,467] In a randomized study of children with high-risk ALL, the cardioprotectant agent dexrazoxane was shown to reduce the incidence of doxorubicin-associated myocardial injury (as measured by cardiac troponin-T during treatment and echocardiographic measures of ventricular wall thickness and function 5 years after therapy) without affecting relapse risk or EFS.[468,469]

Sexual Development and Fertility

Ovarian and testicular function are relatively unaffected by most current childhood ALL regimens,[438,470,471] with the possible exception of programs that use high cumulative dosages of alkylating agents (e.g., cyclophosphamide).[472,473] Prophylactic gonadal radiation can also impair subsequent ovarian or testicular function,[473] but this treatment is no longer included in most regimens. Thus sexual development is normal and fertility is preserved in the vast majority of survivors of childhood ALL.[474] In a study of 949 female childhood ALL survivors, almost all experienced menarche within the normal range, although prior cranial radiation increased the risk for both early and late menarche.[475] In both genders, normal pubertal growth spurts may be blunted.[438,470] No evidence currently indicates that the progeny of survivors of childhood ALL are at increased risk for congenital abnormalities.[476,477]

Cataracts

Survivors of childhood ALL appear to be at increased risk for the development of posterior subcapsular cataracts.[478-480] In general, these cataracts are small and do not impair vision. Cataract formation is believed to be related to the administration of corticosteroids during ALL treatment,[480] although the incidence of cataracts may be higher in ALL survivors who also received cranial radiation.[479]

Second Malignancies

Long-term survivors of childhood ALL are at increased risk for developing second malignant neoplasms, including AML, brain tumors, non-Hodgkin lymphomas, and carcinomas of the parotid and thyroid glands.[481-485] The overall cumulative incidence of second malignant neoplasms reported in the literature ranges from 1% to 6%, depending on the treatment regimen and length of follow-up.[481-484,486] Compared with age-adjusted expected incidence in the general population, survivors of ALL appear to have a 6- to 14-fold increased risk for developing another cancer.[481-484,486] Risk factors for the development of secondary AML include treatment with epipodophyllotoxins and alkylating agents.[487-489] For example, the cumulative risk for secondary AML was 5% to 6% after treatment with epipodophyllotoxin-containing regimens[487,488] but less than 1% after therapy lacking both epipodophyllotoxins and alkylators.[486,490] Cranial and craniospinal radiation have been associated with an increased risk for secondary solid tumors, primarily malignant gliomas and meningiomas.[481-484] In several large studies, the long-term cumulative incidence rates of secondary brain tumors in previously irradiated patients ranged from 1.0% to 1.6%.[481,486,491] It is unclear whether radiation dose is correlated with risk for secondary tumor, although, in one study, patients who had received 18 Gy of cranial radiation appeared to be at lower risk than those who had received 24 Gy of radiation.[482] The cumulative incidence of malignant glioma appears to plateau 15 to 20 years after diagnosis, although, even with 30 years of follow-up, a plateau in the incidence of meningiomas has not yet been observed.[483]

Other Sequelae

Abnormalities of dental and craniofacial development have been observed after cranial irradiation and appear to be related to the age of the patient at the initiation of therapy, with younger patients more severely affected.[492] Elevations of aminotransferase levels are common while patients are receiving therapy but are not associated with chronic liver disease once therapy is completed, and late-occurring hepatotoxicity is a rare phenomenon.[493,494] Chronic liver changes, including portal hypertension and cirrhosis, have been reported after the use of 6-thioguanine.[283,284,495]

RELAPSED ACUTE LYMPHOBLASTIC LEUKEMIA

Relapse subsequently ocurs in 15% to 20% of children with ALL who achieve remission after initial induction chemotherapy. Several factors have been identified that influence the outcome of patients with relapsed ALL, including duration of initial remission, site of relapse, and immunophenotype.

Duration of initial remission is one of the most important prognostic factors, with significantly worse outcomes observed in patients who relapse early—defined as those occurring while on or within 6 months from completion of initial therapy—than in those with longer initial remissions.[496,497,498] T-lineage immunophenotype and age older than 10 years at initial diagnosis have also been associated with an adverse prognosis after relapse.[496-498]

Site of relapse also has prognostic importance, with superior outcomes observed for patients experiencing isolated extramedullary relapses compared with marrow relapses.[497,498] Some studies have suggested that patients with combined marrow and extramedullary relapses have a better prognosis than those with isolated marrow relapses,[496,498] although this has not been consistently demonstrated.[497]

By using these prognostic factors, investigators have identified subsets of relapsed patients with a relatively favorable prognosis (e.g., patients experiencing a late extramedullary relapse) who may be able to maintain durable second remissions with conventional-dose chemotherapy. Patients with a less favorable prognosis, such as those experiencing an early marrow relapse, are considered candidates for more intensive therapies, such as allogeneic SCT.

Marrow Relapse

Relapses involving the marrow account for the majority of recurrences of childhood ALL. Induction of second complete remission can be expected in 70% to 90% of children with relapsed B-precursor cell ALL but is lower in children with T-cell immunophenotype and those whose relapses occur early (<36 months from initial diagnosis).[496,499-501] After a second complete remission has been attained, subsequent treatment may include conventional-dose or high-dose chemotherapy with SCT.

Chemotherapy

Most trials of conventional-dose chemotherapy have been unsuccessful at producing long-term survival in the majority of patients who experience a marrow relapse. In large published series, overall survival rates for children treated with chemotherapy alone (without allogeneic SCT) have ranged from 25% to 40%.[496,502,503]

Duration of initial remission significantly predicts the outcome of children with a marrow relapse who receive chemotherapy alone as salvage therapy. In almost all reported chemotherapy trials, overall survival after an early relapse is less than 20%.[496,503-505] The most successful chemotherapy trial results have been achieved with patients whose initial complete remissions exceeded 30 to 36 months. With intensive chemotherapy regimens, the probability of EFS for this subset of patients has ranged from 40% to 60%.[496,497,503,506]

MRD levels may also help identify patients with late marrow relapses who might respond favorably to chemotherapy-only regimens after a marrow relapse. Several groups have demonstrated that MRD levels measured at the end of the first month of reinduction therapy predict long-term outcome in patients who have experienced a marrow relapse and are treated with chemotherapy only, with significantly higher survival rates associated with lower levels of MRD.[507,508] Patients with a late marrow relapse with high MRD levels at the end of reinduction fare much less with a chemotherapy-only approach. In a study conducted by the BFM group, allogeneic SCT was associated with an improved outcome (compared with historic controls treated with chemotherapy only) in late relapsing patients with high MRD levels at the end of reinduction.[509]

Allogeneic Stem Cell Transplantation

Allogeneic SCT is an important treatment option for children with relapsed ALL.[510-514] In most published reports, the most common cause of failure after allogeneic SCT is recurrence of leukemia.[512-514] Survival after HLA-matched sibling SCT appears to be better when total-body irradiation is included in the preparatory regimen.[506,515] Therapy-related toxicity, including infections and severe graft-versus-host disease, is also a major cause of death, occurring in 15% to 30% of patients.[510-513,515,516] In recent years, the incidence of treatment-related complications appears to be decreasing with improvements in supportive care.[506]

As has been demonstrated in chemotherapy trials for relapsed ALL, duration of initial remission is an important predictor of outcome after matched-sibling SCT, with improved survival observed in patients with longer initial remissions.[506,510] MRD levels, measured at the end of reinduction therapy and just before transplantation, also have been shown to predict outcome after matched-sibling allogeneic SCT, with poorer survival rates observed for patients with higher levels of pretransplantation MRD.[517-519]

The indications for transplantation in second remission after marrow relapse remain controversial. Several studies have attempted to compare outcomes of patients treated with and without allogeneic SCT; however, there are many potential problems inherent in these analyses, including selection bias in terms of which patients receive a transplant. To address these issues, investigators have used matched-pair analyses and other statistical methods to control for time to transplantation and other important prognostic variables when comparing the two treatment modalities. In most of these reports, HLA-matched sibling SCT has been associated with a lower relapse risk and better survival rates compared with chemotherapy-only salvage regimens for patients with early marrow relapse.[506,510,511,514] However, these analyses have not clearly demonstrated that allogeneic SCT is associated with a survival advantage in patients with a late marrow relapse.[506,511] End-reinduction MRD may help to identify late-relapsing patients who might benefit from allogeneic SCT.[509]

HLA-matched siblings remain the donor of choice for allogeneic SCT for relapsed ALL. However, because over 80% of patients do not have histocompatible sibling donors, most patients needing allogeneic transplants rely on alternative donors. Other potential stem cell sources for transplantation include matched unrelated donors and umbilical cord stem cells. Historically, unrelated donor SCT in children with relapsed ALL was associated with a substantial risk for morbidity, with treatment-related mortality rates as high as 40% to 50% in some reports.[520,521] With improvements in supportive care and more accurate genomic typing of HLA antigens, treatment-related mortality after unrelated donor SCT has decreased significantly, making unrelated donor SCT a more viable treatment option.[522-524] Outcomes after unrelated donor SCT and unrelated cord blood SCT appear to be approaching those observed with HLA-matched sibling SCT.[524-526]

Autologous transplantation has also been investigated as an option for patients in second or subsequent complete remission who do not have a matched related donor. Autologous transplantation has been performed in conjunction with ex vivo purging of residual leukemia in harvested marrow via immunologic or pharmacologic techniques.[527-530] Relapse is the most common cause of treatment failure after autologous SCT, with a lower incidence of toxicity-related deaths than that observed after allogeneic SCT.[516] It does not appear that autologous SCT offers any advantage over chemotherapy[531] and, in general, should not be considered in pediatric patients with marrow relapses.

Isolated Extramedullary Relapses

Isolated extramedullary relapses, occurring either in the CNS or testis, are less frequent than marrow relapses in ALL. Such relapse may not actually be isolated; using sensitive molecular techniques, submicroscopic marrow disease can be demonstrated in most children at the time of an isolated extramedullary relapse.[532] The level of this submicroscopic marrow disease may predict response to postrelapse therapy.[532]

Isolated CNS relapse occurs in fewer than 10% of patients with ALL (see Fig. 50-4). Although CNS remission can be successfully induced in more than 90% of such patients with CNS-directed therapy, most patients treated without intensive systemic therapy will subsequently develop a bone marrow recurrence.[533,534] Thus, in addition to CNS-directed therapy, usually irradiation with intrathecal chemotherapy, patients with isolated CNS relapses are also treated with intensive systemic therapy. Several reports have indicated that intensive chemotherapy regimens in conjunction with cranial or craniospinal irradiation may provide adequate postremission therapy for patients with an isolated CNS relapse, with EFS rates as high as 70%.[535,536,537] From the limited published data, it is unclear that allogeneic SCT is associated with a survival advantage for patients with an isolated CNS relapse. In two published comparisons, outcomes after allogeneic SCT were similar to those achieved with chemotherapy and/or irradiation in children with an isolated CNS relapse.[538,539]

As with marrow relapses, patients experiencing late isolated CNS relapses have a better prognosis than those whose relapses occur earlier.[497,502,536,537] On two consecutive clinical trials conducted by the Pediatric Oncology Group, children with the B-precursor cell phenotype and a relatively late isolated CNS relapse (initial remission duration of at least 18 months) had EFS rates of 77% to 83% when treated with intensive chemotherapy and delayed cranial radiation, whereas patients with earlier relapses fared less well with the same regimen.[536,537] Some studies have indicated that patients whose initial CNS prophylaxis included cranial irradiation may have a worse prognosis after an isolated CNS relapse than previously unirradiated patients.[535]

Isolated testicular relapses are uncommon, occurring in 0.5% to 1% of boys with ALL.[24,246] For boys with isolated testicular relapses, systemic chemotherapy and testicular irradiation have resulted in prolonged second remissions in more than 80% of patients with late-occurring relapses.[540,541] This approach has been less successful in patients whose testicular relapses occurred during or soon after cessation of initial therapy, with long-term EFS ranging from 20% to 43%.[540,542]

Other rare sites of extramedullary relapse include eyes and ovaries. Case reports have indicated successful treatment of these patients with local irradiation and intensive systemic therapy.[543,544]

FUTURE DIRECTIONS

Although risk-adapted therapy has resulted in marked improvement in the outcome of children with ALL over the past few decades, molecular techniques may help identify biologic factors that may supplement or replace the currently applied clinical risk factors to identify those patients at highest risk for treatment failure. For example, using transcriptional profiling, single nucleotide polymorphism microarrays, transcriptional profiling, and other genomic assays, biologically distinctive and prognostically relevant subtypes of ALL have been identified, including the *BCR-ABL*–like and early T-cell precursors (ETP) subsets.[29,99,100] In addition, research focused on germline genetic variation and pharmacogenomics may identify patient-related factors that affect outcome and help lead to more individualized therapy.[226,545]

New drug development is essential to improve outcomes. Newer cytotoxic agents, such as clofarabine and nelarabine, may help improve the outcome of some high-risk patients but, like currently available drugs, are associated with significant side effects.[260,546] Imatinib and other tyrosine kinase inhibitors have dramatically altered the therapeutic approach to patients with Ph-chromosome–positive ALL[96] and are the prototypes of a new type of antileukemic agent that is more specific and potentially less toxic than current chemotherapy. Such agents target molecules or pathways that are important in leukemogenesis or leukemic cell survival, and, like imatinib, potentially could be given with cytotoxic chemotherapy to improve outcomes in high-risk subsets. For example, bortezomib, a proteasome inhibitor, was shown to be synergistic with other chemotherapeutic agents in vitro and so was tested in multiply relapsed patients in the context of a standard reinduction treatment platform, with promising preliminary results.[547] Other potential therapeutic targets under investigation include the mammalian target of rapamycin (mTOR), Janus kinase (JAK) family members in *CRLF2*-overexpressing B-cell ALL, and NOTCH pathway inhibitors in T-cell ALL.[548-550] Additionally, epigenetic modifiers, including proteins that regulate chromatin remodeling, DNA cytosine methylation, and covalent histone modifications, are increasingly recognized as potentially important therapeutic targets in leukemia.[551]

In addition to cytotoxic and targeted therapies, there is increasing focus on the role of immune-based therapies in ALL. Novel approaches that aim to promote autologous T-cell lysis of lymphoblasts include: (1) genetic engineering of autologous T cells to express a chimeric antigen receptor specific for a B-cell surface antigen and (2) use of a bispecific monoclonal antibody that both simultaneously engages T cells and targets B-cell surface antigens.[552,553] Toxin-conjugated monoclonal antibodies directed toward leukemia cell surface antigens are also under investigation.[554]

As more children with ALL become long-term survivors, there is an increasingly important need to address issues of late sequelae and the physical and emotional costs of cure.[555,556] While awaiting the development of more specific antileukemic therapeutic modalities, the impact of currently available primary and salvage therapies must be better understood so that rational therapeutic decisions can be made, with the ultimate goal of improving survival and minimizing toxicity.

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CHAPTER 51

Pediatric Myeloid Leukemia, Myelodysplasia, and Myeloproliferative Disease

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CHAPTER OUTLINE

ACUTE MYELOGENOUS LEUKEMIA
- Epidemiology and Etiology
- Biology
- Molecular Genetics
- Morphology and Cytochemistry
- Immunophenotype
- Cytogenetics
- Clinical Presentation
- Treatment
- Prognosis
- Minimal Residual Disease
- Relapsed or Refractory Disease

ACUTE PROMYELOCYTIC LEUKEMIA
- Pathobiology
- Clinical Presentation and Risk Factors
- Treatment
- Central Nervous System Involvement
- Minimal Residual Disease Monitoring
- Relapsed Disease

DOWN SYNDROME WITH TRANSIENT MYELOPROLIFERATIVE DISORDER AND ACUTE MYELOGENOUS LEUKEMIA
- Down Syndrome with Transient Myeloproliferative Disorder
- Down Syndrome with Acute Myelogenous Leukemia

MYELODYSPLASTIC SYNDROMES
- Diagnosis and Classification
- Pathophysiology
- Inherited and Environmental Predisposing Factors
- Clinical Presentation
- Differential Diagnosis
- Treatment

MYELOPROLIFERATIVE DISORDERS
- Juvenile Myelomonocytic Leukemia
- Chronic Myelogenous Leukemia Caused by the *BCR-ABL* Fusion Gene
- Polycythemia Vera
- Essential Thrombocythemia
- Primary Myelofibrosis
- Mastocytosis

CONCLUSION

ACUTE MYELOGENOUS LEUKEMIA

Acute myelogenous leukemia (AML) comprises a heterogeneous group of disorders characterized by the malignant clonal transformation of a hematopoietic stem or progenitor cell. In recent years, deeper understanding has been gained into the chromosomal changes and specific genetic mutations that precipitate this transformation. AML is a relatively rare disorder in children, with between 500 and 600 newly diagnosed patients in the United States each year. Because of the heterogeneous nature of AML, it has proven difficult to find a treatment strategy that is effective for all patients; thus, overall cure rates for AML have improved only modestly in the past few decades, lagging behind those for acute lymphoblastic leukemia (ALL). Drawing from data from the Medical Research Council (MRC) trials, patients with AML are now risk stratified based on cytogenetic features and emerging data on the prognostic significance of minimal residual disease (MRD) early in therapy. Current ongoing clinical trials are attempting to minimize toxicity for patients with low-risk disease and intensify therapy for patients with high-risk disease. As the molecular genetics behind the biology of AML are better elucidated, there is a pressing need to develop new strategies for targeted therapy to improve outcomes in AML.

Epidemiology and Etiology

According to the National Cancer Institute (NCI) Surveillance Epidemiology and End Results (SEER) data, it was estimated that each year, from 2006 to 2010, 5.1 per 100,000 children younger than 14 years and 4.6 per

100,000 adolescents aged 15 to 19 years were diagnosed with cancer.[1,2] AML is less common than ALL: it comprises about 16% of childhood leukemias in children younger than 15 years but 36% of leukemias in adolescents 15 to 20 years, for a total of 500 to 600 new pediatric AML patients annually in the United States. AML has a bimodal distribution, occurring with higher incidence in children younger than 2 years and then again in adolescents 15 to 20 years old.

In ALL, boys are more commonly affected than girls; the incidence of AML is similar for all pediatric age groups, however, regardless of gender.[1,3] Similarly, although the incidence of childhood ALL is higher in white than in black children, the incidence of AML is comparable for both races across all pediatric age groups. With regard to ethnicity, there is a higher incidence of AML in Hispanic versus non-Hispanic children. Of interest, although the incidence of ALL for children younger than 15 years increased from 1977 to 1995, the incidence of AML did not change significantly over this time period.[3]

For a subset of patients with AML, a series of risk factors predisposing to the development of AML has been identified (Box 51-1) These risk factors fall into two general categories: exposures (environmental or toxic) and genetic predisposition. The best understood of the exposures is prior treatment with chemotherapy and radiation. Alkylating agents (e.g., nitrogen mustard, cyclophosphamide, ifosfamide, chlorambucil, and melphalan) are associated with the development of myelodysplastic syndrome (MDS) and secondary AML, with deletions of chromosomes 5 and 7.[4,5] Treatment with topoisomerase II inhibitors such as the epipodophyllotoxins has been linked to a specific type of AML with chromosome band 11q23 translocations, which produce fusion proteins involving the mixed-lineage leukemia (MLL) gene *(MLL)*.[6-10] Treatment with anthracyclines in adults with breast cancer, or in children for leukemias and sarcomas such as osteosarcoma, is associated with the development of secondary leukemias, including ALL, AML, and chronic myelogenous leukemia (CML).[11,12] Although therapy-related AML generally carries a worse prognosis than de novo AML, there have been reported cases of therapy-related AML harboring the better risk cytogenetic abnormalities: t(8;21), inv(16), t(16;16), and t(15;17), often in conjunction with other cytogenetic abnormalities.[13,14] In the case of therapy-related AML with inv(16) and t(16;16), a rare core-binding factor β *(CBFβ)*–myosin heavy chain 11 *(MYH11)* fusion transcript has been reported, with different breakpoints compared with the usual fusion transcript found in better-risk de novo AML.[14] Similarly, there is a case report of a patient with therapy-related AML with t(8;21), and the breakpoint at 21q22 was outside the *AML1* locus.[15]

Radiation exposure may be acquired from the environment or through medical procedures and is well documented to predispose to leukemia. The increased risk for AML and CML in survivors of nuclear blasts in Japan who were exposed to excessive doses of ionizing radiation has been well documented, and these patients have a higher rate of abnormalities of chromosomes 5 and 7.[16-18] An increased risk for AML and ALL in children exposed to radiation in utero has been described, particularly in the past, when diagnostic radiographs were more common during pregnancy,[19,20] although a retrospective study of children with leukemia in Sweden did not confirm this finding.[21] Nuclear power plant workers have a higher risk for leukemia, although there does not appear to be a higher risk in residents living near such plants.[22-24] Air travel is a source of exposure to cosmic radiation, and studies have shown an increase in malignant melanoma and other skin cancers in flight crew members, an increase in breast cancer in female flight crew members, and an increase in AML in male cockpit crew members who logged more than 5000 flight hours annually.[25,26] Studies designed to measure the potential risk for malignancy from residential or occupational exposure to magnetic fields around high-voltage electrical lines have generated conflicting results, and a causal relationship with AML has not been conclusively established.[27-35]

Box 51-1 Predisposing Factors for the Development of Acute Myelogenous Leukemia or Myelodysplastic Syndromes

PRENATAL EXPOSURES

Alcohol
Pesticides*
Foods naturally high in topoisomerase II inhibitors*
Viral infections

ENVIRONMENTAL EXPOSURES

Ionizing radiation
Chemotherapeutic agents
Alkylating agents
Epipodophyllotoxins
Anthracyclines
Organic solvents (e.g., benzene)
Radon*
Pesticides*
Viral infections*

HEREDITARY CONDITIONS

Down syndrome
Noonan syndrome
Neurofibromatosis
Fanconi anemia
Bloom syndrome
Severe congenital neutropenia (Kostmann syndrome)
Shwachman-Diamond syndrome
Klinefelter syndrome (XXY)*

GERMLINE PREDISPOSITION MUTATIONS

NF1 (for JMML)
RUNX1
CEBPA
GATA2
ANKRD26

ACQUIRED DISORDERS

Aplastic anemia
Paroxysmal nocturnal hemoglobinuria
JMML, Juvenile myelomonocytic leukemia.

*Conflicting or limited data.

There are several other environmental exposures with possible links to AML, some of which have yet to be proven definitively. Benzene exposure, which may occur occupationally or through tobacco smoking, has been shown to increase the risk for AML.[25,26,36,37] Several prenatal exposures have been studied as risk factors for the development of AML, particularly in children younger than 3 years. Maternal alcohol consumption during pregnancy was studied in a Children's Cancer Group (CCG) case-control study and found to have an association with increased rates of childhood AML in a dose-dependent matter, with an odds ratio of 2.64 overall and an odds ratio of 7.62 for classic French-American-British (FAB) classification category M1 (myeloblastic with minimal maturation) and M2 (myeloblastic with maturation) AML; other case-control studies have confirmed this finding.[38,39] Some early studies had suggested an association between maternal tobacco and marijuana use and childhood AML, but more recent analyses have not confirmed this association. A Children's Oncology Group (COG) case-control study looked at maternal consumption of foods such as soy, green and black tea, cocoa, red wine, and certain fruits and vegetables that are naturally high in DNA topoisomerase II inhibitors and found an odds ratio of 1.9 to 3.2 for the development of *MLL*-rearranged AML in the setting of high maternal consumption of these foods.[40] Some studies have suggested an increased risk for childhood AML after high exposure to pesticides, either prenatally or postnatally, up to 3 years of age.[41] There is interest in an association between viral infections and AML, but few data. One case-control study has suggested an increase in childhood AML in offspring of mothers who reactivated Epstein-Barr virus during pregnancy.[42] Parvovirus B19 is associated with pure red cell aplasia, and certain human leukocyte antigen (HLA)-DRB1 alleles seem to be associated with symptomatic infection.[43] One study of 16 leukemia patients suggested an association between parvovirus B19 infection and acute leukemia in 4 of the patients, including 1 patient with AML, all of whom carried these particular HLA-DRB1 alleles, although this association needs further investigation.[44] Several retrospective studies have suggested that breastfeeding may be protective against ALL and AML.[45-47] Risk related to indoor radon exposure is controversial. One French study showed a higher rate of AML in children with high exposure but other studies have showed no association.[48,49] Rarely used now, chloramphenicol use in children was formerly associated with an increased risk for AML.[50]

An increased incidence of AML has been observed for patients with certain hereditary disorders. Children with congenital disorders of myelopoiesis, such as Kostmann syndrome, Shwachman-Diamond syndrome, and Diamond-Blackfan anemia, are predisposed to AML.[51-58] Patients with inherited syndromes associated with chromosome fragility and impaired DNA repair mechanisms, such as Fanconi anemia[59-62] and Bloom syndrome,[63-65] also have an increased risk for development of AML. Neurofibromatosis type 1, which is caused by mutations in the neurofibromin tumor suppressor gene on chromosome 17, is associated with juvenile myelomonocytic leukemia (JMML).[66-68] Patients with certain constitutional chromosomal abnormalities carry a higher risk for AML.[69,70] Down syndrome is one of the most clinically prominent examples in this category. It is estimated that over 10% of infants with Down syndrome exhibit a transient myeloproliferative disease associated with a *GATA1* mutation.[71] Down syndrome patients have a 10 to 20 times higher than average risk for acute leukemia. In Down syndrome patients younger than 4 years, AML, usually acute megakaryoblastic (AMKL), is far more common than ALL: an estimated 1 in 500 Down syndrome patients develop AML.[71,72] In contrast to AMKL in the general pediatric population, which carries an extremely poor prognosis, this disease in Down syndrome patients is extremely sensitive to chemotherapy (see later).[71,72] Although early studies did not suggest a higher risk for AML in patients with Klinefelter syndrome (XXY), more recent data suggest that these patients may have a higher risk for hematologic malignancy, including AML.[73-75] More recently, a number of MDS/AML predisposition genes have been identified, including loss of function alterations affecting the key hematopoietic transcription factor genes *RUNX1, CEBPA*, and most recently *GATA2*.[76-80]

Although the predisposing risk factors and genetic conditions described may provide insight into the causative mechanisms that increase the risk for development of AML, most patients with de novo AML have no known predisposing exposures or conditions. Point mutations and chromosomal deletions and translocations occur at a background rate, even in healthy individuals, during hematopoietic stem and progenitor cell expansion. The extent to which inherited, expressed, single-nucleotide polymorphisms in the general population alter mutational rates during myelopoiesis and increase the risk for AML and other cancers remains an area of intense investigation.

Biology

Clonal Origin of Myeloid Leukemia Cells

Normal myelopoiesis is a complex differentiation program whereby primitive hematopoietic stem cells (HSCs) develop along a multistep pathway into fully differentiated, functionally active circulating blood cells.[81] This exquisitely controlled process is regulated by the intricate interactions among the expression levels of various transcription factors, growth factors and their receptors, cytokines, enzymes, and still unidentified novel molecules. A series of sequential genetic abnormalities perturbs this normal developmental progression and leads to AML. Although the relationships between specific morphologic subtypes of AML and their specific recurring genetic abnormalities have provided some insight into the mechanisms of leukemogenesis, understanding of the process whereby these fusion gene products interact with normal signal transduction pathways to subvert hematopoietic cell development is incomplete.[82]

Several strong lines of evidence support the hypothesis that AML progresses from a single transformed hematopoietic stem or progenitor cell. More than 30 years ago, studies were pioneered by Beutler and colleagues[83] and Fialkow[84] using X inactivation patterns in female patients to establish the clonal origin of human malignancies, including leukemia. By showing the presence of a single glucose-6-phosphate dehydrogenase (G6PD) isoenzyme in the leukemic myeloblasts of heterozygous females, the clonal origin of myeloid leukemia cells was demonstrated.[85-87] Females who are heterozygous at the G6PD locus express two isoforms of the enzyme. Approximately half of the cells in normal somatic tissue have randomly inactivated one of the X chromosomes, and approximately half of the cells should therefore express each isoform. Unlike normal somatic cells, AML cells of female patients heterozygous for G6PD expressed only one G6PD isoform, indicating cells of clonal origin.[86,87] In the 1980s, Vogelstein and coworkers[88,89] developed a strategy using X chromosome–linked DNA restriction length fragment polymorphisms to determine the clonal origin of human tumors. They established that maturing granulocytic cells arise from the malignant clone in patients with AML.[90] Later, X chromosome inactivation to determine clonality in malignancies was carried out using the polymerase chain reaction (PCR) assay to distinguish X-linked polymorphic genes.[91-93]

Transformation of Stem Cells with Self-Renewal Capacity

These earlier studies, and those performed by Bonnet and Dick and associates in the 1990s,[94,95] led to the insight that leukemia cell populations form a hierarchy in which leukemia stem cells or leukemia-initiating cells (LICs) are responsible for their own self-renewal and for the generation of the more differentiated progeny within the leukemic clone. This theory was in contrast to the stochastic model, which stated that malignant properties within cancer cell populations follow Gaussian distributions, resulting in the random or stochastic probability that individual cancer cells within the population are able to initiate malignant progression. Whereas AML was the first human cancer in which cancer stem cells were identified, the case has now been made in breast cancer,[96] brain tumors,[97-99] and colon cancer,[100] among others. In general, AML stem cells are believed to arise through the transformation of HSCs, which then retain the capacity for self-renewal, or through transformation of more differentiated hematopoietic progenitor cells that acquire the ability to self-renew by virtue of the particular transforming mutations, such as the formation of an *MLL* fusion gene.

AML is a heterogeneous disease in its clinical manifestations, response to therapy, and molecular genetics; thus insight into the cell of origin would have important ramifications regarding diagnosis and treatment. Moreover, refractory disease and relapse are often attributed to the presence of an LIC population that is resistant to chemotherapy; thus the ability to identify and target this subpopulation may be key to improving overall disease outcome.[101] In the 1990s, Dick's group produced a series of convincing papers[94,95,102] that strongly implicated the primitive, pluripotent HSC as the LIC in some types of AML. "Stemness," or the capacity to function as an LIC, includes self-renewal, proliferation, and differentiation and is tested in vitro by replating limiting dilution studies and in vivo by serial transplantation assays with the ability to regenerate a clonal leukemia in secondary and subsequent recipient animals. The HSC, with its inherent gene programs ensuring self-renewal, proliferation, and survival appears to be an ideal target to become sabotaged through a series of genetic events, leading ultimately to a fully transformed AML LIC that is capable of producing a clonal population of leukemia cells. According to this argument, different AML phenotypes often occur as a result of the gene(s) that are altered, leading to differentiation arrest within the progeny of the LIC, but not necessarily reflecting the degree of the commitment of the initially transformed cell. Support for this hypothesis initially came from several clonality studies in leukemic cells from patients with AML,[103-105] which demonstrated multiple-lineage involvement in a high proportion of cases, and implicated a multipotent HSC as the cell of origin.[104] More supportive evidence has come from studies looking at cytogenetic markers and characteristic cell surface antigen expression patterns.[106] Pluripotent HSCs express CD34 but do not express CD38 or HLA-DR, whereas more committed myeloid progenitor cells are CD34+, CD38+, and HLA-DR+.[81,106] Using fluorescence-activated cell sorting (FACS) of leukemic cell populations, the same two subpopulations, CD34+/CD38– and CD34+/CD38+, were isolated from the bone marrow of patients with different AML subtypes. The two subpopulations were then evaluated using fluorescence in situ hybridization (FISH) to determine the presence of cytogenetic abnormalities. The studies detected the same characteristic cytogenetic abnormalities in the CD34+/CD38– stem cell fraction as in the original leukemic bone marrow samples, implying origin in a very early HSC compartment.[107,108]

Further evidence supporting the stem cell origin model of AML was derived from transplantation experiments in which purified human AML cells were transplanted into mice with severe combined immunodeficiency (SCID). These experiments defined a SCID mouse leukemia–initiating cell (SL-IC) in the bone marrow of patients with AML and showed that these SL-ICs are CD34+/CD38– and that their engraftment produces large numbers of colony-forming progenitors. The CD34+/CD38+ and CD34– fractions did not engraft.[95] Similar results were obtained through transplantation studies in a modified SCID mouse, the nonobese diabetic mouse with SCID (NOD/SCID), with cells from patients with AML.[94] These studies identified and analyzed AML stem cells from samples of different FAB subtypes on the basis of their ability to initiate human AML after transplantation in NOD/SCID mice. These SL-ICs were able to proliferate and differentiate after transplantation, producing disease in the mice identical to that in the donor, as well as being able to renew themselves, reestablishing AML in secondary recipients, demonstrating that SL-ICs are capable of self-renewal. Again, the SL-ICs were found to reside in the CD34+/CD38– fraction and not in the CD34+/CD38+ or CD34– fractions. The SL-IC phenotype was consistent regardless of the FAB subtype (e.g., M1, M2, M4, M5). As few as $2 \cdot 10^4$ CD34+ cells were able to initiate the leukemic clone in recipient mice, whereas 100 times as many CD34– cells failed to engraft. Cells with the CD34 surface antigen were further fractionated on the basis of

CD38 expression. Only the CD34+/CD38− fraction contained SL-ICs.[94]

Whereas the transformation of the HSC provides an attractive mechanism to generate hierarchical myeloid leukemia cell populations, several studies have given new credence to a second mechanism through which committed progenitor cells along the pathway of myeloid differentiation are vulnerable to transforming mutations that confer self-renewal properties to progenitor cells that inherently lack this ability.[109-111] These transformation events create an LIC capable of generating a population of aberrant cells blocked at the differentiation state at which the initial transforming event occurred. Several translocations involving the *MLL* gene have been shown to initiate self-renewal in myeloid progenitor cells and to result in an AML phenotype.[110,112] A head-to-head comparison was conducted by retrovirally transducing the human AML-associated *MLL-ENL* fusion gene into murine HSC, common myeloid progenitors (CMPs), and granulocytic-monocytic–restricted progenitors (GMPs) and evaluating the relative transforming ability and resultant phenotype in each cell type. AML developed in mice transplanted with any of the three transduced cell populations and, in each case, displayed an identical myelomonocytic phenotype as assessed by morphology and flow cytometry.[110] Similarly, the *MOZ-TIF2* fusion gene was able to confer self-renewal properties to committed myeloid progenitor cells and resulted in AML when transplanted into irradiated mice.[111] Although the fusion genes *MLL-ENL* and *MOZ-TIF2* appear to impart leukemogenic capabilities to committed progenitor cells convincingly, other fusion genes such as *BCR-ABL* can induce leukemic transformation only when expressed in an HSC but not a more mature myeloid cell. Similarly, the potent oncogenic combination of *Hoxa9* and *Meis1* only resulted in AML when transduced into HSCs but not in more committed myeloid progenitors. In this case, expression of β-catenin was found to be the necessary factor for oncogenesis. Present in HSCs but absent in more committed progenitors, the addition of β-catenin to progenitors transduced with *Hoxa9* and *Meis1* was sufficient to lead to AML.[113] These findings point to the differing transforming abilities of different oncogenes that are likely cell-type and cell-context specific.[114] Hence both cell-of-origin hypotheses appear to be correct, depending on the specific oncogene or tumor suppressor that provides the initiating genetic lesion. Increased proliferation, survival, and self-renewal are all necessary attributes of a leukemia stem cell. Some genetic abnormalities, such as *MLL-ENL* and *MOZ-TIF2*, may be capable of inducing self-renewal and thus are able to transform a more differentiated myeloid cell into an LIC. By contrast, other oncogene fusions, such as *BCR-ABL*, or oncogenic transcription factor genes, such as *Hoxa9*, do not possess self-renewal capabilities and thus require a cell that inherently has this characteristic, namely, the HSC.

More recently, challenges to the cancer stem cell hypothesis have arisen out of a number of studies, predominantly in solid tumors such as melanoma, where the frequency of cells capable of giving rise to a new tumor in an immunocompromised host is of sufficient frequency to call into question whether the LIC represents a unique subpopulation or the original stochastic model of cancer progression holds true.[115] These studies have again called into question previously accepted tenets. CD34+/CD38+ populations, in addition to the CD34+/CD38−, have also been found to contain LICs when injected into more immune-deficient hosts that lack key components of the innate immune system.[116] These studies and additional syngeneic and cogenic transplant studies in mice as well as zebrafish[117-120] highlight the critical context of the recipient animal in determining the LIC. The heterogeneity of LICs in AML is further demonstrated by an increasing number of cell surface markers in addition to CD34 and CD38, including CD123, TIM3, and CD47.[121-123] Additionally, a number of studies have attempted to identify a specific LIC genetic signature, which has been found at least in some cases to independently predict prognosis. In this case, the LIC signature was reminiscent of the normal HSC, hearkening back to the original predictions of Dick's group highlighting the HSC as a putative LIC cell of origin.[124] However, although these findings are provocative, a consistent cadre of LIC genes has not yet been established. Clonality studies demonstrating the progressive acquisition of mutations in a given cell have also been put forward as an alternative to the cancer stem cell hypothesis.[125,126] Although seemingly at odds, these different concepts can be reconciled into a single integrated framework. Myeloid (and lymphoid) leukemia-initiating events may occur in both HSCs and committed progenitor cells, and each leukemia arises from a combination of multiple genetic abnormalities.[127] Secondary events subsequently are acquired in different subpopulations, generating unique clones with varying malignant potential depending on the genetic lesion and the self-renewal capacity of the cell in which these abnormalities occur. The number of genetic lesions necessary to cause frank AML is being defined based on next-generation sequencing studies recently published by the group at Washington University in St. Louis as well as other centers. The evolution of particular clones appears to determine the eventual phenotype of the AML cells documented at diagnosis (Fig. 51-1).[128,129]

These new discoveries have added additional complexity to our understanding of the contribution of LICs to AML pathogenesis by demonstrating that the heterogeneity inherent in this disease extends to the AML stem cells.[130] However, the identification of new LIC surface markers, genetic signatures, and gene-specific mutations hold out promise that LIC-targeted therapies will be realized in the coming years.

Molecular Genetics

Recent AML protocols have incorporated current knowledge regarding the significance of specific genetic abnormalities to direct risk-stratified approaches to treatment. Chromosomal abnormalities and, in particular, translocations identified on cytogenetic examination of the blast cells represent the initial genetic lesions incorporated into disease taxonomy and formed the basis of the World Health Organization (WHO) classification in 2008.[131-133] Careful analysis of these translocations and other recurring genetic abnormalities in leukemia patients has had a

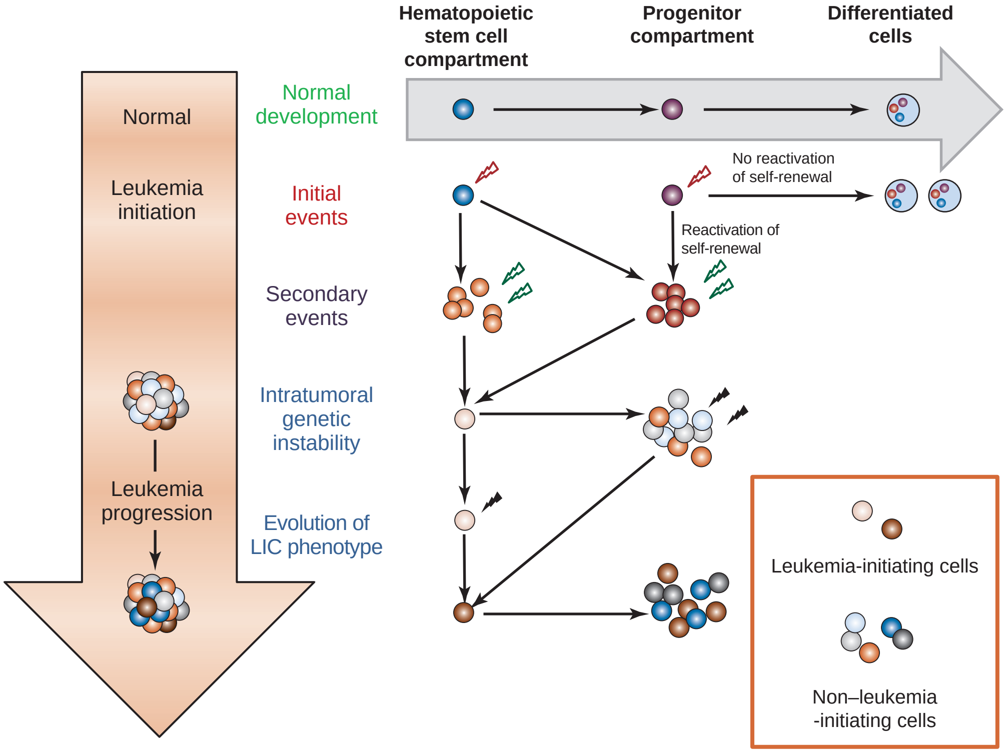
Hematopoietic stem cell compartment
Progenitor compartment
Differentiated cells
Normal
Leukemia initiation
Leukemia progression
Normal development
Initial events
Secondary events
Intratumoral genetic instability
Evolution of LIC phenotype
No reactivation of self-renewal
Reactivation of self-renewal
Leukemia-initiating cells
Non–leukemia -initiating cells

Figure 51-1 Integration of the leukemic initiating cell (LIC) and clonal evolution models of acute myelogenous leukemia pathogenesis. *(Modified from Dick JE: Stem cell concepts renew cancer research.* Blood *112:4793–4807, 2008.)*

profound impact on our understanding of the molecular genetic basis of leukemia (Fig. 51-2). Although many chromosomal abnormalities from AML blast cells have been identified and studied extensively, no single mutation has been shown to be sufficient to cause acute leukemia. These findings prompted Gilliland and colleagues[134-137] to propose what is now considered the classic "two-hit model" of AML pathogenesis. In this model, leukemic transformation results from distinct but collaborative sequential mutations in parallel molecular pathways that affect cell survival, proliferation, differentiation, and self-renewal. Class I mutations frequently involve an activated receptor or cytoplasmic-nuclear tyrosine kinase conferring a proliferative and/or survival ability to a specific cell but not affecting differentiation. By contrast, class II mutations specifically result in differentiation arrest and/or self-renewal and often involve key transcription factor oncogenes but alone do not confer a proliferative advantage. Although attractive, this model likely represents an oversimplification of AML pathogenesis. Initially, microarray studies and more recently, deep-sequencing approaches have revealed the presence of novel mutations and absence of tyrosine kinase lesions, particularly in normal karyotype AML, calling into question the general applicability of the "two-hit" model in all cases of AML. However, although the category of genetic lesions may not always subscribe to this classic paradigm, the number of "hits" required may be more universal. Genomic profiling of individual patient leukemias suggest that in some cases only two to three lesions are required for clonal evolution to frank AML, with fewer "hits" necessary in pediatric AML than in its adult counterpart.[129,138] Regardless, the prevalence of lesions categorized by the "two-hit" model will be outlined here, followed by more recent discoveries of novel genes implicated in AML pathogenesis and the variable frequency of these lesions in pediatric versus adult AML.

Class I Mutations

Among the earliest examples of class I mutations, *RAS* mutations were described in the early 1980s. There are three functional *RAS* gene family members—*NRAS, KRAS,* and *HRAS*—that all act as guanosine nucleotide phosphate (GTP)-binding proteins.[136] Mutations of *NRAS* are the most prominent *RAS* mutations in AML and have been found in as many as 30% of adult patients with AML.[139] Mutations, most frequently in codons 12, 13, and 61, result in the retention of the RAS protein in its active GTP-bound state and lead to the constitutive activation of downstream effector proteins, which causes the transcriptional activation of various target genes that direct cellular differentiation, proliferation, and survival (Fig. 51-3).[140] RAS proteins may be alternatively activated by mutations in genes encoding a number of regulatory factors. Activating point mutations in the *SHP-2/PTPN11* phosphatase provide a stimulatory signal through guanine nucleotide exchange factors, such as "Son of Sevenless" (SOS), resulting in increased RAS pathway signaling, as do inactivating mutations of neurofibromin-1 *(NF1)*, which codes for a GTPase-activating protein (GAP) of the same name.[141,142] NF1, normally functions, at least in part, by increasing the

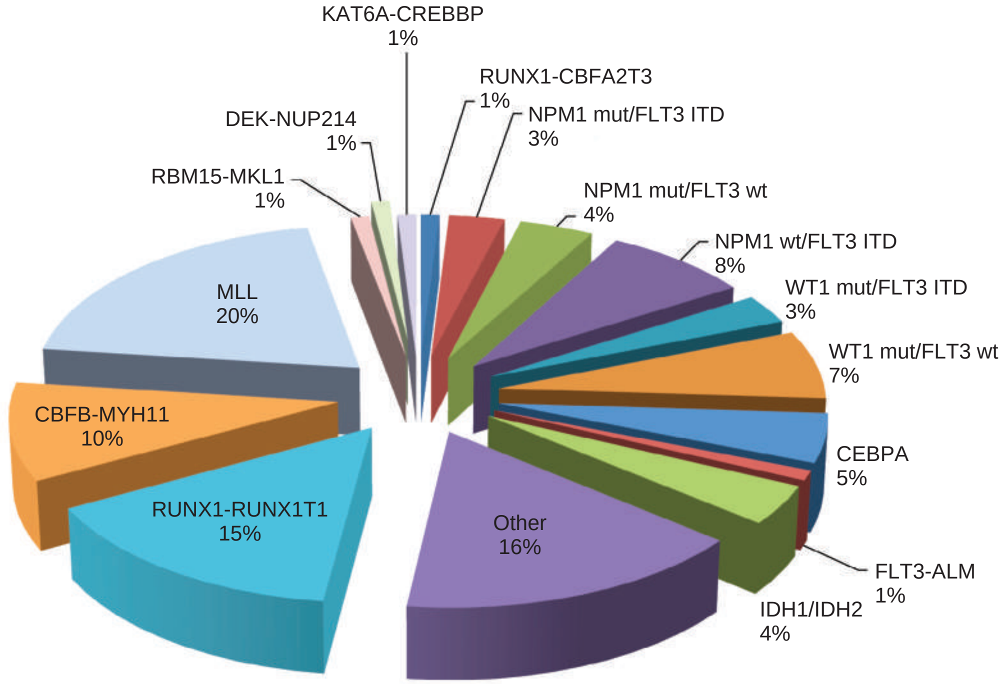

Figure 51-2 Approximate frequencies of recurrent genetic lesions in childhood acute myelogenous leukemia. *(Modified from Rubnitz J, Inaba H: Childhood acute myeloid leukemia.* Br J Haematol *159:259–287, 2012.)*

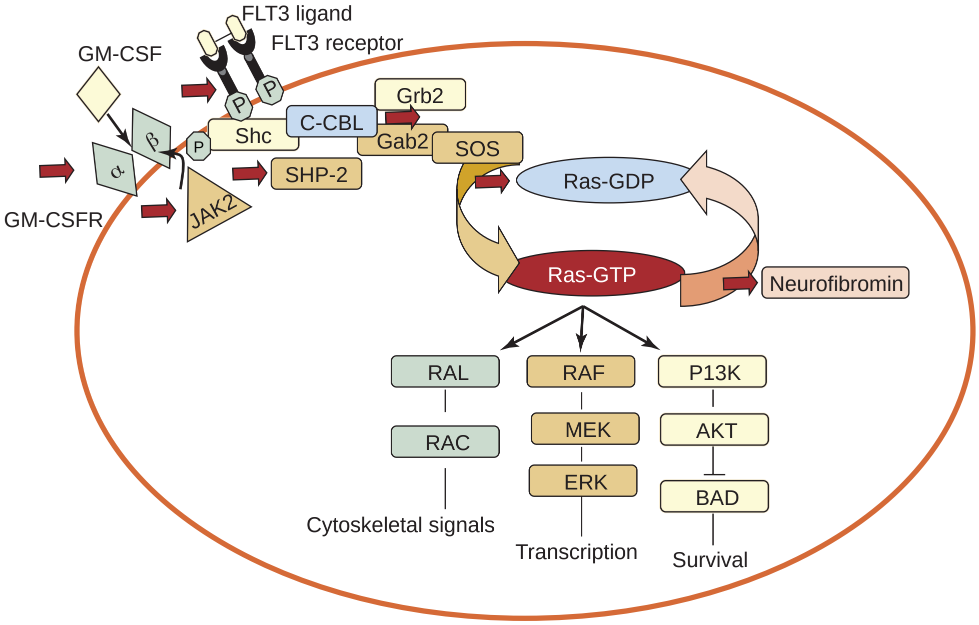
FLT3 ligand
FLT3 receptor
GM-CSF
P
P
Grb2
Shc
C-CBL
Gab2
SOS
β
P
α
SHP-2
Ras-GDP
GM-CSFR
JAK2
Ras-GTP
Neurofibromin
RAL
RAF
P13K
MEK
AKT
RAC
ERK
BAD
Cytoskeletal signals
Transcription
Survival

Figure 51-3 Constitutive activation of the RAS pathway is central to myeloid leukemogenesis. RAS pathway signaling may be constitutively activated via a number of mutually exclusive mechanisms *(horizontal red arrows)*. These include the following: mutations in the GM-CSF or FLT3 surface receptors, resulting in ligand independent activation (found primarily in de novo AML); activating mutations in JAK2 (found in PV, ET, and PM) or SHP-2, c-CBL (found in JMML); activating mutations in RAS itself (found in all types of AML); or inactivating mutations in neurofibromin (found in JMML). *PV,* polycythemia vera; *ET,* essential thrombocytosis; *PM,* primary myelofibrosis; *FLT3,* fms-like tyrosine kinase 3; *GM-CSF,* granulocyte-macrophage colony-stimulating factor; *JAK2,* Janus 2 kinase; *JMML,* juvenile myelomonocytic leukemia. *(Modified from Loh ML, Vattikuti S, Schubbert S, et al: Mutations in* PTPN11 *implicate the SHP-2 phosphatase in leukemogenesis.* Blood *103:2325–2331, 2004; and Proytcheva M: Juvenile myelomonocytic leukemia.* Semin Diagn Pathol *28:298–303, 2011.)*

GTPase activity of RAS, thereby inactivating RAS-GTP by converting it into the inactive RAS-GDP form.[143] These three types of mutations function independently to activate RAS signaling and lead to disturbances in hematopoietic cell differentiation and proliferation that may ultimately contribute to the development of MDS, myeloproliferative disease (MPD), and AML (see Fig. 51-3).[144-146] Mutations in *NF1* and *PTPN11* have been particularly associated with JMML (see later).[142,147]

There has been significant disagreement regarding the prognostic implications of *RAS* mutations in MDS and AML. Several studies have demonstrated that *RAS* mutations in patients with MDS confer a poor prognosis and increase the risk for progression to acute leukemia.[148-155] Other studies have reported no difference in survival in AML patients who carried *RAS* mutations compared with patients without *RAS* mutations,[152] whereas some have suggested improved survival in patients with AML and *NRAS* mutations.[153] A large study of more than 2500 patients with AML demonstrated no difference in prognosis in patients with or without *RAS* mutations. This study also demonstrated an association between *RAS* mutations and a leukemia karyotype containing inv(16), a class II mutation resulting in the CBFβ-MYH11 fusion protein and altered function of the core-binding factor (CBF) transcriptional complex.[156] A recent report from the COG examining 825 pediatric AML samples treated on two recent trials identified *NRAS* mutations in 10% of patients predominantly in codons 12 and 13, with no mutations in codon 61. Interestingly, most of these mutations were associated with good prognosis *NPM1* mutations (see later) and not CBF alterations. However, multivariate analysis confirmed a lack of independent impact of *NRAS* mutations on prognosis.[157]

Another group of class I mutations involves constitutive activation of receptor or cytoplasmic-nuclear tyrosine kinases. The *BCR-ABL* translocation that is the sine qua non of CML is an example of a class I mutation causing the activation of the ABL tyrosine kinase. Similarly, receptor tyrosine kinase mutations activate the human protooncogenes *KIT* and *FLT3* in AML. The KIT protein is activated by the binding of its ligand stem cell factor and is critical for the development and growth of mast cells, melanocytes, HSCs, and the interstitial cells of Cajal.[158] Mutations in *KIT* allow ligand-independent activation of KIT and confer factor-free growth and tumorigenicity in hematopoietic cell lines.[159,160] Blasts from patients with AML express KIT on their cell surfaces in most cases.[161-163] Deletional and insertional mutations of *KIT* have been identified in the blast cells of patients with AML, often in association with the CBF abnormalities inv(16) or t(8;21).[164]

The *FLT3* gene encodes a class III fms-like receptor tyrosine kinase expressed in early hematopoietic progenitors. *FLT3* mutations occur in two varieties, internal tandem duplications (ITDs) in the juxtamembrane region and activation loop mutations (ALMs), primarily at codons 835 and 836. The *FLT3*-ITD is one of the most frequent abnormalities in adult AML, documented in 25% to 30% of patients,[165-169] and generally confers a poor prognosis.[170,171] By contrast, ALMs occur in only 10% of adult patients and have not been associated with inferior survival rates. *FLT3*-ITDs have been observed in 5% to 16% of pediatric AML cases and ALMs in 2% to 5% of cases. Paralleling the adult studies, *FLT3*-ITDs, but not ALMs, were associated with a poor prognosis in children.[172-175] *FLT3*-ITDs result in the constitutive activation of FLT3 and cause interleukin-3–independent growth in Ba/F3 and 32D cells.[165,168,176] This activation, in turn, results in the signaling of multiple downstream pathways, including the aforementioned RAS pathway, and the inhibition of caspase-mediated apoptosis through the stimulation of phosphatidylinositol 3′-kinase (PI3K) and AKT (see Fig. 51-3). However, mice transplanted with FLT3-transformed hematopoietic cells develop a myeloproliferative disorder characterized by splenomegaly and leukocytosis but they do not develop AML.[177] *FLT3*-ITDs often occur in conjunction with other mutations, including *NPM1* (see later) and *WT1* (see later).[178]

Class II Mutations

In class II mutations, the molecular defect appears to be at the level of transcriptional activation, whereby transcriptional repressors cause a dominant negative inhibition of normal hematopoietic cell differentiation. AML can be subtyped based on the type of class II mutations in the blast cells of patients. The type 1 subset of class II mutations occurs in patients with de novo AML. These are typical chromosomal translocations resulting in chimeric oncoproteins that in some cases may cause the inhibition of differentiation. The type 2 subset of class II mutations usually are found in patients with AML arising after a prodrome of MDS and manifest more commonly in older adults or in patients who have received previous chemotherapy. Cytogenetic studies of the blast cell populations in these patients have complex karyotypes, lacking translocations but harboring deletions such as 5q–, monosomy 7, and 20q–.

Type 1 Mutations

Translocations Involving Core-Binding Factor Complex. Several of the translocations that have been identified in adult and childhood de novo AML involve the CBF complex. The CBF complex is the most frequent target of chromosomal translocations in the human leukemias. The CBF regulatory complex consists of a DNA-binding subunit, RUNX1 (also called CBFα or AML1) and CBFβ, a subunit that does not bind DNA independently but heterodimerizes with RUNX1 or one of its closely related family members.[179] Chromosomal translocations that modify the CBF complex in AML include the following: t(8;21)(q22;q22), which generates the AML1-ETO fusion protein, more recently referred to as RUNX1-RUNX1T1 (Fig. 51-4); t(3;21)(q26;q22), which gives rise to AML1-EVI1; and inv(16)(p13;q22), which fuses the CBFβ to smooth muscle myosin heavy chain (SMMHC), resulting in the *CBFB-MYH11* fusion gene. The *AML1* gene was inactivated in the germline of mice and was shown to be essential for definitive hematopoiesis of all cell lineages.[180,181] Homozygous animals die early in embryogenesis of central nervous system (CNS) hemorrhage, but they have normal morphogenesis and yolk sac hematopoiesis lineages.[180,181] Inactivation of the *CBFβ* gene in the mouse has shown a similar phenotype in the homozygous null mice.[179,182] These experiments have demonstrated that the AML1/CBFα complex is essential for normal hematopoiesis and that chromosomal rearrangements involving this complex may interfere with its regulatory function in ways that lead to disruption of cellular differentiation, and eventually malignant transformation.

Evidence primarily from knock-in studies in mice has supported the hypothesis that the AML1-ETO fusion protein functions as a dominant negative inhibitor of wild-type functions.[183-185] In these experiments, the *AML1-ETO* fusion gene is “knocked in” to the wild-type

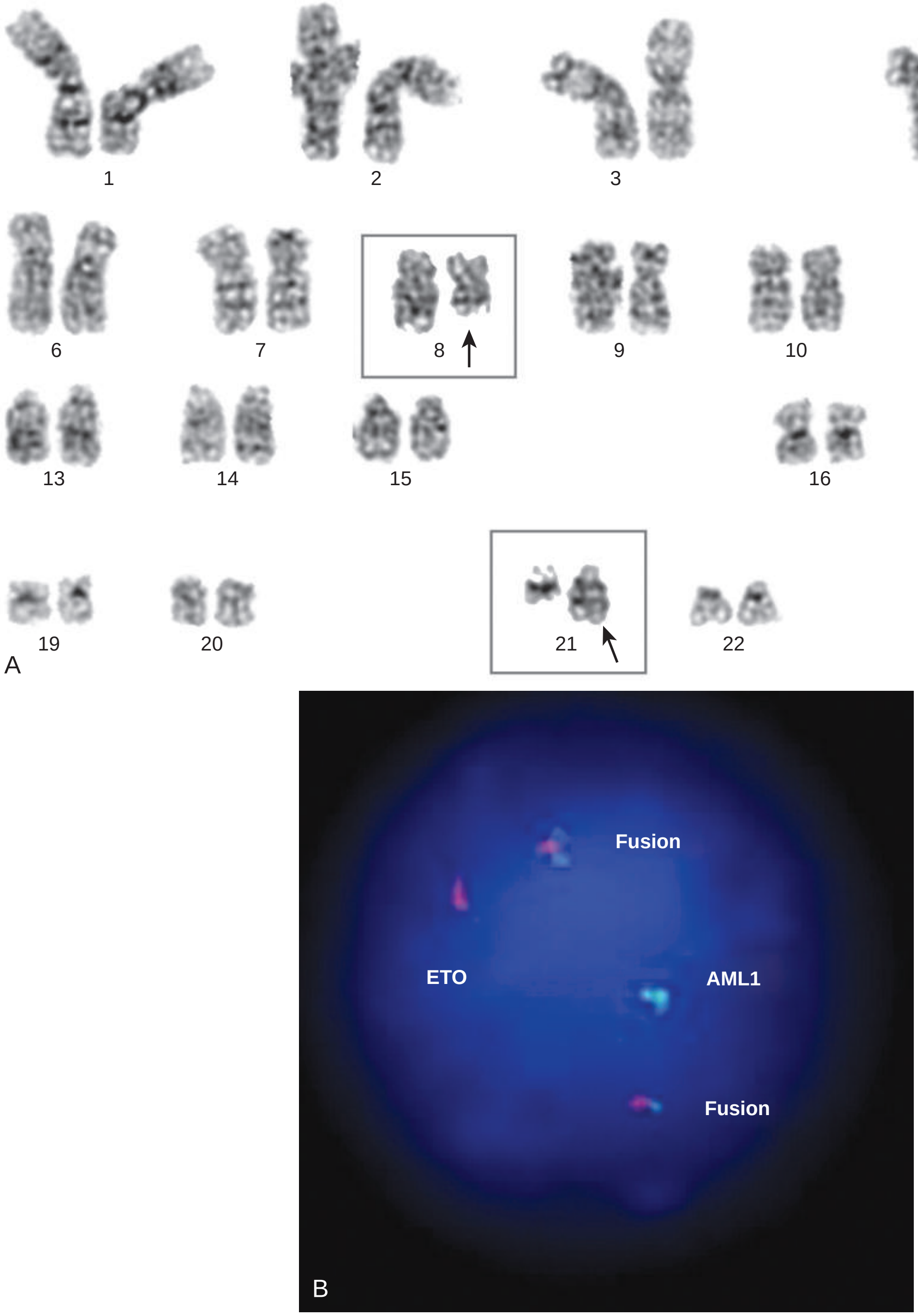

Figure 51-4 Consequences of the t(8;21) AML1-ETO (RUNX1-RUNX1T1) translocation. **A,** Karyotype demonstrating the reciprocal t(8;21)(q22;q22) translocation in an affected patient. **B,** Interphase fluorescence in situ hybridization (FISH) using the AML1/ETO dual-color, dual-fusion Vysis probe demonstrates the novel AML1-ETO fusion by the presence of linked green AML1 and red ETO signals.

Continued

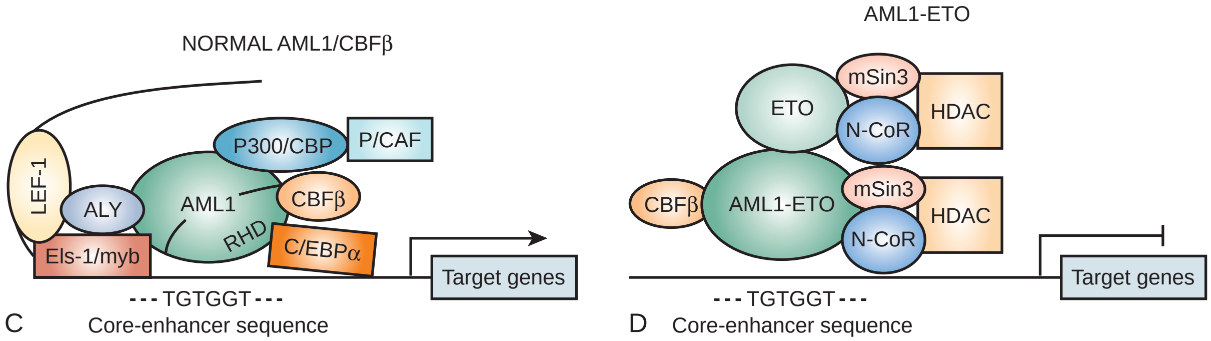

Figure 51-4, cont'd **C,** The normal AML1-CBFβ transcription factor complex. **D,** The molecular consequences of the AML1-ETO fusion; namely, transcription repression may occur in contexts in which the normal AML1/RUNX1-CBFβ acts as to transactivate gene expression. *(Courtesy of Dr. Barbara Morash, Cytogenetics, IWK Health Centre, Halifax, Nova Scotia, Canada;* **D** *modified from Downing JR: The core-binding factor leukemias: lessons learned from murine models.* Curr Opin Genet Dev *13:48–54, 2003.)*

AML1 locus. The phenotype of these mice is identical to the *AML1* knockout; the mice die early in embryogenesis from CNS hemorrhage and lack any evidence of definitive hematopoiesis, although the rare cells that survive and express AML1-ETO have an increased capacity for self-renewal.[185] Biochemical experiments have shown that the ETO portion of the AML1-ETO chimeric protein recruits nuclear corepressor complexes to the CBF promoters, resulting in transcriptional inhibition of target genes normally activated by the AML1/CBFβ heterodimeric complex (see Fig. 51-4D).[186,187] Subsequent studies using inducible murine model systems to bypass the embryonic lethality of *AML1-ETO* overexpression have demonstrated that overexpression of this transgene at later developmental time points confers enhanced self-renewal capacity to bone marrow progenitors, as demonstrated by serial culture experiments. Importantly, the mice do not develop AML but appear to be predisposed to leukemogenesis. Thus after treatment with *N*-ethyl-*N*-nitrosourea (ENU), the mice developed rapid onset of AML compared with similarly exposed wild-type mice. Moreover, as noted, there is an association in myeloid leukemia of *AML1-ETO* translocations and mutant tyrosine kinases, such as KIT.[188] Interestingly, *AML1-ETO* transcripts may persist for a long time in patients with *AML1-ETO*–expressing AML in sustained remission.[189]

Translocations Involving the Retinoic Acid Receptor-α. Reciprocal chromosomal translocations involving the retinoic acid receptor-α *(RARA)* gene locus on chromosome 17 are the defining molecular features of acute promyelocytic leukemia (APL). Before the *RARA* translocations were described, retinoic acid was known to induce myeloid differentiation in vitro[190] and therapy with all-*trans*-retinoic acid (ATRA) in patients with APL was shown to induce complete remission (CR).[191] The intense investigation of the *RARA* gene triggered by these observations culminated in identification of the promyelocytic leukemia PML-RARA translocation breakpoint, t(15;17)(q22;q11), reported simultaneously by several groups.[192-197] Although most APL cases have the associated t(15;17), other reciprocal translocations involving the *RARA* gene have been identified in which the gene is fused to other gene partners. The most common of these include the promyelocytic leukemia zinc finger *(PLZF)* gene on chromosome 11 in t(11;17)(p13;q11) and the nucleophosmin gene *(NPM)* on chromosome 5 in t(5;17)(q31;q11).[198,199]

Normally RARA functions as a ligand-dependent activator of transcription.[200] It forms a heterodimer with the related protein, retinoid X receptor (RXR),[201] and binds through its zinc finger domain to a specific promoter sequence; in the presence of the retinoic acid ligand, the RARA-RXR heterodimer activates transcription of retinoic acid–responsive genes.[194] This process is accomplished in concert with a coactivator complex that includes proteins such as p300 and pCAF. In the absence of retinoic acid, a corepressor complex is recruited, composed of N-CoR (nuclear receptor corepressor) or SMRT (silencing mediator of retinoid and thyroid receptors), which binds to another protein called Sin3 and then to HDAC1.[199,202-204] HDAC1 is a histone deacetylase protein, which epigenetically alters histones to keep DNA in an untranscribable form. The expression of retinoic acid–responsive genes is essential for normal myeloid development.[205]

Much evidence supports the hypothesis that the PML-RARA fusion protein functions as a dominant negative inhibitor of the PML protein and RXR. PML proteins are normally located in macromolecular nuclear organelles, called PML oncogenic domains (PODs).[206] The PML-RARA fusion protein disrupts the PODs, causing normal PML, RXR, and other nuclear proteins to disperse in an abnormal pattern.[207,208] ATRA and arsenic trioxide (As_2O_3)[209] have shown activity in APL blast cells and in patients with APL, and studies of these drugs have afforded important insights into the mechanisms of leukemogenesis in APL.[191,210-212] ATRA and As_2O_3 appear to act through different biochemical pathways, and promyelocytes resistant to ATRA are often sensitive to treatment with As_2O_3. Both drugs induce degradation of the PML-RARA fusion protein but through different mechanisms. Retinoic acid binding to wild-type retinoic acid receptors (RARs) in the cell nucleus causes degradation of the PML-RARA protein through the ubiquitin-proteosome and caspase systems, thus allowing for the terminal differentiation of leukemic promyelocytes.[213-215]

This binding also results in derepression whereby N-CoR is dissociated from the PML-RARA fusion protein and, subsequently, the nuclear coactivator complex is recruited to reverse histone deacetylase-mediated repression. PODs are relocated back to the normal nuclear pattern.[208,216] By contrast, As_2O_3 appears to target the PML portion of the PML-RARA fusion protein preferentially and causes its degradation by inducing apoptosis,[217] potentially through downregulation of the antiapoptotic factor BCL2.[213,218,219] The activity of these agents has led to the inferences that fusion proteins may interfere with normal myeloid cell development in several ways—through inhibitory effects on assembly of the PODs that contain PML or through dominant inhibitory effects on transcriptional targets of dimeric complexes with normal retinoid receptors, leading to arrest of differentiation at the promyelocyte stage.

Studies using transgenic mice have shown that the PML-RARA fusion product is involved in the development of leukemia. Transgenic mice generated with the PML-RARA fusion protein specifically expressed in the myeloid-promyelocytic lineage develop a myelodysplastic-like disorder during the first year of life.[220,221] A subset of these mice then develops a form of acute leukemia that closely mimics human APL and responds to ATRA. Given the relatively long latency period and the development of leukemia in only a fraction of the mice, it is likely that the PML-RARA translocation is insufficient by itself to cause APL and that second mutations are necessary for leukemic transformation.

Mixed-Lineage Leukemia Gene Translocations. Transcriptional coactivators and corepressors have been implicated in leukemogenesis.[134] An example is the myeloid-lymphoid or MLL protein, a large protein containing transcriptional activation and repression domains, which is believed to act epigenetically to modulate gene expression through its effects on chromatin structure and configuration. Wild-type MLL is required for normal hematopoiesis and HSC development.[222] Mice deficient in MLL die on embryonic day 10.5 and have numerous skeletal, neural, and hematologic deficits. Heterozygous mice have defects in embryonic segmentation and yolk sac hematopoiesis, and adults demonstrate a mild anemia and thrombocytopenia.[223,224] Chromosomal translocations of the *MLL* gene on chromosome segment 11q23 have been identified in human AML and ALL and confer a poor prognosis.[225,226] Many gene partners have been identified that are involved in these translocations, including *AF4, AF9, ENL, AF6, ELL*, and *AF10*. In most cases, the fusion proteins incorporate the 5′ end of *MLL* and the 3′ end of the partner.[227,228] Despite the number of different fusion partners, leukemias with *MLL* translocations tend to possess a distinct genetic signature compared with AML and ALL samples lacking an *MLL* translocation. This genetic signature includes the upregulation of a number of specific *HOX* family genes, including *HOXA9, HOXA7,* and *MEIS1*.[225,229-231] Wild-type *MLL* is known to play a major role in *HOX* gene transcriptional regulation.[232] In turn, *HOX* genes play critical roles in embryonic development and normal hematopoiesis. A number of studies have clearly demonstrated that overexpression of specific major *HOX* genes in mouse models and human bone marrow samples, whether by involvement in reciprocal translocations or by gene upregulation, is linked to the transformation of malignant HSCs in AML.[233-241] The mechanism whereby MLL fusion proteins upregulate *HOX* gene expression may differ, depending on whether the fusion partner encodes a nuclear partner with direct transcriptional activity or a cytoplasmic protein that results in MLL protein dimerization and subsequent transcriptional activation.[112]

Transcriptional control may be mediated through the modification of histone proteins, which function to maintain chromatin structure. Recently, the DOTL1 complex was identified as a critical factor in MLL-induced leukemogenesis.[242] DOT1L is a histone-3-lysine-79 (H3K79) methyltransferase, which was previously believed to play a fairly ubiquitous role in methylating gene targets in concert with transcription. However, Zhang and Armstrong's group definitively demonstrated through a series of elegant in vitro and in vivo experiments a specificity for DOT1L in the epigenetic regulation of MLL-translocated downstream targets. When DOT1L was knocked out or absent from *MLL-AF9*–transformed cell lines or transgenic mice, leukemia was prevented.[242-244] The *MLL* gene is rearranged in up to 20% of pediatric cases of AML.[245] Translocations of *MLL* are the single most common genetic alteration in infants with acute leukemia, regardless of phenotype, and account for approximately 70% of all cases of AML and ALL in infants.[246] In pediatric and adult cases of AML, the presence of the 11q23 translocation is generally associated with an unfavorable prognosis.[247]

Although less common than *MLL* translocations, the *MLL* gene can be altered by partial tandem duplications (PTDs) of particular exons. These PTDs occur in adult and pediatric AMLs with a normal karyotype, as well as those with trisomy 11. Similar to *MLL* fusions, the *MLL*-PTD tends to be associated with a poor prognosis and often with the presence of an *FLT3* mutation. The mechanisms underlying MLL-PTD leukemogenesis have not yet been elucidated but appear to be different than those of *MLL* fusions.[228,248] Interestingly, *MLL*-PTD appears to repress wild-type *MLL* expression, which may be an important feature underlying the leukemogenic potential of this abnormality.[248]

Type 2 Mutations. The leukemogenic mutations discussed in the previous sections involve balanced translocations, duplications, or substitutions that appear to disrupt regulatory mechanisms controlling hematopoiesis and result in dysregulated cell differentiation. Another group of mutations involve unbalanced chromosome rearrangements, such as chromosome loss (e.g., monosomy 5, monosomy 7) or large chromosomal deletions (e.g., 5q−, 7q−, 20q−).[249,250] These mutations may lead to leukemic transformation by the loss of a tumor suppressor gene (or genes), which then confers a differentiation block and growth advantage to the mutated cells.[251] Identification of these putative tumor suppressor genes has been problematic because the deleted regions are usually large and include many candidate genes. Moreover, leukemogenesis is triggered by the gene's inactivation rather than by altered structure, making the abnormal gene difficult to identify. However, α-catenin has been identified by our group as a novel tumor suppressor gene on the proximal region of the long arm of chromosome 5.[252] Non–therapy-related AML with these chromosomal losses or deletions occurs most commonly in older patients and is often associated with the prodrome of MDS. In the pediatric population, this class of cytogenetic abnormality is uncommon and is also frequently preceded by MDS.[253] Monosomy 7 is the most frequent chromosomal abnormality documented in children with MDS and is sometimes the sole detectable cytogenetic abnormality.[254,255] Overall, chromosome loss represents about half of the chromosomal abnormalities identified in MDS.[253]

Therapy-Related AML. Chromosomal loss and large chromosomal deletions are common in therapy-related AML in the adult and pediatric populations. Although most cases of MDS and AML arise de novo, without evidence of any leukemogenic exposure, in 10% to 20% of patients the disease arises after previous exposure, particularly to topoisomerase II inhibitors, alkylating agents, and ionizing radiation.[256] The 11q23 translocations producing *MLL* gene fusions are common cytogenetic abnormalities in patients who develop AML after therapy with topoisomerase II inhibitors (e.g., epipodophyllotoxins).[6,7,257] AML developing after the use of topoisomerase II inhibitors has a short median latency period of 30 to 34 months, is usually FAB classification M4 or M5, and lacks the antecedent prodrome of MDS.[258] The blast cells of patients with AML or MDS occurring after exposure to alkylating agents often have large chromosomal losses or deletions and have a poor prognosis.[256,259,260] The most common chromosomal abnormalities in such cases involve chromosome 7 and chromosome 5. In contrast to the therapy-related AML with *MLL* fusion genes that develops after treatment with topoisomerase II inhibitors, MDS or AML related to therapy with alkylating agents typically develops after a longer median latency period, 3 to 5 years from the alkylator exposure. Mutations in the *TP53* tumor suppressor gene have been previously linked to therapy-related AML and were believed to be infrequent in de novo cases. However, more recently, *TP53* mutations have been found to be common in de novo AML with a complex karyotype. In both contexts there is an association with chromosome 5 and 7 copy number alterations.[261-264]

AML with a Normal Karyotype. Chromosomal abnormalities have provided some understanding of the mechanisms of malignant transformation in leukemia. However, in many cases of de novo leukemia, no chromosomal abnormality can be detected and the mechanism behind leukemogenesis in these cases is unknown. Because AML involves a block in myeloid differentiation, evidence for mutations in key transcription factors involved in normal myelopoiesis has been sought. Mutations in the early myeloid transcription factors PU.1 and CCAAT/enhancer-binding protein α (C/EBPα) have been identified in a number of AML subtypes and are generally not associated with a known chromosomal translocation.[265] Mouse models have demonstrated that the degree of transcription factor knockdown is critical to the particular phenotype. Mice expressing PU.1 at 20% of normal levels develop AML, whereas those with complete knockdown or 50% of normal levels do not.[266] GATA1, a critical transcription factor for erythrocyte and megakaryocyte development, is mutated in nearly all cases of acute megakaryoblastic leukemia associated with Down syndrome (see later).[267]

Expression profiling using microarrays first used to distinguish prognostic tumor subclasses in breast carcinoma[268-271] and large B-cell lymphoma[272] has also been applied to leukemia.[233] Using this technology, a set of 50 genes was sufficient to discriminate ALL from AML. More recent validation of this technique in AML has demonstrated the correlation between gene expression profiling studies and the prognostic classification of human AML based on cytogenetic abnormalities.[273] Gene expression profiling has provided valuable new insights into the identification of prognostic subclasses in adult[274] and pediatric[275] AML with a normal karyotype.

Our understanding of the molecular mechanisms underlying de novo AML with a normal karyotype was advanced by the finding of mutations involving the *NPM* gene in 35% of cases of adult AML and 60% of cases with a normal karyotype.[276] This frequency supersedes the incidence of *FLT3* mutations and has established *NPM* mutations as the most frequent genetic abnormality in adult AML. The frequency of *NPM* mutations is lower in pediatric AML, occurring in only 6% to 8% of all pediatric patients with AML, but also occurs in a higher number of pediatric patients with a normal karyotype (approximately 27%).[277,278] The *NPM* gene on chromosome 5q was previously well known as a partner in a number of oncogenic translocations, including *NPM-ALK* (anaplastic lymphoma kinase) in anaplastic large cell lymphoma and *NPM-RARA* in APL. NPM is a molecular chaperone that shuttles between the cytoplasm and nucleus but is found most prominently in nucleoli. It has been shown to function in the prevention of protein aggregation in the nucleolus and the regulation of preribosomal particles through the nuclear membrane. It has also been shown to play a role in the regulation of the alternate reading frame (ARF)-TP53 tumor suppressor pathway. *NPM* mutations are heterogeneous but occur predominantly in exon 12, uniformly affecting the C-terminal of the protein and causing the formation of a neomorphic nuclear export signal in the resulting NPMc protein. This results in a shift of NPM localization, sequestering it exclusively to the cytoplasm. *NPM* mutations occur more frequently in children older than 10 years and, as is the case in adults, tend to be associated with a good prognosis. However, there has been a strong association established between patients with *NPM* mutations and *FLT3*-ITDs, which portend a poor prognosis. Patients with both these abnormalities do not fare as well as those with *NPM* mutations alone.

Similarly, mutations in *CEBPA* have been associated with a good outcome. *CEBPA* encodes the CCAAT/enhancer binding protein-alpha (C/EBPα), which functions as a transcription factor that plays a major role in terminal granulocytic differentiation as well as influencing proliferation. C/EBPα is a basic region leucine zipper transcription factor consisting of two amino-terminal transactivation domains, a basic DNA-binding region, and a carboxyl-terminal leucine zipper. Mutations in pediatric AML commonly occur in the amino-terminal domain and bZip domain of the leucine zipper, and many patients harbor both types of mutations.[279]

Mutations in the Wilms tumor 1 (WT1) transcription factor have been identified in approximately 10% of adult and childhood AML, predominantly in patients with a normal karyotype.[280-284] Mutations are common in exons 7, 8, and 9, comprising three of the four zinc fingers of *WT1*. *WT1* mutations frequently occur together with *FLT3*-ITDs; however, in contrast to the adult studies, they do not independently confer a poor prognosis.[285] Recently, a number of large genomic studies using next-generation sequencing technology revealed novel frequent mutations in DNA methyltransferase 3A (DNMT3A) and IDH1 and IDH2 in adult normal karyotype AML.[128,286,287] In adult disease, these mutations confer a poor outcome. By contrast, these mutations are relatively rare in pediatric AML but similarly occur more commonly in patients with a normal karyotype, highlighting the different molecular spectrum of disease in adult and childhood counterparts.[288-290]

Morphology and Cytochemistry

The initial diagnosis of AML relies on accurate interpretation of the cellular morphology of the bone marrow smears. The diagnosis usually can be made based on the morphologic characteristics of the blasts in a Wright-Giemsa–stained bone marrow smear (Fig. 51-5). In addition to general morphology, the diagnosis can be confirmed in cases exhibiting Auer rods (thin, needle-shaped cytoplasmic deposits that stain pink with Wright-Giemsa and are strongly myeloperoxidase [MPO]-positive). In addition to examination of the morphology of the blasts, the Wright-Giemsa stain is also used to assess for dysplastic features in erythroid, myeloid, and megakaryocytic cell lines.

Additional cytochemical stains are important for the accurate diagnosis of AML. MPO and Sudan black B (SBB) are cytochemical stains for granulocyte, eosinophil, and monocyte lineages. Peroxidase is present in the primary granules of myeloid cells, beginning at the promyelocyte stage and throughout subsequent maturation. Typically, leukemic myeloblasts are also strongly peroxidase-positive. MPO staining of Auer rods is particularly robust in leukemic blasts and can demonstrate Auer rods not recognized using the Wright-Giemsa stain. By contrast, the MPO staining of monoblasts shows a fine granular pattern in the monoblast cytoplasm. The staining pattern of SBB is similar to that of peroxidase, particularly in myeloblasts and in the detection of Auer rods. Monoblasts either do not stain or show a weakly positive, diffuse pattern with SBB.

The esterase stains are useful for distinguishing granulocytic cells from cells of the monocytic lineage. Chloroacetate esterase is a specific stain for granulocytes and mast cells. Results of staining of early myeloblasts are often negative, but promyeloblasts and later granulocytic lineage cells stain strongly, as do Auer rods. Monoblasts do not stain with chloroacetate esterase. In contrast, the nonspecific esterases (NSEs) such as α-naphthyl butyrate esterase and α-naphthyl acetate esterase stain monoblasts and monocytes strongly. Granulocytic and lymphoid cells do not stain with the nonspecific esterases. The blast cells of AML-M4 (i.e., myelomonocytic leukemia) should demonstrate positive chloroacetate esterase activity and α-naphthyl butyrate esterase or α-naphthyl acetate activity; this finding is important for making the diagnosis. The periodic acid–Schiff (PAS) reaction is not as informative as the previously described stains, but it can be useful, particularly in the diagnosis of AML-M6 (i.e., erythroleukemia). Leukemic erythroblasts can stain strongly with PAS, whereas normal erythroid precursors generally demonstrate no PAS activity.

Immunophenotype

Since the 1980s, multiparameter flow cytometric analysis has become an important element in the diagnosis and classification of leukemia. More recently, multiparameter flow cytometry has been used as a tool to detect MRD in acute myeloid leukemia and is being incorporated into clinical trials to clarify prognostic groups and help make treatment decisions.[291-300] Immunophenotyping using a large panel of monoclonal antibodies to myeloid and lymphoid lineage and progenitor cell–associated antigens has been used to discriminate myeloid or lymphoid differentiation correctly in up to 98% of patients.[301,302] Monoclonal antibodies have been classified based on their reactivity with the lineage or differentiation-associated antigens on the surfaces of normal and malignant cells. Because the monoclonal antibodies are not leukemia cell–specific and most of the hematopoietic antibodies are not strictly lineage specific, it is necessary to use panels of antibodies on blast cell populations and to incorporate the immunophenotypic information with the clinical findings and other morphologic, cytochemical, and cytogenetic results for accurate leukemia cell classification.

Blasts of myeloid origin generally express HLA-DR, CD33, and CD34. Monocytic cells usually express HLA-DR at all stages of maturation, whereas promyelocytes and more mature cells of the granulocyte lineage do not. CD33 and CD13 are expressed on neutrophil and monocyte precursors, but CD33 is absent on mature neutrophils. CD14 is relatively specific for cells of the monocytic lineage. As myeloid differentiation continues, CD15 expression increases, whereas CD34 expression decreases. Megakaryocytic cells and platelets express CD41, CD42, and CD61.

Immunophenotyping has been particularly valuable for lineage assignment in undifferentiated leukemias (i.e., AML-M0), identification of acute megakaryocytic leukemia, and analysis of the lineage of leukemias with *MLL* gene translocations.[303-308]

Cytogenetics

Cytogenetic analysis is an essential component in the diagnosis and treatment of AML. The chromosomal aberrations detected by cytogenetic testing also have prognostic value and may be used as tumor markers. In

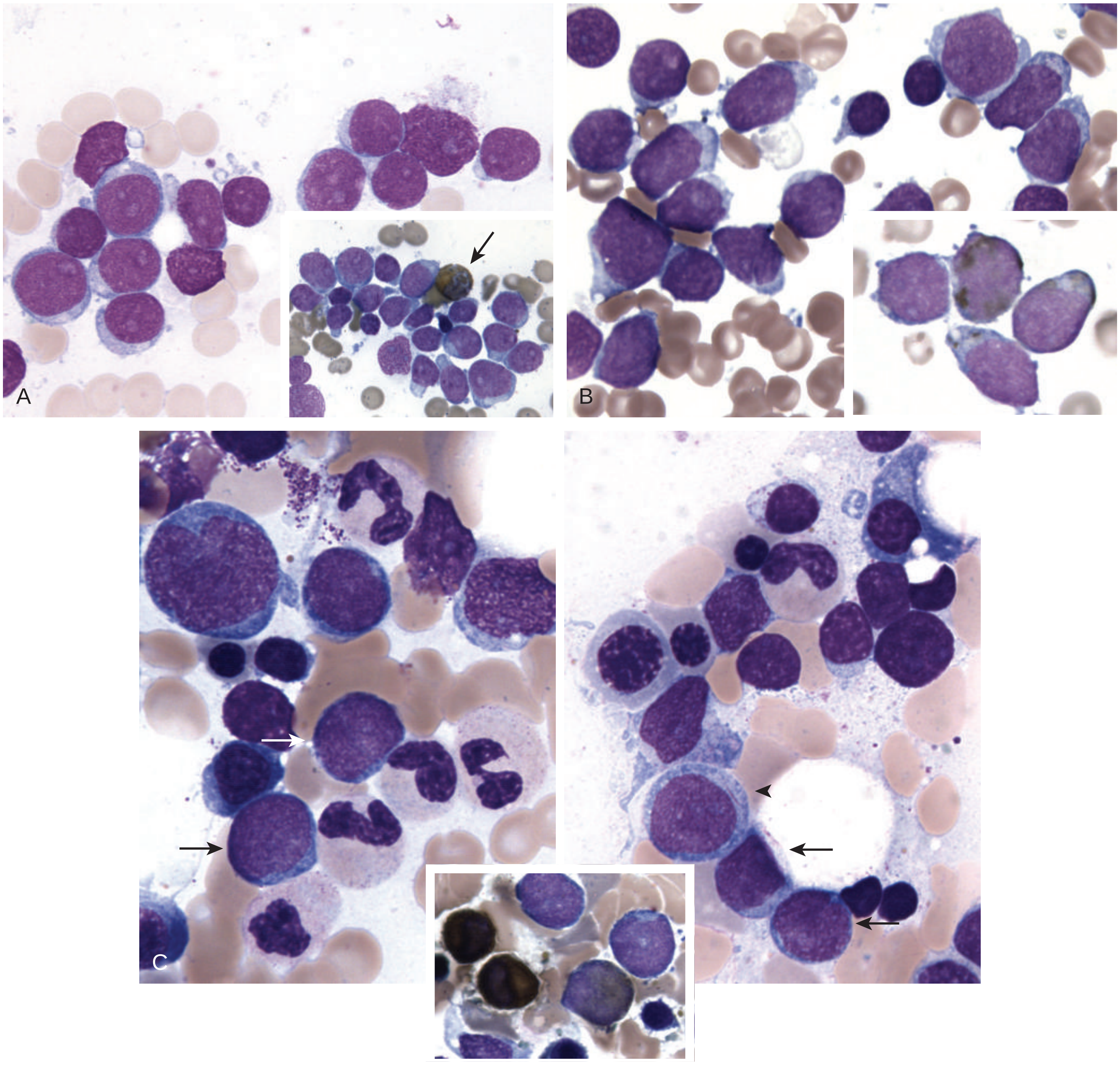

Figure 51-5 Subtypes of acute myeloid leukemia. All images are from Wright-Giemsa–stained bone marrow aspirate smears, except where otherwise designated. **A,** Acute myeloblastic leukemia, minimally differentiated. The blasts may vary in size but have round to oval nuclei, variably prominent single or multiple small nucleoli, and scant cytoplasm that is agranular. *Inset,* The blasts here show no reactivity for myeloperoxidase, although rare reactivity (<3% blasts) may be seen; a neutrophil *(arrow)* serves as a positive internal control. **B,** Acute myeloblastic leukemia, without maturation. Blasts generally predominate within the marrow and comprise 90% or more of nonerythroid cells. The blasts may contain cytoplasmic granules and occasional Auer rods or may resemble lymphoblasts. *Inset,* Myeloperoxidase positivity is present in more than 3% of blasts, although the reactivity itself is of varied intensity. **C,** Acute myeloblastic leukemia, with maturation. Blasts comprise 20% or more of marrow cellularity *(arrows),* whereas maturing neutrophils (e.g., promyelocytes, myelocytes, metamyelocytes, bands, and neutrophils) constitute 10% or more of nucleated elements. Dysmyelopoiesis may be evident. Auer rods are commonly present and may be long and tapered *(arrowhead). Inset,* Myeloperoxidase shows variable reactivity in a subset of the blasts (>3%) and strong reactivity in the maturing neutrophilic forms.

Continued on next page

the WHO classification of hematologic malignancies, specific cytogenetic abnormalities have been included in the diagnostic and prognostic criteria for differentiating subclasses of AML.[309]

Cytogenetic analyses of bone marrow samples have revealed chromosomal abnormalities in most patients with AML.[310-317] In the pediatric population, the incidence of cytogenetic abnormalities is even higher than that in adults.[245,310,318] However, the distribution of the specific cytogenetic abnormalities is different in the various age groups. In infants with AML, the most common chromosomal aberrations are translocations

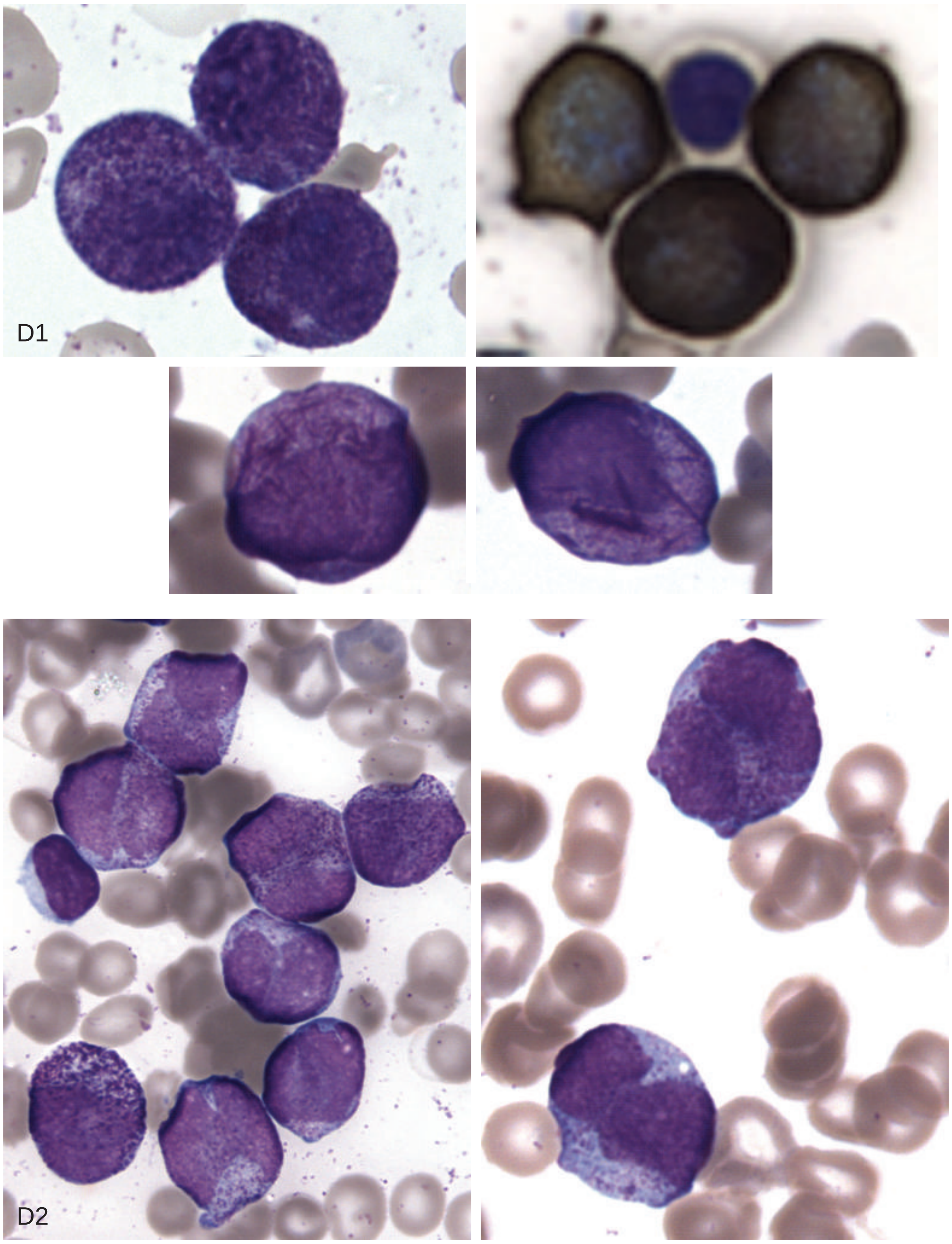

Figure 51-5, cont'd **D1,** Acute promyelocytic leukemia (hypergranular variant). Malignant promyelocytes have eccentric nuclei, prominent nucleoli, and abundant cytoplasm, with intense azurophilic granules. Varied numbers of promyelocytes may contain multiple Auer rods, a characteristic finding *(lower panels)*. Myeloperoxidase shows intense reactivity in the promyelocytes that largely obscures the nucleus *(upper right panel)*. **D2,** Acute promyelocytic leukemia (microgranular variant). These malignant promyelocytes have a bilobed or butterfly-shaped nucleus, fine chromatin, variably prominent nucleoli, and abundant cytoplasm, with sparse to numerous azurophilic granules. Myeloperoxidase shows intense reactivity in these variants similar to that seen in the hypergranular forms.

Continued on next page

involving the *MLL* gene (band 11q23).[319-321] The incidence of 11q23 translocations is as high as 40% in AML blasts of children younger than 2 years. After age 2 years, the frequency of 11q23 translocations decreases with age, and the translocation is detectable in AML blasts from less than 10% of older children and adults.[132,245,310,318] One rare chromosomal abnormality, the t(1;22)(p13;q13), is found almost exclusively in non–Down syndrome infants with acute megakaryocytic leukemia. In a published review of 39 patients with this translocation, 95% were younger than 2 years, and it was not identified in adults with AML.[322] This translocation is now known to

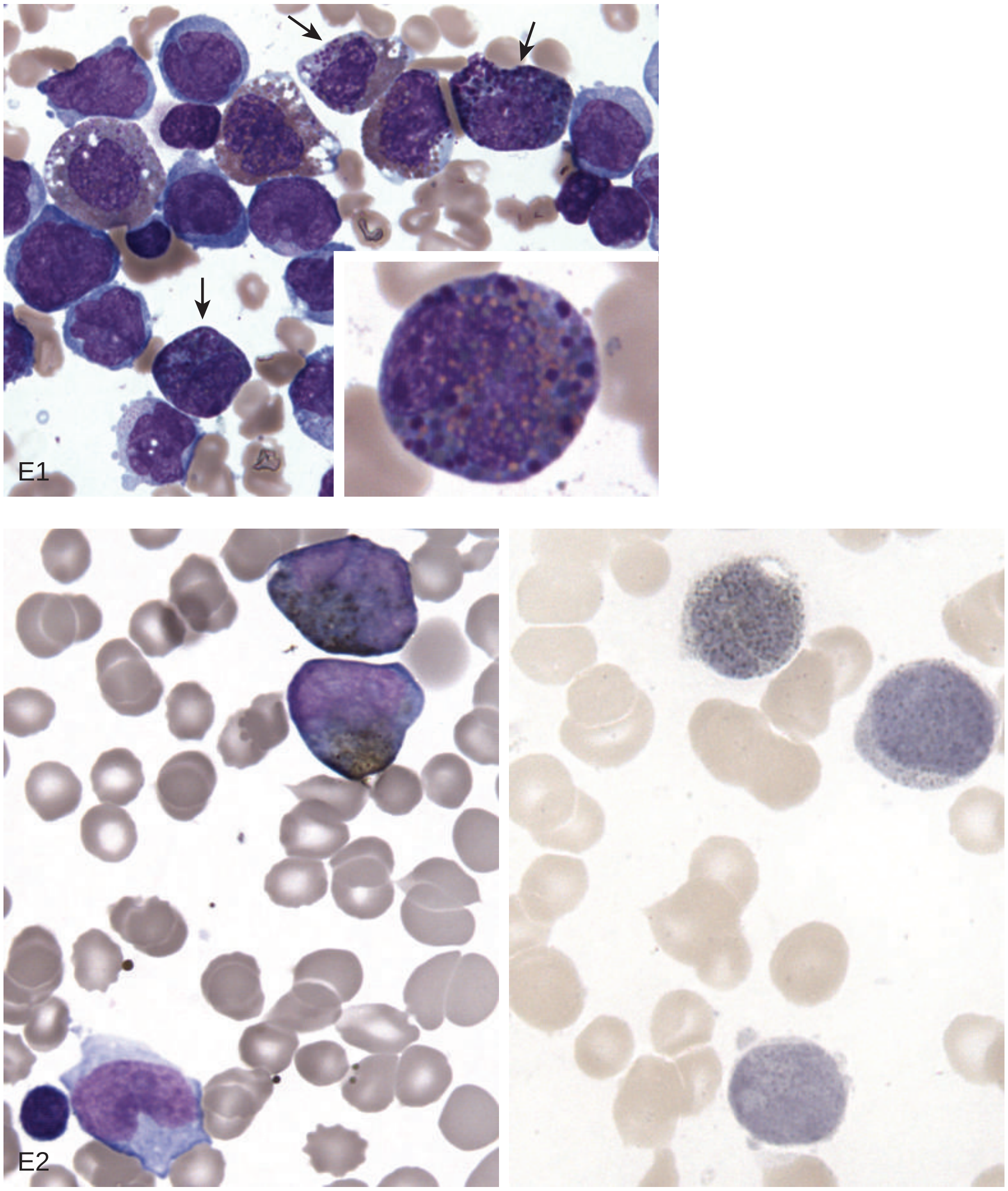

Figure 51-5, cont'd **E1,** Acute myelomonocytic leukemia with eosinophilia. Blasts, maturing neutrophils, and maturing monocytes each constitute 20% or more of marrow cellularity. Additionally, abnormal eosinophils are present in all stages of maturation and contain enlarged granules that are purple-violet as opposed to orangeophilic *(arrows; inset).* E2, Acute myelomonocytic leukemia with eosinophilia (cytochemistries). At least 3% of blasts show myeloperoxidase reactivity *(left),* whereas monoblasts, promonocytes, and monocytes exhibit nonspecific esterase reactivity that may be weak, as seen in this case *(right).*

Continued on next page

result in aberrant expression of the *OTT-MAL* fusion gene.[323]

There are two classification systems for AML (see next section). The FAB classification is based exclusively on morphology. However, there are some morphologic subtypes such as M3 and M4eo that are always associated with particular cytogenetic changes, that is, *RARA* rearrangements for the former and inv(16) or t(16;16) in the latter. In addition, there are certain cytogenetic changes that are often (if not exclusively) associated with a particular subtype and that carry prognostic significance, such as the good prognosis t(8;21) often associated with the M2 subtype or *MLL* rearrangements often associated with the M5 subtype. The WHO classification system takes this into account and has a category of "acute myeloid leukemia with recurrent genetic abnormalities," which includes t(8;21), inv(16) or t(16;16), t(15;17), and 11q23. The fusion proteins thus generated are discussed in more detail earlier (see "Acute Myelogenous Leukemia: Biology"). Some of the more common chromosomal abnormalities and their associated FAB and WHO subtypes are shown in Table 51-1 and Box 51-2. The prognostic significance of certain cytogenetic abnormalities is discussed later (see "Prognosis").

French-American-British Classification

In 1976, the FAB group proposed a system of classification of AML based on morphologic and cytochemical

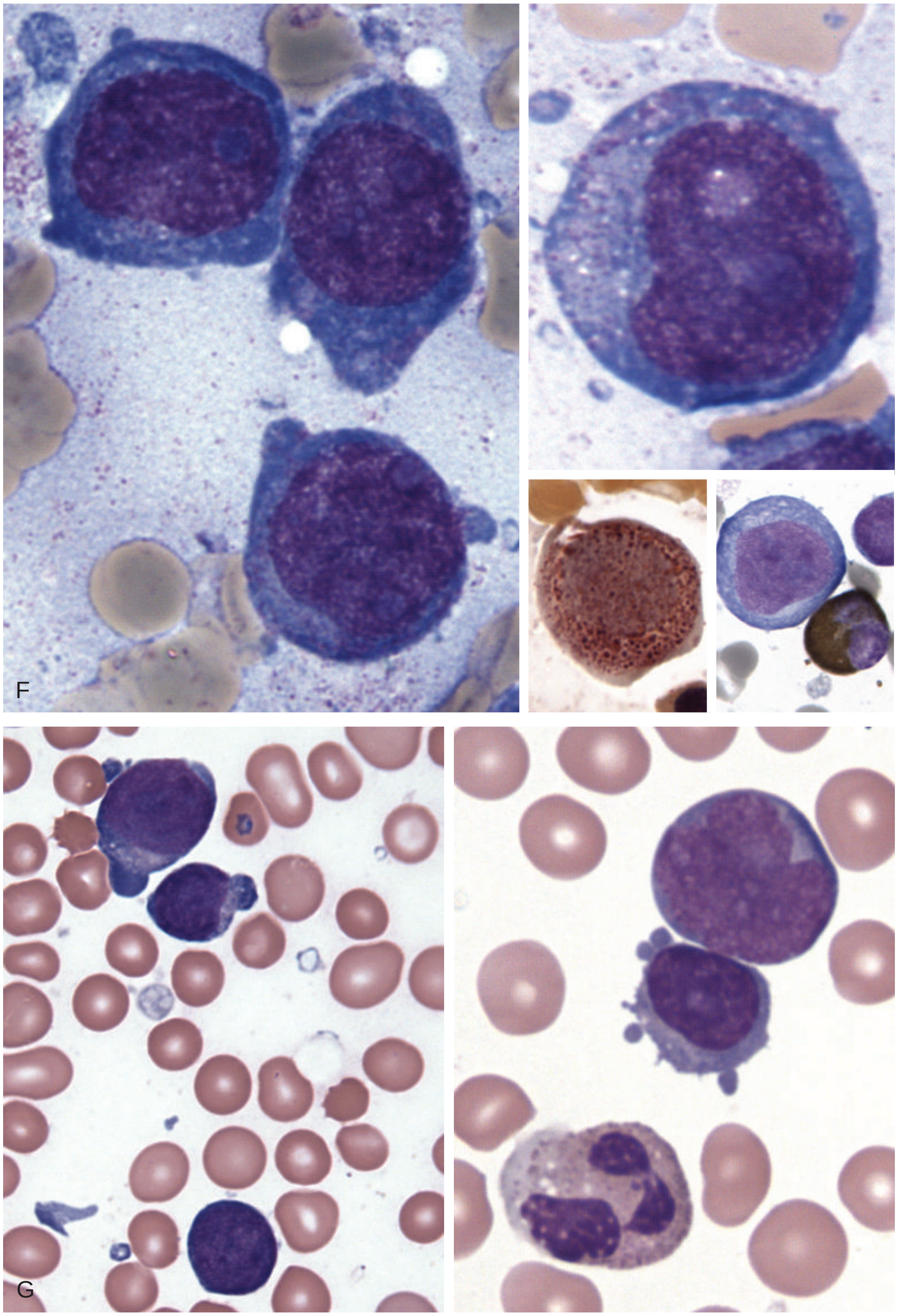

Figure 51-5, cont'd **F,** Acute monoblastic leukemia. Monoblasts are large and have round nuclei, prominent nucleoli, and abundant deeply basophilic cytoplasm, which may contain fine granules and/or vacuoles. Monoblasts show strong reactivity for nonspecific esterase *(lower right, left inset)* and are typically myeloperoxidase negative *(lower right, right inset)*. **G,** Acute megakaryoblastic leukemia. Megakaryoblasts are varied in size and have round, slightly indented or markedly irregular nuclear contours as well as fine chromatin, single to multiple small nucleoli, and scant to moderate basophilic (agranular or granular) cytoplasm, often with cytoplasmic blebs or pseudopod formation. *(Courtesy of Dr. Jo-Anne Vergilio, Assistant Professor in Pathology, Harvard Medical School.)*

TABLE 51-1 French-American-British (FAB) Classification of Acute Myelogenous Leukemia with Corresponding Cytogenetic and Immunophenotypic Characteristics

					MYELOID							ERYTHROID		MEGAKARYOCYTIC			T-CELL LINEAGE				OTHER			
FAB Subtype	**MPO**	**SBB**	**NSE**	**PAS**	CD 11b	CD 13	CD 14	CD 15	CD 33	CD 34	CD 65	Glycophorin A	CD 41/61	CD 42	CD 36	CD 2	CD 3	CD 4	CD 7	CD 56	HLA-DR	CD 117	**Translocations and Rearrangements**	**Genes Involved**
M0	–	–	–	–	–	++	+	+	++	++	+	+	+	+	–	+	–	+ –	+ –	+	++	++	11q23 Trisomy 4, 8,13, –5, –7	*MLL* rearrangements
M1	+	+	–	–	–	+	–	+	+	+	–	–	–	+	–	+	–	–	+	+	++	++	t(8;21)(q22;q22) t(9;22)(q34;q11) Trisomy 8, –5, –7	*AMLI-ETO* *BCR-ABL*
M2	+	+	–	–	+	+	–	+	++	++	–	–	–	+	–	+	–	–	+	+	++	+	t(8;21)(q22;q22) t(6;9) inv3(q21;q26), t(3:3) (q21;q26) Trisomy 8, –5, –7	*AMLI-ETO* *DEK-CAN* *EVI1*
M3	+	+	–	–	+	+	–	+	+	+	–	–	–		–	+	–		+	+	+	+	t(15;17)(q22;q12) t(11;17)(q23;q12), t(5;17) (q32;q12)	*PML-RARA* *PLZF-RARA* *NPM-RARA*
M4	+	+	+	–	++	++	++	+	+	+	–	–	–	++	–	+	–	++	+	+	+++	+	t(6;9) inv3(q21;q26), t(3:3) (q21;q26) t(9;11)(p22;q23) 11q23	*DEK-CAN* *EVII* *AF9-MLL* *MLL* rearrangements
(M4eo)	+	+	+	+	++	+	+	+	+	+	–	–	–	++	–		–				+++	+	inv16(p13;q22), t(16:16)	*MYHII-CBFβ*
M5	–	–	+	–	++	++	++	++	+	++	–	–	–	++	–	+	–	++	+	+	+++	+	t(11;17)(q23;q21) 11q23 11q23,t(9;11) (p22;q23) [M5a] t(8;16), t(10;11) (p12;q23) [M5b]	*MLL-AF17* *MLL* rearrangements *MLL, AF9-MLL* *MOZ-CBP, AF10-MLL*
M6	+	+	–	+	–	+	–	–	+	+	++	–	–			–	–	–	+	+	–	+	Trisomy 8, –5, –7	
M7	–	–	+	+	–	+	–	–	+	+	–	++	++	+ +		++	–	+	+	+	+	+	t(1;22)(p13;q13)	*OTT-MAL*
M7 (DS)	–	–	+	+	–	+	–	–	+	+	–	++	++	++		++	–	+	+	+	+	+	Trisomy 21	*GATA-1*

AML, Acute myelogenous leukemia; *DS*, Down syndrome; *MLL*, mixed-lineage leukemia; *MPO*, myeloperoxidase; *NSE*, nonspecific esterase; *PAS*, periodic acid–Schiff; *SBB*, Sudan black B.

features (see Table 51-1). The system divides AML into seven subtypes, M1 through M7.[305,324,325] An M0 subtype was later added to describe undifferentiated leukemia and, since 1976, immunophenotypic data have also been included.[304,305,326] In general, AML can be differentiated from ALL based on morphologic features and cytochemical stains. Cells of myeloid origin should stain with myeloperoxidase and SBB. Cells of monocytic derivation usually stain with NSE. AML blasts usually do not have PAS activity, except for erythroblasts in AML-M6 and eosinophils of the M4Eo subtype.

M0: Acute Myeloblastic Leukemia with Minimal Differentiation. In patients with AML-M0, the bone marrow is usually hypercellular and more than 90% of the cells are blasts. Most blast cells lack cytoplasmic granules, nucleoli, or Auer rods, and results with MPO and SBB stains are negative.[304] Immunophenotypic analysis shows the presence of myeloid or monocytic cell antigens (i.e., CD13, CD14, CD33, or CD34) that are detectable on the cell surface of most AML-M0 blasts, but some AML-M0 blasts express terminal deoxynucleotidyl transferase, an enzyme usually associated with ALL. AML-M0 is associated with a high incidence of cytogenetic abnormalities, most of which are complex and often involve chromosomes 5 and 7, trisomy 8, or MLL rearrangements.[302,327]

M1: Acute Myeloblastic Leukemia without Maturation. The bone marrow of patients with AML-M1 is hypercellular and filled with myeloblasts (more than 90% blasts). Most blasts stain with MPO and SBB. The blast cells in this subtype display minimal myeloid differentiation. Morphologically, the blast cells may contain scant gray-blue cytoplasm and few or no azurophilic granules or Auer rods. Prominent nucleoli are usually detectable. Flow cytometric analysis usually shows that the blast cells of AML-M1 express HLA-DR, CD13, CD33, and CD34. Cytogenetic abnormalities for this subclass often include monosomy 5 or 7 or trisomy 8.

M2: Acute Myeloblastic Leukemia with Maturation. The bone marrow of patients with AML-M2 usually shows evidence of some maturation beyond the myeloblast, with evidence of maturation beyond the promyelocyte stage in more than 10% of nonerythroid cells. Myeloblasts must represent more than 30% of the bone marrow cells but less than 90% of nonerythroid cells. The blasts generally have a few clusters of primary granules and stain with MPO and SBB. Auer rods and prominent nucleoli are common. Immunophenotypic expression of HLA-DR, CD13, CD33, CD11, and CD15 is typical. This subtype may exhibit monosomy 5 or 7, trisomy 8, t(8;21), t(6;9), or abnormalities of chromosome 3.

M3: Acute Promyelocytic Leukemia. There are two types of AML-M3. The more common type is the hypergranular variant, in which more than 30% of the blasts are promyelocytes and myeloblasts. Most promyelocytes have heavy granulation. Auer rods and Auer rod bundles are common. In the rare microgranular variant, the cells exhibit fine cytoplasmic granules, which are not distinguishable by light microscopy and nuclear morphologic irregularities (i.e., microgranular M3 or M3v). The cells of both subtypes stain strongly with MPO and SBB and also with chloroacetate esterase. The promyelocytes usually do not show PAS and NSE activity. The blasts express CD13, CD33, CD11, and CD15, but they are HLA-DR– and CD14–. This subtype exclusively exhibits *RARA* rearrangements, the vast majority of which are t(15;17) leading to *PML-RARA*.

M4: Acute Myelomonocytic Leukemia. The M4 subtype is defined by the presence of blast cells with both granulocytic and monocytic features. The bone marrow in these patients has more than 30% infiltration by immature myeloid precursors, and there is often extramedullary involvement and a peripheral blood monocytosis. The blasts are usually pleomorphic with regard to size, amount of cytoplasm, granularity, and nuclear morphologic features. Some Auer rods can be seen, and prominent nucleoli are usually present. Staining is variable, with some of the blast cells showing positivity for MPO, SBB, and NSE. M4 AML may be associated with t(6;9), abnormalities of chromosome 3, and *MLL* gene rearrangements, in particular t(9;11). A small proportion of patients with AML-M4 have a moderate eosinophilia in their bone marrow (M4Eo). The eosinophils are notable for the presence of basophilic and eosinophilic granules and stain with chloroacetate esterase and PAS. Cell surface antigen expression includes CD13, CD15, CD33, CD4, CD11c, CD14, CD64, and HLA-DR. M4eo AML exhibits an inv(16) or t(16;16).

M5: Acute Monocytic Leukemia. More than 80% of the nonerythroid bone marrow cells in patients with AML-M5 are monocytic. There are two subtypes: M5a (undifferentiated) and M5b (differentiated). In AML-M5a, more than 80% of the cells are monoblasts. Patients with AML-M5a tend to be younger, have higher presenting white blood cell (WBC) counts, and have a poorer prognosis. In AML-M5b, fewer than 80% of the monocytic cells are monoblasts, and most of the cells are recognizable as monocytes or promonocytes. The peripheral blood in patients with AML-M5b exhibits a profound monocytosis. In both subtypes, the blasts demonstrate strong NSE activity. MPO and SBB staining results are usually negative. Cell surface antigen expression includes CD13, CD15, CD33, CD4, CD11c, CD14, CD64, and HLA-DR. M5 AML often exhibits *MLL* gene rearrangements, t(9;11) or t(11;17) in M5a and t(10;11) in M5b.

M6: Acute Erythrocytic Leukemia. AML-M6 is an uncommon form of AML overall and is rare in children. This form of leukemia is defined by a more than 50% erythroblast infiltration of the bone marrow. M6 AML may exhibit monosomy 5 or 7 or trisomy 8.

M7: Acute Megakaryoblastic Leukemia. The bone marrow exhibits an infiltration with pleomorphic megakaryoblasts that often display cytoplasmic budding and may appear in clusters. Aspiration of the bone marrow

Box 51-2 **World Health Organization (WHO) Classification of Acute Myeloid Leukemia and Corresponding French-American-British (FAB) Subtype***

ACUTE MYELOID LEUKEMIA WITH RECURRENT GENETIC ABNORMALITIES (FAB)

t(8;21)(q22;q22); *(AML1/ETO; RUNX1-RUNX1T1)* **(M2)**
inv(16)(p13;q22) or t(16;16)(p13;q22); *(CBFβ/MYH11)* **(M4E0)**
t(15;17)(q22;q12) *(PML/RARA)* **(M3)**
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*

ACUTE MYELOID LEUKEMIA WITH MYELODYSPLASIA-RELATED CHANGES

Following myelodysplastic syndrome (MDS) or MDS/myeloproliferative disease (MPD)

THERAPY-RELATED MYELOID NEOPLASMS

ACUTE MYELOID LEUKEMIA (FAB)

Acute myeloid leukemia, minimally differentiated **(M0)**
Acute myeloid leukemia without maturation **(M1)**
Acute myeloid leukemia with maturation **(M2)**
Acute myelomonocytic leukemia **(M4)**
Acute monoblastic and monocytic leukemia **(M5)**
Acute erythroid leukemia **(M6)**
Acute megakaryoblastic leukemia **(M7)**
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis

MYELOID SARCOMA

MYELOID PROLIFERATIONS RELATED TO DOWN SYNDROME

BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM

Modified from Vardiman JW, Harris NL, Brunning RD: The World Health Organization (WHO) classification of the myeloid neoplasms. Blood *100:2292–2302, 2002; and Vardiman JW, Thiele J, Arber DA, et al: The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes.* Blood *114:937–951, 2009.*
*Shown in boldface.

may be challenging due to associated fibrosis. M7 blasts generally do not stain with MPO and SBB but show activity with PAS. Immunophenotyping is usually required to distinguish AML-M7 from ALL-L2, documenting the presence of the megakaryocytic antigens, glycoprotein Ib, glycoprotein IIb/IIIa, or factor VIII. Non–Down syndrome infants with M7 AML often exhibit a t(1;22). Down syndrome patients have a very high incidence of M7 AML with a particularly good prognosis, which is in contrast to the poor prognosis of M7 AML in non–Down syndrome children and adults.

World Health Organization Classification

The discovery of cytogenetic and molecular genetic abnormalities in malignant disease has had a significant impact on our understanding of malignant transformation. In myeloid malignancies, some genetic abnormalities have prognostic significance whereas others appear to define specific disease subtypes. The WHO has proposed a classification system for neoplastic diseases of the hematopoietic and lymphoid tissues that includes a classification for AML (see Box 51-2 and Fig. 51-5). This classification uses the traditional FAB-type morphologic categories of disease and includes additional entities such as immunophenotype, molecular genetic, and clinical characteristics that make the system more clinically relevant for diagnosis, prognosis, and treatment.[328] The WHO classification system divides myeloid diseases into several major subtypes—myeloproliferative neoplasms; myeloid neoplasm associated with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1*; myelodysplastic-myeloproliferative neoplasms; MDS, AML and related neoplasms; and acute leukemias of ambiguous lineage. The 2008 revision of the WHO classification has expanded the category of AML to include AML with recurrent cytogenetic translocations, AML with myelodysplasia-related changes, therapy-related myeloid neoplasms, AML not otherwise specified, myeloid sarcoma, myeloid proliferations related to Down syndrome, and blastic plasmacytoid dendritic cell neoplasms.[329]

Within the subtype containing recurrent cytogenetic translocations, seven additional categories have been described:

AML with t(8;21)(q22;q22); *AML1-ETO*; *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
APL with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*

AML with t(6;9)(p23;q34) and AML with inv(3)(q21q26.2) is relatively uncommon in children. Myeloid sarcomas, formerly called chloromas, may precede hematopoietic evidence of disease and so are now classified as a separate entity. The myeloid diseases of Down syndrome were incorporated into the revised WHO classification in 2008 and are described later in this chapter. Plasmacytoid dendritic cell neoplasms were formerly referred to as blastic NK-cell lymphomas, but the nomenclature has changed to reflect the origin of this myeloid malignancy in this subset of dendritic cells. This rare disease can manifest in children with skin lesions, adenopathy, and ultimately bone marrow involvement. Blast cells characteristically express CD4, CD43, CD56, and CD123 without expression of CD34 or CD117.[329]

The FAB standard used to define AML had been 30% replacement of the bone marrow by the blast cells, but later studies have shown that patients with 20% to 30% blasts (previously classified as refractory anemia with excess blasts in transformation [RAEB-T]) have a prognosis similar to that of patients with more than 30% blasts. The blast count for the diagnosis of AML in the WHO classification was therefore changed to 20%, and the category of RAEB-T was eliminated.

Certain specific and recurrent cytogenetic abnormalities are common in MDS, in alkylating agent–related AML, and in de novo AML with a poor prognosis. These abnormalities include 3q–, –5, 5q–, –7, 7q–, +8, +9, 11q–, 12p–, –8, –19, 20q–, +21, t(1;7), t(2;11), and complex karyotypes. In the classification scheme, these cytogenetic abnormalities were considered to indicate a poor prognosis. Prior therapy with topoisomerase II inhibitors (i.e., epipodophyllotoxins and doxorubicin) was also associated with a poor prognosis. Typically, patients in whom AML develops after exposure to these agents have translocations involving 11q23 (*MLL*). The WHO classification includes these patients in the poor-prognosis category but distinguishes them from having alkylating agent–related secondary leukemia.

Patients may have MDS with unilineage or multilineage dysplasia, and these are reflected as distinct diseases in the most recent iteration of the WHO classification. Dysplasia is defined as being observed in greater than or equal to 10% of the cells of a given myeloid lineage. Children with 2% to 19% peripheral blasts or 5% to 19% bone marrow blasts can be classified according to the same criteria as adult MDS, but a new category of refractory cytopenia of childhood has been introduced to define children with persistent cytopenias and dysplasia of two or more lineages but less than 2% peripheral blasts or less than 5% bone marrow blasts.[329]

Clinical Presentation

The presenting signs and symptoms of AML result from leukemic blast cell infiltration of the bone marrow. The leukemia cells overwhelm the processes of normal hematopoiesis, resulting in anemia, thrombocytopenia, and neutropenia. It is rare for AML to be diagnosed incidentally on a routine medical evaluation, but there is considerable variability in the range of presenting signs and symptoms in patients with de novo AML. Usually, patients seek medical attention for fever, fatigue, pallor, skin or mucosal bleeding, bone pain, or infections not responding to appropriate antibiotic therapy.[330] Bone pain is a common symptom, and patients may present with a limp, rib pain, or back pain.[330]

The WBC count of patients with newly diagnosed AML can range from less than 1,000/μL to more than 500,000/μL.[330-332] The leukocyte count is more than 100,000/μL in approximately 25% of pediatric patients with AML, and an elevated count is more common in those with the M4 and M5 subtypes and in those with *FLT3*-ITD mutations.[174,175,330,333,334] The number of circulating granulocytes is often critically decreased, regardless of the total leukocyte count, and, because their function is usually impaired, the risk for overwhelming bacterial infection in patients with newly diagnosed AML is markedly increased. Patients with AML who have a fever require immediate treatment with broad-spectrum antibiotics after appropriate culture samples have been obtained. The hemoglobin level is occasionally normal but is usually less than 9 g/dL, and levels as low as 3 g/dL at diagnosis are not uncommon. About half of children with new-onset AML have platelet counts of 50,000/μL or less, which increases the risk for life-threatening bleeding.[330]

Disseminated intravascular coagulation (DIC) has been observed in patients with all FAB subtypes of AML but is most common in acute promyelocytic leukemia (APL; see later). Before the development of ATRA, treatment with low-dose heparin during induction was initiated for patients with AML-M3 to prevent DIC.[335,336] The later addition of ATRA to induction therapy rapidly resolved DIC in patients with AML-M3 and markedly reduced early mortality.[191,337-340]

Patients with hyperleukocytosis have an increased risk for mortality from leukostasis, particularly in the brain or lung, and urgent leukapheresis or treatment with leukocyte-reducing agents such as hydroxyurea may be necessary (see “Complications”).[341,342] Extramedullary leukemia occurs in 20% to 25% of children with AML and may include chloromas (tumor nodules), skin infiltration, CNS disease, gingival infiltration, hepatosplenomegaly, or testicular involvement.[330,343] Chloromas (e.g., myeloblastomas and granulocytic sarcomas) are solid tumors of myeloblasts that may occur in any area of the body but are most common in the orbit and epidural area.[344] Skin infiltration, or leukemia cutis, usually presents as slightly purple lesions (i.e., “blueberry muffin” spots), is more common in infants with monocytic leukemia, and may be the initial sign of disease.[345] CNS involvement at diagnosis is more common in AML than in ALL. Up to 15% of patients with AML have myeloblasts in the cerebrospinal fluid at diagnosis.[346-349] Gingival infiltration may present as hyperplasia, often accompanied by bleeding.[350] Severe extramedullary disease with massive hepatosplenomegaly is also a more common finding in infants.[345] Extramedullary involvement of the testes can also be documented but is less common than in ALL.[351,352] Extramedullary infiltration, particularly gingival involvement, is more common with the M4 and M5 (acute myelomonocytic and acute monoblastic and monocytic) leukemias.[350,353,354] Chloromas and CNS disease are more common in AMLs with t(8;21). Extramedullary infiltration in pediatric patients is not clearly associated with worse prognosis unless accompanied by a high presenting WBC count.

Treatment

As is true for any pediatric cancer, the goal of therapy for children with AML is to completely eradicate the disease while limiting treatment-induced toxicity. In general, therapy for AML includes immediate supportive care measures followed by intensive remission induction and consolidation chemotherapy. At any given time, several large, multicenter phase III clinical trials are ongoing, each aimed to improve overall survival (OS) through randomized comparisons of treatment options while minimizing short- and long-term toxicities.

Remission Induction

During the past 40 years, survival rates for patients with AML have risen from less than 10% to more than 50% of patients because of intensification of chemotherapy, use of hematopoietic stem cell transplantation (HSCT), and improved supportive care. The first phase of chemotherapy is termed *induction* and consists of two to four cycles of intensive chemotherapy with the goal of inducing remission. Morphologic remission is defined as the presence of fewer than 5% blasts visible on bone marrow aspirate obtained after recovery of counts. Patients may also achieve cytogenetic and FISH-negative remission, as defined by the absence of a previously detected leukemic clone–associated cytogenetic abnormality in the bone marrow cells. Remission is also now being defined by the absence of MRD, assessed by multiparameter flow cytometry that is designed to detect as few as 0.1% leukemic blasts in the marrow. The inclusion of MRD evaluation has not only identified children at risk for relapse who appeared to be in morphologic remission but has also revealed false-positive recovering marrows that had been deemed refractory disease.[355]

Induction chemotherapy should be initiated as soon as the diagnosis of AML is confirmed, preferably by morphologic, immunophenotypic, and cytogenetic studies of the bone marrow. In some cases, particularly in patients presenting with hyperleukocytosis, the patient's condition may be too unstable for bone marrow evaluation, in which case the diagnosis can often be confirmed on studies of the peripheral blood. Induction chemotherapy regimens consist of high-dose myelosuppressive cytotoxic agents that result in prolonged pancytopenia. The period of bone marrow hypoplasia generally lasts from 21 to 30 days from the initiation of therapy but may last considerably longer. During this period, patients have a high risk for life-threatening infection and bleeding. Aggressive supportive care is necessary (see later).

Historical North American Approaches to Treating AML. The basis of modern induction therapy for AML is a combination of cytarabine and an anthracycline. The classic combination therapy was the so-called 7 + 3 regimen, which consists of a continuous 7-day intravenous infusion of cytarabine at 100 to 200 mg/m^2/day and 3 days of bolus infusions of daunorubicin at 45 to 60 mg/m^2/day. Remission is achieved in 60% to 70% of patients with newly diagnosed AML using this regimen.[356-358] In children, several strategies have been used to intensify this regimen to achieve higher rates of CR. CCG trial 213 added etoposide, thioguanine, and dexamethasone to the traditional 7 + 3 regimen to create the five-drug Denver regimen. The Denver and 7 + 3 regimens were compared in a randomized fashion, and there was no significant difference in CR rate, with 79% of children achieving CR with 7 + 3 and 76% achieving CR with Denver regimen.[359]

In CCG trial 2891, the same drugs were used but the timing was intensified in an attempt to improve CR. The hypothesis was that leukemia cells may be recruited synchronously into the cell cycle after chemotherapy, and therefore reexposure at an earlier time point may affect cells while more of them are at a sensitive phase in the cell cycle. Thus the Denver regimen was modified into the five-drug DCTER regimen, again consisting of ***d***examethasone, ***c***ytarabine, ***t***hioguanine, ***e***toposide, and ***R***ubidomycin (daunorubicin). Induction consisted of a total of four cycles of chemotherapy, and patients were randomized to standard or intensive timing. For standard timing, each cycle of induction began with count recovery from the previous cycle. With intensive timing, cycles 2 and 4 began at day 10 after the start of cycles 1 or 3, respectively, regardless of hematologic status. Not surprisingly, the intensive timing resulted in far greater toxicity, with 11% of patients in this arm of the trial dying of toxicity compared with only 4% of patients dying of toxicity in the standard timing trial arm.[360] Because of this, better supportive care measures were instituted for the intensive timing trial arm, including more aggressive empiric antibiotic and antifungal coverage and the use of granulocyte colony-stimulating factor (G-CSF). Interestingly, the CR rates were similar for the two arms in the trial—75% for intensive timing and 70% for standard timing—but the reason for failure to achieve remission was different, with 11% toxic death and only 14% resistant disease in the intensive timing arm versus only 4% toxic death but 26% resistant disease in the standard timing arm. The most dramatic results, however, came later, when it was shown that for patients who achieved a CR there was a 3-year disease-free survival (DFS) of 55% for those who had been treated in the intensive timing arm, versus a DFS of only 37% for patients who had received standard timing. For this reason, the standard timing arm of the trial was closed early and an intensive timing DCTER/DCTER schedule became the new standard combination therapy for newly diagnosed AML patients.

In the meantime, a comprehensive review of five randomized trials comparing idarubicin with daunorubicin as induction therapy for AML had shown improved remission induction rates and OS for patients receiving idarubicin.[361] For this reason, the CCG pilot trial 2941 tried to intensify therapy further by using idarubicin in place of daunorubicin in each cycle of the intensively timed regimen. Because of unacceptably high toxicity, with a toxic death rate of 14%, this was modified to idarubicin only in cycles 1 and 3, and more strict supportive care guidelines were put into place, including mandatory hospitalization during neutropenia, and stricter infection prophylaxis (see later, "Supportive Therapy at Diagnosis and during Therapy").[362] The addition of idarubicin showed no advantage in remission induction or event-free survival (EFS) but did show a decrease in marrow blasts at day 14 of induction. CCG trial 2961 continued to use the hybrid idaDCTER/DCTER intensive timing induction regimen resulting in induction remission rates of 89%, which were similar to historic controls. Most of the improvement was believed to be related to better supportive care rather than to the change in chemotherapy.

Medical Research Council Trials (MRC10, MRC12, and MRC15). During this period, data emerged from the British MRC trials that seemed to show better survival and less toxicity. MRC10 induction chemotherapy randomized DAT (daunorubicin, cytarabine, thioguanine) to ADE (cytarabine, daunorubicin, etoposide) in a 10-day cycle of chemotherapy followed by an 8-day cycle of the same drugs on WBC count recovery. The CR rate for children in this study was 93%, with a 4% rate of toxic death and a 3% rate of resistant disease.[363] Early data suggested a benefit for pediatric patients using ADE versus DAT, but the later data showed no difference in 10-year OS (DAT, 57%; ADE, 51%; $P = .3$), DFS (DAT, 53%; ADE, 48%; $P = .3$) or EFS (DAT, 48%; ADE, 45%; $P = .5$).[363,364] Because of the early results possibly favoring ADE, MRC12 compared ADE with MAE (mitoxantrone, cytarabine, etoposide) in a 10-day cycle followed by an 8-day cycle on count recovery. The CR rates were similar for the two groups (ADE, 92%; MAE, 90%; $P = .3$) with a slight increase in toxic deaths for the MAE group (ADE, 3%; MAE, 6%) but a lower relapse rate (ADE, 39%; MAE, 32%) and better DFS rate for the MAE group (ADE, 55%; MAE, 63%), although the 10-year EFS and OS rates were not significantly different (54% and 63%, respectively).[363,365] In MRC15 there were three randomized induction backbones—ADE; cytarabine and daunorubicin without etoposide, or idarubicin, fludarabine, cytarabine, and G-CSF (Ida-FLAG)—and three randomized consolidation backbones—amsacrine, cytarabine, and etoposide; cytarabine 1.5 g/m^2; or cytarabine 3 g/m^2 with and without the addition of gemtuzumab ozogamicin (GMTZ, GO, Mylotarg).[371] GMTZ, a recombinant humanized anti-CD33 monoclonal agent linked to the tumor antibiotic calicheamicin, was incorporated as a targeted therapy in these studies. Because CD33 is expressed on most AML cells but not on pluripotent HSCs or other tissues, this drug can target leukemia cells more specifically.[367,368] GMTZ was well tolerated overall, but no overall improvements in survival or relapse rate were observed regardless of the chemotherapy backbone in either induction or consolidation.[371]

Recent U.S. Trials. The next series of U.S. trials built on the data from these recent MRC studies. The COG has undertaken three therapeutic trials to date based on an MRC backbone. The COG AAML03P1 pilot and AAML0531 phase III groupwide study backbone included cytarabine/daunorubicin/etoposide (ADE 10+3+5) followed by ADE 8+3+5, cytarabine/etoposide (AE), mitoxantrone/cytarabine (MA), and, finally, high-dose cytarabine (Capizzi II) for children who did not go on to receive HSCT. The COG pilot trial AAML03P1 assessed the safety of adding a single dose of GMTZ to ADE 10+3+5 and MA cycles. Toxicity on this pilot study was acceptable, so GMTZ was added to these same two cycles on AAML0531 in a 1:1 randomization. Subsequent outcome data from the AAML03P1 trial demonstrated a 3-year EFS of 53% and OS of 66% with no increased treatment-related mortality over backbone chemotherapy.[369] Outcome data have not yet been released for AAML0531. However, after data were released from the Southwest Oncology Group (SWOG) Study 106, the U.S. Food and Drug Administration (FDA) removed GMTZ from the U.S. market because this adult-based study showed no benefit in outcome to the addition of GMTZ but an increase in induction deaths.[370] By contrast, subgroup analysis on the MRC 15 trial found that the addition of GMTZ to induction chemotherapy was of benefit to CBF AML and to intermediate-risk patients and did not contribute to increased toxicity.[371] Data from the COG AAML0531 trial demonstrated mixed results. GMTZ improved 3 year EFS and relapse risk for all patients but not OS. Interestingly, positive trends were seen for GMTZ in subgroup analysis, including OS in high risk patients and relapse risk in low risk patients, although this was also associated with increased TRM in the low risk subgroup.[371a] Given this controversy regarding both toxicity and efficacy and the absence of data from the AAML0531 at the time of study initiation, GMTZ was not included on the current COG AAML1031 trial. However, other co-operative groups are planning future trials to examine the optimal dosing of GMTZ in pediatric AML (A. Gamis, personal communication, July 2014). The current COG trial risk stratifies patients into high- and low-risk AML based on cytogenetics and MRD and randomizes the addition of the proteosome inhibitor bortezomib as a potential AML stem cell targeted therapy. In addition, patients harboring the *FLT3*-ITD with sufficient allelic ratio have the option of being nonrandomly assigned to an MRC-based backbone arm with the addition of the FLT3 inhibitor sorafenib. Given recent MRC data that four cycles appear as effective as five cycles of therapy,[365] only four cycles are included on this protocol (ADE 10+3+5; ADE 8+3+5; cytarabine and etoposide; and mitoxantrone and cytarabine) for good responders. Patients with positive MRD after cycle 1, defined as more than 0.1% by multiparameter flow cytometry (MPFC) receive mitoxantrone and cytarabine instead of an additional ADE cycle, and high-risk patients receive high-dose cytarabine for cycle 4.

The St. Jude Children's Research Hospital (SJCRH) trial AML02 randomized patients to standard low- or high-dose cytarabine during the first cycle of induction together with etoposide and daunorubicin (ADE), although this was not found to impact remission rate, EFS, or OS.[372] Toxicity was generally similar in both arms of the trial, although patients treated on the high-dose arm had a greater incidence of serious fungal infections. The SJCRH trial incorporated MRD measurements during induction to guide therapy. MRD by MPFC was determined on a bone marrow sample obtained on day 22 of induction. Patients with MRD greater than 1% on day 22 received induction II before WBC count recovery. Patients with MRD greater than 1% at the end of the first month of induction I received GMTZ in addition to the second cycle of ADE. Patients with CNS-positive disease received triple intrathecal therapy. Patients who were MRD positive after induction I had a significantly worse outcome than patients who were MRD negative (3-year EFS, 43% vs. 74%). However, subgroup analysis revealed that this difference was only found in patients with high-risk AML at diagnosis (*FLT3*-ITD positive, monosomy 7, t(6;9), megakaryoblastic leukemia, therapy-related AML, or AML after MDS) or those with MRD levels greater than 1%. Low- and standard-risk AML patients or those with what was referred to in this study as low-level MRD (0.1% to 1%) did not demonstrate any outcome differences versus those with negative MRD. However, 20 patients who were MRD negative ultimately experienced relapse.[372] In the current SJCRH study, AML08, patients are randomly assigned to receive high-dose cytarabine, etoposide, and daunorubicin versus clofarabine and cytarabine in induction I. Low-risk patients (those with CBF leukemia with negative MRD after induction I) receive 4 courses of chemotherapy, whereas those at high risk for relapse—*FLT3*-ITD positive, monosomy 7, t(6;9), t(8;16), t(16;21), megakaryoblastic leukemia without t(1;22), treatment-related AML, AML after MDS, or MRD greater than 0.1% after induction II—receive two to three courses of chemotherapy followed by allogeneic HSCT. Standard-risk patients receive four courses of chemotherapy and are then eligible to receive one course of natural killer cell therapy if they have a killer cell immunoglobulin-like receptor (KIR)-mismatched haploidentical family member. Patients with *FLT3*-ITD receive sorafenib after each course of chemotherapy (J. Rubnitz, personal communication).

The BFM (Berlin-Frankfurt-Münster) trial for de novo AML, BFM-2010, is for all comers, including patients with APL. Patients are being randomized to ADE ± GMTZ in induction I with MRD measurements on day 8, 15 and 28. Induction II includes cytarabine and idarubicin (AI) for patients with inv(16) and APL (who also receive ATRA). Other favorable-risk patients, including those with t(8;21), *NPM1* mutations, and *CEBPA*, receive HAM (high-dose cytarabine 3 g/m^2 and mitoxantrone), and high-risk patients receive HAM plus targeted therapy, such as sorafenib if *FLT3*-ITD positive, or dasatinib if *KIT* mutant positive. The third course is hAM (high-dose cytarabine at 1 g/m^2) for those who received AI and AI for low-risk patients who received HAM, with AI plus cladribine for the high-risk patients. Course 4 is hAM, except for the inv(16) and APL patients who receive cytarabine or cytarabine and etoposide as a final cycle. For all other patients, course 5 is high-dose cytarabine and etoposide. Liposomal daunorubicin, available in Europe, will be used in this trial. Prior studies have shown it to have equal efficacy in induction to idarubicin with less toxicity[373,374] (G. Kaspars, D. Reinhardt, and D. Johnston, personal communication, January 2013).

Postremission Therapy

Several randomized studies have demonstrated that without postremission therapy almost all patients with newly diagnosed AML suffer a relapse within 2 years.[375-377] The strategies for postremission therapy include autologous HSCT, allogeneic HSCT, or continued courses of chemotherapy. The CCG trials have shown no benefit to autologous HSCT over continued cycles of chemotherapy so, although that strategy continues to be used in some European trials, autologous HSCT is not included in current U.S. trials.[364,378-384] Most studies have shown a lower relapse rate for AML patients treated with matched related donor HSCT versus chemotherapy, but the OS is often no better because of the significant morbidity and mortality of HSCT.[381,383-396] For this reason, until the most recent trials, unrelated donor HSCT was believed to be too morbid for patients in first remission and thus trials used a genetic randomization, wherein all patients with a matched related donor proceeded to HSCT and all others proceeded with further cycles of chemotherapy.[383,385,386]

Based on the success of the MRC trials with chemotherapy alone using a combination of cytogenetic characteristics and response to therapy,[363,364,386,397] the COG and SJCRH adopted risk-based criteria for HSCT. HSCT, even from a matched related donor, is not recommended for patients with low-risk disease. This was previously defined by COG as the presence of t(8;21) or inv(16) in the leukemic clone at diagnosis, but more recently the presence of a *CEBPA* or *NPM1* mutation has been added to this definition, based on the data revealing the better outcome for these patients.[279,398] The COG has classified high-risk disease as monosomy 7, −5/5q−, *FLT3*-ITD with high allelic ratio, MRD positive after induction chemotherapy, or more than 15% blasts in the marrow after one course of induction chemotherapy.* The incorporation of MRD after induction has enabled the COG to relegate normal karyotype patients to high- and low-risk groups, thereby dichotomizing patients and eliminating the intermediate-risk category. Therefore on the current AAML1031 protocol, high-risk patients will receive best available donor HSCT for consolidation, whereas good-risk patients will not go to transplant even if a sibling donor match is available. With recent advances in supportive care and improved therapy to prevent and treat graft-versus-host disease (GVHD), outcomes have improved for matched related and matched unrelated donor HSCT.[381,390,391,393] Therefore for patients with high-risk disease, both trials recommend HSCT in first CR with an unrelated donor if no matched family donor is identified. The optimal number of cycles of chemotherapy before HSCT to balance minimizing toxicity while minimizing MRD is variable, with the MRC10 trial using four cycles, the current SJCRH trial including two to three cycles, and the COG trial using three cycles before HSCT.[363,364]

For patients who do not proceed to HSCT there is controversy as to how many courses of chemotherapy are needed and what chemotherapy should be included. Several trials have shown that cycles of high-dose cytarabine after remission are associated with improved long-term survival.† MRC12 randomized patients to receive four versus five total cycles of chemotherapy, with early results suggesting that five cycles are superior; thus that strategy was subsequently used in the current U.S. trials.[397] However, more recent data from the MRC have shown that the fifth cycle added toxicity with no additional therapeutic benefit.[411] These findings are reflected in the current COG trial in which only four cycles of chemotherapy are used for good-risk patients with elimination of the high-dose cytarabine and asparaginase cycle (Capizzi II) that previously comprised the fifth cycle on AAML0531 and AAML03P1.[409,410,412,413] High-risk patients for whom a donor is not identified also only receive four cycles of chemotherapy, but they do receive Capizzi II as their last consolidation cycle. The SJCRH trial has tried to make up for the lack of amsacrine by tailoring the first postinduction cycle based on the patient's AML subtype and cytogenetics. On AML02, patients with t(9;11) or inv(16) AML receive cytarabine and cladribine, patients with M4/M5 AML receive cytarabine and etoposide, and patients with t(8;21) and all others receive high-dose cytarabine

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*References 131, 255, 310, 321, 364, 399, 400, **401**, 402, **403**, 404, and 405.

†References 311, 316, 389, 394, and 406-410.

and mitoxantrone[311,414-419] followed by two courses consisting of cytarabine and asparaginase followed by cytarabine and mitoxantrone.[409,410,412,413] However, based on small subgroup numbers, AML08 uses a single backbone for all patients with the addition of sorafenib for patients with *FLT3*-ITD (J. Rubnitz, personal communication, January 2013). CNS leukemia in patients with AML is highly sensitive to chemotherapy, and the addition of cranial irradiation does not decrease CNS relapse compared with systemic and intrathecal chemotherapy. Therefore, cranial irradiation is no longer part of the therapy for patients with AML.[420] However, CNS-positive patients defined on the current COG AML study as having even one blast in the cerebrospinal fluid are treated with additional intrathecal cytarabine until the fluid is clear of blasts and for two additional intrathecal treatments beyond. Furthermore, in pediatrics, the addition of prolonged low-intensity maintenance therapy is associated with lower OS because of more resistant disease at relapse, so maintenance therapy is not used in the current U.S. trials.[421-423]

Supportive Therapy at Diagnosis and during Therapy

The diagnosis and treatment of childhood AML require a multidisciplinary approach involving pediatric oncologists, hematopathologists, pediatric surgeons, infectious disease specialists, pediatric intensivists, and pediatric nurses. While studies and procedures are initiated to make the diagnosis, special care must be taken to prevent and treat the life-threatening consequences of leukemia and accompanying pancytopenia. The most serious complications of leukemia at diagnosis are infection and bleeding with pancytopenia, tumor lysis syndrome, and leukostasis. The most common causes of death once remission is achieved are relapse, infection, hemorrhage, and cardiac events.[385,424] The improvement in OS of patients with AML over the past several decades is attributable as much to better supportive care efforts as to more effective treatment regimens.[425]

Infectious Complications. At the time of initial presentation, up to 40% of patients with leukemia exhibit fever, and most of these patients are neutropenic or have impaired neutrophil function.[330] During remission induction and subsequent cycles of chemotherapy, all AML patients experience profound prolonged neutropenia and are at high risk for life-threatening bacterial infections, particularly viridans group streptococcal infections.[424,426-428] Almost all AML patients experience at least one febrile neutropenic episode during therapy, and approximately 40% of these patients will have a documented infection; most commonly these are bacterial infections, followed by viral infections and then fungal infections. Infections account for nearly two thirds of deaths in patients in remission.[385,424,428] Therefore supportive care during induction and postremission therapy must pay particular attention to prophylactic, empiric, and treatment coverage for infectious complications. Several strategies are used to prevent infection or decrease mortality from infection. Some centers isolate patients receiving AML therapy in laminar airflow rooms during episodes of neutropenia.[424,429] The supportive care guidelines for the CCG 2961 study included mandatory hospitalization for all patients, whether febrile or not, until the absolute phagocyte count had risen, and this rule has been strongly recommended in current COG trials. On CCG 2961, almost 50% of patients developed gram-positive infections and approximately 25% developed gram-negative infections across all phases of therapy. Infection-related mortality was 11%, including death from fungus. Bacterial agents included coagulase-negative staphylococci and α-hemolytic streptococci (including viridans streptococci) as well as *Klebsiella* and *Pseudomonas* species.[430] To decrease the rate of bacterial infections, some programs routinely prescribe the use of gut decontamination, mouthwashes, or prophylactic antibiotics.[385,425] A study by SJCRH demonstrated that initiation of antibiotics at the onset of neutropenia prevented sepsis, with the optimal regimen including both vancomycin and ciprofloxacin.[431] This remains an area of controversy because, in some studies, although prophylaxis may decrease the rate of bacterial infection it may also be associated with an increase in drug-resistant organisms. Further investigation is indicated in a prospective randomized fashion before it is widely adopted.[424,429,432-438] For example, in the SJCRH study, an earlier regimen of vancomycin and cefepime resulted in two cases of life-threatening resistant gram-negative bacteria and one of *Bacillus cereus*, leading to the change to ciprofloxacin for gram-negative coverage.[431] The COG is currently conducting a study of a related fluoroquinolone, levofloxacin, for bacterial prophylaxis in AML and patients with relapsed ALL. Patients will be randomized to receive levofloxacin versus no bacterial prophylaxis from the initiation of chemotherapy for two consecutive cycles. In contrast to the controversy regarding bacterial prophylaxis, there is broad agreement that patients who are febrile at initial presentation and patients who experience febrile neutropenia during therapy should be immediately started empirically on broad-spectrum antibiotics, including vancomycin, once cultures have been obtained.[439-441] In many centers, vancomycin is discontinued after 48 to 72 hours if there is no evidence of gram-positive bacteremia.[424] Typhlitis, or generalized bacterial infection of the cecum, is more common in children than in adults and is more common in patients treated with doxorubicin instead of daunorubicin.[442,443] Although this is a potentially life-threatening complication, conservative management with broad-spectrum antibiotics and nothing by mouth is generally preferable to surgical treatment in the setting of profound neutropenia.[444]

AML patients are at high risk for invasive fungal infection, which carries a high risk for mortality. The routine use of fungal prophylaxis is becoming more routine given the intensity of AML protocols. Data from the CCG 2961 study demonstrated *Candida* and *Aspergillus* infections in approximately 13% of patients across all phases of therapy and a strong association with infection-related mortality.[430] Some early studies have shown a decrease in some *Candida* species with fluconazole prophylaxis, but an increase in *Candida krusei* infections was documented, with no decrease in infection by invasive molds.[445-447] Two meta-analyses have concluded that there is a reduction in morbidity because of invasive fungal infections when prophylaxis is used for those with prolonged neutropenia and post-HSCT patients.[448,449] A randomized controlled trial of posaconazole versus fluconazole or itraconazole as fungal prophylaxis in neutropenic patients with AML or MDS has shown a lower rate of invasive fungal infections and improved OS in the posaconazole group.[450] However, posaconazole has not been studied in children younger than age 13 years and, owing to exclusively oral administration, may have limitations during administration of emetogenic AML chemotherapy. Voriconazole has been studied as a prophylactic agent that has activity against both yeast and aspergillus, but the incidence of invasive fungal infections was no different compared with fluconazole or itraconazole.[451-453] Voriconazole, similar to posaconazole, is metabolized through hepatic CYP450, leading to numerous interactions with other drugs and contributing further to a lack of enthusiasm in using either of these agents prophylactically. Thus fluconazole remains the most frequently used agent for fungal prophylaxis but lacks activity against *Aspergillus*.[451,454] Currently the COG is conducting a study randomizing patients with AML to caspofungin versus fluconazole for fungal prophylaxis. Caspofungin is from the echinocandin family of antifungal agents that functions by targeting fungal cell wall biosynthesis of β-1,3-glucan. It is effective against both *Candida* and *Aspergillus* species and has safety data in children as young as 3 months of age.[455-457] Caspofungin is given intravenously and is generally well tolerated but has minimal CNS penetration.

A test to detect galactomannan-containing *Aspergillus* antigens in the sera of immunocompromised patients has become commercially available. At this point, the galactomannan assay has been used most advantageously to follow the status of invasive aspergillosis and its response to therapy.[458,459] However, some centers do not use fungal prophylaxis during AML therapy but rather screen twice weekly with the galactomannan assay and use a low threshold for computed tomography (CT) to detect early invasive disease.[460,461] Whether or not fungal prophylaxis is used, empirical fungal therapy should be initiated for neutropenic patients who remain febrile for longer than 5 days, although some centers use a threshold of 72 hours. Patients who need empiric fungal therapy should be routinely scanned during neutropenia and after the recovery of circulating neutrophils be screened for evidence of fungal disease, and the diagnosis of fungal infection should be made by biopsy whenever feasible. Amphotericin has long been the antifungal agent of choice for empiric therapy, and the liposomal forms appear to be equivalent in efficacy and less toxic to renal function.[462,463] One prospective randomized trial has shown that caspofungin as empiric fungal therapy is as effective as liposomal amphotericin B and is generally better tolerated.[464] Whereas caspofungin is well tolerated with few drug interactions, as mentioned earlier, it does not cross the blood-brain barrier and thus is ineffective at treating suspected or proven CNS fungal infections. Voriconazole as empiric fungal therapy was compared with amphotericin in a prospective randomized trial and found to provide comparable coverage with less renal toxicity but with more visual changes and hallucinations.[465] Voriconazole is seldom used empirically unless *Aspergillus* is highly suspected, such as in the case of prolonged febrile neutropenia with pulmonary lesions on imaging.

All patients undergoing AML therapy should receive prophylaxis against *Pneumocystis carinii* (now called *P. jirovecii*) pneumonia (PCP). The first-line agent in PCP prophylaxis is trimethoprim-sulfamethoxazole, but in some patients this is associated with significant allergic reaction or prolonged bone marrow suppression.[466-468] Dapsone is also effective as PCP prophylaxis in pediatric oncology patients but is associated with significant hemolysis and methemoglobinemia in some patients.[469] Atovaquone has been shown to be highly effective as PCP prophylaxis for pediatric leukemia patients, although it may be associated with gastrointestinal side effects, including pancreatitis.[470] Aerosolized pentamidine has also been shown to be effective as PCP prophylaxis in children with leukemia, although it may be associated with significant bronchospasm and can only be administered to children old enough to comply with the inhalational administration (usually >5 years).[471]

Viral infections can be a significant cause of morbidity and mortality in AML patients as well. CCG 2961 and COG AAML0531 advocate intravenous immune serum globulin (IVIG) administration for low IgG levels, and some centers also recommend respiratory syncytial virus (RSV) prophylaxis for infants. All blood products transfused should be leukoreduced and cytomegalovirus (CMV) negative whenever possible. Acyclovir may be required for patients with recurrent zoster or mucocutaneous herpes simplex.[472]

Growth factors, such as G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF), have been used in the past, both as priming agents to synchronize the cell cycle before chemotherapy and as supportive care to decrease the duration of neutropenia after chemotherapy. Trials have shown that although G-CSF does shorten the duration of neutropenia its use does not lead to fewer episodes of febrile neutropenia, fewer microbiologically documented infections, less infection-related mortality, or improved OS.[425,473-476] Based on these data, most centers no longer advocate the routine use of growth factors as supportive care during AML therapy.

Bleeding Complications. Severe thrombocytopenia is not unusual in leukemia patients, and hemorrhage is one of the leading causes of early mortality in these patients.[477-479] AML patients who present with bleeding and thrombocytopenia should be treated with platelet transfusions. In patients who are thrombocytopenic but do not have active bleeding, concern still remains about spontaneous bleeding, particularly intracranial bleeding, which can be catastrophic and have irreversible sequelae. The level of thrombocytopenia that warrants prophylactic platelet transfusion to prevent bleeding, however, is controversial. Some early observations suggested that platelet counts less than 20,000/μL predispose to increased bleeding, and that number became a standard threshold for transfusion.[480] However, this was primarily in patients also receiving aspirin therapy, and subsequent studies have shown spontaneous bleeding occurs at a similar rate with platelet counts of less than 10,000 or 20,000/μL; and many centers have adopted 10,000/μL as the new threshold for transfusion.[481-487] The threshold for platelet transfusion in patients undergoing procedures such as line placement or lumbar puncture has also not been well studied, and the accepted standard threshold of 50,000/μL may be higher than necessary.[480,488] All blood products should be leukoreduced or CMV negative to prevent CMV infection, and irradiated to prevent transfusion-related GVHD.[489-491] Menstruating women may require hormonal menstrual suppression for the duration of therapy with expected platelet drops to less than 50,000/μL.

Patients with AML, particularly APL, may also present with DIC, and coagulation studies should be a routine part of the initial workup in a patient with AML.[492,493] In APL, overexpression of one of the plasminogen receptors, annexin A2, has traditionally been implicated as the cause of increased production of the fibrinolytic protein plasmin, which may be part of the cause of DIC in these patients.[494] However, recent in vitro studies performed in APL cell lines suggest that, in fact, a different plasminogen receptor, S100A10 (also known as p11), may actually be the culprit. Levels of S100A10 increased concomitantly with *PML-RARA* induction, and targeted inhibition of SA100A10 expression resulted in decreased fibrinolytic activity.[495] Patients whose leukemic cells are successfully treated will exhibit resolution of DIC with the reduction in blast cells but, while the blast count is decreasing, DIC should be treated with fresh-frozen plasma, cryoprecipitate, or recombinant factor VIIa as needed to prevent bleeding. For patients with APL, the initiation of ATRA before chemotherapy leads to rapid resolution of DIC and has markedly reduced the early mortality previously associated with APL.[338-340,496] Since the development of ATRA, patients with APL are no longer routinely treated with low-dose heparin to prevent DIC.[335,336]

Complications of Hyperleukocytosis. Patients with AML, particularly those with high presenting leukocyte counts, have an increased risk for tumor lysis syndrome. In patients with leukemia, the rate of blast cell turnover can exceed the body's ability to metabolize and excrete the contents of dying cells, leading to life-threatening metabolic derangements.[497] Hyperuricemia resulting from the metabolism of excessive amounts of released nucleic acids can cause renal failure. The ensuing hyperkalemia may lead to fatal cardiac arrhythmias, and hyperphosphatemia and hypocalcemia may cause seizures and worsen the renal failure. Elevated blood urea nitrogen levels cause platelet dysfunction, which may exacerbate an existing coagulopathy. Vigorous intravenous hydration and administration of bicarbonate and other agents, such as allopurinol or recombinant urate oxidase (rasburicase), should be started immediately to decrease formation and increase the solubility and renal excretion of insoluble urate.[498-500] Recombinant urate oxidase can dramatically decrease uric acid levels within a few hours but, in patients with G6PD deficiency, it can cause methemoglobinemia and life-threatening hemolysis.[501,502]

Patients with WBC counts higher than 100,000/μL have an increased risk for leukostasis. Leukostasis causes intravascular clumping of blasts, leading to sluggish blood flow and subsequent hypoxia, hemorrhage, and infarction of tissue.[331,332,341,342,503] Leukostasis can be life threatening when it occurs in the organs at greatest risk, the brain and lungs. Patients with pulmonary leukostasis present with significant hypoxia and tachypnea, which may progress rapidly to respiratory failure. CNS leukostasis may produce symptoms of confusion, headache, and somnolence and can lead to coma, stroke, and death. Hydration and hydroxyurea are therapies that can be initiated immediately to decrease the blast count. Leukapheresis or exchange transfusion can reduce the number of circulating blasts quickly, although this effect is transient, and therapy with cytoreductive drugs should be started as soon as possible.[503] Routine pheresis for patients with a WBC count higher than 100,000/μL has resulted in lower mortality rates during induction compared with historic controls. In one study, patients who achieved a higher than 30% decrease in total WBCs through pheresis had a higher CR rate than those who achieved less than a 30% decrease.[341,504,505] Other retrospective reviews have shown a decrease in early mortality after leukapheresis but not an improvement in OS. Cytoreductive chemotherapy should be initiated as rapidly as possible because other measures to reduce leukemic blast numbers have only temporary effectiveness.[506,507]

Chemotherapy Complications. High-dose cytarabine is associated with chemical conjunctivitis, and dexamethasone or prednisolone eye drops were traditionally used to prevent this side effect.[508,509] However, recently owing to the unavailability of this steroid formulation, artificial tear products have been effectively substituted. High-dose cytarabine is also associated with neurotoxicity, including seizures and cerebellar dysfunction, which may not be reversible.[510-512] Patients should be monitored with frequent neurologic examinations, and high-dose cytarabine should be immediately discontinued if cerebellar signs develop. Cytarabine may also be associated with an impressive febrile response, which is difficult to distinguish from an infection-related fever.[513,514] AML chemotherapy is associated with significant nausea and vomiting, and aggressive antiemetic regimens should be instituted. However, because of the high risk for fungal infection in these patients, dexamethasone should be avoided as an antiemetic.[515-517] Mucositis is a significant toxicity during AML therapy, predominantly due to anthracyclines, and almost all patients require parenteral pain medication and nutrition. Malnutrition at the time of diagnosis appears to carry a worse prognosis in children with leukemia and, even in those who are well-nourished at diagnosis, weight loss during chemotherapy should be monitored closely, followed by the initiation of parenteral or enteral feedings in those who lose more than 10% of their initial body weight.[518]

Renal toxicity during AML therapy is common and may be caused by a combination of chemotherapy, tumor lysis syndrome, sepsis, or treatment with antibacterial, antifungal, and antiviral agents. A creatinine clearance or glomerular filtration rate should be checked in patients with abnormal creatinine clearance or prolonged use of medications with renal toxicity. For patients with a creatinine clearance lower than 60 mL/min/1.73 m^2, high-dose cytarabine, etoposide, and other renally cleared chemotherapy agents are usually given at reduced dosages. Significant liver toxicity can also be observed during therapy for AML, particularly for regimens containing the humanized anti-CD33 monoclonal antibody, GMTZ. GMTZ has been associated with sinusoidal obstructive syndrome and veno-occlusive disease, most commonly when used as a component of combination therapy or in patients who are heavily pretreated or who have undergone prior HSCT or undergo subsequent HSCT within 3 months of treatment with GMTZ.[519]

AML regimens rely heavily on anthracyclines and their derivatives. The cumulative anthracycline dose in these regimens is often 300 mg/m^2 or higher in daunorubicin equivalents and therefore carries a high risk for cardiac toxicity. Cardiac deaths are one of the top three causes of death in remission for AML patients.[385,425] All patients should undergo cardiac assessment before administration of chemotherapy cycles containing anthracyclines and yearly after chemotherapy is complete, until growth is completed in adulthood. However, recent pharmacogenomic studies suggest that certain genotypes, such as the rs7853758 variant within the SLC28A3 solute transporter, may particularly predispose children to developing anthracycline-induced cardiotoxicity.[520,521] In vitro evidence has suggested that the cardioprotectant dexrazoxane works synergistically, not antagonistically, with anthracyclines against AML blasts, and there is increasing interest in incorporating this medication into AML clinical trials.[522-524] On the current COG AML trial, use of dexrazoxane is permitted at the discretion of the treating physician and data will be collected to determine the impact on both cardiac outcome and secondary malignancy.

Extramedullary Disease

Between 20% and 40% of children with AML have evidence of extramedullary disease at the time of diagnosis.[350,353] The most common manifestations of extramedullary disease are skin infiltrates, soft tissue or bone involvement, gingival infiltration, and CNS involvement. Some patients, particularly infants, may have isolated extramedullary leukemia without other evidence of disease, but bone marrow infiltration invariably occurs in these patients without systemic therapy.

CNS Involvement. CNS involvement is more common in AML patients with high presenting WBC counts, favorable cytogenetic changes—t(9;11), t(8;21), and inv(16)—and children younger than 2 years.[346,420,525] One recent retrospective review of almost 1500 children enrolled on COG AML trials found an incidence of CNS disease (defined as CNS3 status >5 WBCs per high power field with the presence of blasts) of 11%.[526] AML patients with CNS disease at diagnosis have a higher risk for CNS relapse, but they often have a better overall prognosis because CNS disease is more common in the favorable-risk cytogenetic groups, which carry a lower risk for systemic relapse.[525,527] Specific CNS-directed therapy in AML may also be less important than in ALL because several AML chemotherapy agents, including high-dose cytarabine, have excellent CNS penetrance. Cranial radiation was previously used in AML patients as prophylaxis against CNS relapse, but because of concerns about the myriad side effects of CNS radiation, including secondary malignancy, endocrine and growth impairment, and neurocognitive toxicity, later trials attempted to minimize radiation.[528,529] In the BFM-87 study, all patients received intrathecal cytarabine, but the low-risk patients were randomized to additional therapy with cranial radiation or no further CNS treatment. An interim analysis demonstrated no increase in CNS relapse in the group that did not receive cranial radiation, so the radiation arm of the trial was closed early.[349] CNS radiation therapy is no longer routinely part of AML treatment protocols. Intrathecal chemotherapy with cytarabine alone, methotrexate alone, or triple intrathecal therapy with cytarabine, methotrexate, and hydrocortisone have been reported to be effective as CNS treatment and prophylaxis.[420,525,530,531] As prophylaxis for CNS-negative patients, COG study AAML0531 used intrathecal cytarabine alone at the start of each cycle, except for the Capizzi II cycle, which includes 3 g/m^2 of systemic cytarabine. For patients who had CNS disease at diagnosis, twice-weekly intrathecal therapy with cytarabine alone was used until the cerebrospinal fluid was clear, plus an additional two doses, with a minimum of four doses and a maximum of six doses. If more than six doses were required, the patient was taken off study for refractory CNS disease. These guidelines have been maintained on the current AAML1031 study. The SJCRH AML02 trial used a prophylaxis regimen of triple intrathecal chemotherapy with cytarabine, methotrexate, and hydrocortisone given at the start of each cycle but separated by at least 24 hours from systemic cytarabine dosages greater than or equal to 1 g/m^2 to prevent neurotoxicity. Patients with CNS disease at diagnosis receive once-weekly triple intrathecal chemotherapy for four doses. To decrease mucosal toxicity from the methotrexate, all patients receive leucovorin rescue at 24 and 30 hours after the intrathecal administration.

CNS relapse occurs in 2% to 8.8% of patients with AML.[409,420,532] Most AML patients who suffer a CNS relapse will simultaneously or soon thereafter develop a bone marrow relapse. Truly isolated CNS relapse is rare and is associated with age younger than 2 years, hepatosplenomegaly at diagnosis, elevated presenting WBC count, M5 AML, and chromosome 11 abnormalities.[527] For those with a truly isolated CNS relapse, the optimum treatment plan is unclear. There have been survivors reported after treatment with intrathecal chemotherapy alone, combined intrathecal and systemic chemotherapy, radiation therapy, and HSCT.[346,527]

Chloromas. Extramedullary infiltrations of the soft tissue, also known as chloromas, myelosarcomas, or granulocytic sarcomas, occur in 4% to 5% of children with AML.[344,350,533] They may also be documented in patients with CML, MDS, or rarely in ALL and may be initially confused with non-Hodgkin lymphoma.[534] Chloromas most often present simultaneously with AML or shortly before the systemic evidence of disease, but they may be isolated. The prognostic significance of extramedullary disease is not clear. Some groups have reported an unfavorable prognosis associated with chloromas,[382] but others have demonstrated a more favorable outcome.[407,532] Blast cells from patients with chloromas often exhibit the relatively favorable t(8;21).[353,354,535] Most studies from Europe, Saudi Arabia, Japan, and the United States have reported no influence of chloromas on the OS of children with AML,[343,350,382,387,409,536] and a recent review of four COG studies in fact demonstrated that children with orbital or CNS-based chloromas had significantly higher CR rates and OS compared with counterparts without chloromas treated on these same studies.[526] Accordingly, most centers treat patients who have chloromas with standard AML regimens, because there is no consistent evidence supporting more intensive therapy.[350] A beneficial role for radiation therapy in the treatment of chloromas has not been demonstrated.[536] In a CCG trial, patients with chloromas were treated with local irradiation in addition to systemic therapy. There was no difference in EFS or in the incidence of local recurrence compared with the chemotherapy-only regimen. However, chloromas arising in the orbit or CNS may cause loss of vision or cord compression, and emergency radiation therapy as well as chemotherapy is indicated in these situations.[536]

Prognosis

Although historically based exclusively on morphologic subtype, the ability to predict prognosis for patients with AML in the current genomic era derives from a composite of cytogenetic features, molecular subtype, and response to initial chemotherapy as evaluated by MRD. The recent integration of these features on the background of more intensive therapy with better supportive care has enabled a gradual improvement in pediatric AML outcome (Fig. 51-6). Classically, FAB subtype M0, which commonly harbors a complex karyotype, abnormalities of 5 or 7, or trisomy 8, is associated with a poor prognosis.[537] M7 AML (non–Down syndrome), often associated with monosomy 7, carries a poor prognosis.[538-542] M3 AML with the t(15;17) a group previously associated with a dismal prognosis, has emerged as a highly favorable subtype owing to responses with the addition of ATRA and As_2O_3 to chemotherapy regimens.[543-548] Similarly, CBF AML with t(8;21) or inv(16) and normal karyotype AML with either an isolated *CEBPA* or *NPM1* mutations are favorable subtypes.[403,549-551,552] By contrast, *FLT3*-ITD mutations with high allelic ratios are associated with a poor prognosis, as are patients without good-risk molecular features who have significant residual MRD after induction therapy.[401-403,553]

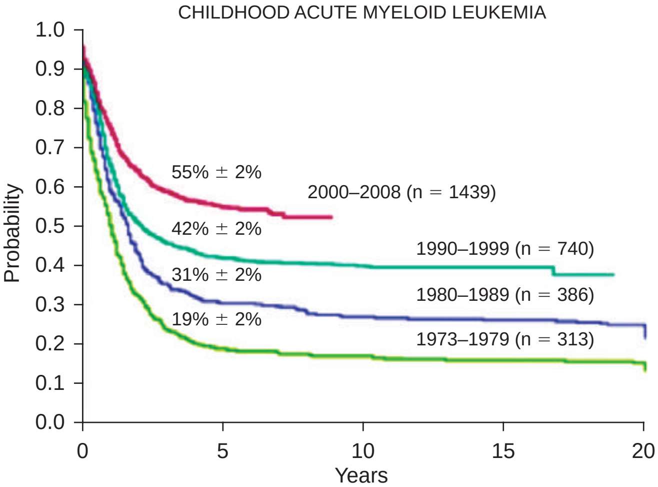

Figure 51-6 Overall survival for pediatric acute myeloid leukemia from 1973 to 2008. *(Modified from Rubnitz J, Inaba H: Childhood acute myeloid leukemia.* Br J Haematol *159:259–287, 2012.)*

Demographic Risk Factors

Age older than 10 years was historically reported to carry a worse prognosis in clinical trials at SJRCH, as well as from the BFM and Japanese groups.[554-556] However, a recent report from the SJCRH AML02 study demonstrated comparable EFS and OS in children as old as age 21 years as in their younger counterparts, although these older children had higher toxic death rates predominantly of infectious etiology.[557] Race and ethnicity play a role in prognosis as well. Patients on CCG2891 who received chemotherapy alone had an OS of 48% for white children versus only 37% for Hispanic children ($P = .016$) and 34% for black children ($P = .007$).[558] On the SJCRH AML97, there was a trend toward inferior outcomes for black compared with white children (hazard ratio, 1.61; 95% confidence interval, 0.76 to 3.42; $P = .22$ with 5-year survival rates of 55.6% vs. 27.3%).[559] In these trials, there was no difference in other prognostic variables between the groups and these patients were hospitalized and received intravenous chemotherapy, received the same supportive care, and had the same compliance rates. Therefore, the observed differences may be attributable to pharmacogenomic differences that affect response to and toxicity from chemotherapy.[538,558] Patients who are overweight (body mass index ≥95th percentile) or underweight (≤10th percentile) also carry a worse prognosis and are less likely to survive and more likely to suffer treatment-related mortality.[560,561]

Cytogenetic Factors

Results of several large multicenter trials have confirmed the correlation between certain cytogenetic abnormalities and clinical outcome and have shown that karyotype in AML represents an independent prognostic variable for attainment of complete response and OS.[310,312,313,316,562] In children and adults, the highest complete response rates and longest survival times have been linked to association with t(8;21) and inv(16) or t(16;16).[403,549-552] These translocations result in modifications to the core-binding complex, a group of proteins that appear to play a vital role in hematopoiesis. AML cells with inv(16) have increased incorporation of cytarabine into nuclear DNA and are more susceptible to cytarabine chemotherapy in vitro.[563] Patients with APL and t(15;17) previously represented a poor-risk group, but, since the advent of ATRA and As_2O_3 therapy (see later), now represent a group with a favorable prognosis.[543-548] Other cytogenetic aberrations have been associated with an unfavorable outcome. These include monosomy 5, del(5q), monosomy 7, abnormalities of 3q, aberrations of 12p, and complex cytogenetic abnormalities.[310,315,564] For pediatric patients with monosomy 7, several studies have shown better OS when HSCT is used as consolidation therapy as opposed to conventional chemotherapy.[541-542] Patients with normal karyotypes have traditionally been grouped together in an intermediate-prognosis group, but now mutations such as the *FLT3*-ITD (poor prognosis) and *NPM1* and *CEBPA* (good prognosis) have been demonstrated to alter prognosis sufficiently to influence the risk assignment of these patients at diagnosis on COG, SJCRH, BFM, and MRC trials. Abnormal karyotypes linked with an intermediate prognosis include del(7q), 11q23 translocations, +21, +8, +22, and del(9q).[310,565] Other karyotypes such as t(6;9)(p23;q34), t(8;16)(p11;p13), and t(16;21)(q24;q22) are uncommon but appear associated with a poorer prognosis.[557,566-568] While more studies with large numbers of patients undergoing cytogenetic evaluation are completed, certain groups of karyotype aberrations are showing heterogeneity in their prognostic significance. For example, all 11q23 translocations were previously grouped together in the intermediate- to poor-prognosis category. However, studies have shown that the 11q23 aberration may be subdivided into groups of lesser and greater prognostic risk.[569] Patients with the t(9;11)(p22;q23) have been found in some studies to have a longer EFS and OS than patients with other 11q23 translocations.[247,312,314,315,565,562] However, this was not found to be true in a recent review of outcome on MRC10 and 12 trials.[366] As new therapeutic agents are developed that target the specific molecular aberrations identified by cytogenetic analysis, accurate detection of these abnormalities becomes even more important.[565]

Molecular Genetic Factors

Analysis of molecular genetics is emerging as an invaluable tool in prognostic determination for patients with AML, as well as for providing insight into the pathogenesis of AML and elucidating possibilities for targeted therapy.[136] *RAS* mutations have been identified in pediatric AML patients, with *NRAS* mutations in 10% to 15% and *KRAS* mutations in 3% of patients.[570] *HRAS* mutations are rare in AML. In both adults and children these mutations are associated with favorable-risk AML. In adults, the association is with CBF leukemias and, in particular, inv(16).[570,571] Although some pediatric studies have also found a link between CBF AML and *NRAS* mutations,[570,571] others have found a distinct absence of *NRAS* with inv(16) and a unique correlation of *NRAS* and *NPM* mutations not seen in adult AML.[157] Independently, however, *NRAS* mutations have no significant impact on prognosis.[153,572,570,573-576] Patients had similar 5-year EFS and OS, although patients with *NRAS* mutations were associated with increased treatment-related mortality on one study.[157] *KIT* mutations have been identified in 11.3% of pediatric AML patients,[570] predominantly in the CBF leukemias, ranging from 17% to 41% of patients.[178,577-580] The role of *KIT* mutations in prognosis has been unclear: in some series, the *KIT* mutations do not seem to have prognostic significance, in others, *KIT* mutations have been shown to confer worse prognosis in patients with t(8;21) but not in patients with inv(16) and, in still other trials, *KIT* mutations confer a worse prognosis in all subsets of CBF leukemias.[570,571,581,582] However, a recent review of more than 200 children with CBF AML enrolled on four COG AML trials concluded that *KIT* mutations do not alter prognosis.[178] Owing to their good outcome with chemotherapy alone, patients with CBF AML are currently nonrandomly assigned to chemotherapy without HSCT in CR1 even if a matched family donor is available. Based on these recent data, on current COG trials the presence of a *KIT* mutation does not alter risk stratification. This is in contrast to adult data which show that *KIT* mutations appear to confer an inferior outcome.[583-585] These prognostic differences in adult versus pediatric CBF AML may reflect more aggressive therapy in children and, potentially, an earlier cell of origin in the adult disease that contributes to increased resistance to therapy.

As noted, *FLT3* mutations include ITDs in the juxtamembrane domain and point mutations in the activation loop (ALM). *FLT3*-ITDs have a worse prognosis, particularly when present in a high allelic ratio (>0.4),[174,586,587-590] whereas *FLT3*-ALMs seem to have no impact on prognosis.[174,586,589] Partial tandem duplications of the *MLL* gene have also been reported in 13% of pediatric AML patients and are associated with a worse prognosis in adult and pediatric patients.[228,586,591,592] Frameshift mutations in *NPM1* lead to cytoplasmic localization of nucleophosmin and have been identified in 6% to 8% of all children with AML and 25% to 30% of pediatric AML patients with normal cytogenetics.[277,278] When identified as an isolated mutation in adults and children, it is associated with improved prognosis, but when found in conjunction with an *FLT3*-ITD, the poor prognostic impact of the *FLT*-ITD prevails.[277,278,593-595] With contemporary chemotherapy, children with AML with a normal karyotype and isolated *NPM1* mutation have outcomes in keeping with CBF leukemia with OS greater than 80%; thus these children have been assigned to the low-risk arm on the current COG trial[557] (R. Aplenc, personal communication, June 2011). *PTPN11* mutations are found in only 4% of pediatric patients with AML (4%), and 18% to 36% of the *PTPN11* mutations are seen in M5 AML. In pediatrics, this mutation does not appear to have prognostic importance.[142,596] The Wilms tumor gene *(WT1)* is aberrantly expressed in AML; in adults, high expression levels of WT1 at diagnosis have been associated with a poor prognosis in many studies.[597-602] In pediatric patients, however, the available data have been contradictory. One small study of 47 patients has shown that low levels of WT1 at diagnosis are associated with good outcome,[603] whereas another small study of 41 patients has shown that high levels of WT1 are more commonly seen in association with the t(8;21) and inv(16) favorable prognosis cytogenetic findings, and therefore associated with a better prognosis.[604] Hollink and colleagues found an association between *WT1* mutations and an inferior outcome.[282] More recently, a large study performed by the COG identifying 70 patients with *WT1* mutations found that although there was significant overlap of the *WT1* mutation with either CBF or *FLT3*-ITD, independently, *WT1* mutations did not influence prognosis.[281] Interestingly, 28% of pediatric AML patients harbor a minor single nucleotide polymorphism (SNP) in exon 7 of *WT1* (rs16754) that was found to result in an improved 5-year OS (62% vs. 44%) for patients treated on CCG 2961 compared with those with leukemic expression of the major *WT1* allele.[289] Levels of expression of wild-type WT1 may also influence prognosis. However, at present, *WT1* mutational status, presence of the minor SNP, and wild-type expression levels continue to be studied but are not being used to make treatment decisions. In addition to its role as a partner in the t(8;21) translocation, *RUNX1* may also be mutated in approximately 5% of pediatric AML patients, specifically with point mutations in exons 3 and 8. Although typically associated with AML secondary to MDS, in de novo AML these patients had less organomegaly, a higher incidence of chloromas, and a trend toward improved outcome with modern intensive AML therapy.[605] However, *RUNX1* mutations are not currently used for risk stratification.

CEBPA mutations have been found to be an independent prognostic marker conferring a good outcome in both pediatric and adult AML and, similar to that seen for CBF AML, at least in children.[279] Interestingly, pediatric patients with AML and *CEBPA* mutations tend to be older (age >10 years) and predominantly have a normal karyotype with an absence of other good- or poor-risk molecular abnormalities. Furthermore, patients with *CEBPA* mutations did not benefit from HSCT in first remission. Thus, in current studies, presence of a *CEBPA* mutation classifies a patient as low risk. Recent next-generation sequencing–based studies have revealed frequent mutations in the DNA methyltransferase gene, *DNMT3A,* the gene coding for the demethylating enzyme, *TET2,* and the genes *IDH1* and *IDH2*, which code for proteins in the Krebs cycle. *DNMT3A* mutations are generally associated with a poor outcome and contribute to a worse prognosis when combined with *FLT3* ITD. *TET2* mutations are similarly associated with a poor prognosis, with or without corresponding *FLT3* abnormalities. By contrast, *IDH1/IDH2* mutations appear to be more positive prognostic features.[606,607] However, these abnormalities are generally rare in pediatric AML[608]; thus their impact cannot be easily determined, and at this time they have been excluded from any current pediatric AML risk stratification schemas.

Minimal Residual Disease

Early response to treatment, as assessed by MRD in the bone marrow and/or the peripheral blood after induction therapy, has been shown to be an independent and powerful prognostic factor in pediatric leukemia. MRD can be detected by molecular means such as PCR assay, which is beneficial for the monitoring of leukemias with fusion genes or recurrent mutations. Alternatively, the use of multiparameter flow cytometry (MPFC) is more universally applicable, but it may be user dependent and vary significantly based on technical aspects, threshold levels, and timing of evaluation.

PCR-based MRD evaluation requires that a cytogenetic abnormality or mutation present at diagnosis will be predictive of relapse or refractory disease. In CBF AML, persistence of the *AML1-ETO* t(8:21) or *CBFB-MYH11* inv(16) transcripts long after completion of therapy has challenged the value of following these lesions either during or after completion of therapy.[557,609] However, a recent adult study[610] has suggested that at least in AML with inv(16), PCR analysis may have more predictive power. Negative bone marrow results of PCR assay for the *CBFB-MYH11* transcript (<10 detectable copies per cell) early in consolidation with two subsequent negative PCR evaluations either in the bone marrow or peripheral blood later in consolidation and within 3 months of completing therapy were highly predictive of remaining in CR, with a high correlation between bone marrow and peripheral blood measurements during surveillance. In APL and CML (see later), levels of *PML-RARA* or *BCR-ABL* transcripts have been found to be extremely valuable for monitoring, although the site of monitoring has been somewhat controversial. Standardized monitoring for *BCR-ABL* levels in the peripheral blood is now accepted as the standard of care in CML, whereas the recently closed COG trial in APL has mandated bone marrow evaluations during maintenance and subsequent follow-up. A number of adult and pediatric studies have examined following *FLT3, CEBPA, NPM1, MLL* rearrangements/PTDs, or *WT1* mutations in those patients harboring these abnormalities at diagnosis.[609,611-617] However, the utility of any of these mutations as a molecular MRD marker remains uncertain owing to stability of the mutation (*FLT3*-ITD) or normal background levels *(WT1)*. Thus, although their presence at diagnosis may be indicative of response, persistence of these lesions is not being routinely used to evaluate disease response in pediatric AML.

By contrast, use of MRD by MPFC has proven to be a more broadly applicable component of risk stratification in both pediatric ALL[618] and AML.[403,404,619] Particularly in the large subset of intermediate-risk AML, MRD has allowed the distinction of standard- and high-risk groups, whereas the use of morphology alone proved insufficient.[355] The ability of MPFC MRD measurement to contribute to the evaluation of early treatment response and improve prognostic evaluation in pediatric AML was demonstrated for CCG 2961. OS for patients in morphologic remission after induction I was 41% if MRD was positive, compared with 69% for those who were MRD negative.[404] A strong impact of MRD was also apparent in AML patients who were in morphologic remission at the end of induction I on the recent AAML03P1 study. The 46 MRD-positive patients of the 188 patients deemed

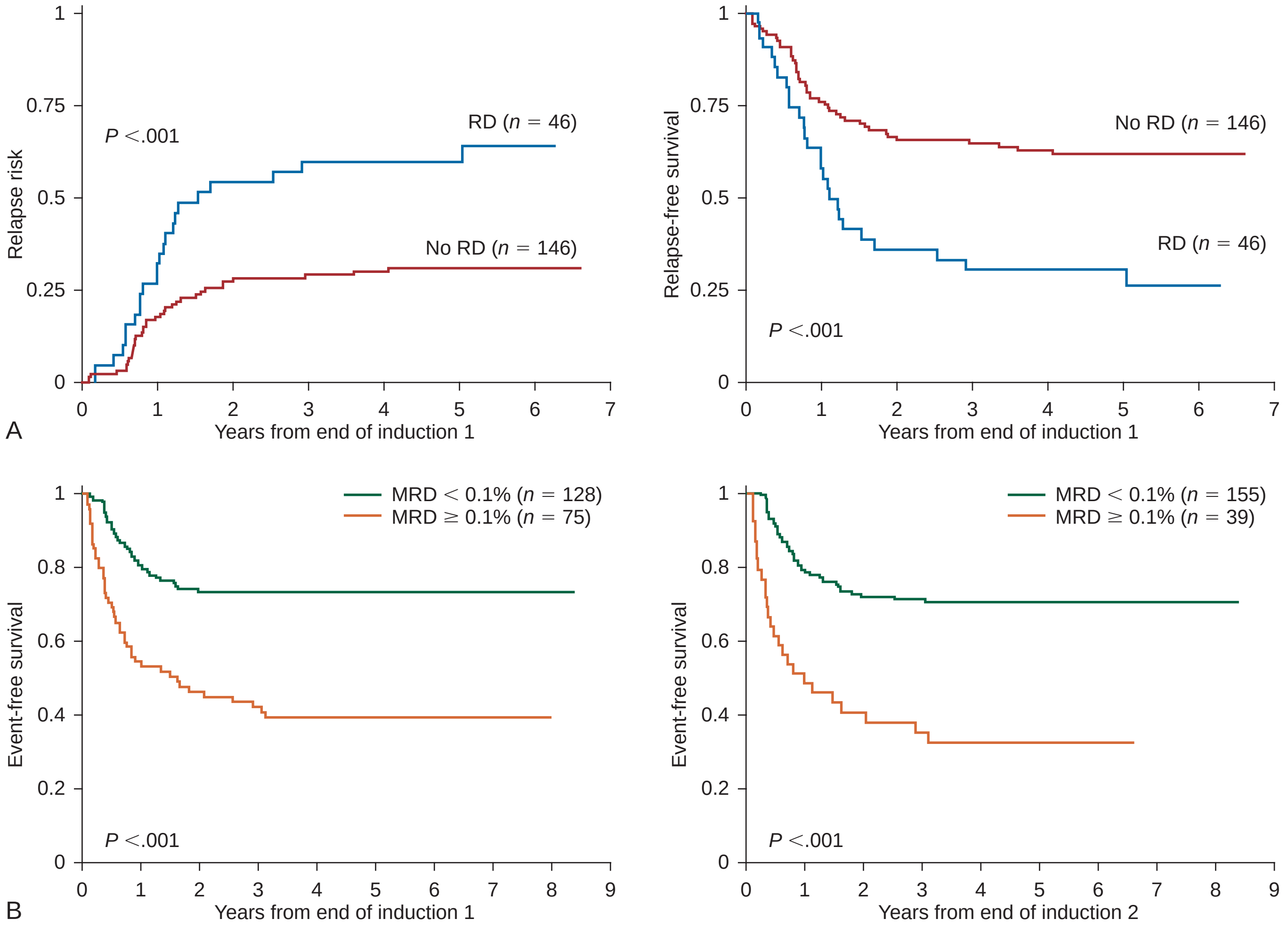

Figure 51-7 **A,** Minimal residual disease *(MRD)* by multiparameter flow cytometry (MPFC) can predict outcome for children with acute myeloid leukemia found to be in morphologic remission (*RD,* residual disease detected by MPFC). **B,** MRD levels less than 0.1% appear most predictive of event-free survival after induction therapy. *(Modified from Loken MR, Alonzo TA, Pardo L, et al: Residual disease detected by multidimensional flow cytometry signifies high relapse risk in patients with de novo acute myeloid leukemia: a report from the Children's Oncology Group.* Blood *120:1581–1588, 2012; and Inaba H, Coustan-Smith E, Cao X, et al: Comparative analysis of different approaches to measure treatment responses in acute myeloid leukemia.* J Clin Oncol *30:3625–3632, 2012.)*

in CR showed an increase in relapse risk (RR) at 3 years (60%, *P* <.001) and lower DFS (30%, *P* <.001) and OS (56%, *P* = .002) compared with those who were MRD negative (3-year RR, 29%; DFS, 65%; OS, 80%) (Fig. 51-7, *A*).[355] Not only did this study demonstrate the adverse prognostic value of positive MRD compared with morphology alone but, conversely, showed that 26% of patients deemed as having disease based on morphologic bone marrow evaluation were in fact MRD negative and had a good outcome.[355] Similar findings were identified by the SJCRH, confirming that recovering normal marrow blasts and leukemic myeloblasts can be challenging to distinguish morphologically even by the most experienced hematopathologist.[609] Multivariate analyses on COG and SJCRH studies confirmed positive MRD as an independent adverse prognostic factor, although the SJCRH study found that this was only significant for MRD levels greater than 1% after induction I, or greater than 0.1% after induction II,[403] whereas this distinction was not specifically identified in the COG studies. Subsequent analysis by the SJCRH demonstrated that an MRD threshold of 0.1% was independently predictive of outcome both after induction I and induction II (see Fig. 51-7, *B*).[609] The impact of MRD was independently confirmed in recent MRC and Dutch trials.[619] Furthermore, COG studies found that MRD levels held their prognostic significance in normal karyotype AML. Based on these data, the COG has used MRD by MPFC to eliminate the intermediate-risk group of patients without prognostic cytogenetic or molecular features. Those patients with residual MRD at a threshold of 0.1% after the first induction cycle are deemed at high risk and relegated to more intensive therapy, whereas those with MRD levels below 0.1% are allocated to a low-risk study arm.

Relapsed or Refractory Disease

The prognosis is grave for children with disease refractory to induction chemotherapy or who have a disease relapse. Although 80% to 90% of pediatric patients with AML will achieve remission, 30% to 40% will later recur, and the OS remains only 40% to 60%.[363,530,531,538,620-624] The bone marrow is the most common site of relapse in AML, and isolated CNS relapse is an uncommon finding, but isolated CNS relapse has been associated with prior CNS involvement.[386,527,625]

Prognosis in Relapsed or Refractory Disease

A durable remission is less likely to be achieved and maintained in patients with relapsed or refractory AML than in patients with newly diagnosed AML, but the salvage rate in these patients has improved in recent years.[538,624,626] The single most important prognostic indicator after relapse is duration of first remission, with less than 1 year to relapse carrying an OS rate of 0% to 21% and longer than 1 year to relapse carrying an OS rate of 33% to 62%.[386,539,624,627-631] Other characteristics that have been associated with better prognosis after relapse are male gender, good-risk cytogenetic features of t(8;21) and inv(16), chemotherapy alone without HSCT as consolidation during first CR, and the use of HSCT as part of relapse therapy. The characteristics associated with worse prognosis after relapse are early recurrence, M5 or M7 morphology, poor-risk cytogenetics, and HSCT during first CR.

Conventional Therapy for Relapsed or Refractory Disease

There is no accepted standard therapy for adult or pediatric patients with relapsed or refractory AML. However, several researchers have demonstrated activity of high-dose cytarabine in patients with relapsed disease, even in those who had been exposed to cytarabine during prior treatment.[632] High-dose cytarabine has been used in combination with other agents, such as mitoxantrone, asparaginase, etoposide, fludarabine, cladribine, and clofarabine.[628,633-638] The combination of cytarabine and mitoxantrone was particularly effective as reinduction with a remission rate of 76%, but now that this regimen has been incorporated into the current standard first-line regimens, its use as a reinduction regimen may provide too much cumulative anthracycline for many patients.[628] Fludarabine, cladribine, and clofarabine are all deoxyadenosine analogues that have been used for the treatment of AML. Trials using high-dose cytarabine in combination with fludarabine and G-CSF (i.e., the FLAG regimen) have demonstrated a 70% remission rate in patients with relapsed AML, and this regimen is frequently used by many centers for patients who have experienced a relapse of their disease.[639-641] The addition of idarubicin to the FLAG regimen has resulted in longer-lasting remissions in some studies, but it has also been associated with more significant toxicity and for this reason is not frequently incorporated.[642-645] The combination of cytarabine and cladribine has been successful as a relapse regimen in some trials for adult patients and pediatric patients with de novo AML, particularly those with the M5 subtype.[417,646-651] However, this combination seems to be of little benefit in pediatric patients with relapsed or refractory AML.[652] Clofarabine was found to be effective as a single agent in relapsed ALL and AML and was subsequently approved by the FDA for use in relapsed ALL.[636,653] Clofarabine has been shown to be safe and effective in combination with cytarabine in regimens for adult patients, and this combination has been tested for pediatric patients with relapsed or refractory AML.[637,638,654] The recently closed COG study AAML0523 examined the combination of clofarabine and cytarabine for relapsed AML and ALL and found that a dose of 52 mg/m^2 of clofarabine was tolerated by patients with relapsed AML. Of the 46 patients who were evaluable, only 21% responded as evaluated by complete response or complete response with incomplete platelet recovery, which did not meet the predetermined statistical threshold for efficacy in overall response rate.[655] Based on these findings, this regimen has somewhat fallen out of favor compared with FLAG. However, as a result of compliance issues and failure to recognize complete response with incomplete count recovery as evidence of response, results from this study may in fact underestimate the efficacy of this combination therapy; thus clofarabine and cytarabine for relapsed AML as a bridge to HSCT (see later) may still be worthy of consideration. For patients who cannot tolerate high-dose cytarabine or further anthracyclines, one alternative regimen that has been used at the Memorial Sloan-Kettering Cancer Center is topotecan, vinorelbine, thiotepa, dexamethasone, and gemcitabine (TVTG), with 5 of 9 AML patients responding to this therapy.[656]

Long-term remission for patients with relapsed or refractory disease requires consolidation therapy with allogeneic HSCT.[359,644] Traditionally, this has included matched family members or matched unrelated donors, but the more recent use of alternative HSC sources, such as banked umbilical cord blood or haploidentical family donors, has increased the number of patients with a potential marrow source. A recent trend has been in using non–total-body irradiation (TBI)–based conditioning regimens for AML in first remission due to equal efficacy and less toxicity, but TBI may still be used in the conditioning of patients with relapsed AML.[657,658] It is possible to cure patients who have persistent, refractory disease at the time of allogeneic HSCT, particularly if they have less than 20% blasts in the marrow. Those patients with shorter remissions and higher blast percentages in their marrow have traditionally been associated with only a 25% chance of cure,[659-664] whereas for patients who have had a long first remission and proceed to HSCT in a second CR the OS rates are much better and may be as high as 40% to 62%.[538,624,664] However, although lower levels of MRD were associated with improved overall outcomes, investigators at the SJCRH found that with contemporary risk-adapted therapeutic regimens, allogeneic HSCT may be curative for patients with AML who continue to be MRD positive or even have persistent marrow blasts, with 5-year OS of 67% and 58%, respectively.[665] This analysis included patients with relapsed AML. These findings are in contrast to the impact of residual disease in ALL, which may be attributed at least in part to the graft-versus-leukemia (GVL) in AML.[615,665] This effect is difficult to measure directly but has been indirectly inferred from the presence of GVHD. The presence of acute and chronic GVHD has been associated with lower relapse rates in AML.[666-672] Donor lymphocyte infusions have been used successfully to induce GVL effect in patients with CML who experience relapse after allogeneic HSCT, but this strategy is much less effective in patients with AML who experience relapse after allogeneic HSCT.[673,674]

There can be GVL effect without GVHD, however, as evidenced by lower relapse rates in patients receiving HSCT from a matched sibling versus an identical twin, even in the absence of GVHD.[675] There is much interest in maximizing the GVL effect, and recent studies have concentrated on the role of alloreactive natural killer (NK) cells in this process. Donors who are mismatched at the KIR ligand produce NK cells that recognize and destroy host AML cells in response to the lack of expression of a self class I ligand on the AML cells.[676-678] Deliberate KIR mismatching through haploidentical HSCT has been shown in small studies to decrease the relapse rate without increasing GVHD, therefore resulting in improved long-term survival. This strategy is being used in clinical trials for patients with high-risk de novo AML or relapsed and refractory disease and is currently incorporated into ongoing pediatric trials of the COG and SJCRH.[677,679] The feasibility of engrafting haploidentical NK cells in children with AML in remission was recently demonstrated.[679]

Novel Agents and Targeted Therapy

Conventional therapy for AML is predominantly a “shotgun” approach in which leukemia cells, as well as normal hematopoietic stem and progenitor cells and many other normal tissues, are injured, with the hope that the normal cells will recover faster and more completely than the leukemia cells. As discussed earlier, this approach has not resulted in superior cure rates in AML and the intensity has been increased to the point that most patients suffer toxicity and treatment-related morbidity; a substantial fraction of patients suffer treatment-related mortality. While the molecular pathogenesis of AML has been elucidated, opportunities have appeared for the development of novel and targeted agents that preferentially destroy leukemia cells, with far less significant effects on normal hematopoietic cells and other normal tissues.[680,681] However, preclinical and adult clinical data, not to mention drug availability, are in rapid flux, given the growing interest in developing novel targeted therapeutics. Thus current therapeutic platforms for relapsed AML are moving to the model of having a standardized backbone to which novel agents can be rapidly inserted to be sufficiently nimble to adapt to the availability and promise of emerging drugs.

Antibody Therapy with Gemtuzumab Ozogamicin. Gemtuzumab ozogamicin (GMTZ, also discussed earlier in the induction chemotherapy section) is a recombinant, humanized anti-CD33 monoclonal antibody linked to the tumor antibiotic calicheamicin. CD33 is expressed on most AML cells but not on pluripotent HSCs or other tissues, thus making it possible to target leukemia cells more specifically.[367,368] GMTZ has been used successfully as a single agent for adults and children in relapse, with infusional side effects of fever and chills, hematologic toxicity resulting from pancytopenia, including some cases of prolonged thrombocytopenia, and hepatic toxicity ranging from transient liver function abnormalities to fatal venoocclusive disease, particularly when used within 3 months of HSCT.[519,682-689] GMTZ as a single agent is not a particularly strong reinduction regimen, but it has now been used successfully in combination with cytarabine-mitoxantrone and cytarabine-asparaginase as part of a reinduction regimen.[558,681,688,690-694] Given the data from the United Kingdom on the limited efficacy of GMTZ in initial adult AML therapy,[370,371] current and future availability of this agent may be very limited. GMTZ may have a role specifically in relapsed APL, however (see later).

Receptor Tyrosine Kinase Inhibitors. *FLT3* (discussed in detail earlier) is a receptor tyrosine kinase that is upregulated in the AML cells of most patients. There are also two types of activating mutations in *FLT3* and, in pediatric patients with AML, ITDs are present in 5% to 16% and ALMs are present in 2% to 5% of cases.[172-175] Several small-molecule inhibitors of the FLT3 tyrosine kinase have been developed, some of which are effective against only the ITDs and some of which are effective against both the ITDs and the ALMs.[695,696] These inhibitors are now in various phases of clinical trials. Overall, they appear to be well tolerated and to induce a decrease in blasts, but as single agents they generally do not result in durable remissions.[172-175,697] The COG completed a pilot study of lestaurtinib in relapsed AML. The drug was well tolerated, but inadequate FLT3 inhibitory plasma concentrations were achieved and the study was closed prior to dose escalation, owing to concerns regarding drug availability. Quizartinib is a tyrosine kinase inhibitor that has shown promise in relapsed adult AML with *FLT3*-ITD with clearance of marrow blasts but incomplete count recovery.[698-700] Rapid resistance to quizartinib is an emerging problem but suggests that the short-term benefits observed are bona fide therapeutic responses. A phase I study conducted by the Therapeutic Advances in Childhood Leukemia and Lymphoma (TACL) consortium demonstrated the tolerability of quizartinib together with cytarabine and etoposide in relapsed and refractory AML and ALL, with remissions both with and without count recovery in four patients, including three with AML and *FLT3*-ITD.[701] Sorafenib is another tyrosine kinase inhibitor that has had documented activity as both a single agent and with cytotoxic therapy in relapsed AML,[702] although several of the mutations that are resistant to quizartinib are also resistant to sorafenib. Sorafenib is currently being incorporated upfront for de novo AML patients with *FLT3*-ITD and high allelic ratio on COG study AAML1031 and SJCRH AML08. With the use of these agents as frontline therapy, it is uncertain whether they will play a significant role in relapsed disease. However, in cases in which a patient without a prior *FLT3*-ITD at diagnosis develops such a lesion at relapse, FLT3 inhibitors should be considered as part of the reinduction-remission strategy. Finally, ponatinib is another new tyrosine kinase inhibitor with activity against *FLT3*-ITD, including those with mutations resistant to other inhibitors, and an impressive response rate in refractory CML harboring resistant *ABL* mutations.[700,703]

This agent may have more broad potential in pediatric myeloid disease; however, the drug has recently been issued a Black Box warning by the FDA due to an association with life threatening thromboses.

Farnesyltransferase Inhibitors. Mutations in *RAS* itself, as well as perturbations in the RAS pathway, are associated with AML. The RAS protein requires isoprenylation by farnesyltransferase to bind to the plasma membrane properly and be fully active. Several farnesyltransferase inhibitors have been developed with the goal of inhibiting upregulated or dysregulated RAS activity. Because these drugs do not specifically target a known mutation in *RAS* and have effect on other proteins that also require farnesylation, they can be effective in patients with or without *RAS* mutations. Tipifarnib (R115777, Zarnestra) as an oral agent has shown activity in adult patients with refractory AML and in older adult patients with de novo AML, with a few transient complete responses, even when used as a single agent.[704-707] The drug was generally well tolerated with toxicities of myelosuppression, neurotoxicity, and reversible renal toxicity.[704,706] In pediatrics, a phase I trial of tipifarnib for refractory solid tumors and neurofibromatosis type 1–related plexiform neurofibromas and a phase II trial for brain tumors both have shown that the drug was tolerated well overall, with dose-limiting toxicities of myelosuppression, rash, nausea, and vomiting; one patient also developed a seizure.[708,709] A phase I trial in patients with refractory leukemia was completed by COG in 2005. The drug has also been used safely in combination with capecitabine for children with advanced solid tumors. Currently, trials are ongoing combining tipifarnib with chemotherapy for adults with AML as well as a COG trial combining tipifarnib with *cis*-retinoic acid, cytarabine, and fludarabine with HSCT for children with JMML.[710] Two other farnesyltransferase inhibitors, lonafarnib (Sarasar, SCH66336) and BMS-214662 have also shown some promise in adults with myeloid disorders, but there are as yet no data for pediatric AML.[711]

Histone Deacetylase Inhibitors. Double-stranded DNA binds to histones to form compact nucleosomes when the DNA is not being actively transcribed. Acetylation of lysine residues by histone acetyltransferases allows the release of DNA from histones for transcription. Deacetylation by histone deacetylase (HDAC) reverses the process. Inappropriate recruitment of HDACs leading to gene silencing has been demonstrated in the CBF leukemic cells bearing translocations t(8;21) and inv(16), as well as in APL cells bearing t(15;17).[712-717] The use of HDAC inhibitors may allow for renewed expression of silenced genes that when activated can induce differentiation or cell death.[718,719] Several HDAC inhibitors have been tested in clinical trials in AML patients, including the antiseizure medication valproic acid. It has been used in clinical trials for adults with MDS or AML as monotherapy and in combination with ATRA, 5-aza-2′-deoxycytidine (DAC), as well as 5-azacytidine and ATRA together.[680,720,721] It was generally well tolerated, with toxicities of myelosuppression, neurotoxicity, and sedation. Some transient responses were seen, even when valproic acid was used as monotherapy, but it was most effective when used in combination with ATRA and 5-azacytidine.[680,720,721] A more potent HDAC, depsipeptide, has been used in clinical trials and has had more toxicity, including fatigue, nausea, and constitutional symptoms. In these trials, it has shown some promise in T-cell lymphoma but thus far only modest antileukemia activity.[722,723] Vorinostat (suberoylanilide hydroxamic acid [SAHA]) is an HDAC inhibitor that has been under discussion for incorporation in pediatric relapse or refractory AML studies. Romidepsin is a newer HDAC inhibitor also under consideration (T. Cooper, personal communication, May 2013). HDAC inhibitors and DNA methyltransferase inhibitors as combination therapy are also being evaluated. In some trials this seems to be a safe and effective strategy,[724] but in at least one trial using decitabine and valproic acid the combination did not show an improved response rate versus decitabine alone and proved too toxic because of encephalopathy.[725] However, this approach may prove more fruitful in the combination of novel HDAC inhibitors and DNA methyltransferase inhibitors with less overlapping toxicities and in subsets of AML patients found to have particularly high levels of methylation and activity of methyl transferase enzymes. In particular, these therapeutic strategies may be specifically applicable for adult patients harboring *DNMT3A* and *TET2* mutations and *MLL* rearrangements in pediatric AML.

Apoptosis Inhibitors. BCL2 is an inhibitor of apoptosis that is highly expressed in some patients with AML and is associated with a poor prognosis.[726-728] A BCL2 antisense oligonucleotide has been tested as a single agent and shown to induce transient remission in some AML patients.[729] It has also been shown to be safe and effective in combination with cytarabine and daunorubicin.[730] This agent is now being used in clinical trials of AML through the Cancer and Leukemia Group B (CALGB) as part of combination therapy during induction and consolidation.[681] Specifically, a trial combining the pan-BCL2 inhibitor obatoclax with vincristine, doxorubicin, and dexrazoxane for relapsed solid tumors and leukemias has just been completed by the COG and results are pending.

Other Targeted Therapies. A number of new promising agents are emerging and being considered for introduction into relapsed AML trials. Some of these agents target recently emerging important molecular pathways in AML cells such as MK2206, which targets the PI3K-AKT pathway, or MK8776, which is a CHK1 inhibitor. AKT pathway activation may contribute to resistance to GMTZ[731] and may be particularly active in AML associated with Down syndrome[732] (see later). Phase I studies of MK2206 have been completed in adults, and a pediatric trial has just been completed. CHK1, or checkpoint kinase 1, regulates the G2 to M phase transition of the cell cycle and may be activated in the phase of DNA damage in AML cells.[733] CHK1-inhibiting agents have been evaluated in a number of adult phase I solid tumor studies[734] and may have efficacy in AML, particularly in children harboring complex karyotypes associated with the highest levels of DNA damage and CHK1 activity.[733]

CHK1 has been shown to suppress apoptosis mediated by caspase 2 in the context of DNA damage, and inhibition of CHK1 may restore apoptosis in AMLs with *TP53* mutations.[735] Another strategy is to interfere with the interaction of AML cells and their microenvironment through the use of drugs such as plerixafor. Plerixafor binds to and antagonizes the interaction of CXCL12 (also known as SDF1) and its receptor CXCR4 found on HSCs and potentially on LICs, affecting LIC mobilization from the stem cell niche and making these cells more accessible to cytotoxic chemotherapies. Thus this agent is believed to target the AML LIC and make these resistant cells more susceptible to conventional therapies. A recent phase I/II adult trial gave plerixafor prior to cytarabine, etoposide, and mitoxantrone for patients with relapsed AML and found that this combination was well tolerated and resulted in approximately 50% complete remission rates.[736]

ACUTE PROMYELOCYTIC LEUKEMIA

Pathobiology

APL or M3 AML is a subtype of AML defined by a specific morphology, a balanced translocation involving the *RARA* gene, and a characteristic coagulopathy.[173,283,737,738] Although most patients with APL have the classic t(15;17) resulting in the *PML-RARA* fusion gene, up to 8% do not. Approximately 5% of patients have a *PML-RARA* because of an insertion or other variant, 1% of patients have a t(11;17) resulting in a *PLZF-RARA*, another 1% have a t(5;17) resulting in an *NPM-RARA* or a t(11;17) resulting in a *NuMA-RARA* or a *STAT5b-RARA*, and 1% of patients do not have an identifiable *RARA* rearrangement.[739] APL provides a clear example of the classic two-hit model for AML (see earlier, "Molecular Genetics").[138] The fusion protein resulting from the pathognomonic translocation in APL, the PML-RARA protein, is an example of a class II mutation, resulting in a block in differentiation. As detailed earlier (see "Biology"), this protein has been shown to interfere with normal myeloid cell development through dominant inhibitory mechanisms as a potent transcriptional repressor that recruits a number of other corepressors to cause differentiation arrest at the promyelocyte stage.[211,740-743] Mutations in the receptor tyrosine kinase *FLT3* occur in up to 43% of cases of APL, and these mutations are classic examples of class I mutations, conferring a proliferative advantage.[744,745] At diagnosis, APL patients with *FLT3* mutations more often present with hyperleukocytosis, microgranular variant morphology, and the bcr3 PML breakpoint.[165,744-749] Although *FLT3*-ITDs clearly confer a worse prognosis in other subtypes of AML, their role in prognosis for APL is less clear. Some groups have reported a worse prognosis for APL patients with an *FLT3*-ITD, whereas other groups have found no statistically significant impact on the generally favorable risk outcome of this disease.[744,745,749-751] A recent report from the COG found *FLT3*-ITD or ALM mutations in 40% of children treated on the CALGB 9710 study and identified a strong correlation with higher WBC count and induction death rates in the *FLT3* mutant subgroup.[401]

Clinical Presentation and Risk Factors

APL is more common in girls and obese children, and obesity is associated with a higher rate of relapse and differentiation syndrome (see later).[561,752] Compared with adults, children with APL have a higher incidence of hyperleukocytosis at presentation, microgranular variant morphology, and *PML-RARA* isoforms bcr2 and bcr3.[753] Moreover, a recent retrospective review of two European studies demonstrated that children younger than age 4 years had a higher incidence of disease relapse than those age 5 to 12, whereas, by contrast, adolescents had higher OS than adults.[754] A presenting WBC count higher than 10,000/μL has been repeatedly shown to carry a worse prognosis,[543,755-757] as has a platelet count less than 40,000/μL, leading to the intensification of therapy with additional anthracycline, cytarabine, and/or ATRA for adult patients treated on recent French and Italian APL trials. Based on improved outcomes, on the recently completed COG trial AAML0631, patients with a WBC count greater than 10,000/μL were designated as high-risk patients who were relegated to additional consolidation cycle with high-dose cytarabine.[758] Before the 1970s, bleeding complications caused significant mortality for patients with APL. Measures that have been used successfully to manage the coagulopathy include platelet and cryoprecipitate transfusions, use of low-dose heparin, and prompt initiation of anthracycline-based chemotherapy.[335,336,354,738] However, the most dramatic decrease in early and induction-related deaths has been the result of the addition of ATRA to the induction regimen, which rapidly leads to the resolution of DIC, and therefore has markedly reduced early mortality.[191,337-340]

Treatment

All-*Trans*-Retinoic Acid

With the introduction of ATRA, the treatment and outcome of APL have changed dramatically. Although previously considered primarily a differentiation agent, a number of recent studies in *PML-RARA* expressing mice and cell lines containing the alternative *PLZF-RARA* fusion have uncoupled differentiation and leukemic cell death, suggesting that exclusive acceptance of a differentiation paradigm belies ATRA's true mechanism of action.[759] In keeping with these recent findings, while even low-dose ATRA induces differentiation in APL blasts in vivo and in vitro and induces remission in up to 90% of patients with newly diagnosed APL,[191,338,760-762] these affects are transient without additional chemotherapy or As_2O_3 (see later). Although ATRA therapy results in a lifting of the repression complex that inhibits APL blast differentiation, higher doses, As_2O_3, or chemotherapy is needed to degrade the PML-RARA fusion protein and halt the self-renewal process, ultimately eliminating the malignant clone. Elimination of PML-RARA may further result in more durable cures by targeting the LIC population in APL in which the initial t(15;17) occurred, as well as restoring the normal function of the intracellular PML bodies disrupted by the presence of the fusion protein.[211,759,763] Thus recent strategies combine ATRA with conventional chemotherapy, resulting in improved prognosis for patients with APL compared with chemotherapy alone.[545,764-768] A long-term analysis has demonstrated significantly higher actuarial EFS rates, lower relapse rates, and improved OS in the group treated with both ATRA and chemotherapy.[769] The addition of ATRA to the chemotherapy regimen has been particularly effective for pediatric patients. The APL93 trial used ATRA in combination with daunorubicin and cytarabine in induction, and the children in this trial had a complete remission rate of 97%.[770] The PETHEMA and GIMEMA-AIEOPAIDA groups have completed pediatric trials using ATRA and idarubicin as induction chemotherapy, with reported CR rates of 92% to 96%.[771,772] As such, this lower dose has become the standard for current North American APL studies in children. Current recommendations are to initiate ATRA therapy early, at the time when a diagnosis of APL first is suspected, to correct and prevent devastating consequences from an evolving coagulopathy.

One potentially life-threatening complication of ATRA therapy is the retinoic acid syndrome. This syndrome occurs in approximately 25% of patients and is characterized by fever, respiratory distress, pleural or pericardial effusions, edema, hypotension, and, in some cases, renal failure.[498,773-775] In most patients, the symptoms are preceded by an increasing WBC count.[761,774] Retinoic acid syndrome can be life threatening, and the mortality rate ranges from 8% to 15% in different reports.[765,766,774,776] The syndrome is also associated with a higher risk for bone marrow and extramedullary relapse.[773,777] The use of chemotherapy concurrently with ATRA therapy during induction, particularly in patients with high presenting WBC counts, results in a significantly lower incidence of fatal retinoic acid syndrome[778]; and given the dose dependence, ATRA syndrome now occurs less frequently with the use of 25 mg/m^2 as the standard therapeutic dosing (see later). The use of high-dose corticosteroids at the first sign of symptoms has also been shown to be effective in preventing ATRA syndrome and reducing its mortality rate. Another serious complication of ATRA therapy is pseudotumor cerebri, presenting as headache and papilledema. True pseudotumor cerebri is observed in up to 16% of children, and an additional 39% of children develop headaches without other signs of increased intracranial pressure.[770,772] Pseudotumor cerebri may require treatment with diuretics such as acetazolamide or serial lumbar punctures to reduce intracranial pressure; and, in some cases, discontinuation or dose reduction of ATRA is required.[779] Close follow-up with an ophthalmologist is advised because these patients may suffer visual loss, which in rare cases may be irreversible.[780] Because of the high rate of ATRA-related side effects, the AML-BFM study group studied dose reduction of ATRA during induction from 45 to 25 mg/m^2/day. They found that this dose was still effective, although in pediatric patients there was still a very high rate of CNS side effects, with 57% of children exhibiting headache, increased intracranial pressure, or frank pseudotumor cerebri.[781,782] The CALGB trial C9710 included pediatric patients and used the higher dose of ATRA, but recent data demonstrated no additional benefit and only increased toxicity compared with 25 mg/m^2 dosing. Therefore this lower dose was incorporated into the recently closed first exclusively pediatric COG APL study, AAML0631, and this dose is now considered the standard of care in pediatric APL.

Arsenic Trioxide

Arsenic trioxide (As_2O_3,) has been used for decades in China as treatment for leukemia. The precise mechanism of action is actively being studied, but As_2O_3 appears to induce both differentiation and apoptosis of APL blasts and to degrade the PML-RARA fusion protein while not affecting transcriptional regulation per se.[213,218,783] As_2O_3 appears to induce nonterminal differentiation followed by apoptosis via the caspase pathway.[213,218,783-785] When As_2O_3 was used as a single agent in patients with APL, remission rates of 65% to 85% were reported and the 10-year survival was as high as 30%.[498] Later studies done in China and the United States showed favorable results, with moderate toxicity, using As_2O_3 in patients with relapsed APL who had previously received ATRA therapy.[210,784,786-788] Several studies have shown promising results using As_2O_3 in remission induction and consolidation therapy for patients with newly diagnosed APL.[789-791] One small series of 11 children with newly diagnosed APL treated with As_2O_3 as a single agent showed mild toxicity, including leukocytosis, skin changes, and mild neuropathy.[791] CR was induced in 91% of these patients, with 1 patient dying of cerebral hemorrhage during induction. With a relatively short median follow-up of 30 months, the DFS was 81% with only 1 relapse; the patient who experienced relapsed achieved a second CR, and thus the OS rate remains 91%.[791] On the U.S. phase I pediatric study of As_2O_3 for relapsed leukemia, 13 patients with relapsed APL received As_2O_3 intravenously daily at 0.15 mg/kg/dose for 5 days per week for 4 weeks with a 2-week break with maximum of 70 doses. A CR rate of 85% was observed after a median of 20 doses. Dose-limiting toxicities included rare vascular leak, anorexia, neuropathic pain, and QTc prolongation (see later).[792]

ATRA and As_2O_3 have been used safely together as induction therapy in children and adults.[213,218,783,793-795] The drugs may have a synergistic effect when used together; one study showed a shorter median time to CR in the group receiving the combination (40.5 days for ATRA alone, 31 days for As_2O_3 alone, 25.5 days for ATRA with As_2O_3).[795] The combination also provided longer duration of remission in this study. Because of these promising data, several groups have included As_2O_3 as part of induction or consolidation therapy for newly diagnosed pediatric patients with APL, with the hope of reducing the total cumulative dose of anthracycline exposure. As_2O_3 was used in a randomized fashion during consolidation in the CALGB 9710 trial that included pediatric patients through the COG and demonstrated a dramatic improvement in outcome. Patients 15 years of age and older who had the addition of As_2O_3 after a common ATRA and chemotherapy induction demonstrated significantly improved 3-year EFS (77% vs. 59%) and OS (86% vs. 77%) compared with patients who received ATRA and chemotherapy alone.[547] These results led to the nonrandom incorporation of As_2O_3 into the successor COG study

AAML0631, such that all patients nonrandomly received As_2O_3 in a dose of 0.15 mg/m^2 for 5 days a week for five courses during consolidation therapy.

As_2O_3 therapy is generally well tolerated, often with only minimal and reversible toxicity.[791,796,797] However, care must be taken with its use, because it can produce a prolonged QTc interval, which may be asymptomatic but can progress to torsades de pointes and fatal cardiac arrhythmia.[797-800] In one retrospective analysis, the rate of arrhythmia was significantly higher in African American patients (3 of 4) versus non–African-American patients (1 of 73).[801] QTc intervals should be followed closely by weekly electrocardiography during As_2O_3 treatment, and hypokalemia and hypomagnesemia should be meticulously avoided.[802] Because of the induction of cellular differentiation, As_2O_3 commonly results in a condition similar to retinoic acid syndrome, most often characterized by fluid retention and pleural and pericardial effusions.[803] This may be successfully treated in the same way as ATRA-induced retinoic acid syndrome. As_2O_3 can also cause a significant polyneuropathy, particularly when given in repeated courses.[804] Less dangerous side effects include skin changes with rash, hyperpigmentation, keratosis, and transient liver function test abnormalities. In a COG phase I study of As_2O_3 in children with refractory or relapsed acute leukemia, dose-limiting toxicities included prolonged QTc, pneumonitis, and neuropathic pain, whereas non–dose-limiting toxicities included elevated liver function test results, nausea, vomiting, abdominal pain, constipation, electrolyte changes, hyperglycemia, dermatitis, and headache.[805]

Owing to their synergistic effects, ATRA and As_2O_3 were both used upfront on the AAML0631 study and were generally well tolerated. Although used together with chemotherapy on this recently completed trial, the efficacy of this combination and recent adult studies demonstrating the ability of this combination therapy to lead to sustained remission without additional cytotoxic chemotherapy are likely to lead to future trials in which additional chemotherapy is significantly reduced or even eliminated to attain a first CR at least in standard patients. Most striking was the recent APL0406 study, which randomized adult APL patients to ATRA and As_2O_3 or ATRA and idarubicin in induction and found a 2-year EFS of 97% versus 86.7% in favor of the ATRA/As_2O_3 trial arm.[806,808] Thus the addition of anthracyclines and cytarabine may be reserved for APL patients designated high risk at diagnosis based on WBC count, those with more resistant disease (PML-RARA positive post-consolidation [see later]), or first recurrence.

Cytarabine

Although cytarabine is the backbone of induction chemotherapy for other subtypes of AML, its role in APL has been less clear.[340] The PETHEMA group completed a pediatric trial using ATRA and idarubicin as induction chemotherapy without cytarabine, and the CR rate was 92%.[771] The GIMEMA-AIEOPAIDA protocol also used ATRA and idarubicin during induction without cytarabine and showed a 96% CR rate in children.[772] However, this question was subsequently studied in a randomized clinical trial in which patients younger than 60 years and with a presenting WBC count less than 10,000/μL were randomly assigned in induction to ATRA and daunorubicin, with or without cytarabine. CR rates were comparably high for the two groups—99% with cytarabine and 94% without; however, in the cytarabine arm, the relapse rate was significantly lower (4.7% vs. 15.9%; $P = .011$), the EFS higher (93.3% vs. 77.2%; $P = .0021$), and the OS higher (97.9% vs. 89.6%; $P = .0066$). Of note, the current trials all use high cumulative anthracycline doses to achieve excellent OS rates but there is concern about long-term cardiac toxicity, particularly in pediatric patients.[807] Considering the improved results with the addition of cytarabine to the induction and consolidation regimens, several groups have been studying the use of more cytarabine with less anthracycline in pediatric patients with APL. On AAML0631, 6 g/m^{2}of cytarabine was given during the second consolidation cycle for all patients, with an additional 6 g/m^2 of cytarabine for high-risk patients. The cumulative anthracycline dose was decreased from 655 mg/m^2 on prior trials to 355 mg/m^2 for standard-risk patients and 405 mg/m^2 for high-risk patients.

Maintenance Therapy

Another difference between APL and other subtypes of AML historically has been an identified benefit from maintenance therapy. The APL93 trial randomized pediatric patients after consolidation to an arm with no further therapy or to arms with 2 years of intermittent ATRA, continuous chemotherapy with 6-mercaptopurine and methotrexate, or both ATRA and chemotherapy. Although 23% of pediatric patients in this trial experienced relapse, none of the patients who received both chemotherapy and ATRA during maintenance therapy did so.[770] In this trial, all patients with relapsed APL achieved a second CR. The 5-year EFS was 71%, and OS was 90%. The PETHEMA group also used ATRA plus 6-mercaptopurine and methotrexate during 2 years of maintenance therapy for pediatric patients and demonstrated a DFS of 82% and an OS of 87%.[771] Since these earlier studies, all recent trials for APL have included a maintenance phase of therapy. To be congruent with parallel European studies, the recently completed AAML0631 study included 2 years of maintenance therapy with ATRA, 6-mercaptopurine, and methotrexate, whereas the CALGB study only included a single year of maintenance with the same agents. However, the future of maintenance therapy in APL is uncertain. The need for chemotherapy versus ATRA alone in maintenance therapy has been raised. Preliminary results of upfront ATRA and As_2O_3 in both Italian and M.D. Anderson Cancer Center adult trials without inclusion of maintenance therapy have prompted consideration of elimination of maintenance therapy altogether.[808] Inclusion of maintenance therapy and whether any chemotherapy is necessary will be key therapeutic goals explored in upcoming trials.

Targeted Therapy

With the use of ATRA and As_2O_3, targeted therapy for APL has become a reality. Other targeted agents are also being used successfully in APL. GMTZ (see earlier) is a recombinant, humanized, anti-CD33 monoclonal antibody linked to the tumor antibiotic calicheamicin. APL cells express very high levels of CD33, making them an attractive target for GMTZ.[809] GMTZ has been used successfully as a single agent to induce remission in patients with multiply relapsed APL.[810,811] In a small series of 12 patients, GMTZ was used safely in combination with ATRA with or without idarubicin, depending on the extent of MRD, as induction therapy for patients with de novo APL with a CR rate of 84%.[812] Some groups are now studying the use of GMTZ as induction therapy in larger numbers of patients. Although not currently used initially for children with APL, GMTZ may be included for the management of high-risk patients and standard-risk patients who have a rise in their WBC count over 10,000/μL during induction therapy or, alternatively, as a strategy to induce remission after relapse in patients who have already received ATRA, As_2O_3, and cytotoxic chemotherapy.

As discussed earlier, mutations in *FLT3* have been identified in up to 43% of patients with APL, making the use of FLT3 inhibitors an attractive option. Most FLT3 inhibitors are oral agents with little toxicity. In one mouse model of APL with the *FLT3* mutation, treatment with doxorubicin alone had no impact on survival, but treatment with the FLT3 inhibitor SU11657, with or without doxorubicin, resulted in prolonged survival.[813] In another mouse model, treatment with ATRA and SU11657 resulted in the rapid regression of APL.[814] There is considerable interest in using FLT3 inhibitors in combination with ATRA and other targeted agents to improve therapy for patients with APL.

Hematopoietic Stem Cell Transplantation

Because the combination of chemotherapy and ATRA, and now ATRA and As_2O_3, results in such high cure rates for APL, HSCT is not recommended for patients in first CR, even with a matched sibling donor. There is evidence for prolonged remission without HSCT, even after relapse for patients treated with agents such as ATRA, As_2O_3, and GMTZ, especially when combined with chemotherapy.[210,784,786-788,810,811,815] Both autologous and allogeneic HSCT have been used successfully in patients with relapsed APL. For autologous HSCT to be successful, peripheral blood stem cells or bone marrow cells must be harvested once the patient has achieved negative MRD by PCR assay.[816,817] The GVL effect appears to play a role in the allogeneic HSCT treatment of APL, as evidenced by the fact that relapse rates are significantly lower after allogeneic HSCT compared with autologous HSCT and that patients who suffer a molecular relapse after allogeneic HSCT may achieve remission after the withdrawal of immunosuppression.[817-819] However, despite the lower relapse rate, because of significantly higher transplantation-related mortality (TRM) after allogeneic HSCT, OS in adults is higher after autologous HSCT than after allogeneic HSCT (postallogeneic HSCT: DFS, 92.3%; EFS, 52.2%; OS, 51.8%; TRM, 39%; and postautologous HSCT with negative preharvest PCR: DFS, 87.3%; EFS, 76.5%; OS, 75.3%; TRM, 6%).[817] In pediatric patients, allogeneic HSCT is much better tolerated. A retrospective analysis demonstrated no difference in OS after allogeneic HSCT versus autologous HSCT, although the allogeneic HSCT group had a lower relapse rate and higher TRM (postallogeneic HSCT: RR, 10%; EFS, 71%; OS, 76%; TRM, 19%; and postautologous HSCT: RR, 27%; EFS, 73%; OS, 82%; TRM, 0%).[818] Given these data, in adults, autologous HSCT is usually the preferred choice versus allogeneic HSCT for patients in second CR, whereas in children, allogeneic HSCT is often the preferred choice, particularly if a fully matched sibling donor is available.

Central Nervous System Involvement

In patients with APL, CNS disease at the time of diagnosis is rare and, because of coagulopathy, diagnostic lumbar puncture prior to the initiation of therapy is not safe for most patients. Therefore if APL is suspected, lumbar punctures should not be performed until the coagulopathy is adequately corrected and ATRA initiated. In the pre-ATRA era, CNS relapse was rarely observed in APL patients. However, while the survival rate for APL improved dramatically with the introduction of ATRA, an increasing number of CNS relapses have been reported. Unlike other subtypes of AML, most APL regimens do not include CNS prophylaxis. CNS relapse is more common in patients with a presenting WBC count higher than 10,000/μL, with microgranular variant morphology, and with bcr3 PML-RARA isoform, all of which are also associated with the presence of an *FLT3* ITD and all of which are more common in pediatric patients.[165,744-749,753,770,809,820,821] Although there was initially concern that ATRA itself was contributing to CNS relapses, it now seems that ATRA does not penetrate the CNS well and, with more patients surviving APL therapy, this likely contributes to the higher rate of CNS relapse currently observed.[820-823] Several groups have now initiated a screening lumbar puncture at the time of CR and use prophylactic intrathecal chemotherapy, usually with cytarabine and methotrexate, for patients at high risk for CNS relapse.[821,824] On AAML0631, prophylactic cytarabine was given intrathecally in consolidation 2, 3, 4 (for high-risk patients) and the first maintenance course but at a lower dose of 30 mg (25 mg for children >2 years of age but <3 years of age) compared with the 70-mg given as standard dosing in other subtypes of AML. Given the infrequency of CNS positivity in APL, future studies may eliminate intrathecal therapy entirely.

Minimal Residual Disease Monitoring

Detection of the *PML-RARA* fusion gene by reverse-transcriptase PCR (RT-PCR) has become a valuable tool for measuring MRD in APL. RT-PCR positivity at the end of induction does not seem to portend a higher risk for relapse and therefore should not be checked at that time point, but RT-PCR positivity after consolidation does carry a significantly higher risk for relapse. RT-PCR positivity during maintenance or after therapy is indicative of impending relapse in almost all patients.[772,825,826] MRD analysis is being used to identify high-risk groups that require more intensive therapy for cure.[772] When detected early by RT-PCR, molecular relapse may also be easier to treat than frank morphologic relapse because of the lower disease burden and the treatment of a healthier asymptomatic patient.[827] In many studies, RT-PCR analysis is carried out on bone marrow cells and evidence regarding analysis of peripheral blood is not as clear,[828] thus *PML-RARA* surveillance on AAML0631 mandated bone marrow samples. However, the frequency of relapse after therapy including As_2O_3 is so rare for standard-risk patients[829,830] that whether follow-up on future trials will require the invasiveness of bone marrow evaluations remains to be determined. There is also controversy regarding the threshold for a "positive" result; some groups do not consider a result a true positive unless there are two positive results separated by 2 to 4 weeks. However, in the event that suspicion is significant for recurrence based on a rising level of PML-RARA transcript in the peripheral blood, a bone marrow evaluation should be conducted to look for molecular, cytogenetic, and hematologic signs of relapse.

Relapsed Disease

In the current ATRA and As_2O_3 era, relapsed APL is a rare occurrence and there is limited experience on what therapeutic strategy to undertake in children who have already received ATRA, As_2O_3, and chemotherapy. Given their efficacy, reinduction with combination ATRA and As_2O_3 is a reasonable strategy if previously well tolerated, although it is unclear whether the addition of chemotherapy provides any further benefit or merely contributes to toxicity. Currently, patients with relapsed APL may have already seen a significant cumulative dose of anthracycline, such that additional anthracycline may push the normally accepted lifetime ceilings with which most clinicians are comfortable. However, the concomitant use of cardioprotectants, such as dexrazoxane, may provide the opportunity to provide further anthracycline therapy at relapse. Furthermore, if future studies show the initial use of chemotherapy and anthracyclines in particular may be significantly reduced owing to the efficacy of combination ATRA and As_2O_3, it may enable chemotherapy to be reserved for relapses and refractory cases. Regardless, particularly with early relapse, given the opportunity for GVL effect and the improvement in both high-resolution HLA typing and better supportive care, after reinduction, best available donor allogeneic transplant would be recommended by most experts.[831] However, on prior studies, TRM with allogeneic HSCT was higher than with autologous transplant, although there was no significant difference in 5-year OS or EFS,[818,832] so this decision should not be made lightly. With later relapses, the ability to induce a second sustained CR with ATRA, As_2O_3, and chemotherapy without HSCT may be a feasible alternative, although with small numbers of patients most of these data are anecdotal.[754]

DOWN SYNDROME WITH TRANSIENT DISORDER AND ACUTE MYELOGENOUS LEUKEMIA

Down Syndrome with Transient Myeloproliferative Disorder

Pathobiology

Down syndrome, characterized by trisomy 21, is the most common congenital chromosomal abnormality, occurring in an estimated 1 in 600 to 1000 live births.[833,834] Up to 10% of infants with Down syndrome are born with a transient myeloproliferative disorder (TMD) (also referred to as transient leukemia [TL] or transient abnormal myelopoiesis [TAM]). TMD is characterized by the presence of immature megakaryoblasts in the liver, bone marrow, and peripheral blood. The disorder usually spontaneously regresses within 3 months.[71,835-837] By morphology, TMD blasts are identical to AMKL blasts and, even though in most cases they regress, up to 30% of Down syndrome infants with TMD subsequently develop AMKL within 3 years.[838,839] Studies have shown that TMD blasts are clonal and that the same clone subsequently evolves into AMKL in a subset of patients.[840-843]

In 2002, Wechsler and colleagues[267] first reported mutations in exon 2 of *GATA1* in Down syndrome–associated AMKL. Soon thereafter, several groups reported that *GATA1* mutations were detectable in almost all patients with Down syndrome–associated TMD.[844-849] Interestingly, *GATA1* mutations have also been detected in approximately 10% of Down syndrome patients who have never been diagnosed with a hematologic abnormality.[850] It is not clear whether these patients have a history of subclinical TMD that regressed without detection or if the mutation simply did not result in the TMD phenotype in these cases. Normal GATA1 plays a vital role in the maturation of erythroid cells and megakaryocytes.[851] The *GATA1* mutations in Down syndrome TMD and AMKL result in GATA1s, a truncated protein lacking the amino-terminal activation domain.[847,852,853] GATA1s appears to be a normal isoform of GATA1 and has been detected in human hematopoietic cell lines and mouse fetal liver. However, because *GATA1* is an X-linked gene, only the truncated GATA1s is expressed in Down syndrome TMD and AMKL blasts. It has been hypothesized that the presence of a trisomy 21 in conjunction with a GATA1s allows abnormal proliferation of megakaryoblasts, but only in the fetal hematopoietic system (i.e., the fetal liver); thus TMD resolves with the loss of fetal hematopoiesis.[835,854] Although the combination of trisomy 21 and a *GATA1* mutation appears sufficient to result in TMD, this is not sufficient to result in frank leukemia, and only if additional mutations are accumulated over time will the disease progress to frank AMKL.

Wilms tumor gene *(WT1)* levels detected by PCR assay are elevated in patients with TMD. Levels normalized in four of five patients studied who had regression of TMD, with no further sequelae, but did not normalize in the fifth, who went on to develop AMKL at 11 months of age, making this an attractive marker for MRD and prognosis.[855] Both gain- and loss-of-function mutations in Janus kinase 3 *(JAK3)* have been identified in a subset of patients with TMD, although the prognostic relevance of this is not yet known.[856,857]

Clinical Presentation

Because Down syndrome infants with TMD are often asymptomatic, the incidence of 10% may be an underestimate, because many patients may have TMD that spontaneously regresses, without ever being detected.[846,858-861]

For this reason, many physicians obtain a routine screening complete blood cell (CBC) count at birth for all infants with known or suspected Down syndrome. However, some infants with Down syndrome have a dramatic presentation of TMD at or shortly after birth, with hydrops fetalis, pleural and pericardial effusions, ascites, and massive hepatosplenomegaly. These patients may rapidly progress to liver fibrosis or multisystem organ failure, both of which are most often fatal.[837,862-865] In the Pediatric Oncology Group (POG) trial 9481, 48 children with TMD were followed prospectively; 19% developed life-threatening disease with hepatic fibrosis or cardiopulmonary failure, with an overall mortality at 3 months of age of 10.4%.[837] Another retrospective analysis from Japan has shown that 22.9% of patients with TMD die before the age of 6 months, and the main causes of death were hepatic or cardiopulmonary failure.[866] In this study, early gestational age, WBC count greater than 100,000/μL, high percentage of peripheral blasts, elevated aspartate aminotransferase (AST), elevated direct bilirubin, and low Apgar score were significantly associated with poor survival. They noted that only 7.7% of term Down syndrome infants with a WBC count less than 100,000/μL died, whereas 54.5% of preterm infants with a WBC count higher than 100,000/μL died, suggesting that risk stratification may be possible to determine high-risk subgroups of patients who require earlier and more aggressive therapy.

Treatment

Because most patients with TMD will exhibit spontaneous regression, treatment often consists of supportive care only. Many groups, including COG protocols 2971 and 08B1, have recommended supportive care only for TMD patients unless they exhibit signs or symptoms of life-threatening disease, including hyperviscosity, with a total blast count higher than 100,000/μL, organomegaly causing respiratory compromise, congestive heart failure not caused by a congenital heart defect, hydrops fetalis, life-threatening hepatic dysfunction, defined as significant (grade 4 level toxicity) DIC, hyperbilirubinemia, ascites, or transaminitis.[837,867] For patients who require therapy, the choices include leukapheresis, exchange transfusion, or low-dose cytarabine.[862] Low-dose cytarabine has been effective when used early in critically ill patients.[868] In patients with hyperleukocytosis or who require therapy with cytarabine, treatment to prevent the consequences of rapid tumor lysis (see earlier) should be promptly initiated.

Down Syndrome with Acute Myelogenous Leukemia

Pathobiology

Children with Down syndrome have a markedly increased risk for leukemia during the first 10 years of life. Acute leukemia is 10 to 20 times more common in children with Down syndrome than in children in the general population, and leukemia in Down syndrome patients younger than 4 years is most commonly AML (specifically AMKL), whereas leukemia in older children with Down syndrome is most commonly ALL.[838,869-878] The ratio of ALL to AML is approximately 1 : 1 in children with Down syndrome, but in the first 4 years of life, AML is 100 times more common than ALL.[72] Although AMKL is a rare subtype of AML in the general population, it is the predominant form of AML in children with Down syndrome, making Down syndrome patients 500 times more likely than other children to develop AMKL. Unlike other children with AML, AMKL in children with Down syndrome often manifests after a period of myelodysplasia, usually characterized by months of thrombocytopenia and later by anemia.[72,879,880] Most cases of AMKL in Down syndrome patients are preceded by TMD (see earlier).[850,881] Several studies have confirmed that when there is a proven antecedent TMD, the same TMD clone subsequently evolves into AMKL.[844,846,882,883]

Also characteristic of AMKL in Down syndrome are *GATA1* mutations (see earlier). Because *GATA1* encodes a transcription factor essential for megakaryocyte development, samples from patients with AMKL were studied for evidence of *GATA1* mutations.[267] Mutations in exon 2 of GATA1 were detected in AMKL blasts from Down syndrome patients but not in other subtypes of AML in Down syndrome patients or in AMKL blasts from non–Down syndrome patients. Several subsequent studies have confirmed *GATA1* mutations leading to a premature stop codon in almost all cases of Down syndrome–associated AMKL.[844-849] *GATA1* mutations have been identified in the blood spots of three of four Down syndrome patients with AMKL who had not been previously diagnosed with TMD, suggesting that in most cases of AMKL the mutation has been present since birth.[846,850,882,883] Down syndrome patients who develop AML after the age of 4 years do not usually have *GATA1*-mutated AMKL but have AML more typical of the general pediatric population (non–Down syndrome AML). This disease is not as sensitive to chemotherapy and does not carry the exceptionally good prognosis carried by Down syndrome AMKL (see later).[884-886]

Additional mutations beyond the *GATA1* mutation in the presence of trisomy 21 are necessary to produce AMKL. The good-risk translocations common in other forms of pediatric AML, including t(8;21), t(15;17), inv(16), and t(9;11), are almost never seen in Down syndrome AMKL, although they are more commonly reported in Down syndrome patients who develop AML after the age of 4.[873,875,886-888] Other than trisomy 21, trisomy 8 is the most common cytogenetic abnormality in Down syndrome AMKL. Other translocations commonly reported in AMKL in the absence of Down syndrome, including t(1;22) and t(1;3), are only rarely documented in Down syndrome AMKL.[889] Monosomy 7, a widely accepted marker of pediatric and adult high-risk AML in the absence of Down syndrome, is also seen in 3.5% to 10% of patients with Down syndrome and AML and, although data are limited, it is generally associated with a worse outcome (see later).[890-892] Approximately 25% of patients have no cytogenetic abnormalities other than the constitutional trisomy 21, although some patients will exhibit tetrasomy 21. As in TMD, overexpression of *WT1* and mutations in *JAK3* have been reported in Down syndrome AMKL.[855-857] More recently, next generation sequencing approaches have identified a number of potential somatic mutational drivers that might contribute to the progression of TMD to AMKL including mutations in the cohesin complex; the epigenetic regulator, EZH2; as well as activating lesions in the RAS, WNT, mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways.[892a,892b]

Clinical Presentation

Children with Down syndrome who develop AML present at a younger age than average for childhood AML, with 60% to 70% of patients diagnosed when they are younger than 2 years.[70,873,875,884,887,888] Down syndrome patients generally present with lower total WBC counts (median, 7,000 to 10,000/µL) and lower platelet counts (median, 25,000 to 30,000/µL) than other children. Hepatosplenomegaly is more common in Down syndrome patients with AML than in other children, whereas lymphadenopathy and CNS involvement are less common. Down syndrome AMKL is frequently preceded by TMD, which is almost absent in non–Down syndrome patients, or MDS, which is far less common in non–Down syndrome patients. Older Down syndrome patients with AML have presenting signs and symptoms that more closely mirror those of the general population, reflecting the virtual lack of AMKL with *GATA1* mutations in patients older than 5 years.

Treatment and Outcome

Many children with Down syndrome have been entered into large clinical trials since 1982, when legislation was passed that prohibits withholding medical intervention from children with disabilities.[72] In the early clinical trials that included patients with Down syndrome and AML, the outcome was poor because of excessive toxicity. The associated congenital abnormalities, increased susceptibility to infection, and altered drug metabolism in patients with Down syndrome made it challenging to find appropriate therapy for this group. However, while general medical care for children with Down syndrome has improved, the outcome for children with AML and Down syndrome has also improved. Multiple studies from around the world have consistently demonstrated that Down syndrome AML patients have a better overall prognosis than children with AML in the general population.[530,873,875,884,887,888,893] A remarkable feature of AML in children with Down syndrome is its extraordinary responsiveness to AML chemotherapy.[72,874] Compared with non–Down syndrome AML, leukemic blasts are 4.5-fold and 12-fold more sensitive in vitro to cytarabine (median half maximal inhibitory concentration [IC_{50}], 77.5 vs. 350.9 nM) and daunorubicin (median $IC_{50,}$ 5.8 vs. 71.2 nM), respectively.[894,895] The increased sensitivity of Down syndrome AML blasts to cytarabine has been explained by a 5.2-fold greater accumulation of the active drug metabolite cytarabine-CTP compared with non–Down syndrome AML blasts.[896] Down syndrome AMKL blasts are significantly more sensitive to other chemotherapy agents, including amsacrine (16-fold), etoposide (20-fold), 6-thioguanine (3-fold), busulfan (5-fold), and vincristine (23-fold).[897] In addition to constitutionally expressed genes encoded on chromosome 21, which modulate drug metabolism in Down syndrome AML blasts, such as cystathionine-β-synthase (CBS),[898-900] the expression of mutant GATA1 protein itself contributes to increased drug accumulation.[901,902] In addition, the cytidine deaminase (CDA) levels in Down syndrome AMKL blasts are low, leading also to increased sensitivity to cytarabine. The CDA promoter contains several binding sites for GATA1, suggesting that *GATA1* mutations may contribute to the sensitivity of these blasts.[900]

Observations of increased in vitro sensitivity of Down syndrome AMKL blasts to cytotoxic drugs have been complemented by pioneering studies in which very low doses of cytarabine, which were administered over a prolonged period of time (10 mg/m^2 subcutaneously for 12 hours for 7 days every 2 weeks for 2 years),[903,904] resulted in durable remissions in as many as 67% of cases (intention to treat analysis[903]). Although outcomes of the very-low-dose cytarabine regimen eventually did not match those of standard dose cytarabine,[904] the observation highlights a unique degree of drug sensitivity of Down syndrome AMKL blasts to cytarabine that is not encountered in pediatric non–Down syndrome AML. Recently, Japanese investigators showed that high-dose cytarabine could successfully be eliminated from a treatment protocol for Down syndrome AML while maintaining a 4-year EFS of 83%.[892] In parallel, studies maximizing dose intensity for the treatment of AML, although successful in the general pediatric population,[411,905] did not translate into any improvements in EFS for patients with Down syndrome and AML owing to excessive mortality during induction therapy in this group.[905] In the German collaborative AML-BFM93 protocol, children with Down syndrome fared worse than other patients because of an increased frequency of infectious complications, but none of the patients experienced relapse and EFS was approximately 70%.[886,906] Therefore, in the subsequent AML-BFM98 protocol, Down syndrome patients received a reduced intensity regimen and the EFS improved dramatically, to almost 90%. In the Nordic Society for Paediatric Haematology and Oncology-Acute Myeloid Leukaemia (NOPHO-AML) trials, the earlier, more intensive NOPHO-AML88 trial showed a high death rate from infectious complications for Down syndrome patients with AML, with an EFS of only 47%, compared with much better outcomes in the later, less-intensive NOPHO-AML93 trial, with an EFS of 85% (76% for those who received full-dose therapy and 92% for those who received reduced-dose therapy).[907] The MRC trials showed similar results with Down syndrome patients receiving the same standard therapy as other patients, resulting in a higher rate of death from toxicity but lower relapse rate when compared with other children.[908] Perhaps the most striking example comes from the CCG 2891 study. In this study, all children were randomly assigned to receive induction chemotherapy with intensive timing (i.e., proceeding with the second cycle, regardless of counts) versus standard timing (i.e., awaiting count recovery before initiating the second cycle). The Down syndrome patients in the intensive timing arm of the study suffered a 32% mortality rate compared with an 11% mortality rate for the remainder of the patients, prompting early closure of that study arm for Down syndrome patients.[875] Standard timing induction for Down syndrome patients resulted in an impressively high CR rate of 95% (2.4% with toxic death and 2.4% with resistant disease).[875] Postremission consolidation with allogeneic HSCT in this trial also resulted in an unacceptably high treatment-related mortality for Down syndrome patients, with only 33% survival at 4 years.[875] In this study, standard timing induction followed by consolidation with chemotherapy was clearly the superior treatment for Down syndrome patients, with a 4-year DFS of 88% compared with a DFS of only 42% for this regimen in the non–Down syndrome patients.[875] Given these collective data, COG trial A2971 used a less intensive approach for Down syndrome patients, with four cycles of standard timing low-dose cytarabine, daunorubicin, and thioguanine, followed by a cycle of high-dose cytarabine with asparaginase and then intrathecal therapy.[909] Five-year outcome data were comparable to those of CCG2891 with OS of 84% and EFS of 79%.[910]

The first toxicity to which DS-AML patients were identified as being particularly susceptible was cardiotoxicity. On the POG9421 AML study in which the total cumulative anthracycline equivalent dose was 535 mg/m^2, 21% of DS children developed congestive heart failure with diminished shortening fractions on echocardiogram requiring chronic diuretics and/or inotropes. Sixty percent of these patients had congenital heart defects, and a third of the patients with cardiac dysfunction died.[911] On both CCG2891 and A2971, the cumulative anthracycline dose was reduced to 350 mg/m^2, but together with the overall reduction in chemotherapy on A2971, grade 3 or higher cardiac toxicity occurred in only 4% of patients during induction and 2% of patients during intensification.[910] The most recent COG Down syndrome AML study, AAML0431, was designed to further reduce the cumulative dose of anthracycline based on A2971 and gave a cumulative dose of only 240 mg/m^2. The study closed in December 2011 and to date no significant cardiac toxicity has been reported (J. Taub, personal communication, June 2014). However, the second cycle of induction chemotherapy during which high-dose cytarabine is given resulted in the greatest number of adverse events, including longest median duration, longest period of myelosuppression, and highest rate of bacterial infections. These toxicities, together with prior data demonstrating the exquisite sensitivity of Down syndrome AMKL blasts to cytarabine, have prompted plans for elimination of high-dose cytarabine for the majority of patients on the upcoming COG Down syndrome AML trial.

Despite the overall favorable outcomes for AML in Down syndrome, 15% to 25% of patients do not achieve long-term survival with current treatment protocols, predominantly owing to relapse or refractory disease.[891,892,912] Lack of response to therapy is associated with very poor outcomes with survival rates of 20-26% even after HSCT.[912a,912b] Prognostic markers in AML in Down syndrome are few because many of the predictive genetic lesions discussed earlier for non–Down syndrome AML are very infrequent findings in Down syndrome patients. To date, age older than 4 years and monosomy 7 appear the only known high-risk factors, both of which are relatively rare, and the outcome in patients harboring a monosomy 7 is still somewhat controversial. In the BFM-93 trial, 3 of 4 patients older than 4 years lacked the typical AMKL of Down syndrome and only 1 of the 3 survived.[886] In a combined analysis of European trials, 17 of 317 Down syndrome patients diagnosed with AML were older than 4 years.[885] DNA was available from blasts of 10 of these patients and, of these, 3 children younger than 7 years had the characteristic AMKL and 1 additional younger child had M0 AML with a *GATA1* mutation. Of the 6 remaining patients without AMKL or a *GATA1* mutation, all were 7 years of age or older and 4 experienced relapse. In CCG 2891, children older than 4 years of age ($n = 9$) had a significantly lower EFS at 6 years of only 33% ± 31%.[891] On A2971, patients younger than 4 years of age fared significantly better than older children (5 year EFS, 81% ± 7% vs. 33% ± 38%), although only 6 children older than age 4 years were included.[913] An international retrospective study of 451 Down syndrome patients with AML found monosomy 7 was associated with a moderately worse outcome, with a 5-year EFS of 69%. CCG studies 2891 and A2971, as well as a recent Japanese study,[892] found that only 11 of 16 patients with Down syndrome and AML and monosomy 7 were in complete clinical remission. These limited data suggest that older children with Down syndrome and AML, particularly given the absence of *GATA1* mutations, have disease more reminiscent of non–Down syndrome AML and should be treated more aggressively. Similarly, the presence of monosomy 7 may contribute to an inferior outcome and justify more intensive therapy for this cohort. However, most children with Down syndrome and AML are younger than age 4 years and lack monosomy 7. For this group, similar to non–Down syndrome AML, MRD after induction may enable the ability to identify high-risk patients. These data will be forthcoming from the recently closed AAML0431 study and will inform future clinical trials.

MYELODYSPLASTIC SYNDROMES

The myelodysplastic syndromes are a heterogeneous group of clonal hematologic disorders characterized by ineffective hematopoiesis, progressive cytopenias, dysplastic transformation of hematopoietic cells, and a propensity for transformation into AML.[914] MDS is most common in patients older than 60 years, but at the same time it is an unusual but clinically important disease of childhood.

Diagnosis and Classification

Morphology

The morphologic features of MDS include bone marrow dysplastic changes involving all three cell lineages (i.e., trilineage dysplasia). A common theme underlying these features is asynchrony between the usual cytoplasmic and nuclear differentiation programs, so that nuclei appear abnormal and less mature than the features of the surrounding cytoplasm. Typical erythrocytic abnormalities

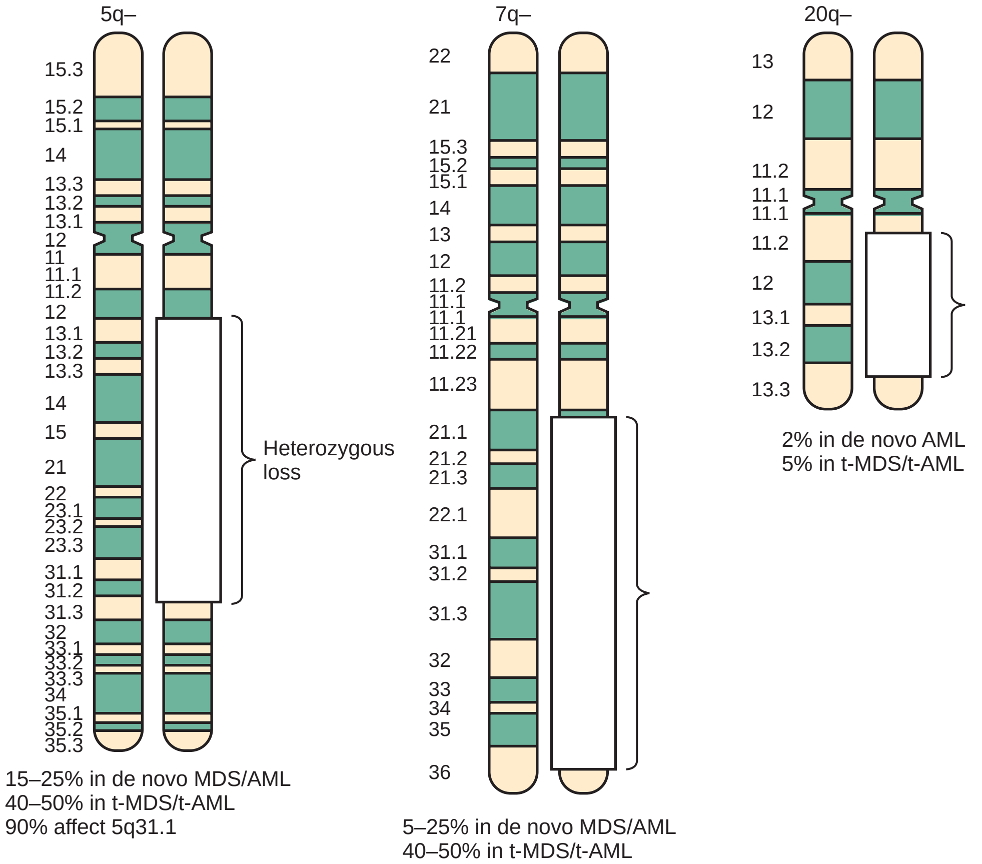
5q–
15.3
15.2
15.1
14
13.3
13.2
13.1
12
11
11.1
11.2
12
13.1
13.2
13.3
14
15
21
22
23.1
23.2
23.3
31.1
31.2
31.3
32
33.1
33.2
33.3
34
35.1
35.2
35.3
Heterozygous loss
15–25% in de novo MDS/AML
40–50% in t-MDS/t-AML
90% affect 5q31.1
7q–
22
21
15.3
15.2
15.1
14
13
12
11.2
11.1
11.1
11.21
11.22
11.23
21.1
21.2
21.3
22.1
31.1
31.2
31.3
32
33
34
35
36
5–25% in de novo MDS/AML
40–50% in t-MDS/t-AML
20q–
13
12
11.2
11.1
11.1
11.2
12
13.1
13.2
13.3
2% in de novo AML
5% in t-MDS/t-AML

Figure 51-8 The hallmark of myelodysplastic syndrome (MDS) is the loss of specific chromosomal regions. Deletions of the long arm of chromosomes 5 and 7 (5q–, 7q–) occur in de novo MDS and acute myelogenous leukemia (AML) but much more frequently in therapy-related MDS and therapy-related AML after treatment with alkylating agents. 20q– abnormalities are less common but also occur in both de novo MDS and AML and after prior chemotherapy. *(Data from Heaney ML, Golde DW: Myelodysplasia.* N Engl J Med *340:1649–1660, 1999; Hofmann WK, Lubbert M, Hoelzer D, et al: Myelodysplastic syndromes.* Hematol J *5:1–8, 2004; Pedersen-Bjergaard J, Andersen MT, Andersen MK: Genetic pathways in the pathogenesis of therapy-related myelodysplasia and acute myeloid leukemia.* Hematol Am Soc Hematol Educ Prog *2007:392–397, 2007.)*

include megaloblastoid changes, perinuclear ringlike deposits of iron (i.e., ringed sideroblasts), multinucleation, nuclear budding and fragmentation, and an increased percentage of immature forms. Dysmegakaryopoiesis is characterized by micromegakaryocytes, abnormal nuclei, and abnormal nuclear lobulation. The dysplastic features of the granulocytic cells comprise immature forms, hypogranulation, and the Pelger-Huët–type abnormality. Monocytic dysplasia includes increased numbers of bone marrow monocytes, abnormal granulation with increased azurophilic granules, hemophagocytosis, abnormal nuclei, and giant forms. Bone marrow cellularity is usually normal or increased, and the reticulin concentration is increased in most cases.

Patients with MDS may present initially with a single peripheral blood cytopenia, which usually progresses to pancytopenia with anemia, leukopenia, and thrombocytopenia. The anemia in MDS is caused by ineffective erythropoiesis with low reticulocyte counts and macrocytosis, teardrop cell formation, and moderate poikilocytosis. In addition, megaloblastoid circulating nucleated red blood cells are frequently detected. Peripheral blood dysgranulopoiesis manifests as circulating myeloblasts, progranulocytes, and Pelger-Huët cells. Thus early signs may be a falling hematocrit with increasing mean corpuscular volume or unexplained thrombocytopenia. The peripheral blood may also exhibit increased numbers of monocytes and monoblasts.

Cytogenetics

Cytogenetic abnormalities are common in children with MDS and were reported in 59% of 227 children tested in one study.[915] Chromosomal deletions are the hallmark of MDS and frequently involve the long arms of chromosomes 5, 7, and 20 (Fig. 51-8). Other common cytogenetic abnormalities include trisomy 8, del(17p), +21, inv(1), and t(7;16).[253,916,917] These chromosomal abnormalities are accompanied by other complex karyotypic changes in many cases. Other abnormalities that have been reported in pediatric cases of MDS include +6, +9, and +11, whereas deletions of chromosomes 11, 12, and 13 and Y are rare.[918] Common balanced chromosomal translocations found in AML, such as t(8;21), t(15;17), and inv(16), are not usually detected in MDS.[919]

Classification

In 1982, the FAB cooperative group defined five categories for the adult myelodysplastic and myeloproliferative syndromes—refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess blasts (RAEB), RA with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMML).[324,325,920,921] Classification of childhood MDS into defined FAB subcategories is not always straightforward. Many children show overlapping features of MPD and MDS, particularly patients with JMML and infant monosomy 7 syndrome.[254] The classification of pediatric MDS is also complicated by the fact that some children develop MDS in the context of inherited predispositions such as neurofibromatosis type 1, severe congenital neutropenia, and Down syndrome.[254] Using the FAB system of classification, the most common subtypes of MDS in children are RAEB and RAEB-T, whereas RA and RARS are uncommon and CMML is rare.

The WHO proposed a revised classification of MDS in 1997. The WHO system includes the following changes to the FAB criteria: elimination of the RAEB-T subtype through a reduction in the marrow blast count to 20% for the diagnosis of AML and the division of the RAEB subtype into RAEB-I, consisting of 5% to 10% marrow blasts, and RAEB-II, comprising 11% to 20% blasts.[309,328,922] Other WHO recommendations include two new categories for patients with refractory cytopenias with multilineage dysplasia and a new category for unclassified MDS. In the new system, CMML is removed completely and reclassified as a myeloproliferative disorder.

A third classification system is used in parallel with the FAB and WHO systems, the IPSS Scoring System.[923] This system assigns a numeric value to each of the number of cytopenias, cytogenetic abnormalities, and percentage of bone marrow blasts, and a higher score is associated with a poor prognosis. The use of the IPSS in children is of somewhat limited value. The only factors found to be predictive were bone marrow blasts greater than 5% and platelet counts greater than 100,000/μL.[924,925]

Pathophysiology

Evidence for clonality in MDS comes primarily from nonrandom X-inactivation studies (see earlier) performed on the bone marrow cells of female patients with MDS. These studies have demonstrated clonal involvement of hematopoietic cells in this disorder.[926-928] The trilineage dysplasia that characterizes MDS strongly suggests that the cell of origin resides within the compartment containing early hematopoietic progenitors or stem cells with multilineage potential.[929] Early mutations in stem cells may cause a failure in myeloid cell maturation, as well as enhanced apoptosis, leading to dysplasia, whereas subsequent defects affecting myeloid cell proliferation and the development of antiapoptotic signals may cause the clonal expansion of aberrant cells and frank leukemia. An increased level of apoptosis has been documented in early MDS, which is followed by decreased blast cell apoptosis in patients whose disease transforms to AML. MDS cells have been found to overexpress certain cell surface death receptors and their ligands, which may be induced as a result of signals from inflammatory cells present in the hematopoietic microenvironment.[919] Patients with more advanced MDS (FAB RAEB) have decreased levels of death receptor expression compared with early-stage MDS and increased expression levels of antiapoptotic factors, such as BCL2.[930,931]

The most common chromosomal abnormalities in childhood MDS involve large deletions, but little is known about the genes contained in the deleted segment[916,932-934] and whether these large genetic aberrations are inciting events leading to the development of MDS or represent secondary events. Ebert and colleagues[935] used an RNA interference screen to identify a putative gene deleted from the distal end of the long arm of chromosome 5 in 5q– syndrome, a subtype of MDS commonly encountered in older women but uncommon in pediatric patients. They found that haploinsufficiency of *RPS14*, a gene that codes for a component of the 40S ribosomal subunit, results in the characteristic phenotype of selectively diminished erythropoiesis, with preservation of megakaryocyte maturation. Moreover, the introduction of a lentiviral vector expressing *RPS14* into the CD34+ cells of MDS patients demonstrates restored erythroid differentiation exclusively in those with a 5q– genotype. These elegant studies convincingly identified a specific genetic lesion underlying the 5q– syndrome and also established this ribosomal protein as critical in erythroid development.[935] This finding is in keeping with the elucidated role of other ribosomal proteins in a number of bone marrow failure syndromes, such as Diamond-Blackfan anemia.[936]

5q– syndrome has a reasonably favorable outcome because of its natural history of slower progression to AML and sensitivity to treatment with lenalidomide, an analogue of thalidomide.[937,938] In this select population, lenalidomide (Revlimid) reduces the need for red cell transfusions and induces significant cytogenetic responses, which has resulted in its recent approval by the FDA for the treatment of MDS patients with 5q– syndrome.[939] Although this therapy has demonstrated impressive efficacy in this condition, deletion of this distal region of 5q is relatively rare. Only a minority of MDS patients without this cytogenetic abnormality respond to lenalidomide, and responders possess no distinguishing clinical or laboratory characteristics compared with nonresponders.[940] However, other studies[941] suggest that responders have a unique genetic signature consisting of low expression levels of genes typically involved in terminal erythroid differentiation. These results imply that the restoration of normal red cell development underlies lenalidomide's mechanism of action, particularly in non-5q–, whereas in 5q– syndrome, lenalidomide may more directly suppress the malignant clone.[942] Clinically, this suggests that genetic profiling could be used to select a group of MDS patients without 5q– deletions who would be more likely to respond to this agent.

These large deletions also likely contain certain important tumor suppressor genes, the elimination of which contributes to MDS pathogenesis. For example, we completed a detailed analysis of the more proximally deleted region of the long arm of chromosome 5 and identified α-catenin as a novel tumor suppressor gene in MDS.[252] In both primary patient samples and AML cell lines with a deletion of 5q–, α-catenin expression levels were found to be specifically and substantially decreased through the combination of a deletion on one chromosome and epigenetic silencing by hypermethylation and histone deacetylation of the promoter of the second allele. Another group has identified the WT1 transcription factor, early growth response 1 *(EGR1)*, as a putative candidate gene in this same region and demonstrated that haploinsufficiency of this tumor suppressor gene can result in MDS or MPD after treatment with an alkylating agent.[943]

In addition to gene deletions, a number of other genes have been found to contribute to MDS pathogenesis. Balanced translocations, such as those seen in de novo AML, are uncommon in MDS, but some of the same genes may be involved. Other genetic lesions appear to be specific for MDS. We and others have shown that certain *HOX* genes, most notably *HOXA9*, are overexpressed in the HSCs of patients with MDS.[944-946] Translocations involving *HOX* genes and the nucleoporin gene *(NUP98)* on chromosome 11, including *NUP98-HOXA9*[947] and *NUP98-HOXA13,*[948] have been found in rare patients with MDS. Certain genetic abnormalities are more characteristic after prior treatment with chemotherapy. Overexpression of the *EVI1* transcription factor on chromosome 3, either through gene rearrangements or rare translocations, has been associated with MDS after chemotherapy.[949-951] Similarly, nucleophosmin and myeloid leukemia factor 1 *(NPM-MLF1)* fusion gene, formed as a result of a t(3;5) chromosomal translocation, occurs in rare cases of MDS or AML arising from MDS.[952,953] Mutations in *RAS* and *FLT3*, such as those seen in de novo AML, are found in patients with MDS, particularly those with 7q– and often after treatment with alkylating agents.[954] These patients may also have mutations in the *AML1* gene, whereas the *AML1-ETO* translocation is rare in MDS.[955,956] Prior treatment with alkylators can also lead to MDS with 5q–, and these patients often have a mutation in *TP53*, a gene that is also frequently mutated in AML cases with a complex aberrant karyotype, which include a 5q deletion, but rarely in classic de novo AML.[955-957]

More recently, owing to responses in MDS to epigenetic regulating agents, such as DNA methyltransferase inhibitors, and the identification of a number of mutations in epigenetic regulating genes in AML, a series of studies using high-throughput genomic approaches have identified similar abnormalities in MDS. Mutations in *TET2* occur in approximately 25% of adults with MDS,[958] whereas *DNMT3A* and *IDH1* and *IDH2* mutations were found in 3% to 10% of patients.[959-962] In addition, mutations have been found in histone modifying enzymes, including *EZH2*, which is rare in de novo AML,[963,964] and *ASXL1*, both associated with a worse outcome in adult MDS.[965,966] Finally, novel mutations in genes involved in the assembly of the spliceosome that removes introns from transcribed pre-mRNA have been identified. The genes most commonly involved include *SF3B1* and *U2AF1*.[967-969] However, MDS is much rarer in children, and all of these mutations appear to be rare in pediatric MDS; thus their impact on pathogenesis and prognosis remain uncertain at this time.[970]

Inherited and Environmental Predisposing Factors

Several environmental exposures and inherited conditions are associated with an increased risk for MDS and AML. As noted, alkylating agents and topoisomerase II inhibitors can predispose to MDS and AML, which can occur as treatment-related second malignancies in patients treated for ALL and many other types of cancer. Secondary disease associated with alkylating agent chemotherapy tends to occur from 3 to 11 years after treatment, after which the incidence appears to plateau.[971-973] The disease often presents as MDS, and an underlying predisposition may involve defective DNA repair mechanisms. Commonly, there are deletions of chromosomes 5 or 7.[972] *MLL* gene rearrangements are often found in AML cases that arise after treatment with topoisomerase II inhibitors such as etoposide, but these patients tend to present with frank AML after a short latency of 1 to 3 years from therapy and without an MDS prodrome.[972,974]

A number of genetic conditions are associated with an increased risk for MDS and subsequent AML, particularly the bone marrow failure syndromes and chromosomal breakage disorders. Some disorders, such as Fanconi anemia, in which patients demonstrate both pancytopenia and increased sensitivity to DNA damage, suggest that impaired DNA repair may be a mechanism for malignant transformation. However, other marrow failure syndromes are not characterized by increased susceptibility to chromosomal breakage such as dyskeratosis congenita or may present predominantly as a single cytopenia, such as neutropenia in Shwachman-Diamond syndrome, anemia in Diamond-Blackfan anemia, and low platelets in amegakaryocytic thrombocytopenia. The evolution of MDS in these conditions is variable (10% to 20% in Shwachman-Diamond syndrome compared with 1% in Diamond-Blackfan anemia), and the mechanism remains elusive, although defective ribosomal processing may be involved, given the identified functions of the target proteins involved in a number of these conditions.[919,975] In addition, there are several MDS/AML predisposition genes that have been identified, including loss-of-function alterations affecting the key hematopoietic transcription factor genes, *RUNX1*, *CEBPA*, and, most recently, *GATA2*. Germline mutations in *RUNX1* can result in familial platelet disorder with a propensity to myeloid malignancy (FPD/AML). These patients have thrombocytopenia or normal platelet numbers but abnormal platelet function and can evolve into MDS or AML.[76-79] A recently identified related disorder termed thrombocytopenia type 2 has been linked to mutations in a gene called *ANKRD26* at 10p11.2-p12 in a number of Italian and American families with a progression to frank myeloid and lymphoid malignancies, with an MDS prodrome rarely described.[976] Deletions of the granulocyte-specifying gene *CEPBA* have been described in a small number of families resulting in an FAB M1 or M2 subtype AML; however, an MDS prodrome has not been described. The *CEBPA* mutations are autosomal dominant, similar to those involving *RUNX1*, but fully penetrant, in contrast to the highly variable penetrance observed for *RUNX1* lesions.[78,977-979] Loss-of-function mutations or deletions involving *GATA2*, which plays a role in early blood, vascular, and lymphatic development, have been described as a cause of a familial MDS/AML syndrome. These patients may present with neutropenia, often with an associated monocytopenia[80] or with so-called monoMAC syndrome/DCML deficiency associated with monocytopenia and dendritic, B-cell, and NK-cell lymphopenias with a predisposition to nontuberculosis mycobacterial infections. Some of these patients may also develop lymphedema.[80,980,981]

Clinical Presentation

Children with MDS generally present with the signs and symptoms associated with bone marrow failure. Abnormalities in the peripheral blood may also be detected on routine medical examinations in asymptomatic children. Symptomatic children often present with some degree of fatigue, fever, malaise, or infections. Physical examination frequently reveals pallor, easy bruising, and petechiae. Splenomegaly, hepatomegaly, and lymphadenopathy are uncommon findings. The peripheral blood cell counts usually reveal anemia, neutropenia, and thrombocytopenia.

Differential Diagnosis

With a careful history and physical examination, as well as the evaluation of peripheral blood and a bone marrow aspirate, biopsy, and cytogenetics, a diagnosis of MDS can be made. Severe nutritional deficiencies of vitamin B_{12} and folate should be excluded, because they may impart a megaloblastic appearance to the bone marrow. Other nutritional deficiencies such as thiamine, pyridoxine, iron, and riboflavin can also present as some degree of bone marrow dysplasia and should be considered in the differential diagnosis. Occasionally, severe aplastic anemia can be difficult to distinguish from MDS, although a bone marrow biopsy should resolve this question. Certain viral infections (e.g., human immunodeficiency virus, parvovirus B19, Epstein-Barr virus, human herpesvirus 6, cytomegalovirus) and exposures (e.g., irradiation, chemotherapy, organic solvents) are associated with bone marrow failure and should be considered before making the diagnosis of MDS. The bone marrow cell karyotype should be determined to identify any clonal abnormalities. In addition, evidence has suggested that whole-genome scan by SNP analysis may detect clonal abnormalities in some patients with a normal bone marrow cell karyotype.[982,983]

Treatment

The rarity and heterogeneity of MDS in children, the lack of consensus on nomenclature, and the need for a risk-based classification system specific for pediatric MDS have hindered the development of appropriate therapy for pediatric MDS. Most clinical trials studying MDS have enrolled adults, in whom the disease may be different, and there have been few meaningful trials enrolling children. Regardless of treatment, the overall clinical outcome of children with MDS is guarded.

The only proven curative treatment for MDS is allogeneic HSCT. AML-based chemotherapy without HSCT has resulted in poor survival in the 30% range.[984,985] Prior to HSCT, or if an appropriate donor was not available, treatment primarily consisted of supportive care with packed red blood cell or platelet transfusions, parenteral antibiotics, and hydration. Other noncytotoxic therapies, including hormone therapy (e.g., androgens, glucocorticoids), recombinant hematopoietic growth factors (e.g., G-CSF, GM-CSF, erythropoietin), and differentiating agents (e.g., 13-*cis*-retinoic acid, ATRA), have been used in adults and have shown minimal benefit.[986-996] Low-dose chemotherapy has been used in an attempt to promote differentiation of the malignant hematopoietic clone. Although studies using low-dose cytarabine have shown improvement in peripheral counts and decreased marrow blast percentages in some patients with MDS, the responses have not been sustained.[997] Other agents, such as melphalan, hydroxyurea, etoposide, topotecan, 6-mercaptopurine, and busulfan, have been used with only partial or temporary responses in small numbers of patients.

A number of new agents have emerged for the treatment of pediatric MDS after randomized clinical trials in adult patients. These therapies have evolved out of a growing interest in the field of epigenetics and the concept that the repression of key tumor suppressor genes in MDS may occur because of aberrant methylation and histone deacetylation, instead of or in concert with gene deletions and mutations.[252] Studies involving the *CDKN2B (p15*^{INK4B}*)* tumor suppressor gene have demonstrated evidence of methylated CpG islands in the promoter region, preventing gene transcription and leading to gene repression.[998,999] Since these initial studies, a number of other genes have been found to have reduced expression because of DNA methylation, thus prompting clinical trials of demethylating agents in MDS.[1000,1001] Based on the impressive response rates and delayed progression to AML compared with supportive care, two demethylating agents, 5-azacytidine and decitabine, have been approved by the FDA for the treatment of MDS in adults.[1002,1003] These agents have shown the greatest efficacy in low-risk MDS. They are given in a low-dosage schedule, and patients may need to be treated for several months before a clinical response is achieved. Although these agents have improved the time to progression in adult MDS, they cannot cure the disease. The role of these therapies in pediatric MDS is currently under active investigation. As indicated earlier, lenalidomide is now approved for the treatment of patients with MDS with the 5q− cytogenetic abnormality and appears to work best in those whose MDS clone lacks any other cytogenetic abnormalites.[937]

For children with MDS, allogeneic HSCT remains the treatment of choice. In the absence of a sibling donor, a matched unrelated donor should be sought. Children with lower-risk MDS are frequently treated with allogeneic HSCT without prior chemotherapy, particularly if a matched sibling is available, to avoid the need for large numbers of transfusions. In more advanced MDS (WHO RAEB1 and RAEB2), AML-type chemotherapy has been often administered to induce a remission or to reduce the disease burden before HSCT. A study from the Fred Hutchinson Cancer Research Center in Seattle in children with MDS demonstrated a 3-year survival rate of patients with lower-stage MDS of 74% compared with 68% for patients with more advanced disease.[1004] The European Working Group on MDS in Children (EWOG-MDS) reported a 55% 5-year EFS rate for 33 pediatric patients with MDS after HSCT.[1005] A recent pediatric study from the University of Minnesota demonstrated an OS of 53% and a DFS of 48% at 3 years for 37 pediatric patients with MDS who received allogeneic HSCT after myeloablative conditioning over a 20-year period ending in 2010. Earlier HSCT without preconditioning chemotherapy was associated with an improved outcome, suggesting that delaying transplant to reduce disease burden with AML-directed therapy is not warranted if an appropriate donor is available.[925] These results are in keeping with those found in a European study of pediatric MDS patients who received a myeloablative allogeneic HSCT in which the OS was 63% at 5 years.[984] This study similarly showed no benefit to pre-HSCT chemotherapy except for the group who had more advanced myelodysplasia-related AML, a subgroup not included in the prior study. A longer interval between diagnosis and HSCT was actually associated with less relapse but may have been confounded by the number of lower-risk MDS patients in this group.[984] TRM during transplant can be a significant issue, resulting in some groups attempting reduced-intensity conditioning regimens. These approaches have had variable success, with some studies reporting lower DFS rates.[925,1006]

MYELOPROLIFERATIVE DISORDERS

Juvenile Myelomonocytic Leukemia

JMML is a rare, clonal, myeloproliferative disorder of the stem cell that usually manifests in the first few years of life. It has also been referred to as juvenile chronic myelomonocytic leukemia (JCML), infantile monosomy 7 syndrome, or juvenile granulocytic leukemia. Although JMML, adult CML, and CMML have some similar features, JMML is a clinically and pathophysiologically distinct disorder of young children. An international consensus panel consisting of the JMML Working Group and the EWOG-MDS has established a set of clinical and laboratory criteria for the diagnosis of JMML (Box 51-3).[1007]

Box 51-3 **Recommended Diagnostic Criteria for Juvenile Myelomonocytic Leukemia**

CATEGORY 1 (ALL OF THE FOLLOWING)

No Philadelphia chromosome or BCR-ABL rearrangement
Peripheral blood monocyte count higher than 1000/μL
Bone marrow blasts less than 20%
Splenomegaly

CATEGORY 2 (AT LEAST ONE OF THE FOLLOWING)

Somatic mutations in *RAS*, *PTPN11*, or *CBL*
Clinical diagnosis of neurofibromatosis type 1 or *NF1* gene mutation
Monosomy 7

CATEGORY 3 (TWO OF THE FOLLOWING IF NO CRITERIA FROM CATEGORY 2)

Increased fetal hemoglobin level
Myeloid precursors in peripheral blood
White blood cell count greater than 10,000/μL
Clonal abnormalities (excluding monosomy 7)
Granulocyte-macrophage colony-stimulating factor hypersensitivity of myeloid progenitors in vitro

Modified from the World Health Organization criteria from the 2008 Juvenile Myelomonocytic Leukemia Symposium, Atlanta, Ga.

Pathobiology

The cause of JMML is unknown. Several recurrent chromosomal abnormalities have been associated with JMML, but their relevance to the pathogenesis of the disease is not well understood. For example, up to 20% of patients meeting the diagnostic criteria for JMML have abnormalities in chromosome 7 (e.g., monosomy 7, 7q−), and the genes involved and their contributions to disease pathogenesis remain an area of intense investigation.[540,1007] At the molecular level, one or more defects involving the NF1/RAS signal pathway have been demonstrated in studies of JMML cells, implicating this pathway in the development of the disease. Activating point mutations in *NRAS* and *KRAS* genes have been demonstrated in 15% to 30% of patients with JMML.[1008,1009] Other patients have loss of *NF1*, which codes for a RAS GTPase-activating protein involved in the GM-CSF signaling pathway.[68,1010] Mutations in *PTPN11* encoding the SHP-2 phosphatase have also been found in patients with JMML.[1011,1012] Similar *PTPN11* mutations have been found to cause Noonan syndrome, an autosomal dominantly inherited syndrome associated with short stature, cardiac abnormalities and characteristic facial features, and, occasionally, a JMML-like hematopoietic picture. More recently, studies using genome-wide SNP arrays have uncovered novel homozygous mutations in the E3 ubiquitin ligase gene, *CBL,* in patients with JMML without mutations in the other identified causative genes.[1013-1015] These mutations are all mutually exclusive. JMML cells grown in culture demonstrate a specific hypersensitivity to the growth factor GM-CSF that is not observed with other growth factors, such as interleukin-3.[1016] Knockout mice lacking expression of *NF1* die at embryonic day 13 or 14, but hematopoietic progenitor cells from these embryos grown in culture also demonstrate a specific hypersensitivity treatment with GM-CSF.[144] Moreover, immunodeficient mice transplanted with these cells develop a myeloproliferative disorder similar to JMML.[146,1017]

An increasing body of evidence has suggested that JMML arises from a pluripotent HSC, but the molecular abnormalities underlying the transformation appear to be distinct from those in the adult-type myeloproliferative disorders.[1017] JMML cells maintain their clonality when grown in long-term culture-initiating assays, in contrast to Philadelphia chromosome–positive CML cells, which yield to polyclonal residual normal cells in similar culture assays. Marrow cells from patients with JMML grow in culture and generate granulocyte-macrophage colony-forming units (GM-CFUs) spontaneously, in the absence of added hematopoietic growth factors. In contrast, GM-CFUs from normal individuals and from patients with adult CML only grow when exogenous growth factors are added to the semisolid culture medium.

Clinical Presentation and Prognosis

The presenting signs and symptoms of JMML result from organ infiltration of malignant cells; the most common symptoms are fever, cough, infection, pallor, malaise, hepatosplenomegaly, lymphadenopathy, rash, bleeding, and failure to thrive.[1007] Splenomegaly has now been incorporated as a category 1 criterion because only 7% of patients with JMML do not have splenomegaly at diagnosis.[1018] Laboratory abnormalities usually include an elevated WBC count with monocytosis, anemia, thrombocytopenia, an elevated fetal hemoglobin level, and hypergammaglobulinemia.[1007]

JMML is a progressive and often rapidly fatal disease, particularly in older children. Children younger than 2 years may have a more indolent course and, in fact, the disease may spontaneously resolve, as has been observed in patients with Noonan syndrome (reminiscent of TMD in Down syndrome) and a proportion of JMML patients with *RAS* or *CBL* mutations.[1018,1019] However, patients with older age at diagnosis (>2 years), higher fetal hemoglobin level, and platelets less than 33,000/μL tend to present with more complex cytogenetic abnormalities and are predisposed to poorer outcome.[1020] Although some studies have not found any prognostic differences in the genetic cause, others have found *PTPN11* mutations to be associated with increased relapse after HSCT.[1021] The accelerated blast phase characteristic of adult CMML is unusual in children with JMML.

Treatment

Several treatment regimens have been used to improve survival, including low-dose chemotherapy, intensive AML-type therapy, and 13-*cis*-retinoic acid.[540,1022-1027] Although durable remissions have been reported in a minority of cases, the response to these agents is usually transient and the long-term survival is poor. The only known curative therapy for JMML is allogeneic HSCT, and neither pretreatment chemotherapy nor splenectomy has not been found to impact survival.[1018] When an HLA-matched related donor is available, HSCT is the recommended treatment for patients with newly diagnosed disease. Survival rates of 35% to 55% have been reported with HSCT, but treatment-related mortality and relapse rates remain high, particularly for patients who undergo unrelated donor HSCT.[1028-1031] Several issues remain controversial, including the management of patients without HLA-matched related donors, GVHD prophylaxis, pre-HSCT chemotherapy, and appropriate myeloablative therapy. Future therapies in JMML are aimed at targeting downstream pathways from RAS, including mTOR- and PI3K-inhibiting agents.

Chronic Myelogenous Leukemia Caused by the *BCR-ABL* Fusion Gene

In 1951, Dameshek first introduced the idea that CML, polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PM) were related myeloproliferative syndromes, even though each has distinct clinical, laboratory, and biologic features.[1032,1033] A common feature is multilineage clonal proliferation involving the entire spectrum of myeloid cells, suggesting that transformation has occurred in a pluripotent stem cell. All these conditions, as well as systemic mastocytosis, hypereosinophilic syndrome, chronic eosinophilic leukemia, and chronic myelomonocytic leukemia, have been linked to a specific mutation or translocation resulting in the constitutive activation of a receptor tyrosine kinase. This accounts for the increased hematopoietic stem and progenitor cell proliferation with differentiation that characterizes these disorders. Patients may occasionally present with mixed manifestations rather than a distinct myeloproliferative disorder or, over the course of their illness, develop problems more typical of one of the other disorders.

Chronic myelogenous leukemia was first recognized as early as 1845, with several described cases of splenomegaly, anemia, and massive granulocytosis.[1034,1035] However, it was not until 1960, with the discovery of the Philadelphia chromosome, that the disease became better understood.[1036] CML constitutes 15% to 20% of all leukemias but accounts for only 1% to 3% of all childhood leukemias. In children, the disorder may present as one of two distinct clinical syndromes, adult-type CML (ACML), which is the same as the disease in older patients (and herein referred to as CML) and juvenile CML (JCML), now known as JMML, a syndrome described in the previous section that is restricted to children and has distinct clinical, laboratory, and cytogenetic characteristics (Table 51-2). The incidence of CML is less than 1 case/100,000 for patients younger than 20 years and 1 to 2 cases/100,000 for those 20 to 50 years, and it rises slowly thereafter. The median age at diagnosis is 67 years, and the male-to-female ratio is 1.8 : 1. It has been difficult to identify environmental factors associated with the pathogenesis of CML. Ionizing radiation is the only clearly identified risk factor for CML. A sevenfold increase has been documented in the survivors of the nuclear bomb–related exposure to radiation in Japan and, notably, the incidence of CML in this population was highest in young people, especially children younger than 5 years.[1037,1038]

Pathobiology

The diagnosis of CML is almost always associated with the t(9;22)(q34;q11) chromosomal translocation in bone marrow cells. This reciprocal translocation is detected by standard cytogenetic techniques in almost 90% of CML cases.[1036,1039] However, the presence of the Philadelphia chromosome in bone marrow cells is not pathognomonic for CML, because this chromosome is present at diagnosis in 25% to 50% of adult patients, 3% to 5% of children with ALL,[1040] and 2% of patients with AML. It is absent in 5% of patients with CML, although these patients' bone marrow cells may harbor a variant of the translocation detectable only by RT-PCR or FISH.[1041] The Philadelphia chromosome of CML involves a reciprocal translocation of the *ABL* gene from its normal position at the end of the q arm of chromosome 9 to a site on the q arm of chromosome 22 within the *bcr*

TABLE 51-2 Clinical Characteristics of Children with Juvenile Myelomonocytic Leukemia (JMML) versus Adult-Type Chronic Myelogenous Leukemia (CML)

Characteristics at Diagnosis	JMML	CML
Age (yr)	Usually <4	Usually >4
Lymphadenopathy	Common	Unusual
Skin lesions	Common	Unusual
Bleeding	Common	Unusual
Bacterial infection	Common	Unusual
White blood cell count >100,000/µL	Unusual	Common
Hemoglobin <12 g/dL	Common	Variable
Platelets	Usually decreased	Usually increased
Monocytosis	Common	Unusual
Circulating pronormoblasts	Common	Unusual
Increased fetal hemoglobin	Common	Unusual
Philadelphia chromosome	Absent	>90%
BCR-ABL fusion gene	Absent	Present
Leukocyte alkaline phosphatase decreased	Variable	Common
Clinical Course		
Median survival (yr)	1-2	Variable, >10 yr in chronic phase
Blast phase	Unusual	Common

region. The reciprocal t(9;22) chromosomal translocation creates a fusion *BCR-ABL* gene detectable in the bone marrow and peripheral blood by PCR assay. The chimeric gene encodes a dysregulated tyrosine kinase that is constitutively active, is expressed in the cytoplasm, and has increased activity compared with the normal ABL protein.[1042] In contrast to cytoplasmic BCR-ABL protein, normal ABL is a nuclear kinase, the activity of which is tightly regulated in vivo.[1043] Although the precise function of normal ABL is not known, available evidence suggests that it regulates pathways that mediate cell cycle arrest after genotoxic damage.[1044-1048] The main clinical manifestation of CML is an overabundance of mature myeloid cells from hyperproliferative progenitor cells. Evidence for the role of the *BCR-ABL* translocation in the pathogenesis of CML is confirmed by the observation that BCR-ABL proteins can also induce a CML-like syndrome in vivo in mice when they are expressed in hematopoietic progenitors.[1049-1052] Mechanistic studies of these fusion proteins have shown that RAS signaling is essential for transformation and that multiple accessory molecules, including the adapters GRB2, SHC, and CRKL, are used to couple the activated ABL kinase to RAS, resulting in activation of Jun kinase.[1053-1055] Oncogenic signaling by BCR-ABL has also been shown to involve the cell cycle–regulated genes *MYC* and *cyclin D1*.[1056,1057] Thus, multiple signaling pathways are activated to mediate leukemic transformation by BCR-ABL. Transformation has also been shown to occur in the pluripotent stem cell. Murine experiments in which sorted HSCs were transduced with *BCR-ABL* and transplanted into irradiated recipients developed a CML-like phenotype, whereas the same experiment performed using more differentiated progenitor cells failed to induce a myeloproliferative disease.[111] Moreover, granulocytes, monocytes, erythroid cells, megakaryocytes, and lymphocytes from patients with this disease have been shown to be clonal progeny of a single cell and to harbor the Philadelphia chromosome.[1058-1060] Probably because of some persistent normal hematopoiesis in patients with CML and because of the long life span of some cell types, such as T lymphocytes, all patients do not have demonstrable involvement of all lineages.[1059] It is worth emphasizing that the *BCR-ABL* fusion gene is acquired somatically by an HSC and its expression is restricted to its clonal progeny, so the nonhematopoietic cells of the patient do not contain the translocation.[1061,1062]

Clinical Presentation

CML can present in one of three phases—chronic phase, accelerated phase, or blast crisis. Most patients present in the chronic phase and may be asymptomatic. When symptoms and signs are present, they are often mild and are caused by the accumulation of mature and immature granulocytic cells. Generalized malaise, weakness, weight loss, fever, pallor, and organomegaly, particularly splenomegaly, are frequent presenting symptoms. Abdominal pain from massive splenomegaly may also bring a patient to medical attention. CNS, retinal, and pulmonary dysfunction can occur, as well as arthritis and priapism. Occasionally, the diagnosis is made from a routine blood cell count.[1063] WBC counts usually exceed 100,000/µL, with the appearance of some circulating immature myeloid forms.[1064,1065] Basophilia and eosinophilia may also occur. In addition, platelet counts above 400,000/µL are frequently observed, although thrombocytopenia may also occur. The WBC and platelet counts in CML patients may fluctuate spontaneously and nonconcordantly, making it difficult to attribute changes to the effects of therapy.[1066] The presence of anemia is also variable in CML.[1067] Leukostasis, a syndrome caused by hyperleukocytosis and frequently presenting as neurologic symptoms, such as dizziness, confusion, and somnolence, or pulmonary symptoms, including tachypnea, dyspnea, and respiratory failure, is observed in AML and ALL secondary to high blast cell counts. However, symptoms of leukostasis are uncommon in adults with CML and are only slightly more common in childhood CML, even in patients with very elevated WBC counts.[1068] Skin nodules, representing extramedullary hematopoiesis, may also be present.[1069] The bone marrow in CML patients typically demonstrates normal myeloid maturation, with granulocytic hyperplasia and an increased myeloid-to-erythroid ratio. Megakaryocytic hyperplasia may also occur.[1063]

Dysplastic marrow basophils or eosinophils are common, and cells of these lineages may be increased in number. About one in six CML patients has lipid-laden macrophages, sometimes called sea-blue histiocytes, similar to Gaucher cells.[1070] Although an increase in reticulin may occur, myelofibrosis is unusual. By definition, in chronic phase CML, the blast cell percentages in both the peripheral blood and bone marrow are less than 10%, and an increase above these levels may indicate a transition from chronic phase to accelerated phase or blast crisis. Other abnormal laboratory findings in chronic phase CML include low leukocyte alkaline phosphatase (LAP) activity and increased vitamin B_{12} levels. The latter occurs because of the increased white blood cell mass, which produces WBC-derived vitamin B_{12}–binding protein. The cause of the low LAP is unclear but may involve imperfect maturation of CML granulocytes. With therapy, the level of vitamin B_{12} falls to normal, whereas changes in LAP are less predictable.[1071]

The accelerated phase is characterized by increased marrow or blood blast cell values of 10% to 19%, peripheral basophilia greater than 20%, persistent thrombocytopenia unrelated to therapy or thrombocytosis unresponsive to therapy, and increasing spleen size or WBC count. If marrow or blood blast cell percentages exceed 19% of total leukocytes, this signifies a transition to blast crisis, which may be myeloid, lymphoid, or mixed. Presentation in blast crisis can mimic an acute leukemia of lymphoid or myeloid lineage, and in this case the cells harbor the Philadelphia chromosome.

Differential Diagnosis

The differential diagnosis of an elevated WBC count (also known as a leukemoid reaction) includes severe infections, congenital heart disease, and metastatic cancer. In disorders other than CML, the peripheral blood rarely contains blasts and promyelocytes, the WBC count is usually somewhat lower, and the LAP scores and cytogenetic studies are normal. In general, patients with CML have a WBC count of more than 100,000/μL, a WBC differential containing promyelocytes and myelocytes (the blood smear often appears similar to a marrow smear), a low LAP, a large spleen, and Philadelphia chromosome or molecular detection of the *BCR-ABL* transcript. Because CML patients can present in blast crisis, the patient with Philadelphia chromosome–positive ALL presents a problem in differential diagnosis. Determination of the size of the abnormal fusion protein (usually 210 kD in CML and 185 kD in Philadelphia chromosome–positive ALL) and the fusion transcript breakpoint by RT-PCR assay can help, as can the response to therapy. An important distinction is that in ALL the Philadelphia chromosome commonly disappears with intensive chemotherapy whereas in CML presenting in blast crisis the disease may revert to the chronic phase, with the Philadelphia chromosome persisting in the recovering bone marrow cells.[1072]

Clinical Course

Approximately 90% of patients are in the chronic phase at diagnosis. Prior to the advent of tyrosine kinase inhibitor therapy, patients sometimes enjoyed unimpaired lifestyles for periods of many months to years, with minimal therapy during the chronic phase of their disease. However, if untreated, most patients will experience progressive disease from 3 to 8 years after diagnosis. This is because of the development of the accelerated phase, which progresses to eventual blast crisis, usually within 3 months. Overall, blast crisis will develop in 75% to 85% of all untreated patients with CML. Blast crisis is a clinically high-risk form of CML that is difficult to control with chemotherapy and often leads to death within a short time, usually months.

Although BCR-ABL alone may be able to induce chronic-phase CML, evolution to accelerated phase or blast crisis is often accompanied by the acquisition of duplicated Philadelphia chromosome or additional cytogenetic abnormalities in the bone marrow, such as trisomy 8 or abnormalities involving chromosome 7, including the t7;11(p15;p15) that creates the *NUP98-HOXA9* fusion.[1073] Transformation to blast crisis appears to follow many of the same multiple mutational steps involved in the molecular pathogenesis of de novo AML or ALL. LAP levels may also rise during the transition to blast crisis. Myeloid blast crisis is somewhat more common and carries a poorer prognosis than lymphoid blast crisis.[1072,1074-1076] About one third of patients have a lymphoblastic crisis, usually precursor B-cell by immunophenotype, although T-lymphoblastic crisis phenotypes can occur.[1077,1078] Patients with lymphoid blast crisis have a younger median age than patients with myeloid blast crisis.[1079] CML blast crisis can also contain subpopulations of blast cells with differing phenotypes (possibly subclones differentiated along both lymphoid and myeloid lineages, biphenotypic leukemias) and blast crisis in which individual cells simultaneously express features of more than one lineage (mixed-lineage leukemias).

Treatment

Historic Chemotherapy. Traditional chemotherapy for CML used busulfan or hydroxyurea.[1080] Busulfan is associated with infertility and, rarely, marrow aplasia and interstitial pneumonitis. Hydroxyurea is less toxic but only induces a decreased granulocyte count. Hydroxyurea does not induce cytogenetic remissions or change the natural history of the disease. Interferon-α (IFN-α) has demonstrated improved rates of hematologic remission over hydroxyurea, with evidence of hematologic control in 70% to 80% of patients and complete cytogenetic responses in 6% to 26% when given as a single agent in different series.[1081-1083] The normalization of bone marrow cytogenetics was shown to persist for many years in some patients, even after stopping the drug. Interestingly, these patients often remain PCR positive for the *BCR-ABL* transcript if sensitive techniques are used.[1084-1086] Despite the efficacy of IFN-α, unwanted side effects are common and include fatigue, myalgias, arthralgias, headaches, weight loss, depression, diarrhea, neurologic symptoms, memory changes, hair thinning, autoimmune diseases, and cardiomyopathy,[1087-1089] leading 14% to 26% of patients to discontinue therapy.[1090-1093] Thus although IFN-α demonstrated some promise in this disease, the toxicity profile made it intolerable to a large fraction of CML patients.

Tyrosine Kinase Inhibitors. The treatment of chronic phase CML has since been revolutionized with the advent of molecularly targeted therapy that specifically targets the BCR-ABL fusion protein. Imatinib mesylate, also known as STI-571 or Gleevec, is a tyrosine kinase inhibitor that blocks enzymatic activity of several tyrosine kinases, including mainly ABL, PDGFR, and KIT, by binding to the adenosine triphosphate (ATP) binding site. The promising results of phase I and II studies that produced impressive activity in patients who had already failed IFN-α[1094] led to the International Randomized Study of Interferon and STI571 (IRIS), which randomized patients to imatinib versus interferon with cytarabine as initial therapy for patients with CML.[1095] This study demonstrated better outcome with longer times to progression and to accelerated phase or blast crisis for patients initially treated with imatinib. Additionally, patients treated with imatinib had higher rates of hematologic and cytogenetic remission and, for the first time, molecular responses were obtained with an undetectable *BCR-ABL* fusion transcript. Treatment with imatinib effectively established new standards for response rates, including complete hematologic response (CHR), implying a normalization of peripheral blood cell counts; major cytogenetic response (MCyR), with the presence of Philadelphia chromosome in 1% to 35% bone marrow cells analyzed by metaphase spread; complete cytogenetic response (CCyR), with the absence of Philadelphia chromosome; and major molecular response (MMR), demonstrating a 3-log reduction in detection of the *BCR-ABL* transcript in bone marrow cell RNA compared with diagnostic levels. The 8-year follow-up of the IRIS study has now been published[1125] and has convincingly demonstrated the long-term benefit and tolerability of imatinib therapy, with increasing rates of MMR and CCyR over time. Imatinib has excellent oral bioavailability, with minimal systemic effects other than myelosuppression. Other side effects include peripheral and periorbital edema, muscle cramps, gastrointestinal intolerance, and rash. Cardiac complications, including congestive heart failure, are rare but have been reported.[1097] However, imatinib must be continued indefinitely because cessation of the drug is generally associated with an increase in bone marrow cell *BCR-ABL* transcript levels and, eventually, hematologic relapse. Given the success of imatinib therapy, a number of studies have strived to determine what cytogenetic or molecular milestones at what specific time points after initiation of imatinib are most predictive of response. CCyR is regarded as the gold standard and considered to correlate with a 2-log reduction of *BCR-ABL* transcript levels (expressed as 1% on the recently accepted international scale that establishes 100% as an initial transcript level). A recent study demonstrated that a transcript level of less than 10% at the 3-month mark had the greatest prognostic value in predicting outcome.[1098] Although current practice includes surveillance at later time points, this manuscript provocatively suggests that changes in management can effectively be made at this early juncture and may preclude the need for further evaluations including invasive bone marrow evaluations. However, the counter argument is that bone marrow evaluations are important for examination of new collaborating cytogenetic abnormalities that may herald the evolution of a clonal population and accelerated disease.

Based on the success, promise, and safety profile of imatinib therapy in adults, studies to determine the efficacy of this drug in childhood CML have been undertaken. The COG published the results of a phase I trial of imatinib in children with Philadelphia chromosome–positive disease who had failed interferon therapy.[1099] Imatinib was well tolerated at escalating dose levels up to 570 mg/m^2 and CHR was demonstrated for all 14 chronic-phase patients within 1 month of starting therapy. Ten of 12 evaluated patients had achieved a CCyR by a median of 3 months. A phase II European pediatric study[1100] demonstrated equally encouraging results for children with chronic-phase CML who failed IFN-α therapy. These studies and extrapolation from adult data have resulted in imatinib becoming first-line therapy in children with chronic-phase CML. Confidence in this approach has been improved by evidence that prior imatinib therapy has no negative impact on the TRM after an allogeneic donor HSCT.[1101] Side effects of imatinib in children include gastrointestinal problems and rashes. If signs of leukostasis are present, more urgent therapy must be used. If the WBC count must be lowered quickly, leukapheresis and immediate chemotherapy with high-doses of hydroxyurea may be used. Proper hydration, alkalinization of the urine, and the use of allopurinol are imperative before the rapid lysis of cells is induced with chemotherapy.

Resistance to imatinib mesylate has been demonstrated.[1102-1106] Mechanisms of resistance include point mutations in *BCR-ABL*, amplification of the *BCR-ABL* gene, overexpression of the BCR-ABL protein, enhanced expression of the multidrug resistance gene, and excessive binding of imatinib by serum proteins. Analysis of a small number of blast crisis patients who experienced relapse while receiving imatinib has frequently shown either *BCR-ABL* gene amplification or *BCR-ABL* kinase domain mutations.[1103] These observations have indicated that resistance to imatinib as a single agent in the treatment of CML involves alterations of the BCR-ABL protein expression level or structure, allowing it to escape inhibition by the drug, rather than mutations in entirely different genes that might have reduced the malignant cells' dependence on BCR-ABL for transformation. A number of approaches have been suggested as interventions after the identification of imatinib resistance. High-dose imatinib has shown some promise for inducing cytogenetic remissions, despite a lack of efficacy of standard-dose therapy.[1107] Additionally, a number of new tyrosine kinase inhibitors have emerged and are more potent inhibitors of BCR-ABL than imatinib. Nilotinib is a structural derivative of imatinib and effective at lower concentrations.[1108] At doses of 300 mg to 400 mg twice daily, it is well tolerated in adults,[1108a] although it requires fasting before and after each dose, a requirement that potentially complicates its administration to children. Dasatinib has a completely different structure and binding configuration, making it active against most imatinib-resistant *BCR-ABL* mutations, as well as a host of other tyrosine kinases. Dasatinib has a higher incidence of thrombocytopenia than imatinib and is associated with a high incidence of pleural effusions in adult patients. Both dasatinib and nilotinib are FDA approved for the treatment of imatinib-resistant chronic-phase CML.[1107,1109,1110] More recently, head-to-head randomized control trials in adult patients with chronic-phase CML have demonstrated that both of these agents are more effective as frontline therapy at achieving CCYR by 1 year than imatinib.[1111,1112] The COG recently completed a phase I trial of dasatinib for imatinib-resistant leukemia and solid tumors. Nine CML patients who had experienced relapse were included and generally tolerated doses as high as 85 mg/m^2; there was one case of hemangiomatosis, and cytogenetic responses were demonstrated in 5 patients (complete response in 3 and partial response in 2).[1113] These results have prompted studies in newly diagnosed patients with Philadelphia chromosome–positive ALL, combining dasatinib in doses of 60 mg/m^2 with intensive chemotherapy and a proposed COG study for dasatinib as a frontline agent in CML. Nilotinib is now being considered for a phase II study in pediatric CML resistant to imatinib or dasatinib.

Hematopoietic Stem Cell Transplantation. Of all malignancies, CML has the most extensive long-term data on the effectiveness of allogeneic HSCT. This approach can provide prolonged DFS in CML and is the only proven curative therapy. The effectiveness of allogeneic HSCT in CML is believed to be caused in part by the effects of the transplanted immune system, rather than solely the result of the high-dose total-body irradiation and chemotherapy in the conditioning regimen. The allogeneic graft creates a new immune system that plays an active role in eradicating CML cells. Immune-mediated antileukemic effects are suspected because patients may remain PCR positive for *BCR-ABL* transcripts for months after HSCT and yet appear to be cured of their disease and eventually convert to PCR negativity without further therapy. Furthermore, there is an extremely high relapse rate for autologous, syngeneic, and T-cell–depleted grafts with the same myeloablative conditioning regimens used for standard, non–T-cell–depleted grafts, a result that suggests alloreactivity is associated with lower relapse rates.[1114-1116]

The role of allogeneic HSCT in CML provides a moving target in the post–tyrosine kinase inhibitor era. The potential toxicity associated with allogeneic HSCT, especially from a matched unrelated donor, compared with the efficacy and tolerability of oral tyrosine kinase inhibitors has raised questions regarding the indications and timing for HSCT in CML patients. Although some patients may go on to long-term survival when HSCT is performed in accelerated phase or blast crisis of CML (15% to 30% of cases), results are substantially better when HSCT is performed in the chronic phase.[1117] If the procedure is performed while the patient is in chronic phase using a matched sibling donor, 1-year survival rates are 60% to 80%, with a 10-year DFS rate of approximately 50%.[1114,1118-1120] Some studies have demonstrated increased survival outcomes if the HSCT was performed within the first year of diagnosis.[1121-1123] Prior to imatinib therapy, these data prompted the initiation of allogeneic HSCT early in the course of treatment. However, HSCT has always been limited to patients who can medically tolerate the procedure and have appropriate donors—30% have matched sibling donors, 5% have matches with additional family members, and 50% have matched unrelated donors in donor registries. Imatinib can be safely given pre-HSCT and has no impact on TRM.[1101,1124] Currently, most adult patients, including even those with sibling donors, are being maintained on imatinib until evidence of resistance develops, which then prompts a change in therapy and consideration of HSCT. In children, the timing of HSCT for CML is even more challenging. Although imatinib has now demonstrated impressive 8-year outcomes,[1125] will these be sustained for the many years that a child with CML would be required to take the drug? Allogeneic HSCT still remains the only curative therapy and is generally better tolerated by children, particularly with modern approaches to supportive care.

Between 10% and 15% of recipients of matched sibling HSCT and less than 5% of matched unrelated donor recipients experience relapse hematologically after allogeneic HSCT. Patients who experience relapse after HSCT may respond to interferon or withdrawal of immunosuppression.[1126-1129] Recent evidence has suggested that relapse rates may be reduced with the addition of imatinib therapy after HSCT whether or not the patient received imatinib before HSCT. However, the recommended duration of imatinib therapy after HSCT remains uncertain. Patients who experience relapse after HSCT may benefit from a second-generation tyrosine kinase inhibitor, such as dasatinib, a second HSCT, or donor-lymphocyte infusions. Sixty to 80 percent of patients who experience relapse cytogenetically or hematologically and return to chronic phase after HSCT can be salvaged by donor lymphocyte infusions without further cytotoxic chemotherapy.[674,1130-1132] Unfortunately, acute and chronic GVHD and marrow aplasia remain significant complications after donor lymphocyte infusion.[1133] Relapse occurs again in 5% to 15% of patients successfully treated with donor lymphocyte infusion, but remissions induced by this modality are durable in most patients.

Blast-Phase Chronic Myelogenous Leukemia

Blast-phase CML is generally resistant to chemotherapy.[1074] As in acute leukemia, leukostasis is more likely in patients with high blast counts, and rapid institution of therapy to lower the WBC count is indicated after initiating measures to protect the patient from tumor lysis syndrome. The blast cell morphology and surface markers should guide therapy in blast crisis of CML. Lymphoid blast morphology or surface markers predict a good initial response to drugs effective in ALL, such as prednisone and vincristine.[1063,1079,1134] However, patients whose blast cells have a myeloid morphology and immunophenotype respond poorly to most therapies. Median survival is only 2 months after transformation to myeloid blast crisis, compared with 6 months if the blast cells have lymphoblastic characteristics.[1135] Allogeneic HSCT in late-stage CML is associated with a poor outcome, primarily because of the high peri-HSCT mortality from regimen-related toxicity and an extremely high relapse rate.

Chronic Myelogenous Leukemia Caused by Other Translocations

Other chromosomal translocations have also been identified in CML. The *TEL* gene belongs to a large family of transcription factors referred to as the *ETS* family. These genes encode related proteins defined by a highly conserved, 90-amino acid, winged helix-turn-helix dimerization motif. This region appears to be an essential requirement for constitutive tyrosine kinase activity and transforming capacity of fusion proteins derived from chromosomal translocations involving *TEL*, generally partnered with a tyrosine kinase gene such as the *PDGF* receptor.[1136-1138] With the exception of the t(12;21)-associated *TEL-AML1* fusion gene exclusively associated with early B-cell lineage ALL, *TEL* gene rearrangements are rare in human leukemia. Because of the small numbers of patients with myeloid malignancy who have *TEL* gene fusions, there is insufficient clinical experience to determine their clinical implications. However, there is a trend toward an unfavorable outcome in patients with atypical CML or CMML accompanied by *TEL* gene rearrangements.

Polycythemia Vera

Polycythemia is defined as an increase in numbers of circulating erythrocytes, hemoglobin, and hematocrit. There are three types of polycythemia: (1) polycythemia vera (PV), or primary polycythemia; (2) secondary polycythemia; and (3) relative polycythemia.[1139] The first two types are associated with an increase in red blood cell mass, whereas in the third the red blood cell mass is normal and plasma volume is reduced. Secondary polycythemia can arise from a multitude of causes, including those that result in excessive erythropoietin production caused by decreased tissue oxygenation (physiologically appropriate), inappropriate erythropoietin secretion from a tumor, a mutant hemoglobin with abnormal oxygen affinity, and decreased erythrocyte diphosphoglycerate.

PV is a myeloproliferative disease caused by clonal expansion of an abnormal multipotent stem cell that produces erythroid progenitors that can proliferate in the absence of erythropoietin (EPO).[1140,1141] In contrast, normal fetal erythroid progenitors and those with mutations in the EPO receptor still require erythropoietin for their function. Growth of erythropoietin-independent erythroid colonies in serum-containing cultures can be a useful diagnostic test.[1141] Because this is an HSC disorder, aberrations in the WBC and platelet counts can accompany the increased red blood cell mass. The diagnosis of PV used to be a diagnosis of exclusion. One had to prove that the elevated red cell mass was not from a secondary cause, such as hypoxia or a high oxygen affinity hemoglobin, and that the *BCR-ABL* transgene was absent. However, in 2005, several groups simultaneously reported the presence of a somatically acquired clonal *V617F* mutation in *JAK2* in more than 90% of cases of sporadic PV in adults.[1142-1145] This seminal discovery linked PV with the other myeloproliferative disorders, such as CML, by demonstrating a primary mutation in a tyrosine kinase as a causative factor and effectively established *JAK2* mutational analysis as a diagnostic test for PV. Interestingly, this same mutation was found in 50% of cases of essential thrombocythemia (ET; see later) and primary myelofibrosis (PM).[1142-1145] These three different phenotypes caused by the same genotype pose many questions, and scientists are currently investigating the role of gene dosage, modifying mutations, or genetic predisposition to account for the difference in disease phenotype. Some studies have suggested that patients with PV have at least a subpopulation of cells that are homozygous for the *JAK2* mutation whereas patients with ET are heterozygous.[1146,1147] Moreover, the *JAK2* mutation may be sufficient to induce PV but other mutations are likely required for the development of ET and PM. Rare cases of familial polycythemia may exhibit autosomal recessive or dominant inheritance, and erythropoietin levels can be low, normal, or elevated.[1148] Studies of these families have revealed that the *JAK2* mutation is acquired, suggesting that there is an inherited predisposition allele that has not yet been identified.[1149] In some of the families, a mutation in the negative regulatory domain of the erythropoietin receptor gene appears to be involved.[1150-1152] Several *JAK2-V617F*–negative patients with PV were found to have a number of different mutations involving exon 12 of *JAK2*.[1153]

The WHO previously established strict criteria for the diagnosis of PV, with recent suggestions for revisions to *JAK2* mutational evaluation.[1154-1156] Evaluation should include a careful history and physical examination, including examination for splenomegaly and measurements of oxygen saturation, a complete blood cell count, and serum erythropoietin level, *JAK2* mutational analysis, and bone marrow aspirate and biopsy, including cytogenetics to rule out *BCR-ABL* or other translocation-induced fusion genes.

PV is extremely uncommon in children. The median age at presentation is 60 years, and fewer than 1% of PV patients are younger than 25 years.[1139,1157,1158] Children with PV are also less likely than adults to exhibit the most prevalent *JAK2* mutation. Erythrocytosis can cause cardiac symptoms, such as dyspnea and hypertension, and symptoms of disturbed cerebral circulation, such as dizziness and paresthesias. Abnormal platelet function and thrombocytosis may lead to thrombosis and hemorrhage. Granulocytic proliferation can cause increased histamine turnover, resulting in gastrointestinal symptoms and pruritus. Hyperuricemia and hypermetabolic symptoms are common, including weakness and weight loss. Most patients have large spleens and may have large livers and increased blood pressure.[1139] The marrow shows hypercellularity, an increase in megakaryocyte number, and decreased iron stores. Serum erythropoietin levels are usually normal or decreased.[1159] A total of 10% to 25% of patients have a clonal chromosomal abnormality evident by cytogenetic analysis, most often a

Box 51-4 **Suggested Criteria for Childhood Polycythemia Vera***

MAJOR CRITERIA

Increased hemoglobin/hematocrit
Presence of *JAK2-V617F* mutation
No cause of secondary erythrocytosis, including:
- a. Absence of familial erythrocytosis (e.g., hereditary mutations of erythropoietin [EPO] receptor)
- b. No elevation of EPO caused by
  - i. Hypoxia (PaO_2 <92%)
  - ii. High oxygen affinity hemoglobin
  - iii. Truncated EPO receptor
  - iv. Inappropriate EPO production by tumor

MINOR CRITERIA

Spontaneous erythroid colony formation
Hypercellular bone marrow with trilineage proliferation
Low serum EPO levels

Modified from Vardiman JW, Harris NL, Brunning RD: The World Health Organization (WHO) classification of the myeloid neoplasms. Blood *100:2292–2302, 2002; Tefferi A, Thiele J, Orazi A, et al: Proposals and rationale for revision of the World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis: recommendations from an ad hoc international expert panel.* Blood *110:1092–1097, 2007; Teofili L, Giona F, Martini M, et al: Markers of myeloproliferative diseases in childhood polycythemia vera and essential thrombocythemia.* J Clin Oncol *25:1048–1053, 2007; Teofili L, Giona F, Martini M, et al: The revised WHO diagnostic criteria for Ph-negative myeloproliferative diseases are not appropriate for the diagnostic screening of childhood polycythemia vera and essential thrombocythemia.* Blood *110:3384–3386, 2007; and Barbui T. How to manage children and young adults with myeloproliferative neoplasms.* Leukemia *26:1452–1457, 2012.*

*Requires the presence of both major and one minor criteria or two to three minor criteria in the absence of the *JAK2* mutation.

20q– deletion. Box 51-4 lists suggested criteria for the diagnosis of pediatric PV.[1160,1161]

Prolonged survival after the diagnosis of PV is common, but spontaneous remission is rare.[1162] Vascular occlusive episodes related to high hematocrit levels are an important cause of morbidity and mortality, with Budd-Chiari syndrome being among the most common manifestations.[1163] Other life-threatening complications of polycythemia vera include bleeding, myelofibrosis with pancytopenia, and acute leukemia.[1164] A poorly defined spent phase may occur, characterized by a decrease in hematocrit (often because of increased plasma volume), variable depression of other blood cell counts, hepatosplenomegaly, and increased bone marrow reticulin and fibrosis.[1165,1166] The decision to treat children with PV who are otherwise asymptomatic is controversial, given the potential side effects and complications of the various therapeutic options. Some experts advocate for completing a thrombophilic workup to assist in determining factors that might contribute to the risk for developing significant thrombi and shift the risk-benefit balance in the direction of treatment.[1167,1168] Treatment includes phlebotomy, although isovolumic erythropheresis may be a safer procedure, but this requires specific resources and is not uniformly available.[1169] The highest risk for thrombotic or hemorrhagic complications is observed immediately after phlebotomy. Iron replacement therapy should be given, because iron deficiency can lead to nonhematologic disturbances and an increase in blood viscosity.[1170] Low-dose aspirin is also recommended for adults with PV and has been adopted in the prophylaxis of thrombosis in children with PV.[1168,1171] Radioactive phosphorus or chemotherapy can also control the hematocrit in PV. However, these treatments have been associated with an increased risk for transformation to AML.[1164] Hydroxyurea, IFN-α, and anagrelide are all agents used in adults to control cell counts and may be useful in pediatric cases.[1172-1174] Hydroxyurea is simpler to administer and has fewer side effects than IFN-α, and the leukemogenic risk of hydroxyurea appears to be low, as demonstrated by a number of long-term studies in sickle cell anemia patients. However, the risk for transformation to AML is still higher in PV, because patients have a primary clonal stem cell disorder.[1175] The main toxicity from hydroxyurea is neutropenia. Anagrelide is a newer agent that inhibits the action of cyclic AMP phosphodiesterase and primarily targets megakaryocyte differentiation and proliferation. It has resulted in the disappearance of thrombocythemia-related symptoms in 80% of patients with PV.[1176] However, it has been found to be inferior to hydroxyurea in ET.[1177] Given their familiarity and experience with the agent, most clinicians would use hydroxyurea in children with PV deemed at higher risk for thrombosis, owing to extremely elevated hematocrit or prior thrombotic event or bleeding, due to features such as esophageal varices, which could be exacerbated by phlebotomy or therapy with acetylsalicylic acid. IFN-α, particularly newer pegylated versions, is both effective and better tolerated[366,1168] and thus a reasonable alternative. A number of JAK2- and combined JAK1/JAK2-inhibiting agents have been developed and have demonstrated some symptom relieve in PM (see later) but have not proven to be more effective than hydroxyurea, IFN-α, and anagrelide in PV.[1178,1179]

Essential Thrombocythemia

ET is a myeloproliferative disorder characterized by an increased platelet count not attributable to any other cause; Box 51-5 lists causes of thrombocytosis.[1157,1180-1184] Similar to the other myeloproliferative diseases, ET is a clonal stem cell disorder that also affects other hematopoietic lineages.[1185] The *JAK2 V617F* mutation is found in approximately 50% of cases, and other cases may be associated with a mutation affecting the genes encoding thrombopoietin or the thrombopoietin receptor (MPL).[1186-1188] ET may occur in childhood, but rarely,[1158,1186,1189-1191] and it may be familial in some cases.[1192] A number of families have been identified who have ET and/or other myeloproliferative diseases with or without acquisition of the *JAK2-V617F* mutation, demonstrating that other mutations are involved in the pathogenesis of the disease and that there is likely an underlying genetic predisposition that has not yet been elucidated.[1149,1193,1194] The requirements for ET are a platelet count greater than 450,000/μL (previously 600,000/μL), a hemoglobin concentration not exceeding 13 g/dL (normal red blood cell mass), normal iron stores, a lack of the Philadelphia chromosome by molecular or cytogenetic analysis, the absence of collagen

Box 51-5 Causes of Thrombocytosis in Children

NUTRITIONAL

Iron deficiency
Megaloblastic anemia
Vitamin E deficiency
Metabolic
Hyperadrenalism

INFECTIOUS

Viral factors
Bacterial
Mycobacterial

TRAUMATIC

Surgery
Fracture
Hemorrhage

INFLAMMATORY

Collagen vascular disease
Inflammatory bowel disease
Sarcoidosis
Any inflammatory condition

NEOPLASTIC

Myeloproliferative disease
Hepatoblastoma
Neuroblastoma
Histiocytosis
Lymphoma
Carcinoma

MEDICATION-RELATED

Corticosteroids
Vinca alkaloids
Citrovorum factor

MISCELLANEOUS

Splenectomy, congenital asplenia
Infantile cortical hyperostosis (Caffey disease)
Cerebrovascular accident
Hemolytic anemia

Data from Addiego JE, Jr., Mentzer WC, Jr., Dallman PR: Thrombocytosis in infants and children. J Pediatr *85:805–807, 1974; Heath HW, Pearson HA: Thrombocytosis in pediatric outpatients.* J Pediatr *114:805–807, 1989; and Chan KW, Kaikov Y, Wadsworth LD: Thrombocytosis in childhood: a survey of 94 patients.* Pediatrics *84:1064–1067, 1989.*

fibrosis of the marrow, and no other identifiable cause of thrombocytosis.[1156,1180] Box 51-6 suggests criteria for the diagnosis of pediatric ET.

ET may be an incidental finding, or patients may present with venous or arterial thrombosis, bleeding, or symptoms of a hypermetabolic state. The bleeding is generally mild, and its cause is unknown, although platelet function is often abnormal.[1195,1196] Splenomegaly is common, as is mild leukocytosis. Bone marrow cytogenetics are usually normal. The clinical course is highly variable, and some patients have been followed up for 10 years without problems.[1184] In adults, 80% survive more than 100 months, with 5 of 95 patients ultimately experiencing a conversion to AML in one study.[1197] Patients with ET can also develop myelofibrosis. Children appear to have a more benign course than adults.[1158,1186,1198] Asymptomatic children do not need to be treated. Treatment options are similar to those available for PV, including hydroxyurea, anagrelide,[1199-1201] or IFN-α.[1202,1203] Treatment is usually recommended to prevent thrombotic complications in patients with platelet counts higher than 1 million/μL or a prior history of thrombohemorrhagic episodes. Hemorrhage may paradoxically occur with these very high platelet counts as a result of an acquired von Willebrand disease.[1204,1205] A randomized clinical trial in adults of hydroxyurea and acetylsalicylic acid or anagrelide and acetylsalicylic acid has demonstrated equal efficacy of these combinations in lowering the platelet count but a higher incidence of arterial thromboses, significant hemorrhage, and transformation to myelofibrosis in the anagrelide-treated group.[1177] More recently, evaluation by the European LeukemiaNet revealed that although anagrelide increased response rates it did not impact OS.[1206] These results suggest that hydroxyurea should serve as frontline therapy, particularly because the theoretical risk for malignant transformation has not been seen.[366,1168] Acetylsalicylic acid has previously been considered to have value in primary prophylaxis for thrombosis, but its utility was called into question in a recent study demonstrating a preventative benefit only in patients with predisposing thrombotic risk factors (*JAK2* mutation or cardiovascular risk factors), where it otherwise increased bleeding risk.[1207] Thus prolonged use of acetylsalicylic acid may be falling out of favor for asymptomatic patients with ET.

Primary Myelofibrosis

PM is a myeloproliferative disease characterized by leukoerythroblastosis, myeloid metaplasia, and varying degrees of myelofibrosis.[1208-1210] The median age at

Box 51-6 Suggested Criteria for Childhood Essential Thrombocythemia

Sustained platelet count greater than 450,000/μL
Bone marrow biopsy specimen showing proliferation mainly of the megakaryocytic lineage, with increased numbers of enlarged mature megakaryocytes; no significant increase or left shift of neutrophil granulopoiesis or erythropoiesis
Not meeting World Health Organization criteria for polycythemia vera, primary myelofibrosis, chronic myelogenous leukemia, myelodysplastic syndrome, or other myeloid neoplasm (e.g., no Philadelphia chromosome or *BCR-ABL* fusion)
Demonstration of *JAK2-V617F* or of another clonal marker (e.g., *MPL* mutation) or, in the absence of a clonal marker, no evidence of reactive thrombocytosis

Modified from Vardiman JW, Harris NL, Brunning RD: The World Health Organization (WHO) classification of the myeloid neoplasms. Blood *100:2292–2302, 2002; Tefferi A, Thiele J, Orazi A, et al: Proposals and rationale for revision of the World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis:* recommendations *from an ad hoc international expert panel.* Blood *110:1092–1097, 2007; Teofili L, Giona F, Martini M, et al: Markers of myeloproliferative diseases in childhood polycythemia vera and essential thrombocythemia.* J Clin Oncol *25:1048–1053, 2007; and Teofili L, Giona F, Martini M, et al: The revised WHO diagnostic criteria for Ph-negative myeloproliferative diseases are not appropriate for the diagnostic screening of childhood polycythemia vera and essential thrombocythemia.* Blood *110:3384–3386, 2007.*

presentation is 60 years, and pediatric cases are rare.[1211] Approximately 22% of patients are younger than 56 years, and approximately 11% are younger than 46 years.[1212] Fewer than 100 cases have been reported in children, and many of these are in patients younger than age 3 years.[1168] Symptoms may include malaise, weight loss, night sweats, and discomfort from splenomegaly. Peripheral blood cell counts are varied, and the smears often show Pelger-Huët cells, leukoerythroblastosis, and teardrop formation, along with occasional immature WBC precursors.[1213] Bone marrow cells are difficult to aspirate, and biopsy specimens show fibrosis in most patients.[1213] Cytogenetic abnormalities are common in PM if enough marrow cells can be obtained for study.[260,1214] PM is a clonal disease involving all myeloid elements,[1214] and the cause of the associated myelofibrosis is unknown.[1215-1217] However, similar to PV and ET, the *JAK2-V617F* mutation has been found in approximately 50% of patients. Another 10% of patients have been found to have a *W515L* mutation in the thrombopoietin receptor.[1218] Because this disorder is very rare in children, other causes of myelofibrosis need to be ruled out. These causes include nutritional, inflammatory, infectious, and neoplastic disorders.[1219]

The natural history of PM can be variable, with patient survival varying from 1.5 to more than 5 years.[1220] Children with PM may have a more favorable natural history than adults.[1221,1222] Young adults tend to have less severe anemia, higher incidence of splenomegaly, lower frequency of thrombocytosis, and lower frequency of chromosomal abnormalities.[1212] Deletions of the long arm of chromosome 20 or 13—(del(20q) or del(13q)—or a normal karyotype have been associated with more favorable outcomes.[1168] Complications of this disease include bleeding, intercurrent infection, transformation to acute leukemia,[1223] and portal hypertension.[1220] Splenectomy will not cause a further reduction of blood cell counts and therefore should be considered only for mechanical problems, portal hypertension, refractory thrombocytopenia, and hemolytic anemia.[1224] The role of chemotherapy in PM is ill defined, although hydroxyurea, anagrelide, and IFN-α have been used, although none of these approaches is curative.[1225] Given the frequency of *JAK2* mutations in PM, a number of recent adult trials have examined the use of JAK2 or combined JAK1/JAK2 inhibitor in PM with evidence of some bona fide clinical responses, including reduction in splenomegaly and relief from constitutional symptoms, such as fatigue and pruritus.[1226,1227] Ruxolitinib, a combined JAK1/JAK2 inhibitor, was approved by the FDA for high- and intermediate-risk PM in 2011. However, none of these JAK inhibitors selectively targets the *JAK2-V617F* mutation, resulting in side effects such as myelosuppression. Moreover, the effectiveness of these agents in abrogating symptoms in PM appear more related to suppression of JAK-STAT pathway–mediated cytokine-related inflammation characteristic of PM than inhibition of the causative mutant clone.[1178] Thus at this time, allogeneic HSCT remains the only cure and should be considered for higher-risk patients with unfavorable cytogenetics or who are transfusion dependent.[1168]

Mastocytosis

Mastocytosis is a pathologic proliferation and accumulation of mast cells in various tissues in the body.[1228] Mast cells are myeloid cells derived from HSCs but, unlike other lineages, they exit the marrow before they are fully matured and complete their development in the tissues in which they eventually reside.[1229-1231] Given their role in allergic and inflammatory conditions, mast cells are commonly found in vascularized connective tissues such as the skin and the mucosal surfaces of the gastrointestinal tract and respiratory epithelium.[1232] The cutaneous form of mastocytosis is most common in children and may consist of an isolated mastocytoma or diffuse lesions termed *urticaria pigmentosa*.[1233] This is a generally benign condition, although in rare cases progression to systemic mastocytosis (SM) can occur. SM implies mast cell proliferation in an extracutaneous site, most commonly the bone marrow. SM is a clonal myeloproliferative disease frequently associated with a *D816V* point mutation in the gene encoding the KIT tyrosine kinase and presents as varying degrees of mast cell hyperproliferation and impaired differentiation, depending on the subtype.[1228] SM is further divided into subtypes—indolent systemic mastocytosis (ISM), systemic mastocytosis with an associated hematopoietic clonal non–mast cell lineage disease (SM-AHNMD), aggressive systemic mastocytosis (ASM), mast cell leukemia (MCL), mast cell sarcoma, and extracutaneous mastocytoma.[1234] Whereas the *KIT* mutations may be sufficient to result in the indolent form of the systemic disease, additional genetic abnormalities appear to be required for progression to the more aggressive forms of the disease.[1228] For example, the *TET2* mutation found in AML has also been identified in approximately 30% of cases of systemic mastocytosis[1235] and may be associated with more aggressive disease and poor prognosis.[1236] SM may also occur in conjunction with the generally favorable prognostic t(8;21) translocation *(AML1-ETO)* but, in this context, it confers a poor prognosis.[1237,1238] Similarly, MCL is associated with a poor outcome.[1234,1239] MCL occurs predominantly in adults, is frequently aleukemic, and may occur de novo or after localized or systemic mastocytosis, including in childhood.[1234] Patients with MCL may harbor the *KIT-D816V* point mutation, but a number of other novel *KIT* mutations have also been recently identified in these patients.[1234,1240,1241] Treatment strategies include combination therapy with steroids and IFN-α,[1239,1242] cladribine,[1243] and, more recently, tyrosine kinase inhibitors.[1244,1245] Imatinib mesylate, which is known to target the KIT tyrosine kinase in addition to BCR-ABL, has been used in patients with SM. Imatinib has shown preferential efficacy for the minority of patients who lack the *D816V* mutation[1246,1247] and has been approved by the FDA for this indication. Other tyrosine kinase inhibitors, including dasatinib[1248] and masatinib,[1240] have shown modest responses in aggressive mast cell diseases, whereas, more recently, midostaurin (PKC412) has shown some promise with improved responses in imatinib-resistant *D816V*-positive mastocytosis and MCL.[1249,1250]

CONCLUSION

Although considerable progress has been made in improving the remission induction rates in AML, remission is not attained in a significant number of children with newly diagnosed AML. The OS of patients with AML is still not optimal, and the toxicity of treatment is often excessive. Myeloid leukemias, myelodysplastic syndromes, and myeloproliferative disorders are heterogeneous in their molecular pathophysiologic course and are often difficult to treat effectively. With the tremendous improvements in outcome using paradigms of targeted approaches in the treatment of APL with ATRA and As_2O_3, and of CML with imatinib mesylate and other tyrosine kinase inhibitors, interest has grown in applying novel molecularly directed therapies to other types of myeloid disease. While more is understood about the molecular biology of the other types of myeloid malignancies, future studies will incorporate new agents that target malignant cells more specifically while sparing normal hematopoietic stem and progenitor cells. Moreover, recent genomic studies highlighting that the driving mutations in AML are likely few in number in any given patient, together with the evolution of more refined approaches to identify and define leukemic initiating cells, suggest that the ability to rationally apply targeted therapies to eliminate the source of the malignant clone may truly be realized. The incredible advancements in understanding the biology of myeloid diseases, coupled with the development of a large number of novel targeted agents, holds great promise to improve the outcome and reduce the toxicity for increasingly large subsets of patients afflicted by myeloid malignancies.

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CHAPTER 52

Infant Leukemias

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CHAPTER OUTLINE

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BIOLOGY OF MIXED-LINEAGE LEUKEMIA TRANSLOCATIONS
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- Prognostic Factors and Outcomes
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ACUTE MYELOID LEUKEMIA
- Clinical and Prognostic Features
- Outcomes and Treatment
- Association with Down Syndrome
- Non–Down Syndrome–Associated Infant AMKL

FUTURE DIRECTIONS IN INFANT ACUTE LEUKEMIA TREATMENT
- Targeting Epigenetic Mechanisms
- Immunotherapy
- Modulators of Glucocorticoid Resistance
- FLT3 Inhibitors

The biologic features and clinical characteristics of infant leukemias differ significantly from those of leukemias in older children. Infant leukemias are distinguished not only by the young age of patients at diagnosis but by their unique morphologic, immunologic, clinical, and genetic presentation.[1] For example, acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) present in infants with a somewhat unique constellation of clinical features, including hyperleukocytosis, massive organomegaly, and central nervous system (CNS) involvement at diagnosis.[2,3] Biologically, the most notable feature distinguishing infant leukemia from leukemia in older children is the high incidence of rearrangements involving the mixed-lineage leukemia (MLL) gene[*] located on chromosome band 11q23.[2,4-7] In the case of infant ALL, unique clinical and biologic characteristics are accompanied by an exceptionally poor prognosis, which is in stark contrast to the cure rates achieved for older children with ALL. In this chapter, the epidemiologic, biologic, and clinical features and the treatment of infant ALL and AML will be discussed.

EPIDEMIOLOGY

The annual incidence of leukemia in the first year of life in the United States is approximately 40 cases per million children.[8] Infants account for 2.5% to 5% of ALL cases and 6% to 14% of AML cases in childhood.[9] Unlike in older children with leukemia, in whom the percentage of ALL cases is approximately four times that of AML, in infants the ratio of ALL to AML is approximately equal. Furthermore, in contrast to an excess of males among older children with leukemia, a slight female predominance is seen in infants with this disease.[8,10,11] Although neuroblastoma represents the most common neoplasm in infants, leukemia is the leading cause of death caused by neoplastic disease in this age group (Table 52-1).[1,8]

PRENATAL ORIGIN OF INFANT LEUKEMIA

The onset of leukemia in infancy strongly suggests a prenatal leukemogenic event, and significant molecular and epidemiologic evidence supports this hypothesis. Molecular studies of monozygotic twins with concordant ALL have demonstrated identical clonal, nonconstitutional rearrangements of the MLL gene and identical oligoclonal, heavy-chain immunoglobulin gene rearrangements in the peripheral blood of these infants.[12-15] This finding suggests the occurrence of a single in utero leukemogenic event in one twin with generation of a clone that is passed to the other fetus by intraplacental metastasis. Further evidence of oncogenesis in utero comes from reports of fetal deaths caused by AML with an MLL gene rearrangement.[16] Moreover, identical MLL gene fusions have been traced back to neonatal Guthrie

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[*] In this chapter, *KMT2A* will be referred to as MLL. *ALL1* and *HRX* are also alternative names for this gene.

TABLE 52-1 Incidence of Cancer in Infants*

Histology	Incidence (per million)
Neuroblastoma	64.6
Leukemias	40.5
Central nervous system	29.7
Retinoblastoma	26.7
Wilms tumor	22.5
Germ cell	15.3
Soft tissue	15.2
Hepatic	9.5
Lymphomas	4.4
Epithelial	2.8
Other, unspecified	1.2
Bone	0.5

From Ries LAG, Smith M, Gurney JG, et al, editors: Cancer incidence and survival among children and adolescents: United States SEER Program 1975-1995 *(NIH publication No. 99-4649). Bethesda, MD, 1999, National Cancer Institute.*

*SEER Program, 1976-1984 and 1986-1994.

genetic screening cards of children who were diagnosed with ALL in their first 2 years of life.[12,17,18] Similarly, leukemia has been diagnosed in fetuses with Down syndrome as early as 33 weeks' gestation, and the characteristic GATA binding protein 1 *(GATA1)* gene mutation associated with megakaryocytic leukemia in young children with Down syndrome has been identified in the neonatal Guthrie genetic screening cards of children in whom leukemia later developed.[19,20]

RISK FACTORS

As with most cancers, the causes of infant leukemia remain largely unknown. However, several epidemiologic risk factors associated with an increased risk of leukemia in young children have been identified. Given the close temporal relationship between embryogenesis and the clinical diagnosis of cancer, most investigations have focused on maternal characteristics and in utero exposures. Maternal alcohol consumption during pregnancy has been correlated with an increased risk of infant AML.[21] Studies have also shown an increased incidence of infant leukemia, particularly ALL, in infants with birth weights higher than 4000 g.[22-24] It has been speculated that high levels of endogenous insulin-like growth factors associated with high birth weight may contribute to leukemogenesis.[9,25] Other proposed risk factors include maternal pesticide and solvent exposure, in utero radiation exposure, and an adverse maternal reproductive history.[26-31] However, given the rarity of infant leukemia, it has been difficult to connect the development of leukemia with any of these exposures or clinical features definitively.

Of particular interest is the potential relationship between maternal consumption of naturally occurring deoxyribonucleic acid (DNA) topoisomerase II inhibitors and the development of infant leukemia. MLL gene rearrangements, similar to those found in many infant leukemias, are common in secondary acute leukemias arising after exposure to DNA topoisomerase II inhibitors, including the epipodophyllotoxins (e.g., etoposide).[32-34] This relationship has led to the hypothesis that transplacental exposure to naturally occurring topoisomerase II inhibitors may be involved in the pathogenesis of infant leukemia.[35] This hypothesis is supported by the finding that both primary infant MLL gene–rearranged leukemias and therapy-related secondary acute leukemias have MLL gene break points that are similarly distributed within the MLL gene break point cluster region (BCR).[33] Furthermore, several dietary bioflavonoids, such as quercetin (found in certain fruits and vegetables) and genistein (found in soybeans) are known topoisomerase II inhibitors and have been shown to induce MLL gene cleavage in vitro.[36] In a study by the Children's Oncology Group (COG), it was found that increased maternal consumption of these and other naturally occurring topoisomerase II inhibitors such as catechins (found in red wine, tea, and cocoa) is associated with an increased risk of MLL gene–rearranged infant AML but not infant ALL.[22,37,38] This association, if true, likely only accounts for a small proportion of infant leukemia cases. Furthermore, the fact that many of these foods are commonly consumed and infant leukemia is rare suggests that this association is either weak or requires a combination of factors. Thus one cannot make recommendations to restrict certain foods to decrease the likelihood of the development of leukemia. Moreover maternal consumption of fruits and vegetables during pregnancy has been associated with a decrease rather than an increase in the risk of infant leukemia overall.[38]

Pharmacogenetic differences in the metabolism of topoisomerase II inhibitors have been hypothesized to modulate the relationship between exposure to these chemicals and the occurrence of MLL gene rearrangements. A common structural feature shared by many topoisomerase II inhibitors, including bioflavonoids, is a quinone moiety. Quinone metabolites generated as by-products of these compounds in the fetal liver have been shown to cleave the MLL gene.[36] Quinones are normally detoxified by reduced nicotinamide adenine dinucleotide phosphatase quinone oxidoreductase (NQO1). The presence of a low-activity variant of NQO1 has been associated with an increased risk of MLL gene–rearranged infant ALL.[39] However, a similar study has also demonstrated a relationship between this NQO1 polymorphism and infant ALL without MLL gene rearrangements.[40]

BIOLOGY OF MIXED-LINEAGE LEUKEMIA TRANSLOCATIONS

The MLL gene located on chromosome band 11q23 is commonly altered in infant leukemia in that it is rearranged in 80% of cases of infant ALL and 60% of cases of infant AML. These chromosomal abnormalities usually

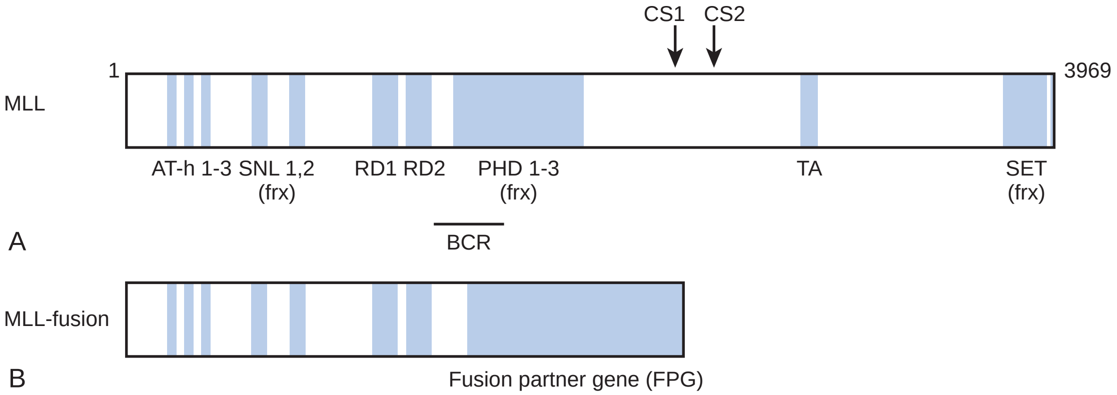

Figure 52-1 Schematic representation of the mixed-lineage leukemia *(MLL)* protein and MLL fusions. **A**, The MLL gene is approximately 89 kb long, consists of 37 exons, and encodes a 3969–amino acid nuclear protein with a complex domain structure (unique domains are highlighted). The N-terminus contains three short AT hook motifs *(AT-h 1-3)*, which are thought to mediate binding to the minor groove of AT-rich genomic deoxyribonucleic acid (DNA) sequences. There are two nuclear localization sites (SNL1 and SNL2) immediately C-terminal to the AT hooks that are followed by a transcriptional repression domain *(RD)* consisting of two functional subunits, RD1 and RD2. RD1 contains a DNA methyltransferase homology domain. The plant homeodomains *(PHD)* zinc finger motifs may mediate binding to a number of different proteins or to chromatin. The transcriptional activation *(TA)* domain recruits the transcriptional coactivator CREB-binding protein and precedes a C-terminal Su(var)3-9, Enhancer of Zeste, Trithorax *(SET)* domain that possesses histone H3 lysine 4 (H3K4) methyltransferase activity. The breakpoint cluster region *(BCR)* spans exons 8 to 13. **B**, Structure of MLL fusion proteins generated by MLL translocations. A typical MLL fusion protein contains the N-terminus of MLL encoded by the first 8 to 13 exons and the C-terminus of one of more than 50 fusion partners. *CS*, Cleavage site.

involve reciprocal translocations, which encode chimeric transcripts that give rise to oncogenic fusion proteins with pronounced transforming potential.[41] A number of groups cloned the MLL-AF4 *(AFF1)* gene in the early 1990s.[42,43,44,45] The MLL-AF4 gene encodes a protein of 2304 amino acids, with the NH2-terminal 1439 amino acids derived from the MLL gene on chromosome 11 and COOH-terminal 865 amino acids derived from the AF4 gene on chromosome 4. Subsequently, close to 100 different translocations have been identified, all of which appear to produce a fusion protein possessing the NH2-terminus of MLL fused in-frame to the COOH-terminus of the fusion partner (Fig. 52-1).[41]

The MLL gene encodes a 3969–amino acid DNA-binding protein that possesses multiple recognizable protein motifs, including an NH2-terminal DNA binding domain, transcriptional activation and repression domains, and a COOH-terminal Su(var)3-9, Enhancer of Zeste, Trithorax (SET) domain that contains histone methyltransferase activity.[46,47] Biochemical studies have identified MLL as a member of a large multiprotein complex that contains members involved in chromatin modification and remodeling. Notably, the complex includes histone deacetylases and members of the switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complex.[47] Also MLL is recruited to the promoters of select cell cycle regulatory genes by the protein product of the multiple endocrine neoplasia 1 *(MEN1)* tumor suppressor gene, suggesting a role for MLL in tumor suppression and cell cycle control.[48,49] These data support the hypothesis that the MLL protein regulates gene expression via chromatin modification.[50]

Analysis of Mll knockout mice has suggested that Mll plays an important role in development and hematopoiesis through maintenance of appropriate homeotic (Hox) gene expression.[51,52] Detailed studies assessing the specific role of Mll in hematopoietic development have shown that Mll is necessary for definitive hematopoiesis and for the expansion of hematopoietic progenitors and stem cells found in the aorta-gonad-mesonephros region of the developing embryo.[53] The defect in hematopoietic progenitor expansion can be rescued by reexpression of Hox genes, confirming the importance of Mll-mediated Hox gene expression during hematopoiesis.

Multiple studies have demonstrated the ability of Hox genes to induce leukemia in mice,[54] and the t(7;11)(p15;p15) translocation found in some human acute myeloid leukemias results in a fusion of the homeobox A9 *(HOXA9)* gene to the nucleoporin 98kDa *(NUP98)*.[55,56] Given the apparent importance of HOX genes in leukemogenesis, it seems likely that translocations involving MLL, a known regulator of HOX genes, alters expression of HOX genes that are important for leukemogenesis. Further support for HOX genes as central regulators of MLL gene–induced leukemogenesis comes from gene expression studies that have found multiple HOXA cluster genes more highly expressed in MLL gene–rearranged myelogenous and lymphoblastic leukemias compared with MLL gene germline leukemias.[57,58,59,60]

Gene expression studies of human MLL gene–rearranged B-precursor ALL have demonstrated that hundreds of genes are differentially expressed when compared with other B-precursor ALLs.[57-59,61] Based on the magnitude of the differences in gene expression, it appears that MLL gene translocations specify a unique lymphoblastic leukemia. Other large gene expression studies have also shown that ALLs with distinct chromosomal rearrangements have unique gene expression profiles, providing support for this hypothesis.[59] The genes that are relatively highly expressed in MLL gene–rearranged B-precursor ALL are those associated with hematopoietic progenitors and developing myeloid cells, whereas the genes expressed at lower levels are genes associated with lymphoid identity. Studies have also defined specific gene expression signatures associated with MLL gene translocations in pediatric—and in some cases infant—AML blasts.[60] It is of interest that even though clear differences exist in expression of lineage-associated genes between MLL gene–rearranged ALL and MLL gene–rearranged AML, a core gene expression profile appears to be found in all MLL gene–rearranged human leukemias, independent of the lineage markers.[60] Presumably, MLL fusion proteins directly regulate a subset of these genes. This theory is further supported by the fact that this MLL-associated signature consists of multiple highly expressed HOX genes.

The observation that leukemic cells bearing an MLL gene translocation often coexpress both myeloid and lymphoid markers raises the possibility that MLL fusion genes selectively transform hematopoietic stem cells (HSCs). If so, this HSC population would have an inherent self-renewal capacity that could be co-opted for leukemogenesis. Xenograft transplantation studies have demonstrated that a rare subpopulation of CD34+ and CD38− human myeloid leukemia cells are able to transfer the disease to immunodeficient mice, providing support for HSCs as the normal compartment from which leukemia-initiating cells (leukemia stem cells) might arise.[62] Alternatively, the translocation event between the MLL gene and a partner gene may affect a more committed population. In this scenario, the MLL fusion protein might confer a self-renewal and proliferative capacity to these short-lived progenitors and allow them to initiate leukemogenesis. Studies have suggested that Mll fusion proteins and potentially other translocation-associated fusion proteins are capable of inducing leukemia when expressed in fully committed myeloid progenitors, such as granulocyte-macrophage progenitors.[63,64] These findings are of particular importance because they suggest that products of chromosomal translocations found in human leukemias are able to induce a program of self-renewal that is not normally present in hematopoietic progenitors.

Multiple lines of evidence now point to a multistep pathogenesis of human acute leukemia. Elegant epidemiologic studies have suggested that childhood leukemias require at least two and probably more genetic events to occur for the development of leukemia. In particular, lymphoblastic leukemias with *ETV6-RUNX1 (TEL-AML1)* rearrangements appear to develop after a multistep process. TEL-AML1 rearrangements have been detected in blood taken at birth from a child in whom ALL then developed 3 to 5 years later. This finding suggests that TEL-AML1 rearrangements are the first genetic event but that other mutations also are required for the development of ALL.[65] Similar studies have been performed on blood spots from children in whom MLL gene–rearranged ALL subsequently developed, and the MLL gene translocations clearly develop in utero, even though the leukemias become apparent at some point during the first year of life.[12]

Receptor tyrosine kinases are attractive candidates as signaling molecules that may cooperate with translocation-associated fusion proteins during leukemogenesis. Ever-increasing evidence has suggested that activated kinases play a central role in the pathogenesis of leukemias and myeloproliferative syndromes.[66] The most dramatic evidence for such a role is activation of the Abelson (ABL) tyrosine kinase by the BCR-ABL fusion produced by t(9;22) and its inhibition by imatinib (Gleevec).[67,68] Other mutant kinases frequently identified in AML and subsets of ALL are the receptor tyrosine kinases fms-related tyrosine kinase–3 (FLT3), c-KIT, and platelet-derived growth factor receptor, among others.[66] Given the high level expression and recurrent mutation of FLT3 in MLL gene–rearranged ALL, studies are under way to assess FLT3 inhibitors in patients with MLL gene–rearranged ALL (discussed later). As approaches to target the rat sarcoma (RAS) signaling pathway are further developed, RAS inhibitors might also be considered in RAS-mutant cases.

Given the central importance of the MLL fusion protein generated by the MLL gene translocation, significant effort has been directed toward defining a unifying mechanism of oncogenesis, because it would facilitate pharmacologic targeting of shared leukemogenic mechanisms. Some broad patterns have emerged that have focused on mechanisms that control MLL target gene expression. The most commonly occurring MLL gene translocations generate chimeric fusion proteins that harbor the NH3-terminus of MLL fused to proteins that are normally part of nuclear complexes, the function for which is now emerging.[69] Nuclear proteins such as AF4, AF9, ENL, ELL, AF10, AF17, and AFF4—fusions of which together account for the vast majority of MLL leukemias—normally directly or indirectly associate with complexes that regulate gene expression.[70-75] A number of complexes linked to transcriptional elongation have been reported, often with overlapping protein components, such as the ENL-associated protein (EAP) complex,[72] the AF4/ENL/P-TEFb (AEP) complex,[75] the super elongation complex (SEC),[74] and the complex comprising the disruptor of telomeric silencing (DOT1)–like histone 3 lysine 79 (H3K79) methyltransferase (DOT1L; DOT1L containing complex [DotCom]; Fig. 52-1).[73] These data point to aberrant control of transcriptional elongation as critical for MLL fusion–mediated oncogenesis.

The histone methyltransferase DOT1 is a non-SET domain containing histone methyltransferase solely responsible for catalyzing the methylation of H3K79.[76] H3K79 methylation is associated with most genes that are highly expressed in hematopoietic cells and is believed to be involved either in activation or maintenance of gene expression. Thus a prominent hypothesis is that MLL fusion proteins induce aberrant gene expression via recruitment of DOT1L (among other protein complexes) to MLL target genes, including the HOXA cluster genes. Genome-wide studies have demonstrated elevated H3K79 methylation at MLL target genes in MLL gene–rearranged ALL and AML cells.[77-79] Several recent studies using conditional loss of function mouse models and ribonucleic acid (RNA) interference approaches have formally demonstrated a critical role for DOT1L in MLL fusion–driven leukemias.[79-82] These studies demonstrate that genetic inactivation of DOT1 leads to a decrease in MLL fusion target gene expression, including a rapid decrease in HOXA cluster gene expression that is correlated with an antiproliferative response. Remarkably, inactivation of DOT1L does not affect the transformation potential of a number of other leukemogenic oncogenes. Furthermore, microarray-based gene expression studies showed that MLL-fusion target gene expression is much more dependent on DOT1L than is gene expression broadly.[79] These studies highlight the importance of aberrant H3K79 methylation for the transforming activity of MLL fusion proteins including MLL-AF4, MLL-AF9, MLL-AF10, and MLL-ENL and show that DOT1L is required for continued HOXA cluster gene expression. These results potentially have profound clinical implications because these fusions comprise the vast majority of MLL gene–rearranged leukemias; small-molecule DOT1L inhibitors have been developed and are entering clinical trials (discussed later).

ACUTE LYMPHOBLASTIC LEUKEMIA

Clinical and Biologic Features

ALL in infants is characterized by a high leukocyte count at presentation, marked hepatosplenomegaly, and a relatively high incidence of CNS involvement.[2,3,83,84] The immunophenotype of infant ALL is usually that of an immature B-lineage precursor and is characterized by a lack of cluster of differentiation (CD)–10 expression, as well as by the coexpression of myeloid-associated antigens, such as CD14, CD15, and CDw65. Lack of CD10 expression and coexpression of myeloid markers correlates with the presence of an MLL gene rearrangement, which is present in 80% of infants who have ALL compared with only 2% to 4% of older children who have ALL.[4,6,85-87] MLL gene rearrangements are more frequent in younger infants, with approximately 90% of infants younger than 6 months at diagnosis having MLL gene rearrangements in their leukemic blasts compared with 30% to 60% of infants aged 6 to 12 months.[4,88,89] The most common MLL gene translocation observed in infant ALL is t(4;11)(q21;q23), resulting in the MLL-AF4 fusion. This translocation is found in approximately 70% of MLL gene–rearranged infant ALL cases. Other common translocations include t(11;19), resulting in the MLL-ENL fusion, and t(9;11), resulting in the MLL-AF9 fusion, which occur in 15% and 4% of MLL gene–rearranged infant ALL cases, respectively.[6,85-87,90] Cases of infant ALL without an MLL gene rearrangement generally resemble the more common B-precursor ALL phenotype (CD19+, CD10+) typical of older children with ALL. Chromosomal abnormalities known to be favorable in older children with ALL, such as high hyperdiploidy (51 to 65 chromosomes, or a DNA index more than 1.16) and the *TEL-AML1* fusion gene, are notably absent in infants.[84] The distinguishing presenting characteristics of infants with ALL are summarized in Table 52-2.

TABLE 52-2 Presenting Characteristics of Infants and Older Children with Acute Lymphoblastic Leukemia

	AGE (yr)	
Characteristic	**0-1 (%)**	**1-18 (%)**
WBC* >100,000[84]	58	6.3
CNS-positive*[84]	14	1.5
Phenotype		
B lineage	96	86.5
T lineage	4	13.5
CD10-negative[84]	54.7	3.3
Myeloid antigen coexpression[96,97]	28	5
DNA index* >1.16[84]	1.5	24.7
TEL-AML1* rearrangement[84]	4.5	24.1
MLL rearrangement[85-87]	70-80	2-4

From Silverman LB: Acute lymphoblastic leukemia in infancy. Pediatr Blood Cancer *49:1070–1073, 2007.*

MLL, Mixed-lineage leukemia; *WBC,* white blood cell.

*B-lineage patients only.

A striking feature of MLL gene–rearranged infant ALL is the paucity of secondary genomic alterations compared with older children who have pre–B-ALL.[91-94] A mutational profiling study of oncogenic RAS alterations in MLL gene–rearranged ALL samples identified mutations in 15 of 109 samples (14%). Of the 38 samples with a t(4;11) MLL gene rearrangement, 9 (24%) were found to have RAS mutations. Identification of a RAS mutation was found to be an independent poor prognostic risk factor.[95]

Prognostic Factors and Outcomes

Despite extraordinary improvements in the cure rates for older children with ALL, the prognosis for infants with this disease remains poor. Historically, infants with ALL who were treated with standard regimens had a less than 20% event-free survival (EFS). During the past decade, intensified regimens designed especially for infants have resulted in improved rates of EFS ranging from 28% to 54% (Table 52-3).[84,85,87,89,97-104] In contrast, EFS rates are greater than 80% for older children with ALL.[105-108] Although 90% to 95% of infants with ALL achieve remission after initial induction chemotherapy, a favorable outcome is hampered by an exceedingly high relapse rate, typically within the first year after diagnosis.

Several closely related adverse prognostic factors have been identified for infants with ALL, including the presence of an MLL gene rearrangement, lack of CD10 expression, coexpression of myeloid markers, age less than 6 months at diagnosis, high white blood cell (WBC) count at presentation, CNS involvement, and poor early response to prednisone therapy.[87,97,109,111] In multivariate analyses, the presence of an MLL gene rearrangement, age younger than 6 months, and a poor early response to prednisone therapy consistently emerge as the most important adverse prognostic features.[89,102] In many studies, the presence of an MLL gene rearrangement is the most important independent predictor of outcome for infants.[88] Long-term EFS for infants with MLL gene–rearranged ALL has ranged from 13% to

TABLE 52-3 Outcomes of Treatment Protocols for Acute Lymphoblastic Leukemia in Infants

Study	Publication Date	Patients Enrolled	CR Rate (%)	EFS Time Point (yr)	EFS (%)
DFCI (1985-1995)[90]	1997	23	96	4	54
Interfant-99[90]	2007	482	94	5	45
AIEOP-91, 95[109]	2006	52	96	5	45
BFM 86, 90, 95[84]	1999	129	95	8	43
CCG-1953[87]	2006	115	97	5	42
P9407[110]	2012	97	n/a	5	41
CCG-1883[101]	1999	135	97	4	39
CCG-107[101]	1999	99	94	4	33
UKALL-92[97]	2002	86	94	5	33
POG 8493[98]	1997	82	93	4	28

AIEOP, Associazione Italiana di Ematologia Oncologia Pediatrica; *BFM*, Berlin-Frankfurt-Münster; *CCG*, Children's Cancer Study Group; *CR*, complete remission; *DFCI*, Dana-Farber Cancer Institute; *EFS*, event-free survival; *POG*, Pediatric Oncology Group; *UKALL*, United Kingdom Acute Lymphoblastic Leukaemia.

34%, versus 52% to 95% for infants with germline MLL (Fig. 52-2).[4,6,87-89,97,99,102,112]

In addition to MLL gene status and age, early response to therapy has been shown to be an important predictor of outcome in infants with ALL. The Berlin-Frankfurt-Munster group has identified a poor response to prednisone as one of the strongest predictors of outcome for infants with ALL, regardless of MLL gene status.[97] Infants with ALL who have a poor response to a 7-day prednisone monotherapy prophase (defined as more than 1000 blasts/μL present in peripheral blood on day 8) had a 6-year EFS of only 15%, compared with a 6-year EFS of 53% for infants with a good response to prednisone. This finding was reproduced in the most recent clinical trial

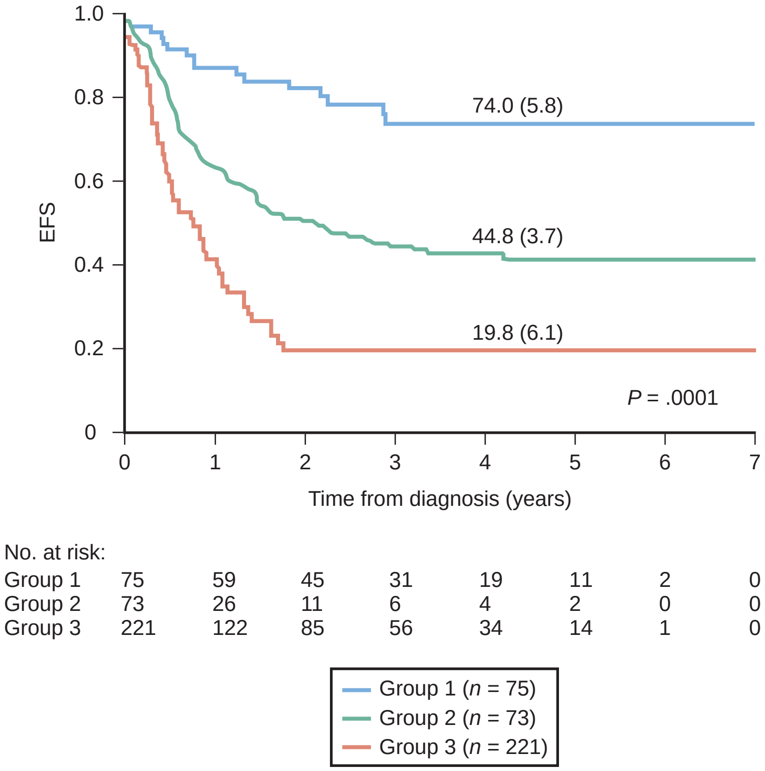

Figure 52-2 Event-free survival *(EFS)* for infants with acute lymphoblastic leukemia who were treated in the Interfant 99 trial.[103] Group 1 consisted of patients with the germline mixed-lineage leukemia (MLL) gene. Group 2 consisted of patients with an MLL gene rearrangement, age younger than 6 months at diagnosis, and white blood cell count higher than 300×10^9 cells/L at diagnosis. Group 3 consisted of all other patients. *(Modified from Pieters R, Schrappe M, De Lorenzo P, et al: A treatment protocol for infants younger than 1 year with acute lymphoblastic leukemia [Interfant-99]: an observational study and a multicentre randomized trial.* Lancet *370:240–250, 2007.)*

conducted by the Interfant study group (Interfant 99), in which infants with a poor response to steroids had a 30% 4-year EFS, compared with a 56% 4-year EFS for good responders.[89] Similarly, the Children's Cancer Group found a threefold excess risk of treatment failure in infants whose bone marrows contained more than 5% leukemic blast cells after 14 days of multiagent chemotherapy.[102]

It has been suggested that the specific MLL gene fusion partner might also have prognostic significance for infants with ALL.[102,109] However, in a large retrospective study that included data from more than 200 cases of MLL gene–rearranged ALL in infants, no significant difference in outcome was found among cytogenetic subgroups, including t(4;11), t(9;11), t(11;19), and other 11q23 rearrangements.[90] The Interfant study group also failed to find any association between the specific MLL gene translocations and treatment outcome.[89] More recently, localization of the fusion break point has been identified as a potential prognostic biomarker, with break points within MLL intron 11 demonstrating poorer outcomes.[113] Gene expression profiling has also identified candidate prognostic markers. Analysis of 97 samples from P9407, a COG trial for infant ALL conducted between 1996 and 2006, identified patients with low FLT3 expression as having superior outcomes, which had previously been observed in patients enrolled in the Interfant 99 trial.[114] Patients with high FLT3 expression, in addition to either high iroquois homeobox 2 (IRX2) or high transforming, acidic coiled-coil containing protein 2 (TACC2) expression, had very poor 4-year EFS rates.[110]

Treatment

Attempts to improve clinical outcomes for infants with ALL have generally involved intensifying treatment regimens and incorporating chemotherapeutic agents more commonly used in AML therapy. An ongoing international trial, Interfant 06, is evaluating the outcome of patients randomly treated with either a standard consolidation approach using mercaptopurine, cyclophosphamide, and cytarabine or two consecutive AML-based cycles that include cytarabine, etoposide, and both daunorubicin and mitoxantrone. Several preclinical and clinical findings have also informed the development of treatment regimens for infants with ALL, including clinical response to prednisone therapy and in vitro drug sensitivity profiles of infant ALL lymphoblasts. When compared with cells from older children with ALL, leukemic blasts from patients with infant ALL are more often resistant to glucocorticoids (prednisone and dexamethasone) and L-asparaginase in in vitro assays. Importantly, both of these agents are central components of current ALL therapeutic regimens.[97,115-117] In vitro studies of sarcoma (Src) kinase inhibition demonstrated sensitization of MLL gene–rearranged ALL cell lines to glucocorticoids, highlighting this strategy as a possible future therapeutic option in these patients.[118,119] Given the prognostic significance of prednisone response in ALL, some infant ALL treatment groups now risk-stratify infants on the basis of prednisone response and other common prognostic factors, including MLL gene status, age, and diagnostic WBC count.[89]

Although infant ALL blasts are relatively resistant to some common ALL therapeutic agents, they are remarkably sensitive to cytosine arabinoside (ara-C; cytarabine), a drug commonly used in the treatment of AML.[115,116] This increased sensitivity may be related in part to elevated expression of the human equilibrative nucleoside transporter–1, which allows cytarabine to permeate the cell membrane at low to moderate concentrations.[120-123] Clinical data are accumulating to suggest that increased use of cytarabine may benefit infants with ALL.[89,99,102] Investigators from the Dana-Farber Cancer Institute ALL Consortium first reported an improved outcome in a small number of infants treated with intensified therapy that included high-dose cytarabine.[124] Subsequently the cooperative groups COG and Interfant incorporated high-dose cytarabine into their intensified therapeutic regimens, a strategy that is likely to have contributed to the improvements in EFS for infants with ALL observed during the past decade.

Hematopoietic Stem Cell Transplantation

The role of hematopoietic stem cell transplantation (HSCT) in first remission for infants with ALL is controversial. Small uncontrolled studies have suggested that HSCT may benefit infants with MLL gene rearrangements.[125-127] Although encouraging, these results must be interpreted with caution, given the small sample sizes, the failure to control for waiting time from diagnosis to transplantation, and the use of total body irradiation in cytoreduction, which many groups are avoiding given the significant morbidity associated with radiation in this age group.[128] The most recent Interfant study group trial (Interfant 99) identified a subgroup of high-risk patients who may benefit from HSCT, including those diagnosed before 6 months of age and who have either a poor steroid response or a high WBC count at diagnosis.[129] In contrast, a larger retrospective analysis did not confirm a benefit of HCST for infants with ALL.[90] Similarly, the COG found no benefit of transplantation compared with intensive chemotherapy alone.[87] Further studies are needed to adequately define the role for stem cell transplantation in infants with ALL.

Toxicity and CNS-Directed Therapy

A major consideration in the treatment of ALL in infants is the significant potential for short- and long-term toxicity, given the intensity of treatment regimens and the lack of pharmacokinetic and pharmacodynamic studies to ensure optimal dosing of chemotherapeutic agents in infants. Of particular concern is the risk of debilitating neuropsychological sequelae, especially in infants receiving cranial radiation. Severe neurologic deficits and learning disabilities have been reported in long-term survivors of infant ALL who received cranial radiation.[3,98,99] In almost all subsequent clinical trials, attempts have been made to reduce neuropsychological complications by minimizing, delaying, or eliminating cranial radiation. Currently most investigators favor eliminating radiation in infants with ALL, even those with CNS leukemia at diagnosis, relying instead on intensive systemic and intrathecal chemotherapy. Several observations have supported this approach, including outcomes from large cooperative group studies documenting very low CNS relapse rates (3% to 9%) with intensive systemic and intrathecal chemotherapy alone.[89,100,102]

ACUTE MYELOID LEUKEMIA

Clinical and Prognostic Features

Infant AML is characterized by a high incidence of myelomonoblastic (French-American-British [FAB] M4) or monoblastic (FAB M5) morphologic features, frequent CNS involvement, extramedullary disease (including skin involvement), and relatively high leukocyte counts at diagnosis.[2]

MLL gene rearrangements are found in about 60% of infant AML cases, with the most common translocation being t(9;11), resulting in the MLL-AF9 fusion, followed by t(11;19), resulting in the MLL-ENL fusion. MLL gene rearrangements in infant AML are associated with a FAB M4 or M5 morphology and hyperleukocytosis.[111,130,131] Other cytogenetic abnormalities in infant AML of the FAB M4 or M5 morphologic subtype include inv(16) and monosomy 7. Translocations commonly seen in older children with AML, such as t(8;21) and t(15;17), are rare in infants with AML.[132]

Unlike in infant ALL, the prognostic factors that predict outcome for infant AML are not clearly defined. Several studies have identified a variety of potential prognostic factors for infants with AML, including presenting WBC count, FAB M4 or M5 morphology, gender, and the presence of an MLL gene rearrangement, but the findings of these studies have varied widely and are often contradictory.[133-138] The difficulty in clearly identifying consistent prognostic factors in infant AML may point to more biologic heterogeneity in infant AML (compared with infant ALL), as well as significant variations in the definition of study groups and treatment regimens.

The prognostic significance of MLL gene rearrangements in infant AML is unclear. Several studies have found that MLL gene rearrangements lack prognostic significance in childhood AML, whereas at least one study has found a trend toward a worse outcome.[131,133,139-142] Alternatively, several groups have demonstrated that the presence of a specific translocation, t(9;11)(p22;q22) or MLL-AF9, confers a favorable prognosis.[138,143-146] For example, the investigators at St. Jude Children's Research Hospital found the presence of t(9;11) to be an important prognostic factor for patients treated in four consecutive pediatric AML trials. The 5-year EFS for infants whose leukemia cells carried t(9;11) was 70% versus 25% for those with other cytogenetic abnormalities, including other MLL gene rearrangements.[147]

The Berlin-Frankfurt-Munster Group recently reported on the clinical characteristics of 62 pediatric patients with AML who had t(8;16)(p11;p13) translocations. The average age of diagnosis was very young (1.2 years), with common features of erythrophagocytosis (70%), leukemia cutis (58%), and disseminated intravascular coagulation (39%). Strikingly, seven patients diagnosed within the first month of life underwent spontaneous remission of their disease, three of whom remained in remission at the time of publication.[148]

TABLE 52-4 Outcomes of Treatment Protocols for Acute Myeloid Leukemia in Infants

Study	Study Period	Patients Enrolled	EFS Time Point	EFS (%)
St. Jude (AML80-91)[138]	1980-1987	28	5	32
BFM (AML 93)[149]	1993-1998	112*	5	41
MRC (AML10, 12)[150]	1988-2002	151	5	58
POG (8821)[151]	1988-1993	122*	5	22
Nordic (NOPHO-93)[152]	1993-2001	57*	5	54
CCG 2891[153]	1989-1995	116	8	71
Japan (ANLL91)[154]	1995-1998	35	3	72
French (LAME 89, 91)[155]	1988-1998	42	5	37.3
FHCRC[156]	1995-1998	35*	3	72

AML, Acute myeloid leukemia; *ANLL,* acute nonlymphocytic leukemia; *BFM,* Berlin-Frankfurt-Münster; *CCG,* Children's Cancer Study Group; *EFS,* event-free survival; *FHCRC,* Fred Hutchinson Cancer Research Center; *LAME,* Leucémie Aiguë Myéloblastique L'Enfant; *MRC,* Medical Research Council; *NOPHO,* Nordic Society of Paediatric Haematology and Oncology; *POG,* Pediatric Oncology Group.

*Children 1 to 2 years of age were included.

Outcomes and Treatment

Whereas age-associated treatment results are clearly evident in childhood ALL, with infants faring significantly worse, similar age-related differences in outcomes have not been routinely observed in AML. In large clinical trials in children with AML, EFS rates of 22% to 73% have been reported for infants, which do not differ significantly from outcomes achieved in older children (Table 52-4).[126,147,149-156] Given that treatment outcomes do not differ by age group, there has been no compelling clinical justification for the development of unique treatment strategies for infants with AML. Current therapeutic regimens for infants and older children focus on intensive remission induction and consolidation chemotherapy, with HSCT reserved for patients with matched sibling donors and those who have poor prognostic features. Although treatment regimens for infants with AML do not currently differ from those for older children, some potential differences have been proposed. The observation that t(9;11) may be a favorable prognostic factor in infants with AML has resulted in controversy with regard to whether bone marrow transplantation in first remission should be considered in this subgroup of patients. Another possible exception includes infants with megakaryoblastic leukemia who are harboring t(1;22)(p13;q13); these infants appear to have a particularly poor prognosis and may be candidates for more aggressive treatment or innovative experimental therapy.[157,158]

Association with Down Syndrome

Several genetic syndromes including monosomy 7, del (7q) syndromes, Noonan syndrome, and neurofibromatosis type 1 are associated with an increased risk of developing myelodysplasia, myeloproliferative diseases, and myeloid leukemia in infancy. A striking example is the increased incidence of leukemia seen in children with Down syndrome. Persons with Down syndrome have a tenfold to twentyfold increased lifetime risk of developing leukemia, including ALL and AML. The most marked increase is observed in young children with Down syndrome in whom acute megakaryoblastic leukemia (AMKL; FAB M7) develops.[159] The risk of AMKL is estimated to be 500 times higher in children with Down syndrome than in the general population. A transient form of megakaryoblastic leukemia, known as transient leukemia, transient abnormal myelopoiesis, or transient myeloproliferative disorder (TMD), often precedes AMKL in newborn infants with Down syndrome (or trisomy 21 mosaicism). Although the true incidence of TMD is unknown, a small study has found that 10% of neonates with Down syndrome have evidence of TMD on examination of their peripheral blood smears.[160] The true incidence of TMD is likely to be higher, given that fetuses who develop TMD in utero may not be identified and neonates with subtle manifestations of TMD may go unnoticed.[161,162]

Clinically the presentation of TMD can be variable, ranging from asymptomatic, with an incidental finding of an elevated WBC count and circulating blasts in the peripheral blood, to fulminating life-threatening disease, with severe liver dysfunction and liver fibrosis, multiple effusions, and respiratory compromise. TMD has also been associated with intrauterine death and hydrops fetalis.[162,163] TMD usually has a relatively benign course, with spontaneous remission within the first 3 months of life. However, in 13% to 33% of infants who recover from TMD, AMKL will go on to develop by age 3 years.[164-166] Often the subsequent leukemia is preceded by a myelodysplastic phase, with progressive cytopenias and increasing marrow fibrosis. In a prospective study of neonates with TMD conducted by the COG, it has been found that 64% of infants with TMD experienced a spontaneous and continuous remission, 17% had an early death (predominantly because of liver failure), and 19% experienced a spontaneous remission but went on to develop AMKL at a mean age of 20 months.[166]

TMD and AMKL in persons with Down syndrome nicely illustrate the multistep process of leukemic transformation. Biologically, the blasts from TMD and AMKL are morphologically and immunophenotypically similar and consistent with megakaryoblastic progenitors.[167-169] Chromosomal abnormalities are more frequent in AMKL than in TMD, with the most common abnormality being trisomy 8.[160,170] The leukemic blasts of TMD and AMKL in patients with Down syndrome have been shown to harbor a unique mutation of the gene encoding the hematopoietic transcription factor *GATA1*.[171,172] These mutations are found exclusively in Down syndrome–associated TMD and AMKL and have not been found in non–Down syndrome–associated AMKL or in patients with Down syndrome who have ALL.[171,173,174] *GATA1* has essential functions during normal erythroid and megakaryocytic development.[175,176] Most *GATA1* mutations cluster within exon 2 and result in the expression of a truncated mutant protein, GATA1s, which retains its DNA binding zinc fingers but lacks the amino-terminal transcriptional activation domain. Studies in model systems have suggested that GATA1 possesses unique activity that leads to expansion of an early megakaryocyte progenitor in the fetal liver, perhaps explaining why *GATA1* mutations are only found in infant AMKL from patients with Down syndrome.[177] Several recent studies have demonstrated that although TMD is caused by constitutional trisomy 21 and *GATA1* mutations, progression to AMKL is caused by the acquisition of additional mutations. These mutations have been identified in multiple cohesin components and epigenetic regulators, as well as wingless type (WNT), Janus kinase–signal transducer and activator of transcription (JAK-STAT), and mitogen-activated protein kinase/phosphatidylinositol 3′-kinase (MAPK/PI3K) pathway genes.[178-180]

Another remarkable characteristic of AMKL in Down syndrome is its extraordinary responsiveness to AML therapy.[135,181-184] In early clinical trials, the outcomes for patients with AML and Down syndrome were not favorable, although poor outcomes were primarily the result of treatment-related toxicity rather than relapsed or refractory disease. Subsequently, patients with Down syndrome and AML who were treated in a series of large multicenter pediatric trials were found to have a better response to chemotherapy and better survival rates than were patients without Down syndrome. EFS rates for children with Down syndrome and AML treated by modern protocols range from 80% to 100%.[135,181,182,184-187] Current treatment protocols for patients with Down syndrome and AML favor less intensive regimens, which aim to maintain high cure rates while minimizing toxicity.

Non–Down Syndrome–Associated Infant AMKL

AMKL also occurs in a subset of infants who do not have Down syndrome. AMKL in these infants frequently harbors the t(1;22)(p13;q13) translocation.[157,158,188] The t(1;22)(p13;q13) translocation is found almost exclusively in infants and young children with AMKL. This disorder is typically accompanied by significant bone marrow fibrosis and organomegaly and has a poor prognosis, with a median survival of only 8 months. The t(1;22)(p13;q13) characteristic of this malignancy is associated with the fusion of RNA-binding motif protein–15 (*RBM15*), an RNA recognition motif-encoding gene, and megakaryoblastic leukemia–1 (*MKL1*), a gene encoding an SAP (scaffold attachment factor A and B [SAF-A/B], Acinus, and protein inhibitor of activated STAT [PIAS]) DNA binding domain.[189] The mechanism by which this fusion contributes to leukemogenesis is not yet fully understood, although it has been shown that Rbm15-Mkl1 can alter SET domain containing 1b (Setd1b) histone methyltransferase activity, leading to epigenetic dysregulation.[190] Alternate translocations have also been identified in non–Down syndrome AMKL. A recent genomic study of non–Down syndrome AML identified a novel cryptic translocation of chromosome 16 involving core-binding factor, runt domain, alpha subunit 2, translocated to 3 *(CBFA2T3)*, a member of the eight-twenty-one (ETO) family of nuclear corepressors, and *GLIS2*, a GLI family transcription factor. This translocation was identified in 27% of studied cases and was associated with significantly poorer outcomes.[191] Translocations joining *NUP98* on chromosome 11 to *KDM5A (JARID1A)* on chromosome 12 are found in approximately 10% of AMKL cases and are characterized by HOXA/B gene overexpression. Unique clinical or prognostic characteristics have not been identified in this subset of patients.[192]

FUTURE DIRECTIONS IN INFANT ACUTE LEUKEMIA TREATMENT

Although morphologic complete remission is achieved in the vast majority of infants with ALL, a favorable outcome is hampered by an exceedingly high relapse rate in the first year after diagnosis. To date, intensification of conventional chemotherapeutic agents has resulted in only incremental improvement in the prognosis of infants with ALL, emphasizing the need for novel, more effective therapies.[193]

Targeting Epigenetic Mechanisms

In the past few years we have seen an explosion in the understanding of the normal function of the MLL protein and the fusion proteins generated by MLL translocations. We now know that the wild-type MLL protein is a histone methyltransferase that influences the expression of important developmental genes such as the HOX genes. Indeed, these proteins that influence gene expression via modification of histones or other components of chromatin (known as epigenetic regulators) appear to be central to the development of a number of different leukemia subtypes and perhaps other cancers. Given that enzymes control many of the epigenetic processes, the possibility that a new generation of therapies that targets epigenetic regulators may be on the horizon is generating tremendous excitement. Histone deacetylase inhibitors and DNA hypomethylating agents may work through the modulation of either DNA or histone modifications, and such approaches are being considered in pediatric leukemias, including infant MLL gene–rearranged leukemias. Although a specific rationale does not exist as to why MLL gene–rearranged leukemias should be more sensitive to these approaches than other leukemias, it is worth considering these agents for clinical trials.

However, new data provide a potential rational approach to target MLL fusion proteins generated by MLL translocation. Recent studies have shown that leukemic transformation by MLL fusion proteins is highly, and somewhat uniquely, dependent upon the histone methyltransferase DOT1L that methylates histone H3 on lysine 79.[77-79] Remarkably, it appears that this enzyme is required for continued proliferation of most if not all MLL gene–rearranged leukemias, and thus a therapeutic agent targeting DOT1L might benefit patients with leukemia who are bearing any MLL translocation.[194] Preclinical studies using specific small molecule DOT1L inhibitors have been completed and demonstrate remarkable activity and minimal toxicity in animal models.[195,196] The phase I trials for such approaches are under way in adults with relapsed/refractory leukemia. The possibilities that this approach may represent are generating tremendous excitement. However, it will be important to thoroughly assess the toxicity of such approaches in adults and children given that this approach to cancer therapy is completely new.

Immunotherapy

Novel therapies directed at proteins on leukemic cells may offer further promise in treating infant leukemia. Three approaches to targeting CD19 for the treatment of pre–B-ALL exist. Blinatumomab is a bispecific antibody that binds both CD19 and CD3, thereby engaging T cells to attack leukemia cells. In a phase II trial of adult patients with persistent minimal residual disease (MRD), 80% of subjects achieved negative MRD after treatment with blinatumomab, and responses in children have already been reported.[197,198] The pediatric phase II trial is under way. Positive results using autologous chimeric antigen cells directed against CD19 have now been reported in both pediatric and adult patients, and further studies in patients with infant ALL will need to be pursued.[199,200] Finally, a novel antibody-drug conjugate, SGN-CD19a, which combines an anti-CD19 monoclonal antibody with an inhibitor of tubulin polymerization (monomethyl auristatin F), is being evaluated in patients with pre–B-ALL and may prove to be effective for treatment of infant ALL. All three therapies target the CD19 protein, which may be a limitation in MLL gene–rearranged infant ALL, given the existence of CD19-negative subpopulations in this leukemia.[201]

Relative to ALL, current options for immunotherapy in AML are fewer. SGN-CD33a, an antibody-drug conjugate in development, targets CD33 and utilizes a pyrrolobenzodiazepine dimer to create DNA damage. This drug is only recently entering into clinical trials but may be worth pursuing in future trials that assess novel infant AML therapy.[202]

Modulators of Glucocorticoid Resistance

Cellular drug resistance may significantly contribute to the poor prognosis of infant ALL.[193] Infants with ALL are more likely to have a poor in vivo response to prednisone than are older children with ALL.[97,117] In addition, leukemic blasts from infants with ALL are highly resistant to glucocorticoids in vitro.[115,116] Both in vivo and in vitro responses to prednisone are highly predictive of clinical outcome in childhood ALL, and thus the mechanisms of glucocorticoid resistance are attractive targets for novel therapeutic agents.[203,204] Gene expression profiling of steroid-resistant and steroid-sensitive lymphoblasts indicates that myeloid cell leukemia–1 (MCL1), an antiapoptotic member of the B-cell lymphoma–2 (BCL2) family, may be an important modulator of glucocorticoid resistance in ALL.[205,206] Furthermore, a gene expression–based chemical genomics screen has identified sirolimus (more commonly known as rapamycin), the mammalian target of rapamycin (mTOR) inhibitor, as an agent capable of reversing a gene expression signature associated with glucocorticoid resistance. It has also been shown that sirolimus sensitizes lymphoid cells to glucocorticoids via the modulation of MCL1 levels.[205] Other modulators of MCL1 have been identified, including honokiol, a naturally occurring compound, and the synthetic inhibitors Seliciclib (CYC202, or R-roscovitine) and R-etodolac (SDX-101).[207-210] The potential to overcome glucocorticoid resistance in infant ALL by compounds that modulate MCL1, or other antiapoptotic molecules such as BCL2, makes agents targeting the apoptotic pathway attractive candidates for further investigation as therapeutic approaches for the treatment of infant ALL.[211]

FLT3 Inhibitors

Lymphoblastic leukemias with rearrangements of the MLL gene display a unique gene expression profile that distinguishes them from other subgroups of ALL and AML.[57,59-61] This distinct gene expression profile has led to the identification of several potential therapeutic targets. One of the most highly expressed genes distinguishing MLL gene–rearranged ALL from other acute leukemias is *FLT3*, the gene encoding fms-like tyrosine kinase 3.[57,212] Activating *FLT3* mutations through internal tandem duplication (ITD) of the juxta-membrane domain (FLT3/ITD) occur in 11% to 17% of de novo pediatric AML cases and are associated with significantly poorer outcomes.[213-216] After identification of these activating mutations, numerous small-molecule FLT3 inhibitors were developed, and clinical trials are under way to evaluate the effectiveness of these compounds in the treatment of AML.[217-222] However, FLT3/ITD–positive AML is rare in infant AML, accounting for fewer than 2% of cases, and therefore may have very limited benefit in this population.[216] High levels of *FLT3* expression have also been demonstrated in MLL gene–rearranged ALL. FLT3 inhibitors have been shown to be active against MLL gene–rearranged cell lines that overexpress *FLT3*, as well as in primary MLL gene–rearranged lymphoblasts.[223-225] Additionally, results from in vitro experiments with MLL gene–rearranged cell lines have suggested that FLT3 inhibitors may work synergistically with several standard chemotherapeutic agents, including ara-C.[225,226] However, the sequence of administration of these agents appears to be important. In vitro studies have found that to achieve maximal synergistic cytotoxicity, the chemotherapeutic agent must be given immediately prior to the FLT3 inhibitor, whereas pretreatment with the FLT3 inhibitor followed by the chemotherapeutic agent results in antagonistic effects.[226,227] Furthermore, disruption of the stromal microenvironment through CXCR4 inhibition leads to increased sensitivity of MLL gene–rearranged ALL xenograft models to FLT3 inhibition.[228] The preclinical data demonstrating activity of FLT-3 inhibitors in infant ALL suggest that FLT3 inhibition may represent a novel therapeutic strategy for infant ALL, and clinical testing is under way. An ongoing COG study (trial AALL0631) is evaluating the benefit of the addition of the FLT3 inhibitor lestaurtinib to an intensive chemotherapy backbone for patients with infant ALL.

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CHAPTER 53

Pediatric Lymphoma

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CHAPTER OUTLINE

LYMPHADENOPATHY
Approach to the Patient
EMERGENCIES IN PATIENTS WITH NEWLY DIAGNOSED LYMPHOMA
HODGKIN LYMPHOMA
Classic Hodgkin Lymphoma
Nodular Lymphocyte-Predominant Hodgkin Lymphoma
Treatment of Relapsed Hodgkin Lymphoma
Late Effects of Therapy
NON-HODGKIN LYMPHOMA
Epidemiology and Causative Factors
Initial Evaluation and Staging
Lymphoblastic Lymphoma
Mature B-Cell Neoplasms
Anaplastic Large-Cell Lymphoma
Rare Lymphomas in Children and Adolescents
Late Effects of Therapy
POSTTRANSPLANTATION LYMPHOPROLIFERATIVE DISEASE
Classification and Epidemiology
Treatment and Outcomes
CONCLUSION AND FUTURE DIRECTIONS IN PEDIATRIC LYMPHOMA

Lymphomas are neoplasms caused by the malignant transformation of the constituent cells of the immune system. Combined, Hodgkin and non-Hodgkin lymphomas are the third most common malignancies in children and adolescents, with Hodgkin lymphoma being the most common cancer in people between the ages of 15 and 18 years.

The treatment of children and adolescents with lymphoma is one of the important success stories in pediatric oncology. The transformation of what were uniformly fatal diseases to those in which cure is the expectation occurred first for those with Hodgkin lymphoma and more recently for the majority of children with non-Hodgkin lymphoma. The understanding of normal and abnormal lymphoid biology has been critical to the develop systems for classification and for the evolution of disease-specific therapy.

This chapter reviews the clinical and pathologic features, including molecular and cellular biology, of the most common pediatric lymphoma subtypes and reviews the current strategies for evaluation and treatment.

LYMPHADENOPATHY

The child with lymphadenopathy poses a relatively common diagnostic challenge for the pediatrician. Over one third of 5-year-old children seen for well-child care and two thirds evaluated for a sick visit have palpable adenopathy.[1,2] In most children, lymph-node enlargement results from transient, self-limited infectious processes that resolve without sequelae. However, serious, life-threatening benign and malignant diseases may present with lymphadenopathy as the first manifestation. Thoughtful decision making is critical regarding further investigation, including which children require biopsy of their nodes for pathologic evaluation.

Various definitions of adenopathy have been described, including specific measurements in the radiologic literature. In general, adenopathy is defined as any node greater than 1 cm in dimension. Epitrochlear nodes are considered enlarged if they are greater than 0.5 cm, and inguinal nodes are enlarged if they are greater than 1.5 cm. Lymphadenopathy is considered localized if it involves a single node or single nodal area and generalized if it involves more than 2 noncontiguous nodal groups and may include hepatosplenomegaly. Chronic adenopathy is variably defined but in general can be considered as enlargement that persists for longer than 3 weeks.

Enlargement of lymph nodes results from expansion and recruitment of normal lymph node cells, expansion of abnormal immune cells, or infiltration of extrinsic cells. Certain considerations narrow the diagnostic possibilities in evaluation of the child with lymphadenopathy. Not all masses in children that appear to be lymphadenopathy represent enlarged lymph nodes; non–lymph-node masses that may mimic cervical lymphadenopathy include thyroglossal duct cysts and branchial cleft cysts. Evidence of recent or current upper respiratory infection and the presence of tender cervical adenopathy suggest inflammation as the cause. The presence of associated systemic symptoms, the chronicity of the adenopathy, and whether the adenopathy is generalized or regional are important considerations in the differential diagnosis. In addition, the age of the patient is important in considering the differential diagnosis, especially as it relates to potential malignant conditions. An abbreviated list of common causes of lymphadenopathy is presented in Box 53-1. More complete listings have been published elsewhere.[3,4]

Approach to the Patient

The management of the child with lymphadenopathy should be focused on obtaining clues to a diagnosis from the history, physical examination, and noninvasive testing, with the goals of ascertaining whether the lymphadenopathy is likely to be a manifestation of a serious illness and determining as early as possible in the workup whether the child should undergo lymph node biopsy. The history should include the duration of the adenopathy, associated symptoms, evidence of recent infection in the regions drained by the involved lymph node, exposure to illnesses, cats, or rodents, and current medications. The physical examination should be focused on ascertaining the location and number of enlarged nodes, their size, and their texture. Involvement of supraclavicular lymph nodes suggests mediastinal pathology and is usually associated with a serious disease mandating a prompt workup including a chest radiograph. In contrast the involvement of upper cervical lymph nodes is more likely to be the result of an upper respiratory tract infection.

For the child with generalized lymphadenopathy initial evaluation to be considered should include a complete blood count and a chest radiograph. Testing for possible infectious pathogens including viruses, fungi, and bacterial and mycobacterial agents should be considered. If no clues emerge, lymph node biopsy is likely indicated.

The workup of a child with localized or regional lymphadenopathy must be tailored to the individual child. Retrospective studies have attempted to identify those children with adenopathy who are more likely to have an underlying malignancy. Of children who underwent node biopsies, predictive factors for the etiology being malignancy included age older than 10 years, node size greater than 2.5 cm, and supraclavicular site.[5,6] Additional factors that were possibly associated included persistence of the node for longer than 6 weeks, having the node be “fixed” by palpation, and having more than one nodal area involved. In asymptomatic children whose adenopathy does not have high risk features, observation with careful measurement of lymph node size and possibly an empirical trial of antibiotic therapy is a reasonable strategy. If the nodes increase in size or fail to decrease to normal size after several weeks of observation, a lymph-node biopsy should be considered.

Box 53-1 **Causes of Lymphadenopathy**

INFECTION

Bacterial: *Staphylococcus aureus,* group A *Streptococcus, Bartonella henselae,* brucellosis, tularemia
Viral: Epstein-Barr virus, cytomegalovirus, human immunodeficiency virus, measles, rubella
Fungal: Histoplasmosis, coccidioidomycosis, *Cryptococcus*
Protozoan: Toxoplasmosis, malaria
Mycobacterial: Tuberculosis, atypical mycobacteria

AUTOIMMUNE DISEASE

Juvenile rheumatoid arthritis, systemic lupus erythematosus, serum sickness, autoimmune lymphoproliferative syndrome

STORAGE DISEASE

Niemann-Pick disease, Gaucher disease

DRUG REACTION

Phenytoin and others

MALIGNANCY

Lymphoma, leukemia, metastatic solid tumors, histiocytic disorders

MISCELLANEOUS

Sarcoidosis, Kawasaki disease, Kikuchi disease

Masses occurring in the mediastinum represent urgent and challenging diagnostic problems, whether the result of enlarged lymph nodes or of the involvement of extralymphatic tissues. Lymphomas account for a significant portion of anterior and middle mediastinal masses in children and adolescents. Their proximity to vital structures and their propensity to cause life-threatening symptoms from vascular or airway compromise mandate an expeditious, systematic approach involving close cooperation among the surgeon, radiologist, oncologist, anesthesiologist, radiotherapist, and pathologist. A differential diagnosis of mediastinal masses according to location within the mediastinum is shown in Box 53-2.

Box 53-2 **Differential Diagnosis of Mediastinal Masses**

ANTERIOR MEDIASTINUM

Lymphoma
Thymic cyst
Thymic “hyperplasia”
Benign teratoma
Malignant germ-cell tumor
Thymoma

MIDDLE MEDIASTINUM

Lymphoma
Tuberculosis
Histoplasmosis
Sarcoidosis

POSTERIOR MEDIASTINUM

Neuroblastoma
Ganglioneuroma
Neurofibroma
Sarcoma
Duplication cyst

EMERGENCIES IN PATIENTS WITH NEWLY DIAGNOSED LYMPHOMA

Children with lymphoma can have life-threatening findings such as respiratory distress and superior vena cava (SVC) syndrome caused by a mediastinal tumor, bowel obstruction from large abdominal masses, cranial nerve palsies, and paraplegia in the case of central nervous system (CNS) involvement and, in rare cases, disseminated intravascular coagulation. Patients may exhibit the metabolic derangements of acute tumor lysis syndrome (ATLS), although more often this problem arises after the initiation of therapy. Invasive diagnostic measures, especially those requiring anesthesia, can create emergencies for children with lymphomas.

A mediastinal tumor can cause respiratory distress as a result of tracheal or other large-airway compression. Patients often have cough, shortness of breath, and orthopnea. If there is considerable respiratory impairment, then all invasive diagnostic procedures need to be completed expeditiously. In these patients general anesthesia carries potential for substantial risks including inability to ventilate the child because of airway compression as well as swelling of the tracheal mucosa secondary to intubation, which may lead to worsening of critical constriction. Diagnostic procedures often need to be completed with local anesthesia alone. The least invasive procedure that will allow for attainment of diagnostic tissue should be undertaken.[7] In emergent situations, completion of staging (e.g., diagnostic lumbar puncture) may need to be deferred. Once tissue has been obtained, then initiation of steroids can be considered until pathologic diagnosis is complete. Very rarely for the patient with impending respiratory failure empirical therapy without specific diagnosis may needed. Cytoreduction with prednisone 60 mg/m^2/day and cyclophosphamide 100 to 200 mg/m^2/day or mediastinal radiation with doses of 10 to 15 Gy may provide a life-saving intervention.

In addition to respiratory impairment, mediastinal tumors can cause SVC syndrome by compression of the SVC and subsequent venous congestion. Patients have dilated neck veins, facial swelling, and discoloration. The life-threatening risk is from CNS venous strokes, often presenting as confusion or somnolence and less often as seizures or focal neurologic deficits. The tempo of progression of SVC syndrome in children tends to be much quicker in children than in adults as a consequence of more rapidly growing tumors. Again, in these patients expeditious evaluation and initiation of therapy is of critical importance.

Patients who have cranial nerve palsies and especially those with signs of incipient paraplegia also require urgent care. As in other pediatric malignancies, interventions may include surgical decompression, radiation, and chemotherapy. In general for children with lymphomas, which tend to be highly and quickly responsive to chemotherapy, initiation of therapy with prednisone or dexamethasone with or without cyclophosphamide is often the best approach. Clinicians should be aware that lumbar puncture in patients with bulky CNS disease may carry the risk of brainstem herniation. Urgent imaging should be considered before the procedure.

ATLS describes the metabolic derangements that occur with tumor-cell breakdown and is characterized by various combinations of hyperuricemia, hyperkalemia, and hyperphosphatemia with or without hypocalcemia and can lead to seizures, arrhythmias, renal failure, and death.[8] ATLS can be present before the initiation of treatment but more commonly occurs immediately after the initiation of chemotherapy. The incidence of ATLS differs among tumor types and depends primarily on the growth fraction, tumor mass, and chemotherapeutic sensitivity of the tumor. Adult and pediatric risk stratification algorithms for ATLS syndrome has been made and includes age, malignancy type, stage/lactate dehydrogenase (LDH) level, and renal function as predictive factors.[8] Patients with non-Hodgkin lymphoma (NHL) are at high risk, particularly those with advanced stage Burkitt lymphoma (BL), Burkitt leukemia, or lymphoblastic lymphoma (LL) with LDH greater than twice normal.

Vigorous hydration, alkalinization, and the administration of allopurinol, an inhibitor of xanthine oxidase, have been considered mainstays of prevention and treatment of ATLS and are aimed at the reduction of uric acid production and prevention of its precipitation in the kidneys. Evidence supporting the use of alkalization is controversial, and some contemporary guidelines do not recommend its use.[8] For patients considered at low risk, no specific intervention is required. For those at intermediate risk, careful monitoring of fluid and electrolytes is critical. Hyperhydration and allopurinol are almost always sufficient.

For those with higher risk for tumor lysis or who have evidence of the syndrome before the initiation of therapy, rasburicase should be considered. This agent is a recombinant urate oxidase that catalyzes the conversion of uric acid to allantoin. Allantoin is an inactive metabolite that is five to ten times more soluble than uric acid, so renal excretion is facilitated. A single dose will result in the rapid reduction in uric acid levels in patients with hyperurecemia.[9] It is contraindicated in patients with a history of glucose-6-phosphate dehydrogenase (G6PD) deficiency because of substantial risks of precipitating severe hemolysis. In a systematic review of published randomized trials, rasburicase was highly effective at reducing elevated uric acid, although the impact on mortality and renal failure was unclear.[10] In a French study of children with advanced stage B-cell NHL that incorporated the use of rasburicase, the incidence of need for dialysis during the first days of treatment was only 2.6% compared with 16% and 23% in other cooperative studies using the same French protocol that used only allopurinol.[11]

In some patients, even with optimal ATLS care renal function is insufficient and early hemodialysis should be instituted. This situation may occur because of direct infiltration of the kidneys, obstruction of the urinary tract caused by lymphomatous compression, established urate or calcium phosphate nephropathy, or a combination of these conditions.

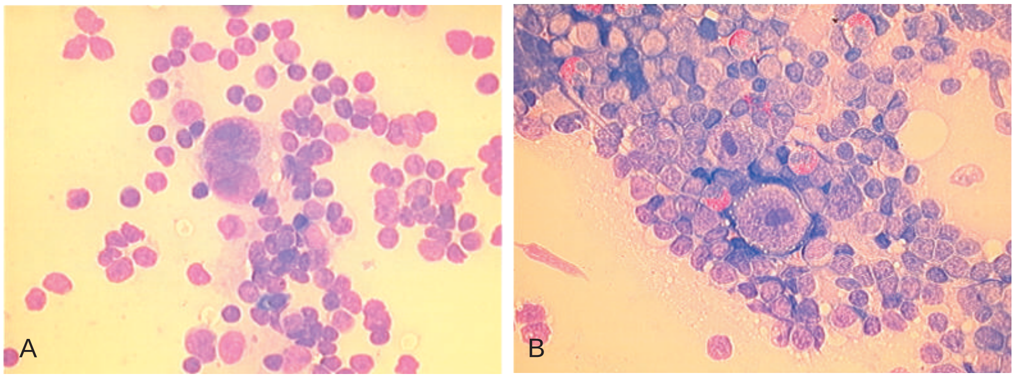

Figure 53-1 **A,** Reed-Sternberg cell. **B,** Hodgkin cell. Reed-Sternberg cells are large cells with abundant slightly basophilic cytoplasm and have at least two nuclear lobes or nuclei containing a prominent inclusion-like eosinophilic nucleolus. Mononuclear variants are known as Hodgkin cells and often have a more intense basophilic cytoplasm.

HODGKIN LYMPHOMA

Hodgkin lymphoma was first described in 1832 by Thomas Hodgkin as a disorder characterized by a peculiar enlargement of the absorbent (lymphatic) glands and spleen and was named in 1865 by Sir Samuel Wilks.[12,13] Sternberg and Reed, in 1898 and 1902, respectively, are credited with the first definitive and thorough description of the binucleate or multinucleated giant cells that when present, are considered pathognomonic of this disorder (Fig. 53-1).[14,15] The malignant nature of the disease was proven when Seif and Spriggs confirmed the clonal origin of the malignant cell by cytogenetic analysis.[16]

Before 1960 Hodgkin lymphoma was an almost uniformly fatal disease. In the 1960s investigators at Stanford and elsewhere developed the use of megavoltage radiotherapy and defined treatment fields including mantle, inverted Y, total nodal, and total lymphoid irradiation.[17] Subsequently in the late 1960s and early 1970s the first randomized trials were initiated.[18,19] Concurrently chemotherapy agents were being investigated. After the observation of the lympholytic effect of nitrogen mustard, this and other agents were used individually in the attempt to treat patients with leukemia and lymphoma. A major step forward toward the use of chemotherapy for Hodgkin lymphoma was the introduction of the four-drug combination of mechlorethamine, vincristine (Oncovin), procarbazine, and prednisone (the MOPP regimen), which led to substantial rates of cure in previously incurable patients.[20]

Over the next 20 years, standard therapy included clinical and surgical staging including laparotomy and splenectomy, as well as extended field radiation with doses of 36 to 44 Gy with or without combination chemotherapy. The probability of cure in children and adolescents using this strategy approached 90%.[21] Early on, however, late effects of therapy were noted. The first to be observed was the impact on musculoskeletal growth of young children receiving high-dose extended field radiation.[22] Subsequently, the long list of therapy-related late effects emerged to include second malignancies, serious cardiac and pulmonary compromise, infectious complications, and sterility.[23] For the last two decades the focus of the development of care for patients with Hodgkin lymphoma has been to maximize the probability of long-term, disease-free survival but to do so while minimizing risks of late effects.

Unlike the complex evolution of classification systems for NHL, the systems used for Hodgkin lymphoma have been more straightforward. The Rye modification of the Lukes-Butler system of classification of Hodgkin disease was universally accepted for 25 years and formed the basis for the current World Health Organization (WHO) classification.[24,25] Studies of the biology and clinical course of the disease have shown that Hodgkin lymphoma should be subdivided into two entities, classic Hodgkin lymphoma (CHL) and nodular lymphocyte–predominant Hodgkin lymphoma (NLPHL) (Table 53-1). The two entities will be discussed separately in the next sections.

Classic Hodgkin Lymphoma

Pathologic Features

CHL is characterized by the presence of mononuclear Hodgkin and multinucleated Reed-Sternberg cells. Hodgkin and Reed-Sternberg cells are usually in the minority, residing in a reactive infiltrate of a variable mixture of nonneoplastic lymphocytes, eosinophils, neutrophils, plasma cells, fibroblasts, and collagen fibers present in response to cytokines produced by the tumor.[26] The Reed-Sternberg cells are large cells with abundant, slightly basophilic cytoplasm and have at least two nuclear lobes or nuclei containing a prominent inclusion-like eosinophilic nucleolus. Diagnostic Reed-Sternberg cells must have at least two nuclei in two separate lobes. Mononuclear variants are termed *Hodgkin cells* and often have a more intense basophilic cytoplasm. Microdissection techniques have enabled the isolation of Reed-Sternberg cells from frozen sections and the investigation of their lineage commitment. In more than 98% of cases, Reed-Sternberg cells are B cells, as defined by monoclonal immunoglobulin gene rearrangements.[27] Only a few cases have shown clonal T-cell receptor (TCR) gene rearrangement in the Reed-Sternberg cells, suggesting T-cell origin.[28] Reed-Sternberg cells of CHL express the B-lineage

TABLE 53-1 Pathologic and Clinical Features of Classic and Nodular Lymphocyte Predominant Hodgkin Lymphoma in Children and Adolescents

	CHL	NLPHL
Percentage of patients	92%-95%	5%-8%
Histologic hallmark	Reed-Sternberg cell	Lymphocyte-predominant cells (also called *popcorn cells*)
Immunophenotype	CD15 and CD30 common, CD20 rare	CD15 and CD30 rare, CD20 common
EBV association	Yes in 40%	Negative
Percentage male	50%	75%
Peak incidence	15-20 years	13 years
Disease at presentation	Majority with stage II or greater; mediastinal involvement common	Majority with stage IA or IIA; mediastinal involvement uncommon

Modified from Shankara A, Saw S: Nodular lymphocyte-predominant Hodgkin lymphoma in children and adolescents: a comprehensive review of biology, clinical course, and treatment options. Br J Haematol *159:288–298, 2012.*

CHL, Classic Hodgkin lymphoma; *EBV,* Epstein-Barr virus; *NLPHL,* nodular lymphocyte-predominant Hodgkin lymphoma.

antigens CD20 and CD79a in variable proportions, whereas the B-cell–specific activator protein (BSAP), a product of the *PAX5* gene, is expressed in about 90% of cases.[29,30] Reed-Sternberg cells are almost invariably positive for the CD30 antigen[29] and usually express the CD15 antigen, whereas the expression of the epithelial membrane antigen (EMA) is rare. In Epstein-Barr virus (EBV)–positive cases of CHL, the Reed-Sternberg cells express the EBV latency type II pattern latent membrane (LMP) protein 1 and Epstein-Barr nuclear antigen (EBNA) 1, but without EBNA2.[31]

Based on the characteristics of the reactive infiltrate and the morphology of the Reed-Sternberg cells, four subtypes of classic Hodgkin lymphoma are distinguished in the WHO classification[25,32]: lymphocyte-rich Hodgkin lymphoma (LRHL), lymphocyte-depleted Hodgkin lymphoma (LDHL), mixed-cellularity Hodgkin lymphoma (MCHL), and nodular sclerosis Hodgkin lymphoma (NSHL). The immunophenotypic and genetic features of the Reed-Sternberg cells are identical in these histologic subtypes, whereas they differ in clinical features and association with EBV. CHL is associated with overexpression and an abnormal pattern of cytokines and chemokines and their receptors by Reed-Sternberg cells and the cells of the reactive background.[33,34] The abnormal cytokine and chemokine expression most likely accounts for the abundant admixture and pattern of inflammatory cells in CHL lesions as well as for distinct clinical features.

The NSHL subtype is characterized by a nodal growth pattern with collagen bands that surround at least one nodule, the formation of clusters of Reed-Sternberg cells, and so-called *lacunar cells* (mononuclear Hodgkin cells with only moderately prominent nucleoli).[26,35] NSHL can be grade 1 or 2, mainly depending on the number of Reed-Sternberg cells in distinct nodules.[36] The EBV-encoded LMP1 antigen is less commonly expressed than in other subtypes.

The MCHL subtype is characterized by scattered classic Reed-Sternberg cells in a diffuse or vaguely nodular mixed inflammatory background without sclerosis. Partial involvement of the lymph node can be observed in MCHL, described as *interfollicular disease.* The EBV-encoded LMP1 antigen is more commonly expressed than in NSHL.

The LRHL and LDHL subtypes are relatively rare in children.[37,38] The LRHL subtype contains scattered Reed-Sternberg cells and a nodular or diffuse background of small lymphocytes but with an absence of neutrophils and eosinophils. The LDHL subtype is a diffuse form of CHL, rich in Reed-Sternberg cells and/or depleted of non-neoplastic lymphocytes and is often sarcomatoid in appearance.

In some patients, distinction between true Hodgkin lymphoma and certain subtypes of NHL can be difficult. Grey-zone lymphomas are those that do not fit into a single disease entity. The WHO classification includes the entity of "B-cell lymphoma, unclassifiable with features intermediate between diffuse large B-cell lymphoma (DLBCL) and classic Hodgkin lymphoma," although this entity is exceptionally rare in children.[25]

Molecular and Cellular Biology

The cell of origin of the malignant cells in CHL was long a matter of controversy because of the low incidence of Reed-Sternberg cells in the tumors and their unusual immunophenotype in that they lack expression of immunoglobulins and other B-cell markers; rather, they express markers characteristic of dendritic cells, granulocytes, monocytes, and T cells.[40] However, the identification of immunoglobulin gene rearrangement[27,41,42] and somatic hypermutation in microdissected Reed-Sternberg cells in almost all cases of CHL has demonstrated that these cells derive from postgerminal B cells. In addition these studies have demonstrated that about 25% of CHL cases carry crippling immunoglobulin-gene rearrangements that destroy the function of the immunoglobulin protein.[27,41] These nonfunctional rearrangements typically induce rapid apoptosis in normal B cells, suggesting that Hodgkin lymphoma may originate from germinal-center B cells that have escaped apoptosis. Analysis of TCR rearrangements has shown that despite the common expression of T-cell markers, CHL originates from T cells in only 1% to 2% of cases.[28]

In NLPHL, the malignant popcorn cells show expression of B-cell markers such as CD20 and CD79a, suggesting a B-cell origin for these tumors as well. As in CHL, the analysis of immunoglobulin genes in microdissected tumors has shown clonal and somatically mutated gene rearrangements.[27,43] In addition, 50% of cases have shown evidence of ongoing somatic hypermutation, which strongly suggests that they derive from germinal center B cells.[27]

Mechanisms of Transformation. EBV has been associated with the pathogenesis of CHL, which shows EBV infection of the Reed-Sternberg cell in 40% of the cases in the Western world and 90% in Central America.[44-46] Importantly, EBV can immortalize human B cells in vitro, and Reed-Sternberg cells are clonally infected by EBV, which suggests that viral infection occurs early in the pathogenesis of the disease.[47] EBV positive Reed-Stenberg cells express EBNA1, LMP1, and the *LMP2a* viral gene, which is characteristic of the viral latency II stage. Mechanistically, LMP1 mimics an active CD40 receptor and can activate the nuclear factor κB (NF-κB) pathway, which is constitutively active in Hodgkin lymphoma (see later).[48] LMP2, a second viral gene product, mimics B-cell receptor signaling and might play a role in rescuing Reed-Sternberg cells from apoptosis with immunoglobulin-crippling mutations.[49] Cytogenetically, Reed-Sternberg cells often show aneuploidy and chromosomal abnormalities, with 20% of the cases harboring chromosomal translocations involving the immunoglobulin loci, a hallmark of many B-cell lymphomas.[50,51]

The most prominent molecular abnormality found in Hodgkin lymphoma is the constitutive activation of the NF-κB pathway. NF-κB functions as an important survival-signaling pathway in B cells in response to the activation of members of the tumor necrosis factor (TNF) receptor family (CD30, CD40). In Hodgkin lymphoma, somatic mutations in different elements of the NF-κB pathway keep it constitutively activated. Thus the *REL* gene, which encodes an NF-κB component, is amplified in the of 30% of the cases.[52,53] Gains of NF-κB–inducing kinase (NIK), a positive regulator of the alternative NF-κB pathway, are also commonly found in Reed-Sternberg cells,[54,55] and translocations and amplifications resulting in overexpression of the *BCL3* gene in Reed-Sternberg cells has been described.[56] Moreover, mutations of the gene-encoding NF-κB inhibitors IKBA and IKBE are found in 10% to 20% of cases of Hodgkin lymphoma.[57-59] Most significantly *TNFAIP3*, which encodes A20, a ubiquitin-modifying enzyme involved in the negative regulation of NF-κB signaling, harbors loss of function mutations and deletions in almost 45% classic Hodgkin lymphomas.[60,61] Notably, *TNFAIP3* mutations and deletions are more common in EBV-negative tumors and are rarely found in lymphocyte-predominant Hodgkin lymphomas.[61,62] Finally, activation of TNF signaling by the microenvironment and activation of NF-κB by the LMP1 EBV oncogene may also contribute to aberrant NF-κB signaling.

Gene amplifications involving the *JAK2* locus have been described in about 20% of CHL cases, suggesting a role for *JAK2* activation in the pathogenesis of this disease.[63] In addition, rearrangements of *JAK2* including a recurrent *SEC31A–JAK2* translocation have been described in rare cases of Hodgkin lymphoma.[64] Moreover, loss of function mutations in *SOCS1*, a negative regulator of Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signaling are present in about 50% of classic and lymphocyte-predominant Hodgkin lymphomas and are associated with increased levels of JAK signaling.[65,66]

Translocations involving the major histocompatibility complex (MHC) class II transactivator gene *CIITA*, resulting in impaired MHC class II expression, are found in 15% of CHL cases[67] and seem to be associated with unfavorable prognosis.[68] In contrast to CHL, the malignant cells of the lymphocyte-predominant type of Hodgkin lymphomas are always EBV-negative, and there is little information on the molecular lesions implicated in the pathogenesis of this group. Chromosomal translocations involving the *BCL6* oncogene can be detected in 30% of cases.[69] *BCL6* is a zinc-finger transcription repressor that functions as a master regulator of germinal-center formation and drives B-cell transformation. Thus *Bcl6*-null mice fail to generate germinal centers in response to immunization, and deregulated expression of *Bcl6* induces B-cell lymphomas in mice.[70] Mechanistically, aberrant expression of *BCL6* in lymphoma hijacks the role of *BCL6* in the control of B-cell activation, differentiation, deoxyribonucleic acid (DNA) damage response, cell-cycle arrest, and apoptosis.[70] Finally, about 40% of lymphocyte-predominant Hodgkin lymphoma tumors show mutations in *SOCS1,*[66] but mutations in *TNAIP3* and *NFKBIA* are rare despite strong NF-κB activity.[62,71]

Cellular Microenvironment: Cytokines and Chemokines. The cellular and cytokine microenvironment surrounding the lymphoid cells in Hodgkin lymphoma plays an essential role in the pathogenesis of this disease. In particular, the expression of soluble factors and their receptors by malignant cells and the reactive microenvironment seems not only to mediate the inflammatory characteristics observed in the histology of Hodgkin lymphoma, but also to contribute to the proliferation and survival of the malignant clone. Thus the cellular microenvironment supports and is supported by a network of cytokines secreted in autocrine and paracrine loops that are essential for the proliferation of Reed-Sternberg cells and the maintenence of a favorable inflammatory environment rich in regulatory T cells (Tregs) and eosinophils.

Both interleukin (IL) 13 and the IL-13 receptor IL-13RA1 are expressed in Hodgkin lymphoma and constitute an important autocrine loop for Reed-Sternberg cells.[72,73] Other cytokines expressed in Hodgkin lymphoma and thought to influence survival of these cells include IL-4, IL-6, IL-7, IL-9, and IL-15.[33,34]

The vast majority of cells in a tumor biopsy of Hodgkin lymphoma are not malignant cells but represent an inflammatory-like cellular infiltrate composed mainly of CD4+ T lymphocytes intermixed with macrophages, eosinophils, plasma cells, and fibroblasts. Most lymphocytes present in this infiltrate are Tregs, which play an important role in protecting the tumor's Reed-Sternberg cells from cytotoxic T cells involved in antitumor immune surveillance.[49]

In contrast with the abundance of Tregs, Th1 CD4+ T cells and CD8+ cytotoxic T cells are rare in Hodgkin lymphoma biopsies and are not detected in the immediate proximity of Reed-Sternberg cells. The recruitment of cells involved in immune tolerance and the exclusion

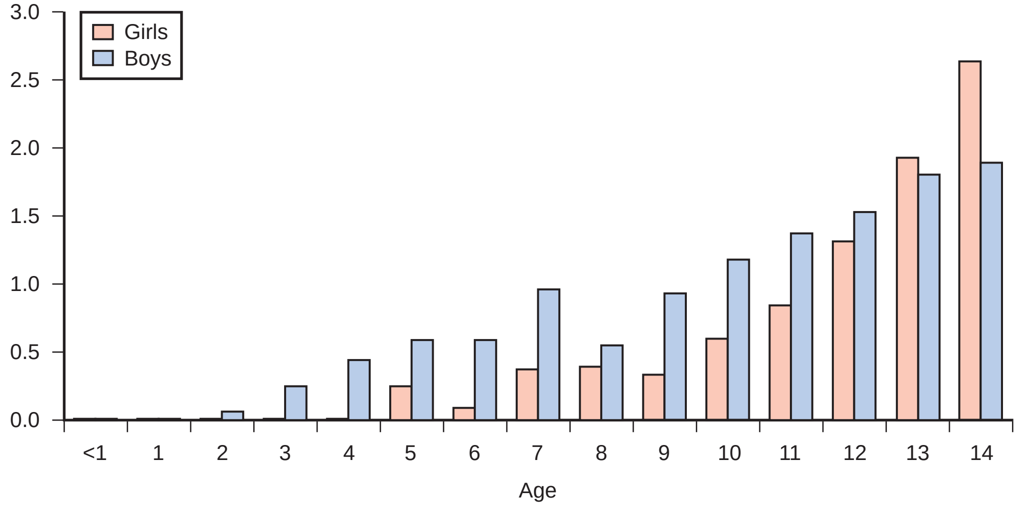

Figure 53-2 Age- and gender-specific incidence rates (per 100,000) of Hodgkin lymphoma during childhood in Germany, 2000 to 2004. *(Modified from Kaatsch P, Spix C:* Annual report, 2005: German Childhood Cancer Registry, *Mainz, Germany, 2007, Institut für Medizinische Biometrie, Epidemiologie, und Informatik, p 52.)*

of lymphoid cells responsible for antitumor immune responses are explained by the expression of Reed-Sternberg cells of pro-Th2–associated cytokines, such as IL-4 and IL-13, and anti-Th1 or CD8 cytokines, such as IL-10 and transforming growth factor (TGF) β.[49] The secretion of these immunosuppressive cytokines creates an immune-privileged microenvironment, allowing the tumor cells to avoid immune surveillance and T-cell–mediated apoptosis.

One of the most prominent features of Reed-Sternberg cells is the expression of CD30. Although coexpression of CD30 and its respective receptor CD153 initially suggested an autocrine mechanism promoting the proliferation of Reed-Sternberg cells via NF-κB, it is now well established that the activation of CD30 is primarily a CD153-independent process.[74] Two additional members of the TNF receptor family, CD40 and receptor activator of nuclear factor κB (RANK), are expressed in Reed-Sternberg cells. Activation of CD40 seems to be mediated by the expression of CD40 ligand (CD40L) on T lymphocytes surrounding the Reed-Sternberg cell.[75,76] Importantly, soluble CD40L induces proliferation and blocks CD95-induced apoptosis in Reed-Sternberg cells.[75-77] Other factors that help rescue Hodgkin and Reed-Sternberg cells from an immunologic attack include the expression of the PD1 ligand and secretion of IL-10, TGF-β, and galectin 1 by the Reed-Sternberg cell,[78-82] as well as inhibition of cytotoxic T cells by regulatory T cells (Tregs).[78] Activation of RANK and osteoprotegerin, a member of the TNF-receptor superfamily, is triggered by the autocrine expression of RANK ligand (RANKL) in Reed-Sternberg cells.[83] Activation of RANK promotes NF-κB activation and contributes to the maintenance of an inflammatory microenvironment by promoting interferon gamma (IFN-γ) and IL-13 secretion.[49] Overall, the activation of TNF receptors by different mechanisms seems to play an important role in promoting the survival of the Reed-Sternberg cell.

Epidemiology and Causative Factors

The epidemiology of Hodgkin lymphoma is complex, with variation across geographic regions and with age, sex, and socioeconomic status. Data from cancer incidence surveys conducted in five continents have suggested that Hodgkin lymphoma has a bimodal peak at ages 15 to 34 years and a second peak in those older than 60 years.[84] In Asian populations the overall incidence is only half that of the incidence in Europe.[85,86] In all regions, Hodgkin lymphoma occurs rarely in children younger than 5 years of age (Fig. 53-2).

Hodgkin lymphoma occurring in patients younger than 15 years of age, referred to as *childhood Hodgkin lymphoma,* has several unique features compared with the more common disease of older adolescents and young adults. In childhood disease there is a 2 to 3:1 male preponderance, whereas in older patients the disease is almost equal in males and females.[87] Within the younger age group, mixed cellularity and nodular lymphocyte-predominant histologies are more common.[88] In addition, in childhood Hodgkin lymphoma increasing family size and lower socioeconomic status are risk factors for disease. Conversely, in Hodgkin lymphoma in adolescents and young adults, higher socioeconomic status and smaller family size are risk factors for disease.[89,90]

The role of EBV as an etiologic agent for Hodgkin lymphoma has been the source of much study.[91] In 1966 MacMahon[92] was the first to suggest an infectious cause of Hodgkin lymphoma, and in 1974 Rosdahl and colleagues[93] reported an increased risk of Hodgkin lymphoma in people with a history of infectious mononucleosis.[87] Molecular studies identified monoclonal EBV genomes in Reed-Sternberg cells, implying that they were infected before malignant transformation.[94,95] Subsequent studies showed EBV genome sequences associated with Reed-Sternberg cells in 59% of cases.[96] The proportion of EBV-positive Hodgkin lymphoma cases varies, however, according to geographic region, age, and histologic subtype. The proportion of EBV-positive Hodgkin lymphoma cases is higher in children than adults. It is most prevalent in mixed cellularity histology and more common in boys. The incidence is higher in the developing world including parts of Africa, Asia, and South America, with an incidence of EBV-positive Hodgkin lymphoma as high as 90% in Peru.[97]

Familial aggregation of Hodgkin lymphoma was first described by Razis and coworkers in 1959[98] and is now well documented.[99-102] The familial risk of Hodgkin lymphoma ranks among the highest in the population-based Swedish Family Cancer Database.[103] In an analysis of 28 reports of familial Hodgkin lymphoma there is only one major peak between 15 and 34 years of age for familial Hodgkin lymphoma instead of the classic bimodal age distribution of sporadic Hodgkin lymphoma.[104] This corresponds to the findings of two large studies of an increased sevenfold risk in siblings of cases diagnosed at ages younger than 45 and 35 years of age, respectively, but little or no increased risk in siblings of cases diagnosed at older ages.[105,106] Strong evidence for a role for genetic susceptibility was provided by the finding that monozygotic twins of patients with Hodgkin lymphoma have a 99-fold increased risk, whereas no increased risk in dizygotic twins was observed.[99]

Familial aggregation of Hodgkin lymphoma may in part reflect inherited abnormalities of the immune response. A familial or personal history of autoimmune conditions and sarcoidosis is associated with an increased risk for Hodgkin lymphoma.[107,108] A 51-fold increased risk for Hodgkin lymphoma was found in kindreds predisposed to autoimmune lymphoproliferative syndrome, a disorder of lymphocyte homeostasis usually associated with germline *FAS* gene mutations.[109] In patients with ataxia-telangiectasia mutation (ATM), the risk of Hodgkin lymphoma was increased, but its risk was much lower than the risk of NHL.[110] In 1975 Svejgaard and coworkers first described an association of certain human leukocyte antigen (HLA) loci with an increased risk of Hodgkin lymphoma, suggesting a disease susceptibility gene within or near the histocompatibility region on chromosome 6.[111,112] Since then, numerous studies have confirmed the association of particular HLA loci in the susceptibility to Hodgkin lymphoma, including HLAs A1, B5, B8, B15, B27, B35, and B37.[113] In a large study on familial Hodgkin lymphoma, Chakravarti and colleagues[114] have found strong evidence for a recessive mode of inheritance for susceptibility to Hodgkin lymphoma, and approximately 60% of associations were caused by an HLA-linked susceptibility gene. Recent genetic studies have identified disruption of *KLHDC8B* in the germline of a family with several cases of Hodgkin lymphoma[115] and a germline frameshift mutation in the *NPAT* gene in a family with four cases of NLPHL.[116] Finally a genome-wide association study has identified risk loci at 2p16.1 (*REL*), 8q24.21 (*PVT1*), and 10p14 (*GATA3*) and confirmed the strong HLA association with this disease.[117]

Clinical Characteristics

The most common clinical presentation of Hodgkin lymphoma in children and adolescents is a persistently enlarged node in the cervical or supraclavicular region. Characteristically the lymph nodes involved with Hodgkin lymphoma are not painful and have a "rubbery" firmness on palpation. Enlarged nodes have often been present for weeks or months, increasing and decreasing in size irrespective of whether antibiotic therapy has been given. Although the majority of patients have painless cervical adenopathy, the clinical presentation varies considerably, ranging from life-threatening airway compression to the coincidental detection of an enlarged node during an otherwise routine examination. Approximately 80% of children have disease in one or both sides of the upper or lower neck. Of those with cervical adenopathy, more than two thirds have intrathoracic disease, most commonly in the anterosuperior mediastinum, paratracheal, and tracheobronchial lymph node groups. Pulmonary parenchymal involvement is rarely observed in the absence of hilar disease. Pleural effusions are uncommon; they are usually an indication of lymphatic obstruction from bulky central disease rather than a sign of advanced-stage disease. Pericardial effusion may occur in cases with pericardial involvement and occurs most often in the setting of bulky mediastinal disease.[118] Hodgkin lymphoma has a strong tendency for contiguous spread along adjacent lymph-node regions. Although about 30% of patients have supradiaphragmatic and infradiaphragmatic disease, Hodgkin lymphoma limited to infradiaphragmatic sites is rare.[38]

Systemic symptoms including fatigue and anorexia are common in patients with Hodgkin lymphoma. At the time of diagnosis, approximately 30% of patients have constitutional signs referred to as *B symptoms* and defined as the presence of fever (temperature higher than 38° C [100.4° F]) for 3 consecutive days, drenching night sweats and unexplained body weight loss of 10% or more over the preceding 6 months. Classically the fever associated with Hodgkin lymphoma, called the *Pel-Epstein fever,* occurs in the evening and becomes more pronounced with time. Severe and unexplained pruritus is associated with Hodgkin lymphoma, sometimes preceding the development of adenopathy. A poorly understood and relatively unusual sign of Hodgkin lymphoma is pain at the sites of disease with alcohol ingestion.

Laboratory findings are often nonspecific with mild anemia and elevated erythrocyte sedimentation rate (ESR) being the most common findings. Nonspecific hematologic laboratory findings include neutrophilia, monocytosis, lymphopenia, and eosinophilia. Acute-phase reactants including C-reactive protein, ferritin, and serum copper may be elevated. High levels of alkaline phosphatase can be associated with bone involvement.

Rarely patients with Hodgkin have findings suggestive of paraneoplastic syndromes such as nephritic syndrome, polymyositis, idiopathic cholestasis and autoimmune hemolytic anemia, neutropenia, and thrombocytopenia, or combinations of these. Limbic encephalitis or subacute cerebellar degeneration have also been reported as very rare paraneoplastic syndromes in patients with Hodgkin lymphoma.[119]

At the time of diagnosis patients may exhibit altered immune function characterized by reduced cellular immunity, whereas humoral immunity is usually relatively intact.[120] The nature of the immune defect is unclear. The severity of the impairment increases with advanced stage, disease progression, and recurrence and after treatment with radiotherapy and chemotherapy. T-cell deficits may persist for a prolonged period in successfully treated patients.

Diagnosis and Staging

Biopsy to provide pathologic tissue for examination is required for the diagnosis of Hodgkin lymphoma. Excisional biopsy of an enlarged node is preferred. Samples from needle biopsies are often insufficient for the diagnosis of Hodgkin lymphoma because of the importance of the information provided by the nodal architecture and background stroma, as well as the need to identify the relatively rare Reed-Sternberg cell. In a case series, one third of children who ultimately were diagnosed with Hodgkin lymphoma and who underwent core needle biopsies of their mediastinal masses were not able to be diagnosed from these initial samples.[121] Needle biopsy should be restricted to situations in which surgery and general anesthesia may carry undue risks for the patient. If needle biopsy is performed, multiple biopsy samples should be taken to augment diagnostic potential.

The purpose of a staging evaluation is to identify all sites and characteristics of Hodgkin lymphoma in each patient to permit accurate stratification of therapy based on risk, the definition of areas to be included in potential radiation fields, and to inform follow-up imaging studies. Table 53-2 gives an overview of suggested evaluations.

TABLE 53-2 Recommended Investigations in Children and Adolescents with Hodgkin Lymphoma

Laboratory studies	1. Complete blood count (CBC) 2. Erythrocyte sedimentation rate (ESR) 3. Renal and liver function studies 4. Albumin 5. Lactate dehydrogenase (LDH) 6. Alkaline phosphatase
Imaging	1. Chest x-ray 2. Computed tomography (CT) of neck and chest 3. CT or ultrasound or magnetic resonance imaging (MRI) of the abdomen and pelvis 4. Positron emission tomography (PET) (if available) 5. Bone scan (if no PET available and focal bony pain or advanced stage disease)
Other investigations	1. Bone marrow biopsy, except stage 1A and 2A, and possibly those without evidence of bone-marrow involvement by PET (see below) 2. Selective biopsy for sites of potential involvement that are ambiguous and whose clarification is important for staging and treatment planning
Other pretherapy considerations	1. Assessment of pulmonary function 2. Assessment of cardiac function 3. Assessment of thyroid function 4. Consideration of fertility preservation (sperm banking) 5. Consideration for need of stable intravenous access

A plain film of the chest is useful in providing preliminary information about mediastinal involvement and is an essential evaluation in a patient who will undergo anesthesia for cervical node biopsy (Fig. 53-3). Computed tomography (CT) of the neck, chest, abdomen, and pelvis are standard initial staging evaluations.[122] In the past, imaging of the neck was considered not necessary given the ability to detect cervical node by physical examination. Imaging of the neck, however, can be of significant importance to current care, because it allows for the most accurate radiation field planning, avoiding potential overtreatment of the neck, and providing baseline data for the measurement of disease response.[23] CT of the chest provides detailed information about sites of disease involvement including the mediastinum, pulmonary parenchyma, pleura, and pericardium. Sites of disease in the abdomen and pelvis are visualized with CT done with oral and intravenous (IV) contrast. Alternatively, magnetic resonance imaging (MRI) or ultrasound can also be used for staging abdominal and pelvis disease.

Functional nuclear imaging is an important component to initial staging though perhaps has an even more significant role in assessment of disease response (see later). Gallium-67 imaging has largely been replaced by 18-fluorodeoxyglucose positron emission tomography (FDG-PET) because of increased sensitivity and specificity.[123,124] Increased ^{18}F-FDG uptake in lymphoma is based on elevated glycolysis and the longer residence time of ^{18}F-FDG in malignant cells compared with most normal tissues.[125] Numerous studies in adults with Hodgkin lymphoma have demonstrated that PET is able to detect an additional number of Hodgkin lymphoma lesions compared with conventional imaging studies, in particular CT and bone marrow biopsy, resulting in a modification of staging in 15% to 20% of patients, with an impact on disease management in 5% to 15% of cases.[126] The data in children are more limited.[127,128] A review of a single-center experience showed that the findings from the diagnostic FDG-PET were more sensitive than conventional imaging and that the findings altered the involved-field radiation treatment fields in 17% of patients.[129] Prospective studies are required to assess whether FDG-PET done at the time of diagnosis has on impact on outcome or on treatment burden.

Historically, surgical staging including splenectomy was considered standard for patients with Hodgkin lymphoma. Staging splenectomy is associated with significant risks. In the German-Austrian experience of 1181 children with Hodgkin lymphoma, the survival was poorer for children younger than age 10 years because of episodes of infection and death caused by sepsis in splenectomized children.[130,131] With the availability of cross-sectional imaging techniques, routine staging laparotomy has now been abandoned. In the relatively rare circumstance of imaging findings that are ambiguous in defining

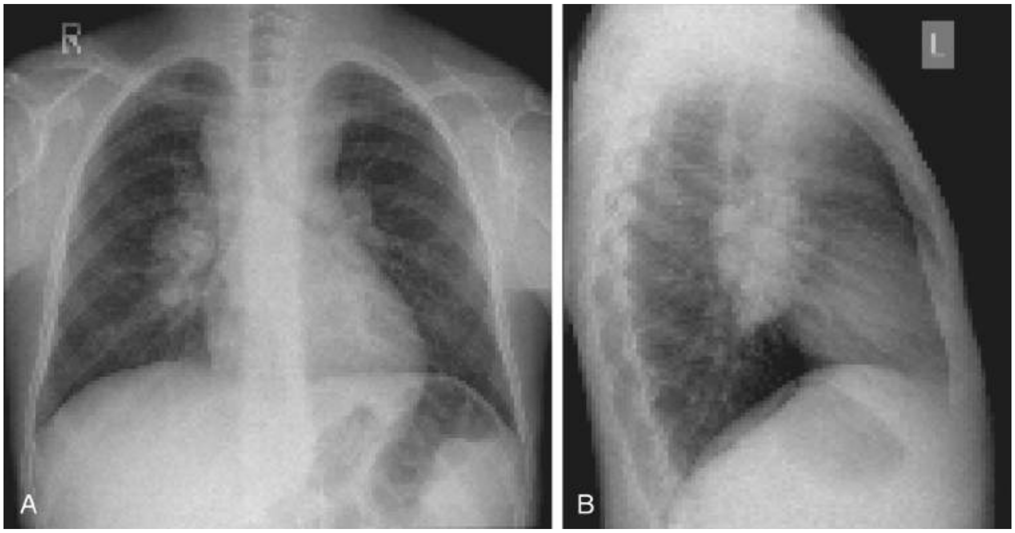

Figure 53-3 Chest x-ray of a patient with Hodgkin lymphoma and involvement of the mediastinal nodes and hilum.

disease involvement or noninvolvement, and when this information affects therapeutic decision making and in particular radiation fields, then staging biopsy may still be warranted.

Until recently bone marrow biopsy has been considered a standard component of the initial staging evaluation for any child with cytopenias at presentation and all patients except for those with stage 1A or 2A disease. Retrospective investigations have suggested that the impact of the findings from diagnostic bone marrow biopsies had a minimal effect on patient-risk assignment and subsequent therapy.[132] FDG-PET is a sensitive and specific method for detection of bone marrow disease. Two recent studies provide data to support the idea that FDG-PET can replace bone marrow biopsies for staging purposes[133,134] in an analogous fashion to cross-sectional imaging of the abdomen replacing staging laparotomy in the past.

The Ann Arbor staging classification for Hodgkin lymphoma, adopted in 1971, was based on the recognized orderly spread of the disease between contiguous lymph nodes, which predominates until late in the course of the disease (Table 53-3).[135] The distinct lymph node regions recognized by the Ann Arbor classification system are shown in Figure 53-4. The substage classifications A, B, and E amend each stage based on distinct features. Stage A designates asymptomatic disease; B indicates the presence of any one of the three B symptoms as defined in Table 53-3; and extralymphatic disease is designated as E, referring to limited extranodal extensions that easily can be encompassed within a radiotherapy portal. Extralymphatic disease is further designated as L (lung), P (pleura), and O (osseous) according to this system. The decision to classify extralymphatic disease either as substage E or as stage IV is based on the clinician's judgment and often depends on whether the extralymphatic disease can be adequately covered in a radiotherapy treatment portal. Multiple E lesions are automatically considered to be stage IV.

Treatment

Centers treating children with Hodgkin lymphoma should have extensive experience and a dedicated multidisciplinary team, including a pediatric surgeon, radiation oncologist, pediatric oncologist, pathologist, and diagnostic radiologist. If such a team is not available at the

TABLE 53-3 Ann Arbor Staging Classification for Hodgkin Lymphoma

Stage	Description
I	Involvement of a single lymph-node region (I) or a single extralymphatic organ or site (I_E)
II	Involvement of two or more lymph node regions on the same side of the diaphragm (II) or localized contiguous involvement of only one extralymphatic organ or site and its regional lymph node(s) on the same side of the diaphragm (II_E)
III	Involvement of lymph-node regions on both sides of the diaphragm (III), which may also be accompanied by involvement of the spleen (III_S) or by localized contiguous involvement of an extralymphatic organ or site (III_E) or both (III_{SE})
IV	Diffuse or disseminated involvement of one or more extralymphatic organs or tissues, with or without associated lymph-node involvement
Designations Applicable to Any Stage	
A	No B symptoms
B	B symptoms, defined as presence of fever (>38° C [100.4° F]) for 3 consecutive days, drenching night sweats, or unexplained loss of 10% or more of body weight in the preceding 6 months
E	Involvement of a single extranodal site that is contiguous or proximal to the known nodal site

Modified from Carbone PP, Kaplan HS, Musshoff K et al: Report of the Committee on Hodgkin's Disease Staging Classification. Cancer Res *31:1860–1861, 1971.*

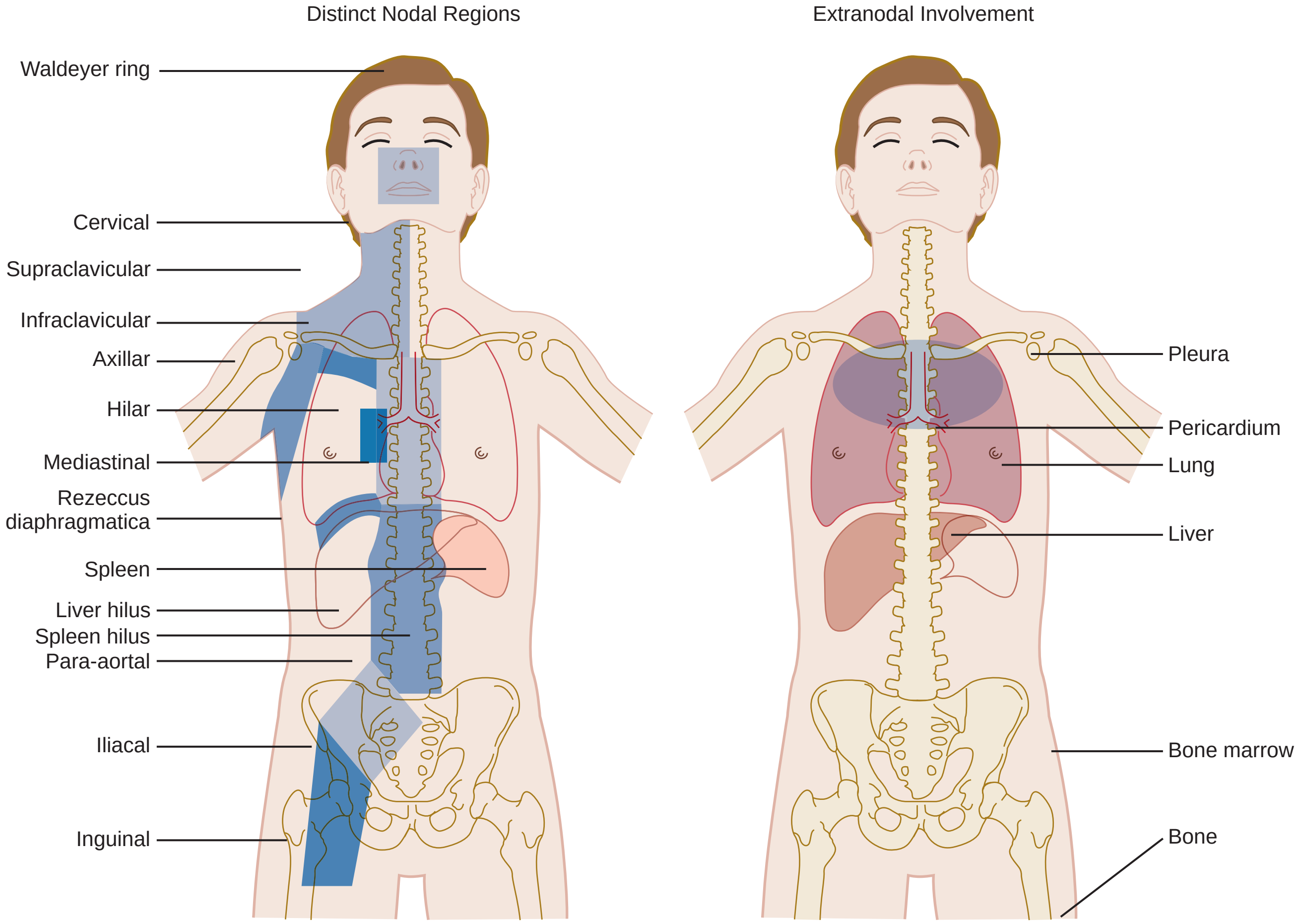

Figure 53-4 Distinct nodal regions according to the Ann Arbor staging classification for Hodgkin lymphoma. *(Modified from Dörffel W, Schellong G: Morbus Hodgkins. In Gadner H, Gaedicke G, Niemeyer C, Ritter J, editors:* Paediatrische Haematologie und Onkologie, *Heidelberg, Germany, 2006, Springer, p. 770–776.)*

facility at which the child is initially seen, prompt referral to a comprehensive childhood cancer center is essential.

Prognostic Factors/Stratification of Treatment. As with other malignancies, prognostic factors are useful as tools for defining risk groups for stratification of treatment intensity. For patients with Hodgkin lymphoma this translates into both the choice of chemotherapeutic regimen and the use of radiotherapy. The stage of disease is a strong prognostic factor and widely used for risk stratification that has been used in all major cooperative groups.[37,130,136] Apart from the stage of disease, a number of factors have been reported to be associated with the risk of treatment failure, including the presence of B symptoms, histologic subtype, bulky disease, gender, anemia, ESR, and evidence of latent EBV infection, some of which have lost significance with improvements in therapy.[137,138] The prognostic value of these variables has not been consistent across studies, in part related to treatment prescribed. For example, mediastinal bulky disease—defined as a mass larger than one third of the maximum chest diameter—was associated with increased risk for relapse in some studies but not in the German-Austrian trial DAL-HD90. However, in this protocol patients with a larger residual mass at the end of chemotherapy received a boost dose in addition to the involved field irradiation.[130,139] Similarly, male gender had an adverse impact on outcome in that trial. For boys, however, procarbazine was partially replaced by etoposide, and girls received a full dose of procarbazine. Race and ethnicity have been identified as risk factors in some pediatric cancers. In a single-center retrospective review, African-American children had an inferior event-free survival (EFS) rate but an identical overall survival (OS) as compared with white children.[140]

The risk-group definitions used in selected clinical trials are described in Table 53-4. In general in addition to stage, disease bulk and presence of B symptoms are the factors used. The kinetics of disease response to chemotherapy, measured by CT and/or PET scan is used in many cooperative group trials as an important additional tool for the stratification of treatment intensity including the use of radiation (see below).

Treatment and Outcomes. In the 1960s extended-field radiation with doses of 35 to 44 Gy were shown to be curative in a significant portion of patients with Hodgkin

TABLE 53-4 Risk Group Definitions in Clinical Trials of Pediatric Hodgkin Lymphoma

	Stage		
Study Group	Low Risk	Intermediate Risk	High Risk
DAL-HD90[130]	IA, IB, IE, IIA	IIEA, IIB, IIIA	IIEB, IIIEA, IIIB, IIIEB, IV
GPOH-HD2002[148]	IA, IB, IIA	IE, IIEA, IIB, IIIA	IIEB, IIIEA, IIIB, IV
CCG 5942[161]	I + IIA, without*	I + II with* IIB, III	IV
Stanford, St. Jude, Boston Consortium[159]	I, II, without†	"Unfavorable" I, II with† III, IV	
POG[160]	I, IIA, IIIA1	"Unfavorable" IIB, IIIA$_2$, IIIB, IV	
COG[157,158]	IA, IIA without‡	IA+IIA with bulk, IB+IIB, IIIA, IVA	IIIB + IVB

CCG, Children's Cancer Study Group; *COG,* Children's Oncology Group; *DAL-HD,* Deutsche Arbeitsgemeinschaft fir Leukamiefarschung-Hodgkin disease; *GPOH-HD,* German Society for Pediatric Oncology and Hematology-Hodgkin disease; *POG,* Pediatric Oncology Group.

*Adverse disease features (one or more of the following): hilar adenopathy, more than four nodal regions, mediastinal tumor >33% of chest diameter, node or nodal aggregate with diameter >10 cm.

†Presence of one or more of the following: bulky disease, peripheral nodal disease 6 cm or larger, and/or mediastinal tumor one third of intrathoracic diameter or more, and B symptoms.

‡No bulk or extranodal extension.

lymphoma.[141,142] In children, the negative sequelae of high-dose extended-field radiation quickly became apparent.[143] Soon thereafter the MOPP combination was created by Devita and coworkers[20] to treat patients with Hodgkin lymphoma. The basic rationale was to combine single drugs with proven efficacy but different mechanisms of action and resistance that had few overlapping toxicities to maximize antitumor effect and limit side effects caused by use of moderate doses of the individual drugs. The second important combination chemotherapy regimen for the treatment of Hodgkin lymphoma was doxorubicin (Adriamycin), bleomycin, vinblastine, and dacarbazine (ABVD), which was developed by Bandanna and coworkers.[144] Variations of these two regimens have formed the cornerstone of almost all subsequent chemotherapy regimens.

Combined-modality therapy using both chemotherapy and radiation has emerged as the standard of care for most children and adolescents diagnosed with Hodgkin lymphoma. The use of six cycles of MOPP chemotherapy combined with involved-field radiotherapy (IFRT) was pioneered by the Stanford investigators in children with pathologically staged disease.[145] This pivotal trial has served as the model for combined-modality treatment programs in children.

Subsequent studies evaluated regimens with modifications incorporated designed to minimize risks of late effects while maintaining efficacy. Changes have included both the substitution of drugs the use of which is associated with lower risks of specific long term toxicities as well as limiting the cumulative dose of drugs that are associated with risks of late sequelae in a dose dependent manner. Some modifications have been gender specific. Table 53-5 gives an overview of chemotherapy combinations. With most of these multiagent combinations, comparable high disease-free survival rates have been achieved.[37,38,130,145,146,147]

The balance of efficacy and late risk of the combined-modality of chemotherapy and radiotherapy adopted by the German-Austrian study group has been investigated over a series of trials (Table 53-6). Modifications investigated have included reduction of the cumulative dose of alkylating agents and replacement of mechlorethamine of MOPP with doxorubicin (vincristine [Oncovin], prednisone, procarbazine, and doxorubicin [OPPA]) and cyclophosphamide (cyclophosphamide, vincristine [Oncovin], procarbazine, and prednisone [COPP]); reduction of radiotherapy (volume, dose, and number of patients who receive radiotherapy); and replacement of procarbazine by etoposide for boys. The seventh of the sequential studies built on findings of the previous six trials.[148] In this study the primary aim was to evaluate modifications of therapy to maintain good outcomes for boys but to do so with less gonadotoxic therapy. In this study procarbazine was replaced with higher dose etoposide in the first two cycles and with dacarbazine in the subsequent two to four cycles. The outcomes for boys and girls on this study were superimposable, supporting the efficacy of this regimen.

The second strategy that is of critical importance in trying to maximize efficacy and minimize toxicity is the stratification of treatment intensity based on the patient's risk for relapse. Cooperative group trials of pediatric and adolescent Hodgkin lymphoma have generally divided patients into low-, intermediate-, and high-risk groups (see Table 53-4). In addition to stratification by stage, studies have also evaluated modifications of therapy based on early disease response. Many reports from these therapeutic studies are confined to a distinct risk group of patients. Comparisons among trials confined to patient subsets need to be done with caution, because the risk group definitions vary both based on the patient characteristics at the time of initial staging and the criteria of disease response.

TABLE 53-5 Combination Chemotherapy Courses for Treatment of Hodgkin Lymphoma

Course	Drugs	Dosage and Route	Days
MOPP[21]	Mechlorethamine	6.0 mg/m^2, IV	1, 8
	Vincristine (Oncovin)	1.4 mg/m^2, IV	1, 8
	Procarbazine	100 mg/m^2, PO	1-15
	Prednisone	40 mg/m^2, PO	1-15
MOPP Derivatives			
COPP[607]	Cyclophosphamide	600 (500) mg/m^2, IV	1, 8
	Vincristine (Oncovin)	1.4 mg/m^2, IV (max 2 mg)	1, 8
	Procarbazine	100 mg/m^2, PO	1-15
	Prednisone	40 mg/m^2, PO	1-15
COMP[608]	Cyclophosphamide	600 mg/m^2, IV	1, 8
	Vincristine (Oncovin)	1.4 mg/m^2, IV (max 2 mg)	1, 8
	Methotrexate	40 mg/m^2, IV	1, 8
	Prednisone	40 mg/m^2, PO	1-15
COP[609]	Cyclophosphamide	600 mg/m^2, IV	1, 8
	Vincristine (Oncovin)	1.4 mg/m^2, IV (max 2 mg)	1, 8
	Procarbazine	100 mg/m^2, PO	1-14
ABVD[144]	Doxorubicin (Adriamycin)	25 mg/m^2, IV	1, 15
	Bleomycin	10 U/m^2, IV	1, 15
	Vinblastine	6 mg/m^2, IV	1, 15
	Dacarbazine	375 mg/m^2, IV	1, 15
MOPP-ABVD Hybrids and Derivatives			
OPPA[148]	Vincristine (Oncovin)	1.5 mg/m^2, IV (max 2 mg)	1, 8, 15
	Procarbazine	100 mg/m^2, PO	1-15
	Prednisone	60 mg/m^2, PO	1-15
	Doxorubicin (Adriamycin)	40 mg/m^2, IV	1, 15
OPA[608]	Vincristine (Oncovin)	1.5 mg/m^2, IV (max 2 mg)	1, 8, 15
	Prednisone	60 mg/m^2, PO	1-15
	Doxorubicin (Adriamycin)	40 mg/m^2, IV	1, 15
OEPA[130]	Vincristine (Oncovin)	1.5 mg/m^2, IV (max 2 mg)	1, 8, 15
	Etoposide	125 mg/m^2, IV	3-6
	Prednisone	60 mg/m^2, PO	1-15
	Doxorubicin (Adriamycin)	40 mg/m^2, IV	1, 15
ChlVPP[610]	Chlorambucil	6 mg/m^2, PO	1-14
	Vinblastine	6 mg/m^2, PO	1, 8
	Procarbazine	100 mg/m^2, PO	1-14
	Prednisone	40 mg/m^2, PO	1-14
VAMP[145,159]	Vinblastine	6 mg/m^2, IV	1, 15
	Doxorubicin (Adriamycin)	25 mg/m^2, IV	1, 15
	Methotrexate	20 mg/m^2, IV	1, 15
	Prednisone	40 mg/m^2, PO	1-14
VBVP[147]	Vinblastine	6 mg/m^2, IV	1, 8
	Bleomycin	10 mg/m^2, IV	1
	Etoposide (VP-16)	100 mg/m^2, IV	1-5
	Prednisolone	40 mg/m^2, PO	1-8
COPP-ABV[161]	Cyclophosphamide	600 mg/m^2, IV	0
	Vincristine (Oncovin)	1.4 mg/m^2, IV	0
	Procarbazine	100 mg/m^2, PO	0-6
	Prednisolone	40 mg/m^2, PO	0-13
	Doxorubicin (Adriamycin)	35 mg/m^2, IV	7
	Bleomycin	10 U/m^2, IV	7
	Vinblastine	6 mg/m^2, IV	7

TABLE 53-5 Combination Chemotherapy Courses for Treatment of Hodgkin Lymphoma (Continued)

Course	Drugs	Dosage and Route	Days
ABC[161]	A		
	Cytarabine	3 g/m^2, IV over 3 hr, q12h	0, 1
	Etoposide	200 mg/m^2, IV over 1 hr, q12h	0, 1
	G-CSF	5 μg/kg, SC	Starting on day 2
	B		
	COPP-ABVD	See COPP/ABVD	21-27
	G-CSF	5 μg/kg, SC	Starting on day 28
	C		
	Vincristine	1.4 mg/m^2, IV	42
	Cyclophosphamide	1200 mg/m^2, IV	42-43
	Doxorubicin	25 mg/m^2/day, continuous infusion	42-44
	Methylprednisolone	250 mg/m^2, IV q6h	42
	Prednisone	60 mg/m^2, PO	43-46
	G-CSF	5 μg/kg, SC	Starting on day 46
BEACOPP[157]	Bleomycin	10 U/m^2, IV	8
	Etoposide	200 mg/m^2, IV	1-3
	Doxorubicin (Adriamycin)	35 mg/m^2, IV	1
	Cyclophosphamide	1200 mg/m^2, IV	1
	Vincristine (Oncovin)	2 mg/m^2 (max 2 mg), IV	8
	Procarbazine	100 mg/m^2, PO	1-7
	Prednisone	40 mg/m^2, PO	1-14
DBVE[611]	Doxorubicin	25 mg/m^2, IV	1, 15
	Bleomycin	10 mg/m^2, SC	1, 15
	Vincristine	1.5 mg/m^2 (max,] 2 mg), IV	1, 15
	Etoposide	100 mg/m^2, IV over 1 hr	1-5
Stanford V[612]	Mechlorethamine	6 mg/m^2, IV	1
	Vinblastine	6 mg/m^2, IV	1, 15
	Doxorubicin	25 mg/m^2, IV	1, 15
	Etoposide	60 mg/m^2, IV	15, 16
	Vincristine	1.4 mg/m^2 (max 2 mg), IV	8, 22
	Bleomycin	5 U/m^2, IV	8, 22
	Prednisone	40 mg/m^2, PO	Every other day
ABVD-PC[158]	Doxorubicin (Adriamycin)	25 mg/m^2 IV	1, 2
	Bleomycin	10 IU/m^2* IV	1, 8
	Vincristine	1.4 mg/m^2 IV (max 2.8)	1, 8
	Etoposide	125 mg/m^2 IV	1-5
	Prednisone	40 mg/m^2, PO	1-7
	Cyclophosphamide	800 mg/m^2 IV	1
	G-CSF	5 μg/kg SC	Starting day 6

G-CSF, Granulocyte colony-stimulating factor; *IV,* intravenous; *PO,* per os; *SC,* subcutaneous
*Modified to 5 IU/m^2 on day 0 mid-study.

Table 53-7 summarizes treatment strategy and results of therapeutic studies in the patient subgroup defined as low risk. Patients at low risk in general are those with stage I and II disease without B symptoms and without bulky disease. These patients have been shown to have excellent outcomes with sequential trials using two to four cycles of multiagent chemotherapy and low-dose involved-field radiation.[38,145,147] These regimens have been designed to minimize exposure to anthracyclines, alkylators, and epipodophyllotoxins in order to minimize risks of cardiac dysfunction, infertility, and secondary acute myelogenous leukemia (AML), respectively.

Table 53-8 summarizes the treatment strategy and results of selected therapeutic studies in patients considered to have intermediate- or high-risk disease. Chemotherapy for these groups generally consists of combinations of ABVD and MOPP or hybrid regimens. Most regimens contain four to six cycles of chemotherapy followed by involved-field radiation with some including high-dose radiation to bulky or residual sites of disease.

Prognostic information for patients with Hodgkin lymphoma is based on information available at the time of the child's initial evaluation. Additionally the importance of the kinetics of disease response to chemotherapy has been shown to be a predictive factor for this disease, as it has been for pediatric leukemias.[149] The premise is that those with more robust responses to initial chemotherapy have more favorable disease and that responsiveness can be included in the factors that are used to decide the intensity of therapy, including whether irradiation is indicated. The definition of a favorable response to chemotherapy, including the timing of assessment of response, the intensity of the chemotherapy regimen used to get the response, and the tools used to measure response have

TABLE 53-6 Development of Treatment Strategy and Outcome for Children and Adolescents with Hodgkin Lymphoma in Seven Sequential Trials of the German-Austrian Multicenter Study Group

		Therapy Group 1			Therapy Group 2			Therapy Group 3		
Study	**No. of Patients (EFS)**	Stages (% of patients) EFS	Chemotherapy	Radiotherapy	Stages (% of patients) EFS	Chemotherapy	Radiotherapy	Stages (% of patients) 5-yr EFS	Chemotherapy	Radiotherapy
DAL-HD78[607]	170 (92%)	I, IIA (43%) 95%	OPPA ×2	EF, 36-40 Gy	>IIA (57%) 89%	OPPA ×2 COPP ×4	EF, 36-40 Gy	Included in TG2		
DAL-HD82[613]	203 (96%)	I, IIA (49%) 99%	OPPA ×2	IF, 35 Gy	IIB, IIIA (26%) 96%	OPPA ×2 COPP ×2	IF, 30 Gy[*]	IIIB, IV (25%) 87%	OPPA ×2 COPP ×4	25 Gy[*]
DAL-HD85[613]	103 (77%)	I, IIA (58%) 59%	OPA ×2	IF, 35 Gy	IIB, IIIA (20%) 62%	OPPA ×2 COMP ×2	IF, 30 Gy[*]	IIIB, IV (22%) 62%	OPA ×2 COMP ×4	25 Gy[*]
DAL-HD87[614]	204 (85%)	I, IIA (51%) 84%	OPA ×2	IF, 30 Gy[*]	IE, IIEA IIB, IIIA (17%) 82%	OPPA ×2 COPP ×2	25 Gy[*]	IIIB, IV (28%) 89%	OPPA ×2 COPP ×4	25 Gy[*]
DAL-HD90[130]	578 (91%)	I, IIA (46%) 94%	OPPA ×2[†] OEPA ×2[‡]	IF, 25 Gy[*]	IE, IIEA IIB, IIIA (21%) 93%	OPPA ×2[†] COPP ×2 OEPA ×2[‡] COPP ×2	25 Gy[*]	IIEB, IIIEA IIIB, IV (31%) 86%	OPPA ×2[†] COPP ×4 OEPA ×2[‡] COPP ×4	20 Gy[*]
GPOH-HD95[38]	1018 (88%)	I, IIA (40%) 94%	OPPA ×2[†] OEPA ×2[‡]	CR: no RT (28%)[§]; non-CR: IF, 20 Gy[*]	IE, IIEA IIB, IIIA (26%), 87%	OPPA ×2[†] COPP ×2 OEPA ×2[‡] COPP ×2	CR: no RT (19%)[§]; non-CR: IF 20 Gy[*]	IIEB, IIIEA; IIIB, IV (33%) 83%	OPPA ×2[†] COPP ×4 OEPA ×2[‡] COPP ×4	CR: no RT (17%)[§]; non-CR: IF, 20 Gy[*]
GPOH-HD02[148]	573 (89%)	1A/B, IIA (34%) 92%	OPPA ×2[†] OE*PA ×2[‡]	CR: no RT (33%)[§]; non-CR: IF, 20 Gy[*]	IE, IIEA, IIB, IIIA (24%) 93%	OPPA ×2[†] COPP ×2 OEPA ×2[‡] COPDAC ×2	20 Gy[*]	IIEB, IIIEA; IIIB, IV (42%) 87%	OPPA, ×2[†] COPP ×4 OEPA ×2[‡] COPDAC ×4	20 Gy[*]

COMP, Cyclophosphamide, vincristine (Oncovin), methotrexate, and prednisone; *COPDAC,* cyclophosphamide, vincristine (Oncovin), prednisone, and dacarbazine; *COPP,* cyclophosphamide, vincristine (Oncovin), procarbazine, and prednisone; *CR,* complete remission; *DAL-HD,* Deutsche Arbeitsgemeinschaft fir Leukamiefarschung-Hodgkin disease; *EF,* extended field; *EFS,* event-free survival; *GPOH-HD,* German Society for Pediatric Oncology and Hematology–Hodgkin disease; *IF,* involved field; *OEPA,* vincristine (Oncovin), etoposide, prednisone, and doxorubicin (Adriamycin); *OE*PA,* vincristine (oncovin) etoposide, prednisone and doxorubicin (adriamycin); *OPA,* vincristine (Oncovin), prednisone, doxorubicin (Adriamycin); *OPPA,* vincristine (Oncovin), prednisone, procarbazine, and doxorubicin (Adriamycin); *RT,* radiotherapy; *TG2,* therapy group 2.

[*] Boost to residual tumor to 30-35 Gy.

[†] Female.

[‡] Male.

[§] Percentage of patients in the treatment subgroup who did not receive radiotherapy.

TABLE 53-7 Combined-Modality Treatment Strategy and Results for Low-Risk Patients with Hodgkin Lymphoma in Selected Multicenter Trials

Group or Institution	No. of Patients	Stage	Chemotherapy[*]	Radiation (Gy), Field	EFS or RFS Overall (%)	Survival (%)
GPOH-HD95[38]	281	I, IIA	Female: OPPA ×2 Male: OEPA ×2	CR[†]: no RT (22%); non-CR: IF, 20 Gy; residual tumor, 30-35 Gy	94	NR
GPOH-HD02[148]	195	IA/B, IIA	Female: OPPA ×2 Male: OE*PA[‡] ×2	CR[†]: no RT (33%); no-CR: IF, 20 Gy; residual tumor, 30-35 Gy	92	99
French Society of Pediatric Oncology[147]	202	I, II I, II	4 VBVP, good responders; 4 VBVP + 1 or 2 OPPA, poor responders	20, IF 20, IF	91 and 78[§]	97.5
Stanford-St. Jude-Boston Consortium[159]	88	1, II[‖]	4 VAMP	CR post 2 cycles, no IF No CR: 25.5 Gy	90 (2 yr)	99
CCG5942[161]	294	I + IIA without[¶]	COPP-ABV × 4	CR,[**] random: IF 21 Gy vs. no RT; PR, 21 Gy IF	95 (3 yr)	100
POG9226[611]	51	I, IIA, IIIA1	4 DBVE	25, IF	91(6 yr)	98

CCG, Children's Cancer Study Group; *COPP-ABV,* cyclophosphamide, vincristine (Oncovin), procarbazine, and prednisone–doxorubicin (Adriamycin), bleomycin, and vinblastine; *CR,* complete remission; *DBVE,* doxorubicin, bleomycin, vincristine, etoposide; *EFS,* event-free survival; *GPOH-HD,* German Society for Pediatric Oncology and Hematology–Hodgkin disease; *IF,* involved field; *NR,* not reported; *OEPA,* vincristine (Oncovin), etoposide, prednisone, and doxorubicin (Adriamycin); *OPPA,* vincristine (Oncovin), prednisone, procarbazine, and doxorubicin (Adriamycin); *POG,* Pediatric Oncology Group; *PR,* partial remission; *RFS,* relapse-free survival; *RT,* radiotherapy; *VAMP,* vinblastine, doxorubicin (Adriamycin), methotrexate, and prednisone; *VBVP,* vinblastine, bleomycin, etoposide (VP-16), prednisolone.

[*] For the composition of combination chemotherapy courses, see Table 53-8.

[†] CR defined as reduction of tumor volume of >95% and residual mass <2 mL.

[‡] OE*PA includes higher dose of etoposide than OEPA.

[§] For good and poor responders respectively.

[‖] Less than 3 nodal sites, no B symptoms, no mediastinal bulk, no extranodal extension.

[¶] Peripheral nodal disease, <6 cm, mediastinal tumor smaller than one third of intrathoracic diameter, absence of extranodal involvement.

[**] CR defined as >70% reduction of initial tumor volume and gallium-negative if initially gallium-positive.

varied widely between study groups and over time. Table 53-9 describes the criteria for disease response evaluations in recent cooperative group trials.

Cross-sectional imaging can define response based on a percentage decrease in size. In Hodgkin lymphoma, a significant proportion of patients have residual masses at the time of disease response assessment and at the end of therapy. Functional imaging methods such as gallium scanning and PET are able to assess metabolically active tissue associated with residual disease (Fig. 53-5). A series of studies primarily performed in adults with Hodgkin lymphoma where therapy was not changed based on interim radiographic results have shown that PET scans performed after two cycles of chemotherapy are highly predictive of outcome.[150-153] The accuracy of PET to predict treatment outcome is higher early in the course of treatment as compared with the end of chemotherapy.[151,154,155] Both the German-Austrian study GPOH-HD2002 and the Children's Oncology Group (COG) studies of intermediate- and high-risk disease addressed the strength of early PET obtained after two cycles of chemotherapy; however the results are still pending.[156]

Several cooperative group studies have focused on modifications of regimens to avoid gender-specific toxicities. The COG evaluated a strategy of stratifying patients with high-risk disease based both on response and gender.[157] All patients received escalated bleomycin, etoposide, doxorubicin (Adriamycin), cyclophosphamide, vincristine, procarbazine, and prednisone (BEACOPP) therapy for 4 cycles. Patients with a rapid early response, defined as a 70% tumor reduction and having a negative gallium scan after four cycles, were then assigned to subsequent therapy based on gender. Boys received two additional cycles of ABVD designed to minimize risks of infertility and involved field radiation. Girls received four cycles of COPP/doxorubicin (Adriamycin), bleomycin, and vinblastine (ABV) and no radiation in order to avoid the risks of secondary breast cancer. Those with disease that met the criteria for slow early response received an additional 4 cycles of BEACOPP. Seventy-seven percent of girls avoided radiotherapy, whereas 68% of boys avoided alkylating agents during consolidation. The EFS and OS rates remained excellent.

The German GPOH-HD2002 study also evaluated sex-based modifications of therapy but did not use patient response for treatment allocation.[148] Boys received two cycles of dose-intensified vincristine, etoposide, prednisone, and doxorubicin (Adriamycin; OEPA) therapy

TABLE 53-8 Combined-Modality Treatment Strategy and Results for Patients with Intermediate- and High-Risk Hodgkin Lymphoma in Selected Multicenter Trials

Group or Institution	No. of Patients	Stage	Chemotherapy	Radiation (Gy), Field	EFS at 5 years	Overall Survival (%)
DAL-HD90 (high risk)[130]	179	IIEB, IIIEA, B, IIIB, IVA, B	2 OEPA-OPPA + 4 COPP	IF, 20-25 Gy residual tumor 30-35 Gy	86%	94%
GPOH-HD95 3-5 ABVD-PC[38]	265	IIEA, IIIEA, B, IIIB, IVA, B	2 OPPA/OEPA + 4 COPP	CR[*]: no RT (22%); non-CR: IF, 20 Gy; residual tumor, 30-35	79% DFS no IFRT 91% DFS with IFRT	NR
GPOH-HD02[148] (intermediate risk)	139	IAE, IB, IIAE, IIB, IIIA	2 OPPA + 2 COPP[†] 2 OE*PA +2 COPAD[‡]	IF, 20 Gy; residual tumor, 30-35	93%	98%
GPOH-HD02[148] (highest risk)	239	IIBE, IIIAE, IIIB, IVA, IVB, IVE	2 OPPA + 4 COPP[†] 2 OE*PA + 4 COPAD[‡]	IF, 20 Gy; residual tumor, 30-35	87% EFS	95% 5 yrs
Stanford-St. Jude-Boston Consortium[609]	159	I-II unfavorable,[§] III, IV	3 VAMP/3 COP	15-25.5 Gy,[‖] IF	76 at 5 yr	93% at 5 yr
POG[160]	179	IIB, IIIA2, IIIB, IV	4 MOPP, 4 ABVD	21 Gy total nodal	79%	92%
POG[138]	216	IB, IIA/IIIA with bulk, IIB, IIIB, IV	3-5 ABVD-PC	21 Gy IF	84% at 5 yr	95% at 5 yr
CCG[37]	394	I/IIB, IIB, III	6 COPP-ABV	CR: randomized to 21 Gy vs. no IFRT, no CR 21 Gy IFRT	87%	95%
CCG[37]	141	IV	COPP-ABV + CHOP + Ara-C	CR: randomized to 21 Gy vs. no IFRT, no CR 21 Gy IFRT	90%	100%
COG[157]	98	IIB with bulk,[¶] IIIB with bulk, IV	BEACOPP × 4 then one of: COPP/ABV × 4[‡] ABVD × 2[†] BEACOPP × 4[#]	Female, RER[**]: no RT Male or SER: 21 Gy Residual tumor, 35 Gy	94% EFS	97% 5 yr

ABV, Doxorubicin (Adriamycin), bleomycin, and vinblastine; *ABVD,* doxorubicin (Adriamycin), bleomycin, vinblastine, and dacarbazine; *Ara-C,* cytarabine; *BEACOPP,* bleomycin, etoposide, doxorubicin (Adriamycin), cyclophosphamide, vincristine, procarbazine, and prednisone; *CCG,* Children's Cancer Study Group; *CHOP,* cyclophosphamide, doxorubicin (hydroxydaunorubicin), vincristine (Oncovin) and prednisone; *COG,* Children's Oncology Group; *COPAD,* cyclophosphamide, vincristine (Oncovin), prednisolone, and doxorubicin; *COP,* cyclophosphamide, vincristine, prednisone; *COPP,* cyclophosphamide, vincristine (Oncovin), procarbazine, and prednisone; *CR,* complete remission; *DAL-HD,* Deutsche Arbeitsgemeinschaft fir Leukamiefarschung-Hodgkin disease; *DFS,* disease-free survival; *EFS,* event-free survival; *GPOH-HD,* German Society for Pediatric Oncology and Hematology–Hodgkin disease; *IF,* involved field; *IFRT,* involved-field radiotherapy; *MOPP,* mechlorethamine, vincristine (Oncovin), procarbazine, and prednisone; *NR,* not reported; *OEPA,* vincristine (Oncovin), etoposide, prednisone, and doxorubicin (Adriamycin); *OE*PA,* vincristine (oncovin) etoposide, prednisone and doxorubicin (adriamycin); *OPPA,* vincristine (Oncovin), prednisone, procarbazine, and doxorubicin (Adriamycin); *PC,* prednisone, cyclophosphamide; *POG,* Pediatric Oncology Group; *RER,* rapid early responder; *RT,* radiotherapy; *SER,* slow early responder; VAMP, vinblastine, doxorubicin (Adriamycin), methotrexate, and prednisone

[*] CR defined as reduction of tumor volume of >95% and residual mass <2 mL.

[†] Males.

[‡] Females.

[§] Defined as presence of one or more of the following: bulky disease, peripheral nodal disease 6 cm or larger, and/or mediastinal tumor one third of intrathoracic diameter or larger; and B symptoms.

[‖] 15 Gy for nonbulky sites and complete response after two cycles of chemotherapy.

[¶] Bulk defined as mediastinal mass greater than intrathoracic diameter at level of T5 or extrathoracic nodal mass greater than 10cm.

[#] Slow early responders.

[**] SER, partial response not meeting criteria for RER; RER, >70%reduction in tumor volume and gallium negative post 4 cycles.

TABLE 53-9 Definitions of Favorable Response to Therapy in Selected Multicenter Trials in Children and Adolescents with Hodgkin Lymphoma

Study	Definition of Favorable Response	Timing of Assessment	Treatment Modification for Those with Favorable Response
CCG5942[37]	>70% reduction in initial tumor volume and gallium negative	At completion of chemotherapy	Randomized to IFRT or no IFRT
GPOH-HD02[148]	≥95% reduction in tumor volume	After 2, 4, or 6 cycles of chemotherapy depending on disease group	No IFRT for patients with low-stage disease only (TG1)
Stanford-St. Jude-Boston Consortium[609]	≥75% reduction in perpendicular diameters of measurable lesions, or return no normal size and gallium or PET negative	After 2 cycles of VAMP	No IFRT
COG[158]	Disappearance of all clinical and disease-related symptoms and PET negative	After 2 cycles of ABVD-PC	All get IFRT; Less than good response: chemo intensified with additional 2 cycles ifos/vinolebine

ABVD-PC, Doxorubicin (Adriamycin), bleomycin, vinblastine, and dacarbazine–prednisone, cyclophosphamde; *CCG,* Children's Cancer Study Group; *COG,* Children's Oncology Group; *GPOH-HD,* German Society for Pediatric Oncology and Hematology–Hodgkin disease; *IFRT,* involved-field radiotherapy; *PET,* positron emission tomography; *TG1,* therapy group 1; *VAMP,* vinblastine, doxorubicin (Adriamycin), methotrexate, and prednisone.

followed by two (for those with intermediate-risk disease) or four (for those with high-risk disease) cycles of cyclophosphamide, vincristine (Oncovin), prednisone, and dacarbazine (COPDAC) designed to attempt to limit gonadal toxicity. Girls received the same number of cycles but of OPPA followed by COPP. EFS and OS rates did not differ between the two sexes with the regimens employed.

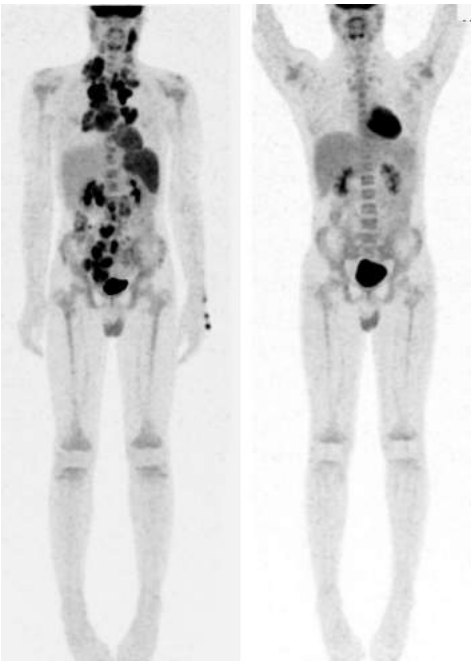

Figure 53-5 PET scan at diagnosis and after two cycles of ABVD-PC chemotherapy in an adolescent with advanced-stage Hodgkin lymphoma.

The Pediatric Oncology Group (POG) evaluated response-based modifications of therapy without modifications for sex in patients with intermediate- and high-risk disease.[158] In this study patients were evaluated for response after three cycles of therapy. Those with a rapid early response defined as a greater-than-70% reduction in disease and gallium negative, received 21 Gy IFRT. Those who were slow responders received an additional two cycles followed by radiation. With this strategy the 5-year EFS rate was the same in both groups, and the OS rate was 95%.

Whether radiotherapy can be omitted from treatment for at least some children and adolescents with Hodgkin lymphoma is one of the important questions in recent and ongoing clinical trials. Three cooperative group trials in the low-risk group each evaluated the removal of IFRT in a subset of patients with largely successful results. In the Stanford-St.Jude-Boston protocol, patients with a complete response, defined as greater than 75% reduction of the sum of the perpendicular of all lesions after two cycles of vinblastine, doxorubicin (Adriamycin), methotrexate (methotrexate), and prednisone (VAMP) chemotherapy were nonrandomly assigned to no radiation. All patients received four cycles of VAMP chemotherapy. Fifty three percent of patients met criteria for complete response. The 2-year EFS rates of this group were no different from those who did receive radiation, at 89% and 92% respectively.[159] The GPOH-HD 2002 study also nonrandomly assigned patients at low risk with a complete response after two cycles, defined as more than 95% volume reduction, to no radiation. Thirty three percent of patients met this criterion, did not receive radiation, and had an outcome identical to those who did receive IFRT, with EFS rates at 5 years of 93% and 91% respectively.[148] The Children's Cancer Group (CCG) study 5942 randomly assigned patients who obtained a complete remission (CR), defined as 70% mass reduction

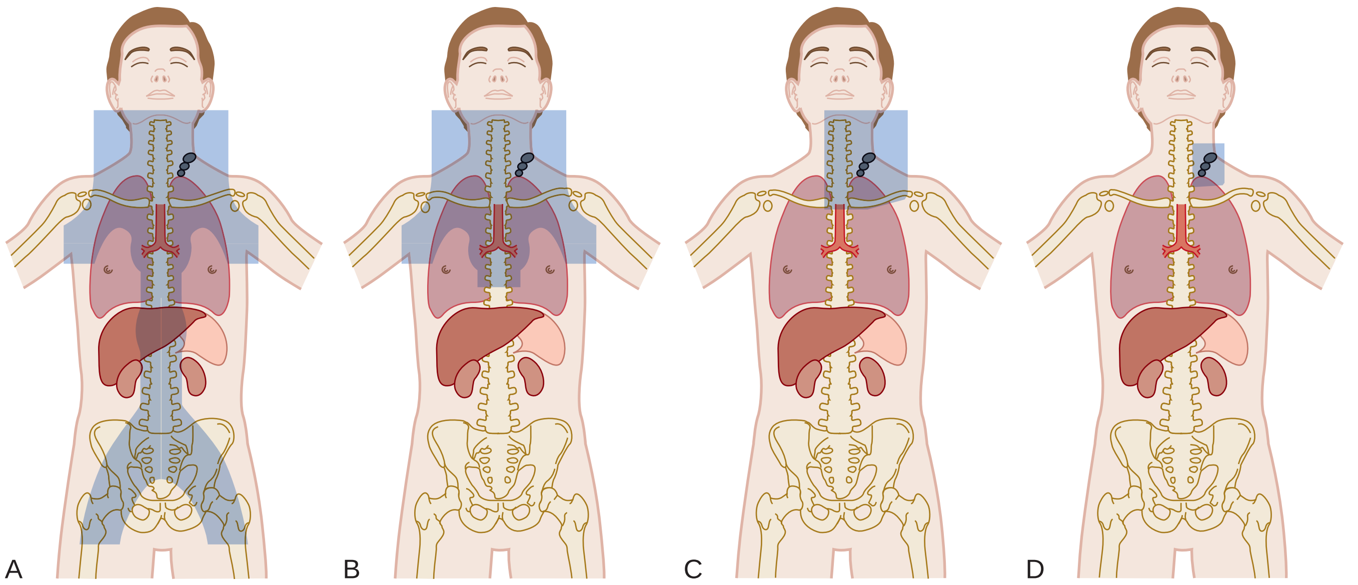

Figure 53-6 Radiation fields for treating Hodgkin lymphoma. **A,** Total nodal irradiation. **B,** Mantle field. **C,** Involved field. **D,** Radiation field individualized to the patient's local disease, taking into account the tolerance of involved or adjacent extranodal organs. *(Courtesy of Dr. W. Dörffel, Berlin, Germany.)*

of tumor volume and negative gallium scan, to radiation or no radiation after four cycles of COPP/ABV therapy. The EFS rates for randomized patients at 3 years were significantly different at 97% versus 91%; however, the OS rate at 3 years in both groups was 100%.[37]

Omission of radiation has also been investigated in subgroups of patients with intermediate- and high-risk disease with mixed results. In the POG trial 8725, patients with stages IIB, IIIA2, IIIB, and IV were randomized to receive eight alternating cycles of MOPP/ABVD chemotherapy followed by total nodal irradiation (21 Gy) or no radiotherapy in cases of CR.[160] There was no difference in EFS or OS between patient groups who did or did not receive radiation. In the GPOH-HD95 trial patients who achieved a complete response received no further therapy, whereas those who did not received 20 to 35 Gy of IFRT.[38] In the intermediate- and high-risk groups, omission of radiation was associated with a significantly decreased EFS rate of 77% for those treated with chemotherapy only versus 92% for those who received combined modality therapy.

The CCG5942 trial included subjects with all disease stages. Based on their risk-group assignment, patients received either four or six cycles of COPP/ABV or six cycles of the mutiagent ABC regimen chemotherapy.[37] Patients with at least a 70% tumor-volume reduction and negative gallium scan at the completion of chemotherapy were randomized to receive or not receive IFRT with 21 Gy. This randomized trial was stopped because of a significantly higher number of relapses in the no-IFRT arm. Most of the relapses were at sites of initial disease. Patients who did not receive IFRT had an EFS rate at 3 years from the time of randomization of 87% compared with 92% ($P = .057$) for patients treated with IFRT in the intent-to-treat analysis. Importantly, longer term follow-up study of this cohort has shown that the OS in the two arms is no different. At 10 years postrandomization, the EFS of those treated or not treated with IFRT were 91.2% and 82.9% ($P = .004$), respectively, whereas the OS of the two groups were 97.2% and 95.5% ($P = .5$), respectively.[161]

Radiation remains a critical component of therapy for the majority of children and adolescents with Hodgkin lymphoma. Radiotherapy planning involves decision making about both dosage and field, with the goals of maximizing efficacy but limiting late effects. Radiation fields have evolved over time (Fig. 53-6). IFRT, used in the majority of contemporary protocols, generally involves radiation of the entire lymph-node region that includes the pathologically enlarged node.[23] One major implication of the change from mantle-field radiation to IFRT for cervical disease is the exclusion of the axilla, which significantly reduces the exposure of lung and breast tissue. Investigations of further limitations to radiation fields are ongoing. The dosages in most contemporary studies used in combination with chemotherapy are generally no greater than 21 Gy, with some studies using higher dosages to sites of bulky or residual disease.

Disease Surveillance after Completion of Therapy

Driven both by required studies as part of clinical trials and as part of standard of care in some pediatric oncology settings, children and adolescents routinely undergo surveillance imaging with CT scans at regular intervals after completion of therapy. Given increasing awareness of the potential risks secondary to imaging-related radiation exposure, recent work has examined the utility of CT imaging as compared with clinical findings in the detection of disease recurrence.[162] A retrospective review of patients treated in a POG study showed that the majority of relapses occurred within the first year and were detected by clinical findings. For the patients with late relapse, detection by either by clinical symptoms or surveillance imaging did not change the OS rate.[163] These authors suggest limiting surveillance-CT imaging to the first 12 months after completion of therapy. FDG-PET scanning, which has utility in staging newly diagnosed patients and in the assessment of disease response (see above) has no established role in off-therapy surveillance.[164] More widespread use of MRI as a replacement for CT may also allow for reduction of radiation exposure in lymphoma patients in the future.[165]

Nodular Lymphocyte-Predominant Hodgkin Lymphoma

Pathologic Features

NLPHL is a monoclonal B-cell neoplasm.[35,166] The histology is characterized by effacement of the lymph node architecture by a nodular or nodular and diffuse infiltrate of small lymphocytes, with an associated follicular dendritic network and scattered or clustered large cells referred to as *lymphocytic and histiocytic (L&H) cells*.[26,35] NLPHL corresponds to Hodgkin paragranuloma of the classification of Jackson and Parker from 1947[167] and to lymphocyte-predominant Hodgkin lymphoma of the Rye modification of Lukes and Butler.[24] Differentiation of NLPHL from the lymphocyte-rich subtype of classic Hodgkin lymphoma can be difficult in some cases. However, the phenotype of L&H cells (also termed *popcorn cells* because of their often multilobed nuclei) is different than classic Reed-Sternberg cells of the CHL subtypes in that they express CD45 and usually retain most normal markers of B-cell differentiation, such as CD20, CD79a, and the B-cell specific transcription factors BOB.1 and Oct-2, but do not express CD30 and CD15 (see Table 53-1).[26,35] Also, markers of EBV infection are consistently absent from L&H cells of NLPLH.[166] In some patients with NLPHL, progressively transformed germinal centers are observed in association with NPLHL. However, it remains uncertain whether these lesions are truly preneoplastic, because most patients with such lesions in reactive lymph-node hyperplasia do not develop a lymphoma.[168]

Clinical Features

NLPHL comprises between 5% and 8% of Hodgkin lymphoma in children and adolescents and accounts for an even higher percentage of prepubertal patients.[169] Unlike in patients with CHL, there is a significant male preponderance in those with NLPHL. The disease course is more indolent, and a higher proportion of patients have low-stage disease, most often presenting as localized peripheral adenopathy, most commonly in the neck and less commonly in the inguinal area. Mediastinal involvement is relatively rare.[170] Unlike CHL, where contiguous nodal spread is the norm, in NLPHD noncontiguous spread is observed in those with high-stage disease. The pattern of relapse of disease also differs from CHL, with relapses occurring late and often repeatedly.[171,172] An additional unique feature of NLPHL is the increased risk of transformation to NHL occurring in 5% to 12% of cases, whereas this is very rarely observed in CHD.[173]

Diagnostic evaluation should include excisional node biopsy. Fine-needle aspirate is often inadequate and can lead to the misdiagnosis of reactive nodal tissue.[174] Staging of patients with NLPHL uses the Ann Arbor system as used for CHL, and staging evaluations to be considered are the same as those for a patient with CHL except that bone marrow biopsies are rarely indicated. The only consistent prognostic factor for patients with NLPHL is stage of disease, although some data suggest that a ratio of lymphocyte count to monocyte count at the time of diagnosis may also have prognostic value.[175,176]

Treatment and Outcomes

Historically patients with NLPHL have been included in most therapeutic studies of childhood and adolescent Hodgkin lymphoma. Uncertainty remains about optimal therapy for these patients. Given the favorable prognosis and the burden of late effects from therapy, the evaluation of less intensive regimens has been undertaken.[177]

The majority of patients with NLPHL have low-stage disease, which is sometimes amenable to complete resection. One of the unique features of NLPHD is that a portion of children with low-stage disease treated with resection alone are cured of their disease. Studies of patients treated in this fashion have reported relapse-free survival rates of 67% to 100%.[178-181] All of the recurrences in patients with relapse were low stage, and the OS has been reported as 100%.[180] For pediatric patients with NLPHD, a watchful waiting approach for those who are in CR after initial surgery is appropriate. However, a careful postoperative staging and meticulous follow-up observation is essential. Other strategies for patients with low-stage disease are the use of radiotherapy alone[175] or low-intensity alkylator-based chemotherapy regimens.[182,183]

Data to evaluate the best therapy for patients with advanced stage NLPHL are relatively scarce. Outcome of these patients is similar to those with CHL, and the usual recommendation is that they receive therapy similar to those with advanced-stage CHL. Investigation of the possible role for therapy with rituximab as a single agent or as part of combination therapy for this disease are ongoing.

Treatment of Relapsed Hodgkin Lymphoma

Although the success of primary therapy for children and adolescents is high, approximately 10% of patients develop relapsed disease.[184] The outcome for those with relapsed disease is variable. The two strongest predictive factors are time to relapse and response to reinduction chemotherapy.[185-187] Time to relapse can be subdivided into three prognostic groups: 1) patients with primary refractory disease or recurrent disease within the first 3 months have the worst outcomes; 2) those with disease relapse within 12 months of diagnosis form an intermediate group; and 3) those with relapses occurring longer than 12 months from diagnosis have the most favorable outcome.[188] Patients who have no response to reinduction therapy have a dismal prognosis, as do those with active disease immediately before autologous stem cell transplantation (SCT).[189,190] Additional prognostic factors include the intensity of primary therapy, stage, and B symptoms at relapse.

There is no consensus on optimal salvage therapy for children with relapsed disease. The options include chemotherapy with or without radiation, autologous SCT, allogeneic SCT, or novel therapies. Data to support treatment choices can be difficult to interpret because there is a lack of randomized trials evaluating these therapeutic options in children; the majority of studies in patients with relapse have been single-arm trials with varying inclusion criteria.

Patients with late relapse who have responsive disease to reinduction therapy have a favorable prognosis with treatment with chemotherapy and radiation.[188] Chemotherapy regimens have been evaluated in this setting including ifosfamide, carboplatin, and etoposide (ICE)[188]; carmustine (BCNU), etoposide, cytarabine (ara-C), and melphalan (miniBEAM)[191]; cytarabine, cisplatinum, and etoposide (APE)[192]; as well as newer regimens including gemcitabine and vinorelbine (GV)[193] among others. Information that should be considered in choosing a salvage regimen should include knowledge of the frontline therapy used, in part to avoid toxicities with cumulative drug exposure.

For patients with high-risk relapsed disease, intensifying therapy with autologous SCT should be considered, although controversy about which patients are likely to benefit from this therapy remains. In a prospective randomized trial of the German Hodgkin Lymphoma Study Group, adults with chemotherapy-sensitive disease had a significantly higher failure-free survival probability when treated with two cycles of dexamethasone, carmustine (BCNU), etoposide, cytarabine (ara-C), and melphalan (Dexa-BEAM) followed by autologous SCT compared with patients receiving four cycles of Dexa-BEAM without SCT.[194] For childhood Hodgkin lymphoma there are very few prospective or randomized trials. In a retrospective study of 51 pediatric patients with recurrent Hodgkin lymphoma treated with different salvage regimens, there was a beneficial impact of autologous SCT on survival only for patients refractory to first-line therapy, but sensitive to second-line therapy disease.[195] The outcome of children with disease that is unresponsive to chemotherapy who undergo autologous SCT is dismal.[184]

The European trial for children with relapsed Hodgkin lymphoma, EuroNet-PHL-C1, divides patients into low-, intermediate- and high-risk groups based on time to relapse and early response assessment. Patients with low-risk disease are treated with chemotherapy and radiation. Those with high-risk disease have their therapy intensified with autologous SCT. Those with intermediate-risk disease have therapy allocated based on response, with those with a good response receiving chemotherapy and radiotherapy only.

The use of allogeneic transplant in relapsed Hodgkin lymphoma, with the hopes of making use of graft-versus-lymphoma effects has been investigated including myeloablative SCT, reduced intensity SCT, and reduced intensity SCT after autologous SCT.[196,197] In this very–high-risk group, both relapsed disease and transplant-related mortality limit the adoption of this therapy except for those patients with adequate performance status and limited curative options.[184]

Novel therapies for relapsed disease are needed. One agent that shows significant promise is the antibody-drug conjugate brentuximab vedotin (also known as *SGN-35*), which combines an anti-CD30 monoclonal antibody with the antimicrotubule agent, monomethyl auristatin E. In a phase 2 study of patients with very–high-risk disease (71% had primary refractory disease, and 100% of patients had previously been treated with autologous SCT), the overall response rate was 75%, with 34% of patients achieving a CR and with a high percentage of these patients remaining without evidence of relapsed disease at 1.5 years. The median age of the patients in this trial was 31 years, with the youngest being 15 years old. Toxicity of the agent was relatively mild, including neuropathy and cytopenias.[198] The safety and effectiveness of adding this agent to standard chemotherapy as well as including it in front-line therapy is being investigated by several cooperative groups.

Late Effects of Therapy

Given the high rate of cure in children and adolescents with Hodgkin lymphoma, the risks of long-term effects of treatment are very important. In the scientific literature, the number of reports on late sequelae after successful treatment of pediatric Hodgkin lymphoma patients has exceeded that of reports on prospective therapeutic trials. Many of these reports document late sequelae associated with earlier treatment approaches. The treatment for pediatric Hodgkin lymphoma has evolved because of a continuous process of attempting to maximize efficacy while limiting the long-term risks of therapy. Therefore many of the reports of sequelae of therapy administered several decades ago may not accurately reflect the risks of late effects associated with current treatment strategies.

Some late effects of Hodgkin lymphoma therapy can be attributed to a distinct treatment modality or a single drug; others may have a multifactorial pathogenesis. Table 53-10 lists late effects that have impact on morbidity and mortality, as well as those that affect the quality of life for survivors.

In two large, single-center series of patients with pediatric Hodgkin lymphoma treated over several decades, the long-term cumulative risk of death from treatment-related sequelae such as infections, cardiac disease, and second cancers approached that of the risk of death from relapsed Hodgkin lymphoma.[199,200] Data from 2742 patients treated between 1970 and 1986 who were under the age of 21 at the time of diagnosis, who were alive longer than 5 years posttherapy, and who were in the Childhood Cancer Survivor Study (CCSS) has delineated the risks and causes of excess mortality.[201] With a median follow-up length of greater than 20 years, of the 500 observed deaths in the cohort, 35% were from Hodgkin lymphoma, 23% from second malignant neoplasms (SMNs), and 14% from cardiovascular disease. The 30-year OS rate from diagnosis was 74%.

SMNs are devastating sequelae of therapy for Hodgkin lymphoma. In the U.S. Childhood Cancer Survivor

TABLE 53-10 Late Effects of Treatment of Children and Adolescents with Hodgkin Lymphoma

Late Effect	Radiotherapy	Chemotherapy
Musculoskeletal growth impairment[201]	X	
Cardiomyopathy[216]		Anthracyclines
Coronary artery disease, valvular disease, and pericardial disorders[214,218]	X	
Stroke[201]	X	
Infections (postsplenectomy or splenic radiation)[219]	X	
Thyroid dysfunction[222]	X	
Female gonadal dysfunction[227-229]	X	
Male infertility[223-226]	Inguinal, pelvic irradiation	Alkylating agents; procarbazine
Lung dysfunction[220]	X	Bleomycin; BCNU; CCNU; busulfan
Second cancer (hematologic)[202-210]		Alkylating agents; topoisomerase II inhibitors
Solid tumors[202-213]	X	

BCNU, Carmustine; *CCNU,* lomustine.

Cohort consisting of more than 13,000 survivors of childhood cancers, patients treated for Hodgkin lymphoma were at the highest risk to develop an SMN.[202] Patients treated with full-dose radiation are at significant risk for SMNs with a cumulative incidence of greater than 25% after 30 years of follow-up study.[203,204] Fifteen years postdiagnosis of Hodgkin lymphoma, the second leading cause of mortality after death from the primary disease is death from an SMN.[205] Breast cancer makes up the largest component of the excess solid tumors in female patients.[203]

Contemporary therapy no longer uses high-dose extended-field radiation. The impact of lowering radiation dosage and the use of combination chemotherapy on risks of SMN continues to be investigated. In a cohort study including both children and adults treated for Hodgkin lymphoma, 459 second malignancies were described in 5798 patients.[206] These patients were treated between 1963 and 2001. All received chemotherapy, and 3432 also received radiation. Of those who received chemotherapy only, there remained significantly increased risks of second malignancies including leukemia, NHL, and lung cancer with the peak period of risk between 5 and 9 years posttherapy. For those who received combined modality therapy including radiation, the absolute risk of an SMN was significantly higher and included a wider arrays of malignancies; the period of risk extended to 25 years and longer. In a small cohort of 112 pediatric patients treated at a single institution between 1970 and 1990 with combined modality therapy including 15 to 25.5 Gy IFRT, the rate of SMN including sarcomas and breast and thyroid carcinomas was similar to that reported in studies of patients who had received higher doses of radiation.[207] Other studies have suggested that low-dose radiation and limiting cumulative alkylator exposure may have a protective effect in adult patients but not in children.[208] Still others have shown that patients who received less than 23 Gy of mediastinal radiation had a lower incidence of breast cancer, which may in part be related to the transition from extended-field radiation to IFRT.[209] The cumulative incidence of second leukemias ranges from 0.6% to 2.1% and reaches a plateau after 10 to 14 years.[131,200,204,210,211] The risk of second leukemias is associated with the cumulative dose of alkylating agents and is almost absent in patients treated with radiotherapy alone.[200,204] The risk of developing thyroid cancer is clearly related to radiotherapy and is highest in young children.[203,204,212] Almost all thyroid tumors arise within the radiation field.[204] A recent study analyzing the genetic factors that may contribute to radiation-induced tumors in survivors of Hodgkin lymphoma has identified variants at chromosome band 6q21, implicating the *PRDM1* gene in the etiology of secondary malignancies.[213]

Cardiovascular disease contributes significantly to the morbidity and mortality of patients who are survivors of Hodgkin lymphoma.[201] Damage to the heart after full dose radiotherapy includes coronary artery disease, valvular pathology, and pericardial disease.[214] Cardiomyopathy with ventricular dysfunction can result from anthracycline therapy.[215] Risks of late anthracycline cardiotoxicity are higher in young children, in girls, and with higher cumulative doses.[216,217] In long-term follow-up observation of a cohort of patients from a single-center combination of exposure to mediastinal radiation and anthracycline was associated with the highest risk of cardiac morbidity.[218] Data regarding the long-term cardiac risks associated with contemporary therapy using low-dose IFRT and chemotherapy regimens that generally limit cumulative anthracyclines exposure to less than 250 mg/m^2 are needed.

Death from overwhelming infections related to splenectomy was a major risk that has been significantly reduced since splenectomy was abandoned from staging procedures.[131,199] Irradiation of the spleen may also induce functional impairment of the spleen, at least if dosages of 20 Gy or more are used.[219]

Chronic lung disease can result from irradiation of the lung and exposure to several chemotherapy agents used in Hodgkin lymphoma regimens, including bleomycin and the nitrosourea derivatives BCNU and lomustine (CCNU). The cumulative dose of these drugs is the most crucial determinant for chronic lung injury. Very young age may be an additional risk factor for possible fatal lung toxicity from BCNU and CCNU.[220] The combination of radiotherapy and drugs with lung toxicity contributes to increased risk of long-term lung impairments. This is of special concern in patients requiring salvage treatment for recurrent Hodgkin lymphoma that includes high-dose chemotherapy.

Survivors of pediatric Hodgkin lymphoma are at increased risk for thyroid malfunction—mainly hypothyroidism but also hyperthyroidism, benign and malignant thyroid nodules, and Graves disease.[221] The risk increases with the dose of neck irradiation, younger age at treatment, and female gender.[222]

Musculoskeletal growth impairment was one of the first sequelae recognized to be a consequence of high-dose radiotherapy for children with Hodgkin lymphoma.[143] Prepubertal children treated with 40 Gy of mantle irradiation would have predictable sequelae of lack of growth of the bone and soft tissues of the neck and clavicles, leading to orthopedic complications as well as significant cosmetic effects. This complication is ameliorated by low-dose IFRT used in most contemporary regimens.

The primary risk factor for gonadal toxicity is exposure to alkylating agents, primarily cyclophosphamide and procarbazine.[223,224] Historically mechlorethamine was the primary cause of chemotherapy-related sterility in males. Dose-dependent testicular damage from germ-cell depletion and Leydig-cell dysfunction has also been documented in prepubertal and postpubertal boys after MOPP; chlorambucil, vinblastine, procarbazine and prednisone (ChlVPP); and OPPA-COPP chemotherapy.[225,226] The ABVD regimen appears to be less toxic to germ cells.[227] Results from the German-Austrian studies on pediatric Hodgkin lymphoma have shown clearly that with moderate doses of cyclophosphamide, male infertility correlates with the cumulative dose of procarbazine. Historically in girls, the most substantial risk of infertility was from pelvic lymph node irradiation and was ameliorated to some extent by ovarian transposition.[143,228] The risks of early menopause for females receiving contemporary therapy is likely fairly low, and small studies have shown no measurable impact of ABVD therapy on fertility in women.[229]

Follow-up care of survivors of Hodgkin lymphoma should follow a structured plan such as that described in the *COG Long-Term Follow-up Guidelines*.[230,231] Availability of a detailed and accurate treatment summary is imperative to determining appropriate care and surveillance investigations in follow-up observation.

NON-HODGKIN LYMPHOMA

NHLs are a diverse collection of malignant neoplasms of lymphoid cell origin, including all the malignant lymphomas that are not classified as Hodgkin lymphoma. They account for 7% of all cancers in those younger than 20 years old.[232] NHLs are heterogeneous in their pathology, clinical features, and responsiveness to therapy. With improved understanding of biology and data from sequential disease-based clinical trials as well as better supportive care, the outcome of children with NHL has improved dramatically over the last several decades. With conventional therapy, depending on subtype and stage, the majority of children with NHL can be cured of their disease.

The classification of NHL has been and continues to be a topic of scientific discussion and a source of confusion for clinicians. The Rappaport system used growth patterns (diffuse vs. nodular) and cytology (undifferentiated vs. differentiated) as the basic criteria for disease definitions.[233] The Lukes Collins classification system was based primarily on splitting the lymphocytic system into T and B lymphocytes.[234] The Kiel classification system attempted to translate information about how the lymphatic system is organized into a classification system for NHL, in which the lymphoma cells were related morphologically and immunophenotypically to the normal cell categories of the immune system.[235,236] The National Cancer Institute Working Formulation for Clinical Usage, established in 1982, did not intend to constitute another classification of NHL; rather it was to be a common language to translate between existing classifications.[237] It took into consideration the clinical course of patients and resulted in a grading of NHL into low-grade, intermediate-grade, and high-grade NHL. This system was widely used by clinicians in North America. In Europe and Asia, the Kiel classification was more broadly used. The use of different classification systems hampered or even made it impossible to compare results of clinical trials. In the 1990s, the International Lymphoma Study Group undertook a major effort to overcome this confusion and to create a uniform classification of NHL based on biologic principles. The result was the Revised European-American Classification of Lymphoid Neoplasms (REAL) classification, which then lead to the WHO Classification of Tumors of the Hematopoietic and Lymphoid Tissues produced in 2002 and revised in 2008.[25] The basic principles of the WHO classification system include subdivision of neoplasms according to the lineage (B, T, or natural killer [NK] cell) and the definition of distinct subtypes within each lineage according to a combination of morphology, immunophenotype, genetic features, and clinical features.

The revisions of the WHO classification system in 2008 included diseases that overlap between entities, such as B-cell lymphomas with features intermediate between DLBCL and BL and B-cell lymphomas with features intermediate between DLBCL and Hodgkin lymphoma. In addition, age-specific factors defined several new disorders including pediatric type follicular lymphoma (FL) and systemic EBV-positive T-cell lymphoproliferative disease of childhood.[238]

Combination chemotherapy is the cornerstone of successful treatment of children and adolescents with NHL. A key moment in the development of current treatment concepts was the recognition that different NHL subtypes require different chemotherapeutic strategies. In children and adolescents with NHL, three main disease groups account for the vast majority of the diagnoses. The three are LL, mature B-cell neoplasms (including BL and DLBCL), and anaplastic large-cell lymphoma (ALCL; Fig. 53-7). In the era of solid organ and SCT, posttransplant lymphoproliferative diseases are becoming more common.

Epidemiology and Causative Factors

There is significant variation in incidence rates according to geographic areas, gender, age, and ethnicity in

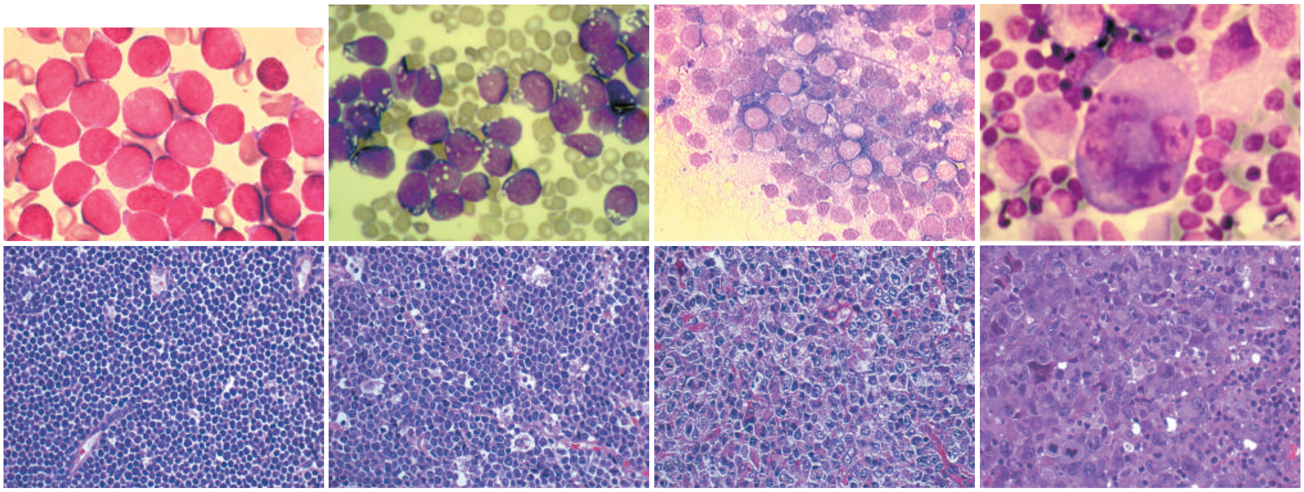

Figure 53-7 Morphology, immunophenotype, and specific cytogenetics of main non-Hodgkin lymphoma subtypes of childhood and adolescence.

childhood NHL. The comparison of incidence rates of NHL in childhood and adolescence in different geographic areas is limited because of a paucity of true population-based registries, different degrees of completeness of data, and the use of various disease classification systems over time. The reported incidence rates range from 6.6 per million population in the United Kingdom to 12.5 per million in Japan. Specific subtypes of NHL, however, occur at a much higher rate in particular regions. Endemic BL accounts for 74% of all childhood malignancies in equatorial Africa, whereas all NHLs combined account for 7% of pediatric cancer in North America.[239-241]

In all registries the incidence rates of NHL are higher in males than in females, usually by a factor of 2.[242-246] The male-to-female ratio varies considerably among different NHL subtypes. Whether male predominance in most NHL subtypes reflects hormonal differences rather than loss of an X-chromosome–linked tumor suppressor gene remains to be determined. The incidence of NHL and of the various subtypes varies widely by age, with substantial differences between children and adults. Among patients younger than 18 years of age there is also considerable variability (Fig. 53-8).[247] NHL as a whole in children younger than 3 years of age is extremely rare. BL or leukemia is the predominant subtype in younger children. The incidence of DLBCL and ALCL increases in those older than 15 years of age.

In the U.S. Surveillance, Epidemiology, and End Results (SEER) program, the incidence rate in Caucasian children is higher as compared with African American children.[242,248] In adults, an increased risk for NHL has been described in first-degree relatives of patients with hematopoietic malignancies.[101,249] Except for classic known hereditary immunodeficiency syndromes predisposing for NHL, only a few specific genes have been associated with risks of childhood NHL. Biallelic mutations in mismatch repair genes have been associated in a series of families

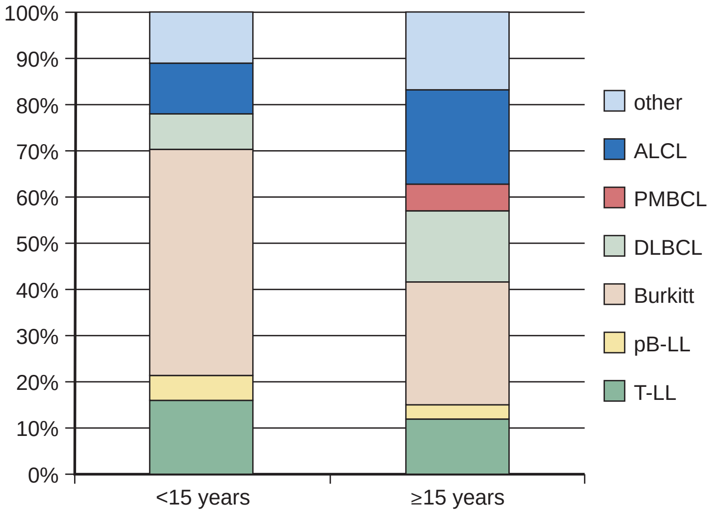

Figure 53-8 Distribution of non-Hodgkin lymphoma subtypes by age. *(Modified from Burkhardt B, Oschlies I, Klapper W et al: Non-Hodgkin's lymphoma in adolescents: experiences in experiences in 378 adolescent NHL patients treated according to pediatric NHL-BFM protocols.* Leukemia *25:153–60, 2011.)*

with several types of childhood malignancies including T-cell lymphomas.[250]

Conclusive data to support the association of environmental factors and risk of childhood NHL are extremely limited. Conversely, data to support the role of infection in a subset of NHLs has been long established. Denis Burkitt not only recognized and described BL as a new disease entity but also established the epidemiologic basics for the current view of its association with EBV, malaria, and possibly arbovirus infections and described the lymphoma belt of Africa."[240,251] In 1964, Epstein and associates[252] described viral particles—later referred to as EBV—in BL samples from African patients. Subsequently the EBV genome was found to be incorporated into 96% of endemic BL cases in the African lymphoma belt, whereas in France and Germany, only 10% to 15% of cases are EBV-positive.[253] It was postulated that EBV infection in addition to exposure to malaria and possibly arboviral infections early in life may be crucial for the pathogenesis of endemic EBV-positive BL.[240,241,254-256] In addition to endemic BL, lymphoproliferative disease, and lymphomas in children with acquired or congenital immunodeficiency are often EBV positive.

Inherited and acquired immunodeficiency is a strong risk factor for developing NHL during childhood.[257] Table 53-11 presents the most common inherited immunodeficiencies associated with lymphoproliferative disorders and lymphoma.[258] For patients with these inherited (also called *primary*) immunodeficiencies, the chance of dying of lymphoma is 10 to 200 times the expected rate for age-matched unaffected children. Many of the lymphomas in this patient group are EBV-positive. There is a male preponderance, partly explained by the fact that some of the inherited immunodeficiencies are X-linked disorders.[259] Children infected with human immunodeficiency virus (HIV) have more than 150 times the risk of developing NHL in comparison with general population rates.[260,261] NHLs are the most common neoplasms occurring in children with HIV, and most are of B-cell lineage.[262,263] For some children NHL may be the first defining condition for acquired immunodeficiency syndrome (AIDS).[262,263] The introduction of highly active antiretroviral therapy has been associated with a significant decrease the incidence of some HIV-related malignancies, such as Kaposi sarcoma, but the effect on the incidence of NHL appears to be more modest.[264]

Initial Evaluation and Staging

As is the case for patients with HL, the most common symptom of childhood NHL is painless lymphadenopathy. In contrast to Hodgkin lymphoma, the nodes may enlarge more rapidly because of a much higher growth fraction of NHL. The variety of presenting features of childhood NHL is even larger than that for Hodgkin lymphoma, ranging from a single painless enlarged lymph node, an asymptomatic asymmetrical enlarged tonsil, or a harmless-looking skin papule to a life-threatening condition. Almost any organ, tissue, and anatomic area can be involved. The most common disease sites are the cervical lymph nodes, abdomen (including the liver and spleen), mediastinum, and head and neck region. Lesions in the head and neck area may involve the tonsils, Waldeyer ring, paranasal sinuses, and any bone structure

TABLE 53-11 Inherited Immunodeficiency Syndromes Associated with Lymphoma in Children

Syndrome	Genetics/Pathogenesis	Immune Defect	Malignancy
Ataxia telangiectasia	*ATM*; impaired DNA repair	Progressive decline in T cells, abnormal immunoglobulins	T-cell ALL and T-cell LL, CHL, BL, DLBCL
Wiskott-Aldrich syndrome (WAS)	*WASP*; abnormal cytoskeletal architecture of hematopoietic cells	Defects in T cells, B cells, neutrophils, macrophages	EBV-associated DLBCL, Hodgkin, lymphomatoid granulomatosis
Severe combined immunodeficiency (SCID)	X-linked: gamma chain mutations with defective IL signaling; *ADA*: defective adenosine deaminase	Dependent of subtype but in general low or absent T and NK cells, nonfunctional B cells	EBV-associated lesions
X-linked lymphoproliferative disease (XLP)	*SH2D1A*; *SAP*, which modulates B- and T-cell interactions	Defective EBV-specific T and NK cells	EBV-associated hematophagocytic lymphohistiocytosis, B-cell lymphoproliferative disease
Autoimmune lymphoproliferative syndrome (ALPS)	Defects in *FAS*-mediated apoptosis pathway	Increased CD4 and CD8 T cells	LPHL, CHL, DLBCL, BL
Common variable immunodeficiency (CVID)	Defects in genes encoding *ICOS*, CD19, *BAFFR*	Low IgG and IgA, decreased B cells	EBV-associated lesions, DLBCL, Hodgkin
X-linked hyperimmunoglobulin M syndrome	CD40 ligand mutation leading to defective B cells	Low or absent IgG and IgA, variable T-cell defects	EBV-associated lesions, DLBCL, Hodgkin

Modified from Tran H: Immunodeficiency associated lymphomas, Blood Rev *22:261–281, 2008.*

ALL, Acute lymphoblastic leukemia; *BL,* Burkitt lymphoma; *CHL,* classic Hodgkin lymphoma; *DLBCL,* diffuse large B-cell lymphoma; *DNA,* deoxyribonucleic acid; *EBV,* Epstein-Barr virus; *Ig,* immunoglobulin; *IL,* interleukin; *LL,* lymphoblastic lymphoma; *LPHL,* lymphocyte-predominant Hodgkin lymphoma; *NK,* natural killer.

including the skull and cranial base. Other sites are bone, soft tissue, kidneys, and skin. Testicular involvement occurs in fewer than 5% of boys and usually manifests as painless unilateral or bilateral enlargement of the testicle. Involvement of ovaries is diagnosed in fewer than 5% of females. The pancreas, adrenal glands, thyroid gland, and salivary glands are rarely affected.

The presenting clinical features, largely based on the site of disease leading to symptoms, vary considerably between different NHL subtypes. Because of the variety of clinical patterns of sites in childhood NHL, careful history and physical examination is essential and should focus on signs and symptoms associated with possible emergent problems such as respiratory compromise from a mediastinal mass or bowel obstruction from abdominal disease. Because CNS involvement is not uncommon in B-cell NHL and LL, careful neurologic examination is necessary to detect signs of cranial nerve involvement, paresis, or increased intracranial pressure. Special attention should be directed to the examination of the skin because of the relatively common involvement, especially in patients with ALCL.

Basic laboratory studies include peripheral blood counts, serum concentrations of LDH, basic coagulation parameters, liver and renal function tests, and serum electrolyte levels. Chest x-ray is critical before anesthesia for invasive procedures to evaluate for mediastinal disease. Other imaging studies vary based on clinical presentation and pathologic information but almost always include CT of the chest. Imaging of the abdomen can be done by CT, although in some centers this is done by ultrasound or MRI. Cranial and spinal imaging is mandatory in any patient for whom there are concerns of possible neurologic impairment. Determination of sites of metastatic disease, except for bone marrow and cerebrospinal fluid (CSF), is largely based on clinical and imaging findings. The use of additional biopsy for staging purposes should be restricted to cases where the findings are uncertain and involvement or noninvolvement would alter treatment.

The involvement of bone marrow and the CNS affects treatment of all subtypes of childhood NHL and therefore needs special attention. Historically, bone-marrow involvement was diagnosed if more than 5% blasts are present in a representative bone-marrow aspiration smear detectable by morphology alone. With the use of flow cytometry and polymerase chain reaction (PCR) for detection of disease-specific genetic alterations, smaller percentages of disease cells in the bone marrow can be routinely detected. A generally agreed upon convention regarding the threshold of detection for allocation of patients as bone marrow-positive or not is lacking. With modern immunophenotyping techniques the additive value of bone-marrow biopsies is somewhat unclear.

There is no internationally standardized definition of CNS involvement in childhood NHL. The definition of CNS involvement includes those patients with an intracerebral mass and/or lymphoma cells in the CSF.[265-269] Opinions differ on how patients with cranial nerve paralysis and paraspinal involvement should be classified, and these patients have been allocated to both CNS-positive and CNS-negative groups in different cooperative group studies.

Despite its increasing use, the role of FDG-PET in the initial evaluation and staging of children with NHL is unclear. FDG-PET is highly sensitive and specific for the detection of sites of disease involvement.[270,271] In adult patients there is evidence of clinical benefit for the use of FDG-PET in disease-response assessment for those patients with Hodgkin lymphoma and DLBCL; however its use for initial staging and for disease response assessment in those with other histologies is less clear.[272] As is true with the introduction of any new, more sensitive testing modality, the risk of its use is "upstaging" patients with the consequence of them receiving potentially unnecessary intensive therapy. Prospective studies are required to evaluate and verify the diagnostic role of FDG-PET in staging and to assess whether the findings of FDG-PET compared with conventional imaging translate into different staging, and more importantly, whether this affects patients' outcomes. The use of FDG-PET as a tool for evaluation of the kinetics of response to therapy as a prognostic marker in children with NHL also requires careful investigation. For this indication, pretreatment FDG-PET is indicated, at least for distinct NHL subtypes with variable FDG uptake.[126]

Skeletal scintigraphy (either FDG-PET or bone scan) can detect bone involvement. Skeletal involvement was not found to be a prognostic factor in several analyses, and therefore bone imaging with skeletal scintigraphy to detect clinically occult bone foci is unnecessary.[273-276] Specific imaging procedures may be indicated in patients with symptoms of bone involvement such as pain and swelling. Larger bone lesions are almost always symptomatic and can be imaged using simple x-ray and evaluated further with MRI if needed.

Obtaining tissue for accurate disease diagnosis is critical. If a malignant lymphoma is suspected, it is important to determine whether the diagnosis can be confirmed with minimally invasive tests. Many children have advanced-stage disease at diagnosis, including bone marrow disease and/or malignant effusions. In many of these cases, correct diagnosis can be made by cytology, immunophenotyping by flow cytometry, and genetic evaluation of bone marrow or effusion samples. A diagnostic surgical procedure should be performed if a definitive diagnosis cannot be achieved by less invasive means. If surgery is to be performed, the procedure should be minimally invasive and should only be used to provide enough biopsy material to characterize the disease fully. A complete resection of a small, localized lymphoma may be beneficial for patients with mature B-cell NHL. However, resections at the expense of functional deficits or risks of significant surgical morbidity, including delay in initiating systemic chemotherapy, are not justified.

With the available routine diagnostic tools, a needle aspiration of a pathologically enlarged node is generally not adequate to provide sufficient material for complete pathologic examination and should be reserved for exceptions, such as when a more invasive procedure would represent an undue risk for the patient.

TABLE 53-12 St. Jude Staging System for Pediatric Non-Hodgkin Lymphoma

Stage	Definition
I	A single tumor (extranodal) or involvement of a single anatomic area (nodal), with the exclusion of the mediastinum and abdomen
II	A single tumor (extranodal) with regional node involvement Two or more nodal areas on the same side of the diaphragm Two single (extranodal) tumors, with or without regional node involvement on the same side of the diaphragm A primary gastrointestinal tract tumor (usually in the ileocecal area), with or without involvement of associated mesenteric nodes, that is completely resectable
III	Two single tumors (extranodal) on opposite sides of the diaphragm Two or more nodal areas above and below the diaphragm Any primary intrathoracic tumor (mediastinal, pleural, or thymic) Extensive primary intraabdominal disease Any paraspinal or epidural tumor, whether or not other sites are involved
IV	Any of the above findings with initial involvement of the central nervous system, bone marrow, or both

Modified from Murphy SB: Classification, staging and end results of treatment of childhood non-Hodgkin's lymphomas: dissimilarities from lymphomas in adults. Semin Oncol *7:332–339, 1980.*

The aim of staging procedures is to identify all disease sites to inform therapeutic decisions, response assessment, and follow-up monitoring. The St. Jude staging system, developed in the late 1970s, is widely accepted for the staging of childhood NHL (Table 53-12).[277] The St. Jude staging system is primarily based on the clinicopathologic features of the main NHL subtypes of childhood LL and BL. It takes into account the different patterns of disease spread of these disorders as compared with Hodgkin lymphoma, especially the more common extranodal involvement. The definition of stages is determined by the number and anatomic pattern of disease sites, their resectability, and the involvement of the bone marrow and CNS. Lymphoblastic neoplasms are considered to be acute lymphoblastic leukemia (ALL) if there are 25% or more lymphoblasts in the bone marrow. For BL, infiltration of the bone marrow with more than 25% lymphoma cells is characterized as B-cell or Burkitt-cell leukemia, also referred to as *B-ALL*. The St. Jude staging system has proven useful for the stratification of therapy intensity for the treatment of these entities. For other entities, such as ALCL, its usefulness for treatment stratification is less well defined.

Lymphoblastic Lymphoma

Neoplasms of the precursor B and T cells occur in two different clinical patterns, as ALL or as LL. Conventionally, ALL and LL are separated by the degree of bone marrow involvement. Cases with at least 25% lymphoblasts in the bone marrow with or without extramedullary disease are diagnosed as ALL, whereas those with extramedullary manifestations and less than 25% bone marrow lymphoblasts are referred to as LL. The WHO classification system categorizes the disease as T-lymphoblastic leukemia/lymphoma and B-lymphoblastic leukemia/lymphoma, with multiple subtypes based on recurrent genetic abnormalities.[25] About 80% of children with T-cell ALL and 90% of those with T-cell LL have a mediastinal tumor at presentation. In a minority of cases, T-cell ALL occurs as bone-marrow disease alone, and 10% of T-cell LL cases have no mediastinal tumor.

Pathologic Features

T-cell LL and B-cell LL are morphologically indistinguishable. The neoplastic cells are uniform in size, with scanty or indistinct cytoplasm and nuclei larger than those of small lymphocytes with finely distributed chromatin. They are historically referred to as the *L1* and *L2 types* according to the French-American-British (FAB) Classification of Acute Leukemias.[278] Nuclear membranes may possess subdivisions of varying prominence, giving rise to a distinction between convoluted and nonconvoluted variants of LL.[237] This distinction may be merely a histopathologic nuance, however,[279] and is not included in the WHO classification.

Both precursor B-cell and precursor T-cell LLs may coexpress myeloid CD13, CD33, and rarely, CD117. A useful marker to distinguish LL of precursor B and T cells from mature B-cell and T-cell neoplasms is terminal deoxyribonucleotidyl transferase (TdT). TdT adds single nucleotides to double-strand DNA breaks during the process of immunoglobulin gene and TCR gene recombination, respectively, a process essential during precursor B-cell and T-cell differentiation.[280,281] TdT is only expressed during this process and thus is a specific marker for neoplasms of precursor B and T cells but not for mature T- and B-cell neoplasms.

ALL cases can be further subdivided according to the maturational stages of the leukemic cells, defined by the sequence of expression of antigens into pro-B, common ALL, and pre-B in the precursor B-cell ALL category and into pro-T, pre-T, thymus cortex type, and thymus medullary type in the precursor T-cell category.[282] Few data exist about whether the same subdivision is possible for LL cases.

Molecular and Cellular Biology

Few data are available on genetic findings in LL, mainly because of the lack of fresh tumor tissue appropriate for cytogenetic and molecular studies. As a result, most molecular and cytogenetic data available are derived from the study of T-cell ALL cases. Globally, xome sequencing and array comparative genomic hybridization analyses of pediatric T-cell ALL shows a broad heterogeneity in the mechanisms of T-cell transformation and a low mutation load with an average of 8.2 coding mutations per sample.[283-285]

Role of T-Cell Receptor Translocations. Classic recurring chromosomal rearrangements found in T-cell ALLs and LLs involve the *TCRB* locus (7q34) or the *TCRA/D* locus (14q11) and are generated from mistakes in the recombination process that generate functional TCRs during the development of normal thymocytes.[286-288] These translocations typically induce the aberrant expression of transcription factor oncogenes in T-cell progenitors, activating an irregular transcriptional program that interferes with normal T-cell development and contributes to leukemic transformation. Transcription factor oncogenes involved in these T-cell ALL-specific translocations include members of the basic helix-loop-helix (bHLH) family (*MYC*, *TAL1*, *TAL2*, *LYL1,* and *BHLHB1*),[289-294] the LIM- domain only (LMO) protein genes (*LMO1* and *LMO2*),[295] homeodomain genes (*TLX1/HOX11*, *TLX3/HOX11L2*, and *HOXA9*),[296-299] *MYB*,[300,301] and *NOTCH1*.[302]

Importantly, a number of chromosomal rearrangements that do not involve the TCR loci are also involved in the aberrant expression of some of these oncogenes. Thus *MYB* can be activated by a t(6;7)(q23;q34) translocation[300] or alternatively by a small intrachromosomal duplication selectively affecting the *MYB* locus in the long arm of chromosome 6.[301] The TAL1d rearrangement, a small intrachromosomal deletion that places the *TAL1* gene under the control of the promoter of the nearby *SIL* gene, is found in about 25% of T-cell ALL cases.[303] Similarly, *LMO2* can be activated by small intrachromosomal deletions in the short arm of chromosome 11.[304] Both *TAL1* and *LMO2* are often expressed biallelically in T-cell ALL cases without known alterations in their respective loci, suggesting that activation of upstream regulatory mechanisms controlling the expression of these genes may also participate in the pathogenesis of T-cell ALL.[305,306]

NOTCH1 Activation. The most characteristic molecular lesion associated with the pathogenesis of T-cell lymphoblastic leukemias and lymphomas is the aberrant activation of the NOTCH1 signaling pathway. The NOTCH signaling pathway plays a critical role in the hematopoietic system by participating at multiple stages of T-cell development.

During early hematopoiesis, NOTCH signaling is required for the commitment of multipotent hematopoietic progenitors to the T-cell lineage.[307-309] Conversely, mice harboring a conditional deletion of *Notch1* in hematopoietic progenitors fail to develop T cells and show ectopic B-cell development in the thymus.[309] In some systems, this occurs between CD4 and CD8 lineages.[310-313]

Mature NOTCH receptors (NOTCH1 through 4) are transmembrane heterodimeric proteins generated from a precursor polypeptide that is postranslationally cleaved by a furin protease in the trans-Golgi network. This first cleavage generates both N-terminal and C-terminal NOTCH fragments, which in the absence of a NOTCH signaling stimulus form a heterodimeric transmembrane protein. The N-terminal fragment of the receptor contains multiple epidermal growth factor (EGF) repeats involved in ligand interaction followed by a series of Lin12-NOTCH repeats (LNRs) that in the absence of ligand, stabilize the heterodimeric association between the N-terminal and C-terminal NOTCH fragments. The latter (membrane-bound) fragment of the receptor contains a transmembrane proximal RBPJ-associated molecule (RAM) domain followed by a series of ankyrin repeats, both of which participate in the interaction with the DNA-binding factor RBPJ, and a carboxy-terminal proline, glutamic acid, serine, and threonine (PEST) sequence responsible for the proteosomal degradation of the activated receptor in the nucleus.

The NOTCH signaling pathway is normally triggered by the interaction of a NOTCH receptor in one cell with a NOTCH Jagged 1,2 and Delta-like 1,3,4 (DSL) ligand expressed in the surface of a neighboring cell. This ligand–receptor interaction induces two consecutive proteolytic cleavages at the cell surface that release the cytoplasmic intracellular domain of NOTCH (ICN1) of the receptor from the cell membrane.[314,315] The first of these ligand-induced cleavages is mediated by extracellular metalloproteases of the A disintegrin and metalloproteinase (ADAM) family. Two ADAM proteases (ADAM10 and ADAM17) have been implicated in the activation of the NOTCH signaling pathway in different organisms.[314,315] After S2 cleavage, the resultant activated membrane-bound form of NOTCH is further processed by the γ-secretase complex that catalyzes the final endomembrane cleavage step and releases the intracellular domains of NOTCH from the membrane.

After γ-secretase cleavage, the consequent activated form of NOTCH rapidly translocates to the nucleus, where it interacts with the RBPJ DNA-binding protein. In the absence of NOTCH signals, the RBPJ transcription factor binds to the DNA in NOTCH target promoters, where it recruits transcription inhibitory factors to form a multiprotein transcriptional corepressor complex.[315] Binding of ICN1 to RBPJ displaces these repressors and recruits the MAML1 coactivator to the complex, thereby switching the activity of RBPJ from repressor to activator and inducing the transcriptional activation of NOTCH-RBPJ target genes.

The human *NOTCH1* receptor gene was originally identified as part of the t(7;9)(q34;q34.3), a rare chromosomal translocation that moves the *NOTCH1* gene next to the *TCRB* locus, leading to the aberrant expression of a truncated and constitutively active form of *NOTCH1* in about 1% of human T-cell ALLs.[302] Despite evidence that NOTCH signaling plays a major role in the development of the T-cell lineage and that aberrant NOTCH1 signaling can transform T-cell progenitors, the significance of activated *NOTCH1* in human T-cell ALL seemed limited to rare human T-cell ALLs harboring the t(7;9). This perception was quickly revised after the identification of activating mutations in *NOTCH1* in 50% of human primary T-cell ALL and LL samples and more than 80% of human T-cell ALL cell lines.[316,317,318]

Activating mutations in *NOTCH1* in human T-cell ALL are concentrated in exons 26 and 27, which encode the homodimerization (HD) domain in the extracellular portion of the receptor and in exon 34, which encodes the PEST domain in the C-terminal region of the protein. In addition, *NOTCH1* juxtamembrane expansion (JME) mutations resulting from small in-frame insertions in exon 28 introduce extra amino acids in the extracellular juxtamembrane region of the receptor in about 5% of T-cell ALLs.[319] *NOTCH1* HD and JME mutations probably operate by inducing ligand-independent activation of *NOTCH1*, whereas *PEST* mutations are predicted to result in increased levels of intranuclear active *NOTCH1* by impairing its degradation by the proteasome.[320,321] Thus both types of mutations result in increased *NOTCH1* activity (five- to tenfold over baseline). Importantly, double-mutant alleles harboring both HD and *PEST* mutations are often found in T-cell ALL samples and increase the activation of *NOTCH1* tenfold more than HD or *PEST* mutations alone (50-fold over baseline).[316]

Aberrant activation of NOTCH1 signaling in hematopoietic progenitors drives commitment to the T-cell lineage and promotes malignant transformation by inducing deregulated cell growth, proliferation, and survival. Genomic studies on the identification of *NOTCH1* direct target genes and pathways in T-cell ALL have uncovered a major role for oncogenic *NOTCH1* as a critical regulator of cell growth and metabolism in T-cell lymphoblasts.[322] Thus *NOTCH1* directly binds the promoters of numerous genes involved in protein biosynthesis and anabolic pathways through RBPJ.[322] In addition, oncogenic *NOTCH1* controls the expression of *MYC*, which further increases the expression of cell-growth genes.[321-324]

The relevance of the NOTCH-MYC regulatory axis in T-cell transformation is further supported by the identification of mutations in *FBXW7* in about 15% of T-cell ALL and LL cases.[317,318,325] This -box protein mediates the proteosomal degradation of activated *NOTCH1, MYC, JUN,* and cyclin E.[326] *FBXW7* mutations found in T-cell ALL are clustered in three critical arginine residues involved in the interaction with its target proteins. Thus mutant *FBXW7* works as a dominant negative factor to promote the stability of activated *NOTCH1,* mimicking the effect of *NOTCH1-PEST* mutations.[325] In addition mutant *FBXW7* also stabilizes *MYC,* which further enhances the NOTCH-MYC axis (see earlier) and blocks the degradation of cyclin E, which may contribute to T-cell ALL transformation by promoting cell-cycle progression.[326]

The identification of activating *NOTCH1* mutations present in patients with T-cell ALL and LL has created great interest in developing anti-NOTCH therapies as treatment of this disease.[316] These include γ-secretase inhibitors (GSIs), which block the release of activated NOTCH1 (ICN1) from the membrane, and have been proposed as potential targeted therapy in T-cell ALL and T-cell LL[316,327,328]; stapled peptides targeting the NOTCH transcriptional complex[329]; and NOTCH1-specific inhibitory antibodies that have been proposed as alternative anti-NOTCH1 therapies for the treatment of T-cell ALL and LL.[330] Notably, blocking NOTCH1 signaling with γ-secretase inhibitors can reverse glucocorticoid resistance in some T-cell ALL and LL tumors, and glucocorticoid treatment abrogates the intestinal secretory-cell metaplasia and gastrointestinal toxicity associated with systemic inhibition of NOTCH signaling, supporting the use of anti-NOTCH1 therapies and glucocorticoids in combination in the clinic.[327,328]

MYC. The *c-MYC* oncogene has been implicated in the pathogenesis of T-cell ALL by the t(8;14)(q24;q11) translocation that induces aberrantly high levels of *MYC* expression in T-cell progenitors.[331,332] Furthermore the prominent role of *MYC* in T-cell transformation is reinforced by the identification of *MYC* as an important direct transcriptional target of *NOTCH1*[322-324] and by the identification of *FBXW7* mutations that contribute to increased *MYC* levels by blocking the proteosomal degradation of the MYC protein.[325,326]

***TAL1, TAL2, LYL1,* and *BHLHB1* bHLH Factors and *LMO1* and *LMO2* Transcriptional Oncoproteins.** *TAL1* is a class II bHLH factor gene aberrantly expressed in more than 60% of T-cell ALL cases.[333] *TAL1* activation can occur by means of chromosomal translocation of the *TAL1* locus in the vicinity of *TCR* genes[334] or by a small intrachromosomal deletion that places the *TAL1* gene under the control of the nearby *SIL* locus, present in 25% of T-cell ALL cases.[291] In addition, sporadic cases of T-cell ALL harbor chromosomal rearrangements that place *TAL1*-related genes such as *TAL2, LYL1*, and *BHLHB1* in the vicinity of the *TCR* loci.[292-294] Class II bHLH factors heterodimerize with the class I bHLH proteins E12, E47, HEB, and E2-2 and modulate their transactivating activities.[335] T-cell tumors expressing *TAL1* contain TAL1-E47 and TAL1-HEB heterodimers.[336] In addition, E2A-deficient mice develop T-cell lymphomas,[337,338] suggesting that TAL1 and related bHLH proteins TAL2, LYL1, and BHLHB1 may contribute to leukemia by interfering with E protein function. However, recent genome-wide mapping of TAL1 binding sites in the leukemia genome and *TAL1*-regulated genes shows a more complex scenario in which TAL1 antagonizes both activating and repressing functions of E2A in T-cell ALL.[339]

Loss of function of E2A and HEB is also believed to be the mechanism of transformation in tumors with chromosomal translocations involving the LMO protein genes, *LMO1* and *LMO2*.[304] *LMO1* or *LMO2* are frequently coexpressed with *TAL1*[333] in T-cell ALL, and TAL1-LMO2-E2A complexes are detected in human T-cell ALL cells.[340,341] Furthermore, aberrant expression of *LMO2* accelerated the leukemogenic process in transgenic mice expressing *TAL1* in the thymus.[342]

***TLX1(HOX11), TLX3(HOX11L2),* and *HOXA9* Homeobox Genes.** Homeobox (*HOX*) genes constitute an evolutionarily conserved family of transcription factors that are expressed in specific patterns during embryogenesis and are responsible for the regulation of various developmental processes in vertebrates.[343] The *TLX1 (HOX11)* family of *HOX* genes includes *TLX2 (HOX11L1)* and *TLX3 (HOX11L2)*,[344] which are characterized by the presence of a threonine in the third helix of the homeodomain; this confers specific DNA-binding properties. Both *TLX1* and *TLX3* have been linked to the pathogenesis of T-cell ALL through their identification as genes activated by chromosomal translocations. In contrast, *HOX11L1* is normally expressed in T-cell progenitors and does not seem to play a role in the transformation of normal thymocytes.[333]

TLX1 was originally isolated at the breakpoints of the recurrent t(10;14)(q24;q11) in T-cell ALL[296,297,345,346] and is aberrantly expressed in 5% of pediatric and up to 30% of adult T-cell ALL cases.[333,347-349] Like other *HOX* genes, *TLX1* plays an important role during embryonic development and acts as a master transcriptional regulator necessary for the genesis of the spleen.[350,351]

Several possible mechanisms through which aberrant *TLX1* expression might lead to malignant transformation have been proposed. In particular, the TLX1 protein is believed to function as a transcriptional regulator, a hypothesis supported by the presence of both a 61-amino acid, helix-turn-helix, DNA-binding domain (or homeodomain) and by the localization of *TLX1* in the cell nucleus.[344] In addition, *TLX1* has been shown to bind to a protein serine-threonine catalytic subunits of phosphatase 2A (PP2AC) and phosphatase 1 (PP1C). Both PP2A and PP1 are targets for oncogenic viruses and chemical tumor promoters. Thus inactivation of PP2A by *TLX1* could contribute to the transformation of T-cell progenitors.[352]

A second *HOX11* family member, *TLX3*, has been implicated in the pathogenesis of human T-cell ALL through characterization of the t(5;14)(q35;q32), a cryptic chromosomal translocation detectable only by fluorescence in situ hybridization (FISH) and chromosome-painting techniques.[298] This translocation leads to the ectopic expression of *TLX3*, possibly by bringing it under the influence of regulatory elements in the *BCL11B* gene, which is highly expressed during T-lymphoid differentiation.[298,353] In contrast to the predominance of *TLX1* expression in adult T-cell ALL cases, both t(5;14) and *TLX3* expression are present in 20% to 25% of pediatric cases but in only 5% of adult T-cell ALL cases.[347,349,354,355] As is the case for *TLX1, TLX3* plays an important role during embryonic development when it is essential for the normal development of the ventral medullary respiratory center.[356]

The TLX1 and TLX3 proteins are closely related in structure and possess a high degree of sequence identity at the amino-acid level, especially in the HOX domain, where their sequences only differ in three amino acids. The high level of structural homology in their DNA binding domains supports the hypothesis that TLX1 and TLX3 may induce T-cell ALL through the regulation of the same transcriptional targets. Gene expression profiling studies analyzing the expression signatures associated with *TLX1* and *TLX3* expressing T-cell ALLs and detailed characterization of a transgenic mouse model of T-cell ALL induced by forced expression of TLX1 in developing T cells have shown a remarkable overlap in the gene expression programs and direct transcriptional targets controlled by TLX1 and TLX3 in T-cell ALL.[357] Moreover, these studies have revealed a transcriptional repressor role for TLX1 and TLX3 in the control of gene expression and uncovered the *BCL11B* and *RUNX1* genes as prominent tumor suppressor genes downregulated by TLX1 and TLX3 in the pathogenesis of T-cell ALL.[358] Additionally, *TLX1* has been implicated in the development of aneuploidy via disruption of mitotic checkpoint mechanisms[357] and in blocking T-cell development via inhibition of *TCRA* recombination.[359]

MYB. The translocation t(6;7)(q23;q34) is a rare recurrent rearrangement that juxtaposes the *TCRB* and *MYB* loci in very young pediatric T-cell ALL cases (median age, 2.2 years).[300] The pathogenic role of *MYB* in T-cell ALL is further supported by the identification by molecular cytogenetic techniques of a short somatic duplication, which includes the *MYB* locus in 15% of T-cell ALL cases.[300,301] These abnormalities induce increased *MYB* expression, particularly in the case of the *TCRB-MYB*–rearranged leukemias. The MYB transcription factor is essential for primitive and adult hematopoiesis, including the T-cell lineage, and the *MYB* locus is a common site of retroviral insertional mutagenesis-induced leukemias; however, the specific mechanisms that mediate *MYB*-induced transformation in T-cell ALL remain to be elucidated. Notably, *MYB* is upregulated downstream of *HOXA9* and *MLL-ENL* T-cell oncogenes and is required for *HOXA9*- and *MLL-ENL*-induced leukemic transformation.[360]

***CALM*-AF10.** The t(10;11)(p13;q14-21) is a recurrent chromosomal translocation found in patients with AML and T-cell ALL. The product of this translocation is a chimeric oncogene resulting from the fusion of clathrin assembly lymphoid myeloid leukemia gene (*CALM*) and AF10.[361,362]

CALM is a ubiquitously expressed protein containing an epsin N-terminal homologous domain involved in the interaction with clathrin during endocytosis.[363] AF10 is a ubiquitously expressed transcription factor originally identified as a partner of *MLL* in AML.[364,365] *CALM*-AF10 rearrangements are present in 5% to 10% of T-cell ALL cases and seem to be particularly common in tumors with a very early block in T-cell development or those expressing TCRad.[366,367] Gene-expression profiling studies have demonstrated that *CALM*-AF10 expression is associated with high levels of *HOXA* genes, including *HOXA9*.[299,368]

MLL-ENL. Rearrangements of *MLL*, located on human chromosome 11q23, are common in infant and therapy-related leukemias and can occur in AML and ALL. These rearrangements typically generate a fusion oncogene containing the N-terminus part of *MLL* and various protein domains from more than 50 different fusion partners. The *MLL-ENL* fusion oncogene is found in rare cases of T-cell ALL and, in contrast with *MLL* rearrangements in other hematologic tumors, is associated with a favorable prognosis.[333] *MLL* is normally responsible for the maintenance of gene expression at the *HOX* paralog groups. *MLL*-fusion oncogene products disrupt the normal function of MLL and result in aberrant expression of *HOX* genes.[369,370] *MLL*-rearranged T-cell ALL cases have high levels of expression of *HOX* genes, including *HOXA9* and *HOXA10*.[333,370]

Transcription Factor Tumor Suppressors. *BCL11B* encodes for a transcription factor critically required for normal T-cell development.[371] Loss-of-function mutations and heterozygous deletions of the *BCL11B* are recurrently found in T-cell ALL, suggesting that *BCL11B* haploinsufficiency is an important pathogenetic event in T-cell leukemogenesis.[357,372] In addition, the Wilms tumor suppressor gene 1 (*WT1*) is deleted and mutated in about 10% of T-cell ALLs.[373,374] Similarly, monoallelic or biallelic deletions involving the *LEF1* locus and mutations in the *LEF1* gene are recurrently present in T-cell ALL cases.[375] Finally, *ETV6*, *RUNX1*, and *GATA3* loss-of-function mutations are characteristically present in a fraction of early-immature T-cell ALLs, a leukemia group characterized by a very early arrest in T-cell development and poor prognosis.[358,376-378]

Cell Cycle Regulators. Deletions of chromosome bands 9p21-229 involving the $p16^{INK4A}$, $p14^{\mathrm{ARF}}$, and $p15^{\mathrm{INK4B}}$ tumor suppressor genes are the most common abnormality found in T-cell lymphoblastic tumors.[379,380] p15 and p16 are involved in cell-cycle regulation by inhibiting cyclin-CDK complexes, whereas $p14^{ARF}$ is a negative regulator of TP53. Thus loss of these tumor suppressors affects both the control of cell proliferation and cell survival.

Chromosomal translocations targeting the cyclin D2 (*CCND2*) locus at chromosome band 12p13 and the *TCRB* or *TCRAD* loci result in high levels of *CCND2* overexpression. *CCND2* expression is normally confined to early (double-negative) stages of thymocyte differentiation and is replaced by *CCND3* in more mature (double-positive) cells, suggesting that sustained expression of CCND2 in *CCND2*-rearranged T-cell ALL cases is oncogenic.[300]

Chromatin Remodeling Tumor Suppressor Genes. The polycomb repressive complex 2 (PRC2) is the “writer” of lysine 27 trimethylation on Histone 3 (H3K27me3), a major chromatin mark associated with transcriptional silencing. Loss-of-function mutations and deletions of *EZH2* and *SUZ12* genes encoding two critical components of the PRC2 complex are found in about 25% of T-ALLs.[377,381] In addition, *PHF6*, a plant homeodomain (PHD)–containing factor involved in epigenetic regulation of gene expression is mutated and deleted about 16% of pediatric T-cell ALL cases.[382]

Genetic Alterations Driving Activation of Oncogenic Signaling Pathways. The *PTEN* tumor suppressor gene, which encodes a central negative regulator of the PI3K–AKT pathway, is mutated and deleted in 5% to 10% of T-cell ALL cases,[383] and prototypical activating mutations in *NRAS*-activating mutations are common in early immature T-cell ALLs.[376,377,384] In addition, deletions and mutations in the neurofibromatosis type 1 (*NF1*) gene, which encodes a negative regulator of Ras signaling, are found in 3% of T-cell ALLs.[385] Aberrant JAK signaling drives T-cell transformation in T-cell ALLs harboring the t(9;12)(p24;p13) translocation, a rare rearrangement encoding a constitutively active *ETV6-JAK2* oncoprotein.[386] Moreover, activating mutations in *JAK1* and *JAK3* and the *IL7R* gene resulting in constitutive activation of the JAK-STAT signaling are present in approximately 15% of T-cell ALLs.[377,387-390]

Other Genetic Alterations. Numerous additional genes and pathways whose mechanism of action remains to be fully clarified are altered in T-cell ALL. Among these, recently identified genetic-lesion mutations affecting the ribosomal proteins RPL5, RPL11, and RPL10 including recurrent alterations of Arg98 in RPL10 are particularly intriguing and suggest a role for disruption of ribosomal function in T-cell transformation.[283]

Clinical Characteristics

Children and adolescents with LL most commonly show signs and symptoms of mediastinal disease such as cough, shortness of breath, or orthopnea. Patients may have severe distress that is sometimes made worse by the effects of pleural or pericardial effusions. Patients may also have painless adenopathy, most often cervical or supraclavicular, although the disease can be widely disseminated and involve the liver and spleen. Those with B-cell LL more often show limited-stage disease of skin, isolated node, or bone. Disease limited to the skin most commonly involves the scalp.[391] Patients may have symptoms related to CNS disease, including signs associated with increased intracranial pressure such as headache, nausea and vomiting, and visual changes or with cranial nerve palsies.[267] Many who are subsequently found to have CNS will not have had preceding symptoms. By definition, patients with LL have less than 25% bone marrow involvement. Most patients therefore do not exhibit signs or symptoms associated with severe pancytopenia, and most commonly the complete blood count (CBC) is normal or near normal.[392] Involvement of the testes for patients with LL is relatively rare. Table 53-13 describes the clinical features at the time of diagnosis in patients with LL in selected cooperative group studies.

Treatment and Outcomes

The treatment of children and adolescents with LL is largely based on the strategies and protocols developed for the treatment of ALL. Outcomes for selected

TABLE 53-13 Presenting Features in Children and Adolescents with T-Cell Lymphoblastic Lymphoma

	% Male	% with Mediastinal Mass	% with CNS Disease	% with Bone Marrow Disease
EORTC-CLG58881[605]	62	81	3	NR
NHL-BFM90[394]	77	89	3	15
NHL-BFM95[403]	63	78	4	26
POG 9404[399]	77	89	4	NR

CNS, Central nervous system; *EORTC-CLG*, European Organization for Research and Treatment of cancer–Children’s Leukemia Group; *NHL-BFM*, Non-Hodgkin Lymphoma–Berlin-Frankfurt-Munster; *NR*, not reported; *POG*, Pediatric Oncology Group.

TABLE 53-14 Treatment Protocols and Results in Recent Multicenter Studies of Childhood and Adolescent Lymphoblastic Lymphoma

Protocol	Stages	Immunophenotype	No. of Patients	Duration of Therapy	Cranial Radiation	EFS	Outcome
NHL-BFM90[244,394]	I-IV	T only	105	24 months	12 Gy prophylaxis 24 Gy if CNS+	90% (5 years)	Excellent outcome with ALL-like therapy and prophylactic cranial radiation, no local radiation
EORTC-CCG58881[605]	I-IV	T only	121	24 months	none	77% (6 years)	No increase in CNS relapse rate observed without cranial radiation, response to prephase therapy predictive of outcome.
POG9404[399]	III-IV	T and B	137	25 months	18 Gy all patients	85% (5 years)	Addition of high-dose methotrexate did not improve outcome for patient with LL (although it did for those with T-cell ALL)
NHL-BFM95[403]	I-IV	T and B	198	24 months	No prophylaxis 18 Gy if CNS +	80% (5 years)	In patients with stage III and IV CNS-positive disease and good response to induction, prophylactic cranial radiation can be avoided
COG A5971[400]	I-IV	T and B	257	24 months	No prophylaxis 18 Gy if CNS +	83% (3 years)	For patients with stage III + IV CNS-negative disease, neither high-dose methotrexate nor intensified anthracycline/ cyclophosphamide improved outcome

ALL, Acute lymphoblastic leukemia; *CNS,* central nervous system; *COG,* Children's Oncology Group; *EORTC-CCG,* European Organization for Research and Treatment of cancer–Children's Cancer Study Group; *LL,* lymphoblastic lymphoma; *NHL-BFM,* Non-Hodgkin Lymphoma–Berlin-Frankfurt-Munster; *NR,* not reported; *POG,* Pediatric Oncology Group.

cooperative groups trials are presented in Table 53-14. Considerations for therapy include immunophenotype, stage including CNS involvement, and more recently, the impact of the presence of minimal disease involvement of the bone marrow at the time of diagnosis.[393] Other clinical factors such as age, gender, and LDH as well as tumor response were not found to be prognostic in the Berlin-Frankfurt-Munster (BFM) studies. Patients with low-stage disease have survival rates that exceed 90%, whereas the rate for those with stage 3 or 4 disease approaches 80%.[394,395]

Patients with stage 1 and 2 disease account for a minority of patients with LL. Approximately 75% of the low-stage group has B-lineage disease.[395] Early studies of localized NHL evaluating 9 weeks versus 8 months of therapy showed that those with LL had a significantly worse outcome than other NHL phenotypes, with evidence of some benefit for longer therapy in this subgroup.[396] Studies that have included patients with all stages of LL have consistently reported favorable outcomes for those with limited disease with EFS rates of 73% to 100%.[397] The largest series of patients with stage 1 and 2 LL disease treated in a uniform manner comes from the COG study A5971. Patients were assigned to a CCG-BFM–like ALL regimen that included 24 months of therapy, intensive intrathecal therapy, and no radiation.[395,398] Seventy five percent of the patients had pre-B immunophenotype, and the majority had disease in their head and neck region, involving skin and nodal tissue with a minority who had isolated bone disease. The 5-year EFS and OS rates were 90% and 95%, respectively.

The majority of patients with LL have advanced-stage disease at diagnosis and have the T-cell phenotype. The most effective regimens for this group of patients are based on regimens adopted from the treatment of children with ALL. Most treatment protocols include corticosteroids, vincristine, anthracyclines, L-asparaginase, and cyclophosphamide; and methotrexate, cytarabine, 6-mercaptopurine, and 6-thioguanine; and some include epipodophyllotoxins. Most involve induction, consolidation, and maintenance phases of chemotherapy and prolonged treatment lasting approximately 2 to 3 years. Radiotherapy to sites of disease outside the CNS is not generally used in contemporary treatment protocols.

In the context of multidrug therapy, isolating the importance of specific agents is challenging. There is mixed evidence that the use of high-dose methotrexate is important in the treatment of children with advanced-stage disease. The BFM90 trial, which included 4 courses of 5 gm/m^2 of methotrexate in consolidation, had excellent outcomes with a 5-year EFS rate of 90% to 95%.[394] The POG9404 study evaluated the addition of high-dose methotrexate to an intensive ALL backbone for patients with T-cell ALL and T-cell LL. The 5-year EFS rate was better for those who received high-dose methotrexate in patients with T-cell ALL but there was no measurable difference for those with T-cell LL.[399] A recent COG study evaluating the impact of induction intensification and the use of high-dose methotrexate in consolidation found no benefit to either intervention.[400] Evidence for the efficacy of L-asparaginase in patients with T-cell LL can be derived from a randomized study conducted by the former POG.[401] Patients with T-cell LL receiving weekly L-asparaginase after induction had a higher EFS rate than controls. Dexamethasone instead of prednisone in induction resulted in improved outcome of children suffering from ALL at the expense of higher toxicity.[402] Randomized BFM-based trials as to whether dexamethasone in induction can also further improve the EFS rate of patients with T-cell LL are ongoing.

Oral 6-mercaptopurine and methotrexate are basic elements of maintenance therapy during remission; they are administered for a treatment duration of 18 to 24 months from the start of chemotherapy. With the exception of the BFM strategy, additional agents (vincristine and steroids) and multiple intensification pulses of different composition are used in many study protocols. When comparing overall results of studies, there is little evidence for a beneficial effect of intensification of maintenance.

CNS-directed chemotherapy including high-dose methotrexate and intrathecal therapy without prophylactic cranial irradiation is sufficient for prevention of CNS relapse in patients with advanced-stage LL without overt CNS disease.[399,403,404,405] In the NHL BFM 95 study, including high-dose methotrexate (5 g/m^2 IV, four times daily) and 11 doses of intrathecal methotrexate but no cranial radiotherapy (CRT), both disease-free and CNS relapse–free survival rates of patients were not significantly inferior to the historic control group of the preceding NHL BFM86 and BFM90 trials, in which patients received prophylactic CRT.[403] High-dose methotrexate therapy also appears efficacious to prevent relapse in the testes.[394]

For patients with overt CNS disease, 18 to 24 Gy CRT, in addition to aggressive systemic chemotherapy, is highly effective in preventing CNS recurrences.[265,394,397] In the BFM studies, patients with CNS-positive LL had EFS rates comparable to patients who were CNS-negative when receiving high-dose methotrexate (5 g/m^2 IV infusion) over 24 hours and intrathecal therapy followed by CRT with 18 Gy.[265]

Therapy for Patients with Relapsed Disease

The outcome for patients with relapsed LL is poor.[406] The general strategy for these patients includes intensive reinduction therapy, and for those patients with responsive disease, ideally a second CR to proceed to SCT. One of the main reasons for poor outcomes is the failure of disease, once it has relapsed, to respond to further therapy. There is no standard reinduction regimen. Various combinations have been described including ifosfamide, carboplatin, and etoposide (ICE)[407] and dexamethasone, etoposide, cisplatin, cytarabine, and L-asparaginase (DECAL).[408]

Nelarabine, a nucleoside analogue, has been shown to have activity as a single agent in children with relapsed and refractory T-cell malignancies.[409] Nelarabine in combination with etoposide and cyclophosphamide has been described in a small case series as being safe and effective. In this series seven of the seven patients had some evidence of disease response and five of seven obtained CR.[410] Nelarabine, in addition to T-cell ALL backbone therapy, is being tested for safety and efficacy in the current phase III randomized trial for children with newly diagnosed high-risk T-cell ALL and T-cell LL within the COG.

The data for SCT in patients with relapsed LL are limited. Unlike for other types of NHL, there is no clear role for autologous SCT in patients with relapsed LL.[411] In a report from the BFM, no patient with relapsed LL who did not proceed to allogeneic SCT was a long-term survivor. The majority of patients did not undergo SCT because of nonresponsive disease. Of the nine patients who were able to undergo allogeneic SCT, four remain in second CR.[406]

Mature B-Cell Neoplasms

Of the numerous subtypes of neoplasms of mature B cells, only two commonly occur during childhood and adolescence, BL and DLBCL. All others are rare in children. Combined, these two entities account for approximately 60% percent of NHL in children and adolescents.

Pathologic Features: Burkitt Lymphoma

Classic BL is composed of monomorphic medium-sized cells with deep basophilic cytoplasm and round to ovoid nuclei, with granular dispersed chromatin and multiple nucleoli corresponding to L3 morphology according to the FAB classification.[278] Lipid vacuoles are commonly present in the cytoplasm and the nuclei but are not mandatory. Numerous mitotic and apoptotic cells are usually seen. On histomorphology, a “starry sky” pattern is usually present, created by the intercalation of numerous macrophages that have ingested apoptotic cells. Using proliferation-associated markers such as Ki67, the growth fraction is high and can be up to 100%.[39]

BL cells invariably express the B-lineage antigens CD19, CD20, CD22, and CD79a and surface immunoglobulin and are negative for CD5, CD23, and TdT.[26] The expression of the germinal center markers BCL6 and CD10 points to a germinal center origin of BL cells.[412]

The 2008 WHO classification eliminated the category of Burkitt-like lymphoma.[238] Two provisional diagnostic groups were added: B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL and B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and CHL[39] that account for disease whose morphologic, immunologic and molecular phenotype lies between classic disease groups.

Molecular and Cellular Biology. BL is universally characterized by a characteristic gene expression signature that includes the aberrant expression of *MYC*.[413] This cellular oncogene encodes a helix-loop-helix transcription factor involved in the control of multiple cellular processes that contribute to cellular transformation, including cell growth, division, death, metabolism, adhesion, and motility. Chromosomal translocations characteristic of BL juxtapose the *c-MYC* locus in chromosome band 8q24 with strong enhancers from the immunoglobulin genes and drive high levels of *MYC* expression.[414,415] In 80% of BL cases, the t(8:14)(q24;q32) translocation places *MYC* in the vicinity of the *IGH* locus.[416] In 15% of cases, the translocation partner is the *IGK* locus at chromosome 2p11, whereas the remaining 5% of cases involve the *IGL* locus at chromosome 22q11. BL exhibits a distinct gene-expression profile characterized by the high-level expression of *MYC* target genes, which allows accurate differentiation of BL from other NHL subtypes such as DLBCLs.[413,417]

Tumors with the t(8;14) show differences in the location of the breakpoints relative to the *MYC* gene and the *IGH* genes, which are strongly associated with the geographic origin of the patient.[418-422] In endemic BL, the chromosome 8 breakpoints are found up to 100 kb 5′ from the *MYC* exon 1, whereas the chromosome 14 breakpoints occur most often in the *IGH* joining regions (J_Hs). In contrast, in sporadic and AIDS-associated BL, the chromosome 8 breakpoints are most often located between *MYC* exons 1 and 2, and those in chromosome 14 are found in the *IGH S*μ switch region. In cases with *MYC* rearrangements involving the light-chain immunoglobulin genes, the breakpoints on chromosome 8 are located at variable distances from the 3′ of the *MYC* locus,[423,424] and the breakpoints on chromosomes 2 and 22 are located 5′ from the constant region of the *IGK* and *IGL* genes. These findings suggest that in sporadic and AIDS-associated BLs with the t(8;14), the chromosome 14 breakpoints are generated during immunoglobulin-class switch recombination. This supports a germinal center B-cell origin for these tumors. In contrast, in endemic BL, the breakpoints on chromosome 14 originate during RAG-mediated variable-diversity-joining (V[D]J) recombination, suggesting that these tumors most probably derive from pre-B cells, which normally express *RAG* genes, or from germinal center B cells undergoing *RAG* gene reexpression.[425] In addition, the breakpoints of the translocation t(8;14) also differ between EBV-positive and EBV-negative cases, with breakpoints outside the *MYC* locus on chromosome 8 associated with EBV positivity and breakpoints within the *MYC* locus being identified in EBV-negative cases.[419] Notably, immunoglobulin gene analysis has revealed two distinct cells of origin for EBV-positive and EBV-negative cases, irrespective of the subtype (endemic, sporadic, or immunodeficiency-associated BL), suggesting that EBV status rather than the geographic distribution determines the biology of the disease.[426]

The first exon and first intron of *c-MYC* contain negative transcription regulatory sequences.[427,428] These negative regulators of *MYC* expression are removed in sporadic BL, with t(8;14) breakpoints located in *MYC* intron 1. In contrast, in endemic BL and sporadic BL with light-chain gene translocations, the chromosomal breakpoints are located 5′ or 3′ to the *MYC* locus, respectively. However, in these tumors, the *MYC* exon 1 and intron 1 are often mutated and show loss of the negative transcriptional regulatory elements located in these regions.[429,430] Thus even though the immunoglobulin enhancers drive high levels of *MYC* ribonucleic acid (RNA) expression, *MYC* levels are further increased in BL by the deletion and/or mutation of negative regulatory sequences within the *MYC* locus.

Additionally, BL tumors often show mutations of *MYC* at codon 58. These mutations stabilize the MYC protein by disrupting a critical threonine phosphorylation site involved in the proteosomal degradation of this transcription factor.[431-434]

Other Recurrent Genetic Abnormalities. Recent genomic studies have uncovered novel, highly prevalent, genetic alterations involved in the pathogenesis of BLs.[54,435-437] Most prominently, 70% of sporadic and immunodeficiency-associated BLs and 40% of endemic tumors show somatic mutations that activate the transcription factor TCF3 or disrupt the activity of its negative regulator ID3, highlighting a major role for *TCF3* activation in B-cell transformation. *ID3* mutations (38% to 68%) are more common than *TCF3* mutations (11%). Mechanistically, *TCF3* modulates essential genes for germinal center function and can activate the prosurvival PI3K pathway by intensifying B-cell receptor signaling. In addition, about 40% of sporadic BLs and a minority of endemic tumors show mutations in the *CCND3* cell-cycle regulator, resulting in highly stable CCND3 isoforms that drive cell-cycle progression. These studies also confirmed the presence of mutations in the *TP53* tumor-suppressor gene in about 30% of BLs.[438,439] Notably, *MYC* overexpression can induce apoptosis by TP53-dependent and TP53-independent mechanisms.[440-442] Thus the loss of *TP53,* which has prominent roles in the control of programmed cell death, may enhance the transforming effects of *MYC*. Additional highly prevalent mutant genes in BL include those encoding the DDX3X RNA helicase, the SMARCA4 SWI/SNF chromatin regulator, the NCOR2 transcriptional corepressor, the PDCD11 ribosomal RNA maturation factor and the GNA13 G protein signaling factor.

Role of Epstein-Barr Virus. EBV infection is present in 90% of endemic BL cases, 20% of sporadic BL cases, and about 40% of HIV-associated BL cases.[423] Although molecular data have indicated that EBV infection precedes or occurs concomitantly with B-cell transformation,[443] the specific mechanisms whereby EBV infection contributes to the pathogenesis of BL have not been fully elucidated. However, the virally encoded *EBNA-1* gene may play an important role, because it can induce B-cell lymphomas in transgenic mice[444] and has been shown to be essential for EBV-induced B-cell transformation in vitro.[445,446]

Pathologic Features: Diffuse Large B-Cell Lymphomas

DLBCLs are composed of large, transformed lymphoid cells derived from germinal-center and postgerminal-center B cells.[39] DLBCL cells consistently express pax5, CD79a, and CD20, whereas expression of all other B-lineage markers varies. Morphologically, DLBCLs are diverse and can be subdivided into three distinct variants. The centroblastic variant is composed of medium-sized to large cells with basophilic cytoplasm, oval to round nuclei, and two to four membrane-bound nucleoli. The immunoblastic variant has a preponderance of immunoblasts with centrally located nucleoli and basophilic cytoplasm. The anaplastic variant has very large, round, oval, or polygonal cells with bizarre pleomorphic nuclei, which may resemble Reed-Sternberg cells and commonly express CD30. However, this variant is biologically unrelated to ALCL. The centroblastic variant is the most common histiologic type of DLBCL in children.

Other subtypes of DLCBL have been described, including T-cell and histiocyte-rich large B-cell lymphoma. Most cells of this type are nonneoplastic T cells, with or without histiocytes; fewer than 10% of the infiltrate are neoplastic B cells, which resemble immunoblasts, centroblasts, Hodgkin cells, or L&H cells of NLPHL. Because of the predominance of reactive cells, this variant may be misdiagnosed as representing a reactive lymph node. Also, the distinction from NLPHL may be difficult, and an overlap between both cannot be completely excluded.[39]

Complex cytogenetic abnormalities are observed in DLBCL. So far, no entity-specific, nonrandom chromosomal translocations have been described for this subgroup, which may reflect its heterogeneity. In many cases, translocations, including the *c-MYC* locus 8q24, are observed, as well as abnormalities in 3q27, where the protooncogene *BCL6* is located.[447] Based on gene expression profiling, two main subtypes of DLBCL can be distinguished: germinal center B-cell–like (GCB) DLBCL and activated B-cell–like (ABC) DLBCL.[448] The subdivision of DLBCL cases into GCB and ABC subtypes by gene expression analysis could be reproduced by means of immunohistochemistry, applying the germinal-center cell markers CD10 and BCL6, and the postgerminal center marker MUM1.[449] CD10 expression assigned cases to the GCB type independently of *MUM1* expression, but in CD10-negative cases, *BCL6* positivity and negativity for *MUM1* were associated with GCB type. Although indistinguishable from adult DLBCL cases in terms of morphology, DLBCLs in children appear to comprise a biologically distinct subgroup of germinal center B-cell lymphomas.[450] In contrast to adult DLBCL, pediatric DLBCL is predominantly of germinal center origin (6 : 1) but differs from adult DLBCL of the GCB type in that it invariably lacks t(14;18).[451] Chromosomal abnormalities in 8q24 including *MYC* rearrangements and gain or amplification of *MYC* are present in 31% and in 50% of pediatric DLBCL cases.[451,452] Additional common chromosomal alterations include losses at 3q and 6q and gains at 1q and 12q.[451,452] In addition, although array-based comparative genomic hybridization (aCGH) analysis has identified genetic lesions shared between adult and pediatric DLBCL (+12q15, +19q13, and −6q), these studies have also highlighted abnormalities unique to the pediatric cases (−4p14, −19q13.32, and +16p11.2).[451]

Clinical Characteristics

In 1958 Denis Burkitt described a “sarcoma involving the jaw in African children,” which a short time later was shown by O’Connor to be a lymphoma. Burkitt recognized the endemic nature of the disease.[251] In 1964 viral particles subsequently known as *EBV* were identified in the tumor.[252] Most NHL cases in children in North America and Europe were morphologically indistinguishable from BL. Because there was no geographic limitation and most cases were not associated with EBV infection, they were referred to as *sporadic BLs*, in contrast to the endemic BL of central Africa. According to the WHO classification, three clinical subtypes are recognized. These are morphologically indistinguishable but differ in clinical presentation and biology and include endemic BL, sporadic BL, and immunodeficiency-associated BL.[39] Endemic BL occurs in equatorial Africa, Papua New Guinea, and equatorial regions of South America. In up to 95% of endemic BL cases, the tumor cells carry the EBV genome.[453] Sporadic BL occurs throughout the world in much lower incidence rates as compared with endemic BL. Nevertheless in industrialized countries, BL lymphoma is the most common subtype of NHL in childhood and adolescence and the most common of the mature B-cell neoplasms. In contrast to areas with endemic BL in central Europe, only 10% to 15% of patients with pediatric BL carry the EBV genome.[253,254] Immunodeficiency-associated BL occurs most often in patients with HIV infection but also in patients after solid organ transplantation. The incidence of EBV infection in immunodeficiency-associated BL in North American patients is less than 40%. The incidence of immunodeficiency-associated BL has fallen somewhat with the use of antiretroviral therapy.[416]

The pattern of disease at presentation varies between sporadic and endemic BL and DLBCL. The most common site of disease at presentation for patients with sporadic BL is the abdomen.[454] Common presenting symptoms include abdominal distension, nausea and vomiting, gastrointestinal bleeding, and abdominal pain. A classic although relatively rare presentation of patients with BL is intussusception, where ultrasound imaging reveals evidence of a tumor as a “lead point mass” in the small bowel.[455] Other common sites include the head and neck including cervical nodes, tonsils, and sinuses.

Patients with Burkitt leukemia most often present as a leukemic disease in combination with large tumors.[456] However, in some cases the disease can be confined to the bone marrow without any extramedullary involvement.

In children with Burkitt leukemia, the peripheral blood may show prominent vacuolate lymphoblasts. However, in contrast to pediatric early B-cell ALL, the blast count in the peripheral blood rarely exceeds 30,000/L. In general the degree of anemia and thrombocytopenia is less severe compared with other types of pediatric leukemia, reflecting a lesser degree of bone-marrow invasion.

Patients with endemic BL have classically presenting symptoms of jaw disease, most commonly found in younger children. In addition periorbital findings and abdominal involvement are common. The incidence of symptomatic CNS disease, particularly paraplegia, is much higher than that in the developed world.[265] In low-income countries the majority of patients present with advanced disease, and many are malnourished at the time of diagnosis.[457] By contrast, patients with DLBCL most often have symptoms of nodal disease including involvement of peripheral nodes as well as those in the abdomen and mediastinum. The majority have advanced stage disease, although CNS and bone marrow involvement is rare.

Treatment and Outcome of Mature B-Cell Neoplasms

In adult patients therapy for those with DLBCL and those with BL differs. In children the chemotherapy regimens developed and utilized for patients with BL, who make up of the majority of patients, have proven to be equally effective for those with DLBCL. Hence in the majority of contemporary pediatric protocols the two diseases are treated identically.

Chemotherapy for BL was pioneered in Africa, where Denis Burkitt achieved remission in children with BL with single or few doses of cyclophosphamide, low-dose methotrexate, or vincristine.[458] He recognized that children with a small tumors were more likely to achieve CR than those with large tumors, which suggests a relationship between tumor mass and response.[459] The next important step was the introduction of the combination chemotherapy of cyclophosphamide, vincristine, methotrexate, and cytarabine, which proved not only efficacious for African children with endemic BL but also for American patients with sporadic BL.[460,461] The high incidence of CNS relapses and the failure of cranial irradiation to prevent them was described by this group, leading to the introduction of combined intrathecal chemotherapy.[460,462,463] Based on the African experience, Ziegler and colleagues first explored the principle of dose intensity and achieved CRs with high-dose chemotherapy in American patients with BL that was unresponsive to lower dose therapy.[464]

In the 1980s, treatment strategies were further improved by the emerging information about the peculiar biologic features of BL. A most important characteristic regarding therapeutic strategy is its high proliferation activity. BL cells cycle extremely rapidly, with an estimated generation time of only 25 hours.[465] It can be estimated that within 48 to 72 hours, every Burkitt cell is likely to traverse the cell cycle, thereby encompassing a period of greatest sensitivity to cytotoxic treatment.[466-468] Based on this knowledge, combination chemotherapy courses were designed based on the principle of maintaining—by means of fractionated administration or continuous infusion—cytotoxically active drug concentrations for the length of time needed to affect as many lymphoma cells as possible during the vulnerable active cell cycle.[469-472] Therapeutic regimens that adhere to the principle of rapidly repeated cycles of dose-intensive combination chemotherapy have resulted in a dramatic increase in the cure rate over the last several decades from less than 40% to greater than 85%.[473]

Although there are few randomized trials testing the impact of individual drugs there is considerable evidence that cyclophosphamide and methotrexate may be key agents. Monotherapy with methotrexate or with cyclophosphamide, with or without low-dose methotrexate and vincristine can induce durable remissions in BL.[458,461,474] The important role of methotrexate can be extrapolated from the effect of different dosages. In patients with stage III disease and high tumor mass (LDH >500 U/L, approximately twice or more the upper limit of normal for age), and in patients with Burkitt leukemia, a 10-fold increase of the dose of methotrexate from 0.5 to 5 g/m^2 resulted in significantly improved outcome.[456,475] Similarly, the elevation of the dose of methotrexate from 3 to 8 g/m^2 in the Societe Francaise d'Oncologie Pediatrque-Lymphoma Maligancy B (SFOP-LMB) protocols resulted in an excellent outcome of patients with advanced-stage BL, including those with CNS involvement.[266,476,477] Comparatively little evidence exists as to the role of corticosteroids and anthracyclines. In a CCG study of the 1980s, addition of daunomycin to the combination of cyclophosphamide, vincristine, methotrexate, and prednisone (COMP) did not improve the outcome of patients with advanced-stage non-LL as compared with COMP alone.[478]

The therapeutic strategies used for children with mature B-cell lymphomas are associated with significant short-term toxicity including severe mucositis and risks of infections. The mucosal toxicity is caused primarily, but not solely, by high-dose methotrexate. In patients with significant mucositis together with the severe neutropenia, risks of bacteremia (often from intestinal flora) are high. Patients with advanced disease stages are at the greatest risk. For these patients, the rate of toxicity-induced death is up to 5%, even in the most recent treatment studies.[266,475] Postchemotherapeutic application of granulocyte colony-stimulating factor (G-CSF) had no effect on treatment-related toxicity, as demonstrated in a randomized trial.[479] Toxicity and the risk of toxic death is greatest during and after the first treatment course.

Risk stratification for patients with BL and DLBCL is of critical importance, allowing for allocation to the most appropriate treatment regimen. Treatment regimens vary in intensity based on the number of drugs used, their dosage, and the number of treatment courses that have obvious implications for both short- and long-term toxicities. Criteria used to stratify patients include stage, LDH level, and CNS involvement but have differed somewhat between cooperative groups (Table 53-15). Other prognostic factors include poor response to "reduction therapy" with cyclophosphamide, prednisone, and vincristine; poor-risk cytogenetics; and mediastinal disease.[473,480] The genetic changes detectable by karyotype associated

TABLE 53-15 Treatment Stratification and Results in Two Large Multicenter Trials of Pediatric Mature B-Cell Neoplasms

Group	Definition	Patients, N (% of all patients)	EFS (%)	Chemotherapy Courses (Randomized Study Questions)	Outcome
FAB-LMB96 Trial[269,483,606]					
A	Stage I, resected; stage II, abdominal, resected	132 (12%)	98 (4 year)	COPAD-COPAD	2 courses of therapy and no intrathecal treatment is curative in patients with resected localized disease.
B	Stage I + II not resected; Stage III Stage IV with bone-marrow blasts <25%	657 (67%)	90 (4 year)	COP-COPADM1-COPADM2-CYM-CYM-M1 (randomization for complete responders after COPADM1 to therapy reduction verus standard)	Therapy reductions of 50% dose reduction of cyclophosphamide and omission of M1 was equally as efficacious as the standard regimen.
C	>25% bone-marrow blasts and/or CNS+ nonresponder to COP of group B	235 (21%)	79 (4 year)	COP-COPADM1-COPADM2-CYVE1-CYVE2-M1-M2-M3-M4 (randomization for complete responders after COPADM1 + M2)	Therapy reductions of reduced intensity “mini-CYVE” and omission of M2, M3, and M4 resulted in inferior outcome.
NHL-BFM95484					
R1	Stages I + II, resected	48 (10%)	94	A-B (randomization: methotrexate IV over 24 hr vs. 4 hr)	Methotrexate over 4 hr less toxic, equally efficacious
R2	Stages I + II, not resected; stage III, LDH <500/U/L	233 (46%)	94	p-A-B A-B (randomization: methotrexate IV over 24 hr vs. 4 hr)	Methotrexate over 4 hr less toxic, equally efficacious
R3	Stage III, LDH 500-999 U/L Stage IV and LDH <1000U/L and CNS–	82 (16%)	85	p-AA-BB-CC-AA-BB (randomization: methotrexate in AA, BB IV over 24 hr vs. 4 hr)	Methotrexate over 4 hr less toxic but in this group less effective
R4	Stage III + IV and LDH ≥1000 U/L and/or CNS+	142 (28%)	81	p-AA-BB-CC-AA-BB-CC (randomization: methotrexate in AA, BB IV over 24 hr vs. 4 hr)	Methotrexate over 4 hr less toxic but in this group less effective

COP, Cyclophosphamide, vincristine, prednisone; *COPAD,* cyclophosphamide, vincristine (Oncovin), prednisolone, and doxorubicin; *CYM,* cytarabine, methotrexate; *CYVE,* cytarabine, etoposide; *EFS,* event-free survival; *FAB-LMB,* French-American-British–lymphoma malignancy B; *LDH,* lactate dehydrogenase; *M,* maintenance; *NHL-BFM,* Non-Hodgkin Lymphoma–Berlin-Frankfurt-Munster.

with an inferior outcome are +7q and deletion (13)q.[452] The presence of minimal disseminated disease in the bone marrow at diagnosis has also been found to be associated with a worse prognosis.[481] The use of bone marrow monitoring for assessment of responses to therapy and persistence of minimal residual disease may be of importance in the future.[482] Within pediatric protocols neither age (older or younger than 15 years) or histology (BL versus DLBCL) is prognostic.

Patients with localized disease have excellent outcomes with an EFS rate approaching 100%. The international study FAB-LMB96 enrolled patients of all disease stages. One hundred and thirty two patients with resected stage I or completely resected abdominal stage II disease were treated with two courses of cyclophosphamide, vincristine, prednisolone, and doxorubicin (COPAD) without intrathecal therapy. The 4-year EFS and OS rates were 98.3% and 99.2%, respectively.[483] In the NHL BFM95 study, 48 patients with completely resected stage I or II disease were treated with two courses of therapy which include dexamethasone, vincristine, ifosfamide, cytarabine, etoposide, cyclophosphamide, doxorubicin, and methotrexate in addition to intrathecal triple therapy and had a 3-year EFS rate of 94%.[484]

Patients defined as having intermediate-risk disease according to the FAB-LMB protocols, the “group B” patients (see Table 53-15) compromise the largest group. These are individuals with unresected stage I or II disease, those with stage III or IV disease without CNS involvement, or those with bone marrow disease meeting criteria for Burkitt leukemia. This patient group also has an excellent outcome with modern intensive therapy. Patients in the FAB-LMB96 study were randomized in a 2-×-2 factorial design to evaluate two-dose reduction questions, reduction of cyclophosphamide dose, and removal of a maintenance cycle of therapy. Six hundred and fifty seven patients with intermediate-risk disease were randomized. The 4-year EFS and OS rates were 90.9% and 93.4%, respectively, with no significant difference across study arms. Patients in the NHL-BFM95 study with stage I or

II unresected disease or those with stage III disease with a low LDH who received four courses of chemotherapy also had a similarly favorable outcome with 3-year EFS rate of 94%.[484]

The use of LDH for stratification of treatment intensity is a major difference between the FAB-LMB and the NHL-BFM studies (see Table 53-15). The serum concentration of LDH, considered a proxy for tumor mass, may vary largely in patients at the same stage, especially for those with stage-III BL. Although not used prospectively for risk assignment on the FAB-LMB study, analysis of patients enrolled showed that LDH greater than two times the institutional upper limit of normal was a significant risk factor for an inferior outcome.[480] The current international study of childhood B-cell lymphoma, InterB-NHL 2010 Ritux defines a group of patients with high-risk disease in part based on LDH.

Patients with high-risk disease in the LMB studies have been defined as those with CNS disease (parenchymal brain involvement, cranial nerve palsies, or CSF disease) or those with Burkitt leukemia (bone marrow with >25% blasts). BL accounts for 70% to 80% of the patients in this group. The EFS and OS rates of this patient group approaches 80%. Reduction of therapy in patients with high-risk disease has not been successful, as it has been for the low- and intermediate-risk groups. In the FAB-LMB96 study the 4-year EFS rate for all patients with high risk was 79%.[269] For those who had responsive disease and therefore were eligible for randomization, the EFS was 90% for those receiving standard therapy and 80% for those receiving reduced intensity therapy. This difference led to the closure of the randomization and assignment of subsequent patients to the standard therapy arm. The NHL BFM95 evaluated the reduction of high-dose methotrexate-related toxicity by shortening the infusion time. Unlike in patients with lower risk where shorter infusion times had similar disease control but less toxicity, in patients with high risk changing the infusion time for the high-dose methotrexate from the standard 24 hours to only 4 hours resulted in an excess of tumor failures in patients with advanced disease.[484] The 3-year EFS rate for R3 and R4 groups (groups described in Table 53-15) overall were 85% and 81%, respectively. Combined, the R3 and R4 groups who received a shorter (4-hour) methotrexate infusion had an EFS rate of 77% versus 93% for those who received the longer (24-hour) infusion.

The group of patients with the worst outcome with current therapy is that of patients with CNS disease, defined by blasts in the CNS, cranial nerve palsies, or an intracranial mass. Among these patients the subgroup with the least favorable prognosis is those with blasts detectable in the CSF at the time of diagnosis, with EFS of 70% to 75% in FAB and BFM trials.[269,484]

Rituximab, the monoclonal chimeric anti-CD20 antibody, when added to conventional chemotherapy for adults with mature B-cell NHL, significantly improves outcomes.[485-487] For children and adolescents it is not part of standard therapy, primarily based on lack of evidence of efficacy in this patient group. Given the favorable outcome of children with conventional chemotherapy, the significant differences between chemotherapy regimens used in children and adults and the biologic difference in pediatric B-cell NHL, the adoption of the data from adult studies cannot be simply applied to pediatric care. Rituximab has shown to be active as a single agent when given upfront in a window trial before conventional therapy for children with B-cell NHL.[488] The COG has established the safety of combining rituximab with FAB-LMB96 therapy in a single-arm pilot trial of patients with high-risk disease.[489] The current international phase III trial for patients with high-risk B-cell NHL, InterB-NHL_2010_ritux will randomize patients to therapy based on the FAB backbone with or without rituximab to gather the needed data regarding efficacy.

Primary Central Nervous System DLBCL. Primary CNS NHL is exceptionally rare in children and adolescents. Most primary CNS NHLs of childhood are of the diffuse large B-cell subtype, though other histologies have been described.[265,490] In a large cohort of 2170 patients of the NHL-BFM studies, only 10 patients were observed with primary CNS lymphoma.[265] The estimated incidence in children in North America is 15 to 20 cases per year.[491] Most cases present with unifocal or multifocal intraparenchymal masses but some patients have meningeal disease only or combinations of both. The clinical features resemble those of brain tumors characterized by signs of increased intracranial pressure with headache, nausea and vomiting, and visual changes, as well as cranial nerve palsies, personality changes, and endocrinologic disturbances.

As with other rare tumors, optimal therapy in children and adolescents with primary CNS lymphoma is not clear. Treatment analogous to what patients who have CNS involvement as a component of more disseminated disease would receive has been effective in the BFM series.[265] The largest case series of pediatric patients with isolated CNS disease included 29 patients treated in various regimens with the majority receiving methotrexate-based regimens and one third receiving radiation in addition to chemotherapy. The 2-year OS rate was 82%.[492] The use of intrathecal rituximab combined with intrathecal methotrexate in adult patients with recurrent B-cell CNS lymphoma in a phase-I trial showed it to be feasible and active, although results have not been reported in children.[493]

Therapy of Relapsed Disease

Given the effectiveness of primary therapy, relapses are relatively rare. When they do occur, patients with BL generally relapse within 6 months from the time of completion of therapy. The period of risk is somewhat longer for those with DLBCL. Patients with primary refractory or relapsed B-cell NHL have a dismal prognosis. In a cohort of patients diagnosed with BL between 1977 and 1997, only 12% of those with relapsed disease survived.[494] There is no standard reinduction regimen for these patients. Commonly used regimens have included ICE as well as ifosfamide, etoposide and high-dose cytarabine.[416] A COG study evaluated the effectiveness of rituximab in addition to ICE therapy and showed a response rate of 60%. Six of the 20 patients were able to proceed in to

SCT. The OS rate at 3 years was 37%, with a majority of the survivors having received either an autologous or allogeneic SCT.[495] Retrospective data from the Center for International Blood and Marrow Transplant Research (CIBMTR) between 1990 and 2005 showed similar outcomes for both autologous and allogeneic SCT for patients with relapsed BL (31% and 27%, respectively) and DLCBL (50% and 52%, respectively).[411]

Anaplastic Large-Cell Lymphoma

ALCL accounts for 10% to 15 % of childhood NHL.[496] ALCL was first described by Stein and colleagues[18] in 1985, followed by the discovery of the nonrandom translocation of t(2:5) (p23; q35), causing the fusion of nucleophosmin (NPM) to the anaplastic lymphoma kinase (ALK) in a majority of the cases.[497,498] The disease has multiple features that are unlike other pediatric NHL subtypes, including the sites of involvement of disease at diagnosis and the responsiveness of relapsed disease to therapy as well as unique biologic features.

Pathologic Features

ALCL is characterized by large pleomorphic cells of a T/null immunophenotype. The morphology is variable but includes cells with eccentric horseshoe-shaped nuclei, called the *hallmark cells*.[39] A number of histologic variants have been described, including the common variant, characterized by sheets of large lymphoid cells; the lymphohistiocytic variant, characterized by a large number of reactive histiocytes that may even mask the anaplastic tumor cell population and can therefore be misdiagnosed as reactive histiocytic disease or malignant histiocytosis[40]; the small-cell variant, characterized by a predominant population of small to medium sized neoplastic cells with irregular nuclei[499,500]; and other rare variants including the giant cell variant, sarcomatoid variant, and neutrophil-rich variant. In some cases, mixtures of histologic subtypes are observed in one lesion, whereas in other patients different subtypes can be seen in different lesions.[500] In addition, in patients with relapsed disease the morphology of disease at relapse may be a different variant than was present at initial diagnosis.[501]

ALCL is CD30-positive on the cell membrane, particularly on large cells and in the Golgi region.[29] ALK detection is reliably measured by immunohistochemistry.[502] Cells express T-cell markers including CD2, CD5, and CD4, although the null-cell phenotype may not express several pan–T-cell antigens.[39]

ALK-negative ALCL is a unique disease category in the WHO classification. It is morphologically indistinguishable from the ALK-positive disease but lacks the expression of the ALK protein.[25] It accounts for less than 10% of pediatric disease and unlike in adult patients, has not been found to be associated with a worse prognosis in children.[273,274,500,503,504]

Molecular and Cellular Biology

The clonal nature of ALCL has been confirmed by the detection of the nonrandom chromosomal translocation t(2;5)(p23;q35) in malignant cells.[497,505,506] This translocation causes the *NPM* gene located at 5q35 to fuse with a gene at 2p23 encoding the receptor tyrosine kinase ALK.[498] The production of monoclonal antibodies directed against fixative-resistant epitopes of ALK allows its detection by immunohistochemistry.[507,508] Although the translocation t(2;5)(p23;q35) is the most common, more than 10 other chromosomal translocations juxtaposing the *ALK* gene at chromosome 2p23 to a partner gene have been detected. The partner gene of *ALK* determines the immunohistochemical ALK staining pattern. Only the NPM-ALK fusion protein is detectable in the cytoplasm and the nucleus because of the heterodimerization of the NPM portion with normal NPM, which functions in the nuclear shuttling of proteins.[509] All other fusion-gene products lacking the NPM portion are detectable only in the cytoplasm. Conversely, the ALK staining pattern, cytoplasmic and nuclear versus extranuclear, distinguishes the NPM-ALK translocation from all others.[510] In children, more than 90% of ALCL cases are ALK-positive.[273]

The cellular origin of ALCL is still not completely clarified. The vast majority express T-lineage antigens, have clonally rearranged *TCR* genes, and express the cytotoxic molecules perforin, granzyme B, and T-cell–restricted intracellular antigen (TIA) 1.[511] However, findings of plasmablastic B-cell lymphoma carrying the translocation t(2 ;5) and expressing NPM-ALK transcripts have suggested that the translocation involving ALK may not be restricted to ALCLs of T-cell lineage.[512] In a minority of cases, the expression of the natural killer (NK) cell–associated marker CD56 and the lack of detectable *TCR* gene rearrangements suggest an origination from NK cells rather than cytotoxic T cells.[511,513,514]

In most cases, ALCL occurs as a de novo malignant lymphoma. On rare occasions, however, ALCL appears as a secondary lymphoma that evolves from a low-grade or high-grade malignant T-cell lymphoma, Hodgkin lymphoma, or lymphomatoid papulosis.[515-518]

As mentioned, the most characteristic molecular feature of ALCL is the presence of gene rearrangements involving the *ALK* locus in chromosome band 2p23. ALK is a transmembrane receptor tyrosine kinase of the insulin receptor superfamily normally expressed only in cells of neural origin. ALCL translocations lead to the aberrant expression and constitutive activation of ALK.[519] The most common of these translocations present in 80% of ALCL cases is the t(2;5)(p23;q35).[520,521] In this translocation, the 5′ region of the *NPM* gene on chromosome 5q35 is fused to the *ALK* gene.[498,521-523] NPM is a ubiquitously expressed nucleolar phosphoprotein involved in the shuttling of ribonucleoproteins between the nucleus and cytoplasm.[524] The *NPM-ALK* fusion oncogene resulting from the t(2;5)(p23;q35) translocation encodes a constitutively active form of ALK,[521] which promotes cell transformation in vitro[509,525] and in vivo[526] by phosphorylating and activating important mediators in a number of critical signaling pathways such as PLCG, PI3K-AKT-mTOR, JAK2, STAT3, STAT5, and MEK-ERK.[519,522,527]

In addition to the t(2;5)(p23;q35) rearrangement, several other chromosomal translocations involving the *ALK* gene have been found in 20% of ALK-positive ALCL cases. All these rearrangements result in the fusion of the catalytic domain of ALK with different partners, such as tropomyosin *(TPM)* 3 and *TPM4, TGF, ATIC,* clathrin heavy chain, moesin, *AL017, TSPYL2, KIAA1618, VCL, KIFB5,* and *MYH9,*[519,527] and result in constitutively active ALK phosphorylation and activation.[521,528] All partners except *MYH9* provide dimerization domains, leading to the dimerization of the fusions and to the constitutive activation of the kinase.[528] The importance of the elucidation of constitutively active ALK as a major mechanism in the transformation of ALCL is highlighted by the development of specific ALK inhibitors. These may provide a targeted therapeutic strategy for treatment of this disease.[529]

In addition to ALK rearrangements, ALCLs also show other chromosomal alterations; however, few recurrent somatic lesions have been reported. About 25% of ALCLs display recurrent deletions affecting the 17p13.3-p12 region where the *TP53* gene is located, and 19% of cases show losses of 6q21 encompassing the *PRDM1* and *ATG5* genes.

Clinical Features

Most children and adolescents with ALCL have presenting symptoms of advanced stage disease. Almost all have lymphadenopathy at diagnosis, but 60% also have extranodal disease, most often involving soft tissue and skin followed by bone and lung.[274,503,504,530-532] Patients may have an indolent phase consisting of lymphadenopathy, which in some instances may be waxing and waning and sometimes associated with a history of illness including fever and other B symptoms.[516,533] Soft-tissue involvement may occur as multiple tumors in the subcutaneous tissue and muscles, or as a single larger tumor resembling a soft-tissue sarcoma. Bone manifestations vary from small osteolytic lesions to large tumors simulating bone tumors. Skin lesions may be single or multiple bluish-colored cutaneous or subcutaneous nodules, large ulcerated lesions or multiple or disseminated papillomatous lesions of a red-yellow color.

Compared with patients with BL or LL, the incidence of microscopically detectable bone-marrow disease at the time of diagnosis is lower. Occasionally, marked hemophagocytosis by macrophages can be observed in the bone marrow of patients with ALCL. In rare cases, disseminated intravascular coagulation of yet unknown pathogenesis has been observed as a paraneoplastic syndrome.[534] CNS disease is rare in patients with ALCL, affecting 1% to 4% of children.

Treatment and Outcomes

Prognostic Factors. Prognostic factors based on standard on clinical information used for informing treatment decisions have been less well-defined for patients with ALCL compared with other pediatric lymphomas. As with other NHLs, lower-stage disease is thought to be favorable. In the BFM90 study, nine patients with stage I and II resected disease had an EFS rate of 100% at 5 years with three courses of therapy.[274] A report including 36 patients with stage I disease on the ALCL99 study showed that those who had completely resected disease and received short-course chemotherapy also had an EFS rate of 100%, whereas those with incompletely resected disease had a relapse rate of 30%, similar to patients with higher-stage disease.[535]

A study that combined patients from three cooperative groups found that poor prognostic risk factors included disease involving the mediastinum, visceral involvement (lung, liver, or spleen), or skin lesions. Factors not significant in multivariate analysis included stage, B symptoms, and level of LDH.[536] In contrast to adults with ALCL, the *ALK1* positivity or negativity had no significant impact on outcome for children and adolescents.[273,274] However, this may be partly related to the relatively rarity of *ALK*-negative disease in children and therefore there is limited power to detect outcome differences.

Two specific tests, both unique to patients with *ALK*-positive ALCL and both done at the time of diagnosis, have been found to have prognostic significance in children with ALCL: the titer of anti-ALK antibodies in blood and copy number of *NPM-ALK* transcripts measured by quantitative PCR of bone marrow. Patients with *ALK*-positive ALCL often have measurable autoantibodies to ALK at the time of presentation with disease.[508] In a study of 95 uniformly treated pediatric patients with *ALK*-positive ALCL, an increased level of ALK autoantibodies in the patient's serum from the time of diagnosis correlated with a significantly lower incidence of relapse, suggesting that a preexisting immune response to the antigen product of the disease-specific translocation was protective from the risk of relapse.[537] Patients with morphologic involvement of their bone marrow make up a small proportion of those with ALCL. In a study of 80 pediatric patients with *ALK*-positive ALCL, only eight had morphologic evidence of bone-marrow involvement, but by assessing these samples with PCR, it was determined that 47.5% had detectable *NPM-ALK* transcript in these bone marrow samples. Using quantitative PCR it was found that those with a higher level of *NPM-ALK* transcript detection had a significantly higher rate of relapse.[538] When the two tests were combined from three patients groups treated in similar though not identical ways, three risk groups could be identified with dramatically different rates of progression survival. Twenty percent of patients had evidence of minimal disseminated disease as determined by PCR and had low or negative antibody titers. This high-risk group had a progression-free survival (PFS) rate of 28%. Conversely those who were negative for minimal disseminated disease and had high antibody titers, 31% of the patients, had a PFS rate of 93%. The intermediate-risk group, which included all others and accounted for 48% of the patients, had a PFS rate of 68%.[539] Replication of these findings and use in patient stratification are components of current and planned cooperative group trials.

Treatment. There is no consensus on optimal therapy for ALCL. Some groups have used brief intensive therapy, similar to that used for mature B-cell disease, whereas others have investigated more prolonged repeated pulse chemotherapy. EFS rates are very similar across studies even with the use of these variable chemotherapy regimens (Table 53-16).

TABLE 53-16 Prospective Randomized Trials of Childhood and Adolescent Anaplastic Large-Cell Lymphoma

Study Protocol (years)	No of Patients	Description of Therapy	Primary Study Question and Design	EFS at 3-5 Years	Primary Study Outcome
NHL-BFM90 (90-95)[274]	89	Short-pulse B NHL-type therapy: Stage I/II resected, 3 courses; Stage II non resected, stage III, 6 courses; Stage IV, 6 intensified courses; 2-5 months	Effectiveness of therapeutic regimen in nonselected group of uniformly treated patients, non randomized	76%	Short-pulse B NHL therapy is effective in ALCL.
POG (94-00)[530]	180	APO-based therapy for patients with large-cell lymphoma, both ALCL and DLBCL 12 months	Evaluation of intensified therapy with intermediate dose methotrexate and high-dose Ara-C, randomized between two regimens postinduction	71%	Intensification of therapy did not improve EFS and increased toxicity.
EICNHL (99-05)[504]	352	Short-pulse B NHL-type therapy; 5 months	Evaluation of modification of methotrexate dose and infusion time with or without intrathecal therapy, of efficacy of therapy with 3 g/m^2 over 3 hours versus 1 gm/m^2 over 24 hours	74% at 2 years	Methotrexate at 3 g/m^2 over 3 hours was as effective and less toxic than a lower dosage with prolonged infusion plus intrathecal therapy. Addition of vinblastine in maintenence delayed relapse but did not impact 2-yr EFS.
ALCL99 (99-06)[503]	110	Short-pulse B NHL-type therapy; 5 months	Evaluation of addition of vinblastine in maintenence versus no maintenence	70%-73% at 2 years	Addition of vinblastine in maintanence delayed relapse but did not impact 2-yr EFS.
COG[542]	125	APO-based therapy; 12 months	Evaluation of vincristine versus vinblastine in postinduction	76% at 3 years	No benefit was found with the use of vinblastine compared with vincristine; vinblastine administration was limited by myelotoxicity.

ALCL, Anaplastic large-cell lymphoma; *APO,* doxorubicin, vincristine, prednisone, 6-mercaptopurine, and low-dose methotrexate; *Ara-C,* cytarabine; *COG,* Children's Oncology Group; *DLBCL,* diffuse large B-cell lymphoma; *EFS,* event-free survival; *EICNHL,* European Intergroup for Childhood Non-Hodgkin Lymphoma; *NHL,* non-Hodgkin lymphoma; *NHL-BFM,* Non-Hodgkin Lymphoma–Berlin-Frankfurt-Munster; *POG,* Pediatric Oncology Group.

Analysis of patients from three consecutive BFM trials showed that the use of B-cell NHL short-pulse therapy was effective for the ALCL group, with a 5-year EFS rate of 76%.[540] In the BFM90 trial, 89 patients with ALCL were treated. In this trial patients were divided into three groups based on stage: K1, stage I and II resected; K2, stage II nonresected and stage III; and K3, stage IV or those with multifocal bone involvement. All groups received a prephase treatment and then 3 to 6 cycles of therapy lasting 2 to 5 months, with the K3 group's therapy intensified primarily by higher dose methotrexate. With this regimen the OS rate at 5 years for the whole group was again 76%.[274] Of the patients that did relapse, the vast majority did so within 15 months of the completion of therapy. Relapses occurred at both original and new sites.

After the BFM90 trial, a large randomized study, ALCL99, was conducted by the European Intergroup for Childhood Non-Hodgkin Lymphoma (EICNHL) and designed to address two questions in patients with ALCL. The first task was to assess the safety and efficacy of two methotrexate regimens (3 g/m^2 over 3 hours with no intrathecal therapy versus 1 g/m^2 over 24 hours with intrathecal therapy). The 2-year EFS rate was identical in the two arms at 74%, although the shorter high-dose methotrexate arm was associated with less toxicity. There was no difference in CNS relapses. Again in this trial it was noted that almost all of the relapses occurred within 2 years from completion of therapy.[503]

The second question addressed in ALCL99 included only the high-risk patients, defined as those with mediastinal, lung, liver, spleen, or skin involvement. Eligible patients were randomized to receive vinblastine concurrent with intensive therapy and as a single-drug maintenance regimen for a total duration of therapy of 1 year versus the standard backbone therapy and no maintenance.[503] At 1 year from the time of randomization the EFS rate for those receiving vinblastine was superior (91% versus 74%), but by 2 years the difference was no longer present with an EFS rate of 73% and 70% respectively. The conclusion was that vinblastine delivered in this manner delayed recurrences but did not impact the overall outcome.[503]

In North America POG performed a series of clinical trials evaluating modifications of the APO therapy backbone.[530,541] APO therapy involves the use of doxorubicin, prednisone, and vincristine in addition to 6-mercaptopurine, low dose methotrextae and intrathecal therapy given as multiple cycles over 2 years in the outpatient setting. This therapy includes a cumulative anthracycline dose of 300 mg/m^2. Patients in these earlier studies included those with advanced-stage disease and with both DLBCL and T-cell phenotype large-cell lymphoma. In these studies, neither intensifying therapy with cyclophosphamide or with intermediate-dose methotrexate and high-dose cytarabine had an impact of EFS, with no statistical difference in outcome between randomized arms in either study. The 4-year EFS rate for the subgroup of ALCL patients, irrespective of randomized therapy, was 71%.[530] Analogous to the pattern in the European trails, the majority of relapses occurred within 2 years of diagnosis.

Subsequent to these studies and analogous to the ALCL99 trial, the COG investigated the impact of vinblastine in the context of APO therapy. In the trial, patients were randomized between treatment with vincristine or vinblastine as part of multiagent maintenance therapy based on the APO backbone. No benefit was found with the use of vinblastine compared with vincristine, although the ability to administer the vinblastine at the originally planned doses was hampered by cytopenias.[542]

The incidence of CNS relapse in patients who are initially CNS-negative is very low, with therapy protocols that involve corticosteroids and systemic methotrexate therapy with or without intrathecal therapy.[265,273,274,516] Prophylactic cranial irradiation is unnecessary. In the European intergroup trial ALCL99 based on the treatment strategy of the NHL-BFM90 study, including dexamethasone as a corticosteroid, it was shown that systemic methotrexate, 3 g/m^2, given as an IV infusion for 3 hours, is sufficient CNS protection for patients without overt CNS disease at diagnosis, and additional intrathecal chemotherapy is not necessary.[534] Because of the small numbers of patients with overt CNS disease at diagnosis, no conclusions can be drawn about optimal treatment in this circumstance. Therapeutic cranial irradiation with doses of 18 to 24 Gy, depending on age, may be efficacious in addition to high-dose methotrexate and high-dose cytarabine combined with triple-drug intrathecal therapy.[273,274]

Treatment of Relapsed Anaplastic Large-Cell Lymphoma

One of the unique features of ALCL compared with other pediatric NHLs is its sensitivity to chemotherapy after recurrence. Of particular interest is the experience of maintenance of prolonged second remissions with single-agent vinblastine. A report of 41 children with relapsed disease enrolled in three consecutive French Society of Pediatric Oncology trials described outcomes with various second-line therapies.[543] Thirty-six of 41 patients (88%) were able to achieve a second complete response. The overall and disease-free survival rates were 69% and 44%, respectively, at 3 years. In this series it was noted that eight of 13 patients had long-lasting remissions with single-agent vinblastine therapy alone. A second report described the experience with 30 patients treated with single-agent vinblastine most commonly given at a dose of 6 mg/m^2 weekly, for varying durations, at varying time points in the patient's disease course, although the majority were treated for first of second relapse.[504] Fifty percent of the patients had a preceding SCT before their vinblastine therapy. The CR rate for the whole group was 83%. The 5-year EFS and OS rates for the whole group were 30% and 65%. Of note, 30% of those treated with vinblastine only remained in CR.

ALCL is characterized in part by its expression of CD30. Monoclonal antibody therapy directed at CD30 has shown to be safe but with limited activity in early phase studies in adult patients.[544] Brentuximab vedotin (SGN-35) is an antibody drug conjugate combing an anti-CD30 antibody with a potent microtubule inhibitor, monomethylauristatin E (MMAE). A phase-II study in adult patients with primary refractory or relapsed ALK-positive or ALK-negative ALCL showed very encouraging results.[545] Eighty-six percent (50/58) of heavily pretreated patients had a response to therapy, and 57% obtained complete response to monotherapy with brentuximab vedotin. Toxicity was modest, including cytopenias and peripheral neuropathy. Studies evaluating the safety and efficacy of this agent in children and in combination with conventional chemotherapies as part of initial therapy and therapy for relapsed disease for patients with both Hodgkin lymphoma and ALCL are being undertaken.

Another extremely exciting agent for the treatment of children and adolescents with ALK-positive ALCL is crizotinib, a small-molecule inhibitor of ALK tyrosine kinase. Two adult patients with refractory ALK-positive ALCL were able to obtain a complete response with crizotinib.[546] In the pediatric setting it is being investigated in early-phase studies for patients with ALK-positive disease including ALCL and neuroblastoma.[547] Given the early indications of safety of the drug and of efficacy in patients with ALCL, plans for evaluating crizotinib in conjunction with standard chemotherapy in newly diagnosed patients are being pursued by the COG.[232]

Rare Lymphomas in Children and Adolescents

LL, mature B-cell lymphomas, and ALCL account for the vast majority of NHLs diagnosed in children and adolescents; however approximately 10% of cases are made up of an array of much rarer entities, some of which are much more commonly seen in adults. A discussion of some of these rare tumors is included below.

Primary Mediastinal (Thymic) Large B-Cell Lymphoma

Primary mediastinal (thymic) large B-cell lymphoma (PMBCL) is a DLBCL with unique clinical, histologic, and genetic characteristics that have justified its being identified as a unique entity in the WHO classification system for lymphomas.[39] Microscopically a prominent compartmentalizing sclerosis dominates the histologic appearance. The malignant cells are of putative thymic B-cell origin and are large, often pleomorphic, lymphoid cells with abundant pale cytoplasm. The cells express CD45 and B-lineage markers such as CD19, CD20, and CD79a, but surface immunoglobulin is usually absent. Contrary to DLBCL, PMLBL cells do not express CD10 or *BCL6* and *BCL2*.[548] The neoplastic cells may express CD30, although usually less strongly than in Hodgkin lymphoma.

PMBCL usually presents with symptoms emanating from the effects of a large mediastinal mass such as cough, shortness of breath, and signs and symptoms of SVC syndrome. The tumor generally spreads locally with invasion into the pleura, pericardium, and lungs but may also involve the liver.[549] Bone-marrow and CNS involvement is exceptionally rare.[550]

The prognosis for pediatric patients with PMBCL has been less favorable than for the other mature B-cell lymphomas. Multiple pediatric international cooperative group studies have reported remarkably consistent outcome data with a 5-year EFS rate of 66% to 67% and an OS rate of 73% to 79%.[551,552] The best results reported to date are from a case series of adult patients treated with rituximab in addition to the combination chemotherapy regime of dose-adjusted etoposide, prednisone, vincristine (Oncovin), cyclophosphamide, and doxorubicin (hydroxydaunorubicin) (EPOCH).[553] The importance of radiotherapy in this group of patients is controversial, with some adult studies reporting improved outcome with IFRT, whereas its impact on patient outcomes in the combined pediatric data showed no advantage.[551,554] The international pediatric B-cell study (interB NHL-ritux-2010) will evaluate the use of dose-adjusted EPOCH with rituximab in a single-arm phase-II component of the larger trial.

Juvenile Follicular Lymphoma

FL is a common diagnosis in adults with NHL but is very rare in children, accounting for fewer than 2% of all pediatric cases. In the 2008 WHO classification of lymphomas, pediatric FL is identified as a unique entity based on patient age but also on the unique biologic features including lack of *BCL2* expression and absence of t(14:18) in the majority of cases.[39] Pediatric FL tends to be of higher grade than its adult counterpart.[555] Children with FL generally have a presentation of low-stage disease, most often involving cervical nodes, other peripheral nodes, Waldeyer ring, or isolated testicular disease. The outcome for children with FL is very favorable including a small number of patients successfully treated with surgical resection alone, although the majority of patients have been treated with brief courses of multiagent chemotherapy.[556-558] Unlike in adult patients, the use of rituximab in the treatment of pediatric FL has not been investigated.[559]

Peripheral T-Cell Lymphomas

Peripheral T-cell lymphomas (PTCLs) other than ALCLs are rare in childhood and account for fewer than 1% of cases. They represent a heterogeneous group of neoplasms, with the most common histology among pediatric patients being PTCL, not otherwise specified. The clinical features, histology, and genetic features are variable by subtype.[39]

Data in children and adolescents with non-ALCL PTCL are limited to several case series and a number of case reports but suggest that the outcome of these patients is inferior compared with that of the more common pediatric NHL histologies.[560-563] Patients with lower-stage disease have a more favorable outcome, although the majority of patients have presenting symptoms of advanced stage disease. The optimal therapy for patients with non-ALCL PTCL is unclear, with some groups suggesting B-cell NHL–type therapy and others T-cell leukemia/lymphoma–like therapy, and with some data supporting specific therapies based on specific histology. In addition, the role of high-dose therapy and autologous or allogeneic transplant in the management of pediatric patients remain unclear.

Late Effects of Therapy

In contrast to pediatric Hodgkin lymphoma, few reports are available on late effects in long-term survivors of childhood and adolescent NHL. The risk of second cancer for survivors of childhood and adolescent NHL appears to be much lower compared with patients with Hodgkin lymphoma. In the CCSS, the excess risk for second cancers was among the lowest for children treated for NHL as compared with all other malignancies, with a cumulative risk for SMN of 1.9% after 20 years.[202,564] However, only events occurring 5 years or later from diagnosis of NHL were included in that analysis, and therefore secondary AML, which usually has a short latency, may have been underestimated in that study. In a single-center study of 497 former pediatric NHL patients, AML was reported to be the most common second malignancy.[565] In that study, the cumulative incidences of SMN at 10 and 20 years were 2.1% and 4.8%, respectively. Seven of 16 SMNs observed were AML with exposure to epipodophyllotoxins a significant risk factor. Another analysis of 103 patients treated as children for NHL, major late effects observed were cardiac toxicity and hepatitis C virus (HCV) infection, the latter presumably resulting from blood transfusions before HCV screening was introduced.[566] Cardiotoxicity was observed in patients who received anthracycline doses of more than 400 mg/m^2. Fertility was not greatly impaired. In long-term survivors of pediatric NHL, the risk for limitations on physical performance and daily activities was among the lowest compared with survivors of other childhood cancers, although higher compared with healthy siblings.[567]

POSTTRANSPLANTATION LYMPHOPROLIFERATIVE DISEASE

Lymphoproliferative disorders are the most common malignancies in children and adolescents with iatrogenic impaired immune function after solid-organ transplantation (SOT) or allogeneic hematopoietic SCT.[568]

Posttransplantation lymphoproliferative disease (PTLD) represents a spectrum of clinically, morphologically, and biologically heterogeneous disorders defined as any lymphoid proliferation or lymphoma developing as a consequence of pharmacologic immunosuppression after SOT or hematopoietic SCT. The initial clinical presentation is extraordinarily broad, ranging from a single, small, asymptomatic lymphomatous tumor, an infectious mononucleosis-like syndrome, and unexplained fever to a widespread disseminated disease with a rapid fatal course. Any organ can be involved by interstitial infiltration of lymphocytes or tumorous lesions, including meninges and the CNS. Occasional spontaneous regression is observed and may be a feature intrinsic to the nature of the disorder as a result of a disturbed balance of immune surveillance. Especially in patients who have had hematopoietic SCT, PTLD may even go unrecognized until autopsy because of the lack of localized tumors and may be clinically misdiagnosed for graft-versus-host disease (GVHD) or infection.[569] In post-SOT PTLD, the malignant cells are almost invariably of recipient origin, whereas in postallogeneic SCT the cell of origin is more commonly of donor origin.

Classification and Epidemiology

According to the WHO classification of hematologic malignancies, PTLD is divided into four subtypes: early lesions, polymorphic PTLD, monomorphic PTLD (classified according to the lymphomas they resemble), and classic Hodgkin lymphoma–type PTLD (Box 53-3).[26,39] In the vast majority of cases, PTLD cells are of B-cell lineage, with a wide spectrum of phenotypes.[570] The B-lineage antigen CD20 is expressed in most cases but not all.[571] In rare cases a T-cell phenotype of PTLD is observed.[572] PTLD can be polyclonal, oligoclonal, or monoclonal, and polymorphic and monomorphic lesions can be observed concurrently in the same patient.[573] Of post-SOT PTLD cases, 70% to 80% are associated with EBV, whereas almost all posthematopoietic SCT PTLD cases are EBV-positive.[26]

Box 53-3 **Classification of Posttransplantation Lymphoproliferative Disease**

EARLY LESIONS

Plasmacytic hyperplasia
Infectious mononucleosis-like lesion

POLYMORPHIC POSTTRANSPLANTATION LYMPHOPROLIFERATIVE DISEASE

MONOMORPHIC POSTTRANSPLANTATION LYMPHOPROLIFERATIVE DISEASE

- B cell neoplasms:
  - Diffuse large B-cell lymphoma
  - Burkitt lymphoma
  - Plasma-cell myeloma
  - Plasmacytoma-like lesions
  - Other
- T-cell neoplasms
  - Peripheral T-cell lymphoma, not otherwise specified
  - Hepatosplenic T-cell lymphoma
  - Other

CLASSIC HODGKIN LYMPHOMA–TYPE POSTTRANSPLANTATION LYMPHOPROLIFERATIVE DISEASE

Modified from Swerdlow SH, Campo E, Harris NL et al: WHO classification of tumours of haematopoietic and lymphoid tissues, *IARC Press, 2008, Lyon.*

The incidence of PTLD in post-SOT varies widely. The two most significant risk factors for PTLD are the organ recipient being EBV-negative at the time of transplant and the degree of immunosuppression.[574] Degree of immunosuppression varies with type of SOT, and consequently risks of PTLD vary with organ type, with the incidence highest in patients who have had heart-lung or intestinal transplants, intermediate for those who have had heart or liver transplants, and lowest for patients after kidney transplant. Children have a higher incidence than adults, explained in part by the increased rate of EBV negativity at the time of organ transplant. Risk of PTLD after allogeneic SCT is increased in those with a T-cell–depleted stem-cell source and in patients whose conditioning regimen included antithymocyte globulin.

Treatment and Outcomes

Preemptive strategies for PTLD in patients with SOT have had mixed results. In contrast to cytomegalovirus (CMV), the prophylactic or preemptive use of antiviral drugs such as acyclovir or ganciclovir appears not to be efficacious to prevent EBV-related disease in transplant recipients.[575] Serial quantitative measurements of EBV DNA by real-time PCR in the blood of patients receiving transplants is a sensitive but not specific marker for the risk of development of PTLD.[576-580] The combined testing for EBV viral load and the amount of EBV-specific T cells that are CD8- and/or CD4-positive in transplant recipients increases significantly the accuracy to predict the risk of PTLD.[581,582] The use of preemptive rituximab may prove to be of benefit.[583]

The mortality of PTLD remains high with survival at 2 years ranging between 54% and 89%,[584-587] with deaths attributed both to progressive disease as well as to graft loss. Principles of therapy include early recognition, risk stratification for therapeutic decisions, and considerations for restoring host immunocompetence.

Reduction of immunosuppression (RIS) should be considered in all newly diagnosed patients. The success of RIS varies widely with increased likelihood of response found in those patients with polymorphic histology, localized nonbulky disease, and a normal LDH.[588-590] Patients with localized disease who undergo complete surgical excision and tolerate RIS have a very good outcome.[588,590] The average time to response has been reported as 3 weeks.[589] For patients with monomorphic or disseminated disease as well as those for whom RIS is initiated but then show evidence of progressive disease or organ rejection, additional interventions need to be undertaken. The utility of RIS in PTLD posthematopoietic SCT is unknown.

Monotherapy with the anti-CD20 agent rituximab has been investigated in patients who are unresponsive to RIS or with recurrent disease. Response rates range from 44% to 85% percent with approximately 2 out of 3 of patients having durable responses.[591,592] A single study of patients with posthematopoietic SCT PTLD strongly favored rituximab, with none of those who did not receive it alive at day 180 versus 46% of those who did.[593] A strategy of "risk-stratified sequential approach" with the use of rituximab and RIS for 4 weeks. Those achieving CR had an additional 4 weeks of rituximab, and those with less than a CR received to rituximab plus cyclophosphamide, doxorubicin (hydroxydaunorubicin), vincristine (Oncovin) and prednisone (CHOP) chemotherapy. All had excellent outcomes with a 1-year progression free survival rate of 93%.[594]

Therapy with low-dose chemotherapy regimens have been investigated using cyclophosphamide and prednisone with or without rituximab with evidence of durable responses in approximately 70% of patients.[595,596] Patients with fulminant PTLD, defined as those with fever, hypotension, and disseminated disease, have a worse prognosis that may be improved by the addition of rituximab to low-dose regimens.[596]

Standard intensive lymphoma therapy should be considered for patients who are unresponsive to low-dose regimens, have EBV-negative PTLD, and for those with Burkitt pathology.[597] Patients with CNS involvement of their PTLD have significantly inferior survival rates. For these patients the addition of high-dose methotrexate should be considered. Responses to intrathecal rituximab have been reported.[598,599] The optimal treatment for the rare patient with T-cell PTLD is unknown, although regimens similar to those used for T-cell ALL have been used.

There is no standard therapy for patients with PTLD after SCT. A number of reports have described the efficacy of rituximab in this setting.[591,600,601] Others have reported responses to donor lymphocyte infusions.[602,603] The adoptive transfer of EBV-specific T-effector cells offers a promising option to treat and or even prevent PTLD.[604]

CONCLUSION AND FUTURE DIRECTIONS IN PEDIATRIC LYMPHOMA

Insights into the pathogenesis and cellular biology have dramatically improved, as have the outcomes for children and adolescents treated for lymphoma. Widely accepted classification systems have allowed for clearer description and comparison of data between investigators. Nevertheless, important issues remain. Knowledge of causative factors is still limited. Although lymphomas were among the first human malignancies for which an infectious cause was hypothesized, definitive clarification of the role of infectious agents in the pathogenesis of childhood lymphoma is still evolving. BL and Hodgkin lymphoma, as well as PTLD, are all associated with EBV and account for a considerable proportion of childhood malignancies worldwide. Thus the clarification of an interaction of genetic predisposition and infectious agents in their pathogenesis merits ongoing efforts. For those lymphomas where curative therapy is now the expectation, investigations into reduction of the treatment burden while maintaining efficacy are warranted. In addition, exploration of new treatment options based on more refined understanding of biology and the availability of targeted therapy such as ALK inhibitors or novel monoclonal antibodies has the potential to change the nature of lymphoma therapy. Refining prognostic indicators such as kinetics of disease response by functional imaging or measurement of minimal disseminated and residual disease may allow for more informed decisions regarding treatment intensity. The outcome of children and adolescents with lymphoma has changed dramatically in the past 50 years. The hope and expectation is that progress will continue in the basic scientific understanding and in its translation to clinical care to continue to improve the outlook for these patients.

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CHAPTER 54

Neuroblastoma

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CHAPTER OUTLINE

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Neuroblastoma is an embryonal tumor of the sympathetic nervous system arising from the neural crest. It is a common extracranial solid tumor of childhood and is a disease distinguished by its clinical and biologic heterogeneity. The prognosis for persons with neuroblastoma is variable and largely dependent on tumor biology. Patients with localized disease and favorable tumor biology may be successfully treated with surgery alone or with minimal therapy with an estimated 5-year survival rate of 90%.[1] However approximately half of all patients present with metastatic disease, adverse tumor-specific biologic features, or both. For these children with "high-risk" disease features, cure rates remain poor, with estimated 5-year survival rates of 50%.[1] The development of resistance to chemotherapy and radiation is the cause of most treatment failures, and neuroblastoma accounts for 10% of childhood cancer mortality. This chapter summarizes our current understanding of neuroblastoma biology and pathogenesis and how this rapidly evolving insight has affected current and future treatment strategies.

EPIDEMIOLOGY

Neuroblastoma accounts for 7% of all childhood cancer diagnoses, with more than 650 new cases diagnosed in the United States each year.[2] The incidence of neuroblastoma is 10.5 per million children younger than 15 years of age per year.[2] It is the most commonly diagnosed cancer of infancy.[2] The median age of diagnosis is 19 months, with a peak incidence in children younger than 4 years.[3] There is no racial preponderance, although African Americans are more likely to have high-risk disease and a fatal outcome.[4,5] Large-scale genetic studies to investigate how this likelihood may influence the disease phenotype revealed that a single nucleotide polymorphism (SNP) within the sperm-associated antigen 16 *(SPAG16)* gene had a higher risk allele frequency in the African reference population and contributed to the ethnic disparities in high-risk disease and survival.[6]

The cause of neuroblastoma is largely unknown. The embryonal origin of the tumor, as well as the young age of onset, suggest that prenatal and perinatal exposures may be important. Studies have investigated a variety of prenatal exposures including tobacco, alcohol, pesticides, and maternal medication or drug use, as well as birth characteristics, including small size for gestational age and maternal history of fetal loss.[7-10,11] The findings from these studies have been inconsistent, and none of these associations has been confirmed in large studies.

Because the majority of neuroblastomas produce catecholamine metabolites that can be detected in the urine, infant screening programs have been conducted to see if it is possible to decrease the mortality from high-risk neuroblastoma by diagnosing cases earlier. In Japan, starting in 1985, a nationwide mass screening program for neuroblastoma was conducted in 6-month-old infants. Results showed that the incidence rate of neuroblastoma increased two- to threefold as a result of the screening, implying that without screening, many of these tumors would have regressed spontaneously and would never have been diagnosed clinically. In addition, the majority of patients had low-stage disease and biologically favorable features, and screening did not decrease overall neuroblastoma mortality.[12-17] Through the Quebec Neuroblastoma Screening Program, infants were screened at 3 weeks and 6 months; the findings confirmed the Japanese results[18,19] and showed a significant complication rate in patients undergoing treatment for tumors found by screening.[20] In the German Neuroblastoma Screening Study, investigators looked at postponing screening until 10 to 19 months of age.[21,22] They found less "overdiagnosis" of neuroblastoma and a greater frequency of patients with unfavorable clinical and biologic features. However, no decrease in the mortality of the patients with unfavorable features was documented. Because of these results, mass screening efforts for neuroblastoma in infants have largely been abandoned.

PATHOGENESIS

Embryology

Neuroblastoma arises during development from neural crest cells committed to the sympathoadrenal lineage.[23,24] The two predominant cell types comprising neuroblastoma are neuroblasts and Schwann cells. Neuroblasts derive from pluripotent cells that arise in the neural crest and eventually form various components of the sympathetic nervous system: the sympathetic ganglia, the chromaffin cells of the adrenal medulla, and the paraganglia, which are typical sites where neuroblastic tumors can arise. What causes persistence of these embryonal cells that develop into peripheral neuroblastic tumors is unclear. Genes that dictate neural development could be altered, causing defects in the normal differentiation and programmed cell death pathways, leading to uncontrolled proliferation and aberrant cell survival.

Microscopic neuroblastic nodules have been reported in fetal adrenal glands during development, peaking at around 17 to 20 weeks' gestation and regressing by birth or within the first few months of postnatal life.[25,26] Beckwith and Perrin[27] (1963) termed these nodules, which were histologically identical to neuroblastoma, "neuroblastoma in situ," which they noted during postmortem examinations of infants younger than 3 months of age who died of other causes. These lesions were detected with a frequency fiftyfold higher than the expected incidence of primary adrenal neuroblastoma and were initially thought to be neuroblastomas that regressed spontaneously. However, with new insights into the development of the adrenal gland, it is clear that these are remnants from normal fetal development.[27] Indeed, the genetic profiles of normal developing neuroblasts and malignant neuroblastomas are similar in many respects, as shown by messenger ribonucleic acid (mRNA) expression profiling of sympathetic neuroblasts from human fetal adrenal glands.[28]

Development of the Sympathetic Nervous System

The sympathetic nervous system is derived from the neural crest, a multipotent embryonic structure that appears early in development and is made up of a transient population of cells that migrate from the neural tube to the region of the dorsal aorta during development.[29-31] These cells develop into sympathetic, parasympathetic, enteric, or sensory neurons governed by transcriptional regulators and growth factors as they exit from the neural tube, during migration or at sites where these cells differentiate. Development of the sympathetic phenotype is summarized in Figure 54-1.[32] Premigratory neural crest cells are specified by the expression of several markers, including FoxD3 and SOX10. *Foxd3* is required for sublineage fate specification, migration, and survival. Zebrafish homozygous for *foxd3* inactivating mutations have nearly a complete loss of sympathetic neurons and their precursor populations.[33] *Sox10* is responsible for the multipotency of neural crest–derived cells and is required for sympathetic nervous system and melanocyte development.[34] At the dorsal aorta, prespecified *SOX10*-positive sympathetic progenitors that are under the influence of extrinsic factors, bone morphogenetic proteins, start the process of differentiation into noradrenergic neurons.[35] The basic helix-loop-helix (bHLH) transcription factor *Ascl1* and the homeodomain transcription factor *Phox2b* are the first transcription factors that are expressed upon initiation of differentiation of sympathoadrenal precursors.[36-40] *Phox2a*, a homolog of *PHOX2B*, is expressed downstream of both *Phoxb* and *Ascl1*,[36,40,41] but its role in the initial stages of the sympathetic lineage remains undefined. Further neuronal differentiation occurs under the influence of the transcription factors *Hand2*,[42,43] *Gata2/3*,[44] and *Tfap2a*,[45,46] which interact in a complex regulatory network to ultimately induce the expression of tyrosine hydroxylase (TH) and dopamine beta hydroxylase. These two enzymes are required for

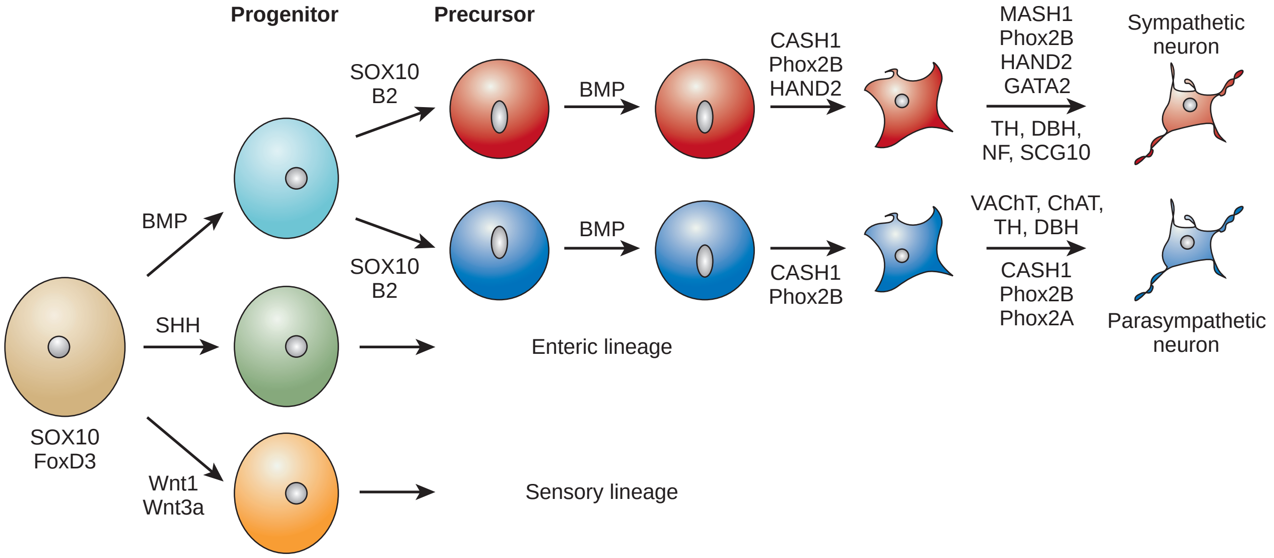


Figure 54-1 The development of the autonomic nervous system. A series of growth factors and transcriptional regulators affect different stages of neurogenesis of neural crest–derived progenitor cells. Neural crest cells segregated from the neuroepithelium can be identified by the expression of forkhead box D3 *(FoxD3)* and sex-determining region Y box–10 *(SOX10)*, among other markers. Progenitor cells differentiate into sympathetic, parasympathetic, enteric, or sensory neurons that are in part dependent upon instructive signals encountered early at or near the time of egress from the neural tube. Additionally, extrinsic cues encountered during migration or at sites where neural crest–derived cells differentiate influence patterns of gene expression. *(Modified from Howard MJ. Mechanisms and perspectives on differentiation of autonomic neurons.* Dev Biol *277:271–286, 2005.)*

catecholamine production and serve as markers of terminal sympathetic neuronal differentiation.[47,48] *Hand2* appears to have more of a role in noradrenergic cell proliferation than differentiation. A massive reduction in sympathetic ganglion size occurs after elimination of Hand2 in the neural crest of model organisms because of a nearly complete block in proliferation of immature sympathetic neurons.[49] Thus different members of the gene regulatory network appear to have selective functions, some in proliferation and others in differentiation.[30]

Neural crest cells from the cranial region migrate from the neural folds into the pharyngeal arches and heart, where they play essential roles in septation of the single outflow of the heart (the truncus arteriosus) and in the formation of the conotruncal portion of the ventricular septum.[50] It follows that coexistence of neuroblastoma with anomalies of other neural crest–derived structures is biologically plausible because of the shared roles of regulatory and developmental genes. For example, ablation of the cardiac neural crest results in congenital cardiovascular malformations in the chick embryo.[50] Indeed, a significantly increased incidence of congenital cardiac malformations, especially those that involve neural crest–derived tissues, was noted in patients with neuroblastoma compared with other malignancies, such as leukemia.[51] These cardiac anomalies were seen primarily in patients with neuroblastoma who were younger than 1 year and had lower stage tumors. Menegaux et al.[52] (2005) confirmed the association of neuroblastoma with conotruncal cardiac anomalies, as well as with various urogenital anomalies; other studies have shown varying results.[53-55] Although the malignant transformation of neuroblasts and the disruption of normal cardiogenesis are likely to be multistep processes, it is possible that shared initiating genetic lesions occur very early in development, causing derangement of common developmental pathways.

Genetic Disorders Associated with Neuroblastoma

Neuroblastoma may occur rarely in association with chromosomal anomalies. In addition to the increased incidence of neuroblastoma in girls with Turner syndrome,[56] neuroblastoma has also been noted to occur in patients with Noonan syndrome.[57] The gene encoding the protein phosphatase PTPN11 is mutated in 50% of patients with Noonan syndrome and is associated with an increased risk of leukemia[58] and neuroblastoma.[57,59] Other neuro-cardio-facial syndromes such as Costello syndrome have been associated with neuroblastic tumors. Mutations in the neurofibromatosis type 1 *(NF1)* gene have been detected in neuroblastoma cell lines,[60,61] and the literature includes one case report of a patient with a germline *NF1* mutation and neuroblastoma who had a homozygous deletion of the *NF1* gene in the tumor.[62] However, germline inactivation of *Nf1* does not predispose to neuroblastoma in the mouse.

Persons affected with overgrowth disorders such as Beckwith-Weidemann syndrome are at risk for embryonal tumors, including neuroblastoma. The increased risk is mainly during the first 4 years of life, and current recommendations include quarterly evaluation with abdominal ultrasound until age 8 years.[63] The molecular aberrations that predispose to neuroblastoma in particular are unknown, although maternal differentially methylated region 2 (DMR2) loss of methylation that occurs in 50% to 60% of cases is thought to confer a 1% to 5%

risk of developing non–Wilm tumors, including neuroblastoma, hepatoblastoma, rhabdomyosarcoma, adrenocortical tumors, gonadoblastoma, and thyroid carcinoma. Mutations of the *CDKN1C* tumor suppressor gene (encoding the cyclin-dependent kinase inhibitor $p57^{KIP2}$), which occur in 5% to 10% of sporadic and 30% to 50% of familial cases, is associated with a 4% estimated risk of non–Wilm tumor formation.[63,64] The X-linked Simpson-Golabi-Behmel syndrome type 1 (SGBS1), associated with mutations in glypican 3 and 4 *(GPC3* and *GPC4)* genes, is characterized by prenatal and postnatal macrosomia, distinctive craniofacies with palatal anomalies, congenital heart defects, and neuroblastoma (reviewed in reference 65). Surveillance is recommended for embryonal tumors starting in the newborn period.[65]

Neuroblastoma Stem Cells

The natural course of high-risk neuroblastoma, which results in relapse and a refractory state after an initial good response, suggests the likely existence of cancer stem cells (CSCs) in these tumors. CSCs are a subpopulation of cells within a tumor with the ability to self-renew, generate differentiated progeny, and reproduce a heterogeneous cancer population.[66] These cells have enhanced tumorigenicity, resistance to chemotherapy, and the ability to establish metastatic foci. Hansford et al.[67] (2007) demonstrated that dissociated cells from neuroblastoma tumors and bone marrow metastases grow as spheres and are capable of self-renewal. Spheres generated from the tumors underwent differentiation under neurogenic conditions to form neurons. Neuroblastoma stem cells from high-risk tumors expressed markers of neural crest stem cells and clinical markers of neuroblastoma, exhibited a higher self-renewal rate than those from low-risk tumors, and formed metastatic tumors that could be serially passaged in xenograft models.[67] Compounds that selectively targeted these tumor-initiating cells (TICs)—DEC14 and rapamycin—were identified; they induced regression of TIC tumor models in vivo and markedly decreased self-renewal or tumor initiation capacity.[68] Moreover, compounds that targeted PI3K/Akt, PKC, aurora kinase, ErbB2, Trk, and polo-like kinase–1 (PLK1) emerged from a small molecule inhibitor library screen designed to identify signaling pathways important for the survival and self-renewal of neuroblastoma TICs. Genetic and chemical inhibition of PLK1 caused regression of TIC tumor models both singly and in combination with irinotecan, suggesting that this kinase may regulate TIC growth and survival.[69]

Cluster of differentiation (CD)–133, in addition to being a marker of hematopoietic stem cells, is also a marker of TICs in several human cancers. CD133-positive subpopulations of cells with tumor-initiating and metastatic properties have been identified in neuroblastoma tumors.[70] Similarly, the stemness-related polycomb repressive complex–1 (PRC1) protein BMI1 is thought to have a role in tumor sphere formation. BMI1 is thought to inhibit neuroblastoma differentiation by repressing expression of the tumor suppressor in lung cancer–1 (TSLC1) and kinesin family member 1B (KIF1B) in these cells.[71]

In a 2013 study,[72] surface expression of the granulocyte colony-stimulating factor receptor CD114 was found to distinguish a subpopulation of neuroblastoma cells in cell lines and primary tumors, the proportion of which was increased in relapsed tumors. These cells were found to have increased responsiveness to signal transducer and activator of transcription–3 (STAT3), a critical transcription factor controlling neural crest specification. This subpopulation of cells was highly tumorigenic, could self-renew, and could give rise to differentiated progeny with characteristics of premigratory neural crest cells. This subpopulation possessed an undifferentiated phenotype similar to that found in early multipotent neural crest precursors.[72] Further studies are required to understand the role of CSCs in neuroblastoma.

Hereditary Predisposition to Neuroblastoma

A family history has been identified in 1% of cases of neuroblastoma and is suggestive of autosomal-dominant inheritance.[73] In these children, neuroblastoma develops at an earlier age and often manifests as multiple primary tumors.

PHOX2B Mutations

The first predisposition mutation identified in neuroblastoma was in *PHOX2B*.[74,75] *Phox2b* is a key regulator of autonomic neuron development, because its complete absence leads to embryonic lethality in mice as a result of the failure of sympathetic nervous system formation.[38,76] This gene is transiently expressed in murine primary sympathetic neuron progenitors from E10.5 to E13[76] and regulates their differentiation.[77-79] *Phox2b* also has growth inhibitory effects, because its overexpression promotes cell cycle exit and inhibits the proliferation of cultured sympathetic neurons.[80,81]

Heterozygous germline mutations of the *PHOX2B* gene occur in pedigrees and are associated with neuroblastoma, ganglioneuroblastoma, congenital central hypoventilation syndrome, and Hirschsprung disease.[74,75,82-85] The human *PHOX2B* gene maps to chromosome 4p13 and consists of three exons encoding a highly conserved 314–amino acid protein with two polyalanine repeats of 9 and 20 residues C-terminal to the homeodomain. Whereas the *PHOX2B* variants in congenital central hypoventilation syndrome largely involve expansions of the second polyalanine repeat within the C-terminus of the protein,[86-88] those associated with neuroblastic tumors are nearly always frameshift and truncation mutations.[74,75,85,86,89] More recently, whole-allele deletions resulting in the reduction of the protein have been reported.[90] Proposed mechanisms contributing to neuroblastoma predisposition include partial loss of function with the preserved ability to suppress cellular proliferation but not to promote differentiation,[83] complete loss of function due to functional haploinsufficiency,[91] and both dominant-negative and gain-of function effects.[81,92,93] Mutated *PHOX2B* also suppresses proliferation in vitro; heterozygous insertion of two frameshift variants, 931del5 and 693del8, into the mouse *Phox2b* locus resulted in impaired proliferation of sympathetic ganglion progenitors and biased differentiation toward the glial lineage.[93] Aberrant *Phox2b* expression in the zebrafish models has been shown to cause an arrest in the normal maturation of sympathetic neurons, leading to immature cells that are resistant to retinoic acid-induced differentiation.[94] Allelic deficiency of *PHOX2B* causes a decrease in the terminal differentiation markers *th* and *dbh* in sympathetic ganglion cells.[94] The neuroblastoma-associated frameshift mutations 676delG and K155X—but not the R100L missense mutation—functioned dominant negatively to impede differentiation.[94] Thus a reduced dosage of *PHOX2B* during development, through either a heterozygous deletion or dominant-negative mutation, imposes a block in the differentiation of sympathetic neuronal precursors, resulting in an immature cell population that is likely to be susceptible to secondary transforming events. The block in differentiation of immature sympathetic neurons caused by certain *PHOX2B* variants could be due to their inability to bind to interacting proteins critical to this process.[95] The neuronal calcium sensor protein HPCAL1 (VILIP-3) exhibits strong binding to wild-type *PHOX2B*, but only weakly or not at all to neuroblastoma-associated frameshift and truncation variants leading to impaired subcellular localization of HPCAL1 and impaired differentiation in vitro.

Anaplastic Lymphoma Kinase Mutations

Dominant mutations in the anaplastic lymphoma kinase (ALK) tyrosine kinase receptor have been identified in approximately 50% of familial neuroblastoma cases.[96,97] Several distinct germline mutations have been described to date—R1275Q, R1192P, T1151R, and G1128A—with R1275Q being the most frequent (Table 54-1). ALK germline mutations generally occur in the context of multifocal tumors, although in one study, affected patients

TABLE 54-1 ALK Mutations in Neuroblastoma

Mutation	Tumor/Cell Line	Effect of Mutation	ALK Domain	Reference(s)
ALKΔ2-3	T/C	GOF, can occur in amplified ALK	Δ224-318	586
ALKΔ4-11	T/C	GOF, can occur in amplified ALK	Δ318-782	587
K1062M	T/C	GOF	JMR	154
G1128A*	T	GOF	P-loop	96, 588
M1166R*	T	Ligand dependent or GOF	αC helix	96, 589
I1171N*	T	GOF	αC helix	96, 588
F1174I*	T	GOF	αC helix	96, 200, 589
F1174L/S*	T/C	GOF	αC helix	96, 154, 155, 200, 590
R1192P*	T	GOF	β4 strand	96, 588
F1245C*	T	GOF	C-loop	96, 155, 588
R1275Q*	T/C	GOF	A-loop	96, 97, 154, 155, 200
T1087I	T	Ligand dependent	JMR	154, 589
D1091N	T/C	Ligand dependent	β1 strand	96, 155, 589
A1099T	T	Ligand dependent	β2 strand	589
T1151M*	T	Ligand dependent	β3 strand	155, 589
A1234T*	T	Ligand dependent	αE helix	155, 589
1464STOP	T	Ligand dependent	C-terminal to kinase domain	589
I1250T*	T	Kinase dead	C-loop	96, 591
R1061Q	T	Unknown	JMR	184
T1151R*	T	Unknown	β3 strand	592
I1170T/S*	T	Unknown	αC helix	186
F1174C/V*	T	Unknown	αC helix	154
R1231Q*	T	Unknown	αE helix	184
L1240V*	T	Unknown	αE helix	186
F1245I/L/V*	T/C	Unknown	C-loop	96, 155, 200
R1275L*	T	Unknown	A-loop	97
*Y1278S**	T	Unknown	A-loop	97

ALK, Anaplastic lymphoma kinase; *A-loop*, activation loop; *C-loop*, catalytic loop; *GOF*, gain of function; *JMR*, juxtamembrane region; *P-loop*, phosphate-binding loop.
*Mutations within the kinase domain (aa 1122-1376).

did not necessarily have younger age of onset. Further guidelines on screening need to be derived from a consensus analysis of inherited cases, but it would seem that in addition to a family history, the presence of multifocal tumors would call for genetic screening. In fact, in the United States, genetic testing for *ALK* and *PHOX2B* mutations is recommended for every newly diagnosed patient with a family history of neuroblastic tumors.

Germline Susceptibility Variants

Genome-wide association studies have identified SNPs within or adjacent to genes that predispose to sporadic neuroblastoma: *BARD1* (BRCA1-associated RING domain 1), *LINC00340* (long intergenic nonprotein coding RNA 340, *CASC15*), *LMO1* (LIM domain only 1), *DUSP12* (dual-specificity phosphatase 12), *DDX4/IL31RA* (DEAD box polypeptide 4/interleukin [IL]31 receptor A), *HSD17B12* (hydroxysteroid [17-beta] dehydrogenase 12), *LIN28B* (lin-28 homolog B), and *HACE1* (HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase–1).[98-102] The *BARD1* SNP effect at chromosome 2q35 has been confirmed in African-American children,[5] and the *BARD1β* isoform[103] and *LIN28B*[104] are oncogenic in neuroblastoma. Patients with neuroblastoma who were homozygous for the risk alleles were more likely to have metastatic disease, amplification of *MYCN* (v-myc avian myelocytomatosis viral oncogene neuroblastoma-derived homolog), and disease relapse.[105] However, the absolute risk conferred by the susceptibility allele is extremely small,[105] and the risk that neuroblastoma may recur in families with these risk alleles is estimated to be very low.[106] A common copy number variation at 1q21.1 was also associated with neuroblastoma, the significance of which is uncertain.[99] The mechanisms through which these risk alleles and associated genes contribute to neuroblastoma initiation and/or maintenance require further investigation.

Genetic Aberrations in Sporadic Neuroblastoma

DNA Ploidy

Neuroblastomas are classified into those with diploid modal chromosomal content (with a deoxyribonucleic acid [DNA] index of 1) or hyperdiploid modal chromosomal content (DNA index greater than 1). Hyperdiploidy is associated with whole chromosome gains and overall favorable biology, whereas diploidy is associated with segmental chromosomal rearrangements and unbalanced translocations. Such segmental chromosomal aberrations seem to affect certain chromosomes—1p, 11q, and 17q—although the biologic significance is unclear at present.[107] Hyperdiploid tumors are often localized and have a favorable outlook, whereas diploid tumors not only tend to have a poor prognosis but also are associated with other high-risk genetic aberrations and *MYCN* amplification. Originally, infants (those younger than 1 year) with a hyperdiploid modal chromosome number were found to respond well to conventional therapy, whereas those with advanced stage disease with diploidy failed to respond to conventional therapy.[108,109] Subsequent studies have shown that tumor cell ploidy predicts responsiveness to therapy in children up to 18 months of age with advanced stage neuroblastoma without MYCN amplification.[110]

MYCN Amplification

MYCN, the first oncogene proven to be of clinical significance in neuroblastoma and gene amplification, occurs in approximately 20% to 30% of primary tumors and 90% of cell lines.[111,112] *MYCN* amplification is generally associated with advanced disease, poor outcome, and rapid tumor progression in neuroblastoma.[112,113] It occurs in 5% to 10% of early stage tumors and in half of the tumors of patients with advanced stage disease.[114,115] The detection of *MYCN* gene amplification in tumor cells at diagnosis is used by most cooperative groups to stratify patients to more intensive therapy. *MYCN* copy number appears to be consistent throughout the natural course of the disease, that is, at diagnosis and relapse and between primary and metastatic tumors in individual patients.[116] The Children's Oncology Group (COG) currently classifies *MYCN* amplification as more than 10 copies per diploid cell; it is unclear whether lower levels of gain (i.e., *MYCN* copy number between 3 and 10) confer an adverse outcome. A correlation usually exists between amplification and increased *MYCN* expression, and tumors with *MYCN* amplification generally express higher levels of MYCN than do nonamplified tumors; however, it has been reported that high expression is not linked to outcome.[117] *MYC* deregulation is common in high-risk tumors without *MYCN* amplification,[118] suggesting that MYC signaling could contribute to the high-risk phenotype.

Amplification manifests cytogenetically as double minutes or homogeneously staining regions; the latter occur mainly in tumors and the former in cell lines (Fig. 54-2).[119-121] However, there is no difference in clinical outcome whether *MYCN* amplification is manifested as double minutes or homogeneously staining regions in tumor samples obtained at diagnosis.[122] The genomic region amplified with *MYCN* is around 500 to 1000 kb, and several genes have been shown to be coamplified with *MYCN* in neuroblastoma. These genes include *DDX1* (DEAD box helicase 1),[123-125] *NAG* (*NBAS* [neuroblastoma amplified sequence]),[126,127] and *NCYM (MYCNOS—MYCN opposite strand)*,[125,128,129] and it is possible that coamplification of these genes may contribute to the tumor phenotype.

Role of MYCN during Development. *MYCN* is a highly conserved bHLH leucine zipper transcription factor located on chromosome 2p24.3 and a member of the *myc* family of oncogenes. The coding regions of both *MYC* and *MYCN* are highly homologous, with each having long 5′ and 3′ untranslated regions and gene products of similar sizes.[130,131] Both dimerize with Max and bind DNA at consensus E-box sequences (CANNTG; reviewed in reference 132).[132] Mice homozygous for disrupted MYCN die at around 10 days of gestation and have multiple organ defects, including tissues of the central and peripheral nervous systems, mesonephros, lung, gut, and heart.[133,134]

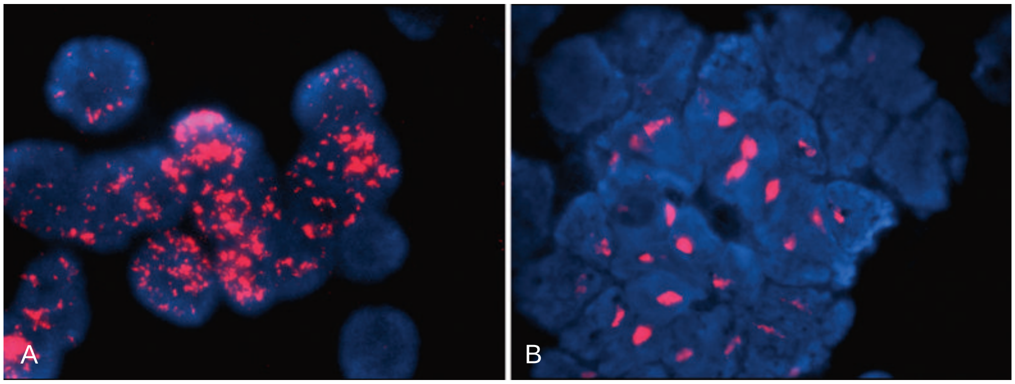


Figure 54-2 V-myc avian myelocytomatosis viral oncogene neuroblastoma-derived homolog *(MYCN)* amplification in neuroblastoma. A fluorescence in situ hybridization image of neuroblastoma tumors depicting *MYCN* amplification manifested as (**A**) double minutes and (**B**) homogeneously staining regions.

MYCN is highly expressed in fetal and neonatal development, specifically in the forebrain, hindbrain, and kidney, but not in adult tissues.[135] MYCN is expressed in the fetal brain up to the onset of differentiation and is also present at high levels in neuroblasts migrating from the neural crest through the adrenal cortex.[136] MYCN regulates overlapping stem-related gene expression programs in neuroblastoma and neural stem cells. MYCN induces a stemlike state by blocking differentiation pathways and activating self-renewal and pluripotency factors such as KLF2 and KLF4 (Kruppel-like factor 2 and 4) and LIN28B.[137,138]

Role of MYCN in Tumorigenesis. Suppression of MYCN expression in neuroblastoma cell lines results in a more differentiated phenotype.[139] Indeed downregulation of MYCN occurs in neuroblastoma cells induced to differentiate with chemical agents, and MYCN overexpression can block retinoic acid–induced differentiation.[140,141] Deletion of MYCN in neural progenitor cells showed decreased brain size and an increase in neuronal differentiation.[142] Enhanced expression of MYCN and the mutant H-*ras* gene cause tumorigenic conversion of primary rat embryo fibroblasts, and these transformed cells elicit tumors in athymic mice and isogenic rats.[143,144] Targeted expression of MYCN to the neuroectoderm of mice using a tyrosine hydroxylase promoter resulted in neuroblastoma tumors with chromosomal copy number abnormalities syntenic to those found in human tumors.[145,146] The prolonged latency together with the additional chromosomal anomalies in these tumors suggests that additional genetic mutations are required for neuroblastoma formation.

MYCN is involved in all aspects of tumor cell metastasis: adhesion, motility, and invasion (reviewed in reference 132). It downregulates integrins α1 and β1 to allow invasion.[147,148] MYCN-amplified tumors have increased angiogenesis: overexpression of MYCN is associated with proangiogenic factors such as angiogenin and vascular endothelial growth factor (VEGF) through the PI3K/mTOR (mammalian target of rapamycin) pathway.[149]

Role of MYCN in Altered Tumor Metabolism. Similar to other cancers, neuroblastomas require abnormally high amounts of glucose and other macromolecules to enable their continued unchecked proliferation. These needs are met through alterations in key metabolic pathways that can potentially be harnessed for the development of new anticancer therapies.[150]

Normal cells generate adenosine triphosphate (ATP) from glucose through oxidative phosphorylation when oxygen is abundant. During oxygen deprivation, which occurs commonly in solid tumors, including neuroblastoma, ATP generation occurs through glycolysis (the Warburg effect). Genes involved in glycolysis are selectively activated by hypoxia-inducible factor–1α (HIF1a), which is upregulated in hypoxic states. HIF1a is preferentially expressed in MYCN-amplified neuroblastoma cells; moreover, high levels of MYCN have been shown to override HIF1a inhibitory effects on cell cycle progression, thus enabling continued proliferation under hypoxic conditions.[151] Therefore both deregulated MYCN and HIF1a cooperate to contribute to the Warburg effect in neuroblastoma cells. Moreover, expression of glycolytic genes phosphoglycerate kinase 1 *(PGK1)*, hexokinase 2 *(HK2)*, and lactate dehydrogenase A *(LDHA)* were higher in MYCN-amplified neuroblastoma than in those without MYCN amplification, with depletion of LDHA inhibiting tumorigenesis in vivo, suggesting that these metabolic pathways could be targeted therapeutically.

MYCN-amplified cells have also been shown to depend on exogenous glutamine for their survival. MYCN-amplified neuroblastoma cells overexpress high-affinity glutamine transporters and other glutaminolytic enzymes that correlate with poor prognosis. Glutamine depletion in MYCN-amplified neuroblastoma cells led to apoptosis through p53-independent PUMA (p53-upregulated modulator of apoptosis) and NOXA induction,[152] an effect that is mediated by activation of the activating transcription factor–4 (ATF4). ATF4 has both context-dependent prosurvival and proapoptotic effects. In MYCN-amplified cells, it appears to have a prodeath role, because ATF4 agonists and glutaminolysis inhibitors potently induced apoptosis in vitro and inhibited tumor growth in vivo, suggesting that ATF4 agonists could be potential therapeutics for MYCN-amplified neuroblastomas.[152]

MYCN in Immune Surveillance. MYCN is also important in immune surveillance in that it represses several chemoattractant proteins that are required for recruitment of natural killer T cells (NKTs).[153] NKTs are important for producing proinflammatory cytokines to recruit immune cells, stimulate the maturation of dendritic cells, and generate antigen-specific T cells to target the tumor. Patients with MYCN-amplified neuroblastoma with bone marrow metastases had fewer bone marrow NKTs. One such protein repressed by MYCN is monocyte chemoattractant protein–1/chemokine (C-C motif) ligand 2 (MCP-1/CCL2).[153] Deletion of MYCN in MYCN-amplified cells rescues MCP-1 production and NKT recruitment. Therefore avenues to improve NKT function are being explored as therapeutic modalities in neuroblastoma.

ALK Amplification and Mutation

The identification of somatic mutations in the ALK receptor in neuroblastoma represents the first therapeutically targetable genetic aberration in this disease.[96,97,154,155] ALK, a cell surface tyrosine kinase receptor of the insulin receptor superfamily, is located in humans at chromosome 2p23 and contains an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (reviewed in reference 156).[156,157] Ligand binding leads to receptor dimerization and activation via trans-autophosphorylation of tyrosine residues.[158] The 177-kDa polypeptide encoded by the human *ALK* gene undergoes posttranslational modification, such as N-glycosylation, to generate a mature protein doublet of 220 and 190 kDa.[158] ALK is preferentially expressed in the central and peripheral nervous systems during development (i.e., thalamus, hypothalamus, midbrain, cranial ganglia and olfactory bulb, and dorsal root ganglia).[159] ALK levels decrease postnatally, so that in adults ALK expression is restricted to rare scattered neural cells, endothelial cells, and pericytes in the brain.[159-161] The physiologic function of ALK is not clear, although its predominant expression in the nervous system during development suggests that it likely plays an important role in the development of the nervous system. Homozygous ALK-null mice are viable and develop without obvious anatomic abnormalities and a normal life span,[162] other than increased basal dopaminergic signaling within the prefrontal cortex and an age-dependent increase in basal hippocampal progenitor proliferation,[162] as well as mild behavioral phenotypes.[163,164] In *Drosophila melanogaster*, the receptor is important for the development of visceral gut musculature because absence of dALK leads to the loss of mesodermal founder cells responsible for gut development.[165] The zebrafish alk ortholog is expressed at high levels in the developing nervous system.[166] Ectopic expression of alk during early neurogenesis results in increased cell proliferation and aberrant neurogenesis in the central nervous system (CNS), leading to mispositioning of differentiated neurons and activation of the MEK/ERK pathway. Morpholino-mediated and pharmacologic depletion of alk, however, did not affect neuron progenitor formation, but it did cause compromised neuronal differentiation and survival in the CNS.[166]

Signaling by the Wild-Type ALK Receptor. Pleotropin (PTN) and midkine (MK) have been reported as candidate ligands of mammalian ALK.[167,168] Whether PTN and MK are true ligands of ALK remains controversial because some groups have failed to reproduce the binding and activation of ALK by PTN.[169-171] Signaling by these two molecules may trigger dimerization and phosphorylation of ALK, leading to activation of downstream signaling pathways. In *D. melanogaster* and *Caenorhabditis elegans*, the ALK ligands are Jelly belly (Jeb)[172] and hesitation behavior 1 (HEN1),[173,174] respectively, with Jeb being structurally different from either PTN or MK.[175] Ligand activation of ALK has been implicated in the inhibition of apoptosis and induction of neuronal cell differentiation through the mitogen-activated protein kinase (MAPK) pathway.[176-178] Signaling through the phospholipase Cγ (PLCγ) pathway by activated ALK, leading to transformation of NIH 3T3 cells, has also been reported.[179]

ALK Mutations in Neuroblastoma. In human tumors the most common mechanism of constitutive ALK activation involves chromosomal translocations that result in the generation of oncogenic *ALK* fusion genes, such as nucleophosmin *(NPM1)-ALK* in anaplastic large cell lymphoma[180] and echinoderm microtubule-associated protein like 4 *(EML4)-ALK* in non–small cell lung cancers (NSCLCs),[181] among others.[182] ALK translocations have not yet been reported in neuroblastomas, apparently because the ALK receptor is normally expressed by sympathetic neuroblasts, and a heterologous promoter does not need to be provided through translocation. Rather, ALK aberrations take the form of point mutations, amplification, and copy number gain. Activating mutations in ALK occur in 7% to 8% of sporadic neuroblastomas.[154,155,97,183-186] ALK is also amplified in neuroblastoma, although always in combination with MYCN amplification, and amplified ALK appears to mediate overexpression alone in that the affected ALK allele does not harbor activating point mutations.[155]

Altogether 12 different residues are known to be affected by ALK mutations (Table 54-1).[156] Mutations range from silent to activating, with the two major mutational hot spots being R1275 and F1174; the R1275Q mutation is found in both familial and sporadic neuroblastoma, whereas the F1174L mutation is restricted to sporadic tumors, presumably because it is embryonic lethal. Activating ALK mutations are associated with constitutive phosphorylation of ALK and that of downstream targets such as ERK, STAT3, and AKT.[156] These ALK variants are for the most part retained intracellularly in the endoplasmic reticulum and Golgi and exhibit impaired maturation with defective N-linked glycosylation.[187] Moreover, the constitutive activity of these variants does not require receptor dimerization.[187] Their oncogenic potential has been demonstrated by cytokine-independent growth,[155,184] transformation of NIH-3T3 fibroblasts in soft agar colony formation assays,[154] and tumor formation in nude mice.[154] Knockdown of ALK in ALK-mutant neuroblastoma cell lines led to significant inhibition of cell proliferation and induction of cell death, further emphasizing the strong oncogenic "addiction" of these cells to mutationally activated ALK.[155]

Elucidation of the crystal structure of ALK in its inactive conformation and mapping of neuroblastoma-associated mutations has shown that many of the ALK residues that become mutated in neuroblastoma play structural roles in autoinhibition of ALK.[188,189] Mutations allow unrestricted mobility of the alphaC helix, phosphate-binding loop, and N-terminal lobe, leading to kinase activation. Both the F1174L and R1275Q mutants show accelerated catalytic efficiency compared with wild-type ALK, with R1275Q exhibiting approximately fourfold increased catalytic efficiency and F1174L exhibiting approximately eightfold increased catalytic efficiency compared with the wild-type enzyme. This increased catalytic efficiency is at least partly due to the enhanced ATP binding affinity of these mutations for both ATP and peptide substrate.[189,190]

The ALK mutants show varying degrees of transformation capabilities. *ALK*^{F1174L} is considered the most aggressive of all *ALK* mutations in neuroblastoma, possessing higher transforming potential and kinase activity.[184] Importantly, *ALK*^{F1174L} also arises secondarily as a mechanism of resistance after an initial response to crizotinib in patients with *ALK*-rearranged cancers.[191] Moreover, the F1174L mutation cosegregates with MYCN amplification in patients with neuroblastoma, and this combination is associated with a particularly poor prognosis.[184] ALK^{F1174L} potentiates the oncogenic activity of MYCN in vitro[192,193] and in vivo,[194-196] although by itself, ALK^{F1174L} appears to be insufficient to cause the malignant transformation of neural crest–derived stem cells.[194,195] When crossed with MYCN transgenic mice, mice overexpressing ALK^{F1174L} in neural crest–derived cells gave rise to doubly transgenic progeny that developed aggressive neuroblastoma with 100% penetrance and a very short latency period.[194] ALK^{F1174L}/MYCN tumors exhibited increased MYCN dosage due to ALK^{F1174L}-induced activation of the PI3K/AKT/mTOR and MAPK pathways, coupled with suppression of MYCN pro-apoptotic effects.[194] Similarly, coexpression of ALK^{F1174L} and MYCN in zebrafish markedly increased the frequency of tumor formation and accelerated the time of onset.[195] MYCN overexpression induces adrenal sympathetic neuroblast hyperplasia, blocks chromaffin cell differentiation, and ultimately triggers a developmentally timed apoptotic response in the hyperplastic sympathoadrenal cells. Coexpression of activated ALK with MYCN provides prosurvival signals that block this apoptotic response and allow continued expansion and oncogenic transformation of hyperplastic neuroblasts, thus promoting progression to neuroblastoma.[195]

ALK mutations show no predilection for particular clinical stages of tumor progression.[185] With the exception of F1174L mutations that occur preferentially in MYCN-amplified tumors, somatic ALK mutations do not appear to be associated with survival,[184] although a more recent study reported that mutation-positive cases had a decreased overall survival (OS) probability.[197] However, ALK overexpression in the absence of mutation or amplification does appear to be associated with OS.[186,198] ALK RNA and protein overexpression is detectable in approximately 90% of primary tumors[199] and in some reports is associated with activation of downstream signaling.[200,201] The significance of this phenomenon, as well as the mechanism underlying activation of ALK, is presently unclear. Passoni et al.[198] (2009) argue that it is the level of expression that dictates oncogenicity, based on the fact that constitutive phosphorylation of ALK was observed in cells with high expression, whereas those with low expression lacked activation. This observation, plus the fact that genetic knockdown of wild-type ALK caused a decrease in cell proliferation,[97] suggest that overexpression of wild-type ALK may have some degree of proproliferative activity. Other studies have shown that neuroblastoma with elevated expression of wild-type ALK had clinical and molecular phenotypes similar to those of tumors with mutated *ALK* gene expression.[186] These data suggest that mechanisms other than mutation and amplification can lead to ALK activation.

ALK Amplification. ALK is amplified in around 2% to 3% of primary neuroblastoma cases.[184] Amplification tends to coexist with MYCN amplification; in fact, ALK amplification has been detected in up to 15% of primary neuroblastomas with MYCN amplification.[155,169,202] ALK amplification does not appear to have any independent prognostic value in neuroblastoma. ALK copy number gain appears to be more common than mutation or amplification, occurring in 15% to 20% of cases,[184,185] and usually involves whole chromosome 2 gain. Such chromosome 2p gains are associated with significantly increased ALK expression, correlating with poor survival.[97,184,198]

ALK Signaling in Neuroblastoma. Constitutively activated ALK as a result of ALK amplification binds to the activated Src homology 2 domain–containing adaptor protein ShcC and thereby activates downstream signaling.[169] Inhibition of this binding impairs survival, differentiation, and motility of neuroblastoma cells through blockage of the MAPK and PI3K/AKT pathways and impairs the induction of apoptosis.[201] Gain-of-function ALK mutations also commonly signal through the MAPK, PI3K/AKT/mTOR, and STAT pathways to regulate cell growth, transformation, and antiapoptotic signaling (reviewed in references 156 and 157).

ATRX Mutations

The alpha thalassemia/mental retardation syndrome X-linked *(ATRX)* gene is mutated primarily in 44% of neuroblastoma tumors in adolescents and young adults, which largely have an indolent tumor phenotype compared with that in children and infants.[203,204] These loss-of-function mutations are associated with absence of the ATRX protein in the nucleus and telomere lengthening, the mechanism of which is unclear. ATRX is involved in the regulation of ATP-dependent chromatin remodeling, nucleosome assembly, and telomere maintenance. ATRX also plays a role in epigenetic regulation of gene expression by controlling the deposition of histone H3.3 at transcriptionally silent regions of the genome, which may potentially lead to increased expression of oncogenes in tumors with ATRX mutations. ATRX mutations correlate with older age at diagnosis (age >5 years) and chronic or indolent disease. The *ATRX* gene is present on the X chromosome, and both males and females were affected. These mutations are not thought to be associated with MYCN amplification, although further studies are required to validate this supposition.[204]

Chromosomal Abnormalities in Neuroblastoma

Chromothripsis

Chromothripsis is a phenomenon that has been recently identified in 18% of high-stage neuroblastomas.[203] Chromothripsis[205] is a local shredding of chromosomes with subsequent random reassembly of the fragments. These structural alterations recurrently affected *ODZ3* (*TENM1* [teneurin transmembrane protein 1]), *PTPRD* (protein tyrosine phosphatase, receptor type, D), and *CSMD1* (CUB and Sushi multiple domains 1) genes involved in neuronal growth cone stabilization.[203] ODZ3 and PTPRD encode transmembrane receptors expressed in the developing nervous system localizing to axon and axonal growth cones.[206] Chromothripsis-related structural aberrations were associated with amplification of *MYCN* or cyclin-dependent kinase–4 *(CDK4)* and loss of heterozygosity (LOH) of chromosome 1p.[203]

1p Loss of Heterozygosity

LOH of the short arm of chromosome 1p is a common occurrence in neuroblastoma.[207] The common region of LOH is within 1p36.2-1p36.3.[208-210] However, this region may be much larger, extending from 1p35 to the terminal end.[211-213] This abnormality has been reported not only in neuroblastoma but also in melanoma, pheochromocytoma, and medullary thyroid carcinoma, all of which are neural crest–derived tumors.[214-216] LOH of 1p occurs in 30% to 40% of neuroblastomas[217] and is positively correlated with older age, advanced stage, MYCN amplification, and a poor outcome.[218] When MYCN amplification and 1p LOH are present together, they define a genetically distinct and very aggressive subset of neuroblastoma.[219] Because cases with MYCN amplification represent a subset of patients with 1p deletion (because MYCN amplification is rarely found in the absence of 1p deletion), it is believed that 1p deletion may precede the development of MYCN amplification. Although 1p loss is also present in almost all samples with MYCN amplification, it is also found in high-risk cases without MYCN amplification.[217]

Therefore it follows that loss or inactivation of a gene or genes on 1p would be critical for the development or progression of neuroblastoma. This scenario has been suggested by reports of constitutional 1p36 alterations in patients with neuroblastoma.[220] Transfection of chromosome 1p into a neuroblastoma cell line restores a differentiated phenotype and abrogates tumorigenicity.[221] In addition, cell fusion experiments between MYCN amplified and single copy cells have resulted in abrogation of MYCN expression, suggesting than MYCN could be regulated by a gene located on 1p.[213] Several candidate tumor suppressor genes have been identified, including *CHD5* (chromodomain helicase DNA binding protein 5),[222-224] *KIF1B* (kinesin family member 1B),[225,226] *TP73* (tumor protein p73),[227] *MIR34A* (microRNA 34A),[228] *CAMTA1* (calmodulin binding transcription activator),[229] and *CASZ1* (castor zinc finger 1).[230]

CHD5. CHD5, a member of the CHD family of proteins involved in chromatin remodeling, is preferentially expressed in the nervous system and maps to a small region of deletion on 1p36.3 in neuroblastomas.[224] Expression of CHD5 has been found to be low or absent in cell lines and tumors with 1p deletion. Low expression of CHD5 is also highly correlated with MYCN amplification, advanced stage, and unfavorable histology.[224,231] When CHD5 was expressed in neuroblastoma cell lines with low or absent expression, clonogenicity and tumor growth were abrogated.[223] Mouse models with loss of a region corresponding to human 1p36 implicated CHD5 as a tumor suppressor that controls proliferation, apoptosis, and senescence via the $p19^{Arf}$/p53 pathway.[222] *CHD5* mutations, however, are rare in neuroblastoma,[224] although it is now becoming clear that complete inactivation of the gene does not appear to be necessary for malignant transformation. Rather, gene dosage may be another mechanism that regulates tumor suppressor activity as shown for some *PHOX2B* mutations.[94] In a study by Bagchi et al.,[222] (2007) increased dosage of CHD5 (as in wild-type or three copies) led to tumor-suppressive properties, whereas decreased dosage (as in heterozygous 1p deletion) enhanced immortalization, spontaneous foci formation, and sensitivity to oncogenic transformation. In addition, it has been suggested that almost complete inactivation of the second allele may occur by an epigenetic phenomenon because the CHD5 promoter was found to be highly methylated in two cell lines that lacked CHD5 expression.[223]

KIF1B. The kinesin family member, KIF1B, is located on 1p36.2 and is thought to function as a tumor suppressor gene in neuroblastoma and pheochromocytoma.[226] Germline loss-of-function mutations have been detected in patients with neuroblastoma, leading to abrogation of the apoptosis that is a requisite to normal neuronal developmental culling when nerve growth factor (NGF) becomes limiting. Neuroblastoma cells have been shown to undergo apoptosis when NGF is withdrawn, which is mediated through the EglN3 prolyl hydroxylase. KIF1B acts downstream of EglN3.[232,233] Similar to the case with CHD5, KIF1B haploinsufficiency may be sufficient for loss of its tumor suppressor activity, especially if combined with loss or abnormalities of other genes on 1p36 such as CHD5.[222] In support of this theory, when KIF1B levels were decreased to 50% using short hairpin (sh)RNA knockdown, inhibition of apoptosis was observed.[226]

CASZ1. Deletion of CASZ1, a neuronal differentiation gene, has been implicated in neuroblastoma tumorigenesis.[229,234-236] CASZ1 is the human homologue of the *Drosophila* zinc finger transcription factor, castor.[237] Low expression of CASZ1 via LOH or epigenetic suppression is associated with poor prognosis in patients with neuroblastoma.[234,238] Moreover, the restoration of CASZ1 in neuroblastoma cells suppresses cell proliferation by activation of pRB in G1 and inhibition of the G2/M regulators cyclin B1 and Chk1 and induces cell differentiation.[234,235] Decreased CASZ1 expression in neuroblastoma is thought to be due to upregulation of one of the three core subunits of the polycomb repressor complex 2, EZH2 (enhancer of zeste 2).[238] This methyltransferase regulates trimethylation of histone H3 on lysine 27 (H3k27me3 [histone H3 trimethyl lysine 27]), which is associated with gene silencing. Analysis of expression data sets revealed that patients with neuroblastoma who have a poor prognosis have increased levels of EZH2 expression.[238] Silencing of EZH2 leads to decreased H3K27me3 and increased expression of CASZ1. Thus a model has been suggested in which one allele of CASZ1 could be lost by 1p LOH, with the remaining allele epigenetically silenced by EZH2-mediated H3k27me3. Moreover, EZH2 also was found to silence a number of tumor suppressors that control differentiation in neuroblastoma, such as clusterin *(CLU)*, runt-related transcription factor–3 *(RUNX3)*, and nerve growth factor receptor *(NGFR)*. Genetic and pharmacologic inhibition of EZH2 inhibited neuroblastoma cell growth and induced differentiation.[238]

MIR34A. The microRNA (miRNA)-34a *(MIR34A)* on 1p36.23 is expressed at lower levels in primary tumors and cell lines with 1p deletion and is also thought to function as a tumor suppressor gene.[228,239,240] Reintroduction of this miRNA into neuroblastoma cell lines with 1p deletion causes a dramatic reduction in cell proliferation through the induction of a caspase-dependent apoptotic pathway and by reducing levels of E2F3, a transcriptional inducer of cell-cycle progression. *MIR34A* also increases during retinoic acid–induced differentiation of neuroblastoma cells.[239,241] Indeed, targeted delivery of miRNA-34a using nanoparticles conjugated to an anti-GD2 antibody that is expressed on the surface of virtually all neuroblastoma cells was shown to result in decreased tumor growth in orthotopic xenograft models, highlighting its therapeutic potential.[242] In addition, *MIR34A* has also been found to directly target and downregulate MYCN and B-cell lymphoma–2 (BCL2).[240] *MIR34A* causes significant suppression of cell growth through increased apoptosis and decreased DNA synthesis in neuroblastoma cell lines with MYCN amplification.[228]

ARID1 Mutations. Deletions and sequence alterations in the chromatin-remodeling genes *ARID1A* and *ARID1B* (AT-rich interactive domain 1A and 1B) have been reported in 11% of tumors.[243] These mutations were associated with early treatment failure and decreased survival. ARID1 family genes are integral components of the switch/sucrose nonfermentable (SWI/SNF) neural progenitor-specific chromatin-remodeling BRG1-associated factor (BAF) complex that is essential for the self-renewal of multipotent neural stem cells.[244] ARID1A was affected by insertional and point mutations predicted to result in premature termination of the protein and deletion of the other allele at 1p36, largely leading to biallelic inactivation.[243] Hemizygous deletions encompassing the whole coding sequence, intragenic hemizygous deletions, and splice-site and missense mutations targeting ARID1B were also reported in this study. High expression of members in the neural progenitor BAF complex correlated with a high-risk neuroblastoma phenotype, whereas high expression of components of the neuron-specific BAF complex or downstream neuritogenesis target genes correlated with low-risk neuroblastoma.[243] Thus it is possible that disrupted BAF complex signaling preserves an undifferentiated progenitor state, although this preservation has not as yet been demonstrated.

LIN28B Overexpression

Overexpression of the *LIN28B* gene that encodes a protein that binds small RNAs has been reported in neuroblastoma.[104] Lin28b is highly expressed in stem cells and developing tissues and regulates germ cell development, skeletal myogenesis and neurogenesis, and glucose metabolism. It is a master regulator of pluripotency in embryonic stem (ES) cells, and in combination with *NANOG* (Nanog homeobox), *OCT4* (organic cation transporter–4), and *SOX2* (sex-determining region Y box–2), it can reprogram differentiated cells to pluripotent stem cells.[245-247] High LIN28B expression was found to be an independent risk factor for adverse outcome in persons with neuroblastoma. In neuroblastoma cells, *LIN28B,* through repression of the let-7 miRNA family, caused elevated MYCN expression and inhibition of normal neuroblast and neuroblastoma differentiation, whereas overexpression of LIN28B in nonmalignant neuroblasts drives proliferation. Targeted expression of LIN28B in the sympathetic adrenergic lineage induced development of neuroblastomas marked by low let-7 miRNA levels and high MYCN protein expression. Mechanisms underlying the overexpression of LIN28B in neuroblastoma are currently unclear.[104] *LIN28B* has also been identified in a genome-wide association study of patients with neuroblastoma as influencing susceptibility to neuroblastoma, and high expression was associated with worse OS.[248]

Chromosome 17q Gain

Gain of chromosome 17q is the most common cytogenetic abnormality in neuroblastoma; it occurs in more than 60% of neuroblastomas and is associated with an unfavorable prognosis[249] and metastatic disease.[250] Gain usually takes the form of one to three extra copies. The break points vary, but in general, gain of a region from 17q22-qter is observed. Partial gain often results from unbalanced translocation of 17q21-25 to another chromosome. Unbalanced 1;17 translocations occur in primary neuroblastoma and result in loss of distal 1p with gain of 17q material.[251] Unbalanced 17q gain is associated with MYCN amplification and most likely harbors an oncogene that contributes to neuroblastoma tumorigenesis. However, 17q translocation break points are not uniform and can involve other partner chromosomes, especially 11q.[252] The break point positions on 11q were found to be variable, whereas all break points on 17q appeared to cluster proximal to position 43.1 Mb on the DNA sequence map.[253]

Isogenic cell lines derived from MYCN-driven murine tumors in transgenic mice showed gains of regions syntenic with human 17q.[254] One of the candidate genes on 17q is survivin, the expression of which is significantly associated with a poor prognosis.[255] Other candidate genes in the 17q region are nm23-H1 and nm23-H2, which are both targets of MYCN. Nm23-H1 binds to Cdc42, which is encoded on 1p36 and prevents neuroblastoma cell differentiation. Overexpression of Nm23 due to gain of 17q and induction by MYCN combined with decreased expression of Cdc42 due to loss of 1p36 can block neuroblastoma tumor differentiation.[256]

Chromosome 11q LOH

Allelic loss of 11q occurs in 35% to 45% of primary tumors.[257,258] Chromosome transfer experiments involving transfer of an intact chromosome 11 into a neuroblastoma cell line induces differentiation.[221] The common region of deletion has been mapped to 11q23, indicating that this could be a location for a neuroblastoma suppressor gene.[259] Chromosome 11q loss is associated with multiple chromosomal breaks, possibly reflecting an underlying DNA repair defect.[260] Chromosome 11q LOH occurs mainly in tumors *without* MYCN amplification and identifies a high-risk subset of patients with advanced stage, older age, and disease with unfavorable histologic features. Unbalanced deletion of 11q occurs in 15% to 20% of cases and is associated with a poor event-free survival (EFS) in patients with otherwise low-risk and intermediate-risk disease (Fig. 54-3).[261]

Chromosome 14q LOH

Deletion of 14q has also been reported in 25% of neuroblastomas,[262-264] with a common region of deletion within 14q23-qter.[265] LOH for 14q is inversely related to 1p36 LOH, MYCN amplification, and 11q LOH.

Other areas of chromosomal gain noted especially in tumors with an aggressive clinical phenotype, but not MYCN amplification, include gain of 1q and 12q.[266] Chromosomal regions that have been deleted are 3p,[267] 4p,[217] 9p,[268] and 18q,[269] but these regions are all less common.

Neurotrophin Receptors in Neuroblastoma

Tropomyosin receptor kinase (TRK) receptors, when activated by their respective neurotrophins, mediate the survival and differentiation of neurons during development (reviewed in reference 270).[270] NGF-induced dimerization of TRKA (NTRK1) or brain-derived neutrotrophic factor (BDNF)–induced dimerization of TRKB receptors leads to activation of the tyrosine kinase domain, which facilitates binding of SHC and phosphotyrosine binding (PTB) domain protein adaptors and PLCγ protein binding, respectively, leading to activation of the major growth factor–regulated signaling pathways—the Ras/MAPK pathway and PI3K/3-phosphoinositide–dependent protein kinase–1 (PDK1)/AKT pathway—and to increases in Ca++ release and activation of the PLCγ pathway. The main ligand for TRKA, NGF, promotes survival and induces differentiation in developing sympathetic neuroblasts. Neuroblastoma tumor cells with high levels of TRKA expression differentiate in the presence of NGF in vitro but will undergo apoptosis in its absence.[271] Depending on the tumor microenvironment, therefore, TRKA signaling could induce differentiation or regression of favorable neuroblastomas. During normal development, depletion of NGF occurs in sympathetic neurons, causing TRKA signaling to activate predetermined apoptotic pathways. It has been postulated that spontaneous regression of neuroblastomas is but a delay in this normal developmental pattern.[92] A neurodevelopmentally regulated splice variant of TRKA, TrkAIII, has been identified that antagonizes the antioncogenic NGF/TRKA signaling and promotes neuroblastoma tumor growth.[272] High levels of TRKA expression are associated with a good prognosis in neuroblastoma and are strongly correlated with favorable tumor stage, younger age, and nonamplified *MYCN*. Patients with hyperdiploid tumors with favorable outcome identified by mass screening were also shown to have very high TRKA expression.[271]

The TRKB *(NTRK2)* transcript is expressed primarily in highly aggressive *MYCN*-amplified tumors.[273] The ligand for TRKB is BDNF, and activation of TRKB by BDNF leads to enhanced proliferation, migration, angiogenesis and resistance to chemotherapy in neuroblastoma.[274] Neuroblastoma cells that survive repeated exposures to cytotoxic agents express increasing levels of BDNF, suggesting that this pathway contributes to a multidrug-resistant phenotype.[275] Moreover, hypoxia induces increased TrkB expression and may contribute to a phenomenon in the tumor microenvironment where autocrine activation of the BDNF/TrkB signaling enables survival of residual tumor cells after repeated rounds of chemotherapy.[276] The full-length TRKB, which is expressed in about one third of tumors tested, is expressed primarily in those with *MYCN* amplification, whereas the truncated form resulting from alternative splicing, which lacks the tyrosine kinase domain, is expressed in ganglioneuromas and ganglioneuroblastoma.[273] The truncated TRKB is thought to sequester BDNF and thus prevent TRKB signaling.[277-279] Studies have shown that survival of neuroblastoma cells exposed to cytotoxic drugs is mediated partly by activation of the Trk receptor via PI3K,[280] activation of Akt,[281] and inactivation of glycogen synthase kinase–3 beta (GSK3B).[282] Thus the response of neuroblastoma cells to chemotherapeutic agents depends on the levels of BDNF; tumor cells expressing low levels of TrkB exhibit minimal effects on exposure to cytotoxic agents in the setting of a BDNF-rich environment. BDNF/TrkB-induced increases in HIF1a may lead to increased VEGF and angiogenesis in neuroblastoma.[283] Thus hypoxia-associated increases in neuroblastoma cell invasiveness can be blocked by inhibition of TrkB.[284] Activation of the BDNF/TrkB signaling pathway stimulates

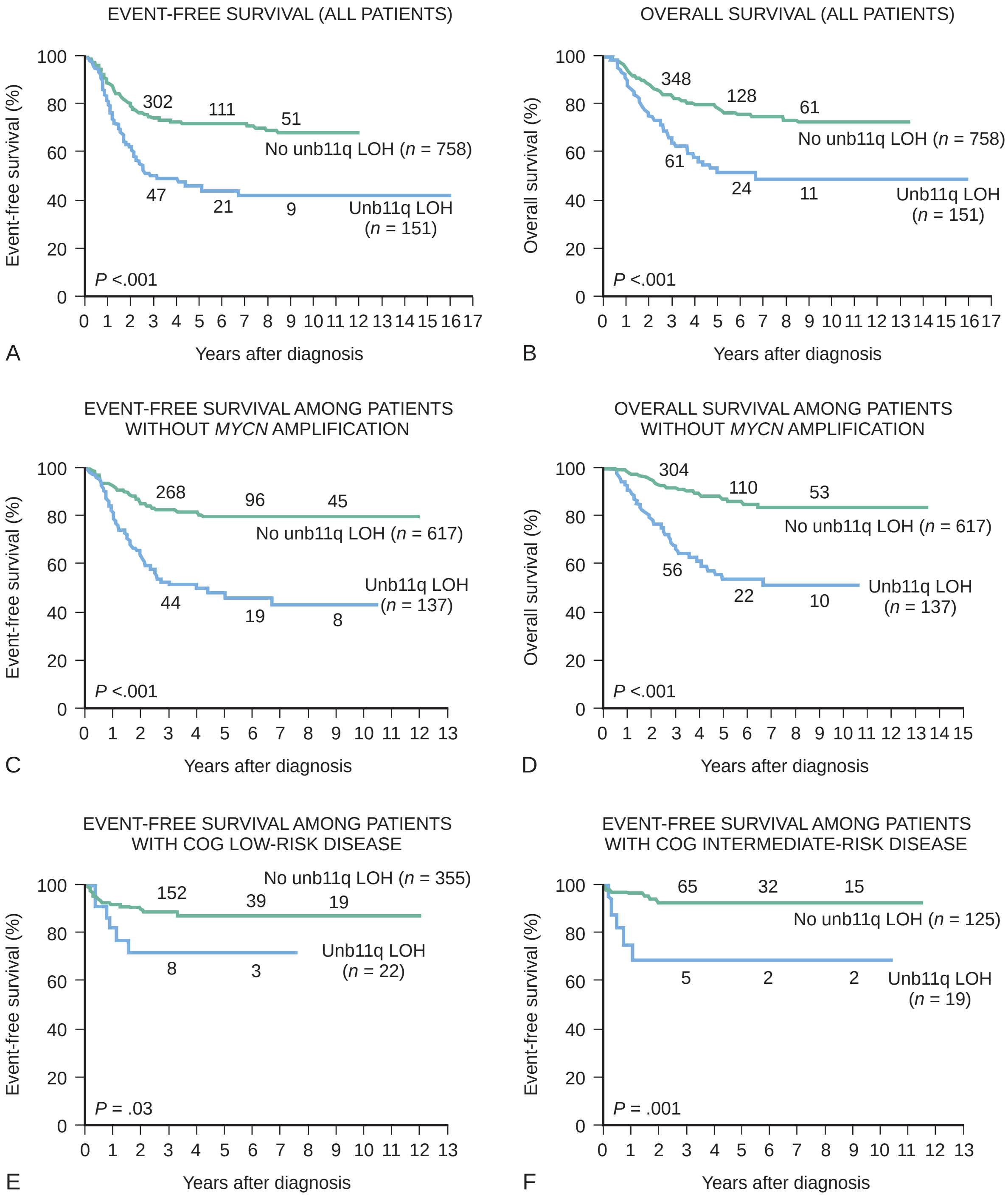


Figure 54-3 Survival according to unbalanced 11q loss of heterozygosity *(unb11q LOH)*. The rates of event-free and overall survival are shown for all patients (**A** and **B**), for patients whose tumors did not have v-myc avian myelocytomatosis viral oncogene neuroblastoma-derived homolog *(MYCN)* amplification (**C** and **D**), and event-free survival for those with low-risk disease (**E**) and intermediate-risk disease (**F**) as defined by the Children's Oncology Group *(COG)*. The numbers of patients at risk for an event are shown along the curves. *(From Attiyeh EF, London WB, Mosse YP, et al: Chromosome 1p and 11q deletions and outcome in neuroblastoma.* N Engl J Med *353:2243–2253, 2005.)*

tumor cell survival and angiogenesis and contributes to chemotherapy resistance and anoikis.[270]

TRKC, like TRKA, is involved in the biology of favorable neuroblastomas, and expression corresponds to lower stages.[285] Although its expression is found in 25% of primary neuroblastomas, it does not have independent prognostic significance, because all tumors with TRKC expression also have TRKA expression.

Apoptosis

In contrast to most human tumor types, the *p53* gene is very rarely mutated in neuroblastoma at diagnosis[286-289] but is found in chemotherapy resistance.[287,286] Other mechanisms of *p53* loss of function, such as cytoplasmic sequestration, mouse double minute 2 (MDM2 protooncogene) amplification, or TWIST-mediated suppression have been proposed, but their contribution appears to be limited.[290-292] Although basal p53 expression in neuroblastoma cells is largely confined to the cytosol, p53 protein levels were found to increase mainly in the nucleus after radiation-induced DNA damage.[293]

Epigenetic modification of the pro-apoptotic gene caspase-8 has been observed in neuroblastomas.[294,295] Up to 70% of human neuroblastoma cell lines and 25% of primary tumors tested lack caspase-8 expression and fail to undergo apoptosis.[294,296,297] Loss of expression of both caspase 8 (apoptosis-related cysteine peptidase, *CASP8*) alleles is correlated with methylation of the caspase-8 gene,[294] and treatment with the demethylating agent decitabine restored caspase-8 expression[296,298] and increased susceptibility to doxorubicin-induced apoptosis.[294] In addition, methylation has been shown to be the mode of silencing of other genes involved in apoptosis: the four tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) apoptosis receptors and the caspase-8 inhibitor FLICE-like inhibitory protein (FLIP).[296,299]

Overexpression of BCL2, an inhibitor of apoptosis, has been found to be associated with unfavorable histology and MYCN amplification.[300] Neuroblastoma cells and tumors are primed for death with sequestration of BIM, a direct activator of apoptosis, either by the prosurvival BCL-2 or myeloid cell leukemia–1 (MCL-1) proteins.[301] This pattern of survival dependency has been shown to predict the pattern of response to Bcl-2 antagonists. The Bcl-2/Bcl-xl/Bcl-w inhibitor ABT-737 was active against Bim:Bcl-2 primed tumor xenografts but not against cells where Bim was sequestered by Mcl-1. In isogenic cell lines at diagnosis and relapse, therapy resistance was shown not to be mediated by upregulation of Bcl-2 family members or loss of Bim priming but by repression of Bak and/or Bax-mediated apoptosis.[301]

Therapy Resistance

The multidrug resistance gene encoding P-glycoprotein is thought to function by causing enhanced drug efflux from the cell, and its overexpression has been found to predict outcome of therapy for neuroblastoma.[302,303] The multidrug resistance-associated protein (MRP), like P-glycoprotein, mediates resistance to a number of drugs.[304,305] MRP is expressed by neuroblastoma tumors of all stages. Tumors with *MYCN* amplification have been shown to have significantly higher expression levels of MRP than do those with normal *MYCN* copy numbers. Reduced MRP expression levels correlate with differentiation of neuroblastoma cells in vitro.[306] In addition, an association has been found between high levels of MRP expression and poor outcome, which is independent of *MYCN* amplification.[307] It is possible that both multidrug resistance protein–1 (MDR1) and MRP function together with several other factors that confer resistance to drug therapy in neuroblastoma such as *MYCN* amplification, TRKB signaling, or loss of p53 expression.[274,308]

MYCN-amplified tumors were found to possess a population of cells with CSC characteristics that was enriched during prolonged drug selection. Treatment with the histone deacetylase (HDAC) inhibitor vorinostat led to histone acetylation and increased the sensitivity of these cells to chemotherapy, with loss of their stemness properties and downregulation of stemlike marker genes.[309]

Acquired Resistance to Targeted Therapies

The dissection of the cancer genome that has resulted in the identification of targetable oncogenic drivers has propelled personalized medicine protocols aimed at treating subsets of patients whose tumors harbor sensitizing mutations. Although pediatric tumors have a relatively silent mutational landscape compared with adult tumors, targeted agents are being used in several cancers, including neuroblastoma. These agents include small-molecule inhibitors of ALK, TRKB, aurora kinase, and PI3K/mTOR, to name a few. Targeted therapies can induce impressive clinical responses, but relapse due to acquired resistance is a major challenge. Several mechanisms lead to acquired resistance to targeted agents: acquisition of secondary mutations or amplification of the drug target itself, activation of alternative or downstream signaling pathways, and changes in drug efflux or bioavailability (reviewed in reference 310).[310] Secondary mutations in the target gene such as gatekeeper mutations preserve pathway activity and confer resistance by interfering with drug binding. Engagement of alternative receptor–mediated pathways has also been reported as causing resistance. Resistance to targeted therapy can also occur through nongenetic mechanisms, such as a reversible drug-tolerant state in a subpopulation of cells that is maintained by a chromatin modification, implicating an epigenetic basis of drug resistance.[311] Another mechanism involves transdifferentiation or histologic transformation with epithelial to mesenchymal transition in the absence of any known genetic mechanism.[312]

Tumor Angiogenesis

Neuroblastoma is characterized by prominent angiogenesis, and neuroblastoma cells have been shown to induce angiogenesis in the chick embryo chorioallantoic membrane assay.[313] Increased tumor vascularity and microvascular proliferation is correlated with widely disseminated disease, MYCN amplification, unfavorable histology, and a poor outcome.[314-316] Such aggressive tumors are associated with high expression of VEGF, basic fibroblastic growth factor, and platelet-derived growth factor A[299,314] and integrins, which are markers of active angiogenesis.[317] The presence of angiogenesis appears to be influenced by the cellular composition of the tumor in that in highly aggressive stroma-poor neuroblastoma, angiogenesis is present as a result of the secretion of angiogenic stimulators, whereas in stroma-rich tumors, numerous angiogenic inhibitors secreted by Schwann cells appear to maintain the inhibitory phenotype.[318] The Schwann cells in neuroblastoma tumors have been shown to have very low tumor vascularity with production of angiogenesis inhibitors, such as tissue inhibitor of matrix metalloproteinase–2 (TIMP-2), pigment epithelium-derived factor, and SPARC (secreted protein acidic and rich in cysteine), a calcium-binding matricellular glycoprotein.[117,314,319] SPARC expression has been found to be inversely correlated with the degree of malignant progression in neuroblastoma tumors, and neutralizing SPARC with antibodies reverses the antiangiogenic activity of Schwann cell–conditioned media.[319]

Metastasis

Metalloproteinases such as matrix metalloproteinase–9 (MMP9), along with CD44 and NM23-H1, which regulate tumor cell adhesion and migration, may play a role in metastasis.[320-322] Overexpression of MMP2 and MMP9 is associated with tumor invasion and metastasis in many types of cancer, whereas inhibitors of MMPs have been shown to suppress tumor invasion and angiogenesis. An association between increased levels of MMP2 and MMP9 and advanced-stage tumors has been observed in neuroblastoma. Caspase-8 has also been shown to be a metastasis suppressor gene.[323] Decreased caspase-8 expression has been shown to occur during the establishment of neuroblastoma metastases in vivo, and reconstitution of caspase-8 expression in deficient neuroblastoma cells suppressed these metastases. Caspase-8 selectively potentiated apoptosis in metastasizing cells, and loss of caspase-8 allowed cellular survival in the stromal microenvironment and promoted metastases.[323,324] In the TH-MYCN mouse model of neuroblastoma, caspase-8 was associated with increased metastasis, specifically to the bone marrow, possibly caused by upregulation of genes involved in epithelial-mesenchymal transition (EMT), decreased cell adhesion, and increased fibrosis.[325]

PATHOLOGY

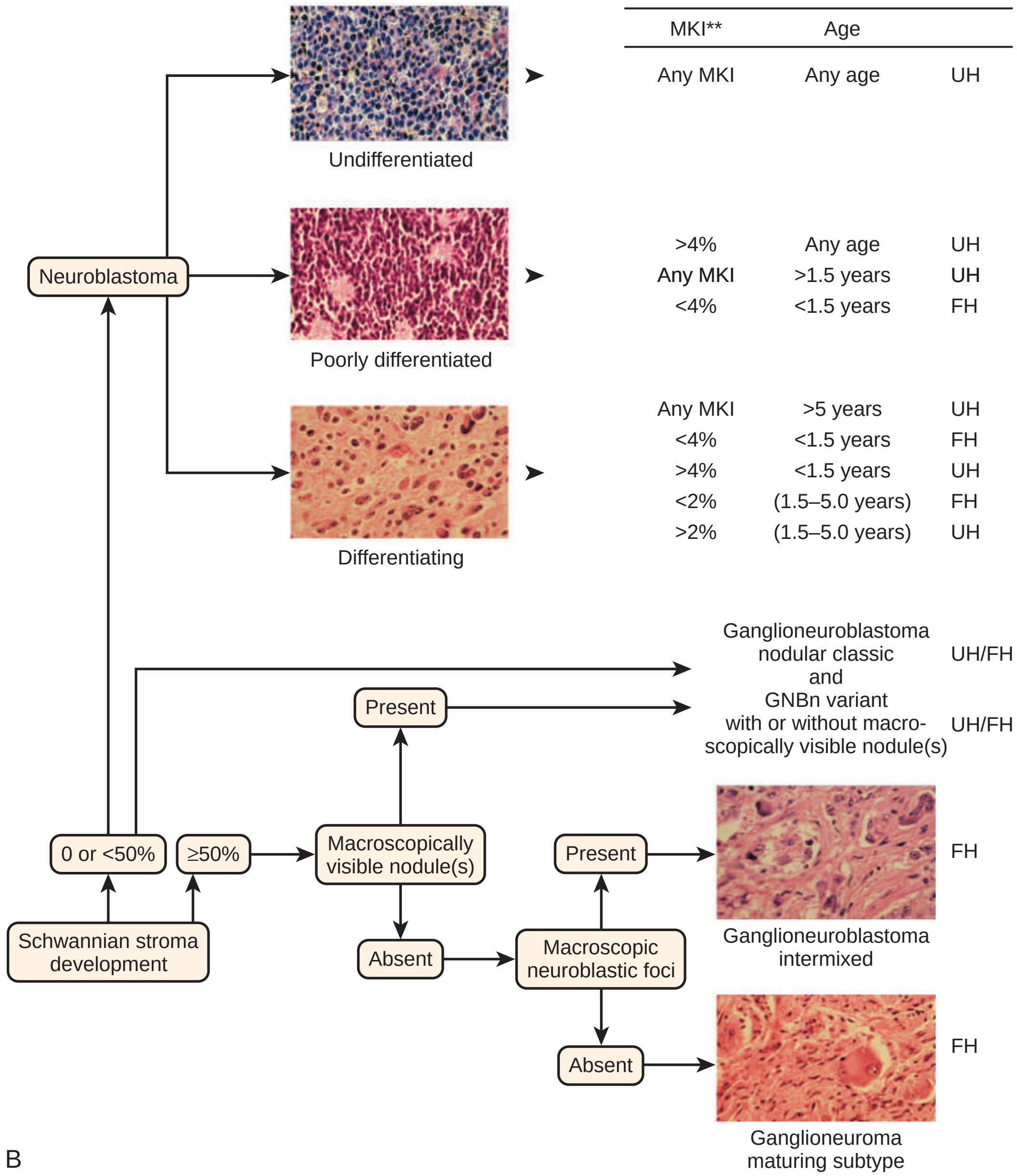
Neuroblastomas arise from primitive sympathetic precursors of the neural crest and belong to the family of small round blue cell tumors. The histopathology of the tumor cells correlates with stages of sympathetic nervous system development. Tumors are composed of small blue round cells that are uniformly sized and contain dense, hyperchromatic nuclei and scant cytoplasm. Surrounding the neuroblasts is stroma, known as Schwannian stroma. A typical feature of neuroblastoma is the presence of neuropil, which is made up of neuritic processes and is found in the majority of neuroblastomas. One of the pathognomonic features of neuroblastoma is the Homer-Wright pseudorosette, a collection of neuroblasts surrounding areas of neuropil, which occurs in up to half of cases (15% to 50%; Fig. 54-4, *A*).[326]

Neuroblastoma manifests as a spectrum of three histologic patterns ranging from neuroblastoma to ganglioneuroblastoma to ganglioneuroma, based on the degree of tumor cell differentiation. Neuroblastomas are composed of mostly small immature blue round cells with scanty cytoplasm, little evidence of differentiation, and high mitotic activity. Ganglioneuroblastomas are tumors with differentiated ganglion cells admixed with neuroblastic tissue. These tumors may vary from predominantly neuroblastic with rare ganglion cells to predominantly maturing cells with rare undifferentiated components, such as neuroblastic cells. If less than 50% of the cells are maturing cells, the tumor is termed a “maturing neuroblastoma,” and if more than 50% of the cells are maturing cells, it is termed a “ganglioneuroblastoma.” Ganglioneuroblastomas can also be focal or diffuse, and both types can exist within a single tumor. Two forms of ganglioneuroblastoma exist: (1) the intermixed variety, in which cells in various stages of differentiation are interspersed with small nests of neuroblasts, predicting a good outcome, and (2) the nodular type, in which hemorrhagic areas and macroscopic nodules are present, which is associated with a worse prognosis. Ganglioneuromas are fully differentiated tumors consisting entirely of maturing ganglion cells, neuropil Schwannian stroma, and nerve fibers.[327,328]

Primary histologic diagnosis may be enabled by hematoxylin and eosin staining and light microscopy. Other techniques also help distinguish neuroblastomas from other small round blue cell tumors of childhood, such as immunohistochemistry with use of antibodies for neural markers, such as neurofilament protein, synaptophysin, neuron-specific enolase, ganglioside GD2, chromogranin A, and tyrosine hydroxylase. Electron microscopy studies may exhibit dense core-membrane–bound neurosecretory granules and microfilaments and parallel arrays of microtubules within the neuropil.[329]

Tumor histology in neuroblastoma has traditionally been determined by the Shimada classification system.[330] Tumors are classified as favorable or unfavorable based on three features: amount of stroma, degree of neuroblastic cell differentiation, and the mitosis-karyorrhexis index (MKI; the percentage of tumor cells in mitosis versus karyorrhexis). This system had one drawback in that it was age-linked, and age itself is a strong independent prognostic feature in neuroblastoma. The Joshi system was simpler in that it examined the presence of calcification and mitotic rate (less than or equal to 10 mitoses per 10 high-power fields) and was designed to be independent of age and stage; however, it did not have the same prognostic power as the Shimada system.[331] Subsequently a unified classification, the International Neuroblastoma Pathology Classification (INPC), was established in 1999[332] and revised in 2003.[333] This classification schema was formulated on the basis of the natural history of neuroblastoma of involution and maturation as described by Beckwith and Perrin (1963).[27] In other words, it is based on the age-dependent normal ranges of morphologic features such as Schwannian stroma, degree of neuroblastic differentiation, and MKI and seeks to divide neuroblastic tumors into those with favorable and unfavorable histology (Fig. 54-4, *B*).[334]

The INPC has four main morphologic categories (Table 54-2).The first category is neuroblastoma (Schwannian-stroma poor), which is a tumor with nests of neuroblastic cells interspersed with little or minimal stroma. There are three subtypes, based on grade of differentiation: (a) undifferentiated, (b) poorly differentiated (some neuropil and <5% of cells exhibiting differentiation), and (c) differentiating (abundant neuropil and >5% of cells showing differentiation toward ganglion cells).


MKI**
Age
Undifferentiated
Any MKI Any age UH
Neuroblastoma
Poorly differentiated
>4% Any age UH
Any MKI >1.5 years UH
<4% <1.5 years FH
Differentiating
Any MKI >5 years UH
<4% <1.5 years FH
>4% <1.5 years UH
<2% (1.5–5.0 years) FH
>2% (1.5–5.0 years) UH
Ganglioneuroblastoma nodular classic and GNBn variant with or without macroscopically visible nodule(s)
UH/FH
UH/FH
Present
Macroscopically visible nodule(s)
Absent
0 or <50%
≥50%
Schwannian stroma development
Present
Macroscopic neuroblastic foci
Absent
FH
Ganglioneuroblastoma intermixed
FH
Ganglioneuroma maturing subtype
B

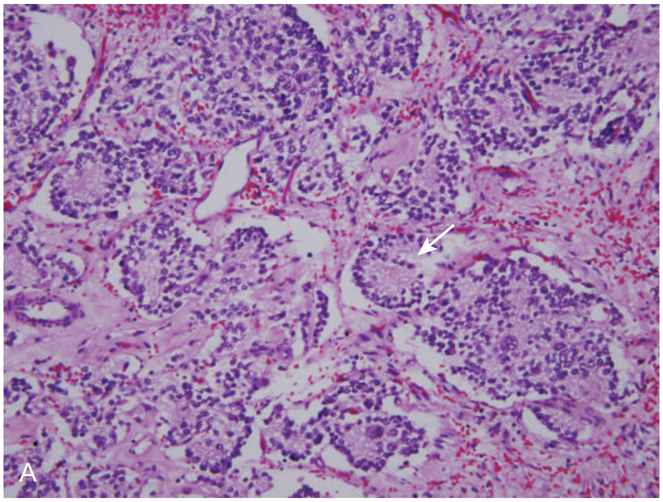


Figure 54-4 Neuroblastoma pathology. **A,** Neuroblastoma tumor showing that aggregates of tumors are composed of small immature blue round cells, uniformly sized, containing dense, hyperchromatic nuclei and scant cytoplasm. Homer-Wright pseudorosettes, which are rings of neuroblasts surrounding eosinophilic neuropil, are seen *(arrowhead)*. **B,** The International Neuroblastoma Pathology Classification. *FH,* Favorable histology; *GNBn,* ganglioneuroblastoma; *MKI,* mitosis-karryorhexis index; *UH,* unfavorable histology. *(From Park JR, Eggert A, Caron H: Neuroblastoma: biology, prognosis, and treatment.* Pediatr Clin North Am *55(1):97–120, x, 2008.)*

TABLE 54-2 International Neuroblastoma Pathology Classification

International Neuroblastoma Pathology Classification		Original Shimada Classification	Prognostic Group
Neuroblastoma	Schwannian Stroma-poor[*]	Stroma-poor	
Favorable		Favorable	Favorable
<1.5 yr	Poorly differentiated or differentiating and low or intermediate MKI tumor		
1.5-5 yr	Differentiating and low MKI tumor		
Unfavorable		Unfavorable	Unfavorable
<1.5 yr	1. Undifferentiated tumor[†] 2. High MKI tumor		
1.5-5 yr	1. Undifferentiated or poorly differentiated tumor 2. Intermediate or high MKI tumor		
≥5 yr	All tumors		
Ganglioneuroblastoma -Nodular	Composite Schwannian stroma rich/ stroma dominant with stroma poor	Stroma-rich nodular (Unfavorable)	Unfavorable
Ganglioneuroblastoma-intermixed	Schwannian stroma rich	Stroma rich intermixed (Favorable)	Favorable
Ganglioneuroma	Schwannian stroma dominant		
Maturing		Well differentiated (Favorable)	Favorable[‡]
Mature		Ganglioneuroma	

Shimada H, Ambros IM, Dehner LP, et al: The International Neuroblastoma Pathology Classification (Shimada) System. Cancer *86:364–372, 1999.*

MKI, Mitosis-karyorrhexis index.

[*] Subtypes of neuroblastoma are described in detail elsewhere.[332]

[†] Rare subtype, especially diagnosed in this age group. Further investigation and analysis are required.

[‡] Prognostic grouping for these tumor categories is not related to patient age.

The second category is ganglioneuroblastoma, intermixed (Schwannian stroma-rich), which is a tumor that contains well-defined microscopic nests of neuroblastic cells intermixed in ganglioneuromatous stroma. The nests are composed of neuroblastic cells in various stages of differentiation, but they are primarily composed of differentiating neuroblasts and maturing ganglion cells in a background of neuropil. The third category is ganglioneuroblastoma, nodular (composite Schwannian stroma-rich/stroma-dominant and stroma-poor). This tumor is composed of biologically different clones, an aggressive clone composed of grossly visible, hemorrhagic neuroblastic nodules (stroma-poor component), and a nonaggressive clone comprising ganglioneuroblastoma, intermixed (stroma-rich component), or with ganglioneuroma (stroma-dominant component). The fourth category is ganglioneuroma (Schwannian-stroma–dominant), of which there are two subtypes, maturing and mature. The maturing subtype is composed predominantly of ganglioneuromatous stroma with scattered differentiating neuroblasts or maturing ganglion cells, as well as fully mature ganglion cells. The mature subtype is composed of ganglion cells and Schwannian stroma.

In the new and revised International Neuroblastoma Risk Group (INRG) classification schema, tumor histology will be classified independent of age and will primarily be based on degree of differentiation and the MKI.

CLINICAL PRESENTATION

Neuroblastomas are tumors of sympathetic nervous system and can arise anywhere along the sympathetic chain or in any sympathetic ganglia. Most primary tumors occur in the abdomen (65%), and half of abdominal tumors occur in the adrenal gland. Other common sites of disease origin include the chest, neck, and pelvis, although rarely a primary tumor cannot be found. Because the sites of origin of neuroblastoma are so diverse, the signs and symptoms of disease at presentation vary widely and depend on both the location of the primary tumor and the degree of disease dissemination.

Localized Disease

Approximately 40% of patients present with localized disease.[335] Primary abdominal disease can present as an asymptomatic abdominal mass or with abdominal pain and fullness. Symptoms of obstruction can occasionally be seen, along with renin-mediated hypertension caused by compression of the renal vasculature.[336] Abdominal or pelvic tumors can also occasionally cause compression of lower extremity venous and lymphatic drainage, leading to lower extremity and scrotal swelling. Rarely abdominal tumors spontaneously hemorrhage, and patients with such hemorrhage present with a sudden, dramatic enlargement of an abdominal mass with increased distension and pain. Lower thoracic tumors are usually identified incidentally when a chest radiograph is obtained for unrelated reasons. Occasionally large thoracic tumors are associated with mechanical obstruction and resultant superior vena cava syndrome. Upper thoracic or cervical tumors may cause Horner syndrome.[337] Congenital tumors arising in this site can also cause hematochromia of the iris as a result of decreased pigmentation of the iris on the affected side.[338] Neuroblastoma arising from the paraspinal ganglia in the chest, abdomen, or pelvis can grow through the intervertebral foramina and compress the spinal cord. Patients may be asymptomatic or present with pain or neurologic deficits resulting from spinal cord compression, which requires emergent treatment as detailed later in this chapter.

Metastatic Disease

Metastatic spread of neuroblastoma occurs through the lymphatics or hematogenously. Regional lymph node metastases are seen in one third of patients with apparently localized tumors, and half of patients present with hematogenous metastases.[335] Hematogenous spread occurs most frequently to the bone, bone marrow, and/or liver; rarely, metastasis to the lungs and brain occurs, usually at relapse rather than at presentation. Classic signs of metastatic neuroblastoma include proptosis and periorbital ecchymoses (commonly referred to as "raccoon eyes") due to tumor infiltration of the periorbital bones. Patients also frequently present with limping and irritability due to bone pain from bone and bone marrow disease, as well as nonspecific symptoms including fever and failure to thrive. The presence of fever is usually associated with extensive bone metastases. Signs and symptoms of bone marrow replacement are also sometimes seen—most frequently pallor (which may also be caused by bleeding within the primary tumor), as well as bruising and increased risk of infection as a result of a low white blood cell count.[339]

Stage 4S Neuroblastoma

Stage 4S (S = special) is a unique presentation of neuroblastoma seen in infants; it was originally described by D'Angio, Evans, and Koop in 1971.[340] Infants with stage 4S neuroblastoma often present with massive hepatomegaly caused by diffuse liver metastases and nontender, bluish subcutaneous nodules as a result of skin nodules. Stage 4S disease accounts for 7% to 12% of neuroblastoma diagnoses and is historically defined as the presence of a small, localized primary tumor with metastases isolated to the liver, skin, and/or bone marrow in an infant younger than 12 months.[341] This special neuroblastoma often spontaneously regresses, although infants younger than 2 months can present with respiratory compromise due to a rapidly enlarging liver and may require cancer-directed treatment.[342] Based on an analysis of a large cohort of international patients diagnosed with neuroblastoma between 1990 and 2002, the INRG Task Force has extended the definition of 4S neuroblastoma to include patients up to the age of 18 months and those with primary tumors crossing the midline.[343]

Paraneoplastic Syndromes

Opsoclonus-myoclonus syndrome (OMS) and vasoactive intestinal peptide syndrome, which are well-described paraneoplastic syndromes associated with neuroblastoma, are detailed in this section, along with a newly described syndrome known as rapid-onset obesity with hypothalamic dysfunction, hypoventilation, and autonomic dysregulation (ROHHAD). Patients with these syndromes usually have localized tumors with favorable biology, perhaps because the syndrome causes early detection of tumor or, more likely, because of a correlation between the syndrome and the tumor biology.

OMS is observed in 2% to 3% of children with neuroblastoma[344] and is usually diagnosed between the age of 1 and 3 years.[345] It is often referred to as "dancing eyes, dancing feet syndrome" and is characterized by the acute onset of rapid eye movements, ataxia, myoclonic jerking of the limbs and trunk, and behavioral disturbances.[345] Although children with neuroblastoma and OMS generally have favorable tumor prognostic features and a high survival rate,[346-348] 70% to 80% experience long-term neurologic sequelae that can include global developmental delay, speech and/or motor delay, behavioral disturbances, and cognitive deficits that can seriously affect quality of life.[344,347,349,350] OMS in children is associated with neuroblastoma more than half of the time.[345] After exclusion of CNS pathology, all children with OMS should be evaluated for neuroblastoma with imaging of their chest and abdomen, an iodine 123–metaiodobenzylguanadine (MIBG) scan, and urine catecholamines.[351] Traditional methods to detect neuroblastoma may be insensitive in patients with OMS,[352,353] and therefore high-resolution computed tomography (CT) or magnetic resonance imaging (MRI) of the chest and abdomen should be considered, even in patients with negative catecholamines or negative results of an MIBG scan, to screen for small tumors.

OMS and its sequelae are thought to be immune mediated and are likely caused by the presence of an antineural antibody that cross-reacts with a common antigen on both neuroblastoma and normal nervous system tissue.[345] Improvement in symptoms can be seen after tumor removal in some cases, but generally immunosuppression with glucocorticoids or adrenocorticotropic hormone is used to relieve acute symptoms.[345,347,354] However more than 80% of patients will have a relapse of symptoms with steroid weaning or in association with a viral syndrome,[351] and more than half of patients will need prolonged steroid treatment, necessitating additional treatment approaches. Intravenous immune globulin has also been used in the treatment of OMS, either with or without steroids, with varying success.[355,356] Case reports have shown efficacy of other immunomodulating strategies including plasmapheresis, rituximab, and mycophenolate.[357-361] Although often effective in treating acute symptoms, none of these treatments has been consistently correlated with improved long-term outcome. Interestingly, a report from the Pediatric Oncology Group (POG) suggested that patients who received chemotherapy to treat their neuroblastoma had a more favorable neurologic outcome.[344] This benefit may be due to the immunosuppressive effects of chemotherapy or to less severe activation of the immune system in patients with more advanced stages of tumor.[345] The same benefit was not observed in a similar review performed by the Children's Cancer Group (CCG),[347] and the benefit of chemotherapy for the treatment of OMS needs to be investigated further. The COG recently conducted a trial for OMS in which patients with low-risk neuroblastoma who would not otherwise receive chemotherapy were treated with cyclophosphamide in addition to steroids. Long-term outcome of these patients compared with historic control subjects should provide insight regarding the role of chemotherapy for the treatment of OMS.

Further investigation is needed to determine the best treatment approach for OMS.[362] Regardless of treatment, physicians caring for patients with OMS should anticipate long-term neurologic abnormalities and use early intervention to help minimize these deficits.

Tumor secretion of vasoactive intestinal peptide can cause a syndrome of chronic watery diarrhea and failure to thrive. Most tumors secreting vasoactive intestinal peptide are histologically mature. Surgical removal of the tumor usually results in complete resolution of symptoms.[363]

ROHHAD syndrome is characterized by rapid-onset obesity, hypothalamic dysfunction, hypoventilation, and autonomic dysregulation.[364] Tumors of neural crest origin have been found in more than one third of cases[364,365] and are usually differentiated (ganglioneuromas or ganglioneuroblastomas).[366] ROHHAD syndrome most commonly affects otherwise normal children between 2 and 4 years of age and typically presents first with rapid weight gain and obesity followed at varying time intervals by autonomic dysfunction and hypoventilation. The prognosis is poor, and at least 25% of children die because of respiratory failure. A genetic cause has been suggested because of the constellation and consistency of symptoms and the reported occurrence in siblings,[366] but several candidate genes (including *PHOX2B, ASCL1* [achaete-scute family bHLH transcription factor–1], *NTRK2 [TRKB]*, and *BDNF*) have been sequenced in affected patients and have not been informative.[364,366] Monozygotic twins discordant for ROHHAD have recently been reported,[367] although this in itself should not preclude a genetic cause because modifier genes or epigenetic variation may play a role in the variable phenotype. Because of the association with neuroblastic tumors and the finding of extensive infiltrates of lymphocytes and histiocytes in the hypothalamus of some patients,[368,369,370] a paraneoplastic cause has been postulated. Immune-modulating agents including intravenous immunoglobulin, rituximab, and cyclophosphamide have been tried in small series of patients with varying success,[367,370a] and collaborative studies are needed to determine the cause of this rare disorder. Surveillance for tumors in patients with this constellation of features is recommended.

DIAGNOSIS AND EVALUATION

Diagnosis

The diagnosis of neuroblastoma is usually established from histopathologic evaluation of primary tumor tissue.[371] In most cases, especially if features of neuronal differentiation are present, a tissue diagnosis of neuroblastoma can be made on the basis of conventional hematoxylin and eosin staining. However, in cases in which there is little differentiation and only small round blue cells are evident, immunohistochemical staining for neuron-specific enolase, chromogranin A, and/or synaptophysin, as well as cytogenetic and molecular analysis, can help differentiate neuroblastoma from other small round blue cell tumors of childhood.[357-359]

In addition to establishing the diagnosis, primary tumor material is essential for risk stratification and prognosis, particularly in children younger than 18 months with locoregional or metastatic spread. In cases in which primary tumor tissue cannot be obtained safely, the diagnosis of neuroblastoma can also be made by demonstrating unequivocal neuroblastoma cells in a bone marrow aspirate or biopsy in conjunction with increased urinary catecholamines. Urinary catecholamine metabolites are increased in 90% to 95% of neuroblastomas using sensitive detection techniques such as high-performance liquid chromatography[372,373] and can be helpful in establishing a diagnosis, as well as in monitoring disease activity and response to therapy in patients who are known to excrete the metabolites. In sympathetic cells the catecholamine precursor 3,4-dihydroxyphenylalanine (DOPA) is converted to dopamine by DOPA decarboxylase, which is converted to norepinephrine and then to epinephrine by the enzyme phenylethanolamine-N-methyltransferase, which is not present in neuroblastoma cells.[374] Instead the enzymes catechol-O-methyltransferase and monoamine oxidase convert DOPA and dopamine to homovanillic acid and norepinephrine and epinephrine to vanillylmandelic acid, both of which are inactive metabolites that are excreted in the urine. Both urinary homovanillic acid and vanillylmandelic acid should be measured for diagnostic purposes. For undifferentiated tumors, urinary dopamine may also be useful.[371] Despite this catecholamine production, symptoms of catecholamine excess such as hypertension, flushing, and sweating are rarely seen in persons with neuroblastoma, likely because of the relatively low concentrations of active catecholamines in the circulation.

Clinical Disease Assessment

The primary tumor and the extent of disease should be evaluated using imaging techniques both at diagnosis and to evaluate response to treatment. Either CT or MRI are routinely used to evaluate the extent and origin of the primary tumor, as well as possible solid organ metastases.[375] CT is more widely available and can rapidly capture images, reducing the need for sedation, but it is associated with substantial radiation exposure. Ionizing radiation is not used to perform MRI but it has a longer imaging acquisition time, making sedation necessary in most young children. Currently no consensus exists with regard to which imaging technique is optimal for evaluating tumors and metastases in the abdomen, pelvis, or mediastinum. Availability and clinical factors, including the risks of sedation, should be taken into account. MRI is preferred for assessing paraspinal lesions, particularly those with possible intraextension and spinal cord impingement. Although abdominal ultrasonography is not as useful for assessing tumor volume and anatomy, it may be useful as a noninvasive method for following tumor response or surveying for disease relapse. Brain imaging is only recommended if clinically indicated by symptoms or examination.

Bone and bone marrow should also be evaluated by imaging to look for the presence and extent of metastatic disease. MIBG is a norepinephrine analogue that is selectively concentrated in more than 90% to 95% of neuroblastomas and can be combined with either the iodine 131 or ^{123}I isotopes for scanning purposes. MIBG scintigraphy is highly sensitive and specific for detecting osteomedullary disease and can also be used to assess the primary tumor, as well as occult soft tissue disease (Fig. 54-5). The ^{123}I isotope provides enhanced image resolution and is the isotope of choice when it is available. MIBG scans are essential for the initial staging of neuroblastoma and should be performed prior to primary tumor resection. International guidelines are available for patient preparation, radiotracer administration, and imaging techniques.[376] A bone scan using technetium 99m–diphosphonate scintigraphy is a relatively sensitive but less specific method to detect bony metastases in patients with neuroblastoma. Bone scans are recommended in cases in which the primary tumor is not MIBG avid, or in cases in which tumor uptake of MIBG cannot be confirmed (i.e., in cases in which the primary tumor is removed prior to imaging for metastatic disease).[375] Fluorine 18–labeled deoxyglucose positron emission tomography (FDG-PET) can also be used to evaluate metastatic disease in persons with neuroblastoma.[377-379] Its sensitivity and specificity in comparison with MIBG scintigraphy is still being evaluated, with studies suggesting that MIBG is more sensitive than FDG-PET for the detection of bone lesions and skull-based lesions, and that FDG-PET is more sensitive than MIBG for the detection of small soft tissue tumors and nodal metastases.[380] The routine use of FDG-PET is not currently recommended, but it may be a helpful tool to follow-up patients with metastatic disease who do not have MIBG-avid tumors.

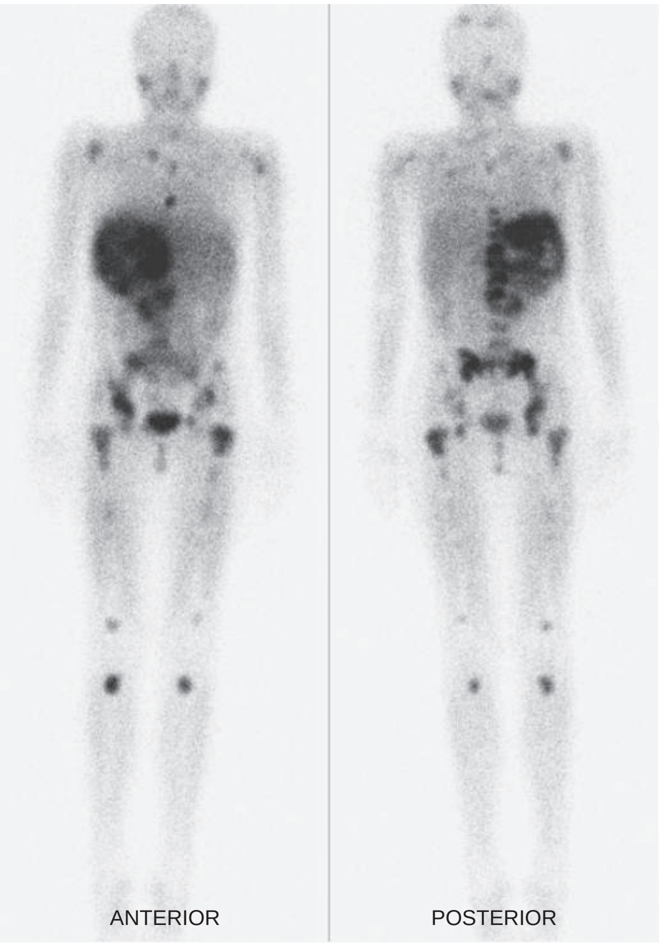


Figure 54-5 Metaiodobenzylguanidine scan. Primary tumor uptake (right adrenal gland), as well as bone/bone marrow metastases, are visualized. *(Courtesy of F. Grant, MD, Division of Nuclear Medicine, Children's Hospital, Boston, Mass.)*

Bone marrow aspirates and biopsies from both iliac crests are recommended to assess for the presence of bone marrow disease using standard histologic analysis.[371,381] Both immunocytochemical and polymerase chain reaction–based technologies can be used to increase sensitivity of marrow detection. Immunocytochemical analysis of bone marrow aspirates with monoclonal antibodies directed against neural-specific antigens (e.g., GD2 and NCAM, neural cell adhesion molecule) increases the sensitivity of detecting marrow involvement to 1:100,000 nucleated cells.[382] Reverse-transcriptase polymerase chain reaction methodologies that target the expression of neuroblastoma-specific messages such as tyrosine hydroxylase, protein gene product 9.5, or GD2 synthase can enhance sensitivity further.[383-388] However, the clinical and prognostic significance of this enhanced detection, remains to be determined, and these studies are generally only recommended within the context of a clinical study.

Staging

The International Neuroblastoma Staging System (INSS) was established in the 1990s to replace three major staging systems used throughout the world in an effort to provide international uniformity so that clinical trials and biology studies performed by different groups in different countries could be compared.[371,381] The INSS (Table 54-3) is largely based on the surgical assessments of a patient at diagnosis and has been used nationally and internationally for more than 20 years. However, because surgical approaches are not uniform, the INSS stage for patients with locoregional disease can vary considerably depending on the degree of initial tumor resection, making comparison of clinical trials conducted in different parts of the world difficult. To address this problem, the INRG Task Force developed a new staging system based on tumor imaging rather than extent of surgical resection.[389] This staging system (known as the INRG staging system) uses radiologic characteristics of the primary tumor to predict surgical risk and resectability (Tables 54-4 and 54-5).[390] Using this approach, in INRG staging, locoregional disease is classified as stage L1 or L2 on the basis of whether the tumor is locally invasive by imaging at the time of diagnosis. Stage M indicates metastatic disease that is assessed in the same

TABLE 54-3 International Neuroblastoma Staging System[371,381]

Stage	Description
1	Localized tumor with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (nodes attached to and removed with the primary tumor may be positive)
2A	Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically
2B	Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor; enlarged contralateral lymph nodes must be negative microscopically
3	Unresectable unilateral tumor infiltrating across the midline,* with or without regional lymph node involvement; *or* localized unilateral tumor with contralateral regional lymph node involvement; *or* midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement
4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined for stage 4S)
4S	Localized primary tumor (as defined for stage 1, 2A, or 2B), with dissemination limited to skin, liver and/or bone marrow† (limited to infants younger than 1 year of age)

Multifocal primary tumors (i.e., bilateral adrenal primary tumors) should be staged according to the greatest extent of disease, as previously defined, followed by a subscript "M" (e.g., 3_M).

*The midline is defined as the vertebral column. Tumors originating on one side and "crossing the midline" must infiltrate to or beyond the opposite side of the vertebral column.

†Marrow involvement in stage 4S should be minimal, that is, less than 10% of total nucleated cells identified as malignant on bone marrow biopsy or aspirate. More extensive marrow involvement would be considered to be stage 4. The metaiodobenzylguanidine scan (if done) should be negative in the marrow.

way it was assessed by INSS criteria, via both imaging and pathology.

The INRG system also differs from the INSS system in that the midline and lymph node status are not included in the staging criteria. In addition, INRG stage MS includes patients up to 18 months of age and those with L2 tumors, whereas INSS stage 4S has an upper age limit of 12 months and includes only patients with stage 1 or 2 primary tumors.

Because imaging can be retrospectively reviewed, the INRG staging system should provide data that are more uniform than the INSS system. The INRG staging system is currently used in Europe and will be adopted into the COG studies as well.

TABLE 54-4 Image-defined Risk Factors in Neuroblastic Tumors[375,593]

Anatomic Region	Description
Multiple body compartments	Ipsilateral tumor extension within two body compartments
Neck	Tumor encasing carotid artery, vertebral artery, and/or jugular vein Tumor extending to skull base Tumor compressing trachea
Cervicothoracic junction	Tumor encasing brachial plexus roots Tumor encasing subclavian vessels, vertebral artery, and/or carotid artery Tumor compressing trachea
Thorax	Tumor encasing aorta and/or major branches Tumor compressing trachea and/or principal bronchi Lower mediastinal tumor infiltrating costovertebral junction between T9 and T12 vertebral levels
Thoracoabdominal junction	Tumor encasing aorta and/or vena cava
Abdomen and pelvis	Tumor infiltrating porta hepatis and/or hepatoduodenal ligament Tumor encasing branches of the superior mesenteric artery at the mesenteric root Tumor encasing the origin of the celiac axis and/or the origin of the superior mesenteric artery Tumor invading one or both renal pedicles Tumor encasing the aorta and/or vena cava Tumor encasing the iliac vessels Pelvic tumor crossing the sciatic notch
Intraspinal tumor extension	Intraspinal tumor extension provided that more than one third of the spinal canal in the axial plane is invaded, the perimedullary leptomeningeal spaces are not visible, or the spinal cord signal intensity is abnormal
Infiltration of adjacent organs and structures	Pericardium, diaphragm, kidney, liver, duodenopancreatic block, and mesentery

TABLE 54-5 International Neuroblastoma Risk Group Staging System[389]

Stage	Description
L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment
L2	Locoregional tumor with the presence of one or more image-defined risk factors
M	Distant metastatic disease (except stage MS)
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow

Patients with multifocal primary tumors should be staged according to the greatest extent of disease as defined in the table.

RISK STRATIFICATION

Neuroblastoma is a tumor in which biologic factors are consistently used to influence disease risk stratification and treatment. In 1998 the COG established a risk-stratification system that stratifies patients into low-, intermediate-, or high-risk categories based on prognostic features including age at diagnosis, stage, tumor histology, DNA index (ploidy), and MYCN amplification status (Table 54-6). Treatment recommendations are based on this risk grouping, as described in the next section, and prognosis is distinct for each risk group (Fig. 54-6).

In an effort to unify risk stratification internationally and facilitate comparison of clinical trials performed in different parts of the world, the INRG Task Force was formed with representatives from the major pediatric cooperative groups. The INRG Task Force retrospectively analyzed the prognostic effect of 13 variables in a cohort of 8800 patients diagnosed with neuroblastoma between 1990 and 2002 with respect to EFS. Based on these analyses, a consensus INRG classification scheme was created using the most statistically significant and clinically relevant factors, which include INRG stage, age, histology, grade of tumor differentiation, MYCN and 11q status, and tumor ploidy (Table 54-7).[343] The significance of the risk factors used in both the INRG and COG risk stratification systems is described later.

Clinical Variables

Age is an important prognostic factor in neuroblastoma. Traditionally it was thought that infants (those younger than 1 year or 365 days of age) had a more favorable outcome, whereas children older than 1 year had a

TABLE 54-6 Current Children's Oncology Group Risk Group Classification

INSS	Age	MYCN*	Ploidy	Shimada†	Risk Group
1	Any	Any	Any	Any	Low
2	Any	Not amp	Any	Any	Low
2	Any	Amp	Any	Any	High
3	<547 days	Not amp	Any	Any	Intermediate
3	≥547 days	Not amp	Any	FH	Intermediate
3	≥547 days	Not amp	Any	UH	High
3	Any	Amp	Any	Any	High
4	<365 days	Amp	Any	Any	High
4	<365 days	Not amp	Any	Any	Intermediate
4	365 - <547 days	Not amp	>1	FH	Intermediate
4	365 - <547 days	Any	1	Any	High
4	365 - <547 days	Any	Any	UH	High
4	365 - <547 days	Amp	Any	Any	High
4	≥547 days	Any	Any	Any	High
4S	<365 days	Not amp	1	FH	Low
4S	<365 days	Not amp	1	Any	Intermediate
4S	<365 days	Not amp	Any	UH	Intermediate
4S	<365 days	Amp	Any	Any	High

Courtesy of the Children's Oncology Group.

FH, Favorable histology; *MYCN*, v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog; *INSS*, International Neuroblastoma Pathology Classification; *UH*, unfavorable histology.

*MYCN amplification status: *Amp*, amplified; *non-amp*, not amplified.

†International Neuroblastoma Pathology Classification.

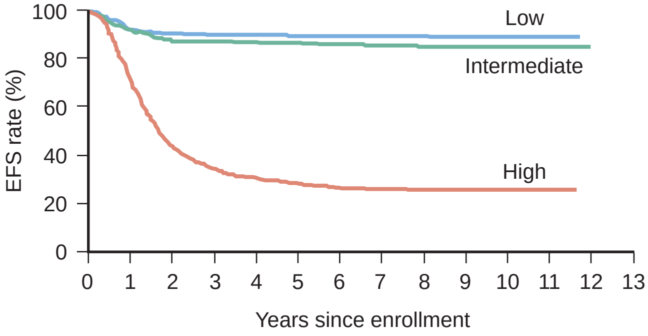


Figure 54-6 Event-free survival *(EFS)* of patients with neuroblastoma stratified according to risk group. *(Courtesy of W. B. London, PhD, Children's Oncology Group.)*

much more dismal outcome, especially those with metastatic disease at diagnosis.[391,392] However, recent studies of a large series of cases have demonstrated that age is a continuous prognostic variable in neuroblastoma, and 18 months has been identified as the age under which patients with stage 3 and 4 disease share the same favorable outlook as those younger than 1 year of age (Fig. 54-7).[3,110,393] Whether children aged between 12 and 18 months who have advanced stage disease but otherwise favorable biologic features can be cured with less aggressive therapy is currently under investigation. Fewer than 5% of cases of neuroblastoma are diagnosed in children older than 10 years. Experience suggests that older patients with high-risk–type disease can have a more indolent course but ultimately a dismal outcome, implicating a unique tumor biology.[394-397] Age older than 10 years is not currently used in risk stratification but may be in the future when the biology is more clearly understood.

Stage has always been considered an important prognostic factor, with low stage or localized disease having a better outcome than high stage or metastatic disease; the overall prognosis for patients with stage 1, 2, and 4S disease is 80% to 90%, whereas patients with disease between stages 3 and 4 have a 2-year survival rate of 20% to 40%.[374] As described previously, the INRG staging system, which determines local-regional disease extent based on image-defined risk factors rather than surgical risk factors, is in the process of being adopted internationally.

Pathologic features are also used to classify neuroblastomas. The Shimada classification divides tumors as having "favorable" and "unfavorable" histology by incorporating age with tumor differentiation, MKI, and Schwannian stroma.[330] However, because age itself is a strong independent prognostic variable, new classification systems such as the INRG examine MKI and differentiation as independent variables, along with established clinical and biologic variables.

Biologic Variables

Biologic factors currently used for neuroblastoma risk stratification include *MYCN* copy number by fluorescence in situ hybridization,[397a] DNA ploidy by flow cytometry,[108] and tumor histology using the INPC.[332] *MYCN* amplification automatically stratifies children into the high-risk category in a majority of cases, except for completely resected INSS tumors, which remains controversial. DNA index or ploidy is considered prognostic

TABLE 54-7 International Neuroblastoma Risk Group Consensus Pretreatment Classification[343]

INRG Stage	Age (mo)	Histologic Category	Grade of Tumor Differentiation	MYCN	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very low
				Amp			K High
L2	<18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	<18			NA		Hyperdiploid	F Low
	<12			NA		Diploid	I Intermediate
	12 to <18			NA		Diploid	J Intermediate
	<18			Amp			O High
	≥18						P High
MS	<18			NA	No		C Very low
					Yes		Q High
				Amp			R High

AMP, Amplified; *GN,* ganglioneuroma; *GNB,* ganglioneuroblastoma; *INRG,* International Neuroblastoma Risk Group; *NA,* not amplified.

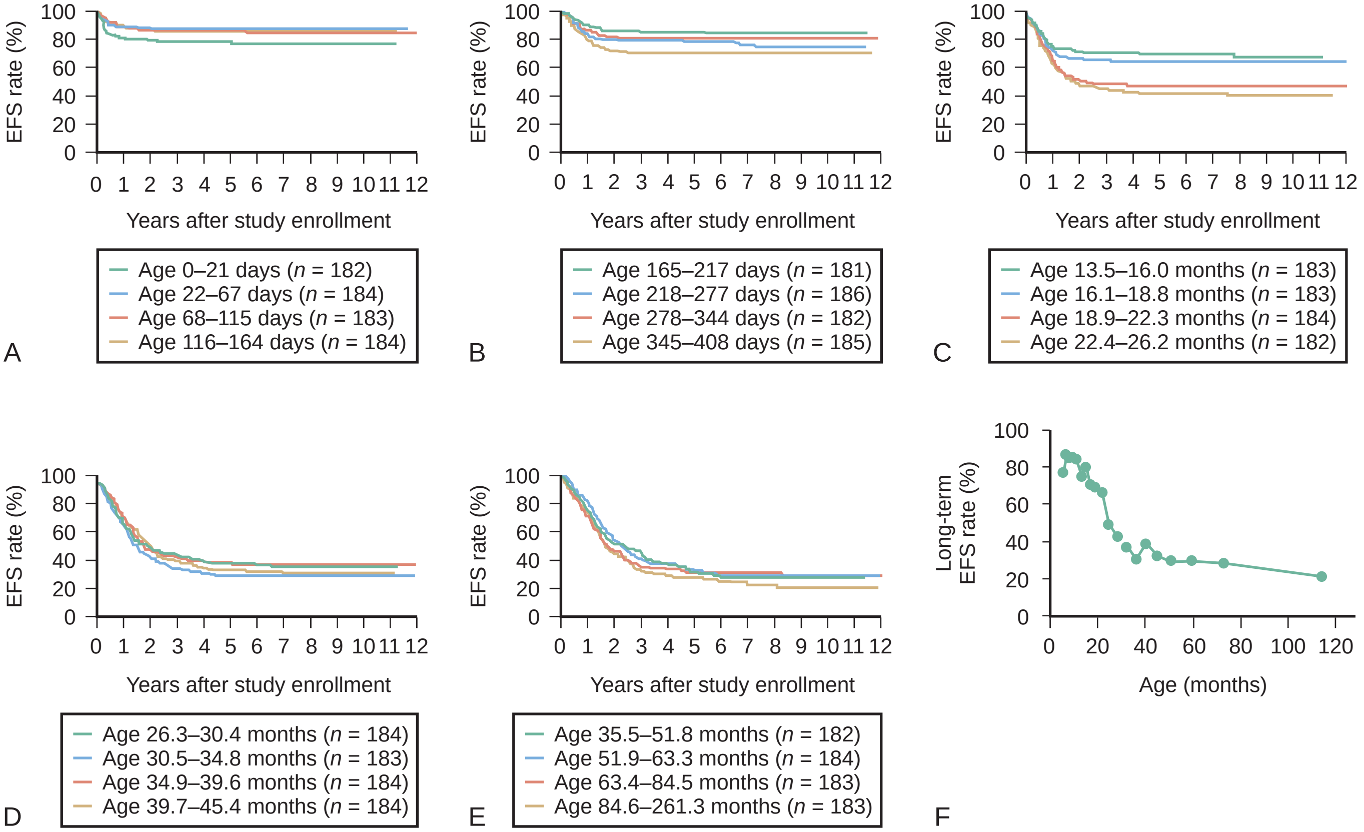


Figure 54-7 Kaplan-Meier event-free survival *(EFS)* curves by age group. **A,** 0 to 164 days. **B,** 165 to 408 days. **C,** 13.5 to 26.2 months. **D,** 26.3 to 45.4 months. **E,** 35.5 to 261.3 months. **F,** Long-term EFS rate by age group (not a Kaplan-Meier curve). *(From London WB, Boni L, Simon T, et al: The role of age in neuroblastoma risk stratification: the German, Italian, and Children's Oncology Group perspectives.* Cancer Lett *228:257–266, 2005.)*

in children up to 18 months of age.[110] Neuroblastoma tumors are classified as diploid (DNA index [DI] = 1) and hyperdiploid (DI > 1). In the COG schema, a DI of 1 would stratify a patient to the high-risk group if the patient was between 12 and 18 months of age with stage 4 disease, regardless of *MYCN* amplification status or histology. Diploid DNA content in infants (<12 months) with *MYCN* nonamplified, stage 4S disease will signify intermediate risk, regardless of tumor histology.

Other genetic features such as evaluation for LOH of 1p36 and 11q23[261] were used to stratify therapy in prior studies and could be important for stratification of non–high-risk patients with neuroblastoma. In patients with neuroblastoma whose tumors did not have MYCN amplification, the prognostic impact of the three most common segmental chromosomal alterations—1p deletion, 11q deletion, and/or 17q gain—was studied.[107] Segmental chromosomal anomalies were more frequent in children older than 18 months and with stage 4 disease. In multivariate analysis, the presence of a segmental genotype significantly correlated with a poorer EFS, although age and stage were still more significant; therefore the utility of such a segmental genomic profile for prognostic risk assessment is questionable, given that age and stage were still more significant in this study ($P < .0001$ and $P = .0002$, respectively, vs. $P = .01$ for the segmental genotype).[107]

APPROACH TO TREATMENT

The modalities used in the treatment of neuroblastoma include surgery, chemotherapy, radiotherapy, and biotherapy, as well as observation alone in a small subset of patients. The determination of treatment is largely based on prognostic factors and subsequent risk group assignment, as previously described. A major goal of ongoing neuroblastoma clinical trials is to prospectively validate the existing risk group assignments, as well as to evaluate newer prognostic markers for future refinement of risk stratification and treatment assignment. The general principles of therapy for each of the current risk groups are detailed below.

Treatment of Low-Risk Disease

Low-risk neuroblastoma is defined as INSS stage 1 disease in patients of any age, INSS stage 2 in infants 365 days of age or older with any MYCN status and histology, INSS stage 2 in children older than 365 days with MYCN of any status and favorable histology, and INSS stage 4S MYCN—not amplified, favorable histology, and hyperdiploid. For patients with INSS stage 1 disease, surgical resection is almost always the only treatment required, and these patients have an excellent OS rate. The COG study P961 prospectively evaluated 915 eligible patients with low-risk neuroblastoma. The 453 patients with INSS stage 1 disease had a 5-year EFS and OS of 93% ± 2% and 99% ± 1% with surgery as their primary treatment, which is consistent with findings of previous studies.[398] The same COG study showed that chemotherapy can be omitted for most patients who have biologically favorable but incompletely resected localized tumors (INSS stage 2A and 2B). Patients with asymptomatic INSS stage 2 disease were observed after surgery and had 5-year EFS and OS of 87% ± 2% and 96% ± 1%, respectively. The EFS was significantly better for patients with stage 2A disease compared with stage 2B disease (92% ± 3% and 85% ± 3%; $P = .0321$), but the OS did not differ. The majority of patients with INSS stage 2B disease had unfavorable histology and/or diploid tumors, which independently had a significant impact on EFS and OS. Similar results were published by the International Society of Pediatric Oncology neuroblastoma group.[399] For low-risk disease, chemotherapy should only be used in cases in which the tumor is causing organ- or life-threatening symptoms or in cases of recurrent of progressive disease not amenable to surgery. Although disease recurrence is rarely seen, it usually occurs within a year of diagnosis, and the majority of recurrences are local.[400-402] Local recurrences in low-risk patients can usually be managed with second surgeries. Metastatic recurrences are rare but are often salvageable with more intensive systemic therapy.

Neuroblastoma is known to have one of the highest rates of spontaneous regression among malignant tumors, which has been well documented in patients with stage 4S disease.[403] Data from mass screening studies in Japan show a two- to threefold higher incidence of neuroblastoma in areas with screening programs.[404] This increase in the rate of diagnosis supports the hypothesis that a subset of non–stage 4S neuroblastomas in young infants can undergo spontaneous regression as well. The German Society of Pediatric Oncology and Hematology reported a prospective trial supporting this hypothesis.[405] Ninety-three infants with localized neuroblastoma were observed without use of surgery or chemotherapy. Spontaneous regression was seen in 44 of the 93 infants (47%), and 17 infants had a complete regression. The 3-year EFS (56% ± 5%) was low because approximately half of the infants required an intervention, but the overall survival remained excellent (99% ± 1%). This study included patients with locally invasive disease and any tumor biology except for MYCN amplification. The COG recently completed an observation study for perinatally detected adrenal tumors to further explore the hypothesis of spontaneous regression in young infants with localized tumors.[406] This study had more restricted eligibility than did the German study, limiting enrollment to patients 6 months of age or younger with tumors limited to the adrenal gland, but biopsies were not performed to confirm diagnosis and determine tumor biology. Parents could choose observation or immediate surgical resection at enrollment. Eighty-seven eligible patients were enrolled, and for 83 patients, the parents elected observation. For the observation arm, 56 patients completed observation and 27 discontinued observation (13 were lost to follow-up). The majority of the patients who completed observation had a decrease in the volume of their adrenal mass, and 27 patients (48%) had no residual mass at the end of the observation period. Sixteen observation patients had surgery (one at the end of the observation period); 10 patients had neuroblastoma, 2 had low-grade adrenal cortical neoplasms, 2 had adrenal hemorrhages, and 2 had extralobar pulmonary sequestrations. Nine of the patients with neuroblastoma underwent surgery as a result of progression, seven had INSS stage 1 disease, one had INSS stage 2B disease, and one had INSS stage 4S (neither of the latter two patients required further treatment). For the overall cohort, the 3-year EFS for a neuroblastoma event was 97.7% ± 2.2%, and the 3-year OS was 100%. The results of this study confirm that expectant observation results in excellent survival rates and spares surgery in a majority of infants with adrenal masses. The COG has plans to extend observation to all L1 tumors in infants in a future clinical trial.

The optimal treatment of the rare patient with *MYCN*-amplified, localized neuroblastoma is controversial. Most studies have demonstrated a worse outcome for patients with localized tumors with *MYCN* amplification. A CCG prospective analysis of 374 patients with Evans stage I and II disease showed that four of seven patients (four with stage I disease and three with stage II disease) who had MYCN amplification experienced a relapse (in three cases, the disease was metastatic) between 2 and 22 months from diagnosis, and three of these patients died as a result of disease progression.[400] A POG analysis of 329 patients with stage A neuroblastoma showed that 4 of 11 patients with MYCN amplification remained disease free 22 months to 68 months after initial surgery. Additional treatment of four of seven patients with recurrent disease was successful. In the COG P9641 study previously described, 10 patients with INSS stage 1 disease had MYCN amplification. Five-year EFS was 93% ± 2% and 70% ± 17%, and OS was 99% ± 1% and 80% ± 15% for MYCN nonamplified and MYCN-amplified tumors, respectively ($P < .001$).[398] Although these results clearly support a worse outcome for MYCN-amplified localized disease, they also show (based on a small sample size) that a subset of patients may achieve long-term remission with surgery alone.[401] A retrospective review of 600 patients (32 with MYCN-amplified tumors) who had localized neuroblastoma and were enrolled in a POG neuroblastoma biology study from 1990 to 1999 suggests that ploidy might be useful for further risk stratification of these rare patients. In this study ploidy was significantly correlated with outcome for patients with *MYCN*-amplified localized disease (7-year OS 87% + 9% for patients with hyperdiploid tumors vs. 38% + 12% for patients with diploid/hypodiploid tumors).[407] The same outcome was found in a retrospective analysis of the INRG database, which contains 2600 patients with low-stage neuroblastoma, known MYCN status, and available follow-up data. In this database 87 patients had low-stage disease with MYCN-amplified tumors, and they had a less favorable outcome compared with patients who had nonamplified tumors (5-year OS 72% ± 7% vs. 98% ± 1%).[408] As in the POG analysis, the outcome of patients with hyperdiploid tumors was significantly better than in patients with diploid tumors (5-year OS 94% ± 11% vs. 54% ± 15%). Further prospective evaluation is needed to determine a consensus treatment strategy for these patients.

Cord compression from intraspinal tumor extension can occur in children who otherwise would be considered low risk and is an oncologic emergency requiring rapid intervention. The choice of emergent therapy is somewhat controversial, with pediatric oncologists favoring chemotherapy and neurosurgeons favoring laminectomy with spinal cord decompression.[409] Retrospective analyses show similar neurologic outcomes for patients treated with chemotherapy versus laminectomy.[410-413] The severity of motor impairment at diagnosis seems to be the strongest predictor of short-term response to treatment and the development of long-term functional outcome. Studies have also shown that because of the young age of most of these patients, laminectomy can result in significant long-term orthopedic problems.[414] For these reasons, intermediate-risk–type chemotherapy is preferred in most cases for emergent treatment of spinal cord compression. Neurosurgery should be reserved for patients with rapid neurologic deterioration or those in whom worsening symptoms develop while they are receiving chemotherapy. Radiation therapy can also be used but is usually reserved for patients with progressive neurologic abnormalities despite chemotherapy and surgical decompression because of the long-term morbidity of spinal irradiation in infants and young children.[409]

Most patients with INSS stage 4S disease have favorable biologic features (single-copy MYCN, hyperdiploid, and favorable Shimada histology) and are categorized as having low-risk disease. The POG reviewed 110 patients with stage D(S) neuroblastoma diagnosed between 1987 and 1996 and found a 3-year OS rate of 85% ± 4% with a significantly worse prognosis for infants younger than 2 months of age or those with tumor cell diploidy, unfavorable histology, or *MYCN* amplification.[341] A report from the CCG showed similar results for 80 infants with a 5-year OS of 92%.[342] Patient with suspected INSS stage 4S neuroblastoma should undergo biopsy of the primary tumor or an accessible metastatic site (such as a subcutaneous nodule) to establish the diagnosis and to determine biologic features of the tumor. Patients with favorable biologic features usually do not require therapy and should be observed closely.[340] Resection of the primary tumor is not necessary to achieve an excellent outcome in these patients.[341] Patients with progressive or symptomatic disease and those who are too ill to undergo a biopsy at diagnosis are generally treated with moderately intensive chemotherapy, such as that currently recommended for intermediate-risk disease.[341,342] Patients with hepatomegaly that progresses despite chemotherapy and causes respiratory compromise are treated with small doses of radiation—450 to 600 cGy in 150-cGy fractions. The rare patient with INSS stage 4S disease who has unfavorable ploidy and/or histology tumors should be treated with intermediate-risk–type therapy. Patients with INSS stage 4S disease who have *MYCN* amplification should be treated with high-risk–type therapy.

Treatment of Intermediate-Risk Disease

The intermediate-risk classification group includes a wide spectrum of disease. The current approach to treatment utilizes a combination of moderate-dose multiagent chemotherapy and surgery with an expected OS of greater than 90%. The CCG 3881 study was one of the first to prospectively stratify patients according to risk group. In this study intermediate-risk patients (defined by MYCN status, age, histology, and ferritin level) were treated with moderately dose-intensive chemotherapy (cyclophosphamide, doxorubicin, cisplatin, and etoposide), along with local radiation for any gross residual disease after surgery. Patients who had favorable biology (i.e., MYCN that was not amplified, a favorable Shimada classification, and a low serum ferritin level) had an OS of 92% to 100% depending on stage, and infants with unfavorable features had an OS of 93%.[415,416] After this study, in the COG A3961 study, investigators attempted to reduce acute and long-term toxicity of the CCG regimen by substituting carboplatin for cisplatin and decreasing total cumulative doses of chemotherapy while maintaining dose intensity and eliminating radiation therapy in all patients except those with unresectable primary tumors and unfavorable biologic features at the end of chemotherapy. This study used the COG risk stratification described previously and enrolled 479 eligible patients between 1997 and 2005. Intermediate-risk neuroblastoma was defined as INSS stage 3 or 4 disease in an infant (<365 days of age) without MYCN amplification; stage 3 disease and favorable histopathologic features in a child (≥365 days of age); and stage 4S disease with unfavorable histopathologic features and/or a diploid DNA index. Patients received four to eight cycles of chemotherapy depending on risk factors. Among all patients, 3-year EFS and OS were 88% ± 2% and 96% ± 1%, proving that therapy could be substantially reduced for the majority of intermediate-risk patients.[417] The outcome for patients with INSS stage 4 disease (for 176 patients, EFS and OS 81% ± 3% and 93% ± 2%, respectively) was inferior to that of patients with INSS stage 3 or 4S but did reach the overall study goal of maintaining a 3-year OS greater than 90% for all patients. Ploidy was significantly associated with outcome for patients of all ages. This study was followed in the COG by a study that prospectively used 1p and 11q status to further stratify patients and reduce therapy for patients whose tumors lack LOH at 1p and 11q and who have other favorable biologic factors.[261] The results of this study are still pending, but early reports suggest that excellent OS can be maintained despite the reduction of therapy in intermediate-risk patients who have favorable risk factors.

Existing data suggest that adjuvant therapy could be further reduced despite the presence of gross residual tumor in patients with locoregional tumors who have favorable biology. Traditionally, patients receive chemotherapy with the goal of facilitating surgical resection.

Experience from Memorial Sloan Kettering Cancer Center showed that patients with non–stage 4, non–*MYCN*-amplified disease who had gross residual disease after initial surgery could be safely observed and could maintain an excellent survival rate without use of cytotoxic therapy.[418] In this report only 1 of 22 patients required chemotherapy, and 4 of 13 patients with gross residual disease required a second surgery. This outcome is also supported by the European experience. In the German neuroblastoma 97 protocol, infants who had stage 2 or 3 MYCN nonamplified tumors with incomplete resections were observed postoperatively without use of adjuvant chemotherapy. Regression without further treatment was seen in 32 of 55 patients, and salvage therapy was effective for patients who progressed or experienced a recurrence, with a 3-year OS rate of 98%.[405] On the basis of this information, the most recent COG intermediate-risk study used partial response as the treatment end point in patients with favorable biology and INSS stage 2 or 3 disease.

Treatment of High-Risk Disease

Treatment of disseminated neuroblastoma remains one of the greatest challenges in pediatric oncology. High-risk neuroblastoma represents more than half of all newly diagnosed neuroblastoma cases and is the cause of most of the mortality associated with the disease. Treatment approaches that utilize a combination of intensive induction therapy, myeloablative consolidation therapy with stem cell support, and biologic therapy for minimal residual disease have improved 5-year survival rates from less than 15% to 30% to 40%.[259,419] This modest improvement in survival, however, has come at the cost of increased doses of both chemotherapy and radiotherapy, significantly increasing treatment-related morbidity in disease survivors.

Induction Therapy

The goal of induction therapy is to maximally reduce the overall tumor burden by using a combination of chemotherapy, surgery, and radiation therapy. High-risk neuroblastoma is generally sensitive to initial chemotherapy, even in cases with MYCN amplification. A retrospective meta-analysis of 44 trials showed that a correlation existed between dose intensity and treatment efficacy, including improved response and survival.[420] Induction chemotherapy for high-risk neuroblastoma generally consists of five to seven alkylator and platinum-based cycles using agents selected to reduce cross-resistance. A number of combinations have been used in the United States and Europe, including a regimen developed at Memorial Sloan-Kettering Cancer Center that has been widely used. This regimen consists of cycles of cyclophosphamide, vincristine, and doxorubicin alternating with cycles of cisplatin and etoposide. In a single-institution study of this regimen, Kushner et al.[421] reported a complete response rate of 63% and a complete plus very good partial response rate of 87% in 24 patients. The French Society of Pediatric Oncology Neuroblastoma Study Group used this induction regimen in a multiinstitution study of 47 patients and found a complete response rate of only 45%.[422] Similar results were obtained in the COG A3973 phase III trial, where 242 of 477 patients (51%) attained a complete or very good partial response, suggesting that chemotherapy resistance during induction remains an obstacle to cure.[423]

One strategy to improve induction response is to decrease treatment intervals during induction to lead to more rapid cell death with less chance of drug resistance. Pearson and colleagues[424] (2008) reported a European randomized clinical trial comparing treatment of 262 patients with stage 4 neuroblastoma using rapid-timing chemotherapy (i.e., a cycle every 10 days) versus standard-timing chemotherapy (i.e., a cycle every 21 days) using the same cumulative doses of five chemotherapeutic drugs given over 10 weeks or 18 weeks. The rapid regimen, also known as COJEC (cisplatin, vincristine, carboplatin, etoposide, and cyclophosphamide) was feasible without markedly increased toxicity. Complete or very good partial responses were achieved in 53% of patients assigned to conventional treatment and in 71% of patients assigned to rapid treatment ($P = 0.002$), although no significant difference in OS was found between the two regimens.

The COG piloted an alternative strategy of using non–cross-resistant chemotherapeutic agents to further improve induction response rates. Topotecan is a topoisomerase-1 inhibitor that has shown antineuroblastoma activity in both phase I and II trials with a limited toxicity profile, including dose-limiting myelosuppression and mild to moderate nausea, vomiting, and mucositis.[425,426] Thirty-one patients were enrolled in a COG feasibility trial that incorporated high-dose topotecan into the Memorial Sloan-Kettering backbone.[427] The induction regimen was well tolerated, without unexpected toxicities. Fifteen of 31 patients (48%) achieved a complete or very good partial response. This regimen was recently studied by the COG in a large phase III trial in a large patient population to more accurately access efficacy; the results are pending.

A growing body of evidence suggests that the quality of induction response correlates with outcome. Ladenstein and colleagues[428] performed a multivariate analysis of 549 patients with high-risk neuroblastoma included in the European Bone Marrow Transplantation Solid Tumor Registry and showed that persistent cortical bone lesions ($P = .004$) and bone marrow involvement ($P = .03$) were the only independent adverse prognostic factors. This report included patients treated over a 14-year period, leading to significant interpatient treatment variability. More recent smaller retrospective studies have shown that MIBG response at the end of induction assessed by semiquantitative scoring methods directly correlates with EFS after myeloablative therapy.[429-431] Yanik and colleagues[432] assessed a semiquantitative MIBG score using the Curie scoring system for patients with INSS stage 4 disease who were treated in the COG A3973 trial at diagnosis ($n = 280$) and at the end of induction ($n = 237$). They found that patients who had a Curie score greater than 2 at the end of induction ($n = 52$) had a significantly decreased EFS compared with patients who had Curie scores of 2 or less (3-year EFS 15.4% ± 5.3%

vs. 44.9% ± 3.9%, $P < .001$). Patients who had MYCN amplification with a Curie score greater than 2 ($n = 6$) at the end of induction were particularly affected, with a 3-year EFS of 0% compared with 44.2% ± 6.9% for patients with MYCN amplification who had a Curie score of 2 or less ($n = 65$). These results support previous studies that showed that residual disease at the end of induction measured using Curie scoring of MIBG scans is clearly associated with a worse outcome in patients with high-risk disease. Efforts to further improve survival may need to include response-based end-of-induction treatment stratification, and alternative treatment strategies should be strongly considered for patients with Curie scores greater than 2.

Local Control

Patients with high-risk disease usually have large and locally invasive primary tumors that chemotherapy alone is unlikely to eradicate. Local control is obtained using a combination of surgery and local radiation therapy. Definitive surgery on the primary tumor is usually performed near the end of induction therapy after four to five cycles of chemotherapy. Delaying surgical resection until after chemotherapy has been shown to improve resectability and may reduce the surgical complication rate.[433,434] However, the benefit of complete resection in the treatment of high-risk neuroblastoma remains controversial because the impact of aggressive treatment of the primary tumor site on EFS is unclear in the face of metastatic disease. Several retrospective studies have shown contradictory results. La Quaglia and colleagues[435] reviewed 141 patients treated at Memorial Sloan-Kettering Cancer Center with sequential protocols between 1979 and 2002 and showed evidence of a significant improvement in survival with a gross total resection. Similarly, Adkins and colleagues[433] reviewed the 210 patients (out of 539 total patients) entered in the CCG 3891 study who achieved a complete response by resection and showed a trend toward improved 5-year EFS and OS. However, a report from St. Jude Children's Research Hospital that examined the extent of resection compared with outcome in 107 children older than 1 year with stage 4 neuroblastoma treated with one of four consecutive protocols between 1984 and 2001 showed no evidence of association between survival and the extent of surgery.[436] In addition, Von Allmen and colleagues[437] reported on the local control of 76 patients treated at two institutions using the same treatment protocol with a consistent surgical approach. They found that aggressive surgery followed by local radiation provided excellent local tumor control with no isolated local occurrences in patients who had greater than 90% resection (60 of 76 patients). However the completeness of resection was not correlated with a difference in EFS or OS. Further prospective evaluation of the role of surgery in high-risk neuroblastoma is needed. It is likely that resectability is a surrogate for tumor biology. Gross total resection with nodal resection should be the goal to provide adequate local control, but it should not be performed if it is done at the cost of vital organs or significant postoperative morbidity that would lead to a delay in further therapy.

Neuroblastoma is a highly radiosensitive tumor, and local radiation directed at the primary tumor site in persons with high-risk disease has been shown to decrease the risk of local recurrence.[377,438,439] The total dose generally used in the United States is 2160 cGy in daily 180-cGy fractions to the postinduction chemotherapy, preoperative primary tumor volume. Local radiation is generally used either at the end of induction or after recovery from consolidation chemotherapy. Patients whose radiation field includes their liver may be at higher risk of veno-occlusive disease if radiation is used prior to stem cell transplant.[440] To reduce the possibility of local recurrence after an incomplete surgical resection, the most recent COG study investigated giving patients who have an incomplete resection of the primary tumor an additional boost of 1440 cGy to the gross residual volume in addition to the standard 2160 cGy (for a total dose of 3600 cGy to the gross residual tumor volume). Results of this intervention are still under analysis.

Proton therapy, which can result in improved sparing of normal tissue, is becoming more widely available for pediatric patients. Preliminary studies show no difference in local control compared with photons.[441,442] Assessment in regard to improvement in late toxicity will require long-term follow-up of a large group of patients.

Consolidation Therapy

The goal of the consolidation phase of therapy for patients with high-risk neuroblastoma is to consolidate the response obtained during induction by eliminating any remaining tumor cells using myeloablative chemotherapy and stem cell rescue. The role of dose intensification to overcome tumor drug resistance mechanisms followed by bone marrow or peripheral blood stem cell support has been investigated for more than 20 years. Early nonrandomized studies suggested a survival advantage compared with historic control subjects but may have been influenced by selection bias.

The CCG conducted a randomized study from 1991 to 1996 to test the hypothesis that consolidation with myeloablative chemotherapy followed by purged autologous bone marrow rescue would improve EFS probability compared with nonmyeloablative consolidation chemotherapy.[443] A total of 539 eligible patients were enrolled in this clinical trial, and 379 patients were randomly assigned to myeloablative therapy (consisting of carboplatin, etoposide, melphalan, and 1000 cGy of total body irradiation) with autologous marrow rescue ($n = 189$) or continuation chemotherapy ($n = 190$). An intent-to-treat analysis showed a significant improvement in 3-year EFS for the patients assigned to myeloablative therapy (34% ± 4% vs. 22% ± 4%; $P = 0.03$; Fig. 54-8, *A*). A follow-up report of this study assessing long-term outcomes confirmed the benefit of myeloablative therapy, with a 5-year EFS of 30% ± 4% for patients treated with myeloablative chemotherapy versus 19% ± 3% for patients treated with conventional chemotherapy.[444] The German Society of Pediatric Oncology showed similar results in a randomized study of 295 patients receiving either myeloablative therapy with autologous stem cell rescue ($n = 149$) or consolidation with oral maintenance chemotherapy

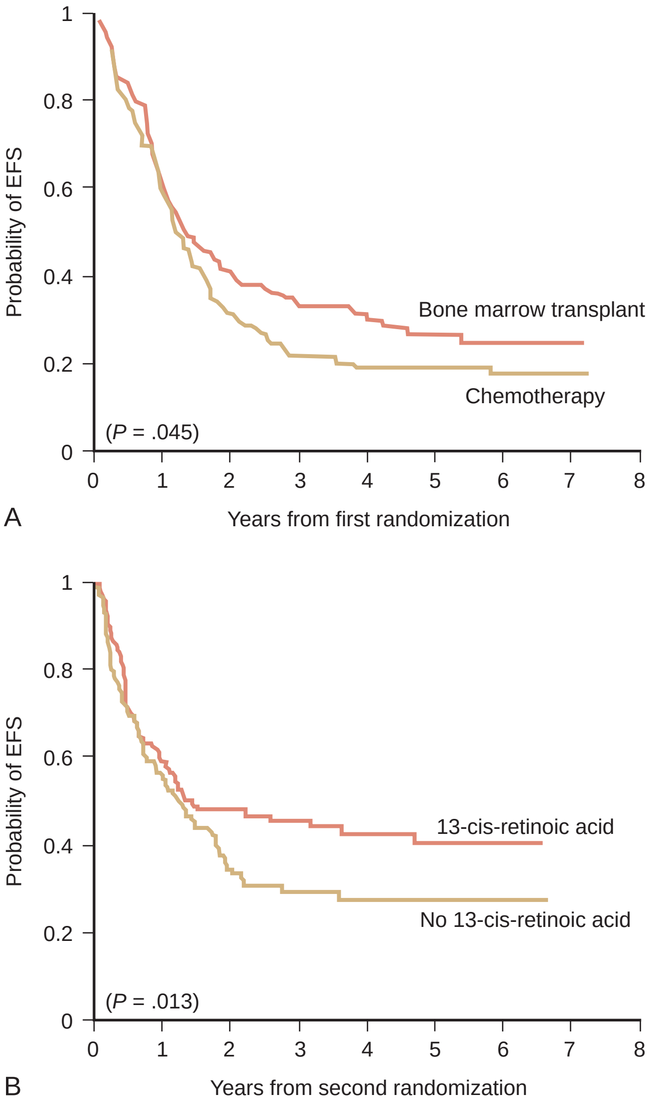


Figure 54-8 Children's Cancer Group study 3891 showing that autologous bone marrow transplant (ABMT) and 13-cis-retinoic acid improve event-free survival for patients with high-risk neuroblastoma. Patients were treated with identical induction chemotherapy, then randomized to ABMT or continuation chemotherapy and then further randomized to receive 13-cis-retinoic acid or no further therapy. **A**, Event-free survival *(EFS)* from time of randomization for patients randomized to ABMT (*n* = 189) or continuation chemotherapy (*n* = 180; *P* = .03). **B**, EFS from time of randomization to 13-cis-retinoic acid (*n* = 130) or no further therapy (*n* = 128; *P* = .03). *(Modified from Matthay KK, Villablanca JG, Seeger RC, et al: Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group.* N Engl J Med *341:1165–1173, 1999.)*

(*n* = 146). An intent to treat analysis again showed an improvement in 3-year EFS for patients assigned to myeloablative therapy (47%, 95% confidence interval [CI] 38-55, vs. 31%, 95% CI 23-39).[445]

Early myeloablative regimens for high-risk neuroblastoma used bone marrow as rescue with allogeneic marrow showing no survival advantage compared with autologous marrow.[446,447] The use of peripheral blood stem cells results in more rapid engraftment and less transplant-related morbidity.[448,449] In addition, peripheral blood stem cells may be less likely to contain contaminating tumor cells.[450] Peripheral blood stem cells are now the standard for myeloablative autotransplant regimens and are typically harvested after two to four cycles of induction chemotherapy.

An important concern relating to autotransplant is potential tumor cell contamination of the stem cell product contributing to relapse. Strategies to purge tumor cells from harvested marrow or peripheral blood stem cells include ex vivo immunomagnetic purging based on neural-specific antigens or CD34+ selection of hematopoietic progenitor cells.[449,451] A randomized clinical trial designed to test the efficacy of ex vivo purging of immunocytochemically negative peripheral blood stem cell products was performed by the COG. No difference in survival was seen in patients who received immunomagnetic purged stem cells compared with patients who did not receive these stem cells (5-year EFS 40% vs. 36% and 5-year OS 50% vs. 51%).[423]

Given the improvement in EFS observed with single transplant myeloablative strategies, tandem and even triple transplant regimens have been evaluated in limited institution and cooperative group pilot studies. The theoretic advantages to multiple transplants include exposure to multiple non–cross-resistant therapies at maximal doses in a relatively rapid sequence and possible modulation of the tumor microenvironment during the first transplant, potentiating cell killing with subsequent transplant(s).[449] Grupp and colleagues[449] and George and colleagues[452] treated 97 patients with high-risk neuroblastoma between 1994 and 2002 with a tandem rapid sequence myeloablative consolidation regimens using peripheral blood stem cell support in a limited institution single-arm study. They showed that tandem transplant was feasible with rapid myeloid cell recovery and low treatment-related mortality (five treatment-related deaths; 82 patients completed two transplants). The progression-free survival (PFS) rate at 5 years from diagnosis was 47% (95% CI, 36% to 56%), and the PFS at 7 years was 45% (95% CI, 24% to 55%) and the overall survival rate at 5 and 7 years, respectively, was 60% (95% CI, 42% to 64%) and 53% (95% CI, 40% to 64%; Fig. 54-9). This study and several others[453-455] suggest that further intensification of consolidation therapy may affect survival of patients with high-risk neuroblastoma. The COG recently completed a large randomized trial of single versus tandem myeloablative consolidations to further evaluate the true impact of a tandem high-dose myeloablative consolidation strategy, the results of which are still pending.

Using different chemotherapy for myeloablation is an alternative strategy that is being explored for high-risk consolidation therapy. A recent European high-risk neuroblastoma trial randomly compared two myeloablative regimens—busulfan and melphalan versus carboplatin, etoposide, and melphalan. Randomization was stopped early because of an interim analysis that showed that the regimen containing busulfan had a superior outcome (a 3-year EFS of 49% vs. 33% and a 3-year OS of 60% vs. 48%).[456] In this study the rapid COJEC induction regimen

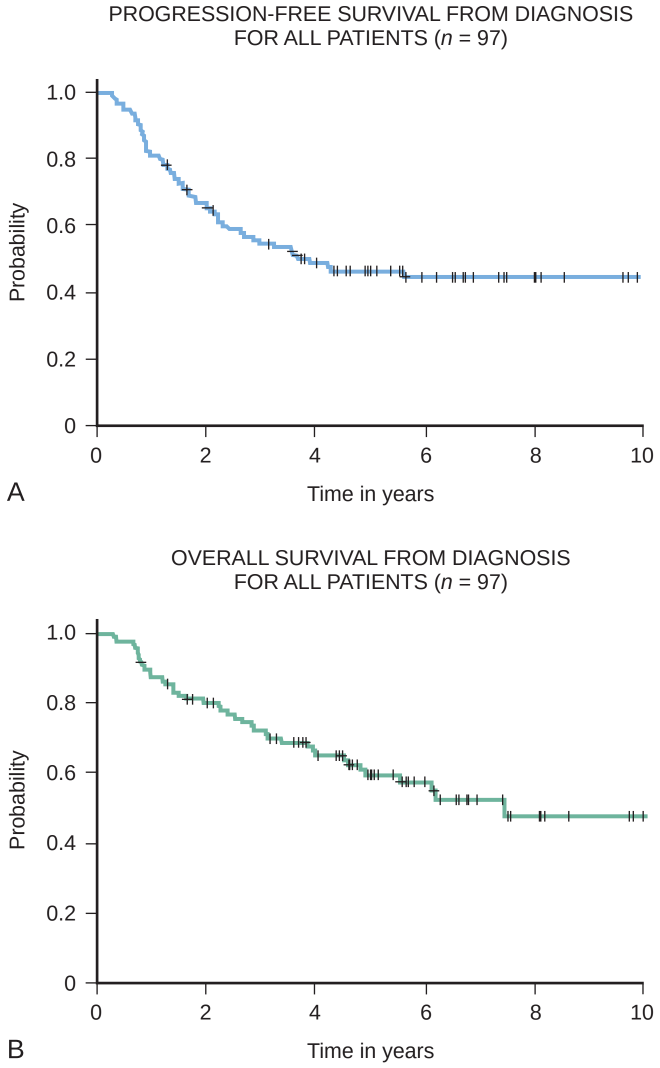


Figure 54-9 Tandem transplant for high-risk neuroblastoma. Progression-free survival (**A**) and overall survival (**B**) from diagnosis of 97 patients. *(From George RE, Li S, Medeiros-Nancarrow C, et al: High-risk neuroblastoma treated with tandem autologous peripheral-blood stem cell-supported transplantation: long-term survival update.* J Clin Oncol *24:2891–2896, 2006.)*

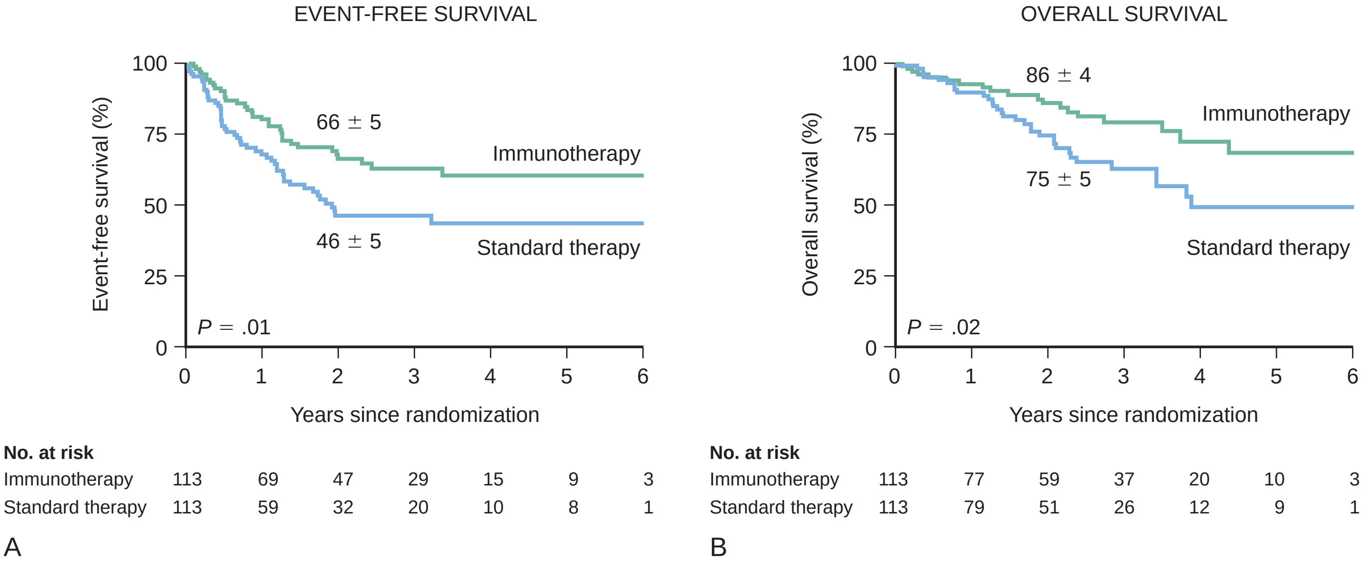
was used for all patients.[457] The high cumulative dose of platinum in this regimen may help explain the lower-than-expected survival for the arm containing carboplatinum, etoposide, and melphalan. Although results are still being analyzed, conditioning with busulfan and melphalan had less associated acute toxicity. The COG is running a pilot study to determine the feasibility of using busulfan and melphalan as consolidation therapy after the COG induction used in the last randomized trial. If feasible, this regimen will likely be used in the next COG phase III high-risk study.

Treatment of Minimal Residual Disease

Even when a complete remission is obtained after myeloablative therapy and stem cell rescue, relapse remains a significant problem, suggesting the presence of highly chemotherapy-resistant minimal residual disease. Alternative strategies targeting unique biologic features of neuroblastoma have therefore become an important part of current high-risk neuroblastoma treatment strategies after consolidation therapy. Minimal residual disease therapy with 13-cis-retinoic acid has been a standard in high-risk neuroblastoma care since the late 1990s. Immunotherapy targeted against the GD2+ antigen is now being more widely adopted as standard therapy.

The retinoids have been shown to decrease proliferation, decrease expression of the MYCN oncogene, and induce differentiation in neuroblastoma cell lines, including those established from refractory tumors.[458,459] A phase I study of children with high-risk neuroblastoma showed that an intermittent schedule of high-dose 13-cis-retinoic acid after transplant had minimal toxicity (primarily cheilitis, dry skin, and hypertriglyceridemia) and resulted in the clearing of tumor cells in the bone marrow by morphologic assessment in 3 out of 10 patients.[460] The efficacy of 13-cis-retinoic acid was tested in the previously mentioned CCG randomized trial using a factorial design after randomization to myeloablative or continuation chemotherapy.[443] A total of 130 patients were randomly assigned to receive either six cycles of 13-cis-retinoic acid or no further therapy. The cohort of patients assigned to receive posttransplant therapy with 13-cis-retinoic acid had a significantly improved EFS probability (46% ± 6% vs. 29% ± 6% from second randomization; $P = .03$; see Fig. 54-8, *B*). Ongoing studies of 13-cis-retinoic acid are focusing on the pharmacokinetics and proper dosing of 13-cis-retinoic acid, particularly in the youngest patients, as well as potential late toxicities of chronic dosing.[461,462]

Another biologic strategy that has been evaluated in the minimal residual disease phase of therapy is antibody therapy directed against the GD2 disialoganglioside that is uniformly expressed on neuroblastoma cells. Murine, chimeric, and humanized antibodies have shown activity in preclinical models, particularly in the minimal residual disease setting,[463-465] as well as phase I and II clinical trials.[377,466-468] The COG completed a randomized clinical trial testing the efficacy of ch14.18 with IL-2 and granulocyte-macrophage colony-stimulating factor and 13-cis-retinoic acid versus 13-cis-retinoic acid alone after myeloablative chemotherapy. The results showed a significant improvement in 2-year EFS and OS in the immunotherapy group compared with the patients who received isotretinoin alone (EFS, 66% ± 5% vs. 46% ± 5%, and OS, 86% ± 4% vs. 75% ± 5%[469]; Fig. 54-10). A nonrandomized trial of ch14.18 given alone that was performed by Simon and colleagues[470] showed no benefit of antibody therapy, suggesting that cytokine-mediated activation of antibody-dependent cell-mediated toxicity may be necessary. GD2 has restricted distribution in normal tissue to neurons, skin melanocytes, and peripheral sensory nerve fibers, and thus the toxicities usually associated with chemotherapy are not seen with immunotherapy. Instead, neuropathic pain can be a significant adverse effect in addition to cytokine-mediated capillary leak and hypersensitivity reactions associated with either the antibody or cytokines. Ongoing studies are exploring alternative antibodies, as well as alternative methods of cytokine delivery, as discussed later.



No. at risk

Immunotherapy	113	69	47	29	15	9	3
Standard therapy	113	59	32	20	10	8	1

No. at risk

Immunotherapy	113	77	59	37	20	10	3
Standard therapy	113	79	51	26	12	9	1

Figure 54-10 Children's Oncology Group study showing the efficacy of chimeric antiGD2 antibody and immunocytokine in high-risk patients. Kaplan-Meier Estimates of survival for patients randomly assigned to immunotherapy versus standard therapy (13-cis-retinoic acid alone). **A,** Event-free survival. **B,** Overall survival. *(From Yu AL, Gilman AL, Ozkaynak MF, et al: Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma.* N Engl J Med *363:1324–1334, 2010.)*

Assessment of Response to Treatment

For neuroblastoma, response to treatment is assessed using the International Neuroblastoma Response criteria, which are detailed in Table 54-8.[371] Determination of overall response requires assessment of both primary and metastatic sites. This assessment should be made using the same modalities that were used at diagnosis. The timing of response assessments varies according to protocol and risk group, but they are generally performed at the end of induction, before and after surgical procedures, and at the end of treatment. In addition to these response criteria, semiquantitative scoring systems for evaluating MIBG-positive disease have been developed.[429,471] As previously mentioned, the Curie scoring system has prognostic significance in patients with high-risk neuroblastoma. The ability to objectively quantify MIBG response is also useful in clinical trials for relapsed disease. Patients who have had a relapse often have only bone and/or bone marrow disease that can be seen with a MIBG scan but cannot be measured by CT or MRI using standard response criteria.

Late Effects of Therapy

Improvements in survival of patients with high-risk neuroblastoma during the past 20 years has come at the cost of intensification of therapy, putting these patients at increased risk of experiencing late effects of treatment. Hearing loss caused by exposure to high cumulative doses

TABLE 54-8 Definitions of Response to Treatment[371,381]

Response	Primary*	Metastases*	Markers*
1. CR	No tumor	No tumor (chest, abdomen, liver, bone, HVA/VMA bone marrow, nodes, etc.)	HVA/VMA normal
2. VGPR	Reduction >90% but <100%	No tumor (as above except bone); no new bone lesions, all preexisting lesions improved	HVA/VMA decreased >90%
3. PR	Reduction 50%-90%	No new lesions; 50-90% reduction in measurable sites; 0-1 bone marrow samples with tumor; bone lesions same as VGPR	HVA/VMA decreased 50%-90%
4. MR	No new lesions; >50% reduction of any measurable lesion (primary or metastases) with <50% reduction in any other; <25% increase in any existing lesion†		
5. NR	No new lesions; <50% reduction but <25% increase in any existing lesion†		
6. PD	Any new lesion; increase of any measurable lesion by >25%; previous negative marrow positive for tumor		

CR, Complete response; *HVA,* homovanillic acid; *MR,* mixed response; *NR,* no response; *PD,* progressive disease; *PR,* partial response; *VGPR,* very good partial response; *VMA,* vanillylmandelic acid.

*Evaluations of primary and metastatic disease as outlined in the text.

†Quantitative assessment does not apply to marrow disease.

of platinum chemotherapy at a young age is a particular problem that can put survivors at risk for learning problems and psychosocial difficulties.[472] Use of high doses of alkylating agents and topoisomerase inhibitors often results in sterility and puts patients at an increased risk of secondary leukemias.[473,474] Neuroblastoma survivors who received total body irradiation are also at risk for growth delay, cataracts, and thyroid insufficiency, as well as the risk of secondary cancers.[475] Survivors of high-risk neuroblastoma therapy should be followed-up regularly throughout their lifetimes with anticipatory management for these potential problems.[476]

NOVEL THERAPIES

Despite the intensive multimodal treatment that was previously described, more than half of children with high-risk neuroblastoma have a 5-year EFS of less than 50%.[343] Currently no standard treatment regimen exists for patients with relapsed neuroblastoma. Cytotoxic chemotherapy regimens for relapsed neuroblastoma are typically based on the camptothecin derivatives topotecan and irinotecan. Topotecan has been combined with both cyclophosphamide and with vincristine and doxorubicin in phase II studies.[477,478] Irinotecan is most commonly given with temozolamide[479] and is being used more frequently in the United States now that topotecan is being used in induction therapy for high-risk patients in the COG. Neither topotecan nor irinotecan-based therapies led to ultimate cure. Using novel strategies that are targeted to tumor-specific genetic alterations up front in conjunction with standard chemotherapeutic agents may improve cure rates without increased toxicity. Several of these targeted therapies are in clinical or preclinical development, as described later.

Targeted Radiotherapy

In addition to its use for staging and response evaluations, MIBG can be labeled with ^{131}I and used to deliver targeted radiotherapy. ^{131}I-MIBG has undergone extensive single-institution and, more recently, cooperative group clinical trials for the treatment of refractory neuroblastoma.[416,32] The results from a phase II study of 164 patients show that MIBG is active against refractory neuroblastoma with an objective response rate of 36% and disease stabilization in 34% of patients with frequent palliation of pain.[480] Treatment-related toxicity, even at substantial doses, is usually minimal. The most significant reported toxicity is hematologic with prolonged thrombocytopenia, which is most marked in patients with tumor in the bone marrow, and myelosuppression, both of which can be abrogated by autologous stem cell infusion.[480,481] Nonhematologic toxicities include brief nausea and vomiting, transient hepatic dysfunction, and chemical hypothyroidism. More recent clinical trials have focused on combining MIBG with potential radiosensitizing agents with known activity in neuroblastoma to enhance efficacy. The New Approaches to Neuroblastoma Therapy (NANT) consortium completed two phase I studies combining MIBG with a radiosensitizer. In the first study, MIBG was combined with irinotecan and vincristine, and it was shown that MIBG could safely be given at the single-agent maximal tolerated dose along with standard doses of chemotherapy.[482] In the other study, MIBG was combined with vorinostat, a histone deacetylase inhibitor that has been shown to increase the expression of functional norepinephrine transporters in neuroblastoma preclinical models and has completed accrual.[483] The NANT consortium next plans on performing a three-way phase II comparison of both combinations and MIBG as a single agent. In addition to MIBG, radiolabeled anti-GD2 antibodies are also under investigation to provide targeted radiotherapy.[484]

A phase I dose escalation study of ^{131}I-MIBG followed by myeloablative carboplatin, etoposide, and melphalan with stem cell rescue showed a response rate of 27% in patients who were refractory to standard therapy. Based on these promising results in patients who were refractory to treatment, a phase II study at the maximal tolerated dose was completed.[485,486] The COG is currently performing a pilot study to determine the feasibility of giving ^{131}I-MIBG prior to consolidation therapy in newly diagnosed high-risk patients.

Immunotherapy

As previously discussed, a monoclonal antibody directed against the GD2 antigen in combination with cytokines has been shown to significantly improve survival and is now being used as a standard part of the minimal residual disease phase of treatment in newly diagnosed high-risk patients. Current research is focusing on enhancing efficacy and making the regimen less toxic. To this end, the chimeric anti-GD2 antibody is being piloted with subcutaneous (rather than intravenous) IL-2[487] and with lenolidomide, which has been shown to enhance NK cell–mediated antibody dependent cellular cytotoxicity in preclinical models.[465,488] Humanized antibodies are also being developed that are designed to cause less hypersensitivity-type reactions and have increased tumor specificity. One of these antibodies, hu14.18K332A, has a single amino acid mutation that limits its ability to fix complement and thereby reduced the pain associated with ch14.18 while retaining the antibody-dependent cellular toxicity capabilities.[488a] Additionally, preclinical studies have shown that antibody efficacy can be enhanced by creating a fusion protein consisting of antibody linked to a cytokine such as IL-2.[463] A phase II study of a humanized GD2 antibody fused to IL-2 (hu14.18-IL2) showed a complete response in 5 of 24 patients with MIBG and/or bone marrow disease with tolerable toxicity, warranting further investigation.[489]

One limitation of monoclonal antibody therapy in patients with neuroblastoma is that it seems to be most active in the setting of minimal residual disease, with questionable benefit in patients who have bulky disease. More active immunotherapy strategies are also being developed to try to address this problem. These strategies include use of immunostimulatory antibodies such as ipilimumab (anti-cytotoxic T-lymphocyte antigen 4) and vaccination with anti-idiotype antibody or dendritic cells.[490-492] In addition, engineered cytolytic T lymphocytes that are directed against tumor-associated antigens (chimeric antigen receptor T cells) are under development with encouraging preliminary results.[493,494]

Molecular Targeting

Several large-scale sequencing efforts have concluded that sequence alterations are not represented in neuroblastoma in a major way, especially those that can be readily targeted therapeutically. However strategies focused on targeting genetic pathways relevant to proliferation of neuroblastoma cells hold promise. Additionally, alterations in chromatin remodeling and other epigenetic regulators may provide novel avenues for therapeutic intervention.

ALK

ALK mutations have been established as oncogenic drivers in neuroblastoma and offer bona fide therapeutic targets.[495] ALK small molecule inhibitors may be beneficial not only to 10% to 12% of patients with neuroblastoma who have mutation and amplification, but also to the proportion whose tumors overexpress the wild-type, phosphorylated ALK receptor and that appear to respond to genetic and chemical ALK depletion by undergoing apoptosis (Fig. 54-11).[186,198]

Small Molecule Inhibitors. The first ALK inhibitor to be developed was NVP-TAE684 (Novartis), a 5-chloro-2, 4-diaminophenylpyrimidine compound that induces significant cytotoxicity in neuroblastoma cells expressing different ALK mutations (Fig. 54-12).[155,496,497] This highly selective ATP-competitive inhibitor was identified by screening a kinase-targeted small molecule library against Ba/F3 cells expressing oncogenic kinases, specifically NPM-ALK.[496] TAE684 is especially selective for ALK because the orthomethoxy group of TAE684 projects into a small groove located between the side chains of residues L258 and M259 of the NPM-ALK kinase domain; bulkier amino acids at the L258 position (which are seen in other kinases) would lead to a steric clash with TAE684.[496] Treatment of neuroblastoma cells harboring the F1174L and the R1275Q ALK mutations induces rapid inhibition of phosphorylated ALK and its downstream signaling, leading to apoptosis and cell cycle arrest.[155,497] However TAE684 could not be taken forward to clinical development because it tends to form an extensive number of reactive adducts upon metabolic oxidation, which creates the potential for significant toxicity.[498] Nevertheless, it remains a valuable compound for preclinical studies of ALK kinase inhibition.

Crizotinib (PF-2341066; Xalkori), an orally bioavailable, 2,4-pyrimidinediamine derivative, is an ATP-competitive dual inhibitor of MET and ALK.[499,500] Crizotinib binds to the inactive conformation of both MET and ALK[501] and has shown striking efficiency against ALK-rearranged tumors, both in mouse models

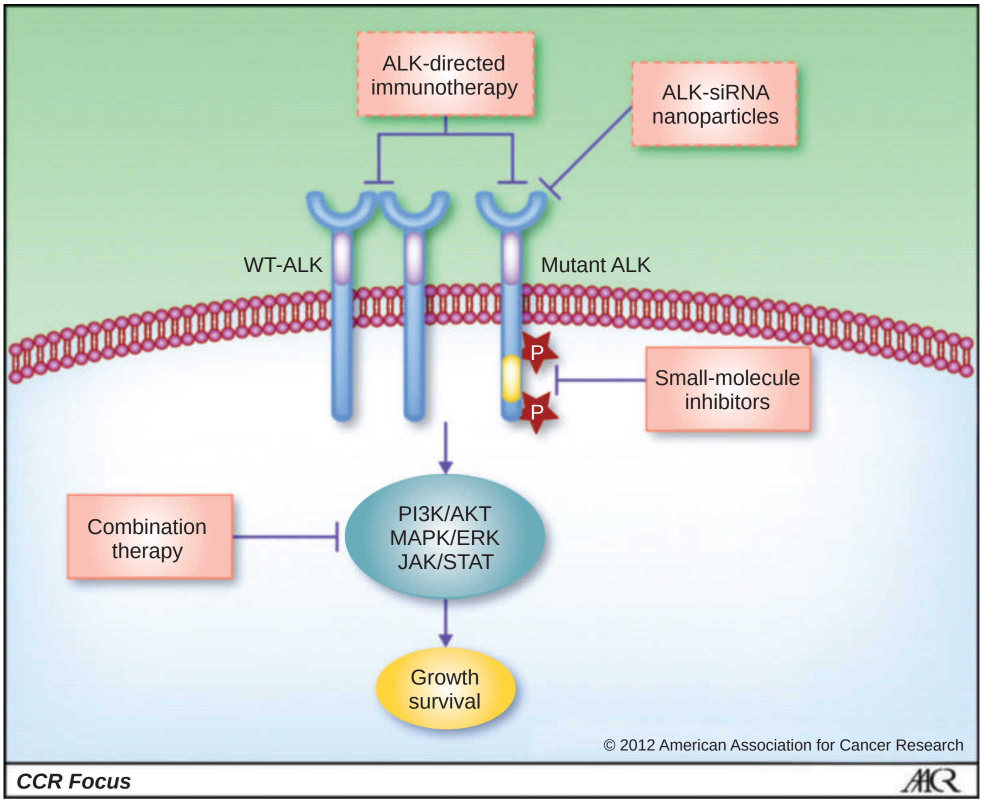
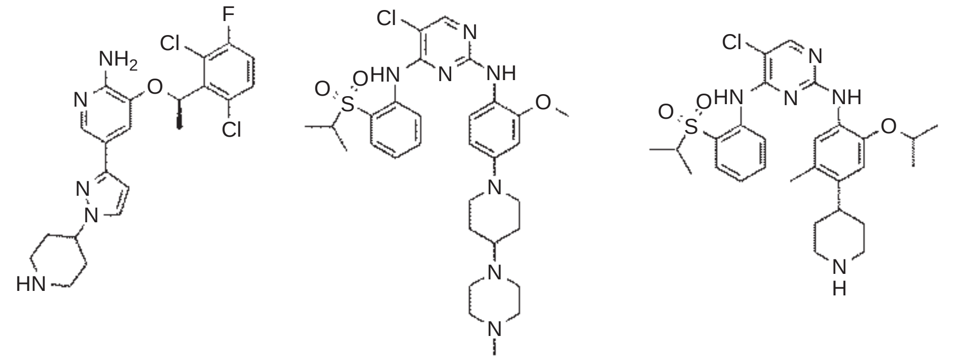
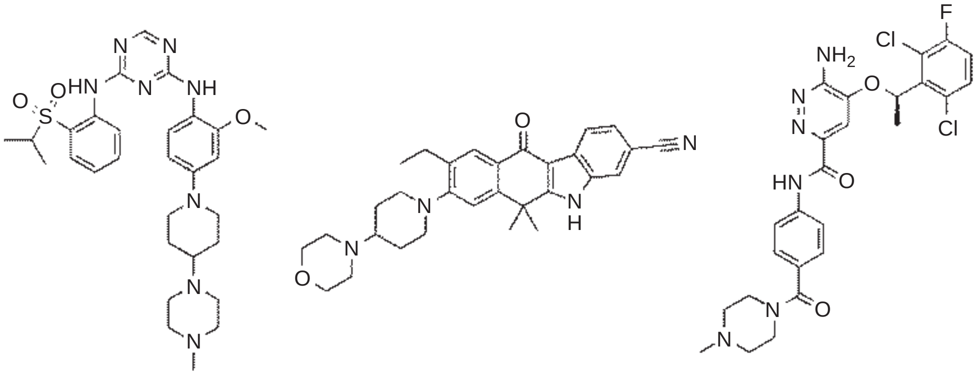


Figure 54-11 Current and future prospects for targeting anaplastic lymphoma kinase *(ALK)* in neuroblastoma. *JAK/STAT,* Janus kinase/signal transducer and activator of transcription; *MAPK/ERK,* mitogen-activated protein kinase/extracellular signal-regulated kinase; *PI3K,* phosphoinositide-3-kinase; *siRNA,* small interfering ribonucleic acid; *WT,* wild-type. *(From Matthay KK, George RE, Yu AL: Promising therapeutic targets in neuroblastoma.* Clin Cancer Res *18(10):2740–2753, 2012.)*



crizotinib **TAE684** **LDK378**



ASP3026 **CH5424802** **X-396**

Figure 54-12 Chemical structures of representative anaplastic lymphoma kinase inhibitors.

and in the clinic.[500,502] In early-phase clinical testing, for example, at a mean treatment duration of 6.4 months, the overall response rate was 57% in patients with EML4-ALK–positive NSCLC.[502] Crizotinib has also shown striking responses in adult patients with other ALK-rearranged cancers such as inflammatory myofibroblastic tumor (IMT) and anaplastic large-cell lymphoma (ALCL).[503,504] A phase III trial showed a superior PFS and overall response rate for crizotinib compared with chemotherapy in patients with NSCLC who had disease progression after standard chemotherapy.[505] Because of this result, crizotinib was granted approval for the treatment of patients with ALK-positive metastatic NSCLC by the U.S. Food and Drug Administration (FDA).

Unfortunately, the same positive results have not been observed in patients with neuroblastoma. When tested against neuroblastoma cells bearing either of two of the more common mutations, crizotinib inhibited growth and induced apoptosis in cells expressing ALK^{R1275Q} but failed to inhibit the growth of ALK^{F1174L}-positive cells.[194,506] The relative resistance of ALK^{F1174L} to crizotinib has been attributed to either lack of direct contact with the crizotinib-binding site within the ATP-binding pocket or the increased ATP-binding affinity of the mutant, with complete inhibition of constitutively active ALK attainable only at very high doses of the drug.[191] Crizotinib was tested in a COG phase I trial that included patients whose tumors expressed aberrant ALK. With results similar to those in adult patients and as expected from preclinical studies, patients whose tumors expressed translocated ALK (ALCL and IMT) responded very well to the drug.[507] However, the results for patients with neuroblastoma whose tumors harbored point mutations within the full-length receptor were less encouraging, with only 1 of 11 patients with ALK-mutated neuroblastoma showing an objective response to this agent.[507] Moreover, ALK mutations exhibit differential sensitivity to crizotinib. In this study, three of the four patients with ALK^{F1174L}-positive neuroblastoma tumors experienced progressive disease, compared with two of five whose tumors had a missense mutation at the R^{1275} locus.[507] Therefore crizotinib as a single agent does not appear to be sufficient to induce tumor regression in neuroblastoma. Other studies have shown that the cytotoxicity of crizotinib could be significantly enhanced by targeting the critical downstream pathways that mediate ALK signaling.[194,508,509] The ALK^{F1174L} mutation signals through the PI3K/mTOR pathway,[156] and combined inhibition of ALK and mTOR induces tumor regression and prolongs the survival of mice both in xenografts and transgenic models of neuroblastoma.[194,509] Therefore clinical trials of crizotinib in combination with downstream pathway inhibitors may enhance the antitumor activity of the former, and in particular, their ability to overcome the resistance of ALK^{F1174L}-mutated neuroblastoma cells to crizotinib.

Several other compounds are in development or are even in early-phase trials in adults with ALK-aberrant cancers.[498,510] LDK378 (Novartis Pharmaceuticals, Basel, Switzerland), which is derived from TAE684,[496] is an orally active inhibitor of the ALK kinase. In cellular assays, LDK378 inhibits ALK at an inhibitory concentration of 50% of 40 nM against wild-type ALK and of 22 nM against NPM-ALK. It also has an excellent pharmacokinetic profile in rodents, with an oral bioavailability of greater than 50%. LDK was shown was shown to induce a response rate of 80% in patients with NSCLC who had experienced disease progression after an initial response to crizotinib, and therefore this drug has received "breakthrough therapy" designation from the U.S. FDA for patients with ALCL or metastatic NSCLC whose disease progressed while being treated with crizotinib or who are intolerant to it. LDK is currently in phase II trials for NSCLC. It is currently being tested in phase I trials in pediatric patients (NCT01283516, ClinicalTrials.gov), and it may hold promise for the treatment of patients with neuroblastoma who have mutated ALK.

Other ALK inhibitors in development include CH5424802 (Roche/Chugai Pharmaceuticals, Ltd., Tokyo, Japan),[510] an orally available inhibitor that was shown to potently inhibit the growth of neuroblastoma cells expressing amplified ALK. This agent also displayed in vitro inhibitory activity against neuroblastoma cells expressing $\text{ALK}^{\text{F1174L}}$, which was shown to be comparable to that achieved in cells with wild-type ALK.[510] Whether CH5424802 will show efficacy for in vivo models of neuroblastoma with mutated ALK remains to be seen. Other ALK inhibitors in phase I/II clinical trials include ASP3026 (Astellas Pharma, Northbrook, Ill.),[511] CEP-28122 (Cephalon Inc., Frazer, Pa.),[512] AP26113 (ARIAD, Cambridge, Mass.), a small molecule that has exhibited activity as a potent dual inhibitor of ALK and epidermal growth factor receptor,[513] and X-396 (Xcovery, West Palm Beach, Fla.).[514]

ALK-Based Immune Therapy. The abundant expression of ALK on the surface of most neuroblastoma tumor cells and its restricted tissue expression in normal tissues render it an ideal tumor-associated antigen.[199] Treatment of neuroblastoma cell lines with ALK-specific monoclonal antibodies has been shown to induce enhanced immune cell-mediated cytotoxicity with inhibition of cell proliferation and cell death.[515] Moreover exposure to crizotinib induced accumulation of ALK on the cell surface, thus increasing the accessibility of the antigen for antibody binding. If validated in vivo, this strategy may be useful in cases of neuroblastoma that constitutively express ALK, either alone or in combination with small-molecule inhibitors. Other avenues such as treatments with an ALK-based chimeric antigen receptor are being actively explored.

RNA Interference-Based Therapeutic Strategies. Nanoparticles carrying ALK small interfering RNA have been shown to be effective in inducing antitumor activity in ALK-expressing neuroblastoma models.[516] In this study, the use of a special anti-GD2 targeted liposomal formulation of nanoparticles ensured the death, as well as specific and nontoxic delivery, of ALK-small interfering RNA GD2 expressing neuroblastoma cells. Although still far removed from the clinic, these preliminary results have the potential to be developed into therapies for children with neuroblastoma.

Resistance to ALK Inhibitors. One issue with molecularly targeted agents is the inevitable development of resistance, as has been observed with crizotinib.[508,517] Even if the small-molecule inhibitor is potent enough to block tumor growth once a tumor has acquired additional aberrations, this strategy generally becomes ineffective, leaving the patient with static disease that begins to progress once treatment is stopped.[518] Resistance to targeted agents usually develops as a result of the development of secondary mutations or activation of compensatory signaling pathways. Secondary mutations are frequently acquired in the "gatekeeper" position, where the presence of amino acids with bulky side chains such as methionine interferes with the binding of the kinase inhibitor. The ALK L1196 mutation has been identified in a patient with lung cancer with an EML4-ALK tumor translocation, after an initial response to crizotinib.[517] The F1174L mutation that occurs de novo in neuroblastoma also developed in a patient with IMT bearing the RAP2-ALK translocation who experienced a relapse after therapy with crizotinib that was initially successful.[191] Another mechanism by which cancer cells with activated mutant kinases evade treatment with kinase inhibitors is to switch to alternative signaling pathways. Thus NSCLC cells with MET gene amplification develop resistance to crizotinib by overexpressing the epidermal growth factor receptor.[519] Preclinical models of resistance will enable prediction of resistance mechanisms to newer ALK inhibitors and aid in the design of drug combinations to delay or even prevent resistance.

Targeting Amplified MYCN

Because deactivation of MYCN in MYCN-expressing neuroblastoma tumors causes proliferative arrest and tumor regression, treatment strategies focused on silencing MYCN would be expected to affect an appreciable proportion of patients with high-risk neuroblastoma. Yet direct targeting of MYCN has proved difficult because (1) it lacks an obvious "druggable" domain, and (2) its requirement in normal cells imposes a high risk of prohibitive nonspecific toxicity. However, it is possible to target MYCN indirectly through the molecules that regulate MYCN activation or stability either at the mRNA or protein level. These strategies include inhibition of epigenetic proteins such as BRD4,[520,521] decreasing MYCN protein stability through inhibition of aurora kinase,[522] or targeting molecules that are synthetic lethal to the growth and proliferative activity of MYCN.[523] Other methods include activation of p53-induced apoptosis,[524] differentiation induction,[141] and inhibiting MYCN-dependent metabolic dependencies.[525,526] A major consideration in thinking about strategies to inhibit MYCN regulation and function is to have a therapeutic window sufficient for antitumor activity without inducing severe toxicity to normal tissues.

BET Bromodomain Inhibitors. The bromodomain and extraterminal (BET) family of transcriptional coactivators are epigenetic reader proteins that recognize and bind to covalent modifications of chromatin[527] to promote gene activation. BET proteins BRD2, BRD3, and BRD4 have been found to be critical in promoting transcription of MYC and MYCN through their recognition of lysine side-chain acetylation on histone tails.[520,528] JQ1 is a novel thienotriazolo-1,4-diazepine compound that displaces BET bromodomains from chromatin by competitively binding to the acetyl lysine recognition pocket.[528] JQ1 suppresses the growth of MYCN-amplified neuroblastoma in both orthotopic models and transgenic *TH-MYCN*-MYCN models, with a significant increase in survival. Tumors showed increased apoptosis and decreased proliferation concomitant with decreased expression of MYCN.[520] JQ1 displaces BRD4 from the MYCN promoter, with depletion of MYCN mRNA and protein after its administration. Downregulation of the MYCN transcriptional program was induced in JQ1-treated cells, with genes involved in proliferation, apoptosis, neural crest stemness, and cellular differentiation being particularly affected. Therefore BET bromodomain inhibition may be a promising option for patients with MYCN-amplified neuroblastoma, and clinical trials are being developed.[520] Similar results were also seen with another BRD4 inhibitor GSK1324726A (I-BET726), which induced potent growth inhibition and cytotoxicity in most neuroblastoma cell lines tested irrespective of MYCN copy number or expression level.[521]

Although MYCN amplification predicted sensitivity to BRD4 inhibition in these two studies, cell lines that were sensitive to JQ1 did not always have MYCN amplification, suggesting that MYC or MYCN is not the sole predictor of sensitivity to BET inhibition. Wyce et al.[521] (2013) reported that BRD4 inhibition led to direct suppression of BCL2 in addition to MYCN, although neither protein fully accounted for the sensitivity of I-BET726 in neuroblastoma cells.

Aurora Kinase Inhibitors. The aurora serine/threonine protein kinase–A is critical for the stabilization of MYCN. Aurora kinase A (AURKA) associates with MYCN and prevents its Fbxw7 ubiquitin ligase-mediated proteosomal degradation.[522,529] AURKA also plays an important role in centrosome maturation and spindle formation during mitosis.[530] AURKA overexpression has been reported in MYCN-amplified neuroblastoma[531]; these cells depend on high levels of this protein for maintaining MYCN function.[522,531] The Aurora-A inhibitors, MLN8054 and MLN8237, have shown promising activity in neuroblastoma models.[529,532,533] These agents disrupt the MYCN-AURKA complex and promote degradation of MYCN. The disruption of the complex inhibits MYCN-dependent transcription, correlating with tumor regression and prolonged survival in a transgenic MYCN neuroblastoma model.[529] Additionally, additive effects have been observed with the combination of MLN8237 and vorinostat, an HDAC inhibitor.[534] AURKA inhibition has also been shown to inhibit angiogenesis in neuroblastoma.[535] MLN 8237 is currently in phase II trials in patients with neuroblastoma (NCT01154816).

PI3K inhibition. Small-molecule inhibitors of PI3K have been shown to block the growth of transgenic neuroblastoma models, and they also induce cell cycle arrest and apoptosis in human neuroblastoma cells with MYCN amplification.[536,523] MYCN is a critical target for PI3K inhibitors.[523] The MYCN protein is stabilized through PI3K signaling,[537] and blockage of PI3K and mTOR can destabilize MYCN, leading to decreased MYCN protein levels and blocked proliferation of tumors driven by TH-MYCN.[536,538] In addition to promoting MYCN degradation, PI3K/mTOR inhibitors have been shown to induce secondary paracrine blockage of angiogenesis. The PI3K inhibitor NVP-BEZ235 improved survival in both human (highly pretreated MYCN-amplified orthotopic xenograft) and transgenic mouse models of MYCN-driven neuroblastoma.[523] Clinical trials of the mTOR inhibitor temsirolimus, in combination with irinotecan and temozolomide, have shown tolerability in patients with relapsed or refractory solid tumors, including neuroblastoma,[539] and phase II trials of the combination are ongoing.

p53-Pathway Inhibition. Another way of inhibiting the effects of MYCN is by targeting the p53-dependent apoptotic pathway activated by MYCN. MDM2, an E3 ubiquitin ligase, mediates p53 degradation, as well as cytoplasmic localization and transcriptional inactivation. Deregulation of the ARF/MDM2 pathway is seen in neuroblastoma tumors, resulting in high MDM2 activity and subsequent p53 deactivation.[540] Uncoupling p53 from MDM2 with small molecules that compete for respective docking sites may offer another avenue for targeted therapy by activating gp53. Nutlin-3, a cis-imidazoline small molecule that mimics a p53-binding peptide, has been shown to interfere with MDM2 binding to p53 and has proven effective in reducing growth and inducing apoptosis in neuroblastoma cells.[541,542] Nutlin-3 has also been shown to prevent tumor metastasis in xenograft models[540] with accumulation of p53 protein, as well as induction of p53 target gene expression. Synergy has been shown between nutlin-3 and DNA-damaging chemotherapeutic agents,[543] as well as with the pan CDK inhibitor roscovitine (Seliciclib)[544] and the anti-VEGF antibody bevacizumab.[545] Nutlin-3 has also been shown to be effective in relapsed neuroblastoma by inhibiting drug efflux activities of P-glycoprotein and multidrug resistance–associated protein 1.[546] Thus nutlin agents may offer benefit in patients with neuroblastoma.

Polyamines. A number of genes involved in polyamine homeostasis are known to be MYCN or c-MYC targets, and the expression of others is linked to *MYCN* status.[525,526] Approaches that target the polyamine pathway may be effective in treating high-risk neuroblastoma, especially those with MYCN amplification. Polyamines are positively charged multifunctional polycations that are critical for cell growth, differentiation, and survival. Polyamine depletion leads to growth arrest, whereas overexpression is cytotoxic; therefore tight regulation of intracellular levels is critical (reviewed in reference 547).[547] Polyamines are elevated in neuroblastoma cells and the gene encoding ornithine decarboxylase–1 (ODC1), the rate-limiting enzyme in polyamine biosynthesis, is a direct transcriptional target of MYCN. ODC1 is coamplified or overexpressed with MYCN and is associated with a poor prognosis in MYCN-amplified tumors.[525] a-difluoromethylornithine (DFMO), which acts as a specific inhibitor of ODC1 and is the most widely studied inhibitor of polyamine metabolism, serves both as a chemotherapeutic and a chemopreventive agent.[548] DFMO treatment in neuroblastoma cell lines inhibited proliferation and increased tumor latency and overall survival in MYCN-driven transgenic neuroblastoma models.[525] It also enhances the effects of the cytotoxic chemotherapeutic agents cyclophosphamide and cisplatin[525] and induces synergistic and antiproliferative effects when combined with the cyclooxygenase 2 inhibitors celecoxib and sulindac.[549] A phase I clinical trial coordinated by the NANT consortium for the treatment of refractory neuroblastoma using high-dose DFMO and celecoxib in combination with standard chemotherapy (cyclophosphamide and topotecan) is under way. Polyamine enhancement is also seen in nonamplified tumors and is associated with high-risk disease, and therefore these strategies may also be effective in the latter group as well.[525,550]

Retinoids

Fenretinide (N-[4-hydroxyphenyl] retinamide; 4-HPR) is a synthetic retinoid that is cytotoxic to neuroblastoma cells in vitro. Unlike 13-cis-retinoic acid, it does not induce tumor differentiation of cells but is cytotoxic by a variety of other mechanisms.[551-553] Its mechanism of action is thought to be through de novo ceramide synthesis induction,[554-556] generation of reactive oxygen species,[557] angiogenesis inhibition,[558,559] and increased NK cell activity.[560] Fenretinide has been tested in preclinical and early phase clinical studies and showed wide interpatient variability of plasma levels with suggested antitumor activity in relapsed disease.[23-26] To improve plasma levels, an oral powder in an organized lipid complex was developed that increased 4-HPR levels in plasma and tissues.[560a] A phase I trial of this formulation of fenretinide confirmed attainment of higher plasma levels with minimal toxicity. Complete tumor responses were observed in patients with tumor involvement limited to bone marrow and/or bone metastasis, suggesting that relapsed patients who commonly present with similar disease may benefit most from this agent.[561]

TRKB Inhibitors

In animal models, CEP-701 (lestaurtinib), a small-molecule inhibitor, has shown activity against the TRKB tyrosine kinase,[562,563] which acts as an oncogenic kinase in a subset of neuroblastomas. In animal models, lestaurtinib alone significantly inhibited tumor growth, and it also enhanced the antitumor efficacy of the cyclophosphamide/topotecan combination.[564] Lestaurtinib was tested in a phase I trial and was found to be well tolerated, with evidence of tolerability of a dose level sufficient to inhibit phospho-TRKB activity in plasma. Additionally, lestaurtinib in combination with 13-cis-retinoic acid was found to be additive or synergistic in neuroblastoma cells.[565]

CDK4/CDK6 Inhibitors

The cyclin D/CDK4/CDK6/RB pathway is hyperactive in many cancers.[566] CDK4 and CDK6 encode serine threonine kinases that complex with D-type cyclins to phosphorylate or inactivate the RB protein. This in turns induces the release of RB from E2F transcription factors, thus allowing progression of G1-S phase progression and cell proliferation. Moreover, CDK4/CDK6 also suppresses senescence through the forkhead box M1 (FOXM1) transcription factor.[566a] Genomic amplification of cyclin D1 and CDK4 has been reported in a subset of neuroblastomas.[567-571] Others have evaluated CDK4/CDK6 inhibitors in neuroblastoma and have shown sensitivity to inhibition, with sensitivity attributed to G1 arrest and cellular senescence. Treatment with the Novartis inhibitor LEE011 reduced proliferation in 12 of 17 lines, although the mean inhibitory concentration of 50% was in the 300-nM range in sensitive lines. It caused cell cycle arrest and cellular senescence that was attributed to dose-dependent decreases in phosphorylated RB and FOXM1, respectively. Treatment resulted in delay of subcutaneous tumor xenografts.[572] Given that CDK4/CDK6 inhibition causes a cytostatic response, it is unclear what the effects would be if it were used in combination with conventional cytotoxic agents that rely on S-phase DNA replication,[573] and further studies may be required to provide elucidation. Therefore CDK4/CDK6 inhibition may be most effective in postchemotherapy maintenance treatment.

HDAC Inhibitors and Demethylating Agents

It is becoming increasingly clear that not only in other cancers but also in neuroblastoma, a large portion of the cancer genome undergoes DNA methylation and histone modification, leading to silencing of tumor suppressor genes. Methylation of caspase-8[294] and that of the tumor suppressor gene *CHD5* on chromosome 1p,[223] for example, have been reported in neuroblastoma. HDAC agents have also shown preclinical activity against neuroblastoma[280,574] and are of potential therapeutic benefit because they cause apoptosis of human neuroblastoma cells, with an enhanced inhibitory effect when combined with retinoic acid.[574-577] It is thought that HDAC inhibitors potentiate the derepression of retinoid responsive genes in the presence of ligand.[578] Vorinostat, an oral class 1 and II HDAC inhibitor, has shown efficacy in solid tumor models with additive or synergistic preclinical and/or clinical activity in combination with the demethylating agent 5-aza-2-deoxycytidine (decitabine)[579] and 13-cis-retinoic acid.[580] In pediatric phase I trials, no objective responses were seen, although a complete response was seen in one patient with neuroblastoma who received the combination of retinoic acid and vorinostat.[581] Increased acetylated-H3 histones in peripheral blood lymphocytes were identified as a surrogate marker of drug target activity.[581] Based on preclinical evidence that low-dose decitabine is sufficient to induce demethylation and subsequent expression of caspase-8 in neuroblastoma cells,[582] decitabine was tested in combination with cytotoxic chemotherapy in a phase I trial.[583] Although this dose resulted in caspase-8 promoter demethylation and gene expression in bone marrow samples, the dose of decitabine capable of producing clinically relevant biologic effects were not well tolerated with cytotoxic chemotherapy in these patients. Demethylation and reexpression of melanoma antigen-encoding gene (MAGE)–1 in peripheral blood mononuclear cells as a marker of demethylation was seen in the majority of patients.[583] Decitabine has also been shown to increase the expression of other cancer/testis antigens—MAGE-A3 and NY-ESO-1—on neuroblastoma cells, facilitating cytotoxic T-lymphocyte–mediated tumor cell killing,[490,584] and forms the basis of a cancer antigen vaccine therapy for patients with relapsed neuroblastoma (NCT01241162).

EZH2 Inhibitors

MYCN has been shown to induce EZH2 expression and activity,[237,585] and therefore inhibition of EZH2 could potentially cause reexpression of tumor suppressor genes that are suppressed as a result of stimulation of EZH2 by MYCN. In neuroblastoma cells, inhibition of MYCN expression causes decreased EZH2 protein levels, which is accompanied by a decrease in global H3K27me3.[237] EZH2 inhibition was shown to cause induction of genes having tumor suppressive or differentiation-inducing capacity. Inhibition of EZH2 using small hairpin RNA knockdown or a small-molecule inhibitor of EZH2, 3-Deazaneplanocin A, led to decreased growth and induction of neurites and induction of cell death as a result of increased activated caspase-3/7 activity.[237] The small-molecule inhibitor also inhibited the growth of neuroblastoma xenografts, suggesting that this may be a potential avenue to investigate in neuroblastoma.

CONCLUSIONS AND FUTURE DIRECTIONS

The clinical heterogeneity of neuroblastoma is clearly correlated with tumor biology. The significant progress to date in understanding neuroblastoma biology has led to the ability to stratify patients and adapt treatment according to prognostic variables, resulting in a significant reduction in therapy for low- and intermediate-risk patients and the intensification of treatment for high-risk patients. A remaining challenge for low- and intermediate-risk disease is to better understand the biology of the small subset of patients who have a poor outcome and tailor their therapy accordingly. High-risk disease remains a significant clinical problem. We have likely reached the limits of dose intensification, and innovative approaches are needed to improve survival and decrease treatment-related morbidity. Future progress will likely be made by translating new discoveries regarding the underlying pathobiology of neuroblastoma into targeted therapies that can then be integrated into current treatment strategies for newly diagnosed patients. The challenge will be to determine how these individually targeted agents can be combined with cytotoxic chemotherapy, whether up front, concomitantly, or in the setting of minimal residual disease. Finally, an understanding of relapsed high-risk tumors can be achieved only by analysis of the molecular aberrations that have developed during the evolution of resistance either by direct examination of relapsed tissue or through the validation of surrogate markers.

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CHAPTER 55

Pediatric Renal Tumors

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CHAPTER OUTLINE

PATHOLOGY OF PEDIATRIC RENAL TUMORS
Staging
Wilms Tumor (Nephroblastoma)
Clear Cell Sarcoma of the Kidney
Congenital Mesoblastic Nephroma
Malignant Rhabdoid Tumor of the Kidney
Renal Carcinomas in Children
Primitive Neuroectodermal Tumor of Kidney
Synovial Sarcoma of the Kidney
WILMS TUMOR (NEPHROBLASTOMA)
Epidemiology
Molecular Biology and Genetics
Wilms Tumor Subsets Defined by Genetic and Epigenetic Features
Clinical Presentation and Diagnostic Evaluation
Treatment
Anaplastic Wilms Tumor
Bilateral Wilms Tumor
Recurrent Wilms Tumor
CLEAR CELL SARCOMA OF THE KIDNEY
Epidemiology
Molecular Biology and Genetics
Clinical Presentation and Diagnostic Evaluation
Treatment
MALIGNANT RHABDOID TUMOR
Epidemiology
Molecular Biology and Genetics
Clinical Presentation and Diagnostic Evaluation
Treatment
RENAL CELL CARCINOMA
Epidemiology
Molecular Biology and Genetics
Xp11.2 and t(6:11) (p21:q12) Translocation Carcinomas
Clinical Presentation and Diagnostic Evaluation
Treatment
CONGENITAL MESOBLASTIC NEPHROMA
Epidemiology
Molecular Biology and Genetics
Clinical Presentation and Diagnostic Evaluation
Treatment
FUTURE DIRECTIONS

Pediatric renal tumors account for approximately 5% of malignancies in children younger than 15 years old and 3.6% of malignancies in children younger than 20 years old.[1] Among 9731 patients registered with the National Wilms Tumor Study Group (NWTSG) (1969–2002), Wilms tumor (WT) composed the vast majority of childhood renal tumors (92%), followed by clear cell sarcoma of the kidney (3.4%), congenital mesoblastic nephroma (1.7%), malignant rhabdoid tumor (1.6%), and rare miscellaneous neoplasms, including primitive neuroectodermal tumor, synovial sarcoma, neuroblastoma, and cystic nephroma (1.1%). Although not historically included on NWTSG studies, renal cell carcinoma accounts for 8% of renal tumors in children from birth to 19 years, according to data from the Surveillance, Epidemiology, and End Results (SEER) program.[1]

The study of pediatric renal tumors has had a significant impact on the field of oncology. WT has provided a paradigm for multidisciplinary treatment approaches and the conduct of cooperative group studies. WT investigators took the lead in establishing one of the leading biologic-sample banks, which now contains several thousand annotated tumor, blood, and urine samples. Tenets of cancer biology, including Knudson's two-hit model and loss of imprinting in tumorigenesis, were pioneered in WT. This chapter reviews the pathology, epidemiology, genetics and biology, and treatment of pediatric renal tumors.

PATHOLOGY OF PEDIATRIC RENAL TUMORS

Staging

Stage is the major determinant of prognosis and therapy for all pediatric renal neoplasms. Two staging systems are widely used (Table 55-1). The Children's Oncology Group (COG) staging system reflects tumor extent after surgery

TABLE 55-1 Staging Systems for Pediatric Renal Tumors

	Children's Oncology Group (COG) (Prechemotherapy)	International Society of Pediatric Oncology (SIOP) (Postchemotherapy)
I	Tumor is limited to kidney and is completely resected Renal capsule intact, not penetrated by tumor No tumor invasion of veins or lymphatics of renal sinus No nodal or hematogenous metastases No prior biopsy Negative margins	Tumor is limited to kidney and is completely resected Renal capsule may be infiltrated by tumor, but tumor does not reach the outer surface Tumor may protrude or bulge into the pelvic system or ureter, but does not infiltrate Vessels of renal sinus not involved
II	Tumor extends beyond kidney but completely resected • Tumor penetrates renal capsule • Tumor in lymphatics or veins of renal sinus • Tumor in renal vein with margin not involved • No nodal or hematogenous metastases • Negative margins	Tumor extends beyond kidney but completely resected • Tumor penetrates the renal capsule into perirenal fat • Tumor infiltrates the renal sinus or invades blood and lymphatic vessels outside renal parenchyma (or both) but is completely resected • Tumor infiltrates adjacent organs or vena cava but is completely resected
III	Residual tumor or nonhematogenous metastases confined to abdomen • Involved abdominal nodes • Peritoneal contamination or tumor implants • Tumor spillage of any degree occurring before or during surgery • Gross residual tumor in abdomen • Biopsy of tumor (including fine needle aspiration) before removal of kidney • Resection margins involved by tumor	Incomplete excision of the tumor which extends beyond the resection margins (gross or microscopic residual) • Involved abdominal lymph nodes, including necrotic tumor or chemotherapy-induced changes • Tumor rupture before or intraoperatively • Tumor has penetrated the peritoneal surface • Tumor thrombi present at resection margins • Surgical biopsy before resection (does not include needle biopsy)
IV	Hematogenous metastases or spread beyond abdomen	Hematogenous metastases or spread beyond abdomen
V	Bilateral renal tumors Each side should be substaged separately according to the preceding criteria (e.g., stage V, substage II [right], substage I [left])	Bilateral renal tumors Each side should be substaged separately according to the above criteria (e.g., stage V, substage II [right], substage I [left])

before chemotherapy is given. By contrast, the International Society of Pediatric Oncology (SIOP) system reflects tumor extent after 4 to 6 weeks of chemotherapy and surgery. The COG and SIOP staging systems are applied to all pediatric renal tumors except renal cell carcinoma, for which the TNM (tumor, nodes, metastasis) system is used.[2]

The COG staging schema is very similar to that used in the National Wilms Tumor Study 5 (NWTS-5), although one change distinguishes the current COG staging criteria from the prior NWTS-5 criteria.[3,4] Localized spillage or rupture, or any form of biopsy before removal of the kidney, is no longer considered stage II but instead is considered stage III. The SIOP system was recently revised to classify tumors with local and regional lymph node involvement as stage III. Previously lymph node involvement was considered stage II, with separate designations for node-positive and node-negative disease.[5]

Wilms Tumor (Nephroblastoma)

Gross Features

WT typically presents as a unicentric, spherical mass that is sharply demarcated from the renal parenchyma.[6] Approximately 10% of WTs are multicentric, a finding that is associated with an increased likelihood of WT formation in the contralateral kidney. Approximately 5% to 6% of WTs are bilateral at presentation.

The cut surface of WT is generally pale gray and uniform, but hemorrhage, necrosis, and cyst formation are common. The texture is usually soft and friable, but stroma-rich neoplasms may have a dense, myomatous consistency. Calcification is relatively uncommon. Prominent septa frequently impart a multinodular appearance.

WT may arise anywhere in the cortex or medulla, frequently compressing and distorting renal parenchyma around its margin. Rarely exophytic tumors connected to the renal surface by a narrow stalk mimic extrarenal WT. Polypoid masses in the pelvicalyceal lumen may occur either as extensions from the primary intrarenal neoplasm or as separate neoplasms arising within the pelvic wall. The renal vein and its branches not uncommonly are filled by tumor thrombus that can extend via the inferior vena cava into the right atrium.

Cystic Variants of Wilms Tumor

Scattered cysts are commonly encountered in conventional WT, but rarely the encapsulated, well-delineated neoplasm is composed entirely of cystic spaces and delicate septa, without an expansile solid component. For neoplasms that are entirely composed of mature cells, the term is cystic nephroma (CN). If the septa contain embryonal cell types, the designation cystic, partially differentiated nephroblastoma (CPDN) is appropriate. In contrast, cystic WT is distinguished by the presence of solid, expansile regions that replace or distort the cystic spaces, rather than being passively molded by the cysts. This distinction is best made by careful examination of the gross specimen.[7]

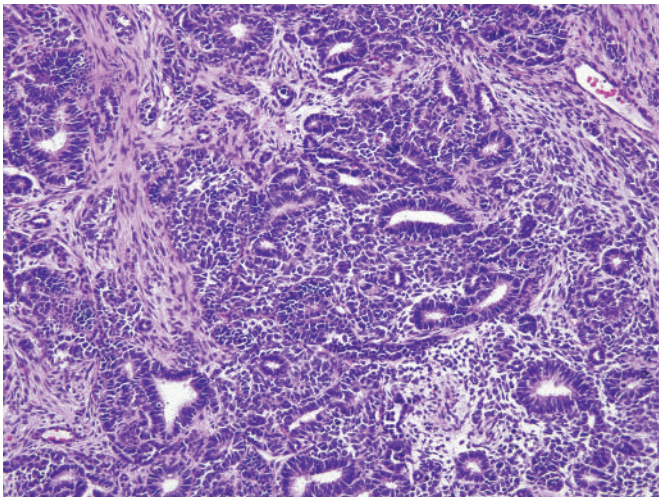

Figure 55-1 Wilms tumor, favorable histology, triphasic pattern.

CPDN has traditionally been thought to be a transitional stage in a continuum ranging from CN to WT. However recent studies show that CN is associated with *DICER1* mutations, whereas such mutations are not observed in CPDN. This suggests that the two cystic renal tumors are distinct entities.[8] The distinction of CN from CPDN has little clinical importance when the lesion has been completely resected because both lesions are curable by resection alone.

Microscopic Features

WTs are surpassed only by teratomas in their diversity of cell and tissue types and degrees of differentiation. Most WTs exhibit, at least focally, the so-called triphasic appearance, including cells of blastemal, stromal, and epithelial lineage (Fig. 55-1). However monophasic and biphasic WTs are relatively common, consisting of only one or two of these cell lineages. Each of the three major cell types can demonstrate a spectrum of patterns and degrees of differentiation, accounting for the remarkable diversity of appearances characterizing various WTs.

Blastemal Patterns. The blastemal cells of WT are small, tightly packed cells with a high nucleus-to-cytoplasm ratio, demonstrating little or no evidence of differentiation toward epithelial or stromal cell types at the light-microscopic level. Their nuclei are usually round or oval, with moderately coarse chromatin, and mold to one another in the same fashion as do the nuclei of small cell carcinoma of the lung. Nucleoli are relatively inconspicuous. Blastemal cells form several distinctive aggregation patterns, which can be divided into two broad categories, diffuse and nested, depending on their structure and degree of invasiveness. Monomorphous, relatively dyscohesive sheets of blastemal cells with aggressively invasive margins characterize the diffuse blastemal pattern. This is the most consistently aggressive pattern of WT. Stage I WTs rarely show this pattern; the majority of diffuse blastemal WT present at advanced stage, stage III or IV. Fortunately most neoplasms with the diffuse blastemal pattern are highly responsive to modern therapeutic protocols, so this pattern remains in the "favorable histology" category.[9] The diffuse blastemal pattern can be readily confused with other small blue cell tumors of childhood, and ultrastructural or other special studies may be required to establish the correct diagnosis (Fig. 55-2).

Nested blastemal patterns are characterized by sharply outlined clusters of blastemal cells in a myxoid mesenchymal background. They usually lack the invasive behavior seen with the diffuse blastemal pattern, and these neoplasms have sharply defined margins at their advancing edge. Several nested patterns may be seen. The serpentine blastemal pattern features long, serpiginous, anastomosing cords of blastemal cells in a loose, stellate, spindled stroma. This is a highly distinctive pattern of WT and helps distinguish WT from other small blue cell neoplasms of childhood. The nodular blastemal pattern resembles the serpentine pattern but has rounded blastemal nests instead of long cell cords. The basaloid blastemal pattern resembles the serpentine or nodular patterns, except that the blastemal cell clusters are outlined by a palisaded layer of cuboidal or columnar cells, reminiscent of the architecture of cutaneous basal cell carcinoma.

Epithelial Patterns. Epithelial differentiation in WT produces a variety of cell types and degrees of differentiation. Most of these recapitulate events in normal nephrogenesis (homologous differentiation). Others, such as squamous differentiation or mucinous differentiation, do not occur in the normal kidney at any stage of development (heterologous differentiation). Tubular differentiation is the most frequent epithelial pattern. This ranges from vague hints at tubular formation in blastemal foci (which may resemble rosettes) to highly differentiated tubules resembling those of the mature kidney. WTs in which tubular differentiation is predominant tend to be less aggressive, and most of these present at stage I. Although not usually invasive, tubular-predominant WTs often grow rapidly, and it is not uncommon to find huge tumors that remain confined to the kidney. Glomerular

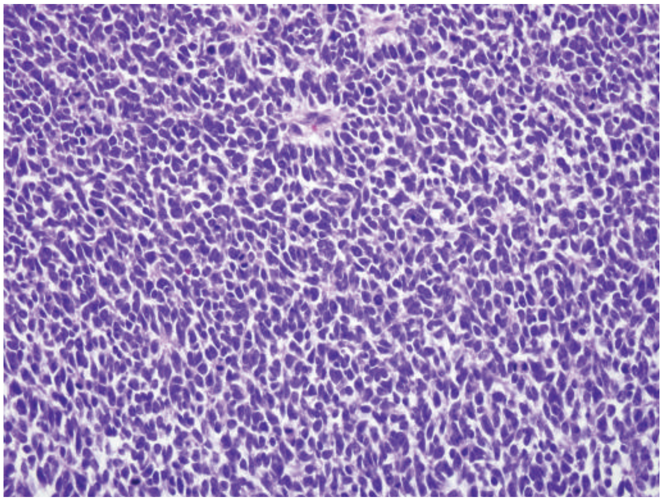

Figure 55-2 Wilms tumor, favorable histology, diffuse blastemal pattern.

differentiation, present in many WTs, ranges from simple papillary formations barely suggesting glomerulogenesis to mature tumor glomeruli closely resembling those of normal developing kidneys.

Stromal Patterns. Immature myxoid and spindled mesenchymal cells are the most common stromal cell types seen in WTs. Skeletal muscle is the most common heterologous cell type. The presence of skeletal muscle (rhabdomyoblastic differentiation) in WT must not be confused with renal rhabdoid tumor, which is discussed later in this chapter. In fact, the presence of skeletal muscle in a pediatric renal neoplasm is very strong evidence supporting the diagnosis of WT.

Anaplasia: the Sign of Unfavorable Histology in Wilms Tumor

Approximately 5% of WTs demonstrate anaplastic nuclear change, the only criterion of "unfavorable histology."[10] All WTs lacking this feature are designated as having "favorable histology." Anaplasia is almost never seen in WT diagnosed during the first year and is rare in the second year of life. The relative frequency of anaplasia increases after that age, and it is found in approximately 10% of WTs diagnosed after the age of 5 years. Anaplastic nuclear change reflects extreme polyploidy and is usually apparent under low magnification (10× objective). The features of anaplasia include (a) markedly enlarged tumor cell nuclei with increased chromatin content (hyperchromasia) and (b) multipolar mitotic figures (Fig. 55-3). The former criteria reflect polyploidy, whereas the latter criterion helps exclude degenerative nuclear changes, as are commonly seen in cells showing skeletal muscle differentiation, from the anaplastic category. Anaplasia is tightly correlated with the presence of *TP53* gene mutations.[11,12] Whereas favorable-histology WT virtually never harbors *TP53* mutations, the majority of anaplastic WTs do. At a practical level, p53 protein overexpression by immunohistochemistry correlates well but not perfectly with morphologically defined anaplasia.[13]

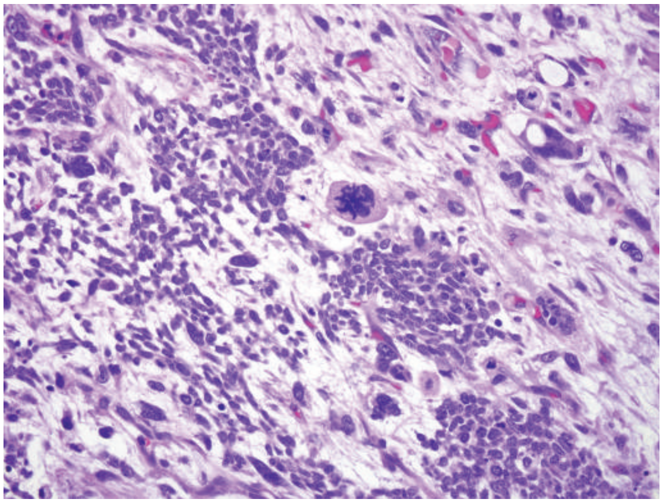

Figure 55-3 Wilms tumor, anaplastic.

Focal and Diffuse Anaplasia. Initially the presence of anaplastic nuclear changes in any region of a WT was considered prognostically unfavorable. It subsequently was suggested that anaplasia is an indicator of increased resistance to adjuvant chemotherapy and not necessarily a marker of increased tumor aggressiveness; therefore stage I anaplastic WT and WT with limited intrarenal foci of anaplasia (focal anaplasia [FA]) would be predicted to have an excellent prognosis. This concept implies that the prognosis for a patient with anaplastic WT is determined by the completeness of surgical removal of anaplastic cells.[14] However recent data on patients with stage I anaplastic WT have challenged this concept.[15] Regardless the definition of FA includes only those WTs meeting the following criteria:

1. Anaplasia is confined to one or more discrete sites within the primary tumor and is not present in extrarenal sites.
2. Tumor cells outside anaplastic foci show no "nuclear unrest" (nuclear or mitotic abnormalities that approach, but do not quite attain, the degree of severity required for a designation of anaplasia).

Any WT not meeting this definition of FA is designated as having diffuse anaplasia (DA). Any of the following situations merits assignment to the DA category:

1. Nonlocalized anaplastic change
2. Anaplastic change or nuclear unrest in invasive sites or any extrarenal deposits
3. Localized anaplastic change in a tumor that also has severe nuclear unrest elsewhere
4. Anaplasia in a random biopsy specimen
5. Anaplasia involving the edge of one or more sections, and the site(s) from which the sections were taken cannot be determined. (The latter emphasizes the importance of mapping the sections taken from a WT, best done on a photograph of a cut section of the tumor.)

Anaplastic foci are usually clearly demarcated from adjacent nonanaplastic tumor. Most cases in the FA category have a single focus of anaplasia. Anaplastic foci are usually only a few millimeters in diameter but can be larger if their localized nature can be convincingly documented.

The SIOP Postchemotherapy Histologic Classification System

Patients treated on SIOP studies receive several weeks of chemotherapy before undergoing nephrectomy. Chemotherapy usually affects necrosis of immature and actively proliferating cell types in WT, whereas slowly replicating and differentiated cell types are usually unaffected. For example, tumors composed mostly of mature skeletal muscle or renal tubules show little clinical regression with chemotherapy because so few cells are proliferating. The microscopic appearance of the tumor after chemotherapy has prognostic significance. Approximately 5% to 10% of WTs are completely necrotic after chemotherapy, a finding associated with a 98% 5-year relapse-free survival rate.[16] By contrast, WTs with a predominance of blastemal cells after chemotherapy, defined as viable cells in more than one third of the tumor mass and blastemal cells in

TABLE 55-2 Comparison of COG and SIOP Histologic Risk Classification Schemas

Children's Oncology Group (COG)	International Society of Pediatric Oncology (SIOP)
Favorable-histology Wilms tumor • No evidence of anaplasia Focal anaplastic Wilms tumor • Anaplasia confined to one or more discrete sites within the primary tumor with no extrarenal involvement • No nuclear unrest outside anaplastic foci Diffuse anaplastic Wilms tumor • Nonlocalized anaplasia • Anaplasia in invasive sites or extrarenal deposits • Localized anaplasia with severe nuclear unrest elsewhere • Anaplasia in a random biopsy specimen. • Anaplasia involving the edge of one or more sections Non–Wilms renal tumors not included in this classification schema	Low risk • Mesoblastic nephroma • Cystic partially differentiated nephroblastoma • Completely necrotic Wilms tumor Intermediate risk • Wilms tumor of epithelial, stromal, mixed, or regressive types • Focal anaplastic Wilms tumor High risk • Blastemal-type Wilms tumor • Diffuse anaplastic Wilms tumor • Clear cell sarcoma of the kidney • Rhabdoid tumor of the kidney

at least two thirds of the viable component, have relapse rates of nearly 40%.[5,17] Based on these observations, the SIOP histologic risk classification schema divides renal tumors into three risk categories: low risk, intermediate risk, and high risk. The COG and SIOP histologic classification systems are compared in Table 55-2.

Ultrastructural and Immunohistochemical Studies

Ultrastructural study is rarely necessary to establish a diagnosis of WT but can occasionally be very helpful in distinguishing blastemal WT from other undifferentiated neoplasms.[18,19] The diversity of differentiation in WT creates a correspondingly varied profile of immunohistochemical results. Blastemal cells may or may not label for vimentin and cytokeratin, whereas various differentiating elements will label according to their patterns of differentiation. For example, primitive rhabdomyoblasts within a tumor will label for the myogenic transcription factor myogenin and for cytoskeletal proteins such as actin and desmin. WT blastemal cells characteristically label for desmin but not for actin, myogenin, or other muscle markers.[20] Immunoreactivity for WT1 protein is typically limited to the blastemal and epithelial components of WT, with the stroma being negative.[21] Hence absence of labeling for WT1, particularly in a stroma-rich tumor, does not exclude the diagnosis of WT. Although WT1 immunoreactivity may help distinguish WT from PNET, it should be noted that desmoplastic small round cell tumor typically labels for WT1. Although useful, WT1 immunoreactivity cannot in and of itself establish or disprove a diagnosis of WT.

Nephrogenic Rests and Nephroblastomatosis

In more than 30% of kidneys resected for WT, the renal parenchyma contains one or more regions of persistent embryonal tissue, representing potential precursors of WT. These lesions have been given many names in the past; however, the term suggested by Dr. J. Bruce Beckwith, nephrogenic rests (NRs), is the currently accepted terminology.[22,23] The presence of multiple or diffusely distributed NRs is called nephroblastomatosis. This term is most commonly applied when the NRs are in an active state of cellular proliferation and are large enough to be apparent on imaging studies.

NRs have a dynamic life history that can yield a variety of appearances. Dr. Beckwith's classification is based on the assumption that the structure of NRs reflects both the dynamic state and the history of an individual rest. A brief summary is provided here.

Two fundamental categories of NRs are recognized, based on their topographic relation to the renal lobe. These are designated perilobar nephrogenic rests (PLNRs) and intralobar nephrogenic rests (ILNRs). ILNRs may occur anywhere in the renal lobe, including the peripheral cortex. They may also occur within the renal sinus, including in the walls of the pelvicalyceal system. ILNR have a more varied structure than do PLNRs and typically intercolate between nephrons, whereas PLNRs usually are discrete structures that are well delineated from adjacent nephrons. Rests of either major category may be further classified on the basis of their developmental fates. An individual NR may undergo any of the following fates, several of which often occur sequentially over time:

1. It may remain unchanged in size or composition, even for many years, as a tiny, microscopic blastemal focus (dormant NR).
2. It may undergo maturation, sclerosis, and eventual disappearance (sclerosing NRs and obsolescent NRs). This is the most common fate of NRs.
3. It may undergo hyperplasia, or coordinated proliferation of all susceptible cells of the rest, as distinguished from a clonal neoplastic process originating in a single cell of the rest. Hyperplastic NR may produce large, actively growing masses that have numerous mitotic figures, and a section from the interior of a hyperplastic NR may be indistinguishable from WT. Several features do help make this distinction. First diffuse hyperplastic growth, involving all or most cells of a rest, tends to preserve the original shape of the rest. When PLNRs form a continuous layer of embryonal cells at the lobar surface, hyperplastic proliferation will produce a thick "rind" of abnormal tissue at the renal surface. Ovoid and lenticular masses result from hyperplasia of NRs that originally had these shapes. An irregular-shaped, multinodular appearance will result if only some of the cells are capable of proliferation. In contrast, WT, a neoplasm presumably arising from a single cell, tends to form spherical masses. The second important feature distinguishing actively hyperplastic NRs from WT is the usual

absence of a pseudocapsule at the interface between hyperplastic NRs and the renal parenchyma.

4. Neoplastic induction is assumed to represent a clonal event originating in single cells of a rest, resulting in WT or benign adenomas. As mentioned previously rapidly growing tumors originating at a single point (or cell) will tend to grow equally in all directions, forming spherical, expansile nodules with compressed rest remnants often present at the periphery.
5. Very rarely, anaplasia may develop in nephrogenic rests.[24]

An individual rest commonly progresses through several of these processes sequentially. For example, an incipient or dormant rest may undergo hyperplasia, followed by phases of growth arrest and maturation. This will result in a large but inactive-appearing lesion. Ultimately, one or more cells within the regressing rest may be induced to form a WT.

ILNRs are most often found at the tumor-kidney interface, where they can be misinterpreted as infiltrating tumor cells or effaced by tumor compression. A helpful feature distinguishing ILNRs at the edge of a WT is the poorly defined, irregular outer border of the ILNR, which contrasts with the sharp, pushing interface between the WT and the rest within which it arose. Also ILNRs are usually less cellular than the adjacent WT that they surround.

The presence of NRs in a kidney removed for WT is correlated with an increased risk for subsequent WT formation in the remaining kidney. The type of rest and the age of the patient modify this risk.[25] When a carefully sampled kidney is free of rests, the risk of contralateral WT is extremely low. The possibility of subsequent WT developing in the remaining kidney should be considered in planning the follow-up of patients whose nephrectomy specimen reveals the presence of NRs in addition to WT.

Differential Diagnosis of Wilms Tumor

Triphasic Wilms Tumor. The triphasic pattern rarely presents a problem in diagnosis, except when small biopsies are obtained from large retroperitoneal masses of uncertain origin. In this setting other mixed neoplasms might deserve consideration, including teratoma, hepatoblastoma, pancreatoblastoma, teratoma, mesothelioma, synovial sarcoma, and intraabdominal desmoplastic small round cell tumor. In the absence of nephrogenic differentiation or the distinctive nested blastemal patterns described previously, ancillary studies such as immunohistochemistry, molecular biology, or electron microscopy may be required to distinguish some of these lesions from WT. WT with extensive heterologous differentiation ("teratoid WT") is easily confused with immature teratoma. Renal teratomas are extremely rare, and some of the reported cases likely represent teratoid WT.

Blastemal Wilms Tumor Versus Other Small Blue Cell Tumors of Childhood. The problem of distinguishing blastemal WT from other small blue cell tumors is most likely to arise when dealing with biopsies from metastatic sites or from large abdominal tumors of uncertain origin. The distinctive aggregation patterns of blastemal WT, the presence of nuclear molding, or the focal presence of tubular differentiation will often reveal the diagnosis. Early tubular differentiation in WT lacks lumens and therefore can sometimes be confused with rosettes of neuroblastoma or primitive neuroectodermal tumor (PNET). The presence of true lumens even focally confirms tubular differentiation, whereas neurofibrils are diagnostic of a neuroblastic pseudorosette. Neuroblastic rosettes do rarely occur in WT, but usually only in teratoid WTs, which are not likely to be confused with neuroblastomas or PNETs. Immunohistochemistry, electron microscopy, molecular diagnostic techniques, cytogenetics, circulating tumor markers, and other special studies are often required to confirm the nature of a small blue cell tumor.

Epithelial Predominant Wilms Tumor Versus Papillary Renal Cell Carcinoma. The challenge of distinguishing epithelial predominant WT from papillary renal cell carcinoma (PRCC) is most often encountered in tumors from adolescents or adults. Papillary adenoma, the benign precursor of PRCC, may resemble epithelial predominant NRs, and epithelial predominant WTs can have a predominantly papillary architecture. Sometimes PRCCs have a predominant tubular or solid component. Molecular or cytogenetic studies may be helpful because PRCC characteristically contains increased copies of chromosomes 7 and 17 and, in tumors from male patients, deletion of Y.[26] Unequivocal glomerular differentiation, characteristic blastemal aggregation patterns, or the presence of heterologous cell types can confirm the diagnosis of WT. Immunoreactivity for cytokeratin 7 has been advocated as a useful marker for PRCC, but focal positive labeling for this marker occurs in many WTs. Only when diffusely positive is cytokeratin 7 labeling likely to be discriminatory. Nuclear labeling for WT1 protein may distinguish WT, NRs, and metanephric adenoma from PRCC, insofar as only the latter does not label.[27]

Clear Cell Sarcoma of the Kidney

Gross Appearance

Clear cell sarcoma of the kidney (CCSK) is always unicentric, with a distinct tumor-kidney junction. Tumors are usually relatively large, with a mean specimen diameter of approximately 11 cm.[28] The cut surface is often glistening and gelatinous. Cysts are almost always present and may be so prominent as to suggest cystic nephroma on gross examination or imaging studies.

Microscopic Appearance

Under low magnification, most CCSKs appear monomorphous, without the prominent lobulation usually seen in WT. CCSK usually has a scalloped border that appears fairly sharp under low power. Under higher magnification, the border appears less sharply defined because of the penetration of neoplastic cells a short distance into the surrounding kidney or the tumor capsule. This growth pattern tends to surround and isolate individual single nephrons, which is rarely if ever seen with WT. The entrapped tubules are usually confined to the peripheral 2 to 3 cm of a CCSK. Their epithelium commonly shows embryonal metaplastic changes similar to those entrapped in congenital mesoblastic nephroma, and the resultant basophilic epithelium creates confusion with WT. Dilation of these entrapped tubules produces intratumoral cysts that may mimic cystic nephroma. CCSK may show a wide variety of morphologic patterns, described in the subsequent sections.[28-31]

Classic Pattern. The hallmark of the classic pattern is an evenly distributed network of vascular septa, which has a branching, "chicken-wire" pattern similar to that seen in myxoid liposarcoma or oligodendroglioma. These fibrovascular septa subdivide the tumor into a conspicuous pattern of cords and nests, averaging six to ten cells in width, composed of polygonal cells that usually lack distinct cytoplasmic borders. Cells within the cords are less densely packed than those of blastemal WT, and overlapping nuclei are less frequent. Nuclear chromatin is usually finely granular, with inconspicuous nucleoli (Fig. 55-4). In well-fixed specimens, the fine nuclear chromatin pattern is the most helpful clue to the diagnosis. However, it is influenced markedly by the timing and type of fixation. Mitotic figures are variable in number but are usually less numerous than those in WT. The cytoplasm usually lacks distinct borders and is surrounded by extracellular mucopolysaccharides, which creates the illusion of clear cytoplasm.

CCSK is easily confused with other neoplasms because the classic pattern is often modified, presenting alterations that mimic other neoplasms to a sometimes striking degree. Fortunately the classic pattern of CCSK predominates in most specimens and is present at least focally in more than 90% of tumors. However the pathologist who is unaware of these variant patterns is likely to diagnose CCSK as another neoplasm, with different therapeutic and prognostic implications. These variant patterns are described in the subsequent sections.[28-30]

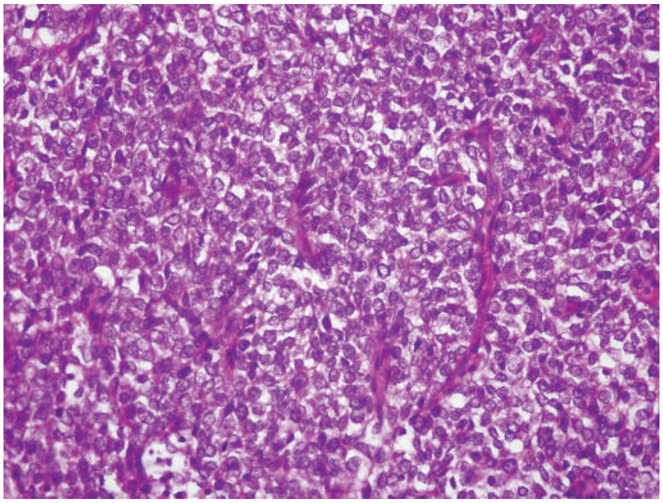

Figure 55-4 Clear cell sarcoma of the kidney.

Epithelioid Patterns. Condensation of cord cells of the classic CCSK pattern creates striking epithelioid arrangements in approximately 15% of cases.[28] These condensations usually form trabeculae or rosettes. These epithelioid formations conform to the pattern of the original cell cords and can be either straight or undulating in configuration. The epithelioid variants of CCSK are most likely to be mistaken for WT. In all of the examples analyzed by immunohistochemistry, these epithelioid formations have been negative for epithelial markers, in contrast to the epithelial cells of WT.

Spindle Cell Patterns. Spindle cell patterns are observed in approximately 10% of cases and result from two mechanisms.[28] Proliferation of septal cells produces wide spindle cell septa that compress or obliterate the cell cords. Intersections of these hyperplastic septal cells often resemble the storiform patterns seen with fibrohistiocytic neoplasms. Spindled transformation of cord cells yields a spindled pattern with preservation of thin fibrovascular septa.

Myxoid and Sclerosing Patterns. Most CCSKs contain abundant mucopolysaccharide, apparently produced by the cord cells.[28] This material separates cord cells and creates the appearance of clear cytoplasm. In approximately 30% of cases, mucopolysaccharide occupies more volume than the neoplastic cells themselves and forms large pools or cystic spaces.[28] While it accumulates, the tumor cells become progressively more isolated and eventually degenerate. In time the mucoid material becomes denser, eosinophilic, and hyaline in appearance. Hyaline sclerosis is found in 35% of CCSK. Complete replacement of cell cords by hyaline sclerosis preserves the original cord pattern with retention of the vascular septa, preserving the chicken-wire appearance. More diffuse zones of dense stromal sclerosis surrounding individual tumor cells may create an osteosarcoma-like appearance. Dense stromal sclerosis is relatively uncommon in untreated WT, and this finding can be a clue to the diagnosis of CCSK or rhabdoid tumor in limited biopsy material.

Palisading (Verocay-Body) Pattern. Nuclear palisading resembling schwannoma is focally prominent in approximately 10% of CCSK specimens.[28] Unlike schwannomas, these areas are not immunoreactive for S-100 protein.

Monstrocellular (Anaplastic) Pattern. Approximately 3% of CCSK contain foci with enlarged, pleomorphic nuclei and bizarre mitotic figures, resembling the appearance of anaplastic WTs or pleomorphic sarcomas. This is the only pattern of CCSK that frequently overexpresses p53 protein.[28]

Posttherapy Patterns. Recurrences of CCSK after therapy may have a deceptively hypocellular and bland appearance, suggesting low-grade fibromatosis or myxoma. A lump appearing anywhere in a child with a history of CCSK should be viewed as a potential metastasis until proven otherwise.

Ultrastructure, Immunohistochemistry, and Cytogenetics

Ultrastructural studies yield few clues as to the putative cell of origin of CCSK.[32] The tumor cells are characterized by a high nuclear-to-cytoplasmic ratio, with nuclear shapes that are more irregular and variable than would be expected from light microscopy. The cytoplasm is usually tenuous, with elongated, irregular processes surrounding abundant intercellular matrix. This latter feature is responsible for the vacuoles often seen with the light microscope. The cytoplasm tends to be poor in organelles. Immunohistochemistry has afforded little new insight into the histogenesis of CCSK, except to exclude various potential lines of differentiation for this enigmatic neoplasm. Vimentin is positive in nearly all specimens and BCL2 in some, but other markers are consistently negative. These include stains for epithelial markers (cytokeratins and epithelial membrane antigen [EMA]), neural markers (S-100 protein), neuroendocrine markers (chromogranin, synaptophysin), muscle markers (desmin), CD34, CD117 (c-kit), and CD99 (MIC2).[28] A subset of CCSK harbor a t(10;17)(q22;p13) chromosome translocation, resulting in a *YWHAE-FAM22* gene fusion.[33]

Differential Diagnosis of Clear Cell Sarcoma of the Kidney

The distinction of CCSK from other renal neoplasms can be extremely difficult, even for those with extensive experience in pediatric renal neoplasm pathology. The distinction of CCSK from rhabdoid tumor of the kidney is covered in the section on the latter. The major differential diagnostic concerns are discussed in the text that follows.

Clear Cell Sarcoma of the Kidney Versus Wilms Tumor. Some blastemal WT are richly vascular, and the vascular pattern may perfectly mimic that of CCSK. Several features help in this differential diagnosis. First, under low magnification, blastemal WTs often show distinctive nested patterns, whereas CCSKs are not typically nested. Second, blastemal WTs are more densely cellular than are CCSKs. When cells are closely packed, most nuclei are overlapping, and nuclei mold to one another, WT is more likely than CCSK. Third, heterologous tissues such as skeletal muscle are never seen in CCSK. The presence of a focus resembling CCSK in an otherwise unequivocal WT can safely be ignored. Fourth, multicentricity is common in WT, whereas multicentric and bilateral CCSKs have not been reported. Fifth, sclerotic, hyalinized stroma in an untreated tumor favors CCSK over WT. Finally, a fine nuclear chromatin pattern favors a diagnosis of CCSK over WT, although this feature is susceptible to fixation and processing artifacts.

Clear Cell Sarcoma of the Kidney versus Congenital Mesoblastic Nephroma. Cellular congenital mesoblastic nephroma (CMN) may resemble spindled variants of CCSK, and the age ranges of these two entities overlap. Small foci resembling CCSK may occur in CMN, but CMN lacks the diversity of morphologic patterns that typifies CCSK. The absence of the t(12;15) chromosome translocation that is characteristic of cellular CMN in all CCSKs examined to date indicates that these two neoplasms are unrelated.

Congenital Mesoblastic Nephroma

Gross Appearance

CMN arises unicentrically and usually appears to have arisen deep within the medial parenchyma, near the renal sinus. The renal sinus and adjacent structures on the medial side of the kidney are major sites of extrarenal spread of CMN. Surgeons and pathologists must pay particular attention to the medial margin of the CMN resection specimen. However because of the predominance of freely mobile fat in this location and retraction artifact, the medial specimen margin is notoriously difficult to evaluate, and it is rarely possible to be certain that it is free of involvement by CMN.

The appearance of the sectioned surface of CMN is variable and depends on the subtype. Classic CMNs have a tough, whorled appearance resembling that of leiomyoma, but cellular CMNs may be soft, friable tumors. Hemorrhage, necrosis, and cyst formation are present to some degree in a majority of cellular CMNs.

Microscopic Appearance

CMN is a low-grade fibroblastic sarcoma of the infantile kidney. The classic type of CMN has a fibrous, whorled gross appearance. Morphologically, classic CMN is identical to infantile fibromatosis; it is composed of bland fibroblastic or myofibroblastic cells, arranged in fascicles that dissect into the native kidney (Fig. 55-5).[34] Mitoses are usually rare, and necrosis is absent. The leading edge of the tumor characteristically induces angiomatous vascular proliferation where it abuts perirenal fat. Entrapped renal tubules may acquire a primitive appearance (embryonal metaplasia), which may cause confusion with true neoplastic tubular differentiation that would suggest a diagnosis of biphasic stromal-epithelial WT. In contrast, the cellular variant of CMN typically has a softer, more fleshy and hemorrhagic appearance with necrosis and cystification. Microscopically, cellular CMN is identical to infantile

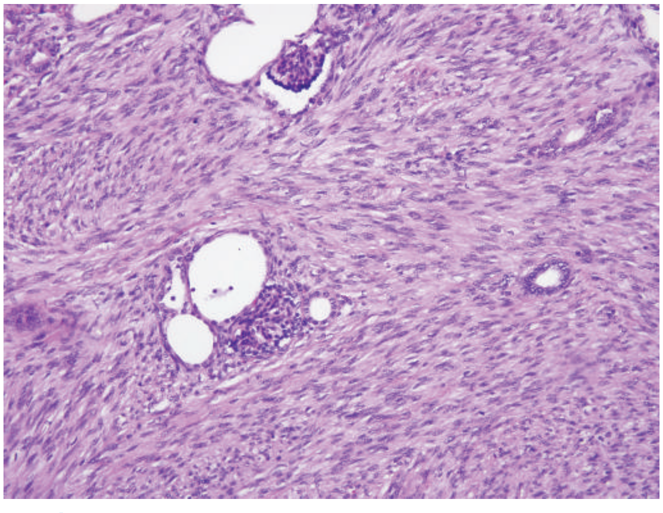

Figure 55-5 Congenital mesoblastic nephroma, classic type.

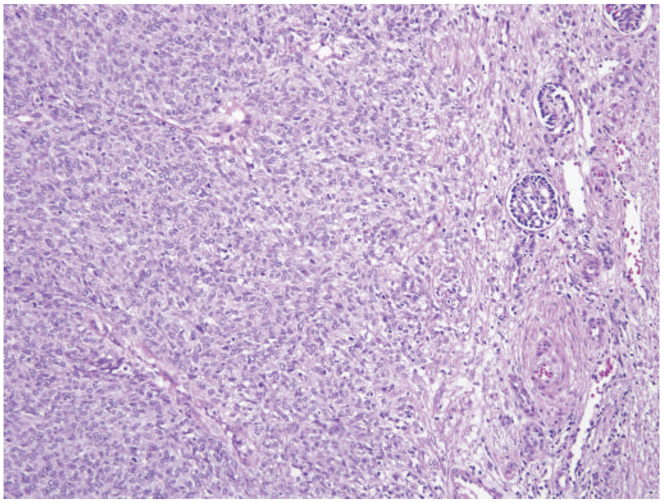

Figure 55-6 Congenital mesoblastic nephroma, cellular type.

fibrosarcoma (IFS).[35] Compared with the classic variant, cellular CMNs more often have a "pushing" border, demonstrate sheetlike growth patterns, are less often fascicular, are more cellular, and have a higher mitotic rate (Fig. 55-6). Tumor cells may be polygonal with well-demarcated pink cytoplasm and nuclei with vesicular chromatin and prominent nucleoli; this raises the differential diagnosis of rhabdoid tumor of the kidney. Alternatively the tumor cells may be thinner, primitive, and spindled, yielding a more embryonal "blue cell" appearance. Mixed CMNs have areas identical to both cellular and classic CMN in varied proportions within the same tumor.

Immunohistochemistry and Ultrastructure

Immunohistochemistry is of limited value in the diagnosis of CMN. The cells of CMN label in a fashion consistent with fibroblasts or myofibroblasts.[36] Whereas actin may be focally positive, desmin and CD34 are typically negative (P. Argani, unpublished observations). INI1 protein is retained. Ultrastructural studies also reveal features consistent with fibroblasts or myofibroblasts.[37] Most CMN cells show abundant rough endoplasmic reticulum with branching and anastamosing profiles, and primitive cell junctions are often found.

Differential Diagnosis of Congenital Mesoblastic Nephroma

The differential diagnosis for CMN includes stromal-predominant WT, CCSK, metanephric stromal tumor (MST) and malignant rhabdoid tumor (MRT). These distinctions are crucial because most children with CMN do not require adjuvant chemotherapy, whereas those with WT, CCSK, and MRT are treated on specific chemotherapy protocols. Features that distinguish CCSK and MRT from CMN are covered in the sections on CCSK and MRT, respectively. The features that distinguish CMN from WT and MST are expanded on in the subsequent sections.

Congenital Mesoblastic Nephroma Versus Wilms Tumor. As mentioned previously, embryonal metaplastic changes in nephrons surrounded by CMN cells are sometimes misinterpreted as tubular or papillary elements in a WT. Rarely untreated WT will be composed predominantly of stromal cells. In most of these the presence of immature or mature skeletal muscle readily excludes the diagnosis of CMN. Specimens removed after chemotherapy are particularly challenging. Treatment often ablates the embryonal, proliferating elements of a WT but tends to spare stromal cells. The resulting appearances can readily be confused with CMN. The following features are helpful in the differential diagnosis:

1. A diagnosis of CMN should be considered suspect in a patient who has received prior therapy, unless the clinical and pathologic features are characteristic.
2. Most CMNs are diagnosed in the first 3 months of life, whereas WT is relatively uncommon at this age. Only 10% of CMNs are diagnosed after 1 year, and virtually none are diagnosed after 2 years.
3. Most WTs express a diversity of cell types and tissue patterns. In contrast blastemal foci do not occur in a CMN, and skeletal muscle is never seen. However nodules of entrapped cartilage and squamous pearls may occur in CMN (likely reflecting coexisting renal dysplasia), and this should not be taken as evidence for WT.
4. The interdigitating, irregular border of classic CMN distinguished it from most WTs. However, cellular CMN often has a sharp, pushing border, similar to that of most WTs.
5. Bilaterality or the presence of NRs (or both) strongly favors WT over CMN.

Congenital Mesoblastic Nephroma Versus Metanephric Stromal Tumor. Many tumors previously considered to be CMNs in children older than age 3 years are in fact distinct from CMN and represent the newly recognized entity MST.[38] MST is identical to the stromal component of metanephric adenofibroma [39] (previously known as nephrogenic adenofibroma),[40] a biphasic tumor containing an epithelial component that is identical to metanephric adenoma. MST is a benign lesion composed of spindled to stellate cells featuring thin hyperchromatic nuclei and thin indistinct cytoplasmic extensions. Several of the characteristic features of MST distinguish it from CMN. MST characteristically surrounds and entraps renal tubules and blood vessels to form concentric "onion-skin" rings or collarettes around these structures. Most MSTs induce angiodysplasia of entrapped arterioles, consisting of epithelioid transformation of medial smooth muscle and myxoid change. One fourth of MSTs feature juxtaglomerular cell hyperplasia within entrapped glomeruli, which may occasionally lead to hypertension associated with hyperreninism. Finally, unlike CMNs, MSTs are typically immunoreactive for CD34, but labeling may be patchy.

Malignant Rhabdoid Tumor of the Kidney

Gross Appearance

Most MRTs are relatively small because metastasis occurs early in the evolution of the neoplasm. On gross

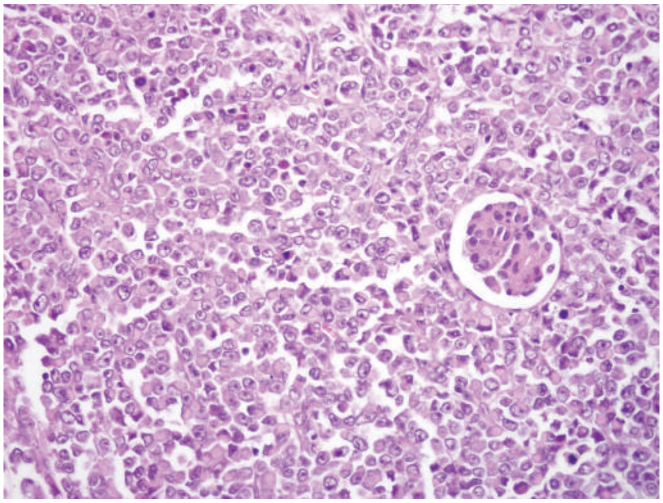

Figure 55-7 Malignant rhabdoid tumor of the kidney.

examination MRTs are typically fleshy or hemorrhagic neoplasms, with ill-defined borders and frequent satellite nodules that reflect the tumor's highly invasive nature.[41]

Microscopic Appearance

MRT was so named because of the resemblance of the tumor cells to rhabdomyoblasts. However this highly lethal infantile neoplasm does not demonstrate muscle differentiation. Microscopically these neoplasms typically engulf native renal elements at their periphery and demonstrate extensive vascular invasion. MRT classically consists of sheets of monotonous cells featuring a characteristic cytologic triad: vesicular chromatin, prominent nucleoli, and hyaline cytoplasmic inclusions (Fig. 55-7). These features are variably well developed within a given tumor, which means that it may be necessary to examine multiple microscopic fields from many sections to find them.

Ultrastructure and Immunohistochemistry

Ultrastructurally the cytoplasmic inclusions correspond to whorled intermediate filaments.[42] The whorled filaments may trap antibodies used for immunohistochemistry, yielding a nonspecific polyphenotypic pattern. With this caveat, strong vimentin and focal EMA labeling are characteristic immunohistochemical findings. Recently, an immunohistochemical assay for INI1 protein has become available. Loss of INI1 protein expression reflects *INI1* genetic status and, within the kidney, is a sensitive and nearly specific marker for MRT.[43]

Differential Diagnosis of Rhabdoid Tumor of the Kidney

The diagnosis of MRT is often challenging because a wide variety of renal and extrarenal tumors of adult and children may feature fully developed "rhabdoid" cytology, at least focally.[44,45] In the pediatric kidney these include cellular CMN (of the plump cell type), CCSK, blastemal WT, PNET, renal medullary carcinoma, and rhabdomyosarcoma.[44] In general, thorough sampling of such tumors is most helpful, insofar as it typically reveals specific differentiation that excludes MRT. Additionally, the age of the patient is very helpful because MRT is not seen outside of early childhood. Finally, in the kidney loss of INI1 protein expression by immunohistochemistry is highly specific for MRT or renal medullary carcinoma.[46] Several specific differential diagnoses are covered in the subsequent sections.

Malignant Rhabdoid Tumor Versus Wilms Tumor. Both blastemal and myogenic elements of WT may contain cytoplasmic inclusions resembling those of MRT. A distinctive serpentine blastemal pattern, other distinctly nephrogenic features, or heterologous cells such as skeletal muscle supports the diagnosis of WT. The prominent nucleoli characteristic of MRT are not typically seen in WT, with the exception of some anaplastic specimens.

Malignant Rhabdoid Tumor Versus Cellular Congenital Mesoblastic Nephroma. The plump cell variant of cellular CMN often has vesicular nuclei with prominent nucleoli, and a rare CMN contains foci with hyaline cytoplasmic inclusions. A predominance of spindled patterns and a less invasive tumor periphery favor a cellular CMN over a rhabdoid tumor. Cytogenetic or molecular analysis (or both) may be invaluable in this setting; demonstration of chromosome 22q loss or loss of INI1 protein expression by immunohistochemistry supports MRT, whereas the presence of the ETV6-NTRK3 gene fusion supports cellular CMN.

Malignant Rhabdoid Tumor Versus Clear Cell Sarcoma of the Kidney. CCSK may rarely show prominent nucleoli, which can lead to difficulty in distinguishing CCSK from MRT. The presence of classic cytologic features of CCSK elsewhere and the presence of characteristic CCSK patterns are the major clues to the correct diagnosis in such instances. CCSKs lack the extreme invasiveness of MRTs and are generally more subtly invasive neoplasms.

Renal Carcinomas in Children

All of the common renal cell carcinomas (RCCs) of adulthood (clear cell, papillary, chromophobe, and collecting duct) may occasionally affect the pediatric kidney, but the clinical and differential diagnostic considerations differ in this setting. Children with von Hippel-Lindau syndrome are predisposed to develop conventional (clear cell) RCC. Children with tuberous sclerosis are at increased risk for developing both RCC and epithelioid angiomyolipoma,[47] and immunohistochemical analysis may be needed to distinguish these two. As discussed previously, PRCC may overlap morphologically with epithelial predominant WT or metanephric adenoma. However most pediatric RCCs represent distinctive neoplastic entities. Four of these entities are described in the subsequent sections.

Xp11.2 Translocation Renal Cell Carcinomas

RCCs with chromosome translocations involving Xp11.2 and resulting gene fusions involving the *TFE3* transcription factor gene were officially recognized in the 2004 World Health Organization Renal Tumor Classification.[48] Although this subtype of RCC is relatively uncommon on

TABLE 55-3 MiTF/TFE Translocation Neoplasms

Gene Fusion	Chromosome Translocation	Age (Years)	Tumor
ASPL-TFE3	der(17)t(X;17)(p11.2;q25)	5-40	ASPS
ASPL-TFE3	t(X;17)(p11.2;q25)	2-68	RCC
PRCC-TFE3	t(X;1)(p11.2;q21)	2-70	RCC
PSF-TFE3	t(X;1)(p11.2;p34)	5-68	RCC
NoNo-TFE3	inv(X)(p11;q12)	39	RCC
CLTC-TFE3	t(X;17)(p11.2;q23)	14	RCC
Alpha-TFEB	t(6;11)(p21;q12)	6-53	RCC

ASPS, Alveolar soft part sarcoma; *RCC*, renal cell carcinoma.

a percentage basis in adults, it most likely accounts for the majority of pediatric RCCs. One distinctive subtype bears a t(X;17)(p11;q25), which results in the identical *ASPL-TFE3* gene fusion, as was initially identified in alveolar soft part sarcoma (ASPS), and has been designated the *ASPL-TFE3* RCC.[49] Other subtypes are listed in Table 55-3.

Xp11.2 translocation RCCs closely resemble conventional (clear cell) renal carcinomas on gross examination, usually being tan-yellow and often necrotic and hemorrhagic. A papillary carcinoma composed of clear cells is the most distinctive histopathologic appearance (Fig. 55-8), because this combination is uncommon in other defined types of renal carcinomas. However the Xp11.2-translocation RCCs often have nested architecture and often contain cells with granular eosinophilic cytoplasm. The histologies of Xp11-translocation RCC associated with different *TFE3* gene fusions differ. The *ASPL-TFE3* RCCs feature cells with voluminous, clear to eosinophilic cytoplasm, discrete cell borders, vesicular nuclear chromatin, and prominent nucleoli. In contrast, the *PRCC-TFE3* RCCs, characterized by a t(X;1)(p11.2; q21), typically have less abundant cytoplasm; fewer psammoma bodies; fewer hyaline nodules; and a more nested, compact architecture.[50]

Renal carcinomas with Xp11.2-associated translocations characteristically underexpress epithelial immunohistochemical markers such as cytokeratin and epithelial membrane antigen. Only approximately one half of cases will be positive with these markers, and the labeling is often focal. Vimentin immunoreactivity is also often focal compared with that of the adjacent blood vessels; this also differs from conventional RCCs, which are diffusely positive. Rare Xp11.2-translocation carcinomas, especially ones with variant gene fusions such as *PSF-TFE3* and *CLTC-TFE3*,[51] express melanocytic markers HMB45 and Melan A, creating confusion with epithelioid angiomyolipoma. Cathepsin K, a serine protease normally expressed in osteoclasts, is expressed in approximately 50% of translocation RCC but no other subtype of RCC.[52,53] The most distinctive immunohistochemical feature of these neoplasms is nuclear labeling for TFE3 protein using an antibody to the C-terminal portion of TFE3, which is retained in the gene fusions. Nuclear labeling for TFE3 is a common feature of all Xp11.2-translocation RCC and ASPS but does not occur in other RCCs (Fig. 55-9). Because native TFE3 is known to be ubiquitously expressed but is not detectable in normal tissues by immunohistochemistry, it is postulated that the different *TFE3* gene fusions consistently lead to overexpression of the fusion protein relative to native TFE3, such that the protein becomes detectable by this assay.[54] However TFE3 immunohistochemistry is difficult to optimize in the setting of variable fixation, and a break-apart fluorescence in situ hybridization (FISH) assay for *TFE3* gene rearrangements is a more reliable assay in formalin-fixed, paraffin-embedded tissue.[55]

When analyzed by electron microscopy, Xp11.2-associated carcinomas feature cell junctions, microvilli, intracytoplasmic fat, and glycogen and thereby most closely resemble conventional renal carcinomas. However a subset of cases demonstrates distinctive ultrastructural

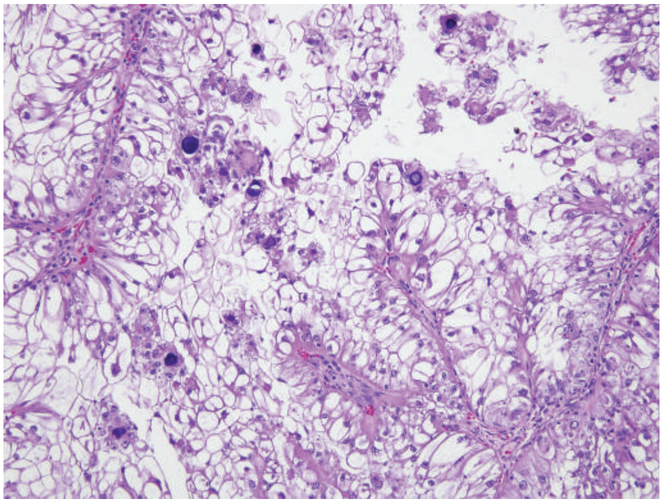

Figure 55-8 Xp11.2 translocation renal cell carcinoma.

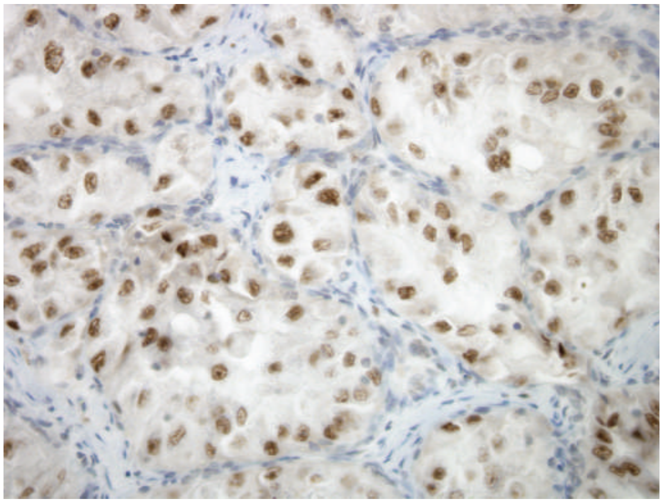

Figure 55-9 Xp11.2 translocation renal cell carcinoma, TFE3 immunohistochemistry.

features. Most of the *ASPL-TFE3* RCCs contain membrane-bound cytoplasmic granules, and a few have membrane-bound rhomboidal crystals that are identical to those seen in soft tissue ASPS. Some *PRCC-TFE3* RCCs have had distinctive intracisternal microtubules similar to those seen in malignant melanoma and extraskeletal myxoid chondrosarcoma.

t(6;11)(p21;q12) Renal Cell Carcinomas

Another distinctive type of RCC bears a t(6;11)(p21;q12). The distinctive clinicopathologic features of these neoplasms were not described until 2001.[56] On microscopic examination these neoplasms usually feature nests and tubules of polygonal epithelioid cells, separated by thin capillaries. Some cases have had papillary formations. The majority of the tumor cells have abundant clear to granular eosinophilic cytoplasm, well-defined cell borders, and round nuclei with small nucleoli. However a second population of smaller epithelioid cells is also characteristic, typically (but not always) clustered around nodules of hyaline basement membrane material within larger acini (Fig. 55-10). The cases examined have generally been negative for cytokeratins by immunohistochemistry, but almost all have labeled at least focally for HMB45, Melan A, and Cathepsin K, again creating confusion with epithelioid angiomyolipoma.

The t(6;11)(p21;q12) has been shown to result in a fusion of the intronless, untranslated *Alpha* gene with *TFEB,* a gene belonging to the same transcription factor family as *TFE3*.[57,58] The consequence of the *Alpha-TFEB* fusion is dysregulated expression of the normal full-length TFEB protein. Along these lines, the t(6;11) RCCs (also known as *Alpha-TFEB* RCCs) demonstrate specific nuclear labeling for TFEB protein by immunohistochemistry (IHC), whereas other neoplasms and normal tissues do not (Fig. 55-11). Hence nuclear labeling for TFEB is a sensitive and specific diagnostic marker for this neoplasm with a *TFEB* gene fusion, just as nuclear labelling for TFE3 is a sensitive and specific marker for neoplasms bearing *TFE3* gene fusions.[59] A break-apart FISH assay for *TFEB* gene rearrangements has recently been developed for formalin-fixed, paraffin-embedded tissue and is proving to be more sensitive than TFEB IHC assay.[60]

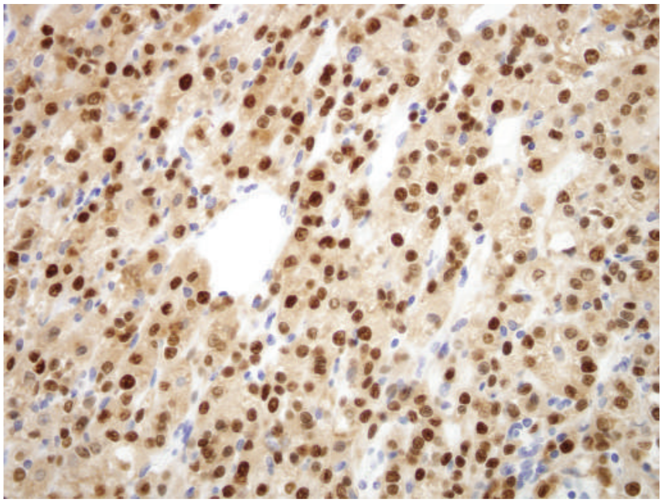

Figure 55-11 Renal cell carcinoma with t(6;11) (p12:q12) translocation, transcription factor EB immunohistochemistry.

Renal Medullary Carcinoma

Renal medullary carcinoma (RMC) was first described in 1995 by Davis and colleagues as “the seventh sickle cell nephropathy.”[61] These tumors characteristically affect young patients (mean age, 22 years) with sickle cell trait. Tumors are typically centered in the renal medulla but demonstrate an aggressively infiltrative growth pattern with frequent vascular permeation. The tumor cells feature vesicular chromatin and prominent nucleoli, frequently associated with hyaline intracytoplasmic inclusions, therefore imparting a rhabdoid cytologic appearance. The tumor cells of RMC demonstrate diffuse (sheetlike), cribriform, gland-forming, microcystic, or reticular growth patterns, the latter two simulating yolk-sac tumor (Fig. 55-12). The stroma is typically desmoplastic and

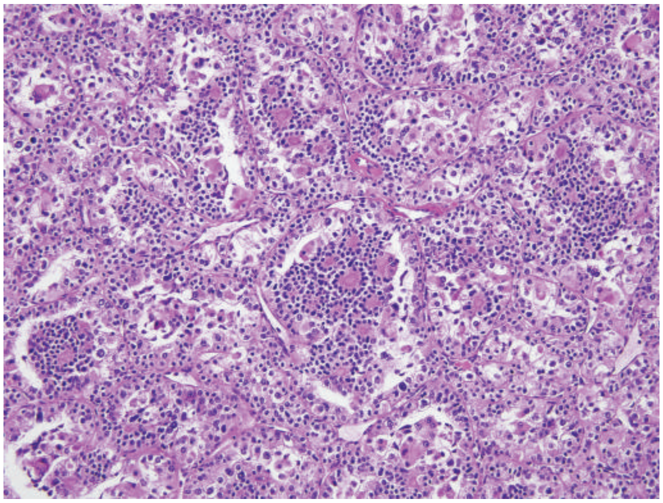

Figure 55-10 Renal cell carcinoma with t(6;11) (p12;q12) translocation.

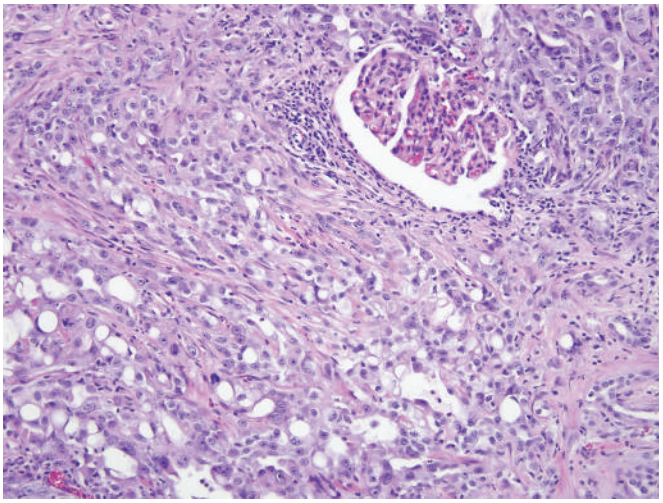

Figure 55-12 Renal medullary carcinoma.

infiltrated by neutrophils, and close examination of extravasated red blood cells may reveal sickling. However, hemoglobin electrophorosis is more reliable than the latter for determining that the patient has sickle cell trait. These tumors frequently present at high stage and are highly lethal; Davis and colleagues reported a mean survival of only 15 weeks. Given their medullary epicenter and high-grade cytology, RMCs are considered by some to be variants of collecting duct carcinoma, although the latter entity is a notoriously elusive one to define. As noted previously, RMCs consistently show loss of expression of INI-1 protein by IHC, similar to RTK.

Oncocytic Renal Carcinomas in Neuroblastoma Patients

Meideros and coworkers have described oncocytic renal carcinomas arising in children who had previously had neuroblastoma.[62] The genetic alterations identified in these lesions did not match those of known renal carcinomas, suggesting that these are another distinctive entity.

Primitive Neuroectodermal Tumor of Kidney

Primary renal PNET should be considered in the differential diagnosis of any undifferentiated blue cell tumor of the kidney. This neoplasm is encountered mainly in adolescent and young adult patients and tends to present at advanced stage.[63] Tumors consist of sheets of undifferentiated small blue cells with frequent necrosis and occasional rosettes. Renal PNETs are frequently mistaken for blastemal WTs of adolescent or adult patients and might account for some of the adverse prognosis attributed to "adult WT." Compared to WT, tumor cell nuclei are less hyperchromatic and more evenly placed. Molecular detection of the *EWS-FLI1* gene fusion that results from the characteristic and specific t(11;22)(q24;q12) chromosome translocation is a useful way to confirm the diagnosis of PNET in suspected cases.[64] Also helpful is immunohistochemical labeling for CD99; the characteristic membrane staining pattern observed in PNETs is rarely if ever observed in WT blastemal cells or cellular CCSKs, which are the main differential diagnosis.[65]

Synovial Sarcoma of the Kidney

Similar to renal PNET, primary renal synovial sarcoma has been established as a distinctive neoplastic entity by demonstration of its specific chromosome translocation and gene fusion.[66,67] Genetically confirmed cases have affected young adults. Many have previously been classified as adult blastemal WT, embryonal sarcoma of the kidney, adult cellular mesoblastic nephroma, or sarcoma arising in cystic nephroma. Renal synovial sarcomas are typically large and often cystic, and they present at advanced stage. The typical appearance is that of a monomorphic, highly cellular neoplasm composed of plump, spindled cells growing in short fascicles. Tumor cells have ovoid nuclei and minimal cytoplasm and may be associated with sclerosis. Grossly identified cysts are lined by mitotically inactive polygonal eosinophilic cells with apically oriented nuclei ("hobnailed epithelium"), and the cellularity of their walls may be deceptively low, creating confusion with cystic nephroma in limited biopsy samples. Overall, the appearances are best conceptualized as monophasic spindle-cell synovial sarcoma encircling native renal collecting ducts. True epithelial differentiation yielding a biphasic synovial sarcoma may occur, but this is less common. Immunohistochemically, tumor cells label focally for epithelial markers (EMA more likely than cytokeratin), but many cases are completely negative for all epithelial markers. Many cases have labeled for CD99. The diagnosis can be confirmed in such cases by molecular demonstration of the *SYT-SSX* gene fusion that results from the t(X;18)(p11;q11) that is characteristic of this neoplastic entity. As with renal PNET, renal synovial sarcoma is more aggressive than WT and also may account for some of the adverse prognosis attributed to "adult WT."

WILMS TUMOR (NEPHROBLASTOMA)

Epidemiology

The age-adjusted incidence of pediatric renal tumors in the United States is 7.5 per million children younger than 15 years of age and 6 per million children younger than 19 years of age.[1] Although WT is the most common renal tumor overall, its relative frequency compared with other pediatric renal neoplasms varies according to patient age. Between 0 and 1 month of life, CMN is more common than WT, and after age 14 years RCC surpasses WT as the most prevalent renal cancer.[1,68] In North America the average age of diagnosis for unilateral WT is 42 months for boys and 47 months for girls.[69] Children with clinical malformations, genetic abnormalities, and tumor predisposition syndromes have a significantly earlier median age of diagnosis of WT.[70] For bilateral WT the average age of diagnosis is 30 months for boys and 33 months for girls.[69] The younger age of presentation for bilateral WT is due to genetic predisposition, an observation that provided the basis for Knudson's two-hit model.[71] The reason for the later age of presentation in girls is more elusive. An analysis from the NWTSG revealed that, compared with boys, girls had a relative excess of PLNRs, WT precursor lesions associated with genetic alterations of the Beckwith-Wiedemann syndrome locus at chromosome 11p15.[72] Conversely boys had a relative excess of ILNRs, distinct precursor lesions associated with mutations of the *WT1* gene at chromosome 11p13. ILNR-associated WTs arise at an earlier age than PLNR-associated WTs, partially explaining the gender difference in age distribution. However even in the absence of NRs, girls presented with WTs a few months later, on average, than boys. In North America there is a slight preponderance of WT in girls compared with boys, with a female-to-male ratio of 1.1 to 1.

There also are racial and ethnic differences in the predisposition to WT.[69] Most Caucasian populations have an age-adjusted incidence of six to nine cases per million children annually. Black populations in North America and some parts of Africa exceed 10 cases per million children annually. In Japan, the Philippines, and China, the incidence is fewer than four cases per million children per year. Interestingly, in these East Asian populations, there is an excess of male subjects with WTs, the age at diagnosis is earlier than in North America, and PLNRs are rarely seen.[73] There may also be biologic differences associated with racial differences in WT. A small study of WT in 15 Kenyan children demonstrated differences in molecular signature by imaging mass spectrometry and immunostaining compared with histology and age-matched North American controls. The Kenyan tumors exhibited more aggressive histology and worse outcome, although it is difficult to distinguish truly biologic aggressive disease from inadequate care related to resource limitations.[74]

Several studies have uncovered maternal and paternal environmental associations with WT. Maternal exposures that have been associated with WT include cigarettes, coffee or tea, oral contraceptives, hormonal pregnancy tests, hair-coloring products, hypertension, household pesticide use, and vaginal infections.[75,76] However none of these factors was confirmed by subsequent case-control studies conducted by the NWTSG.[77,78] In a COG study breastfeeding was found to be associated with reduced risk of WT, although the effect was restricted to mothers with less than a college education.[79] On the paternal side the occupations of machinist, welder, and other jobs involving exposure to hydrocarbons or lead were associated with increased risk of WT.[80,81] A subsequent case-control study found an association with the paternal occupations of vehicle mechanics, auto-body repairmen, and welders, although no unifying exposure could be established.[82] A correlation of a low Apgar score (0-5) with a higher risk (46%) of cancer diagnosis was found in a nationwide population-based study in Denmark and Sweden that followed 5,091,188 children from age 0 to 14 years from 1978 to 2006 (excluding children with birth defects). The potential impact of the low Apgar score and cancer diagnosis was largely confined to children diagnosed with cancer before 6 months of age, and the highest risk was observed for the occurrence of WT.[83]

Molecular Biology and Genetics

Retinoblastoma and WT were the original tumors used by Knudson to establish the two-hit hypothesis of oncogenic transformation.[71] Unlike retinoblastoma, in which alterations of a single gene (*Rb*) account for the vast majority of tumors, several genes have been implicated in the genesis of WT, and a sizable proportion of WTs have indeterminate genetic etiology. The genes that are most commonly mutated or epigenetically altered in sporadic WTs are *WT1, IGF2,* β-catenin (*CTNNB1*), *WTX,* and *TP53*. These five genes are discussed in detail in the subsequent sections, whereas other genes and loci implicated in WT are summarized in Table 55-4.

WT1

WT1 was the first gene found to be specifically mutated in WT.[84-86] The gene was originally identified via study of patients with the WAGR syndrome. Children with WAGR suffer from the constellation of *W*T, *a*niridia, *g*enitourinary abnormalities, and mental *r*etardation. Chromosomal analysis of these patients revealed heterozygous constitutional deletions at 11p13. Subsequent investigation of the genes contained within these deletions identified disruption of the *PAX6* gene as the basis of aniridia and *WT1* as the key tumor suppressor. The remaining allele of *WT1* is mutated in the WTs from these patients.

WT1 encodes an approximately 45-kd DNA-binding protein that contains domains rich in glutamine and proline in the amino terminus and four zinc fingers in the carboxy terminus, collectively suggesting that it acts as a transcription factor. The *WT1* gene is quite complex insofar as it encodes numerous distinct isoforms and contains an evolutionarily conserved cryptic splice site that can result in the insertion of three amino acids, lysine, threonine, and serine (KTS), between zinc fingers 3 and 4. Notably distinct functions have been identified for the different KTS isoforms. Patients with Frasier syndrome, characterized by male pseudohermaphroditism, progressive glomerulopathy, and an increased risk of WT, have a congenital donor splice-site mutation in *WT1* that results in loss of the +KTS isoform.[87] Furthermore mice that express only the +KTS or only the −KTS isoform have distinct phenotypes.[88]

Congenital point mutations in *WT1* are also the basis of Denys-Drash syndrome.[89] Patients with this syndrome suffer from renal failure, pseudohermaphroditism, and a susceptibility to WT. Denys-Drash syndrome is caused by missense mutations in *WT1* that disrupt zinc finger binding to DNA. The phenotype of patients with Denys-Drash is quite severe, and evidence suggests that these mutations may lead to a dominant-negative protein that impairs function of the normal protein encoded by the remaining allele. Nonetheless the remaining *WT1* allele is mutated in the WTs that arise in these patients, suggesting that mutation of both alleles is required for tumor formation. Notably whereas mutations in *WT1* are the basis of several syndromes that predispose to the formation of WT, *WT1* is mutated in only 10% to 20% of sporadic tumors.[90] Mouse models have provided insight into the contributions of *WT1* to development and tumor suppression. *WT1* is expressed at high levels in the developing kidney, gonads, spleen, and mesothelium and at lower levels in several other tissues. Mice deficient for *WT1* die midgestation with abnormalities of the metanephric blastema, gonads, mesothelium, heart, and lungs.[91] Cells from the metanephric blastema in these mice undergo apoptosis, and consequently the metanephric kidney never develops. Based on these data it has been postulated that the WT1 protein regulates gene expression pathways required for morphogenesis of the kidney, and that its disruption results in aberrant stimulation of tissue-specific growth. This role in differentiation may also explain why WT is largely a disease of early childhood insofar as kidney morphogenesis is initiated in utero and is completed in early postnatal life. Indeed the level of *WT1* plummets within a few weeks after birth in mice. Whereas $WT1^{-/-}$ mice are not viable, $WT1^{+/-}$ mice live but do not develop WTs. This differs from the human condition in which individuals with germline *WT1* deletions or mutations have a greatly increased risk for developing WT compared with the general population.

β-catenin (CTNNB1)

The Wnt signaling pathway regulates the cellular processes of proliferation, differentiation, motility, and

TABLE 55-4 Genes and Loci Associated with Wilms Tumor

Locus	Gene	Gene Function	Comments	Key References
11p13	*WT1*	Transcription factor	WAGR, Denys-Drash, and Frasier syndromes associated with WT predisposition; somatic mutations in 10% to 20% of WT	84-86, 90
11p15	*WT2 (IGF2/ H19)*	Insulin-like growth factor pathway	Beckwith-Wiedemann syndrome associated with WT predisposition; somatic genetic or epigenetic alterations seen in 70% of WT	113-115
Xq11	*WTX (AMER1/ FAM123B)*	Wnt signaling pathway	Osteopathia striata congenita with cranial sclerosis syndrome has not been associated with WT predisposition but somatic mutations seen in 20% to 30% of WT	97-99
3p21	*CTNNB1 (β-catenin)*	Wnt signaling pathway	Somatic mutations seen in 15% of WT; strong correlation with *WT1* mutations	93, 94
17q12-q21	*FWT1*	Unknown	Locus associated with familial WT; gene not identified	305, 306
19q13	*FWT2*	Unknown	Locus associated with familial WT; gene not identified	307
17p13	*TP53*	Cell cycle checkpoint and apoptosis	Li-Fraumeni syndrome associated with WT predisposition; somatic mutations seen in 75% of anaplastic WT but uncommon in favorable-histology WT	11, 12, 120
13q12	*BRCA2 (FANCD1)*	DNA repair	Fanconi anemia; biallelic mutations associated with WT predisposition	308, 309
16p12	*PALB2 (FANCN)*	DNA repair	Fanconi anemia; associated with WT predisposition	310
15q26	*BLM*	DNA helicase	Bloom syndrome; associated with WT predisposition	311-313
15q15	*BUB1B*	Mitotic spindle checkpoint	Mosaic variegated aneuploidy; associated with WT predisposition	311, 314
Xq26	*GPC3*	Wnt signaling pathway	Simpson-Golabi-Behmel syndrome; associated with WT predisposition	311, 315-318
2q37	*DIS3L2*	Micro-RNA processsing	Perlman syndrome; associated with WT predisposition	319
7p14-15	*POU6F2*	Transcription factor	None	320, 321
1q25-q32	*HRPT 2* (parafibromin)	RNA processing; histone modification	Hyperparathyroidism-jaw tumor	322
6q21	*HACE1*	Ubiquitin ligase	None	323, 324
2p24	*MYCN*	Transcription factor	Somatic gene amplification seen in 5% to 10% of WT	325
4q31	*FBXW7*	Ubiquitin ligase	Somatic mutations seen in 4% of WT; germline mutation described	326

WAGR, Constellation of *W*ilms tumor, *a*niridia, *g*enitourinary abnormalities, and mental *r*etardation; *WT,* Wilms tumor.

survival. Central to the pathway is the transcriptional activator β-catenin, which is actively degraded in the absence of Wnt signaling.[92] Activating mutations in the *β-catenin (CTNNB1)* gene occur in approximately 15% of WTs.[93,94] Interestingly mutations in *β-catenin* occur predominantly in tumors in which *WT1* is also mutated, suggesting that these two events cooperate in the genesis of WT.[94,95] In the developing kidney *Wnt4* is an inducer of the mesenchymal to epithelial transition that underlies normal nephron development; disruption of normal renal development may be the means by which *β-catenin* mutation contributes to the formation of WT.[96]

WTX (AMER1/FAM123B)

WTX was discovered by an array comparative genomic hybridization screen that identified deletions of the Xq11.1 locus in male patients with WT. Further investigation found deletions and mutations in girls. Intriguingly *WTX* is inactivated by a single hit affecting either the sole *WTX* copy in male subjects or the copy located on the active X chromosome in female subjects.[97] According to the initial report, *WTX* was deleted or mutated in approximately one third of WTs, although other groups have reported a lower mutation frequency.[97-99] Germline *WTX* mutations have been observed in individuals with osteopathia striata congenita with cranial sclerosis, a rare slerosing bone dyspasia.[100] Individuals with osteopathia striata congenita with cranial sclerosis do not have increased risk for WT or other cancers, but NRs have been observed in one individual with a germline *WTX* mutation.[101]

WTX is alternatively spliced to encode two isoforms of a protein with disparate functions.[100] The short isoform of the protein is nuclear and forms a complex with β-catenin, AXIN1, APC, and β-TrCP2, ultimately promoting ubiquitination and degradation of β-catenin, thereby acting as a negative regulator of the WNT signaling pathway.[102] The full-length WTX protein binds to the adenomatous polyposis coli protein and regulates its distribution between microtubules and the plasma membrane.[103] WTX also interacts with WT1 protein and enhances its function as a transcriptional activator.[104] It was recently shown to bind p53 and modulate p53 function through its activator CBP/p300.[105]

Although *WT1* and *β-catenin* mutations are highly concordant, there is no clear association between *WT1* and *WTX* mutations.[97,98] This suggests that *WTX* and *β-catenin* mutations are not functionally equivalent even though both proteins reside within the WNT pathway.

IGF2

Cytogenetic analyses have long suggested the presence of an additional WT gene at 11p15 distinct from the *WT1* locus at 11p13.[106] Loss of heterozygosity (LOH) at 11p15 occurs in some cases of sporadic WT. Additionally, this locus is linked to Beckwith-Wiedemann syndrome (BWS), an overgrowth disorder associated with macroglossia, umbilical hernia, gigantism, neonatal hypoglycemia, and predisposition to WT and other cancers. BWS arises from alterations in several different genomically imprinted genes at the 11p15 locus.[107] Genomic imprinting is the preferential expression of a gene from either the maternal or paternal allele. The molecular basis of genomic imprinting is epigenetic modification, most notably DNA methylation. The 11p15 locus has two imprinting domains (Fig. 55-13). The telomeric domain (domain 1) includes the genes *insulin-like growth factor 2 (IGF2)* and *H19*. *IGF2* encodes a growth factor expressed at high levels in the developing kidney and WT.[108,109] *H19* encodes a biologically active untranslated RNA that may function as a tumor suppressor.[110] In normal cells these genes are oppositely expressed such that *IGF2* is expressed only from the paternal allele and *H19* is expressed only from the maternal allele. The centromeric imprinting domain (domain 2) contains several imprinted genes, including *KCNQ1* (*KvLQT1*), *KCNQ1OT1 (LIT1)*, and *CDKN1C* (*p57*^{KIP2}). *KCNQ1* encodes a subunit of a voltage-gated potassium channel that is implicated in several cardiac arrhythmia syndromes, including long-QT syndrome 1. *KCNQ1OT1* encodes a noncoding RNA with antisense transcription of *KCNQ1*. *CDKN1C* encodes a member of the cyclin-dependent kinase inhibitor family of proteins that negatively regulates cell proliferation and is a tumor suppressor. Molecular subgroups of BWS include those with (1) loss of methylation in domain 2, which is associated with decreased expression of *CDKN1C* (~50%); (2) duplication of domains 1 and 2 of the paternal allele with no maternal contribution (uniparental isodisomy) (~20%); (3) mutation of CDKN1C (~10%); (4) gain of methylation of domain 1, resulting in increased IGF2 expression and decreased H19 expression (~2% to 7%); 5) small duplications or chromosome rearrangements (<2%); and (5) unknown genetic etiology (~13% to 15%).[107] Interestingly, the Silver-Russell syndrome, associated with growth retardation, is caused by loss of methylation at domain 1 and maternal 11p15 duplication (i.e., the opposite of BWS).[111,112]

In the past decade correlations between BWS genotypes and cancer predisposition have emerged. Uniparental isodisomy and gain of methylation at domain 1 carry the highest risk of WT. Loss of methylation at domain 2 is associated with omphalocele, macrosomia, and a distinct lack of WT, although these individuals develop other tumor types.[113-115] A common thread in the patients most susceptible to WT is increased expression of *IGF2*.

Wilms Tumor Subsets Defined by Genetic and Epigenetic Features

A traditional model of WT development purports that WTs fall into two discrete subtypes: type I and type II.[72,116] Type I WT is caused by *WT1* mutation, resulting in intralobar NRs, which then accumulate second hits

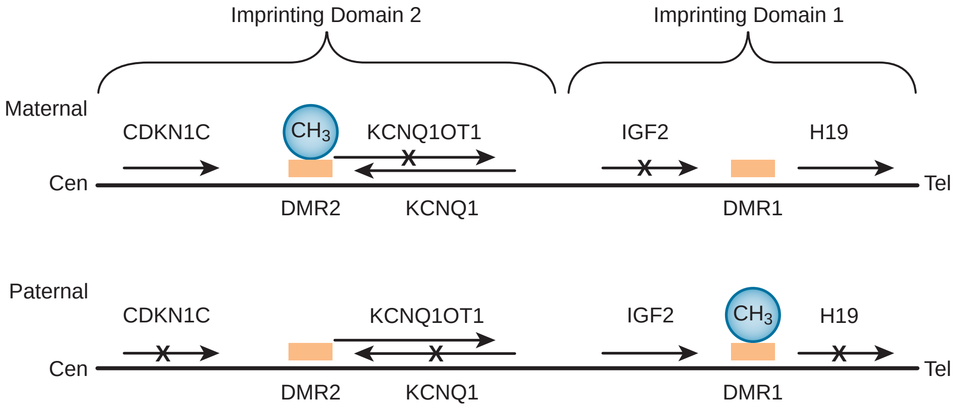

Figure 55-13 The Beckwith-Wiedemann syndrome locus. The chromosome 11p15.5 locus contains several imprinted genes clustered in two imprinting domains: centromeric *(cen)* and telomeric *(tel)*. Each domain has a differentially methylated region *(DMR)* that controls expression of surrounding genes. In normal individuals the DMR of domain 1 is methylated in the paternal allele, resulting in expression of *IGF2* and silencing of *H19*. In normal individuals the DMR of domain 2 is methylated in the maternal allele, resulting in expression of *CDKN1C (p57)* and *KCNQ1* and silencing of *KCNQ1OT(LIT1)*. Beckwith-Wiedemann syndrome can arise from decreased methylation of domain 2, increased methylation of domain 1, duplication of the paternal 11p15 allele with no contribution of the maternal allele (uniparental isodisomy), or small deletions or rearrangements. Increased methylation of domain 1 and uniparental isodisomy carry the greatest risk of Wilms tumor.

such as Wnt activating mutations (β-catenin or WTX mutations). Type II WT is caused by genetic or epigenetic changes in the 11p15 locus that result in IGF2 overexpression, perilobar NRs, and presumably other second-hit genetic changes that lead to WT. Comprehensive mutation analyses of WT1, β-catenin, WTX, and the 11p15 locus in WT samples indicate that whereas some tumors fit the classic paradigm, other tumors have evidence of both *IGF2* overexpression and *WT1* mutation. Indeed transgenic mice with *Igf2* overexpression and *Wt1* deletion developed WT, whereas neither genetic abnormality alone was sufficient to cause WT.[117] Other WTs lack evidence of either *IGF2* or *WT1* alteration. The requirement for coexisting mutations likely depends on the developmental stage of the cell of origin of a particular tumor.[118]

TP53

Constitutional mutations of the *TP53* gene are responsible for Li-Fraumeni syndrome (LFS), which predisposes carriers to cancer.[119] *TP53*, one of the most commonly mutated genes in human cancer, encodes a transcription factor that is a master regulator of cell cycle arrest, apoptosis, and DNA repair in response to a variety of stimuli. Although WT is not a defining criterion for LFS, several LFS families with WT have been identified.[120] It is important to note that *TP53* mutations are associated with the anaplastic histology subtype of WT; approximately 75% of anaplastic WTs have detectable *TP53* mutations, whereas *TP53* mutations are uncommon in favorable histology tumors.[11,121,122] Several lines of evidence suggest that anaplastic WT arises from the acquisition of a *TP53* mutation in a favorable histology WT. First, patients with bilateral WT often have discordant histologies between contralateral tumors.[15] Second, some WTs have favorable histologic features at diagnosis but are found to have anaplastic histology at relapse.[121] Finally, microdissected WTs were found to have *TP53* mutations restricted to areas of anaplasia within the tumor tissue.[12]

Clinical Presentation and Diagnostic Evaluation

The most common presenting signs and symptoms of WT are abdominal mass (75%), abdominal pain (28%), hypertension (26%), gross hematuria (18%), microscopic hematuria (24%), and fever (22%).[123] The hypertension, which can be severe, may be due to ectopic renin production by the tumor, compression of the renal artery by the tumor with resulting physiologic renin production, or ectopic adrenocorticotropic hormone production. An analysis of 31 patients with WT with hypertension at diagnosis found increased plasma renin levels in 81% of patients.[124] Some patients have persistent hypertension after the tumor has been removed, suggesting underlying renal pathology. There are no tumor markers pathognomic for WT, although associated laboratory findings include hematuria and anemia. An association between WT and acquired von Willebrand disease has been described.[125]

WT can spread locally or hematogenously. Locally the tumor has the potential to spread into structures surrounding the kidney and can occasionally invade other organs such as the liver. If the tumor ruptures, peritoneal tumor deposits can be detected. WT also spreads to lymph nodes near the renal hilum and in the paraaortic chain. Between 6% and 8% of WTs spread through the renal vein and form a tumor thrombus in the inferior vena cava, which can propagate into the heart.[126,127] The most common sites of distant metastasis are the lung and liver. There are reports of WT spread to brain or bone, but this is very uncommon. About 7% of patients have synchronous or metachronous bilateral tumors, and 10% have unilateral multifocal tumors.[69]

Radiologic evaluation of WT focuses on the identification of local and distant sites of spread. A computed tomography (CT) scan of the abdomen and pelvis is recommended to visualize the primary tumor, the contralateral kidney, lymph nodes, and intraabdominal or pelvic tumor deposits. CT also provides information valuable to surgical planning. Assessment of the diagnostic performance of CT in identifying the presence or absence of preoperative rupture found moderate specificity but relatively low sensitivity when CT findings were compared to operative notes or pathologic examination. The single best indicator of rupture was the finding of ascites beyond the cul-de-sac, followed by perinephric fat stranding and retroperitoneal fluid.[128] Doppler ultrasound has historically been used as a complementary test to evaluate for tumor thrombus in the renal vein and inferior vena cava. The detection of tumor thrombus by CT versus ultrasound was compared in a study population drawn from the first 1015 patients enrolled on the COG AREN03B2 Renal Tumor Biology and Risk Classification study. CT scans from 62 children with and 111 children without tumor thrombus identified at nephrectomy were reviewed. Comparable sensitivity and specificity of CT and ultrasound were found for detection of cavoatrial thrombus. These findings suggest that CT can adequately identify tumor thrombus that will affect surgical approach and that Doppler ultrasound evaluation is not a diagnostic requirement.[129] CT imaging can also aid in surgical planning by allowing an estimation of tumor weight before surgery.[130,131] In the COG AREN03B2 risk-stratification process, tumors less than 550 gm are considered for treatment with observation only, and preoperative weight estimation can be used in determining the appropriateness of central line placement at time of nephrectomy. On the other end of the spectrum, tumors greater than 1000 gm have also been associated with an increased risk of intraoperative spill; therefore estimation of larger size can also be a factor in surgical planning.[132]

Chest x-rays have historically been used to assess patients for lung metastases, but CT scans are more sensitive in detecting nodules or effusions.[133,134] On NWTS-5, 129 patients had lung lesions detected on CT scan but not chest x-ray. Forty-two of these patients with "CT-only" nodules had lung biopsies, and tumor was confirmed in 31 patients (73.8%).[135] Retrospective studies of the prognostic significance of CT-only lung nodules have yielded conflicting results.[133,136-139] The largest analysis to date included 186 patients with CT-only nodules from

NWTS-4 and -5. These patients were treated heterogeneously (+/– doxorubicin, +/– lung radiation) because the treatment decisions were left to the discretion of the local physicians. Outcome analysis found that patients with CT-only nodules who were treated with doxorubicin had superior relapse-free survival compared with patients who did not receive doxorubicin, but the study found no impact on overall survival. The use of lung radiation did not have a significant effect on outcome.[140,141] Based on these findings, the first generation of COG renal tumor studies recommended CT scans, but not chest x-rays, for the diagnostic staging evaluation for WT. Lung nodules are considered to contain tumor unless biopsy proves otherwise.

Bone scan and brain magnetic resonance imaging are not routinely recommended in the evaluation of patients with newly diagnosed WT. Fluorodeoxyglucose (FDG) positron emission tomography (PET) scanning is not a routine part of the WT workup. Initial experience with PET scans indicates that most WTs are FDG avid.[142,143] Correlations of PET avidity and measures of standardized uptake value with histology and treatment response have been reported in small patient series. These studies suggest that FDG PET may have value in assessing response to chemotherapy as well as a role in identifying tumor viability in lesions with no radiologic evidence of change.[144-146]

Treatment

The treatment approach to WT has evolved along two distinct pathways. The NWTSG, which became the Renal Tumor Committee of COG in 2002, advocates the upfront nephrectomy approach whenever surgically and medically feasible, followed by chemotherapy. This approach provides for accurate histologic diagnosis and staging information. In contrast, SIOP uses preoperative chemotherapy, which shrinks the tumor, thereby reducing the risk of surgical complications such as intraoperative tumor spillage. It is difficult to compare the two approaches side by side because of differences in the staging systems and treatment plans, but both approaches produce excellent outcomes. Regardless of the timing of initial surgery, the three pillars of WT treatment are surgery, chemotherapy, and radiation therapy.

Surgery

In the SIOP treatment protocols, patients 6 months of age or older receive chemotherapy before surgery unless there is evidence of tumor rupture, in which case they undergo immediate nephrectomy. Open or needle biopsy is not routinely performed. Infants younger than 6 months old are treated with upfront nephrectomy because non-Wilms renal tumors, such as mesoblastic nephroma or malignant rhabdoid tumor, are prevalent in this age group.

In the COG treatment protocols, all patients are considered candidates for immediate surgery, although there are several indications to give preoperative chemotherapy: (1) bilateral WT; (2) tumor thrombus in the inferior vena cava above the level of the hepatic veins; (3) tumors that invade adjacent organs such that resection of the tumor would involve resection of the other structures (except the adrenal gland); (4) tumors that, in the surgeon's judgment, would result in significant morbidity or mortality if resected before chemotherapy; and (5) respiratory distress resulting from extensive pulmonary metastatic disease. If preoperative chemotherapy is given, it is recommended not to delay definitive surgery beyond week 12 of treatment.

A generous transabdominal, transperitoneal, or thoracoabdominal incision is recommended for adequate exposure. An increased rate of rupture and complication is associated with a midline laparotomy.[147] Complete exploration of the abdomen should be performed, followed by a radical nephrectomy, dividing the ureter as distally as possible. Routine exploration of the contralateral kidney is not recommended if imaging is satisfactory and does not suggest a bilateral process because the chance of detecting a contralateral kidney tumor during surgery that is undetected with modern imaging techniques is less than 1%, and the outcome of these patients is excellent.[148] An important component of WT surgery is lymph node sampling, even if the nodes appear normal. NWTS-4 showed that patients with positive lymph nodes had better outcomes than patients whose lymph nodes were not sampled, indicating that some patients who did not undergo lymph node sampling were understaged and undertreated.[149] Another important facet of surgery is to remove the tumor en bloc, avoiding tumor spillage. Intraoperative spillage was shown to increase the risk of tumor recurrence.[149] In a review of the first 2000 patients enrolled on COG Biology and Risk Stratification study AREN03B2, an intraoperative rupture rate of 9.7% was found in 1131 primary nephrectomies for unilateral WT. Risk factors for intraoperative spill were diameter (>12 cm) and laterality (right).[150]

Surgical resection or biopsy of lung nodules plays an important role in the diagnosis of metastatic WT. Current COG renal tumor studies consider circular lung nodules, regardless of size, to be metastatic disease unless proven benign by biopsy. The resection of lung metastases may also provide a therapeutic benefit. In the SIOP protocols, patients whose lung nodules are resected completely do not receive pulmonary radiation therapy (XRT). The COG AREN0533 study tested a new strategy for favorable-histology WT with lung metastases. Lung nodules were assessed after 6 weeks of chemotherapy and XRT omitted for patients with a complete response. With this approach it is appropriate not to resect all lung nodules upfront to assess chemosensitivity.

Radiation Therapy

Radiation therapy is a mainstay of WT treatment. Initially used in all patients with WT, successive studies from the NWTSG and SIOP demonstrated that only patients with stage III or IV disease require radiation therapy (Table 55-5). Among these patients radiation doses have been reduced over time, thereby decreasing the potential for late adverse effects.

The radiation guidelines for favorable-histology WT that were used in NWTS-5 are commonly applied as standard practice in North America. Patients with positive lymph nodes, positive tumor margins after surgery,

TABLE 55-5 Key Findings of National Wilms Tumor and SIOP Studies

NWTS Study	Key Findings	SIOP Study	Key Findings
NWTS-1 (1969-1973)	Group I: XRT not beneficial for patients <2 years of age treated with AMD Group II/III: VCR/AMD superior to either agent alone; anaplastic histology strongly predictive of adverse outcome	SIOP 1 (1971-1974)	Preoperative XRT reduces the number of tumor ruptures and produces more low-stage tumors
NWTS-2 (1974-1978)	Group I: reduction of chemotherapy duration from 15 to 6 months did not adversely affect RFS; XRT not needed for this group Group II: inferior outcome for those with lymph node involvement Groups II-IV: addition of DOX to VCR/AMD improved outcome	SIOP 2 (1974-1976)	9 months of VCR/AMD equivalent to 15 months Reduction of tumor ruptures in preoperative treatment group confirmed
NWTS-3 (1979-1986)	Stage I FH: RFS similar whether duration of VCR/AMD was 10 or 26 weeks Stage II FH: Addition of DOX to VCR/AMD did not improve RFS; XRT not necessary Stage III FH: • Without DOX, 20 Gy flank XRT superior to 10 Gy • Addition of DOX to 10 Gy flank XRT improved RFS • Addition of DOX to 20 Gy flank XRT did not improve RFS Stage IV FH: Addition of CYCLO did not improve RFS Stage II to IV diffuse anaplasia: Addition of CYCLO appeared to improve RFS	SIOP 5 (1977-1979) SIOP 6 (1980-1987)	Preoperative chemotherapy equivalent to XRT in preventing tumor ruptures Stage I: 17 weeks VCR/AMD equal to 38 weeks Stage IIN0: Withholding XRT triggered stopping rule because of abdominal relapses; however, DFS and OS were not affected by withholding XRT Stage IIN+/III: DOX improved DFS but not OS
NWTS-4 (1986-1994)	Stage I to IV FH: No difference in RFS when AMD was given as a single dose rather than as 5 doses; toxicity profile and cost better with single dose Stage III to IV FH: No difference in RFS when DOX given as a single dose rather than as 3 doses; toxicity profile and cost better with single dose Stage II to IV FH: No difference in RFS between 6 and 15 months of therapy Stage II to IV diffuse anaplasia: Improved RFS with addition of CYCLO confirmed	SIOP 9 (1987-1991)	4 weeks of preoperative VCR/AMD is equivalent to 8 weeks in terms of % of stage I tumors, EFS, and OS
NWTS-5 (1995-2002)	Stage I FH, age <2 years, tumor <550 gm: Without chemotherapy, 2-year RFS rate was only 86% but OS was 100% Stage I to IV FH: LOH at 1p and 16q predict decreased RFS and OS Stage I anaplasia: RFS and OS inferior to stage I FH Stage II-IV diffuse anaplasia: Best reported outcomes to date using VCR/DOX/CYCLO/ETOP	SIOP 93-01 (1993-1999)	Patients with stage I WT do just as well with 4 weeks of postoperative VCR/AMD as with 18 weeks Postchemotherapy histology predictive of relapse: complete necrosis = low risk; blastemal predominance = high risk

AMD, Dactinomycin; *CYCLO,* cyclophosphamide; *DFS,* disease-free survival; *DOX,* doxorubicin; *EFS,* event-free survival; *ETOP,* etoposide; *FH,* favorable histology; *LOH,* loss of heterozygosity; *NWTS,* National Wilms Tumor Study; *OS,* overall survival; *RFS,* relapse-free survival; *SIOP,* International Society of Paediatric Oncology; *VCR,* vincristine; *WT,* Wilms tumor; *XRT,* radiation therapy.

or tumor spillage receive 10.8 Gy to the flank. Patients with diffuse tumor spillage or peritoneal seeding receive 10.8 Gy to the whole abdomen. Sites of gross residual disease after surgery receive a boost of 10.8 Gy. Early reports from the NWTSG demonstrated that XRT delay beyond 9 days from surgery was an adverse prognostic factor.[151,152] NWTSG studies recommended that radiation commence by day 9 after surgery. However a retrospective analysis of NWTS-3 and -4 did not detect a difference in local recurrence rate between patients who received radiation within 9 days and patients whose radiation was delayed beyond day 9, although very few patients had XRT delayed much beyond day 10.[153] To allow for real time central review and risk stratification, COG renal tumor therapeutic protocols allow up to 14 days from surgery to initiation of abdominal or flank radiation. Patients whose tumors are initially unresectable have radiation therapy delayed until after definitive surgery.

The local radiation doses that the SIOP treatment protocols use are higher than those in COG studies. Patients with stage III intermediate-risk histology receive 14.4 Gy to the abdomen or flank rather than 10.8 Gy. Patients with stage II or III high-risk histology receive 25.2 Gy.

The radiation guidelines for patients with distant metastatic disease are evolving. The most common metastatic site is the lung. In the NWTSG studies all patients with lung metastases received whole lung radiation to a dose of 12 Gy at the beginning of treatment, yielding 16-year relapse-free survival rates of 80%.[154] In SIOP studies patients whose lung nodules resolve with chemotherapy and surgery did not receive lung XRT. A number of studies using this approach have showed varied outcome.

With this approach the 4-year survival rate was 83%.[155] However the United Kingdom Wilms Tumor Study used the SIOP approach and observed a 6-year survival rate of only 65%.[156] In the SIOP 93-01 protocol, patients were treated with 6 weeks of three-drug (vincristine/dactinomycin/doxorubicin) preoperative chemotherapy. Patients in complete remission (CR) after 6 weeks of chemotherapy +/– surgical metastasectomy did not receive pulmonary radiation and continued with three-drug therapy. Patients without CR at 6 weeks received intensified chemotherapy with etoposide/carboplatin and ifosfamide/doxorubicin. If after 9 weeks of this therapy, CR was obtained, no pulmonary radiation was given. If CR was not obtained, patients received 15 Gy of lung radiation with a boost to residual disease. In the study 84% of patients were in CR after the initial 6 weeks of therapy, 17% requiring metastectomy. Only 14% of patients received radiation as front-line therapy. Patients in CR after chemotherapy only or chemotherapy and metastectomy had good overall survival rates (88% and 92%), whereas patients with residual pulmonary metastatic disease had a significantly lower overall survival rate of 48%. This disparity may relate to differences in patient populations and treatment, but it raises the question of whether more stringent selection criteria should be used for withholding lung XRT.

The COG AREN0533 Higher Risk Favorable Histology Wilms Tumor study also stratified therapy according to the lung nodule response after 6 weeks of three-drug chemotherapy. The study asked whether XRT can be omitted without compromising event-free survival for patients whose lung nodules disappear after 6 weeks of chemotherapy and whether outcomes can be improved by intensifying chemotherapy therapy for patients whose lung nodules do not resolve. Patients without pulmonary complete response received 1200 cGy (1050 cGy for ages younger than 12 months) of pulmonary XRT and an intensified chemotherapy with cyclophosphamide/etoposide added to the three-drug therapy. That study closed in 2013 after reaching accrual of 395 patients, and results have not yet been published.

Chemotherapy

In the mid-1950s Farber and colleagues pioneered the use of dactinomycin as an adjunct to surgery and radiation therapy for the treatment of WT. With the introduction of dactinomycin, unprecedented survival rates were observed, particularly in patients with metastatic disease.[157] In the early 1960s the Southwest Oncology Group found that vincristine produced regression of metastatic WT and that the response to vincristine plus XRT was similar to that reported for dactinomycin.[158] In 1969 the first National Wilms Tumor Study (NWTS-1) found the combination of vincristine and dactinomycin to be superior to either agent alone for groups II and III tumors.[159] Since then vincristine/dactinomycin has been the backbone of WT therapy worldwide, although subsets of patients with stage I disease fare well with vincristine alone or even surgery only, as discussed later. In the early 1970s the third stalwart of WT chemotherapy regimens, doxorubicin, was introduced. NWTS-2 demonstrated the benefit of doxorubicin for patients with groups II to IV disease, and NWTS-3 showed that radiation doses can be reduced when doxorubicin is used.[160,161] NWTS-4 refined how dactinomycin and doxorubicin are given. Both agents were as effective when delivered as a single dose rather than as separate doses over 5 (dactinomycin) or 3 (doxorubicin) days.[162] Not only was this "pulse-intensive" dosing more convenient and cost-effective, but it also was associated with less hematologic toxicity.[163,164] The key findings of the NWTSG studies are summarized in Table 55-5.

By the end of NWTS-4 the survival rate for patients with favorable histology WT was approximately 90%. Given this success the priority for NWTS-5 was to identify novel prognostic markers to help stratify patients into risk-appropriate treatment groups. Pilot data from NWTS-3 and -4 showed that LOH at chromosomes 1p and 16q was associated with adverse outcome in patients with WT.[165] Following this lead NWTS-5 prospectively evaluated the prognostic significance of LOH in more than 2000 patients.[166] There was no significant association between LOH and outcome in patients with anaplastic WT, CCSK, or MRT. However LOH at 1p and 16q was associated with increased risk of relapse and death in patients with favorable-histology WT, with the greatest effect seen in tumors with LOH at both loci (Table 55-6).

A second objective of NWTS-5 was to determine whether therapy can be reduced in selected populations of patients with truly outstanding outcomes. Earlier studies suggested that young patients with small stage I tumors with favorable histology have excellent outcomes, so NWTS-5 asked if these patients could be treated with

TABLE 55-6 Effect of Loss of Heterozygosity on Patient Outcome (NWTS-5)

Stage	4-Year RFS (%)	RR	*P*	4-Year OS (%)	RR	P
Favorable Histology Stage I/II						
No LOH	91.2	1.0		98.4	1.0	0.02
LOH 1p only	80.4	2.19	0.02	91.2	4.03	0.6
LOH 16q only	82.5	1.91	0.01	98.1	1.40	0.01
LOH 1p and 16q	74.9	2.88	0.001	90.5	4.25	
Favorable Histology Stage III/IV						
No LOH	83.0	1.0		91.9	1.0	0.36
LOH 1p only	89.0	0.69	0.37	97.6	0.52	0.76
LOH 16q only	85.3	0.89	0.67	92.0	0.88	0.04
LOH 1p and 16q	65.9	2.41	0.01	77.5	2.66	

LOH, Loss of heterozygosity; *NWTS-5*, National Wilms Tumor Study 5; *OS*, overall survival; *RR*, relative risk, with group with no LOH as a baseline; *RFS*, relapse-free survival.

TABLE 55-7 NWTS-5: Treatment Overview

Tumor Histology and Stage	Chemotherapy Regimen	Radiation Therapy
Favorable-histology Wilms tumor		
Stage I/II	VCR/AMD × 18 weeks	None
Stage III/IV	VCR/AMD/DOX × 18 weeks	Flank/abdomen if local stage III and to metastatic sites
Focal anaplastic Wilms tumor		
Stage I	VCR/AMD × 18 weeks	None
Stage II-IV	VCR/AMD/DOX × 18 weeks	Flank/abdomen and to metastatic sites
Diffuse anaplastic Wilms tumor		
Stage I	VCR/AMD × 18 weeks	None
Stage II-IV	VCR/DOX/CYCLO/ETOP × 24 weeks	Flank/abdomen and to metastatic sites
Clear cell sarcoma of the kidney		
Stage I-IV	VCR/DOX/CYCLO/ETOP × 24 weeks	Flank/abdomen and to metastatic sites
Malignant rhabdoid tumor		
Stage I-IV	CARBO/ETOP/CYCLO × 24 weeks	Flank/abdomen and metastatic sites

AMD, Dactinomycin; *CARBO,* carboplatin; *CYCLO,* cyclophosphamide; *DOX,* doxorubicin; *ETOP,* etoposide; *LOH,* loss of heterozygosity; NWTS-5, National Wilms Tumor Study 5; *VCR,* vincristine.

surgery alone, without adjuvant chemotherapy and radiation therapy.[167] Among 75 patients treated with surgery only, eight patients relapsed and three patients developed metachronous tumors in the contralateral kidney.[168] The 2-year disease-free survival rate was only 86%, which triggered a stopping rule, and the study was discontinued. However 74 of the 75 patients survived, suggesting that the nephrectomy-only approach should be revisited. Although the standard treatment for stage I favorable-histology WT in North America is 18 weeks of vincristine and dactinomycin, the United Kingdom WT group treated with 10 weeks of vincristine only and the SIOP 93-01 study demonstrated that 4 weeks of postoperative treatment is equivalent to 18 weeks.[169,170] The treatment regimens from NWTS-5 are summarized in Table 55-7, and the corresponding outcomes are summarized in Table 55-8.

With the foundation of the findings from the NWTS studies, the first COG renal tumor studies (opened 2006) aimed to further refine the treatment of favorable-histology WT. For entry into any COG Renal Tumor Therapeutic study, patients first had to enroll in the COG Renal Tumor Biology and Risk Stratification study AREN03B2, which continued the NWTSG tradition of central pathology and surgery review and tumor banking. More than 4000 patients enrolled in the first 6 years of the study, with a steady accrual of 600 patients annually. Submission of pathology slides, radiology images, operative reports, and tumor and blood samples for analysis was required within 7 days of surgery to meet enrollment deadlines stipulated by the therapeutic studies. Central pathology review for histology and stage, radiology review for presence of bilateral lesions and pulmonary metastases, and surgical review for operative stage were completed electronically by a panel of expert reviewers. An Initial Risk Assignment was made by the study chairs on the basis of the multidisciplinary central reviews. Final Risk Assignments included LOH status of 1p and 16q for patients with favorable-histology WT, and response of pulmonary metastases for patients with stage IV favorable-histology WT. Real-time central review was shown to be feasible, with a median time to Initial Risk Assignment of 8 days from initial surgical procedure.

The AREN0532 study for the treatment of Very Low and Standard Risk Favorable-Histology WT reassessed the feasibility of surgery without adjuvant therapy for patients younger than 2 years old with stage I favorable-histology WTs less than 550 grams. Participation in this study arm required a real-time central review with verification of lymph node sampling confirmed negative for tumor, and the patient could not have an underlying predisposition syndrome, contralateral rests, or multifocal tumors. The study completed accrual of 116 patients with very low-risk disease in August 2013, and outcome data are pending. AREN0532 also assessed the benefit of therapy augmentation for patients with stage I and II favorable-histology WT with LOH at 1p and 16q. Instead of receiving the standard therapy with vincristine and dactinomycin, patients with LOH receive vincristine, dactinomycin, and doxorubicin. Likewise, the AREN0533 study (higher-risk favorable-histology WT) augmented therapy for patients with stage III and IV favorable-histology WT with LOH at 1p and 16q. Instead of receiving vincristine, dactinomycin, and doxorubicin, these patients received a new regimen that added four cycles of cyclophosphamide and etoposide to the standard treatment. AREN0533 also introduced a new approach to patients with lung metastases. Patients whose lung nodules responded completely after 6 weeks of vincristine, dactinomycin, and doxorubicin chemotherapy did not receive lung radiation therapy (see previous section on radiation therapy). By contrast, patients whose lung nodules did respond completely received radiation therapy and augmented chemotherapy with vincristine, dactinomycin, doxorubicin, cyclophosphamide, and etoposide.

The SIOP 2001 study aimed to evaluate whether doxorubicin is necessary for patients with stage II and III intermediate-risk tumors; patients were randomized to receive vincristine and dactinomycin with or without doxorubicin. Preliminary analysis showed equivalent 5-year overall survival of 96% in both treatment groups.[171] The study also adjusted chemotherapy according to

TABLE 55-8 NWTS-5: Treatment Results

Stage	4-Year Relapse-Free Survival Rate (%)	4-Year Overall Survival Rate (%)
Favorable Histology without LOH 1p		
I (<24 months/tumor weight <550 g)	95.6	100
I (≥24 months/tumor weight ≥550 g)	94.2	98.4
II	86.2	97.7
III	86.5	94.4
IV	76.4	86.1
V	64.8	87.1
Focal Anaplastic Histology		
I	67.5	88.9
II	80.0	80.0
III	87.5	100
IV*	61.4	71.6
V	76.2	87.5
Diffuse Anaplastic Histology		
I	68.4	78.9
II	82.6	81.5
III	64.7	66.7
IV	33.3	33.3
V	25.1	41.6
Clear Cell Sarcoma†		
I	100	100
II	86.8	97.3
III	73.8	86.9
IV	35.6	45.0
Malignant Rhabdoid Tumor‡		
I	—	33.3
II	—	46.9
III	—	21.8
IV	—	8.4

LOH, Loss of heterozygosity; *NWTS-5*, National Wilms Study 5.
*Results are for patients who received preoperative chemotherapy because very few patients in this group had upfront nephrectomy.
†Results are reported as 5-year relapse-free and overall survival.
‡Results are combined results for NWTS 1-5.

histologic response to preoperative treatment. Patients with stage I blastemal predominance after 4 weeks of preoperative chemotherapy received a regimen containing doxorubicin instead of just vincristine and dactinomycin. Patients with stage II and III blastemal predominance received a regimen of alternating cyclophosphamide and doxorubicin with carboplatin and etoposide.

Anaplastic Wilms Tumor

In 1978 Beckwith and Palmer published a detailed histopathologic review of the patients entered in the first NWTSG.[172] Approximately 6% of the WT specimens contained anaplasia, as defined in the pathology section of this chapter. The presence of anaplasia was prognostically significant; 11 of 25 (44%) patients with anaplasia died as a result of tumor, whereas only 26 of 364 (7.1%) patients without anaplasia died as a result of tumor. Subsequent National Wilms Tumor Studies, as well as SIOP and the United Kingdom Wilms Tumor Study, confirmed the adverse prognostic significance of anaplastic histology.[156,173,174]

The first NWTSG trial to stratify patients with anaplastic WT into a distinct treatment group was NWTS-3. In this study and in NWTS-4, patients received 15 months of vincristine, dactinomycin, and doxorubicin and were randomized to receive or not receive cyclophosphamide. Patients with stages II through IV diffuse anaplastic WT had a 4-year relapse-free survival rate estimate of 27.2% when treated without cyclophosphamide, compared with 54.8% when treated with cyclophosphamide ($P = .02$).[175] Patients with all stages of focal anaplastic histology and stage I diffuse anaplastic histology had excellent outcomes regardless of treatment regimen.[161,162]

On the basis of these results, cyclophosphamide was incorporated into the treatment plan of NWTS-5 for patients with stages II and IV diffuse anaplastic WT. Patients received a regimen consisting of vincristine, doxorubicin, and cyclophosphamide alternating with cyclophosphamide and etoposide for 24 weeks of treatment and flank radiation therapy to a dose of 10.8 Gy.[15] With this regimen the 4-year relapse-free survival rate and overall survival rate compared favorably with historic data from NWTS-3 and -4 (see Table 55-8). Patients with stage I diffuse anaplastic histology were treated with vincristine and dactinomycin according to the regimen used for favorable-histology WT. With this approach the 4-year event-free survival was only 68.4%, significantly worse than that seen for stage I favorable-histology tumors.[15] Patients with stage II through IV focal anaplastic WT were treated with vincristine, doxorubicin, dactinomycin, and radiation therapy and had outcomes intermediate between favorable histology and diffuse anaplasia (see Table 55-8).

AREN0321, the COG study for high-risk renal tumors, sought to build on the NWTS-5 experience by adding carboplatin to the treatment regimen for diffuse anaplastic WT. Patients were scheduled to receive 31 weeks of alternating vincristine, doxorubicin, and cyclophosphamide, and cyclophosphamide, carboplatin, and etoposide with flank radiation therapy. Because of the poorer-than-expected outcomes for stage I anaplastic WT, the AREN0321 study treated such patients with vincristine, dactinomycin, doxorubicin, and radiation therapy instead of just vincristine and dactinomycin. Patients with stage IV diffuse anaplastic WT had a 4-year relapse-free survival rate estimate of only 33% in NWTS-5. For this group the priority was to identify new chemotherapy combinations. The AREN0321 study included a phase 2 "window" study to evaluate the antitumor activity of vincristine and irinotecan. This combination was selected on the basis of preclinical and clinical data showing activity of the camptothecins (topotecan and irinotecan) against WT.[176] The study was opened in 2006 and permanently closed to accrual in 2013. Outcome results are pending.

Bilateral Wilms Tumor

Between 4% and 6% of patients with WT have tumor involvement of both kidneys at initial presentation (synchronous bilateral WT).[69] These patients pose a dual challenge, with the need to eradicate the tumor cells while preserving renal function. Although renal failure occurs in fewer than 1% of patients with unilateral WT, the long-term incidence of renal failure in patients with bilateral WT has been reported to range from 11% to 20%.[177] Historically synchronous bilateral WT was managed with surgical resection followed by chemotherapy. Typically a unilateral nephrectomy of the more involved kidney was performed with biopsy or partial nephrectomy of the contralateral kidney, followed by adjuvant therapy. With the recognition that chemotherapy could decrease tumor volume and facilitate partial nephrectomy, preoperative chemotherapy has become the standard approach to treatment. Data from the NWTSG showed no difference in outcome whether the tumor was resected at the time of diagnosis or after preoperative chemotherapy.[178-180] Despite the effectiveness of preoperative chemotherapy, approximately 60% of patients continue to undergo nephrectomy of at least one kidney. In NWTS-4 only 19% of patients underwent bilateral nephron-sparing procedures, but more recent data from single-institution series suggest that more patients are candidates for bilateral partial nephrectomies.[181-183] NWTS data also showed that children with bilateral WT or WT and contralateral NRs who had complete radiologic resolution lesions in a kidney after initial treatment did well without surgical intervention to that kidney.[184] An aim of the current COG AREN0534 study for bilateral WT is to decrease the percentage of patients who require nephrectomy.

Chemotherapy regimens for bilateral WT use the same agents as for unilateral WT. In the NWTSG and SIOP studies, patients started treatment with vincristine and dactinomycin therapy and doxorubicin was reserved for patients known to have distant metastatic disease, locally advanced disease, or poor response to therapy. With this treatment approach long-term survival rates for patients with synchronous bilateral WTs were approximately 70% to 80%.[178-180] The COG AREN0534 study for bilateral WT treats all patients with vincristine, dactinomycin, and doxorubicin before surgery to promote improved local control. Given the high likelihood of bilateral renal lesions representing WT and the known limitations of biopsy, upfront biopsy or resection is discouraged in this study. However open biopsy or definitive surgical resection is mandated by week 12 for all patients in the study. Review of the NWTS-4 cohort of patients with synchronous bilateral WT identified 38 patients with progressive or nonresponsive disease, who had a median of 7 months of therapy before surgery. Pathology at eventual surgery revealed histologic subtypes (anaplasia or rhabdomyomatous or differentiated stromal tumors) that could have benefited from earlier surgery.[185] In the patients with bilateral WT with anaplasia from the NWTS-4, core needle biopsy did not identify anaplasia in seven of seven children, whereas open biopsy or complete nephrectomy identified anaplasia in 10 of 18 at initial diagnosis. In 20 of 24 there was discordance in histology between kidneys.[186] In the AREN0534 study chemotherapy after surgery is tailored according to histologic response, on the basis of the postchemotherapy risk classification schema developed by the SIOP group.

Recurrent Wilms Tumor

Approximately 10% of patients with favorable-histology WT and 50% of patients with anaplastic WT develop recurrent disease. The survival rate after recurrence depends on several factors, including histology, time from diagnosis to recurrence, initial stage and treatment regimen, and site of recurrence.[187,188] The NWTS-5 study included treatment of recurrent WT. Patients with favorable-histology WT whose initial treatment consisted of vincristine and dactinomycin received vincristine, doxorubicin, cyclophosphamide, etoposide, and radiation therapy as salvage therapy, resulting in 4-year event-free and overall survival estimates of 71.1% and 81.8%, respectively.[189] Patients whose initial treatment was vincristine, dactinomycin, doxorubicin, and radiation therapy received a salvage regimen of alternating cyclophosphamide and etoposide, and carboplatin and etoposide, resulting in 4-year event-free survival rates and overall survival rates of 42.3% and 48.0%, respectively.[190] Interestingly female patients had better event-free survival rates and overall survival rates (54.7 and 58.7%) than male patients (28.6% and 38.0%). Other studies have demonstrated the efficacy of ifosfamide, carboplatin, and etoposide chemotherapy in the treatment of recurrent WT.[191,192] A phase II trial of topotecan demonstrated a response rate of 48% in patients with multiply recurrent favorable-histology WT.[176] The outcomes of patients with recurrent anaplastic WT are poor, with salvage rates on the order of 10%. Because upfront therapy for anaplastic WT involves the known active agents, patients with recurrent disease are candidates for phase 1 and 2 studies.

An unresolved question is whether high-dose chemotherapy with autologous stem cell rescue provides a benefit for patients with recurrent WT. Several series have reported survival rates of approximately 60% in patients with recurrent WT using single or tandem transplants.[193-195] However similar results have been attained without high-dose therapy, so the benefit of high-dose therapy remains undefined.[196] Efforts toward a randomized clinical trial to answer this question have been hampered by patient numbers. A meta-analysis of 100 patients from six studies attempted to determine characteristics of patients that predict survival with high-dose therapy and autologous stem cell rescue, comparing these patients to the published outcomes of the patients in the NWTS-5 relapse study who were treated for relapse without autologous stem cell transplant. The 4-year survival rate in the group of patients who underwent transplant was 54%. Patients who relapsed in the lung only who underwent transplant had a significantly higher 4-year survival rate (77.7%) than those with relapse in other locations or multiple relapses (4-year survival rate: 41.6%) who were treated with high-dose therapy and stem cell transplant. In comparison with the NWTS-5 cohort, 4-year survival rates were higher for stages I and II patients treated with salvage chemotherapy rather than with transplant, but the outcomes for patients with stages III through IV disease were comparable with both treatment approaches.[197] A second meta-analysis divided patients with relapse WT into three risk groups. Only patients who received four or more drugs for initial therapy or for previous relapse seemed to benefit from high-dose therapy and stem cell rescue.[198]

Most WT relapses occur within 2 years from diagnosis, but a small number of patients experience late relapse. A review of children registered in 10 national or international cooperative clinical trials identified 70 of 13,330 with relapse more than 5 years after initial diagnosis. The median time to diagnosis of this group was 13.2 years. Outcome after late relapse was most influenced by site of relapse, with an 87% survival rate in the 15 patients with relapse in the contralateral kidney and 47% for all other patients.[199]

Late Effects of Wilms Tumor Treatment

The excellent relapse-free survival rate for favorable-histology WT heightens the importance of minimizing long-term effects of treatment. Follow-up of the NWTSG cohort has identified several treatment-related effects that current studies are aiming to minimize.

One of the most feared complications of WT therapy is cardiotoxicity. The cumulative frequency of congestive heart failure in patients treated in NWTS-1 through -4 was 4.4% at 20 years for patients treated initially with doxorubicin and 17.4% for patients treated with doxorubicin for recurrent disease.[200] The risk for congestive heart failure was strongly associated with female sex (relative risk 4.5 compared with male subjects), cumulative doxorubicin dose (relative risk 3.3 for each 100 mg/m^2); lung radiation (relative risk 1.6), and left abdominal radiation (relative risk 1.8).[201]

A second major complication of WT therapy is second malignant neoplasms (SMNs). The 15-year cumulative incidence of SMN in WT survivors treated in NWTSG studies between 1969 and 1991 was 1.6% and increasing steadily.[202] The SMNs observed in this series were sarcomas, carcinomas, leukemias, lymphomas, and brain tumors. Although no cases of leukemia or lymphoma were seen after 8 years, the risk of developing a solid tumor continued to increase with time. Abdominal radiation increased the risk of SMNs, with doxorubicin potentiating the effect. Each 10 Gy of abdominal radiation therapy was estimated to increase the SMN incidence by 22% without doxorubicin and by 66% with doxorubicin. An international cohort of North American, British, and Nordic subjects between 1960 and 2004 found similar results, with a standardized incidence ration of 5.1 and 5 for solid tumors and leukemias, respectively. The leukemia risk was highest in the first 5 years after treatment, but the solid tumor risk increased with age after diagnosis. Leukemia rates were highest for those diagnosed after 1990, and rates of solid tumors were lower for those diagnosed after 1980. This may reflect the increase in intensity of chemotherapy regimens and decreased use of radiation therapy.[203]

Renal failure is a concern for WT survivors because patients undergo nephrectomy or partial nephrectomy as part of their treatment. However the cumulative incidence of end-stage renal disease (ESRD) at 20 years after WT diagnosis is only 0.6% among patients with nonsyndromic unilateral WT.[177] A small cohort of patients followed up to 5 decades after nephrectomy for childhood unilateral renal tumor demonstrated mild to moderate renal function loss, increasing from the third to fifth decade.[204] The cumulative incidence of ESRD is much higher for patients with unilateral WT and WAGR syndrome (36%), Denys-Drash syndrome (74%, but this is probably an underestimate due to inaccurate classification of the syndrome), and isolated genitourinary anomalies (7%).[177] The cumulative incidence of ESRD is also high among patients with non-syndromic bilateral WT (12%). The high rate of renal failure among patients with bilateral WT is related mainly to bilateral nephrectomies performed for tumor control.[205] However the renal failure seen with WAGR and Denys-Drash syndromes is caused by underlying renal pathology.[206,207] In the follow-up of 173 children enrolled in NWTS who developed ESRD, treatment and outcomes were very different in the 55 patients who developed ESRD from progressive bilateral WT than in the 118 who had ESRD from other causes (grouped as chronic kidney disease).[208] Of the 55 subjects with PBWT, 25 died while on initial ESRD treatment with dialysis, all but four of these as a result of WT. Only 47% of these patients had a renal transplant at 5 years. In the 118 patients with ESRD from chronic kidney disease, 77% had received a transplant at 5 years, and the survival at 10 years was 73%. Once renal transplant had been performed, the differences in outcome between the patients with progressive bilateral WT and those with chronic kidney disease were markedly reduced, suggesting that renal transplant should be considered earlier for some patients rather than waiting the 1- to 2-year tumor-free interval, as is commonly recommended.

A review of pregnancy outcomes in patients treated in NWTS-1 through -4 revealed that fetal malposition and early or threatened labor were more frequent among women who received abdominal irradiation compared with unirradiated women.[207] There were an excessive number of infants born before 36 weeks of gestation to women who received flank radiation and an excessive number of infants with birth weight below 2500 g. Most of the birth weights were appropriate for gestational age. Congenital malformations were more frequent among the offspring of women who received flank radiation compared with those of unirradiated women. Fertility was related to the dose and field of radiation therapy.[209] In general fertility is preserved in WT survivors after upper abdominal radiation that does not include the pelvis and flank, rather than whole-abdominal radiation.

Dental abnormalities (e.g., root stunting, enamel hypoplasia, microdontia) in excess of the expected incidence in control patients were observed in WT survivors.[210] A small study of 49 WT survivors revealed osteopenia in 27%.[211] Stature loss has been observed in WT survivors, with a predicted height deficit at age 18 years of 1.8 cm for children treated with 10 Gy to the flank at age 4 years.[212] Height deficits were negatively correlated with age (greater deficit with younger age of irradiation) and positively correlated with radiation dosage. Unlike cardiac effects or second malignancies, doxorubicin did not exacerbate reduction in stature.

Additional late effects were assessed in the Childhood Cancer Survivor Study, through the 25-year follow-up of childhood WT.[213] This study assessed chronic health conditions, health status, health care utilization, socioeconomic status, SMNs, and mortality of WT survivors diagnosed from 1970 to 1986. In this cohort there was a cumulative incidence of all and severe chronic health conditions of 65.4% and 24.2% at 25 years. WT survivors reported more adverse general health status than the sibling group, but mental health status, socioeconomic outcome, and health care utilization were similar. There was a 3% cumulative incidence of SMN and 6.1% incidence of mortality. Radiation exposure increased the likelihood of congestive heart failure (with and without doxorubicin), as well as occurrence of SMN and death.

CLEAR CELL SARCOMA OF THE KIDNEY

Epidemiology

The average age of onset of CCSK in the NWTSG experience was 36 months, with a range of 2 months to 14 years. In contrast to WT, a male predominance was noted, with a male-to-female ratio of 2 to 1. In a review of published series and cases reports of CCSK, no familial cases were described, and only three cases were bilateral. Because these were all in patients with widely disseminated disease, they may have represented metastatic disease rather than second primary tumors.[28,214]

Molecular Biology and Genetics

The genetic etiology of CCSK is poorly understood. Most cases of CCSK have normal karyotypes, although a recurrent t(10;17) translocation has been identified in 10% to 15% of CCSK, resulting in a fusion transcript of the *YWHAE* and *FAM22* genes.[33] Although the *TP53* gene is located at the 17p13 breakpoint of these translocations, p53 is not thought to play a prominent role in the pathogenesis of CCSK because most CCSK tumors lack detectable p53 protein by immunostaining,[28,215,216] *TP53* mutations are infrequently detected by sequencing analysis,[216] and CCSK primary cell cultures have a functional p53 pathway in response to DNA damage.[217] Other recurrent abnormalities detected by either conventional karyotyping or comparative genomic hybridization include chromosome 14q deletion (two cases) and 1q gain (three cases).[217-219]

Gene expression analysis of CCSK tumors has revealed frequent upregulation of neural markers with activation of the sonic hedgehog and phosphosinositide-3-kinase/Akt pathways.[220] Among the upregulated members of the Akt signaling pathway was the epidermal growth factor receptor *(EGFR)* gene. A subsequent study found EGFR protein immunoreactivity in 12 of 12 CCSK samples, *EGFR* gene amplification in 1 of 12 samples, and a somatic *EGFR* mutation in one sample.[221] Two samples had mutations of *PTEN*, a negative regulator of the Akt pathway.[221] These results provide a rationale for evaluating EGFR inhibitors in patients with CCSK that is refractory to current treatment modalities.

Clinical Presentation and Diagnostic Evaluation

Although CCSK has been termed the "bone-metastasizing renal tumor of childhood," metastatic disease at the time of initial presentation is distinctly uncommon. Only 4% of patients have distant metastatic disease at diagnosis, with bone, lung, and liver as the most common sites.[28] However approximately 30% of patients who undergo local lymph node sampling are found to have tumor involvement in the renal hilar and periaortic lymph nodes.[28] The value of bone scan versus skeletal survey to detect bone metastases has been addressed. Most lesions are detectable by both modalities, but some lesions are seen by only one of the two modalities.[222] Despite the predilection of this tumor to bone, bone marrow involvement is uncommon and bone marrow aspiration is no longer recommended as part of the staging workup.

Treatment

Although considered one of the unfavorable-histology renal tumors, significant progress has been made in the treatment of CCSK. Data from NWTS-1, -2, and -3 suggested that the addition of doxorubicin to the combination of vincristine and dactinomycin improved the 6-year relapse-free survival rate percentage.[223] The beneficial effect of doxorubicin was confirmed in a retrospective review of 351 cases, including 182 cases from NWTS-1 through -4.[28] NWTS-3 demonstrated no improvement in outcomes of patients with CCSK when cyclophosphamide was added to the combination of vincristine, dactinomycin, and doxorubicin.[223] However, the cyclophosphamide was delivered at a relatively low dosage and intensity. NWTS-4 asked whether the duration of therapy affects outcome for patients with CCSK. Compared with 6 months of vincristine, dactinomycin, and doxorubicin therapy, 15 months of therapy was associated with a trend toward improved 8-year relapse-free survival rate (87.8% versus 60.6%, $P = .08$), although there was no difference in 8-year overall survival rate (87.5% vs. 85.9%).[224] NWTS-5 treated patients with 6 months of a combination of vincristine, doxorubicin, and cyclophosphamide alternating with a combination of cyclophosphamide and etoposide. Preliminary analysis showed 5-year event-free and overall survival estimates of 79% and 89%, respectively.[225] Interestingly 14 patients had stage I disease, and none of these patients relapsed. An aim of the COG AREN0321 study was to assess whether the outstanding outcomes for stage I patients can be maintained without flank radiation therapy. This study closed to accrual in 2013, and outcome data are pending.

The treatment and outcomes of 191 patients with CCSK were reported from the SIOP 93-01 and SIOP 2001 protocols.[226] Significant discordance in initial pathology diagnosis and SIOP panel pathology diagnosis was noted. Of the CCSK patients 27% were initially diagnosed as a different renal tumor type, and seven cases of CCSK were changed to another tumor type by panel pathologists. Of 191 subjects, 169 received SIOP preoperative chemotherapy (recommended therapy vincristine and dactinomycin for 4 weeks for localized disease and vincristine, dactinomycin, and doxorubicin for 6 weeks for metastatic disease). Through evaluation with Response Evaluation Criteria in Solid Tumors (RECIST) criteria, 21% had a partial response after preoperative chemotherapy, 15% had minor response, 31% had stable disease, and 33% had progressive disease. All patients underwent total nephrectomy, and 189 of 191 received postoperative chemotherapy. Recommended postoperative chemotherapy was a three- or four-drug therapy regimen. Abdominal radiation therapy (mean dose of 25.2 Gy) was given to 85 patients, 2 of 71 stage 1 patients, 33 of 43 stage II patients, 44 of 50 stage III patients, and 4 of 13 stage IV patients. Five-year event-free survival and overall survival rates were 78% and 86%, respectively. The brain and lung were the most common sites of relapse, followed by bone and primary site. Five-year event-free survival and overall survival rates varied by stage as follows: stage I 79% and 87%, stage II 92% and 91%, stage III 69% and 82%, stage IV 58% and 73%. The timing of full staging in patients varied, insofar as almost all received preoperative chemotherapy for a presumed diagnosis of WT, and bone scans and brain imaging were not done routinely before starting therapy. Patients younger than 12 months were noted to have worse outcomes, with five-year event-free survival and overall survival rates of 49% and 61%.

An interesting observation on recent CCSK trials has been a shift in the site of recurrent disease. Although CCSK is associated with bone metastases, the brain has now surpassed bone as the most common site of recurrence in North American and European studies.[225,227,228] The reason for this shift is unclear, but it is possible that improved disease control outside the central nervous system has uncovered the brain as a sanctuary site for tumor cells.

MALIGNANT RHABDOID TUMOR

Epidemiology

MRT occurs primarily in infants, with a mean age of diagnosis of 15 months.[229] However, older children and rare adults with MRT have been reported. MRT is associated with mutations of the *SNF5* gene at chromosome 22q11.1, and familial MRT has been described.[230] A recent report of three individuals with MRT whose parents shared a common site of employment raises the possibility of an environmental etiology.[231]

Molecular Biology and Genetics

MRTs most frequently occur in the kidney, where they are referred to as *rhabdoid tumor of the kidney,* and the central nervous system, where they are referred to as *atypical teratoid/rhabdoid tumors,* but they can also occur in soft tissues throughout the body, where they are generally referred to as *extrarenal rhabdoid tumors*. It had long been debated whether renal, central nervous system, and extrarenal rhabdoid tumors are distinct cancers that possess similar histologic appearances or whether they are the same cancer in different anatomic locations. This issue was largely resolved when the majority of rhabdoid tumors from all anatomic sites were found to have specific biallelic inactivating mutations in *SMARCB1* (also known as *INI1*, *BAF47* and *HSNF5*).[232,233] Inactivating mutations in both copies of *SMARCB1* occur in 70% of MRT and an additional 20% to 25% of the tumors do not express SMARCB1 protein even though a specific mutation cannot be identified.[234] Whereas loss of *SMARCB1* occurs in the majority of MRTs, mutation of other genes such as *SMARCA4(BRG1)* account for a minority of cases.[235,236]

Constitutional mutation in one allele of *SMARCB1* is the basis of familial MRT, a condition termed *rhabdoid predisposition syndrome*.[237,238] Carriers have a markedly increased risk for MRT in all sites and can present with more than one primary tumor. Invariably, the second *SMARCB1* allele has been inactivated in these cancers. Constitutional mutation also accounts for the approximately 15% of patients who, after treatment for MRT, develop a second primary MRT. The frequency of germline mutations in patients presenting with new onset rhabdoid tumor is somewhat unclear. It has been reported to be present in as many as 33% (16 of 49) of cases, although this number is likely inflated by selection bias.[234] Patients who carry a germline *SMARCB1* mutation typically present with early onset rhabdoid tumor. Most, but not all, of these individuals are diagnosed before 1 year of age. Given the relatively high frequency of germline *SMARCB1* mutations, patients with new-onset MRT should have their brain and kidneys imaged to rule out additional tumors. Sequencing the *Smarc* gene from the peripheral blood of newly diagnosed patients should also be considered to rule out a constitutional mutation. If a constitutional mutation is identified, other family members should be screened for this mutation. Notably although familial studies have identified cancer-free carriers, three families with multiple siblings affected by *SMARCB1* mutant MRT have been identified in which neither of the parents carried a *SMARCB1* mutation. This suggests that gonadal mosaicism in a parent can be the basis of familial cases. Consequently the absence of a constitutional mutation in the parents of a patient with a germline *SMARCB1* mutation does not fully exclude the possibility that siblings may be affected.

SMARCB1 encodes a core member of the SWI/SNF chromatin remodeling complex. Tightly compacted chromatin provides an organizational structure for DNA but constitutes a significant barrier to gene expression. Nucleosomes, consisting of 147 base pairs of DNA wrapped around an octamer of histones, are a fundamental unit of chromatin. The SWI/SNF complex regulates transcription of specific targets by using the energy of adenosine triphosphate hydrolysis to mobilize nucleosomes and thereby control access of the transcriptional machinery to promoters. Mice heterozygous for *Snf5* are predisposed to the formation of rhabdoid tumors that display a histologic appearance essentially indistinguishable from human MRT.[239-241] Further, conditional inactivation of both Snf5 alleles leads to the extremely rapid formation of cancer, with 100% of mice developing either lymphoma or MRT at a median onset of only 11 weeks. Because the SWI/SNF complex regulates expression of many genes, the precise genes involved in the development of MRT remain to be elucidated. Evidence suggests that the cyclinD1/p16INK4a, aurora kinase A, interferon, and sonic hedgehog pathways are downstream of SMARCB1 and are dysregulated in MRT.[242-248]

Clinical Presentation and Diagnostic Evaluation

Children with MRT of the kidney present with signs and symptoms related to an intrarenal mass. Because of the young age at presentation, it is difficult to assess pain, yet most parents report fussiness. About 60% of patients have gross hematuria, which contrasts with WT in which only 20% have hematuria.[249] Fever is a presenting feature in 50% of patients, and hypertension is seen in up to 70%. Because MRT of the kidney can be associated with involvement of the central nervous system, patients may present with focal neurologic signs or signs of increased intracranial pressure.

The diagnosis of MRT must be made by histologic confirmation, although several laboratory features are suggestive of MRT. Approximately 25% of patients have hypercalcemia and 55% have hemoglobin levels less than 9g/dL. Gross or microscopic hematuria is evident in 75% of patients. The most common sites of MRT metastasis are the lung, bone, and brain. Many of the central nervous system lesions likely represent second primary tumors rather than metastatic disease. The staging evaluation for MRT therefore includes a CT scan of the chest, abdomen, and pelvis; ultrasound of the abdomen to evaluate for tumor thrombus; bone scan; and magnetic resonance imaging of the brain. Bone marrow aspirates and biopsies are not routinely required.

Treatment

MRT is one of the most lethal and aggressive pediatric cancers. Patients with rhabdoid tumor of the kidney historically were treated in NWTSG trials with vincristine, dactinomycin, and doxorubicin, with or without cyclophosphamide. The outcomes attained with these agents were poor (see Table 54-8).[229,250] In an attempt to improve on these results, NWTS-5 tested a treatment regimen consisting of carboplatin and etoposide, alternating with cyclophosphamide, but this regimen did not yield improved survival rates compared with previous regimens.[229] Case reports have highlighted patients with metastatic rhabdoid tumor who had durable survival after treatment with various combinations of ifosfamide, etoposide, carboplatin, vincristine, doxorubicin, and cyclophosphamide.[251-254] Survivors treated with high-dose therapy and autologous stem cell rescue have also been reported,[254] but it is unclear whether this provides a benefit compared with intensive regimens of standard-dose chemotherapy. On the basis of this limited experience, the COG AREN0321 study aimed to test the efficacy of the combination of cyclophosphamide, carboplatin, and etoposide alternating with vincristine, doxorubicin, and cyclophosphamide. The protocol opened in 2006 and accrued 39 patients with MRT of the kidney before permanent closure in 2013. Outcome data are still pending. A window phase of treatment with vincristine and irinotecan was trialed on this protocol for stage IV MRT, but it did not demonstrate activity in these patients. SIOP reported survival data on 107 patients with MRT treated on the SIOP 93-01 and 2001 (1993-2005).[255] Of these patients 22% had surgery performed at time of diagnosis, 62% received vincristine/dactinomycin (VA) therapy for 4 weeks prior to surgery, and 38% received vincristine/dactinomycin/doxorubicin (VAD) for 6 weeks before surgery. Postoperative treatment recommendations included four-drug therapy with etoposide, carboplatin, cyclophosphamide or ifosfamide, and doxorubicin or epirubicin. Five-year event-free survival and overall survival rates were 22% and 26%. Most patients demonstrated evidence of chemosensitivity with marked tumor reduction with initial chemotherapy, but this did not correlate with increased survival. The median time from diagnosis to relapse was 8 months, and survival after relapse was markedly poor (4 of 60, 1 to 5 years from relapse). Advanced tumor stage and young patient age were significant poor prognostic factors.

The lack of treatment uniformity among reported patients makes it difficult to determine if radiotherapy is effective for MRT. In NWTS-1 through -5, radiation therapy was given to the flank or abdomen at total doses of 1080 to 3500 cGy, yet no relationship was observed between dose and outcome.[229] Radiation therapy is a cornerstone of treatment for central nervous system rhabdoid tumors, and some suggest that the high doses delivered to the posterior fossa improve patients' outcomes.

RENAL CELL CARCINOMA

Epidemiology

Two European registries of childhood RCC revealed that the median age of diagnosis in children and adolescents was 10 to 11 years, with a male-to-female ratio of 1 to 1.1.[256,257] Among the first 3250 patients enrolled in the COG Renal Tumor Biology Study AREN03B2, 3.8% had RCC. In adults clear cell RCC, associated with mutations of the von Hippel Lindau gene, is the predominant type of RCC. In children the clear cell subtype is uncommon.[258,259] An association between oncocytoid RCC and a previous history of neuroblastoma has been described.[62,260] Translocation RCC, associated with translocations involving the *TFE3* (chromosome Xp11.1) or *TFEB* (chromosome) genes, has been associated with previous exposure to chemotherapy.[261] The evolving characterization of translocation RCC revealed that a large percentage (up to two thirds) of pediatric RCC falls within this subtype.[258,259]

Molecular Biology and Genetics

The molecular pathogenesis of the Xp11.2 and t(6:11) translocation carcinomas is an area of active study.

Xp11.2 and t(6:11) (p21:q12) Translocation Carcinomas

The fusion partners of *TFE3* have variable functions. *PSF* and *NonO* are splicing factor genes.[262] *PRCC* and *ASPL* are novel genes of unknown function, but the former may also be involved in splicing.[263-266] The clathrin heavy chain gene (*CLTC*) trimerizes with a single light chain to form clathrin, the major protein constituent of the coat that surrounds organelles (cytoplasmic vesicles) to mediate selective protein transport.[267] All TFE3 fusion proteins retain the C-terminal portion of TFE3, including its leucine-zipper dimerization domain, nuclear localization signal, and DNA binding domain. It is likely that the genes fused 5' to *TFE3* contribute strong promoters that cause overexpression of the chimeric protein, as suggested by results with TFE3 immunohistochemistry. Both PRCC-TFE3[268] and ASPL-TFE3 fusion proteins[269] localize to the nucleus and can act as aberrant transcription factors. PRCC-TFE3 may also interfere with splicing and mitotic checkpoint control through protein-protein interactions.[270]

Both ASPS and the t(X;17) renal carcinomas feature one of two types of *ASPL-TFE3* fusion transcript, in which the *ASPL* gene is fused to *TFE3* exon 4 (type 1 fusion) or *TFE3* exon 3 (type 2 fusion). Four types of *PRCC-TFE3* fusion transcripts have been described. Significant differences in clinicopathologic features between cases with different fusion types have yet to be established. Both *ASPL-TFE3* and *PRCC-TFE3* fusion transcripts are readily detected by conventional RT-PCR using appropriate primers, as described in detail elsewhere.[49,50]

A recent gene expression study has identified several novel genes that are differentially expressed between the Xp11 translocation carcinomas and conventional renal carcinomas and has shown that Xp11 translocation carcinomas may be more similar to ASPS than to conventional renal carcinomas.[271] Additionally gene expression profiling has identified potential therapeutic targets in the Xp11 translocation RCC. For example the ASPL-TFE3 fusion protein transactivates the promoter of the MET receptor tyrosine kinase, leading to MET protein overexpression. Inhibition of the MET receptor tyrosine kinase may therefore be a potential avenue of targeted therapy for these RCCs.[272]

The characteristic t(6;11)(p21;q12) translocation fuses the *Alpha* gene, an intronless gene of unknown function at 11q12, with the first intron of the *TFEB* transcription factor gene at 6p21. The breakpoint on *TFEB* is just upstream of the *TFEB* initiation ATG codon, which results in retention of the entire *TFEB* coding region in the fusion. Although the *Alpha* promoter drives expression of the fusion gene, the *Alpha* gene does not contribute to the open reading frame. Therefore the consequence of the *Alpha-TFEB* fusion is dysregulated expression of the normal full-length TFEB protein. These findings explain why the t(6;11) renal carcinomas demonstrate specific nuclear labeling for TFEB protein by IHC whereas other neoplasms and normal tissues do not. The association of both the Xp11.2 and the t(6;11) renal translocation carcinomas with exposure of the child to prior chemotherapy raises the possibility that the *TFE3* and *TFEB* loci may be particularly susceptible to DNA damage that promotes chromosome translocations.[261]

It is now thought that the t(6;11) renal carcinomas are related to the Xp11-translocation carcinomas based on the following findings:

1. Their clinical predilection to affect young patients is similar.
2. Their morphology is similar. Both neoplasms are composed of nests of predominantly clear to eosinophilic epithelioid cells, typical of a conventional RCC on routine hematoxylin and eosin sections.

3. Their immunohistohemical profiles are similar. Given their epithelioid morphology, both tumors underexpress epithelial IHC markers (cytokeratins, EMA). Several Xp11-translocation carcinomas (specifically, those with PSF-TFE3 and CLTC-TFE3 fusions) have expressed melanocytic markers by IHC, just as the t(6;11) renal neoplasms characteristically do. Both RCCs express cathepsin K in contrast to other RCC subtypes. Both RCCs overexpress proteins in the MiTF/TFE transcription factor family (TFE3 fusion proteins or native TFEB), and in each case a routine IHC assay detects this overexpression in a highly sensitive and specific fashion.
4. Their molecular pathologies are related. TFEB, TFE3, TFEC, and Mitf are members of the microphthalmia subfamily of basic helix-loop-helix transcription factors, which have homologous DNA binding domains and in fact bind to a common DNA sequence.[273] Members of the MiTF/TFE transcription factor family homodimerize and heterodimerize in all combinations to bind similar or identical DNA sequences. Therefore it seems likely that the transcription factors that are overexpressed in these two tumors (TFE3 fusion proteins and native TFEB) have similar downstream targets. Most of these targets remain to be determined; however, genes normally expressed in melanocytic differentiation may be one such example. In cell line transfection assays, overexpression of TFE3 activates the promoter of the tyrosinase gene, whereas both TFEB and TFE3 activate the promoter of the tyrosinase-related protein 1 gene.[274] The genes encoding tyrosinase and tyrosinase-related protein 1 are normally expressed in melanocyte differentiation, where they are regulated by another MiTF/TFE family member, MiTF. These results suggest that aberrant overexpression of TFEB may be responsible for the expression of melanocytic markers, a characteristic and distinctive feature of the t(6;11) renal carcinomas, and that some specific TFE3 fusion proteins may do so as well. Mouse knockout studies provide further evidence that MiTF family members may functionally overlap in certain cell types. In these studies severe osteopetrosis occurs in mice with combined TFE3 and MiTF inactivation, but there is no effect of TFE3 or MiTF loss individually on osteoclasts.[275]

Therefore, based on the preceding facts, it has been suggested that the t(6;11) renal carcinomas and the Xp11.2 translocation carcinomas should be classified as members of the "MiT translocation carcinoma family."[276] This suggestion was adopted by the 2012 International Society of Urologic Pathology Consensus Conference in Vancouver, British Columbia.

Clinical Presentation and Diagnostic Evaluation

The most common presenting signs and symptoms of pediatric RCC are flank or abdominal pain, hematuria, fever, nausea and vomiting, abdominal mass, anemia, pallor, and malaise.[256,257] It is unusual for children to present with the typical adult RCC triad of abdominal mass, hematuria, and hypertension. Approximately 20% of patients have locoregional lymph node metastases, and as many as 30% have distant metastatic disease at initial presentation. The lung, mediastinum, and liver are the most common sites of distant spread, but osseous, central nervous system, and other soft tissue metastases are also possible.[257,277] The imaging workup for pediatric RCC therefore includes CT of the chest, abdomen, and pelvis, with consideration of a bone scan and magnetic resonance imaging of the head. The role of PET scan has not been fully determined, but some forms of pediatric RCC have been found to be PET active. CT imaging has been demonstrated to have a high specificity but relatively low sensitivity for detection of local lymph nodes in pediatric RCC, underlining the importance of lymph node sampling at surgery, insofar as pathologically confirmed nodal disease can be observed even with small primary tumors (COG AREN03B2 study, unpublished observations).

Treatment

Several series of pediatric RCC have demonstrated stage-for-stage outcomes that mirror outcomes observed in adult patients.[256,257,277] Survival rates for patients with stage I, II, III, and IV disease are about 92%, 85%, 73%, and 13%, respectively.[277] A difference between adult and pediatric RCC is the prognostic significance of local lymph node involvement in the absence of distant metastases.[277] Whereas the survival rate for adult patients with local lymph node involvement is in the 20% to 30% range, approximately 75% of children with local lymph node involvement survive.[257,277] This difference likely reflects the disparate histologies between adult and pediatric RCC. Given the good outcomes of children with localized RCC and the lack of agents with known efficacy, the COG AREN0321 study recommended treating such patients without adjuvant therapy. Patients with metastatic RCC are treated according to the physician's professional judgment. The study was open from 2006 to 2013, and outcome data are pending.

Historically, the only therapy with proven benefit for metastatic RCC was immunotherapy using interleukin-2, interferon-alpha, or a combination of these agents. However immunotherapy produces tumor responses in only 10% to 20% of patients, most of whom achieve a transient response and experience substantial toxicity.[278] With the development of drugs targeted to signal transduction and tumor-related angiogenesis, multiple trials have response rates and impact on progression-free survival in adult RCC. Agents that have demonstrated benefit in some studies of adult RCC include vascular endothelial growth factor pathway inhibitors (sorafenib, sunitinib, pazopanib, axitinib, and bevacizumab) and mTOR inhibitors (temsirolimus and everolimus). In the wake of these studies, molecularly targeted therapy has become the frontline treatment for advanced RCC in adult patients. However in considering treatment of pediatric RCC, the clinician must remember that childhood and adult RCC are distinct entities. Most of the patients enrolled in adult studies had the clear cell subtype of RCC, whereas the majority of pediatric RCC patients have the translocation or papillary subtypes. A retrospective series from the Juvenile RCC Network indicated that tyrosine kinase inhibitors, most notably sunitinib, have produced objective responses or disease stabilization in translocation RCC.[279] A prospective trial of tyrosine kinase inhibition therapy for metastatic or recurrent translocation RCC is under development by the COG.

A category of pediatric RCC with a distinctly poor outcome is renal medullary carcinoma, which is observed in patients with sickle cell trait.[61] Patients with renal medullary carcinoma almost always present with metastatic disease and have fatal outcomes. Transient responses have been observed after chemotherapy treatment with a combination of methotrexate, vinblastine, doxorubicin, and cisplatin[280-282] and a combination of platinum, gemcitabine, and taxane.[283,284] Agents that target the ABL tyrosine kinase may be considered for investigation in renal medullary carcinoma because these tumors have been reported to have ABL gene amplification and BCR-ABL rearrangements.[281,282] A tumor also recently was found to have a translocation involving the *ALK* gene.[285] A patient with renal medullary carcinoma was shown to have a complete tumor response after treatment with the proteosome inhibitor bortezomib.[286]

CONGENITAL MESOBLASTIC NEPHROMA

Epidemiology

The average age of diagnosis of CMN is 3.4 months. The vast majority of cases are diagnosed in infancy, but in rare cases children up to 9 years of age have been diagnosed. Congenital malformations accompanied 14% of CMN cases, including genitourinary and gastrointestinal anomalies, polydactyly, and hydrocephalus.[287] There is a strong correlation of age at diagnosis and histologic subtype; the cellular type is seen more commonly in children older than 3 months of age.[288]

Molecular Biology and Genetics

IFS and cellular CMN have much in common. The two neoplasms have virtually indistinguishable morphology and affect the same young age group. Both also are associated with a prognosis that is better than that expected given their ominous-appearing morphology; both IFS and cellular CMN only rarely metastasize. There had also been molecular hints of a relationship between IFS and cellular CMN, insofar as both tumors are characterized by polysomies, particularly of chromosomes 11, 17, and 20.[289,290]

A major advance occurred in 1998, when it was discovered that IFS is characterized by a specific t(12;15)(p13;q25). The t(12;15) results in fusion of the *ETV6* gene on chromosome 12 with the *NTRK3* gene on chromosome 15.[291] The *ETV6 (TEL)* gene had previously been implicated in the t(12;21)(p13;q22) of pediatric precursor B-cell acute lymphoblastic leukemia and encodes a transcription factor with a helix-loop-helix protein dimerization domain. NTRK3 is a receptor tyrosine kinase that is activated by ligand-dependent dimerization and is involved in neural development. The chimeric ETV6-NTRK3 protein is postulated to be constitutively dimerized and therefore to affect constitutively active tyrosine kinase growth pathway signaling. Significantly, neither infantile fibromatosis nor adult-type fibrosarcomas, two entities in the histologic differential diagnosis of infantile fibrosarcoma, contain this gene fusion.

Given the clinicopathologic similarities of IFS and cellular CMN, the question arose as to whether cellular CMN bore the same chromosome translocation and gene fusion. Two studies have conclusively proven this to be true.[292,293] Of 11 cellular CMNs studied, 10 contained the fusion transcript, whereas all five classic CMNs (the renal counterpart of infantile fibromatosis) did not. Hence it is tempting to consider cellular CMN to be essentially an IFS of the renal sinus. Indeed there is no clinical, morphologic, histopathologic, or genetic reason to think otherwise. Additionally these studies highlight the fact that classic and cellular CMNs are molecularly distinctive neoplasms, not just two morphologic patterns of one neoplasm, as their names would suggest. The data on mixed CMN is conflicting, but most recent studies have found an absence of the *ETV6-NTRK3* gene fusion in mixed CMN.[67,289,294]

Further studies have addressed the function of the *ETV6-NTRK3* fusion gene. Wai and colleagues have shown that ETV6-NTRK3 chimeric tyrosine kinase is capable of transforming NIH3T3 cells.[295] Tognon and colleagues have subsequently shown that this requires activation of both the Ras-Raf1-Mek1-ERK1/2 mitogenic pathway and the phosphatidylinositol 3’-kinase (PI3K)-Akt cell survival pathway, such that inhibition of either pathway abolishes transformation.[296] These findings raise the possibility that MEK or PI3K inhibitors could be potentially useful in treating the minority of patients who develop metastatic disease. Jin and coworkers have shown that the *ETVG-NTRK3* chimeric tyrosine kinase suppresses transforming growth factor(TGF) β tumor suppressor activity by binding to the type II TGF-β receptor.[297] Finally the *ETV6-NTRK3* gene fusion has also been found to be consistently present in secretory carcinoma of the breast, a rare epithelial cancer that tends to affect young people, and mammary analogue secretory carcinoma of the salivary gland.[298,299] Rare cases of acute myeloid leukemia have been shown to bear the same gene fusion.[300,301] Hence the *ETV6-NTRK3* gene fusion is remarkable in that it may apparently transform cells of the mesenchymal, epithelial, and hematopoietic lineages.

Clinical Presentation and Diagnostic Evaluation

A palpable abdominal mass is the most common presenting symptom in infants with CMN. Other signs and symptoms at presentation include hematuria (18%), hypertension (4%), anemia (4%), and vomiting (6%).[287] Hypercalcemia has been reported as a presenting feature of CMN.[302] Patients with CMN do not present with metastatic disease, although the cellular histologic type has the potential to metastasize to the lung and, rarely, the brain later in the course of the disease. A baseline chest, abdomen, and pelvis CT scan is recommended.

Treatment

Outcomes for patients with CMN are generally excellent without adjuvant therapy, with overall survival rates of 95%.[287,303] The few tumors that recur are almost exclusively the cellular type of the CMN. In addition to cellular histologic subtype, age greater than 3 months and stage III disease have been associated with relapse.

The standard therapy for all stages of classic CMN (noncellular) and stage I and II cellular CMN is surgical resection followed by observation with serial imaging studies. It remains to be established whether patients with stage III cellular CMN benefit from adjuvant chemotherapy. In a series published by the German Pediatric Oncology Group, two of five patients with stage III cellular CMN developed recurrent disease, whereas only one of the remaining 45 patients experienced recurrence.[303] Experience with presurgical treatment of CMN shows that most of these tumors respond to a combination of vincristine and dactinomycin therapy.[303] Recurrent cellular CMN tumors are responsive to combinations of vincristine, doxorubicin, and dactinomycin; vincristine, dactinomycin, and cyclophosphamide; vincristine, doxorubicin, and cyclophosphamide; and ifosfamide , carboplatin, and etoposide.[304] There is no established role for radiation therapy in the treatment of CMN.

FUTURE DIRECTIONS

A broad view of the field of pediatric renal tumors provides cause for both celebration and trepidation. Treatment of favorable-histology WT has been a terrific success, with cure rates in the 90% range. Remarkably the success has been coupled with a diminishment in treatment, thereby sparing patients of acute and long-term adverse effects. Clinicians have a more complete understanding of renal tumor biology than ever before, with the discovery of several new genes (*WTX*, *BRCA2*, *INI1*, *TFE3*, and others) and increased understanding of previously identified genes (*WT1* and the *11p15* locus). The availability of a new pipeline of molecularly targeted agents provides the opportunity to advance renal tumor therapies beyond the traditional cytotoxic agents.

On the other hand challenges remain. Outcomes for children with anaplastic WT, bilateral WT, recurrent WT, RCC, and MRT remain suboptimal (all with survival rates of less than 75%). For some of these tumor types, such as anaplastic WT and MRT, increased chemotherapy doses and intensity are approaching the maximum level of patient tolerance. Novel treatment approaches, such as molecularly targeted agents, differentiating agents, or immunotherapy, are required. A pitfall to the introduction of new agents is that the patient populations are small, so clinical trials take years to execute. New initiatives for international collaboration between the COG and SIOP will facilitate the conduct of clinical research on the uncommon pediatric renal tumors. For tumor types such as pediatric RCC, the tumor biology and natural clinical history have only recently begun to be understood. Broad support for pediatric renal tumor research is essential for further progress.

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8. Doros LA, Rossi CT, Yang J, et al: DICER1 mutations in childhood cystic nephroma and its relationship to DICER1-renal sarcoma. *Mod Pathol* 2014 [epub ahead of print].

   This study found loss-of-function DICER1 *mutations in 14 of 20 (70%) cystic nephromas evaluated, whereas none of six CPDNs had* DICER1 *mutations. CPDN previously was considered to lie on a spectrum between cystic nephroma and WT, but these data suggest that the two cystic renal tumors are distinct entities. The authors also describe sarcomas developing within cystic nephromas, analagous to the progression from the cystic type 1 pleuropulmonary blastomas to the solid and sarcomatous type PPBs, also associated with* DICER1 *mutations.*

11. Bardeesy N, Falkoff D, Petruzzi MJ, et al: Anaplastic Wilms' tumour, a subtype displaying poor prognosis, harbours p53 gene mutations. *Nat Genet* 7:91–97, 1994.

    This study first showed an association between TP53 *mutations and anaplastic histology WT. Several lines of evidence indicate that anaplastic WT arises on a background of favorable-histology WT. About 75% of anaplastic WTs have* TP53 *mutations. Studies are under way to assess whether prognosis and biology differ between anaplastic WTs based on their TP53 mutation status.*

15. Dome JS, Cotton CA, Perlman EJ, et al: Treatment of anaplastic histology Wilms' tumor: results from the fifth National Wilms' Tumor Study. *J Clin Oncol* 24:2352–2358, 2006.

    This study demonstrates that patients with stage II through IV diffuse anaplastic WT had improved survival rates compared with historic controls using a new regimen consisting of vincristine, doxorubicin, and cyclophosphamide alternating with cyclophosphamide and etoposide. The study indicates that although anaplastic WT is relatively resistant to therapy compared with favorable-histology WT, outcomes were improved with a more intensive chemotherapy regimen. The study also showed that patients with stage I anaplastic WT fared significantly worse than patients with stage I favorable-histology WT, contrary to the previous belief.

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    This was the first study to correlate postchemotherapy histologic changes with prognosis in WT. The work here formed the basis of the SIOP histologic classification schema for pediatric renal tumors (see Reference 5).

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carcinomas of children and adolescents. *Am J Pathol* 159:179–192, 2001.

This paper describes molecular genetic abnormality seen in the majority of cases of childhood RCC. "Translocation-type" RCC is now recognized as a distinct RCC subtype by the World Health Organization.

68. van den Heuvel-Eibrink MM, Grundy P, Graf N, et al: Characteristics and survival of 750 children diagnosed with renal tumors in the first seven months of life: a collaborative study by the SIOP/GPOH/SFOP, NWTSG, and UKCCSG Wilms tumor study groups. *Pediatr Blood Cancer* 50:1130–1134, 2008.

    This large international series of 750 infants with renal tumors describes the epidemiology and treatment results for this patient subgroup.

69. Breslow N, Olshan A, Beckwith JB, et al: Epidemiology of Wilms tumor. *Med Pediatr Oncol* 21:172–181, 1993.

    This classic paper contains valuable data on age, tumor laterality, gender, ethnicity, and congenital malformations in WT.

71. Knudson AG, Strong LC: Mutation and cancer: a model for Wilms' tumor of the kidney. *J Nat Cancer Inst* 48:313–324, 1972.

    Knudson first described the "two-hit" model of mutagenesis and cancer using retinoblastoma as a model. This paper, published 1 year after the retinoblastoma paper, applied the same principles to WT. Many of the observations made in this seminal paper hold true today, although we now recognize that more than two hits are required and that several distinct pathways are involved in Wilms tumorigenesis.

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86. Gessler M, Poustka A, Cavenee W, et al: Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature* 343:774–778, 1990.

    The preceding three papers describe the initial identification of the WT1 *gene and provide the first insights into its function as a transcription factor. Subsequent papers indicate that there are multiple splice variants of this gene with differing functions.*

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93. Koesters R, Ridder R, Kopp-Schneider A, et al: Mutational activation of the β-catenin proto-oncogene is a common event in the development of Wilms tumors. *Cancer Res* 59:3880–3882, 1999.

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97. Rivera MN, Kim WJ, Wells J, et al: An X chromosome gene, WTX, is commonly inactivated in Wilms tumor. *Science* 315:642–645, 2007.

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117. Hu Q, Gao F, Tian W, et al: Wt1 ablation and Igf2 upregulation in mice result in Wilms tumors with elevated ERK1/2 phosphorylation. *J Clin Invest* 121:174–183, 2010.

    WT has traditionally been categorized into two subtypes: those associated with WT1 mutations and those associated with alterations of the Beckwith-Wiedemann 11p15/IGF2 locus. It is now clear that the lines are blurred between the two subtypes. This paper describes one of the first genetically engineered mouse models of WT obtained by combined Wt1 ablation and Iff2 upregulation.

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    This paper describes a detailed analysis correlating gene expression patterns with WT1 mutations, 11p15 copy number and methylation, CTNNB1 (β-catenin) mutations, and WTX mutations in more than 300 WT samples. The analysis led to a revised ontogenic model of WT consisting of five subsets with distinct clinical and biologic features.

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    This paper describes the largest study to date on the significance of WT lung nodules detected by CT scan but not chest x-ray. The findings suggest that so-called CT-only nodules have clinical significance because patients treated with only two-drug therapy (vincristine and dactinomycin) had a significantly lower event-free survival rate compared with patients treated with three-drug therapy (vincristine, dactinomycin, and doxorubicin). The overall survival rate, however, was not different between the two groups.

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166. Grundy PE, Breslow NE, Li S, et al: Loss of heterozygosity for chromosomes 1p and 16q is an adverse prognostic factor in favorable-histology Wilms tumor: a report from the National Wilms Tumor Study Group. *J Clin Oncol* 23:7312–7321, 2005.

    This is the primary paper reporting outcomes for favorable-histology WT in the NWTS-5. It describes a prospective analysis of LOH at 1p and 16q as a prognostic marker. Combined LOH at both loci was found to predict inferior event-free and overall survival in all stage groups. These findings influenced the risk stratification schema for WT on the most recent generation of COG studies.

168. Green DM, Breslow NE, Beckwith JB, et al: Treatment with nephrectomy only for small, stage I/favorable histology Wilms' tumor: a report from the National Wilms' Tumor Study Group. *J Clin Oncol* 19:3719–3724, 2001.

    One of the primary objectives of the NWTS-5 was to assess whether young patients with small stage I favorable-histology WT can be treated with surgery only, without adjuvant chemotherapy. The study closed early after the predefined stopping rule for number of relapses was reached. However the salvage rate after recurrence exceeded expectations, so the overall survival rate with this approach was outstanding. This question was revisited in the recently completed COG AREN0532 study.

171. Pritchard Jones K, Graf N, Bergeron C, et al: Doxorubicin can be safely omitted from the treatment of Stage II/III intermediate risk histology Wilms Tumour: results of the SIOP 2001 randomized trial. *Pediatr Blood Cancer* 57:741, 2011.

    Although published only in abstract form at the time this chapter was written, these results are important because they will lead to a reduction in therapy for a large group of patients. The study found that withholding doxorubicin does not affect overall survival in patients with stage II and III intermediate-risk WT. It is important to recognize that these findings cannot be applied to *patients treated in COG studies because the staging and histologic classification systems differ.*

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The preceding two papers describe the outcomes of patients with relapsed WT treated in NWTS-5. Two primary prognostic categories emerged. Patients who were treated initially with only two-drug therapy had overall survival rates after relapse af approximately 80%. Patients treated initially with three drugs or more had overall survival rates of approximately 50% with a more intensive salvage regimen. Initial treatment overshadowed other prognostic factors for survival after recurrence, including site of recurrence and time to recurrence.

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CHAPTER 56

Retinoblastoma

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CHAPTER OUTLINE

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Retinoblastoma is the most frequent neoplasm of the eye in childhood and the third most common intraocular malignancy in all ages, following malignant melanoma and metastatic carcinoma. Retinoblastoma represents 2.5% to 4% of all pediatric cancers but 11% of cancers in the first year of life.[1] The average age-adjusted incidence rate of retinoblastoma in the United States and Europe is 2 to 5 per million children (approximately one in 14,000 to 18,000 live births).[2,3] Thus retinoblastoma develops in an estimated 8000 children each year worldwide.[4]

Retinoblastoma is a cancer of the very young; two thirds of all cases of retinoblastoma are diagnosed before 2 years of age, and 95% of cases are diagnosed before 5 years of age.[1] For these reasons, in addition to cure of the disease, therapeutic approaches require consideration of the need to preserve vision with minimal long-term adverse effects.

Retinoblastoma presents in two distinct clinical forms. The first form is bilateral or multifocal and heritable (occurring in 25% of all cases of retinoblastoma); it is characterized by the presence of germline mutations of the retinoblastoma-1 *(RB1)* gene. Multifocal retinoblastoma may be inherited from an affected survivor (25%), or it may be the result of a new germline mutation (75%). The second form is unilateral (representing 75% of all cases); about 85% to 90% of the cases are nonhereditary, and the remainder carry a germline *RB1* mutation.

EPIDEMIOLOGY

The incidence of retinoblastoma is not distributed equally around the world. It appears to be higher (6 to 10 cases per million children) in Africa[2,5] and India[6] and among children of Native American descent in the North American continent.[7,8] The increased incidence in those groups occurs primarily at the expense of unilateral cases. Whether these geographic variations are due to ethnic or socioeconomic factors is not known. However the fact that even in industrialized countries an increased incidence of retinoblastoma is associated with poverty and low levels of maternal education[9] suggests a role for the environment. Differences in the incidence of embryonal tumors between countries and ethnic groups have been consistently reported during the past decades, particularly for retinoblastoma[2]; however the deficiencies inherent to suboptimal cancer registries in low-income countries have made proper estimates difficult. In a country as ethnically diverse and socioeconomically heterogenous as Brazil, De Camargo and colleagues[10] calculated the age-adjusted incidence rates (AAIR) of retinoblastoma in 20 population-based cancer registries and noted higher AAIRs than those of developed countries. The AAIR for children 0 to 4 years of age was as high as 15 and 27 in Salvador and Bahia, respectively, two of the most deprived states in the country (versus 10 to 12 in the United States and Europe).[10] In a broader study testing the differences in incidence for embryonal tumors and their correlation with socioeconomic status, the same group documented an inverse correlation between the incidence of retinoblastoma and socioeconomic index. A similar phenomenon has been reported in Mexico, where a higher incidence of retinoblastoma has been documented, particularly in deprived states such as Chiapas.[11,12]

Decreased dietary intake of vegetables and fruits during pregnancy, resulting in decreased intake of nutrients such as folate and carotenoids, which are necessary for deoxyribonucleic acid (DNA) methylation and synthesis and for retinal formation, has also been associated with an increased risk of unilateral sporadic retinoblastoma.[13] In a case-control study, the risk of developing retinoblastoma was associated with a maternal polymorphism in dihydrofolate reductase (*DHFR* 19bpdel), particularly in women taking prenatal synthetic folic acid supplements.[14]

It is well known that exposure to certain toxic agents during gestation increases the frequency of germinal mutations in animals. The vast majority of germline mutations in sporadic heritable retinoblastoma are paternally derived,[15] and studies have suggested an association between paternal age[9,16,17] and occupation[18] with the occurrence of sporadic heritable retinoblastoma. Reports have also suggested an association between retinoblastoma and increased sunlight exposure,[19,20] air toxins from gasoline and diesel combustion,[21] or in vitro fertilization.[22-24] In a case-control study of sporadic retinoblastoma, radiologic studies of the abdomen leading to scattered radiation exposure of the gonads was associated with an increased risk of bilateral retinoblastoma in the child.[25]

Retinoblastoma tumors arise from fetal retinal cells that have lost function of both allelic copies of the *RB1* gene, the first of the tumor suppressor genes to be cloned. The first event may be either a germline or a somatic mutation, but the second and subsequent events are always somatic. Not all tumors have mutations in *RB1*, suggesting that there is either another gene or alternate mechanisms for inactivation of *RB1* function.[26,27] For example, the *RB1* gene can be epigenetically silenced through hypermethylation of the promoter.[28,29] In recent years, studies have suggested a role for human papillomaviruses (HPVs) in the pathogenesis of retinoblastoma. The viral oncoprotein E7 of high-risk HPV types has been shown to bind to and inactivate the *RB1* gene product. Therefore it is plausible that HPV infection could be functionally equivalent to the biallelic loss of *RB1*.[30] Transgenic mice expressing HPV16 E6 and E7 proteins develop retinoblastoma.[31] Presumably exposure to HPV would occur peripartum from genital infection of the mother. In this regard the use of barrier methods of contraception is associated with a reduced incidence of both retinoblastoma and HPV infection,[9,32] and studies have also shown a correlation with sexually transmitted diseases during pregnancy.[17] Interestingly an overlap occurs between countries in which the relative incidence of retinoblastoma is greatest and those in which the incidence of cervical carcinoma is highest.[32] High-risk HPV sequences have been detected in 28% to 36% of tumors in some populations.[32,33]

BIOLOGY OF RETINOBLASTOMA

Cellular Origins of Retinoblastoma

It is widely believed that the molecular and cellular features of a tumor reflect its cell of origin and can thus provide clues about treatment targets. Since 1897 when Wintersteiner first proposed that rosettes in retinoblastoma are reminiscent of photoreceptor differentiation,[34] the cell of origin and the cellular features of retinoblastoma have been debated. Initially the focus was on the cellular features that could be identified by histologic analyses, such as the presence of rosettes. With advances in molecular biology and biochemistry, the debate about the retinoblastoma cell of origin has been revisited, with a strong predilection toward photoreceptors being the cell of origin.[35-43] In addition to photoreceptors, a number of independent studies suggested that retinoblastomas may also have features of progenitor cells or interneurons such as amacrine cells.[38,44,45,46,47-51] The possibility of tumor cells with a hybrid molecular/cellular phenotype was not considered in those previous studies.

In a recent study of the molecular, cellular, neuroanatomic, and neurochemical features of mouse and human retinoblastoma, it was shown that they have features of multiple cell classes, principally amacrine/horizontal interneurons, retinal progenitor cells, and photoreceptors.[52] Indeed, single-cell gene expression array analysis showed that these multiple cell type-specific developmental programs are coexpressed in individual retinoblastoma cells, which creates a progenitor/neuronal hybrid cell. It is widely believed that the initiating genetic lesion in the *RB1* gene occurs in proliferating retinal progenitor cells during fetal development. Retinal progenitor cells may be the cell of origin for retinoblastoma, and the multiple differentiation programs that are found in human and mouse retinoblastomas may reflect the multipotent competence of retinal progenitor cells. Indeed in single-cell gene expression array analyses of mouse retinal progenitor cells, photoreceptor and interneuron genes were often expressed in proliferating retinal progenitor cells.[53]

The finding that retinoblastoma tumor cells express multiple neuronal differentiation programs that are normally incompatible in development suggests that the pathways that control retinal development and establish distinct cell types are perturbed during tumorigenesis. This discovery underscores the challenges of assigning cell-of-origin status without first establishing the full molecular and cellular signature of a tumor. It is important to emphasize that these studies do not necessarily indicate that a photoreceptor, interneuron, or progenitor cell is the cell of origin for retinoblastoma. Detailed cell-lineage studies combined with live imaging approaches will be required to definitively identify the cell of origin for retinoblastoma. Nonetheless, this detailed characterization of the neuronal pathways that are deregulated in retinoblastoma may provide novel targets for therapeutic intervention. This example highlights the importance of comprehensive molecular, cellular, and physiologic characterization of human cancers with single-cell resolution as we incorporate molecular targeted therapy into treatment regimens.

Retinoblastoma Tumor Cell Differentiation

Many of the previous studies on retinoblastoma tumor cell differentiation have focused on photoreceptor features, including a recent study linking the origin of retinoblastoma to cone photoreceptors.[43] As previously described, a detailed cross-species gene expression array analysis indicated that retinoblastomas express molecular features of photoreceptors and that the rod signature is more robust than the cone signature.[52] However morphometric and neuroanatomic studies of mouse and human retinoblastomas showed few cellular features of photoreceptors, which raises the possibility that the gene regulatory network that controls photoreceptors is deregulated in human retinoblastomas. Interestingly a large number of photoreceptor genes are also expressed in medulloblastomas[54,55] despite their distinct cellular origins, which may indicate that the transcriptional pathways involved in photoreceptor differentiation are nonspecifically deregulated in a variety of tumors of the central nervous system (CNS). It is not known if this "photoreceptor signature" has any prognostic or therapeutic significance in retinoblastoma or other tumors of the CNS.

Human and mouse retinoblastomas have molecular, morphometric, neuroanatomic, and neurochemical features of interneurons, including amacrine and horizontal cells.[52] Indeed, the cell-type specific features that are most common across human and mouse retinoblastomas are those related to amacrine cell differentiation. As previously discussed, the tumor's cell of origin may be completely independent of amacrine interneurons, and because tumor suppressor or oncogenic pathways are deregulated during tumorigenesis, the amacrine differentiation program may be aberrantly activated during retinoblastoma tumorigenesis.

Nonetheless, the amacrine differentiation of human and mouse retinoblastoma may be functionally significant for tumor survival and progression. The most striking evidence for amacrine differentiation at the cellular level came from the identification of dense core vesicles in human and mouse retinoblastomas in electron micrographs. Retinoblastomas produce catecholamines and other monoamines,[56-58] and several of the receptors for monoamines are expressed, as are the genes involved in the biosynthesis of monoamines. When monoamine receptors were blocked with pharmacologic agents, retinoblastoma growth was reduced in culture and in vivo. In contrast, agents that block the other major neurotransmitter signaling pathways (γ-aminobutyric acid, glycine, and glutamate) had no effect on retinoblastoma, nor did the inhibition of selective reuptake of catecholamines with pharmacologic agents. One interpretation of these data is that retinoblastoma cells are engaged in an autocrine induction of the catecholamine-signaling pathway. All of the major catecholamine/monoamine receptors are G-protein–coupled receptors and signal through adenyl cyclase–mediated cyclic adenosine monophosphate induction. This pathway can directly or indirectly stimulate the mitogen-activated protein kinase pathway and provide an important mitogenic signal for retinoblastoma. It may also explain why retinoblastomas form in the retina. That is, this tissue is uniquely susceptible to tumorigenesis because the amacrine-differentiation pathway can be co-opted for mitogenic signaling after *RB1* gene inactivation. Additional studies will be required to determine whether catecholamine signaling influences survival and growth through the mitogen-activated protein kinase pathway or if it is required for some other aspect of tumorigenesis.

Retinoblastoma Genomics and Epigenomics

Most retinoblastomas initiate with biallelic loss of the *RB1* gene.[59] *RB1* inactivation confers limitless replicative potential to retinoblasts, and these preneoplastic cells can progress to retinoblastoma by acquiring additional somatic mutations that contribute to the acquisition of new cellular properties, including evasion of cell death and senescence, sustained angiogenesis, and activation growth–signaling pathways. Several different mechanisms have been proposed to explain the rapid progression of retinoblastoma after *RB1* inactivation. In a series of studies using genetically engineered murine cells and immortalized human cells, it was shown that RB1 may play an important role in maintaining genomic stability.[60-62] Thus in some cellular contexts, inactivation of the *RB1* gene could lead to chromosome instability, allowing secondary and tertiary mutations in key cancer pathways to be rapidly acquired. Alternatively RB1 has also been implicated in a variety of epigenetic processes, so it is also possible that perturbations in the epigenetic landscape may contribute to tumorigenesis in the retina. In support of an epigenetic mechanism, recent whole-genome sequencing and integrated epigenetic analysis of human retinoblastoma revealed that the tumors have relatively stable genomes, and several cancer genes were epigenetically deregulated.[63] At least one of those epigenetically deregulated genes (spleen tyrosine kinase; *SYK*) is required for retinoblastoma tumor cell survival in vivo (discussed later).[63] These two alternative mechanisms (genome instability and epigenetic deregulation) of retinoblastoma progression are not necessarily mutually exclusive, and some tumors may show evidence of both chromosomal instability and epigenetic deregulation.

Although epigenetic deregulation of key cancer pathways has been shown to be directly relevant for retinoblastoma tumor progression, the functional significance of recurrent secondary genetic lesions is still poorly understood. During the 27 years since the *RB1* gene was cloned, researchers have focused on identifying genetic lesions in retinoblastoma that contribute to tumor progression after *RB1* inactivation.[64] Cytogenetic and array comparative genome hybridization studies have led to the identification of regions of the genome that are gained or lost in retinoblastomas and may contribute to tumorigenesis.[64] Those studies have led to the identification of candidate oncogenes and tumor suppressor genes whereby copy number variations (CNVs) correlate with changes in gene expression.[65,66] However the major limitation of those studies is their relatively small number of tumors analyzed and the modest effects on gene expression for tumors with CNVs versus those without the corresponding lesion. Even for the most common recurrently mutated gene in retinoblastoma, BCL6 corepressor *(BCOR)*, there have been no direct in vivo studies showing that it contributes to tumorigenesis.

Carefully designed in vivo experiments to explore the role of putative oncogenes and tumor suppressor genes in retinoblastoma tumorigenesis are important for advancing our understanding of the molecular mechanisms of retinoblastoma progression. For example, a recent study showed that in a small proportion (1.5%) of human unilateral nonfamilial retinoblastomas, no *RB1* gene mutations are present and they have v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog *(MYCN)* amplification (≥10 copies).[27] The authors suggested that in those patients, *MYCN* amplification may initiate tumorigenesis.[27] This observation was recently validated and extended in an independent study with a separate cohort of patients.[67]

Recent whole-genome sequencing of human retinoblastomas and their matched germline DNA demonstrated that at least some retinoblastomas have relatively stable diploid genomes with few CNVs or somatic nucleotide variations,[63] and thus genome instability may not be required for retinoblastoma progression. One of the most common misconceptions related to cancer genomics is that tumors with few CNVs have stable genomes and tumors with more CNVs have unstable genomes. Chromosome instability is a dynamic process that cannot be accurately measured at a single time point because it involves the acquisition of sequential chromosomal lesions over time. It is important to distinguish between such dynamic processes that reflect the continuous accumulation of genetic lesions versus more acute genomic events such as multiple chromosome trisomies as seen in hyperdiploid acute lymphoblastic leukemia (ALL), multiple chromosome loss as seen in hypodiploid ALL, and the more recently described process of chromothripsis.[68,69] The best way to directly analyze chromosome instability is to sample the same tumor at multiple time points and perform a comprehensive analysis of the genetic landscape at each time point. Orthotopic xenografts of human retinoblastoma in immunocompromised mouse eyes have proved to be very useful for analysis of chromosome instability in retinoblastoma because the genomic landscape can be analyzed sequentially over time.[63] Clearly this does not preclude the possibility that some retinoblastomas have unstable genomes, but genome instability is not a universal hallmark of *RB1*-deficient retinoblastomas, nor is it required for rapid tumor progression.

In the most recent genomic analysis of retinoblastoma, single nucleotide polymorphism 6.0 analysis was performed on 94 human retinoblastomas and matched normal germline DNAs. These data were used to identify regions of loss of heterozygosity and copy number changes, including whole chromosomes, regional changes, and focal changes (<3 Mb). Specifically, the majority (70%) of retinoblastomas have relatively few (≤10 per tumor) chromosomal, regional, or focal CNVs.[67] No correlation was found between the number of CNVs and the heritable or sporadic form of disease, nor was there any relationship between the type of *RB1* mutation and the number of lesions.[67] A much larger study will be required to determine if more subtle associations exist between the clinicopathologic features of retinoblastoma and the rate of CNVs.

The overall low rate of CNVs was consistent with the paucity of focal recurrent lesions in genes. As reported previously, inactivation of the *RB1* gene and the *BCOR* gene were the most common deletion events, and amplification of the *MYCN* gene was the most common focal recurrent gain.[63] In this most recent study, a new recurrent focal amplification of orthodenticle homeobox 2 *(OTX2)* was discovered in 3% of retinoblastomas. *OTX2* is a homeobox gene that is involved in photoreceptor and retinal pigment epithelium development.[70-73] Retinoblastomas express a variety of rod and cone photoreceptor genes,[38,52] and in future studies, it will be important to determine if OTX2 plays a role in modulating the photoreceptor differentiation program in retinoblastoma.

One of the most important aspects of the study was analysis of the relationship between mutations in *MYCN, BCOR,* and *OTX2* and the mechanism of *RB1* inactivation by sequencing all 27 exons of *RB1* in the cohort.[67] In total, 10 tumors were identified that had no single nucleotide variations or insertions or deletions in the coding region of *RB1*. Whole-genome sequencing on those 10 tumors and their matched normal germline DNA led to the identification of one tumor with a wild-type *RB1* gene and *MYCN* amplification. That tumor sample showed robust RB1 nuclear protein expression, suggesting that retinoblastoma can initiate in the absence of *RB1* mutation. Gene expression array analysis showed that the tumor with wild-type *RB1* was indistinguishable from all other retinoblastomas with *RB1* mutations. Those data confirm a previous report from the Gallie laboratory that a small subset of retinoblastomas (~1% to 2%) have wild-type *RB1*.[27] More importantly this larger more recent genomic analysis also identified three tumors that had focal chromothripsis, disrupting the *RB1* gene and leading to loss of RB1 protein expression. The chromothriptic lesions are not detected by routine genetic analyses because all the exons are present in the genome. This is the first example of chromothripsis in the *RB1* gene in human cancer and the first clear example of chromothripsis as a mechanism for tumor initiation in a well-defined developmental cancer—retinoblastoma. Whole-genome sequencing combined with break-apart fluorescence in situ hybridization analysis of the *RB1* locus and RB1 immunohistochemistry (IHC) can be used to identify this unique subset of retinoblastoma tumors.

Retinoblastoma Preclinical Models

Mouse models of cancer have become increasingly important in the fields of cancer genetics and translational research. The importance of mouse models is particularly true for pediatric cancers because the patient population is relatively small and preclinical models are essential for validating the efficacy of new combinations of chemotherapy before initiating clinical trials in children. In addition, animal models of cancer can provide important new insight into the molecular, cellular, and genetic mechanisms underlying tumor initiation and progression.

One challenge of modeling pediatric cancer in mice is that these tumors initiate in the context of developing tissues that change rapidly, and the cells that give rise to the tumors can display an extraordinary degree of plasticity and heterogeneity. This situation is further complicated by the fact that many tumor suppressor genes and protooncogenes play essential roles in regulating cell-fate specification and differentiation during development. Specifically, the genetic lesions that contribute to tumor initiation and progression may also alter the intrinsic cell-fate specification and differentiation programs in the tumor cells, thereby making it very difficult to infer the cell of origin for that tumor.

Retinoblastoma is a relatively simple tumor that initiates with a common genetic lesion (*RB1* inactivation) and progresses rapidly in children. Moreover many features of retinal development are also conserved across species, making retinoblastoma an ideal tumor for cross-species comparison.

During the past decade a series of knockout mouse models of retinoblastoma have been generated by conditionally inactivating multiple Rb family members in the developing retina.[52,74,75] Knockout mouse models of retinoblastoma have also been valuable for studying the contribution of other tumor suppressor pathways such as the p53 pathway[74] and for testing novel therapeutic agents for the treatment of retinoblastoma.[76,77] In one study six different strains of mice that developed retinoblastoma were analyzed side by side using the same retinal progenitor–specific Cre transgene (*Chx10-Cre*) to inactivate tumor suppressor genes in the developing retina.[52] Histopathologic analysis, gene expression profiling, and morphometric, neuroanatomic, and neurochemical analyses[52] showed that mouse retinoblastomas faithfully recapitulate the molecular and cellular features of human retinoblastomas. However whereas the timing of retinoblastoma initiation was indistinguishable across the six strains, tumor penetrance and the rate of progression varied dramatically.[52] These data raise the possibility that the genetic and epigenetic changes that accompany human retinoblastoma progression may not be faithfully recapitulated in the mouse, despite the remarkable interspecies similarities at the molecular and cellular level.

To more directly assess the similarities and differences across species for retinoblastoma, a recent study analyzed the aneuploidy, CNVs, somatic nucleotide variations, and epigenetic landscape of murine retinoblastoma. Similar to human retinoblastoma, mouse tumors have a low rate of single-nucleotide variations in genes.[78] However mouse retinoblastomas have a higher rate of aneuploidy and regional and focal copy number changes, and this is dependent on the genetic lesions that initiate tumorigenesis in the developing murine retina.[78] In addition the epigenetic landscape in mouse retinoblastoma was significantly different from human tumors, and some pathways that are candidates for molecular targeted therapy for human retinoblastoma such as SYK or myeloid cell leukemia–1 are not deregulated in genetically engineered mouse models (GEMMs). Taken together these data suggest that important differences exist between mouse and human retinoblastomas with respect to the mechanism of tumor progression, and those differences can have significant implications for translational research to test the efficacy of novel therapies for this devastating childhood cancer.

In fact, preclinical testing of combination chemotherapy (etoposide, carboplatin, and vincristine) showed dramatic differences in response between GEMMs and human orthotopic xenografts; virtually all of the GEMMs were cured of their disease, whereas no improvement was seen in progression-free survival or overall survival in the orthotopic xenograft model.[79] It is possible that such species-specific differences could be further amplified when testing molecular targeted therapeutics directed toward processes important for maintaining genomic stability or the epigenetic landscape of retinoblastoma or targeting pathways such as SYK/myeloid cell leukemia–1 that are not deregulated in murine retinoblastoma. A careful assessment of the pathways that are deregulated in murine retinoblastomas is essential to make an accurate evaluation of the efficacy of novel therapeutics in preclinical studies focused on selecting the most promising new drugs for testing in clinical trials.

Understanding the intra- and interspecies differences is paramount when choosing the right model for the study of retinoblastoma. Unlike orthotopic xenograft models, genetic mouse models present the advantage of understanding the developmental processes that support tumor formation arising from a single cell and provide an ideal tool for genetic characterization of the contribution of individual pathways in retinoblastoma progression. On the other hand, orthotopic xenografts from patients faithfully recapitulate the mechanisms by which gene deregulation occurs in the human disease and can be more accurate for testing novel therapeutics. As a result, orthotopic xenografts are an important tool for target identification and target validation during preclinical trials, and GEMMs are important for understanding tumor initiation and progression in the developing retina.

Retinoblastoma Translational Research

Worldwide retinoblastoma is diagnosed in approximately 8000 children each year, including 250 to 300 patients in the United States. In developed countries more than 90% of patients with localized retinoblastoma disease are cured using a combination of chemotherapy, focal therapies, surgery (enucleation), and radiation therapy.[80-85] However many patients in developing countries present with advanced intraocular or metastatic disease. Although mortality is low with aggressive multimodal therapy, partial or full loss of vision occurs in approximately 50% of patients with advanced bilateral retinoblastoma.[86] In addition, significant late effects of therapy occur, including facial deformities and increased incidence of secondary malignancies.[87,88] A vigorous effort is now under way to develop targeted therapies to improve ocular salvage and vision preservation and reduce late effects of therapy without compromising therapeutic outcomes.[63,89]

Major advances have recently been made in understanding the biology of retinoblastoma, including identification of several molecular targets such as the inhibition of the MDM4-p53 interaction, SYK, histone deacetylase (HDAC), and the B-cell lymphoma–2 (BCL2) family of proteins. MDM4 is overexpressed in most human retinoblastomas and has been shown to contribute to tumor formation by suppressing p53. Consequently specific inhibition of the MDM4-p53 interaction represents a promising drug target for retinoblastoma treatment. The small-molecule MDM2/MDM4 antagonist nutlin-3a has been shown to efficiently induce p53-mediated cell death in retinoblastoma cells and mouse models of retinoblastoma.[79] Subsequent characterization of the genetic and epigenetic landscapes of retinoblastoma revealed profound increases in expression of the protooncogene *SYK*. Although *SYK* is not expressed in the normal human retina, it is upregulated in all retinoblastomas analyzed to date.[63] *SYK* is also expressed at high levels in metastatic and posttreatment retinoblastomas (Brennan and Bahrami, unpublished data). Many small-molecule *SYK* inhibitors with diverse physiochemical properties are in development for rheumatoid arthritis and oncology applications, and some of these novel therapeutics may eventually benefit patients with retinoblastoma.[90-93] Moreover, previous studies of *SYK* inhibition have implicated a number of downstream signaling molecules, including the BCL2 family of proteins, as mediators of the *SYK* survival signal.[94] Thus small-molecule BCL2 inhibitors currently in development for other cancers, such as Obatoclax, ABT-737, and TW-37,[95] may also prove to be effective therapeutic agents for retinoblastoma. Retinoblastomas are also highly sensitive to HDAC inhibition, and many HDAC inhibitors are currently in clinical trials.[96]

Retinoblastoma is unique because several well-established routes of drug delivery exist that provide flexibility when considering novel therapeutics. Systemic routes can be used for some drugs but are typically limited by poor penetration across the blood-retinal barrier (BRB) and systemic toxicity concerns.[89] Local ocular delivery routes include topical (transcorneal), periocular (transscleral), intravitreal (direct injection) and intraarterial infusion. However all routes are not amenable to all compounds because of the physiochemical property of the compounds, the effects on normal tissues, and the schedule of drug administration. Some chemotherapy drugs such as topotecan effectively cross the BRB, and equivalent intraocular pharmacokinetic (PK) profiles result from either systemic or local topotecan.[77,97] Topotecan is routinely administered daily for 5 days in children with cancer, providing repeat tumor exposure in an outpatient setting. In contrast carboplatin and nutlin-3a[79,98] cannot be administered systemically because of insufficient penetration of the BRB, and in these cases, subconjunctival injections have been a significantly more effective delivery route. However subconjunctival injections are only performed during examination with use of an anesthetic once every 3 weeks, precluding the opportunity for repeat dosing of the drug. Moreover reports of significant and dose-limiting local ocular toxicities of carboplatin have prevented widespread use of subconjunctival delivery for children with retinoblastoma. These studies highlight the importance of pharmacokinetics and toxicokinetics in drug-specific route selection for intraocular targets.

Because of the young age of the patient population and the relatively rarity of the disease, preclinical testing is a critical component of developing retinoblastoma therapeutics. A standardized approach for evaluating efficacy and safety using mouse genetic models of retinoblastoma and human orthotopic xenograft models has been developed, but the large number of potential compounds, routes, and formulations precludes preclinical testing of all possible combinations. Previous work suggests that, given the unique challenges of drug delivery to the eye, one way to streamline the candidate selection process is to determine which route and formulation achieve optimal PK before conducting preclinical studies. When administered using the routes and formulations that, according to PK studies, achieved target intraocular exposure, topotecan, nutlin-3a, and carboplatin were all efficacious. In contrast, despite promising in vitro cytotoxicity, subconjunctival delivery of a SYK inhibitor failed to provide efficacy in preclinical studies; this failure was related to insufficient exposure. However alternative formulations and routes of local delivery did improve PK compared with systemic delivery; intraocular concentration never reached levels required for cytotoxicity.

In conclusion, effective translational research for retinoblastoma is built on a foundation of outstanding basic research to advance our understanding of the pathways that are deregulated in this devastating childhood cancer. From there, suitable targets for therapy are identified, such as MDM4, HDAC, or SYK, and small molecules that disrupt signaling through those pathways are identified and tested for in vitro cellular effects. Next pharmacokinetic, pharmacodynamic, and toxicokinetic studies are combined with approaches to optimize formulation of the drugs for the different routes of delivery. Finally when suitable exposure is achieved with minimal toxicity, comprehensive preclinical testing using validated preclinical models is performed to compare efficacy of the new combination with current standard of care and other possible novel therapeutic approaches. Variations in schedule and integration with upfront standard of care therapy can also be tested at this stage to achieve an optimal route, dose, and schedule for a new retinoblastoma clinical trial.

GENETIC COUNSELING FOR FAMILIES WITH RETINOBLASTOMA

Retinoblastoma is a unique neoplasm in that the genetic form imparts a predisposition to developing tumor in an autosomal-dominant fashion with almost complete penetrance (85% to 95%).[99] The majority of such children acquire the first mutation as a new germline mutation, with only 15% to 25% having a positive family history. However some families display an inheritance pattern characterized by reduced penetrance and expressivity.[26] These low-penetrance retinoblastoma mutations either cause a reduction in the amount of normal protein produced or result in a partially functional mutant protein.[100] An MDM2 polymorphism has also been shown to modify the clinical penetrance of the RB1 mutation, either by enhancing RB1 haploinsufficiency or by increasing resistance to p53-mediated apoptosis.[101] Also the *RB1* gene mutation can occur at a late stage of embryogenesis, resulting in a variable expression depending on the tissue, causing mosaicisms in 10% to 15% of the patients or their progenitors.[102] In general, however, based on the inheritance pattern and considering the existence of mosaicisms, the following risk estimates can be made,[99] which may be refined on the basis of DNA sequence analysis of the *RB1* gene in the patient with retinoblastoma and his or her offspring or siblings.

1. Risk for offspring of survivors of retinoblastoma:
   a. The risk of retinoblastoma arising in the offspring of survivors of bilateral disease is 45%.
   b. In the case of patients with unilateral disease, investigators have estimated the risk of retinoblastoma overall to be 2.5%. However this estimate includes offspring of survivors of unilateral retinoblastoma who have a positive family history and whose risk is similar to that of bilateral cases—45%. If the family history is negative and genetic screening has not been performed, the actual risk is probably less than 2%.
2. Risk for siblings of patients with retinoblastoma: In the case of siblings of bilaterally affected children whose parents are also affected, the risk of developing retinoblastoma is 45%; if the sibling is unilaterally affected, the risk is 30%. For cases without a family history, the empirically derived risk is 2% for siblings of bilateral cases and 1% for siblings of unilateral cases.

Genetic counseling is of the utmost importance to assist parents in understanding the genetic consequences of each form of retinoblastoma and to estimate the risk in relatives. Counseling is relatively straightforward when a parent is affected, or when the child presents with bilateral disease; these patients all have the heritable form. For children without an affected parent who have only a single tumor, there is always a question about whether they carry the mutated gene. Children with unilateral disease who are older than 2 years at the time of diagnosis are not likely to be gene carriers, but we recommend that all children with retinoblastoma be screened for mutations in *RB1,* regardless of laterality and age at diagnosis. With the refinement in methods of mutational analysis during the past decade, detection rates have increased from 20% to 30%,[103,104] to 70% to 80%,[105,106] to greater than 90% at present.[26] Given the heterogeneity in the site and type of gene defects, no single technology will be sensitive and effective, and a multistep approach must be taken. More than 80% of the mutations can be detected with sequencing of the 27 exons of the *RB1* gene using a quantitative multiplex polymerase chain reaction.[26,105] However 10% to 20% of the defects are due to large deletions,[104,105] and therefore deletion scanning and Southern blotting is required for cases with no detectable mutations by quantitative multiplex polymerase chain reaction. Finally a small proportion of cases (probably less than 5%) may result from gene inactivation by promoter methylation, and therefore screening for constitutional[26,105] methylation should be considered if the other methods do not reveal a mutation. Finally 1% to 2% of retinoblastomas have germline *RB1,* and in these cases *MYCN* amplification and chromothripsis of the *RB1* gene may be the initiating events.[27]

With the recent improvements in the detection rates, genetic testing could be performed in the offspring of retinoblastoma survivors who are known to have the mutated gene (detection of the mutation is relatively easily accomplished if the parental mutation is known), and screening can be tailored appropriately. Even if genetic screening is negative for the mutation, newborn siblings of children with retinoblastoma should be examined periodically until they are about 2 years of age, because inherited retinoblastoma would be extremely rare beyond that age. In the future if the false-negative rate is negligible, one might expect that molecular diagnosis of mutations would lead to earlier treatment and better health outcomes for patients with retinoblastoma, with lower cost than conventional surveillance for children at risk.[26]

PATHOLOGY OF RETINOBLASTOMA

Retinoblastoma arises from the photoreceptor elements of the inner layer of the retina and usually extends into the vitreous cavity as a fleshy nodular mass (endophytic retinoblastoma).[107] Less frequently it extends externally, causing a secondary retinal detachment, in which case there is no localized visible vitreous nodule (exophytic retinoblastoma). Macroscopically retinoblastoma is soft and friable and it tends to outgrow its blood supply, with resulting necrosis and calcification. Because of its friability, dissemination within the vitreous and retina in the form of small, white nodules (seeds) is common. In those cases it may be difficult to distinguish a multicentric primary tumor from a disseminated tumor.

Microscopically the appearance of retinoblastoma depends on the degree of differentiation. Undifferentiated retinoblastoma is composed of small, round, densely packed cells with hypochromatic nuclei and scant cytoplasm. Several degrees of photoreceptor differentiation have been described and are characterized by distinctive arrangements of tumor cells. Homer-Wright rosettes are composed of irregular circlets of tumor cells arranged around a tangle of fibrils with no lumen or internal limiting membrane. They are infrequently seen in retinoblastoma and are most often seen in other neuroblastic tumors such as neuroblastoma and medulloblastoma. Flexner-Wintersteiner rosettes, on the other hand, are specific for retinoblastoma. These structures consist of a cluster of low columnar cells arranged around a central lumen that is bounded by an eosinophilic membrane analogous to the external membrane of the normal retina. The lumen contains an acid mucopolysaccharide similar to that found around normal rods and cones. These rosettes are seen in 70% of tumors. The fleurettes are less often seen. In this case the cells exhibit even more ultrastructural characteristics of photoreceptor differentiation. They are composed of larger cells with abundant eosinophilic cytoplasm arranged in a distinctive fleur de lis pattern. Especially well-differentiated tumors composed almost entirely of fleurettes have been called retinomas or retinocytomas. Ultrastructurally retinoblastoma cells also demonstrate photoreceptor differentiation with the presence of the 9-0 microtubule doublet pattern, abundant cytoplasmic microtubules, synaptic ribbons, and neurosecretory granules.[108,109]

Dissemination of retinoblastoma occurs via several routes. Choroidal invasion provides access to a rich vascular network that serves as a potential route for distant metastases. In advanced cases direct extension occurs through the sclera into the orbit. Retinoblastoma can also invade the iris and the ciliary body and metastasize to the regional lymph nodes. Finally retinoblastoma can extend along the optic nerve, gaining access to the subarachnoid space and intracranial cavity. In cases of disseminated disease, positive cone-rod homeobox (CRX) staining may help differentiate retinoblastoma from other common small, blue, round cell malignancies of childhood.[110] The typical macroscopic and microscopic characteristics of retinoblastoma are depicted in Figure 56-1. Guidelines for processing and evaluation of the enucleated eye have been published.[111]

CLINICAL MANIFESTATIONS OF RETINOBLASTOMA

Retinoblastoma is by definition a tumor of the young child, and the age at presentation correlates with laterality. Patients with bilateral retinoblastoma tend to present at a younger age (usually before 1 year) than do patients with unilateral disease (often in the second or third year of life).[99,112] Half of the cases of retinoblastoma diagnosed during the first year are bilateral, compared with fewer than 10% of cases diagnosed after 1 year of age.[1] It is rare for retinoblastoma to be diagnosed during the first month of life, except in familial cases in which examination has been recommended early; however, regardless of the family history, in more than 90% of neonatal cases, either bilateral disease is present at presentation or asynchronous bilateral retinoblastoma will develop.[107]

In more than half of cases the presenting sign is leukocoria, which occasionally is first noticed after a flash photograph (Fig. 56-2). Strabismus, the second most common presenting sign, usually correlates with macular involvement. Very advanced intraocular tumors may become painful as a result of secondary glaucoma.[112] Other childhood diseases that can present with leukocoria must be considered, such as persistent hyperplastic primary vitreous, retrolental fibrodysplasia, Coats disease, congenital cataracts, toxocariasis, and toxoplasmosis. In some series these nonmalignant conditions account for a significant proportion of enucleated eyes.[113,114] In familial cases, the diagnosis is usually made through screening, although almost 50% of familial cases are diagnosed later in life, when patients present with the typical signs of retinoblastoma, underscoring the importance of genetic counseling.[112]

The successful management of retinoblastoma depends on the ability to detect the disease while it is still intraocular. Disease stage correlates with delay in diagnosis.[115,116] In developing countries, late referrals are strongly associated with orbital and metastatic disease (Fig. 56-2).[116-118] For this reason, eye assessment should be performed in all newborns and at all subsequent health supervision visits by the primary care provider.[119] Eye assessment is encouraged in all newborns and at subsequent health visits. The consensus guidelines by the American Academy of Pediatrics and Bright Futures for Pediatric Preventive Care include health supervision visits at birth and at 3 to 5 weeks of life, at 1, 2, 4, 6, 9, and 12 months during the first year, at 15, 18, and 24 months during the second year, and at 30 months and 3, 4, and 5 years of life. Importantly evaluation of vision and ocular health, including red reflex examination, is a component of each health supervision visit. Mass screening is also being considered, especially where the tumor presents in advanced stages, such as in areas of South America and Asia. Photoscreening is a system by which a photograph is produced by a calibrated camera under prescribed lighting conditions, which shows a red reflex in both pupils. A trained observer can identify ocular abnormalities by recognizing characteristic changes in the photographed pupillary reflex. This technique is fast, efficient, and reproducible, but it is still evolving.[120]

Most patients with bilateral retinoblastoma carry a germline mutation of the *RB1* gene. However a small proportion (5% to 6%) carry a deletion involving the 13q14 locus, which is large enough to be detected by karyotype analysis, either as a deletion or as part of a balanced translocation, most typically t(X;13).[121] In those cases retinoblastoma is part of a more complex syndrome resulting from the loss of additional genetic material. Patients with the 13q deletion syndrome are characterized by typical facial dysmorphic features, subtle skeletal abnormalities, and different degrees of mental retardation and motor impairment.[122-124] Dysmorphic features more consistently found include thick anteverted ear lobes, a high and broad forehead, a prominent philtrum, and a short nose.[122,123] A proportion of patients also

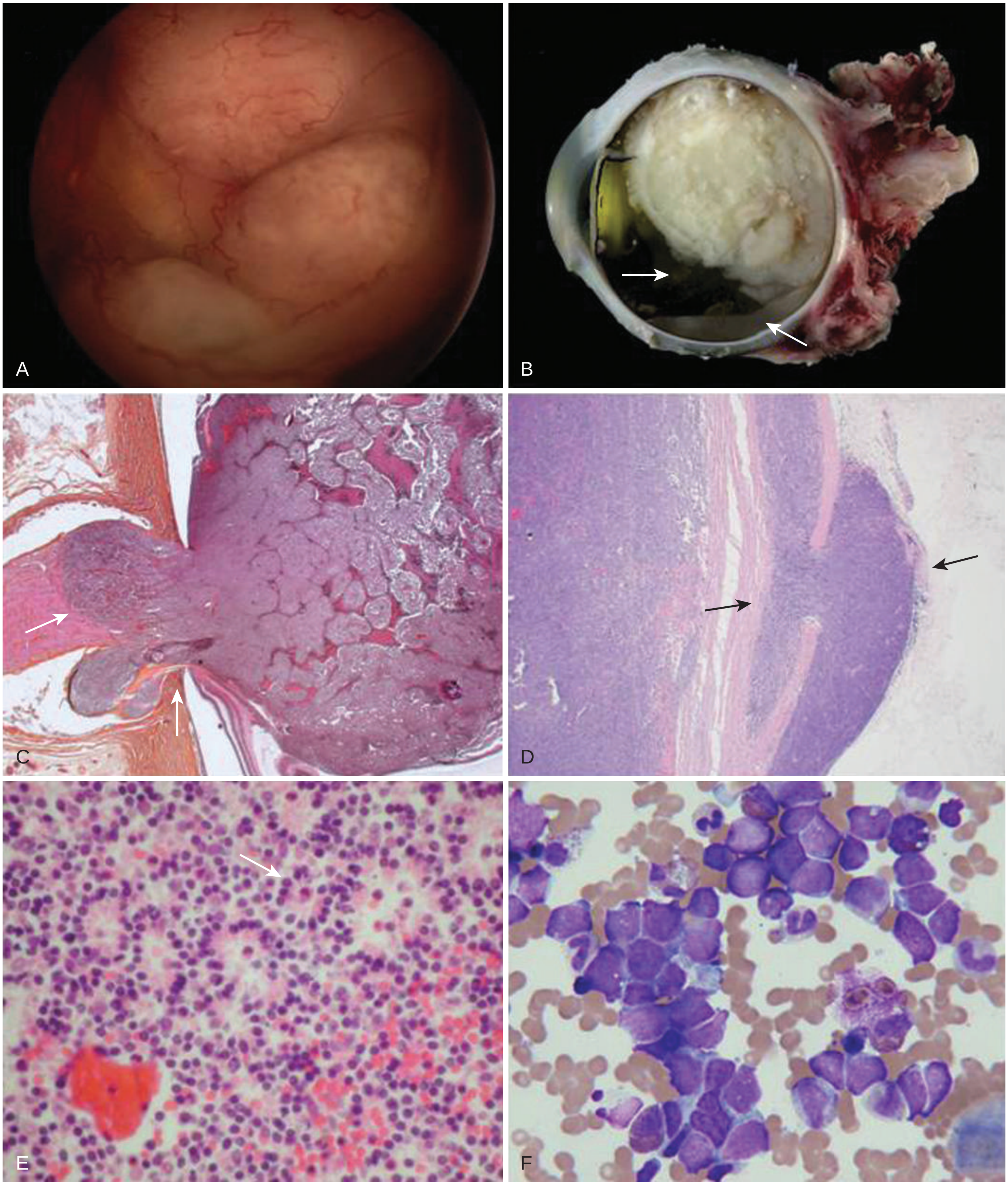

Figure 56-1 Pathology of retinoblastoma. **A,** Dilated eye examination of a group E retinoblastoma. **B,** Gross appearance of an enucleated eye showing a large retinoblastoma filling the posterior chamber, vitreous seeds and retinal detachment *(arrows)*. **C,** Retinoblastoma invasion of the optic nerve *(arrow)*. **D,** Transscleral invasion *(arrows)*. **E,** Microscopic appearance of retinoblastoma showing distinctive rosettes *(arrow)*. **F,** Metastatic retinoblastoma to the bone marrow.

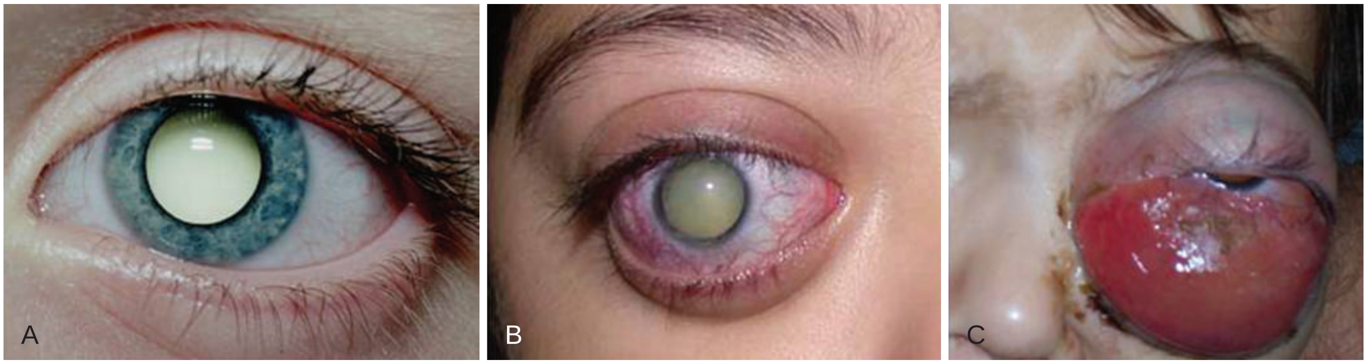

Figure 56-2 Clinical presentation of retinoblastoma showing progressive delay in diagnosis and referral. Retinoblastoma usually presents as leukocoria (**A**), but as disease advances, patients present with buphthalmos (**B**) and extraocular disease (**C**).

have overlapping fingers and toes, microcephaly, and delayed skeletal maturation. The severity of the deficits correlate with the size of the deletion; normal psychomotor development may be seen in patients in whom the deletion is restricted to the 13q14 band.[122]

Trilateral retinoblastoma refers to the association of bilateral retinoblastoma with an asynchronous intracranial tumor (Fig. 56-3).[125-127] Tumors comprising trilateral retinoblastoma are primitive neuroectodermal tumors (PNETs) exhibiting varying degrees of neuronal or photoreceptor differentiation, suggesting an origin from the germinal layer of primitive cells.[128] This association can occur in 3% to 9% of patients with the heritable form and appears to be more common in familial cases. Until recently the prognosis has been almost uniformly fatal; trilateral retinoblastoma has been the principal cause of death from retinoblastoma during the first decade of life in the United States.[129] The majority of these tumors are pineal region PNETs (pineoblastomas), but in 20% to 25% of cases, the tumors are suprasellar or parasellar. Rare cases of quadrilateral retinoblastoma have been reported, in which bilateral retinoblastoma is associated with both pineal region and suprasellar intracranial primary PNETs.[130] In most cases, the intracranial PNETs in association with retinoblastoma resemble undifferentiated retinoblastomas with the more frequent formation of Homer-Wright rosettes. The median age at diagnosis of trilateral retinoblastoma is 23 to 48 months,[125,126,129,131] and the interval between the diagnosis of bilateral retinoblastoma and the diagnosis of the brain tumor is usually more than 20 months.[127,131] Suprasellar tumors are usually diagnosed earlier,[127] and in 15% to 20% of cases, the intracranial tumor antecedes the diagnosis of retinoblastoma.[126] In recent years with the more widespread use of chemoreduction treatments and the decrease in the use of radiation therapy for patients with bilateral retinoblastoma, the incidence of trilateral retinoblastoma has decreased dramatically.[132,133] However pineal cysts

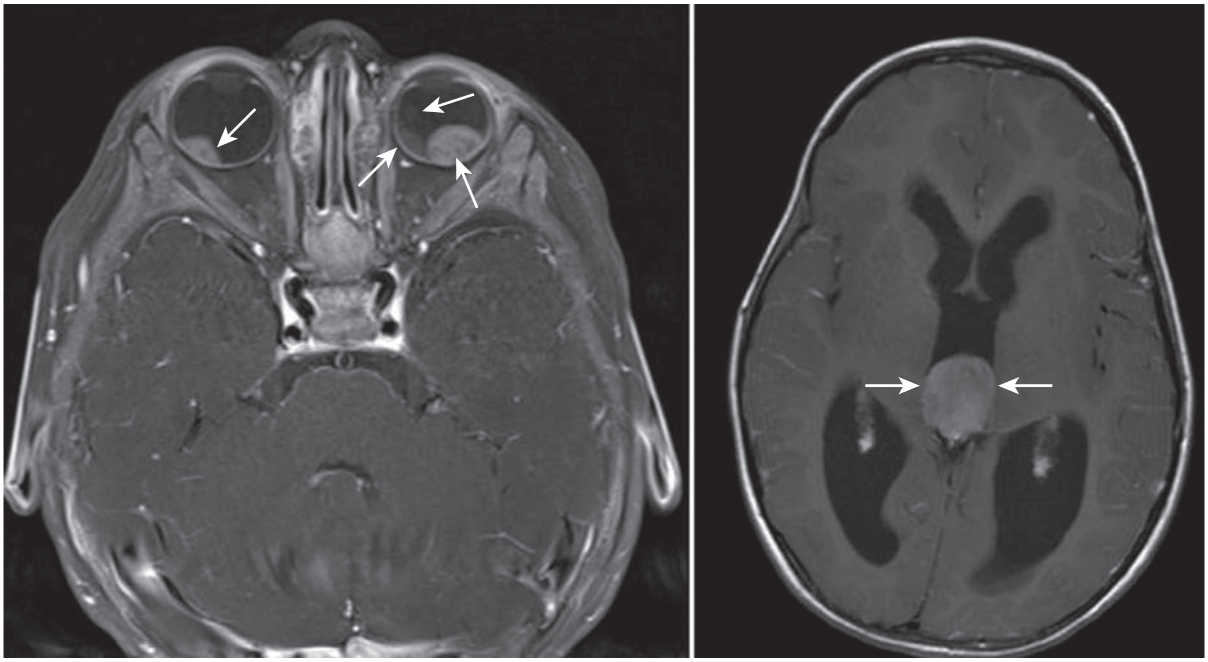

Figure 56-3 **A,** An axial T1-weighted magnetic resonance image showing bilateral intraocular masses in an 8-month-old infant with retinoblastoma *(arrows)*. **B,** A pineal gland tumor *(arrow)* in a 3-year-old child with a history of bilateral retinoblastoma and presenting with hydrocephalus.

develop in approximately 5% to 8% of patients with bilateral disease; these cysts appear to be a forme fruste of trilateral retinoblastoma.[133,134,135]

DIAGNOSIS AND EXTENT OF DISEASE EVALUATIONS IN RETINOBLASTOMA

The diagnosis of intraocular retinoblastoma is usually made without pathologic confirmation. An examination with use of an anesthetic with a maximally dilated pupil and scleral indentation is required to examine the entire retina. A careful examination of the iris and the anterior chamber is first performed, and the intraocular pressure is measured. Retinoblastoma usually appears as a mass projecting into the vitreous, although the presence of retinal detachment or vitreous hemorrhage may make its visualization difficult. Endophytic tumors are those that grow inward to the vitreous cavity. Because of its friability, endophytic retinoblastoma may seed the vitreous cavity (Fig. 56-4, *D*). Exophytic retinoblastoma grows into the subretinal space, thus causing progressive retinal detachment and subretinal seeding (Fig. 56-4, *C*). Exophytic tumors frequently resemble Coats disease. Less frequently retinoblastoma can adopt an infiltrative pattern without an obvious mass; this infiltrative pattern appears to be more frequent among older children. A very detailed documentation of the number, location, and size of tumors, the presence of retinal detachment and subretinal fluid, and the presence of vitreous and subretinal seeds must be performed. Wide-angle real-time retinal imaging systems such as RetCam (Clarity Medical Systems, Pleasanton, Calif.) provide a 130-degree field of view and digital recording, facilitating diagnosis and monitoring.

Additional imaging studies that aid in the diagnosis include bidimensional ultrasound, computerized tomography (CT), and magnetic resonance imaging (MRI). These imaging studies are particularly important to evaluate extraocular extension and to differentiate retinoblastoma from other causes of leukocoria. CT is very helpful to detect calcifications, although it is generally avoided to limit radiation exposure, particularly in children with bilateral disease, and MRI is very helpful in considering the differential diagnosis of Coats disease and other inflammatory conditions, along with persistent fetal vasculature of hyperplastic primary vitreous (Fig. 56-3).[136] In the absence of extraocular disease, MRI is not useful in the estimation of microscopic or optic nerve involvement.[137-139]

Evaluation for the presence of metastatic disease also needs to be considered in a subgroup of patients. Metastatic disease occurs in approximately 10% to 15% of patients, and it usually occurs in association with distinct intraocular histologic features, such as massive choroidal and scleral invasion, or with involvement of the iris or ciliary body and optic nerve beyond the lamina cribrosa.[140] In these cases additional staging procedures must be performed, including bone scintigraphy, bone marrow aspirates and biopsies, and lumbar puncture. In as many as one third of high-risk patients, the synthase of ganglioside GD2 messenger ribonucleic acid may be detected in the cerebrospinal fluid by reverse transcriptase-polymerase chain reaction, and it appears to correlate with massive involvement of the optic nerve, the presence of glaucoma at diagnosis, and a high risk of cerebrospinal fluid relapse.[141] In general in the absence of high-risk disease in patients undergoing enucleation, and in patients with intraocular disease who are undergoing ocular salvage therapies, a metastatic workup is usually not necessary. In patients with extraocular disease the use of immunocytology with GD2 or CRX staining may increase the yield for detection of small clumps of metastatic cells.[142,110]

STAGING OF RETINOBLASTOMA

The Reese-Ellsworth (R-E) grouping system has been generally accepted as the standard for intraocular disease. This grouping system was initially designed to predict the outcome after external beam radiation therapy (EBRT). It divides eyes into five groups on the basis of the size, location, and number of lesions and the presence of vitreous seeding (Box 56-1).[143] However developments in the conservative management of intraocular retinoblastoma have made the R-E grouping system less predictable of eye salvage and less helpful in guiding treatment.[85,144] A new staging system (International Classification of Retinoblastoma) has been developed, with the goal of providing a simpler, more user-friendly classification that is more applicable to current therapies. This new system is based on the extent of tumor seeding within the vitreous cavity and subretinal space rather than on tumor size and location, and it seems to be a better predictor of treatment success (Box 56-2; Fig. 56-4).[144,145]

For patients undergoing enucleation, pathologic staging that incorporates other features known to influence the modality of treatment and the prognosis, such as choroidal and scleral involvement, optic nerve extension, and the presence of metastatic disease, is used.

Box 56-1 **Reese-Ellsworth Grouping for Suitability for Treatment of Retinoblastoma by Radiation Therapy**

GROUP I. VERY FAVORABLE

Ia. Solitary tumor smaller than 4 dd at or behind the equator
Ib. Multiple tumors, none larger than 4 dd, all at or behind the equator

GROUP II. FAVORABLE

IIa. Solitary tumor, 4 to 10 dd, at or behind the equator
IIb. Multiple tumors, 4 to 10 dd, at or behind the equator

GROUP III. DOUBTFUL

IIIa. Any lesion anterior to the equator
IIIb. Solitary tumor larger than 10 dd behind the equator

GROUP IV. UNFAVORABLE

Iva. Multiple tumors, some larger than 10 dd
IVb. Any lesion extending anteriorly to the ora serrata

GROUP V. VERY UNFAVORABLE

Va. Massive tumors involving more than half the retina
Vb. Vitreous seeding

From Reese AB, Ellsworth RM: The evaluation and current concept of retinoblastoma therapy. Trans Am Acad Ophthalmol Otolaryngol *67:164–172, 1963.*

dd, Disk diameter.

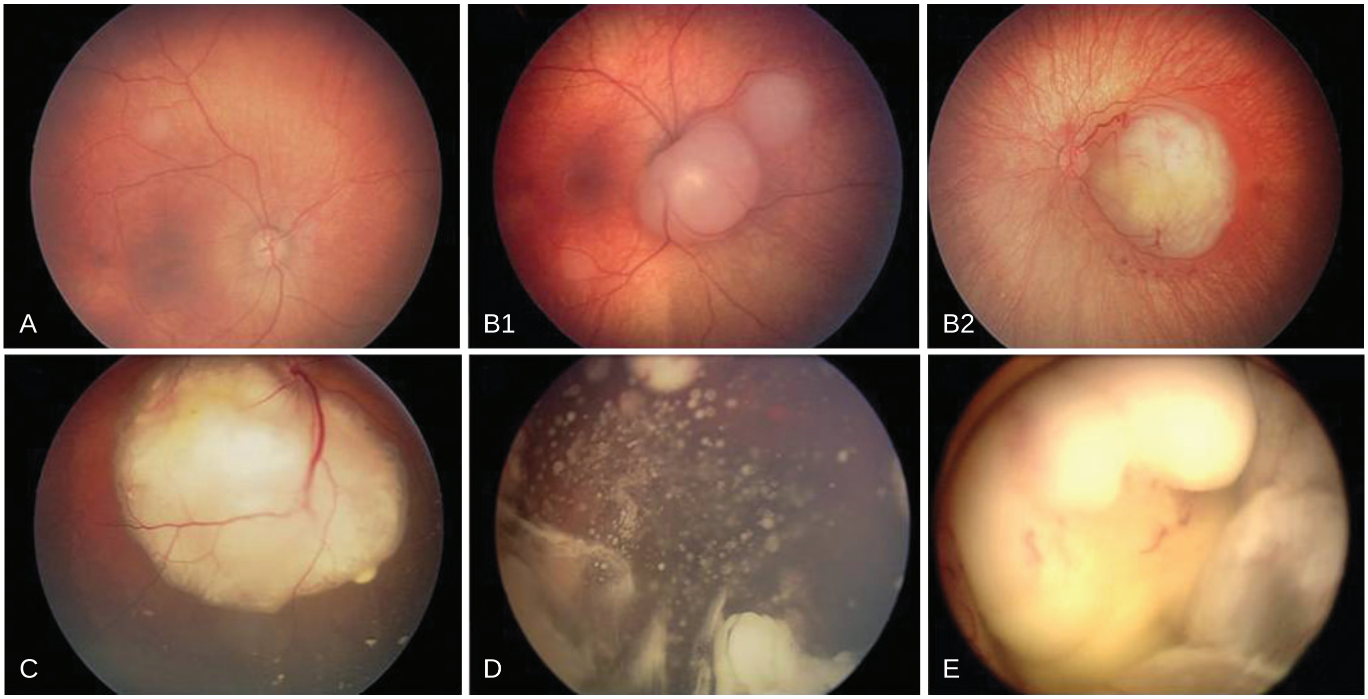

Figure 56-4 International Classification for Intraocular Retinoblastoma. **A,** A small tumor confined to the retina and distant from the foveola and the optic nerve (group A). **B1,** Two small tumors confined to the retina but adjacent to the optic nerve (group B). **B2,** Tumor with a small amount of subretinal fluid and no subretinal seeding (group B). **C,** Exophytic retinoblastoma with subretinal fluid and seeding (group C). **D,** Endophytic retinoblastoma with massive vitreous seeding (group D). **E,** Large retinoblastoma filling more than two thirds of the globe (group E).

Growth and invasion occur as a sequence of events, and extraretinal extension occurs only after the tumor has reached large intraocular dimensions.[146] As part of this process, retinoblastoma extends into the ocular coats (choroid and sclera), the optic nerve, and the anterior segment. Extraocular disease is the next step in this progression; locoregional dissemination occurs by direct extension through the sclera into the orbital contents and preauricular lymph nodes, and extraorbital disease manifests as intracranial dissemination and hematogenous metastases. Different staging systems have been classically used, including the Grabowski-Adamson,[147] the St. Jude Children's Research Hospital,[148] the American Joint Committee on Cancer (AJCC),[149] and the International Retinoblastoma Staging System (IRSS).[150] The IRSS is a newly proposed staging system developed by an international consortium of ophthalmologists and pediatric oncologists that incorporates the most important elements of the older systems (Box 56-3). The IRSS (Box 56-3) and AJCC (Box 56-4) systems appear to be the most reliable for grouping patients according to their risk of extraocular relapse.[151]

Although the clinical significance of extraocular extension is obvious, there is no uniform agreement on the prognostic implications of the different histologic characteristics, resulting in the use of different staging

Box 56-2 **International Classification for Intraocular Retinoblastoma**

GROUP A

SMALL TUMORS AWAY FROM THE FOVEOLA AND DISC

- Tumors ≤3 mm in the greatest dimension confined to the retina, *and*
- Located at least 3 mm from the foveola and 1.5 mm from the optic disc

GROUP B

ALL REMAINING TUMORS CONFINED TO THE RETINA

- All other tumors confined to the retina not in group A
- Subretinal fluid (without subretinal seeding) ≤3 mm from the base of the tumor

GROUP C

LOCAL SUBRETINAL FLUID OR SEEDING

- Local subretinal fluid alone >3 to ≤6 mm from the tumor
- Vitreous seeding or subretinal seeding ≤3 mm from the tumor

GROUP D

DIFFUSE SUBRETINAL FLUID OR SEEDING

- Subretinal fluid alone >6 mm from the tumor
- Vitreous seeding or subretinal seeding >3 mm from the tumor

GROUP E

PRESENCE OF ANY OR MORE OF THESE POOR PROGNOSIS FEATURES

- More than two thirds of the globe is filled with tumor
- Tumor in the anterior segment
- Tumor in or on the ciliary body
- Iris neovascularization
- Neovascular glaucoma
- Opaque media from hemorrhage
- Tumor necrosis with aseptic orbital cellulitis
- Phthisis bulbi

From Linn Murphree AL: Intraocular retinoblastoma: the case for a new group classification. Ophthalmol Clin N Am *18:41–53, 2005.*

Box 56-3 International Retinoblastoma Staging System

Stage 0. Patients treated conservatively
Stage I. Eye enucleated, completely resected histologically
Stage II. Eye enucleated, microscopic residual tumor
Stage III. Regional extension
  a. Overt orbital disease
  b. Preauricular or cervical lymph node extension
Stage IV. Metastatic disease
  a. Hematogenous metastasis (without CNS involvement)
    1. Single lesion
    2. Multiple lesions
  b. CNS extension (with or without any other site of regional or metastatic disease)
    1. Prechiasmatic lesion
    2. CNS mass
    3. Leptomeningeal and CSF disease

From Chantada G, Doz F, Antonelli CBG, et al. A proposal for an international retinoblastoma staging system. Pediatr Blood Cancer *47:801–5, 2006.*
CNS, Central nervous system; *CSF,* cerebrospinal fluid.

systems. Even in the absence of extraocular disease, a variable risk of developing metastatic disease exists.[152-160] Many studies have attempted to evaluate the risk associated with the different histologic variables. Two thirds of patients have exclusive retinal disease, and invasion of the anterior segment, choroid, and optic nerve occur in variable proportions and combinations in the remaining patients. Extension of tumor into the sclera and across the line of transection of the optic nerve are associated with elevated mortality and by definition are considered to represent extraocular disease.[157,159,160] The question arises when interpreting the other variables, because the risk associated with each variable is confounded by the lack of standardized grading methods and biased by the use of adjuvant chemotherapy.

Optic nerve involvement is common (occurring in 25% to 45% of all cases), but its impact on outcome appears to be limited to the involvement beyond the lamina cribrosa (where the meninges insert) and to the extension up to the transection line.[152,153,159-164] The mortality rates for untreated patients with those features are 40% to 60% and greater than 80%, respectively.[153-156,159,160]

Choroidal involvement is found in up to 40% of patients, although massive invasion occurs in fewer than 10% of cases.[156,158,161,164,165] This distinction is important, because although choroidal invasion might have prognostic implications,[140,152,162,166] its impact appears to be limited to cases with massive replacement by tumor.[158,161] However contrary to optic nerve evaluation, criteria for determining the extent of choroidal disease are more subjective, and the grading of invasion is seldom reported. Consensus criteria for definition of massive or significant choroidal invasion include a maximum diameter (thickness or width) of invasive focus of tumor of 3 mm or more in any diameter and reaching the inner fibers of the sclera. A tumor focus of less than 3 mm and not reaching the sclera is considered focal choroidal invasion.[111]

Evaluating the risk for each histologic variable individually is insufficient; different combinations of simultaneously occurring factors are very frequent.[146,167] When choroidal invasion is present, half the cases will also show optic nerve involvement, whereas optic nerve invasion is uncommon (20%) if the choroid is intact.[152,155,168] Conversely, when the optic nerve is invaded, 30% to 40% of cases will have choroidal replacement (more than 80% if the tumor has spread to the transection line), but significant choroidal invasion is quite rare (less than 20%) if the optic nerve is free of tumor.[152,154,155,167,168] Therefore the risk associated with each histologic variable can only be estimated in the light of its association with others. Most available data support the notion that choroidal invasion alone is not associated with increased risk of extraocular spread, although massive choroidal replacement by tumor may be an exception.[158,161] Similarly retrolaminar optic nerve invasion appears to be of prognostic significance only when there is concomitant significant choroidal invasion,[155,159,165,168,169] but this represents fewer than 20% of cases.[152,155] The significance of other histologic features, such as invasion of the anterior segment or grade of differentiation, remains unclear.[161,162] The presence of extensive tumor necrosis appears to correlate with high-risk histologic features, such as postlaminar optic nerve involvement and choroidal invasion.[170]

Clinical presentation correlates with disease only in cases of advanced intraocular disease; group E eyes, and those presenting with increased intraocular pressure or buphthalmos, have a higher frequency of high-risk histology.[164,171]

PRINCIPLES OF TREATMENT OF RETINOBLASTOMA

Treatment of retinoblastoma aims to save life and preserve useful vision, and thus it needs to be individualized. Factors that need to be considered include laterality of the disease and *RB1* germline status, potential for preserving vision, and intraocular and extraocular staging. For patients presenting with intraocular disease, particularly those with bilateral eye involvement, a conservative approach consisting of tumor reduction with intravenous or ophthalmic artery chemotherapy coupled with aggressive focal therapy may result in high ocular salvage rates. Radiation therapy, one of the most effective treatments in retinoblastoma, is usually reserved for cases of intraocular or extraocular disease progression.

Surgery

Enucleation is indicated for large tumors filling the vitreous for which there is little or no likelihood of restoring vision, and in cases of tumor present in the anterior chamber or in the presence of neovascular glaucoma. This corresponds to group E eyes and a significant proportion of group D eyes; upfront enucleation should be offered to those patients. Enucleation should be performed by an experienced ophthalmologist. The eye must be removed intact, without seeding the malignancy into the orbit and avoiding globe perforation. For optimal staging, a long section (10 to 15 mm) of the optic nerve needs to be removed with the globe. An orbital implant is usually fitted during the same procedure, and the extraocular muscles are attached to it. In the past, orbital

Box 56-4 **American Joint Committee on Cancer Staging System**

CLINICAL CLASSIFICATION (cTNM)

PRIMARY TUMOR (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumors no more than 2/3 the volume of the eye with no vitreous or subretinal seeding
T1a	No tumor in either eye is greater than 3 mm in largest dimension or located closer than 1.5 mm to the optic nerve or fovea
T1b	At least one tumor is greater than 3 mm in largest dimension or located closer than 1.5 mm to the optic nerve or fovea. No retinal detachment or subretinal fluid beyond 5 mm from the base of the tumor
T1c	At least one tumor is greater than 3 mm in largest dimension or located closer than 1.5 mm to the optic nerve or fovea, with retinal detachment or subretinal fluid beyond 5 mm from the base of the tumor
T2	Tumors no more than 2/3 the volume of the eye with vitreous or subretinal seeding. Can have retinal detachment
T2a	Focal vitreous and/or subretinal seeding of fine aggregates of tumor cells is present, but no large clumps or "snowballs" of tumor cells
T2b	Massive vitreous and/or subretinal seeding is present, defined as diffuse clumps or "snowballs" of tumor cells
T3	Severe intraocular disease
T3a	Tumor fills more than 2/3 of the eye
T3b	One or more complications present, which may include tumor-associated neovascular or angle closure glaucoma, tumor extension into the anterior segment, hyphema, vitreous hemorrhage, or orbital cellulitis
T4	Extraocular disease detected by imaging studies
T4a	Invasion of optic nerve
T4b	Invasion into the orbit
T4c	Intracranial extension not past chiasm
T4d	Intracranial extension past chiasm

REGIONAL LYMPH NODES (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node involvement
N1	Regional lymph node involvement (preauricular, cervical, submandibular)
N2	Distant lymph node involvement

METASTASIS (M)

M0	No metastasis
M1	Systemic metastasis
M1a	Single lesion to sites other than CNS
M1b	Multiple lesions to sites other than CNS
M1c	Prechiasmatic CNS lesion(s)
M1d	Postchiasmatic CNS lesion(s)
M1e	Leptomeningeal and/or CSF involvement

PATHOLOGIC CLASSIFICATION (pTNM)

PRIMARY TUMOR (pT)

pTX	Primary tumor cannot be assessed
pT0	No evidence of primary tumor
pT1	Tumor confined to eye with no optic nerve or choroidal invasion
pT2	Tumor with minimal optic nerve and/or choroidal invasion:
pT2a	Tumor superficially invades optic nerve head but does not extend past lamina cribrosa *or* tumor exhibits focal choroidal invasion
pT2b	Tumor superficially invades the optic nerve head but does not extend past lamina cribrosa *and* exhibits focal choroidal invasion
pT3	Tumor with significant optic nerve and/or choroidal invasion:
pT3a	Tumor invades optic nerve past lamina cribrosa but not to surgical resection line *or* tumor exhibits massive choroidal invasion
pT3b	Tumor invades optic nerve past lamina cribrosa but not to surgical resection line *and* exhibits massive choroidal invasion
pT4	Tumor invades optic nerve to resection line or exhibits extra-ocular extension elsewhere
pT4a	Tumor invades optic nerve to resection line but no extra-ocular extension identified
pT4b	Tumor invades optic nerve to resection line and extra-ocular extension identified

REGIONAL LYMPH NODES (pN)

pNX	Regional lymph nodes cannot be assessed
pN0	No regional lymph node involvement
pN1	Regional lymph node involvement (preauricular, cervical)
N2	Distant lymph node involvement

METASTASIS (pM)

cM0	No metastasis
pM1	Metastasis to sites other than CNS
pM1a	Single lesion
pM1b	Multiple lesions
pM1c	CNS metastasis
pM1d	Discrete mass(es) without leptomeningeal and/or CSF involvement
pM1e	Leptomeningeal and/or CSF involvement

From American Joint Committee on Cancer: Retinoblastoma. In Edge SB, Byrd DR, Compton CC, et al, editors: AJCC Cancer Staging Manual*, ed 7, New York, 2010, Springer, p. 562–63.*

CNS, Central nervous system; *CSF,* cerebrospinal fluid.

implants were avoided because it was believed that they would interfere with the palpation of the socket and clinical detection of orbital recurrence. However with improved understanding of the histologic risk factors and the availability of better imaging techniques to detect orbital disease, implants should be placed at the time of the enucleation. Different orbital implants are available, including polymethylmethacrylate, polyethylene, and coralline and bovine hydroxyapatite spheres. A tissue wrap to the implant is placed, which will allow the four rectus muscles to be anatomically reattached, thus providing implant motility with little resistance in the orbit. The size and type of implant are important to stimulate orbital growth. A ceramic false eye is later fitted in the orbital socket. Orbital exenteration is very seldom indicated, although it should considered in cases of tumor recurrence after radiation. For patients presenting with orbital disease, a judicious use of chemotherapy, surgery, and radiation therapy will result in effective tumor control, avoiding the need for orbital exenteration.

Focal Therapies

Focal treatments are used for small tumors (less than 3 to 6 mm) and in combination with chemotherapy.[172] In this setting of a patient undergoing chemotherapy for cytoreduction, focal treatments should be applied to tumors that fail to calcify; it is usually recommended to allow maximal reduction in tumor size with one or two cycles of chemotherapy prior to proceeding with aggressive focal consolidation to minimize the damage to the surrounding retina, particularly when the tumor is close to visually critical structures, such as the fovea and the optic nerve.[173] The focal therapies available to treat retinoblastoma include argon green laser, diode laser, cryotherapy, and brachytherapy. The use of each modality depends on the tumor location and size. Lasers are more commonly used to treat posterior tumors, whereas more anterior tumors are easily accessed with cryotherapy. Brachytherapy is reserved for tumors that cannot be consolidated with either of those two approaches, usually because of their size.[172]

Two wavelengths of light are used to treat retinoblastoma. The argon green laser, with a wavelength of 532 nm, photocoagulates tissue by inducing a temperature in excess of 65°C; it has been traditionally used to treat the retina edge surrounding a tumor in an effort to deprive the tumor of its blood supply and subsequently induce tumor regression, and for the treatment of retinal neovascularization due to radiation therapy.[172,174] Using an indirect ophthalmoscope, a double row of white burns is used to encircle the tumor, and powers of 250 to 350 mW and burn durations of 0.3 to 0.5 seconds are used.[172,175] This technique is limited to tumors measuring no greater than 4.5 mm in base and no greater than 2.5 mm in thickness. Debate exists as to whether the tumor surface should be directly treated for fear of releasing tumor cells into the vitreous. Newer generations of the 532-nm laser permit continuous delivery similar to that of the diode laser; such modalities have led some persons to advocate its use in the treatment of retinoblastoma.[172] If the tumor surface is treated, the desired end point remains a subtle whitening of the treated areas. Laser energy should be kept at the lowest possible level necessary to achieve the desired end point; powers in excess of 500 mW will result in tumor disruption and should be avoided. As with all focal therapies, treatment should be continued until the residual fish-flesh tumor regresses into a flat chorioretinal scar. Typically treatment with lasers is administered every 3 to 4 weeks. The optimal indication for direct treatment with the argon green laser is a residual fish-flesh mass overlying a largely calcified tumor. In such situations, the uptake of the diode laser is unpredictable. With use of the argon green laser, a whitening of the tumor can be seen, ensuring an adequate treatment. The argon laser has both direct and indirect cytotoxic effects. Photocoagulation directly kills tumor cells, and the thermal spread from the laser may have secondary hyperthermic effects that act synergistically with ongoing chemotherapy. Argon laser photocoagulation should not be performed if there is a retinal detachment overlying the tumor.[172]

The diode laser, with a wavelength of 810 nm, was adapted for the treatment of retinoblastoma based on its success in the treatment of choroidal malignant melanoma. The laser energy is readily absorbed by melanin pigment and proved successful in the treatment of small melanocytic tumors.[172] Low-power settings coupled with a large spot size (2 to 3 mm) and prolonged delivery achieved hyperthermia (45°C to 65°C) at subphotocoagulation temperatures. The end result was a sustained penetrant burn with direct cytotoxic effects.[172] The term *transpupillary thermotherapy*, or TTT, was coined to describe this technique.[176,177] The methods of TTT were subsequently adapted to treat retinoblastoma. The infrared laser is focused directly on the tumor surface using either an ophthalmic microscope adapter or a large spot size indirect ophthalmoscope adapter. When using the indirect ophthalmoscope for TTT, it is important to ensure that the correct adapter is being used, because adapters designed for diode photocoagulation will not provide a continuous wavelength delivery necessary for hyperthermia.[172,178] For small tumors the underlying retinal pigment epithelium aids in absorption and transfer of the heat to the tumor. The desired end point is a subtle whitening of the tumor free of hemorrhage. Powers are titrated based on the tumor size and pigmentation of the underlying retinal pigment epithelium but should not exceed 600 mW when there is visible uptake by the surrounding retinal pigment epithelium. Tumors with significant elevation or residual elements that rest on adjacent calcified tumor do not readily whiten with TTT. Prolonged treatment sessions of 5 to 10 minutes using powers of 600 to 800 mW may be needed to achieve a therapeutic effect. Even with such prolonged treatment, a visible change in the tumor may not be seen until follow-up examination 3 to 4 weeks later. If available the argon green laser may provide a more effective treatment.[172] The sequential administration of thermotherapy with carboplatin enhances the antitumor effect by increasing the platinum-DNA adducts, for which reason thermochemotherapy is becoming a very important component in the treatment of intraocular retinoblastoma.[81,83]

Cryotherapy is used for the treatment of small equatorial and peripheral lesions, measuring no more than 3.5 mm in base and no more than 2 mm in thickness.[172,179,180] Cryotherapy is directly cytotoxic, causing cell lysis by disruption of the cell membrane that occurs after the formation of cytoplasmic ice crystals. Nitrous oxide gas is used to deliver a transscleral freeze via a cryoprobe under direct visualization. The lesion to be treated is visualized with an indirect ophthalmoscope, and the scleral underlying the tumor is indented with a cryoprobe. A succession of three freezes with three intervening thaws is applied to the tumor. An “ice ball” should be seen to encompass the tumor and adjacent vitreous with each freeze. Treatment should be limited to the area of the tumor to minimize damage to adjacent normal retina. Multiple tumors may be treated at one examination, but extensive treatments should be avoided. Aggressive cryotherapy may cause serous retinal detachments and iatrogenic retinal breaks. Although each cryotherapy unit may vary, we target a freezing temperature of −70°C during treatment. Treatments should be repeated at 3- to 4-week intervals until the lesion regresses to a flat chorioretinal scar. Posterior tumors can also be treated with cryotherapy. The conjunctiva is opened in the quadrant of the tumor to be treated. The curved cryoprobe is passed along the curvature of the eye and the tumor is indented. Once the tumor has been identified, the triple freeze-thaw is applied. This “cut-down” cryotherapy is usually reserved for recurrent posterior tumors not amenable to laser treatment.[172] Also in addition to its effect on tumor control, cryotherapy contributes to increase the intraocular penetration of chemotherapy agents, presumably through disruption of the blood-vitreous barrier.[181,182] In general local control rates of 70% to 80% can be achieved.[174,180,83] Complications of focal treatments include transient serous retinal detachment, retinal traction and tears, and localized fibrosis.[179]

Chemotherapy

Chemotherapy is indicated in patients with extraocular disease, in the subgroup of patients with enucleated eyes with intraocular disease and high-risk histologic features, and in patients with bilateral disease in conjunction with aggressive focal therapies. Agents with documented efficacy include microtubule inhibitors (vincristine and paclitaxel), platinum compounds (cisplatin and carboplatin), topoisomerase II inhibitors (teniposide and etoposide), alkylating agents (cyclophosphamide and ifosfamide), anthracyclines (doxorubicin and idarubicin), and topoisomerase I inhibitors (topotecan).[183,184] The first clinical experience with chemotherapy in the treatment of retinoblastoma was reported with the use of nitrogen mustard in 1953.[185] Institutional experiences in the management of extraocular retinoblastoma were reported subsequently, usually applying regimens modeled after the treatment regimens for metastatic neuroblastoma.[186,187] Since then the role of chemotherapy has continued to expand, and it is now a main component in the management of intraocular disease.

Ocular Pharmacokinetics

Application of new therapeutic possibilities for cancer treatment involves drug delivery in many forms, but ocular drug delivery is hampered by the barriers protecting the eye. The eye is protected from the xenobiotics of the bloodstream by blood-ocular barriers. The anterior blood-eye barrier (blood-aqueous barrier) is constituted by the endothelial cells in the uvea. This barrier limits access of hydrophilic drugs from plasma into the aqueous humor; however, inflammation may disrupt the integrity of this barrier, causing unlimited drug distribution to the anterior chamber. The posterior blood-eye barrier (BRB) is formed by the neural retina, the retinal pigment epithelium (RPE), and the tight walls of retinal capillaries. The inner border of the neural retina faces the vitreous, and the outer border is next to the RPE. The neural retina is composed of nine layers, and the RPE is composed of a monolayer of polarized cells. The blood supply to the inner two thirds of the retina is from retinal vessels; the retinal endothelial cells have basal lamina, and they are surrounded by pericytes, thus forming tight junctions. The blood supply to the outer third of the retina and the RPE comes from the choroidal circulation. Unlike the retinal capillaries, the vasculature of the choroid has extensive blood flow and leaky walls; drugs easily gain access to the choroid extravascular space, but thereafter distribution into the retina is limited by the RPE and retinal endothelia.[188,189] Lipophilicity, molecular weight, and protein binding (it is believed that only unbound drugs can pass through the BRB) are the main factors for penetration through the barrier. Inflammation and trauma also have important roles in breaking the BRB by disrupting tight junctions, increasing transendothelial vesicles, and increasing pinocytosis.[190] It is possible that a similar phenomenon occurs in retinoblastoma.

Ocular Drug Transporters

The BRB restricts the movements of substances after systemic and periocular administration to the retina. The tight junctions form the penetration barrier to hydrophilic substances, thereby limiting the transfer of compounds both inward (blood to vitreous) and outward (vitreous to blood). The RPE has many important features essential for normal retinal function, such as transport of nutrients and waste products, regulation of ion, and fluid balance. RPE thus expresses many transporters and channels related to these physiologic functions. Among those the RPE and the endothelial cells also express efflux transporters such as P-glycoprotein, multidrug resistance–associated protein–1, –4, and –5, and breast cancer resistance protein. This efflux protein activity restricts movements of certain drugs to the retina. In most cases this is an important protective system for the retina, but it also limits drug delivery to the posterior eye segment.[188] Many antineoplastic agents are known substrates of efflux proteins.

Mechanisms for Transscleral Drug Delivery

Transscleral drug delivery is a potential solution to the need to achieve high intraocular concentrations of chemotherapeutic agents. Traditionally subconjunctival, subtenon, and retrobulbar injections have been used to administer drugs such as steroids and local anesthetic agents. The high permeability of the sclera to macromolecules has revived the interest in this route of drug administration.[191] For transscleral drug delivery, the drug is placed into a periocular space (subconjunctival, subtenon, peribulbar, retrobulbar, or posterior juxtascleral), and it may permeate from the periocular space into the vitreous via the anterior chamber, via a direct penetration pathway across the sclera, or through the systemic circulation. In the anterior chamber route, the drug reaches the aqueous humor either directly across the sclera and ciliary body or indirectly via the tear fluid and cornea, and it subsequently diffuses to the posterior chamber and into the vitreous. In the direct penetration pathway, the drug permeates into the vitreous through the underlying tissues; in the anterior eye, the drug may diffuse through the ciliary body and then to the posterior chamber and vitreous, whereas in the posterior eye, the drug has to permeate across the choroid, retinal pigment epithelium, and neural retina to reach the vitreous. Finally a small proportion of the drug enters the general circulation via conjunctival, episcleral of choroidal vessels and returns into the eye with the systemic blood flow.[189] The relative importance of each route to vitreal drug delivery has been investigated in animal models. The direct penetration pathway is the most important route in the vitreal delivery of most compounds with widely differing physicochemical properties.[191]

The drug must permeate across several tissues, and some of the barriers are different in the anterior and posterior eye. The RPE is the rate-limiting permeation barrier in the retinal delivery of hydrophilic drugs and macromolecules through the transscleral route. Permeability in RPE is determined both by physical-chemical factors (e.g., molecular weight, lipophilicity, and charges) and affinity to active transporters. The transporters may decrease or increase the drug transfer across the RPE depending on the type of transporter (influx or efflux) and its location (vitreal or blood side). The permeating drug also faces active barriers such as the clearance via choriocapillaris blood flow. The choroidal blood flow is higher than in any other tissue (relative to the tissue weight), and choriocapillaris walls contain large fenestrations. Large macromolecules are able to diffuse through the fenestrations in rabbit choriocapillaris. In a rabbit model for administration of triamcinolone acetonide, elimination of the blood flow with cryotherapy did not result in improved penetration of the drug after subtenon administration.[61,192] The orbital and conjunctival vasculature and lymphatics seem to have a larger role in drug clearance than does the choroid. The elimination of conjunctival lymphatic and blood flow by incising a small window in the conjunctiva was more effective in increasing the vitreous concentration than was the elimination of the choroidal blood flow. When considering the orbital clearance, the posterior subtenon location appears to be the best periocular route, because it is closer to the sclera and farther from the orbital vasculature.[193] After periocular injection, the drug must penetrate across the sclera, which is more permeable than the cornea. However for the drug to reach the retina, it must pass across the choroid and RPE (a tighter barrier than the sclera), at which point drugs may be cleared significantly to the bloodstream.[194]

The sclera has a large surface area (16.3 cm^2), and scleral permeability shows no dependence on lipophilicity but dependence on molecular radius. The sclera is composed of collagen and proteoglycan fibers with few protein-binding sites. Drugs permeate through the aqueous intercellular media of the sclera, occupying the pores between the collagen fibers. Pore diameter and intercellular space are important determinants of transscleral drug delivery, particularly for drugs such as large hydrophilic peptides and oligonucleotides. Scleral permeability is affected by an increase in intraocular pressure.[190] Scleral permeability may be enhanced in several ways. Treatment of many ocular diseases incorporates the use of transscleral depots, either as scleral implants (used for antiinflammatory agents) or collagen matrix and fibrin sealants, or the use of microspheres and other nanoparticles.[190,195]

Periocular Chemotherapy in Retinoblastoma

Control of the intraocular tumor burden requires achieving high levels of chemotherapeutic agents in all the intraocular compartments, including the vitreous, the subretinal space, and the ocular coats. Although it is effective systemic administration of chemotherapy is limited by the blood-ocular barriers previously described. In recent years methods to increase the intraocular concentrations of chemotherapeutic agents have been extensively investigated, and periocular administration of carboplatin has been incorporated into most regimens for the treatment of advanced intraocular retinoblastoma. In the transgenic murine retinoblastoma treated with intravitreous injections of carboplatin, inhibition of tumor growth occurs in a dose-dependent manner,[196] thus suggesting that higher intraocular levels of chemotherapy would be desirable. In this retinoblastoma model, subconjunctival administration of carboplatin resulted in tumor control in a dose-dependent manner,[197] suggesting good intraocular penetration of this agent when given periocularly. Pharmacokinetic studies performed in the rabbit eye have validated this method.[198] Mendelsohn and colleagues[199] compared the intraocular concentrations of agents after local or systemic administration in primates. Intravenous carboplatin was given at the standard dose used in humans (18.7 mg/kg), and systemic and intraocular pharmacokinetics were compared with peribulbar and episcleral injections of 10 mg of carboplatin. The intravenous administration resulted in higher levels in the aqueous humor than did peribulbar or episcleral injections. However vitreous levels were significantly higher when carboplatin was administered periocularly. Importantly systemic absorption after periocular administration was minimal; plasma levels of carboplatin were 3% of those achieved after intravenous administration. In the same study no etoposide was detected in the aqueous or vitreous humor when given systemically. Etoposide is a protein-bound drug, and it likely stays in the plasma and thus has limited penetration. Studies in the mouse model have also shown little intravitreal penetration of etoposide when given intravenously. The vitreous/plasma ratios when measured as area under the curve concentrations were 0.59 for carboplatin and 0.07 for etoposide.[76] However these models may not recapitulate the natural history of retinoblastoma because the blood-vitreous barrier is likely disrupted by tumor. In fact intraocular carboplatin concentrations in the human eye with retinoblastoma are higher after intravenous administration than what the primate eye model shows; levels in the vitreous are much higher than what the animal studies would predict and are similar to levels measured in unaffected animals when the drug is given after cryotherapy.[200] The periocular route has also been explored in animal models for topotecan[97,201] and for new investigational agents such as SYK and MDM4 inhibitors.[63,79]

Given the promising preclinical data, periocular administration of carboplatin has been progressively incorporated into the clinical practice. Patients tolerate the administration of 2 mL of the 10 mg/mL solution, although acute orbital toxicity is significant. In the only phase II study reported, responses were seen in three of the five eyes with vitreous disease and in two of the five eyes with retinal tumors.[202] The role of this form of administration in the management of retinoblastoma is still being evaluated. Patients with advanced intraocular disease (particularly those with massive vitreous or subretinal seeding) are candidates to receive up to three doses of periocular carboplatin concomitantly with systemic chemotherapy to maximize intraocular levels of the agent. However great caution should be exerted if periocular carboplatin is administered; acute toxicities (e.g., periorbital edema and inflammation) and long-term toxicities (e.g., orbital fat necrosis, soft tissue and muscle fibrosis, and optic neuritis) are common.[203,204] Although periocular carboplatin has a very limited role as a single agent, it may lead to long-term disease control when combined with other treatment modalities (Box 56-4).[205] Topotecan has also been used via periocular administration, although its intraocular penetration may be lower[97,201] and its potential role in therapy may be more limited.[206] In the animal model periocular administration of topotecan reached potentially active levels of its active lactone moiety without significant toxicity.[97] However systemic absorption was significant. A recently completed phase I study has documented the feasibility of administering 2 mg of topotecan periocularly, although no significant responses were seen.[160]

Although effective in increasing intraocular levels, subconjunctival administration of carboplatin or topotecan in aqueous media has many limitations. Most of the drug delivered is dispensed quickly throughout the subconjunctival space and surrounding orbit, thus exposing extraocular tissues to high transient concentrations of the drugs and potential toxicities. Methods for improving periocular delivery while minimizing toxicity include the use of a collagen matrix or fibrin sealants. Studies have shown that carboplatin may enhance fibrin clot formation.[207] One benefit of the sealant is that the anhydrous form of carboplatin can be overloaded beyond its solubility limit to provide higher levels of drug for extended periods. The scleral drug exposure is maximized, extending the duration of therapeutically significant intraocular drug penetration, and decreasing the need for repeated periocular injections.[208] However in the animal models, as the intraocular levels increase, so does the risk of retinal toxicity.[196,208,209]

Intravitreal and Intraarterial Chemotherapy for Intraocular Retinoblastoma

Administration of chemotherapy drugs directly into the tumor vasculature is a common practice in oncology. A small, localized tumor such as retinoblastoma would be an excellent candidate for such an approach. Japanese investigators have pioneered the administration of intravitreal and intraarterial melphalan for patients with advanced or recurrent intraocular retinoblastoma.[210,211] Preclinical data suggest that retinoblastoma is very sensitive to melphalan and that there is a synergistic effect with thermotherapy[212] and with other agents such as topotecan.[213] Direct delivery to the ocular vasculature has been limited by the technical difficulties in the cannulation of the retinal artery. Kaneko and Suzuki[210] initially reported the feasibility of injecting melphalan into the ipsilateral carotid artery, with documented efficacy. The technique was later perfected by Suzuki and colleagues[211] and Mohri[214] using a balloon catheter that allowed for selective injection into the ophthalmic artery. More recently Abramson and colleagues[215] reported a variation of this technique that includes a direct cannulation of the ophthalmic artery using a microcatheter (supraselective). Successful catheterization and delivery of chemotherapy can be achieved in 98% of cases,[216,217] although in 16% of patients an alternative vascular route is required—often the orbital branch of the middle meningeal artery.[218] This approach has also been documented as being feasible as tandem therapy in patients with bilateral disease.[219]

Although melphalan has remained the most commonly used agent and the most effective agent, it is often combined with topotecan or carboplatin when responses are suboptimal or the intraocular disease is very advanced.[216,217,220-223] The doses of the chemotherapeutic agents are determined by patient age (and ocular volume) and by the angioanatomy; doses reported to result in a significant antitumor effect and limited toxicity are 2.7 to 7.5 mg for melphalan, 0.3 to 0.6 mg for topotecan, and 25 to 50 mg for carboplatin.[216,220,221,223,224] In pigs, the infusion of 7 mg of melphalan or 1 mg of topotecan to the ophthalmic artery results in peak vitreous levels higher than the inhibitory concentration of 50%, and with limited systemic exposure.[213,225] In children a large variability of systemic exposure occurs, and patients receiving tandem administrations of melphalan have a higher risk of the development of neutropenia, which correlates with plasma levels.[213]

Patients typically receive one to six (median, three) intraarterial administrations of chemotherapy including melphalan alone or in combination, and responses are usually seen immediately after the first administration. For patients with treatment-naive eyes, the 2-year radiation-free ocular survival rate is 80% to 90%.[216,220,223] Similar outcomes are also being reported with single-agent carboplatin and in combination with topotecan.[221] Outcome correlates with the intraocular burden; patients with early intraocular disease (group B and C eyes) have an excellent outcome and may be treated with single-agent therapy.[221,222] Eyes with significant vitreous or subretinal seeding have a worse outcome, with radiation-free survival rates of 80% for eyes with subretinal seeding and 65% for eyes with vitreous seeding.[226] For patients with very advanced intraocular disease, an alternative is the use of systemic chemotherapy followed by consolidation with intraarterial melphalan.[227] The presence of massive retinal detachment is not a contraindication to intraarterial chemotherapy; studies have shown that this approach promotes retinal reattachment and may result in ocular salvage rates in excess of 85%.[228] Ocular salvage rates when intraarterial chemotherapy administration is used as salvage for patients with recurrent or progressive disease are consistently lower, with globe survival rates of 50% to 60%.[216,217,223,226] However the use of a more intensive three-drug regimen with melphalan, topotecan, and carboplatin in patients whose disease has progressed after undergoing standard systemic and focal therapies has been reported to result in radiation-free ocular salvage rates of 75% at 2 years.[224]

Patients with bilateral disease can receive tandem administrations.[219] In those circumstances patients are at a higher risk of systemic toxicity as a result of melphalan exposure,[213] and single-agent carboplatin may be used to treat the eye with less advanced disease during the tandem procedure.[229] For neonates and very young infants for whom the cannulization of the ophthalmic artery is not feasible, a "bridge" treatment with single-agent systemic carboplatin until the baby is 3 months old or weighs 6 kg followed by consolidation with intraarterial chemotherapy has been shown to be very effective, with 1-year radiation-free ocular survival of 95%.[230]

Direct administration of high doses of chemotherapy to a sensitive organ such as the eye of a young child is not devoid of complications. In the nonhuman primate model, treatment with three cycles of melphalan or carboplatin results in a significant inflammatory response, with leukostasis and retinal arteriole occlusion with ultrastructural changes in the endothelial cells and surrounding pericytes. Eyes also show nerve fiber infarcts and optic nerve hemorrhage and leukostasis, central retinal artery thrombosis, choroidal inflammation, and birefringent intravascular foreign bodies.[231] Similar histopathologic evidence of vasculopathy is seen in enucleated eyes of children with retinoblastoma after intraarterial chemotherapy.[232] Clinically a transient chorioretinal ischemia has been documented during the procedure, the impact of which is not fully understood.[233,234] However approximately 15% of the eyes show a long-term sectorial choroidal atrophy, and 15% have delayed vitreous hemorrhage leading to enucleation.[222,235] The impact of the intraocular vascular changes on vision has not been fully assessed because of the young age of the first cohorts of patients treated. The majority of patients do not have substantial electroretinographic changes,[236] and preservation of central vision has been reported.[237] However in patients with heavily pretreated eyes, intensive intraarterial chemotherapy may result in worsening of retinal function.[224]

Acute and usually transient adverse events include mild eye edema, blepharoptosis, and orbital congestion with temporary dysmotility.[221,222,238] Systemic effects are minimal; grade III neutropenia develops in only 10% of patients,[216] although this proportion is higher in children with bilateral disease who undergo tandem therapy.[213] Major vascular complications are very rare; no strokes or significant acute neurologic events have been reported when the procedure is performed at a state of the art treatment center.[211,216,217] However stenosis of the ophthalmic artery and occlusion of the retinal artery have been documented.[238] The risk of thrombosis is significantly increased in children with thrombophilia.[239]

One additional risk associated with intraarterial chemotherapy is the exposure to ionizing radiation during fluoroscopy. In very experienced hands, the mean fluoroscopy time per procedure is 7 minutes.[240] Radiation doses per procedure can be as high as 191 mGy to the affected eye and 35 mGy to the contralateral eye,[241] although doses as low as 1 mGy per procedure have been reported by the center with the most experience in performing the procedure.[240] After multiple procedures, cumulative doses can reach 0.1 to 0.2 Gy, which can be cataractogenic and potentially carcinogenic in this susceptible population.[241] Long-term outcome data reported by Japanese investigators seem to indicate that no increase occurs in the incidence of second malignancies[211]; however, longer follow-up will be required to fully ascertain the risks associated with the procedure.

To achieve the maximum concentrations of chemotherapy close to the tumor and in the vitreous, investigators have attempted direct intravitreal delivery. This approach was pioneered by Swedish investigators in the 1960s.[242,243] Thiotepa was the agent selected in those initial studies, and repeated injections of 1 to 1.5 mg were administered, often in conjunction with EBRT. Regressions were obtained, but long-term control of the intraocular disease was generally not possible. This approach was rekindled by Seregard et al.[244] in three patients with bilateral disease with recurrent retinoblastoma in a single remaining eye. Patients received repeated intravitreal injections of thiotepa, followed by vitrectomy and weekly injections, for a total cumulative dose of 10 to 14 mg of thiotepa; no obvious clinical responses were observed.

Direct administration of melphalan into the vitreous is a promising approach for patients with vitreous disease. Clinical responses in patients with progressive retinoblastoma were obtained with use of intravitreal melphalan followed by hyperthermia,[210] and more recent data seem to indicate that intravitreal chemotherapy may have a role in the management of patients with progressive vitreous disease.[245,246] This approach is currently being explored in combination with systemic chemotherapy for patients with advanced intraocular disease.

Radiotherapy

Retinoblastoma is a very radiosensitive tumor. Radiotherapy in combination with focal treatments can provide excellent tumor control.[247,248,249] However because radiation therapy increases the risk of second malignancies, contemporary management of intraocular retinoblastoma is designed to avoid or delay its use. The role of irradiation is mainly as salvage management for eyes that have failed to respond to chemotherapy and focal treatments, usually because of progression of vitreous and subretinal seeding.[250] Because most patients with intraocular retinoblastoma who undergo radiotherapy have multifocal disease, the entire retinal surface needs to be irradiated to a uniform dose. Several techniques can be used, usually through lateral or anterior fields.[247-249] Recommended total doses are 40 to 45 Gy, in 180 to 200 cGy fractions, although doses of 36 Gy and even lower may be effective in conjunction with other techniques.[82,251-253] Radioactive plaque technique is useful when treating localized tumors, both because the procedure time is short and because a high dose of irradiation is delivered to the areas of interest while minimizing irradiation effects in extraocular structures.

Episcleral Plaque Brachytherapy

Indications for plaque therapy include solitary tumors with a diameter ranging between 6 and 15 mm, tumor thickness of 10 mm or less, and location of the lesion more than 3 mm from the optic disc or fovea.[250] Since the early use of cobalt-60 plaques in the 1950s, the field has evolved to the more common use of iodine-125, iridium-192, and ruthenium-106.[254-258] In combination with appropriate use of chemotherapy and focal consolidation, brachytherapy can be very effective, even in the presence of focal vitreous seeding overlying the tumor.[250,259]

The most commonly used radioisotope is ^{125}iodine, a low-energy γ-ray emitter (27 to 35 keV; half-life, 60 days) that is readily available in encapsulated form, allowing custom fabrication of gold plaques of varying sizes.[254] ^{125}Iodine seeds are loaded within the wells of the plaque to achieve the dose and distribution desired based on the size and location of the tumor; lesions of up to 10 to 12 mm in height and 10 to 15 mm in diameter can be treated using this approach. ^{192}Iridium and ^{106}ruthenium are alternative isotopes of higher energy (295 to 612vkeV and 3.5 MeV, respectively) and longer half-life (74 and 374 days, respectively), which makes dosimetry calculations and safety measures more complex. The dose is prescribed to reach the tumor apex and overlying localized vitreous seeds if present. For ^{125}iodine the target dose is usually 40 Gy at the apex at 40 to –80 cGy per hour; the dose at the tumor base may approximate 120 Gy. Tumor control using this approach may be close to 80%. In approximately 25% of the eyes, long-term complications may occur, including nonproliferative retinopathy, papillopathy, and nonproliferative maculopathy.[254,260] Similar results are achieved with ^{106}ruthenium.[255]

External Beam Radiation Therapy

EBRT is commonly used as consolidation therapy in conjunction with chemotherapy for intraocular retinoblastoma when the disease is not responding well to conservative management, usually because of the presence of vitreous or subretinal seeding or progression of the retinal tumors, and in the management of extraocular disease.[250] Traditionally the opposed "D" fields have been used to treat bilateral retinoblastoma, with the isocenter placed 2 to 3 mm behind the lens; with this approach, aimed at sparing the lens, the retina anterior to the equator may be relatively underdosed.[261,262] Currently the use of conformal and intensity-modulated radiation therapy (IMRTs) can provide coverage of the entire retina and vitreous cavity while limiting the dose to the lacrimal gland and soft tissues and orbital bones.[262,263] The standard EBRT dose is 40 to 45 Gy, delivered in 180 to 200 cGy fractions; doses higher than 45 Gy do not seem to be associated with an increased benefit.[261] One study also showed the potential role of low-dose (26 Gy) EBRT to consolidate advanced intraocular retinoblastoma upon completion of chemotherapy, in the absence of disease progression.[253] IMRT may be superior to three-dimensional (3D) conformal radiation therapy and conventional two-dimensional irradiation in terms of the dose delivered to normal orbital and extraorbital tissues.[264] However although increasing the conformity of the treatment results in a sharp decline of the dose-volume curve at the higher doses, this gain still comes at the expense of increasing the volume of normal tissue that receives the lowest doses. Even with optimal IMRT planning, 50% of the orbit receives 50% of the prescribed dose.[250] The use of fractionated stereotactic radiation therapy may allow for a significant decrease in the orbital volume exposed.[265] Proton beam therapy allows for exquisite dosimetry, thus providing a theoretic advantage in sparing radiation exposure to orbital tissues.[263,266] A proton beam has exquisite stopping power and may produce essentially no lateral scatter, and thus protons can be used to control tumors at any depth without the entrance and exit doses associated with photon beam irradiation that are responsible for the complications associated with radiation therapy in patients with retinoblastoma.[266] In a study comparing protons with conformal therapy, electron therapy, and IMRT, protons had the best orbital bone-sparing dose distribution. Only 10% of the bone exceeded 5 Gy, compared with 25% for 3D–conformal radiation therapy electrons, 69% for IMRT, 41% for a single 3D lateral beam, 51% for a 3D anterolateral beam with a lens block, and 65% for a 3D anterolateral beam without a lens block.[250,266] Very importantly preliminary long-term data seem to indicate that the cumulative incidence of second malignancies in the irradiated field is significantly lower with protons when compared with standard photon therapy.[267]

Radiation therapy is indicated for extraocular retinoblastoma and after enucleation when high-risk features are present (e.g., transscleral involvement and involvement of the cut end of the optic nerve). The target volume is the orbit and may extend to include regional sites and lymph nodes based on clinical and imaging evidence of tumor extension. The dose typically used for treatment of the orbit or regional extraocular sites is 45 Gy. Radiation therapy may be indicated for patients with metastatic disease based on response to chemotherapy. In most treatment protocols, patients with hematogenous metastases or CNS involvement who achieve a complete response to induction chemotherapy do not undergo irradiation; however, patients with an incomplete response usually undergo irradiation at the completion of therapy, which often includes consolidation with high-dose chemotherapy and autologous hematopoietic stem-cell rescue. This sequence is also recommended for patients who require craniospinal irradiation. Based on age older patients who require craniospinal irradiation should receive 36 Gy to the neuraxis and 45 Gy to residual disease. Younger patients should receive at least 23.4 Gy to the neuraxis. Evidence suggests that clinically defined disease has limited microscopic tumor infiltration so that the clinical target volume surrounding the clinically identifiable residual tumor (gross tumor volume) may be as small as 5 mm.[250]

TREATMENT OF INTRAOCULAR RETINOBLASTOMA

Treatment of retinoblastoma is tailored to the intraocular and extraocular disease burden, disease laterality and germline status, and potential for vision (Fig. 56-5).

Unilateral Retinoblastoma

In the absence of extraocular disease, enucleation alone is curative for 85% to 90% of children with unilateral retinoblastoma.[113,161,165,268] The outcome for patients with unilateral disease that has been enucleated is excellent, with good functional results and minimal long-term effects.[269] However in view of the success in the treatment of bilateral intraocular disease with systemic or intraarterial chemoreduction and focal treatments, more patients

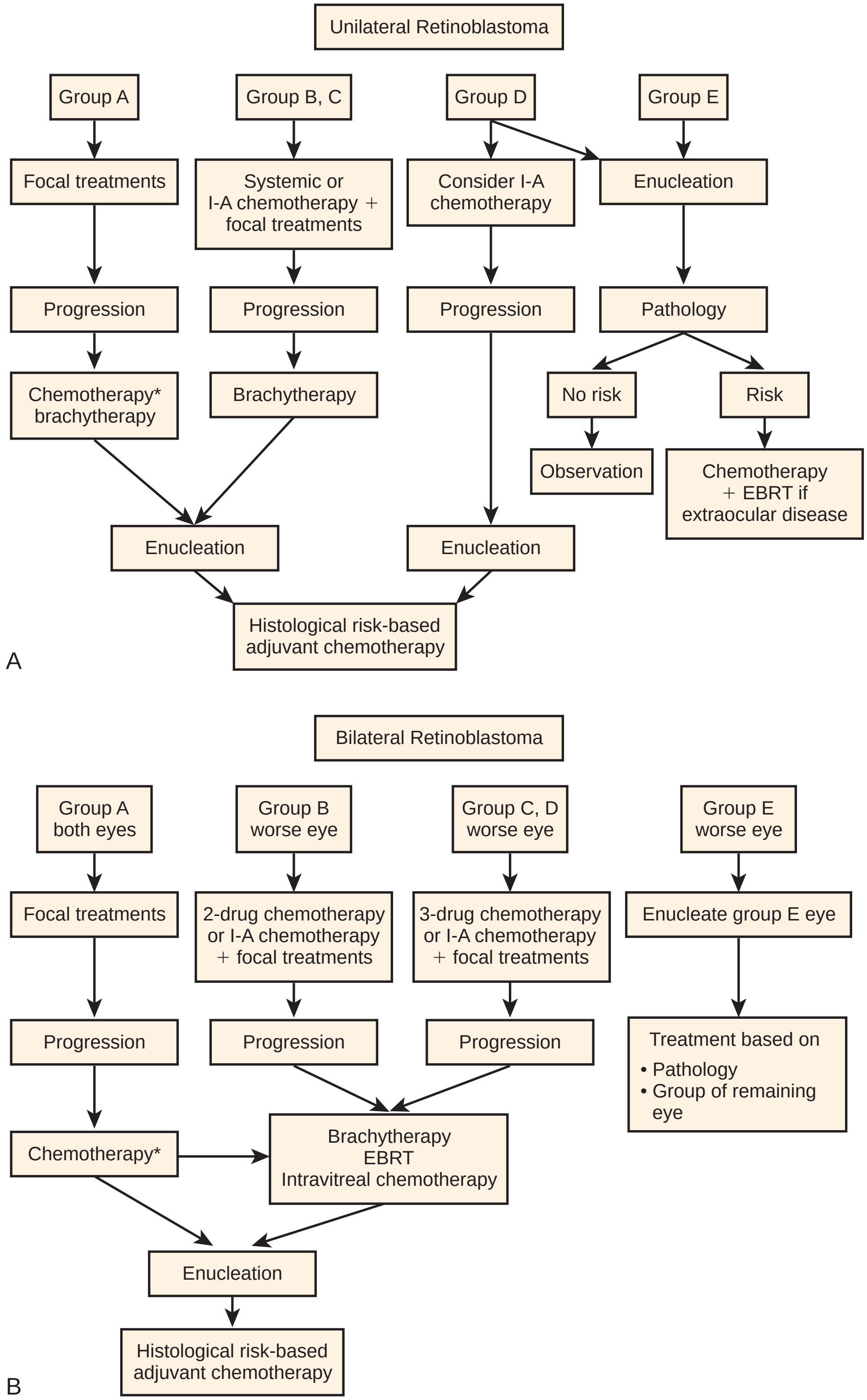
Unilateral Retinoblastoma
Group A
Group B, C
Group D
Group E
Focal treatments
Systemic or I-A chemotherapy + focal treatments
Consider I-A chemotherapy
Enucleation
Progression
Progression
Progression
Pathology
Chemotherapy* brachytherapy
Brachytherapy
No risk
Risk
Observation
Chemotherapy + EBRT if extraocular disease
Enucleation
Enucleation
Histological risk-based adjuvant chemotherapy
A
Bilateral Retinoblastoma
Group A both eyes
Group B worse eye
Group C, D worse eye
Group E worse eye
Focal treatments
2-drug chemotherapy or I-A chemotherapy + focal treatments
3-drug chemotherapy or I-A chemotherapy + focal treatments
Enucleate group E eye
Progression
Progression
Progression
Treatment based on
• Pathology
• Group of remaining eye
Chemotherapy*
Brachytherapy
EBRT
Intravitreal chemotherapy
Enucleation
Histological risk-based adjuvant chemotherapy
B

Figure 56-5 Algorithms for the management of unilateral (**A**) and bilateral (**B**) retinoblastoma. *EBRT,* External beam radiation therapy; *I-A,* intra-arterial. *Systemic or I-A.

are considered candidates for ocular preservation treatments. This is particularly important in very young children in whom metachronous bilateral disease may develop. Using the standard three-drug regimen (vincristine, carboplatin, and etoposide) and aggressive focal consolidation, more than two thirds of patients with unilateral disease do not respond to therapy and ultimately require either enucleation or radiation therapy.[270] Therefore the use of upfront systemic chemotherapy in this group of patients with unilateral disease needs to be

TABLE 56-1 Recommended Approach to the Treatment of Intraocular Retinoblastoma

		TREATMENT			
R-E Group	**ABC Group**	**Focal Treatment**	**Intraarterial Chemotherapy**	**Systemic Chemotherapy**	**Radiation**
I-II	A	+	If PD	If PD	If PD
I-III	B	+	MEL 3-5 mg × 3-6 courses	VCR 0.05 mg/kg d 1 CBP 18.6 mg/kg d 1 × 2-6 courses	If PD
IV-V	C-D*	+	MEL 3-5 mg × 3-6 courses Consider addition of second agent (TOP, CBP)	VCR 0.05 mg/kg d 1 CBP 14 mg/kg d 1, 2 ETO 6 mg/kg d 1, 2 × 6 courses ± subconjunctival CBP	If PD Consider early EBRT if massive vitreous seeding at completion of chemotherapy
V b	E	Enucleation			

CBP, Carboplatin; *EBRT,* external beam radiation therapy; *ETO,* etoposide; *MEL,* melphalan; *PD,* progressive disease; *R-E,* Reese-Ellsworth; *TOP,* topotecan; *VCR,* vincristine.

*Consider upfront enucleation if unilateral.

considered very judiciously and only in cases with low intraocular disease and when focal consolidation is available. Preenucleation chemotherapy results in downstaging with underestimation of extraretinal and extraocular disease, leading to an increased risk of metastatic spread.[271] However patients presenting with group E eyes and buphthalmos, in whom enucleation might be associated with surgical risks, may benefit from preenucleation chemotherapy; in these cases, given the inability to perform proper histologic risk evaluation, patients should be considered to have high-risk disease and be treated accordingly with postenucleation chemotherapy.[164,171]

With the advent of intraarterial chemotherapy, many patients with groups B, C, and D disease are now good candidates for ocular salvage, and long-term eye preservation may be achieved in more than 75% of cases (Box 56-4, Table 56-1, and Fig. 56-5).[216]

For patients undergoing upfront enucleation, a careful histologic evaluation is needed to assess high-risk disease. Adjuvant treatment is indicated in cases with transscleral invasion and in patients with positive tumor at the transection line of the optic nerve (see the discussion in the "Treatment of Extraocular Retinoblastoma" section). Adjuvant treatment for the remaining patients with intraocular disease is more debatable. In the absence of randomized studies, available information would suggest that the use of adjuvant chemotherapy may be beneficial for the selected group of patients with higher risk of extraocular dissemination, which includes involvement of the anterior chamber, ciliary body, or iris, massive infiltration (>3 mm) of the choroid, retrolaminar optic nerve infiltration, or focal choroidal disease in combination with any degree of nonretrolaminar optic nerve involvement (Table 56-2 and Fig. 56-5).[146,156,157-159,161,165,167,272] Adjuvant chemotherapy is not indicated for patients with isolated prelaminar involvement[156,251] or isolated focal choroidal involvement.[158,161,165,251,268] Different chemotherapy regimens have been proposed. Six-month treatment with vincristine, cyclophosphamide, and doxorubicin; vincristine, carboplatin, and etoposide; or a hybrid with alternating courses of both regimens appears to be effective (Table 56-2). Idarubicin is also an effective agent in persons with retinoblastoma and can be substituted for doxorubicin.[273] Radiation therapy is not indicated in these cases.

Bilateral Retinoblastoma

In patients with germline mutation of the *RB1* gene, multiple, bilateral retinoblastomas develop at an earlier age, and these patients are at risk of development of new tumors until the completion of retinal differentiation.[87] In the past treatment for patients with bilateral retinoblastoma has been enucleation of eyes with advanced intraocular disease and no visual potential, and the use of EBRT for the remaining eyes. However, several complications are associated with radiation therapy. Irradiation of the orbit during a period of rapid growth results in a major decrease in orbital volume, resulting in midfacial deformities.[274,275] More importantly however is the greatly increased risk for the development of a sarcoma within the radiation therapy field, compared with the underlying increased risk of secondary neoplasms in these predisposed persons. This risk may be age related and decreases as irradiation is delayed.[276] These concerns have resulted in the development of new and more conservative approaches. The treatment of patients with bilateral retinoblastoma now incorporates upfront chemotherapy, which is intended to achieve maximum chemoreduction of the intraocular tumor burden early in the treatment, followed by aggressive focal therapies (Fig. 56-5). Chemoreduction coupled with intensive use of sequential focal therapies (e.g., cryotherapy, laser photocoagulation, thermotherapy, and brachytherapy) has resulted in an increase in the eye salvage rates and in a decrease (and delay) in the use of radiation therapy. Intraocular retinoblastoma is extremely chemosensitive, and disease progression during treatment is rarely seen. Noticeable responses are seen in more than 90% of the eyes; these responses are maximum after two cycles, and tumor regression after subsequent cycles is less pronounced (Fig. 56-6).[134,277,278] However chemotherapy alone can only save fewer than 10% of

TABLE 56-2 Adjuvant Chemotherapy for Retinoblastoma with High-Risk Histology

Regimen	Drugs	Doses	Comments
COG-ARET0332 protocol	VCR	0.05 mg/kg day 1	6 cycles
	CBP	18.6 mg/kg day 1	
	ETO	5 mg/kg days 1, 2	
Chantada et al.[165]	VCR	0.05 mg/kg day 1	Cycles 1, 3, 5, 7
	CYC	65 mg/kg day 1	
	IDA	10 mg/m^2 day 1	
	CBP	18.7 mg/kg (<10 kg) days 1, 2 560 mg/m^2 (≥10 kg) days 1, 2	Cycles 2, 4, 6, 8
	ETO	3.3 mg/kg (<10 kg) days 1, 2, 3 100 mg/m^2 (≥10 kg) days 1, 2, 3	
SJCRH*	VCR	0.05 mg/kg (<12 mo) day 1 1.5 mg/m^2 (≥12 mo) day 1	Cycles 1, 3, 5
	CYC	40 mg/kg (<12 mo) day 1 1,200 mg/m^2 (≥12 mo) day 1	
	DOX	1.5 mg/kg (<12 mo) day 1 45 mg/m^2 (≥12 mo) day 1	
	VCR	0.05 mg/kg (<12 mo) day 1 1.5 mg/m^2 (≥12 mo) day 1	Cycles 2, 4, 6
	CBP	AUC 6.5 mg/mL/min day 1	
	ETO	3.3 mg/kg (<12 mo) days 1, 2, 3 100 mg/m^2 (≥12 mo) days 1, 2, 3	

AUC, Area under the curve; *CBP,* carboplatin; *CYC,* cyclophosphamide; *DOX,* doxorubicin; *ETO,* etoposide; *IDA,* idarubicin; *SJCRH,* St. Jude Children's Research Hospital; *VCR,* vincristine.

*SJCRH regimen from Sullivan EM, Wilson MW, Billups CA, et al: Pathologic risk-based adjuvant chemotherapy for unilateral retinoblastoma following enucleation. *J Pediatr Hematol Oncol* 36:e335–40, 2014.

the eyes.[134] Macular tumors are more sensitive to chemotherapy, probably because of the richer vascular supply that maximizes exposure to chemotherapy. Up to two thirds of macular tumors can be controlled with chemotherapy alone,[23,279] and with the addition of thermotherapy, this proportion may increase to greater than 80%.[23,24,279,280] Chemoreduction coupled with intensive use of sequential focal therapies (e.g., cryotherapy, laser photocoagulation, thermotherapy, and brachytherapy) has resulted in an increase in eye salvage rates and in the decrease (and delay) in the use of radiation therapy.

Different chemotherapy combinations are used, although the best results are achieved with a combination of vincristine, carboplatin, and etoposide (or teniposide).[80,83,281-283] An alternative chemotherapy regimen includes the addition of cyclosporin to the three-drug regimen.[284] The rationale for the use of cyclosporin originates from the documentation of the presence of the P-glycoprotein efflux pump in a significant proportion of retinoblastomas, an expression that appears to correlate with treatment failure.[285] The concurrent administration of cyclosporin could potentially abrogate the efflux of drugs from the cancer cell. However the expression of P-glycoprotein does not always seem to predict response,[286] and cyclosporin adds toxicity. For patients with early intraocular stages (R-E groups I to III or International group B), a less intensive regimen with vincristine and carboplatin alone appears to be effective (Box 56-4 and Figs. 56-5 and 56-6).[134,287]

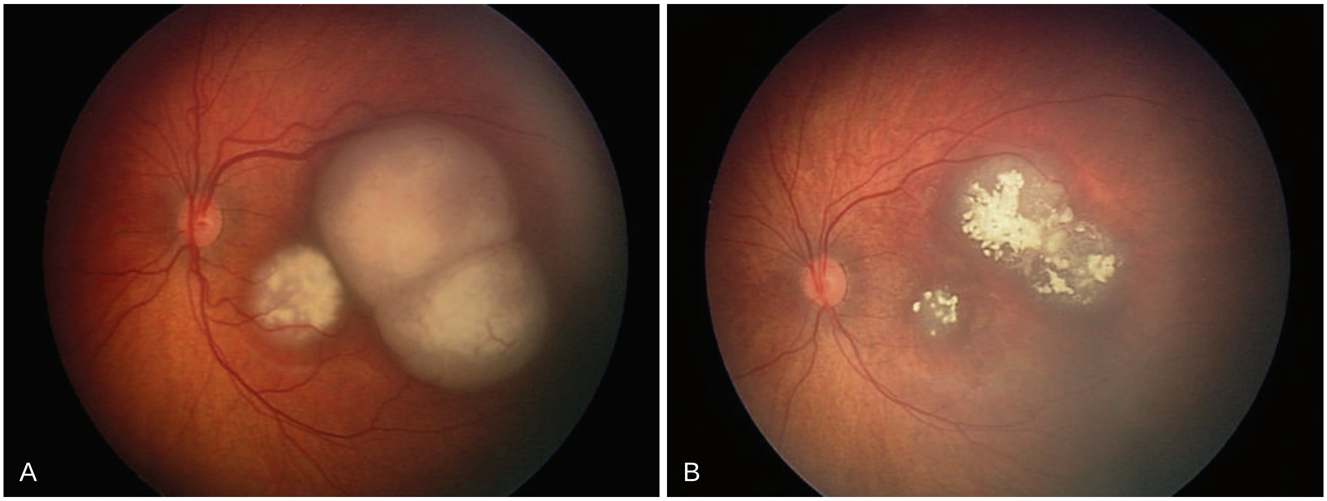

Figure 56-6 Response of a group B retinoblastoma (**A**) to two courses of vincristine and carboplatin (**B**).

Salvage rates for eyes classified as R-E groups I to III approaches 100% with use of these techniques. For patients with advanced intraocular tumors (International groups C and D), ocular salvage rates are not better than 50% to 70% when systemic chemoreduction is used, and EBRT is usually required.[281] However the use of radiation therapy is usually delayed for several months, which allows for better orbital growth and a decrease in the risk of second malignancies. A major proportion of failures occur because of progression of tumor in the vitreous, or as subretinal implants, two areas of difficult access for antineoplastic agents.[80] For these patients, if systemic chemotherapy is used, the addition of periocular carboplatin may be beneficial.[205]

Thus the treatment of patients with advanced intraocular disease (R-E groups IV and V or International groups C and D) remains a major challenge. Although randomized studies have not been performed, compared with radiation therapy and focal treatments alone, chemoreduction does not seem to improve overall ocular salvage significantly for patients with very advanced intraocular disease.[247-249] Chemotherapy intensification appears to correlate with outcome, and better results are obtained with protocols that include at least six courses of vincristine, etoposide, and carboplatin.[281,288,289] Central retinal tumors usually respond better to chemotherapy than tumors in the peripheral retina,[290] but large central tumors may be associated with subretinal seeds, which ultimately may cause treatment failure.[291] Despite the addition of aggressive sequential focal therapies, globe retention is still no better than 50% for patients with group D eyes, and most patients eventually require irradiation. A major proportion of failures occur because of progression of tumor in the vitreous or as subretinal implants, two areas of difficult access for antineoplastic agents.[80] Radiation therapy appears to be the only valid alternative for these patients, and with the incorporation of lower doses of irradiation (26 Gy) early in the treatment, in a situation of minimal disease, before disease progression occurs, ocular salvage rates for International group D eyes may improve.[253]

New agents with better intraocular penetration are under investigation. Topotecan, a topoisomerase-I inhibitor with well-documented efficacy against pediatric tumors, is a promising alternative. Studies performed in the animal model have shown that topotecan has excellent intraocular penetration and antitumor effect.[76] In a phase II study using an upfront window of vincristine and topotecan in patients with advanced bilateral disease, responses were documented in 90% of the patients.[292]

Direct administration of chemotherapy drugs into the ophthalmic artery to maximize drug exposures is a very effective approach and has become the standard of care at many centers, particularly for children with advanced bilateral disease, with ocular survival rates in excess of 80% (as previously discussed).

New Tumor Formation during Treatment with Chemotherapy

In the natural history of patients with germline *RB1* mutation, new tumors continue to form after diagnosis. It is not clear whether the use of chemotherapy may alter this process. Several authors have evaluated the impact of primary systemic chemotherapy on the rate of new tumor formation in patients with hereditary retinoblastoma. For patients receiving treatment with the standard two- or three-drug regimens that include six to eight cycles of chemotherapy, the cumulative incidence of new tumors after the start of chemotherapy is 10% to 24%,[293,294] although higher incidences have also been reported.[295] However most new tumors develop after chemotherapy is discontinued.[134,294] The incidence of new tumors is higher if a regimen of shorter chemotherapy duration is used or only focal treatments are administered.[296-298] Younger age at diagnosis, a family history of retinoblastoma, and lower grouping at diagnosis are factors that significantly increase the likelihood of new tumor formation.[293-295] These risk factors are intrinsically tied together; patients with a family history are more likely to be screened early and be diagnosed at a younger age, early in the natural history of the disease. Importantly the majority of the new tumors after the start of chemotherapy develop in the equatorial or peripheral retina, and fewer than 20% of the tumors form in the macula.[293] This tumor distribution is likely tied to retinal development rather than to the use of chemotherapy, but it is possible that small tumors in the richly vascularized central retina are effectively treated with chemotherapy before they become visible. New tumors may still retain sensitivity to the drugs used, because they likely represent new somatic mutations that occur after the start of treatment. With the recent implementation of intraarterial chemotherapy as primary treatment for patients with bilateral disease, a similar preventive phenomenon has been observed.[299] All in all these data seem to suggest that small tumors undetectable by ophthalmoscopy may be present at diagnosis that can be effectively treated before becoming apparent.

TREATMENT OF EXTRAOCULAR RETINOBLASTOMA

Extraocular dissemination of retinoblastoma bears a close relationship with the socioeconomic conditions that result in delayed diagnosis and treatment. In Europe and the United States, fewer than 5% of patients present with extraocular disease,[152,300,301] in contrast to 12% to 20% in South America,[268,302] 25% to 40% in Mexico and India,[118,303,304] and more than 50% in less developed countries.[305]

Four patterns of extraocular disease have been recognized: (1) locoregional dissemination, including orbital disease, tumor extending to the cut end of the optic nerve, and lymphatic spread to the preauricular lymph nodes; (2) CNS dissemination; (3) metastatic retinoblastoma; and (4) trilateral retinoblastoma (Figs. 56-2 and 56-3).

Orbital and Locoregional Retinoblastoma

Orbital retinoblastoma occurs as a result of progression of the tumor through the emissary vessels and sclera. For this reason, scleral disease is considered to be extraocular and should be treated as such. Orbital retinoblastoma is isolated in 60% to 70% of cases; lymphatic, hematogenous, and CNS metastases occur in the remaining patients.[306] Treatment should include systemic chemotherapy and radiation therapy; with this approach, 60% to 85% of patients can be cured. Because most recurrences occur in the CNS, regimens using drugs with well-documented CNS penetration are recommended. Different chemotherapy regimens have proven to be effective, including vincristine, cyclophosphamide, and doxorubicin, platinum- and epipodophyllotoxin-based regimens, or a combination of both.[307,308,309-311] For patients with macroscopic orbital disease, it is recommended that surgery be delayed until response to chemotherapy has been obtained (usually with two or three courses of treatment). Enucleation should then be performed, and an additional four to six courses of chemotherapy should be administered. Local control should then be consolidated with orbital irradiation (4000 to 4500 cGy). Using this approach, orbital exenteration is not indicated.[312,313] Similar management should be followed for patients with scleral disease, including radiation therapy, although good outcomes without irradiation have also been reported.[302] Patients with isolated involvement of the optic nerve at the transection level should also receive similar systemic treatment, and irradiation should include the entire orbit (3600 cGy) with a 1000 cGy boost to the chiasm (for a total of 4600 cGy). Also because metastases in parameningeal bones have been associated with intracranial dissemination,[307] irradiation fields should be adjusted carefully. The preauricular and cervical lymph nodes should be explored carefully, because 20% of patients with orbital retinoblastoma have lymphatic metastases.[306] Lymphatic dissemination does not carry a worse prognosis, provided that the involved lymph nodes are also irradiated.[301,312,314] Patients with spontaneous or accidental ocular perforation and patients with intraocular surgery for unsuspected retinoblastoma should be considered to have orbital disease by definition and treated accordingly.

Central Nervous System Disease

Intracranial dissemination occurs by direct extension through the optic nerve, and its prognosis is dismal.[308,315,316] Treatment for these patients should include platinum-based intensive systemic chemotherapy and CNS-directed therapy. Although traditionally intrathecal methotrexate (with or without cytosine arabinoside) has been used, no preclinical or clinical evidence exists to support its use.[317] Other intrathecal agents with documented effect against retinoblastoma include topotecan[76,292,318] and thiotepa.[314,319] However no evidence indicates that their use can have an impact on outcome. Although the use of irradiation in these patients is controversial, responses have been observed with craniospinal irradiation, with a dose of 2500 cGy to 3500 cGy to the entire craniospinal axis, and a boost (1000 cGy) to sites of measurable disease.[307,311,312,314,315] Therapeutic intensification with high-dose, marrow-ablative chemotherapy and autologous hematopoietic progenitor cell rescue has been explored,[307] but its role is not yet clear. Despite the intensity of the treatment and the documented responses of the intracranial disease,[316] most patients succumb to their disease, and reports of survivors are anecdotal.[307,309]

Trilateral Retinoblastoma

The prognosis for patients with trilateral retinoblastoma is very poor; most patients die of disseminated neuroaxis disease in less than 9 months.[125,127,131] The rare survivors are usually those diagnosed with screening imaging and treated with intensive chemotherapy with or without craniospinal irradiation.[67,131] Pineal primary neuroectodermal tumors (PNETs; pineoblastoma) occurring in patients without retinoblastoma are also associated with a poor prognosis in younger patients. However with an appropriately aggressive multimodal approach, these patients can be cured. PNETs are chemosensitive tumors, and they appear to have a steep dose-response curve for alkylating agents. Studies in older patients with pineoblastoma have recently shown that a treatment with complete resection and intensive alkylator- and cisplatin-based therapy, followed by craniospinal irradiation (3600 cGy with a boost to the pineal gland to 5900 cGy) and consolidation with high-dose, myeloablative chemotherapy and autologous hematopoietic progenitor cell rescue, may produce survival rates in more than two thirds of patients.[320] It is therefore possible that similar treatment guidelines could be used for trilateral retinoblastoma. However one must consider the serious long-term toxicities of such doses of irradiation in the very young child. Therefore current strategies are directed toward avoiding irradiation and instead using intensive chemotherapy followed by consolidation with myeloablative chemotherapy and autologous hematopoietic progenitor cell rescue, an approach similar to those being used in the treatment of brain tumors in infants. With use of the Head Start regimen, which takes this approach, improved outcomes have been reported in young children who have supratentorial PNETs, including pineoblastomas.[321]

Because of the poor prognosis of trilateral retinoblastoma, screening neuroimaging is a common practice. One fourth of the cases in the literature correspond to cases found during screening.[131] Given the short interval between the diagnosis of retinoblastoma and the occurrence of trilateral retinoblastoma, routine screening might detect the majority of cases within 2 years.[131] Although it is not clear whether early diagnosis can have an impact on survival,[322,323] it is usually recommended that neuroimaging be performed every 6 months until 5 years of age.[127,131,323,324]

(Extracranial) Metastatic Retinoblastoma

Hematogenous metastases may develop in the bones, bone marrow, and, less frequently, in the liver.[302,308,311,312,315,325,326] Although long-term survivors treated with conventional chemotherapy have been reported,[326] these cures should be considered anecdotal; metastatic retinoblastoma is not curable with conventional chemotherapy.[302,312] In recent years, however, small series of patients have shown that metastatic retinoblastoma can be cured with use of high-dose, marrow-ablative chemotherapy and autologous hematopoietic progenitor cell rescue (Table 56-3).[307,308,311,315,325,327,328] The approach is similar to that used with metastatic neuroblastoma; patients receive short and intensive induction regimens that usually contain alkylating agents, anthracyclines, etoposide, and platinum

TABLE 56-3 Recommended Chemotherapy Regimen for Metastatic Retinoblastoma

		DOSES		
Phase	**Drugs**	**<36 months**	**>36 months**	**Days**
Induction (4 cycles)	VCR	0.05 mg/kg	1.5 mg/m^2	1, 8, 15
	CDDP	3.5 mg/kg	105 mg/m^2	1
	CYC	65 mg/kg	1950 mg/m^2	2, 3
	ETO	4 mg/kg	120 mg/m^2	2, 3
Consolidation	CBP	AUC 7 (maximum 16.7 mg/kg)	AUC 7 (maximum 500 mg/m^2)	−8, −7, −6
	TT	10 mg/kg	300 mg/m^2	−5, −4, −3
	ETO	8.3 mg/kg	250 mg/m^2	−5, −4, −3

From Dunkel IJ, Khakoo Y, Kernan NA, et al: Intensive multimodality therapy for patients with stage 4a metastatic retinoblastoma. Pediatric Blood Cancer *55:55–59, 2010.*

AUC, Area under the curve; *CBP,* carboplatin; *CDDP,* cisplatin; *CYC,* cyclophosphamide; *ETO,* etoposide; *TT,* thiotepa; *VCR,* vincristine.

compounds, and consolidation is then performed with marrow-ablative chemotherapy and autologous hematopoietic cell rescue. With use of this approach, the outcome appears to be excellent. As with any "megatherapy" consolidation, the agents selected may be important. In general recurrences are intracranial, and for this reason, agents with proven efficacy in intracranial retinoblastoma should be used. In this regard the combination of carboplatin and etoposide has been shown to be effective against CNS disease,[316] and for this reason it should be part of the regimen. In one of the largest series, seven patients underwent consolidation with the CARBOPEC combination (i.e., carboplatin, 1250 to 1750 mg/m^2; etoposide, 1750 mg/m^2; and cyclophosphamide, 6.4 g/m^2). Five of these patients were cured, and the regimen failed in two patients because of CNS relapse. In two other series a thiotepa-based consolidation regimen has been used (i.e., thiotepa, 900 mg/m^2; etoposide, 750 to 1200 mg/m^2; and carboplatin, 1500 mg/m^2). A strong rationale exists for using thiotepa: retinoblastoma is responsive to alkylating agents such as thiotepa, a group of agents for which dose escalation is shown to overcome resistance. Furthermore thiotepa has excellent CNS penetration.[308,315,329] An interesting observation is that patients with distant bone metastases (i.e., outside the orbit and skull) that show good response to induction chemotherapy may not require radiation therapy when they are treated with marrow-ablative chemotherapy.[307,327]

LONG-TERM EFFECTS OF RETINOBLASTOMA AND ITS TREATMENT

Retinoblastoma is a cancer of the very young, and patients are very susceptible to the long-term effects that the disease and the treatments administered may have with regard to organ development and function. More importantly patients with bilateral retinoblastoma are born with a germline mutation of the *RB1* gene, probably the most potent tumor suppressor gene, and are at risk of the development of cancers throughout their lives; in this regard, retinoblastoma may be just the beginning.

Second Malignancies

The cumulative incidence of second cancers in patients with germline mutations of the *RB1* gene increases steadily with age, to up to 30% to 60% at 40 to 50 years of age, although more recent studies estimate a considerably lower risk.[87,330-333] Second malignancies are responsible for an excess risk of mortality in survivors of bilateral disease;[334] up to 50% of the deaths of survivors are due to second cancers.[333] The type of mutation, whether de novo or inherited, appears to influence the risk of second cancers—patients with a family history of the disease have a higher cumulative incidence of second neoplasms when compared with survivors of bilateral retinoblastoma who do not have a family history of the disease.[335] Patients with nonhereditary retinoblastoma are not at increased risk. Most information on second malignancies in retinoblastoma survivors is derived from the prospective follow-up of a cohort of 1601 survivors of retinoblastoma who were diagnosed between 1914 and 1984 at institutions in Boston and New York. The sequential updated analyses of this cohort have provided (and continue to provide) the most reliable description of cancer risk in this population.[87,335-337]

In the most recent analysis, the median follow-up for patients with hereditary and nonhereditary retinoblastoma was 25.2 years and 29.5 years, respectively.[87] The standardized incidence ratio (SIR) in these studies was calculated as the ratio of the observed number of cancers to the expected number from the Connecticut Tumor Registry. The incidence of second cancers was significantly elevated in survivors of hereditary retinoblastoma (SIR, 19 vs. 1.2 in patients with nonhereditary retinoblastoma), and almost any neoplasm has been described in this population[87,338]; similar data have been reported using population-based registries.[333] For hereditary retinoblastoma, the greatest risk (SIR >100) was for neoplasms of bone, connective, and soft tissue, eye and orbit, and nasal cavities. An increased risk (SIR >10) was also noted for pineoblastoma, melanoma, CNS malignancies, and neoplasms of the buccal cavity and corpus uteri. The lowest risk (SIR <10) was seen for neoplasms of the lung, breast, and colon (Table 56-4).

The cumulative incidence of a second neoplasm after 30 years from diagnosis of retinoblastoma is greater than 30%.[87,330,331,333,338] Reports differ on the risk, and cumulative incidences in excess of 50% have been reported.[331,338] The more accurate data probably derive from the Boston–New York cohort, which illustrates the changes in cancer

TABLE 56-4 Risk and Type of New Cancers in 963 Survivors of Hereditary Retinoblastoma

Cancer Site	Observed (%)	SIR (95% CI)
All sites	260 (100)	19 (16-21)
Bone	75 (28.8)	360 (283-451)
Soft tissue	34 (13)	122 (84-170)
Nasal cavities	32 (12.3)	1111 (760-1569)
Melanoma	29 (11.1)	28 (18-40)
Eye and orbit	17 (6.5)	266 (155-426)
Brain	10 (3.8)	13.6 (6.5-25)
Breast	10 (3.8)	3.96 (1.9-7.3)
Corpus uteri	7 (2.7)	20 (8.0-41)
Buccal cavity	7 (2.7)	20 (8.2-42)
Lung	5 (1.9)	5.94 (1.9-14)
Pineoblastoma	5 (1.9)	90.8 (29-212)
Colon	3 (1.1)	6.28 (1.3-18)
Hodgkin lymphoma	3 (1.1)	3.4 (0.7-10)
Bladder	2 (0.7)	6.15 (0.7-22)
Thyroid	2 (0.7)	3.34 (0.4-12)
Leukemia	2 (0.7)	2.25 (0.3-8.1)

Modified from Kleinerman RA, Tucker MA, Tarone RE, et al: Risk of new cancers after radiotherapy in long-term survivors of retinoblastoma: an extended follow-up. J Clin Oncol *23:2272–2279, 2005.*

CI, Confidence interval; *SIR,* standardized incidence ratio.

risk estimates with longer follow-up. The cumulative incidence of second cancers at 50 years in survivors of hereditary retinoblastoma was estimated to be 51% in the analysis reported by Wong et al.[331] in 1997. This figure decreased to 36% in the analysis of the same cohort reported by Kleinerman et al.[87] in 2005, probably reflecting the lower doses of scatter radiation received by patients treated in more recent eras. Although patients have a lifetime risk of developing additional malignancies, the second malignancy usually occurs in the first decades after the diagnosis of retinoblastoma. The median age at diagnosis of a second neoplasm is 15 to 17 years.[276,331] However survivors of retinoblastoma continue to have an increased risk of second malignancy–associated death throughout their life.[333,337] Furthermore survivors of a second neoplasm appear to have an increased risk of developing a third and a fourth malignancy, which occur at shorter intervals. The cumulative incidence of a third neoplasm is 22% at 10 years from the time of diagnosis of the second cancer, and it occurs at a median of 5.8 years.[339]

Radiation therapy plays a major role in the risk of second neoplasms in patients with hereditary retinoblastoma. The second tumorigenic event ("second hit") is usually chromosomal in nature, often as a result of mitotic recombination errors.[100,340] This second hit is very sensitive to environmental factors such as ionizing radiation, thus explaining the increased risk of radiation-induced malignancies in survivors of retinoblastoma.[341] Radiation results in a significant increase in the incidence of second cancers in patients with hereditary disease; the cumulative probability of developing a second malignancy is 38% in patients who have been treated with radiation versus 21% in patients who have not been treated with radiation.[87] The greatest risk (SIR >100) is for neoplasms of bone, soft tissues, nasal cavities, and orbits, and for pineoblastoma. The risk of second cancer appears to correlate as well with timing of radiation; children receiving radiation therapy during their first year may have a higher risk of developing a second cancer.[276,337] However the need for earlier radiation may also be an indication of a biologically and clinically more aggressive disease. Changes in radiation techniques over the years are having an impact on cancer risk. As shown in the cohort reported by Kleinerman and colleagues,[87] the use of orthovoltage prior to 1960 was associated with a higher risk of second malignancies than radiation techniques used in subsequent decades; the cumulative incidence of second cancers at 40 years was 32.9% and 26.3%, respectively. With the evolving treatment approaches for intraocular retinoblastoma, which may decrease the overall need for external beam radiation, and the development of newer radiation techniques, such as conformal, intensity-modulated, and proton-beam radiation, it is likely that a further decrease in cancer risk will be observed. In a cohort of British retinoblastoma survivors born before 1950, prior to the routine use of radiation therapy, fewer than 15% of second tumors were sarcomas, highlighting the important role of radiation therapy in the development of those malignancies.[338] Population-based studies have shown a significant decline in the use of radiation therapy during the past decades, from 30.5% of patients in the 1980s to less than 3% after 1999.[333] This change in practice is likely to lead to a further decline in the risk of second cancers in this population.

Traditionally an excess risk has been attributed solely to the use of radiation therapy; the added risk of the use of chemotherapy is not fully understood. In survivors of childhood cancer, an increased risk of second malignancies has been documented, particularly osteosarcoma, which is associated with the combined use of chemotherapy and radiation therapy.[342] A similar phenomenon may occur in retinoblastoma survivors, and the most recent analyses suggest a synergistic effect in carcinogenic risk with the use of chemotherapy. The overall risk of cancer for persons with hereditary retinoblastoma who received radiation and chemotherapy was 25, compared with 19 for persons who received only radiation. Most of this difference was accounted for by the higher risk noted for osteosarcomas; the SIR was 539 for combined treatment and 302 for radiation only.[87]

Sarcomas of bone and soft tissues account for more than 40% of all second neoplasms, followed by neoplasms of the nasal cavities and melanoma (Table 56-4).[87,331] Sarcomas in general and osteosarcoma in particular are cancers of the young retinoblastoma survivor; 76% of all cancers in the irradiated patients occurring at less than 25 years are sarcomas versus 33%

in nonirradiated patients. Conversely 76% of cancers diagnosed at less than 25 years of age are sarcomas, whereas sarcomas represent fewer than 50% of cancers in older patients; in irradiated patients not treated with radiation, the proportion was 33% in persons younger than 25 years and 8% in persons older than 25 years.[87,338]

The role of imaging screening for the early detection of second malignancies has not been fully addressed. One study using whole-body MRI concluded that although this approach had good specificity, its sensitivity was low (66.7%).[343] However age- and disease-specific screening methods may need to be evaluated.

Osteosarcoma

Osteosarcoma as a second malignancy is often associated with retinoblastoma. In the general population only 7% of osteosarcomas follow other malignancies, and almost half of these cases occur in retinoblastoma survivors.[344] Similarly although multifocal osteosarcoma accounts for only 4% of all osteosarcomas, 25% of those occur in retinoblastoma survivors.[345] Osteosarcoma is the most common malignancy in survivors of retinoblastoma, both in the irradiated and the nonirradiated areas, and account for 25% to 40% of all second malignancies.[87,276,337,346-348] One half to two thirds of osteosarcomas occur in the irradiated fields of the skull and face,[87,276,346-348] one third of tumors develop in the extremities, and fewer than 10% develop in the trunk.[87] The SIR is 360; it is higher in irradiated fields (SIR 406), but it is still significantly increased in nonirradiated fields (SIR 69). Osteosarcoma occurring in patients with retinoblastoma who have been treated with radiation therapy accounts for 30% of all osteosarcomas of the head and neck.[349]

The clinical presentation is similar to that of conventional osteosarcoma, and the clinical behavior does not seem to be different; fewer than 20% of the cases are metastatic.[350] Osteosarcomas occur in young patients, both in irradiated and nonirradiated fields, usually peaking during the adolescent years.[276,346,350] However osteosarcomas that occur during the first decade of life are usually in the irradiated fields.[350] With appropriate multidisciplinary treatment, secondary osteosarcoma occurring in retinoblastoma survivors is curable.[351,352] However a major limitation is the common occurrence in the head and neck, which are sites where local control is challenging.[347-349]

Ewing sarcoma, although rare, has also been described in retinoblastoma survivors. It accounts for 15% to 25% of all bone tumors, almost always occurring outside the radiation field.[330,348] Ewing sarcoma in retinoblastoma survivors accounts for approximately 16% of cases of Ewing sarcoma that occur as a second malignancy.[353]

Soft Tissue Sarcomas

Soft tissue sarcomas are the second most common malignancy in retinoblastoma survivors, with an SIR of 122, and they account for approximately 10% to 15% of all second malignancies.[337,354,355,356] Soft tissue sarcomas are closely associated with the use of radiation; the SIR is much higher in irradiated fields (140) than in nonirradiated fields (23). In the Boston–New York cohort, the cumulative risk for soft tissue sarcomas at 50 years after radiation therapy was 13.1%.[354,356] Approximately 10% of all secondary sarcomas in children surviving cancer occur in retinoblastoma survivors.[355]

As with osteosarcoma, most soft tissue sarcomas occur in the irradiated fields; sarcomas of the head and face account for 70% of the cases, with the remaining being in the trunk (20%) and limbs (<5%).[276,346-348,354,356] A very characteristic spectrum of histologies has been described (Table 56-5). Leiomyosarcoma is the most common subtype, accounting for approximately one third of the cases, followed by fibrosarcoma, malignant fibrous histiocytoma, rhabdomyosarcoma, nonspecified sarcomas, and liposarcoma. When compared with the normal population, SIR is significantly elevated for leiomyosarcoma (390), fibrosarcoma (398), and rhabdomyosarcoma (279), but it is also higher for malignant fibrous histiocytoma (100) and liposarcoma (99).[356] A correlation exists between radiation exposure and histologic subtype; fibrosarcoma, rhabdomyosarcoma, malignant fibrous histiocytoma, and nonspecified subtypes almost always occur

TABLE 56-5 Risk and Type of Soft Tissue Sarcomas in 963 Survivors of Hereditary Retinoblastoma

	TREATMENT					
	All Treatments		Radiation		No Radiation	
Histology	Observed (%)	SIR (95% CI)	Observed	SIR (95% CI)	Observed	SIR (95% CI)
Total	69 (100)	184 (143-233)	66	212 (164-270)	3	47 (9.4-137)
Soft tissue sarcoma NOS	10 (14.5)	96 (46-177)	10	115 (55-211)	0	0.0 (0.0-218)
Fibrosarcoma	13 (18.8)	398 (211-681)	13	475 (253-813)	0	0.0 (0.0-691)
Malignant fibrous histiocytoma	12 (17.4)	100 (52-175)	11	110 (55-197)	1	51 (0.66-284)
Liposarcoma	3 (4.3)	99 (20-286)	3	120 (24-351)	0	0.0 (0.0-329)
Leiomyosarcoma	23 (33.3)	390 (247-585)	22	476 (298-720)	1	78 (1.0-436)
Rhabdomyosarcoma	8 (11.6)	279 (120-551)	7	281 (112-578)	1	271 (3.5-1510)

Modified from Kleinerman RA, Tucker MA, Abramson DH, et al: Risk of soft tissue sarcomas by individual subtype in survivors of hereditary retinoblastoma. J Natl Cancer Inst *99:24–31, 2007.*

CI, Confidence interval; *NOS,* not otherwise specified; *SIR,* standard incidence ratio.

in the irradiated fields. Conversely leiomyosarcoma and liposarcoma commonly arise in the nonirradiated fields. Although secondary sarcomas in childhood cancer survivors are associated with increasing doses of anthracyclines or alkylators,[357] the use of chemotherapy does not appear to increase the risk of soft tissue sarcomas in retinoblastoma survivors; however the combined use of radiation and chemotherapy appears to increase the risk for leiomyosarcoma when compared with radiation alone.[356]

Soft tissue sarcomas are a malignancy of the young survivor, and more than half of the tumors are diagnosed in the first 30 years after retinoblastoma diagnosis.[276,346,356] Fibrosarcoma, rhabdomyosarcoma, rhabdomyosarcoma, and malignant fibrous histiocytoma predominantly occur 10 to 20 years from retinoblastoma diagnosis, whereas nonspecified sarcomas occur across all time intervals. An exception to the rule is leiomyosarcoma, which more commonly (78%) occurs after 30 years from retinoblastoma diagnosis, and typically outside the irradiated fields; the SIR for this subtype after 30 years is 435.[28] Very common locations for leiomyosarcoma are the uterus and bladder,[330,337,354,356,358,359] and these tumors may also occur as a radiation-induced malignancy in the paranasal sinuses.[352]

The liposarcoma risk also increases with age.[356] Alteration of the *RB1* gene has been demonstrated in both lipomas and liposarcomas. An interesting observation is the increased incidence of lipomas in survivors of hereditary retinoblastoma. Lipomas were present in 3.6% of patients with hereditary retinoblastoma, compared with only 0.6% in patients with sporadic disease.[360] The incidence of a second neoplasm appears to be higher in patients with lipomas; 30% of patients with lipoma experienced a second malignant neoplasm, compared with 12% of patients without lipoma. These data suggest that the presence of lipomas could be a clinical marker of susceptibility to second neoplasms.[360]

Sarcoma risk and location do not seem to differ by sex, except for leiomyosarcomas. In males, 64% of primaries were in the face, whereas for females, 58% of tumors were in the pelvic area, highlighting the relevance of uterine primary tumors.[356]

Skin Cancers

Skin cancers account for 16% to 19% of all second cancers in survivors of hereditary retinoblastoma, and they occur both inside and outside the irradiated fields, usually within the first three decades of life.[276,330,337,354] The highest risk is for melanoma, but basal cell and squamous cell carcinomas are also common. The SIR for melanoma is 28 in the radiation field and 15 outside the irradiated areas. The risk of developing melanoma is higher in survivors with a family history, compared with persons with de novo mutations.[335] Skin cancer also accounts for approximately one third of all third tumors.[276]

Lung Cancer and Other Common Cancers of Adulthood

In recent years with improvements in treatment that have resulted in longer survival rates, it has become apparent that patients with hereditary retinoblastoma are also at risk of developing epithelial cancers late in adulthood.[337,338] The SIR is elevated for lung, breast, colon, buccal cavity, bladder, and corpus uteri cancers (see Table 56-4).[354] Lung cancer appears to be one of the most common second neoplasms that occur in adulthood, which is not surprising because somatic mutations of the *RB1* gene are known to contribute to the development of lung cancer.[332,361] Compared with the general population, hereditary retinoblastoma survivors have a higher mortality from lung cancer and all other epithelial cancers combined (standard mortality ratio 7.01 and 3.29, respectively).[338] Rather than being epithelial, most bladder and uterine cancers (and a large proportion of colon neoplasms) are probably leiomyosarcomas.[354,356] An increased incidence of breast cancer occurs in patients who have been treated with radiation therapy, but this increased risk is similar for patients with hereditary and nonhereditary retinoblastoma; the dose to the breast from scatter radiation is approximately 0.4 Gy. New reported excess risks include carcinomas of the salivary glands and tongue; low doses of radiation (<5 Gy) have been associated with secondary salivary cancers.[354] It is possible that most of the excess cancer risks might be preventable by limiting exposure to agents that damage the DNA, such as tobacco and ultraviolet light.

Hematologic Malignancies

Patients with hereditary retinoblastoma have a slightly increased risk of developing hematologic malignancies, with an SIR of 2.25 (and an SIR of 1.27 in nonhereditary cases).[354] The use of etoposide and other topoisomerase-II inhibitors such as anthracyclines may add an additional risk to this patient population.[362] However the cumulative doses of those agents are relatively low, and it is therefore assumed that the risk of treatment-related leukemia is minimal. Therapy-related acute myeloid leukemia after the treatment of retinoblastoma has been reported, although its occurrence does not appear to be higher than in other patients receiving etoposide.[363,364]

Trilateral Retinoblastoma

Trilateral retinoblastoma refers to the association of bilateral retinoblastoma with an asynchronous intracranial tumor (Fig. 56-3).[126,129,131] The SIR for trilateral retinoblastoma is 90.8, and it seems to be associated with the use of radiation therapy (SIR is 0 in patients who have not been treated with radiation therapy). Tumors comprising trilateral retinoblastoma are PNETs that exhibit varying degrees of neuronal or photoreceptor differentiation, suggesting an origin from the germinal layer of primitive cells.[128] This association can occur in 3% to 9% of patients with the genetic form and appears to be more common in familial cases. The prognosis is almost uniformly fatal. Trilateral retinoblastoma was the principal cause of death from retinoblastoma during the first decade of life in the United States.[129] The majority of these tumors are pineoblastomas, but in 20% to 25% of the cases, the tumors are suprasellar or parasellar. The association of bilateral retinoblastoma with a tumor in the pineal gland and a fourth primary tumor of suprasellar location is called “quadrilateral retinoblastoma.”

In most cases trilateral retinoblastoma resembles undifferentiated retinoblastomas, with the more frequent formation of Homer-Wright rosettes. The median age at diagnosis of trilateral retinoblastoma is 23 to 48 months,[125,126,129,131] and the interval between the diagnosis of bilateral retinoblastoma and the diagnosis of the brain tumor is usually more than 20 months.[127,131] Suprasellar tumors are usually diagnosed earlier,[42,127] and in 15% to 20% of the cases, the intracranial tumor antecedes the diagnosis of retinoblastoma.[126] In recent years with the more widespread use of chemoreduction treatments for patients with bilateral retinoblastoma and a decrease in the use of radiation, the incidence of trilateral retinoblastoma has decreased dramatically, to the point that patients with the genetic form of retinoblastoma are now considered to be almost protected against this fatal complication.[132] However pineal cysts develop in approximately 5% to 8% of patients with bilateral disease; these cysts appear to be a forme fustre of trilateral retinoblastoma.[133,134,135]

The prognosis for patients with trilateral retinoblastoma is dismal—patients die of disseminated neuroaxis disease in fewer than 9 months.[125,127,131] The rare survivors are usually those diagnosed with screening imaging and treated with intensive chemotherapy with or without craniospinal radiation.[131] Because of the poor prognosis of trilateral retinoblastoma, screening neuroimaging is a common practice. One fourth of the cases in the literature correspond to cases found during screening.[131] Given the short interval between the diagnosis of retinoblastoma and the occurrence of trilateral retinoblastoma, routine screening might detect the majority of cases within 2 years.[131] Although it is not clear whether early diagnosis can have an impact on survival,[322] it is usually recommended that neuroimaging be performed every 6 months until 5 years of age.[127,131,323,324]

OTHER LONG-TERM EFFECTS

Orbital Growth and Facial Asymmetry

Because their orbital growth is still in progress, children treated for retinoblastoma are at risk of functionally and cosmetically significant bony orbital abnormalities. These sequelae become evident by early adolescence, when orbital growth is largely complete, and results in the “hourglass facial deformity.”[274] Both enucleation, which causes orbital contraction, and radiotherapy, which induces arrest of bone growth, adversely affect orbital growth. In children treated for bilateral retinoblastoma, the impact of enucleation in orbital development is not different from that of irradiation. Final orbital volumes after enucleation correlate with the size of the prosthetic implant, but there are no differences in final orbital volumes based on the type of implant.[275,365]

Delay of radiation therapy should therefore be a goal when designing treatment for children with bilateral retinoblastoma. Studies show that the therapeutic strategy of chemoreduction and aggressive focal treatments can successfully delay the use of radiation therapy for at least 6 or 7 months (median age, 21 months).[134,281] In addition to theoretically decreasing the risk of second cancers, delaying radiation therapy may also allow more complete facial and orbital growth to occur, thus reducing the degree of midfacial deformities.[274,275] However with the use of a multidisciplinary approach, the dose of radiation needed for disease control may also be reduced.[82]

Visual Outcome

Visual outcome is frequently good in children treated for retinoblastoma[366]; however maculopathy and deficits in acuity, visual fields, dark adaptation, and visuomotor integration have been reported for many patients.[269,366] Visual outcome correlates with the intraocular stage and, more importantly, tumor location. Visual acuity of 20/40 or better can be achieved in 90% of eyes with extramacular tumors and in 24% of eyes with macular tumors. Overall a final visual acuity of 20/40 or better may be achieved in half of the eyes, and two thirds have a visual acuity of 20/200 or better.[367] However visual outcome is not always easily predicted on the basis of the initial presentation.[368] These findings suggest that visual function in survivors of retinoblastoma is influenced by more distal elements of the visual system, including the primary visual cortex. Because retinoblastoma affects the visual system at an age at which the CNS is still developing,[369] differences in visual outcome among patients with retinoblastoma may be due, at least in part, to disease- or treatment-induced differences in visual field development in the primary visual cortex. New imaging technology such as functional MRI and diffusion tensor imaging may be used to evaluate changes in the visual pathways and the primary visual cortex.[370,371]

Cognitive and Functional Development of Patients with Retinoblastoma

Patients with retinoblastoma may experience a restriction in their development because of their visual limitations. However few studies have addressed the development of these young children. Interestingly the earliest articles describing cognitive development in this population suggested that children with retinoblastoma might have above average or superior intelligence.[372-375] These findings were based on a small number of patients and were never confirmed or unconfirmed, but the idea of superior intelligence became part of the “lore” among clinicians working with the retinoblastoma population. In the report of Ek and colleagues,[366] a population-based study from Sweden, 22 children with retinoblastoma were assessed at 4 and 6 years of age. Patients with both bilateral and unilateral disease showed above-average developmental quotients, with the bilateral group showing higher scores in the superior range. Only two children in the cohort demonstrated below-average development, and both of these patients had bilateral disease and received radiation therapy in the first month of life. The authors concluded that bilateral retinoblastoma is associated with superior cognitive capacities. The other recent series involved 54 patients who were assessed prior to the age of 42 months with the Bayley Scales of Infant Development.[269] No indication of superior intelligence was found in this cohort, but neither was there an indication of increased difficulties. The average mental and motor development scores were in the normal range and did not differ from population norms. Thus although children with retinoblastoma do not appear to be at-risk developmentally, questions regarding the developmental trajectory for this population remain.

Other Late Effects

Platinum compounds are commonly used to treat retinoblastoma. Although the effects of cisplatin on hearing are well documented, evidence is conflicting with regard to the potential ototoxic effects of carboplatin. Although it is usually assumed that carboplatin ototoxicity is extremely rare, very few studies have investigated the cumulative incidence of and prognostic factor for ototoxicity as a result of the use of this platinum agent. In two of the studies reported, no cases of carboplatin-induced hearing loss were documented.[376,377] However infants younger than 6 months appear to be at a higher risk of hearing loss.[378] Thus very careful age-appropriate evaluations of hearing function, including tympanometry, otoacoustic emission measurement, and visual reinforced audiometry, must be performed.[376,379]

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CHAPTER 57

Tumors of the Brain and Spinal Cord

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CHAPTER OUTLINE

EPIDEMIOLOGY
NEURODEVELOPMENT
Neural Tube
Development of Brain Structures
Cancer Stem Cells
Genetic and Signaling Pathways Implicated in Development and in Pediatric Tumors
CONCEPTUAL ORGANIZATION OF PEDIATRIC BRAIN TUMORS
Obstructive Hydrocephalus and Raised Intracranial Pressure
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Chemotherapy
Immunotherapy
Gene Therapy
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Suppression of Tumor Resistance
High-Dose Chemotherapy with Stem Cell Rescue
PEDIATRIC BRAIN TUMORS
Gliomas
Oligoastrocytomas
Embryonal Tumors
Medulloblastomas
Pineocytomas and Pineoblastomas
Supratentorial or Central Nervous System Primitive Neuroectodermal Tumors
Atypical Teratoid Rhabdoid Tumors
Neurocytomas
Choroid Plexus Tumors
Germ Cell Tumors
Craniopharyngiomas
Infant Tumors
Other Tumors
LATE EFFECTS OF TREATMENT
Late Effects of Surgery
Late Effects of Radiation
Late Effects of Chemotherapy
PALLIATIVE CARE

Tumors of the central nervous system (CNS) account for approximately 25% of pediatric cancer but are now the leading cause of cancer-related mortality in children. The complexities of tumors in this site are related to the large number of different histologies within the CNS and a historic nomenclature that is confusing, even to those in this field. With the need to modify therapies to spare important neurocognitive function in the youngest patients and the presence of the blood-brain barrier (BBB), which restricts the delivery of effective therapies, improvement in outcome has lagged well behind that of many other cancers, especially childhood leukemia. The molecular revolution offers the chance to begin classifying tumors by the signals that drive their phenotype rather than by their appearance under the microscope.[1] In this chapter we will discuss the different types of brain tumors in children, their diagnoses, and their treatments, while incorporating the expanding knowledge of tumor biology.

Although clinical studies often focus on progression-free survival (PFS) and overall survival (OS), successful therapy incorporates much more. Accepting a lower overall cure rate but preserving neurocognitive function is the norm for many types of brain tumors, especially those of infants and young children. Optimization of outcome requires expertise in multiple subspecialties that play a role in the care of these children. The skill of the neurosurgeon, the sophistication of the radiation planning, and the safe administration of chemotherapy are all important factors in improving the long-term outcome

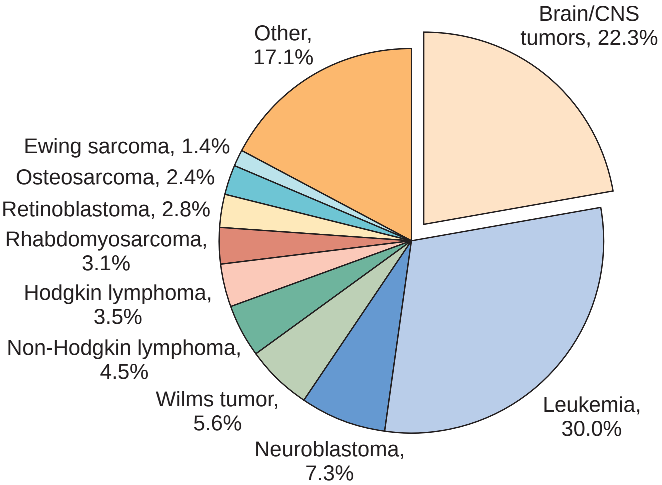

Figure 57-1 Incidence of pediatric cancer. *CNS*, Central nervous system. *(Data from American Cancer Society:* American Cancer Society Cancer Facts & Figures, 2007*, Atlanta, Ga, 2007, American Cancer Society.)*

for these patients.[2] In fact, many centers now use neurooncologists who have completed additional training in outcome optimization. When combined with a large number of subspecialty services (e.g., endocrinology, neurology, neuropsychology, social work, back to school, and physical and occupational therapy), truly optimal care is now possible for this patient population. Needs of the family continue to evolve as patients transition from diagnosis to treatment to posttherapy follow-up.[3,4] A comprehensive understanding of pediatric neurooncology and the delivery of comprehensive care to these patients and their families will be the focus of this chapter.

To assist the reader, a number of important review articles summarizing different aspects of the care of children with CNS tumors are referenced.[5-18]

EPIDEMIOLOGY

Primary CNS (PCNS) tumors rank second behind leukemia as the most common pediatric cancer diagnosed in the United States each year (Fig. 57-1). Brain tumors are the most common form of solid tumors in children and are now the leading cause of death from solid tumors in children. The spectrum of adult brain tumors[19] based on location, histology, and outcome differs significantly from that in pediatrics, suggesting that the causative events are different from those for pediatric brain tumors. No single standard classification system has been implemented for pediatric brain tumors, although development of a standardized platform for epidemiologic studies has been attempted.[20,21] The 2013 Central Brain Tumor Registry of the United States Statistical Report included primary nonmalignant (1.93 per 100,000 children) and malignant (3.33 per 100,000 children) pediatric brain and CNS tumors with a total incidence of 5.26 per 100,000 children.[22] Similarly in the 2013 National Cancer Institute Surveillance Epidemiology and End Results (SEER) Cancer Statistics Report, the annual age-adjusted incidence rate of pediatric malignant brain and other nervous system tumors was listed as 3.1 cases per 100,000 children.[23] The rate is higher in males (3.2 per 100,000) compared with females (2.9 per 100,000). Approximately 4100 new cases of childhood PCNS tumors are diagnosed in the United States each year. Of these, an estimated 3007 will be in children younger than 15 years. The incidence for all brain tumors is highest among 0- to 4-year-olds (5.77 per 100,000) and lowest among 10- to 14-year-olds (4.78 per 100,000), results that are similar to those reported in 2005.[21] The age-adjusted mortality rate for pediatric CNS tumors in 2010 was 0.6 per 100,000 children, resulting in an estimated 500 deaths per year in the United States for those aged 0 to 19 years. The prevalence rate for all malignant and benign pediatric CNS tumors (ages 0 to 19 years) is estimated at 9.5 per 100,000, with more than 26,000 children estimated to be living with this diagnosis in the United States in 2000. However the prevalence rate for patients with only malignant brain tumors was 7.9 per 100,000, with more than 21,000 children estimated to be living with a diagnosis of primary malignant CNS tumor in the United States in 2000.[24] The distribution of pediatric brain tumors by site is presented in Figure 57-2. Different brain tumor histologies have different age distributions (Fig. 57-3). The most common histologies in the younger age group (ages 0 to 14 years) include pilocytic astrocytomas (PAs) and medulloblastomas, which account for 20% and 16% of cases, respectively. The broad category of glioma accounts for 56% of tumors in children younger than 15 years. The most common histologies in adolescents ages 15 to 19 years include PA and pituitary tumors, which account for 15% and 14% of cases, respectively. The broad category of glioma accounts for 45% of tumors in adolescents ages 15 to 19 years. The rates among boys are slightly higher than those in girls, and brain tumors are more common in whites (4.7 per 100,000) than in blacks (3 per 100,000).

The histologic-specific differences in brain and CNS tumor distribution by age and gender suggest that childhood tumors have different mechanisms whereby normal cells, possibly somatic CNS stem cells, are susceptible to oncogenic mutation. Although certain histologic subtypes can also differ by race,[25,26] the overall concordance of tumor histologies among different ethnic groups and different locations suggest that specific local environmental factors are not the cause of most cancers in children.[27-29] The incidence of common pediatric brain tumors such as medulloblastoma, malignant gliomas, and diffuse pontine glioma do not differ significantly in industrialized versus nonindustrialized countries, in vegetarian versus meat-eating societies, and in areas where smoking and drinking are permitted versus where they are not permitted. Similarly death rates for children with CNS tumors between different ethnic groups within the United States (Hispanics, Asians, blacks, and whites) do not differ significantly for most CNS tumor types.[30]

In the mid 1990s an increase in the incidence of childhood brain cancer appeared to occur compared with that in the previous two decades. This increase is now thought to reflect the introduction and widespread use of

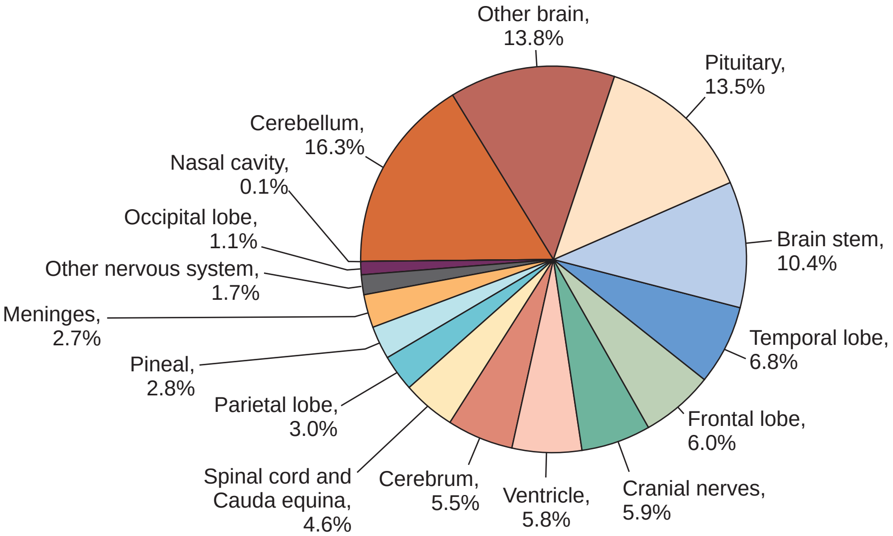

Figure 57-2 Distribution of childhood (ages 0 to 19 years) primary brain and central nervous system tumors by site ($n = 21{,}512$). *(Data from Ostrom QT, Gittleman H, Farah P, et al: CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2006-2010.* Neuro Oncol *15[Suppl 2]:ii1–ii56, 2013; and Central Brain Tumor Registry of the United States:* 2007-2008 Statistical Report: Primary Brain Tumors in the United States Statistical Report, 2000-2004 [Years of Data Collected], *Chicago, Ill., 2008, University of Illinois at Chicago School of Public Health.)*

magnetic resonance imaging (MRI) technology in the mid 1980s, resulting in improved detection and reporting of pediatric brain tumors. More precise classification of brain tumors and diagnostic capabilities, such as stereotactic biopsy, also may have contributed to the increase in incidence. The rise in incidence was followed by the establishment of a new baseline that has remained stable. Mortality rates have not mirrored the increase in incidence.[31]

PCNS tumors develop from an accumulation of genetic changes. Such changes can result from inherited mutations or develop from exposure to chemical, physical, or biologic agents that damage deoxyribonucleic acid (DNA). Unlike in adults, for whom lifetime exposure is significant, most pediatric tumors are believed to be the result of random genetic mutations that occur during normal cellular proliferation. Today molecular biologic techniques are used to unravel the complex genetic errors that lead to the development of CNS tumors. To date most pediatric brain tumors have demonstrated a limited number of mutations, even in highly aggressive tumors, suggesting that their presence during development or within the stem cell compartment is an important aspect of tumorigenesis in pediatric patients.[32]

The search for causative factors that place children at risk for developing CNS tumors has not yielded clear answers.[33] Numerous epidemiologic studies have evaluated potential risk factors. Similar to most pediatric cancers, no specific risk factor explains more than a small proportion of tumors.[34] Factors studied but not conclusively found to increase risk include exposure to tobacco and smoke,[35] alcohol, traffic-related air pollution, electromagnetic fields, pesticides, and occupational and industrial chemicals; diet; drugs and medications; infections and viruses; epilepsy; and consumption of cured meats during pregnancy.[36-45] The dramatic increase in the use of cellular telephones has generated concerns about the potential risk for the development of brain tumors. A meta-analysis of nine case-control studies concluded that cellular phone users have no overall increased risk of brain tumors. The potential risk after long-term cellular phone use awaits analysis in future studies.[46] A seasonal variation unique to medulloblastoma incidence by month of birth may provide evidence for an environmental exposure cause, although further studies are needed.[47] An association between atopic disease and a reduced risk of glioma has been observed in adult epidemiologic studies, with the implication that heightened immune surveillance decreases the risk of brain tumor development.[33,48] Prenatal multivitamin use has been associated with a protective effect in the development of pediatric brain tumors in a large meta-analysis.[49] Confirmation of this result in a prospective trial is needed.

The role of viruses in the pathogenesis of tumors has been documented in experimental animals, and adenovirus serotypes have been shown to induce tumors in rodents. Adenoviral sequences were evaluated in more than 500 tumors derived from 17 different pediatric cancer entities. Although most leukemias and solid tumors were negative for the presence of adenoviral sequences, tumor material from 25 of 30 glioblastomas, 22 of 30 oligodendrogliomas, and 20 of 30 ependymomas, as well as normal brain, were positive by polymerase chain reaction assay for adenoviral gene sequences. This finding raises important questions about the contribution of this infectious agent to pediatric brain tumorigenesis.[50] In contrast, tests for polyomavirus sequences in adult and pediatric CNS tumors were rarely positive.[51]

Ionizing radiation, immunosuppression, and certain hereditary genetic disorders are the only factors that have been proven thus far to increase a child's risk for CNS malignancy. Ionizing radiation exposure is a

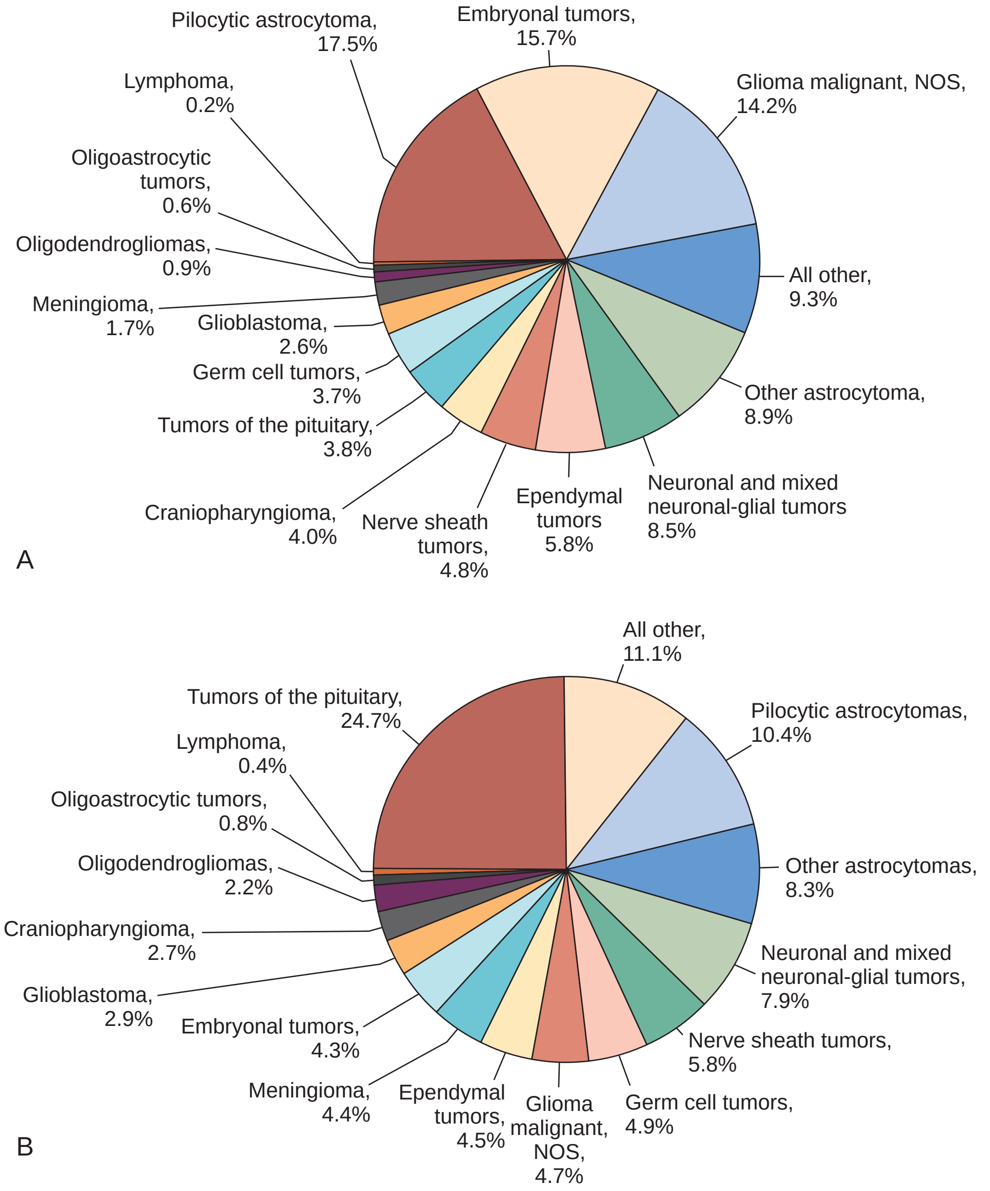

Figure 57-3 **A,** Distribution of childhood primary brain and central nervous system (CNS) tumors by histology and age (ages 0 to 14 years; $n = 15{,}398$). **B,** Distribution of childhood primary brain and CNS tumors by histology and age (ages 15 to 19 years; $n = 6114$). *NOS,* Not otherwise specified. *(Data from Ostrom QT, Gittleman H, Farah P, et al: CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2006-2010.* Neuro Oncol *15:ii1–ii56, 2013.)*

well-documented cause of brain tumors.[52] Children who undergo therapeutic irradiation to the CNS for the treatment of malignancy are at risk for development of a second tumor, specifically meningioma, high-grade glioma (HGG), or sarcoma. Since its introduction in the 1970s, computed tomography (CT) has become an essential tool in the diagnosis and monitoring of disease. The growing use of CT scans has raised concerns about potential risks. Pediatric CT scans may result in a small but not negligible increased lifetime risk for cancer mortality.[53]

Immunocompromised children are at increased risk for PCNS lymphoma. The risk for developing CNS lymphoma is 1% to 5% higher for adults and children undergoing transplantation and for those with congenital immunodeficiencies. The risk is 2% to 6% higher for persons with acquired immunodeficiency syndrome (AIDS). This risk will probably increase with longer survival because of improved AIDS treatment.

In summary although a few environmental factors are associated with an increased risk of developing a pediatric CNS tumor, the vast majority of patients have no easily identifiable risk factors. For a small percentage, inherited genetic mutations contribute to the onset of these tumors, but for the remainder, CNS tumors are likely the result of spontaneous mutations.

NEURODEVELOPMENT

Our understanding of the steps in hematopoietic development, together with the pathways and markers that distinguish precursors along each blood cell lineage, has been instrumental in allowing better classification and subsequently better treatment of leukemias. In the same way that leukemias can be viewed as deregulated expansion of hematopoietic precursor cell pools, pediatric brain tumors may similarly be considered as the proliferation of neuropoietic precursors. Thus knowledge of the steps and intermediates in neural development may help us understand and treat pediatric brain tumors. A current schema for neuropoiesis relies heavily on models for hematopoiesis but introduces two additional aspects of developmental regulation, the first of which is the importance of regionalization. During neural development a rostral-caudal gradient delineates distinct zones for proliferation and differentiation, while the orientation of proliferating cells relative to the dorsal-ventral axis provides the basis for determining the progeny of each proliferative event.

The second aspect of neuropoiesis is the concept of mitogenic niches. Although hematopoietic stem cells can proliferate and give rise to the entire array of blood cell types when exposed to the environment of the immature or mature bone marrow, neural stem cells use a number of distinct niches, some of which are eliminated before birth, some that persist through early childhood, and some that remain extant into adult life. These niches provide important cues for proliferation and differentiation but may also provide an environment that fosters the growth of tumor cells.

Neural Tube

The nervous system develops as a specialized zone of the epithelium. In the third week after fertilization, the midline zone of the epithelium becomes specialized as the neural plate. This distinctive zone extends from the caudal to the rostral portion of the embryo. While the embryo turns, the neural plate grows and folds (Fig. 57-4). Subsequent fusion of the folds creates a discrete neural tube that zips up from both the top and bottom. The two last places where the fold fuses are the hindbrain (the incipient cerebellum) and the lumbar spine. Cells at the crest of the developing neural tube are the neural crest cells, which give rise to the peripheral nervous system, including sympathetic ganglia, dorsal root ganglia, and Schwann cells, as well as to melanocytes in the developing skin. The neural crest cells are the precursors to neuroblastomas,[54] neurofibromas,[55] and melanomas.[56]

After the neural tube fuses, rapid expansion of cell number continues, but this expansion occurs very differently along the rostral-caudal axis. The dramatic expansion of cell number in the rostral neural tube provides the building blocks for the brain, whereas the more caudal regions undergo more limited growth and engender the spinal cord.

Development of Brain Structures

Along the rostral-caudal axis of the neural tube, three outpouchings can be seen at the end of the fourth week after fertilization—the forebrain, midbrain, and hindbrain (Fig. 57-5). Subsequent branching of the forebrain forms two lateral protrusions that are destined to become the left and right cortex, and the midline portion of the forebrain gives rise to the thalamus. The midbrain does not undergo much expansion, but the hindbrain undergoes massive proliferation to give rise to the cerebellum and underlying pons, as well as the medulla. Cerebral cortical tumors, including supratentorial primitive neuroectodermal tumors (CNS PNETs) and cortical and subependymal astrocytomas, all derive from the forebrain. Posterior fossa tumors, including the distinctive pontine gliomas, medulloblastomas, and cerebellar PAs, all derive from the hindbrain. Thus the areas of the brain that undergo rapid expansion in early life engender most pediatric brain tumors. Along the dorsal-ventral plane of the neural tube, greater proliferation of neural tube precursors occurs in the dorsal part of the tube than in the ventral tube. Proliferation of more dorsal precursors gives rise to the multilayer structures of the cerebral and cerebellar cortex; these areas are also the regions that give rise to many pediatric brain tumors.

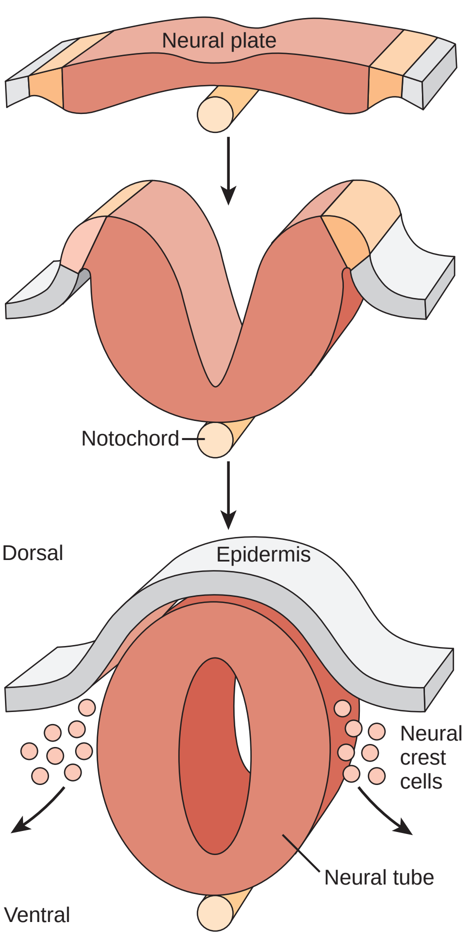

Figure 57-4 Growth and folding of the neural plate.

Forebrain and Cerebral Cortex

In the forebrain the open spaces within the lateral outpouchings of the neural tube become the lateral ventricles, and proliferation largely occurs adjacent to the ventricular zone (VZ) in the medial and lateral ganglionic eminences, in the subventricular zone (SVZ) or subependymal zone. Early in development, neural stem cells/precursors divide extensively to provide the cellular elements of the cerebral cortex, thalamus, and basal ganglia (see Fig. 57-5, *A*).

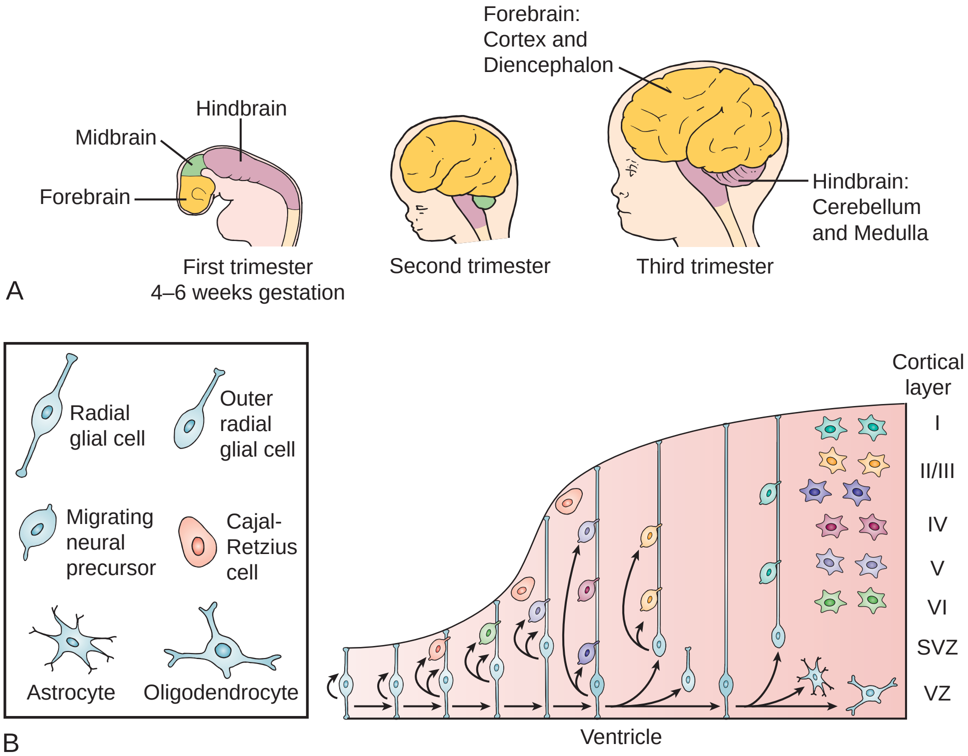

Figure 57-5 **A**, Outpouching of the neural tube. **B**, Cortical Development process. Radial Glia (RG) function as neural stem/precursor cells. Initially, the population of RG expands by proliferation, and the early RG produce a largely transient population of Cajal-Retzius cells. RG continue to proliferate and produce: (1) outer RG located in the Subventricular zone *(SVZ)*, (2) more RG, and (3) early cortical neurons. Neurons produced by the RG and the outer RG migrate along the RG to the cortex. Neurons are generated in an inside-out pattern so that the earliest neurons are found in the deep cortical layers. After most of the neurons have been generated, RG give rise to astrocytes and oligodendrocytes. The cell of origin for astrocytomas may be residual RG, outer RG, or astrocytes.

Although the VZ in the forebrain initially contains the dividing neural stem/progenitor cells, during development the size of the VZ progressively decreases while the adjacent SVZ increases in size. The early generated cells migrate radially from the VZ and SVZ to become the neurons of the innermost cortical layer (layer VI), and later mitoses give rise to neurons that also migrate radially and occupy increasingly superficial layers. Inhibitory neurons are derived from precursors in the medial ganglionic eminence. They migrate tangentially throughout the cortex. Thus the cortex develops in an inside-out pattern. Toward the end of the prenatal neurogenic phase, the precursors of the VZ and SVZ generate glial cells, including astrocytes, oligodendrocytes, and ependymal cells.[57,58]

A small population of precursors remains in the SVZ, just above the ependymal cells, throughout life. These neural stem cells continue to generate glial cells and oligodendrocytes and also continue to give rise to a limited number of neuronal cells through adult neurogenesis. An additional zone of adult neurogenesis is located in the hippocampus, adjacent to the granular zone. Thus the neural stem cell represents a common precursor for glial and neuronal cells. Accumulating data suggest that many, if not most, brain tumors arise from such stem cells or their early derivatives.

Cerebellar Cortex

The cerebellar cortex develops in a way that has some similarities to, but some differences from, the pattern in the cerebral cortex. The hindbrain is the site where the neural tube closes last. While closure occurs, the neural folds pucker to form the rhombic lips. These two protrusions will give rise to many cell types of the cerebellum and pons. Cells in the VZ of the upper rhombic lip proliferate and then begin an unusual migration pattern. The precursor cells of the rhombic lip migrate over the top of the rhombic lip and disperse by moving from the caudal aspect of the pucker to cover the rhombic lip or incipient cerebellum. The rhombic lip–derived precursors settle in a zone that covers the developing cerebellum, which is called the external granule cell (external germinal cell [EGL]) layer. This layer constitutes a site of extensive postnatal proliferation and is a specialized mitogenic niche where precursors divide and give rise almost exclusively to cerebellar granule cells, the most numerous neuronal cells in the brain. This secondary proliferative zone may be necessary to generate this vast number of granule cells. To generate the 60 to 80 billion granule cell neurons, the granule cell precursors in the EGL undergo many rounds of cell divisions, beginning in the ninth week after fertilization and continuing through the first 18 months of life in humans. Other stem/precursors in the VZ follow distinct differentiation paths. Radial glia adjacent to the cerebellum provide one source of these multipotential stem/precursors. Some VZ stem/precursors migrate toward the cerebellar white matter, where they can give rise to cerebellar interneurons and glia. Others migrate past the cerebellar white matter and form the Bergmann glia of the cerebellar cortex.

It has been suggested that four distinct subtypes of cerebellar stem/precursors each give rise to a distinct subtype of medulloblastoma. A great deal of evidence has indicated that the granule cell precursors of the EGL are a cell of origin for the sonic hedgehog (SHH) subtype of medulloblastoma.[59] First, in very young children, medulloblastomas are often continuous with the EGL, and an intermediate zone of dysplastic cells can sometimes be seen to join the EGL and tumor tissue. Second, the appearance and pattern of gene expression of the granule cell precursors resemble those of SHH-type medulloblastoma. Finally, SHH signaling pathways that normally regulate proliferation of granule cell precursors are constitutively active in one type of medulloblastoma (discussed later).

The precursor cells adjacent to the ventricle that resemble radial glia have been suggested to be the cells of origin of Wingless (Wnt)-subtype medulloblastomas. In mouse models, activation of the Wnt pathway and/or activation of phosphoinositide 3′ kinase (PI3K) in these radial glial precursors can mimic this group of medulloblastomas.

The cellular origin and the oncogenic mutations responsible for initiating other medulloblastoma subtypes have also been identified. For example, it has been suggested that the stem cells of the white matter and earlier stem cells may provide the cellular origin for group III and IV medulloblastomas, respectively.[60] It has been suggested that less specialized stem cells can give rise to medulloblastomas. Indeed molecular characterization of medulloblastomas suggests that genetically distinct tumor types exist that may represent the oncogenic transformation of cerebellar precursor cells at different locations and stages.[61,62] This scenario would be analogous to leukemias, in which oncogenic transformation of hematopoietic precursors at distinct developmental stages leads to distinct types of leukemias.

Cancer Stem Cells

Brain tumors have predominantly been classified as neuronal or glial in nature. The neuronal tumors include CNS PNETs, pineoblastomas, and medulloblastomas, as well as ganglion cell tumors. Glial tumors include many different gliomas, such as juvenile PA, subependymal giant cell astrocytoma, other low-grade astrocytomas, pontine glioma, malignant astrocytoma (including glioblastoma multiforme), and tumors that resemble other glial cell types, such as oligodendroglioma and ependymoma. Although this classification schema remains useful, it appears that many brain tumors are generated by oncogenic mutations in neural stem–precursor cells, rather than more mature cell types. Furthermore although cancers traditionally have been viewed as clonal, increasing evidence indicates that this is not the case. Instead the concept of the existence of a subpopulation of cancer stem cells has developed—distinctive cells within the tumor that are uniquely capable of regenerating the cancer.[63] Recent studies of brain tumors have identified cluster of differentiation (CD)-133–positive cells as radioresistant, slowly proliferating cancer stem cells that are particularly prevalent in high-grade tumors, such as glioblastoma multiforme. The ability of these cancer stem cells to survive surgical resection, radiation, and cytotoxic chemotherapy is a major reason for the difficulty in curing high-grade brain tumors.

Genetic and Signaling Pathways Implicated in Development and in Pediatric Tumors

Inherited disorders that cause a familial propensity for brain tumors have provided an important method for identifying genetic pathways that contribute to these cancers.[64-68] Neurofibromatosis, tuberous sclerosis, Gorlin syndrome, Turcot syndrome, Cowden syndrome, and the *SMARCB1* (switch/sucrose nonfermentable [SWI/SNF]–related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1) mutation all represent heritable disorders associated with an increased risk of brain tumors (Table 57-1).[69]

Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF-1) is an autosomal-dominant neuroectodermal disorder characterized by café-au-lait spots and fibromatous tumors of the skin. Additional clinical features that can be seen in NF-1 (Box 57-1) include Lisch nodules in the iris, scoliosis, cognitive problems, and epilepsy.[70-72] Several tumors occur with greater frequency in persons with this disorder, including pheochromocytoma, ependymoma, meningioma, and glioma; this characteristic may relate to dysregulation of specific stem cell populations.[73] Among these tumors, gliomas of the optic pathway are the most common tumors seen.[74] The unique biology of these tumors is slowly being elucidated through studies of the role of NF-1 in the development of the optic pathway in animal models.[75] Neurofibromatosis is caused by heterozygous mutations in neurofibromin, and the cancers observed in this disorder result from loss of heterozygosity at chromosome 17q11.2, leaving only the mutant NF-1 allele.[76] Although this disorder is inherited as an autosomal-dominant disease, as many as 50% of patients represent new germline mutations and therefore do not have a family history of the disorder.

The neurofibromin protein is a 250-kD tumor suppressor that functions as a guanosine triphosphatase activator for the small G protein Ras.[77] In this way, active neurofibromin decreases the ratio of guanosine triphosphate–bound (active) to guanosine diphosphate–bound (inactive)

TABLE 57-1 Common Chromosomal Abnormalities Associated with Pediatric Central Nervous System Tumors

Chromosomal Abnormality	Tumor
Monosomy 22	Atypical teratoid-rhabdoid tumor Acoustic neuromas Meningioma Ependymoma
1p and/or 22q loss	Oligodendroglioma
Isochrome i17	Medulloblastoma
9q22 loss (*PTCH1* gene)	Medulloblastoma
Loss of chromosome 10, 9p, 17p	Progression to high-grade glioma

Ras or Ras-like protein. While activated Ras stimulates mitogen-activated protein kinases (MAPKs) and PI3Ks, the change in Ras activity leads to unregulated proliferation and survival (Fig. 57-6).[78] Although the incidence of brain tumors, particularly optic nerve gliomas, is significantly increased in persons with NF-1 (approximately 5% to 15%), the tumors that develop in these patients tend to be less aggressive than other gliomas. These tumors are more susceptible to chemotherapeutic interventions and thus can be treated differently than other gliomas. In fact many tumors stop growing spontaneously. The unique developmental environment of the optic pathways may account for the differential occurrence of these tumors in patients with NF-1, as well as their increased responsiveness.[79] In addition to optic pathway gliomas (OPGs), non-OPGs occur at frequencies 100 times greater than expected, with the most common sites being the brainstem (49%), cerebral hemispheres (21%), and basal ganglia (14%). Other MRI signal abnormalities within the brain are often observed in patients with NF-1.[80,81] The most characteristic abnormality is the unidentified bright object.[82] Unlike low-grade gliomas (LGGs), these lesions are bright on T2-weighted imaging, do not demonstrate contrast enhancement, and usually produce neither mass effect nor symptoms. They often come and go and should not be biopsied or treated. The varied intracranial localization of lesions and variable need for neurosurgical intervention in a subset of children with NF-1 suggests that radiologic surveillance should be based on careful and regular neurologic and ophthalmologic examinations.[83]

Box 57-1 Diagnostic Criteria of Neurofibromatosis Type 1*

Six or more café-au-lait spots ≥1.5 cm in postpubescent individuals or >0.5 cm in prepubescent individuals
Two or more neurofibromas or one or more plexiform neurofibromas
Freckling in the axillae or groin
Optic glioma
Two or more Lisch nodules
Dysplasia of the sphenoid bone or dysplasia or thinning of the cortex of long bones
A first-degree relative with neurofibromatosis type 1

*The diagnosis of neurofibromatosis type 1 requires any two or more of these criteria.

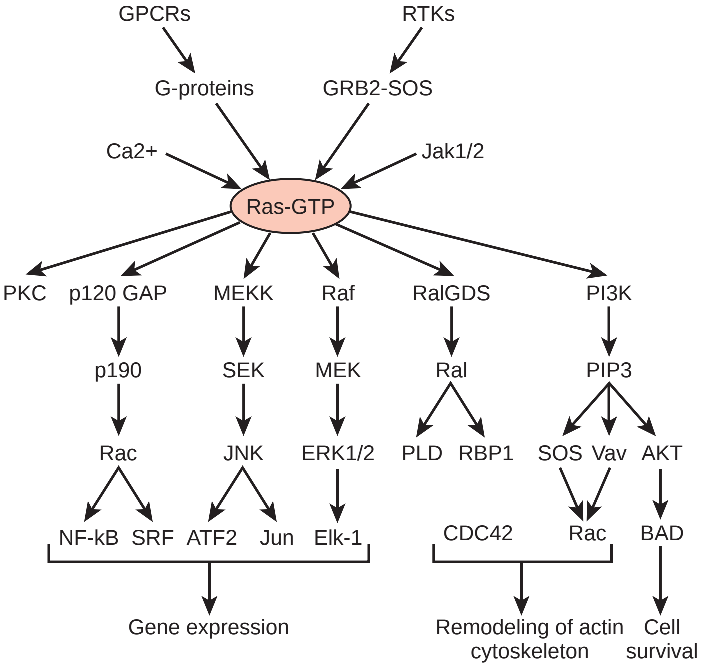

Figure 57-6 Ras and downstream pathway. *AKT,* V-Akt Murine Thymoma Viral Oncogene Homolog; *ATF2,* activating transcription factor 2; *Bad,* bcl-2-associated death promoter; Ca2+, Calcium; *CDC42,* cell division cycle 42; *Elk-1,* ETS domain-containing protein; *GPCRs,* G-Protein Coupled Serpentine Receptors; *G proteins,* guanosine nucleotide-binding proteins; *GRB2-SOS,* Growth Factor Receptor-Bound Protein-2—Son of Sevenless; *Jak1/2,* Janus kinase inhibitor1/2; *JNK,* c-Jun N-terminal kinases; *Jun,* jun proto-oncogene; *MEK,* mitogen-activated protein kinase kinase 1; *ERK1/2,* Extracellular Signal-Regulated Kinase 1/2; *MEKK,* mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase; *NF-KappaB,* Nuclear Factor-KappaB; *PI3K,* phosphatidylinositol 3-kinase, catalytic subunit type 3; *PIP3,* Phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3); *PKC,* Protein Kinase-C; *PLD,* Phospholipase D; *p120 GAP,* Ras GTPase activating protein; *p190,* RhoGAPp190; *Rac,* ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2); *Raf,* v-raf murine sarcoma 3611 viral oncogene homolog; *Ral,* v-ral simian leukemia viral oncogene homolog B (ras related; GTP binding protein); *RalGDS,* Ral Guanine Nucleotide Dissociation Stimulator; *Ras-GTP,* v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog guanosine triphosphate (GTP); *RBP1,* Retinoblastoma binding protein1; *RTKs,* Receptor tyrosine kinases; *SEK,* Dual specificity mitogen-activated protein kinase kinase 4; *SOS,* son of sevenless homolog 1; *SRF,* serum response factor (c-fos serum response element-binding transcription factor); *Vav,* vav 3 guanine nucleotide exchange factor.

Patients with NF-1 appear to be at increased risk of moyamoya syndrome,[84,85] and this risk becomes especially high after cerebral radiation therapy. Patients with NF-1, even in the absence of a brain tumor, are also affected by a number of other problems as a result of their disease, in particular neurocognitive impairment, which can range from mild to severe.[78]

Neurofibromatosis Type 2 (Merlin)

Neurofibromatosis type 2 (NF-2) is characterized by familial, bilateral acoustic neuromas and is caused by mutations in the gene that encodes merlin, or schwannomin, localized to chromosome 22q12.2.[86,87] Merlin interacts with cytoskeletal components and appears to be important in adhesion-dependent growth control. Persons with germline mutations also have skin tumors with both peripheral schwannomas and neurofibromas and have a propensity to develop intracranial meningiomas or, more rarely, gliomas and spinal tumors.[88] The onset of symptomatic tumor growth is uncommon in childhood, and in most patients the condition is identified in adulthood.

Tuberous Sclerosis

A third neurocutaneous disorder associated with an increased propensity for brain tumors is tuberous sclerosis (TS). This condition can be caused by mutations in either of two genes, *TSC1* (hamartin, at chromosome 9q34)[89] or *TSC2* (tuberin, at chromosome 6p), and it is characterized by hamartomata in multiple organs. The most common clinical manifestations include epilepsy, cognitive and behavioral problems, and characteristic skin lesions. The white leaf-shaped skin lesions can best be seen under a Wood light; adenoma sebaceum (facial angiofibroma) can also be seen. Renal manifestations include angiomyolipomas, renal cysts, and, more rarely, renal cell cancer. Brain tumors develop in between 5% and 14% of patients, the most common being the subependymal giant cell astrocytoma (SEGA); other gliomas and ependymomas are also relatively frequent. Careful serial evaluations are required because of the possibility of additional tumor development in this patient population.[90] Cortical tubers can cause seizures and require specialized neurosurgical approaches in children.[91] Resection of tubers does not always control seizures and suggests that extratuberal epileptogenic brain abnormalities may be present that require more specialized imaging.[92]

The phenotypic similarity of mutations in Tsc1 and Tsc2 is explained by the finding that these two proteins interact directly with one another.[89] This complex acts as a guanosine triphosphatase–activating protein for Ras homolog enriched in brain (Rheb). The decreased activity of Rheb inhibits the mammalian target of rapamycin (mTOR) and p70 ribosomal S6 kinase–1. As a result there is diminished translation by eukaryotic translation initiation factor 4E-binding protein–1 (EIF4EBP1; 602223). The hamartin-tuberin complex thereby regulates growth and proliferation of subependymal and subventricular neural stem cells. The Tsc-mTOR pathways may normally be regulated by Wnt and insulin-like growth factor (IGF) ligands during development. Patients with TS who have SEGA or LGGs have demonstrated responses to mTOR inhibitors, confirming the clinical relevance of these findings.[93]

Gorlin Syndrome

Gorlin syndrome (also known as basal cell nevus syndrome or nevoid basal cell carcinoma syndrome) is characterized by multiple basal cell carcinomas or basal cell nevi before the age of 30 years, odontogenic keratocysts or polyostotic bone cysts, and palmar and plantar pits. Other manifestations include rib or vertebral anomalies, large head circumference with frontal bossing, cardiac or ovarian fibroma, and lymphomesenteric cysts.[94] Gorlin syndrome is caused by mutations in PTCH1 (chromosome 9q22.3), the receptor for the SHH ligand.[95-100] Medulloblastoma develops in approximately 4% to 10% of persons with Gorlin syndrome. This syndrome also predisposes to other tumors, such as rhabdomyosarcoma and meningioma.[101,102]

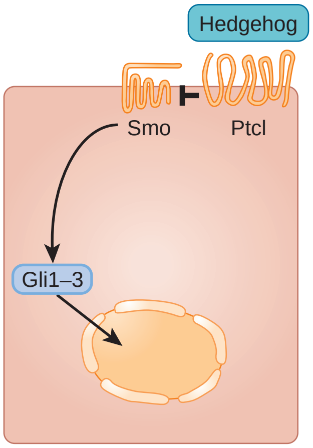

Figure 57-7 The PTCH1 gene product functions as both a receptor and negative regulator of signaling initiated by sonic hedgehog or the related ligands, Indian hedgehog and desert hedgehog. These ligands initiate an unusual and incompletely understood signaling pathway that culminates in Gli1-3 initiating transcription of cell cycle genes, including cyclin D1 and D2. *Smo,* Smoothen.

The PTCH1 gene product functions as both a receptor and negative regulator of signaling initiated by SHH or the related ligands, Indian hedgehog and desert hedgehog. These ligands initiate an unusual and incompletely understood signaling pathway (Fig. 57-7). When a hedgehog ligand binds to Ptc, this alters the activation state of smoothened, or Smo, a seven-transmembrane protein. Normally Ptc represses the activity of Smo; however, when a ligand binds to Ptc, this derepresses Smo activity. Active Smo translocates to a distinctive subcellular organelle known as a primary cilium. Here active Smo enables the dissociation of a signaling complex containing suppressor of fused homolog (SUFU) and Glioma-associated Oncogene Homolog (Gli) transcription factors. The dissociation of this complex results in the nuclear relocalization of Gli family members and increased expression of Gli family members, as well as the expression of v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN), D-type cyclins, and the stem cell–associated chromatic complex component, B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1). The active pathway thereby potentiates proliferation and inhibits apoptosis.

Good evidence indicates that constitutive activity of the SHH pathway can cause medulloblastoma. Gorlin syndrome is associated with increased incidence of medulloblastoma, as is the analogous mutation in mice. Activating mutations in Smo, or mutations in suppression of fused (SuFu), can also lead to these brain tumors. Recent studies have suggested that specific inhibitors of Smo may provide valuable biologic therapies for medulloblastoma.

The value of understanding developmental pathways that normally regulate neural precursor proliferation to decipher the mechanisms that cause pediatric brain tumors is reinforced by data showing that SHH ligand stimulates and regulates proliferation of granule cell precursors and neural stem cells. Thus it is perhaps not surprising that SHH-responsive genes are also expressed in gliomas, including diffuse intrinsic pontine gliomas (DIPG) and glioblastoma. Thus inhibitors of SHH signaling may play a role in treating several types of brain tumors.[103]

Turcot Syndrome

Turcot syndrome is characterized by familial polyposis of the colon, together with malignant brain tumors. This disorder can be caused by mutations in the adenomatous polyposis coli gene (*APC*, on chromosome 5q21) or in the mismatch repair genes *MLH1* (120436) or *PMS2* (600259). The distinction between the clinical entities that result from mutations in *APC* and mutations in repair genes[104] include the nature of the brain tumors seen; the characteristic brain tumors seen are medulloblastomas or astrocytomas, respectively.[105] APC is a large protein whose activity is critical in the Wnt signaling pathway. Therefore inactivating mutations of *APC* result in the aberrant accumulation of β-catenin and increased transcription of transcription factor 4 (Tcf4)-dependent genes, including *c-myc*. Mutations in β-catenin and in APC have also been reported in sporadic medulloblastoma,[104] highlighting the importance of this pathway for this malignant cerebellar tumor.

Wnts constitute a family of ligands that can act through two distinct signaling pathways. The canonical Wnt pathway is initiated when Wnt proteins bind to cell-surface receptors of the Frizzled family. This binding leads to activation of Dishevelled (DSH) family proteins. When DSH becomes activated, it inhibits a protein complex that includes axin, glycogen synthase kinase–3 (GSK3), and APC (Fig. 57-8). The axin–GSK3–APC complex promotes the proteolytic degradation of β-catenin. After this β-catenin destruction complex is inhibited, cytoplasmic β-catenin becomes stabilized and β-catenin is then able to enter the nucleus. Nuclear β-catenin interacts with TCF-LEF (lymphoid enhancer-binding factor 1) family transcription factors to promote the expression of a gene program that includes c-myc, N-myc, and cyclin D1 and thereby stimulate cell cycle progression (Fig. 57-9). It is not clear whether the non-canonical Wnt pathway, which does not involve APC, also contributes to brain tumors.

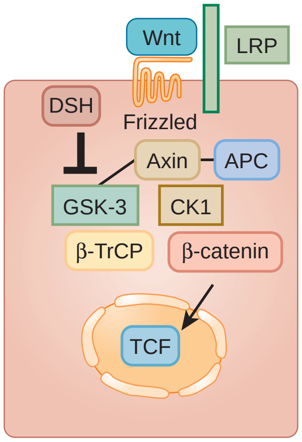

Figure 57-8 Wnts are a family of ligands that can act through two distinct signaling pathways. The canonical Wnt pathway is initiated when Wnt proteins bind to cell-surface receptors of the Frizzled family, which leads to activation of Dishevelled family proteins. When Dishevelled *(DSH)* is activated, it inhibits a protein complex that includes axin, glycogen synthase kinase 3 *(GSK-3)*, and adenomatous polyposis coli *(APC)*. The axin/GSK-3/APC complex usually promotes degradation of β-catenin. After this β-catenin destruction complex is inhibited, cytoplasmic β-catenin becomes stabilized so that it is able to enter the nucleus. Nuclear β-catenin interacts with the transcription factor *(TCF)* family transcription factors to promote expression of a gene program that includes cyclin-D and thereby stimulates cell cycle progression. *CK1*, Casein kinase 1; *LRP*, lipoprotein receptor-related protein; *β-TrCP*, beta-transducin repeat containing protein.

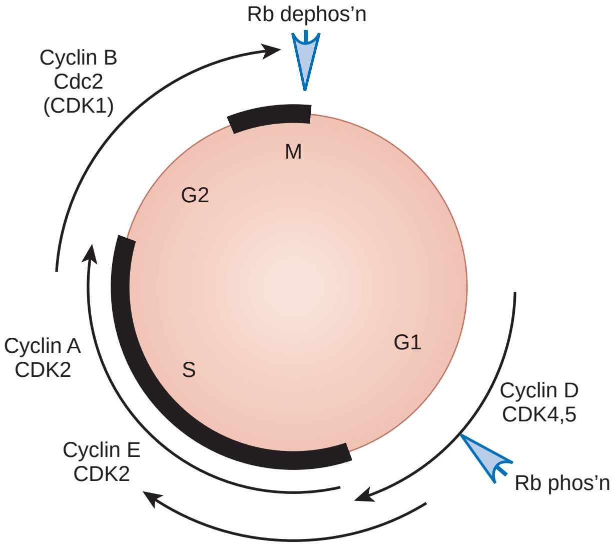

Figure 57-9 Cell cycle diagram. Oncogenic events can increase cyclin-D (i.e., hedgehog or Wnt signals) or alter other cell cycle regulators. *Cdc2,* Cell division cycle protein 2 homolog; *CDK,* cyclin-dependent kinase; *Rb dephos'n,* retinoblastoma 1 dephosphorylated; *Rb phos'n,* retinoblastoma 1 phosphorylated.

As is the case for SHH signaling, the ability of the deregulated Wnt pathway to cause medulloblastomas highlights the relevance of understanding neurodevelopment. In the absence of Wnt1, the cerebellum does not form properly.[106] The Wnt and SHH pathways cooperate during normal development to generate normal cerebellar neurons. It is likely that these pathways also synergize in tumor formation, particularly during medulloblastoma oncogenesis.

Lhermitte-Duclos Disease, Cowden Syndrome, and *PTEN* Mutation

Activation of PI3K results in phosphorylation of phospholipids at the 3′ position; this phosphorylation is removed by the phosphatase and tensin homolog *(PTEN)* phosphatase. Therefore mutations in *PTEN* result in excess and/or incorrectly localized activation of the PI3K pathway. PI3K is critical in several signaling pathways that regulate proliferation, survival, migration, and cell size. The multiplicity of functions explains the diverse spectrum of disorders seen in *PTEN* mutations, including neurologic, cutaneous, and oncologic syndromes.

Molecular studies on Lhermitte-Duclos disease (LDD) tissue have revealed *PTEN* gene mutations in 83% of cases, with immunostaining showing lost or reduced *PTEN* expression in 78% of cases. As a consequence, Akt phosphorylation is increased.[107] Initially Cowden syndrome was described as a familial predisposition for breast cancers, thyroid cancers, brain tumors, and other neoplasia. This syndrome was subsequently recognized as a spectrum of disorders that includes LDD and Bannayan-Ruvalcaba-Riley syndrome. The diagnosis of LDD depends on characteristic hamartomas of the cerebellum. These lesions in the cerebellar cortex exhibit thickened cerebellar folia, with misplaced cerebellar granule cells and enlarged size of the cerebellar neuronal cell bodies. In addition to cerebellar ataxia, these hamartomas can cause hydrocephalus and herniation. Another manifestation of the *PTEN* mutations is seen in Bannayan-Ruvalcaba-Riley syndrome, with macrocephaly, seizures, cognitive dysfunction, and autistic behaviors. Different manifestations of the mutation can be observed even within a family, and thus a careful consideration of family history is warranted.

RB1 Mutations

The retinoblastoma gene was the first tumor suppressor gene identified. In addition to the retinal tumors seen in persons with germline mutations in *Rb1*, “trilateral” retinoblastoma has been described. In these persons, bilateral retinoblastoma is accompanied by a pineal tumor (pineoblastoma) with similar characteristics to retinoblastoma. The other secondary tumors that occur in patients with retinoblastoma are osteosarcomas. The Rb1 protein is required for the G1 checkpoint, and studies on this pathway have provided key insights into the mechanisms of growth regulation.

Atypical Teratoid Rhabdoid Tumor

Mutations in the sucrose nonfermentable 5/integrase interactor 1 (SNF5/INI1) component of the SWI-SNF DNA remodeling complex cause rhabdoid tumors. These tumors include renal and soft tissue tumors and brain tumors. In the CNS, these brain tumors, called atypical teratoid rhabdoid tumors (ATRTs), are characteristically found in the cerebellopontine angle or in supratentorial locations. Prior to the identification of deletions or mutations of the *SMARCB1* gene on chromosome 22, these tumors were historically grouped with medulloblastomas and PNETs. However their histology is distinct, with a mixture of atypical spindle cells, poorly differentiated small round blue cells, and rhabdoid cells with prominent cytoplasmic inclusions, large eccentric vesicular nuclei, and adjacent whorls of intermediate filaments. The nature of the cell of origin for these tumors, and why they predominantly arise in very young children, is as yet poorly understood.

CONCEPTUAL ORGANIZATION OF PEDIATRIC BRAIN TUMORS

Leukemias are considered in the context of their lineage and stage of development, whereas neuroblastoma is evaluated by the extent of spread, age of the child, and molecular phenotype. Neither of these approaches is well suited to CNS tumors. Although brain tumors share an anatomic site, a number of unique cell types, significant heterogeneity in distribution, and differences in the consequence of therapy differ according to the age of the patient and location within the CNS. These factors, combined with a complicated historic nomenclature, require a different approach to understanding these tumors.

The CNS is made up of three major elements, and therefore three major groups of tumors are commonly observed:

1. Glial cells—responsible for structural support and maintenance of the CNS, and composed of three cell subtypes:
    a. Astrocytes—structural support for the CNS → astrocytoma
    b. Ependymocytes—help regulate homeostasis of the CNS → ependymoma
    c. Oligodendrocytes—myelination for the neural axons → oligodendroglioma
2. Neurons—electrical activity → medulloblastoma, pineoblastoma, CNS PNETs
3. Choroid plexus—production of cerebrospinal fluid (CSF) → choroid plexus carcinoma (CPC)

Tumors arising from glia, neurons, or the choroid plexus account for approximately 90% of all pediatric CNS tumors. The remaining 10% of pediatric brain tumors arise from cells that are derived from extracranial sources but become entrapped in the developing CNS during embryogenesis (Fig. 57-10).

4. Germ cells, which arise in the primordial gonadal ridge and normally migrate down to their final resting place in the abdomen (ovaries) or scrotum (testes), can occasionally migrate upward and become enveloped in the developing brain → germinoma, nongerminomatous germ cell tumor (NGGCT)
5. Cells from the Rathke pouch, which normally gives rise to structures of the head and neck, can become trapped within the developing brain → craniopharyngioma

Two additional tumor types that are rare in children but account for approximately 80% of CNS tumors in

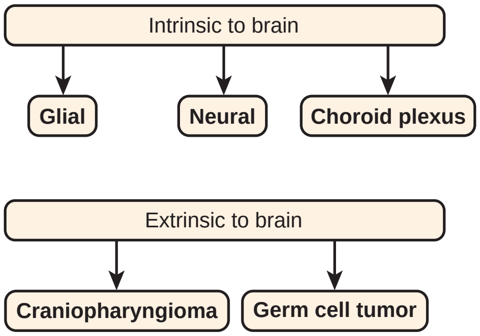

Figure 57-10 Most pediatric brain tumors can be classified into one of five different categories, based on cell of origin. Glial, neuronal, and choroid plexus tumors account for those that derive from cells within the central nervous system (CNS), while germ cell tumors and craniopharyngiomas arise from cells that are enclosed in the developing CNS in early development as a result of abnormal migration.

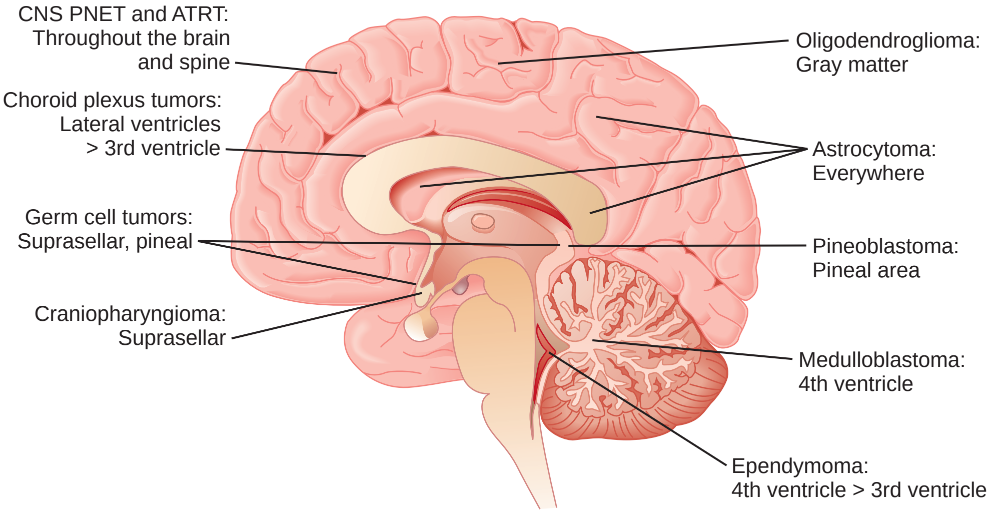
CNS PNET and ATRT: Throughout the brain and spine
Choroid plexus tumors: Lateral ventricles > 3rd ventricle
Germ cell tumors: Suprasellar, pineal
Craniopharyngioma: Suprasellar
Oligodendroglioma: Gray matter
Astrocytoma: Everywhere
Pineoblastoma: Pineal area
Medulloblastoma: 4th ventricle
Ependymoma: 4th ventricle > 3rd ventricle

Figure 57-11 Common distributions of different pediatric central nervous system *(CNS)* tumor histologies. *ATRT,* Atypical teratoid/rhabdoid tumor; *CNS PNET,* primitive neuroectodermal tumor.

adults include metastatic carcinoma, especially of the breast, colon, lung, and prostate. Metastatic lesions to the brain in pediatric patients are exceptionally rare, and when they occur, they are usually in the context of end-stage disease.[108-110] Meningiomas are the other common adult brain tumor that is rarely observed in children.

The five primary cell types of the brain are not evenly distributed in the CNS (Fig. 57-11):

1. Glia.
    a. Astrocytes are found throughout the entire brain and spine.
    b. Ependymocytes line each of the ventricles, and hence these cells are most predominant in the 4th ventricle > 3rd ventricle.
    c. Oligodendrocytes are found around the junction of the gray-white matter.
2. Neural tumors are defined by location rather than histology; molecular characterization indicates that these distinctions based on location are biologically important.
    a. Medulloblastoma is found within the posterior fossa.
    b. Pineoblastoma is found within the pineal region.
    c. CNS PNETs can be found anywhere in the brain or spine.
3. Choroid plexus is predominantly located in the lateral ventricles.
4. Germ cell tumors (GCTs) are localized to the suprasellar region, the pineal area, or both.
5. Craniopharyngiomas are found within the suprasellar region.

Brain tumors tend to spread in one of two ways, by direct invasion into adjacent regions with focal expansion of the primary mass, or by dissemination (seeding) of cells through the CSF, with resultant multifocal disease. Of the five tumor types listed, glial tumors and craniopharyngiomas tend to grow by direct extension and the other three tend to grow by seeding cells into the CSF. The workup of patients with newly diagnosed brain tumors will therefore require MRI of the involved area for glial tumors and craniopharyngioma, and craniospinal imaging and CSF cytology will be needed for seeding tumors (neural, choroid plexus, and germ cell).

Three major treatment strategies are considered for all CNS tumors: (1) surgery, (2) radiotherapy, and (3) chemotherapy. Certain general principles can be applied to their use (Table 57-2):

1. Surgery is important for making the diagnosis, achieving rapid reduction in tumor size, and relieving elevated pressure from obstructive hydrocephalus.
2. Radiotherapy is effective for a wide range of tumors but has significant morbidity on the developing CNS, which frequently limits its applicability. In general focal tumors (gliomas and craniopharyngiomas) are treated with focal radiation therapy, whereas seeding tumors (medulloblastomas, pineoblastomas, CNS PNETs, CPCs, and GCTs) are treated with craniospinal radiotherapy with a boost to the primary site and areas of metastatic disease, particularly in children 3 years and older.
3. Chemotherapy is effective for most seeding tumors and has become part of the initial therapy for these tumors (neural, choroid plexus, and germ cell). By contrast chemotherapy has had limited success for most focal tumors (malignant gliomas and craniopharyngiomas).

The classification of tumors based on their histologic characteristics is important to provide prognostic information, although the unique environment of the brain makes the classification of benign versus malignant less important than for most other sites in the body. The brain and spine are critical for the control of basic autonomic response, as well as higher order function, and therefore limit the ability to obtain complete resection with a wide margin in most cases. Even benign tumors located in critical and inoperable structures may result in death if their growth cannot be stopped or slowed. Conversely many highly malignant World Health Organization (WHO) grade IV brain tumors (defined histologically)

TABLE 57-2 Common Types of Treatment for Pediatric Central Nervous System Tumors*

Type of Tumor	Method of Spread	Attempted Surgery	Type of Radiation Therapy†	Chemotherapy
Glial	Local	Yes	Focal	No‡
Neuronal	Seeding	Yes	CSI	Yes
Choroid	Seeding	Yes	CSI	Yes
Germ cell	Seeding	Yes	CSI	Yes
Cranio	Local	Yes	Focal	No

CSI, Craniospinal irradiation.

*Tumors that exhibit focal growth receive attempted resection and focal radiation therapy. Tumors that are at high risk for early dissemination are treated with focal surgery, CSI, and chemotherapy.

†Radiation therapy is often deferred in children younger than 3 years.

‡Symptomatic unresectable low-grade gliomas are treated with chemotherapy to delay radiation therapy.

that are responsive to radiation treatment (CNS germinoma) or radiation and chemotherapy (medulloblastoma) have an excellent prognosis. Because patients and their parents have preconceived notions about the importance of benign versus malignant, clarifying these terms early can be important.

The presenting symptoms for patients with CNS tumors can usually be categorized into one of two patterns: (1) direct compression of nerves or (2) obstructive hydrocephalus. The location of the tumor, histologic subtype, and age of the patient are major determinants in the length of clinical symptoms before the diagnosis is made.[111] Although dependent on the location and rapidity of growth, the time to diagnosis for many children may range from 3 to 8 months, and multiple visits to primary care providers is not infrequent.[112] For younger children who cannot verbalize their symptoms and for whom fine motor coordination, speech, and gait are still developing, even greater delays may result.

Symptoms related to the direct compression of adjacent nerves by a tumor will cause a unique constellation of symptoms that can be localized to an area as a result of the highly organized structure of the CNS.

- The posterior fossa contains the brainstem, 12 cranial nerves, and the descending and ascending fibers connecting the upper and lower aspects of the CNS, in addition to the cerebellum, which is responsible for movement and balance. Tumors in this area result in cranial nerve dysfunction such as diplopia, choking, or facial asymmetry. Tumors of the brainstem can compress the descending motor tracts, resulting in lower motor deficits. Compression of the cerebellum will lead to ataxia or dysmetria.
- The thalamus is the major relay station of coordinated function from the motor strip and other areas of the cortex. Tumors in this area will often lead to significant hemiparesis.
- The frontal lobe regulates mood and behavior and contains the motor cortex. Patients with tumors in this area will often present with changes in behavior (more aggressive or more passive), worsening school performance, or specific motor deficits (except those controlled by the cranial nerves).[113,114] In some patients, more subtle signs of frontal lobe dysfunction such as fatigue, lack of interest, or decreased energy can be mistaken for the behaviors frequent in adolescence.
- The parietal lobe possesses the centers for sensory function. Tumors in this area can often compress a specific area of the sensory cortex, resulting in a focal sensory deficit that does not follow classic dermatomal or peripheral nerve patterns.
- The hypothalamus and suprasellar regions contain the area that coordinates endocrine function (i.e., growth hormone, regulation of salts, pubertal development, and stress hormones). This area is near the optic nerves and chiasm. Tumors in this area often present as a change in growth (accelerated or delayed), hormonal dysfunction, or change in vision.
- The occipital lobe organizes and interprets vision. Tumors in the occipital lobe will present with homonymous defects in vision.
- The pineal area sits adjacent to the centers of upward gaze (supranuclear tectal or pretectal areas). Lesions in this area can result in Parinaud syndrome (paresis of upward gaze, enlarged pupils that are poorly reactive to light, and poor or limited convergence).
- The spinal cord possesses all the ascending and descending tracks for sensory and motor function to all areas innervated from that segment of the cord and below. Mass lesions in this area will reduce the motor and/or sensory activity of those areas below the lesion and can consist of motor, sensory, temperature, position, and vibration abnormalities.
- Gray matter is where neuron bodies are concentrated. Lesions in the gray matter of the frontal, parietal, temporal, and occipital lobes can result in seizure activity.[115] Although initially focal in nature, seizures can rapidly become generalized, obscuring the initial presenting focality.
- White matter tracts are the myelinated axons of neurons. Lesions in white matter tracts typically result in focal neurologic deficits that correspond to the tracts compressed.

The differential diagnosis of a new tumor of the CNS should be developed using the preceding information. Symptoms will help localize the probable site of the tumor. In turn the site will assist in developing a limited differential diagnosis of possible tumors at that site. Staging and treatment can then be considered in the context of focal versus seeding tumors. Although this exercise will not obviate the need for a definitive biopsy, it can help organize the large array of CNS tumors and ensure that appropriate presurgical planning and staging have been completed.

Obstructive Hydrocephalus and Raised Intracranial Pressure

The brain and spinal cord are supported in the cranium and spinal canal by the CSF, which is largely localized to

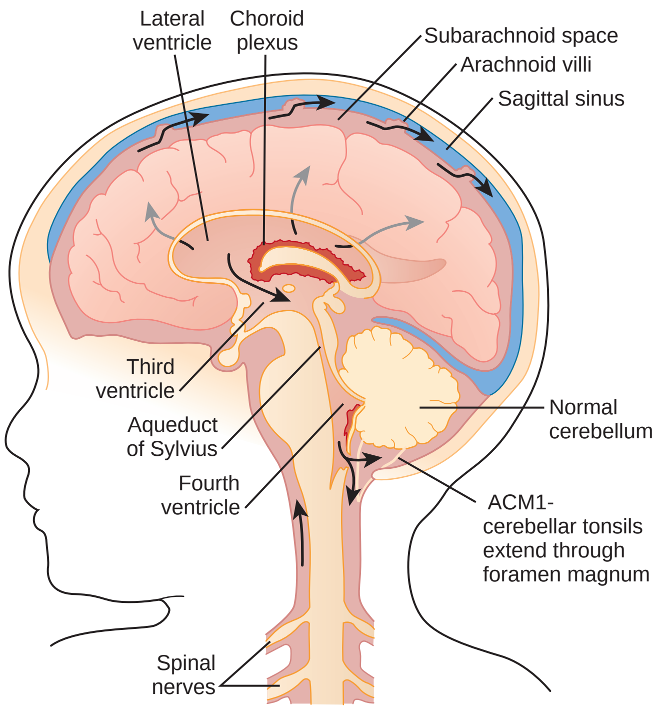

Figure 57-12 Normal flow of cerebrospinal fluid. *Blue arrows* show compression of cortex as a result of obstructive hydrocephalus. *ACM1,* Arnold-Chiari Malformation Type 1.

the subarachnoid space. CSF is initially made by the choroid plexus in the lateral ventricles and, to a lesser degree, in the third and fourth ventricles. The production of CSF is not linked to its passage from the lateral and third ventricles to the fourth ventricle and, finally, through the foramen of Magendie or foramen of Luschka, where it is eventually reabsorbed by the arachnoid villi (Fig. 57-12). The ventricles hold approximately 50 mL of CSF and, with approximately 500 mL of CSF produced each day, any failure to remove old CSF in the context of continued production will cause the fluid-filled ventricles to expand like water balloons in the closed cranium. Obstruction anywhere above the exit from the ventricles to the subarachnoid space (posterior fossa or above) will therefore result in obstructive hydrocephalus. The speed with which the accumulation of fluid occurs will in part determine the rapidity of the symptoms, as well as their severity. Obstructive hydrocephalus is considered a medical emergency because progressive expansion of the ventricular volume will force the brain to be compressed in all directions, including downward, resulting in tonsillar herniation.

The three common symptoms of obstructive hydrocephalus include headaches, often severe in nature, that are thought to arise as a result of stretching of vessels and the pial surfaces. These headaches can often be worsened by changes in body position or head motion. They can be dull, aching, or stabbing in nature. Because of the prevalence of headaches in the general population, it is their persistence and worsening in the context of other symptoms (e.g., morning vomiting and focal neurologic deficits) that usually trigger further investigation. Patients will often have vomiting (often associated with a headache), especially early in the morning upon wakening. Whether this symptom results from hydrostatic pressure changes when first getting up, resulting in compression of the area postrema, or changes in CNS homeostasis upon wakening as a result of more rapid breathing and carbon dioxide release is unknown. Unfortunately the significance of morning vomiting is often overlooked and thought to be related to school avoidance or the flu. Many patients present with prolonged histories of intermittent morning vomiting and headache, suggesting that this process can be partial and relieved by the vomiting, which itself causes raised intraabdominal pressure and equilibration of the CSF pressure gradient. A third common symptom in children with hydrocephalus is blurring of the optic discs, related to the increase in intracranial pressure (ICP). Patients often report blurring or double vision, as well as difficulty in upward gaze. These symptoms likely result from a constellation of factors, including compression of the brainstem and cranial nerves, as well as edema and swelling of the optic discs (papilledema) and pathways leading to vision. The final common symptom of obstructive hydrocephalus is the presence of lower motor deficits, likely because of compression of motor tracts within the brainstem and difficulties with balance and gait related to pressure on the cerebellum.

The symptoms of obstructive hydrocephalus differ in infants, in whom the presence of open sutures permits the head to expand. This expansion relieves the buildup of pressure and thus the associated symptoms. While their head size expands, infants may begin to show some signs of delay in gaining milestones. Careful attention to head circumference changes will help identify these infants early, independent of the cause of the obstruction.

CT imaging of the brain is a rapid method for confirming the presence of obstructive hydrocephalus. Images (without the need for contrast material) will demonstrate enlargement of the ventricles above the area of obstruction. Increasing concerns of radiation dose regarding repeat CT imaging in young children has resulted in a shift to rapid-sequence MRI vent checks.[116] These images are performed without contrast material and are not useful for assessing changes in the tumor. However they have the advantage of assessing changes in fluid within the CNS and without exposure of the child to ionizing radiation.[117,118] MRI will show large ventricles on T1- and T2-weighted images. Best seen on fluid-attenuated inversion recovery (FLAIR) sequences, the presence of a bright signal around the ventricles is suggestive of transependymal flow, which is thought to result from the backward transduction of pressure from the ventricle to the brain parenchyma (Fig. 57-13).[119] Obstructive hydrocephalus is a surgical emergency and requires urgent intervention. For posterior fossa tumors such as medulloblastoma, ependymoma, or low-grade astrocytoma, relief from obstruction can be achieved with resection of the tumor in most patients, thus avoiding the need for a separate CSF diversion procedure.[120]

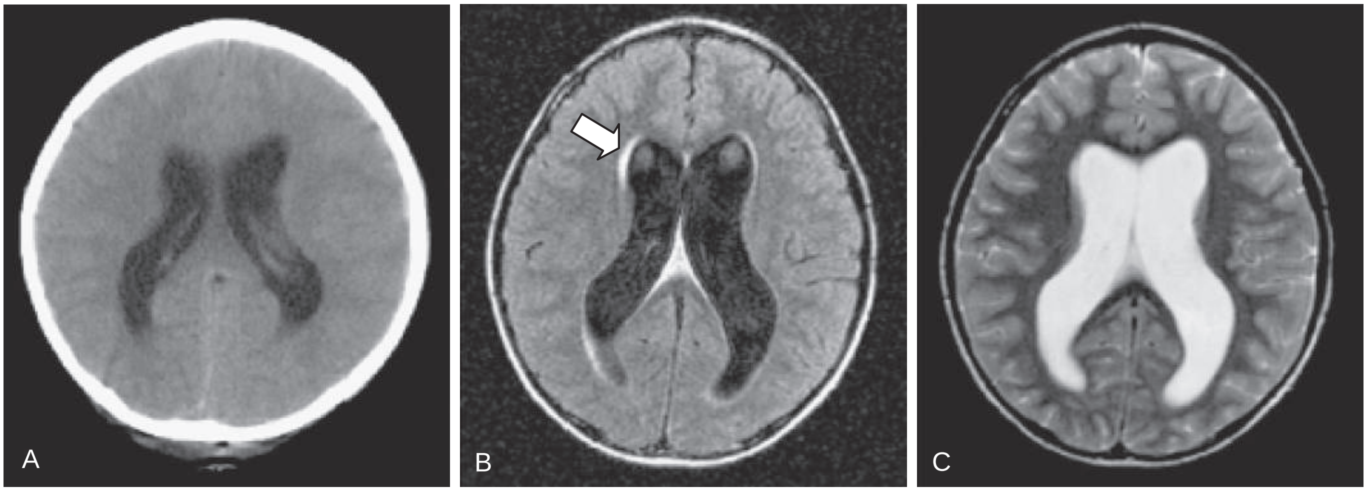

Figure 57-13 Hydrocephalus. **A,** An axial computed tomography noncontrast scan demonstrating significant enlargement caused by obstructive hydrocephalus. **B,** An axial fluid-attenuated inversion recovery image demonstrates enlarged ventricles and transependymal flow *(arrow)*, suggestive of raised intracranial pressure. **C,** An axial T2-weighted magnetic resonance image demonstrating enlarged ventricles.

IMAGING STUDIES

Neuroimaging

Imaging of brain tumors entails determining the size and site of origin of the lesion, establishing primary diagnosis, and planning treatment. Neuroimaging is critical for the appropriate placement of catheters for stereotactic biopsy, resection, planning of radiation, guided application of experimental therapeutics, and delineation of tumor from functionally important neuronal tissue. After treatment, imaging is used to quantify response and the extent of residual tumor. At follow-up imaging helps determine tumor progression and differentiate recurrent tumor growth from treatment-induced tissue changes, such as radiation necrosis.[121]

Imaging brain tumors in children presents unique challenges not encountered in adult imaging, including the need for sedation and consideration of the long-term effects on a growing child. Cranial CT and MRI remain the main modalities for the primary diagnosis of brain tumors. However several other techniques are being increasingly used in the evaluation of this patient population, including positron emission tomography (PET), MR perfusion and diffusion, and MR spectroscopy (MRS). Assessment of response in pediatrics is a critical component in the appropriate treatment of patients, as well as in identifying active regimens. Recently adult neurooncologists have adopted a new series of guidelines (developed by the International Radiologic Assessment in Neuro-Oncology Committee) to aid in better differentiating tumor progression from pseudoprogression and similarly tumor response from pseudoresponse. Although some of these adult criteria will be useful in pediatrics, the types of tumors common in the pediatric population will require some modifications to the adult approach. Currently an international working group is developing recommendations for children with brain tumors (Radiologic Assessment in Pediatric Neuro-Oncology).[122]

CT is a rapid and inexpensive modality for assessing fluid, blood, and calcification in the central CNS. As such it has typically been the first imaging procedure in children with a presumed intracranial bleed or raised ICP that might require immediate neurosurgical intervention. Other than for lesions arising from the skull vault and to assess calcified tumors, CT scans are used less frequently for routine surveillance of pediatric patients because of an increase in long-term cancer risk caused by CT imaging.[53,116]

MRI is currently the modality of choice for localization and assessment of the size of brain tumors. MRI provides valuable information about secondary phenomena such as mass effect, edema, hemorrhage, necrosis, and signs of increased ICP. In addition MRI provides excellent tissue contrast and high spatial resolution. Standard T1- and T2-weighted MRI sequences detect brain tumors with high sensitivity. Varying acquisition parameters such as T1 or T2 weighting, techniques such as diffusion- and perfusion-weighted images, and FLAIR sequences reveal a characteristic pattern of each tumor, depending on tumor type and grade.[123] Susceptibility-weighted MRI is useful for detecting areas of hemorrhage, calcifications, and increased vascularity associated with brain tumors.[124] Recently rapid-sequence MRI for the assessment of bleeds or hydrocephalus has been developed, which can avoid the radiation doses associated with CT scans and, like CT scans, can be performed without sedation, even in young children.[117,118,125] However it should be noted that these studies are limited in evaluation of the tumor burden and should only be used to assess for acute findings.

MRS can also be helpful for the initial characterization of tumors[126,127]; it can also be used to differentiate tumor tissue from other tissue in children with CNS tumors in certain circumstances.[128] MRS has continued to evolve, and the development of multivoxel and two-dimensional techniques has resulted in improved spatial resolution, thereby supplying additional information regarding tumor heterogeneity and intratumoral metabolite distribution. Although MRS is a sensitive technique, it still lacks specificity as a stand-alone technique in the clinical setting. In a recent study, brain proton MRS biomarkers were shown to predict survival of children with CNS tumors better than standard histopathology. More accurate prediction using this noninvasive technique represents an important advance and may suggest more appropriate therapy, especially when a diagnostic biopsy is not feasible.[129] In general, decreased *N*-acetylaspartate (NAA) and creatine concentrations and increased choline concentrations correlate with tumor grade. Reduction of NAA is likely because of neuronal death or damage, although the reduction in creatine is likely to be a result of changes in cell energetics. The increase in choline is believed to reflect increased membrane synthesis. Increases in lipid and lactate concentrations have been observed in some gliomas. Lactate accumulation is believed to be a result of central tumor necrosis.

Diffusion-weighted MR pulse sequences enable a quantitative and reproducible assessment of the diffusion changes, not only in areas exhibiting signal abnormality in conventional MRI but also in areas of normal signal.[130] MR diffusion using predominantly echoplanar techniques has been useful in the characterization of tissue, tumor cellularity, tumor grading, tumor response to treatment, and distinction of tissue types.[131,132] Diffusion tensor imaging (DTI) provides visualization of fiber bundle direction and integrity, with in vivo characterization of the rate and direction of white matter diffusion. DTI is useful for presurgical planning or coregistration of tractography data with radiosurgical planning and functional MRI data.[133] Fractional anisotropy using DTI may prove helpful for the assessment of treatment-induced white matter changes in children.[134,135]

The usefulness of diffusion-weighted imaging (DWI) for characterizing intracranial cystic or cystlike lesions has been demonstrated in a number of studies.[136-139] DWI has long been used to differentiate between epidermoid and arachnoid cysts.[139,140] Arachnoid cysts are characterized by free diffusion, whereas epidermoids have an apparent diffusion coefficient similar to that of brain parenchyma, thereby demonstrating restricted diffusion.[137] The usefulness of DWI in the distinction between ring-enhancing cerebral lesions such as brain abscesses, cystic or necrotic HGG, or metastasis has been shown in multiple studies during the past decade, although this differentiation continues to be a challenge.[141] The ring enhancement of a brain abscess can be indistinguishable from that of a cystic or necrotic HGG or metastasis. Other lesions that may also have a similar appearance are subacute ischemic infarction, resorbing hematoma, and demyelinating disease.[130] Abscesses demonstrate high signal on DWI and a reduced apparent diffusion coefficient (ADC) in a cystic ring-enhancing cerebral lesion.[140,141] ADC values have been assessed between tumor types; however, considerable overlap exists between certain tumor types, requiring additional evaluations.[132,142] Authors of a retrospective study of ADC values of 275 adult and pediatric brain tumors have reported a significant negative correlation between ADC and WHO astrocytic tumor grades II through IV.[142] Other comparisons included a higher ADC in dysembryoplastic neuroepithelial tumors (DNETs) than in astrocytic grade II tumors (100% accuracy) or other glioneuronal tumors, a lower ADC in malignant lymphomas compared with glioblastomas and metastatic tumors, a lower ADC in CNS PNETs compared with ependymomas, and a lower ADC in meningiomas compared with schwannomas. The ADC of craniopharyngiomas was higher than that of pituitary adenomas, whereas the ADC of epidermoid tumors was lower than that of chordomas. In meningiomas the ADC was not indicative of malignant grade or histologic subtype. DWI has also been used to obtain additional information regarding tumor type and grade. The reduction in extracellular space, as well as high nuclear-to-cytoplasmic ratios of some cancer cells, causes a relative reduction in ADC values.[131,143] In some studies overlap was seen in ADC values of HGGs and LGGs.[132] The presence of glycosaminoglycans such as hyaluronan in the extracellular space of some HGGs may decrease water content and cause a reduction in ADC values.[144] In addition, one pitfall of DWI is that high-grade tumors that may exhibit necrosis can lead to higher ADC values.[145]

DWI and proton MRS have been evaluated as diagnostic tools, and in a study of children with posterior fossa lesions in which these techniques were combined, MRI was successful in correctly identifying the histologic diagnosis in every case.[146] Although this approach does not replace the pathologic diagnosis, it demonstrates the increasing accuracy of biologic-based imaging.[147,148] Similar results have been reported for ADC analysis.[149,150] DWI may also be helpful in differentiating postsurgical changes from tumor recurrence.[148] DWI can also detect acute changes in white matter from methotrexate administration, which must be differentiated from progressive disease.[151]

Determination of the tumor margins is considered by many investigators to be extremely important for the management of brain tumors. Complete resection of tumors with minimal neurologic deficit is the ultimate goal of surgical resection. In some studies DWI has been shown to discriminate among tumor, infiltrating tumor, peritumoral edema, and normal brain parenchyma.[152,153] However other studies have not found DWI to be helpful for evaluating tumor margins.[132,154]

MR diffusion imaging has also been assessed as a biomarker for early prediction of treatment response in patients with brain tumors. Recent studies have indicated the possibility of using functional diffusion map analysis as an early biomarker for treatment response preceding decrease in tumor size.[155,156] Increasingly MR perfusion imaging is being used to evaluate cerebral perfusion dynamics by analysis of the hemodynamic parameters of relative cerebral blood volume (CBV), regional cerebral blood flow (CBF), and mean transit time. CBV is the parameter most commonly quantified in brain tumors.[157] CBV is defined as the volume of blood in a region of brain tissue, commonly measured in milliliters per 100 g of brain tissue. CBF refers to the volume of blood/unit time passing through a given region of brain tissue, measured in milliliters per minute per 100 g of brain tissue. Mean transit time refers to the average time it takes blood to pass through a given region of brain tissue and is commonly measured in seconds.[158] Perfusion imaging techniques include T2-weighted dynamic susceptibility techniques, arterial spin labeling (ASL) techniques, and T1-weighted dynamic contrast-enhanced perfusion techniques. These techniques use exogenous tracer agents, such as paramagnetic contrast material, or endogenous tracer agents, such as magnetically labeled blood (arterial water).[159] The most common method currently performed in the clinical setting is dynamic contrast-enhanced perfusion MRI with an exogenous tracer, such as gadopentetate dimeglumine. It is assumed that the tracer is restricted to the intravascular compartment and does not diffuse into the extracellular space. Imaging is performed dynamically (rapid imaging over time during a bolus injection) using echoplanar imaging–based spin echo or gradient echo sequences. It is thought that the spin echo sequences are more sensitive to capillary level blood vessels, whereas gradient echo techniques are more sensitive to the larger vessels.[160] Although gradient echo sequences are associated with more magnetic susceptibility artifacts, particularly in the posterior fossa, they are the more common of the two techniques. For young children and infants, challenges exist relating to intravenous (IV) access, smaller intravenous catheters, and limitations of the contrast medium dose.

DTI is an adaptation of DWI and is performed by acquiring diffusion data in six or more directions, enabling determination of the direction and magnitude of water diffusion. Connecting the directions of diffusion in each voxel to those of neighboring voxels using a variety of mathematical algorithms enables creation of a three-dimensional (3D) white matter tract map, termed “tractography.” This technique is used to delineate important white matter tracts affected by tumor[161] and help guide surgical resection. In conjunction with functional MRI, tractography can be used to predict possible postoperative deficits resulting from white matter tract damage.

Dynamic T1-weighted contrast imaging can be used to assess microvascular permeability (measured as the transendothelial transfer constant, or Kps) in brain tumors.[162] Kinetic modeling of the dynamic signal changes can yield estimates of regional fractional blood volume and Kps, which is an indicator of BBB disruption and correlates with angiogenesis. This technique can be successfully performed in children, and applications of this technique may be useful for monitoring antiangiogenic therapies in pediatric patients with brain tumors.[163-165] ASL is an MR perfusion technique that does not use an IV contrast agent.[166] The perfusion contrast in the image results from the subtraction of two successively acquired images, one with and one without proximal labeling of arterial water spins, with a magnetic gradient used to invert the magnetization of inflowing blood.[167] The signal-to-noise ratio, anatomic coverage, and shorter imaging time are currently better for the dynamic contrast perfusion techniques compared with ASL. However ASL may have a future role in the imaging of pediatric brain tumors, particularly because it relies on a noninvasive endogenous contrast agent.[168,169]

The use of PET and single-photon emission CT (SPECT) imaging continues to improve[170] and can be important in helping to differentiate treatment effect from tumor recurrence.[171-174] The usefulness of PET imaging is especially evident when a baseline evaluation is performed so that postoperative changes can be evaluated in the context of the pretherapy PET avidity, thus requiring consideration of nuclear imaging early in the workup of these patients.[175,176]

Standardization of neuroimaging parameters for children with CNS tumors and the testing of novel sequences that can be adapted to specific molecular inhibitors now being evaluated in this population are being developed.[130,177]

A further advance in MRI of brain tumors has occurred with the availability of intraoperative scanners. These scanners enable preoperative guidance for stereotactic biopsy and for planning tumor resection, and they provide a review of the resection site for residual tumor prior to closure of the craniotomy.[178] Intraoperative DTI has been proposed to aid in the preservation of fiber tracts and to minimize postoperative deficits.[179]

Somatostatin receptor scintigraphy has been used to differentiate the presence of residual or recurrent tumor from scar and necrosis and is better than MRI scans for a number of pediatric tumor types.[180,181] Molecular imaging is likely to play an expanding role in neurooncology as more pathway-specific inhibitors become available.

Posterior reversible leukoencephalopathy (PRES) is increasingly identified in children with brain tumors, particularly in children with episodes of hypertension. Patients present with headaches that are usually severe, mental and visual status changes, and seizures concurrent with hypertension and characteristic MRI findings, including T2 signal abnormalities.[182] MRI findings are those of vasogenic edema with T2 and FLAIR hyperintensities involving predominantly the parietal and occipital regions bilaterally (Fig. 57-14). The diffusion changes in PRES are traditionally thought to be represented by higher ADC values, consistent with vasogenic edema. Focal areas of restricted diffusion (likely representing infarction–tissue injury with cytotoxic edema) are uncommon (11% to 26%) and may be associated with an adverse outcome.[183-187] Hemorrhage (focal hematoma, isolated sulcal-subarachnoid blood, or protein) is seen in approximately 15% of patients.[188,189] The parietal and occipital lobes are most commonly affected, followed by the frontal lobes, the inferior temporal-occipital junction, and the cerebellum.[188] Lesion confluence may develop as the extent of edema increases.

The mechanism of PRES remains controversial, although the hypertension-hyperperfusion theory is favored because of the common presence of elevated blood pressure and perceived response to hypertension management. Key issues remain problematic, including PRES in normotensive patients with pressures rarely reaching autoregulatory limits, and brain edema that is lower in patients with severe hypertension. Hypertensive

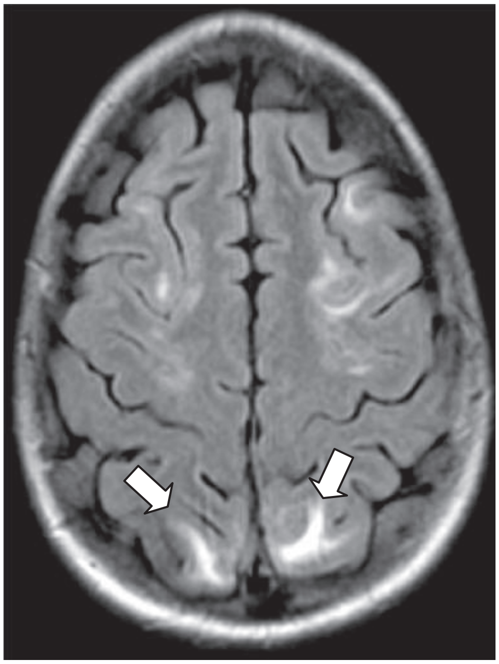

Figure 57-14 An axial fluid-attenuated inversion recovery image in a patient after radiation therapy with new-onset seizure and hypertension with bilateral hyperintense signal in the occipital lobes *(arrows)*. Also note increased signal to a lesser extent in the frontal lobe.

encephalopathy animal models do not reflect the systemic toxicity that is present, and hyperperfusion has not conclusively been demonstrated in patients.

Intracranial vasospasm has been seen with conventional and MR angiography, suggesting vasospasm as a possible pathophysiologic mechanism for the observed findings.[190] MR DWI was instrumental in establishing and consistently demonstrating that the areas of abnormality represent vasogenic edema. Prompt treatment with antihypertensive therapy or discontinuation of immunosuppressive agents can lead to complete recovery in some cases. However, if untreated, permanent neurologic deficits or even death may occur as a result of cerebral infarctions or hemorrhages,[191] and 20% to 40% of patients with PRES can be normotensive.[192,193] PRES can be associated with a number of inciting events, including chemotherapy, radiation therapy, and antiangiogenic drugs.[194] This latter group of drugs may cause PRES as a result of their direct effect on vascular endothelial growth factor (VEGF) and raised blood pressure. Rapid recognition of this entity is critical to prevent permanent damage from occurring.

Surveillance Imaging

The role and usefulness of surveillance imaging for patients with a brain tumor remain controversial and depend on a number of factors, such as the age of the patient, histology of the tumor, time from diagnosis, and type of treatment. For example, in one study only nine of 318 imaging encounters identified an asymptomatic recurrence.[195] Other studies have demonstrated the cost-effectiveness of surveillance imaging,[196] recognizing that decisions are often made on the basis of insurance coverage. A common practice has been imaging every 3 months while the patient is undergoing therapy (to assess continued response while undergoing therapy), and then every 3 months for the first year after the completion of therapy. Beginning in the second year, scans are performed every 6 months for a year and then annually afterward. With time the risk of tumor recurrence will go down, although the risks of radiation-induced vasculopathy and second tumors begin to increase. Modification of these guidelines for children with tumors at low risk of recurrence (e.g., completely resected craniopharyngioma or low-grade astrocytoma) or those who did not receive radiation therapy can be made on a case-by-case basis.

NEUROPATHOLOGY

The neuropathologic classification of pediatric brain tumors has evolved greatly during the past century. Categorizing tumors is helpful to guide therapy and estimate prognosis. A number of outstanding reviews on the classification of CNS tumors have been written.[197-206] When attempting to determine the treatment and/or prognosis of a tumor based on published reports or meeting abstracts, the classification schema used in those reports will become critical before applying this information to other patients.

Most current classification systems are based on the pioneering work of Cushing and Bailey almost 100 years ago. The major premise of this approach was to define tumors by their presumed cell of origin and cell lineage based on morphologic similarity to normal immature or mature brain cells. This system was adapted by Kernohan when he proposed that certain tumors, especially those with a glial appearance, such as astrocytomas, ependymomas, and oligodendrogliomas, could be further classified by the degree of anaplasia, which related to prognosis. Most current systems now use these two criteria—presumed cell lineage and degree of anaplasia—as the primary basis for classification of adult and pediatric CNS tumors. In spite of the usefulness of this classification schema, it is becoming progressively clear that most brain tumors do not derive from mature cell types but rather from primitive precursors or stem cells that can differentiate down many different pathways, obscuring the cell lineage. In comparison with their adult counterparts, pediatric brain tumors are exceptionally diverse in their morphologic appearance and therefore represent a particular challenge for classification by morphologic criteria alone. This diversity likely stems from the fact that pediatric brain tumors are derived from a wide spectrum of proliferative cell types at many developmental stages not present in adult brains.

Significant advances have been made in the field of neuropathology. The WHO classification of CNS tumors has been recently revised, with a number of important modifications based on histopathologic recognition of new pediatric brain tumors.[207] A synopsis of the WHO 2007 classification system is provided in Table 57-3. Increasingly the field has begun to include use of molecular alterations for many tumors and the use of highly specific immunohistochemical markers.[198-201,206,208-212] When

TABLE 57-3 Simplified World Health Organization Classification of Pediatric Central Nervous System Tumors

Type	Subtype	Example(s)	Grade
Glial tumors	Astrocytic tumors	Pilocytic astrocytoma	I
		Subependymal giant cell astrocytoma	I
		Pilomyxoid astrocytoma	II
		Pleomorphic xanthroastrocytoma	II
		Diffuse (fibrillary) astrocytoma	II
		Anaplastic astrocytoma	III
		Glioblastoma multiforme	IV
		Gliosarcoma	IV
		Gliomatosis cerebri	III-IV
	Oligodendroglial tumors	Oligodendroglioma	II
		Oligoastrocytoma	II
		Anaplastic oligodendroglioma	III
		Anaplastic oligoastrocytoma	III
	Ependymal tumors	Subependymoma	I
		Myxopapillary ependymoma	I
		Ependymoma	II
		Anaplastic ependymoma	III
Neural/embryonal tumors		Medulloblastoma	IV
		Pineocytoma	I
		Pineal parenchymal tumor of intermediate differentiation	II-III
		Papillary tumor of the pineal region	II-III
		Pineoblastoma	IV
		Primitive neuroectodermal tumor (medulloepithelioma, ependymoblastoma)	IV
		Atypical teratoid-rhabdoid tumor	IV
Choroid plexus tumors		Choroid plexus papilloma	I
		Atypical choroid plexus papilloma	II
		Choroid plexus carcinoma	III
Germ cell tumors	Germinoma		III
	Nongerminoma	Embryonal carcinoma	III
		Yolk sac tumor	III
		Choriocarcinoma	III
		Mature teratoma	0
		Teratoma	I
		Immature teratoma	III
		Teratoma with malignant transformation	III
Craniopharyngioma	Craniopharyngioma	Adamantinomatous	I
		Papillary	I
Other	Mixed glial neuronal tumor	Ganglioglioma	I
		Gangliocytoma	I
		Anaplastic ganglioglioma	III
		Dysembryoplastic neuroepithelial tumor	I
		Desmoplastic infantile astrocytoma	I
		Central neurocytoma	II
		Extraventricular neurocytoma	II
	Neuroepithelial	Astroblastoma	—
	Nerve tumors	Schwannoma	I
		Neurofibroma	I
		Malignant peripheral nerve sheath tumor	II-IV
	Meningeal	Meningioma	I
		Atypical meningioma	II
		Anaplastic meningioma	III
		Hemangioblastoma	I

combined with immunohistochemical analysis and the increasing use of cytogenetic classification and molecular profiling, the classification of tumors continues to become more reproducible and predictive of clinical outcomes. A simplified overview of the chromosomal abnormalities associated with pediatric brain tumors is presented in Table 57-1. For many large consortium-based studies in both Europe and North America, molecular profiling of tumors to ensure proper classification is now required, especially for medulloblastoma and ATRTs.

The immunohistochemical patterns used to classify CNS tumors require considerable experience on the part of the neuropathologist, as well as appropriate control subjects. Most markers lack specificity and can be identified in a wide array of histologies, requiring correlation with other clinical or molecular data.[213] Common

TABLE 57-4 Immunohistochemical Markers of Pediatric Central Nervous System Tumors

Marker	Tumor
Glial fibrillary acidic protein	Astrocytoma, oligodendroglioma, ependymoma, choroid plexus papilloma, PNET, ATRT
Synaptophysin/NeuN	PNETs, ganglial tumor, neurocytoma
MIB1/Ki-67	Measures all cells not in G0
Mitotic rate	Measures cells in mitosis
Neurofilament proteins	Ganglial tumor, PNET, neurocytoma, subependymal giant cell tumor, ATRT
S-100 and neuron-specific enolase	Normal and neoplastic glial and neuronal in origin
Retinal S-antigen	Pineal parenchymal tumor, PNET, retinoblastoma
Desmin	Muscle tumor, teratoma, PNET
Smooth muscle actin	Muscle tumor, ATRT
Cytokeratin	Chordoma, choroid plexus tumor, meningioma, some malignant gliomas, nongerminomatous germ cell tumor, PNET, ATRT
Epithelial membrane antigen	Meningioma, ependymoma, teratoma, ATRT
Vimentin	Mesenchymal tumor, meningioma, sarcoma, melanoma, ependymoma, astrocytoma, chordoma, schwannoma, PNET, ATRT
Alpha-fetoprotein	Embryonal carcinoma, endodermal sinus (yolk sac) tumor
Human chorionic gonadotropin	Germinoma, choriocarcinoma
Placental alkaline phosphatase	Germ cell tumor
INI-1	ATRT

ATRT, Atypical teratoid/rhabdoid tumor; *PNET,* primary neuroectodermal tumor.

markers used to classify pediatric CNS tumors are provided in Table 57-4. Four commonly used immunohistochemical markers are the oligodendrocyte lineage transcription factor 2 (OLIG2)[214] and glial acidic fibrillary protein (GFAP), which stain glial cells (Fig. 57-15); synaptophysin, which stains neurons (Fig. 57-16); and Ki-67, which stains cells that have left G0 cell cycle and are at some stage of cellular division (Fig. 57-17).

TREATMENT STRATEGIES

Neurosurgery

The neurosurgeon is often the first of the specialized team of caregivers called to see a child with a brain tumor, with

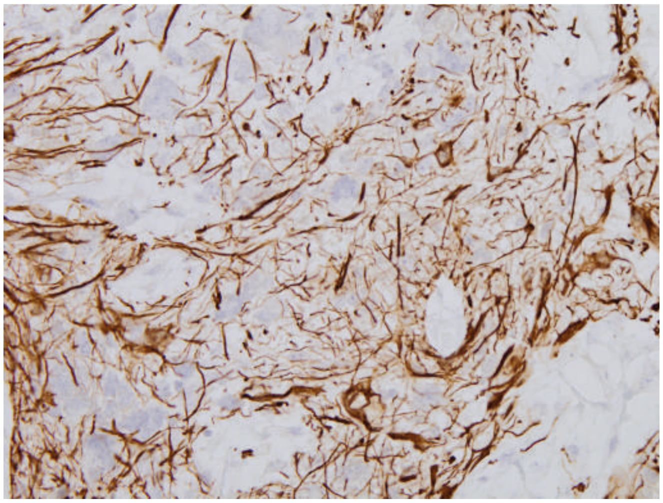

Figure 57-15 Glial acidic fibrillary protein staining of astrocytic cells in a child with glioblastoma multiforme (×400).

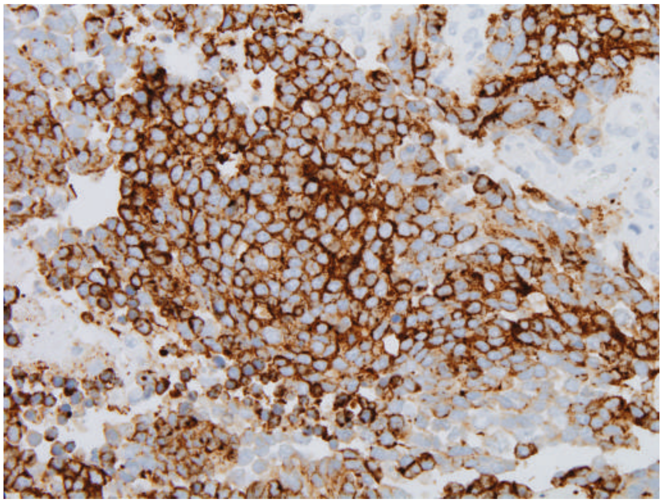

Figure 57-16 Synaptophysin immunohistochemical staining of medulloblastoma (×400).

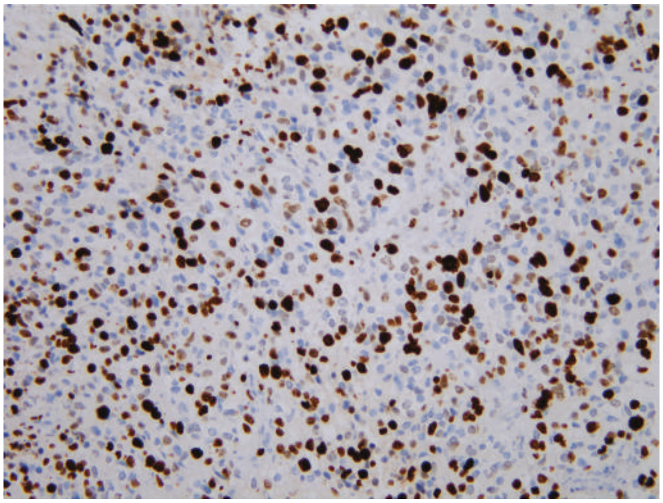

Figure 57-17 Ki-67 immunohistochemical staining in glioblastoma multiforme (×200).

the call frequently coming from the emergency department or radiology department. The initial evaluation consists of acute management—that is, assessment of the patient's clinical condition with respect to neurologic stability and the possibility of acute neurologic decompensation requiring immediate intervention, and subsequent to that the development of a plan that includes surgical interventions geared toward obtaining tissue for pathologic diagnosis, resection of tumor as part of the overall management of the tumor, and dealing with the secondary effects of the tumor.[215-217]

In the field of neurosurgery remarkable advances have occurred in technology, from intraoperative microscopes, robotics, and computer-assisted navigation to intraoperative MRI, endoscopic techniques, and minimally invasive techniques.[218,219] Each of these advances has been instrumental in reducing the morbidity of neurosurgical procedures while ensuring maximal surgical resections.

Acute Management Issues

Hydrocephalus. Hydrocephalus is the most common clinical presentation of posterior fossa brain tumors, which are the most common pediatric brain tumors. Historically the initial evaluation of children suspected of harboring a posterior fossa brain tumor has been via CT scan. However with improved technologies and more recent concerns regarding the long-term effects of radiation exposure, there is a trend toward less utilization of CT scans. If possible the initial assessment should be an MRI of the brain, which can provide the dual information regarding the brain tumor and the extent of hydrocephalus. The cause of the hydrocephalus is obstruction to the CSF flow as a result of the tumors in and around the fourth ventricle. The evaluation should consist of assessing the severity of the hydrocephalus (via the presence of periventricular capping/edema and tonsillar herniation) and the need for immediate intervention. High-dose steroids will often produce significant relief in symptoms as peritumoral edema is suppressed, and they are often utilized as a temporary measure for symptom relief. In infants, hydrocephalus can be asymptomatic and only evidenced by a rapidly enlarging head circumference. Supratentorial brain tumors can also produce hydrocephalus by causing mass effect on the ventricles, as well as by obstructing CSF flow through the ventricular system. Patients with suprasellar tumors can also have inadequate cortisol production or electrolyte disturbances, which need to be addressed as quickly as possible and often in concert with preparation for surgery.[220]

Raised ICP is a medical emergency. Immediate relief can be accomplished by temporary placement of a catheter (drainage tube) in the ventricles, although this step is rarely needed, primarily because of the fact that steroids in the preoperative period followed by surgery to remove the tumors within 24 to 48 hours after diagnosis usually relieves the obstruction. Permanent techniques of hydrocephalus treatment include the placement of a ventriculoperitoneal (VP) shunt that drains CSF around the obstruction into the peritoneal space via a permanently implanted tube and more recently via an endoscopic third ventriculostomy (ETV).[221,222] The latter has become the treatment of choice in the management of obstructive hydrocephalus and is not associated with the long-term complications of shunt revisions and infections.[223] Most pediatric centers with sufficient expertise now routinely perform ETVs as the primary management of hydrocephalus in children with brain tumors.[224] In addition the overall incidence of shunting for brain tumors has decreased during the past two decades.[225,226] Like all complex procedures, rare but significant neurologic risks are associated with this procedure in approximately 1% of cases.[227] The possibility of closure of a third ventriculostomy and resultant acute hydrocephalus requires long-term follow-up with neurosurgery, as with all children who have had placement of shunts for the management of the hydrocephalus.

Visual and Endocrine Evaluation. Children with tumors in the sellar and suprasellar area can present with blindness, which rarely can be acute in onset. In those circumstances, although extremely rare, it is possible to obtain some recovery of vision by immediate treatment of the tumor. If a large mass or cyst is causing compression, acute drainage in conjunction with steroid administration can lead to some recovery of vision. Tumors in this location can also interfere with endocrine function and may lead to electrolyte disturbances and decreased steroid production, and these effects can be critical if they are unrecognized, especially if acute surgical intervention is indicated.

Surgical Management

Surgical management after treatment of hydrocephalus consists of obtaining tissue for diagnosis and resecting the tumor. The decision to perform these procedures depends on the location of the tumor, the known biology, and the goals of treatment management.

Certain tumors do not require routine biopsy or attempted resection at diagnosis (e.g., diffuse pontine gliomas, classic optic pathway tumors in children with NF-1, and some suprasellar or pineal lesions with positive serum or CSF tumor markers). Tectal gliomas are rare tumors centered in the tectum with a classic radiographic appearance and presentation of hydrocephalus and, rarely, neurologic deficits. Their management consists solely of treatment of the hydrocephalus via ETV and radiographic and clinical follow-up.

The leading factor in the outcome of a child with a brain tumor is not the presenting symptoms, age of the child, or the final disease. Rather it is the experience of the neurosurgeon performing the operation and the number of similar procedures performed during the prior few years.[228] With increasing cure rates in children with CNS tumors, careful consideration of the neurosurgical morbidity, which can often be lifelong, needs to be taken into account while a treatment plan is developed.[229]

Advances in Surgical Techniques. Recent advances in image-guided neurosurgical techniques have been considerable. Endoscopic procedures provide minimally invasive techniques to address not just obstructive hydrocephalus but also biopsy or resection of intracranial masses, with minimal morbidity in experienced hands.[230] New techniques using intraoperative ultrasound have been developed and are being used in pediatric patients.[231] 3D laser-guided maps can assist in the orientation and surrounding environment of the tumor during a procedure. More recently intraoperative MRI facilities now allow surgeons to use MRI procedures with the same magnet strength as that used for diagnostic imaging while operating.[232] Real-time MRIs can assist the neurosurgeon in the detection of residual disease or hemorrhage before closure of the resection site.[233]

The surgical approach used by the neurosurgeon will depend on a number of factors that must balance the need for diagnosis with the potential risks of operating in a given area.[234] While the relative risks of general anesthesia and neurosurgical procedures continue to diminish with improving techniques, it is now common to perform staged operations in which a limited resection is performed to confirm the diagnosis. Based on the pathologic results obtained, decisions about more complicated or risky surgery can be considered for lesions in which complete resection is a critical component of improved outcome and can be deferred in lesions in which additional resection would not significantly alter prognosis but could cause significant morbidity. Specialized techniques such as endonasal endoscopic approaches can allow a minimally invasive approach, with excellent outcome in centers that have expertise in this approach.[235]

The need of neurosurgeons to be aware of the evolving treatment strategies for children reinforces the role of the multidisciplinary team. During the past 5 years this need has become even more important, because many pediatric brain tumors now require molecular classification to guide therapy. For example in spite of their similar appearance, the treatment of posterior fossa medulloblastoma now differs significantly from posterior fossa ATRT. Treatment on national protocols therefore requires submission of fresh-frozen material obtained at the time of surgery for proper stratification on the basis of therapy protocols. As our molecular classification of pediatric brain tumors expands and newer and better molecular inhibitors of specific pathways become available, the role of the neurosurgeon and neuropathologist in ensuring the proper processing of these samples will continue to expand.

Perioperative Issues. A number of factors must be considered for a child after a tumor-based procedure has been completed. The rate of weaning steroids after an operation will depend on many factors, including the histology, degree of resection, postsurgical edema, and patient status. Although most patients can be weaned off steroids rapidly after an operation, it is important that the members of the team have a coordinated structure so that as children pass from neurosurgical care to the radiation therapist or oncologist, overall management of these issues is seamless. A similar discussion holds true for anticonvulsant agents. Whereas many patients will receive preoperative or perioperative anticonvulsant therapy, most can be weaned rapidly, and communication among team members will therefore be required.

Seizures can be a presenting symptom for many patients with brain tumors or a result of electrolyte disturbances in the perioperative period. Management includes anticonvulsant agents and surgical resection geared not only toward the tumor but also toward dealing with the seizures. Intraoperative electrocorticography is particularly useful in the management of temporal and frontal lobe tumors with seizures and requires a multidisciplinary approach, which can lead to excellent overall outcomes. Choices of antiepileptic drugs (AEDs) are important because some may interfere with metabolism of chemotherapeutic agents, which requires a comprehensive approach toward the management of the patient's seizures and tumor.

Hyponatremia is a common problem that can occur after neurosurgical intervention and requires immediate recognition.[236] Two common conditions, both resulting in hyponatremia but needing different interventions, can occur. Cerebral salt wasting (CSW) occurs because of excess renal loss of sodium with volume depletion and has been associated with abnormally high atrial natriuretic peptide or brain natriuretic peptide levels, which block all stimulators of zona glomerulosa steroidogenesis, resulting in mineralocorticoid deficiency.[237] CSW usually occurs 1 to 2 days after neurosurgical intervention, and patients typically demonstrate polyuria, dehydration, a serum sodium level less than 130 mEq/L, and excess urine sodium or urine osmolarity. Duration of CSW was 1 to 9 days in a study of 12 pediatric patients.[238] CSW is generally treated with salt repletion, although fludrocortisone supplementation has also been successful.[239] Syndrome of inappropriate diuretic hormone (SIADH), by contrast, results when water is preferentially retained, causing dilution of serum sodium. Patients present with hyponatremia and an elevated urine sodium level.[240] The major clinical difference between CSW and SIADH is that in the former, dehydration is common, whereas in SIADH, symptoms of dehydration are lacking. SIADH is treated with water restriction. CSW was much more common than SIADH in one study of 30 patients.[241]

Posterior fossa syndrome (or cerebellar mutism syndrome) is a complex and heterogeneous disorder that tends to occur 24 to 48 hours after resection of a posterior fossa tumor,[242] usually medulloblastoma, ependymoma, or low-grade astrocytoma. Posterior fossa syndrome will develop in up to 25% of patients undergoing surgical resection in the posterior fossa and, in most patients, it will be severe.[242] The pathophysiologic basis for this syndrome is unclear, with many hypotheses proposed. It is likely related to pressure effects on the deep cerebellar nuclei and is more frequently associated with large tumors extending into those areas. Gross total resection (GTR) of most posterior fossa brain tumors is associated with better long-term outcomes, and it is thought that the more aggressive surgical resections for those reasons are associated with the perceived increase in the frequency of this syndrome. The exact clinical patterns of posterior fossa syndrome can vary among patients both in constellation and severity. Usually the disorder includes loss of speech in patients who were capable of talking immediately after surgery. Other symptoms include irritability, which may relate to difficulties in communication, emotional withdrawal, and motor difficulties with ataxia.[242] In a review of 450 children from two large Children's Cancer Group (CCG) protocols for high-risk and standard-risk medulloblastoma, 107 patients (24%) had posterior fossa syndrome. It was classified as severe in 43%, moderate in 49%, and mild in 8%. As with many neurologic insults, most patients demonstrated significant improvement, although neurologic abnormalities persisted in a large proportion of patients.[243] No uniform diagnostic criteria exist for posterior fossa syndrome, and radiologic imaging with SPECT has failed to define a specific controlling neurologic region.[244]

Venous thrombosis in adults with brain tumors is common, especially when compared with adults undergoing operations for causes not related to brain tumors,[245,246] and typically requires therapy. A similar predilection to significant symptomatic thrombosis in children with CNS tumors has not been identified.[247] Because of the risks of spontaneous hemorrhage in patients taking anticoagulants, their routine use is discouraged for pediatric patients.[248] When thromboses in this patient population are identified, other precipitating factors such as the presence of a central venous access device[247] are usually evident.[249] CNS hemorrhage is rare in pediatric patients undergoing tumor resection. Use of recombinant factor VIIa has been used successfully when bleeding was difficult to control.[250]

Long-Term Follow-Up

Long-term follow-up is extremely important because neurosurgical sequelae of the treatment of brain tumors exist. The management of hydrocephalus is lifelong, and all survivors of childhood brain tumors who have treated hydrocephalus should undergo frequent evaluations by a neurosurgeon and radiographic evaluation of the patency of the ETV (via specialized MRIs) or of their shunt systems.

A relatively rare but significant complication of radiation therapy and surgery for tumors in the sellar/suprasellar area is moyamoya disease. This condition is manifested by the development of occlusive vascular disease at the base of the skull with subsequent ischemic events and stroke. The management includes surgical revascularization, which is effective in stopping the clinical symptomatology and preventing new ischemic events and is performed in centers with specialized interest in this condition and expertise in the surgical procedure.

Radiotherapy

A detailed section on the fundamentals of radiation oncology is included elsewhere in this text (see Chapter 48). The primary goal of this brief discussion is to focus on radiation therapy issues that are specific to pediatric neurooncology.[251] An understanding of the basic principles of radiation therapy is critical because it remains one of the most effective, albeit toxic, therapies for this patient population.

Like chemotherapy, radiation therapy targets dividing cells, one of the hallmarks of cancer therapy. Unlike chemotherapy, however, delivery of radiation therapy is not limited by the BBB. The biologic effects of ionizing radiation are the result of damage to cellular DNA, primarily through irreparable double-strand breaks. Photon radiation can cause damage through direct interaction with DNA or through the formation of free radicals, which damage the DNA. Charged particles can cause damage through direct interactions with the nucleus. This damage results in complex cascades of molecular events, affecting cell cycle checkpoints, apoptosis, DNA damage response, and DNA repair. Tumor cells have lost the regulation required to repair DNA damage before entering cell replication. In contrast normal adjacent cells that receive radiation therapy will repair the damage between fractions. After weeks of continued radiation therapy, normal cells will have repaired themselves, although the repair process is not perfect and accounts for the toxicity to normal brain caused by radiation therapy. Tumor cells, on the other hand, will have accumulated significant damage, leading to cell death. The concept of fractionation is the hallmark of radiation therapy.

Technical Aspects of Radiation Therapy

Radiation therapy technology has advanced continually since the discovery of x-rays. The most commonly used modalities in the treatment of CNS tumors are photon radiotherapy and proton radiotherapy. Both these forms of irradiation provide the same treatment dose to the tumor tissue, and therefore their differences are not related to efficacy. Rather the differences in the therapeutic beams and the physical properties associated with them result in different dose distributions. The major clinical difference between the two techniques relates to potential toxicities to the normal brain.

Photon Therapy. Photons are packets of high energy that enter tissue, depositing their energy as they pass through both normal tissue and the tumor, eventually exiting the brain. The presence of an exit dose is one of the major differences between this modality and proton therapy. The generation of photon beams is easily achieved with a large array of commercially available machines and, with more than 60 years of clinical experience, photon radiotherapy remains the most widely used form of radiation therapy. Significant advances in this modality have occurred through the development of better imaging (MRI and functional imaging such as PET and metaiodobenzylguanidine), planning algorithms, and machine delivery that has allowed the radiation oncologist to target tumors more accurately and to minimize the dose delivered to normal tissue.

Opposed Lateral Fields. Used largely before 1990, opposed lateral fields typically rely on two wide beams, usually one from the left and the other from the right. This approach is no longer considered the standard of care in children with a brain tumor because of excessive toxicity to normal brain tissue. At a minimum conformal fields should be used.

Conformal Radiation Therapy. The conformal radiation technique uses the principle of tumor volume definition through MRI and CT scans. In the treatment of CNS tumors, the anatomic localization of the bony structures of the skull using CT scans are fused to the MRI with specialized fusion software. This technique allows accurate tumor definition and submillimeter accuracy in treatment. Radiation therapists choose beam placement to maximize tumor coverage while avoiding normal brain tissue. With 3D conformal radiation therapy, conformal beams are used to shape the dose delivered to the target, and wedges or compensators can be used to optimize the dose distribution. With 3D conformal radiation therapy, variable field weighting and use of different energies (higher beam energies are more penetrating) are additional tools that enable optimization of the dose distribution. With the development of more rapid and powerful computers, the ability to generate a large number of different beam orientations has allowed for diffusion of the radiation dose over normal tissue, thus reducing long-term damage while ensuring complete coverage of the tumor volume.[252]

Stereotactic Radiotherapy. Stereotactic radiotherapy is a further improvement on 3D conformal therapy by ensuring improved head immobility so that beam configuration may be further reduced without having to worry about head position and tumor location.[253] In the normal delivery of the radiation beam, some head movement may occur; therefore, the target volume must be expanded in all directions to ensure that at no time is part of the tumor outside the targeted area. To overcome this difficulty, a series of techniques have been developed that use head immobilization to ensure less head movement and consequently smaller target volumes. Options for such immobilization include frames bolted to the skull and then fixed to the radiation device, which is the standard procedure for stereotactic radiation surgery (discussed later). The major limitation of this procedure, however, is that radiation therapy is delivered over approximately 6 weeks, and bolting screws into the skull for this length of time is not practical. A second option is the use of a head mask made for each patient, which fits around the face and skull and can sometimes use the ear canals or palate to ensure exact and reproducible fits. A new development is the use of real-time CT scans that constantly reevaluate the position of the skull, with computer software that corrects for changes in head position to reset the beams accordingly. In children the additional dose of radiation therapy from CT scans is not trivial and needs to be considered in the choice of method.

Intensity-Modulated Radiation Therapy. Intensity-modulated radiation therapy (IMRT) gives radiation therapists the opportunity to modulate the intensity of a radiation beam so that instead of uniform dosing throughout a volume, areas of decreased intensity may spare critical structures.[254] For example if a critical nerve runs through or adjacent to a tumor, IMRT allows the radiation therapist to spare the middle, like a doughnut, while still treating the entire surrounding tumor. IMRT is a device that fits onto the gantry of a radiation machine and moves small metal slots in and out of the beam path as it moves in arcs to deliver the required fields. Large metal (macroleaf) and small metal (microleaf) pieces are available. The microleaf collimators allow for a more refined shaping of the field. An important limitation of this technique is the development of hot spots in areas within the field. As a general rule this technique can be added to 3D conformal and stereotactic radiation therapy treatment plans.

Stereotactic Radiosurgery. Stereotactic radiosurgery (SRS) techniques (e.g., gamma knife, cyber knife, or X-knife) use a single fraction (or occasionally several fractions) rather than the prolonged treatment courses that are standard with radiation therapy. As the names imply in these techniques, all develop a focused beam of energy that covers a tight volume and causes the cells within the volume to die.[255] Unlike traditional radiation therapy, in which normal cells recover between successive doses while tumor cells do not, the principle for this technique is similar to focusing sunlight with a magnifying glass to burn a small area. Because of the significant toxicities possible with a technique designed to kill a targeted area, a specific volume that excludes normal adjacent structures is critical. To achieve this requirement, after induction of general anesthesia, the head is often bolted into a metal head frame, and then the metal frame is bolted to the radiation device. In this way no additional or unintended movement can occur that would result in the targeted beam missing part of the tumor and damaging uninvolved adjacent normal brain tissue. New methods that can avoid the need for fixed head localization are being developed.[256]

Proton Radiotherapy. The use of proton beam radiation therapy as an alternative to high-energy x-rays (photons) has the potential to limit some of the late effects of radiation therapy by reducing the exposure of normal tissue to the radiation.[257,258] Physically, photon beams deliver their maximum radiation dose near the surface, followed by a continuously reducing dose with increasing depth. Tissues outside the target area receive an exit dose of the radiation beams. For example when a single posterior field is used to treat the spinal axis, critical organs along the path such as the heart, lung, bowel, and ovaries may receive significant exposure. In contrast in proton beam radiotherapy, while the charged particles—namely, protons—move through tissue, they ionize particles and deposit their radiation dose along the path. The maximal dose, called the Bragg peak, occurs shortly before the point of greatest tissue penetration, which is dependent on the energy of the proton beam. Because the energy can be precisely controlled, the Bragg peak can be placed within the tumor targeted to receive the radiation dose. Because the protons are absorbed at this point, normal tissues beyond the target receive little irradiation.[259] Proton beam radiotherapy is becoming more readily available but remains a more limited and costly modality. Children most likely to benefit from proton beam treatment are those with favorable or curable brain tumors such as craniopharyngiomas, medulloblastomas, LGGs, ependymomas, and GCTs. With the increasing complexity of radiation planning, a dedicated pediatric radiation oncology team is required to ensure appropriate care for the pediatric patient.[260-262]

Toxicity of Radiation Therapy

Even with the significant advances in the highly precise delivery of radiation therapy, a number of circumstances limit its usefulness for children. Humans achieve their maximum number of brain cells shortly after birth. From that time forward, a steady loss of cells continues throughout life. While we age our neurocognitive development is related to the development of new connections and interactions between cells, not the addition of cells. For reasons that are poorly understood, irradiation can affect not only the proliferation of new cells in infancy but also the ability of cells already present to form or maintain connections in children and adolescents.

Late effects of radiotherapy in the treatment of children with brain tumors include neurocognitive sequelae, ototoxicity, hormonal dysfunction, vascular complications, growth disturbance, and secondary malignancies.[263-273] The severity of these effects depend upon many factors. The three major factors that determine the severity of the impairment after radiation therapy are the age of the patient, the volume of the brain to be irradiated, and the dose of radiation therapy required.

1. *Age*. Because the age of the patient at diagnosis is not mutable, simply withholding radiation therapy until a child has had an opportunity to grow older would reduce the long-term morbidity, but this approach may allow a tumor to recur. As such, the decision between accepting toxicity or foregoing efficacy is not uncommon in pediatric practice.
2. *Volume*. Although neurocognitive development is most active from birth until the age of 3 years, significant development occurs up to the age of 10 years and even into adulthood, which implies that radiation therapy to large parts of the brain can cause detrimental effects on cognition throughout life. Because the volume of brain to be irradiated is determined by the extent of tumor spread, radiation therapists must treat the required volumes with all the associated long-term morbidity or reduce the volume to be treated with a corresponding reduction in the efficacy that radiation provides.
3. *Dose*. The third factor related to radiation toxicity is the dose used. Because most brain tumors require the use of maximal tolerated doses to have a significant clinical impact on outcome, reducing the dose would again require a tradeoff between toxicity reduction and efficacy reduction.

Further discussion of the toxicities of radiation therapy is found in Chapter 47.

Chemotherapy

The approach and use of chemotherapy, like radiation therapy, does not differ significantly for most children with brain tumors when compared with children with other cancers. Similarly the effects of antiseizure medications on chemotherapy mirror those of other patient populations, and agents susceptible to altered metabolism by enzyme-inducing anticonvulsants, such as irinotecan, require appropriate dose adjustments or a switch to a nonenzyme-inducing anticonvulsant.[274-276] This requirement has resulted in the development of many pediatric treatment regimens modified from adult studies.[277] The increasing recognition of the unique nature of pediatric tumors in general and pediatric brain tumors specifically has led to the development of pediatric-specific preclinical models of cancer.[278-281] Incorporation of information from these models is likely to be slow as different combinations of chemotherapy, biologic therapies, and radiation therapy undergo evaluation. The need for drugs to cross into the brain raises questions about agents that showed no activity in prior clinical trials. Although the routine approach was to discard such agents as inactive, the ability to modify such agents to improve their CNS penetration has resulted in the need to retest some of these chemotherapeutic classes.[282-284]

Blood-Brain Barrier

The BBB results from the tight junctions of endothelial cells and astrocytic projections surrounding the brain that limit the penetration of substances, especially infections and inflammatory responses, from gaining access to the CNS. A slightly different barrier exists between the blood and CSF (called the blood-CSF barrier), although the primary role of the two systems remains the same—to isolate the brain from the entry of as many foreign chemicals and pathogens as possible.

While tumors develop, they grow and invade normal structures, which can disrupt the BBB. Tumors also need to secure a blood supply, which can be achieved through the secretion of a large number of cytokines, of which the best characterized is VEGF. Before its discovery in stimulating angiogenesis, this molecule was initially discovered as vascular permeability factor (VPF) because of its ability to open up endothelial junctions, allowing for changes in fluid shifts. The secretion of VEGF (VPF) by tumors is responsible for significant peritumoral edema and leakage of the BBB. For reasons that remain poorly understood at present, many tumors, or areas within tumors, do not demonstrate disruption of the BBB, making their detection on contrast-enhanced MRI scans more difficult.

General principles of chemotherapy administration in tumors of the CNS are similar to those of other tumors of the body. The CNS lacks a lymphatic system and, because of the presence of the BBB, extraneural metastases are uncommon. Thus the goal of treatment remains focused on the brain and spine. Some agents may not fully penetrate through the BBB,[285,286] depending on the characteristics of the drug and local breakdown of the BBB. Although the chemical structure of compounds should be an important consideration in their predicted ability to penetrate the BBB, and thus have the potential for clinical activity, many hydrophilic drugs have demonstrated activity in brain tumors. However some hydrophobic agents, which should easily traverse the BBB, do not traverse it. Even with extensive knowledge of the hydrophobicity of a drug, one cannot predict with certainty whether it will have activity in CNS tumors. The platinum drugs, for example, which would not be predicted to penetrate into the brain significantly, are active agents for various tumors in the brain and spine. This outcome may in part relate to the breakdown of the BBB around tumors, resulting in penetration of drugs into restricted areas. One important variable that can significantly affect BBB penetration is the degree of protein binding. To overcome this problem of drug delivery, direct application of drugs into surgical cavities or cysts is possible. Hydrostatic pressure gradients moving from tissue into an empty cavity draw most of the drugs away from the tumor and likely account for the limited activity of chemotherapeutic agent–impregnated wafers along the resection margin.

Intrathecal or Intra-Ommaya Chemotherapy

Intrathecal administration of chemotherapy can overcome the blood-CSF barrier and is of importance in tumors with a predilection to seeding of the brain and spine. This technique is not safe in patients with obstructed CSF flow, but for patients without this problem who also lack diversional shunts that would draw the drugs out of the CSF spaces, high concentrations can be delivered.[287] Because repeated access to the lumbar spine can be uncomfortable, insertion of an Ommaya or Rickham reservoir may reduce the difficulties of repeated administration. To assist in the delivery of chemotherapeutic agents into the CSF, insertion of reservoirs that sit on top of the skull or in the subcutaneous tissue of the abdomen or flank can make repeated administration more practical. Although a number of new agents have been investigated for intrathecal or intra-Ommaya/Rickham administration, including busulfan, etoposide, and mafosfamide,[288-291] overall a limited number of agents may be safely administered to this compartment, including standard intrathecal agents used in leukemia (e.g., methotrexate and cytarabine), etoposide,[292] topotecan,[293] and liposomal cytarabine.[294,295]

An important approach to increasing the penetration of chemotherapy into the brain has been the use of high-dose systemic therapy followed by stem cell rescue. The efficacy of this approach and management of the associated toxicities continue to be an area of significant study (discussed later).

Newer methods of targeting penetration of drugs into the CNS include BBB disruption agents. These agents are in clinical trial and are designed to temporarily open up the tight junctions protecting the CNS.[296] They are typically administered just before the active anticancer agent is administered. Although this approach is promising and deserving of additional evaluation, a common problem to date has been the opening of the BBB in normal areas of the brain, resulting in greater toxicities to uninvolved areas. In a similar approach new lipophilic carrier molecules have been designed to help transport drugs across the BBB, and further advances in these areas are expected.

Convection-Enhanced Delivery

With the movement of fluids away from areas of high interstitial pressure to areas of low interstitial pressure, passive diffusion of drugs deep into tumors is unlikely to occur in sufficient concentrations to be effective. To overcome this problem developments in convection-enhanced delivery have been reported. These techniques require the implantation of small catheters that can be tunneled under the scalp, which then penetrate the solid tumor parenchyma or adjacent brain. By injecting drugs under high pressure, these agents move through the interstitial space between cells, giving the drugs an opportunity to kill tumor cells, even in critical areas of the CNS such as the pons.[297] Because tumors usually penetrate along the pathway of least resistance, similar to fluids under pressure, this technique allows the drug to spread out in a fashion similar to that of the infiltrating tumor. A number of candidate molecules designed for convection-enhanced delivery are being developed. This technique should be equally well suited to small-molecule inhibitors, chemotherapeutic agents, biologic drugs (including large protein molecules), and gene vectors. Advances in drug packaging may permit control of drug delivery, improving the activity of this approach.[298]

Novel Chemotherapeutic and Biologic Agents

The revolution in the molecular classification of adult and pediatric tumors has significantly advanced our understanding of pathways implicated in tumor initiation, progression, and metastases. These pathways have also become important targets for new therapy approaches that are included in the realm of chemotherapy but differ in many fundamental ways. The unique mechanism of action of these inhibitors, and their lack of typical chemotherapy-related toxicity (e.g., myelosuppression) make them ideally suited for combination with traditional chemotherapy and radiation therapy.[5,299] In addition to classic cytotoxic agents, the use of agents that modulate the epigenome, such as histone deacetylases, which modify DNA methylation, a process critical in gene regulation, may allow apoptotic or differentiation genes to be reactivated while turning off proliferative pathways.[300,301]

The development of new formulations of old drugs also deserves comment. Many drugs that were tested in children with tumors of the CNS did not demonstrate activity, which may have been the result of poor penetration or unknown pharmacokinetics. Modifications to older agents such as doxorubicin by pegylation and liposomal encapsulation will require additional clinical testing.[302] Even vincristine, which is commonly used in pediatric patients with CNS tumors despite a lack of clear data demonstrating its activity in these diseases, is being redeveloped to improve its potential activity.[284,303]

Numerous targeted agents are now available, and each will require some early pediatric clinical experience regarding dosing and tolerability.[304] Unfortunately most biologic agents are unlikely to possess significant single-agent activity or resistance will develop, although some exciting responses have been observed in a number of different pathways, including SHH and BRAF.[305] Many of these drugs may be better at slowing tumor progression and will need to be used in combination with other molecular inhibitors, radiation therapy, and chemotherapy. These targets may also have important roles as prognostic markers, as well as therapeutic targets.[306,307]

Small-Molecule Inhibitors. The sequencing of the human genome and the identification of a number of critical signaling pathways, especially the receptor tyrosine kinases, have provided the basis for a whole new class of anticancer agents.[308] Normal cells transmit signals from the external environment to the nucleus via receptor tyrosine kinases. These molecules sit on the cell surface and homodimerize or heterodimerize in the presence of ligand, which results in a conformational change in the intracellular domain of the receptor. This process allows a phosphorylation event on the cytoplasmic component of the receptor that begins a complex cascade that results in the alteration of cell function. Many tumors use these receptors or their pathways to drive cell proliferation and migration, as well as decouple cell repair and apoptosis.[309]

The activation of receptor tyrosine kinases results from the phosphorylation of a tyrosine residue in the intracellular domain of the receptor. This process can be blocked by the steric interference of small molecules designed to fit into the phosphorylation pocket. By carefully designing the shape of these molecules, the ability to define the specificity of these drugs to related receptors means that some inhibitors can disrupt only a single receptor or entire families of receptors. A number of experimental studies of these inhibitors have been tested in children with brain tumors, including those targeting the platelet-derived growth factor receptor (PDGFR)[310] and ras pathways.[311] One problem with small-molecule inhibitors, as with other agents targeting the CNS, is the need to get drugs across the BBB; small molecules are ideally suited for this purpose, but efflux pumps are present that may expel the agents, resulting in limited activity.[312] Unfortunately this mechanism must be evaluated on a drug-by-drug basis.

Immunotherapy

Although the brain and spine are considered immunologically privileged sites, the presence of lymphocytic infiltrates in many brain tumors suggests that activation of the immune system is possible. Significant attempts to activate the immune system against these tumors,[313] especially malignant gliomas, have followed two main approaches. Some groups have attempted to increase the immune activation of lymphocytes by cytokine stimulation of the innate immune system. In the second approach a patient's own tumor is required to generate tumor-specific immune-activated cells.[314] While more is learned about how immune cells interact with and are activated by tumors, opportunities to develop immunotherapies may become more feasible,[315,316] and some early encouraging results are already being reported.[317,318]

Gene Therapy

The use of gene vectors for the treatment of brain tumor allows for the expression of a large array of different molecules.[319] Many experimental approaches have focused on modulators of the immune response.[320] An important component in the expansion of these approaches will be the identification of different vectors,[321] as well as improvement in direct delivery of large molecules directly into the brain to bypass the BBB.[322]

Antiangiogenic Agents

During the past few decades the role of angiogenesis in the development of cancer has evolved from a novel hypothesis to a fundamental area of research and therapeutic intervention. Although not brain tumor–specific, angiogenesis has been a hallmark of the progression of malignant gliomas. A large number of antiangiogenic inhibitors are now in clinical trials and have focused on targeting the cytokine vascular endothelial growth factor,[323,324]; other inhibitors of the angiogenic cascade are being tested as well.[325,326] Although bevacizumab has been extensively tested in adults with malignant gliomas, its role in pediatric tumors remains unclear.[327,328] A developing area ideally suited for pediatric patients has been the use of oral antiangiogenic chemotherapy.[329-331] The principle behind this approach is the use of very-low-dose chemotherapy that targets dividing endothelial cells rather than tumor cells. A number of low-dose chemotherapy approaches are being tested, and preliminary results are encouraging.[226,332-336]

Suppression of Tumor Resistance

The innate resistance of many tumors to cytotoxic agents, especially those of glial origin, is well documented by the near-complete and rapid progression of HGGs after upfront radiation and chemotherapy.[337] Tumors can use a number of pathways to avoid cell death when confronted with DNA-damaging agents. Temozolomide has become widely used in conjunction with radiation therapy in adults and has demonstrated clear activity in these patients. Although this combination has demonstrated prolongation in time to progression of the disease, in the vast majority of patients the disease will eventually progress as resistance mechanisms are activated. To help overcome this problem recent clinical trials have begun testing molecules that can bind and inactivate the enzymes responsible for resistance. One such example is O(6)-benzylguanine (O(6)-BG), a small molecular compound that can bind the enzyme O(6)-methylguanine-DNA methyltransferase (MGMT), which functions to remove the methyl group on the O(6) position of guanine after temozolomide treatment.[338] By treating the patient in advance with O(6)-BG, all free MGMT can be consumed, at which point administration of temozolomide can damage the DNA. Although still early in pediatric testing, this approach offers considerable opportunity.[339,340] Additional resistance pathways have been identified in pediatric and adult brain tumors that may guide therapeutic approaches, as well as the development of new inhibitors of the resistance pathways.[341,342] For example mutations within the *PMS2* mismatch repair gene, which can lead to tumorigenesis and treatment resistance, may be influenced by the addition of retinoic acid.[343]

High-Dose Chemotherapy with Stem Cell Rescue

The principles of high-dose chemotherapy and stem cell rescue are similar to those for other malignant diseases. Many brain tumors, especially those of primitive neuroectodermal origin, such as medulloblastoma and CNS PNETs, have demonstrated dose-dependent chemotherapy responses.[344-346] This technique has therefore been used extensively in children with relapsed disease after standard upfront therapy[347-349] and in young children and infants.[350-353] In light of the long-term neurocognitive effects of radiation therapy for young children, particularly for diseases that require craniospinal radiation therapy, high-dose chemotherapy with autologous stem cell rescue (ASCR) currently serves as the backbone treatment in many infant protocols around the world. Although the conditioning regimens, disease histologies, and patient characteristics have differed across multiple clinical trials and retrospective studies, this approach has demonstrated positive results in patients who could achieve minimal residual disease prior to transplant.[354-356] The addition of this modality with other therapies remains to be tested and may influence the utility of this approach in persons without minimal residual disease or the ability to maintain the disease-free state.[357] The role of high-dose chemotherapy with stem cell rescue is debatable, however, in the recurrent setting.[358] The evaluation of patients after transplantation can be complicated by the presence of therapy-related signal changes on MRI scan, including heterogeneously enhancing lesions, often causing clinical symptoms related to their location. Although these lesions can appear consistent with disease progression early after transplantation, they do not progress and need to have long-term follow-up with regard to their clinical significance.[359]

PEDIATRIC BRAIN TUMORS

Gliomas

Glial tumors are usually classified according to the type of glial cell that constitute the tumors—astrocytomas, ependymomas, and oligodendrogliomas. Each is further divided by morphologic features, degree of invasiveness, and location and is assigned a grade ranging from I to IV as the features of malignancy increase. In pediatrics, the grading of astrocytomas has been defined by the WHO or St. Anne-Mayo system and is predictive of patient survival.[213,360] In pediatrics, the modified WHO classification of CNS tumors[197] has become the standard classification system (see Table 57-3). Astrocytomas can be classified as low grade (WHO grades I and II) or high grade (WHO grades III and IV). LGGs may consist of relatively pure tumors such as a juvenile pilocytic (grade I) or fibrillary (grade II) astrocytoma or mixed populations of both glial and neuronal lineages, such as ganglioglioma or glioneurocytoma. The classification of several other subtypes is still being debated. Although the differentiation of HGG from LGG is universally used and based on degree of atypia, mitoses, necrosis, and vascular proliferation, with greater molecular definition of astrocytic tumors, the need to separate grade I from II and grade III from IV tumors regarding treatment and prognosis is increasing. Pediatric gliomas can also be discussed in the context of location, rather than grade. This approach recognizes some of the unique aspects of the environment in which tumors of similar histologies can grow and its effect on treatment and prognosis (Box 57-2).

Box 57-2 Histologic Classification of Low-Grade Gliomas

- Astrocytic tumors
  - Pilocytic astrocytoma
  - Pilomyxoid astrocytoma
  - Diffuse astrocytomas (fibrillary, protoplasmic, gemistocytic)
  - Pleomorphic xanthoastrocytoma
  - Subependymal giant cell astrocytoma
- Oligodendroglial and mixed glial tumors
  - Oligodendrocytoma
  - Oligodendroglioma
  - Oligoastrocytoma
- Mixed glial-neuronal tumors
  - Gangliocytoma
  - Ganglioglioma
- Dysembryoplastic infantile astrocytoma and dysembryoplastic infantile ganglioglioma
- Dysembryoplastic neuroepithelial tumor
- Special locations that are often not biopsied
  - Optic pathway gliomas
  - Tectal gliomas
- Cervicomedullary gliomas

Low-Grade Glioma

Supratentorial, Cerebellar Pilocytic, and Other Low-Grade Astrocytomas. LGGs represent the most frequent group of brain tumors that develop during childhood. According to the latest statistical report from the Central Brain Tumor Registry of the United States,[22] the annual incidence of pediatric LGGs in the United States is 2.1 per 100,000 persons. PAs, which make up the majority of LGGs, are well-circumscribed tumors classified as WHO grade I.[361] These tumors were formally referred to as juvenile PAs but are now classified simply as PAs. Grade I tumors are the most common LGGs found in children, representing 20% to 30% of all childhood brain tumors. PAs typically appear in the first two decades, with no clear gender predominance. They usually grow slowly, although their presentation can occur as acute deterioration as a result of obstructive hydrocephalus. NF-1 is the best example of a condition associated with an increased risk of PA in up to 15% of these patients.[362] The localization of PAs in the context of NF-1 and their improved long-term prognosis is well established,[363,364] although the molecular basis for this difference is unclear. Other predisposing factors such as common cytogenetic abnormalities are uncommon.[365] Low-grade astrocytomas typically lack epidermal growth factor receptor (EGFR) amplification,[366] although defects in the BRAF pathway have recently been reported and are present in the majority of cases.[367]

Clinical Presentation. Low-grade astrocytomas in children have a varied course, ranging from dissemination and persistent recurrence to spontaneous regression without therapy, and they behave differently from low-grade astrocytomas in adults.[368,369] The biologic factors that account for these differences remain investigational, although telomere length may play an important role.[370] PAs commonly occur throughout the brain, including the optic pathways, optic chiasm–hypothalamus, thalamus and basal ganglia, cerebral hemispheres, cerebellum, and brainstem (dorsally exophytic brainstem glioma). PAs of the spinal cord are less common.

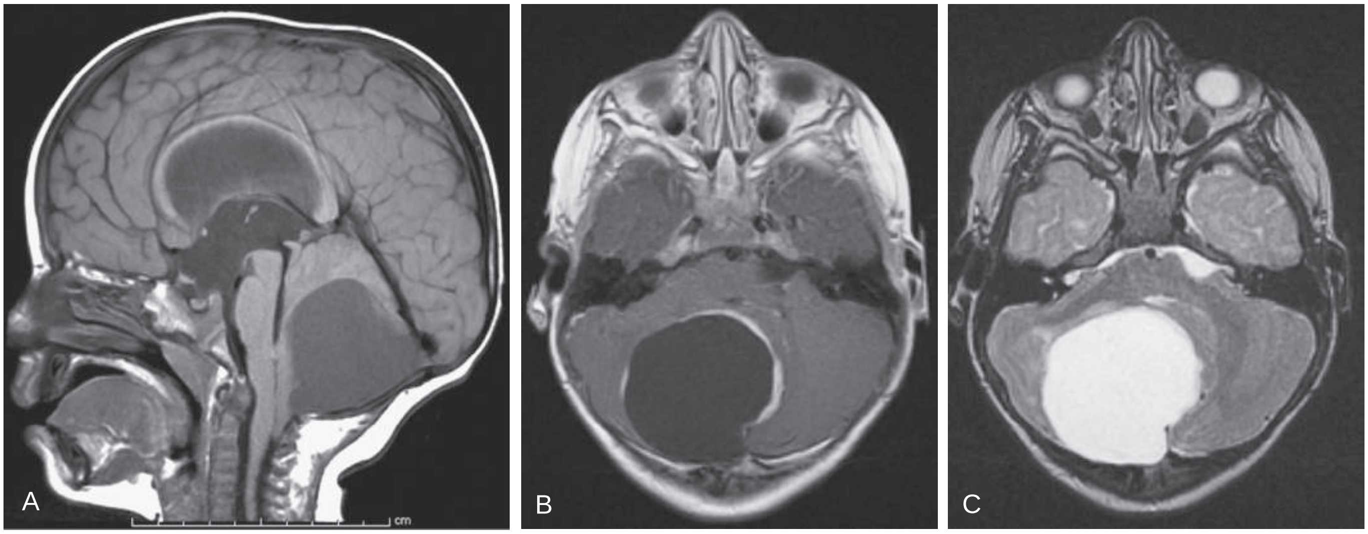

Figure 57-18 Posterior fossa pilocytic astrocytoma (juvenile pilocytic astrocytoma). **A,** A sagittal T1-weighted image without use of contrast material. **B,** An axial T1-weighted image with use of contrast material. **C,** An axial T2-weighted image.

The spectrum of clinical manifestations of a PA depends on the site of origin, its size, the age of the patient, and the presence of raised ICP. Cerebellar PAs and dorsally exophytic brainstem PAs usually present with symptoms of increased ICP, such as headache, nausea, and vomiting. Children may also present with a relatively long history of progressive focal neurologic deficits, including gait disturbance, and infants may present with progressive secondary macrocephaly. Signs of chronicity such as bone remodeling, scoliosis, or hemihypertrophy may be present, depending on the primary tumor location. The diencephalic syndrome is unique to low-grade astrocytomas, both PA and pilomyxoid astrocytoma, is typically seen in infants whose tumors arise from the hypothalamus or optic pathways, and consists of emaciation, emesis, euphoria, and normal linear growth.[371,372] Although many other deep-seated low-grade glial tumors cannot be resected, patients with diencephalic syndrome appear to have a worse prognosis, suggesting that subtle biologic differences among these tumors and other PAs may exist. Leptomeningeal dissemination is associated at diagnosis in 3% to 5% of cases.[373,374]

Imaging and Histology. The typical MRI appearance of a grade I astrocytoma is that of an intensely homogeneous, well-circumscribed, contrast-enhancing lesion with minimal surrounding edema. Lesions are typically bright on both T1- and T2-weighted images. Tumoral cysts are more prevalent in the cerebellum than in the cerebrum (Fig. 57-18) and often possess a contrast-enhancing mural nodule. Apparent diffusion coefficient imaging may be useful in differentiating PAs from higher grade astrocytomas.[375] Imaging characteristics can help with the differential diagnosis of low-grade lesions preoperatively but are not specific enough to be used without biopsy confirmation.[376]

Histologic examination reveals a biphasic pattern, with a compacted component containing bipolar cells (Fig. 57-19) and Rosenthal fibers and a loose cellular array containing microcysts and eosinophilic granular bodies. Rosenthal fibers and eosinophilic granular bodies are pathologic hallmarks of pilocytic astrocytomas, although they can be observed in other diseases of the CNS. Rosenthal fibers are brightly eosinophilic, hyaline masses composed of alpha-B-crystalline and are best seen on tumor smear preparations (Fig. 57-20). Eosinophilic granular bodies are globular aggregates within astrocytic processes and are also best visualized with smear preparations. PAs stain intensely with the GFAP immunoreagent. Invasion of the overlying meninges and adjacent brain parenchyma is commonly observed. Mitoses are rare, and the MIB1 labeling index is usually lower than 4%.[377] Although the pathologic criteria for anaplastic astrocytoma include the identification of mitoses, their presence in PAs does not indicate a higher grade.

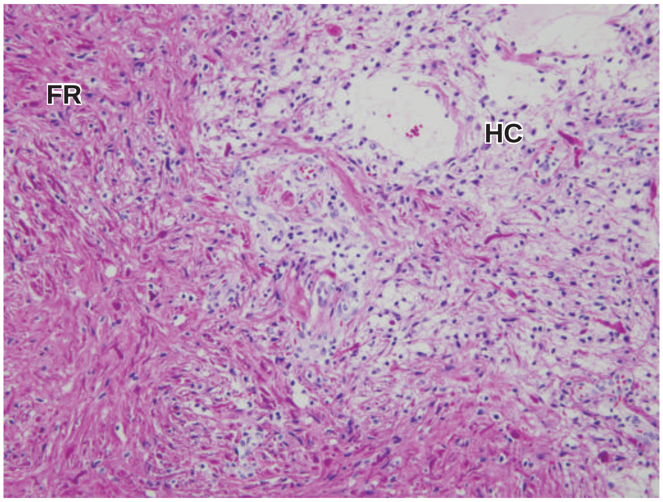

Figure 57-19 Pilocytic astrocytoma. Shown is a biphasic pattern of compact, fiber-rich *(FR)* tumor and hypocellular *(HC)* areas with microcysts (×200).

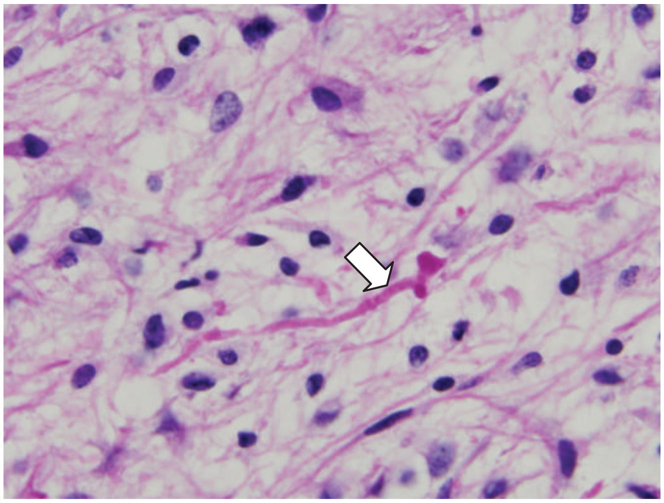

Figure 57-20 Rosenthal fiber *(arrow)* in a pilocytic astrocytoma (hematoxylin and eosin; ×1000).

Inexperienced pathologists can often misinterpret these mitoses, resulting in a diagnosis of a malignant rather than an LGG. Similarly PAs can have vascular proliferation, a hallmark of glioblastoma multiforme (grade IV astrocytoma), although, again similar to that of the presence of mitoses, this does not indicate transformation to a more malignant phenotype. Rarely PAs can present with diffuse leptomeningeal dissemination, especially in the variant of pilomyxoid astrocytoma.[378-381] Characteristic of the unique biology of PAs, each of the metastatic lesions continues to behave as a low-grade astrocytoma with a slow, indolent course. These tumors therefore are not difficult to treat as a result of their metastatic phenotype but rather as a result of their slow, persistent recurrences.

Molecular and Genetic Characteristics of Pediatric LGGs. Recent efforts in the characterization of genomic alterations in pediatric LGGs have significantly expanded our understanding of the biology of those tumors.[210] Genomic alterations of the *BRAF* gene, resulting in alteration of the MAPK pathway, are prominent in pediatric but not adult LGGs.[382] Other genomic alterations affecting PI3K/AKT, EGFR, PDGFRa, FGFR1, TrkB, MybL1 and VEGF signaling pathways have been described in a subset of pediatric LGGs.[383,384]

Importantly pediatric LGGs present distinct molecular alterations compared with adult LGGs. Tumor protein p53 *(TP53)* mutations are frequent in adult LGGs (up to 60% to 70%)[385] but are rare in pediatric LGGs.[386-388] Deletion of 1p and 19q is the most frequent copy number alteration in adult oligodendrogliomas.[389,390] In contrast very few 1p-19q codeletions have been reported in pediatric LGGs.[391,392] IDH1 and IDH2 mutations occur in about 70% of adult LGG,[393] whereas these mutations are very rarely described in the pediatric population.[394,395]

BRAF Truncation-Duplication. The high incidence of LGGs in patients with NF-1 prompted initial investigation into the role of the MAPK pathway in pediatric LGG tumorigenesis.[396] Early comparative genomic hybridization studies performed on pediatric LGGs, especially PAs, identified a significant recurrent gain of the 7q34 region containing the BRAF locus. This region is amplified in 50% to 90% of pediatric PAs, with the highest frequency in tumors of the posterior fossa and of the hypothalamic/chiasmatic region and less frequently in fibrillary astrocytomas and oligodendroglial tumors,[397-399] and they are thought to have a better prognosis than other LGGs.[400] Further genomic studies found that the 7q34 gain corresponded to a *BRAF* duplication and *KIAA1549* insertion.[401,402] In vitro validation showed that this gene product is constitutively active, leading to downstream upregulation of effectors of the MAPK pathway, MEK and ERK.[403] One short form of the *KIAA1549-BRAF* fusion induces anchorage independent growth in vitro.[404,405] Also short-term cultured pediatric LGG lines showed significant diminution of cell proliferation rate using pharmacologic inhibitors of MEK1 and MEK2.[401] Other partners of BRAF fusion have recently been identified that involve either SRGAP3 or FAM131B, all causing MAPK pathway activation.[404-408] Although break points between *BRAF* genes differ in those variants, they all result in the loss of the N-terminal inhibitory domain of *BRAF*, leading to the constitutive activation of the BRAF kinase. Recent functional studies using BRAF fusion variants have highlighted the importance of the context in which this genomic alteration drives tumor growth. Kaul et al and colleagues[409] demonstrated that in vivo transfection of the BRAF fusion transcript in mature astrocytes was not able to induce glioma tumors, whereas transfected neural stem cells were able to develop tumors. Another recent study comparing the overexpression of the BRAF-KIAA transcript in neural stem cells of different regions within the brain showed that tumors appeared only in neural stem cells located in the third ventricular region.[73]

BRAF V600E Point Mutation. BRAF V600E mutation has been described in a variety of cancer subtypes including melanoma, colorectal cancers, papillary thyroid carcinoma, non–small-cell lung carcinoma, and leukemia. This mutation enhances BRAF kinase activity and leads to the constitutive activation of the MAPK pathway. In contrast to BRAF truncation-duplication, which is strongly associated with grade I histology, the BRAF V600E mutation occurs more frequently in grade II pediatric LGGs (PLGGs), especially fibrillary astrocytomas, gangliogliomas, pilomyxoid astrocytomas, and pleomorphic xanthoastrocytomas (PXAs).[388,410-413] V600E mutation was also described as transforming fibroblasts in vitro,[405] suggesting that this specific genomic alteration drives cell proliferation in a subset of PLGGs. Interestingly a recent study showed that BRAF V600E mutation promotes neural stem cell transformation followed by senescence, which may parallel the natural history of PLGGs.[414]

Other Genomic Alterations Affecting Key Pathways. In addition to the MAPK pathway, other pathways such as PI3K/AKT/mTOR, EGFR, SHH, and VEGF have been described to be altered in PLGGs. Although PTEN deletions and p16 deletions were previously identified in anaplastic astrocytomas and therefore associated with a more aggressive phenotype of astrocytic tumors,[415] a recent study of 32 PLGGs showed that 44% had PI3K/Akt/PTEN/mTOR activation, mostly through PTEN promoter methylation.[416] Additionally BRAF fusion transcript transfection in vivo is associated with mTOR pathway activation through the S6-kinase cascade.[409] EGFR amplification, assessed by genomic hybridization and fluorescence in situ hybridization, was described in a subset of disseminated PLGGs, suggesting that the EGFR pathway may play a role in a fraction of invasive LGGs.[374] Evidence of WNT pathway activation in a subset of PAs, especially in young children, has been highlighted by a recent study showing that the patched *(PTCH1)* gene, coding for the PTCH receptor, was highly expressed in a subset of PAs, especially in patients younger than 10 years.[417] PAs might also carry abnormal function of the VEGF pathway, supported by the observation that vessel architecture of those tumors is often immature and unstable, comparable with that of HGGs.[418] The active phosphorylated forms of the VEGF receptors 1, 2, and 3 have been shown to be highly expressed in tumor vasculature in PAs.[419]

Recently genomic alterations of the transcriptional activator MYB, a known oncogene in other tumor subtypes, especially T-cell acute lymphoblastic leukemia,[420] have been identified in PLGGs. MYB amplification and focal deletions have been described in fibrillary astrocytomas and in angiocentric gliomas, respectively.[384,421]

Management. Surgery is the mainstay of therapy for most pilocytic and other low-grade astrocytomas.[422] GTR is often curative, even though residual microscopic disease may often be left behind.[423,424] Radiation therapy and chemotherapy are typically not required as part of upfront therapy after a complete resection.[425] Surgery is also an effective method of seizure control in patients with LGGs, especially those of the temporal lobes.[426,427] PLGGs in more eloquent areas may not be amenable to surgical resection. Because the OS even of nonresectable PLGG is very high, surgical resection should not be attempted when a significant risk of morbidity exists.[428] Unfortunately even in patients with complete resection, the presence of a tumor and surgical intervention can be associated with some long-term adverse effects.[429] PAs of the optic pathway in patients with NF-1 do not require surgical confirmation unless atypical radiographic or clinical features are present.[430] Patients with tectal gliomas require CSF diversion but do not benefit from biopsy; they can be diagnosed on the basis of the presence of hydrocephalus and MRI appearance of the lesion alone. In the largest prospective series of patients with PLGG stratified to observation, chemotherapy, or radiation therapy, the overall outcome of these patients was similar to that of patients reported in other independent series and is an important milestone in the assessment of more than 1000 patients with PLGG.[431]

Progressive or unresectable PAs, or those arising in infants or children that cause alterations of vision or other neurologically relevant symptoms, may require adjuvant treatment.[432] Chemotherapy is assuming an increasingly important role in the management of unresectable and/or progressive LGGs, diencephalic LGGs in younger patients, and other unresectable tumors (Table 57-5). Various combination regimens, such as carboplatin and vincristine or thioguanine, procarbazine, lomustine (CCNU), and vincristine (TPCV) have produced consistent, durable responses,[433-435] reviewed by Perilongo.[436] Monthly carboplatin is more easily administered but may have less activity[437-439] and requires additional study.

With multiagent combinations, stabilization of tumor occurs in almost 50% of patients, and radiographic response is observed in an additional 40%. Median time to progression is approximately 3 years, and up to 60% of patients will eventually demonstrate tumor growth. The Children's Oncology Group (COG) recently completed a prospective randomized phase III clinical trial examining outcomes of children younger than 10 years treated with vincristine and carboplatin versus TPCV. The TPCV arm showed a trend toward a superior 5-year event-free survival (EFS) compared with the vincristine and carboplatin arm (52% vs. 39%, respectively), although no statistically significant difference was found.[440] The ability to retreat these patients with multiple regimens has allowed most patients to avoid radiation therapy, especially early in life when the long-term morbidity of this modality is greatest. Although the time to progression may appear short and the overall progression rate of 50% to 60% appears high, these chemotherapy regimens are well tolerated, with few long-term complications. This outcome is in contrast to radiation therapy, which demonstrates a significantly improved response rate (85% response or stable disease) and duration of disease control (longer than 10 years)[441]; however, radiotherapy also entails significant long-term morbidities, such as neurocognitive, vascular, hormonal, and second tumor risks. Because most children with LGGs will be long-term survivors, this is exactly the population that would benefit from the avoidance of the late effects of radiation therapy.[442] To reduce the volume of normal tissue, stereotactic conformational external radiotherapy,[443] stereotactic radiosurgical techniques,[444] and proton radiotherapy[445] have been evaluated in the treatment of recurrent and progressive PLGG tumors. However more focused delivery of radiation in patients with PLGGs only marginally mitigates the long-term sequelae, which include malignant transformation and second malignancy, vascular injury, and, depending on location, neurocognitive decline, endocrinopathies, and other neurologic deficits. These significant and largely irreversible iatrogenic sequelae, conferred in the treatment of a disease whose natural history is self-limiting in most cases, provide an argument in favor of a radiation-avoidance strategy for children with PLGG.

The overall improved outcome for patients with NF-1 has also been confirmed,[440] indicating that these tumors may have a unique biologic phenotype. Temozolomide (TMZ), an orally active alkylating agent with a favorable adverse effect profile, has been shown to have some activity as monotherapy for adult LGGs.[446] Although it has not been widely studied, it appears to have a low response rate in LGGs in children, with a median time to

TABLE 57-5 Chemotherapy for Pediatric Low-Grade Gliomas

Treatment	No. of Patients	Objective Response, % (CR + PR)	Overall Response, % (CR + PR + SD)	EFS or OS
Carboplatin[437]	80	2 CR, 17 PR	2 CR, 17 PR, 4 MR, 46 SD	72% 3-yr EFS in patients with NF-1; 62% 3-yr EFS in patients without NF-1
Carboplatin[1911]	12 ND	4 PR	4 PR, 6 SD	
Carboplatin[1912]	4 ND, 2 PD		6 SD	
Carboplatin[1913]	13 ND/PD	1 CR/PR		
Iproplatin[1913]	15 ND/PD	1 CR/PR	1 CR/PR, 9 SD	
Cyclophosphamide[449]	15 ND	1 CR	1 CR, 9 SD	
Cyclophosphamide[1914]	1 PD, 3 ND with leptomeningeal dissemination		2 PR/MR, 2 SD	
Ifosfamide[1915]	6	1 PR	1 PR, 3 SD	
Temozolomide[1916]	21 PD	1 PR	1 PR, 20 SD	
Temozolomide[447]	13 PD	2 CR, 3 PR	2 CR, 3 PR, 3 MR, 4 SD	57% 3-yr EFS
Temozolomide[1917]	10 ND, 20 PD	3 PR	3 PR, 1 MR, 25 SD	51% 2-yr PFS and 17% 4-yr PFS
Temozolomide[1918]	2 PD		2 SD	
Methotrexate[1919]	10 PD	2 PR	2 PR, 5 SD	
Topotecan[1920]	2 PD	1 PR	1 PR, 1 SD	
Topotecan[1921]	11 PD		5 SD	
Etoposide[1922]	14 PD	1 CR, 4 PR	1 CR, 4 PR, 3 SD	
Etoposide[1923]	12 ND		6 PR/SD	
Vincristine–actinomycin-D[1924]	24 ND	3 PR	3 PR, 6 MR, 15 SD	
Vincristine-carboplatin[489]	123 ND		105/123 CR, PR or SD	61% 5-yr PFS
Vincristine-carboplatin[433]	78 ND	4 CR, 22 PR	4 CR, 22 PR, 18 MR, 29 SD	68% 3-yr PFS
Vincristine-carboplatin[434]	24 PD	7 PR	7 PR, 5 MR, 5 SD	
Carboplatin-etoposide[1925]	13 ND	1 CR	1 CR, 3 MR, 6 SD	69% patients alive at mean of 30 mo
Cisplatin-etoposide[469]	31 ND, 3 PD	1 CR, 11 PR	1 CR, 11 PR, 12 MR, 11 SD	3-year PFS 100% in patients older than 5 yr, 66% in patients younger than 5 yr
Vincristine-etoposide[1926]	11 ND, 9 PD	1 PR	1 PR, 4 MR, 9 SD	
Tamoxifen-carboplatin[1927]	12 ND, 1 PD	2/13 PR	2/13 PR, 9/13 SD	47% 3-yr PFS, 69% 3-year OS
6-Thioguanine, vincristine, CCNU, dibromodulcitol, procarbazine (TPDCV)[435,492]	15 ND (4 patients were treated with other therapy and are not included), 42 ND	11 PR, 15 CR/PR	11/15 PR, 15 CR/PR + 25 SD	Median TTP not reached at 79 wk,[312] median TTP at 132 wk[313]
Procarbazine, carboplatin, vincristine, etoposide, cisplatin, cyclophosphamide[1928]	85 ND	36/85	74/88; 36 CR or PR, 15 MR, 23 SD	34% 5-yr PFS, 89% 5-yr OS

TABLE 57-5 Chemotherapy for Pediatric Low-Grade Gliomas (Continued)

Treatment	No. of Patients	Objective Response, % (CR + PR)	Overall Response, % (CR + PR + SD)	EFS or OS
Carboplatin, etoposide, cyclophosphamide, vincristine, CCNU, procarbazine[460]	7 ND, 3 PD	2/10	70% CR + PR + MR; 100%, including SD	70% PFS at 5.6 yr
5-Fluorouracil, vincristine, cyclophosphamide, etoposide[450]	13 (12 ND, 1 PD)	6/13, 1 CR, 5 PR	8/13, 1 CR, 5 PR, 2 SD	6-yr PFS 67%
Vinblastine[332]	9 (ND with carboplatin allergy; nonprogressive at time of therapy)	2/9	9/9; 1 CR, 1 PR, 5 MR, 2 SD	Median follow-up of patients, 10 mo
Thioguanine, procarbazine, CCNU, vincristine[491]	9 (5 ND with carbo allergy; nonprogressive at time of therapy, 4 PD)	0/9	7/9; 7 SD	78% progression-free at 13 mo
Cisplatin, etoposide, vinblastine[1929]	16 ND	4/16 4 PR	9/16 4 PR, 5 SD	5-yr PFS 56%

Modified from Perilongo G: Considerations on the role of chemotherapy and modern radiotherapy in the treatment of childhood low-grade glioma. J Neurooncol *75:301–307, 2005.*

CR, Complete remission; *EFS,* event-free survival; *MR,* minor response; *ND,* newly diagnosed; *NF-1,* neurofibromatosis type 1; *OS,* overall survival; *PD,* progressive disease; *PFS,* progression-free survival; *PR,* partial remission; *SD,* stable disease; *TTP,* time to progression.

progression of 6.7 months, although many patients appear to have prolonged stable disease.[447,448] Responses with TMZ in disseminated low-grade astrocytomas have also been reported,[378] although another alkylator, cyclophosphamide, when given every 3 weeks, lacked significant activity.[449] The role of TMZ in combination with vincristine and carboplatin for PLGGs is currently being investigated. Other investigators have successfully used novel combinations containing 5-fluorouracil.[450] The metronomic application of vinblastine, a mitotic inhibitor, has resulted in some clinical responses or stable disease in children with LGGs who are unable to tolerate carboplatin.[332] Vinblastine is also being evaluated alone[451] and in combination with carboplatin in newly diagnosed patients. Although more information on the activity of these approaches is needed, responses in refractory LGGs have been reported with other metronomic-based chemotherapy approaches,[326] suggesting that angiogenesis may be an important pathway in these tumors. Antiangiogenic approaches, such as bevacizumab, have also been tested with some responses,[452-455] which is also consistent with the presence of vascular proliferation observed in these tumors. Chemotherapy may also allow for improved surgical resection of previously unresectable lesions and therefore should be continuously reevaluated as a therapeutic option.[456] Tumors treated with chemotherapy, even if they are not smaller, can be easier to resect because of less bleeding and a firmer texture at the time of the procedure. The recent identification of BRAF mutations in the majority of PLGGs offers the potential for targeted approaches to these patients. Phase II studies of the downstream inhibitor of mTOR, called everolimus, have recently been completed in this patient population with encouraging activity. In addition BRAF V600E small-molecule inhibitors are also being evaluated for patients with this specific mutation. Importantly the majority of patients with LGGs have the truncated fusion of BRAF (often referred to as the KIAA1549 fusion because this is the most common translocation). Treatment with a BRAF drug such as sorafenib or other V600E targeted agents would be expected to stimulate tumor growth as a result of a complex feedback loop, not to inhibit tumor growth.[457,458] Patients with truncated fusions will require therapy that accounts for this biologic pathway, and MEK inhibitors are just entering clinical trials for this patient population.

Although most chemotherapy regimens for LGGs have been reserved for children younger than 10 years, older children seem to derive equal benefit with the use of these regimens, potentially avoiding radiation therapy and the risks of second tumors, vasculopathy, and hormonal dysfunction.[459] The use of dose-intensive chemotherapy has been pilot tested in a small series of children, with results similar to those observed using standard doses.[460]

Radiotherapy is considered to be contraindicated in children with NF-1 and is usually deferred in children with PAs and other LGGs, especially in diencephalic and optic pathway tumors. Even highly focused radiation therapy in these locations cannot avoid the potential cognitive, endocrine, or vascular risks associated with radiation therapy. In spite of this highly focused radiation therapy is effective for LGGs and is without significant marginal failures, suggesting that these lesions have not deeply penetrated into surrounding brain.[461] Long-term concerns about second tumors, hormone dysfunction, and cognitive impact, however, still make this approach questionable for children.[462,463] Additional late effects of radiation therapy when used in younger children with diencephalic gliomas may include strokes related to a moyamoya-like syndrome.[85,464,465]

Prognosis. The prognosis for surgically resectable tumors is excellent after GTR. For patients with PAs whose tumors can be completely surgically resected, depending on location, the 10-year PFS approaches 90%.[380,466,467] Even in patients with incompletely resected lesions, treatment is not always required and, depending on the patient and clinical scenario, observation can be considered unless tumor or symptom progression is documented.[468] The most critical variable in the treatment of PAs is the anatomic location of the tumor. Complete resections are most difficult for tumors located in the brainstem, spinal cord, optic pathways, thalamus, and hypothalamus. As such, the PFS of children with centrally located tumors (e.g., in the optic chiasm, thalamus, or hypothalamus) is less than 50%.[467] Given the favorable toxicity profile of chemotherapy versus radiation therapy in young children, chemotherapy is preferred as the first therapeutic modality in young patients with tumors not amenable to gross total resection.[433] The use of chemotherapy as initial treatment in patients with centrally located or unresectable lesions allows for the delay of radiation therapy until the child is less likely to incur the serious developmental and neuropsychological sequelae of radiation therapy. Patients demonstrating radiographic response to chemotherapy have PFS similar to those whose tumors remained stable.[434,469] Ultimately the quality of survival depends on multiple factors, including the tumor location, the extent to which the tumor can be resected, timing of any radiotherapy, and adverse effects of surgery, chemotherapy, and radiotherapy.[470] Transformation of PAs into higher grade malignant gliomas is highly unusual.[471]

Optic Pathway Gliomas

Optic pathway gliomas (OPGs) represent approximately 4% to 6% of all primary pediatric brain tumors; the incidence is higher when asymptomatic lesions in patients with NF-1 are also included.[472] OPGs are evenly distributed between boys and girls. These tumors may involve various parts of the optic pathway, such as the optic nerves, chiasm, optic tract, and optic radiations. They may also infiltrate the adjacent hypothalamus and temporal lobes. Optic nerve gliomas are strongly associated with NF-1, although sporadic lesions are not uncommon. OPGs in patients with NF-1 have a more indolent course than those arising in patients without NF-1[473] and likely represent a different biology than sporadic tumors.[75,474] Although OPGs may be considered a subset of PAs, their unique features and management necessitate a separate discussion.[475,476]

Clinical Presentation. Most OPGs consist of PAs (WHO grade I).[477] The clinical course of OPGs can vary considerably, from an indolent mass in a child with NF-1 to a relatively aggressive, invasive, and expansile diencephalic tumor. Unilateral optic nerve gliomas often present with the classic triad of visual loss, proptosis, and optic atrophy. Optic nerve tortuosity, which can be present alone or in the context of OPGs in children with NF-1, does not necessarily define the presence of disease or the need for therapy.[478] Chiasmatic involvement may lead to unilateral or bilateral visual loss, a bitemporal visual field defect, and obstructive hydrocephalus as the tumor grows dorsally to obstruct CSF flow in the third ventricle. Further invasion into brain parenchyma may result in more pronounced visual field deficits, as well as hemiparesis. Chiasmatic tumors in infants present as large suprasellar masses that may also extend into the hypothalamus and third ventricle, producing hydrocephalus and endocrine abnormalities. In cases of hypothalamic extension, in addition to nystagmus, visual loss, and hydrocephalus, the diencephalic syndrome may occasionally be seen. This syndrome consists of hyperkinesia, euphoria, and emaciation, with preserved normal linear growth.[372] Failure to thrive is a common presentation of this syndrome, but in the context of a number of other conditions that may also cause failure to thrive during infancy, a delay in diagnosis of a brain tumor is not uncommon.[371] Endocrine deficiencies commonly accompany PAs in this location, and these tumors are more likely to have CSF dissemination in association with diencephalic syndrome, in spite of their histopathologic classification as benign.[479] Many patients with NF-1 will have long-standing, subtle ophthalmologic abnormalities, and thus regular visual evaluation is required. For these patients routine surveillance MRI scans are not indicated because many asymptomatic and clinically irrelevant tumors will be diagnosed, creating a treatment dilemma.[480]

Imaging and Histology. The clinical diagnosis of an OPG is suspected when a child presents with visual impairment, nystagmus, and/or optic atrophy. MRI of the brain or orbits typically shows a solid, cystic, or mixed type of tumor, with strong gadolinium enhancement. The imaging characteristics of LGGs of the optic pathway are similar to those of low-grade astrocytomas in other locations. The typical MRI appearance of a grade I astrocytoma is an intensely homogeneous, well-circumscribed, enhancing lesion with minimal surrounding edema (Fig. 57-21). Lesions are typically bright on both T1- and T2-weighted images. A higher ADC may predict a greater chance of progression.[481] MRI studies and clinical presentation may distinguish an OPG from other childhood tumors that arise in the suprasellar location, such as a GCT or craniopharyngioma. Histologically OPGs are usually grade I PAs or, less frequently, grade II fibrillary astrocytomas. Mixed LGGs have been reported in this region, but the overall therapeutic approach is not altered, because most of these lesions are not resectable at diagnosis. Tumors with an elevated MIB1 labeling index of more than 1% and p53 expression were more likely to be WHO grade II and had a worse outcome compared with tumors with an MIB1 and p53 labeling index less than 1%, all of which were PAs. PAs with a more aggressive behavior had an MIB1 labeling index of 2% to 3% but retained a low p53 labeling index of less than 1%.[477] Like LGGs in other locations, patients with sporadic OPGs have both BRAF V600E and truncated fusion KIAA1549 abnormalities.[399,482,483] BRAF abnormalities are rare in patients with NF-1 because of the existence of constitutive activation of the ras/raf/mek/MAPK pathway.

Management. The unpredictable clinical course of patients with optic pathway tumors has led to controversy

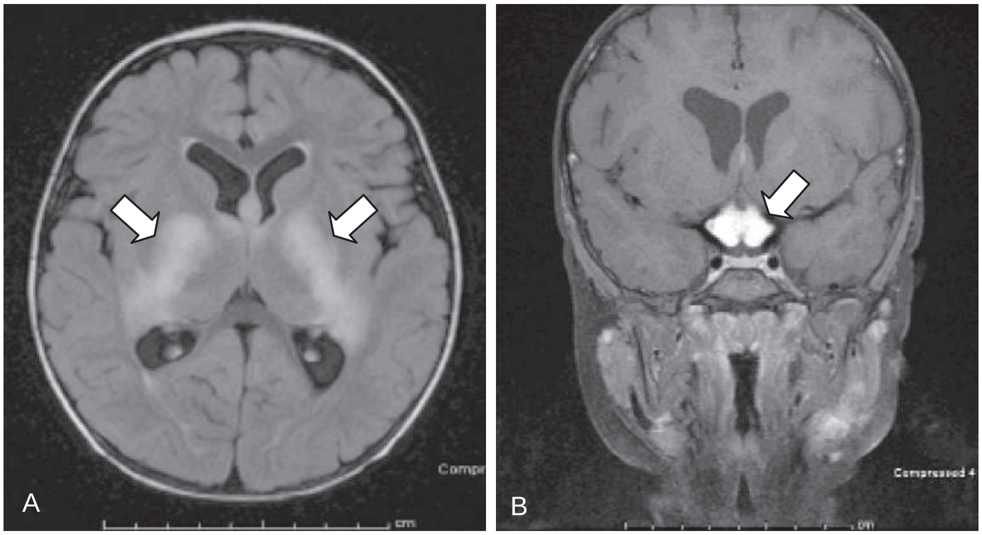

Figure 57-21 A large optic pathway glioma in a 2-year-old boy who does not have neurofibromatosis type 1. **A,** An axial T2-weighted fluid-attenuated inversion recovery image that demonstrates bilateral involvement of the optic tracts posterior to the chiasm *(arrows)*. **B,** A T1-weighted gadolinium-enhanced coronal image demonstrating the enhancing tumor expanding the optic chiasm *(arrow)*. The patient has no functional vision and is legally blind.

regarding the optimal management of these tumors. The clinical course, age of onset, severity of symptoms, size and extent of the tumor, and presence of NF-1 may all affect management decisions. Treatment is frequently started promptly in younger patients, in patients with progressive symptoms, and in those with more extensive CNS involvement, with the paramount concern being preservation of vision. Although progression can be easily quantified on an MRI scan, correlation between MRI findings and visual outcome is poor.[484] The initial treatment of choice is chemotherapy (see Table 57-5),[436] which may cause stabilization or regression[485] even in older children and adolescents[486] and is not associated with the neurocognitive decline observed with radiation therapy.[487] Surgery can be used upfront in selected cases, but the tumor may not be removed from the optic nerve without sacrificing vision.[488] Combination therapy with carboplatin and vincristine[433,489,490] or with TPCV[491] has been considered to have comparable beneficial effects. TPCV should not be used in children with NF-1 because of the increased risks of secondary tumors associated with alkylator-based treatment. When progression of the tumor occurs after treatment with vincristine and carboplatin, vincristine and actinomycin can be considered for patients with NF-1 to avoid further risks of alkylator therapy.[492,493] As for other LGGs, a number of new chemotherapy regimens are being developed (see Table 57-5) and can include bevacizumab and irintoecan,[452] vinblastine,[451] metronomic therapy,[335] and low-dose cisplatin and etoposide.[494] Current clinical trials targeting the mTOR pathway are under way, as is a phase II trial of lenalidomide.[495] Although delaying radiation therapy is paramount in young children, older patients may also benefit from these chemotherapy approaches and from delaying or avoiding radiation therapy.[459]

A randomized phase III COG study comparing the two regimens, carboplatin and vincristine versus TPCV for the treatment of progressive low-grade astrocytoma in children younger than 10 years, closed to accrual in 2005.[440] The results have shown that both regimens are well tolerated and can delay or obviate the need for radiation therapy in most patients, and that some patients will show improvement in vision with therapy.[496,497] Newer chemotherapy clinical trials for OPGs combine a number of agents such as TMZ with vincristine and carboplatin, and vinblastine and carboplatin. Although a definitive role for radiotherapy exists for the management of OPG,[498,499] current trends in treatment favor a delay in the initiation of radiotherapy in young patients.[500] Newer surgical techniques with direct administration of radiotherapy are also being explored and have demonstrated usefulness for selected patients.[501] Unlike LGGs in other areas, optic pathway tumors affect vision directly, and changes in visual fields and acuity can be a better determinant of both disease response or progression than are changes on MRI. Recent consensus criteria for the assessment of visual function in patients with NF-1 have been reported, and these criteria should provide a useful platform for the assessment of therapies that target OPGs.[502]

Prognosis. Although optic pathway tumors are almost always of low-grade histology, their location often results in serious morbidity. In patients with significant visual deterioration, progressive vision loss can continue for many years after the tumor has become stable on routine surveillance imaging.[503] The growth rate of these tumors, however, often slows during late childhood so that by adulthood, tumors may become quiescent and do not require further therapy.[442] Children with NF-1 have been shown to have a better PFS, although an age of younger than 1 year is clearly associated with a higher risk of tumor progression.[504] Patients with NF-1 appear to be at high risk for the development of moyamoya disease,[84] as well as radiation-induced second malignancies.[505] Patients with NF-1–associated optic nerve gliomas may remain stable for several years. Close observation and symptomatic management are recommended for these patients.

Low-Grade Astrocytomas of the Brainstem

Although most brainstem tumors are DIPGs, approximately 20% of brainstem tumors are low-grade astrocytomas involving the medulla, midbrain, tectum, or cervicomedullary, pontomedullary, or midbrain-pontine junction. Although 20% of nonpontine tumors in these locations can be classified as grade III or IV malignant gliomas, 80% are low-grade glial lesions with a much better prognosis.[506] These lesions differ from diffuse pontine gliomas by their clinical presentation and their imaging characteristics. Early identification of the type of brainstem lesion is critical in the workup, especially for consideration of neurosurgical intervention.[507]

Presentation. Patients with brainstem lesions can present with various clinical symptoms, depending on the location of the tumor. Medullary tumors are more common than midbrain tumors, and the male to female ratio is approximately 1 : 1.[508] In spite of the eloquent function of the brainstem, most patients with low-grade astrocytomas in this location have an indolent course with subtle neurologic findings. Most commonly patients present with cranial nerve dysfunction or head tilt. Lower motor weakness with subtle hemiparesis is also seen. Most parents have difficulty defining the start of the symptoms and refer to their child as having always been clumsy or weak. Rarely these tumors can be multifocal in nature.[509]

Histology and Imaging. Most low-grade brainstem tumors are either grade I or II astrocytomas; fewer than 20% are malignant astrocytomas. Imaging characteristics are similar to those of other LGGs; PAs (grade I) tend to be bright on T1- and T2-weighted images and enhance after administration of contrast material. Edema tends to be minimal. Fibrillary astrocytomas (grade II), by comparison, enhance to a lesser degree after administration of contrast material. The considerable overlap and variability in the imaging characteristics of grade I versus grade II astrocytomas, however, prevents accurate diagnosis based on MRI characteristics alone. The histologic features of brainstem low-grade astrocytomas are identical to grades I and II astrocytomas in other locations.

Management. Brainstem low-grade astrocytomas that remain focal can often be surgically resectable if they possess a plane between the brainstem and tumor.[510] If the tumor is completely resected, these patients are unlikely to need additional therapy. Given the good long-term outcome of this patient population, aggressive surgery should not risk damage in areas with poor tumor boundaries.[511] Incompletely resected brainstem low-grade astrocytomas have a high recurrence rate, and most of these patients will need additional therapy,[512] usually chemotherapy rather than radiation therapy, given the good long-term prognosis of these patients (see Table 57-5).[436] White matter tracts are often displaced by these tumors, and preoperative DTI can assist the neurosurgeon regarding the optimal approach to maximize resection while minimizing morbidity in this patient population.[513] Although postsurgical morbidity such as problems with swallowing can be significant, many patients eventually recover function, although this process can take many months and requires extensive physical and occupational therapy.[514] The chemotherapy options for these tumors are identical to those for low-grade astrocytomas in other locations and include vincristine and carboplatin or TPCV chemotherapy. Recurrence and need for retreatment are common, but most tumors will eventually stop growing while these patients enter adulthood.

Prognosis. Because most patients with a low-grade brainstem glioma will be long-term survivors,[510] it is important that the workup and treatment of these patients be adapted with this likely outcome in mind. Differentiation from DIPGs is usually easily made on the basis of imaging and clinical criteria. Surgical intervention must be based on the expectation that minimal long-term morbidity will result, given the large number of other treatment options available for these patients.

Low-Grade Thalamic Astrocytomas

Thalamic tumors are rare in pediatric patients, accounting for fewer than 5% of intracranial tumors. Most thalamic tumors are unilateral, and approximately 50% are low-grade astrocytomas.[515,516] Thalamic tumors occur at a slightly older age than do many other LGGs of childhood. Low-grade thalamic tumors present as unilateral or bithalamic in location, which appears to have prognostic significance.

Clinical Presentation. Thalamic tumors can present with a number of different clinical findings.[517] Raised ICP, tremors, motor deficits, seizures, and mood changes are the most commonly observed presenting symptoms.[518] Unlike in adult tumors, dementia is a rare presenting symptom.[519]

Imaging and Histology. Imaging characteristics of thalamic tumors are similar to those of gliomas in other locations (Fig. 57-22). Low-grade and high-grade histologies are approximately equally distributed, although pathologic classification of these tumors can be influenced by sampling error from small biopsy specimens.[520] The use of PET or SPECT imaging can help identify areas to be biopsied.[521] Although astrocytic histologies predominate, oligodendroglial tumors have also been identified.[516]

Management. The presence of tumor in the thalamus presents a number of management difficulties. Because of their location, complete resection is difficult,[519] although surgery can result in symptom improvement in selected cases.[522] Even attempts at biopsy can result in significant morbidity. The small amount of surgical material often raises concerns of sampling error in attempting to determine the histopathologic grade of the tumor. Biopsy of the most malignant component of these deep-seated

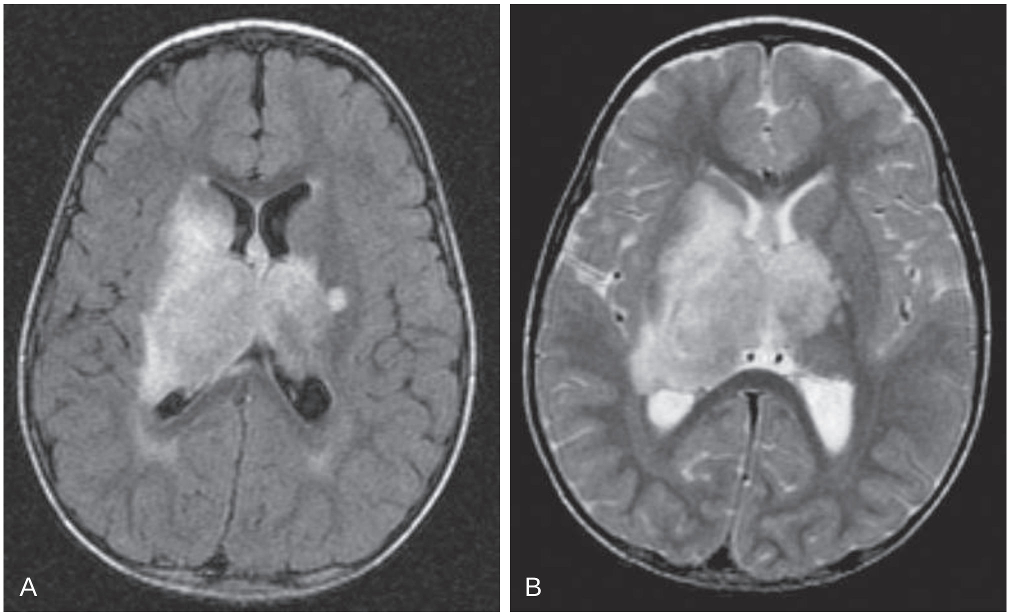

Figure 57-22 Bithalamic astrocytoma. **A,** An axial fluid-attenuated inversion recovery image. **B,** An axial T2-weighted image.

lesions can be guided with PET imaging.[521] This guidance is particularly important for astrocytic tumors, for which samples of sufficient size and content are needed to define the elements of tumor grade. In the analysis of children with thalamic lesions, most tumors are unilateral in nature, and approximately 50% to 60% are low-grade astrocytomas; the remaining 40% are high-grade lesions.[515] Some tumors are amenable to significant resection.[523,524] The response of low-grade thalamic tumors to chemotherapy and radiation is not known to be different from that of similar tumors in other locations. Thus younger patients are often treated initially with chemotherapy used for other low-grade astrocytomas (Table 57-5).[436] In those with rapid progression or histologic verification of high-grade features, radiotherapy can be used, although a significant portion of the brain will receive a substantial dose, resulting in long-term toxicity for survivors.

Prognosis. Contrary to some reports of very poor outcome for bithalamic astrocytomas of any grade,[525] five of nine patients were long-term survivors in one retrospective series.[515] Part of the difficulty in assigning an accurate prognosis to these tumors is the limited biopsy material available for assessment. With a number of reports of survivors now available, even in the context of bilateral disease, all young patients should be given a trial of chemotherapy.

Low-Grade Diencephalic Astrocytomas (Diencephalic Syndrome)

Diencephalic tumors remain a unique and poorly understood subtype of PLGGs. They typically occur in young infants, although a few patients have presented late in the first or second decade of life.[526] A high incidence of dissemination throughout the neural axis has been recognized[509] and may relate to the increased frequency of the pilomyxoid variant in this location. The presence of failure to thrive in infants often leads to an extensive and prolonged workup for gastrointestinal abnormalities before the correct cause is identified.[527] The presence of diencephalic tumors with early age of onset and the propensity to disseminate suggest that unique biologic differences may exist among these tumors and other low-grade astrocytomas, although no specific abnormalities have yet been documented.

Clinical Presentation. Diencephalic low-grade astrocytomas are differentiated from other LGGs in children by their presence around the hypothalamus–optic chiasm and a unique constellation of symptoms, including the three “E’s”—emaciation, emesis, and euphoria. In spite of severe failure to thrive that is often seen, most infants retain normal growth rates and maintain normal pituitary secretion prior to surgical resection.[526] Although an accurate clinical picture for these patients continues to be defined, because many lack the typical constellation,[372] their management is difficult because of the deep-seated nature of these lesions and the frequent presence of leptomeningeal dissemination at diagnosis.[528] As such, these patients require full craniospinal imaging at diagnosis, along with CSF analysis. Any patient presenting with symptoms of spinal disease needs to undergo immediate restaging.

Imaging and Histology. The imaging characteristics of diencephalic LGGs do not differ from those of low-grade astrocytomas in other locations. Lesions are centered around the hypothalamus and chiasm, are bright on T2-weighted images, and usually show homogeneous enhancement after administration of contrast material.[529] The lesions are typically PAs or fibrillary astrocytomas, although the pilomyxoid variant can also be observed in this location.[520] These tumors cannot be differentiated by mutational status of BRAF because some patients have the BRAF V600E mutation, some have the BRAF truncated fusion KIAA1549, and other patients have neither.[413]

Management. The approach to therapy follows that used for other low-grade astrocytomas. Because of the young patient age and deep location of these tumors, radiation therapy is usually contraindicated and will result in significant cognitive impairment over the long term.[464] Most patients undergo maximal safe surgery, followed by chemotherapy with vincristine and carboplatin or TPCV (see Table 57-5).[436] Radiographic response or stable disease accompanied by weight gain is common.[371,530,531] High-dose chemotherapy has also been used successfully[532] as an investigational approach. Radiographic responses and improvement is weight have also been reported with bevacizumab and irinotecan.[452]

Prognosis. In spite of the low-grade nature of diencephalic tumors, patients with these tumors do less well than do patients with similar tumors located in the optic pathway, brainstem, or cerebellum.[431] In addition to the continued progression of these tumors, which leads to compression of vital structures, patients are at high risk for surgery-induced hypothalamic damage resulting in obesity.

Fibrillary Astrocytomas

Fibrillary (WHO grade II) astrocytomas are low-grade astrocytomas that are distinct from PAs. Precise determination of the incidence of fibrillary astrocytomas is difficult because many tumors, particularly those in deep structures of the diencephalon or brainstem, cannot be resected sufficiently to provide the material required to classify the tumor accurately. Thus the terms “fibrillary astrocytoma” and “low-grade astrocytoma” are often used interchangeably. Although all fibrillary astrocytomas are LGGs, most LGGs are PAs. Many tumors are classified as grade II astrocytoma with piloid features because a biopsy specimen may have been too small to identify all the elements required for classification as a grade I PA. Similarly the histologic term “pilocytic astrocytoma with fibrillary features” is used to indicate tumors that have all the components of grade I pilocytic astrocytomas but with areas of infiltration suggesting that they could be grade II. Other LGGs include pilomyxoid astrocytomas, oligodendrogliomas, and gangliogliomas. Fibrillary astrocytomas localized to the posterior fossa represent 3% to 15% of cerebellar tumors[533]; their incidence is lower in the remainder of the brain and spine. No gender predilection exists, and the peak age at diagnosis is 6 to 10 years. Genetic abnormalities in LGGs are uncommon.[365] The reported incidence of LGGs has increased during the past several years. Although an association with paternal workplace exposure in the chemical and electrical industries is purported, a more likely explanation is the increased use and availability of MRI and the diagnosis of presymptomatic lesions.[19,534]

Clinical Presentation. The initial symptoms of fibrillary astrocytomas vary, depending on the location of the tumor. Patients with medullary tumors may present with a long history of dysphagia, hoarseness, ataxia, and hemiparesis. Cervicomedullary tumors may cause medullary or upper cervical symptoms such as neck discomfort, weakness or numbness of the hands, and an asymmetrical quadriparesis. Patients with midbrain tumors such as a tectal glioma often present with signs and symptoms of raised ICP. Other symptoms include diplopia and hemiparesis. In children with dorsally exophytic brainstem glioma, a component of the tumor arises in the medulla and expands in a dorsal direction, resulting in noncommunicating hydrocephalus. Supratentorial fibrillary astrocytomas more commonly present with seizures, although thalamic lesions present with motor deficits. Hypothalamic lesions can present as diencephalic syndrome.[520] Low-grade astrocytomas in the brainstem are usually focal rather than diffuse. They tend to arise in the midbrain, cerebellar peduncles, medulla, or cervicomedullary region. Because of the slow rate of progression of these lesions, most patients have subtle neurologic changes that only become evident over a long period. Unlike adult fibrillary astrocytomas, with which degeneration to malignant gliomas is common, pediatric fibrillary astrocytomas remain low grade, even after multiple recurrences.[515,535] Symptoms may exist for months to years prior to the diagnosis of a low-grade astrocytoma.

Imaging and Histology. Most low-grade fibrillary astrocytomas appear isodense on CT without significant contrast enhancement. The tumors are hypointense on T1-weighted and hyperintense on T2-weighted MRI, with minimal or no gadolinium enhancement (Fig. 57-23) except for dorsally exophytic brainstem tumors. This appearance is in contrast to that of most PAs, in which homogeneous contrast enhancement is common. Pathologic examination may demonstrate some cellular pleomorphism, but no mitoses, necrosis, or endothelial proliferation are present (i.e., no histologic features of malignancy are present). Although PAs are usually well-circumscribed lesions with a biphasic pattern of areas of bipolar cells and Rosenthal fibers and other areas with microcysts, fibrillary astrocytomas have greater cellularity and infiltrating boundaries.[536] In contrast to grade III or IV astrocytomas, fibrillary astrocytomas must lack features of malignancy, such as significant atypia and pleomorphism, mitoses, vascular proliferation, and palisading necrosis. A number of other subtypes of grade II astrocytomas have been identified, including the lipoastrocytic,[537] protoplasmic, gemistocytic,[538] and xanthomatous types.[536] These subtypes are much less common in pediatric patients compared with adults, and may have a worse outcome compared with fibrillary astrocytomas and PAs. The rarity of these lesions prevents formal studies of these subtypes.

Management. Management of most fibrillary astrocytomas is similar to that of PAs and depends on the clinical prodrome, age, and location of the primary tumor.[539]

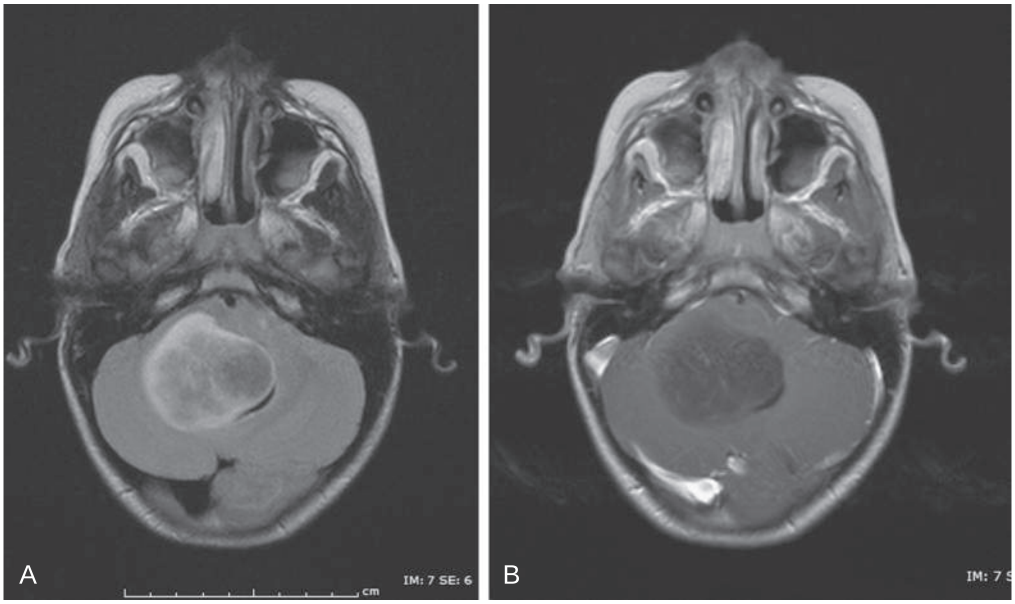

Figure 57-23 Grade II fibrillary astrocytoma. **A,** An axial fluid-attenuated inversion recovery image. **B,** An axial T1-weighted image with use of contrast material showing a noncontrast-enhancing fibrillary tumor.

Rapidly evolving clinical symptoms in the setting of an operable tumor usually warrant prompt neurosurgical intervention. On the other hand, a lesion with a long history of indolent and mild symptoms is often managed with close MRI and clinical surveillance.[468] In patients who subsequently show progressive neurologic symptoms and for whom MRI studies suggest tumor growth, therapeutic intervention is required.[540] Diffuse fibrillary astrocytomas in eloquent locations such as the thalamus or motor regions are often biopsied to confirm the diagnosis and to exclude higher grade glial tumors. They do not lend themselves to radical resections, as is commonly performed for PAs. However in certain cases, such as dorsally exophytic brainstem astrocytomas, radical resection can often confer a long symptom-free outcome. In cases in which resection is not feasible, chemotherapy or radiotherapy may be indicated.[432] As in the case of progressive or unresectable PAs, chemotherapy is the preferred first approach for patients (see Table 57-5).[491,436,541-543] Patients respond well to either vincristine and carboplatin or TPCV chemotherapy. A recent randomized clinical trial of grades I and II astrocytomas has been completed in which these two treatment regimens were compared.[440] Although recurrences occur in most patients, necessitating retreatment with other chemotherapeutic agents, the overall prognosis remains good. Experience with novel combinations of agents[469,450] or high-dose chemotherapy is limited.[460] Radiotherapy is reserved for older children or younger patients whose tumors progress and are refractory to chemotherapy. Reduced radiation doses to adjacent normal tissue, with equivalent tumor control to that of standard photon therapy, can be achieved with conformal proton therapy.[498] Radiation therapy delivered at the time of initial diagnosis does not appear to provide additional benefit for OS.[425]

Prognosis. The long-term survival rate for completely resected pediatric supratentorial low-grade astrocytomas is excellent, especially when compared with similar tumors in adults.[369] Even partially or unresected fibrillary astrocytomas may remain stable for many years. An example of this is the focal midbrain or tectal glioma, which is rarely biopsied or resected.[544,545] Response to chemotherapy appears to be similar to that observed in PAs, although most patients will experience one or more episodes of progression, requiring retreatment with additional chemotherapy.[546,547] However children with primary tumors arising in the pons or thalamus have a worse prognosis.[506,548] The presence of a gemistocytic component characterized by a predominance of large astrocytes, with thick processes and dramatic accumulation of GFAP, represents a histologic variant of low-grade fibrillary (grade II) astrocytoma. This variant may be associated with p53 mutations and may also convey a higher predisposition to malignant transformation.[549,550]

Although most patients with low-grade astrocytomas will survive,[442] patients with fibrillary astrocytomas have a poorer outcome than patients with grade I astrocytomas.[551] Because PAs are more focal and easier to resect than are fibrillary astrocytomas, prognosis may be related to the ease of resection rather than to biologic differences between these histologic variants. Overall survival after chemotherapy of children and adolescents with low-grade astrocytomas at 10 years is 70% to 80%.[425,489,459] The role of MIB1 expression in the prognosis of low-grade astrocytomas is not established as a prognostic factor, after age and degree of resection are excluded.[552] Survivors of low-grade astrocytomas still have a number of limitations.[470,553] Future treatment efforts for these tumors therefore will need to combine effective therapy with improved quality of survivorship in these patients.

Tectal Gliomas

Tectal gliomas are typically hamartomas or low-grade astrocytomas, and patients present with acute hydrocephalus in most cases. These tumors appear to represent a unique variant of low-grade tumors, based on their positive long-term outcome, with relief of hydrocephalus as the sole therapeutic intervention. Because biopsy or surgical resection of these lesions is rarely required, little is known about the pathways that drive their activation. No known genetic syndromes give rise to these tumors.

Presentation. Most patients with tectal gliomas present with the symptoms of obstructive hydrocephalus because of expansion of these lesions adjacent to the periaqueductal space. When the presence of these tumors is picked up incidentally, many patients will demonstrate prolonged periods of stability and do not require treatment.

Imaging and Histology. The radiographic appearance of most tectal gliomas is similar to that of other LGGs. Most tumors lack contrast enhancement (Fig. 57-24). Based on their location and presenting symptoms, histologic verification is not required to make the diagnosis and can cause significant morbidity if attempted.

Management. Biopsy or resection of tectal gliomas is usually not required. Rather, most patients require immediate CSF diversion through a third ventriculostomy.[554,555] Failures of third ventriculostomies have been reported, even many years after the initial procedure, necessitating the proper education of families regarding symptoms that should precipitate immediate evaluation.[556] Reduction in ventricular size is often incomplete and the ventricular size continues to change during the first year after diversion.[557] Rarely tectal gliomas larger than 10 cm at presentation will continue to progress over time and may require surgical debulking and chemotherapy.[558] Tumors that have atypical radiographic features, rapid progression, or repeated recurrence may require a biopsy. Tumors with asymptomatic progression can often be observed carefully.[559] When treatment is required, approaches similar to those used for other low-grade astrocytomas are recommended. Chemotherapy is usually initiated. Radiation therapy is not required for most patients, and continued progression of these lesions into adulthood is rare.

Prognosis. The long-term outcome for patients with tectal gliomas remains excellent, which is surprising given the unresectable nature of the lesion and the absence of therapy for most patients. Overall survival approaches 100% in this population,[560] and thus avoidance of unnecessary surgical or radiation-related long-term morbidity is critical.

Pilomyxoid Astrocytomas

Pilomyxoid astrocytomas are a newly stratified group of grade II pediatric tumors in the 2007 WHO classification[207,361] that were previously grouped together with PAs.[561,562] Although no specific molecular pathway responsible for these tumors has yet been identified, the presence of defects in the *BCR* gene on chromosome 17[563] provides possible clues that require further analysis. Other chromosomal aberrations have also been identified.[564] Pilomyxoid astrocytomas are found most commonly in the midline of the brain and spine.[565] These tumors are usually identified in infants and young children, although their presence in adults has been

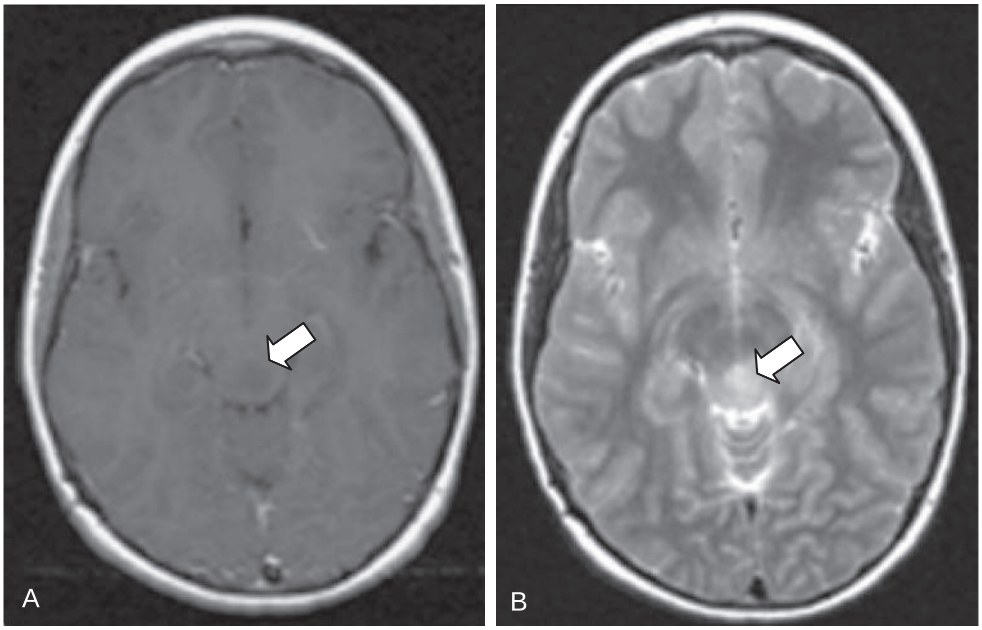

Figure 57-24 Tectal glioma. **A,** An axial T1-weighted image with use of contrast material showing a noncontrast-enhancing tectal tumor *(arrow)*. **B,** An axial T2-weighted image shows T2 hyperintense lesion in the tectum *(arrow)*.

reported.[566] At recurrence, tumors can appear as classic PAs, suggesting a developmental relationship between the two types of tumors.[567]

Clinical Presentation. Although pilomyxoid astrocytomas present in a manner similar to that of other LGGs, the incidence of metastatic disease at presentation appears higher and requires a full workup, including spinal MRI and CSF for cytologic evaluation. Patients with pilomyxoid astrocytomas may be at greater risk for spontaneous hemorrhage at the time of diagnosis or resection or during follow-up.[568,569]

Imaging and Histology. On MRI pilomyxoid astrocytomas appear similar to PAs, with well-circumscribed margins and little peritumoral edema.[570] They are usually found in the midline, including the brainstem[571] and spine,[572] and can be solid or solid and cystic. They are usually bright on T1- or T2-weighted and FLAIR sequences, and they enhance with administration of contrast material.[573] In approximately 50% of cases, the contrast enhancement is heterogeneous.[574,575] Adjacent areas of brain can demonstrate elevated choline-to-creatinine ratios suggestive of an infiltrative margin.[575] Although their location, young patient age, and increased presence of dissemination provide clues to the diagnosis before a biopsy is performed, imaging characteristics do not clearly differentiate these tumors from other LGGs in the pediatric population.[576] Histologically these lesions lack many features of PAs, such as Rosenthal fibers and a biphasic pattern. Rather a monophasic pattern and myxoid background is seen, with strong GFAP and synaptophysin staining.[577] Like other glial tumors, mixed lineages may also be possible for pilomyxoid astrocytoma.[578] The variable histologic appearance compared with PAs,[579] MRS signal changes,[580] and a higher incidence of progression and dissemination support their distinction from PAs.[561] A predilection for younger age appears to exist when the tumor is localized to the pituitary-hypothalamic region.[581]

Management. Because of the more aggressive nature of these tumors compared with PAs, pilomyxoid astrocytomas are now classified as grade II gliomas. Treatments typically follow those of other LGGs, including vincristine and carboplatin and TPCV chemotherapy. Other therapies used for PLGGs have also been used for patients with pilomyxoid astrocytomas (Table 57-5).[582,583] Upfront chemoradiation therapy combinations have also been tried for these tumors.[584] Formal clinical trials for this rare subtype are not possible, however. Because of the young age of the patient and the deep location of these tumors in many patients, radiation-based strategies will lead to significant long-term morbidity in most survivors. Many patients with pilomyxoid astrocytomas are infants and have diencephalic syndrome with dissemination, and thus complete surgical resection is not possible. Thus most patients will start with LGG chemotherapy. The use of intrathecal therapy for patients with disseminated disease who are unable to receive craniospinal radiation can be considered.

Prognosis. Without well-defined studies, the prognosis of pilomyxoid astrocytoma is difficult to assess with certainty. Confounding issues of deep-seated lesions, especially around the hypothalamic region in which surgical options are limited, along with the young age of these patients and the presence of metastatic disease, likely affect the overall poor prognosis. A mean OS of 60 months for patients with pilomyxoid astrocytoma versus 220 months for patients with grade I PAs has been reported.[581] Whether their unique biology affects survival is unknown, although these tumors can recur as typical grade I pilocytic astrocytomas.[567] As in other LGGs, nestin expression may correlate with a worse prognosis.[585]

Ganglioglioma and Glial-Neuronal Tumors

Gangliogliomas are low-grade (WHO grade I) glioneuronal tumors. These tumors represent 4% to 8% of primary brain tumors in children, with 80% occurring before age 30 years and at a mean age less than 10 years.[586] These tumors are frequently associated with seizures and tend to grow slowly. Gangliogliomas can occur throughout the CNS,[587,588] although most are localized to the temporal and parietal lobes.[586] A significant proportion of these tumors have the BRAF V600E mutation,[388,411,413,589] which is now being targeted in open international clinical trials. The presence of this oncogenic mutation may result is a more aggressive behavior and worse outcome.[590]

Clinical Presentation. Seizures are the first manifestation in 50% of cases of ganglioglioma, and many patients have a prolonged history of seizures of longer than 2 years.[586] Complex partial seizures are common because gangliogliomas are frequently located in the temporal lobe, particularly the temporomesial region,[591] although they can occur anywhere in the brain or spine.

Imaging and Histology. Gangliogliomas are typically contrast-enhancing cystic lesions on CT scans. The MRI appearance of these tumors can be variable but is frequently hypointense on T1-weighted sequences and hyperintense on T2-weighted images. Gadolinium enhancement varies in intensity from absent to significant and can be nodular, solid, or circumferential.[592] An infantile variant of ganglioglioma, desmoplastic infantile ganglioglioma (DIG), can occur (discussed later). Pathologic studies show synaptophysin and neuronal nuclear antigen (NeuN)–positive ganglion cells, as well as a GFAP-positive astrocytic component.[593] Lesions associated with the ganglion cell but lacking the astrocytic component are called gangliocytomas. Gangliogliomas are frequently WHO grade I tumors, although some may show anaplastic features in the glial or neural component.[594,595] MIB1-positive cells are usually localized to the astrocytic component.[596] Glial-neuronal tumors likely represent a spectrum of LGGs that include gangliogliomas and may also have PI3K pathway activation.[597]

Management. Complete or near-total resection is the treatment of choice[591] and, in most cases, can eliminate or significantly improve seizure frequency in this patient population.[598,599] Recurrent or unresectable tumors may be treated with radiation therapy,[586,600,601] although these lesions may respond to LGG chemotherapy, and thus patients may avoid some of the long-term toxicity of radiation.[459] In the case of recurrent or unresectable ganglioglioma, response to LGG chemotherapy has been demonstrated.[586] Treatment for recurrent or unresectable gangliogliomas is included in current LGG chemotherapy trials. It should be noted that these tumors have been shown to undergo malignant transformation over time, usually involving the astrocytic component. Clinical trials of BRAF V600E inhibitors are under way for persons with this mutation, and significant durable responses have been reported.[305]

Prognosis. GTR is often curative, making tumor location and extent of resection the most important prognostic factors.[591] Most patients are rendered seizure-free after GTR.[599] Gangliogliomas of the posterior fossa have also been reported, and their outcomes appear to be similar to those for supratentorial lesions. Patients whose tumors can be fully resected to remove the enhancing components are likely to remain disease free, although persons with subtotal resected disease often require additional resection, chemotherapy, and/or radiation therapy.[587] The uncommon presentation of these tumors in the infratentorial location and the limited sample sizes in case reports limit the confidence with which therapeutic approaches can be recommended. Spinal cord ganglioglioma appear to have an indolent course. Patients with subtotal resection can remain stable for prolonged periods. Therefore treatment should be deferred until lesions show clear evidence of progressive disease.[602] Very rarely, gangliomas and glial neuronal tumors can degenerate into a CNS PNET (the neural component) or into a malignant glioma (the astrocytic component).

Desmoplastic Infantile Gangliogliomas

DIGs are supratentorial tumors involving the leptomeningeal surface and are identified predominantly in children younger than 2 years,[603] although reports in older children are available.[604] These lesions are often very large, in part because of the presence of cysts, and they frequently involve the dura. Although precise estimates of their incidence are lacking, they make up fewer than 1% of pediatric brain tumors. A number of chromosomal abnormalities have been reported, although only in a small number of cases, particularly at chromosome 7q31 (corresponding to *MET* gene). One case containing the BRAF V600E mutation was also noted in this series[605] and was reported in two other patients in a different analysis.[606]

Clinical Presentation. Most patients present with increasing head circumference, bulging fontanelle, and lethargy. Older patients present with focal motor deficits.

Imaging and Histology. DIGs are large cystic structures on CT scans, with contrast enhancement of the solid component. T1-weighted MRI sequences demonstrate an isointense tumor; T2-weighted intensity of the tumor is variable. The tumors usually enhance after administration of contrast material. Peritumoral edema is uncommon. DIGs possess a desmoplastic stromal background, with neoplastic neurons and astrocytes. They often have areas with an elevated MIB1 labeling index, although this characteristic does not usually represent transformation to a more malignant phenotype.[607] Tumors lacking the neural component are called desmoplastic infantile astrocytomas.

Management. Cure can be achieved with complete resection. Many patients have remained progression-free after total resection without additional treatment.[608] Chemotherapy, as for other low-grade astrocytomas, can be considered for patients with symptomatic or progressive cases for whom surgical removal is not feasible.[593,609]

Prognosis. Most patients will be long-term survivors if a complete resection is achieved.[608] Lesions that are more deep-seated may have a poorer outcome, and initiation of chemotherapy may be considered at the first signs of progression in these unresectable tumors.[603,610] Metastatic lesions have been reported, suggesting that in rare cases, a more malignant phenotype can occur.[593,611]

Dysembryoplastic Neuroepithelial Tumors

DNETs are recently described tumors that may comprise as many as 1% of all brain tumors in patients younger than 20 years.[612] Two thirds of all DNETs are located in the temporal lobe, and DNETs are found in 5% to 15% of temporal lobe resections for intractable epilepsy. These lesions are classified as WHO grade I tumors.[613] Thought to be developmental in nature, they are treated with surgery. They are considered to have limited proliferative potential, suggesting that complete surgical resection is not required for long-term disease control[614]; nonetheless, recurrences can occur.[615]

Clinical Presentation. The diagnosis of DNET should be a consideration in children and young adults with new-onset seizures or a long history of epilepsy. Patients with these tumors typically present with a long history of complex partial seizures, with an average age of onset of 9 years. In many patients the seizures are refractory to anticonvulsant medications. The superficial cortical location of DNETs may account for the high risk of seizures.

Imaging and Histology. Contrast-enhanced cranial MRI typically shows a temporal or frontal lobe lesion, absent peritumoral edema, and only minimal, if any, gadolinium enhancement. The tumors are bright on T2-weighted sequences and hypointense on T1-weighted images (Fig. 57-25). Mass effect is minimal to absent. Although routine fluorine 18–labeled deoxyglucose–PET imaging has not been helpful in the preoperative identification of these lesions, Carbon 11–methionine–PET was able to distinguish these lesions from other low-grade lesions.[616] The pathologic findings include a specific glioneuronal element manifested by GFAP-negative oligodendroglia-like cells and neurons in a mucinous eosinophilic background that give the appearance of floating neurons.[617] Because

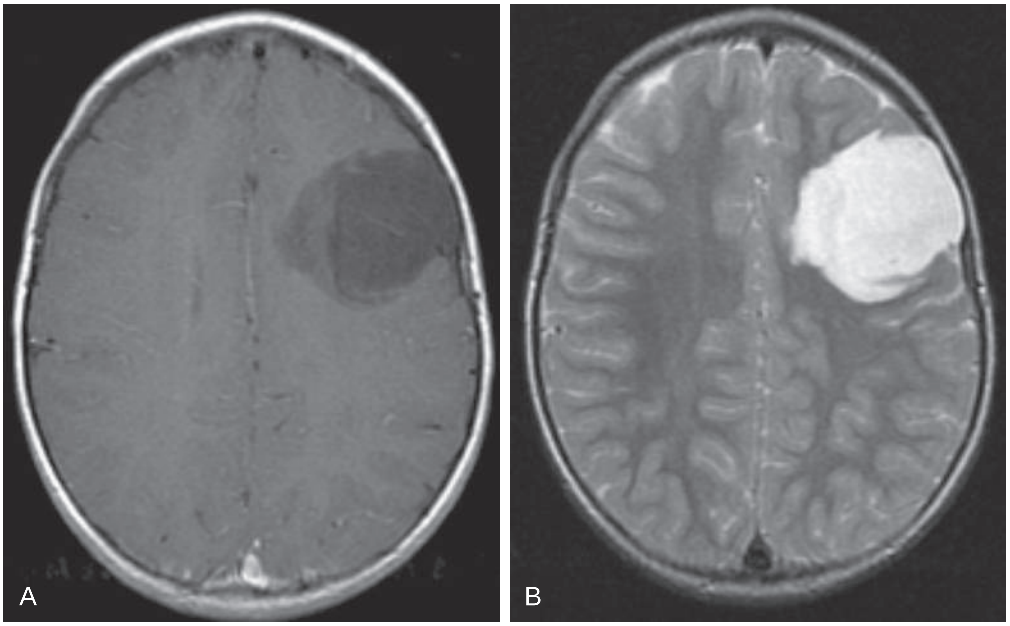

Figure 57-25 Left frontal dysembryoplastic neuroepithelial tumor. **A,** An axial T1-weighted image with use of contrast material showing a large nonenhancing tumor. **B,** An axial T2-weighted image.

oligodendrocytes, astrocytes, or both are found on histopathologic analysis, the pathologic differential diagnosis often includes oligodendroglioma, mixed oligoastrocytoma (OA), and ganglioglioma. Like gangliogliomas and PXAs, DNETs have been reported to have the BRAF V600E mutation in approximately 30% of cases.[618] Differentiation of DNETs from other LGGs can be difficult.

Management. Although these tumors have a benign course, the associated seizures may be refractory to antiepileptic drugs because of the increased expression of multidrug transporters.[619] Gross total resection is often curative and typically alleviates the seizures, especially if the areas of cortical dysplasia can also be removed,[620] although some case series have shown a significant number of patients who continue to have residual seizures.[614,615] Adjuvant chemotherapy or radiation therapy is not recommended. Rare cases of malignant transformation of DNETs after radiation and chemotherapy have been reported.[621]

Prognosis. The stable behavior of these tumors over time results in an excellent prognosis after gross total or partial resection.[614] Patients often have improved seizure control after surgery, especially if a complete resection was achieved,[622] and some improvement in neuropsychologic functioning has been observed.[623]

Pleomorphic Xanthoastrocytomas

Pleomorphic xanthroastrocytomas (PXAs) are uncommon grade II cortical tumors that mainly occur in children and adolescents and account for fewer than 1% of CNS tumors. The median age at the time of diagnosis is 14 years. The molecular pathogenesis of these tumors is poorly understood, and no causative chromosomal abnormalities have been identified. The BRAF V600E activating mutation has been detected in more than 50% of cases, and drugs targeting this mutation are now in clinical trials.[412,413] A recent analysis of 50 PXAs by comparative genomic hybridization, however, has revealed that loss of chromosome 9 is the most common chromosomal lesion.[624] A few reports of PXA in conjunction with NF-1 may implicate the Ras pathway in this disease.[625]

Clinical Presentation. PXAs are typically large and superficially located, especially in the temporal lobes. Seizures are the most common presenting feature. For tumors of the cerebellum, pineal region, or spine, direct nerve compression results in focal deficits, and obstruction of CSF flow results in raised ICP.[626] Rare cases of dissemination have been reported.[627,628]

Imaging and Histology. PXAs typically manifest enhancement on MRI, with occasional intratumoral cysts and calcification (Fig. 57-26). Peritumoral edema is uncommon. Tumors usually extend to the meninges. The typical histopathologic picture includes a pleomorphic appearance of the astrocytic component, with significant cellular atypia and bizarre multinucleated giant cells with intracellular lipid accumulation. The proliferative indices are usually low, although necrosis, endothelial proliferation, and mitoses have been described. PXA can be confused with glioblastoma multiforme[629] because of the presence of multinucleated cells and occasional foci of necrosis.[630] The expression of CD34 on most tumors may be useful in the histologic classification of difficult or atypical cases.[631] Prognosis appears to correlate with MIB1 expression.[632] In lesions with mitoses, an elevated MIB1 labeling index, and necrosis, the term “pleomorphic xanthoastrocytoma with anaplastic features” is used.

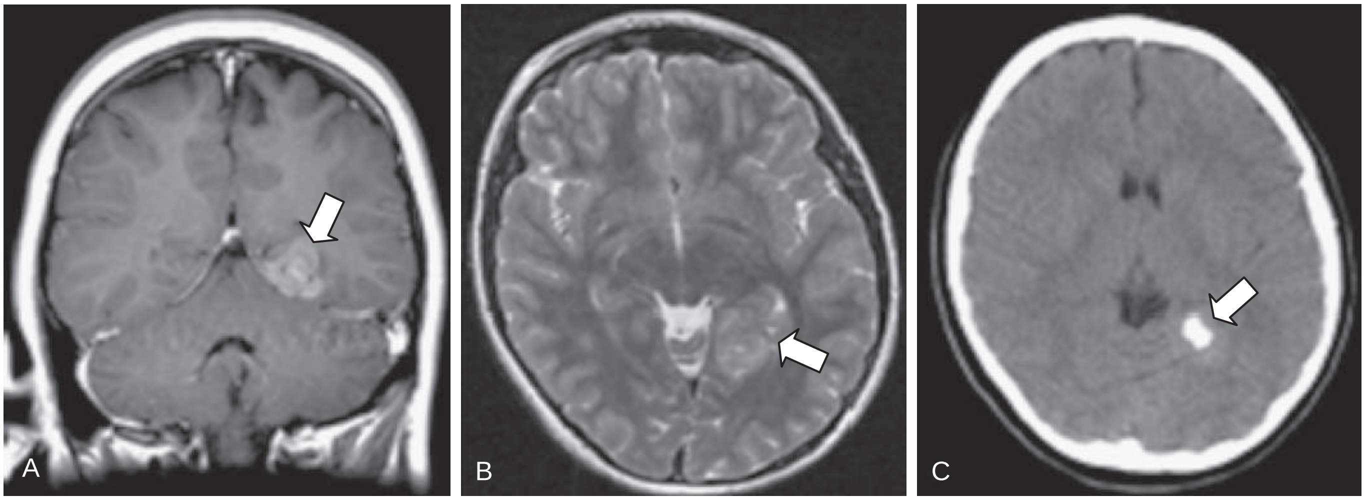

Figure 57-26 Pleomorphic xanthoastrocytoma. **A,** A coronal T1-weighted image with use of contrast material *(arrow)*. **B,** An axial T2-weighted image *(arrow)*. **C,** A noncontrast computed tomography image *(arrow)*.

Management. The clinical diagnosis of PXA should be suspected in children who present with new-onset seizures, focal deficits, and a large, enhancing cortical mass on brain imaging. The goal of surgery is to achieve a GTR, which is usually curative.[633,634] Adjuvant therapy can be deferred, although the patient should be followed expectantly. Tumors that recur and incompletely resected lesions with anaplastic features may be treated with chemotherapy[635] or radiotherapy,[636] although significant activity for these modalities has not been demonstrated. Few formal clinical trials for this rare subtype have been performed.

Prognosis. Several series have reported a 5-year PFS of greater than 70%. However the presence of mitoses, endothelial proliferation, or necrosis on the pathologic specimen, although rare, may significantly alter the clinical behavior and prognosis.[632] Cases of anaplastic PXA with subsequent malignant transformation have been reported.[637,638] Radiation therapy does not alter the poor outcome in these cases.

Subependymal Giant Cell Astrocytomas

SEGAs usually originate in the ependymal walls of the lateral ventricles and are associated almost exclusively with TS, an autosomal-dominant disorder.[639] Patients with TS have hamartomas and benign tumors of multiple organs, including the brain. The CNS manifestations include cortical tubers (hamartomas), subcortical glioneuronal hamartomas, subependymal glial nodules, and subependymal giant cell astrocytomas.[640] Two genetic defects, one in the *TSC1* gene on chromosome 9q and the other in the *TSC2* gene on chromosome 16p, account for TS. Sporadic cases are rare.[641]

Clinical Presentation. SEGA is sometimes the presenting feature of TS in patients without the typical physical stigmata of the syndrome. Because of the variable phenotype of TS, a consensus on diagnostic criteria has been developed. Presentation in older children and adolescents is common. Although patients with TS typically have multiple periventricular SEGAs, those that produce symptoms of hydrocephalus arise in close proximity to the foramen of Monro, although lesions around the pineal gland can also result in symptoms.[642] Most patients with known TS are followed up with regular neuroimaging and the SEGAs are removed serious neurologic syndromes, such as headaches, altered sensorium, or weakness are present.[324] Of note, patients with TS may have other significant neurologic symptoms, such as seizures and cognitive deficiency related to cortical tubers.[643]

Imaging and Histology. CT and MRI are essential for early and accurate diagnosis. Although CT is superior to MRI for detecting small calcified lesions, MRI is superior to CT for identifying areas of gliosis, heterotopia, and SEGAs, which have the typical radiographic appearance of candle dripping.[644] SEGAs typically show diffuse contrast enhancement on CT and MRI studies (Fig. 57-27).[90] SEGAs are considered grade I astrocytomas. They are well-circumscribed tumors that are positive for GFAP, neurofilament, neuron-specific enolase, and synaptophysin.[645,646] Mitoses can be identified but do not indicate a higher grade or more malignant phenotype. They can occasionally be associated with bleeding.[647]

Management. The management of patients with TS is complex and requires adherence to recent consensus conference guidelines.[648] GTR is the treatment of choice for SEGAs that are progressive, and cause obstructive hydrocephalus[649] or seizures.[650,651] The major risk of surgery is injury to the forniceal columns, resulting in memory disturbance. The benign behavior of these tumors warrants reoperation in the case of recurrence or progression after subtotal resection. Inhibition of the mTOR pathway in SEGAs has resulted in a very high response rate, and mTOR inhibitors have now been approved for the treatment of these lesions.[93,652]

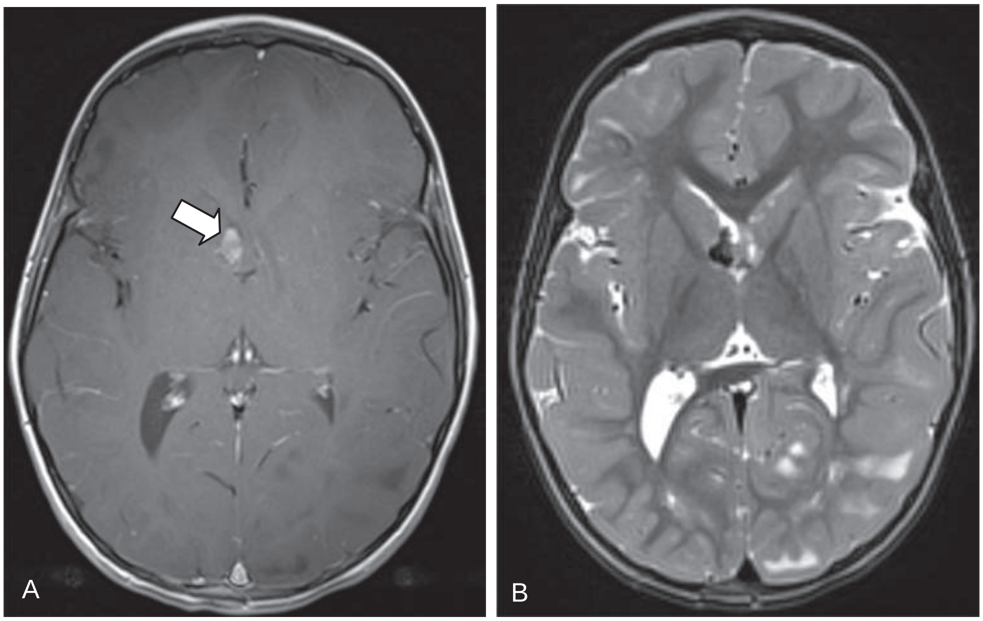

Figure 57-27 Subependymal giant cell astrocytoma. **A,** An axial T1-weighted image with use of contrast material showing an enhancing tumor *(arrow)*. **B,** An axial T2-weighted image showing an area of dark signal corresponding to tumor.

Prognosis. SEGAs are essentially benign tumors, and gross total resection is generally curative. However multiple tumors may arise in some patients with TS, requiring ongoing radiologic surveillance.[90] The overall prognosis for patients with TS is good, despite increased susceptibility to other tumor types, including rhabdomyomas of the myocardium and angiomyomas of the kidney, liver, adrenals, and pancreas.[653]

Hemangioblastomas

Hemangioblastomas are WHO grade I lesions associated with von Hippel-Lindau (VHL) disease. They occur primarily in adults and are centered in the cerebellum, brainstem, and spinal cord.[654] Although sporadic tumors can arise, pediatric cases are usually associated with the inherited syndrome. Cases associated with VHL disease can be single or multifocal, although most sporadic cases involve isolated nodules. Hemangioblastomas are highly vascularized lesions, although the genetic defect is localized to the stromal elements rather than the vasculature.[655] Tumor development appears to be related to the deregulation of VEGF. Patients with hemangioblastomas need to undergo complete genetic evaluation for VHL disease and, for those with the inherited disease, careful lifelong screening is necessary.[656]

Clinical Presentation. Obstruction of CSF flow is the primary reason for symptomatology in patients with VHL disease who are diagnosed with hemangioblastomas. Headaches, morning vomiting, and long tract signs are therefore common. Ataxia and cranial nerve dysfunction can also be observed.[657]

Imaging and Histology. Hemangioblastomas are typically well-circumscribed cystic lesions with a solid tumor nodule. The solid component tends to enhance brightly with contrast and is usually located peripherally within the cyst (Fig. 57-28). Flow voids are commonly observed on MRI and can be well delineated with angiography. Peritumoral edema is commonly observed. Tumors are usually composed of two elements—the stromal cells, which express vimentin and VEGF, and the endothelial cells, which express the VEGF receptor VEGFR2. Hemangioblastomas typically have low proliferative levels based on MIB1 immunoreactivity and are considered grade I tumors.

Management. The management of these tumors requires specialized neurosurgical expertise because of the high risk of bleeding. The primary approach to these lesions, which tend to occur most commonly in the posterior fossa and cervicomedullary area, is maximal surgical resection.[656] This approach provides long-term control in most patients,[658] although, because of the underlying genetic predisposition, new tumors may continue to develop throughout the life of the patient. Embolization can reduce the intraoperative risks of bleeding.[659,660] Radiosurgery can successfully treat the mural component of these tumors, although control is negatively influenced by the presence of the cysts.[661] VEGF inhibitors have been tested in this population with initial encouraging results, although follow-up of these reports is still pending.[662] A case of prolonged stable disease with thalidomide treatment was reported, although whether this outcome was related to its antiangiogenic or immune modulatory effects is unknown.[663]

Prognosis. Because of the genetic basis of these lesions, recurrences are common and likely represent new tumors rather than recurrences of previously resected ones, which

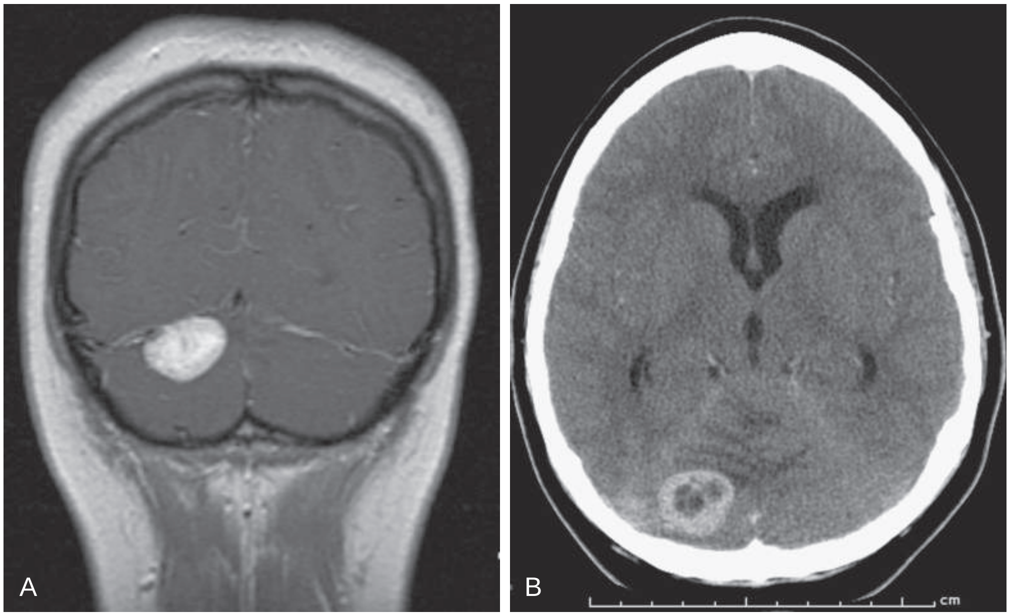

Figure 57-28 Cerebellar hemangioblastoma. **A,** A coronal T1-weighted image with gadolinium. **B,** A computed tomography image.

indicates the importance of continued careful evaluation in this patient population.

Rare LGGs

Tanycytic Astrocytomas. Tanycytic astrocytomas are rare and poorly characterized tumors. They are most common in adults and usually occur in the hypothalamic region.[664] Their malignant potential is not well defined, although recurrences after complete resection have been observed. One reported case occurred in the context of an adolescent with NF-2.[665]

Lipoastrocytomas. Lipoastrocytomas are rare cortical tumors that, as the name suggests, possess fat droplets that give them the appearance of adipocytes in the context of a low-grade astrocytic lesion. These tumors express GFAP.[537] Recurrences can occur and are treated with resection. Malignant transformation has not been reported in this tumor type, although one case was identified on autopsy in a patient with diffuse intrinsic pontine glioma.[666]

Dysplastic Cerebellar Gangliocytomas. Dysplastic cerebellar gangliocytomas are rare tumors of the cerebellum and fourth ventricle region that are of limited proliferative potential. They have a characteristic imaging pattern on MRI, with an abnormal laminated pattern in the cerebellum, although this pattern is not pathognomonic and can be mimicked by medulloblastoma. As such biopsy is recommended to differentiate the two entities.[667] Dysplastic cerebellar gangliocytomas do not typically require therapeutic intervention, although seizure activity can occur as a result of their presence.[668]

Disseminated Oligodendroglial-Like Leptomeningeal Tumor of Childhood. Disseminated oligodendroglial-like leptomeningeal tumor of childhood is a newly described entity that typically demonstrate leptomeningeal enhancement on MRI and associated cystic or nodular T2 hyperintense lesions within the spinal cord/brain along the subpial surface. A discrete intraparenchymal lesion, usually in the spinal cord, is found in the majority of cases. These tumors are thought to have a greater risk of malignant transformation.[669]

High-Grade Astrocytomas

High-grade astrocytomas (HGAs) encompass anaplastic astrocytoma (WHO grade III) and glioblastoma (previously glioblastoma multiforme, WHO grade IV) of the brain and spinal cord. This category also includes as a subgroup DIPG, even though these tumors occasionally show lower grade histology. HGAs, unlike LGGs, are much less common in children than adults. Whereas more than 30% of all pediatric brain tumors are LGGs, non-DIPG HGAs represent only 6% to 12% of all primary pediatric brain tumors, and DIPGs represent another 10%. In the past several years, many key discoveries have been made about the biology of these tumors, and increasingly, important distinctions from adult HGA biology are being found.[670] Unfortunately major treatment advances have not yet followed the biologic discoveries, and most children with these tumors eventually succumb to their disease. Because of key differences between DIPGs and other pediatric HGAs, most notably in prognosis and management, DIPG is discussed separately from the remainder of tumors in this category.

Supratentorial HGAs (Excluding DIPG). Although adult and pediatric HGAs are similar histologically, increasing evidence indicates key biologic differences, even within shared pathways of tumorigenesis. Common pathways include the overexpression or mutation of EGFR,[671] although both changes are less frequent in pediatrics,[672] activation of the PI3K signaling pathway via silencing of the PTEN tumor suppressor,[673-675] most often by methylation in pediatrics,[676] and the MGMT DNA repair pathway, which is commonly methylated in adult HGAs but is more often overexpressed in pediatric tumors,[677,678] perhaps helping to explain TMZ resistance in children with HGAs.[342]

Recent findings have also demonstrated involvement of new pathways and features of pediatric HGA, further distinguishing them from adult tumors. These new pathways include overexpression of PDGFRα[679,680] and novel histone mutations in *H3F3A* (observed in a minority of these tumors vs. a majority of DIPGs).[681,682] Comprehensive profiling of HGA genetics has shown gains in chromosome 1q in pediatric HGA on copy number analyses,[683,684] which is unusual in adults; fewer cases show chromosome 7 gain and 10q loss, two features seen in the majority of adult HGAs.[684] One study appeared to show two distinct gene expression profiles in pediatric HGA, one that is similar to adult HGA, and one that is divergent.[685] The PARP DNA repair pathway is also overexpressed in many pediatric HGAs,[686] and this feature is associated with a poor prognosis[687]; PARP inhibitors may be useful as radiosensitizers.[687] Aurora kinase B is overexpressed in a majority of pediatric HGAs.[688] Unlike in adults, *IDH1* mutations have not been found in pediatric HGAs.[689] Pediatric tumors have demonstrated abnormalities in the Ras and Akt pathways, as well as Y-box protein-1.[690,691] Microsatellite instability, which has been associated with a number of adult tumors, is very rare in pediatric HGAs.[692-694]

Overexpression of many proteins distinguish pediatric HGAs from LGGs, including the DNA repair protein insulin-like growth factor binding protein 2 (IGFBP2),[695] the cancer-testis antigen and possible immunotherapy target NY-ESO-1,[696] the p53 inhibitor HDMX,[697] the angiogenic marker endoglin,[698] and the catalytic subunit of human telomerase reverse transcriptase (HTERT)[699]; the differentiation marker NF1A is underexpressed in pediatric HGA compared with LGG.[700] In the CCG study of children with malignant HGGs (CCG945), p53 status was an important independent prognostic variable, with a 5-year PFS of 44% in tumors expressing a low level of p53 versus 17% in those overexpressing p53.[701] Finally new epigenetic findings in pediatric HGAs include hypermethylation of the LIM homeobox 9 (LHX9) transcription factor,[702] downregulation of class II and IV histone deacetylases (HDACs),[703] acetylation of histone H3.3,[703] and a decrease in adenosine deaminase, ribonucleic acid (RNA)-specific, 2 (ADAR2)–mediated RNA editing.[704]

Clinical Presentation. The clinical manifestations of HGAs depend on the anatomic location and the age of the patient. The clinical prodrome is usually short and rapidly evolving, with signs and symptoms of elevated ICP and focal neurologic deficits. A protracted course may evolve in the usual case of malignant transformation of a low-grade glioma,[705] but in general, transformation of low-grade gliomas to high-grade gliomas is extremely rare in children, unlike in adults. Dissemination of HGA into the cerebrospinal fluid is less common than for medulloblastoma and other neural tumors but is being recognized more frequently,[706] particularly as patients survive longer. Approximately 3% of pediatric patients with an HGA will have CNS metastatic disease at presentation.[707]

Imaging and Histology. Typical HGAs have an MRI appearance of heterogeneous enhancement or diffuse nonenhancing tumor with significant edema on the T1-weighted image, compressing or displacing adjacent ventricular structures and occasionally causing hydrocephalus. The T2-weighted signal is often more diffuse, consistent with both infiltrative tumor and edema (Fig. 57-29). MRS demonstrates a markedly elevated choline-to-NAA ratio; a 40% increase in choline over normal brain tissue indicates an HGA with 90% sensitivity and specificity. Lesions tend to be ^{18}F-labeled deoxyglucose–PET avid, although ^{18}F-fluoro-ethyl-tyrosine (FET)–PET may be superior for treatment planning and distinguishing between LGGs and HGAs.[708,709] Technetium 99m–sestamibi SPECT (MIBI SPECT) imaging can also detect

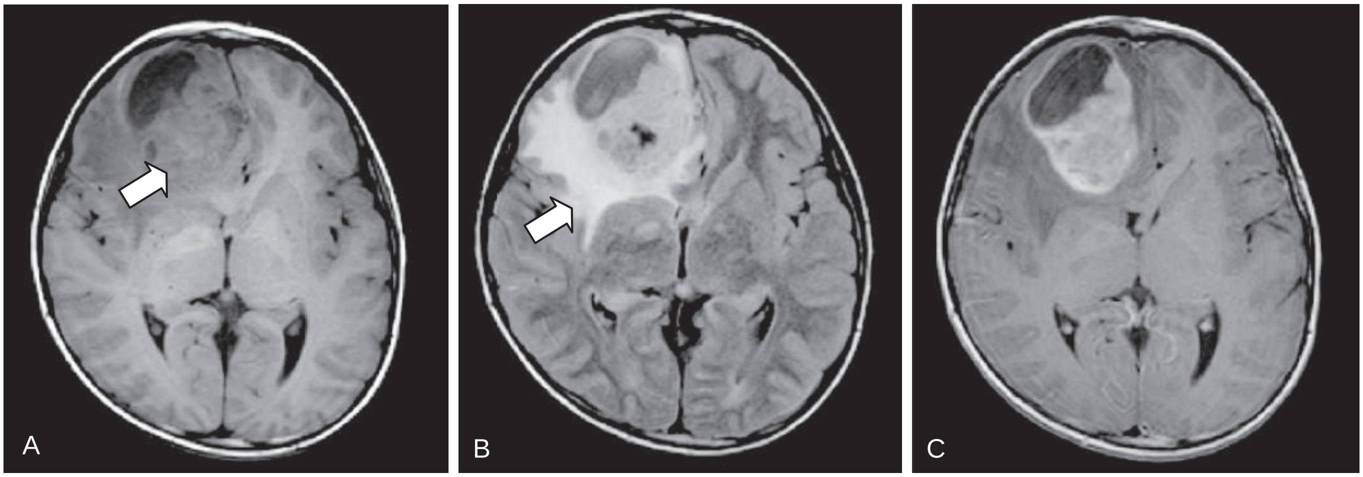

Figure 57-29 Right frontal glioblastoma. **A,** An axial T1-weighted image without use of contrast material showing the tumor *(arrow)*. **B,** An axial fluid-attenuated inversion recovery image showing peritumoral edema and infiltrating tumor *(arrow)*. **C,** An axial T1-weighted image with use of contrast material.

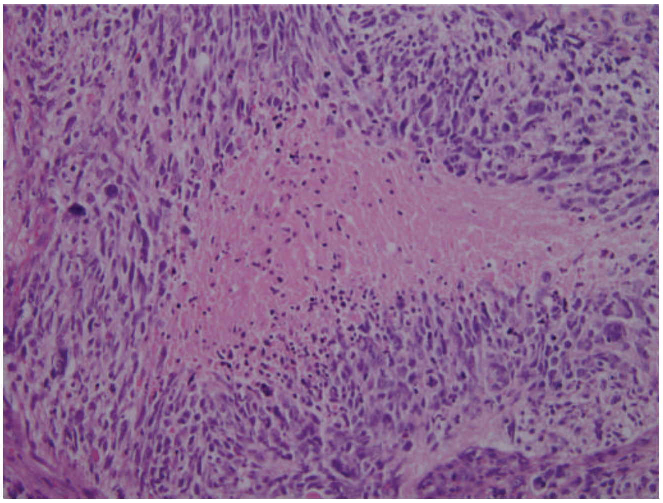

Figure 57-30 Palisading necrosis in a child with glioblastoma (hematoxylin and eosin; ×400).

HGAs and can be used to follow up for response or recurrence,[174] as can serial diffusion weighted assessment.[710] Areas of hemorrhage within pediatric HGAs at diagnosis are not uncommon.[711] The presence of an HGA within the volume of a previously irradiated field is difficult to differentiate from a radiation-induced high-grade transformation of a low-grade astrocytoma, although preliminary studies have identified some molecular differences distinguishing radiation-induced glioblastoma.[712] Malignant features on histopathologic examination of HGA include nuclear pleomorphism, mitoses, palisading necrosis (Fig. 57-30), endothelial proliferation (Fig. 57-31), and a high Ki-67 or MIB1 labeling index (see Fig. 57-17), although high interobserver variability exists between pathologists in judging these features.[713] Most pediatric HGAs are nestin-positive on immunohistochemistry.[714] Distinguishing between grade III and IV tumors relies on differences in histologic features; all HGAs must demonstrate nuclear atypia and mitoses, whereas grade IV tumors must also show either endothelial proliferation, necrosis, or both. It has now been shown that a proportion of CD133+ HGA cells costain for CD105 (endoglin), an angiogenesis marker, suggesting that vasculogenic mimicry occurs in pediatric HGAs, which may help increase angiogenesis in HGA through the formation of fluid-conducting vessels of tumor cells. CD105 staining is an unfavorable prognostic marker.[698]

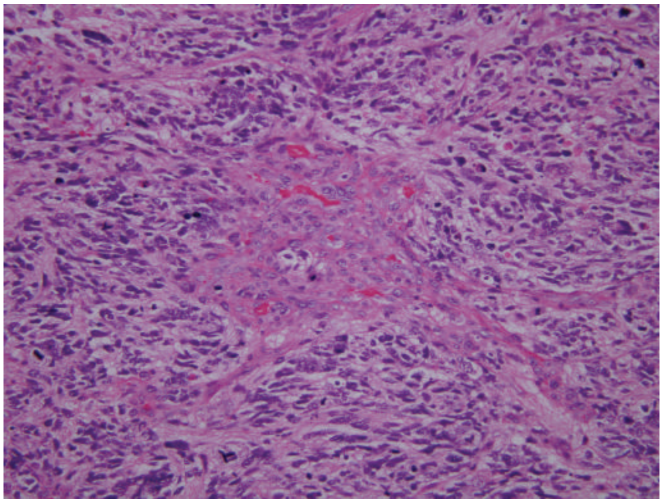

Figure 57-31 Vascular proliferation with a glomeruloid tuft in a child with glioblastoma (hematoxylin and eosin; ×400).

Management. The initial management of pediatric HGA after neuroimaging is to administer high-dose corticosteroids to achieve symptomatic relief. Subsequently the goals of neurosurgery include establishing a histologic diagnosis and, whenever possible, achieving GTR.[715] GTR is often not possible given the infiltrative properties of HGA, but even subtotal resection that achieves internal decompression or debulking makes subsequent radiotherapy more tolerable and probably more effective, given the lower tumor burden. Resection also diminishes the duration of corticosteroid therapy.[716] Intraoperative MRI appears to increase the likelihood of GTR.[717] The role of radiation therapy in the treatment of older children has been clearly established during the past 30 years,[718,719] and the current standard in patients 3 years of age and older is a conformal dose of 5400 cGy to the primary tumor volume with a boost to 5940 cGy in the tumor bed.

Ever since the first national randomized trial comparing radiation versus radiation with chemotherapy in pediatric HGAs (CCG-943) showed a survival benefit for patients treated with chemotherapy, the use of adjuvant chemotherapy has been standard in the treatment of pediatric HGAs.[720] However subsequent trials have failed to show a similar benefit, and the role of chemotherapy in the disease remains unclear. One area of recent focus has been the use of TMZ, which has become the standard of care in adult HGA. However a primary pediatric HGA phase II trial of concomitant TMZ and radiation therapy, followed by adjuvant TMZ, failed to show an improvement in overall or EFS compared with historic control subjects.[721] Other phase II trials of the mTOR inhibitor temsirolimus,[722] the VEGF inhibitor bevacizumab,[723] and TMZ in combination with the DNA repair inhibitor O6-benzylguanine,[338] all in recurrent or progressive pediatric HGA, also failed to meet their aims. Radiosensitizers, including inhaled carbogen, have also failed to show efficacy.[724] A strategy that has shown potentially more promise is multiagent chemotherapy, with or without ASCR. Multiagent, multicourse chemotherapy with radiation therapy improved survival over historic control subjects in patients with primary HGA who had undergone GTR.[725] Another trial of multiagent chemotherapy given over a long period showed promise in delaying radiation therapy for patients with HGA who were younger than 3 years, given the especially severe neurodevelopmental effects of radiation in this age group.[726] Treatment with high-dose chemotherapy and ASCR has also been attempted in this group.[727,728] The risk-benefit ratio is still under investigation,[729] with initial reports of a 23% overall response but a mortality rate of 16%.[730] A trial of high-dose thiotepa and carboplatin followed by ASCR produced one long-term survivor out of four children treated for recurrent or progressive HGA; remission at the time of treatment appeared to be a key positive prognostic factor in this trial, which also included children with high-grade nonastrocytic brain tumors.[507]

Clearly, further study of the role of chemotherapy in pediatric HGAs is needed, as are new strategies. A current COG trial is comparing three potential chemotherapy regimens (the HDAC inhibitor vorinostat, TMZ, and bevacizumab) to be given with radiation therapy, followed by maintenance chemotherapy with TMZ and bevacizumab. Recent phase I trials have used the EGFR inhibitor erlotinib[731] and a prolonged dosing schedule of TMZ.[732] An autologous dendritic cell–based vaccine has shown potential efficacy.[733] Oral valproic acid has shown some effect retrospectively and is well tolerated, making it an attractive potential additive agent for future trials.[734] New mouse models of pediatric HGA inspire hope for more effective preclinical testing that could help bring more novel treatments to clinical trials.[735,736]

Prognosis. The prognosis for children with HGA is poor but is better than for adults.[737-739] The degree of surgical resection is associated with better PFS.[740,741] Patients with anaplastic astrocytoma have a more favorable prognosis than those with glioblastoma.[742,743] A report from the CCG[740] showed that the 5-year PFS rates for anaplastic astrocytoma were 44% ± 11% and 22% ± 6% for children who underwent GTR versus other types of surgery, respectively. The 5-year PFS rates for glioblastoma were 26% ± 9% and 4% ± 3% for children who underwent GTR versus other types of surgery, respectively. Patients younger than 3 years appear to have a survival advantage compared with older patients.[744] One study showed worse PFS for patients initially treated with dexamethasone, raising the question of whether tumor inflammation may be important to treatment.[745] Poor performance at diagnosis on the FMH health status scale correlates with a worse prognosis.[746] The size of the treating medical center has no effect on prognosis.[747] In terms of biologic markers, PTEN expression[748] and MGMT methylation[749] favorably affect survival, whereas p53,[701] endoglin,[698] and PARP[686] positivity are negative prognostic markers. MIB1 staining is usually elevated in pediatric HGA and correlates with grade and prognosis. Patients with MIB1 levels greater than 36% had a 5-year PFS of 11% compared with 33% in persons with MIB1 levels less than 18%.[742]

SPECIAL CASES. HGAs occur more commonly in persons with certain genetic syndromes. These syndromes include hereditary nonpolyposis colorectal carcinoma[104] and Li-Fraumeni syndrome, a dominantly inherited syndrome involving mutations of the p53 tumor suppressor protein.[750] *TP53* mutations are rare in sporadic pediatric CNS tumors lacking a typical family history.[751] HGAs may also arise in persons with NF-1, although low-grade lesions are far more common; in one large case series, HGAs developed in 0.5% of patients.[752]

Cases of de novo congenital HGAs have been observed at birth or within the first 3 months of life and show similarities in gene expression profiling compared with both pediatric and adult HGA.[753] Prognosis, however, appears to be far better. Long-term survival, occasionally even without treatment,[754] occurs in the majority of these patients and supports the unique characteristics of these otherwise fatal tumors.[738,755-759]

Thalamic gliomas can occur as high-grade or low-grade lesions.[516] Poor prognostic factors in these patients include a short interval of symptoms, poor early response to chemoradiotherapy, high-grade histology, and inability to resect the lesion.[515,760] For spinal HGA the median age of presentation is 4 years. Positive prognostic factors include age younger than 5 years, anaplastic astrocytoma histologic features, lack of hemorrhage, and GTR.[761] Multimodality treatment may have some effect.[762] Another rare subtype of pediatric glioblastoma is giant cell glioblastoma. Although time to progression in this variant may be slightly longer, overall prognosis is not significantly better than in other pediatric HGAs.[763]

Gliosarcomas are glioblastomas with a sarcomatous component that can affect children of any age but seem to be most frequent in infants and toddlers, as well as patients who have received prior radiation therapy. They most often present with increased ICP and are located in the cerebrum. Gliosarcoma also carries a poor prognosis.[764] Optic nerve gliomas show HGA features on pathologic examination in approximately 17% of patients. School-aged children are most often affected. The 5-year OS rate is approximately 20% with high-grade histologic features, apparently irrespective of treatment with radiation or surgery.[765]

Diffuse Intrinsic Pontine Gliomas

Tumors of the brainstem occur in approximately 10% of pediatric patients with CNS tumors. The majority of these tumors, approximately 80%, are classified as DIPG and are infiltrative, expansile lesions of the pons. Most of these tumors are anaplastic astrocytoma or glioblastoma on histopathologic examination, although grade II DIPG has been reported; the dismal prognosis is not affected by grade.[766,767] This diagnosis must be distinguished from other pediatric brainstem tumors[768] that are usually WHO grade I, well circumscribed as opposed to infiltrative, and carry a much improved prognosis. The median age at diagnosis of DIPG is 5 to 9 years, but these tumors may occur throughout childhood.

Lack of understanding of DIPG biology was a major roadblock in the past, mostly because researchers had minimal tissue to study because these tumors were rarely biopsied; the procedure was believed to be highly morbid. In addition many samples that were obtained were from areas at the periphery of the tumor and included normal cells and reactive gliosis. Even autopsy specimens were rare. However in more recent years, two major trends have emerged. First, researchers determined that obtaining DIPG tissue by autopsy was feasible, and provided an opportunity that families of these children generally greatly appreciated and did not regret.[769-772] This tissue, generally preirradiated, led to new DIPG culture lines[769] and biologic studies. Second, new neurosurgical techniques emerged, along with targeted therapies based on initial biologic studies,[773] that could be offered to families who consented to upfront biopsy so that patients' tumors could be tested for susceptible genetic changes.

With tissue available, studies of DIPG biology have emerged at a rapid pace. One theme across several studies is amplification of the region containing PDGFR alpha (PDGFRA),[684,774] and protein expression analysis has confirmed the importance of PDGFRA deregulation in DIPG.[684] Thirty-six percent of DIPG samples in another study had PDGFRA amplification, far more than in pediatric and adult HGA.[775] Another key finding has been the discovery of novel mutations in *H3F3A*, encoding histone H3.3, or histone cluster 1, H3b *(HIST1H3B)*, encoding histone H3.1.[776] These mutations were isolated by whole genome sequencing of DIPG samples. Of note, the *H3F3A* mutation was also found in 22% of nonbrainstem pediatric HGAs but was not found in adult HGAs. In a subsequent study in which investigators looked for these mutations in a targeted manner, it was found that the H3.3 mutation occurred in 71% of DIPGs.[681] Although the biologic consequences of this mutation are not yet clear, it does seem to correlate negatively with prognosis.

Other new findings have included overexpression of Aurora kinase B,[688] WEE1 G2 checkpoint kinase (WEE1),[777] B7-H3 (CD276),[778] and interleukin (IL)-13Rα2.[779] Common mutations have been found in TP53, PI3K catalytic subunit (PI3KCA),[780] and EGFR, which surprisingly showed expression of the vIII mutant in a majority of cases, although confirmation of this finding is needed.[781] Authors of one study suggested two biologic subgroups of DIPG, one driven by proangiogenic changes and one driven by PDGFRA amplification or mutation; this latter group seemed to have more deaths before 10 months from diagnosis.[782] Finally, with establishment of a plausible cell of origin that depends on the hedgehog pathway,[783] as well as development of new preclinical models including mouse intracranial xenograft models,[784,785] further understanding of the disease should be forthcoming.

Clinical Presentation. The clinical presentations of children with brainstem tumors appear to fall into two groups. Patients with fewer than 3 months of symptoms, multiple cranial neuropathies (unilateral or bilateral), long tract signs (e.g., Babinski sign, hyperreflexia, and weakness), and cerebellar signs (e.g., ataxia, dysmetria, and dysarthria) are likely to have DIPG. Symptomatic hydrocephalus is present in fewer than 10% of patients with DIPG at presentation. Although the pons is not typically associated with behavior changes, pathologic laughter or separation anxiety have been symptoms noted in persons with DIPG.[786] In contrast children with low-grade brainstem gliomas tend to manifest a more insidious history of isolated cranial nerve palsy or weakness, usually for longer than 3 months.

Imaging and Histology. The diagnosis of DIPG may be made on the basis of the classic MRI appearance of a diffusely expanded pons with encirclement of the basilar artery.[787] Most DIPGs appear to be hypointense on T1-weighted MRI and hyperintense on T2-weighted imaging. A prominent edema signal is common (Fig. 57-32). The ventral pons may appear swollen and infiltrated. Contrast enhancement is variable, from homogeneous rim enhancement to patchy enhancement to complete absence of enhancement. In contrast to DIPGs low-grade focal lesions of the brainstem present with a more circumscribed appearance and associated contrast enhancement that occupies less than 50% of the axial diameter of the brainstem. These tumors may be composed of cystic and solid components. Leptomeningeal dissemination at diagnosis and recurrence may be more common in DIPGs than previously understood,[788] and given the negative effect on prognosis, spine MRI at diagnosis should be considered. Diagnosis by imaging alone puts DIPG in a unique category, apart from nearly every other pediatric tumor, and some studies showing variability in reading of MRIs have called the practice into question.[789] Another study of MRI-biopsy correlation showed that all diffuse, nonenhancing tumors were DIPG pathologically, whereas the diagnosis was more variable with focal or enhancing tumors.[790]

New MRI techniques have been found to have specific roles in diagnosing DIPGs. DTI is helpful in differentiating DIPG from demyelinating disease when the diagnosis is in question.[791] Susceptibility-weighted imaging appears to be the best technique for detecting hemorrhages in DIPGs, which appear to be present in 47% of patients at diagnosis, although most hemorrhages are petechial and asymptomatic.[792] MRS has identified a specific spectrum consistent with the malignant potential of DIPG. More importantly this method can detect positive responses in patients after radiation therapy, as well as changes indicative of progression in advance of MRI changes.[793] The Cho : NAA ratio and lactate measurement on MRS appear to correlate with the length of survival.[794,795] Elevated tumor perfusion on MRI after radiation therapy appears to predict a longer OS, and perfusion has been shown to decrease prior to clinical progression.[796] Serial measurement and estimation of response may be best achieved with FLAIR imaging,[797] although no radiologic methodology is optimal for disease assessment or response. Time to progression or OS, rather than radiographic response, may therefore be the best determination of treatment activity in clinical trials for this population.[798] Ventriculomegaly without obstruction appears to be a common finding after radiation therapy in persons with DIPG, suggesting communicating hydrocephalus.[799]

The histologic appearance of DIPG is similar to that of other HGAs.[800] Most biopsy samples show evidence of atypia and mitoses and qualify as grade III anaplastic astrocytomas. Differentiation between anaplastic astrocytoma and glioblastoma in the pons is not relevant because all these patients appear to have an equally poor outcome.

Management. During the past 30 years, little progress has been made in the treatment of DIPG.[801] Radiation therapy has improved the median OS from weeks to months, but it can at best be considered palliative therapy. Despite many clinical trials, no chemotherapeutic agent has shown an improvement compared with radiation alone. Few patients survive more than 2 years.[802]

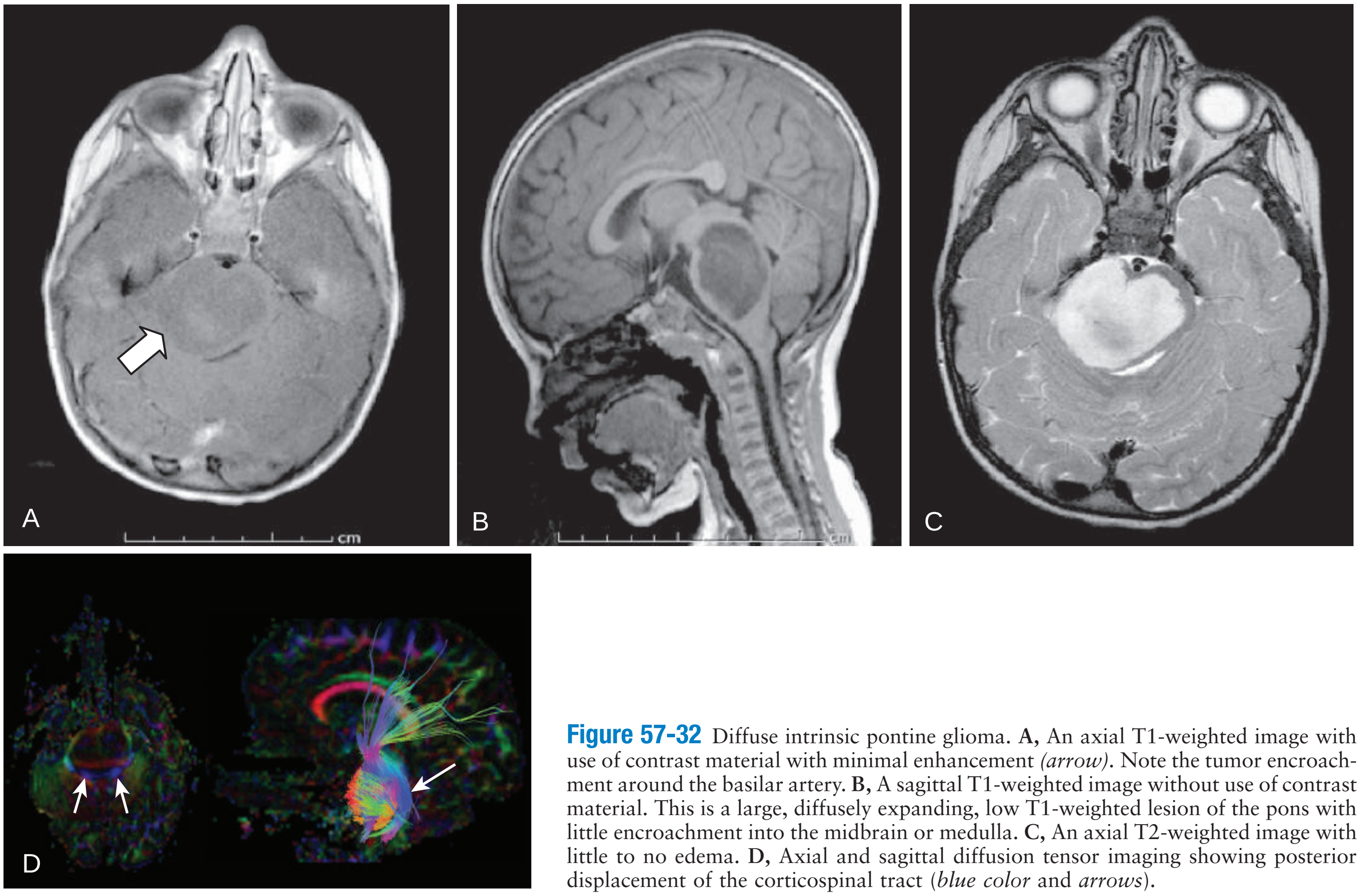

Figure 57-32 Diffuse intrinsic pontine glioma. **A,** An axial T1-weighted image with use of contrast material with minimal enhancement *(arrow)*. Note the tumor encroachment around the basilar artery. **B,** A sagittal T1-weighted image without use of contrast material. This is a large, diffusely expanding, low T1-weighted lesion of the pons with little encroachment into the midbrain or medulla. **C,** An axial T2-weighted image with little to no edema. **D,** Axial and sagittal diffusion tensor imaging showing posterior displacement of the corticospinal tract (*blue color* and *arrows*).

Corticosteroids are generally started at the time of diagnosis, because peritumoral edema may contribute significantly to symptoms, and steroids may therefore offer some relief. Patients with DIPG often require steroids for a long duration, and investigation of agents that can offer relief from peritumoral edema without the adverse effects of steroids is ongoing.

The tide is clearly shifting toward diagnostic biopsy in patients with suspected DIPG. Several retrospective studies of open and stereotactic biopsy showed that the procedure is feasible and safe.[803-805] A prospective trial of 24 patients undergoing stereotactic biopsy showed that transient cranial nerve palsies in two children were the only complications, with tissue obtained in all patients, including two for whom the biopsy results changed the management approach.[806] A consensus statement in support of biopsy on behalf of a large group of DIPG experts has now been published.[807] The procedure still carries risk, however, and should be performed at experienced centers by experienced neurosurgeons. The ideal locations for stereotactic biopsy appear to be areas of focal anaplasia, represented by T2 hypointensity, contrast enhancement, and diffusion restriction.[808]

Radiation therapy remains the mainstay and standard treatment for DIPG. The tumor volume and a 1- to 2-cm volume of surrounding brainstem are generally included in the field, and conventional doses range from 5400 to 5940 cGy given over 6 weeks. This treatment is generally effective at decreasing tumor size, decreasing symptoms, and allowing patients to stop taking steroids, but these effects generally last only a matter of months, and the treatment is still considered palliative instead of curative. Multiple studies have attempted hyperfractionation techniques, ranging from 6600 to 7800 cGy, without improvement in survival[809-815]; this outcome may relate to the inherent resistance of these tumors.[816] More recently in an attempt to reduce the impact of the radiation therapy schedule for these children with a limited life span, hypofractionation schedules have been tested, usually 3900 to 4500 cGy over 3 to 4 weeks, with a noninferior response rate and duration.[817,818] Reirradiation is also the only therapy that has shown any potential efficacy at recurrence: two retrospective reports showed transient responses of some patients to reirradiation doses of 1800 to 2000 cGy, with children who had a prolonged duration of response from initial radiation therapy most likely to benefit.[819,820]

Many adjuvant chemotherapy agents and strategies have been tried and have failed to improve outcomes in patients with DIPG compared with radiation therapy alone. Many recent phase II trials have centered on the use of TMZ, given that this agent is now standard of care in adults with HGG. However three phase II trials of TMZ given with radiation therapy, followed by TMZ alone, have failed to show an increase in survival time compared with historic standards from radiation therapy alone,[721,821,822] and the addition of TMZ worsened toxicity. Other recent phase II trials have shown no benefit of tamoxifen,[823] pegylated interferon α-2b,[824] a regimen of cisplatin, etoposide, ifosfamide, vincristine, and valproate,[725] and inhaled carbogen as a radiosensitizer.[724] Other past failed agents and strategies have included preirradiation multiagent chemotherapy,[825,826] topotecan as a radiosensitizer,[827,828] etanidazole as a radiosensitizer,[829] chemotherapy concurrent with radiation therapy including thalidomide,[830] trofosfamide and etoposide,[831] carboplatin,[832] carboplatin and etoposide,[833] TMZ and cis-retinoic acid,[834] chemotherapy with BBB disruption,[835] intraarterial bevacizumab injection,[836] and a postradiation high-dose chemotherapy regimen.[837] New strategies that have been the subject of recent phase I reports include the use of the PDGFR inhibitor dasatinib in combination with the VEGFR2 inhibitor vandetanib,[838] vandetanib alone,[839] and the use of arsenic trioxide.[840] The development of nonhuman primate models of direct drug delivery into the brainstem opens another avenue for developing improved therapies for these tumors.[841,842]

Prognosis. DIPG remains the most challenging of pediatric tumors to cure. Despite all approaches most survival curves are superimposable with regard to OS.[843] Meta-analyses of numerous clinical reports indicate that median PFS ranges from 5 to 9 months, with a median OS of 6 to 16 months. One-year and 2-year survival ranges from 17% to 50% and 7% to 29%, respectively, with less than 10% survival at 3 years.[843,844] Neither MRI characteristics nor WHO grade has an impact on prognosis in persons with DIPG.[845] Poor performance at diagnosis on the FMH health status scale correlates with a worse prognosis.[746] The size of the treating medical center has no effect on prognosis.[747] One report indicated an improved prognosis for patients with DIPG who are younger than 3 years,[846] although questions of misclassification of patients with low-grade brainstem glioma as having DIPG inevitably arise with reports of improved survival—yet another reason for the use of diagnostic biopsy. Very few cases of neonatal DIPG have been reported, and outcomes in these reports have been inconsistent, ranging from spontaneous regression to death within weeks.[847-849] Retrospective studies have shown worse prognoses for patients initially treated with dexamethasone or rofecoxib, raising the question of whether tumor inflammation may be important to treatment.[745,850] Patients with leptomeningeal dissemination at diagnosis appear to have a shorter PFS than do patients who do not have this finding.[788] It is hoped that with time, the greatly improved understanding of DIPG biology and promising preclinical models will soon lead to successful clinical trials and an improved outlook for patients afflicted with this terrible disease.

Gliomatosis Cerebri

Although much more common in adults, gliomatosis cerebri has also been identified in children.[851] Similar to adults, in children these lesions are often grade III astrocytomas that diffusely infiltrate the brain and appear to favor white matter tracts,[852] and they can easily cross the corpus callosum. In spite of the widespread presence of disease, often involving both hemispheres and multiple lobes, a large primary focal mass is usually absent. All parts of the brain and spine can be involved. The overall prognosis is very poor by virtue of the disease's diffuse nature and unresectability.[853] Patients can be kept alive with palliative care for longer than would be expected for widely diffuse lesions, suggesting a different biology from that of typical grade III astrocytomas.[854] Genetic aberrations, including loss of chromosomes 13q and 10q or gains of chromosome 7q, have been shown to be markers of poor outcome.[855] Some tumors demonstrate abnormalities in the p53 pathway.[856,857] Recently IDH1 mutations have also been found in adult but not pediatric gliomatosis cerebri.[858]

Clinical Presentation. The clinical presentation of children with gliomatosis cerebri is varied and often subtle, mimicking a number of neurologic conditions.[859,860] Children present more frequently than adults with refractory seizures that can be palliated with surgical intervention.[861] Other presentations of this disease include raised ICP and cognitive impairment.[862] Tumors involving the brainstem can result in cranial nerve deficits, and those in the optic pathway can present with visual field deficits.

Imaging and Histology. Gliomatosis cerebri is diagnosed on MRI scan and demonstrates a characteristic diffuse infiltrative pattern.[863] T1-weighted sequences are usually isointense to hypointense and underestimate the extent of disease, whereas T2-weighted sequences are hyperintense and highlight the full extent of the disease (Fig. 57-33). Signal abnormality usually involves multiple lobes.[864] The presence of contrast enhancement is strongly correlated with a poorer prognosis in both adult and pediatric patients.[854,855] A solid area of tumor is usually not evident. MRS demonstrates elevation of choline and a decrease in NAA levels.[865] Although the hallmark of gliomatosis cerebri is an infiltrative astrocytic tumor, areas of oligodendroglial cells are not uncommon for adult and pediatric patients.[866,867] Most tumors are consistent with grade III histology, and mitoses are often present. MIB1 staining can vary considerably. The absence of mitoses is more likely to be because of sampling error. GFAP and S-100 can be positive or negative. Nestin and vimentin can be positive.[868] Because of their infiltrative growth and co-opting of existing blood vessels, these tumors lack the abundant neovascularization observed in other malignant gliomas,[869] which suggests that they will not be effectively treated with antiangiogenic agents.

Management. Currently patients with gliomatosis undergo biopsy to confirm the diagnosis. Given the diffuse nature of these tumors, complete resection is rarely feasible. The mainstay of therapy is irradiation to the involved region, which, for pediatric patients, may require almost complete whole-brain therapy. In addition to significant morbidity, large-volume radiation therapy for

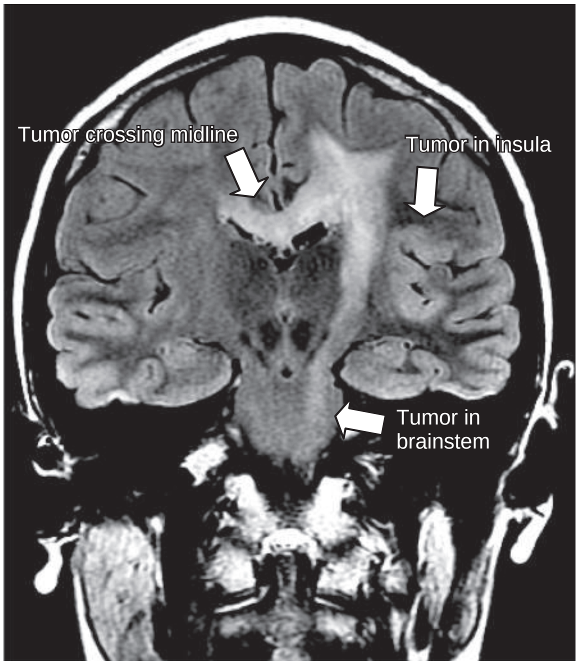

Figure 57-33 Gliomatosis cerebri. This coronal fluid-attenuated inversion recovery image demonstrates the diffuse infiltration. Note that a solid uniform mass is absent.

gliomatosis cerebri has not been proven to be effective,[867] although it may delay the time to progression.[870] Objective responses to TMZ have been documented in adults[871,872] and, in oligodendrogliomas, correlate with chromosome 1p and/or 19q loss.[873] The impact of MGMT expression on response and outcome has not yet been determined.

Prognosis. Pediatric patients with gliomatosis cerebri have a poor outcome.[853,854] In a large series of pediatric and adult patients, a median survival of 14.5 months was demonstrated. Younger age, lower grade histology, and chemoresponsiveness were associated with slightly longer survival times.[874] Although long-term survival is unlikely, adults whose tumors have chromosomal 1p and/or 19q loss can show more chemoresponsiveness to agents such as TMZ.[855] Similar activity in pediatric patients has not been reported because of the rarity of these tumors in this population.

Ependymomas

Ependymomas are glial tumors that arise from ependymal cells that line the ventricles in the CNS. Ependymal cells play an essential role in the transport of CSF. Experimental evidence has indicated that these cells may be derived from radial glia.[875,876] Ependymal tumors represent approximately 10% of all childhood intracranial neoplasms, constituting the third most common pediatric brain tumor, after astrocytomas and medulloblastoma. They are equally distributed between males and females, and the median age at diagnosis is approximately 6 years. They are significantly less common in blacks than whites.

Ninety percent of pediatric ependymomas are intracranial, with 66% to 75% arising from the posterior fossa.[877] Supratentorial ependymomas are often located in the brain parenchyma, away from the ependymal surface, in contrast to infratentorial ependymomas, which are usually located in relation to the fourth ventricle. Spinal cord ependymomas represent fewer than 10% of pediatric intramedullary spinal tumors. In contrast ependymomas represent more than 50% of intramedullary spinal tumors in adults.[878]

The WHO classification system[879] recognizes four major types: subependymoma and myxopapillary ependymoma (WHO grade I), classic ependymoma (WHO grade II), and anaplastic ependymoma (WHO grade III). Differentiation of grade II and grade III ependymoma does not clearly have an influence on outcome,[880] in contrast to the grade in astrocytic tumors. Subependymomas are well circumscribed, slow-growing, and often asymptomatic intraventricular neoplasms that are usually found incidentally in middle-aged and older adults or at autopsy. Myxopapillary ependymomas (WHO grade I) are also slow-growing tumors of the conus medullaris, cauda equina, and filum terminale and manifest primarily in young adults. They account for approximately 10% of all ependymomas and portend an overall good prognosis with surgery-only approaches.

Recent advances in molecular studies have found that ependymomas from different compartments of the brain and spine have unique molecular signatures with distinct patterns of gene expression,[881] chromosomal changes, and protein expression.[882] Deletion of tumor suppressor P16INK4A (located at chromosome 9p21.3) was frequently observed in supratentorial ependymoma. Witt et al[883] analyzed the transcriptome of 177 patients with posterior fossa ependymoma and proposed two distinct subtypes of posterior fossa ependymoma based on different clinical and molecular characteristics (i.e., age, tumor location, degree of genomic instability, alterations in molecular pathways, and prognosis). The "pediatric" subgroup (median age of 2.5 years), designated as group A in the study, showed relatively less genomic instability but commonly harbored chromosome 1q gain and 22q loss. A balanced genomic profile and chromosome 1q amplification have been previously described as two of three defining molecular features of posterior fossa ependymoma.[884,885] The older subgroup (median age of 20 years), designated as group B, exhibited a much higher degree of genomic instability and extensive cytogenetic aberrations. Interestingly a balanced genomic profile, seen in the pediatric subgroup, was paradoxically associated with a worse prognosis. Posterior fossa ependymomas in young children were much more likely to recur, metastasize, or result in mortality. Even accounting for whether GTR was achieved, group A tumors exhibited worse PFS and OS compared with group B (18% 5-year PFS and 52% OS in group A vs. 91% 5-year PFS and 100% OS in group B; $P < .0001$).[882,883] The identification of immune markers in these analyses may provide important opportunities for the development of immunotherapy-based treatments.[886] Chromosome 22q loss is commonly associated with spinal ependymoma in adults and children.[887,888]

The most consistent genetic defects in ependymoma are monosomy 22 or structural abnormalities of chromosome 22q,[889] which raises the possibility that a tumor suppressor gene may be located on chromosome 22.[890-892] As previously noted, comparative genomic hybridization has demonstrated significant differences between infant and childhood ependymoma, suggesting that the pathogenesis of this disease differs by age[893] and location.[894] A number of other molecular defects have been described in ependymomas, including the abnormal expression of ERBB2 and ERBB4 receptors,[895] defects in the p53 homologue p73,[896] increased expression of the VEGF protein,[897] Nestin expression,[898] PI3K activation,[899] immune targets such as EphA2, IL-13Rα2, and Survivin,[900] amplification or overexpression of the p53 regulator MDM2,[901] overexpression of cyclooxygenase-2 (COX-2),[902] the presence of telomerase activity,[903,904] and expression of protein 4.1.[905] Other molecules implicated in the pathogenesis of ependymoma have been reported by using micro RNAs[906] and proteomic fractionation of tumor tissue. Future studies will be required to determine the significance of these molecules and their potential use as diagnostic markers and targets for therapy.[306]

Significant progress has also been made in the use of these markers in attempting to understand and characterize the putative ependymoma stem cell[907,908] with expression of OLIG2.[909] The role of telomerase may also be an important factor in the recurrence of ependymomas,[910,911] and additional markers are being developed.[891,912] The advances in assessment of the epigenome of ependymoma have also recently been reported and give important clues about the role of DNA methylation in the genesis and possibly targets of ependymoma.[913-915] Finally, the presence of JC (John Cunningham) viral sequences identified in 5 of 18 ependymomas (but 0 of 32 medulloblastomas) raises the possibility that this agent is associated with ependymal tumorigenesis.[916] Simian virus 40 (SV40) viral, large, T-antigen sequences have also been identified in ependymomas and choroid plexus papillomas (CPPs)[917] but are negative in adjacent normal brain.[918] Although these results have generated significant discussion, they have not been confirmed.[919,920] Further studies will be needed to validate these findings.

In myxopapillary ependymoma, a WHO grade I ependymoma that is confined to the conus medullaris-cauda equina-filum terminale of spinal cord, upregulation of 20 members of the homeobox (HOX) family that are not typically observed in intracranial ependymoma has been described.[921] EGFR has also been identified in ependymomas of the brain[922] and myxopapillary ependymoma and correlates with progression.[923]

Persons with NF-2 have an increased susceptibility to intramedullary spinal cord ependymomas.[924] Although the *NF2* gene is located at chromosome 22q12, mutations in *NF2* (Merlin) are rarely found in sporadic ependymomas.[925,926] A recent microarray analysis of pediatric ependymomas has identified a cluster of genes distinct from *NF2* that may be involved in ependymoma tumorigenesis.[891] Expression profiling has indicated that histologically similar ependymomas from different parts of the CNS are molecularly and clinically distinct disease subgroups.[875,927] Certain familial colon cancer syndromes may also be associated with an increased incidence of ependymoma in offspring.[928]

Clinical Presentation. The presenting symptoms of infratentorial ependymomas are a result of their origin from ependymal tissue lining the fourth ventricle. Hydrocephalus results when the tumor fills the fourth ventricle, causing obstruction of the normal spinal fluid flow and subsequent development of headache, irritability, nausea, vomiting, and ataxia. Papilledema can often be found on physical examination. Tumors that extend out of the foramen of Luschka may compromise lower cranial nerve function and cause hearing impairment, hoarseness, and/or dysphagia. If the tumor extends through the foramen of Magendie, the patient may report neck discomfort or be noted to have torticollis. The most common signs of the tumor in infants are irritability, decreased oral intake, vomiting, ataxia, head pain, lethargy, and increased head circumference.[929] Supratentorial ependymomas which represent approximately one third of ependymomas, are more common in older children and adults. These patients present with focal neurologic deficits and seizures. Spinal ependymomas are typically located in the cervical region. The most common presenting symptom is pain or motor deficits localized to the level of the spinal cord lesion. The pain is typically described as being worse at night, presumably because of congestion of the spinal venous plexus that occurs when the patient is in the recumbent position. The second most common symptom is radicular dysesthesias, and a late manifestation of this symptom is progressive spastic quadriparesis. Thoracic ependymomas are associated with scoliosis. Myxopapillary ependymomas of the conus medullaris and filum terminale may cause low back pain, radicular pain, saddle anesthesia, and sphincter dysfunction.[930] When these tumors disseminate, they usually remain in the spine,[931] although cranial metastases have been reported; therefore craniospinal imaging at diagnosis should be undertaken.[932] Pediatric myxopapillary ependymoma may have a higher propensity to spread compared with adult tumors.[932]

Imaging and Histology. A typical MRI appearance of a fourth ventricular ependymoma is that of a homogeneously enhancing well-circumscribed solid mass, extending out one of the foramina of Luschka or Magendie with obstructive hydrocephalus. These tumors classically wrap around the brainstem or herniate through the foramen magnum (Fig. 57-34). Hemorrhage and calcifications can be observed and are more common in supratentorial lesions. Ependymomas may occasionally spread via CSF, seeding the leptomeninges or ventricles, either at diagnosis or at recurrence.[933] Careful attention to staging of patients is therefore important.

The characteristic microscopic feature of a classic ependymoma is that of dense cellularity, intermixed with perivascular pseudorosettes consisting of tumor cells and surrounding a neoplastic blood vessel (Fig. 57-35). True ependymal rosettes representing abortive canals are relatively uncommon.[934] Histologically the anaplastic variant is recognized by the presence of mitoses, necrosis, and

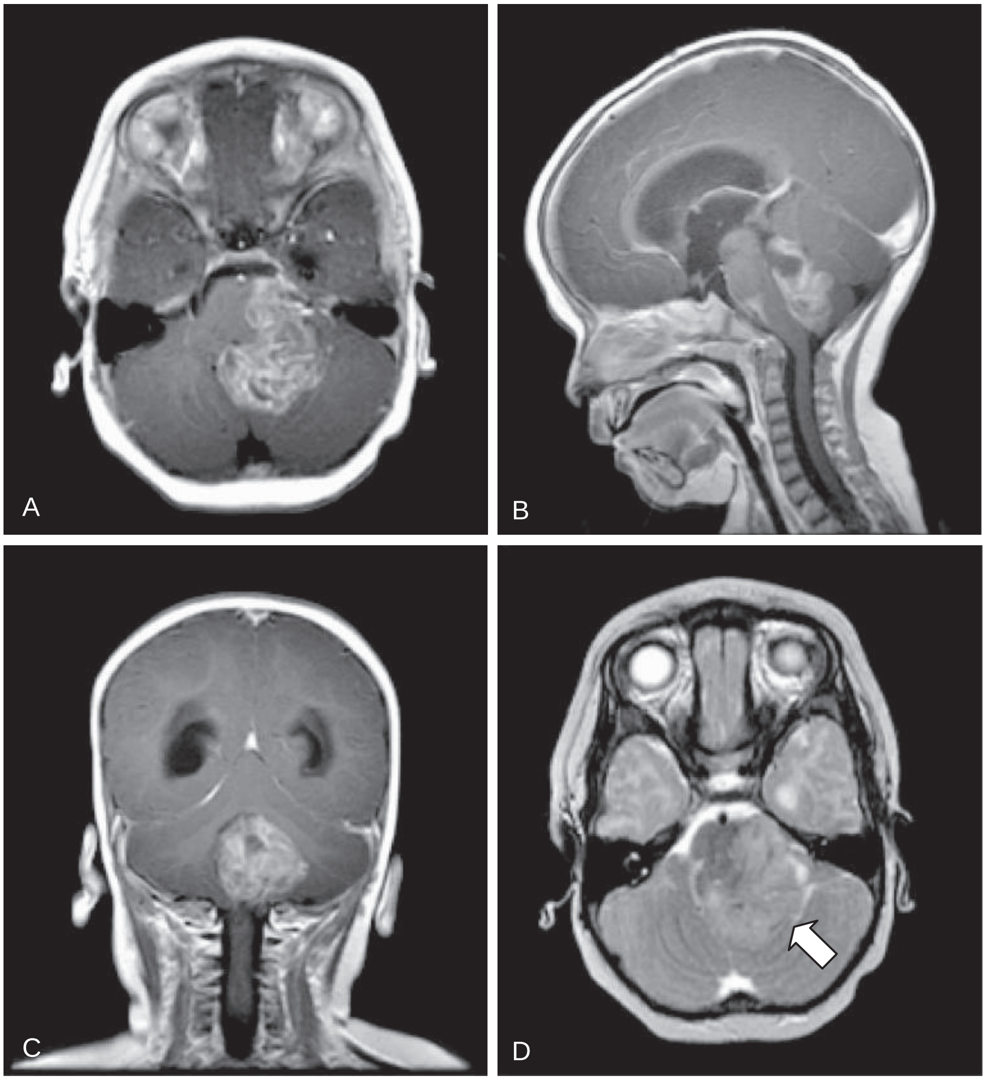

Figure 57-34 Posterior fossa ependymoma. **A,** An axial T1-weighted image with use of contrast material. **B,** A sagittal T1-weighted image with use of contrast material. **C,** A coronal T1-weighted image with use of contrast material. **D,** An axial T2-weighted image showing a T2 isodense lesion *(arrow)*.

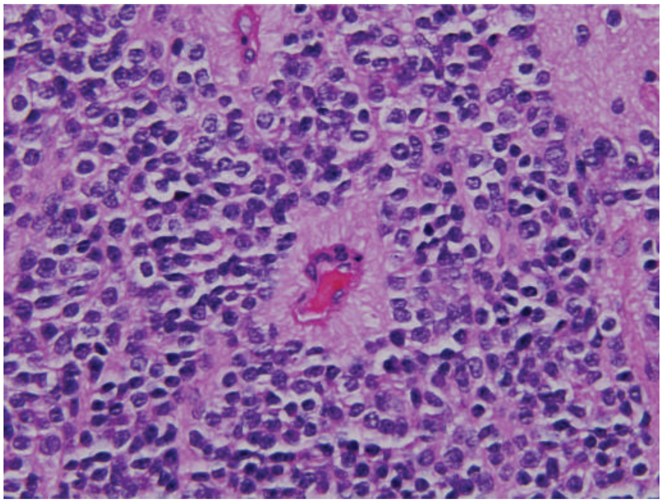

Figure 57-35 Ependymal pseudorosette (hematoxylin and eosin; ×600).

vascular proliferation. They tend to be more cellular than grade II ependymomas but usually remain well demarcated. MIB1 rates greater than 5% in incompletely resected tumors or greater than 15% in completely resected tumors correlate with more aggressive behavior.[935] GFAP and S-100 are positive in most ependymomas, and epithelial membrane antigen (EMA) stains are positive in anaplastic variants. The presence of perivascular elastic fibers may also be a diagnostic feature of these tumors.[936] In patients in whom the diagnosis of ependymoma is indeterminate, electron microscopic analysis of cilia structure and junctional complexes, which are found in most ependymomas, can be used to confirm or exclude the diagnosis. Myxopapillary ependymomas have a mucinous appearance and arise almost exclusively in the cauda equine.[930] Their MIB1 labeling index is typically low. Tanycytic ependymomas are a rare subtype of ependymoma that can occur throughout the brain or spine.[664,937] Other rare subtypes include cellular ependymoma, papillary ependymoma,[938] and clear cell ependymoma[939] variants.

Management. Evaluation of patients at diagnosis or recurrence should include an MRI of the brain and spinal cord, as well as cytologic evaluation of the CSF, usually performed at least 14 days after the operation.[940] Attempting CSF analysis during the surgery or shortly after could give false-positive results. The usefulness of CSF sampling in patients may not be particularly sensitive.[941,942] A number of factors have been associated with an unfavorable outcome, including younger age at diagnosis, subtotal resection, and a high MIB1 labeling index. Anaplastic histology remains controversial as a negative prognostic factor. Of these factors the single most important factor in the determination of prognosis appears to be whether a complete resection can be accomplished.[943]

The first line of treatment is surgery with the goal of GTR. If a complete resection with clear margins of a grade II spinal cord or supratentorial ependymoma can be achieved, adjuvant therapy can be deferred in some patients.[944] Technologic advances such as the operating microscope, Cavitron ultrasonic aspirator, intraoperative ultrasound, and intraoperative MRI, as well as electrophysiologic monitoring, have facilitated the safety of resections of intraaxial spinal cord tumors while reducing operative morbidity.[945] Overall, spinal cord ependymomas are more easily resectable than astrocytomas because of the presence of a better demarcated cleavage plane. Tumors with an infiltrative boundary, or those with anaplastic histology, require adjuvant therapy. All posterior fossa tumors, independent of grade and degree of resection, should also receive adjuvant treatment. Spinal myxopapillary ependymomas with a complete surgical resection have an excellent prognosis[946] and do not usually require adjuvant therapy even in the presence of drop metastases in the thecal sac.[947] Given the overall good prognosis of these tumors, the need for radiation in incompletely resected disease is unclear, and careful surveillance imaging is needed.[948] Early radiation therapy is not always routinely used but is recommended by some persons.[949,950]

Regarding radiation therapy, there is a long history of deferring radiation therapy for infants and young children who have posterior fossa ependymomas, with use of various conventional chemotherapy regimens instead. Previous treatment recommendations included radiation therapy for patients in whom gross resection was not achieved or for patients with recurrent disease. However partially resected tumors almost invariably recur, requiring further surgery, radiotherapy, and conventional chemotherapy. As such, in the United States the current strategy for ependymoma is to recommend deferral of adjuvant therapy in persons with completely resected supratentorial ependymoma, use of radiotherapy in the involved fields alone for completely resected posterior fossa ependymoma, and use of multiagent chemotherapy for subtotally resected ependymoma, followed by second-look surgery and involved field radiation therapy.[951-953] The benefit of hyperfractionation has not been established.[954] Wide-field pulsed reduced dose rate radiotherapy is currently being evaluated.[955] Craniospinal radiation therapy does not appear to be superior to focal radiation therapy.[877] Several studies have suggested that radiotherapy prolongs PFS after subtotal resection of an ependymoma. Deferral of radiation therapy after initial complete resection can result in reduced cure, even when complete resection is achieved a second time.[956] Proton radiation therapy appears to have equal efficacy when compared with photon therapy; it can reduce the potential long-term toxicity by decreasing the volume of normal brain tissue that is also targeted and is therefore recommended over photon therapy if it is available locally.[957,958]

Until recently there was no clear role for chemotherapy in the management of ependymomas outside of clinical trials.[959] Several small series in newly diagnosed and recurrent disease have shown objective responses to the following drugs: carboplatin, cisplatin, ifosfamide, and etoposide.[960] Chemotherapy is more often used for infants and younger children with incompletely resected or disseminated disease.[961-963] Encouraging results have been reported from the most recent cooperative group clinical trial involving preirradiation chemotherapy,[964] which showed a 40% complete response (CR) rate in patients who received preirradiation chemotherapy for residual postoperative tumor.[965,966] The two factors associated with a favorable outcome were the achievement of a complete resection and supratentorial disease. Chemotherapy should therefore be used to achieve a CR or improve the chances of a complete surgical resection at second-look surgery. It is not highly effective as sole treatment for this disease, and recurrences occur relatively rapidly in a significant percentage of patients if radiotherapy is not administered early. Infants may be more responsive, and 23% to 40% can be cured with chemotherapy alone.[967,968] The use of high-dose chemotherapy and stem cell rescue in infants with ependymoma has failed to demonstrate a significant advantage compared with standard modalities in most,[969] although not all, studies.[970] Although uncommon, up to 10% of patients can have leptomeningeal spread of ependymoma. Response to intrathecal liposomal cytarabine has been reported.[961]

Recurrences of intracranial ependymoma can occur throughout the first decade after initial therapy, although most recur within the first few years.[971] Surveillance scanning is important because it allows smaller asymptomatic tumors to be identified that may be more amenable to reresection. Most recurrences are within the original radiation field[972] and do not appear to be related to marginal failures.

Prognosis. The most important prognostic factors for intracranial and spinal cord[973] ependymoma are age, tumor grade,[974] extent of surgical resection, and delivery of radiation therapy with doses of at least 5400 cGy.[975-980] Children younger than 3 years, those with WHO grade III disease, or those with less than a GTR have been shown to have lower survival rates.[981] The 5-year PFS and OS for patients with subtotal versus total resection of posterior fossa ependymomas is 25% and 66%, respectively. The prognosis of patients with disseminated disease is worse.[539,976,982] Patients with a supratentorial nonanaplastic ependymoma can be treated with surgery only if the tumor can be completely resected. Recently a molecular assay for HTERT expression has been shown to predict the likelihood of progression and survival of pediatric intracranial ependymoma.[910] Additional studies on the molecular profile of ependymomas will likely lead to improved stratification and prognostication of patients as previously discussed.[891,912] Patients with an elevated MIB1 labeled index of more than 5% in incompletely resected ependymomas or of more than 15% in completely resected ependymomas were reported to have a worse prognosis in one study.[935] Patients with a deletion of 6q15.3 appear to have an improved outcome.[983] Molecular profiling has identified that the group A ependymomas (posterior fossa, infants) have a more aggressive biologic behavior and worse prognosis when compared with the group B ependymomas (supratentorial),[882] although this classification is not currently being used to guide therapy intensity at the present time.

Most patients with completely resected ependymoma followed by focal radiation therapy will be long-term survivors. Consideration of advanced radiation planning and the type of radiation delivered (i.e., proton vs. photon) are critical to minimize long-term neurocognitive morbidity in this population.[984]

At the time of recurrence, no standard salvage regimen has been proven to be effective.[985] Reoperation to achieve a complete resection has been curative in some patients and is typically the first modality considered[986]; this approach can be combined with reirradiation.[987,988] SRS has also been used with some success for focal recurrences of ependymoma.[989,990] Patients with metastatic disease or infiltration into the brainstem or other critical structures have a poor salvage rate, presumably because of the lack of good therapies for recurrent disease and the inoperable nature of the lesions. Oral etoposide alone[991] or in combination as a metronomic therapy combination has demonstrated responses in this disease.[341,335] Bevacizumab in combination with irinotecan did not show any significant activity.[992] A single case report of a response to the mTOR inhibitor sirolimus has also been published.[993]

Recurrence of myxopapillary ependymoma can occur after complete resection and can be associated with dissemination. Although cranial metastases have been reported, most cases result in regional metastases of the spine but not beyond the foramen magnum.[994] Many of these patients can undergo salvage therapy with radiation. Consequently frequent surveillance scanning of patients with myxopapillary ependymoma is recommended to identify recurrent disease early.

Oligodendrogliomas

Oligodendrogliomas are rare brain tumors in children, representing fewer than 1% of all pediatric brain tumors.[995,996] The mean age at diagnosis is approximately 10 to 13 years, with a male predominance.[997,998] The WHO grading system recognizes two grades for oligodendroglial tumors: well-differentiated grade II oligodendroglioma and grade III anaplastic oligodendroglioma.[999] As with other glial tumors, higher grade is a predictor of decreased OS. Other reported markers in oligodendrogliomas include deletions of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene on chromosome 9p, mutations in *TP53* and *PTEN*, and amplification of the EGFR.[1000,1001] However it is not known whether these factors are predictive of outcome in pediatric patients. Unlike adult tumors, pediatric oligodendrogliomas do not typically have the chromosome 1p or 19q deletions or mutations in *TP53* or *IDH1*.[382]

Clinical Presentation. Oligodendrogliomas are diffusely infiltrating tumors that tend to occur in the white matter of the cerebral hemispheres. Patients frequently present with seizures, although higher-grade tumors may present with evidence of increased ICP because of more rapid growth.[1002] Other symptoms at presentation can include headache, visual field defect, paresis, and cranial nerve palsy.[998] Although uncommon, oligodendrogliomas can also occur throughout other areas of the brain, including the posterior fossa, brainstem, and spine.[1003-1007]

Imaging and Histology. MRI evaluation of oligodendrogliomas typically reveals increased T2-weighted signal and decreased T1-weighted signal.[1002] Gadolinium contrast enhancement is more common in tumors that grow as solid masses and less common in purely infiltrative tumors. Contrast enhancement is also more common in grade III than in grade II oligodendrogliomas.[1008] Although oligodendrogliomas bear morphologic similarity to oligodendrocytes, the cellular origin of these tumors has remained difficult to prove. Histologic examination of fixed specimens of oligodendrogliomas reveals a monotonous pattern of cells with round nuclei and clear perinuclear halos (a "fried egg" appearance). This appearance is an artifact of formalin fixation and is not seen in frozen sections or tumor smears. Higher-grade anaplastic oligodendrogliomas (WHO grade III) are characterized by increased nuclear variability, increased mitotic activity, and/or microvascular proliferation. These features can occur diffusely throughout the tumor or in discrete foci. The differentiation between oligodendrogliomas and other diffuse gliomas such as astrocytomas and OAs is difficult because of a lack of reliable molecular markers that distinguish oligodendroglial tumors from astrocytic tumors. Mature oligodendrocyte-specific markers such as myelin basic protein, 2′3′–cyclic-nucleotide 3′-phosphodiesterase, and myelin-associated glycoprotein are not expressed in oligodendrogliomas, although astrocytic markers such as GFAP and S-100β are expressed in both astrocytomas and oligodendrogliomas.[999] More recently the lineage markers OLIG1 and OLIG2 have been evaluated in the hope that they may enable specific identification of oligodendroglial tumors.[1009] However despite the restriction of OLIG2 expression to normal oligodendrocytes and their precursors, OLIG2 stains all diffuse gliomas and precludes specific immunohistochemical classification of these tumors.[1010] The loss of chromosome 1p and/or 19q is commonly seen in adult oligodendrogliomas and correlates strongly with chemotherapy responsiveness and outcome.[1011] Pediatric oligodendrogliomas have a much lower incidence of chromosomes 1p and/or 19q deletions.[1012,1013] Even in children in whom these deletions are identified, the greater chemoresponsiveness observed in adults is less evident.

Management. Treatment of pediatric oligodendrogliomas, like other glial tumors, involves surgical resection, radiation therapy, and chemotherapy. The goal of surgery is to facilitate accurate diagnosis and to remove as much of the tumor as safely possible. In the adult literature, there is uncertainty as to whether the extent of resection correlates with OS. Although some larger case series have shown no prognostic value in the extent of resection,[1011] most evidence seems to indicate that the extent of resection may be associated with better prognosis.[1014,1015] In pediatric patients, it appears that the extent of resection is a sensitive predictor of outcome.[547] Fewer pediatric low-grade oligodendroglial tumors progress to high-grade tumors, so it is possible that patients with subtotally resected tumors may still have a good outcome. For example in one series of 20 patients with oligodendrogliomas treated with surgery only, 70% remained progression-free at a median of 5 years.[1016]

Despite the fact that oligodendroglial tumors are radiosensitive,[1017] the use of adjuvant radiation therapy in asymptomatic children with incompletely resected oligodendrogliomas is not favored because of the well-described late effects of radiation therapy.[425] Children who are symptomatic, who have tumors in critical locations such as the brainstem, or who have progressive disease despite resection and/or chemotherapy may benefit from radiotherapy.[466] However it is now clear that a subset of these children can be effectively treated with chemotherapy, allowing for a delay in, or avoidance of, radiation therapy.[1018] The combination of carboplatin and vincristine chemotherapy has demonstrated activity in various low-grade astrocytomas, regardless of histologic subtype. This therapy should therefore be considered for patients with oligodendroglioma.[433] Older children may also benefit from chemotherapy.[459] Based on overlapping activity in children with low-grade astrocytomas, the combination of TPCV might also be of use in patients who do not respond to or have disease progression after vincristine and carboplatin therapy. The combination of procarbazine, lomustine, and vincristine or TMZ monotherapy could also be considered based on the responses seen in adults, but these treatments have not yet been adequately tested in children.[492,1019,1020]

In patients with anaplastic oligodendroglioma or progressive disease, despite surgery and adjuvant chemotherapy, the risks of radiation therapy may be offset by the risk of further tumor growth. A recent survey of adult neurooncologists has found that the most commonly recommended treatment for anaplastic oligodendroglioma is the use of concurrent TMZ and radiotherapy followed by adjuvant TMZ.[1021] In rare cases of dissemination, the overall prognosis for these patients is poor, although transient chemotherapy responsiveness can be achieved.[1022] Patients with oligodendroglial gliomatosis cerebri have a very poor prognosis.

Prognosis. Overall long-term survival from low-grade oligodendrogliomas appears comparable with that of other LGGs.[1023] It should be noted, however, that only limited numbers of case series are available, making comparisons among studies difficult. Bowers and colleagues[1016] have reported an OS of at least 5.5 years in a series of 20 patients with low-grade oligodendrogliomas treated initially with surgical resection alone. Six of 20 patients in this series had disease progression at a median of 2.2 years after initial resection. Other studies have reported 5-year OS rates of 65% to 84.4%,[997,998] although the proportion of patients treated with radiation therapy and/or chemotherapy differed among these studies. Children younger than 3 years and children with incompletely resected tumors may do worse.[1024] The rarity of anaplastic oligodendroglioma in children makes specific estimates of survival in pediatric patients difficult. Oligodendrogliomas that are more centrally located do not appear to do as well as those located more peripherally in the cortex.[997]

A number of studies from adult patients have indicated that allelic losses at chromosomes 1p and 19q, typically involving the entire chromosomal arm at both sites, correlate with histologic classification, response to treatment, and prognosis.[1025] Loss at chromosome 1p is a predictor of chemosensitivity, and loss at combined chromosomes 1p and 19q is associated with both chemosensitivity and longer recurrence-free survival.[1026] Recent evidence has indicated that loss at chromosomes 1p and 19q is associated with MGMT promoter methylation and lower expression of MGMT.[1027] Interestingly these chromosomal features appear to be less common in pediatric oligodendrogliomas.[1011,1012] One study has reported the lack of allelic loss in children younger than 9 years, although a modest number of tumors from children older than 9 years had losses of chromosome 1p (45%) and/or 19q (27%).[1013] Nevertheless the incidence of chromosomal losses is significantly less than that seen in adults (50% to 90%). Taken together these studies suggest that the oncogenesis of these tumors is distinct from their adult counterparts.

Oligoastrocytomas

OAs are tumors containing a mixture of two distinct neoplastic cell types that morphologically resemble the tumor cells of oligodendrogliomas and diffuse astrocytomas. These tumors correspond histologically to WHO grade II. The demography of OAs and anaplastic OAs (WHO grade III) is difficult to ascertain because of a high variability in the histopathologic criteria used for classification of these tumors. Clinically these tumors present with signs and symptoms similar to those of astrocytomas and oligodendrogliomas. Tumors arise most frequently in the cerebral hemispheres and lack any neuroradiologic features that would facilitate distinguishing them from oligodendrocytomas. Histologic diagnosis requires the recognition of the two different glial components, both of which must be neoplastic.[1028] Anaplastic OAs are WHO grade III oligoastrocytomas with histologic features of malignancy, including high cellularity, high mitotic activity, and increased nuclear atypia and pleomorphism. Currently treatment for OAs and anaplastic OAs is similar to that for other grade II and III glial lesions—surgical resection with adjuvant chemotherapy and/or radiation therapy for higher grade or progressive lesions.

Embryonal Tumors

Embryonal tumors represent a large and important fraction of pediatric brain tumors, both for their clinical impact and the importance of the scientific insights gained through their study. The most common embryonal tumors include medulloblastoma, pineoblastomas, and CNS PNETs. A number of other tumors are grouped into this category, such as ATRTs, ependymoblastomas, and medulloepitheliomas (see Table 57-6). Recent advances in gene expression array technologies have facilitated a new understanding of the molecular features of tumors within this set of diseases.[1029] The grouping of these tumors into the larger category of CNS PNETs will continue to evolve while a better understanding of their origin progresses. Currently all these tumors share a common property: high risk of dissemination and therefore the need to treat the entire craniospinal axis. It is expected that new technologies will soon allow for a better understanding of the cell of origin of the different tumor types. These considerations are no longer intellectual exercises. Gene-based classification systems may be the key to improved stratification and management and will likely augment, if not replace, the current histopathology-based classification system that underlies current treatment protocols.

The history of small round blue cell tumors is a fascinating story that spans the evolving approach to cancer during the past century. Histopathology was, and remains, the cornerstone of all classification schemas. Tumors from different sites that appeared the same under microscopic examination were assumed to be similar tumors arising from different organs. With the introduction of immunohistochemical techniques of pathologic specimens, pathologists began to define important differences among populations. Small round blue cell tumors within the body were now divided into unique categories based on the presumed cell of origin. Tumors such as lymphoma, neuroblastoma, Ewing sarcoma, rhabdomyosarcoma, and others became easily differentiated. The classification of small round blue cell tumors of the brain, by contrast, took a different direction. Medulloblastoma, a small round blue cell tumor of the cerebellum, was previously differentiated from other similarly appearing tumors based on its location within the posterior fossa,[1030] but some investigators thought that this arbitrary separation was unwarranted. Hart and Earle[1031] attempted to classify all embryonal tumors based on the expanding array of immunohistochemical markers and variations in the patterns of staining among these tumors. Rather than clarifying the classification of embryonal tumors, further blurring of the understanding of these tumors resulted. One contributing factor involved differing definitions for the grouping of embryonal tumors. For some investigators, medulloblastoma was considered a PNET and thus was lumped with pineoblastomas and sPNETs, whereas for other investigators, medulloblastoma remained a separate category. Another factor was the considerable variability in the quality of the staining pattern and heterogeneity within different areas of a single tumor and within different tumors of the same type. With advances in molecular pathology, classification has moved away from the simple microscopic appearance of a tumor to one that incorporates the pathways active in the tumor. Molecular signatures for these different tumors have been demonstrated; these signatures have shown that the tumors arise from different cell populations and, as a result, these tumors will likely require different treatments. Many prior treatment protocols grouped all these heterogeneous tumors together, which has confounded the true efficacy of the therapy under consideration and the prognoses for this heterogeneous group of tumors.

TABLE 57-6 Classification of Embryonal Tumors

Tumor Location	Histologic Classification	Histologic Subtype
Fourth ventricle	Medulloblastoma	Classic or nondesmoplastic Desmoplastic Anaplastic Large cell Melanotic
	Cerebellar neuroblastoma* Atypical teratoid-rhabdoid tumor	
Pineal	Pineocytoma Pineoblastoma Atypical teratoid-rhabdoid tumor	
Supratentorial or intratentorial	Primitive neuroectodermal tumor* Cerebral neuroblastoma Atypical teratoid-rhabdoid tumor Ependymoblastoma†	

*The nomenclature for neuroblastoma and primitive neuroectodermal tumor (PNET) in the central nervous system results from the historic grouping of these tumors with other small round blue cell tumors of the body. Primary cerebral or cerebellar neuroblastoma are unrelated to similarly named tumors in the body and do not possess the abnormalities in the N-Myc pathway. Similarly central nervous system PNET is unrelated to Ewing sarcoma PNET and does not possess the classic chromosome 11;22 translocation.

†Ependymoblastomas were previously grouped with ependymomas but are now differentiated from this group as a result of their embryologic development and patterns of spread.

Medulloblastomas

Medulloblastomas represent approximately 15% of all pediatric brain tumors and approximately one third of posterior fossa tumors.[1032,1033] In addition medulloblastoma accounts for more than 50% of pediatric embryonal intracranial tumors. The incidence in males is twice that of females, and the median age at diagnosis is 5 to 7 years, with most cases diagnosed in the first decade of life.[1034] The current standard of care for children diagnosed with medulloblastoma involves maximal surgical resection and adjuvant therapy with radiotherapy and chemotherapy. Radiation therapy involves a boost to the tumor site with craniospinal prophylaxis. Chemotherapy is administered both concurrently with radiation therapy and as multiagent maintenance chemotherapy after the completion of radiation therapy. Approximately 80% of children with medulloblastoma are cured,[1035] but current treatment strategies result in significant long-term morbidity.

With recent advances in transcriptome and genomic profiling of medulloblastoma, it has become apparent that medulloblastoma encompasses a heterogenous group of tumors with distinct pathologic features and clinical outcomes.[1036-1039] These findings represent exciting prospects for the development of novel tumor-directed therapies and treatment strategies that are stratified according to the genetic profiles of the individual medulloblastoma.

Different subtypes of medulloblastoma are thought to originate from distinct germinal zones within the cerebellum—the EGL, which contains committed granule cell precursors, and the ventricular zone, which contains multipotent stem cells that give rise to most cerebellar neurons. Several cancer predisposition syndromes carry an increased risk of medulloblastoma, including nevoid basal cell carcinoma syndrome or Gorlin syndrome, Turcot syndrome, and Li-Fraumeni syndrome.[1040-1042] Gorlin syndrome, although rare, is an important factor that must be considered in the treatment of these patients, because they are at high risk for the development of nevoid basal cell carcinoma, especially within the involved radiation fields.[1043] In addition Gorlin syndrome provided the first clues as to potential pathways that may be involved in the pathogenesis of medulloblastoma, with the finding of mutations in SHH pathway members, such as *PTCH1*, increase the risk of tumorigenesis.[1044]

Recent cooperative studies have comprehensively profiled the biology of medulloblastoma to reveal four distinct subgroups of tumors.[1036-1039] The groups are distinguishable from each other on the basis of gene expression, methylation, copy number, and mutation profiles.[1045-1049] These groups have been designated as Wnt-positive medulloblastoma, SHH-positive medulloblastoma, and group 3 and group 4 medulloblastoma.[1037] Wnt-positive medulloblastomas are characterized by mutations of β-catenin, resulting in activation of Wnt pathway signaling. Children with Wnt-positive medulloblastoma rarely have metastatic disease and have an excellent overall prognosis. The SHH group is characterized by tumors that carry mutations of the SHH pathway, with resultant pathway activation. The types of SHH pathway mutations differ between infants, children, and adults and may affect the use of targeted therapies for this group.[1050] These tumors can encompass multiple histologic subtypes, although nodular desmoplastic medulloblastomas are more likely to carry SHH pathway mutations. Group 3 medulloblastomas carry a poor prognosis and are largely characterized by amplification of *MYC* or amplification/overexpression of the *OTX2* oncogene. Group 4 medulloblastomas carry an intermediate prognosis and are likely to have isochrome of 17q, and gene expression studies show enrichment of genes involved in neuronal differentiation and development. *TP53* mutations have been described to be enriched in the SHH subgroup of medulloblastoma, and when present, they are associated with inferior responses to therapy.[1051] Profiling studies of recurrent medulloblastomas have revealed that the subgroup determinations are stable, with recurrent tumors maintaining the same subgroup profile as they were at diagnosis.[1052]

In addition to the driver genomic alterations identified in medulloblastoma, evidence is growing that epigenetic dysregulation may play a role in the pathogenesis of these tumors.[1045] This belief is supported by the observation of enrichment in genomic alterations of chromatin modifiers in large-scale profiling projects for medulloblastoma.[1049,1053,1054] These alterations include inactivating mutations of the histone methyltransferase genes MLL2 and MLL3,[1055] *KDM1A* a histone demethylase[1056] and *SMARCA4*, a member of the SWI/SNF chromatin remodeling complex.[1053,1057] Mutations and aberrant hypermethylation of the telomerase reverse transcriptase (TERT) promoter have also been recently described in large-scale sequencing projects of medulloblastoma.[1058] Mutations in TERT promoters have been found to be particularly enriched in the SHH subtype of medulloblastoma.[1059] In addition altered expression of genes regulated by the polycomb repressor complex have also been described in medulloblastoma.[1060,1061] Alterations in chromatin modifiers have been described in medulloblastoma, and novel agents that target chromatin modification are being investigated as potential therapeutic for medulloblastoma. These agents include drugs that suppress expression of the MYC isoforms by inhibiting bromodomain containing 4 (BRD4), a bromodomain and extraterminal (BET)–containing protein,[1062] and inhibitors of enhancer of zeste 2 (EZH2).[1060]

Alterations in genes involved in cell cycle regulation have also been described in medulloblastoma.[1049] These alterations include amplification of cyclin-dependent kinase (CDK)/cyclin D genes[1063] and loss of the cell cycle inhibitor p27Kip1.[1063] Inhibition of *CDK6* has been shown to reduce cell proliferation in medulloblastoma cell lines,[1059] and small molecule CDK inhibitors may have a potential therapeutic role in this disease. In addition strategies that target G2-M regulators have also been investigated in model systems of SHH medulloblastoma using Aurora and Polo-like kinase inhibitors.[1064,1065]

No specific single environmental factor has been demonstrated to be associated with the development of medulloblastoma, although the nonrandom occurrence of disease detected in children born in the fall raises the possibility that some environmental or infectious pathogen may be implicated.[47] The association between the development of medulloblastoma and viruses remains controversial. Multiple reports implicating polyomaviruses (including JC[1066] and SV40[1067,1068] virus in animal models) as a causative agent in medulloblastoma have been published. Human medulloblastoma samples have been found to possess JC and SV40 viral sequences.[918,1069,1070] However independent confirmation of JC and SV40 sequences in human tumors could not be duplicated.[51,920] Prematurity was identified as a significant risk factor in one study.[1071] Whites are affected 42% more often than blacks.[1072] In contrast several genetic syndromes (e.g., nevoid basal cell carcinoma syndrome or Gorlin syndrome, Turcot syndrome, and Li-Fraumeni syndrome) are associated with a significantly increased risk for the development of medulloblastoma.[1040-1042] Gorlin syndrome, although rare, is an important factor that must be considered in the treatment of these patients, because they are at high risk for the development of nevoid basal cell carcinoma, especially within the involved radiation fields. Children of African-American descent require especially careful skin evaluation for the early detection of these lesions.[1043]

Clinical Presentation

Many of the clinical manifestations of medulloblastoma are similar to those of other tumors that arise in the fourth ventricular region and are related to obstructive hydrocephalus. Headache, nausea, and vomiting, characteristically in the morning, are the most common initial symptoms and usually precede diagnosis by 4 to 8 weeks, although longer intervals are not uncommon, especially if the headaches and vomiting caused by obstructive hydrocephalus are intermittent.[1073] Interestingly it appears that the duration of presenting symptoms may correlate inversely with disease state at the time of presentation; patients with low-stage disease were shown in one series to have a longer median duration of symptoms than were patients with high-stage disease.[1074-1076] Personality changes (irritability) are an early feature but may be difficult to recognize as a sign of a brain tumor. Other features that can lead to diagnosis include lethargy, diplopia, head tilt, and truncal ataxia. The common signs found on physical examination are papilledema, ataxia, dysmetria, and cranial nerve involvement. Abducens nerve (cranial nerve VI) palsy as a result of raised ICP may cause diplopia and head tilt. Torticollis can be a sign of cerebellar tonsil herniation. Less commonly, intratumoral hemorrhage may lead to acute onset of confusion, headache, and loss of consciousness. The presentation in infants may include an abnormal rate of increase in head circumference. Other presenting symptoms can include loss of previously achieved milestones and failure to thrive. With regard to metastatic disease, at the time of diagnosis, although lumbar CSF analysis or MRI-visualized leptomeningeal metastases occur in 20% to 30% of children with medulloblastoma,[1077,1078] clinical manifestations of metastases are uncommon. Back pain and radicular pain may indicate the rare complication of spinal canal dissemination. Attention to the clinical manifestation of posterior fossa tumors is important because the presenting signs mimic many other common ailments in children, including viral infections and stress. Because tumors have a significant propensity to metastasize early, careful evaluation of associated neurologic signs (e.g., papilledema and diplopia) can help identify children in need of referral for imaging sooner.

Imaging and Histology

Most medulloblastomas arise in the cerebellar vermis and extend into the fourth ventricle, resulting in obstructive hydrocephalus. The remainder are localized to the cerebellar hemisphere, especially in older patients and those with the desmoplastic subtype.[1079] The signs and symptoms of obstructive hydrocephalus, such as severe headache, morning vomiting, long tract signs, and papilledema, can be a medical emergency and require immediate evaluation. CT scanning of the head or rapid sequence MRI is the immediate choice of imaging to rule out an obstructive mass. This rapid study provides good differentiation between the fluid cavities and brain and will be sufficient to allow the neurosurgeon to determine whether immediate action is required. Often CSF diversion can be avoided if tumor resection is immediately possible. When required third ventriculostomy is preferred to VP shunts. All patients will require high-quality MRI scans of the brain and spine, including the entire thecal sac at the base of the spine. Lumbar puncture for CSF cytologic examination in newly diagnosed patients should be deferred if any possibility of elevated ICP exists; lumbar puncture is usually better performed after the tumor has been resected and CSF flow has been restored.

The MRI findings may differentiate between medulloblastoma types,[1080] as well as from other cerebellar tumors, such as ependymoma and pilocytic astrocytoma, and may be helpful before surgical resection.[1081] However atypical appearances of these three tumors can also occur, and thus a diagnosis based solely on imaging characteristics currently is not feasible. The appearance of a vermian location, hypercellularity (often demonstrated as dark areas of tumor on T2-weighted imaging), and intratumoral hemorrhage is more compatible with a cellular medulloblastoma or an ependymoma. A tumor that fills the fourth ventricle and extends through the foramina of Luschka and Magendie is more likely to be an ependymoma, although one with a large cyst and mural nodule (with a small area of solid tumor) is more consistent with a PA. PAs typically arise in the cerebellar hemisphere and are imaged on T2-weighted MRI as areas of homogeneous high-signal intensity, with the fluid collections defining the less intense tissue components of the tumor.

Medulloblastoma has T1-weighted MRI signal characteristics more similar to those of gray matter, reflecting hypercellularity and typically resulting in a relatively homogeneous image. On T2-weighted images these tumors can appear to be hyperintense or, more frequently, they can display mixed signal characteristics indicative of small intratumoral cysts, calcification, or small areas of hemorrhage.[1082,1083] The background signal intensity of medulloblastoma on T2-weighted images is characteristically lower than in other tumor types, indicating a dense packing of cells and a high nuclear-to-cytoplasmic ratio. Because medulloblastoma typically arises in the roof of the fourth ventricle, a cleft of CSF beneath the tumor in the fourth ventricle helps distinguish this tumor from ependymoma. Tumors are typically enhanced with administration of gadolinium (Fig. 57-36). The presence of characteristic gyriform morphology and a well-circumscribed appearance on an MRI scan suggest the presence of the nodular subtype of medulloblastoma.[1084] Functional imaging with PET and SPECT are helpful for the improved diagnosis of medulloblastoma. A number

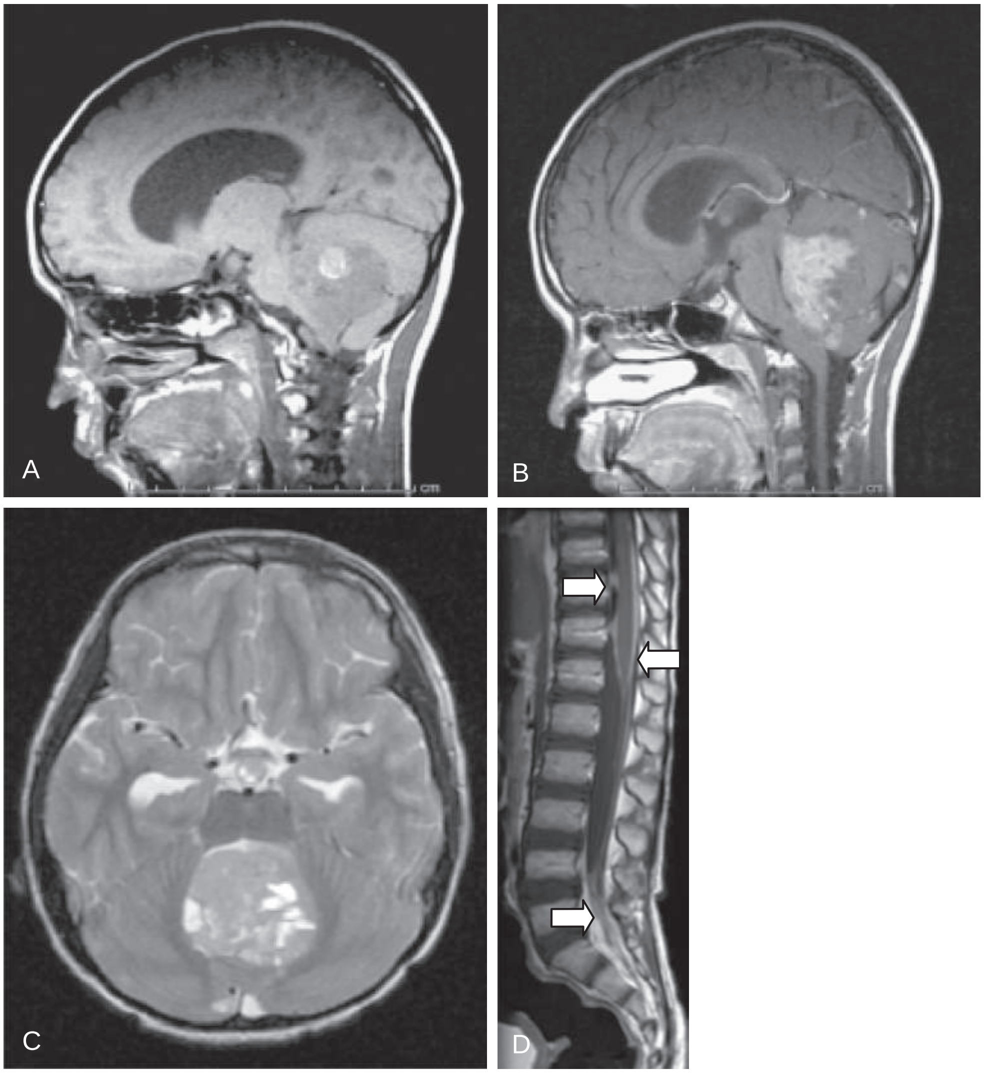

Figure 57-36 Medulloblastoma. **A,** A sagittal T1-weighted image without use of contrast material. An area of hemorrhage is present. **B,** A sagittal T1-weighted image with contrast of a medulloblastoma. **C,** An axial T2-weighted image. The areas of low T2 signal suggest a cellular lesion. **D,** A saggital T1-weighted image with use of contrast material. The lumbar-sacral-enhancing nodules *(arrows on left)* and leptomeningeal coating *(arrow on right)* represent metastatic disease.

of agents such as oxidronate (OctreoScan) can directly assess for the presence and activity of certain cellular constituents, which helps differentiate recurrent disease from scarring.[1085]

Histopathologic analysis of resected tumor specimens is essential for diagnosis and treatment planning. It should be noted that the histopathologic classification and nomenclature of medulloblastoma, within the broader context of embryonal tumors, has long been controversial and even today shows signs of continued evolution.[1086] The most current WHO classification schema considers medulloblastoma to be an independent entity in the group of embryonal tumors,[1040] separate from sPNET (now called CNS PNET in the revised WHO classification[207]). Within medulloblastoma, it is also clear that these tumors are not a homogeneous entity from a histopathologic viewpoint. At present five main histologic subtypes are recognized—classic, desmoplastic-nodular, anaplastic, large cell, and medulloblastoma with extensive nodularity. In addition any of these five variants of medulloblastoma can have areas of myogenic or melanotic differentiation.[1087] Classic (nondesmoplastic) medulloblastoma is the most common subtype, with approximately two thirds of tumors being classified as such, followed by the desmoplastic subtype (25%). This distribution is not uniform and is influenced by factors such as age and the presence of Gorlin syndrome. Individual tumors are often heterogeneous with regard to the extent of desmoplasia.[1088,1089]

Classic medulloblastoma consists of uniform sheets of densely packed small round blue cells with round to oval hyperchromatic nuclei and scant cytoplasm[1040] (Fig. 57-37). Desmoplastic medulloblastoma is characterized by so-called pale islands, or reticulin-free nodules, that are surrounded by reticulin-producing proliferating cells. Desmoplastic medulloblastoma is linked to the

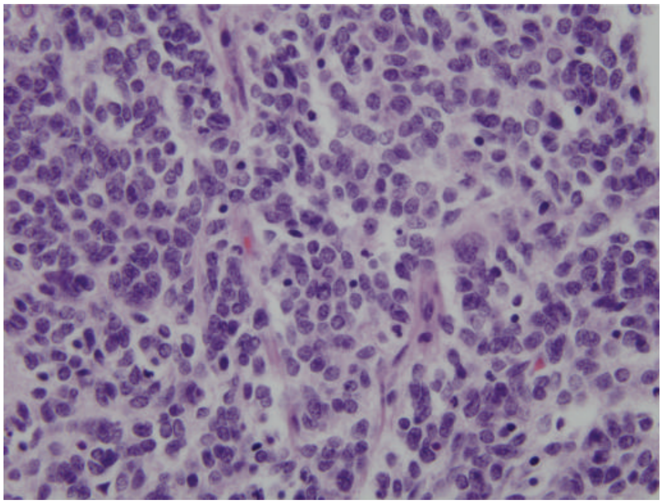

Figure 57-37 Nonanaplastic medulloblastoma. Lesions consist of sheets of homogeneous-appearing undifferentiated tumor cells (hematoxylin and eosin; ×600).

nevoid basal cell carcinoma (Gorlin) syndrome, caused by mutations in the *PTCH1* gene and leading to dysregulation of SHH signaling. Only recently have the large-cell and anaplastic variants of medulloblastoma been identified. These variants are defined by light microscopic findings of prominent nuclei with a high degree of pleomorphism, high mitotic indices, increased cytoplasm, and a higher rate of necrosis than in other variants.[1090] These tumors are uniformly positive for synaptophysin and are generally positive for chromogranin. Anaplastic and large-cell medulloblastomas tend to have higher rates of metastasis at presentation, loss of chromosome 17p13.3, and *MYC* amplification, and both have relatively poor prognoses.[1091] These two variants can occur together and contain cells with markedly increased nuclear atypia and/or large nuclei with prominent nucleoli (Fig. 57-38).[1091-1093]

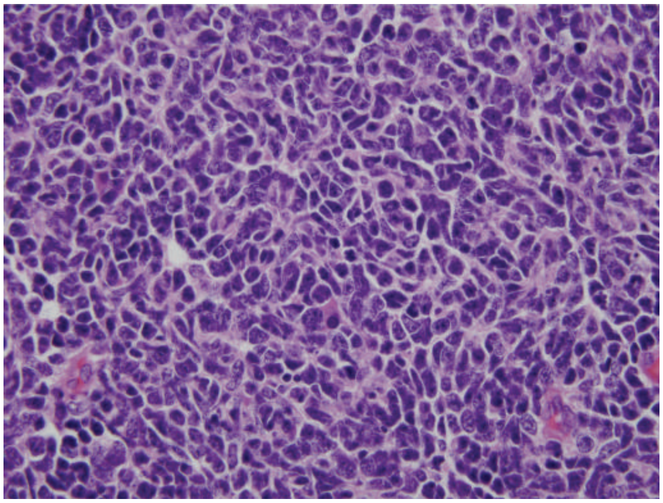

Figure 57-38 Anaplastic medulloblastoma. Tumor cells are pleomorphic with atypia, increased nuclear size, nuclear molding, and cell-cell wrapping (hematoxylin and eosin; ×600).

Wnt-positive medulloblastomas are usually classic histology. SHH group tumors include classic histology tumors, and nodular desmoplastic medulloblastomas most commonly belong in this group. Group 3 and 4 tumors have higher rates of MYC or MYCN amplification and isochrome 17q.

One of the cardinal features of medulloblastoma is its tendency to differentiate along one or more pathways, most commonly neuronal, astrocytic, and ependymal lineages. Synaptophysin expression, a characteristic feature of medulloblastomas, indicates neuronal lineage.[1094] The expression of certain intermediate filament proteins, such as nestin, vimentin, and GFAP, can also be seen in medulloblastomas.[1095] Medulloblastoma is divided into five variants:

1. Nondesmoplastic (classic) medulloblastoma may be strongly immunoreactive for vimentin. Although GFAP expression is typically restricted to developing and mature astrocytes, most classic medulloblastomas contain GFAP-positive cells, which could indicate astrocytic differentiation.[1040]
2. Nodular-desmoplastic medulloblastoma has been shown to have an increased degree of reticulin staining,[1096] with reticulin-free areas that can be identified throughout the lesion or only focally. The nodules represent regions of neuronal maturation.[1040] This rare subtype makes up only 5% of medulloblastomas but accounts for 57% of medulloblastomas in infants[1089] and accounts for the particularly good outcome in this age group.[1097]
3. Anaplastic medulloblastoma has marked nuclear pleomorphism, a high mitotic rate, apoptotic cells, and significant atypia. If only small areas of anaplasia are identified, the tumor does not meet the diagnostic criteria for the anaplastic subtype. Experienced pediatric neuropathologists are therefore required for difficult cases. Anaplasia may be related to abnormalities in *c-Myc*.[1098,1099]
4. Large-cell medulloblastoma is a rare subtype with abundant mitoses and apoptotic bodies.[1093] The name derives from the large nuclei that are present.[1100] It often contains areas of anaplasia, possibly related to specific aberrant signaling pathways,[1101] and thus many refer to these two variants as large-cell/anaplastic medulloblastoma.
5. Medulloblastoma with extensive nodularity, previously called cerebellar neuroblastoma, is typically identified in infants and has very large reticulin-free zones in comparison with desmoplastic medulloblastoma.[1102]

Medulloblastoma with myogenic differentiation refers to the presence of rhabdomyoblastic elements[1103,1104] and can be seen with any of the five variants of medulloblastoma discussed earlier (classic, desmoplastic, anaplastic, large cell, and medulloblastoma with extensive nodularity).[1105] The historic term for medulloblastoma with rhabdomyoblastic elements was *medullomyoblastoma*,[1106] although this latter term is no longer recommended. Similarly medulloblastoma with melanotic differentiation refers to the presence of melanin and does not represent an additional unique variant of the disease.[1107,1108] Rather

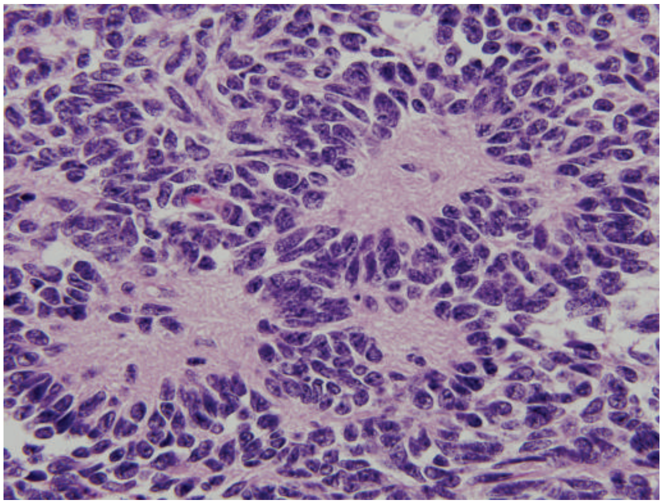

Figure 57-39 Homer-Wright rosettes (hematoxylin and eosin; ×600).

melanotic differentiation can occur with the other recognized variants. This tumor type was previously called “melanocytic medulloblastoma.”

Mitoses and other markers of proliferation such as the MIB1 labeling index are common features of these tumors and do not alter the prognosis.[1109] A mitotic index of 0.5% to 2%, an MIB1 index higher than 20%, and the presence of Homer-Wright rosettes, that is, cells arranged around a central lumen or hub (Fig. 57-39),[1110] are often associated with increased atypia and mitotic activity and are seen in approximately one third of cases. Pseudorosettes—that is, cells arranged around a central clearing with a vessel in the middle—are a common feature of medulloblastoma but are variable; they are not prognostic. Vascular proliferation and necrosis are uncommon.

Determining the cellular origin of medulloblastoma may be a key to the proper stratification and management of these tumors.[1111] As noted earlier current evidence indicates that patients with the desmoplastic variant, which is thought to originate from cerebellar granule cell precursors, have a better prognosis than patients with the classic or large-cell/anaplastic variants.[1112] Similarly patients with the large-cell and anaplastic variants appear to have a worse prognosis, and our developing understanding of the unique pathways involved will likely allow better classification and therapy in future studies.

Historic Perspective

Like most other treatments, the development of our current approaches to multimodality therapy for medulloblastoma is the result of a series of overlapping clinical trials. It is beyond the scope of this chapter to give a detailed recounting of the progression to our current understanding of medulloblastoma therapy. Rather a few key events will provide some idea of why we do what we currently do and indicate some of the weaknesses on which our current assumptions are based.

For decades after the first description of medulloblastoma was published by Bail and Cushing in 1925, the prognosis for this highly malignant tumor was extremely poor. Cushing’s development of neurosurgical techniques to remove tumors of the fourth ventricle, coupled with a dramatic decline in surgical morbidity, was critical to the development of effective therapies.[1113] With the introduction of radiation therapy and further refinements in neurosurgical techniques between 1950 and 1970, significant improvements in survivorship were achieved. For the first time patients with standard-risk disease had a 5-year EFS approaching 50%, whereas those with high-risk disease were estimated to have a 5-year EFS of approximately 20%. It was demonstrated that radiation therapy could cure a significant percentage of patients, thus securing its place in the standard approach for these tumors. The craniospinal dose for all patients at that time was 3600 cGy (craniospinal irradiation; CSI), with a posterior fossa boost to an approximate total of 5400 cGy. The promise of EFS was tempered, however, by the poor functioning levels seen in surviving patients.

Based on these outcomes and late effects, treatments were modified on the basis of risk categories. For high-risk patients, chemotherapy was added to full-dose 3600 cGy craniospinal radiotherapy, and the resulting improvement in outcome was easily measured, with 3- and 5-year EFS rates of approximately 60%. By contrast the approach to patients with standard-risk disease was to reduce the dose of craniospinal radiotherapy from 3600 to 2400 cGy to spare neurocognitive function, while maintaining the boost to the posterior fossa of 5400 cGy. A national clinical trial in the United States was undertaken, but as the study progressed, there was concern that the number of recurrences in the 2400-cGy treatment arm was greater than that for the 3600-cGy treatment arm. Consequently the study was terminated early. With further follow-up, however, the survival curves for the two treatment arms began to approach one another, such that no statistical difference in outcome was identified, and the early termination of the trial hindered the determination of the statistical conclusion. The results from these treatment studies suggest that chemotherapy in combination with craniospinal radiation therapy could positively affect outcome. The strategy for standard-risk disease thus became the lower-dose radiation therapy of 2400 cGy CSI with the addition of chemotherapy. In follow-up studies of these combinations, PFS of 86% and 79% at 3 and 5 years, respectively, was achieved for standard-risk patients.[1114] The two approaches for high- and standard-risk disease have now become the basis of most North American treatment trials.

Current Treatment

Management. A high level of clinical suspicion is critical for an early diagnosis of medulloblastoma. Neuroimaging is usually the first step, with CT or MRI scanning of the brain frequently performed in the acute setting. MRI of the brain and spine should be performed as soon as is feasible. In children in whom the diagnosis of medulloblastoma is suspected, an MRI of the spine should be obtained because the incidence of CSF dissemination at diagnosis ranges between 20% and 30%.[1077] Corticosteroids are usually used to control increased ICP. A lumbar

Box 57-3 Modified Chang Staging System in Medulloblastoma

Stage M0: No evidence of subarachnoid or hematogenous metastases
Stage M1: Microscopic tumor cells found in the cerebrospinal fluid
Stage M2: Gross nodular metastatic seeding in the subarachnoid space or ventricular system distant from the primary site of disease
Stage M3a: Gross nodular seeding in the spine subarachnoid space without evidence of intracranial seeding
Stage M3b: Gross nodular seeding in the spinal subarachnoid space, as well as intracranial seeding
Stage M4: Extraneural metastases

puncture should be deferred until after intracranial hypertension has been relieved by surgery. Because extraneural spread of medulloblastoma is possible, bone marrow aspiration and biopsy should be considered for the complete evaluation of patients with medulloblastoma,[1115] although these procedures are no longer required for participation in cooperative clinical trials. Although the exact incidence of extraneural metastases is evolving, current estimates suggest that such metastases occur in fewer than 5% of patients. Because patients will require general anesthesia for insertion of a central line for the administration of chemotherapy, bone marrow samples and a baseline lumbar sample can be easily obtained with minimal trauma to the child or family. CSF obtained from the ventricular or cisternal fluid is not considered an adequate sample upon which to base staging decisions; thus lumbar sampling is recommended for all patients in whom such sampling is not contraindicated pretherapy.[1116]

At present risk stratification and treatment assignment are based primarily on clinical factors, although this will change with greater reliance on molecular profiling.[1111] Recently histology has been added in the risk assignment of medulloblastoma. The modified Chang criteria[1117] are based on the extent of tumor and the degree of metastasis (Box 57-3). After completion of the staging evaluation (i.e., MRI of the brain and spine, CSF cytology, and bone marrow studies), histology, and degree of resection, patients are stratified to the standard-risk, high-risk, or infant-risk groups. The major determinants of clinical risk categorization are age at diagnosis (younger than 3 years vs. 3 years or older),[1072] metastasis or M stage (M0 versus higher than M0), volume of residual postoperative disease (less than 1.5 cm^3 residual disease vs. greater than 1.5 cm^3 residual disease),[1118] and histology (anaplastic–large cell versus other).[1119] Although these criteria are widely used in the staging of medulloblastoma, the role of complete resection versus less than 1.5 cm^3 residual disease, the age of the patient, and the significance of anaplastic histology are still in question, and entry criteria may vary from those indicated.

Histology has just recently begun to be considered in risk stratification. Most prior published series of patients with medulloblastoma had incorporated patients with anaplasia–large cell medulloblastoma into standard-risk treatment if they met the other criteria for standard risk. The recognition that infants with the desmoplastic variant may do better than infants with the nondesmoplastic variant may provide an opportunity to deescalate therapy in this particular group. Newer protocols are now incorporating these variables. Tolerability of therapy has also recently come to light as more adult programs begin to treat older patients with regimens similar to those that have produced significant advances in pediatric patients. Older patients, including adolescents, do not tolerate therapy as well as younger children or infants, which provides an argument for dose modifications in this group.[1120]

ATRTs of the posterior fossa often resemble medulloblastomas. In prior cooperative group studies, up to 30% of patients with ATRTs were misclassified as having a medulloblastoma. With the use of INI1 immunohistochemical analysis of the tumor sample, rapid differentiation of these two histologies is required because the treatment for ATRT differs from that of medulloblastoma in most large cooperative groups.

Treatment

Surgery. Treatment of medulloblastoma, as well as of other embryonal tumors, is multimodal, consisting of surgery, radiation therapy, and chemotherapy, with current therapies guided primarily by age and stage at diagnosis. The initial goal of surgery is to control raised ICP, if present. Once the safety of the patient is ensured, consideration of tumor resection becomes the next objective. If metastatic tumor is already known to be present, immediate aggressive surgery within the posterior fossa will not reduce the need for more intensive radiation therapy and chemotherapy. Rather the goal of surgical resection should be to achieve maximal tumor volume reduction without significant damage to adjacent areas. In balance it is more important for the neurosurgeon to preserve function rather than to maximize resection. Given the excellent prognosis for patients with medulloblastoma, even with metastatic disease, damage to the brain in a high percentage of patients means a high percentage of survivors with permanent neurologic damage.[1121] A third ventriculostomy (preferred) or VP shunt should be deferred prior to initial surgery but may be necessary if sufficient resection to reopen CSF flow is not achieved.

A number of surgical approaches are possible to optimize resection of posterior fossa and cerebellar tumors. Most fourth ventricular lesions will require interruption of the cerebellar vermis, although resections through the vermis using horizontal or vertical incisions have both been associated with posterior fossa syndrome. Even attempts at going under rather than through the vermis have been associated with this morbidity. The brainstem must also be protected while removing medulloblastomas within the fourth ventricle. Because these tumors can invade the brainstem, intraoperative decisions regarding the aggressiveness of resection must be made. Similarly resection must protect the nearby cranial nerves. New neurosurgical techniques are now available that allow for greater guidance in resection and in physiologic monitoring so that damage to important anatomic structures can be avoided. The use of intraoperative MRIs has improved the neurosurgeon's ability to ensure adequate yet safe resection.[1122,1123]

Potential complications of surgery in this location, regardless of tumor type, include cerebellar mutism (also called posterior fossa syndrome)[1124] and aseptic meningitis. The posterior fossa syndrome, or cerebellar mutism, occurs in approximately 10% to 20% of cases, although it is being reported in an increasing percentage of patients as more attention is given to the quality of survivorship. The characteristics are reduced speech output or mutism, personality changes, hypotonia, ataxia, and reduced oral intake. Symptoms typically appear 1 or 2 days after surgery, may range between mild to severe, and may last from days to months with varying degrees of recovery.[1125]

Radiation Therapy. Radiotherapy has become an important modality in the long-term outcome of patients with medulloblastoma. During the past 20 years, a great deal of effort has been focused on the reduction of craniospinal doses as a result of significant long-term neurocognitive damage, as well as secondary tumor risk and stroke. Radiotherapy is currently risk adapted, and additional modifications to dose and volume continue.[1126] For standard-risk patients, 5400 to 5580 cGy are administered to the tumor bed. This volume continues to evolve, from inclusion of the entire posterior fossa, even when the tumor is small and focal, to more limited fields that encompass only structures in contact with the tumor and a small margin. Previous standard-radiation therapy included craniospinal doses of 3600 cGy. A national clinical trial evaluating standard-risk patients with a craniospinal dose of 2400 cGy demonstrated equal efficacy to historic reports, although this study did not randomize between the two CSI doses; 2400 cGy has been accepted as the new standard for this patient population.[1127] An important conclusion from this study was the poor outcome of patients with high-risk disease treated with lower dose craniospinal radiotherapy, reinforcing the importance of proper staging and radiation planning for all patients. A COG clinical trial for patients with standard-risk medulloblastoma is ongoing; this trial randomizes children younger than 8 years to receive either 2400 or 1800 cGy CSI and randomizes all children to receive either a full posterior fossa boost or an involved field boost. This trial will help define the dose and field of radiation therapy required for this population.

For patients with high-risk disease that is caused either by the presence of bulk unresectable disease within the posterior fossa or by the presence of metastatic disease, treatment remains 3600 cGy to the craniospinal axis and a focal dose to the involved posterior fossa of 5400 to 5940 cGy. Although this dose to the entire brain and spine increases the morbidity of therapy, it is an important component of effective treatment, as noted in the previously described randomized trial for standard-risk patients who, on central review, were found to have high-risk disease. A number of cooperative group trials are currently evaluating drugs combined with radiotherapy (radiation sensitizers) to augment the activity of radiation therapy in children with high-risk medulloblastoma.

Infants with medulloblastoma (and other CNS PNETs) continue to pose the greatest therapeutic dilemma. Even in the presence of standard-risk disease, the contraindication for craniospinal radiation therapy limits the treatment options for this group and their long-term prognoses. The omission of craniospinal radiotherapy has required the acceptance of a lower cure rate but a much higher functionality for those who do survive.[1128] The use of treatment protocols that rely only on surgery and chemotherapy demonstrate a relatively poor outcome for this group in all but a select group (e.g., those with nodular desmoplastic medulloblastoma[1097]), again supporting the importance of radiation therapy for the treatment of medulloblastoma. Because many relapses occur locally, many centers have tested focal radiotherapy to the posterior fossa, forgoing the craniospinal component of therapy.[727,1129] This approach attempts to improve local control to the cerebellum, which is thought to be an area less important for neurocognitive development than the cortex. However in a recently completed COG study (trial P9934) that pilot-tested focal radiation therapy to the posterior fossa, it was noted that many recurrences occurred in sites that were outside the radiation field.[1130]

Hyperfractionation of radiotherapy—that is, twice-daily dosing of radiation therapy—has not demonstrated significant improvement in outcome,[1131] although one pilot trial has reported such an improvement.[1132] By contrast a significant delay in the completion of the radiation therapy appears to negatively affect survival.[1133,1134] Proton radiation therapy is increasingly used for children with medulloblastoma. Although its efficacy is similar to that of conventional photon radiotherapy, the absence of an exit dose can reduce radiation exposure to areas uninvolved with tumor. The limited number of proton beam facilities, however, restricts the wider application of this technique. Other more readily available radiation therapy options include IMRT. In particular, tighter margins within the posterior fossa and better 3D conformal planning with IMRT-based techniques can help avoid the auditory apparatus and thus long-term hearing impairment.

The potential use of chemotherapy before delivery of craniospinal radiotherapy has been pilot tested[1134,1135] and has been shown to be associated with inferior EFS and OS.[1135] A national clinical trial with randomization between standard-dose radiation therapy (3600 cGy) and reduced-dose (2340 cGy) CSI in patients with low-stage (M0) disease who were not receiving adjuvant chemotherapy resulted in an increased rate of recurrence outside of the posterior fossa.[1136] However it should be noted that no statistically significant difference was found between the two groups at 6 to 7 years after treatment.

Chemotherapy. The chemosensitivity of medulloblastoma and the benefits of adjuvant chemotherapy in treatment regimens have been demonstrated by a number of studies.[1137-1139] During the past 20 years studies have shown a nearly 20% to 30% improvement in EFS and OS by using chemotherapy during and/or after radiation therapy. Most regimens have included vincristine, cisplatin, etoposide, and an alkylator (cyclophosphamide or CCNU). Although platinum-containing regimens are considered important components of the treatment of medulloblastoma, the ultimate cumulative dose required for optimal activity while minimizing renal and audiologic impairment remains to be defined.[1140] Chemotherapy is currently a standard adjuvant therapy in children. Similarly infant studies with chemotherapy alone have shown the importance of chemotherapy as an effective strategy for patients with medulloblastoma. For most of these infant studies the removal of CSI has had a negative effect on survival, although a significant proportion of survivors can achieve cure.[1097] Recently methotrexate has been added into multiagent chemotherapy for medulloblastomas and other PNETs with excellent tumor response. In one phase II study consisting of four cycles of carboplatin, etoposide, and methotrexate, CR and partial response (PR) rates of 71% and 81% were achieved in patients younger than 3 years and older than 3 years, respectively.[1141]

The concurrent use of craniospinal radiation therapy and multiagent chemotherapy for medulloblastoma can result in significant impairment of nutrition during therapy for these patients.[1142] Patients who present with persistent vomiting likely initiate therapy in a negative nutritional state. A large proportion of patients will require nutritional support through a nasogastric tube or gastric tube. Constant surveillance of nutrition is important because this issue is often dismissed early and is only recognized when severe weight loss has occurred.

Impairment in hearing function continues to be a major concern in survivors of medulloblastoma. The overlap between posterior fossa radiation therapy and the use of ototoxic drugs such as cisplatin has resulted in significant toxicity for this patient population. Conformal radiation planning with tighter margins, the use of IMRT, and the use of proton beam radiation therapy can all significantly reduce the damage to the auditory apparatus. Recent medulloblastoma treatment protocols have also reduced the cumulative doses of cisplatin. The use of amifostine continues to be controversial, with some studies showing efficacy at protecting hearing and other studies not showing efficacy.[1143,1144] Concerns about protecting the tumor from the chemotherapy remain, and only a well-controlled randomized trial will resolve this question.

STANDARD-RISK DISEASE. The components of combination chemotherapy used during and after CSI continue to evolve. A large national clinical trial involving 2400 cGy CSI with a boost to 5400 cGy to the posterior fossa and randomizing between vincristine, cisplatin, and either CCNU or cyclophosphamide (COG trial A9961) demonstrated that both treatment arms have approximately similar activity. The OS for both groups was approximately 90%, with only a slight difference in EFS between the two chemotherapy regimens (85% for patients who received CCNU vs. 83% for those who received cyclophosphamide).[1035] The emphasis in the ongoing clinical study has been to reduce the CSI dose further in a randomized fashion for children younger than 8 years (i.e., those most at risk for the severe neurocognitive impact of radiation therapy) while combining the CCNU and cyclophosphamide agents to reduce the total exposure to each. The safety of 1800 cGy craniospinal radiotherapy was pilot tested in 10 patients with standard-risk medulloblastoma, and in this small cohort, the survival outcome appeared equally effective to that for 2400-cGy therapy and with less neurocognitive impairment.[1145] Radiation- and chemotherapy-adapted approaches are especially important for younger patients given the significant dose-dependent neurocognitive impact of radiation therapy in this population.[1146] A large European cooperative group trial of standard versus hyperfractionated radiation therapy showed no significant outcome difference between these two approaches and has led to the adoption of daily fractionated radiation therapy as the standard for children with medulloblastoma.[1147]

HIGH-RISK DISEASE. Treatment strategies for this population have focused on the addition of new agents, including radiosensitizing agents and novel biologic inhibitors, targeting pathways important for these tumors in the hopes of further improving outcome.[1148] It should be noted that the sequence of treatment has been shown to be of major importance, with the best survival rates being achieved if radiation therapy is not delayed,[1149] although a recent study (Pediatric Oncology Group [POG] trial 9031) reported no difference in EFS in children who received radiation therapy first compared with those who received chemotherapy first in the presence of high-risk disease.[1150]

INFANT CHEMOTHERAPY. Patients younger than 3 years at diagnosis are considered to have higher risk disease at all stages and degrees of resection, related to the limitations of delivering craniospinal radiation therapy to this group. There is also an increased frequency of leptomeningeal dissemination at the time of diagnosis in young children (27% to 43%) versus older children (20% to 25%) with similar histologic diagnoses.[1151] After surgical resection, infants with medulloblastoma are often treated with chemotherapy alone or chemotherapy with involved field radiotherapy in an effort to reduce the high incidence of developmental and neuropsychological sequelae caused by craniospinal irradiation. It is clear that the risks of craniospinal radiation therapy in infants and young children are significantly greater than the benefits of therapeutic response in terms of neurocognitive development.[1152] Currently several chemotherapy regimens designed to delay or eliminate the need for whole-neuraxis radiation therapy are being investigated, including regimens using intraventricular chemotherapy.[1097] In addition high-dose regimens have been found to be feasible and effective for young children with disseminated medulloblastoma.[1153] The relative merits and risks of conventional multiagent chemotherapy compared with high-dose submyeloablative or myeloablative chemotherapy with stem cell support is under investigation.[348,728] An important finding in a recent study for infants with medulloblastoma is the particularly good outcome in infants with the desmoplastic variant, even without radiation therapy,[1097] suggesting that nodular desmoplastic medulloblastoma may represent a low-risk group that does well with less intensive therapy. These tumors are also commonly of the SHH subtype, and it is possible that integration of novel agents targeting this pathway may have a potential therapeutic role. Ongoing studies to confirm this finding are under way but raise the possibility that desmoplastic medulloblastoma may form a new low-risk group.

Prognosis

Patients with standard-risk medulloblastoma have a 5-year survival of approximately 75% to 90%, whereas 5-year survival in patients with high-risk medulloblastoma remains at between 40% and 60%.[1035,1129,1154] Infants with metastatic disease or bulky unresectable disease continue to do poorly, with a 3-year EFS of less than 40%, except in those with the desmoplastic variant. Patients with other rarer subtypes of medulloblastoma, including the large-cell, anaplastic, large-cell/anaplastic, and melanotic variants, continue to do poorly, even with maximal upfront therapy.[1092] Various histologic features associated with improved prognosis have been evaluated, of which nodular appearance appears to be most important.[1088] The presence of dissemination is the single most important factor that correlates with poor outcome.[1155] Lifelong repeated surveillance imaging is required in anticipation of radiation-induced secondary malignancies, such as a meningioma or HGG.[1156] Radiotherapy, particularly in young children, can also cause significant adverse late effects in neurocognitive development, growth, and endocrine function.[1146,1157,1158] Gene profiling of medulloblastoma was an accurate method of predicting outcome, even when evaluated in the context of clinical data. Variables such as age, M stage, and degree of resection did not significantly improve the predictive ability of this independent data set.[1159]

Relapsed Medulloblastoma

Relapses occur as failures at the primary site, at distant sites, or both. MRI is not always accurate when lesions are identified, and pathologic correlation is important in avoiding toxic therapy for patients whose disease has not actually progressed.[1160] Tumors occurring at the primary site within the posterior fossa may have a greater chance for salvage. Patients treated without upfront radiation therapy (infants, in particular) also have a good salvage rate.[1161] Retrieval therapy is rarely curative, but long-term disease control may occur with high-dose myeloablative chemotherapy in a small proportion of patients.[348,1162] Past experience has shown that patients entering high-dose chemotherapy without chemotherapy-responsive disease, or with residual disease at the time of transplantation, are unlikely to benefit from this approach. A large number of new intrathecal,[1163] biologic, and antiangiogenic agents are being evaluated for patients with relapsed medulloblastoma.[335,1164-1166] Although these agents are likely to be more effective when moved up early in the upfront setting, additional testing is still required. Most exciting are preliminary results with SHH inhibitors in this subgroup of patients.[1167] Given the distribution of mutations within the SHH pathway that differ between infants, children, and adults,[1050] as well as concerns for premature bone closure in infants and children taking these inhibitors, careful selection of patients and a balance of prognosis versus toxicity is required.

Future Directions

The recent discovery of genetic alterations defining different subtypes of medulloblastoma presents novel and exciting opportunities for the development of targeted therapies for children with medulloblastoma. Trials examining the efficacy of SHH inhibitors for those of the SHH subtype are currently under way, and novel agents that target the *MYC* transcription factor or activation pathways also are currently being investigated for MYC or MYCN amplified tumors. In addition studies that determine potential mechanisms by which medulloblastomas may acquire resistance to targeted agents are also important to provide insights into potential combination therapies.[1052,1168] Conversely treatment strategies that minimize long-term therapy-related morbidity in children with Wnt-positive medulloblastoma are also being investigated. The challenge in the coming era will be to develop strategies that incorporate risk stratification and biologically targeted agents in the upfront treatment strategy for children with medulloblastoma.

Pineocytomas and Pineoblastomas

Tumors of the pineal region are often grouped together based on their location rather than on their cellular origins and behavior. Embryonal tumors of the pineal region include pineocytoma, pineal parenchymal tumor of intermediate differentiation (PPTID), pineoblastoma, and papillary tumor of the pineal region (PTPR). Together they account for less than 5% of all pediatric CNS tumors. Other pineal-based lesions include GCTs and, less commonly, astrocytic tumors.[642] GCTs are discussed in a separate section. In a recent series of children diagnosed with pineal parenchymal tumors, the extent of surgical resection has been reported to be a significant prognosticator.[1169]

Pineocytomas

Pineocytomas arise from the pinocyte, the primary function of which appears related to photoreceptor activity and neuroendocrine function.[1170] They are classified as low-grade (grade I) neoplasms that are most common in adults and in late adolescence, although they can be observed in young children. They account for approximately 50% of the tumors of pineal origin, although pineal region neoplasms account for only 1% to 5% of all pediatric CNS tumors.[1171]

Clinical Presentation. Like other pineal region lesions, pineocytomas often present with a constellation of symptoms related to their location. This constellation includes obstructive hydrocephalus and Parinaud syndrome,[1172] a cluster of abnormalities of eye movements and papillary dysfunction characterized by paralyzed upgaze, pseudo–Argyll Robertson pupils, convergence-retraction nystagmus, eyelid retraction, and a conjugate downgaze “sunsetting” sign. Impairment of hypothalamic-pituitary, brainstem, and cerebellar function is also possible. Unlike malignant pineoblastomas, metastases are rare.

Imaging and Histology. On neuroimaging, pineocytomas are typically small focal lesions that can contain cysts and/or calcifications; they are best seen by CT. They are similar to other low-grade tumors on MRI, with strong contrast enhancement, hypointensity on T1-weighted sequences, and high signal intensity on T2-weighted sequences. Surgically these tumors tend to be well demarcated from surrounding tissue. Areas of hemorrhage are not uncommon. Microscopically pineocytomas are made up of small, round, mature-looking cells that have maintained features of pinocytes, including rosettes.[1173] These tumors demonstrate characteristic pineocytomatous rosettes that differentiate them from pineoblastomas. Mitoses are rare, as are other features of malignancy. Pineocytomas are usually strongly synaptophysin and neuron-specific enolase (NSE)–positive. If areas of pineoblastoma are identified with a pineocytoma, treatment is dictated by the most malignant element (i.e., pineoblastoma).

Management. Surgery remains the mainstay of the therapeutic approach for these lesions. Treatment directed at the hydrocephalus with third ventriculostomy may provide an opportunity to obtain a biopsy before aggressive and potentially morbid surgical resection is attempted.[1174,1175] Tumors with persistent growth can be irradiated, although the benefit of this treatment has not been clearly demonstrated. A role for chemotherapy has not been defined. Patients with incompletely resected tumors do not require therapy unless clear progression is demonstrated. Even patients with metastatic disease can remain untreated. The overall benign course observed with pineocytomas is in stark contrast to that of pineoblastomas.

Prognosis. The long-term prognosis remains excellent, even with incompletely resected disease. Conservative management should be followed with regard to attempted surgical resection and radiation therapy. Because almost all patients with pineocytomas will be long-term survivors, the need to avoid toxicity is paramount.

Pineal Parenchymal Tumor of Intermediate Differentiation

PPTIDs are tumors of the pineal region that occur at all ages, although they are frequent in middle age, and are classified as grade II or III, depending on pathologic features. They account for 20% of pineal region tumors and were formally identified in 1993. Although reports are limited, there appears to be a slight female preponderance. Genetic evaluation has demonstrated a large number of abnormalities.[1176]

Clinical Presentation. The presentation of patients with PPTID is similar to that of pineocytomas and includes obstructive hydrocephalus, as well as Parinaud syndrome,[1172] a cluster of abnormalities of eye movements and papillary dysfunction characterized by paralyzed upgaze, pseudo–Argyll Robertson pupils, convergence-retraction nystagmus, eyelid retraction, and conjugate downgaze. Impairment of hypothalamic-pituitary, brainstem, and cerebellar function is also possible. Unlike pineocytomas, metastatic disease appears to be slightly more frequent.

Imaging and Histology. Imaging characteristics are similar to those of other lesions of pineal origin. They are cellular lesions with moderate nuclear atypia and a low mitotic index.[1177] Because of sampling error, diagnosis can be difficult with limited tissue, especially tissue obtained via third ventriculostomy.

Management. Because of the increasing degree of malignant potential when compared with pineocytomas, attempts at surgical resection should be considered. Although formal pediatric studies are lacking, radiation therapy and chemotherapy should be considered. Intensity of treatment can be based on the localization of the tumor (focal, invasive, or disseminated), as well as the proportion of mitoses, necrosis, atypia, and possibly neurofilament protein expression.[1178]

Prognosis. A definite prognosis for PPTID has not been clearly established. Grade is likely to play an important part in the long-term outcome of patients, as is the size and metastatic stage of patients.[1179] Because these tumors span all age groups, large homogeneous studies are difficult to perform.

Pineoblastoma

Unlike pineocytomas, pineoblastomas are highly malignant embryonal tumors. Like other small round blue cell tumors, these lesions are considered WHO grade IV and have a high propensity to metastasize. Typically they are observed in children younger than those seen with pineocytomas, although considerable overlap exists. Pineoblastomas can be associated with the genetic form of retinoblastoma and in such cases are often referred to as trilateral retinoblastomas.[1180] In a recent study of 408 patients with retinoblastoma, pineoblastoma was detected in 1% of all patients.[1181] An association with familial adenomatous polyposis has also been reported.[1182] Little is known about the molecular classification of these tumors with respect to their genesis or their association with other neural tumors of the CNS (PNET or medulloblastoma).[1183] Defects in *TP53* have not been reported.

Clinical Presentation. Presenting symptoms of pineoblastoma are virtually identical to those for pineocytomas, although the duration of symptoms may be shorter than that seen for other tumors in this location. Pineoblastomas often present with a constellation of symptoms related to their location, including obstructive hydrocephalus in most patients,[1184] as well as Parinaud syndrome,[1172] a cluster of abnormalities of eye movements and papillary dysfunction, as previously described. Impairment of hypothalamic-pituitary, brainstem, and cerebellar function is also possible. Pineoblastomas have a much greater propensity to metastasize and thus may demonstrate symptoms beyond the pineal region.

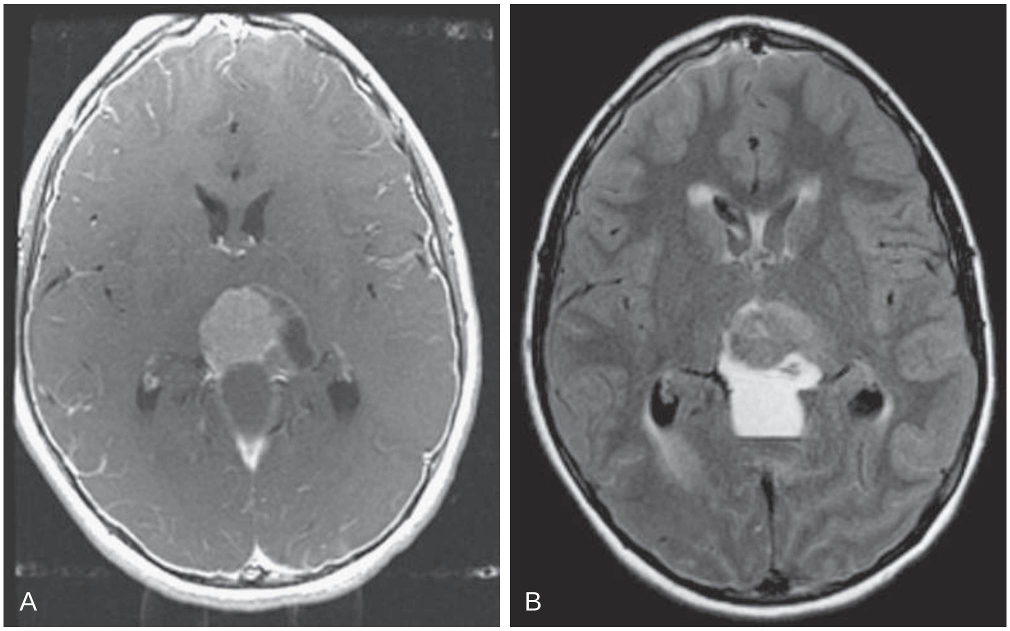

Figure 57-40 A large contrast-enhancing pineoblastoma. **A,** An axial T1-weighted image with use of contrast material. **B,** An axial fluid-attenuated inversion recovery image demonstrating a bright T2-weighted signal.

Imaging and Histology. The neuroimaging characteristics of pineoblastomas are not pathognomonic for these tumors, but they are differentiated from pineocytomas by a significant increase in the volume of the tumor and the presence of much less distinct boundaries. MRI characteristics include low signal on T1-weighted sequences and heterogeneous areas of contrast enhancement (Fig. 57-40).[1185] T2-weighted signals may be lower than in pineocytomas because of the higher nuclear-to-cytoplasmic ratio evident in these more malignant tumors.[1186] On histologic analysis these tumors are similar to other small round blue cell tumors, composed of sheets of cells but lacking the characteristic pineocytomatous rosettes of pineocytomas.[1187] Pineoblastomas are typically synaptophysin- and NSE-positive and other markers, including those for photoreceptor pathways, can be present, consistent with the developmental role of the pineal gland.[1178,1188] Homer-Wright rosettes (i.e., cells with a central zone of cytoplasm; see Fig. 57-39) and Flexner-Wintersteiner rosettes (i.e., cells with a central zone of cytoplasm and a central space; Fig. 57-41) are common, but these rosettes can be seen in a large number of other embryonal tumors. These tumors often have significant atypia, and mitotic activity can be high. When additional pathways of differentiation are present (e.g., melanin, cartilage, and muscle), the tumors are called pineal anlage tumors. Regions of pineoblastoma can also be identified as part of a pineocytoma and are treated as a pineoblastoma. Although sporadic pineoblastoma has no clear genetic association, patients with hereditary (bilateral) retinoblastoma have a significant incidence of pineoblastoma (trilateral retinoblastoma). The clinical course of retinoblastoma-associated pineoblastoma can differ from that of sporadic pineoblastoma.[1189]

Recent profiling of genomic alterations have reported copy number alterations observed in pineoblastoma. These alterations include loss of chromosome 16q and amplification of PCDHGA3, and FAM129A.[1169] In addition DNA copy number profiles have reported more similarities between sPNETs and pineoblastoma than between these tumors and the lower grade tumors of the pineal region.[1190] The development of pineoblastoma has also been reported in a young patient with a germline mutation of *DICER1*, which is also associated with the development of pleuropulmonary blastoma.[1191]

Management. The therapeutic approaches for pineoblastoma have followed those of other embryonal seeding

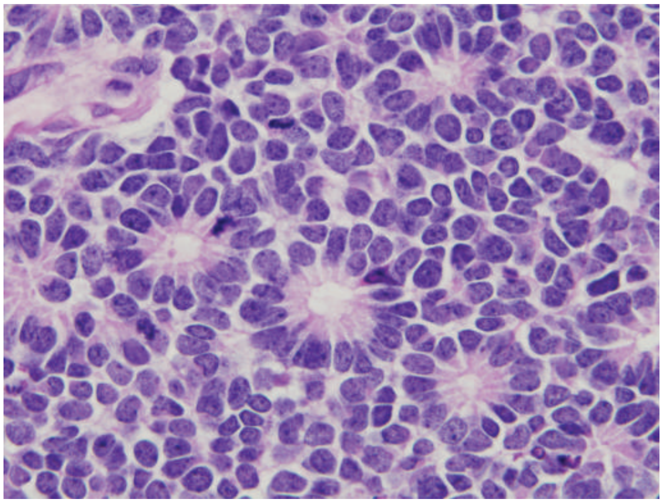

Figure 57-41 Flexner-Wintersteiner rosette in a patient with pineoblastoma (hematoxylin and eosin; ×600).

tumors of the CNS. The workup of patients with pineoblastoma includes immediate intervention for obstructive hydrocephalus and assessment of risk for herniation. A rapid CT or MRI scan, in conjunction with the examination and presenting history, will permit the neurosurgeon to assess these risks. If urgent CSF shunting is not required, then completion of the imaging baseline workup, including MRI of the brain and spine, can be performed and surgical resection can be planned. Unlike medulloblastoma, the location of pineoblastomas with adjacent vascular structures makes exploratory surgery much more risky, and referral to a highly specialized center should be considered. A third ventriculostomy or VP shunt should be deferred prior to initial surgery but may be necessary if sufficient resection with reestablishment of CSF flow is not achieved. In many patients an endoscopic biopsy of the tumor to confirm the diagnosis and concurrent third ventriculostomy can be performed with a planned open craniotomy and resection once the patient is clinically stable, the disease has been defined, and possible presurgical chemotherapy has been provided.[1174] Patients with metastatic disease at diagnosis should not undergo upfront aggressive surgery because adjuvant therapy will be required. For patients with focal disease at diagnosis based on initial MRI and documentation of negative results of a lumbar puncture, complete removal of the tumor ultimately will be an important component of long-term prognosis[1192] but should not be undertaken at any costs. Consideration for staged surgeries (second- and even third-look approaches) may be equally effective without causing permanent neurologic damage.

As with other embryonal seeding tumors of the CNS, craniospinal radiotherapy is an important modality in treating microscopic metastatic disease at presentation. Unlike medulloblastoma, pineoblastomas do not have a standard-risk category. All patients require aggressive therapy, including a craniospinal dose of 3600 cGy and a focal boost to the primary site of 5400 to 5940 cGy. Sites of metastatic disease require radiation boosts as well. Even with focal resectable disease, the prognosis for pineoblastoma falls significantly below that of medulloblastoma.[1184] In persons with disseminated disease, especially diffuse leptomeningeal spread, and infants, for whom craniospinal radiotherapy is contraindicated, the prognosis remains especially poor. The use of SRS or other similar modalities to deliver single-fraction ablative radiation therapy may provide added disease control of unresectable tumor, although these approaches are limited to small areas of disease.

Chemotherapy is an important modality for patients with pineoblastoma, and significant responses to chemotherapy have been observed, including in patients with trilateral retinoblastoma.[1193] Results from infant studies, which deferred radiotherapy, demonstrated that although chemotherapy can be effective at reducing bulk disease, few patients are long-term survivors with this modality alone.[1194] Because of the rarity of this entity most protocols have combined these patients with patients who have other embryonal tumors, including CNS PNETs and, occasionally, medulloblastoma. Specific estimates of survival from different therapies have therefore been difficult to determine. Current approaches continue to include initial craniospinal radiotherapy in combination with chemotherapy, followed by postradiation chemotherapy. Most regimens have used combinations of vincristine, cyclophosphamide, cisplatin, and etoposide.[1192] Because of the chemoresponsiveness of these tumors, pilot clinical trials with high-dose chemotherapy with stem cell transplantation have also been undertaken. The results of the German HIT2000 clinical trial, which included children with CNS PNET and pineoblastoma, recently reported 5-year EFS and OS of 24% ± 10% and 40% ± 12%, respectively, with multiagent chemotherapy and radiation therapy.[1195] Children with high-risk disease received intensified therapy.

The concurrent use of craniospinal radiation therapy and multiagent chemotherapy for pineoblastoma has resulted in a significant impairment of nutrition during therapy for these patients.[1142] A large number of patients will require nutritional support through a nasogastric tube or, more commonly, a gastric tube. Constant surveillance of nutrition is important because these issues are often dismissed early and are only recognized when severe weight loss has occurred. Patients with persistent vomiting usually initiate therapy in a negative nutritional state.

Prognosis. The outcome of patients with pineoblastoma remains poor. Patients with focal disease that can be completely resected have 3- and 5-year EFS of approximately 50%.[1184,1192] Infants, patients with incompletely resected disease by the end of therapy, or patients with metastatic disease continue to do poorly, with 3- and 5-year EFS estimates of 20% or lower. Progression tends to occur early and can occur at the primary site, throughout the CNS, or both. A recent study of recurrence patterns in children with embryonal intracranial tumors noted a high rate of relapses that involved the spine in children with pineoblastoma.[1196] Extracranial metastases are uncommon. Some patients with metastatic disease, however, are long-term survivors.[1192] After recurrence, no standard therapy has been documented to be effective, and most patients will succumb to their disease, usually within months.

Papillary Tumors of the Pineal Region

PTPRs are rare tumors of children and adults that were separated from other pineal tumors in 2003.[207,1197] As the name implies these neuroepithelial lesions possess papillary structures and are thought to be derived from specialized ependymal cells. Because of their varied appearance, they can be confused with ependymomas, choroid plexus tumors, and pineocytomas.[1198] PTPRs appear to fall into the WHO grade II or III category.

Clinical Presentation. The clinical presentation of PTPRs is similar to that of other lesions of the pineal area. Hydrocephalus is present in almost all patients, and Parinaud syndrome[1172] is common. Impairment of hypothalamic-pituitary, brainstem, and cerebellar function is also possible. Metastatic disease does not appear to be as prevalent as in pineoblastomas.

Imaging and Histology. PTPRs are well-circumscribed, often cystic lesions. They are hyperintense on T1- and T2-weighted sequences and enhance with administration of contrast material.[1199,1200] Histologically these tumors stain strongly for keratins and are usually negative or weakly positive for GFAP (in contrast to ependymomas) and synaptophysin.[1201] Most of the tumor cells show strong expression of NSE, cytokeratins (particularly CK18), S-100 protein, and vimentin. The mitotic index and MIB1 labeling index are intermediate but of unclear prognostic significance.[1202]

Management. In spite of their differentiated appearance, PTPRs are aggressive tumors that are not highly responsive to therapy.[1203] In a recent report of 31 patients, 21 of whom achieved a gross total resection, adjuvant radiation therapy alone resulted in a PFS rate of only 27%.[1204] The ability of achieve a gross total resection has been reported in some studies to be associated with improved PFS.[590,1203]

Prognosis. In spite of complete resection and focal radiation therapy, local recurrences are common,[1205] although patients with complete removal of these tumors may do better.[1206] Dissemination appears rare, and craniospinal radiation therapy therefore does not appear to be required. Patients with an elevated MIB1 labeling index or mitotic rate may tend to do worse.[1207]

Supratentorial or Central Nervous System Primitive Neuroectodermal Tumors

Supratentorial or CNS PNETs are tumors of neuroepithelial origin.[1208] The classification of CNS PNET (previously called sPNET) as separate from medulloblastoma and other embryonal tumors, which was initially proposed in 1973,[1031] has not been without controversy. Historically these tumors have been called by various names, including cerebral medulloblastoma, cerebral neuroblastoma, and cerebral ganglioneuroblastoma. Rorke[1209] (1983) proposed that these tumors are the supratentorial equivalent of medulloblastoma, another common embryonal neoplasm. Although these tumors are histologically similar to medulloblastoma, they respond poorly to medulloblastoma-specific therapy. Part of the confusion in the approach to CNS PNETs has been in the nomenclature—the overlap in the term "PNET" for tumors of the body as well. These tumors do not share the molecular pathways,[1183] metastatic sites, or treatment responses of the extracranial PNET tumors. Even within the CNS, PNETs likely represent a heterogeneous group of tumors.[1029] According to the WHO classification schema,[1210] a number of subtypes of PNET are recognized: classic PNET, PNET with neuronal differentiation (called cerebral neuroblastoma), and, if ganglion cells are present as part of the tumor, cerebral ganglioneuroblastoma. Neither of the latter two entities is related to classic neuroblastoma, which is typically identified around the adrenal gland and shares a common name as a result of historical nomenclature only. New molecular determinants associated with PNETs of the brain have been identified using molecular and proteomic analysis and will, it is hoped, be useful for better subclassification of the diseases that fall into this broad category of tumors.[306,1211-1213] Recently gene expression and genomic profiling efforts of CNS PNETs have confirmed that this disease entity is heterogenous and consists of multiple different tumor types. Copy number analyses of PNETs have in general revealed a complex karyotype with frequent copy number alterations.[1190] In addition these efforts have highlighted the inherent difficulty in classifying tumors based on histology. Molecular profiles have been found to match those of other tumor types, including ATRT (characterized by loss of INI1) and HGGs (characterized by the presence of mutations in the histone genes). Importantly, clustering of profiles have revealed that tumors have distinct profiles based on the underlying lineage of the tumor, including primitive neural, oligoneural, and mesenchymal lineages.[1214] These groups are associated with differences in clinical courses and prognosis, with tumors with an underlying primitive neural profile exhibiting the worse prognosis. Further, these profiling studies have shed light on other genes that may be involved in the pathogenesis of these brain tumors and thus may represent candidates for targeted therapies. These therapies include amplification of genes involved in cell cycle regulation.[1063]

CNS PNETs are relatively rare, occurring at a rate that is approximately 10% to 20% that of medulloblastoma.[1215] Precise statistics regarding incidence are difficult to ascertain because of historically different views regarding classification and nosology. These tumors are more common in early childhood, with most patients diagnosed before the age of 10 years,[1216] and white persons make up most of the reported cases. Most cases are identified in early childhood, but they span all ages. Although they are classified as supratentorial in nature, these tumors have been identified in the infratentorial and spinal compartments. A better term has recently been adopted by WHO[207]—*CNS PNET*.

Clinical Presentation

Presenting features of CNS PNETs depend on the location of the lesion. Those adjacent to the ventricular flow path will usually include signs and symptoms of raised ICP as a result of obstructive hydrocephalus. Specific neurologic signs are dependent on the anatomic structures adjacent to the tumor and can include seizures, mood changes, or hemiparesis. Metastases are frequent in CNS PNETs, and clinical symptoms unrelated to the primary site of disease require investigation.

Imaging and Histology

CNS PNETs appear similar, independent of location within the CNS. As in medulloblastoma and pineoblastoma, these tumors can have areas of cyst or necrosis evident on CT or MRI. Lesions are dark on T1-weighted sequences unless hemorrhage is present and dark on T2-weighted sequences, reflecting their high nuclear-to-cytoplasmic ratio.[1217] CNS PNETs are usually contrast-enhancing after administration of gadolinium (Fig. 57-42). Edema, as evident on T2-weighted or FLAIR sequences, is often prominent. All CNS PNETs are

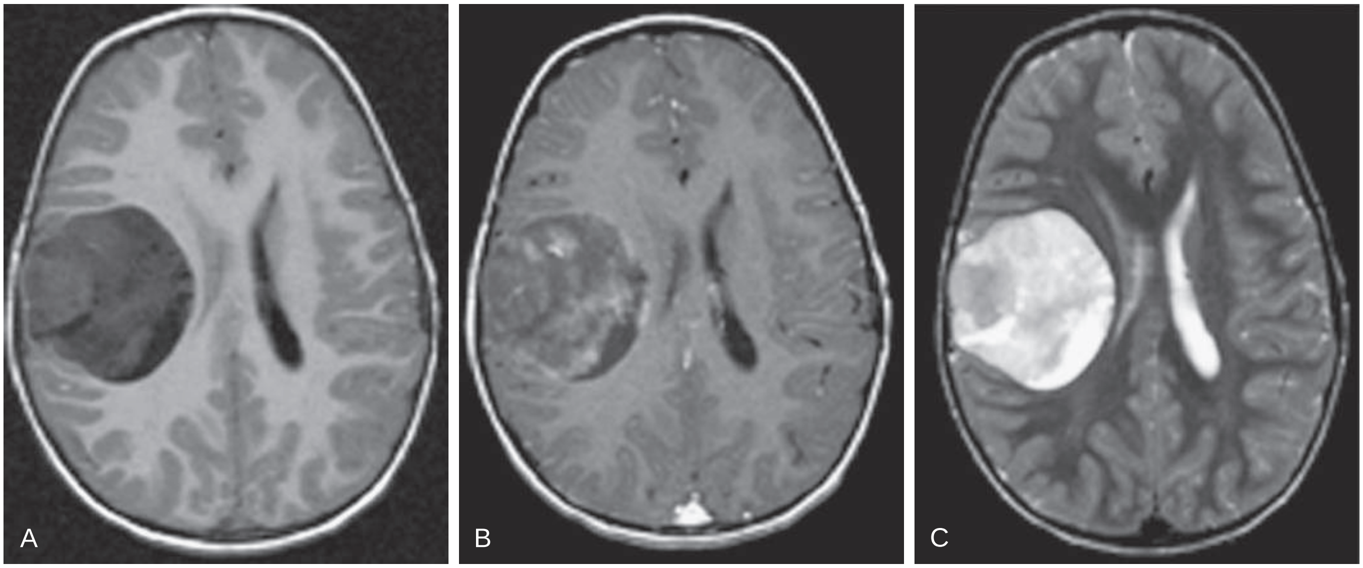

Figure 57-42 Primitive neuroectodermal tumor. **A,** An axial T1-weighted image without use of contrast material. **B,** An axial T1-weighted image with use of contrast material. **C,** An axial T2-weighted image.

considered WHO grade IV. The light microscopic features of CNS PNETs can be similar to those of medulloblastoma or pineoblastoma. Tumors are characterized by the presence of neuroepithelial cells. Many CNS PNETs have evidence of differentiation along glial, neuronal, ependymal, or oligodendroglial lines, although most (approximately 60%) appear undifferentiated.[1218]

Immunohistochemical markers of CNS PNETs include synaptophysin, B-tubulin, and S-100. Occasionally GFAP can be identified in these tumors and indicates their ability to undergo divergent differentiation. Mitotic rates are often high and, although variable, the MIB1 labeling index is usually abundant. Mutations of p53 are rare[1219] and were identified in only 3 of 28 cases of CNS PNET in one study, although many tumors had overexpression of wild-type p53, which correlated with a poor prognosis.[1220] Specific markers for CNS PNETs that distinguish them from medulloblastoma do not exist. Other features, such as Homer-Wright rosettes and perivascular pseudorosettes (i.e., cells around a central blood vessel) are observed in many different tumors (see Figs. 57-35 and 57-39). ATRTs may resemble PNETs, but the presence of cells that stain positively for myosin but lack the protein produced by the *SMARCB1* (*hSNF5/INI1)* gene are diagnostic features for ATRT.[1221,1222]

Medulloepitheliomas, Ependymoblastomas, and Embryonal Tumors with Abundant Neuropile and True Rosettes

Historically medulloepitheliomas, ependymoblastomas, and embryonal tumors with abundant neuropile and true rosettes (ETANTRs) have been considered distinct subtypes of CNS PNETs. However recent genomic and gene expression profiling efforts, including genome-wide DNA methylation and copy number profiling, have revealed similarities in genomic profiles, suggesting that they may represent spectrums of the same biologic entity.[1223] In this study, which included 97 tumor samples, all tumors were found to strongly express LIN28A, which had previously been described in ETANTRs.[1224,1225] No difference in clinical features was observed, and unsupervised hierarchical clustering did not reveal subgroups for 41 tumors with sufficient material to perform the analysis, while as a group the tumors clearly separated from other pediatric brain tumors. Finally 95% of tumors were found to harbor amplification of chromosome 19q13.42, previously associated with embryonal tumors with multilayered rosettes.[1226-1228] This study suggests that these entities share the same biology and can be considered spectrums of the same disease. However we will describe the traditional classification and clinical features of these tumors, in keeping with the current WHO classification.

CNS PNET tumors that have re-created features of the neural tube, called medulloepitheliomas, are typically found in very young children[1229] and have even been reported in newborns. They can occur throughout the brain, spine, and optic tract[1230] and can also occur outside the CNS. Their imaging characteristics differ somewhat from other CNS PNETs, with bright appearance on T2-weighted imaging.[1231] They tend to be large on macroscopic imaging. Additional immunohistochemical markers include nestin and vimentin staining.[1210] As with other CNS PNETs, maximal surgical resection, chemotherapy, and radiation therapy are the cornerstone of therapy.[1232] The predilection for young age in this subset of PNETs limits the use of craniospinal radiation.

PNET tumors with ependymoblastic rosettes are called ependymoblastomas and have features that overlap other CNS PNETs.[1223] These tumors predominate in infants and young children[1233] and can be found throughout the CNS, although most are associated with the ventricular system.[1210] Dissemination throughout the CNS is common,[1234] and symptoms include obstructive hydrocephalus, enlarging head circumference in infants, and focal neurologic deficits. The MRI appearance is similar to that of other PNETs, and edema is commonly observed.[1235] Treatment mirrors that of other CNS PNETs. Maximal resection is paramount, followed by multiagent chemotherapy. Although craniospinal radiation therapy is likely important for outcome, the prevalence of ependymoblastoma in infants precludes its use. Prognosis is poor, with rapid disease progression in most patients.

ETANTR is another rare CNS PNET tumor with a spectrum of histologic features and chromosomal abnormalities.[1210] These lesions can be large, cystic, and calcified and come to clinical presentation as a result of mass effect.[1236] Their rarity makes treatment recommendations difficult, although most are approached in a manner similar to that of other PNETs of the brain. The presence of isochrome 17[1237] in only a few cases suggests that they are biologically distinct from medulloblastoma.[1238] Various cytogenetic abnormalities have been defined.[1239]

Cerebral Neuroblastomas

PNETs with neuronal differentiation are called cerebral neuroblastomas and are rare tumors of infancy and childhood. They are unrelated to the more prevalent peripheral sympathetic nervous system form of neuroblastoma and predominate in young infants,[1240] occurring in both the cerebrum[1241] and posterior fossa.[1242] Lack of immunohistochemical staining with IGF2 and IGF type 1 receptor, which is positive in many other infant CNS tumors, can help with the diagnosis.[1210,1243]

A related entity of cerebral neuroblastoma is termed *ganglioneuroblastoma*, which has the presence of ganglion cells in addition to the neuronal component.[1210] These tumors cannot be differentiated from other malignant seeding tumors of infancy by MRI characteristics.[1244] Unlike most other CNS PNETs, cerebral neuroblastoma and CNS ganglioneuroblastoma may have a better prognosis[1245] with surgery and chemotherapy.

Treatment Strategies

A number of treatment protocols based on the successful therapeutic approaches of medulloblastoma have been applied to CNS PNETs.[1148] Repeated studies using maximal surgery, high-dose craniospinal radiotherapy to 3600 cGy with a boost to the primary site of at least 5400 cGy, and multiagent chemotherapy have not fared as well for CNS PNETs[1246,1247] as for high-risk medulloblastoma, with 4-year survival of approximately 40% in one retrospective series.[1216] In one study of 55 patients treated with maximal surgery, craniospinal radiation therapy, and CCNU, vincristine, and prednisone versus 8-in-1 chemotherapy sandwiched in the middle, OS and EFS were comparable. The 3-year EFS was approximately 50% in the two groups and thus appeared similar to that reported in other studies. Poor prognostic variables included the presence of metastases and age younger than 2 years. Patients with pineoblastoma did better, with an estimated 3-year PFS of 65%.[1247] Compared with medulloblastoma, CNS PNETs often respond poorly to medulloblastoma-specific therapy, despite the fact that they are histologically similar.[1248] The use of high-dose chemotherapy with stem cell rescue has been successful for some young children in eliminating the need for radiation therapy, with 5-year EFS of 39%.[1249] The exact conditioning regimen and number of cycles of intensive chemotherapy remain under investigation.[1250] Recurrences are often disseminated,[1248,1251] even when craniospinal radiation therapy is delivered upfront, and the salvage rate for these patients is exceedingly poor. In spite of these overall poor results, there have been reports of survivors after surgery, especially GTR, and radiation therapy.[1252] In one study patients treated with surgery, radiation therapy, and high dose stem cell–supported chemotherapy were reported to have a 5-year EFS of 75% ± 17% and an OS of 88% ± 13%.[1253] For patients with high-risk metastatic disease, the use of adjuvant carboplatin with radiation therapy as a radiosensitizer has been pilot tested in a phase I/II trial with promising results[1254] and is currently undergoing further investigation by the COG.

The concurrent use of craniospinal radiation therapy and multiagent chemotherapy for CNS PNETs has resulted in a significant impairment of nutrition during therapy for these patients.[1142] Therapy is intensive, and supportive care is important. A large number of children will require nutritional support through a nasogastric tube or, more commonly, a gastric tube. Constant surveillance of nutrition is important because these issues are often dismissed early and are only recognized when severe weight loss has occurred. Patients with persistent vomiting may initiate therapy in a negative nutritional state.

Prognosis

Although the prognosis of CNS PNET is well below that of medulloblastoma, 40% of patients will be long-term survivors. The most important variable for prolonged survival is the ability to achieve a complete resection in patients with nonmetastatic disease, followed by CSI and multiagent chemotherapy. The success of salvage therapy in patients who have had a relapse is exceedingly poor, even with high-dose chemotherapy and stem cell rescue.[1255,1256]

Atypical Teratoid Rhabdoid Tumors

ATRTs are uncommon, highly malignant tumors seen primarily in infants and in young children,[1257-1259] with a peak incidence between birth and 3 years. Cases throughout childhood[1260] and in adults have been reported,[1261] however, as has hereditary transmission of the susceptibility gene, resulting in congenital disease.[1262] Although CNS ATRTs account for approximately 1% to 2% of childhood brain tumors, they represent almost 10% of CNS tumors in infants.[1263,1264] Rhabdoid tumors may arise anywhere in the body but are most common in the kidney, CNS, and soft tissues. CNS ATRTs are most commonly identified in the posterior fossa (60%) at the cerebellopontine angle, although supratentorial ATRTs are also frequently seen.[1265] Pineal, spine, and suprasellar area ATRTs have also been reported. This tumor has a strong male preponderance, and metastases at diagnosis are common.[1265]

ATRTs have a spectrum of morphologic and molecular variants,[1266] but the hallmark of this disease is the loss of the *SMARCB1* (also known as *INI1*, *SNF5*, and *BAF47*) gene product on chromosome 22 as a result of biallelic inactivating mutations.[1267] Since the original description of monosomy 22 in ATRTs, this tumor suppressor gene has been cloned from this region[1268] and encodes a core subunit of the SWI/SNF chromatin remodeling complex.[1267] Recent publications have reported on the absence of other canonical pathway mutations and the extremely low mutation rates found in these tumors, thus demonstrating the remarkably simple genome of malignant rhabdoid tumors.[1269,1270]

Mutations and loss of the *SMARCB1* gene and protein expression may not be confined to ATRTs alone because reports on choroid plexus carcinoma, CNS PNET, and medulloblastoma have been reported,[1257] along with a recently identified entity, cribriform neuroepithelial tumor.[1271] Whether these later reports represent other diseases with *SMARCB1* mutations or atypical cases of rhabdoid tumors is controversial. Other diseases such as familial schwannomatosis, multiple meningiomas, epithelioid sarcomas, and extraskeletal myxoid chondrosarcomas are known to have loss of SMARCB1.[1272-1275] In addition, mutations in other subunits of the SWI/SNF chromatin remodeling complex (AT rich interactive domain 1A [ARID1A], polybromo 1 [PBRM1], and BRG1) have also recently been identified in neuroblastoma and ovarian, kidney, and lung cancers.[1276-1280]

Clinical Presentation

Presenting symptoms are dependent on the age and location of the tumor. In persons with posterior fossa tumors, obstructive hydrocephalus with headaches, morning vomiting, and long tract signs is common. Because most patients are young, irritability, lethargy, or failure to thrive may be evident. In patients without closed fontanelles, rapidly enlarging head circumference is observed. Cranial nerve dysfunction is common, resulting in head tilt and diplopia. Patients with supratentorial tumors will often have obstructive hydrocephalus because of the rapid growth and concurrent cysts that obstruct CSF flow. Additional symptoms can be referred to direct compression of neural structures adjacent to the tumor and thus are dependent on location.[1265,1281] Although uncommon spinal ATRT can occur and present with focal motor deficits. Because of the high incidence of metastatic disease, patients with symptoms related to a specific area away from the primary tumor site require thorough investigation. Although rare constitutional loss of the *SMARCB1* gene (or the *INI1* gene; the nomenclature is interchangeable) can occur,[1263] resulting in intracranial and metachronous extraneural disease. Patients should therefore undergo CT evaluation of the chest, abdomen, and pelvis.

Imaging and Histology

Imaging characteristics are similar to those of other neuroectodermal tumors (medulloblastoma and PNET) of the CNS,[1282] including isointense appearance on T1-weighted MRI and heterogeneity on FLAIR and T2-weighted sequences.[1283,1284] Cystic areas are common. Tumors are usually heterogeneously enhancing with contrast administration. Because of their high cellular composition, restricted diffusion is often observed. The presence of hemorrhage and calcifications is not uncommon.[1285]

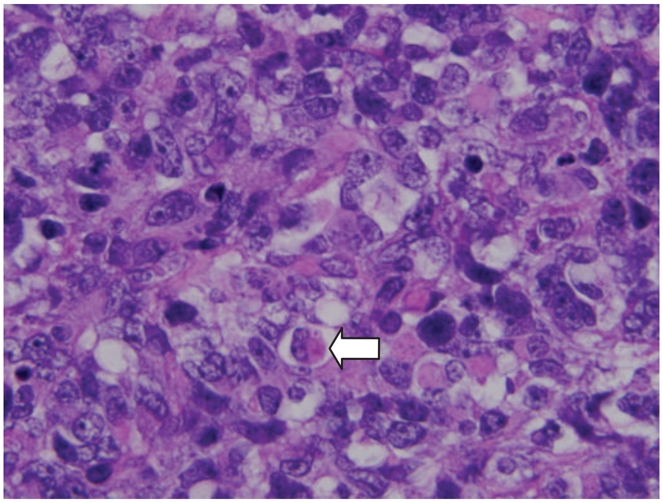

Figure 57-43 An atypical teratoid rhabdoid tumor *(arrow)* demonstrating prominent nucleoli and eosinophilic globular cytoplasmic inclusions (hematoxylin and eosin; ×600).

ATRT has been recognized as a distinct pathologic entity, and it is differentiated from medulloblastoma and other CNS PNETs.[1286] This distinction has been supported by findings of deletions or loss at chromosome 22q11.2, the identification of the tumor suppressor gene *SMARCB1*, and the finding of germline and somatic mutations of *SMARCB1* in approximately 75% of cases of CNS ATRTs,[1287] although ATRTs based on morphologic criteria with normal expression of *SMARCB1* have been identified.[1288] Rhabdoid cells have the characteristic appearance of an eccentric nucleus, prominent eosinophilic nucleoli, and abundant cytoplasm, with an eosinophilic globular cytoplasmic inclusion (Fig. 57-43).[1289] These cells stain with EMA and vimentin. GFAP and synaptophysin can also be positive.

SMARCB1 (also known as *INI1* or *BAF47*) staining is routinely used to confirm the diagnosis and has become a standard assessment tool for making the diagnosis of ATRT in atypical cases.[1222] The expression of this nuclear protein, which remains expressed in endothelial cells, is characteristically lost in tumor cells (Fig. 57-44). Tumors without the presence of rhabdoid cells but with loss of *SMARCB1* staining are still considered ATRTs.[1288] Whether choroid plexus tumors can have loss of *SMARCB1* expression or whether these tumors represent actual ATRTs is controversial.[1221,1290] ATRTs are classified as WHO grade IV[1289] lesions with an elevated MIB1 labeling index and are composed of primitive neuroepithelial, epithelial, and mesenchymal components. Large areas of small round blue cells can predominate, resulting in the misdiagnosis of medulloblastoma or CNS PNET. In a review of 55 cases from a POG study, the small round blue cell component resembled medulloblastoma,[1291] and ATRT was misclassified as medulloblastoma in more than 50% of cases.[1257] These findings emphasize the need for confirmatory testing with *SMARCB1* immunohistochemical staining or fluorescence in situ hybridization to look

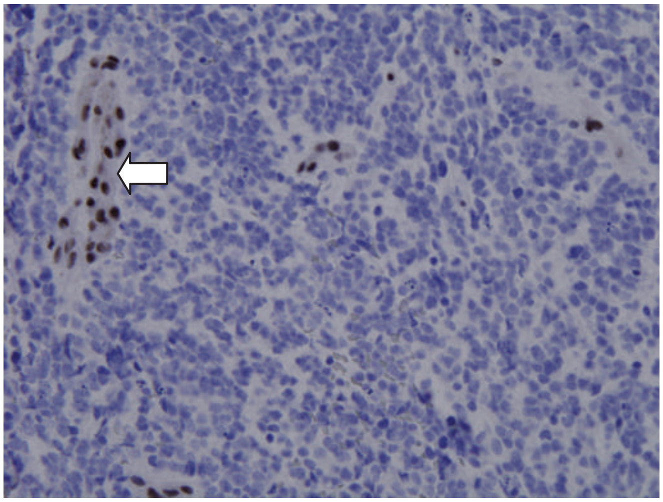

Figure 57-44 An atypical teratoid rhabdoid tumor with absence of switch/sucrose nonfermentable–related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1 (SMARCB1; INI1) immunoreactivity in the tumor cells. Normal endothelial cells within the blood vessels have normal SMARCB1 expression *(arrow;* SMARCB1 immunostaining; ×400).

for monosomy chromosome 22 to help confirm or exclude the diagnosis of ATRT.[1291] Similarly a central pathology review of 227 of 284 eligible infants with brain tumors from a CCG clinical trial for infant patients (trial 9921) showed that ATRT is a significant component of the tumors in this population and that frequent misdiagnoses occur.[962] Given the predominance of *SMARCB1* loss of expression in ATRT, it is now standard practice to evaluate *SMARCB1* expression for treatment stratification onto the appropriate disease-specific protocols.

Management

Multimodality protocols developed for the treatment of medulloblastoma have not been effective for ATRTs. Some success has been achieved by using treatment protocols based on therapy for children with rhabdomyosarcoma[1281,1292] and high-dose myeloablative chemotherapy.[1265,1293,1294] Currently the optimal therapy for pediatric ATRT remains maximal surgical resection, multiagent chemotherapy, and radiation therapy.[1260,1295] Older patients appear to have a better prognosis and, with complete surgical resection, multiagent chemotherapy, and craniospinal radiotherapy, they have 2-year EFS and OS of 78% and 89%, respectively. In contrast children younger than 3 years have a dismal outcome with EFS and OS of 11% and 17%, respectively.[1260] A recent risk-adapted treatment protocol in which patients received maximal surgery, multiagent chemotherapy, both systemic and intrathecal, and focal radiation therapy in children younger than 3 years and CSI in older patients has demonstrated encouraging results. The 2-year EFS and OS in this group of patients, most of whom were younger than 3 years, was 53% and 70%, respectively.[1295] These results indicate that ATRT can be effectively treated and that even patients with metastatic disease can be long-term survivors. The need for radiation therapy in the treatment of ATRT remains controversial, especially for the youngest patients, although some centers have encouraged its use.[1296] The overall positive outcomes seen in infants treated with 3D-conformal therapy, as well as those treated with high-dose chemotherapy and a stem cell transplantation approach without radiation therapy, suggest that craniospinal radiotherapy is not needed in all patients. The identification of a radiation-resistant CD31-positive stem cell may explain some of the innate resistance of this tumor to current therapies.[1297] It is hoped that the continued discovery of the function of the *SMARCB1* tumor suppressor gene, coupled with an improved understanding of the pathways aberrant in these tumors, will lead to more specific targeted therapies.[1298]

Prognosis

Until recently estimates of survival rates for ATRT ranged from 6 to 11 months.[962,1291,1299,1300] CNS ATRT tumors tend to have specific mutational hotspots in the *SMARCB1 (INI1)* gene,[1301] although treatment response and outcome are not known to be influenced by specific mutations. Although tumors tend to be initially responsive to chemotherapy, recurrence rapidly occurs, both locally and with metastatic disease.[1287] However the prognosis for patients with ATRT may be improved dramatically with intensive multimodality treatment.[1260,1295] Although survival is extremely uncommon, some survivors of relapsed disease have been reported.[1281]

Neurocytomas

Central neurocytomas are rare tumors, comprising between 0.25% and 0.5% of all brain tumors.[1302,1303] First described in 1982 by Hassoun and colleagues, neurocytomas typically occur in adolescents and young adults. These tumors are of neural lineage based on the expression of neuronal markers such as βIII-tubulin, neural cell adhesion molecule, NSE, and synaptophysin.[1304,1305] A gene expression profile comparison of central neurocytoma cells with normal adult VZ progenitors has demonstrated significant overlap, indicating that central neurocytoma is most consistent with proneural cells and that these neurocytoma cells differ from progenitor cells by the increased expression of IGF2 and receptors and effectors of the canonical Wnt signaling pathway and *PDGFR*.[1306] Neurocytomas usually arise in the lateral ventricles, especially in proximity to the third ventricle and foramen of Monro, and are called central neurocytomas. Those localized to the brain parenchyma are called extraventricular neurocytomas. Historically neurocytomas have been frequently described as benign lesions because of the positive outcome of patients with complete surgical resections, although aggressive behavior and poor outcome are not uncommon.[1307,1308] Furthermore numerous reports have been made of tumors with MIB1 labeling indices greater than 3%[1309] and/or atypical histologic features such as increased mitotic activity, vascular proliferation, and/or focal necrosis.[1310] The mean age at presentation in adults is 28 to 29 years, with most patients between the ages of 20 and 40 years,[1302] and a male-to-female ratio of 3:2.[1311] A review of neurocytoma in 60 children younger than 18 years, compiled primarily from reports in the medical literature, has demonstrated a median age of 16 years, with a similar male predominance (61%).[1312]

Clinical Presentation

Most neurocytomas are located in the ventricular system,[1313,1314] specifically the anterior portion of the lateral ventricles near the foramen of Monro, although extraventricular tumors have been described.[1308,1315] Symptoms of raised ICP are common as a result of tumor obstruction of CSF flow at the level of the foramen of Monro or cerebral aqueduct.[1307,1314] Patients with neurocytoma typically present with symptoms including headache, visual changes, nausea, and vomiting.[1316] Focal neurologic deficits are less common presenting signs. Hemorrhage can result in acute symptoms, leading to the diagnosis. Primary disease in the fourth ventricle[1317] or spinal cord has been reported in children.[1318]

Imaging and Histology

The typical MRI appearance of central neurocytoma is that of a well-circumscribed lobulated mass in the anterior portion of the lateral ventricles, making it difficult to distinguish from other intraventricular neoplasms such as ependymomas, astrocytomas, and oligodendrogliomas. Tumors often have an isointense or slightly hypointense signal relative to the cerebral cortex on T1-weighted images and a hyperintense signal on T2-weighted images with enhancement upon administration of gadolinium (Fig. 57-45).[1319-1321] Proton MRS and DWI have recently been described as a possible diagnostic tool in central neurocytoma.[1322] A characteristic MRS appearance of a high choline peak, a low NAA peak, and a glycine peak at 3.55 ppm is seen in a subset of patients.[1323,1324]

Histopathologic analysis of neurocytomas often reveals benign-appearing tissue composed of uniform round cells. Neurocytomas are classified as WHO grade II.[1325] Features such as large fibrillary areas, calcifications, and perivascular pseudorosettes are commonly observed.[1326] Routine hematoxylin and eosin staining of a neurocytoma can reveal the presence of cells with perinuclear halos suggestive of oligodendroglioma. However the reactivity with immunohistochemical staining for neuronal markers such as synaptophysin, βIII-tubulin, neural cell adhesion molecule, and NSE helps establish the diagnosis of a neurocytoma.[1327] As noted earlier atypical forms with mitoses, necrosis, and endothelial proliferation have been reported.[1328,1329] In tumors lacking synaptophysin or Neu-N staining, electron microscopy can assist in confirming the diagnosis.[1330] An MIB1 labeling index is useful for prognostic purposes. Patients with an MIB1 labeling index greater than 2% to 3% are referred to as having atypical neurocytoma and have a worse prognosis for local control and survival.[1309,1310,1328,1329] Subtotal resection, atypical histologic features, and older patient age have been associated with an increased risk of recurrence.[1331,1332]

Management

GTR can be curative for central neurocytomas and is the treatment of choice for pediatric patients.[1333,1334] A review of 73 children younger than 18 years pooled from multiple studies has shown an OS of 100% at 10 years for children who underwent a GTR.[1335,1336] Local failure was demonstrated in approximately 15% of patients who underwent complete resection alone versus 0% in patients who underwent complete resection with adjuvant radiation therapy. However given the absence of survival

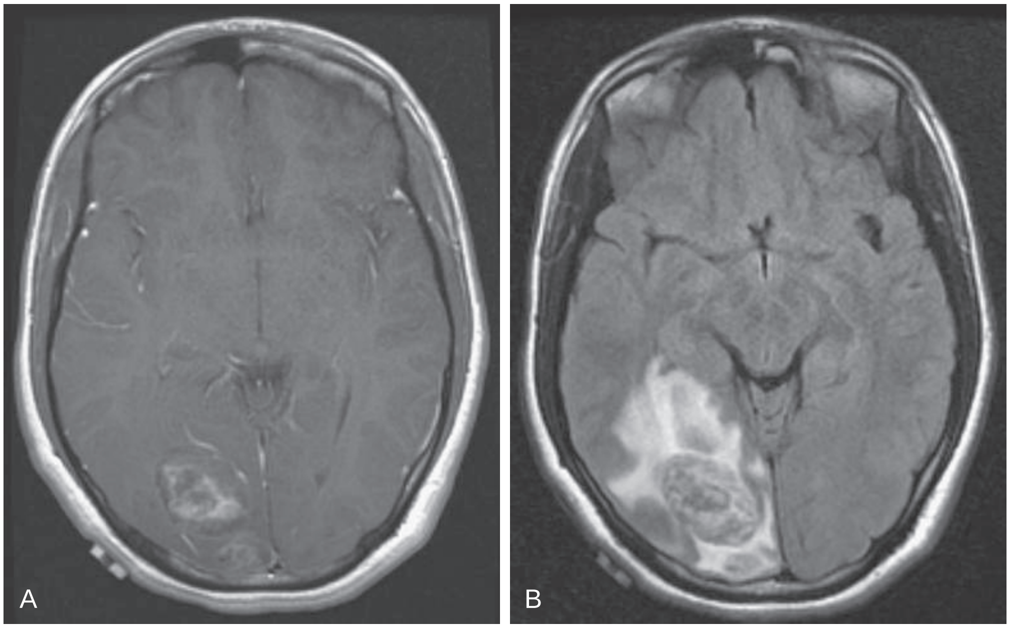

Figure 57-45 Right occipital extraventricular neurocytoma. **A,** An axial T1-weighted image with use of contrast material. **B,** An axial fluid-attenuated inversion recovery image. This tumor is an isointense to hyperintense extraventricular neurocytoma with surrounding edema.

difference and the potential neurocognitive toxicities, adjuvant radiation therapy is not recommended as initial treatment.[1312] Children with incomplete resections have a significantly higher risk of recurrence (mean, 100 months) and a lower OS (82%). Adjuvant radiation therapy has been shown to reduce the risk of recurrence but not the risk of death.[1312] In the case of incomplete resection, radiotherapy may be beneficial at a dose of 5400 cGy,[1337] although it should be noted that the number of patients available for evaluation in these studies is small. Radiotherapy should be more seriously considered for atypical neurocytomas, such as those with a high MIB1 labeling index (higher than 3%). A recent meta-analysis has shown that patients with lesions that have an MIB1 labeling index less than 3% have less than a 15% risk of recurrence and a 95% 5-year OS, compared with 38% and 66%, respectively, for patients with tumors that have an MIB1 labeling index higher than 3%.[1309,1310] In a review of 85 adult and pediatric patients, Rades and colleagues[1338] have demonstrated a marked difference in 5-year survival for incompletely resected atypical neurocytoma (43%). The addition of adjuvant radiation therapy increased 5-year survival rates to 78%. Complete resection was again found to be the best treatment, with 5-year survival rates of 93%. Adjuvant radiotherapy held no additional benefit in cases of completely resected atypical neurocytomas. Stereotactic radiosurgery has been explored as a potential first-line treatment for neurocytoma, but only in limited numbers of adult and pediatric patients,[1339] and it requires careful attention to the radiation therapy margins.[1340] Historically the use of chemotherapy for central neurocytoma has been limited, with only case reports and small case series being reported.[1341] Brandes and associates[1342] have described three adult patients with progressive neurocytoma treated with etoposide, cisplatin, and cyclophosphamide, which led to disease stabilization in two patients and complete remission in one patient.

Prognosis

Complete surgical resection and an MIB1 labeling index of less than 2% are the most important prognostic markers.[1309] Even with extraventricular location and atypical features, most patients will be long-term survivors. Thus judicious use of therapeutic modalities that can lead to significant long-term morbidity, such as radiation therapy, is indicated.

Choroid Plexus Tumors

Choroid plexus tumors usually arise from the epithelium of the choroid plexus in the lateral or fourth ventricles, where the choroid plexus is found. Although choroid plexus lesions represent only 3% of pediatric brain tumors, they comprise 10% to 20% of tumors that develop in the first year of life and account for a considerable percentage of in utero diagnoses.[1343] The median age at diagnosis for choroid plexus tumors is younger than ten years.[1344] Three histologic choroid plexus variants have been described: papillomas, atypical papillomas, and carcinomas.[1345] CPPs outnumber CPCs by a ratio of at least 5:1. Both tumors typically arise in the lateral ventricles in 50% of cases and in the fourth ventricle in 40% of cases. Tumors arising from multiple ventricles represent only 5% of cases. Because of their rarity, little is known about the molecular abnormalities that give rise to these tumors,[1346] although their strong association with the Li-Fraumeni p53 cancer susceptibility gene is well established.[1347] A significant percentage of choroid tumors will have abnormalities on array comparative genomic hybridization analysis.[889] The presence of normal *SMARCB1* staining in these tumors can be used to exclude ATRTs, which share many histologic features with choroid plexus tumors.[1221]

Little is known at present about the developmental or molecular biology of choroid plexus tumors, although a number of markers have been identified using gene array analysis.[1348,1349] Recent findings have implicated the transcription factor, TWIST1, notch3 signaling pathway, and tumor necrosis factor–related apoptosis-inducing ligand.[1350,1351] Patients with Li-Fraumeni syndrome who have a germline mutation of p53 are at increased risk for CPCs.[1347] Epigenetic analysis looking at DNA methylation patterns has shown increased methylation in CPC.[1352] DNA sequences from sporadic papillomas may harbor the human neurotropic JC virus.[916] The role of SV40 (another polyomavirus) in the development of pediatric CNS tumors, particularly ependymomas and choroid plexus, is an interesting one. SV40 sequences have been found in the tumor tissue of up to 50% of patients but not in adjacent areas of normal brain. SV40 is known to induce brain tumors in preclinical models, and SV40 can transform human cells in vitro, which may support a causative role for this polyomavirus in choroid plexus tumors.[917] Polio vaccines used in certain countries in the 1950s and 1960s were contaminated with SV40. In countries that have used uncontaminated vaccines, the identification of SV40 sequences in choroid plexus tumors is rare. However the incidence of choroid plexus tumors is similar between the two populations and thus appears independent of both SV40 exposure and SV40 detection within the tumors.[919,1353] Because of the strong association of Li-Fraumeni syndrome and *p53* mutations with CPC, patients need a comprehensive family history to ascertain the incidence of associated cancers and assist in the screening of family members. The presence of a *p53* germline mutation may affect the choice of radiation therapy as a therapeutic option and would indicate the need for genetic counseling in other family members.[1347] Additional mutations in the *TP53* gene have been discovered that lead to an increased incidence of CPC and can have an impact on the survival of patients with choroid plexus tumors.[1354-1356] The presence of PDGFR expression in a high percentage of cases (87%) is a rationale for the use of targeted inhibitors of this pathway in the context of a clinical trial.[1357]

Clinical Presentation

Initial symptoms are usually secondary to elevated ICP and hydrocephalus and include headaches, nausea, and vomiting, with papilledema. Other possible manifestations include lethargy, seizures, and failure to thrive. Because these tumors tend to arise in infants who retain

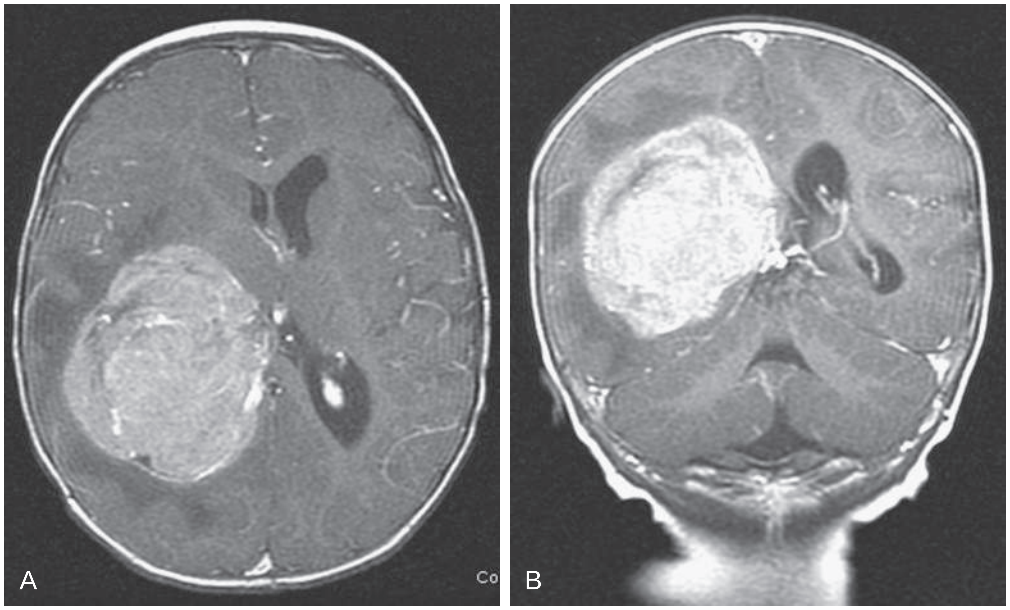

Figure 57-46 Choroid plexus carcinoma. **A,** An axial T1-weighted image with use of contrast material. **B,** A sagittal T1-weighted image with use of contrast material.

open sutures, the presentation may be relatively delayed, and the tumors may reach an exceptional size. Infants typically demonstrate irritability, lethargy, vomiting, a tense fontanelle, and macrocephaly, with splayed sutures. Although most choroid plexus tumors arise in the lateral ventricles, predominantly in infants, fourth ventricular choroid plexus tumors occur in persons of all ages, including adults. Metastatic disease can occur in CPPs, although these patients still have a good prognosis and many of the tumors will not progress. Leptomeningeal dissemination is common with CPCs and is a poor prognostic marker because of the limited use of craniospinal radiation therapy in this very young population. Dissemination through a VP shunt has been reported.[1358]

Imaging and Histology

A choroid plexus tumor should be suspected when a large enhancing tumor in the lateral ventricle is visualized on gadolinium-enhanced MRI (Fig. 57-46). Multilobular, calcified, well-delineated contrast-enhancing intraventricular masses are characteristic of choroid plexus tumors. Tumors are usually isodense on T1-weighted images and bright on T2-weighted images.[1359] CPCs often have more heterogeneous enhancement and edema signal on FLAIR than is routinely seen with CPPs.[1360] Unique characteristics of MRS can differentiate CPP from CPC at diagnosis although, for most patients, this differentiation will not obviate the need for surgery because of the high incidence of CSF flow obstruction in these patients, necessitating neurosurgical intervention.[1361]

CPPs (WHO grade I) have the lowest proliferative rate and are composed of fibrovascular fronds covered by a single layer of epithelial cells. They closely resemble the normal choroid plexus. Cytokeratin, S-100 protein, podoplanin, and vimentin are typically expressed on immunohistochemistry. GFAP, which is typically not seen in the normal choroid plexus, is found in approximately 25% to 50% of CPPs. In rare cases CPPs can transform into CPCs.[1362] In contrast CPCs (WHO grade III) manifest higher cell density, nuclear pleomorphism, frequent mitoses (more than 5 of 10 high-power fields), high nuclear-to-cytoplasmic ratios, necrosis, and invasive appearance (i.e., blurring of the papillary structures). Up to 20% of CPCs can be positive for GFAP.[1363] Atypical CPPs (WHO grade II) refer to cases in which a CPP has increased mitotic activity. Clear diagnostic criteria for these atypical tumors have not been established. These tumors can possess greater pleomorphism, increased cellularity, and areas of necrosis, but these elements are not required for diagnosis. However it appears that the presence of mitotic activity (2 mitoses or more per 10 high-power fields) is the sole atypical histologic feature independently associated with recurrence.[1363] Atypical CPPs accounted for approximately 15% of all CPPs in one large series.[1363]

Management

After diagnostic neuroimaging studies, high-dose corticosteroids are often administered when elevated ICP or tumor edema is suspected. Because the extent of surgical resection is the single most important factor that determines the prognosis in choroid plexus tumors, the goal of the neurosurgeon is to perform a GTR.[1364] In patients with CPP, complete resection is usually sufficient therapy.[1365] Achieving complete resection may require more than one surgical procedure but appears to improve outcome.[1366] One obstacle to the surgical removal of choroid plexus tumors is the rich vascular network that is often located within the tumor. The choroid plexus receives its blood supply from the anterior and posterior choroidal arteries, branches of the internal carotid artery, and the posterior cerebral artery. Achieving a GTR in cases of CPP and atypical papilloma is often curative, and adjuvant therapy may be deferred after normal results of a restaging evaluation are found.

Because most children diagnosed with CPC are younger than 3 years, chemotherapy is the treatment of choice in those for whom a complete resection cannot be achieved.[1194,1344] Various multiagent chemotherapy regimens have been explored, and preliminary evidence suggests that CPCs are chemosensitive tumors. Currently an International Society for Pediatric Oncology protocol exists that is exploring different chemotherapeutic options. Agents such as carboplatin and etoposide have shown good activity. In addition, intrathecal therapy has been incorporated into treatment with ara-C and etoposide. The role of radiotherapy is controversial and is usually reserved for children older than 3 years who have had a subtotal resection, malignant features within the tumor, or dissemination of the tumor along the neuraxis.

Prognosis

GTR is often curative for CPP. Even in patients with a subtotal resection, 50% of the residual tumor will not demonstrate progression.[1367] The 5- and 10-year PFS and OS rate for patients with papillomas are 81% and 77%, respectively, versus 41% and 35%, respectively, for persons with carcinomas.[1344] Atypical CPP can recur, and thus careful follow-up is needed. The long-term prognosis for this group of patients remains excellent.[1363] Patients with dissemination at diagnosis do less well. Several adjuvant platinum-based chemotherapy regimens have been used with some measure of success,[1368] but at present no standard protocol has been established for CPC. Development of an international protocol for the prospective treatment and outcome of choroid papilloma, atypical CPP, and CPC is under way.

Germ Cell Tumors

CNS GCTs are the most prevalent tumors of the pineal region and represent approximately 3% to 5% of intracranial childhood malignances in the United States. These tumors are much more common in Asia, particularly in Japan.[1369,1370] The reason for this geographic variability is unknown. Most GCTs occur in early adolescence, and males are affected significantly more often than are females.[1371] Males are much more likely to have both pineal and suprasellar GCTs, with a reported male to female ratio of approximately 14 : 1.[1372] Germinomas are much more common than nongerminomas.[1373] Histologically these tumors resemble the GCTs that arise in the gonads.

Most (95%) GCTs arise in midline sites such as the infundibular region (40%) and pineal region (50%), although 5% arise simultaneously in both regions without an apparent connection.[1374] Occasionally they are isolated to the basal ganglia[1375] but can be approached in a similar fashion to those in the pineal or suprasellar area.[1376] CNS GCTs are presumed to result from the abnormal migration of primitive germ cells early in embryogenesis within the gonadal ridge.[1377,1378] Occasionally metachronous lesions at both sites are detected at the time of diagnosis.[1377,1379] CNS GCTs are divided into two clinical groups, which reflect sensitivity to cytotoxic therapies—pure germinomas (60%) and NGGCTs (40%).[1369,1380] NGGCTs include yolk sac tumors (endodermal sinus tumors), embryonal carcinomas, choriocarcinomas, immature teratomas, teratomas with malignant transformation, and mixed GCTs. Mixed GCTs are defined as tumors that contain any two of the elements previously listed and can include mixtures of germinoma and nongerminoma. Germinomas are the most common GCT of the pineal region,[1372] although NGGCTs and germinomas arise with equal frequency in the suprasellar region. A male predominance occurs in pineal region tumors, although both genders are equally affected by tumors in the suprasellar region. Mature teratomas of the brain causing significant mass effect have been reported.[1381] Their peak age of occurrence is in the second and third decade of life.

No clear genetic predisposition to CNS GCTs has been identified other than males with Klinefelter syndrome (47XXY).[1382] Of interest a number of CNS GCTs have an abnormal karyotype, including gain of chromosome X, which raises the possibility that patients with Klinefelter syndrome are uniquely at risk because of the presence of their additional X chromosome. A number of cases of GCTs in patients with Down syndrome have also been reported.[1383,1384]

Clinical Presentation

The clinical presentation between suprasellar and pineal region GCTs can differ. Patients diagnosed with suprasellar GCTs often have a long prodrome, often several years in duration.[1385] The earliest symptoms usually involve endocrine dysfunction, most frequently symptoms of diabetes insipidus (DI).[1386] This presenting symptom can also be observed in other lesions of the pituitary region, such as lymphocytic hypophysitis.[1387] In some patients with DI, a mass is not identified on MRI scan but over time a mass becomes evident, leading to the diagnosis. Eventually other endocrine manifestations may occur, such as growth impairment, delayed puberty, and hypothyroidism. Visual loss and symptoms of raised ICP are late manifestations when the tumor has reached appreciable size or spread in a periventricular distribution. Even in patients without MRI evidence of pituitary or hypothalamic involvement of tumor, endoscopic evaluation can often detect subtle areas of tumor.[1388] It is not yet clear whether patients with endoscopically identified regional disease should be considered to have metastatic disease.

Tumors arising in the pineal region often produce headache, nausea, and vomiting because of obstructive hydrocephalus. Limitation of vertical gaze, convergence nystagmus, impaired pupillary reflexes, and double vision may occur because of tectal compression (Parinaud syndrome). Atypical presentations occur when GCTs arise in unusual locations such as the basal ganglia, when they present with widespread leptomeningeal metastases, or when they diffusely infiltrate deep white matter structures.

GCTs can cause precocious puberty because of release of β-human chorionic gonadotropin (β-HCG).[1389,1390] Atypical presentations, such as movement disorders or

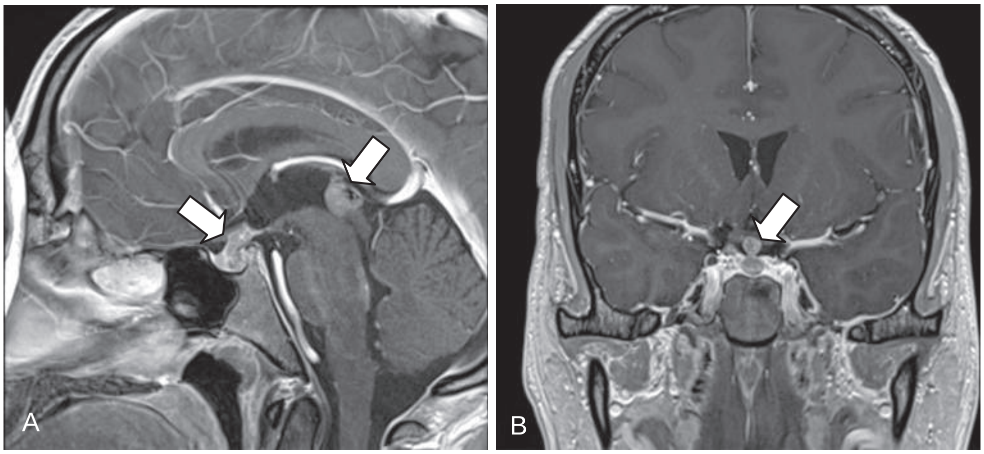

Figure 57-47 Pituitary and pineal germinomas. **A,** A sagittal T1-weighted image with use of contrast material showing two lesions, one in the pituitary and one in the pineal region. A coronal T1 fat-saturated image. **B,** A coronal T1-weighted image with use of contrast material showing the same pituitary/suprasellar lesion.

depression as the presenting symptom, can result in significant delays in diagnosis.[113,1385]

Imaging and Histology

GCTs can have a heterogeneous appearance on CT and MRI and can differ between the pineal and suprasellar regions.[1391] Most are contrast-enhancing solid lesions, with isointense signal on T1-weighted sequences and a bright signal on T2-weighted sequences (Fig. 57-47).[1392] Cysts are commonly observed, especially in nongerminomas.[1393] These signal characteristics overlap with other common tumors of the pineal region and require biopsy or hormone marker analysis for accurate diagnosis.[1380,1394] An important exception is CNS teratoma. These lesions are composed of all three mature tissue elements and therefore will demonstrate calcified areas with adjacent cysts and low-density regions of fat. Areas of bright signal on T1-weighted images, consistent with hemorrhage, are suggestive of choriocarcinoma.[1393]

The NGGCTs are unique among CNS tumors in that they can be diagnosed solely on the basis of expression of tumor markers.[1395,1396] Either β-HCG, a normal product of syncytiotrophoblasts, α-fetoprotein (AFP), a normal product of yolk sac endoderm, or both can be detected in the blood and CSF of patients with NGGCTs. β-HCG is associated with choriocarcinoma, although low levels can also be associated with germinomas. CSF levels tend to be higher than levels in the serum.[1397] AFP is expressed by yolk sac tumors, although low immunohistochemical expression can be observed in some teratomas. Although placental alkaline phosphatase (PLAP) and lactate dehydrogenase isoenzymes and CSF c-kit have been used as markers of germinoma,[1395,1398-1400] immunohistochemical staining for these markers is more commonly used for diagnosis. Because AFP is normally expressed in newborns, considerable attention must be given to interpretation of these levels in infants.[1401]

The histologic identification of CNS GCTs is critical for proper therapeutic assignment and prognostic counseling. Seven major categories are recognized by the WHO.[1402] Clinically mixed GCTs are encountered in most patients, except those with pure germinomas or mature teratomas, and the most malignant component typically dictates the type and intensity of the therapy.

Germinoma is the most common CNS GCT encountered and is considered the equivalent of the testicular seminoma. The appearance of these large cells on hematoxylin and eosin staining, frequent mitoses, and lymphocytic infiltrates is characteristic and can easily be observed on smear preparations. Cells stain brightly for both c-kit and octamer-4.[1403,1404] Placental alkaline phosphatase has been less reliable. SALL4 has been studied as a novel marker and appears sensitive in the diagnosis of CNS GCT.[1405] Even pure germinomas can possess syncytiotrophoblastic giant cells, resulting in low-level expression of β-HCG, which does not upstage their classification to the more malignant choriocarcinoma.[1390]

NGGCT is typically used to refer to one of three different malignant germ cell tumor variants, as well as teratomatous elements with atypical or malignant degeneration. The first, yolk sac tumor (endodermal sinus tumor), is an epithelial tumor that can mimic the appearance of germinomas but highly expresses AFP throughout the cytoplasm. Embryonal carcinoma is an epithelial-derived tumor that has a characteristic appearance with abundant mitoses and diffuse presence of cytokeratin. This latter feature can help differentiate these tumors from germinomas because both can express PLAP and octamer-4. Lastly, choriocarcinoma is a trophoblastic tumor of extraembryonic origin. It requires the identification of cytotrophoblastic elements and syncytiotrophoblastic giant cells. Hemorrhagic necrosis is a common feature of this tumor, and the giant cells stain brightly for β-HCG.

Teratoma refers to a group of three lesions that have evidence of differentiation along the three embryonic germ cell layers—ectoderm, endoderm, and mesoderm:

1. Mature teratoma possesses fully differentiated tissue elements of all three layers. The ectodermal components often contain skin, brain, and/or choroid plexus. The mesodermal component can frequently include cartilage, bone, fat, and/or

muscle, although the endodermal component often contains respiratory or gastrointestinal cysts lined by epithelium. Teratomas usually have an absent or low mitotic index.

2. Immature teratoma is similar to mature teratoma but possesses some elements from one or more of the germ cell layers that are not fully differentiated. Most commonly seen are mitotically active stromal elements or neuroectodermal cells. These immature elements are at considerable risk for malignant degeneration and thus require additional therapy unless a complete resection has been performed.[1406]
3. Teratomas with malignant transformation are rare lesions in which an area of malignant cells of any histologic type is observed. Rhabdomyosarcoma or undifferentiated sarcoma is the most commonly seen, although almost any tumor can occur in the context of teratomas because teratomas already possess all three germ cell layers.[1407]

The diagnosis of an intracranial GCT is suspected in any patient with acquired DI or Parinaud syndrome. Although the location of the primary tumor on neuroimaging studies and certain features of the clinical presentation are supportive of the diagnosis of a CNS GCT, the diagnosis must be confirmed by histology or CSF tumor markers (Table 57-7). Patients with a thickened pituitary stalk with DI are more likely to have an infiltrative tumor process than Langerhans cell histiocytosis or hypophysitis, and the appropriate workup should be initiated.[1408] Histologic confirmation may not be necessary in patients with elevated serum and/or CSF concentrations of tumor markers (AFP or β-HCG) consistent with an NGGCT.[1395,1396]

The tumor markers β-HCG and AFP are useful in not only confirming the presence of a CNS GCT but also in monitoring response to therapy and in suspecting earlier recurrence. Elevation of AFP alone suggests yolk sac (endodermal sinus tumor), whereas a high level of β-HCG suggests choriocarcinoma. Pure germinomas may have modest elevations of CSF β-HCG, usually lower than 50 mIU/mL (100 mIU/mL at some institutions). Lactate dehydrogenase isoenzymes, the soluble c-kit oncogene product, and PLAP are also detectable in the CSF of patients with a germinoma.[1395,1399,1400] Serum markers of β-HCG at diagnosis can be negative, even in the presence of significantly elevated CSF levels, demonstrating the importance of a comprehensive workup for this patient population, including MRI of the spine and CSF for markers and cytology.[1395,1409] Proper prospective studies using ventricular CSF rather than lumbar CSF have not been completed and therefore should not be used for staging at present.[1116]

Management

Preoperative evaluation should include contrast-enhanced brain and spine MRI, serum and CSF tumor markers (if lumbar puncture can be safely performed), CSF cytology, assessment of endocrine function and visual acuity, and visual field examinations for suprasellar disease. In the absence of elevated lumbar CSF tumor markers, a biopsy should be performed. Aggressive resection leading to morbidity is neither necessary nor advisable in cases of germinoma because of the tumor's exquisite sensitivity to cytotoxic and radiation therapies. Thus less invasive neurosurgical procedures to obtain a tissue sample, such as an endoscopic or stereotactic biopsy, are used more frequently.[1174,1388,1410] A third ventriculostomy is frequently performed at the same time as an endoscopic biopsy in patients with noncommunicating hydrocephalus and a pineal region tumor.[1411] Radical resections of an NGGCT may be easier to accomplish after several courses of chemotherapy.[1412]

Patients with germinomas have a number of nonsurgical treatment options.[1413,1414] With radiation therapy alone for pure germinoma, the 5-year OS rate has approached 90% to 95% in clinical series[1415]; however, radiation therapy alone is inadequate for NGGCT, with 5-year OS ranging from 0% to 30%.[1416,1417] Several new treatment strategies are under investigation in an attempt to minimize some of the late effects of radiation therapy, as seen in long-term survivors of CNS germinomas.[1418] Administering an intermediate radiation dose of

TABLE 57-7 Common Marker Analysis in Central Nervous System Germ Cell Tumors*

Type of Tumor	Placental Alkaline Phosphatase	Oct-4	c-kit	AFP	β-HCG	Cytokeratin	Cerebrospinal Fluid Marker Positivity
Germinoma	+	+	+	–	+	–	c-kit, low level of β-HCG†
Yolk sac tumor	–	–	–	+	–	+	AFP
Embryonal carcinoma	–	–	–	–	–	+	–
Choriocarcinoma	–	–	–	–	+	+	β-HCG
Mature teratoma	–	–	–	–	–	–	–
Immature teratoma	–	–	–	–	–	–	–
Teratoma with malignant transformation	–	–	–	–	–	–	–

AFP, α-Fetoprotein; *β-HCG*, β-human chorionic gonadotropin; *Oct-4*, octamer-4.

*The common staining patterns provided do not include positive reactions in small subsets of cells.

†Germinomas can express low concentrations of β-HCG (50-100 mIU/mL), depending on institutional preference.

approximately 4000 to 5000 cGy encompassing the brain and ventricles is curative treatment for most patients with localized intracranial germinomas. Craniospinal radiation therapy is no longer routinely used for this population. Although whole-brain radiotherapy (WBRT) has been used in the past, whole ventricular fields are now recommended to spare the outer margin of the cortex without increasing the risk of recurrence. Typically 3000 cGy is administered to the whole ventricular region and the primary tumor receives an additional 1500 cGy. This therapy is effective, with 10-year EFS in excess of 90% and distant recurrences such as spinal metastases rarely occurring.[1419] Concurrent pineal and pituitary tumor locations represent regional rather than metastatic disease and can also be adequately treated with whole ventricular radiation therapy.[1420] Like other patients with brain tumors receiving radiation therapy, these children often experience late consequences of radiation therapy, such as cognitive and endocrine deficiencies and radiation therapy–induced secondary tumors.[1418] This outcome has led to the development of treatment regimens using adjuvant chemotherapy followed by response-based radiotherapy to permit a selective reduction in dose (from 4500 to 3000 cGy) in patients whose tumors completely disappear after two to four courses of chemotherapy.[1421-1424] A similar approach has been used with success by the Japanese cooperative group, including an intermediate-risk group of germinomas with elevated β-HCG.[1425] Decreasing the volume from whole ventricle to involved field is not sufficient because a number of failures have been noted with this approach.[1426,1427] The optimal dose and field of radiation and the best combination of chemotherapy are still being investigated and will require further follow-up to confirm efficacy and decreased toxicity. Proton radiation therapy is favored over photon therapy when it is available because efficacy seems equivalent but potential toxicity may be reduced.[1428] In one study attempts at treating patients with chemotherapy alone have demonstrated significant responses but a recurrence rate of approximately 50%.[1429,1430] Although these patients had an excellent salvage rate with radiation therapy, a higher craniospinal dose of 3600 cGy is then required. For this reason chemotherapy alone is not used for germinomas outside of formal clinical trials.

Relapse germinoma is rare, but patients who have a relapse may still respond well to treatment, including additional radiation, chemotherapy, and high-dose chemotherapy with autologous transplant.[356,1431]

For patients with NGGCT, more aggressive chemotherapy, radical tumor resection, and high-dose and high-volume radiotherapy are required for survival.[1421,1432-1435] NGGCTs are highly responsive to chemotherapy, as was seen in one study with 16 of 17 patients achieving a CR or PR with two cycles of cisplatin and cyclophosphamide-based therapy, one third of whom were long-term survivors.[1432] The use of consolidation with high-dose chemotherapy and stem cell rescue is being developed in patients with residual disease[348] and appeared to further improve outcome in a small pilot series.[1436] It is not clear if patients with focal NGGCT require CSI, and this question is currently being evaluated in a large prospective cooperative group trial. Many patients initially treated with chemotherapy who have progressive disease can undergo salvage therapy with high-dose chemotherapy and craniospinal radiation therapy.[1437] Immature teratomas are classified as NGGCTs but can often be treated with a more conservative approach. A complete resection may be sufficient therapy, and the prognosis is good. Patients with incomplete resection likely require adjuvant therapy.[1406]

After treatment GCTs can demonstrate significant progressive growth on an MRI scan with concurrent clinical decline in the setting of normal tumor markers (β-HCG and/or AFP). Although a nonsecreting germ cell recurrence cannot be excluded, most of these cases represent growing teratoma syndrome.[1438,1439] These lesions are nonmalignant progression of the teratomatous component of lesions that, when resected, will relieve the clinical symptoms.[1438,1440] Patients with growing teratomatous syndrome are not considered to have progressive disease, in spite of the dramatic increase in the size of the lesion on MRI. Neither radiation therapy nor chemotherapy should be discontinued in these patients.

Prognosis

Patients with germinoma have an excellent prognosis because of the tumor's sensitivity to radiation and chemotherapy. For these patients the major treatment challenge is maximizing the quality of life and limiting long-term sequelae of therapy. The COG is currently conducting a randomized phase III study comparing standard radiation therapy alone versus chemotherapy followed by response-dependent reduced-intensity radiation therapy, in which the primary outcome measures are PFS, OS, and quality of life. For patients with germinoma who progress after radiation therapy, many can undergo salvage therapy with high-dose chemotherapy, with or without stem cell transplantation, followed by a higher dose and volume of radiation therapy.[1441,1442] Chemotherapy and radiation therapy for patients not previously treated with radiation therapy can also lead to a high salvage rate.[1437]

Nongerminomas have a less favorable prognosis and thus clinical trials are evaluating conventional multiagent chemotherapy followed by dose-intensive myeloablative chemotherapy and second-look surgery for poor responders, followed by high-dose craniospinal radiation therapy. A small fraction of these patients will respond to high-dose chemotherapy and stem cell rescue,[1442] and long-term survival will improve, particularly if they can be brought back to a state of minimal residual disease.[1443] The response rate of recurrent NGGCT to second-line chemotherapy is poor. Poor prognostic factors in NGGCTs include degree of resection, presence of hemorrhage, and metastases.[1444] A rise in CSF β-HCG can be a sensitive marker of pending relapse and was identified well in advance of elevated levels in the serum.[1397]

Craniopharyngiomas

Craniopharyngiomas are benign nonglial tumors in children and account for 3% to 5% of all pediatric brain tumors, with a peak age ranging between 6 and 14 years.

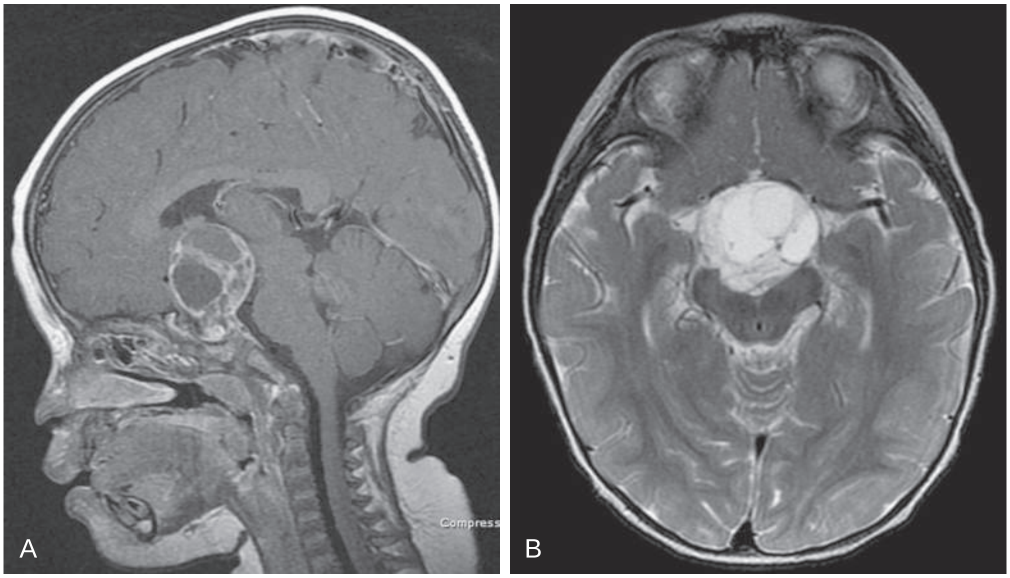

Figure 57-48 Craniopharyngioma with multiple cysts. **A,** A sagittal T1-weighted image with use of contrast material. **B,** An axial T2-weighted image.

These tumors arise from Rathke pouch epithelium and are classified as WHO grade I tumors.[1445] Craniopharyngiomas are slow-growing tumors that arise in the sella and parasellar regions; they are composed of both solid and cystic components, which often extend into the parasellar cisterns and occasionally invade adjacent cortical and vascular structures.[1446] Calcification is a common finding of these lesions,[1447] although it is not pathognomonic for these tumors. Compression of critical intracranial structures can lead to pituitary, hypothalamic, and optic dysfunction. As a result these patients often have complicated medical courses and long-term sequelae. Nigerian and Japanese children appear to have a greater risk for these tumors.[1370,1448] A strong association between β-catenin mutations and adamantinomatous craniopharyngioma has been identified.[1449,1450]

Clinical Presentation

The typical onset of craniopharyngioma is insidious and can extend over several years. The diagnosis is often made in retrospect upon recognizing one or more slowly evolving symptoms, such as progressive visual loss, delay in sexual maturation, growth failure, weight gain, and DI.[1451] Eventually clinical recognition is heralded by a change in mental status as enlargement of the cystic component causes obstructive hydrocephalus. More than 70% of children have growth hormone deficiency, obstructive hydrocephalus, short-term memory deficits, and/or psychomotor slowing at the time of diagnosis. The presenting feature in young adults also includes other symptoms of hypopituitarism, such as galactorrhea or amenorrhea in females and impotence in males. The differential diagnosis of a suspected craniopharyngioma is limited because of the characteristic radiographic findings of these lesions.[1452] Congenital craniopharyngioma associated with a paraneoplastic expression of parathyroid hormone–related protein expression has been reported.[1453]

Imaging and Histology

MRI features (Fig. 57-48) usually include a multicystic and solid enhancing suprasellar mass.[1454] The T1-weighted signal is usually isointense. The chiasm is often stretched over the suprasellar mass and, if dorsal extension is sufficient, hydrocephalus will be apparent.[1455] A classic neuroimaging distinction of craniopharyngiomas from other suprasellar tumors such as a diencephalic glioma or GCT is the presence of calcifications on a noncontrast CT scan. With use of contrast material, the solid components of craniopharyngiomas are usually bright on CT. Transcranial Doppler can also be used in the diagnosis and surveillance of these tumors.[1456]

Histologically craniopharyngiomas are divided into adamantinomatous and papillary subtypes. Adamantinomatous craniopharyngiomas are the more common variant in adults and children and typically consist of cystic and solid areas, with frequent calcifications. Papillary tumors, which are seen almost exclusively in adults, are predominantly solid, without calcification, and are less infiltrative. The cysts seen in craniopharyngioma usually contain a dark liquid with the consistency of machine oil. The diagnosis of adamantinomatous craniopharyngioma requires the presence of squamous epithelium bordered by palisading columnar epithelium.[1445] Gliosis and Rosenthal fibers can be observed but rarely result in misdiagnosis because of the other characteristic features of craniopharyngioma. Palisading columnar cells can have a high MIB1 labeling index rate ranging from 0% to 15%, but this finding does not correlate with a poorer outcome than for patients with a low MIB1 labeling index rate.[1457,1458]

Management

Surgical resection remains the standard approach for craniopharyngioma in many centers, both through an

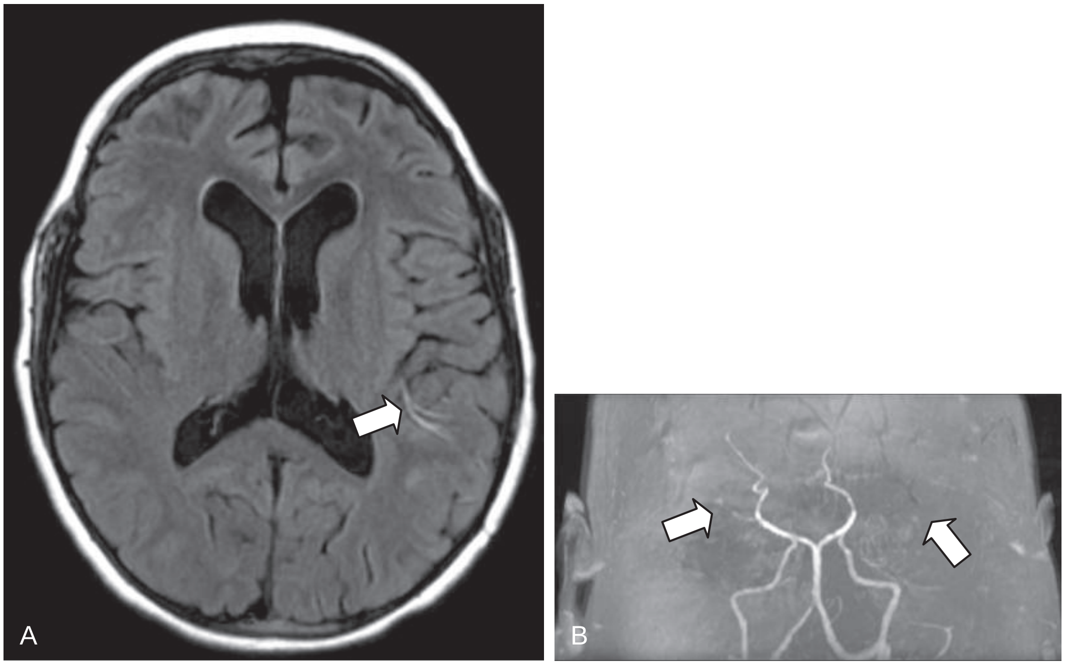

Figure 57-49 Radiation-induced moyamoya disease. **A,** An axial fluid-attenuated inversion recovery image showing the "IVY" sign of slow flow *(arrow)*. **B,** Coronal reconstruction from a maximal intensity projection image from magnetic resonance angiography demonstrating missing lateral vessels *(arrows)*.

open approach and via an endoscopic endonasal approach.[1452,1459,1460] When the child presents with acute raised ICP, the initial management will relate to measures to alleviate this condition with high-dose corticosteroids and monitoring in the intensive care unit. Because patients may have unappreciated panhypopituitarism, baseline hormonal and electrolyte levels should be obtained and the child should be given empiric stress-dose steroids.

Although it is somewhat controversial, a complete microsurgical resection of the entire tumor is the treatment of choice for newly diagnosed craniopharyngiomas.[1461] A multistaged operative approach may be planned, including a combination of transsphenoidal, pterional, and transcallosal approaches, depending on the extent and location of the tumor. Many tumors are adherent to adjacent structures, which will either limit the degree of resection or result in potentially significant postsurgical morbidity.[1446]

Despite the surgical accessibility of many of these tumors, radical resection does not guarantee recurrence-free survival, and the 3-year EFS after a GTR is 60%.[1462] Aggressive resection can result in more extensive hypothalamic deficiencies and visual complications,[1459,1463] even in patients without evidence of hypothalamic involvement at diagnosis.[1464] Careful management of DI can be required immediately after surgical resection in these patients,[1465] which may lead to a permanent condition. Postoperative behavioral sequelae include altered regulation of appetite and weight control, impulsivity, hypersexuality, and changes in memory.

An alternative treatment strategy for the long-term control of craniopharyngiomas is subtotal resection[1466] followed by high-dose radiotherapy of the involved field.[1467-1469] Whether this approach is superior in terms of tumor control and preservation of quality of life remains to be established, but several studies have reported comparable or even improved PFS and OS statistics in retrospective, uncontrolled institutional series.[1462,1470-1473] In patients with recurrent craniopharyngioma, an attempt at a complete resection can be considered, although the need for focal radiation therapy will be required for most of these patients. Proton therapy, based on the lack of an exit dose, is considered the preferred method of radiation therapy if it is available locally.[1474,1475] Long-term complications of radiation therapy include secondary malignancies, optic neuropathy, and vascular injury leading, rarely, to moyamoya disease (Fig. 57-49).[85,1476-1478] The recent identification of the BRAF V600E activating mutation in almost all papillary craniopharyngiomas, which are usually seen in adults,[1479] offers this patient population the option of targeted therapy.[1480] The adamantinomatous subtype, which is found predominantly in children and is characterized by mutations in B-catenin, will need to await development of improved inhibitors of this pathway.

A temporizing approach to control the cystic components of the tumor is the intracystic instillation of sclerosing agents such as bleomycin or phosphorus-32.[1481-1483] The use of intralesional bleomycin has also been shown to help delay the need for aggressive surgery or radiation therapy, although prospective clinical trials of this approach are needed.[1484] This procedure has the risk of leakage of the bleomycin from the cyst into adjacent brain tissue and should be reserved for patients for whom all other management approaches have failed.[1485] However the solid tumor usually continues to grow and make new cysts, thus making subsequent resections more difficult. In addition subsequent external beam radiotherapy treatment planning may be difficult because of the unpredictable dosimetry of ^{32}P.[1482] Recently pegylated interferon alpha 2b has been used to stabilize cystic progression in patients with craniopharygioma.[1486]

Recurrent or progressive craniopharyngiomas may be treated with surgery, radiosurgery, fractionated radiotherapy, or chemotherapy. Responses to chemotherapy have been reported with interferon, vinblastine, cisplatin, pegylated interferon,[1487] and other agents,[1471,1488-1493] although well-designed studies to test different agents for craniopharyngioma are lacking.[1494]

Prognosis

The most important factors that correlate with PFS are the extent of resection and postoperative radiation therapy. Larger tumors and those with cysts are more likely to recur.[1495] In a surgical series, recurrence or progression occurred in 7% to 15% of cases after total resection and in 50% of cases after subtotal resection.[1496] The recurrence rate of 15% is observed when subtotal resection is followed by radiation therapy.[1497] The outcomes between adult and pediatric patients does not appear to be different.[1498] Recurrences along the surgical tract have been reported but are thought to be related to the procedure rather than representing metastatic disease and are treated with surgery and focal irradiation, if needed.[1499] Unfortunately many long-term survivors experience significant morbidity related to panhypopituitarism,[1500] cognitive impairment, sleep pattern disturbance, and obesity.[1498,1501-1505] Growth hormone deficiency is present in 100% of patients,[1506] but the early use of growth hormone in these patients is not thought to be contraindicated.[1507,1508] Although narcolepsy is uncommon,[1509] control of weight and regulation of sleep can be difficult because their circadian rhythms are disrupted and melatonin production is severely diminished.[1510] The use of stimulants has been investigated, with preliminary positive results,[1511] although clear efficacy for these interventions is lacking.[1512-1514] Although still in the early stages, attempts to regulate obesity in these patients have also demonstrated some success.[1515] Finally, psychosocial problems can be significant in this patient population, and a comprehensive team approach is beneficial.[1516]

Infant Tumors

Infants and young children with brain tumors present specific therapeutic challenges because of the morbidity associated with current treatment strategies. As a group infants and young children with brain tumors have poor outcomes, with 5-year EFS and OS rates as low as 19% and 25%, respectively.[1517] This poor prognosis can be related to unique characteristics of the tumors in this age group, as well as the significant limitations with regard to therapy. Evidence from numerous sources has suggested that neural development extends from the embryonic period through adolescence and is especially prominent during the first 3 years of life, making young children most susceptible to neurotoxic insults.[1518] These findings are in keeping with early observations that the most devastating consequences of radiotherapy in children, in terms of neuropsychological sequelae, were greatest in children younger than 3 years.[1519] Although early observations have led to the arbitrary age limit of 3 years as the cutoff for introduction of radiation therapy, numerous subsequent studies have shown that even older children can experience cognitive, endocrine, behavioral, and many other medical problems after undergoing radiation therapy.[1520,1521] Consequently several strategies have emerged to delay or eliminate radiotherapy through the use of chemotherapy in an attempt to preserve cognitive, neurologic, and endocrine function in younger children.[1522,1523]

A significant proportion of pediatric brain tumors occur in infants and young children. Data from the Childhood Brain Tumor Registry of the United States from 2000 to 2004 have shown that approximately 28% of childhood brain tumors occur in children younger than 4 years[1524] and 18% of brain tumors occur in children younger than 2 years.[1525] Tumor location in infants and young children is different from that in the older child, with a predominance of supratentorial tumors.[1525-1527] Seventy percent of tumors that present in the first week of life are also located supratentorially[1528] compared with 56% for the pediatric population.[1529] The male to female ratio in this age group is also different in that there is a lower male predominance in this age group[1526] when compared with childhood brain tumors overall. Histopathologic subtypes in infants and young children are also different from those in the older child, but accurate determination of this difference has been difficult because early epidemiologic data collected by the traditional data collection agencies such as the SEER registry and National Brain Tumor Registry did not have standardized histologic subtype information. For example PNET was not previously recognized as a separate entity in the SEER Registry. In the large cooperative groups brain tumors were categorized differently, and thus the POG excluded medulloblastoma from PNET protocols, although the CCG routinely included medulloblastoma in PNET protocols.[1522] The distribution of tumor types treated in the infant POG clinical trial ("Baby POG") was medulloblastoma, followed by ependymoma, PNET, and malignant glioma.[1530] However in a review of 1289 patients aged younger than 1 year reported in the literature, astrocytoma was the most common diagnosis, comprising 31% of cases (of which 75% were low grade and 25% were reported as high grade), followed by medulloblastoma at 12%, ependymoma at 11%, choroid plexus tumors at 11%, sPNET at 7%, and teratoma at 5%.[1526] Astrocytoma and medulloblastoma remain the most common in the young age group, although a peak of ependymoma is seen within the first 2 years of life, along with an increased incidence of PNET.[1517] A recent analysis of the SEER database has revealed that the incidence of brain tumors in infants and young children has remained stable since 1986. The most common histologies in the dataset were glioma, medulloblastoma, and sPNET and ependymomas.[1531]

Two important histologic diagnoses in infants include teratomas and ATRTs, which account for 4.9% and

0.3%, respectively, of infant brain tumors.[1526] Briefly, ATRT is a rare tumor of childhood (see the earlier section on ATRT), which has only recently been recognized as a separate entity; 75% of these tumors carry a unique chromosome 22q11.2 cytogenetic abnormality.[1291] An initial description of malignant rhabdoid tumors of the kidney,[1532] followed by the description of the simultaneous appearance of renal and CNS tumors with similar histologic features,[1533] eventually resulted in the recognition that this unique histology represents a separate entity.[1257,1300] ATRT primarily affects young children at a mean age of 17 to 24.5 months, with 75% occurring in children younger than 3 years.

Clinical Presentation

The clinical presentation of brain tumors in infants and young children is most often associated with the symptoms of obstructive hydrocephalus, which in this age group manifests as persistent vomiting, irritability, lethargy, abnormal gait and coordination difficulties, failure to thrive, bulging and splayed fontanelles, seizures, and loss of developmental milestones. In a meta-analysis of 13 studies reported in the literature consisting of 332 children younger than 4 years presenting with intracranial tumors, increasing head circumference and macrocephaly was reported as the most common presentation (41%). Other presentations included focal motor weakness, head tilt, squint, abnormal eye movements, altered levels of consciousness, and hemiplegia.

Management

The historic approach to treating infants and young children with brain tumors had focused on the use of surgery and radiation. In the late 1960s serious functional and neurocognitive sequelae that were incompatible with a normal productive life, especially for children younger than 2 years, were recognized.[1519] As a result several different strategies to delay or exclude radiotherapy from the treatment of young children with brain tumors were developed and were centered on the use of chemotherapy.[1522,1523,1534,1535] van Eys and coworkers[1536] (1985) treated 12 young children with medulloblastoma using mechlorethamine, Oncovin (vincristine), procarbazine, and prednisone chemotherapy without radiotherapy and found that 8 of 12 patients were long-term survivors. This strategy led to other clinical trials, including those investigated by the POG, which worked on the premise of giving chemotherapy until the child achieved an age at which radiotherapy was accepted as less detrimental and its benefits could still be realized.[1522,1523] The "Baby POG" protocol, initiated in 1986, enrolled 198 children, 132 of whom were younger than 24 months, who were treated with chemotherapy for 2 years, and an additional 66 children between the ages of 24 and 36 months were enrolled, who received chemotherapy for 1 year prior to receiving radiotherapy.[1530] Chemotherapy consisted of two 28-day cycles of cyclophosphamide plus vincristine followed by one 28-day cycle of cisplatin and etoposide; these cycles were given for 1 or 2 years, depending on the age of the child at diagnosis or until there was disease progression, after which radiotherapy was administered. After two cycles of cyclophosphamide and vincristine, 39 of 109 evaluable patients showed a CR or PR, although patients with brainstem glioma and PNET did poorly. The PFS at 1 year for children diagnosed between the ages of 24 to 36 months and PFS at 2 years in children diagnosed at younger than 24 months were 41% and 39%, respectively. The 5-year PFS was reported as 30% ± 5%, and the 5-year OS was 39% ± 4%. The single most important predictor of survival was the degree of surgical resection, because children with a GTR had a 5-year OS of 62% ± 7% compared with those with a subtotal resection, whose 5-year survival was only 31% ± 5%. Of great importance in this landmark study was its demonstration that a small subset of children could achieve disease control without radiotherapy, and that even a delay of 1 or 2 years did not adversely affect outcome in all patients.[1522,1523,1534] Other study groups have adopted a similar approach of delaying radiotherapy through use of chemotherapy. The national CCG-9921 clinical trial for infants with medulloblastoma, CNS PNET, and ependymoma used upfront chemotherapy for 1 year followed by focal radiotherapy to the involved site or craniospinal radiotherapy.[1537] This was an "8-drugs-in-1-day" clinical trial that included 46 children with medulloblastoma who were younger than 18 months at the time of diagnosis, most of whom had gross total resections and were able to avoid radiotherapy; 22% were progression-free at 3 years.[1537] The German Pediatric Brain Tumor Study Group pilot tested an intensive chemotherapy regimen consisting of procarbazine, ifosfamide, cisplatin, cytarabine, vincristine, and high-dose methotrexate for children to delay or avoid radiotherapy (trial HIT-SKK87). A direct comparison to the results of Baby POG cannot be made because the results of HIT-SKK87 have not yet been published. The United Kingdom Children's Cancer Study Group piloted a Baby Brain protocol in 1989 using weekly alternating cycles of myelosuppressive and nonmyelosuppressive chemotherapy consisting of vincristine and carboplatin, vincristine and methotrexate, vincristine and cyclophosphamide, and cisplatin to delay or avoid radiotherapy. Although only 28 children were treated with the protocol, the 4-year OS was 35%.[1538]

The overall results of these "Baby" chemotherapy protocols were thus somewhat disappointing, with EFS rates of 20% to 40%, although the ability to defer radiation therapy in this patient population was an important advance. Given the high response rate for infants treated according to these protocols, strategies centered on high-dose chemotherapy with ASCR were developed to improve the response rates already being observed.[728,1535] The French Society of Pediatric Oncology used this approach of high-dose chemotherapy with autologous bone marrow rescue (ABMR) in their BBSFOP protocol in an attempt to extend survival of young children with relapsed or progressive disease who received conventional chemotherapy. Twenty children (median age, 23 months) with medulloblastoma in whom progressive disease developed while they were treated with conventional chemotherapy were then treated with high-dose busulfan and thiotepa followed by ASCR. Of these patients 75% demonstrated a radiographic response to chemotherapy.

For patients with initial local relapses only, an EFS rate of 50% and a median survival rate of 39.5 months were achieved.[1539] A follow-up report of this study demonstrated a 69% 5-year OS in patients with local relapse while eliminating the need for craniospinal radiotherapy in 30% to 50% of patients. The need for a complete resection limits use of this strategy for many patients, especially those with metastatic disease.[1534]

Another chemotherapy-based approach has been the “Head Start” protocols, in which conventional induction chemotherapy followed by high-dose chemotherapy with autologous stem cell transplantation is used in the newly diagnosed setting to delay or defer radiation therapy in infants and young children. “Head Start I” consisted of five induction cycles of cisplatin, vincristine, etoposide, and cyclophosphamide. After this treatment patients with no radiologic evidence of disease, or after second-look surgery or reresection of tumor, underwent myeloablative therapy with carboplatin, thiotepa, and etoposide with ABMR. Radiotherapy was deferred in patients who achieved complete remission. Of 62 children enrolled (median age, 30 months), 37 patients proceeded to ABMR, of whom 15 patients were free of disease and required no further treatment; this outcome resulted in a 3-year EFS and OS of 40% and 25%, respectively. Seven deaths occurred as a result of treatment-related complications. These results are similar to those noted earlier and again demonstrate that a subset of patients can be effectively treated with chemotherapy and avoid radiotherapy.[1540] Head Start II further intensified the induction regimen with the addition of high-dose methotrexate for the patients with the highest risk, specifically patients with high-risk tumors (nonmedulloblastoma) or those presenting with evidence of metastatic spread. A total of 21 patients with disseminated medulloblastoma were treated, 17 of whom had a CR yielding a 3-year EFS and OS of 49% and 60%, respectively.[1153] In this cohort of patients, 13 had a GTR and 10 patients received radiotherapy as part of their upfront or salvage therapy; six patients avoided radiotherapy altogether. For patients with CNS PNETs, this approach appears to confer an improved 5-year EFS and OS of 39% and 49%, respectively.[1249] Patients with nonpineal CNS PNET fared better than those with pineal-based tumors, although metastatic disease at diagnosis, age, and extent of resection were not significant prognostic factors; 60% of survivors were able to avoid radiotherapy. In contrast, this approach has not demonstrated equivalent results for patients with ependymoma with an estimated 5-year EFS and OS of 12% and 38%, respectively, with younger age as the only significant prognostic factor.[969] In this cohort of 29 children with ependymoma, 22 were younger than 36 months and only 8% of patients were radiation-free at 5 years.

To further increase chemotherapy dose intensity, other investigators have followed induction chemotherapy with tandem cycles of high-dose chemotherapy with stem cell rescue. In a small series of 15 infants between 4 and 38 months old with varied histologies, including five medulloblastomas, four CNS PNETs, five malignant gliomas, and one ependymoma, patients were treated with induction chemotherapy. This treatment consisted of three cycles of cisplatin, cyclophosphamide, and etoposide followed by three cycles of tandem high-dose therapy with carboplatin and etoposide and ASCR.[1541] Although the follow-up period was short, the 2-year PFS and OS rates of 52% and 72%, respectively, is encouraging. Furthermore, of the 10 patients who were alive, only five received local radiotherapy, and only one patient with M1 stage medulloblastoma received craniospinal radiotherapy.[1541] Similar results have also been reported by the French Society for Pediatric Oncology in a small series of seven patients with medulloblastoma and CNS PNET using high-dose therapy with busulfan and melphalan.[1542] Although three patients received additional treatment with thiotepa and two patients received additional treatment with topotecan, all patients were able to avoid radiotherapy. The PFS was 71% ± 17%, although longer follow-up will be needed.

With regard to the long-term benefits of adjuvant chemotherapy strategy of delaying or deferring radiotherapy, authors of one infant study evaluated their neuropsychological outcomes in a follow-up report. Of the six children who did not receive radiotherapy, all had intelligence quotient (IQ) scores within the normal range, with a mean of 101, although the five children who had received radiation therapy had lower IQs, with a mean of 85 at 5.8 years and a continued decline to a mean of 63 by 10 years.[1543]

In addition to the use of intensive chemotherapy to defer or omit radiation therapy, radiation techniques themselves have also been optimized in attempts to minimize long-term morbidity. This optimization includes the administration of involved field radiation for infants with brain tumors[291,1130,1535] and the use of proton beam radiation therapy.[261,1544] The Pediatric Brain Tumor Consortium also completed a pilot study to assess the feasibility of adding intrathecal mafosfamide to multisystemic chemotherapy followed by conformal radiation therapy.[291] This study included 93 newly diagnosed infants with embryonal CNS tumors and resulted in a 5-year OS rate of 51% ± 11%.

Specific Management Issues in Infants and Young Children

A more detailed description of the diagnosis and management of individual disease categories was provided earlier, but management issues specific to infant tumors are described here.

Infant Medulloblastoma. Despite significant improvements in the treatment of standard-risk medulloblastoma in older children, the outcome of infants and young children with medulloblastoma remains poor, mainly because of limitations in the use of radiotherapy to the developing brain, increased risk of metastatic disease at presentation, and difficulty in achieving a complete surgical resection in infants.[1545] Although the strategies of delayed radiotherapy with chemotherapy and intensification of treatment with stem cell rescue have shown some promising results in infants and young children, the small number of patients, heterogeneous histologies, and varied staging limit their immediate widespread adaptation.[1153,1541,1542] Salvage therapy in infants and young children has also been shown to be feasible in up to 50% of patients but requires the use of radiotherapy. Thus some cognitive impact and compromise will be required in these patients if disease control is to be achieved with this modality.[1546] Attempts at reduction in radiotherapy dose to reduce neurodevelopmental damage can significantly compromise OS.[1547] Alternative therapeutic strategies are therefore needed in the treatment of medulloblastoma of the infant and young child.

With increasing understanding of the biology of medulloblastoma, it has been recognized that the frequency of different histologic subgroups varies with different stages of development. The nodular-desmoplastic subtype of medulloblastoma occurs most frequently in infants and young children and has been found to be most frequently associated with the SHH subtype and is associated with favorable prognosis.[1548] It has also been reported that children younger than 3 years of age who have nodular-desmoplastic medulloblastoma have a high frequency of germline mutations in the negative regulator of SHH pathway signaling, SUFU.[1549] Although SHH-positive desmoplastic medulloblastomas also occur in adults, those that occur in infants have been found to be molecularly distinct to their adult counterparts.[1550] Desmoplasia is not prognostic in the adult tumors, which have an inferior outcome compared with those that occur in infants.

The incorporation of high-dose methotrexate in chemotherapy regimens has been investigated. In the German HIT-SKK study, intrathecal methotrexate and systemic methotrexate were administered to 43 children younger than 3 years who had medulloblastoma, including 26 patients with M+ disease. The 5-year OS for patients with completely resected tumors, residual tumor, and macroscopic metastases was 93% ± 6%, 56% ± 14%, and 38% ± 15%, respectively, an improvement compared with historic control subjects.[1097] Only 38% of survivors received radiotherapy. Of note desmoplastic histology was highly predictive of good outcome, with an OS of 95% ± 4% versus an OS of 41% ± 11% for patients with classic histology; histology was found to be an independent prognostic marker, along with age younger than 2 years. Although outcomes for children with localized disease or favorable histology was excellent without the use of radiation therapy, the poor outcomes of patients with metastatic disease or unfavorable histology led to the inclusion of involved-field radiation therapy to this treatment protocol.[1551] High-dose methotrexate has also been investigated in the "Head Start II" protocol and was shown to be particularly effective for young patients with high-risk medulloblastoma.[1153] The major concern with the use of methotrexate is that this agent has been associated with white matter changes on MRI, especially when methotrexate is administered after radiation therapy.[1552,1553] In spite of this finding the use of methotrexate continues to be investigated, given the overall poor prognoses for this subset of patients. Stratification of risk groups through better histologic diagnosis, together with an improved understanding of the biology of medulloblastoma, may help improve survival in this age group.[1545,1554]

A recent study that included a cohort of 12 patients with medulloblastoma assessed outcomes of infants and very young children treated with surgery, with adjuvant high-dose chemotherapy followed by proton beam radiation.[1544] Longer-term follow-up of this cohort is required to document both survival outcomes and long-term morbidity. In COG study P9934, children were treated with induction chemotherapy after surgery, followed by age- and response-adapted conformal radiation therapy to the posterior fossa and maintenance chemotherapy.[1130] Four-year EFS and OS were 50% ± 6% and 69% ± 5.5%, respectively. Consistent with other infant studies, children whose histologies were desmoplastic medulloblastoma had superior outcomes compared with those without desmoplasia. Of note the children who experienced recurrence after administration of conformal radiation therapy had progression of their disease outside the radiation field.

Infant CNS PNETs and Pineoblastoma. CNS PNET is a neuroepithelial tumor similar to medulloblastoma but with a significantly worse prognosis.[1210,1555] The most recent WHO classification clarifies the nomenclature of this heterogeneous group of tumors. It was traditionally referred to as sPNET and is now called CNS PNET, not otherwise specified, and includes biologically similar tumors that can also be found in the brainstem and spinal cord.[207] Even though medulloblastoma and CNS PNET are classified together as embryonal tumors, cytogenetic[1238,1239] and molecular evidence from microarray analyses[1029] suggests that biologic differences exist between these tumors. Pineoblastomas are also tumors of embryonal origin with a clinically aggressive course.[1187]

CNS PNETs are the fourth most common brain tumors of infants[1526] and account for approximately 10% of all brain tumors occurring in children younger than 3 years.[1556] It is also the most common malignant brain tumor in the first year of life[1525] and, according to SEER data, has a dismal OS of 19% in this age group.[1517] In a national clinical trial, POG 8633, 13 patients with nonpineal CNS PNET who were younger than 3 years were treated. These patients presented with large, well-demarcated tumors with limited edema. In this study CNS PNET was the most common supratentorial tumor in this age group. Presenting symptoms included seizures, nausea, vomiting, lethargy, irritability, headache, focal motor weakness, and increased head circumference; 12 of 13 subjects had symptoms for less than 1 month at the time of presentation.[1557] For unknown reasons, younger children with CNS PNETs and pineoblastomas present with more aggressive disease and a higher frequency of leptomeningeal disease.[1558,1559] Whether this manifestation is inherent to the biology of the disease or related to clinical factors remains controversial. In a retrospective review of 50 patients with CNS PNET, metastatic disease at diagnosis, extent of initial resection, and tumor site did not affect OS, although it was affected by age younger than 2 years and the use of radiotherapy and chemotherapy.[1216] Similar findings were also reported in 46 patients with CNS PNET registered in the CCG-9921 study; treatment regimen, M stage, extent of resection, and age were not found to be significant prognostic factors.[962] In contrast in the "Baby POG" study, 28 patients with CNS PNET, including 11 with pineoblastoma, were treated, all of whom received chemotherapy and delayed radiotherapy.[1522] This study showed a considerable difference in the 3-year survival between the four patients in whom GTR was achieved (100% survival) compared with the nine patients with a subtotal resection (11% survival). Authors of this study also concluded that chemotherapy did not provide benefit.[1522] No benefit from chemotherapy was seen in the French BBSFOP protocol for 25 patients with supratentorial embryonal tumors, which included 17 patients with CNS PNET treated with chemotherapy in order to delay or omit radiotherapy. Of the five patients with CNS PNET who survived, four underwent salvage therapy with high-dose chemotherapy and/or surgery and radiotherapy.[1560] Results from the Head Start studies have also suggested a benefit from intensification of chemotherapy because 60% of this cohort of 43 patients with CNS PNET avoided radiotherapy and achieved an estimated 5-year EFS and OS of 39% and 49%, respectively.[1249] Interestingly metastasis at diagnosis, age, and extent of resection were not significant factors, although numbers were small and the interpretation of individual subgroups is limited.

Pineoblastoma in infants has been associated with a dismal outcome. In the "Baby POG" study 11 patients were treated, ranging from 1 to 35 months (eight patients were younger than 12 months), and all patients had local recurrences, the majority with metastatic disease. All children died 4 to 13 months from the time of diagnosis, despite the use of radiotherapy in six patients.[1194] Similar dismal results have been reported in the CCG 921 protocol in which eight infants with predominantly nonmetastatic, subtotally resected tumors all had progression of their disease within 3 to 14 months (median, 4 months), with a median time to death of 10 months.[1561] Similarly five children younger than 3 years with pineoblastoma, treated according to the German Pediatric Oncology Group HIT-SKK87 and HIT-SKK92 protocols, all had progression of their disease within 6 months and had a median overall survival of 0.9 year.[1558] Although there have been reports of better survival rates in patients with pineal region PNETs, these patients tended to be older and more often received radiotherapy.[1192,1561]

A treatment strategy for infants and young children with CNS PNETs may involve intensification of treatment, as demonstrated by the "Head Start I and II" experience in which 5-year EFS and OS rates of 39% and 49%, respectively, were achieved.[1249] High-dose chemotherapy and ASCR have also been reported to be successful in a series of newly diagnosed children and adults, which included two infants who avoided radiotherapy. The 4-year PFS and OS rates of 69% and 71%, respectively, were observed.[1562] In contrast the German HIT-SKK87 and HIT-SKK92 studies had PFS and OS rates of only 15% and 17%, respectively, for 29 children between the ages of 3 and 37 months who were treated with chemotherapy to delay radiotherapy. A positive impact on survival was seen in patients who were able to achieve complete resection and those who received radiotherapy.[1563] The use of radiotherapy in some of these patients may improve survival, but radiation therapy diminishes the quality of survivorship.[1564] Findings from the CCG 921 study showed that all children with CNS PNET who received radiotherapy and were younger than 9 years experienced significant developmental delays.[1561] The current recommendations for infants with CNS PNETs include maximal safe surgery, multiagent chemotherapy, consideration of high-dose chemotherapy with stem cell rescue, and focal 3D conformal radiation therapy, if permitted by the field size and tumor location.

Infant Ependymoma. Ependymoma is the third most common tumor of infants and young children and the second most common CNS malignancy in children younger than 3 years who are referred for treatment.[1530] Approximately 43% of ependymomas occur in children younger than 2 years.[1233,1517] Unlike adult ependymoma, most ependymomas in infants and young children occur intracranially and, unlike other infant brain tumors, are predominantly infratentorial.[1565] Negative prognostic factors from several studies have demonstrated the importance of the extent of resection (less than GTR or biopsy) and young age (younger than 3 years at diagnosis).[981,1566,1567] Another prognostic factor that has been reported includes anaplastic histology. However the heterogeneity of anaplasia seen in tumors and the differences in the diagnostic criteria for anaplastic histology by pathologists, which have ranged from 7% to 89%, have made a full prognostic assessment of this factor difficult.[943] An MIB1 or Ki-67 index rating of 20 or higher[1568] may also be a negative prognostic marker.[1569] Although the 5-year OS for children with ependymoma has been estimated at 55% to 66%,[976,978] the 5-year survival for infants and young children remains around 25% and is worse if radiotherapy is delayed or a complete resection is not achieved.[963] The role of chemotherapy remains controversial in this population, even though cisplatin is an active agent against ependymoma.[1570] Several studies incorporating chemotherapy have shown lack of efficacy,[1570] including those with dose escalation such as in "Head Start."[969] In the "Head Start III" protocol, which consisted of maximal surgical resection followed by five cycles of induction chemotherapy with a myeloablative consolidation, excellent outcomes were reported only for infants with supratentorial ependymomas. Infants with infratentorial ependymomas had poor outcomes in the absence of radiation therapy.[1571] POG study 9233 ("Baby POG 2") reported improvement in PFS in infants with ependymoma after the administration of dose-intensified chemotherapy, but no difference in OS was observed.[968]

The importance of radiotherapy in the treatment of ependymoma has been clearly established, and stereotactic radiation therapy administration has been demonstrated to be safe and effective.[461] A phase II study of conformal radiotherapy treating 88 young children, many of whom were younger than 18 months (after chemotherapy and maximal surgical resection were completed), yielded a PFS of 75% ± 6% and reduced early neurocognitive deficits, even though younger patients received 5400 cGy of conformal radiotherapy, supporting the use of the modality in these patients.[951] Although long-term neurocognitive follow-up of this younger population is not yet known, this study formed the basis for the COG clinical trial of conformal radiotherapy, including short-duration chemotherapy in the subset of patients who achieved only subtotal resections. A previous CCG clinical trial (study 9942), in which children with incompletely resected ependymoma received upfront chemotherapy before radiation therapy, has also been completed. Experience from the United Kingdom Children's Cancer Study Group–International Society of Pediatric Oncology collaboration for patients with ependymoma has suggested some benefit from the administration of alternating courses of nonmyelosuppressive chemotherapy with myelosuppressive chemotherapy in patients with incomplete resections who do not have metastatic disease.[1572] In this series of 89 children younger than 3 years, in which nine patients had metastatic disease, preirradiation chemotherapy resulted in a 5-year EFS and OS of 42% and 63%, respectively. In addition the 5-year cumulative incidence of freedom from radiotherapy was 42% in the nonmetastatic cohort. Also patients who received the highest dose intensity had a 5-year OS of 76% compared with those with the lowest dose intensity, who had a 5-year OS of 52%.[1572] All patients with metastatic disease in this study experienced a relapse, and the 5-year OS in this group was approximately 25%.[1572] The outcome of patients with metastatic disease at presentation compared with those with localized disease has ranged from no difference in outcome[963,978] to relapse and death in all patients within 2 years of surgery.[1573] Fortunately fewer than 10% of patients with ependymoma present with metastatic disease, although this rate is slightly higher in infants.[963,978] In a retrospective meta-analysis of prognostic factors of 40 patients with metastatic ependymoma, including 29 children younger than 3 years, those in whom GTR was achieved had the best 5-year EFS and OS of 35% ± 13% and 59% ± 13%, respectively, compared with the 5-year EFS and OS of 25% ± 9% and 32% ± 10%, respectively, for those who did not have GTR.[1574] The EFS for patients who received radiotherapy was 57% ± 19%, and it was 40% ± 22% for those who received combined chemoradiotherapy. For patients treated with chemotherapy alone, the EFS was 20% ± 9%. Because no statistical difference was found in the 5-year OS between patients with M1 and M3 disease (51% ± 16% and 40% ± 9%), even patients with bulky disease can be treated if a GTR can be achieved.[1574]

Infant Glioma. Astrocytoma is the most common tumor in children younger than 3 years,[1556] as well as in infants younger than 1 year, and constitutes 30% of the total, of which 75% are low-grade tumors and 25% are reported as high-grade tumors.[1526] Management of these tumors in infants and young children mirrors the approaches used in older children, including observation for patients with completely resected low-grade lesions, chemotherapy for patients with symptomatic, progressive low-grade tumors, and chemotherapy and radiotherapy for patients with high-grade lesions. One major difference between young children and adults with HGGs is the improved prognosis observed in infants. In the "Baby POG series," 18 children younger than 3 years who were diagnosed with HGG (13 of whom were younger than 12 months) had 5-year PFS and OS rates of 43% ± 23% and 50% ± 14%, respectively.[758] Four survivors completed the 24 months of therapy but did not undergo irradiation because of parental refusal and remained without evidence of recurrence. Similar results have been reported in the French BBSFOP trial, in which 21 children younger than 5 years (13 with WHO grade III tumors and 8 with grade IV tumors) received postoperative chemotherapy and had 5-year PFS and OS rates of 35% and 59%, respectively. Of the 12 survivors, 10 did not receive radiotherapy at the time of reporting.[739] Another retrospective review of 16 patients younger than 3 years showed similar results, with 5-year EFS and OS rates of 29% (standard error [SE], 12%) and 66% (SE, 12%), respectively, although six patients received upfront radiation therapy and an additional six patients received radiation therapy at the time of progression.[1575] In the CCG-945 trial for children with newly diagnosed HGGs, children younger than 3 years were treated with the intent to avoid radiation therapy, including 49 children younger than 3 years. In this cohort of 49 children, the 10-year EFS and OS rates were 29% ± 6% and 37.5% ± 7%, respectively.[1576]

Infant CPC. Choroid plexus tumors account for 1% to 3% of childhood brain tumors, and approximately 30% to 40% are CPCs,[1577] with 70% occurring before the age of 2 years.[1578] Because of the association with Li-Fraumeni syndrome, analysis for the presence of *TP53* mutations is recommended.[1356] GTR has been shown to be prognostic[1579] although technically difficult to achieve because of the highly vascular nature of these lesions.[1526] The role of radiotherapy for the treatment of this highly invasive tumor is controversial.[1580,1581] In the "Baby POG" study it was reported that eight patients had choroid plexus tumors, two of whom were alive without use of radiotherapy.[1579] Other investigators have also reported long-term survival without use of radiotherapy in this group.[1582] Because the number of patients with this rare disease is so small, international collaboration is needed to determine the best treatment strategy for this group of patients. Currently maximal safe surgery and adjuvant chemotherapy constitutes a reasonable approach to avoid or delay radiotherapy,[1526] and salvage therapy is possible, not necessarily including craniospinal radiation therapy.[1583]

Other Tumors

Meningiomas

Meningiomas are rare pediatric tumors that are often seen in conjunction with either NF-2 or after radiation therapy[1584]; they account for 1% to 3% (range, 0.4% to 4.1%) of all childhood tumors of the CNS.[1585,1586] Familial meningiomas have been reported, and thus a detailed family history is required.[1587] In one study of children who received radiation therapy to the CNS for childhood leukemia, meningiomas were found in 20% upon routine

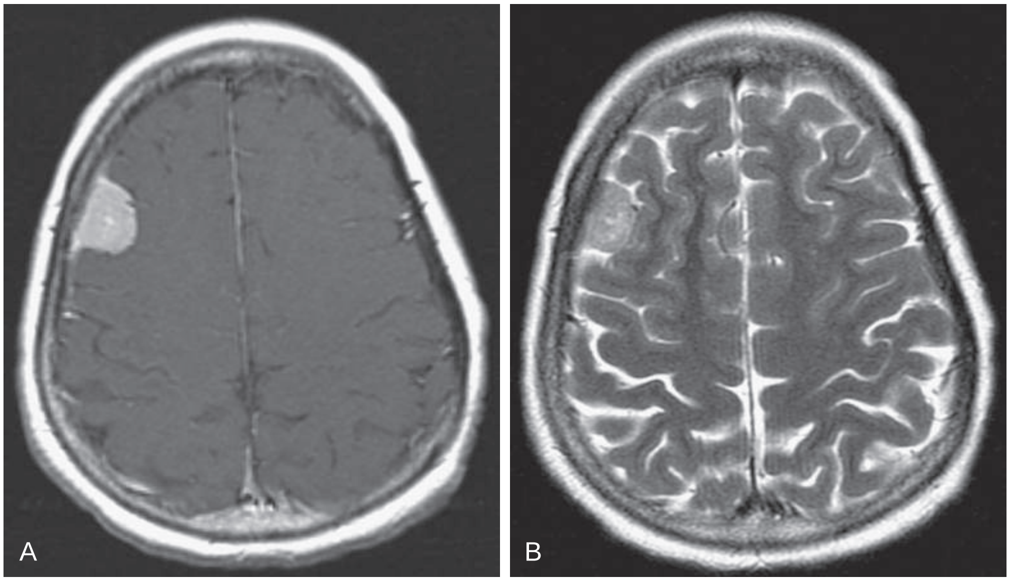

Figure 57-50 Peripheral contrast-enhancing meningioma. **A,** An axial T1-weighted image with use of contrast material. Note the dural tail. **B,** An axial T2-weighted image—bright T2 signal of a right frontal meningioma.

surveillance imaging.[1588] Although meningiomas are uncommon in pediatric patients, they are the second most common primary brain tumor in adults, after gliomas. Meningiomas are frequently identified as an incidental finding at autopsy, suggesting that many of these tumors remain small and asymptomatic. Molecular differences between symptomatic lesions and those that remain asymptomatic suggest that they may be distinct entities.[1589] Meningiomas are known to express hormone receptors, and a number of cell cycle pathways have been implicated in their dysregulated growth.[1590] In a review of the literature, radiation-induced meningiomas were the second most common radiation-induced malignancy after malignant glioma in children treated for a primary malignant neoplasm, with a mean latency of 21.1 and 13.7 years for atypical and benign meningiomas, respectively.[1591] Similarly in a cohort of children with acute lymphoblastic leukemia (ALL) who received cranial radiotherapy (1800 to 2400 cGy) and were monitored by serial scans, meningiomas were the most common tumor, with a mean latency of 21 years.[1588] In one study of patients who were treated with radiation therapy to the CNS for childhood leukemia, 20 years after treatment, it was reported that the incidence of meningioma was as high as 47%.[1592]

Clinical Presentation. The most common clinical presentations in children are seizures (33%), headaches (13%), ataxia (10%), and hemiparesis (10%). Tumor locations are primarily supratentorial (64%), with the remainder distributed infratentorially (16%), intraventricularly (12%), and in the spinal cord (8%).[1585] A few small series have indicated that the location and behavior of meningiomas in children may differ from those in adults, with a greater male preponderance, intraventricular location, increased multiplicity on first presentation, and increased atypical or anaplastic subtypes (15% in childhood).[1586,1593,1594]

Imaging and Histology. Meningiomas are usually well-circumscribed dural lesions on CT and MRI. Areas of calcification are common and are best seen on CT scans. Tumors tend to have low T1-weighted and bright T2-weighted and FLAIR signals. They are usually brightly contrast-enhancing and can be associated with edema.[1595] A “dural tail” is a common finding on MRI scans in which there is dural thickening, tailing away from the lesion (Fig. 57-50). This finding does not indicate a greater propensity of the tumor to invade.[1596] Although most pediatric meningiomas are low-grade lesions (WHO grade I), a higher number of pediatric meningiomas have features of atypia or anaplasia (WHO grades II and III) compared with adult patients, especially in spontaneously arising tumors. This trend can be more pronounced with younger age.[1584] In adult meningiomas good correlation exists between histologic atypia, recurrence, and staining for the Ki-67 monoclonal antibody, a marker of proliferation. Such correlation is not seen in pediatric meningiomas, suggesting that the aggressiveness of pediatric meningioma is not related to proliferation alone.[1597] A number of histologic variants of meningioma can occur in children.[1598-1601] Most meningiomas stain for EMA and vimentin. Loss of chromosome 22 is commonly observed in grade I meningiomas. Loss of heterozygosity of chromosomes 14 and 18 has been associated with tumor grade.[1602] Additional chromosomal abnormalities can be observed in atypical and anaplastic meningioma. In patients without NF-2, a high incidence of mutations in this gene are still detected.[1603] A subset of meningiomas lacking NF-2 mutations have recurrent oncogenic mutations in AKT1 (E17K) and SMO (W535L).[1604]

Management. The management of pediatric meningiomas is difficult to define because of their rare occurrence and association with other mitigating factors, such as prior radiation therapy or association with NF-2.[1584] Treatment guidelines from cooperative groups such as the British Children's Cancer and Leukemia Group are beginning to emerge.[1605]

Meningiomas that are asymptomatic are usually left untreated and followed up carefully for the development of neurologic sequelae, radiologic progression, or radiologic evidence of transformation, at which point neurosurgical intervention should be considered. GTR is usually curative, although the appearance of other lesions is not uncommon. In patients with progressive or symptomatic lesions, radiotherapy can be delivered and can often stop tumor progression. For small unresectable lesions, SRS is also being used with increasing frequency. Meningiomas that develop after radiotherapy in the pediatric population can take on a more malignant phenotype, and early surgical resection should therefore be considered, which may lead to a better outcome, especially if a GTR can be achieved.[1588] The role of chemotherapy in the treatment of meningiomas is limited. Recurrent malignant and atypical meningiomas are difficult to treat successfully, and treatment with chemotherapy has been unsuccessful. Brachytherapy has been proposed for the treatment of patients who have had a relapse, but with high accompanying morbidity.[1606] Partial responses of refractory meningiomas to hydroxyurea[1607,1608] and tamoxifen[1609] have been reported in the literature but need to be confirmed in larger clinical trials.[1610,1611] A phase II trial of combination therapy of imatinib and hydroxyurea in adult patients with relapsed or refractory meningioma demonstrated only modest activity.[1612] A number of new molecularly targeted therapies for meningiomas are now being developed and tested.[1613]

Prognosis. Because of the peripheral nature of many of these lesions, complete resection is achieved in a high percentage of cases. Surgery remains the primary modality of treatment of these tumors and is a major determinant of outcome.[1586] Histologic grade is a further determinant of EFS.[1585] Even though the OS of patients with meningioma has been reported to be close to 90%, length of follow-up is limited or unknown.[1614] In one series extending between 1935 and 1984, long-term survival of 35% in children was reported, with a mean follow-up of 10 years after diagnosis.[1615] The true extent of the morbidity and mortality of this tumor in children is not accurately known[1614] and needs to be the subject of a prospective study by national and international cooperative groups.

Pituitary Adenomas

Although infrequent in pediatric patients compared with adults, pituitary adenomas account for approximately 2.7% of pediatric brain tumors.[1616] The differential diagnosis of pituitary fossa tumors includes craniopharyngioma, GCTs, and Langerhans cell histiocytosis. The vast majority of childhood pituitary adenomas are hormone-secreting or functional, meaning that they produce active hormones, including adrenocorticotropic hormone (ACTH), prolactin, growth hormone, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), or follicle-stimulating hormone (FSH).[1617] Nonfunctioning adenomas account for only 3% to 6%.[1618,1619] Depending on the series, the two most commonly secreted hormones are prolactin and ACTH, followed by growth hormone,[1618,1620-1623] and finally TSH, LH, and FSH.[1617,1624]

Clinical Presentation. The main clinical manifestations of pituitary adenomas are determined by their location and function and usually consist of headaches (with or without signs of raised ICP), visual field defects, endocrine dysfunction, and menstrual irregularities. Other endocrine-specific presentations can be related to hyperprolactinemia, causing secondary amenorrhea and galactorrhea. Cushing disease resulting from ACTH oversecretion presents with hypertension, weight gain, and occasionally diabetes, although gigantism and acromegaly are rarely observed as a result of growth hormone oversecretion. Hypopituitarism can manifest as hypogonadism with delayed or arrested puberty, amenorrhea, and hypoadrenalism, which can manifest as fatigue, hypoglycemia, and/or weight loss. Short stature or growth failure can result from growth hormone deficiency. Hypothyroidism with a low thyroxine and a low-normal TSH level can also be a manifestation, although hyperthyroidism from a thyrotropinoma is a rare finding in childhood.[1624] Nonfunctioning tumors can also have endocrine manifestations, but these manifestations are typically related to pituitary insufficiency.[1625]

Imaging and Histology. The diagnostic workup and management of these tumors includes MRI of the pituitary region with and without contrast, although a CT scan can be useful in extensive disease or in an emergency to rule out local invasion or hemorrhage.[1626] Loss of the pituitary bright spot can indicate compression of the pituitary stalk.[1627] Microcystic and macrocystic structures are also easily identified on MRI (Fig. 57-51).[1627] A detailed biochemical workup in conjunction with an endocrinologist is vital to making a diagnosis and should include determination of serum prolactin, thyroxine and TSH, LH, FSH, serum urea, electrolyte, and morning cortisol levels. Determination of growth hormone, IGF1 and its binding partner IGF-BP3, and urine-free cortisol levels and a low-dose dexamethasone suppression test may be indicated if a growth hormone–secreting tumor or Cushing disease is suspected. Occasionally bilateral inferior petrosal sinus sampling for ACTH may be necessary for the diagnosis of Cushing disease.[1628] Visual field testing should be performed as part of a baseline assessment, and serum β-HCG and AFP should also be part of an initial assessment to rule out a GCT.

Management. Elevated secretion of prolactin can be associated with hyperplasia of TSH and prolactin-secreting cells but without the presence of a pituitary adenoma. Hypothyroidism should be managed before surgical intervention in cases in which hyperplasia may be evident.[1629] In terms of managing patients with

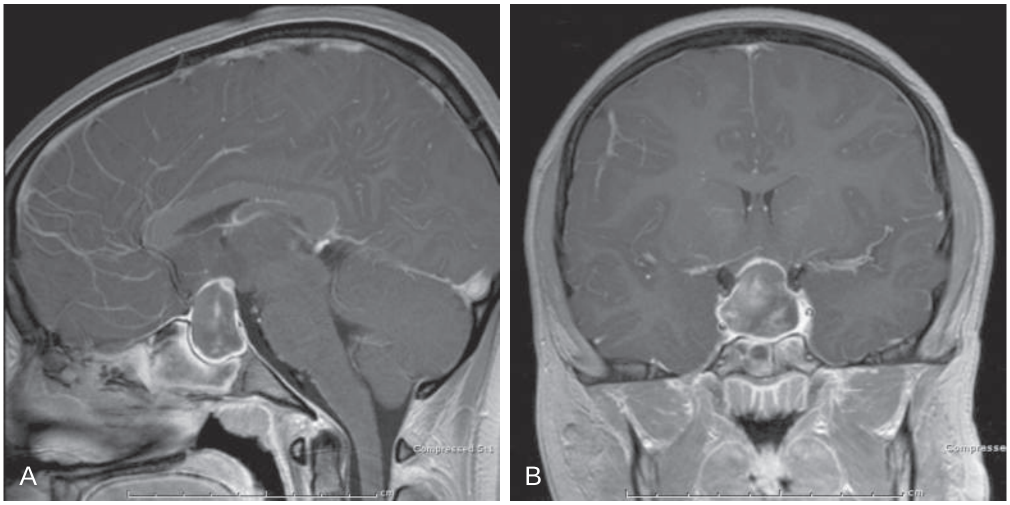

Figure 57-51 Large hemorrhagic pituitary adenoma. **A,** A sagittal T1-weighted image with use of contrast material. **B,** A coronal T1-weighted fat-saturated image.

pituitary adenoma, medical management alone with agents such as bromocriptine or similar analogues may be all that is needed for persons with prolactinoma.[1630] Transsphenoidal surgical resection is typically used for the treatment of growth hormone–secreting and ACTH-secreting tumors,[1631] although a potential role for somatostatin analogs such as octreotide has been proposed for the medical treatment of acromegaly.[1632] Multimodal therapy with medication, surgery, or radiotherapy may be necessary where initial therapy has failed.[1623,1624] Fortunately endocrinologic remission can be achieved in most patients with growth hormone- and ACTH-secreting tumors.[1622]

Multiple endocrine neoplasia type I (MEN1) is an autosomal-dominant disorder characterized by hyperparathyroidism, enteropancreatic tumors, pituitary adenomas, and, rarely, carcinoid, adrenal adenoma, and lipoma. Pituitary adenomas are observed at first presentation in 10% of cases.[1633] Familial isolated pituitary adenoma, characterized by familial pituitary adenomas in the absence of other tumors, is associated with germline mutations in the aryl hydrocarbon receptor-interacting protein gene *(AIP)*.[1634] The prevalence of germline mutations in *MEN1* or *AIP* in seemingly sporadic cases of pituitary adenoma in the pediatric population is high. One study demonstrated that 22% of patients younger than 18 years with sporadic pituitary adenoma had either *MEN1* or *AIP* germline mutations.[1635] In another study *AIP* mutations were reported in 20% of patients younger than 30 years who had pituitary adenomas.[1636] Although formal guidelines are lacking, because there is an increased incidence of genetic disorders such as MEN1 in pituitary tumors in children compared with adults,[1637] the families of index cases of childhood pituitary adenoma should be referred for screening and long-term follow-up.

Prognosis. The long-term prognosis of children with pituitary adenomas is excellent. Remission from abnormal hormone secretion is common; in one review it was achieved in 100% of patients with Cushing disease and 89% of patients with growth hormone secretion.[1622] Although disease control can be achieved in most patients with surgery, 40% may require radiation therapy.[1638] Given the central location of these tumors, this patient population must be carefully followed up for neurocognitive impact, radiation-induced tumors, and additional endocrinopathies because of pituitary dysfunction and radiation-mediated vascular damage.

Hamartomas

Hamartomas are an excessive but focal growth of cells and tissues native to the organs within which they occur. They have been described in the brain, eye, liver, lung, kidney, pancreas, heart, and gastrointestinal tract and in the lymphatic and vascular systems. Although they are benign lesions, hamartomas are regarded by pathologists as a link between developmental malformations and neoplasia. Because of the difficulty in distinguishing these two entities, hamartomas can sometimes be tenuously and variously interpreted as benign neoplasms.[1639] However despite their benign histology, their location and dysregulated growth in the brain can be the source of great morbidity, causing intractable seizures, obstructive hydrocephalus, and developmental delay.

Among the best-known conditions associated with hamartomas in the CNS are NF-1 and TS.[1640] NF-1–associated Lisch nodules are pigmented hamartomas in the iris and are found in more than 95% of affected adults and children older than 6 years; they are thought to be virtually pathognomonic for the condition.[1641] Although Lisch nodules remain asymptomatic and do not usually interfere with vision, they are important as a diagnostic criterion for NF-1. These lesions may be associated with unidentified bright spots seen in patients with NF-1[1642] and have been hypothesized ultimately to be related to neurofibromin levels.[1643] NF-2 results from loss of the tumor suppressor merlin and is characterized by predisposition to the development of multiple tumors, including schwannoma (particularly of the vestibular nerve), meningioma, and spinal cord glioma. Although not a commonly described association, combined pigment epithelial and retinal hamartoma have also been reported in patients with NF-2, although as a rare finding.[1644-1646] Their clinical significance is not clear. TS, like NF-1 and NF-2, results from the loss of the *TSC1* and *TSC2* tumor suppressor genes, which encode for hamartin and tuberin, respectively. These act by downregulating the Akt-mTOR pathway,[1647] as well as by regulating transcription through cross-talk with the Wnt signaling pathway through the ability of TSC1 and TSC2 to form a β-catenin degradation complex.[1648] A pathologic feature of TS within the CNS is the formation of cortical tubers, which are hamartomatous growths, in addition to subependymal nodules and the propensity to develop SEGAs. The clinical manifestation of this disorder is often seizures, cognitive disability, and other systemic manifestations, including dermatologic and visceral changes.[1640]

Several other rare inherited syndromes are associated with hamartomatous lesions in the brain, including Cowden disease, Bannayan-Zonana syndrome, Bannayan-Riley-Ruvalcaba syndrome, and LDD. In considering other conditions that are associated with hamartomatous lesions in the brain, Cowden disease (Online Mendelian Inheritance in Man [OMIM] 158350) is important because it is characterized by the familial predisposition to multiple benign hamartomas. These patients are also at increased risk of malignant lesions in all three germ layers involving most major organs, including the brain, mucocutaneous tissue, breast, thyroid, and uterus,[1649] with multiple tricholemmomas or benign neoplasms of the hair follicle, which are considered pathognomonic.[1650] The gene responsible for Cowden disease on chromosome 10 was cloned by linkage analysis and found to be a phospholipid phosphatase *PTEN* (*p*hosphatase *ten*ascin).[1651,1652] It is mutated in the germline in 80% of patients with Cowden disease, as well as in the Bannayan-Zonana syndrome.[1653] The same gene is also mutated in 60% of patients with Bannayan-Riley-Ruvalcaba syndrome (OMIM 153480), which is manifested by macrocephaly, developmental delay, and intestinal hamartomas. Several authors have reported mutations in the *PTEN* gene in patients with Proteus or Proteus-like syndrome; hamartomatous growths develop in both syndromes. Although rare LDD is associated with hamartomas of the brain resulting in ataxia, increased ICP, and seizures. The underlying lesion is a dysplastic gangliocytoma of the cerebellum, which is thought to be a hamartomatous overgrowth of Cowden disease.[1654,1655] In fact adult-onset of LDD is considered pathognomonic for Cowden disease by the National Comprehensive Cancer Network Guidelines.[1640,1649] Some authors have suggested that these familial hamartomatous syndromes be clustered with Cowden disease and the Bannayan-Riley-Ruvalcaba syndrome into a new category called PTEN hamartoma tumor syndrome, although this suggestion remains controversial.[1656] Recently a consortium was established, which published revised diagnostic criteria for PTEN hamartoma tumor syndrome to address this issue.[1657]

Imaging and Histology. Considerable variability exists in the imaging characteristics and pathologic appearance of hamartomas in the brain. Certain commonly observed features include areas of calcification on CT scan. On MRI scan, most lesions tend to be hypointense to isointense on T1-weighted sequences and bright on T2-weighted sequences and FLAIR (Fig. 57-52).[1658] Hamartomas of the brain are normally noncontrast-enhancing, which can help differentiate them from low-grade astrocytomas. The load of signal abnormality can be correlated with the severity of the symptoms and prognosis in many patients, such as those with TS.[1659] Using magnetoencephalography and concurrent SPECT imaging, precise tuber localization can be achieved and can assist in surgical planning.[1660,1661] Histologic features of hamartomas are highly dependent on their location.

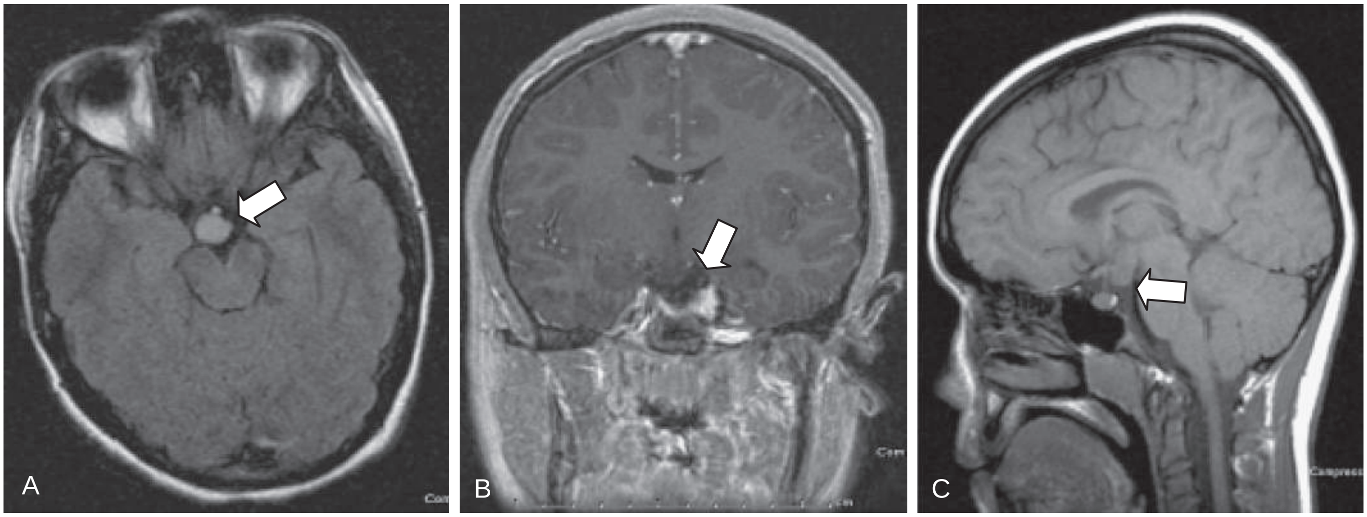

Figure 57-52 Hypothalamic hamartoma. **A,** An axial fluid-attenuated inversion recovery (FLAIR) image of a FLAIR bright lesion *(arrow)*. **B,** A coronal T1-weighted image with use of contrast material demonstrates little contrast enhancement *(arrow)*. **C,** A sagittal T1-weighted image without use of contrast material with hypointensity to isointensity *(arrow)*.

Management. The detailed management of tumors arising from the hamartoma conditions of LDD and Cowden disease, tuberous sclerosis, and neurofibromatosis is beyond the scope of this text. Multidisciplinary team management involving input from the geneticist, neurologist, neurooncologist, neurosurgeon, and affiliated specialists such as the neuropsychologist is highly recommended to provide comprehensive care. Molecular testing of patients and tumor tissues is also mandatory as targeted therapies with new agents emerge. Rapamycin and its analogs and derivatives specifically target mTOR, which is downstream from the PTEN/PI3K/Akt pathway and is common to neurofibromatosis and TS.[1647] Everolimus has been shown in a randomized phase III trial to be effective for the treatment of subependymal giant cell astrocytomas in patients with TS.[1662] Similar therapeutic agents are also available for targeting Ras through the inhibition of farnesyltransferase and are the subject of ongoing clinical trials for progressive plexiform neurofibromas.[1663] Pallister-Hall syndrome, which results in hypothalamic hamartomas, involves abnormalities in the SHH–Gli 3 pathway,[1664] and a number of new molecular inhibitors of this pathway are beginning to undergo clinical investigation.[1665]

Optimal management of hamartomatous lesions of the CNS is typically directed at the presenting symptoms.[1666] Seizures are a common presenting symptom, and treatment with antiepileptic agents is the first approach. Hamartomatous lesions are often refractory to these medications, even when multiple drugs are coadministered. Complete resection of hamartomas is not always required to improve seizure control, because disconnection of the epileptiform focus without gross surgical resection has resulted in significant clinical improvement in hypothalamic hamartomas.[1667]

Prognosis. Although isolated hamartomatous lesions that can be resected have a good prognosis, patients usually present with a number of comorbid symptoms related to the hamartomas and to the underlying associated genetic syndrome. Mental retardation, autism, and behavioral issues can persist. Seizures are not always amenable to surgical intervention, especially in deep structures of the diencephalon. Even in patients who are treated successfully, their underlying genetic susceptibility places them at risk for the development of new lesions.

Astroblastoma

Astroblastoma is a rare glial tumor of the CNS that accounts for 0.45% to 2.8% of all primary brain gliomas[1668] and approximately 0.92% of all pediatric brain tumors.[1669] The tumor was first described by Bailey and Cushing in their 1924 classification of brain tumors,[1030] but some persons argue that the term *astroblastoma* is confusing because the tumor is neither overtly astrocytic nor blastic in nature.[1670] The current widely accepted pathologic description of astroblastoma is that of a generally solid, well-circumscribed mass that is histologically defined by the presence of astroblastic pseudorosettes and perivascular hyalinization.[1671] Ependymoma is an important differential diagnosis because of the clinical and histopathologic similarity between these tumors, which includes perivascular orientation, pseudorosette formation, and immunohistochemical staining with GFAP and EMA. These histopathologic similarities have led to the suggestion that the tanycyte, or ependymal cell, may be a common precursor of ependymoma and astroblastoma.[1672] Even though astroblastoma does have some distinctive features, including broader tapering perivascular processes, lack of fibrillarity, and perivascular hyalinization, which is not seen in ependymoma, this rare tumor continues to be classified under "other neuroepithelial" tumors in the latest WHO classification of CNS tumors.[1673]

Clinical Presentation. The clinical presentation of astroblastoma is usually in children and young adults in the first three decades of life,[1670] with several reports in children 5 years or younger,[1669,1674-1677] as well as a congenital form.[1668] Several case series have shown a female preponderance.[1670,1678,1679] Presenting signs and symptoms are usually of headache and raised ICP because of mass effect or obstruction of CSF flow. The site of the tumor is most often supratentorial, peripherally located in the brain.

Imaging and Histology. Radiologically, astroblastomas appear as large, well-circumscribed, heterogeneous, lobulated, solid and cystic masses, with little vasogenic edema disproportional to their size. This lack of edema is a feature that can also help distinguish astroblastoma from ependymoma and HGGs. The solid component of the mass is described as bubbly, and the MRI T2-weighted signal is isointense with gray matter. Enhancement of the solid component is seen in 75% of cases, and punctate calcification is occasionally seen.[1678,1679]

The histopathologic features of astroblastoma include strong immunoreactivity for S-100, GFAP, and vimentin, with focal staining with EMA.[1673] Histologic grade is considered as either well differentiated, which correlates with the ability to achieve a GTR,[1670] or malignant, with features of anaplasia, which is a poor prognostic marker.[1677] MIB1 immunoreactivity has been observed over a wide range (1% to 18%), although it is unclear whether an increasing MIB1 index rating is related to a poorer outcome.[1670] A formal WHO grade has not yet been assigned.[1673]

Management. Treatment of these tumors includes maximal surgical resection followed by focal or WBRT, especially when features of anaplasia are present. The role of TMZ,[1680,1681] intensive chemotherapy in younger children,[1677] or other forms of chemotherapeutic agents[1669] has not been determined. The establishment of an international registry to gain more information on treatment responses and a detailed understanding of the biology of these tumors has been proposed to optimize treatment approaches for this rare tumor.[1682] To this end studies of chromosomal abnormalities, such as gains of chromosome 20q and chromosome 19[1670] or loss of heterozygosity of chromosome 9p,[1683] are important and help distinguish this tumor from ependymomas. The description of variants of astroblastoma that have rhabdoid features and a better prognosis[1680,1684] warrants further study of this rare tumor.

Prognosis. The prognosis of astroblastoma is in part dependent on the histologic features. Anaplastic lesions do less well than those with low-grade features.[1671,1677] Complete resection is important for long-term survival and may be sufficient therapy for most patients, including some lesions with more of an anaplastic appearance.[1670]

Primary CNS Lymphoma

CNS lymphoma is a rare group of tumors that can arise in the CNS. Although primarily associated with immunosuppression from human immunodeficiency virus (HIV) or other immunodeficiency states, CNS lymphoma has also been detected in patients lacking a prior infectious or immunocompromised history. These tumors are often very responsive to therapy, including radiation therapy and/or chemotherapy.[1685]

PCNS lymphoma in childhood and young adults is an extremely rare tumor, accounting for about 1.2% to 1.5% of all brain tumors,[1686,1687] with an annual incidence rate of 0.02 per 100,000 based on data collected by the Childhood Brain Tumor Trust between 2000 and 2004. These data suggest that approximately 16 new cases per year are expected in the United States.[1032] Children with congenital immunodeficiency syndromes face an inherent 4% overall risk of developing cancer, which is 10,000 times higher than the expected rate. The highest risk groups are ataxia-telangiectasia and common variable immunodeficiency.[1688] Data collected by the Immunodeficiency Cancer Registry have shown a median age of presentation in this group of children to be 10 years, and more than 50% of the cases consist of non-Hodgkin lymphoma, 30% of which were extranodal.[1689] Patients with Wiskott-Aldrich syndrome have the highest proportion of CNS lymphoma among the congenital immunodeficiencies, which is estimated at approximately 3%,[1690] followed by immunoglobulin (Ig)A deficiency, hyper-IgM syndrome, and severe combined immunodeficiency.[1691] Immunodeficiency after organ transplantation also carries a risk of between 1% and 5% for PCNS lymphoma, and increasing rates are being observed in renal, cardiac, lung, and liver transplant recipients.[1692] AIDS, particularly HIV-AIDS in adults, was responsible for a peak in CNS lymphoma in the mid 1990s, with 2% to 13% of adult patients with AIDS affected.[1693] In one study of pediatric patients with AIDS, the Centers for Disease Control and Prevention reported 18 of 6209 patients presenting with CNS lymphoma.[1694,1695] The overall risk of CNS lymphoma was estimated at 17% to 42% in pediatric patients with AIDS.[1688,1696,1697] Subsequent reporting by the SEER program has not shown an increase in the rate of PCNS lymphoma in the 0- to 19-year-old age group, unlike in adults, whose rates have fallen dramatically since the peak in the 1990s.[1698] This finding is probably attributable to the introduction of multiantiretroviral therapies (e.g., highly active antiretroviral therapy) and other therapeutic improvements.[1699]

Although patients with immunodeficiencies are at higher risk of developing PCNS lymphoma, most adult and pediatric patients in whom this malignancy develops are immunocompetent.[1685,1692,1700] The pathogenesis of PCNS lymphoma in the immunocompetent host is unknown. One hypothesis is that systemic inflammatory cells have become trapped in the brain and subsequently transform, isolated from the remainder of the immune system by virtue of the immunologic sanctuary status of the brain. This hypothesis does not fully explain the fact that normally only T lymphocytes traffic through the CNS, although most cases of PCNS lymphoma are of B-cell origin.[1701]

Clinical Presentation. Although the clinical presentation of PCNS lymphoma is in keeping with the diffuse infiltrative growth seen in adult patients and is dominated by cognitive dysfunction, psychomotor slowing, behavioral changes, raised ICP, and seizures,[1702,1703] most pediatric patients present with symptoms of increased ICP, hemiparesis, ataxia, and cranial nerve dysfunction.[1685,1686,1700]

Imaging and Histology. Radiologic features of PCNS lymphoma include enhancing unifocal or multifocal lesions, usually in the periventricular area, although cortical lesions are not uncommon. MRI is the best modality to use because these lesions are characteristically hypointense on T1-weighted sequences and T2 hyperintense tumor with evidence of peritumeral edema.[1702] Most tumors are brightly enhancing upon administration of contrast material. PET and SPECT imaging can also help confirm the presence of lymphomas and identify metastases in the CNS and outside of the brain.[1704-1706] Using the Revised European American Lymphoma classification system, the diffuse large B-cell phenotype accounts for most cases, followed by the small, noncleaved Burkitt phenotype.[1707] Anaplastic large T-cell lymphoma is rare.[1700,1703] Pediatric patients with AIDS, on the other hand, have been found to have small noncleaved B-cell tumors, both of the Burkitt and non-Burkitt types, and are more frequently associated with Epstein-Barr virus infection.[1688,1708]

Management. The treatment of PCNS lymphoma has followed the adult practice because pediatric-based studies to define optimal therapy have not been conducted. Although PCNS lymphomas are aggressive tumors and require intensive treatment, they are responsive to radiation therapy and chemotherapy. Historically treatment for adults with CNS lymphoma consisted of radiotherapy alone, but these patients also had high relapse rates, which led to the introduction of combination chemotherapy using high-dose methotrexate (greater than 1 g/m^2), a treatment that has proven to be effective.[1709] The role of WBRT as part of consolidation therapy in adults is controversial given its toxicity, especially in older adults and when given in combination with high-dose methotrexate. Some persons consider it to be an essential part of therapy and believe it should be excluded only in persons of advanced age or with neurocognitive impairment.[1710] In contrast to the data with high-dose methotrexate, evidence suggests that consolidation with WBRT and high-dose cytarabine or high-dose cytarabine alone does not improve survival in patients who achieve complete remission with high-dose methotrexate-based combination treatment.[1711] With effective salvage regimens such as re-treatment with high-dose methotrexate or treatment options with TMZ, rituximab, and other agents,[1709] a compelling argument exists to exclude WBRT as part of first-line treatment.

Although pediatric patients have been successfully treated with WBRT alone,[1685,1686] this treatment is often too toxic, especially in younger children. In a multicenter retrospective review of 12 pediatric patients who had a 5-year EFS of 70% and OS of 75% (median follow-up of 79 months), only two patients received radiotherapy, and one patient was treated with high-dose chemotherapy and ASCR, confirming that radiotherapy can be excluded from the upfront therapy of these patients.[1700] Most patients in this study received multiagent chemotherapy including high-dose methotrexate (8 g/m^2), high-dose cytarabine, and steroids, with patients having been treated according to the French-American-British LMB-96 protocol (group C for persons with CNS disease). All patients in this retrospective study had received intrathecal chemotherapy, with the exception of one immunocompromised patient who was treated with hydroxyurea only. Similar results have been reported in a recent single-institution retrospective analysis of six pediatric patients with PCNS lymphoma.[1712] All patients were treated with systemic, methotrexate-based chemotherapy and intrathecal chemotherapy. The estimated 5-year survival rate was 83.3% in this cohort. Organ transplantation patients can be treated with multimodality therapy, as discussed earlier, in addition to a decrease in their immunosuppressive regimens,[1713] although care must be taken to protect their grafts.

Prognosis. A recent, relatively large ($n = 29$) multicenter retrospective analysis of pediatric patients with PCNS reported a 3-year OS of 82%.[1714] It should be noted that these results are based on a cohort of mixed immunocompetent and immunocompromised patients. Further improvements in treatment outcome will require a large prospective multinational study that separates immunocompetent and immunocompromised patients with PCNS lymphomas to determine optimum therapy for this rare malignancy.

Dermoid, Epidermoid, and Arachnoid Cysts

A vast array of cysts can occur throughout the CNS,[1715-1717] and although they are not classified as tumors, these CNS lesions can cause significant morbidity and often require surgical intervention. If they are completely removed, recurrences are uncommon, although incompletely resected lesions can lead to a reaccumulation of fluid, resulting in recurrence of symptoms and necessitating further resection[1718] or cyst drainage.[1719]

The benign cysts of the CNS are often divided into two main categories. The first are those that arise from tissue within the CNS, including porencephalic cysts, arachnoid cysts, ependymal cysts, and hemangioblastomas. The second group of cysts consists of those that arise from extracranial tissue and includes teratomas, dermoid and epidermoid cysts, craniopharyngioma, and endodermal, colloid, enterogenous, and Rathke cleft cysts.[1715] Cysts may also arise from infectious origins, such as cysticercosis or toxoplasmosis.

Arachnoid Cysts. Arachnoid cysts are CSF-filled cavities lined by arachnoid cells[1720,1721] that arise from the arachnoid layer of the meninges. They account for approximately 1% of all intracranial space-occupying lesions and are more common in boys.[1716] They are usually congenital and occur in early infancy[1722] but can also occur as a result of inflammation in older children. Arachnoid cysts usually occur in the supratentorial compartment, arising in areas that are rich in arachnoid tissue, with 50% occurring in the Sylvian fissure,[1723] although they can occur anywhere in the CNS.[1724] Arachnoid cysts are often incidental findings on CT or MRI scans obtained for unrelated symptoms. Although usually isolated in their occurrence, multiple arachnoid cysts can be part of inherited syndromes such as the acrocallosal syndrome (a mutation in the *GLI3* gene, OMIM 200990) or the Chudley-McCullough syndrome (OMIM 604213), both of which are associated with agenesis of the corpus callosum. A familial pattern associated with cysts in multiple family members has been reported, one of which includes abnormalities on chromosome 16.[1725] Arachnoid cysts need to be distinguished from cysts associated with malignant primary or metastatic brain tumors, which are thought to develop as a result of a disrupted BBB as a result of the malignant process.[1726]

Clinical Presentation. In infants and children, arachnoid cysts tend to present with increasing head circumference or hydrocephalus, seizures, headaches, and psychomotor retardation.[1716] In the suprasellar region, 90% of patients present with obstructive hydrocephalus,[1727] although precocious puberty has also been recognized.[1728] In this location, it may be necessary to distinguish arachnoid and Rathke cleft cysts from craniopharyngioma, especially because the clinical management of craniopharyngioma differs significantly. Although differentiating these lesions can be difficult, arachnoid and Rathke cleft cysts are usually uniformly cystic and lack a solid component. In contrast craniopharyngiomas are solid in 10% of patients and solid and cystic in 43% of patients. Another distinguishing feature is the presence of calcification in approximately 90% of craniopharyngiomas, a finding that is rare in arachnoid cysts.[1729] Although the neurologic presentation of craniopharyngioma and arachnoid cysts can be similar, 95% of craniopharyngiomas have accompanying endocrine dysfunction, which is usually absent in patients with arachnoid cysts.

Imaging and Histology. The radiologic appearance of arachnoid cysts is usually nonenhancing, and they appear hypodense on CT and isointense to CSF on MRI (Fig. 57-53).[1730] Several theories have been proposed to explain the expansion of arachnoid cysts, which vary from the pulsatile movement of CSF into cyst cavities to osmotic gradients between cysts and CSF.[1716] Analysis of cyst fluid confirms that it is CSF, although the protein content tends to be elevated.[1731]

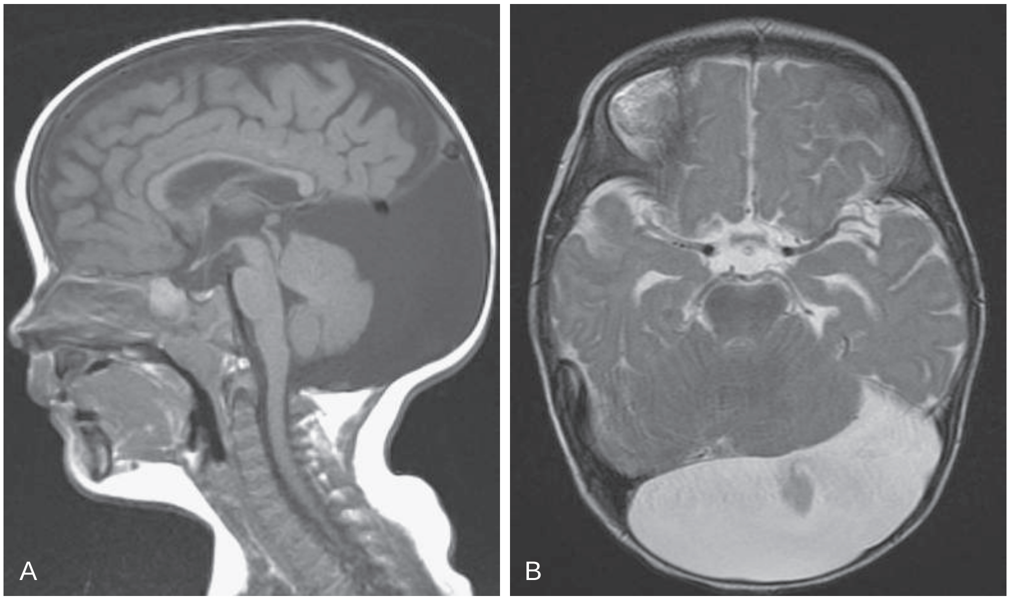

Figure 57-53 Arachnoid cyst. **A,** A sagittal T1-weighted image without use of contrast material. **B,** An axial T2-weighted image.

Management. The management of arachnoid cysts is usually conservative, especially if the patient is asymptomatic.[1732] However approximately 6% of patients with arachnoid cysts will experience complications, including cyst rupture or hemorrhage. Risk factors for cyst rupture or hemorrhage include large cyst size (greater than 5 cm) and head trauma.[1733] A clearly expanding lesion or worsening signs and symptoms will necessitate surgical intervention with cyst fenestration[1728,1734] or cystoventricular shunting.[1719] Newer surgical approaches with endoscopic management have been developed and have been successful.[1727,1735] Retrospective analysis of 1324 cases of pediatric arachnoid cysts demonstrated that open craniotomy for cyst excision or fenestration, endoscopic fenestration, and cystoperitoneal shunting have similar outcomes.[1736] Most patients will have resolution of their presenting symptoms after surgical intervention, although approximately 20% will not experience resolution.[1737,1738] Potential postsurgical complications include infections, subdural hygroma, and CSF leak.[227]

Prognosis. The long-term prognosis of children with arachnoid cysts is excellent. Because fluid reaccumulation can recur, repeat surgical procedures will be required in approximately 20% of patients.[1738] Patients requiring shunting from the ventricles or cysts may require additional procedures as they grow.[227,1739,1740] Seizure control can require lifelong anticonvulsants, especially if complete surgical resection of the lesion is not feasible. Patients with a history of arachnoid cysts may be at increased risk of chronic subdural hematomas.[1741]

Dermoid and Epidermoid Cysts. Dermoid and epidermoid cysts account for 0.5% and 0.8%, respectively, of all intracranial space-occupying lesions[1742] and result from the intrusion of nonnervous tissue, mainly epithelia, into the neuraxis at the time of closure of the neural tube, between the third and fifth weeks of embryonic life.[1718,1743,1744] These cysts are most often located in the major subarachnoid cisterns of the brain—namely, the suprasellar cistern, pineal region, and cerebellopontine angle cistern.[1715] Although benign and slow growing, these lesions can be problematic if they cause local mass effect or obstructive hydrocephalus, and they have a tendency to recur if they are not completely resected or are complicated by secondary infection. In general dermoid cysts are primarily located in the midline in the suprasellar, frontobasal, and cisterna magna areas, although epidermoid cysts can also be found in the suprasellar area. Epidermoid cysts are more common in the lateral skull base around the cerebellopontine angle and are rare in the brain parenchyma or intraventricular region. Both dermoid and epidermoid cysts can occur in the brainstem and spinal cord.[1717,1718,1742,1745]

The presentation of intracranial dermoid cysts occurs in younger patients (10.5 years) compared with epidermoid cysts, which present in adulthood with a mean age of 27 years.[1717] Although usually sporadic in their presentation, intracranial dermoid cysts can have a familial predisposition affecting several generations (OMIM 600679).[1746] Unlike arachnoid cysts, which have a more clearly defined association with intracranial syndromic malformations such as Aicardi syndrome, dermoid and epidermoid cysts do not appear to follow the same pattern. However patients with dermoid cysts can also have associated CNS malformations, such as agenesis of the corpus callosum (OMIM 600679).[1747,1748] In Goldenhar syndrome, which is composed of oculoauricular dysmorphic features and hemifacial microsomia, associated epibulbar dermoid cysts have been reported (OMIM 164210) but do not have a link with cancer predisposition. In contrast Gardner syndrome, a variant of familial adenomatous polyposis with associated predisposition to colorectal carcinomas, includes hyperostosis of the skull and subcutaneous and intraabdominal dermoid cysts (OMIM 175100). Turcot syndrome is characterized by familial polyposis coli with a predisposition to the development of malignant neuroepithelial tumors[1042] and is also associated with mutations in the APC and mismatch repair genes.[1749,1750] The coexistence of Gardner and Turcot syndromes in a family,[1751,1752] coupled with the report of an intracranial epidermoid cyst in a patient with Gardner syndrome,[1753] has suggested that dermoid and epidermoid cysts may share a common pathogenesis. Finally, epidermoid cysts can present as a late effect of a lumbar tap.[1754]

Clinical Presentation. Dermoid and epidermoid cysts can present with a wide array of symptoms, including recurrent fevers, headaches, signs of raised ICP, cerebellar ataxia, behavioral changes, seizures, cranial nerve palsies, increased head circumference, Parinaud syndrome, deafness, long tract signs, visual loss, and cord compression.[1717,1718,1742,1755]

Imaging and Histology. The MRI appearances of dermoid and epidermoid cysts are of well-circumscribed lesions with an accompanying sinus tract that is often visualized on imaging. They produce moderate mass effect but no perilesional edema. They are usually hypointense on T1-weighted imaging and hyperintense on T2-weighted imaging, with enhancement of the solid component (Fig. 57-54).[1718] In a recent study of intraparenchymal epidermoid cysts, the imaging characteristics were similar to extracerebral epidermoid cysts with isointensity to CSF on both T1- and T2-weighted images, with additional diagnostic information from DWI.[1756] Histologically dermoid and epidermoid cysts show overlap consistent with their ectodermal origin. Both are lined by squamous epithelium and some degree of keratin deposition and both lack features of invasion. In addition dermoid cysts can have skin adnexa present in the cyst wall, including hair follicles, sebaceous glands with sebum, and keratin flakes in the cyst fluid.[1717,1757]

Management. The management of dermoid and epidermoid cysts is surgical when intervention is required. Maximal safe resection should be attempted but can be complicated by adherence of the cyst wall to vital structures, limiting the resection.[1718] Subtotal resection is associated with a high rate of recurrence, requiring repeated surgery.[1746] Potential complications include aseptic meningitis in cases of spillage of cyst contents and even malignant transformation.[1743]

Prognosis. Dermoid and epidermoid cysts can recur and continued surveillance is required, especially for lesions that are incompletely resected.

Spinal Cord Tumors

Spinal cord tumors of infants and children are rare and account for 1% to 10% of all tumors of the CNS. They can occur as extradural, intradural but extramedullary, or intramedullary.[1758] There is a slight male predominance, with 58% of cases occurring in males.[1759] Approximately 80% of tumors are low-grade neoplasms. Predominant histologies in the spine from one large series include ependymoma (19%), schwannoma (neurilemmoma; 17%), and astrocytoma[565] (15%).[27] Benign cysts are also common in this location.[1760] Rare histologies identified in this region include neuroblastic tumors,[1761] oligodendroglioma,[1003] gangliogliomas,[602] anaplastic ganglioglioma,[588] nongerminomatous germ cell tumor,[1762] teratoma,[1763] cavernous angioma,[1764] clear cell meningioma,[1765] lipoma,[1766] PNET,[1767] and ATRT.[1768] In a review of 164 patients with spinal cord tumors undergoing resection, the median age was 8.6 years.[1769]

Clinical Presentation. The clinical symptomatology of pediatric spinal cord tumors relates to neurologic impairment concordant with the level of the lesion. Because most spinal tumors grow along the cord, a number of segments are commonly involved. Each segment of the

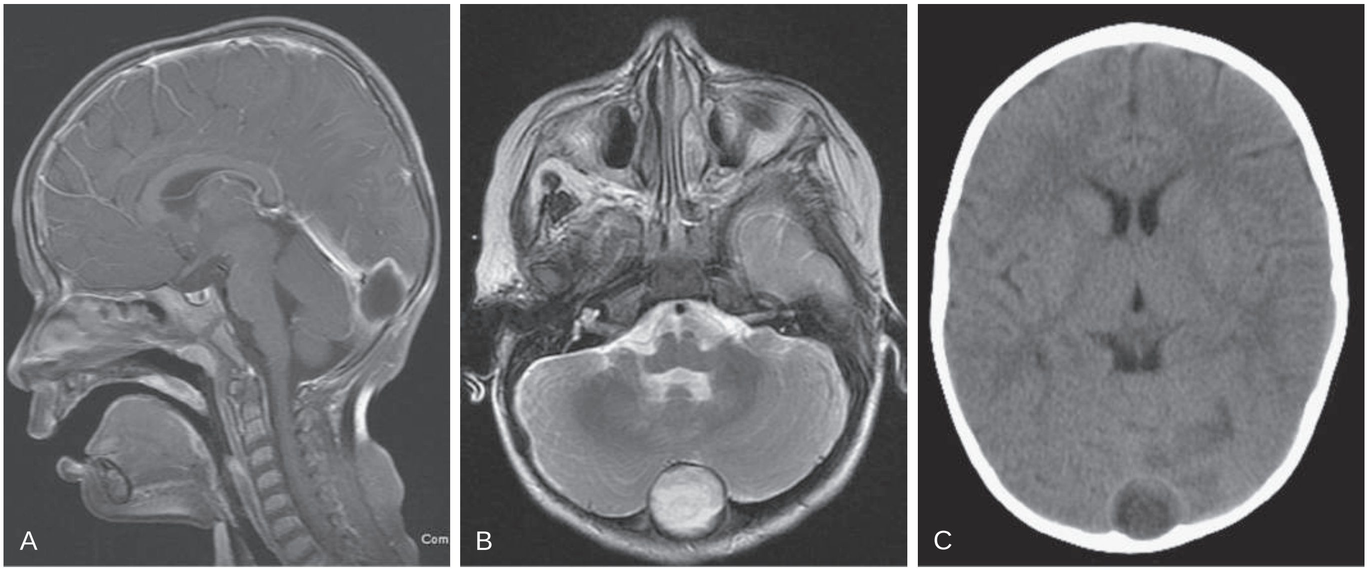

Figure 57-54 Occipital dermoid cyst. **A,** A sagittal T1-weighted image with use of contrast material. **B,** An axial T2-weighted image. **C,** A computed tomography noncontrast image.

spinal cord (cervical, thoracic, lumbar, and sacral) can be involved by tumors, and no histologic type is localized to a single area except myxopapillary ependymomas, which are predominantly found in the conus medullaris and filum terminale. Common symptoms of spinal cord tumors include pain (the most common symptom and identified in up to 80% of cases in most series), motor weakness, sensory loss, torticollis, and bladder and bowel incontinence.[1770-1773] Delay in diagnosis is common because of the nonspecific nature of the symptoms early in the disease process.[1774] Patients with evidence of a cerebral mass and spinal cord symptoms must undergo spinal imaging for metastatic disease.

Imaging and Histology. MRI is important in differentiating the location of tumors in the spine (extradural, intradural extramedullary, and intramedullary), as well as differentiating the possible histologies.[1775] As a general rule the imaging characteristics of spinal cord lesions are similar to those of the same histology in the brain. Histologic evaluation of spinal cord tumors follows that of the same lesions in the brain.

Management. Complete or near-complete resection of intraspinal tumors can be achieved in a significant majority of patients. In one large series of 164 patients, a greater than 95% resection was obtained in 76% (125 patients).[1769] Because most spinal tumors are low-grade tumors and can be cured with complete resection, having an experienced neurosurgeon perform the procedure is of utmost importance in achieving a complete resection. Other adjuvant therapies may be considered on the basis of the histology of the tumor. Myxopapillary ependymomas are best managed with complete surgical resection, which is achievable in most patients. Radiation therapy is administered to patients with progressive disease.[947] Patients with spinal ependymoma may have better outcomes than those with similar tumors of the posterior fossa in that many children can be treated effectively with surgery alone, without the need for radiation therapy. In a group of 20 children, a complete resection was achieved in 14 patients, and 6 patients had subtotal resections. None of these six patients received radiation therapy, and yet three remained without progressive disease.[1776] Children with high-grade astrocytomas of the spine who are treated with radiation therapy do as poorly as those with supratentorial tumors.[1777] Low-grade astrocytomas of the spine generally respond to vincristine and carboplatin[1778] or focal radiotherapy.[1779] Chemotherapy (irinotecan and cisplatin) has been used in infants with astrocytomas to delay the need for radiation therapy, with good results in three patients.[1780] Radiotherapy should be reserved for those in whom a complete resection cannot be obtained.[1781] Gangliogliomas are often cured with resection alone; patients with incomplete resection or recurrence can be treated with LGG therapy or focal radiotherapy.[602]

Prognosis. The long-term outcomes for patients with spinal cord tumors are not significantly different from those with brain tumors of the same histology and degree of resection. The morbidities of the tumor and therapies, however, may be significant. Direct neurologic consequences of spinal cord tumors include pain and motor and sensory deficits, as well as urinary and bowel incontinence. Many patients will demonstrate significant improvement in these deficits after treatment. Negative predictive factors for prolonged or permanent motor deficits include older age, unilateral symptoms, preoperative urinary symptoms, and other preoperative deficits.[1782] High-grade astrocytic histology and a short history of symptomatology can also be poor prognostic markers.[1783] Patients who present with sensory deficits do worse than those without this symptom at presentation.[1783] In the case of spinal cord ependymomas, location can effect prognosis, with lesions in the lower half of the spinal cord carrying a poorer prognosis.[1784] Persons with symptoms related to cyst enlargement are most likely to regain function.[945] More than one fourth of patients (27%) experience long-term spinal deformity as a result of their initial laminectomy.[1785,1786] Chronic pain can be particularly difficult in this patient population and requires aggressive management.[1787]

Patients with radiation therapy to the cervical spine can experience paresthesias a few months after therapy that are often described as a feeling of electric shock in the spine. This phenomenon, called Lhermitte sign, is related to transient demyelinization from radiation therapy.[1788] Patients are treated symptomatically, and the sensations typically resolve without the need for intervention.

Chordomas

Chordomas are rare tumors of the skull base that are thought to derive from remnants of the primitive notochord.[1789] Although most cases arise in adults, they can be observed at all ages, including in infants.[1790] Whites appear to be more commonly affected than are African Americans, and males are affected more often than are females.[1791,1792]

Clinical Presentation. Chordomas can present along the axial skeleton, including the skull base, spine, and sacral area. Skull-based lesions typically present with headaches and diplopia. Spinal lesions often result in pain, motor weakness, or sensory deficits. Although initially minor these symptoms tend to progress and eventually result in referral for imaging. Rarely metastatic disease identified on CSF cytology is detected and provides an argument for complete staging of patients, particularly those with atypical or poorly differentiated disease.[1793]

Imaging and Histology. Chordomas are well-circumscribed lesions on CT imaging that can demonstrate areas of calcification. They are hypointense on MR T1-weighted images and bright on T2-weighted images and FLAIR sequences (Fig. 57-55). Chordomas can brightly enhance after administration of gadolinium.[1791] The histologic appearance of chordomas can vary.[1794] Although conventional chordomas (58%) predominated in one pediatric series of 73 cases, 23% were chondroid chordomas and

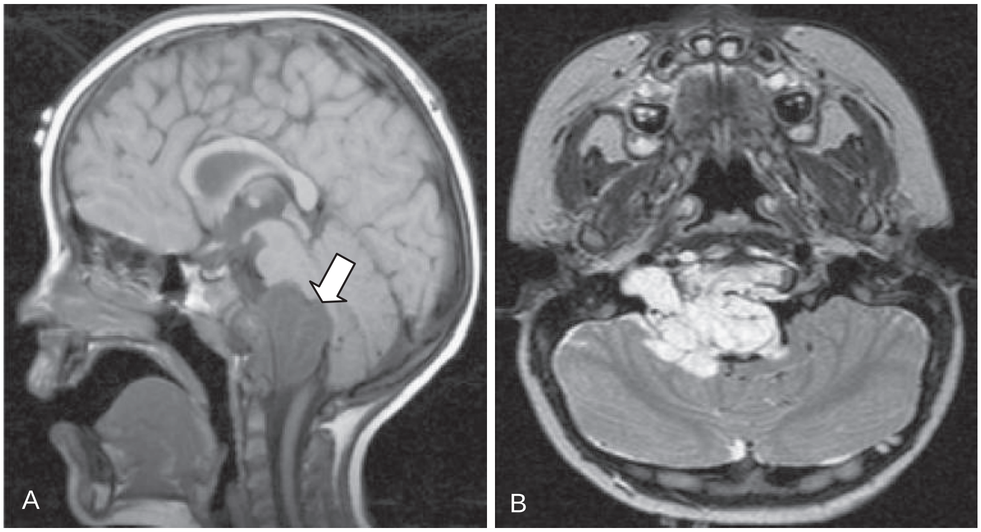

Figure 57-55 A large skull-based chordoma. **A,** A sagittal T1-weighted image without use of contrast material showing a hypointense lesion *(arrow)*. **B,** An axial T2-weighted image.

an additional 19% had high cellularity.[1795] Tumors stained positively for keratin, EMA, S-100, and vimentin. Mitoses and necrotic areas were also commonly observed.[1795]

Management. Radiation therapy can be effective in the treatment of chordomas. In addition to reducing the toxicity of photon radiation therapy to normal structures,[1796] proton beam radiation therapy in combination with IMRT can provide excellent delivery of a high dose to the lesion.[1797] Chordomas are largely resistant to chemotherapy. In patients with more aggressive lesions, or for those refractory to radiation therapy, chemotherapy can be offered. Response to ifosfamide and doxorubicin was reported in one patient with metastatic disease in conjunction with triple intrathecal therapy (cytarabine, hydrocortisone, and methotrexate).[1798] The use of a combination of actinomycin D, cyclophosphamide, and methotrexate or cisplatin and 5-fluorouracil demonstrated no activity in this report.[1798] A retrospective review of six pediatric patients with clival chordomas reported some success with ifosfamide and regimens containing etoposide.[1799] Repeat irradiation with proton therapy and SRS have also been used as salvage therapy in patients who have had a relapse.[1800,1801]

The recent identification of PDGFR-α and PDGFR-β in tumor cells and concurrent expression of the receptors in adjacent stromal cells suggest a possible role for therapeutic intervention with imatinib mesylate.[1802,1803] Another molecular target identified in these tumors includes EGFR; one patient with metastatic disease achieved a partial response with cetuximab and gefitinib.[1804] Modest activity in recurrent chordomas has also been observed with 9-nitrocamptothecin.[1805]

Prognosis. Patients who have chordomas with poorly differentiated features have a worse prognosis than those with classic forms of the disease. Complete resection remains an important prognostic factor. In one large pediatric series, 81% of patients were alive at a median of 7 years after treatment with proton beam radiation therapy.[1795] An elevated MIB1 rating has also been reported as an important predictor of recurrence and OS.[1806]

LATE EFFECTS OF TREATMENT

The long-term and late effects of therapy are a critical concern in pediatric neurooncology.[1807,1808] The developing nervous system is uniquely vulnerable to the long-term effects of radiation therapy, and these effects can become apparent and progressive throughout the life span of the child into adulthood.[1809] Although much of the interest about late effects has refocused on the consequences of radiation therapy, increasing evidence indicates that the presence of the tumor compressing the brain, the surgery needed to remove it, and the chemotherapy provided to treat it all add to the experience of late effects by survivors of pediatric brain tumors.[1810,1811] The improving prognoses of patients with CNS tumors means that more children will be at risk of developing a wide range of late effects of therapy,[1812] including secondary malignancies.[1813] Of particular importance is the focus on the cognitive outcome of children treated for CNS tumors as we shift from the number of survivors to the number of functioning survivors. In association with cognitive deficits, patients can experience depression, anxiety, and social isolation. Survivors have increased risk for suicidal ideation and increased need for psychosocial intervention, and all patients should have follow-up with psychosocial providers to ensure that psychosocial symptoms are not having a significant impact on their lives.[1814,1815] This need for follow-up has required the development of specialized neurocognitive assessment tools[1816-1820] and will also require the development of markers to identify which patients are at risk for late effects from different therapies. Genetic constitution, pharmacogenomics, and individualized pharmacokinetics will need to be assessed when therapy is planned, and appropriate modifications will need to be made to adapt to the unique variables of each individual patient.[1821]

Not all late effects result solely from therapy. Many late effects are the direct result of the tumor or the raised ICP at presentation. For example seizures can occur as a function of the location of the tumor, the therapy, or both. The occasional breakthrough seizure that is relatively well-controlled and not a major concern in childhood can become a significant problem with respect to work and driving while the survivor enters adulthood.[1822] The effect of having had a brain tumor on body image can also be significant. Facial asymmetry caused by tumor infiltration of the brainstem, visual impairment from tumors in the optic pathway, or gait abnormalities from involvement of the motor cortex provide daily reminders to patients that they continue to live with the effects of having had a brain tumor. Raised ICP can also lead to significant vision loss.[1823]

Late Effects of Surgery

Although GTR is the goal of surgery for most types of brain tumors, the consequences of cerebellar or brainstem injury after resection of posterior fossa tumors or the behavioral and endocrine alterations after resection of suprasellar tumors may have a serious impact on the long-term quality of life for a patient. Many surgical morbidities may be acute and can improve over time but other deficits may persist, directly affecting the quality of life for survivors.[1824] The association between surgical dissection of the vermis for tumors involving the fourth ventricle and cerebellar mutism (posterior fossa) syndrome has been well documented, although a precise mechanism has yet to be elucidated. In a report from two large cooperative clinical trials for patients with medulloblastoma, up to 25% of patients were retrospectively identified as having cerebellar mutism syndrome and 92% of cases were deemed moderate or severe. More concerning, 1 year after surgery a significant proportion of these patients were reported to have continued nonmotor speech, language, and neurocognitive deficits, and/or ataxia.[242] Changes in respiratory function related to cerebellar dysfunction can also persist.[1825] Long-term balance problems after posterior fossa surgery remains a major issue for patients and, although this adverse effect may be improved with sparing of the deep cerebellar nuclei when resecting posterior fossa lesions, a significant proportion of children will still have residual deficits.[1826]

The increasing use of third ventriculostomies to treat hydrocephalus has helped reduce the need for prolonged VP shunt management. Patients with VP shunts will need long-term assessment of shunt function and, for patients who were very young at the time of VP shunt placement, repeat surgery to reposition and lengthen tubing may be required. Patients must be aware of the risks of shunt failure (both VP shunts and third ventriculostomies) throughout their lives.

Children with brain tumors who undergo surgical resection as their sole treatment—many with benign histologies—are expected to have few long-term effects from their tumor or treatment. However detailed neuropsychiatric and neurocognitive assessments have revealed that these patients often demonstrate significant abnormalities, including mood, behavioral, and academic difficulties.[429,1827] These results indicate that all brain tumor survivors, including those receiving minimal intervention, require long-term evaluation and support.

Late Effects of Radiation

Radiation therapy can produce acute, subacute, and late effects on the CNS.[1828] The subacute effects of radiation therapy typically become evident 2 to 6 months after treatment and include the radiation somnolence syndrome (RSS), Lhermitte sign, and radiation necrosis.

RSS typically follows within 1 to 2 months of large-volume cerebral irradiation. In a series of 19 adult patients treated with radiation therapy for their CNS tumors, 16 experienced RSS.[1829] Patients typically become lethargic and anorexic and may report fever and headaches. Drowsiness and inability to concentrate are also common.[1829] Many of the symptoms recapitulate those at presentation of the initial tumor and can be very distressing to patients and their parents. The syndrome spontaneously resolves within 1 month in most cases, but occasionally low-dose corticosteroids are required.[1830]

Lhermitte sign is described by patients as an electric shock sensation traveling down the spine on neck flexion, usually arising within several months of cervicothoracic spinal irradiation.[1788] Occurrence after administration of chemotherapy, especially cisplatin, has also been reported.[1831] It is estimated to occur in 3% to 13% of adults[1832] but has not been well studied in the pediatric population. It usually resolves spontaneously within 2 to 3 months, although a few rare cases of prolonged sensory symptoms have been reported.[1832]

Radiation necrosis (Fig. 57-56) frequently occurs within months of treatment, although it can be observed

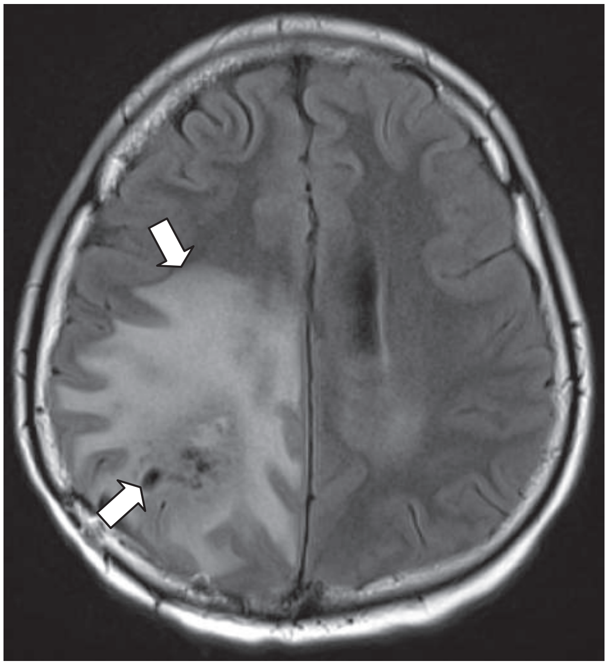

Figure 57-56 Radiation-induced necrosis demonstrating edema *(anterior arrow)* and central necrosis and blood products *(posterior arrow)*.

even years later; a median of 9 months for onset was reported in one retrospective series.[1833] The incidence can increase in association with chemotherapy.[1834] Common findings on MRI include an enlargement of the tumor region, with a central area of necrosis and strong edema signal best demonstrated on T2-weighted or FLAIR sequences. Because of the overlapping symptoms and MRI appearance of radiation necrosis with tumor progression, surgery is sometimes required to confirm the diagnosis and to relieve symptoms. A number of newer imaging modalities are being evaluated to better differentiate tumor necrosis from tumor progression; these modalities include perfusion MRI,[1835] DTI,[1836] and ^{11}C-methionine or ^{13}N-NH3-PET imaging.[1837,1838] On histopathologic examination, if a biopsy is performed, a significant lymphocytic infiltration can be observed.[1839] The occurrence of radiation necrosis is associated with increasing doses of radiation therapy and is most common with doses greater than 6000 cGy and fraction sizes greater than 180 cGy. It is commonly associated with SRS, especially in highly cellular tumors. Small round blue cell tumors of the CNS and glioblastoma multiforme are at particular risk. The treatment of radiation necrosis has historically relied on the use of high-dose steroids to suppress the inflammatory response and resulting edema. More recently the use of antiangiogenic agents has demonstrated activity in this regard. VEGF (initially identified as vascular permeability factor) regulates endothelial cell integrity. Thus agents that target VEGF signaling may become important therapeutic options in this regard.[1840,1841]

The late effects of radiation therapy can occur over a wide time range, and patients have an increase in mortality rate as adults as a result of secondary malignancies, recurrence, and vasculopathy.[1842,1843] On MRI transient T2-weighted signal abnormalities have been identified in patients approximately 1 year after radiation and chemotherapy. These lesions are smaller than 1 cm, and patients are generally asymptomatic. They occur in the high-dose radiation volumes and typically resolve on subsequent MRIs.[1844] These MRI findings can be a cause of concern for tumor recurrence but will usually resolve without therapy.

Late consequences of CNS radiotherapy include subtle or symptomatic, progressive, cognitive deficiencies in areas such as attention[1845] and memory impairment; learning disabilities become apparent[1158] and are related to the dose and volume delivered.[1846] Younger age at the time of radiation therapy is a critical variable. Patients younger than 3 years are at severe risk; those aged 3 to approximately 10 years can anticipate some cognitive decline. Even older children and adults will demonstrate some cognitive deficits after large-volume, high-dose cranial irradiation. It is unclear whether this effect reaches a plateau. Abnormal patterning of the hippocampus in patients undergoing radiation therapy have been observed and may account for some of the memory difficulties in this group of survivors.[1847] Similarly distinct patterns of volume loss in the posterior components of the corpus callosum in patients treated for medulloblastoma have also been reported and follow the radiation dosimetry in these regions.[1848] A detailed evaluation and assessment of the neurocognitive effects caused by therapy requires standardized and validated tools.[1820,1849]

Leukoencephalopathy has been described in children with ALL who received intrathecal and intravenous methotrexate[1850] and whole-brain irradiation.[1851] Similar findings are reported in association with high-dose cytarabine. Leukoencephalopathy may manifest subtle but progressive cognitive decline, as well as seizures. In its severe form, the child becomes demented and incapacitated. MR and CT imaging show diffuse periventricular leukomalacia, with patchy necrosis and calcification. The administration of high-dose intravenous methotrexate after craniospinal irradiation may lead to an especially high risk of leukoencephalopathy and transverse myelitis, and this sequence of treatments should be avoided. Myelopathy may arise as a late consequence of spinal irradiation. Changes in white matter as a result of radiation therapy can have a dramatic impact on cognitive function later in life, and serial assessment of these changes may be used to guide survivorship issues and intervention.[1852,1853] Many patients with significant cognitive decline after radiation therapy, particularly of the temporal lobes, may not demonstrate specific white matter or vascular changes to explain the clinical deterioration.[1854]

Radiation injury to vascular endothelium may lead to ischemic strokes.[1855,1856] If the process is slowly progressive, multiple small collateral vessels may arise consistent with a moyamoya pattern, as seen on MRI or angiogram. Patients with NF-1 are particularly at risk.[1828] This damage to vascular structures of the brain can result in long-term cerebrovascular problems, even decades later,[1857] ranging from acute hemorrhages and cavernous angiomas[1858] to slow and progressive loss of the vasculature and compensatory development of new vessels. The new vessels associated with moyamoya, however, are insufficient to replace the damaged ones.[85] When diagnosed, moyamoya can be corrected with a procedure called pial synangiosis and is well tolerated.[1859,1860]

Secondary malignancies including HGGs, atypical meningiomas,[1861] osteosarcomas,[1862] thyroid carcinoma,[1863] cavernous malformations, and schwannomas have been observed within the treatment field several years after the completion of radiation therapy (Fig. 57-57). Most radiation-induced tumors are not observed before 5 years and can often occur in the marginal areas that received intermediate doses (Fig. 57-58). From a molecular basis, radiation-induced secondary tumors in children do not appear significantly different than those that arise de novo. The prognoses for secondary HGG, atypical meningiomas, and osteosarcomas remains exceedingly poor,[712] thus arguing for the judicious use of radiation therapy, especially for patients with low-grade tumors.[621] Patients with Turcot syndrome, Gorlin syndrome, and NF-1 are more likely to have a secondary malignant glioma after radiation therapy compared with patients who do not have these conditions.[1864]

Pediatric patients with CNS tumors are at high risk for various endocrinologic abnormalities as a result of their tumor[1865] if they needed surgery (especially for pituitary region tumors) or radiation therapy that involves the

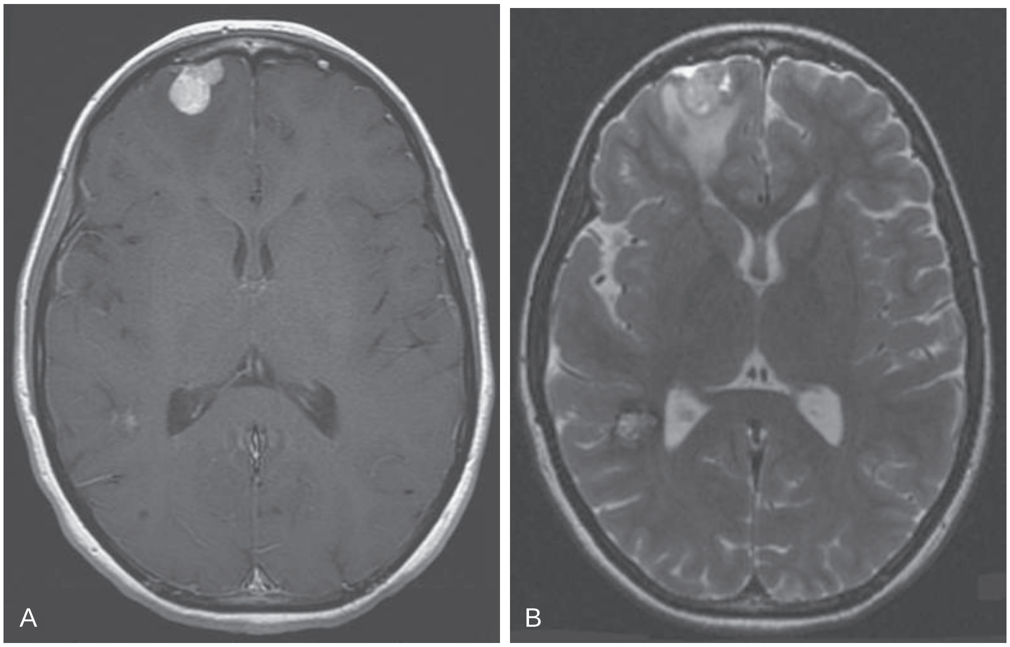

Figure 57-57 Radiation-induced meningioma and cavernous malformation. **A,** An axial T1-weighted image with use of contrast material. **B,** An axial T2-weighted image.

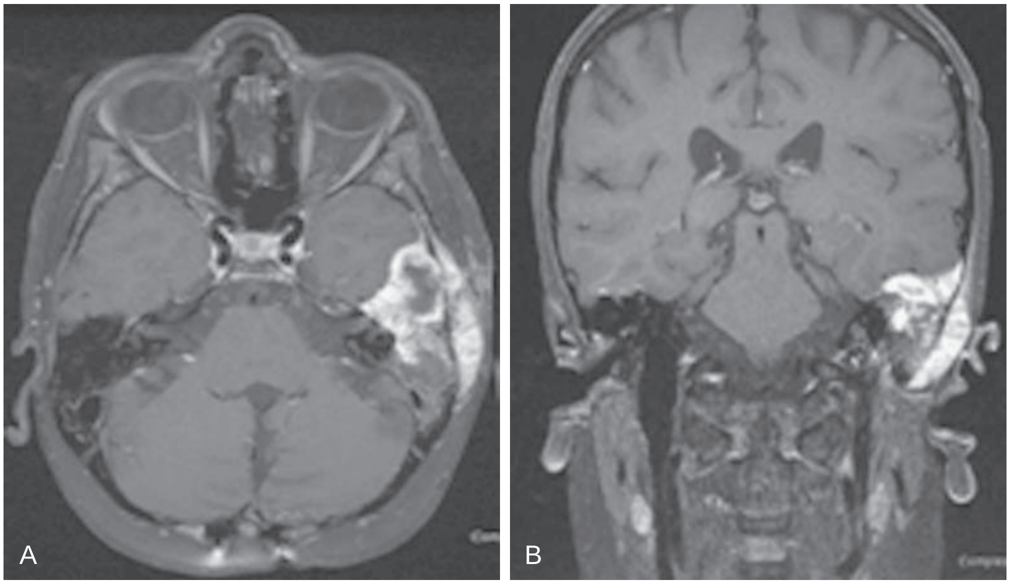

Figure 57-58 A radiation-induced left cranial osteosarcoma. **A,** An axial T1-weighted image with use of contrast material, with fat suppression. **B,** A coronal T1-weighted image with use of contrast material, with fat suppression.

hypothalamus and/or pituitary region.[1866,1867] Growth retardation is usually multifactorial.[1868] Radiation to the pituitary gland may affect the production and release of growth hormone.[1869] Pituitary function may also be impaired by direct invasion of the gland by adjacent tumor. Obesity can be a direct result of radiation therapy on the hypothalamus.[1870] In addition spinal radiation (as part of craniospinal treatment) affects the growth of the vertebral bones and overall bone density, within the radiation field and throughout the body.[1871] Ovarian function can be protected with laparoscopic oophoropexy preirradiation therapy in prepubertal girls in whom the ovaries are likely to be near the midline.[1872] Growth failure after treatment requires active and continued participation by endocrinologists as the cause of growth failure changes from chemotherapy-induced cachexia to radiation-mediated growth hormone deficiency.[1873] Although many patients are discouraged from initiating growth hormone replacement because of concerns for reactivation of the primary tumor or a second malignancy, no evidence exists that these phenomena occur.[1874,1875] In contrast the morbidity related to not replacing growth hormone for persons who are deficient can be severe and lifelong.[1876] Although most centers start growth hormone 1 year after completion of therapy, no evidence suggests that earlier treatment would increase the risk of tumor recurrence. Sleep problems and daytime fatigue[1503,1877] are also highly prevalent, and hypothyroidism and adrenocortical dysfunction can augment these problems and need to be corrected before any additional interventions are initiated.

Proton beam radiation therapy is becoming more common, and children needing radiation therapy for treatment should be evaluated for proton beam radiation therapy if possible because of the decrease in exit dose that would further minimize exposure of normal tissue to radiation.[1878]

Late Effects of Chemotherapy

Although most chemotherapy-related toxicities occur acutely, a number of long-term adverse effects are well recognized. Many of the neurologic effects of chemotherapy become evident at the time of drug administration and will improve after cessation of the drug. In some patients, however, these toxicities can become long-term issues. Peripheral neuropathy is a common late effect of several chemotherapy agents,[1879] particularly vincristine.[1879] Cisplatin and carboplatin mainly affect proprioception and spare pain and temperature sensation. The usual presenting symptoms are painful dysesthesias and tingling sensations in the toes and later in the fingers. Motor fibers are spared. In contrast vincristine produces sensorimotor neuropathies. The first symptoms are usually tingling in the toes and fingers. Loss of ankle reflexes is typically the first objective sign. Continued treatment with the drug leads to loss or decrease in reflexes and motor weakness involving the dorsiflexors of the feet. Patients with preexisting neuropathies may become quadriparetic after treatment with vincristine. Cerebellar syndromes of acute onset may be seen with high-dose cytarabine and occasionally with 5-fluorouracil. These complications are usually reversible within 2 weeks, but severe irreversible damage to Purkinje cells may occur if the drug is given for several months or if the drug is reintroduced at a later time.[1880] Transverse myelopathy is seen with prolonged treatments with intrathecal methotrexate or cytarabine. The risk is higher when combined with spinal irradiation.

Speech, language, and hearing are important components of daily functioning and can often be significantly impaired as a result of the brain tumor itself and its treatment.[1881] Many patients can have significant impairment that requires early and aggressive diagnosis and management.[1882] Radiation therapy can cause long-term hearing impairment,[1883] but platinum agents such as cisplatin and, to a lesser extent, carboplatin can cause significant sensorineural hearing loss in a dose-dependent manner. Although the higher frequencies of hearing, those above the normal hearing range, are usually affected initially, progressive loss can occur over time, including frequencies within the normal range. In one retrospective institutional study, 4% of patients treated with carboplatin and 57% of patients treated with cisplatin experienced significant hearing loss.[1884] The question of whether agents such as amifostine can reduce cisplatin-mediated hearing loss is controversial.[1143,1144] Patients at risk for hearing loss should therefore be followed up regularly by audiologists so that early corrective action (e.g., dose reduction of platinum agents and hearing aids) can be instituted.[1885]

Renal dysfunction is a rare complication of cisplatin toxicity; it can lead to lifelong proteinuria and salt wasting.[1886] Various electrolytes can be lost with renal impairment and may require daily oral supplementation.

Secondary malignancies are a rare but significant late effect of chemotherapy. In children acute myelogenous leukemia is the most common type of secondary malignancy induced by chemotherapy, and it is most frequently caused by intravenous etoposide.[1887] The risk of acute myelogenous leukemia appears to be dependent on the frequency and dose administered and is highest when etoposide is administered weekly, is less when administered monthly, and is very low when given daily in low-dose oral administration. These leukemias tend to occur 3 to 5 years after etoposide administration. Alkylating agents and platinum-based drugs are less commonly implicated. The transient genotoxic damage to chromosomes by chemotherapy may account for this risk.[1888]

A critical measure of the effectiveness of treatment of pediatric brain tumors is the patient's quality of life. Although this broad measure is difficult to define across all heterogeneous tumor types, age at presentation, and presenting morbidities, a greater emphasis on understanding how to optimize each child's potential has become the focus of many treatment protocols. The development of new assessment tools will also be important as new therapies are evaluated based on their efficacy and the quality of life.[1889]

Survivors have indicated the constant stress they feel after the medical team, family, and friends have declared success in curing them and their brain tumors.[1890] They recognize their own impaired social functioning much of the time[1891] and yet can also be unaware of important psychosocial and behavioral issues evident to others.[1892] Depression is a frequent and often poorly recognized outcome in these patients.[1893] Even patients with excellent prognoses, such as those with a history of LGGs, demonstrate chronic medical problems, as evidenced in one report of 87 patients in which 100% of patients had such issues.[1894] Declines in attention and concentration in survivors of pediatric brain tumors are a major problem,[1895] even when compared with other populations that have received therapy directed toward the CNS, such as survivors of childhood ALL.[1896] Part of this effect likely reflects the dosimetry of radiation used for patients with brain tumors, which increases neurocognitive impairment.[1897] Verbal memory difficulties associated with the treatment of tumors in the third ventricular region are significantly greater than those seen in patients with posterior fossa tumors, in whom attention difficulties predominate.[1898] The development of models to assist in the risk assessment of having a poor outcome is needed.[1899] These issues become particularly acute for this population, especially when they reach adulthood, when they are often faced with insurance barriers preventing them from receiving much-needed therapy.[1900]

Given the broad issues faced by brain tumor survivors, it is difficult to develop a single all-encompassing guideline for follow-up care. Multidisciplinary care in dedicated survivor programs can focus resources needed to identify and address issues in this patient population. From the imaging standpoint, many centers have adopted the standard of scanning patients who have completed therapy every 3 months for the first year, every 6 months for the second year, and then yearly thereafter. Routine surveillance of renal and audiologic assessments depends on the field and dose of radiation therapy, as well as the cumulative dose of platinum chemotherapy agents. The frequency of cardiac and pulmonary assessments also depends on particular agents previously used and their cumulative doses. Continued radiologic and blood chemistry surveillance is recommended for most patients as the risk of tumor recurrence decreases and is replaced by increased second tumor risk.

PALLIATIVE CARE

Although clinicians and caregivers focus a great deal of attention on attempts to eradicate disease, a significant proportion of patients with pediatric brain tumors will not survive and, in the process, many often suffer. The early introduction of palliative services, including discussions on the management of all symptoms[1901] and resuscitative measures (e.g., do not resuscitate orders),[1902] can allow patients and families in the terminal phase of care a more peaceful and dignified death.[1903-1905] Early and aggressive management of pain must be anticipated and the necessary expertise put in place.[1906] Children can often participate in discussion of end-of-life care.[1907] Decisions about where the child will die, either at home or in the hospital, as well as other aspects of terminal care must be addressed with the family and caregivers and require institutional support and resources.[1908] Finally, the discussion about the possibility of autopsy is never an easy one. The more time that patients, parents, and families have to consider an autopsy, separated in time from the actual death and grieving, the more likely they are to be able to make a decision that is right for themselves and their families. To understand the patient's disease better so that other children might be spared the same fate is comforting to families who consent to an autopsy.

Although limited by local governing policy, there is a low risk of transmission of cancer via transplanted organs from children with primary CNS tumors because of their infrequent spread outside the brain and spine. Given the severe shortage of organs, consideration of this option should be presented to the patient, family, and transplantation team.[1909]

Many families have expressed the importance of their relationship with the caregiving team. Many families actually feel abandoned after the child has died when communication and interaction with the medical team suddenly ends.[3] A number of palliative care programs and the expanding literature in this area are now available to help parents and caregivers through the grieving period.

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CHAPTER 58

Hepatoblastoma and Other Liver Tumors in Children

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CHAPTER OUTLINE

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INTRODUCTION AND EPIDEMIOLOGY

Liver tumors in children account for approximately 1.1% of all malignancies in children younger than 20 years of age according to the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) reports. The age-adjusted incidence rate of liver tumors is approximately 11 per million in infants younger than the age of 1 year and approximately 6 per million in children between 1 and 4 years of age.[1] This translates to approximately 100 to 150 new cases of liver cancer annually in children in the United States.

Liver malignancies in children can be divided into three broad classifications: hepatoblastoma, which accounts for two thirds of malignant liver cancers and the predominant malignant tumor type in young children; hepatocellular carcinoma (HCC), the predominant tumor type in adolescents; and other rare hepatic malignancies of childhood. A spectrum of benign tumor histologies broadens the differential diagnosis of the infant or child with a liver mass.

In an early meta-analysis of eleven separate series totaling 1256 primary liver tumors in children reported by Weinberg and Finegold, 43% were hepatoblastoma, 23% HCC, 13% benign vascular tumors, 6% mesenchymal hamartomas, 6% sarcomas, 2% adenomas, 2% focal nodular hyperplasia, and 5% other tumors.[2] Rare tumors of the liver also include rhabdoid tumors and hepatic germ cell tumors. An international working group recently provided guidelines regarding the histologic classification of pediatric liver tumors.[3]

Most liver tumors in childhood are more common in boys than girls. The male-to-female ratio observed for hepatoblastoma in cooperative group trials has ranged from 1.5 to 1 to 2 to 1; a combined analysis of multiple studies suggests that the male-to-female ratio in hepatoblastoma is 1.65,[4-7,8] consistent with SEER data.[1]

Hepatoblastomas have a unique age distribution (Fig. 58-1). Two peak ages occur, one at birth or within the first month of life and a second broad peak between 16 and 20 months of age. Ninety percent of hepatoblastomas occur in children younger than 4 years of age, and 95% of hepatoblastomas occur by age 5. Although rare, hepatoblastoma has been seen in adults, as described in multiple sporadic case reports.[9-31] A systematic review of hepatoblastoma in adults suggests that these tumors are associated with a particularly poor prognosis, although the time frame over which these cases were gathered

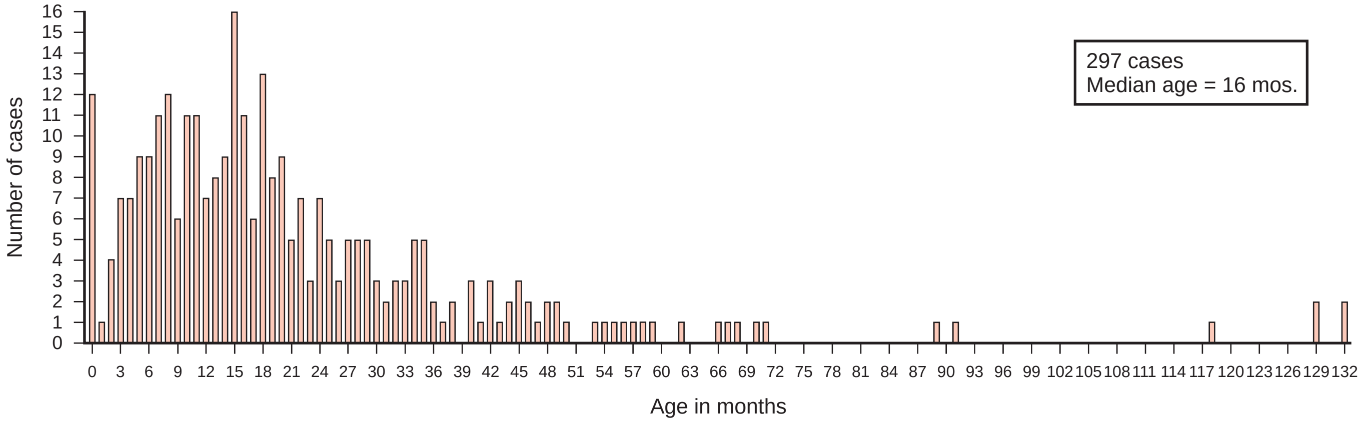

Figure 58-1 Age distribution of hepatoblastoma derived from 256 sequential cases representing all stages and histologies of hepatoblastoma. An initial peak is seen at birth, reflecting tumors developing during gestation. The median age of all children with hepatoblastoma is 18 months, and the oldest age is 78 months. In 99% of cases hepatoblastoma was apparent by 5 years of age.

spanned multiple decades.[32] In contrast hepatoblastomas diagnosed very early in life (i.e., in the neonatal period) are associated with a favorable outcome.[33]

Hepatoblastoma in children older than 5 years of age is often more aggressive than the typical hepatoblastoma, has characteristics of HCC, and has been termed *transitional liver cell tumor*.[34] More recently pathologists prefer to designate this poorly understood entity as *hepatocellular tumor, not otherwise specified*, pending a much-needed and more thorough understanding of the biology of these tumors with features of both hepatoblastoma and HCC.[3] HCCs make up the bulk of liver tumors seen after age 10 years and throughout adolescence and adulthood. As with hepatoblastoma, HCC is more common in male subjects than female subjects.[35-37] The fibrolamellar variant of HCC is a notable exception, with equal occurrence in male and female subjects.[38,39,40]

The incidence of liver tumors has increased over recent decades, perhaps more than any other type of childhood tumor. During a single decade the incidence rates of hepatic tumors in infants younger than 1 year of age increased from 4 to 8 per million.[41] The increase in rates was especially pronounced in female infants, in whom rates of hepatic cancer increased from 2 to 12 per million in a single decade. Ross and Gurney estimated that the average annual percentage change in incidence of hepatoblastoma between 1973 and 1992 was 5.2% for males and 8.2% for females.[42] The rise in incidence of hepatoblastoma has been estimated to be 4% per year between 1992 and 2004[43] and continues to rise in recent years compared with even recent SEER data.[1,44] The largest contributing factor to the increased incidence is thought to be the increased survival rates of premature infants who are at markedly increased risk of hepatoblastoma, as discussed in subsequent sections of this chapter.

ORIGINS OF HEPATOBLASTOMA

Hepatoblastoma has long been thought to have prenatal origins,[45,46] and increasing evidence points to prematurity, prenatal exposures, and overgrowth in early infancy as factors contributing to hepatoblastoma. It has long been presumed that embryonal tumors derive from primitive cells and that hepatoblastoma, as an embryonal tumor of the liver in which cells morphologically resemble cells in the developing embryonal and fetal liver, derives from a primitive hepatic cell. In rodents immature oval cells that proliferate after exposure to hepatocarcinogens express both biliary and hepatocytic markers.[47] These cells are characterized by staining with OV-1 and OV-6 markers. Ruck and colleagues demonstrated the existence of a similar cell type in hepatoblastoma on the basis of marking with OV-1 and OV-6 in a small population of cells within the tumor.[48-50] These OV-1 and OV-6 staining cells account for fewer than 1% of the cells within the tumor and provided evidence to support the existence of a population of stem cells in hepatoblastoma. Fiegel and coworkers analyzed hepatoblastomas for stem cell markers including CD34, Thy1, and c-kit and demonstrated the co-occurrence of these stem cell markers with hepatobiliary markers in ductal cells phenotypically resembling stem cells, which suggests that stemlike cells play a role in the histogenesis of hepatoblastoma.[51] Other markers of hepatic progenitor cells including epithelial cell adhesion molecule (EpCAM), cytokeratin 19 (CK19), octamer-binding transcription factor 3/4 (Oct-3/4), and delta-like 1 homologue (DLK1) have been shown to be expressed in hepatoblastoma tumor cells.[52] NOTCH2, which peaks in the second half of fetal development and then declines toward birth, is expressed in most hepatoblastomas.[53] CITED1, expressed in embryonic mouse liver and a marker of hepatic progenitor cells, is shown to be present in hepatoblastoma, providing another similarity of hepatoblastoma to hepatic precursor stem cells.[54]

GENETIC PREDISPOSITION AND LIVER TUMORIGENESIS

Most cases of childhood liver malignancy are not associated with an apparent major genetic syndrome. However numerous syndromes have been observed in association with hepatoblastoma or HCC, as well as some benign

TABLE 58-1 Genetic Syndromes' Predisposition to Liver Tumors

Disease	Tumor Type	Gene
Familial adenomatous polyposis	Hepatoblastoma, adenoma, hepatocellular carcinoma, biliary adenoma	*APC*
Beckwith-Wiedemann syndrome	Hepatoblastoma, hemangioendothelioma	Multiple candidates
Simpson-Golabi-Behmel syndrome	Hepatoblastoma	*GPC3*
Trisomy 18	Hepatoblastoma	No single gene
Li-Fraumeni syndrome	Hepatoblastoma, undifferentiated sarcoma	*TP53*
Glycogen storage disease types I-IV	Hepatocellular adenoma, carcinoma hepatoblastoma	Glucose-6-phosphatase
Hereditary tyrosinemia	Hepatocellular carcinoma	Fumarylaceto-acetate hydrolase
Alagille syndrome	Hepatocellular carcinoma	*JAGGED-1, NOTCH2*
Other familial cholestatic syndromes	Hepatocellular carcinoma	*FIC1, ABCB11*
Neurofibromatosis	Hepatocellular carcinoma, malignant schwannoma, angiosarcoma	*NF-1*
Ataxia—telangiectasia	Hepatocellular carcinoma	*ATM*
Fanconi anemia	Hepatocellular carcinoma, fibrolamellar cancer, adenoma	*FAA, FAC,* others (20%)
Tuberous sclerosis	Angiomyolipoma	*TSC1, TSC2*

hepatic tumors. A complete summary of genetic syndromes and liver tumors in children is provided in Table 58-1. The major genetic syndromes associated with childhood liver tumors are discussed in the subsequent sections.

Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP) is caused by germline mutation of the *APC* gene,[55] FAP is best characterized by the presence of colonic polyps, which without intervention are associated with the virtual certainty that adenocarcinoma of the colon will develop. Germline mutation of the *APC* gene is, however, also associated with a marked increased risk of hepatoblastoma. The risk of hepatoblastoma in children in FAP kindred is estimated to be 800-fold.[56] The risk that a parent with an *APC* mutation will have a child with hepatoblastoma is 0.4%.[57] Because FAP is an autosomal dominant disorder, genetic testing should demonstrate a predisposing mutation in 50% of offspring, such that the risk of hepatoblastoma in an infant who is a documented mutation carrier would be slightly less than 1%. Within a series of consecutive hepatoblastoma patients for whom family histories were obtained, approximately 8% of sequential cases of hepatoblastoma have a family history suggestive of FAP, and abnormalities of the *APC* gene were detectable in all cases.[58]

Also reported in children from FAP kindreds, albeit less commonly, are other liver tumors, including HCCs.[59-62] A benign precursor lesion, hepatocellular adenoma has been reported in a patient with a germline *APC* mutation and colonic polyps.[63] Within resected liver tissue from children with germline *APC* mutations and hepatoblastoma, multiple hepatic nodules were consistent with precursor lesions with potential to progress to either hepatoblastoma or HCC.[64] Presumably the timing of additional mutations or key developmental processes could determine the resulting histologic tumor type in the predisposed liver.

Significantly of nine reported cases of hepatoblastoma in siblings, at least four were in families with FAP;[65-68] Of the remaining reported sibling pairs, one was affected with type 1a glycogen storage disease,[69] and two sibling pairs had no apparent familial syndrome,[70,71] and one recent report of a sibling pair with hepatoblastoma demonstrated an *APC* mutation in only one of the siblings.[72] These reports suggest that whereas *APC* mutation accounts for a significant percentage of familial cases of hepatoblastoma, additional predisposition genetic changes may exist either as a distinct underlying cause of hepatoblastoma or as a modifying factor in infants with an existing *APC* mutation.

The percentage of sporadic hepatoblastoma patients (those without an obvious family history) who carry a germline mutation of the *APC* gene is not well defined. However it has been suggested both that children with hepatoblastoma be screened for germline *APC* mutations and that asymptomatic children in families with known polyposis be screened for hepatoblastoma.[73] Within the *APC* gene genotype-phenotype correlations suggest regions of the gene that predict both the severity of polyposis as well as the presence of extracolonic manifestations. The literature suggests that germline mutations in FAP families with children who are affected by hepatoblastoma can occur throughout the gene (Fig. 58-2) and can also include whole gene deletions or chromosome rearrangements such that the site or type of the individual *APC* mutation cannot be used to predict the occurrence of hepatoblastoma.[58,65,74] This finding implies a risk to all *APC* mutation carriers.

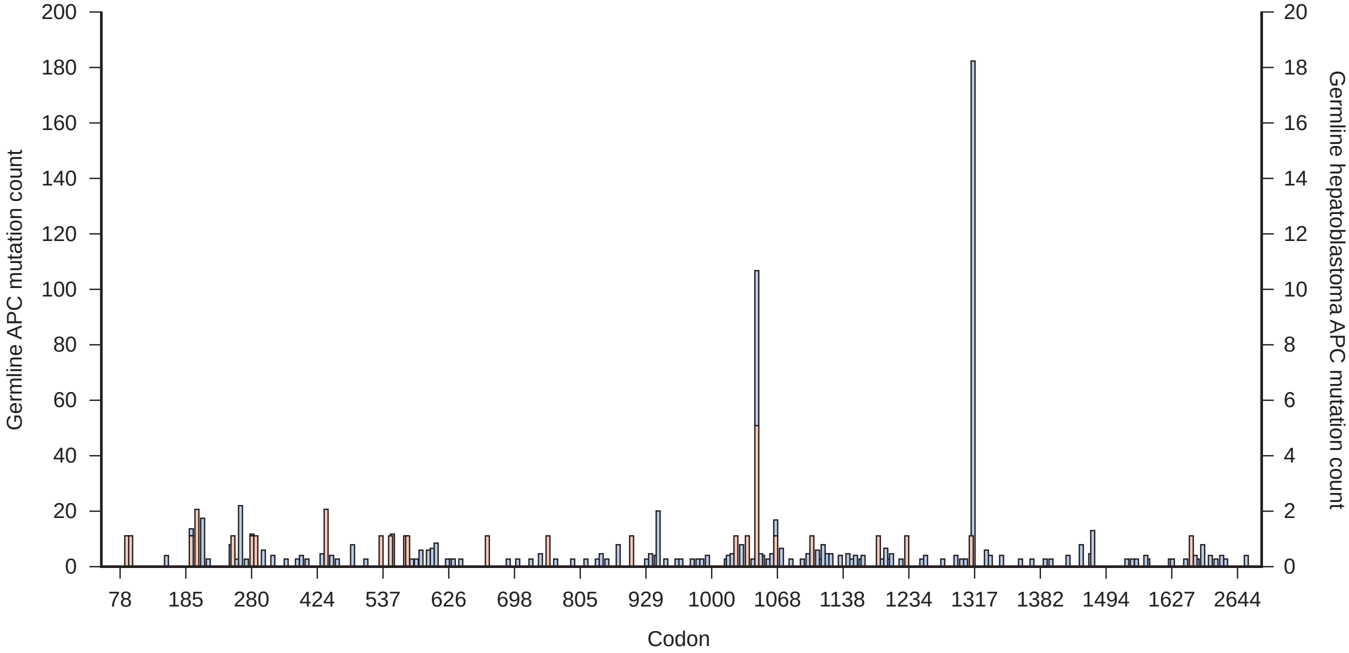

Figure 58-2 Frequency histogram of mutations in the adenomatous polyposis coli *(APC)* gene. Left axis and black lines represent all known APC germline mutations. Right axis and orange lines represent APC germline mutations in children with hepatoblastoma. Germline mutations associated with hepatoblastoma are observed throughout the gene. *(From Hirschman B, Pollock B, Tomlinson G: The spectrum of APC mutations in children with hepatoblastoma from familial adenomatous polyposis kindreds.* J Pediatr *147:263–266, 2005.)*

Overgrowth Syndromes and Hepatoblastoma

Beckwith-Wiedemann syndrome (BWS) is an overgrowth disorder classically characterized by large birth weight, macroglossia, omphalocele, and visceromegaly.[75,76] It is associated with a markedly increased risk of all embryonal tumors, including Wilms tumors, hepatoblastoma, neuroblastoma, and adrenal cortical adenoma.[75,77,78] A registry of children with BWS followed at the National Cancer Institute determined that the relative risk of hepatoblastoma in children with BWS was 2280, higher than the relative risk of any other type of cancer in BWS.[79] Unlike hepatoblastoma and FAP, hepatoblastoma has not been seen in siblings with BWS, despite the extraordinarily high relative individual risk in a patient with BWS. Presumably, this is because of the low incidence of familial occurrence of BWS.

BWS maps to chromosome 11p15, a region known to show loss of alleles in hepatoblastoma[80,81] as well as parental-specific expression of genes (i.e., imprinting), including *IGF2* and *H19*. Molecular subtyping demonstrated that genetic alterations underlying BWS include mutations, epigenetic events involving imprinted genes, or uniparental disomy and that imprinting defects are of two types—those with imprinting defect at the imprinting control element 1 (IC1) at distal 11p15.5 and involving IGF2 and H19 hypermethylation and those with a defect at the imprinting control element (IC2), which is more proximal and involved imprinting changes of KvDMR1 and KCN11OT1.[82,83] One series demonstrated that significantly more neoplasia is noted with either uniparental disomy or imprinting defects at IC1,[82] although hepatoblastoma has been observed in BWS patients with mutations at the IC2 locus.[83] Although differences may exist specific to the type of underlying genetic alteration, it is not possible at this time to tailor screening recommendations to genetic subtype. Present screening recommendations involve periodic ultrasonography of the abdomen and measurement of serum alpha-fetoprotein (AFP).[84-86] In a series of tumors detected in children with BWS who underwent such surveillance, all tumors were small and resectable at diagnosis.[84] Because hepatoblastoma has been noted to occur in the early neonatal period in BWS, some experts recommend screening at an early age.[87] Even though AFP levels are normally very high in the neonatal period, screening with AFP should confirm a downward trend in the absence of tumor.

Simpson-Golabi-Behmel syndrome (SGBS) is an X-linked syndrome characterized by overgrowth with clinical features reminiscent of BWS, including an increased risk of embryonal tumors. Individual cases of SGBS with hepatoblastoma have been reported.[88-90] The underlying gene for SGBS, *GPC3,* is highly expressed in hepatoblastoma compared with normal liver, which is consistent with a potential role in the tumorigenesis of hepatoblastoma.[91-93] Although no longitudinal tumor surveillance studies have been carried out for SGBS, the overlap in tumor types observed suggests that surveillance similar to that used for BWS may be appropriate.

Sotos syndrome, an overgrowth syndrome that has clinical features overlapping with BWS and is associated with a predilection for tumors, has been reported in a child with hepatoblastoma.[94,95] No large series have been reported, however, and the benefit of screening is unclear at this time.

Other Syndromes

Li-Fraumeni syndrome predisposes affected individuals to multiple tumor types in childhood and early adulthood.[96,97] Although liver tumors are not typically included in Li-Fraumeni syndrome, hepatoblastoma has been reported in two Asian Li-Fraumeni kindreds.[98,99] A kindred has also been reported in which a child developed undifferentiated (embryonal) sarcoma of the liver.[100] Acquired mutations of the *TP53* gene in hepatoblastoma tumor specimens, however, are rare or absent but have been reported in the undifferentiated embryonal sarcoma of the liver.[101-103]

Both hepatoblastoma and HCC have occurred in children with primary biliary atresia.[104,105] However it is unclear whether liver tumor development in children with biliary atresia is a complication resulting from liver disease or whether a common predisposition to both biliary atresia and tumor development exist. Significantly the same immature cells thought to share similarities to stem cells seen in hepatoblastoma are also seen in biliary atresia.[106] Multiple cases of hepatoblastoma and trisomy 18 have been reported.[107-113] This is somewhat notable in that trisomy of chromosome 18 is not seen as an acquired event despite the observation of multiple other recurring trisomies.[114]

Hepatoblastoma has been reported in a female subject with monosomy 7 myelodysplasia, a predisposition syndrome not previously seen with hepatoblastoma.[115] Hepatoblastoma has also been reported in neurofibromatosis type 1, although it is not characteristic of this disorder.[116]

A recent report has noted a possible association of hepatoblastoma with prune-belly syndrome, a congenital disorder of lax abdominal musculature and urogenital abnormalities.[117] A review of data from the Children's Oncology Group (COG) demonstrated a significant association with kidney and bladder congenital abnormalities.[118]

Genetic Syndromes and Hepatocellular Carcinoma

HCC, in contrast to hepatoblastoma, is seen in genetic syndromes that are characterized by hepatic cirrhosis. Progressive familial intrahepatic cholestasis, a condition marked by neonatal hepatic cirrhosis, is associated with liver tumor development in children. Both HCC and cholangiocarcinoma are known to occur in this disorder. The disorder, also known as Byler disease, is a heterogeneous syndrome.[119] The underlying genetic etiology is a germline mutation of the *ABCB11* gene, which encodes a bile salt export pump.[120] In a series of 11 cases of HCC in children younger than 5 years with neonatal hepatitis with clinical characteristics of familial intrahepatic cholestasis, 10 had immunohistochemical evidence of a deficiency in bile acid transporter bile salt export pump.[121] In all patients in whom genotyping was possible, a germline mutation of the *ABCB11* gene encoding bile salt export pump was detected.

Alagille syndrome is an autosomal-dominant disorder characterized by a characteristic facies, congenital heart disease, intrahepatic cholestasis with neonatal jaundice, and a paucity of hepatic bile ducts resulting in neonatal jaundice. The liver pathology may progress to frank cirrhosis. HCC has been reported in children with Alagille syndrome.[122-127] The vast majority of these Alagille syndrome–associated HCCs occur in boys. In one family three children were reported with HCC in the absence of any other type of liver disease other than the Alagille syndrome.[123] Alagille syndrome is associated with germline mutations in the *JAGGED1* gene, the endogenous ligand for *NOTCH2*, a developmental gene involved in controlling maturation of the heart and liver.[128] In a minority of cases of Alagille syndrome, in which the *JAGGED1* gene is not found to be mutated, the *NOTCH2* gene itself is mutated.[98,129] It has been shown that the mechanism of liver disease in Alagille syndrome may relate to an increased expression in hepatocyte growth factor in cells that carry *JAGGED1* mutations.[130]

The family of glycogen storage disorders has been associated with liver tumorigenesis. Glycogen storage disease type I has been reported in association with benign adenomas, HCC, and hepatoblastoma, although the latter has been reported only in adolescents.[69,131,132] Similarly glycogen storage disorder type III has been associated with HCC,[133] and type IV has been associated with hepatocellular adenoma.[134] Because HCC is known to occur in diseased liver, with long-term follow-up HCC may be considered a complication of increased survival of patients with glycogen storage disease.[132,135]

Hereditary tyrosinemia type 1, characterized by the deficiency of fumarylacetoacetate hydrolase is likewise associated with HCC in the setting of severe liver disease.[136]

Fanconi anemia is associated with liver tumors, although the association has been almost always reported in association with the use of androgenic steroid treatment.[137-143] In a review of 1301 reported cases of Fanconi anemia, 2.8% of subjects were reported to have a liver tumor.[144] The most common type of liver tumor reported in Fanconi anemia is HCC, but adenomas are also seen in these patients. The incidence appears to increase with age and does not appear to plateau. In multiple instances liver malignancy was seen in patients with Fanconi anemia who also had a diagnosis of leukemia. The extent of the associated androgen treatment with development of hepatic malignancy versus the effect of the underlying genetic defects in DNA repair as manifested by chromosome breakage in Fanconi anemia is unclear, but a review of the association of androgens and liver tumors suggests that patients with Fanconi anemia develop liver tumors after smaller and briefer androgen exposure than do individuals without Fanconi anemia.[145] It is also unclear whether there is a difference among the genetic subtypes of Fanconi anemia with regard to the predisposition to hepatic and other malignancy.[146]

Genetic Syndromes and Other Benign Liver Tumors

In addition to syndromes predisposing to malignant liver tumors, several syndromes are associated with benign liver tumors. Tuberous sclerosis has been linked to development of benign hepatic angiomyolipomas.[147,148]

Although these hepatic growths are not as common as renal angiomyolipomas, which may develop in as many as 80% of patients with tuberous sclerosis, the hepatic lesions are evident in imaging studies in approximately 13% of patients with tuberous sclerosis, according to one study.[149]

PREMATURITY AS A RISK FACTOR

One of the most intriguing aspects of hepatoblastoma is its association with premature or low-birth-weight infants. This association was initially reported by Japanese investigators who analyzed patient diagnoses and birth weights in the Japan Children's Cancer Registry over the 9-year period from 1985 to 1993.[150] It was found that 3.9% of patients with hepatoblastoma were of very low birth weight. Moreover it was observed that when data were subdivided over 4-year periods, the percentage of hepatoblastoma cases of low birth weight rose from 0.7% in 1985 to 1989 to 8.6% in 1990 to 1993 and demonstrated a linear increasing trend. The risk of hepatoblastoma has also been shown to be inversely proportional to birth weight.[151] The relative risk for an infant between 2000 and 2999 grams is 1.21, whereas the relative risk for an infant of less than 1000 grams is 15.64.

Data from the Children's Cancer Group survey confirmed the preponderance of former premature infants in a series of 76 patients with hepatoblastoma.[152] A second confirmation in the United States comes from the California population-based cancer registry, which also reported an elevated risk of hepatoblastoma in children of very low birth weight.[153] This study also pointed out that the age of diagnosis of hepatoblastoma in children of low birth weight was actually older than that of children of normal birth weight, which could partly be explained by gestational age.

It has been speculated that the increase in the percentage of low-birth-weight infants among children with hepatoblastoma may be partially caused by the increased survival rates of low-birth-weight infants. It is also believed that the increase in survival rates of premature infants at risk for hepatoblastoma may contribute to the overall increase in childhood liver tumors.

Indeed the rise in the rate of hepatoblastoma has been found to be consistent with the risk in births of low-birth-weight infants.[154] However whether the pathways by which low-birth-weight infants develop hepatoblastoma involve key exposures in the newborn period, increased sensitivity of the liver in premature infants to potential carcinogens, disruption of the normal process of liver development by premature birth, or a combination of these factors has yet to be defined. One small single-institution study, in which treatment records of five infants who developed hepatoblastoma were compared with those of infants of similar birth weights who did not develop hepatoblastoma, suggests that perinatal intensive long-term treatments contribute to the development of hepatoblastoma.[155] A somewhat larger study comparing 12 hepatoblastoma cases and 75 birth weight–matched controls suggests that the duration of oxygen therapy, use of furosemide, and length of time taken to regain body weight at birth were factors in predicting the likelihood that hepatoblastoma would develop in premature infants.[156] A study from the United Kingdom reported that polyhydramnios and either eclampsia or preeclampsia are more common in children with hepatoblastoma compared with those without hepatoblastoma, and that the eclampsia or preeclampsia observed was also associated with low birth weight.[157]

A recent case-control study of 600 COG patients with hepatoblastoma (AEPI04C1) has examined exposures in low-birth-weight and other infants with hepatoblastoma and has not found an association with maternal illness or medication use during pregnancy or infertility treatment and hepatoblastoma.[158,159] Previously use of assisted reproductive technology was postulated to be a contributing factor, although it is thought that it may contribute to hepatoblastoma development only through the associated risk of BWS and assisted reproductive technology (ART), but not as an independent risk factor.[159,160]

ENVIRONMENTAL EXPOSURES AND HEPATOBLASTOMA

An early epidemiologic case-control study of risk factors for hepatoblastoma by Buckley and colleagues, based on parental interviews, revealed an association of hepatoblastoma with maternal occupational exposure to metals, such as those used in welding or soldering (odds ratio [OR] = 8); petroleum products such as lubricating oils or greases (OR = 3.7); and paints or pigments (OR = 3.7). There was also a significant association with paternal occupational exposure to metals (OR = 3, $P = .01$) and a marginally significant association with paternal occupational exposure to petroleum products (OR = 1.9).[161] Although the aforementioned study did not find an association of parental smoking with hepatoblastoma, this has been reported in several subsequent studies.[162-164] The risk of hepatoblastoma in children of parents who smoked was approximately double in all three studies. Indeed in 2009 the International Agency of Research on Cancer declared that parental smoking is a carcinogen in the developing liver. Subsequent findings have been variable, however. A recent report from COG, the largest etiologic study to date, reported no association between smoking and hepatoblastoma. Variability among studies, however, could be based on small numbers and differences in reporting and overall smoking prevalence in the different populations. Although alcohol clearly plays a role in cirrhosis of the liver and HCC in adults, the COG study reported no association with parental alcohol use and hepatoblastoma in offspring.

VIRAL HEPATITIS AND LIVER TUMORS IN CHILDREN

Viral hepatitis infection has historically accounted for a large percentage of HCC cases. Before the introduction of the hepatitis B vaccine in Taiwan, HCC accounted for 80% of cases of liver tumors in children.[165] With the introduction of the hepatitis B vaccination in 1984, rates of liver tumors in Taiwanese children older than 6 years dropped significantly, from 0.70 per 100,000 from 1981 to 1986 to 0.36 per 100,000 between 1990 and 1994.[166] The decrease in rates of HCC after introduction of the vaccine was more pronounced in boys than in girls. In fact the incidence of HCC decreased in boys but not in girls.[167] The male-to-female incidence of HCC in Taiwan steadily decreased from 4.5 per 100,000 in 1981 to 1986 to 1.9 per 100,000 in 1990 to 1996, 6 to 12 years after the introduction of the vaccine. The reasons for these sex-related differences in responses to the vaccine are not known.[168] It has been demonstrated that among children with hepatitis B the presence of a deletion of the pre-S region of the hepatitis B virus was an additional risk factor for the development of HCC (OR = 36.69).[169]

PRESENTATION AND EVALUATION OF THE CHILD WITH A LIVER MASS

Aside from patients with known genetic syndromes (discussed in the preceding sections), most cases of hepatoblastoma occur in otherwise well children who present with an abdominal mass, often with some degree of abdominal discomfort.

Isosexual precocious puberty has been observed in some boys as a presenting finding in hepatoblastoma and occurs secondary to tumor production of chorionic gonadotropin.[170-182] In one study of 48 children, the incidence of precocious puberty in children with liver tumors was 6%.[183] To the authors' knowledge, there are no known cases of precocious puberty associated with hepatoblastoma in girls.

Laboratory evaluation of the child with a liver mass should include a complete blood count and liver function tests, as well as AFP and β-human chorionic gonadotrophin tumor marker levels. The platelet count is often high in hepatoblastoma but is not diagnostic of hepatoblastoma among children with liver tumors.[184] This thrombophilia is associated with high serum levels of thrombopoietin (TPO), also known as c-Mpl ligand. Normally synthesized in the liver, TPO has also been shown to be expressed in hepatoblastoma tumor tissues and is present at higher than normal levels in the serum of patients with hepatoblastoma.[185] In a large series of patients of various ages (13 to 84 years) who had hepatic tumors, thrombocytosis was noted in 2.7%. The high platelet count was correlated with higher serum TPO levels.[186] Other components of the complete blood count are usually normal insofar as bone marrow involvement with liver tumors has not been reported.

AFP is the primary serum marker that is used diagnostically and prognostically, as well as in surveillance. A sample should be obtained in the initial evaluation of any child with a liver mass.[187] AFP levels are markedly elevated in more than 90% of hepatoblastomas and more than 50% of HCCs. Care should be taken in interpreting AFP levels in the young infant because levels are normally very high at birth and decline to less than 10 ng/dL over the first year of life. Of note is that some liver malignancies—notably the small cell undifferentiated subtype of hepatoblastoma as well as the fibrolamellar variant of HCC—are not associated with elevation of the AFP.[188,189] Likewise, sarcomas and rhabdoid tumors are not associated with elevated AFP. The International Society of Oncology Epithelial Liver Group (SIOPEL) has demonstrated that hepatoblastomas with low AFP levels at diagnosis (less than 100 ng/mL) tend to present at a higher stage and are associated with a poor outcome.[188] A low AFP level has also been noted to be a marker of poor outcome across multiple studies compiled and analyzed by the Children's Hepatic Tumor International Consortium (CHIC) group.[190] The β-human chorionic gonadotrophin level is only occasionally elevated in hepatoblastoma, usually in cases that present with precocious puberty. The β-human chorionic gonadotrophin level is also elevated in the rare choriocarcinoma of the liver, as well as teratomas.

Evaluation of a child with a liver tumor should include a thorough family history to document specific cancers in relatives, with notation made of any early-onset colon cancers or colectomies, in addition to the presence of thyroid cancers or medulloblastoma, which can be part of FAP syndrome. Testing for germline mutation of the APC gene may be indicated; if performed, it should include appropriate genetic counseling. These patients with hepatoblastoma and germline APC mutations will require long-term surveillance for colonic polyps and tumor development.

In addition the medical history should note the birth weight, history of prematurity, and presence or absence of stigmata of neonatal overgrowth, including omphalocele and hemihypertrophy, which would suggest BWS and a risk for other embryonal tumors.

STAGING

Although North American trials have traditionally used a postsurgical diagnostic staging group, applied after the initial surgical procedure (biopsy or resection), the European system, based on the appearance by imaging before chemotherapy and surgical resection (PRETEXT), is being adopted worldwide. PRETEXT stages I through IV reflect the number of sectors that are involved with tumor. Other components of the staging system reflect extension into the vena cava , extension into the portal vein, extrahepatic abdominal disease, or distant metastases. The SIOPEL staging is shown schematically in Figure 58-3. A comparison of the prior North American staging and the current SIOPEL staging is shown in Table 58-2.

Figure 58-4 demonstrates a small hepatoblastoma tumor image that was initially diagnosed by surveillance ultrasound in a patient with BWS. This tumor involved two sectors and as such is classified as PRETEXT II, but because it was completely surgically excised after diagnosis, it would be stage 1 using the North American staging system. Figure 58-5 demonstrates a multifocal hepatoblastoma that involves all four sectors and had metastatic lesions in the lung; therefore it is PRETEXT IV by the North American staging system. If the patient had not had metastatic lesions, the tumor would be stage 3 according to the North American system but still PRETEXT IV.

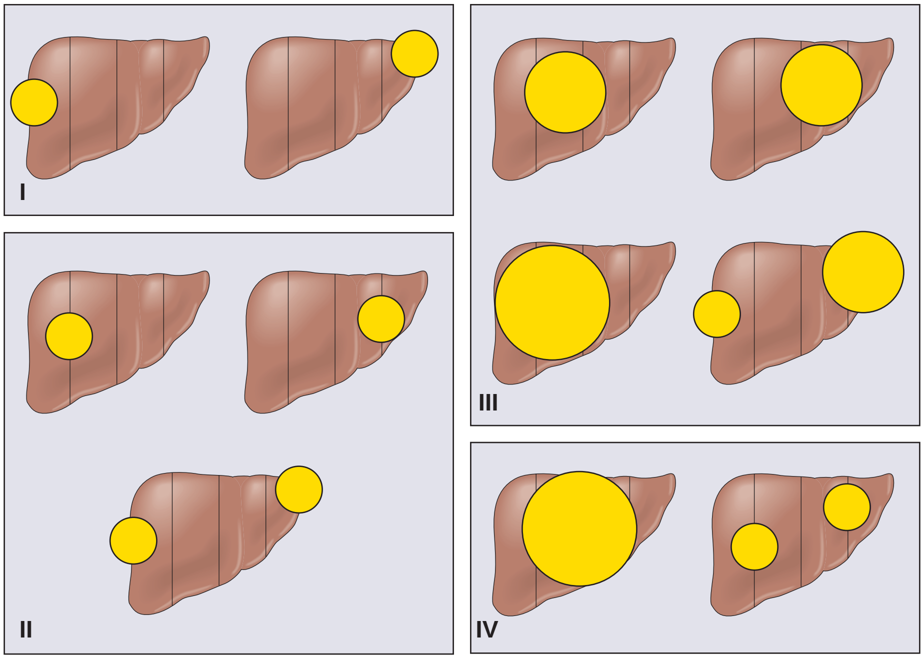

Figure 58-3 Pretreatment imaging-defined staging protocol presurgical staging system used by the International Society of Pediatric Oncology to predict outcome. The liver image is divided into four sectors, and tumor stage is defined as the number of free sectors. In this system, stage is further defined by extension of tumor beyond the liver as *V* indicating extension into the vena cava or all three hepatic veins (or both), *P* indicating extension into the main or both left and right branches of the portal vein, *E* indicating extrahepatic disease proved by biopsy, and *M* indicating the presence of distant metastases.

TABLE 58-2 North American Postsurgical Staging Versus European Presurgical Staging

	North American Staging	European Staging SIOPEL/PRETEXT
	Postsurgical Staging	Presurgical Staging
Stage 1	No metastases; tumor completely resected	Tumor involves only one quadrant; three adjoining liver quadrants free of tumor
Stage 2	No metastases, tumor grossly resected with microscopic residual disease (i.e., positive margins, tumor rupture, or tumor spill at the time of surgery)	Tumor involves two adjoining quadrants; two adjoining quadrants are free of tumor
Stage 3	No distant metastases, tumor unresectable or resected with gross residual tumor, or positive nodes.	Tumor involves three adjoining quadrants or two nonadjoining quadrants; one quadrant or two nonadjoining quadrants are free of tumor
Stage 4	Distant metastases regardless of the extent of liver involvement.	Tumor involves all four quadrants; there is no quadrant free of tumor

PRETEXT, Pretreatment Imaging-Defined Staging Protocol; *SIOPEL,* International Society of Pediatric Oncology Epithelial Liver Group.

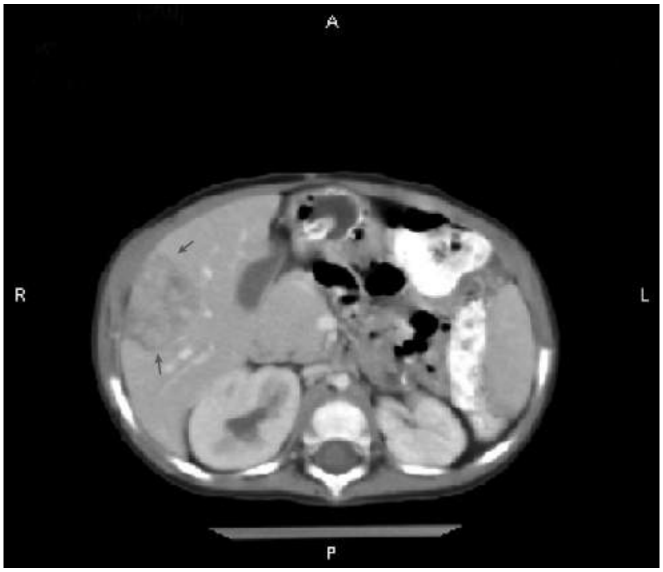

Figure 58-4 Computerized tomography scan of a low-stage hepatoblastoma detected by periodic surveillance with ultrasound in a patient at high risk of hepatoblastoma because of Beckwith-Wiedemann syndrome. The tumor was fully resected and classified as stage 1. *(Courtesy of Children's Medical Center, Dallas, Tex.)*

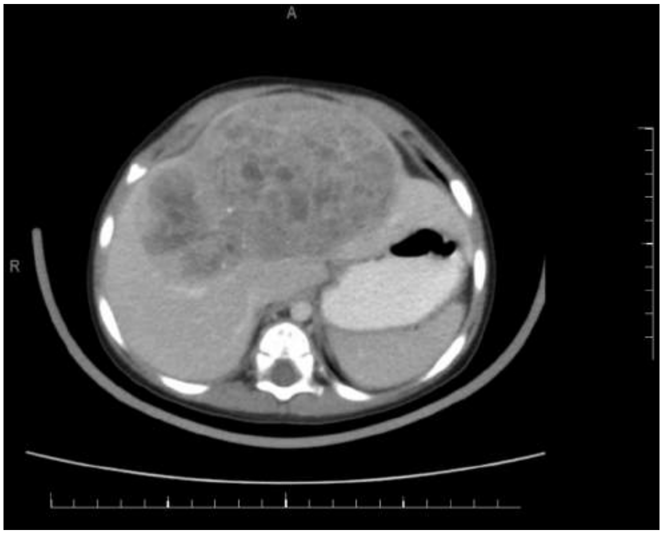

Figure 58-5 Computerized tomography of a high-stage hepatoblastoma. The tumor occupies most of the left lobe and a portion of the right lobe. Preoperative chemotherapy rendered this tumor resectable. This tumor was also accompanied by pulmonary metastases (not shown) and therefore was classified as stage 4 by North American staging system. *(Courtesy of Children's Hospital, Boston, Mass.)*

Both staging systems have been shown to have prognostic significance, but it is difficult to compare studies using different staging systems.[191] In the future the PRETEXT system will be used by most groups internationally.

PATHOLOGY OF HEPATOBLASTOMA AND HEPATOCELLULAR CARCINOMA

Grossly hepatoblastomas occur as single expansile masses, usually in the right lobe of the liver. A series of international discussions originating at the Los Angeles COG International Pathology Liver Tumors Symposium in 2011 resulted in a consensus classification of liver tumors in children, summarized in Table 58-3. A description of immunohistochemical properties currently recommended to aid in the classification of pediatric liver tumors is provided in Table 58-4.

A representative resected hepatoblastoma tumor is shown in Figure 58-6. Hepatoblastomas are composed primarily of epithelial cells that resemble different stages of the developing liver. Most hepatoblastomas are a mixture of cells resembling fetal cells and cells resembling embryonal cells. A typical hepatoblastoma of fetal and embryonal histology is shown in Figure 58-7, *A*. Because hepatoblastomas derive from immature cells that may retain the potential to differentiate, tumors often have foci of other types of more differentiated cells, including hematopoietic cells, as demonstrated in Figure 58-7, *B*. A subset of hepatoblastomas are characterized by cholangiocyte-like cells, which suggest the differentiation of the neoplastic cells along the cholangiocyte lineage as opposed to the hepatocyte lineage.[192] When the epithelial cells are aligned in thick cordlike structures, the tumor is described as having macrotrabecular features. In approximately 5% of hepatoblastomas, only fetal-type cells are seen histologically, as shown in Figure 58-7, *C*. These fetal-only tumors are now further classified according to the degree of mitoses.[3]

Two histologic subtypes of hepatoblastoma have significant implications for predicting clinical outcome. Approximately 7% of hepatoblastomas are composed of pure, well-differentiated fetal cells. The well-differentiated histology, previously referred to as *pure fetal histology*, has been associated with an exceptionally favorable outcome. A recent COG study by Malogolowkin and coworkers demonstrated that if the hepatoblastoma is completely resected surgically at the time of diagnosis, further therapy may be unnecessary.[193] The presence of this subtype and the desire to avoid the toxicity of

TABLE 58-3 International Consensus Classification of Pediatric Tumors

Epithelial Tumors	Mesenchymal tumors
Hepatocellular Benign and tumor-like conditions Hepatocellular adenoma Focal nodular hyperplasia Macroregenerative nodule Premalignant lesions Dysplastic nodules	Benign Vascular tumors Infantile hemangioma Mesenchymal hamartoma PEComas
Malignant Hepatoblastoma Epithelial variants: Pure fetal with low mitotic activity Fetal, mitotically active Pleomorphic, poorly differentiated Embryonal Small-cell undifferentiated INI1-negative INI1-positive Epithelial mixed (any/all above) Cholangioblastic Epithelial macrotrabecular Mixed epithelial and mesenchymal: Without teratoid features With teratoid features Hepatocellular carcinoma Classic Fibrolamellar Hepatocellular neoplasm not otherwise specified Biliary Benign—bile duct adenoma/hamartoma Malignant Cholangiocarcinoma	Malignant Embryonal sarcoma Rhabdomyosarcoma Vascular tumors Epithelioid hemangioendothelioma Angiosarcoma Other malignancies Tumors of uncertain origin Malignant rhabdoid tumor INI1-negative (or documented *INI1* mutation) Nested epithelial stromal tumor Germ cell tumors Teratoma Yolk sac tumor Desmoplastic small round cell tumor Peripheral primitive neuroectodermal tumor

From Lopez-Terrada D, Alaggio R, de Davila MT, et al: Towards an international pediatric liver tumor consensus classification: proceedings of the Los Angeles COG liver tumors symposium. Mod Pathol *27:472–91, 2014.*

TABLE 58-4 Immunohistochemical Evaluation of Pediatric Liver Tumors

	Fetal WDF	Fetal with Mitoses	Pleomorphic	Embryonal	Small Cell Undifferentiated	Mesenchymal	Cholangioblastic
GPC3	Finely granular	+++ Coarse	++ Coarse	+++ Coarse/ rare −	−, Rare+cell	−	−
β-cat	Variably +/+++ nuclear or normal	+/+++ Nuclear	+/+++ Nuclear	+/+++ Nuclear, can be−	+++ Nuclear	+++ Nuclear on osteoid/ blastema/neg in teratoid elements	Variable/positive nuclei
GS	+++	+++	Variable	Variable, can be neg	−	−	−
Hep Par	+++	+++	Variable	Usually −	−	−	−
Cyclin D1	−	+/++	+/+++	+/+++	+/++	Variable/neg in teratoid	
CK7	−	−		−	−/+	Variable/weak in blastema	+++
CK19	−	−	−	−	+/++ Variable	Neg/weak in blastema	+++
CD34	+ Endo	+ Endo					
Vimentin	−	−	−	−	+/++	+++	Usually −
INI1	+++	+++	+++	+++	Negative in pure SCU; variable when mixed	+++	+++

From Lopez-Terrada D, Alaggio R, de Davila MT, et al: Towards an international pediatric liver tumor consensus classification: proceedings of the Los Angeles COG liver tumors symposium. Mod Pathol *27:472–91, 2014.*

chemotherapy remain compelling reasons to pursue upfront resection whenever possible.

The small cell undifferentiated variant accounts for approximately 5% of hepatoblastoma and also carries clinical significance. Histologically the cells are small and pleomorphic and show high mitotic rates, as shown in Figure 58-7, *D*. There are several reports in small cell undifferentiated hepatoblastoma of unique chromosome translocations involving chromosome 22q12, the region of *SMARCB/INI1*, the gene most often associated with rhabdoid tumors and atypical teratoid tumors. Emerging evidence suggests that from histologic, cytogenetic, molecular genetic, and clinical perspectives these tumors resemble rhabdoid tumors to varying degrees.[194] Immunohistochemistry demonstrated loss of the INI1 protein in the small cell undifferentiated variant, and it is now recommended that INI1 staining be performed as part of the histologic characterization (see Table 58-4).[3] Mounting evidence suggests that the small cell variant is associated with a poor outcome.[188,194,195]

HCCs consist of cells that are well-differentiated and resemble normal hepatocytes. A typical HCC is shown in Figure 58-8. The fibrolamellar variant of HCC is characterized by larger, polygonal cells mixed with fibrous stroma and often eosinophilic cytoplasm, occasionally with cystic degeneration (Fig. 58-9).[196-198] In contrast to hepatoblastoma, the adjacent liver tissue in patients with HCC is often marked by cirrhosis. The fibrolamellar variant is an exception in which surrounding liver tissue is usually noncirrhotic.

ACQUIRED GENETIC CHANGES IN HEPATOBLASTOMA

Karyotypic changes in hepatoblastomas can be classified into two broad categories: numeric changes and structural changes of individual chromosomes, the most common of which involve translocations of chromosome

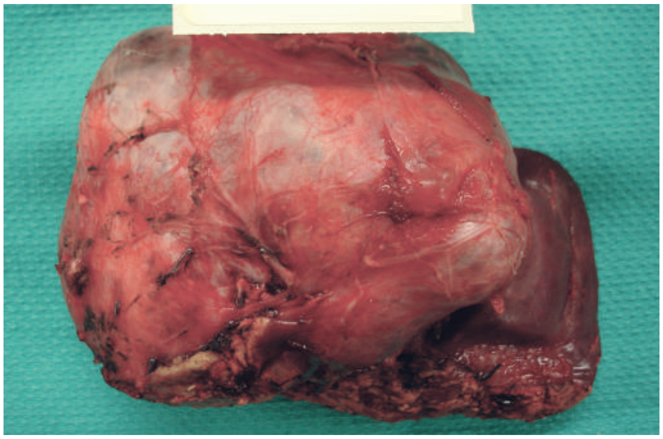

Figure 58-6 Gross pathology of resected hepatoblastoma tumor specimen. Tumor appears as a large expansile mass. The tumor has a whitish appearance compared with the darker adjacent liver in background. The cut surface is shown at the bottom of the image and has a variegated appearance. *(Courtesy of Victor Saldivar, MD, Children's Hospital of San Antonio, Tex.)*

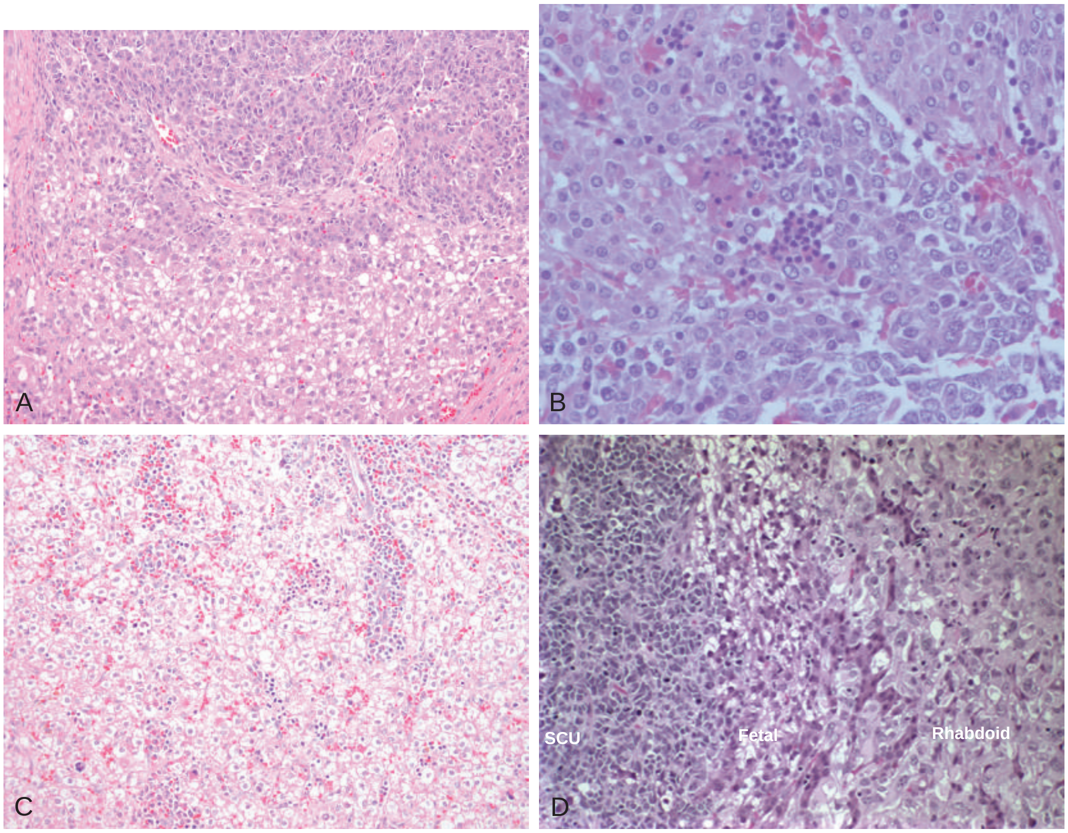

Figure 58-7 **A,** Mixed fetal and embryonal epithelial hepatoblastoma. This hepatoblastoma shows two different morphologic patterns. The fetal pattern, seen in the lower part of the image, is composed of uniform, small cuboidal cells with distinct cell membranes, whereas the embryonal pattern, seen in upper part of the image, consists of smaller angulated cells with hyperchromatic nuclei, higher nuclear-to-cytoplasmic ratios, and indistinct cell membranes. This is the most common histologic pattern of hepatoblastoma. **B,** Hepatoblastoma with extramedullary hematopoesis. As is often observed in hepatoblastoma, islands of hematopoietic cells are observed as clusters of small dark cells with prominent nuclei within surrounding tumor tissue. **C,** Pure fetal epithelial hepatoblastoma. This is composed of relatively uniform, small cuboidal cells. The cells have a small round nucleus with inconspicuous nucleolus, abundant cytoplasm that may be clear or finely granular, and distinct cell membranes. The pure fetal variant accounts for approximately 5% of all hepatoblastomas and is associated with a favorable outcome. **D,** Small cell undifferentiated hepatoblastoma. The central portion shows cells resembling fetal hepatoblastoma. On the *left,* cells appear undifferentiated with round nuclei and minimal cytoplasm and fail to form tubules. At *right,* cells are more typical of rhabdoid-like histology. *(**A**, Courtesy of Dinesh Rakheja, MD, Children's Medical Center, Dallas, Tex.; **B**, Courtesy of Victor Saldivar, MD, Children's Hospital of San Antonio, Tex.; **C**, Courtesy of Dinesh Rakheja, MD, Children's Medical Center, Dallas, Tex.; **D**, Courtesy of Milton Finegold, MD, Texas Children's Hospital, Houston, Tex.)*

1.[114] The initial recurring translocation described was the translocation involving chromosomes 1 and 4, t(1;4)(q12;q34).[199,200] It has since been reported in a large series that hepatoblastoma is characterized by a family of chromosome translocations with similar breakpoints on either chromosome 1q12 or 1q21.[114] Each such translocation observed is unbalanced, resulting in a gain of the long arm of chromosome 1, and is also frequently associated with numerous whole chromosomal gains.

Most of the numeric aberrations result in addition of whole chromosomes, but occasionally they result in loss of chromosomes. The specific numeric changes are nonrandom. Trisomies of chromosome 2, 8, and 20 are the most common recurring numeric aberrations. The most commonly observed chromosomal losses are chromosomes 4 and 18. Although these whole chromosome changes have been described previously by classical karyotype analysis, they can perhaps be better visualized by whole genome comparative genomic hybridization, as shown in Figure 58-10. The clinical significance of these chromosomal abnormalities is not yet known..

HCC is also characterized also by chromosomal gains and losses, with loss of the Y chromosome a notable finding.[201] Duplication of chromosome 1q is also seen in HCC with increased expression of numerous chromosome 1 genes.[202,203] Cytogenetic data on the fibrolamellar variant of HCC is sparse, but one childhood fibrolamellar carcinoma has been characterized by a hypertriploid karyotype with clonal evolution and multiple additional chromosomal gains as well as loss of the Y chromosome.[204]

MOLECULAR ABERRATIONS

The most common genetic alteration in hepatoblastoma is alteration of the Wnt signaling pathway. *CTNNB1* alteration is detected in the vast majority of hepatoblastomas, either as a point mutation in exon 3 or a large gene deletion.[205,206] Less commonly alterations are observed in other members of the Wnt signaling pathway, including *APC* and *AXIN* genes.[207] CTNNB1 accumulates in the nucleus of tumor cells with alterations of the Wnt signaling pathway.

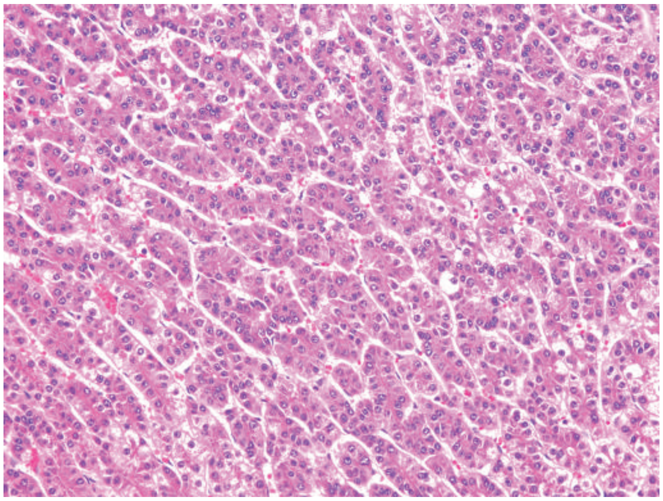

Figure 58-8 Hepatocellular carcinoma. This well-differentiated hepatocellular carcinoma is composed of two- to four-layer thick cords or trabeculae of cells resembling normal hepatocytes. Sinusoidal-like spaces lined by endothelial cells are present between the trabeculae, further mimicking the architecture of normal liver. No portal tracts are present. *(Courtesy of Dinesh Rakeja, MD, Children's Medical Center, Dallas, Tex.)*

In a preliminary whole exome study by Trevino and colleagues, mutation of the *NFE2L2* gene was present in approximately 6% of hepatoblastoma tumors.[208] Of note this same gene mutation is observed in 6% of HCCs in adults.[209]

Cairo and coworkers have demonstrated that upregulation of MYC in conjunction with Wnt signaling aberration is a hallmark of aggressive hepatoblastoma and have described a 16-gene signature in a posttreatment resected specimen that was associated with a more aggressive clinical behavior.[210] These and other molecular findings will need to be examined in a prospective study to determine the impact on patient risk stratification and management.

TREATMENT: CHEMOTHERAPY IN HEPATOBLASTOMA

Chemotherapy has been used extensively in the management of hepatoblastoma to reduce tumor bulk and thereby render tumors resectable and to treat microscopic disease after resection. Surgical resection, as discussed later, is essential for all malignant liver tumors to achieve cure. Although great strides have been made in the development of chemotherapeutic approaches for hepatoblastoma, challenges remain in eradicating unresectable disease as well as in determining new effective agents for refractory disease. Success in the past three decades has been obtained primarily through the four major cooperative groups based in North American (COG and its predecessors), Europe (SIOPEL), Germany (Cooperative German Paediatric Liver Tumor Study), and Japan (Japanese Study Group for Pediatric Liver Tumor [JPLT]). The legacy North American cooperative group approach has been to resect, if possible, before the administration of chemotherapy, whereas the European approach has been to use neoadjuvant chemotherapy with initial biopsy to shrink tumors before therapy. Regimens used in the treatment of hepatoblastoma have centered largely on platinum agents and doxorubicin. Other agents studied and incorporated into protocols have included vincristine, 5-fluorouracil, ifosfamide, etoposide, and recently irinotecan. A summary of results of published treatment regimens is given in Table 58-5.

Early reports documented the effectiveness of vincristine as a single agent,[211,212] and subsequent Pediatric Oncology Group and Children's Cancer Group trials used vincristine along with 5-fluorouracil and cisplatin. Doxorubicin was shown to be effective in a phase II trial of refractory pediatric solid tumors, including hepatoblastoma, using either a 4-day course of 15 to 20 mg/m^2 daily or a 3-day course of 30 mg/m^2 daily.[213]

A study from St. Jude Children's Research Hospital reported a response to cisplatin (DDP) at a dose of

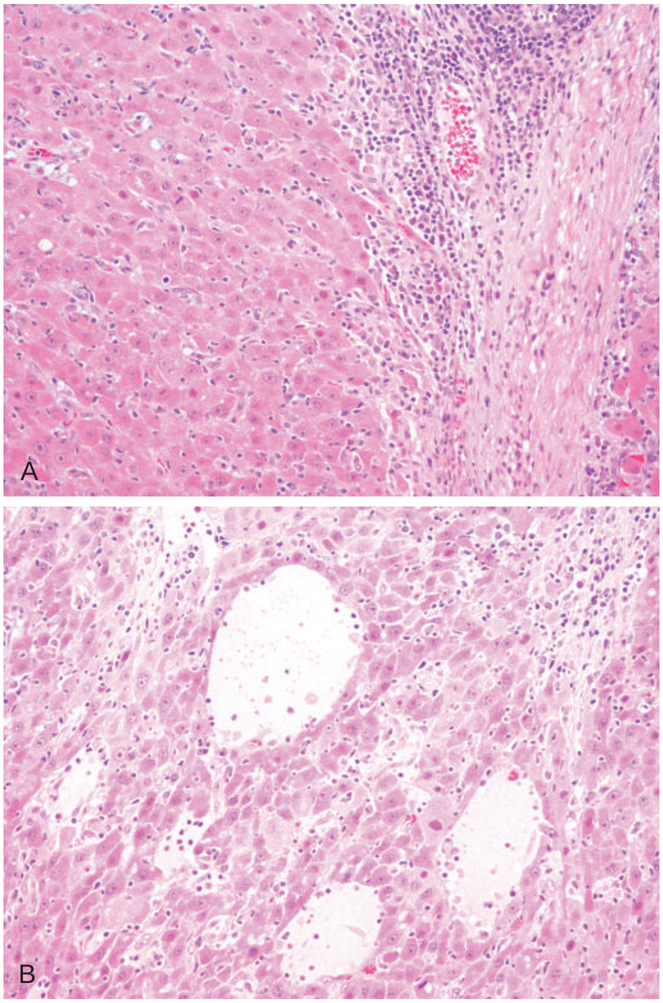

Figure 58-9 Fibrolamellar hepatocellular carcinoma. Fibrolamellar hepatocellular carcinoma is composed of sheets of polygonal tumor cells that are larger than normal hepatocytes and have a deeply eosinophilic cytoplasm, a large nucleus with prominent eosinophilic nucleolus. As shown prominently in **A,** the cells are embedded in collagenous stroma, and variably thick fibrous septa course through the tumor. Often there are focal infiltrates of mononuclear inflammatory cells. **B,** Foci of cystic degeneration (pseudogland formation) are apparent. *(Courtesy of Dinesh Rakeja, MD, Children's Medical Center, Dallas, Tex.)*

TABLE 58-5 A Summary of Recent Cooperative Group Studies in Europe and North America

Study Protocol	Presurgical chemotherapy	Chemotherapy agents	N =	Outcome	Reference
POG—#8697	No	Cisplatin, vincristine, fluorouracil (C5V)	73	3-year EFS: stage I (UH) / II: 91% stage III: 67% stage IV 12.5%	305
COG—#9645	Yes	C5V vs. intensified platinum[*]	33	5-year EFS: stage III: 73% stage IV: 27%	306
INT—0098	Selected patients	C5V vs. cisplatin and doxorubicin	182	5-year EFS: stage I (UH) / II: >90% stage III/IV: >50%	4
HB—89	Yes	Ifosfamide, cisplatin, doxorubicin	72	3-year EFS: stage I: 100% stage II: 50% stage III: 74% stage IV: 29%	225
HB—94	Selected patients	Ifosfamide, cisplatin, doxorubicin	69	3-year EFS: stage I: 96% stage II: 100% stage III: 76% stage IV: 36%	226
SIOPEL—1	Yes	cisplatin, doxorubicin	154	5-year OS: 75%	222
SIOPEL—2	Yes	Cisplatin/carboplatin, doxorubicin—risk stratified	150	3-year OS[†] SR: 90% HR: 78%	7
SIOPEL—3	Yes	Cisplatin/ carboplatin	151 (HR)[‡]	3-year OS: 69% (HR)	223
SIOPEL—4	Yes	Cisplatin—dose dense	61 (HR)	3-year OS = 83% (HR)	224

COG, Children's Oncology Group; *EFS,* event-free survival; *HR,* high risk; *OS,* overall survival; *POG,* Pediatric Oncology Group; *SIOPEL,* Childhood Liver Tumours Study Group; *SR,* standard risk; *UH,* unfavorable histology.

[*] Only Stage II and IV patients enrolled.

[†] SR: Standard risk. Defined as hepatoblastoma confined to the liver and involving no more than three hepatic sectors.

[‡] HR: High risk. Defined as hepatoblastoma extending into all four sectors or lung metastases or extrahepatic involvement (or a combination thereof).

90 mg/m^2 in 10 of 11 patients with hepatoblastoma.[214] A subsequent study at MD Anderson Cancer Center used high-dose cisplatin (150 mg/m^2 as a single dose) in patients with unresectable hepatoblastoma, showing a marked response in primary tumor size as well as metastatic disease.[215] Platinum compounds subsequently emerged as the most effective means to treat hepatoblastoma, although treatment has been associated with considerable toxicity. Ototoxicity was observed in 37% of children receiving platinum agents, according to a European study that included 120 children with cancer diagnosed at a median age of 2.6 years, including 11 patients with hepatoblastoma; the ototoxicity was most severe in patients younger than 36 years of age at diagnosis and was progressive over time.[216]

The use of doxorubicin and cisplatin together in the treatment of hepatoblastoma has long been shown to be effective. The first report of this combination described a 15-month-old girl with unresectable hepatoblastoma of mixed histology who received doxorubicin and cisplatin.[217] Quinn and colleagues reported four patients with hepatoblastoma, three of whom had unresectable tumors that were treated with cisplatin and doxorubicin.[217a]

The successful combination of cisplatin with doxorubicin was further studied in Toronto. In this study 15 children were treated with cisplatin (20 mg/m^2 daily for 5 days) in combination with doxorubicin (25 mg/m^2 daily for 3 days), with unresectable disease in 13 of the original 15.[8] A second report, also from Toronto, documented success in administering similar doses of cisplatin and doxorubicin simultaneously as a continuous infusion in an attempt to further increase cell kill while minimizing toxicity.[218] Significant tumor reduction allowing surgical resection was attained in all six of the patients studied, with five surviving over the long-term and one child dying perioperatively as a result of surgical complications.

The Children's Cancer Study Group undertook a study administering cisplatin at a dose of 100 mg/m^2 daily together with continuously infused doxorubicin and reported efficacy in initially unresectable hepatoblastoma.[219] Building on this successful combination of doxorubicin and cisplatin, the two North American cooperative groups designed an intergroup study to determine the efficacy and safety of this regimen in a large number of patients. The doxorubicin and cisplatin regimen was

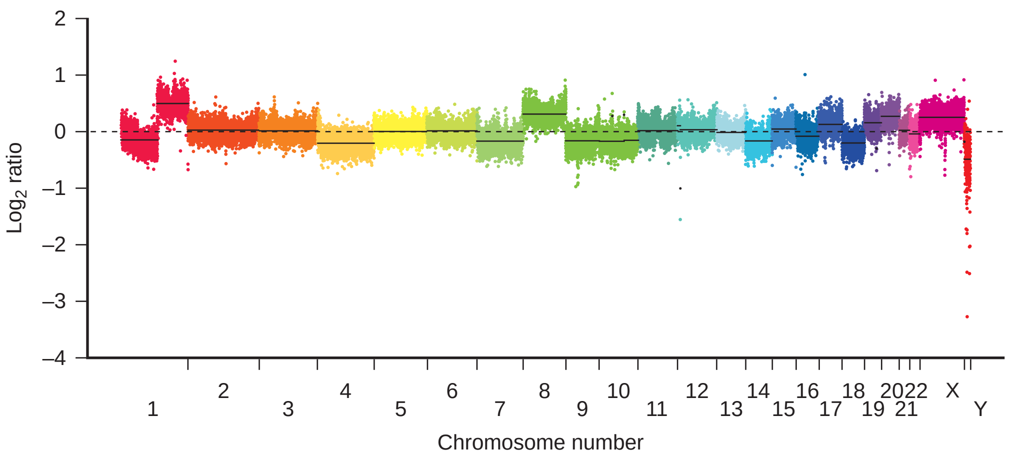

Figure 58-10 Genome-wide copy number comparative genomic hybridization (CGH) scan of hepatoblastoma tumor compared with standard normal male control DNA. Hepatoblastoma is characterized by whole chromosome gains and losses as well as unbalanced translocations of chromosome 1 resulting in duplication of the long arm of chromosome 1q. This CGH plot demonstrates increased copy number of chromosome 8, 17, 19, and 20 as well as whole chromosome losses of chromosomes 4 7, 9, 10, 14, and 18. In addition a duplication of chromosome 1q and loss of chromosome 1p is observed. Numeric aberrations of X and Y reflect the female sex of the child rather than tumor-specific changes.

found to be as effective as the cisplatin, vincristine, and 5-fluorouracil regimen (C5V); however toxicity was greater, particularly in infants, in the patients treated with the doxorubicin and cisplatin regimen.[4] Subsequent studies in North America have omitted doxorubicin and concentrated on platinum-based regimens.

The use of alternating platinum analogues cisplatin and carboplatin without concomitant use of other agents was attempted in a randomized trial conducted by the Pediatric Oncology Group P9645, but this resulted in a dismal outcome of a 37% event-free survival rate compared with a 57% event-free survival rate for patients receiving the C5V regimen. In addition, patients randomized to receive intensified platinum therapy only experienced more toxicity and a greater need for transfusions. Therefore the standard of care in North America returned to the C5V regimen, with the recent trials (AHEP0731) re-incorporating the use of doxorubicin in addition to C5V in intermediate-risk patients. This trial also explored the efficacy of irinotecan as an upfront window in the patients at highest risk.

Because early reviews suggested that tumors of pure fetal histology had a more favorable outcome,[220,221] a concentrated effort has been made to reduce therapy in this group. The first attempt to reduce therapy in this subset of tumors was in the intergroup study POG 8945/CCG 8881, in which patients with stage 1 tumors of pure fetal histology were treated with four courses of doxorubicin only, compared with patients with other tumors, which were treated with multiagent therapy. In the recent AHEP0731 study, tumors of pure fetal histology, if fully resected, received no further therapy. The ability to avoid chemotherapy in this select group of tumors, based on pathology, argues strongly for the need for histologic characterization before embarking on any course of chemotherapy.

The Europe-based SIOPEL group also used a regimen of cisplatin and continuous infusion of doxorubicin in the SIOPEL-1 study, with 5-year survival rates of PRETEXT I, II, III, and IV disease observed to be 100%, 83%, 56%, and 46%, respectively.[222] SIOPEL-2 used cisplatin for standard-risk patients and cisplatin alternating with carboplatin and doxorubicin in higher-risk patients.[7] SIOPEL-3 demonstrated notable improvement in patients with metastatic disease, with a 5-year survival rate of 56% following intensive treatment of 10 cycles of cisplatin alternating with carboplatin and doxorubicin.[223] The recent SIOPEL-4 study used a dose-dense cisplatin-based regimen that also included doxorubicin for high-risk patients (i.e., those with extrahepatic abdominal disease, major vascular invasion, tumor rupture, tumor in all segments, or metastatic disease). The overall survival rate at 3 years was 83%, a significant improvement over past regimens; however moderate to severe ototoxicity occurred in 50% of patients.[224] Given the efficacy of cisplatin along with the frequent occurrence of ototoxicity now documented in multiple studies, families of children currently treated for hepatoblastoma should be advised of the risk of hearing loss, and children should be closely monitored during treatment and for years afterward. A current priority is the development of therapies that are less toxic.

Exploration of treatment by the German cooperative groups has included the use of ifosfamide in conjunction with cisplatin and doxorubicin (IPA). The overall survival rate was 75%, which is comparable to that of SIOPEL and North American studies and indicates a possible alternative approach. However the combination of etoposide, ifosfamide, and carboplatin used in stage IV tumors had a survival rate of 21%, a dismal result similar to those of previous regimens.[225,226]

Treatment of Recurrent or Refractory Disease

Despite the often successful treatment of newly diagnosed hepatoblastoma, treatment of recurrent hepatoblastoma remains a significant therapeutic challenge. The relative rarity of tumor recurrence poses additional challenges to retrieval protocol development strategies. As with the treatment of primary disease, the ability to surgically resect recurrent disease may influence outcome, and surgical approaches to recurrent disease either for local hepatic recurrence or for pulmonary metastases have met with some success.

Feusner et al reported in 1993 from the early Children's Cancer Group study CCG-881 that of 10 initially low-stage hepatoblastoma tumors that later recurred with lung metastases, the most effective treatment was surgical resection of metastatic lesions in addition to systemic chemotherapy.[227] In a retrospective study by Meyers and colleagues of 20 patients with initial clearance of pulmonary disease, using INT-0098 data, 13 had a surgical approach, with 4 of 13 surviving.[228] These studies suggest that surgical resection may play a role in survival, but they also underscore the need for better systemic therapies to control metastatic disease. The SIOPEL group reported 59 patients from SIOPEL studies 1,2, and 3.[229] All of these patients received doxorubicin as part of their initial therapy. Retrieval strategies were attempted in 52 patients and varied, including chemotherapy plus surgery in 25 (42%), chemotherapy alone in 21 (36%), and surgery alone in 7 (12%). Palliative care only was provided in five cases. Slightly more than half of the patients achieved a second complete response, but the 3-year event-free survival rate was only 34 %. Factors associated with successful outcome included PRETEXT group I through III at diagnosis and the use of both surgery and chemotherapy as a retrieval strategy.

High-dose chemotherapy with stem cell rescue has been attempted for hepatoblastoma with little success, as summarized elsewhere.[230] The Japanese Study Group for Pediatric Liver Tumor, JPLT-2, has also reported limited success with high-dose chemotherapy and stem cell transplant.[231]

TREATMENT OF HEPATOCELLULAR CARCINOMA

Compared with the successes in treating hepatoblastoma with chemotherapy, the progress in treating HCC has been for the most part disappointing in both children and adults. Although an early study indicated that doxorubicin induced remission in HCC in 14 of 44 (32%) patients[232] subsequent studies with doxorubicin in HCC have been disappointing. Within the cooperative groups, because of the small numbers seen, HCC has been treated similarly to hepatoblastoma, with acknowledgment that it is a distinct entity. Survival for initially unresectable HCC has remained dismal. When patients treated with regimens of cisplatin, vincristine, and 5-fluorouracil were compared to patients treated with continuously infused cisplatin and doxorubicin using the same regimens shown to be effective in hepatoblastoma[4] the results demonstrated that less than 20% of stage III and IV patients survived.[37] However among eight patients with HCC that was resected at diagnosis, the long-term survival rate was 88%. This is the largest treatment study of HCC in children. Thus early resectability is an important factor in predicting the likelihood of survival in children who have HCC, which is similar to that observed in adults with HCC.

In 2007 the FDA approved the use of sorafenib, an oral tyrosine kinase inhibitor previously shown to be effective in treating advanced renal cell carcinoma, in the treatment of HCC after a large trial of 602 adult patients demonstrated a median 10.7-month survival rate and a 5.5-month time to tumor progression in patients treated with 400 mg sorafenib twice daily compared with a median 7.9-month survival rate and 2.8-month time to tumor progression in patients treated with placebo. The dose-limiting toxicity of sorafenib appears to be hand-foot skin reaction with other toxicities, such as diarrhea and lipase or amylase elevation. A previous recent study in the United States, from Memorial Sloan Kettering, demonstrated in a phase II, nonrandomized study that sorafenib was well-tolerated, albeit with a modest efficacy, which would suggest an indication for its future inclusion in multiagent trials for HCC.[233] Interestingly this study also performed gene expression microarray analysis on tumors treated with sorafenib and placebo and determined a pattern of gene expression that correlated with a response to sorafenib; this information could conceivably serve as a means of treatment stratification in future trials. Further exploration of the role of sorafenib in pediatric liver tumors is warranted.

SURGICAL ASPECTS OF LIVER TUMORS

Surgical resection is an important component in the management of both benign and malignant liver tumors. In the case of benign lesions such as adenoma, focal nodular hyperplasia, and mesenchymal hamartoma, complete resection of the tumor is usually curative. Resection may involve anatomic lobectomy when necessary or nonanatomic wedge resection in the case of smaller peripheral lesions. In the case of hemangiomas or other vascular malformations of the liver, specific diagnosis is essential to avoid unnecessary surgical intervention or inappropriate use of steroids or chemotherapy agents.[234]

In the assessment of hepatoblastoma for resectability, a high-quality computed tomography (CT) scan or magnetic resonance imaging (MRI) is essential, especially in the adoption of the PRETEXT staging system, which relies on presurgical imaging.[235-238] These studies should be performed with particular attention to the vascular anatomy of the liver. A dual-phase CT scan with both arterial and venous phases may be used to assess vascular involvement and resectability. MRI, sometimes supplemented with magnetic resonance angiography, provides an excellent depiction of vascular anatomy.[238]

Although the PRETEXT staging system is a useful guide for deciding whether a lesion is resectable, the ultimate decision regarding surgical management rests with the surgeon. If possible primary resection should be undertaken for PRETEXT I or II tumors if there is no vascular involvement. Involvement of all three hepatic veins or both branches of the portal vein will usually preclude surgical resection. However a PRETEXT III or IV lesion that appears unresectable at the time of diagnosis may respond very well to chemotherapy, and repeat imaging after two or three cycles of chemotherapy might demonstrate adequate regression of the tumor from vital vascular structures, enabling resection. Very rarely will arterial or biliary involvement preclude tumor resection. In some cases of extensive tumor involving the bifurcation of the bile ducts, biliary reconstruction may be necessary. At the time of surgical resection, any suspicious porta hepatis, hepatic artery, celiac, or periaortic lymph nodes should be sampled. When complete tumor resection is not feasible despite adequate chemotherapy, liver transplantation should be performed.[239-242] Therefore early referral of patients with PRETEXT III or IV tumors to a center that performs liver transplantation is essential.

Surgery remains the mainstay of treatment for HCC given the poor response to chemotherapy and other modalities, including chemoembolization, radiofrequency ablation, and cryotherapy. When HCC develops in the context of cirrhosis, surgery may be limited by the lack of liver physiologic reserve.[243] In these cases transplantation may be the only safe option even for early-stage disease. In the fibrolamellar variant, the remaining adjacent liver is usually normal and therefore resection is more often possible even with extensive disease; this may be a factor contributing to the impression that fibrolamellar HCC has a better prognosis than standard HCC, although stage for stage, fibrolamellar HCC is not different from other types of HCC and depends on resectability for cure.[39,244] Other malignant tumors of the liver, including angiosarcoma, embryonal sarcomas, rhabdoid tumors, and rhabdomyosarcoma, like other malignant liver tumors, require complete resection if there is to be any hope for cure.[245-255]

Transplantation has become increasingly common in the treatment of unresectable hepatoblastoma. In most cases involving malignant liver tumors, complete surgical resection is a necessary component of therapy. Whether benign or malignant, when complete surgical resection by way of partial hepatectomy is not possible because of the extent of disease or lack of hepatic reserve, liver transplantation is the only remaining option. Approximately 2% of children undergoing liver transplants received the livers as treatment for hepatoblastoma,[256] although clearly this number is increasing in recent years. Although liver transplantation for malignant disease was used during the early years of liver transplantation, the survival rate was poor because of transplantation complications as well as recurrent disease. More recent data demonstrate significantly improved survival rates in select patients after transplantation to treat both hepatoblastoma and HCC.[257,258-262] The combined experience of SIOPEL studies was quite favorable: 25 patients with otherwise unresectable hepatoblastoma confined to the liver underwent transplantation after chemotherapy, with the survival rate for patient and graft at 100% after 1 year for living related donor transplants (N = 7) and 91% for cadaveric transplants (N = 18). At 10 years after transplant, the survival rate was 77.6% for cadaveric transplants and 83% for living related donor transplants.[263] In a recent overview of SEER data involving patients younger than 20 years of age undergoing surgery for hepatoblastoma, of a total of 318 patients with hepatoblastoma, 16.7% underwent transplant; of 80 patients with HCC, 25% underwent transplant. The survival rate at 5 years was 86.5% for hepatoblastoma and 85.3% for HCC.[264] PLUTO (Pediatric Liver Unresectable Tumor Observatory) is an international registry established to capture data, including detailed clinical parameters and outcome statistics, and will be a source of additional information on transplantation of pediatric liver tumors.[265]

In the planning of therapy for patients with advanced hepatoblastoma that may require liver transplantation, early referral to a liver transplant center is key. Options for transplantation include the more traditional deceased donor whole organ or donor split liver transplants and also, more recently, living-donor transplants.[256] Although there are no data to suggest optimal timing for transplantation, most centers will administer at least two rounds of chemotherapy before transplantation in an attempt to shrink the tumor and determine whether resection without transplantation may be an option. Given the small window of opportunity to perform a transplant between cycles, close communication between the oncology team and the transplant team is critical. Evaluation by an experienced transplant team should take place as soon after diagnosis as possible. Current United Network for Organ Sharing (UNOS) liver allocation policy prioritizes children who require liver transplantation for hepatoblastoma. A child listed for transplantation with a diagnosis of hepatoblastoma is automatically assigned 30 PELD (Pediatric End-Stage Liver Disease) points, with the opportunity to be upgraded to status 1B after 30 days. This high priority listing allows patients a reasonable chance of receiving transplants between cycles when hematologic counts are not prohibitive. In the case of living-donor transplantation, the surgery can be scheduled electively. At the time of transplant an initial early abdominal exploration to rule out metastatic disease is required. If intraabdominal metastatic disease is discovered and is not completely resectable, transplantation should be aborted.

Liver transplantation for HCC has evolved significantly over the past several years, and as stated previously, SEER data indicate an 85% 5-year survival rate for HCC patients younger than 20 years undergoing liver transplantation.[264] Centers have used the Milan criteria to select patients for liver transplantation; these criteria allow for increased MELD (Model for End-Stage Liver Disease) points in patients with stage II disease.[260] However data from the University of California San Francisco (UCSF) group demonstrate acceptable patient survival rates after transplantation for patients who exceed the Milan criteria, generally established for adult patients.[266]

LESS COMMON MALIGNANT TUMORS OF THE LIVER

Embryonal sarcoma of the liver, also termed *undifferentiated embryonal sarcoma of the liver* (UESL), is the third most common malignant liver tumor in children.[100] UESL was first recognized as a distinct entity in 1978.[267] Histologically these tumors are composed of mixtures of cells with histiocytoid, fibrohistiocytoid, myofibroblastic, and undifferentiated (primitive mesenchymal) morphologies.[268] They share a chromosome translocation similar to that seen in mesenchymal hamartoma (discussed later) and are thought to arise from the benign mesenchymal hamartoma as a malignant counterpart.[252,269-273] UESL can be distinguished from biliary tract rhabdomyosarcomas by several immunohistochemical stains, including myogenin and MyoD1, which are negative in UESL and positive in rhabdomyosarcoma.[274] UESL differs from rhabdomyosarcoma of the liver in that the median age of UESL is 10.5 years, compared with 3.6 years for rhabdomyosarcoma.

The male-to-female ratio in UESL is 1:1. USEL is considered an aggressive malignancy that should be treated with multiagent regimens used for soft tissue sarcomas of other sites.[275] A European Cooperative Group study from Italy and Germany treated a series of 17 patients with UESL with chemotherapy, conservative surgery, and (in two patients) radiotherapy. Of the 17 patients 12 survived (70%), and all of those who underwent complete resection survived.[276] A more recent series reported 10 patients with UESL, all receiving both combination chemotherapy and surgery, three with upfront resection, and seven with resection after neoadjuvant chemotherapy. Nine of the ten (90%) survived.[277]

True rhabdomyosarcomas, when they are seen in the hepatobiliary system, occur in the biliary tract. These tumors are mesenchymal in origin and histologically resemble rhabdomyosarcomas of other sites with prominent myoblastic cells that stain for myogenin.[274] The tumors may have a botryoid appearance.[278,279] Obstructive jaundice is the usual presenting sign, with or without abdominal distention.[279,280] In an early report from the Intergroup Rhabdomyosarcoma Study, only 4 of 10 patients with biliary tract tumors survived.[280] However additional reports cite better long-term survival rates with chemotherapy regimens and without the need for extensive surgery.[281,282]

Angiosarcomas of the liver are rare but aggressive high-grade malignancies derived from endothelial cells.[246,248,283] Angiosarcoma of the liver in children was previously known as *infantile hemangioendothelioma type 2*.[284] Fewer than 40 cases have been reported. Some cases have been preceded by a diagnosis of infantile hepatic hemangioendotheliomas.[246,285,286] In contrast to other malignant liver tumors in children, angiosarcomas are more common in girls than boys. The pathology of pediatric angiosarcoma is distinct from angiosarcomas in adults in that kaposiform spindle cells are present. The overall prognosis is poor.[246] Although liver transplantation had been reported as successful in one child, a recent European study indicated an overall exceptionally poor outcome in a series of angiosarcomas after liver transplantation.[287,288]

Choriocarcinoma of the liver is an extremely rare tumor. Most cases of infantile choriocarcinoma are actually metastases from gestational choriocarcinoma of the placenta that spreads to the child; however, primary tumors of the liver have been reported. This can be an aggressive and devastating tumor, and although the overall outcome has been historically poor, there are recent reports of cures with chemotherapy, including one case with liver transplant.[289-292] Other primary germ cell tumors of the liver have been reported but are extremely rare.[293]

BENIGN TUMORS OF THE LIVER

Hepatic mesenchymal hamartoma (HMH) is a benign tumor with an age distribution similar to hepatoblastoma, with almost all cases occurring by 5 years. Patients with these tumors present with an enlarged abdomen. Clinical presentation can resemble that of hepatoblastoma, although the serum AFP level is not elevated.[294] As with hepatoblastoma, the majority of HMHs arise in the right side of the liver. HMHs are characterized by the presence of cysts, which are less characteristic of hepatoblastoma. Figure 58-11 demonstrates the cystic appearance on imaging. These benign tumors are characterized by massive growth and cause morbidity by compressing adjacent structures. Occasionally these tumors are diagnosed prenatally and can be a cause of fetal demise.[294] Microscopically HMH consists of spindle cells in a myxoid background with occasional areas of extramedullary hematopoiesis similar to that seen in hepatoblastoma. Figure 58-12 demonstrates the histologic pattern characteristic of HMHs with fluid-filled cystic areas. Cytogenetically these tumors are characterized by translocations involving 19q13.4, as described in multiple cases.[252,271,295-297] The breakpoints involved in one such translocation have been characterized.[273] An interstitial deletion at 19q13.4 has also been reported.[298] HMH can show malignant transformation into malignant mesenchymoma or embryonal sarcoma.[270,299] Treatment of HMH is usually surgical removal. Long-term follow-up of patients after resection is generally favorable.[300]

Hemangioendothelioma is a term that has been used to denote a type of benign vascular tumor. Previously

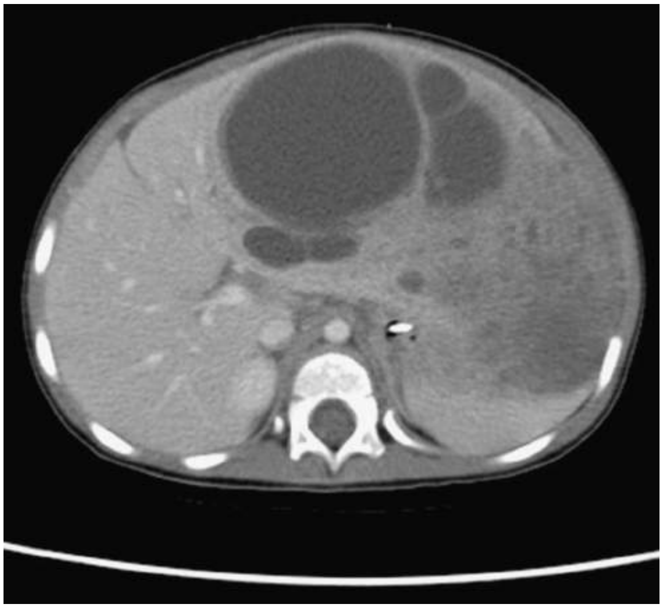

Figure 58-11 Computerized tomography image of an child with mesenchymal hamatoma. Note the multiple cysts with little solid component.

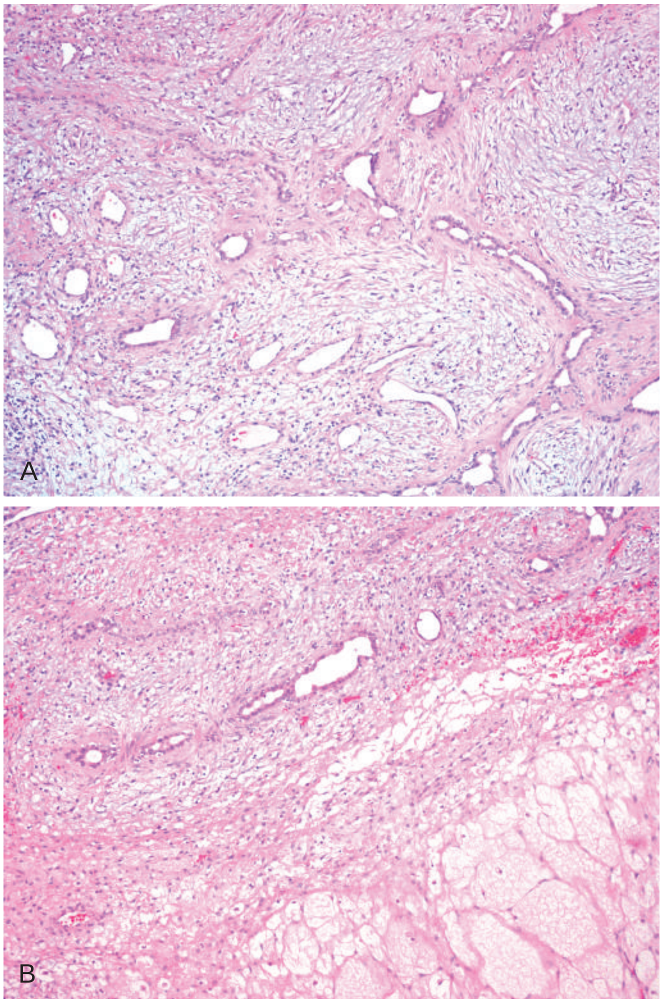

Figure 58-12 Mesenchymal hamartomas. Mesenchymal hamartoma is composed of haphazardly arranged and architecturally distorted elements that are normally found in the liver. In **A,** dilated and serpentine biliary ducts, each surrounded by a variably thick cuff of collagen, are apparent. The bile ducts are lined with low cuboidal epithelial cells without cytologic atypia. The second image (**B**) also shows fluid-filled lymphatic-like cystic spaces. All of these elements are embedded in a myxoid and edematous mesenchyme containing delicate fibroblasts. *(Courtesy of Dinesh Rakheja, MD, Children's Medical Center, Dallas, Tex.)*

grouped together as *infantile hepatic hemangioendothelioma,* the pediatric benign hepatic vascular tumors have recently been shown to be at least two different lesions—hepatic infantile hemangioma and congenital hepatic vascular malformation with associated capillary proliferation. One type (infantile or juvenile hemangioma) often manifests as multiple masses that usually involute and regress. The other type, which has been called a vascular malformation, is usually a large single mass that often undergoes central infarction, does not regress, and may be associated with consumption coagulopathy caused by platelet trapping in the abnormal vasculature. GLUT1 endothelial reactivity distinguishes the two entities histologically, with hepatic infantile hemangiomas staining positively for GLUT1 whereas the hepatic vascular malformations do not exhibit GLUT1 immunoreactivity.[301] Figure 58-13 demonstrates these two benign hepatic vascular disorders. Patients with hepatic hemangioendothelioma usually exhibit abdominal enlargement with or without congestive heart failure. Hemangioendothelioma may be distinguished from hepatoblastoma by imaging studies, particularly Doppler flow studies.[302] Many hepatic hemangioendotheliomas involute without intervention. Although steroids have been used in the past, propranolol has been used successfully to treat some hemangiomas of the liver, although the precise indications for its use have not yet been well-defined.[303,304]

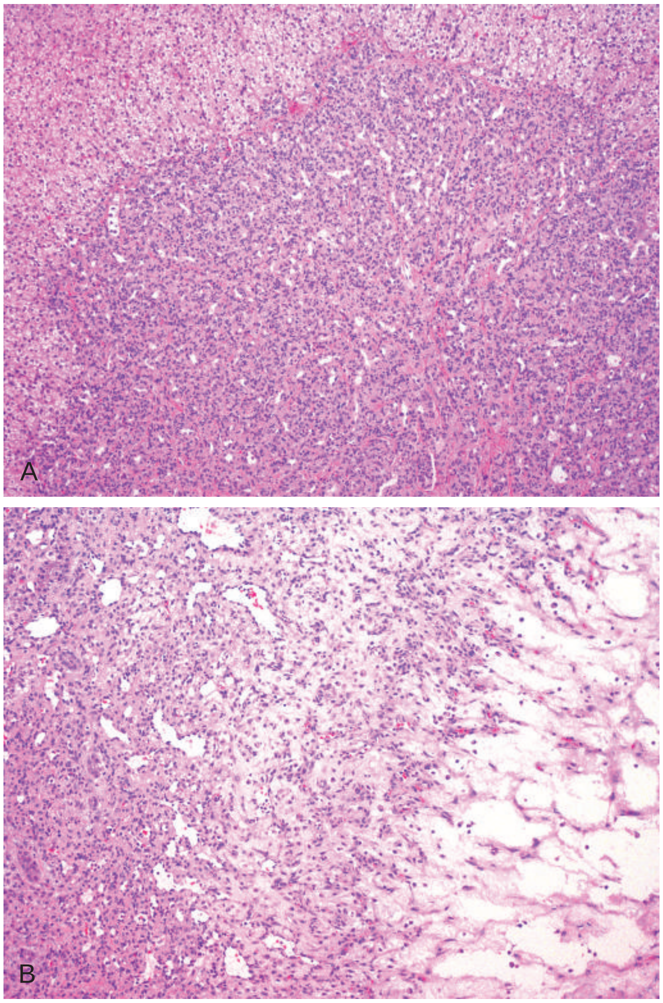

Figure 58-13 Benign hepatic vascular tumors. Previously grouped together as infantile hepatic hemangioendothelioma, the pediatric benign hepatic vascular tumors have recently been identified as at least two different lesions. Hepatic infantile hemangioma can be distinguished from congenital hepatic vascular malformation with associated capillary proliferation by the presence or absence of GLUT1 staining. The infantile or juvenile hemangioma, shown in **A,** often presents as multiple masses that usually involute and regress. Microscopically the lesions are sharply demarcated lobules composed of closely packed capillary-sized vessels that stain for the glucose transporter GLUT1. The other type, also known as hepatic vascular malformation, is usually a large single mass that often undergoes central infarction, does not regress, and may be associated with consumption coagulopathy. As shown in **B,** the area of infarct is surrounded by variably dilated thin-walled vessels with entrapped bile ducts and hepatocytes. The lesional vessels are negative for GLUT1. *(Courtesy of Dinesh Rakheja, MD, Children's Medical Center, Dallas, Tex.)*

FUTURE CHALLENGES IN TREATMENT OF PEDIATRIC LIVER TUMORS

Although cure rates for liver tumors, particularly hepatoblastoma, have increased over recent decades, many significant challenges remain. The rarity of these malignancies has contributed to the difficulty in the design and implementation of clinical trials, and each treatment hypothesis proposed may take many years to test. Agents of current interest for clinical application for hepatoblastoma include irinotecan and incorporation of sorafenib. Clinical trials for childhood HCC will likely parallel those for adult HCC and use multikinase inhibitors such as sorafenib in addition to chemotherapy.

Additional biologic markers for predicting prognosis and stratifying risk are needed. Although several molecular pathways have been identified in pediatric liver tumors, the integration of molecular characteristics in disease risk stratification and prognosis needs to be developed. In addition, more therapies specifically targeting relevant pathways are needed, with the additional goal of limiting toxicities.

The recently formed Children's Hepatic International Consortium will foster an exciting international collaboration in the analysis of risk factors and outcomes in hepatoblastoma, as well as accelerating new clinical approaches to the treatment of liver tumors in children.[190] This consortium has to date established a series of prognostic risk factors based on a compilation of international trials and will design future treatment strategies based on these classifications.

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CHAPTER 59

Rhabdomyosarcoma

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CHAPTER OUTLINE

PATHOLOGIC CLASSIFICATION OF RHABDOMYOSARCOMA
RHABDOMYOSARCOMA IN CANCER PREDISPOSITION SYNDROMES
GENETICS OF SPORADIC RHABDOMYOSARCOMA
Gene Fusions Generated by Chromosomal Rearrangements
Chromosome Copy Number Changes
Amplification of Specific Chromosomal Regions
Allelic Loss Events
Oncogene and Tumor Suppressor Gene Mutations in Rhabdomyosarcoma
Hypermethylation as an Alternative Mechanism of Gene Inactivation in Rhabdomyosarcoma
APPLICATIONS OF MOLECULAR GENETIC APPROACHES TO DIAGNOSIS AND PROGNOSIS
Detection of Recurrent Gene Fusions
Pathologic and Clinical Differences between Fusion Subtypes
Gene Expression Profiling with Microarrays
Detection of Rhabdomyosarcoma in Sites Other Than Primary Tumor
MOLECULAR AND CELLULAR BIOLOGY OF RHABDOMYOSARCOMA
Myogenic Pathways in the Tumorigenesis of Rhabdomyosarcoma
MicroRNAs
Epigenetic Regulation
Role of Insulin-like Growth Factors in Rhabdomyosarcoma
Modulation of Growth and Apoptotic Pathways by Fusion Oncoproteins
Metastatic Pathways in Rhabdomyosarcoma
PRECLINICAL INVESTIGATION OF EMERGING DRUG TARGETS FOR RHABDOMYOSARCOMA
Preclinical Models to Study Rhabdomyosarcoma Tumorigenesis
Inhibitors of Receptor Tyrosine Kinases
Inhibitors of Intracellular Signaling Pathways
Inhibitors of Developmental Pathways
Inhibitors of Other Cellular Processes That Support Rhabdomyosarcoma Tumorigenesis
Immunotherapy for Rhabdomyosarcoma
CLINICAL RESEARCH ON RHABDOMYOSARCOMA
INCIDENCE AND EPIDEMIOLOGY
CLINICAL PRESENTATION AND EVALUATION
RISK GROUP CLASSIFICATION
Histology and Fusion Status
Site
Age
Invasiveness and Size (Tumor Stage)
Clinical Group
Lymph Node Involvement
Metastases at Diagnosis
Interactions
TREATMENT
Chemotherapy
Primary Tumor Treatment
Special Situations
FOLLOW-UP AFTER TREATMENT
Surveillance and Patterns of Recurrence
Late Effects
FUTURE DIRECTIONS

PATHOLOGIC CLASSIFICATION OF RHABDOMYOSARCOMA

The term *rhabdomyosarcoma* (RMS) comprises a heterogeneous family of soft tissue cancers that are related in poorly understood ways to the skeletal muscle lineage. Some of these tumors occur in the vicinity of skeletal muscle, but others occur in areas without obvious skeletal muscle; thus these cancers cannot be defined solely as tumors of skeletal muscle. Instead, this family is derived from unknown mesenchymal precursors and is characterized by a shared program of skeletal myogenesis. Despite this shared program, some precursors may intrinsically undergo myogenesis whereas others may have developed this ability aberrantly.

Histopathologic classification has evolved over time, but two principal histopathologic subtypes have been recognized consistently in children: alveolar RMS (ARMS) and embryonal RMS (ERMS).[1] The criteria for these categories were refined over time as these subtypes were associated with clinically distinct phenotypes. As described in detail later, ARMS more often occurs in the extremities and axial musculature in older children and is associated with a less favorable prognosis whereas ERMS tends to occur in the head, neck, and genitourinary tract of younger patients and is associated with a favorable prognosis. ARMS and ERMS account for 20% to 30% and 70% to 80% of RMS cases, respectively.

The diagnosis of RMS is often difficult because of the paucity of features of striated muscle differentiation. A variety of pediatric solid tumors, including RMS, neuroblastoma, Ewing sarcoma, and non-Hodgkin lymphoma, can present as collections of poorly differentiated cells (small round blue cell tumors). To detect more subtle evidence of myogenic differentiation, immunohistochemical reagents are used to identify muscle-specific proteins, such as MYOD, myogenin, desmin, myoglobin, and skeletal muscle-specific actin.[2] In addition, the detection of myofilaments by electron microscopy provides further evidence of the myogenic phenotype.

In ARMS, the tumor cells are small blue round cells with only small amounts of cytoplasm (Fig. 59-1, *A*).[3] The prominent round nuclei generally have monotonous, coarse chromatin, and central nucleoli are often present. These tumor cells form highly cellular aggregates separated by fibrovascular septa. Within these aggregates, discohesive areas tend to form, resulting in spaces or clefts lined by rhabdomyoblasts. ARMS was named for these open spaces, which have an appearance reminiscent of pulmonary alveoli. In addition to these features, the tumor cells tend to align along septa in a "picket-fence" pattern and tumor giant cells are often present. In a subset of cases there is a paucity of fibrovascular stroma, no evidence of spaces, and the predominance of a highly cellular small round cell population with cytologic features similar to the classic alveolar pattern; such cases are referred to as the solid variant of ARMS.

ERMS is so named because of its histologic similarity to developing skeletal muscle. The tumor cells show varying degrees of differentiation along the myogenic spectrum, from small primitive round cells to larger oblong cells with eccentric oval nuclei and varying amounts of eosinophilic cytoplasm (see Fig. 59-1, *B*).[3] ERMS nuclei are often notable for a relatively bland chromatin pattern. These differentiated cells can elongate to assume a straplike appearance and occasionally show cross-striations and multinucleation. In addition to the characteristic cytology, ERMS tumors classically have variable cellularity, in which areas of hypercellularity alternate with areas of hypocellularity in a loose myxoid stroma.

Within the ERMS category, there are several histologic variants. In sclerosing RMS, there is abundant desmoplasia and the tendency to form microalveolar tumor cell nests within the desmoplastic areas[4]; in some cases, this microalveolar appearance has been mistaken for ARMS. In a second variant, there are dense clusters of ERMS cells that can be confused with the solid variant of ARMS[5]; this dense ERMS variant can be distinguished based on cytologic features and other special studies. Finally, two variants of ERMS have been associated with

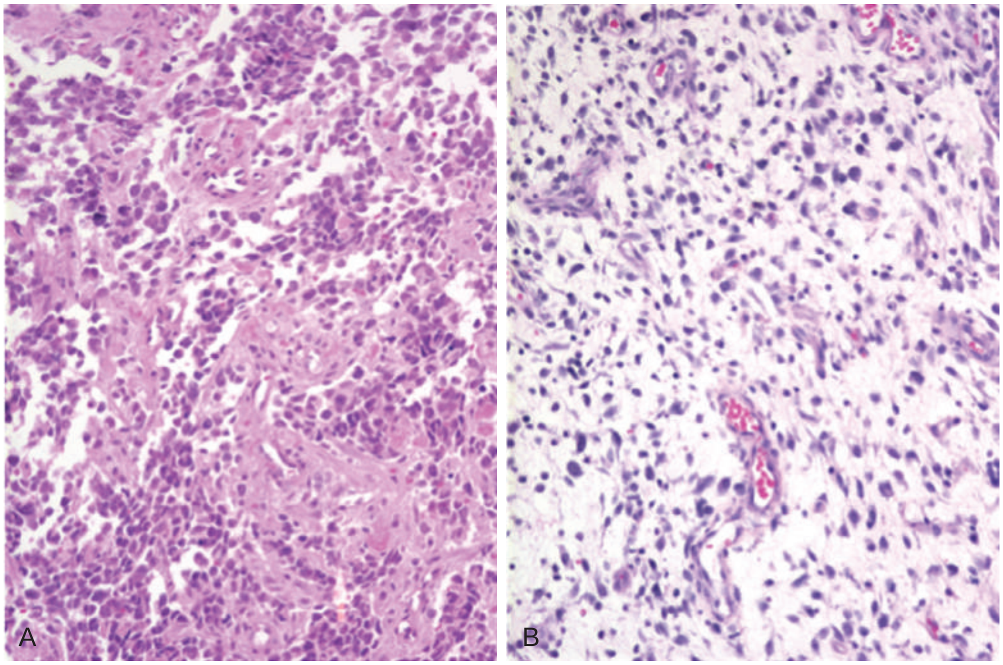

Figure 59-1 Histopathology of RMS subtypes. **A,** Alveolar rhabdomyosarcoma (hematoxylin-eosin, original magnification ×100). **B,** Embryonal rhabdomyosarcoma (hematoxylin-eosin, original magnification ×100). *(Courtesy Dr. Bruce Pawel, Children's Hospital of Philadelphia.)*

an improved prognosis.[3] Botryoid RMS typically occurs in the lumen of a hollow viscus, such as the urinary bladder, vagina, or extrahepatic bile ducts, and grossly has multiple polypoid nodules. At the microscopic level, this tumor typically has a dense cambium layer of tumor cells under an intact epithelial surface. Spindle-cell RMS has dense bundles or whorls of spindle-shaped cells that resemble smooth muscle. In children, these lesions commonly occur in the paratesticular region, but in adults, in whom the prognosis is worse, these lesions tend to occur in the head and neck and are often associated with a sclerosing pattern. In both of these latter two variants, the tumors cells can show marked rhabdomyoblastic differentiation.

Another small subset of RMS cases demonstrates discrete areas with ERMS-like histology and separate discrete areas with ARMS-like histology.[5] The ERMS-like and ARMS-like areas can have any one of the various histologic patterns described earlier. Although these cases were previously diagnosed as ARMS or ERMS based on prevailing histopathologic criteria, these cases are currently classified as mixed RMS.

A final RMS subset shows features similar to anaplastic Wilms tumors.[6] Although the anaplasia can be seen in both ARMS and ERMS, it is more prevalent in the latter. Anaplastic RMS tumors have large, lobated hyperchromatic nuclei and atypical mitoses. In some cases anaplastic cells are scattered in small patches (focal anaplasia), whereas other cases have larger clusters or sheets of anaplastic cells (diffuse anaplasia).

RHABDOMYOSARCOMA IN CANCER PREDISPOSITION SYNDROMES

The majority of RMS cases arise as sporadic nonheritable tumors, but a small fraction of cases are associated with heritable genetic syndromes (Table 59-1). In some cases the proband with RMS inherited a mutant gene as part

TABLE 59-1 Familial Cancer Predisposition Syndromes Associated with Rhabdomyosarcoma

Cancer Syndrome	Locus	Gene	Nonneoplastic Findings	Characteristic Tumors	Frequency of RMS
Costello syndrome	11p15.5	*HRAS*	Facial anomalies, cardiac hypertrophy, cognitive defects, developmental delay	*Benign:* skin papillomas *Malignant:* RMS, neuroblastoma, bladder cancer	7%
Li-Fraumeni syndrome	17p13.1 22q12.1	*TP53* *CHEK2*	None	Sarcoma, breast cancer, brain tumor, adrenocortical cancer, leukemia	6%
Hereditary retinoblastoma	13q14	*RB1*	None	Retinoblastoma, osteosarcoma	2%
Neurofibromatosis type 1	17q11.2	*NF1*	Café au lait spots, skinfold freckling, Lisch nodules, cognitive deficits	*Benign:* neurofibroma *Malignant:* MPNST, AML	1%
Constitutional mismatch-repair-deficiency syndrome	7p22.2 and others	*PMS2, MLH1, MSH2, MSH6*	Café au lait spots	hematologic cancers, brain tumors, colorectal cancer	1%
Beckwith-Wiedemann syndrome	11p15.5	Unknown	Macrosomia, macroglossia, hemihyperplasia, visceromegaly	Wilms tumor, hepatoblastoma	<1%
Nevoid basal cell carcinoma syndrome	9q22	*PTCH*	Macrocephaly, skin cysts, palmar and plantar pits, rib anomalies	Basal cell carcinoma, medulloblastoma	<1%
Rubinstein-Taybi syndrome	16p13.3	*CREBBP*	Mental retardation, facial anomalies, and broad thumbs	Leukemia, brain tumors	<1%
Noonan syndrome	12q24 and others	*PTPN11, SOS1, RAF1,* and others	short stature, facial anomalies, congenital heart defects	Neuroblastoma, ALL, glioma, RMS	<1%
DICER1 syndrome (formerly PPB family tumor and dysplasia syndrome)	14q32	*DICER1*	None	PPB, cystic nephroma	Unknown

ALL, Acute lymphoblastic leukemia; *AML,* acute myelogenous leukemia; *MPNST,* malignant peripheral nerve sheath tumor; *PPB,* pleuropulmonary-blastoma; *RMS,* rhabdomyosarcoma.

of an established familial syndrome, and in other cases a new germline mutation occurred in one germ cell that ultimately produced the proband. The penetrance of RMS in individuals with new or inherited germline mutations varies from as high as approximately 7% in Costello syndrome to 0.1% to 1% in other syndromes. Although there are rare cases of RMS found in association with other syndromes, the determination that such syndromes increase predisposition to RMS is limited by the overall low frequency of these syndromes. In a similar way, the small contribution of known RMS susceptibility syndromes to the overall incidence of RMS is related to the low incidence of these syndromes in the general population. Neurofibromatosis type 1 has the highest prevalence, occurring in approximately 1 in 3500 individuals and accounting for about 1% of all RMS cases.[7,8] Although *TP53* germline mutations occur less frequently (~1 in 20,000 individuals), there is a higher prevalence of RMS in these mutation carriers; thus this susceptibility syndrome has been reported to occur in up to 10% all RMS cases.[9]

Among the syndromes predisposing to RMS, three syndromes (termed *RASopathies*) are caused by alterations of the RAS signaling pathway. Costello syndrome is caused by mutations in *HRAS* codons 12 or 13.[10] These same mutations commonly occur in many types of sporadic tumors, have transforming activity in cell culture, and thus are gain-of-function alleles. Similarly, Noonan syndrome is caused by activating mutations of one of several oncogenes encoding downstream RAS pathway components with growth stimulatory function.[11] In contrast, neurofibromatosis type 1 is caused by inactivating mutations of the *NF1* tumor suppressor gene, which encodes neurofibromin, a guanosine triphosphatase–activating protein that negatively regulates RAS signaling.[12] The presence of a common affected signaling pathway is also indicated by the significant overlap in nonneoplastic phenotype in both Costello and Noonan syndromes. In contrast, neurofibromatosis type 1 is associated with different nonneoplastic phenotypic features. Analysis of these heritable syndromes has thus shown that activation of RAS signaling by one of several genetic changes leads to a low and variable frequency of RMS susceptibility.

The RMS tumors associated with these hereditary predisposition syndromes display a number of notable properties. In many of these syndromes, the RMS tumors occur at a younger age than sporadic RMS tumors, which is in accord with the two-hit tumor susceptibility gene model.[13] Of note, RMS cases in hereditary retinoblastoma occur at a later age but are associated with radiation treatment of the primary retinoblastoma, thus suggesting the need for a postnatal DNA damage event for RMS in this setting.[14] From a pathology perspective, most of these syndromic RMS tumors are diagnosed as ERMS, the subtype that is not associated with the *PAX3-FOXO1* and *PAX7-FOXO1* gene fusions (described later).[11] In the few cases in which tumors are described as ARMS, such as in hereditary retinoblastoma or Beckwith-Wiedemann syndrome, molecular testing reveals that these RMS tumors are fusion negative.[15,16]

GENETICS OF SPORADIC RHABDOMYOSARCOMA

Gene Fusions Generated by Chromosomal Rearrangements

Cytogenetic studies established that nonrandom chromosomal translocations distinguish most ARMS tumors from ERMS and other pediatric solid tumors (Fig. 59-2). A translocation involving chromosome 2 and 13, t(2;13)(q35;q14), was found in 56% of 99 published ARMS cases, and a variant translocation involving chromosome 1 and 13, t(1;13)(p36;q14), was identified in 6% of cases (http://cgap.nci.nih.gov/Chromosomes/Mitelman). This latter fraction is probably an underestimate because the 1;13 translocation is usually followed by an amplification event (see later). In contrast to ARMS, the 2;13 and 1;13 translocations were found in 3% and 0% of 77 published ERMS cases, respectively. Such rare translocation-positive ERMS cases may represent tumors with mixed histology or misdiagnosed ARMS tumors.

At the molecular level, the 2;13 and 1;13 translocations break and rejoin portions of transcription factor–encoding genes to generate fusion genes expressed as fusion transcripts (see Fig. 59-2). The rearranged genes on chromosomes 2 and 1 are *PAX3* and *PAX7*, which encode related members of the paired box family.[17,18] The chromosome 13 locus in these translocations is *FOXO1 (FKHR)*, which encodes a member of the forkhead family.[19] Although two reciprocal fusion genes, *PAX3-FOXO1* and *FOXO1-PAX3* (or *PAX7-FOXO1* and *FOXO1-PAX7*), are formed by these translocations, the higher and more consistent expression of *PAX3-FOXO1* or *PAX7-FOXO1* supports the premise that the *PAX3-FOXO1* or *PAX7-FOXO1* products are involved in ARMS pathogenesis.

The *PAX3-FOXO1* and *PAX7-FOXO1* fusion transcripts are translated into novel chimeric transcription factors (see Fig. 59-2).[18,19] The wild-type *PAX3, PAX7,* and *FOXO1* genes encode transcription factors organized with an amino-terminal (N-terminal) DNA-binding domain and a carboxy-terminal (C-terminal) transcriptional activation domain. The translocations break within *PAX3* or *PAX7* intron 7 and maintain an intact DNA-binding domain but separate it from an essential part of the transactivation domain. In addition, the translocations break within *FOXO1* intron 1 and disrupt the DNA-binding domain but maintain an intact transactivation domain. In the chimeric transcripts, the 5′ *PAX3* (or 5′ *PAX7*) and 3′ *FOXO1* coding sequences are fused in-frame to generate a 2508- or 2484-nucleotide open reading frame encoding an 836- or 828-amino acid fusion protein, respectively. These fusion proteins contain the first seven exons of *PAX3* or *PAX7,* including an intact DNA-binding domain, and the last two exons of *FOXO1,* including an intact transactivation domain. A functional fusion protein cannot be created by other combination of *PAX3* and *FOXO1* exons because of incompatible reading frames or loss of functional domains. These rearrangements of *PAX3* or *PAX7* intron 7 and *FOXO1* intron 1 thus appear to be selected because of functional constraints related to genomic organization of *PAX3, PAX7,* and *FOXO1.*

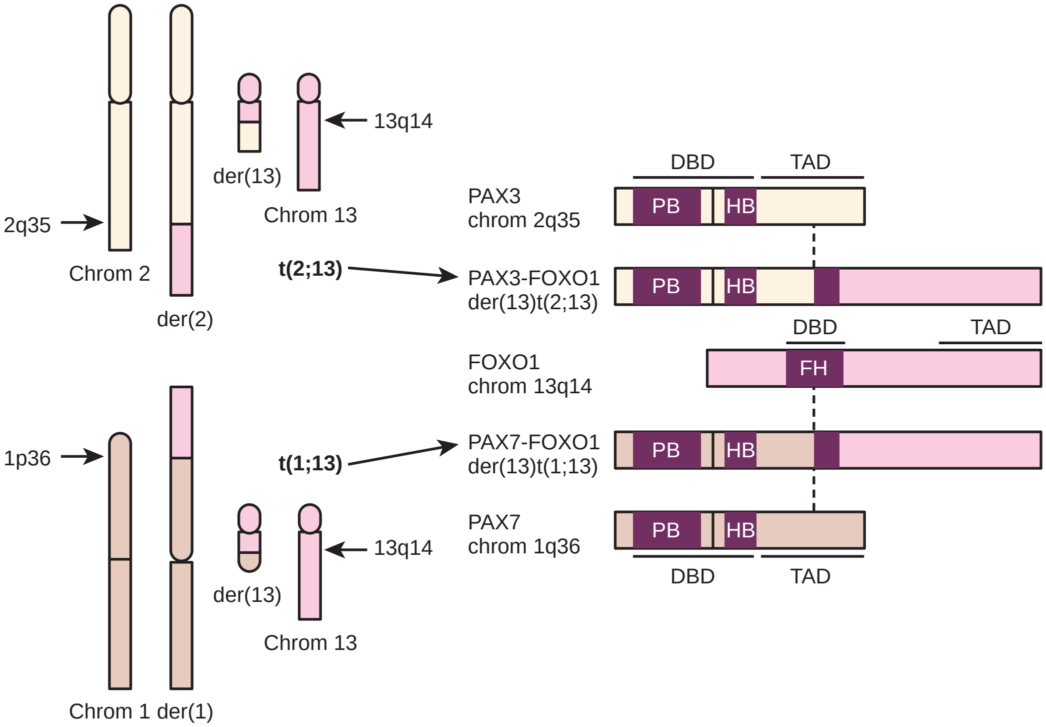

Figure 59-2 Diagrams of 2;13 and 1;13 chromosomal translocations and associated fusion products. A schematic representation of the normal and derivative chromosomes associated with t(2;13) and t(1;13) is shown on the left. The translocation breakpoints are indicated by *short horizontal arrows* with the location of involved chromosomal region. The wild-type and fusion products associated with 2;13 and 1;13 translocations are shown on the right. The paired box (PB), octapeptide, homeobox (HB), and forkhead (FH) domains are indicated as *dark purple boxes,* and transcriptional domains (DBD, DNA binding domain; TAD, transcriptional activation domain) are shown as *solid bars.* The *vertical dashed line* indicates the translocation fusion point.

There are changes in both function and expression caused by the fusion events in ARMS. The chimeric transcription factors activate transcription from PAX3/PAX7-binding sites but are much more potent as transcriptional activators than the wild-type PAX3 and PAX7 proteins.[20,21] This difference in transcriptional activity appears to be caused by a decreased sensitivity of the FOXO1 activation domain to the inhibitory effects of N-terminal PAX3 (or PAX7) domains, when compared with the effect of these N-terminal domains on the PAX3 (or PAX7) activation domain. The fusion proteins may also have a relaxed capability to bind and activate target genes owing to increased structural flexibility in the N-terminal DNA-binding domain.[22] In addition to changes in transcriptional function, the PAX3-FOXO1 or PAX7-FOXO1 fusion products are expressed in ARMS tumors at higher levels compared with the corresponding wild-type PAX3 or PAX7 products.[23] The gene-specific mechanisms for this enhanced fusion product expression are described later. Therefore these chromosomal changes result in high levels of chimeric transcription factors that inappropriately activate transcription of genes with PAX3/PAX7 DNA-binding sites. These biologic effects contribute to tumorigenesis by modulating myogenic differentiation, altering growth and apoptotic pathways, and stimulating motility and other metastatic pathways.

Several methodologies, including reverse-transcriptase polymerase chain reaction (RT-PCR) assay and fluorescent in situ hybridization (FISH), were applied to detect the *PAX3-FOXO1* and *PAX7-FOXO1* fusions in clinical material.[24-27] A single large study of RMS cases from the Intergroup Rhabdomyosarcoma Study (IRS) IV protocol showed that all 93 cases of RMS or undifferentiated sarcoma with a diagnosis other than ARMS were fusion negative, whereas 77% of the 78 ARMS cases were fusion positive.[28] Of these ARMS cases, 55% expressed *PAX3-FOXO1,* 22% expressed *PAX7-FOXO1,* and 23% were fusion negative.[28] These findings were confirmed by several other large cooperative group studies. In other studies of mixed RMS, most but not all cases were fusion negative.[27,29] Furthermore, when areas with ARMS-like and ERMS-like histology were individually examined by FISH, there was concordance for fusion status among the regions in a single tumor.

These molecular pathology studies establish that there is a significant subset (~20%) of ARMS cases that do not express either the *PAX3-FOXO1* or *PAX7-FOXO1* fusion. The IRS IV protocol and subsequent cooperative group studies used centralized pathology review and uniform tissue banking, and thus these fusion-negative results cannot be explained by inaccurate histopathologic diagnosis or suboptimal tissue samples. Subsequent analysis of these fusion-negative cases revealed that a small subset have variant fusions of *PAX3, PAX7,* or *FOXO1* with other genes, including fusion of *PAX3* with the *FOXO1*-related locus *FOXO4 (AFX1)* and fusion of *PAX3* with *NCOA1* or *NCOA2,* which encode two similar transcription factors unrelated to the forkhead family.[30,31] However, these variant fusions are uncommon; most fusion-negative ARMS cases do not have any rearrangements of *PAX3, PAX7,* or *FOXO1* and thus appear to represent true fusion-negative cases with respect to these loci.

Although genetic studies have not revealed any chromosome rearrangements that occur in most ERMS cases, gene fusions have been identified in a small number of ERMS cases. In particular, rearrangements of the *NCOA2* gene in the 8q11-13 chromosomal region were detected in four ERMS tumors.[31,32] The pathologic subtype in three of the four cases was a spindle cell variant of ERMS. Furthermore, all four cases occurred in infants younger than 1 year of age and thus are characterized as congenital/infantile RMS. Further analysis of three cases demonstrated fusions to different partners in each case—*PAX3* (2q35), *SRF* (8q11), and *TEAD1* (11p15). Finally, there is a report of an unrelated fusion, *EWSR1-DUX4,* in an ERMS in a 19-year-old patient.[33]

Chromosome Copy Number Changes

Various studies, including classic cytogenetics (http://cgap.nci.nih.gov/Chromosomes/Mitelman), chromosome-based comparative genomic hybridization (CGH), and DNA-based microarrays established that a second set of differences between the RMS histopathologic subtypes is at the level of whole chromosome gains and losses.[34,35-37] ERMS tumors have multiple numerical changes of whole chromosomes. The most frequent chromosome change in ERMS involves gains of chromosome 8. This change is characterized by polysomy, or multiple copies, of chromosome 8 and occurs in 50% to 90% of ERMS tumors but only 10% to 20% of ARMS tumors. This chromosome gain results in elevated expression of genes along chromosome 8 and cannot be localized to any specific chromosomal region. Additional common gains in ERMS involve chromosomes 2, 7, 11, 12, 13, and 20. In contrast to chromosome 8 gains, each of these latter changes is often a low-level gain, most likely a trisomy, and occurs in 20% to 50% of ERMS cases. Several of these gains, such as chromosome 2, 11, and 13, occur at significantly lower frequencies (2- to 10-fold) in ARMS tumors. Whole chromosome losses in ERMS commonly involve chromosomes 6, 9, 10, 14, 15, and 16 and occur in 15% to 25% of ERMS tumors. A study of intermediate-risk ERMS reports higher frequencies of these chromosome losses, suggesting that these events may vary among RMS risk groups.[35] There are also quantitative differences in chromosome losses between ERMS and ARMS, but these differences are not as striking as the differences involving gains.

Although the chromosome gains and losses are not prevalent in the ARMS cases as a group, the fusion-negative ARMS subset has a pattern of gains and losses that is much more similar to ERMS than to the fusion-positive ARMS subset.[37] The most striking finding involves chromosome 8; these events occur in more than 50% of fusion-negative ARMS tumors but not in fusion-positive ARMS tumors. As a second example, chromosome 11 gains occur in 30% of fusion-negative ARMS tumors but only 10% of fusion-positive ARMS tumors.

TABLE 59-2 Frequently Amplified Chromosomal Regions in Rhabdomyosarcoma

		FREQUENCY		
Chromosome Region	**Genes Involved**	*PAX3-FOXO1*	*PAX7-FOXO1*	Fusion-negative
1p36	*PAX7*	0%	>90%	0%
2p24	*MYCN*	20%	20%	0-6%
12q13-14	*CDK4* and others	24	<5%	<5%
13q14	*FOXO1*	<10%	>90%	0%
13q31	*MIR17HG*	<10%	70%	<10%

Amplification of Specific Chromosomal Regions

CGH and DNA-based microarray studies revealed that ARMS has significantly more genomic amplification events than ERMS. In CGH studies, one or more amplification events were detected in 47 of 84 ARMS cases (56%) compared with 7 of 43 ERMS cases (16%).[34,38,39] The most common amplicons in ARMS tumors were derived from chromosomal regions 1p36, 2p24, 12q13-q14, 13q14, and 13q31 (Table 59-2).[38,40-42] The pattern of changes in the fusion-negative ARMS subset was again more similar to the pattern in ERMS than that in fusion-positive ARMS.[37] This difference is exemplified by the finding of 12q13-q14 amplification in 24% of fusion-positive ARMS, 4% of fusion-negative ARMS, and 0% of ERMS. In addition to differences between fusion-positive and fusion-negative tumors, many of these high-frequency amplicons have a marked preference for either the *PAX3-FOXO1*– or *PAX7-FOXO1*–positive subset .

The amplification events at 1p36 and 13q14 generally occur in the same cases and are indicative of *PAX7-FOXO1* amplification (see Table 59-2).[41] In particular, *PAX7-FOXO1* is amplified in more than 90% of *PAX7-FOXO1*–positive cases whereas *PAX3-FOXO1* is amplified in less than 10% of *PAX3-FOXO1*–positive cases. As a further difference, there is a higher number of cells containing the amplicon in amplified *PAX7-FOXO1*–positive cases than in amplified *PAX3-FOXO1*–positive cases. At the expression level, the fusion transcript is expressed at higher levels in *PAX7-FOXO1*–positive cases than in *PAX3-FOXO1*–positive cases. Despite these differences, both fusion products are expressed in ARMS tumors at higher levels than the corresponding wild-type *PAX3* or *PAX7* products.[23] This high expression is postulated to generate a fusion product level above a critical threshold for oncogenic activity. Although there is a common feature of fusion overexpression in fusion-positive ARMS, the mechanism of overexpression differs between the two subtypes. In *PAX7-FOXO1*–positive tumors, the fusion is generally overexpressed because of genomic amplification, a copy number–dependent mechanism. In contrast, *PAX3-FOXO1* is generally overexpressed by a copy number–independent increase in transcriptional rate.

For amplification of the 2p24 chromosomal region, the minimal amplified region contains the *MYCN* protooncogene (see Table 59-2).[40] This genomic region corresponds to the same region that is frequently amplified in neuroblastoma. In ARMS there is a comparable incidence of 2p24 amplification (20%) in *PAX3-FOXO1*– and *PAX7-FOXO1*–positive tumors. In contrast, this amplification occurs very infrequently in ERMS and fusion-negative ARMS tumors (0% to 6% of cases).[37] In addition to amplification, low copy number gains, which may be explained in part by whole chromosome 2 gains, were found in 50% to 60% of ARMS and ERMS cases.[43] In fusion-positive tumors, 2p24 amplification generally results in high expression of the *MYCN* transcript and an increase in MYCN protein expression. The lower-level gains may also be associated with moderate increases in *MYCN* expression.

Amplification of the 12q13-q14 chromosomal region was found in numerous cancers, including bone and soft tissue sarcomas (e.g., osteosarcoma and liposarcoma), brain tumors (mostly glioblastoma), and carcinomas (including breast cancer).[44] In ARMS, the minimal amplified region was localized to a 0.5-Mb region containing more than 20 genes, including CDK4, which encodes a cell cycle regulatory protein (see Table 59-2).[40] The 12q13-q14 region is amplified preferentially in *PAX3-FOXO1*–positive cases; there is a 24% and 4% frequency of 12q13-q14 amplification in *PAX3-FOXO1*– and *PAX7-FOXO1*–positive cases, respectively. This amplification also occurs at a low frequency in fusion-negative RMS tumors. In fusion-positive RMS tumors, 12q13-q14 amplification is associated with increased expression of multiple (but not all) genes in the minimal amplified region. Of note, the nearby 12q15 region (containing *MDM2*) is independently amplified at a low frequency in both fusion-positive and fusion-negative RMS tumors.[40,45-47]

The 13q31 amplification event was localized to a 150-kb region surrounding the *MIR17HG* gene (see Table 59-2).[42] This gene contains the miR-17-92 cluster, and the transcript expressed from the *MIR17HG* gene is processed to six microRNAs (miRs). This 13q31 region is also amplified in diffuse large B-cell lymphoma and small cell lung carcinoma.[48] In RMS, this region is preferentially amplified in *PAX7-FOXO1*–positive cases; this amplicon is present in 70% of *PAX7-FOXO1*–positive cases and less than 10% of *PAX3-FOXO1*–positive RMS and fusion-negative RMS cases. In RMS tumors with 13q31 amplification, five of the six microRNAs in the cluster (miR-17, miR-19a, miR-19b, miR-20a, and miR-92a) are expressed at high levels. Transcripts from a number of tumor suppressor genes are targets of these microRNAs, and thus these microRNAs are postulated to be oncogenic by decreasing tumor suppressor expression.

Allelic Loss Events

RMS studies using polymorphic markers to distinguish between the two alleles at a locus reveal that allelic loss (or conversion to homozygosity) occurs commonly in ERMS tumors.[49] With the use of DNA-based arrays with polymorphic markers, the genome-wide incidence of allelic loss (termed *fractional allelic loss*) was twofold higher in ERMS compared with ARMS tumors.[50] Furthermore, the fusion-negative ARMS subset demonstrated a fractional allelic loss similar to that of ERMS tumors that was more than twofold higher than that of fusion-positive ARMS tumors.

In ERMS, chromosome 11 is most commonly affected by allelic loss, and the smallest region of consistent allelic loss is 11p15.5 (Fig. 59-3).[51] This 11p15.5 allelic loss is also detected in a small subset (24%) of fusion-positive ARMS tumors but is much more common (77%) in ERMS and fusion-negative ARMS tumors.[50,52] The probable presence of a tumor suppressor gene in this region, which is inactivated in RMS, is supported by several pieces of evidence. First, the gene or genes responsible for Beckwith-Wiedemann syndrome (which predisposes to several cancers, including RMS) were localized to this chromosomal region.[53] Second, transfer of wild-type copies of chromosome 11 or fragments containing the 11p15 region suppresses growth of ERMS cells.[54,55]

Determination of the parent of origin of the two alleles from the 11p15.5 region revealed that ERMS tumors preferentially maintain the paternal allele and lose the maternal allele (see Fig. 59-3).[56] This preference suggests the action of genomic imprinting, a normal epigenetic developmental process that selectively inactivates expression of alleles in a gamete-of-origin–dependent process. Several genes in the human 11p15 chromosomal region and the corresponding mouse region show imprinting.[53] For example, *IGF2*, encoding an embryonic growth factor, is preferentially expressed from the paternal allele, whereas *CDKN1C*, which encodes a cyclin-dependent kinase inhibitor (p57/KIP2) that negatively regulates cell-cycle progression, is preferentially expressed from the maternal allele. Two additional genes with preferential maternal expression and tumor suppressor function derive from opposite strands of the *H19* locus, which produces the nontranslated H19 RNA, and the HOTS nucleolar protein.[57] These combined studies of allelic loss in the 11p15.5 region suggest that ERMS tumorigenesis frequently involves inactivation of an imprinted tumor suppressor by allelic loss of the active maternal allele and retention of the inactive paternal allele.

Oncogene and Tumor Suppressor Gene Mutations in Rhabdomyosarcoma

The earlier-described studies of RMS patients with cancer predisposition syndromes revealed genes that are mutated and corresponding pathways that are altered in the pathogenesis of rare RMS cases. In particular, RMS cases occurred in syndromes in which the RAS pathway was activated whereas other cases occurred in syndromes in which the RB1 or TP53 signaling pathway was inactivated. The corresponding pathways thus are strong candidates for alterations in sporadic tumors, either by mutating the same genes that are altered in the predisposition syndromes or mutating other genes in these pathways.

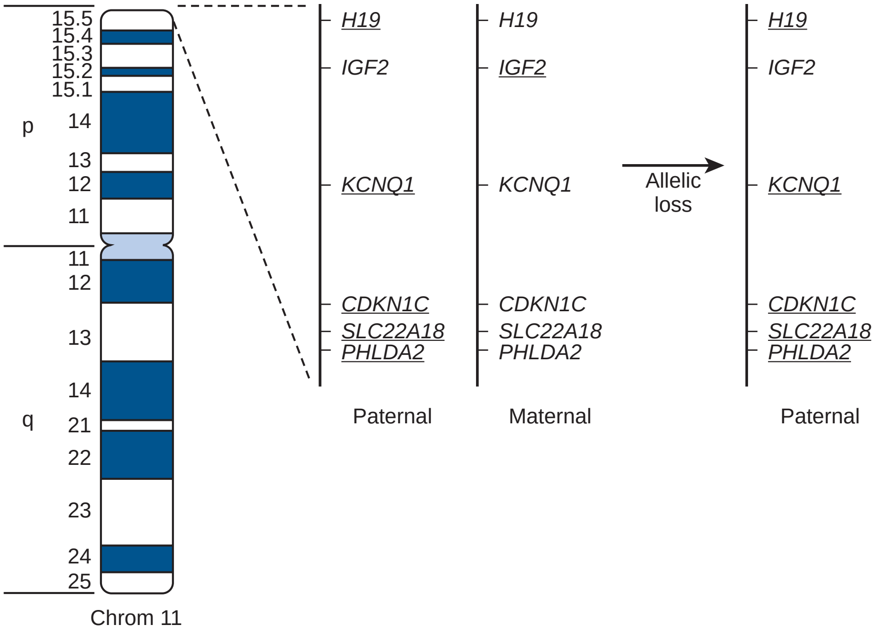

Figure 59-3 Allelic loss of imprinted region at 11p15.5 in RMS. In the 11p15.5 chromosomal region, there is parent-of-origin-specific expression (imprinting) of multiple genes, such that *underlining* indicates an expressed allele and lack of underlining indicates an unexpressed allele. In many cases of ERMS, a few cases of ARMS, and other tumors, the maternal alleles in the 11p15.5 region (and variable amounts of contiguous regions) are lost by one of a variety of genomic mechanisms in a process termed *allelic loss, loss of heterozygosity,* or *conversion to homozygosity.*

Several studies suggest that small mutations are more common in ERMS than ARMS. In a recent next-generation sequencing (NGS) study of 147 RMS cases (53 fusion-positive and 94 fusion-negative), the mean rate of verified nonsynonymous mutations was fourfold higher in fusion-negative tumors (17.8 mutations/tumor) compared with fusion-positive tumors (6.4 mutations/tumor).[58] Recurrent deleterious point mutations involving known genes were found in more than 50% of fusion-negative RMS cases compared with less than 5% of fusion-positive cases. Of note, only two recurrent point mutations (*PIK3CA* and *BCOR*) were found in the 48 fusion-positive ARMS cases whereas three recurrent point mutations were found in 11 fusion-negative ARMS cases. These overall findings in this NGS study were very similar to a second smaller NGS study[59] as well as a directed mutation study.[60] In this latter directed mutation study of 31 genes, mutations were detected in 14 of 57 (25%) ERMS cases compared with 1 of 21 (5%) ARMS cases.[60] Except for one ERMS case with two mutations, the other 13 ERMS cases had only one mutation per case from this panel of 31 genes. An insufficient number of cases was examined to comment on mutation frequency in the fusion-negative ARMS subset.

The RAS pathway is frequently activated in sporadic ERMS tumors by mutations in one of several genes (Table 59-3). In recent studies analyzing 147 ERMS cases, *RAS* family genes were mutated in 20% of ERMS cases.[35,60-63] More than half of these *RAS* family mutations (19/31, 61%) occurred in the *NRAS* gene and the majority (15/19, 79%) of these *NRAS* mutations involved codon 61. In contrast, only one *RAS* family gene mutation (in *KRAS*) was detected in 41 cases of ARMS (2%). Of various other RAS pathway components, *PTPN11* and *BRAF* mutations were found in 3% and 1% of ERMS cases but not in any ARMS cases.[60-65] In 1 of 12 ERMS cases (8%), a mutation was also found in the *SPRY1* gene, which encodes a modulator of RAS signaling.[63] Finally, homozygous deletions involving the *NF1* locus were found in 15% of ERMS cases.[35] The *RAS*, *PTPN11*, and *BRAF* point mutations as well as the *NF1* deletions are mutually exclusive, consistent with the premise that these changes activate the same pathway. Therefore, one of several components in the RAS pathway can be altered to activate this pathway in more than 40% of sporadic ERMS cases but in very few (<5%) sporadic ARMS cases.

Alterations in genes encoding RB1 or proteins regulating RB1 function occur in sporadic ERMS and ARMS tumors, providing several mechanisms to alter RB1 signaling. Although small *RB1* mutations were not conclusively investigated in sporadic RMS, homozygous deletions were found in 22% (6/27) of ERMS and 10% (2/20) of ARMS cases (see Table 59-3).[66] A higher frequency of *RB1* gene alterations in ERMS is suggested by the finding of significantly lower RB1 expression in ERMS compared with ARMS tumors. As described earlier, 12q13-q14 amplification in a subset of ARMS cases results in overexpression of CDK4, which phosphorylates RB1 and contributes to RB1 functional inactivation. In another subset of RMS cases, homozygous deletions and/or point mutations occur in *CDKN2A* and *CDKN2B*, which encode inhibitors of CDK4 and CDK6.[35,67-69] These alterations occur in both ARMS and ERMS, with an overall homozygous deletion frequency of 13% (11/82) and a mutation frequency of 7% (6/88).

There is also evidence for genetic changes that alter the TP53 pathway in sporadic RMS tumors (see Table 59-3). In multiple studies examining a total of 159 RMS cases,

TABLE 59-3A Point Mutations in Sporadic Rhabdomyosarcomas*

Gene	ERMS	ARMS	Other†	All RMS
HRAS	5/147‡ (3%)	0/41 (0%)	NA	5/188 (3%)
KRAS	7/147 (5%)	1/41 (2%)	NA	8/188 (4%)
NRAS	19/147‡ (13%)	0/41 (0%)	NA	19/188 (10%)
BRAF	1/93 (1%)	0/31 (0%)	0/29 (0%)	1/153 (1%)
MAP2K1	0/80 (0%)	0/21 (0%)	NA	0/101 (0%)
MAP2K2	0/23 (0%)	NA	NA	0/23 (0%)
PTPN11	3/108 (3%)	0/31 (0%)	NA	3/139 (2%)
SPRY1	1/12 (8%)	0/7 (0%)	NA	1/19 (5%)
SOS1	0/20 (0%)	NA	NA	0/20 (0%)
CDKN2A	0/27 (0%)	0/17 (0%)	6/44 (14%)	6/88 (7%)
CTNNB1	2/57 (4%)	0/21 (0%)	NA	2/78 (3%)
DICER1	4/57 (7%)	NA	NA	4/57 (7%)
FGFR4	11/112 (10%)	2/47 (4%)	2/12 (17%)	15/171 (9%)
PIK3CA	3/57 (5%)	0/21 (0%)	1/12 (8%)	4/90 (4%)
TP53	2/50 (4%)	0/41 (0%)	10/68 (15%)	12/159 (8%)

ARMS, Alveolar rhabdomyosarcomas; *ERMS*, embryonal rhabdomyosarcomas; *NA*, not available.
*RMS cell lines were not included in this compilation.
†Other includes Botryoid, pleomorphic, mixed, and unclassified cases.
‡In one ERMS case, both a HRAS and a NRAS mutation were detected.

12 missense mutations (8%) were detected in the *TP53* gene.[46,47,70-72] Although many cases were not subclassified for histopathologic subtype, mutations were found in both ARMS and ERMS.[46,47,71,73] Homozygous deletions involving *TP53* were also detected in a few cases.[73,74] In addition to these direct changes in *TP53*, changes in the TP53 pathway may be caused by *MDM2* gene amplification (described earlier), which encodes a protein that binds to and inactivates TP53. Finally, deletion of the *CDKN2A* locus (described earlier) also eliminates expression of p14ARF, which is a protein that normally blocks MDM2 function and stabilizes the TP53 protein.[69]

Activation of several additional signaling pathways is also implicated in sporadic RMS tumors (see Table 59-3). Mutation in the tyrosine kinase domain of the membrane receptor fibroblast growth factor receptor 4 *(FGFR4)* was detected in 15 of 171 (9%) RMS tumors, including 11 in 112 (10%) ERMS and 2 in 47 (4%) ARMS cases.[35,60,75] These mutations activate downstream signaling from FGFR4 and increase proliferation, invasion, and metastatic potential in mutation-bearing cells. Other genes in which mutations were found only in ERMS tumors include *PIK3CA* (3/57, 5%) and *CTNNB1* (2/57, 4%); these mutations will activate phosphatidylinositol-3-kinase and Wnt signaling pathways, respectively.[60,64] Although activation of the Hedgehog signaling pathway has been noted in a substantial subset of ERMS tumors, point mutations were not found in genes encoding several classic pathway components.[76-78] As a possible alternative explanation of this increased Hedgehog signaling, low-level gains of the *GLI1* locus were found in numerous ERMS cases.[35] Finally, *DICER1* mutations have been detected in 2 of 52 (4%) sporadic ERMS tumors and result in deregulated microRNA processing.[79,80]

Hypermethylation as an Alternative Mechanism of Gene Inactivation in Rhabdomyosarcoma

In a final set of genomic changes in RMS, genetic loci can be silenced by epigenetic events involving tumor-specific hypermethylation of CpG islands. The cytosine residues in CpG dinucleotides are ordinarily unmethylated in normal cells but can become methylated during

TABLE 59-3B Homozygous Deletions in Sporadic Rhabdomyosarcomas*

Gene	ERMS	ARMS	Other†	All RMS
CDKN2A/B	8/32 (25%)	1/6 (17%)	2/44 (5%)	11/82 (13%)
NF1	4/26 (15%)	NA	NA	4/26 (15%)
RB1	6/27 (22%)	2/20 (10%)	NA	8/47 (17%)
TP53	1/4 (25%)	0/2 (0%)	1/31 (3%)	2/37 (5%)

ARMS, Alveolar rhabdomyosarcoma; *ERMS*, embryonal rhabdomyosarcoma; *NA*, not available.
*RMS cell lines were not included in this compilation.
†Other includes Botryoid, pleomorphic, mixed, and unclassified cases.

tumorigenesis, thereby altering the nearby chromatin structure and silencing transcription of associated genes. Studies of small numbers of RMS cases indicated that several genes often hypermethylated in other tumor types, such as *CDKN2A,* are not affected in a significant subset of RMS cases. However, hypermethylation of the CpG islands in the *RASSF1A, HIC1,* and *CASP8* genes was detected in both ARMS and ERMS tumors.[81-83] In contrast, there is evidence of differential methylation of the CpG islands in the *MYOD* and *PAX3* genes such that hypermethylation occurs in most ERMS cases and hypomethylation occurs in most ARMS cases.[84,85] A genome-wide approach also identified 140 promoter regions hypermethylated in at least four of ten RMS tumors compared with skeletal muscle, including genes implicated in development and tumorigenesis, such as *GATA6* and *HES5*.[86] Unsupervised hierarchical cluster analysis indicates that DNA methylation pattern correlates with RMS subtype. These hypermethylation events are generally associated with loss of expression of the corresponding proteins, providing an alternative nonmutagenic mechanism for inactivating genes and altering associated pathways.

APPLICATIONS OF MOLECULAR GENETIC APPROACHES TO DIAGNOSIS AND PROGNOSIS

Detection of Recurrent Gene Fusions

As described earlier, RT-PCR and FISH methodologies were developed to detect the *PAX3-FOXO1* and *PAX7-FOXO1* gene fusions in clinical material.[24-27] In contrast to Southern blot approaches, both RT-PCR and FISH can be applied to detect these fusions in small amounts of input tissue. Furthermore, both RT-PCR and FISH approaches have been developed for formalin-fixed paraffin-embedded tissues as well as fresh and frozen tissues. Another favorable feature of the RT-PCR assays is high technical sensitivity, which permits detection of micrometastatic spread of rare fusion-positive cells to tissues such as bone marrow.[87] Although FISH has lower technical sensitivity, a break-apart *FOXO1* FISH assay has simplified detection of *FOXO1* rearrangements resulting from either *PAX3-FOXO1* or *PAX7-FOXO1*.[41] In addition, break-apart *PAX3* and *PAX7* assays facilitate detection of variant fusions in ARMS cases that do not contain either of the two usual fusions.[27]

Pathologic and Clinical Differences between Fusion Subtypes

Clinical-molecular correlative studies revealed several differences between fusion-negative and fusion-positive tumors. In comparisons of outcome among the fusion subsets, the fusion-negative ARMS cases have a failure-free survival (FFS) and overall survival (OS) similar to ERMS cases and significantly better than fusion-positive ARMS cases.[37,88] There is a similar frequency of other parameters, such as unfavorable sites and metastasis, in ERMS and fusion-negative ARMS, and this frequency is lower in fusion-negative ARMS than in fusion-positive ARMS. Finally, in an examination of histopathologic parameters, nearly half of fusion-negative cases lack cystic foci and show totally "solid alveolar" architecture.[89]

Although there are no detectable histopathologic differences between *PAX3-FOXO1*– and *PAX7-FOXO1*–positive cases,[89] there are clinical differences between these two fusion-positive subsets. The OS rate in the *PAX7-FOXO1*–positive subset is superior to that in the *PAX3-FOXO1*–positive subset and similar to the OS rate in the ERMS and fusion-negative ARMS categories.[41,88,90] In contrast, failure free survival in the two fusion-positive subsets is similar. In addition to OS, there are other clinical differences between these two fusion-positive subsets. In particular, the *PAX7-FOXO1*–positive tumors are typically diagnosed at a younger age, more often present with an extremity primary tumor, and are associated with a lower frequency of local invasion and nodal metastasis.[28,41] A multivariate analysis of the contributions of prognostic parameters found that fusion status was an independent predictor of OS. A risk-based stratification incorporating fusion status, stage, and age has potential utility for stratifying the Children's Oncology Group (COG) intermediate-risk group into distinct subgroups.[90]

Gene Expression Profiling with Microarrays

Microarray-based analyses of genome-wide gene expression compared RMS with other pediatric tumors included in the differential diagnosis of small blue round cell tumors. Cases were clustered based on differences and similarities in expression patterns. Statistical analyses identified lists ranging from 20 to 93 genes that can correctly classify all tumors.[91-94] These lists contain genes specifically expressed in each tumor category, including muscle-specific and myogenesis-related genes that are specifically expressed in RMS. With the use of minimization strategies, the criteria needed for classification were reduced in size to lists ranging from 7 to 31 genes. To extend these findings, 39 genes were selected to design a multiplex RT-PCR assay, which generates findings comparable to the microarray platform.[95]

Microarray studies also developed classifiers for subsets within the RMS family. With the use of clustering algorithms, it was shown that the *PAX3-FOXO1*– and *PAX7-FOXO1*–positive subsets of ARMS cluster together, indicating no major expression differences between the two fusion-positive subsets.[37,50,96,97] In contrast, there was a striking difference in expression pattern between the fusion-positive and fusion-negative ARMS cases. There were four published cases with variant fusions: two clustered with fusion-positive tumors and two clustered with the fusion-negative tumors. These analyses also demonstrated a striking difference in expression pattern between ERMS and fusion-positive ARMS cases. Comparison of ERMS and fusion-negative ARMS cases did not reveal any recurrent expression differences between these two groups of fusion-negative RMS cases. Therefore, based on expression pattern, RMS cases can be divided into two major categories: the fusion-positive and fusion-negative cases. Furthermore, when viewed on a multidimensional scaling plot, the fusion-positive group showed a dense cluster whereas the fusion-negative group showed a cluster with more heterogeneous spread.

The fusion-negative RMS category is proposed to be further divisible into three novel subsets, which are related to expression of muscle differentiation and chromosome 11 genes.[50] A well-differentiated RMS subset expressed both gene sets, whereas a moderately differentiated RMS subset expressed the chromosome 11 genes with decreased levels of muscle differentiation genes. A final subset containing approximately 25% of ERMS tumors as well as undifferentiated sarcomas and non-RMS soft tissue tumors (NRSTS) did not express either gene set. In addition, tumors in this last subset had a fractional allelic loss (including 11p loss) similar to that in fusion-positive RMS tumors and twofold lower than that in the well-differentiated and moderately differentiated subsets. The significance of these subsets is highlighted by the finding of good, intermediate, and poor outcomes in well-differentiated, moderately differentiated, and undifferentiated sarcoma/NRSTS categories, respectively.

For prospective diagnosis of fusion-positive or fusion-negative RMS, genes differentially expressed between the two fusion subsets were identified to generate initial signatures ranging from 121 to 534 genes.[37,50,96,97] The genes preferentially expressed in fusion-positive tumors were overrepresented in PAX3-FOXO1 downstream targets (including genes involved in neurogenesis) and chromosome 6 genes, whereas the genes preferentially expressed in fusion-negative tumors were overrepresented in chromosome 8 genes. Additional statistical approaches identified minimal signatures consisting of 10, 5, or even 2 genes with predictive accuracy of 95% or greater.[50,97,98] To develop an assay applicable to formalin-fixed paraffin embedded samples, biomarkers were identified that correspond to differentially expressed genes with commercially available antibodies suitable for immunohistochemistry.[50,99] These efforts identified epidermal growth factor receptor (EGFR), fibrillin-2, and high-mobility group AT-hook 2 (HMGA2) as markers expressed in fusion-negative RMS cases and transcription factor AP-2β (TFAP2B) and P-cadherin as markers expressed in fusion-positive RMS cases. The combination of two markers for each RMS subset achieved specificity of 90% or greater and a sensitivity of 60% or greater.

Several microarray and other genomic approaches were used to identify fusion protein downstream targets. In one approach, transduction of *PAX3-FOXO1* or *PAX7-FOXO1* in an ERMS cell line modulated expression of 334 genes (266 increased and 68 decreased); 81 of these genes (24%) were also differentially expressed between fusion-positive and fusion-negative RMS tumors.[100] In the second approach, siRNA-mediated downregulation of *PAX3-FOXO1* in an ARMS cell line decreased expression of 1834 genes after 24 hours of treatment; only 51 (3%) of these genes overlapped the differential expression signature in RMS tumors.[101] In a final approach, ChIP-Seq analysis identified 1463 genomic regions bound to the fusion protein in ARMS cells; these genomic regions correspond to 24% of genes expressed at higher levels in fusion-positive RMS and 6% of the genes expressed at higher levels in fusion-negative RMS.[102] These findings indicate that only a fraction of expression differences between fusion-positive and fusion-negative tumors is directly attributable to the fusion protein; many of the expression differences may result from other genetic and environmental differences between these RMS subsets.

Microarray analysis also elucidated genes whose expression predicts outcome in RMS patients. In particular, Cox regression proportional hazards modeling of OS with RMS microarray expression data identified 15 or 34 gene sets whose aggregate pattern of expression was correlated with OS.[90,103] The performance of these two gene sets was high in the initial patient cohort and lower but still significant in a validation cohort. For the 34 gene set, this expression-based stratification of RMS cases was related to the current COG risk classification system. In addition, this expression-based stratification for both the 15- and 34-gene sets was also correlated with *PAX3-FOXO1* and *PAX7-FOXO1* fusion status. Multivariate analysis showed that these 15- or 34-gene sets were not independent predictors of outcome.

A hypothesis was also proposed that fusion protein downstream targets may predict outcome.[100] Starting with 81 target genes that were both modulated by exogenous fusion protein in ERMS cells and differentially expressed between the fusion-positive and fusion-negative tumors, Cox regression modeling elucidated a 28-gene predictor. An aggregate score calculated from expression of these 28 genes is predictive of OS in ARMS and independent of known prognostic variables. This finding suggests that modulation of downstream fusion protein target expression is associated with differences in biologic aggressiveness of ARMS.

Detection of Rhabdomyosarcoma in Sites Other Than Primary Tumor

Molecular markers in conjunction with high-sensitivity detection methodologies have been applied to screen for RMS dissemination in accessible and commonly involved sites such as bone marrow, peripheral blood, and lymph nodes (Table 59-4). In general, these approaches screen for molecular markers within cells to identify cases that are not detected by conventional radiology or pathologic examination. One strategy for minimal disseminated disease detection focuses on tumor-specific markers, such as *PAX3-FOXO1* and *PAX7-FOXO1*.[87,104] A second strategy, which is broadly applicable to all RMS subtypes, assays lineage-specific markers, such as myogenic markers of determination and differentiation (including MYOD1 and MYOG).[104-106] In a third strategy, sites of disease spread were assayed for proteins involved in metastatic pathways, such as CXCR4 and MET.[107] RT-PCR assays were developed to detect several of these markers and demonstrate analytical sensitivity in the range of one positive cell per 10^4 to 10^5 normal cells.[87,104,105] More recent PCR protocols use real-time technology to quantify the extent of disseminated disease and can thus monitor changes in disease load over time and compare results to an empirically determined clinical threshold. In addition to PCR assays, antibodies have been used to

TABLE 59-4 Assays for Detecting RMS in Sites Away from Primary Tumor

Marker	Molecule	Target	Assay
ACHRA	mRNA	Tumor cells	RT-PCR
ACHRG	mRNA	Tumor cells	RT-PCR
PAX3/PAX7-FOXO1	mRNA	Tumor cells	RT-PCR
MYOD1	mRNA protein	Tumor cells	RT-PCR Immunocytology
MYOG	mRNA protein	Tumor cells	RT-PCR Immunocytology
CXCR4	Protein	Tumor cells	Immunocytology
MET	Protein	Tumor cells	Immunocytology
CD45/CD56	Protein	Tumor cells	Flow cytometry
miR-206	miRNA	Plasma/serum	RT-PCR
IGFBP2	Protein	Plasma/serum	ELISA
VEGF-A	Protein	Plasma/serum	ELISA

ELISA, Enzyme-linked immunosorbent assay; *mRNA,* messenger ribonucleic acid; *RT-PCT,* reverse-transcriptase polymerase chain reaction assay.

detect various protein markers in immunocytochemical or flow cytometry assays.[106,107] These assays also have a high level of analytical sensitivity (~10^{-4}), which is significantly better than conventional morphology-based evaluation.

These minimal disseminated disease assays were applied to clinical material from RMS patients in several different settings (see Table 59-4). Multiple studies addressed the feasibility of detecting occult disease in bone marrow.[87,104,105] These studies showed that histologically positive bone marrows are consistently detected, regardless of the methodology or marker. For histologically negative marrows, various methodologies and markers detected submicroscopic disease, with the frequency tending to be higher for ARMS relative to ERMS. In ARMS, which often metastasizes to the bone marrow, the frequency of a positive result in a histologically negative marrow ranges from 15% to 60%. In ERMS, in which bone marrow is not a common site of metastasis, the frequency of a positive result in a histologically negative marrow ranges from 7% to 33%. The presence of these marrow-infiltrating RMS cells is not consistently associated with subsequent overt marrow involvement, suggesting that the marrow can act as a transitory reservoir for disseminated RMS cells.[107] Despite this finding, a significant association was found between poor outcome and detection of RMS cells in bone marrow; marrow involvement was associated with a significantly higher risk for recurrence (50% vs. 11%, $P = .011$) and poorer OS rate (47% vs. 92%, $P = .01$).[106] In addition to bone marrow, studies also evaluated peripheral blood samples at diagnosis, during treatment, and after treatment.[105,108] Comparison with outcome revealed that a positive result at the end of treatment was associated with poor outcome, and persistent positive results preceded a metastatic relapse.

Recent studies also examined expression of molecular markers in the serum or plasma. These markers include miR-206 and other muscle-specific miRNAs (see section below on microRNAs), which were measured by RT-PCR, and RMS-associated proteins, such as insulin-like growth factor–binding protein 2 (IGFBP2) and vascular endothelial growth factor (VEGF)- A (VEGF-A), which were assessed by enzyme-linked immunosorbent assay (ELISA).[109-111] The finding of higher abundance of these markers in the plasma or serum of RMS patients relative to normal controls indicates a possible use in noninvasive diagnosis. Furthermore, an application in disease monitoring is suggested by the finding of decreased levels after treatment. Finally, an association of plasma IGFBP2 levels with metastatic RMS suggests a potential role in patient stratification for risk-based therapy.

MOLECULAR AND CELLULAR BIOLOGY OF RHABDOMYOSARCOMA

Myogenic Pathways in the Tumorigenesis of Rhabdomyosarcoma

Based on the premise that RMS is related to the skeletal muscle lineage, the expression pattern of muscle-specific proteins has been extensively examined in RMS. In particular, many studies have focused on the family of myogenic transcription factors (MyoD, Myf5, myogenin, and MRF4) that are responsible in part for the determination of stem cells into myoblasts and then differentiation into myocytes. Assays of the corresponding genes detected RNA expression of *MYOD* and *MYF6* in all ARMS and ERMS tumors and RNA expression of *MYOG* (myogenin) and *MYF5* in all ARMS and most ERMS tumors.[112] Antibodies directed to MyoD and myogenin have proved to be suitable for immunohistochemistry on paraffin-embedded, formalin-fixed tissues.[113] With the use of these antibodies the vast majority of RMS cases (about 97% in a study of 956 cases) showed positive nuclear immunostaining for each protein.[2] Because positive staining also occurred in multiple cases of pleuropulmonary blastoma, the specificity of these reagents was estimated to be between 90% and 91%. There are differences between ARMS and ERMS tumors in the myogenin staining patterns such that most cells within an ARMS tumor stain positive, whereas fewer cells within an ERMS tumor stain positive. This differential staining between ARMS and ERMS has also been shown for MyoD. There is a statistical difference in the extent of MyoD or myogenin expression among the RMS subtypes, but there is still overlap. Hence the immunohistochemical pattern of these myogenic proteins is not sufficient to classify cases but may be helpful in selecting cases for further testing.

Functional studies in RMS cell lines determined that MyoD is not an active transcription factor in the RMS environment (Fig. 59-4).[114] In particular, although MyoD is able to bind DNA at its specific binding sites in RMS cells it is not capable of acting as a transcriptional activator on model genes with these DNA-binding sites. These

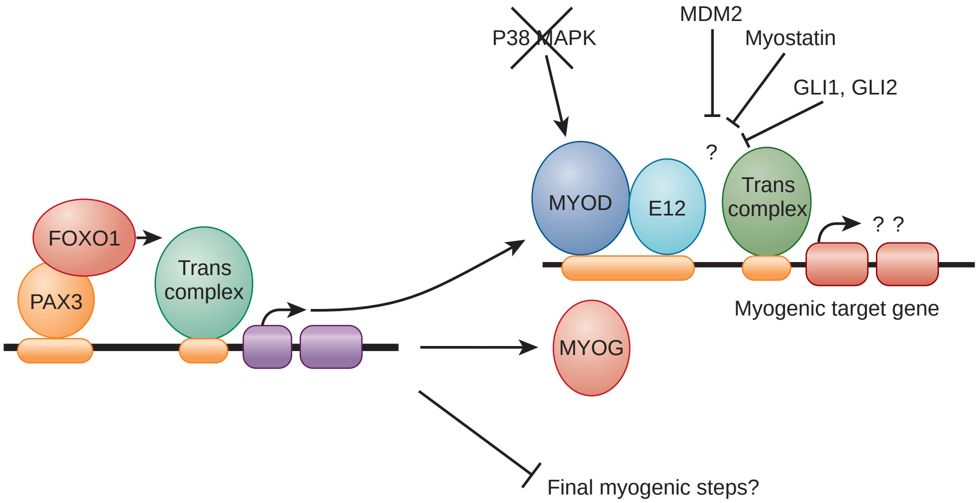

Figure 59-4 Influences on myogenic differentiation in ARMS. A model *PAX3-FOXO1* target gene is shown on the left, with PAX3-FOXO1 binding to a positive acting *cis*-element and interacting with the transcriptional machinery at the promoter to upregulate transcription of mRNA and ultimately increase production of the target protein. Two such targets are postulated to be MYOD and myogenin (MYOG). In turn, MYOD and its binding partner E12 bind to *cis*-elements of myogenic target genes and stimulate the transcriptional machinery to upregulate transcription of the corresponding mRNAs. In alveolar rhabdomyosarcoma (and embryonal rhabdomyosarcoma), various upstream changes can influence the transcriptional activity of the MYOD protein and thereby inhibit myogenic differentiation.

effects may contribute to the failure of RMS cells to undergo terminal differentiation. Various factors may contribute to this inhibition of transcriptional activation by MyoD. Experiments fusing RMS cell lines to a second normal cell line resulted in some instances in which the transcriptional activation block was overcome, suggesting that the RMS line was deficient in a *trans*-acting factor. In other instances, the block was not overcome by this cell fusion, suggesting a dominant-acting inhibitory factor. As an example of the former recessive scenario, the p38 mitogen-activated protein kinase (MAPK) pathway is deficient in some RMS cell lines, resulting in the absence of an essential activator of MyoD during myogenic differentiation.[115] Examples of the latter dominant scenario include amplification of *MDM2* in an RMS cell line that inhibited MyoD activity by a mechanism involving competition for the transcription factor Sp1[116,117] and upregulation of musculin and a novel splice variant of E2A that inhibited MyoD activity by competing for heterodimer formation and preventing formation of functional MyoD:E-protein heterodimers, required for the switch to differentiation.[118] In addition, increased expression of the negative muscle growth regulator myostatin, which is found in both ERMS and ARMS, is also capable of inhibiting MyoD-mediated transcriptional activity, possibly via Smad3 interactions.[119] In RMS associated with activation of the Hedgehog pathway, expression of the transcription factor GLI1 or GLI2 inhibits MyoD-mediated transcriptional activation by reducing heterodimerization with its partner E12 and DNA binding.[120] Finally, as discussed in the later section "Epigenetic Regulation," MyoD activity can also be inhibited by decreased expression associated with KMT1A methyltransferase activity.[121]

The identification of *PAX3-FOXO1* and *PAX7-FOXO1* gene fusions in ARMS also highlight PAX3 and PAX7 as important proteins in normal and aberrant myogenic transcriptional control. In murine systems, both Pax3 and Pax7 are involved in early embryonic development of cells giving rise to axial musculature, and Pax3 also has a necessary role in the early embryonic development of the limb musculature.[122-124] In the adult mouse, Pax7 has an important role in the development of the major population of myogenic satellite cells and Pax3 is expressed in satellite cells in a subset of muscles.[125,126] This developmental biology of *PAX3* and *PAX7* is relevant to ARMS tumors, which express *PAX3* or *PAX7* as a fusion with *FOXO1*,[127] and to ERMS tumors, which express wild-type *PAX3*, *PAX7*, or both.[128]

The wild-type and fusion PAX proteins can variably affect the myogenic status of the cells expressing them. When wild-type Pax3 is introduced into explanted embryonic tissues, it can activate the myogenic program as shown by expression of MyoD, Myf5, and myogenin as well as myogenic differentiation products.[129] In contrast, Pax3 could not induce this myogenic program in NIH 3T3 murine fibroblasts, suggesting that this murine fibroblast represents a less permissive environment.[130] Studies of the PAX3-FOXO1 fusion protein have demonstrated a range of activities on the myogenic program (see Fig. 59-4). First, studies focusing on the *MyoD* and *myogenin* promoters provide evidence that both of these genes are transcriptional targets of PAX3-FOXO1. Of two studies of NIH 3T3 cells, one indicated that PAX3-FOXO1 induced the myogenic program, including the upstream products MyoD, myogenin, Six1, and Slug as well as downstream myogenic products, such as troponins and myosin light chain.[130] However, a second study of NIH 3T3 cells demonstrated induction of the upstream products MyoD, myogenin, and desmin but no induction of the downstream product myosin heavy chain.[131] In contrast to these findings, transduction of *PAX3-FOXO1* into two additional murine fibroblast lines (10T1/2 and Plus) resulted in expression of both upstream and downstream myogenic products and fusion into multinucleated myotubes. In contrast, introduction of *PAX3* or *PAX3-FOXO1* into two myogenic cell lines, C2C12 myoblasts and MyoD-expressing 10T1/2 cells, inhibited terminal myogenic differentiation after stimulation of differentiation by growth factor withdrawal.[132] *PAX3-FOXO1* was more potent than wild-type *PAX3* in this phenotypic activity. Finally, repression of *PAX3-FOXO1* expression in human ARMS cell lines with RNA interference (small interfering RNA [siRNA] or short hairpin RNA [shRNA] constructs) results in decreased proliferation without apparent apoptosis, decreased motility and invasion, and increased differentiation as demonstrated by morphologic and myogenic gene and protein expression.[101,133,134] This finding indicates that *PAX3-FOXO1* normally represses expression of this myogenic pathway in this cell type.

The finding of both stimulatory and inhibitory effects of PAX proteins on myogenic events may be explained by the hypothesis that these wild-type or fusion PAX proteins facilitate entry into the myogenic pathway and variably inhibit the final steps of the pathway depending on the cellular environment. In support of this hypothesis, data suggest that PAX3-FOXO1, by inducing MyoD expression but attenuating its activity, and inducing FGFR expression to block differentiation, simultaneously stimulates but blocks completion of myogenesis.[135] This intermediate commitment to differentiation may be partially explained by recent studies in which expression of *PAX3-FOXO1* in muscle satellite cells did not inhibit expression of MyoD but did inhibit expression of MyoD target genes.[136]

MicroRNAs

Although decades of study shed light on the intrinsic cellular signaling pathways and extrinsic microenvironmental stimuli that control skeletal muscle differentiation during embryogenesis, postnatal life, and muscle repair, it was not until 2005 that another layer of control was identified. In work examining the spatiotemporal control of cardiogenesis, it was discovered that specific microRNAs were expressed in cardiac and skeletal muscle and served to balance proliferation and differentiation of muscle precursor cells.[137] Since that time, a great deal of information on these small noncoding RNAs that enable the regulation of protein dosage through binding to 3′ ends of mRNA and affecting stability or translation to protein has been gathered and understood not only in the framework of normal skeletal myogenesis but also in tumors related to the skeletal muscle lineage such as RMS.

MicroRNAs influencing skeletal myogenesis can generally be divided into myomirs (miRs specific to skeletal and cardiac muscle) and nonmyomirs (miRs expressed in nonmuscle tissue but that can also be found in muscle.) The myomirs (nomenclature miR-1 to miR-206, which includes the miR-133 group) are encoded in bicistronic clusters on chromosomes 6, 18, and 20 and control such diverse developmental processes as somite organization, muscle precursor cell migration, survival, differentiation, fusion, and cross talk with epigenetic processes that control differentiation (see next section). Similar to other human cancers, RMS tumors are noted to have lost expression of miRs and their tumor suppressive properties. When miR-206 is expressed in human RMS cells, xenograft growth is halted and accompanied by differentiation,[138] presumably by the orchestrated downregulation of hundreds of miR-206 target genes,[139] including *MET*.[138] Mir-206 expression levels also correlate with clinical behavior of RMS.[140] Regarding the nonmyomirs, the reader is referred to two reviews on microRNAs and their role in RMS.[141,142] For example, miR-185 suppresses RMS tumor growth and progression by targeting the *Six1* oncogene[143] (see later discussion under "Metastatic Pathways in Rhabdomyosarcoma").

Finally, as was discussed in the section on "Rhabdomyosarcoma in Cancer Predisposition Syndromes," germline or somatic mutations of DICER1, the enzyme that cleaves pre-microRNA to mature microRNA, have been found associated with ERMS.[80,144,145] To date these mutations appear to be in the RNase IIIb domain, resulting in a decrease in its activity.

Epigenetic Regulation

Although the block to myogenic differentiation in RMS cells has been apparent for decades, only recently is there insight into some of the epigenetic changes enforcing this inhibition. As mentioned earlier, whereas the muscle-specific transcription factor MyoD can bind DNA in RMS, some targets fail to activate. For example, although MyoD binds similarly at the genome-wide level in both normal and RMS (in this case the human embryonal RMS cell line RD was studied), in RMS there is a subset of genes to which MyoD binds poorly and thus does not activate.[146] This subset includes the transcription factors *MEF2C, RUNX1, JDP2,* and *NFIC,* found expressed at lower levels in RMS compared with normal muscle cells. There was also differential DNA accessibility, including changes in DNA methylation status. Therefore it has become clear that epigenetic changes contribute as much to RMS as changes in the DNA sequence itself. Below are summarized some of the epigenetic changes found in RMS, including changes in DNA methylation (introduced earlier in "Hypermethylation as an Alternative Mechanism of Gene Inactivation in Rhabdomyosarcoma"), changes in histone methylation, and changes in nucleosome positioning.

Methylation of gene promoters at CpG islands by DNA-specific methyltransferases serves to silence expression of target genes. Thus it would follow that tumors should demonstrate different DNA methylation profiles compared with nonmalignant tissue, and indeed a study of the genomes of RMS compared with normal skeletal muscle showed this to be the case.[147] In both ERMS and ARMS, genes involved in development and differentiation were hypermethylated compared with normal skeletal muscle. However, there were also differences between ERMS and ARMS, with ARMS showing more hypermethylation of Polycomb target genes (see later.) As methods to improve the speed and resolution of the detection of DNA methylation patterns improve, further changes are expected to be identified.

Methylation of lysine residues in histones by Polycomb group remodeling complexes (known as PRC1 and PRC2) also serves to silence chromatin, by influencing nucleosome formation and function. Although this process occurs in a carefully controlled manner during development, if Polycomb complexes are inappropriately recruited to target genes or inappropriately activated due to component mutation, impaired commitment to cell lineage or a frank block in differentiation can ensue, resulting in a primitive cellular phenotype. For example, in both ERMS and ARMS the NF-κB-YY1-mir-29 regulatory circuit that ordinarily controls skeletal myogenesis is usurped; YY1 recruits PRC2 to silence target genes including *MIR29*, so that muscle differentiation cannot occur.[148] In ARMS, PAX3-FOXO1 transcriptionally activates JARID2 expression, which recruits PRC2 to the promoter regions of target genes including *MYOG* and *MYL1*, again impairing differentiation.[149] Recently, there has been some focus on the catalytic component of PRC2 known as the EZH2 methyltransferase. EZH2 was found upregulated at the protein level in RMS tumors[150]; and in loss-of-function studies in RD cells, silencing of EZH2-induced MyoD activation and partial reversal of the tumorigenic phenotype.[151]

There are also methyltransferases independent of Polycomb group remodeling complexes that appear dysregulated in RMS. For example, the histone methyltransferase KMT1A (formerly known as Suv39h1), which methylates histone H3 on the lysine 9 residue, represses myogenic gene expression directed by MyoD.[152] In ARMS this keeps the cells in an undifferentiated state. In support of its presumed oncogenic role, suppression of KMT1A by siRNA decreases ARMS cell and xenograft growth.[121] In independent studies, a screen for tumor suppressors in a Kras- driven model of zebrafish ERMS revealed that point mutations in KMT1A suppress RMS formation.[153]

Regarding the epigenetic changes that occur in response to nucleosome repositioning, components of the SWI/SNF chromatin remodeling tumor suppressor complex have been found mutated in RMS. An early hint of the involvement of the SWI/SNF complex was in the study of TPA-mediated differentiation of RD cells, which was shown to work by recruiting proteins including BRG1 (a subunit of SWI-SNF) to the myogenin promoter,[154] driving differentiation. Later work showed that the BAF53a subunit of SWI/SNF is not downregulated in RMS and thus contributes to a differentiation block.[155]

Role of Insulin-like Growth Factors in Rhabdomyosarcoma

Insulin-like growth factor 2 (IGF-2) is an important growth factor during the fetal period and is highly expressed in fetal skeletal muscle but not detectably expressed in adult skeletal muscle.[156] High-level IGF-2 expression in murine myoblasts results in an increased proliferative rate, impaired myogenic differentiation, and anchorage independence, indicating a potential role of IGF-2 in neoplasia of the myogenic lineage.[157] In accord with these findings, IGF-2 is highly expressed by both ERMS and ARMS tumors as well as in derived RMS cell lines[158] (Fig. 59-5). In addition to the growth factor, both RMS subtypes express the IGF-1 receptor (IGF-1R), which is a cell surface receptor for IGF-2 as well as IGF-1. This simultaneous expression of growth factor and receptor creates an autocrine loop that stimulates the growth and motility of RMS cells. No mutations have been detected in these genes; hence this autocrine situation may reflect the fetal muscle expression pattern that is maintained or induced by the other genetic alterations. The growth response is mediated through IGF-1R, whereas the motility response is mediated through the distinct IGF-2/mannose-6-phosphate receptor.[159] Numerous studies have explored different strategies for interfering with the action of IGF-1R. These strategies include an antibody directed to this receptor, a kinase-deficient mutant that acts as a dominant negative, and an antisense construct directed to this receptor.[160-162] Each of these strategies decreases the response of the cells to IGF-2 stimulation, inhibits cell growth and anchorage independence in culture, and inhibits tumorigenicity in vivo. Exploitation of this downregulation for preclinical and clinical studies is described in "Preclinical Investigation of Emerging Drug Targets for Rhabdomyosarcoma" and "Clinical Research in Rhabdomyosarcoma".

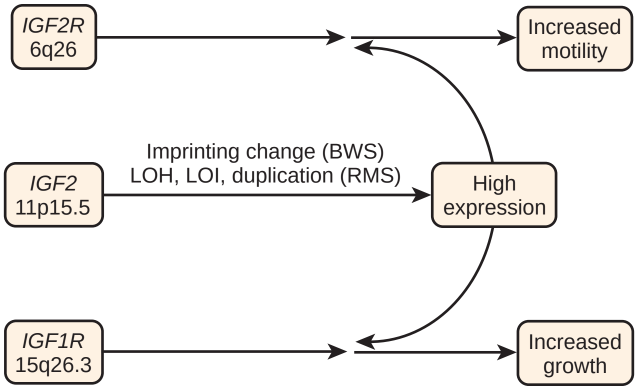

Figure 59-5 Changes in IGF pathway and associated phenotypic consequences in RMS. The three involved genes and their chromosomal locations are shown on the *left*. Genetic or epigenetic changes are indicated above the *horizontal arrows*. Changes in expression or phenotype are indicated on the *right*. *BWS,* Beckwith-Wiedemann syndrome; *LOH,* loss of heterozygosity; *LOI,* loss of imprinting.

As described earlier, the *IGF2* gene is located in the 11p15.5 chromosomal region, which is implicated in allelic loss events that occur in RMS tumors and in genetic alterations in the inherited disorder BWS, which predisposes to malignant tumors, including RMS.[49,52,53] The *IGF2* gene is part of an imprinted region and is preferentially expressed from the paternally inherited alleles. The result of the allelic loss events and genetic changes in BWS is loss of the nonexpressed maternal allele and maintenance or gain of the expressed paternal allele. In addition to these genetic changes, other alterations of 11p15 involving the *IGF2* locus have been identified in both ARMS and ERMS tumors. In one study of 11 RMS tumors (including 4 ARMS tumors, 6 ERMS tumors, and 1 RMS tumor of uncertain classification), 1 ARMS and 3 ERMS cases demonstrated evidence of duplication of an *IGF2* allele.[163] Furthermore, although *IGF2* is normally expressed from only one allele, loss of imprinting can occur and result in a biallelic expression pattern. In RMS, loss of imprinting at the *IGF2* locus has been observed in 5 of 12 ERMS and 9 of 16 ARMS cases.[163-165] These duplication or imprinting changes increase the number of active *IGF2* alleles and may thereby contribute to the high expression of *IGF2* mRNA and protein found in RMS tumors.

In mammalian cells, the subcellular localization of the wild-type FOXO1 (previously FKHR) and related FOXO3 and FOXO4 proteins is regulated by a PI3K-AKT (PKB-dependent) signaling pathway that is activated by survival- and growth-related signals such as IGF-2.[166] In this signaling pathway, AKT-mediated phosphorylation of FOXO proteins leads to protein transfer from the nucleus to the cytoplasm and thus inactivates transcriptional function. Of the three AKT phosphorylation sites in FOXO1, two are retained in PAX3-FOXO1 and PAX7-FOXO1. If the fusion proteins are regulated by this pathway, the high level of IGF-2 in ARMS cells would cause these proteins to be phosphorylated and sequestered in the cytoplasm. However, experiments show that PAX3-FOXO1 is retained in the nucleus and is transcriptionally active in cultured cells in the presence of active AKT and in ARMS cells.[167] All studies performed to date find that PAX3-FOXO1 is constitutively nuclear, even in the presence of activated AKT.[167,168] However, whereas one transfection study did not find any changes in PAX3-FOXO1 transcriptional activity in the presence of activated AKT, a more recent study of a murine model of ARMS found that AKT hyperactivation results in PAX3-FOXO1 phosphorylation and decreased transcriptional activity. Studies in human ARMS cells are needed to resolve these issues and discern whether AKT activation has any effect on PAX3-FOXO1 function.

Modulation of Growth and Apoptotic Pathways by Fusion Oncoproteins

Gene transfer experiments have investigated specific functions of the PAX3-FOXO1 and PAX7-FOXO1 fusion proteins and suggest that these proteins exert an oncogenic effect through multiple pathways. In initial studies of growth control, transduction of PAX3-FOXO1 into murine NIH 3T3 or chicken embryo fibroblasts resulted in transforming activity, whereas wild-type Pax3 did not transform these cells.[169,170] In a complementary study, a protein consisting of the N-terminal region of PAX3 fused to the KRAB transcriptional repression domain reverted the transforming activity of ARMS cells in culture and suppressed tumor formation of ARMS cells in mice.[171] In mutagenesis analyses, the homeodomain but not the paired box is needed by PAX3-FOXO1 for transformation; thus target genes with paired box binding sites may not be required for cellular transformation. In addition, transforming activity is activated when the VP16 activation domain is substituted for the C-terminal domain of PAX3, suggesting that other activation domains can mimic the effect of the C-terminal FOXO1 domain.[172]

More recent studies indicate that there is an antagonistic balance between transforming activity and growth-suppressive or toxic activity in many cell types in which PAX3-FOXO1 is expressed.[173,174] Transforming activity is optimally exerted at low expression levels of exogenous fusion protein. In contrast, at higher expression levels, comparable to the levels in human ARMS tumors, PAX3-FOXO1 causes cell death or growth suppression in various nontransformed cell lines. Therefore human ARMS cells can tolerate these high “physiologic” expression levels, whereas the non-ARMS cells do not tolerate these higher levels. The hypothesis proposed is that additional genetic alterations are necessary in ARMS to attenuate the toxic and growth-suppressive effects of the fusion protein. One such event may be inactivation of the *CDKN2A* tumor suppressor gene that collaborates with PAX3-FOXO1 to permit primary human skeletal muscle cell precursors to bypass the senescent growth arrest checkpoint.[175] It is noteworthy that mutation studies show that the growth suppressive and toxic activity is at least partly dependent on an intact paired box but does not require an intact homeodomain.[173] These findings suggest that there may be two separate sets of target genes mediating the transforming and toxic phenotypes as a result of the complex function of the PAX3-FOXO1 DNA-binding domain.

Despite the ability of PAX3-FOXO1 to induce toxic effects when introduced into many cultured cells, an important function of the PAX3-FOXO1 oncoprotein in ARMS cells is maintenance of cell viability by inhibiting apoptosis. Treatment of ARMS cells with either an antisense oligonucleotide directed against the *PAX3* translational start site or with siRNAs directed against the 5′ *PAX3* region resulted in a decrease in PAX3-FOXO1 protein expression.[101] This expression change was associated with a significant decrease in cell number as well as with morphologic and biochemical characteristics of apoptosis. Similarly, expression of a tamoxifen-inducible PAX3-KRAB construct in ARMS cells demonstrated evidence of apoptosis when induced in low serum conditions or tumor xenografts.[176] One downstream transcriptional target of PAX3-FOXO1 that at least partially mediates this apoptotic function is the gene that encodes the transcription factor TFAP2B.[101] The functional role of TFAP2B is indicated by the induction of apoptosis in ARMS cells when this gene is downregulated by siRNA and by the prevention of apoptosis mediated by siRNA directed against the 5′ *PAX3* region when a construct constitutively expressing TFAP2B is introduced into ARMS cells.

Metastatic Pathways in Rhabdomyosarcoma

Early studies of RMS metastasis examined subclones of the RD ERMS cell line. The metastatic capacity of different subclones did not correlate with the tumorigenic or proliferative capability of the subclones.[177] After exposure to conditions that stimulate myogenic differentiation, some subclones showed increased differentiation manifested by an increased fraction of myosin-positive cells.[178] Although proliferative capability did not correlate with this in vitro differentiation, the metastatic efficiency was reduced in subclones demonstrating differentiation.

Additional studies with the RD line showed that its metastatic efficiency is affected by alterations of cell surface molecules. In the case of the cell surface glycoprotein NCAM (neural cell adhesion molecule), this protein is posttranslationally modified by the addition of polysialic acid on its extracellular domain.[179] When the enzyme endoneuraminidase-N was injected intraperitoneally to cleave the polysialic acid from the surface of intraperitoneally implanted RD cells, there was a decrease in the frequency of lung and liver metastases. When the integrin VLA-2 (α2β1) that is not normally expressed by RD cells was introduced into these cells, there was increased adhesion to collagen and laminin in vitro and more metastatic foci in mice after either intravenous or subcutaneous injection.[180]

Among the various RMS subtypes, there are differences in matrix metalloproteinase (MMP) expression that may contribute to differences in metastatic behavior. Immunohistochemical analysis of 5 MMPs in 33 RMS cases found higher expression of MMP1, MMP2, and MMP9 in ARMS than in ERMS cases.[181] After confirming the higher MMP2 expression in ARMS in a comparison of ARMS and ERMS cell lines, the high MMP2 expression was correlated with more invasive behavior in the ARMS cell lines.[182] In a human RMS cell line with spontaneous metastatic progression, there was upregulation of MMPs and downregulation of tissue inhibitors of metalloproteinases.[183] Finally, attachment of RMS cells to fibronectin results in increased MMP2 expression and in vitro invasive behavior along with increases in COX-2 expression and prostaglandin E_2 production.[184] Treatment with exogenous prostaglandin E_2 can recapitulate this effect and increase MMP2 expression at the level of the MMP2 promoter. In contrast, treatment of cells with COX-2 inhibitors can reverse the fibronectin effects on MMP expression and invasive behavior. Another soluble factor released by RMS cells is heparanase, which is found upregulated in several human RMS cell lines. Heparanase cleaves heparan sulfates, thus releasing and likely making more bioactive heparan-bound growth factors. shRNA-mediated suppression of heparanase in RD and Rh30 cells (a commonly studied human ARMS cell line) inhibited their invasive properties as assessed by Matrigel invasion assays. Parallel studies examining heparanase mRNA expression in human tumor samples and human serum in order to validate the upregulation were suggestive but not statistically significant because total numbers were small and error bars wide, thus suggesting that further studies will be required.[185]

Three cell surface receptors, MET, CXCR4, and LIFR, are expressed in RMS and function in signaling pathways that impact metastatic behavior[186-188] (Fig. 59-6). Although *MET* and *CXCR4* genes are downstream targets of the PAX3-FOXO1 fusion protein, all three genes are expressed in both ARMS and ERMS tumors. CXCR4 is a G-protein–coupled chemokine receptor whose ligand is stromal derived factor-1 (SDF1), whereas MET is a member of the tyrosine kinase family of receptors whose ligand is hepatocyte growth factor/scatter factor (HGF/SF). Finally, leukemia inhibitory factor (LIF) binds to a heterodimeric membrane receptor composed of the LIF-specific LIFR and the gp130 receptor chain, which is also used as the receptor for interleukin (IL)-6, oncostatin M, and several other cytokines. All three ligands are secreted by the bone marrow, an important site of ARMS metastasis. The CXCR4-SDF1 signaling pathway is involved in the homing of normal cells to hematopoietic sites, and the MET-HGF/SF signaling pathway is involved in the

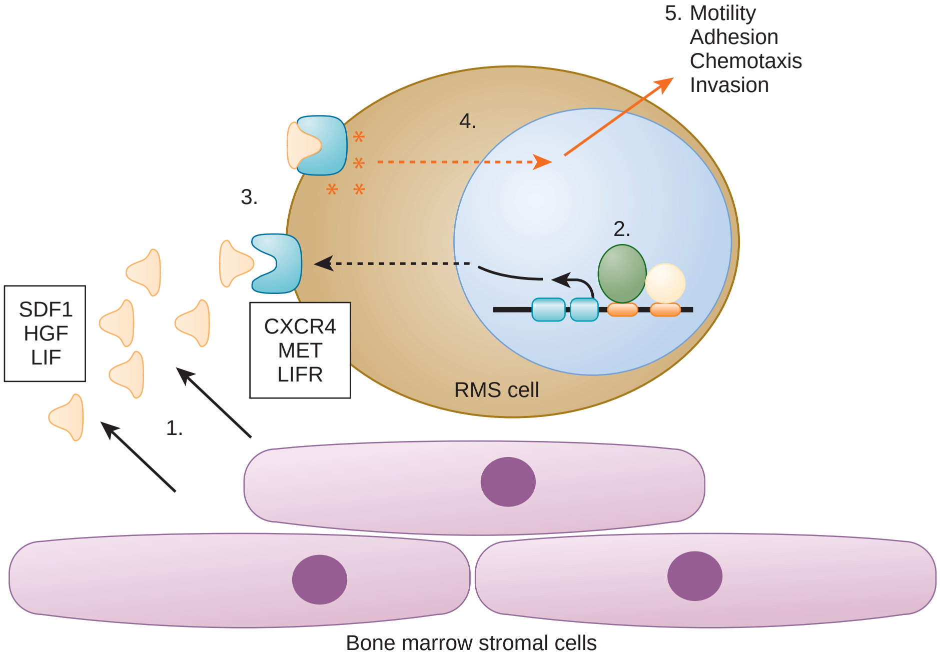

Figure 59-6 Role of secreted protein mediators in metastatic behavior of RMS cells. Bone marrow stromal cells secrete various protein mediators, including SDF1, HGF, and LIF (step 1). The RMS cells transcribe genes and then express cell surface receptors for these protein mediators (step 2). Binding of protein mediators to cognate receptors (step 3) activates various signaling pathways in the RMS cells (step 4). These signaling pathways ultimately modulate expression pathways in the nucleus that result in phenotypic changes (step 5).

proliferation and motility of various cell types. The LIF-LIFR signaling pathway has a variety of roles in multiple tissues, including proliferation of hematopoietic cells and muscle satellite cell production. For these reasons, usurpation of these signaling pathways has been proposed in the metastasis of various cancers.

Cell culture studies have explored the influence of CXCR4-SDF1, MET-HGF/SF, and LIFR-LIF signaling on the metastatic behavior of ARMS cells. SDF1, HGF, or LIF treatment of RMS cell lines induced cell culture changes in relevant properties, including motility, adhesion, chemotaxis, and invasion. In an in vivo experiment, more ARMS cells than ERMS cells are chemoattracted to and seed lethally irradiated bone marrow in association with the upregulation of HGF and SDF1 in irradiated bone marrow stroma. In addition, a selected ARMS subclone that preferentially responds to LIF demonstrates increased seeding of bone marrow, liver, and lymph nodes after intravenous injection and increased cells in bone marrow and lung 6 weeks after intramuscular injection. Recently, another receptor for SDF-1 called CXCR7 has been identified. This receptor binds not only SDF-1 but also interferon-inducible T-cell α chemoattractant (ITAC) chemokine. It is expressed in both ARMS and ERMS cells and appears to be independent from CXCR4, suggesting that CXCR4-SDF-1 axis targeting alone may not be enough.[189] Finally, as models of pathway-specific potential therapeutic strategies, a CXCR4-specific inhibitor blocked SDF1-directed adhesion and chemotaxis in ARMS cells, an siRNA directed against LIFR inhibited in vivo metastasis of ARMS cells, and suppression of MET receptor by shRNA in RMS cells decreased the migratory response in vitro.[190]

Recently, three additional cell surface receptors including IL-4 receptor-α (IL-4Rα), FGFR4 (discussed in "Oncogene and Tumor Suppressor Gene Mutations in Rhabdomyosarcoma"), and cannabinoid receptor 1 (CNR1) have been implicated in RMS metastasis. IL-4Rα is found upregulated in human and murine ARMS, and blockade by a neutralizing antibody inhibited both lymphatic and hematogenous metastasis in a genetically engineered murine model of ARMS.[191] Because there are neutralizing antibodies to IL-4R (and cognate ligands IL-4 and IL-13) in clinical use for nononcologic human diseases, this may be a feasible approach toward blocking RMS metastasis. FGFR4 gain of function (K535 and E550 tyrosine kinase domain) mutants enhanced the metastatic phenotype when expressed in both murine RMS cell lines and NIH 3T3 cells, as assessed by lung metastasis burden in xenograft assays.[75] Lastly, CNR1, which is also a PAX3-FOXO1 target, was shown to be dispensable for PAX3-FOXO1–driven cell proliferation and transformation but required for invasion and metastasis. In transformed primary mouse myoblasts expressing PAX3-FOXO1, either genetic or pharmacologic suppression of CNR1 reduced cell invasion in vitro and lung metastasis in vivo.[192]

Studies of a murine metastatic RMS model system elucidated an important pathway involving the *Vil2* (encoding Ezrin) and *Six1* genes. In this model system, RMS tumors developed in transgenic mice that were deficient in *CDKN2A (Ink4/Arf)* and overexpressed *HGF/SF*.[193] In a comparison of gene expression profiles of highly and poorly metastatic cell lines derived from this system, 44 differentially expressed genes were identified (28 overexpressed and 16 underexpressed in highly metastatic cells), including the overexpressed genes *Vil2* and *Six1*.[194] Ezrin is an adhesion molecule and member of the ERM family that promotes cytoskeletal reorganization in pathways linked to survival, motility, invasion, and adherence. Six1 is a homeodomain-containing transcription factor involved in the development of several lineages, is a downstream target of PAX3-FOXO1 in NIH 3T3 cells, and is expressed by ARMS cells.[130] Furthermore, the gene encoding Ezrin is a direct transcriptional target of Six1.[195] These gene expression relationships were confirmed by RMS tumor studies in which both genes were generally expressed at higher levels in fusion-positive RMS tumors than in fusion-negative RMS tumors.

Expression of *Vil2* and *Six1* in RMS tumors was correlated with clinical stage, which is consistent with a role in tumor progression and metastatic behavior.[194] There is direct proof of this role: transfer of either gene into low metastatic RMS lines from the HGF transgenic system caused increased metastatic activity. Similarly, when expression of either protein was suppressed by shRNA or repressed by a dominant-negative inhibitor, metastatic activity in the highly metastatic lines decreased. Finally, Ezrin is needed for Six1 to exert its metastatic function, thus further confirming that Ezrin acts downstream of Six1.[195] These studies provide the framework of a pathway that leads from the fusion proteins to Six1 to Ezrin to metastatic activity.

There are surely additional genes and proteins supporting the metastatic phenotype in RMS, and to this end a European collaborative group evaluated in RMS the expression of genes known to support metastasis using an Affymetrix approach.[196] After examining 19 primary human RMS tumor samples, they focused on two genes known as *LMO4* and *FOFOXF1*. Although they did not evaluate for expression of the protein products of these genes in RMS samples, they did assess their role in metastasis by showing that siRNA suppression of these genes in RD and Rh30 cells decreased migration as assessed by Matrigel invasion.

PRECLINICAL INVESTIGATION OF EMERGING DRUG TARGETS FOR RHABDOMYOSARCOMA

Preclinical Models to Study Rhabdomyosarcoma Tumorigenesis

Human Tumor Cell Xenografts in Immunodeficient Mice

Although the identification of mutations and/or dysregulated signaling in RMS is accelerating, the value of these entities as therapeutic targets must be evaluated in preclinical models before application in humans. The original models to study cancer treatments other than in cell lines were xenograft models, in which human cell lines were injected as xenografts into immunocompromised mice. Historically for RMS this began at St. Jude's Research Children's Hospital in the 1980s and has evolved into the Pediatric Preclinical Testing Program (http://pptp.nchresearch.org/). This program is a large-scale effort to systematically evaluate new agents against a panel of human RMS and other pediatric cancer cell lines by testing novel drugs against not only classic oncogene and tumor suppressor pathways including TP53 and receptor tyrosine kinases (RTKs) but also against newly identified targets found dysregulated in childhood cancer. Recently, the feasibility of adding radiation therapy to the Pediatric Preclinical Testing Program was demonstrated, using RMS as a pilot.[197]

There are obvious advantages to examining the efficacy of novel agents against pediatric tumor cell lines, including that they are of human origin and have been validated in prior biologic studies. But there are disadvantages as well, including that many cell lines have been passaged in culture for decades, acquiring genetic changes not representative of the original tumor. There is also the practical problem of cell line annotation[198]; however, with the development of methods to fingerprint cell lines, identification has been more reliable. Finally, the intrinsic property that enables evaluation of human cell lines in a whole murine organism— their immunodeficient state—does not permit the evaluation of the role of the immune system in tumorigenesis, a problem that can be overcome by the use of genetically engineered mouse models.

Genetically Engineered Murine Models of RMS

With the advent of genetic manipulation of the murine genome, it is now possible to examine the genesis and pathophysiology of RMS at the organism level. Indeed there are more than two dozen genetically engineered murine models that give rise to RMS. The earliest models were serendipitously discovered, for example that ERMS occurs in TP53 knockout mice or SV40 transgenic mice. Later models were deliberate in their design and incorporated genetic lesions found in RMS, including overexpression of the cMET signaling pathway or *PAX3-FOXO1* fusion gene. These and others are reviewed elsewhere.[199] More recently, sophisticated models have emerged in which Cre-Lox (or Flp-Frt) recombinase technology has been harnessed to turn on or turn off expression of specific genes or negative regulatory components such as shRNAs. This has been especially useful in examining the cells of origin of RMS, which may range from an undifferentiated mesenchymal stem cell to the myonucleus of a differentiated myofiber.[200] These approaches have led to some surprising discoveries, such as the finding that RMS can arise from the adipocyte lineage[201] and that ERMS is on a continuum with a common adult sarcoma known as undifferentiated pleomorphic sarcoma.[202] Last, these genetically engineered mouse models have also been useful as additional models for preclinical studies, such as the *PAX3-FOXO1*–based ARMS model.[203]

Lower Eukaryote Models of RMS

Although murine models have been the mainstay of modeling human cancer including RMS, lower organisms including zebrafish *(Danio rerio)* and the fruitfly *(Drosophila melanogaster)* are more amenable to genetic manipulation and screening and contribute a unique understanding of RMS genesis and metastasis. For example, zebrafish models of ERMS[204] have been useful for in vivo imaging of tumor-propagating cells and their properties[205] and understanding the impact of oncogenic RAS expression at different stages of muscle development.[206] *Drosophila* models of ARMS have provided insight into the pathophysiology of *PAX-FOXO1* fusion genes[207] and the role of defective myoblast fusion in RMS.[208]

A comprehensive listing of RMS animal models and their utility is available.[199] Along with the xenograft and genetically engineered mouse models, these models have enabled testing of pharmacologic agents in a preclinical fashion before to moving to phase I clinical trials. Below are listed some examples of agents that have been tested in a variety of in vivo preclinical models.

Inhibitors of Receptor Tyrosine Kinases

RTKs are embedded in the cellular plasma membrane and transmit signals from the environment to the cell interior. When a ligand binds to an RTK, dimerization occurs and this leads to internal phosphorylation events and signaling through a variety of canonical intracellular signaling pathways such as AKT, MAPK, mammalian target of rapamycin (mTOR), and many others. Because RTKs are at the cell surface, they can be blocked with interfering antibodies (or their ligands can be inhibited with neutralizing antibodies). Given the expansion in our understanding of the RTKs that are dysregulated or upregulated in RMS,[209] there are several monoclonal antibodies that have been evaluated in RMS. The prototype was a monoclonal targeting IGFR-1, known as figitumumab (previously CP-751871).[210] This agent not only blocked binding of ligand to the IGFR-1 but also downregulated the receptor, suppressed signaling to AKT, inhibited tumor-derived VEGF, and synergized with rapamycin. Several other studies followed, including testing of other anti–IGFR-1 monoclonal antibodies,[211,212] some of which advanced to clinical trials. However, tumor cells were able to activate bypass routes, rendering resistance to IGFR-1 blockade. Bevacizumab, a monoclonal antibody to VEGF, has been evaluated in a phase II clinical trial for RMS (see "Clinical Research in Rhabdomyosarcoma"). Still in development and preclinical testing are monoclonal antibodies to FGFR4[213] and NCAM,[214] both upregulated in a significant percentage of RMS tumors and implicated in RMS metastasis (see "Metastatic Pathways in Rhabdomyosarcoma"). Other new agents are also being considered for RMS.[215]

Given their enzymatic activity, RTKs can also be inhibited with small molecules. In a manner similar to the prototype small molecule imatinib, which was developed to inhibit the BCR-ABL fusion protein in chronic myelogenous leukemia,[216] there are also small molecule inhibitors being evaluated for RMS-associated RTKs. An early example was BMS-754807, an oral small molecule inhibitor of IGFR-1,[217] which showed intermediate efficacy in RMS. Later examples include sunitinib, cediranib, and pazopanib, which are small molecules that inhibit the kinase activities of platelet-derived growth factor receptor (PDGFR) and other RTKs, VEGF receptors 1 to

3 (VEGFR1 to VEGFR3), and VEGFR1 to VEGFR3 and other RTKs, respectively.[209] Given the known resistance that emerges with RTK monotherapy, these small molecules are now being tested in conjunction with traditional chemotherapeutic agents. For example, pazopanib has been tested in combination with topotecan, and in Rh30 xenografts this combination inhibits tumorigenesis.[218] Indeed, this has supported the incorporation of pazopanib into human clinical trials (see "Clinical Research in Rhabdomyosarcoma"). Whereas the upregulation of a specific RTK in RMS warrants investigation of that RTK as a drug target, not all RTK inhibitors have efficacy. For example, although EGFR is found upregulated in some RMS tumors, erlotinib (an EGFR inhibitor) had no effect on blocking tumorigenesis in a genetically engineered mouse model of ARMS.[219]

In addition to inhibiting RTKs, whether through monoclonal antibodies or small molecules, there are also efforts to inhibit heparanases (discussed in "Metastatic Pathways in Rhabdomyosarcoma"). Pharmacologic inhibition of heparanases using SST0001, a chemically modified heparin, in RD and Rh30 xenografts showed inhibition of tumor growth. The precise mechanism is not clear, but there was evidence of decreased angiogenic factors secreted from the RMS cell lines in the in vitro studies.[220]

Inhibitors of Intracellular Signaling Pathways

Regarding downstream signaling pathways, it is clear that blockade of only one pathway is not durably antitumorigenic because of compensatory upregulation of other pathways. Therefore there are ongoing efforts to simultaneous block two or more pathways. Dual blockade of the PI3K/AKT/mTOR and RAS/MEK/ERK pathways with small molecules synergistically inhibits RMS cell growth in vivo.[221] Similarly, treatment of RMS xenografts with rapamycin was shown to block tumor growth by inhibiting not only the mTOR pathway but also Hedgehog signaling,[222] and indeed rapamycin analogues are in human clinical trials (see "Clinical Research in Rhabdomyosarcoma"). Attesting to the power of utilizing lower eukaryotes for target discovery, a screen performed in an oncogenic RAS-driven model of zebrafish RMS identified dual MEK and mTOR/S6K1 inhibition as effective.[223]

Finally, there is RAS itself, which is found mutated in a significant portion of ERMS tumors (see previous section, "Oncogene and Tumor Suppressor Gene Mutations in Rhabdomyosarcoma"). Targeting the RAS protein has proven difficult. However, silencing of Sprouty1, an upstream modulator of RAS that is also found in ERMS tumors and associated with upregulated ERK signaling, using shRNA triggers complete regression of ERMS xenograft tumors carrying a mutated oncogenic *RAS* gene,[224] suggesting that there will be alternate ways to block RAS signaling in the future.

Inhibitors of Developmental Pathways

Pharmacologic blockade of RTK signaling by small molecule inhibitors became possible because of a deep structural understanding of their enzymatic activity. As the role of the evolutionarily conserved developmental pathways (e.g., Hedgehog, Notch, Wnt, transforming growth factor-β [TGF-β]) in RMS tumorigenesis has become apparent,[225] it has become similarly imperative to understand the molecular basis for their dysregulation. For the most part, it appears that in RMS these pathways, which during normal development and tissue homeostasis direct myogenesis by promoting myogenic stem cell maintenance, expansion, and tight control of differentiation, are usurped and prevent proper myogenic lineage progression. In this regard, agents developed to inhibit members of these pathways must be evaluated for unanticipated effects on normal tissue, especially considering the continual growth and development of pediatric patients. Studies in *Ptch* mutant mice, which through increased Hedgehog signaling develop both medulloblastoma and RMS, showed that combined dosing with the epigenetic drugs 5-aza-2′-deoxycytidine and valproic acid inhibited tumor formation when treated early.[226] In human RMS xenografts, treatment with betulinic acid (a naturally occurring plant product), GANT61 (a Gli antagonist), or forskolin (also derived from a naturally occurring plant product) suppressed Hedgehog signaling and RMS tumor growth.[227-229] Recently, several additional small molecule inhibitors of Smoothened and Gli have been developed, raising the expectation that blockade of Hedgehog signaling will be possible in human cancers.[230]

Regarding Notch signaling, given its critical role in normal muscle development and the upregulation of many components of this pathway in RMS, this pathway has gained attention, and several Notch pathway genes and proteins have been suppressed using genetic (shRNA) or pharmacologic (γ-secretase inhibitors) approaches and when suppressed shown to inhibit the growth of RMS xenografts, including Notch1, Notch3, RBPJ, and Hey1.[231-233] Although as important in skeletal muscle development as Notch, the Wnt pathway is not as well understood in the pathobiology of RMS and therefore in vivo preclinical studies using genetic or pharmacologic approaches to target the Wnt pathway are lagging. Finally, regarding TGF-β signaling, this pathway, too, is under investigation in RMS. However, recently a miR that is suppressed by TGFB1 known as miR 450b-5p, when overexpressed in RMS cells inhibits cell growth in vitro and tumor xenografts in vivo and promotes myogenic differentiation.[234]

Inhibitors of Other Cellular Processes That Support Rhabdomyosarcoma Tumorigenesis

Apoptosis

In addition to specific signal transduction pathways, there are efforts to target specific cellular processes. For example, as described earlier, RMS cells can resist apoptosis (programmed cell death) through upregulation of anti-apoptotic proteins or the suppression of pro-apoptotic proteins. Therapeutic approaches therefore may include inhibiting anti-apoptotic signaling, for example through neutralizing Bcl-2 family proteins (although caution must be taken because some Bcl-2 family members have pro-survival roles), or activating pro-apoptotic signaling, for example through stimulation of the extrinsic apoptotic pathway via members of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors.[235] Drozitumab is a monoclonal antibody to death receptor 5 that supports assembly of death-induced signaling complexes and has shown efficacy in RMS xenografts.[236] It may also be possible to reestablish normal function of mutated pro-apoptotic pathways. For example, in ARMS xenografts the MDM2 inhibitor RG7112 interferes with MDM2-TP53 binding, releasing TP53 from negative control and permitting its normal cell surveillance function.[237] Finally, although apoptosis has historically been the major focus of inducing cell death, the process of necroptosis (nonapoptotic cell death) has been found to be induced in RMS cells via obatoclax, which inhibits BCL2.[238]

Although involved not only in avoidance of apoptosis but also many other pro-tumorigenic processes, it is important to note that *MYCN* has been proven targetable through the use of antigene therapy in which a peptide nucleic acid oligonucleotide (tethered to a nuclear localization signal peptide and targeted against the antisense strand of *MYCN* exon 2) was delivered to ARMS cells. This antigene therapy caused a decrease in proliferation and an increase in apoptosis in vitro and eliminated 75% of tumors in Rh30 RMS xenografts, with 25% showing a reduction in tumor.[239]

Cell Cycle

Although apoptosis is a cellular event that can be triggered by many distinct stimuli, direct manipulation of the cell cycle and its checkpoints also provides opportunities for pharmacologic manipulation and treatment of RMS. Of long-standing interest are antimitotic agents, which interfere with transit through mitosis, including spindle assembly, chromosome positioning, and chromosome separation. Classic chemotherapy agents such as vincristine, which is a backbone of RMS therapy, interfere with the mitotic spindle by binding tubulin. Indeed, a more potent synthetic antitubulin agent, eribulin, which is distinct from the *Vinca* alkaloids in how it binds tubulin, has shown some complete responses in RMS xenografts.[240] More recently, kinases and kinesin motor proteins that control mitosis are now able to be pharmacologically targeted and agents blocking these proteins are being evaluated against human cancer panels, including RMS. The agents MLN8237 and B16727, Aurora A, and Pololike kinase inhibitors, respectively, have both demonstrated some response in RMS xenograft studies.[241,242] The kinesin spindle protein inhibitor ispinesib was toxic and ineffective in inhibiting RMS xenograft growth,[243] whereas the CENP-E kinesin inhibitor GSK923295A showed some efficacy.[244]

Regulation of Protein Stability and Turnover

The heat shock and ubiquitin-proteasome systems, which contribute to homeostasis of protein stability and turnover, have also been mined for their potential as anticancer targets. 17-DMAG, a first-in-class derivative of geldanamycin that inhibits Hsp90, showed some antitumor activity in xenograft models of ARMS,[245] whereas a non-geldanamycin Hsp90 inhibitor, AT13387, showed no objective tumor responses.[246] Bortezomib, which is approved by the U.S. Food and Drug Administration (FDA) for the treatment of several human diseases, showed activity in ERMS and ARMS xenografts in vivo.[247] MLN4924, a first-in-class NEDD8-activating enzyme inhibitor that affects the ubiquitin-proteosome pathway also showed efficacy in RMS.[248]

Epigenetic Modifiers

Currently there are two groups of agents under study that qualify as epigenetic modifiers. The first group of agents is composed of histone deacetylase (HDAC) inhibitors. Agents in the second group inhibit DNA methylation and include 5-aza-2′-deoxycytidine. Although results of early in vitro studies were promising,[249,250] there are few in vivo studies demonstrating inhibition of RMS tumorigenesis in response to epigenetic modifiers. As mentioned earlier, combined dosing with the epigenetic drugs 5-aza-2′-deoxycytidine and valproic acid, which target Dnmt and HDAC, respectively, in a genetically engineered mouse model of *Ptch*-mutant mice inhibited tumor formation, but only when the mice were treated early during tumorigenesis.[226] Additionally, drugs that target proteins involved in epigenetic regulation can have unanticipated protumorigenic effects, as in the case of the HDAC inhibitor trichostatin A and DNA demethylating agent (5-Aza), which reactivated the expression of Ezrin, a known prometastatic protein in RMS.[251]

Immunotherapy for Rhabdomyosarcoma

In addition to pharmacologic agents, there are efforts to stimulate or harness the immune system to target and kill RMS tumor cells. However, like most other cancers, RMS is remarkably successful at evading the immune system. One approach to overcome this evasion is isolation and expansion of natural killer cells obtained from normal healthy donors, which can kill human sarcoma cells in vitro and Ewing sarcoma xenografts in vivo.[252] Recently, IL-15 cytokine–induced killer cells from donors were shown to be active in xenograft models of RMS.[253] Another novel approach is chimeric antigen receptor therapy, which although still in early phases has been effective in the treatment of leukemias. Thus there is hope that this approach can be similarly used in solid tumors. To prepare for these efforts, a recent study identified candidate cell surface proteins on pediatric tumors including RMS that could be future therapeutic targets for immunotherapy.[254] Finally, there are ongoing efforts to develop vaccines to solid tumors including RMS. Given the specific expression of the PAX3-FOXO1 fusion protein in ARMS, an epitope-enhanced peptide bearing the PAX3-FOXO1 breakpoint was designed and generated. Because this peptide represents a neoantigen exclusively found in ARMS, the immune system should recognize it as such; and, indeed, dendritic cells pulsed with this peptide generated a CTL line that lysed human ARMS tumor cells in vitro.[255] Similar proposals have been made to use the fetal acetylcholine receptor and other antigens for RMS immunotherapy.[256,257]

CLINICAL RESEARCH ON RHABDOMYOSARCOMA

RMS may be the most clinically complex tumor in pediatric oncology. In addition to its histologic and biologic diversity, RMS has a remarkable variety of sites and clinical behaviors.

Much of our clinical knowledge of RMS has come from well-organized multiinstitutional clinical trials in North America and Europe. In the United States and Canada (and later Australia, New Zealand, and Switzerland) the first Intergroup Rhabdomyosarcoma Study (IRS-I) opened in 1972 and closed in 1978, recruiting patients from the pediatric division of the Southwest Oncology Group (SWOG) and the Children's Cancer Study Group (CCSG). There were five further IRS studies (II, III, IV-pilot, IV, and V), during which the pediatric division of SWOG became the Pediatric Oncology Group (POG) and the CCSG became the Children's Cancer Group (CCG). Finally, the IRS, POG, CCG, and the National Wilms Tumor Study merged to form the Children's Oncology Group (COG), and that group's Soft Tissue Sarcoma Committee completed the IRS-V studies before launching its own ARST series. Publications from the first generation of COG RMS studies (ARST series) are beginning to appear.

Meanwhile, in Europe, the International Society of Pediatric Oncology (SIOP) has conducted a series of Malignant Mesenchymal Tumor studies (SIOP 75 and MMT-84, MMT-89, and MMT-95). The German CWS *(Cooperative Weichteilsarkom Studie)* conducted four trials, CWS 81, 86, 91, and 96. The Italian Rhabdomyosarcoma Group had two clinical trials, RMS 79 and 88, before joining the CWS studies. Limited patient numbers made randomized controlled trials difficult, so the Europeans joined to form the European Paediatric Soft Tissue Sarcoma Study Group (EpSSG), which has launched a randomized controlled trial for patients with localized RMS (RMS-2005). Unfortunately, the loss of Germany for regulatory reasons has slowed accrual, and the results of many of the earlier European studies have never been published.

INCIDENCE AND EPIDEMIOLOGY

RMS accounts for about half of the soft tissue sarcomas in children and adolescents and about 3% of childhood cancer cases. This varies with age, so that they are 60% of soft tissue sarcomas in patients aged younger than 5 years and 23% in the 15- to 19-year age group.[258] They occurred in 4.6 children per million per year in 1996 to 2003 according to the Surveillance, Epidemiology and End Results (SEER) database,[259] and in the period 1973 to 2005 the incidence increased at a mean annual rate of 0.58%.[260] The highest incidence (8.4 per million children) is in the 1- to 4-year age group, after which the incidence is stable at about 4 per million children per year. SEER data show that the incidence of ERMS peaks at about age 3 and then declines until about age 10, whereas the incidence of ARMS is fairly constant from birth to age 19.[261] Most studies report a slight male preponderance (about 5:4); recent analyses of SEER data found no significant differences in incidence by race[260] or a slightly higher incidence in black children.[261]

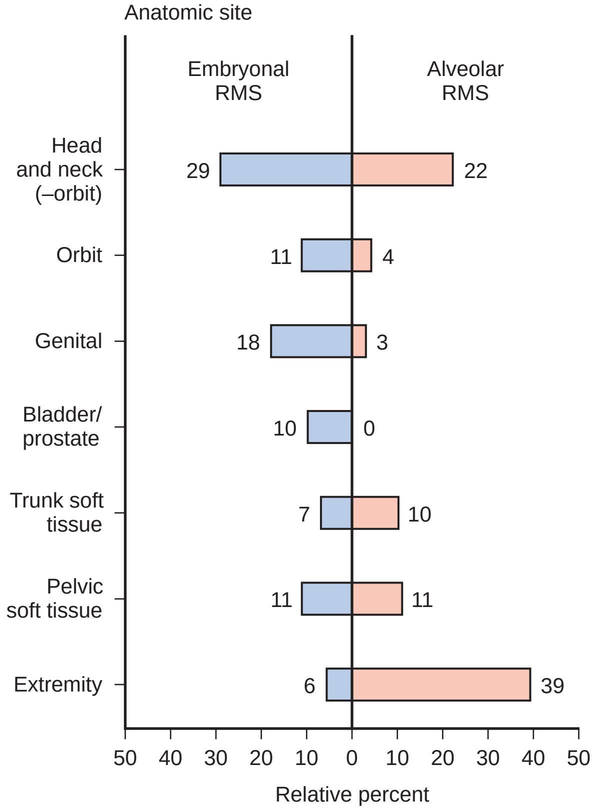

Figure 59-7 Primary sites of embryonal and alveolar RMS, percent of distribution by histology. *(From Gurney JG, Young JL, Roffers SD, et al:* Soft Tissue Sarcomas, SEER Pediatric Monograph, *Bethesda, Md., 1999, National Cancer Institute, p. 116.)*

CLINICAL PRESENTATION AND EVALUATION

RMS occurs in virtually all parts of the body outside the central nervous system (although they may involve it). ERMS often occurs in areas where there is very little skeletal muscle, such as the orbit, pharynx, sinuses, vaginal wall and paratesticular tissues. ARMS has a greater tendency to occur where there is skeletal muscle, such as in the trunk and limbs (Fig. 59-7).

The clinical presentations of either histology are myriad and vary with the primary site. For example, tumors of the parameningeal region (e.g., the sinuses and pharynx) may have cranial nerve deficits as initial symptoms, orbital tumors often produce proptosis and diplopia, and tumors of the bladder base and prostate usually cause urinary retention. Often, however, the tumor is a painless mass without associated symptoms. Usually it is hard or firm, smooth, fixed to surrounding tissues, and not tender. There may be palpable regional lymphadenopathy and, occasionally (with alveolar histology tumors), distant involved nodes and metastases to the skeleton or marrow.

The initial evaluation of a patient with a suspected RMS has three components: the evaluation of the extent and anatomic relationships of the primary tumor; the

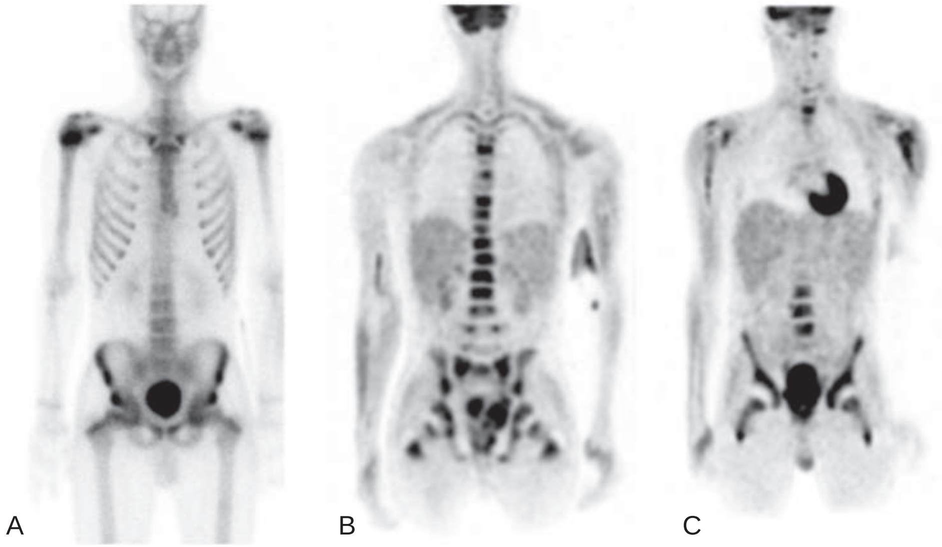

Figure 59-8 Metastatic alveolar rhabdomyosarcoma. Radionuclide bone scan (**A**) and two images from a tomographic coronal PET scan (**B** and **C**). The abnormalities in the bone scan are few and subtle; the PET scan clearly shows multiple skeletal metastases.

detection of metastases; and the identification of other problems that may be related to the tumor (e.g., a cranial nerve palsy or urinary retention) or that are unrelated but could affect therapy (e.g., a cancer predisposition syndrome).

The most appropriate time for referral to a pediatric oncology center is at the first suspicion of a malignancy. Experienced radiologists should perform the imaging of the primary tumor before a biopsy has produced local hemorrhage and edema, using equipment that can share images with computerized radiation therapy planning systems. If possible, the surgeon who will perform the definitive excision of the tumor should perform, or at least supervise, the diagnostic biopsy of a suspected sarcoma. Violation of tissue planes or neurovascular structures with inexpertly placed biopsy tracts (whether open or closed) may limit excision and reconstruction options, prevent limb salvage procedures, or increase the chances of local recurrence. Because sarcomas are often very heterogeneous and contain large areas of necrosis and because molecular techniques are increasingly important in diagnosis, generous fresh specimens are important, with frozen section confirmation of specimen adequacy before the procedure is declared complete. Many passes of the needle (10 or more) are usually required if closed techniques are used. Fine-needle aspirates do not provide the tissue architectural information currently necessary for pediatric sarcoma diagnosis or sufficient tissue for advanced diagnostic techniques.

For evaluation of the primary tumor, the current standard of care is magnetic resonance imaging (MRI) with contrast enhancement, encompassing the primary tumor and regional lymph nodes. Traditionally, patients have also undergone a spiral chest computed tomographic (CT) scan without administration of a contrast agent (although contrast enhancement is necessary to find hilar or mediastinal adenopathy or to distinguish a mass from a surrounding pleural effusion) and a radionuclide bone scan to detect skeletal metastases, as well as bilateral bone marrow aspirates and biopsies. However, a recent analysis indicates that these studies are unnecessary in patients with noninvasive tumors without regional node involvement (T1N0), which are about one third of cases.[262]

The use of fluorodeoxyglucose-18–labeled positron emission tomography (FDG-PET) is particularly promising for the detection of involved regional nodes and distant metastases[263,264] and skeletal and marrow disease not revealed by conventional techniques (Fig. 59-8). The combination of PET and CT (PET/CT) can help discern the extent of tumor versus peritumoral edema or whether a remote mass is a tumor. A retrospective study of 23 RMS patients showed that side-by-side evaluation of PET/CT and conventional imaging (primary site MRI, bone scintigraphy, marrow biopsy, chest CT) provided more accurate staging than either conventional imaging or PET/CT alone.[265] A larger single-institution study of 30 pediatric RMS patients retrospectively compared the findings of conventional imaging (MRI, chest CT, bone scintigraphy), marrow aspirates and biopsies, biopsies of selected possible metastases, and PET/CT. For regional nodes, PET/CT was equally sensitive to conventional imaging but much more specific (95% v. 14%); for bone and marrow disease PET/CT was more sensitive (detecting 4 of 4 patients with bone or marrow disease, versus 1 of 4 with bone scintigraphy); and PET/CT detected soft tissue metastases not visible on conventional imaging in 2 patients. Chest CT revealed more nodules than PET/CT (seven nodules in 6 patients vs. one nodule in 1 patient).[266] A retrospective comparison of PET/CT and conventional imaging in 35 pediatric and adult RMS patients (two thirds with alveolar histology) similarly found PET/CT more sensitive and specific for both regional node and metastatic assessment.[267] The potential role of PET/CT in assessing response to therapy will be discussed later.

Whole-body MRI for detection of metastasis was tested in several single-institution studies. In particular, a large single-institution study compared whole-body MRI, FDG-PET, and bone scintigraphy in the detection of skeletal metastases in 39 pediatric patients with a variety of tumors (including Ewing sarcoma, RMS, osteosarcoma, lymphoma, melanoma, and Langerhans cell histiocytosis), with biopsy of all detected lesions for histologic confirmation. PET had the highest sensitivity (90%), followed by whole-body MRI (82%), and bone scintigraphy (71%), although all three modalities had both false-positive and false-negative results that varied with anatomic location (for example, the high glucose uptake in the brain made PET relatively insensitive in the skull). The greatest sensitivity (96%) came from combining any two imaging modalities.[268] A recent large multicenter study enrolled a cohort of 188 children with soft tissue sarcoma, lymphoma, or neuroblastoma. With a complex system of central review, the investigators compared the findings of whole-body MRI with those of CT and disease-appropriate scintigraphy (metaiodobenzylguanidine, gallium, or [in some patients] FDG-PET). Whole-body MRI detected more skeletal metastases per patient, but this did not change the staging of any patient's tumor; and it was less sensitive than conventional imaging in detecting liver and lung metastases. The investigators concluded that whole-body MRI "is not justified" as "the initial study of choice" for staging pediatric cancers.[269]

RISK GROUP CLASSIFICATION

RMS varies tremendously in prognosis, from almost 100% survival (for orbital embryonal tumors) to virtually no prospect of survival (for disseminated alveolar tumors). There has been a variety of schemes for classifying RMS to try to match treatment intensity to prognosis. Some of the schemes are prospective, relying on tumor characteristics before any therapy, whereas others are dynamic, subject to change based on response to surgery or chemotherapy. We will consider individual prognostic variables and then the ways in which they have been combined.

Histology and Fusion Status

Patients with ARMS are less likely to survive than patients with ERMS, even if all other factors are equal. Alveolar tumors are also more likely to be regionally or metastatically disseminated at the time of diagnosis and are more likely to be invasive (Table 59-5) The difficulties in classifying tumors with alveolar histology that lack a *PAX-FOXO1* gene fusion and the differences between alveolar tumors with fusions involving *PAX3* and *PAX7* have already been discussed. So far, histology has taken precedence over fusion status, but this appears to be changing with accumulating evidence that fusion status is the key determinant of clinical behavior.[270,271]

TABLE 59-5 Clinical Features of Embryonal and Alveolar Rhabdomyosarcoma

	Embryonal Tumors	Alveolar Tumors
Age	Younger	Older
Location	Central, not muscular	Peripheral, muscular
Invasiveness	Low/moderate	High
Metastases	Lung	Everywhere
Prognoses	40% to 90% + PFS	0 to 60% PFS

PFS, Progression-free survival.

Site

The clinical behavior and prognosis of RMS varies greatly with the site of the primary tumor, independently of histology. For example, an ERMS of the orbit is almost always curable whereas an ERMS arising in the ethmoid sinus, just a few centimeters away, carries about 25% mortality despite more intense therapy. The favorable sites for RMS are the orbit, superficial head and neck, biliary tree, vagina, and paratestis; all other sites are considered unfavorable. The biologic basis for this variability remains mysterious, with many different hypotheses (size at diagnosis, access to lymphatics, ploidy, and many others) having been explored and discarded.

Age

The prognosis for patients with RMS is best in preschool and middle childhood and worse for infants, adolescents, and adults. An analysis of data from the IRS-III, IRS-IV pilot, and IRS-IV (spanning 1984 through 1997) showed that when infants younger than 1 year of age are assigned a risk of treatment failure of 1.0, the relative risk for older children is 0.4 to 0.6 and that for adolescents (≥10 years) is 0.8.[272] Although other prognostic variables such as histology, nodal status, stage, site, and clinical group differed between the three age groups, the impact of age remained when all other factors were controlled. A more recent analysis of infants enrolled in the IRS-IV and COG D9602 and D9803 studies largely confirmed these findings and noted that the outcome for infants with group III tumors (gross residual disease at the beginning of chemotherapy) have a particularly poor outcome, perhaps related to fewer than half receiving protocol-assigned radiation therapy. Overall, FFS at 5 years was 57%, 81%, and 67% for patients aged younger than 1, 1 to 9 years, and 10 years and older, respectively.[273] Prognosis varies with age gradually, however, so the 1- and 10-year boundaries are somewhat arbitrary.

Adults with RMS have a much worse prognosis than children and adolescents. The SEER registry analyzed the records of 2600 RMS patients diagnosed from 1973 to 2005. The OS rate of patients younger than age 19 was 61% at 5 years versus 27% for patients older than 19. Hazard ratios for death increased steadily with increasing age past 19 years; on multivariate analysis the other significant independent variables were histology, primary site, and extent of tumor (local, regional, metastatic).[274] A multicenter study of 169 pediatric and adult RMS diagnosed between 1977 and 2009 (ages birth to 73 years) identified through the Dutch National Histological Database System considered a variety of tumor, host, and treatment variables, many of which were significantly associated with outcome in univariate analysis. On multivariate analysis, increasing age was the most important factor influencing relapse-free survival (RFS), with histology the only other factor retaining significance.[275]

Invasiveness and Size (Tumor Stage)

Invasive tumors, those that cross tissue planes or extend beyond the organ of origin (designated T2 in the TNM system), have a worse prognosis that noninvasive tumors (T1). Similarly, patients with tumors 5 cm in diameter or larger (Tb) have a worse prognosis than those with tumors less than 5 cm (Ta). The International Rhabdomyosarcoma Workshop pooled data from four studies conducted in the late 1970s and 1980s (IRS-II, CWS-81, SIOP RMS 75, and the Italian Cooperative Group study) to examine prognostic factors in 951 patients with localized disease. In multivariate analysis, site and invasiveness were the surviving factors.[276] Histology was not among the variables considered, but it is closely related to site and invasiveness. European studies have used invasiveness for risk classification, but North American clinical trials have not, largely because a definition that would be usable in a large cooperative group has been elusive.

Size is highly correlated with invasiveness in multivariate analyses[276] and is much simpler to measure. The current COG risk classification uses stage, which depends on both site and size (Table 59-6).

Clinical Group

The extent of tumor when chemotherapy begins determines the clinical group (see Table 59-6). It is not a true staging system, because it depends on the initial surgery; stage and group only overlap in patients with metastases (stage 4, group IV). IRS-I showed clinical group to be a powerful prognostic variable, but it is a complex one: site, invasiveness, the aggressiveness of the surgeon, the attitude of the family toward mutilating surgery, and the confidence of the treating team in chemotherapy and radiation therapy can all influence whether a tumor is excised with negative margins at diagnosis (group I) or only sampled (group III).

TABLE 59-6 Comparison of IRS Clinical Group and Stage for Rhabdomyosarcoma

GROUP I: Complete excision No gross or microscopic residual disease	STAGE 1: Favorable site Orbit, superficial head and neck, biliary tree, paratestes, vagina
GROUP II: Microscopic residual disease IIa: Microscopically positive excision margins IIb: Excision margins negative; involved nodes but completely excised; most distant node negative IIc: Microscopically positive margins at primary site or grossly excised nodes; or most distant node positive	STAGE 2: Unfavorable site, diameter ≤5 cm, and nodes clinically negative or unknown All sites except the favorable ones above are unfavorable.
GROUP III: Gross residual disease Biopsy only or incomplete excision	STAGE 3: Unfavorable site and diameter >5 cm or clinically involved nodes
GROUP IV: Distant metastases	STAGE 4: Distant metastases

Lymph Node Involvement

Lymph node positivity is variably defined as clinical involvement on physical examination or imaging or histologic involvement at biopsy; some reports provide no definition. The International Rhabdomyosarcoma Workshop found lymph node involvement (undefined) to be a prognostic factor in univariate analysis but not as strong as tumor stage, size, or site.[276] Clinical involvement of local or regional lymph nodes was prognostically significant among IRS-III and IRS-IV patients with stage 3, group III ARMS (5-year OS of 66% with negative nodes vs. 34% for positive nodes, $P = .01$), but not for patients with embryonal tumors.[277] A later study using IRS-IV cases with localized tumors or single metastatic sites came to the same conclusion about ERMS, but for ARMS multivariate analysis showed nodal positivity (whether by physical examination, imaging, or histology) to be a potent adverse factor, with N0 and N1 5-year FFS rates of 73% and 43%, respectively.[278] The investigators also found that 80% of N1 primary ARMS overexpressed immune-related genes such as β_2-microglobulin, immunoglobulins, complement, and histocompatibility complex components.

Metastases at Diagnosis

Patients with metastases at diagnosis fare poorly, and there has been little change over the years. Patients with group IV tumors had a 5-year OS of 20% in IRS-I, 23% in IRS-II, 20% in CWS-86, and 23% in MMT-98 and across a range of IRS studies beginning with IRS-III.[279-283] However, they are not a uniform population. Patients younger than the age of 10 years with metastatic ERMS were classified as intermediate-risk (rather than high-risk) patients in the IRS-V series of studies because of a 51% FFS rate in the previous three studies.[272,284] An analysis of patients with metastases at diagnosis in IRS-IV found that the number of metastatic sites and histology defined two risk groups: patients with one or two metastatic sites, or three metastatic sites with embryonal histology, had a 3-year OS of 43% to 47%, but patients with three or more metastatic sites and alveolar histology had a 3-year survival of only 5%.[285] After pooling 788 patients with metastatic RMS from nine cooperative group studies (IRS, SIOP MMT, and Italian), a multivariate analysis identified four unfavorable prognostic factors: unfavorable site, bone or marrow metastases, age younger than 1 or older than 9 years, and three or more metastatic sites. Applying these factors divided the population into two groups: the first had none to one unfavorable factor, accounted for 42% of the patients, and had an event-free survival (EFS) of 44%; the second had two or more factors, accounted for 58% of the patients, and had an EFS of 14%.[286]

Interactions

All of these variables interact, which complicates classification. For example, large extremity primary tumors are usually alveolar histology, often have involved regional nodes, and often occur in teenagers, and ARMS are usually invasive.

A recent multivariate analysis of 1164 CWS study patients, enrolled between 1980 and 2002, identified histology, age (≤10 years vs. >10 years), tumor size (5-cm cutoff), clinical group, site, and the use of radiation therapy in treatment as significant factors. Further distillation led to three groups of patients, those with (1) embryonal tumors 5 cm or less, whose 5-year EFS was 76%, whose relapses were only 19% systemic, and whose postrelapse survival was 46%; (2) embryonal tumors larger than 5 cm, with an EFS of 64%, whose relapses were 32% systemic, and postrelapse survival was 18%; and (3) alveolar tumors, with an EFS of 46%, systemic relapse rate of 52%, and postrelapse survival of 13%.[287]

Many different risk classification schemes exist for RMS, and they change from one clinical trial to another. In the system used by the COG for current studies, stage (which depends on site and size), clinical group, and histology are combined to produce a three-way risk classification (Fig. 59-9 and Table 59-7) . The system used in the current European studies assigns patients to four risk groups (low, standard, high, and very high) based on histology, clinical group (referred to as stage), site, nodal involvement, tumor size, and age; these four risk groups are then divided into eight subgroups based on clinical group, nodal status, age, and tumor size (Table 59-8). In view of recent information on the overarching importance of *PAX-FOXO1* fusion status,[271] risk classifications are likely to change in the next generation of cooperative group studies.

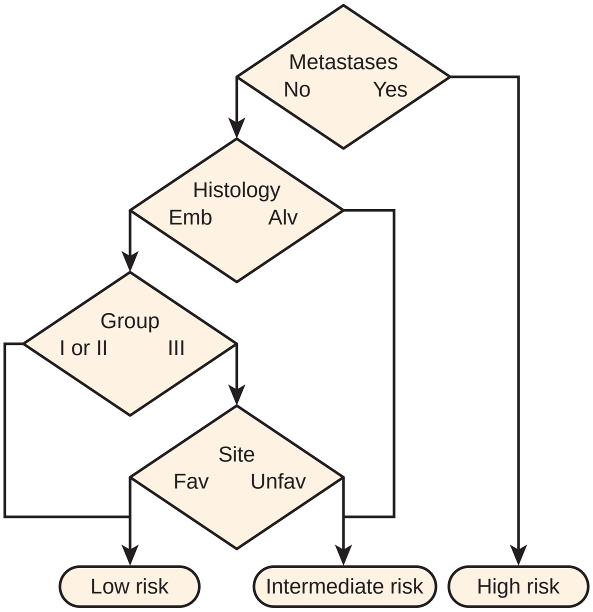

Figure 59-9 Risk group assignment algorithm for the first generation of Children's Oncology Group ARST studies (ARST0331, ARST0431, ARST0531). The results of those studies and the use of molecular rather than histologic criteria for alveolar RMS are likely to change this considerably. *Alv,* Alveolar; *Emb,* embryonal.

TABLE 59-7 Children's Oncology Group Risk Stratification for Rhabdomyosarcoma

	Low Risk	Intermediate Risk	High Risk
Metastases	No	No	Yes
Histology	Embryonal	Any	Any
Stage	Any if group I or II; 1 if group III	1, 2, or 3 if alveolar histology; 2 or 3 if embryonal histology	4
Group	I, II, or III if stage 1; I or II if stage 2 or 3	III if stage 2 or 3; I, II, or III if alveolar histology	IV

TREATMENT

Before the advent of chemotherapy for RMS, survival was rare—a retrospective review of 64 CCSG RMS cases with "complete" surgical excisions revealed a survival rate of less than 8%.[288] The dramatic progress in treatment and survival of RMS patients over the past 40 years has flowed mostly from multidisciplinary care, in which surgeons, oncologists, radiologists, radiation oncologists, and pathologists approach treatment in a planned, coordinated fashion. Such care, which was pioneered in the late 1960s,[289] has been the foundation for clinical trials in RMS in the United States and Europe.

Chemotherapy

Systemic chemotherapy for all patients with RMS began with a Children's Cancer Study Group randomized controlled trial, which ran from 1967 to 1971. Patients with complete excisions (group I) were randomly assigned to chemotherapy with vincristine and dactinomycin (actinomycin D) for 6 to 13 nine-week cycles or to observation only, whereas patients with microscopic residual disease were nonrandomly assigned to the chemotherapy arm of the trial. RFS at 4 years was 82% in the chemotherapy group and 47% in the control group ($P = .03$); RFS was 90% in group II.[288] This trial established the role of chemotherapy in treating RMS and also established the feasibility and importance of coordinated multidisciplinary care on a national, multiinstitutional scale.

Cyclophosphamide

Cyclophosphamide was part of combination chemotherapy for RMS from some of the earliest trials, based on its efficacy as a single agent in patients with advanced RMS.[289,290] The vincristine-dactinomycin (actinomycin D)-cyclophosphamide (VAC) combination has been in continuous use since IRS-I[280] and the first European

TABLE 59-8 European Paediatric Soft Tissue Sarcoma Study Group Risk Stratification for Rhabdomyosarcoma

Risk Group	Subgroups	Pathology	Postsurgical Stage (IRS Group)	Site	Node Stage	Size and Age
Low risk	A	Favorable	I	Any	N0	Favorable
Standard risk	B	Favorable	I	Any	N0	Unfavorable
	C	Favorable	II, III	Favorable	N0	Any
	D	Favorable	II, III	Unfavorable	N0	Favorable
High risk	E	Favorable	II, III	Unfavorable	N0	Unfavorable
	F	Favorable	II, III	Any	N1	Any
	G	Unfavorable	I, II, III	Any	N0	Any
Very high risk	H	Unfavorable	I, II, III	Any	N	Any

cooperative group trials. The standard North American chemotherapy combination for patients with intermediate-risk tumors is still the VAC regimen. The exact composition of the VAC regimen has varied considerably over time (Table 59-9), but no randomized controlled trial has shown any combination to be more effective.

In IRS-I, patients with group II tumors were randomized between 2-year regimens consisting of vincristine-dactinomycin (VA) (with six doses of vincristine and 5 consecutive days of dactinomycin per 9-week cycle) or VAC (12 weekly doses of vincristine at the start of therapy only, dactinomycin every 12 weeks, and daily oral cyclophosphamide). There was no significant difference between the two study arms,[280] but the design of the comparison allows one to conclude only that cyclophosphamide could substitute for 60 doses of vincristine and three cycles of dactinomycin.

The IRS-II study addressed the role of cyclophosphamide in two randomizations. In group I patients, the VAC regimen from IRS-I (for 2 years) was compared with the VA regimen alone for 1 year, with the same 12 initial weekly doses of vincristine and dactinomycin every 12 weeks. The VAC regimen provided a statistically insignificant advantage in disease-free survival (DFS) but no advantage in OS. For group II patients there was a randomization between two 1-year regimens: the VA regimen with 9-week cycles incorporating six weekly doses of vincristine and one 5-day series of dactinomycin doses per cycle and a VAC modification with 4-week cycles each containing a single dose of vincristine, a single 5-day series of dactinomycin injections, and intravenous cyclophosphamide. The contest was a draw,[281] and because the VA regimen had three times the vincristine and half the dactinomycin of the cyclophosphamide-containing regimen, inferences regarding the role of cyclophosphamide are impossible.[291] There is evidence that cyclophosphamide (or ifosfamide) can allow omission of radiation therapy for some patients with vaginal RMS, however (discussed later).

There is also no evidence of a relationship between the dose of cyclophosphamide and its efficacy. For intermediate-risk patients getting the VAC regimen, IRS-III prescribed the equivalent of 900 mg/m^2 per 3-week cycle in regimen 34[292]; IRS-IV used 2.2 g/m^2 per cycle in its VAC regimen[293]; and a pilot IRS study tried 3.6 g/m^2 of cyclophosphamide per cycle (a dose of 4.5 g/m^2 proved too toxic).[294] However, there was no change in FFS rates across these three trials despite a fourfold increase in cyclophosphamide dose and dose intensity,[293,294] and the most recent COG ARST studies use 1200 mg/m^2 of cyclophosphamide in their VAC arms.

Doxorubicin

Doxorubicin is probably the single most effective agent against sarcomas generally, but its role in RMS remains been both unclear and controversial despite three decades of study. Concern about its cardiotoxicity (especially in young patients) has limited its use. The value of doxorubicin in treating RMS, and particularly its relative efficacy against embryonal and alveolar tumors, remain open questions.

The investigators of the IRS tried doxorubicin in a variety of ways. In IRS-I, patients with group III and IV tumors were randomized between VAC (with dactinomycin every 12 weeks and continuous oral cyclophosphamide) and the same regimen with doxorubicin doses halfway between the dactinomycin cycles. The addition of doxorubicin made no difference to either DFS or OS.[280] The IRS-II study treated group III and IV patients with more modern, every-4-week intravenous regimens, randomizing between VAC and a regimen in which doxorubicin replaced dactinomycin in alternate cycles. Again, there were no significant differences in DFS or OS, although the results were much better than those of IRS-I.[281] Unfortunately there was no analysis to see whether doxorubicin might have affected ERMS or ARMS patients differently.[291]

IRS-III used doxorubicin in two randomizations. For group II patients with favorable (embryonal) histology, vincristine-dactinomycin-doxorubicin was superior to VA alone (89% vs. 54% 5-year survival, $P = .03$); however, patients in the VA arm of the study had worse results than those in IRS-II, and when the IRS-II and III results were combined the statistical significance disappeared (89% vs. 73% for survival, $P = .14$).[292] Doxorubicin was also part of all three randomized regimens for clinical group III and IV patients in complex ways that make its contribution to the results impossible to assess.[291]

The COG compared six "phase II window" regimens to IRS-III in 420 patients with group IV (metastatic)

TABLE 59-9 Evolution of Vincristine/Dactinomycin (Actinomycin D)/Cyclophosphamide (VAC) Chemotherapy

	VCR Dose	VCR Schedule	AMD Dose	AMD Schedule[†]	CPM Dose	CPM Schedule	Duration	Total VCR	Total AMD	Total CPM
IRS-I	2 mg/m^2; max: 2 mg	Weekly × 12	0.015 mg/kg/day × 5 days; max: 0.5 mg/dose	q12 wk	2.5 mg/kg	Daily PO	2 yr	12 doses	10 cycles	51.5 g/m^2
IRS-II*	2 mg/m^2; max: 2 mg	Weekly × 12, then q4 wk	0.015 mg/kg/day × 5 days; max: 0.5 mg/dose	q4 wk	10 mg/kg/day × 3	× 1, q3-4 wk	1 yr for group II, 2 yr for others	35 doses	24 cycles	27 doses, 24.3 g/m^2
IRS-III	1.5 mg/m^2; max: 2 mg	Weekly × 12, then q3-4 wk	0.015 mg/kg/day × 5 days; max: 0.5 mg/dose	q3-4 wk	10 mg/kg/day × 3	× 1, q3-4 wk	2 yr	36 doses	21 cycles	27 doses, 24.3 g/m^2
IRS-IVP	1.5 mg/m^2; max: 2 mg	Weekly × 12, then q3 wk with q1 wk intervals	1.35 mg/m^2 (1 dose)	q3 wk	3600 to 4500 mg/m^2 (first four cycles), then 2200 mg/m^2 IV	× 1, q3 wk	38 wk	32 doses	11 doses	13 doses, 34.2 to 37.8 g/m^2
IRS-IV	1.5 mg/m^2; max: 2 mg	Weekly × 12, then q3 wk with q1 wk intervals	0.015 mg/kg/day × 5 days; max: 0.5 mg/dose	q3 wk	2200 mg/m^2 IV	× 1, q3 wk	44 wk	32 doses	9 cycles	12 doses, 26.4 g/m^2
IRS-V	1.5 mg/m^2; max: 2 mg	Weekly × 12, then q3 wk with q1 wk intervals	1.5 mg/m^2; max: 2 mg	q3 wk	2200 mg/m^2 IV	× 1, q3 wk	40 wk	30 doses	12 doses	14 doses, 30.8 g/m^2
COG ARST[‡]	1.5 mg/m^2; max: 2 mg	Weekly × 12, then q3 wk with q1 wk intervals	0.045 mg/kg (max 2.5 mg)[‡]	q3 wk	1200 mg/m^2 IV	× 1, q3 wk	40 wk	30 doses	12 doses	14 doses, 16.8 g/m^2

AMD, Actinomycin (dactinomycin); *ARST*, Rhabdomyosarcoma and Soft Tissue; *CPM*, cyclophosphamide; *IV*, intravenously; *PO*, orally; *VCR*, vincristine.

*Repetitive-pulse VAC.

[†] Except during radiation therapy.

[‡] Lower doses for infants.

RMS. Patients receiving the doxorubicin-ifosfamide and ifosfamide-etoposide combinations and the IRS-III treatment did best in terms of FFS; there was no ultimate difference in OS, however.[279] Again, this analysis failed to shed any specific light on the role of doxorubicin.

In Europe, doxorubicin was part of the vincristine-doxorubicin (Adriamycin)-cyclophosphamide/dactinomycin (actinomycin D) (VACA) and vincristine-doxorubicin (Adriamycin)-ifosfamide-dactinomycin (actinomycin D) (VAIA) regimens of CWS-81 and CWS-86, respectively, was part of retrieval regimens for poor responders in MMT-84, and was widely used in the Italian RMS 79 and RMS 88 studies, but there were no randomized comparisons.[282,295] The addition of epirubicin (a doxorubicin analogue) along with carboplatin and etoposide proved to be no improvement over ifosfamide-vincristine-dactinomycin (actinomycin D) (IVA) in EFS or OS in the MMT-95 study of patients with incompletely excised RMS of unfavorable sites; and the six-drug regimen was considerably more toxic.[296] We still do not know the value of doxorubicin (or other anthracyclines) in treating RMS.

Ifosfamide

DeKraker and Voute reported promising phase II results for the combination of vincristine and ifosfamide and went on to substitute ifosfamide for cyclophosphamide in the VAC combination in an study of 18 previously untreated patients.[297] The German CWS-86 study substituted ifosfamide for cyclophosphamide to change its VACA combination to VAIA, and the results were slightly improved[282]; however, it was not a randomized controlled trial. A randomized controlled comparison of cyclophosphamide and ifosfamide had to await IRS-IV; 457 patients were randomly assigned to either VAC (with cyclophosphamide as a single 2.2 g/m^2 dose) or VAI (with ifosfamide as five daily doses of 1.8 g/m^2 each); the Kaplan-Meier plots of FFS were identical.[293] Thus, although the IVA regimen has long been the European standard for intermediate-risk RMS, there are no data demonstrating its superiority to VAC; and ifosfamide has more urothelial, renal, and neurologic toxicity than cyclophosphamide.[298,299]

Etoposide

A phase II study of etoposide showed some activity in RMS.[300] The IRS-III had a three-way randomization for intermediate-risk patients that included a comparison of VAC with VAC plus cisplatin, plus or minus etoposide. Neither the cisplatin nor the cisplatin-etoposide combination added to the efficacy of VAC.[292]

In a comparison of “phase II window” regimens for patients with metastatic disease at diagnosis, in contrast, ifosfamide-etoposide was significantly superior to the IRS-III combination therapy, ifosfamide-doxorubicin, irinotecan, vincristine-melphalan, topotecan, or topotecan-cyclophosphamide in producing FFS. Survival of the ifosfamide-etoposide–treated patients was also longer, although the proportion surviving at 5 years was not improved.[279]

The combination of ifosfamide and etoposide produced complete or partial responses in 9 of 13 patients with recurrent or refractory RMS in a phase II National Cancer Institute study,[301] a remarkable level of activity. In the IRS-IV study, intermediate-risk patients were randomly assigned to chemotherapy with vincristine-ifosfamide-etoposide, VAC, or VAI; the three regimens had almost identical efficacy.[293] This showed that not only cyclophosphamide and ifosfamide are interchangeable (as given in the trial) but so are etoposide and dactinomycin. Because dactinomycin can be given in a single dose rather than three to five daily doses, and because all four patients who developed myeloid leukemia or myelodysplasia as a second malignancy in IRS-IV had received etoposide,[293] VAC was carried forward as the control regimen for the IRS-V study.

Oral etoposide as a single agent, given daily for 21 days in every 28-day cycle, produced stable disease after two cycles in 3 of 11 patients with refractory or relapsed RMS or other soft tissue sarcoma in a British phase II trial.[302] Responses to oral etoposide appeared to be independent of previous etoposide exposure. A Turkish phase II study of a similar regimen included 4 patients with RMS; 1 had a durable complete response (87+ months), and another had a partial response lasting 10 months.[303] There have been no reports of regimens incorporating oral etoposide into initial therapy or any combination regimens using it in RMS or other pediatric sarcomas.

Topotecan

Topotecan is a camptothecin analogue and as such is a topoisomerase I poison that appeared to be active against a variety of pediatric solid tumors in preclinical trials. In a phase II Pediatric Oncology Group study, the topotecan-cyclophosphamide combination produced complete or partial responses in 10 of 15 patients with RMS.[304] The IRS-COG conducted a “phase II window” study of topotecan alone,[305] and then topotecan with cyclophosphamide,[306] in patients with group IV (metastatic at diagnosis) RMS, but neither distinguished itself in either EFS or OS.[279] More patients with alveolar histology responded to topotecan alone,[305] but the ERMS and ARMS patients responded similarly to topotecan-cyclophosphamide.[304,306]

The IRS-V intermediate-risk study randomized 516 patients between VAC and VAC alternating with vincristine-topotecan-cyclophosphamide. There were no differences in EFS or OS between the two regimens and no differences in toxicity apart from a slightly higher incidence of febrile neutropenia in the VAC arm of the study.[307]

Irinotecan

Irinotecan is another topoisomerase I poison with promising preclinical activity against pediatric solid tumors in preclinical models.[308] A British-French phase II study of irinotecan given as a single bolus dose every 3 weeks to patients with relapsed RMS produced only one complete response and three partial responses among 35 patients.[309] The results of a COG phase II study were similar, with one partial response among 18 RMS patients using a “daily times five” schedule (5 consecutive days of treatment every 3 weeks).[310] A “daily times five times two” schedule (5 days of treatment, 2 days off, then 5 more days of treatment, repeated every 3 weeks) was shown to be promising in both a xenograft model and children with refractory solid tumors in a phase I study,[311] but in an Italian phase II study only 2 of 12 RMS patients had a complete or partial response.[312]

Vincristine and topotecan appeared to be synergistic in mouse xenografts,[313] inspiring a combination of vincristine and irinotecan in an IRS (later COG) study.[314] In the first part of the study, which enrolled patients with RMS metastatic at diagnosis, patients received 6 weeks (two cycles) of irinotecan using the "daily × 5 × 2" schedule, followed by VAC; in the second part patients were treated with vincristine-irinotecan. The response rate for the irinotecan window was 42%, but 32% had progressive disease; and the response rate to the combination vincristine-irinotecan window was 70%, with only 8% having progressive disease. Initial responses did not translate into improved survival, however: patients who declined "phase II window" therapy received VAC alone and had a similar response rate and FFS (23%) to patients receiving vincristine-irinotecan.

A subsequent COG study randomized patients with relapsed or refractory RMS between the "daily × 5 × 2" schedule (10 doses over 2 weeks) and a "daily × 5 × 1" schedule (five doses in 1 week) and found no significant differences in response or toxicity; the authors recommended carrying the more-convenient shorter regimen forward.[315] The most recent COG study for patients with intermediate-risk RMS randomized patients between VAC and alternating 6-week blocks of vincristine-irinotecan and VAC; results are anticipated soon.

Other Agents

Despite good results when the cisplatin-etoposide combination was tested in a phase II study,[316] cisplatin was shown not to add to the efficacy of VAC in intermediate-risk patients in IRS-III, either alone or in combination with etoposide (see earlier). There has been no evaluation of carboplatin in a phase III study, but the addition of carboplatin to the ifosfamide-etoposide combination (the ICE regimen) in a phase II study produced 51% complete and partial responses in patients with a variety of relapsed sarcomas,[317] which was very similar to the response rate to ifosfamide-etoposide, and with greater toxicity. The SIOP MMT-98 study included a "phase II window" of carboplatin in 16 RMS patients with metastases at diagnosis. Five patients had complete or partial responses after two cycles (31%), but 4 (25%) had progressive disease,[318] and the carboplatin-treated patients had significantly worse 3-year EFS than those treated with doxorubicin "window" therapy or those treated with standard four-drug initial therapy.[283] These results, along with the prolonged myelosuppression it produces, have limited enthusiasm for carboplatin.

Melphalan with vincristine was a "phase II window" combination in an IRS-IV trial for patients with metastases at diagnosis; however, it produced an unimpressive response rate and more toxicity than ifosfamide/etoposide.[279,319]

Combinations

Two attempts to improve outcome for patients with intermediate- or high-risk RMS with intense combinations of several agents have failed in randomized controlled trials. IRS-III compared VAC with the same drugs plus doxorubicin and cisplatin, etoposide, or both; the outcomes were identical for the three arms of the study.[292] The MMT-95 study randomly assigned similar patients to IVA or IVA plus carboplatin, epirubicin, and etoposide; the EFS and OS were the same at 3, 5, and 10 years, although the toxicity of the intensified regimen was significantly greater and it was associated with twice as many second malignancies.[296]

The success of alternating vincristine-doxorubicin-cyclophosphamide and ifosfamide-etoposide (VDC-IE) in Ewing sarcoma inspired a limited-institution trial of that therapy in 46 patients with intermediate-risk RMS and a comparison with the results with similar patients in IRS-IV. FFS for VDC-IE and IRS-IV were 82% and 72%, respectively (P = .26), and OS at 5 years was 76% for both. However, a comparison of the VDC-IE patients in a stratified analysis with 346 IRS-IV patients matched for site, histology, age, and clinical group showed a relative risk of failure of 0.5 with VDC-IE (P = .06).[320] The COG has completed a study of interval-compressed VDC-IE combined with vincristine and irinotecan in patients with metastatic RMS (ARST0431), but the results are not yet available.

Alternating topotecan-cyclophosphamide with VAC did not improve outcomes in patients with intermediate-risk RMS in the COG D9803 study[307]; and the results of the ARST0531 study comparing VAC with VAC alternating with vincristine-irinotecan are awaited.

A French phase II study of vinorelbine and continuous oral cyclophosphamide in relapsed pediatric soft tissue sarcomas showed an overall response rate of 36% among 50 patients with RMS.[321] This combination is under study in a phase III EpSSG study (see later).

Duration of Chemotherapy

The IRS-I, II, and III studies prescribed 2 years of treatment for most patients; in IRS-IV the duration of treatment was trimmed to 44 weeks for patients with intermediate-risk tumors and 32 weeks for patients with low-risk tumors.[293] In contrast, the European MMT-84 and CWS-81 and CWS-86 studies used 30 to 56 weeks of therapy.[282,322] The recent COG trials used only 22 weeks of therapy for patients with the lowest-risk tumors and 42 and 51 weeks for intermediate- and high-risk patients respectively. Although there have been no apparent adverse consequences of shorter durations of therapy, there have been no systematic controlled comparisons.

The current EpSSG study is testing the efficacy of 24 weeks of "maintenance" therapy with vinorelbine and oral cyclophosphamide after 26 weeks of intense cyclic chemotherapy for high-risk patients who achieve a complete response.

Treatment Strategies

There have been three broad approaches to the treatment of RMS: the SIOP MMT studies have used intense initial chemotherapy and then SIOP tried to tailor primary tumor treatment according to responses to initial chemotherapy, so as to minimize the use of surgery and radiation (with their accompanying late effects). The Intergroup Rhabdomyosarcoma Studies in North America (now the Soft Tissue Tumor Committee of the COG) has sought to maximize EFS through the use of chemotherapy, surgery, and radiation therapy in almost all patients. The German-Italian CWS studies have taken an intermediate approach. The European groups established a joint European Paediatric Soft Tissue Sarcoma Study Group (EpSSG), which has launched a protocol for localized RMS (RMS-2005), although the Germans have withdrawn from the consortium.

These different philosophies are illustrated in the groups' treatments for orbital RMS. The MMT-84 and MMT-89 studies treated patients with intense multiagent chemotherapy and no radiation therapy for those achieving a complete response. The IRS gave all patients radiation therapy (45 to 55 Gy, depending on the study) and evolving chemotherapy that was either relatively mild (vincristine and dactinomycin) or intense (the IRS-IV randomization between VAC, VIE, and IVA). The CWS studies modulated the radiation dose according to the response to intense multiagent chemotherapy: 40 or 50 Gy in CWS-81 and the Italian ICG-88 study and 32 or 54.4 Gy in CWS-86 for good and poor responders, respectively. Although the three approaches led to very different EFS rates at 10 years (57% for MMT to 86% for IRS, $P < .001$), the OS rates were almost identical (85% and 88%, $P = .67$). The incidence of radiation-associated late effects was higher in the IRS patients than in the MMT patients, although the total burden of therapy in the MMT approach includes the treatment of relapse in 37% of patients.[323]

A recent comparison of Intergroup Rhabdomyosarcoma Study Group (IRSG), SIOP MMT, Italian ICG, and German CWS approaches to bladder-prostate RMS reflected similarly divergent treatment philosophies. Forty-eight percent of SIOP-ICG patients received radiation as part of their initial therapy, compared with 85% of IRSG-CWS patients. However, there were no significant differences in FFS and OS between the groups.[324]

A comparison of RMS of all sites treated on MMT-89 and IRS-IV gave a considerable advantage to the IRS approach, however. Overall, IRS-IV had higher EFS and OS than MMT-89 (78% vs. 57% and 84% vs. 71%, respectively). For some sites there was no difference (e.g., genitourinary tract, not including bladder and prostate: IRS-IV vs. MMT-89 83% vs. 82% EFS, 90% vs. 94% OS), but for others the difference was large (e.g., limbs, IRS-IV vs. MMT-89 64% vs. 35% EFS, 71% vs. 46% OS). For patients with ERMS, the IRS approach had a small advantage for OS (87% vs. 78%) but a large advantage for patients with ARMS (71% vs. 38%), orbital tumors (100% vs. 85%), and nonparameningeal head and neck tumors (89% vs. 64%).[325] Another review found that the four major RMS clinical trial groups enjoyed roughly equal success in preventing metastatic relapse, but there were considerable differences in local relapse, ranging from 13% for the IRS to 30% for the MMT; the CWS and Italian groups were in the middle, with 19% and 22%.[287]

Response Assessment

Recent results call into question the CWS strategy of modifying treatment according to primary tumor response assessed by conventional imaging (CT or MRI). A review of group III patients treated on IRS-IV demonstrated no relationship between response to the first 8 weeks of therapy (complete, partial, or none) and 5-year FFS. This was true for patients who were initially treated with chemotherapy alone, as well as for those with parameningeal tumors with intracranial extension who received early radiation therapy. The only statistically significant difference found was among patients with ARMS, in whom patients with no response had better FFS (81%) than patients with complete (71%) or partial (39%) responses ($P = .04$).[264] More recently, a review of imaging and second-look surgery results from IRS-IV patients with group III tumors showed a weak relationship between imaging response and histologic response at second-look surgery, with tumor viability at second-look surgery associated with better FFS.[326]

FDG-PET imaging might prove to be useful, because it can differentiate between viable tumor and a residual mass of necrosis or fibrosis.[327] A single-institution retrospective study reviewed RMS patients who had PET/CT performed at diagnosis, after initial chemotherapy, and after radiation therapy. For the 68 patients who had scans at all three times, PET negativity after initial chemotherapy was weakly associated with better local RFS (97% vs. 81% at 3 years, $P = .06$), although the association was stronger for postirradiation PET negativity (94% vs. 75%, $P = .02$). Among patients with a complete response by CT, PET response predicted better local RFS (97% vs. 43%, $P = .01$); but among patients with a residual mass on CT, PET response was not useful (3-year local RFS 80% vs. 64%, $P = .72$).[328]

Current Practice and Protocols

Most RMS patients in North America are enrolled on COG studies, when they are open for accrual. Patients are classified as of low, intermediate, or high risk based on whether there are metastases, the tumor stage, histology, and clinical group (see Table 59-7). Low-risk patients were treated with four cycles of VAC (using a single 1200-mg/m^2 dose of cyclophosphamide), followed by vincristine-dactinomycin for a total of 6 months or 1 year of chemotherapy. Patients with group II or III tumors receive radiation therapy. Intermediate-risk patients were randomized on the COG ARST-0531 study between the standard therapy of VAC for 1 year, or a regimen in which blocks of vincristine-irinotecan therapy alternate with blocks of VAC; results are pending. The results of treatment in high-risk patients are poor enough that it is difficult to designate any treatment as "standard"; the COG recently completed a single-arm study using vincristine-irinotecan and interval-compressed (every-2-week) vincristine-doxorubicin-cyclophosphamide alternating with ifosfamide and etoposide, but the data are not yet available.

The EpSSG is using a complex algorithm to classify patients without metastases into eight risk groups and subgroups, with further treatment subdivisions based on response to initial chemotherapy (see Table 59-8). Low-risk patients are being treated with eight cycles of vincristine-dactinomycin. Standard-risk patients are receiving regimens including IVA and vincristine-dactinomycin, whereas high-risk patients are being enrolled in two randomized controlled trials, one evaluating the role of doxorubicin added to IVA and the other exploring "maintenance" chemotherapy with vinorelbine and cyclophosphamide. "Very high risk" patients (those with alveolar histology and involved nodes; EpSSG excludes patients with metastases) are being treated with IVA, doxorubicin, and maintenance vinorelbine-cyclophosphamide. All patients except those with group I ERMS are also receiving radiation therapy.

Primary Tumor Treatment

When only local therapy with surgery and irradiation was available, the survival rate of RMS patients was dismal. Chemotherapy has dramatically improved survival, and it has also changed the surgical and radiotherapeutic approaches, with minimization of late effects becoming a new priority. Optimal outcomes require multidisciplinary care.

Surgery

The surgeon is often involved with an RMS patient from the time of initial biopsy. This is best done at the cancer center where treatment will occur and where a full complement of diagnostic tools (including molecular techniques) is available. The biopsy needs careful planning and execution, because diagnosis, treatment, and late effects can all depend on it.

The earliest classification system for RMS, the IRS clinical grouping system, is based on the amount of tumor remaining after the first surgical intervention, be it a complete excision with clear margins (group I) or only a biopsy with gross residual disease remaining (group III) (see Table 59-5). IRS-I showed that the clinical group is a powerful predictor of survival[280] and established the role of surgery in the treatment of RMS in the chemotherapy era. Although initial complete surgical excision can involve disfigurement or loss of function, it may have a smaller late effects burden than primary tumor treatment with radiation in some patients, so multidisciplinary consultation is essential.

When a patient's initial excision leaves microscopically positive margins (group II), or when the initial excision was performed outside the pediatric cancer center, there may be value in a "primary reexcision" before chemotherapy begins. An analysis of patients with trunk or limb RMS in IRS-I and IRS-II showed that group II patients converted to group I had the same survival as patients who were rendered group I in their initial procedure. Patients who were referred as group I patients and had primary reexcisions had superior outcomes to those who did not.[329]

Regional lymph node sampling is important in limb primary tumors, which often have alveolar histology and a predilection for nodal involvement. Patients age 10 years or older with paratesticular RMS also have an increased risk of paraspinal nodal relapse and should have node sampling. Otherwise, lymph node sampling may be limited to nodes that are suspicious by physical examination or imaging.[330,331]

Traditional surgical teaching is that an attempt at excision is not valuable unless the patient can be left with microscopic residual disease at most (group I or II). Among patients with unresectable tumors (group III), debulking surgery may be beneficial even if the patients remain in clinical group III. In a retrospective study of IRS patients treated between 1984 and 1991 with large (≥5 cm) group III or IV retroperitoneal tumors, patients with embryonal histology who underwent debulking surgery before chemotherapy fared better than those who did not (FFS 72% vs. 39% at 4 years, $P = .03$); too few patients with alveolar histology tumors had debulking surgery to permit analysis.[332] It is not yet clear whether this principle applies only to retroperitoneal tumors or whether it is generally applicable. However, a retrospective analysis of the Italian RMS-79, RMS-88, and RMS-96 protocols that compared the fate of 323 patients with biopsy only and 71 patients with debulking surgery (defined as more than 50% excision but still group III) found no overall benefit. One subgroup, patients 10 years or older, appeared to benefit from debulking surgery (EFS 73% vs. 50%, $P = .04$; OS 83% vs. 62%, $P = .06$), but no other subgroup defined by site, size, histology, invasiveness, or response to initial chemotherapy did so.[333]

In patients with gross residual disease when chemotherapy begins (group III and IV), second-look operations and delayed excision of residual tumor masses, even after radiation therapy and chemotherapy, may improve the chances for survival. In a retrospective study of second-look operations in IRS-III, 12% of patients clinically believed to have had a complete response had residual viable tumor and three fourths of patients clinically believed to have had a partial response or no response either had no viable tumor or were converted to a complete response at surgery. These "converted" patients had the same 3-year survival as those whose complete responses were confirmed at second-look surgery (73% and 80%, respectively).[331] A single-institution study of 48 group III patients showed that those who had a delayed complete surgical excision (n = 22) had superior RFS and OS to those who did not (approximately 90% vs. 60% RFS, $P = .013$).[334] Patient numbers were too small to permit a multivariate analysis, however, and histology and nodal status were also significant variables on univariate analysis.

Radiation Therapy

Because RMS can often occur in sites that are not amenable to surgery, radiation therapy has a key role in primary tumor treatment. Advances in radiation therapy techniques are reducing the acute and late morbidities of treatment. However, there are large gaps in our knowledge of how best to use radiation to treat RMS.

The first knowledge gap is to determine who needs radiation therapy. A retrospective analysis of 439 group

I patients in IRS-I, IRS-II, and IRS-III found no benefit from irradiation for patients with embryonal tumors for OS (95% at 10 years for both groups, $P = .83$). For alveolar and undifferentiated tumors, however, each of the IRS studies showed a benefit from radiation therapy, analyzing by treatment received.[335] Subsequently, an analysis of IRS-III and IRS-IV data found no EFS or OS advantage for radiation therapy in alveolar stage 1 and 2 tumors but it remained important for patients with stage III tumors.[336]

A retrospective analysis of the CWS-81, CWS-86, CWS-91, and CWS-96 studies showed that the use of radiation therapy roughly halved the risk for death for patients with embryonal tumors smaller than 5 cm ($P = .02$) and for patients with larger embryonal tumors ($P = .001$) but had a smaller effect for patients with alveolar tumors (risk of death ratio 0.7, $P = .09$).[287] Another analysis of the same studies focused on 203 patients with group II tumors (microscopic residual disease at diagnosis). It compared the 110 patients who received radiation therapy with the 93 who did not (for a variety of reasons). The two groups of patients differed significantly in histology (more irradiated patients had alveolar tumors), site (more extremity and parameningeal tumors, and fewer vaginal-paratesticular tumors, among the irradiated patients), and age (fewer infants among the irradiated patients). All patients received combination chemotherapy according to one of several regimens. Despite the greater number of unfavorable characteristics among the irradiated patients, their local control was better (83% vs. 65%, $P < .04$), and their EFS was better (76% vs. 58%, $P < .005$), although the OS rates of the irradiated and unirradiated patients were similar (84% and 77%).[337] In the IRS studies I through IV there were 37 patients with group II tumors who did not receive radiation; their FFS was 69%, and OS 85%, which was very similar to the 73% and 78%, respectively, for the entire cohort.[338] Thus radiation therapy probably confers an advantage to group II patients but it is far from indispensable for local control and survival.

The management of patients with initially unresected tumors (Group III) who have delayed excisions is also a contentious issue. The IRS and subsequent COG protocols have required radiation therapy for these patients, although European protocols have not. A single institution study of 48 patients with group III RMS identified 22 who had complete excisions at second-look surgery, of whom 13 were treated with subsequent radiation and 9 were not. One patient in each group experienced relapse ($P = .67$), neither at the primary site.[334] In the MMT-89 study, 58% of the 289 survivors with "incomplete" initial surgery or biopsy only had either chemotherapy alone or chemotherapy plus "conservative" local therapy (defined as no functional deficits and no external-beam irradiation).[339] Unfortunately, these patients are not classified by stage or site, but radiation therapy was not indispensable for cure. Similarly, the CWS-86 study had 31 group III patients who did not receive radiation therapy (16 because of age <3 years), again not classified by site or histology. All but 1 had a complete response to chemotherapy and surgery. The local relapse rate was 27%, versus 10% for patients receiving radiation ($P = .2$), and the overall relapse rate was 40%, versus 28% for irradiated patients ($P = .001$).[282] Thus, although radiation therapy seems to reduce the local recurrence risk and improves the survival rate for patients with group III tumors, it is not indispensable.

Assuming the use of radiation therapy, the question becomes how much radiation the different presentations of RMS require to maximize the chances for cure. At the low side of the dose-response curve, it is unclear how much radiation is advisable. A single-institution study of 32 patients with IRS group II RMS (all but two embryonal histology) used chemotherapy (vincristine, doxorubicin, actinomycin, cyclophosphamide, with or without methotrexate, bleomycin, and carmustine) and from 30 to 60 Gy of radiation. Nineteen patients received 40 Gy or less, with only one local relapse; 14 received 36 Gy or less, with the same single local failure.[340] The CWS-86 study compared 67 group III patients who received 25 to 35 Gy (mean 31.6 Gy) with 49 patients who received 40 to 67 Gy (mean 52 Gy); 31% of the lower-dose group and 26% of the higher-dose group experienced relapse, although a higher proportion of the relapses in the higher-dose group were metastatic rather than local or regional.[282] In this study, the higher doses of radiation seem to have reduced the risk for local recurrence. The COG D9602 study of low-risk RMS used lower doses of radiation for selected patients: 36 Gy for patients with microscopic residual, N0 disease (41.4 Gy in earlier studies), and 45 Gy for patients with orbital tumors (had been 50.4 Gy). The local relapse rates were higher than in IRS-IV, but changes in chemotherapy for orbital and vaginal tumors (elimination of cyclophosphamide or ifosfamide) complicated the analysis. The authors concluded that the lower radiation doses do not lead to poorer local control, provided that cyclophosphamide is part of the chemotherapy.[341,342]

At the high side of the dose-response curve, IRS-IV randomized patients with group III tumors between 50.4 Gy by conventional fractionation (180 cGy/day) and 59.4 Gy by hyperfractionation (110 cGy twice daily). The hyperfractionated regimen was designed to allow a higher dose without an increase in late effects. There was no difference in any outcome measure between the two regimens (FFS, OS, local relapse, distant relapse) and no difference according to histology.[293] This indicates that 50.4 Gy is sufficient to control most gross residual RMS; less radiation may be equally efficacious.

In an effort to spare normal tissues from radiation, some institutions have used brachytherapy at the time of second-look surgery.[343-346] This technique usually involves the placement of catheters in the tumor or tumor bed during surgery; the catheters are subsequently loaded with radioactive pellets and then removed when the dose is complete. The brachytherapy dose often needs to be supplemented with external-beam radiation, and we have found that intensity-modulated radiation therapy (IMRT) and proton therapy have largely replaced brachytherapy.

Some institutions have also used intraoperative radiation therapy (IORT), which involves the delivery of a single large fraction of external-beam radiation with the tumor or tumor bed surgically exposed. Generally, larger radiation fractions decrease the therapeutic index, especially for late effects, and this may be the case in pediatric IORT. One series of seven children treated with IORT for neuroblastoma followed for 4 to 143 months (median 38 months) after irradiation; all 7 had hypertension, 5 had vascular stenosis in the radiation field, and 2 patients died in remission (one of mesenteric artery stenosis and bowel ischemia and another of massive ascites).[347] Similarly, of eight surviving patients in another series treated for a variety of pediatric solid tumors, 2 patients required surgery for hydronephrosis and small bowel obstruction, a third developed severe renovascular hypertension and superior mesenteric artery stenosis, and 2 developed neuropathies.[348] In another series, 3 of 14 children treated with IORT for recurrent brain tumors developed radiation necrosis within 6 to 12 months.[349] However, the largest reported series of IORT-treated children (n = 59) who had a variety of malignancies, recorded no long-term toxicities among the 14 long-term survivors.[350]

Proton therapy minimizes the irradiation of normal tissues surrounding the target, and its resulting potential to decrease late effects makes it particularly attractive for treating children. Proton therapy is currently available only at a small (but growing) number of centers, and there are few systematic evaluations. A comparison of conformal radiation, IMRT, and proton plans for a patient with parameningeal RMS calculated that protons reduced the risk for a second malignant neoplasm by a factor of two or more.[351] A retrospective study of 17 consecutive children with parameningeal RMS found 4 with local recurrences (of whom 3 had had intracranial extension at diagnosis), and late effects that were similar to or less frequent than those reported in other studies.[352] Ten of those patients had both proton and IMRT plans, and a comparison of them showed that the two techniques provided equally good coverage of the target volumes but the proton fields greatly reduced the irradiation of normal tissues (by factors of twentyfold to over fiftyfold for median doses to contralateral structures), except for the ipsilateral cochlea and mastoid.[353] Another study compared the proton and three-dimensional conformal radiotherapeutic plans of seven patients treated with protons for orbital RMS. Proton therapy reduced the radiation dose to the hypothalamus, pituitary, temporal lobes, and optic chiasm by 82% to 94% and reduced the dose to the ipsilateral lens by 65%.[354] Similarly, an analysis of 7 consecutive patients with bladder-prostate RMS showed that proton therapy statistically significantly reduced radiation doses to the bladder, testes, femoral heads (sixfold), growth plates, pelvic bones, and bone volume compared with IMRT. Rectal, prostatic, and bowel doses were not significantly affected.[355] More patients, longer follow-up, and application of objective criteria will be necessary to determine whether the lower doses lead to fewer or milder late effects.

The last radiation therapy variable is timing, which has been most controversial in parameningeal RMS. Different IRSG and COG protocols have called for radiation for parameningeal RMS to begin at times ranging from the first week of treatment to the twelfth. Earlier radiotherapy was associated with better survival in IRS-II and IRS-III, although there were so many other changes in treatment that its role cannot be isolated.[291] A recent analysis of consecutive studies has settled the question. In IRS-IV, patients with parameningeal RMS and cranial nerve palsies or cranial base bony erosion had irradiation during the first week, and similar patients in COG D9803 had irradiation beginning in the twelfth week; patients with no such risk factors had irradiation beginning at weeks 6 to 9 in IRS-IV and week 12 in COG D9803. For all parameningeal RMS patients, the 5-year local failure rate, FFS, and OS were almost identical in the two studies (19% local failure in both, $P = .67$; FFS 70% vs. 67%, $P = .32$; OS 75% vs. 73%, $P = .45$). There were also no statistically significant or consistent differences in local failure rates within the groups of patients with and without intracranial extension or cranial nerve palsies.[356] There appears to be no advantage to earlier radiation therapy in RMS; in fact, there may be an advantage to later irradiation through reduction of late effects. A single-institution study of 22 patients with parameningeal RMS showed that the use of delayed irradiation with a shrinking field technique reduced the mean treated area by half, from 99 cm^2 before chemotherapy to 48 cm^2 after chemotherapy, with no apparent effect on RFS.[357]

Special Situations

Infants and Toddlers

Infants with RMS differ from older children in the characteristics of their tumors, their treatment, and their responses to treatment and outcomes. An analysis of 76 infants (age <1 year) treated on IRS-IV and IRS-V showed fewer embryonal and more undifferentiated and other histologies in infants than in older children (ages 1 to 9 years). Infants also had more tumors of the limbs, trunk, bladder-prostate, and abdomen and fewer tumors of the parameningeal sites and orbit.[273]

Treating small children with unresectable (group III) tumors without devastating late effects can be difficult, because tissues irradiated to the doses used for RMS will not grow. Results with very young patients treated without radiation challenge the dogma that radiation therapy is essential for cure of all but group I patients. In the CWS-86 study, 30 patients with stage III (equivalent to group III) tumors, about half of them younger than age 3 years, were treated without radiation therapy, having achieved a complete response with chemotherapy and surgery. Twelve of the 30 (40%) experienced relapse, and 8 (27% of the 30 patients) had local recurrences, for a progression-free survival (PFS) rate of about 60%.[282] Although this was similar to the EFS rate for stage III patients with a complete response to initial therapy in this study, the patients are not classified according to other risk factors (such as site), and the numbers are too small for statistically meaningful comparison. In the IRS-IV and IRS-V analysis, 23 of 72 infants with group III tumors received no radiation; their 5-year FFS was inferior to that of the group receiving radiation (32 vs. 47%, $P = .17$), but the OS was similar (58% vs. 63%, $P = .49$).[273]

Infants and toddlers also appear to tolerate aggressive chemotherapy less well than older children. In the COG D9803 intermediate-risk study, which used VAC with a single dose of dactinomycin and 2.2 g/m^2 of cyclophosphamide, there were 18 cases of hepatopathy resembling sinusoidal obstructive syndrome, 4 of them fatal.[358] The total incidence of hepatopathy among 339 eligible patients was 6%, but it was 14% for patients younger than 36 months and only 4% for older patients. A modification of dactinomycin and cyclophosphamide doses for young patients ameliorated the problem. Cooperative group protocols have varying schemes for calculating infant chemotherapy doses (such as using a weight basis rather than a surface area basis), but none has a foundation in data.

The effects of these compromises on outcome is unclear. A retrospective analysis of IRS-COG studies from 1984 through 1997 (IRS-III, IRS-IV pilot, and IRS-IV) found that patients younger than 1 year of age have a much lower FFS than patients aged 1 to 9 years and one similar to that of adolescents (age ≥ 10 years) (53% v. 72% vs. 52%, $P < .001$)[272]; the infants with nonmetastatic RMS in IRS-IV and IRS-V did similarly.[273] In contrast, the SIOP MMT-84 and MMT-89 studies compared infants (<1 year of age) with localized RMS to children aged 1 to 9 years and adolescents 10 years and older and found identical EFS and OS rates between the infants and older children, both of whom did better than the adolescents (EFS 57% vs. 58% vs. 42%; OS 72% vs. 72% vs. 59%, $P < .001$ between adolescents and the other groups).[359] The infants more often had alveolar histology than older children and also more often had bladder-prostate and limb primary sites. In the earlier IRS study and the MMT studies there was no analysis of deviations from protocol therapy, whereas the later COG analysis showed many deviations from protocol-prescribed radiation therapy but few from prescribed chemotherapy. It remains unclear how reductions in therapy and biologic differences contribute to the inferior outcomes.

Bladder and Prostate Tumors

Because RMS can occur anywhere in the pelvis, and because pelvic tumors are often very large at the time of diagnosis, the exact primary site may be difficult to determine. One should not assume that a pelvic RMS is arising from the bladder or prostate without definitive imaging or cystoscopy. Bladder-prostate tumors usually produce urinary symptoms and are rarely alveolar histology, so one should be especially suspicious that tumors without these characteristics are arising in nongenitourinary pelvic sites. This distinction can have large implications for primary tumor treatment.

The evolution of approaches to bladder-prostate RMS can be traced through the IRS trials. Early approaches included extensive surgery (often complete or partial pelvic exenteration) in addition to chemotherapy. In IRS-I, more than half of the patients with bladder-prostate RMS had initial excisions (group I or group II), often involving anterior exenteration or cystectomy. At 5 years the DFS rate was 67% and the OS rate was 76%. The effectiveness of chemotherapy led to efforts to use initial chemotherapy and postpone and reduce surgery in favor of radiation therapy in hopes of preserving genitourinary anatomy and function. When this approach was taken in IRS-II, the proportion of group I or II patients decreased to 5%. However, the DFS rate declined substantially (to 51%), although the OS was comparable (71%). The new approach had no effect on the proportion of patients alive and with intact bladders, however, which were 23% in IRS-I and 22% in IRS-II.[360] The IRS-III study used more intense chemotherapy (all patients receiving VAC plus doxorubicin, cisplatin, and etoposide), radiation therapy beginning at week 6, and second-look surgery at week 20. The fraction of patients retaining their bladders rose to 60%, with PFS and OS rates improving to 74% and 83%, respectively.[292,361] IRS-IV used different chemotherapy, moved the radiation therapy from week 6 to week 9, and encouraged second surgical procedures short of complete cystectomy or prostatectomy unless there was biopsy-proven residual tumor at the end of therapy or progression. The OS rate of the 88 reviewed patients was 82%, the FFS rate was 77%, and 63% of patients retained their bladders.[362] A retrospective study of 63 CWS patients produced similar results, with an approach that varied the radiation dose and fractionation for group II and III patients according the response to initial chemotherapy.[363] As discussed previously, a comparison of results from the Italian, SIOP MMT, German CWS, and IRS/COG studies showed statistically insignificant variations in EFS despite the groups' different approaches, and no difference in OS (range, 79% to 85%; $P = .93$).[324]

There have been two problems with these bladder-preserving strategies: often the bladders do not work very well, and the collateral side effects can be considerable. Radiation therapy can cause fibrosis of the bladder and sphincters, leading to limited bladder capacity, dribbling, or incontinence. Radiation therapy to the pelvis limits bony growth, and even with modern IMRT techniques it can be difficult to avoid irradiation of the hip joints, leading to future orthopedic problems. A review of IRS-I and IRS-II patients, which involved asking physicians whether their patients' urinary function was normal, found "normal" urinary function in 73% of the 52 patients who had retained all or part of their bladders; the remaining 27% had incontinence, frequency, or nocturia. Radiation therapy and cyclophosphamide (before mesna's availability) combined to produce hematuria after completion of treatment in 20%. Outside the urinary tract, spontaneous reporting disclosed 4 patients with colostomies to repair fistulas or relieve strictures and 6% with fecal incontinence, chronic diarrhea, or rectal stenosis. Eight percent had orthopedic problems, including slipped femoral epiphyses, and pelvic fibrosis or hypoplasia.[364]

A more recent study of IRS-IV patients used routine follow-up forms and questionnaires to physicians. Of 55 patients with preserved bladders, two thirds were reported to have normal urinary function while the balance had late diversions, dribbling, incontinence, enuresis, hydronephrosis, or strictures. Interestingly, no differences in relapse rate or pattern could be found between patients who had protocol-specified radiation therapy and those who had major deviations in radiation therapy (including 9 who received none), although 2 irradiated patients developed second malignancies.[362]

The cooperative group data may underestimate the frequency and severity of late effects in this population, however. A single-institution study of 26 girls cured of pelvic RMS (including nonbladder sites) found that 23 (88%) had grade III or IV late effects at a median of 20 years follow-up, with over half requiring surgery for these complications. The most common problems were musculoskeletal hypoplasia, recurrent urinary tract infections, incontinence, urinary reflux, and short stature. There were three secondary solid tumors in radiation fields. Radiation therapy was associated with more late effects per patient ($P = .002$), and patients treated since 1984 had about twice as many late effects per patient than patients treated earlier (12.5 vs. 6.5, $P = .041$).[365]

None of these studies used urodynamics to measure bladder function objectively. One single-institution study that did so had 11 patients with pelvic RMS (7 with tumors of the bladder or prostate) and found that all 7 patients who had received radiation therapy had markedly reduced bladder capacity (11% to 48% of age norms) and abnormal voiding patterns; 4 had upper tract abnormalities, and 2 had severe bilateral hydronephrosis.[366]

Bladder-prostate RMS is unusual in that one can monitor the tumor easily with cystoscopy and biopsy. In view of the considerable late effects of radiation therapy in particular, some centers use cystoscopic and histologic monitoring to delay and minimize the use of irradiation, moving in the direction of the SIOP MMT studies.[367] This requires expert pathologic interpretation, however, because distinguishing between viable RMS and harmless differentiated rhabdomyoblasts can be very difficult, especially in the field of previous surgery or radiation therapy.[367,368]

Vaginal Tumors

Excellent survival rates make the female genitourinary tract a favorable site in RMS trials but one fraught with anatomic, late effects, and emotional difficulties. In the young girls in whom this tumor most often occurs, the vagina is very small, very thin-walled, deep in the pelvis, and sandwiched between the urethra (and bladder, if distended with tumor) and the rectum. The late effects of complete or partial excision are severe, as can be the effects of radiation therapy (on both the soft tissues and nearby hips). The use of chemotherapy and conservative surgery alone is thus very attractive.

A retrospective survey of female genitourinary RMS patients on the MMT-84 and MMT-89 studies, which used the SIOP strategy of relying on chemotherapy (IVA regimen) and avoiding local therapy whenever possible, included 38 patients. Four had initially complete excisions with conservative surgery and survived event free; 13 had incomplete excisions and chemotherapy with no further local therapy and were event-free survivors; and 17 required local therapy for microscopic or gross residual tumor after initial chemotherapy. This was radical surgery in 6 patients, radiation in 10, and conservative surgery in 1. Although it is extremely difficult to trace the 38 patients through their treatment, the data showed that about half of girls with genitourinary RMS were cured with the IVA chemotherapy regimen without radical surgery or irradiation.[369]

More recently, the COG reviewed vaginal RMS treated on the consecutive low-risk studies D9602 and ARST0331. On the former study, group I and IIA (microscopic local disease) patients received 45 weeks of vincristine-dactinomycin, whereas patients with regional nodal involvement or gross residual tumor (groups IIB, IIC, and III) received 45 weeks of VAC. In ARST0331, patients received four cycles of VAC (with a lower dose of cyclophosphamide; see Table 59-9) and then VA to a total of 24 weeks (groups I and IIA) or 46 weeks (all others). Local therapy was based on response assessments beginning at week 11 to reduce or avoid radiation. Of 37 group IIA or III patients, only 4 received radiation; 4 had tumor progression before local treatment. However, the local recurrence rates were 26% and 43% on D9602 and ARST0331, respectively, and there appeared to be a strong relationship between total cyclophosphamide dose and risk of local recurrence. Therapy after recurrence was not collected systematically, but OS was excellent despite the local recurrences (100% on D9602 and 94% on ARST0331).[370] Successful treatment of vaginal RMS without mutilating surgery or irradiation appears possible using alkylating agent–containing chemotherapy (VAC or IVA regimens) and careful local surveillance. Fortunately, females are much less likely than males to be sterilized by alkylator chemotherapy.

Parameningeal Tumors

The parameningeal sites include the nasal cavity and nasopharynx, parapharyngeal space, paranasal sinuses (ethmoid, maxillary, and sphenoid), the pterygopalatine and infratemporal fossae, and the middle ear and mastoid sinuses. Parameningeal RMS has a tendency to invade the cranium and intracranial contents, either by eroding through the skull base or by extending through a foramen. Curiously, tumors arising in the orbits are much less aggressive. Complete excision of parameningeal tumors at diagnosis is almost never possible, so that they are at best stage II, group III in the IRS-COG staging system. The vast majority are of embryonal histology.

In the IRS-I study, 5-year OS was only 47%[280]; it was identical in CWS-81.[371] The IRS-II study intensified therapy for patients with parameningeal tumors with craniospinal radiation and intrathecal chemotherapy (methotrexate, cytarabine, and hydrocortisone) beginning immediately after diagnosis, and OS improved to 67%.[281,372] IRS-III substituted whole-brain for craniospinal radiation therapy and began it early only for patients with cranial nerve palsies or intracranial extension or base-of-skull erosion on imaging; chemotherapy was also considerably more intense. Survival improved again, to 73%, but 5 patients (3.4%) developed ascending myelitis, which was fatal in 3.[373] In IRS-IV, intrathecal chemotherapy was eliminated, and the radiation field was reduced to the involved area with a 5-cm margin, with no loss of efficacy found on a retrospective analysis of 611 IRS II-IV patients.[372]

A univariate analysis of parameningeal RMS patients in IRS II and IRS-IV identified age between 1 and 9 years; site in the nasal cavity, nasopharynx, parapharynx, middle ear, or mastoid; and lack of bony erosion or intracranial extension as favorable features.[372] Patients with entirely favorable features had a 92% OS at 5 years, compared with 57% for those with entirely unfavorable features. Size and histology were not statistically significant factors.

Results in Europe lagged far behind, with 5-year OS 55% in SIOP, 47% in Germany, and 39% in Italy.[374] An international workshop in 1994 combining the American and European experience recommended routine use of MRI to define the extent of the tumor; treatment of all parameningeal RMS patients with radiation therapy (in the SIOP-84 study 18 of 44 had not been irradiated, with only 5 surviving); a minimum dose of 50 Gy with a 2-cm margin; and routine external quality control of radiation.[374]

Recent results have largely settled the long-running controversy over the timing of radiation in parameningeal RMS, as discussed in an earlier section.

Relapsed Rhabdomyosarcoma

The prognoses of patients whose RMS relapses are highly variable, but mostly depend on initial tumor characteristics and treatment. An analysis of survival after relapse in 605 IRS-III, IRS-IV pilot, and IRS-IV patients showed 5-year survival rates ranging from 3% (for patients with alveolar histology and originally group II through group IV tumors) to 72% (for patients with originally stage I or group I tumors and embryonal histology).[375] The median survival for all relapsed patients was 9.8 months, and OS was 17% at 5 years. Postrelapse survival for patients with embryonal histology was far better than for those with alveolar or undifferentiated tumors, but other prognostic factors were different for the two histologic groups.

An analysis of relapsed patients from Italian RMS studies (n = 125) identified metastatic relapse, unfavorable primary site, alveolar histology, and relapse or progression during initial therapy to be unfavorable features. When each of those was used as a risk factor, the 5-year survival ranged from 72% with no risk factors to nil with three or four factors.[376] The analysis only included patients with initially localized tumors, which may be why the median survival after relapse (15.4 months) and 5-year OS (28%) were better than those observed in the IRSG study.

The most sophisticated analysis of postrelapse survival was based on nonmetastatic patients who experienced relapse after treatment on the SIOP MMT-84, MMT-89, and MMT-95 studies (n = 474). Two thirds of the relapsed patients had not had irradiation as part of their original treatment, reflecting the philosophy behind the studies. In a multivariate analysis, the prognostic variables were the type of chemotherapy (2, 3, or 6 drugs), histology, nodal status, primary site, original size, metastatic or local recurrence, radiation therapy as part of initial treatment, and time to recurrence. These eight variables were combined into a nomogram, permitting estimation of prognosis for an individual patient.[377] The remarkably high OS rate (36% at 5 years) reflects the exclusion of patients whose tumors were metastatic at diagnosis, and the omission of initial radiation therapy in a large proportion of patients.

For treatment of relapsed RMS, after a COG trial in patients with metastases at diagnosis demonstrated that vincristine-irinotecan was superior to vincristine alone,[314] the COG conducted a randomized controlled trial in relapsed patients of two schedules of vincristine-irinotecan “window” therapy followed by five-drug alternating chemotherapy (vincristine-doxorubicin-cyclophosphamide/ifosfamide-etoposide). This showed that a 5-day schedule with irinotecan 50 mg/m^2/day was less toxic, and at least as effective, as a “daily × 5 × 2” schedule that used 20 mg/m^2/day irinotecan for 5 days on 2 consecutive weeks. Unfortunately, the median survival remained dismal at 1.3 and 1.4 years, as did FFS (14% and 15% at 3 years).[315] The most recent COG study (ARST0921) was a randomized phase II trial comparing bevacizumab and temsirolimus added to concurrent vinorelbine and cyclophosphamide. The results are pending.

Megatherapy for Metastatic and Recurrent Rhabdomyosarcomas

A perennial question in the treatment of patients with metastases at diagnosis, or who have experienced relapse, is the role of megatherapy: high-dose chemotherapy, with or without total-body irradiation, with autologous (occasionally allogeneic) stem cell or marrow transplantation. The analytic problems are large: there are vast differences between different populations of patients with metastases, with 3-year survivals ranging from 5% to 47% and an even wider range of prognoses for patients with relapsed RMS (see earlier). There are also wide variations in entry criteria for megatherapy studies, ranging from having a complete response to initial salvage chemotherapy, to failing to achieve a complete response to initial chemotherapy. Often, the entry criteria and key terms (e.g., “high risk”) remain undefined. For all these reasons, reports of results of megatherapy in RMS merit careful scrutiny (see the excellent review by Meyers[378]).

Two recent systematic reviews of high-dose therapy in metastatic RMS could find no randomized controlled trials, and only two historically controlled trials, each with serious methodologic limitations and potential biases. The reviews concluded that there is no evidence to justify the use of megatherapy in metastatic RMS patients outside a controlled clinical trial.[379,380] Until randomized controlled trials are performed, the role of megatherapy in RMS will remain dubious.

FOLLOW-UP AFTER TREATMENT

Surveillance and Patterns of Recurrence

Examination of Kaplan-Meier plots of many studies of newly diagnosed RMS shows that the vast majority of failures (progression or relapse) occur in the 3 years after diagnosis, although they can occur even 9 years after diagnosis. The complex nature of RMS persists as complex patterns of failure, varying by primary site and histology, and being local, regional (lymph nodes), metastatic, or any combination. In IRS-III, for example, local failures outnumbered distant ones by 7:1 among patients with orbital primary tumors; but among patients with extremity primary sites, distant and combined failures outnumbered local ones by almost 7:1. Among patients with embryonal histology tumors, local failures outnumbered combined and distant failures by about 2:1; but among patients with alveolar and "other" histology, combined and distant failures were more common.[381]

In the same analysis of failure patterns in IRS-III, univariate analysis identified N1 (clinically positive) nodal status as the key risk factor for local or combined local and regional or distant failure; N1 nodal status was, in turn, associated with older age, alveolar histology, large (>5 cm) tumors, and unfavorable primary sites.[381] Distant failure was most likely in patients with N1 nodes and primary tumors in limbs.

A review of IRS-IV data showed that achievement of a complete response at the end of therapy (no residual tumor present on examination and conventional imaging) appears to have no effect on prognosis in group III RMS patients; nor does it influence local, regional, or distant failure rates (e.g., complete vs. partial or no response 13.5% vs. 13.5% for local failure). Similarly, resection of a residual mass at the end of therapy did not appear to improve outcome.[382]

Failure patterns appear to have changed across the first three IRS studies, but this may be due to shifts in definitions and classifications. Attention to definitions is again necessary when assessing reports, because, for example, failures in regional lymph nodes may be considered local (if they were within the radiation therapy field), regional, or distant.

The follow-up intervals and procedures prescribed in COG RMS protocols reflect the changes in risk over time. They include a history, physical examination, and chest imaging (radiograph or CT) every 3 months during the first year off therapy, every 4 months during the second and third years, and every 6 months during the fourth year, with primary site imaging depending on the site and the primary tumor treatment used. During and annually after this follow-up for tumor recurrence, monitoring for late effects of treatment is also necessary, especially for tissues in a radiation field.

Late Effects

The late effects of childhood cancer and its treatment are discussed thoroughly in Chapter 71. In patients treated for RMS, most observed late effects are from the tumor itself (e.g., cranial nerve palsies from parameningeal tumors), surgery, and radiation therapy. Radiation therapy sequelae are particularly problematical because of the young ages of many of the patients and the locations of their tumors.

Chemotherapy late effects particularly relevant to RMS include late hemorrhagic cystitis from cyclophosphamide and ifosfamide and secondary myelodysplasia or leukemia. Ifosfamide may cause a nephropathy, which is occasionally progressive.[383] Relatively few patients treated on current protocols receive anthracyclines (with their attendant risk of late heart disease) or cisplatin (with its hearing loss and renal dysfunction). Sterility in males and early menopause in females may result from the high doses of alkylating agents used.

FUTURE DIRECTIONS

Among ERMS there is an enormous range of clinical behavior that varies with site and age. Fusion-bearing ARMS is a highly aggressive tumor, with the PAX7-FOXO1–bearing variant perhaps being less so. However, we remain far from having molecularly based classification of RMS that correlates with, much less explains, the enormous variations observed in clinical behavior, especially among embryonal (fusion-negative) tumors. Techniques such as gene expression profiling, application of epigenetic analyses, and increasingly sophisticated evaluation of combined clinical and laboratory data may allow us to discern the combinations of tumor and host variables that make, for example, a paratesticular ERMS much more aggressive in a teenager than in a preschooler.

More sophisticated classification opens opportunities for "tailoring" chemotherapy but also presents the challenge of smaller populations of patients for study. In the COG, it is already barely statistically feasible to carry out randomized controlled trials with intermediate-risk patients in 5 years. International collaboration has the benefit of providing larger patient numbers, but having fewer studies means that fewer ideas are tested. The bureaucratic and regulatory barriers are also formidable. The path through these obstacles is not obvious.

Therapeutically, there are two challenges: identifying promising new agents and integrating them into existing therapy. One cannot predict which of the many newly discovered biochemical pathways and small molecule inhibitors will lead to improvements in outcome in RMS patients. The numbers of patients are too small to provide an attractive market for the pharmaceutical industry, so an agent that would, for example, specifically block the action of the PAX-FOXO1 fusion proteins is unlikely to become available. Downstream of the fusion proteins lay potential pharmacologic targets that may well be commercially interesting, however. Immunotherapy is a possibility for the future if appropriate antigens can be found and paired with the appropriate technology.[384]

The quest for new targets and agents is very unlikely to lead to a "magic bullet" that makes current therapy obsolete. Thus we must continue our efforts with "traditional" chemotherapy and carefully integrate new agents. The closest to clinical application are probably the tyrosine kinase inhibitors (particularly pazopanib) and "rapalog" mTOR inhibitors, but the former have only been tested in mice, and one of the latter is in a phase II trial in combination with chemotherapy. Large improvements in outcome have occurred in the leukemias in the past decade, with a collection of drugs that are at least 25 years old; one of the advantages of that disease system is easy evaluation of response through marrow aspiration. Good early response markers (perhaps PET) could extend the same advantage to RMS and other solid tumors.

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CHAPTER 60

Nonrhabdomyosarcoma Soft Tissue Sarcomas and Other Soft Tissue Tumors

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CHAPTER OUTLINE

EPIDEMIOLOGY
PRESENTATION AND EVALUATION
STAGING AND GRADING
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Fibrosarcoma Arising in Dermatofibrosarcoma Protuberans and Giant Cell Fibroblastoma
Low-Grade Fibromyxoid Sarcoma
Myxofibrosarcoma
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Inflammatory Myofibroblastic Tumor
SO-CALLED FIBROHISTIOCYTIC TUMORS
Plexiform Fibrohistiocytic Tumor
SMOOTH MUSCLE TUMORS
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Leiomyosarcoma Associated with the Epstein-Barr Virus
VASCULAR TUMORS
Epithelioid Hemangioendothelioma
Giant Cell Angioblastoma
Kaposiform Hemangioendothelioma
Kaposi Sarcoma
Angiosarcoma
CHONDROOSSEOUS TUMORS
Mesenchymal Chondrosarcoma
Extraskeletal Myxoid Chondrosarcoma
TUMORS OF UNCERTAIN DIFFERENTIATION
Synovial Sarcoma
Alveolar Soft Part Sarcoma
Clear Cell Sarcoma
Desmoplastic Small Round Cell Tumor
Angiomatoid Fibrous Histiocytoma
Extrarenal Rhabdoid Tumor
Epithelioid Sarcoma
Extraosseous Ewing Sarcoma
Gastrointestinal Stromal Tumor
Ectomesenchymoma
NEUROECTODERMAL TUMORS
Malignant Peripheral Nerve Sheath Tumors
Melanotic Neuroectodermal Tumor of Infancy
UNDIFFERENTIATED TUMORS
Undifferentiated Pleomorphic Sarcoma and Undifferentiated Round Cell and Spindle Cell Sarcoma

Soft tissue sarcomas of childhood and adolescence constitute a heterogeneous group of tumors that exhibit features of mesenchymal differentiation (Tables 60-1 and 60-2). From a pediatric oncology perspective, the various types of rhabdomyosarcoma, the most common soft tissue sarcoma in younger children, are grouped separately such that the remainder of these tumors are collectively termed the *nonrhabdomyosarcoma soft tissue sarcomas* (NRSTS). This term seems an odd eponym, defining a category of soft tissue sarcoma by exclusion and grouping together widely disparate clinicopathologic entities distinguished by clinical features, molecular pathogenesis, and biologic behavior. However, the term *NRSTS* carries a unique value in pediatric oncology, enabling the study of significant cohorts of children with uncommon tumors. Sarcomas in children represent a somewhat distinct set of tumors that diverge in both incidence and histology from those in adults. The types of sarcomas that develop in adolescents and young adults are similar to those seen in older individuals; however,

TABLE 60-1 World Health Organization Classification of Soft Tissue Tumors: Intermediate (Rarely metastasizing) and Malignant

	Intermediate (Rarely Metastasizing)	Malignant
Adipocytic		Dedifferentiated liposarcoma Myxoid liposarcoma Pleomorphic liposarcoma Liposarcoma, not otherwise specified
Fibroblastic/Myofibroblastic	Solitary fibrous tumor Dermatofibrosarcoma protuberans (fibrosarcomatous and pigmented) Inflammatory myofibroblastic tumor Low-grade myofibroblastic sarcoma Myxoinflammatory fibroblastic sarcoma (including atypical) Infantile fibrosarcoma	Adult fibrosarcoma Myxofibrosarcoma Low-grade fibromyxoid sarcoma Sclerosing epithelioid fibrosarcoma
So-Called Fibrohistiocytic	Plexiform fibrohistiocytic tumor Giant cell tumor of soft tissues	
Smooth Muscle		Leiomyosarcoma (excluding skin)
Pericytic (Perivascular)		Malignant glomus tumor
Skeletal Muscle		Embryonal rhabdomyosarcoma Alveolar rhabdomyosarcoma Pleomorphic rhabdomyosarcoma Spindle cell/sclerosing
Vascular Tumors	Retiform hemangioendothelioma Papillary intralymphatic angioendothelioma Composite hemangioendothelioma Pseudomyogenic (epithelioid-like) hemangioendothelioma Kaposi sarcoma	Epithelioid hemangioendothelioma Angiosarcoma of soft tissue
Chondroosseous		Extraskeletal mesenchymal chondrosarcoma Extraskeletal osteosarcoma
Nerve Sheath Tumors		Malignant peripheral nerve sheath tumor Epithelioid malignant nerve sheath tumor Malignant triton tumor Malignant granular cell tumor Ectomesenchymoma
Gastrointestinal Stromal Tumors		Gastrointestinal stromal tumors, malignant (AFIP groups 3b, 5, 6a, 6b)
Uncertain Differentiation	Ossifying fibromyxoid tumor Myoepithelioma Parachordoma	Synovial sarcoma (including spindle cell and biphasic) Epithelioid sarcoma Alveolar soft part sarcoma Clear cell sarcoma of soft tissue Extraskeletal myxoid chondrosarcoma Extraosseous Ewing sarcoma Desmoplastic small round cell tumor Extrarenal rhabdoid tumor Neoplasms with perivascular epithelioid cell differentiation (PEComa) (includes clear cell myomelanocytic tumor Intimal sarcoma
Undifferentiated/Unclassified		Undifferentiated spindle cell sarcoma Undifferentiated pleomorphic sarcoma Undifferentiated round cell sarcoma Undifferentiated epithelioid sarcoma Undifferentiated sarcoma, not otherwise specified

Modified from Fletcher CD, Bridge JA, Hogendoorn PCW, et al, editors: World Health Organization classification of tumors of soft tissue and bone, *Lyon, 2013, IARC Press.*

AFIP, Armed Forces Institute of Pathology.

TABLE 60-2 World Health Organization Classification of Soft Tissue Tumors: Benign and Intermediate (Locally Aggressive)

	Benign	Intermediate (Locally Aggressive)
Adipocytic	Lipoma Lipomatosis Lipomatosis of nerve Lipoblastoma/lipoblastomatosis Angiolipoma Myolipoma Chondroid lipoma Extrarenal angiomyolipoma Extraadrenal myelolipoma Spindle cell/pleomorphic lipoma Hibernoma	Atypical lipomatous tumor/ well-differentiated liposarcoma
Fibroblastic/Myofibroblastic	Nodular fasciitis Proliferative fasciitis Proliferative myositis Myositis ossificans Fibroosseous pseudotumor of digits Ischemic fasciitis Elastofibroma Fibrous hamartoma of infancy Fibromatosis coli Juvenile hyaline fibromatosis Inclusion body fibromatosis Fibroma of tendon sheath Desmoplastic fibroblastoma Mammary-type myofibroblastoma Calcifying aponeurotic fibroma Angiomyofibroblastoma Cellular angiofibroma Nuchal-type fibroma Gardner fibroma Calcifying fibrous tumor	Palmar/plantar fibromatosis Desmoid-type fibromatosis Lipofibromatosis Giant cell fibroblastoma
So-Called Fibrohistiocytic	Tenosynovial giant cell tumor (localized and diffuse types and malignant) Deep benign fibrous histiocytoma	Plexiform fibrohistiocytic tumor Giant cell tumor of soft tissues
Smooth Muscle	Deep leiomyoma	
Pericytic (Perivascular)	Glomus tumor (and variants) Myopericytoma	
Skeletal Muscle	Rhabdomyoma (adult, fetal and genital types)	
Vascular Tumors	Hemangioma (synovial, venous, arteriovenous hemangioma/ malformation, lymphangioma) Epithelioid hemangioma Angiomatosis Lymphangioma	Kaposiform hemangioendothelioma
Gastrointestinal Stromal Tumors	Benign gastrointestinal stromal tumor (AFIP prognostic groups 1, 2, 3a)	
Nerve Sheath Tumors	Schwannoma Melanotic schwannoma Neurofibroma (including variants and plexiform neurofibroma) Perineuroma (malignant perineuroma) Granular cell tumor Dermal nerve sheath myxoma Solitary circumscribed neuroma Ectopic meningioma Nasal glial heterotopia Benign triton tumor Hybrid nerve sheath tumors	
Chondroosseous	Soft tissue chondroma	
Uncertain differentiation	Acral fibromyxoma Intramuscular myxoma Juxtaarticular myxoma Deep ("aggressive") angiomyxoma Pleomorphic hyalinizing angiectatic tumor Ectopic hamartomatous thymoma	Hemosiderotic fibrolipomatous tumor

Modified from Fletcher CD, Bridge JA, Hogendoorn PCW, et al, editors: World Health Organization classification of tumors of soft tissue and bone, *Lyon, 2013, IARC Press.*

AFIP, Armed Forces Institute of Pathology.

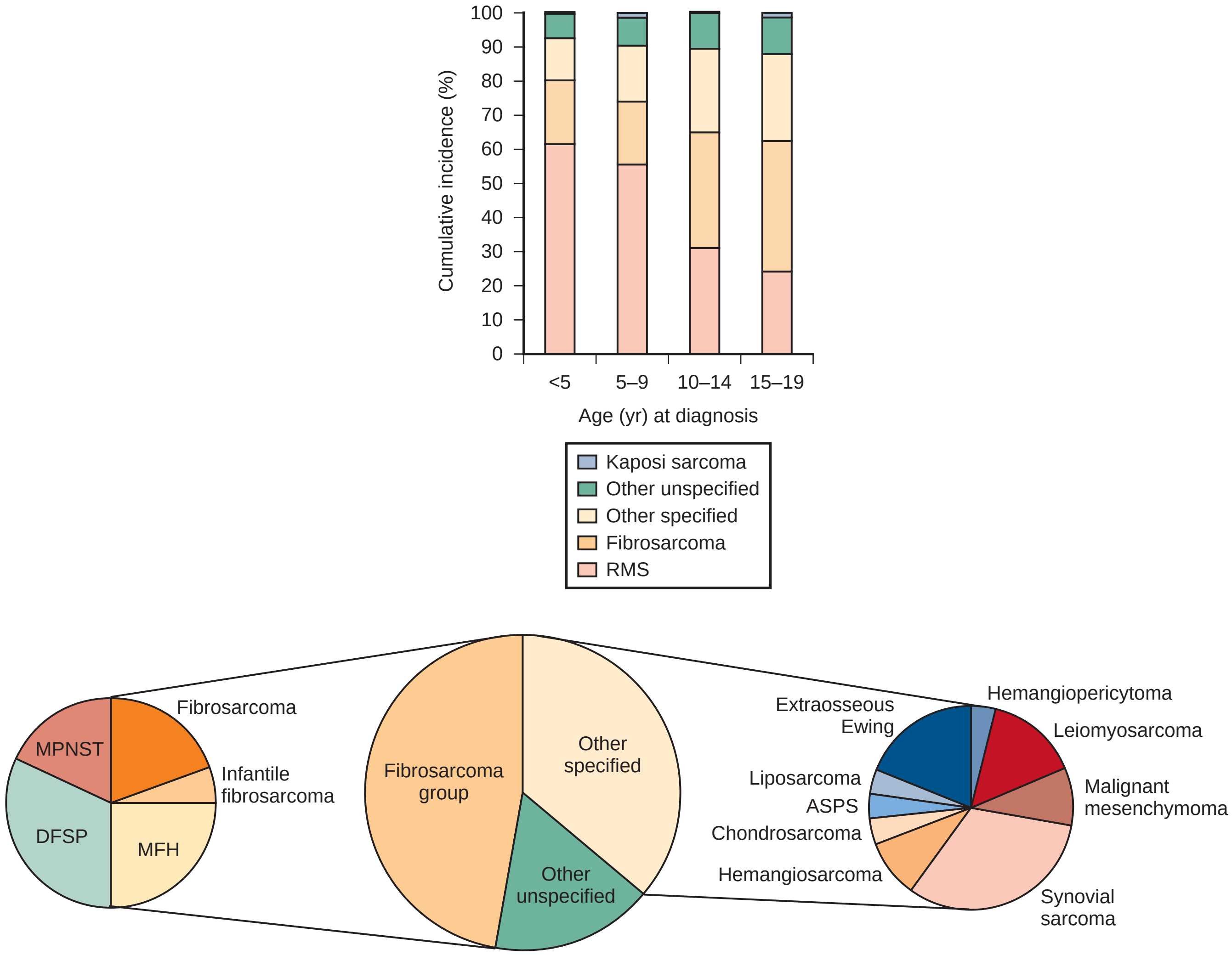

Figure 60-1 Incidence and distribution of pediatric soft tissue sarcomas. **A,** Age-adjusted incidence for soft tissue sarcomas of childhood grouped according to the International Classification of Childhood Cancer. **B,** Depiction of age-adjusted incidence for the specific tumors that comprise the major classifications of NRSTS. *ASPS,* Alveolar soft part sarcoma; *DFSP,* dermatofibrosarcoma protuberans; *MFH,* malignant fibrous histiocytoma; *MPNST,* malignant peripheral nerve sheath tumor; *RMS,* rhabdomyosarcoma. *(Data from Kramarova E, Stiller CA: The International Classification of Childhood Cancer.* Int J Cancer. *68:759–765, 1996; and Gurney JG, Young JL, Jr., Roffers SD, et al: Soft tissue sarcomas. In Ries LAG, Smith MA, Gurney JG, et al, editors:* Cancer incidence and survival among children and adolescents: United States SEER Program 1975–1995. *NIH publication No. 99-4649, Bethesda, Md., 1999, National Cancer Institute, p. 111–124.)*

the relative incidence and response to therapy may differ.[1] In addition, the term *NRSTS* permits the flexibility to include undifferentiated tumors and tumors that, based on treatment, are typically excluded from other groupings (e.g., desmoplastic small round cell tumor [DSRT]). Finally, by evoking those tumors prevalent in the pediatric population, the term *NRSTS* also permits consideration of the unique toxicities specific to children when evaluating treatment approaches and designing clinical trials.

NRSTS are often characterized by distinct, consistent cytogenetic, transcriptional, and intracellular signaling changes. Exploring these molecular alterations may offer insights into the biology of mesenchymal development and the pathogenesis of these tumors, thus leading to the development of new treatment strategies.

EPIDEMIOLOGY

In children younger than 5 years, rhabdomyosarcoma constitutes the most frequent type of soft tissue sarcoma (STS), however by late school age and continuing into adolescence the incidence of NRSTS rises to exceed that of rhabdomyosarcoma (Fig. 60-1). The total incidence for STS in children and adolescents younger than 20 years is approximately 11 per million, which represents 7.4% of cancer in this population.[2] NRSTS constitute about one half of these cases or 3% to 4% of childhood malignancies. Within the class of NRSTS, specific tumor types demonstrate a strong age association. Although the median age at diagnosis is approximately 13 years, NRSTS prevalent in younger children include infantile fibrosarcoma and rhabdoid tumor, whereas synovial sarcoma and malignant peripheral nerve sheath tumor (MPNST) are more common in adolescents.[3-9]

Although the vast majority of NRSTS occur spontaneously, several cancer predisposition syndromes are associated with the development of STS. Families that harbor mutations of the tumor suppressor gene *TP53* (i.e., those with Li-Fraumeni syndrome) are prone to the development of many types of cancer.[10,11] STS as well as osteosarcoma can be associated with germline *TP53* mutation.[12] The cell cycle regulatory protein RB has also been implicated in STS development. *RB* was originally identified as the locus deleted in hereditary and sporadic retinoblastoma.[13] However, retinoblastoma survivors are at risk for the development of STS, including fibrosarcoma, pleomorphic sarcoma, and leiomyosarcoma, and molecular analysis of these tumors may demonstrate homozygous *RB* deletion.[14,15] In addition to other tumors, neurofibromatosis type 1 (NF1, von Recklinghausen disease) is associated with the development of MPNSTs and multifocal gastrointestinal stromal tumors (GISTs).[16,17] Radiation therapy for optic nerve glioma in patients with NF1 increases the risk for subsequent MPNST development.[18,19] Carney triad is associated with multifocal GIST (as well as paragangliomas and pulmonary chondromas).[20,21] Individual Carney triad tumors are associated with specific nongermline mutations, although a possible common association with chromosome 1 changes has been reported.[22] Carney-Stratakis dyad, characterized by paraganglioma and GISTs, is associated with germline mutations in *SDHA, SDHB,* or *SDHC.*[23-25] Gardner syndrome, a phenotypic variant of familial adenomatous polyposis (FAP), results from mutation of the *APC* gene and is associated with an increased risk for abdominal desmoid tumor.[26-30] Gorlin syndrome, caused by mutation of the Hedgehog signaling pathway regulator *PTCH,* is associated with undifferentiated sarcoma.[31] Werner syndrome is caused by mutations of the deoxyribonucleic acid (DNA) helicase gene *RECQL2* or nuclear lamin gene *LMNA* and is associated with several cancers, including STS in early and middle adulthood.[32-34] Typically resulting from mutations in *TSC1* or *TSC2,* dominantly inherited tuberous sclerosis is associated with spontaneously resolving cardiac rhabdomyomas in infants and renal angiomyolipoma, including the malignant epithelioid variant, as well as pulmonary lymphangiomyomatosis.[35-38] Pleomorphic sarcoma in adults has infrequently been associated with Lynch syndrome, which is characterized by mutations in the DNA mismatch repair genes *MLH1* and *MLH2.*[39]

In addition to cancer predisposition syndromes, environmental exposures have been linked to sarcoma development. Although osteosarcoma predominates, radiation exposure is associated with NRSTS, including fibrosarcoma and pleomorphic sarcoma, with an incidence associated with the dose of radiation.[40-43] Of the several environmental chemical exposures that have been linked to the development of sarcomas in adults, vinyl chloride exposure demonstrates the strongest association to the development of angiosarcoma.[44] Childhood cancer survivors also appear to be at ninefold higher risk for the development of sarcomas (particularly NRSTS) in comparison with the general population, with a mean time from primary cancer diagnosis of 11 years.[45] Although an initial diagnosis of sarcoma was associated with the greatest risk, perhaps indicating an underlying predisposition, treatment with radiation or alkylating agents was also positively associated with sarcoma development. Epstein-Barr virus infection in individuals infected with human immunodeficiency virus (HIV) predisposes to leiomyosarcoma.[46]

PRESENTATION AND EVALUATION

NRSTS typically presents as a persistent or enlarging, asymptomatic mass. Although NRSTS may arise in any location, extremities constitute the most common sites. However, within the first year of life, soft tissue tumors tend to develop in the trunk and head and neck.[3] A significant fraction of tumors may be associated with pain that likely indicates invasion or compression of surrounding structures.[7]

The evaluation of NRSTS is typically initiated with appropriate imaging then followed by biopsy. For most tumors, plain radiography and magnetic resonance imaging (MRI) are justified. Technetium-99m–labeled methyldiphosphonate (^{99m}Tc MDP) nuclear scintigraphy can localize sites of bony metastatic disease. Because as a group these tumors tend to metastasize to the lungs, chest computed tomography (CT) is commonly indicated. Imaging may narrow the differential diagnosis and will aid in assessing resectability by identifying tumor margins, involved tissue planes, and relationship to neurovascular structures. Fluorine-18 (^{18}F)-labeled fluorodeoxyglucose (FDG) or ^{18}F-labeled fluorothymidine (FLT) positron emission tomography (PET) has been used for sarcoma staging, and combined PET/CT can identify primary tumors as well as metastatic spread and may detect metastatic lesions missed by bone scintigraphy.[47] However, small lesions (<1 cm) are difficult to visualize by PET. FLT-PET has been used to distinguish between low- and high-grade sarcomas.[48]

Accurate tissue diagnosis is critical to establish a treatment plan and to predict prognosis.[5] Because of the diverse range of sarcoma subtypes as well as their rarity, arriving at a definitive diagnosis can be challenging.[49] The complexity of NRSTS diagnosis and grading requires the integration of histopathology with immunohistochemical, ultrastructural, cytogenetic, and molecular studies (Table 60-3). Advance communication between the surgeon or interventional radiologist, pathologist, and pediatric oncologist can help ensure that sufficient material is obtained and suitably processed. Fine-needle aspiration often generates an inadequate specimen to yield a specific diagnosis. Multiple core needle biopsies may be sufficient to initiate the evaluation, with additional tissue resulting from eventual resection. Although sentinel lymph node biopsy is not a standard component of NRSTS evaluation, it could be considered for high-grade sarcomas such as clear cell sarcoma, synovial sarcoma, vascular sarcomas, and undifferentiated sarcomas.[50-52]

In general, sarcomas can be classified as those with a recognizable recurrent chromosomal rearrangement

TABLE 60-3 Diagnostic Studies in the Evaluation of Nonrhabdomyosarcoma Soft Tissue Sarcomas

		RECURRENT CYTOGENETIC CHANGES	
Tumor	**Immunohistochemical Markers**	Associated Karyotype	Genes Implicated
Alveolar soft part sarcoma	TFE3, MyoD1 (cytoplasmic), desmin (50%), S100 protein (25%), MSA (in some)	der(17)t(x;17)(p11;q25)	*ASPSCR1 (ASPL, TUG, RCC17), TFE3*
Angiomatoid fibrous histiocytoma	Vimentin, desmin (50%), CD68 (50%), CD99 (50%), EMA (40%)	t(2;22)(q33;q12) t(12;16)(q13;p11) t(12;22)(q13;12)	*EWSR1, CREB1* *FUS, ATF1* *EWSR1, ATF1*
Angiosarcoma	ERG, factor VIII–associated (VWF), CD34, FLI1, CD31		
Congenital infantile fibrosarcoma	Vimentin, actins (30%), desmin (20%), cytokeratins (20%)	t(12;15)(p13;q25)	*ETV6, NTRK3 (TRKC)*
Clear cell sarcoma	MITF, HMB45, S100, melan A	t(12;22)(q13;q12) t(2;12)(q32.3;q12)	*EWSR1, ATF1 EWSR1, CREB1*
Dermatofibrosarcoma protuberans	CD34, apolipoprotein D	t(17;22)(q22;q13)	*COL1A1, PDGFB*
Desmoplastic small round cell tumor	WT1 (carboxy terminus), desmin (dotlike), EMA, cytokeratin, vimentin	t(11;12)(p13;q12)	*EWSR1, WT1*
Desmoid-type fibromatosis	Vimentin, actins, β-catenin (nuclear)	+8, +20	
Ectomesenchymoma	S100 protein, synaptophysin, GFAP, neurofilament protein, rhabdomyosarcoma markers (MSA, desmin, myogenin, MyoD1)	+2, −6, +11, +20	
Epithelioid sarcoma	Cytokeratins, EMA, vimentin, CD34 (60%), CA-125		
Epithelioid hemangioendothelioma	VWF, CD31, CD34 Cytokeratins (25% weak)	t(1;3)(p36.3;q25) t(X;11)(p11.23;q22.1)	*WWTR1, CAMTA1* *YAP1,TFE3*
Extraosseous Ewing sarcoma	CD99 (membranous), NSE, vimentin, cytokeratins (25%), FLI1 (nuclear)	t(11;22)(q24;q12) t(21;22)(q21;q12) t(7;22)(p22;q12) t(17;22)(q12;q22) t(2;22)(q36;q12)	*EWSR1, FLI1* *EWSR1, ERG1* *EWSR1, ETV1* *EWSR1, ETV4 (E1AF)* *EWSR1, FEV*
Extrarenal rhabdoid tumor	Vimentin, cytokeratins, EMA, CD99, NSE, synaptophysin	Abnormalities of 22q11	*SMARCB1 (hSNF5, INI1, BAF47)*
Gastrointestinal stromal tumor	CD117 (KIT), CD34		
Extraskeletal myxoid chondrosarcoma	S100 protein (50%), CD117 (KIT, 30%), cytokeratin (focal), EMA (30%) Neuroendocrine markers (chromogranin, NSE, synaptophysin) in t(9;17) tumors	t(9;22)(q22;q12) t(9;17)(q22;q11) t(9;15)(q22;q21)	*EWSR1, NR4A3 (TEC, CHN, CMSF, NOR1)* *TAF15 (RPB56, TAF2N), NR4A3* *TCF12 (HTF4), NR4A3 (TEC)*
Giant cell angioblastoma	Actins, vimentin		
Inflammatory myofibroblastic tumor	ALK, vimentin, actins, desmin (50%), cytokeratins (focal), CD68 (25%)	Multiple translocations involving 2p23	*ALK,* multiple partners
Kaposiform hemangioendothelioma	D2-40, CD31, CD34, FLI1, SMA, D2-40		
Kaposi sarcoma	D2-40, CD31, CD34, FLI1, HHV-8 (KSHV) (in situ hybridization)		
Leiomyosarcoma	SMA, desmin, h-caldesmon	Complex	

Continued on following page

TABLE 60-3 Diagnostic Studies in the Evaluation of Nonrhabdomyosarcoma Soft Tissue Sarcomas (Continued)

		RECURRENT CYTOGENETIC CHANGES	
Tumor	**Immunohistochemical Markers**	Associated Karyotype	Genes Implicated
Lipoblastoma	S100 protein, desmin	del(8)(q12;q24),r(8) t(7;8)(p22;q13)	*HAS2, PLAG1* *COL1A2, RAD51L1*
Low-grade fibromyxoid sarcoma	Vimentin, actins (focal)	t(7;16)(p33;p11)	*FUS, CREB3L1, CREB3L2 (BBF2H7)*
Malignant peripheral nerve sheath tumor	S100 (50-90%), GFAP (50%-90%), CD57 (50%), myelin basic protein (40%), TP53	Numerical and structural abnormalities of chromosomes 1, 4, 7, 11, 12, 14, 16, 17, and 22	
Mesenchymal chondrosarcoma	S100 protein (cartilaginous component) NSE, CD57, CD99 (membranous) (undifferentiated component)	Complex cytogenetics alterations del(8)(q13.3;q21.1)	*HEY1, NCOA2*
Myxoid liposarcoma	S100 protein	t(12;16)(q13;p11) t(12;22)(q13;q12)	*FUS (TLS), DDIT3 (CHOP)* *EWSR1, DDIT3 (CHOP)*
Plexiform fibrohistiocytic tumor	CD68, actins (70%), EMA (30%), BCL2 (30%)		
Solitary fibrous tumor	STAT6 (nuclear), CD34, CD99 (cytoplasmic)		*NAB2, STAT6*
Synovial sarcoma	EMA, cytokeratins, S100 protein (30%), CD99 (cytoplasmic), BCL2, TLE1	t(x;18)(p11;q11)	*SS18 (SYT), SSX1* *SS18 (SYT), SSX2*
Undifferentiated round cell and spindle cell sarcomas			*EWSR1, NFATC2, PATZ1, POU5F1, SMARCA5, SP3* *CIC, DUX4*

EMA, Epithelial membrane antigen; *GFAP,* glial fibrillary acidic protein; *HHV-8,* human herpesvirus 8; *MITF,* microphthalmia-associated transcription factor; *MSA,* muscle-specific actin; *NSE,* neuron-specific enolase; *VWF,* von Willebrand factor.
Note: Immunohistochemical markers and cytogenetic changes found in a large percent of tumors. Less common abnormalities are not listed.

(typically translocation) and those without. The identification of cytogenetic abnormalities, many of which are highly specific of certain pediatric and young adult NRSTS, is helpful in arriving at or confirming a diagnosis (see Table 60-3). However, the same gene can be translocated in different tumor types. For example, the *EWSR1* gene is rearranged in DSRT, clear cell sarcoma, myxoid liposarcoma, and a subset of undifferentiated sarcomas in addition to Ewing sarcoma. In contrast, the rearrangement of certain genes is highly restricted, such as *SS18* (*SYT*) rearrangement in synovial sarcoma. Molecular studies (e.g., polymerase chain reaction [PCR] assay and interphase fluorescence in situ hybridization [FISH]) may be required to evaluate for specific cytogenetic abnormalities, especially if there a high index of suspicion and standard cytogenetics are unavailable or unremarkable.[53] High-throughput DNA sequencing and other comprehensive genomic approaches have rapidly emerged as strategies to identify genetic alterations in sarcomas. Ongoing efforts to systematically identify mutations in select STS include the Pediatric Cancer Genome Project and the Cancer Genome Atlas. Although translocations can be detected relatively easily when sequencing the whole genome, owing to significant expense and technical considerations, most current high-throughput sequencing applications focus on exonic sequence variation and consequently are not designed to detect intronic breakpoints. However, RNA sequencing as well as whole exome and targeted sequencing of cancer-associated genes offers the potential to identify individuals with specific and therapeutically actionable mutations. To take advantage of emerging techniques for molecular analyses and biomarker identification, additional tumor material, if possible, should be preserved at −70° C for DNA or ribonucleic acid (RNA) analysis. For those sarcomas that may metastasize to the bone marrow, aspiration and biopsy may be performed to complete the evaluation for distant spread.

STAGING AND GRADING

As with all cancers, appropriate recommendations for therapy depend on accurate predictions of clinical course and prognosis. Clinically informative classification schemes will accurately predict tumor outcome and will aid in the development and interpretation of multicenter trials necessary to study these uncommon cancers. Studies of adult extremity STS suggest that grade followed by tumor depth and size are of independent prognostic value for the rates of distant metastases and tumor mortality.[54] However, the clinicopathologic classification of pediatric NRSTS poses unique challenges. The low incidence of these tumors results in grouping potentially disparate tumors in clinical trials. Patient age may influence outcome. For some tumors, high grade does not predict clinically aggressive behavior, whereas other tumors behave

TABLE 60-4 Children's Oncology Group Histologic Grading System

Grade	Description
1—Low	Myxoid and well-differentiated liposarcoma Well-differentiated or infantile (≤4 years old) fibrosarcoma Well-differentiated or infantile (≤4 years old) hemangiopericytoma Well-differentiated malignant peripheral nerve sheath tumor Extraskeletal myxoid chondrosarcoma
2—Intermediate	Sarcomas not specifically included in grade 1 and 3 and in which: • <15% of the surface area shows necrosis • Mitotic count is ≤5/10 high-power fields using a 40× objective • Absence of marked nuclear atypia* • Tumor is not markedly cellular*
3—High	Pleomorphic or round cell liposarcoma Mesenchymal chondrosarcoma Extraskeletal osteosarcoma Malignant triton tumor Alveolar soft part sarcoma Sarcomas not included in grade 1 with >15% of surface area with necrosis or ≥5 mitoses/10 high-power fields using a 40× objective

Modified from Parham DM, Webber BL, Jenkins JJ, III, et al: Nonrhabdomyosarcomatous soft tissue sarcomas of childhood: formulation of a simplified system for grading. Mod Pathol *8:705–710, 1995; and Khoury JD, Coffin CM, Spunt SL, et al: Grading of soft tissue sarcoma in children and adolescents.* Cancer *116:2266–2274, 2010.*

*Secondary criteria. Tumor diagnoses specified in grade 1 or 3 are excluded from grade 2.

TABLE 60-5 Surgical-Pathologic Grouping System of the Intergroup Rhabdomyosarcoma Study

Group	Definition
Low-Stage	
I	Localized disease, completely resected
II	Total gross resection with evidence of regional spread
IIa	Grossly resected tumor with microscopic residual disease
IIb	Regional disease with involved nodes, completely resected with no microscopic residual disease
IIc	Regional disease with involved nodes, grossly resected but with evidence of microscopic residual disease and/or histologic involvement of the most distal regional lymph node from the primary site in the dissection.
High-Stage	
III	Incomplete resection with gross residual disease
IV	Distant metastatic disease present at onset

Modified from Meza JL, Anderson J, Pappo AS, et al: Analysis of factors in patients with nonmetastatic rhabdomyosarcoma treated on Intergroup Rhabdomyosarcoma Studies III and IV: the Children's Oncology Group. J Clin Oncol *24:3844–3851, 2006.*

aggressively regardless of histologic appearance. To address these issues, the Pediatric Oncology Group (POG) grading system incorporates histology, cytologic features, and age to classify NRSTS as low, intermediate, or high grade (Table 60-4).[55] Low grade is defined by histologic type, whereas intermediate grade takes into account necrosis and mitosis with secondary consideration of nuclear atypia and tumor cellularity. High-grade tumors include those of specific histologies as well as tumors with more than 15% necrosis or five or more mitoses/10 high-power field. The National Cancer Institute (NCI) and Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) grading specifications are alternative methodologies validated on sarcomas in adults.[56-59] Similar to the POG system, they take into consideration mitotic index, necrosis, and histologic type or tumor differentiation. The POG and FNCLCC perform similarly in their ability to correlate with outcome.[60] Gene expression signatures have also been used to predict outcomes in non–translocation-associated sarcomas in adults.[61]

Pediatric multicenter studies also make use of the surgical-pathologic grouping system of the Intergroup Rhabdomyosarcoma Study (IRS) (Table 60-5), which incorporates tumor spread and outcome of surgical resection, or the International Society of Pediatric Oncology (SIOP)-International Union Against Cancer TNM classification system for pediatric tumors (Table 60-6).[62-64] STS may also be classified according to the American Joint Commission on Cancer (AJCC) or Enneking staging systems, which integrate tumor size, location relative to the superficial fascia, regional lymph node status, presence of distant metastases, and histologic grade (Table 60-7).[65,66]

NRSTS most commonly spread locally and hematogenously but may exhibit regional lymph node metastases. The lung is the most common site for distant metastases, but with less frequency these tumors can metastasize to

TABLE 60-6 SIOP-UICC TNM Soft Tissue Sarcoma Clinical Staging System

Stage	Description
I	T1—Tumor confined to organ or tissue of origin N0—No evidence of regional lymph node involvement M0—No evidence of distant metastasis
II	T2—Tumor involving one or more contiguous organs or tissues or with adjacent malignant effusion N0 M0
III	Any T N1—Evidence of regional lymph node involvement M0
IV	Any T Any N M1—Evidence of distant metastases

Modified from Rodary C, Flamant D, Donaldson SS; the SIOP-IRS Committee: An attempt to use a common staging system in rhabdomyosarcoma: a report of an international workshop initiated by the International Society of Pediatric Oncology (SIOP). Med Pediatr Oncol *17:210–215, 1989.*

TABLE 60-7 American Joint Committee on Cancer Staging

Primary Tumor (T)		**Regional Lymph Nodes (N)**	
TX	Primary tumor cannot be assessed	NX	Regional lymph nodes cannot be assessed
T0	No evidence of primary tumor	N0	No regional lymph node metastasis
T1	Tumor 5 cm or less in greatest dimension[*]	N1	Regional lymph node metastasis[†]
T1a	Superficial tumor		
T1b	Deep tumor		
T2	Tumor more than 5 cm in greatest dimension[*]		
T2a	Superficial tumor		
T2b	Deep tumor		

Distant Metastasis (M)		**Histologic Grade (G)**	
		(FNCLCC System Preferred)	
M0	No distant metastasis	GX	Grade cannot be assessed
M1	Distant metastasis	G1	Grade 1
		G2	Grade 2
		G3	Grade 3

Anatomic Stage/Prognostic Groups				
Stage IA	T1a	N0	M0	G1, GX
	T1b	N0	M0	G1, GX
Stage IB	T2a	N0	M0	G1, GX
	T2b	N0	M0	G1, GX
Stage IIA	T1a	N0	M0	G2, G3
	T1b	N0	M0	G2, G3
Stage IIB	T2a	N0	M0	G2
	T2b	N0	M0	G2
Stage III	T2a, T2b	N0	M0	G3
	Any T	N1	M0	Any G
Stage IV	Any T	Any N	M1	Any G

From American Joint Committee on Cancer: AJCC cancer staging manual, *7th ed. New York, 2002, Springer-Verlag.*

[*] Note: Superficial tumor is located exclusively above the superficial fascia without invasion of the fascia; deep tumor is located either exclusively beneath the superficial fascia, superficial to the fascia with invasion of or through the fascia, or both superficial yet beneath the fascia.

[†] Note: presence of positive nodes (N1) in M0 tumors is considered Stage III.

bone or brain. Metastatic spread at the time of diagnosis likely occurs in alveolar soft part sarcoma, high-grade MPNST, clear cell sarcoma, high-grade angiosarcoma, and epithelioid sarcoma.[5]

Several factors have been shown to be prognostic for tumor-associated mortality. In both univariate and multivariate analyses of patients with grossly resected tumors, tumor invasion into contiguous structures and size (>5 cm) are the most highly predictive of decreased 5-year event free survival (EFS) and overall survival (OS). Other factors that correlate with poor outcome including female sex, older age, high-grade tumors, high-grade MPNST histology, and tumors in nonextremity locations.[5]

TREATMENT

Surgical resection constitutes the mainstay of treatment of localized tumors. Complete resection for large tumors may result in significant functional compromise, and amputation should be considered for those patients in whom limb-sparing surgery would leave residual tumor. Radiation therapy is typically reserved for those tumors for which complete resection is not possible. However, the high radiation dose required for sarcoma therapy is associated with significant growth-associated side effects in children. Although surgery with or without radiation results in good outcome for localized and low-grade disease, high-grade disease is associated with significant morbidity and mortality. With the exception of synovial sarcoma and extraskeletal Ewing sarcoma, the role of conventional chemotherapy remains ill defined. However, in pediatric NRSTS, responses can be observed and may reduce tumor burden prior to surgical resection. Biologically targeted therapies have altered the management of individual tumor types (e.g., GISTs in adults), and their roles are evolving as they enter clinical trial.

Local Control

Surgical resection is typically the initial therapy for operable nonmetastatic NRSTS. Complete resection of small (<5 cm) NRSTS results in a 5-year OS in excess of 80%.[60,67-69,70] However, large, high-grade or incompletely resected tumors have a significant chance of local recurrence. Tumor location (e.g., abdomen vs. extremity) and invasion may limit the ability to perform a wide local excision necessary to achieve tumor-free margins. For these patients, external-beam radiation therapy has been used to reduce the risk for local recurrence based on adult studies.[71-73] Pediatric studies seem to confirm that radiation therapy reduces the risk for local recurrence associated with an inability to achieve a wide surgical margin (>1 cm) for high-grade disease.[74] The impact of postoperative radiation therapy may be most significant for IRS group II patients, although grade and size may mitigate this effect.[75] Higher radiation doses are associated with increased rates of local control in NRSTS.[76,77] However, these doses are associated with abnormalities of bone development, complicating the delivery of this modality to growing children.

Brachytherapy—interstitial, intercavitary or surface placement of radioisotopes—constitutes an alternative approach to deliver radiotherapy.[78,79] Brachytherapy can precisely deliver high doses of radiation to the tumor or resection site while sparing normal tissue. Brachytherapy can also be administered concurrently with external-beam radiation, and a study of adult STS suggests that the combination may result in increased rates of local control when surgical margins are positive.[80] Other techniques to augment local control include radiofrequency ablation, cryoablation, and embolization.[81-83] Strategies for local control that incorporate site-directed chemotherapy include intralesional chemotherapy followed by electroporation at accessible sites and intraperitoneal chemotherapy infusion.[84]

Chemotherapy

Because complete resection of NRSTS is associated with a high rate of cure, chemotherapy is principally employed when tumors are unresectable, incompletely resected, or metastatic. The infrequency of each of the heterogeneous tumors that constitute the class of NRSTS makes clinical trials of chemotherapy particularly challenging. To enable multicenter groups to accrue sufficient subjects, the nonselective inclusion of NRSTS complicates the interpretation and extrapolation of most trials. Nonetheless, several consistent themes emerge. In contrast to the striking successes of chemotherapy for Ewing sarcoma and rhabdomyosarcoma, NRSTS tend to be relatively chemoresistant, with synovial sarcoma and extraosseous Ewing sarcoma as exceptions.[5,69,85,86] For patients with fully resected tumors, the inclusion of chemotherapy fails to increase survival.[87,88] The impact of chemotherapy may be most significant for patients harboring tumors with the highest risk for metastatic spread. For high grade, grossly resected tumors, chemotherapy was associated with a 5-year metastasis-free survival of 49.5% compared with 0% for untreated patients.[89] Chemotherapy may also be beneficial for patients with unresectable tumors. For both chemotherapy-sensitive and chemotherapy-resistant tumors, neoadjuvant treatment may permit the subsequent surgical resection of initially nonresectable tumors.[85] Patients with unresected or metastatic tumors have a poor outcome, although 40% to 50% of patients with advanced disease demonstrate some response (partial + complete response) to a regimen containing either ifosfamide and doxorubicin or cyclophosphamide and doxorubicin.[86,88,90] Treatment with doxorubicin, cyclophosphamide, and vincristine alternating with ifosfamide and etoposide together with aggressive local control for incompletely resected tumors or those metastatic at presentation, resulted in a partial + complete response of 80%.[90] Approximately 40% of children and adolescents with NRSTS responded to ifosfamide and doxorubicin, results similar to those for cyclophosphamide and doxorubicin.[86,88] Based on the principle that advanced sarcomas may respond to intensive therapies, the feasibility of dose-intensified neoadjuvant chemotherapy has been an end point of several trials using doxorubicin, ifosfamide, and dacarbazine in adults and doxorubicin, cyclophosphamide, ifosfamide, etoposide, and vincristine in children.[86,91-94] However, evidence in support of high-dose chemotherapy with autologous stem cell infusion remains limited.[95]

Regarding specific chemotherapeutic agents, topoisomerase 1 inhibitors may also have activity against these tumors. Topotecan with high-dose cyclophosphamide as well as irinotecan demonstrated limited efficacy against recurrent NRSTS, and the effect of irinotecan together with temozolomide is currently being studied.[96-99] The combination of vinorelbine with low-dose cyclophosphamide may also have activity against these tumors.[100] Gemcitabine and docetaxel, both of which have some activity against adult sarcomas, were more effective together than gemcitabine alone in adult patients with metastatic sarcoma.[101] In adults, advanced disease may also respond to the combination of gemcitabine and vinorelbine.[102] In children, however, the efficacy of gemcitabine or docetaxel administered individually in the setting of relapsed pediatric solid tumors seems small.[103-105] As previously mentioned, synovial sarcoma is considered an exception to the general chemoresistance of NRSTS. Children and adolescents exhibit a 5-year EFS of about 70% with decreased EFS, progression-free survival (PFS), and OS associated with tumor size (>5 cm), local invasion, and IRS grouping (III and IV).[106-108,109] Another exception is extraosseous Ewing sarcoma, which is generally grouped with bony Ewing sarcoma for treatment purposes (see Chapter 61, "Ewing Sarcoma").

Insights regarding the biology of STS are beginning to identify novel targetable pathways. The management of GISTs in adults has been altered by treatment with KIT and PDGFR-targeted tyrosine kinase inhibitors. Multitargeted tyrosine kinase inhibitors have demonstrated efficacy in nonadipocytic STS in adults, and sorafenib has activity against desmoid tumors.[110,111] Insulin-like growth factor 1 (IGF-1) inhibition has demonstrated efficacy in a fraction of patients with DSRT.[112] Targeting the mammalian target of rapamycin (mTOR) pathway demonstrated a significant, although brief, delay in disease progression in a large adult trial of sarcomas previously treated with cytotoxic chemotherapy.[113]

GENERAL BIOLOGIC FEATURES

STS can be classified into two groups based on cytogenetics. Tumors that occur in children and young adults tend to reveal relatively simple karyotypes often characterized by an easily recognizable and frequently pathognomonic reciprocal chromosomal translocation. In contrast, STS of adults typically demonstrate complex karyotypes without recurrent cytogenetic abnormalities. Cellular mechanisms for telomere maintenance may correlate with translocation status. Tumors harboring single translocations tend to have normal to short telomeres, suggestive of active telomerase, whereas tumors with complex karyotypes have longer telomeres, suggesting the use of alternative mechanisms of telomere lengthening.[114-118] Although other cellular changes, in addition to translocations, are likely to be necessary for sarcoma development, the difference between sarcomas of children and young adults and those of older individuals may reflect divergent cellular origins. Experimentally, haploinsufficiency of the nonhomologous end-joining DNA repair pathway in the context of *Ink4a/Arf*-deficient mice leads to the development of sarcoma with varied histology and karyotype.[119] Although the tumors that develop in these mice demonstrate translocations, amplifications, and deletions, the translocated regions are not recognizably syntenic with known human translocations suggesting that these sarcomas more closely reflect adult sarcomas. This finding also highlights the issue of whether complex cytogenetic abnormalities themselves result in oncogenesis or are a manifestation of genomic instability associated with the loss of cell cycle and DNA repair checkpoints.

ADIPOCYTIC TUMORS

Liposarcoma is the most common of the STS in adults.[120] In children, however, approximately 95% of adipose tumors are benign lipomas and lipoblastomas with liposarcomas accounting for the remainder.[121] Lipoblastomas tend to occur in children younger than 3 years of age, whereas liposarcomas predominate in older school-aged children and adolescents. Liposarcoma is classified into three morphologic types: well-differentiated/dedifferentiated, myxoid/round cell, and pleomorphic.[122] Morphologic types correspond to a developmental progression bearing similarities to that seen during induced mesenchymal differentiation into adipocytes.[123] Dedifferentiated/well-differentiated and pleomorphic liposarcomas occur predominantly in the retroperitoneum, whereas myxoid/round cell histology tends to occur in the extremities.[124] High-grade, dedifferentiated liposarcomas carry the highest risk for recurrence and metastasis.[125,126] In contrast to adults, most children present with the myxoid variant.[127,128] Myxoid liposarcoma is histopathologically characterized by proliferation of round to spindled primitive mesenchymal cells and scattered lipoblasts in an abundant faintly eosinophilic, homogeneous myxoid matrix (Fig. 60-2). A network of delicate straight and curvilinear capillaries is distinctive. The presence of a round cell component may increase the risk for disease progression.[129] Complete resection is associated with prolonged tumor-free survival. When margins are microscopically positive, the addition of radiation therapy may increase disease-free survival. The histologic variants of liposarcoma are associated with differences in response to chemotherapy. Myxoid liposarcoma demonstrates the highest likelihood of response to a doxorubicin and ifosfamide regimen.[130] Myxoid liposarcoma also exhibits sensitivity to trabectedin (ecteinascidin-743; ET-743), a DNA minor groove–binding small molecule.[131,132]

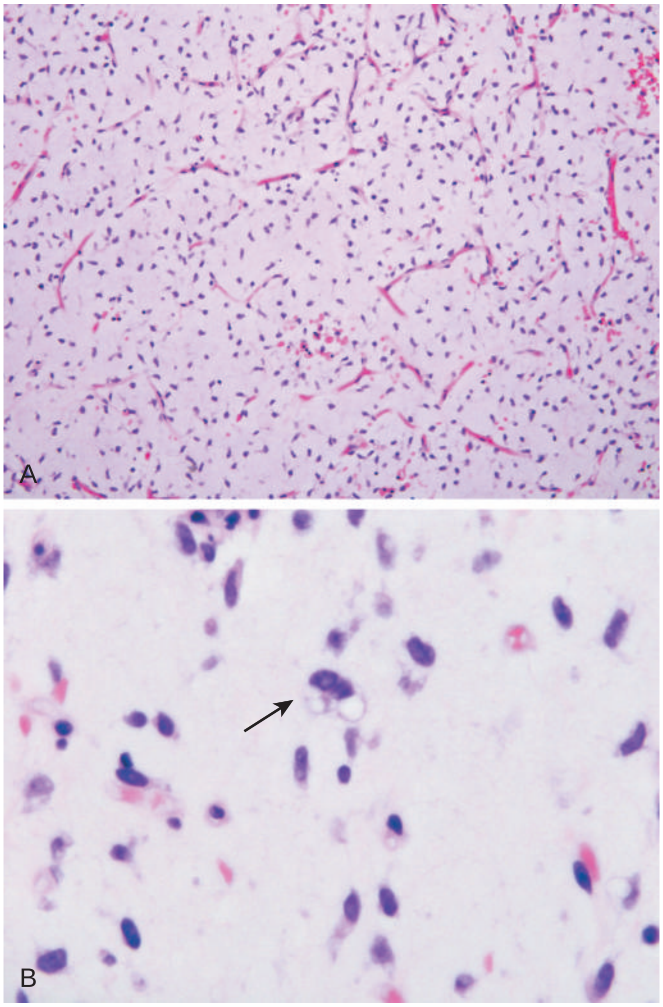

Figure 60-2 Myxoid liposarcoma. **A,** Uniform stellate and spindled cells in a prominent myxoid stroma and with characteristic branching capillaries. **B,** Primitive tumor cells and scattered lipoblasts *(arrow)* in a myxoid background.

Myxoid and round cell liposarcomas share recurrent chromosomal translocations that distinguish them from other liposarcoma histologies. t(12;16)(q13;p11) results in fusion of *FUS* (also known as TLS, an abbreviation for translocated in liposarcoma) with the transcription factor *DDIT3 (CHOP)*, and t(12;22)(q13;q11) fuses *EWSR1* with *DDIT3*.[133-135] DDIT3 is a member of the b-ZIP family of transcriptional regulatory proteins and seems to function as a negative transcriptional regulator. Chimerism with both EWSR1 and FUS (TLS) suggests that the retained domains of EWSR1 and FUS confer similar activities to the DNA-binding domain of *DDIT3*. Ectopic expression of either FUS-DDIT3 or EWSR1-DDIT3 in NIH-3T3 cells resulted in characteristics of oncogenic transformation, including growth in soft agar and tumor formation in immunocompromised mice.[136] Furthermore, implantation of mice with primary murine mesenchymal progenitor cells transduced with FUS-DDIT3 led to the development of tumors that morphologically and immunohistochemically resemble human myxoid liposarcoma.[137]

Well-dedifferentiated liposarcomas are associated with complex karyotypes. However, a frequent finding is amplification of 12q, which includes *MDM2* and *CDK4*.[138,139] MDM2 binds TP53 to inhibit its activity. In the context of wild-type *TP53*, inhibition of MDM2 by the small molecule nutlin3 results in increased apoptosis in liposarcoma cell lines.[140,141] Transgenic mice expressing FUS-DDIT3 in mesenchymal progenitor cells developed sarcomas only in the absence of TP53. Interestingly, treatment of these mice with trabectidin resulted in adipocytic differentiation, which was increased by concurrent treatment with the peroxisome proliferator-activated receptor γ (PPARγ) agonist rosiglitazone.[142] Comprehensive genomic analysis of several sarcomas demonstrated *TP53* and *NF1* mutation in a subset of pleomorphic liposarcomas whereas *PIK3CA* was mutated in nearly 20% of myxoid/round cell liposarcomas.[143]

Chromosomal rearrangements of 8q12 are found in lipoblastoma, and their detection can aid in diagnosis. These rearrangements have implicated the zinc finger DNA-binding protein PLAG1. In lipoblastoma, translocation places the PLAG1 coding exons under the transcriptional control of the *HAS2, COL1A2,* or *RAD51L1* promoters.[144,145] PLAG1 was originally identified based on its translocation in pleomorphic adenoma of the salivary gland.[146] In contrast to lipoblastoma, *PLAG1* is translocated near *CTNNB1, CHCHD7* or *TCEA1* in pleomorphic adenoma.

FIBROBLASTIC AND MYOFIBROBLASTIC TUMORS

Desmoid Tumor

Desmoid tumor, also known as deep musculoaponeurotic fibromatosis, presents from infancy through adulthood with a median age at diagnosis in the early 30s and a twofold higher incidence in women. Although most tumors occur spontaneously, FAP patients, and in particular those with Gardner syndrome, have reported incidences of desmoid tumors ranging from 3.5% to 32%.[27,147] Desmoid tumors in the pediatric population tend to occur in the second decade and are located in the extremities and head and neck, in contrast to the adult population in whom abdominal and truncal sites are common.[148,149] Abdominal desmoids are more common in FAP patients, whereas limb and trunk locations predominate in non-FAP patients.[150]

The tumors consist of an infiltrating, uniform, spindled cell proliferation lacking nuclear pleomorphism and mitotic activity (Fig. 60-3). Although desmoid tumors have a high potential for local invasion and recurrence, they never metastasize. Surgery is the primary treatment; however, tumors have a significant chance of local recurrence even if negative margins can be achieved.[149] Achieving negative margins without severe disfigurement or functional impairment may be impossible. In these circumstances, preoperative chemotherapy or partial resection and postoperative chemotherapy should be considered, although, given a highly variable clinical course, close observation may be justified. Multiple drug regimens have been shown to shrink or stabilize desmoid tumor growth. These include methotrexate with vinblastine or tamoxifen with diclofenac.[151-154] Other potentially active treatments include the combination of doxorubicin, vincristine, dactinomycin, and cyclophosphamide or the combination of doxorubicin and dacarbazine.[155-157] Responses to imatinib and sorafenib have been observed in about half of desmoid patients.[111,158,159]

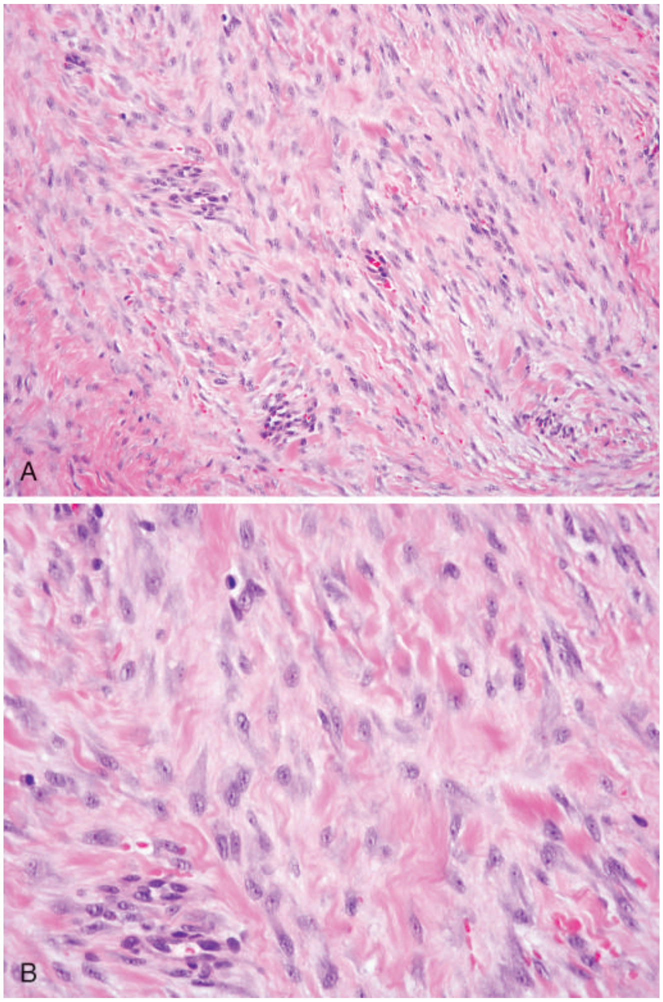

Figure 60-3 Desmoid tumor. **A,** Spindle cell neoplasm with prominent collagenization. **B,** Tumor cells are histopathologically bland with ovoid uniform nuclei, open chromatin, and single small nucleolus. Some cells have abundant eosinophilic cytoplasm.

Although most desmoid tumor cells are karyotypically diploid, abnormalities of the Y chromosome and 5q and gains in chromosome 8 and 20 may be noted.[160-163] Abnormalities have been identified in a subset of tumor cells, and the finding of trisomy 8 may suggest an increased risk for recurrence.[161] Gardner syndrome, which is associated with colonic polyposis and abdominal desmoid tumors, is a phenotypic variant of FAP, which is caused by mutation of *APC*. APC regulates β-catenin, the downstream mediator of WNT signaling. Stabilized β-catenin forms a multiprotein complex with members of the T-cell factor/lymphoid enhancer factor family (TCF-LEF) resulting in transcriptional activation of TCF-LEF target genes.[164-167] *APC* mutations resulting in increased β-catenin or stabilizing mutations in the β-catenin gene (*CTNNB1)* have been implicated in the proliferation of sporadic desmoid tumors.[168-170] Eighty-five to 90 percent of sporadic desmoid tumors are associated with somatic mutation of *CTNNB1*.[171] Mice expressing either mutant *APC* or stabilized β-catenin develop aggressive fibromatoses.[172,173]

Infantile Myofibroma/Myofibromatosis and Hemangiopericytoma

In recent World Health Organization (WHO) classifications of soft tissue tumors, the term *hemangiopericytoma* is no longer used; instead, a majority of these tumors are currently classified as solitary fibrous tumors.[174,175] Infantile hemangiopericytoma, recognized as a distinct category of benign hemangiopericytoma by Enzinger, is currently regarded in the category of infantile myofibroma/myofibromatosis.[176] Infantile myofibromatosis is a benign proliferation of myofibroblastic cells affecting almost exclusively young children. The tumors are solitary or multicentric and may involve the skin, muscle, viscera, or bone. Sixty percent are noted at birth, and 88% occur before the age of 2.[177] More commonly found in males, solitary lesions typically develop in the soft tissues of the head and neck and trunk. In contrast, the multicentric form is more common in females and in addition to affecting soft tissues it can develop in bones and viscera. Histopathologically, infantile myofibroma and myofibromatosis have similar features. They are multinodular and biphasic with alternating hemangiopericytoma-like,

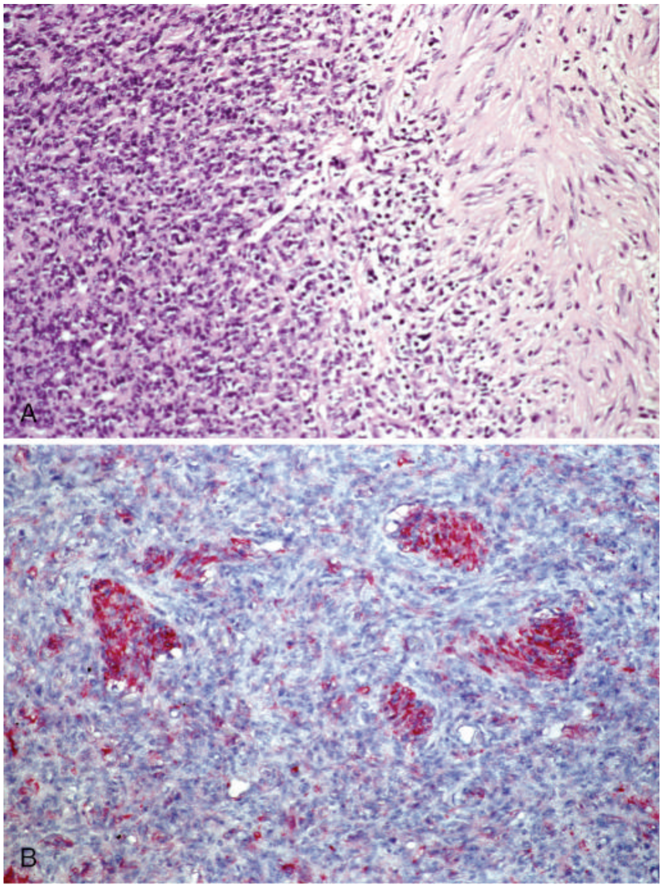

Figure 60-4 Infantile myofibromatoses. **A**, Biphasic tumor with an area of bland smooth musclelike proliferation *(right)* sharply demarcated from an area of cellular hemangiopericytoma-like tumor. **B**, Characteristic nests of strongly actin-immunoreactive cells *(red)* are scattered through a cellular hemangiopericytoma-like area.

densely cellular areas and less cellular areas with smooth muscle differentiation immunoreactive to actin (Fig. 60-4). Solitary lesions can rarely recur. Multicentric lesions undergo spontaneous regression; however, multiple visceral lesions may increase in size and number prior to regression, compromising vital structures and, rarely, leading to death.[178] Optimal initial therapy involves complete surgical excision. Life-threatening multifocal disease has been treated successfully with low-dose vincristine and dactinomycin or low-dose vinblastine and methotrexate.[179] Urinary basic fibroblast growth factor has been shown to be elevated in an infant during the proliferative phase, and connective tissue growth factor is expressed in both tumor and stromal cells.[180,181] An autosomal dominant pattern of inheritance has been associated with mutations in the growth factor receptor PDGFRB.[182-186]

Infantile myofibroma/myofibromatosis should be distinguished from pericytoma with t(7;12), a distinct tumor with histologic, immunohistochemical, and ultrastructural features of pericytic differentiation and a translocation involving t(7;12)(p22;q13).[187] Although typically developing in soft tissue, this entity can develop in bone.[188] The translocation brings together the β-actin gene *(ACTB)* with the zinc finger transcription factor *GLI1*.[189] Originally identified based on amplification in human glioma, GLI1 is activated by Sonic hedgehog signaling through the transmembrane receptor *PTCH*.[190-192] In t(7;12) pericytoma the carboxyl terminus of GLI1 is fused with the amino-terminal domain of ACTB. Intriguingly, *GLI1* has also been found to be amplified in a subset of rhabdomyosarcoma and osteosarcoma that lack typical markers of differentiation.[193]

Infantile Fibrosarcoma

Infantile (congenital) fibrosarcoma is one of the most common NRSTS in young children.[194] In contrast to fibrosarcoma in adults, this low-grade tumor displays a benign clinical behavior and is typically cured by surgical excision. However, large tumors for which surgical excision would result in significant morbidity may be treated with preoperative chemotherapy (Fig. 60-5, *A*). Regimens with activity against these tumors include vincristine and dactinomycin alone or together with either cyclophosphamide, ifosfamide, or doxorubicin.[195-202]

Histopathologically, the tumor is characterized by intersecting bundles with a herringbone pattern composed of cellular spindle to ovoid cells with a high proliferative rate, frequent mitoses, and areas of necrosis (see Fig. 60-5, *B* and *C*). Some tumors may have a hemangiopericytoma-like pattern of growth. Scattered chronic inflammatory cells are frequently present. Tumor cells have a fibroblastic and myofibroblastic ultrastructure (see Fig. 60-5, *D*) and immunophenotype with variable positivity for desmin and actin. Aberrant cytokeratin expression is observed in 15% to 20% of the tumors.

Infantile fibrosarcoma is characterized by t(12;15)(p13;q25).[203] This translocation fuses the amino-terminal helix-loop-helix oligomerization domain of the ETV6 transcription factor (also known as *TEL*) with the carboxyl-terminal tyrosine kinase domain of the neurotrophic tyrosine kinase receptor, type 3 (*NTRK3*, also known as *TRKC*). The detection of ETV6-NTRK3 by reverse-transcriptase PCR is highly specific and can distinguish infantile fibrosarcoma from more aggressive spindle cell sarcomas.[204] Intriguingly, the same translocation also occurs in congenital mesoblastic nephroma, secretory breast carcinoma, and acute myeloid leukemia.[205-208]

Chimerism results in constitutive activation by autophosphorylation of NTRK3 tyrosine residues. Translocation also results in aberrant *NTRK3* expression. *ETV6* is widely expressed whereas *NTRK3* is normally restricted to neuronal cells and fetal kidney.[209-211] ETV6-NTRK3 expression can transform NIH3T3 cells as assayed by growth in soft agar and in xenografts in immunocompromised mice.[212] ETV6-NTRK3 is associated with several signaling pathways that may be important in oncogenesis. ETV6-NTRK3 activates both the MAPK and AKT pathways, with AKT activation mediated by direct interaction of ETV6-NTRK3 with SRC.[213,214] Activation of signaling also leads to phosphorylation of YAP1 and WWTR1, Hippo pathway transcriptional coactivators, and a characteristic transcriptional profile.[215] ETV6-NTRK3–mediated fibroblast transformation also requires the insulin-like growth factor receptor (IGF1R) possibly

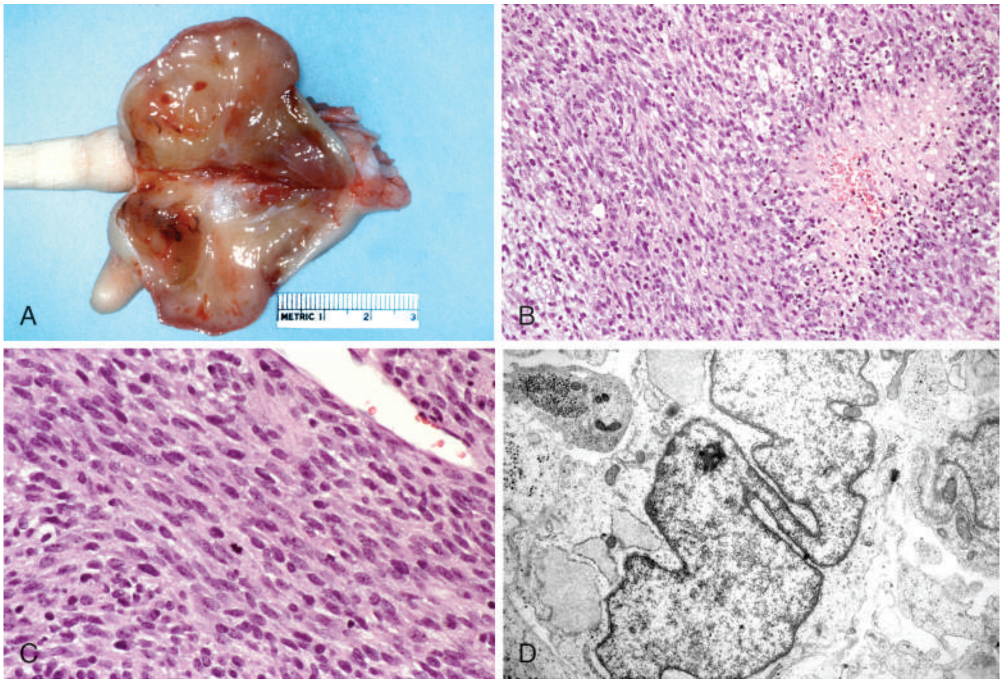

Figure 60-5 Infantile fibrosarcoma. **A,** Partial amputation of the hand in an infant with a rapidly growing soft tissue mass. On cut section the mass is fleshy and soft with areas of hemorrhage and necrosis. **B,** Histopathology shows a cellular spindle cell sarcoma with frequent mitoses, apoptosis, and confluent areas of necrosis. **C,** At higher magnification, nuclei appear uniform and hyperchromatic and cytoplasm is elongated and pink. Scattered inflammatory cells are also present. **D,** Ultrastructurally, tumor cells have the features of fibroblasts with prominent secretory-type rough endoplasmic reticulum.

through directly interacting with the IGF1R substrate IRS-1.[216-218] The ETV6-NTRK3 fusion may also act by suppressing transforming growth factor-β (TGF-β) signaling through interaction with the type II TGF-β receptor.[219]

Fibrosarcoma Arising in Dermatofibrosarcoma Protuberans and Giant Cell Fibroblastoma

Dermatofibrosarcoma protuberans (DFSP) is a rare cutaneous fibrohistiocytic tumor characterized by locally aggressive behavior. DFSP may begin as a plaquelike, raised red-blue lesion followed by a rapid growth phase that may be associated with the development of one or more nodules. DFSP appears as a cellular, spindle cell proliferation, frequently with a distinct storiform pattern that infiltrates the surrounding dermis and subcutaneous fat (Fig. 60-6, *A*). Tumor cells are typically immunoreactive for apolipoprotein D and CD34 (see Fig. 60-6, *B*). The Bednar tumor refers to an uncommon DFSP variant with scattered dendritic cells containing melanin.[220] The majority of the tumors develop on the trunk and upper extremity. Although typically diagnosed during the fourth and fifth decades, DFSP may occur in children and adolescents.[221] Inadequate resection of the tumor is associated with a significant risk for local recurrence.[222] Radiation therapy can be used effectively as an adjuvant for the treatment of resection sites with positive margins.[223-225] Rarely, DFSP can evolve into a high-grade fibrosarcoma often associated with a loss of CD34 staining and an increased risk for metastasis (Fig. 60-7, *A* and *B*).[226] Giant cell fibroblastoma, a rare tumor predominantly of males younger than 10 years of age, is in the spectrum of DFSP. Histopathologically, giant cell fibroblastoma is composed of variably cellular spindle or stellate cells often in a myxoid background containing pseudovascular spaces and multinucleated giant cells (see Fig. 60-7, *C*).[227-229]

Like DFSP, giant cell fibroblastoma is characteristically immunoreactive for CD34 and apolipoprotein D.[230] Both lesions also harbor a pathognomonic translocation between the collagen, type I, alpha 1, gene *(COL1A1)* and the platelet-derived growth factor-β gene *(PDGFB)*.[231-233] In contrast to the other sarcoma-associated translocations, the translocated *COL1A1-PDGFB* locus may be present as a low-level amplification on a ring chromosome. Detection of the resulting *COL1A1-PDGFB* transcript can provide confirmation of the diagnosis.[234] The fusion of *PDGFB* with *COL1A1* preserves the full coding region of *PDGFB,* and the resulting chimeric polypeptide is subject to intracellular processing that results in the secretion of a native PDGFB molecule that is capable of activating its receptor, PDGFR.[235,236] Although the mechanisms for autocrine-activated PDGFR signaling are not known, it may function through STAT3 and ERK activation.[237] Presumably through the inhibition of autocrine PDGFR activation, the growth of xenografted primary human DFSP cells can be inhibited with the kinase inhibitor imatinib (Gleevec).[238] Treatment with imatinib has also been shown to be effective in children and in patients with metastatic or unresectable disease.[239-245] Imatinib-resistant DFSP may respond to sorafenib or sunitinib.[246,247] Response to vinblastine and methotrexate has also been reported.[248]

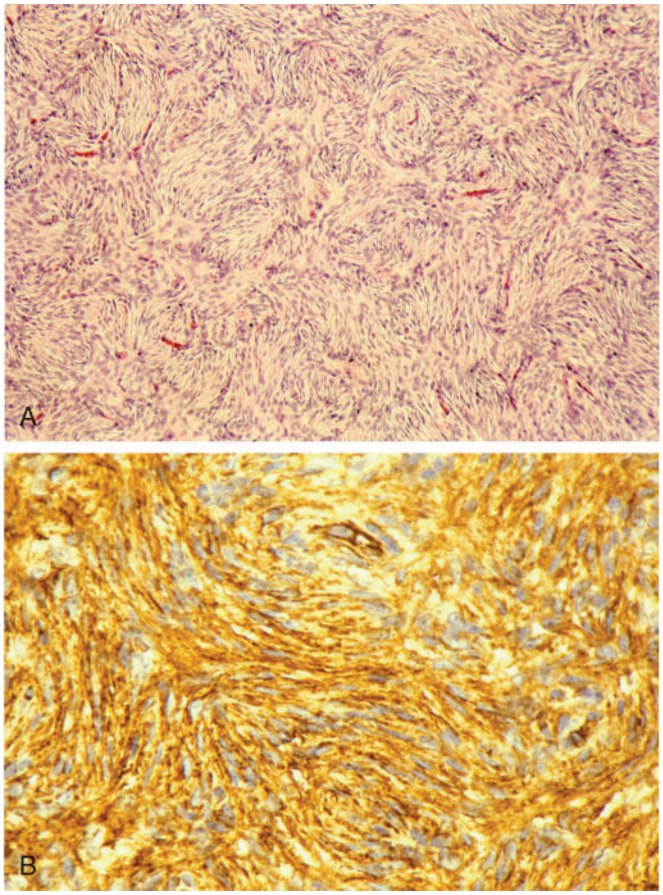

Figure 60-6 Dermatofibrosarcoma protuberans. **A,** Cellular spindle cell neoplasm with distinct storiform (cartwheel) pattern of growth. **B,** Tumor cells are diffusely immunoreactive with CD34.

Low-Grade Fibromyxoid Sarcoma

Low-grade fibromyxoid sarcoma (LGFMS) typically develops in the lower extremities of young adults and occasionally in adolescents (Fig. 60-8). Described by Evans in 1987, it is characterized by bland, uniform spindle cells with a short fascicular or whorly pattern of growth in a dense collagenous or variably myxoid stroma (Fig. 60-9).[249,250] An abrupt transition between cellular myxoid and hypocellular collagenous areas is frequently present. Dense collagenous areas mimic desmoid tumor. Myxoid areas contain characteristic arcades of curvilinear vessels that may be sclerotic or be surrounded by more densely cellular spindle cells. Mitotic count is characteristically low. Occasionally there are giant collagenous rosettes (hyalinizing spindle cell tumor with giant rosettes). Vimentin is the only constant marker present in tumor cells. Some tumors may be focally immunoreactive for actin, CD34, cytokeratins, or epithelial membrane antigen (EMA). MUC4 reactivity is common in LGFMS and can distinguish it from other histologically similar tumors.[251] A superficial location occurs more frequently in children.[252] Although primary resection typically results in disease control, local recurrences and pulmonary metastases can develop in 5% to 10% of patients after many years.[253,254] However, resection of recurrent disease can result in long-term remission. LGFMS seems to be chemoresistant, although limited responses to trabectedin have been reported.[255,256]

LGFMS is associated with t(7;16)(q34;p11) or t(11;16)(p11;p11), which results in the fusion of *FUS (TLS)* with *CREB3L2* or *CREB3L1*.[257-260] These translocations are also found in sclerosing epithelioid fibrosarcoma, suggesting that these tumors may be related.[261] *EWSR1-CREB3L1* has also been noted.[262] Native CREB3L2 is a member of the CREB basic leucine zipper family of transcription factors and acts as a transducer of endoplasmic reticulum (ER) stress signals. Normally tethered through a transmembrane domain to the ER, ER stress results in cleavage of the cytoplasmic amino terminal b-ZIP domain, enabling it to translocate to the nucleus and to activate

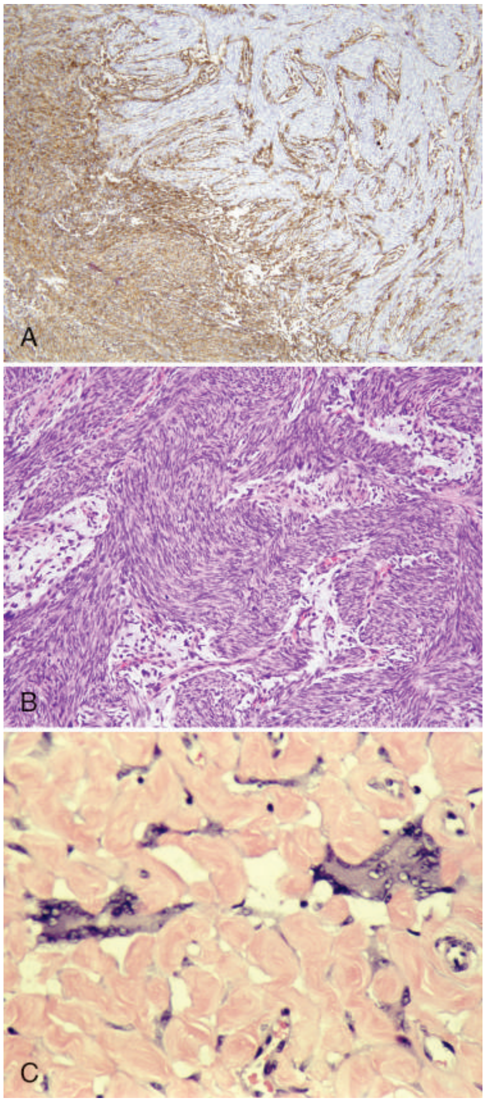

Figure 60-7 Fibrosarcoma in dermatofibrosarcoma protuberans/giant cell fibroblastoma. **A,** Loss of CD34 immunoreactivity in dedifferentiated fibrosarcoma area, which appears sharply demarcated from adjacent CD34-positive dermatofibrosarcoma protuberans *(lower left corner)*. **B,** Highly cellular fibrosarcoma with fascicular pattern and numerous mitoses. **C,** Giant cell fibroblastoma with characteristic multinucleated giant cells and spindled cells with collagenization.

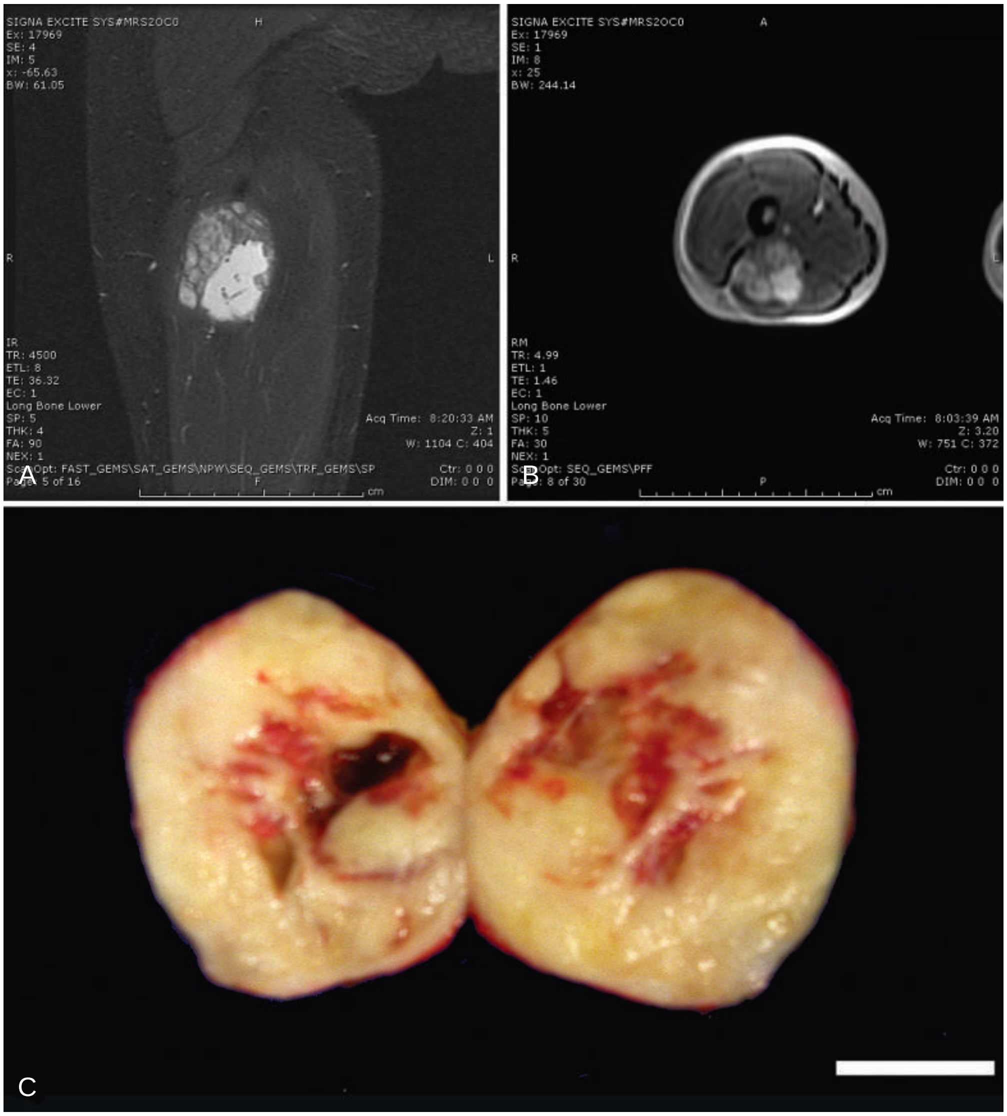
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Figure 60-8 Low-grade fibromyxoid sarcoma. **A** and **B**, MR images of enhancing well-delineated heterogeneous thigh mass. **C**, Grossly the tumor is well circumscribed and shows a soft white and tan cut surface with areas of hemorrhage and cystification.

transcription from cyclic adenosine monophosphate (AMP)–responsive elements.[263] Chimerism with FUS, a translocation partner with CHOP in myxoid liposarcoma, likely deregulates CREB3L2 transcriptional activity.[264] PCR-based identification of the fusion gene product from paraffin sections can be used to confirm the diagnosis.[261] LGFMS is associated with a gene expression profile that distinguishes it from similar tumors and is marked by the expression of *FOXL1*.[265]

Myxofibrosarcoma

Myxofibrosarcoma (MFS) is one of the most common sarcomas of the elderly but has been reported in adolescents and young adults.[266,267] Typically developing in the sixth and seventh decades of life, MFS exhibits a predilection for the dermal or subcutaneous tissues of the extremities.[268-270] MFS may be alternatively referred to as low-grade myxoid malignant fibrous histiocytoma or fibrosarcoma, myxoid type. Histopathologically, it is characterized by a multinodular growth of spindled or stellate cells in a myxoid stroma (Fig. 60-10). MFS grading has prognostic value and is based on cellularity, nuclear pleomorphism, and mitotic activity.[271] Resection is typically curative for low- or intermediate-grade tumors; however, recurrent tumors may show morphologic and molecular multistep tumor progression.[272,273] Large tumor size, necrosis, and decreased myxoid stroma in low-grade tumors are associated with a significant risk for local and distant recurrence.[274] Intermediate- and high-grade tumors have a significant risk for recurrence and metastasis.[275] In a study of adults, 23% of patients with intermediate- or high-grade tumors developed metastases, most commonly to the lung and lymph nodes and rarely to the brain. A subset of intermediate- or high-grade tumors exhibiting an epithelioid morphology seems to behave more aggressively than other high-grade histologies.[276] In addition to grade, an initial incomplete resection and infiltrative MRI T2-weighted signal

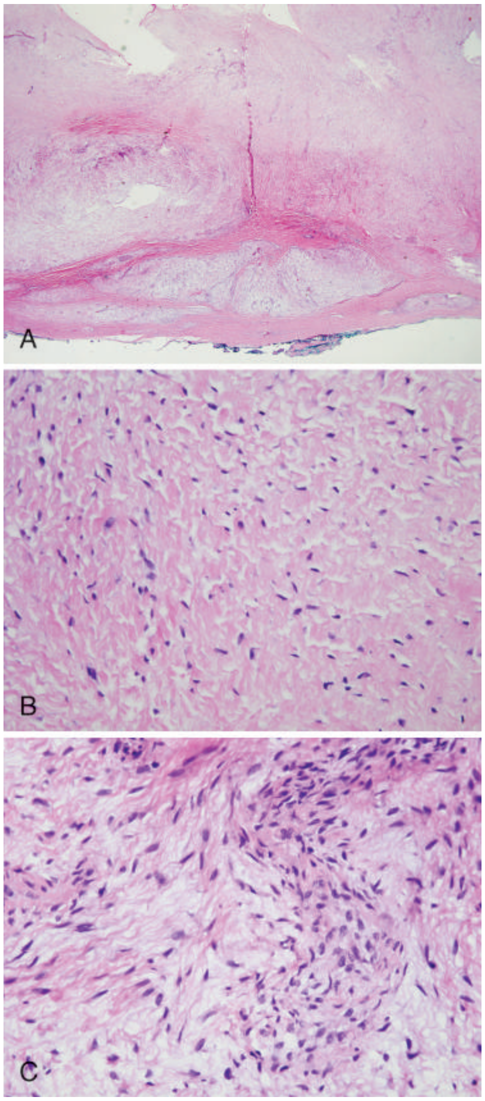

Figure 60-9 Low-grade fibromyxoid sarcoma. **A,** Multinodular, infiltrative growth at the tumor periphery and a variably myxoid stroma. **B,** Fibromatosis-like area with bland spindled cells immersed in a collagenous background. **C,** Characteristic curvilinear blood vessels, bland spindled cells, and myxoid background.

correlates with an increased risk for local recurrence.[277] Combined chemotherapy and irradiation has efficacy for MFS.[278] Cytogenetically, MFS may be diploid but is typically complex. Numerous chromosomal gains and losses as well as ring chromosomes have been observed.[273,279] Large-scale 7q gains were associated with increased expression of the hepatocyte growth factor receptor MET and CDK6, and increased expressed of these proteins was associated with lower metastasis-free survival.[280,281] Ezrin expression also correlates with decreased metastasis-free survival.[282] Isolated chromosomal translocations have been noted.[283,284]

Solitary Fibrous Tumor

Solitary fibrous tumor (SFT) is a fibroblastic tumor that was originally described in the pleura but can occur at other sites, most frequently the abdomen and pelvis. Most of the tumors formerly classified as hemangiopericytomas are currently categorized as SFT. SFT can be classified as classic, malignant, or dedifferentiated.[285,286] Five to 10 percent of soft tissue SFTs are malignant. SFT may be associated with hypoglycemia owing to synthesis by tumor cells of insulin-like growth factor 2 (IGF-2).[287,288] The histopathology is described as a patternless proliferation of spindle cells with a hemangiopericytoma-like growth (Fig. 60-11). Areas of sclerosis and hyalinization are characteristic. Mitotic count is generally low. Although the behavior of SFTs cannot be always predicted by histopathology, the presence of frequent mitoses, hypercellularity, pleomorphism, and necrosis is generally associated with malignant behavior. Large tumors and those with a mitotic index greater than 4 indicate an increased risk for recurrence locally or, more frequently, metastatically, even in the presence of negative resection margins.[289] Tumor cells are immunoreactive with CD34 and CD99 (see Fig. 60-11, *inset*). Some tumors also express EMA, actin, BCL2, apolipoprotein D, or nuclear β-catenin.[290-292] Recurrent NAB2-STAT6 fusions have

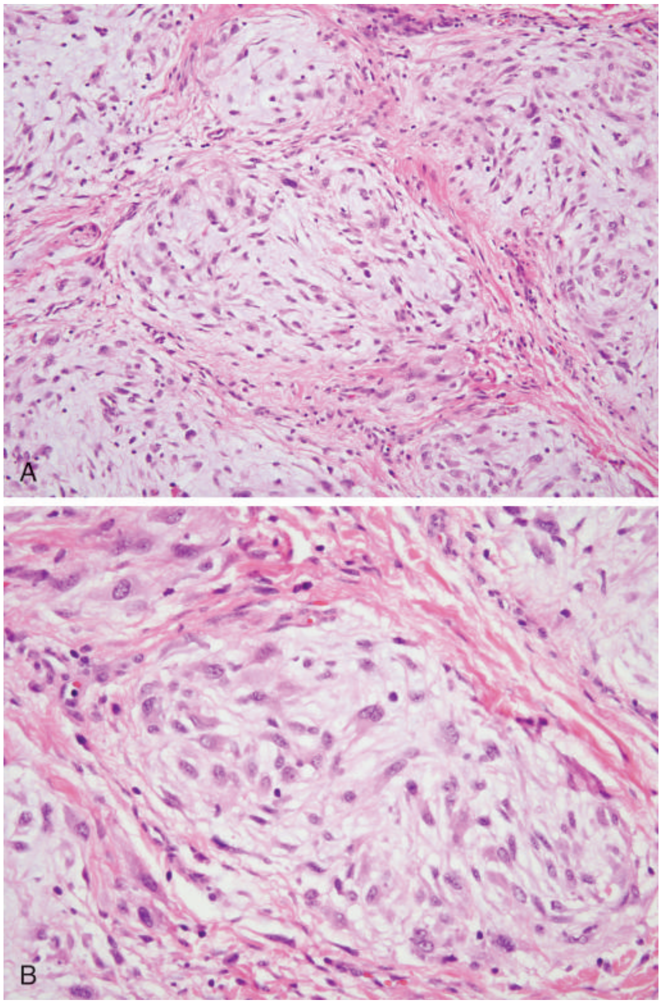

Figure 60-10 Myxofibrosarcoma. **A,** Multinodular growth pattern and prominent myxoid stroma. Numerous curvilinear vessels are present at the periphery of the nodules. **B,** Atypical myofibroblasts with large mildly pleomorphic nuclei and abundant cytoplasm.

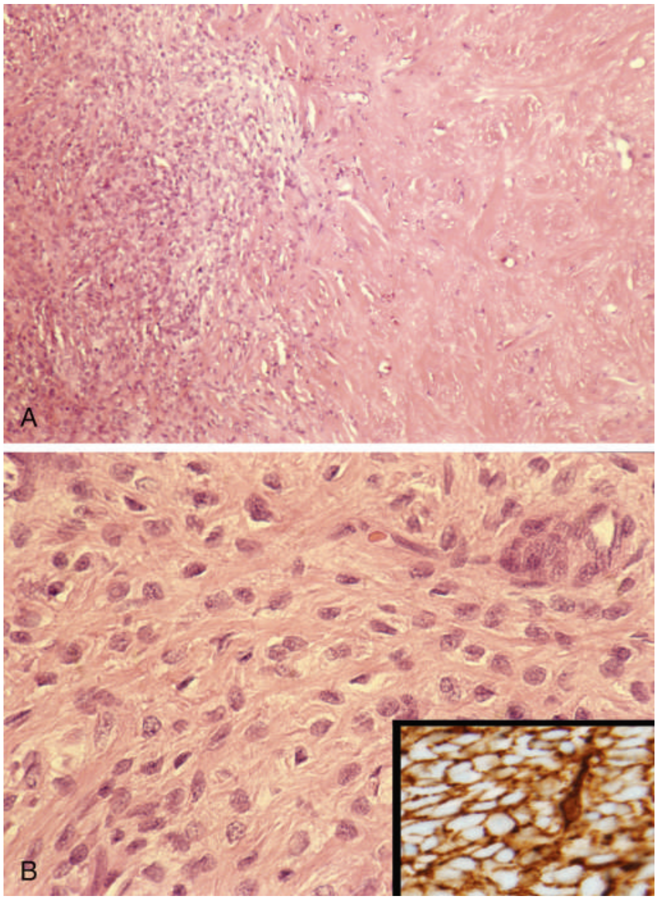

Figure 60-11 Solitary fibrous tumor. **A,** Spindle cell tumor with a prominent area of sclerosis. **B,** Patternless proliferation of spindled cells with uniform, ovoid nuclei, inconspicuous nucleoli, and eosinophilic cytoplasm with poorly defined borders. Tumor cells are strongly immunoreactive with CD34 *(inset)*.

been identified in the majority of SFTs regardless of the site of origin or SFT classification.[293,294] In this fusion protein, NAB2 contributes an amino-terminal EGR1 binding domain and STAT6 contributes a carboxyl terminal transcriptional activation domain with or without an associated DNA-binding domain. The resulting protein may cooperate with EGR1 to activate growth factor pathways. Based on this chromosomal rearrangement, virtually all SFTs demonstrate nuclear expression of STAT6 by immunohistochemistry.[295] Dacarbazine has demonstrated efficacy in SFTs as has treatment with bevacizumab or one of several tyrosine kinase inhibitors, including sorafenib, sunitinib, and pazopanib.[110,296-300]

Inflammatory Myofibroblastic Tumor

Inflammatory myofibroblastic tumors (IMTs) occur primarily in the viscera and soft tissue (particularly lungs) of children and young adults with a mean age of 13.2 years.[301] Of intermediate biologic potential, these tumors often recur locally but rarely metastasize. They are characterized by a proliferation of myofibroblastic spindled cells and an inflammatory, predominantly lymphoplasmacytic infiltrate (Fig. 60-12, *A*). Approximately half of IMTs show immunoreactivity for anaplastic lymphoma kinase (ALK) (see Fig. 60-12, *B*). ALK-positive IMTs are more common in younger patients and seem less likely to metastasize than ALK-negative disease, although local recurrence occurs with equal frequency.[301]

Clonal cytogenetic changes that activate ALK occur in 50% to 70% of IMTs.[302] The most common chromosomal translocation results in a fusion of the *ALK* receptor tyrosine kinase with the amino-terminal coiled domain of tropomyosin 3 or tropomyosin 4 (*TPM3* and *TPM4*). ALK may also be fused to the amino-terminal domains of clathrin heavy chain *(CTLC)*, cysteinyl-tRNA synthetase, RAN-binding protein 2 *(RANBP-2), SEC31L1,* or *PPFIBP1.*[303-307] ALK rearrangements were first identified in anaplastic large cell lymphoma, in which the fusion of *ALK* with nucleophosmin is most common, although chimerism with *CTLC* has also been observed.[308,309] Possibly based on ALK hyperactivation, the ALK/MET inhibitor crizotinib has demonstrated activity, although the response may be limited by mutation-associated resistance.[310-312] IMTs frequently demonstrate expression of MYC, cyclin D1, and MCL1 regardless of ALK status. Cyclooxygenase 2 inhibitors may be effective for IMT.[313,314]

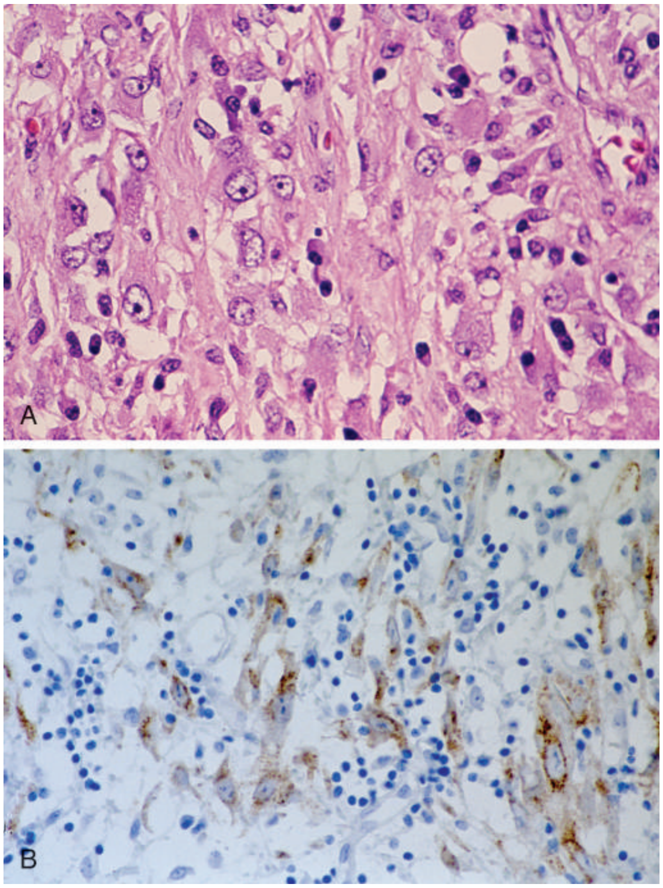

Figure 60-12 Inflammatory myofibroblastic tumor. **A,** Spindled and plump myofibroblasts with abundant eosinophilic cytoplasm, round to ovoid nuclei, and prominent single nucleolus. Intermingled are numerous lymphocytes and plasma cells. **B,** Neoplastic myofibroblasts show cytoplasmic immunoreactivity for anaplastic lymphoma kinase.

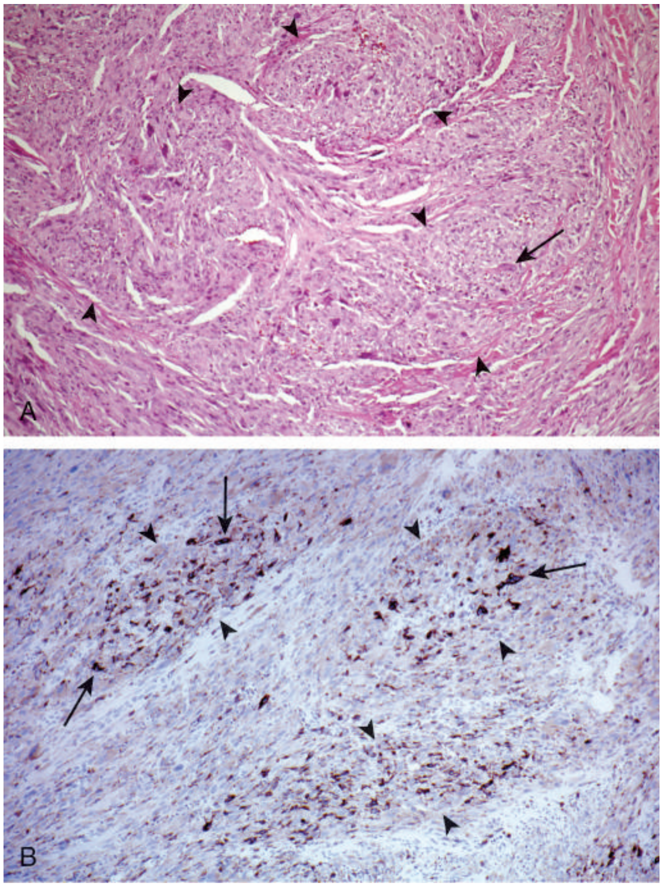

Figure 60-13 Plexiform fibrohistiocytic tumor. **A,** Multiple micronodules *(between arrowheads)* of fibrohistiocytic cells and multinucleated giant cells separated by collagenous stroma. **B,** Strong CD68 immunoreactivity of multinucleated giant cells *(arrows)* within micronodules *(between arrowheads).*

SO-CALLED FIBROHISTIOCYTIC TUMORS

Plexiform Fibrohistiocytic Tumor

Plexiform fibrohistiocytic tumor is a fibrohistiocytic neoplasm of intermediate malignant potential with a multinodular (plexiform) pattern of growth that occurs in the deep dermis and subcutaneous tissue primarily in children, adolescents, and young adults, with a mean age of 14.5 years.[315,316] Histopathologically these tumors are characterized by a plexiform growth of small nodules composed of mononuclear histiocyte-like cells, fibroblasts, and multinucleate giant cells (Fig. 60-13). Mitoses are infrequent, and pleomorphism is minimal. Immunohistochemical findings reflect the proportion of histiocytic to fibrous elements, with CD68 marking osteoclast-like multinucleated giant cells and smooth muscle actin (SMA) focally marking the fibrous component.[317] These tumors are histologically similar to some neurothekomas, but immunohistochemical staining for microphthalmia-associated transcription factor (MITF) may discriminate these entities.[318,319] This tumor has a tendency to locally recur and in about 5% of cases may metastasize, particularly to regional lymph nodes.[320] Cytogenetic abnormalities in the few cases reported are heterogeneous.

SMOOTH MUSCLE TUMORS

Sporadic Leiomyosarcoma

Leiomyosarcoma (LMS) is one of the most common STS in adults.[321] LMS can develop in uterus, bowel, vascular structures, and skin, as well as soft tissue and bone.[322,323] In children, LMS most commonly develops in the gastrointestinal tract and skin. In infants, intestinal LMS can present as perforation and obstruction.[324,325] In the head and neck, trunk, and extremities LMS has an equal incidence and tends to exhibit a more low-grade morphology and a clinical behavior comparable to that in adults.[326,327] Secondary LMS may develop in the radiation field of childhood cancer survivors.[328] Histopathologically, LMS is characterized by intersecting fascicles of spindle cells with prominently eosinophilic cytoplasm and elongated cigar-shaped nuclei (Fig. 60-14). In some tumors, nuclear pleomorphism and hyperchromatism are present. Mitoses are usually easily found, and their frequency is often uneven throughout the tumor. Tumor cells are immunoreactive for SMA, desmin, and h-caldesmon.

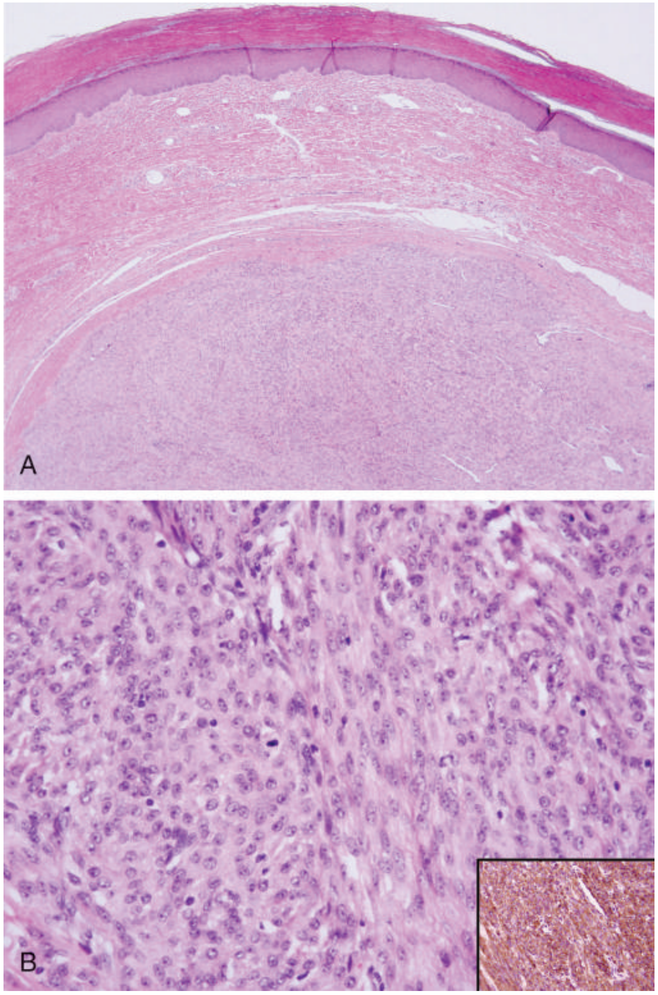

Figure 60-14 Leiomyosarcoma. **A,** Large tumor nodule is centered in the deep dermis and subcutaneous tissue. **B,** Tumor cells are spindled and have brightly eosinophilic cytoplasm and prominent nuclei with distinct centrally located nucleoli. Note strong and diffuse immunoreactivity for smooth muscle actin *(inset).*

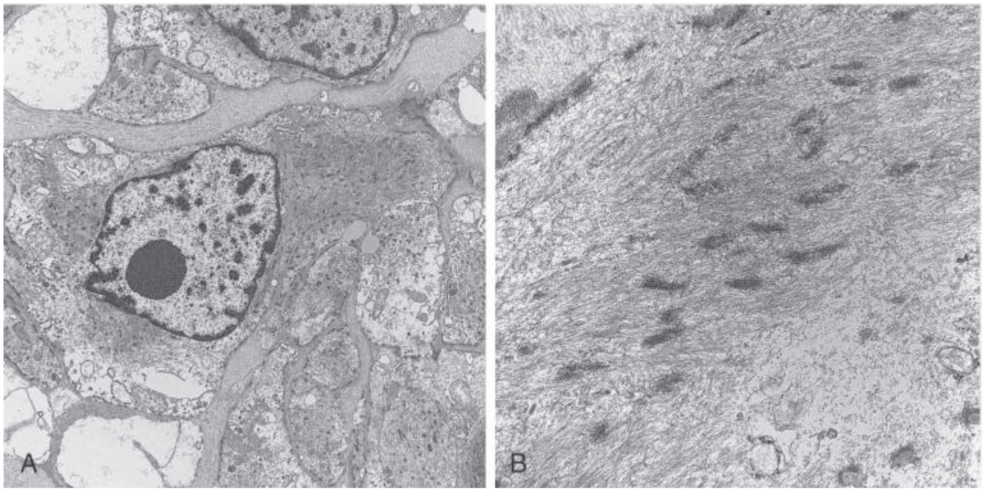

Figure 60-15 Leiomyosarcoma, electron microscopy. **A,** Tumor cells are individually invested by a continuous, well-developed basal lamina, and the abundant cytoplasm is filled with smooth muscle–type filaments. **B,** At higher magnification, note actin-type filaments with uniformly distributed dense bodies, typical of smooth muscle.

Ultrastructurally, tumor cells are invested by a continuous basal lamina and have subplasmalemmal densities and their cytoplasms contain abundant smooth muscle–type myofilaments with evenly distributed dense bodies (Fig. 60-15). Owing to the morphologic continuum of smooth muscle tumors, mitotic activity is the most reliable way to distinguish a leiomyoma from an LMS.

LMS has been treated with doxorubicin or ifosfamide as a single agent but with limited efficacy.[329,330] Patients with unresectable LMS treated with gemcitabine together with docetaxel demonstrated an overall response rate of 53%.[331] In recurrent or progressive STS, including LMS, the combination of gemcitabine and docetaxel was superior to gemcitabine alone.[101] Gemcitabine with docetaxel followed by doxorubicin in uterus-limited LMS resulted in a 57% PFS at 3 years.[332] When vinorelbine or dacarbazine is combined with gemcitabine the response rates have been reported as 15% to 20%.[102,333] Treatment with bendamustine resulted in stable disease in 40% of patients with LMS refractory to other agents.[334] Treatment with trabectedin is associated with disease stabilization in a significant fraction of patients in several clinical trials, including in pretreated LMS patients.[132,335-338] The multikinase inhibitor pazopanib also has limited disease-stabilizing activity in LMS.[110,339]

Germline mutation of the fumarate hydratase gene *(FH),* resulting in fumarate hydratase deficiency, is associated with a cancer predisposition syndrome with an increased risk for renal cell carcinoma, benign leiomyomas of the skin and uterus, and uterine leiomyosarcoma.[340,341,342] These tumors exhibit complex and unbalanced karyotypes. Genome-wide examination of DNA copy number variation and gene expression segregates a class enriched in the conventional LMS histologic subtypes with chromosome 10 and 13 losses and gains at 17p11-12. The remainder exhibit gene expression and copy number variation patterns similar to those of undifferentiated pleomorphic sarcomas and MFS.[343,344] *MED12,* which is mutated in the majority of uterine leiomyomas, is occasionally mutated in leiomyosarcoma.[345,346] MED12 is a component of the mediator transcriptional complex and can regulate TGF-β receptor signaling, influencing cellular responses to tyrosine kinase inhibition.[347] Vascular endothelial growth factor (VEGF) overexpression detected by immunohistochemistry was associated with a significantly shorter survival, suggesting a mechanism for the activity of pazopanib.[348] Tissue and expression microarray-based studies comparing uterine LMS with uterine leiomyoma demonstrated increased expression of CDKN1A, TP53, PDGFR, KIT, and CDKN21A.[349-354] Approximately 20% of transgenic mice expressing teratocarcinoma-derived growth factor 1 (also known as CRIPTO) from an MMTV promoter developed uterine leiomyosarcoma.[355,356] Chromosome 10q deletion is associated with *PTEN* loss and hyperactivation of the phosphatidylinositol 3′-kinase/serine–threonine kinase (PI3K/AKT) pathway. Consistent with the importance of this pathway in LMS, conditional *Pten* inactivation in smooth muscle led to the development of abdominal LMS in mice.[357] Decreased tumor growth after treatment with the rapamycin derivative everolimus suggested the importance of mTOR activation in this model. However, clinical trials failed to demonstrate significant treatment responses to mTOR inhibition by temsirolimus or ridaforolimus in LMS.[113,358,359]

Leiomyosarcoma Associated with Epstein-Barr Virus

Immunosuppression associated with solid transplantation and HIV infection can result in EBV-associated LMS, particularly in children and young adults.[46] Multicentricity is common. The histopathologic, immunohistochemical, and ultrastructural features are similar to the sporadic ones (Fig. 60-16). In situ hybridization with EBV-encoded RNA shows nuclear positivity in nearly all tumor cells (see Fig. 60-16, *inset*). EBV can infect smooth muscle cells, resulting in expression of latent and replicative viral products and production of infectious virus.[360] Reducing immunosuppression can lead to long-term remission.[361-363]

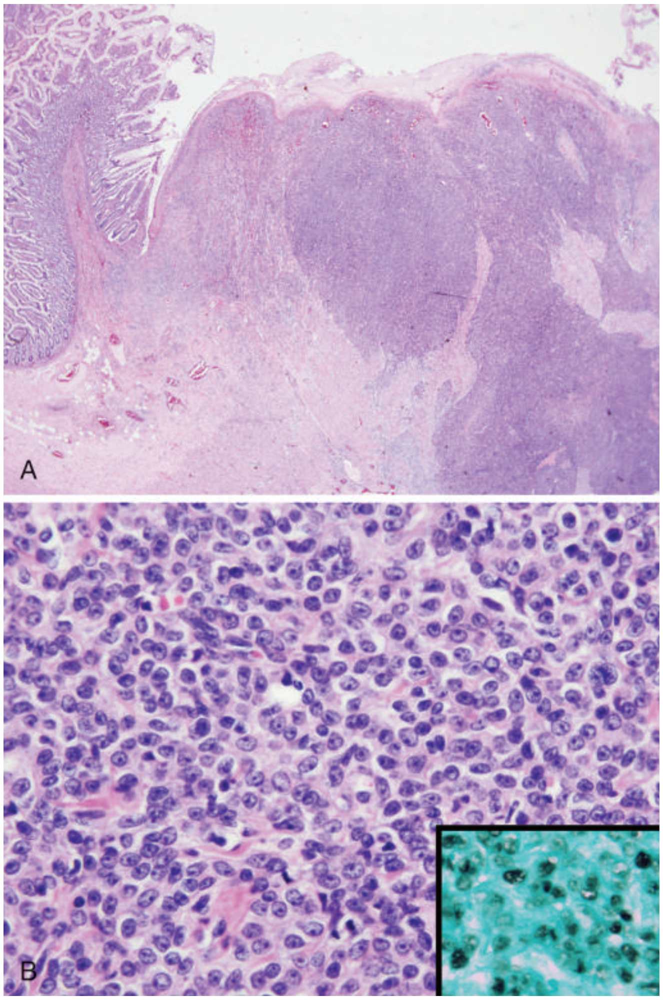

Figure 60-16 Epstein-Barr virus–associated smooth muscle tumor. **A,** Small intestine with large ulcerating and infiltrative submucosal mass. **B,** The tumor is cellular with spindled and round tumor cells with uniform nuclei and a distinct single nucleolus. Diffuse nuclear positivity for EBV-EBER is seen by in situ hybridization *(inset)*.

VASCULAR TUMORS

Epithelioid Hemangioendothelioma

Epithelioid hemangioendothelioma (EHE) is a vascular tumor that most commonly develops in superficial or deep soft tissue of the extremities.[364] Other sites include the liver, bone, lung, skin, lymph nodes, and central nervous system.[365-370] EHE often seems to arise from a medium to large vein. Unlike epithelioid hemangioma, vascular differentiation in EHE is primitive with a solid growth pattern or in strands (Fig. 60-17). The stroma is usually myxoid. Tumor cells are large with abundant eosinophilic cytoplasm. Intracellular vacuoles are characteristic and represent early lumen formation sometimes containing erythrocytes. Occasionally there are associated eosinophils and lymphocytes. Focal cellular spindling, atypia, and necrosis are associated with a more aggressive course; however, a bland-appearing lesion may also metastasize.[371] The differential diagnosis includes metastatic carcinoma, melanoma, or various sarcomas with epithelioid appearance, in particular, epithelioid angiosarcoma and epithelioid sarcoma. Tumor cells are immunoreactive with vascular markers, such as CD31, factor VIII–related antigen, and CD34. Ultrastructurally, tumor cells reveal vascular differentiation with prominent cytoskeletal intermediate filaments, Weibel-Palade granules, pinocytosis, and basal lamina. Two translocation-associated gene fusions have been reported in EHE. t(1;3)(p36.3q25) in EHE results in the *WWTR1-CAMTA1* gene fusion.[372-374] CAMTA1 is a member of a family of calmodulin-binding transcriptional regulatory proteins, and WWTR1 (also known as TAZ) is a component of the Hippo pathway. Interestingly, this pathway has been implicated in infantile fibrosarcoma. In t(1;3)-negative tumors, RNA sequencing identified a *YAP1-TFE3* gene fusion.[375] *TFE3* translocation has also been described in alveolar soft part sarcoma (ASPS) and translocation-associated renal cell carcinoma, although with different fusion partners.

Extended periods of stable disease as well as partial spontaneous resolution have been noted.[376] Lower-grade lesions can be conservatively resected, whereas malignant lesions likely justify radical surgical resection. Although surgical resection can result in cure, about one third of patients will develop a local recurrence or distant metastasis, most commonly to the lung. Although EHE is considered to be chemoresistant, responses to carboplatin with etoposide and carboplatin with paclitaxel for aggressive pulmonary disease and to interferon-alfa 2 (IFN-2α) for multifocal or disseminated liver disease have been reported.[377-381] Targeting the VEGF pathway with sorafenib and bevacizumab has shown limited efficacy.[382-384] Thalidomide and lenalidomide have also demonstrated activity.[385,386]

Giant Cell Angioblastoma

Giant cell angioblastoma is a rare neonatal vascular tumor. Although the tumor grows slowly, it can be locally

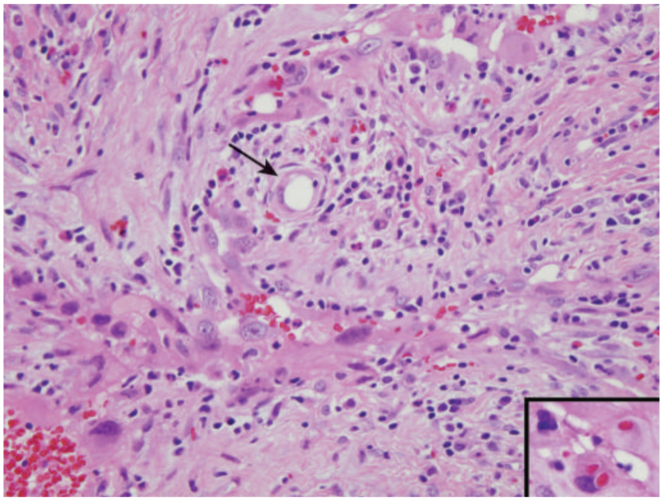

Figure 60-17 Epithelioid hemangioendothelioma. Poorly formed vascular channel lined by large epithelioid cells in an inflammatory stroma. Tumor cells have cytoplasmic vacuoles *(arrow)*. Red blood cells can be seen within cytoplasmic vacuoles *(inset)*.

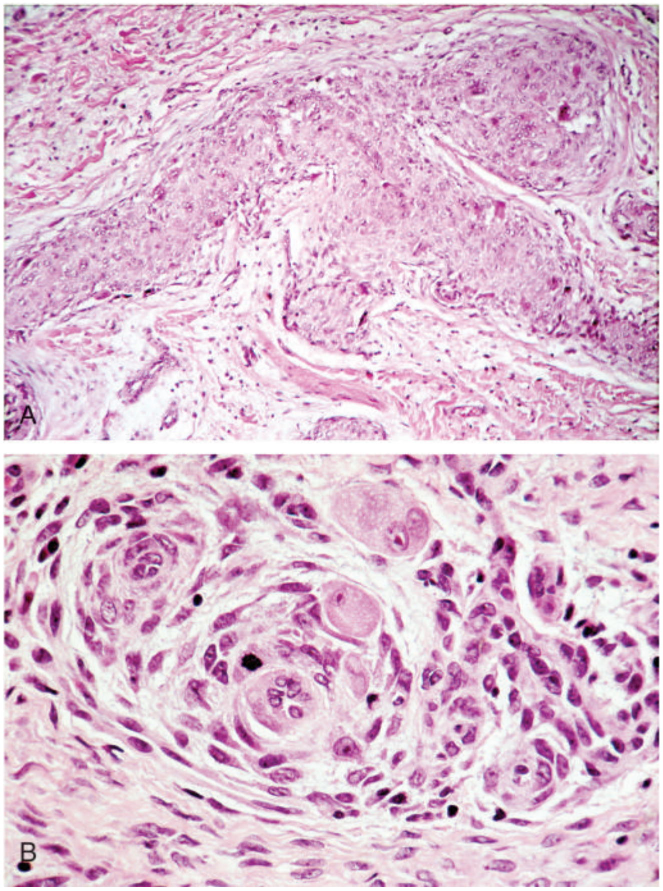

Figure 60-18 Giant cell angioblastoma. **A,** Nodular and plexiform aggregates of oval to spindle cells, mononuclear cells, and multinucleated giant cells in a perivascular distribution. **B,** Perivascular giant, spindled, and epithelioid cells.

invasive and highly destructive.[387,388] Histopathologically it consists of nodular and plexiform aggregates of oval to spindle cells, mononuclear cells, and multinucleated giant cells in a perivascular distribution. Like epithelioid sarcoma, this tumor mimics granulomatous inflammation (Fig. 60-18). Based on previous success with hemangiomas, two patients have been effectively treated with IFN-α2b.[389]

Kaposiform Hemangioendothelioma

Kaposiform hemangioendothelioma (KHE), a vascular tumor of infancy and childhood, occurs in either superficial or deep soft tissue. Large lesions, particularly in the retroperitoneum, are aggressive and present as consumption coagulopathy and thrombocytopenia (Kasabach-Merritt syndrome).[390] The lesions usually develop postnatally with a mean age at diagnosis of 3 years, 9 months. The lesions are multinodular, often with desmoplasia and vasoformative spindling of tumor cells resembling Kaposi sarcoma and glomeruloid structures (Fig. 60-19). Droplets of hyalin, fragmented erythrocytes, hemosiderin granules, and fibrin thrombi are characteristic. A lymphatic component is usually present. A majority of tumor cells are immunoreactive to CD31, CD34, and D2-40.[391] In contrast to infantile hemangiomas, GLUT1 is negative.[392] Tumors can spread locally but do not metastasize distantly.[393]

The mortality rate associated with KHE approaches 30%, typically resulting from Kasabach-Merritt phenomenon, which presents as consumption coagulopathy characterized by moderate microangiopathic hemolytic anemia, severe thrombocytopenia, and hypofibrinogenemia.[394] Although Kasabach-Merritt phenomenon is believed to be a consistent association with KHE, smaller lesions (<8 cm) may not result in sufficient platelet trapping to cause thrombocytopenia.[395] Large and invasive lesions are more likely to be associated with platelet trapping.[396] Surgical resection may be effective for superficial lesions. Unresectable tumors may respond to sirolimus, propranolol, ticlopidine, IFN-α, corticosteroids, vincristine, embolization, or low-dose radiation therapy.[397-402] For extensive disease, combination therapy with vincristine, cyclophosphamide, and dactinomycin with or without methotrexate may result in long-term remission.[403,404]

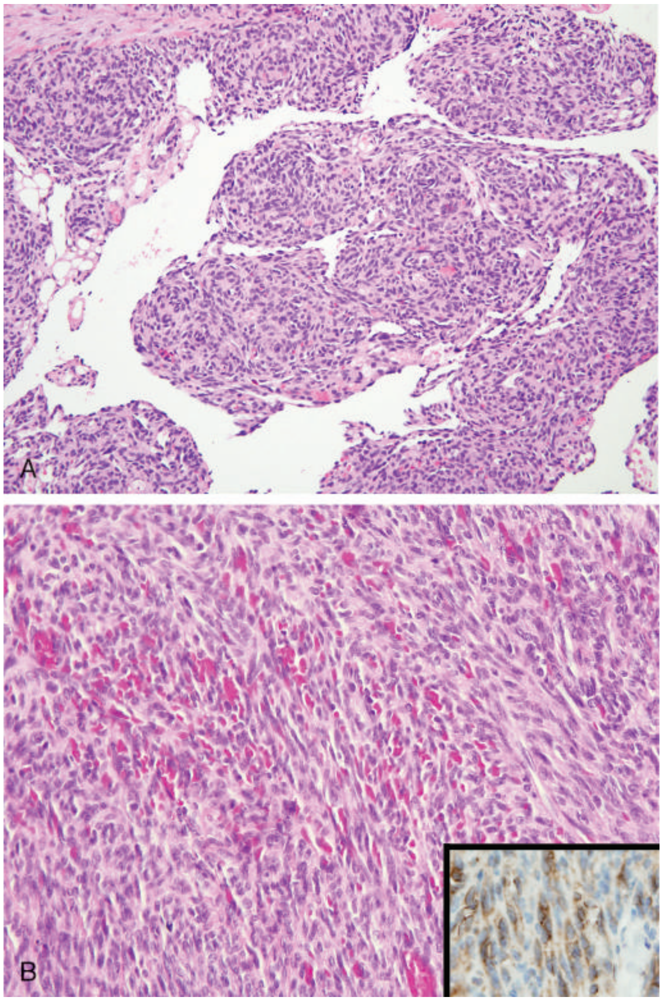

Figure 60-19 Kaposiform hemangioendothelioma. **A,** Irregular nests of tumor with spindled cells and glomeruloid structures. **B,** Kaposi-like area with cellular spindled cell growth and erythrodiapedesis. Spindled cells are immunoreactive with D2-40, a marker associated with lymphatic differentiation *(inset)*.

Kaposi Sarcoma

Kaposi sarcoma (KS) is an endothelial cell malignancy that results from transformation by human herpesvirus 8 (HHV-8, also known as Kaposi sarcoma herpesvirus).[405-407] KS can be multifocal and represent clonal or oligoclonal disease.[408,409] Four clinicoepidemiologic variants of KS are recognized: African (endemic), classic, acquired immunodeficiency syndrome (AIDS)-related (epidemic), and immunosuppression associated. Immunosuppression-associated KS can develop after solid-organ or bone marrow transplantation.[410] The incidence of AIDS-related KS has significantly decreased, likely owing to improvements in anti-HIV therapies.[411] KS in immunosuppressed individuals tends to behave aggressively, presenting as multifocal skin lesions and involvement of mucosal surfaces with spread to lymph nodes, brain, lung, gastrointestinal tract, and bones.[412-415]

The lymphadenopathic form of KS is most common in children; it is characterized by replacement of lymph nodes by a monotonous spindled cell proliferation, variable presence of slitlike vascular channels, and a lymphoplasmacytic infiltrate (Fig. 60-20, *A*). Spindled cells show striking nuclear reactivity for HHV-8 by in situ hybridization (see Fig. 60-20, *B*). HHV-8, like EBV, is a gammaherpesvirus, and, similar to immunosuppression-related EBV-associated lymphoproliferative disorder, KS may respond to reduced immunosuppression. KS also responds to pegylated liposomal doxorubicin and pegylated INF-2α.[416-419] Radiation therapy is effective for cutaneous disease.[420] Bevacizumab treatment was associated with an approximately 50% response rate in a phase II trial.[421] Preclinical evidence supports a role for mTOR inhibition acting through VEGF secretion and HSP90 inhibition acting through the viral latency-associated nuclear antigen (LANA).[422,423] Clinical responses to mTOR inhibitors have been observed.[424,425]

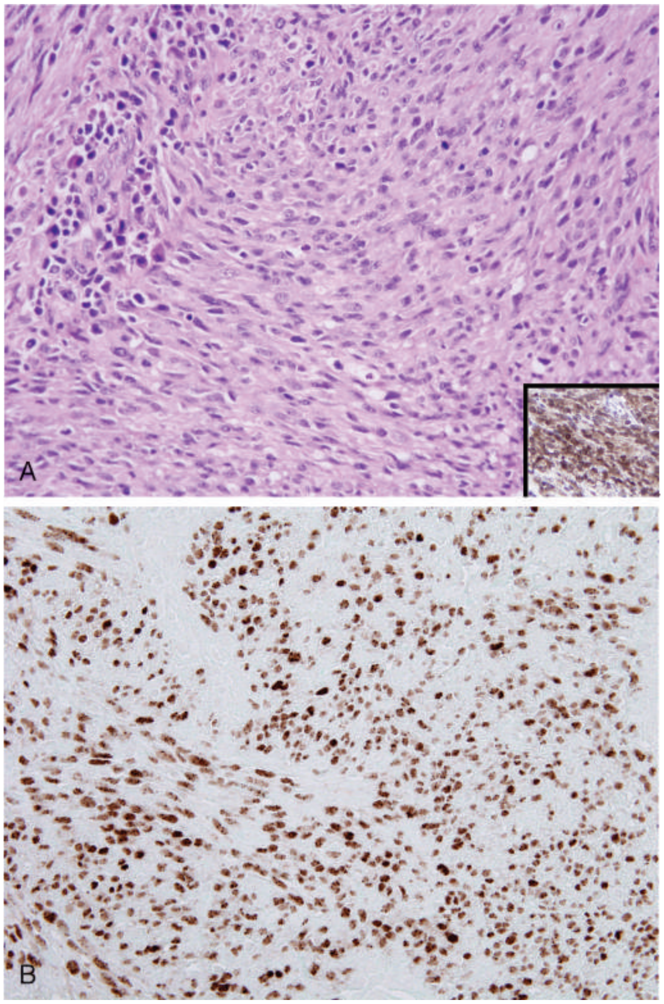

Figure 60-20 Kaposi sarcoma. **A,** Fascicles of monomorphic spindle cells and clusters of inflammatory cells, particularly lymphocytes and plasma cells. Spindled cells are strongly immunoreactive for D2-40 a lymphatic marker *(inset)*. **B,** The majority of tumor cells show nuclear staining for human herpesvirus 8 by in situ hybridization.

Angiosarcoma

Angiosarcomas are aggressive high-grade malignancies with endothelial differentiation that constitute approximately 2% of vascular tumors in children.[426] Angiosarcomas typically develop in skin or soft tissues.[427] In young children, angiosarcomas tend to occur in soft tissues and internal organs, including the liver, spleen, heart, and testes, and may be associated with Aicardi and Klippel-Trenaunay-Weber syndromes.[428-431] In adults, angiosarcoma development is associated with chronic lymphedema, prior radiation exposure, and exposure to vinyl chloride, arsenic, and thorium oxide.

Angiosarcomas are aggressive tumors with a 5-year survival of 20% to 40%.[432-435] Complete surgical resection is associated with survival.[436] Angiosarcomas have a high rate of local and distant recurrence and can metastasize to the regional lymph nodes, lungs, soft tissue, bones, liver, and brain.[427] Although tumors are typically resistant to chemotherapy and radiation, a partial response by unresectable tumors to chemotherapy may facilitate resection.[437] Docetaxel with radiation, paclitaxel, liposomal doxorubicin, vinorelbine, and metronomic trofosfamide may have efficacy in the treatment of these tumors.[438-442] Sorafenib or bevacizumab may be transiently effective for a subset of patients.[443,444] Radiation therapy is associated with decreased incidence of local recurrence.[433] Liver transplantation for hepatic angiosarcoma does not seem to result in increased survival, although long-term survival after transplantation has been reported for type II infantile hepatic hemangioendothelioma, which is considered a form of angiosarcoma.[445]

Angiosarcomas are vasoformative tumors with varying degrees of differentiation (Fig. 60-21). Tumor cells stain positive for factor VIII, CD31, CD34, and ERG, supporting an endothelial nature.[446,447] The cellular target of the antiangiogenic compound angiostatin, annexin II, is expressed in angiosarcoma.[448] Chromosomal gains at the *MYC* and *FLT4* loci can be detected in radiation-associated angiosarcoma.[449,450] Angiosarcomas are consistently positive for TEK (Tie2), and this kinase may serve as a therapeutic target.[451,452]

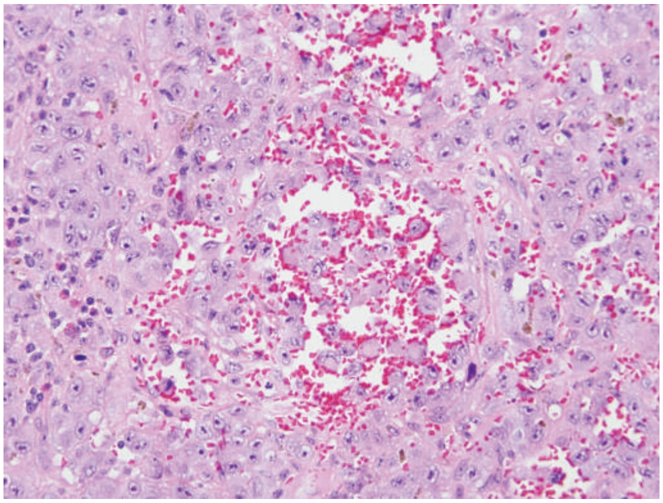

Figure 60-21 Angiosarcoma. High-grade angiosarcoma composed of large epithelioid cells with large pleomorphic nuclei, inclusion-like nucleoli, and abundant eosinophilic cytoplasm. There is vasoformation and hemorrhage.

CHONDROOSSEOUS TUMORS

Mesenchymal Chondrosarcoma

Mesenchymal chondrosarcoma is a rapidly growing, highly metastatic cartilaginous tumor. Although the majority develop in bone, more than 30% can develop in extraskeletal sites.[453] In contrast to most chondrosarcomas, mesenchymal chondrosarcoma tends to occur in young adults and, infrequently, children. Histopathologically, mesenchymal chondrosarcoma has two components: one consists of well-differentiated cartilage and the other of small cells resembling Ewing sarcoma (Fig. 60-22). Transition between these two components is usually abrupt. The small cell component frequently shows a hemangiopericytoma pattern of growth. The small cell component is immunoreactive with CD99, and the islands of cartilage are immunoreactive with S100 protein. Given its malignant behavior, patients with this tumor have a 38-month median survival with 5- and 10-year survival rates of 54.6% and 28%, respectively.[454,455] SOX9 expression can distinguish mesenchymal chondrosarcoma from small round blue cell malignancies.[456] Chromosomal translocation results in *HEY1-NCOA2* gene fusion in the majority of mesenchymal chondrosarcoma but not in other types of chondrosarcoma.[457] *HEY1* encodes hairy/enhancer of split, a component of the Notch signaling pathway. *NCOA2* encodes a transcriptional coactivator that functions with nuclear hormone receptors. *NCOA2* is a relatively promiscuous translocation partner contributing a carboxyl-terminal domain to gene fusion partners *ETV6 (TEL)* and *MYST3 (MOZ)* in a small number of leukemias, to *PAX3* in alveolar rhabdomyosarcoma, and to *AHRR* in soft tissue angiofibroma.[458-461] t(1;5)(q42;q32), resulting in *IRF2BP2-CDX1* fusion, has also been described.[462]

Extraskeletal Myxoid Chondrosarcoma

Rare in children and adolescents, extraskeletal myxoid chondrosarcoma (EMC), also known as chordoid sarcoma, typically develops in older adults, with a peak incidence in the fifth or sixth decade.[463-465] EMC is a slowly growing, occasionally painful, deep mass commonly in the extremities. Histopathologically, it exhibits a multinodular growth pattern with a prominent chondroid appearance. Tumor cells are uniform, are arranged in cords or strands, and are ovoid. The cytoplasm is usually eosinophilic and rarely has rhabdoid-type inclusions. Tumor cells are consistently immunoreactive to vimentin. About half weakly express S100 protein, and a lesser proportion are immunoreactive to cytokeratins, synaptophysin, and EMA.[466-469] SMARCB1 (INI1, SNF5) is deficient in about 10% of EMCs, and these tumors can be difficult to discriminate histologically for extrarenal rhabdoid tumor.[470] Like other STS, EMC has a significant propensity for late recurrences and pulmonary metastases.[471,472] Responses to sunitinib have been observed.[473]

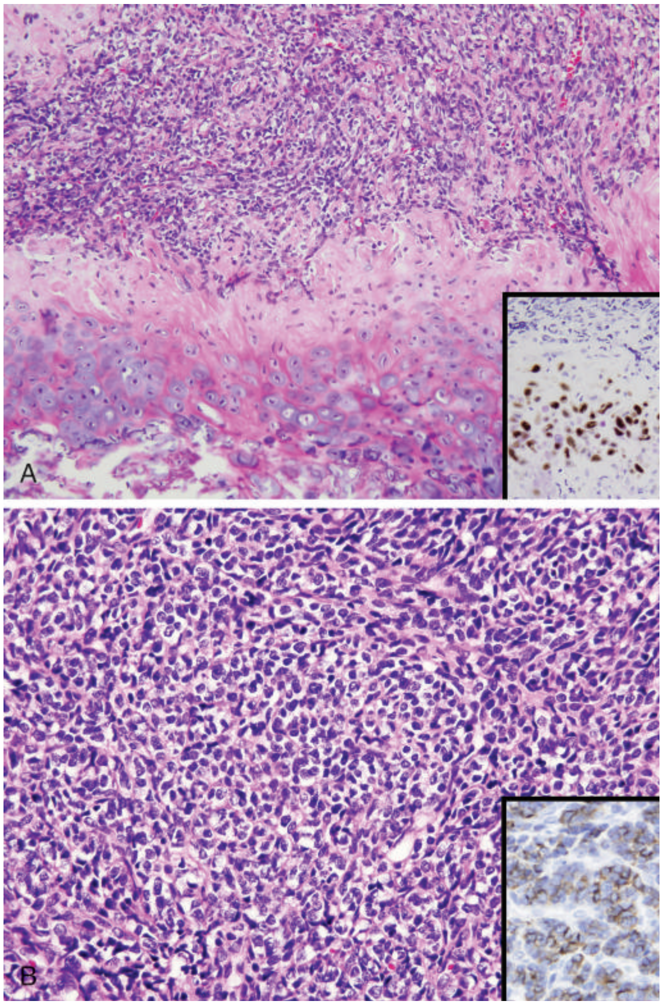

Figure 60-22 Mesenchymal chondrosarcoma. **A,** Biphasic tumor with a small cell Ewing sarcoma–like component at the top of the photograph and well-differentiated cartilage at the bottom. Cartilage component is strongly immunoreactive to S100 protein *(bottom of inset)*. **B,** The small cell component is shown at higher magnification demonstrating undifferentiated small cells with membranous CD99 immunoreactivity *(inset)*.

EMC is characterized by several recurrent clonal translocations. Approximately 75% harbor t(9;22)(q22;q12), which brings together the Ewing sarcoma gene *EWSR1* with *NR4A3 (CHN, TEC, NOR-1, MINOR)*.[474,475] Other translocations fuse *TAF15 (TAF2N, TAFII-68, RBP56)* and *TCF12* with *NR4A3*.[476-478] In contrast to EWSR1 and TAF15, which are ubiquitously expressed RNA binding proteins and translocated in many sarcomas, TCF12 is a basic helix-loop-helix transcription factor that contributes its DNA-binding domain to the *NR4A3* fusion.[479,480] NR4A3 is a member of a family of orphan nuclear receptors that includes NUR77 (NR4A1) and NURR1 (NR4A2).[481-484] In contrast to typical nuclear receptors such as the estrogen receptor that require binding to a ligand for activity, the NR4A family of transcription factors regulate transcription in a ligand independent fashion.[485] Their activity is controlled by regulated expression and posttranslational modification by phosphorylation.[486-490] The NR4A family has been implicated in a variety of cellular processes, including T- and B-cell apoptosis and hepatic glucose metabolism.[491-493] Mice deficient for both *Nr4a1* and *Nr4a3* develop acute myeloid leukemia in the early postnatal period.[494] The fusion of *EWSR1* with *NR4A3* results in an increase in transactivation activity over the native *NR4A3*.[495] Targets of the oncogenic fusion are not known; however, expression profiling of EMC with differing histologies and translocations demonstrated a consistent RNA profile distinct from that of myxoid liposarcoma (which is characterized by an *EWSR1-DDIT3* fusion).[496] *PPARG* and *NDRG2* have been identified as transcriptional targets for EWS-NR4A3.[497]

TUMORS OF UNCERTAIN DIFFERENTIATION

Synovial Sarcoma

Synovial sarcoma typically originates in proximity to major articular structures, bursae, and tendon sheaths, particularly of the proximal lower extremity. In spite of its name, a true relationship with synovium is no longer believed and intraarticular location is exceptional. Other sites for synovial sarcoma development include the trunk, head and neck, and mediastinum.[498-500] Synovial sarcoma is classified into two histologic subtypes, the more common monophasic spindle cell morphology subtype and a biphasic subtype (Fig. 60-23). Both subtypes exhibit an undifferentiated spindled cell component. In addition, the biphasic subtype has a variable degree of epithelial differentiation in the form of glandular structures or clusters of poorly organized epithelial cells. A small proportion of cases are characterized by small round cells with an organoid, hemangiopericytomatous pattern mimicking Ewing sarcoma (see Fig. 60-23, C). This group is associated with a worse prognosis. Glandular structures, epithelial clusters, and focally the spindle cell component show intense immunoreactivity for keratin and EMA.

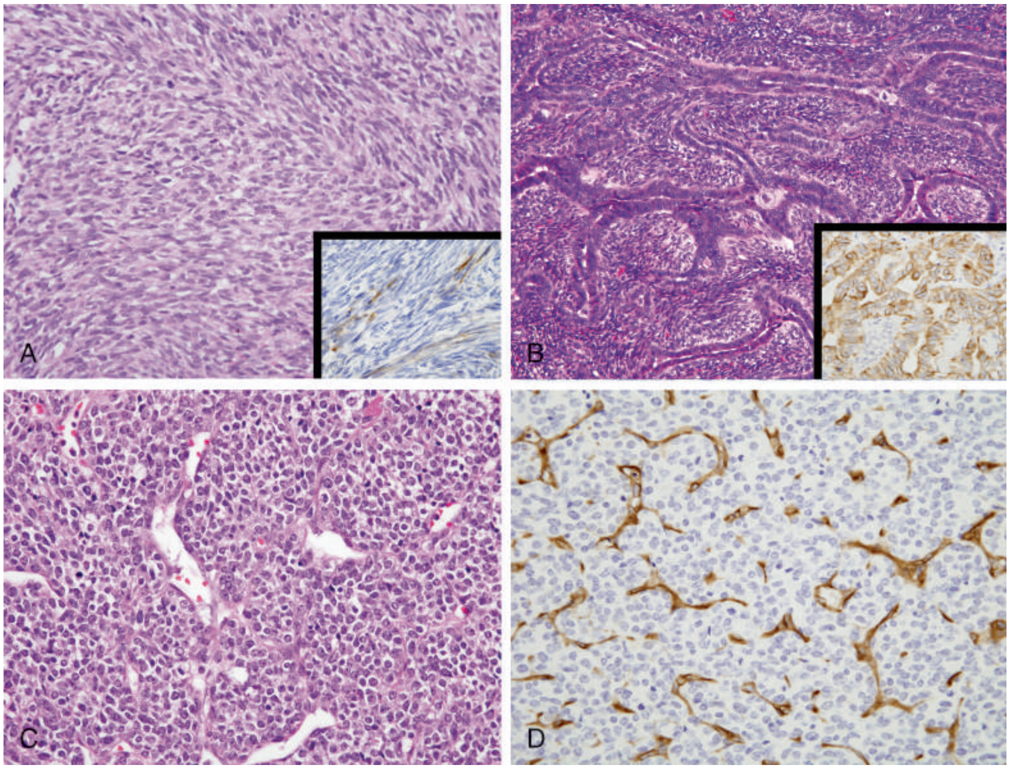

Figure 60-23 Synovial sarcoma. **A,** Monophasic synovial sarcoma composed of cellular undifferentiated spindled cells. Focal immunoreactivity for epithelial membrane antigen *(inset)*. **B,** Biphasic synovial sarcoma with prominent slitlike glandular component and intervening undifferentiated spindled cells. The glandular component is strongly immunoreactive to cytokeratins *(inset)*. **C,** Small round cell synovial sarcoma with a hemangiopericytoma-like pattern mimicking a malignant primitive neuroectodermal tumor. **D,** Malignant primitive neuroectodermal tumorlike synovial sarcoma showing characteristic vessels highlighted by CD34-immunoreactive endothelium.

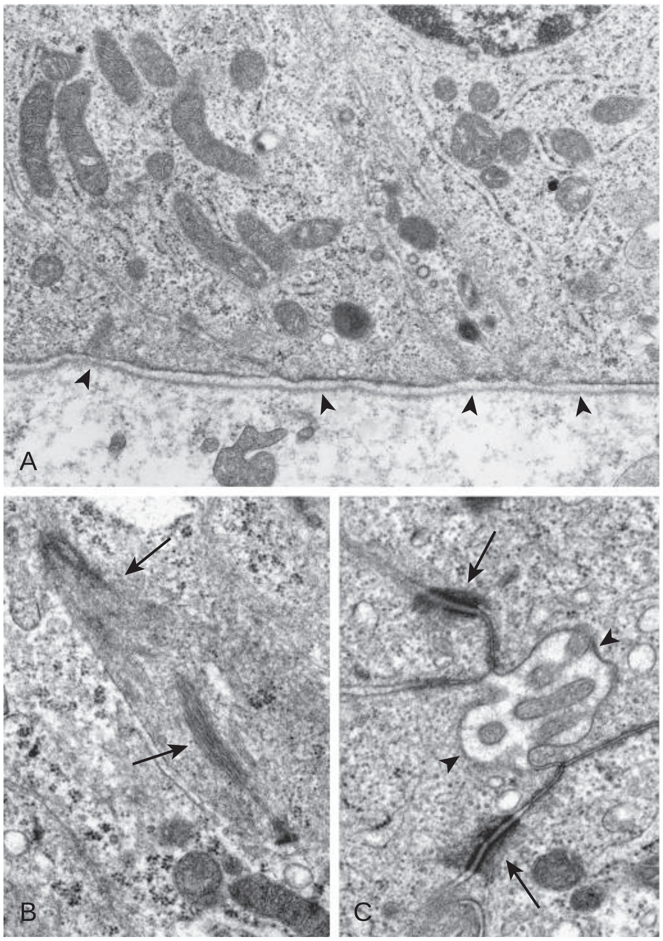

Figure 60-24 Synovial sarcoma. **A,** Groups of tumor cells are invested by a well-formed, continuous basal lamina *(arrowheads)*. **B,** Well-developed cytoplasmic tonofilaments *(arrows)*. **C,** Rudimentary intercellular lumen with microvilli *(arrowheads)* and well-developed desmosomes *(arrows)*.

Ultrastructurally there is evidence of epithelial differentiation with basal lamina, cell junctions, and microvilli in both glandular and spindle cell components (Fig. 60-24).

Cytogenetically, more than 95% of synovial sarcomas are characterized by t(X;18)(p11;q11), which results in the fusion of *SS18* (also known as *SYT*) with *SSX1, SSX2,* or *SSX4*.[501-506] A variant fusion of the SS18-related gene *SS18L1* at 20.q13.3 with *SSX1* has also been reported.[507] Identification of the fusion by RT-PCR assay in paraffin-embedded tissue is an important diagnostic tool with a sensitivity of 95% and a specificity of 100%.[508-512] Nuclear immunostaining of SS18 in paraffin-embedded tissue has a sensitivity of 85%.[513]

SSX1, SSX2, and *SSX4* are distinct genes that are located in proximity on the X chromosome.[514] SSX1 and SSX2 are highly conserved proteins characterized by acidic carboxyl and amino termini with homology to the Kruppel-associated box (KRAB), which, in the context of a DNA-binding protein, has been implicated in transcriptional repression.[515] The translocations found in synovial sarcoma encode nearly full-length *SS18* fused to the carboxyl terminus of SSX.[506] SS18 and the SS18-SSX chimeras demonstrate nuclear localization in a speckled distribution.[516,517] SS18 is an integral component of the SWI/SNF (BAF) chromatin remodeling complex.[518-521] SS18-SSX competes with normal SS18, resulting in an aberrant BAF complex lacking SMARCB1 (SNF5). The aberrant *BAF* complex activates *SOX2,* and *SS18* overexpression displaces *SS10-SSX* binding, decreasing *SOX2* expression and cell proliferation.[521] SS18-SSX–associated SWI/SNF disruption places synovial sarcoma in a class of tumors that share aberrancy of this large multiprotein complex, including malignant rhabdoid tumors (see later) and a growing number of epithelial cancers. Moreover, an increasing number of proteins have been found to be components of the mammalian SWI/SNF complex, increasing the number of tumors in which mutations in the genes that encode for these proteins have been identified.[522] Gene expression studies in human pluripotent stem cells expressing SS18-SSX indicated that expression changes virtually always represented gene activation.[523] Intriguingly, only half of the genes are regulated by SS18-SSX in mesenchymal stem cells, supporting an emerging theme in chromatin biology that cell lineage–specific epigenetic differences are critical in determining the gene expression consequences. Human synovial sarcoma likely derives from an early stem cell because tumors and cell lines consistently express stem cell marker genes *OCT3/4, NANOG,* and *SOX2*.[524] SS18 was also found to interact with a nuclear coactivator (SIP/CoAA) that shares regional homology with EWS and TLS/FUS.[525,526] The SS18-SSX1 fusion demonstrates transforming activity in a rat fibroblast cell line not shared by SS18 or SSX1 alone.[527] Tissue-specific activation of *SS18-SSX2* fusion controlled by the ubiquitous ROSA26 promoter in a conditional knock-in system by myoblast Myf5-activated Cre results in tumor development in 100% of surviving mice between 3 and 5 months of age (100% penetrance).[528] The mouse tumor shares histopathology with human synovial sarcoma, exhibiting both monophasic and biphasic components, and it has been shown that some mouse tumors shared gene expression profiles. Intriguingly, the timing of SS18-SSX2 expression plays a critical role in the resulting phenotype. Expression of SS18-SSX2 in early embryos was lethal, whereas expression in developing myocytes resulted in myopathy.

Several studies have attempted to identify pathologic and clinical differences between tumors harboring the most common fusions. Pathologically, tumors harboring the *SSX1* fusions demonstrate biphasic morphology and increased Ki67 staining, whereas those with the *SSX2* fusions are monophasic.[529,530] Although a large multicenter study comparing the outcome of patients with tumors harboring the *SS18-SSX1* fusion to those with the *SS18-SSX2* fusion demonstrated that the *SSX1* fusion predicted a worse outcome,[531,532] a more recent multicenter study failed to confirm this finding but demonstrated that histologic grade was a strong predictor of survival.[533,534] An analysis of cell cycle–related proteins demonstrated that tumors with the *SS18-SSX1* fusion express higher levels of cyclin A and D1 relative to those with *SSX2* fusion.[535,536] Although there are biologic and epidemiologic differences between these fusions, these differences are likely to be subtle.

In addition to the *SSX* fusions, other genes have been associated with synovial sarcoma biology. The expression of IGFR1 has been shown to correlate with a high incidence of lung metastases.[537] A significant fraction of synovial sarcoma also express hepatocyte growth factor (HGF) and/or its receptor MET.[538] Expression of HGF or coexpression of MET and HGF were associated with large, high-grade and high-stage tumors and correlated with decreased survival.[539] Synovial sarcoma–specific gene expression patterns have been identified. Compared with undifferentiated pleomorphic sarcoma, synovial sarcoma shows increased expression of several growth factors and receptors, notably insulin-like growth factor–binding protein 2 (IGFBP2), the receptor tyrosine kinase HER2 (ERBB2), IGF-2, and fibroblast growth factor receptor 3 (FGFR3).[540] Activation status of AKT/mTOR correlates with decreased EFS.[541] Compared with other STS, synovial sarcoma demonstrated increased expression of genes involved in the Wnt or notch signaling pathway, including *TLE1,* which was shown to immunohistochemically discriminate synovial sarcoma from other sarcomas.[542,543] SS18-SSX mediates Wnt signaling by interacting with β-catenin in the nucleus, and small molecule inhibition of Wnt signaling in transgenic mice resulted in decreased tumor growth.[544,545]

Recent studies have begun to explore the role of immune-directed therapy for synovial sarcoma based on the consistent expression of the tumor antigen NY-ESO-1.[546,547] However, a pilot study of the anti-CTLA4 antibody ipilimumab was discontinued owing to disease progression.[548]

Alveolar Soft Part Sarcoma

ASPS is a malignant tumor that usually presents in the thigh and trunk of young adults but can develop in the orbit or tongue in children.[549-551] Unusual primary locations include breast, heart, bladder, vagina, and uterine cervix.[552-556] About 25% of patients present with metastatic disease.[557] Because ASPS can metastasize to the brain in addition to lung and bone, the staging evaluation should include head CT. Complete surgical resection is associated with long-term survival.[558,559] ASPS is generally considered to be resistant to chemotherapy, although a small fraction of patients may respond.[557] Although pulmonary metastases may demonstrate response to treatment with vincristine, cyclophosphamide, and doxorubicin alternating with etoposide and ifosfamide, chemotherapy does not seem to extend survival.[560] Although considered an indolent tumor, ASPS carries a poor prognosis, given the insidious propensity for late metastases. Despite localized disease at presentation, subsequent distant recurrence results in a 77% survival at 2 years, 60% at 5 year, 38% at 10 years, and 15% at 20 years in adults.[561,562] Long-term survival in the pediatric population is significantly better with 5- and 10-year OS of 87% and 78%, respectively.[557] Given the protracted course of ASPS, metastatic disease can be palliated by resection of metastatic disease to lung and brain.[563-565] ASPS seems particularly responsive to VEGF-directed therapy, an observation consistent with gene expression profiling demonstrating elevated expression of several genes associated with angiogenesis.[566] Becvacizumab, sunitinib, and cediranib have efficacy in advanced ASPS.[567-571] Trabectedin may also be effective.[572,573]

Histologically, ASPS is characterized by an organoid, pseudoalveolar proliferation of large polygonal cells that range from eosinophilic to clear (Fig. 60-25). Intravascular tumor growth is often present. A characteristic of these tumors is the presence of cytoplasmic periodic acid–Schiff-positive crystalline structures visible on light and electron microscopy. Ultrastructurally these membrane-bound crystals consist of a latticework with distinctive periodicity (Fig. 60-26). The crystals are composed of monocarboxylate transporter protein MCT1 and its chaperone CD147.[574] The cell of origin for ASPS remains unclear, with refuted evidence suggesting a myogenic or neural crest derivation.[575,576] Tumor cells may show focal

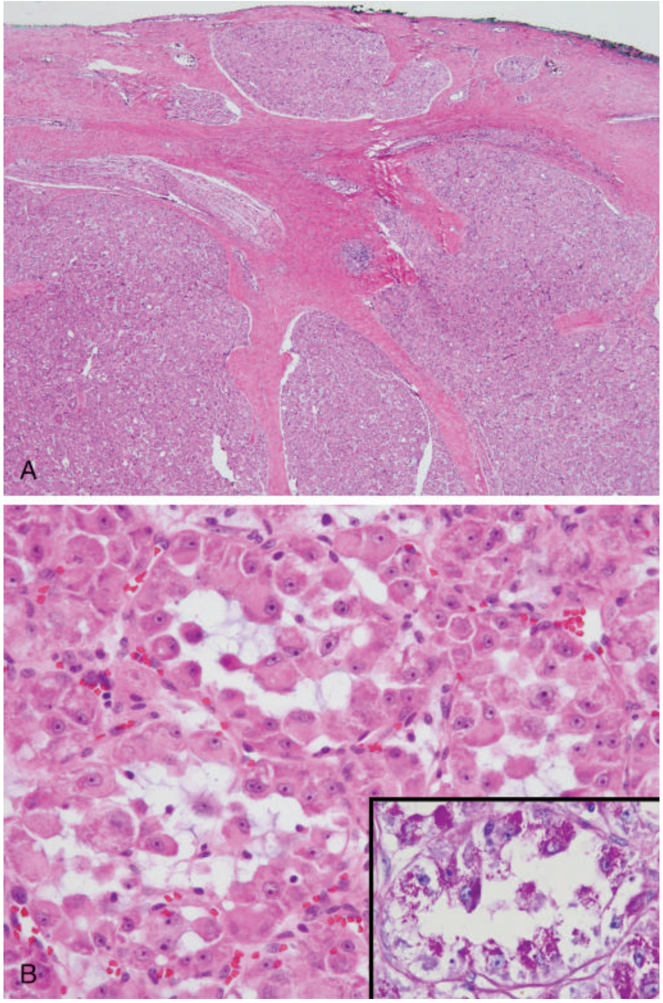

Figure 60-25 Alveolar soft part sarcoma. **A,** Multinodular growth with intervening fibrous septa. **B,** Large tumor cells with abundant eosinophilic cytoplasm are arranged with pseudoalveolar structures. Nuclei are large and round and contain a single prominent nucleolus. Note characteristic cytoplasmic periodic acid–Schiff-positive diastase-resistant inclusions *(inset)*.

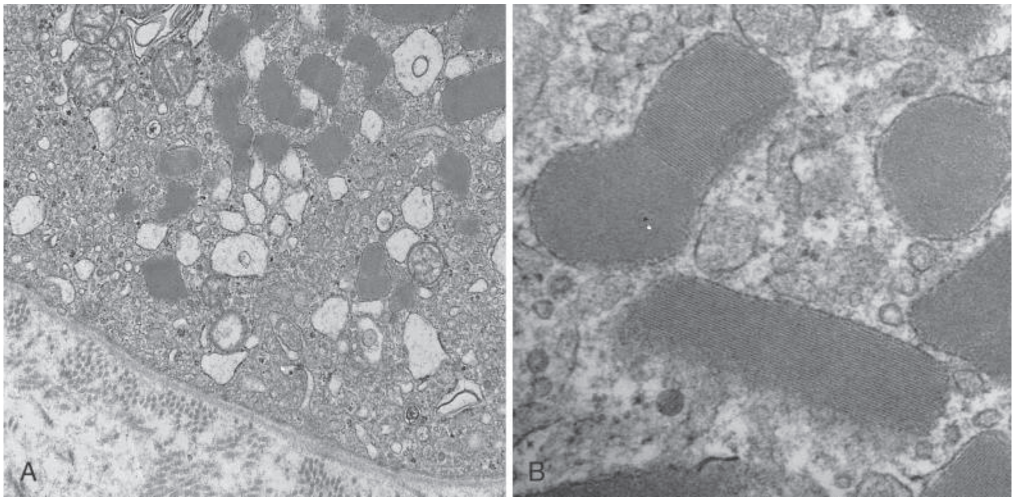

Figure 60-26 Alveolar soft part sarcoma. **A,** Pseudoalveoli are invested by a well-developed basal lamina, and tumor cells contain prominent endoplasmic reticulum cisternae filled with dense proteinaceous material. **B,** At higher magnification, proteinaceous material shows a paracrystalline periodicity, typical of this tumor.

positivity for actin or desmin, but these markers are not muscle specific.

ASPS is characterized by a unique chromosomal translocation, t(X;17)(p11;q25).[577,578] The translocation is frequently unbalanced, consistently resulting in the fusion of *TFE3* with *ASPSCR1* (also known as *ASPL*).[579] *TFE3* is a member of the MiT basic helix-loop-helix leucine zipper transcription factor family.[580,581] ASPSCR1 is not well characterized. However, amino acid sequence similarity suggests that ASPSCR1 may be the human homolog of mouse Tug, a protein that regulates intracellular trafficking of the glucose transporter GLUT4.[582] The *ASPSCR1-TFE3* translocation may dysregulate *TFE3* expression by placing it under the control of the ubiquitous *ASPSCR1* promoter. Alterations in the *TFE3* locus can be detected cytogenetically, by FISH or PCR assay. In addition, nuclear immunoreactivity for TFE3 provides a sensitive and possibly specific marker.[583] ASPSCR1-TFE3 likely functions by altering target gene transcription. *ASPCR1-TFE3* translocation can also be found in pediatric translocation-associated renal carcinoma, and *TFE3* can also be translocated in perivascular epithelioid cell neoplasm and epithelioid hemangioendothelioma.[375,584,585] One potential target gene with therapeutic potential is the receptor tyrosine kinase MET, although a clinical trial of tivantinib demonstrated modest effect.[586,587] TFE3 fusions may also regulate the mTOR pathway, suggesting activity of inhibitors of this pathway.[588] The biologic importance of aberrant *TFE3* in ASPS classifies it together with a growing number of other MiT family–associated tumors, including clear cell sarcoma (see later), translocation-associated renal carcinoma, and melanoma.

Clear Cell Sarcoma

Clear cell sarcoma (CCS) is a slowly growing soft tissue tumor that typically arises in the tendons, aponeuroses, and fascial structures of the extremities of adolescents and young adults.[589,590] It often presents as a small (<5 cm) painful nodule around the ankle or foot. CCS has a propensity for late metastasis, regionally to lymph nodes and distantly to lungs and bone. Overall, CCS is associated with a 5-year survival of 40% to 70%, with children faring somewhat better than adults.[591-594] Aggressive surgical management is critical to achieve disease control. In a pediatric population, fully resected and small tumors (<3 cm) are associated with a 5-year EFS of 90% to 100% whereas larger tumors (>10 cm) result in a 20% 5-year EFS.[593] Postoperative radiation therapy is probably not necessary for fully resected CCS but may result in improved local control when negative margins cannot be achieved, a situation that frequently arises because complete surgical excision with wide margins at common locations is often not feasible without amputation. Unresectable, metastatic, relapsed CCS is refractory to conventional chemotherapy; however, postoperative chemotherapy may be of benefit for localized resectable disease.[85,595] Responses to sorafenib and sunitinib have been observed.[596,597] Although MET activation by HGF was demonstrated in clear cell sarcoma, clinical trials of tivantinib have had limited efficacy.[587,598]

Clear cell sarcoma has an infiltrative, nesting and fascicular pattern of growth (Fig. 60-27). The nesting pattern is highlighted by a reticulin framework. Tumor cells are usually ovoid or spindle with ovoid to elongated nuclei and distinct prominent eosinophilic nucleoli. CCS shares several histologic, immunophenotypic, and ultrastructural similarities with cutaneous melanoma, leading to the frequent designation of this tumor as “malignant melanoma of the soft parts.”[590] Melanocytic differentiation of CCS is demonstrated by the expression of melanoma markers, including MITF, HMB45, S100, neuron-specific enolase (NSE), and melastatin.[599,600] Furthermore, about 70% of CCSs reveal melanin pigment by hematoxylin and eosin or Fontana stains and, ultrastructurally, they may contain premelanosomes and melanosomes.[600-602] By microarray expression analysis, CCS segregates from other sarcomas based on a

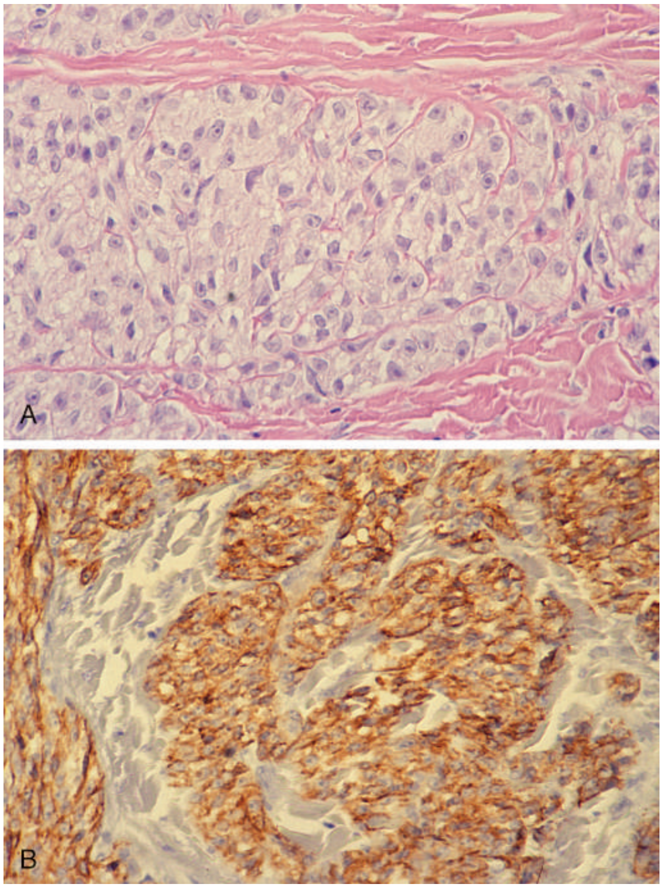

Figure 60-27 Clear cell sarcoma. **A,** Well-defined nest of tumor cells surrounded by fibrous septa. Tumor cells have abundant pale-staining cytoplasms and rounded vesicular nuclei with prominent single centrally located nucleolus. B, Tumor cells are strongly immunoreactive for HMB45, a melanoma marker. Strong staining for S100 protein is also characteristic of clear cell sarcoma *(not shown)*.

melanocytic expression pattern.[603,604] However, clinically and cytogenetically, CCS and cutaneous melanoma are distinct. CCS presents as isolated masses in deep soft tissue, often originating from tendons and aponeuroses. CCS rarely shows the degree of anaplasia or microsatellite instability associated with melanoma and, unlike with melanoma, *BRAF* is not mutated.[605-607] The vast majority of CCS tumors are distinguished by t(12;22)(q13;q12) that results in fusion of the Ewing sarcoma gene *EWSR1* with the CREB transcription factor family member *ATF1*.[608] The *EWSR1-CREB1* gene fusion is less commonly observed in soft tissue CCS but is more common in gastrointestinal CCS. These translocations are not found in cutaneous melanoma. Native ATF1 is regulated by serine phosphorylation in response to cyclic AMP levels by protein kinase A.[609,610] In CCS, translocation dysregulates ATF1 activity by replacing the kinase-inducible domain of *ATF1* with the amino-terminal region of *EWSR1*.[611] *Ewsr1-Atf1* expression in mice resulted in the high penetrance development of tumors that shared histologic and immunohistochemical features with the human tumor.[612,613]

Induction of MITF expression by EWSR1-ATF1 links the melanocytic phenotype of CCS with tumor proliferation/survival.[607] MITF, a member of the MiT basic helix-loop-helix leucine zipper transcription factor family, is the master regulatory factor of melanocyte differentiation and survival.[581,614] EWS-ATF1 coopts the typically melanocyte-stimulating hormone (MSH)-regulated cyclic AMP response element in the *MITF* promoter, aberrantly activating *MITF*. A critical transcriptional target, MITF mediates both the survival and proliferation of the melanocytic phenotype of CCS. TFE3 and TFEB, other members of the MiT transcription factor family, can rescue the effect of inhibited MITF expression in CCS.[607] This functional redundancy places CCS among a family of tumors that share aberrant MiT transcription factor family expression, including cutaneous melanoma, ASPS, and translocation-associated renal cell carcinoma. A gastrointestinal form of CCS is associated with a translocation that brings together *EWSR1* with *CREB1*.[615] Despite the related activity of *CREB1* with *ATF1*, this form of clear cell sarcoma does not express MITF or markers of melanocytic differentiation. Microarray expression profiling of the gastrointestinal CCS demonstrates a pattern that clusters more closely with angiomatoid fibrous histiocytoma (AFH) than soft tissue CCS (see later).[616]

Epidermal growth factor signaling may be important for CCS development. Clear cell sarcoma cell lines express ERBB3 and either ERBB2 or ERBB4.[603,617] Furthermore, ERBB3 is constitutively phosphorylated in cells, expressing its ligand neuregulin (NRG1), and can be activated by exogenous neuregulin in those cells that are not activated in an autocrine fashion. Treatment with the pan-ERBB inhibitor CI-1033 decreases cell proliferation.[617]

Desmoplastic Small Round Cell Tumor

Desmoplastic small round cell tumor (DSRT) typically arises in intraabdominal soft tissues. It has a strong predilection for males. Although its most common site is the peritoneum, DSRT can arise in kidney, testes, ovaries, pleura, scalp, ethmoid sinuses, and pancreas.[618-622] DSRT usually presents as abdominal pain and weight loss and is frequently locally or distantly metastatic at the time of diagnosis. The diffuse paraserosal spread of DSRT may prevent resection with negative margins. A high tumor response rate in untreated DSRT and previously treated DSRT to high-dose cyclophosphamide, doxorubicin, and vincristine (HD-CAV) alternating with ifosfamide and etoposide may cytoreduce bulky disease, permitting surgical resection and radiation therapy, if necessary.[623] Although the results are mixed, patients with high-risk disease may benefit from consolidation with high-dose chemotherapy and autologous stem cell rescue.[624,625] Relapsed disease may respond to irinotecan and temozolomide or low-dose cyclophosphamide and vinorelbine.[626,627] Because DSRT tends to develop and spread widely in the peritoneum, based on its efficacy for peritoneal carcinomatosis, the use of continuous hyperthermic peritoneal infusion of chemotherapy is being studied, although initial results do not appear promising.[626,628] Multimodality therapy was associated with a 3-year

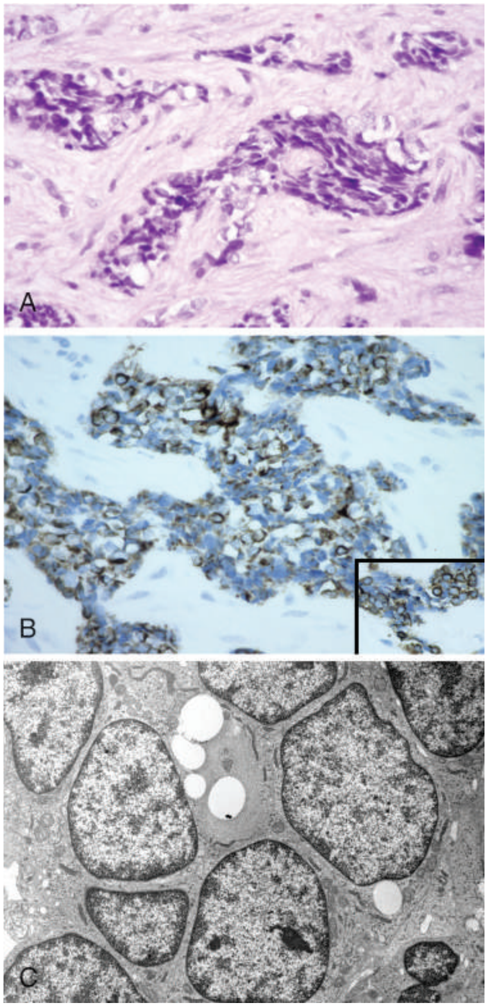

Figure 60-28 Malignant desmoplastic small round cell tumor. **A,** Infiltrative growth of elongated nests of variable size immersed in a densely collagenous stroma. Tumor cells are small and round or ovoid with hyperchromatic nuclei and poorly defined cytoplasmic borders. **B,** Tumor cells show strong diffuse and dotlike cytoplasmic immunoreactivity for desmin and diffuse cytoplasmic immunoreactivity for cytokeratin *(inset)*. **C,** Ultrastructurally, a cluster of undifferentiated small tumor cells with uniform round nuclei and occasional cytoplasmic whorls of intermediate filaments is evident.

survival of 55% compared with 27% when all three modalities were not used. Gross tumor resection was associated with a 58% 3-year survival compared with no survivors in the nonresection control.[629] However, despite multimodality therapy, long-term survival remains very low.

As the name implies, DSRT appears histologically as malignant undifferentiated small round cells typically associated with a prominent desmoplastic reaction (Fig. 60-28).[630] Desmoplastic stroma, however, is not always present, and location of the tumor may play a role in this regard. For example, DSRT originating in the kidney characteristically lacks desmoplastic stroma.[618] Some tumors have glandular or rosettelike epithelial differentiation. Nuclei are usually hyperchromatic with little pleomorphism, dispersed chromatin, and small nucleolus. The scant cytoplasm is poorly outlined. A rhabdoid-type cytoplasmic inclusion can be observed focally in about half of the cases. Areas of necrosis and frequent mitoses are common. Multiphenotypic differentiation is characteristic immunohistochemically. Most cases show immunoreactivity for cytokeratin, EMA, vimentin, and desmin. Desmin and vimentin often show a dotlike intracytoplasmic pattern corresponding to the rhabdoid-type inclusion. Nuclear WT1 expression with antibodies to the carboxyl terminus is usually observed. Ultrastructurally, tumor cells are primitive, are organelle poor, and may show paranuclear whorls of intermediate filaments (see Fig. 60-28, C). Cell junctions including desmosomes may be observed focally.

Molecularly, DSRT is characterized by t(11;22)(p13;q12), which fuses the Ewing sarcoma gene *EWSR1* with *WT1*.[631-634] Although there is variability in the structure of the resulting chimera, the most common fusion includes *EWSR1* exons 1 to 7 and *WT1* exons 8 to 10.[634,635] WT1 is a Cys$_2$-His$_2$ zinc finger DNA-binding protein that shares a high degree of homology and DNA recognition motif with the growth factor–stimulated zinc finger DNA-binding protein EGR1.[636-639] The most common translocations result in *EWSR1-WT1* chimeras that preserve three of the four zinc finger motifs; however, owing to molecular heterogeneity, chimeras may lack *EWSR1* or *WT1* exons.[635] The *EWSR1-WT1* fusion has been detected in two intraabdominal tumors with histologic features of epithelioid leiomyosarcoma but lacking desmoplastic stroma, suggesting either a variant DSRT or another tumor type marked by *EWSR1-WT1*.[640] Based on the paradigm of the other transcription factors fused to the Ewing sarcoma gene, *EWSR1-WT1* is believed to exert its oncogenic activity through transcriptional mechanisms, and several putative transcriptional targets have been identified. The EWSR1-WT1 fusion activates platelet-derived growth factor alpha (PDGFA), T-cell acute lymphoblastic leukemia–associated antigen 1 (TALLA-1), and interleukin-2/15 receptor expression.[641-643] DSRTs stain positive for PDGF and IGF-2.[644] As a mitogen potentially acting on tumor-associated fibroblasts, PDGF seems a plausible cause for the desmoplastic reaction characteristic of these tumors. However, immunohistochemical analysis demonstrated an inverse correlation of PDGF expression with desmoplasia.[645] Although PDGF expression might suggest therapeutic PDGFR inhibition, imatinib demonstrated little to no activity as a single agent for DSRT.[646] About one third of DSRTs stain positive for androgen receptor, and androgen blockade may result in some response.[647]

Angiomatoid Fibrous Histiocytoma

Angiomatoid fibrous histiocytoma (AFH) was first described by Enzinger.[648] Histopathologically, it consists of a nodular fibrohistiocytic proliferation that is usually surrounded by lymphoid follicles and a lymphoplasmacytic infiltrate (Fig. 60-29). The lesion often undergoes focal cystification and hemorrhage. In contrast to the more common (and similar sounding) adult tumor malignant fibrous histiocytoma (currently classified as high-grade undifferentiated pleomorphic sarcoma [see later]), the current WHO classification of STS no longer

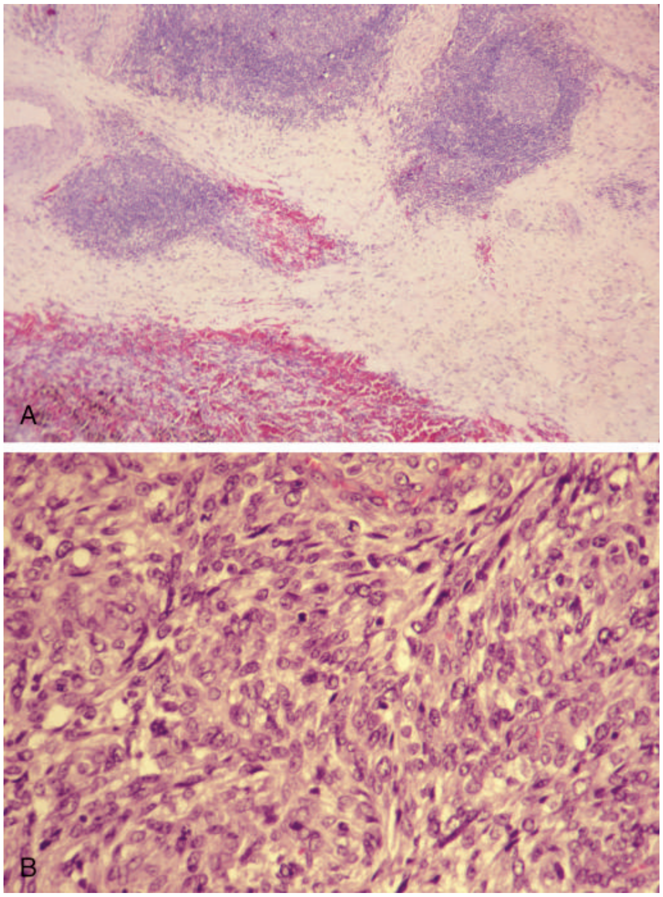

Figure 60-29 Angiomatoid fibrous histiocytoma. **A,** Nodular spindle cell proliferation with hemorrhage *(bottom)* and peripheral fibrous tissue with well-formed lymphoid follicles. **B,** Histiocyte-like cells and fibroblasts poorly arranged in short fascicles.

considers AFH a malignancy. Surgery is considered curative treatment. However, incomplete resection is associated with a significant rate of local recurrence. Metastases to lung and brain have been reported in a single case in an adult with a large tumor.[649]

AFH is associated with a translocation of *EWSR1* or its related protein FUS1 with *CREB1* or the related CREB family member *ATF1*.[616,650,651] These translocations are also characteristic of clear cell sarcoma (see earlier) and have been reported in primary pulmonary myxoid sarcoma.[652] In contrast to typical CCS, these tumors do not express markers of melanocytic differentiation, including MITF. Because *SOX10* is required for *CREB1/ATF1*-mediated *MITF* activation, the differences in expression may reflect the absence of *SOX10* in AFH.[607]

Extrarenal Rhabdoid Tumor

Soft tissue rhabdoid tumor is a high-grade malignant tumor that occurs almost exclusively in infants and children. A subset is congenital, and some of these may be disseminated at presentation.[653-655] Similar to its counterpart of renal origin, extrarenal rhabdoid tumor is an aggressive tumor with a very high rate of distant metastases, most commonly to the lung, lymph nodes, and liver.[656] Soft tissue rhabdoid tumors usually occur in the deep soft tissue, particularly in the paraspinal and neck regions. Some tumors arise adjacent to cutaneous benign mesenchymal lesions.[657,658] Tumor cells have little cohesiveness and grow in sheets or in a pseudoalveolar pattern, often mimicking alveolar rhabdomyosarcoma. Tumor cells are of medium size and are rounded with large eccentric nuclei and a prominent eosinophilic nucleolus. Typically, the cytoplasm contains a paranuclear eosinophilic inclusion. Although extrarenal and renal rhabdoid tumors in children form a discrete entity, soft tissue rhabdoid tumors in adolescents and adults are a heterogeneous group without consistent cytogenetic aberrations.

A majority of tumor cells are immunoreactive for vimentin, EMA, and cam5.2. CD99, synaptophysin, and NSE are also frequently positive. Actin, S100 protein, desmin, myogenin, and CD34 are negative. Ultrastructurally, the rhabdoid inclusion corresponds to a paranuclear whorl of intermediate filaments.

Cytogenetic analysis of rhabdoid tumor consistently demonstrates abnormalities of 22q11.[659] These changes include deletions and translocations. Mapping the deleted region in rhabdoid tumor cell line implicated the *SMARCB1 (hSNF5, INI1, BAF47)* gene, which was found to be mutated and truncated in other tumors.[660,661] SMARCB1 is a component of the multiprotein SWI/SNF adenosine triphosphate (ATP)-dependent chromatin remodeling complex. Mutations that disrupt SWI/SNF function have been identified in a growing number of human cancers in children and adults.[522] Loss of SMARCB1 results in polyploidization and chromosomal instability of rhabdoid cells. Expression of wild-type SMARCB1 results in cell cycle arrest, possibly through restoration of DNA damage-responsive cell cycle checkpoints or replicative senescence, processes mediated by CDKN2A and CDKN1A.[662,663] For further discussion of rhabdoid tumor of the kidney and central nervous system atypical teratoid/rhabdoid tumor, see Chapters 55 and 57.

Epithelioid Sarcoma

Epithelioid sarcoma is a distinctive high-grade soft tissue neoplasm that arises most commonly in the distal extremities of adolescents and young adults, particularly in the fingers, wrists, and hands. In young children, the tumor is more likely to arise in the head and neck as well as the pelvis.[664-667] The tumor can present as a superficial ulcerated lesion or a deep, often painful, mass. Dermis, subcutaneous tissue, fascia, and tendons can be involved, and on recurrence the tumor has a propensity for local spread along fascial planes and neurovascular bundles, resulting in the formation of proximal tumor satellites. Histologically, these tumors may resemble MPNSTs, synovial sarcoma, CCS, rhabdoid tumor, and melanoma.[668] The occurrence of intravenous extension or lymph node involvement is predictive of a very high rate of pulmonary metastases.[669,670] In adults, epithelioid sarcoma demonstrates a 77% risk for local recurrence and a 45% risk for metastatic disease.[671] Survival in children with epithelioid sarcoma exceeds that in adults.[672] In children, epithelioid sarcoma is associated with a 5-year EFS of 61% and an OS that declines from 92.4% at 5 years to 86.9%

and 72% at 10, and 15 years, respectively, indicating the propensity for late recurrence.[673] A small series of children with epithelioid sarcoma demonstrated that after lymphadenectomy with or without irradiation, all patients were free of disease approximately 10 years after diagnosis.[6] Tumor size is the best predictor of prognosis, with tumors larger than 5 cm associated with a worse prognosis.[673,674] Wide local excision with negative margins provides no advantage over primary amputation for patients with localized disease or regional metastases.[675] In a palliative setting, systemic chemotherapy offers a limited and brief response, although an extended response was observed with navelbine.[676,677] Disease stabilization with sunitinib has been described.[678]

Histologically, the more aggressive "proximal" type variant exhibits a distinct rhabdoid phenotype with a pattern of growth that is more sheetlike than nodular.[679] Nodular aggregates consist of medium-sized to large epithelioid and plump spindled cells with central necrosis and collagenization simulating granulomas or metastatic carcinoma (Fig. 60-30, *A*).[680] Immunohistochemically, tumor cells are commonly positive for keratin 8 and 19, EMA (see Fig. 60-30, *B*), vimentin, and CD34.[681,682] Tumor cells are typically diploid, although deletions of the long arm of chromosomes 22 and 1, monosomy 21, and isochromosome 8q as well as gains at 22q have been noted.[683-688] t(8;22)(q22;q11) and t(6;8)(p25;q11.2) have been reported in isolated cases of epithelial sarcoma.[689,690] As in malignant rhabdoid tumors, the majority of epithelioid sarcoma lose *SMARCB1,* which is located at 22q12, through homozygous deletion, although this loss may be less common in pediatric cases.[691-694] Epidermal growth factor receptor (EGFR) overexpression has been noted in epithelioid sarcoma, and one cell line derived from a recurrent malignant tumor demonstrates interleukin-6 secretion and receptor expression, suggesting autocrine activation.[695-697] In preclinical studies, EGFR modulation affected tumor cell proliferation and motility and combined EGFR and mTOR inhibition decreased tumor cell growth.[698] Autocrine MET activation by HGF production has been speculated based on detection of both proteins by immunohistochemistry.[538]

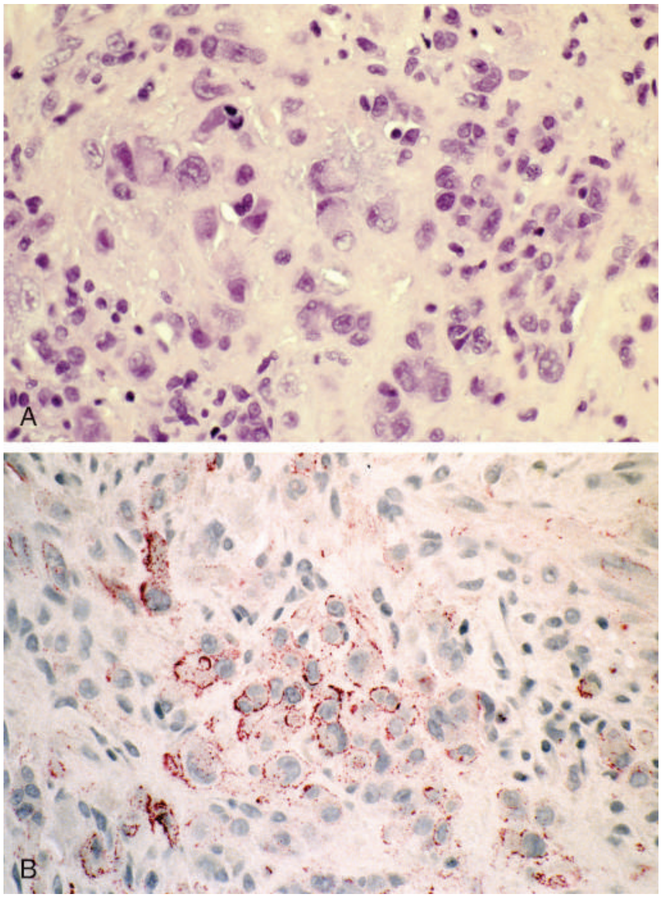

Figure 60-30 Epithelioid sarcoma. **A,** Granuloma-like cluster of large atypical cells (some with rhabdoid morphology), scattered inflammatory cells, and collagenization. **B,** Tumor cells are strongly immunoreactive for epithelial membrane antigen *(membranous pattern).*

Extraosseous Ewing Sarcoma

Although more commonly associated with an osseous origin, extraosseous Ewing sarcoma (peripheral primitive neuroectodermal tumor) can arise in many locations, including the small intestines, kidney, and spinal epidural space. Like its osseous counterpart, extraosseous Ewing sarcoma consists of undifferentiated small round cells with minimal neuronal differentiation. It is characterized by membranous immunoreactivity for CD99 and harbors rearrangement of the *EWSR1* gene on chromosome 22 with a variety of partner genes, but most commonly with *FLI1* on chromosome 11. Other partner genes include *ERG, ETV1, EIAF,* and *FEV. EWSR1* break-apart FISH can be diagnostic. Extraosseous Ewing sarcoma is treated similarly to the osseous form.[699,700] Given differences in treatment and prognosis, care should be made to distinguish central nervous system Ewing sarcoma from central primitive neuroectodermal tumors. For a more complete discussion of Ewing sarcoma see Chapter 61, "Ewing Sarcoma."

Gastrointestinal Stromal Tumor

Most commonly originating from the stomach or small intestine, GISTs are mesenchymal neoplasms that can develop throughout the gastrointestinal tract. Typically, GISTs occur in middle-aged to older adults; however, approximately 3% of GISTs are identified in patients younger than 21 years of age.[701,702] Congenital GIST has been reported.[703,704] In children, the majority of tumors are identified in girls. Although isolated GIST predominates, multifocal epithelioid GIST is associated with Carney triad and together with paraganglioma in Carney-Stratakis syndrome.[702] GIST is also associated with NF1 patients in whom small multifocal but indolent tumors tend to be located in the small intestine.[705] Pediatric GIST typically presents as gastrointestinal hemorrhage that can result in anemia. Although the majority of tumors identified in children are surgically resectable and associated with a good prognosis, GIST is frequently metastatic. The most common sites of metastases at the time of presentation or recurrence are the liver and omentum.[706]

The cell of origin for GIST is uncertain, but the observation that tumor cells have immunohistochemical[548] and

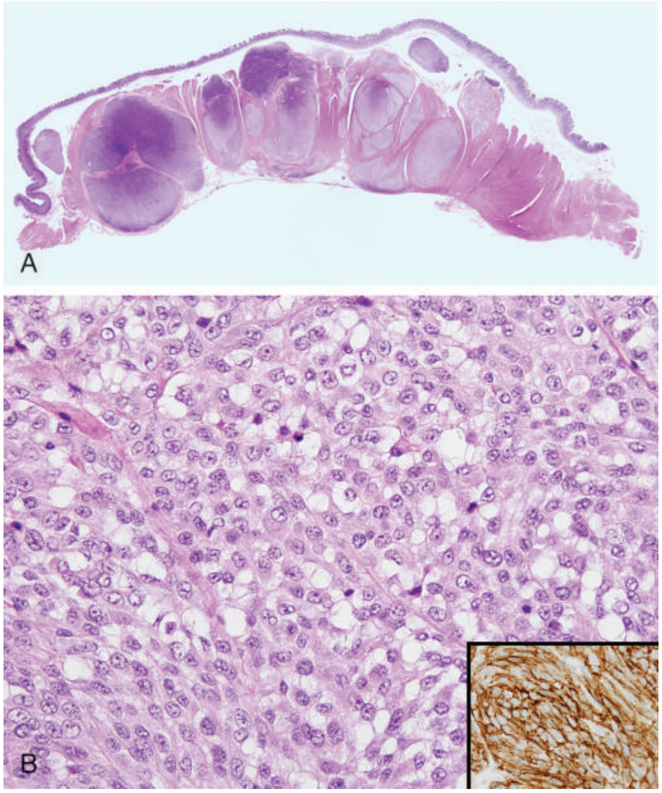

Figure 60-31 Gastrointestinal stromal tumor. **A,** Gastric antrum showing multifocal GISTs centered within the muscularis propria. **B,** Nests of tumor are composed of spindled and epithelioid cells, diffusely immunoreactive for *KIT (inset)*.

ultrastructural[707,708] similarities to the interstitial cells of Cajal led to the proposition that GIST derives from these cells.[707,708] Histopathologically, most GISTs are composed of spindled cells with blunt-ended, elongated nuclei and moderately abundant cytoplasm (Fig. 60-31). They resemble smooth muscle or peripheral nerve sheath tumors. Unlike these tumors, however, immunohistochemistry of GIST in adults and children almost uniformly demonstrates the presence of the receptor tyrosine kinase KIT (CD117) (see Fig. 60-31, *B inset*) and, less frequently, CD34.[709,710]

Several molecular features distinguish adult and pediatric GIST. Whereas GIST in adults is often associated with chromosomal losses of 1p, 14q, and 22q with additional chromosomal gains and losses in metastatic tumors, pediatric GIST typically lacks cytogenetic aberrancy.[711-713] GIST in adults is frequently associated with activating mutations identified in multiple regions of the stem cell factor (also known as mast cell growth factor) receptor *KIT*.[714-716] Mutations include in-frame deletions and amino acid substitutions. A significant fraction of tumors that lack *KIT* mutations harbor activating mutations in *PDGRFA*.[717] The ETS family transcription factor ETV1 is highly expressed in GIST and cooperates with activated KIT.[718] Strikingly, in pediatric GIST, *KIT* and *PDGFRA* are infrequently mutated.[702,719,720] GIST in children and young adults is more commonly associated with mutations that affect the succinate dehydrogenase (SDH) complex.[23,721] The SDH complex, located in the mitochondrial inner membrane, consists of four proteins and catalyzes the oxidation of succinate to fumarate in the citric acid cycle, and mutations significantly decrease complex activity.[24,722] Loss of SDH activity leads to accumulation of fumarate and succinate, which inhibits α-ketoglutarate–dependent processes, including histone demethylases and the TET family of 5-methylcytosine hydroxylases that may alter chromatin in tumors.[723] In addition, SDH loss can increase oxidative stress and promote DNA mutagenesis.[724] Mutations in *SDHB, SDHC,* and *SDHD* have been associated with GIST as well as paraganglioma and pheochromocytoma.[725-729] Mutation in *SDH* genes have been also associated with other tumors, including renal cell carcinoma. IGF1R is frequently expressed in *SDH* mutation–negative tumors, and IGF1 and IGF2 expression seems to correlate with disease-free survival.[730,731]

Historically, nonmetastatic GIST was managed surgically.[732] However, treatment with the KIT and PDGFR targeted inhibitor imatinib demonstrates a significant and durable response in a majority of patients harboring *KIT*- and *PDGFR*-activating mutations.[733,734] Although a significant fraction of tumors progress owing to acquired resistance to imatinib, they may respond to second-line treatment with sunitinib.[735,736] Consequently, *KIT* and *PDGF* genotyping is important in determining initial treatment. For localized tumors without *KIT* or *PDGF* mutation, surgical resection should be performed. For asymptomatic patients with unresectable or metastatic disease, frequent surveillance is reasonable, with resection reserved for disease progression or development of clinical symptoms.[737] In children, the demonstration of KIT activation in the absence of mutation suggests that KIT inhibition may be effective.[713]

Ectomesenchymoma

Ectomesenchymoma is a rare tumor that affects primarily young children. Histopathologically, ectomesenchymomas are composed of a mixture of embryonal rhabdomyosarcoma, ganglioneuroma, and other neuroectodermal or mesenchymal components.[738-740] These tumors are believed to derive from neural crest cells. Treatment of these tumors typically involves surgical resection followed by sarcoma- or rhabdomyosarcoma-directed chemotherapy.[739,741]

NEUROECTODERMAL TUMORS

Malignant Peripheral Nerve Sheath Tumors

MPNSTs are sarcomas that ultrastructurally and immunohistochemically demonstrate nerve sheath differentiation.[742] An origin from a nerve or within a benign nerve sheath tumor or ganglioneuroma as well as a history of NF1 are also important in the diagnosis of this tumor. Previously referred to as malignant schwannomas and neurofibrosarcoma, the term MPNST is preferred given the heterogeneity of tumor cell morphology. MPNSTs typically arise in the deep soft tissues of the extremities, head and neck, and trunk and may present as a mass or symptoms of nerve compression, including

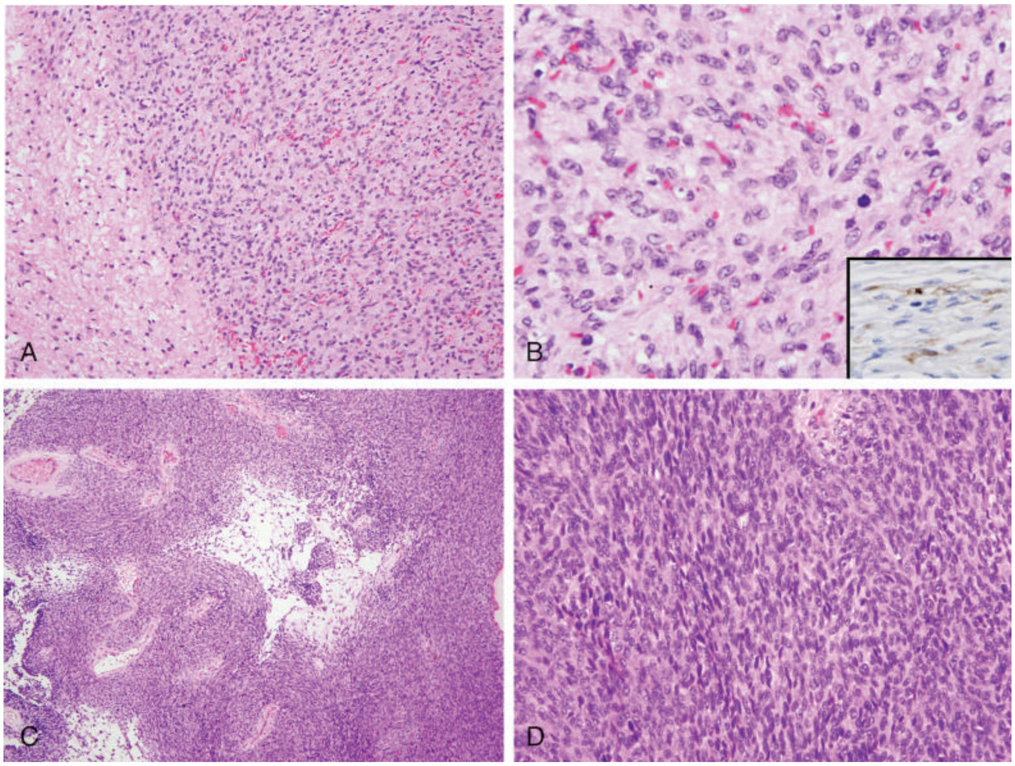

Figure 60-32 Malignant peripheral nerve sheath tumor. **A,** Low-grade MPNST showing a cellular area *(at right)* sharply demarcated from a less densely cellular component *(left)*. **B,** At higher magnification, low-grade MPNST showing mild atypia and occasional mitoses. Low-grade MPNST shows focal strong nuclear and cytoplasmic immunoreactivity for S100 protein *(inset)*. **C,** High-grade MPNST showing densely cellular spindled cell tumor. **D,** At higher magnification, tumor cells in high-grade MPNST are densely arranged and have hyperchromatic nuclei and scattered mitoses and apoptosis.

pain, dysesthesia, or motor disturbance.[742,743] Fifty to 80 percent of MPNSTs develop spontaneously. Patients with NF1 have a lifetime incidence of MPNSTs between 2% and 29%.[16,18,744-747] In a pediatric population, MPNSTs in NF1 patients tend to develop at an older age and tumors tend to be larger, less likely to be resectable, and less likely to respond to chemotherapy than those that develop spontaneously.[743] MPNST is associated with a 5-year OS of 40% to 50% and a PFS of 35% to 37%.[743,748] However, IRS grouping strongly predicts both OS and PFS with complete surgical resection associated with an OS of 80% at 10 years. Histologically, MPNST is composed of fascicles of spindled cells with oval to wavy, slender nuclei with tapering poles and a pale cytoplasm with a poorly defined border (Fig. 60-32).[742] Perivascular hypercellularity and abrupt changes from dense cellular areas to zones containing abundant myxoid matrix are features that suggest nerve sheath differentiation. Pleomorphic, hyperchromatic nuclei are frequently present. Mitoses are usually frequent except in low-grade tumors arising in preexisting neurofibromas in patients with neurofibromatosis (see Fig. 60-32, *A* and *B*). High-grade MPNSTs are densely cellular with frequent mitoses and areas of necrosis (see Fig. 60-32, *C* and *D*). MPNST with rhabdomyoblastic differentiation is referred to as malignant triton tumor and has a worse prognosis than the typical MPNST. Osteosarcomatous or glandular differentiation may also occur. Immunohistochemically, MPNST may show focal positivity for S100 protein, EMA, and glial fibrillary acidic protein (GFAP). For unresectable or metastatic disease or for large tumors, chemotherapy with doxorubicin and ifosfamide or ifosfamide alone can be of limited benefit.[743] Radiation therapy can be beneficial for higher-grade lesions and incomplete resections and is often administered concurrently with chemotherapy. MPNST in the context of NF1 tends to be more refractory to chemotherapy and more likely to recur locally.

The development of MPNST is associated with loss of the tumor suppressor *NF1* at 17q11.2, which encodes neurofibromin. Neurofibromin is a guanosine triphosphatase (GTPase)-activating protein that modulates RAS signaling.[749-753] MPNSTs also frequently harbor mutations of *TP53* and the *CDKN2A*/*CDK2AP2* (*p16*^{INK4a}/*p14*^{ARF}) locus affecting both the TP53 and RB pathways in 75% of tumors.[754] *CDKN2A* or *CDK2AP2* promoter inactivation by CpG island methylation may also downregulate expression.[755] Mice with homozygous *Tp53* and heterozygous *Nf1* deficiency develop predominantly MPNST and malignant triton tumor–appearing sarcomas, and these tumors demonstrate loss of heterozygosity at the *Nf1* locus.[756,757] Multiple signaling pathways appear to be involved in MPNST development. EGFR is consistently expressed in MPNST- and MPNST-derived cell lines, and 26% of MPNSTs demonstrate amplification of the *EGFR* locus.[758,759] *IGF1R* is amplified in 24% of tumors and expression correlates with survival.[760]

Enforced EGFR expression in transgenic mice results in Schwann cell hyperplasia with rare tumor development, whereas EGFR haploinsufficiency in *Nf1+/–*, *Tp53+/–* mice significantly reduces tumor formation.[761] Mutagenesis screening in mice identified Wnt/β-catenin as well as PI3K/AKT/mTor pathway alterations in MPNST.[762,763] PTEN, which modulates PI3K signaling, is involved in malignant transformation.[764]

Melanotic Neuroectodermal Tumor of Infancy

Melanotic neuroectodermal tumor (retinal anlage tumor, melanotic progonoma) is a dysembryoplastic tumor that recapitulates embryonic retinal development. Most cases occur in the head and neck region, particularly in the maxilla, and usually affect male children younger than 1 year of age. The tumors are typically benign but grow rapidly, invade locally, and may metastasize in about 5% of cases.[765] Although complete surgical resection is required for cure, chemotherapy has been shown to decrease tumor mass, thus improving resectability.[766,767] Local recurrence after resection is common, with virtually all recurrences within 4 months and justifying close follow-up.[768] Balancing the desire for negative margins with disfigurement supports a staged surgical approach, and multiple resections may be required to ultimately establish disease control.

The tumor is classified in the family of neuroblastic tumors, and rare cases are associated with elevated urinary levels of vanillylmandelic acid. The histopathology is characteristically biphasic with a component of neuroblast-like cells with neuropils arranged in nests and an epithelioid melanocyte-like component containing melanin (Fig. 60-33). The melanocyte-like cells are usually arranged at the periphery of the neuroblastic-like cells, forming pseudoglandular or pseudoalveolar structures in a densely fibrous stroma. Both cellular components are immunoreactive with neuroectodermal markers such as synaptophysin, GFAP, and Leu7. Melanocyte-like cells are also immunoreactive with HMB45 and cytokeratins.

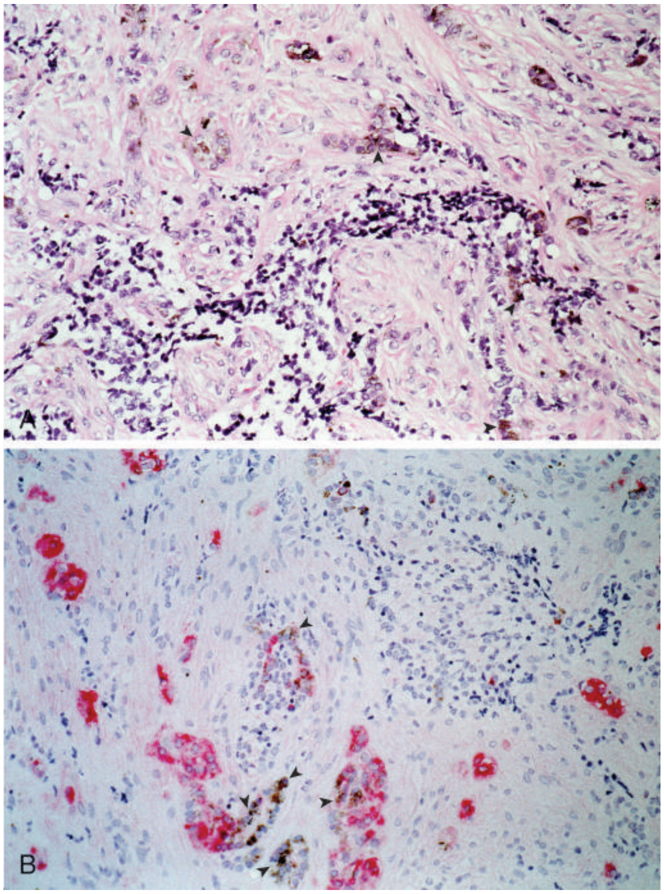

Figure 60-33 Retinal anlage tumor (progonoma). **A,** Biphasic tumor with a component of neuroblastic-like cells with neuropil arranged in nests and an epithelioid melanocyte-like cell component containing melanin *(arrowheads)*. The melanocyte-like cells arranged at the periphery of the neuroblastic-like cells form pseudoglandular or pseudoalveolar structures in a densely fibrous stroma. **B,** Strong cytokeratin immunoreactivity in melanocyte-like cells *(red)*. The dark cytoplasmic granules represent melanin pigment *(arrowheads)*.

UNDIFFERENTIATED TUMORS

Undifferentiated Pleomorphic Sarcoma and Undifferentiated Round Cell and Spindle Cell Sarcoma

Advances in immunophenotyping, microscopy, and molecular-based techniques have resulted in improved classification of soft tissue tumors. Historically, for the purposes of therapeutic trials in childhood cancers, undifferentiated sarcoma had been grouped with rhabdomyosarcoma. However, the application of MyoD1 and myogenin immunohistochemistry enabled segregation of rhabdomyosarcoma from undifferentiated sarcoma. A similar reclassification also occurred for adult undifferentiated sarcomas. Many of the tumors previously described as malignant fibrous histiocytoma have been reclassified to specific categories on the basis of immunohistochemistry, electron microscopy, and molecular genetics.[769-771] However, as a diagnosis of exclusion, undifferentiated sarcomas represent a collection of tumors that lack identifiable morphologic, immunohistochemical, or genetic changes, thus permitting classification as a specific subtype of sarcoma using currently available technologies (Fig. 60-34). Classification of these otherwise undifferentiated tumors rests exclusively on their histology, including undifferentiated round cell and spindle cell sarcoma and undifferentiated pleomorphic sarcoma.

In infants younger than 1 year of age, undifferentiated sarcoma constitutes the most common nonrhabdomyosarcoma malignant mesenchymal tumor. Surgery and chemotherapy result in a 10-year OS of 75%.[8] Undifferentiated pleomorphic sarcoma constitutes a common soft tissue tumor of older adults, with a peak incidence in the seventh decade, and rarely occurs in children or adolescents.[772] A high-grade malignancy, this tumor demonstrates a significant incidence of local and distant recurrence. Pulmonary and lymph node metastases are most common. In a trial of docetaxel and gemcitabine compared with docetaxel alone in metastatic sarcoma, undifferentiated pleomorphic sarcoma was among the most responsive tumor type and demonstrated superior response to both agents in combination.[101]

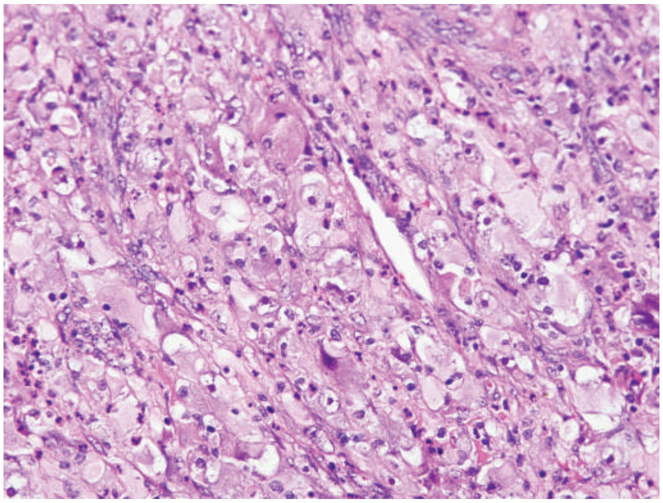

Figure 60-34 High-grade undifferentiated sarcoma. Undifferentiated, pleomorphic sarcoma composed of large highly anaplastic tumor cells. Immunohistochemistry, electron microscopy, and cytogenetics defied further classification.

Pediatric undifferentiated sarcomas may express vimentin, KIT, and VEGF.[773] Hierarchical clustering of adult STS based on messenger RNA expression array profiling revealed an undifferentiated sarcoma class, distinct from, among other tumors, fibrosarcoma and liposarcoma.[542] Expression analyses performed by other groups have also clustered undifferentiated sarcomas but with intermingling of liposarcoma and leiomyosarcoma to varying degrees.[774,775] Cytogenetic studies have also demonstrated that a subset of undifferentiated sarcoma share some features with liposarcoma, but tumors with 12q14-15 amplification, which includes MDM2 and CDK4, are now classified as dedifferentiated liposarcoma.[61,776] Murine modeling suggests that some undifferentiated pleomorphic sarcomas exist on a developmental continuum with embryonal rhabdomyosarcoma and arise from muscle satellite cells.[777]

Undifferentiated round cell and spindle cell sarcomas are occasionally characterized by *EWSR1* translocations and fusion with genes other than those of the ETS transcription factor family typical in Ewing sarcoma. Gene partners include *PATZ1, POU5F1 (OCT3/4), SMARCA5, NFATC2,* and *SP3*.[778] Although the involvement of *EWSR1* as a translocation partner in these tumors suggests that they constitute Ewing sarcoma variants, genomic targeting of these fusions would be expected to differ from the ETS transcription factor containing fusions characteristic of Ewing sarcoma.[779] Other translocations include *CIC-DUX4* and *BCOR-CCNB3*. WNT signaling seems to play a central role in undifferentiated sarcoma development. DKK1, the secreted inhibitor of WNT signaling, is expressed in undifferentiated sarcoma, and it inhibits differentiation by blocking Wnt2/β-catenin signaling.[780] Conversely, treatment of an undifferentiated sarcoma cell line with either exogenous Wnt2 or Wnt5a induced markers of adipogenic or osteogenic differentiation. JUN deregulation may also play a role in undifferentiated sarcoma. *JUN* and *JNK* can be amplified in undifferentiated sarcoma, and JUN can inhibit adipogenic differentiation.[781]

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This study compares FNCLCC with POG grading systems for NRSTS and demonstrates that both systems perform similarly while proposing a system that incorporates histology-based grading as well as incorporation of mitotic figures for intermediate-risk clinical grouping.

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This study demonstates that for children with completely resected tumors, chemotherapy offers limited additional benefit.

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CHAPTER 61

Ewing Sarcoma

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CHAPTER OUTLINE

EPIDEMIOLOGY

BIOLOGIC FEATURES
- Chromosomal Rearrangements in Ewing Sarcoma
- The Cloning of EWS/FLI
- EWS/FLI and Other TET/ETS Fusions in Ewing Sarcoma
- Ewing Sarcoma Cell of Origin
- EWS/FLI Target Genes
- Cooperative Pathways in Ewing Sarcoma Oncogenesis
- Apoptotic Pathways in Ewing Sarcoma
- Biological Features Summary

CLINICAL PRESENTATION AND DIAGNOSIS
- Clinical Presentation
- Imaging Features
- Pathologic Diagnosis

PROGNOSTIC FACTORS

TREATMENT OF PATIENTS WITH NONMETASTATIC EWING SARCOMA
- Local Control
- Systemic Treatment with Chemotherapy
- Management of Tumors Arising at Special Sites
- Summary of Outcomes for Patients with Nonmetastatic Ewing Sarcoma

TREATMENT OF PATIENTS WITH METASTATIC EWING SARCOMA
- Systemic Therapy
- Role of Surgery and Radiation in Patients with Metastatic Disease
- Role of High-Dose Therapy with Hematopoietic Stem Cell Rescue for Metastatic Disease
- Summary of Outcomes for Patients with Metastatic Ewing Sarcoma

RECURRENT EWING SARCOMA
- Location and Timing of Disease Recurrence
- General Approach to Patients with Recurrent Ewing Sarcoma
- Systemic Therapies for Recurrent Ewing Sarcoma
- Role of Surgery and Radiation for Recurrent Ewing Sarcoma
- Role of High-Dose Therapy with Hematopoietic Stem Cell Rescue for Recurrent Disease
- Outcomes for Patients with Recurrent Ewing Sarcoma

LATE EFFECTS IN PATIENTS TREATED FOR EWING SARCOMA

FUTURE DIRECTIONS

In 1921 James Ewing described the cancer that came to carry his name: a primary bone tumor composed of small round blue cells and devoid of the malignant osteoid that characterizes osteosarcoma.[1] Subsequently, pathologists described other clinicopathologic entities initially thought to be distinct, such as peripheral primitive neuroectodermal tumor (PNET) of bone or soft tissue or the Askin tumor of the chest wall.[2] However, biologic study and clinical responses to chemotherapy have now linked these tumors into one entity or family of tumors, a grouping that also includes extraosseous Ewing sarcoma. Commonly used terms for this tumor include Ewing sarcoma, Ewing tumors, and Ewing sarcoma family of tumors. In this chapter the World Health Organization–approved term, Ewing sarcoma, is generally used. When appropriate, specific characteristics that differentiate Ewing sarcoma, PNET, and Askin tumors are described.

The chapter begins with a discussion of the epidemiology of Ewing sarcoma. Next, the current understanding of the cellular and molecular features of these tumors is reviewed. An overview of the clinical presentation and prognostic features of patients with this disease are presented. This overview is followed by a discussion of the management of patients with Ewing sarcoma. The chapter concludes with a review of the late effects seen in patients treated for these tumors.

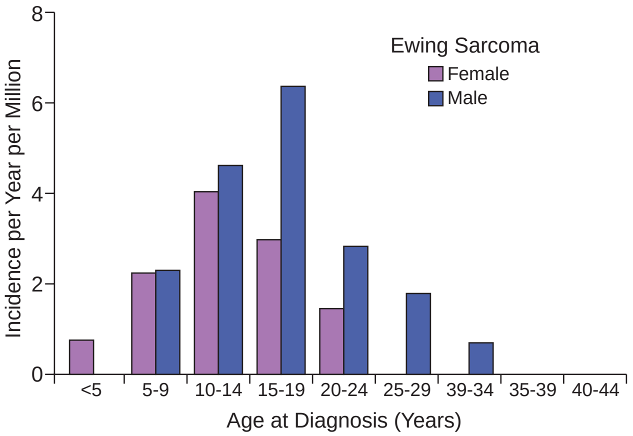


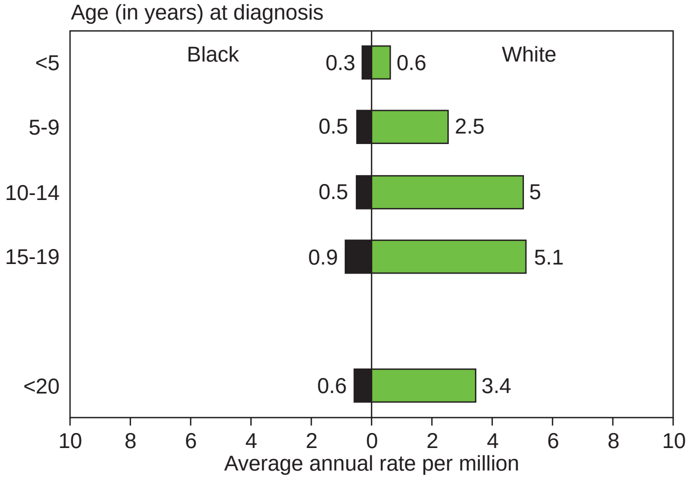
Figure 61-1 Incidence of Ewing sarcoma for male and female subjects according to age at initial diagnosis. *(From Mascarenhas L, Siegel S, Spector L, et al., Malignant bone tumors. In Cancer epidemiology in older adolescents and young adults 15 to 29 years of age, including SEER incidence and aurvival 1975-2000. Bleyer A, O'Leary M, Barr R, and Ries L, Editors. 2006,* National Cancer Institute: *Bethesda. p. 98–109.)*

EPIDEMIOLOGY

Ewing sarcoma is the second most common type of primary bone cancer in the United States and Europe, accounting for approximately 25% to 34% of malignant bone tumors.[3-5] Approximately 250 new cases of Ewing sarcoma are diagnosed in the United States each year.[4,5] In the United States Ewing sarcoma has had a relatively stable average incidence of 2.5 to 3 cases per million per year from 1975 to 2000.[4,6] A similar age-adjusted incidence has been noted in Europe.[3] As shown in Figure 61-1, the peak incidence occurs during adolescence, with an average incidence of 4.6 cases per million per year for the 15- to 19-year age range.[4] Younger children are affected less frequently, and the disease is distinctly uncommon in adults older than 35 years of age. A male predominance has been noted, with a male-to-female ratio of approximately 1.25 : 1.[5,7] However, one study reported a female predominance in patients younger than 3 years of age at initial diagnosis.[8] There is no seasonal variation in the appearance of this disease.[7,9]

The incidence of Ewing sarcoma varies markedly among races and countries, with the highest frequency reported from Australia and Sao Paolo, Brazil.[10] People of African ancestry appear to have the lowest incidence of this disease. Figure 61-2 demonstrates the dramatically lower incidence of these tumors in the U.S. African-American/Black population.[55] A similar low incidence in sub-Saharan Africa has been reported and suggests a possible genetic difference in the risk of developing Ewing sarcoma.[10,11] Patients of African ancestry with Ewing sarcoma have higher rates of soft tissue tumors and an older age distribution compared with patients of European ancestry, indicating that racial differences in this disease extend beyond differences in incidence.[12] Ewing sarcoma also appears to be uncommon in people of East Asian ancestry, based on data from China, Japan, and Thailand.[10,13,14] Data from the Middle East, North Africa, and the Indian subcontinent suggest that Ewing sarcoma is at least as common in these populations as in people of European ancestry.[10,15-17] In small studies from Kuwait and Bombay, for example, Ewing sarcoma was identified as the most common primary malignant bone tumor.[16,17]

Based on these geographic differences in the incidence of Ewing sarcoma, several groups have investigated the genetic epidemiology of this disease. One group reported differences between African and European populations in the number of Alu repeat sequences in intron 6 of the *EWSR1* gene, upstream of the breakpoint region involved in translocations at this locus seen in most cases of Ewing sarcoma.[18] Two studies further focused on this possibility that differences in the incidence of Ewing sarcoma may be due to differences in polymorphisms in the *EWSR1* locus that could result in different rates of translocation.[19,20] Both studies each identified a different single nucleotide polymorphism with low-level statistical association with Ewing sarcoma, although the effect of each polymorphism on the risk of Ewing sarcoma was small. Another study investigated the incidence of other tumors that harbor *EWSR1* translocations across racial groups and observed higher rates of desmoplastic small round cell tumor in the U.S. African-American population compared with the U.S. European-American population.[21] This finding of higher rates of an *EWSR1*-associated tumor in a population with a lower rate of Ewing sarcoma provides additional evidence that differential susceptibility to translocation at the *EWSR1* locus does not account for racial differences in the incidence of Ewing sarcoma. More recently, a genome-wide association study identified and validated polymorphisms in three loci, 1p36, 10q21, and 15q15, associated with increased risk of



*Adjusted to the 1970 US standard population

Figure 61-2 Incidence of Ewing sarcoma by age according to race, demonstrating the marked rarity of the disease in the United States African-American/Black population. *(From Gurney JG, Swensen AR, Bulterys M: Malignant bone tumors, in cancer incidence and survival among children and adolescents: United States SEER Program 1975-1995. Ries LAG, Smith MA, Gurney JG, et al, editors.* National Cancer Institute, SEER Program, *NIH Pub. No. 99-4649, Bethesda, MD, 1999, p. 99–110.)*

Ewing sarcoma. These risk variants are present at lower frequencies in African populations, providing one potential explanation for the lower rates of Ewing sarcoma in this population.[22]

The etiology of Ewing sarcoma remains largely obscure. These tumors are not generally believed to be familial, although rare cases of sibling pairs with Ewing sarcoma have been reported.[23-25] First- and second-degree relatives of patients with Ewing sarcoma do not appear to have an increased overall risk of cancer, although a higher rate of stomach cancer, melanoma, renal cancer, and brain tumors has been reported.[26,27]

Patients with Ewing sarcoma appear to have an increased incidence of specific congenital anomalies. The most consistent finding has been an association between Ewing sarcoma and congenital hernias, particularly inguinal hernias.[28-30] A pooled analysis of the available literature has confirmed this association, with an odds ratio of developing Ewing sarcoma of 2.8 for children with congenital hernias compared with children without congenital hernias.[31] Whether a common hormonal or environmental mechanism underlies the development of both congenital hernias and Ewing sarcoma remains unclear. Patients with Ewing sarcoma have also been noted to have an increased incidence of bone anomalies, particularly rib and vertebral anomalies.[30,32,33] Other congenital anomalies, including cataracts and genitourinary anomalies, have been reported in patients with Ewing sarcoma.[30,32,34] These anomalies occur at a low incidence in these patients and without a consistent pattern, suggesting that their occurrence with Ewing sarcoma may be coincidental.

Various parental exposures have been associated with the risk of developing Ewing sarcoma. The most consistent finding has been an association between parental farm exposure around the time of conception through birth and the subsequent development of Ewing sarcoma. This association has been reported in a series of case-control studies and affirmed in a meta-analysis, with an increased risk if either parent was involved in farming.[28,35-38] Specific exposures related to agricultural work that have been suggested as driving this increased risk include parental fertilizer, pesticide, solvent, and wood dust exposure.[36,37,39] Less consistently reported associations include an increased risk of Ewing sarcoma with parental cigarette smoking and parental occupation in manual labor.[28,36] Family income does not appear to be associated with the risk of Ewing sarcoma.[28,37]

Additional attention has focused on features of physical growth and development that might distinguish patients with Ewing sarcoma. Differences in birth weight between patients with Ewing sarcoma and controls have not been observed.[40] Given its peak incidence during adolescence, some groups have investigated differences in the onset of puberty between patients with Ewing sarcoma and controls. One group reported that boys with Ewing sarcoma began shaving earlier than boys without Ewing sarcoma.[29] Other differences related to development of secondary sex characteristics have not been observed.[29,40] At the time of diagnosis, patients with Ewing sarcoma do not seem to be taller than their peers.[40-42]

Several viruses have been suggested as playing a role in the pathogenesis of Ewing sarcoma. One group reported that the adenovirus *E1A* gene could specifically induce the chromosomal translocation characteristic of these tumors.[43] Follow-up studies failed to corroborate this finding.[44-47] Based on increased Epstein-Barr virus titers in patients with Ewing sarcoma, other groups evaluated Ewing tumors for Epstein-Barr virus infection and found no viral involvement.[48,49] Both BK and SV40 viral sequences have been variably identified in cases of Ewing sarcoma, but the functional importance of these findings remains unknown.[50-52]

Ewing sarcoma rarely develops as a second malignancy. Several case reports describe the development of Ewing sarcoma years after the successful treatment of children with a range of hematologic malignancies and solid cancers.[53-58] More formal studies indicate that Ewing sarcoma as a second malignancy occurs only infrequently. In a population-based study, 2.1% of all Ewing sarcoma cases arose as second malignancies.[59] In a large institutional review, only 1.3% of second malignancies following treatment of pediatric cancer were Ewing sarcoma or PNET.[60] Several large series of patients with secondary bone sarcomas have reported only a small number of patients with Ewing sarcoma.[61-64] These secondary Ewing tumors do not appear to be radiation-related.[59] In addition, the wide range of primary malignancies described in these patients suggests that these tumors do not arise as part of a specific cancer predisposition syndrome.

BIOLOGIC FEATURES

Chromosomal Rearrangements in Ewing Sarcoma

In the mid-1980s the presence of a recurrent reciprocal chromosomal translocation, t(11;22)(q24;q12), was reported in Ewing sarcoma tumors and cell lines.[65,66] The same translocation was also identified in PNET, supporting the hypothesis that these two tumors represented the same disease exhibiting varying levels of differentiation along a neuronal pathway.[67] Subsequent studies demonstrated that t(11;22)(q24;q12) was present in approximately 83% of Ewing sarcomas.[68]

The early analysis of translocations in Ewing sarcoma demonstrated that the derivative chromosome 22 was always maintained as these tumors and cell lines undergo clonal evolution, whereas the derivative chromosome 11 could be lost.[66] This indicated that the “22;11” derivative is the key chromosomal abnormality, whereas the reciprocal “11;22” is unnecessary. The t(11;22)(q24;q12) is a tumor-specific or somatic mutation because it is not found in the germline DNA of patients.[66]

In addition to the t(11;22)(q24;q12) rearrangement (and related translocations with similar molecular consequences, discussed later), other chromosomal abnormalities have also been reported in Ewing sarcoma.[66,69-75] A careful analysis of these sometimes complex karyotypes has revealed a small group of recurrent, nonrandom chromosomal abnormalities. Gains (usually trisomies) of chromosome 8 may be observed in up to 50% of cases, and gains of chromosomes 12 and 1q are seen in approximately 25% of cases.[69,73,74,76,77] Gains in chromosome 20 have also been reported in 10% to 20% of cases.[78] Interestingly, a chromosomal translocation that is unrelated to the t(11;22)(q24;q12) has been observed in approximately 20% of cases: der(16)t(1;16).[69,73,74,76,77] This translocation is often found as an unbalanced rearrangement and thus results in partial gains (trisomies and occasionally tetrasomies) of 1q and losses (monosomies) of 16q.[69,73,74,76,77] The breakpoints of this translocation appear to be variable and range from q11 through q32 on chromosome 1 and from q11.1 through q24 on chromosome 16.[69,73,74,76,77] This suggests that the translocation does not alter particular target genes at the breakpoint but rather introduces gains of 1q and losses of 16q. Finally, losses at 1p36 have also been observed.[75] More recent high-resolution copy number studies have identified copy number alterations in specific genes or regions of the genome, although the role of specific genomic gains or losses has yet to be determined.[79-82] There has also been a report of genomic instability, as measured by microsatellite instability and loss of heterozygosity, in Ewing sarcoma.[83] This finding, however, is somewhat controversial, insofar as a second group was not able to replicate the microsatellite instability findings.[84]

The Cloning of EWS/FLI

A major advance in the understanding of the pathogenesis of Ewing sarcoma occurred when the breakpoint of the (11;22)(q24;q12) translocation was cloned in 1992.[85] The translocation breakpoint was localized roughly in the middle of two genes: *EWSR1* and *FLI1*. *EWSR1* had not been previously identified and so was named on the basis of its involvement in the Ewing sarcoma translocation breakpoint (*Ew*ing *s*arcoma *r*earrangement domain *1*).[85,86] *FLI1* had been previously identified in mice as the *F*riend *L*eukemia Virus *i*ntegration site.[87] As a result of the translocation event, these two genes become fused and the expressed transcript encodes the EWS/FLI fusion protein (Fig. 61-3).[85]

Wild-Type EWS Protein

EWSR1 encodes the EWS protein (see Fig. 61-3).[85] Wild-type EWS contains 656 amino acids and appears to be ubiquitously expressed.[85,88,89] EWS is primarily localized to the nucleus,[90-92] although it has also been detected in the cytoplasm[93] and on the cell surface.[94] The amino terminus of EWS (sometimes referred to as NTD-EWS) contains 285 amino acids consisting of 31 pseudorepeats rich in tyrosine, glutamine, serine, threonine, glycine, alanine, and proline.[85] This region bears some similarity to the carboxyl-terminal domain (CTD) of RNA polymerase II.[85] This similarity suggested that the region could modulate RNA transcription. The carboxyl terminus of EWS contains an RNA recognition motif (an RRM domain), as well as three arginine-glycine-glycine (RGG) domains that are often found in RNA binding proteins. Indeed, it has been demonstrated, in vitro, that EWS can bind to RNA.[89,95] These findings suggest that wild-type EWS may be involved in RNA transcription or processing. The carboxyl-terminal region also contains a potential C_2C_2 zinc finger of uncertain significance.[96] There is evidence that EWS may undergo posttranslational modifications, but the significance of these alterations is not well understood.[94,95,97-100]

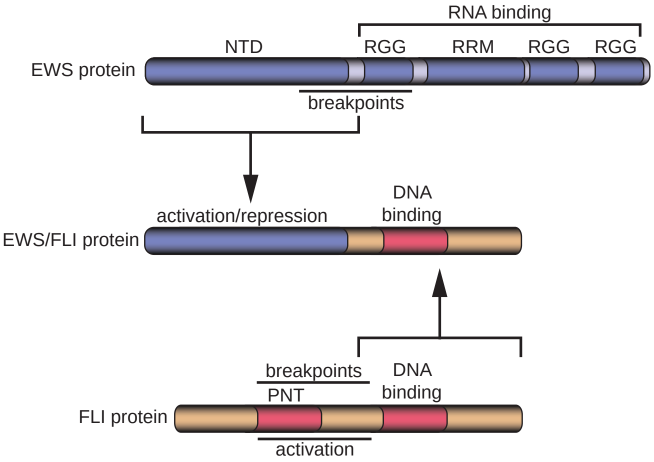


Figure 61-3 Fusion of EWS and FLI produces the EWS/FLI protein. Wild-type EWS is characterized by an amino-terminal domain *(NTD)*, along with three RGG regions and an RNA recognition motif *(RRM)* that confer RNA-binding properties to the protein. Wild-type FLI contains a pointed domain *(PNT)* that overlaps with a transcriptional activation domain and an ETS-type DNA-binding domain. The translocation breakpoint regions of each protein are shown. After a translocation event, EWS/FLI is formed. EWS/FLI maintains the NTD of EWS (which functions as both a strong transcriptional activation domain and a transcriptional repression domain in this context), and the DNA-binding domain of FLI.

EWS is closely related to two additional sarcoma-associated translocation partners, TLS (*t*ranslocated in *l*ipo*s*arcoma; also called FUS) and TAF15 (also known at $TAF_{II}68$, TAF2N, and RBP56), as well as to a *Drosophila melanogaster* protein named cabeza (also known as SARFH; originally called P19).[96,101-104] TLS was first identified in human myxoid liposarcoma in the context of the t(12;16)(q13;p11) translocation, which encodes a TLS/CHOP fusion protein (Table 61-1).[102,104] TAF15 is a member of the TFIID general transcription complex.[101] It is found fused to NR4A3 (also called CHN, TEC, CSMF, or NOR1) in extraskeletal myxoid chondrosarcomas and to ZNF384 (also called CIZ or NMP4) in acute leukemias (see Table 61-1).[105-107] TAF15 also has significant homology and domain organization to EWS and TLS.[101,103] The three human proteins are sometimes described as the TET family of proteins (*T*LS, *E*WS, *T*AF15) or the FET family (*F*US, *E*WS, *T*AF15). Although the normal function of these proteins is not completely understood, some evidence suggests that they may be involved in RNA splicing, gene expression, microRNA processing, and formation of RNA-containing intracellular granules.[90,101,108-125] Interestingly, constitutional mutations in TLS result in familial amyotrophic lateral sclerosis (ALS), and mutations in TAF15 may also be associated with the disease.[126,127]

Wild-Type FLI Protein

FLI is a member of the ETS family of transcription factors.[128] This family is defined by the presence of an ETS type DNA-binding domain and consists of 27 different members in humans.[129] As with most transcription

TABLE 61-1 Translocations in Ewing Sarcoma, Ewing Sarcoma-Like, and Other Tumors that Contain *EWSR1*, *TLS* (also called *FUS*), or *TAF15* Family Members*

Tumor Type	Translocation	Fusion Gene
Ewing sarcoma	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12) t(17;22)(q12;q12) t(2;22)(q35;q12) t(16;21)(p11;q22) t(2;16)(q35;p11)	*EWSR1/FLI1* *EWSR1/ERG* *EWSR1/ETV1* *EWSR1/ETV4* *EWSR1/FEV* *TLS/ERG* *TLS/FEV*
Ewing-like sarcoma	t(20;22)(q13;q12) (NB: can occur in ring chromosome and may be amplified) t(6;22)(p21;q12) t(4;22)(q31;q12) Submicroscopic inv(22) in t(1;22)(p36.1;q12) t(2;22)(q31;q12) t(4;19)(q35;q13) inv(X)(p11.4;p11.22)	*EWSR1/NFATC2* *EWSR1/POU5F1* *EWSR1/SMARCA5* *EWSR1/ZSG* *EWSR1/SP3* *CIC/DUX4* *BCOR/CCNB3*
Clear cell sarcoma	t(12;22)(q13;q12)	*EWSR1/ATF1*
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWSR1/WT1*
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12) t(9;17)(q22;q11) t(9;15)(q22;q21)	*EWSR1/NR4A3* *TAF15/NR4A3* *TCF12/NR4A3*
Myxoid liposarcoma	t(12;16)(q13;p11) t(12;22)(q13;q12)	*TLS/DDIT3* *EWSR1/DDIT3*
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11)	*TLS/ATF1*
Low grade fibromyxoid sarcoma	t(7;16)(q33;p11)	*TLS/CREB3L2*
Acute myelogenous leukemia	t(16;21)(p11;q22)	*TLS/ERG*
Acute myelogenous, lymphoblastic, or undifferentiated leukemia	t(12;22)(p13;q12) t(12;17)(p13;q11)	*EWSR1/ZNF384* *TAF15/ZNF384*

*Gene names are provided rather than their corresponding protein names.

factors, ETS family members have a modular architecture with separate domains that contribute DNA binding and transcriptional functions.[130] The DNA-binding ETS domain of FLI is present in the carboxyl-terminal portion of the protein (see Fig. 61-3).[128] Most ETS family members (including FLI) bind to sequences containing a GGAA or GGAT core sequence.[129,130] In addition to DNA-binding function, FLI contains two separate transcriptional activation domains, one in the amino terminus and a second in the carboxyl terminus (distal to the ETS DNA-binding domain; see Fig. 61-3).[131] Wild-type FLI also contains a PNT (pointed) domain in the amino terminal portion of the protein.[132] PNT domains are thought to mediate protein-protein interactions.[133]

The normal role of FLI appears to be primarily in the hematopoietic lineage, where it regulates megakaryocytic development.[134,135] Knockout of murine Fli-1 results in abnormal megakaryocytic differentiation and a loss in vascular integrity that results in embryonic lethality from central nervous system hemorrhage.[136,137] Hemizygous deletion of FLI is found in patients with Paris-Trousseau or Jacobsen thrombocytopenia syndromes.[136,138,139] FLI also appears to be important for vascular development in the mouse and zebrafish.[136,140] FLI is also expressed in neural crest–derived mesenchyme in both the mouse and quail.[141,142] This finding is particularly interesting in light of the neural crest phenotype of Ewing sarcoma.

FLI itself can function as an oncogene, at least in the hematopoietic context. Indeed, the murine *Fli* locus was first defined as a predominant *F*riend *mu*rine *l*eukemia *v*irus (F-MuLV) insertion site.[87] Thus insertion of F-MuLV at the *Fli* locus results in upregulation of the *Fli* gene and subsequent development of erythroleukemia.[87,128] The oncogenic activity of FLI appears to be limited to the hematopoietic system as introduction of the protein into other models of oncogenesis, such as the NIH3T3 cell model, does not result in the transformed phenotype.[90]

EWS/FLI and Other TET/ETS Fusions in Ewing Sarcoma

EWS/FLI consists of the amino terminus of EWS fused, in frame, to the carboxyl terminus of FLI (see Fig. 61-3).[85] The EWS portion retained in the fusion contains the pseudorepeat domain that harbors a strong transcriptional activation function as well as a transcriptional repression function.[85,90,119,143] The RNA-binding domain of EWS is lost in the fusion protein and instead is replaced by the portion of FLI that contains its ETS DNA-binding domain.[85]

EWS/FLI functions as an oncoprotein. EWS/FLI can induce NIH3T3 cells (an immortalized mouse fibroblast cell line) to exhibit both anchorage independent growth and growth as tumors when injected subcutaneously into immunodeficient mice.[144-146] Conversely, blockade of EWS/FLI expression or function (via antisense RNA, dominant-negative blocking alleles, or RNA interference) causes Ewing sarcoma cells to lose their transformed phenotype.[147-155] These data demonstrate that EWS/FLI expression is required for the oncogenic phenotype of Ewing sarcoma. Interestingly, it has been reported that after therapy-induced neural differentiation of Ewing sarcoma, EWS/FLI expression may also be lost.[156] Although these data support an important role for EWS/FLI expression in Ewing sarcoma proliferation and transformation, they do not address whether EWS/FLI formation is the first step in Ewing sarcoma development or whether other oncogenic events occur first and are followed by EWS/FLI formation.

There are three main consequences of the t(11;22)(q24;q12) rearrangement. First, it places the transcriptional regulation of the EWS/FLI fusion under the control of the *EWSR1* promoter. This is important because the activity of the *FLI1* promoter is limited to hematopoietic (and perhaps neural crest) lineages, whereas the *EWSR1* promoter appears to be active in most, if not all, cell types (see previous sections). Second, the translocation results in a loss of one wild-type *EWSR1* allele and one wild-type *FLI1* allele. Loss of one *FLI1* allele is probably of no consequence because wild-type *FLI1* is not expressed in Ewing sarcoma.[155] Whether loss of one *EWSR1* allele is important in Ewing sarcoma development is not known. The third consequence of the translocation is that it creates the EWS/FLI fusion protein. This is important because although EWS/FLI functions as an oncoprotein, neither wild-type EWS nor wild-type FLI could induce transformation of NIH3T3 cells, thus demonstrating a gain of function for the EWS/FLI fusion.[90] Structure-function analyses of EWS/FLI demonstrated that both the amino terminal EWS domain and the ETS DNA-binding domain of FLI were required for oncogenic transformation of NIH3T3 cells and Ewing sarcoma cells.[143,144] These results indicate that the fusion protein functions as an aberrant transcription factor. Comparisons of EWS/FLI with wild-type FLI demonstrated that the EWS domain included in the fusion protein could function as a much stronger transcriptional activation domain than the domain of FLI that was lost in the fusion.[90] These results suggested that EWS/FLI induced oncogenic transformation by binding to target genes through its ETS DNA-binding domain and upregulating the expression of those genes through its EWS domain.

The mechanisms by which the EWS portion of the fusion functions as a transcriptional activation domain are only now beginning to be understood. Because wild-type EWS has been shown to interact with TFIID, RNA polymerase II, or the p300/CBP coactivators, it appears likely that these interactions are also important for EWS/FLI function.[109,111,116,117] RNA helicase A (RHA) has also been shown to be important for the transcriptional activity of EWS/FLI.[157] Thus RHA may also function as a coactivator for gene expression mediated by the fusion protein.

More recently, it has been appreciated that EWS/FLI also functions as a transcriptional repressor. For example, analysis of the transcriptional program triggered by EWS/FLI in Ewing sarcoma cells has revealed that more genes are downregulated by the fusion than are upregulated.[152,155] Additional analysis has demonstrated that EWS/FLI functions as a direct transcriptional repressor at some target genes, like *LOX* and *TGFBR2*.[143,158] The mechanism of repression appears to be through binding of a transcriptional corepressor complex called NuRD, that includes nucleosome remodeling function, as well as histone deacetylase and histone lysine-specific demethylase activities.[143] Both transcriptional activation and repression functions are required for the oncogenic activity of EWS/FLI in Ewing sarcoma cells.[143]

Analysis of EWS/FLI binding sites in the human genome revealed that the fusion protein binds to sites that contain "classic" ETS-type high-affinity sequences (e.g., ACCGGAAGTG).[159-161] However, a more interesting and unusual finding was that EWS/FLI was also found to bind to GGAA-containing microsatellite sequences.[159-162] EWS/FLI binds to these microsatellites and uses them as response elements to activate the expression of target genes, and the transcriptional function is proportional to the length of the microsatellite.[159,162,163] This raises the intriguing possibility that polymorphisms in GGAA-microsatellites associated with key EWS/FLI target genes (e.g., *NR0B1, CAV1,* or *GSTM4*) may alter susceptibility to Ewing sarcoma or perhaps outcome in patients with the disease.[162,164,165] It has also been suggested that the lack of GGAA-microsatellites near critical EWS/FLI target genes, such as *NR0B1*, in organisms other than humans might be the reason that only humans develop Ewing sarcoma.[159,162]

In addition to EWS/FLI, other less frequent Ewing sarcoma translocation breakpoints were cloned and revealed a very similar structural organization (see Table 61-1). As described, approximately 83% of Ewing sarcoma tumors contain an (11;22)(q24;q12) translocation.[66-68] Approximately 10% of cases demonstrate an alternate translocation, t(21;22)(q22;q12).[166] Cloning of the breakpoint of this rearrangement revealed that EWS was fused, in frame, to another member of the ETS family, ERG.[166] As in the case of EWS/FLI, the EWS/ERG fusion contains the amino terminus of EWS fused to the carboxyl terminus of ERG, which harbors its ETS DNA-binding domain.[166] Interestingly, the ETS DNA-binding domains of FLI and ERG are 98% identical at the amino acid level.[166,167] Subsequent investigations demonstrated that EWS/ERG also functioned as an oncoprotein in the NIH3T3 cell model.[146,168] In subsequent years additional translocations were identified in Ewing sarcoma, including a t(7;22)(p22;q12), t(17;22)(q12;q12), and t(2;22)(q33;q12).[169-172] Cloning of transcripts from these translocations demonstrated that in each case EWS was fused to another member of the ETS family.[169-172] Currently, five EWS/ETS fusions have been described (see Table 61-1).[85,166,169-173] It is assumed that all EWS/ETS fusion proteins bind similar (if not identical) target genes to induce oncogenic transformation.[173]

Overall, most cases of Ewing sarcoma appear to harbor fusions between EWS and various ETS family members. Rarely, fusions between TLS and ETS family members may be present in Ewing sarcoma instead, as shown by the identification of TLS/ERG fusions in four patients with Ewing sarcoma and the identification of a TLS/FEV fusion in another case (see Table 61-1).[174,175] These findings highlight the similarities across the TET family and across the ETS family. Perhaps the most generic manner of describing the fusion proteins in Ewing sarcoma would be as "TET/ETS fusions."[173] Because EWS/FLI is the most common fusion identified in Ewing sarcoma, most of the experimental work has been performed using this fusion protein. Experimental findings based on EWS/FLI have only occasionally been confirmed using other Ewing sarcoma fusion proteins.

In addition to variability of fusion partners, variability also exists in the exonic structure of some of the fusion proteins (Fig. 61-4). The breakpoints found in Ewing sarcoma translocations occur in the introns of *EWSR1* and *FLI1* (and presumably the other ETS factors as well, although in most cases this has not been experimentally

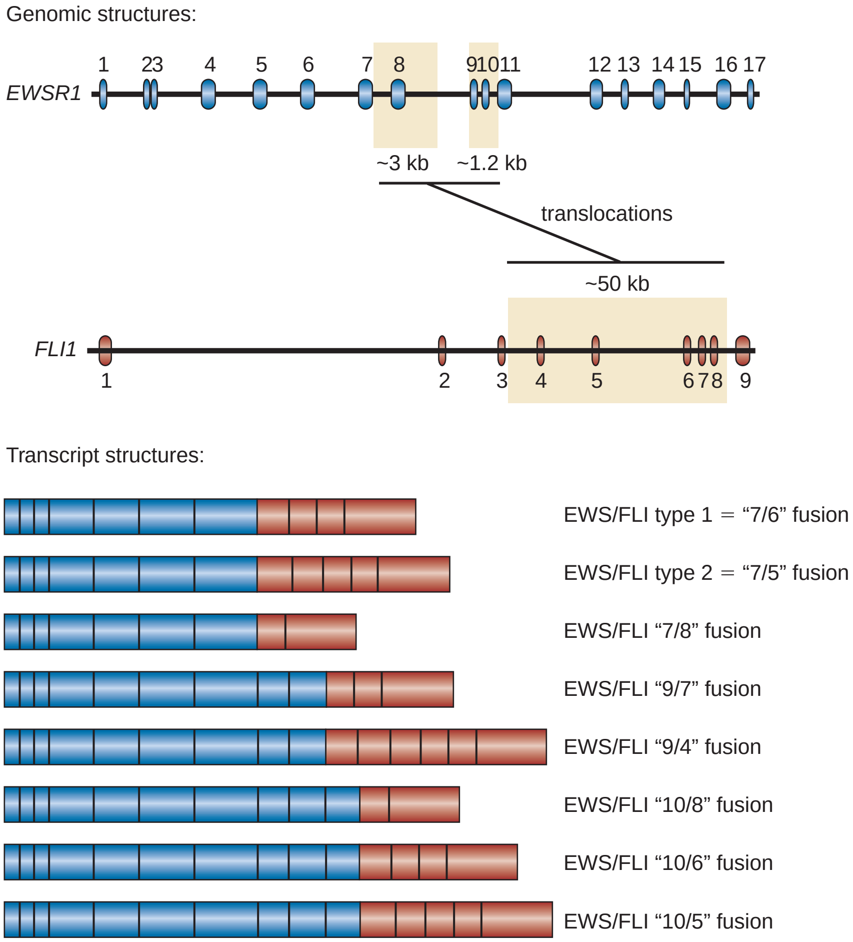


Figure 61-4 Translocation breakpoints may occur within one of two portions of a 6-kb region of the *EWSR1* gene and in a 50-kb region of the *FLI1* gene. This variety results in the observed heterogeneity of EWS/FLI fusion transcripts, as indicated.

confirmed).[85,86,176,177] The breakpoints in the *EWSR1* gene are found in one of two regions (3 kb and 1.2 kb in size) present in an approximately 6 kilobase (kb) region that includes four of the gene's 16 introns.[85,86,176] The breakpoints in *FLI1* occur over an approximately 50-kb region, which includes six of eight *FLI* introns.[85,86,176] Splicing events join adjacent exons together in the fusion transcript.[85,86,176,177] As a result of this variability in breakpoint and subsequent RNA splicing, at least 10 different EWS/FLI isoforms are generated (see Fig. 61-4).[85,86,144,176-178]

The most common Ewing sarcoma translocation fuses exon 7 of *EWSR1* to exon 6 of *FLI1*.[85,177] This "7/6" fusion is commonly referred to as a "type 1" EWS/FLI fusion and accounts for approximately 50% to 60% of EWS/FLI fusions.[177,179] A "type 2" fusion joins exon 7 of *EWSR1* to exon 5 of *FLI1* and is the second most common EWS/FLI fusion, accounting for approximately 20% to 30% of EWS/FLI fusions.[85,177,179] Laboratory studies suggested that the type 1 EWS/FLI fusion was a weaker oncoprotein than the type 2 fusion, and originally it was thought to have prognostic significance.[180,181] However, these functional differences were not reflected on changes in gene expression between type 1 and type 2 fusions, and large-scale clinical correlative data did not indicate a prognostic difference based on fusion subtype.[182-184]

Although there is general agreement regarding the designation of the type 1 and type 2 fusions, there is no generalized nomenclature for the remaining fusions, and so these are best described in terms of which exons are fused to one another. In addition to variability in the EWS/FLI breakpoint, EWS/ERG has also been reported to demonstrate variability based on fusion points, with at least four fusions reported.[166,177] Because the other fusions are so rare, there is only minimal information as to whether they also show similar levels of breakpoint heterogeneity.

Ewing Sarcoma Cell of Origin

The cell of origin of Ewing sarcoma is unknown, and the subject has generated intense debate.[185] The original description by James Ewing in 1921 suggested that this "round cell sarcoma" was of endothelial origin and hence properly referred to as a "diffuse endothelioma of bone."[1] Dr. Ewing later suggested that it may arise from perivascular lymphatic endothelium.[186] A hematologic origin was proposed in the early 1970s following an in-depth evaluation of light and electron microscopic characteristics and cytochemistry of both primary tumor and cell culture specimens.[187] In the early 1980s a fibroblastic-mesenchymal derivation was proposed based on, among other things, the patterns of collagen expression of the tumor.[188,189] In the late 1980s, however, a neural crest derivation was proposed because of the occasional identification of certain neural features, such as the presence of Homer Wright rosettes, neural processes, neurosecretory granules, and neural immunohistochemical markers.[190-195] Additionally, undifferentiated Ewing sarcoma cell lines can occasionally be induced to express markers of neural differentiation, including the expression of neuritelike elongated processes, neuron-specific enolase (NSE), cholinesterase, and neural filament triplet protein.[191,193,196-199] Interestingly, there are a few examples of neural differentiation of Ewing sarcoma occurring after therapy, which further supports this theory.[156,200-202] More recently, it was shown that introduction of EWS/FLI into murine mesenchymal stem cells could result in oncogenic transformation, suggesting that these may be the cell of origin (although the same does not hold true for human mesenchymal stem cells).[203,204] This hypothesis was further supported by microarray and cell biological studies performed on Ewing sarcoma cell lines in which EWS/FLI expression was reduced using RNA interference techniques.[205,206] In the absence of EWS/FLI expression, these cells had a gene expression pattern that was reminiscent of mesenchymal stem cells and a cellular structural organization and adhesive and migratory properties that were also consistent with a mesenchymal phenotype.[205,206] Furthermore, Ewing sarcoma cells with reduced EWS/FLI expression could be induced to differentiate into other mesenchymal cell types, such as fat and bone.[205] This is a characteristic that is shared by mesenchymal stem cells.

It is important to note, however, that the phenotype of Ewing sarcoma (at least the neural crest component) may be a *consequence* of the (11;22) translocation rather than being related to the tumor's cell of origin. Introduction of EWS/FLI into NIH3T3 murine fibroblasts induces a phenotype that is highly similar to Ewing sarcoma, including the small round blue cell morphology and features of neural differentiation.[145,146] Similarly, expression of EWS/FLI in human rhabdomyosarcoma cells, neuroblastoma cells, or even normal human fibroblasts causes those cells to express genes that are similar to the genes expressed in Ewing sarcoma.[207-209] This suggests that EWS/FLI may cause transdifferentiation of its target cells. Thus the neural crest phenotype of Ewing sarcoma may be induced by EWS/FLI.

Transcriptional profiling experiments (e.g., those using oligonucleotide or complementary DNA microarrays and, more recently, RNA sequencing) in Ewing sarcoma tumors and cell lines have allowed for a comprehensive analysis of the gene expression patterns associated with this disease. One of the earliest studies compared Ewing sarcoma tumors and cell lines with other small round cell tumors of childhood, including neuroblastoma, non-Hodgkin lymphoma, and rhabdomyosarcoma.[210] These studies demonstrated that these histologically similar tumors could be distinguished from one another using gene expression data.[210] A more recent analysis compared the expression of approximately 180 sarcoma tumor samples and again showed that tumors could be distinguished using gene expression patterns.[211] Additional studies in which the gene expression pattern of Ewing sarcoma was compared with that of a variety of normal tissues supported the neural crest derivation but also suggested some similarity to endothelial cells.[212] As discussed in the preceding section, analysis of some model systems has suggested a mesenchymal stem cell origin. A definitive understanding of the cell of origin of these tumors will require additional studies. It should be noted that as of yet no genetically engineered mouse model of Ewing sarcoma has been reported, and therefore tissue-specific oncogene expression or lineage-tracing approaches have not been used to identify the Ewing sarcoma cell of origin.

EWS/FLI Target Genes

Because most studies have suggested that EWS/FLI functions as a transcription factor, the two biggest questions in the field have been (1) what genes are dysregulated by EWS/FLI and (2) what are the roles of these gene targets in oncogenic transformation? These questions are beginning to be answered. Because the cell of origin of Ewing sarcoma is unknown, most laboratory-based studies of EWS/FLI have used heterologous cell types with varying results.[155,213-218] More recent approaches have benefited from the use of "knockdown" (reduction) of gene expression using RNA interference (RNAi).[152,154,155,219-222] The RNAi technique has allowed for the analysis of EWS/FLI in patient-derived Ewing sarcoma cell lines themselves, thus avoiding the concerns associated with using heterologous cell types.[152,154,155,219-222]

Gene expression analysis of Ewing sarcoma cells in which EWS/FLI has been knocked down has allowed a comprehensive identification of the thousands of genes that are modulated by the fusion protein.[152,155,219,221] For example, increased expression of NKX2.2 and NR0B1 are required for the oncogenic phenotype of Ewing sarcoma, although the specific mechanisms by which these transcriptional regulators facilitate the transformed phenotype remains poorly understood.[155,221,223,224] Similarly, repression of TGFBR2 (the receptor for transforming growth factor β) and lysyl oxidase (LOX) by EWS/FLI are also required for tumorigenicity of Ewing sarcoma cells.[143,158]

Gene expression studies also demonstrated that EWS/FLI represses the expression of the insulin-like growth factor binding protein 3 (IGFBP-3).[152] IGFBP-3 appears to be involved in Ewing sarcoma apoptosis, and thus decreased expression of this protein appears to prevent apoptosis.[152] These data complement a growing body of literature that supports a critical role for IGF-1 signaling in Ewing sarcoma development. For example, it was shown that Ewing sarcoma tumors and cell lines express IGF-1 and IGF receptors.[225,226] IGF-1 and its receptor appear to function in an autocrine/paracrine fashion in Ewing sarcoma, and disruption of this signaling pathway blocks various functions that contribute to tumorigenesis.[225,227-232] This occurs both in Ewing sarcoma cell lines as well as in model systems.[225-239] Indeed, disruption of this pathway has shown some efficacy as a molecularly targeted therapeutic approach for this disease (discussed later).

NPY1R was identified as an EWS/FLI target gene through microarray analysis.[155] Neuropeptide Y (NPY) is a small polypeptide neurotransmitter with diverse functions.[240,241] Engagement of NPY receptors by NPY in Ewing sarcoma cell lines is growth inhibitory, suggesting a potential therapeutic role for these receptors.[241,242] This result was surprising, insofar as Ewing sarcoma cells also express the NPY ligand.[241,242] It appears likely that Ewing sarcomas are protected from the growth-inhibitory effect of NPY/NPY1R interactions by the expression of dipeptidyl peptidase IV, which cleaves NPY into a form that is unable to bind NPY1R.[243,244] This cleaved form, $NPY_{3\text{-}36}$, functions as an angiogenic factor by binding to NPY2R receptors on endothelial cells, thus providing a likely explanation for the maintenance of this growth-inhibitory autocrine pathway in Ewing sarcoma cells.[243]

Another protein that has been shown to be upregulated by EWS/FLI is vascular endothelial growth factor (VEGF).[208,245] VEGF levels are increased in Ewing sarcoma cell lines and xenografts.[246] VEGF levels are also increased in the blood of patients with Ewing sarcoma and VEGF has been shown to be expressed in 55% of Ewing sarcoma tumor samples by immunohistochemistry.[245,247,248] In the case of Ewing sarcoma, VEGF may induce not only angiogenesis but also vasculogenesis.[249,250] Blockade of VEGF function in Ewing sarcoma model systems prevents tumor growth.[246,250-252] These preclinical studies demonstrate the potential for VEGF inhibition as a therapeutic strategy for Ewing sarcoma.

Most, but not all, studies of Ewing sarcoma have shown that these tumors typically express telomerase activity.[253-256] Interestingly, it appears that hTERT, the catalytic subunit of the telomerase holoenzyme, is upregulated by EWS/ETS proteins.[245,257,258] This upregulation appears to be caused by specific binding of the ETS portion of the fusion to the *TERT* promoter.[245,258,259] These data suggest that EWS/ETS fusions regulate telomerase activity by direct activation of the *TERT* promoter.

One key protein whose expression may be regulated by EWS/FLI is CD99 (also called MIC2).[208] In human fibroblasts engineered to express EWS/FLI, the expression of CD99 closely mimicked that of the fusion protein.[208] In the early 1990s it was found that CD99 is expressed at high levels in Ewing sarcoma cells but is uncommonly found on other tumor types.[260-262] The CD99 antigen is recognized by a variety of monoclonal antibodies, including 12E7, HBA71, and O13, and these antibodies have an important role in the diagnosis of Ewing sarcoma (discussed later).[260-269] The CD99 antigen is a membrane glycoprotein that plays a role in T-cell development and activation and migration of monocytes through endothelial junctions.[270-279] In Ewing sarcoma CD99 inhibits neural differentiation and thus contributes to oncogenic transformation.[280] Significantly, emerging data suggest that antibody-mediated engagement of CD99 may eventually be exploited as a new therapeutic approach to Ewing sarcoma.[281,282] CD99 engagement in Ewing sarcoma results in apoptosis of the tumor cells.[281,282] This effect enhances tumor cell killing by chemotherapeutic agents.[281,283] Gene expression studies of CD99-mediated apoptosis suggested a role for the cytoskeletal protein zyxin in this process, and this finding was confirmed through functional studies.[284] The identification of zyxin in this process was interesting in light of subsequent studies demonstrating that zyxin acts as a tumor suppressor in Ewing sarcoma.[285] Taken together, these studies suggest that CD99 may be an effective therapeutic target in Ewing sarcoma by modulating cytoskeletal actin function.

Cooperative Pathways in Ewing Sarcoma Oncogenesis

Multiple potentially cooperating mutations in addition to TET/ETS fusions have been identified in Ewing sarcoma. The most widely assessed are mutations in the p53 and RB pathways. The p53 pathway includes proteins such as $p14^{ARF}$ and MDM2, as well as p53 itself.[286-290] When expressed in some heterologous systems, such as primary human fibroblasts, EWS/FLI induces the expression of p53.[208] This finding suggests that there is selective pressure to inhibit the p53 pathway. This hypothesis appears to be at least partially true. Deletions in the *CDKN2A* locus (commonly called the *INK4a* locus encoding both the $p14^{ARF}$ and $p16^{INK4a}$ proteins; note that $p14^{ARF}$ is involved in the p53 pathway, whereas $p16^{INK4a}$ is involved in the RB pathway) have been identified in approximately 10% to 30% of primary Ewing sarcoma tumors and more than 50% of Ewing sarcoma cell lines.[291-293] Amplifications or overexpression of *MDM2* is occasionally seen in Ewing sarcoma.[294-297] Mutations in p53 are found in 5% to 20% of Ewing sarcomas.[293,294,297-299] Taken together, mutations in the p53 pathway may be present in nearly 70% of Ewing sarcomas, and these mutations may have prognostic significance in this disease (discussed later). Because there are likely additional components of the pathway yet to be identified or analyzed, the frequency of alterations in this pathway may be even higher.

The RB pathway regulates the cell cycle and includes proteins such as CDK4, D-type cyclins, and $p16^{INK4a}$.[300-302] Mutations have been found in RB itself, and deletions of the *INK4a* locus have been documented as well in Ewing sarcoma.[291,292,303] As was observed in the case of the p53 pathway, mutations in the RB pathway also appear to be relatively common in this disease.[291,292,303]

Whereas mutations in the p53 and RB pathways are relatively common in Ewing sarcoma, mutations in other genes or pathways have only rarely been identified. For example, mutations in BRAF (which is also a member of the RAS/MAPK pathway) have been identified in 1 of 22 cell lines examined.[304] No mutations in other components of the pathway, including RAS or receptor tyrosine kinases, have been identified, as of yet. This suggests that the RAS/MAPK pathway is only rarely activated by mutation in this disease.

Although the RAS/MAPK pathway does not appear to be frequently activated by mutation, there is some evidence for a requirement of pathway activation in Ewing sarcoma tumor development. In the NIH3T3 model system, it was shown that expression of EWS/FLI induced phosphorylation of the ERK1 and ERK2 proteins (downstream members of the MAPK pathway).[305] This effect may result from autocrine stimulation of these transformed cells by PDGF-C, which was also shown to be induced by EWS/FLI expression in the NIH3T3 model, and expressed in Ewing sarcoma cell lines and tumor specimens.[215,306] There is some controversy as to whether PDGF-C plays a role in the human disease, as PDGFR-α, which is required for PDGF-C responsiveness, is not expressed in Ewing sarcoma.[307] Although PDGF-C may not be involved in Ewing sarcoma, inhibition of the MAPK pathway does block oncogenic transformation mediated by EWS/FLI in the NIH3T3 model.[305] This finding was not replicated in Ewing sarcoma cells, thus raising the question as to whether this is an NIH3T3-specific pathway.

Many members of the Wnt signaling pathway are expressed in Ewing sarcoma, including soluble Wnt ligands, Wnt receptors (frizzled), and Wnt coreceptors (LRP5/6).[308] Additionally, microarray analysis of EWS/FLI target genes has demonstrated downregulation of the Wnt inhibitor DKK1 and upregulation of various Wnt signaling components.[155,207,221] These data suggest that Wnt signaling occurs in Ewing sarcoma. In contrast, analysis of the pathway in Ewing sarcoma cell lines in tissue culture has not demonstrated evidence of active Wnt autocrine signaling.[207,308] If the pathway is involved in Ewing sarcoma, it seems likely that it is important for processes such as migration and metastasis, rather than proliferation and transformation.[308] Current studies have not addressed this possibility.

Finally, genes involved in chromosomal stability or chromatin remodeling have also been implicated in Ewing sarcoma. For example, the STAG2 protein helps regulate chromosomal separation during mitosis and was found to be deleted in a number of human tumor types, including Ewing sarcoma.[309] It was suggested that this may be a mechanism of chromosomal instability in Ewing sarcoma and other tumors.[309] Deletion of *SMARCB1* (encoding the SMARCB1/INI1/SNF5 protein) was found in approximately 10% of Ewing sarcoma cases.[81] SMARCB1 is a member of the SWI/SNF chromosome remodeling complex whose deletion is tightly associated with atypical teratoid/rhabdoid tumor.[310] Whether SMARCB1-deleted Ewing sarcoma tumors represent a unique subset of Ewing sarcoma or instead indicate that alterations in chromatin remodeling complexes are widespread in Ewing sarcoma is currently unknown. Interestingly, a newly identified chromosomal rearrangement, t(4;22)(q31;q12), encodes an EWS/SMARCA5 fusion protein in tumors that appear consistent with Ewing sarcoma, supporting the idea that alterations in this chromatin remodeling complex may indeed be widespread in this disease.[311] Another possibility is that the EWS/SMARCA5 fusions are found in tumors that are similar, but not identical, to Ewing sarcoma.[173] Indeed, the most recent World Health Organization guidelines have named these (and other non-TET/ETS translocation-bearing tumors that have similar appearance to Ewing sarcoma; see Table 61-1) as *Ewing-like sarcomas*.[312]

Apoptotic Pathways in Ewing Sarcoma

As described previously, mutations in the p53 pathway are relatively common in Ewing sarcoma. These mutations likely confer resistance to the growth effects of TET/ETS fusions in the disease and may also protect against apoptosis.[208,313,314] Whereas p53 is an important mediator of apoptosis, other signaling pathways are involved as well. These other pathways have been evaluated in Ewing sarcoma in a number of studies.

Both Fas and the Fas ligand (FasL) are expressed in the majority of Ewing sarcomas.[315-317] The status of the FasL in Ewing sarcoma is somewhat controversial; one study reported soluble FasL in Ewing sarcoma–conditioned media,[317] whereas another study reported that the FasL is only found intracellularly and thus is not accessible to cell surface receptor.[315] In terms of the receptor, Fas at the cell surface can be functional, but Ewing sarcoma cells can be divided into Fas-sensitive, Fas-inducible, and Fas-resistant groups.[315] Fas-sensitive lines are killed by coincubation with a FasL-expressing effector cell.[315] Fas-inducible lines are killed by this effector cell line only after pretreatment with interferon-γ (IFN-γ) or cycloheximide (or both).[315] The Fas-resistant lines were resistant regardless of pretreatment.[315] The differences between sensitive and resistant cells are not completely known but may involve differences in Fas expression or expression of proapoptotic or antiapoptotic mediators, such as BAD and BAR.[315,318] Strategies to improve Fas-mediated killing of Ewing sarcoma cells have been investigated, such as allowing for accumulation of more FasL through the use of metalloproteinase inhibitors[319] and upregulation of Fas by administration of interleukin-12.[320,321]

In addition to Fas/FasL, evaluations of tumor necrosis factor–related apoptosis-inducing ligand and tumor necrosis factor α as inducers of Ewing sarcoma apoptosis have also been conducted, in vitro and in vivo, alone and in combination with other agents.[322-333] Taken together, these data support a potential use of proapoptotic ligands in the treatment of Ewing sarcoma. However, the variability of the effects seen demonstrate the need for an ongoing mechanistic evaluation of these pathways in this disease.

Biological Features Summary

The identification of t(11;22)(q24;q12) and subsequent cloning of the EWS/FLI fusion protein represented a major advance in the understanding of the pathogenesis of Ewing sarcoma. EWS/FLI appears to function primarily as an aberrant transcription factor to dysregulate gene targets involved in the development of this disease. Multiple gene targets and cooperating molecular pathways have been identified. These represent a wide array of functions, including growth factor signaling, survival and apoptosis pathways, angiogenesis, cellular immortalization, and cellular differentiation. Despite these myriad molecular functions, a unified mechanistic understanding of Ewing sarcoma development has not yet been reached. Similarly, the cell of origin of the disease has not yet been clearly identified. Nonetheless, there is great hope that a detailed understanding of the molecular mechanisms involved in Ewing sarcoma development will lead to new diagnostic, prognostic, and therapeutic approaches for this disease.

CLINICAL PRESENTATION AND DIAGNOSIS

Clinical Presentation

The most common presenting symptoms of Ewing sarcoma are pain, a palpable mass, or both. In one series 89% of patients with Ewing sarcoma reported pain at the time of initial presentation.[334] Pain does not necessarily occur at night; this pattern was reported in only 19% of patients in one series.[334] Patients may report that their pain began at the same time as a minor musculoskeletal injury. This presentation, reported in approximately 25% of patients, may delay the diagnosis.[334] Unlike patients with osteosarcoma, patients with Ewing sarcoma may report constitutional symptoms such as fever and weight loss. Approximately 15% to 20% of patients have fever at presentation.[335-337] Other symptoms depend on the site of disease. For example, 40% to 94% of patients with paraspinal tumors have presenting symptoms of spinal cord compression.[338-341] Patients with large pelvic tumors may complain of an alteration in voiding. Extensive bone marrow metastatic disease may cause symptoms related to anemia, thrombocytopenia, or neutropenia. Patients may have symptoms for many weeks or months before seeing a physician for evaluation, with most studies reporting an average time from symptom onset to diagnosis of 3 to 5 months.[342-344]

Ewing sarcoma can arise from any bone but has a predilection for pelvic bones and long bones of the leg. Approximately 45% of Ewing sarcomas develop in the axial skeleton. More than half of these axial tumors arise from pelvic bones, and a quarter of axial tumors arise from a rib.[4,5] Up to 30% of Ewing sarcomas arise from long bones in the leg, and up to 15% of tumors develop in long bones in the arm.[4,5] Tumors of the hands, feet, and head develop only rarely, accounting for 10% or fewer of all tumors.[4,5] The distribution of primary tumor site does not vary among racial groups.[10] Soft tissue tumors may arise in any location, although axial sites predominate.[345,346]

Laboratory studies in patients with newly diagnosed Ewing sarcoma may reveal nonspecific abnormalities. More than a third of patients have an elevated erythrocyte sedimentation rate (ESR) at the time of diagnosis.[337,347] Similarly, serum lactate dehydrogenase (LDH) levels are elevated in approximately one third of patients.[336,347-349] Laboratory manifestations of tumor lysis syndrome are uncommon with these tumors. Unlike osteosarcoma, alkaline phosphatase is not typically elevated in patients with Ewing sarcoma of the bone. Patients with advanced bone marrow metastatic disease may have anemia, thrombocytopenia, and neutropenia detected on a complete blood count. In one large series 12% of patients had anemia even in the absence of metastatic disease.[336]

Box 61-1 summarizes the differential diagnosis of Ewing sarcoma. In most cases of Ewing sarcoma of the bone, the main alternative diagnosis is osteosarcoma. Table 61-2 displays features that may help differentiate Ewing sarcoma of the bone from osteosarcoma. Compared with osteosarcoma, Ewing sarcoma is more likely to occur in the axial skeleton. Those Ewing tumors that arise in long bones have a greater tendency to occur in the diaphysis, compared with the tendency of osteosarcoma to arise in the metaphysis of long bones.[350] Patients with Ewing sarcoma are more likely to report constitutional symptoms than are patients with osteosarcoma. Other malignant tumors in the differential diagnosis of Ewing sarcoma of the bone include primary bone lymphoma, giant cell tumor of the bone, and bone metastasis of an extraosseous malignancy. The differential diagnosis of Ewing sarcoma arising from the soft tissues is more broad because the site of origin for these tumors is not restricted to the bone. The site of origin may help narrow the differential diagnosis. Paraspinal tumors may be confused with neuroblastoma, particularly in younger patients. Pelvic tumors may suggest rhabdomyosarcoma or malignant germ cell tumor. Lymphoma and other soft tissue sarcomas can arise at any site and should be included in the differential diagnosis.

Box 61-1 Clinical Differential Diagnosis of Ewing Sarcoma

EWING SARCOMA OF BONE

Osteosarcoma
Primary bone lymphoma
Ewing-like sarcoma
Langerhans cell histiocytosis
Osteomyelitis
Metastasis of an extraosseous malignancy
Benign bone tumor
Osteoblastoma
Chondrosarcoma
Giant cell tumor of the bone

EWING SARCOMA OF SOFT TISSUE

Rhabdomyosarcoma
Nonrhabdomyosarcoma soft tissue sarcoma
Lymphoma
Benign soft tissue tumor
Ewing-like sarcoma
Neuroblastoma
Malignant germ cell tumor

Reports from several cooperative group studies have defined the metastatic behavior of Ewing sarcoma. Approximately 25% of patients exhibit distant metastases at presentation.[351,352] Rates of metastatic disease do not appear to differ between patients with skeletal tumors and those with soft tissue primary tumors.[346] Patients with pelvic primary tumors have an increased incidence of metastatic disease at initial diagnosis.[353] The lung is the most common metastatic site, with pulmonary dissemination reported in 50% to 60% of patients with metastatic disease.[354,355] Patients with isolated pulmonary metastasis represent between 26% and 36% of cases of metastatic disease.[354,356] Patients usually have multiple lung nodules identified in both lungs.[351]

The bone is the next most common metastatic site at presentation. One group reported cortical bone metastases in 43% of patients with metastatic disease.[355] A large

TABLE 61-2 Clinical Differentiation of Ewing Sarcoma of Bone from Osteosarcoma

	Ewing Sarcoma	Osteosarcoma
Age distribution	Peak in adolescence Occurs in young children Very rare in adults >40 years	Peak in adolescence Very rare in children <5 years Occurs in older adults
Racial distribution	Rare in patients of African or East Asian ancestry	No racial predilection
Predisposing factors	Not associated with radiation No known familial predisposition	Radiation exposure Li-Fraumeni syndrome History of retinoblastoma
Constitutional symptoms	Yes	No
Involved bones	Both flat and long bones	Long bones more common
Location in long bones	Diaphyseal more common	Epiphyseal/metaphyseal more common
Type of periosteal reaction	Laminated/layered "Onion-skinning"	Spiculated "sunburst"
Laboratory findings	Normal alkaline phosphatase Abnormal CBC if marrow disease	Elevated alkaline phosphatase Normal CBC
Histologic findings	Small round blue cells No malignant osteoid	Malignant spindle cells Malignant osteoid

CBC, Complete blood count.

cooperative group reported that the bone marrow is involved in approximately 19% of metastatic patients at initial diagnosis.[355] A smaller series noted bone marrow involvement in 52% of patients with metastatic disease.[357] This group performed up to 10 bone marrow aspirates on patients at initial presentation, suggesting that routine sampling may underestimate the incidence of bone marrow involvement. Brain metastasis at initial diagnosis appears to be extremely uncommon. In two series of patients with brain metastasis from Ewing sarcoma, all cases were reported at the time of disease recurrence and not at initial presentation.[358,359]

Fewer than 10% of patients have lymph node involvement at initial diagnosis.[360] The incidence of node involvement is higher in patients with soft tissue rather than bone primary tumors.[355,360,361] In one population-based study, the incidence of regional node involvement among patients with soft tissue tumors was 12.4% compared with 3.2% in patients with bone primary tumors.[360] The incidence of regional node involvement is also higher among patients with distant metastatic disease.[360]

This metastatic pattern helps determine the appropriate staging evaluation for patients with Ewing sarcoma (Box 61-2). In addition to computed tomography (CT) or magnetic resonance imaging (MRI) scans of the primary tumor, all newly diagnosed patients should be evaluated with a chest CT scan. Assessment for bone metastasis has conventionally been with radiolabeled-technetium bone scan. More recent data indicate that positron emission tomography (PET) scans are superior for this indication (discussed later), prompting wider adoption of PET imaging at initial diagnosis.[362] At least bilateral bone marrow aspirates and biopsies should routinely be performed in newly diagnosed patients, with biopsy more sensitive for detection of marrow involvement compared with aspirate.[363] Attention to regional nodes on imaging studies is particularly warranted in patients with soft tissue primary tumors. These evaluations will indicate whether a patient has metastatic or nonmetastatic Ewing sarcoma at initial diagnosis. This distinction serves as the main staging classification in practice for these patients. Although a staging classification for musculoskeletal tumors has been devised by Enneking, this system is not routinely used in the clinical care of patients with Ewing sarcoma.[364]

Imaging Features

Most patients with Ewing sarcoma of the bone are initially evaluated with a plain radiograph. Two representative radiographs are shown in Figure 61-5. These tumors typically appear as poorly circumscribed lesions arising from the bone, but with an associated soft tissue mass.[350] Both lytic and sclerotic areas may be seen within the bony component of the tumor. Given the aggressive nature of these tumors, periosteal reaction is commonly observed. A layered or laminated periosteal reaction is most typical, often giving an onion-skin appearance (see Fig. 61-5, *A*).[350,365] A spiculated "sunburst" periosteal reaction is more commonly associated with osteosarcoma but may be occasionally observed in Ewing sarcoma (see Fig. 61-5, *B*).[350] Codman triangle, a triangular area forming under the periosteum in the setting of a rapidly growing bone lesion, may also be seen in some cases. Cortical thickening is present in approximately 20% of tumors.[350] Pathologic fractures occur in 15% of patients with Ewing sarcoma of bone.[337,350,366]

CT scans and MRI scans are used to better define the extent of local tumor in patients with both osseous and

Box 61-2 Recommended Evaluations for Newly Diagnosed Patients with Ewing Sarcoma

ASSESSMENT OF PRIMARY TUMOR

Magnetic resonance imaging (MRI) scan and/or
Computed tomography (CT) scan

EVALUATION FOR METASTATIC DISEASE

CT scan of the chest
Whole body fluorodeoxyglucose positron emission tomography scan or radiolabeled-technecium bone scan
Bone marrow aspirate and biopsy

EVALUATION BEFORE INITIATING CHEMOTHERAPY

Echocardiogram
Serum creatinine (with formal creatinine clearance if renal function in question)

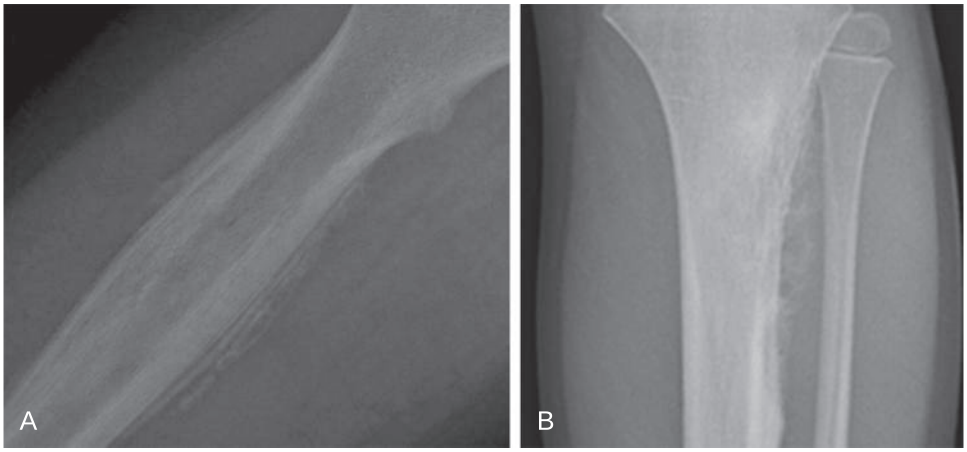


Figure 61-5 **A**, Plain radiograph of a femoral Ewing sarcoma demonstrating a laminated periosteal reaction, resulting in an “onion-skin” appearance. **B**, Plain radiograph of a tibial Ewing sarcoma with a spiculated periosteal reaction.

extraosseous tumors. CT scans most commonly demonstrate a heterogeneous mass with heterogeneous contrast enhancement.[367-371] The soft tissue component generally has lower attenuation than muscle on non-enhanced CT scans.[367,369,372] MRI scans typically reveal a mass that is heterogeneous with respect to both signal intensity and gadolinium contrast enhancement.[370,371,373] Typically T1- and T2-weighted MRI scans demonstrate areas of increased signal intensity of the soft tissue component compared with skeletal muscle.[367,370,371] Although both CT and MRI are able to delineate the soft tissue component of the tumor, MRI may be better able to determine the extent of bone marrow extension and the degree of growth plate involvement.[374]

Emerging evidence suggests that fluorodeoxyglucose PET (FDG-PET) may play an increasing role in the management of patients with Ewing sarcoma. In one series all 32 patients with Ewing sarcoma evaluated by FDG-PET imaging before initiation of chemotherapy had FDG-avid tumors.[375] Figure 61-6 demonstrates an FDG-PET scan of a patient with widely metastatic Ewing sarcoma at initial presentation. A group of studies has evaluated the role of FDG-PET imaging in screening for metastatic disease and for disease recurrence. Several groups have demonstrated that FDG-PET imaging has superior sensitivity and specificity compared with conventional bone scans for detecting bone metastases in patients with Ewing sarcoma.[376-378] In contrast, FDG-PET appears to be inferior to spiral CT scans in screening for pulmonary metastases in these patients.[379] In screening patients with Ewing sarcoma for overall disease recurrence at any site, FDG-PET imaging may have lower sensitivity but higher specificity compared with conventional imaging.[380] Finally, one group has reported that a decrease in FDG-avidity in response to neoadjuvant chemotherapy correlates with improved progression-free survival.[375]

Pathologic Diagnosis

Once imaging studies have localized the tumor, diagnostic tissue must be obtained. The current pathologic diagnosis of Ewing sarcoma depends on tumor morphology, immunohistochemistry findings, and demonstration of a Ewing sarcoma–specific translocation (by standard cytogenetics, fluorescence in situ hybridization [FISH], or reverse transcriptase polymerase chain reaction assays [RT-PCR]).[381] In planning for biopsy, the physician must ensure that adequate tissue will be available to perform each of these tests. For example, one series reported successful cytogenetic analysis in only 59% of fine needle aspirations performed for Ewing sarcoma.[382] The biopsy should be planned with an eye toward ultimate local control of the tumor. If surgical resection is ultimately planned, the biopsy tract should be included in the resected specimen.[383]

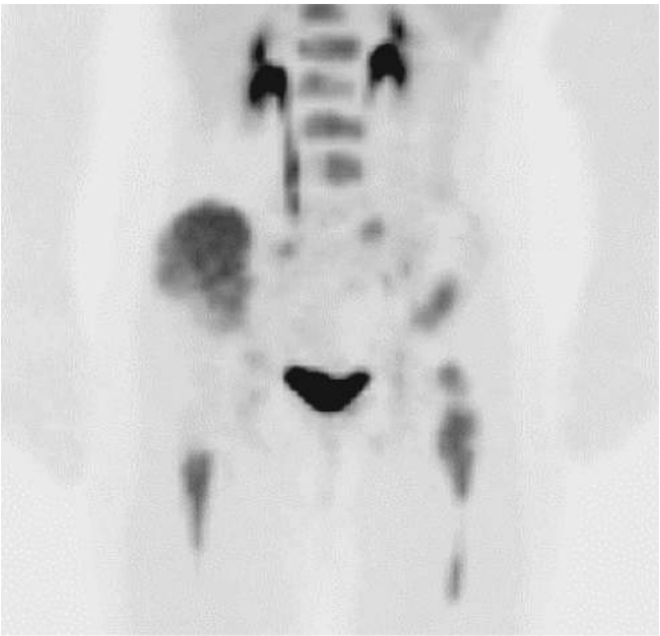


Figure 61-6 Whole-body fluorodeoxyglucose positron emission tomography (FDG-PET) scan of a patient with widely metastatic Ewing sarcoma at initial diagnosis. FDG uptake is evident in bones, soft tissue, and bone marrow.

Ewing sarcoma and PNET form a morphologic continuum ranging from tumors with no apparent neural differentiation to tumors with evidence of early neural differentiation. Of note, the histologic classification of these tumors does not imply site of origin because tumors with either histology may arise in the bone or soft tissue. Ewing sarcoma appears as relatively monomorphic small round blue-staining cells with scant cytoplasm (Fig. 61-7).[384,385] The nuclei tend to be round without prominent nucleoli.[384,385] These cells demonstrate positive periodic acid–Schiff staining owing to cytoplasmic glycogen.[381,385] Electron microscopy may reveal these glycogen stores.[381,385] The tumor cells grow in sheets without additional structure.[384-386] A large cell or atypical Ewing sarcoma subset has been described with large irregular nuclei and more conspicuous nucleoli.[384-386] As many as 30% of tumors with Ewing sarcoma histology arise in soft tissues rather than bone.[384,386] PNETs typically display primitive Homer Wright pseudorosette formation.[384,386] Electron microscopy may reveal additional features suggestive of neural differentiation, such as neurosecretory granules.[381,385,387] Tumors without pseudorosette formation may also be classified as PNETs if they express two or more neural markers (discussed later).[386] Approximately 50% of tumors with PNET histology arise in bone.[384,386] Despite these morphologic differences, Ewing sarcoma and PNET are now viewed as the same biological entity.

Immunohistochemistry plays a key role in differentiating Ewing sarcoma and PNET from other small round cell tumors of children and young adults. The most useful antigen in the diagnosis of Ewing sarcoma appears to be CD99. This protein product of the *MIC2* gene is expressed in a limited number of normal tissues, including strong staining of ependymal cells, pancreatic islet cells, anterior pituitary gland, testicular Sertoli cells, ovarian granulosa cells, and maturing T lymphocytes.[261,388] Less intense staining has been noted in scattered endothelial cells.[261] Ewing sarcoma and PNET both display prominent CD99 immunostaining in a membranous pattern (see Fig. 61-7). Multiple series have found strong CD99 immunostaining in more than 90% of cases of Ewing sarcoma and PNET.[261-263,268,269,388-391] Staining for neural markers, such as synaptophysin, S-100, NSE, and neurofilament, is variable and helps to characterize tumors as Ewing sarcoma or PNET.[381,384-386] These tumors also commonly stain with vimentin, whereas desmin staining is distinctly uncommon.[384,386,392] Approximately 30% of Ewing sarcomas and PNETs demonstrate diffuse c-kit (CD117) staining.[393-395]

Several tumors also express CD99 and may confuse the diagnosis. Strong CD99 immunostaining has been observed in ependymoma, glioblastoma, and pancreatic islet cell tumors.[261,388] These tumors are not typically within the clinical differential diagnosis of Ewing sarcoma or PNET. In contrast, lymphoblastic lymphoma (particularly T-cell), neuroblastoma, alveolar rhabdomyosarcoma, synovial sarcoma, and desmoplastic small round cell tumor may demonstrate variable levels of CD99 staining and are frequently considered in the differential diagnosis of these tumors.[261,392,396-400] Whereas both Ewing sarcoma and lymphoblastic lymphoma may demonstrate strong CD99 staining, Ewing sarcoma and PNET should be negative for lymphocyte markers, particularly leukocyte common antigen and terminal deoxynucleotidyl transferase.[386,392,398] Ewing sarcoma and PNET are also

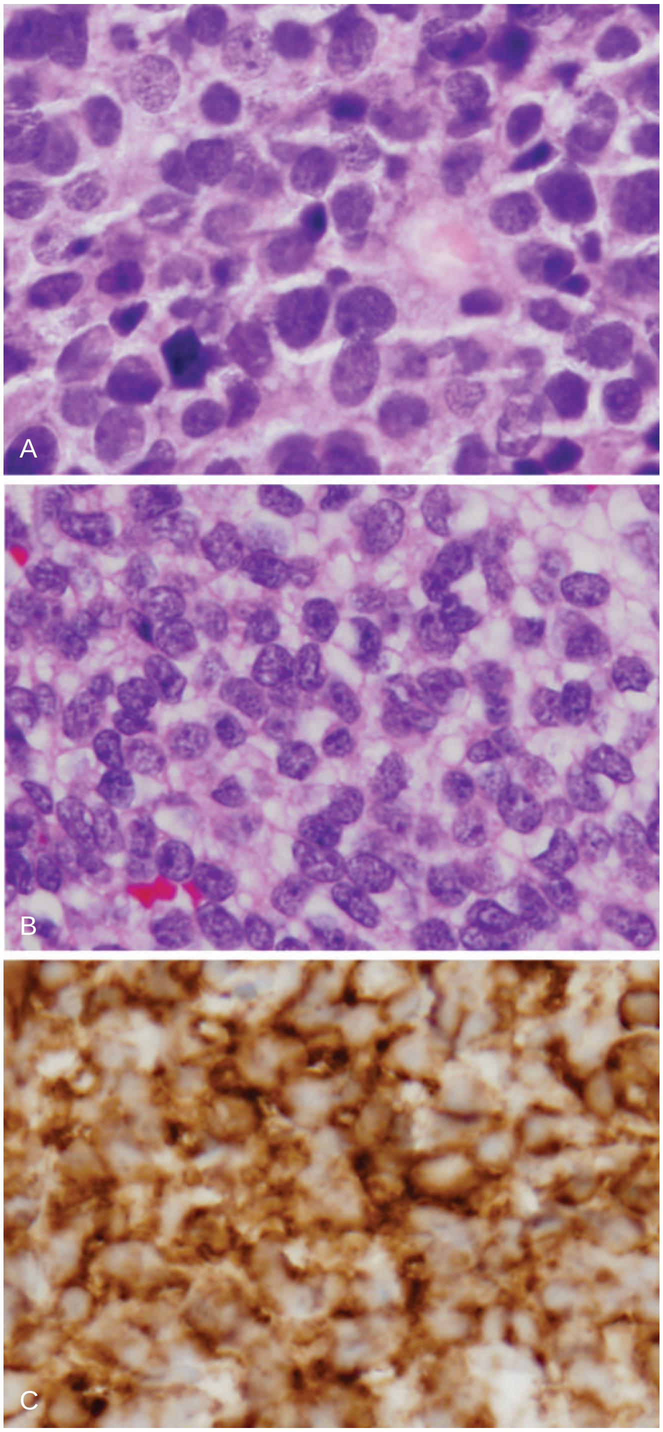


Figure 61-7 **A**, Characteristic morphology of Ewing sarcoma with monomorphic small round blue cells without pseudorosette formation. **B**, Morphologic features of a primitive neuroectodermal tumor (PNET), including a pseudorosette formation in the center of the panel. **C**, The characteristic strong membranous CD99 immunohistochemical staining of Ewing sarcoma. *(Courtesy Dr. Antonio Perez-Atayde, Department of Pathology, Children's Hospital, Boston, Mass.)*

more likely to demonstrate strong vimentin staining compared with lymphoblastic lymphoma.[392] The immunohistochemical differentiation of Ewing sarcoma and PNET from neuroblastoma is typically more straight-forward largely because CD99 staining in neuroblastoma, if present, is weak and patchy.[261,397] Neuroblastomas commonly demonstrate strong staining with NSE, whereas Ewing sarcoma and PNET occasionally will show weak to moderate NSE staining.[263,397] Poorly differentiated synovial sarcoma and desmoplastic small round cell tumor may both also demonstrate CD99 staining and morphologically be difficult to differentiate from Ewing sarcoma and PNET.[396,399,400] In both cases strong membranous staining is more consistent with Ewing sarcoma and PNET. In contrast, weak staining for CD99 and strong staining for cytokeratin and epithelial membrane antigen suggest synovial sarcoma.[396,399] Desmoplastic small round cell tumors reliably demonstrate nuclear staining for WT1, whereas Ewing sarcoma and PNET typically do not stain with WT1.[400] Nuclear staining for FLI1 may also help differentiate these tumors from other pediatric CD99 small round cell tumors. Most cases of Ewing sarcoma and PNET demonstrate nuclear FLI1 staining, whereas synovial sarcoma, neuroblastoma, and rhabdomyosarcoma only rarely show positive nuclear FLI1 staining.[384,401,402] Significantly, however, a high proportion of lymphoblastic lymphomas also exhibit nuclear FLI1 staining.[401] Finally, a number of small round cell tumors of bone harboring novel translocations have been described (see Table 61-1). These have recently been classified as Ewinglike sarcomas.[312] As an example, a *BCOR/CCNB3*-containing tumor has recently been described. This entity has a gene expression profile that is distinct from that of Ewing sarcoma, demonstrates membranous CD99 positivity in only a minority of cases, and can be identified by intense immunohistochemical staining for CCNB3.[403]

The combination of characteristic morphology and immunohistochemistry typically allows an experienced pathologist to render the diagnosis of Ewing sarcoma or PNET. The identification of recurrent chromosomal translocations and their associated oncoproteins in these tumors has allowed for the incorporation of molecular testing into their diagnosis. These methods play a role as confirmatory tests in the setting of typical morphology and immunohistochemistry. In more unusual cases these molecular tests may serve as the key feature on which the diagnosis is based. Although these molecular approaches have been very effective, it is worth recognizing their strengths and weaknesses when interpreting diagnostic studies.

Karyotypic analysis is one approach to confirming the diagnosis of Ewing sarcoma. The presence of one of the characteristic translocations can be considered strong supportive evidence for the diagnosis.[66-68,404,405] It is important to recognize that false-negative results may occur from a number of sources, including overgrowth of nonmalignant elements during culture, complex translocations in which the characteristic Ewing-associated rearrangement is partially masked by the presence of an additional fusion partner,[68,406-408] and cryptic rearrangements in which the translocation cannot be identified using typical techniques.[407,409] FISH can be very helpful as a complementary technique to demonstrate that the *EWSR1* locus is “split,” which suggests a translocation event.[407,410-414] FISH for the *FLI1* and/or *ERG* loci that demonstrates a split signal from either of these genes or fusion between *EWSR1* and *FLI1* or *ERG* signals provides additional strong evidence for involvement of these genes in the rearrangement.[407,410-414] A false-negative FISH result may occur when the fusion involves a rarer translocation partner, such as TLS (in lieu of EWS) or ETV1, ETV4, or FEV (in lieu of FLI or ERG).[169-172,175] These rare translocations are not typically included in the molecular diagnostic evaluation of these tumors.

A positive *EWSR1* “split” FISH assay is not specific for the diagnosis of Ewing sarcoma because of the presence of *EWSR1* rearrangements in tumor types other than Ewing sarcoma (see Table 61-1). For example, clear cell sarcoma (also called malignant melanoma of the soft parts) usually harbors a t(12;22)(q13;q12) rearrangement.[415] This translocation fuses EWS to the ATF1 protein to form the EWS/ATF1 protein.[415] Similarly, desmoplastic small round cell tumor harbors a t(11;22)(p13;q12) rearrangement.[416] This fuses the EWS protein to the Wilms tumor suppressor WT1 to form the EWS/WT1 protein.[416] EWS or TAF15 can join the NR4A3 orphan nuclear receptor to form EWS/NR4A3 and TAF15/NR4A3 fusion proteins in extraskeletal myxoid chondrosarcoma.[107,417-420] EWS is occasionally fused to DDIT3 (also called CHOP) to form EWS/DDIT3 in t(12;22)(q13;q12) positive myxoid liposarcoma.[421] An EWS/ZNF278 fusion was identified in a small round cell sarcoma as a result of a t(1;22)(p36;q12) rearrangement.[422] In some cases there may be diagnostic confusion between some of these tumor types and Ewing sarcoma, and thus the presence of a rearranged *EWSR1* locus may not be adequate for diagnostic purposes. In rare instances tumors may show biphenotypic differentiation with both neural and myogenic patterns. At least some of these express EWS rearrangements (EWS/FLI or EWS/ERG fusions) as well as the classic PAX3/FKHR fusion protein associated with alveolar rhabdomyosarcoma.[423-425]

The use of RT-PCR may provide greater specificity in the diagnosis of Ewing sarcoma. RT-PCR is a useful method for detection of EWS/ETS fusions.[414,426-428] As with any PCR-based method, false-positive results may occur from contamination. False-negative results may occur because of degradation of the RNA, alternate fusion partners, or because an RT-PCR primer pair may be designed to detect some EWS/FLI transcripts but not others.[426-428] False-negative results may also occur when biopsies do not capture viable tissue or primarily include nonmalignant elements. Despite their shortcomings, molecular techniques have become an important addition to the diagnostic approach for Ewing sarcoma.

PROGNOSTIC FACTORS

A number of clinical and molecular features have been identified as prognostic variables in patients with Ewing sarcoma. The presence of metastatic disease at initial diagnosis is widely regarded as the most important adverse clinical prognostic feature. In the largest analysis of prognostic features in this disease, patients with metastatic disease at presentation had a 5-year relapse-free survival rate of 21% compared with 55% for nonmetastatic patients.[353] Patients in this analysis received therapy according to several sequential treatment protocols. Patients with metastatic disease treated in the North American study INT-0091 had a similar 5-year event-free survival rate of 22% regardless of their chemotherapy regimen.[352] Patients with isolated lung metastases have a better outcome than patients with dissemination to other sites or with dissemination to lungs plus other sites.[351,353,356,429] Within this group of patients, patients with unilateral lung metastases may fare better than patients with bilateral lung metastases.[351] Dissemination to regional lymph node is also an adverse prognostic factor, even among patients with otherwise localized disease.[360]

Tumor size has long been recognized as a predictor of outcome in patients with Ewing sarcoma. Patients with larger tumors are at increased risk for having metastatic disease at initial diagnosis. For example, a pooled analysis of German patients treated in the European Intergroup Cooperative Ewing's Sarcoma Study (EICESS) and Cooperative Ewing's Sarcoma Study (CESS) indicated that tumor volume greater than 100 mL was an independent predictor of metastatic disease at initial diagnosis.[430] The effect of tumor size on outcome does not depend entirely on this increased risk of metastatic disease, however. Tumor size appears to be prognostic even among patients with initially nonmetastatic tumors. The CESS-81 study demonstrated that tumor volume greater than 100 mL was a significant adverse prognostic factor for patients treated with either surgery or radiation.[431] The follow-up CESS-86 study found that tumor volume greater than 200 mL was a major adverse prognostic feature for patients with localized Ewing sarcoma.[432,433] The North American INT-0091 study reported that nonmetastatic patients with tumors greater than 8 cm in maximal dimension had an inferior outcome compared with patients with smaller tumors.[352] The French EW88 study reported that tumor size, using either tumor volume or longest dimension, was prognostic only among patients who received radiotherapy alone as the mode of local control.[347] Although some smaller studies have not observed this effect of tumor size on outcome,[434] most investigators consider tumor size prognostic. Indeed, a large pooled analyses of prognostic factors in Ewing sarcoma identified large tumor volume as an independent predictor of poor outcome.[353]

Tumor site is also widely considered a prognostic factor in Ewing sarcoma, with axial tumors carrying a worse prognosis. The Intergroup Ewing Sarcoma Study 1 (IESS-1) established pelvic site as an unfavorable prognostic feature.[435] Several other cooperative group studies have confirmed this finding and indicated that patients with axial tumors generally have a worse outcome compared with patients with nonaxial tumors.[343,352,436,437] In many of these studies, patients with pelvic primary tumors have been shown to have a particularly poor outcome compared with patients with other axial locations.[343,352,436] However, the inferior outcomes in patients with pelvic tumors may be confounded by a higher incidence of both large tumor size and metastatic disease.[353,430] For example, in the CESS-86 trial, axial tumor location was a significant adverse prognostic feature on univariate analysis.[433] After controlling for tumor size and chemotherapy response, however, the prognostic impact of axial site became insignificant. Several groups evaluated only patients with nonmetastatic tumors and found that patients with pelvic tumors still had an inferior outcome compared with patients with nonpelvic tumors.[352,435,436] Two other groups used multivariate methods to evaluate the prognostic impact of axial tumor location in nonmetastatic patients.[336,353] Both groups reported that axial tumors have an inferior outcome compared with extremity tumors, even after controlling for potential confounders. Neither analysis controlled for tumor size, however. Therefore whether axial tumor location portends poor outcome independent of large tumor size remains unclear.

The prognostic impact of soft tissue rather than bone primary tumor origin remains unclear. In one population-based study, soft tissue origin was independently associated with improved outcomes compared with bone origin.[346] In contrast, a cooperative group clinical trial that included both patients with soft tissue and bone tumors showed equivalent outcomes.[438] As more trials include patients with soft tissue tumors, the prognostic impact of tumor origin will become clearer.

A number of groups have specifically evaluated histologic response to neoadjuvant chemotherapy as a predictor of outcome. Patients with a favorable chemotherapy response (typically 90% to 100% tumor necrosis depending on the grading scale used) at the time of surgery have been found to have a decreased risk of disease recurrence compared with patients who exhibited less necrosis.[255,347,431,433,439-442] For patients with nonmetastatic tumors of the extremity, chemotherapy response is prognostic independent of age and tumor size.[440] Despite these results, grading of chemotherapy response in Ewing sarcoma has not yet become routine practice, perhaps at least in part because this variable is not evaluable in those patients who receive definitive radiotherapy as their mode of local control. An alternative to histologic grading is radiographic grading of chemotherapy response. Two groups have reported that favorable radiographic response to chemotherapy is a predictor of favorable outcome.[433,437,439] Evaluation of radiographic response may also not be possible in all patients, particularly in patients with tumors confined to bone without an associated soft tissue mass.

Age at initial diagnosis has been increasingly recognized as an important prognostic feature in Ewing sarcoma. Several studies have indicated that younger patients have an improved outcome compared with older patients.[336,348,352,353,434,435] Older patients have an increased risk of presenting with large tumors, but interestingly they do not have a higher incidence of metastatic disease at initial diagnosis.[430] Even controlling for these potential confounders, age has been identified as an independent predictor of outcome in a number of studies.[336,348,436]

Most studies have used a single cut-point in the 12- to 15-year age range. One study divided patients into three groups (younger than 10 years, 10 to 17 years, and 18 years or older) and found that nonmetastatic patients 18 years of age or older at initial diagnosis had a particularly poor outcome (44% 5-year event-free survival rate).[352] A number of studies have focused on outcomes in older adolescents and adults with Ewing sarcoma. These studies have typically identified the same adverse prognostic features (metastatic disease, large size, axial location, and poor chemotherapy response) in older patients that have been identified in larger studies in patients at a wide range of ages.[443-446] The mechanism underlying these differences in outcome with age remains unclear. Possible explanations include biological differences in tumor and host as well as health care delivery differences in compliance and dose intensity. For example, at least two reports have noted higher rates of soft tissue tumors in adults older than 40 years of age at initial diagnosis, which suggests biological differences in this disease when it arises in older patients. Another series noted lower treatment intensity and inferior outcomes in older patients,[447,448] whereas another series reported similar treatment intensity and outcomes in older patients.[448,449] These results highlight the importance of optimizing treatment intensity to maximize outcomes in adults with this disease.

Serum LDH at initial diagnosis also appears to be prognostic. Several early studies reported that patients with elevated serum LDH levels at diagnosis had an increased risk of disease recurrence.[450-452] More recent studies have confirmed this finding and have found that the negative prognostic impact of an elevated LDH remains even after controlling for other possible confounding variables.[336,348,453] A meta-analysis evaluating a range of tumor markers in Ewing sarcoma also concluded that elevated LDH at diagnosis is associated with inferior outcomes.[454] Presumably, elevated LDH levels indicate greater tumor cell turnover and therefore more aggressive clinical behavior.

A variety of other clinical and demographic features at diagnosis have been variably reported to be prognostic in Ewing sarcoma. Two groups have reported that female sex is an independent predictor of favorable outcome.[336,348] Other groups have not corroborated this finding.[352,353,432] Other studies have demonstrated superior outcomes among the U.S. white, non-Hispanic population compared with members of other racial and ethnic groups.[12,455,456] A greater degree of neural differentiation has been reported as an unfavorable marker by some groups but not by others.* Fever, anemia, leukocytosis, elevated erythrocyte sedimentation rate, and hypoalbuminemia at initial diagnosis have also been suggested as unfavorable prognostic features, but their prognostic value has not yet been validated.[336,347,453,462]

The nature of the Ewing sarcoma–specific translocation does not appear to provide prognostic information. One group has demonstrated that patients with any type of EWS/FLI translocation have similar outcomes to patients with any type of EWS/ERG translocation.[463] Previous studies suggested that patients with a type 1 EWS/FLI translocation had better outcomes compared with other types of EWS/FLI or EWS/ERG translocations.[179] However, two large cooperative group trials both demonstrated that translocation subtype does not affect survival in the setting of modern therapy.[183,184]

Several studies have measured EWS/FLI fusion transcripts in the peripheral blood or bone marrow to predict outcome. These studies use RT-PCR techniques to identify occult metastatic disease, with 25% of clinically nonmetastatic patients noted to have detectable EWS/FLI fusion transcripts in blood or bone marrow in one early study.[464] The results of studies correlating these findings to clinical outcome have been mixed. In one report patients with clinically localized disease but positive peripheral blood EWS/FLI RT-PCR at diagnosis had a higher risk of relapse compared with patients with negative peripheral blood EWS/FLI RT-PCR.[465] A second study evaluating bone marrow EWS/FLI RT-PCR at diagnosis was not able to confirm this finding.[466] Patients in this study had follow-up EWS/FLI RT-PCR performed on blood or bone marrow (or both). Patients with positive blood or bone marrow EWS/FLI RT-PCR at their last evaluation had a significantly increased risk of clinical disease recurrence.[466] The utility of this methodology for predicting outcome in patients with Ewing sarcoma will require further prospective evaluation.

Chromosomal changes beyond the characteristic TET/ETS translocations may also provide prognostic information. Although it is the most common additional karyotypic abnormality in Ewing sarcoma, at least three groups have reported that trisomy 8 does not appear to influence outcome.[467-469] A small series of 21 patients reported that all three patients with gain of chromosome 1q died as a result of disease.[470] Another study of 88 patients with complete cytogenetic data did not observe an association between 1q gain and poor outcome, although only 12 patients had 1q gain.[469] A larger study of 124 patients with Ewing sarcoma included 26 patients with gain of chromosome 1q and found that patients with gain of chromosome 1q have a worse outcome.[467] One study reported that more global karyotypic anomalies may also be prognostic, including complex karyotypes (more than five chromosomal anomalies) or hyperdiploidy (more than 50 chromosomes).[469]

Aberrations in p53 and p16 have also been correlated with outcome in patients with Ewing sarcoma. The 10% to 15% of patients with mutations in the *TP53* gene (encoding the p53 protein) have consistently been shown to have a significantly worse outcome compared with patients who have wild-type *TP53*, even after controlling for other prognostic factors.[293,471] Additional work has suggested that mutations or deletions of the *CDKN2A/INK4A* gene may also confer a poor outcome in these patients.[292,293,472] For example, aberrant *TP53* or *CDKN2A/INK4A* gene status in one study was shown to be an independent negative prognostic feature.[293] However, another study focused exclusively on *CDKN2A/INK4A* deletion failed to show an association with prognosis.[473] A formal meta-analysis of this issue concluded that *CDKN2A/INK4A* aberration is a strong negative prognostic factor.[474]

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*References 194, 263, 361, 385, 386, 433, 457-461.

A number of other molecular markers have been proposed to have prognostic impact in Ewing sarcoma. In one study telomerase activity in the peripheral blood during follow-up, but not at initial diagnosis, was strongly associated with disease recurrence.[255] None of the patients in this study with low telomerase activity during follow-up had disease recurrence. CD56 co-expression has been reported to be a favorable prognostic indicator in one series.[475] One report suggested that serum IGF-1R levels were prognostic,[476] although two other studies found no prognostic impact of IGF-1R levels.[477,478] Two studies identified expression of the genes encoding glutathione S-transferases, enzymes involved in chemotherapy metabolism, as novel prognostic factors.[165,479]

TREATMENT OF PATIENTS WITH NONMETASTATIC EWING SARCOMA

Local Control

Patients with nonmetastatic Ewing sarcoma require aggressive multimodality therapy to achieve long-term disease control. Local control of the primary tumor plays a key role in modern treatment for these patients. Historically, patients received radiation therapy alone as local control. Surgical local control was less commonly used because therapy was generally viewed as palliative. With improved outcomes thanks to chemotherapy, however, the late effects of radiotherapy on long-term survivors of Ewing sarcoma became apparent. In addition, limb-salvage surgical techniques became more advanced. As a result, surgical local control of resectable tumors has become the standard of care. Radiotherapy for local control of the primary tumor is now generally reserved for unresectable tumors or tumors that have been resected with inadequate margins. This section reviews the principles of radiotherapy and surgical resection in the care of patients with Ewing sarcoma.

Surgical resection is generally recommended for those tumors deemed completely resectable with wide or radical margins. For extremity tumors, surgical resection may take the form of an amputation or a limb-sparing procedure. Rotationplasty is another option for patients with femoral tumors.[383] Technical advances, such as expandable implanted prostheses, have made limb-sparing procedures increasingly appealing.[383,480] However, the choice of a limb-sparing procedure over other surgical options must not compromise the adequacy of the resection.[383] Tumor infiltration of surrounding neurovascular structures, muscle, or skin should raise concern that a limb-sparing procedure will not result in the desired wide resection.[383] Often neoadjuvant chemotherapy will produce sufficient response to allow for limb salvage when this option did not appear feasible at initial presentation.[383] In a cooperative group analysis of chest wall tumors, resection after neoadjuvant chemotherapy was associated with higher rates of negative margins compared to initial resection.[481] However, data from a single institution suggested that neither the likelihood of an adequate margin nor the overall outcome differs between patients who had surgical resection at initial diagnosis compared with patients who had surgical resection after a period of neoadjuvant chemotherapy.[482,483] Nevertheless, most available data support the current standard approach of neoadjuvant chemotherapy before planned surgical resection.

Investigators from the CESS group and the Rizzoli Institute evaluated the impact of surgical margins on outcome in Ewing sarcoma in two retrospective case series.[484,485] These groups both reported the rate of obtaining an adequate surgical margin as approximately 75%, among those patients selected for planned definitive resection.[484,485] Both groups observed that an adequate margin is more difficult to obtain in axial tumors. For example, the Rizzoli group reported that approximately one quarter of the extremity tumors treated with a limb-salvage procedure had inadequate margins. In contrast, none of the paraspinal or sacral tumors in their large series of surgically treated patients had an adequate margin.[484] The Rizzoli group observed that the 5-year event-free survival rate was higher for patients with an adequate surgical margin, although this finding may reflect improved outcome with smaller, more resectable tumors.[484] In both series a small group of patients with inadequate surgical margins did not receive postoperative radiotherapy. Only 14% to 21% of these patients experienced a local recurrence.[484,485] However, the risk of local recurrence was diminished by the addition of postoperative radiotherapy, which is the current standard practice.

Radiation therapy alone can provide adequate local control in Ewing sarcoma. In fact, the radiosensitivity of these tumors was one of the early characteristics that distinguished Ewing sarcoma from osteosarcoma.[1] Local control rates with definitive radiation therapy in large series have been reported to range from 53% to 86%.[486] For patients treated with radiation therapy alone, axial tumors (particularly pelvic tumors) have a higher rate of local failure than extremity tumors.[436,487-489] One group reported that patients with metastatic disease treated with radiation alone or surgery plus radiation have a higher rate of local failure compared with patients who have nonmetastatic disease.[490]

Several studies have evaluated the appropriate treatment volume, dose, timing, and schedule of radiotherapy in Ewing sarcoma. The IESS-1 study group reported on their experience with radiotherapy.[488] All patients received radiotherapy as the mode of local control in this study. Across the range of doses administered (30 Gy to more than 60 Gy), no differences in local failure rates were observed, although very few patients received less than 40 Gy. Most patients received radiation to the whole bone involved with tumor, but some patients were treated with more limited fields. Patients who received radiotherapy to the primary tumor plus a 5-cm margin had the same local failure rate as patients treated with whole bone radiotherapy (8% for both groups). Patients treated with less than a 5-cm margin had an inferior local control rate.

The Pediatric Oncology Group conducted a study from 1983 and 1988 with the specific aim of determining the appropriate radiation volume for patients with Ewing sarcoma of the bone.[487] Patients with tumors in expendable bones were recommended for surgical resection. Other patients were randomized to receive whole bone irradiation or involved field radiation. Patients treated with whole bone irradiation received 39.6 Gy to the entire bone containing tumor plus a boost to 55.8 Gy to the tumor with a 2-cm margin. Patients treated with involved field radiation received 55.8 Gy to the tumor with a 2-cm margin. Because of enrollment issues, 20 patients were randomized to whole bone radiation and 20 patients to involved field radiation. An additional 54 patients were nonrandomly assigned to involved field radiation. The 5-year event-free survival rate did not differ between patients randomized to whole bone radiation and patients randomized to involved field radiation (37% and 39%, respectively). The local control rate was 53% in both treatment arms. Of note, patients who received radiotherapy according to protocol guidelines for dose and volume had a superior local control rate (80%) compared with patients with major deviations (16% local control rate) or minor deviations (48% local control rate). Deviations in treatment volume seemed to be more critical. These results indicate that involved field radiation is an acceptable mode of local control for these patients but that treatment to the initial tumor volume plus an adequate margin is necessary.

Several retrospective single-institution case series have evaluated the impact of radiation dose on local control in Ewing sarcoma.[490-492] In one series patients treated with less than 49 Gy had a 5-year local control rate of 37% compared with 89% for patients treated with 49 Gy or more.[491] In this study higher doses seemed particularly important for larger tumors. In another series patients with larger tumors had a uniform local failure rate regardless of radiation dose, whereas patients with smaller tumors treated with more than 40 Gy had a higher local control rate compared with patients who had smaller tumors treated with less than 40 Gy.[492] A third case series reported no difference in outcome between patients treated with radiation doses above and below 54 Gy.[490] The CESS-81 trial randomized extremity tumors to receive 46 Gy or 60 Gy. The local failure rate was similar in these two groups.[431]

Three studies have specifically commented on the use of lower-dose radiotherapy.[462,493,494] The first study administered approximately 30 Gy in patients mainly in conjunction with some type of surgical resection, although five patients received radiotherapy only.[493] No local failures were noted in this group. The other two studies administered 30 to 36 Gy as definitive local control to patients with an objective response to neoadjuvant chemotherapy.[462,494] This strategy resulted in an unacceptably high local failure rate.

The timing of radiotherapy may be important for patients receiving definitive radiotherapy and not for patients receiving radiotherapy after surgery. A retrospective analysis of patients treated with surgery followed by radiation therapy in the CESS-86 and EICESS-92 trials demonstrated that the interval between surgery and radiation did not affect the local control rate or event-free survival rate, even up to intervals beyond 90 days.[495] In contrast, a retrospective analysis of patients with pelvic Ewing sarcoma suggested that initiating definitive radiotherapy earlier may result in a lower local failure rate.[496] A pooled analysis has also suggested that longer periods (15-18 weeks) of neoadjuvant chemotherapy before starting radiation therapy may have a negative impact on the overall survival rate.[497]

Two retrospective case series evaluated hyperfractionated radiotherapy in the management of Ewing sarcoma.[490,498] Both series concluded that local control rates were not improved by hyperfractionation. One group reported better functional results with the hyperfractionated schedule.[498] The CESS-86 trial randomized patients to conventional or hyperfractionated radiation schedules.[499,500] Patients on the conventional schedule had chemotherapy held during radiotherapy, while patients on the hyperfractionated schedule continued to receive chemotherapy during radiation. For patients treated with radiotherapy alone, the local control rate was 82% for patients on the conventional schedule and 86% for patients on the hyperfractionated schedule. The radiation schedule did not affect overall or relapse-free survival rates. The radiation schedule did not affect disease control in patients treated with surgery followed by radiation.[500]

On the basis of this experience with radiotherapy for Ewing sarcoma, current practice is for patients with unresectable tumors to receive definitive radiotherapy after 4 to 6 cycles of chemotherapy. Most groups administer 54.4 to 60 Gy as definitive radiotherapy. Patients typically receive treatment as 45 Gy to the pretreatment volume plus a safety margin of at least 2 cm followed by a boost to full-dose radiotherapy to the treatment volume remaining after neoadjuvant chemotherapy.[486] Tumors with soft tissue extension, but not infiltration, into the chest or pelvic cavities often receive treatment to the postchemotherapy volume plus a safety margin. Some centers advise full-dose preoperative radiotherapy to tumors that may become fully resectable with such therapy. Patients with inadequate surgical margins are recommended to receive postoperative radiation. The EURO-EWING group also recommends postoperative radiotherapy for patients with adequate surgical margins and a poor histologic response to chemotherapy, although this practice is not standard in North America. The EURO-EWING group recommends a hyperfractionated schedule of radiotherapy, whereas the standard in North America remains conventional fractionation.

Several groups have compared the three available modes of local control: surgery, definitive radiation, and surgery plus radiation. Several studies have clearly documented that patients who undergo surgical resection have higher local control rates or improved overall outcomes (or both) compared with patients who receive definitive radiotherapy only.* These studies must be interpreted with caution because of the presence of confounding by indication. For example, because small extremity tumors are typically selected for surgery and large axial tumors are typically managed with radiotherapy alone, the outcome between these two groups might be expected to differ on account of reasons other than the chosen mode of local control. Indeed, not all studies have concluded that surgery provides superior disease control compared with radiation. A retrospective single-institution study of 76 patients with nonmetastatic Ewing sarcoma concluded that the overall survival rate and the rate of local control were equivalent between patients who received local control as surgery, radiation, or surgery plus radiation, although the power to detect a difference may have been limited by the small size of this study.[502] A secondary analysis of data from the United Kingdom Children's Cancer Study Group (UKCCSG) ET-2 trial reached the same conclusion.[503] In addition, the overall rate of disease recurrence did not differ between local control groups. In the SE 91-CNR Italian cooperative group trial, the 3-year event-free survival rates did not differ between patients who received surgery alone and those who received radiotherapy alone.[434]

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*References 336, 343, 347, 348, 352, 484, 485, 487, 500, 501.

Although a randomized trial might resolve the issue of optimal mode of local control, such a randomization would be untenable to patients. Instead, several analyses have evaluated this issue by attempting to control for the known confounding factors influencing both outcome and choice of local control modality. An analysis from the Rizzoli Institute showed that patients treated with definitive radiotherapy had inferior event-free survival rates compared with patients who received surgery alone, even after controlling for age, tumor size, and serum LDH level.[484] This group did not control for tumor site in this analysis. Two similar previous analyses by this same group controlled for tumor site, among other factors.[336,348] In these analyses patients treated with radiotherapy did not have an increased risk of relapse. The EICESS/CESS experience with local control has also been retrospectively reviewed in several analyses.[500,501] On univariate analyses, these studies have demonstrated that patients treated surgically have improved local control compared with patients treated with definitive radiotherapy. Although this group has not reported a multivariate analysis of outcome based on local control, they have reported a subgroup analysis based on tumor size (above and below 100 cm^3) and site (axial versus appendicular).[501] Patients treated with radiation alone had inferior local control for all combinations of tumor size and site except small central tumors. However, patients treated with surgery appeared to have a higher rate of systemic failure compared with patients treated with radiation alone, such that the overall rate of disease recurrence was the same between groups.[485,501] This finding has not been reported by other groups. An analysis of 220 patients treated at St. Jude Children's Research Hospital also demonstrated that surgical resection was associated with lower rates of local failure, even after controlling for tumor size and year of treatment.[504] This analysis did not control for tumor site, however. Significantly, this analysis and a prospective randomized trial demonstrated lower rates of local failure in patients treated with more modern chemotherapy regimens that included ifosfamide and etoposide.[352,504]

TABLE 61-3 Major Chemotherapy Regimens Evaluated for Ewing Sarcoma

Abbreviation	Regimen
VAC	Vincristine, actinomycin-D, and cyclophosphamide
VACA	Vincristine, actinomycin-D, cyclophosphamide, and doxorubicin
VDC	Vincristine, doxorubicin, and cyclophosphamide
IE	Ifosfamide and etoposide
VAI	Vincristine, actinomycin-D, and ifosfamide
VDI	Vincristine, doxorubicin, and ifosfamide
VAIA	Vincristine, actinomycin-D, ifosfamide, and doxorubicin
VIDE	Vincristine, ifosfamide, doxorubicin, and etoposide

Systemic Treatment with Chemotherapy

The use of chemotherapy for patients with Ewing sarcoma has revolutionized the care of these patients. Before the 1970s, standard therapy for this disease consisted solely of local control directed at the primary tumor. Most patients treated in this manner ultimately died from local or systemic recurrent disease.[505-507] In one series patients treated with local control alone had a median survival rate of 11 months.[506] In the 1960s and 1970s, evidence emerged that systemic chemotherapy could prevent recurrent disease in a substantial number of patients with Ewing sarcoma. This section reviews the development of modern chemotherapy regimens for this disease. Table 61-3 lists major chemotherapy regimens evaluated for Ewing sarcoma along with abbreviations for these regimens. Table 61-4 summarizes the major cooperative group trials that have contributed to current treatment approaches to this disease.

Early Improvements with Vincristine, Actinomycin-D, Doxorubicin, and Cyclophosphamide

Given the poor outcomes observed with local control alone, investigators began using single-agent chemotherapy for Ewing sarcoma. Although single-agent therapy did not substantially improve the long-term outcomes for these patients, these studies did identify a group of drugs capable of inducing tumor regression. Some of the most effective agents included vincristine, actinomycin-D, doxorubicin, and cyclophosphamide. Very quickly, investigators began to evaluate the use of these drugs in combination for patients with Ewing sarcoma.

One group evaluated two different combinations: vincristine with cyclophosphamide given in 8-week cycles for five cycles and vincristine, actinomycin-D, doxorubicin, and cyclophosphamide (VACA) given in weekly components for 18 months.[506] Most patients received

TABLE 61-4 Outcome for Patients with Localized Ewing Sarcoma of Bone in Cooperative Group Trials of Nonmyeloablative Therapies

Trial	Years	Regimen(s)	5-year EFS or RFS	Reference(s)
VACA Trials				
IESS-1	1973-1978	VACA VAC + WLI VAC	60% 44% 24%	435
IESS-2	1978-1982	High dose VACA Protracted VACA	73% nonpelvic 55% pelvic 56% nonpelvic	513 531 513
First French	1978-1984	VACA	52% (4 year)	437
UKCCSG ET-1	1978-1986	VACA	41%	343
CESS-81	1981-1985	VACA	55%	431
French EW88	1988-1991	Protracted VACA	58%	347
VACA/IE Trials				
Second French	1984-1987	VAI/VDI	52%	335
CESS-86	1986-1991	VAIA for high risk VACA for low risk	53% (10 year) 49% (10 year)	433
UKCCSG ET-2	1987-1993	VAI/VDI	62%	436
INT-0091	1988-1992	VACA/IE VACA	69% 54%	352
SSG IX	1990-1996	VDI/PDI	58%	344
Italian SE 91-CNR French EW-93	1991-1997 1993-1999	VACA/IE VACA for low risk VACA/IE for intermediate risk	69% 70% 54%	434 519
EICESS-92	1992-1999	VAIA for low risk VACA for low risk VAIA for high risk VAIAE for high risk	68% 67% 44% 52%	520
Dose Intensified VACA/IE Trials				
INT-0154	1995-1998	VACA/IE standard VACA/IE intensified	72% 70%	438
AEWS0031	2001-2005	VACA/IE 3-week VACA/IE 2-week	65% 73%	522
EURO-EWING 99	Ongoing	VIDE	Ongoing	
AEWS1031	Ongoing	VDC/IE 2-week VDC/IE/TC 2-week	Ongoing	

EFS, Event-free survival; *PDI,* cisplatin, doxorubicin, and ifosfamide; *RFS,* relapse-free survival; *TC,* topotecan and cyclophosphamide; *VAIAE,* vincristine, actinomycin-D, ifosfamide, doxorubicin, and etoposide; *WLI,* whole lung irradiation. See Table 61-3 for additional abbreviations.

radiotherapy as the mode of local control. In both regimens chemotherapy and radiotherapy started simultaneously at the initiation of treatment. Patients who received either regimen had a statistically significant improvement in outcome compared with historical controls who received only local control, suggesting that the VACA regimen resulted in better outcomes than the vincristine-cyclophosphamide regimen.

Another group reported on 30 patients with nonmetastatic Ewing sarcoma treated with 18 months of vincristine, doxorubicin, cyclophosphamide, and procarbazine.[507] Chemotherapy and radiotherapy were given concurrently at the start of therapy. The 6-year disease-free survival rate was 49%, which was a substantial improvement on outcomes in historical controls treated with radiotherapy alone.

The Memorial Sloan Kettering Cancer Center treated 20 patients with localized Ewing sarcoma with 18 to 20 months of VACA as adjuvant therapy.[508] The cumulative dose of doxorubicin used in this study typically exceeded 500 mg/m^2, higher than in most other studies. The 5-year disease-free survival rate was 75%, perhaps highlighting the sensitivity of Ewing sarcoma to doxorubicin. The cardiotoxicity associated with these doses of doxorubicin resulted in a reduction in the cumulative doxorubicin exposure in subsequent studies by this group.[509]

The St. Jude Children's Research Hospital evaluated the use of neoadjuvant therapy with cyclophosphamide and doxorubicin for five cycles. An early report of this regimen reported that 19 of 23 evaluable patients had a complete response to therapy.[510] After these five cycles of neoadjuvant therapy, patients received local control, usually with radiotherapy. With the start of local control, patients began adjuvant therapy with vincristine and actinomycin-D for approximately 11 weeks followed by an additional six cycles of cyclophosphamide and doxorubicin. At the time of a follow-up report of 50 evaluable patients with nonmetastatic disease treated in this manner, 17 patients had developed recurrent disease.[462] A larger cooperative group study evaluating radiotherapy in Ewing sarcoma used a similar chemotherapy regimen and reported a 5-year event-free survival rate of 51% in patients with nonmetastatic disease.[487]

The Rizzoli Institute performed a series of trials evaluating both adjuvant (after local control) and neoadjuvant (before local control) VACA regimens. In the first trial patients received adjuvant therapy with vincristine, doxorubicin, and cyclophosphamide (VDC) for 2 years.[505] At the time of diagnosis, patients began chemotherapy in concert with local control of the primary tumor. In the 85 patients with localized disease treated in this manner, the 5-year event-free survival rate was 34%.[342] In contrast, only 5% of historical controls treated with local control only remained continuously free of disease.[505] The successor study treated 59 patients with localized disease with 18 months of VACA.[511] In this study the 5-year event-free survival rate was 59%, suggesting a benefit to the addition of actinomycin-D. The next study by this group evaluated VDC given as three neoadjuvant cycles followed by VACA chemotherapy after local control for a total of 15 months.[342,512] The 5-year event-free survival rate for the 108 patients with localized disease treated in this study was 49%. Compared to VACA given exclusively as adjuvant therapy, this result suggests that the change to neoadjuvant therapy did not improve outcomes for these patients.

Based on these promising results, several cooperative groups set out to evaluate vincristine, actinomycin-D, and cyclophosphamide (VAC) and VACA regimens in large groups of uniformly treated patients. IESS-1 was the first randomized study in patients with Ewing sarcoma.[435] This study enrolled patients from 1973 and 1978. The 342 patients with nonmetastatic Ewing sarcoma were randomized to one of three treatment arms. Patients in the first and second treatment arms received VAC, with patients in the second treatment arm also receiving prophylactic whole lung irradiation. Patients in the third treatment arm received VAC plus doxorubicin. Patients in this arm received doxorubicin every 12 weeks and actinomycin-D every 12 weeks, such that they received one of these agents every 6 weeks. Patients in the VAC arms received actinomycin-D every 12 weeks. All patients received radiotherapy to the primary tumor. Patients who received doxorubicin had a 5-year relapse-free survival rate of 60% compared with only 24% for patients who received VAC only. Patients who received VAC with whole lung irradiation had an intermediate 5-year relapse-free survival rate of 44%. These results indicated that the addition of doxorubicin to VAC significantly improved outcomes compared with either VAC alone or VAC with whole lung irradiation. The contribution of increased dose intensity in the doxorubicin arm to this improved outcome is not clear. Subgroup analysis showed that outcomes were inferior for patients with pelvic primary tumors and did not differ by treatment arm for these patients. Doxorubicin performed as well as whole lung irradiation in preventing lung metastasis. Based on these results, four-drug therapy with VACA became the standard chemotherapy approach for patients with Ewing sarcoma.

The IESS-2 study expanded on these results by randomizing patients with nonmetastatic disease not involving the pelvis to two different VACA dosing schedules.[513] Patients on one schedule received cycles of therapy every 3 weeks, and patients on the other schedule received more moderate doses of the same drugs at more frequent intervals. All patients received treatment for approximately 20 months. A total of 214 patients were enrolled in this study from 1978-1982. The 5-year relapse-free survival rate for patients with nonpelvic tumors who received higher-dose intermittent VACA was 73%, compared with 56% for patients who received more protracted VACA. Overall survival rate results showed a similar pattern. These results indicated a significant survival advantage for the higher-dose intermittent dosing schedule, at least for patients with tumors arising outside the pelvis.

The first German cooperative group trial for this disease (CESS-81) also used VACA administered in 3-week cycles.[431] This trial enrolled 93 patients with nonmetastatic disease from 1981 to 1985. Treatment duration was only 10 months, which was shorter than treatment on the IESS protocols. The 5-year disease-free survival rate was 55%, including patients with pelvic tumors. This result is similar to the outcome of VACA-treated patients on IESS-1, which also included patients with pelvic tumors. This result suggested that a shorter treatment duration using higher-dose regimens every 3 weeks does not compromise outcome.

The first Ewing sarcoma study of the United Kingdom Children's Cancer Study Group (UKCCSG ET-1) sought to standardize the treatment of newly diagnosed Ewing sarcoma using VACA-based chemotherapy.[343] This nonrandomized trial enrolled 142 patients from 1978 to 1986. Patients received one or two induction cycles of chemotherapy with VDC. Local therapy, with a preference for definitive radiotherapy, occurred next while patients received weekly cyclophosphamide and vincristine. Patients then received cycles of VDC alternating every 3 weeks with VAC for a total of 1 year of therapy. The 5-year relapse-free survival rate was 41%, and the 5-year overall survival rate was 44% for patients with nonmetastatic disease. The study authors suggest that the inferior outcome in this trial compared with that of other VACA-based trials at the time may have been due in part to decreased early doxorubicin dose intensity.[343]

The first French cooperative group study of Ewing sarcoma also used VACA-based chemotherapy as well as radiotherapy as the preferred mode of local control.[437] This study enrolled 95 patients between 1978 and 1984. Patients received cycles of VDC alternating with VAC for a total of 16 months of treatment. The 4-year disease-free survival rate for patients with nonmetastatic disease was 52%.

A follow-up French cooperative group study (EW88) enrolled 141 patients with nonmetastatic disease from 1988 to 1991.[347] This study administered cyclophosphamide in a more protracted manner, following a regimen piloted at St. Jude's Children's Research Hospital in the 1970s. Patients received initial chemotherapy with cyclophosphamide given daily for 7 days followed by doxorubicin on day 8. Patients received five cycles of this therapy before proceeding to local control. Surgery was recommended when possible. Radiotherapy was reserved for patients with unresectable tumors, incompletely resected tumors, or tumors with more than 5% viable tumor after initial chemotherapy. Following local control, patients received vincristine and actinomycin-D for 12 weeks followed by six additional cycles of cyclophosphamide and doxorubicin using the same schedule used neoadjuvantly. Administering these standard four drugs in this manner resulted in a 5-year disease-free survival rate of 58%.

Addition of Ifosfamide and Ifosfamide with Etoposide to VACA-Based Therapy

An initial report from the U.S. National Cancer Institute (NCI) demonstrated a response rate of 45% in 20 patients with recurrent Ewing sarcoma treated with a 5-day course of ifosfamide.[514] A follow-up study by this group evaluated ifosfamide combined with etoposide (IE) for patients with recurrent pediatric tumors.[515] Of the 17 evaluable patients with recurrent Ewing sarcoma included in the preliminary report, 16 patients had a partial response to therapy, for a response rate of 94%. A larger cooperative group study sponsored by the Pediatric Oncology Group included 55 patients with recurrent Ewing sarcoma treated with ifosfamide and etoposide.[516] The response rate in this study was 25%. Based on these promising results in patients with recurrent disease, the St. Jude Children's Research Hospital evaluated IE in patients with newly diagnosed high-risk disease.[517] This group gave three cycles of IE as an initial window before proceeding with doxorubicin and cyclophosphamide chemotherapy. Only 1 of 26 patients failed to respond to IE, for a response rate of 95%.

A number of single-institution studies incorporated ifosfamide and etoposide into the VACA backbone of therapy for newly diagnosed patients. One study from the NCI administered IE alternating with VDC or VAC for a total of 18 3-week cycles.[518] Ifosfamide was administered both before and after local control. A total of 31 patients with nonmetastatic disease were treated and had a 5-year event-free survival rate of 64%. This outcome was significantly better than historical controls with nonmetastatic disease treated with VDC and total body irradiation with stem cell rescue. Two studies from the Rizzoli Institute introduced ifosfamide at different times and reached different conclusions. In the REN-2 study, patients with localized disease received ifosfamide for the first time after local control.[512] The cumulative ifosfamide dose was 54 grams/m^2. The 5-year event-free survival rate for the 82 patients treated was 51%. This result was not different from historical controls treated with VACA-based therapy. In the REN-3 study, ifosfamide was introduced before local control with the cumulative dose held constant at 54 grams/m^2.[342] The 157 patients with nonmetastatic disease had a 5-year event-free survival rate of 71%, which was significantly better than any of the previous chemotherapy regimens evaluated at their center. These results suggest that early introduction of ifosfamide may improve outcome.

Several cooperative group studies have evaluated the efficacy of ifosfamide both with and without etoposide. One of the first cooperative group studies incorporating ifosfamide was the second French cooperative group study of Ewing sarcoma.[335] Patients enrolled from 1984 to 1987 and received initial therapy with alternating 3-week cycles of vincristine, actinomycin-D, and ifosfamide (VAI) and vincristine, doxorubicin, and ifosfamide (VDI) for a total of six cycles. Patients then underwent local control, with a preference for complete surgical resection. All patients were to receive radiotherapy, with the dose dependent on the extent of surgical resection. During radiotherapy patients received two cycles of vincristine with doxorubicin and two cycles of vincristine with ifosfamide. Following local control, patients received alternating 3-week cycles of VAI and vincristine with doxorubicin to complete a total of 1 year of therapy. The 5-year disease-free survival rate was 52% for 95 patients with nonmetastatic disease. This result prompted this group to conclude that substituting ifosfamide for cyclophosphamide did not improve outcomes. In addition, patients in this study had a higher than expected incidence of diminished cardiac function, prompting early closure of the study.

The CESS-86 trial was the first cooperative group attempt to risk-stratify patients and alter therapy based on risk.[433] Patients with small (less than 100 mL tumor volume) extremity tumors received VACA given as 3-week cycles for 36 weeks. Patients with large (100 mL or greater) tumors or axial tumors received identical therapy except ifosfamide was substituted for cyclophosphamide (VAIA). For the 301 nonmetastatic patients treated on this study, the ten-year event-free survival did not differ between patients who received VACA or VAIA (49% and 53%, respectively). However, multivariate analysis demonstrated that, controlling for tumor size and site, VACA chemotherapy was less effective than VAIA.

The French EW-93 study prescribed doxorubicin and cyclophosphamide for all patients with localized disease and reserved IE for patients with a poor response to three cycles of that therapy.[519] Patients with less than 50% regression of soft tissue mass on first evaluation were nonrandomly assigned to receive two cycles of IE before surgical resection. Of 214 patients, 33 received IE, and only eight of these refractory patients responded to IE before local control measures.

The UKCCSG ET-2 study evaluated the substitution of ifosfamide for cyclophosphamide in newly diagnosed patients.[436] This nonrandomized trial enrolled 243 patients from 1987 to 1993. Patients received VDI for four 3-week cycles before undergoing local control. In contrast to ET-1, ET-2 suggested a preference for surgery as mode of local control. Following local control, patients received VAI to complete 1 year of therapy. Patients received a cumulative doxorubicin dose of 420 mg/m^2 and cumulative ifosfamide dose of 114 grams/m^2. The 5-year relapse-free survival rate was 62% for patients with nonmetastatic disease. These results demonstrate a substantial improvement over the results of the UKCCSG ET-1 study. These improvements may be due to increased doxorubicin dose intensity, use of ifosfamide, preference for surgery as local control, or a combination of these factors.

An Italian cooperative group study (SE 91-CNR) enrolled 160 evaluable patients with nonmetastatic Ewing sarcoma from 1991 to 1997.[434] Patients treated on this protocol received initial therapy with alternating 3-week cycles of VDC and VAI. After 24 weeks of therapy, patients received alternating 3-week cycles of IE and VAC to complete 36 total weeks of therapy. Surgery was the preferred mode of local control for resectable tumors. The 5-year event-free survival rate was 69.4%.

A Scandinavian Sarcoma Group trial (SSG IX) enrolled patients with metastatic and nonmetastatic Ewing sarcoma from 1990 to 1996.[344] All patients received 35 weeks of chemotherapy given as 3-week cycles. Patients received cycles of VDI every 3 weeks, with cisplatin, doxorubicin, and ifosfamide substituted at cycles 2, 5, 8, and 11. Surgery was the preferred mode of local control. The 5-year metastasis-free survival rate was 58% for the 73 patients with initially nonmetastatic disease. The 5-year overall survival rate for these patients was 70%. The 5-year overall survival rate for patients presenting with metastatic disease was 28%. These results compare favorably to those from other contemporary trials.

Two randomized cooperative group trials have directly compared VACA-based chemotherapy to ifosfamide-containing chemotherapy. The North American intergroup study INT-0091 enrolled 518 patients with metastatic and nonmetastatic Ewing sarcoma from 1988 to 1992.[352] The standard arm on this study consisted of 3-week cycles of VDC. The experimental arm consisted of 3-week cycles of VDC alternating with IE. In both treatment arms, actinomycin-D was substituted for doxorubicin after a cumulative doxorubicin dose of 375 mg/m^2. All patients received 17 cycles of therapy, for a total study duration of 49 weeks. Local control occurred at week 12 of the therapy, with mode of local control determined on an individualized basis. The addition of IE significantly improved the outcome for patients with nonmetastatic disease, with a 5-year event-free survival rate of 69% compared with 54% for patients receiving VDC only. The incidence of local failure was also lower for patients who received IE, indicating that improved systemic therapy also improves local control. Based on these results, the combination of VDC and IE has become the standard therapy for patients with nonmetastatic Ewing sarcoma treated in North America.

The EICESS-92 study represents a collaborative effort between the UKCCSG and German cooperative groups. This trial enrolled 647 patients with either localized and metastatic Ewing sarcoma from 1992 to 1999. Patients with small (less than 100 mL tumor volume) localized tumors were deemed standard risk. These patients received initial chemotherapy with VAIA given as 3-week cycles followed by a randomization between continued VAIA or VACA. All other patients were deemed high risk and were randomized to receive VAIA or VAIA plus etoposide. Among standard-risk patients there were no statistically significant differences in event-free or overall survival rates between the randomized arms, although it is important to note that all patients received ifosfamide before randomization.[520] Among high-risk patients there were trends suggesting improved outcomes with the addition of etoposide to VAIA, although no statistically significant differences were found.

Dose-Intensified VACA/IE Regimens

The next generation of chemotherapy regimens emphasizes increasing the dose intensity of known active agents in this disease. The P6 protocol from the Memorial Sloan Kettering Cancer Center used an augmented dose of cyclophosphamide given in combination with vincristine and standard-dose doxorubicin.[521] Patients received a total of seven cycles of alternating VDC and IE along with local control of the primary tumor. The 4-year event-free survival rate for the 44 patients with localized disease was 82%, suggesting an improvement in outcome with higher-dose cyclophosphamide.

Two recent North American cooperative group studies have investigated strategies for dose-intensifying therapy. The first trial, known as INT-0154, included 478 eligible patients with nonmetastatic Ewing sarcoma diagnosed from 1995 to 1998.[438] Patients were randomized at study entry to receive alternating 3-week cycles of VDC and IE using either a dose-intensified or standard-dose approach. The standard-dose arm emulated the therapy given to patients assigned to the experimental arm of INT-0091. Patients in the dose-intensified arm received higher individual doses of the alkylating agents than patients in the standard-dose arm, but cumulative drug doses were approximately equivalent between arms. Patients in the dose-intensified arm completed therapy in 30 weeks, whereas patients in the standard-dose arm completed therapy in 48 weeks. Dose-intensified treatment did not improve event-free or overall survival rates and was associated with increased toxicity, such that this approach is not recommended for this population.

In Children's Oncology Group (COG) protocol AEWS0031, 568 patients with nonmetastatic Ewing sarcoma were randomized at study entry to receive cycles of standard-dose VDC and IE alternating every 3 weeks or every 2 weeks.[522] Individual and cumulative drug doses were equivalent between treatment arms. All patients received 14 cycles of therapy, requiring 42 weeks in standard arm and 28 weeks in the interval compression arm. Interval compression was feasible, with a median cycle length of 15 days in the interval compression arm. Patients randomized to the interval compression arm had a superior event-free survival rate compared with patients randomized to the standard arm (73% versus 65% at 5 years). Toxicity was equivalent between both arms. Based on these findings, this approach has been adopted in North America as a standard practice for patients with localized Ewing sarcoma.

Several groups have evaluated chemotherapy regimens that combine multiple active agents given together during a cycle of therapy. In a protocol at St. Jude Children's Research Hospital, 51 patients with Ewing sarcoma received ifosfamide, etoposide, cyclophosphamide, and doxorubicin given together for three cycles before local control.[523] Patients then received alternating cycles of IE and cyclophosphamide with doxorubicin for a total of 41 weeks of therapy. The doses of ifosfamide, etoposide, and cyclophosphamide given after local control were higher than in previous studies. Patients were able to complete the planned neoadjuvant therapy, but myelosuppression limited the ability of patients to receive the planned adjuvant therapy on schedule. Specifically, only 66% of patients received all planned chemotherapy, and only 25% of patients received all planned chemotherapy on schedule. Despite difficulties in achieving the desired degree of dose intensification, outcomes remained favorable. The 3-year event-free survival rate for patients with localized disease was 78%.

A British group has piloted the use of vincristine, ifosfamide, doxorubicin, and etoposide (VIDE) as neoadjuvant therapy for this disease.[524] In this study 30 patients with newly diagnosed Ewing sarcoma were treated with six 3-week cycles of VIDE. Because of hematologic toxicity, etoposide was dose reduced or omitted from 50% of the planned cycles. All attempts at collecting peripheral blood stem cells after a cycle of VIDE were successful. Of the 24 patients evaluable for radiographic response, only one patient had progressive disease. Patients in this pilot study received additional therapy following local control. The final results of this study are not yet available.

The European cooperative groups have initiated a combined trial known as EURO-EWING 99 that relies on VIDE as neoadjuvant therapy. All patients receive initial treatment with six 3-week cycles of VIDE. This trial then tailors therapy according to risk. Patients with small (less than 200 mL tumor volume) nonmetastatic tumors with good chemotherapy response are randomized to receive additional therapy with seven cycles of VAC or VAI. Patients with large tumors, pulmonary metastases, or poor chemotherapy response are randomized to receive additional therapy with seven cycles of VAI or high-dose therapy with stem cell rescue (discussed later). This randomized trial is ongoing.

Two nonrandomized trials have reported on the use of high-dose chemotherapy as a consolidation strategy for patients with localized Ewing sarcoma and adverse prognostic factors. In the French EW93 trial, patients with localized Ewing sarcoma were considered high risk if they had either a 30% or greater viable tumor at time of surgical resection or a 50% or less reduction in tumor volume by imaging for patients not candidates for surgical resection.[519] These patients were nonrandomly assigned to receive high-dose chemotherapy with busulfan and melphalan. There were 48 patients who were classified as high risk, 34 of whom received high-dose chemotherapy. For the entire cohort of patients with high-risk disease, the 5-year event-free survival rate was 48%. A joint Italian-Scandinavian protocol adopted a similar approach, with busulfan and melphalan high-dose chemotherapy prescribed for patients with localized disease and macroscopic foci of tumor at the time of surgery or incomplete regression of soft tissue masses in patients not candidates for surgical resection.[525] Outcomes for the 126 patients with these poor risk features who received high-dose therapy were similar to those of patients without poor risk features who did not receive high-dose therapy. Given the nonrandomized study designs and risk of selection bias for patients who proceeded to high-dose therapy in these two trials, the role of this approach in patients with localized Ewing sarcoma remains unclear.

Management of Tumors Arising at Special Sites

Pelvic Ewing Sarcoma

Ewing sarcoma frequently arises in the pelvis. These pelvic tumors tend be large and associated with crucial viscera, nerves, and bones (particularly of the acetabulum), making complete surgical resection difficult. The local failure rate for patients with pelvic primary tumors ranges from 15% to 27%, which is higher than the local failure rate typically reported in appendicular tumors.[526-530] In one large series, even after exclusion of patients with a local relapse, the outcome for patients with pelvic primary tumors was still inferior to outcomes at other sites.[527] This result suggests that both systemic control and local control are inadequate for these patients.

Recognizing the poor outcome of patients with pelvic primary tumors, the IESS-2 trial nonrandomly assigned patients to the higher-dose intermittently scheduled VACA arm of that study.[531] In addition, an increased emphasis was placed on surgical control of pelvic tumors. With this strategy, patients with pelvic tumors had a 5-year relapse-free survival rate of 55% compared with only 23% for patients in the IESS-1 trial. Because the higher dose VACA arm resulted in better outcomes in the randomized nonpelvic patients treated in IESS-2, the improvements in outcome seen in patients with pelvic tumors may have been attributable to better systemic control, better local control, or both. Indeed, patients with pelvic tumors in IESS-2 were more likely to undergo complete surgical resection and had a lower local failure rate compared to patients with pelvic tumors in IESS-1. Compared with patients who did not have a complete resection, complete resection did not improve the overall or relapse-free survival rate in these patients in IESS-2.[531]

The optimal mode of local control for pelvic tumors has been difficult to determine with rigorous studies. A report from the Mayo Clinic found that patients with pelvic Ewing sarcoma treated with surgery had a statistically significant superior overall survival rate compared with patients treated without surgery.[532] In several other series of patients with pelvic primary tumors, the mode of local control has not clearly been shown to affect outcome,[527-529,533] although some trends have suggested improvements in survival rates among patients treated surgically.[528,529,533] Some groups have also reported a trend toward a lower local recurrence rate in patients treated surgically,[496,527,528,532] although not all groups have reported this trend.[529] The results of these studies are often difficult to evaluate insofar as patients treated with complete surgical resection typically have small resectable tumors. However, two groups have attempted to control for this and other confounding factors. The French cooperative group reported on 53 patients with pelvic Ewing sarcoma.[534] After controlling for size and other potential confounding factors, researchers found that patients treated with radiotherapy alone had an increased risk of recurrence and death compared with patients who received surgery or surgery with radiotherapy. The COG retrospectively analyzed 75 patients with pelvic primary tumors treated on INT-0091.[530] In contrast to the results of the French group, the mode of local control did not affect event-free survival or local failure rates after controlling for confounding factors such as size. As observed in the main analysis of all tumor sites, patients with pelvic tumors who were randomized to receive VACA plus IE on this study had a lower risk of local failure compared with patients randomized to receive VACA alone.[530] Given this uncertainty and the risk of second tumors from radiotherapy, many groups choose to resect pelvic tumors that can be resected without functional consequences. For tumors that cannot be resected without functional consequences, options include either definitive radiotherapy alone or preoperative radiotherapy with the possible addition of wide resection.

Chest Wall Ewing Sarcoma

Chest wall tumors also require specialized management, particularly insofar as these tumors often invade the pleura or have associated pleural effusions. Some data suggest that surgical resection of chest wall tumors provides superior local control, although these analyses do not account for differences in tumor size that influence resectability.[535,536] Indeed, other analyses have not shown a difference in outcome based on mode of local control for chest wall tumors.[481,537,538] Results from INT-0091 and INT-0154 indicate that neoadjuvant chemotherapy before attempted resection of a chest wall tumor increases the likelihood of obtaining negative margins compared with results from attempted resection before chemotherapy.[481,538] Delayed resection at this site therefore decreases the need for postoperative radiotherapy in these patients.[481,538] Avoiding chest wall radiation is particularly appealing given the added pulmonary and cardiac toxicities associated with this therapy.

Whereas several reports have suggested that an associated pleural effusion did not worsen outcome, patients with chest wall tumors and pleural effusions were often recommended to receive hemithorax irradiation in these studies.[536,537,539] Indeed, a report from the French cooperative group suggested that patients with rib primary tumors and an associated pleural effusion had an increased risk of local relapse without radiotherapy.[537] In the EICESS/CESS studies, patients who received hemithorax irradiation had more unfavorable features than patients who did not receive hemithorax irradiation.[540] Despite this difference, the rate of local relapse was the same in both groups, whereas the rate of systemic relapse was higher in the group of patients who did not receive hemithorax irradiation.[540] This result may be interpreted as evidence of the direct benefit of hemithorax irradiation in preventing dissemination of residual tumor cells from the pleural space. Alternatively, hemithorax irradiation may reduce the volume of lung at risk for subsequent relapse, because the IESS-1 researchers noted that whole lung irradiation can prevent lung metastasis in patients with initially nonmetastatic disease. Patients with chest wall primary tumors and positive pleural fluid cytology or pleural-based nodules treated according to the most recent COG protocol received hemithorax radiation.

Ewing Sarcoma of the Spine

Management of patients with tumors arising along the spinal column requires careful planning by a multidisciplinary team, including oncologists, radiation oncologists, orthopedic surgeons, general surgeons, and neurosurgeons. Small case series have suggested that local control and overall outcomes for patients with primary tumors at this site do not differ from outcomes for patients with tumors arising from other sites.[341,541] One group reported that sacral primary tumors fare less well than other vertebral tumors,[340] although this result was not replicated in other series.[338,339,341] The largest reported experience with vertebral Ewing sarcoma comes from the EICESS/CESS studies.[542] A total of 116 patients had tumors arising from cervical, thoracic, or lumbar vertebrae. Given the difficulties in obtaining a complete resection at this site, all but four patients received radiotherapy as part of local control of the tumor. The local failure rate for patients treated with radiotherapy alone was not significantly different from that of patients treated with surgery plus radiotherapy (22.6% and 18.7%, respectively). Local relapses typically occurred within the radiation field, suggesting that inadequate selection of radiotherapy fields was not to blame for these local relapses. Despite the fact that the spinal cord limits radiotherapy dosing at this site, the local failure rate for patients with vertebral tumors treated with radiotherapy alone was not higher than the local failure rate for patients with tumors of any location treated with radiotherapy alone.[501,542] This effect may result from the generally smaller size of paraspinal tumors compared with that of other tumors selected for local control with definitive radiotherapy. It should be noted, however, that patients with a vertebral tumor treated with radiotherapy who experience local recurrence often have few local control options because additional radiotherapy may compromise the spinal cord.[542]

Soft Tissue Ewing Sarcoma

Historically, patients with soft tissue Ewing sarcoma were not included in cooperative group clinical trials for patients with Ewing sarcoma of bone. Instead, patients with soft tissue Ewing sarcoma have often been included in protocols designed for patients with other soft tissue sarcomas, including rhabdomyosarcoma.[543,544] More recent work suggests that these patients should receive treatment following Ewing sarcoma protocols. For example, one study compared patients treated according to International Society of Paediatric Oncology (SIOP) protocol MMT89 without doxorubicin to patients treated on the French EW93 protocol with doxorubicin.[543] Although it was a nonrandomized comparison, baseline patient characteristics were similar between both groups, but event-free and overall survival rates were superior for patients treated according to the EW93 protocol. In North American study INT-0154, patients with soft tissue disease had outcomes equivalent to those of patients with skeletal primary tumors.[438] These results support the current practice of including these patients in modern trials for Ewing sarcoma.

Summary of Outcomes for Patients with Nonmetastatic Ewing Sarcoma

Patients with Ewing sarcoma have shown great improvements in outcome over the past 4 decades. For patients with localized tumors, expected survival rates have increased to greater than 70% with modern multimodality therapy. Figure 61-8 demonstrates these improvements graphically, using Surveillance, Epidemiology, and End Results (SEER) data from 1975 to 2000.[5,545] A similar pattern of steady improvement over time has also been reported using data from a large European registry.[546]

TREATMENT OF PATIENTS WITH METASTATIC EWING SARCOMA

Systemic Therapy

Despite the recognition that these patients have a poor prognosis, early studies often treated patients with metastatic disease with the same combined modality therapy used for nonmetastatic patients. Because the outcomes for these patients have not improved in concert with the gains seen in nonmetastatic patients, more aggressive strategies have been added to this backbone. These strategies are discussed here.

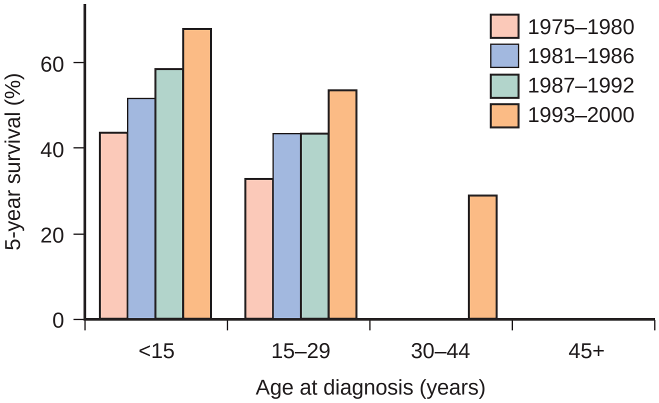

Figure 61-8 Overall survival rates at 5 years from initial diagnosis of Ewing sarcoma according to age at initial diagnosis and treatment era. *(Gurney JG, Swensen AR, Bulterys M: Malignant bone tumors. In Ries LAG, Smith MA, Gurney JG, et al, editors:* Cancer incidence and survival among children and adolescents: United States SEER Program 1975-1995, Bethesda, MD. *1999, National Cancer Institute, SEER Program, NIH Pub. No. 99-4649, p. 99–110.)*

Attempts to improve outcomes for these patients through changes in chemotherapy regimens have been largely unsuccessful. The IESS-2 study attempted to improve on the 30% 5-year overall survival rate for metastatic patients treated on IESS-1.[355] This group added 5-fluorouracil to the VACA backbone of IESS-1. As in IESS-1, radiation to all metastatic sites was prescribed. The 5-year overall survival rate was 28% despite the addition of 5-fluorouracil.[355] Single-institution studies suggested that the addition of IE to VACA did not improve the outcome for patients with initially metastatic disease.[518,521,523] The INT-0091 study confirmed these disappointing results.[352,356] INT-0091 also included a nonrandomized arm in which patients with newly diagnosed metastatic disease received dose-intensified VDC and IE.[547] Patients received higher doses of doxorubicin, cyclophosphamide, and ifosfamide compared with patients treated in the randomized portion of INT-0091. Although this was a nonrandomized comparison, the 60 patients treated with dose-intensified therapy had similar outcomes to patients with metastatic disease treated with standard-dose therapy on INT-0091. The Pediatric Oncology Group and Children's Cancer Group conducted a phase II study of alkylator-intensive chemotherapy for patients with newly diagnosed metastatic disease.[548] Patients in this study received topotecan (N=36) or topotecan and cyclophosphamide (N=37) in an upfront window. After the window phase, patients received IE alternating with VDC, with higher doses of ifosfamide and cyclophosphamide used than in standard protocols. This strategy did not improve the outcomes for these patients. A regimen developed in Seattle combines vincristine, doxorubicin, cyclophosphamide, ifosfamide, and etoposide given together each cycle.[549] All five patients with metastatic Ewing sarcoma and evaluable disease obtained a complete radiographic response to this therapy. Only one of these patients remains free of disease 33 months from initial diagnosis.

Patients with metastatic disease at initial diagnosis are candidates for novel approaches to the treatment of Ewing sarcoma. COG evaluated the addition of metronomic chemotherapy with vinblastine and celecoxib to the VACA/IE backbone used in INT-0091 in 35 patients with metastatic disease.[550] This approach used an antiangiogenic approach together with known active cytotoxic chemotherapy in an attempt to improve the outcome for these patients. This approach was feasible, although small patient numbers precluded conclusions about the impact of vinblastine and celecoxib on disease control.

Patients with metastatic disease who do not respond to initial therapy may receive second-line chemotherapy (described later, in the section on patients with recurrent disease). These patients may also be candidates for other investigational therapies, including high-dose therapy with hematopoietic stem cell rescue (discussed later).

Role of Surgery and Radiation in Patients with Metastatic Disease

Patients presenting with metastatic disease typically receive local control of their primary tumor. Because of their disseminated disease and poor prognosis, the local control strategy for these patients tends to differ from that of patients with nonmetastatic disease. In particular, patients with metastatic disease are more likely receive definitive radiotherapy for local control because they often require radiotherapy to other sites of disease. Indeed, at least one analysis has demonstrated the importance of local control measures directed against both primary and metastatic sites of disease in improving outcomes for this high-risk patient population.[551]

Whole lung irradiation appears to be an effective therapy for patients with lung metastases. The St. Jude Children's Research Hospital reported on patients at their center with lung metastases at initial diagnosis. Patients in this series received whole lung irradiation if they had residual lung metastases after initial chemotherapy. Patients in this series who received whole lung irradiation for residual lung metastases had an outcome equivalent to that of patients whose lung metastases resolved with initial chemotherapy and who did not receive whole lung irradiation.[552] The authors argue that the patients with residual lung metastases after initial chemotherapy would have had an even worse outcome without the addition of whole lung irradiation. Data from the CESS studies suggest that a dose-response relationship may exist for whole lung irradiation.[553] This group reported that patients with initial lung metastases who remained in clinical remission had received higher doses of whole lung irradiation than patients who relapsed. The number of patients reported in this series was small, but the group reported that four of ten patients who received 12 to 16 Gy remained in remission compared with five of six patients who received 18 to 21 Gy. Only one of six patients who did not receive whole lung irradiation remained in clinical remission.[553]

The strongest evidence in favor of whole lung irradiation for patients with lung metastases comes from the EICESS group. This group reported on 100 evaluable patients with isolated lung metastases, 75 of whom received 15 to 18 Gy whole lung irradiation as part of their combined modality therapy.[351] The risk of pulmonary recurrence was significantly decreased with the addition of whole lung irradiation. Moreover, the overall risk of disease recurrence diminished with this intervention. The 5-year event-free survival rate for patients treated with whole lung irradiation was 38%, compared with 27% for patients who did not receive whole lung irradiation. This result is even more striking in light of the fact that the indication for whole lung irradiation in this trial was residual lung metastasis after chemotherapy. Although whole lung irradiation was not prescribed in a randomized manner, potential confounding variables appeared to be balanced between patients who received whole lung irradiation and patients who did not receive whole lung irradiation. In addition, a Cox regression model indicated that whole lung irradiation improved the event-free survival rate after controlling for potential confounding variables.[351] An additional report from the EICESS group indicated that whole lung irradiation improves the outcome even for patients with lung metastases combined with metastases at other sites.[354] Currently, whole lung irradiation is commonly being employed for patients with lung metastases at initial presentation regardless of the radiographic response to initial chemotherapy. Patients with bone metastases also typically receive radiotherapy directed to these sites, although data supporting this strategy are lacking.

Pulmonary metastasectomy plays little role in the management of patients with lung metastases at initial diagnosis. One small case series suggested that pulmonary metastasectomy might improve the outcome for these patients, although this result was confounded by the fact that only patients with resectable lung metastases underwent resection.[554] Surgical resection of pulmonary metastases was not shown to improve outcomes in patients treated by the EICESS group who had residual lung metastases after initial chemotherapy.[351]

Role of High-Dose Therapy with Hematopoietic Stem Cell Rescue for Metastatic Disease

Patients with initially metastatic Ewing sarcoma may be candidates for high-dose therapy with stem cell rescue. The role of high-dose therapy for these patients remains unclear, with most studies relying on comparisons with historical controls consisting of patients treated without high-dose therapy. For example, a study using data from a bone marrow transplant registry observed similar outcomes between patients treated with and without high-dose therapy.[555] Numerous groups have demonstrated the feasibility of delivering myeloablative therapy as consolidation therapy after initial chemotherapy and local control in these high-risk patients. No completed studies have evaluated high-dose therapy in a randomized manner. As such, studies suggesting a benefit from this modality are subject to selection bias in which only patients with good overall physical condition who attain a certain level of disease control are considered for high-dose therapy. Results from larger single-institution and multicenter studies are summarized here.

In the late 1980s and early 1990s, two American groups investigated the use of 8 Gy total-body irradiation with autologous bone marrow rescue as consolidation for patients with high-risk Ewing sarcoma.[556,557] Patients received VDC with or without actinomycin-D as initial chemotherapy along with radiotherapy to their primary site. Patients who attained a complete response to this therapy proceeded to consolidation therapy. Despite selecting therapy-responsive patients, high-dose therapy did not improve the outcome for patients with metastatic disease. The 6-year overall survival rate for patients with metastatic disease in one study was only 14%.[557]

Two EICESS centers evaluated 12 Gy total-body irradiation combined with melphalan and etoposide as consolidation therapy in patients with bone metastases or early relapsed disease.[558,559] Patients in this series received either autologous or allogeneic transplant if they had responded favorably to initial chemotherapy. Although an initial report showed a relapse rate of only 52%, a follow-up report indicated that the event-free survival rate from the time of transplant for this cohort of patients was 24%. The outcomes did not differ significantly between patients who received autologous stem cells and those who received allogeneic stem cells, although patients who underwent allogeneic transplant had a higher rate of complications. A follow-up study from this same group evaluated two consolidation courses of myeloablative melphalan and etoposide in 17 patients with bone metastases.[560] The 5-year event-free survival rate for these patients was 21%.

A later Children's Cancer Group study evaluated melphalan, etoposide, and 12 Gy total-body irradiation for patients with newly diagnosed Ewing sarcoma with bone or bone marrow metastases.[561] These patients received VDC alternating with IE for five cycles along with radiotherapy to their primary tumor and bone metastases. Patients with a complete response or very good partial response to initial therapy received the planned myeloablative therapy. The 2-year event-free survival rate for all 32 patients enrolled in this study was 20%. For the group of patients who were able to receive myeloablative therapy, the 2-year event-free survival rate was 24%. The outcome did not differ from a cohort of historical controls treated in protocol INT-0091. As such, the authors concluded that myeloablative therapy did not improve the outcome for patients with bone and bone marrow metastases.

A French multicenter trial prospectively evaluated the role of high-dose therapy in patients with newly diagnosed metastatic disease.[429] Patients in this trial received initial chemotherapy with cyclophosphamide and doxorubicin alternating with IE. Of the 97 patients enrolled in the study, 75 had a favorable response to initial therapy and proceeded to consolidation therapy with busulfan and melphalan followed by stem cell rescue. The 5-year event-free survival rate was 37% for all 97 patients and 47% for the 75 patients who received high-dose therapy. Much of the improvement after high-dose therapy appeared to be in patients with isolated lung metastases. These patients had a 5-year event-free survival rate of 52% compared with 24% for patients with metastases involving other sites.

A joint Italian and Scandinavian trial treated 102 patients with newly diagnosed metastatic disease, the majority of whom had isolated pulmonary metastatic disease.[562] Patients initially received multiagent chemotherapy and local control measures followed by high-dose chemotherapy with busulfan and melphalan. Patients with lung metastasis at study entry also received 12 to 15 Gy whole lung radiation. High-dose chemotherapy was administered to 79 patients, and the primary reason that the remaining patients did not receive high-dose chemotherapy was early disease progression. The 5-year event-free survival rate for the entire study population was 43%. For patients with isolated pulmonary metastatic disease who were able to receive high-dose chemotherapy and whole lung radiation, the 5-year event-free survival rate was 53%.

A report of 171 patients treated by the EICESS group indicated that high-dose therapy did not improve the outcome among patients with metastatic disease at initial diagnosis.[354] Further analysis suggested that patients with multiple sites of metastases require some form of intensified therapy, either as high-dose therapy or whole lung irradiation. The 4-year event-free survival rate for patients with multiple metastatic sites treated with high-dose therapy, whole lung irradiation, or both modalities was 27% compared with 0% for patients who did not receive at least one form of intensified therapy.[354]

The only randomized study comparing high-dose therapy with other treatment for high-risk patients is ongoing. This trial, known as the EURO-EWING 99 study, randomizes newly diagnosed high-risk patients to receive consolidation therapy with busulfan and melphalan followed by stem cell rescue or continuation chemotherapy. Patients with isolated pulmonary metastases are randomized to consolidation therapy with high-dose chemotherapy and stem cell rescue or to continuation chemotherapy followed by whole lung irradiation. Patients with metastases beyond the lung are nonrandomly assigned to high-dose chemotherapy with stem cell rescue. Of note, patients with nonmetastatic disease with poor response to neoadjuvant chemotherapy are also randomized to high-dose chemotherapy or to continuation chemotherapy. Results of the randomized portion of this trial will not be available for several years. However, patients with extrapulmonary metastatic disease were nonrandomly assigned to receive high-dose chemotherapy and their outcomes have been reported.[563] Of 281 enrolled patients, 169 patients went on to receive high-dose therapy, mainly with busulfan and melphalan conditioning. The event-free survival rate at 3 years for the entire cohort of 281 patients was 27%. The selected group of patients in complete remission at the time of high-dose therapy had a 3-year event-free survival rate of 57%, compared with 32% and 24% for patients with partial response or stable disease/progressive disease, respectively. These results indicate the importance of remission status if high-dose therapy is to be considered in this high-risk patient population.

The use of autologous stem cell infusion raises the concern of re-infusion of Ewing cells contaminating the stem cell product. Several groups have investigated this issue using PCR techniques with mixed results. The incidence of tumor cell contamination ranges from 6% to 100% of pheresis products, depending on the study.[564-567] The largest and most recent evaluation of this issue studied 88 patients with high-risk Ewing sarcoma treated with high-dose therapy with stem cell rescue.[567] Only seven patients had tumor cell contamination of their infused product. The outcome of these seven patients did not appear to differ from the outcome of patients without tumor cell contamination of their infused product.

Summary of Outcomes for Patients with Metastatic Ewing Sarcoma

Despite improved outcomes for patients with nonmetastatic disease over time, patients with initially metastatic disease have not been as fortunate. Most reports suggest that no more than 30% of patients with metastatic Ewing sarcoma are long-term survivors, although this number may be slightly higher for patients with isolated lung metastases.[351,356] Even with combined modality therapy, many patients never attain a complete remission. One recent study reported a complete response rate of only 43% after these patients received chemotherapy, surgery, radiation, and combinations thereof.[548] As such, a number of these patients have treatment-refractory disease. Among patients who do attain a complete remission, disease recurrence remains a major barrier to durable cure.

RECURRENT EWING SARCOMA

Location and Timing of Disease Recurrence

Among patients whose disease recurs, recurrence involving distant sites is more common than isolated local recurrence. Isolated distant relapse accounts for 48% to 71% of recurrences,[568,569] although this appears to be more common in patients with initially metastatic disease.[570-573] Another 10% to 29% of recurrences are combined local plus distant recurrences, and estimates of isolated local recurrence vary widely among studies, ranging from 11% to 35%.[352,570-573] Most initial distant relapses involve the lung, the bones, or both.[571,572]

Most episodes of disease recurrence occur after patients have completed their planned initial therapy. Disease recurrence within 2 years of initial diagnosis is more common than later recurrence, with more than 70% of relapses occurring during this time frame in two large analyses.[568,569] The median time to disease recurrence across several series ranges from 17 to 27 months.[570-573] Patients with initially metastatic disease that recurs have a shorter time to recurrence compared with patients who have initially localized disease that recurs.[569] An analysis of data from the UKCCSG ET-1 and ET-2 studies indicated that patients have a relatively stable rate of death during the first 2 years after diagnosis.[574] Between 2 and 5 years after diagnosis, the rate of death is lower but remains relatively constant during this interval. Only after 5 years from initial diagnosis does the risk of death plateau at an even lower rate that remains constant with extended follow-up. However, Ewing sarcoma can recur very late. In one series 5 of 31 patients who remained disease free for 5 years from the time of initial diagnosis later recurred.[575] In another report 25 of 326 patients who had been in complete remission for 5 years from initial diagnosis experienced late disease recurrence.[576] Other series of patients with recurrent disease include patients with disease recurrence beyond 10 years from initial diagnosis.[570,571,576,577] For example, a report from the Childhood Cancer Survivor Study observed that 26% of late relapses (beyond 5 years from initial diagnosis) occurred 10 or more years from initial relapse.[577] These results highlight the need for extended follow-up in patients with this disease.

General Approach to Patients with Recurrent Ewing Sarcoma

Patients with progressive or recurrent disease after initial therapy present a particular challenge to the clinical team. The time to disease recurrence provides an important indication of the likelihood of long-term survival for these patients (discussed later). More than one quarter of patients with late (more than 2 years from initial diagnosis) recurrence may achieve long-term disease control with aggressive therapy.[568-573] Most clinicians initiate systemic chemotherapy for these patients to assess their sensitivity to the chosen chemotherapy regimen. For patients with chemotherapy-responsive disease, the use of surgery or radiation (or both) for sites of recurrence is often considered. For a local recurrence another attempt at local control may be undertaken with surgery, radiation, or both. For patients with pulmonary recurrence, many clinicians will recommend whole lung irradiation. Patients who respond well to second-line therapy may also be candidates for high-dose therapy with stem cell rescue, although this approach remains unproved (discussed later). Patients with late recurrent disease that does not respond to initial chemotherapy are managed as for poor-risk recurrent disease, according to the strategies outlined in subsequent paragraphs.

For patients with an early (less than 2 years from initial diagnosis) recurrence, the likelihood of long-term disease control is less than 10%.[570-573] Given these data, the appropriate treatment for these patients must be highly individualized, with a clear understanding of the patient's and family's goals for therapy. A variety of strategies are considered for these patients, ranging from palliative measures to aggressive multimodality therapy with curative intent. These patients often receive treatment with the second-line chemotherapy regimens described later in this chapter and are also candidates for investigational phase I and phase II clinical trials. For patients with chemotherapy-responsive disease, more aggressive measures can then be considered.

Systemic Therapies for Recurrent Ewing Sarcoma

Historically, very few chemotherapy options existed for patients with recurrent Ewing sarcoma. As such, patients with late-relapse disease were often treated with agents that they had received at initial diagnosis. In one small series a substantial number of patients who relapsed when they were off therapy responded again to the same agents they received as initial therapy; some of these patients had a durable second remission.[578] Another group reported on 35 patients with disease recurrence after ifosfamide-containing chemotherapy, of whom 34% responded to higher-dose ifosfamide (15 grams/m^2/course).[579] Nevertheless, because of concerns regarding resistance to previously used agents, most clinicians now use newer chemotherapy regimens for patients at the time of relapse. However, for patients who experience late relapses, some clinicians will incorporate previously used active agents into an overall treatment plan that also includes newer second-line regimens. Some of these more common second-line regimens are reviewed here.

Camptothecin-containing regimens have demonstrated efficacy for patients with relapsed Ewing sarcoma. Topotecan monotherapy appears to have limited activity against Ewing sarcoma, with objective response rates of 0% to 10%.[580,581] In contrast, topotecan in combination with cyclophosphamide has a response rate of approximately 30% in patients with relapsed or refractory disease.[582,583] Patients with disease that was refractory to standard alkylator-containing therapy responded to this regimen, which suggests that this response rate was not attributable solely to cyclophosphamide but rather to the combination of topotecan with cyclophosphamide. Patients with newly diagnosed metastatic disease treated in an upfront window study had a response rate of 57% to topotecan and cyclophosphamide.[548] On the basis of this activity, the combination is being evaluated in a COG trial for patients with newly diagnosed localized disease (AEWS1031).

A second camptothecin-containing regimen has shown promise in patients with Ewing sarcoma. An initial phase I study of irinotecan and temozolomide found that this well-tolerated regimen induced one complete response and one partial response among seven patients with Ewing sarcoma treated in this study.[584] A follow-up report that included these seven patients plus seven additional evaluable patients reported an objective response in four patients (28%), although an additional four patients had a mixed response or stable disease.[585] A single-institution series of this same regimen reported a 63% response rate in 19 evaluable patients, confirming the activity of this combination in patients with relapsed disease.[586]

Two carboplatin-based regimens have benefited patients with relapsed or refractory Ewing sarcoma. One group reported a 26% response rate among 39 patients treated with carboplatin, cyclophosphamide, and etoposide.[587] A second group evaluated carboplatin, ifosfamide, and etoposide in patients with a range of relapsed or refractory sarcoma.[588] Of the 21 patients with Ewing sarcoma, 48% had an objective response. The contribution of carboplatin to these responses is unclear because both regimens contain first-line agents that are also effective in the treatment of this disease (ifosfamide, etoposide, and cyclophosphamide).

The combination of docetaxel and gemcitabine has also been tested in patients with recurrent Ewing sarcoma, with limited success. In three retrospective case series that included 11 patients with relapsed disease, only one patient had a partial response to standard doses of this combination.[589-591] A formal phase II study of this same regimen demonstrated a 14% response rate in 14 patients with relapsed disease.[592] One group used higher doses of gemcitabine and docetaxel and observed a 66% response rate, suggesting that if there is activity of this combination, dose intensity may be critical.[593]

A number of novel therapies have been evaluated in patients with recurrent Ewing sarcoma. Of these, the most promising and consistent results have been with monoclonal antibodies targeting the IGF-1R. In initial phase I studies, an early signal of objective responses emerged in a subset of patients with relapsed disease.[594,595] Follow-up phase II studies of several different IGF-1R monoclonal antibodies confirmed this activity, with response rates of approximately 10% to 15% between studies.[596-598] Another study evaluated an IGF-1R monoclonal antibody together with an mTOR inhibitor in 17 patients with Ewing sarcoma and reported tumor regressions greater than 20% in 29% of patients.[599] Follow-up studies are necessary to evaluate these agents in combination with cytotoxic chemotherapy and to identify predictors of response to IGF-1R inhibition.

Role of Surgery and Radiation for Recurrent Ewing Sarcoma

Patients with recurrent Ewing sarcoma typically must respond to systemic therapy for long-term disease control to be possible. However, surgery and radiation also play a role at the time of disease recurrence. One study reported that patients who received surgery for local recurrence had a superior outcome compared with patients who did not receive surgery, although this analysis did not control for the size of the recurrent tumor.[570] This same study reported that whole lung irradiation may benefit patients with isolated pulmonary recurrence.[570] Radiotherapy also plays an important role in controlling pain for patients with refractory Ewing sarcoma.

Pulmonary metastasectomy at relapse has a much more limited role in Ewing sarcoma compared with other diseases, such as osteosarcoma. One group reported that pulmonary metastasectomy did not improve the outcome for patients with Ewing sarcoma and isolated pulmonary relapse.[570] Two groups compared patients who underwent pulmonary metastasectomy and patients with lung metastases that were not resected.[554,600] Both groups suggested that metastasectomy improved the outcome for these patients. However, in both studies patients were selected for resection only in the presence of resectable disease, such that the comparison group may have had a greater burden of disease. Another center described 12 patients with isolated pulmonary relapse treated with surgery alone.[601] Five of these patients remained disease free for at least 3 years. The authors noted that these 12 patients were selected for resection alone on the basis of favorable characteristics, such as a small number of pulmonary nodules and a long period of time from diagnosis to relapse. Whether this strategy would benefit a less favorable group of patients remains to be demonstrated. Currently, pulmonary metastasectomy at relapse is considered mainly for diagnostic purposes and only rarely for therapeutic purposes.

Role of High-Dose Therapy with Hematopoietic Stem Cell Rescue for Recurrent Disease

Some clinicians recommend high-dose therapy as consolidation therapy for patients with recurrent Ewing sarcoma who respond favorably to salvage therapy. Data supporting this approach are relatively limited, particularly because many of the reported studies include both patients with metastatic disease and patients with recurrent disease. Although most studies do not report specific outcomes for patients with recurrent disease, the EICESS group reported that patients treated with high-dose therapy for early relapse had a 5-year event-free survival rate of approximately 45%.[560]

Two reports retrospectively evaluated the impact of high-dose therapy on outcome in patients with recurrent disease. In one series of 64 patients with relapsed disease, seven patients received high-dose therapy with stem cell rescue.[573] The outcomes for these seven patients did not differ from outcomes for patients who received other types of relapse therapy. These results differ from a single-institution report of 55 patients with relapsed Ewing sarcoma, of whom 13 received high-dose therapy with busulfan, melphalan, and thiotepa.[572] Patients who received high-dose therapy had a 5-year progression-free survival rate of 61% compared with 7% for other patients. Because high-dose therapy was offered only to patients who responded to second-line therapy, this report also compared the outcomes of patients who received high-dose therapy and those of patients who had responsive disease but did not receive high-dose therapy. In this analysis high-dose therapy resulted in improved 5-year progression-free survival rate over other therapy (61% versus 21%). A multivariate analysis confirmed that high-dose therapy was an independent predictor of favorable outcome.

Outcomes for Patients with Recurrent Ewing Sarcoma

The outcome for patients with recurrent disease is generally poor. The median overall survival rate from the time of recurrence is 9 to 14 months with current therapies.[568,573] Estimates of 5-year overall survival rates from the time of disease recurrence range from 8% to 23%.[570-573] For patients with recurrent disease, the most widely recognized determinant of outcome is time to disease recurrence. An initial single-institution study noted that four of five patients with relapse more than 5 years from diagnosis were salvaged compared with only 2 of 44 patients with earlier relapses.[575] Follow-up studies have all confirmed this association, including two large cooperative group analyses that both demonstrated that time to relapse was the strongest independent predictor of outcome after first relapse.[568-573] Estimates of 5-year progression-free survival rate for patients with recurrence more than 2 years from initial diagnosis were approximately 30% compared with 7% for earlier relapses.[568,569] Another common finding is that patients with a combined local and distant relapse fare poorly compared with patients who experience either isolated local failure or isolated systemic failure.[568,569] Metastatic site at recurrence has not consistently been associated with outcome after relapse.[568-573]

LATE EFFECTS IN PATIENTS TREATED FOR EWING SARCOMA

Given the nature of the therapy required to treat Ewing sarcoma, patients treated for this disease have an increased risk of developing second cancers. In one large European cohort study, patients with Ewing sarcoma had a thirty-fold increased cumulative risk of developing a solid second cancer 25 years from initial diagnosis.[602] The relative risk that patients will develop a second solid cancer or a bone cancer after Ewing sarcoma was second only to that of patients with an initial primary cancer diagnosis of retinoblastoma.[61,602] In another series survivors of Ewing sarcoma had a risk of second malignancy that was second only to that of survivors of Hodgkin lymphoma.[603] A number of groups have reported that the risk of developing any second cancer continues to increase with increasing time from initial diagnosis of Ewing sarcoma. Estimates of the cumulative incidence of developing any second malignancy from the time of initial diagnosis range from 0.7% to 0.9% at 5 years, 2.9% to 6.5% at 10 years, 4.7% at 15 years, 4.7% to 12.7% at 20 years, and 9% at 25 years.[604-608,609-611] Figure 61-9 demonstrates this trend in one representative report from the Childhood Cancer Survivor Study.[609]

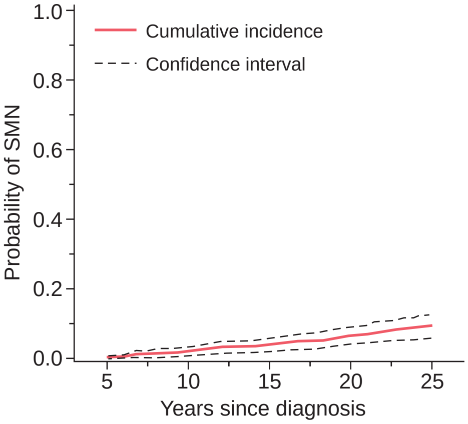

Figure 61-9 Cumulative incidence of second malignancies in patients treated for Ewing sarcoma. *(Ginsberg JP, Goodman P, Leisenring W, et al: Long-term survivors of childhood Ewing sarcoma: report from the childhood cancer survivor study.* J Natl Cancer Inst *102:1272–83, 2010.)*

Radiation-induced sarcomas and chemotherapy-induced acute leukemia constitute the majority of second malignancies seen in these patients. Osteosarcoma accounts for approximately 25% of the secondary sarcomas, with a variety of other bone and soft tissue sarcomas also observed.[604,606,607,612] Estimates of the cumulative incidence of secondary solid cancers are 1.7% to 1.8% in 10-year survivors and 6% to 22% in 20-year survivors.[61,605,606,611,613] This wide range likely results from variations in sample size, follow-up time, and second malignancy surveillance programs between studies. In almost all cases, these secondary sarcomas arise in the initial radiation field. One group has reported that the incidence of these secondary sarcomas increases with increasing radiation dose, with no cases of secondary sarcoma reported in those patients who received less than 48 Gy.[606]

Secondary acute leukemia occurs significantly earlier than secondary sarcomas in these patients.[604,612] Reported mean latency periods from initial diagnosis of Ewing sarcoma to onset of acute leukemia range from 2.3 to 4.8 years, although some patients have developed secondary acute leukemia while still receiving chemotherapy for Ewing sarcoma.[604,612,614,615] Most of these cases of secondary acute leukemia are acute myeloid leukemia or myelodysplastic syndrome. In one large clinical trial, the cumulative incidence of secondary acute myeloid leukemia or myelodysplastic syndrome was approximately 2% 7 years from the initial diagnosis of Ewing sarcoma, with no additional cases observed beyond that time.[614] These cases are characterized by high frequencies of monosomy

5, monosomy 7, and abnormalities at 11q23.[614,615] These cytogenetic findings suggest a role for alkylating agents (cyclophosphamide and ifosfamide associated with monosomy 5 and monosomy 7) and topoisomerase II inhibitors (doxorubicin and etoposide associated with 11q23 abnormalities) in the pathogenesis of these cases. Interestingly, in protocol INT-0091, the incidence of secondary leukemia, including leukemias with the 11q23 abnormality, did not increase with the addition of ifosfamide and etoposide to the regimen of vincristine, doxorubicin, and cyclophosphamide.[614] However, a report from a single institution suggested that the risk of secondary leukemia was increased with more modern treatment protocols that included etoposide.[611] Increased rates of secondary acute myeloid leukemia have been reported in patients with Ewing sarcoma who received augmented doses of alkylating agents and anthracyclines compared with patients who received conventional doses.[614,615] By way of contrast, in protocol INT-0154 increasing the dose rate of alkylating agents while holding the cumulative dose constant did not increase the rate of secondary leukemias.[438]

Patients treated for Ewing sarcoma are at risk for a variety of late effects in addition to second cancers. Cardiac toxicity from anthracycline therapy can result in significant morbidity and mortality rates for these patients. In one small series 6 of 30 Ewing sarcoma survivors developed echocardiographic shortening fractions less than 20% compared with only 1 of 26 patients with soft tissue sarcomas treated with anthracyclines.[616] Both groups of patients received the same mean doxorubicin dose, but patients with Ewing sarcoma received higher individual doses of doxorubicin at shorter intervals. The addition of ifosfamide to chemotherapy regimens for Ewing sarcoma has resulted in the development of chronic renal tubular dysfunction in a subset of these patients.[617] Patients treated with radiation therapy to the bone are at risk for fractures in the radiation field, with an incidence of approximately 20% in one series of Ewing sarcoma survivors.[618] Use of whole lung radiation for patients with lung metastases may result in acute and late pulmonary toxicity, with one series reporting a 7% incidence of severe pulmonary complications.[619] Patients treated for Ewing sarcoma may have hematologic changes after treatment, including mild thrombocytopenia and macrocytosis.[615] Fertility is impaired among survivors of Ewing sarcoma. In one study 29.7% of female survivors reported a pregnancy compared with 40.1% of female sibling controls.[609] The impact of fertility in male survivors may be even greater, with this same series reporting 11.3% of male survivors siring a pregnancy compared with 33.2% of male sibling controls.[609] Indeed, among 23 male survivors of Ewing sarcoma who underwent sperm analysis, 13 were azospermic, 8 were oligospermic, and only 2 were normospermic.[610]

Several studies have evaluated the psychosocial ramifications of treatment for bone sarcomas, including Ewing sarcoma. A study from the Childhood Cancer Survivor Study reported that survivors of lower-extremity bone tumors overall have excellent self-reported orthopedic function and quality of life.[620] Approximately 5% of these patients reported severe or complete disability. Long-term function and quality of life do not appear to differ between patients who underwent amputation and patients who underwent limb-salvage procedures.[620,621] In another report from the Childhood Cancer Survivor Study, survivors of lower-extremity bone tumors were less likely to be married than sibling controls.[622] In addition, adolescents who underwent amputation were less likely to complete high school and were more likely to have difficulties with health insurance issues compared with sibling controls. Some of these findings were replicated in a single-institution study specific to Ewing sarcoma survivors.[623] In this study survivors had lower rates of employment and marriage than sibling controls, although educational attainment and insurance access did not differ between the two groups. All these studies highlight the importance of evaluation for late effects in survivors of Ewing sarcoma.

FUTURE DIRECTIONS

Remarkable gains have been achieved in the treatment of patients with Ewing sarcoma over the past 40 years. During this same period the understanding of the biology of these tumors has also greatly progressed. Future improvements in the management of this disease will require taking advantage of this improved understanding of the events involved in Ewing sarcoma tumorigenesis. Specific avenues of investigation should include therapies directed at key targets in Ewing sarcoma, including IGF-1R, CD99, mTOR, PARP, and EWS/FLI itself. These therapies will likely be attempted first in those patients with the worst prognosis: patients with metastatic disease and patients with early relapse. Effective targeted therapies in these populations will then be applied to patients with lower-risk disease. Targeted therapy holds the promise not only of improving outcomes for patients with Ewing sarcoma but also of reducing exposure to conventional chemotherapy and the late effects of that therapy. For now, further study of the clinical and molecular prognostic features of this disease is required so that current active agents may be applied in a more risk-adapted approach.

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CHAPTER 62

Osteosarcoma

Katherine Janeway

CHAPTER OUTLINE

EPIDEMIOLOGY
ETIOLOGY
CLINICAL PRESENTATION AND EVALUATION
Clinical Manifestations
Radiologic Evaluation
Biopsy
Laboratory Evaluation
PATHOLOGY AND STAGING
Pathology
Staging
NATURAL HISTORY AND PROGNOSIS
Localized Disease
Metastatic and Relapsed Disease
BIOLOGY
Cell of Origin
Genomics
Tumor Suppressors
Developmental Pathways and Stem Cell Programs
Oncogenes
Determinants of Metastasis
Biologic Markers of Response to Chemotherapy
Translational Science
TREATMENT
Local Control
Chemotherapy for Localized Disease
Initially Metastatic Osteosarcoma
Recurrent Osteosarcoma
Radiation Therapy
Complications of Therapy and Late Effects
CONCLUSION AND FUTURE DIRECTIONS

Osteosarcoma is the most common primary bone tumor. There are approximately 600 cases/year in the United States.[1] The term *osteosarcoma* was first used in the early 1800s by Alexis Boyer,[2] the imperial family surgeon for Napoleon. Although low-grade forms of osteosarcoma do exist, more than 90% of osteosarcomas are high-grade malignant lesions. Low-grade osteosarcomas will be discussed, but most of what follows pertains to high-grade osteosarcomas.

Osteosarcoma usually occurs in adolescents and young adults. Presenting symptoms can include tumor-related pain with or without a mass, a painless mass, and a pathologic fracture. The distal femur is the most frequent site of primary disease, followed by the proximal tibia and then the proximal femur, hip, and proximal humerus.[3] As early as 1818 it was recognized that untreated patients with osteosarcoma often developed metastatic disease.[2] The most common site of metastasis is the lungs, followed by bone. Metastatic disease is present in approximately 15% of patients at the time of diagnosis.[4]

Prior to the 1970s, when chemotherapy was first used to treat osteosarcoma, overall survival (OS) was approximately 20%. With modern chemotherapy and surgical control, the 10-year OS of patients with localized osteosarcoma is around 70%.[5] The OS is much worse for patients with metastases at diagnosis and for those with relapsed disease. In both groups, the 5-year OS rate is approximately 25%.[4,6,7] The most active chemotherapy agents in osteosarcoma are doxorubicin (Doxo), cisplatin (CP), and high-dose methotrexate (HDMTX). Ifosfamide and etoposide are also active against osteosarcoma and are typically used in addition to Doxo, CP, and HDMTX in patients with metastatic disease and in patients with recurrence.

In the past three decades, a number of discoveries have led to a better understanding of osteosarcoma biology. For example, the tumor suppressor gene *TP53* and the retinoblastoma gene *RB1* are altered in osteosarcoma. Oncogenes, including *MYC, MET,* and *FOS,* have also been implicated in osteosarcomagenesis. These tumor suppressors, oncogenes, and other biologic factors will be discussed in detail. Unlike most childhood cancers, there has been minimal improvement in OS over the past three decades.[8,9] Consequently, one of the research priorities in osteosarcoma is the identification of additional active therapies.

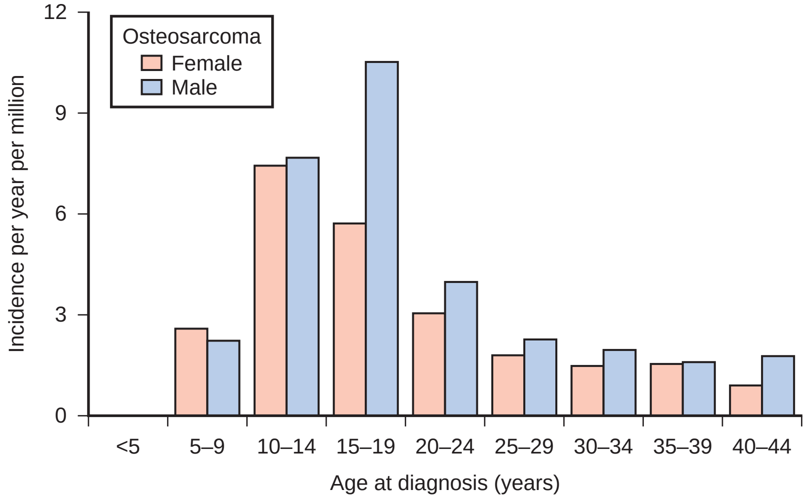


Figure 62-1 Age Distribution of osteosarcoma based on U.S. cases between 1975 and 1999. *(Data from Bleyer A, O'Leary M, Barr R, et al, editors:* Cancer epidemiology in older adolescents and young adults 15 to 29 years of age, including SEER incidence and survival: 1975-2000. *NIH publication No. 06-5767. Bethesda, Md., 2006, National Cancer Institute.)*

EPIDEMIOLOGY

Osteosarcoma is the most common primary bone tumor in children.[1] The average annual incidence of osteosarcoma in children younger than 20 years in the United States is 4.8/million. Approximately 3% of all malignancies in this age group are osteosarcomas. In 2000, the most recent year for which data are available, there were 440 cases of osteosarcoma in children from birth to 19 years of age in the United States. There were an additional 135 cases in young adults aged 20 to 29.[1] Internationally, incidence rates approximate the U.S. rate, with the exception of a higher incidence in some African countries. In Sudan and Uganda, the osteosarcoma incidence in children younger than 14 years is 5.3 and 6.4/million respectively, compared with an incidence of between 2 and 3/million in this age group in the United States, Europe, and Asia.[10,11]

Osteosarcoma occurs rarely in children younger than 5 years. After age 5, the incidence increases steadily, reaching a peak at age 15 years.[12] After the adolescent peak, the rate of osteosarcoma steadily declines, leveling off at a rate of 1 to 2/million. A second peak in incidence, approximately half the magnitude of the adolescent peak, occurs in the sixth to seventh decade.[13,14] Osteosarcoma in older patients is associated with Paget disease and prior radiation therapy, although approximately half of older patients with osteosarcoma have neither condition.[15] The adolescent peak occurs at age 13 years in girls and between ages 15 and 17 years in boys (Fig. 62-1). The age at peak incidence corresponds to the age of greatest growth velocity in each gender. This association contributes to the evidence supporting a role for growth in the cause of osteosarcoma (see "Etiology"). Osteosarcoma is slightly more common in males, particularly in the 15- to 19-year-old age group.

In the United States, osteosarcoma is slightly more common in African Americans, Hispanics, and Asians and Pacific Islanders (Fig. 62-2). Several studies have reported that rates of osteosarcoma in black children younger than 14 years are about twice those in white children of the same age.[16,17] In the most recent report on osteosarcoma from the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute, U.S. blacks younger than 20 years continue to have a slightly higher rate of osteosarcoma.[12] As shown in Figure 62-2, for the age group 15 to 29 years, the racial group with the highest rate of osteosarcoma is Hispanic. The relative risk of developing osteosarcoma for Hispanic children younger than 14 years of age, based on data collected between 1975 and 2003, is 1.3.[18] Asians and

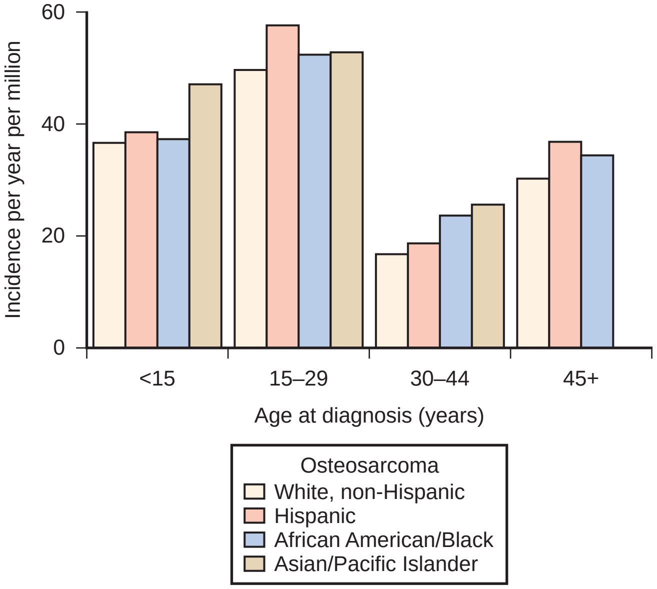


Figure 62-2 Incidence of osteosarcoma by racial group based on U.S. cases between 1990 and 1999. *(Data from Bleyer A, O'Leary M, Barr R, et al, editors:* Cancer epidemiology in older adolescents and young adults 15 to 29 years of age, including SEER incidence and survival: 1975-2000. *NIH publication No. 06-5767. Bethesda, Md. 2006, National Cancer Institute.)*

Pacific Islanders have the highest rate of osteosarcoma in the age groups younger than 15 years and 30 to 44 years. However, the reported incidence in Hispanics and in Asians and Pacific Islanders is likely to be inaccurate given the small absolute number of affected individuals and uncertainty in the denominator (i.e., the number of Hispanics and Asians and Pacific Islanders within each age group living in the United States). In summary, there is evidence for a slightly increased incidence of osteosarcoma among U.S. blacks and Hispanics. Recent SEER data show an increased rate in Asians and Pacific Islanders younger than 15 years, but this finding needs further investigation.

The most common anatomic sites of osteosarcoma at initial presentation are the long bones of the lower limbs. Fifty percent of osteosarcomas are located around the knee, in the distal femur, proximal tibia, or proximal fibula (Fig. 62-3). Approximately 10% of tumors occur in the mid or proximal femur, and 9% occur in the proximal humerus.[3,12] Of patients with osteosarcoma, 10% to 20% have metastatic disease at initial presentation. The most common site of metastasis is the lungs. Isolated pulmonary metastases are present in 61% of patients with metastatic disease at presentation. Isolated metastases to bone are present in 16% of patients, and 14% have both pulmonary and bone metastases. Seven percent of patients have metastases to other rare sites plus bone and/or pulmonary metastases, and 2% of patients have isolated metastases to rare sites. Rare sites of metastasis in patients with primary metastatic disease, as opposed to metastatic disease in patients with recurrent disease, are lymph nodes, central nervous system, liver, adrenal gland, and soft tissues.[4]

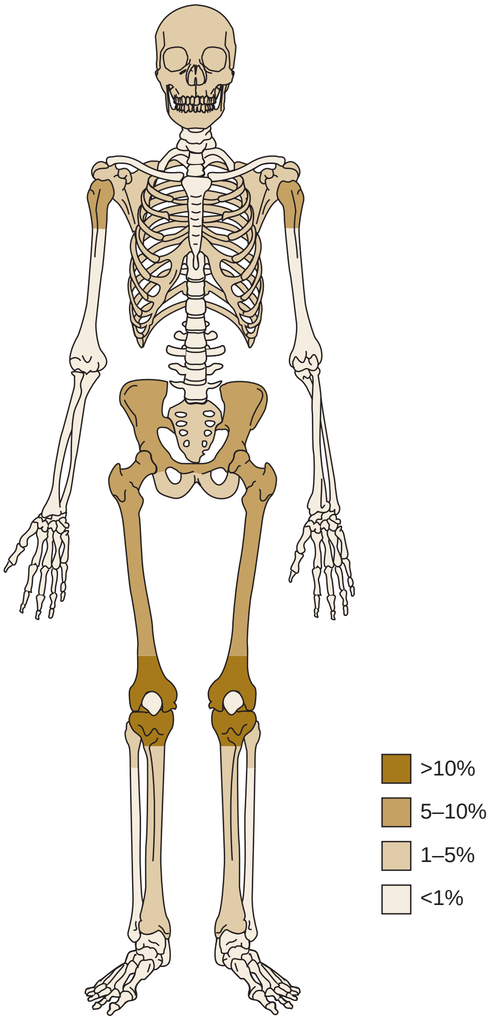


Figure 62-3 Anatomic sites of osteosarcoma at initial presentation. *(Modified from Dahlin DC, Unni KK: Osteosarcoma of bone and its important recognizable varieties.* Am J Surg Pathol *1:61–72, 1977.)*

ETIOLOGY

When a patient presents with osteosarcoma, it is often difficult to identify a cause. However, recognition of causative factors has led to the discovery of biologic mechanisms of transformation, as in the case of predisposition to osteosarcoma in Li-Fraumeni syndrome, or the identification of exposures to be avoided, as in the case of radiation-induced osteosarcoma. Inherited syndromes in which there is an increased occurrence of osteosarcoma are hereditary retinoblastoma and Li-Fraumeni, Rothmund-Thomson, Bloom, and Werner syndromes. There is a clear association between the risk for osteosarcoma and prior radiation therapy or treatment with alkylating chemotherapy. Epidemiologic studies and clinical observation have suggested that increased height may be a risk factor for the development of osteosarcoma. Finally, environmental exposures to radium and beryllium likely increase the risk for osteosarcoma, but exposure to high doses of these agents is rare.

As noted, peak rates of osteosarcoma coincide with the period of maximal linear growth during puberty. Most osteosarcomas occurring in the adolescent age group are in the long bones of the axial skeleton, the bones in which the most active growth occurs. These clinical observations have led to the hypothesis that the hormonal milieu during maximal linear growth encourages the development of osteosarcoma and that, by extension, osteosarcoma would be more common in taller adolescents. Multiple epidemiologic studies have addressed this question,[19-24] but results are contradictory. Approximately 50% of the studies have found that osteosarcoma patients are taller at diagnosis than age-matched controls or the normal population.[22-24] The remaining studies have not supported an association between height and the occurrence of osteosarcoma.[19-21] The larger studies[23] and those with more reliable sources of height data[22] have shown a positive association between tall stature and osteosarcoma. These data, in part, have led to the investigation of the insulin-like growth factor axis in osteosarcoma (see "Biology").

Approximately 1% of patients develop secondary bone tumors after treatment of a primary pediatric malignancy[25]; of these secondary bone tumors, 50% to 80% are osteosarcomas. On the other hand, secondary Ewing sarcoma is rare.[25,26] The relative risk of secondary osteosarcoma increases greatly with radiation doses to the bone higher than 1000 cGy. In patients who received between 3000 and 5000 cGy to the bone during treatment of their primary pediatric malignancy, the relative risk of secondary osteosarcoma was variably reported, but it may be as high as 100.[25,27] A study in patients with primary Ewing sarcoma has concluded that only higher radiation doses increase the risk for secondary sarcomas, with no tumors developing in patients who had received less than 4800 cGy. The highest risk, 130 cases/10,000 person-years of observation, was observed among patients who had received 6000 cGy or more.[28] In the 92 published cases of secondary osteosarcoma for whom radiation dose was reported, the median radiation dose delivered was 4500 to 5400 cGy.[29] The average time between diagnosis of the primary malignancy and development of secondary osteosarcoma is 10 years.[26] Another factor that increases the likelihood that survivors of a primary cancer will develop secondary osteosarcoma is treatment with alkylating agents. This class of chemotherapy increases the relative risk of secondary osteosarcoma by a factor of 5.[25]

Data regarding an increased risk for cancer in people exposed to low levels of radiation from the atomic bombs in Hiroshima and Nagasaki have led to increased concern about the risk of diagnostic imaging with x-rays and computed tomographic (CT) scans, particularly in pediatric patients.[30] However, current evidence does not suggest that osteosarcoma risk would be increased from commonly used imaging modalities.[31] In particular, the radiation dose of a CT scan is approximately 6 cGy and the risk for bone cancer in pediatric cancer survivors who received less than 1000 cGy to the bone is not increased.[25]

Osteosarcoma is seen in a number of inherited cancer predisposition syndromes. These syndromes have often illuminated mechanisms of transformation in sporadic osteosarcoma. Children with hereditary retinoblastoma have a germline heterozygous, inactivating deletion, and/or a frameshift or point mutation of the tumor suppressor gene retinoblastoma 1 *(RB1)*. Affected children develop retinoblastoma usually in one or both eyes. The cumulative incidence of a nonretinoblastoma malignancy in hereditary retinoblastoma is 51%, and approximately 40% of these second malignancies are osteosarcomas.[32] Both osteosarcomas and retinoblastomas in these patients have loss of heterozygosity of the *RB1* allele, resulting in a complete loss of RB1 function. As discussed later (see "Biology"), loss of RB1 function also occurs in sporadic osteosarcomas.[33,34]

In the Li-Fraumeni syndrome, patients carry a heterozygous inactivating mutation of the tumor suppressor gene *TP53*. Affected individuals have a greatly increased cancer risk at a young age. The most common malignancies are breast cancer and bone and soft tissue sarcomas, brain tumors, and leukemia.[35] Slightly more than 10% of the malignancies in patients with Li-Fraumeni syndrome are osteosarcomas. Among patients with Li-Fraumeni syndrome, mutations in the *TP53* DNA-binding domain (as compared with mutations outside the DNA-binding domain or null mutations) are associated with early sarcomas.[36] Patients with osteosarcomas and other cancers that develop in Li-Fraumeni syndrome have loss of the normal *TP53* allele consistent with the tumor suppressor function of the TP53 gene product. Loss of TP53 function by *TP53* mutation or amplification of *MDM2* or *COPS3* is also present in most sporadic osteosarcomas (see "Biology").[37]

Rothmund-Thomson, Bloom, and Werner syndromes are members of a family of autosomal recessive disorders caused by mutations in one of the RECQ DNA helicases. Rothmund-Thomson syndrome, caused by mutations in the *RECQL4* gene, is characterized by rash, skeletal dysplasias, and sparse hair. Up to 30% of patients with Rothmund-Thomson syndrome develop osteosarcoma at a slightly younger age than in sporadic osteosarcoma.[38,39] Bloom syndrome, caused by mutations in the *BLM (RECQL2)* gene, is characterized by short stature, telangiectasia, and an increased risk for developing a wide variety of cancers at a young age.[40] Unlike in Rothmund-Thomson syndrome, only a small percentage of cancers occurring in affected individuals are osteosarcomas. Werner syndrome is caused by a mutation in the *WRN (RECQL3)* gene. Patients have scleroderma-like skin changes, premature aging, and a typical body habitus with short stature, long spindly limbs, and a stocky trunk. Like in Bloom syndrome, the risk for developing cancer at a young age is increased in Werner syndrome. Also like in Bloom syndrome, osteosarcoma constitutes fewer than 10% of the malignancies occurring in patients with Werner syndrome.[41] For a further discussion of the role of RECQ DNA helicases in osteosarcoma, see "Biology".

The risk for developing osteosarcoma is increased in adults with Paget disease. In Paget disease, which affects up to 1% of adults older than the age of 40 years, bone resorption by osteoclasts is increased. As a result, there is a compensatory increase in osteoblast activity and bone formation.[42] Osteosarcoma develops in less than 1% of patients with Paget disease, but this represents a higher rate than that seen in the general population. The average age of patients with Paget disease–related osteosarcoma is 70 years.[15]

A single genome-wide association study has been conducted in osteosarcoma. There are two susceptibility loci achieving statistical significance in osteosarcoma. One locus occurs in a gene desert and the other, at 6p21.3, implicates *GRM4,* a glutamate receptor gene involved in inhibition of the cyclic adenosine monophosphate (AMP) cascade.[43]

Previously, some believed that trauma may cause osteosarcoma. However, modern epidemiologic studies have not supported a relationship between trauma and osteosarcoma.[20] Prior observations linking trauma and osteosarcoma were likely the result of the fact that a traumatic event may result in a previously existing osteosarcoma being brought to medical attention.

Environmental exposures, other than ionizing radiation, for which there is evidence of an association between exposure and osteosarcoma risk, include exposure to radium and beryllium. Radium is a radioactive element that is variably present in well water. When ingested, radium retained in the body is deposited in bone. Studies of those exposed to high levels of radium while painting dials on clocks in the 1920s strongly indicated that exposure to high doses of radium causes osteosarcoma.[44] The risk for osteosarcoma from exposure to low levels of radium in drinking water is less clear. Two studies have shown a slight increase in risk for bone sarcoma or osteosarcoma in those born in areas with drinking water containing relatively high concentrations of radium.[45,46] Conversely, two studies have shown no link between mean levels of radium in water and osteosarcoma.[46,47] Beryllium is a metallic element with limited use in the production of aerospace structural material, x-ray tubes, and nuclear reactors. Although intravenous injection of beryllium salts causes osteosarcoma in rabbits, there is limited evidence of an increased risk for osteosarcoma caused by beryllium exposure in humans.[48] Case-control studies have failed to identify other environmental or occupational exposures that increase the risk for osteosarcoma.[24] Because fluoride can stimulate bone formation there has been concern about a possible relationship between fluoride in drinking water and osteosarcoma. This is a very difficult question to study adequately given that multiple factors beyond water fluoridation may impact fluoride intake. So far, there is no substantial evidence to support a causative association between fluoride exposure and osteosarcoma.[49]

CLINICAL PRESENTATION AND EVALUATION

Clinical, laboratory, and radiologic findings are often highly suggestive of osteosarcoma but are not pathognomonic. Biopsy is always required for diagnosis. Atypical imaging characteristics, including a benign appearance in high-grade osteosarcoma, have been described.[50] The differential diagnosis of osteosarcoma includes benign and malignant bone tumors (Box 62-1; Fig. 62-4).

Box 62-1 Differential Diagnosis of Osteosarcoma

OTHER MALIGNANT PRIMARY BONE TUMORS

Ewing sarcoma
Chondrosarcoma
Fibrosarcoma
Langerhans cell histiocytosis

OTHER MALIGNANCIES PRESENTING AS BONE TUMOR(S)

Lymphoma
Neuroblastoma
Metastatic rhabdomyosarcoma
Metastatic melanoma

BENIGN BONE TUMORS

Aneurysmal bone cyst
Osteoblastoma
Osteoid osteoma
Giant cell tumor
Unicameral bone cyst

INFECTION

Osteomyelitis

Clinical Manifestations

Clinical symptoms of osteosarcoma are nonspecific; 25% of patients have pain and a palpable mass at presentation,

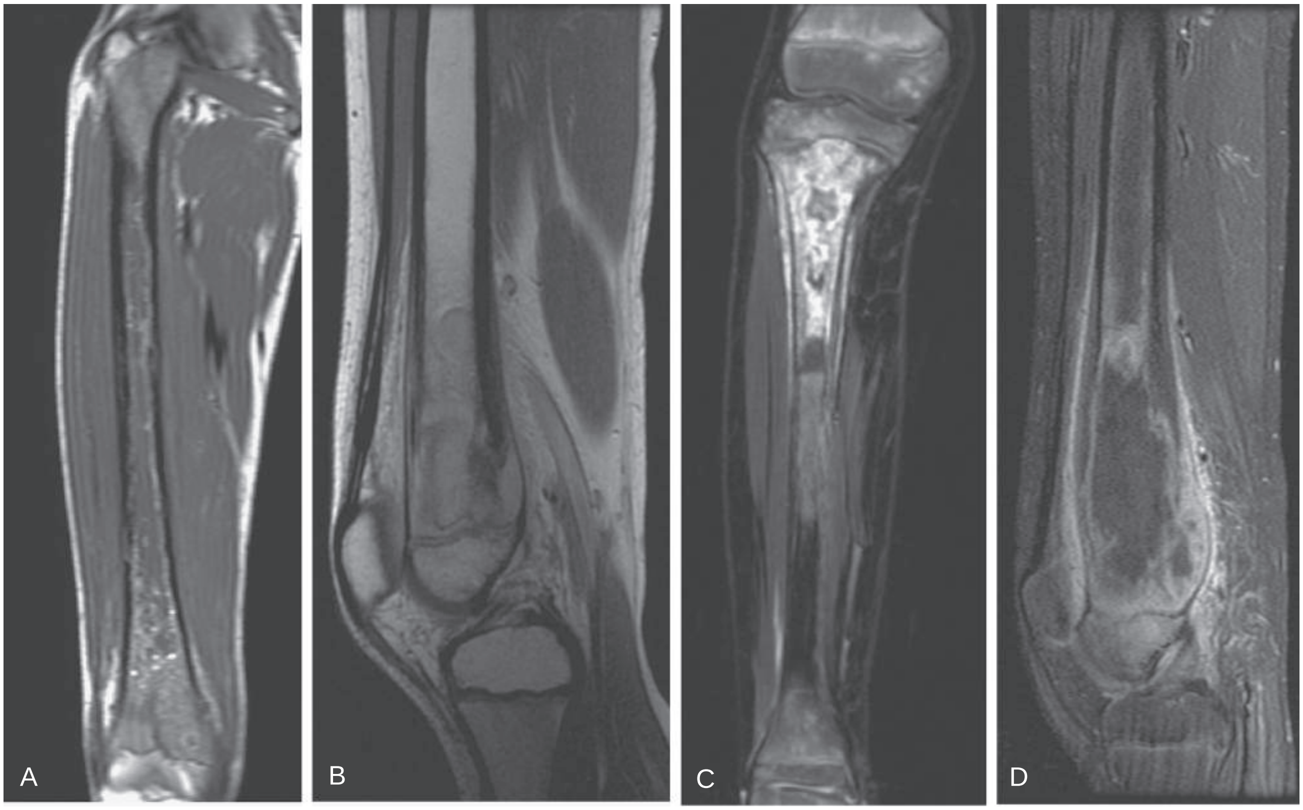


Figure 62-4 MRI appearance of osteosarcoma and lesions that can be confused with osteosarcoma in clinical presentation and radiographic appearance. **A,** Osteomyelitis of the femur. **B,** Lymphoma of the distal femur. **C,** Ewing sarcoma of the proximal tibia. **D,** Osteosarcoma of the distal femur.

and 70% of patients present with pain as their only complaint. Pain tends to be intermittent and exacerbated by activity. The remaining patients present with a painless mass.[51] The duration of symptoms before diagnosis ranges from 1 day to 5 years, with a median of about 2½ months. In approximately 10% of patients, symptom duration is longer than 6 months.[52] A pathologic fracture is present in 8% of patients at the time of diagnosis.[53]

Radiologic Evaluation

Radiologic evaluation in osteosarcoma informs the initial diagnostic impression, aids in the determination of the best approach for biopsy, facilitates surgical resection, and identifies sites of metastatic disease, if present. The recommended imaging studies for osteosarcoma at the time of diagnosis are plain radiography and magnetic resonance imaging (MRI) of the primary tumor site to evaluate the extent of local disease, a bone scan to screen for bony metastases, and a CT scan of the chest to determine whether pulmonary metastases are present. Whenever possible, these imaging studies should be performed before diagnostic biopsy because postsurgical atelectasis interferes with evaluation of the lung parenchyma. MRI of the primary tumor should include the entire bone from which the tumor arises and the closest adjacent joint to screen for skip metastases properly. An additional MR image should be obtained after neoadjuvant chemotherapy has been given but before definitive resection to determine the best plan for the surgical approach.

A plain radiograph of the primary tumor has usually been obtained before referral to an oncologist or orthopedist. Most appendicular osteosarcomas are located in the metaphyseal portion of the long bone. Both lytic destruction of bone and sclerotic irregular new bone formation can occur. Osteosarcoma usually has a mixed pattern of bone lysis and sclerosis, but one of these patterns can predominate. Regardless of the predominant pattern, the boundary between tumor and normal bone is usually irregular. The bone cortex is often disrupted by tumor growth into the surrounding soft tissue. Osteoid formation within the tumor results in areas of calcification visible in the bony and soft tissue components of the tumor. Periosteal new bone formation in osteosarcoma can be irregular or can occur in a sunburst pattern. "Sunburst" refers to linear new bone growth that is perpendicular to the bony cortex and arranged in a fanlike pattern (Fig. 62-5).[54] In contrast, in Ewing sarcoma, periosteal new bone growth typically occurs in an onionskin pattern in which linear new bone is oriented parallel to the bony cortex.[3] Periosteal new bone formation in

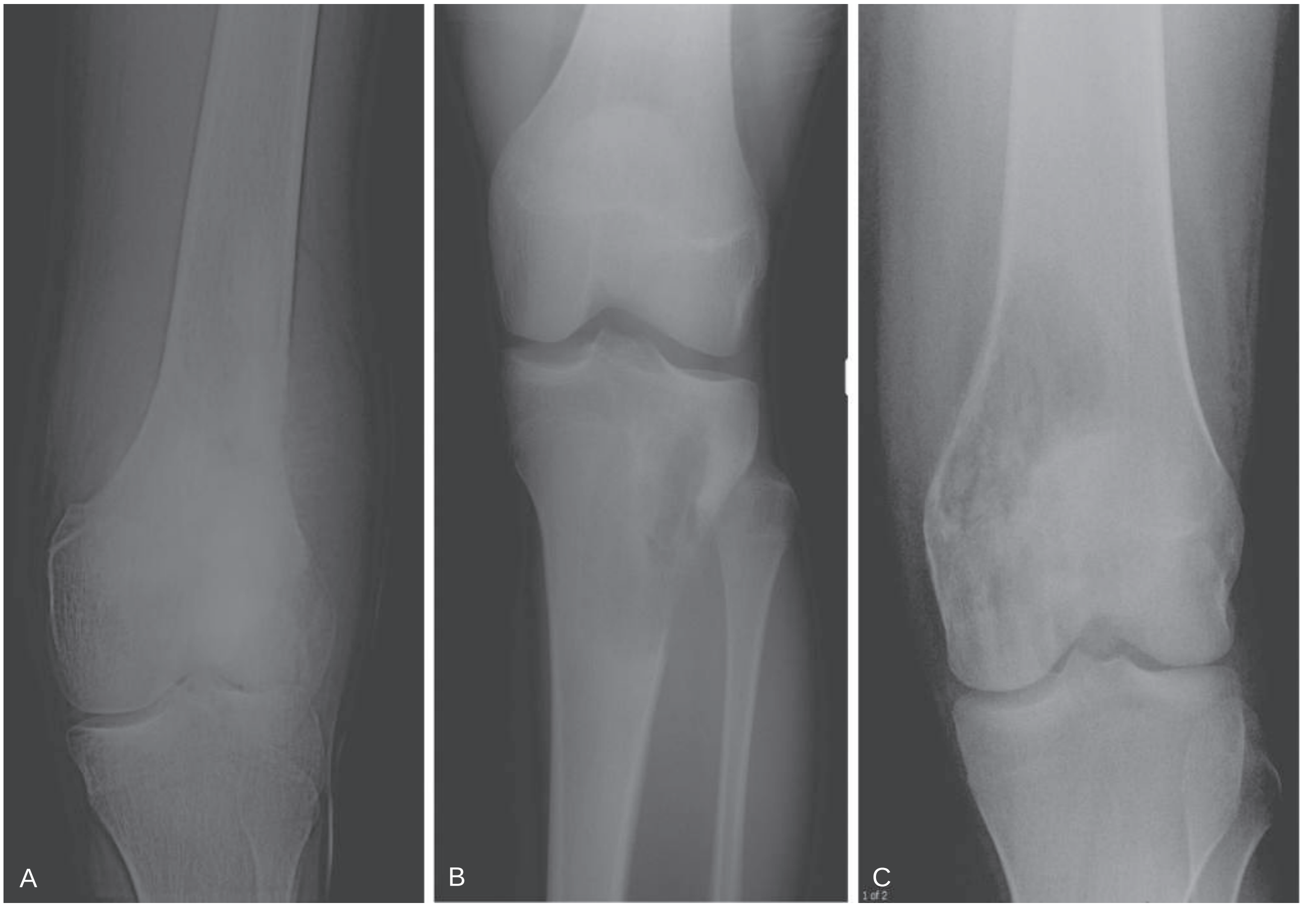


Figure 62-5 Three typical patterns observed in plain radiographs of osteosarcoma. **A,** Predominantly sclerotic pattern in an osteosarcoma of the distal femur with sunburst periosteal new bone formation. **B,** Predominantly lytic pattern in an osteosarcoma of the proximal tibia. **C,** Mixed lytic and sclerotic pattern in an osteosarcoma of the distal femur.

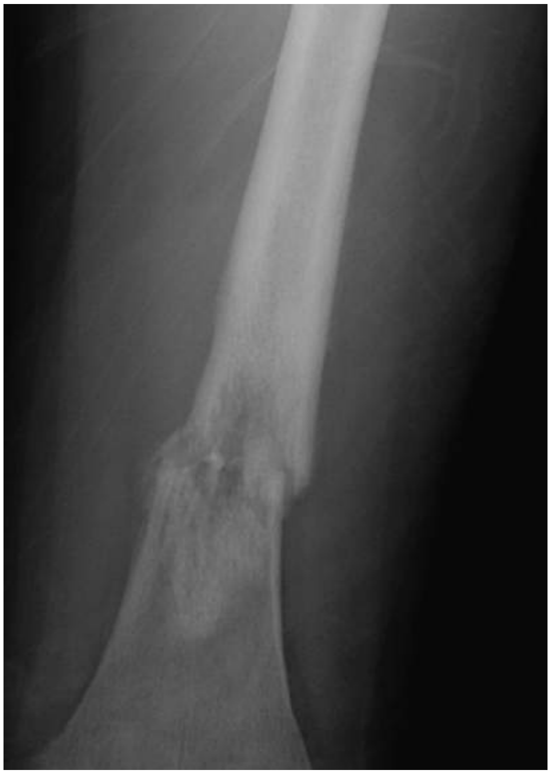


Figure 62-6 Pathologic fracture in an osteosarcoma of the distal femur.

osteosarcoma can also be present at the edge of the preserved cortex, resulting in the Codman triangle. Plain radiographs also assist in the diagnosis of pathologic fractures (Fig. 62-6).

Although plain radiographs of the primary tumor are informative, MRI is preferable for local staging, presurgical planning, and identification of skip metastases. CT is an acceptable alternative to MRI, but most orthopedic surgeons and oncologists prefer MRI. In two studies directly comparing preoperative CT and MRI results with pathologic findings in patients with osteosarcoma, MRI was found to be superior to CT in defining the longitudinal extent of intraosseous tumor,[55,56] extension into adjacent muscle compartments, and involvement of the neurovascular bundle. The two modalities were equivalent in their ability to define cortical bone and joint involvement. However, a more recent study in a larger patient population did not find a statistically significant difference between CT and MRI in their ability to predict the involvement of bone, muscle, joints, and the neurovascular bundle.[57] One possible explanation for the contradictory results is improvements in CT quality during the interval between the studies. In addition, the later study grouped all primary bone tumors together for analysis but, given that 60% of the bone tumors were osteosarcoma, results would likely be similar for osteosarcomas alone. In comparison to CT and technetium-99 m (^{99m}Tc) bone scanning, MRI more accurately predicts the intraosseous extent of osteosarcoma.[56] The presence of significant edema has been associated with inaccurate assessment of the margins of tumor on MRI. Because edema can resolve with chemotherapy, imaging performed for the purpose of surgical planning should be obtained after the administration of neoadjuvant chemotherapy.[58]

A *skip metastasis* is defined as a smaller distinct focus of osteosarcoma that is either within the same bone as the primary tumor or on the other side of the joint adjacent to the primary tumor.[59] There must be normal structures not involved with tumor present between the primary tumor and skip metastasis. Skip metastases are synchronous with the primary tumor and thus are present at initial presentation. Diagnosis of skip metastases is of critical importance for prognostication and because complete surgical remission in osteosarcoma with skip metastases requires complete resection of both the primary tumor and skip metastases. Over 80% of skip metastases are visible on MR images (Fig. 62-7). In contrast, skip metastases are visible on bone scan, CT scans, and plain radiographs in only 46%, 45%, and 36% of cases, respectively.[60] Interestingly, a common reason for failure to identify skip metastases by bone scanning is merging of the signal from the primary tumor with the signal from the skip metastasis.[61] To ensure that skip metastases are identified, MRI of the primary tumor should include the entire bone and adjacent joint. Bony metastases that are not skip metastases are best identified with a ^{99m}Tc bone scan (Fig. 62-8).[61] The usefulness of ^{18}F-labeled fluorodeoxyglucose positron emission tomography (FDG-PET) in osteosarcoma has not yet been adequately evaluated. Results of preliminary studies have suggested that FDG-PET is more useful for assessing tumor response to chemotherapy and for posttherapeutic

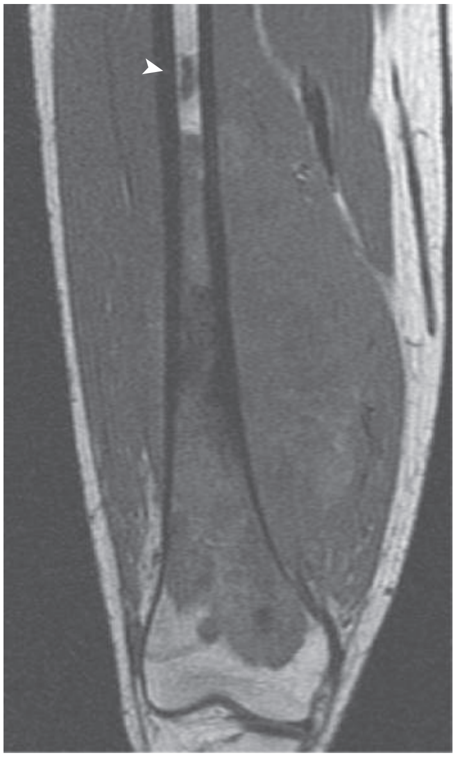


Figure 62-7 Skip metastasis *(arrowhead)* of an osteosarcoma of the distal femur as seen on T1-weighted MR image.

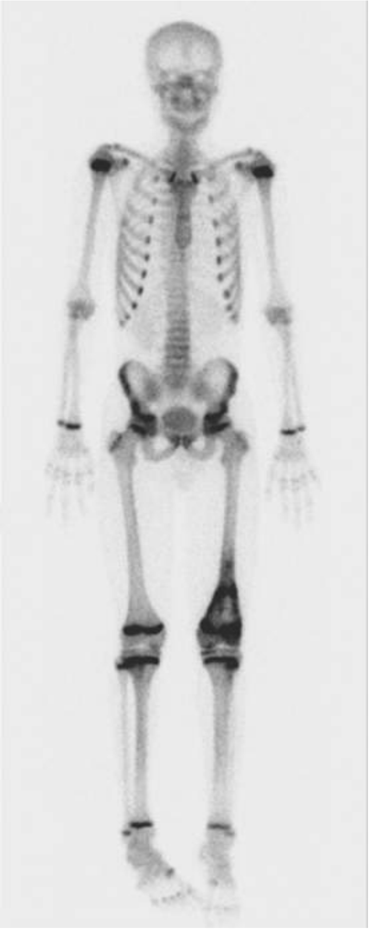


Figure 62-8 Technetium-99 m bone scan showing an osteosarcoma of the distal femur without distant bony metastases.

monitoring for recurrence than for assessing the initial tumor.[62] Because edema can resolve with chemotherapy, imaging performed for the purpose of surgical planning should be performed after the administration of neoadjuvant chemotherapy.[58]

CT of the chest is the most sensitive radiologic study for detection of pulmonary metastases. A generally accepted definition of definitive pulmonary metastatic disease is three or more lesions 5 mm or larger or one lesion 1 cm or larger.[63] The interpretation of very small lesions (<5 mm) and solitary lesions between 5 mm and 1 cm detected by high-resolution CT can be difficult. In some cases, it is not possible to determine whether such CT findings represent metastatic disease or a nonmalignant lung process, and biopsy may be needed. Compared with a CT scan, a plain radiograph of the chest has a sensitivity of 57% and a ^{99m}Tc bone scan has a sensitivity of 41%.[64] Although CT is highly sensitive, evaluation at the time of thoracotomy usually reveals more pulmonary metastases than are appreciated on the CT scan.[65] Importantly, chest CT scans should be obtained before biopsy of the primary tumor because postanesthesia atelectasis makes chest CT scans more difficult to interpret.

Whole-body MRI screening as a method of surveillance in patients with cancer predisposition is currently being evaluated. In a small group or individuals (n = 25) with hereditary retinoblastoma, whole-body MRI detected two cases of osteosarcoma. An additional individual was diagnosed with osteosarcoma due to symptoms.[66] A screening protocol including whole-body MRI utilized in individuals with Li-Fraumeni syndrome detected no cases of osteosarcoma but did detect one case of malignant fibrous histiocytoma.[67]

Biopsy

Traditionally, bone tumor tissue for pathologic examination was obtained by open biopsy. However, percutaneous core needle biopsies have become more common as the availability and sophistication of interventional radiology services have increased. A number of retrospective series have reported on the diagnostic yield and accuracy of core needle biopsies in osteosarcoma.[68-73] Although none specifically studies pediatric osteosarcoma, all series that provide patient age included children. In these published series, core needle biopsy resulted in a definitive, correct diagnosis in 78% to 94% of cases. The more recently published series have reported a diagnostic accuracy of 90% or greater.[69,73] However, even those percutaneous biopsies that yield sufficient tumor material for a diagnosis often do not yield sufficient tumor specimen for other biologic studies.

The outcome of percutaneous biopsy varies depending on the provider, technique, and patient population. In the published series reporting a high diagnostic yield, biopsies were performed by experienced interventional radiologists in consultation with orthopedic surgeons in tertiary or referral centers with considerable expertise in the diagnosis and management of bone tumors. In the more recent series, patients underwent conscious sedation or were given general anesthesia. Specific techniques included the use of fluoroscopic, CT, or ultrasound guidance and use of at least a 14-gauge needle. The tumor component targeted for biopsy was the soft tissue mass, if present, followed by the lytic aspect of an intraosseous lesion.

The most common reason for failure to obtain diagnostic tissue by core needle biopsy is the presence of a highly sclerotic tumor.[70,71] Of the various types of osteosarcoma, telangiectatic osteosarcoma most often results in a nondiagnostic specimen or is misdiagnosed.[69,70] Development of osteosarcoma in a percutaneous biopsy tract has been reported.[74] Therefore, it is important that the biopsy site be located so that it can be resected en bloc with the primary tumor. Because fine-needle aspiration has a diagnostic yield lower than core needle biopsy[68] and does not obviate the need for sedation in the pediatric population, it is not a recommended approach for diagnosis.

Surgical or open biopsy of bone tumors can be incisional or excisional. Excisional biopsy is almost never performed in osteosarcoma because neoadjuvant chemotherapy can decrease tumor edema and, in doing so, simplify resection. In addition, time is required for adequate surgical planning, especially for younger children in whom placement of a growing prosthesis is being considered. Complications of open biopsy include anesthesia-related events, infection, bleeding, and tumor seeding in the biopsy tract.[75] In addition, disruption of anatomic compartments and incorrect localization of the biopsy incision can lead to a more complex or more extensive tumor resection and can occasionally result in an amputation that would not have otherwise been necessary.[76] As with percutaneous biopsy, the incision for an open biopsy should be placed and oriented in a way that allows it to be excised en bloc with the primary tumor. Complications of open biopsy, including those that result in more extensive resections, are unusual when the biopsy is performed at a center specializing in the diagnosis and treatment of malignant bone tumors.[77]

In summary, the percutaneous and open biopsy routes are both acceptable. Given the implications for ultimate surgical resection of a poorly performed biopsy, biopsy should be performed or planned by an orthopedic surgeon with bone tumor expertise, ideally by the same orthopedic surgeon who will perform the ultimate resection. Percutaneous biopsies should only be performed by experienced interventional radiology staff working in conjunction with an experienced orthopedic surgeon. If the percutaneous route is recommended, it is important to counsel patients and families about the possible risks of core needle biopsy, including the potential for an inconclusive specimen. For a further discussion of the debate regarding the correct route for bone tumor biopsy, see Box 62-2.

Laboratory Evaluation

Laboratory test results are often normal in osteosarcoma. When laboratory abnormalities are present, they are nonspecific, having many other potential causes. The serum alkaline phosphatase level is elevated in approximately 50% of patients presenting with osteosarcoma.[78,79] A higher percentage of patients with metastatic disease have a high alkaline phosphatase level, but this is not a reliable test of metastatic disease, because some patients with metastatic disease will have a normal result. As will be discussed later, alkaline phosphatase elevations are associated with a poorer prognosis. Lactate dehydrogenase (LDH) is elevated in approximately 30% of osteosarcoma patients.[80] The erythrocyte sedimentation rate is generally not elevated in patients with osteosarcoma, whereas it is often elevated in those with Ewing sarcoma.

PATHOLOGY AND STAGING

Pathology

On gross examination, osteosarcoma usually presents as a large mass located in the metaphysis (Fig. 62-9) arising in the medullary cavity and crossing the bony cortex, with invasion into the adjacent soft tissues. Microscopically, the malignant cells are pleomorphic but spindle cells typically predominate. Epithelioid, small round cells, multinucleated giant cells, and plasmacytoid cells may also be present and occasionally are the predominant morphology.[81] Osteoid, which can be plentiful or sparse, is currently essential for the diagnosis of osteosarcoma. Sometimes, it is not present in the initial biopsy sample but is seen in the definitive resection specimen. If not

Box 62-2 **Controversies in Osteosarcoma: Open vs. Core Needle Biopsy**

There are two possible approaches to obtaining a diagnostic specimen in suspected osteosarcoma—open biopsy and core needle biopsy. Considerable controversy exists regarding which diagnostic approach is best. An open biopsy is performed in the operating room by an orthopedic surgeon. As discussed in more detail in the text, an open biopsy should be performed by orthopedic surgeon with experience treating bone tumors and ideally by the same orthopedic surgeon who will ultimately perform the tumor resection. In a core needle biopsy, while imaging the tumor with CT, an interventional radiologist obtains several needle cores of tumor material. As with an open biopsy, a core needle biopsy should be performed by an interventional radiologist with expertise in the diagnostic evaluation of bone tumors. Planning for core needle biopsy should include a discussion with the orthopedic surgeon who will ultimately perform the resection. The relative merits of core needle and open biopsy as they relate to several core issues are discussed below:

1. DEFINITIVE DIAGNOSIS

Because orthopedic surgeons directly visualize a tumor during an open biopsy and because pathologists often view frozen sections during open biopsies, nondiagnostic open biopsies are uncommon. In retrospective series,[68-73] core needle biopsy results in a definitive, correct diagnosis in 78% to 94% of cases. The more recently published series report a diagnostic accuracy of 90% or more.[69,73] The interventional radiology-guided core needle biopsies in these studies were performed in centers with considerable experience with the diagnosis of bone tumors and similar diagnostic accuracy would not be expected in inexperienced centers. Less tumor material is obtained from core needle biopsies, which can compromise the pathologist's ability to perform additional testing, such as cytogenetics, in some cases.

2. POSTPROCEDURE RECOVERY

Both core needle and open biopsies require general anesthesia in children. In general, open biopsies are longer procedures, requiring a greater length of general anesthesia. Open biopsies are also more invasive and result in a longer postprocedure recovery.

3. IMPACT ON ULTIMATE RESECTION

Whether the biopsy approach is core needle or open, definitive surgical treatment of osteosarcoma, which is essential for cure, requires excision of the biopsy tract or site en bloc with the primary tumor. A poorly placed biopsy can convert a tumor amenable to limb-sparing surgery into one that requires amputation. This impact on ultimate resectability is one of the greatest potential negative aspects of core needle biopsy. Because interventional radiologists are less knowledgeable about surgical approaches for definitive tumor resection in osteosarcoma, the risk of a poorly placed biopsy is potentially higher with a core needle biopsy. However, supporters of core needle biopsy argue that this risk can be reduced through discussion of the biopsy with the patient's treating orthopedic surgeon.

4. IMPACT ON BIOLOGIC STUDIES OF OSTEOSARCOMA

Although the availability of tumor material for the study of osteosarcoma biology is not an important issue from the perspective of the individual patient with osteosarcoma, it is an important issue from the perspective of researchers and future patients who could benefit from research discoveries. Because tumor resection occurs after the administration of chemotherapy, osteosarcoma resection specimens are often not suitable for laboratory investigation. After open biopsy there is usually sufficient prechemotherapy tumor material available for biologic studies after the diagnosis is made. This is not the case with core needle biopsies. Thus, progress in osteosarcoma research would likely be negatively affected if the majority of centers were to perform core needle biopsies.

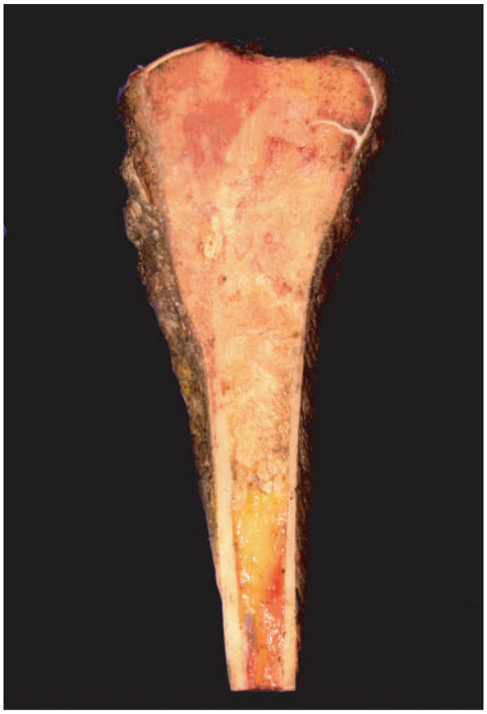


Figure 62-9 Osteosarcoma: gross pathologic appearance.

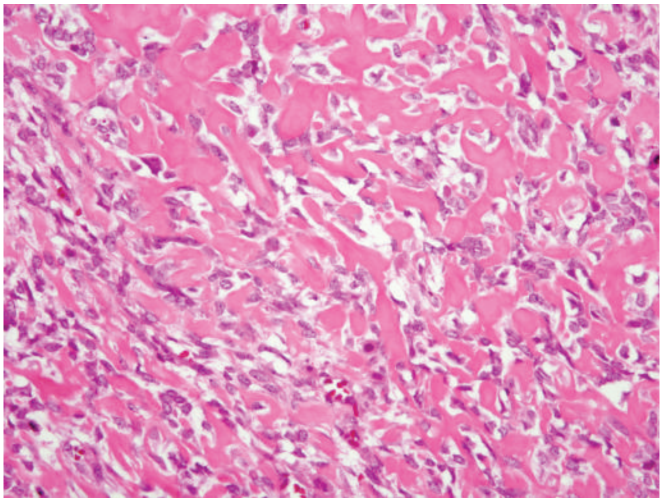


Figure 62-10 High-grade osteosarcoma composed of malignant-appearing tumor cells having a high nuclear-to-cytoplasmic ratio, hyperchromatism, and prominent nucleoli and an abundant eosinophilic osteoid matrix.

present in either, the diagnosis of osteosarcoma cannot be made with certainty. The histologic appearance of osteoid is dense, pink, and amorphous. Classic or conventional osteosarcoma comprises 70% to 80% of cases of osteosarcoma.[79] A number of other types of osteosarcoma are distinguished from classic osteosarcoma based on clinical or pathologic features (Table 62-1).

Classic osteosarcoma has a highly malignant appearance, with prominent anaplasia and frequent mitoses. There is variability in the histologic appearance of conventional osteosarcoma. To facilitate pathologic diagnosis and best describe this variability in appearance, classic osteosarcoma is divided into three subtypes based on the predominant matrix produced by the malignant cells.[79] In the first, 50% of typical osteosarcoma is osteoblastic osteosarcoma, in which osteoid is the predominant matrix (Fig. 62-10). Next, 25% of typical osteosarcoma is chondroblastic osteosarcoma in which cartilaginous islands are the predominant matrix. This subtype is differentiated from chondrosarcoma by the presence of osteoid. Because osteoid can be sparse in chondroblastic osteosarcoma, distinguishing chondroblastic osteosarcoma from chondrosarcoma can require extensive biopsy material, which may necessitate rebiopsy. Sixty-one percent of chondrosarcomas have mutations in the isocitrate dehydrogenase 1 and 2 genes (*IDH1* and *IDH2*). Mutations in these genes have not been seen in osteosarcoma. Therefore, the identification of an *IDH1* or *IDH2* mutation in a malignant bone tumor with a chondroid matrix would strongly suggest a diagnosis of chondrosarcoma.[82,83] The remaining 25% of typical osteosarcoma is fibroblastic. In fibroblastic osteosarcoma, osteoid is minimal and spindle cells grow in a herringbone pattern.[3]

TABLE 62-1 Pathologic Features and Relative Frequency of Osteosarcoma Subtypes

Type	Pathology	Approximate % (of all Osteosarcoma)
Classic	Anaplastic, numerous mitoses	70-80
Osteoblastic	Predominant matrix osteoid	35
Chondroblastic	Predominant matrix chondroid	18
Fibroblastic	Spindle cells in herringbone pattern	18
Jaw	Similar to classic osteosarcoma	3-7
Secondary	Similar to classic osteosarcoma	7
Telangiectatic	Cysts and septa, scant osteoid, giant cells present	4-10
Small cell	Small round malignant cells, osteoid present but quantity variable	1-4
Low-grade intramedullary	Minimal atypia, few mitoses	1-2
Parosteal	Located on bone surface, low grade, matrix osteoid and, in 50%, cartilage	4
Periosteal	Located on bone surface, intermediate grade, predominant matrix cartilage	1-2

Many osteosarcomas have a mixture of different histologies, but in mixed histology cases one histology usually predominates. Of note, the subtypes of typical osteosarcoma were originally designated for the purpose of diagnosis, and their prognostic significance is uncertain. A better histologic response to chemotherapy in fibroblastic osteosarcoma and a poorer histologic response to chemotherapy in chondroblastic osteosarcoma have been consistent findings in several studies.[80,84,85] Telangiectatic osteosarcoma also has a better histologic response to chemotherapy (see later). Whether these differences in histologic response to therapy are correlated with differences in OS is not entirely clear. In the largest study to evaluate this question, there was a slight, statistically significant 5-year OS advantage in patients with fibroblastic osteosarcoma. Unlike other types of osteosarcoma, histologic response to chemotherapy may not be correlated with outcome in chondroblastic osteosarcoma.[85]

The remaining 20% to 30% of osteosarcomas that are not classic osteosarcomas are divided into secondary (occurring in Paget disease or after irradiation), jaw, telangiectatic, small cell, parosteal, periosteal, and low-grade intramedullary osteosarcoma. Telangiectatic osteosarcoma accounts for 4% to 11% of all osteosarcoma cases.[79,86-88] The age and gender distribution are the same as in conventional osteosarcoma. Pathologic fracture is more common, occurring in 15% to 25% of cases.[79,87,88] Radiographically, a lytic pattern is present because of the presence of large or small cystic areas and minimal osteoid formation. Microscopically, cysts are divided by septa that are lined with malignant cells producing scant osteoid. Cysts are also lined by benign, multinucleated giant cells. The presence of cysts and multinucleated giant cells can lead to misdiagnosis as an aneurysmal bone cyst or giant cell tumor, both benign bone tumors.[79] Although case series published prior to the modern era of multiagent chemotherapy had reported that telangiectatic osteosarcoma has an adverse prognostic implication, more recent studies have reported a better histologic response to therapy and improved survival as compared with typical osteosarcoma.[85-87]

Small cell osteosarcoma is a rare but histologically distinct variant representing 1% to 4% of osteosarcomas.[89,90] The clinical and epidemiologic characteristics are the same as conventional osteosarcoma. Microscopically, tumors are composed of small round cells that produce variable amounts of osteoid. Differentiation from Ewing sarcoma and lymphoma can be difficult.

Osteosarcomas of the jaw, located in the maxilla or mandible, deserve specific mention. Even when secondary osteosarcomas are excluded, the mean age of patients with osteosarcoma in this location is the mid-30s, which is older than in classic osteosarcoma.[79,91,92] These tumors are more often low grade; approximately 50% are low grade in most series.[92,93] Histologically, the appearance is similar to that of classic osteosarcoma but a larger proportion of tumors are chondroblastic. In concordance with the greater proportion of low-grade tumors, prognosis is better for osteosarcoma of the jaw. Although local recurrence does occur, especially when the initial resection is inadequate, metastasis is unusual.

Low-grade intramedullary osteosarcoma is relatively rare, making up 1% to 2% of all osteosarcomas. Patients developing this form of osteosarcoma tend to be older than those with conventional osteosarcoma, with 30 years being the mean age at diagnosis. Microscopic examination reveals spindle cells permeating bony trabeculae, with minimal atypia and few mitoses. A variable amount of osteoid is present. Recurrence and metastasis are rare. With adequate resection, chemotherapy is generally not indicated.[94,95]

Parosteal osteosarcoma is another low-grade osteosarcoma distinguished from low-grade intramedullary osteosarcoma by its location on the cortical surface of the bone. It is generally a sclerotic tumor attached to the bone by a broad base. Microscopically, the stroma is sparsely populated by minimally atypical spindle cells. The matrix contains osteoid and, in 50% of cases, cartilage.[96] A limited degree of medullary involvement is seen in 25% of cases. Areas of dedifferentiation can be present at diagnosis or at the time of recurrence and portend a poorer prognosis. Of patients with dedifferentiation, 31% will die of disease, usually with pulmonary metastasis.[97] Parosteal osteosarcoma makes up approximately 4% of all osteosarcomas. Like intramedullary low-grade osteosarcoma, parosteal osteosarcoma occurs in slightly older patients than conventional osteosarcoma and recurrence and metastasis are rare in adequately resected cases without dedifferentiation.[98]

Parosteal osteosarcoma must be distinguished from periosteal osteosarcoma. Periosteal osteosarcoma also arises from the surface of the bone but is an intermediate- or high-grade lesion. Periosteal osteosarcoma has an age distribution similar to that of typical osteosarcoma but almost all tumors arise in the proximal tibia. The appearance on plain radiography is highly characteristic and suggests the diagnosis. Histology is of a spindle cell neoplasm with osteoid and cartilaginous islands present, usually in large amounts. The prognosis is better than in conventional osteosarcoma but probably not as good as in parosteal osteosarcoma without dedifferentiation or intermedullary low-grade osteosarcoma.[99] There is controversy regarding the need for chemotherapy for periosteal osteosarcoma. The entity is too rare to permit conduct of prospective randomized controlled trials. Interpretation of retrospective series is difficult because variability in chemotherapy administration likely correlates with variability in clinicopathologic features related to outcome.[100,101]

Secondary osteosarcoma arises in previously irradiated bone or in patients with Paget disease. In both cases, patients are older than those with conventional osteosarcoma. Pathology is similar to conventional osteosarcoma. Prognosis is poorer in Paget disease than in conventional osteosarcoma.[84] There is some evidence that radiation-induced osteosarcomas have similar outcomes to de novo osteosarcoma if standard osteosarcoma chemotherapy is given.[102,103]

Staging

Staging of osteosarcoma follows the Enneking[104] or musculoskeletal society staging system (Table 62-2). The

TABLE 62-2 Enneking Staging System for Osteosarcoma: Distribution of Osteosarcoma and Indicated Treatment by Stage

Stage	Grade	Site	Metastasis	% of Patients[107]	Surgical Margin	Chemotherapy	Representative Subtype
IA	G1	T1	M0	5	Wide	No	Parosteal
IB	G1	T2	M0	3	Wide	No	
IIA	G2	T1	M0	5	Wide	Yes	Periosteal
IIB	G2	T2	M0	74	Wide	Yes	Classic
III	G1, G2	T1, T2	M1	13	Variable	Yes	Classic

Modified from Enneking WF, Spanier SS, Goodman MA: A system for the surgical staging of musculoskeletal sarcoma. Clin Orthop Relat Res *(153):106–120, 1980.*

G1, Low grade, characterized by few mitoses and a relatively well-differentiated appearance; *G2,* high grade characterized by higher mitotic rate and a less differentiated appearance; *M0,* no distant metastases present; *M1,* distant metastases present; *T1,* tumor is intracompartmental or confined to the anatomic compartment of origin; *T2,* tumor is extracompartmental or extends beyond the anatomic compartment of origin.

TNM system is not used for several reasons. The TNM system lacks biologic relevance in that osteosarcoma seldom metastasizes to lymph nodes. In addition, it is more complex than needed for predicting outcome, because patients in different TNM stages have overlapping prognoses.[104]

The Enneking staging system is based on tumor grade (G), site (T), and metastases (M). Low-grade (G1) tumors are well differentiated and have few mitoses, corresponding to Broders grades I and II.[95] Osteosarcoma is high grade (G2) if it is poorly differentiated and has numerous mitoses, corresponding to Broders grades III and IV. As noted, most osteosarcomas are high grade (G2). Surgical site (T) is determined by whether the tumor is contained within its anatomic compartment of origin (T1) or extends beyond its compartment of origin (T2). For example, a typical osteosarcoma that arises in the medullary space and extends through the cortex into the soft tissue is T2. A parosteal osteosarcoma that does not extend from the bone surface, its site of origin, into the medullary space and does not invade the surrounding soft tissue is T1. Most classic osteosarcomas are T2. Metastases are absent (M1) or present (M2). Stages IA and IB are low-grade (G1) tumors. Stages IIA and IIB are high-grade tumors. Stage IA tumors are low-grade osteosarcomas that are T1 or lack extension beyond the compartment of origin. Stage IB tumors are low-grade osteosarcomas that are T2 or demonstrate extension beyond the compartment of origin. Similarly, stage IIA tumors do not have extracompartmental extension, whereas IIB tumors do. Stage III osteosarcomas are tumors of any grade and any site that have distant metastases.[104-106] The vast majority of osteosarcomas are stage IIB. The next most common stage for osteosarcoma at presentation is stage III. Stages IA, IB, and IIA are uncommon, with 3% to 5% of osteosarcomas falling into each of these categories.[107]

NATURAL HISTORY AND PROGNOSIS

The most significant predictor of survival in osteosarcoma is the presence of residual disease caused by incomplete resection of primary tumor in localized osteosarcoma, local recurrence, or the presence of metastases. Consequently, the natural history, prognosis, and prognostic factors of localized, relapsed, and metastatic osteosarcoma differ significantly.

Localized Disease

Natural History

Prior to the use of chemotherapy for osteosarcoma, the OS of patients with localized disease who had a complete resection was 20%. Death was almost always caused by complications of pulmonary metastases.[79,108] With wide or radical surgical margins, local recurrence was rare. The development of pulmonary metastases in 80% of patients treated with only complete resection of the primary tumor has been interpreted as evidence of the presence of micrometastatic foci in the lungs at the time of diagnosis[109] and was one rationale for adjuvant chemotherapy.

Five-year event-free survival (EFS) and OS of localized osteosarcoma in the era of modern therapy are 65% to 75% and 70% to 80%, respectively.[110,111-113] Approximately 80% of relapses are pulmonary either alone or, in 25% of patients, with a second disease site, which is most often bone. Bone, distant from the initial resection, is the initial relapse site in 8% of patients; 1% to 2% of patients have recurrence isolated to unusual sites such as brain, kidneys, heart, or liver.[6,7,114] Local recurrence is seen in 2% to 8% of patients, despite having had adequate surgical margins.[53,115,116] Most relapses occur during the first 2 years after completion of chemotherapy. However, relapses occur as late as 10 years and the EFS decreases slowly between 2 and 10 years. In the few studies reporting long-term follow-up data, EFS decreases to 60% at 10 years from 65% at 5 years.[115,117]

Prognostic Factors

The most consistent predictor of outcome in localized, fully resected osteosarcoma is the extent of tumor necrosis after neoadjuvant chemotherapy.[52,53,118-121] When investigators at the Memorial Sloan-Kettering Cancer

TABLE 62-3 Grading of Histologic Response to Neoadjuvant Chemotherapy

Response	Grade	Histology
Poor	I	Necrosis minimal or absent
	II	Necrosis is <90% of the tumor but greater than minimal
Good	III	Scattered areas of viable tumor but >90% of tumor necrotic
	IV	No viable tumor

Modified from Rosen G, Caparros B, Huvos AG, et al: Preoperative chemotherapy for osteogenic sarcoma: selection of postoperative adjuvant chemotherapy based on the response of the primary tumor to preoperative chemotherapy. Cancer *49:1221–1230, 1982.*

Center first gave neoadjuvant, or preoperative, chemotherapy to facilitate surgical resection without amputation, they noted varying degrees of necrosis on pathologic examination of resected tumor specimens.[122] It was later observed that patients with significant necrosis had a better EFS and OS than those with lesser degrees of necrosis.[123] However, tumor response is not a true prognostic factor because it cannot be determined until after the initiation of chemotherapy.

The generally accepted grading system for tumor necrosis (Table 62-3) assigns higher grades to tumors with greater evidence of necrosis. In most studies, grades III and IV, in which more than 90% of the tumor is necrotic, define the group of patients whose tumors have a good response to chemotherapy. Patients whose tumors have a poor response have 10% or more viable tumor, corresponding to grades I and II. Patients with a poor response have disease-free survival (DFS) rates of 40% to 50%, whereas those with a good response have DFS rates of 70% to 80% (Fig. 62-11).[111,124-126] An improved outcome for poor responders is an objective of past and current trials (see "Chemotherapy for Localized Disease").

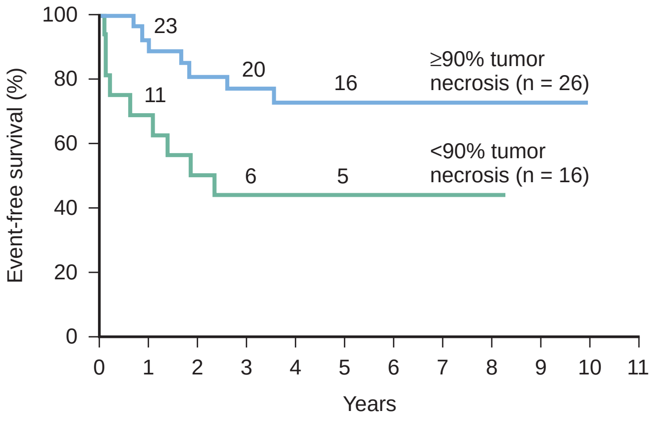


Figure 62-11 Representative event-free survival curve of patients with localized osteosarcoma greater than or equal to 90% *(blue line)* or less than 90% *(green line)* tumor necrosis after neoadjuvant chemotherapy. *(Modified from Goorin AM, Schwartzentruber DJ, Devidas M, et al: Presurgical chemotherapy compared with immediate surgery and adjuvant chemotherapy for nonmetastatic osteosarcoma: Pediatric Oncology Group Study POG-8651.* J Clin Oncol *21:1574–1580, 2003.)*

Radiographic parameters have been investigated for correlation with tumor necrosis and outcome. A decrease in FDG-PET standard uptake value (SUV) before and after neoadjuvant chemotherapy is correlated with histologic response. Neither the change in SUV nor absolute SUV after neoadjuvant chemotherapy has a statistically significant association with OS in localized osteosarcoma. The lack of statistical significance may be due to investigation in only a small population of patients,[127] and larger studies are needed to determine whether FDG-PET responses are predictive of outcome in osteosarcoma. Various MRI techniques including dynamic contrast-enhanced MRI[128] and diffusion-weighted MRI[129] show promise for potential to predict histologic response.

Additional prognostic factors have been identified in localized osteosarcoma. In many cases these factors are not consistently demonstrated to be statistically significant across studies. This may be due to a small effect size and, as a result, the need for large studies to identify a factor as being statistically related to outcome. In other cases, differences in measurement or categorization contribute to variable results across studies.

Tumor size has been identified as a significant prognostic factor by multivariate analysis in several studies.[52,53,113,120] However, other studies have not found this patient variable to be predictive of outcome.[118,130-132] One possible explanation for this conflict is variability in the definition of tumor size and measurement techniques. Some studies evaluate absolute tumor length, others use relative (to the entire bone) tumor length, and still others base analyses on tumor volume. Most studies showing size to be a significant predictor of outcome on multivariate analysis have measured tumor volume.[53,113,120] However, the volume that distinguishes small, low-risk tumors from large, high-risk tumors varies among studies. In summary, further studies using standard measurement techniques and risk criteria need to be conducted before tumor size can be used as a clinically significant predictor on which to counsel patients or base therapeutic decisions. The prognostic significance of tumor size might be caused by the influence of tumor size on resectability. In other words, larger tumors may confer a poorer prognosis because it is less likely that the tumor will be completely resected with wide margins.

Several studies have found an association between tumor site and outcome, with axial and proximal appendicular primary tumors having a worse prognosis than distal extremity tumors.[52,53,110,113,120,133-136] However, there are potential interactions between tumor site and other osteosarcoma prognostic factors. In particular, site and size are likely to be associated. A distal extremity osteosarcoma is likely to be noticed at a smaller size because of the relative lack of soft tissue. More importantly, the higher risk sites, such as axial skeleton, are also those for which a complete resection is less likely.

Elevated LDH and alkaline phosphatase levels at the time of diagnosis are associated with a poorer prognosis.[53,118,119] In one study,[118] the EFS of patients with a normal alkaline phosphatase level at diagnosis was 88%, whereas it was 46% for patients with an elevated alkaline phosphatase level. There are several issues that limit the usefulness of LDH and alkaline phosphatase as prognostic indicators. First, elevated levels are not consistently associated with a poorer prognosis in all studies. Second, elevations in LDH and alkaline phosphatase levels confer only a modestly increased relative risk (1.5 for LDH and 2 for alkaline phosphatase) of a poor outcome.[119]

Other prognostic factors that have been reported to be significant in a limited number of studies are age and gender, with older age and male gender being associated with a worse outcome.[52,53,137,138] Chemotherapy toxicity, represented by grade 3 or 4 mucositis, has been found to be correlated with improved OS.[138,139] A single study from the Children's Oncology Group (COG) identified a high body mass index at the time of diagnosis as a risk factor for inferior survival.[140]

As noted earlier, histologic subtype may be a prognostic factor. P-glycoprotein positivity, loss of heterozygosity at the *RB1* locus, and high ezrin expression have been identified as possible biology-based predictors of a poor outcome.[141,142] These prognostic factors are discussed more extensively (see "Biology").

In summary, the extent of tumor necrosis is the most reliable prognostic indicator in localized, completely resected osteosarcoma. Additional factors correlated with a poorer outcome include older age, less chemotherapy toxicity, larger tumor size, axial tumor site, elevated LDH and alkaline phosphatase levels, and high body mass index. However, for prognostic factors other than tumor necrosis to be utilized for risk stratification, further validation is required in large populations who received uniform therapy. Identification of reliable prognostic factors available at the time of diagnosis is a research priority in osteosarcoma. Biologic efforts such as TARGET (see "Genomics" under "Biology") and further investigation of imaging characteristics are most likely to provide robust predictors of outcome.

Metastatic and Relapsed Disease

The OS for patients with relapsed disease is poor, with most series showing a 25% OS at 5 years.[6,7,114] As with primary osteosarcoma, the most significant prognostic factor for postrelapse survival is the ability to achieve complete resection of all disease sites. In one report of 162 patients with initially localized osteosarcoma who developed recurrent disease, the 5-year projected OS was 39% for those who had complete resection of all disease and 0% for those who did not.[6] With isolated pulmonary metastases, prognosis is significantly influenced by the number of pulmonary nodules and whether there is unilateral or bilateral pulmonary disease.[6,7,114] In particular, having more than one pulmonary nodule at relapse conveys a worse prognosis. An additional prognostic factor is the duration of remission. Patients experiencing relapse less than 24 months after initial diagnosis have a worse outcome.[6,114] Finally, use of chemotherapy is associated with a slightly improved postrelapse prognosis,[7] particularly in patients with unresectable disease.[6] Patients who experience second and subsequent recurrences have a 5-year OS rate of about 15%. As with the initial recurrence, the duration of relapse-free interval, the number of lesions at recurrence, and the ability to resect recurrent disease are all associated with outcome.[143]

Local recurrence warrants special mention because of its dismal prognosis. The 5-year DFS is 0% to 15%.[115,116,144,145] Local recurrence is almost always associated with concurrent or delayed pulmonary metastasis.[115] In one series, the outcome of patients with local recurrence was strongly influenced by the presence or absence of systemic metastases at the time of local recurrence. The 5-year DFS was 25% for patients without systemic metastases at the time of local recurrence, and it was 0% for those with systemic metastases.

The outcome and prognostic factors for metastatic disease present at diagnosis are similar to those for relapsed disease. The 5-year OS of patients presenting with primary metastatic disease is 20% to 30%.[4,146] One exception to the poor prognosis for patients with metastatic disease is skip metastases. In patients with skip metastases, OS is 50%, even when pulmonary metastases are also present.[60] Higher OS has been reported in some chemotherapy trials, possibly because of therapy efficacy or shorter periods of follow-up.[147] An important predictor of a poor outcome is the inability to achieve complete surgical remission. Similarly, patients with metastases to more than one site or more than one organ are at increased risk for death from disease.[4,146] Lymph node metastasis at the time of diagnosis is uncommon, occurring in less than 5% of patients. Lymph node involvement at the time of diagnosis is associated with larger tumor size and an extraskeletal primary lesion. OS at 5 years for those with lymph node metastasis at diagnosis is 10%.[148] In one study, an alkaline phosphatase level higher than 500 IU/L was significantly associated with poor prognosis on multivariate analysis.[146]

BIOLOGY

Structural chromosomal alterations, loss of tumor suppressor function, and oncogene amplification have all been described in osteosarcoma. In addition, alterations in specific proteins contribute to chemotherapy resistance and metastatic behavior. A summary of genes altered in osteosarcoma is presented in Table 62-4.

Cell of Origin

Genetically engineered murine models of osteosarcoma have helped define the cell of origin for osteosarcoma. Lineage-specific models have demonstrated that osteosarcoma can arise from mesenchymal stem cell progenitors, osteoblast progenitors, and osteoblasts. In these lineage-specific genetically engineered mouse models, osteosarcoma develops with the highest penetrance and shortest latency when derived from osteoblast progenitors whereas mesenchymal stem cell progenitors give rise to a larger proportion of poorly differentiated soft tissue sarcomas.[149] A case report of osteosarcoma arising from donor cells after allogeneic stem cell transplantation suggests that mesenchymal stem cells can give rise to osteosarcoma in humans as well.[150]

TABLE 62-4 Genes Implicated in Osteosarcoma Pathogenesis and Biologic Progression

Gene	Alteration	Gene Function	% of Sporadic Osteosarcoma
RB1	Deletion, inactivation	Cell cycle control	70
TP53	Mutation, inactivation	Apoptosis Response to DNA damage Cell cycle control	50
MDM2	Gene amplification	TP53 regulation	10-15
COPS3	Gene amplification	TP53 regulation	25
CDKN2A	Deletion, promoter methylation	Cell cycle control through inhibition of CDK4-CCND1	15-20
MET	Increased expression	Motility Invasion	60
FOS	Increased expression	Proliferation Possibly, differentiation	60
MYC	Gene amplification	Proliferation	40
ABCB1	Increased expression	Efflux of chemotherapeutic agents	25-45
DHFR	Increased expression	Folate metabolism; MTX target	60% relapsed tumors
ERBB2	Increased expression	Growth factor receptor	40
Ezrin	Increased expression	Membrane cytoskeleton linker, facilitates metastasis	92
FAS	Decreased expression	Apoptosis	60% pulmonary metastases lack FAS
Reduced folate carrier	Decreased expression	MTX transport	65
IGF1R	Increased expression	Growth factor receptor, proliferation	Unknown

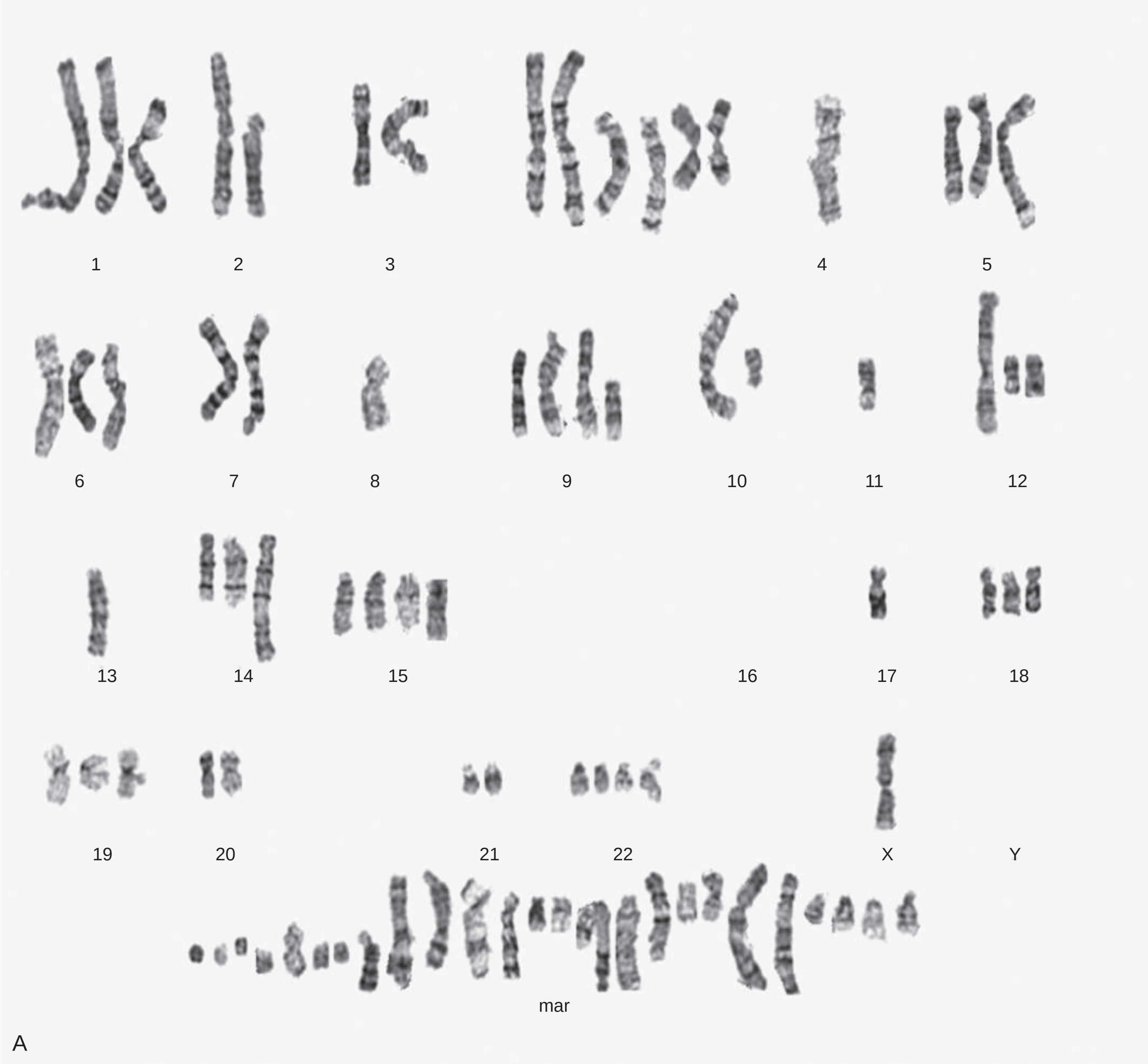
MTX, Methotrexate.

Genomics

Osteosarcomas contain complex genomic aberrations, suggesting the involvement of multiple genes in osteosarcoma development. Copy number abnormalities, changes in ploidy, and structural rearrangements are seen in all high-grade osteosarcomas. It is a tumor type in which chromothripsis has found to occur frequently.[151] Homogeneously staining regions and marker, double-minute, and ring chromosomes are seen in most tumors.[152,153] A representative karyotype and Circos plot are shown in Figure 62-12. Nonclonal changes, often superimposed on clonal aberrations, are present in many tumor specimens.[81] Several comprehensive next-generation sequencing projects are underway, including one being conducted as part of the National Cancer Institute sponsored Therapeutically Applicable Research to Generate Effective Treatments (TARGET) project. The nonclonal complex nature of the osteosarcoma genome makes the interpretation of genomic data difficult and the identification of candidate genes implicated in a significant proportion of osteosarcomas challenging.[154,155]

Osteosarcoma DNA ploidy ranges from diploid to hexaploid.[152] Spectral karyotyping (SKY) of osteosarcomas reveals an average of 39 chromosomal rearrangements/tumor.[156] Chromothripsis is the simultaneous occurrence of multiple somatic rearrangement events during one single cellular episode. In nine osteosarcoma samples, three (33%) had evidence of chromothripsis.[151] Chromoplexy is a related mechanism of chromosomal rearrangement in which series of interrelated rearrangements affect multiple chromosomes.[157] Preliminary results of an ongoing comprehensive next-generation sequencing study found evidence of chromoplexy in most osteosarcoma samples studied.[158] Pilot studies have demonstrated the feasibility of using detection of patient-specific translocations to track disease burden in patients with osteosarcoma and other solid tumors.[159] Whether this method for detection of minimal residual disease burden will have prognostic implications in osteosarcoma as it has in leukemia remains to be determined.

Multiple genomic techniques have been applied to the assessment of copy number alterations in osteosarcoma. Traditional and spectral karyotype permitted the initial identification of genomic complexity and copy number alterations in osteosarcoma. More sophisticated techniques such as array comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays have permitted the identification of recurrently amplified and deleted regions.[154] In almost all CGH analyses, gains are more common than losses.[160-164] Amplified regions consistently seen in a significant proportion of tumors and across studies include 1q21-q24, 6p11.2-p12, 8q21.3-q24, 17p11.2-p12 (high-level amplification), 1p, 1q21,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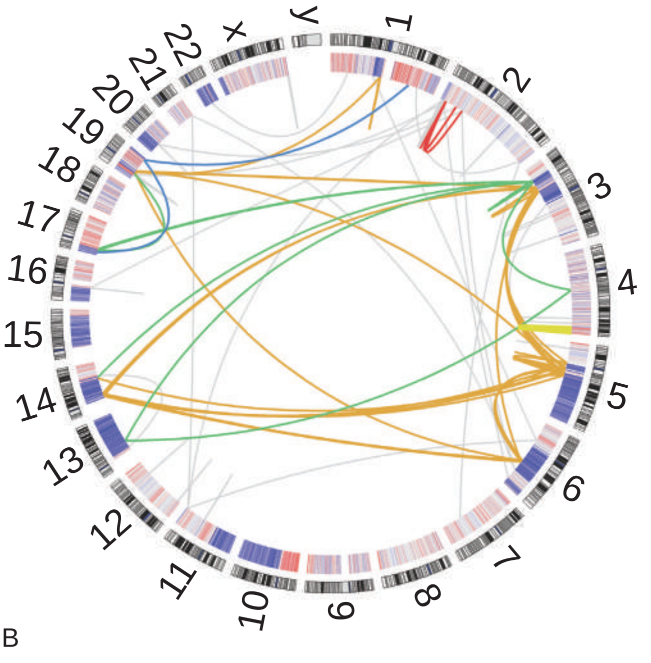

Figure 62-12 **A,** Representative osteosarcoma karyotype showing chromosome 16 loss, multiple marker chromosomes, monosomies, trisomies, and tetrasomies. **B,** Circos plot from whole-genome sequencing of an osteosarcoma sample.

5p14, and 6p12-21.3. Commonly seen deletions include 2q and 6q and chromosomes 9, 10, and 13q. Copy number alterations are also present in other regions but have been reported in a small proportion of cases or single studies only. Loss of 13q14[161] and gain of 8q (8q21.3-q22 or 8cen-q13)[160] have been associated with a worse prognosis, and 5q loss[161] has been associated with a better prognosis, although these findings have not been replicated. Higher-detail analysis with fluorescence in situ hybridization (FISH), quantitative polymerase chain reaction (PCR) assay, or array CGH has revealed that amplification and deletion patterns are complex, in that individual genes in an amplified region may not be amplified.[165]

Importantly, candidate genes have been identified via assessment of copy number in osteosarcoma. The region 12q13-14, which is amplified in 10%[160,162] of osteosarcomas, contains multiple potential oncogenes, including *MDM2*. In the commonly amplified region 17p11.2-p12, candidates *PMP22, TOP3A, MAPK7,* and *COPS3* display high levels of amplification in more than 50% of cases using semiquantitative PCR assay and microsatellite markers.[166,167] *RB1* is located at 13q14, a region that is lost in many osteosarcomas.[152,153] *MDM2* and *COPS3* amplifications and *RB1* loss are discussed further later. Three groups have identified a recurrent focal deletion at 3q13.3 including the candidate tumor suppressor gene *LSAMP.*[168-170] The *MYC* gene, located at 8q24.1, is amplified in as many as 40% of osteosarcomas. Amplification of 8q24 containing *MYC* and of 12q14 harboring CDK4 and a higher overall degree of loss of heterozygosity have been associated with a poorer outcome.[171] As with other prognostic factors in osteosarcoma, the prognostic significance of these copy number alterations needs to be validated in larger, prospectively conducted clinical studies.

Investigation into the relationship between aberrations in microRNAs and osteosarcoma pathogenesis and prognosis are in their infancy.[172-174] Definitive conclusions await research with large numbers of osteosarcoma samples and more robust statistical methodologies.

Tumor Suppressors

RB1

The retinoblastoma gene *RB1*, located at 13q14, was the first tumor suppressor gene identified and is one of the most frequently altered genes in osteosarcoma. As noted earlier (see "Etiology"), the second most common tumor in patients with hereditary retinoblastoma who carry germline *RB1* mutations[33] is osteosarcoma. *RB1* abnormalities are also present in most sporadic osteosarcomas. *RB1* inactivation is believed to contribute to osteosarcoma tumorigenesis through its role in cell cycle control and possibly through its regulation of differentiation and apoptosis.

Hereditary retinoblastoma is a condition in which individuals with a heterozygous germline *RB1* mutation develop retinoblastoma, a malignant tumor of the embryonal neural retina, with 90% penetrance.[175] Germline *RB1* mutations can be sporadic or inherited. *RB1* germline mutations in patients who develop bilateral tumors are usually small deletions or frameshift mutations resulting in a null protein,[176] whereas 40% of *RB1* germline mutations in patients with unilateral tumors are in-frame point mutations.[176] The cumulative incidence of a second malignancy by 50 years after the initial retinoblastoma diagnosis is 51%. Approximately 50% of the second malignancies developing in patients with hereditary retinoblastoma are osteosarcomas.[32,177] Both osteosarcomas and retinoblastomas occurring in patients with hereditary retinoblastoma have somatic loss of the normal *RB1* allele, with resulting complete absence of a functional RB1 protein.[33,178,179]

Somatic alterations in the *RB1* gene are seen in most sporadic osteosarcomas. Loss of heterozygosity at the *RB1* locus is seen in 65%[34,142] of sporadic osteosarcomas. Structural changes, usually associated with loss of heterogeneity, are present in 30% to 40%[34,180,181] of tumors, and homozygous deletions are seen in 23%[182] of tumors. Point mutations are rare and are present in only 6% of cases.[34] Overall, 70% of sporadic osteosarcomas have at least one *RB1* abnormality,[34] and many tumors have a combination of *RB1* alterations. *RB1* gene alterations are generally not seen in low-grade bone tumors.[34] There is some evidence that the initially mutated allele is usually of paternal origin and the allele that undergoes deletion is maternally derived.[183] Decreased RB1 expression is seen in about 50% of osteosarcomas,[184] but changes in RB1 expression are not correlated with *RB1* gene alterations.[34] For example, in some tumors with *RB1* loss of heterogeneity, RB1 protein levels are not reduced.

Some studies have found an association between *RB1* loss of heterogeneity and a poorer prognosis.[34,142] However, these studies were based on univariate analyses in relatively small populations and another study had contradictory results.[185] Theoretically, *RB1* inactivation could contribute to a poorer prognosis through increasing the expression of thymidylate synthase and dihydrofolate reductase, consequently increasing resistance to antimetabolites,[186] an important component of osteosarcoma therapy.

As reviewed by Classon and Harlow,[187] RB1 is a key regulator of the G1 to S cell cycle transition. RB1, by binding and thereby inhibiting the E2F family of transcription factors, blocks the transition from the G1 to the S phase of the cell cycle. RB1 is regulated by cyclin-dependent kinases (CDKs), particularly CDK4 and CDK6, which interact with CCND1. These CDK-CCND1 complexes phosphorylate RB1. RB1 phosphorylation inhibits the RB1-E2F interaction, allowing transcriptional activation by E2F, which leads to progression from G1 to S (Fig. 62-13). RB1 probably also controls cell cycle progression through chromatin remodeling, as reviewed by Khidr and Chen.[188] In particular, the RB1-E2F complex, by interacting with histone deacetylases, histone methyltransferases, and DNA methylases, contributes to the inactivation of E2F target genes during G0 and G1.

Genetically engineered *Rb1* null mouse models implicate Rb1 in regulation of cellular differentiation and

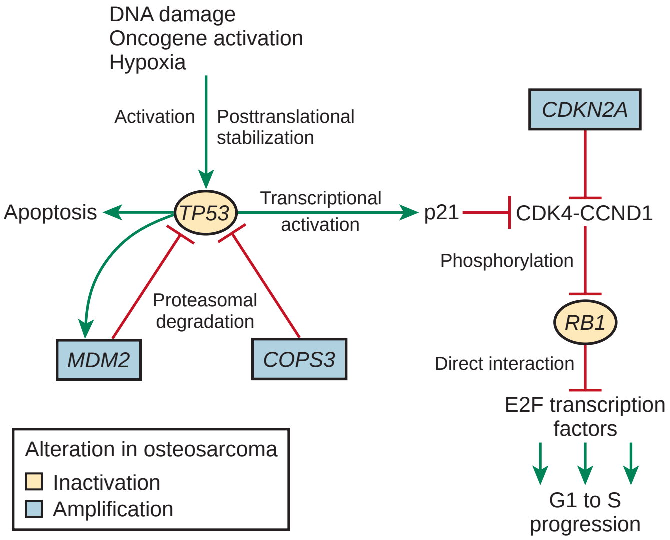

Figure 62-13 Selected regulators and effectors of *TP53* and *RB*. Alterations present in osteosarcoma are highlighted in *yellow* and *blue*.

apoptosis. *Rb1* deletion in mice results in embryonic lethality due to a placental defect.[189] When rescued from the placental defect, abnormal bone development is noted, and this appears to be due to increased proliferation in osteoblast cells combined with a failure in terminal osteoblastic differentiation.[190] Through interaction with CBFA1, a key transcriptional regulator in osteoblasts, RB1 induces osteogenic differentiation.[191] Loss of RB1 in osteoblast precursors appears to result in dedifferentiation, as demonstrated by an increased propensity for adipogenic differentiation.[192] The significance of RB1 differentiation and apoptosis roles in tumorigenesis in general and osteosarcomagenesis in particular is not clear.

TP53

There is convincing evidence that abnormal TP53 function is one of the central events in osteosarcomagenesis. Patients with Li-Fraumeni syndrome, who carry heterozygous germline *TP53* mutations, are predisposed to osteosarcoma. Somatic alterations in the *TP53* gene are found in a significant percentage of sporadic osteosarcomas. Furthermore, several different transgenic mouse models, with altered or absent TP53 function, develop osteosarcoma. Finally, components of the TP53 regulatory and effector pathways, *MDM2* (murine double-minute 2), *CDKN2A* (cyclin-dependent kinase inhibitor 2A), and *COPS3* (COP9 constitutive photomorphogenic homolog subunit 3) are also frequently perturbed in osteosarcoma, particularly in tumors lacking *TP53* mutations.

Of cancers arising in patients with Li-Fraumeni syndrome, 10% are osteosarcoma, making osteosarcoma the second most common tumor in Li-Fraumeni syndrome.[33] In addition to osteosarcoma, patients with Li-Fraumeni syndrome are predisposed to breast cancer, various soft tissue sarcomas, brain tumors, and various carcinomas. *TP53* germline mutations are present in 7% of osteosarcoma patients who have a personal history of multiple cancers but who do not meet Li-Fraumeni diagnostic criteria.[193] Individuals with Li-Fraumeni syndrome have germline mutations in *TP53* located throughout the gene, although approximately 75% of the mutations are in exons 5 to 8,[194] which encode the DNA-binding domain of the TP53 protein. Tumors arising in patients with Li-Fraumeni syndrome typically have loss of the normal *TP53* allele.[195]

Somatic alterations of the *TP53* gene are present in 20% to 50% of sporadic osteosarcomas.[37,196-204] Despite the prevalence of somatic *TP53* mutations in sporadic osteosarcomas, germline *TP53* mutations are rare in patients with sporadic osteosarcoma without a personal or family history of multiple cancers.[205,206] The wide range in the reported frequency of somatic *TP53* alterations in sporadic osteosarcoma is the result of small study size, differences in techniques of ascertainment, and varying definitions of an abnormal result. *TP53* point mutations are present in 20% to 30% of osteosarcomas.[37,197,200-202] As with *TP53* mutations in other cancer types, missense mutations constitute approximately 75% of osteosarcoma point mutations.[207] Point mutations are present throughout the *TP53* coding sequence, although, as with Li-Fraumeni syndrome germline mutations, approximately 73% occur in exons 5 to 8.[201] The initial *TP53* abnormality to be recognized in osteosarcoma was gross rearrangements of the *TP53* gene.[199] Gross rearrangements are present in 15% to 25% of cases.[197,198,200] Studies that evaluated *TP53* for both point mutations and gross gene rearrangements have found that almost 50% of osteosarcomas examined have aberrant *TP53*.[200,203] Because mutant TP53 protein usually accumulates in cells because of an increased half-life,[208] TP53 immunohistochemistry has been used to screen for *TP53* mutations in osteosarcoma. By immunohistochemistry, 15% to 25% of osteosarcomas have TP53 abnormalities,[196,204] reflecting the fact that immunohistochemistry has a relatively low sensitivity for detecting *TP53* mutations. Approximately 50% of osteosarcomas with *TP53* point mutations have loss of the normal allele.[201]

Several genetically engineered mouse models with dysfunctional or absent *Tp53* develop osteosarcoma. In a genetically engineered mouse model in which *Tp53* and *Rb1* deletion is restricted to osteoblasts, osteosarcoma develops with a high penetrance and short latency.[209,210] Mice homozygous for null *Tp53* develop a variety of tumors. Although lymphoma and hemangiosarcoma are the most common tumor types seen, 4% of the mice develop osteosarcoma.[211] Mice heterozygous for null *Tp53* develop tumors at 25% the rate and at an older age than homozygous null mice, but 25% of the tumors that develop are osteosarcomas.[212] Osteosarcomas are much more common in mice genetically engineered to be heterozygous for a common cancer-associated *Tp53* mutation, R172H. Almost 50% of these mice develop osteosarcoma, usually associated with hematogenous metastases.[213] Several other mouse models heterozygous for mutant *Tp53* develop osteosarcoma at a rate of 20% to 30%. Metastases are common in these models as well.[214,215] Transgenic mice expressing the SV40 T antigen from different promoters[216,217] develop osteosarcoma at a rate of approximately 70%. The mechanism of transformation in the SV40 T antigen–expressing models probably involves the inactivation of Tp53 and Rb1.

In other cancers, *TP53* mutation status has been associated with proliferation, response to chemotherapy, and outcome. In osteosarcoma, the response to chemotherapy is equivalent in *TP53* mutant and *TP53* wild-type osteosarcomas.[218] Several studies have examined the impact of *TP53* mutation on survival in osteosarcoma. Only one study, in 30 patients, showed a worse survival in patients whose tumors had *TP53* mutations.[203] The remainder of the studies did not show a relationship between *TP53* mutation status and outcome.[196,200,218,219] Finally, *TP53* mutations are not more common in metastatic disease than in localized disease.[201] Actually, the *TP53* mutation status of primary and metastatic tumors tends to be concordant.[201]

As reviewed by Levine[208] and Vogelstein and colleagues,[220] *TP53* mutations and structural alterations lead to transformation by interfering with TP53 tumor suppressor functions and, possibly, by producing a TP53 protein that has gained oncogenic function. TP53 tumor suppressor activities include cell cycle control and induction of apoptosis. Several forms of cellular stress, including DNA damage, hypoxia, shortened telomeres, and oncogene activation lead to TP53 posttranslational stabilization and TP53 activation. After activation, TP53 binds DNA and promotes the transcription of several genes, including *CDKN1A* and *MDM2*. As in other tumor types, the *TP53* mutations seen in sporadic osteosarcomas and Li-Fraumeni syndrome are concentrated in the DNA-binding domain and interfere with the transcriptional activity of the protein.[207] TP53 prevents progression from the G1 phase to the S phase of the cell cycle through the actions of one of its target genes, *CDKN1A*. The CDKN1A protein inhibits the CDK4-CCND1 complex, which in turn keeps RB1 in an unphosphorylated state. Unphosphorylated RB1 binds E2F transcription factors, preventing progression into the S phase (see Fig. 62-13).[220] TP53 also plays a regulatory role in the G2 to M cell cycle checkpoint. Another inhibitor of CDK4-CCND1 is CDKN2A. CDKN2A abnormalities are also seen in osteosarcoma (see later).

As with TP53-mediated cell cycle control, TP53-mediated apoptosis is triggered by DNA damage. In addition, oncogene overexpression and *RB1* inactivation stimulate TP53-mediated apoptosis. The pathways involved in TP53-mediated apoptosis are not fully understood. However, they likely involve proapoptotic proteins whose genes are targets of TP53 transcriptional activation, including *BAX, PMAIP1,* and *TP53AIP1*.[220]

TP53 inactivation allows cells to progress through the cell cycle and escape apoptosis, despite the presence of DNA damage. Consistent with this observation, the presence of a *TP53* mutation in osteosarcoma is significantly correlated with a greater level of genomic instability.[37] Thus promotion of genomic instability and the resultant accumulation of secondary mutations in oncogenes or tumor suppressors is yet another mechanism of transformation in *TP53* mutant tumors.

TP53 Regulators: *MDM2* and *COPS3*

The regulators of *TP53* activity, *MDM2* and *COPS3,* are amplified in a proportion of osteosarcomas. MDM2 inhibits TP53 activity by direct interaction with the TP53 DNA-binding domain and by transporting TP53 to the cytoplasm.[221-223] In addition, MDM2 ubiquitinates TP53, targeting it for proteasomal degradation. MDM2-mediated ubiquitination is enhanced by DNA damage.[224] The *MDM2* gene, located at 12q14, is amplified in 10% to 15% of osteosarcomas.[197,203,204,225-227] Usually, *MDM2* amplification occurs in tumors without *TP53* mutations, and thus *MDM2* amplification acts as an alternative mechanism of TP53 inactivation. There is limited evidence that *MDM2* amplification is more common in parosteal and other forms of low-grade osteosarcoma.[165,228,229]

COPS3, located at 17p11.2, a frequently amplified region in osteosarcoma, is a subunit of the COP9 signalosome. The COP9 signalosome interacts directly with TP53 and, through phosphorylation, targets TP53 for interaction with MDM2 and degradation by the 26S proteasome.[230] *COPS3* gene amplification is present in 25% to 30% of osteosarcomas.[231,232] Increased COPS3 expression through alternative mechanisms is present in some tumors lacking *COPS3* gene amplification.[231] Like *MDM2* amplification, COPS3 overexpression may be an alternative mechanism of *TP53* inactivation. *TP53* mutations are present in a small proportion of *COPS3*-amplified osteosarcomas.[231,232]

CDKN2A and *CDKN2D*

As described earlier, CDKN2A, like the TP53-inducible protein CDKN1A, is an inhibitor of the CDK4-CCND1 complex. As with CDKN1A, inhibition of CDK4-CCND1 by CDKN2A prevents progression from the G1 to the S phase. Ten to 20 percent of osteosarcomas have *CDKN2A* homozygous deletion,[197,202,203,233] and in another 5% to 10% the *CDKN2A* promoter is methylated. Similarly, by immunohistochemistry, 16% of osteosarcomas lack CDKN2A protein expression.[234] *CDKN2A* deletions appear to be more common in osteosarcomas grown in cell culture[235] but seem to be less common in osteosarcomas with *RB1* abnormalities, possibly because the two alterations have a similar effect on cell cycle control.[233] Few studies have evaluated the coexistence of *TP53* mutations and *CDKN2A* deletions in osteosarcomas. There is limited evidence that the two abnormalities occur simultaneously.[203]

CDKN2D is an alternatively spliced product of the gene encoding *CDKN2A*. CDKN2D inhibits MDM2 by direct binding.[236] Thus loss of CDKN2D leads to increased MDM2-mediated TP53 degradation. As a consequence of the gene structure, the 10% to 20% of osteosarcomas with *CDKN2A* homozygous deletion also lack functional CDKN2D.

RECQ DNA Helicases

As noted earlier, osteosarcoma incidence is increased in three autosomal recessive cancer predisposition syndromes caused by RECQ DNA helicase mutations, Rothmund-Thomson syndrome, Werner syndrome, and

Bloom syndrome. The overlapping but distinct clinical features and specific nature of osteosarcoma predisposition in these syndromes has already been discussed (see "Etiology"). Whereas germline *RECQL4* mutations predispose patients with these syndromes to osteosarcoma, somatic *RECQL4* mutations are not present in sporadic osteosarcomas.[237]

Patients with Rothmund-Thomson syndrome caused by mutations in *RECQL4*[238] develop osteosarcoma with a prevalence of approximately 30% and probably do not have an increased susceptibility to other types of cancer.[239] Interestingly, osteosarcoma occurs only in the 60% of patients with Rothmund-Thomson syndrome who have truncating or inactivating *RECQL4* mutations.[38] On the other hand, patients with Bloom and Werner syndromes, caused by mutations in *RECQL2* and *RECQL3,* respectively,[240,241] are prone to a wide variety of cancers, and osteosarcoma represents less than 10% of the cancers occurring in these patients.[40,41] Why *RECQL4* mutations, in contrast to *RECQL2* and *RECQL3* mutations, specifically increase the risk for osteosarcoma is unknown.

The mechanism whereby loss of function of RECQ DNA helicases results in tumor development generally and in osteosarcoma in particular are not well understood. Proposed RECQ functions that could be related to tumor formation include participation in DNA recombination, maintenance of chromosome stability, recovery from DNA replication collapse, initiation of DNA replication, and telomere processing. The three RECQ DNA helicases mutated in cancer predisposition syndromes have overlapping but not identical functions. In vitro cell culture and mouse models of *RECQL4* mutant Rothmund-Thomson syndrome display increased chromosomal instability with high rates of aneuploidy.[242] Bloom and Werner syndrome models have abnormally high rates of homologous recombination events, with accumulation of recombination intermediates.[243] Studies with the *Xenopus RECQL4* homologue have suggested that RECQL4 participates in the initiation of DNA replication.[244] Several lines of evidence have indicated that the RECQ helicases likely play an essential role in reestablishing DNA replication after disruption of the replication fork.[243] Finally, WRN may be involved in telomerase-independent telomere maintenance.[243,245]

WWOX

WWOX encodes a WW domain containing oxoreductase. This class of proteins has a wide variety of functions including regulation of protein degradation, transcription, and splicing. There is evidence from other cancers that *WWOX* can act as a tumor suppressor. Mice carrying a homozygous deletion of *Wwox*[246] develop osteosarcoma at a rate of 30% prior to premature death at 4 weeks postnatal age.[246] Thirty percent of human osteosarcoma samples have *WWOX* deletions, and 60% have reduced or absent WWOX expression. WWOX interacts directly with RUNX2, a regulator of osteoblast differentiation. In osteosarcoma cell lines deficient in WWOX expression, forced WWOX expression results in decreased RUNX2 expression, decreased proliferation, decreased invasion, and a reduction in xenograft viability.[247,248]

Developmental Pathways and Stem Cell Programs

Wnt Pathway

The Wnt pathway is active in bone development. Activating mutations in *LRP5* (low density lipoprotein receptor–related protein 5), which encodes an Wnt coreceptor (with frizzled4), cause high bone mass whereas inactivating *LRP5* mutations result in decreased bone mass. Despite multiple investigations into Wnt pathway activity in osteosarcoma, it remains unclear how and whether perturbations in Wnt pathway activity contribute to osteosarcomagenesis.[249] The majority studies conclude that overactive Wnt signaling contributes to osteosarcoma formation. For example, deletion of the Wnt inhibitory factor, *Wif1,* in mice increased osteosarcoma formation after irradiation.[250] However, there are conflicting reports such as a genome-wide expression study finding downregulation of the Wnt pathway in osteosarcoma.[251]

USP1

USP1 encodes a deubiquitinating enzyme that deubiquitinates inhibitor of DNA-binding/differentiation (ID) proteins, preventing their degradation. ID proteins or inhibitors of DNA binding are basic helix-loop-helix transcription factors that have been shown to inhibit differentiation and maintain a stemlike state. In a small group of osteosarcoma tumors, expression of USP1 and ID2 were correlated and 7 of 14 tumors had high USP1 and ID2 expression. In U2OS and 143B osteosarcoma cell lines USP1 knockdown decreased ID1, ID2, and ID3 protein levels; decreased expression of the stem cell markers e-cadherin and fibronectin; and increased expression of markers of mature osteoblasts. *USP1* knockdown decreased the number of cells in S phase of the cell cycle and decreased xenograft formation.[252] If the results of this single study are validated, USP1 inhibition is a potential therapeutic avenue for osteosarcoma. Development of USP1 inhibitors is of interest owing to potential for activity in a wide variety of cancers, including lung cancer.[253,254]

Oncogenes

Multiple cell surface receptor oncogenes are expressed and activated in osteosarcoma. It appears as though coexpression and coactivation of multiple cell surface receptors is a feature of osteosarcoma cell lines.[255,256] Whether this phenomenon is also present in primary osteosarcoma tumors is not yet known. *MET, FOS, ERBB2,* and *MYC* are oncogenes that have been extensively investigated in osteosarcoma.

MET

MET, a receptor tyrosine kinase, was first identified and noted to be oncogenic in a transformed human osteosarcoma cell line. In the 1970s, Rhim and colleagues[217] treated a human osteosarcoma cell line with *N*-methyl-*N′*-nitronitrosoguanidine (MNNG), a chemical carcinogen. MNNG treatment resulted in a morphologic change and the acquisition of in vivo tumor-forming capacity. Subsequent studies have shown that the transforming event is formation of a fusion oncogene, *TRP-MET,* which leads to high levels of MET expression.[257,258]

MET is highly expressed in human osteosarcomas and osteosarcoma cell lines. The exact proportion of osteosarcomas that have high MET expression varies, depending on the detection method used. By Western blotting, 60% to 88% of osteosarcoma tumor specimens and 100% of osteosarcoma cell lines display high levels of MET protein.[259,260] Immunohistochemical analysis yields more variable results, with 35% to 75% of osteosarcomas highly expressing MET.[261,262] The quantitative PCR assay also demonstrates elevated MET expression in all osteosarcoma tumor specimens and cell lines studied.[263] Of note, the benign bone tumors osteoblastoma and nonossifying fibroma do not express MET but chondroblastoma and giant cell tumors do express MET.[259,260,262] When primary and metastatic tumors from the same patient are compared, metastatic tumors have higher MET expression than the primary tumors, suggesting a role for MET in osteosarcoma metastasis.[259,261,264] The mechanism of elevated MET expression in osteosarcoma is not known. When evaluated, *MET* gene amplification does not appear to be present in osteosarcoma.[259]

Functional studies in cell lines have contributed to the understanding of MET-transforming roles in osteosarcoma. Expression of MET carrying an activating mutation or overexpression of MET by lentiviral transfection transforms normal osteoblasts.[263] Despite a high level of MET expression, MET is not activated in the absence of its ligand, hepatocyte growth factor (HGF).[265] An autocrine-paracrine loop may cause MET activation in human osteosarcomas in vivo.[260] Addition of HGF to MET-expressing osteosarcoma cell lines results in MET activation, activation of downstream signaling intermediates MAPK and AKT, and increased invasive growth.[265,266] MET-HGF interaction in other model systems induces proliferation and enhances motility, cellular changes that frequently precede metastasis.[267]

As reviewed by Trusolino and Comoglio,[267] the MET receptor, and HGF, turn on cellular programs that lead to invasive growth. Downstream mediators of MET activity include cadherins, integrins, and metalloproteinases. The ultimate outcome of MET activation includes loss of cell-cell adhesion, increased cell motility, and extracellular matrix degradation. Physiologic requirements for invasive growth include organogenesis, tissue regeneration, and wound healing. Inappropriate activation in cancer cells leads to tissue invasion and metastasis.

FOS

FOS was initially identified as osteosarcomagenic through investigation of the FBJ murine virus. Mice inoculated with FBJ murine virus develop osteosarcomas that are pathologically similar to human tumors.[268] The viral protein responsible for transformation after infection with FBJ murine virus has almost complete homology with the murine c-Fos protein expressed from the murine Fos gene.[269] Transfection of a functional, highly expressed *FOS* gene transforms fibroblasts.[270]

Further evidence that c-Fos overexpression can induce osteosarcomas has been provided by transgenic mouse models. Transgenic mice in which *Fos* is expressed from a major histocompatibility complex (MHC) class I promoter develop osteosarcoma with 100% penetrance.[271] In these mice, *Fos* is highly expressed in both osteoblasts and osteosarcomas. Transgenic mice in which the gene is expressed from a different universally expressed promoter, human metallothionein, also develop osteosarcomas but at a much lower frequency,[272] suggesting that there is a dose-response relationship between the extent of FOS overexpression and the development of osteosarcoma.

FOS is overexpressed in human osteosarcoma tumor specimens. By immunohistochemical analysis, 50% to 60% of human osteosarcomas express high levels of FOS protein.[273,274] Only 20% of benign bone tumors have immunohistochemical positivity for FOS, and the level of FOS immunohistochemical staining in benign tumors is not as high as in osteosarcomas.[234] When compared with normal tissues and nonosteosarcomatous lesions, FOS expression, as assessed by immunohistochemistry, is 150%.[274] FOS RNA levels are increased in 40% of osteosarcomas.[275] One study has found an association between FOS expression and the occurrence of metastasis or recurrence,[275] but another did not.[273]

Although it is known that FOS, a member of the AP1 transcription factor complex, is an important mediator between extracellular signaling and transcriptional activation in normal bone development, it is not known exactly how FOS overexpression contributes to osteosarcomagenesis. Induction of *Fos* expression in osteoblasts alters CCND1 levels and increases entry into the S phase.[276] Wagner[277] has noted that AP1 complex activity is regulated by vitamin D, transforming growth factor β, and parathyroid hormone, all of which are important factors influencing bone formation, growth, and healing. In particular, FOS appears to play an essential role in enchondral ossification. *Fos* null mice have significant osteopetrosis, suggesting that FOS activity is required for normal osteoclast function.[278,279]

Insulin-like Growth Factor

As noted earlier (see "Etiology"), there is epidemiologic evidence for a link between normal bone growth and osteosarcoma, including the coincidence of osteosarcoma peak incidence with maximal linear growth in adolescence and the higher rate of osteosarcoma in larger dog breeds.[280] The observed epidemiologic links between osteosarcoma and bone growth could have alternate explanations. Osteosarcoma in large-breed dogs could be caused by the underlying genetics of breed founders rather than by rates of bone growth. Nevertheless, as central mediators of normal linear bone growth and cellular proliferation in general, insulin-like growth factor 1 (IGF-1) and its receptor, insulin-like growth factor 1 receptor (IGF1R), have been investigated in osteosarcoma. In vitro and in vivo data have supported a possible role for IGF-1 and IGF1R in osteosarcoma proliferation and invasion.

IGF-1 and IGF1R are members of the family of insulin-like growth factors and insulin-like growth factor receptors. The other ligands in the IGF-1 family are IGF-2 and insulin. There are three tyrosine kinase receptors in the family, IGF1R, IGF2R, and the insulin receptor. IGF1R is the primary target of IGF-1 and the most thoroughly studied insulin-like growth factor receptor in osteosarcoma. IGF2R also appears to be highly expressed across a number of osteosarcoma cell lines.[256] Other key modulators of IGF1R activity are the insulin-like growth factor binding proteins (IGFBPs).[281] Knockout mice lacking IGF-1 or IGF1R are approximately half the size of normal mice and have delayed formation of their ossification centers.[282] Several humans with fetal growth restriction and postnatal short stature were found to have mutations in IGF1R.[283,284]

Competitive binding assays, affinity labeling experiments, and Northern and Western blot studies have demonstrated expression of IGF1R by osteosarcoma cell lines.[285,286] IGF-1 expression is variable across osteosarcoma cell lines.[287] Although less thoroughly studied than IGF-1, IGF-2 appears to be expressed by osteosarcoma cells.[286,288] There has been limited investigation into IGFBPs in osteosarcoma, but one study has shown that IGFBP3 transcription is augmented in MG-63 osteosarcoma cells in response to IGF-1.[289]

In osteosarcoma cell lines, IGF1R is not activated in the absence of its ligand.[290] Addition of IGF-1 to osteosarcoma cells in culture results in IGF-1R activation,[291] activation of IGF-1R downstream signaling, including AKT and ERK activation,[291] and increased proliferation[285,287,288] One study has shown that IGF-2 is also mitogenic for osteosarcoma cell lines and that this effect is mediated by binding to IGF2R.[288]

Multiple mechanisms of IGF1R inhibition in vitro and in vivo suppress osteosarcoma growth and invasion. In a number of different osteosarcoma cell lines, antibodies to IGF1R decrease osteosarcoma proliferation in a dose-dependent manner.[285,287,288,292] In some cases, antibody treatment causes 100% inhibition of IGF-1–induced proliferation. Forced expression of IGFBP5 or IGFBP3 by genetic or chemical mechanisms results in decreased IGF-1 expression, increased expression of differentiation markers, and decreased proliferation of osteosarcoma cells.[293,294] In vivo interruption of growth hormone by hypophysectomy or growth hormone–releasing hormone antagonism leads to a dramatic decrease in osteosarcoma xenograft tumor size and metastasis.[248,256,286,295]

IGF-1 and IGFBP3 levels in patients with osteosarcoma are different than established age- and gender-matched normal values, with 68% of patients having low IGF-1 levels. IGF-1 and IGFBP3 levels are not correlated with clinical variables such as metastasis and outcome.[296] Phase I studies of octreotide pamoate, a growth hormone antagonist, in patients with osteosarcoma have shown a 50% decrease in serum IGF-1 levels. However, there were no clinical responses to octreotide pamoate. There are several possible explanations that could reconcile these clinical findings, with evidence suggesting an important role for the IGF axis in osteosarcoma. The behavior of IGF-1 and IGF1R in osteosarcoma models may not accurately represent osteosarcoma in patients. More likely, IGF-1 may act in a paracrine rather than endocrine manner, so that IGF-1 serum levels are not representative of the tumor milieu. Alternatively, IGF1R activation in osteosarcoma in vivo may be mediated by IGF-2 or the extent of IGF1R activation may not be determined by ligand concentration. Finally, osteosarcoma biology is likely to be heterogeneous, with the IGF axis being important in some tumors but not in others. Early phase IGF1R antibody trials included an insufficient number of osteosarcoma patients to determine whether these drugs are effective in osteosarcoma.[297] It is not clear whether IGF1R antibodies will continue to be developed given lack of activity of these drugs in carcinomas. IGF1R kinase inhibitors continue to be developed.

ERBB2

The *ERBB2* protooncogene encodes the human epidermal growth factor receptor 2, a receptor tyrosine kinase in the epidermal growth factor receptor family. *ERBB2* is amplified in 20% of breast cancers,[298] and ERBB2 protein is expressed in a similar proportion of breast cancers.[299] In breast cancer, *ERBB2* amplification or ERBB2 expression is associated with a poorer prognosis. Trastuzumab, an anti-HER2 therapeutic antibody, improves survival in patients with *ERBB2*-amplified tumors.[300]

ERBB2 protein levels are increased in 30% to 40% of osteosarcomas.[196,301-303] Initial studies raised the possibility of an association between ERBB2 expression and a worse prognosis, but this finding was not confirmed in later studies.[304] Some investigators have found evidence of *ERBB2* gene amplification in osteosarcoma,[302] but others have not.[301,305,306] As discussed later (see "Treatment"), these findings led to a COG study, AOST 0121, that evaluated the feasibility of adding trastuzumab to multiagent chemotherapy. Eligible patients were those with high-risk metastatic osteosarcoma whose tumors were ERBB2 positive. Outcome in ERBB2-positive patients was similar to that of historical controls, suggesting limited activity of trastuzumab in ERBB2-positive osteosarcoma.[303]

MYC

The *MYC* gene, located at 8q24.1 is amplified in 7% to 10% of osteosarcomas when assessed by Southern blotting.[275,307] The more sensitive technique, array CGH, detects *MYC* amplification in 44% of osteosarcomas.[164,275,307,308] MYC mRNA and protein levels are increased in a similar proportion of osteosarcomas, and MYC expression might be correlated with metastasis. MYC, when complexed with MAX, activates the transcription of a number of genes, perhaps as many as 15% of all genes. Downstream effects of MYC transcriptional activation include proliferation, cell growth, inhibition of differentiation, and apoptosis. Exactly how MYC regulates these diverse cell processes and how *MYC* amplification leads to cancer is an area of active investigation.[309,310] One percent of mice with conditional *Myc* overexpression from the EμSRa promoter develop osteosarcoma.[311] With the recent development of BET bromodomain inhibitors, MYC has become a potential therapeutic target.[312]

Determinants of Metastasis

One of the defining features of osteosarcoma is its tendency to metastasize early, hence the need for systemic chemotherapy in all patients. Metastasis is a complicated process that involves motility, migration, degradation of extracellular matrix, extravasation, survival during transit through vasculature, invasion, and growth. Given the complicated nature of the process, many factors are likely to play a role in metastases, but it is difficult to decipher the specific contribution of each. Several of the factors that have been more extensively studied in the context of osteosarcoma will be discussed.

Chemokine ligand 12 (CXCL12) is a cytokine-like protein expressed on the surface of vascular endothelial cells. Through binding to its chemokine receptor, CXCR4, it plays a role in cytoskeleton rearrangement, adhesion to endothelial cells, and chemotaxis.[313,314] Involvement of the CXCR4/CXCL12 pathway has been implicated in the metastatic potential of several cancers, including rhabdomyosarcoma and lymphoma.[315-317] CXCR4 mRNA has been shown to be expressed in osteosarcoma and associated with the presence of metastases at the time of diagnosis.[318-320] In vitro assays have shown that migration of osteosarcoma cells expressing CXCR4 follows an CXCL12 gradient and that their adhesion to endothelial and bone marrow stromal cells is promoted by CXCL12 treatment.[320] This study also provided a rationale for the propensity of osteosarcoma to metastasize to the lung, where CXCL12 concentration is high, and demonstrated prevention of pulmonary metastasis in a murine model by the administration of a CXCR4 inhibitor, suggesting molecular strategies inhibiting this axis as a therapeutic target.[320]

Ezrin is a membrane-cytoskeleton linker protein that allows direct cellular interactions with the microenvironment, facilitating signal transduction through growth factor receptors and adhesion molecules, thereby regulating cell migration and metastasis, among other processes.[141] In an orthotopic model of murine osteosarcoma, ezrin expression was threefold higher in the more aggressive K7M2 cell line, which correlated with its metastatic potential when compared with the less aggressive K12 cell line.[321,322] In follow-up experiments, ezrin expression was found to provide an early survival advantage for pulmonary metastatic osteosarcoma, which is in part mediated by AKT.[141] Ezrin exists in phosphorylated (active) and unphosphorylated (inactive) states.[323] Establishment of osteosarcoma metastases in a mouse model is dependent on fluctuation between the active and inactive forms of ezrin.[324] Ezrin phosphorylation at threonine 567 is mediated in part by protein kinase C in osteosarcoma cell lines. The identification of protein kinase C as a mediator of ezrin phosphorylation and the discovery of small molecules that inhibit ezrin phosphorylation make ezrin a potential therapeutic target for osteosarcoma.[325] A significant correlation between high ezrin expression and poor outcome in osteosarcoma has been shown, with shorter DFS and higher risk for metastatic relapse, supporting the animal model data.[141]

Overexpression of MET and its ligand hepatocyte growth factor, in osteosarcoma cell lines suggests a role for MET in its metastatic phenotype. Binding of the ligand to the MET ligand receptor stimulates both cell proliferation and motility, features associated with metastasis.[259,326] In a study of 17 osteosarcoma tumor samples, 60% expressed the MET ligand receptor at high levels.[259] When primary and metastatic tumors from the same patient are compared, metastatic tumors have higher MET expression than the primary tumors, indicating its role in potentiating the metastatic phenotype of osteosarcoma.[259-261]

FAS is a transmembrane receptor that activates the extrinsic cell death pathway on stimulation by its ligand, FASL. There is increasing evidence that a variety of tumor types that escape from apoptosis may enhance the ability of tumor cells to metastasize.[327] Studies in an osteosarcoma murine xenograft model with a high rate of pulmonary metastasis have suggested that decreased FAS expression enhances osteosarcoma metastasis. Therapies enhancing FAS expression inhibit the development of metastases in this model system. However, evidence that perturbations in FAS are a generalized mechanism of enhanced metastatic potential in osteosarcoma is limited.

The SAOS2 LM6 cell line was produced by serially passaging a human osteosarcoma cell line, SAOS2, through immunodeficient mice until pulmonary metastasis developed reliably and rapidly after tail vein injections. FAS protein and mRNA levels are lower in the SAOS2 LM6 metastasis–producing cell line compared with the seldom-metastasizing parental cell line SAOS2.[328] Furthermore, when SAOS2 LM6 FAS expression is increased through transfection, the number and size of pulmonary metastases developing after tail vein injection decrease.[329] FAS expression can also be induced by interleukin-12 (IL-12).[328] Intrapulmonary aerosolized IL-12 gene therapy after SAOS2 LM6 tail vein injection increases FAS expression in metastatic tumors and inhibits the development of pulmonary metastases.[330-333] Interestingly, ifosfamide and IL-12 display a synergistic relationship when used together in this model system, possibly because of ifosfamide-induced expression of FASL.[330]

FAS expression has been evaluated in human osteosarcoma pulmonary metastases from 28 patients. FAS was not expressed in 60% of the tumors, and the remaining 40% had only weak FAS expression.[334] Further study is needed to confirm that decreased FAS expression contributes to the metastatic potential of osteosarcoma occurring in humans and in osteosarcoma models other than the SAOS2 LM6 model.

Other factors that likely play a role in osteosarcoma metastases include expression of matrix metalloproteinases[335] and other degradative enzymes. The expression of these enzymes is necessary to degrade the extracellular matrix, permitting extravasation into the vasculature.

Biologic Markers of Response to Chemotherapy

The response of osteosarcoma to chemotherapy is evident in the change in patient survival resulting from its administration. Defining "response" is somewhat more difficult. Chemotherapy response in osteosarcoma is typically used to refer to the degree of necrosis in the definitive surgical resection specimen. The degree of necrosis seen in that specimen is a consistent predictor of patient survival across studies, as described elsewhere in this chapter. In the original description of the grading system, a grade I response was considered indicative of no histologically visible response to chemotherapy. Subsequently, this was specified as being less than 50% necrosis, because untreated osteosarcomas could have as much as 25% spontaneous necrosis. It was therefore believed that necrosis more than double this amount would be attributable to a chemotherapy effect. Numerous biologic studies have been performed correlating a particular marker with the necrosis grading. In some of these studies, the necrosis grading was used as a surrogate for EFS, with the anticipation that these will correlate. The advantage of necrosis grading in this context is that the data are available much sooner than mature EFS data. In other contexts, the necrosis grading is used as a marker of chemotherapy response. Complicating these analyses, the standard of care in osteosarcoma is multiagent chemotherapy. Factors that only affect responsiveness to a single agent may not be predictive in the context of multiagent chemotherapy. Given these limitations, relating a genetic alteration to necrosis grading may provide a more robust relationship with chemotherapy response than survival, because survival may be influenced by many factors other than chemotherapy response. Similarly, necrosis grading may be a better marker of response than radiographic response in osteosarcoma, because the mineralized matrix of an osteosarcoma prevents radiographically visible shrinkage of the tumor.

Because chemotherapy has had a dramatic effect on the outcome of osteosarcoma, it is perhaps intuitive to assume that genetic alterations that produce drug resistance would be associated with inferior chemotherapy response and patient survival. Along these lines, perhaps the most extensively studied prognostic marker is the expression of P-glycoprotein (PGP) or multidrug resistance protein 1 (MDR1). This is a transmembrane adenosine triphosphate–dependent efflux pump protein encoded by the multidrug resistance gene (*ABCB1,* formerly *MDR1*), which is responsible for the efflux of numerous chemotherapeutic agents from the malignant cell. In the context of osteosarcoma, the most important drug that can be effluxed by MDR1 is doxorubicin, although etoposide is also a substrate. Immunohistochemistry and reverse-transcriptase (RT)-PCR quantification studies have explored MDR1 and *ABCB1* expression in osteosarcoma and demonstrated overexpression of MDR1 in 23% to 45% of tumors, with decreased survival reported for patients with MDR1-positive tumors[336-341] in initial studies. Of interest, although many of these studies demonstrated a correlation with outcome, several key studies did not demonstrate a relationship with the necrosis grade. This suggests that use of necrosis grading as an early marker of outcome may not always be appropriate in the context of some biologic markers. A meta-analysis has proposed that MDR1 is associated with an increased risk for disease progression.[342] Although the early literature suggested that MDR1 may be a marker of drug resistance and aggressiveness in osteosarcoma, this finding has not been verified consistently across studies.[336-339,341] In addition, a prospective clinicopathologic cooperative group study has concluded that there is no correlation between MDR1 expression and percentage of osteosarcoma tumor necrosis after induction chemotherapy or EFS in localized osteosarcoma.[343] Overexpression of *ABCB1* has also been explored with regard to its prognostic relevance. Although a small pilot study has shown a trend toward a worse outcome in patients exhibiting high levels of *ABCB1* expression,[344] a larger prospective investigation did not identify a correlation between ABCB1 messenger RNA expression and prognosis in osteosarcoma patients, with patients with either very low or very high levels of ABCB1 having a relatively poor outcome.[345]

High-dose methotrexate (HDMTX) with leucovorin rescue is a major component of current protocols for the treatment of osteosarcoma.[110,346] HDMTX is vastly more effective than conventional dose methotrexate in the treatment of osteosarcoma, a finding that is not observed in other malignancies treated with MTX, implying a mechanism of intrinsic MTX resistance within osteosarcoma tumor cells.[347] In experimental systems, resistance to methotrexate can occur through a variety of mechanisms, including impaired intracellular transport of the drug via the reduced folate carrier, upregulation of dihydrofolate reductase, and diminished intracellular retention caused by decreased polyglutamylation. Studies have demonstrated that impairment of drug influx as a result of decreased expression and mutations in the reduced folate carrier gene is the major basis of intrinsic resistance; 65% of osteosarcoma tumor samples were found to have decreased reduced folate carrier expression at the time of initial biopsy.[347] In contrast, dihydrofolate reductase overexpression was seen relatively infrequently at initial biopsy, in only 10% of tumor samples, compared with 62% of the tumors examined at the time of definitive surgery or relapse, suggesting dihydrofolate reductase overexpression as the major mechanism of acquired MTX resistance in osteosarcoma.[347]

In vitro induction of HMGB1 expression in osteosarcoma cells prevented apoptosis. HMGB1 is a chromatin-binding nuclear protein that may be involved in chemotherapy resistance in other cancers.[348] Evaluation of HMBG1 in human osteosarcoma samples has not been performed. Other potential mechanisms of drug resistance include alterations in multidrug resistance protein expression, topoisomerase II, glutathione *S*-transferases, DNA repair, DNA damage response, drug metabolism or inactivation, and reduced intracellular delivery. Few studies have been performed in osteosarcoma to indicate whether these processes play a role in defining chemotherapy response.

In other studies, necrosis after chemotherapy has been used as a marker of response, and investigators have attempted to identify genetic signatures predictive of response. In each of these studies, a large number of genes were required to define a profile of response. The studies reported using 60 genes,[349] 104 genes,[350] and 45 genes[351] to distinguish between responders and nonresponders. This may be related to the small sample sizes used in each of these studies. Alternatively, the lack of robust clustering may be caused by the heterogeneity of osteosarcoma itself. Lack of robust clustering on gene expression profiling appears to be a property of non–translocation–associated sarcomas, a result of their high levels of genetic complexity.

Translational Science

Bone metabolic pathways are of interest in osteosarcoma given that this is a tumor arising from osteoblasts and osteoblast precursors. Zoledronate is a nitrogen-containing bisphosphonate and the most potent of its class. It inhibits the development of osteolytic and osteoblastic bone lesions.[342,352] Preclinical models have shown cytotoxicity against osteosarcoma cell lines.[353,354] In vivo models in mice inoculated intravenously with the spontaneous murine osteosarcoma cell line POS-1 have shown prolonged survival when treated with single-agent zoledronate.[355] Moreover, combination with ifosfamide has shown more activity than either agent alone in preventing tumor recurrence, improving tissue repair, and increasing bone formation.[356] Bisphosphonates may also improve bone integrity after limb salvage surgery.[357,358]

Receptor activator of nuclear factor κB ligand (RANKL, also known as tumor necrosis factor ligand superfamily member 11 [TNFSF11]) and its receptor RANK have the physiologic function of regulating bone turnover. In response to RANKL-RANK binding, osteoclast precursors differentiate and become activated resulting in bone resorption.[359] An antibody against RANKL, denosumab, has been approved by the U.S. Food and Drug Administration (FDA) for treatment of osteoporosis. RANK is expressed in 57% of human osteosarcomas, most human osteosarcoma cell lines, and 70% of canine osteosarcomas, including two of two canine osteosarcoma skin metastases.[360,361] RANKL is expressed in 75% of human osteosarcoma tumor specimens, and the strength of expression is associated with prognosis.[362] In osteosarcoma cell lines, RANKL activates downstream signaling and modulates gene expression.[361,363] Further evidence supporting activity of the RANK-RANKL pathway in osteosarcoma is provided by a transgenic mouse model of osteosarcoma. In an osteosarcoma mouse model created by expression of the SV40 T/t antigen from the osteocalcin promoter, heterozygous deletion of *Prkar1a* accelerates development of osteosarcoma via increased *Rankl* gene expression. A subset of human osteosarcomas have low *PRKAR1A* expression and high *RANKL* expression.[365] In vivo studies of denosumab are limited by the fact that the antibody does not recognize murine or canine Rankl. To circumvent these limitations, in vivo studies have utilized other approaches to inhibit Rankl activity, including Rank gene therapy, which creates a decoy receptor for Rankl, RANK-Fc, a chimeric protein that efficiently binds Rankl, or SiRNA directed against Rankl. In a murine orthotopic OS model, Rank gene therapy decreased primary and metastatic tumor burden: mice receiving this therapy did not develop osteosarcomatous lung metastases whereas 80% of control mice developed lung metastases.[365] A study by the same group using RANK-Fc confirmed these original findings.[366]

The phosphatidylinositol 3’ kinase/mammalian target of rapamycin (PI3K/mTOR) pathway is a central signaling pathway contributing to proliferation and survival in many cancers. The pathway has been recognized to contribute to proliferation, drug resistance, and metastasis in osteosarcoma. Expression of mTOR and p70S6K, an indicator of mTOR activation, is present in 70% to 80% of osteosarcomas, and the level of expression has been correlated with overall and disease free survival.[367] Activation of PI3K-AKT signaling is downstream of several biological processes involved in osteosarcoma metastasis such as Ezrin expression.[368] Micro-RNA-221, expressed at high levels in osteosarcoma targets PTEN, increases cell survival, decreases apoptosis, and induces cisplatin resistance.[369] The dual PI3K/mTOR inhibitor BEZ235 and the alpha selective PI3K inhibitor BYL719 induce cell cycle arrest and decrease tumor formation in vivo.[370,371] The clinical trial experience with mTOR inhibition in osteosarcoma is limited, and PI3K inhibitors have not been evaluated in this disease. There have been rare but dramatic responses to mTOR inhibition in patients with advanced, refractory osteosarcoma.[372] In a study using a sequenom assay to identify cancer-associated mutations in osteosarcoma, 3 of 89 tumors were found to have mutations in *PIK3CA*, the PI3K catalytic subunit.[373] A comprehensive next-generation sequencing study of 59 osteosarcoma samples thus far presented only in abstract form, identified PI3K/mTOR pathway alterations in 25% of osteosarcomas.[158]

GD2 is a cell surface sialic acid containing glycosphingolipid expressed normally in the central nervous system, peripheral nerves, and melanocytes. In neuroblastoma, anti-GD2 antibody given with cytokines to promote antibody-dependent cell-mediated cytotoxicity significantly improves EFS and OS.[374] More than 90% of osteosarcomas express GD2, and the intensity of expression, in most samples, is greater than that seen in neuroblastoma.[375,376] In the phase I/Ib trial of the murine anti-GD2 antibody, 14.2G2a, plus interleukin-2, one of two osteosarcoma patients treated had a complete response persisting for 8 months.[377]

There is mixed evidence regarding the role for immunotherapy in osteosarcoma. Completed clinical trials utilizing general immune stimulation with interferon α (IFN-α)[378] and inhaled granulocyte-macrophage colony-stimulating factor (GM-CSF)[379] did not demonstrate significant activity of either approach. Dendritic cell therapy has shown anti-osteosarcoma activity in one mouse model, particularly when pulsed in vitro with irradiated tumor and administered in a minimal residual disease setting.[380] However, in a clinical study of dendritic cell vaccination there was a lack of T-cell response and no evidence of clinical benefit.[381] Immune surveillance by T cells may delay or prevent the development of metastasis, as seen in a mouse model.[382] In a single study in a murine xenograft model, chimeric antigen receptor T cells expressing interlukin-11Rα treatment resulted in regression of pulmonary metastases.[383]

TREATMENT

Local Control

Definitive surgical treatment of osteosarcoma (discussed more extensively in Chapter 49 "Pediatric Surgical Oncology") has two components: tumor resection and reconstruction. Tumor resection is essential for cure of osteosarcoma. Ten-year survival in patients with macroscopic residual tumor is less than 15%, even with multiagent chemotherapy.[52,384] Tissue margins on the resection specimen should be wide. Although the exact tissue margin required is not known, wide margins are generally defined as 2 to 5 mm for soft tissue and 2 to 3 cm for bone marrow. Marginal and intralesional margins are associated with a poor outcome and an increased risk for local recurrence.[53,385] As noted earlier (see "Natural History and Prognosis"), an additional factor increasing the risk for local recurrence and poor outcome is a poor histologic response to chemotherapy. In one study, inadequate margins and a poor histologic response to therapy were independent predictors of local recurrence.[386] In this study, having both a poor histologic response to therapy and inadequate margins dramatically increased the risk for local recurrence.[386]

Options for limb reconstruction include limb-sparing techniques that use allografts or fixed or expandable endoprostheses. Currently, 75% to 80% of patients with nonaxial tumors are treated with limb-sparing surgery.[110,115] As long as the resection margins are adequate, limb-sparing techniques do not change EFS or OS.[119,132] Because of the significant cosmetic impact, amputation and rotationplasty are often reserved for those patients whose tumors are not amenable to limb-sparing techniques. Occasionally, however, patients will opt for amputation or rotationplasty because of their advantages, which include a lower incidence of repeated surgical revisions and a greater ability to participate in high-impact activities.[387] Benefits of endoprostheses over allografts include a shorter postoperative recovery time and a lower incidence of perioperative complications. Allografts have the important advantage of allowing preservation of the growth plate. The relative strengths and weaknesses of the various surgical options are discussed further later (see "Complications of Therapy") and in Chapter 49, "Pediatric Surgical Oncology."

The optimal surgical approach should be determined by a multidisciplinary team that includes a radiologist, oncologist, and orthopedic surgeon with experience in the treatment of bone tumors. Adequate imaging, usually MRI, is required to determine which surgical procedures will produce sufficient margins. Finally, a good understanding of the patient's and family's priorities is needed to provide appropriate counseling.

Chemotherapy for Localized Disease

The use of neoadjuvant and adjuvant chemotherapy has increased the OS of patients with localized high-grade osteosarcoma from 20% to 70% to 75%. The most active chemotherapeutic agents for localized osteosarcoma are HDMTX with leucovorin rescue, doxorubicin (Doxo), and cisplatin (CP). In addition, ifosfamide and etoposide have demonstrated efficacy in metastatic osteosarcoma. Institutional and cooperative group studies have attempted to identify the optimal combination of active agents, the most effective drug dosing, and the impact of dose intensity and schedule on outcome and toxicity (Tables 62-5 and 62-6). Much has been learned from these studies. However, important questions remain, including the ability to improve high-risk disease outcomes through chemotherapy intensification, the role of immune modulators in osteosarcoma therapy, and whether there is additional benefit of adding ifosfamide and etoposide to first-line therapy for localized disease.

Chemotherapy and Outcome

It has not always been clear that chemotherapy is effective in osteosarcoma. Successful treatment of osteosarcoma with chemotherapy was first reported in the literature in the early 1970s. Jaffe and colleagues[109] treated 20 patients with adjuvant HDMTX and vincristine (VCR). They noted 1 death in 20 patients compared with 14 expected deaths, based on historical controls. In 12 patients with completely resected primary tumors, there was 1 death, which was 6 fewer than predicted by historic controls.[109] Cortes and colleagues[388] saw similar dramatic improvements in OS and EFS in 21 patients treated with surgery and Doxo. The Memorial Sloan-Kettering Cancer Center (MSKCC) has reported an OS of 75% with neoadjuvant HDMTX containing multiagent chemotherapy.[123] Other groups reported improved survival compared with historical controls with adjuvant CP and Doxo[389] or adjuvant multiagent chemotherapy containing Doxo.[390] However, in other studies, chemotherapy did not appear to improve outcome. Lange and colleagues[391] treated 20 patients with completely resected nonmetastatic osteosarcoma with HDMTX, VCR, and Doxo and observed an OS of only 26%, a rate similar to that of untreated historical controls.

Randomized controlled trials comparing chemotherapy and observation have been conducted for several reasons, despite the positive results of some early chemotherapy studies. First, the improved outcome observed with chemotherapy could have been caused by an improved outcome for all patients unrelated to chemotherapy. Advances in diagnosis and surgical approach could have improved outcome, irrespective of other therapies provided. Results of a randomized controlled trial at the Mayo Clinic,[134,392] in which the EFS was 42% in both treated and untreated patients, supported this theory. Second, because of the lack of a control group, the favorable results of these early chemotherapy trials could have been caused by patient selection.[393]

Two randomized controlled trials published in the mid-1980s confirmed the effectiveness of chemotherapy

TABLE 62-5 Randomized Controlled Trials of Chemotherapy for Nonmetastatic Osteosarcoma

Group	Year	Regimen(s)	No. Patients	EFS/ DFS	Primary Objective(s)	Results/Conclusions
Mayo[134]	1984	HDMTX/VCR vs. observation	38	42%	Demonstrate superior outcome with adjuvant chemotherapy.	1. EFS and OS equivalent in chemo and observation arms. 2. EFS/OS in observation arm better than historic controls. 3. Power inadequate to detect a difference. 4. Natural history may be improving.
POG[394]	1986	HDMTX/Doxo/BCD vs. Observation	36	66% 17%	Demonstrate superior outcome with adjuvant chemotherapy.	1. Adjuvant chemotherapy improves outcome. 2. Natural history is not improving.
UCLA[395]	1987	HDMTX/VCR/Doxo/BCD vs. Observation	59	55% 20%	Demonstrate superior outcome with adjuvant chemotherapy.	1. Adjuvant chemotherapy improves outcome. 2. Natural history is not improving.
EORTC-SIOP[450]	1988	HDMTX/Doxo/CTX + adjuvant HDMTX/Doxo/CP vs. HDMTX/Doxo/CTX + adjuvant pulmonary XRT	205	40%	Compare efficacy of adjuvant chemotherapy vs. adjuvant pulmonary XRT.	1. EFS and OS equivalent in all arms. 2. Pulmonary XRT less toxic than chemotherapy.
MSKCC (T10)[412]	1982	HDMTX/BCD/Doxo ±VCR Good response: Continue same Poor response: –HDMTX, +CP, ± VCR	57	93% 96% 91%	Evaluate the effect of VCR on EFS.	1. Improved EFS compared with historic controls 2. VCR has no impact on EFS.
COSS[125]	1988	MTX/Doxo/CP Good response: Continue same Poor response: ifosfamide/CP/ BCD vs. IFN-α MTX/BCD Good response: Continue same Poor response: Doxo/CP	141	58% 49%	Improve survival or decrease toxicity by altering chemotherapy intensity based on tumor response.	1. Lower intensity therapy results in a reduced EFS. 2. Higher-intensity therapy for poor responders does not result in an increased EFS (compared with historic controls).
Rizzoli[118]	1990	MTX/CP Good responders: Continue same Fair response: + Doxo Poor response: + Doxo/BCD HDMTX: 7500 mg/m^2 vs. MDMTX: 750 mg/m^2	127	58% 42%	Compare efficacy of HDMTX and MDMTX.	1. EFS worse in patients receiving MDMTX although result is not statistically significant. 2. Lower intensity therapy for good responders results in a reduced OS of 20%.
EOI[126]	1992	HDMTX/Doxo/CP vs. Doxo/CP	198	41% 57%	Compare efficacy of multiagent regimen and two-agent regimen.	Two-drug regimen as efficacious as multiagent regimen.
EOI[422]	1997	HDMTX/VCR/BCD/Doxo/CP vs. Doxo/CP	407	44%	Compare efficacy of multiagent, prolonged (44 wk) regimen and two-agent shorter (18 wk) regimen.	1. EFS and OS equivalent in both arms. 2. Compliance better in shorter, two-agent regimen.

TABLE 62-5 Randomized Controlled Trials of Chemotherapy for Nonmetastatic Osteosarcoma (Continued)

Group	Year	Regimen(s)	No. Patients	EFS/ DFS	Primary Objective(s)	Results/Conclusions
MSKCC (T12)[112]	1998	HDMTX/BCD Poor response: + Doxo/CP Good response: + Doxo vs. Neoadj.: HDMTX/BCD/Doxo/CP Poor/good response: Continue	73	73% 78%	Improve outcome by intensifying preoperative chemotherapy.	1. Preoperative chemotherapy intensification increases percentage of good responders. 1. EFS is equivalent in both arms. 2. Intensifying preoperative chemotherapy does not increase DFS.
POG[111]	2003	Neoadj.: None Adj: HDMTX/BCD/Doxo/CP vs. Neoadj.: HDMTX/Doxo/CP Adj.: + BCD	106	65%	Compare outcomes of patients treated with or without neoadjuvant chemotherapy.	1. Neoadjuvant chemotherapy does not improve or worsen EFS. 2. Limb salvage rates are similar in both arms.
COG[5,110]	2005	HDMTX/Doxo/CP ± MTPPE ± Ifosfamide	667	63%	Improve outcome by intensifying chemotherapy with ifosfamide, MTPPE or both.	1. OS and EFS were not statistically different between arms on initial analysis. 2. On follow-up analysis OAS is slightly better in patients receiving MTPPE.
EOI[423]	2007	Doxo/CP q3 wk cycles vs. Doxo/CP q2 wk cycles (w/G-CSF)	497	39% 41%	Improve survival by increasing dose intensity.	1. EFS/OAS equivalent in both arms. 2. Toxicity equivalent in both arms. 3. Outcome with two-agent, shorter regimen (this study) significantly worse than with multiagent prolonged regimen (other studies).

Adj., Adjuvant; *BCD*, bleomycin, cyclophosphamide, and dactinomycin; *CP*, cisplatin; *DFS*, disease-free survival; *Doxo*, doxorubicin; *EFS*, event-free survival; *G-CSF*, granulocyte colony-stimulating factor; *HDMTX*, high-dose methotrexate; *IFN-α*, interferon-α; *MDMTX*, moderate-dose methotrexate; *MTP-PE*, muramyl tripeptide phosphatidylethanolamine; *Neoadj.*, neoadjuvant; *OS*, overall survival; *VCR*, vincristine; *XRT*, radiation therapy.

TABLE 62-6 Nonrandomized, Uncontrolled Trials of Chemotherapy in Nonmetastatic Osteosarcoma

Group	Date	Regimen(s)	No. Patients	EFS
CHOP[391]	1982	HDMTX/VCR/Doxo	20	26%
CCG[132]	1997	Neoadj.: HDMTX/VCR/BCD Good responders: Doxo Poor responders: Doxo/CP	268	53% 81% 46%
COSS[133]	1998	Low-risk: HDMTX/Doxo/CP High-risk: HDMTX/Doxo/CP/I	171	66%
Rizzoli[115]	2000	Neoadj.: HDMTX/Doxo/CP Good response: Continue same Poor response: + IE	164	61%
St. Jude[405]	2001	HDMTX/Doxo/I	69	72%
SSG[404]	2003	Neoadj.: HDMTX/Doxo/CP Good response: Continue same Poor response: + I/E	113	63%
ISG/SSG[113]	2005	HDMTX/Doxo/CP/I	182	64%

BCD, Bleomycin, cyclophosphamide, and dactinomycin; *CP*, cisplatin; *Doxo*, doxorubicin; *EFS*, event-free survival; *HDMTX*, high-dose methotrexate; *I*, ifosfamide; *IE*, ifosfamide and etoposide; *Neoadj.*, neoadjuvant; *VCR*, vincristine.

and an unchanged osteosarcoma natural history. In a multiinstitutional study, 36 patients with nonmetastatic fully resected osteosarcoma were randomized to observation or treatment with HDMTX, Doxo, and a regimen of bleomycin, cyclophosphamide, and dactinomycin (BCD).[394] The EFS was 66% in the group treated with chemotherapy but only 17% in the observed patients. Investigators at the University of California, Los Angeles, randomized 59 patients to a similar chemotherapy regimen or observation and obtained almost identical results.[395] Of treated patients, 55% were free of disease at 2 years whereas only 20% of observed patients remained free of disease. Statistically significant improvement in EFS and OS with chemotherapy has remained after long-term follow-up of this study population.[396]

Chemotherapeutic Agents

Cisplatin. In addition to the initially identified agents, HDMTX and Doxo, CP was identified as an active antiosteosarcoma drug in the late 1970s[397] and was rapidly incorporated into multiagent protocols. Adjuvant CP, in combination with Doxo, results in DFS rates at least as high as those seen with adjuvant HDMTX and Doxo.[389] In the past, it was theorized that intraarterial administration of cisplatin directly to the primary tumor might improve local control and survival. However, several randomized trials have failed to show survival gains from the intraarterial administration route,[133,398] despite an increased proportion of tumors with a good histologic response in some studies. Thus, it is now generally accepted that intraarterial administration of cisplatin is not of sufficient benefit to be worth the increased risk and inconvenience, although countervailing opinions remain.[399] Carboplatin has successfully replaced cisplatin in the treatment of some other cancers, but it may be less efficacious than CP in osteosarcoma. In a Pediatric Oncology Group study, presurgical carboplatin produced pathologic tumor responses in only 1 of 37 chemotherapy-naïve patients with metastatic osteosarcoma.[400] On the other hand, patients enrolled on a single-arm St. Jude Children's Research Hospital study in which chemotherapy consisted of carboplatin, ifosfamide, and Doxo[401] had similar outcomes compared with historical populations treated with CP-containing chemotherapy regimens. Carboplatin may have adequate activity in osteosarcoma when combined with some chemotherapy regimens, particularly those containing ifosfamide.

A recently published trial conducted by the Société Française d'Oncologie Pédiatrique (SFOP)[402] has underscored the importance of CP in osteosarcoma therapy. This study had two arms, with different preoperative chemotherapy regimens. In one arm, preoperative chemotherapy was HDMTX and Doxo, and patients in the other arm received HDMTX and combined ifosfamide and etoposide (IE). Patients with a poor histologic response to preoperative chemotherapy in the HDMTX-IE arm received postoperative CP-Doxo and had an EFS of 60%, whereas patients with a poor histologic response in the HDMTX-Doxo arm received postoperative IE without CP and had an EFS of 49%.

Ifosfamide and Etoposide. After demonstration of antiosteosarcoma activity in unresectable or metastatic disease,[403] ifosfamide was incorporated into multiagent treatment protocols for localized disease with the goal of improving outcome[113,115,133,404] or minimizing toxicity.[405] In two separate nonrandomized studies, one conducted at the Rizzoli Institute (Italy) and one by the Scandinavian Sarcoma Group (SSG), patients with a poor histologic response were treated with the IE regimen. In both studies, outcome was improved in patients with a poor response when compared with historical controls.[115,404] However, in a follow-up joint study by the SSG and the Italian Sarcoma Group (ISG), ifosfamide was given to all patients regardless of tumor response and the outcome was similar to that of historical controls.[113] Because none of these trials was randomized, a definitive conclusion regarding the additional benefit gained by adding IE to first-line therapy of localized disease is not possible. In a randomized controlled COG trial, the addition of ifosfamide to Doxo, CP, and HDMTX for patients with localized osteosarcoma did not increase survival.[110] Of note, in this trial, patients were treated with ifosfamide not in combination with etoposide, and ifosfamide was administered in a relatively low dosage (9 g/m^2/course). In another approach to incorporating ifosfamide into osteosarcoma regimens, the ISG conducted the ISG/OS-1 trial in which ifosfamide was administered to all enrolled patients in the neoadjuvant period. The use of ifosfamide in the neoadjuvant period did not improve the proportion of patients with a good histologic response or EFS or OS when compared with appropriate historical populations.[406]

A meta-analysis of osteosarcoma trials found that three-drug regimens, most of which do not contain ifosfamide, and four-drug regimens, most of which do contain ifosfamide, produce equivalent EFS and OS. This outcome does not eliminate a potential role for ifosfamide in osteosarcoma chemotherapy regimens.[407] Ifosfamide may be utilized to decrease the cumulative dose of other chemotherapy agents such as Doxo to decrease toxicity. In addition, studies conducted to date have not adequately addressed whether there is a role for ifosfamide in improving outcomes in high-risk patients. An international, multigroup study, the European and American Osteosarcoma Study Group (EURAMOS), is examining the efficacy of the addition of postoperative IE for improving EFS in patients whose tumors have a poor histologic response to chemotherapy.

Methotrexate. In early protocols, MTX was administered at dosages between 3 and 7.5 g/m^2.[109,123,131] However, methotrexate pharmacokinetics in children result in lower peak MTX levels for a given dosage per kilogram of body weight partly because of faster renal clearance.[408] Since this information became known, most regimens have used a MTX dosage of 12 g/m^2.[110,111] Several retrospective studies have shown a correlation between higher peak methotrexate levels (700 to 1000 μmol/L) and tumor necrosis[80] and DFS.[409] At a methotrexate dosage of 12 g/m^2, more than 90% of patients achieve peak methotrexate levels above 1000 μmol/L.[409,410] However, the only study directly comparing 7.5 g/m^2 and 12 g/m^2

did not demonstrate improved survival with the higher dosage.[411] Moderate MTX dosages (600 to 750 mg/m^2) do not seem to be as effective as high MTX dosages.[118,130] Alkaline intravenous fluids and leucovorin are administered with HDMTX, and MTX levels should be monitored after MTX administration.

Microtubule Inhibitors Vincristine and Eribulin. VCR was used in combination with MTX in the initial chemotherapy study by Jaffe and colleagues[109] because of preclinical data suggesting that VCR potentiated cellular MTX uptake. It is no longer used because a randomized controlled trial has shown no effect on EFS compared with MTX alone.[412] Eribulin is a synthetic analogue of a natural product, halichondrin B, and has a novel mechanism of action when compared with other microtubule inhibitors. Unlike other antimicrotubule drugs that suppress the shortening and growth phases of microtubule dynamic instability, eribulin works by inhibition of microtubule growth.[413,414] When evaluated by the Pediatric Preclinical Testing Program (PPTP), a platform for assessing the activity of novel agents in xenografts of common pediatric malignancies, eribulin had significant activity in osteosarcoma xenografts. Three of six patients with osteosarcoma receiving xenografts had a complete response, one had stable disease, and the remaining two had progressive disease. In a preclinical study with the osteosarcoma cell line U2OS, eribulin affected mitotic spindle centromere dynamics.[415] On the basis of these promising preclinical results, a phase II trial of eribulin in recurrent osteosarcoma is planned.

Bleomycin, Cyclophosphamide, and Dactinomycin. The combination of bleomycin, cyclophosphamide, and dactinomycin (BCD) was initially used by investigators at MSKCC[123] in combination with other active agents. BCD was included in a number of other treatment regimens in a nonrandomized fashion. However, in a direct comparison of BCD and CP in combination with HDMTX, VCR, and Doxo conducted by the Cooperative Osteosarcoma Study Group (COSS), the results of therapy with BCD were equivalent to those with CP.[124] In addition, BCD does not cause tumor regression in patients with previously treated metastatic disease.[416] Because of the equivalency with CP, lack of demonstrated efficacy in metastatic disease, and potential pulmonary toxicity, BCD is no longer included in most osteosarcoma chemotherapy regimens.

Immune Modulators. In vitro, IFN-α decreases the proliferation of osteosarcoma cell lines.[417] In vivo, growth of mouse osteosarcoma xenografts is inhibited by IFN-α.[418] Based on these preclinical data, investigators at the Karolinska Institute in Sweden gave IFN-α monotherapy to patients with localized fully resected osteosarcoma. The EFS was 53%.[419] Because of the impressive EFS in this study, a randomized trial evaluating IFN-α as maintenance after multiagent chemotherapy was conducted by EURAMOS. Results of this study have been presented, and IFN-α maintenance therapy did not improve EFS or OS.[378]

Another immunomodulatory therapy that might have activity in osteosarcoma is muramyl tripeptide phosphatidylethanolamine (MTP-PE). MTP-PE is a liposomal encapsulated bacille Calmette-Guérin cell wall component. It activates macrophages and, in mouse xenografts, induces tumoricidal activity.[420] Adjuvant treatment of osteosarcoma in dogs with MTP-PE alone led to a significantly increased DFS.[421] The addition of MTP-PE to HDMTX, Doxo, and CP was evaluated in a COG randomized controlled trial.[110] In the initial analysis of this study, neither EFS nor OS was statistically significantly improved in patients treated with the addition of MTP-PE or MTP-PE and ifosfamide to standard three-drug HDMTX, CP, and Doxo when compared with those treated with the three-drug combination of HDMTX, CP, and Doxo alone. Interpretation of the study results remains controversial, especially in light of a recently published follow-up analysis of the study data. In this analysis, the addition of MTP-PE to the standard three-drug regimen of HDMTX, CP, and Doxo significantly improved 6-year OS from 70% to 78% but did not significantly improve EFS.[5] At present, the FDA application for approval of MTP-PE is pending and MTP-PE is therefore not currently available.

Neoadjuvant Chemotherapy

Neoadjuvant chemotherapy is an approach in which some of the chemotherapy cycles are given prior to tumor resection and the remaining cycles are given postoperatively. Neoadjuvant chemotherapy in osteosarcoma has several potential benefits. Theoretically, neoadjuvant chemotherapy could increase DFS and OS through earlier treatment of microscopic metastatic foci.[123] From a practical perspective, neoadjuvant chemotherapy provides sufficient time for custom endoprosthesis preparation and other surgical planning. In addition, if sufficient tumor shrinkage occurs, neoadjuvant chemotherapy could make a patient who would have required amputation eligible for limb-sparing surgery. Postoperative healing could also be improved by neoadjuvant chemotherapy because there is less urgency to resume chemotherapy.

Theoretically, the greatest benefit of neoadjuvant chemotherapy is that it permits evaluation of tumor response to chemotherapy, the only clear predictor of outcome in osteosarcoma. Identifying patients with a poorer prognosis combined with subsequent therapeutic interventions could improve outcome. However, at present, there are no known therapeutic interventions that improve outcomes for patients with a poorer prognosis based on histologic response to chemotherapy. If therapeutic interventions that improve outcomes in patients with a poor tumor response to chemotherapy are not identified, the value of neoadjuvant chemotherapy will be diminished.

Neoadjuvant chemotherapy was initially used in MSKCC protocols.[123,412] The OS in patients treated with the MSKCC neoadjuvant protocols was 75% to 93%. Because an OS of 93% was significantly better than outcomes reported with adjuvant chemotherapy, there was initial excitement that neoadjuvant therapy would improve survival. However, a COSS protocol using the neoadjuvant approach, COSS-80, had an EFS of 68%, similar to EFS in ongoing studies of adjuvant chemotherapy.[124] Nevertheless, essentially all protocols after 1980 included, in a nonrandomized fashion, neoadjuvant chemotherapy. In general, EFS in these neoadjuvant protocols was similar to EFS seen in protocols giving only adjuvant therapy.

In the only randomized controlled trial comparing adjuvant and neoadjuvant chemotherapy,[111] OS and EFS were not significantly different for patients receiving adjuvant or neoadjuvant chemotherapy. Other than allowing the identification of a group of patients with a poorer prognosis, there did not appear to be significant additional benefits to neoadjuvant chemotherapy. The number of limb salvage procedures was the same in the adjuvant and neoadjuvant groups. Surgical complications were not reduced by neoadjuvant chemotherapy. Several patients had progression of the primary tumor during neoadjuvant chemotherapy, and a few of these patients developed pulmonary metastasis. Thus patients with clearly documented tumor progression during neoadjuvant chemotherapy should proceed to resection without completing the planned course of neoadjuvant therapy. This is especially true in the case of proximal tumors at risk for becoming inoperable (e.g., a proximal humeral lesion crossing the shoulder joint). On the other hand, chemotherapy-induced swelling of the tumor can sometimes be difficult to distinguish from progressive disease.

Response-Based Therapy

Multiple protocols have attempted to improve outcome in patients with a poorer prognosis by intensifying chemotherapy for this group of patients. In general, poor histologic tumor response after neoadjuvant chemotherapy has been used to stratify patients into an intensified treatment arm. Treatment intensification has generally been through the addition of one or two agents in the adjuvant phase not given during the neoadjuvant phase. For example, in Children's Cancer Group, Rizzoli Institute, and COSS protocols using neoadjuvant therapy consisting of HDMTX and BCD, adjuvant treatment was intensified by adding CP and Doxo.[118,125,132] Similarly, in the MSKCC T10 protocol, CP was administered to intensify therapy after neoadjuvant treatment with HDMTX, BCD, and Doxo.[412] With the exception of the T10 protocol, patients in the poor responder group had a similar outcome to historical controls (who did not get intensified chemotherapy), despite chemotherapy intensification.

In protocols in which both Doxo and CP are given neoadjuvantly, ifosfamide with or without etoposide has been used to intensify adjuvant chemotherapy for patients with a poor histologic response.[113,115,125,404] In the SSG protocol SSGVIII[404] and a recent Rizzoli protocol,[115] HDMTX, Doxo, and CP were given neoadjuvantly and IE was given adjuvantly to poor responders. In the Rizzoli trial, patients did not have a better outcome than historical controls (who did not receive IE intensification), whereas in the SSGVIII trial, outcome was better than historical controls, with an OS of 70% in patients with a poor histologic response.

In a different approach to chemotherapy intensification, COSS divided patients into high-risk and low-risk groups based on tumor size, pathologic subtype, and clinical response to neoadjuvant chemotherapy, measured by results of a bone scan. Patients in the high-risk group were treated with additional ifosfamide, added to the HDMTX, Doxo, and CP therapy. High-risk patients achieved the same outcome as low-risk patients. These results can be interpreted either as an indication that ifosfamide is effective at improving survival in high-risk patients or as indicating that the factors used to define the high-risk group do not actually convey a worse prognosis.[133] In summary, there is no convincing evidence that intensifying adjuvant chemotherapy for patients whose tumors have a poor histologic response results in an improved outcome. The lack of randomized studies addressing this question prevents a definitive conclusion about the usefulness of this approach.

On the other hand, there is fairly clear evidence that attempting to decrease toxicity for low-risk patients whose tumors have a good chemotherapy response by de-intensifying chemotherapy results in a worse prognosis.[118,125] In one arm of COSS-82, neoadjuvant chemotherapy consisted of HDMTX and BCD and, during adjuvant therapy, a lower dose of Doxo (30 mg/m^2 × 2 days) was given than had been used in previous protocols. The EFS was 41% in this arm of the study, which was significantly less than in both the other study arms and in historical controls. In a trial at the Rizzoli Institute, after neoadjuvant therapy with HDMTX and CP, patients with a good response received only further HDMTX and CP. These patients had an EFS of only 27%.

The conclusion of a series of studies by the European Osteosarcoma Intergroup (EOI)[126,422,423] is that a two-drug regimen of Doxo and CP is inferior to regimens that include three or more drugs. In the first and second randomized studies, outcomes in patients treated with CP and Doxo and those treated with regimens containing at least the three drugs were equivalent.[117] However, in these two studies and a third study, in which all patients were treated with Doxo and CP, EFS was approximately 40%, a lower rate than is seen in studies of multidrug regimens conducted by other groups.

Non–Risk-Based Chemotherapy Intensification

There are gains to be made in outcome for all osteosarcoma patients, not just those with high-risk disease. With this in mind, some groups have evaluated the impact of intensifying chemotherapy for all patients. The MSKCC T12 trial randomized patients to a more- or less-intensive neoadjuvant chemotherapy regimen. Patients randomized to the more-intensive study arm received HDMTX, BCD, Doxo, and CP neoadjuvantly and adjuvantly, whereas patients randomized to the less-intensive arm received HDMTX and BCD neoadjuvantly, with adjuvant therapy based on tumor response. Doxo was continued for patients with a good response, with Doxo and CP used for poor responders. EFS was 73% for patients in the less-intensive arm of the study and 78% for those in the more-intensive arm of the study,[110] a difference that is neither clinically nor statistically significant.

The COG conducted a randomized trial to evaluate the impact of adding adjuvant ifosfamide and/or MTP-PE to the initial HDMTX, Doxo, and CP. As noted earlier, in the initial analysis of this trial, neither the addition of ifosfamide nor the addition of MTP-PE significantly improved survival.[110] However, in a follow-up analysis, MTP-PE was shown to improve 6-year OS significantly, by a small margin.[5] As noted, interpretation of these data remain uncertain. As with chemotherapy intensification for poor responders, randomized controlled trials are needed to determine whether additional benefit can be gained from adding ifosfamide, etoposide, or other active agents such as immunomodulators to the core anti-osteosarcoma agents HDMTX, Doxo, and CP.

Dose Density

The most recent EOI study has evaluated the impact on toxicity and survival of shortening the interval between CP and Doxo courses from 3 to 2 weeks. In this randomized trial, patients on the every-2-week arm were supported with granulocyte colony-stimulating factor (G-CSF) between chemotherapy cycles. The EOI found that the shortened interval dosing was equivalent in both toxicity and outcome to the longer interval dosing.[423]

Current Approach to Therapy

Most of the osteosarcoma research groups, COG, SSG, COSS, and the EOI, have collaborated on an international trial, EURAMOS 1. The chemotherapy administered to all patients in the trial initially is neoadjuvant and adjuvant HDMTX, Doxo, and CP (Table 62-7). The objectives of this randomized trial are the following: (1) to determine whether the postoperative addition of IE improves EFS of patients with a poor response to neoadjuvant chemotherapy; and (2) to evaluate whether the postoperative addition of IFN-α after neoadjuvant chemotherapy improves outcome in patients with a good histologic response. All patients will receive a total of 10 weeks of neoadjuvant chemotherapy with HDMTX (four cycles) and Doxo and CP (two cycles). Patients with a poor response will be randomized to continue HDMTX, Doxo, and CP or to continue HDMTX, Doxo, and CP with the addition of IE. Patients with a good response will be randomized to continue HDMTX, Doxo, and CP or to continue HDMTX, Doxo, and CP, with the addition of IFN-α. The results of the good histologic response study arm were reported at the American Society of Clinical Oncology Annual Meeting in 2013. There was no difference in event free or overall survival between those who received IFN-α and those who did not. Only 55% of enrolled patients completed IFN-α therapy per protocol; 45% stopped early due to toxicity. The conclusion of this arm of the study is that, when given as tolerated, IFN-α does not improve outcome in osteosarcoma. The results of the poor histologic response study arm are still not available.[423a]

Initially Metastatic Osteosarcoma

As noted earlier (see "Natural History and Prognosis"), the presence of metastatic disease at the time of initial diagnosis is the most unfavorable prognostic feature. In view of the unfavorable outlook of these patients, strategies that included higher toxicity regimens have been used in an effort to improve patient outcome. The Pediatric Oncology Group (POG)[400] administered carboplatin, 1 g/m^2 as a 48-hour continuous infusion, to patients with initially metastatic osteosarcoma as initial therapy. Only 1 patient had an overall partial response. After researchers separately analyzed the primary and metastatic sites of disease, they found that 3 patients had a partial response of their primary tumor (2 had histologic evaluation of the tumor at definitive surgery, showing between 50% and 90% necrosis), 3 had complete responses, and 1 had partial response of their pulmonary disease. However, 20 of 37 patients had unequivocal progressive disease while receiving carboplatin. After induction, multiagent chemotherapy was administered with a combination of high-dose methotrexate, ifosfamide (2.4 g/m^2/day for 5 days, as a single agent), CP, and Doxo, for a total of 40 weeks. Overall outcome was poor, with an EFS of 24% and OS of 40% at 3 years. Patients with isolated pulmonary metastasis had a more favorable outlook, as in the COSS series, with a 44% 3-year survival. Those with unresectable bony metastases had a particularly poor outcome, 6% survival at 3 years.

A subsequent POG study began therapy with two cycles of high-dose ifosfamide (3.5 g/m^2/day for 5 days) and etoposide (100 mg/m^2/day, also for 5 days).[147] This was based on a prior phase I study of etoposide with escalating dosage of ifosfamide, in which 6 of 13 patients with recurrent osteosarcoma responded to therapy with higher dosage levels of ifosfamide.[424] Induction with ifosfamide and etoposide was followed by multiagent chemotherapy with high-dose methotrexate, CP, and Doxo, as well as three additional cycles of a slightly lower dosage of ifosfamide, 2.4 g/m^2/day for 5 days, and etoposide, 100 mg/m^2/day for 5 days. Surgery of the primary and resectable metastatic sites was encouraged. Of 41 evaluable patients, 2-year progression-free survival was 43%, with an OS probability of 55%. In the limited number of patients studied, survival was similar for

TABLE 62-7 Schema of Typical Administration Scheduled for Doxorubicin (A), Cisplatin (P), and High-Dose Methotrexate (M) Therapy for Osteosarcoma

Week*	1	4	5	6	9	10	11	12†	15	16	17	20	21	22	24	25	26	28	29
Therapy	A, P	M	M	A, P	M	M	Surgery	A, P	M	M	A, P	M	M	A	M	M	A	M	M

*Weeks 2, 3, 7, 8, 13, 14, 18, 19, 23, and 27 no therapy scheduled.

†Week 12 chemotherapy is administered after adequate postoperative healing has occurred.

patients with isolated pulmonary metastases, 52% at 2 years, as compared with patients with bone metastases, with or without lung metastases, 58% at 2 years. Toxicity was significant, however, with two toxic deaths, one from gram-negative sepsis and the second from congestive cardiac failure. Eighty-three percent of patients experienced severe neutropenia, with sepsis in 10% (including the septic death) and other bacterial infections in an additional 7%.

In view of the relatively favorable outcome of the IE-containing regimen, it served as the model for the core regimen in the COG study AOST 0121. The ifosfamide dosage utilized was one level lower (2.8 g/m^2/day for 5 days), along with etoposide 100 mg/m^2/day for 5 days because of toxicity observed on the previous trial, and the ordering of the cycles was slightly different. As noted earlier (see "Biology"), the presence of ERBB2 expression in osteosarcoma was believed to confer an unfavorable prognosis and to represent a tumor target. Trastuzumab is a humanized antibody that binds specifically to the ERBB2 protein. Preclinically, it was found to antagonize the function of the growth-signaling properties of the ERBB2 system, signal immune cells to attack and kill tumor targets, and augment chemotherapy-induced cytotoxicity.[425-429] A pivotal randomized phase III trial in women with previously untreated ERBB2-positive metastatic breast cancer has shown a significantly improved response rate and survival for those who initially received trastuzumab along with chemotherapy.[300] Trastuzumab increased the risk for cardiotoxicity when given with anthracyclines. However, trastuzumab cardiotoxicity appears to be reversible on discontinuation of the drug.[430] In view of the possible increased risk for cardiotoxicity, entry into AOST 0121 was limited to the highest-risk patients—those with bone metastases, bilateral lung metastases, or unilateral lung metastases with at least four nodules. In addition, dexrazoxane was administered as a cardioprotective agent prior to bolus doxorubicin. Approximately one third of patients had tumors that expressed ERBB2, and only these patients received trastuzumab. Toxicity data demonstrated that it is feasible to combine trastuzumab with multiagent chemotherapy in osteosarcoma. Clinically significant cardiac events did not occur. Outcome in ERBB2-positive patients was similar to that for ERBB2-negative patients treated with chemotherapy alone and to that for historical controls, suggesting limited activity of trastuzumab in ERBB2-positive osteosarcoma.[303]

More recently the COG conducted a study in patients with initially metastatic osteosarcoma incorporating zoledronate into the same background chemotherapy utilized in AOST 0121. The study assessed the feasibility and safety of adding zoledronate to multiagent chemotherapy. Zoledronate could be successfully combined with chemotherapy. Similar results were obtained in a related study in which pamidronate was combined with chemotherapy.[431] The 5-year EFS and OS in patients with metastatic osteosarcoma treated with chemotherapy and zoledronate were 45% and 62%, respectively.[432] Because of differences in study populations these outcomes could not be compared with prior prospectively studied patients and thus determining the effectiveness of inhibiting bone turnover in osteosarcoma will require additional studies. A trial being conducted by the SFOP may shed some light on this question.

The trial evaluating the addition of MTP-PE to chemotherapy also enrolled patients with metastatic disease (see "Immune Modulators" under "Chemotherapy for Localized Disease"). As in patients with localized osteosarcoma, the 5-year OS in patients with metastatic osteosarcoma randomized to receive MTP-PE plus chemotherapy was slightly better than in those randomized to receive chemotherapy alone. However, this result was not statistically significant.[433] Therefore, as with MTP-PE in localized osteosarcoma, the results of this initial randomized study of MTP-PE are not conclusive. An additional clinical trial of MTP-PE would help clarify the role of this therapy in metastatic osteosarcoma.

In the absence of positive results from any of the clinical trials that have been conducted in the metastatic patient population, the standard approach to chemotherapy in patients with metastatic osteosarcoma is to use treatment protocols utilized in patients with localized disease. Some clinicians will intensify therapy such as by adding ifosfamide and etoposide to a HDMTX, Doxo, and CP based regimen as was done for the COG studies AOST 0121[303] and AOST 06P1.[432]

As with localized osteosarcoma, complete resection of all gross disease, when feasible, is an important part of therapy in metastatic osteosarcoma. For patients with isolated pulmonary metastases, metastasectomy of all pulmonary disease improves prognosis.[4] The surgical technique to best accomplish resection of pulmonary metastases in osteosarcoma has not been investigated with clinical trials and is a question open to debate. Because CT has been demonstrated to have less sensitivity for presence of pulmonary metastases than surgical exploration, some researchers believe that the surgical standard of care for osteosarcoma with isolated pulmonary metastases should be open thoracotomy with resection of all palpable pulmonary disease.[434] However, others argue a video-assisted thoracoscopic surgery approach is adequate in some cases.[435]

Recurrent Osteosarcoma

The recurrence of osteosarcoma is an unfavorable event, with subsequent outcome most influenced by the time to recurrence, burden of disease at the time of recurrence, and resectability of the recurrent disease. In a series from the COSS group,[7] 576 of an initial 1702 patients developed recurrent disease at a median time of 1.6 years (range, 0.1 to 14.3 years). EFS was 0.13 (standard error [SE], 0.01) at 5 years and 0.11 (SE, 0.02) at 10 years, with an OS of 0.23 (SE, 0.02) at 5 years and of 0.18 (SE, 0.02) at 10 years. The most important factor associated with survival was achievement of a second surgical complete remission. The ability to achieve second surgical complete remission was likely related to the number and bilaterality of pulmonary lesions, as well as pleural disruption by the pulmonary metastasis. Patients with multiple lesions, bilateral lesions, and lesions that disrupted the pleura fared less well. Chemotherapy was of limited benefit, with multiagent therapy probably more effective than single-agent therapy.

Similar results have been reported from the Rizzoli Institute[6] in a study of 175 patients. Again, achievement of a second surgical remission was key to survival and was more readily accomplished in patients with recurrence limited to the lungs (97 of 125 [75%] of those with isolated pulmonary recurrence as compared with 21 of 37 [57%] of those with other than lung metastasis). EFS was 16%, with postrelapse survival of 39% at 5 years for those who achieved a second complete surgical remission but 0% among those who did not. A longer initial relapse-free interval (>24 months) and the presence of only one or two lung metastases were favorable prognostic indicators in patients with isolated pulmonary metastases who achieved a second complete surgical remission. Chemotherapy slightly prolonged survival in those who did not achieve a second surgical complete remission and possibly in those with three or more pulmonary nodules who underwent resection. Other groups have reported comparable findings in smaller numbers of patients.[114,431,435,437] Aggressive and, if necessary, repeated thoracotomies have also been advocated by many.[438-441]

As discussed earlier (see "Determinants of Metastasis" under "Biology"), the development of pulmonary metastasis may be facilitated by the absence of FAS on the malignant cell surface. Tumor cells expressing FAS are eliminated by the lung endothelium, which constitutively expresses FASL. The metastatic potential of osteosarcoma cells in model systems has been shown to be inversely related to FAS expression, and metastatic osteosarcoma samples often lack FAS expression.[334,442,443] It was hypothesized that the administration of GM-CSF by the inhalation route may enhance FAS expression on osteosarcoma cells. A study at the Mayo Clinic in 40 patients with pulmonary metastases from a variety of malignancies has shown benefit in at least 50%.[444] Dose escalation in patients with melanoma has shown melanoma-specific T cells at the higher dosage levels of 1750 and 2000 µg administered by the inhalation route twice daily, 1 week on and 1 week off (S. Markovic, personal communication, 2008). A COG study (AOST 0221) investigated the feasibility of inhaled GM-CSF monotherapy in patients with recurrent osteosarcoma, with biologic end points including analysis of the FAS pathway and study of infiltrating dendritic cells. Metastatic nodules resected after inhaled GM-CSF did not display induction of FAS expression and had limited evidence of dendritic cell infiltrate.[379]

Chemotherapy administration via the inhalation route is one approach being considered for both recurrent and primary metastatic osteosarcoma. Advantages of inhaled delivery include the ability to circumvent first pass metabolism in the liver, decreased systemic absorption, and, consequently, decreased systemic symptoms. A phase Ib/IIa study of inhaled lipid cisplatin demonstrated this therapy to be feasible and to have minimal systemic toxicity. Activity in patients with gross pulmonary metastases was limited.[445] An ongoing phase II study is evaluating the activity of this therapy in patients with recurrent osteosarcoma limited to the lungs whose pulmonary nodules have been completely resected.

In a phase II study of the pan-kinase inhibitor sorafenib in 35 patients with recurrent osteosarcoma with measurable disease, 3 patients (8%) had a partial response according to RECIST (Response Evaluation Criteria in Solid Tumors) and progression-free survival at 4 months was 46%.[446] The mechanism of action of sorafenib in osteosarcoma is not known. Data from preclinical models suggests sorafenib inhibits angiogenesis, which is not unexpected given that sorafenib is a potent inhibitor of vascular endothelial growth factor receptors (VEGFRs).[369] This hypothesis is supported by the observation of partial responses in osteosarcoma patients enrolled on phase I trials of potent VEGFR inhibitors cediranib[447] and regorafenib.[448] Sorafenib may also function via inhibition of ERK1/ERK2 signaling.[369]

In summary, recurrent osteosarcoma carries a grave prognosis. Cure is most likely in the minority of patients who have a limited number of metastases that are completely resected, leading to a second complete surgical remission, especially if the first remission was of relatively long duration. The role of adjuvant therapy for patients with recurrent osteosarcoma is uncertain. Enrollment of such patients in clinical trials should be encouraged.

Radiation Therapy

Radiation therapy is relatively ineffective against osteosarcoma, especially when compared with the combination of adequate surgery and multiagent chemotherapy. In vitro studies comparing osteosarcoma with other human cell lines have demonstrated that osteosarcoma cells are more resistant to irradiation.[449] In addition, those osteosarcomas with loss of TP53 function would be expected to survive radiation-induced DNA damage.[208,220]

Adjuvant pulmonary radiation therapy has been compared in a randomized fashion with adjuvant VCR plus HDMTX.[450] Although outcomes were the same in patients receiving chemotherapy and those receiving pulmonary irradiation, DFS in both arms of the study was much lower (at 24%) than with the current standard approach of wide surgical resection and multiagent chemotherapy.[110] In addition, 14% of patients receiving adjuvant pulmonary irradiation experienced decreased pulmonary function. Thus adjuvant pulmonary irradiation is not recommended.

Radiation therapy has been used for local control of osteosarcoma. When radiation therapy is the only local control measure (i.e., no operation is performed), the local recurrence rate is almost three times greater than is seen with surgical resection.[450] Nevertheless, when surgical resection of the primary lesion is not an option, there may be a role for radiation therapy. Machak and colleagues[451] have reported a 5-year OS survival of 60% in 31 patients who refused surgical resection of the primary tumor and were treated instead with local irradiation and multiagent chemotherapy. Similarly, patients with osteosarcoma of the spine who had intralesional margins or no surgery tended to have a better outcome if they received local irradiation plus multiagent chemotherapy than chemotherapy alone, although the difference was not statistically significant.[452] Proton-based radiotherapy used in the setting of incomplete or no resection produced reasonable local control rates, but only 35% of patients in the series had high-grade osteosarcoma.[453] Contrasting results have been presented by Hug and associates,[454] who saw local failures in 2 of 3 patients who did not undergo surgical resection of the primary tumor but were treated with local irradiation and multiagent chemotherapy.

Preoperative irradiation, in combination with neoadjuvant multiagent chemotherapy, has been given in an attempt to increase the rate of limb salvage surgery.[455] Although this approach has resulted in a higher percentage of limb salvage procedures compared with historical controls, local complications were also increased. Given the increased rate of local complications, this approach is not recommended.

Whether postoperative irradiation is useful when inadequate surgical margins are present is controversial. There are no randomized trials comparing postoperative irradiation with observation in patients whose tumor resection specimens had marginal or intralesional surgical margins. Studies that have compared such patients with similar historical controls show an improved local control rate and OS in patients treated with local postoperative irradiation.[452,456] However, a great diversity of tumor burden is present in patients with intralesional and marginal tumor margins, and chemotherapy regimens have changed over time. Thus, comparison with historical controls might not be valid.

Samarium 153-labeled ethylenediaminetetramethylene phosphonate (^{153}Sm-EDTMP) is a pharmaceutical radioisotope that is efficiently and specifically taken up by bone. Patients with osteosarcoma bone metastases treated with ^{153}Sm-EDTMP experience a rapid decrease in pain symptoms.[457] At high doses of ^{153}Sm-EDTMP, peripheral blood stem cell rescue is required.

Complications of Therapy and Late Effects

Patients receiving osteosarcoma therapy can experience acute complications from chemotherapy and surgery. As noted, the usual initial osteosarcoma chemotherapy comprises HDMTX, Doxo, and CP. Ifosfamide and etoposide are also given in many protocols. The acute toxicities of HDMTX, Doxo, and CP combination chemotherapy with and without ifosfamide have been well documented in numerous osteosarcoma treatment protocols.[111,113,133,404,422,423] The most common acute side effects, occurring in 75% of patients, are grades 3 or 4 leukopenia and neutropenia. Significant nausea and vomiting are also common and occur in 60% to 70% of patients, particularly during Doxo-CP cycles; 20% to 30% of patients experience mucositis. A similar percentage develops significant infections. Reversible aminotransferase elevations occur in about 15% of patients, usually because of HDMTX. Renal toxicity, which occurs in 1% to 2% of patients, presents as renal insufficiency or tubular dysfunction, with accompanying electrolyte abnormalities. Renal dysfunction can be acute or chronic. Neurologic toxicity presenting as seizures, headache, or radiologic changes is rare. The rate of therapy-related mortality varies from 0% to 4%, with most protocols reporting a 1% to 2% rate of therapy-related mortality, usually caused by infection and, less frequently, by cardiotoxicity.

A potential complication of HDMTX therapy in all disease settings is renal damage from insoluble MTX metabolites. Because MTX is primarily excreted by the kidneys, renal damage can lead to delayed MTX clearance, which in turn worsens renal dysfunction and further delays MTX clearance. Delayed MTX clearance, even in the setting of adequate leucovorin rescue, can lead to severe mucositis and prolonged myelosuppression. With adequate hydration, alkalinization, and monitoring of drug levels, the incidence of severely delayed MTX clearance is low. Delayed MTX clearance can be treated with increased hydration, adjustments in leucovorin dosing, hemodialysis, or carboxypeptidase (glucarpidase), an enzyme that converts MTX to inactive metabolites that are readily excreted in the urine.[458]

Acute complications of osteosarcoma primary resection surgeries are reviewed extensively in Chapter 49, "Pediatric Surgical Oncology." Limb salvage procedures have a much higher rate of perioperative complications than amputations. In one study, 41% of limb-sparing procedures had wound complications compared with only 13% of amputations.[111] Acute complications of surgery include infection, flap necrosis, damage to the neurovascular bundle, and venous thrombosis.[111,459]

Late effects of chemotherapy in patients with osteosarcoma include cardiac dysfunction, hearing loss, secondary malignancy, and decreased fertility.[115] Guidelines regarding posttreatment monitoring for late effects and relapse are presented in Table 62-8. Many osteosarcoma protocols use a cumulative doxorubicin dosage of 450 mg/m^2. Published rates of cardiac toxicity after treatment for osteosarcoma range from 0% to 5%.[111,120,404,423] Cardiac dysfunction caused by Doxo is irreversible and can be severe enough to cause death or require transplantation. Doxo-related cardiotoxicity is more common in women and is more likely with higher cumulative Doxo dosages.[460] Younger children treated with Doxo may develop delayed cardiac failure during rapid somatic growth in adolescence, and women may similarly develop cardiac failure as a result of the increased cardiac workload presented by pregnancy. The long-term cardiac outcome of patients treated with Doxo especially at a relatively high cumulative dosage remains to be defined. Administration of dexrazoxane in conjunction with Doxo in pediatric sarcoma patients has led to preservation of left ventricular function, without any change in outcome, compared with placebo control in an initial small clinical trial.[461] Recently, dexrazoxane administration in patients receiving therapy for Hodgkin disease has been associated with an increased risk for subsequent acute myeloid leukemia and myelodysplastic syndrome.[462] It is uncertain whether these findings pertain to patients with other disease types.

Second malignancies occur at an increased rate in osteosarcoma survivors compared with the general population or patients with benign bone tumors.[463,464] The incidence of second malignancies ranges from 2% to 4%.

TABLE 62-8 Typical Monitoring after Completion of Therapy*

Routine Monitoring for Tumor Recurrence (also imaging for symptoms, as indicated)	
First Year	
Primary site	Plain radiograph every 3 months
Site(s) of bone metastases	Plain radiograph film every 3 months
Chest	Chest CT every 3 months
Second Year	
Primary site	Plain radiograph every 6 months
Site(s) of bone metastases	Plain radiograph every 6 months
Chest	Chest CT every 6 months
Third Year	
Primary site	Plain radiograph every 6 months
Site(s) of bone metastases	Plain radiograph every 6 months
Chest	Chest radiograph every 6 months
Fourth and Fifth Years	
Primary site	Plain radiograph every 12 months
Site(s) of bone metastases	Plain radiograph every 12 months
Chest	Chest radiograph every 6 months
Sixth to Tenth Years	
Primary site	Plain radiograph every 12 months
Site(s) of bone metastases	Plain radiograph every 12 months
Chest	Chest radiograph every 12 months
Monitoring for Late Effects of Chemotherapy	
Cardiac	Echocardiogram every 12 months for 2-3 years then at the time of pubertal growth spurt and pregnancy
Nephrotoxicity	Monitor electrolytes, blood urea nitrogen, creatinine every 12 months
Ototoxicity	Audiology at end of therapy
Secondary malignancy	Complete blood cell count every 12 months
Fertility	Semen analysis offered to postpubertal males
All	Physical examination every 3 months × 1 year, every 6 months until 5 years after completion of therapy then yearly

CT, Computed tomography.

*Recommendations for posttreatment surveillance vary. For further reading see references 475 to 477.

Because a number of genetic cancer syndromes increase osteosarcoma risk, it is difficult to determine whether second malignancies are caused by an underlying genetic predisposition or a complication of osteosarcoma therapy. Certainly some of the osteosarcoma survivors who develop a second malignancy have no family history of cancer. No particular type of secondary malignancy predominates.

Ototoxicity as a result of osteosarcoma therapy is predominantly caused by CP. In some patients, aminoglycoside treatment of infectious complications of chemotherapy may be a contributing factor. Mild ototoxicity, which does not involve the frequencies of audible speech, is relatively common. Significant ototoxicity involving the audible speech frequencies is rare, reported in 1% or fewer patients.[465] Amifostine, administered concurrently with CP, has been tested in a small group of pediatric osteosarcoma patients. Compared with untreated controls, patients receiving amifostine had less severe myelosuppression but the incidence of renal and ototoxicity was not decreased by amifostine therapy. Amifostine was emetogenic but was otherwise well tolerated.[466]

Azoospermia is known to be caused by alkylating agents and is common in osteosarcoma patients treated with ifosfamide. Azoospermia has also been seen in patients who were treated with HDMTX, Doxo, and CP; and it is likely that this is chemotherapy related.[467] Data on female infertility after osteosarcoma therapy are limited. In one study, 6% of female patients treated with HDMTX, Doxo, and CP plus ifosfamide had early menopause.[468]

Long-term orthopedic complications, which depend on the type of surgical resection performed, are discussed further in Chapter 49, “Pediatric Surgical Oncology.” Patients who have limb-sparing operations are more likely to have late orthopedic complications. Most late complications are caused by endoprosthesis or allograft failure, although late infections also occur.[459] Thirty percent of endoprostheses will require surgical revision or replacement by 10 years after the initial surgery.[469] Other problems that arise in patients undergoing limb-sparing surgery include poor joint mobility and leg-length discrepancy, both of which can impair limb function. Although limb-sparing procedures have a higher complication rate, the functional outcome is superior when assessed by oxygen consumption during walking. Functionally, rotationplasty is equivalent to a below-knee amputation.[459] In long-term follow-up studies, quality of life did not differ in those who had an amputation when compared with those who underwent a limb-sparing procedure. Poorer lower limb function does have an adverse impact on quality of life, and failure of a limb-sparing procedure had an impact on body image.[470] Compared with survivors of other childhood cancers and their siblings, survivors of osteosarcoma have greater activity limitations.[471,472] Despite these limitations, those who are long-term survivors after osteosarcoma appear to have similar social and occupational outcomes as their siblings.[473]

CONCLUSION AND FUTURE DIRECTIONS

Osteosarcoma treatment and prognosis have not changed significantly during the past 2 decades. The standard basic chemotherapy for localized osteosarcoma—Doxo, CP, and HDMTX—has remained the same. Consequently, outcomes in patients with localized osteosarcoma are similar today to outcomes 20 years ago. New therapies, particularly ifosfamide and etoposide, have been used for patients with metastatic and recurrent disease, and there are early indications that this may result in a slight improvement in outcome. In contrast, surgical approaches have changed significantly over the past 20 years. Previously, amputations were common; currently, many patients have limb-sparing surgeries. In addition, there has been increasing recognition of the ability to increase survival via complete resection of pulmonary metastases.

This relatively slow rate of progress in treatment of osteosarcoma is partly the result of the biologic complexity of the disease. There have been a number of biologic insights over the past 2 decades, but none has revealed obvious drug targets that are of central relevance in a large proportion of osteosarcomas. An additional factor slowing the rate of progress in osteosarcoma is the small patient population available for clinical studies.

Several ongoing clinical studies should provide important clinical insights in the next 5 to 10 years. It is hoped the results of the international cooperative study EURAMOS1 will determine whether the postoperative addition of ifosfamide and etoposide improves the EFS of patients with a poor response to neoadjuvant chemotherapy.

Identification of new therapeutic agents with activity in osteosarcoma is a major research priority. Several novel agents are likely to be evaluated in phase II clinical trials in osteosarcoma in the next several years. The Sarcoma Alliance for Research through Collaboration (SARC) is studying an SRC inhibitor in patients with metastatic osteosarcoma. Phase II studies of the microtubule inhibitor eribulin, of the anti-GD2 antibody CH14.18, and of the RANKL antibody denosumab have been proposed by the COG.[474]

Breakthroughs in the understanding of osteosarcoma biology are likely in the next 10 years as the number and sophistication of tools and techniques for biomedical research continue to increase. For example, improved transgenic mouse models of osteosarcoma are likely to be developed, and these models will facilitate basic and applied studies. Results of comprehensive next-generation sequencing studies are expected at the time of publication. Genomic and proteomic techniques requiring minimal amounts of tumor tissue will continue to facilitate the study of human osteosarcoma samples, which are in limited supply. It is hoped the combination of these new techniques, with the proliferation of new drug compounds and the recently established international collaboration for clinical studies, will lead to significant advances in osteosarcoma in the near future.

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CHAPTER 63

Pediatric Germ Cell Tumors

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CHAPTER OUTLINE

DEVELOPMENT OF THE GERMLINE
Specification of Primordial Germ Cells
Primordial Germ Cell Migration
Erasure of Imprinting
Gonadogenesis
HISTOPATHOLOGY OF GERM CELL TUMORS
Overview of Testicular Germ Cell Tumors
Overview of Ovarian Germ Cell Tumors
Intratubular Germ Cell Neoplasia, Unclassified
TERATOMA
Histopathology of Teratoma
Testicular Teratoma
Ovarian Teratoma
Immature Teratoma
Monodermal Teratomas
Teratoma with Malignant Transformation
YOLK SAC TUMOR
Histopathology of Yolk Sac Tumor
Testicular Yolk Sac Tumor
Ovarian Yolk Sac Tumor
SEMINOMA AND DYSGERMINOMA
Histopathology of Seminoma and Dysgerminoma
Seminoma
Dysgerminoma
EMBRYONAL CARCINOMA
Histopathology of Embryonal Carcinoma
Testicular Embryonal Carcinoma in Adolescents and Adults
Ovarian Embryonal Carcinoma
CHORIOCARCINOMA
Testicular Choriocarcinoma in Adolescents and Adults
Ovarian Choriocarcinoma
MIXED OVARIAN GERM CELL TUMOR
Pathology of Mixed Germ Cell-Sex Cord Tumors
SUMMARY OF PATHOLOGY
EPIDEMIOLOGY OF PEDIATRIC GERM CELL TUMORS
Incidence
Possible Environmental Etiologies
Predisposing Syndromes
MOLECULAR BIOLOGY OF GERM CELL TUMORS
The GCT Transcriptome
Noncoding RNAs
Chromosomal Constitution and Copy Number Aberrations
Genome-Wide Association Studies
The Role of Developmental Signaling Pathways in Germ Cell Tumor Pathogenesis
DIAGNOSIS AND STAGING OF PEDIATRIC GERM CELL TUMORS
Clinical Presentation of Pediatric Germ Cell Tumors
Evaluation for Metastatic Disease
Tumor Markers
SURGERY AND GERM CELL TUMOR
Surgical Approach to Sacrococcygeal Tumors
Surgical Approach to Testes Tumors
Surgical Approach to Ovarian Tumors
Surgical Approach to Mediastinal Germ Cell Tumors
Surgical Approach to Extragonadal Tumors at Other Sites
PROGNOSTIC FACTORS AT DIAGNOSIS OF PEDIATRIC GERM CELL TUMORS
TREATMENT
Overview
Explanation of the Nomenclature
Infantile Choriocarcinoma: A Rare but Rapidly Fatal Condition if Not Recognized
Seminoma and Dysgerminoma
TREATMENT OF STAGE I MALIGNANT NONSEMINOMATOUS GERM CELL TUMORS
Chemotherapy for Pediatric Germ Cell Tumors (> Stage I)
Treatment of Pediatric Germ Cell Tumors in the United States
Treatment of Pediatric Germ Cell Tumors in the United Kingdom
Treatment of Pediatric Germ Cell Tumors in Germany

The Dose of Bleomycin in Treatment of Pediatric Germ Cell Tumors
Mature and Immature Teratomas
Extragonadal Tumors
Postchemotherapy Management
RELAPSE AND SALVAGE THERAPY
Salvage Therapy
Molecular Basis of Cisplatin Sensitivity and Resistance in Germ Cell Tumors
Prospects for Targeted Therapy of Germ Cell Tumors
LATE EFFECTS
Late Effects Among Men Treated for Testicular Cancer
Cisplatin Levels are Measurable for Years After Treatment
Risk of Cardiovascular Disease
Risk of Second Malignant Neoplasm
Risk of Secondary Leukemia
Other Significant Late Effects
CONCLUSIONS

Germ cell tumor (GCT) is the designation given to neoplasms arising from the cells of the germline—the cells that are destined to become either the egg or the sperm. A number of unique features of these tumors including their bimodal and wide age distribution, remarkable phenotypic diversity, and varying biologic behavior make GCTs a particular challenge for the surgeon, pathologist, and oncologist. Successful treatment regimens developed over the last several decades have focused current clinical research on ways to maintain efficacy and minimize toxicity for most patients and intensify treatment for patients who fail first-line therapy. Recent advances in understanding the underlying aberrations in germline development shed light on the genesis of these tumors and may provide insight into new avenues for treatment.

DEVELOPMENT OF THE GERMLINE

The pathogenesis of GCTs can best be understood through an analysis of the mechanisms of germline development.[1] The role of the germ cell is to ensure the continuation of a species by producing the gametes, cells that will give rise to the next generation. Reflecting this unique role, germ cells are set aside from somatic cells very early in development. In humans, germ cells arise in an extraembryonic position and must migrate to the site at which the gonads will form. The pluripotency of germ cells is reflected in the wide histopathologic diversity of GCTs.

Specification of Primordial Germ Cells

Much of what is known about early molecular events in the mammalian germline comes from examination of germ-cell development in the mouse.[2-5] Similar molecular mechanisms of germ cell development appear to operate in humans, based on studies of human embryos and human embryonic stem cells differentiating into germ cells in vitro.[6-10] Development of the germline begins at the time of blastocyst implantation, when the extraembryonic ectoderm and the visceral endoderm send signals to cells in the proximal epiblast, also known as the *embryonic ectoderm* (Fig. 63-1). The major inductive signals are the bone morphogenetic proteins (BMPs), which are members of the transforming growth factor β (TGF-β) superfamily.[11-14] In response to BMPs, some of the epiblast cells begin to express the marker *fragilis*,

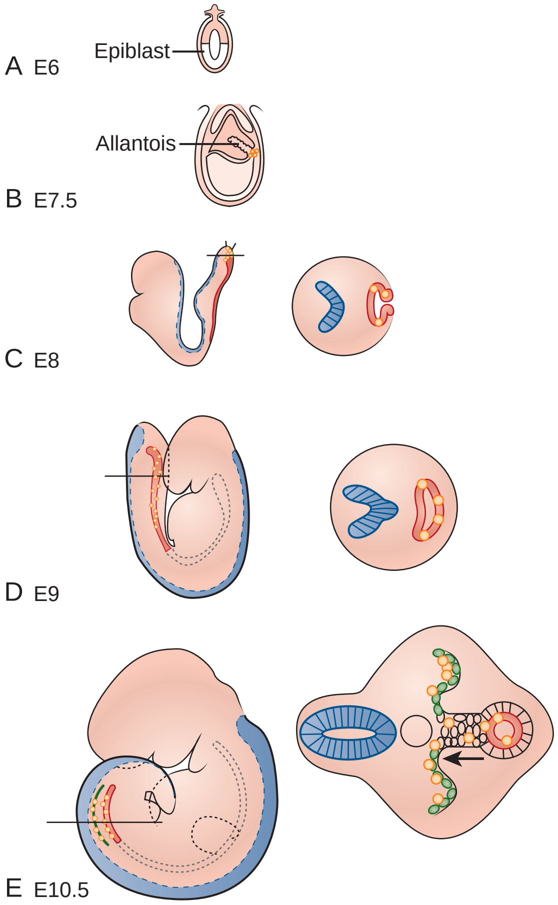

Figure 63-1 Development and migration of PGCs. Stages of embryonic development and primordial germ cell (PGC) migration in the mouse. **A,** At E6 the epiblast *(white)* and extraembryonic tissue *(gray)* are discernable. **B,** By E7.5 the allantois has developed and the PGCs *(yellow)* appear. **C,** At E8 the neural tube *(blue)* and hindgut[70] can are visible. In cross-section *(right side)*, the PGCs can be seen colonizing the hindgut. **D,** At E9 the embryo has turned and the posterior section is to the left; the PGCs are migrating through the hindgut. **E,** At E10.5 the PGCs begin exiting the hindgut and migrate through the dorsal mesentery *(white cells)* to enter the genital ridges *(green)*. The equivalent stages of human embryonic development are: **A,** Weeks 1 and 2; **B,** Week 2.5; **C,** Week 3; **D,** Week 4; **E,** Week 5. *(Modified from Starz-Gaiano M, Lehmann R: Moving towards the next generation.* Mech Dev *105:5–18, 2001.)*

signaling their competence to become germ cells.[2,15] Of these *fragilis*-expressing cells, a few begin to express the transcriptional repressor *Blimp1/Prdm1*.[16] These cells, in which expression of somatic genes such as *Hoxb1, T/Brachyury,* and *Snail* is repressed, will become the primordial germ cells (PGCs). Unlike other epiblast-derived cells, PGCs regain or maintain expression of certain genes associated with pluripotency such as *STELLA, OCT3/4,* and *NANOG*.[2,17-25] Certain pluripotency genes can be reactivated in GCTs and may contribute to malignant potential; for example, *NANOG* and *OCT3/4* have been used as sensitive markers of malignant germ cells in studies of GCTs.[26-30] In humans, PGCs can be identified in the wall of the yolk sac by their intrinsic alkaline phosphatase activity beginning at about day 24. The PGCs begin to proliferate as they migrate out of the yolk sac into the embryo.[31]

Primordial Germ Cell Migration

In humans, as in many other organisms, the PGCs arise in an extraembryonic location, distant from the eventual site at which the gonad will form. This physical separation may serve to insulate the germ cells from various proliferation and differentiation signals in the developing embryo or to enforce a quality control by selecting for healthy PGCs capable of successfully navigating to the developing gonad. Proper PGC migration is critical to the survival of the germ cells and formation of the gonad, and failure of this migration can result in ectopic germ cells. Persistence and malignant transformation of these ectopic germ cells is one possible mechanism by which extragonadal GCTs are thought to arise.[32]

Toward the end of gastrulation, morphogenetic movements in the developing embryo bring the PGCs in proximity to the hindgut.[31] Invading the endoderm, the PGCs colonize the hindgut and begin vigorous, apparently random migratory movements, remaining confined to the gut. The receptor tyrosine kinase c-kit, expressed in PGCs, and its ligand, Steel, expressed in somatic cells, are required for the colonization of the hindgut and for the survival and migration of PGCs within the gut.[33-36] Wylie and co-workers have shown that in mice, PGCs deficient in c-kit signaling undergo apoptosis through the action of the p53-target gene, *Bax*.[36-39] At 5 to 6 weeks after fertilization, the PGCs upregulate expression of the adhesion factor E-cadherin[40] and exit the hindgut on the dorsal side to begin colonization of the genital ridge primordia. The genital ridges are bilateral swellings of mesenchyme covered by coelomic epithelium and situated on either side of the dorsal aorta on the posterior wall of the embryo in the lower thoracic and lumbar regions.[41] The genital ridges appear to attract the PGCs because of their expression of stromal cell–derived factor 1 (SDF-1, or CXCL12), which is a ligand for the chemokine receptor CXCR4, which is expressed in the PGCs.[42,43] As the PGCs exit the hindgut, they divide into two lateral streams to colonize the genital ridges. PGCs that fail to colonize the ridges and remain in the midline are eliminated by apoptosis, as the midline cells downregulate expression of Steel.[39] Once they have entered the genital ridges, PGCs become much less motile but continue several more rounds of division. This proliferation is dependent on continued Steel/c-kit signaling.[31,38,39,42,44]

Erasure of Imprinting

Imprinting refers to the epigenetic modification of certain genes (typically by cytosine methylation) such that only the maternal or paternal allele of the gene is expressed.[45-47] Lineage-specific patterns of imprinting are established in different tissues, including the germline, around the time of gastrulation.[46,48] Upon entering the gonadal ridges, however, PGCs actively erase these genomic methylation patterns. This erasure is necessary in order to allow the maternal and paternal imprinting patterns to be established in the oocytes and sperm, respectively. Imprinting is reestablished in sex-specific patterns during gonadogenesis.[49-51] GCTs exhibit partial or total erasure of imprinting, implying their origin from early germ cells.[1]

Gonadogenesis

Beginning shortly before the arrival of the PGCs in the genital ridges, the coelomic epithelium begins to proliferate and invade the underlying mesenchyme, forming the primitive sex cords. Migrating PGCs entering the gonad are surrounded by the cords. At this early stage, the appearance of the developing gonad is identical in males and females, and the tissue is referred to as the *indifferent gonad*. Subsequently, changes occur in both the germ cells and the gonadal somatic cells according to the genetic sex of the embryo. In genetic males, sex determination occurs under the influence of the testis-determining *SRY* gene on the Y chromosome.[52,53] After the initial rounds of cell division, male germ cells enter a mitotic arrest that persists until after birth.[14] In males, the primitive sex cords proliferate and penetrate deeper into the mesenchyme, forming the testis or medullary cords, which connect proximally to form the rete testis. By the fourth month of development, the cords consist of germ cells and the supporting Sertoli cells, which are derived from the surface epithelium of the genital ridge. The cords remain solid until puberty, when they form lumens to become seminiferous tubules. By the eighth week of development the mesenchyme of the gonadal ridge gives rise in males to Leydig cells, which secrete testosterone. Secretion of antimüllerian hormone by the Sertoli cells and testosterone by the Leydig cells leads in males to degeneration of the paramesonephric ducts and the development of the mesonephric ducts into the vas deferens and epididymis. Testosterone is also necessary for male differentiation of the external genitalia.

Female gonadal development proceeds along different lines. In the absence of the Y chromosome, the sex cords initially degenerate and are replaced by a new set of cortical sex cords derived from the surface epithelium. By the fourth month the cortical sex cords have become isolated clusters with each cluster surrounding a single germ cell. In females the primitive germ cells, called *oogonia,* continue rapid proliferation, reaching maximal numbers by the seventh month. After that point, most of the oogonia degenerate; the remaining cells, now called *primary oocytes,* enter meiosis and arrest at the diplotene stage of the first meiotic prophase. Granulosa cells (derived from the surface epithelium) and thecal cells (derived from the mesenchyme) together form the follicle cells that surround each primary oocyte. Beginning at adolescence, groups of oocytes periodically resume meiosis. In females, the paramesonephric (müllerian) ducts develop into the oviducts, uterus, cervix, and upper part of the vagina. The mesonephric ducts degenerate.

In summary, development of the germline requires the proper specification of PGCs and their migration through the embryo to the gonadal ridges, where the gonads are formed through interactions of the germ cells with somatic cells. Concomitant with this process, the inherited pattern of genomic imprinting is erased, and a new, sex-specific pattern is formed. The complex process of gonadal organogenesis is subject to both genetic and environmental influences. Abnormal development of the gonads during the embryonic and fetal periods leads to defects such as cryptorchidism and gonadal dysgenesis, which are strongly associated with the risk of developing GCTs.

HISTOPATHOLOGY OF GERM CELL TUMORS

GCTs are a heterogeneous group of neoplasms with a wide variety of histopathologic features. This variety reflects the pluripotent nature of the PGCs from which GCTs arise.[1,54] Adding to the complexity of this tumor type, GCTs with apparently similar histopathology can have very different biologic behaviors when presenting at different ages or anatomic sites. The five major histologic subtypes of GCTs are teratoma, yolk sac tumor (YST), germinoma, embryonal carcinoma, and choriocarcinoma. The World Health Organization (WHO) classification of GCTs is presented in Boxes 63-1 and 63-2.

Overview of Testicular Germ Cell Tumors

Before puberty in infants and children, teratoma and/or YST account for the vast majority of GCTs.[55-57] *Seminoma* (the term used for a testicular germinoma), embryonal carcinoma, and choriocarcinoma are very rare in this age group. GCTs of the prepubertal testis differ from their adult counterparts not only in the distribution of histologic subtypes, but also in their spectrum of cytogenetic abnormalities. After the early peak of testicular YST and teratoma in the 0-to-4–years age group, testicular tumors are relatively rare until the onset of puberty. Between ages 10 and 40 years, there is a second peak in testicular tumor incidence, comprised of seminoma and nonseminomas (including embryonal carcinoma, teratoma, YST, and choriocarcinoma). Postpubertal tumors demonstrate a wider array of histopathologic differentiation than do the GCTs of the prepubertal testis. Moreover, adolescent and adult GCTs share a common precursor cell of origin (the carcinoma in situ [CIS] or intratubular germ cell neoplasia, unclassified [IGCNU]), as well as characteristic cytogenetic abnormalities including the near-universal presence of amplifications of chromosome 12p. These histopathologic and molecular cytogenetic differences have lent support to the hypothesis that different pathogenic mechanisms underlie juvenile and adult testicular GCTs.[58-60]

Overview of Ovarian Germ Cell Tumors

In neonates and infants, the most common ovarian lesions are ovarian cysts.[61] Cysts can occur any time from late gestational stages onwards, and they occur at a greater rate in babies of mothers with diabetes, preeclampsia, and Rh immunization. Ovarian cysts in children often resolve

Box 63-1 **World Health Organization Classification of Germ Cell Tumors: Male**

GERM CELL TUMORS

Intratubular germ cell neoplasia, unclassified
Other types

TUMORS OF ONE HISTOLOGIC TYPE

- Seminoma
  - Seminoma with syncytiotrophoblastic cells
  - Spermatocytic seminoma
  - Spermatocytic seminoma with sarcoma
- Embryonal carcinoma
- Yolk sac tumor
- Trophoblastic tumors
  - Choriocarcinoma
  - Trophoblastic neoplasms other than choriocarcinoma
    - Monophasic choriocarcinoma
    - Placental site trophoblastic tumor
- Teratoma
  - Dermoid cyst
  - Monodermal teratoma
  - Teratoma with somatic type malignancies

TUMORS OF MORE THAN ONE HISTOLOGIC TYPE

- Mixed embryonal carcinoma and teratoma
- Mixed teratoma and seminoma
- Choriocarcinoma and teratoma/embryonal carcinoma
- Others

SEX CORD/GONADAL STROMAL TUMORS

PURE FORMS

- Leydig cell tumor
- Malignant Leydig cell tumor
- Sertoli cell tumor
  - Sertoli cell tumor lipid rich variant
  - Sclerosing Sertoli cell tumor
  - Large cell calcifying Sertoli cell tumor
- Malignant Sertoli cell tumor
- Granulosa cell tumor
  - Adult type granulosa cell tumor
  - Juvenile type granulosa cell tumor
- Tumors of the thecoma/fibroma group
  - Thecoma
  - Fibroma
- Sex cord/gonadal stromal tumor, incompletely differentiated
- Sex cord/gonadal stromal tumor, mixed forms
- Malignant sex cord/gonadal stromal tumor
- Tumors containing both germ cell and sex cord/gonadal stromal elements
  - Gonadoblastoma
  - Germ cell-sex cord/gonadal stromal tumor, unclassified

Box 63-2 **World Health Organization Classification of Germ Cell Tumors: Female**

GERM CELL TUMORS

- Dysgerminoma
- Yolk Sac Tumor
  - Polyvesicular vitelline tumor
  - Glandular variant
  - Hepatoid variant
- Embryonal carcinoma
- Polyembryoma
- Nongestational choriocarcinoma
- Mixed germ cell tumor

BIPHASIC OR TRIPHASIC TERATOMA

- Immature teratoma
- Mature teratoma
  - Solid
  - Cystic
    - Dermoid cyst
  - Fetiform teratoma

MONODERMAL TERATOMA AND SOMATIC-TYPE TUMORS ASSOCIATED WITH DERMOID CYSTS

- Thyroid (Struma ovarii)
- Carcinoid
- Neuroectodermal
- Carcinoma
- Melanocytic
- Sarcoma
- Sebaceous tumors

GERM CELL SEX CORD-STROMAL TUMORS

- Gonadoblastoma
  - Variant with malignant germ cell tumor
- Mixed germ cell sex cord-stromal tumor
  - Variant with malignant germ cell tumor

SEX CORD-STROMAL TUMORS

- Granulosa-stromal cell tumor
  - Granulosa cell tumor
    - Adult type granulosa cell tumor
    - Juvenile type granulosa cell tumor
- Tumors of the thecoma/fibroma group
  - Thecoma
  - Fibroma
- Sertoli-stromal cell tumors
  - Sertoli cell tumor
  - Stromal-Leydig cell tumor
- Sex cord-stromal tumors of mixed or unclassified types
- Steroid-cell tumors
  - Leydig cell tumor
  - Steroid cell tumor, not otherwise specified

spontaneously without intervention.[62] GCTs account for 75% of ovarian tumors in the first 2 decades of life and up to 90% of ovarian tumors in premenarchal girls. The majority of these (95%) are benign, mature teratomas, mostly commonly "desmoid" tumors.[63] Five percent of ovarian GCTs are malignant; these include dysgerminomas, immature teratomas, mature teratomas with somatic malignancies, YSTs, choriocarcinoma, embryonal carcinoma, polyembryoma, and mixed GCTs. Finally, gonadoblastoma is a mixed germ cell–sex cord stromal tumor occurring in cases of mixed gonadal dysgenesis with ambiguous genitalia, or 45,X Turner syndrome with Y chromosome material.[64-67] The histopathology of ovarian GCT types is similar to that of their testicular counterparts. After the third decade of life, ovarian GCTs occur rarely, and carcinomas derived from the coelomic epithelial covering of the ovary are much more common.[68]

Intratubular Germ Cell Neoplasia, Unclassified

IGCNU,[69] also called *testicular CIS,*[70] is considered the precursor for all invasive testicular GCTs (TGCTs) of postpubertal males[59,71-76] except spermatocytic seminoma.[77] According to one source, if left untreated IGCNU has a up to a 50% probability of progressing to invasive TGCT, either seminoma or nonseminoma, within 5 years.[74] Nonseminomas can be composed of embryonal carcinoma (the stem cell component), teratoma (somatic differentiation), and YST and choriocarcinoma (extraembryonic lineages). In IGCNU, germ cells with abundant vacuolated cytoplasm; large, irregular nuclei; and prominent nucleoli are found within the seminiferous tubules, showing similarities to PGCs and early gonocytes.[69] IGCNU is found in 1% of cases of male infertility and in 2% to 4% of cryptorchid testes in adults.[69,73,76,78-81] In men with a TGCT, the prevalence of IGCNU is about 80% in the ipsilateral testis (range, 63% to 99%],[79,82] predominantly in men with nonseminomas,[83] and 5% in the contralateral testis.[71,84,85] Various markers such as placental alkaline phosphatase (PLAP), c-kit, transcription factor AP-2γ, OCT3/4 (POU5F1), and testis-specific protein Y-encoded (TSPY) have been extensively used for immunohistochemical detection of IGCNU.[26,30,79,86-93] All these markers are also found in PGCs and/or gonocytes, which supports the model that IGCNU derives from PGCs or gonocytes.[1,30,94]

Whether an identifiable precursor lesion exists for GCTs of children is much less clear. In prepubertal children, the incidence of IGCNU appears to be very low,[58,95,96] although several cases have been reported.[94,97-99] These data must be interpreted with caution, however, because of the difficulty of distinguishing IGCNU in the developing gonad, in which primitive germ cells normally express markers such as *OCT3/4* and *c-KIT*.[94] The precursor lesion for the teratomas and YSTs of neonates and infants is yet to be identified. These pediatric tumors show partially erased imprinting, suggesting that the cell of origin is most likely a germ cell at an earlier development stage than that of IGCNU.[100-102]

IGCNU has been reported in a high percentage of gonadal biopsy specimens from children with disorders of sexual development (DSDs) who are at risk for the development of germ cell malignancies.[103,104] These disorders include gonadal dysgenesis,[105,106] partial androgen insensitivity syndrome (AIS),[107] and less often, complete AIS.[66,108-110] Development of these tumors in patients with DSDs is linked to the presence of a specific fragment of the Y chromosome, known as the *GBY region.*[29,93,111-114] For these patients with an increased risk of germ cell malignancy, prophylactic gonadectomy is often indicated.[103,115] However, overdiagnosis of IGCNU is possible

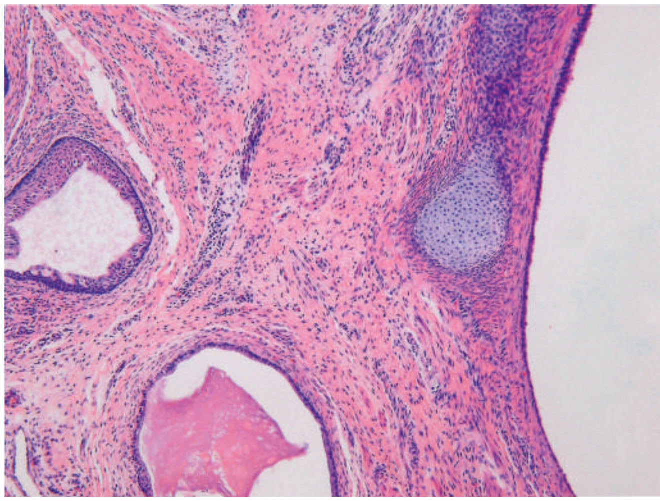

Figure 63-2 Mature teratoma. Low-power view showing cystic tumor with areas of cartilaginous differentiation.

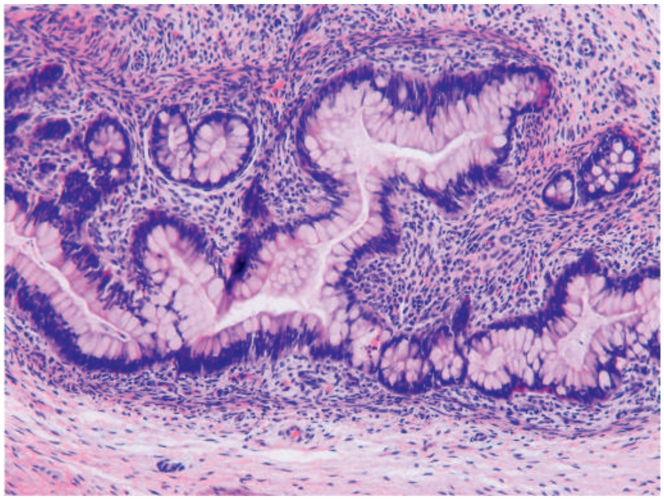

Figure 63-3 Mature teratoma with glandular differentiation.

in this setting because of the common maturation delay of germ cells in patients with DSDs, resulting in retained presence of *c-kit*-positive germ cells.[21,116,117] Therefore careful examination of the distribution pattern of *OCT3/4*-positive cells and their position within the seminiferous tubule are essential to making the diagnosis of IGCNU.[66,110,116]

TERATOMA

Histopathology of Teratoma

Teratomas are tumors composed of multiple tissues that are normally foreign to, and able to proliferate in excess of, the site in which they occur.[118] In the usual definition of *teratoma,* tissue elements of all three germ layers (endoderm, mesoderm, and ectoderm) are present; however, teratomas also occur in which only one or two of the germ layers are present (referred to as *monodermal* or *bidermal teratomas,* respectively). The clinical and biologic behavior of teratomas varies significantly with differing anatomic location, degree of maturity of the tumor tissue, and age of onset. Grossly, teratomas are nodular and heterogeneous with solid and cystic areas depending on the types of differentiated tissue present. Cartilage, bone, hair, and pigmented areas may be recognizable (Fig. 63-2). At the histopathologic level, ectodermal derivatives such as neuroepithelium, skin, and hair are the most common tissues in teratomas of infants and children. However, practically any tissue type can be seen, including muscle, bone, cartilage, and well-differentiated glands (Fig. 63-3).

Mature teratomas are typically cystic and contain differentiated adult-type tissue from one or more of the germ layers. The term *dermoid cyst* refers to the common finding in mature teratomas of tissue resembling the adult epidermis and its appendages. Immature teratomas are those in which embryonal-appearing tissue is present, typically in the form of neuroepithelial rosettes and tubules and often mixed with mature tissue (Fig. 63-4). A loose, myxoid stroma of immature mesenchyme with focal differentiation into osteoid, fat, cartilage, and rhabdomyoblasts may be present.[119] Hypercellularity, increased mitotic index and nuclear atypia can be present. The prognostic significance of these findings is different for ovarian and testicular teratomas,[56] and these are discussed separately in the next sections.

Testicular Teratoma

Testicular Teratoma in Infants and Children

In most series, testicular teratoma is the second most common GCT of the prepubertal testis, representing about 35% of GCTs.[56,60,120-123] However, the actual incidence may be higher, because prepubertal testicular teratomas are uniformly benign and thus may be underreported to tumor registries.[122-125] Histologically, 85% of prepubertal testicular teratomas are mature, and 15% are immature. Unlike the case of ovarian teratomas, the

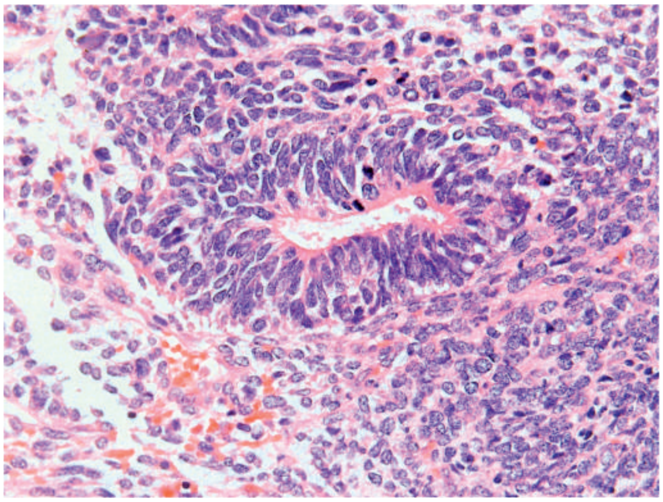

Figure 63-4 Immature teratoma. Immature teratomas are graded according to their content of primitive neuroepithelium. In this high-power view, the rosette formation characteristic of these tumors can be seen.

degree of histologic immaturity of childhood testicular teratoma does not carry prognostic significance. In this age group, pure testicular teratomas lack metastatic potential and have not been found to recur after surgical removal of the tumor.[56,126-129] A review of cases of immature teratoma from Pediatric Oncology Group (POG)/Children's Cancer Study Group (CCG) protocols concluded that the presence of microscopic foci of YST, rather than the grade of immature teratoma, is the only valid predictor of recurrence in pediatric immature teratoma at any site.[130] The benign nature of these tumors has permitted the widespread use of testis-sparing enucleation surgery for prepubertal testicular teratomas.[58,124,131-133] In agreement with the benign clinical behavior of pure teratomas of the prepubertal testis, these tumors display normal karyotypes and normal results from comparative genomic hybridization (CGH).[123,134,135] Epidermoid cysts are rare tumors in the prepubertal testis and are most likely monodermal teratomas showing ectodermal differentiation.[63,124,136,137] These tumors, which have a characteristic ultrasonographic appearance, are hormonally inactive and do not produce α-fetoprotein (AFP);[124] as with other teratomas of the prepubertal testis, they are managed conservatively with testis-sparing surgery.[124,136,138] In the prepubertal testis, the seminiferous tubules surrounding the teratoma may contain germ cells with atypical features such as enlarged nuclei. However, these features are distinct from the seminoma-like changes of IGCNU, and do not signify malignant transformation of the germ cells.[139,140]

Testicular Teratoma in Adolescents and Adults

Teratoma occurs as a component of 50% of mixed nonseminomas in the postpubertal testis. Pure teratoma is rare, accounting for only 2% to 3% of postpubertal TGCTs.[141,142] Testicular teratomas in adolescents and adults may spread, giving rise to teratomatous and nonteratomatous metastases.[55,69,133] Furthermore, adult testicular teratomas typically are associated with IGCNU and exhibit characteristic cytogenetic abnormalities such as isochromosome 12p that are also found in seminomas and in other types of nonseminomatous TGCTs. Based on these findings, Ulbright proposed that teratomas in the adult testis arise from a germ cell that has already undergone malignant transformation; thus the histogenesis of adult teratoma is distinct from that of teratomas arising in the ovary or the prepubertal testis.[133,143] Postpubertal teratomas may contain syncytiotrophoblastic giant cells, and patients with teratoma may have gynecomastia[144] or elevated serum β-human chorionic gonadotropin (β-HCG) or AFP.[145,146]

Ovarian Teratoma

Teratoma is the most common GCT of the ovary, comprising more than 95% of all ovarian GCTs.[63] Ovarian teratomas differ in important ways from testicular teratomas. Teratomas in the prepubertal testis are uniformly benign. In adolescents and adults, testicular teratomas are nearly always malignant and occur as a component of mixed malignant GCTs (MGCTs), the exception being dermoid cysts of the testis. In the ovary, in contrast, the phenotype of teratomas does not vary as strikingly by age, and ovarian teratomas are usually pure tumors.[147,148] The great majority of ovarian tumors are mature, benign tumors (often referred to as *dermoid cyst* or *mature cystic teratoma*).[133,143,149,150] Less than 5% of ovarian teratomas are malignant; these are discussed separately in the following sections.

Benign, Mature Ovarian Teratoma

Mature ovarian teratomas are diploid, cytogenetically normal tumors.[123,151-154] This contrasts to postpubertal testicular teratomas, which are aneuploid and demonstrate cytogenetic abnormalities such as i12p. Interestingly, when polymorphic molecular markers are assayed, a high percentage of mature ovarian teratomas show a homozygous pattern, indicating that these tumors can arise from a germ cell that has completed meiosis I but not meiosis II.[107,134,151,155-159] Mature ovarian teratomas are usually cystic, though solid tumors can occur, especially in the first two decades.[160,161] Other characteristics of mature ovarian teratomas include a well-ordered, "organoid" appearance to the differentiated tissues within the teratoma, and the absence of cytologic atypia.[162] Mature ovarian teratomas are uniformly benign, except in the rare case of postteratomatous "malignant degeneration" of a dermoid cyst.[133,162,163]

Malignant Ovarian Teratoma

In children, immature teratoma is the most common type of malignant ovarian teratoma. Also in this category are other tumors that are rare in children, such as monodermal teratomas with malignant elements (e.g., papillary thyroid carcinoma in struma ovarii), dermoid cysts with malignant degeneration, and the rare case of mixed malignant ovarian GCT (MOGCT) with a teratoma component.

Immature Teratoma

The term *immature teratoma* refers to teratomas containing immature-appearing tissues, principally neuroepithelium.[164] Additionally, foci of mitotically active glia may be present. A grading system has been developed for immature teratomas based on the amount of neuroepithelial tissue present.[119,165] Mature teratomas containing only fully differentiated tissue are considered grade 0, and immature teratomas range from grade 1 (comprised of less than 10% immature neuroepithelium) to grade 3 (more than 50% immature neuroepithelium present). A two-component grading system (low grade and high grade) has also been proposed and shown to have improved reproducibility.[166] In adult ovarian teratomas, the finding of grade 2 or 3 immature teratoma predicts the likelihood of metastasis and confers a worse prognosis.[119,160,166,167] In children, however, it is not clear that the same relationship of grade to outcome of immature teratomas holds true. A POG study concluded that surgery alone was curative in children and adolescents with stage I immature teratomas of any grade and that chemotherapy should be reserved for cases of relapse.[130,168]

Immature teratomas may be associated with implants of glial tissue on the peritoneum, known as *gliomatosis* *peritonei*.[169-171] If the implants are composed solely of mature tissues, the presence of gliomatosis peritonei does not "upstage" the patient.[172,173] Interestingly, data suggest that the implants arise from metaplastic transformation of pluripotent müllerian stem cells in the peritoneum or underlying mesenchyme, rather than representing metastasis of the teratoma tissue.[174-176]

Monodermal Teratomas

Monodermal teratomas consist largely or exclusively of a single type of tissue, such as thyroid, carcinoid, and neuroectodermal tissues.[63,177-180] Monodermal teratomas with neuroectodermal differentiation include indolent types such as ependymoma and poorly differentiated forms such primitive neuroectodermal tumor (PNET), as well as anaplastic tumors resembling glioblastoma.[123,181-185] These tumors are rare in children.

Teratoma with Malignant Transformation

Teratoma with malignant transformation makes up 0.2% to 1.4% of mature cystic tumors of the ovary.[133,163,186] In these tumors, more commonly seen in older women, somatic-type malignancies are seen among the individual tissues in the teratoma. Squamous cell carcinomas are the most common tumor type,[187,188] but others including melanoma, adenocarcinoma, small cell carcinoma, sarcomas (including PNET), and malignant glioma may also occur.[133,162]

YOLK SAC TUMOR

Histopathology of Yolk Sac Tumor

YST is the most common malignant GCT in infancy and childhood.[56,57,118,122,189] The differentiation of the tumor cells is predominantly along endodermal lines and can take the form of both intraembryonic endodermal derivatives, such as primitive gut and liver, and extraembryonic structures such as allantois and yolk sac. There are many synonyms for YST, including *orchioblastoma, Teilum's tumor,* and *clear cell adenocarcinoma*. Although *endodermal sinus tumor* is commonly in use as a synonym for YST, some consider the term problematic, because the endodermal sinus is not a component of normal human embryogenesis.[190] In macroscopic appearance, YSTs are yellowish, with multiple cysts and areas of hemorrhage, necrosis, and liquefaction.[162] Microscopically, YSTs display a loose, myxoid stroma containing a reticulated pattern of microcystic spaces (Fig. 63-5). The cysts are lined by a flattened, periodic acid-Schiff (PAS)–positive, diastase-resistant epithelium. About 20% of YSTs exhibit characteristic Schiller-Duval bodies (a clustering of cells around a small, central blood vessel) (see Fig. 63-5). In addition to the microcystic/reticular pattern, several variant histologies of YST are described, including the solid, polyvesicular, and parietal types (corresponding to primitive endoderm and extraembryonic structures such as the allantois and yolk sac) and the glandular and hepatic types (corresponding to the intraembryonic endodermal derivatives, primitive lung and liver).

YSTs are typically cytokeratin-positive, which can help differentiate the solid YST variant from germinomas.[162]

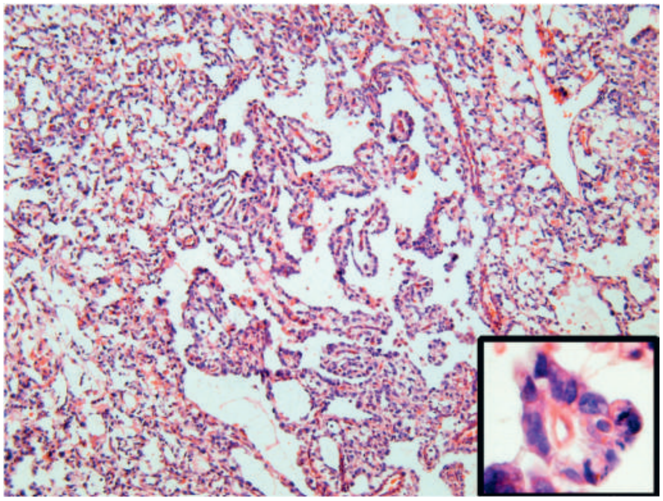

Figure 63-5 YST. A loose, myxoid stroma is filled with a labyrinthine network of cysts lined by clear, flattened epithelial cells. *Inset,* Schiller-Duval body.

Alpha-fetoprotein (AFP) expression is characteristic of these tumors, and AFP is a valuable tumor marker. Neonates and infants have high physiologic levels of AFP in serum, which can confound the use of serum AFP levels for diagnosis of YST or for monitoring therapy. Tables of normal serum AFP levels in neonates and infants have been published (Fig. 63-6).[191-193] In addition, at least 70% of YSTs have detectable AFP expression by immunohistochemical assays.[194-198]

Testicular Yolk Sac Tumor

Testicular Yolk Sac Tumor in Infants and Children

YST[199] is the most common malignant GCT in infants and children, comprising approximately 65% of prepubertal testicular GCTs.[121,200] YST is most common in males aged 0 to 2 years and can occur in pure form or as a malignant component of teratoma.[56] Histologically, prepubertal YSTs show the same spectrum of patterns as their adult counterparts: pseudopapillary, reticular, polyvesicular vitelline, and solid, with the pseudopapillary pattern being most common in children.[201] IGCNU is not a feature of childhood YST, and it is unlikely that P53 mutations play a role in pathogenesis.[95,96,135] Unlike childhood teratomas, YSTs in children are aneuploid, with characteristic, nonrandom chromosomal abnormalities including gains at 3p and losses at 1p and 6q.[135,202,203] Immunohistochemistry detects AFP expression in more than 90% of YSTs, and in most cases the serum AFP level is also elevated.[56,195,198,204-208]

Testicular Yolk Sac Tumor in Adolescents and Adults

YST in the adolescent and adult testis is extremely rare in the pure form but occurs in approximately 40% of mixed TGCTs.[209] In one series of primary mediastinal GCTs, YST was present in nearly 50% of the tumors.[210] As in childhood YST, hematogenous metastases are common, especially to the liver. The majority of tumors

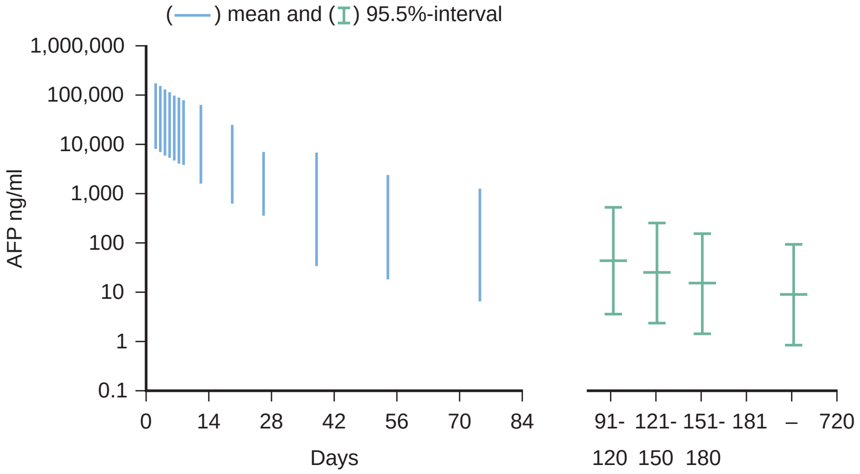

Figure 63-6 Serum AFP values in term babies, mean *(–)* and 95.5% interval *(I)*. *(From Blohm ME, Vesterling-Horner D, Calaminus G, Gobel U: Alpha 1-fetoprotein [AFP] reference values in infants up to 2 years of age.* Pediatr Hematol Oncol *15:135–142, 1998.)*

stain positive for AFP, and serum AFP is commonly elevated.[211]

Ovarian Yolk Sac Tumor

YST of the ovary is a clinically aggressive tumor that often presents at advanced stages with metastasis to lymph nodes or peritoneal structures. The tumors are rarely bilateral.[118,196,212,213] The median age at diagnosis is 18 to 19 years.[61,214-216] In a series of 26 pediatric patients, reticular type was the most common histology identified.[196]

SEMINOMA AND DYSGERMINOMA

Histopathology of Seminoma and Dysgerminoma

Seminomas are a class of GCTs in which the tumor cells resemble primitive, undifferentiated germ cells, both in morphology and in expression of pluripotency genes such as *OCT3/4, NANOG,* and *STELLAR/DPPA3*.[28,30,91,217-219] These tumor are referred to as *germinomas* when arising in extragonadal locations (such as the pineal gland, the mediastinum, or the retroperitoneum), *dysgerminomas* when arising in the ovary, and *seminomas* when arising in the testis. The histology of seminoma is identical regardless of the site in which the tumor occurs. Grossly, these are rounded, nodular tumors, separated into lobules by fibrous bands. Microscopically, seminomas are characterized by a monotonous proliferation of large, uniform cells with large central nuclei and prominent nucleoli (Fig. 63-7). The cytoplasm is clear or granular and often PAS-positive because of high glycogen content. A lymphocytic infiltrate is a typical feature of seminomas.

Seminoma

Seminoma is extremely rare in children. In the postpubertal testis, seminoma can occur in pure form or as a component of mixed GCT.[69,143,220] The peak age of onset of seminoma is 34 to 45 years, slightly later than that of nonseminomatous tumors.[69,221] Several distinct variants of seminoma have been recognized. Up to 7% of classic seminomas contain syncytiotrophoblastic giant cells, and almost 25% of seminomas contain foci that stain positive for β-human chorionic gonadotropin (HCG).[222-225] These tumors may be accompanied by an elevation in serum β-HCG; however, the presence of syncytiotrophoblasts or elevated β-HCG does not worsen the prognosis.[226-228] Marked elevations of β-HCG or persistently elevated β-HCG after orchiectomy may indicate the presence of choriocarcinoma.[229] *Anaplastic* or *atypical seminoma* is the designation used for seminomas displaying increased mitotic activity, nuclear pleomorphism, and sparse lymphocytic infiltrate on histologic examination.[230-233] Whether these aggressive features portend a worse prognosis or a higher likelihood of metastasis remains controversial. Whereas some studies have suggested that anaplastic seminomas are clinically more aggressive tumors,[234,235] others have found no evidence of a worse prognosis when these features are present.[226,230,236]

Dysgerminoma

Dysgerminoma is the most common MOGCT of children and adolescents, and makes up one third of

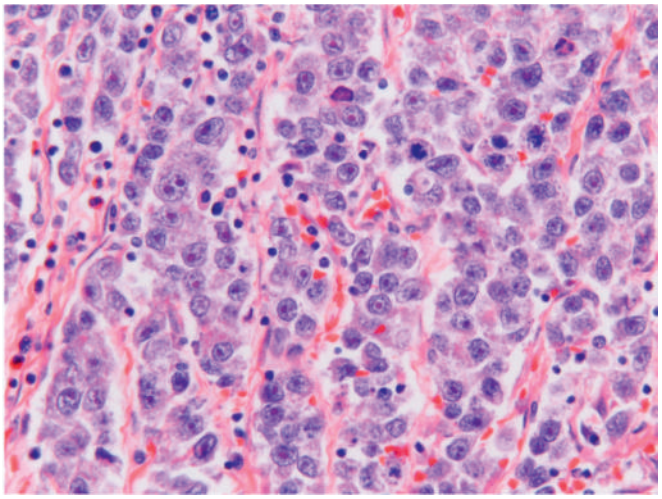

Figure 63-7 Dysgerminoma. Germinomas consist of sheets of large, uniform cells with clear or granular cytoplasm, large central nuclei and prominent nucleoli. A lymphocytic infiltrate is a typical feature of germinomas. The histologies of ovarian dysgerminoma, testicular seminoma, and extragonadal germinoma are identical.

MOGCTs.[237-239] Pathologically, dysgerminoma is the ovarian counterpart of the seminoma of the testis and the germinoma of extragonadal sites. Unlike seminomas of the testis, which are rare in the prepubertal period, dysgerminomas can occur at any age, though the peak incidence is age 15 to 19 years.[239-242] Also unlike seminomas, which develop from IGCNU, dysgerminomas do not appear to arise from a precursor cell.[162] Dysgerminomas stain positively for *OCT4* on immunohistochemistry, which can be useful for distinguishing these tumors from nondysgerminomas.[243,244] As with seminomas, the majority of dysgerminomas also stain positive for *c-KIT.*[245]

EMBRYONAL CARCINOMA

Histopathology of Embryonal Carcinoma

Embryonal carcinoma is very rare in infants but can occur in prepubertal females and adults of both sexes. Embryonal carcinomas commonly have areas of hemorrhage and necrosis and can be locally invasive into the epididymis and spermatic cord in males.[69,162] At the microscopic level, the tumors consist of large, epithelial-appearing cells that resemble the early embryonic cells of the inner cell mass (Fig. 63-8). The tumor cells may grow in solid, papillary, and reticular patterns, forming many clefts and glandlike structures. The tumors are typically cytokeratin and CD30-positive, but negative for epithelial membrane antigen (EMA), carcinoembryonic antigen, and vimentin.[162,246] Syncytiotrophoblast cells, when present in the tumor, can produce β-HCG and cause precocious pseudopuberty in premenarchal girls or vaginal bleeding in older women.[194]

Testicular Embryonal Carcinoma in Adolescents and Adults

Embryonal carcinoma is the most common pure nonseminomatous TGCT in adults, representing about 2% to 10% of cases in this age group. Additionally, 80% of mixed malignant TGCTs contain embryonal carcinoma as one component.[247,248]

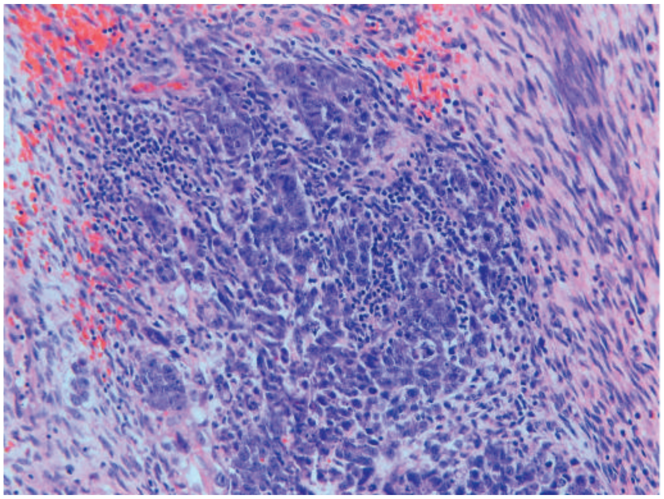

Figure 63-8 Embryonal carcinoma. Embryonal carcinomas consist of large, irregular epithelial-like cells with indistinct borders. The designation *embryonal carcinoma* derives from the resemblance of the tumor cells to the early embryonic cells of the inner cell mass.

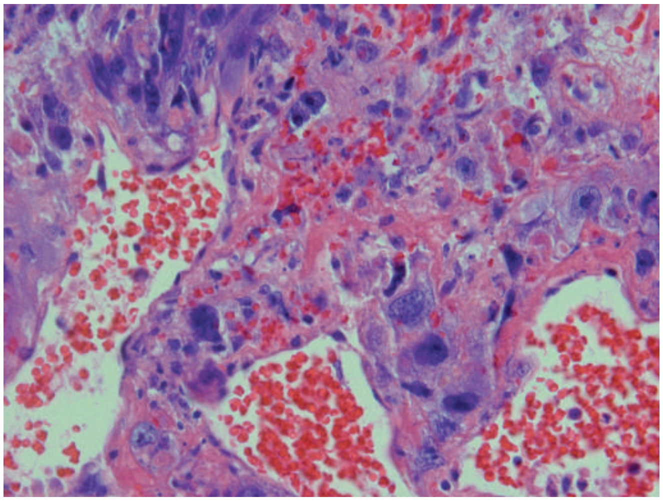

Figure 63-9 Choriocarcinoma. Choriocarcinomas are characterized by a mixture of syncytiotrophoblastic, cytotrophoblastic, and intermediate trophoblastic cells, with multiple areas of hemorrhage.

Ovarian Embryonal Carcinoma

Embryonal carcinoma occurs in the ovary less commonly than in the testis, accounting for 4% of malignant ovarian tumors.[194,196] The median age at diagnosis is 14 years, somewhat younger than ovarian YST. Ovarian embryonal carcinoma is more often associated with β-HCG production and with hormonal manifestations such as precocious puberty, amenorrhea, and hirsutism.[194] Owing to the totipotent nature of embryonal carcinoma cells, differentiation into various histologies may be seen including teratomas and syncytiotrophoblastic giant cells resembling choriocarcinoma. Polyembryoma is a rare tumor in which appear multiple embryoid bodies resembling presomite stage embryos. Polyembryoma is considered by some to be an organoid variant of embryonal carcinoma,[162] though others describe it as one component of mixed GCTs.[249,250]

CHORIOCARCINOMA

Choriocarcinoma is a rare tumor composed of cytotrophoblast, syncytiotrophoblast, and extravillous trophoblast. The tumor can cause anemia and bleeding symptoms, as well as pseudoprecocious puberty because of expression of β-HCG by the tumor cells.[118] Choriocarcinomas are usually large tumors with prominent foci of hemorrhage. The histopathology of choriocarcinoma is a mixture of syncytiotrophoblastic, cytotrophoblastic, and intermediate trophoblastic cells, often mixed in random fashion surrounding areas of hemorrhage and necrosis (Fig. 63-9).[69] Vascular invasion is a common feature of choriocarcinomas.

Testicular Choriocarcinoma in Adolescents and Adults

Choriocarcinoma, like YST, is rare in pure form but occurs in approximately 8% of mixed TGCTs in adults.[247,251]

Ovarian Choriocarcinoma

Primary (nongestational) choriocarcinoma occurs rarely as a pure tumor and more often as a component of mixed ovarian GCT, usually in association with teratoma.[238] Choriocarcinoma in infants may arise as a primary tumor in the lung, liver, brain, kidney, or other location.[118,252,253] Treatment of infantile choriocarcinoma is a medical emergency (see "Infantile Choriocarcinoma: A Rare but Rapidly Fatal Condition if Not Recognized.") Gestational choriocarcinoma arises in the placenta and may metastasize widely in the mother; case reports describe metastasis of gestational choriocarcinoma to the infant.[254] In patients in the reproductive years, deoxyribonucleic acid (DNA) analysis to detect the presence of paternal sequences can be helpful in determining the placental origin of gestational choriocarcinoma.[255,256]

MIXED OVARIAN GERM CELL TUMOR

Mixed ovarian GCTs are composed of two or more of the malignant germ cell histologic subtypes. These tumors comprise 8% of malignant GCTs of the ovary in children and adolescents and 20% of ovarian malignant GCTs overall.[61,257,258] Mixed GCTs thus represent only about 1% of all ovarian GCTs in contrast to the postpubertal testis, in which one third of GCTs are of the mixed type.[63,69,143] Dysgerminoma, YST, and teratoma are the most common germ cell components, with embryonal carcinoma and choriocarcinoma more rarely present.[63,216]

Pathology of Mixed Germ Cell-Sex Cord Tumors

Gonadoblastoma

Gonadoblastomas contain mixtures of two cell types: large cells resembling primitive germ cells and smaller cells, similar to immature Sertoli and granulosa cells (Fig. 63-10). It occurs most exclusively in cases of mixed gonadal dysgenesis with ambiguous genitalia, or 45,X Turner syndrome with Y chromosome material.[64,65,67,110] Gonadoblastoma can occur as single or multiple nodules; foci of calcification are a characteristic finding.[118] Microscopically, nests of germ cells within basement membrane material are surrounded by supportive cells (i.e., Sertoli-granulosa cells). The stroma may have elements resembling Leydig or luteinlike cells.[259] The presence of gonadoblastoma substantially increases the risk that a malignant GCT will develop in a dysgenetic gonad. From several studies, the average incidence of GCTs in gonadal biopsy specimens from patients with mixed gonadal dysgenesis is 12%.[66] Gonadoblastoma gives rise most often to dysgerminoma, and less commonly to embryonal carcinoma, teratoma, YST, and choriocarcinoma. The germ cells in dysgenetic gonads abnormally retain their embryonic characteristics, as demonstrated by their marker expression profile, including *OCT3/4* and *TSPY*. These cells give rise to gonadoblastoma in areas of undifferentiated gonadal tissue within the dysgenetic gonad, whereas IGCNU arises in regions with testicular differentiation.[*] Thus just as IGCNU is the precursor lesion for invasive TGCTs, gonadoblastoma can be considered a precursor lesion to the invasive GCTs of dysgenetic gonads.

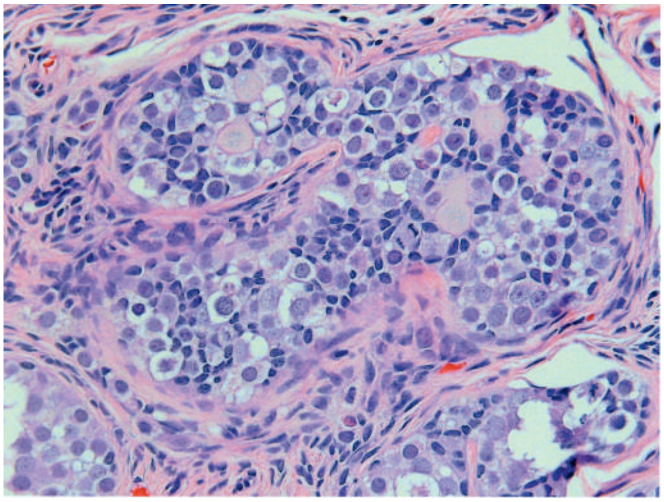

Figure 63-10 Gonadoblastoma. Gonadoblastomas are mixed germ cell-stromal tumors consisting of nests of primitive germ cells surrounded by immature-appearing Sertoli and Leydig cells.

SUMMARY OF PATHOLOGY

The five major histologic subtypes of GCTs are teratoma, YST, germinoma, embryonal carcinoma, and choriocarcinoma. In infants and children, teratoma and YST are the most common GCTs. YSTs occurring at any age are malignant tumors. Teratomas are benign in the prepubertal testis; in the postpubertal testis teratomas occur as one component of nonseminomatous GCTs and are uniformly malignant. Most ovarian teratomas are benign; however, ovarian teratomas with a significant immature component are considered malignant. Germinomas in the ovary are known as dysgerminomas and may occur in children and adults. The testicular counterpart is known as seminoma and occurs exclusively in the postpubertal setting. Extragonadal germinomas can occur in both sexes, typically in the midline of the retroperitoneum or mediastinum or in the pineal gland. The remaining tumor types (embryonal carcinoma and choriocarcinoma) are very rare in children but can occur in the testis or the ovary in adolescents and adults, either as pure tumors or as one component of a mixed GCT.

EPIDEMIOLOGY OF PEDIATRIC GERM CELL TUMORS

Incidence

Pediatric GCTs account for only a small proportion of all cancer diagnosed in children under age 20, but incidence fluctuates dramatically with age. Teratomas, which are benign, are the most common neoplasm of the newborn. However, there are malignant forms of GCT that arise in early childhood between the ages of 0 and 4 years, particularly extragonadal GCT in girls and TGCT in boys.[260]

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[*] References 21, 26, 29, 30, 66, 110.

During adolescence, rates of GCT rise substantially for both males and females. Whereas in children younger than 15 years old, GCTs represent only 3.5% of all cancer diagnoses, during adolescence between ages 15 and 19 years, GCTs represent 16% of the total cancer burden. The incidence of testicular GCTs continues to rise throughout young adulthood, peaking in the third decade, and are the most common malignancy in men between the ages of 15 and 34 years of age.[261] Approximately 8300 cases of testicular cancer are diagnosed among adult males annually.

Extrapolating data from the Surveillance, Epidemiology, and End Results (SEER) cancer registry of childhood cancer collected between 1976 and 2006, it is estimated that in the United States, approximately 850 children and adolescents under the age of 20 are diagnosed with a gonadal or extragonadal malignant GCT each year (see Tables 63-1 and 63-2).[260] Incidence of GCT in childhood has a bimodal distribution; there is a peak in diagnosis at birth and up to age 4, and then a second peak after the onset of puberty. Histology varies dramatically with age. YST is the predominant histology in newborns and younger children, whereas tumors that arise in the peripubertal period contain a wider range of histologies, including YST, embryonal carcinoma, and choriocarcinoma, as well as germinomatous tumors (testicular seminoma and ovarian dysgerminoma).

The site of GCT varies by gender. Under age 10 years, the overall rates are similar between boys and girls, but the extragonadal site is much more common in girls than boys. Over age 10 years, incidence increases in both males and females, but the magnitude differs. Rate increases sixfold in boys but only 2.5 fold in girls. Site, however, is primarily gonadal in both sexes after puberty.

Incidence rates vary dramatically by race as well. Nonwhite boys have higher rates than white boys under age 10, but over age 10, African-American men have much lower rates than white males. In contrast, the rates of GCT are higher among African-American women at all ages. These striking differences in rates of diagnosis by race persist into adulthood; adult African-American males also have a very low likelihood of being diagnosed with testicular cancer.[262]

Incidence not only varies by race and ethnicity, but the overall incidence of GCT has been steadily increasing over the last several decades. Among pediatric patients, incidence rates between 1975 and 2006 increased among boys ages 10 to 19 years (annual percentage change, 1.2; 95% confidence interval [CI], 0.4 to 2.1) and among girls ages birth to 9 years (annual percentage change, 1.9; 95% CI, 0.3 to 2.5). In England and Wales from 1962 to 1990, investigators examined trends in cancer registry data for testicular cancer in males younger than 15 years of age and reported that on average incidence increased 1.3% per annum over the interval.[263] German investigators also report an increase in the incidence of pediatric GCTs over the last several decades.[264,265]

An increase in testicular cancer has also been noted in adult males in many of the countries of the developed world since at least 1940. In the most recent analysis of U.S. data, the incidence of testicular GCTs in adult men rose 44% between 1973 and 1998, with a more pronounced rise in seminoma (64%) than in nonseminoma (24%).[266] In a study of 22 European countries, incidence was noted to be increasing between 1% (Norway) to 6% per year (Spain and Slovenia).[267] The fact that similar trends in incidence have been observed in both young children and adults strengthen the hypothesis that it is exposures that occur either prenatally or in

TABLE 63-1 Average Annual Aged-Adjusted* Incidence Rates per Million for Germ Cell Trophoblastic and Other Gonadal Cancers by Sex And Subtype, Age <20 All Races, SEER, 1986-95

ICCC Group X, a-e	Description	Total	Males	Females
	Germ cell trophoblastic and other gonadal tumors	11.6	12	11.1
Xa	Intracranial and intraspinal GCTs	1.6	2.3	0.9
Xb	Other and unspecified nongonadal GCTs (This category includes the tumors of infants and young children that originate in the sacrococcygeal region as well as mediastinal tumors primarily developing in older children.)	1.6	1.5	1.8
Xc	Gonadal GCTs	6.7	8	5.3
	Testis	4.1	8	—
	Ovary	2.6		5.3
Xd	Gonadal carcinoma	1.4	0.1	2.9
	Ovary	1.3	—	2.6
	Other	0.1	0.1	0.03
Xe	Other and unspecified malignant gonadal tumors	0.2	0.1	0.3

GCT, Germ cell tumor; *SEER,* Surveillance, Epidemiology, and End Results.
*International Childhood Cancer Classification (ICCC).

TABLE 63-2 Average Annual Age-Adjusted* Incidence Rates per Million for Germ Cell Trophoblastic and Other Gonadal Cancers by Race, Sex, and Subtype Age <20, SEER, 1975-95

ICCC Group X	ICCC Germ cell Tumor Category	White Male	Black Male	White Female	Black Female
X a-e	All	12.3	3.2	9	10.8
Xc	Gonadal GCTs	9.1	1.2	4.5	5.6
	Testis	9.1	1.2	—	—
	Ovary	—	—	4.5	5.6
Xa,b,d,e	Other than gonadal GCTs	3.2	2	4.5	5.2

GCT, Germ cell tumor; *ICCC,* International Childhood Cancer Classification; *SEER,* Surveillance, Epidemiology, and End Results.
*Adjusted to the 1970 U.S. standard population.

early childhood that are responsible for this increase in incidence.

Possible Environmental Etiologies

Given the worldwide increase in testicular cancer of almost 100% in the last 3 decades, and the concomitant rise in pediatric GCTs, there has been significant interest in identifying potential environmental exposures that could be implicated. The parallel rise among both children and young adults has pointed to early life exposures as likely being related to development of GCTs.

One of the primary hypotheses has been that induction of GCTs occurs in the setting of prenatal exposure to high levels of maternal estrogens that could affect gonadal development.[268] Several lines of evidence suggest this is a plausible hypothesis. When diethylstilbestrol (DES) is administered to pregnant mice, it causes maldescent of the testes and testicular hypoplasia. Male offspring of women who took DES during pregnancy have higher rates of cryptorchidism, a known risk factor for TGCT in humans. Maternal use of DES has also been directly associated with increased risk of testicular cancer in some but not all studies.[269]

The fetus is also exposed to high levels of endogenous maternal estrogens during pregnancy (versus exogenous sources such as DES or oral contraceptives). Several conditions of pregnancy are known to be associated with higher levels of serum estrogens and have also been linked to increased risk of GCT, including hyperemesis gravidum and preeclampsia. Testicular cancer is more common among first-born males and twins, especially dizygotic twins, and again, both conditions of pregnancy are known to be associated with higher levels of circulating estrogens.[268,270-273] Increased birthweight, which has been associated with higher risk of testicular cancer, is also associated with higher estradiol and estriol levels in both the first and third trimesters of pregnancy.[274] Maternal weight gain is a predictor of higher birthweight and is also associated with higher insulin levels, leading to lower sex hormone–binding globulin levels, which thereby increases levels of bioavailable estrogens.

Furthering the hypothesis of the importance of early life exposures playing a role in testicular carcinogenesis, it has been noted that men born during World War II in Norway, Sweden, and Denmark have a decreased risk of testicular cancer compared with men born before or after the war. Lack of maternal weight gain during wartime may explain in part this temporal association. Rationing during WWII in Norway resulted in a 15% to 20% energy restriction and a resultant decrease in maternal weight in the period from 1941 to 1945.[275] Height, determined in part by childhood nutrition, has been found to be strongly associated with risk of testicular cancer in two case-control studies.[276,277]

Because GCTs are rare in children, the number of case-control studies conducted among children has been quite limited. The Children's Cancer Group (CCG) conducted an epidemiologic study that included all children with cancer registered for treatment with CCG between 1982 and 1989.[278] Parents were asked to complete a 22-page self-administered questionnaire. Approximately 50% of those with children who had eligible cancer cases completed the questionnaire; controls were obtained through a random-digit–dialing procedure. Among all the cases, there were 105 cases of children younger 15 years of age with a malignant GCT and 639 controls. In this study, several factors were associated with risk of GCT. Birthweight of more than 4000 g was related to 2.4-fold increase in risk (CI, 1.0 to 5.8) compared with a birthweight of less than 3000 g after adjustment for gestational age. Pregnancy conditions such as nausea and vomiting or hypertension or eclampsia were not associated with risk. An inverse association between maternal smoking and risk was noted; among mothers who smoked more than 15 cigarettes per day, risk of GCT was reduced by 70%. Among mothers who ever reported being exposed to chemicals or solvents or to plastic and resin fumes, an increased risk was observed (odds ratio [OR] 4.6; CI, 1.9 to 11.3); (OR 12.0; CI, 1.9 to 75).

A more in-depth follow-up study that specifically focused on GCTs in children was conducted under the aegis of the Children's Oncology Group (COG).[279] The study included 278 cases and 423 controls identified through random-digit dialing. Subjects completed a self-administered questionnaire, a telephone interview, and a medical record review of the maternal records before and during the index pregnancy. The study did not confirm a relationship between maternal exposure to exogenous hormones (either DES or oral contraceptives) and risk of pediatric germ tumor. None of the pregnancy-related conditions including birthweight, hyperemesis, preeclampsia, pregnancy-induced hypertension, or maternal weight gain that can alter circulating levels of endogenous estrogen were related to risk.[279] The study also failed to replicate the finding from the prior case-control study in CCG that showed a relationship between maternal smoking and decreased risk of GCT[280]; neither environmental or occupational exposure to pesticides and other chemicals increased risk of GCT in offspring.[281]

Predisposing Syndromes

Cryptorchidism and Testicular Cancer

Many studies have shown a definitive relationship between cryptorchidism and testicular cancer with relative risks that range from 2.1 to 17.6.[282] Of note, cryptorchidism is three to four times more common in white males than African-American males, which mirrors the difference in incidence by race. The etiology of the relationship between cryptorchidism and testicular cancer remains unclear. It has been suggested that exposure of the testis to higher temperatures in the abdomen as compared to in the scrotum in some way promotes cancer growth. In this case, orchiopexy should minimize risk, especially if it is done before the hormonal stimulation and proliferation of puberty. Others have suggested that the etiology of the undescended testicle and the cancer is the same, and in this case, orchiopexy should not significantly decrease risk. It has been observed that there is an increased risk of testicular cancer in the descended contralateral testicle, which supports this hypothesis for a common etiology. In a case-control study from Kaiser-Permanente of 183 patients with testicular cancer matched to 551 controls, there were 20 men who had had a cryptorchid testes.[283] Interesting, if orchiopexy had been performed successfully by age 11, there was no increase in risk of testicular cancer. However, among men whose cryptorchid testis was not treated until after their eleventh birthday, who had a failed orchiopexy, or who had never had treatment, the odds of testicular cancer were increased 32 fold. Although the study was limited by small numbers, it would suggest that orchiopexy in early childhood before puberty may obviate the risk of subsequent testicular cancer. In the case in which an abdominal testes is discovered after the onset of puberty, an orchiectomy may be the most prudent procedure.

Turners Syndrome and Gonadoblastoma

Turner syndrome, the most common sex-chromosome abnormality in girls (1:2000 live births), is caused by partial or total loss of an X chromosome, resulting in a 45,XO karyotype. Most girls with Turner syndrome have short stature and many have primary amenorrhea. Approximately two thirds of girls have only the remnant of an ovary or “streak gonad.” In these dysgenetic and often nonfunctional ovaries, there is a high risk of developing a gonadoblastoma, particularly in girls who also retain a portion of the Y chromosome. Gonadoblastoma is itself a benign lesion, but some of these tumors undergo malignant degeneration, and therefore gonadectomy is usually recommended, although this recommendation has become more controversial. In a Danish study that examined 114 girls with Turner syndrome, presence of the Y chromosome was documented in 13 girls (12%).[284] In the 10 girls with Y-chromosome material who subsequently underwent gonadectomy, gonadoblastoma was present in only one specimen. Therefore the authors suggest that close follow-up examination by ultrasound may be sufficient for monitoring rather than routine gonadectomy but urged that more prospective data is necessary.

Klinefelter Syndrome

Klinefelter syndrome, a condition present in between 1:500 and 1:1000 males, is caused by an extra X chromosome resulting in the 47, XXY karyotype. Men with Klinefelter syndrome have abnormal testicular development and are usually infertile. Men with Klinefelter syndrome also have a substantially increased risk of developing GCTs, particularly mediastinal GCTs, beginning in early adolescence.[285]

Androgen Insensitivity Syndromes

Mutations either in the androgen receptor itself or its downstream effectors results in phenotypic females with an XY karyotype. The prevalence of this disorder is estimated to be approximately 1:20,000 females. Oftentimes, these patients do not come to medical attention until they undergo evaluation of primary amenorrhea. Patients with complete androgen insensitivity have little to no axillary or pubic hair, no uterus, and serum testosterone levels of a male. Testes are abdominal or inguinal but can also present on rare occasions as labial masses. These cryptorchid testes are at high risk for development of gonadoblastoma and/or more malignant GCTs and gonadectomy is advised.

MOLECULAR BIOLOGY OF GERM CELL TUMORS

Analysis of the GCT genome and transcriptome has begun to yield insight into the pathogenesis of these tumors in recent years. Genome-wide association studies (GWAS) comparing GCT cases with unaffected controls have identified a number of loci linked to GCT susceptibility. Several different types of analyses, described in the following sections, lend support to the idea that prepubertal and postpubertal GCTs arise from different molecular mechanisms. In the coming years, identification of specific gene expression signatures, mutations, copy number aberrations, and other abnormalities in different subclasses of GCTs will undoubtedly help refine risk stratification in newly diagnosed patients, as well as provide new targets for novel therapies, especially in chemotherapy-resistant disease.

The GCT Transcriptome

In the last decade studies have begun to illuminate the messenger ribonucleic acid (mRNA) expression pattern of TGCTs in adult men.[218,286-290] These analyses have revealed gene signatures distinguishing GCTs from other types of malignancies and further identified differences in gene expression among different histologic subtypes of GCTs. A major unresolved question of whether pediatric GCTs exhibited the same gene expression patterns was addressed by Palmer, Coleman, and co-workers in an investigation of the pediatric GCT transcriptome, the largest such study to date.[291] This study demonstrated striking differences in the gene expression repertoire of malignant YSTs compared to seminomas, with the former enriched for genes associated with the self-renewing or pluripotent phenotype and the latter enriched for genes associated with differentiation and proliferation. In addition, Palmer et al compared gene expression in childhood GCTs with that in adult TGCT, as described in a study by Korkola et al.[292] The analysis of Palmer and colleagues showed clear differences between the transcriptional profile of pediatric and adult GCTs, even within the same histologic subtype (Fig. 63-11, *A* and *B*).

A key additional question is whether particular gene expression patterns are associated with clinical outcome in GCTs, a point addressed by some studies of adult TGCT.[287,293] A study by Korkola and co-workers identified and validated a prognostic mRNA gene expression signature that defined 5-year overall survival in both a training and validation cohort of nonseminomatous TGCTs.[293] Further work will be required to translate this finding into a robust, clinically useful prediction tool.

Noncoding RNAs

Micro RNAs (miRs) are short, 21-nucleotide, noncoding RNAs that have major roles in regulating gene expression and are often misexpressed in cancer.[294] A seminal study in 2006 identified miR-372 and miR-373 as oncogenes in TGCTs—one of the first demonstrations that miRs play a role in human cancer.[295] In childhood GCTs, Palmer and co-workers demonstrated that relative to normal gonadal tissue, MGCTs overexpress miRs from the miR-371~373 and miR-302 clusters, regardless of histologic subtype, patient age, or gonadal vs. extragonadal origin.[296] This study also demonstrated that expression of these miRs causes specific downregulation of their target miRs in GCTs. In a subsequent study, Murray et al. further defined miRs with differential expression that distinguished YSTs from germinomatous GCTs and identified a core set of transcription factors that may be regulated by these miRNAs and contribute to the specific cellular phenotypes of different subtypes of GCT.[297]

Micro RNAs have been shown to be shed from tumor cells into the circulation.[298] The translational significance of the finding that miR-371~373 and miR-302 family members are universally overexpressed in GCTs became clear with the demonstration that these same miRs were present at elevated levels in the serum of a child with YST at diagnosis and fell in parallel with declining AFP levels during treatment.[299] Further studies have confirmed that serum levels of these miRs are increased in patients with GCT at diagnosis, regardless of age group, tumor histology, or location (Fig. 63-12).[300,301] This is a particularly important finding, since many patients with GCT do not manifest elevation of serum tumor markers such as AFP, HCG, or lactate dehydrogenase (LDH).[302] Serum miRs may thus represent sensitive and specific new tumor markers for MGCTs.

Chromosomal Constitution and Copy Number Aberrations

The analysis of tumor karyotypes and genomic copy number aberrations (CNAs) in GCTs provides some of the most important evidence for distinct pathogenic mechanisms of GCT in children compared with adolescents and adults.[60,154,202,303-305] In addition, the gain or loss

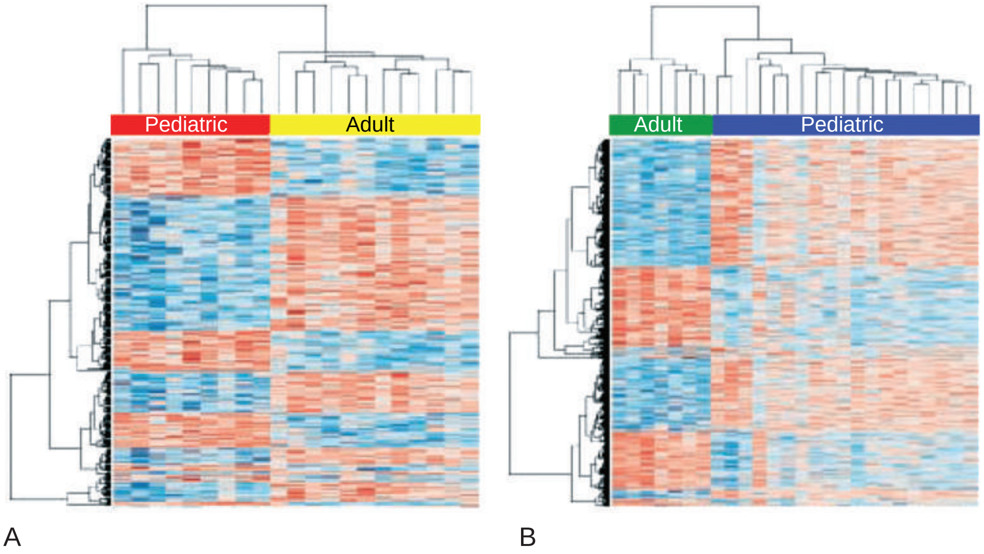

Figure 63-11 Pediatric and adult germ cell tumors (GCTs) display different gene expression patterns. Heat maps comparing the transcriptomes of 27 pediatric GCTs with that of pure histologic diagnosis adult TGCTs. **A**, Seminomas. **B**, YSTs. *Red* represents relative overexpression and blue represents underexpression. *(Modified from Palmer RD, et al: Pediatric malignant GCTs show characteristic transcriptome profiles.* Cancer Res *68:4239–4247, 2008.)*

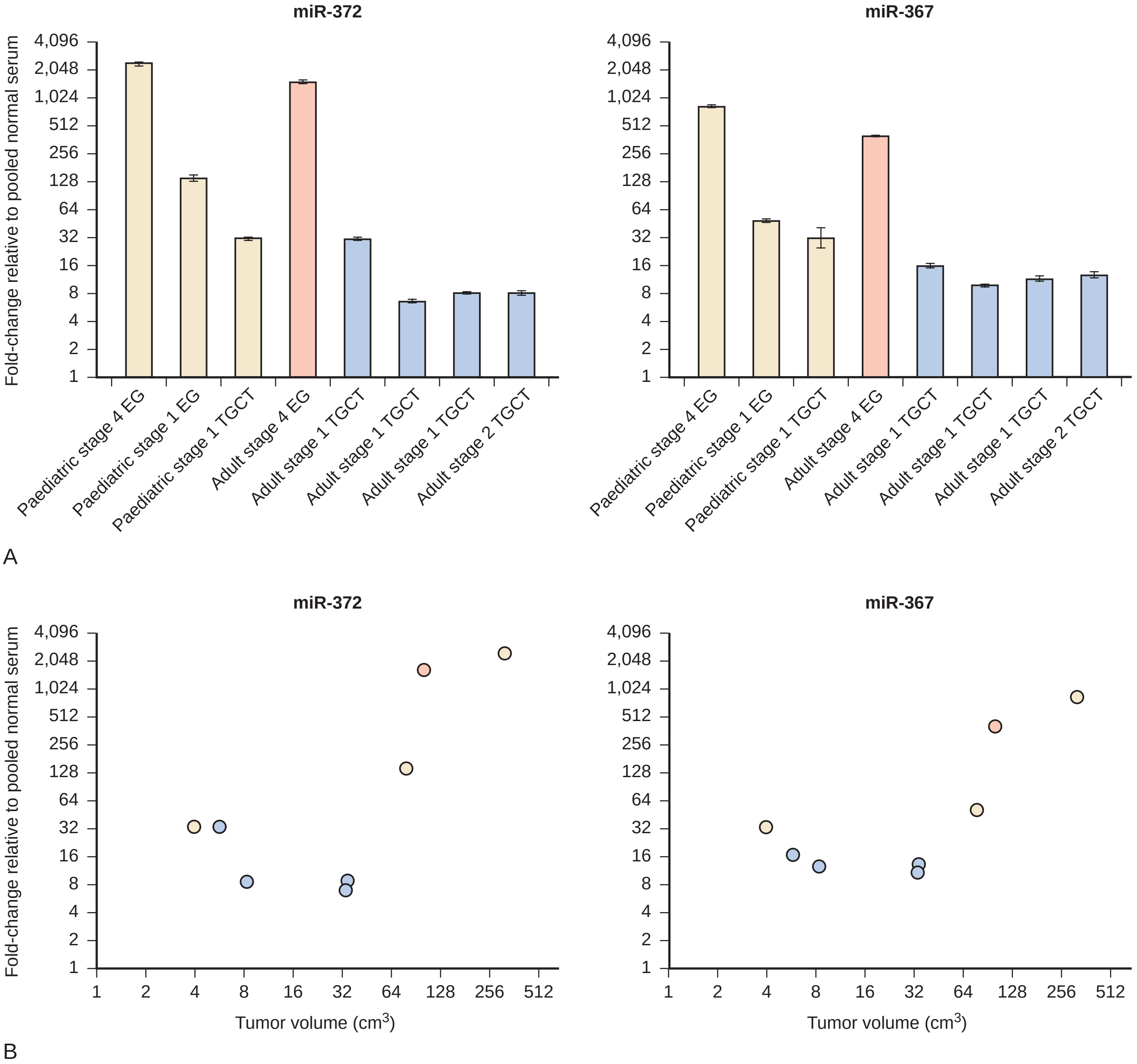

Figure 63-12 MiRs as serum biomarkers of malignant germ cell tumors (GCTs). **A**, Relative levels of miR-372 *(left)* and miR-367 *(right)* in the serum of patients with GCTs at diagnosis, compared with levels in control serums. MiR-372 and miR-367 are elevated in all patients regardless of age, site or tumor histology. **B**, Serum expression of miR-372 and miR-367 at diagnosis correlates to volume of the primary tumor. *(Modified from Murray MJ, Coleman N: Testicular cancer: a new generation of biomarkers for malignant germ cell tumours.* Nat Rev Urol *9:298–300, 2012.)*

of specific chromosomal regions may provide important clues as to the molecular mechanisms of germ-cell tumorigenesis.[1,69,94]

Adolescent and adult malignant TGCTs are universally aneuploid, and polyploidization of germ cells has been emphasized as an early event in testicular germ-cell tumorigenesis.[306,307] IGCNU and seminoma cells are hypertriploid, whereas nonseminomas of all histologic types are hypotriploid. TGCTs consistently exhibit biallelic expression of imprinted genes.[102,308-312] Erasure of parental imprinting occurs in mammalian embryonic germ cells,[313,314] implying the origin of TGCTs from an early germline cell.[1]

The most common cytogenetic aberrations in TGCTs are relative loss of all or parts of chromosomes 4, 5, 11, 13, 18, and Y and gain of material from chromosomes 7, 8, 12, and X.[307,315-321] Of these, the most consistent chromosomal aberration in adolescent/adult malignant GCTs is the overrepresentation of chromosome 12p, which is found in all histologic subtypes and primary sites (ovarian, testicular, and extragonadal).[322-331] A characteristic isochromosome 12p (i[12p]) can be detected cytogenetically in 80% of cases;[59] in other cases, an increased copy number of 12p sequences can be detected on derivative chromosomes.[332] Along with tumor markers such as β-HCG, the detection of i(12p) can identify a germ-cell origin in cases of carcinoma of unknown primary.[333] Further analysis has identified a particular amplicon, 12p11-12p12.1, that is present in many TGCTs.[334,335] Candidate genes in the 12p region include the oncogenes *KRAS* and *CCND2*, as well as the stem cell–specific genes *NANOG* and *STELLAR/DPPA3*; however, the precise contribution of 12p amplification to the pathogenesis of GCTs remains unclear.[*] 12p amplification appears to be a late event in GCT pathogenesis, because it is not present in the precursor lesion, IGCNU.[342]

Molecular genetic analysis of teratomas further supports the hypothesis of different histogenetic origins of different types of teratomas.[133] The malignant teratoma component of mixed ovarian or TGCTs uniformly exhibits the same genomic aberrations as the other malignant components of the tumor, including i(12)p. In contrast, mature, cystic ovarian teratomas and dermoid and epidermoid cysts of the testis exhibit normal karyotypes.[†] Based on these observations, Ulbright has proposed that mature ovarian teratomas develop directly from transformed germ cells, whereas the post-pubertal testicular teratomas develop through a malignant IGCNU stage, as do the other malignant post-pubertal TGCTs.[133]

A recent study of genome-wide copy number variation (CNV) in seminomas using high-resolution single-nucleotide polymorphism (SNP) arrays confirmed many of these reported findings and identified novel CNVs, including both amplifications and homozygous deletions.[344] Several genes identified in regions of recurrent CNV are potential candidate seminoma susceptibility genes, including *FAT3, EDNRB1*, and *RASSF8*.

Genomic aberrations seen in MGCTs of infants and children are generally distinct from those occurring in postpubertal tumors.[19,60,154,202,303] Pediatric GCTs show only partial erasure of parental imprinting, indicating that prepubertal GCTs likely arise from an earlier stage of embryonic germ cell development than do the postpubertal tumors.[‡] A similar pattern of imprinting is seen in gonadal and extragonadal pediatric GCTs, suggesting that gonadal and nongonadal tumors share a common pathogenesis and cell of origin.[100,102,154,305,346]

In analyses of chromosomal structure and copy number imbalances, differences between the pediatric and adolescent/adult GCTs are again consistently found. In the prepubertal period, pure teratomas of the testis or of extragonadal sites nearly always exhibit a normal profile in genomic analyses, including classical cytogenetics, fluorescence in situ hybridization, loss-of-heterozygosity analysis, and array CGH.[§] These data contrast sharply with the universally abnormal cytogenetic profile of postpubertal teratomas arising as a component of a mixed malignant GCT. In adolescents and adult GCTs, only mature teratomas of the ovary and dermoid and epidermoid cysts of the testis exhibit normal cytogenetics, suggesting that these tumors may share a common pathogenesis with the prepubertal teratomas.[123,133]

In children under the age of 5 years, YST is the most common malignant GCT of the testis and of extragonadal sites. Unlike teratomas, cytogenetic and other genomic aberrations are consistently reported in analyses of YSTs in infants and children. The most common imbalances reported are gains at chromosomes 1q and 20q and losses at chromosome 1p and chromosome 6q.[¶] More recently, the advent of higher-resolution techniques such as array-based CGH has further defined chromosomal aberrations in this group of tumors. Veltman et al[203] reported the array CGH profiles of a series of 24 GCTs from patients aged younger than 5 years, including 16 teratomas and eight YSTs. Most of the teratomas had normal CGH profiles, with two teratomas exhibiting loss of chromosome 20p as the only recurrent change. In contrast, all of the YSTs had abnormal profiles. The recurrent changes, seen in at least three of the eight YSTs, included gains of chromosomes 1q (1q32-1qter), 3p (3p21-pter), and 20q (20q13) and loss of chromosomes 1p (1p35-pter), 6q (6q24-qter), and 18q (18q21-qter). In the Veltman et al study, only one tumor from a child aged younger than 5 years exhibited gain of chromosome 12p, whereas five of seven cases from children older than 5 years showed gains at chromosome 12 p. Most of these tumors occurred in the ovary, supporting the hypothesis that ovarian and testicular MGCTs have a common pathogenesis, at least in older children and adults.[1,353] Another study from the U.K. Children's Cancer and Leukaemia Group examined the metaphase CGH profile of 34 malignant GCTs in children aged younger than 16 years.[354] This study supported the previous reports of an increased frequency of loss of chromosomes 1p and 6q and gain of chromosome 3p in YSTs. Intriguingly, 4 of 14 MGCTs from children younger than 5 years of age demonstrated gain of 12p, including in two cases gain of the 12p11 locus that is strongly associated with adolescent and adult MGCTs. Therefore childhood and adolescent/adult MGCTs may share more pathogenic mechanisms in common than has been previously appreciated based on the analysis of a relatively small number of cases.

Loss of chromosomes 1p and 6q correlates with loss-of-heterozygosity analysis indicating true allelic loss in these regions in pediatric GCTs.[349,355] However, the most common chromosomal aberrations of pediatric GCTs 1p–, 1q+, 6q–, and 20q+ are not highly specific for GCTs but are seen in other cancers of childhood, including neuroblastoma and Wilms tumor, as well as carcinomas.[356,357] Deletion of chromosome 1p is associated with *MYCN* amplification and poor prognosis in neuroblastoma,[358,359] which has led to the speculation that one or more genes in this interval may act as a tumor-suppressor in neuroblastoma.[360] Several candidate genes have been proposed, including *CHD5, TNFRSF25, CAMTA1,* and

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[*] References 1, 6, 94, 219, 243, 311, 336-341.

[†] References 123, 151, 152, 183, 203, 328, 331, 343.

[‡] References 100, 102, 123, 154, 305, 345, 346.

[§] References 123, 134, 153, 203, 347-349.

[¶] References 91, 134, 135, 154, 303, 304, 348, 350-352.

AJAP1.[361] *CHD5* was shown to act as a tumor-suppressor in vivo.[70] Whether these or other genes in the 1p and 6q deleted regions play a pathogenic role in pediatric MGCTs is not known.

Genome-Wide Association Studies

A number of genome-wide studies have used SNP arrays to analyze the constitutional (germline) DNA of men with TGCT, resulting in the identification of a large number of novel GCT susceptibility loci. In 2009 two groups reported strong linkage of GCT susceptibility to loci on chromosomes 5, 6, and 12, suggesting roles for *SPRY4* and *KITLG*, both components of receptor tyrosine kinase signaling, as well as for the proapoptotic *BAK1* gene.[362,363] A follow-up study by Turnbull and co-workers identified additional susceptibility loci near the *DMRT1*, *TERT,* and *ATF7IP* genes.[364] The studies have subsequently been replicated in other patient populations.[365-367] More recently, further GWAS studies have identified new candidate GCT susceptibility loci, including genes with potential roles in telomere regulation, such as *PITX1*, or germ-cell development, such as *TEX14, RAD51C, PRDM14,* and *DAZL*.[368,369]

The Role of Developmental Signaling Pathways in Germ Cell Tumor Pathogenesis

Compared with many other solid malignancies, relatively few somatic mutations have been described in GCTs. The most commonly reported mutated gene is *KIT*, a tyrosine kinase growth factor receptor that plays important roles in germ cell development.[199,243,370-373] Mutations have also been reported in *NRAS* and *KRAS*, signaling components of the mitogen-activated protein (MAP) kinase pathway that act downstream of *KIT*.[374-377] Interestingly, somatic mutations in *BRAF*, another MAP kinase pathway member, have been associated with cisplatin resistance in adult TGCTs[378]; however, *BRAF* mutations appear to be rare in pediatric GCTs.[379]

Large-scale genomic profiling projects, now underway in several centers, will be required to fully delineate the mutational landscape of MGCTs. Other mechanisms impairing normal gene expression in GCTs such as CNV and aberrant miR expression are being explored (described previously), as is differential methylation. Epigenetic modulation of gene expression through promoter DNA methylation is commonly found in adult and pediatric GCTs. Smiraglia and co-workers first reported that seminomas are globally hypomethylated compared to nonseminomas,[380] whereas Netto et al. extended these observations to show that IGCNU cells, like seminomas, appear globally hypomethylated. Other groups have examined the potential contributions of differential methylation to TGCT susceptibility[381] or cisplatin resistance.[382] Two recent studies examined global DNA methylation patterns specifically in pediatric GCTs. Jeyapalan and co-workers found that pediatric YSTs exhibited a "methylator" phenotype, perhaps attributable to higher expression of DNA methyltransferase 3B in these tumors. Among the genes predicted to be silenced were several associated with caspase-8–dependent apoptosis.[383] Amatruda et al. found a similar pattern of differential methylation comparing YSTs to seminomas and further identified effects on the expression of genes associated with stem cell pluripotency and Wnt/β-catenin signaling.[384]

In addition to these efforts, many studies have focused on understanding how aberrant control of normal developmental pathways may play a role in GCT pathogenesis. Fritsch and co-workers showed that pediatric YSTs and teratomas express active Wnt/β-catenin signaling and Wnt target gene expression.[385] Pediatric YSTs express high levels of *BMP2*, a component of the BMP signaling pathway,[291] and expression of *BMP7* is associated with poor outcome in TGCTs.[293] The importance of BMP was further revealed in the analysis of zebrafish bearing inactivating mutations in a cell-surface BMP receptor, which develop GCTs strongly resembling human seminomas.[386] Thus the state of BMP signaling distinguishes undifferentiated tumors such as seminomas from the more differentiated nonseminomas.[387]

Another key developmental pathway concerns the pluripotency gene *LIN28*, which is universally expressed in GCTs.[388-390] Whereas *LIN28* expression is critical for normal embryonic development, prolonged or aberrant *LIN28* expression in germ cells may be pathogenic, owing to *LIN28*'s ability to block maturation of the let-7 family of tumor-suppressing miRs.[390] Let-7 normally regulates a number of genes associated with proliferation such as *MYCN*, *RAS*, *AURKB,* and *CCNF*, and Murray et al. have shown that *LIN28* expression drives the upregulation of many of these oncogenes in GCT cells.[391]

Although the above studies have begun to yield important insight into the mechanisms by which perturbations in the Wnt, BMP and *LIN28*/let-7 pathways may contribute to GCT pathogenesis, strategies to target these defects are still at fairly early stages of development. There may potentially be more immediate translational impact from the finding that GCTs express cell-surface proteins that have been successfully targeted by monoclonal antibodies in other solid tumors, including *ERBB2/Her-2*, *CD30*, and epithelial cell adhesion molecule (EpCAM).[392-396]

DIAGNOSIS AND STAGING OF PEDIATRIC GERM CELL TUMORS

In this section, the clinical presentation of GCTs, evaluation of the patient for metastatic disease, and the use and interpretation of the tumor markers in the diagnosis and staging of GCTs will be discussed. The surgical approach, for purpose of both diagnosis and staging, will also be delineated.

Clinical Presentation of Pediatric Germ Cell Tumors

The clinical presentation of a pediatric GCT is determined by the site of origin. Site of origin varies by age. Gonadal tumors, particularly testicular tumors, do present during infancy but are more common after the onset of puberty. The site of the extragonadal GCT also varies as a function of age at diagnosis. Sacrococcygeal tumors, much more common in females than males, present either at birth (typically mature/immature teratomas) or between 6 months and 4 years of life (typically

MGCT with a large component of YST). Mediastinal tumors are rare in infancy; incidence begins to rise at onset of puberty, particularly among males. Site-specific features of the presentation are discussed in the sections that follow.

Presentation of Testicular Germ Cell Tumors

Testicular GCTs most often present with painless generalized swelling of the scrotum. Gynecomastia and infertility are rarely present,[220] though subfertile and infertile men do appear to have an increased risk of developing TGCT.[397,398] Local extension to the spermatic cord and the epididymis may occur, and the presenting symptoms can be mistaken for epididymitis. Symptoms of metastasis, including back or abdominal pain, cough or dyspnea, are present at diagnosis in about 10% of adult patients.[69]

An initial testicular ultrasound that shows the lesion to be intratesticular with areas of microcalcification is often helpful in clarifying the differential diagnosis, which includes lymphoma and paratesticular rhabdomyosarcoma. Eighty-five percent of children younger than age 4 have a tumor that is confined to the testes (stage I), as compared with only 35% of adults.[399]

Choriocarcinoma is the most aggressive histologic subtype of testicular GCT, with a propensity to hematogenous metastasis. Patients may have an occult primary tumor and few or no testicular complaints but rather symptoms caused by hemorrhage of visceral metastases, such as hemoptysis or neurologic symptoms resulting from brain metastases. Because of the risk of hemorrhage, the suspected diagnosis of metastatic choriocarcinoma is a medical emergency that requires prompt initiation of treatment, often without confirmatory biopsy and just on the presumption of the diagnosis given the presenting signs, symptoms, radiologic appearance, and markedly elevated β-HCG. However, it has been hypothesized that chemotherapy may actually precipitate hemorrhage, and therefore patients need very careful monitoring; some have advocated initiation of chemotherapy in an intensive care–unit (ICU) setting. With the extreme elevation of the serum β-HCG that can be seen in choriocarcinoma, occasional patients manifest thyrotoxicosis or gynecomastia resulting from the cross-reactivity of β-HCG with the thyroid and luteinizing hormone receptors.[220,400-404]

Presentation of Ovarian Germ Cell Tumors

Ovarian GCTs typically present with abdominal pain and a palpable pelvic-abdominal mass (85%).[405] Symptoms of acute abdomen occur in about 10% to 25% of patients and are usually caused by rupture, hemorrhage, or torsion of the ovarian tumor that requires immediate surgical exploration.[258] Other less common presenting symptoms include abdominal distension (35%), fever (10%), and vaginal bleeding (10%). Rarely, patients can experience precocious puberty resulting from the production of β-HCG by the tumor. Not uncommonly, a young girl with an abdominal/pelvic mass and and an elevated β-HCG is assumed to be pregnant. This assumption is especially distressing for young females who are peripubertal and have not yet have initiated sexual intercourse.

If the diagnosis of dysgerminoma is made, it is important to recognize that 10% to 15% of cases are bilateral; therefore the finding of dysgerminoma on frozen section during surgical exploration of an ovarian mass generally warrants close inspection and sometimes biopsy of the contralateral ovary.[406-409] Most dysgerminomas present as stage I (disease confined to the ovary),[406-409] but lymphatic metastasis of these tumors to pelvic, paraaortic, mediastinal, and supraclavicular lymph nodes can occur.[216,410,411] Five percent of dysgerminomas arise in dysgenetic gonads of girls with 46XY (Sweyer syndrome or testicular feminization) or who have 45X/46XY mosaicism.[412] For this reason, karyotyping should be considered for all girls with a prepubertal pelvic mass.[216] If dysgenetic gonads and/or the presence of Y-chromosome material are detected, removal of both of the gonads is indicated because of the 25% to 50% risk of developing malignancy such as a gonadoblastoma or dysgerminoma in the contralateral ovary.[66,110,413,414] See "Epidemiology of Pediatric Germ Cell Tumors" for more information.

Presentation of Sacrococcygeal Germ Cell Tumor

Sacrococcygeal teratomas (SCTs) occur predominately in females. In Altman's classic 1974 review of 405 clinical cases, 74% occurred in females.[415] However, even though SCTs are much more common in females, rate of malignancy does not vary by gender. Of note, 18% of patients with SCT had associated congenital anomalies. The most prevalent defects were in the musculoskeletal system, but other organ systems that were involved included renal, cardiovascular, gastrointestinal, and central nervous systems. More than half of the patients were diagnosed on the first day of life, but approximately 10% were diagnosed after 1 year of age.

Altman classified sacrococcygeal tumors by the degree of internalization vs. externalization of the tumor (Fig. 63-13).[415] An "Altman type I" SCT is a tumor that is entirely external; an Altman type IV SCT is entirely internal. Altman was the first to note the correlation between rate of malignancy, age at diagnosis, and degree of internalization. At younger than 2 months of age, 7% of girls and 10% of boys had malignant tumors, whereas after 2 months of age, more than half of the tumors were malignant (48% of SCT in girls and 67% of SCT in boys).[415]

Most neonatal SCTs have a significant external component (Altman type I or II) and can be completely excised in the neonatal period. Over 90% are benign lesions (either mature or immature teratoma) that do not require further therapy. Recurrence rates after resection of neonatal mature or immature teratoma have been estimated to range from 4% to 21% with a malignancy rate of 50% to 70%.[192,416] The reason that a fraction of neonatal tumors do recur could either be because of oversight of a malignant element during the initial pathologic review, such as a microscopic foci of YST, or because malignant transformation of a small benign remnant left in situ after the initial procedure. Presacral tumors that are predominately internal present later in infancy through age 4 years. Usual presenting symptoms of an internal lesion are either constipation or a buttock/abdominal mass; probability is quite high that these lesions are malignant.

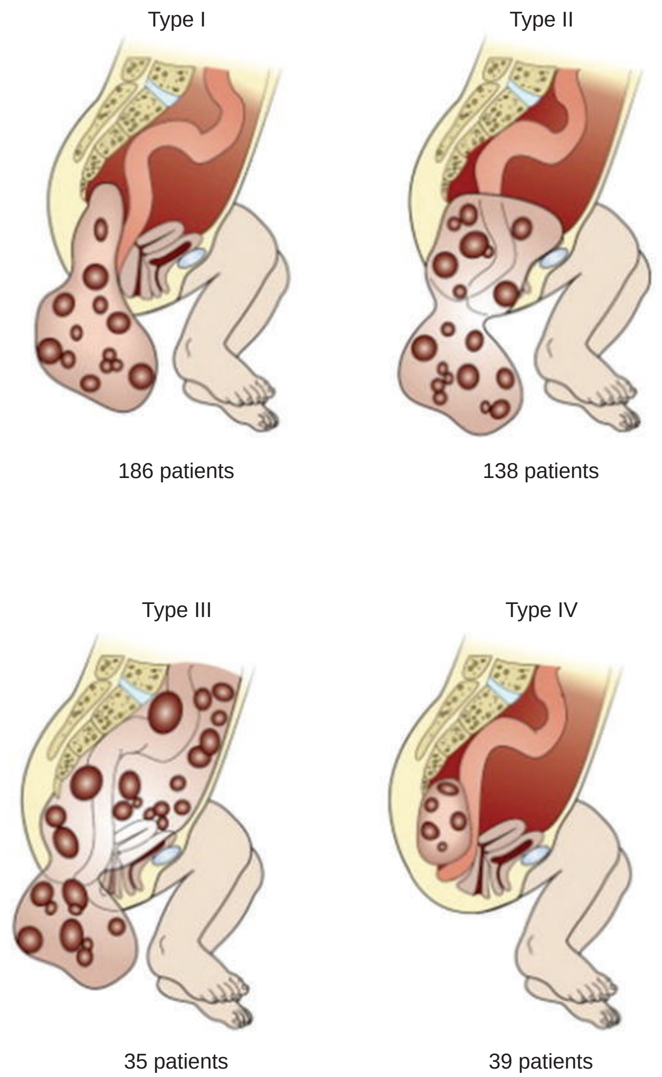

Figure 63-13 Altman classification of sacrococcygeal germ cell tumors. SCTs are classified according to their degree of internalization. Type I tumors are nearly entirely external; in type II tumors the mass extends equivalently into and external to the pelvis. Type II tumors have only minimal external component, and Type IV tumors are entirely internalized. The numbers of patients with each type of tumor in this series of 398 patients is shown. *(From Altman RP, Randolph JG, Lilly JR: Sacrococcygeal teratoma: American Academy of Pediatrics Surgical Section Survey, 1973.* J Pediatr Surg *9:389–398, 1974.)*

Presentation of Mediastinal Germ Cell Tumors

In a series of patients with mediastinal GCTs registered on the German MAKEI protocols, chest pain or respiratory symptoms such as cough or dyspnea were the most common presenting clinical symptoms.[417] Other presenting clinical scenarios included discovery of the mass as an incidental finding on fetal ultrasound or during a workup for pneumonia that did not resolve with standard antibiotics. In the MAKEI experience, all mediastinal tumors that presented at younger than 1 year of age (n = 9) were either mature or immature teratomas.[417] Mediastinal tumors presenting after infancy but before puberty were either pure YSTs or had predominant yolk sac components. Embryonal carcinoma was more prevalent after the age of 10 years. Mediastinal seminoma was not diagnosed before age 10 years.

Presentation of Extragonadal Germ Cell Tumor at Other Sites

Extragonadal GCTs are the most common fetal and neonatal neoplasm; perinatal teratomas present at wide array of sites. In a review of all perinatal GCTs reported in the literature from 1965 through 2004, the author identified 534 cases.[56] The sacrococcygeal site is the most common (40%), but perinatal GCTs have also been reported in the cervical area (30%), in the oropharynx and nasopharynx (8%), and in other sites including cardiac (7.5%), gastric (2.6%), orbital (2.4%), facial (1.5%), mediastinal (2.6%), and placental (1.5%) locations. In addition, there were 17 teratomas reported in other sites, including tongue, tonsil, liver, retroperitoneum, ileum, mesentery, vulva, and the anorectal area. In addition, 25 cases of fetus in fetu were reported. Most common reasons for diagnosis included detection of a mass on prenatal ultrasound, polyhydramnios, or respiratory distress. Other symptoms were specific to site; for instance, pericardial effusion and tamponade were the most common signs in neonates with intracardiac teratomas. The vast majority of perinatal GCTs are benign, either mature or immature teratomas. Overall the incidence of YST within the neonatal teratoma was 5.8%; the highest rate of YST (10%) was documented in SCTs. Teratomas detected antenatally had three times the mortality rates as those diagnosed postnatally. Fetal survival rate was 53%; neonatal survival rate was 85%. Cause of death was most commonly either because of prematurity or to hydrops. Survival rate was only 7% among those patients whose tumors were diagnosed as a result of fetal distress before 30 weeks of gestation.

Evaluation for Metastatic Disease

The current system for staging pediatric GCTs in use by the COG is shown in Table 63-3.[418] All patients suspected of having a GCT should have a radiologic evaluation of the extent of the disease including either an ultrasound (if testes is the primary site), computed tomography (CT), or magnetic resonance imaging (MRI) of the primary site; abdominal/pelvic CT if the tumor is likely to drain to the lymph nodes of the retroperitoneum and abdomen; and a chest CT. Radiologic staging is preferably obtained preoperatively so that the clinician does not have to differentiate between malignant involvement and postoperative changes (i.e., atelectasis on the chest CT or postoperative lymph node enlargement after surgery). GCTs can also metastasize to brain and bone. Although bone and brain metastases are generally considered quite rare, German investigators reported that up to 10% of patients have bone metastasis, and that incidence was as high as 26% among stage IV patients.[419] On the last combined U.S. intergroup GCT study, 6% of patients overall had bone metastases, but the incidence increased among stage IV patients, of whom 13% had bone metastases at diagnosis.[420] Brain metastases are present in about 4% of adults with GCT. In a review of the St. Jude experience in 206 cases of pediatric GCT from 1962 to 2002, 16 patients in the series had brain metastases at some point

TABLE 63-3 Staging of Testicular, Ovarian, and Extragonadal Tumors

Testicular	
I	Limited to testis, completely resected by high inguinal orchiectomy; no clinical, radiographic or histologic evidence of disease beyond the testis; tumor markers normal after appropriate half-life decline: patients with normal or unknown markers at diagnosis must have negative ipsilateral retroperitoneal lymph node sampling to confirm stage I disease.
II	Transscrotal orchiectomy; microscopic disease in scrotum or high in spermatic cord (<5 cm from proximal end): retroperitoneal lymph node involvement (<2 cm) and/or increased tumor markers after appropriate half-life decline
III	Tumor-positive retroperitoneal lymph node(s) >2 cm diameter: no visceral or extra abdominal involvement
IV	Distant metastases that may include liver
Ovarian	
I	Limited to ovary, peritoneal washings negative for malignant cells; no clinical, radiologic, or histologic evidence of disease beyond the ovaries (gliomatosis peritonei did not result in upstaging): tumor markers negative after appropriate half-life decline
II	Microscopic residual or positive lymph nodes (<2 cm); peritoneal washings negative for malignant cells (gliomatosis peritonei did not result in upstaging); tumor markers positive or negative
III	Gross residual or biopsy only; tumor-positive lymph node(s) >2 cm diameter; contiguous visceral involvement (omentum, intestine, bladder); peritoneal washings positive for malignant cells
IV	Distant metastases that may include liver
Extragonadal	
I	Complete resection at any site; coccygectomy included as management for sacrococcygeal site; negative tumor margins
II	Microscopic residual; lymph nodes negative
III	Gross residual or biopsy only; regional lymph nodes negative or positive
IV	Distant metastases that may include liver

during treatment.[421] Only two patients (1%) had brain metastases at diagnosis. Twelve patients were diagnosed at relapse, and 2 patients were discovered to have brain metastases at autopsy. Most patients with brain metastases had clinical symptoms (12/16) and also had concurrent pulmonary metastases (14/16). The data may overestimate the incidence of brain metastases, however, because 11/16 were diagnosed before 1982 when cisplatin-based chemotherapy was not routine. Risk factors for the development of brain metastases in the St. Jude's study included extragonadal site (P = .013), advanced stage (P = .02) and choriocarcinoma (P < .001). In comparison, in a more recent cohort of the patients treated from 1990 through 1996 in the U.S. pediatric intergroup study, only three out of 299 patients were diagnosed with brain metastases; two of these patients were stage IV at diagnosis.[420] All patients had other sites of metastatic disease; the histology of all three patients included choriocarcinoma. Two of three patients are alive and well; one was treated with PEB alone and one patient received bleomycin, etoposide, and cisplatin (PEB) plus x-ray therapy (XRT). Therefore a bone scan should be obtained in all patients with advanced stage disease (stage III or IV), but head CT or brain MRI are recommended only for patients with stage IV disease, particularly in patients who have choriocarcinoma.

The use of fluorodeoxyglucose F 18 positron emission tomography (PET) has not been systematically evaluated in pediatrics, but its use is becoming more routine among adult patients with GCTs in the setting of a residual mass postchemotherapy. In a German study that compared the sensitivity and specificity of three different modalities for the evaluation of the residual mass, PET vs. CT vs. tumor markers, the results were as follows: PET, 59% sensitivity and 92% specificity; CT, 55% sensitivity and 86% specificity; and tumor markers, 42% sensitivity and 100% specificity.[422] The positive and negative predictive values for PET were 91% and 62%, respectively. Teratomas are not PET-avid. The authors concluded that PET did not add to the preoperative inference in the setting of a mass in which viable tumor was suspected by CT and tumor marker, but it was a useful adjunct in the setting in which tumor markers had normalized or the patient had marker-negative disease at diagnosis. PET-negative masses also require resection because of the possibility of residual teratoma that is PET-negative.

Tumor Markers

Specific subtypes of GCTs secrete proteins that can be used as markers of tumor presence. The pattern of these tumor markers and degree of elevation provide an indication of the likely histology (Table 63-4).[423] AFP is a glycoprotein that is synthesized by the fetal liver and yolk sac. AFP is elevated in patients with YST,[199] although low levels of AFP (generally <100 IU) are often observed in immature teratoma (perhaps because of occult microscopic foci of YST within the tumor). When an elevated AFP level is detected, one must consider other reasons for an elevated AFP in the differential diagnosis, including synthesis by a liver tumor, such as a hepatoma

TABLE 63-4 Histologic Subtype and Tumor Marker Levels

Term	Synonym	Comments*	Incidence Pre- vs. Post-Puberty	Tumor Marker
Seminoma	Dysgerminoma, germinoma	Seminoma-testes tumor; dysgerminoma-ovarian tumors; germinoma-extragonadal tumors	Post	Low levels of AFP; low levels of β-HCG in dysgerminoma caused by presence of multinucleated syncytiotrophoblastic giant cells
Endodermal Sinus Tumor	Yolk sac tumor	Most common form before puberty, besides teratoma	Pre	AFP +++
Embryonal Carcinoma			Post	AFP + β-HCG +++
Choriocarcinoma		Pure forms rare; most commonly one component of mixed GCT	Post	β-HCG +
Teratoma			Pre and Post	
Immature Teratoma			Pre and Post	≈⅓ of immature teratoma produce low levels of AFP
Dermoid cyst				
Polyembryoma				AFP +; β-HCG +
Mixed GCT		Tumors with more than one component are categorized by the most malignant component, not by the most common component	Pre and Post	Depends on the components

AFP, α-Fetoprotein; *GCT,* germ cell tumor; *HCG,* human chorionic gonadotropin.

*Much of the therapy for pediatric GCTs has been gleaned from experience treating testicular GCTs in adult males. An excellent guide for the workup and treatment of adult testicular tumors is the National Comprehensive Cancer Network (NCCN) Practice Guidelines in Oncology (http://www.nccn.org/).

or hepatoblastoma, or other disease states such as hypothyroidism, folate deficiencies, autoimmune disorders, acquired immunodeficiency syndrome (AIDS), congenital heart defects, cystic fibrosis, and platelet aggregation disorders. β-HCG is a peptide hormone produced in pregnancy, that is made by the embryo soon after conception and later by the syncytiotrophoblast (part of the placenta). Its role is to prevent the disintegration of the corpus luteum of the ovary and thereby maintain progesterone production that is critical for a pregnancy in humans. In tumors that originate in extraembryonic tissues, like choriocarcinoma, β-HCG can be significantly elevated. β-HCG is also sometimes mildly elevated in seminoma/dysgerminoma (<50 IU/L).

Interpretation of AFP levels must incorporate knowledge of age-related norms (see Fig. 63-6).[191,192] AFP is elevated in all infants at birth because there of continued synthesis of AFP by the fetal liver. Over the course of the first 2 years of life, AFP declines in normal infants as the synthesis in the liver ceases. To establish normal values for infants, Blohm et al analyzed data on 414 full-term infants, 90 preterm infants, and 259 children up to age 2 years from the University Hospital Düsseldorf (Germany).[192] Using regression analysis, the authors estimated that the half-life of AFP increases with age over the first 2 years of life. From birth to day 28, the half-life is estimated to be 5.1 days. The half-life of AFP increases thereafter to 14 days at 1 to 2 months of age, 28 days from 2 to 4 months of age, and 42 days from 4 to 6 months of age. The increase in the half-life of AFP with age also results in a "shouldering" of the AFP curve during the first 2 years of life. Not all children have adult levels of AFP by 2 years of age. In the Blohm study, the mean AFP at age 2 was 8 ng/mL; however, the 95% range extended between 0.8 and 87 ng/mL. Similar results were reported in an earlier and smaller study by Wu et al.[191] Values of AFP obtained in children under age 2 ought to be plotted on the nomogram developed by Blohm et al to determine whether the value falls within the normal range.[192]

Tumor markers may also be prognostic. In adult males with testicular cancer, the prognostic significance of postoperative tumors markers is well established. An international consortium pooled data on over 5000 men with testicular cancer and used multivariate analysis to investigate prognostic factors that created the International Germ Cell Consensus Classification (IGCCC), which divided patients into good, intermediate, and poor risk groups based on the extent of elevation of tumor markers, the presence of nonpulmonary visceral metastases or a nontesticular primary site of disease.[424] Although elevated tumor markers are one of the primary variables that determine risk group assignment in adult men, evaluation of the prognostic significance of tumor markers in pediatrics has been limited. In adult males, the failure of tumor markers to decline appropriately among poor-risk patients has also been shown to be a poor prognostic feature. Further discussion can be found in "Prognostic Factors at Diagnosis of Pediatric Germ Cell Tumors."

Tumor markers are also used prospectively to differentiate between stage I and II tumors. For patients with gonadal GCTs who have had all disease completely removed surgically and in whom no evidence of further disease is noted on pathologic or radiologic staging evaluations a watch-and-wait strategy is often recommended. However, if tumor markers fail to decline according to the expected half-life, the patient by definition has occult metastatic disease. The patient should be upstaged to stage II and therapy commenced.

There is one situation in which tumor markers may increase and not be cause for alarm. During the first cycle of chemotherapy, it has been observed that tumor markers can increase transiently. However, levels should have declined to the expected level by the start of the second cycle of chemotherapy.

SURGERY AND GERM CELL TUMOR

Surgery is an integral part of the management of GCTs. For benign tumors, complete excision is curative. For malignant tumors, proper staging and appropriate timing of excision are important for optimal outcome. At all sites, primary excision should be considered if the tumor can be safely removed without significant risk of morbidity. For tumors that pose risk to adjacent structures or expectation of incomplete excision, biopsy should be performed for diagnosis and neoadjuvant chemotherapy should be given. Reduction in tumor size allows greater expectation of safe and complete excision. Evidence is mounting that delayed resection of extragonadal tumors does not just decrease morbidity but also more importantly may increase survival, because of the ability to achieve complete resection at the time of the delayed surgery. In the case of expected malignancy, a child should be referred to a center with surgeons with experience in oncologic surgery, preferably a site that participates in COG clinical trials so that the proper staging can be done to allow for trial entry.

Surgical Approach to Sacrococcygeal Tumors

Over 90% of neonatal sacrococcygeal tumors are benign (mature or immature teratoma), and require complete excision at diagnosis should be attempted without harming adjacent structures. There is a 9% to 14% risk[425,426] of malignant recurrence in benign tumors, and long-term follow up care is important. Beyond the neonatal period, the risk of malignancy increases, and the tumor configuration is more likely to have an internal component (Altman types III and IV; see Fig. 63-13). Children with a large sacrococcygeal tumor suspected to be malignant should be evaluated for feasibility of resection. Gastrointestinal and genitourinary complications are far more common than lower extremity neurological sequelae, with up to 41% patients affected in one study.[39] Long-term follow up observation is mandatory in these children to ensure that normal bowel, bladder, and sensory-motor function is preserved.[40] If a problem is encountered, these children are best cared for by a multidisciplinary team that simultaneously initiates symptom management (such as management of constipation) with investigational studies including functional evaluations such as anorectal manometry and urodynamic studies (including direct electromyography [EMG] evaluation of the urinary sphincters) to define the extent of the problem at discovery as well as any progress (or lack thereof) in response to the prescribed interventions.[427] One final note is recognition of the recommendation by Ozkan and colleagues,[41] who suggest the need for defining the extent of neurologic, gastrointestinal, and genitourinary dysfunction before any intervention so as to better categorize any impairment of function at baseline and not just after surgery.[428] This suggestion is especially valuable for those patients whose tumors are discovered outside of the neonatal period.

Therefore an initial biopsy with a delayed resection must be seriously considered if morbidity is likely to be reduced.[429] In patients whose lesions involve the rectum or extend into the sacral bone or in patients already shown to have metastatic disease, biopsy rather than resection is the more appropriate initial surgical approach. If a needle biopsy is used to obtain diagnostic material, multiple passes should be obtained because of the histologic heterogeneity of these tumors and the possibility that the malignant foci will not be found in every sample. The histologic diagnosis should also be evaluated in the context of the information from tumor markers. A benign histologic diagnosis in the setting of elevated tumor markers should lead one to question whether there was a sampling error in the sample obtained or in the pathologic review. Lin28 is a new histochemical reagent that can be used to detect YST in the specific and sensitive way that should be used to detect occult YST.[388]

Delayed resection of a SCT has been definitively demonstrated not to adversely affect survival. In fact, German investigators have shown that patients with locally advanced and metastatic tumors actually had better overall survival when treated with chemotherapy before tumor resection. Overall survival for patients with delayed resection was 83%, whereas overall survival for patients who had an initial resection was only 45% ($P = 0.01$).[419] Preoperative chemotherapy allowed for more complete primary resection of the tumor; 19 of 31 patients had a complete resection after preoperative chemotherapy vs. only 11 of 35 patients who had a complete resection when definitive surgery was attempted before chemotherapy. Complete resection of the tumor, either at diagnosis or delayed until after chemotherapy, was the most significant predictor of event-free survival (EFS) in the German series of sacrococcygeal tumors (Fig. 63-14).[419] Second-look surgery was recommended for those with incomplete resection and had therapeutic benefit. Analysis of the data from the United States Pediatric Intergroup Trial (POG9049/CCG8882) also confirms that delayed resection did not have an adverse effect on prognosis.[416]

Most presacral lesions will be initially approached through a posterior transsacral incision. Superior

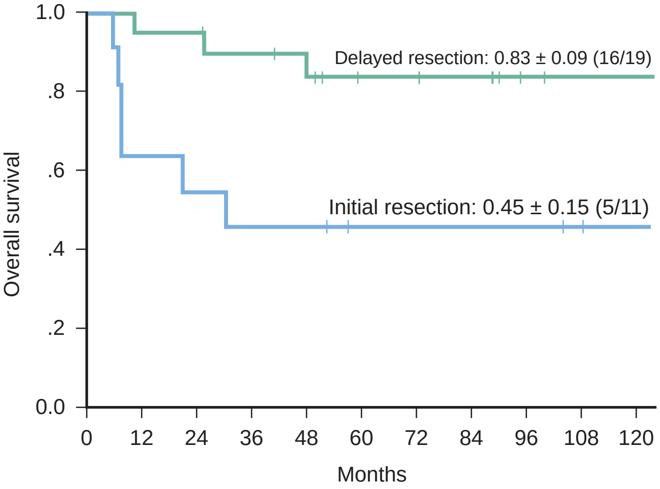

Figure 63-14 Five-year overall survival of 30 patients with locally advanced and metastatic malignant sacrococcygeal GCT (stage T2b M1) treated with initial resection versus delayed resection ($P = .01$, log-rank test). *(From Gobel U, Schneider DT, Calaminus G, et al: Multimodal treatment of malignant sacrococcygeal GCTs: a prospective analysis of 66 patients of the German cooperative protocols MAKEI 83/86 and 89.* J Clin Oncol *19:1943–1950, 2001.)*

extension of the tumor into the pelvis often requires a laparotomy. If laparotomy is performed, retroperitoneal lymph nodes should be examined and biopsied if enlarged. In all cases, the coccyx must be completely removed en bloc with the primary tumor. The importance of the resection of the coccyx was first noted by Gross, who reported a recurrence rate of 38% of those who had not had coccygectomy and 0% of those who had had a coccygectomy.[430]

Surgical Approach to Testes Tumors

Risk of malignancy is age dependent with 38% to 74% of testicular tumors in the prepubertal age group having benign histology.[121,122] In prepubertal boys with localized tumors, those with normal tumor markers and imaging studies can be considered for testis sparing enucleation of the tumor. Because the most common sites of metastasis of a testicular GCT are the retroperitoneal lymph nodes, a pelvic/abdominal CT scan should be obtained preoperatively. If the CT scan is delayed until after surgical exploration of the inguinal region, reactive retroperitoneal lymphadenopathy may be noted on CT scans and may be difficult to differentiate from metastatic disease. Evaluating the lungs for pulmonary metastases with chest CT before surgery is advisable, because postoperative atelectasis can make evaluation for malignant disease more difficult. Tumor markers should also be obtained preoperatively, because these levels are important to confirm the initial diagnosis and to provide prognostic information. In addition, the rate of decline is important to be able to track postoperatively in patients, especially in patients with stage I disease who are being expectantly observed, reserving chemotherapy only for those patients who have demonstration of residual malignant disease.

The surgical approach for a scrotal tumor should be through an inguinal incision. Control of the testicular vessels and vas deferens is gained at the level of the inguinal ring. The testis is then mobilized from the scrotum. Occasionally, a large testicular mass cannot be mobilized out of the scrotum into the inguinal region, and in this situation the inguinal incision should be enlarged to the superior aspect of the scrotum in order to avoid tumor rupture during mobilization. A radical orchiectomy is then performed with high ligation of all cord structures at the level of the internal inguinal ring. In prepubertal boys who have a focal lesion with normal markers and metastatic imaging, tumor enucleation protecting the tumor capsule followed by frozen section should be considered. If the frozen section is consistent with teratoma, the testis can then be placed back in the scrotal position, because enucleation is adequate therapy for prepubertal testicular teratoma. In the postpubertal boy, however, even localized teratoma has malignant potential and orchiectomy ought to be undertaken.

Rate of adherence to surgical guidelines was evaluated among the 63 patients with stage I GCT of the testes who were treated with observation only on the last U.S. intergroup trial from 1990 through 1996.[431] Adherence to guidelines could be verified in only 69% of the patients. Likelihood of adherence was directly related to whether or not the surgeon thought the diagnosis was tumor preoperatively. In patients in whom the preoperative diagnosis was malignant tumor, the surgical guidelines were followed in 80% of patients, whereas among patients in whom another preoperative diagnosis was suspected (i.e., hydrocele, hernia, torsion), the guidelines were followed in only 17%. There was no difference in recurrence rate among those patients in whom the surgical guidelines were followed and among those for whom the guidelines were not followed, with the exception of patients who had had scrotal violations. Among the four patients with transscrotal violation, three had recurrence of disease.

Based on detailed review of the surgical procedures followed in the CCG-POG Intergroup Study (INT0098), the surgical guidelines in the case of scrotal violation were revised. In cases in which a scrotal orchiectomy has been performed without violation of the tumor capsule, the patient may still remain in stage I. If the cord structures show evidence of tumor spread, the patient should be upstaged to stage II. However, if a scrotal biopsy was performed as the initial procedure, the scrotum is considered to be contaminated and the patient should be treated as having stage II disease. Hemiscrotectomy, however, is no longer required. In addition, a "completion orchiectomy" with removal of all cord structures to the level of the internal ring should be performed.

Evaluation of Retroperitoneal Nodes in Pediatric Testicular Germ Cell Tumors

Lymphatic drainage of the testes is to the retroperitoneal nodes and to the nodes below the renal vessels. Right testicular tumors usually metastasize to the nodes between the aorta and the inferior vena cava (interaortocaval nodes). Left testicular tumors metastasize to the nodes lateral to the aorta (paraaortic).[432] Left supraclavicular nodes and pulmonary nodules have been observed in the absence of retroperitoneal adenopathy. In adults, it is estimated that up to 30% of patients have false negative CT scans; in the most recent U.S. intergroup study of stage I testicular cancer among boys younger than 10 years of age, 5% of patients had failure of normalization of tumor markers postoperatively (i.e., patients had occult disease that was not detected on initial CT scan (false-negative initial CT scan). In total, 22% of patients presumed to be in stage I at diagnosis eventually relapsed, but all were salvaged with chemotherapy with 100% survival.[399]

If the preoperative CT scan of the abdomen has identified enlarged (≥4 cm) retroperitoneal lymph nodes, biopsy is not necessary, and one may assume that this represents metastatic disease; the patient should be treated as having stage III disease. If retroperitoneal lymph nodes are between 2 and 4 cm, a biopsy should be performed. If the retroperitoneal nodes are smaller than 2 cm, a biopsy is not required; however, the tumor markers (AFP and β-HCG) should be observed and return to normal with appropriate decline according the half-life (see previous discussion of tumor markers). In adult males, 10% to 20% of nodes between the size of 1 and 2 cm contain metastatic disease.

If the decision is made to biopsy a suspected node of the prepubertal boy, the retroperitoneal procedure should simply be a lymph node excision, not a full retroperitoneal lymph node dissection. If no biopsy is performed and the nodes are enlarged (>2 cm), that patient should be treated as a patient with stage III disease. But in adolescents (as in adults), if a biopsy is being undertaken to evaluate nodal disease, then a nerve-sparing modified template retroperitoneal lymph node dissection (RPLND) should be employed, which some surgeons would expand to a full bilateral nerve-sparing RPLND if any of the initial nodes are positive on frozen section. RPLND is both diagnostic and potentially therapeutic. For further discussion of RPLND please see the section on treatment of adolescent boys with stage I testicular cancer.

Metachronous Testicular Cancer

In adults, 3% to 5% of men with testicular cancer have a metachronous tumor in the other testicle at diagnosis, and 2% of men subsequently develop a tumor in the contralateral testicle. These risks have not been quantified for pediatric patients and no formal evaluation of the contralateral testis is recommended besides that continued thorough physical examinations, although a patient should be advised of the likely need for continued surveillance of the contralateral testis. Patients who have developed a testicular tumor in a cryptorchid testis are at increased risk of malignancy in the normally descended contralateral testes.

Surgical Approach to Ovarian Tumors

The largest series of ovarian GCTs (66 cases) estimated rate of malignancy in an ovarian mass as 19% at ages 0 to 5 years, 14% at ages 6 to 10 years, and 33% at ages 11 to 15 years.[258] AFP and β-HCG should be determined preoperatively for ovarian tumors. If not done preoperatively, these markers should be sent from the operating room once the diagnosis is apparent. The goals of the initial exploration for a suspected ovarian tumor are to completely resect the tumor if it can be accomplished without sacrifice of adjacent organs and to completely evaluate the extent of disease.

Surgical guidelines for ovarian GCTs were originally based on the experience with ovarian epithelial carcinoma, in which aggressive surgical resection is associated with higher chance of survival. Application of those surgical guidelines to ovarian GCTs, which have a different pattern of spread and are also highly chemosensitive, is not warranted. It is clear from studies in adult women with GCTs that fertility-sparing surgery (i.e., performing an unilateral instead of bilateral salpingo-oophorectomy) had no adverse outcome on survival.[238] On the most recently completed U.S. Intergroup Trial for gonadal tumors (CCG8892/POG9048), surgeons adhered to the guidelines (derived from adult epithelial cancer) in only 2% of the patients,[418] but overall EFS and overall survival (OS) rates were nonetheless excellent.[433] Surgical omissions resulting in protocol noncompliance resulted from failure to biopsy bilateral nodes (97%), no omentectomy (36%), no peritoneal cytology (21%), and no contralateral ovary biopsy (59%). Among the patients in whom peritoneal cytology was examined, 25% of patients had malignant tumor found in what would otherwise been declared to be stage I disease. Biopsy of normal-appearing lymph nodes, omentum, and/or contralateral ovary very rarely, if ever, showed occult malignant disease.

Given the excellent overall survival, despite low adherence to the surgical guidelines on the Pediatric Intergroup Trial, the current surgical guidelines for pediatric ovarian GCT have been substantially revised. Given the high rate of positivity in the examination of peritoneal fluid, all patients with ascites present should have the fluid collected for cytology. In the absence of ascites, peritoneal washings should be conducted and sent for pathologic review. Any patient in whom cytology is not available should not be considered to be in stage I. The pelvic viscera should be examined, and pelvic and retroperitoneal lymph nodes should be palpated bilaterally. Only suspicious or enlarged lymph nodes should be biopsied. The omentum and peritoneal surfaces should also be palpated and visualized. If the omentum is adherent or has nodules or implants, partial or complete omentectomy that includes the lesions should be done. If the omentum does not appear grossly involved, an omentectomy does not need to be performed. Any nodules studding the peritoneal surfaces should be biopsied.

If only one ovary is involved, the tumor should be removed by unilateral oophorectomy. Attempts to preserve completely uninvolved fallopian tubes and the uterus should be made in all patients. Lesions adherent to the uterus may be managed by separation of the adherence if feasible or by biopsy only with planned second-look surgery after chemotherapy if necessary. The tumor capsule should not be aspirated. If the capsule is violated, either by the surgeon or because of rupture by the tumor or if there is microscopic capsule penetration seen by the pathologist in an apparently intact tumor, the patient should be upgraded to stage II (this is in contrast to adult staging guidelines in which a patient with capsular involvement is International Federation of Gynecology and Obstetrics [FIGO] stage IC). The ovary must be delivered intact and not removed in fragments. The contralateral ovary should only be biopsied if it appears involved with tumor; biopsy of a normal-appearing contralateral ovary is discouraged because of concern regarding possible adverse impact on future fertility. Bilateral ovarian lesions were present in only 6% of patients (n = 10) on the U.S. Pediatric Intergroup Study. Of these, four were benign teratomas and six were malignancies. If a patient has bilateral disease, preservation of as much normal ovarian parenchyma as possible should be the goal.

Surgical Approach to Mediastinal Germ Cell Tumors

The German MAKEI experience with mediastinal GCTs was analogous to the experience with sacrococcygeal tumors. Delayed resection increased the odds of complete resection of the primary tumor (10 of 11 patients at delayed resection vs. 6 out of 12 patients at primary resection) and complete resection was a strong predictor of more favorable outcome (EFS 0.94 ± 0.06 vs. 0.42 ± 0.33).[417] Therefore the surgeon must decide whether it is more prudent to attempt a primary resection or to obtain a biopsy and plan for delayed resection.

The resection may be through a lateral thoracotomy or median sternotomy, as determined by the site of the lesion. The margin of resection should include adherent nonvital structures such as the thymus and pericardium. Regional lymph nodes should be evaluated and biopsied when possible in all patients.

Surgical Approach to Extragonadal Tumors at Other Sites

YST (also referred to as *endodermal sinus tumor*) of the vagina is a rare tumor occurring exclusively in children younger than 3 years of age. The mass can be confused with sarcoma botryoides. Only small lesions that can be resected with vaginal preservation should be excised at initial presentation. In others, a careful vaginal examination and limited biopsy should be performed. After completion of chemotherapy, repeat evaluation and complete excision of residual disease should be considered, although oftentimes residual tissue is just scar tissue. A biopsy to guide the need for resection is a reasonable approach.

Malignant GCTs found at other locations should be completely excised if possible without sacrifice of adjacent organs. Biopsy only may be appropriate at the initial operation, with planned secondary procedures for delayed excision after chemotherapy. Any enlarged regional lymph nodes in the area should be sampled.

PROGNOSTIC FACTORS AT DIAGNOSIS OF PEDIATRIC GERM CELL TUMORS

Tumor markers have prognostic significance. In pediatric GCT, preoperative tumor markers have been evaluated, whereas in adult testicular cancer tumor markers evaluated at the time of initiation of chemotherapy are used as the benchmark, which is why it is important to obtain preoperative levels. In adult males with testicular cancer, the prognostic significance of tumor markers is well established. IGCCC pooled data on over 5000 men with testicular cancer; multivariate analysis identified prognostic factors that could divide patients into good, intermediate, and poor risk groups. Elevated tumor markers are one of the primary variables that determine risk group assignment in adults. Other adverse prognostic factors include mediastinal primary site and presence of nonpulmonary visceral metastases. LDH has been shown to be prognostic in adult males with testicular cancer but has not been shown to have prognostic significance in pediatric patients, although analyses to date have been underpowered to examine this issue.

Several retrospective studies in adult males with testicular cancer have shown that slower than expected decline in tumor markers after the initiation of treatment predicts both lower EFS and OS.[434-436] In an analysis of adult men with testicular cancer treated between 1986 and 1998 at Memorial Sloan Kettering, those with satisfactory decline in their tumor markers had an excellent 2-year survival rate (EFS 91%; OS 95%) compared with those who had unsatisfactory decline (EFS 69%, OS 72%).[436] A recent randomized trial among patients classified as having “IGCCC intermediate or poor risk” testicular cancer compared standard therapy, four cycles of BEP, to two cycles of BEP followed by 2 cycles of high-dose carboplatin, etoposide, and cyclophosphamide. Although the overall results of the trial did not show a difference in survival between the two arms, in the subgroup of 67 patients with unsatisfactory tumor marker decline, the 1-year durable complete response was 61% for the patients who received high-dose chemotherapy (HDCT) compared with 34% for those who received BEP alone.[437]

Evaluation of the prognostic significance of tumor markers in pediatrics has been limited by relatively small numbers enrolled on clinical trials. Nonetheless in data from the last U.S. pediatric intergroup trial, data from the United Kingdom’s GC2 trial, and from the French TGM85 and TGM90 trials, an AFP of 10,000 IU/L or higher portends a worse prognosis for EFS.[418,438-441] In multivariate analysis of the U.K. data, AFP higher than 10,000 IU/L was a stronger predictor of relapse than site or stage; those with AFP greater than 10,000 IU/L had four times the risk of relapse. In two pediatric studies, AFP was not found to be prognostic, but both analyses were restricted only to patients with extragonadal primaries.[420,442] β-HCG and LDH was evaluated in the U.S. data and not shown to be of prognostic significance when using the cutpoints developed for adult men in the IGCCC data set.[418,438] Extragonadal tumor site was of prognostic significance in the United States, British, and French studies, although it was defined slightly differently in each study. Common to each study was the inclusion of mediastinal site, which is clearly associated with reduced EFS in all studies. Relative impact of other sites of extragonadal disease will require examination in a larger data set. The impact of satisfactory vs. unsatisfactory tumor marker decline has not been evaluated in pediatric patients with GCTs.

TREATMENT

Overview

Survival has improved markedly since the landmark publication by Einhorn in 1979 noting that widely metastatic testicular GCTs were curable with cisplatin-based regimens.[443] In 1981 Einhorn declared that "testicular cancer is the model of a curable neoplasm."[444] As it becomes more and more established, at least from long-term studies of adult men who have been successfully treated for testicular GCTs, that treatment is associated with significant long-term morbidities and even increased chance of mortality, oncologists need to concede that initial cure is not necessarily synonymous with normal life expectancy. Although highly curable, less toxic therapies need to be determined. Many patients require no treatment besides complete surgical resection; chemotherapy can be reserved to salvage only those patients who have recurrence. On the other end of the spectrum it is also apparent that for a subset of approximately 15% to 20% of patients, the standard cisplatin-based regimens are insufficient and more intensive chemotherapeutic strategies must be devised. A key part of future treatment strategies will be the ability to identify those patients at high risk of relapse at the time of their initial treatment and to design chemotherapeutic regimens that increase the odds of long-term survival both by optimal first-line therapies and salvage therapies for those who fail to respond.

Explanation of the Nomenclature

GCTs are generally divided into several categories when treatment is being discussed. The categories include: 1) teratoma, 2) immature teratoma, 3) seminoma/dysgerminoma, and 4) "other malignant" GCTs that include the histologies of YST (or endodermal sinus), embryonal carcinoma, and choriocarcinoma. GCTs can either present as a single histology, such as pure YST, or with a mixture of different histologies including histologies from all four categories (teratoma, immature teratoma, seminoma/dysgerminoma, and "other malignant"). The terminology is somewhat confusing in that a "malignant" GCT is shorthand for a GCT that contains at least one of the other malignant components, which means yolk sac, embryonal carcinoma, or choriocarcinoma. These histologies do have a more aggressive nature; however, both immature teratoma and seminoma/dysgerminoma are also malignant forms of GCTs. The general proviso is that the one must treat the most malignant form of the GCT. However, it is important to keep in mind what other histologies are present. For instance, if there is a significant portion of the original tumor that is mature or immature teratoma at diagnosis, this part of the tumor is not likely to respond to chemotherapy and it will likely be necessary to surgically excise the remaining teratoma at the end of the chemotherapeutic regimen.

Infantile Choriocarcinoma: A Rare but Rapidly Fatal Condition if Not Recognized

Infantile choriocarcinoma is an extremely rare entity that is a medical emergency and requires immediate institution of therapy. The disease is believed to be transplacentally acquired from a focus of choriocarcinoma that has arisen in the placenta as a variant of gestational trophoblastic disease. Choriocarcinoma is composed of cytotrophoblast, syncytiotrophoblast, and extravillous trophoblast, often mixed in random fashion surrounding areas of hemorrhage and necrosis.[69] Vascular invasion is a common feature of choriocarcinomas.

The median age at presentation was 1 month of age (range 0 days to 5 months).[11] Typical symptoms at diagnosis include severe anemia, failure to thrive, hepatomegaly, and seizures. Less commonly, infants can also have with hemoptysis and respiratory distress or signs of precocious puberty.[445,446] Marked elevations of β-HCG are typical (often $>1 \times 10^6$ IU/L).[12]

The disease is rapidly fatal if the syndrome is not recognized. Among 30 cases reviewed by Blohm and Gobel, two cases were diagnosed in stillborn children; in 19 cases, the diagnosis was made after death at postmortem examination.[445] Among those children who succumbed to the disease, the median time from initial symptoms to death was 21 days. Because of the rapid progression of this disease, treatment should not be delayed for histologic confirmation in an infant who shows a markedly elevated β-HCG and a clinical presentation consistent with infantile choriocarcinoma.

Single agent chemotherapy with methotrexate, which is usually sufficient as a single agent to treat gestational trophoblastic disease in the mother has never been curative in an infant with choriocarcinoma; multiagent chemotherapy is required.[447,448] In Blohm's series of 30 children, the five cases successfully treated received multiagent chemotherapy that included a platinum compound, either cisplatin or carboplatin, used in combination with etoposide. However, because of the rapid growth of these tumors, in several cases the platinum-etoposide combination was coupled with methotrexate administered either the week before or after the platinum regimen. Surgical resection of residual masses has been carried out in most patients at the end of the platinum-based therapy. Upfront surgical resection of tumor is definitely not advised in these patients because of the friability of the tumors, the chance of uncontrolled bleeding, and the delay that would be incurred in starting chemotherapy, as well as the usual fragile state of the infant at diagnosis.

If an infantile choriocarcinoma is diagnosed, the mother should also be screened for the disease. In the review by Blohm and Gobel, 60% of the mothers also had metastatic choriocarcinoma (17/30).[445] Since β-HCG is uniformly elevated in this disease, serial screening of the maternal β-HCG should be sufficient, although some physicians have advocated for radiologic examination of likely metastatic sites (lung, liver, brain) as well.

Seminoma and Dysgerminoma

The nomenclature of this subtype of pediatric GCT can be confusing. It is important to recognize that a tumor with the same histologic appearance has a different name depending on the site in which it arose. *Germinoma* typically refers to an intracranial lesion, usually in the pineal gland. *Dysgerminoma* describes a lesion in the ovary. *Seminoma* describes a lesion that arises in the testis.

Seminomas and dysgerminomas are much more common histologies in young and middle-aged adults than in adolescents. Diagnosis of a seminoma/dysgerminoma is exceedingly rare before the onset of puberty. However, in men overall seminomas account for approximately 50% of all testicular cancers, occurring most commonly in the fourth decade (Fig. 63-15).[261]

Because these tumors are exquisitely sensitive to radiotherapy, radiation was an important component of the treatment regimen. In patients with ovarian tumors, radiation resulted in sterilization of the remaining ovary; therefore since the 1980's chemotherapy with cisplatin-based therapy has replaced radiotherapy in the treatment of ovarian GCT. With longer follow-up observation of patients treated with radiotherapy, it became clear that there was reason for serious concerns about induction of secondary malignancies and cardiovascular disease in patients who received radiation therapy. In large prospective study, risk of cardiac events was 2.4 (95% CI, 1.04 to 5.5).[449] Risk of second malignant neoplasm is 2 (95% CI, 1.9 to 2.2).[450] An accepted standard now for stage I disease is surveillance with expected 15% to 20% relapse rate. With seminomas, relapses can occur quite late; 5% occur after 5 years, and there are anecdotal reports of relapses occurring after 10 years. Most pediatric radiotherapists would prefer not to use radiotherapy to treat a seminoma or dysgerminoma because of the risk of late effects; chemotherapy has replaced radiation as first-line therapy.

Treatment of these histologies has never been explicitly studied in pediatric clinical trials given their rarity, and hence treatment recommendations derive directly from the adult experience. The pediatric oncologist who is referred a patient with one of these diagnoses should consult with an adult oncologist and refer to an organization like National Cancer Care Network (NCCN) that maintains updated clinical practice guidelines for these tumors (http://www.nccn.org/).

TREATMENT OF STAGE I MALIGNANT NONSEMINOMATOUS GERM CELL TUMORS

Because much of the understanding about treatment options for pediatric stage I tumors derives from the adult experience, this work is reviewed in the following section.

Active Surveillance of Adult Men with Stage I Malignant Nonseminomatous Germ Cell Tumor Disease

The watch-and-wait strategy of active observation is based on a plethora of data from adult trials of men with stage I malignant GCT. Over 2000 adult patients have been enrolled on clinical trials of surveillance with a median follow-up range of at least 10 years.[451] Relapse rates range from 27% to 35%; 95% of relapses occur in the first 2 years after orchiectomy; almost all patients who relapse have "good risk" disease as defined by the IGCCC criteria; overall survival with chemotherapy is 98% to 99%.

An increasing concern is that patients who undergo surveillance are obligated to serial CT scans at regular intervals, which expose the patients to a dose of radiation that may subsequently be the cause of secondary malignancy. COG recommends scans every 3 months for the first year and every 6 months for the second year after diagnosis. However, a whole-trunk CT scan (chest/abdomen/pelvis) produces a radiation dose of 10 to 30 mSv.[452] It is recommended that exposure be limited to 100 mSv over 5 years.[453] Although it has been suggested that risk related to low doses of radiation may have been overestimated, a recent retrospective cohort study using data from 15 countries estimated that a cumulative exposure of 100 mSV would lead to a 9.7% increase in risk of all mortality from cancer, excluding leukemia, and a 19% increase in mortality because of leukemia.[454] Rustin et al. postulated that five total-body CT scans could therefore induce one second cancer in every 200 patients, which is the risk of death after stage I testicular cancer.[455] Therefore Rustin and colleagues designed a trial in adult men with stage I testicular cancer comparing a surveillance policy of two CT scans over the first year (at 3 and 12 months) with the standard surveillance policy of five CT scans over the first 2 years (3, 6, 9, 12, and 24 months).[455] Patients continued to have frequent clinical evaluations that included measurement of tumor markers and chest radiography (CXR; once per month in the first year, every 2 months in the second year, and every 3 months in the third year). There was no difference in overall relapse rate between the two surveillance groups, no increase in more advanced stage at relapse between the two groups, and no difference in overall survival. A major limitation of the study was that only 10% of the patients were at high risk for relapse on the basis of the presence of lymphovascular invasion (LVI), so it is not clear whether this schedule is vigilant enough for this group of patients. Given the increased radiosensitivity of children to the low-dose of ionizing radiation associated with CT scans, the schedule of surveillance in children with stage I GCTs deserves serious scrutiny.

Factors that Predict Relapse in Adult Stage I Nonseminomatous Germ Cell Tumor Patients on Surveillance Studies. Multiple studies have confirmed that the single most important prognostic factor is LVI, bifurcating risk into low risk (15% to 20%) and high risk (40% to 50%) for relapse. Although embryonal carcinoma is a significant prognostic variable in univariate analyses, its importance in multivariate models is subsumed by presence of LVI. No immunohistochemical markers, including MIB-1, p53, bcl-2, cathepsin D, or E-cadherin have added to the prognostic significance of LVI alone.[456-460]

Retroperitoneal Lymph Node Resection in Adult Men with Stage I Nonseminomatous Germ Cell Tumor

There is no doubt that surgical removal of retroperitoneal lymph nodes is known to comprise the "landing zones" for metastatic disease from the testes is the most accurate way to determine the stage of patient's cancer and is potentially therapeutic as well. Relapse in the surgical field is less than 1% after RPLND. The consensus of a

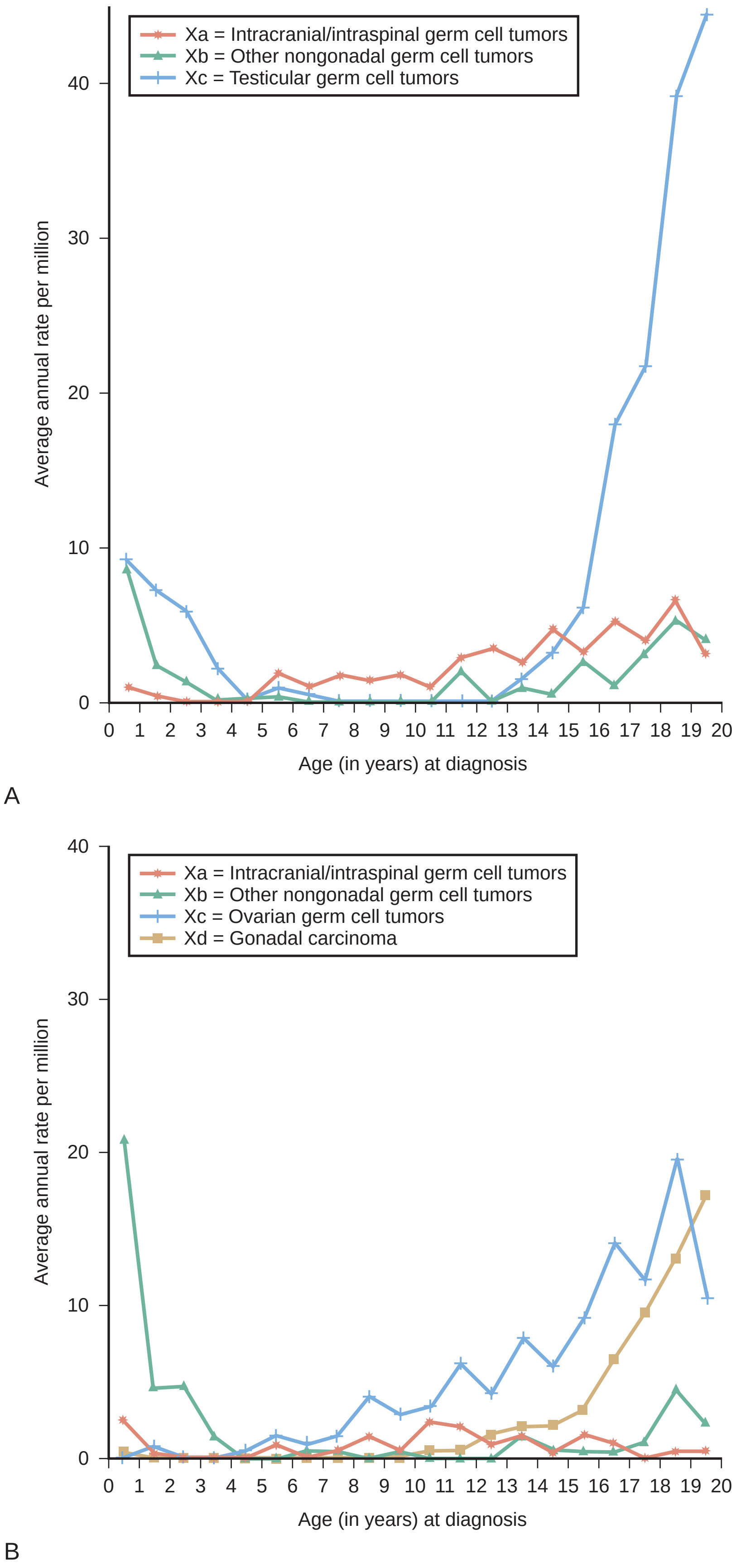

Figure 63-15 Incidence of germ cell tumors in the SEER program from 1973 to 1978 and 1994 to 1998. **A,** Males. **B,** Females. *(From McGlynn KA, Devesa SS, Sigurdson AJ, et al: Trends in the incidence of testicular GCTs in the United States.* Cancer *97:63–70, 2003.)*

series of clinical trials has shown that between 30% and 50% of adult males with testicular stage I nonseminoma have occult metastases at RPLND that were not detected by radiologic scans. Surveillance for patients without an elevation of one of the tumor markers at diagnosis relies heavily on radiologic scans, and some patients prefer the certainty of RPLND in this particular situation.

A serious concern for any patient undergoing RPLND is nerve injury that interferes with the ability to ejaculate, causing retrograde ejaculation, or ejaculatory failure. Because of refinements in surgical technique and the development of so-called "nerve-sparing" approaches, it is estimated that preservation of ejaculatory function is as high as 90% to 95%, especially if the surgery is performed by an urologist who performs a high volume of these procedures per year (i.e., 10 to 20). The surgery can be the cause of uncommon but nonnegligible complications such as bowel complications, lymphocele, and chylous ascites. Referral of the adolescent male who chooses RPLND as primary therapy to a cancer center with a urologist who specializes in RPLND is warranted.

However, RPLND does not mean that chemotherapy will be avoided. Ten percent of patients with pathological stage 1 cancer experience relapse after RPLND (primarily in the lungs) and approximately one third of patients with stage II disease (or 10% to 15% of patients who undergo RPLND) have evidence of sufficiently extensive disease (multiple nodes, any node >2 cm, or any evidence of extranodal spread) that chemotherapy will be initiated immediately after RPLND. Usually at least 2 cycles of BEP is required. One argument in favor of primary (upfront) RPLND is that it may reduce the odds of having to perform RPLND after chemotherapy if there is residual disease. After chemotherapy, the operation is much more technically challenging because of the desmoplastic reaction to chemotherapy. Another advantage of primary RPLND is that it removes any concurrent teratoma, which is a chemoresistant form of GCT. Because a small percentage of teratomas can undergo malignant degeneration to non–germ cell malignancies that are much more difficult to treat, it is beneficial to resect these components of GCT early in course of treatment.

Adjuvant Chemotherapy for Stage I Testicular Nonseminomatous Germ Cell Tumors

Another option employed in men with clinical stage I nonseminomatous germ cell tumors (NSGCT) is adjuvant chemotherapy with one or two cycles of BEP. Although 11 studies have shown that the risk of relapse can be reduced to less than 2%, the concern remains that the majority of patients, conservatively at least 50%, have no further need for chemotherapy after the orchiectomy, and therefore treatment with BEP is overtreatment for a large percentage. Moreover, accumulating evidence of significant long-term side effects associated with three or four cycles of cisplatin-based therapy, including a marked increase in cardiovascular disease and risk of second malignancy (see "Late Effects") should invoke caution about the administration of even two cycles of the same therapy, because the relation between dose and late effect has not been quantified.

Active Observation of Pediatric Germ Cell Tumor Patients with Stage I Disease

On the U.S. Pediatric Intergroup Study (POG09048/CCG8891), boys younger than 11 years of age with stage I testicular malignant GCTs were observed after surgery (watch-and-wait strategy) and only given chemotherapy if tumor markers did not normalize as expected or if imaging revealed recurrent disease. Among the 63 patients enrolled on the protocol, 11 patients had progression of disease and received chemotherapy (four cycles of standard-dose PEB; see the following section on treatment); 80% of the recurrences occurred in the first year after surgery. The 6-year EFS of active observation was 78.5% ± 7%; however, all patients were salvaged with subsequent chemotherapy (6-year OS 100%).[431]

On the subsequent COG GCT study (AGCT0132), an initial strategy of surveillance after surgical resection was extended to girls with pediatric stage I malignant ovarian GCT (MOGCT) to determine whether overall survival could be preserved.[461] Between November 2003 and July 2011, 25 girls, ages 0 to 16 years (median age 12 years old) with stage I MOGCT were enrolled into the trial. Twenty-three patients had elevated AFP at diagnosis. Predominant histology was YST. Twelve patients had evidence of persistent or recurrent disease (EFS 52%; 95% CI, 31% to 69%). Median time to recurrence was 2 months. All patients had elevated AFP at recurrence; six had localized disease, two had metastatic disease, and four had tumor marker elevation only. Eleven of 12 patients with relapse were salvaged with chemotherapy (OS 96%; 95% CI, 74% to 99%) consisting of three cycles of "compressed PEB" (cisplatin 33 mg/m^2 and etoposide 167 mg/m^2 on days 1 through 3, bleomycin 15 U/m^2 on day 1; cycles repeated every 21 days). This trial demonstrated that 50% of stage I pediatric MOGCT can be spared with chemotherapy; a larger study is needed to ascertain whether prognostic factors exist for relapse of ovarian GCT, similar to LVI in adult male testicular cancer, and whether this strategy can be extended to older adolescents and young adults.

In the U.K. Germ Cell Tumor Study II that was conducted between 1989 and 1997, all gonadal (both testicular and ovarian) stage I tumors were observed without chemotherapy. Forty of 51 TGCT patients (78%) and 6 of 9 (66%) patients with ovarian stage I GCT were successfully treated with surgery alone. All patients who experienced recurrence after observation were cured with 4 to 6 cycles carboplatin, etoposide, bleomycin (JEB) chemotherapy. For details of JEB chemotherapy please see the section on treatment.

The Germans have used separate protocols for treating testicular tumors (MAHO protocols) and nontesticular, extracranial pediatric GCTs (MAKEI protocols). In the latest update of the MAHO protocols from 2002, the authors report on results from 1982 to 2001.[423] A watch-and-wait approach was taken for patients with stage I nonseminomatous testicular tumors, but only if the malignant histology was pure yolk sac, pure teratoma, pure immature teratoma, or pure seminoma. Boys with testicular tumors that included a malignant component other than yolk sac were treated with standard chemotherapy that consisted of platinum, vinblastine, and bleomycin (PVB). Of the 140 boys with stage I YST, 16 (13%) progressed and required standard chemotherapy 6 to 60 weeks after orchiectomy.[423] All but one of the 140 patients are alive and well. All boys with either mature teratoma (n = 40), immature teratoma (n = 19), or seminoma (n = 2) were cured with surgery alone.

On the MAKEI96 protocol, the observational strategy was extended to include stage I ovarian and extragonadal GCTs. Excluded from this surgery-only approach were extragonadal tumors that arose in the sacrococcygeal region. Children with primary sacrococcygeal tumors, regardless of stage, were treated with four cycles of cisplatin, etoposide, and ifosfamide (PEI), except for infants younger than 4 months of age, who were treated with four cycles of cisplatin and etoposide (PE; see "Treatment of Pediatric Germ Cell Tumors in Germany"). Results of this approach have not yet been published.

Chemotherapy for Pediatric Germ Cell Tumors (>Stage I)

Prechemotherapy Evaluation

Before initiating chemotherapy, studies should be done to establish a baseline for monitoring toxicity, and procedures should be undertaken to minimize risk of late effects. The postpubertal male patient should be encouraged to use a sperm bank, because a small percentage of male patients will be infertile after chemotherapy. Oocyte cryopreservation (egg freezing) should be considered in postpubertal females because of an ill-defined but apparently increased risk of infertility. In addition, patients who are to receive standard chemotherapy with PEB should have baseline measurements of their renal function by measuring the glomerular filtration rate (GFR), preferably with a chromium ethylenediaminetetraacetic acid (EDTA) clearance, pulmonary function tests (PFTs), including diffusing capacity of the lung for carbon monoxide (DLCO), and baseline audiometry that includes accurate measurements of the high-frequency region, because this is the region that is affected first by cisplatin-induced hearing loss.

History of Use of Chemotherapy Among Adult Men with Testicular Cancer

Treatment of pediatric GCTs derives in large part from knowledge gained in the treatment of adult men with testicular cancer. In 1979 Lawrence Einhorn, MD, reported in *Cancer Treatment Reports* that PVB (also referred to as the *Einhorn regimen*) cured a substantial portion of men with widely disseminated testicular cancer. A follow-up study showed that substitution of etoposide for vinblastine (PEB vs. PVB) was shown to be a superior regimen both in terms of increased efficacy in poor-risk patients and reduced neuromuscular toxicity,[462] and PEB became the standard of care. Finally, it was shown that three cycles of PEB was equivalent to four cycles of PEB among patients with good prognoses as defined by the IGCCC.[463] On this backbone of experience in the treatment of adult testicular cancer, the pediatric trials reviewed in the next section were developed.

Treatment of Pediatric Germ Cell Tumors in the United States

Before the use of chemotherapy, the outcome of children with GCTs was dismal; 3-year survival rates did not reach 20%.[212,464] In the mid 1970s, the first reports of effective chemotherapy regimens for pediatric GCTs were published.[465,466] These regimens were based on vincristine, dactinomycin, and cyclophosphamide (VAC) but also included radiation therapy as well. Although the survival for patients of average risk improved to approximately 60%, children with advanced-stage disease continued to have a very poor prognosis. After the Einhorn regimen of PVB was published in the late 1970s, showing that a high rate of cure was possible for men with metastatic testicular cancer,[443] cisplatin-based regimens were quickly adopted in pediatrics with marked improvement in outcome, even for patients with advanced stage disease. Ablin et al. initiated a regimen in the CCG (CCG861) that combined PVB with the drugs that had previously been shown to have activity: cyclophosphamide, actinomycin, and adriamycin.[453] At 18 weeks, patients in partial remission (PR) received radiation therapy and maintenance chemotherapy until 2 years after diagnosis. This protocol enrolled 93 patients with pediatric germ cell tumors, excluding patients with TGCTs and including patients with ovarian (n = 30), sacrococcygeal (n = 37), and mediastinal tumors (n = 17). Ovarian tumors had a 4-year OS rate and EFS rates of 67% and 63%, whereas extragonadal tumors had lower rates of survival (EFS, 48%; OS, 42%). Even though this protocol did include cisplatin, it was given at a low dose (60 mg/m^2) and dose intensity (every 9 weeks) so that its effectiveness was likely compromised.

Subsequently, the CCG and the POG initiated an intergroup study from 1990 to 1995. The intergroup study divided patients into two risk groups: the low-/intermediate-risk group included stage I/II gonadal MGCT patients (treated on POG9048/CCG8891),[467] and the high-risk group included stage III/IV gonadal MGCT and stages I through IV extragonadal MGCT (treated on POG9049/CCG8892).[433]

On the POG9048/CCG8891 protocol for treatment of low-stage gonadal tumors, only boys younger than 11 years of age were enrolled. Older boys were to be treated according to adult standards. Boys with stage I disease were treated with active observation (see "Treatment of Stage I Malignant Nonseminomatous Germ Cell Tumors"); boys with stage II testicular MGCT (including relapses of initially stage I testicular MGCT) and girls with stage I/II ovarian MGCT younger than 21 years of age received four cycles of standard-dose pediatric BEP (bleomycin 15 U/m^2 on day 1, etoposide 100 mg/m^2/day on days 1 through 5, and cisplatin 20 mg/m^2/day on days 1 through 5). On both the low-/intermediate-risk and high-risk arms of the intergroup study, bleomycin was reduced from a weekly dose used on adult protocols to once per cycle, or every 3 weeks, in order to reduce the incidence of pulmonary fibrosis. Of note, both the British and German investigators have similarly reduced the dose of bleomycin without apparent adverse results. It is important to realize, however, that this reduction in dosage has never been studied in a randomized trial that compares weekly dose, as bleomycin is administered in the adult trials, versus the once-every-3-week regimen introduced by pediatric oncologists. Patients were reevaluated with tumor markers and radiologic scans after four cycles. If either imaging or tumor markers suggested residual disease, patients underwent surgical exploration, and if viable tumor was found pathologically and/or if tumor markers did not normalize, the patient was given an additional two cycles of PEB.[467]

Patients with pure dysgerminoma/seminoma were excluded from the U.S. Pediatric Intergroup Trials. The treatment of pure immature teratoma on the U.S. Pediatric Intergroup Trial is discussed in "Mature and Immature Teratoma."

On the low-/intermediate-risk arm of the study, overall 6-year EFS was 94.5%, and 6-year OS was 95.7%.[467] Six-year EFS and OS by primary site were as follows: testicular stage II (100% and 100%), ovarian stage I (95% and 95%), and ovarian stage II (87% and 94%). Two patients underwent second-look surgery; one patient had evidence of residual malignant disease. Although 23% of patients had grade 3 to 4 hematologic toxicity, no delay in therapy was reported. No patient had grade 3 to 4 ototoxicity, renal toxicity or pulmonary toxicity. Two patients with primary ovarian tumors developed acute myelogenous leukemia (AML) at 5 and 32 months from start of therapy, but neither patient had the topoisomerase-induced 11q23 cytogenetic abnormality.[467]

To follow this trial the COG protocol AGCT0132, was opened in 2002 for low-risk (gonadal stage I patients, both ovarian and testicular) and intermediate-risk (stage II through IV gonadal tumors and stage I/II extragonadal tumors) patients. The aims of this trial were to evaluate a watch-and-wait strategy for low-risk patients, reserving chemotherapy for those who fail observation after surgery (see prior summary of results of the low risk trial). For patients who were judged to be of intermediate risk, the protocol evaluated the efficacy of three courses of PEB (rather than four courses, which had been used on the previous protocol) and whether the drugs can be given in a compressed manner over 3 days rather than over 5 days. Enrollment was completed in June 2012; analyses are ongoing.

The high-risk protocol of the previous Pediatric Intergroup Trial (POG9049/CCG8892) was a randomized clinical trial comparing standard-dose PEB (total cisplatin dose 100 mg/m^2) with high-dose PEB (HDPEB; total cisplatin dose 200 mg/m^2).[433] The results of the prior intergroup high-risk clinical trial for stage III/IV gonadal tumors and stage I to IV extragonadal MGCT did not show a significant difference in 6-year OS between HDPEB and PEB (92% vs. 86%); however, there was evidence of an increase in 6-year EFS among patients receiving HDPEB (89.6% ± 3.6% vs. 80.5 ± 4.8%; %, $P = 0.03$).[433] Overall 6-year EFS and OS by site and stage are as follows: testicular stage III (94% and 100%), testicular stage IV (88% and 91%), ovarian stage III (97% and 98%), ovarian stage IV (87% and 93%), extragonadal stage I and II (89% and 93%), extragonadal stage III (75% and 80%), and extragonadal stage IV (78% and 81%). The study was not powered to be able to examine response by site and stage, but in each subgroup there was a trend towards higher EFS and OS for patients treated with HDPEB. This difference was most pronounced in patients with extragonadal MGCT, in which it achieved borderline statistical significance. Patients with stage III/IV extragonadal tumors treated with HDPEB had a 2-year EFS of 84% compared with 74% for patients treated with standard-dose PEB ($P = 0.09$).

Toxicity was much greater on the HDPEB arm of the study. Fatal infections accounted for 7 deaths on the study, and 6 deaths occurred on the HDPEB arm. Hearing aids were needed in 67% of the patients treated with HDPEB. Three patients who initially had been diagnosed with a mediastinal MGCT developed hematologic malignancies posttreatment. Two patients developed AML; neither had the characteristic 11q23 abnormality usually associated with etoposide-induced leukemias. One patient developed erythrophagocytic syndrome.

Given the trend observed for better survival among patients with high-stage extragonadal GCTs on the HDPEB arm, a follow-up pilot study was designed incorporating amifostine (825mg/m^2/day) with the goal of reducing the observed toxicities of the higher dose of cisplatin, particularly the ototoxicity.[468] Among the 20 patients enrolled, 17 were females, median age was 1.6 years, and the primary site was sacrococcygeal in 15 patients. Although the EFS and OS were acceptable (83% and 85%), amifostine at this dose and schedule did not protect against ototoxicity; 75% of patients had significant hearing loss.

A second pilot study for patients with high-risk advanced stage extragonadal tumors has been completed by COG. In this trial, standard-dose PEB was combined with escalating doses of cyclophosphamide.[469] The three dose levels of cyclophosphamide were 1.2 gm/m^2, 1.8 g/m^2, and 2.4 gm/m^2. Nineteen patients were enrolled; 3 patients were nonevaluable for the assessment of dose limiting toxicity (DLT). Sixteen patients completed four cycles of induction, 11 had complete response, one had progressive disease, and four had a partial response. All four with partial response had a residual mass and underwent second look surgery and then completed two additional cycles of their assigned regimen. Only one patient, enrolled on the dosage of 1.8 g/m^2, experienced DLT during the first cycle of therapy manifested as grade 3 hyperglycemia. No other patient experienced DLT. The 4-year EFS and OS (± standard deviation) were 74% ± 7% and 89% ± 10%, respectively.[469] This observed EFS is not apparently different than what was observed with standard dose PEB in the prior intergroup study.

Treatment of Pediatric Germ Cell Tumors in the United Kingdom

From 1977 through 1988, patients in the United Kingdom were treated on United Kingdom Children's Cancer Study Group (UKCCG) Germ Cell Tumor Studies 1 (GC1).[470] This protocol compared five regimens that were used sequentially over the time interval: low-dose VAC, high-dose VAC, high-dose doxorubicin plus VAC (Adria-VAC), PVB, and PEB. GC1 enrolled 52 patients who received chemotherapy until complete response was documented and then two more cycles after achieving complete remission (CR; CR +2). Outcome varied significantly by regimen; PEB was clearly the most effective.

In 1989 based on preliminary data showing efficacy of carboplatin in pediatric MGCT, the UKCCG initiated their second GCT study (GC2) in which cisplatin was replaced by carboplatin.[471] JEB consists of etoposide 120 mg/m^2/day for days 1 through 3 (total dose 360 mg/m^2); carboplatin on day 2 in at an area under the curve (AUC) of 7.9 (AUC formula: [6 × *uncorrected GFR*] + [15 × *surface area*]), equivalent to approximately 600 mg/m^2; and bleomycin 15 mg/ m^2 on day 3. Clinicians were urged to use the AUC formula to calculate the carboplatin dose rather than use a dose based on surface area, but 75% of the patients enrolled on the study were dosed according to the square-meter dose. It is important to note that this is a higher dose and dose-intensity of carboplatin than used in the French study of carboplatin in pediatric GCTs (TGM90), in which the results with carboplatin were inferior to those in a previous trial that had utilized cisplatin (TGM8589).[472,473] As was the practice with GC1, patients in GC2 received chemotherapy until a complete response was documented and then two further cycles.

The U.K. investigators have published an interim analysis of the patients treated between 1989 and 1997.[471] During this time, 184 patients younger than 16 years of age with either localized or metastatic extracranial GCTs were enrolled in GC2 and eligible for analysis. In total, JEB chemotherapy was administered to 137 patients. GC2 remained open to recruitment after analysis in 1997, and an additional 84 patients received JEB in this period, resulting in a total of 221 patients who eventually received chemotherapy. Patients with gonadal stage I tumors were observed without chemotherapy (watch-and-wait strategy). Forty of 51 patients with TGCTs (78%) and six of nine (66%) patients with ovarian stage I GCTs were successfully treated with surgery alone. All patients who experienced recurrence after observation were cured with JEB chemotherapy. The overall survival for all 137 JEB-treated patients at 5 years was 90.9% (95% CI, 83.8% to 95%) and EFS at 5 years was 87.8% (95% CI, 81.1% to 92.4%). Outcome by site, stage, histologic classification, and initial presurgical AFP level are summarized in Figure 63-6.[75] EFS according to site was 100% for testis, 90.7% for ovary, 86.5% for sacrococcygeal, and 75% for mediastinal. EFS according to stage was 100% for stage I, 93.8% for stage II, 84.8% for stage III, and 78% for stage IV. Toxicity was minimal with JEB. In the GC2 study, only one child had severe deafness (likely attributable to a middle-ear hemorrhage when the child had thrombocytopenia), and none had severe renal toxicity. However, carboplatin is more myelotoxic than cisplatin; 36% of the total number of courses were delayed more than 28 days. Second-look surgery was undertaken in 45 patients (33%) in GC2. Necrotic or fibrotic tissue was found in 24 patients (53%), mature or immature teratoma was found in 19 patients (42%), and only 2 patients were found to have viable tumor.

The United Kingdom Children's Cancer and Leukemia Group (UKCCLG) protocol (GC3) opened in May 2005. This protocol continues to use JEB chemotherapy as first line treatment. Patients with low-risk disease (stage 1 gonadal tumors) are treated surgically with close surveillance. Other patients receive four or six courses of JEB according to risk group stratification determined by site, stage, age, and AFP level. The study has closed; analyses are ongoing but not yet reported.

The results are similar to those reported by the U.S. POG/CCG Intergroup Study[433] and raise the question of whether it is necessary to continue treatment with cisplatin in pediatric patients, given the higher toxicity profile of cisplatin. The concern is that carboplatin has been shown to be inferior to cisplatin in five randomized clinical trials in adults with metastatic "good risk" testicular cancer.[474,475] However, the maximum dose used in the adult trials was 500 mg/m^2, and in two of the trials, the carboplatin was given every 28 days in a less dose-intense fashion.[476] Likewise in pediatric GCTs, the French showed that carboplatin was inferior to previous results of treating with cisplatin (TGM90 vs. TGM85); however both the dose (400 mg/m^2) and dose-intensity (once every 6 weeks) were low on the French trial.[472,473] Consideration of carboplatin as first-line therapy for low and intermediate risk pediatric germ cell patients is warranted.

Treatment of Pediatric Germ Cell Tumors in Germany

The German Society of Pediatric Oncology has used separate protocols for treating testicular tumors (MAHO protocols) and nontesticular, extracranial pediatric GCTs (MAKEI protocols). In the latest update of the MAHO protocols published in 2002, the authors report on results from 1982 through 2001.[477] A watch-and-wait approach was taken for patients with stage I testicular YSTs. All patients with stage I tumors who had a malignant component other than yolk sac, as well as patients with higher than stage I testicular tumors, were treated with "standard chemotherapy" that consisted of PVB (vinblastine 3.0 gm/m^2 on days 1 and 2, bleomycin 15 U/m^2 on days 1 through 3 as continuous infusion, and cisplatin 20 mg/m^2/day on days 4 through 8). Four cycles were given every 28 days. If there was not a complete response after 2 cycles, however, patients underwent exploratory laparotomy, and if viable disease was discovered, patients received four cycles of salvage therapy that consisted of PEI (etoposide 80mg/m^2/day on days 1 through 3, ifosfamide 1500 mg/m^2/day on days 1 through 4, and cisplatin 20 mg/m^2/day on days 2 through 5). If there was no evidence of disease at laparotomy, the patient received two more cycles of the standard PVB for a total of four courses. In toto, 59 patients received chemotherapy with an overall survival rate of 95%. Survival among patients with stage I and II disease was excellent; 41 of 42 patients survived. Among patients with stage III testicular cancer, 5 of 17 patients died despite the use of salvage chemotherapy.

The German MAKEI protocols (MAKEI83/86, MAKEI89, MAKEI96) specifically include only patients with either an ovarian or extragonadal primary tumor and exclude patients with a testicular primary tumor (treated on MAHO). In MAKEI83/86, patients were stratified into either low risk or high risk categories. The low-risk classification included only stage I ovarian tumors; these patients received four cycles of PVB. Patients in the high-risk group had stage II through IV ovarian and stage I through IIb extragonadal primary tumors. These patients received four cycles of PVB followed by four cycles of PEI. Because of concerns about the pulmonary toxicity associated with bleomycin and the neurotoxicity associated with vinblastine in the original Einhorn regimen, a modified Einhorn regimen was developed. Bleomycin was given before cisplatin, because cisplatin had been shown to reduce the clearance of bleomycin, as a continuous infusion over 3 days at 15 mg/m^2/day or 45 mg/m^2 per cycle. Cumulative dose of bleomycin was limited to 180 mg/m^2. This is substantially higher than the total dose of bleomycin patients receive on either U.S. or U.K. protocol, in which the per-cycle dose of bleomycin is only 15 units/m^2, and most patients receive between three and six cycles (or total cumulative dose of 45 to 90 U/m^2. The German dose of bleomycin is more analogous to the cumulative doses of bleomycin one would find on an adult protocol for treating GCTs, in which 30 units of bleomycin is administered weekly for 9 weeks for total cumulative dose on average of 270 units or 135 U/m^2 in a average adult with a body surface area (BSA) of 2 m^2. Vinblastine was administered at 50% the dose of the original Einhorn regimen to reduce the associated neurotoxicity.

In MAKEI89, changes were made to risk stratification, and the overall amount of chemotherapy administered was reduced. Risk level was stratified into three groups. The low-risk category remained stage I ovarian tumor; medium risk included ovarian stages II and III and extragonadal stage I and IIa tumors, and the high-risk group was comprised of extragonadal stage IIb, coccygeal stage I and IIb, and ovarian stage IV tumors. Therapy was reduced for these low- and intermediate-risk groups by 25% compared to MAKEI83/86. Patients in the low-risk group received three cycles of PEB, and patients at medium risk received three cycles of PEB followed by 3 cycles of PIV.

Outcome of the earlier MAKEI trials are as follows: on MAKEI83/86, the low-risk group achieved 94% EFS, whereas the high-risk group achieved 76% EFS. In MAKEI89, EFS on the low-risk arm was 91%, medium risk was 86%, and high risk was 83%. In terms of site, sacrococcygeal tumors had the lowest EFS without much significant change over time (74%, 80%, and 77% in MAKEI83, 86, and 89, respectively), whereas ovarian tumors had the best outcome with significant improvement over time (EFS 77%, 92%, and 90% on MAKEI83, 86, and 89, respectively).

In MAKEI96, several changes have been made to the risk stratification and recommended therapies. Chemotherapy is determined by site, stage, and degree of resection. Bleomycin was removed from all chemotherapy protocols. All patients with ovarian and extragonadal stage I tumors (except patients with stage I sacrococcygeal tumors) are initially treated with a watch-and-wait strategy. Children with sacrococcygeal primary tumors, regardless of stage, are treated with four cycles of PEI (cisplatin 20 mg/m^2/day on days 1 though 5, etoposide 100 mg/m^2/day on days 1 through 3), and ifosfamide 1500 mg/m^2/day on days 1 through 5 as a continuous infusion). Infants younger than 4 months of age with sacrococcygeal primary tumors are treated with four cycles of PE, omitting the ifosfamide. Patients with primary ovarian tumors receive two cycles of either PE (completely resected) or PEI (incompletely resected), and patients with extragonadal primaries receive three cycles of either PE (completely resected) or PEI (incompletely resected). Patients with advanced stage ovarian and extragonadal primary tumors with poor prognosis are treated with four cycles of PEI. If the tumor was not completely excised at the onset of therapy, three cycles of PEI are given and then the patient undergoes resection. Postoperatively, an additional one to four cycles of PEI are administered depending on whether the tumor was completely or incompletely resected. In all MAKEI protocols diagnosis is based on imaging, tumor markers, and histology. In advanced/bulky disease, diagnosis could be based on markers and imaging, and a preoperative chemotherapy could be applied for downstaging.

The Dose of Bleomycin in Treatment of Pediatric Germ Cell Tumors

In adults, investigators have shown that bleomycin can be safely omitted in patients at "good risk" if the patient receives an additional (fourth) cycle of etoposide and cisplatin.[478] Concern about the possibility of bleomycin-induced pulmonary fibrosis has caused pediatric oncologists to evaluate the same issue. The British and American collaborative groups have reduced the dose of bleomycin from once a week to once per cycle (or once every 3 weeks).[433,471] This dose of bleomycin represents a 67% reduction in the total dose of bleomycin when compared to adult regimens. Since the institution of these changes (1989 in Britain and 1990 in the United States), there have been no reported cases of fatal pulmonary fibrosis in over 137 British cases of patients treated with JEB and 373 cases in the United States of patients treated with PEB. On MAKEI89, after two deaths occurred in children younger than age 2 years from pulmonary fibrosis, German investigators no longer treated children under 1 year of age with bleomycin (using instead etoposide and cisplatin [EP]), and children ages 1 to 2 years received half of the standard dose of bleomycin. However, the dose of bleomycin for older children on MAKEI83/86 and 89 was quite significant: 15 mg/m^2/day for 3 days with each cycle (total 45 mg/m^2/cycle). In MAKEI96, bleomycin was omitted entirely from the chemotherapeutic regimens for all patients. No decrement in survival has been observed since its deletion.

Other countries besides Germany have decided to delete bleomycin from use in pediatric patients. A Brazilian study has reported preliminary results using four cycles of EP with 5-year survival rates of 83% in patients at high risk.[479] The Italians also did not use bleomycin in the Italian cooperative study (TCG91), but their regimen, which alternated carboplatin and etoposide with vincristine, dactinomycin, and ifosfamide, produced suboptimal results, particularly in patients with higher stages of disease, compared with other pediatric germ cell trials.[480]

Mature and Immature Teratomas

Complete surgical excision is adequate therapy for pure mature teratomas. Mature teratomas are much more common than immature teratomas. It is estimated that mature teratomas of the ovary, also called *cystic teratoma* or *dermoid cyst,* account for 95% to 99% of all ovarian teratomas. Mature teratomas that contain an element of a malignant GCT (yolk sac, endodermal-sinus, choriocarcinoma, embryonal carcinoma) should be treated according to the guidelines for the treatment of malignant nonseminomatous GCTs discussed previously.

Immature teratomas are made up elements from the three germ layers and graded from 0 to 3 depending on the amount of immature neuroepithelium present in the tumor.[119] Grade has been shown to correlate with both stage and prognosis. Treatment for immature teratoma is more controversial and evolving.

A classic study by Norris et al reviewed 58 adult women with ovarian immature teratomas who had been treated with surgical resection alone and reported that 70% of women who had grade 3 tumors at diagnosis subsequently relapsed.[119] The recommendation was thus made that stage I grade 3 ovarian immature teratomas ought to be treated with chemotherapy.

Concern that the biology and behavior of immature teratoma might be different in childhood than adulthood led the U.S. pediatric oncology groups to investigate whether surgical resection was sufficient for immature teratoma, even among patients who had grade 3 tumors. Between 1990 and 1995, as part of the U.S. Intergroup Pediatric Study (POG9048/CCG8891), 73 patients with immature teratoma who had undergone a gross total resection were enrolled in the study.[168] Central review of grade showed 31.5% were grade 1; 38.4% were grade 2, and 30.1% were grade 3. Primary site of disease included ovarian (n = 44), testicular (n = 7), sacrococcygeal (n = 5), retroperitoneal (n = 7), and head/neck (n = 7). All pathology was centrally reviewed; 50 patients were confirmed to have pure immature teratoma, whereas 21 had microscopic foci of YST and two had microscopic foci of PNET. Because significant time had elapsed between the initial surgery and the central review of the pathology (typically at least 6 months), it was decided to continue to observe patients discovered to have a malignant focus at central pathology review. Tumor markers were elevated in 25 patients at diagnosis (AFP alone, 18; β-HCG alone, 5; both, 2). Overall, only one half of the patients with microscopic foci of YST had an elevated AFP at diagnosis (9 out of 20 patients). However, in ovarian tumors there is a tight correlation between an elevated AFP and presence of YST; 70% of patients shown to have microscopic foci of YST had an elevated AFP at diagnosis, whereas only 25% of patients with pure immature teratoma had an elevated AFP at diagnosis.[130] Elevation of AFP at diagnosis was also strongly correlated with stage and grade of the immature teratoma.[130]

With a median follow-up of 35 months, the overall 3-year EFS was 93%, with 3-year EFS of 97.8%, 100%, and 80% for ovarian, testicular, and extragonadal tumors respectively.[168] Five patients relapsed, all within 7 months of the original diagnosis; one had an ovarian primary tumor and 4 had extragonadal primary tumors. In retrospect at central pathology review, three of the five patients had YST present at diagnosis; one patient had PNET. The patient with PNET who had recurrence was resistant to platinum-based therapy. All of the other patients were successfully treated with PEB.

How to treat pure immature teratoma higher than stage I lacks clinical consensus and published data. There is generally a belief in the pediatric oncology community that the diagnosis of immature teratoma has different meaning and clinical significance in the newborn vs. the peripubertal period. In the newborn period, patients with incompletely resected immature teratomas are usually closely observed, reserving treatment (usually reoperation) for those who have recurrence. Immature teratoma in the peripubertal stage (older child and adult) is generally considered to be a malignant disease that warrants treatment with chemotherapy. Treatment with 3 to 4 cycles of standard-dose PEB is generally recommended for patients with higher than stage I pure immature teratoma in the United States, whereas in the United Kingdom all patients with immature teratoma, regardless of stage, site, and age are observed after surgical resection, even in the face of residual disease.[481]

In Germany, the German Society for Pediatric Oncology and Hematology (GPOH) has prospectively studied all extracranial testicular and nontesticular mature and immature teratomas since 1982 on successive MAHO and MAKEI protocols.[482] All pathology is centrally reviewed at a single institution, and grade of immaturity is classified according to Gonzalez-Crussi.[165] AFP and β-HCG are measured on all patients and by definition have to be within normal limits for age in order to establish the diagnosis of teratoma. Treatment of immature teratoma has varied over time. On MAKEI83/86, patients with incompletely resected immature teratoma grade 2 or 3 were treated with three cycles of VAC chemotherapy. On MAKEI89, chemotherapy (three cycles of PEB) was given to patients with incompletely resected immature teratomas of grade 3.

Overall, the completeness of the original tumor resection was the chief predictor of relapse. EFS after complete resection was 96%, whereas EFS was only 55% among those with incomplete resection at diagnosis. The Germans use a very strict definition of a complete resection. Complete resection of a coccygeal tumor was defined as resection of the tumor with its pseudocapsule and the coccygeal bone in one piece. Relapses occurred in 20% of patients with sacrococcygeal tumors, compared with 7% among patients with tumors at other extragonadal sites and only 2% of patients with ovarian primaries. Relapse rate was similar among patients treated with surgery alone vs. patients treated with chemotherapy on the basis of higher grade of immaturity. However, no malignant components were found at the time of relapse among the seven patients who relapsed after initial chemotherapy, whereas 15 of 29 patients who relapsed after surgery only as first treatment had a malignant component detectable in their recurrence.

Immature teratomas were more likely to relapse than mature teratomas; higher grade of immature teratoma was more likely to relapse than lower grade (grade 0 [mature], 10%; grade 1, 14%; grade 2, 21%; grade 3, 31%.).

On MAKEI96, patients with incompletely resected immature teratomas of grade 2 or 3 were stratified to either a watch-and-wait approach or to two courses of adjuvant chemotherapy. The therapy was switched from PEB to PEI, substituting ifosfamide 1500 mg/m^2/day on days 1 through 5 for the bleomycin. However, because of low accrual, the randomization was stopped in the year 2000, and patients have been treated according to physician preference. 261 evaluable patients were enrolled.[482] Because the criteria for complete vs. incomplete resection were more highly scrutinized, the percentage of incompletely resected tumors rose from 10% to 34% on MAKEI96. However, this change was not associated with a reduced relapse rate in the patients whose tumors were completely resected (4.2%). However, the overall relapse rate decreased from 13.3% to 9.5% over that time. The authors are not certain whether this reflects better surgical procedures or a change in the distribution of the primary site of the tumor. The rates are similar to those reported in other studies.[425] It is easier to achieve a complete resection for a gonadal teratoma. Extragonadal teratomas are often adjacent to or infiltrating vital structures such as the sacral plexus in the case of the SCT or large vessels. Probability of relapse on MAKEI96 was not linked to the histologic grade of immaturity, as it had been on MAKEI 83/86/89. One-half of the relapses on MAKEI83/86/89 had a malignant component, whereas two thirds of the relapses on MAKEI96 had a malignant component, illustrating the malignant potential of the teratoma.

Growing Teratoma Syndrome

Growing teratoma syndrome describes the situation in which tumors are noted to enlarge either during chemotherapy or after chemotherapy in a patient with normalized tumor markers. Often mistaken as progression or recurrence, when these tumors are resected and examined pathologically, these tumors are comprised entirely of mature teratoma. The incidence of this phenomenon has been described in approximately 3% of men with testicular cancer[483] and in as high as 12% of women with ovarian GCT, particularly among women originally diagnosed with immature teratoma.[484] The hypothesized etiology is that the chemotherapy might induce differentiation of residual immature teratoma into a mature teratoma. Median interval between chemotherapy and first detection of growing teratoma syndrome was 9 months in a retrospective study of women with ovarian immature teratomas from the Institut Gustave-Roussy; however, the range extended from 1 month after treatment to 12 years after treatment.[484] The disease usually occurs at the site of prior disease; for instance, women known to have peritoneal deposits of tumor at diagnosis were noted to have peritoneal location for the growing teratoma syndrome. In most cases, the recurrence is treated with surgery only. In cases where total resection is not possible, patients require continued surveillance. These patients can become quite ill if the tumor is not amenable to resection and impinges on organ function. A report in the *New England Journal of Medicine* described disease stabilization in three patients with nonresectable tumors who were treated with a CDK4/6 inhibitor; two patients had stable disease for 18 and 24 months at the time of publication.[362]

Extragonadal Tumors

Mediastinal Tumors

The Germans have reported on the outcome of pediatric mediastinal GCT as a separate entity.[417] Included in their report are all patients treated between 1983 and 1999 on German treatment protocols, including MAKEI83/86, 89, and 96. The authors examined the outcome of both mediastinal teratomas (both mature and immature; n = 21) and malignant GCTs of the mediastinum (n = 26). All the mediastinal GCTs diagnosed in the first year of life were teratomas (Fig. 63-16). The incidence of teratoma declined during infancy and adolescence; seminoma was not observed until after age 10.

Teratomas were surgically resected; no adjuvant chemotherapy or radiotherapy was given. Sixteen of the teratomas were mature; five were immature. All patients with teratoma underwent primary resection; surgery was microscopically complete in 17 of 21 patients. All patients with teratomas, regardless of the completeness of the tumor resection, were cured with surgery only.

Twelve of the 26 patients with MGCT had primary resection of the tumor that was complete in six of the 12. Four of the six patients with incomplete resections subsequently underwent second-look surgery, and all achieved a complete resection of the residual tumor. Eleven patients underwent delayed resection of the primary tumor after preoperative chemotherapy, and a complete resection was achieved in 10 of the 11 patients. Three patients did not undergo resection of the tumor;

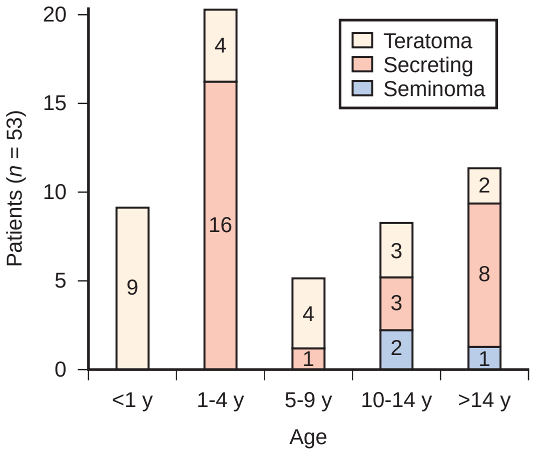

Figure 63-16 Age and histologic differentiation in all registered patients with mediastinal GCTs (n = 53; one patient with insufficient histopathology was excluded). *(From Schneider DT, Calaminus G, Reinhard H, et al: Primary mediastinal germ cell tumors in children and adolescents: results of the German cooperative protocols MAKEI 83/86, 89, and 96.* J Clin Oncol *18:832–839, 2000.)*

TABLE 63-5 Summary of Treatment of Nonseminomatous Malignant Germ Cell Tumors in the United States, Germany, and the United Kingdom

	United States	Germany	United Kingdom
Testicular			
Stage I	W&W[*]	W&W for pure yolk sac Other: 2-3 cycles PVB	W&W
Stage II	3 cycles compressed PEB	2-3 cycles PVB	JEB[†]
Stage III-IV	3 cycles compressed PEB	2 cycles PVB, 4 cycles PEI	JEB
Ovarian			
Stage I ovarian	W&W	W&W	W&W
Stage II	3 cycles compressed PEB	2 cycles PE if completely resected 2 cycles PEI if incompletely resected	JEB
Stage III-IV	3 cycles PEB	4 cycles PEI	JEB
Extragonadal			
Stage I extragonadal	3 cycles compressed PEB	W&W except sacrococcygeal tumors; Stage I SCT: 4 cycles PE, PEI[‡]	W&W
Stage II	3 cycles compressed PEB	3 cycles PE if completely resected. 3 cycles PEI if incompletely resected	JEB
Stage III-IV	High risk protocol	4-5 cycles PEI Poor responder: 1 cycles PEI, 3 cycles HDPEI	JEB
Relapse	TIC +/– stem cell transplant	Local: 4 cycles local deep hyperthermia + PEI Disseminated: 1 cycles PEI, 3 cycles HDPEI	

HDPEI, High-dose cisplatin, etoposide, ifosfamide; *JEB,* carboplatin, etoposide, bleomycin; *PE,* cisplatin, etoposide; *PEB,* bleomycin, etoposide, cisplatin; *PEI,* cisplatin, etoposide, ifosfamide; *TIC,* taxol, ifosfamide, carboplatin; *W&W,* watch-and-wait strategy.

[*] Observation only after surgical excision of Stage I tumor with chemotherapy reserved for those who recur.

[†] 4-6 Cycles; number of cycles determined by risk group stratification.

[‡] Depending on age: <4 months, PE; >4 months, PEI.

two patients had a CR to first-line therapy; and one patient had progressive disease on chemotherapy. Patients were treated with cisplatin-based regimens for a total of three to eight courses (details are shown in Table 63-5).[417] Excellent results on MAKEI83/86 encouraged the German investigators to reduce the maximum number of courses from eight to four or five cycles with no apparent detriment to overall survival. Overall 5-year EFS was 0.83 ± 0.05 at a median follow-up period of 41 months. Complete resection of the tumor either initially, at delayed resection, or at second-look surgery was the strongest predictor of survival. Only one patient of 20 with complete resection relapsed, and that patient was cured with salvage therapy. Analysis of stage of tumor at diagnosis was limited by small sample size, but EFS for patients without distant metastases was 0.93 ± 0.07 vs. 0.65 ± 0.17 for those with distant metastases at diagnosis (P = 0.09). Histology at diagnosis and level of AFP at diagnosis were not prognostic.

Sacrococcygeal Tumors

The Germans have also reported on 71 patients with malignant sacrococcygeal tumors treated in MAKEI83/86 and 89 from 1983 to 1995.[419] Of the 66 evaluable patients, there were 14 boys and 52 girls; median age was 17.4 months. Twelve patients had lymph node metastases, and 30 patients had distant metastases (lungs, 21; liver, 8; bones, 8). On histological examination, 45 patients had pure YST and 21 patients had mixed GCT. Treatment details of MAKEI83/86 and 89 are summarized in Table 63-5. Patients in MAKEI83/86 received a total of eight cycles of cisplatin-based therapy: four cycles of PVB followed by four cycles of PEI. Patients in MAKEI89 received a total of 6 cycles of cisplatin-based therapy: three cycles of PEB followed by three cycles of etoposide, ifosfamide, and cisplatin (VIP). EFS for the entire group was 0.76 ± 0.05, and OS was 0.81 ± 0.05. OS did not differ by protocol (MAKEI83/86 vs. 89) or by stage (T1 vs. T2). The most significant predictor of favorable outcome was the completeness of tumor resection. The completeness of this resection could either be established by tumor resection at initial diagnosis or at delayed resection after chemotherapy is given. Among patients with advanced and metastatic disease (T2b M1), those who had a delayed resection fared better than patients with an initial resection (5-year OS 0.83 ± 0.09 vs. 0.45 ± 0.15; *P* = 0.01). The presence of metastatic disease at diagnosis was only an adverse risk factor among those who had initial resection of their tumor at diagnosis. AFP levels at diagnosis were not prognostic, even when the analysis was restricted to children older than 1 year of age. The authors conclude that the therapeutic strategy of preoperative chemotherapy with delayed resection results in superior outcomes that abrogate prior reports that higher stage and higher pretherapy levels of AFP portend a worse prognosis.

An analysis of the prognostic factors among children with SCT treated on the U.S. intergroup study confirms the conclusions of the German report.[416] Seventy-four children with malignant SCT were randomized to receive either standard-dose PEB or HDPEB. Prognosis was excellent in this subgroup overall (4-year EFS 84% ± 6%; 4-year OS 90% ± 4%). Neither presence of metastatic disease nor delayed resection had an adverse effect on outcome. In this group of patients, AFP greater than 20,000 IU/L was associated with a higher than expected rate of failure.

Postchemotherapy Management

At the end of chemotherapy, full restaging should be undertaken, including radiologic imaging of the primary site and the most likely sites of nodal spread, chest CT, and measurements of tumor markers (AFP and β-HCG). If tumor markers are elevated and chest CT and pelvic/abdominal CT do not reveal the site of metastasis, a bone scan and brain MRI are indicated to look for other possible sites of metastatic disease. In the case in which tumor markers are elevated but no mass is detectable, a PET scan can sometimes be helpful in identifying occult disease. The recommended surveillance postchemotherapy is similar to that recommended for patients with stage I disease (see "Treatment of Stage I Malignant Nonseminomatous Germ Cell Tumors").

Postchemotherapy Retroperitoneal Lymph Node Dissection

If there is either a residual mass detected, surgery is recommended to remove the mass or to explore and biopsy any potentially involved residual lymph nodes. There is limited data on findings from second-look surgeries in children with GCTs. In the United Kingdom after completion of JEB chemotherapy, 45 out of 137 patients required surgery for residual mass seen on radiologic evaluation.[471] Half of the patients (53%) had necrosis or fibrosis; 42% were found to have immature or mature teratoma, and only two patients had viable tumors. The two patients who had viable tumors did not receive any further therapy after complete resection, and both remain in complete remission.

Postchemotherapy Retroperitoneal Lymph Node Dissection in Adult Men with Testicular Cancer

In a retrospective review from Memorial Sloan Kettering, 11% of men who underwent postchemotherapy RPLND had viable residual GCT; 49% had fibrosis, and 40% were found to have teratoma in the postchemotherapy surgical specimen.[437] In the subgroup of patients with teratomas, 85% had pure mature teratomas, 7% had immature teratomas, and 8% had teratomas with malignant transformation (to a non-GCT histology).[437] The median size of the node at postchemotherapy RPLND was 3.0 cm, but 18% of the patients discovered to have teratoma had a node smaller than 2 cm and 11% had a node smaller than 1 cm. The 5- and10-year probabilities of relapse-free survival were 83% and 80%, respectively. There is debate in the adult testicular cancer community as to the optimal surgical approach: a modified RPLND template vs. bilateral infrahilar dissection. The complexity of these issues argue strongly for referral of the adolescent patient to a urologist well-versed in the data.[437,485]

Second-Look Surgery in Ovarian Malignancies

In adult women with epithelial ovarian carcinoma, second-look surgery postchemotherapy is performed on all patients to cytoreduce and has been found to improve prognosis. In adult women with malignant ovarian GCTs, however, it has been shown that the yield of second-look surgery, in the setting of a negative radiologic evaluation and normal tumors markers, has a very low probability of discovering occult residual malignant disease. A report by Gershenson et al. states that only 1 of 53 women with negative scans and tumor markers had viable tumor at second-look surgery.[164]

What should be chemotherapy if tumor is found? Standard of care if viable tumor is found at postchemotherapy lymph node resection has been two more cycles of the original chemotherapy. However, persistence of viable tumor after standard chemotherapy is increasingly viewed as evidence of resistant disease and anecdotally, most adult oncologists are now treating patient with salvage therapy in this setting.

Of note, in adults a higher-than-expected incidence of sarcoidosis has been noted in patients previously treated for GCTs.[434] Sarcoidosis should be considered in the differential diagnosis of any adolescent patient with pulmonary nodules or infiltrates or mediastinal adenopathy in the setting of negative tumor markers.

RELAPSE AND SALVAGE THERAPY

In the German experience, more than 90% of relapses occurred locally.[423] In an analysis of 22 patients with recurrent sacrococcygeal tumors previously treated on the German MAKEI protocols, the majority of patients (17 of 22) presented with local recurrence only. Two patients had only distant metastases, and three patients had combined local and distant recurrences.[346] Most relapses occurred within 6 months of completing primary therapy (median 5.5 months; range 0 to 21 months). Complete surgical resection of the relapse was associated with higher likelihood of continuous CR (5 of 7 patients in first relapse who had a microscopic CR of the relapsed tumor remained in CR). Only 1 of 5 patients who had distant metastases at relapse achieved a second continuous CR. Early vs. late relapse (<1 year vs. >1 year after diagnosis), AFP serum levels at relapse, and tumor size at relapse were not prognostic. These data contrast with those from adult men with TGCT in whom favorable prognostic factors at relapse include IGCCC "good risk" group at first diagnosis, testis as the primary site (vs. retroperitoneal or mediastinal), previous complete response to first line therapy, low tumor markers at relapse, and low-volume disease.

Salvage Therapy

Surgery

Unlike many other forms of cancer, surgical excision can be curative in chemorefractory GCTs in patients who have anatomically confined disease.[486] Most patients also require chemotherapy to achieve a second durable remission.

Chemotherapy

Several different regimens have been used in adult males with relapsed or refractory GCT. Vinblastine, ifosfamide, mesna, and cisplatin (VeIP; vinblastine 0.11mg/kg, cisplatin 25 mg/m^2 × 5, and ifosfamide 1.2 gm/m^2 × 5) results in a complete response in 50% of patients, but responses are durable in only 20% to 30% of cases.[460] More recently, investigators from Memorial Sloan Kettering have reported on a paclitaxel, ifosfamide, and cisplatin (TIP) regimen that substitutes paclitaxel for vinblastine (paclitaxel 250 mg/m^2, ifosfamide 1.2 gm/m^2 × 5, and cisplatin 20 mg/m^2 × 5), in which up to 70% of patients experience CR with durable CR rate of 63%.[487] Oxaliplatin and gemcitabine have both shown activity in phase II trials as single agents in men with relapsed testicular cancer.[488-490] Two recent single-institution reports of treatment of relapsed patients with high-dose chemotherapy (HDCT) have shown encouraging results. Kondagunla et al reported on the treatment of 48 patients with a poor response to standard BEP therapy who were treated with two cycles of paclitaxel and ifosfamide followed by three cycles of carboplatin and etoposide with peripheral blood stem cell (PBSC) support administered at 14-to 21-day intervals.[491] CR was obtained in 55% of patients, and 51% remain in remission at a median follow-up period of 40 months. Einhorn et al reported on the treatment of 184 patients with relapsed testicular cancer who were treated with two cycles of carboplatin and etoposide with PBSC support.[492] CR was obtained in 63% of patients with median follow-up time of 48 months; a high response rate was observed even among the subset of patients who had cisplatin-refractory disease, or had failed prior salvage regimens.

Limited data exist on optimal therapy for pediatric patients whose GCT has relapsed. A trial of paclitaxel, ifosfamide, and carboplatin (TIC; paclitaxel 135 mg/m^2, ifosfamide 1.8 gm/m^2 × 5, carboplatin 560 mg/m^2) is under investigation in the COG. The decision was made to use carboplatin rather than cisplatin for two reasons. First, children are more sensitive to the ototoxicity of cisplatin, and it is likely that many children would have serious hearing impairment if further courses of cisplatin were administered after PEB. Secondly, carboplatin has been shown to be potentially equally as efficacious as cisplatin in children with GCTs in the GC2 study conducted in the United Kingdom.[471] The study is evaluating response to two cycles of TIC. Additional therapy after two cycles of TIC (for instance, either to continue with TIC or to proceed to HDCT with stem cell transplant) is left to the physician's discretion.

Radiotherapy

Radiotherapy is not a standard part of salvage therapy in the United States and certainly not until standard second-line therapy has been attempted. However, in an analysis of outcome of patients with sacrococcygeal GCT tumors treated in Germany, Schneider et al report that 8 out of 22 patients received local irradiation after relapse, most in the situation of an incompletely resected tumor. None of the patients who received less than 45 Gy achieved a stable second CR, whereas 3 of 5 patients who received more than 45 Gy achieved a stable continuous remission.[346]

The Germans have also used regional deep hyperthermia successfully in the control of local recurrence, especially if it is applied early in the course of recurrence.[493] Cisplatin is a thermosensitizer, and thus this approach may be of use in overcoming cisplatin-resistant tumors.

Molecular Basis of Cisplatin Sensitivity and Resistance in Germ Cell Tumors

Compared to most other malignancies, GCTs are unusually responsive to chemotherapy, especially to cisplatin-based regimens (with the exception of teratomas, which are chemoresistant). The reasons for this exquisite sensitivity are not fully known, precluding a complete understanding of the mechanisms of cisplatin resistance, and hampering efforts to overcome chemoresistance in GCTs. Several features of GCTs are thought to contribute to their relative chemosensitivity.[494,495] In particular, GCTs may differ from other malignancies regarding uptake, efflux, and conjugation of cisplatin, as well as the cellular DNA repair and apoptotic response to cisplatin exposure. Uptake of cisplatin into GCTs may occur by passive or facilitated diffusion. Recent work has highlighted the role of copper transporter *CTR1* in cisplatin uptake, and the significance of altered *CTR1* expression in cisplatin-resistant solid tumors.[496-498] However, it is unclear whether *CTR1* significantly affects cisplatin uptake into germ cells. Similarly, overexpression of proteins capable of exporting cisplatin, such as lung resistance protein (LRP) and multidrug resistance-related protein (MRP) 2 is not a consistent feature of cisplatin-resistant GCTs.[494,495,499,500] Conjugation of cisplatin to glutathione can limit its cytotoxicity and facilitate export; in germ cells this conjugation is mediated principally by the isoform of glutathione-*S*-transferase (GST) θ.[501-503] Strohmeyer et al reported an overall low level of GST enzyme activity in GCTs, and Mayer et al found consistent GST-θ expression in only 5 of 36 nonteratoma GCTs.[494,502] Thus low levels of GST activity may contribute to the activity of cisplatin in GCTs. However, in the Mayer et al study the differences between cisplatin-responsive and refractory tumors were not significant.

In the absence of compelling evidence that import or export mechanisms predominantly determine cisplatin's effect on GCTs, attention has focused on the role of DNA damage repair and apoptosis pathways in cisplatin-mediated cytotoxicity. Cisplatin causes bulky DNA adducts, which are removed via nucleotide excision repair (NER).[504] Most GCT cell lines exhibit low NER activity, which may be attributable to low expression levels of the NER proteins XPA, XPF, and ERCC1.[505-507] However, one study found no difference in *XPA* expression between cisplatin-sensitive and resistant tumors,[508] and restoring *XPA* expression to a testicular tumor cell line did not increase cellular resistance to cisplatin,[509] calling into question whether NER is an important factor in GCT response to cisplatin. Another DNA damage-repair pathway, mismatch repair (MMR), has also been investigated in relation to cisplatin treatment of GCTs. A hallmark of defects in the MMR pathway is the presence of microsatellite instability (MSI). Several studies have indicated a low level of MSI in GCTs, implying that the MMR system is generally intact in these tumors.[378,510,511] In the presence of DNA damage, the MMR pathway can trigger p53-dependent apoptosis,[512,513] and this response may explain the sensitivity of most GCTs to cisplatin. On the other hand, loss of MMR may confer chemoresistance. In one series, only 6% of unselected GCTs displayed MSI, whereas 45% of chemoresistant tumors exhibited MSI or low levels of MMR proteins.[378] In another study of GCTs, Velasco and co-workers found that low MMR expression and a high degree of MSI were associated with chemotherapy resistance, shorter time to recurrence, and poor survival.[514] Loss of MMR function is associated with genomic instability and the acquisition of secondary mutations that may enhance tumor survival or aggressiveness.[451,515,516] Thus the MMR system is an important determinant of cisplatin sensitivity in GCTs, and loss of MMR may signal a worse prognosis.

As mentioned, the MMR pathway not only functions to maintain genome integrity but is also one of the factors that triggers apoptosis in response to genotoxic damage such as that caused by cisplatin. Apoptosis, or programmed cell death, is a key determinant of the death or survival of cancer cells and the effectiveness of chemotherapy.[517-522] The tumor-suppressor protein p53 plays a central role in coordinating the apoptotic response to genotoxic damage,[523,524] and attention has focused on the role of the p53 pathway in chemotherapy of GCTs. Mutations in p53, which are commonly found in other tumor types, are rare in GCTs.[494,525] The presence of high levels of wildtype p53 protein in GCTs has been suggested to account for the unusual chemoresponsiveness of these tumors.[526,527] However, p53 deficiency per se does not prevent an apoptotic response to cisplatin in GCT cell lines,[528] and the presence or absence of wildtype p53 correlates poorly with clinical response to chemotherapy.[525] Thus the importance of wildtype p53 expression in determining the cisplatin response of GCTs is not clear. Also unclear is why GCTs treated with cisplatin predominantly exhibit apoptosis rather than undergoing cell-cycle arrest, senescence, or other p53-mediated responses. Despite the absence of p53 mutations, other mechanisms exist in GCTs to interfere with p53 function, including overexpression of the p53 inhibitor MDM2[529,530] and epigenetic silencing of p53 target genes.[531] Particularly interesting in this regard is the recent finding that the miR cluster miR-371-3 acts in GCTs to neutralize the p53 response to oncogenic stress.[295] In summary, there is suggestive evidence that specific features of GCTs, including their retention of a normal DNA damage repair, p53, and apoptosis pathways, may account for the unusual chemosensitivity of these cells. However, a simple loss of these mechanisms does not seem to account for the majority of cases of chemoresistance. Recently, CGH analysis has revealed genomic CNV of specific chromosomal regions associated with acquired cisplatin resistance,[532,533] and further exploration of the genes involved in these regions may shed light on the mechanisms underlying resistance.

Prospects for Targeted Therapy of Germ Cell Tumors

Cisplatin-based chemotherapy of GCTs has been highly effective and will continue to be a mainstay of treatment. However, the toxicity of these regimens, as well as the poor prognosis of cisplatin-resistant disease, points up the need for more specific, effective therapies. Such therapies will depend on advances in research focusing on the molecular mechanisms of germ cell tumorigenesis. To date, these mechanisms are only incompletely understood, but some case reports and small trials have raised intriguing prospects for targeted therapy of GCTs. The receptor tyrosine kinase *c-kit* oncogene is commonly overexpressed in seminomas, and activating mutations in *c-kit* have been described in seminomas,[199,243,372] suggesting that GCTs may be susceptible to treatment with the kinase inhibitor imatinib. Whereas case reports have documented efficacy of imatinib in seminoma[534] the majority of the described *c-kit* mutations in seminomas are insensitive to imatinib,[370] and a phase II trial of imatinib in six patients with *c-kit*–positive metastatic GCT failed to show significant antitumor activity.[490] Although *c-kit* remains a promising target in GCTs and other malignancies, it is clear that not all *c-kit* mutations will be susceptible to imatinib or other tyrosine kinase inhibitors (TKIs) currently in use.[535] Another potential TKI target, the epidermal growth factor receptor (EGFR) has also been investigated in relapsed or refractory GCTs. Several studies reported that EGFR is expressed in nonseminomas,[422,536,537] and a phase II study has been initiated to treat GCTs with erlotinib, a small-molecule EGFR inhibitor. Early reports that GCTs overexpress HER2/Neu and might be susceptible to trastuzumab (a chimeric anti-HER2/Neu antibody)[439] were not borne out by subsequent analyses.[538]

Differentiation therapy is another potential route to targeted therapy of GCTs. Based on the differentiating effect of retinoic acid on GCT cell lines in vitro, 16 patients with chemoresistant GCTs were treated with retinoic acid; no partial or complete responses occurred.[539]

Inhibition of tumor angiogenesis is a promising avenue under intense investigation for many tumor types. In GCTs, increased expression of the proangiogenic vascular endothelial growth factor (VEGF) correlates with tumor metastasis.[540,541] Trials are underway to evaluate the effect of inhibiting VEGF in patients with GCTs by using the small-molecule TKI sunitinib or the combination of oxaliplatin with the anti-VEGF antibody, bevacizumab.

It is important to note that most targeted therapy trials are based on preclinical models of adult GCTs; because the molecular pathogenesis of pediatric tumors likely differs from that of adults, targeted therapy developed for adult GCTs may not translate directly into clinical efficacy in children. More effort is required to define the "targets" for pediatric GCTs and to develop animal models that accurately model the human tumors. In both children and young adults, GCTs appear to arise from defects in early development.[1] Therefore particular attention should be given to examining developmental and stem-cell pathways such as the Wnt/β-catenin, BMP, notch, and hedgehog pathways. Intriguingly, a recent report describes activation of the Wnt/β-catenin pathway in immature teratomas and YSTs but not germinomas, embryonal carcinomas, or choriocarcinomas.[385] The emerging role of miRs in human cancer is another area with potentially great implications for understanding GCT pathogenesis.[390,542,543] Continued progress in molecular cytogenetics and pathway discovery in pediatric GCTs will be critical to the development of improved therapies.

LATE EFFECTS

There is limited data on both the short- and-long term side effects of treatment for GCTs among patients treated as children. GCTs are not included in the Childhood Cancer Survivor Study (CCSS), an invaluable source of information about late effects among children treated for other diagnoses. The only comprehensive survey of late effects of pediatric GCTs is a single-institution study from St. Jude's of 73 patients treated from 1962 through 1988; two thirds of patients had at least one major complication.[544] This study is limited for several reasons; first, the treatment of GCTs has changed radically since 1962 and even since 1988. In the era of the St. Jude study, the majority of patients received radiotherapy as part of their treatment; radiotherapy is seldom used today. The chemotherapy for GCTs during this era included VAC either alone or in combination with a cisplatin-based regimen. The inclusion of an alkylator, cyclophosphamide, in the older regimens radically changes the profile of expected toxicities. Cumulative doses of drugs were also much higher on the older regimens, because "maintenance" therapy often continued for a year, whereas total duration of current regimens is generally only 9 to 12 weeks. For these reasons, the toxicities described in the St. Jude report, such as the high prevalence of delayed pubertal development and ovarian failure, are related to either radiation or treatment with an alkylators, neither part of standard care today.

Other pediatric studies have focused primarily on short-term toxicity either occurring during the clinical trial or within the window of follow-up observation used to establish relapse rate. For instance, among 33 children treated with PEB in the GC1 study in the United Kingdom, 45% developed some degree of renal impairment and 10% developed auditory impairment (grades 1-3). One child developed AML while receiving PEB. There have been no deaths from bleomycin-induced pulmonary fibrosis after the dose of bleomycin was reduced to once per cycle every 3 weeks. There had been four deaths from pulmonary toxicity among children receiving weekly bleomycin. Of the 17 evaluable patients with SCT, four had neuropathic bladder and/or bowel and one child had shortening of one leg.[470,471] Among the 137 patients treated on GC2, which replaced carboplatin for cisplatin, only one patient had significant hearing loss, but this occurred after middle ear hemorrhages, and only patient died of pulmonary failure, and that patient had preexisting bronchopulmonary dysplasia.[470] All other renal and pulmonary toxicity noted on study was reversible. On the U.S. intergroup study, no patient died of pulmonary toxicity.

Late Effects Among Men Treated for Testicular Cancer

Physicians who care for long-term survivors of pediatric GCTs must be aware of and extrapolate from data emerging from follow-up studies of men treated for testicular cancer. Studies of adult men treated for testicular cancer are consistently demonstrating an increased risk of both cardiovascular disease and second malignant neoplasm. However, the limitations of this approach are twofold. One is that age at treatment likely has an important bearing on type and severity of toxicities. For instance, adults are much more likely to develop tinnitus or Reynaud phenomenon after cisplatin than children. Younger age at treatment, however, increases the risk of development of second malignant neoplasm. A concern about extrapolating from the data on men who have been treated with testicular cancer is that one must assume that the experience of men can be applied without revision to the experience of women, an assumption proven to be false in other clinical scenarios.

Cisplatin Levels Are Measurable for Years After Treatment

Cisplatin is a derived from platinum, a heavy metal like iron or lead. Recently it has become apparent that cisplatin is not completely excreted after treatment but is stored and slowly leaches back into the circulation over time. At autopsy, cisplatin can be measured in every tissue of the body,[545] and cisplatin levels are measurable for years after treatment.[546] In 45 patients, platinum plasma concentrations ranged between 1.42 and 2.99×10^{-3}ng/L.[547] Among adult testicular cancer survivors, the circulating level of cisplatin is highly correlated with the amount of persistent neuropathy reported after treatment.[548] The correlation of cisplatin levels with second malignant neoplasm (SMN) and cardiovascular disease has not been reported to date.

Risk of Cardiovascular Disease

In a nationwide cohort in the Netherlands of 2707 5-year survivors of testicular cancer, the standardized incidence ratio of myocardial infarction, angina pectoris, or congestive heart failure was 1.4.[549] In another study of long-term survivors, the relative risk (RR) of cardiovascular disease was estimated to be 2.59 for patients treated with chemotherapy compared with patients under surveillance (with median follow-up period of 10 years).[550] A partial explanation for this excessive morbidity and mortality is the higher prevalence of cardiovascular risk factors among survivors, including a 30% incidence of metabolic syndrome including hypertension, dyslipidemias, obesity, insulin resistance, and gonadal dysfunction[546,551] as compared with either patients treated with surgery and surveillance only or with normal population controls. However, the reason that patients treated with chemotherapy have higher prevalence of cardiovascular risk factors is not well understood. A possible factor contributing to cardiovascular morbidity may be the chemotherapy induces vasospastic disease, as evidenced by the high incidence (up to 50%) of Raynaud phenomenon postchemotherapy in adult survivors of testicular cancer.[552-555] Another explanation for premature onset of cardiovascular events may be direct damage to the endothelial cell. Evidence of endothelial cell damage is the high prevalence (22%) of microalbuminuria observed in long-term survivors.[556] Other authors report an increase in levels of von Willebrand factor in plasma and in the intimamedia thickness of the carotid artery after treatment as further evidence of endothelial cell toxicity.[557]

Risk of Second Malignant Neoplasm

Several studies of the risk of second malignancy among adult men treated for testicular cancer have been conducted.[450,558,559] In the first two studies published (n = 1025 and 1909) an increased risk of secondary malignancy, principally gastrointestinal cancers, was observed, but secondary cancers occurred only in patients who had received radiotherapy.[558,559] A subsequent study that utilized population-based cancer registries identified 40,576 survivors of testicular cancer who had developed a total of 2285 secondary solid cancers.[450,560] In this cohort, patients treated with chemotherapy alone exhibited a 1.8 fold excess risk of developing a second solid cancer, unlike prior studies in which risk appeared to be restricted to those who had been treated with radiotherapy. Treatment with radiotherapy did incur a higher risk (RR 2.0) than that observed with chemotherapy alone, and a synergistic effect between chemotherapy and radiation therapy was also observed (RR 2.9). This study was also the first to report an increased risk of supradiaphragmatic cancers (esophagus, pleura, and lung). Interestingly, cumulative risk at any given age increased with decreasing age at testicular cancer diagnosis. The authors estimated that a patient diagnosed with seminoma at age 20 would have a cumulative risk of solid cancer of 47% by age 75, compared with 36% for patient diagnosed at age 25 and 28% for a patient diagnosed at age 50. There was no plateau in the upward trend for risk throughout the 40 years of follow-up study, with serious implications for those diagnosed and treated at a young age.[450]

Risk of Secondary Leukemia

The development of secondary leukemia or myelodysplastic syndrome after treatment with epipodophyllotoxins is a well-described clinical syndrome usually involving a translocation of the *MLL* gene.[561] The risk of secondary leukemia among adult patients with testicular cancer has been estimated to be 0.6%.[562,563] An analysis of data that included children treated on the last U.S. intergroup national protocol for pediatric GCTs estimated the risk to be virtually identical (0.7%).[561] In a report of the German pediatric experience, 442 patients received chemotherapy, and 174 patients received chemotherapy and radiotherapy.[564] Six patients subsequently developed AML; four of the six had the classic cytogenetic abnormalities associated with topoisomerase II inhibitor-induced leukemias. Cumulative risk at 10 years for AML was 1% for children treated with chemotherapy alone and 4.2% for children treated with both chemotherapy and radiotherapy.

Other Significant Late Effects

Bleomycin and Pulmonary Toxicity

Bleomycin is known to cause fatal pulmonary fibrosis in 1% to 2% of patients, but all patients experience some measurable decrement in pulmonary function while undergoing treatment. However, in adults, almost universally, pulmonary function tests recover to pretreatment values after completion of treatment.[565] Of more concern is the development of rapidly fatal adult respiratory distress syndrome (ARDS) in long-term survivors after general anesthesia. High concentrations of inspired oxygen have been implicated.[566,567] However, in a study reporting on the surgical management of patients that had been treated with bleomycin, the most significant predictor of postoperative oxygen saturation was the amount of blood transfused; intraoperative fractional oxygen was not significant in multivariate analysis.[568] These authors suggest that careful and conservative fluid management is at least as important as restriction of inspired oxygen during surgical procedures.

Most pediatric protocols have either deleted or significantly reduced the amount of bleomycin used so as to completely avoid the complication of fatal pulmonary fibrosis. It is not known how the reduction in dose will affect the development of ARDS during anesthesia post-treatment, and pediatric patients should be made aware of the risk.

Renal Toxicity

The glomerular filtration rate is decreased during treatment in almost all adult male patients, but most studies report restoration of renal function to approximately 85% of pretreatment value by 4 years off therapy.[475,565,569-571] Persistent salt-wasting (Ca, Mg, phosphate) has been reported in 20% of patients.[475,570] Men have also been reported to have elevated renin and aldosterone after therapy.[572]

Gonadal Function

Abnormal sperm counts and motility, and elevated follicle-stimulating hormone (FSH) indicative of impaired spermatogenesis have been documented in two thirds of men after orchiectomy and before any adjuvant treatment.[573,574] One study showed a similar clinical picture even before the initial orchiectomy.[571] Despite these abnormalities in spermatogenesis at diagnosis, a significant proportion of men recover both sperm number and function after treatment.[573,575,576] In one study, 50% of men who were azoospermic at baseline recovered sperm counts to at least 10×10^6 /mL, and 80% of patients who were oligospermic recovered normal sperm counts.[577] Elevated FSH (>2× upper limit of normal) was predictive of abnormal semen analysis both at baseline and also after treatment.[577] Semen recovery usually occurs in the first 2 years after completion of treatment, but improvement has been observed as late as 4 years after treatment.[571] Encouragingly, paternity rates posttreatment are quite high; most men (67% to 76%) who wished to father a child have succeeded.[578]

Persistent endocrine dysfunction has been documented in men after treatment for testicular cancer, particularly in the pituitary-gonadal axis.[553] Several authors note elevations of FSH (up to 75% of men), luteinizing hormone (LH; up to 50% of men).[208,553,569,579] and decreased testosterone (10% to 15%) in long-term survivors of testicular cancer.[553] One author noted that the volume of the remaining testis was reduced in those men who received chemotherapy compared with those who underwent surgery only.[580] Moderate testosterone deficiency, as documented in some long-term survivors, may have a broader systemic impact. For instance testosterone deficiency has been correlated with higher levels of serum cholesterol,[581] and lower testosterone levels are associated with lower levels of bone mineral density.[582] Site apparently was not related to endocrine dysfunction; patients with extragonadal primary tumors had the same profile of endocrine abnormalities.

CONCLUSIONS

Pediatric GCTs are a highly curable form of cancer. Several challenges remain. One objective of current research is to elucidate a better understanding of the underlying biologic etiology of these tumors. Work is also underway to improve upon the current ability to predict who is likely to fail first-line therapy. Better prognostication will allow a more refined approach to therapy, reducing the amount of therapy needed for those likely to be cured. Given the seriousness of the emerging late effects of therapy on rates of cardiovascular disease and SMN in men treated for testicular cancer, physicians must reweigh the risks of adjuvant chemotherapy versus either observation or primary RPLND. Conversely, the field is also challenged to find ways to intensify therapy for those for whom standard therapy is not sufficient. Finally, it is imperative that researchers and practitioners catalogue the late effects of treatment for children with GCTs so that they can adequately care for the majority of pediatric patients who will be cured.

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CHAPTER 64

Histiocytoses

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CHAPTER OUTLINE

TYPES OF DENDRITIC CELLS
ORIGINS OF DENDRITIC CELLS
LANGERHANS CELL HISTIOCYTOSIS
Incidence
Clinical Features
Management
Refractory Langerhans Cell Histiocytosis
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Overview of Therapeutic Options
NON–LANGERHANS CELL HISTIOCYTOSES
Juvenile Xanthogranuloma
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MONOCYTES AND MACROPHAGES
Origins of Macrophages
HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS
Incidence
Clinical Features
Familial Hemophagocytic Lymphohistiocytosis
Secondary Hemophagocytic Lymphohistiocytosis
Management
CONCLUSIONS

The histiocytoses constitute a collection of rare hematologic diseases that resist easy classification, at least in part because of the imprecise definition of "histiocyte." "Histiocyte" broadly refers both to cells of the macrophage lineage and to dendritic cells (DCs), only some types of which are derived from macrophages. The numerous descriptive and functional subsets of macrophages and DCs only add to the confusion. Nonetheless, their collective consideration is justified by a degree of uniformity in these childhood diseases that present with infiltration of bone, secondary lymphoid organs, or the liver, with or without concomitant involvement of other visceral organs. However, prognosis and treatment options are specific to the subtype, and thus an appreciation of the individual varieties of histiocytoses is mandatory.

This chapter is divided into two main sections that correspond to the two most common clinical histiocytic disorders, Langerhans cell histiocytosis (LCH) and hemophagocytic lymphohistiocytosis (HLH). Also included is a relatively brief discussion of non–Langerhans cell histiocytoses and Rosai-Dorfman disease. Each section will begin with a description of the normal counterpart(s) of the relevant histiocytes and their ontogeny to provide a context for describing how these cells are pathologically involved in the histiocytic disorders. Each section will also include a description of their clinical manifestations and current approaches to treatment.

TYPES OF DENDRITIC CELLS

The pathologic cells of LCH share some features with normal epidermal Langerhans cells (LCs), which are the primary antigen-presenting cells (APCs) of skin.[1] LCs are one type of the general class of APCs known as DCs because of the characteristic dendritelike structure they assume when activated. DCs are, in turn, just one of several types of APCs that are called "professional" because they can, by themselves, fully activate naive T lymphocytes by providing both the antigen/major histocompatibility complex ligand for T-cell receptor binding and the accessory signals required for full activation.[2,3]

Because DCs can also render T cells tolerant to specific antigens,[4] they have become a major focus of basic immunologic research. This research has revealed a surprising level of functional and phenotypic diversity among DCs, which will undoubtedly be relevant to the proliferative disorders affecting these cells. Several authors have provided helpful guides to thinking about and classifying DCs.[2,3,5,6] Overall, DCs can be divided into conventional and inflammatory types (Box 64-1). Conventional DCs are present in lymphoid and nonlymphoid organs in their basal state, and their primary function is to collect antigens for presentation to T cells in order to activate or tolerize them, depending on the antigen's source (i.e., nonself vs. self). Inflammatory DCs arise in response to

Box 64-1 **Dendritic Cells**

CONVENTIONAL DENDRITIC CELLS

MIGRATORY DENDRITIC CELLS

Migratory dendritic cells (DCs) gather antigen in peripheral tissues, then migrate to regional lymph nodes to present antigen to T lymphocytes

Examples: Langerhans cells, dermal DCs, interstitial DCs

LYMPHOID TISSUE DENDRITIC CELLS

Lymphoid tissue DCs gather antigen within lymphoid tissue and present antigen to T lymphocytes within the same lymphoid tissue

Examples: thymic DCs, splenic DCs

INFLAMMATORY DENDRITIC CELLS

Inflammatory DCs arise in response to inflammatory signals; they can gather antigen and present antigen to T lymphocytes and can secrete cytokines

Example: tumor necrosis factor and inducible nitric oxide synthase–producing DCs

MUCOSAL DENDRITIC CELLS

Mucosal DCs are resident in mucosal surface tissues; they can gather antigen and present antigen to T lymphocytes and help direct responses toward activation or tolerance

PREDENDRITIC CELLS

Predendritic cells can develop DC function directly in response to inflammatory stimuli

Example: plasmacytoid DCs, monocytes

Modified from Shortman K, Naik SH: Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol *7:19–30, 2007.*

specific inflammatory or infectious signals and are ordinarily not identifiable in the nonchallenged state. In addition to presenting antigen, these cells secrete cytokines and other mediators, including tumor necrosis factor (TNF), that enhance host defense.

Within the conventional DC category, one can distinguish between migratory DCs and DCs that reside in lymphoid tissue. Migratory DCs fulfill the sentinel role of this leukocyte class and include LCs, dermal DCs, and interstitial DCs of other organs. Migratory DCs shuttle between end organs that interface with the external world (e.g., skin or mucosa), in the case of LCs, and regional lymph nodes. This trafficking occurs at a low level in the basal state but can be greatly enhanced in the presence of foreign antigen or inflammatory challenges.[7-10] The migratory DC takes up antigen in the periphery and, once activated, travels to regional nodes either to present antigen itself or to transfer antigen to resident DCs in the node, which then perform the presentation function to T cells. Although the molecular signals that control this migratory behavior are not understood in detail, chemokines and their receptors play a large role.[11,12] In the case of LCs, resting cells express the chemokine receptor CCR6 whose ligand, CCL20, is secreted by cutaneous keratinocytes. Upon antigen uptake and activation, LCs downregulate CCR6 and in its place upregulate another chemokine receptor, CCR7, whose ligands, CCL19 and CCL21, are secreted by cells in regional lymph nodes. This receptor switch has the dual effect of neutralizing the LC's anchor to the skin and attracting them to regional lymph nodes.

In contrast, DCs that reside in lymphoid tissue are nonmigratory. They make up most of the DCs populating the thymus and spleen and about half of the DCs in lymph nodes.[8,13] Unlike migratory DCs, which are mature when they appear in lymph nodes, resident DCs are immature, which allows them to take up, process, and present local antigens. Surface markers can distinguish several subsets of resident DCs, which are presumed to have specialized functions. In the mouse, these subsets include cluster of differentiation (CD)8+ and CD8− cells, and among the CD8− population are CD4+ and CD4− subpopulations.[14] Specific DC subsets also have characteristic gene expression profiles.[15]

LCs bear distinctive intracellular and extracellular markers that permit their distinction from DCs that reside in lymphoid tissue. The most characteristic surface marker is CD1a,[16] and the most characteristic ultrastructural feature is Birbeck granules, which are pentilaminar "tennis racket"–shaped cytoplasmic organelles that appear to be uniquely present in LCs.[17] Langerin, also known as CD207, is a cell surface marker for LCs that associates with Birbeck granules when internalized.[18] Langerin is a C-type lectin with specificity for sugars that contain mannose, suggesting that it may be involved in processing and trafficking of antigens that bear these sugars. Dectin-2 is another C-type lectin restricted to DCs,[19] and DEC-205 (CD205) is yet another lectin that is often used to identify LCs, although its expression is not as restricted to LCs as are the other lectins.[20] In the appropriate histologic context, from a diagnostic standpoint, CD1a and Langerin are the most reliable lineage-specific markers.[16]

Some authors also distinguish a group of so-called "predendritic cells" that do not ordinarily have DC functions or structure.[21,22] However, these cells can develop directly into DCs upon stimulation without the need for proliferation. One well-studied example is the plasmacytoid DC, which circulates as a nondescript mononuclear cell but acquires APC function and secretes large amounts of alpha interferon when activated.[23,24] Technically, using this definition, monocytes would also be predendritic cells. After exposure to interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (CSF), monocytes become functional DCs, although their phenotype is closer to inflammatory DCs than conventional DCs.[25]

ORIGINS OF DENDRITIC CELLS

The availability of genetically modified mice that carry lineage-restricted markers has greatly expanded our understanding of the origins and development of DCs. Although mouse models have some limitations,[26] the major insight these animals provide is that a salient characteristic of DC ontogeny is flexibility. For example, although DCs generally have myeloid characteristics, all DC subtypes in the mouse can arise from either committed lymphoid or committed myeloid precursors.[27-29] Even though half of thymic DCs show evidence for immunoglobulin rearrangements, suggesting a lymphoid phenotype, they also transcribe the macrophage colony-stimulating factor (M-CSF) receptor gene, which is a myeloid characteristic.[30] Activation of fms-like tyrosine kinase–3 (FLT3), a growth factor receptor, is required for DC development, and it has been suggested that committed myeloid or lymphoid precursors expressing FLT3 can differentiate into DCs in the presence of FLT3 ligand.[31]

Splenic DCs have a relatively rapid turnover rate (every 3 days).[32] These cells are repopulated both by DC division and splenic precursors, as well as by bone marrow precursors. In contrast, LCs are very long-lived.[32] After their migration from the skin, the population is replenished from a pool of LC precursors in the skin, as well as by circulating monocytes.[33] The latter is dependent on the action of the chemokine CCL2 acting on its receptor CCR2.[34,35] Thus the ontogeny of LCs is distinct from that of splenic DCs. Immature DCs are more closely related to LCs but appear to be derived from noninflammatory monocytes that do not express CCR2.[36] Plasmacytoid DCs split off from conventional DCs early in development, consistent with their distinct functional characteristics.[37]

LANGERHANS CELL HISTIOCYTOSIS

The hallmark of LCH is the accumulation of LC-like DCs in one or more tissues or organs. The clinical manifestations of LCH are highly diverse. They result both from the direct, local effects of the growth and accumulation of pathologic LCs and the indirect, secondary effects these activated cells have on normal tissues, particularly cells of the immune system. Although the most commonly involved sites of disease are the bone and skin, virtually any organ or system may be involved. The pattern of involvement is not necessarily related to patterns of normal LC or DC migration.

Although the histiocytes in LCH share certain characteristics with LCs, such as expression of CD1a and the presence of Birbeck granules, recent reports suggest that mature LCs may not be the cell of origin for LCH histiocytes. In particular, a global analysis of gene expression suggested that LCH cells are more closely related to myeloid dendritic precursors than to LCs.[38] Whether this pattern of gene expression reflects a non-LC cell origin for LCH or is a consequence of LC transformation remains to be determined.

The fact that pathologic LCs are related to immunomodulatory cells and that they elicit inflammatory infiltrates suggests that LCH might be a reactive rather than a neoplastic disease. Indeed, ample precedent for this mechanism exists among the histiocytoses, including the secondary HLH syndromes that arise in the context of viral infection.[39] However, no reproducible reports exist of viral genomes recovered from LCH cells, and epidemiologic studies are not consistent with an infectious or environmental cause of LCH.[40,41,42,43] Rather, the preponderance of evidence indicates that LCH arises as a consequence of intrinsic genetic abnormalities. The most salient arguments that LCH is a neoplasm and not a reactive disease are as follows: (1) pathologic LCs are clonal, as demonstrated by nonrandom X chromosome inactivation both in whole LCH tissue (in a proportion corresponding to the proportion of CD1a+ cells in the lesion)[42] and in sorted CD1a+ cells,[44] and (2) nearly 60% of LCH samples carry the oncogenic BRAF V600E variant.[45,46] Notably, the MAPK pathway, which would be activated constitutively in the presence of BRAF V600E, is uniformly activated in LCH cells regardless of the presence or absence of BRAF V600E, suggesting that alternative mechanisms of pathway activation are present in LCH samples that lack BRAF V600E. In some cases, other activating mutations of *BRAF* have been found.[46,47] Additional known mechanisms of *BRAF* activation, such as gene amplification or translocation, which occur in other diseases, have not been described in LCH.[45] Notably, although pulmonary LCH in adults is generally considered to be a polyclonal disease, more than 40% of pulmonary LCH cases contain BRAF V600E.[45] This finding suggests that a significant proportion of these cases are clonal[48] or that multiple independently developed clones of pulmonary LCH have BRAF V600E, giving the overall appearance of polyclonality.

Although *BRAF* mutations are among the most common molecular abnormalities found in all cancers, they are not present in other histiocytoses that affect children, such as juvenile xanthogranuloma (JXG) or Rosai-Dorfman disease.[45,49] However, BRAF V600E mutations are present in the histiocytes of about 50% of patients with Erdheim-Chester disease, a rare and aggressive non-LCH of adults.[49] Furthermore, treatment of patients who have BRAF V600E–positive Erdheim-Chester disease with a *BRAF* inhibitor led to dramatic clinical responses, indicating the pathogenetic importance of this mutation.[50] Interestingly, some of these patients had concomitant LCH that also responded to *BRAF* inhibition, suggesting that this therapeutic approach may be effective in patients who have BRAF V600E–positive LCH as well.

Activating mutations of *BRAF* are insufficient by themselves to produce neoplastic disease, and therefore additional molecular alterations are likely to be required for the development of LCH. One of the most common molecular abnormalities in LCH is overexpression of *TP53*,[51] suggesting that mutational inactivation of this pathway may contribute to pathogenesis. More such changes in other genes are likely to be discovered as advanced genomic analyses are applied to LCH samples.

Incidence

The incidence of LCH is difficult to determine precisely because of the rarity and marked clinical variability of the disorder. Estimates are in the range of 2.6 to 8.9 cases per million per year for children younger than 15 years,[52-55] corresponding to roughly one tenth the incidence of acute leukemia in childhood. LCH occurs in people of all races and all ages. Although the peak age at diagnosis is 2 years, LCH can present at any time from birth to old age. No evidence of seasonal variation has been noted in the time of presentation of LCH. Several studies have shown a slight male predominance.[56,57]

No known predisposing factors exist for the development of LCH in the majority of cases.[58] Studies have demonstrated concordance of the disorder in monozygotic twins, suggesting an inherited predisposition to LCH that might account for ~1% of cases.[59] However, a positive family history is lacking in most cases. Interestingly, isolated pulmonary LCH in adults is closely linked to cigarette smoking, and it often resolves with smoking cessation.[60] However, exposure to cigarette smoke has not been linked to pediatric LCH.[61] As previously noted, a significant proportion of pulmonary LCH cases also carry the activated BRAF V600E allele.

An intriguing (although incompletely understood) association exists between malignancy and LCH. Children with a history of cancer have an increased risk of LCH. Conversely, children with a history of LCH appear to have an increased risk of cancer. This finding suggests the possibility that underlying genetic abnormalities may place individual patients at increased risk for LCH.[62] Alternatively, the association between LCH and cancer may fall within the spectrum of secondary posttherapy effects, because irradiation and drugs such as etoposide are used to treat LCH and are known to induce tumor-promoting genotoxic injury. Several cases of LCH arising in the setting of T-cell acute lymphoblastic leukemia have been described, typically when the leukemia is in remission while the patient is receiving therapy or in the early posttherapy period.[63] A clonal link between the initial leukemia and pathologic LCs of LCH has been established in some cases, suggesting that the LCH represents a manifestation of the malignant clone rather than a distinct reactive or neoplastic process related to the leukemia or its therapy.[64,65] An intriguing link may exist to hairy cell leukemia, which also harbors BRAF V600E in the majority of cases.[66]

Clinical Features

The clinical manifestations of LCH are protean. Although patterns of clinical presentation have been described, the disease is appropriately viewed as a spectrum. LCH may involve a single site, multiple sites in a single organ system, or multiple organ systems. Many patients present with localized pain, soft tissue swelling, or skin rash. Less commonly, patients have symptoms of diabetes insipidus (DI), respiratory insufficiency, cytopenias, lymphadenopathy, liver dysfunction, or organomegaly.

In the past, three distinct clinical syndromes were described: eosinophilic granuloma, Hand-Schüller-Christian disease, and Letterer-Siwe disease.[67,68] "Eosinophilic granuloma," a term still commonly used by radiologists and orthopedists, refers to the presence of one or more lytic bone lesions. Hand-Schüller-Christian disease consists of the triad of bone defects, exophthalmos, and polyuria. Letterer-Siwe disease, a fulminant disorder of the reticuloendothelial system, is characterized by hepatosplenomegaly, lymphadenopathy, skin rash, bone lesions, anemia, and the tendency to bleed. This classification has important historic significance, but its applicability in the clinical setting is limited. Once it was recognized that the diverse manifestations of the disease share common histopathologic features, the three syndromes were unified under the term "histiocytosis X."[69] Categorizing patients based on the number and location of lesions and the presence or absence of organ involvement was shown to be useful in predicting prognosis and determining therapy.[70-74] Single-system LCH (SS-LCH) disease—which usually affects bone, less frequently affects skin, and rarely affects the lymph nodes, lung, or central nervous system—accounts for approximately two thirds of pediatric LCH cases. Involvement of two or more organ systems, referred to as multisystem LCH (MS-LCH), accounts for the remaining one third of cases. In about half of MS-LCH cases, "risk" organs (i.e., the liver, spleen, and hematopoietic system) are affected.[55,75] Thus far the presence of BRAF V600E does not correlate with disease extent or prognosis.

Pathology

A biopsy of lesional tissue is required to establish the diagnosis of LCH. The pathologic diagnosis is usually straightforward, but because the disease is rare, varied, and may mimic many other conditions, delay in diagnosis is common. LCH should be considered in any patient who presents with skeletal lesions, a persistent rash, chronically draining ears, central DI, unexplained lymphadenopathy, respiratory insufficiency with a reticulonodular pattern on a chest radiograph, hepatosplenomegaly, or cytopenias.

Once clinical suspicion is raised, the workup should proceed with a biopsy of the most accessible site of disease. Complete surgical excision of the lesion is generally unnecessary either for diagnosis or treatment. LCH lesions at all sites share common histopathologic features: accumulation of large neoplastic LCs with a moderate amount of dense pink cytoplasm and plump, distinctive "C-shaped," "coffee bean," or cleaved nuclei admixed with variable numbers of inflammatory cells, including T lymphocytes, macrophages, plasma cells, and eosinophils. The composition and architecture of the infiltrate characteristically differ according to the location. Osteolytic bone lesions often contain many nonneoplastic osteoclasts, as well as multinucleated tumor giant cells and large numbers of eosinophils, the latter speaking to the origin of the term "eosinophilic granuloma." Mature, "burned out" bone lesions may be difficult to distinguish from chronic osteomyelitis, which is often in the clinical and radiographic differential diagnosis. LCH infiltration of the skin typically involves the superficial dermis, with tumor cells infiltrating the epidermis (so-called "epidermotropism" or "exocytosis"), thus altering epidermal barrier function and leading to superinfection and the characteristic appearance of some LCH rashes. In all cases, the neoplastic LCs express the LC markers CD1a[16] and langerin,[76,77] as well as S100 protein[78] and fascin[79] (Fig. 64-1). Electron microscopy, which historically is used to demonstrate the presence of Birbeck granules indicative of the LC lineage, has little diagnostic utility at the present time. Cytogenetics or molecular studies are predominately needed to exclude other diseases with similar clinical presentations or histologies. Furthermore, no immunohistochemical or other laboratory methods are useful in

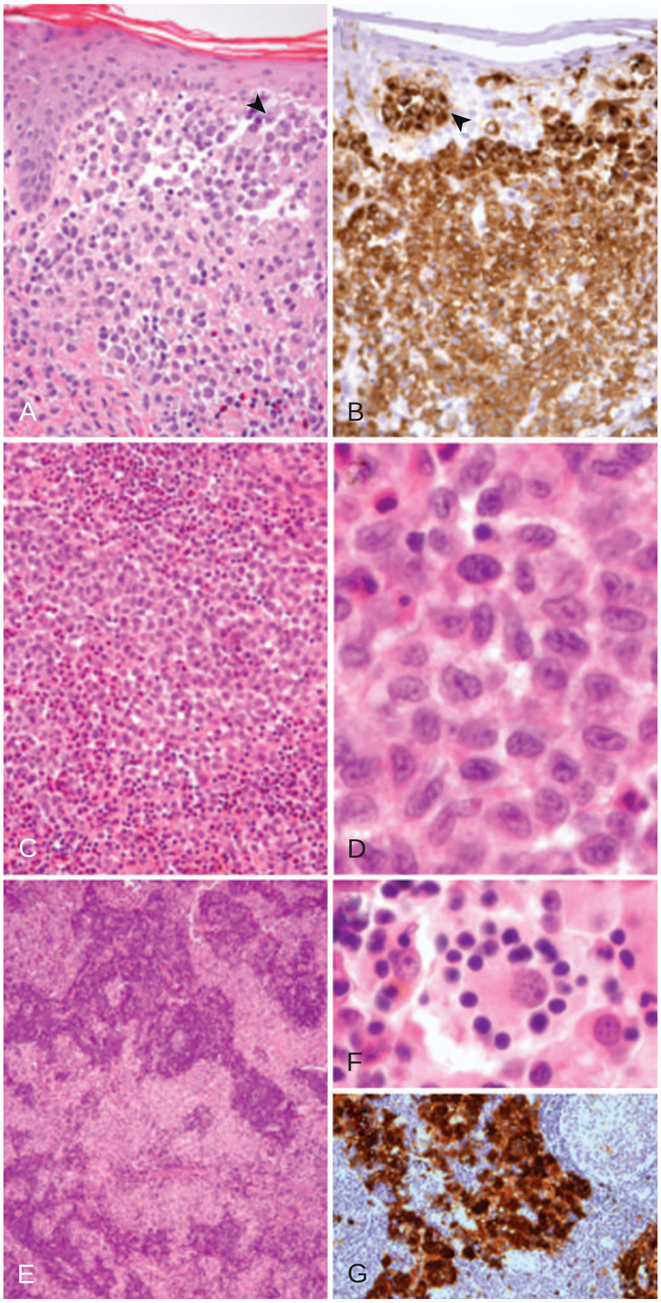

Figure 64-1 Histopathologic features of Langerhans cell histiocytosis (LCH) and sinus histiocytosis with massive lymphadenopathy (SHML). **A** and **B**, Hematoxylin and eosin (**A**) and CD1a immunostain (**B**) of LCH involving the skin. Lesional cells infiltrate the upper dermis and focally *(arrowheads)* involve the epidermis. **C** and **D**, Low-power (200× magnification, **C**) and high-power (1000× magnification, **D**) views of a hematoxylin and eosin–stained section of an LCH bone lesion. The low-power view (**C**) demonstrates tumor cells present in association with a mixed inflammatory infiltrate rich in eosinophils. The high-power view (**D**) illustrates the histologic features of the lesional Langerhans cells, including the characteristic "C-shaped" nuclei and abundant pink cytoplasm. **E, F,** and **G,** Low-power (40× magnification, **E**) and high-power (1000× magnification, **F**) views of a hematoxylin and eosin–stained section of SHML involving a lymph node. Pale areas in panel **E** represent dilated sinuses replaced by lesional histiocytes that are highlighted by an S100 immunostain in panel **G**. Panel **F** demonstrates a histiocyte that contains numerous lymphocytes, which is typical of the emperipolesis characteristic of SHML.

distinguishing clinically indolent from clinically aggressive forms of the disease.

Diagnostic Evaluation

In suspected LCH cases, evaluation for disease extent is indicated, starting with a history and physical examination.[80] A skeletal radiographic survey is recommended to comprehensively assess skeletal involvement. Abnormalities detected on plain radiographs may be followed by axial imaging to document the presence of a soft tissue mass.[81,82] Nuclear imaging (i.e., a bone scan or fluorodeoxyglucose positron emission tomography [FDG-PET]) complements the skeletal survey. Compared with plain radiographs, nuclear imaging tests detect active lesions on the basis of increased local metabolic activity. Active bone lesions usually show increased radiotracer uptake, whereas inactive lesions may be undetectable or appear "photopenic." FDG-PET appears to be the most sensitive imaging modality for the detection of lesions in persons with LCH, and it is particularly useful for following up on patients. Unlike a bone scan, PET is capable of detecting extraosseous lesions.[83-85]

Complete blood cell counts and liver function studies should be performed, and liver and spleen size should be assessed by physical examination. Abdominal ultrasound is indicated when laboratory studies are abnormal or if hepatosplenomegaly is present or suspected. The erythrocyte sedimentation rate may be elevated, but it is not a sensitive or specific disease indicator. Measurement of specific gravity of an early morning urine sample is an easy and inexpensive screening test for DI. A formal water deprivation test may be necessary to establish the diagnosis of DI. Magnetic resonance imaging (MRI) of the head should be performed in children with cranial bone lesions and when DI is suspected or confirmed. A chest radiograph should be performed in all cases, and a computed tomography (CT) scan of the chest should be performed if the radiograph findings are abnormal or if the patient has respiratory signs or symptoms. Additional evaluations such as upper/lower endoscopy and biopsies of bone marrow, skin, lymph nodes, and the liver should be considered in the appropriate clinical context.

Management

The approach to management of LCH must account for the variability in its clinical behavior. On one hand, LCH is an uncontrolled accumulation of a clonal population of cells, with the capacity to behave extremely aggressively and the potential to involve multiple sites and organ systems. The process may be driven, in part, by oncogenic variants of *BRAF*. In addition, it may be effectively treated with cytotoxic chemotherapy and radiotherapy and, based on preliminary reports, *BRAF* antagonists.[50] On the other hand, LCH is histologically benign, it sometimes resolves spontaneously, and it may respond to immunomodulatory or immunosuppressive agents. The historic uncertainty about the fundamental nature of LCH is reflected in the broad range of therapies that have been used to treat it.

Performing controlled clinical trials in persons with LCH has been challenging. As a consequence, the LCH literature is often descriptive, anecdotal, and retrospective. Since the 1980s, several collaborative groups have conducted prospective clinical trials that have led to standardization and improvements in treatment. An overall observation of these studies is that the treatment of LCH should match the clinical scenario. Patients with localized disease have an excellent prognosis with little or no intervention. For these children, the goal of therapy is to minimize symptoms and avoid long-term disability and potential late effects. In contrast, children with MS-LCH, especially those with involvement of the hematopoietic system or liver, are at significant risk for morbidity and mortality. For these children, aggressive treatment is justified.

Bone

Bone is the most commonly involved site, with bone involvement occurring in approximately 75% of children with LCH.[57] Although any bone(s) may be affected, the bones of the skull, pelvis, and vertebral bodies are most commonly affected[56] (Fig. 64-2). Bone pain (which is often worse at night), swelling, and a limp are typical presenting symptoms. Sometimes a history of local trauma precedes or coincides with the onset of symptoms. The typical plain radiographic appearance is of a smooth-edged, "punched-out" hole in the bone. Lesions of the long bones typically involve the diaphysis, but metaphyseal and epiphyseal lesions also occur. Involvement of a vertebral body may manifest as flattening or loss of height (vertebra plana) or as a wedge deformity. Axial imaging (CT and MRI) may show an associated soft tissue mass (Fig. 64-3). The radiographic differential diagnosis of osseous LCH lesions includes malignancy, especially Ewing sarcoma, leukemia or lymphoma, and osteomyelitis.

LCH of the bone may be unifocal (monostotic) or multifocal (polyostotic), and it may present in association with disease in other organ systems. Monostotic disease, which is the most common presentation of LCH (especially in older children), occurs in more than 50% of cases.[75,86] Children with LCH that is limited to a single bone have an excellent prognosis regardless of the treatment administered. Although various local and systemic therapies appear to be efficacious,[87] retrospective trials have failed to demonstrate a comparative advantage for any specific intervention or modality, including observation only, biopsy, curettage, simple excision, intralesional steroid instillation, local radiotherapy, or systemic therapy.[88,89]

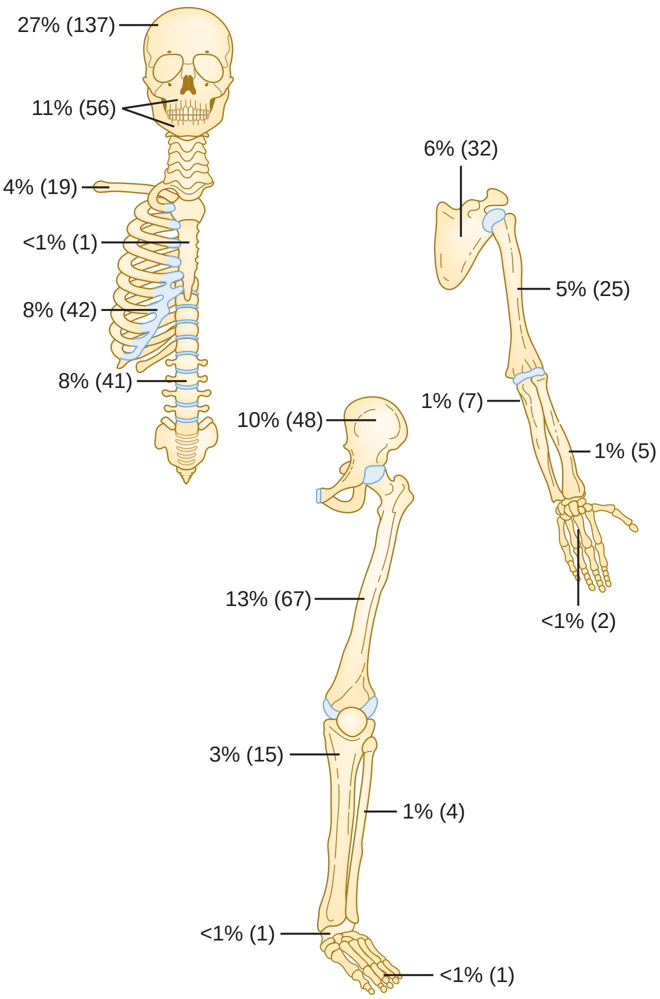

Figure 64-2 Anatomic distribution of 503 osseous lesions among 263 patients with Langerhans cell histiocytosis of bone. *(From Kilpatrick SE, Wenger DE, Gilchrist GS, et al: Langerhans' cell histiocytosis [histiocytosis X] of bone. A clinicopathologic analysis of 263 pediatric and adult cases.* Cancer *76:2471–2484, 1995.)*

Often the biopsy procedure performed to establish the diagnosis of LCH of the bone provides therapeutic benefit. In many cases, adequate tissue for diagnosis may be

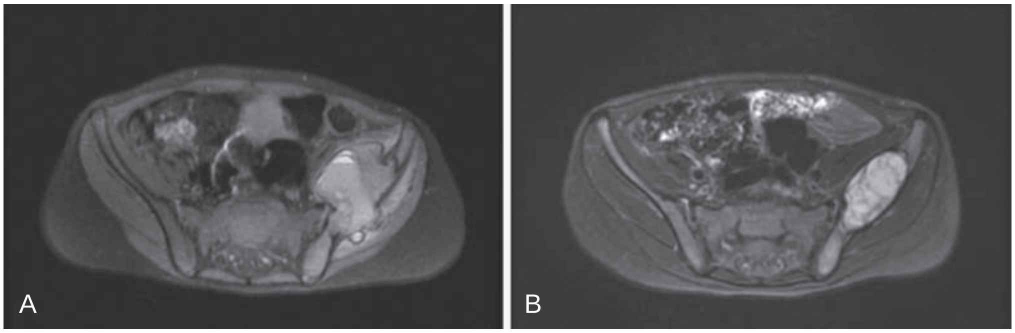

Figure 64-3 Magnetic resonance imaging scans of the pelvis of a 2-year-old boy with unifocal Langerhans cell histiocytosis of the left ilium. An expansile, destructive iliac lesion and large soft tissue mass (**A**) decreased dramatically in size within 3 months after an intralesional glucocorticoid injection (**B**).

obtained via percutaneous needle biopsy with ultrasound or CT guidance. Neurosurgeons tend to prefer open biopsy of orbital and skull base lesions and simple excision or curettage of calvarial lesions. Regardless of the surgical approach, most patients experience progressive healing after the procedure, even when complete excision of the lesion is not achieved. In cases in which LCH is strongly suspected or confirmed intraoperatively via frozen section, methylprednisolone can be instilled directly into the lesion.[90,91] This intervention yields rapid relief of pain and may hasten resolution.[92,93] Intralesional steroid injection is less commonly used in the management of cranial and vertebral lesions.

In the past, radiotherapy was routinely used to treat bone lesions. Even though a relatively low dose in the range of 600 to 1000 centigray is effective,[56,94,95] the role of radiotherapy in pediatric LCH has waned because the long-term risk of radiotherapy, especially the risk of secondary malignant neoplasms, is rarely justified in treatment of a benign disease. In the setting of disseminated disease, local radiotherapy contributes little benefit.[96]

Systemic therapy may be required for patients with monostotic LCH who experience unrelenting symptoms or are at significant risk for serious or permanent disability related to the location or extent of their lesion. This principle applies in patients with unifocal craniofacial bone involvement, including the orbit, temporal bone, mastoid, sphenoid, zygomatic, ethmoid, maxilla, paranasal sinuses, or cranial fossa (so-called "central nervous system [CNS]-risk" lesions), because these children are at increased risk for DI and other neurologic sequelae.[97] Currently it is standard practice to administer chemotherapy in "CNS-risk" cases, even though it has not been definitively established that treatment decreases the risk of neurologic complications.

Systemic therapy is also recommended for children with polyostotic (i.e., multifocal bone) LCH. In these patients, an important goal of treatment is to mitigate the risk of future reactivation,[71,75] because reactivation risk is approximately 40% for patients with multifocal bone disease versus 10% for patients with unifocal bone disease.[98] Children with SS-LCH of the bone who require systemic therapy—that is, those with CNS-risk lesions or multifocal bone involvement—are typically treated with the combination of vinblastine and prednisone (discussed later).

Skin

The skin is the second most commonly involved system in persons with LCH, with skin involvement occurring in about one third of patients.[54,55,71] Skin rashes may be extremely variable, and misdiagnosis is common. Cutaneous LCH may present as single or multiple nodules, vesicles, or scaly, seborrhea-like patches or plaques. Although the rash may be located anywhere on the body, it is typically most intense in the flexural areas, such as the neck, axillary, and inguinal folds. Involvement of the scalp, the ear canals, and the posterior auricular areas is also characteristic. Ear canal involvement may lead to chronic ear drainage that may be malodorous. Perineal and perianal rashes may be severe, persistent, and refractory to topical preparations.

Cutaneous involvement is more common in infants than in older children. Some infants with isolated cutaneous LCH prove to have a benign, self-healing disorder known as Hashimoto-Pritzker disease.[99,100] However, in infants who initially present with apparently isolated cutaneous LCH, MS-LCH may proceed to develop, and therefore self-healing cutaneous LCH is a diagnosis that can be made with accuracy only in retrospect.[101,102] The differential diagnosis of cutaneous LCH may include superficial candidiasis, seborrheic dermatitis ("cradle cap"), eczema, contact dermatitis, and viral exanthem. A skin biopsy is required to establish the diagnosis.

When treatment for cutaneous LCH is required, topical medications such as corticosteroid or nitrogen mustard may suffice.[103,104] Occasionally, systemic therapy is necessary to control extensive or severe skin involvement, especially when it is associated with pain, disfigurement, or infection. Alternatives such as psoralen ultraviolet A[105,106] or interferon[107] have been used with success in some patients who have resistant cutaneous involvement.

Central Nervous System

The brain and skull can be affected by LCH in a variety of ways, including extension of extraaxial bone-based lesions, hypothalamic-pituitary disease, intraparenchymal mass lesions, and later onset neurodegeneration.[108]

Hypothalamic-pituitary disease is the most common and best-characterized CNS manifestation of LCH. Infiltration of the pituitary stalk by lesional cells leads to deficiency of antidiuretic hormone and clinical DI.[109] Anterior pituitary deficiencies or panhypopituitarism may develop in some cases.[110] The diagnosis of DI may precede, coincide with, or occur years after the diagnosis of LCH. Estimates of the frequency of DI in persons with LCH vary considerably because of variability in study populations. One large retrospective review reported a 12% incidence of DI, with 6% having DI present at the time of LCH diagnosis. In this analysis, the risk of DI correlated significantly with the location and extent of LCH involvement. Children who have craniofacial lesions (CNS risk) or MS-LCH are at significantly higher risk for DI.[97]

Among children with a new presentation of DI, LCH is the cause in a significant proportion (approximately 15%) of cases.[111] Absence of posterior pituitary hyperintensity (the pituitary "bright spot") or thickening of the pituitary infundibulum[112] may be noted on brain imaging; however, these radiographic findings are neither sensitive nor specific for LCH. Their differential diagnosis includes brain tumors, especially germinoma, and inflammatory conditions such as hypophysitis. Investigation for other sites of LCH is worthwhile because detection and biopsy of extracranial lesions may allow histopathologic confirmation of the diagnosis without performing a pituitary stalk biopsy.[113]

Screening for DI in children who have LCH with use of a careful history is essential, and referral to an endocrinologist should be considered if symptoms of polyuria, polydipsia, nocturia, or dehydration are elicited. Urinary specific gravity greater than 1.015 weighs against a diagnosis of DI, and this reading can often be easily determined by analysis of a first-morning voided urine specimen. In cases in which the history or laboratory studies raise suspicion, a formal water deprivation test should be performed.

In general, DI is irreversible once it appears in patients with LCH. However, improvement has been reported in "early" DI cases when patients were treated promptly with chemotherapy.[114,115] At one time, emergent radiotherapy was recommended in patients with new onset DI. This recommendation has fallen out of practice because it is rarely, if ever, effective and is associated with potential long-term consequences.[116] Chemotherapy has no role in patients with established, long-standing DI who have no evidence of active LCH within or outside of the CNS.

Parenchymal CNS mass lesions in persons with LCH are composed of granulomas of CD1a+ cells admixed with other inflammatory cells. These lesions can occur in isolation or in association with disease in other organ systems. Depending on the extent and location, headaches, seizures, and focal neurologic symptoms may result. CNS mass lesions are managed with chemotherapy,[117] surgery, or radiotherapy.

Progressive neurodegeneration is an uncommon and poorly understood late event that arises years after LCH diagnosis.[118,119] Radiographically, it is characterized by signal abnormality confined to the brain stem and cerebellum on MRI.[120,121] Posterior fossa symptoms or neurocognitive dysfunction may be clinically apparent. Biopsy results show gliosis, neuronal cell loss, and lymphocytic infiltration without active CD1a+ cell infiltration.[109,122] The timing and course of this manifestation of LCH is quite variable, but it may be severe and progressive. No established treatment exists for neurodegeneration in patients with LCH. Retinoic acid[123] or low-intensity chemotherapy with or without immunoglobulin[38,124] may stabilize the disease or slow the rate of neurologic decline.

Other Sites

Lymph node involvement is occasionally present in the setting of MS-LCH, and sometimes it represents a single site of disease. The pathologic pattern of lymph node involvement is characteristically interfollicular and subtle.

The gastrointestinal tract is another uncommon site of disease in LCH, but its incidence may be underappreciated. When it does occur, it is usually in the context of multisystem involvement.[125] Clinical signs include diarrhea, bloody stools, failure to thrive, and hypoalbuminemia.[125-127] Gastrointestinal biopsies typically demonstrate a sparse infiltrate of LCs in the lamina propria.

Pulmonary involvement was historically thought to be an adverse prognostic feature, warranting its classification as a "risk" organ in early Histiocyte Society clinical trials. Involvement of the lung may be asymptomatic in up to half of affected children, with diffuse micronodular interstitial disease or cyst formation. Overt respiratory symptoms including tachypnea, chronic cough, dyspnea, and pneumothorax are seen in some patients. In children, pulmonary involvement occurs in the context of MS-LCH and is often accompanied by hematologic or hepatic dysfunction. Recent retrospective studies have shown that in the absence of other risk organ involvement, pulmonary involvement is not an independent negative prognostic indicator.[128-130] Interestingly, adults with LCH often present with disease limited to the lung, and their illness is closely linked to cigarette smoking. Cessation of smoking leads to resolution in the majority of patients.[60] Although traditionally described as a polyclonal disease, as many as 30% of cases may be clonal,[48] and a significant proportion harbor BRAF V600E.[45]

Risk Organs

Although the behavior of LCH is unpredictable, it is known that children with disease involving the hematopoietic system or liver are at highest risk for severe illness and death.[72,73] Conventionally, involvement of "risk" organs is defined by the presence of organ dysfunction, such as cytopenias, hyperbilirubinemia, and impaired hepatic synthetic function.[73] Risk organ involvement is typically a component of MS-LCH and is often associated with overt constitutional symptoms. Involvement of one or more risk organs occurs in approximately half of children with MS-LCH. Risk organ involvement is usually present at diagnosis, but it can develop later. It is clearly associated with young age, because more than half of children younger than 2 years manifest risk organ involvement. The association between young age and aggressive disease accounts for the perception that young age is an adverse prognostic feature.[53]

The presence of peripheral blood cytopenias in a patient with LCH is referred to as hematologic dysfunction and is taken to be synonymous with bone marrow involvement. Interestingly, however, evidence of bone marrow infiltration by morphologically apparent LCs is not typically seen. Most often the marrow contains a lymphohistiocytic infiltrate with hemophagocytosis that can be highly reminiscent of hemophagocytic syndromes (which will be discussed later), further illustrating that the neoplastic LCs retain some of the immunomodulatory function of their normal counterparts.[131-134] Splenomegaly is commonly seen in association with hematologic dysfunction as a consequence of organ infiltration, extramedullary hematopoiesis, and hemophagocytosis. Hypersplenism may contribute to cytopenias in these patients.

Hepatic dysfunction, like hematologic dysfunction, is associated with serious illness and a high risk of morbidity and mortality. Hepatic dysfunction is associated with any of the following findings: hepatomegaly, ascites, hyperbilirubinemia, increased serum concentrations of hepatic transaminases (especially γ-glutamyl transferase) and impaired hepatic synthetic function (hypoalbuminemia and hypoproteinemia). The pathogenesis of liver involvement is heterogeneous. In some cases hepatomegaly or transaminitis is a consequence of macrophage activation in a sinusoidal pattern in the liver and is associated with multisystem disease. In other cases, a cholestatic laboratory picture predominates, presumably resulting from the infiltration of hepatic bile ducts by CD1a+ LCs, even if these cells are not demonstrable upon a percutaneous liver biopsy.[135] In these patients, progressive hepatic dysfunction may lead over time to irreversible hepatic cirrhosis that requires liver transplantation, even after resolution of active LCH.[136]

Treatment of Multisystem LCH

Systemic therapy is indicated for all patients with MS-LCH and for some patients with SS- LCH, as previously described. The optimal therapy for this diverse group of patients is still not established. Over the years, a wide variety of immunosuppressive and cytotoxic drugs have demonstrated activity. Encouraging results were first published in the early 1960s using corticosteroids[137] and vinblastine.[138] Later, alkylating agents[139,140] and antimetabolites[141] were used. In the 1980s, the epipodophyllotoxin etoposide was shown to have particular cytotoxicity for cells of the monocyte/macrophage lineage. This agent showed promising results in children with refractory LCH and was subsequently studied in newly diagnosed patients.[142,143,144-146] More recently, the nucleoside analogs 2-chlorodeoxyadenosine (2-CdA)[147-150] and clofarabine[151,152] have shown promise in children with refractory LCH and in adults.[153] Regardless of the treatment regimen, survival among low-risk patients is excellent. However, a substantial proportion of high-risk patients still experience a progressive and fatal course despite therapy.[73,139,154,155]

The Deutsche Arbeitsgemeinschaft fur Leukamieforschung und Behandlung im Kindersalter (German Working Group for Research and Treatment of Leukemia in Childhood) performed two consecutive multicenter studies (known as DAL-HX 83 and DAL-HX 90) in which children with multifocal bone or MS-LCH were treated with a nonrandomized, risk-adapted, prospective, multidrug regimen. After initial therapy with prednisolone, vinblastine, and etoposide, children who had multisystem disease went on to receive 1 year of continuation therapy with prednisolone, vinblastine, etoposide, and mercaptopurine. In the DAL-HX 83 study, patients with organ dysfunction also received methotrexate. In these trials, approximately 67% of the patients who had organ dysfunction responded to therapy, with 42% of these patients experiencing subsequent disease reactivation. In contrast, the patients with multisystem disease who did not have organ dysfunction and the patients with multifocal bone disease had a roughly 90% response rate and a 20% reactivation rate.[71,156] Unfortunately, death due to refractory or progressive LCH occurred in about 20% of subjects in the DAL-HX studies. Mortality was restricted to children with disseminated disease, organ dysfunction, and an unfavorable response to initial therapy.

In 1991, The Histiocyte Society initiated LCH I, the first international, randomized clinical trial in persons with LCH. This study compared the efficacy of 6 months of vinblastine (arm A) versus etoposide (arm B) in children with multisystem disease.[142] The results showed that the two treatment arms, arms A and B, were equivalent in all respects, including response at week 6 (57% and 49%), toxicity (47% and 58%), probability of survival (76% and 83%), probability of disease reactivation (61% and 55%), and probability of developing permanent sequelae (39% and 51%), including DI (22% and 23%). Of the 29 children in the study who died as a result of their disease, all had risk organ involvement. The probability of survival among the children older than 2 years who did not have risk organ involvement was 100%. Secondary acute myeloid leukemia developed in one patient in arm B. In light of the fact that etoposide was not superior to vinblastine in any measure, the study suggested that the added risk associated with it may not be justified.

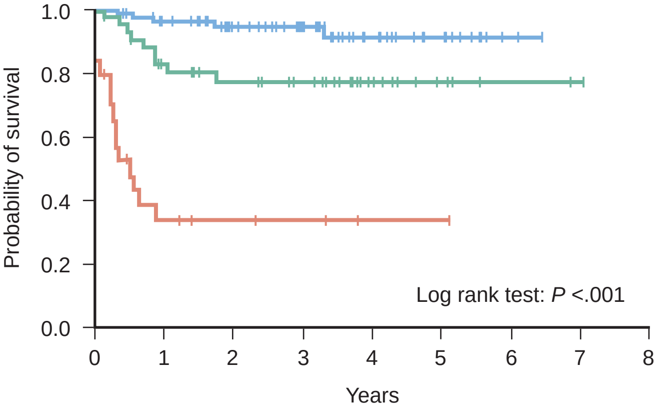

Figure 64-4 Results of the Langerhans cell histiocytosis (LCH) I clinical trial for the treatment of multisystem LCH in children. Survival related to response to treatment at week 6. The *solid line* represents responders ($n = 71/76$), the *dashed line* represents intermediate responders ($n = 32/41$), and the *dotted line* represents nonresponders ($n = 10/25$). *(From Gadner H, Grois N, Arico M, et al: A randomized trial of treatment for multisystem Langerhans' cell histiocytosis.* J Pediatr *138:728–734, 2001.)*

Importantly, analysis of the LCH I study revealed that early response to therapy is an extremely powerful prognostic indicator in persons with MS-LCH. Children younger than 2 years who had MS-LCH and failed to respond to 6 weeks of initial therapy had a dismal 17% survival rate compared with an 88% survival rate in children who responded favorably (Fig. 64-4). The prognostic value of early response was confirmed in a retrospective analysis of the DAL-HX 83 and DAL-HX 90 studies.[157]

The Histiocyte Society's successor study, LCH II, intensified treatment for all patients and further explored the role of etoposide in persons with MS-LCH. All children received 6 weeks of initial therapy that consisted of continuous oral prednisone and weekly administration of vinblastine. Low-risk children who were risk organ negative received continuation therapy with pulse prednisone and vinblastine every 3 weeks for a total of 6 months. High-risk children who were risk organ positive also received mercaptopurine and were randomly assigned to either receive etoposide (arm B) or not receive etoposide (arm A) during both the initial and continuation phases. LCH II did not demonstrate a statistically significant benefit of the addition of etoposide in patients with MS-LCH; response at week 6 (63% vs. 71%), survival (74% vs. 79%), disease reactivation (46% in both arms) and permanent consequences (43% vs. 37%) were all similar in arms A and B, respectively. However, in comparison with the less-intensive therapy studied in the LCH I trial, the more intensive LCH II regimen did show a somewhat higher rapid response rate and lower mortality in children with risk organ involvement.[158]

LCH III opened for enrollment in 2001. For the highest risk group of subjects in this study—that is, the children with MS-LCH who had involvement of risk organs—methotrexate was added to the backbone of prednisone, vinblastine, and mercaptopurine in a randomized fashion. The total duration of therapy was lengthened to 12 months for all high-risk patients. Children without risk organ involvement were randomly assigned to receive vinblastine and prednisone for 6 versus 12 months. The study failed to demonstrate a benefit as a result of the addition of methotrexate to the standard backbone in multisystem high-risk patients. However, prolongation of therapy from 6 to 12 months in multisystem low-risk patients is associated with a significantly lower reactivation rate (a 3-year cumulative incidence of reactivation of 50% vs. 35%).[159]

Refractory Langerhans Cell Histiocytosis

Children with MS-LCH whose disease does not respond promptly to vinblastine and prednisone have an unfavorable prognosis, with survival rates in the range of 10% to 34%.[142,156] Unfortunately, LCH that is resistant to vinblastine and corticosteroid is often unresponsive to other conventional agents. 2-CdA, a nucleoside analog, has shown significant promise as salvage therapy in children with reactivated or refractory disease.[147,160,161] Dramatic responses to 2-CdA combined with cytarabine have been observed in children with refractory LCH who have hematologic dysfunction.[150] Also, the nucleoside analog clofarabine has demonstrated activity in a smaller number of reported cases of refractory MS-LCH.[151,152]

A small but growing number of children with refractory MS-LCH have been successfully treated with allogeneic hematopoietic cell transplantation (HCT).[162-166] Not surprisingly, toxicity is a significant obstacle in these very ill patients. Autologous transplantation has not resulted in durable responses.[167] Transplant regimens that incorporate reduced intensity conditioning are especially appealing in persons with refractory LCH and appear to be feasible, with lower transplant-related morbidity and mortality.[168,169]

Reactivation

Some children experience LCH reactivation after initial improvement or resolution of their disease. Most reactivations occur within 1 year of diagnosis, and almost all develop within 2 years.[75,170] Patients with MS-LCH are clearly at higher risk of reactivation than are patients with SS-LCH. Patients occasionally experience a chronic, relapsing, and remitting course with repeated reactivations. Aggressive radiographic surveillance for reactivation is generally discouraged, because the site of reactivation is unpredictable and the risk of imaging may outweigh the benefit of early diagnosis. However, monitoring physical examination and growth and development is advised, along with prompt evaluation of any new signs or symptoms. Elevation of the erythrocyte sedimentation rate and the platelet count can be a tip-off to disease reactivation.[171]

Bone is the most frequent site of LCH reactivation. Fortunately, reactivation in risk organs is distinctly uncommon in children who did not have risk organ involvement at presentation.[172] Localized reactivations, such as a single bony site or recurrent rash, may be effectively managed with local therapy alone (i.e., surgery or intralesional or topical administration of a corticosteroid). However, systemic therapy is often necessary in persons with reactivated disease to control symptoms, prevent permanent sequelae, and decrease the severity and frequency of subsequent reactivations. In contrast to recurrent malignancies in which acquired drug resistance limits the efficacy of cytotoxic drugs after previous exposure, in reactivated cases of LCH, medications used successfully in the setting of newly diagnosed disease often remain effective in the treatment of reactivation. When cytotoxic therapies such as corticosteroids, vinca alkaloids, antimetabolites, or nucleoside analogues fail to provide durable disease control or when adverse effects are limiting, more intensive and less conventional options are worth exploring. For these relatively rare cases, therapy must be individualized, keeping in mind the specific site(s), symptoms, and risk of the reactivation, as well as the specific adverse effects of therapy.

Alternatives to conventional therapy that have been used in bone reactivation include nonsteroidal antiinflammatory agents (e.g., indomethacin)[106] and bisphosphonates (e.g., pamidronate).[173,174] These agents may act to ameliorate pain but may not directly inhibit disease activity. Thalidomide has shown some efficacy in patients with low-risk LCH that is refractory to conventional therapy. The presumed mechanism of action is by inhibition of TNF or other cytokines.[175] Many children who undergo repeated reactivations experience permanent disabilities related to their LCH. Nonetheless, their overall likelihood of survival approaches 100%.[76]

Long-Term Consequences

Roughly half of survivors of LCH experience irreversible long-term consequences of their illness or, to a lesser extent, of the therapy they received. The incidence and pattern of sequelae strongly correlate with the initial extent and pattern of disease involvement; patients who experience multisystem disease and multiple reactivations are at highest risk.[170,176,177] A considerable fraction of irreversible complications observed in survivors of LCH were present at the time of diagnosis, and thus not all long-term complications are preventable.

Orthopedic complications are the most frequently observed permanent sequelae in survivors of SS-LCH. Examples include spinal deformity leading to scoliosis or kyphosis, dental loss due to involvement of the jaw, and facial asymmetry or proptosis due to involvement of orbital or facial bones. Although conductive hearing loss attributed to chronic inflammation of the middle and external ear is reversible, sensorineural hearing loss due to involvement of osseous structures is usually permanent when it occurs. Although permanent orthopedic and cosmetic consequences are relatively frequent, they do not necessarily adversely affect the quality of survival.[76]

DI is an irreversible condition in almost all cases in which it occurs, and a lifelong need for desmopressin is to be expected. Similarly, other endocrinopathies such as growth hormone deficiency and panhypopituitarism are not responsive to LCH-directed therapy but are managed with hormone supplementation. Awareness and close attention to growth and development are very important in the follow-up care of these patients.[97]

Rarely, survivors of LCH experience progressive end organ damage in the absence of demonstrable activity of LCs. Progressive hepatic cirrhosis with liver failure requiring liver transplantation is a rare but well-recognized late event.[136] A somewhat analogous process may occur in the lungs, leading to pulmonary fibrosis, cystic changes, or spontaneous pneumothorax. Adult survivors of pediatric LCH should be counseled to avoid exposure to cigarette smoke because of the potentially increased risk of developing pulmonary abnormalities.[178] Neurodegeneration, which was previously described, is the most dreaded late event in survivors of LCH.

Overview of Therapeutic Options

Approach to SS-LCH

Children with localized SS-LCH have an excellent prognosis. Most children with localized disease may be safely observed after a biopsy or resection is performed, without the need for additional intervention. Local therapy may be added to accelerate resolution of symptoms. In patients with craniofacial bone or multifocal bone disease or isolated CNS involvement, systemic therapy is indicated to control symptoms and to prevent reactivations and long-term (especially orthopedic and neuroendocrine) sequelae.

Approach to Low-Risk MS-LCH

Children with MS-LCH who do not have involvement of risk organs have an excellent prognosis. Because their course is generally favorable, maximizing response while minimizing short- and long-term toxicities is the primary objective. When possible, enrollment in a clinical trial is encouraged. The current standard of care in the United States is the combination of vinblastine and prednisone for a total duration of 12 months.

Approach to High-Risk MS-LCH

Thus far, up-front intensification of therapy has not been shown to improve outcome in children with MS-LCH who have involvement of risk organs. Children with high-risk features who respond favorably to standard chemotherapy have a good prognosis, but children who do not respond rapidly to standard treatment have a poor prognosis. For this group of patients, intensive chemotherapy, investigational agents, or HCT should be considered.

The role of *BRAF* inhibitors in patients whose LCH cells harbor oncogenic *BRAF* variants remains to be established. The increased risk of squamous cell carcinomas of the skin associated with the currently available inhibitors will likely restrict their use to patients with a poor prognosis in whom the benefits may outweigh the risks.

NON–LANGERHANS CELL HISTIOCYTOSES

In addition to LCH, a spectrum of other even rarer histiocytic lesions exists that affect both children and adults. In general they include a large number of clinically benign, not infrequently progressive dermatologic disorders that are pathologically characterized by proliferations of Langerhans-like cells, often with distinctive cytosolic features[179,180] (see Fig. 64-1). Other rare non-LCHs, most notably Erdheim-Chester disease and the “true” malignancies such as Langerhans cell sarcoma and histiocytic sarcoma,[181] have high mortality rates. With the exception of JXG and sinus histiocytosis with massive lymphadenopathy (SHML), these disorders will not be discussed further because of their rarity, and the reader is referred to several authoritative reviews on these diseases.[179,180]

Juvenile Xanthogranuloma

JXG is a generally benign tumoral proliferation of histiocytes with a CD1a–, S100–, Langerin–, CD68+, CD163+, fascin+ immunophenotype that, like LCH, is believed to be derived from dermal interstitial DCs. Lesions characteristically present as reddish to yellowish-brown cutaneous nodules in the head and neck region of children younger than 1 year of age; the median age of onset is 5 months, and tumors may be congenital. Multifocality is not uncommon, particularly in male infants.[180,182] An association exists with neurofibromatosis 1, Noonan syndrome, and juvenile myelomonocytic leukemia.[183] The natural history of most lesions is spontaneous regression, but they are often diagnosed by excisional biopsy, leaving little or no residual tumor. Extranodal involvement of the deep soft tissues, liver, spleen, conjunctivae, and the CNS—so-called systemic JXG—with or without cutaneous involvement occurs in nearly 5% of cases.[182,184] Although systemic lesions also typically regress, local disease, as in the eye and CNS, may lead to significant morbidity. Patients with systemic disease involving the CNS or liver have occasionally died of their disease.[182,184,185] In cases requiring treatment, single agents[186,187] or multiagent regimens similar to those used for persons with LCH have been employed with success.[188]

Sinus Histiocytosis with Massive Lymphadenopathy

SHML, also known by the eponym Rosai-Dorfman disease,[189] is an enigmatic histiocytic disorder associated with a proliferation of CD1a–, Langerin–, S100+, CD68+, CD163+, fascin+ histiocytes with abundant foamy cytoplasm.[180] Most characteristically, a subset of lesional histiocytes contains a large number of lymphocytes and other cells, but unlike in the hemophagocytic syndromes (discussed later), these cells are not contained within phagolysosomes. Rather, they are “just passing through” in invaginations of the cell membrane, in a process termed *emperipolesis*[190] (see Fig. 64-1). In lymph nodes, these histiocytes preferentially involve the nodal sinuses and secondarily the interfollicular areas, leading to remarkably huge lymphadenopathy, which gives the disease its name.

Because the SHML literature is largely retrospective, particularly since 1990 when a patient registry was last updated,[191] the reported patterns of clinical presentation and natural history of the disease are undoubtedly biased. Nevertheless, the prototypical clinical presentation is massive, painless cervical lymphadenopathy. In addition, up to 40% of patients present with extranodal disease, most commonly in the skin, upper respiratory tract, and bone.[191] The orbit and meninges are also not uncommon extranodal sites.[192,193] Although extensive nodal and extranodal (especially kidney, lower airway, and liver) disease and coexisting immunologic disorders tend to correlate with an adverse prognosis, it is apparent that many if not most cases remit spontaneously without therapy.[191,194,195] Constitutional symptoms including fever associated with neutrophilia, increased erythrocyte sedimentation rate, and polyclonal hypergammaglobulinemia that are often associated with SHML usually respond to systemic corticosteroids. In cases in which disease has compromised organ function, requiring therapy, no treatment modality is clearly preferred; surgical debulking and radiation therapy generally result in some amelioration, but the response to systemic chemotherapy—often a combination of alkylators, vinca alkaloids, and steroids—is inconsistent.[191,196]

MONOCYTES AND MACROPHAGES

Resident tissue macrophages play essential roles in host defense, especially when they populate tissues that face the external environment (e.g., alveolar macrophages in the lung). However, macrophages also contribute to tissue homeostasis through their repair and clearance activities. Functional and morphologic heterogeneity among macrophages has been long recognized, and identification of specific subsets has been aided by antibody-based identification of characteristic cell surface markers.[197] Some of these subsets may be involved in the histiocytoses of macrophage origin.

Among the macrophages active in host defense and inflammation, alveolar and gut macrophages are best understood. These cells express pattern recognition receptors and scavenger receptors that enhance recognition of microbial products,[198] are highly phagocytic, and are efficient antigen presenters. However, even within this category some degree of heterogeneity exists because gut macrophages tend to secrete lower amounts of proinflammatory cytokines. Kupffer cells are derived from macrophages and may also play a primarily defensive role in the host. In contrast, macrophages involved in homeostasis include osteoclasts and microglia.

Macrophages in secondary lymphoid organs show additional degrees of heterogeneity, with specific cell types occupying characteristic anatomic niches.[198,199] In the spleen, red pulp macrophages, which express high levels of the murine surface marker F4/80, are distinguishable from tingible body macrophages in the white pulp. Also present in the white pulp are metallophilic macrophages, which align adjacent to the marginal sinus.[200] Within the marginal zone itself are functionally distinct macrophages that express pattern recognition and scavenger receptors they may use to clear pathogens from the bloodstream.[201-203] These macrophages express much lower levels of the F4/80 marker than do red pulp macrophages. Similar subanatomic heterogeneity is observed in lymph nodes: macrophages in cortical regions express low levels of F4/80, whereas macrophages in the medullary sinuses, paracortex, and subcapsular sinuses express high levels of F4/80.[204]

In the absence of specific stimuli, tissue macrophages remain in a low-functioning, resting state. Activation induces functions that are characteristic of their host protection or homeostasis roles. Based on in vitro analyses, four distinct pathways of macrophage activation have been described.[205-207] "Classical" activation via interferon-γ or lipopolysaccharide induces microbicidal activities and upregulation of major histocompatibility complex class II expression. "Alternative" activation via IL-4 or IL-13 induces expression of genes that are involved in tissue repair or suppression of inflammation. "Innate" activation via toll-like receptor ligands also induces microbicidal activities. Finally, "deactivation" via IL-10 or transforming growth factor–β stimulation reduces class II expression and increases secretion of antiinflammatory cytokines. As clearly defined as these pathways are in vitro, little evidence exists as yet that they are relevant in vivo. However, secretion of cytokines by activated macrophage-derived cells may be a major pathophysiologic factor in histiocytoses.[208] The first and third mechanisms lead to the production of what are sometimes called "M1" macrophages, whereas the second and fourth mechanisms produce "M2" macrophages.[209]

Origins of Macrophages

For the most part, steady-state renewal of tissue macrophages appears to be accomplished by local proliferation of similar cell types. However, under conditions of rapid turnover such as inflammatory states, macrophages are replaced by bone marrow precursors—that is, circulating monocytes.[210] Recent findings have revealed substantial heterogeneity among circulating monocytes and the roles played by specific subsets in macrophage development.

Broadly defined, circulating monocytes can be separated into "classical" or inflammatory cells versus "resident" cells, which have a phenotype that closely resembles tissue macrophages.[197,211] Cell surface markers can distinguish these subsets: inflammatory monocytes are CD14hi CD16− in humans and Ly6C+ in mice; resident monocytes are CD14+ CD16+ in humans and Ly6C− in mice. Experiments in mice have tracked the fate of some of these cells.[212] For example, bone marrow–derived Ly6C+ cells, which express high levels of the chemokine receptor CCR2 and low levels of the chemokine receptor CX3CR1, enter the circulation. Ordinarily, these cells are very short-lived, but in inflammatory states, they are attracted to affected sites where they can differentiate into macrophages or DCs. Meanwhile, the precise process whereby circulating monocytes replenish resident macrophages (which are CCR2−, CX3CR1hi, Ly6C−) is unknown. Nonetheless, some of the signals that dictate tissue-specific macrophage differentiation are understood. For example, circulating mononuclear precursors that localize to bone will become osteoclasts under the influence of m-CSF and receptor activator of nuclear factor kappa-B ligand,[213] which may have pathophysiologic relevance in histiocytic disorders that attack bone.

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

HLH is not a single disease entity but rather a syndrome consisting of clinical signs and laboratory abnormalities that result from the uncontrolled proliferation and activation of cells of the monocyte/macrophage lineage. Normally, monocytes and macrophages are responsible for phagocytosis of antigen and activation of other immune cells through the production of cytokines and chemokines. By way of their interactions with natural killer (NK) cells, macrophages are an important feature of the innate immune response. Additionally, they participate in adaptive immunity via their complex interactions with T cells. When pathogens, especially viral pathogens, are not rapidly eliminated by the innate immune response, the chemokines and cytokines produced by activated macrophages stimulate and perpetuate T-lymphocyte activation. Activated macrophages are themselves regulated by stimulatory and inhibitory factors that they and other immune cells produce. When this complex process escapes control, extremely potent and destructive inflammatory forces are unleashed. Uncontrolled macrophage activity is the hallmark of HLH. Although the pathogenetic mechanisms underlying the hyperinflammatory state in HLH are heterogeneous, the clinical consequences are shared.

The earliest published reports of hemophagocytic syndrome by Farquhar described four siblings who were afflicted in early infancy with fever, irritability, hepatosplenomegaly, and pancytopenia. Autopsy demonstrated histiocytic proliferation throughout the reticuloendothelial system with prominent hemophagocytosis.[214,215] The disorder was dubbed "familial haemophagocytic reticulosis" and was recognized as distinct from Letterer-Siwe disease (i.e., the disseminated form of LCH).[216] An autosomal-recessive pattern of inheritance was proposed. Subsequently, sporadic cases with similar clinical features were seen in association with viral infections,[217] bacterial infections,[218] and a host of other infections.[219] Concomitantly, it was observed that certain systemic illnesses, notably rheumatologic conditions and malignancies, occasionally led to the development of clinical features of hemophagocytic syndrome. In 1987, the Histiocyte Society adopted the unifying term *hemophagocytic lymphohistiocytosis* and defined a set of diagnostic criteria to assist clinicians and researchers. The criteria have subsequently been refined to account for advances in our understanding of the syndrome[220,221] (Box 64-2).

Incidence

HLH is broadly categorized into two forms: familial (FHL) and secondary (sHLH). Undoubtedly the delineation is an oversimplification, because significant clinical and perhaps even genetic overlap exists between the two forms. Importantly, FHL and sHLH may be indistinguishable at presentation.

Box 64-2 Diagnostic Guidelines

DIAGNOSTIC GUIDELINES (1994)*

CLINICAL CRITERIA

- Fever
- Splenomegaly

LABORATORY CRITERIA

- Cytopenias (hemoglobin <9 g/L; platelets <100 × 109/L; neutrophils <1.0 × 109/L)
- Hypertriglyceridemia and/or hypofibrinogenemia (fasting triglycerides ≥3 SD, fibrinogen ≤3 SD of normal for age)

HISTOPATHOLOGIC CRITERIA

- Hemophagocytosis in marrow, spleen, or lymph nodes
- No evidence of malignancy

REVISED DIAGNOSTIC GUIDELINES (2004)

Molecular diagnosis consistent with hemophagocytic lymphohistiocytosis OR five of the following eight clinical, laboratory, and histopathologic criteria

CLINICAL CRITERIA

- Fever
- Splenomegaly

LABORATORY CRITERIA

- Cytopenias (hemoglobin <9 g/L; platelets <100 × 109/L; neutrophils <1.0 × 109/L)
- Hypertriglyceridemia or hypofibrinogenemia (fasting triglycerides ≥3 standard deviations, fibrinogen ≤3 standard deviations of normal for age)
- Hyperferritinemia (>500 µg/L)
- Elevated CD25 (≥2400 U/L)
- Low/absent natural killer function

HISTOPATHOLOGIC CRITERIA

- Hemophagocytosis in marrow, spleen, or lymph nodes with no evidence of malignancy

Data from Henter JI, Horne A, Arico M, et al: HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer *48:124–131, 2007; and Henter JI, Elinder G, Ost A: Diagnostic guidelines for hemophagocytic lymphohistiocytosis. The FHL Study Group of the Histiocyte Society.* Semin Oncol *18:29–33, 1991.*

*All criteria required. If hemophagocytic activity is not proven at presentation, further search is encouraged.

In persons with FHL, an ordinary immune stimulus, such as a common viral exposure, triggers an unrestrained hyperinflammatory state as a result of the existence of an inherited, intrinsic defect in the patient's immunologic effector function. FHL is genetically heterogeneous, resulting from mutations that disturb the function of one or more of several proteins that participate in lymphocyte cytotoxicity. Approximately 1 in 50,000 live births are estimated to be affected by FHL.[222] A slight preponderance of males may be attributable to the occurrence of FHL in association with X-linked lymphoproliferative disorders.

In contrast, sHLH develops as a consequence of an intense immunologic stimulus in the form of an infection, malignancy, or inflammatory process in a person without an inherited immune defect. sHLH is more prevalent than

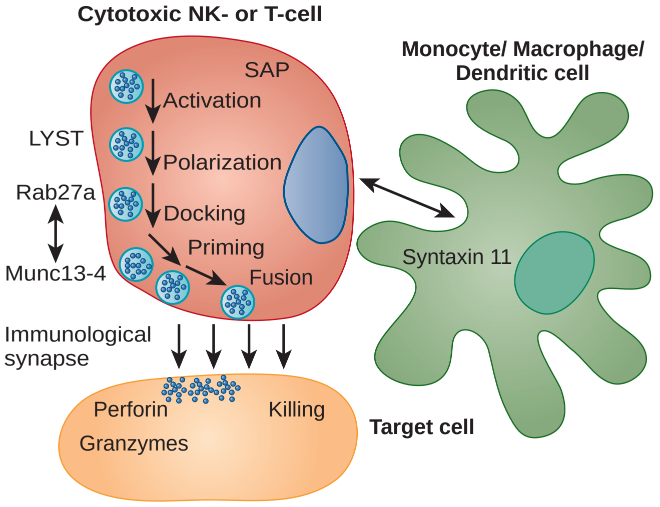

Figure 64-5 Mechanics of cytotoxic function revealed by hemophagocytic lymphohistiocytosis (HLH)-associated gene mutations. HLH-associated genetic abnormalities (in the indicated genes) may affect granule-dependent lymphocyte cytotoxicity by impairing trafficking, docking, priming for exocytosis, or membrane fusion of cytolytic granules. The function of this pathway may also be severely impaired by loss of functional perforin, the key delivery molecule for proapoptotic granzymes. Diverse mutations in this pathway all give rise to similar clinical phenotypes (albeit of variable severity). *LYST* (the gene affected in Chédiak-Higashi syndrome) is not portrayed because its function is not entirely clear, although it appears to play an important role in the maintenance of normally sized (and functional) cytolytic granules. *(From Jordan MB, Allen CE, Weitzman S, et al: How I treat hemophagocytic lymphohistiocytosis.* Blood *118:4041–4052, 2011.)*

FHL,[223] but its true incidence in children and adults is not defined.

Clinical Features

The cardinal clinical features of HLH are persistent high fever, cytopenias, and splenomegaly, with or without hepatomegaly. Unquestionably, this constellation of signs and symptoms is not rare or specific. Furthermore, establishing the diagnosis is made more difficult by the fact that all clinical features of the disorder may not be initially or simultaneously present (Fig. 64-5).

Persistent and prolonged fever is essentially universal and is usually the first sign of illness. Signs of a routine childhood upper respiratory or gastrointestinal illness often accompany the fever. However, instead of resolving in the typical time course, constitutional symptoms generally progress in children who have HLH. At presentation, affected patients are usually acutely ill and require urgent evaluation and intervention.

Cytopenias that affect at least two cell lines are a key feature of HLH. Thrombocytopenia is almost always present. The platelet count may be normal or mildly depressed initially, but it often falls precipitously as the disease progresses. Normocytic anemia with reticulocytopenia is also common. In cytopenic patients, less than the expected increment may be seen in response to transfusions because of shortened survival of transfused blood cells. Leukopenia and neutropenia are more variably present.

Abdominal distention and hepatosplenomegaly are usually present and may be accompanied by jaundice and ascites. Biochemical evidence of liver dysfunction is typical, although it is not a universal feature of HLH. Hypertriglyceridemia,[224,225] increased transaminases, or hyperbilirubinemia may be present and may in a very young child raise the possibility of primary liver disease or a metabolic disorder. Impaired hepatic synthetic function and clotting factor consumption leads to hypofibrinogenemia and prolongation of the prothrombin and partial thromboplastin times. When coagulopathy is combined with thrombocytopenia, the risk of bleeding escalates.[226] Spontaneous hemorrhage, especially intracranially, may lead to acute decompensation and may raise an erroneous suspicion of child abuse.[227]

Typically, the serum ferritin level is markedly elevated in patients who have active HLH.[228,229] A serum ferritin level exceeding 10,000 μg/L is a highly sensitive and specific marker that supports the diagnosis.[230,231] However, although most patients with HLH have an increased ferritin level, not all patients demonstrate extreme hyperferritinemia. Furthermore, mild to moderate elevation in the serum ferritin level may be seen in disorders other than HLH, such as inflammatory conditions, infections, liver disease, hemochromatosis, and metabolic disorders. The precise mechanism of ferritin elevation in persons with HLH is unknown, but it is probably related both to increased synthesis of ferritin mediated by high levels of cytokines[232] and increased release of ferritin due to liver injury and red blood cell turnover.

In keeping with a state of generalized, uncontrolled inflammation, the levels of various cytokines are increased in patients with active HLH, including IL-2, IL-6, IL-8, IL-10, interferon, and TNF.[233-235] Cytokine levels are not routinely measured, but the soluble IL-2 receptor (also known as CD25) is available as a clinical laboratory test. CD25 elevation is a sensitive and reliable marker of HLH activity,[236] and it has been incorporated into the diagnostic criteria for HLH.[220] CD25 levels are also elevated in other disorders, especially lymphoid malignancies, but typically to a lesser degree.

The term *hemophagocytosis* describes the pathologic finding of activated, histologically benign macrophages engulfing erythrocytes, leukocytes, platelets, and their precursor cells (Fig. 64-6). This process is often assessed in the bone marrow, but it occurs throughout the reticuloendothelial system (i.e., in the liver, spleen, and lymph nodes) and sometimes in the CNS. Destruction of blood cells and their precursors leads to peripheral blood cytopenia(s), often in association with bone marrow hypercellularity. Analysis of the bone marrow is always recommended when a diagnosis of HLH is suspected, both to demonstrate hemophagocytosis and to rule out the presence of leukemia/lymphoma. Although the finding of hemophagocytosis in the bone marrow supports the diagnosis of HLH in the proper clinical context, it is not essential for the diagnosis or pathognomonic. In a series of 122 children with HLH from the Histiocyte Society's International Registry, only 75% had evidence of hemophagocytosis at diagnosis.[237] In some cases, repeated bone marrow analyses may be necessary to document hemophagocytosis. Neither the pathophysiologic basis for hemophagocytosis nor its precise mechanism is well

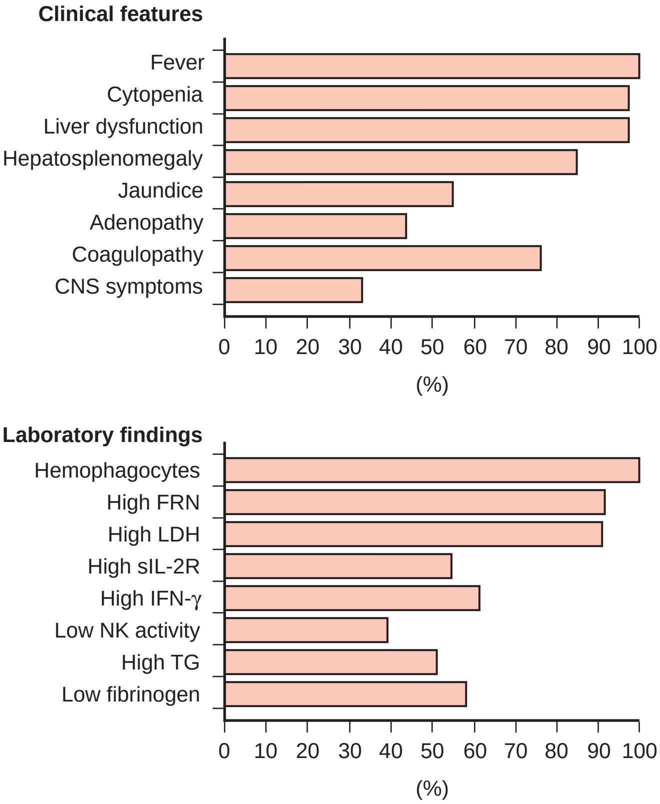

Figure 64-6 Incidence of clinical features and abnormal laboratory findings in 82 cases of hemophagocytic lymphohistiocytosis. *CNS,* Central nervous system; *FRN,* ferritin (normal range 8 to 78 μg/L); *IFN-γ,* interferon gamma; *LDH,* lactate dehydrogenase (normal range, 234 to 471 IU/L); *NK,* natural killer; *sIL-2R,* CD25 (normal range, <1090 U/mL); *TG,* triglycerides.[328]

understood. In HLH, hemophagocytosis is a reactive process that presumably results from the dysregulated immune response.

Despite the hyperactivity of the immune response in HLH and the presence of normal numbers of effector cells in many cases, targeted killing by these cells is depressed or absent. In the laboratory, impaired effector function may be demonstrated with use of a standard chromium release assay against K562 target cells. In the setting of active HLH of any variety, the functional activity of NK cells is usually impaired.[238] Manipulation of the conditions of the NK cell functional assay by the addition of mitogen or IL-2 or prolonged incubation times results in restoration of NK cell function in some patient samples.[239] It appears that the inability to reconstitute NK functional activity in vitro using modified assay conditions is associated with the familial form of the disease.[240] Some degree of correlation exists between the pattern of NK functional impairment and the molecular abnormality present in patients with FHL.[241,242]

Normally, when stimulated cytotoxic T cells and NK cells undergo exocytosis of lytic granules, expression of CD107a is induced on the cell surface, where it can be detected by flow cytometry. In a very high proportion of patients with most forms of FHL, CD107a degranulation is abnormal. Importantly, however, the assay is normal in the majority of patients with sHLH and in patients with FHL that is related to perforin deficiency (FHL2) and XLP. The CD107a degranulation assay, combined with flow cytometry for perforin and SAP and XIAP in males, may allow for rapid identification of patients with FHL.[243]

Evaluation for infectious agents should be undertaken as part of the initial workup in patients with the clinical features of HLH. Bacterial cultures and tests for other pathogens such as Epstein-Barr virus (EBV), cytomegalovirus, herpes simplex virus, respiratory syncytial virus, adenovirus, fungi, and protozoa should be performed on the basis of the patient's symptoms and exposure history. Identifying and treating infection when possible is a very important part of managing the condition of these critically ill patients. However, evidence of a bacterial, viral, or other infection in a patient with HLH does not discriminate between the familial and acquired forms of the disease. In fact, patients with FHL commonly present after an identifiable infectious trigger.[237] Conversely, a specific infectious trigger may not be identified in all patients who turn out to have infection-associated hemophagocytic syndrome. The possibility of a secondary infection must be assessed and managed conservatively in these patients, who are likely to be febrile and neutropenic and may be hemodynamically unstable.

A range of CNS symptoms including irritability, lethargy, seizures, and focal deficits may be seen. Analysis of the CSF may show pleocytosis (especially with activated monocytes present) or elevated CSF protein. Evidence of hemophagocytosis is present in a minority of cases. MRI of the brain may demonstrate a wide range of abnormalities.[244,245] CNS manifestations including neurologic symptoms, abnormal CSF, or both are reported in up to 63% of children at diagnosis,[246] and they develop in most if not all cases of advanced FHL.[247,248] Occasionally, neurologic symptoms represent the most conspicuous manifestation of illness, obscuring and delaying the correct diagnosis of HLH.[249] Pathologically, CNS involvement is variable, with meningeal inflammation at the mild end and multifocal brain infiltration with necrosis at the severe end of the clinical spectrum.[250] CNS involvement occurs in both FHL and sHLH.[251]

Familial Hemophagocytic Lymphohistiocytosis

FHL is predominantly a disease of early childhood. Although congenital cases do occur, most affected infants are born healthy and grow normally until the onset of symptoms in the first few months of life, often in association with an otherwise routine childhood infection. The peak age at diagnosis is 1 to 6 months, and it is estimated that approximately 70% are diagnosed within the first year of life. However, the age range at diagnosis is wide. FHL has been reported in patients beyond the fourth decade of life.[252-254]

As previously described, familial inheritance was recognized in the earliest descriptions of the clinical phenotype, and an autosomal-recessive pattern of inheritance

TABLE 64-1 Familial Hemophagocytic Lymphohistiocytosis Genes

Disease	Locus	Gene	Gene Symbol	Syndromic Associations
FHL1	9q22.1-23	Unknown	Unknown	None
FHL2	10q22	Perforin	*PRF1*	None
FHL3	17q25	Mammalian homologue of *Caenorhabditis elegans* (Unc13)	*UNC13D*	None
FHL4	6q24	Syntaxin-11	*STX11*	None
FHL5	19p13	Syntaxin binding protein 2	*STXBP2*	None
Griscelli syndrome type 2	15q21	RAS-associated protein (RAB27A)	*RAB27A*	Hypopigmentation, +/– neurodegeneration
Lysinuric protein intolerance	14q11.2	Solute carrier family 7, member 7	*SLC7A7*	Protein intolerance, malabsorption, failure to thrive
Chédiak-Higashi syndrome	1q42.1-42.2	Lysosomal trafficking regulator	*LYST*	Partial albinism, giant lysosomes/melanosomes, neutropenia, susceptibility to infection
XLP1	Xq25	Signaling lymphocyte activation molecule (SLAM)-associated protein (SAP)	*SH2D1A*	Sensitivity to Epstein-Barr virus infection, hypogammaglobulinemia, lymphoma
XLP2	Xq25	X-linked inhibitor of apoptosis	*XIAP*	Splenomegaly, hypogammaglobulinemia

was inferred for many cases.[255] It is evident that the familial forms of HLH can be subdivided into nonsyndromic forms, in which the only or predominant clinical phenotype is a predisposition to hemophagocytic syndrome, and syndromic forms, in which hemophagocytosis is only one, often inconstant phenotype present in association with other systemic abnormalities, particularly pigmentation defects or immune deficiency (Table 64-1).

Early on, genetic linkage studies performed with large nonsyndromic FHL families indicated substantial genetic heterogeneity with potential linkage to several different loci, systematically named FHL1 to FHL5. FHL1, which was among the first to be mapped, continues to defy molecular characterization.[256] However, the genes responsible for FHL2, FHL3, FHL4, and FHL5 have been cloned and have led to remarkable insights into the pathogenesis of this group of disorders. Each encodes a component required for development or trafficking of a mature, fully capacitated cytotoxic lymphocyte or NK cell cytotoxic granule.

FHL2 is due to mutations in the gene that encodes perforin (*PRF1*).[257,258] Perforin is a component of T-lymphocyte and NK cell cytotoxic granules that is homologous to the pore-forming complement component C9, and like its homologue, it facilitates lysis of target cells by creating holes in cell membranes.[259] FHL2-associated mutations may lead to a complete lack of protein or a defect in protein maturation/transport, leading to varying degrees of perforin deficiency and lymphocyte-mediated cytotoxicity.[258,260]

Whereas FHL2 is attributed to primary defects in the effector function of cell-mediated toxicity itself, FHL3, FHL4, and FHL5 are attributed to mutations in protein components of the trafficking machinery required for exocytosis of cytotoxic granules. Prior to exocytosis, vesicles must associate or "dock" with the membrane, a process facilitated by soluble NSF-attachment proteins (SNAP) on the vesicular compartment surface and SNAP receptors (SNAREs) on the target membrane (Fig. 64-7). Mutations in the gene that encodes the putative vesicular SNAP, syntaxin-11 (STX11), are responsible for FHL4.[261] A mammalian homologue of the *Caenorhabditis elegans* protein UNC13, known as UNC13D (also known as MUNC13-4), is mutated in patients with FHL3.[262,263] UNC13 family members are thought to participate in preparing vesicles docked at the cell surface for fusion with the membrane—a process called "priming."[264,265] The binding of STX11 with STXBP2 regulates granule docking and initiation of SNARE complex formation. Mutations in STXBP2, also known as MUN18-2, are associated with FHL5, which is also often associated with colitis and hypogammaglobulinemia.[266-268] Thus it would appear that STX11, STXBP2, and UNC13D are necessary to deliver *PRF1* extracellularly.[269] In toto, then, the deficiency of NK cell function in patients with FHL can be ascribed to a defect in the primary cytotoxicity effector pathway, leading to poor clearance of target cells and a

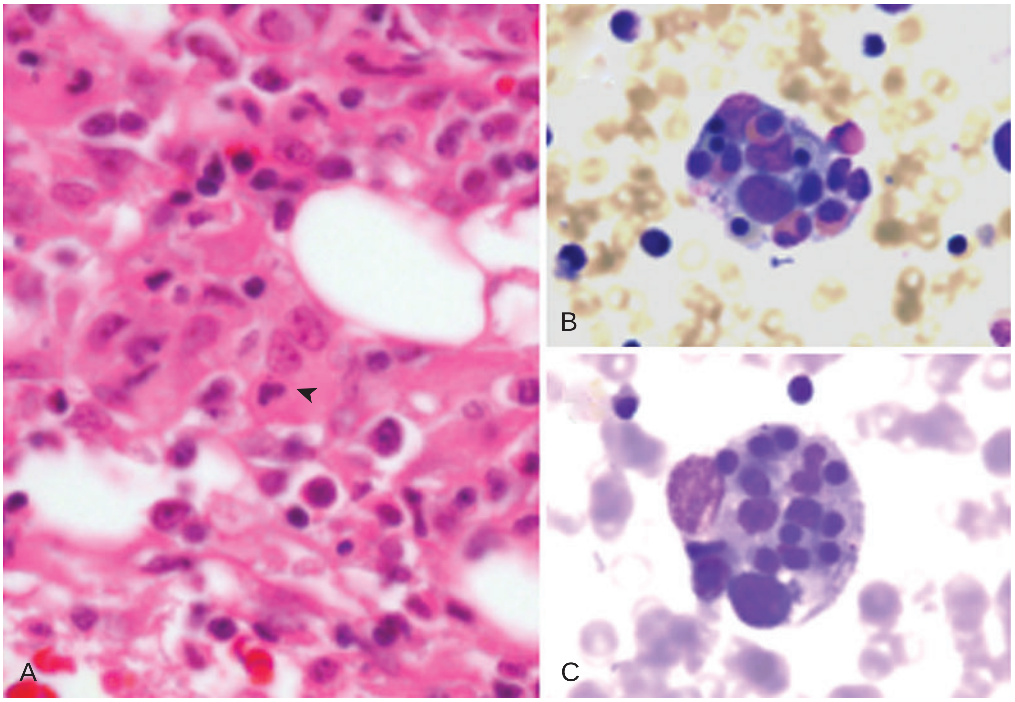

Figure 64-7 Histopathologic findings of hemophagocytic lymphohistiocytosis. **A,** A hematoxylin and eosin–stained bone marrow biopsy section (400× magnification) showing marrow infiltration by benign-appearing macrophages. A macrophage containing a phagocytosed neutrophil is indicated by the *arrowhead*. **B** and **C** show individual macrophages from a Wright-Giemsa–stained bone marrow aspirate smear (1000× magnification) that contain phagocytosed marrow elements, including mature eosinophils and erythroid precursors in **B** and numerous erythroid precursors and a band neutrophil in **C**.

continued state of immune activation, which is characteristic of the hemophagocytic syndromes.

Whereas the absolute frequency of disease-causing mutations in each of these genes varies greatly from publication to publication and population to population, it is evident that up to 80% of *molecularly defined* cases of FHL are attributable to mutations in *PRF1*, *UNC13D*, or *STXBP2*. *STX11* mutations are rare.[263,270-272] Despite the recent progress in the understanding of the genetic basis of FHL, a subset of presumptive FHL cases evade genetic characterization.

HLH Associated with Other Genetic Syndromes

HLH may occur in association with other inherited anomalies, including Chédiak-Higashi syndrome,[273] Griscelli syndrome type 2 (GS2),[274,275] lysinuric protein intolerance (LPI),[276] and X-linked lymphoproliferative syndrome (XLP1 and XLP2).[277,278] In these patients, hemophagocytosis is a variable feature, and it is not the sole manifestation of the disease. These disorders are diverse, with each disorder resulting from defects in distinct genes (Table 64-1).

Not coincidentally, the proteins encoded by the genes involved in several of these disorders each play a role in the process of cellular cytotoxicity. The GS2 protein labeled RAB27A, which is a small guanosine triphosphatase of the RAS-associated family, directly associates with the FHL3 protein labeled UNC13 and is similarly thought to regulate exocytosis.[279] Chédiak-Higashi syndrome, like GS2, gives rise to pigmentation defects and is also due to mutations in a protein, LYST, that is involved in vesicular trafficking. Patients with XLP1 as a result of mutations in the Src homology 2 domain–containing gene 1A (*SH2D1A*), which functions as a lymphocyte signaling adapter, have a multifactorial humoral and cellular immune deficiency most saliently characterized by susceptibility to EBV infection, lymphoproliferative disorders, and dysgammaglobulinemia (see Chapter 24).[280] Defects in NK cell and T-cell development and function, including cytotoxicity, appear to predispose patients with XLP1 to HLH, particularly in the setting of primary EBV infection. How abnormalities in dibasic amino acid transport[281] should result in HLH is completely obscure; however, several persons with LPI have presented with HLH.[276]

Secondary Hemophagocytic Lymphohistiocytosis

Infection-Associated HLH

Virus-associated hemophagocytic syndrome was first described in a report of 19 adult patients, 14 of whom were immunocompromised.[217] Subsequently, it was shown that this disorder also may develop in immunocompetent persons. Although viruses, especially EBV and other members of the herpes virus group, are the most commonly identified infectious triggers, a host of other infectious agents have been implicated, including bacteria,[218] fungi, and protozoa (e.g., leishmania).[282] In a review of the 219 cases of infection-associated hemophagocytic syndrome reported in the medical literature between 1979 and 1995, 55% were attributed to EBV.[39] The clinical features of infection-associated HLH are identical to FHL, although the median age of onset is higher; about 50% of cases occurred in children younger than 3 years and 18% of cases occurred in children younger than 1 year. An interesting but as yet unexplained fact is that about half of reported cases are in people of Asian ancestry.

Malignancy-Associated HLH

HLH occurs rarely in association with a malignant neoplasm in children, and in these rare instances it is most frequently associated with T-cell malignancies. The association presents in two uncommon but somewhat distinct forms: during treatment for a known cancer in clinical remission (e.g., acute lymphoblastic leukemia)[283] or as a manifestation of an aggressive uncontrolled cancer that may be masked (e.g., T-cell or NK-cell leukemia/lymphoma). In the first case, the clinical features of HLH arise unexpectedly in a patient undergoing cancer chemotherapy, presumably as a result of iatrogenic immune dysregulation. An infectious trigger may or may not be identified.[284] In the latter case, which occurs more commonly in adults, the hemophagocytic syndrome is a feature of the hematologic malignancy itself.[285,286] It is proposed that the malignant clone potentiates a state of hypercytokinemia that results in the clinical signs and symptoms of HLH. Interestingly, some of these cancers are associated with EBV infection. The majority of the reported cases are in persons of Asian descent.

Macrophage Activation Syndrome

Macrophage activation syndrome (MAS) is a life-threatening complication of systemic inflammatory conditions that is characterized by all of the clinical symptoms and laboratory findings of HLH, leading to its classification as a form of sHLH. The syndrome was first described in 1985 in a report of seven children with systemic onset juvenile idiopathic arthritis (S-JIA).[287] Rheumatologic conditions other than JIA, such as systemic lupus erythematosus, may also be associated with the syndrome in both children and adults. Typically the syndrome develops years after the diagnosis, but it may occur early on or even concomitantly with the diagnosis of the rheumatologic disease.[288] With the onset of MAS, the chronically ill child may experience unremitting fever, a decrease in blood cell counts, and organomegaly. Interestingly, the erythrocyte sedimentation rate may decrease concomitant with the development of MAS. Flare-ups of the underlying disease, infection, and medication adverse effects not only mimic MAS but might also trigger the events.[289,290] Ravelli[290] proposed MAS diagnostic guidelines after examining clinical and laboratory characteristics of patients with active S-JIA compared with S-JIA–associated MAS. According to the guidelines, the diagnosis of MAS rests on the presence of two or more laboratory criteria (platelets $\leq 262 \times 10^9$/dL; aspartate aminotransferase >59 U/L; white blood cell count $\leq 4.0 \times 10^9$/dL; fibrinogen ≤ 2.5 g/L) or any two or more laboratory or clinical criteria (e.g., CNS dysfunction, hemorrhages, or hepatomegaly).[291] Distinguishing MAS from FHL and forms of sHLH can also be a challenge.[292]

It has been suggested that inherited defect(s) in one or more of the genes associated with FHL might confer predisposition to the development of MAS in the setting of rheumatologic disease or, moreover, to the development of the rheumatologic condition itself.[293] A review of 133 children with S-JIA and 384 matched unrelated control subjects failed to demonstrate an increased incidence of detectable abnormalities in the genes encoding perforin, MUNC13D, Granzyme B, or RAB27A.[294]

Recently, a form of sHLH has been observed in some children and adults treated with T-cell activating therapies using blinatumomab[295] and chimeric antigen receptor bearing T cells[296] for refractory leukemia. The cytokine release syndrome that occurs in this context may be successfully managed with corticosteroid and the IL-6 receptor antagonist tocilizumab. Remarkably, the development of cytokine release syndrome is associated with antileukemia efficacy in treated patients.

Management

The development of specific and effective therapy for HLH is hampered by the fact that affected patients demonstrate common clinical features despite variable underlying pathophysiologic conditions. Additionally, there may be little time to wait for diagnostic clarity in affected patients. Whether the patient has FHL or sHLH, the first objective of therapy is to cool down an overheated immune response. At the same time or later, the underlying basis of the patient's disease process must be determined and addressed.

Without therapy, FHL is rapidly and invariably fatal, usually within a month or two of presentation. A comprehensive literature review performed prior to the discovery of effective chemotherapy showed that only 12% of children survived 6 months and just 3 of 101 children survived long term.[297] sHLH is also a life-threatening condition, although the outcome is much more variable. It is estimated that without therapy, the mortality rate for EBV-associated HLH is approximately 50%.[39]

In 1980, Ambruso and colleagues[298] first reported the successful treatment of two infants who had presumed FHL with etoposide (VP-16). This report was an enormous step forward because no effective therapy for the disease had previously existed.[299] However, disease control proved to be transient in these and subsequent patients, with the CNS being a major site of treatment failure. To address the problem of CNS disease, cranial irradiation or intrathecal methotrexate was added to the combination of VP-16 and corticosteroid.[300] Systemic and CNS relapses remained a significant problem and few, if any, patients survived long term. In most of the reported cases, patients who received maintenance therapy with etoposide eventually died of refractory disease within 1 to 3 years.[300,301] Teniposide, also an epipodophyllotoxin, has been used in some patients who have HLH with roughly equivalent results.[302]

In 1986, the first successful matched sibling donor bone marrow transplant in a boy with FHL was described.[303] HCT was quickly recognized as the sole potentially curative modality for this group of patients who previously had no real possibility of cure. However, allogeneic transplantation, which is always a high-risk procedure, is particularly toxic in children with HLH. Control of the clinical signs of HLH prior to transplantation significantly improves the likelihood of a successful outcome.[304,305] Additionally, absence of CNS involvement and infection are favorable indicators.[237] These factors drive the preference for early transplantation, which should be performed as soon as possible after the achievement of disease control and prior to the onset of CNS manifestations or the acquisition of an opportunistic infection.

Initial Therapy

To standardize, improve, and study the treatment of this rare disease, the Histiocyte Society sponsored an international clinical trial that was named HLH-94.[306] This study and its successor, HLH-2004, defined a set of clear diagnostic criteria and laid out a nonrandomized treatment strategy that combined chemotherapy (etoposide, glucocorticoids, and intrathecal methotrexate in select cases), immunotherapy (cyclosporine A), and HCT (when possible in familial, refractory, or relapsing cases). The HLH-94 study enrolled 249 eligible children between 1994 and 2003. Because it is impossible to differentiate FHL from sHLH at onset in many cases, confirmation of familial disease was not required, and therefore both familial and secondary cases were included. Overall, 86% percent of the children responded to 8 weeks of initial therapy, approximately 60% of whom achieved nonactive disease. Factors associated with early death included marked hyperferritinemia, hyperbilirubinemia, and CSF pleocytosis at diagnosis and persistent fever, thrombocytopenia, and hyperferritinemia after 2 weeks of treatment.[307] Half of the patients taking HLH-94 underwent transplantation, with a median time between onset of therapy and HCT of 6 months. Overall survival at 5 years among the 124 patients who underwent HCT was 66% versus 54% for the entire study population. No patients with verified FHL survived without HCT.[307]

The combination of antithymocyte globulin (ATG), corticosteroid, and cyclosporine A (CSA) is an alternative to HLH-94 chemoimmunotherapy, although experience with it is more limited. Among 38 children with confirmed FHL who received one or two courses of rabbit ATG, complete resolution was achieved in 73% and partial improvement was noted in 24%. This finding includes responses seen among 10 children who had received prior immunosuppressive treatment (including steroids, CSA, or chemotherapy). All of the patients received maintenance therapy with CSA. Sixteen of 19 patients who proceeded to receive an allogeneic transplant after being treated with ATG as first-line therapy are long-term survivors. All patients who did not undergo transplantation died. The therapy was relatively well tolerated, although infusional and infectious complications were not trivial.[308,309]

Newer agents with inhibitory effects on T cells and cytokine production are being developed. These approaches are attractive because they may directly interfere with the hyperinflammation that is responsible for the clinical features of HLH without the adverse effects of cytotoxic chemotherapy. Alemtuzumab is a therapeutic monoclonal antibody that is directed against CD52, an antigen expressed on lymphocytes, NK cells, monocytes, and macrophages. Alemtuzumab seems to be effective as a bridge to transplant in patients with refractory HLH,[310] and it has been incorporated in reduced-intensity conditioning regimens in patients undergoing HCT for HLH.[310]

As stated previously, the CNS is not infrequently involved in children with HLH at diagnosis, and it is a major site of treatment failure in patients with advanced FHL. Prompt and aggressive initiation of systemic chemoimmunotherapy, followed by allogeneic stem cell transplant in familial, refractory, and relapsing cases, is the best strategy for minimizing the potential for CNS damage.[248] The role of CNS-directed therapy is not well established. Intrathecal methotrexate and cranial irradiation have been used in the context of systemic therapy. In the HLH-94 study, intrathecal methotrexate was reserved for patients in whom neurologic symptoms progressed or CSF abnormalities did not resolve after 2 weeks of systemic treatment. Interestingly, the same fraction of children (10 of 15) experienced normalization of neurologic symptoms by 2 months, whether or not they received intrathecal therapy. Intrathecal methotrexate does not appear to prevent the development of CNS manifestations in patients who do not undergo transplantation. Furthermore, intensive intrathecal methotrexate is not sufficient to reverse CNS manifestations in children with advanced FHL.[248] Cranial irradiation is now rarely used because of the lack of established efficacy and the potential long-term toxicities in these very young patients.

Management of Secondary HLH

HLH that is associated with EBV infection is the most common and best-studied type of infection-associated hemophagocytic syndrome, and it is the prototype of sHLH. Compared with children with infectious mononucleosis, children with EBV-HLH have higher EBV genome copy numbers as measured by quantitative polymerase chain reaction. The EBV copy number quickly decreases with the initiation of effective therapy and can be used as a marker of disease activity in these patients.[311] The role of antiviral agents (e.g., acyclovir) is not well established but is probably of limited benefit during the phase of active viral replication. Importantly, the need for immunosuppressive therapy is well established in these patients. The efficacy of HLH-94-based immunochemotherapy has been clearly demonstrated, yielding a survival rate of up to 90% in patients who are treated promptly.[312] Because of the potential short-term (myelosuppression) and long-term (secondary myelodysplastic syndrome/acute myelogenous leukemia) toxicities linked to etoposide,[313] its role has been specifically evaluated. Mortality was 14 times higher for patients with EBV-HLH who did not receive etoposide within the first 4 weeks after diagnosis.[314] The risk associated with a relatively short course of etoposide appears to be justified when weighed against the high risks of morbidity and mortality in these patients and the knowledge that delayed treatment yields inferior outcomes. In a series of 78 patients with EBV-HLH, most of whom were treated according to the HLH-94 study protocol, 75% remain disease free, with a median follow-up of 43 months. Reactivations occurred in approximately 20% of cases, and many of these patients were eventually cured by HCT.[315]

A complementary therapeutic strategy for EBV-HLH is the use of B-cell directed therapy, which reduces the burden of EBV-infected B cells. Two young men known to have XLP and who were diagnosed with acute infectious mononucleosis were treated with rituximab (anti-CD20 monoclonal antibody) in combination with corticosteroids and responded favorably.[316] Recently, a retrospective review of 42 patients with EBV-HLH, some of whom proved to have FHL, demonstrated that rituximab in combination with conventional HLH-directed therapy is well tolerated and leads to improvement in HLH disease manifestations and a reduction in the EBV viral load.[317]

When sHLH occurs in association with visceral leishmaniasis, a very uncommon infection in the United States, treatment of the underlying infection with amphotericin B is usually lifesaving.[282]

Treatment of HLH and MAS that is associated with malignancy must be individualized. When an active malignancy is responsible for the hemophagocytic syndrome, administration of tumor-directed cancer chemotherapy is the best treatment choice. The clinical features of HLH can be expected to resolve if the triggering cancer is controlled. For the very rare patients in whom HLH develops while they are receiving immunosuppressive therapy for a cancer that is in remission, the best strategy is not known.[318] Discontinuation of immunosuppressive therapy is beneficial in some cases but is not always possible. Treatment with corticosteroids and etoposide may be indicated. When standard therapy for HLH fails, HCT should be considered.

MAS and rheumatologic disease-associated HLH fall into a clinical spectrum within which there are no generally accepted distinctions or definitions. MAS is a term that arose in the rheumatology literature and has become more or less synonymous with reactive HLH. It is likely that many patients in this disease spectrum do not come to the attention of pediatric hematologists/oncologists because their cases are managed by pediatric or adult rheumatologists. Some differences in approach exist between the two groups of specialists. High-dose, parenteral, pulsed corticosteroids are usually used in the initial management of MAS. CSA may be effective when corticosteroids lack efficacy.[319] More recently, inhibitors of TNF—etanercept[320] and infliximab[321]—have been used successfully in patients with MAS that is refractory to steroids and cyclosporine.

In patients in whom HLH resolves completely and for whom a cause of the syndrome is not determined (i.e., they lack a positive family history or an identified genetic defect), HCT is not indicated. Very close monitoring in the period after cessation of therapy is warranted. Patients who remain well are presumed to have had sHLH. In the subset of patients who manifest signs of disease reactivation, chemoimmunotherapy should be promptly reinstituted to control the disease prior to HCT.

Supportive Care

Some of the improvement in prognosis observed in the past decades in persons with HLH is attributable to advances in supportive care. New onset and uncontrolled cases of HLH are typically characterized by the presence of multiple organ system dysfunction, including immunologic compromise, pancytopenia, hemorrhagic tendency, and hepatic and neurologic dysfunction. Hemodynamic instability, respiratory insufficiency, renal dysfunction, and electrolyte imbalance may be present. Immunosuppressive and myelosuppressive chemotherapy in these critically ill patients magnifies their risk for serious infection. A significant fraction of children with HLH succumb to opportunistic infection. Empiric antibiotics for febrile neutropenia and pneumocystis, along with fungal prophylaxis and intravenous immunoglobulin repletion, are suggested. These interventions—along with other supportive care measures, including aggressive blood product support—contribute substantially to the likelihood and quality of survival.

Hematopoietic Cell Transplantation

Multiple studies have demonstrated the necessity for an allogeneic transplant in the management of FHL. In the International Registry report of 122 children with HLH, HCT was performed in 29 children, for whom the 5-year survival after HCT was 66%. Most of the donors were fully matched or mismatched family donors, and only three children received transplants from unrelated donors. Among patients who did not undergo transplantation, the 5-year survival rate was only 10%, with just three children surviving long term.[237] Because only a minority of the patients received HCT, the 5-year event-free survival for the entire cohort of 122 patients was just 21%.

The fact that only approximately 20% of children in need of an allogeneic stem cell transplant have an available matched family donor accounts in part for the poor overall survival among patients in the International Registry. Aside from human leukocyte antigen compatibility, another limitation to the applicability of matched sibling donor transplantation in a genetic disorder such as HLH is the possibility that the sibling donor is at risk for developing the disease. These factors, combined with the knowledge that FHL is invariably fatal without transplantation, has led to the increased use of unrelated and alternative donors.[305,322,323] The 3-year overall survival rate was 44% among 16 children with HLH who underwent transplantation with marrow from unrelated donors. Among the 65 children on the HLH-94 protocol who underwent transplantation, approximately half of whom received marrow from an unrelated donor, the 3-year probability of survival was 62%. The impact of expanded access to HCT by increased use of alternative donors (among other factors) was borne out in the relatively high overall survival rate of 55% at 3 years in the HLH-94 study. Disease remission at the time of transplantation is associated with a superior outcome after HCT.[322] However, HCT should not be viewed as futile in a child

TABLE 64-2 Comparison of Langerhans Cell Histiocytosis and Hemophagocytic Lymphohistiocytosis

	LANGERHANS CELL HISTIOCYTOSIS		**HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS**	
	Single System	**Multisystem**	**Primary**	**Secondary**
Pathologic cell	Dendritic cell		Macrophage	
Histologic features	Clonal Langerhans cell proliferation (CD1a+, Langerin+) Mixed cellular infiltrate		Reactive macrophages (CD68+) hemophagocytosis	
Typical age at onset	Childhood	Infancy	Infancy	Childhood
Inheritance	Sporadic	Sporadic	Autosomal recessive	Sporadic
Clinical presentation	Bone pain/swelling Skin rash	Bone pain/swelling Skin rash, FTT, DI	Fever, hepatosplenomegaly, irritability, jaundice	
Laboratory/ radiographic features	Laboratory tests usually normal/lytic bone lesions	Cytopenia(s), liver dysfunction, bone lesion(s)	Cytopenias, ↑ferritin, ↓fibrinogen, ↑triglycerides, ↓NK function, ↑soluble CD25, abnormal CSF	
Treatment	Observation, vinblastine/steroids	Vinblastine/steroids	Etoposide/steroid/ CSA, HCT	Etoposide/steroid/CSA
Prognosis	Excellent	Variable Fatal in ≈25%	Fatal without HCT, Survival ≈50% with HCT	Variable Fatal in ≈20%

CD, Cluster of differentiation; *CSA,* cyclosporin A; *CSF,* cerebrospinal fluid; *DI,* diabetes insipidus; *FTT,* failure to thrive; *HCT,* hematopoietic cell transplantation; *NK,* natural killer.

with active disease, even involving the CNS, because many children with active disease at the time of HCT have done well.[324] Despite high early mortality rates, late reactivation of disease is very uncommon in patients who have undergone allogeneic HCT for FHL.[324]

The most commonly used myeloablative conditioning regimen for HCT in patients with HLH is the combination of busulfan, cyclophosphamide, etoposide, and ATG. Transplant-related mortality is very high with this approach; roughly one third of recipients of transplants die after myeloablative conditioning within the first few months as a result of infection, hepatopathy, pneumonitis, or uncontrolled disease.[325] Reduced-intensity conditioning is increasingly recommended in this setting because it is associated with a significantly higher likelihood of survival.[168,326] Establishment of stable, long-term donor chimerism is an issue, and donor lymphocyte infusion is indicated in many cases. For this reason, reduced-intensity conditioning is not suitable for patients for whom the only acceptable donor source is umbilical cord blood.

CONCLUSIONS

HLH is an acute, rapidly progressive, potentially life-threatening syndrome. The clinical features are the consequence of hyperinflammation and immune dysregulation, but the underlying mechanisms are diverse. Prompt recognition and initiation of therapy is warranted, even when diagnostic certainty is elusive. Initial therapy with etoposide and a corticosteroid with or without the addition of CSA is the standard of care in the United States. Analysis for defects in the genes known to be associated with the condition should be undertaken, although not all familial cases will have a confirmatory genetic test. Assessment for triggers of secondary HLH should be pursued, but identification of an infectious trigger does not reliably differentiate FHL and sHLH. Children who have FHL based on a positive family history, the finding of a genetic abnormality, or a refractory or relapsing course should be referred for allogeneic hematopoietic stem cell transplant as soon as possible. Children not known to have FHL based on these criteria may be observed if they achieve disease resolution after 2 to 3 months of initial therapy. A comparison of some of the cardinal features of LCH and HLH is shown in Table 64-2.

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CHAPTER 65

Rare Tumors of Childhood

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CHAPTER OUTLINE

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This chapter covers the rare tumors of childhood. No attempt has been made to consider the rarest of the rare tumors, and the focus is on the more common of the rare pediatric tumors that do not fall into other categories covered in this text. The chapter is generally organized from top to bottom, with cancers of the head and neck at the beginning and cancers of the gastrointestinal tract discussed later in the chapter. The tumors discussed afflict mostly adults, and some are actually common cancers in older patients. Many are carcinomas and are more familiar to the oncologist who treats adults. These cancers tend to increase in incidence as children reach adolescence.

Evaluation of Surveillance, Epidemiology, and End Results (SEER) data has shown that 9.2% of all childhood cancers are carcinoma, and 75% of these cases are diagnosed in children between the ages of 15 and 19 years.[1] Of the cases in the study, 36% were thyroid carcinomas and 31% were melanomas. The next most common single carcinoma in children younger than age 19 years was nasopharyngeal carcinoma, which comprised 4.5% of these cases. Thus this chapter emphasizes discussion of melanoma, thyroid carcinoma, and nasopharyngeal carcinoma and reviews other rare tumors of childhood.

OROPHARYNGEAL CANCER

Oropharyngeal cancer of squamous cell histology in children remains uncommon.[2] Most of the children who develop epithelial cancer of the nose, ears, and throat are diagnosed with nasopharyngeal carcinoma, which is considered separately in this chapter. The use of smokeless tobacco, cigarette smoking, and alcohol consumption are well established risk factors for the development of squamous cell carcinoma in adults, and the continued use of these substances in Western children warrants monitoring for the development of adult types of squamous cell carcinoma of the oropharynx in children. Although such habits ultimately lead to increased risk for a variety of cancers and early death from heart disease, it remains a theoretical concern that the use of smokeless tobacco by children, particularly by male adolescents, could increase the incidence of these tumors during adolescence and young adulthood.

Duration of smoking increases risk for tobacco-related morbidity and mortality, including several cancers, and significant public health efforts have been aimed at reducing adolescent initiation of tobacco use.[3] Educational interventions have been shown to decrease the prevalence of cigarette and smokeless tobacco use in Massachusetts youth,[4] but restrictions on tobacco sales have a mixed record, because even a complete ban on sales of smokeless tobacco in Finland did not lead to a decrease in its use by adolescents.[5] Conversely, restrictions on smoking in public places have been demonstrated to decrease overall tobacco consumption (e.g., by up to 8% in Italy).[6] Overall trends of cigarette smoking among adolescents have decreased steadily since the mid-1990s. Rates of smoking for white, non-Hispanic twelfth grade students are significantly higher in comparison to Hispanic and African-American students.[3,7,8] Although smokeless tobacco use also declined during the late 1990s and early 2000s, recent data from Monitoring the Future and the National Youth Tobacco Survey have demonstrated increased use in recent years, with concurrent decreased reporting of perceived risk. These changes in trends are synchronous with aggressive marketing campaigns by tobacco companies of novel delivery forms of smokeless tobacco such as “snus” and dissolvable tobacco.[7,9]

The increasing prevalence of human papillomavirus (HPV) infection in adolescents and young adults has raised new concerns about the development of HPV-associated oral squamous-cell carcinoma in younger patients. HPV has recently been detected in approximately 26% of head and neck squamous-cell carcinomas; HPV16 was highly prevalent among these tumors.[10] Trends in adults show an increasing incidence in oral squamous-cell carcinomas despite declining use of tobacco in the United States because of the increasing prevalence of oral HPV infection.[11,12] A recent study showed 1.7% of 14- to 17-year-old adolescents and 5.6% of 18- to 24-year-old adults had oral HPV; male sex, number of sexual partners, tobacco use, open-mouth kissing, and oral sex have been associated with infection.[13,14] HPV is most often transmitted in adolescence and young adulthood; a quadrivalent vaccine for HPV that includes strains likely to cause oral cancers is now available and is recommended for both boys and girls.[15,16]

Few existing studies report outcomes of pediatric squamous-cell carcinomas. A population-based study of 54 pediatric patients with oral-cavity squamous-cell carcinoma had a 5-year disease-specific survival rate of 75%.[17] It is unlikely that many pediatric oncologists will be confronted by adult-type squamous-cell carcinoma of the head and neck, but it is likely that pediatric oncologists will be confronted with tobacco use in young adult and adolescent survivors of pediatric cancer. A St. Jude Children’s Research Hospital study has found that 29% of surviving children treated on an acute myelogenous leukemia (AML) protocol were found to be cigarette smokers at follow-up examinations.[18] Although survivors of childhood cancer may not be seen often in the pediatric oncology clinic, tobacco use and prevention still merit mention during such visits.

Mucoepidermoid carcinoma of the salivary glands is still another type of head and neck carcinoma from which children suffer and is commonly associated with prior therapy for cancer, including radiation.[19] A study of childhood cancer survivors identified 23 cases of secondary head and neck malignancies; of the 14 secondary cancers that arose in the parotid gland, 10 were identified as mucoepidermoid carcinoma.[20] Most children are cured with surgery and sometimes surgery and radiation therapy. Any mass lesion or presenting complaint potentially related to a mass lesion in the salivary glands, particularly if found in a previous radiation field, should prompt a thorough evaluation with suspicion for this tumor.

NASOPHARYNGEAL CARCINOMA

Nasopharyngeal carcinoma is a cancer of the epithelial lining of the nasopharynx. It shows varying degrees of differentiation but is a type of squamous-cell carcinoma. Most cases have undifferentiated histology; the undifferentiated type is associated with Epstein-Barr virus (EBV) infection of the nasopharyngeal epithelium, and virus is found in the tumor cells.[21] It is the most common type of epithelial tumor of the head and neck in children, accounting for up to 50% of head and neck tumors in children. Incidence varies widely among populations and in relationship to exposure to EBV, environmental exposures such as preserved or salted fish, and geographic location. Rates in China are particularly high, and ethnic Chinese born in China who move to the West have a higher incidence of the disease than ethnic Chinese who are born in Western countries.[21] In the United States, this cancer is more common in the South and more prevalent in African-American children, who have incidence rates of the disease that approach those of the Chinese. These findings suggest interplay of genetic, viral, and environmental factors in the carcinogenesis of nasopharyngeal carcinoma.

Analysis of incidence and survival rates from SEER data has demonstrated improved survival for Asian-American patients compared to Caucasian and African-American patients. However, nasopharyngeal carcinoma-specific mortality is worse for adults than for children and adolescents, and survival rates in the adolescent and young-adult age ranges tend to be similar among races.[22-25]

Epidemiology

Nasopharyngeal carcinoma is more common in Asia and is strongly associated with Chinese ethnic origin within Asia, with some parts of China having incidence rates from 15 to 30 cases per 100,000 population.[21] There is also a higher incidence of the cancer in Turkey.[26,27] A bimodal age distribution has been suggested in nonendemic areas, with an early peak in adolescents age 15 to 19 years.[28] The actual cancer cells are infected by EBV, suggesting a possible role for immunosuppression in the pathogenesis of the tumor, or at least that immune augmentation could help prevent recurrence of nasopharyngeal carcinoma.[21] In Greenland and other regions with similar native populations, high rates of nasopharyngeal carcinoma have been found. Additionally, high rates for other cancers associated with EBV infection, such as various forms of uterine cancer, are more common in families with EBV and nasopharyngeal carcinoma.[29,30] Population-based screening based on EBV serology in endemic areas can help detect nasopharyngeal carcinoma early. However, this would be impractical in nonendemic areas.

Presentation

The typical presenting signs and symptoms of nasopharyngeal carcinoma include evidence of tumor mass in the nasopharynx such as epistaxis, nasal obstruction and discharge, Eustachian tube dysfunction, palsy of the fifth and sixth nerves relating to skull-based extension, and neck masses.[21] In children with epidemiologic factors making this disease more likely, clinicians must maintain a high index of suspicion for nasopharyngeal carcinoma and obtain imaging studies and consultation as necessary for these signs and symptoms. However, most children diagnosed with nasopharyngeal carcinoma in the United States are unlikely to have obvious epidemiologic associations for the cancer, and even in the United States EBV infection in childhood is common enough that screening based on evidence of EBV infection would be impractical.

Staging and Evaluation

Proper evaluation of patients suspected of having nasopharyngeal carcinoma includes computed tomography (CT) scan and/or magnetic resonance imaging (MRI) of the head and biopsy of appropriate tissue for diagnosis. A clinical examination is key to evaluate for any apparent lymph node spread. Although systemic evaluation, including laboratory testing, positron emission tomography (PET) scanning, whole-body anatomic scanning, and bone marrow biopsy can be considered, they have not been shown to improve staging accuracy in patients with nasopharyngeal carcinoma.[21]

All nasopharyngeal carcinoma cases are considered squamous-cell cancers, but the World Health Organization (WHO) classification of nasopharyngeal carcinoma defines type 1 histology as keratinizing squamous-cell carcinoma, a form of cancer more common in adults, versus the type 2 histology, which does not show keratinization on light microscopy but does show some differentiation. Type 3 histology is nonkeratinized and nondifferentiated and is associated with endemic nasopharyngeal carcinoma.[21] The majority of children with nasopharyngeal carcinoma have a presentation of undifferentiated or WHO type 2 or 3 histology.[23]

The American Joint Commission on Cancer (AJCC) tumor-nodes-metastasis (TNM) staging system (2010) is considered the most relevant for patients in the Western countries. The WHO staging system has fewer local (T) staging categories but seems to be adequate for patients in endemic areas.[21,31] The latest AJCC staging system is shown in Tables 65-1 and 65-2.

TABLE 65-1 Tumor-Nodes-Metastasis Definitions for Nasopharyngeal Carcinoma*

Primary Tumor (T)	Regional Lymph Nodes (N)	Distant Metastasis (M)
TX: Primary tumor cannot be assessed T0: No evidence of primary tumor Tis: Carcinoma in situ T1: Tumor confined to the nasopharynx, or tumor extends to oropharynx and/or nasal cavity without parapharyngeal extension† T2: Tumor with parapharyngeal extension† T3: Tumor involves bony structures of skull base and/or paranasal sinuses T4: Tumor with intracranial extension and/or involvement of cranial nerves, hypopharynx, orbit, or with extension to the infratemporal fossa/masticator space	NX: Regional lymph nodes cannot be assessed N0: No regional lymph node metastasis N1: Unilateral metastasis in cervical lymph node(s), ≤6 cm in greatest dimension; above the supraclavicular fossa and/or unilateral or bilateral; retropharyngeal nodes, ≤6cm in greatest dimension‡ N2: Bilateral metastasis in cervical lymph node(s), ≤6 cm in greatest dimension; above the supraclavicular fossa‡ N3: Metastasis in a lymph node(s)‡ >6 cm and/or to supraclavicular fossa N3a: >6 cm in dimension N3b: Extension to the supraclavicular fossa§	M0: No distant metastasis M1: Distant metastasis

From Edge SB, Byrd DR, Compton CC et al, editors: AJCC: pharynx. In: AJCC cancer staging manual, *ed 7, New York, 2010, Springer, p. 41–56.*

*The distribution and prognostic impact of regional lymph-node spread from nasopharynx cancer, particularly of the undifferentiated type, are different from those of other head and neck mucosal cancers and justify the use of a different regional lymph-node classification scheme.

†Parapharyngeal extension denotes posterolateral infiltration of tumor.

‡Midline nodes are considered ipsilateral nodes.

§Supraclavicular zone or fossa is relevant to the staging of nasopharyngeal carcinoma and is the triangular region originally described by Ho. It is defined by three points: 1) the superior margin of the sternal end of the clavicle; 2) the superior margin of the lateral end of the clavicle; and 3) the point where the neck meets the shoulder. Note that this would include caudal portions of levels IV and VB. All cases with lymph nodes (whole or part) in the fossa are considered N3b.

TABLE 65-2 American Joint Committee on Cancer Stage Groupings for Nasopharyngeal Carcinoma*

Stage	Groupings
0	Tis, N0, M0
I	T1, N0, M0
II	T1, N1, M0 T2, N0, M0 T2, N1, M0
III	T1, N2, M0 T2, N2, M0 T3, N0, M0 T3, N1, M0 T3, N2, M0
IVA	T4, N0, M0 T4, N1, M0 T4, N2, M0
IVB	Any T, N3, M0
IVC	Any T, any N, M1

From Edge SB, Byrd DR, Compton CC et al, editors: AJCC: pharynx. In: AJCC cancer staging manual, *ed 7, New York, 2010, Springer, p. 41–56.*

*Results of radiation therapy for nasopharyngeal carcinoma (locoregional control and survival) are usually reported by T stage and N stage separately or by specific T and N subgroupings rather than by numerical stages I to IV. Outcome also depends on various biologic and technical factors related to treatment.

Treatment

Although nasopharyngeal carcinoma can be treated by radiation therapy only, it has been shown to be sensitive to chemotherapy in adults and children. Using chemotherapy may ultimately allow for curative radiotherapy at lower doses with less risk for late effects from head and neck radiotherapy.[21,24,26-27,32] A Pediatric Oncology Group (POG) study used four cycles of preirradiation chemotherapy with methotrexate with leucovorin, 5-fluorouracil (5-FU), and cisplatin to treat children with AJCC stage III or IV nasopharyngeal carcinoma. Children with stage I or II disease were treated with irradiation only. The 4-year event-free and overall survival rates were 77% ± 12% and 75% ± 12%, respectively.[24] Other agents used to treat nasopharyngeal carcinoma include bleomycin and doxorubicin.[27] More recently, the use of neoadjuvant chemotherapy with concomitant chemoradiation has demonstrated improved outcomes.[21,26-27] Xerostomia and dental problems are common adverse effects of treatment. Other long-term morbidities include sensorineural hearing loss, endocrinopathies, trismus, and recurrent infections.[33] Sensorineural hearing loss incidence has been noted to increase with the use of concomitant chemoradiotherapy.[34] Although both children and adults with nasopharyngeal carcinoma are at risk of developing secondary malignancies after treatment, the risk for children is 4.3 times the general population, compared with 1.4 for adults.[23] Current treatment strategies are investigating the use of intensity-modulated radiation therapy (IMRT) and amifostine to preserve salivary function[35,36] and to decrease acute and chronic sequelae of therapy.

THYROID CARCINOMA

This section summarizes the clinical-pathologic and therapeutic aspects of differentiated thyroid carcinoma in children and adolescents. Medullary thyroid carcinoma (MTC) and associated multiple endocrine neoplasia (MEN) syndromes are not discussed.

Epidemiology

The prevalence of palpable thyroid nodules in children is lower than that seen in adults (1.5% vs. 5%); despite this, thyroid nodules in children are five times more likely to be malignant than those in adults (25% vs. 5%).[37-39] Of the estimated 56,000 cases of thyroid cancer diagnosed in 2012, only 1.8% occur in patients under 20 years of age.[40] Furthermore, thyroid carcinoma accounts for 3% of all cancers in patients younger than 20 years, and 75% of these occur in patients between the ages of 15 and 19 years (Fig. 65-1).[41] Epidemiological studies suggest that the incidence of thyroid carcinoma has steadily increased over time and that it affects both low and high socioeconomic counties that were surveyed by the SEER registries.[42,43] In pediatrics, an increasing rate has been documented in the SEER registry for the years 1973 through 2007, with the rates being higher in whites, those aged between 15 and 19 years, girls, and in registries with predominantly white or Hispanic populations.[44]

Radioactive exposure to the neck is a well-established risk factor for the development of thyroid carcinoma.[45] Survivors of childhood cancer have up to a 14-fold excess risk of developing thyroid cancer if their thyroid gland received 20 to 25 Gy, and this malignancy accounts for 10% of all subsequent neoplasm in the survivor population.[46,47] A risk-prediction model for subsequent primary thyroid carcinoma in survivors of childhood cancer has been developed.[48] This model incorporates several variables including radiation and therapy information, age, sex, and a history of a thyroid nodule; the software to compute risk projections can be downloaded at http://dceg.cancer.gov/tools/riskassessment.[48]

Exposure to radioactive iodine and cesium isotopes after the Chernobyl accident resulted in a markedly increased rate of thyroid cancer in children; the estimated relative excess risk was estimated to be 5.6 per Gy of exposure in subjects with estimated doses of less than 1 Gy.[49-51] Other risk factors associated with the development of pediatric thyroid cancer include various genetic syndromes such as familial adenomatous polyposis (FAP), Cowden disease, Carney syndrome, and immune thyroid disorders such as Hashimoto thyroiditis.[37,52-54]

Pathology and Molecular Pathology

More than 90% of pediatric thyroid carcinomas have well-differentiated histology; papillary histology accounts for over 70% of cases, and follicular histology represents about 20% of cases. Medullary thyroid cancer is seen in fewer than 10% of pediatric cases of thyroid cancer and can be found in association with MEN syndrome types

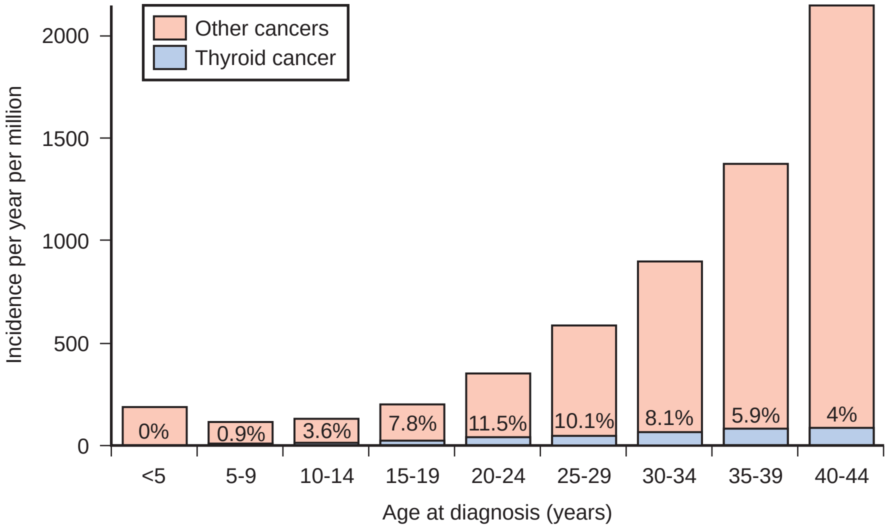

Figure 65-1 Incidence of thyroid carcinoma relative to all cancers, SEER 1975-2000. *(Data from Bleyer A, O'Leary M, Barr R, Ries LAG, editors:* Cancer epidemiology in older adolescents and young adults 15 to 29 years of age, including SEER incidence and survival: 1975-2000 [NIH Publication No. 06-5767]. *Bethesda, Md., 2006, National Cancer Institute.)*

2A and 2B.[55] The distribution of histologies varies slightly in cases with known radiation carcinogenesis; in a study of 740 children with thyroid cancer, of whom 92% were exposed to radiation at Chernobyl, 90% of cases were papillary, about 5% were follicular, and 0.4% were medullary.[56]

RET-PTC rearrangements have been documented in 50% to 60% of pediatric cases of papillary thyroid carcinoma and in up to 70% of radiation-induced pediatric papillary thyroid carcinoma.[54,57] *NTRK1* rearrangements and *AKAP9-BRAF* fusions have also been described in a small number of radiation-induced papillary tumors.[58-60] Unlike in adults, *BRAF* mutations are rarely seen in pediatric papillary tumors.[61-62] Follicular tumors are characterized by *RAS* mutations and peroxisome proliferator–activated receptor γ (PPARγ) rearrangements, whereas MEN-associated medullary thyroid carcinomas are characterized by germline-activating mutations of *RET*.[54,63]

Clinical Presentation and Staging

Most patients with thyroid carcinoma are older than 10 years and initially have an asymptomatic palpable thyroid nodule.[64] Younger age (<15 years), large fixed nodules, palpable adenopathy, history of radiation exposure, and diseases associated with an increased incidence of thyroid carcinoma such as FAP, Carney complex, Cowden syndrome, MEN types 2A or 2B, pheochromocytoma, and hyperparathyroidism should raise the suspicion for the presence of malignancy.

Children and adolescents with thyroid carcinoma are more likely to have nodal regional involvement, extrathyroid disease, and pulmonary metastases and are less likely to die from their disease than adults.[64] Ultrasound is a useful diagnostic technique, and most investigators prefer this method for evaluating thyroid nodules (Fig. 65-2).[37,64] Initial evaluation of a thyroid nodule should include measurements of serum thyroid-stimulating hormone (TSH), antithyroid peroxidase, antithyroid globulin antibodies, and a neck ultrasound that evaluates the contralateral thyroid lobe and cervical nodes.[39] Screening for MTC using plasma calcitonin may not be cost-effective in children given its low prevalence in the pediatric population.[37,39,65] If the TSH level is low, raising the possibility of a hyperfunctional nodule, a thyroid technetium or iodine scan should be performed. Because of the higher likelihood of malignancy in children, it is recommended that all hyperfunctioning nodules be surgically removed in children, with further evaluation and treatment should the pathology be consistent with malignant disease.[65,66] Fine-needle aspiration biopsy is the most cost-effective and expeditious preoperative procedure to help distinguish benign from malignant thyroid nodules; a meta-analysis of 12 pediatric studies found that this procedure has a sensitivity of 94% and a specificity of 81% (see Fig. 65-2).[37,67] Preoperative staging should include a chest x-ray and a comprehensive neck ultrasound to evaluate the whole thyroid and lymph nodes.[64]

Prognostic Factors

Factors associated with an increased risk of progression include capsular invasion, soft-tissue invasion, positive margins of resection, age younger than 10 to 15 years, male sex, nonpapillary histology, distant metastases, and residual disease after surgery.[68,69,70] Survival for children with differentiated thyroid carcinoma approaches 100% (Fig. 65-3).

Treatment and Outcome

Surgery

Surgery is the mainstay of therapy for pediatric and adolescent thyroid carcinoma, and this procedure should be performed by a highly experienced thyroid surgeon.[64] Total thyroidectomy is the initial treatment of choice. especially in younger patients (<10 years) or those with a history of radiation exposure or a family history of

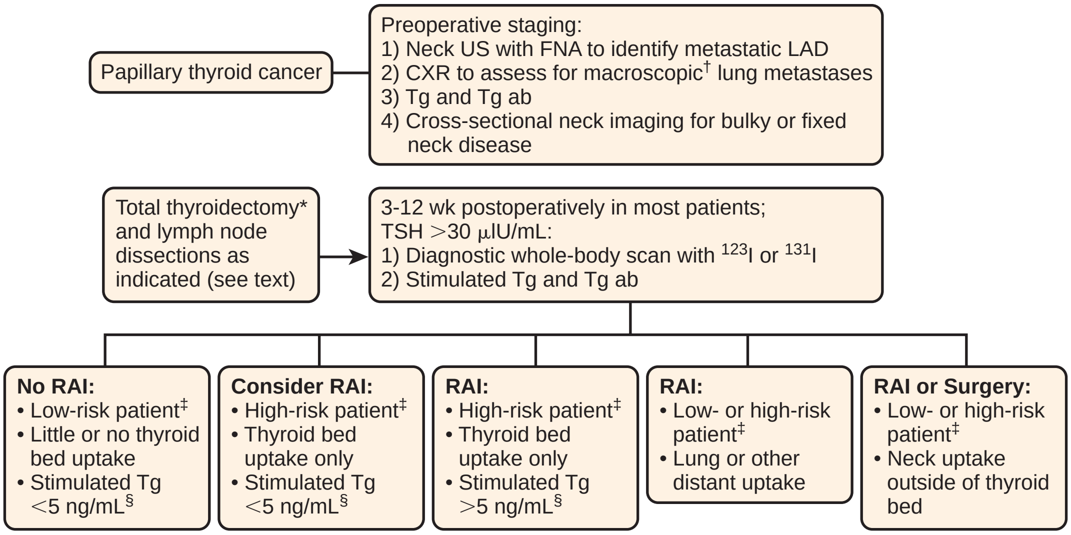
Papillary thyroid cancer
Preoperative staging:
1) Neck US with FNA to identify metastatic LAD
2) CXR to assess for macroscopic† lung metastases
3) Tg and Tg ab
4) Cross-sectional neck imaging for bulky or fixed neck disease
Total thyroidectomy* and lymph node dissections as indicated (see text)
3-12 wk postoperatively in most patients; TSH >30 μIU/mL:
1) Diagnostic whole-body scan with 123I or 131I
2) Stimulated Tg and Tg ab
No RAI:
• Low-risk patient‡
• Little or no thyroid bed uptake
• Stimulated Tg <5 ng/mL§
Consider RAI:
• High-risk patient‡
• Thyroid bed uptake only
• Stimulated Tg <5 ng/mL§
RAI:
• High-risk patient‡
• Thyroid bed uptake only
• Stimulated Tg >5 ng/mL§
RAI:
• Low- or high-risk patient‡
• Lung or other distant uptake
RAI or Surgery:
• Low- or high-risk patient‡
• Neck uptake outside of thyroid bed

Figure 65-2 Initial evaluation and treatment of papillary thyroid cancer in children. *CXR,* Chest radiography; *FNA,* fine-needle aspiration; *LAD,* lymphadenopathy; *RAI,* radioactive iodine; *Tg,* thyroglobulin; *Tg ab,* thyroglobulin antibody; *TSH,* thyroid-stimulating hormone; *US,* ultrasound. *Rare cases where lobectomy may suffice. †Macroscopic lesions are considered to be larger than 1 cm. ‡Low-risk: primary tumor does not grossly invade the trachea, recurrent laryngeal nerve, esophagus, or other vital structures; nonbulky lymph node presentation; no evidence of distant metastatic disease. High risk: any of the previous features present. §Assumes negative Tg ab. ¶RAI if no macroscopic disease on US; surgery if macroscopic disease on US. *(From Waguespack SG: Initial management and follow-up of differentiated thyroid cancer in children.* J Natl Compr Canc Netw *8:1289–1300, 2010.)*

thyroid cancer.[64] Many surgeons also advocate the upfront use of this procedure because pediatric thyroid carcinoma is associated with a high incidence of multifocal and metastatic disease, and follow-up screening with serum thyroglobulin is most useful after total thyroidectomy.[64,66] Lobectomy and isthmectomy can be considered in patients who have a low risk for recurrence such as adolescents with localized small (<1 cm) lesions.[64,71-73]

Lymph Node Dissection

Upfront nodal dissection has been shown to decrease the risk of surgical reintervention and nodal recurrence in children with thyroid carcinoma.[56,74] Current recommendations favor the use of central compartment neck dissection, reserving less extensive surgeries for patients with small tumors and no evidence of nodal involvement by ultrasound (see Fig 65-2).[64]

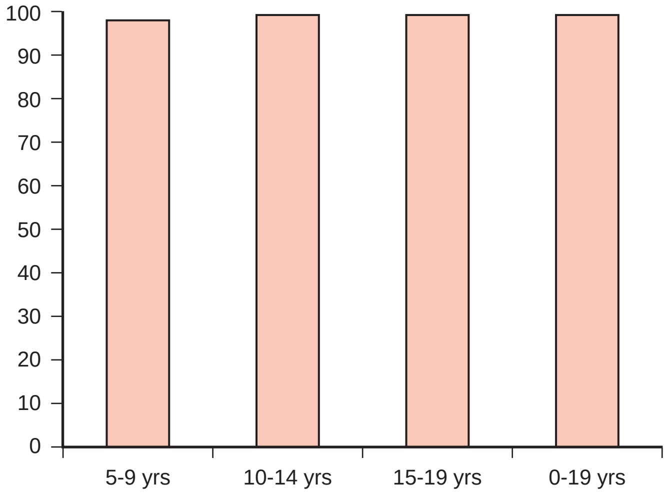

Figure 65-3 Five-year relative survival rates for thyroid carcinoma by age group, SEER 2002-2008.

Use of Radioactive Iodine

In adults, the use of radioactive iodine treatment to ablate residual thyroid or iodine-avid metastatic disease decreases the risk of distant metastases.[75] The goals for the use of this therapy in children includes ablation locoregional recurrence, elimination of residual sources of thyroglobulin production, and high remnant uptake that can obscure the presence of metastases and accurate detection of pulmonary metastatic disease.[76] The use of this therapy in children has been associated with a reduced recurrence risk in the thyroid and nodes independent of the surgical approach.[76] However, a recent study by Hay failed to show a significant difference in local and distant recurrence when this modality was used, and 73% of patients who died from a subsequent malignant neoplasm had received therapeutic irradiation postoperatively.[77] Radioactive iodine treatment should be reserved for children at high risk for disease recurrence, and a suggested measured approach for the postoperative use of this agent is shown in Figure 65-2.[64] When radioactive treatment is prescribed, TSH should be greater than 30 μIU/mL.[64] The dosing of this agent is not well established in children, and various centers follow the American Thyroid Association (ATA) guidelines for adults (*http://thyroidguidelines.net/revised/differentiated*). A posttherapy thyroid scan is usually obtained about 1 week after treatment, and this may identify additional sites of disease that were not apparent initially. Because of the risk of regrowth after TSH exposure, thyroid hormone suppression to achieve serum TSH levels between 0.1 and 0.5

μIU/mL is recommended in all pediatric patients with differentiated thyroid cancer.[64]

Targeted Therapies

A variety of "targeted" therapies have been developed for iodine-refractory differentiated thyroid carcinoma, and many of these agents have been approved for adult use.[78] Most of these therapies target RET and the vascular endothelial growth factor (VEGF) receptors but some also target platelet-derived growth factor β receptors (PDGFRβs), FGFR, MET, and endothelial growth factor (EGF) receptors.[78] Some of these agents include sorafenib, axitinib, motesanib, sunitinib, pazopanib, cabozantinib, and vandetanib.[78] In children sorafenib has been successfully used in a patient with refractory papillary thyroid carcinoma, and in a phase I/II study, vandetanib produced two partial responses in children with MEN2b-associated medullary thyroid carcinoma.[79,80]

Follow-Up Observation

Lifelong surveillance is recommended for children with thyroid carcinoma. A recent article recommends determination of suppressed thyroglobulin levels and antibodies every 3 to 6 months and a neck ultrasound every 6 to 12 months for the first 2 years of follow-up care.[64] If a patient was treated with radioactive iodine, a stimulated thyroglobulin test and whole body scan is recommended 1 year after initial treatment. About 25% of patients develop antibodies, and these interfere with the thyroglobulin assays. Declining antibody titers usually correlate with lower disease burden.[64]

BREAST TUMORS

Breast masses are rare in young children. During puberty, benign fibroadenomas are the most common cause of a palpable breast lump (Table 65-3).[81] The estrogen stimulation during adolescence may cause exaggerated growth of the stroma and epithelium in a localized breast lobule that characterizes a fibroadenoma. They have been found in as many as 20% of women between the ages of 15 and 25 years on autopsy studies.[82] Fibroadenoma occurring in adolescence does not appear to be associated with concurrent or future breast carcinoma. Any dominant discrete breast mass in an adolescent should be reexamined at midcycle after one or two menstrual cycles. Persistent masses should be imaged by ultrasound (not mammography) and referred to a surgeon. Typically, malignant breast masses in children have similar imaging characteristics to malignant breast masses in adults, which can help guide the decision to perform more invasive evaluation.[83] Lesions typical for fibroadenoma are sometimes observed clinically; fine-needle aspiration or core biopsy can also be performed to confirm the diagnosis.[84] Most fibroadenomas in adolescents self-resolve or shrink.[85] Most boys with breast masses have gynecomastia that requires only reassurance and noninvasive supportive care. This is borne out by review of pathology specimens from teenage male patients who underwent resection of breast masses; nearly all such evaluations were negative.[86]

TABLE 65-3 Breast Lesions in Adolescent Girls.

Abnormality	Incidence (%)
Fibroadenoma	68
Miscellaneous	27
Juvenile hypertrophy	2
Giant fibroadenoma	1
Phyllodes tumor	<1
Cancer	<1
Metastatic	38
Adenocarcinoma (lobular or ductal)	31
Lymphoma	13

Data from Neinstein LS: Breast disease in adolescents and young women. Pediatr Clin North *Am 46:607–629, 1999.*

Phyllodes tumors are stromal tumors that present as rapidly growing but clinically benign masses. Although the median age of presentation is 45 years, they can occur in adolescence.[87] Benign phyllodes tumors of the breast are pathologically similar to a large fibroadenoma with more stromal cellularity. They are treated by resection with at least 1 cm margins.[88] Malignant phyllodes tumors are sarcomas with nuclear pleomorphism and mitotic activity; treatment requires a multimodality sarcoma approach. Five-year disease-free survival rates have been reported to be 96%, 74%, and 66% for benign, borderline, and malignant phyllodes tumors, respectively, in adults[89]; it is thought that adolescents have a more favorable outcome.[90]

Breast masses can also represent primary or metastatic sites of pediatric cancers such as rhabdomyosarcoma,[91] non-Hodgkin lymphoma, Hodgkin lymphoma, or neuroblastoma.[92]

Breast carcinoma in a patient under the age of 20 years is rare; five cases were seen at M.D. Anderson Cancer Center over 10 years, compared with 180 cases in patients between ages 20 and 30 years and 1347 in patients between ages 31 and 50 years.[93] The most common pathology is a juvenile secretory carcinoma that secretes mucin and mucopolysaccharide-containing material. It is treated by excision and lymph node biopsy. No studies have evaluated outcomes in patients younger that the age of 20 years with breast carcinoma, but multiple studies have shown that age younger than 30 or 35 years is an independent predictor of poor response.[93] The two younger age groups at particular risk of breast cancer are women who have received radiation to the breast, usually for lymphoma, and women who have a familial predisposition. These women should be taught breast self-examination and awareness at a young age.

PULMONARY TUMORS

Primary tumors of the lung are extremely rare in childhood, and metastatic pulmonary lesions far exceed the number of primary tumors.[94] Table 65-4 lists the most common pulmonary tumors in children.

TABLE 65-4 Pulmonary Tumors in Children

Malignant	Benign
Bronchial adenomas (40% to 60%)	Hamartomas
Carcinoid	Hemangiomas
Mucoepidermoid carcinoma	Lymphangioma
Adenoid cystic carcinoma	Leiomyomas
Pleuropulmonary blastoma (15%)	Myofibroblastic tumors
Bronchogenic carcinoma (10% to 15%)	Inflammatory myofibroblastic tumor
Adenocarcinoma	Myofibromatosis
Small-cell carcinoma	Congenital peribronchial myofibroblastic tumor
Bronchoalveolar carcinoma	Neurogenic tumors
Squamous-cell carcinoma	Mature teratoma
Undifferentiated carcinoma	
Fetal-lung adenocarcinoma	
Pulmonary mesothelioma	
Sarcomas (20% to 25%)	
Rhabdomyosarcoma	
Synovial sarcoma	
Hemangiopericytoma (solitary fibrous tumor)	
Leiomyosarcoma	
Angiosarcoma	
Bronchopulmonary fibrosarcoma	

Bronchial Adenomas

A large percentage of malignant pulmonary tumors in children are called bronchial adenomas, and although not benign, as the term implies, they are predominantly low grade in behavior. They include carcinoid tumor, mucoepidermoid carcinoma, and adenocystic carcinoma. Although pulmonary carcinoid tumors account for only 2% of all primary lung tumors in adults, between 40% and 85% of pediatric malignant lung lesions are carcinoids.[94-97] See "Carcinoid" for a more complete description of carcinoid and its management. Mucoepidermoid carcinomas (MECs)[98] and adenoid cystic carcinomas[99] are more often seen in the head and neck but can arise from mucous glands located in the respiratory submucosa.

Most bronchial adenomas are endobronchial; patients often have recurrent infections or obstructive pneumonitis before their diagnosis is determined (Fig. 65-4), and the most common presenting symptoms are cough, fever, or wheezing.[100-102] Although over 25% of adults with pulmonary carcinoid and MEC are asymptomatic, the figure is lower in children, who are less likely to have a radiographic study that would detect an incidental lesion.[103] Aggressive lesions can present with hemoptysis, chest pain, or pneumothorax in rare cases. It is extremely rare for patients with pulmonary carcinoid to show evidence of carcinoid syndrome in the absence of metastatic disease,[103] but a reported 2% to 4% of patients have Cushing syndrome.[101,104,105] A mass is usually detected by chest x-ray or chest CT, but endobronchial lesions may only be suspected by recurrent localized obstruction and volume loss (Fig. 65-5, *A* and *B*). Octreotide scans have a sensitivity of 80% to 90% for carcinoids and can be useful at diagnosis to assess for metastatic disease.[106] More recently, PET CT imaging with the somatostatin analog ^{68}Ga-DOTATOC has shown sensitivity of 97% and specificity of 92% in identifying neuroendocrine tumors.[107] Diagnostic biopsy is usually obtained by flexible fiberoptic bronchoscopy (see Fig. 65-5, *C*). Lymph nodes can be involved, especially with higher grade tumors, but may also be enlarged as a reaction to obstructive pneumonitis rather than tumor.

The typical carcinoid tumor and low-grade MEC are pathologically bland and have a low rate of postresection recurrence or metastasis, even with local invasion.[95] The rarer atypical carcinoids and high-grade MEC have a higher mitotic rate and more necrosis and are more likely to behave aggressively.[97,108] The appropriate therapy is open resection of tumor and involved lymph node, the extent of which is determined by the centrality of the mass. Lobectomy or even pneumonectomy may be required for tumors of the main stem bronchus. Because adenoid cystic carcinomas can spread submucosally with circumferential bronchial involvement, the margin of resection should be determined intraoperatively by frozen sections of the bronchial margins.[94] Local recurrences of bronchial adenomas can occur, especially for MECs, but metastases are rare. Adenoid cystic carcinoma has a greater likelihood of distant metastases than MECs and a poorer overall survival rate.[94] Estimates for metastases from bronchial carcinoid range from 5% to 27%.[101,109,110]

When surgery is not curative, other therapies can be used but have shown limited success.[111] Octreotide, a somatostatin analogue used to treat the symptoms of carcinoid syndrome, has been known to stabilize carcinoid tumor growth but not result in regression; similarly, interferon has been used with a modest response. Radionuclide therapy with radiolabelled somatostatin analogs have also been investigated for carcinoids in adults and have demonstrated control of disease and symptom improvement in patients with metastatic disease.[112]

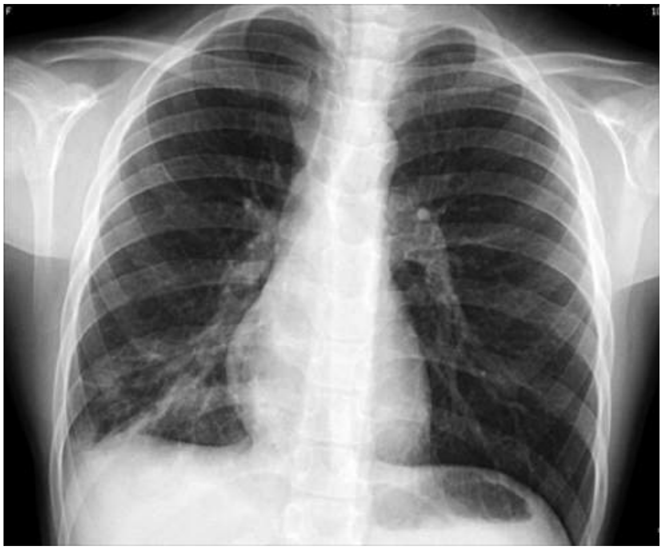

Figure 65-4 Plain radiograph of 9-year-old patient showing right lower-lobe infiltrate persistent over 1 month. Further workup revealed a right lower-lobe bronchus mucoepidermoid carcinoma.

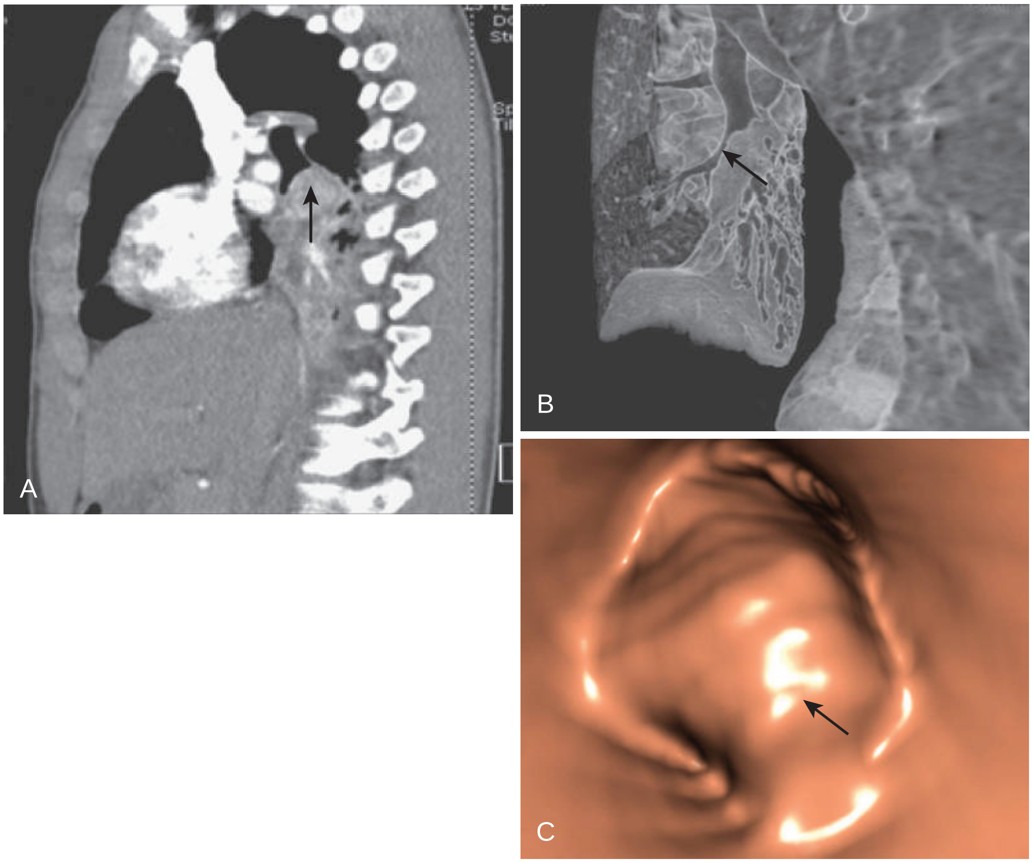

Figure 65-5 **A,** Carcinoid tumor *(arrow)* obstructing bronchus intermedius and causing peripheral consolidation of lung. **B,** Carcinoid tumor obstructing bronchus intermedius *(arrow)*. **C,** Carcinoid tumor *(arrow)* obstructing bronchus intermedius.

Cytotoxic chemotherapy for carcinoid and MECs has been used, but response rates, even with combination agents, are under 20%.[97,111] Novel molecular agents are being tested, including agents directed against VEGF, PDGFR, and mammalian target of rapamycin (mTor). Prognosis remains excellent for bronchial adenomas, even for locally invasive or high-grade tumors.

Pleuropulmonary Blastoma

Pleuropulmonary blastoma (PPB) in young children is distinct from pulmonary blastoma in adults; the pediatric tumor is composed of primitive blastema and neoplastic mesenchymal cells without epithelial malignant cells.[94,113] Since its initial description, there has appeared to be an increase in malformations and cancer in the afflicted child and family (Fig. 65-6).[114] Many pleuropulmonary blastomas are now known to be a part of a spectrum of dysplastic and neoplastic lesions that can arise from a constitutional mutation in *DICER1,* which is located on chromosome 14q and encodes a ribonuclease (RNase) endonuclease essential in the production of micro–ribonucleic acids (miRs). Other lesions that may arise as a result of germline *DICER1* mutations include ovarian sex-cord stromal tumors, cystic nephroma, embryonal rhabdomyosarcoma, ciliary body medulloepithelioma, nasal chondromesenchymal hamartoma, and multinodular goiter.[115-117] The tumor is located peripherally and can be locally invasive. Most patients have symptoms of wheezing, respiratory distress, or pneumothorax. Pathologically there are three subtypes: type 1 PPB, which is exclusively cystic, without a macroscopically detectable solid component); type 2 PPB with solid and true cystic areas; and type 3 PPB, a true solid tumor.[113] PPBs (particularly type 1 cystic lesions) are often misdiagnosed as congenital pulmonary airway malformations (CPAMs) because of similar appearance on chest x-ray and CT imaging. Echocardiography may occasionally demonstrate extension into the thoracic great vessels and heart, and fatal embolic events have been reported in rare occurrences.[118]

Treatment involves resection. Adjuvant chemotherapy (doxorubicin, actinomycin, vincristine, and cyclophosphamide or ifosfamide), especially for types 2 and 3, may reduce the risk of recurrence[119] or can be used neoadjuvantly to improve the resectability of large lesions.[120] Radiation has been used adjuvantly or palliatively; its role is unclear. If left unresected, type 1 lesions may eventually progress to more aggressive subtypes with development of anaplasia. Prognosis correlates with subtype; type 1 PPB that is completely resected has a 5-year survival rate of 85% to 90%, whereas type 2 and 3 tumors have 45% to 60% survival rates.[121,122] Metastases can develop in up to 55% of patients with type 3 PPB; the most common sites are the brain and spinal cord and

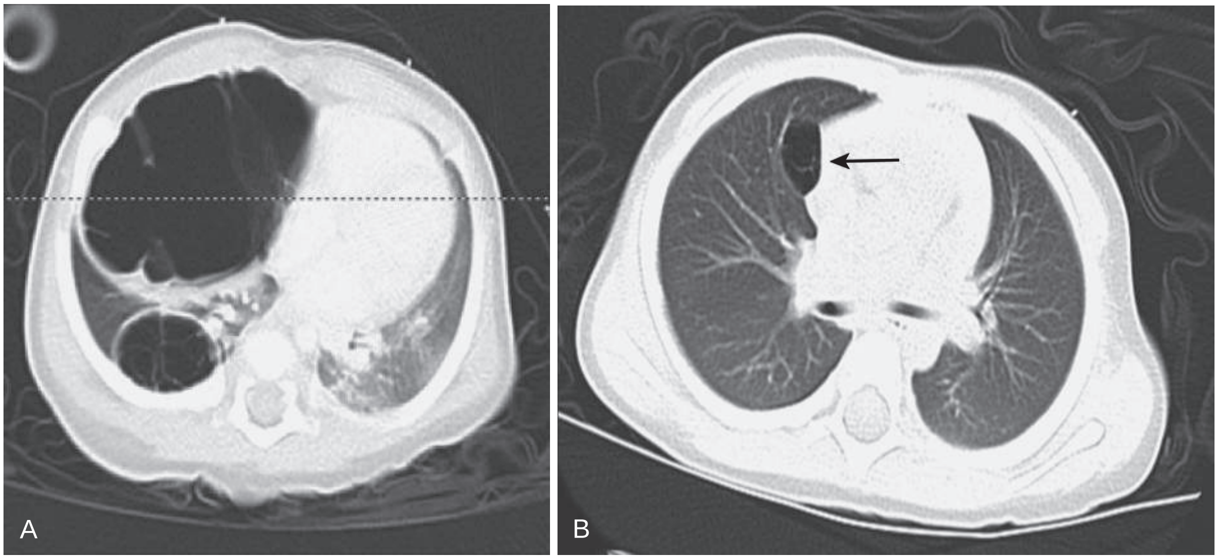

Figure 65-6 **A,** Two cystic lesions in 3-month-old infant found to be pleuropulmonary blastoma (PPB) on resection. **B,** Brother of patient with PPB in **A** with a benign cystic-adenomatoid carcinoma malformation *(arrow)*.

bone.[119,123] An international PPB registry has been established that collects clinical data on incident cases in an attempt to define the outcomes of patients with this rare disease. Families should be referred for genetic counseling to evaluate for the presence of a *DICER1* predisposition syndrome and risk of developing other related lesions.

Bronchogenic Carcinoma

Primary epithelial cancers of the lung are rarely seen in children; fewer than 100 cases have been reported in the literature. They are pathologically indistinguishable from the same tumors occurring in adults. Therapy should be planned after consultation with a medical oncologist experienced with lung cancers and in general should mimic the standard of care for adult tumors. When these histologies occur in children, they are often metastatic at diagnosis and have an aggressive course, high mortality, and average survival time of 7 months after diagnosis (Fig. 65-7).[94,97] Bronchioalveolar carcinoma (BAC) is a histology that accounts for less than 10% of lung malignancies in adults, with a mean age of presentation between the sixth and seventh decades. In children, mucinous BAC has been associated with CPAM type 1[94,124,125] and has also been reported as a second malignancy in osteosarcoma survivors.[126] Surgery is the primary treatment for BAC. Prognosis depends on nodal status and the resectability of the tumor; it appears to be slightly better for children than for adults with BAC or for children with other primary bronchogenic carcinomas.[127] Squamous cell carcinoma,[128] adenocarcinoma,[97] and small-cell lung cancer[129,130] are occasionally seen in children.[100] There is no clear causative pathway or association with environmental exposure, and most patients have no family history to suggest a genetic component. A rare form of adenocarcinoma in children is well-differentiated fetal adenocarcinoma (WDFA), which histologically resembles fetal lung and has an excellent prognosis.[97,131,132]

Other Pulmonary Tumors

Sarcomas arise from mesenchymal cells and therefore can develop from connective tissue cells in the lung. The most common types seen are hemangiopericytoma, synovial sarcoma, leiomyosarcoma, bronchopulmonary fibrosarcoma, and rhabdomyosarcoma.[94,100] Ewing sarcoma—peripheral primitive neuroectodermal tumor in the lung (Askin tumor)—is often pleural-based. Given its marked chemosensitivity, it should be treated with chemotherapy

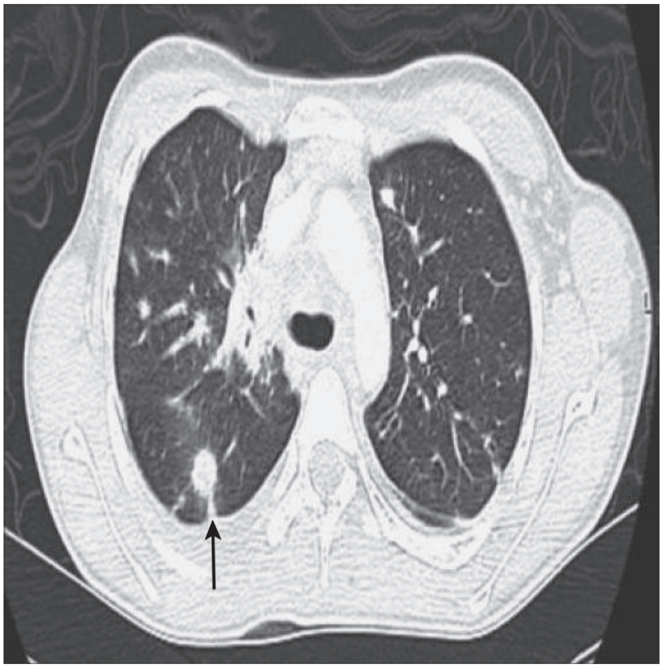

Figure 65-7 A 14-year-old patient with primary pulmonary poorly differentiated carcinoma *(arrow)* and lung, bone, lymphatic, hepatic, and adrenal metastases.

before surgical resection in contrast to almost all other lung tumors (see Chapter 61). Pleural mesothelioma is occasionally seen in children, with fewer than 100 cases in the literature and even fewer that have been confirmed by pathologic review.[133] Although there have been reports of assumed causation by asbestos exposure and chemotherapy and/or radiation for another malignancy, most cases appear in healthy children.[133,134] The tumor is similar to that in adult cases in its pathologic appearance and poor prognosis, with a median survival length of approximately 10 months.[135,136] Most cases are treated with a multimodality approach, but tumors are often diffuse and unresectable and are poorly responsive to chemotherapy and radiation. Current concepts in the therapeutic approach of adults with mesothelioma include the use of cisplatin and multitargeted antifolate chemotherapy[137] and aggressive surgery (extrapleural pneumonectomies).[138]

GASTROINTESTINAL STROMAL TUMORS

Epidemiology

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasm of the gastrointestinal tract in adults, with an incidence of 11 to 19.6 cases per million, which translates into 3300 to 6000 new cases per year.[139-141] GISTs are presumed to originate from the interstitial cells of Cajal, which regulate gastrointestinal motility.[142] Bardsley has characterized a putative interstitial cell of Cajal stem cell. This cell is capable of self-renewal and differentiation, has a KIT$^{\text{low}}$CD44+CD34+ phenotype, and is not dependent on KIT signaling for survival and proliferation.[143]

Gastrointestinal Stromal Tumors in Adults

The median age at diagnosis of GIST is approximately 60 years, and the disease has no sex predilection.[141,142] The most common presenting symptoms are abdominal pain, palpable mass, and gastrointestinal bleeding.[144] In adults, tumors range in size from 1 to 40 cm with a median size of 5 cm and most commonly arise in the stomach (60%) followed by the small intestine (30%) colon, rectum (5%), and esophagus (5%). About 95% of GISTs stain positive for CD117, and activating mutations of *KIT* (70% to 80%) and *PDGFRα* (~7%) are seen in nearly 90% of adult GISTs. About 12% to 15% of adult GISTs are wild-type for *KIT* and *PDGFR*, but mutations of other genes such as *BRAF, SDHB, KRAS,* and *NRAS* have been increasingly recognized (Table 65-5).[144]

Chemotherapy for unresectable or metastatic GISTs produces responses in fewer than 10% of patients, and the median survival for adults with metastatic disease is historically 20%.[145] The use of the tyrosine kinase inhibitor imatinib has revolutionized the treatment of GIST. Administration of this drug to adults with unresectable or metastatic GISTs produce responses in up to 85% of patients, and the median survival time for this group has increased to 58 months.[146] The likelihood of response to imatinib is correlated with the mutational genotype of the tumor; patients with *KIT* exon 11 mutations have significantly higher response rates to imatinib than patients with exon 9 mutations or no mutations.[147] A similar pattern has been documented for patients with *PDGFR* mutations; two thirds of patients with *PDGFR* lesions have the imatinib-resistant isoform D842V.[148] Suggested initial management of GISTs based on mutational status is shown in Table 65-5.

TABLE 65-5 Genetic Subtypes and Use of Imatinib in Selected Subsets of Adult Gastrointestinal Stromal Tumors

Genetic Type	Frequency of Total	Adjuvant Imatinib	Imatinib for Metastatic Disease
KIT mutated	80%		
Exon 11	67%	Yes, 400 mg/d	Yes, 400 mg/day
Exon 9	10%	Yes, 400 mg/day	Yes, 800 mg/day
Other	2%	Yes, 400 mg/day	Yes, 400 mg/day
PDGFR mutated			
Exon 12	5% to 8%		
Exon 14	1%	Yes, 400 mg/day	Yes, 400 mg/day
Exon 18	<1%	Yes, 400 mg/day	Yes, 400 mg/day
D842V	5%	No	No
Wild-type	12% to 15%		
BRAF V600	7% to 15%	?	?
SDH A,B,C,D	2%	?	?
HRAS/ NRAS	<1%	?	?
Other	2%	?	?

Modified from Corless CL, Barnett CM, Heinrich MC: Gastrointestinal stromal tumours: origin and molecular oncology. Nat Rev Cancer *11:865–878, doi:10.1038/nrc3143, 2011; and Blay JY, Le Cesne A, Cassier PA, Ray-Coquard IL: Gastrointestinal stromal tumors (GIST): a rare entity, a tumor model for personalized therapy, and yet ten different molecular subtypes.* Discov Med *13:357–367, 2012.*

Pediatric Gastrointestinal Stromal Tumors and Succinate Dehydrogenase–Deficient Gastrointestinal Stromal Tumors

Epidemiology and Presenting Features

Pediatric GISTs account for 1% to 2% of all GISTs, but their true incidence in the United States is largely unknown.[149,150] Review of the SEER database from 2000 through 2009 revealed 23 cases of malignant GIST, and 14 were seen in patients between 15 and 19 years of age for an expected incidence of 0.06 per million among this age group. In children and adolescents these tumors present at a median age of 14 years and predominantly affect females. The most common presenting symptoms are gastrointestinal bleeding, abdominal pain, anemia, weight loss, vomiting and early satiety. About 20% of patients have metastatic disease at diagnosis, and approximately 10% have a history of Carney triad or Carney Stratakis syndrome (see below).[151,152,153] GISTs in younger patients more commonly arise in the stomach, tend to be multifocal and involve lymph nodes, more often contain epithelioid or mixed histology, and have a more indolent clinical course. Despite surgical resections, 30% to 80% of patients develop metastatic disease to lymph nodes, liver, or locoregional sites, but despite multiple recurrences, survival is close to 90%.[154]

Biology

In children and adolescents, GISTs can occur within the context of two genetic syndromes: Carney triad and Carney Stratakis syndrome.[155,156] Carney triad is characterized by pulmonary chondroma, GIST, and paraganglioma, whereas Carney Stratakis syndrome lacks the pulmonary chondromas. Furthermore, Carney triad is sporadic and its cause is not known, whereas Carney Stratakis syndrome is caused by germline inactivating mutations of succinate dehydrogenase (SDH) B, C, or D.[157] Unlike adult GIST in which *KIT* and *PDGFR* mutations are key for disease development, only about 15% of GIST tumors found in children have activating mutations of one of these two genes. Despite this their tumors still express *KIT* at comparable levels to those seen in adults.[151,158] In contrast to adult GISTs, pediatric tumors lack large-scale chromosomal or genomic aberrations and overexpress *IGF1R*, suggesting that this pathway should be investigated as a possible therapeutic target in these patients.[150,158-160]

About 10% of children with wild-type GIST and no history of paraganglioma have a heterozygous germline mutation of SDH B or C.[161] Additionally, wild-type GIST in patients under 20 years of age almost lack *SDHB* expression as determined by immunohistochemistry, and nearly 90% of SDHB-negative cases express *IGF1R* by immunohistochemistry.[162,163] *SDHD* germline mutations have been reported in cases of sporadic GIST and in association with familial paraganglioma.[157,164] Germline *SDHA* mutations have also been reported in sporadic GIST.[165] Interestingly, about 30% of SDHB-negative cases by immunohistochemistry also lack *SDHA* expression, and immunohistochemical loss is strongly associated with germline mutations of *SDHA*.[164] Given the markedly different biology of GIST in younger patients, some investigators prefer using the term *SDH-deficient GIST* to identify a preferentially younger population that has wild-type GIST and SDH-deficient driven tumors.

Treatment

GIST in younger patients has a more indolent course, and recurrences are common. For this reason, ultimate cure in this group of patients is unlikely and treatment options that combine the judicious use of surgery combined with selected medical therapies offer the best chance for disease control. Because of the rarity of this disease, it is recommended that children and adolescents with GIST be preferentially managed by a multidisciplinary team with expertise in sarcoma and gastrointestinal tumors.[166] Initial management of localized disease should include surgical resection with the goals of achieving a gross resection with an intact pseudocapsule and negative margins. Lymph-node sampling in younger patients should be considered. Gastrectomy and other extensive surgeries should be avoided, because recurrence is common; therefore wedge resections are preferred. The use of adjuvant imatinib in patients with wild-type high-risk tumors is not recommended. For patients with unresectable or metastatic disease who have a *KIT* or *PDGFR* mutation, treatment according to Table 65-5 or the National Comprehensive Cancer Network (NCCN) guidelines is recommended.[167] Wild-type or SDH-mutated GISTs do not usually respond to tyrosine kinase therapies used in adults, and in selected asymptomatic patients with active disease, careful observation and follow up care might offer the best approach. In patients who develop significant symptoms or progression, a trial of imatinib or sunitinib is warranted; in one series one partial response and five cases of disease stabilization were seen in children refractory to imatinib.[168] A trial using sunitinib in patients 6 to 21 years if age with wild-type GIST and a trial using the tyrosine kinase inhibitor directed against insulin-like growth factor 1 receptor (IGF1-R) OSI-906 in patients over 18 years with wild-type GIST are ongoing. Follow-up care should include imaging and examinations every 3 to 6 months for 5 years. The use of CT should be limited when possible, and use of MRI and plain chest radiographs encouraged.

CARCINOID TUMORS

Carcinoid tumors are tumors of neuroendocrine cells that derive from embryonic divisions of the gut. The most common locations for carcinoid tumors are in locations arising from the midgut: the appendix, small intestine, proximal colon, and rectum. Carcinoid tumor of the appendix is speculated to be the most common tumor of the gastrointestinal tract in children.[169] Tumors of the epithelial component of the bronchial mucosa arising from the foregut appear as carcinoid tumors of the lung and bronchus. Biologically, carcinoid tumor cells contain membrane-bound neurosecretory granules that contain hormones and biogenic amines. When serotonin, 5-hydroxyindole acetic acid (5-HIAA), and other metabolites of the granules are released, a syndrome of flushing, diarrhea, and abdominal cramps can occur. The syndrome rarely occurs in children, because it is associated with large tumor mass, hepatic metastases, or distant metastases.[170] Carcinoid tumors are one of the rarer tumors (less than 10%) seen in the autosomal dominant syndrome MEN type 1 (MEN1), which is caused by a mutation in the tumor suppressor gene *MEN1* located at 11q13.[171,172] *MEN1* may be involved in sporadic carcinoid tumors as well, as evidenced by loss of heterozygosity (LOH) on chromosome 11 or inactivated copies of the *MEN1* gene.[173,174] Other possible contributors may be p53, K-ras-2, C-raf-1, and Bax/Bcl2.[172]

In a review of a 22-year period at St. Jude Children's Research Hospital, 0.08% of patients evaluated for malignancy had carcinoid tumors.[175] In that series, like others, the majority (approximately 60%) arose in the appendix. Pathologic examination of appendectomy specimens reveal carcinoid tumors in 0.23%, with the figure slightly lower in children than adults (Fig. 65-8).[170] Most patients with appendiceal carcinoid tumors have

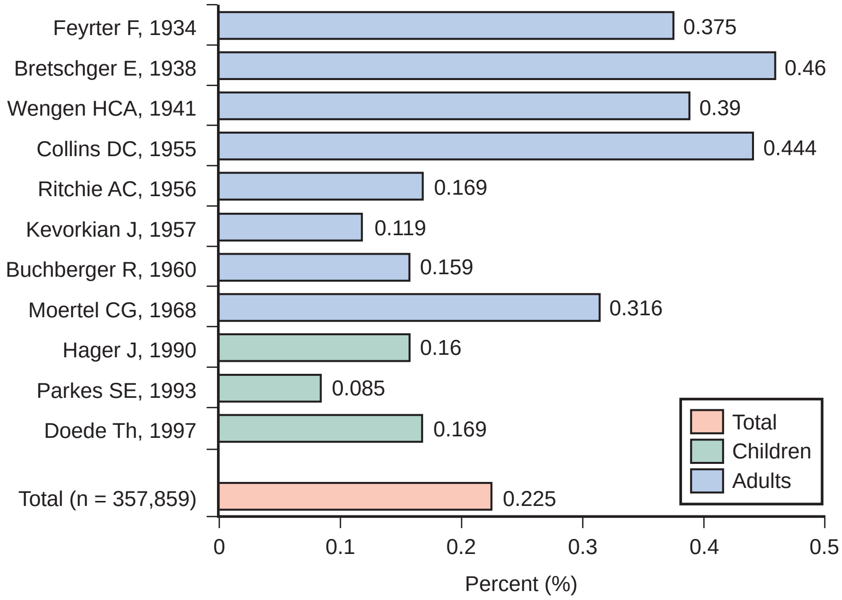

Figure 65-8 Percentage of appendectomy specimens found to have carcinoid tumors on pathologic examination. *(Modified from Doede T, Foss HD, Waldschmidt J: Carcinoid tumors of the appendix in children-epidemiology: clinical aspects and procedure.* Eur J Pediatr Surg *10:372–377, 2000.)*

symptoms of acute appendicitis; others have chronic abdominal pain, or an incidental appendectomy is done during another surgical procedure. The diagnosis is rarely made preoperatively.

Appendiceal carcinoid tumors smaller than 1 cm rarely metastasize, and appendectomy alone is the appropriate therapy.[176] After a diagnosis of an appendiceal carcinoid tumor, it is reasonable to check levels of serum serotonin and chromogranin A and 24-hour urinary 5-HIAA, and abdominal imaging should be performed to look for metastases. Octreotide scans have a sensitivity of 80% to 90% for carcinoid tumors and can be useful at diagnosis to assess for metastases,[106,177] although there is a low incidence of distant disease in children. For appendiceal tumors larger than 2 cm, right hemicolectomy is advocated in adults[178]; there is controversy regarding its use for tumors between 1 and 2 cm and for children, for whom cecal or ileocecal resection may suffice.[179-181] Additionally, there are only rare cases of carcinoid tumors of the appendix with perforation described, but it is presumed that perforation in any carcinoid tumor would increase the risk for dissemination. The decision for hemicolectomy, particularly in those children with tumors having some high-risk features but size between 1 and 2 cm, must be made on a case-by-case basis.[182]

No deaths from pediatric appendiceal carcinoid have been reported in several large series with extended follow-up study.[169,180,181] In addition to increasing size, location at the base of the appendix and the presence of mucin-producing cells are thought to be poor prognostic indicators.[178] Given its rarity and benign behavior, recommended follow-up care is controversial. Several authors have recommended a schedule of monitoring serotonin metabolites, with or without abdominal ultrasonography, in patients whose tumor diameter was more than 5 mm.[170,176] The findings that 14.6% of patients with appendiceal carcinoids in the SEER registry had synchronous or metachronous noncarcinoid malignant tumors,[183] that the risk of offspring carcinoid tumors (SIR 4.31) and secondary cancers (standardized incidence ratios [SIR] 2.15-3.31) was elevated in a Swedish registry study,[184] and several case reports of patients with secondary adenocarcinoma of the colon[175,185,186] have raised other questions of appropriate counseling and follow-up care for these patients. For the rare metastatic carcinoid tumor in childhood, treatment recommendations are based on experience in adult patients. Octreotide, a somatostatin analogue that has been used to treat the symptoms of carcinoid syndrome, has stabilized carcinoid tumor growth but not resulted in regression.[187] Similarly, interferon has been used with a modest response.[188] Recent efforts have demonstrated possible clinical activity of mTOR inhibitors and certain receptor tyrosine kinase inhibitors, but these initial findings await more follow up investigation and larger studies.[189]

COLORECTAL CARCINOMA

In Western and developed countries, colorectal carcinoma is one of the most common malignant cancers and is responsible for significant morbidity and mortality. Conversely, colorectal carcinoma is rare in populations that do not have high rates of obesity and meat consumption. In the United States alone, approximately 153,000 patients per year are diagnosed with colorectal carcinoma, and approximately 52,000 deaths each year are attributed to it.[190] However, colorectal carcinoma is rare in children, with fewer than 100 cases per year in the United States as estimated by SEER data and approximately one case per million in those younger than age 20

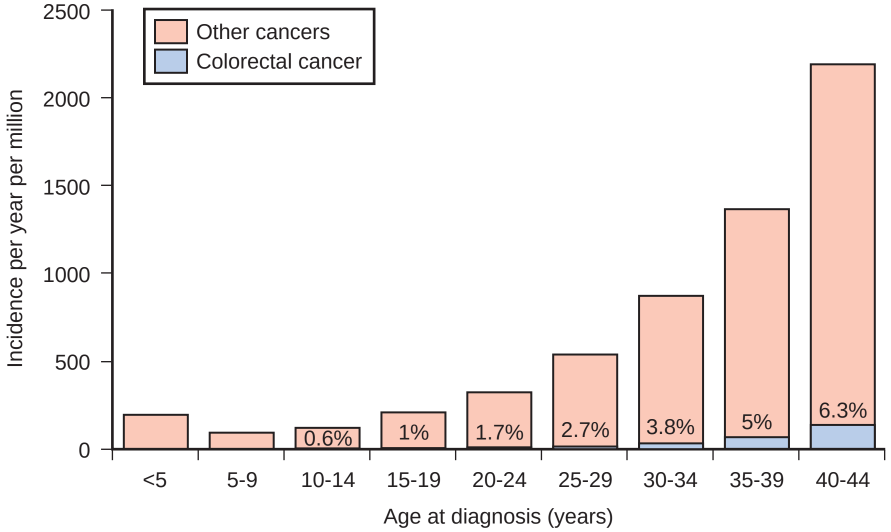

Figure 65-9 Incidence of colorectal cancer relative to all cancer, SEER 1975-2000. *(Data from Bleyer A, O'Leary M, Barr R, Ries LAG, editors:* Cancer epidemiology in older adolescents and young adults 15 to 29 years of age, including SEER incidence and survival: 1975-2000 [NIH Publication No. 06-5767]. *Bethesda, Md., 2006, National Cancer Institute.)*

years. The incidence of colorectal carcinoma relative to other cancers by age is shown in Figure 65-9, the 5-year survival rates of colorectal carcinoma by age are shown in Figure 65-10, and 5-year survival rates by age and stage are shown in Figure 65-11. Although case series offer suggestions about the biologic nature of this tumor in patients younger than 20 years, the rarity of this diagnosis in children means that comprehensive population-wide data are not available. Any case series from a large institution will be limited in number and by referral bias. Although many case series have suggested a higher stage at diagnosis for pediatric colorectal carcinoma and a prevalence of mucinous histology,[191,192] SEER data suggest that this disease in children more closely mirrors the adult presentation and that referral bias could lead to the findings in the case series.

Most cases of colorectal carcinoma in adults occur sporadically and in association with lifestyle choices. Only approximately 10% of adults with colorectal carcinoma have an identified inherited or acquired predisposition to it. These include patients with the dominantly inherited FAP syndrome who have a mutation in the *FAP* tumor suppressor gene, patients with Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer [HNPCC]) characterized by one of several defects in DNA mismatch repair genes, or patients with long-standing inflammatory bowel disease of more than 10 years' duration. It is not clear from the data whether

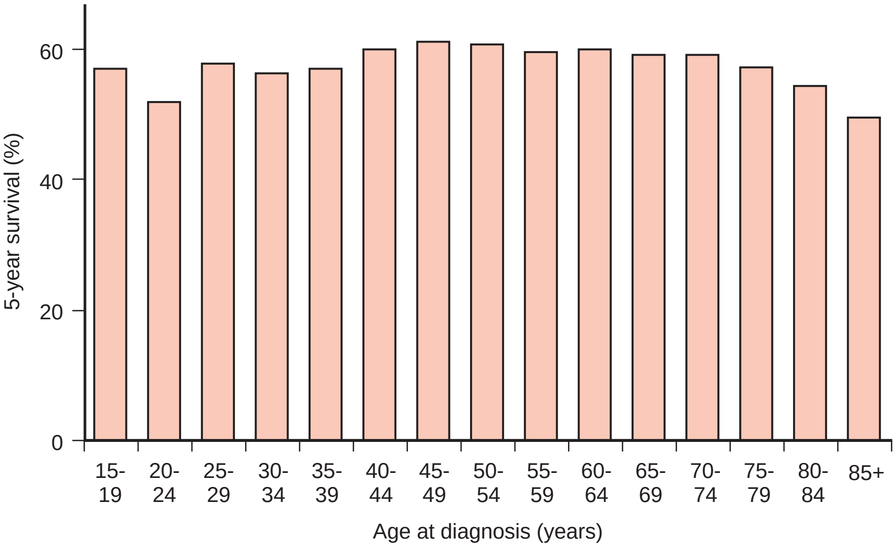

Figure 65-10 5-year survival rate for colorectal carcinoma, SEER 1975-1999. *(Data from Bleyer A, O'Leary M, Barr R, Ries LAG, editors:* Cancer epidemiology in older adolescents and young adults 15 to 29 years of age, including SEER incidence and survival: 1975-2000 [NIH Publication No. 06-5767]. *Bethesda, Md., 2006, National Cancer Institute.)*

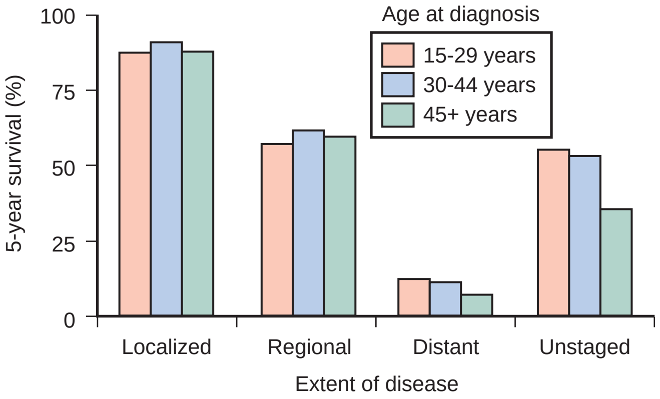

Figure 65-11 5-year survival rate for colorectal cancer by extent of disease, SEER 1975-2000. *(Data from Bleyer A, O'Leary M, Barr R, Ries LAG, editors:* Cancer epidemiology in older adolescents and young adults 15 to 29 years of age, including SEER incidence and survival: 1975-2000 [NIH Publication No. 06-5767]. *Bethesda, Md., 2006, National Cancer Institute.)*

children diagnosed with colorectal carcinoma are likely to have one of these common predispositions to the disease or if there is some other causative factor in these patients. Children with FAP, also known as Gardner syndrome, are commonly found to have myriad polyps on colonoscopy and many ultimately undergo prophylactic total colectomy before the age of 21 years because of symptoms (e.g., continued blood loss) or because of a strong family history of colon cancer. The vast majority of patients with FAP syndrome develop colorectal cancer by the age of 40 years. Many children who do undergo prophylactic colectomy are found to have dysplasia that was not apparent on routine screening colonoscopy.[193] Prophylactic colectomy is not clearly indicated in patients with Lynch syndrome, although in cases where there is lack of compliance with routine colonoscopy it may be appropriate.[194] Other syndromes associated with colorectal carcinoma include Bloom, Peutz-Jeghers, Turcot, Oldfield, and Rendu-Osler-Weber syndromes.[195-199]

Clinical Presentation

Colorectal carcinoma arises from the mucosal surface of the bowel, often growing from within a polyp. The tumor can cause intestinal obstruction through growth into the lumen of the bowel or can erode through the serosa and spread intraperitoneally. The early signs of colorectal carcinoma are difficult to separate from more common causes of abdominal complaints. Anemia, vague abdominal pain, bleeding, weight loss, and change in bowel habits have all been reported as the presenting complaint for children with colon cancer.[191] Many of these presenting complaints are common in pediatric care; it is possible that children who seek care with higher stages of colon cancer would have earlier diagnoses if they were adults, because an older adult with these complaints would likely be referred for colonoscopy. In two relatively large series reporting on this rare tumor in children, 0 of 20 and 3 of 30 patients had modified Dukes stage B tumors.[191-192] All other patients were stage C or D, and only 1 of 20 and 2 of 30 patients were alive without evidence of disease when these reports were published. In these two series, 16 of 20 and 25 of 30 patients had tumors with mucinous histology, a higher rate than that reported for adults with colorectal carcinoma. Again, these dire statistics likely reflect referral patterns, although they demonstrate that children with colorectal carcinoma can have high-stage disease at diagnosis. Although vigilance for pediatric colorectal carcinoma remains important, in the absence of known risk factors for the disease, adult-oriented screening examinations such as colonoscopy, routine testing of stool for occult blood, and sigmoidoscopy are not indicated, because they would pick up many false-positives and would not be cost-effective.

Diagnosis and Staging

The diagnosis of colorectal carcinoma in children requires a high index of suspicion in patients with the typical presenting signs or symptoms and tissue acquisition. The exact procedure required to obtain tissue for diagnosis is best determined in consultation with surgical colleagues and depends on the patient's clinical situation. For some patients, complete resection before chemotherapy may be appropriate, but for others, particularly those known to have metastatic disease, chemotherapy or radiotherapy may make resection possible in previously unresectable cases, so a less invasive approach to diagnosis is warranted after careful consultation with a multidisciplinary team. Any patient undergoing resection should have up to 12 lymph nodes sampled in an attempt to determine whether the cancer has spread.[200] An expert panel convened by the American Society of Clinical Oncology has not recommended adjuvant chemotherapy for those adult patients with stage II colorectal carcinoma that can be fully surgically resected. Children tend to have more widespread disease at presentation; however in the absence of data to support a contrary approach, for children with disease that can be fully resected at initial diagnosis, surgical intervention and subsequent observation seem to be indicated.[201]

For pediatric patients discovered to have colorectal carcinoma, full staging should involve functional and anatomic imaging (see later) as would be carried out in adult patients. In adults, linked PET and CT scans are increasingly becoming the norm, because this modality helps link fluorodeoxyglucose (FDG)-avid lesions with actual sites of disease more efficaciously than separate PET and CT scans that cannot be linked because of bowel movement. Most large pediatric oncology centers now have access to linked PET and CT. Barium enema is sometimes used to help identify areas of concern before the diagnosis is made, and a bone scan can be used to identify bone metastases if there is concern that PET is not picking up lesions.[202] The AJCC staging for colorectal carcinoma is shown in Tables 65-6 and 65-7.

Treatment

Initial management of the patient is focused on the diagnostic and staging workup and on any surgical interventions that are urgently needed in the context of bowel-related emergencies such as perforation or obstruction. Consultation with medical oncologists experienced

TABLE 65-6 Tumor-Nodes-Metastasis Definitions for Colorectal Carcinoma

Primary Tumor (T)	Regional Lymph Nodes (N)[§]	Distant Metastasis (M)
TX: Primary tumor cannot be assessed T0: No evidence of primary tumor Tis: Carcinoma in situ: intraepithelial or invasion of the lamina propria[*] T1: Tumor invades submucosa T2: Tumor invades muscularis propria T3: Tumor invades through the muscularis propria into the subserosa or into nonperitonealized pericolic or perirectal tissues T4: Tumor directly invades other organs or structures and/or perforates visceral peritoneum[†,‡]	NX: Regional nodes cannot be assessed N0: No regional lymph node metastasis N1: Metastasis in one to three regional lymph nodes N2: Metastasis in four or more regional lymph nodes	MX: Distant metastasis cannot be assessed M0: No distant metastasis M1: Distant metastasis

From Colon and rectum. In Edge SB, Byrd DR, Compton CC, et al, editors: AJCC cancer staging manual, *ed 7, New York, 2010, Springer, p. 143–164.*

[*] Tis includes cancer cells confined within the glandular basement membrane (intraepithelial) or lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa.

[†] Direct invasion in T4 includes invasion of other segments of the colorectum by way of the serosa; for example, invasion of the sigmoid colon by a carcinoma of the cecum.

[‡] Tumor that is adherent macroscopically to other organs or structures is classified T4. If no tumor is present in the adhesion microscopically, however, the classification should be pT3. The V and L substaging should be used to identify the presence or absence of vascular or lymphatic invasion.

[§] A tumor nodule in the pericolorectal adipose tissue of a primary carcinoma without histologic evidence of residual lymph node in the nodule is classified in the pN category as a regional lymph node metastasis if the nodule has the form and smooth contour of a lymph node. If the nodule has an irregular contour, it should be classified in the T category and also coded as V1 (microscopic venous invasion) or as V2 (if it was grossly evident), because there is a strong likelihood that it represents venous invasion.

in evaluating adult patients with colorectal carcinoma may help guide the workup.[203] Because of the potential for anemia from bleeding and liver involvement, as well as the potential for peritoneal seeding, complete blood counts, liver function tests, and kidney function tests with electrolyte levels should be obtained at diagnosis.[191] Carcinoembryonic antigen (CEA) should also be determined, although most series have found that children tend not to have heightened CEA at diagnosis,[191-192] and subsequently followed to survey for occult recurrence. Many chemotherapeutic agents can increase CEA, so care should be taken when interpreting these values, especially when the patient is receiving oxaliplatin.[204] These patients should generally not have values checked until they have recovered from chemotherapy. If the patient did not have an elevated CEA level at diagnosis, the decision not to monitor subsequent levels to screen for occult relapse is reasonable but should be made in close consultation with medical oncology colleagues.

TABLE 65-7 American Joint Committee on Cancer Stage Groupings for Colorectal Carcinoma

Stage	Groupings
0	Tis, N0, M0
I	T1, N0, M0 T2, N0, M0
IIA	T3, N0, M0
IIB	T4, N0, M0
IIIA	T1, N1, M0 T2, N1, M0
IIIB	T3, N1, M0 T4, N1, M0
IIIC	Any T, N2, M0
IV	Any T, any N, M1

From Colon and rectum. In Edge SB, Byrd DR, Compton CC, et al, editors: AJCC cancer staging manual, *ed 7, New York, 2010, Springer, p. 143–164.*

The treatment for children with colorectal carcinoma should be planned in conjunction with reports in the adult literature and with a medical oncologist experienced in treating gastrointestinal cancer in adults. Adult patients with stage II disease have not been shown to benefit from adjuvant chemotherapy if they have no evidence of disease after resection[201] and should undergo wide surgical resection with anastomosis if possible. It is reasonable to recommend careful observation after surgical treatment for children in this scenario in the absence of a clinical trial, because there is no standard of care therapy for these patients that has proven to reduce the risk for relapse. Although these patients should be considered for randomized controlled clinical trials, there are likely to be few trials available that include children younger than 18 years. If details of the case or of the surgical resection increase the concern that the patient could suffer a relapse, adjuvant therapy could be considered because the risk for recurrence could be from 51% to 73%.[205] The situation should include careful discussion between the oncologist and family. For patients with stage III or higher disease, chemotherapy has demonstrated a survival benefit for adults with colorectal carcinoma, and children should be treated in a similar fashion.[206] Patients with metastatic colorectal carcinoma should have their tumors tested for *KRAS* in codons

12 or 13 in a Clinical Laboratory Improvement Amendment (CLIA)–certified laboratory. Those with tumors demonstrated to have *KRAS* mutations should not receive anti–epidermal growth factor receptor (EGFR) antibody therapy with chemotherapy, because it is not of benefit in these cases in adults.[207]

SEER data have suggested that the proportion of children with colorectal carcinoma who have high-stage disease at diagnosis mirrors that of adults with colorectal carcinoma. Thus at diagnosis many children have a higher stage disease that is fully resectable and that requires chemotherapy. These patients tend to have a poor prognosis. There are several regimens associated with prolonged survival for patients with metastatic colorectal carcinoma in adults based on the use of 5-fluorouracil (5-FU), oxaliplatin, and irinotecan in a variety of combinations. Bevacizumab (Avastin) is also being used in conjunction with these regimens, often as second-line therapy in adults, with the benefit of an improvement in survival.[208] Children with high-stage colorectal carcinoma have a poor prognosis, and any child successfully cured may have years of life added, so it may be reasonable for pediatric oncologists to add bevacizumab to the established regimens as frontline therapy. However, this depends somewhat on the clinical scenario. Bevacizumab has been tested in children with cancer in combination therapy, and a pediatric phase I trial of bevacizumab has been completed, so there is experience administering this agent to children.[209] New data also suggest that blocking VEGF with aflibercept has therapeutic benefit in colorectal carcinoma.[210] Further information on chemotherapy regimens for patients with colorectal carcinoma can be found at http://www.cancer.gov/cancertopics/pdq/treatment/colon/HealthProfessional/page4.

Screening and Preventive Therapy for At-Risk Children

Children with a family history of FAP syndrome should generally begin undergoing colonoscopy by the age of 10 or 12 years and should be observed closely by geneticists and pediatric gastroenterologists.[211,212] For such patients, prophylactic colectomy is essential to prevent death from colorectal carcinoma later in life. The decision to undergo total colectomy must be made in conjunction with the family history and timing of cancer onset in the pedigree, the clinical situation, and the child's wishes. Patients with HNPCC are unlikely to develop colorectal carcinoma before the age of 18 years, but the accepted standard for such patients is to begin regular colonoscopy at 20 years of age or at least 10 years earlier than the earliest onset of colorectal carcinoma in their pedigree.[213] For patients with inflammatory bowel disease, care should be based on standard practices for pediatric gastroenterology. Cyclooxygenase-2 (COX-2) inhibitors have been shown to reduce the risk for developing colorectal carcinoma in adults but are known to increase the risk for cardiovascular side effects, so they are not universally recommended for adults at risk of developing colorectal carcinoma.[214] These agents have been used in the pediatric oncology setting and may in the future be appropriate chemoprophylaxis for children at high risk for colorectal carcinoma.[215]

Follow-Up Observation

For the child with the sporadic occurrence of colorectal carcinoma who is cured by surgery or surgery and chemotherapy, careful observation must be recommended. However, there is little evidence available on the risk for such children to suffer recurrence or the risk for second malignancies later in life. Colorectal carcinoma is rare in children and often difficult to cure, so survivors are few. No large-scale randomized trials have documented the efficacy of a standard, postoperative monitoring program in adults to date. Therefore given the long latency period for relapse for young children, they should probably be screened with regular colonoscopy and radiologic evaluation of the lungs and perhaps PET scanning as well.[216,217] Because pediatric colorectal carcinoma is so rare, the child may suffer from a cancer predisposition syndrome and consideration should be given to such a diagnosis. The child may benefit from yearly colonoscopy and should be brought to medical attention with little delay for any blood in the stool or other symptoms of colon cancer. Screening for CEA at a reasonable interval could be considered but is associated with a high rate of false-positives in adults. It should probably only be used for children who had high levels at presentation and are healthy enough to undergo risky surgery if any liver or lung metastases are found early by screening.[216] Although the 5-year survival rate for low-stage colon cancer is excellent (approximately 90%), patients with metastatic disease have less than a 10% 5-year survival rate. For those who survive metastatic pediatric colorectal cancer, it is unclear for how long surveillance against relapse should be done, but follow-up care in a long-term survivor clinic is indicated.

URINARY BLADDER CARCINOMA

Rhabdomyosarcoma is a relatively common tumor affecting the urinary tract and is discussed elsewhere in this text (see Chapter 59). Malignant epithelial tumors of the bladder are extremely rare in children in the Western world; the incidence of bladder cancer in general is higher in Africa because of the inflammation caused by chronic parasitic infection with bilharzial and other organisms.[218] Boys are more likely than girls to be diagnosed with this.[219] The presenting signs and symptoms of transitional cell tumors of the bladder in children include gross painless hematuria, urinary tract infections, voiding difficulties, and even hematospermia.

If a child is suspected of having a bladder tumor, the differential diagnosis must include malignancies such as epithelial bladder cancer and rhabdomyosarcoma, as well as benign lesions such as inflammatory masses. In the absence of obvious clinical signs of a mass, these patients are usually seen first by the pediatric urologist. The initial workup should include urinalysis and culture, urine cytology, and imaging studies. CT or MRI can help define the lesion. Cystoscopy can help reveal the nature of the tumor and allow for biopsy of lesional tissue. Attempts at transurethral biopsy are a primary mode of obtaining a tissue diagnosis, with open procedures to be considered

Box 65-1 Ovarian Neoplasms

MALIGNANT TUMORS

1. Germ cell (65% to 70%)
2. Epithelial tumors (15%, including low malignant potential or borderline tumors)
    a. Adenocarcinoma
    b. Cystadenocarcinoma: mucinous, serous
    c. Endometrioid
    d. Clear-cell tumor
    e. Undifferentiated carcinoma
3. Stromal tumors (15%)
    a. Juvenile granulosa-cell tumors (JGCTs)
    b. Sertoli-Leydig cell tumors
    c. Sclerosing stromal tumors
    d. Gynandroblastoma
4. Miscellaneous (<5%)
    a. Burkitt lymphoma
    b. Carcinosarcoma (malignant mixed müllerian tumor)

BENIGN TUMORS

1. Germ cell
    a. Cystic teratoma (dermoid)
2. Epithelial
    a. Cystadenomas
    b. Mucinous
    c. Papillary and nonpapillary serous
3. Stromal tumors
    a. Granulosa cell
    b. Theca cell
    c. Fibromas

as a backup maneuver.[220] Because bladder cancer is uncommon in younger patients, delay in diagnosis is not uncommon for younger patients. However, the outcome for children and young adults with bladder carcinoma seems to be better than for adults, explained in part by more indolent clinical and biological features. Patient younger than 20 years of age at diagnosis, for instance, have a lower incidence of chromosome 9 abnormalities, *FGFR3* mutations, and few epigenetic alterations. Therefore treatment decisions must include careful consideration of late effects.[221]

OVARIAN TUMORS

Approximately 50% of tumors occurring in the ovary in children are nonneoplastic. Furthermore, the minority of neoplastic lesions are malignant, so that only 10% to 20% of ovarian masses in children are cancerous (Box 65-1).[222-224] In children and even in adolescents, surgical management plans should take into consideration the lower rate of malignancy than in adults and minimize the risk of compromising hormonal function and fertility.[225] Ovarian masses usually present with abdominal pain, bloating, distention, or dysmenorrhea. The most appropriate and widely used initial evaluation tool for an ovarian mass is ultrasonography, which can distinguish the size, origin, and cystic nature of the tumor.[226] If cystic, unilateral, and presumed benign, a laparoscopic procedure is appropriate. Otherwise, further workup with chest, abdomen, and/or pelvic CT and tumor markers such as β-human chorionic gonadotropin (β-hCG), α-fetoprotein (AFP), cancer antigen-125 (CA-125), and CEA is required, and open laparotomy should be the surgical approach.[226] Ovarian tumors are staged by the International Federation of Gynecology and Obstetrics (FIGO) system (Table 65-8).

Of the neoplastic tumors, germ-cell tumors account for 65% to 70% (see Chapter 63). Epithelial carcinomas, the

TABLE 65-8 Carcinoma of the Ovary

Stage	Extent of Growth
I	Growth limited to the ovaries
IA	Growth limited to one ovary; no ascites present containing malignant cells; no tumor on the external surface; capsule intact
IB	Growth limited to both ovaries; no ascites present containing malignant cells; no tumor on the external surface; capsules intact
IC[*]	Tumor either stage IA or IB, but with tumor on surface of one or both ovaries or with capsule ruptured, ascites present containing malignant cells, or positive peritoneal washings
II	Growth involving one or both ovaries with pelvic extension
IIA	Extension and/or metastases to the uterus and/or tubes
IIB	Extension to other pelvic tissues
IIC[†]	Tumor either stage IIA or IIB, but with tumor on surface of one or both ovaries or with capsule(s) ruptured, ascites present containing malignant cells, or positive peritoneal washings
III	Tumor involving one or both ovaries, with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes; superficial live metastases equals stage III; tumor limited to the true pelvis but with histologically proven malignant extension to small bowel or omentum
IIIA	Tumor grossly limited to the true pelvis, with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces or histologically proven extension to small bowel or mesentery
IIIB	Tumor of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surface not exceeding 2 cm in diameter; nodes are negative
IIIC	Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive retroperitoneal or inguinal nodes
IV	Growth involving one or both ovaries with distant metastases; if pleural effusion is present, there must be positive cytology to allot a case to stage IV; parenchymal liver metastasis equals stage IV

From Heintz AP, Odicino F, Maisonneuve P, et al: Carcinoma of the ovary: FIGO 6th Annual Report on the Results of Treatment in Gynecological Cancer. Int J Gynaecol Obstet *95(Suppl 1):S161–S192, 2006.*

[*] International Federation of Gynecology and Obstetrics (FIGO) nomenclature, Rio de Janeiro, 1988.

[†] To evaluate the impact on prognosis of the different criteria for allotting cases to stage IC or IIC, it would be of value to know if rupture of the capsule was spontaneous or caused by the surgeon and if the source of malignant cells detected was peritoneal washings or ascites.

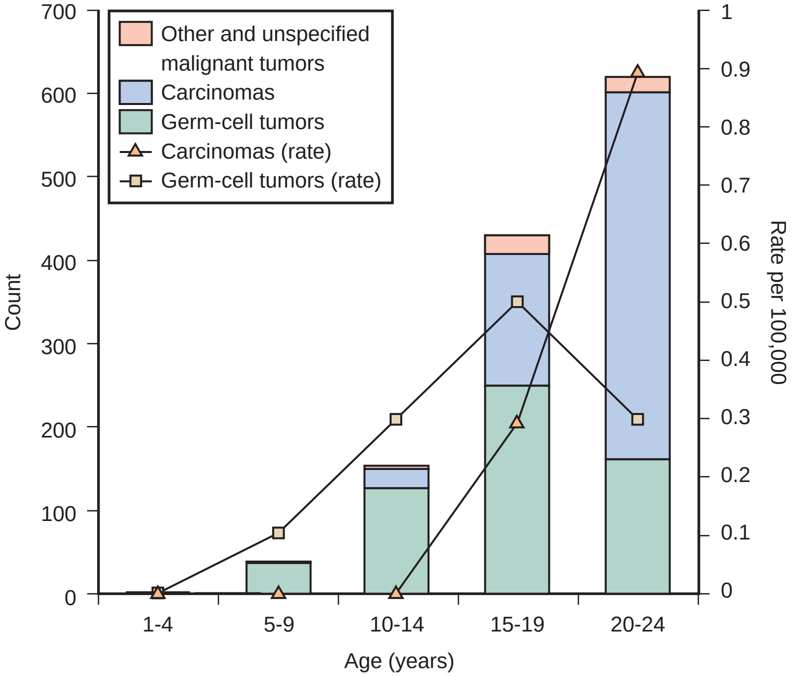

Figure 65-12 Incidence and rate per 100,000 cases of ovarian cancer in children and young adults, SEER-17 1998-2002.

most common ovarian tumor in adults, account for 15% to 20% of pediatric ovarian tumors, with most occurring after menarche when the rate increases markedly (Fig. 65-12). Unlike in adults, most pediatric epithelial ovarian tumors are localized, and pathologically most are of low malignant potential (borderline) or well differentiated.[224,227] Stage I tumors of potential low malignancy are appropriately treated by unilateral salpingo-oophorectomy alone, and have almost a 100% survival rate.[227] Surgery should include careful inspection of the peritoneum and contralateral ovary and lymph nodes.[228] For those rare children with invasive or advanced disease, platinum-based therapy developed for adult epithelial carcinomas has proven effective. Although a thorough family history should be obtained as a matter of course, studies suggest that breast cancer genes 1 and 2 (*BRCA1/2*), breast and ovarian cancer syndrome, and HNPCC syndrome account for few cases of epithelial cancer in women younger than age 30 years.[229-231]

Stromal Carcinomas

Tumors of the mesenchymal tissue of the ovary account for 15% of ovarian malignancies in pediatrics. Most present at an early stage and have a prognosis for survival higher than 85%.[232] Sertoli-Leydig tumors often cause overproduction of androgen precursors, producing virilizing symptoms of amenorrhea, masculinization, and hirsutism. Juvenile granulosa-cell tumors can overproduce estrogen and progesterone and present with precocious puberty. These tumors are sometimes classified with benign tumors, and many act in a benign fashion but have malignant potential and have been reported to metastasize and be fatal.[233] Nonlocalized tumors should be treated with adjuvant platinum-based chemotherapy with or without radiation.[234]

TUMORS OF THE CERVIX AND VAGINA

The most common nonovarian tumor of the female genital tract is rhabdomyosarcoma (82% in a registry series over 25 years in Great Britain), and the appearance of a protruding cluster-of-grapes vaginal mass in a young child is classic for sarcoma botryoides.[235,236] Cervical and vaginal carcinomas in children can be adenocarcinomas or squamous carcinomas; the ratio is skewed more toward adenocarcinoma in children than in adults. There is no known difference in causative factors, treatment, or prognosis between the two pathologies. Mesonephric adenocarcinoma is rare in adults but is a relatively more common subtype in children; it is thought to arise from mesonephric duct remnants and tends to arise deeply without reaching the endocervical surface.[237]

Cervicovaginal clear-cell adenocarcinomas are not associated with HPV infection. Rather, a peak of incidence in children and adolescent girls was a historic phenomenon caused by the maternal use of diethylstilbestrol (DES) therapy in pregnancy. Since its proscription, the tumor is again relatively uncommon in pediatrics. However, rates of non-DES cervical cancer in adolescents have been rising because of increasingly early sexual activity and higher rates of HPV infection.[238] HPV types 16 and 18 are the causative agents in more than 70% of cervical cancer. Around puberty the cervix has a particular biologic vulnerability to HPV infection, so that the sexually active adolescent has one of the highest risks of infection after exposure,[239] creating a risk for the development of squamous intraepithelial lesions that can progress over 10 to 15 years to invasive cervical cancer.[240] Recent data have shown that 27.8% of ninth-grade and 63.6% of twelfth-grade girls report having had sexual intercourse.[241] These sexually active female adolescents have high rates of genital HPV infection (13% to 64%) and abnormal Papanicolaou (Pap) smear results (as high as 38%).[239,240,242] The recent formulation of a vaccine against multiple strains of HPV is recommended for administration to 12-year-old girls—before anticipated initiation of sexual activity.[243] However, a 2010 survey found that only 32% of adolescent females ages 13 to 17 years complete the 3-dose series. In order to improve herd immunity and cost-effectiveness, the quadrivalent HPV vaccine is now recommended for adolescent males as well.[15-16] The natural history of progression of cervical abnormalities (from squamous intraepithelial lesions to cervical intraepithelial neoplasia) appears similar in adolescents and adults.[244] Recommendations for adolescents with abnormal Pap smears are available on the American Society for Colposcopy and Cervical Pathology website.

The most common clinical presentation of a cervical or vaginal tumor is vaginal bleeding, which may be difficult to distinguish from irregular menses in prepubescent and early pubescent girls. In one series evaluating causes of vaginal bleeding in children, tumors accounted for 11%.[245] These tumors often present at later stages in children and adolescents than adults because of the lack of screening Pap smears and hesitancy to perform pelvic examinations.[246]

Treatment includes surgical resection with lymph-node exploration and adjuvant radiation for residual disease or lymph nodes. This preference of surgery over radiation differs from therapy for adults because of the need to avoid radiation toxicity in younger, growing children. Novel uterus-sparing techniques (e.g., radical abdominal/vaginal trachelectomy) first adopted in adults to preserve fertility have been used successfully in children.[247,248] In a retrospective study of women younger than 25 years, localized cervical cancer had a 5-year survival rate of 91% and regional disease had a 5-year survival rate of 46%.[249] A series of 37 pediatric cases of vaginal and cervical adenocarcinoma from St. Jude Children's Research Hospital has found a 71% 3-year survival rate.[246] Other rare tumors reported in the cervix and vagina are alveolar soft parts sarcoma,[250,251] germ-cell tumors such as endodermal sinus (yolk sac) tumor and embryonal carcinoma,[236,252] and Wilms tumor.[253] These tumors are covered elsewhere in this text.

GESTATIONAL TROPHOBLASTIC TUMOR

Sexually active adolescents are at risk for these rare tumors, which arise as tumors of the products of conception. A hydatidiform mole, or molar pregnancy, is a uterine mass consisting only of placental-like tissue without an intact fetus. Pregnancies in those younger than 15 years have a sixfold risk of being molar.[254] Hydatidiform moles are treated by dilation and curettage with careful follow-up monitoring for resolution of elevated β-hCG levels.[255] Persistent or increasing β-hCG levels after a molar pregnancy, abortion, or term pregnancy indicate a malignant gestational trophoblastic tumor (GTT), the most aggressive of which is choriocarcinoma, a malignant tumor of the trophoblastic epithelium. The most common symptom is abnormal vaginal bleeding, but patients can also have asymptomatic pulmonary metastases or neurologic symptoms caused by brain metastases.[256] Patients with nonmetastatic or good-prognosis metastatic GTT are treated with single-agent chemotherapy (methotrexate, dactinomycin, or etoposide), and have an excellent prognosis.[257,258] Those with single-agent resistant or high-risk metastatic disease (high β-hCG, delay in start of chemotherapy, brain metastases, multiple sites of metastases) require multiagent cytotoxic chemotherapy.[259-261] The use of platinum and etoposide-based regimens has significantly improved salvage rates for high risk patients who fail initial multiagent chemotherapy.[262]

MELANOMA

Epidemiology

Pediatric melanoma, which has been classified as an epithelial malignancy by the International Classification of Childhood Cancer (ICCC), accounts for 30% of epithelial cancers in children and adolescents.[41,55] Although rare, particularly among prepubertal patients, it is estimated that nearly 450 cases of pediatric melanoma are diagnosed each year in the United States in patients younger than age 20 years[31] and most cases affect 15- to 19-year-old adolescents, in whom this malignancy accounts for 7.1% of all cancers (Fig. 65-13).[41]

The incidence of melanoma in children younger than age 20 years increased by 2.9% per year from 1973 to 2001, with the highest rates of increase in adolescents.[263] Most melanomas in pediatric patients affect white females, and over 80% are localized. The female-to-male ratio increases with age, especially during adolescence, and there is a preponderance of nonwhite patients, nodular histology, and head and neck sites in patients younger than 10 years of age.[263]

Risk Factors

Melanoma can be transmitted transplacentally, but this is a very rare phenomenon that is associated with a dismal clinical outcome.[264] Other risk factors associated with the risk of melanoma in the pediatric population include the presence of a large congenital nevus, retinoblastoma, Werner syndrome, prior history of childhood cancer, xeroderma pigmentosum, neurocutaneous melanosis, immunosuppression, familial melanoma, and dysplastic nevi.[265-267]

Clinical Molecular and Pathologic Features

Pediatric melanoma resembles adult melanoma in its clinical presentation and can arise in a preexisting nevus or de novo. The classic ABCDEs of melanoma (asymmetry, border irregularity, color variegation, diameter larger than 6 mm, and an evolving lesion) are commonly reported in childhood and adolescent cases, but the presence of other lesions such as Spitz nevus or amelanotic lesions may make diagnosis difficult and delay the diagnosis. It is estimated that up to 60% of pediatric patients with melanomas are initially misdiagnosed.[266,268] The pathologic diagnosis of melanoma may be often difficult and challenging. Increasing evidence has suggested that

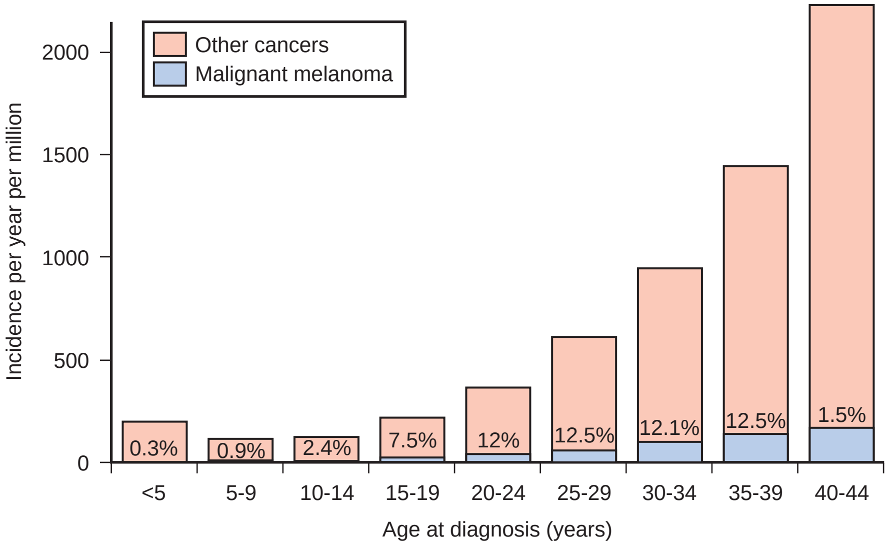

Figure 65-13 Incidence of malignant melanoma relative to all cancer, SEER 1975-2000.

pediatric melanoma may have a significant variability in its pathologic spectrum. The term *melanocytic lesion with unknown metastatic potential* has gained popularity in recent years, adding to the uncertainty and confusion of diagnosing pediatric melanoma.[269] The use of fluorescence in situ hybridization (FISH) probes sets that target 6 distinct chromosomal loci has identified 9p21 deletions and 6p25, 11q23 gains as markers of aggressive behavior.[270] About 50% of pediatric melanomas have a *BRAF V600* mutation that can be targeted with available therapies for adults.[271,272]

Diagnosis and Staging

Since there is no validated system for children, guidelines for diagnosing and staging children with melanoma have been extrapolated from adult studies. Adhering to the current AJCC guidelines for staging all pediatric patients with melanoma is recommended.[273] The use of routine imaging in children with melanoma has not been studied prospectively, but in one study 25% of patients with thicker lesions had evidence of distant disease on CT of the chest and abdomen. The routine use of PET imaging for staging pediatric melanoma has not been studied prospectively, but in adults it has not revealed a significant patient-relevant benefit.[274] Early detection and biopsy are imperative, and when melanoma is suspected the pathology report should include the type of melanoma, depth, mitotic count, and surgical margins. Nodal staging using sentinel node biopsy has become commonplace in pediatrics and should be performed in children with lesions greater than1 mm thick and may be considered in children with thinner lesions if they display one or more high risk features such as Clarks level of invasion IV or V, ulceration, and high mitotic rate ($>0/\text{mm}^2$).[275]

Treatment and Outcome

Surgery is the treatment of choice for pediatric melanoma. For patients with lesions greater than 1 mm a wide excision with a 1-cm margin is appropriate. For those that are 1 to 2 mm, a 1- to 2-cm margin is recommended, and for those thicker than 2 mm a 2-cm margin should be used.[276] After a positive sentinel node it is recommended that a patient be offered a complete lymph node dissection, because about 16% of these patients have occult nodal metastases; however, a survival advantage after this procedure has not been clearly demonstrated.[277,278] The administration of adjuvant high-dose interferon improves relapse-free survival in adult patients with high-risk melanoma, and this option should be offered to patients at high risk for recurrence.[279] Although single-institution studies suggest that high-dose interferon is well tolerated in children with melanoma, its use has not been studied prospectively in large numbers of patients.[280-282] Subcutaneous pegylated interferon for up to 5 years has been shown to prolong disease-free survival in adults with resected microscopic nodal disease and ulceration.[283] Its use in pediatrics has been limited, and results of clinical trials using this agent in children have not been yet published. Because *BRAF V600* mutations are seen in up to 50% of pediatric melanomas, the use of targeted therapies such as vemurafenib and dabrafenib should be explored further. There is a pediatric trial using vemurafenib for patients 12 to 17 years of age with measurable *BRAF V600* mutated melanoma. Ipilimumab, a monoclonal antibody directed against the inhibitory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4), has improved survival in adults with metastatic melanoma, and a pediatric trial is being conducted.[284] In addition, other novel immunotherapies such as BMS-936558, a monoclonal antibody directed against programmed death receptor 1 (PD1), and BMS-936559, a monoclonal antibody directed against programmed cell death 1 ligand 1 (PDL1), has produced encouraging responses in adults with metastatic melanoma, but a pediatric trial is not available.[285,286] The overall survival of pediatric melanoma is stage-dependent and overall excellent, with

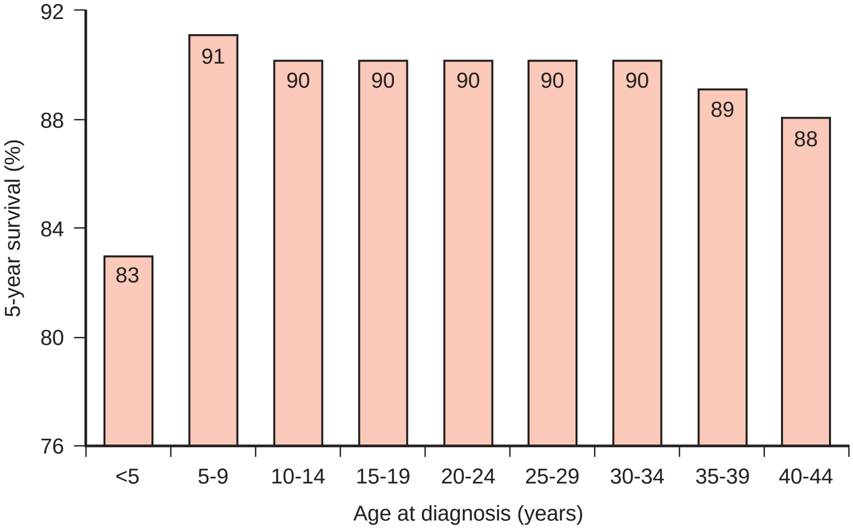

Figure 65-14 5-year survival rate for melanoma, SEER 1975-1999.

5-year survival rates in excess of 90% except in those who are younger than 10 years of age (Fig. 65-14).

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CHAPTER 66

Imaging in the Evaluation and Management of Childhood Cancer

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CHAPTER OUTLINE

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Computed Tomography
Magnetic Resonance Imaging
Nuclear Medicine
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Supratentorial Tumors
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Infratentorial Tumors
PULMONARY AND INTRATHORACIC TUMORS
Thyroid and Parathyroid Tumors
Mediastinal Masses
Chest-Wall/Pleural-Based Neoplasms
GASTROINTESTINAL TUMORS
Liver
Spleen
Biliary tract
Pancreas
Alimentary Tract Tumors
GENITOURINARY TUMORS
Renal Masses
Bladder Neoplasms
ADRENAL/SUPRARENAL TUMORS
Neuroblastomas
GERM-CELL TUMORS
Ovarian Germ-Cell Tumors
Testicular Germ-Cell Tumors
Sacrococcygeal Germ-Cell Tumors
MUSCULOSKELETAL NEOPLASMS
Primary Bone Tumors
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Sites of Relapse in Hematologic Malignancies
IMAGING TREATMENT COMPLICATIONS
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NEW IMAGING TECHNIQUES
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SURVEILLANCE IMAGING
CONCLUSION

The incidence of cancer in childhood is relatively rare when compared to the incidence in adults; approximately 6500 new cases are diagnosed in the United States each year. Nevertheless, cancer remains the second most common cause of death during childhood. Beginning in the mid-1960s treatment of childhood cancer has witnessed remarkable advances in chemotherapy, radiotherapy, and surgical intervention, with improvements in survival for the majority of pediatric malignancies. The imaging of children with malignancy has also evolved, with significant technical advances in both diagnostic and interventional radiology techniques. Faster magnetic resonance (MR) and computed tomography (CT) scanners, three-dimensional (3D) acquisition and postprocessing techniques, and an increasing number of hybrid imaging technologies allow practitioners to image children more quickly, accurately, and safely, in addition to providing options for image-guided diagnostic and therapeutic procedures that historically required surgical intervention. This chapter provides an introductory overview of the various diagnostic imaging techniques available and a review of the imaging findings commonly seen in a broad spectrum of pediatric tumors. The chapter concludes with a discussion of diagnostic and therapeutic interventional

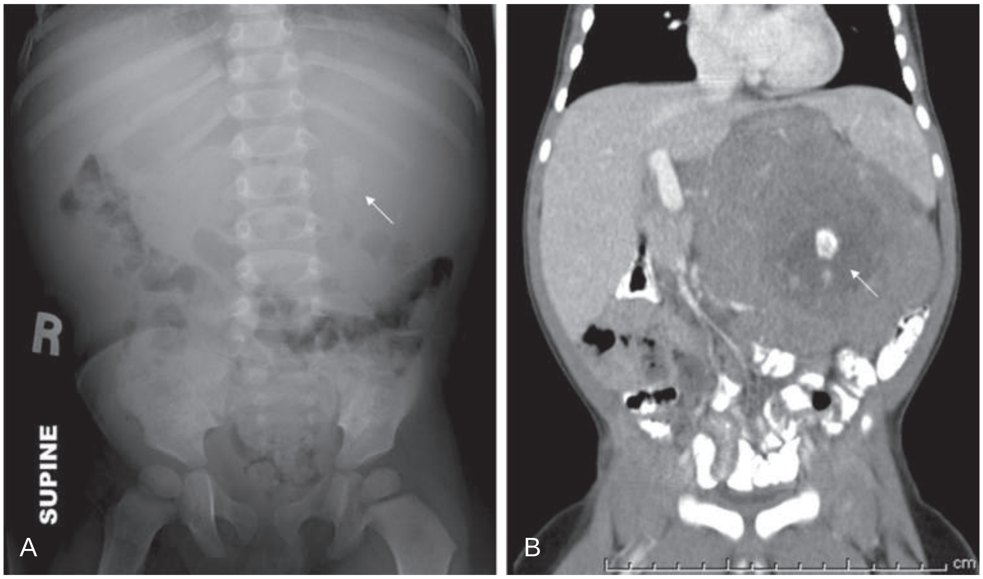

Figure 66-1 Neuroblastoma, detected incidentally. A 20-month-old infant fell, and caregivers felt a firm abdomen with no other symptoms. Abdominal radiograph (**A**) shows a calcified abdominal mass *(arrow)*, further characterized by CT (**B**) and found to be neuroblastoma.

radiologic techniques, surveillance imaging considerations, and finally a brief introduction to the evolving field of molecular imaging and novel imaging technologies.

TECHNICAL CONSIDERATIONS

Conventional Radiography

The decision to perform an imaging study on a child and the type of imaging that is selected very much depend on the patient's clinical history and physical examination findings. In young children with cancer, initial presenting symptoms and complaints are often nonspecific. An easily performed, relatively inexpensive imaging assessment like an x-ray is therefore appropriate as a first step in the evaluation. Conventional radiography is still commonly used as the first imaging examination undertaken both in children with suspected malignancy and in patients with nonspecific complaints that ultimately turn out to be related to neoplastic disease. These initial imaging studies provide valuable initial information that can help suggest a differential diagnosis and inform decision makers about subsequent imaging evaluations.

Although it is tempting to forego obtaining conventional radiographs in favor of cross-sectional imaging modalities such as ultrasound and CT, it is still appropriate in most instances to obtain conventional radiographs as a first step. Normal x-rays in certain settings may provide needed reassurance. Abnormal radiographs, on the other hand, provide a sensitive and accurate initial means of identifying pathology. For example, abdominal films allow assessment for bowel obstruction or perforation and may show solid mass lesions, abdominal calcifications, or areas of bone destruction (Fig. 66-1). Chest radiographs often reveal primary intrathoracic or mediastinal tumors and pulmonary metastases. Chest radiographs are also critical in suggesting impending airway compromise, pneumothorax, or pneumomediastinum and are rapidly obtained, easy to perform, and fairly quickly interpreted. Oncologic emergencies are fortunately uncommon; however, cardiorespiratory emergencies in a child, such as pneumothorax or pneumomediastinum, airway compression secondary to a large mediastinal mass (Fig. 66-2), and malignant pericardial or pleural effusions can be effectively evaluated in the emergent setting by properly executed chest and abdominal radiographs. Indeed, a CT scan may be contraindicated if radiographs indicate the presence of significant airway compromise in the setting of a large mediastinal mass, because supine positioning with or without sedation may lead to further airway compression, impaired venous return, and acute cardiorespiratory collapse.[1]

Radiographs are essential for initial imaging of nonspecific orthopedic complaints such as pain and limp and may lead to the diagnosis of marrow infiltrative disorders such as leukemia and neuroblastoma (Fig. 66-3, *A*). Radiographs are also necessary for evaluation of primary bone tumors such as Ewing sarcoma and osteogenic sarcoma, where the presence of calcifications, periosteal reaction, bone destruction, and soft-tissue involvement are important in formulating an initial impression and plan. Conventional radiography also plays a role in specific conditions such as Langerhans cell histiocytosis, where skeletal surveys are still used to detect the lytic lesions characteristic of this disease (see Fig. 66-3, *B*). Whether whole body MRI or 18-fluorodeoxyglucose positron emission tomography (FDG-PET) scanning can be used to improve the sensitively and specificity of detecting these lesions has been the subject of several investigations.[2-5]

The role of fluoroscopy in the primary evaluation of pediatric oncology patients is limited and has been largely replaced by CT. CT provides the advantage of visualizing both endoluminal and surrounding mesenteric/intraperitoneal/retroperitoneal processes, for which traditional fluoroscopic evaluations are limited.

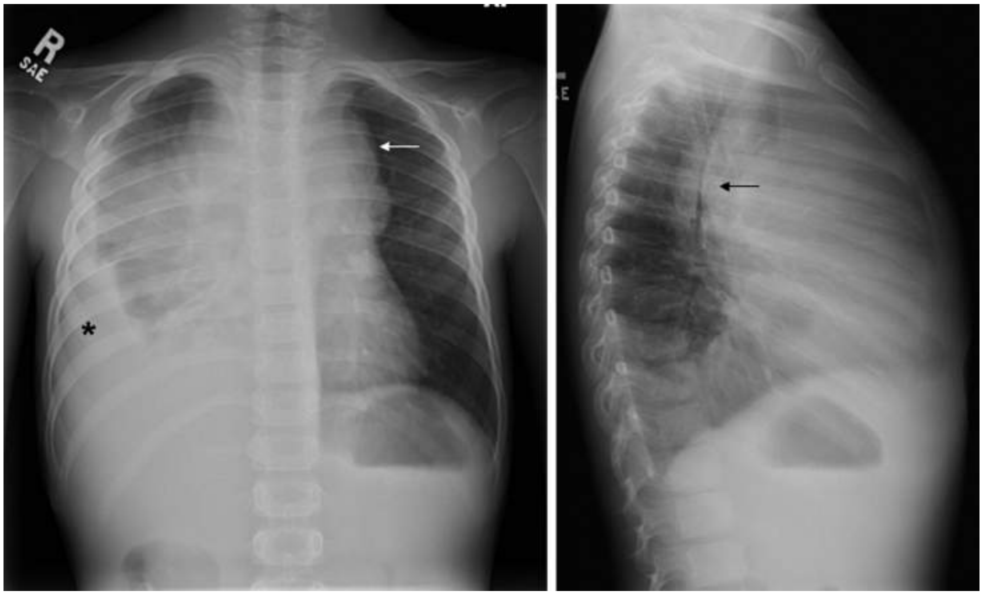

Figure 66-2 Incidentally discovered lymphoma. A 5-year-old boy, otherwise well, developed fever and cough and was treated for pneumonia. The chest x-ray, obtained for persistent symptoms, showed a large mediastinal mass *(white arrow)*. Note large pleural effusion *(asterisk)* and marked tracheal narrowing *(black arrow),* placing this child at considerable risk for acute cardiorespiratory compromise. The biopsy showed T-cell lymphoblastic lymphoma.

Ultrasound

The use of ultrasound in pediatric radiology is ubiquitous. Ultrasonography is relatively inexpensive, can be done portably, involves no radiation, and in skilled hands can be used to accurately assess an anxious, moving child for whom sedation may pose additional risk. Technically ultrasound relies on the use of variable penetration and attenuation of sound waves by different tissue types. The routine ultrasound examination typically involves the combination of B-mode (brightness mode) scanning and either color or pulsed wave/duplex Doppler sonography to assess flow within vascular structures. As an initial screening modality, ultrasound is very sensitive at evaluating intraabdominal solid organ viscera and is the initial imaging modality of choice in the evaluation of Wilms tumor, neuroblastoma, and hepatoblastoma. Pelvic neoplasms, intraperitoneal free fluid, and musculoskeletal soft-tissue tumors can all be effectively evaluated by ultrasound.

Newer ultrasound imaging techniques rely on a large array of transducers, which afford high-resolution images independent of the size of the patient and depth of the lesion. Extended field-of-view image-processing algorithms offer the capability of visualizing large masses in their entirety along with their relationships to normal structures. Although not in routine clinical use, 3D ultrasound allows images to be displayed in multiple planes and may be useful in demonstrating relationships between solid-organ malignancies and adjacent normal structures.[6] Ultrasound contrast agents are in common use experimentally and have the potential to add to the diagnostic information obtained by conventional ultrasound, but they are not yet being used routinely in clinical practice.[7-9]

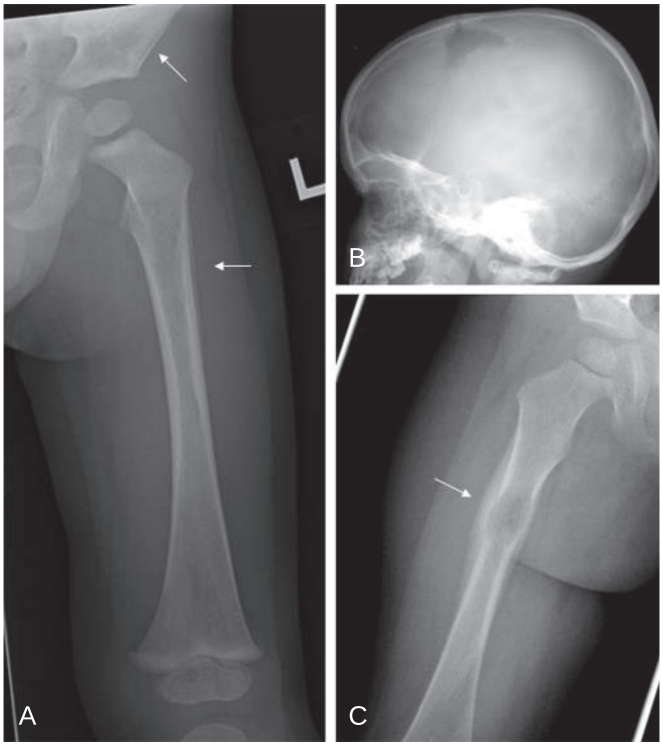

Figure 66-3 Two children with limp. **A**, 23-month-old child; hip and femur radiographs showed periosteal reaction involving femur and supraacetabular ilium *(arrows)*. The ultrasound revealed an abdominal mass, which on biopsy was neuroblastoma. **B** and **C**, 25 month-old-child with lytic skull lesion (**B**) that was revealed on a skeletal survey, which was obtained after extremity radiographs taken for limp identified a lytic femoral lesion with periosteal reaction (**C**). Biopsy showed Langerhans cell histiocytosis.

Ultrasound is also used intraoperatively to help identify lesions that are not visible, not palpable, or undetectable using other imaging techniques. Intraoperative ultrasound can also play an essential role in helping the surgeon identify a plane between tumor and adjacent normal tissue, which can be vital when tissue-sparing surgical techniques are desirable, such as nephron-sparing partial nephrectomy (see "Renal Masses") and partial hepatectomy.[10]

Computed Tomography

After ultrasound, CT scanning remains the primary imaging modality for evaluating most pediatric solid tumors. It is readily available in the majority of institutions, can be performed rapidly (often without sedation), and with newer generation scanners provides high spatial resolution and multiplanar viewing capabilities. CT scanning involves the use of a fan-shaped or cone-shaped x-ray beam generated by the x-ray tube rotating 360 degrees around the body part being examined. At the same time the x-ray tube rotation is occurring, the patient is moving longitudinally through the scanner, allowing the sequential and nearly simultaneous examination of multiple contiguous body parts. Once the x-rays pass through the patient, they strike an array of photodetectors that convert the x-ray energy into electrical signals, which are then used to reconstruct images from the region of the body through which the x-ray beam penetrated. The current generation of scanners are called *multidetector-row helical CT scanners* and rely on multiple detector rows (up to 256) to simultaneously acquire imaging information from larger segments of tissue.[11,12] This allows for more rapid scanning and also allows the reconstruction of imaging data in multiple planes with minimal artifact and image distortion. These multiplanar reconstructions also allow volumetric data to be evaluated, which may be important in assessing response to therapy.[13-14,15,16] In addition, the ability to view images in multiple planes with two-dimensional (2D) and 3D reconstructions and the ability to export images to robotic-assisted surgical devices is increasingly used by surgeons for both preoperative planning and intraoperative guidance in complicated cases.

For infants and anxious young children, it may be necessary for patients to be sedated in order to acquire high-quality imaging. In the majority of patients, sedation can be performed safely and effectively with skilled nursing staff and monitoring equipment.[17,18] In particular, sedation is usually necessary before infusion of intravenous contrast agents, because the contrast infusion may be startling to an otherwise calm child, and motion artifact severely compromises the image quality.

The use of intravenous contrast agents, particularly for the majority of pediatric solid tumors, is necessary to properly show relationships between primary neoplasms and adjacent structures. Although centers less experienced in the care of pediatric patients may consider performing scans without the use of intravenous contrast based on perceived increased risks of contrast reactions in children, a retrospective review of CT scanning results from over 12,000 contrast-enhanced studies performed over 5 years showed less than 0.5% incidence of contrast reactions (57 reported adverse events), with the vast majority being minor reactions. No severe contrast reactions occurred in infants or very young children.[19] Based on this and the results from other pediatric centers[20] there is little data to support the routine omission of intravenous contrast in the evaluation of children suspected of having malignancy. In particular, the use of nonionic low osmolar agents can be performed very safely in the majority of patients and are the contrast agents of choice in most large pediatric radiology departments. Enteric contrast is also recommended to opacify the bowel lumen, allowing better discrimination between normal viscera and potential sites of mesenteric or retroperitoneal lymph-node enlargement. Historically barium had been used, but this has been largely replaced by low-osmolar water-soluble contrast agents that can be mixed with water or juice, produce fewer streak artifacts, and do not affect other imaging studies such as PET and single-photon emission CT (SPECT) imaging.

Optimal imaging of the thorax for the purpose of evaluating the mediastinum and hilar structures requires the use intravenous contrast, whereas CT scanning of the chest to assess for pulmonary metastatic disease can generally be performed without the use of intravenous contrast. The use of multiplanar reconstructions and the ability to review slices in very thin sections improves detection of pulmonary nodules,[21,22] although the ability to discriminate benign from malignant pulmonary nodules based on CT is still limited.[23] In infants and small children who are either asleep or in whom quiet breathing can be encouraged, the speed with which current generation scanners can acquire images may allow noncontrast examinations of the chest to be performed without sedation. In older children for whom breath-holding is possible, image quality and sensitivity/specificity of pulmonary nodule detection is improved when images are obtained at the end of inspiration. If breath-holding is required for a child requiring sedation, anesthesia consultation should be obtained to determine the need for general anesthesia and endotracheal intubation versus using positive pressure laryngeal mask ventilation.[24]

The use of intravenous contrast agents also requires skill on the part of the radiologist in interpreting the examination, with awareness of potential causes of artifact. In particular, the spleen has a variable pattern of enhancement that is well appreciated but can be confused for splenic disease, particularly in the setting of lymphoma. Delayed scanning can usually help in discriminating artifact from disease.[25] In addition, acquisition of images at multiple phases after contrast infusion is often helpful in detecting subtle foci of disease that may only be seen at early arterial phases of enhancement, particularly in the liver (Fig. 66-4). Other lesions, in contrast, may only become evident after delayed enhancement, and knowledge of the typical patterns of enhancement for the particular disease that is being evaluated or suspected is important in determining the scanning technique.[26] It is still common to perform evaluations for hepatoblastoma in both hepatic arterial and portal venous phases in order to maximize sensitivity of lesion

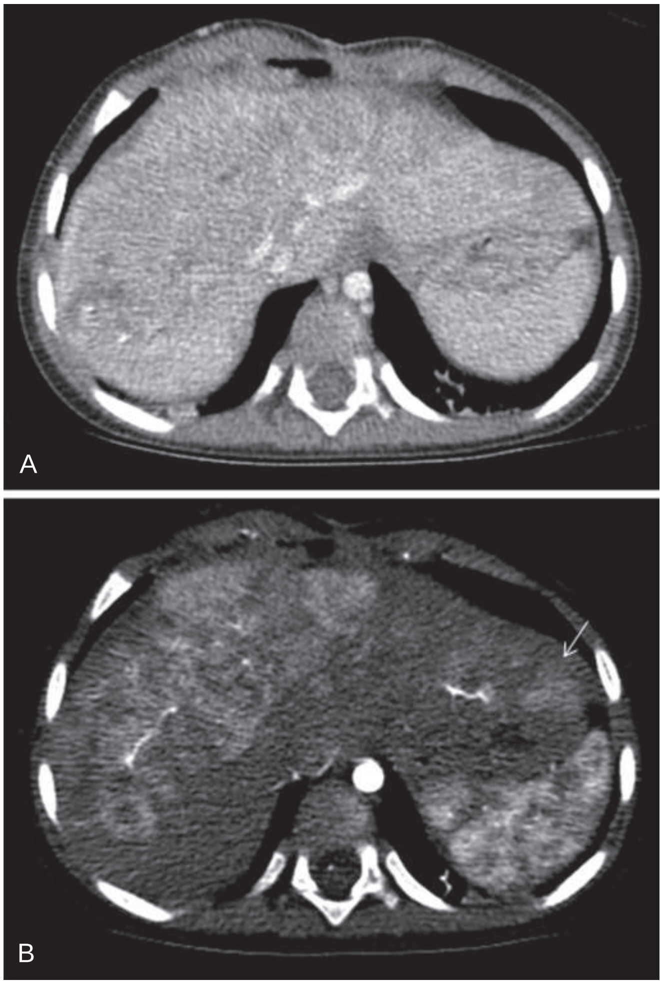

Figure 66-4 A 20-month-old child with unresectable hepatoblastoma, emphasizing the importance of multiphase scanning for optimal lesion detection. The presence of the left lateral segment lesion *(arrow)* was only seen on early arterial-phase scanning. Its identification determined surgical unresectability in this patient.

detection, delineate vascular anatomy, and provide optimal images to aid in surgical planning, although in many centers, improvements in abdominal MR image quality together with concerns about radiation related to repeated CT scanning have resulted in increasing use of MR imaging (MRI).[27]

Discussions of radiation dose in the setting of diagnostic CT scanning have become widespread in both the imaging and wider medical literature.[28-33] The data indicate measurable increases in incidence of malignancy attributable to radiation doses during diagnostic CT scanning, with cumulative increases in risk with increasing numbers of scans. Although these concerns are not to be underemphasized, particularly for otherwise well patients undergoing screening examinations, for patients with known malignancy or in whom malignancy is suspected, the relative benefit derived from the high-quality information achieved after diagnostic CT scanning almost always outweighs the modest incremental increase in risk associated with these techniques.[34] Nonetheless every effort should be made to limit the examination to involved regions of the body and to minimize the frequency of scanning in these heavily evaluated patients, using the "as low as reasonably achievable" (ALARA) principle as guidance.[31,32,35-37]

Magnetic Resonance Imaging

The physical principles of MR image formation are beyond the scope of this chapter and can be reviewed in articles devoted to this subject.[38,39] MRI involves the use of an external magnetic field to orient protons (primarily contained in water molecules) within the body, after which radiofrequency pulses are applied with specific frequencies and orientations. These radiofrequency pulses force the protons aligned in the magnetic field to come out of alignment. After the radiofrequency energy is withdrawn, the protons gradually reassume their original alignment, or resonate, within the magnetic field. The process of relaxation is multidimensional, and the rate with which longitudinal and transverse relaxation (T1 and T2 relaxation times, respectively) occurs is dependent on the properties of the specific tissue. The energy released during this realignment process can be measured much in same way that a standard radio receiver acquires the signal (radiofrequency energy) from a radio station transmitter. This radiofrequency energy can be characterized both in terms of its spatial location and frequency, and this information in turn can be used to reconstruct images. The result is high resolution MR images obtained in multiple orientations and tissue planes with signal properties that reflect the tissue microenvironments within which the protons reside.

High field-strength and low field-strength magnets are in common clinical use. In general terms, with higher field strength there is a higher signal-to-noise ratio.[38,40] Despite this, the high field-strength magnets are susceptible to some image degradation and magnetic field artifact, and most imaging can be reliably performed at 1.5 and 3 Tesla. With lower field strengths, the signal-to-noise ratio is considerably diminished. There are a variety of radiofrequency coils that are utilized as receivers for the radiofrequency energy emitted during proton relaxation and reorientation. Newer coil designs allow for rapid scanning techniques and for high-resolution imaging of specific body parts to be performed.[38,41]

Multiple MR images of the same organ or tissue are typically generated; however, the imaging technique chosen results in different patterns of tissue contrast, allowing the unique features that characterize distinct components of an organ or tissue to be depicted. The tissue contrast that one observes is the result of the interplay between the externally applied magnetic field, the inherent properties of the tissue (i.e., freely mobile water protons or highly ordered soft-tissue protons), and the radiofrequency pulse-sequence parameters chosen to acquire the image. The tissue properties that have the greatest influence on image contrast are the proton density (PD) and the T1 and T2 proton relaxation times. Conventional MRI techniques are acquired with "T1- and T2- weighting," in which the tissue contrast is weighted to reflect differences in the T1 and T2 relaxation times among the different tissues. This is accomplished by varying the interval between which the radiofrequency pulses are applied (the pulse repetition time, or TR) and the time that is allowed before the emitted signal is received (the echo time, or TE). T1-weighted images typically have a short TR (300 to 600 msec) and short TE (10 to 20 msec) and emphasize T1 characteristics of tissues. On T1-weighted images, fat is typically bright, fluid has low signal intensity, and complex or proteinaceous fluid is of intermediate to high signal intensity. Conventional contrast agents used in MRI result in T1 shortening and therefore produce increased signal on T1-weighted images.

T2-weighted images have longer TR (greater than 2000 msec) and longer TE (greater than 80 msec). T2-weighted images obtained using standard techniques display simple fluid such as cerebrospinal fluid (CSF) or urine as bright in signal intensity, whereas fat is low in signal intensity. Muscle and solid organs, depending on their tissue make-up, have variable signal intensity on T2-weighted images. Beyond these simple T1- and T2-weighted images, specific pulses sequences can be created that eliminate fluid signal (fluid-attenuated inversion recovery, FLAIR) or provide fat suppression either using chemically selective fat suppression techniques or so-called *inversion recovery techniques,* in which the TE is selected to minimize signal from fat and maximize signal from non–fat-containing structures.

Diffusion-weighted MR imaging (DWI) has also emerged as an important technique for evaluation of malignancy.[42,43] DWI is sensitive to the molecular motion of water within the imaging volume and historically was developed for neuroradiologic applications, where it became routine for evaluation of CNS ischemia, identifying early areas of potentially reversible cellular damage by virtue of restricted water diffusion across ischemic cellular membranes. Tumors also commonly demonstrate restricted diffusion relative to surrounding tissue because of increased cellularity and reduced extracellular space. With improvements in gradient echo imaging techniques, DWI can help identify subtle sites of disease (Fig. 66-5) and in some instances distinguish benign from malignant disease.[44] DWI changes in response to therapy, with increasing free diffusion of water molecules in areas of cellular necrosis as opposed to the more restricted movement of water in cellular regions of a tumor, has also been shown to correlate with tumor necrosis induced by treatment, and is emerging as an important technique in assessing response to therapy.[45,46,47,48]

MRI angiography and MR spectroscopy are all standard imaging techniques in common practice in the evaluation of the central nervous system (CNS). MR angiography takes advantage of rapidly flowing blood moving in and out of the imaging slice. Alternatively, gadolinium (Gd)-enhanced MR angiography relies on the use of Gd-based contrast agents to display the vasculature. The use of dynamic enhanced MRI allows multiple acquisitions in a specific region such as a tumor to be obtained, and there is evidence to suggest that the relative rates of contrast uptake and perfusion in tumors may be a reflection of tumor cellularity and/or necrosis.[49,50]

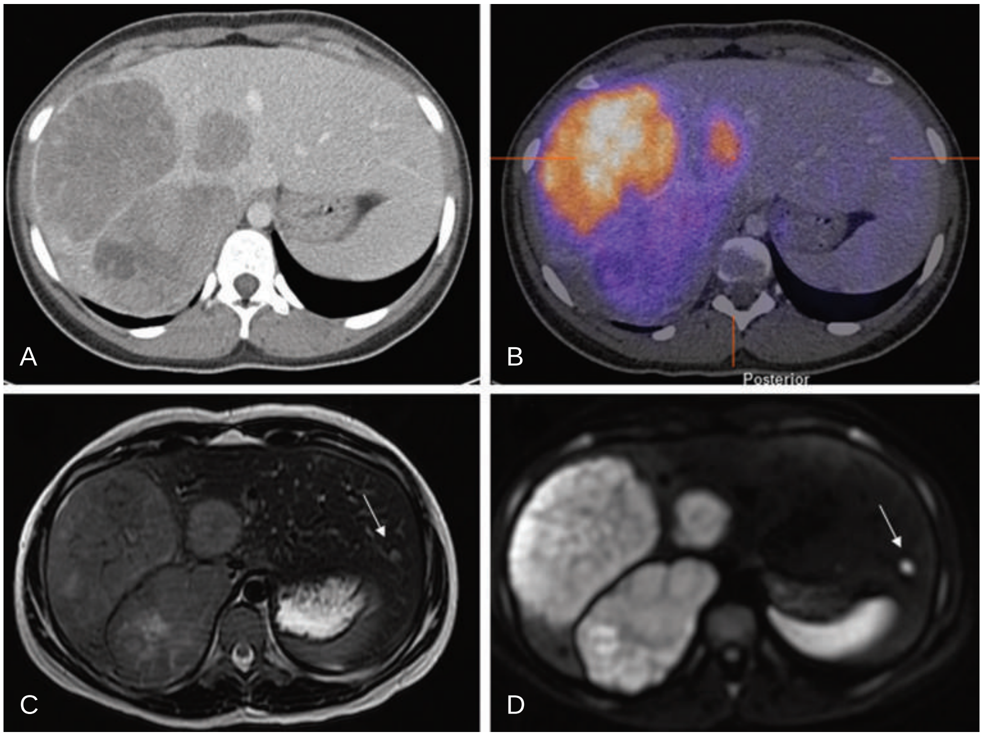

Figure 66-5 A 17-year-old adolescent with adrenocortical carcinoma primarily involving the right lobe. A small, metastatic deposit in the left lateral segment of the liver was not detectable by CT (**A**) or PET (**B**), is barely detectable on conventional T2-weighted MRI (**C**, *arrow*), but is easily seen on DWI (**D**, *arrow*).

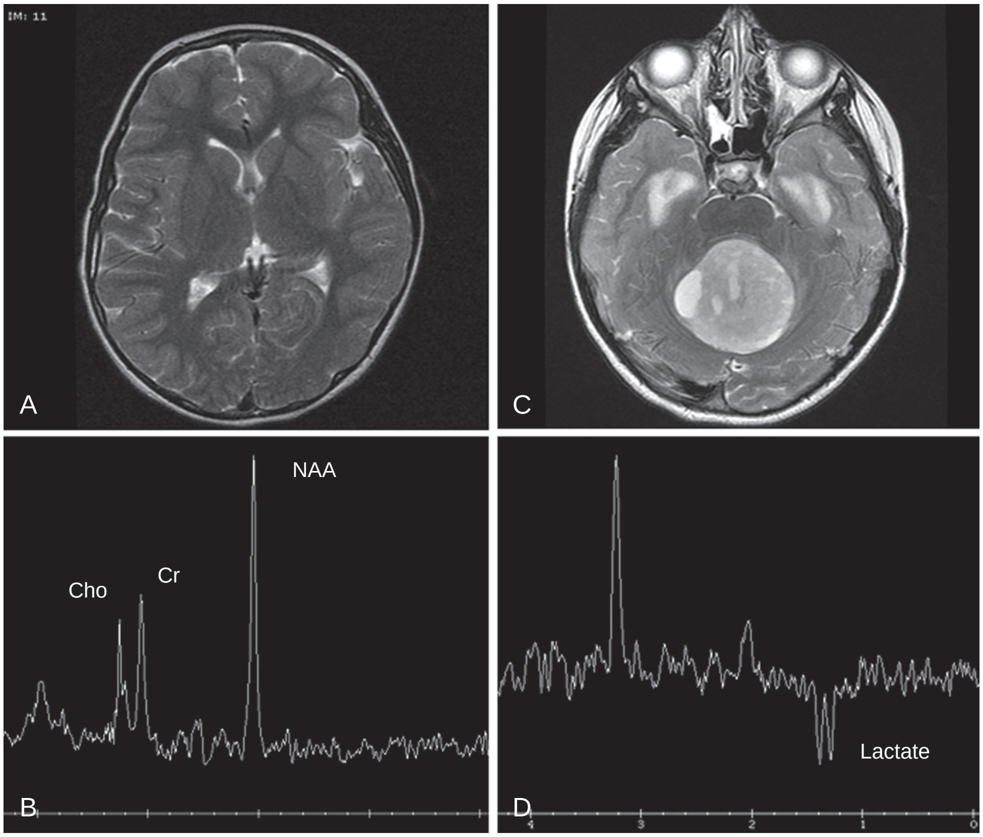

Figure 66-6 MRI and MR spectroscopy in a patient with medulloblastoma (**C** and **D**) compared with the normal MRI and spectrum on the left (**A** and **B**), demonstrating the increased Cho peak, decreased/absent NAA peak, and lactate peak characteristically seen in malignant lesions. *Cho,* Choline; *Cr,* creatine; *NAA,* N-acetylaspartate.

Contrast-enhanced angiography in particular may be useful as well for characterizing relationships of abdominal and mediastinal masses to critical adjacent vascular structures.

MR spectroscopy is used routinely in the evaluation of CNS tumors, where the metabolites N-acetylaspartate (NAA), creatine (Cr), and choline (Cho) have been defined and have specific and characteristic spectra. The use of spectroscopy, although not entirely diagnostic, adds additional information in evaluation of CNS tumors.[51,52] For example NAA is generally thought to correlate with neuronal density, whereas increases in Cho levels usually signify increases in cell density and membrane turnover, reflecting the relatively rapid metabolic rate and division of actively dividing tumors. NAA peaks are typically decreased or absent in pediatric brain tumors, and a decreased NAA/Cho ratio is a characteristic finding.[53,54] The presence of a prominent lactate peak, which is not usually present in the normal brain spectrum, signifies an increase in cellular hypoxia and necrosis (Fig. 66-6) and has been described in more malignant CNS neoplasms.[52] Because the obtainment of good spectral data relies on relatively static body parts, proton spectroscopy has not been used routinely outside the brain; however, investigations are underway to develop a better understanding of the spectra achieved in pediatric neoplasms outside the CNS.[55]

In addition to the use of spectroscopy and diffusion-weighted imaging for characterization of brain tumors, diffusion MR tractography based on diffusion tensor imaging (DTI) has rapidly become an important clinical tool that can delineate functionally important white matter tracts for surgical planning (Fig. 66-7). Tractography based on diffusion MR utilizes the correlation between water diffusion and brain structure to delineate the course of white matter pathways.[56] White matter tracts in the brain are highly organized into fasciculi comprised of densely packed axons. Axonal membranes, myelin, and other structures affect the pattern of Brownian motion of water within white matter. Tractography involves following the trajectory of the white matter tract from voxel to voxel in three dimensions by assuming that the direction of least restricted diffusion corresponds with the axon orientation.

While quantitative assessments of tissue microstructure are essential for many applications of diffusion MR, these measurements are not directly used for presurgical tractography.[57] Instead, the aim of presurgical tractography is to delineate the position of eloquent pathways such as the motor, sensory, visual, and language tracts. One of the goals of brain surgery is to avoid damage to eloquent cortex and subcortical white matter. DTI is the only noninvasive method available to segment the subcortical course of a white matter tract. Subcortical motor evoked

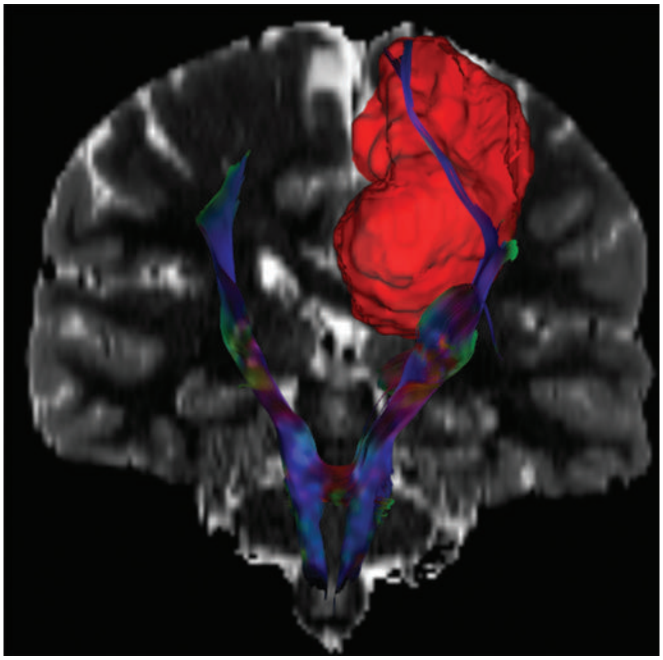

Figure 66-7 Diffusion MR tractography of the corticospinal tracts in a patient with a left-sided tumor showing displacement of the tracts by the tumor (*segmented and shown in red*).

potentials are often combined with tractography to preserve motor function.

Functional MRI (fMRI) using the blood oxygenation level–dependent (BOLD) method allows mapping out of the eloquent areas and is a noninvasive, repeatable, and flexible technique for studying brain function in the clinical setting (Fig. 66-8). It is often critical in surgical planning before resection of brain tumors and has become the standard of care for neurosurgical planning in centers where it is available. Although paradigms to measure eloquent cortices have not been standardized, simple tasks allow visualization of reliable maps for planning neurosurgical procedures. Current research indicates that patient-specific paradigm design can help refine the utility of fMRI for prognostication and recovery of function.[58] It should be noted that certain pathologic conditions and technical issues limit the interpretation of fMRI maps in clinical use and should be considered carefully.

Nuclear Medicine

PET has emerged as an important imaging tool in the evaluation of both adult and pediatric patients with malignancy. A number of textbooks and reviews have been dedicated to this topic and should be consulted for a more in depth discussion of this exciting technology.[59-68] The use of ^{18}F-fluorodeoxyglucose (^{18}F-FDG) has been routine in adult practice and is becoming increasingly commonplace in the evaluation of children with cancer.[69,70,72,128] FDG-PET scanning relies on the differential uptake of glucose by metabolically active tumor cells relative to surrounding tissues. The use of FDG-PET has been shown to be feasible in the majority of pediatric tumors[64,70] and is likely to play an important role in monitoring responses to therapy, particularly with agents that do not produce immediate tumor shrinkage or cellular necrosis but do block specific metabolic pathways. Although flourine-18 (^{18}F) is the most common PET radionuclide used clinically, other agents are being developed and are in preclinical testing, including copper-64 (^{64}Cu)[73,74] and iodine-124 (^{124}I)[75,76] and gallium-68 (^{68}Ga).[76] The use of biomarkers other than fluorodeoxyglucose is starting to emerge clinically, with compounds such as fluorinated thymidine (FLT) allowing deoxyribonucleic acid (DNA) synthesis and cellular proliferation to be directly imaged.[77]

Multiple studies have shown that the use of PET imaging alone, without the simultaneous review of anatomic imaging data acquired either by CT or MRI, results in high sensitivity of lesion detection but has an increased incidence of false positives and an overall decreased specificity. The use of correlative cross-sectional imaging improves the specificity of lesions detected by PET scanning and also increases the sensitivity with which lesions are detected by conventional imaging techniques.[78-80,81,82] As a result, integrated PET/CT scanners have rapidly replaced stand-alone PET imaging equipment in most centers, allowing the PET scan and CT scan to be obtained on the same instrument with the patient in the same position and orientation. This has revolutionized the use of PET scanning in evaluation of oncology patients, and in many institutions it is considered standard of care for PET scans and CT scans to be reviewed simultaneously by skilled radiologists and nuclear medicine physicians, who use the complementary information available from both imaging modalities to achieve greater confidence in rendering a diagnosis.[79,83] Current state-of-the

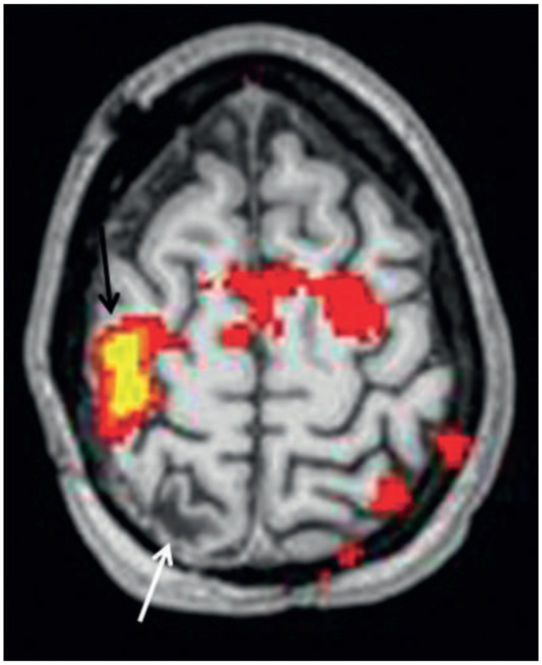

Figure 66-8 Functional MRI (fMRI) in an 18-year-old patient with a history of right posterior parietal oligoastrocytoma resection *(white arrow)*, now showing epilepsy localized to around the surgical cavity. fMRI activation using left-finger tapping shows robust, right-lateralized activation of the primary motor cortex *(black arrow)* and subtle activation of midline supplementary motor cortex.

art equipment utilizes high-sensitivity multirow PET detectors and multidetector-row CT scanners, allowing imaging to be performed more rapidly, with greater sensitivity, and, depending on the equipment, can incorporate respiratory and cardiac gating functions to improve image quality. Postprocessing techniques, relying on newer software, also allow the functional data from PET scanners to be fused to MRI images acquired separately, much in the same way that the PET images are fused to their correlative CT data.

In 2011 the first integrated PET/MRI scanners were approved by the FDA for clinical use and have now been installed at a number of major academic centers in the United States, Europe, and Asia. As with PET/CT, the PET/MRI scanners allow the PET scan and MRI scan to be simultaneously acquired.[84-86] The advantages include lower radiation exposure, the ability to integrate quantitative MRI data with concurrently acquired functional PET data, and the increasing use of MRI for characterization of many pediatric malignancies. As additional PET tracers become available as well, PET/MRI is likely to have a significant impact on the imaging evaluation of pediatric oncology patients.[87]

In addition to PET scanning, conventional γ-emission planar and SPECT imaging is still an important element of pediatric oncology imaging.[88-90] In particular, ^{123}I-metaiodobenzylguanidine (MIBG) and technetium-99m (^{99m}Tc)-methylene diphosphonate (MDP) bone scintigraphy are routinely used in the evaluation of neuroblastoma patients.[91] Bone scintigraphy is also an important component of the sarcoma patient evaluation.[90] As with PET scanning, SPECT images can be fused to anatomic data acquired by either CT or MRI in order to provide correlative information and to confirm sites of abnormality and clarify areas of equivocal disease.[92] Integrated SPECT/CT systems are also available, which allows both SPECT and CT images to be acquired concurrently. Attenuation and scatter correction techniques can be used to further optimize SPECT image quality and potentially reduce tracer dose, as well as improve the accuracy of lesion colocalization.[93] In many instances performing the SPECT and CT simultaneously may also obviate the need for two sedations, further enhancing patient safety and the overall imaging experience.

STAGING CONSIDERATIONS

A detailed discussion of the different staging classifications used in pediatric oncology is beyond the scope of this chapter. Specific staging considerations are discussed in the disease-specific chapters elsewhere in this text. It is important to note, however, that there are unique staging systems in pediatric oncology, such as the International Neuroblastoma Staging System (INSS), the Wilms tumor staging system, the Hodgkin lymphoma staging system (Ann Arbor Staging), the non-Hodgkin lymphoma staging system (St. Jude Classification), and the Pretreatment Extent of Disease (PRETEXT) staging system for hepatoblastoma. Because these different staging systems do not simply rely on tumor size and nodal spread in determining disease stage (i.e., tumor-nodes-metastasis [TNM] staging), the imaging evaluation must be tailored to reflect the type of malignancy being evaluated. The radiologist should work closely with the oncologist during the review of the initial imaging data in order to accurately determine the patient's disease stage and risk classification.

CENTRAL NERVOUS SYSTEM TUMORS

Primary CNS malignancies are the most common pediatric solid tumors, exceeded only by leukemia as a cause of pediatric cancer.[94,95] Although many of the pediatric CNS tumors are also seen in adults, there are several tumors that are unique to infancy and childhood and have characteristic imaging features and intracranial locations. The imaging evaluation should be directed at characterizing the location of the lesion (supratentorial or infratentorial) and determining whether the lesion is intraaxial or extraaxial. CT is usually the first imaging study performed to investigate children with suspected CNS tumors. Although the role of CT has been diminished by the increased use of MRI for imaging the brain, CT is still widely available and readily accessible in nearly all institutions. CT can effectively identify foci of hemorrhage or necrosis and can identify critical abnormalities such as brain edema or impending brain herniation. CT may also provide clues as to the histologic nature of the tumor, for example showing either a cystic or solid mass or the presence of subtle calcifications. Bone invasion into the adjacent skull is typically better depicted by CT, and particularly lesions at the base of the skull should include an evaluation by CT.

MRI of the brain, as with other parts of the body, provides enhanced spatial resolution and, with the variety of newly developed pulse sequences and imaging coils, can give highly specific functional and anatomic information about the lesion in question and the condition of the surrounding brain.[52,96] MR angiography can be performed during the same imaging evaluation and allows assessment of both arterial and venous systems. Obstruction to CSF flow can also be directly evaluated by MRI using specialized techniques aimed at monitoring CSF flow dynamics.

As described, advanced imaging techniques including MR spectroscopy, fMRI, and DTI[52] allow biochemical and metabolic characterization of focal lesions, assessment of functional activity in the brain, and direct evaluation of the structural integrity of CNS white-matter tracts, respectively. FDG-PET and PET scanning with newer radiotracers such as ^{18}F-thymidine may also be of additional value in delineating postsurgical margins from residual tumor and in predicting response to therapy.[97,98]

There are several classification systems for pediatric brain tumors. The most commonly used World Health Organization (WHO) classification of intracranial tumors is based on histopathologic characteristics and clinical course, with tumors further classified based on their intracranial location, specifically whether lesions are supratentorial or infratentorial. This classification system both allows a practical assignment of differential diagnoses to new CNS tumors based on their location and provides prognostic information related to histologic subtype and grade when this additional data becomes available.

Supratentorial Tumors

Astrocytomas

The most common supratentorial brain tumor is the astrocytoma, representing between 50% and 60% of all primary pediatric brain tumors.[99] Astrocytomas are seen in all age groups, and presenting symptoms are typically of intracranial pressure, with headache, nausea, and vomiting commonly seen. Astrocytomas can be reliably diagnosed by CT and MRI (Fig. 66-9). They range from benign to malignant in their histologic grade, with grade 1 lesions classified as benign, grade 2 as low-grade glioma, grade 3 as anaplastic glioma, and grade 4 as glioblastoma. The lesions are usually quite large with both solid and cystic components. Calcifications can occur but are unusual. Parietal, frontal, and temporal lobes are common sites of astrocytoma. On fluid-sensitive sequences, the cystic component of the lesion may show features of complex fluid, with decreased signal intensity relative to CSF on T2-weighted sequences. After contrast infusion, homogeneous enhancement of the solid component of the lesion is characteristic. Higher-grade, more malignant tumors often have less distinct margins, more heterogeneous enhancement, and increased peritumoral edema and mass effect.[100] Newer techniques including perfusion-weighted MRI and MR spectroscopy have been evaluated as noninvasive techniques to determine tumor angiogenesis and capillary permeability. For example, in higher-grade gliomas increased blood flow to the tumor lesion has been demonstrated using this technique.[101] Also, as the tumor grade increases to a more malignant variant, there is an increase in the Cho-to-NAA ratio, reflecting the increased metabolic activity present in higher grade lesions.[101,102] Complete surgical resection is often not possible because of involvement of adjacent major neural structures. The 5-year survival rate for low-grade astrocytomas is between 40% and 50%, with less than 5% survival for the higher-grade malignancies. Because of a propensity to spread to the spine via the CSF, the staging evaluation must also include imaging of the entire spine.

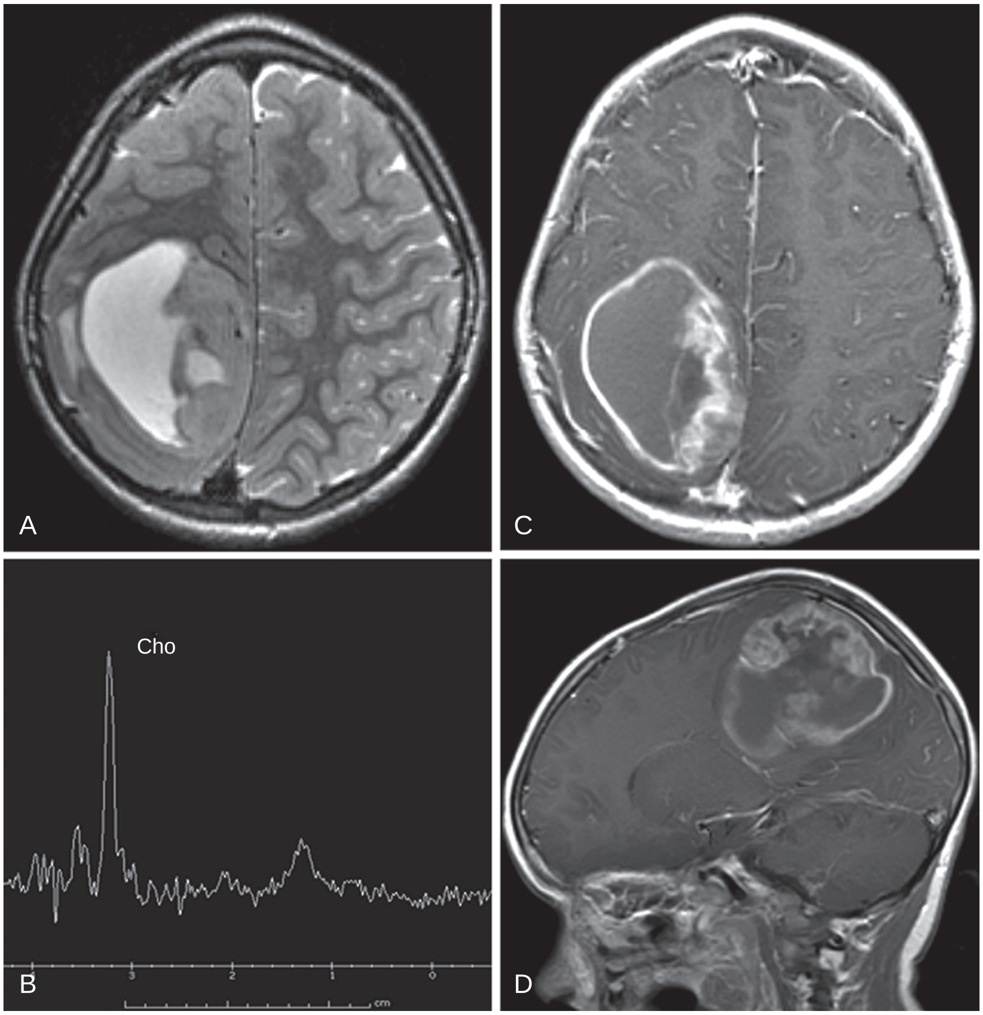

Figure 66-9 A 4-year-old patient with hemispheric astrocytoma. T2-weighted (**A**) and post-Gd T1-weighted images (**C** and **D**) show cystic and solid mass with peripheral enhancement located in the frontoparietal region. MRS from the solid component is notable for decreased N-acetylaspartate and elevated choline *(Cho)* and lipid peaks, typical of a highly cellular tumor (**B**).

The infratentorial/cerebellar form of astrocytoma, also known as *juvenile pilocytic astrocytoma,* is distinct from the diffuse supratentorial astrocytoma discussed here and is described in further detail below.

Oligodendrogliomas

Oligodendrogliomas, although common in adults, are relatively rare in children and adolescents.[103] The most common location is in the frontotemporal region, with less common involvement of the posterior fossa and spinal cord. Imaging evaluation shows a hypodense lesion on CT. Because the majority of these contain varying degrees of calcification, they are distinct as being the most common supratentorial tumor in childhood to calcify. The noncalcified solid component is typically isodense to the adjacent brain on CT. On MRI, the lesions are characterized by low signal intensity on T1-weighted images, hyperintensity on T2-weighted images, and minimal enhancement after infusion of gadolinium contrast (Fig. 66-10). Although these tend to be slow growing lesions, depending on their size there may be edema evident in the surrounding brain.

Gangliogliomas

Gangliogliomas and ganglioneuromas comprise about 5% of the pediatric brain tumors.[99] As with their counterparts in the peripheral nervous system, calcification is seen in up to 40% of the cases. The temporal lobes, frontoparietal lobes, and hypothalamic regions are the most common sites of involvement. The brainstem, posterior fossa, and spinal cord are less commonly involved. MRI demonstrates a low signal-intensity lesion on T1-weighted images with hyperintensity on T2-weighted images and hyperintense homogeneous enhancement after gadolinium infusion (Fig. 66-11).

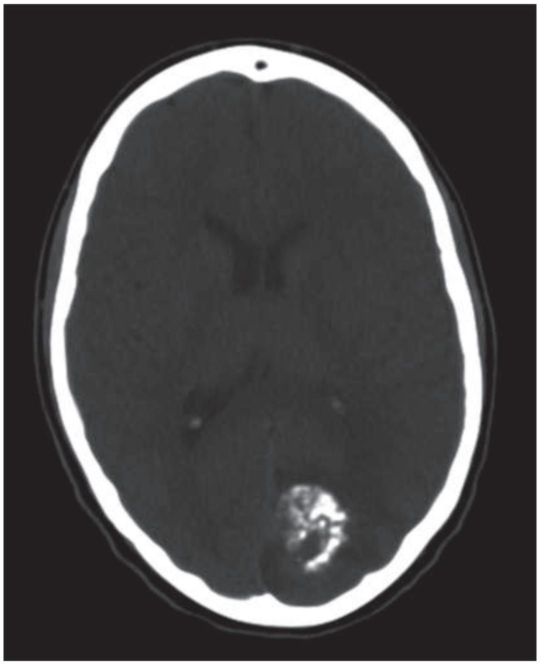

Figure 66-10 Oligodendroglioma. CT showing calcified low-attenuation lesion in the occipital cortex, with imaging features characteristic of oligodendroglioma.

Tumors of the midline deep gray matter, including the basal ganglia, thalamus, hypothalamus, and chiasmatic/optic pathway region tend to be astrocytomas.[95,104] As with the cerebral hemispheric astrocytomas, tumor grades range from benign to highly anaplastic. The imaging features are similar to the lesions located in the cerebral hemispheres with decreased signal intensity on T1-weighted images, hyperintensity on T2-weighted images, and variable contrast enhancement. Because of their location, behavioral changes, emotional and memory changes, movement disorders, visual abnormalities, and hydrocephalus are all common symptoms associated with these deep gray-matter tumors. Surgical resectability is often not possible, and the imaging evaluation is directed at identifying involvement of adjacent critical structures and monitoring posttherapy changes.[95]

Intraventricular tumors include choroid plexus papillomas, neurocytomas, and dermoid/epidermoid tumors. Choroid plexus papillomas/carcinomas are relatively common, making up between 10% and 20% of the intraventricular tumors identified in the first year of life and comprising approximately 5% of all CNS neoplasms.[103] The imaging features are characteristic and typically reveal large lobulated intraventricular lesions (Fig. 66-12). These tumors are highly vascular and are often associated with calcification and occasionally hemorrhage.[100,104] Depending on the size and location of the lesion, there may be obstructive hydrocephalus. MRI shows a low signal-intensity lesion on T1-weighted images with variable hyperintensity on T2-weighted images. Magnetic susceptibility is seen if hemorrhage is present, and after contrast infusion these lesions typically show marked contrast enhancement. The combination of a lobulated, intensely enhancing intraventricular lesion is nearly diagnostic of choroid plexus papilloma. Five percent to 10% of these lesions degenerate into choroid plexus carcinomas,[104] and this latter diagnosis is suggested by accompanying necrosis within the lesion and metastatic spread into the adjacent brain, either by direct extension into the adjacent brain or by CSF dissemination. Because of their intraventricular location, metastatic spread throughout the CNS via CSF dissemination is unfortunately common for choroid plexus carcinomas, resulting in a uniformly poor prognosis.[95]

Central Neurocytomas

Neurocytomas are rare in children and have imaging features that mimic oligodendroglioma, showing an intraventricular calcified mass with heterogeneous contrast enhancement. These tumors typically involve the lateral ventricle in the midline; however, surgical resection is necessary to distinguish this lesion from other intraventricular tumors such subependymal astrocytomas and ependymomas.

Midline Supratentorial Tumors

Tumors of the hypothalamic/suprasellar region include craniopharyngiomas, hypothalamic hamartomas, and pineal region tumors.

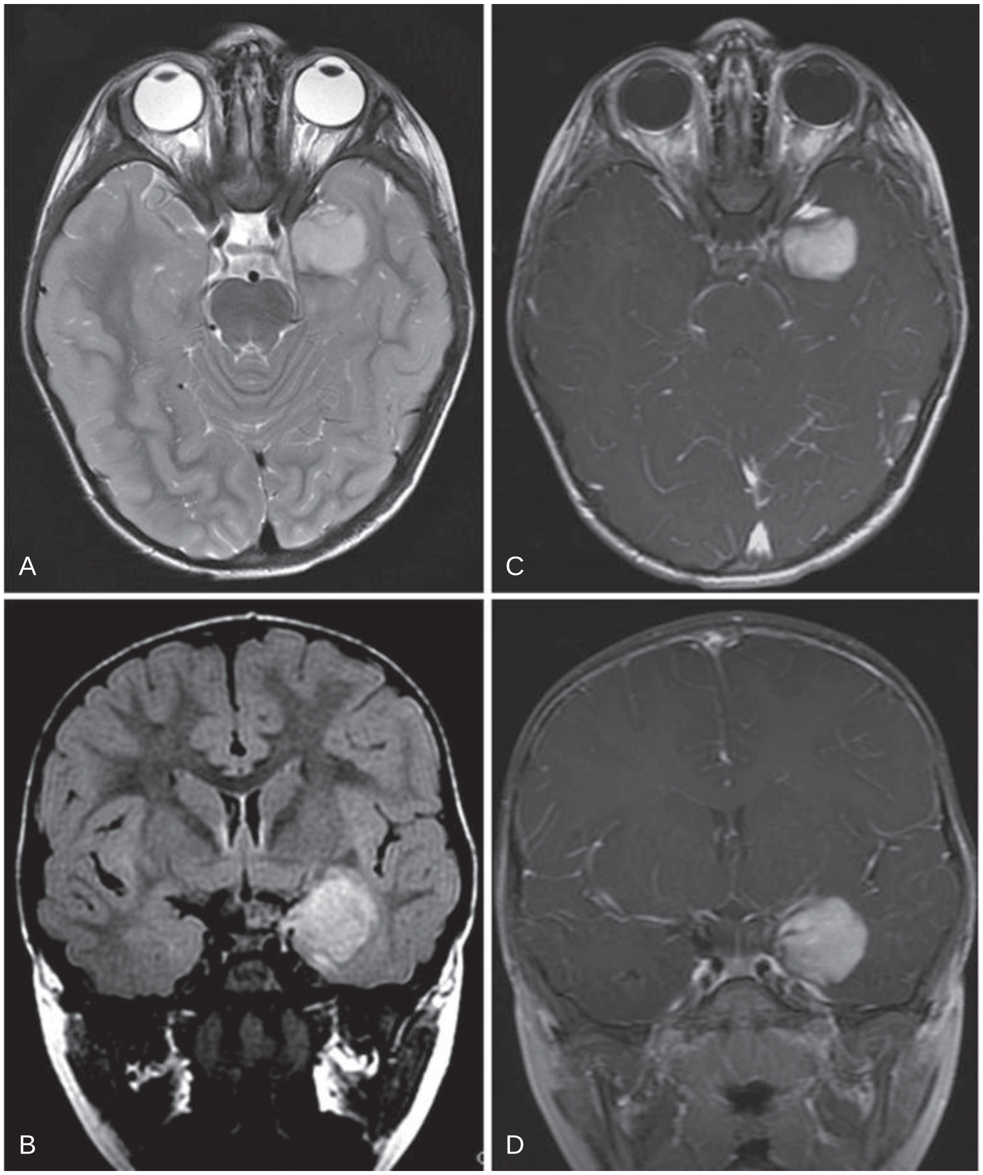

Figure 66-11 Ganglioglioma. A 4-year-old patient with left temporal-lobe ganglioma showing increased signal on T2-weighted images (**A**), and bright enhancement after gadolinium administration (**C** and **D**). Depending on their location, these lesions may be more conspicuous on fluid-attenuated FLAIR images (**B**).

Craniopharyngiomas

Craniopharyngioma is the most common suprasellar tumor.[100,104,105] It has fairly characteristic imaging features with lesions containing solid, cystic, and calcified elements. Symptoms usually result from increased intracranial pressure and local effects upon the hypothalamic pituitary region and optic chiasm. Because these are relatively slowly growing lesions, they may be quite large at diagnosis. The presence of a calcified suprasellar mass at CT scan is usually suggestive of the diagnosis; however, MRI is superior in evaluating the extent of involvement (Fig. 66-13). Depending on the type of material present in the cystic component of the mass, variable signal intensity on T1- and T2-weighted images may be seen.[106] It is common, for example, for the complex proteinaceous, cholesterol-laden fluid to be variably hyperintense on both T1- and T2-weighted images (see Fig. 66-13). After contrast infusion, the cyst wall commonly enhances. However, the solid components of the cyst often demonstrate inhomogeneous enhancement as well. Of note, craniopharyngiomas commonly cause T2 prolongation along the optic tracts. This is a nonspecific sign and has been reported in other tumors centered in this region. It is thought to signify optic tract edema caused by compression by the tumor.[107]

Pineal-Region Tumors

Pineal-region masses are characterized by their location in the quadrigeminal plate cistern. These tumors typically arise from primordial germ cells and include pineoblastomas, pineocytomas, and immature germ-cell tumors.[95,100,103,104] It is important to distinguish these

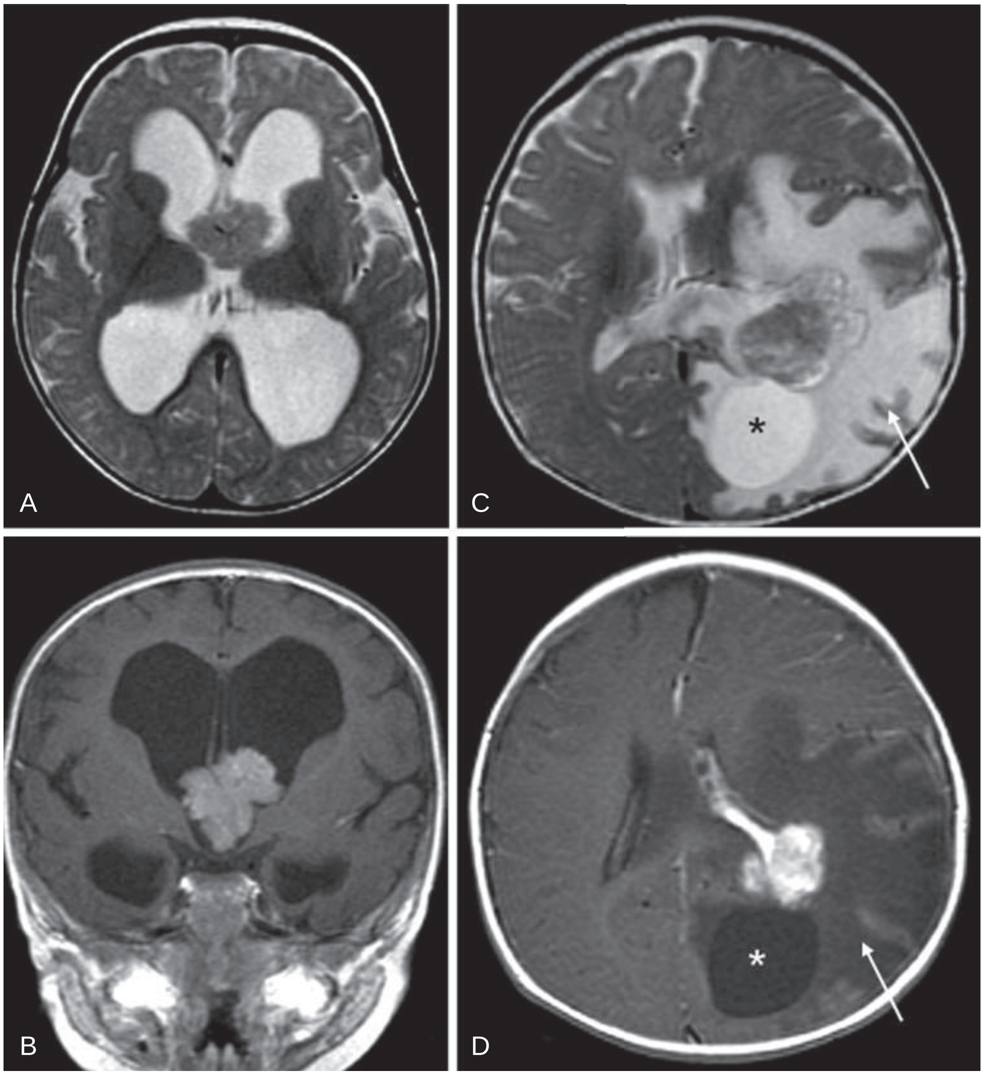

Figure 66-12 Choroid plexus papilloma. 8-month-old and 10-month-old infants, both with large lobulated lesions causing obstructive hydrocephalus. **A** and **B**, In one patient the tumor is centrally located, producing severe global hydrocephalus. **C** and **D**, In the other patient the peripheral location is accompanied by extensive vasogenic edema *(arrow)* and left occipital-horn hydrocephalus *(asterisk)*.

neoplastic lesions from benign pineal cysts, which are commonly seen within the pineal gland. Pineal cysts have a characteristic appearance on both CT and MRI and do not distort or compress the adjacent ventricles. Simple pineal region cysts typically do not achieve sizes greater than 1 cm. Also of note is normal calcification within the pineal gland. Calcification is unusual before the age of 10 but is commonly seen in adolescents and older children.[108] Therefore the presence of calcification in the pineal region in an infant or young child should raise suspicion for a pineal region mass/germ-cell tumor and prompt further investigation.

Pineal-gland tumors, including pineocytomas and pineoblastomas, are relatively rare. Pineocytoma is benign and usually nonaggressive, whereas the pineoblastoma, representing an immature undifferentiated lesion, often metastasizes. Pinealoblastoma is also a component of the "trilateral retinoblastoma" and is seen with increased incidence in patients diagnosed with bilateral retinoblastoma. Pineocytomas are more often more calcified than pinealoblastomas. Pinealoblastomas are typically larger with low signal intensity on T1-weighted images, variable hyperintensity on T2-weighted images, and fairly homogeneous contrast enhancement (Fig. 66-14).[109] Very large pinealoblastomas frequently produce mass effect on the adjacent quadrigeminal cistern, and because of their large size often show areas of necrosis.

Germ-Cell Tumors

The pineal region is the most common location for intracranial germ-cell tumors.[110,111] Histopathologically these are most often germinomas and are usually associated with calcification. This is well demonstrated by CT. MRI is indicated to better delineate the extent of the noncalcified solid portion of the mass as well as heterogeneous cystic portions that may also be present. On T2-weighted images, the solid portion is typically hyperintense with bright enhancement after contrast infusion. Other tumors

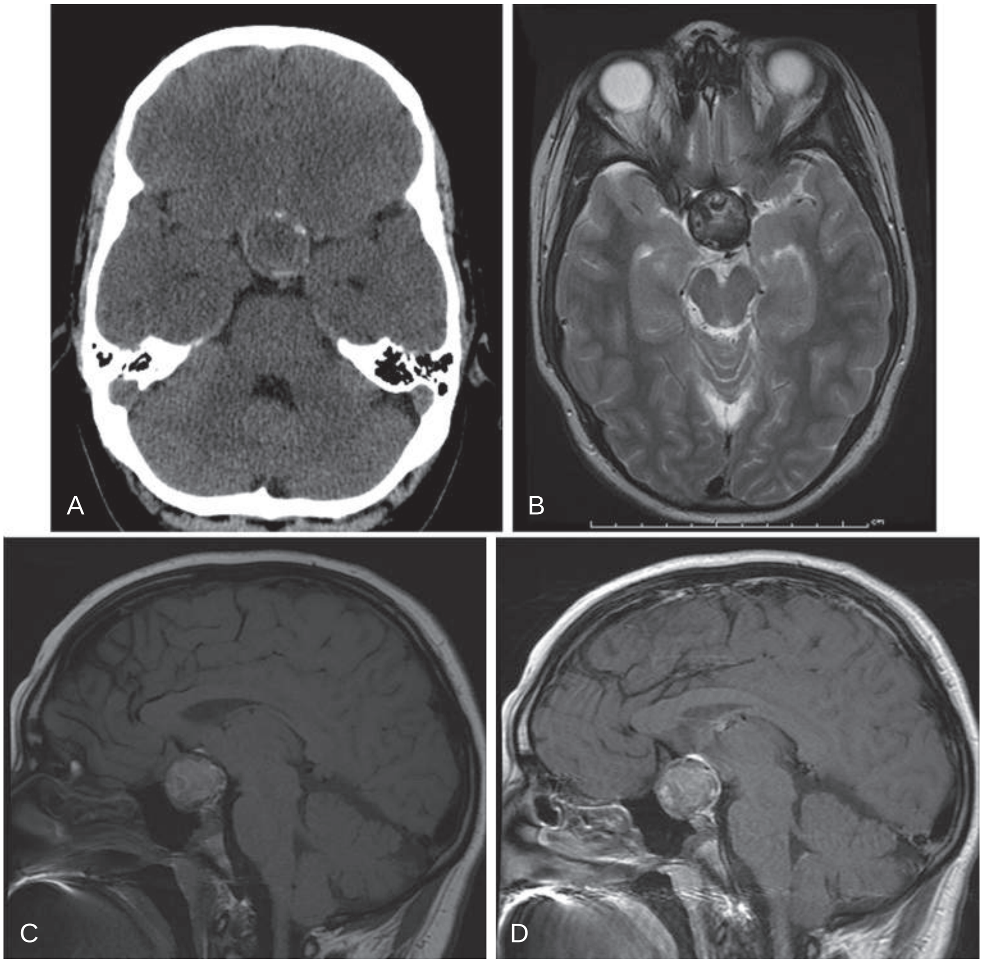

Figure 66-13 Craniopharyngioma. CT showing calcified suprasellar mass (**A**), with heterogeneous increased signal on both T2-weighted images (**B**) and T1-weighted images (**C**), with peripheral enhancement after gadolinium (**D**).

of germ-cell origin are also seen, with choriocarcinomas more commonly showing hemorrhagic components and displaying more aggressive features. Benign teratomas, in contrast, display imaging features more in keeping with their well-differentiated dermoid/epidermoid components, including low attenuation fluid on CT, hyperdense fluid on T1-weighted sequences, and heterogeneous contrast enhancement (Fig. 66-15).

Hypothalamic Hamartoma

This is a rare congenital lesion, usually identified in infants under the age of two. Seizure disorders are common, and these patients often have the presenting symptoms of intractable gelastic seizures. The presence of precocious puberty or diabetes insipidus may provide clinical clues to suggest this diagnosis.

On imaging, CT scanning shows an isodense mass in the region of the hypothalamus. There is little to no contrast enhancement either on CT or MRI.[112] On MRI, the lesion is usually isodense to the brain on T1-weighted images, minimally hyperintense on T2-weighted images, and interestingly may be hyperintense on FLAIR images (Fig. 66-16). Because of the location of these tumors, surgical resection may be challenging, and MR- or CT-guided ablation techniques are now increasingly being used (Fig. 66-17).

Infratentorial Tumors

The most common lesions of the posterior fossa/cerebellum are juvenile pilocytic astrocytomas (JPAs) and medulloblastomas.[99]

Medulloblastomas

Medulloblastomas are slightly more common in incidence than cerebellar astrocytomas.[95] They typically occur in the first decade of life. Presenting symptoms usually relate to increased intracranial pressure secondary to obstructive hydrocephalus, most often related to mass effect upon the fourth ventricle. Medulloblastomas are highly malignant and have a propensity to disseminate and seed the CNS via the CSF route.[103] Hematogenous spread to other sites may also occur in medulloblastoma.

CT scanning is usually the first imaging study performed to evaluate new-onset nausea, vomiting, headache, or seizures. CT scanning usually demonstrates a hyperintense solid lesion within the cerebellum, often

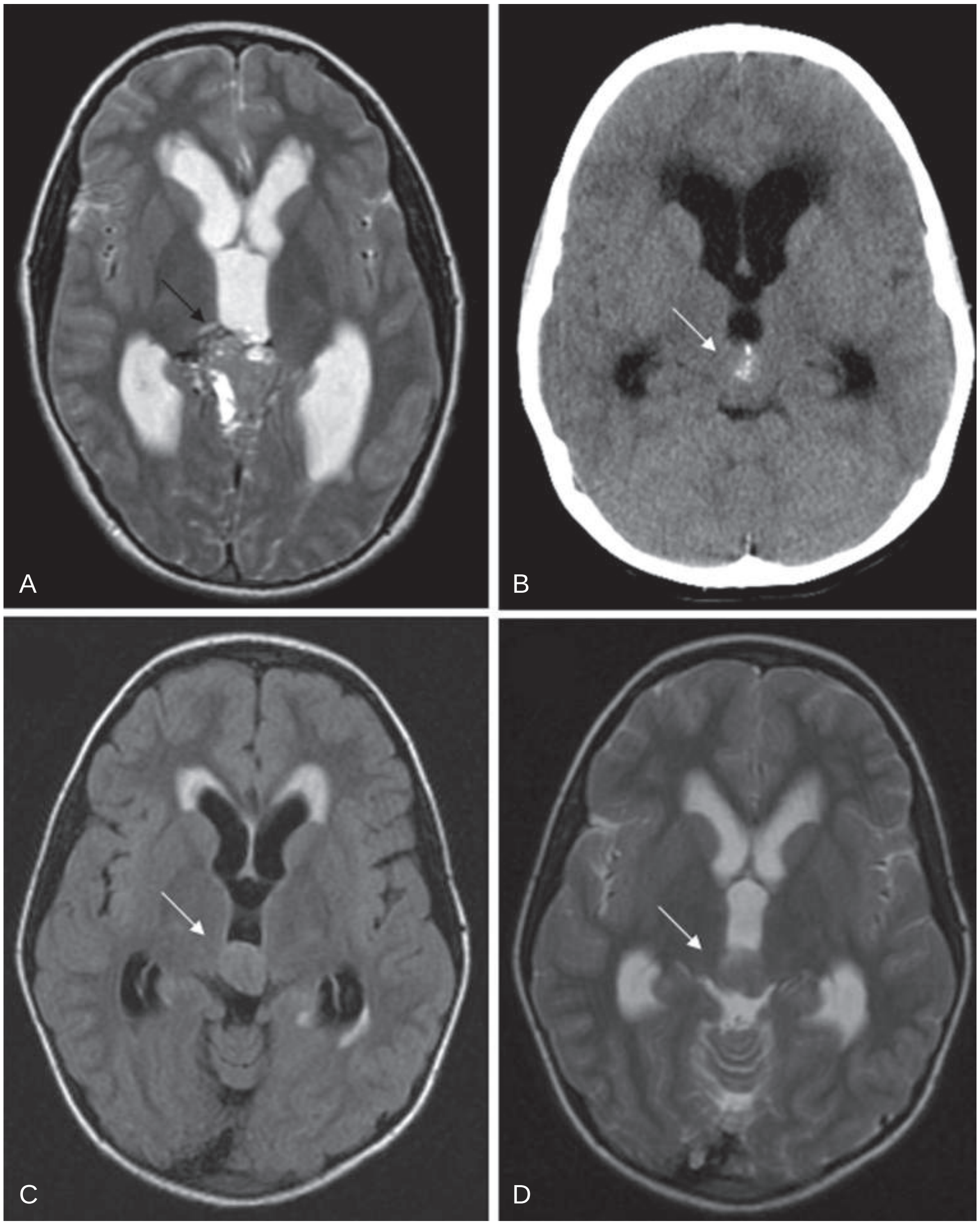

Figure 66-14 Pinealoblastoma. Two patients with pinealoblastoma. The larger cystic/solid mass seen in one patient causes obstruction and hydrocephalus at the level of the quadrigeminal plate cistern (**A**). In the accompanying case (**B**), even the smaller calcified mass causes hydrocephalus as a result of its central location abutting the ventricles. As noted in the text the lesions are often hyperintense on fluid-attenuated sequences (**C**, *FLAIR images*) and isointense to cortex on T2-weighted images (**D**).

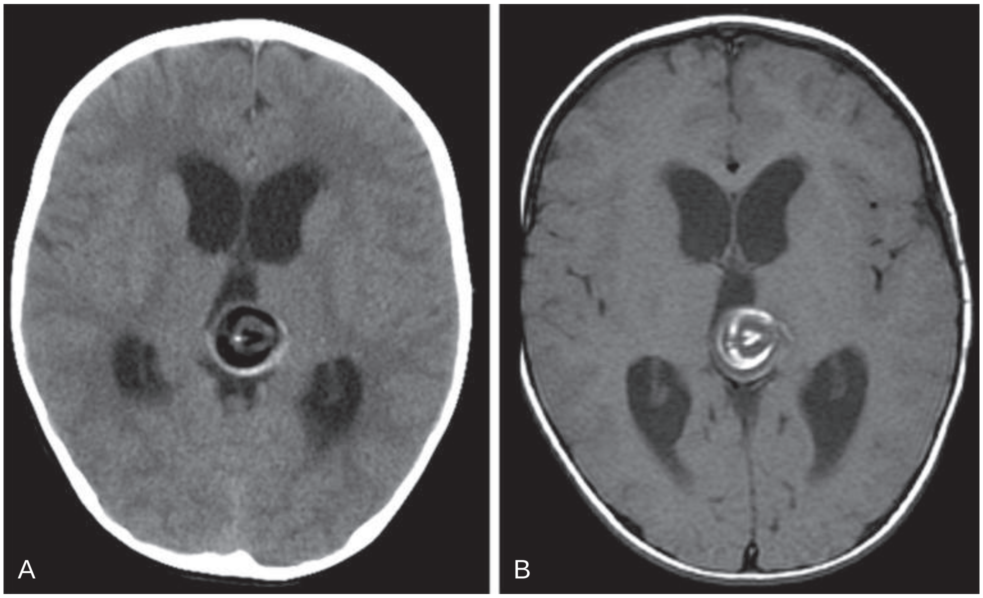

Figure 66-15 Pineal germ-cell tumor. CT (**A**) and T1-weighted axial MRI (**B**) showing low attenuation fluid on CT and hyperintense fluid on T1-weighted sequences, typical of a well-differentiated pineal germ-cell tumor (teratoma).

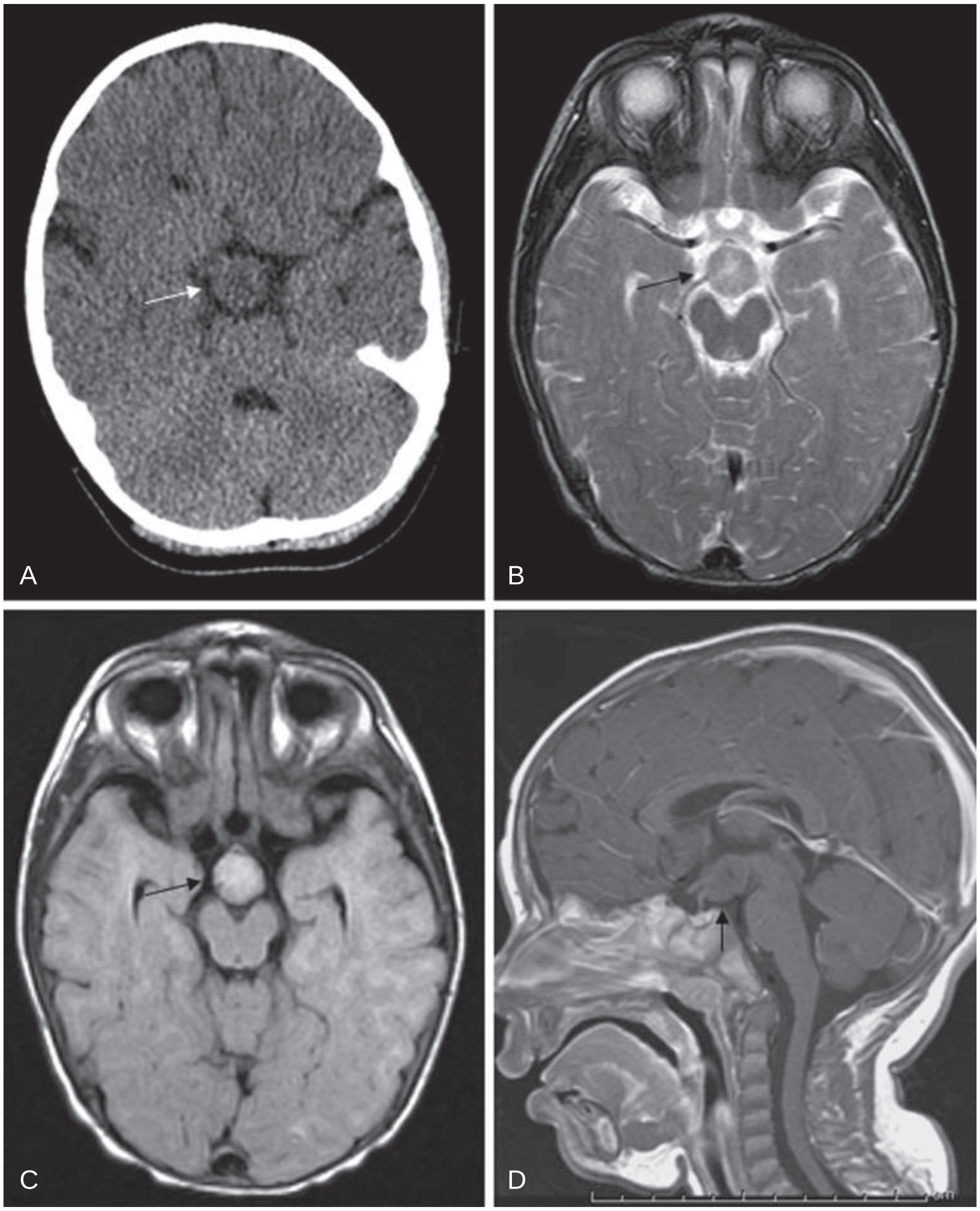

Figure 66-16 Hypothalamic hamartoma. **A**, CT shows a low density suprasellar/hypothalamic lesion. MRI shows the lesion to be isointense to brain on T2-weighted sequences (**B**), mildly hyperintense on FLAIR images (**C**), with no significant enhancement after gadolinium infusion (**D**).

near the midline (Fig. 66-18). MRI imaging is indicated to better characterize the lesions and to establish sites of local and metastatic spread. As with other highly cellular CNS tumors, on T1-weighted images medulloblastomas are usually hypointense relative to the adjacent cerebellum, with increased signal intensity on T2-weighted images (see Fig. 66-18) and homogeneous enhancement after gadolinium infusion.[100,104] Calcification and cystic areas are unusual in medulloblastoma. Because of the propensity to disseminate to other sites via the CNS, the staging evaluation must include a contrast-enhanced evaluation of the entire spine.

Cerebellar Astrocytomas

Astrocytomas are the next most common posterior fossa tumor.[95,99] As with their intracerebral counterpart, cerebellar astrocytomas range in histologic grade from benign to anaplastic. The characteristic juvenile pilocytic form makes up the majority of the cerebellar astrocytomas. Both cystic and solid components are normally present in this type of astrocytoma (Fig. 66-19). Calcification is unusual and occurs in 10% of tumors.[113] After resection, survival is excellent with a 10-year overall survival rate greater than 90%. As with medulloblastoma, CT scanning is usually the first imaging modality used to evaluate initial symptoms. CT shows a heterogeneous, predominantly cystic lesion in the posterior fossa, typically with a small mural nodule eccentrically along the margin of the tumor. These lesions are often midline, near the vermis, but may also be eccentric in location. MRI imaging is indicated to better characterize the extent of cerebellar involvement. Although the presence of a

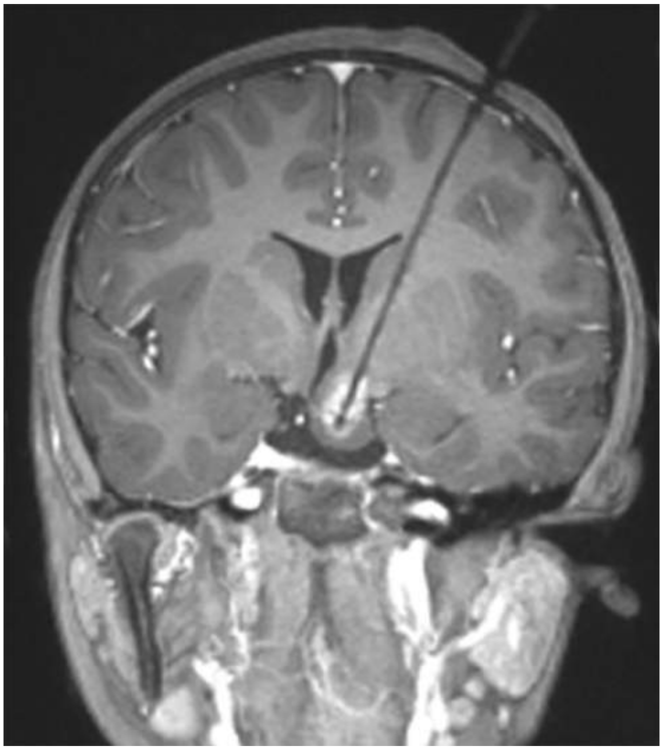

Figure 66-17 MRI-guided laser ablation of hypothalamic hamartoma in a 4-year-old female with medically intractable gelastic seizures. Coronal reformat of a magnetization-prepared rapid acquisition with gradient echo shows the needle tip within the lesion in the hypothalamus with enhancement around the tip indicating the areas that have been ablated by the procedure.

predominantly cystic component suggests a lower-grade neoplasm, foci of local/metastatic spread may be present and will be manifest as a focus of increased enhancement after contrast administration.[103] Therefore the imaging evaluation should include conventional T1- and T2-weighted imaging as well as postcontrast enhanced scanning in all three imaging planes. Because higher-grade neoplasms may also disseminate via the CSF, as with medulloblastoma, the initial staging evaluation should also include evaluation of the entire spine.

Brain-Stem Gliomas

Brain-stem gliomas make up the majority of the neoplasms involving the brain stem. These tumors are of astrocytoma origin and because of their location result in nearly uniformly poor survival.[95] These predominantly cellular solid lesions are best evaluated by MRI, and they typically appear as hypodense lesions on T1-weighted images, with increased signal intensity on T2-weighted images, and show heterogeneous enhancement.[100,103,104] Lower-grade lesions, or lesions with larger cystic components may only show very minimal contrast enhancement (Fig. 66-20). As with the other tumors in these locations, spread is most often via the CSF to other portions of the brain.

Ependymomas

After medulloblastomas and juvenile pilocytic astrocytomas, the next most common posterior fossa tumor is the ependymoma, constituting approximately 8% to 15% of intracranial neoplasms.[99] The majority of these tumors are benign and arise from the ependyma of the fourth ventricle. They typically present as a calcified intraventricular mass. Symptoms result from obstruction of CSF flow at the level of the fourth ventricle leading to obstructive hydrocephalus. There are two histologic types, with the

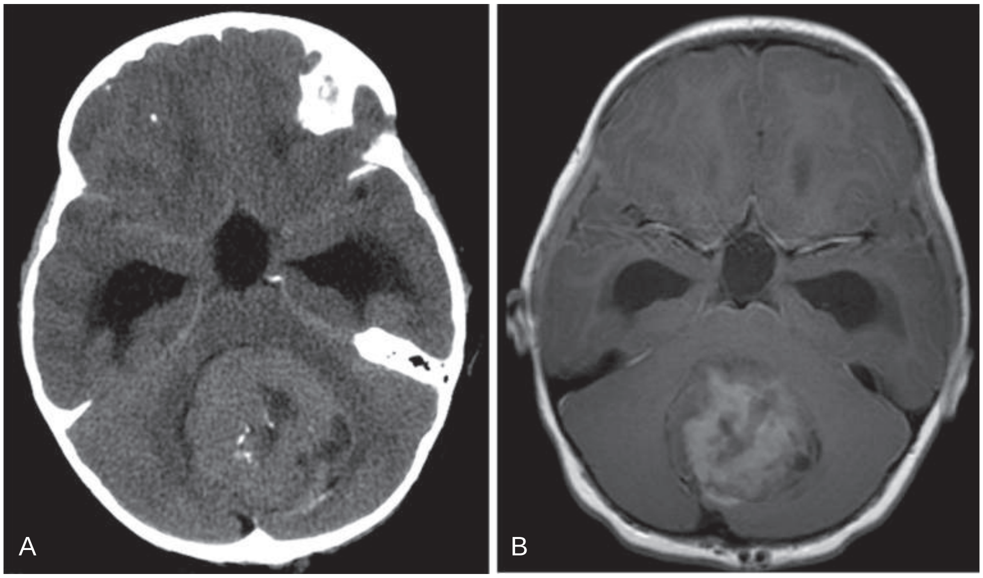

Figure 66-18 Medulloblastoma. **A**, Unenhanced CT shows hyperdense mass in the posterior fossa, compressing the fourth ventricle with resultant obstructive hydrocephalus. **B**, Gadolinium-enhanced T1-weighted MRI shows enhancement of the majority of the mass; these features are all consistent with a highly cellular medulloblastoma.

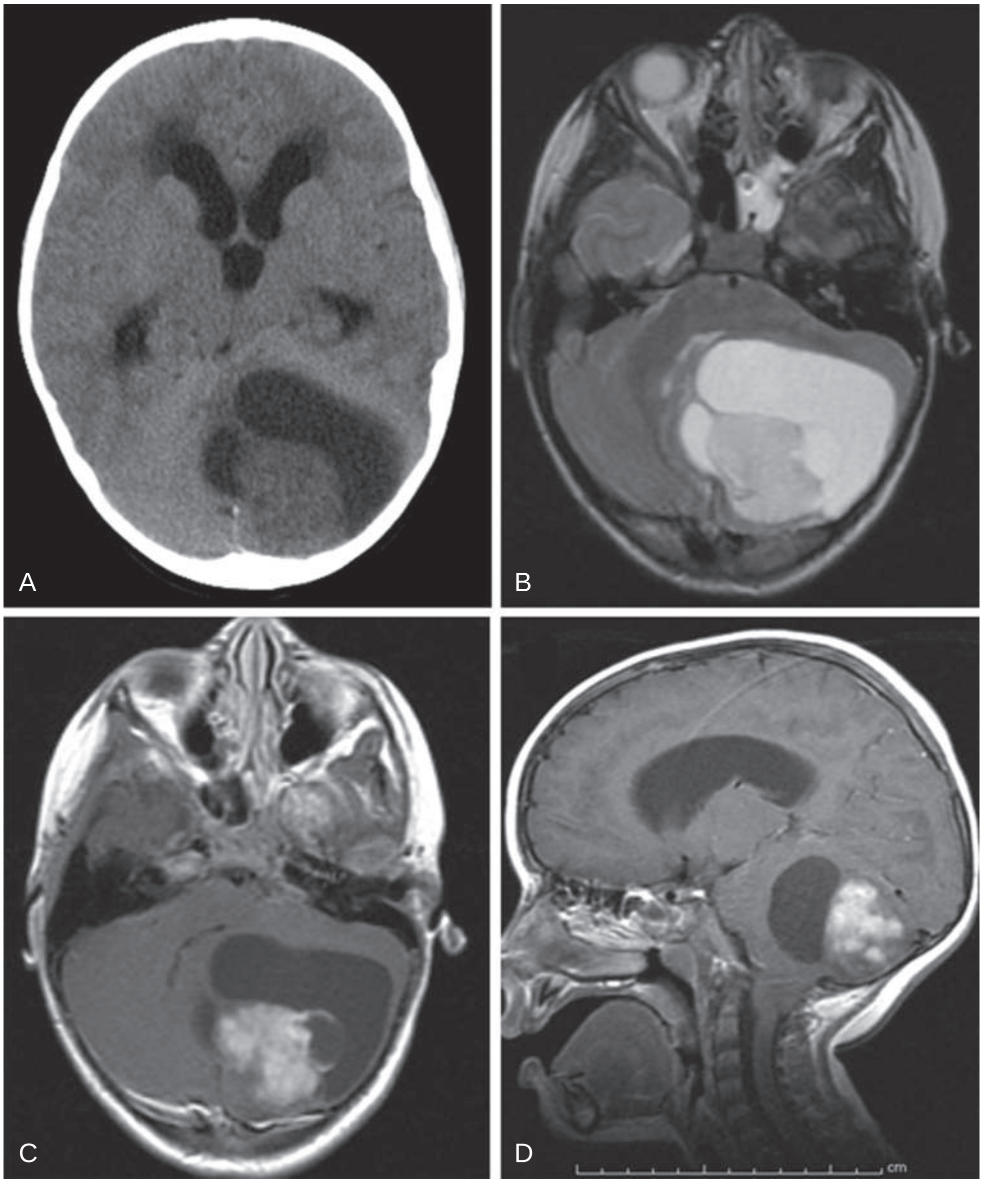

Figure 66-19 Cerebellar pilocytic astrocytoma. **A**, Unenhanced CT shows a large, predominantly cystic mass with a solid mural component in the posterior fossa with moderate hydrocephalus. **B**, T2-weighted MRI shows high signal-intensity cystic fluid and intermediate signal mural nodule. **C** and **D**, Only the mural nodule enhances after gadolinium infusion.

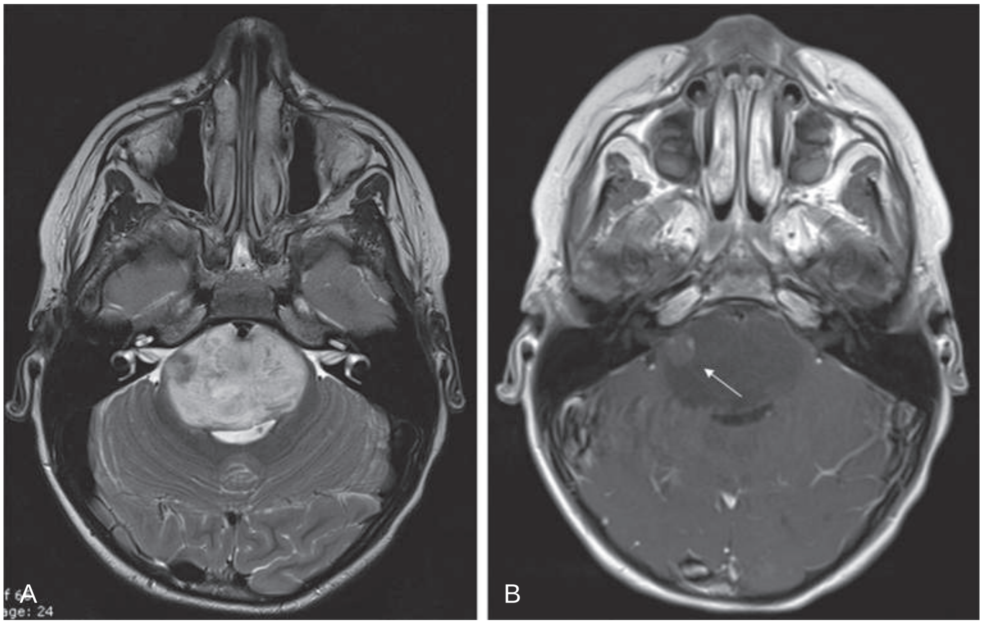

Figure 66-20 Low-grade pontine glioma. Axial T2 (**A**) and post-Gd T1-weighted (**B**) images show T2-bright lesion with faint enhancement of a small nodular component of the tumor along the lateral margin *(arrow)*.

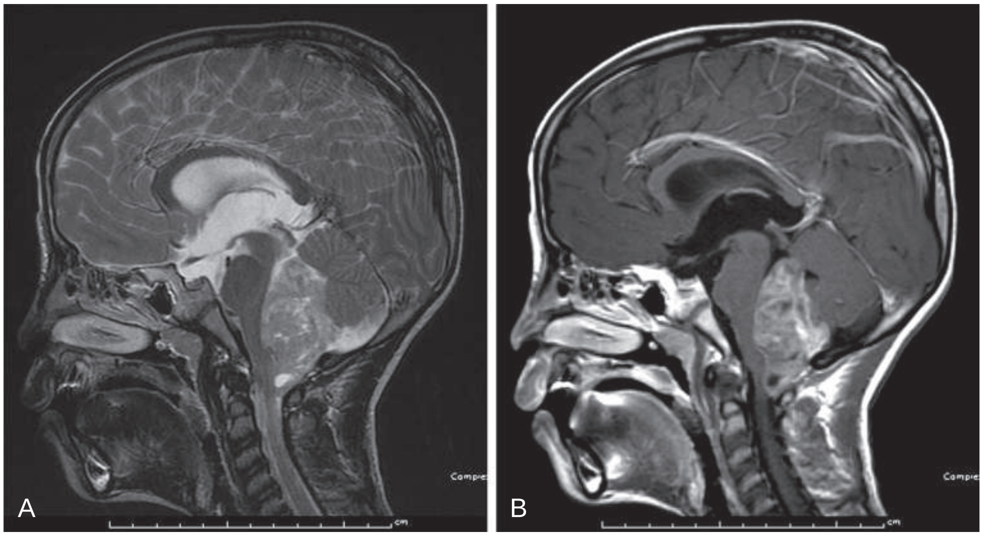

Figure 66-21 Ependymoma. A 7-year-old patient with the symptom of lethargy. T2-weighted (**A**) and gadolinium-enhanced (**B**) MRI show a large heterogeneously enhancing posterior fossa mass compressing the brain stem and beginning to extend out through the foramen magnum.

benign undifferentiated type more common, typically less invasive locally, and with a lower likelihood of CSF dissemination. Malignant anaplastic ependymomas, in contrast, tend to spread via the CSF early in the disease course to other sites within the CNS. Because of their propensity to insinuate through neural foramina, resulting in a lobulated appearance, ependymomas have a fairly characteristic appearance on imaging (Fig. 66-21). Depending on the degree of calcification, these lesions may be predominantly hypointense on T1- and T2-weighted images. In the absence of calcification, these are usually hyperintense lesions on T2-weighted images with heterogeneous intense enhancement after gadolinium infusion.[104] Because of their propensity to seed throughout the CSF and ventricular system, the imaging evaluation must include examination of both the brain and spinal canal.

Hemangioblastomas

Hemangioblastomas are benign vascular lesions that are more common in adults than children. However, the cerebellum is still the most common location in children, and multiple hemangioblastomas may be encountered in familial predisposition syndromes such as the von Hippel-Lindau disease.[114] The imaging features demonstrate a predominantly cystic lesion with an enhancing mural nodule that may be difficult to distinguish from JPAs. As with JPAs, these lesions show cystic characteristics on MRI with low signal intensity on T1-weighted images and T2 hyperintensity, with intense enhancement of the solid mural nodular component (Fig. 66-22). Depending on the size of the mural nodule, the degree of enhancement, and its location, these lesions may also be difficult to discriminate from a benign posterior fossa arachnoid cyst.

Metastatic Disease

Metastatic disease to the CNS may result from leptomeningeal seeding, hematogenous dissemination, or direct extension. Leptomeningeal seeding results from a number of primary brain tumors, most notably medulloblastomas.[115] Leptomeningeal seeding can also result from non-CNS primary tumors and systemic malignancies.[116] The imaging appearance in this setting is similar to that of leptomeningeal dissemination occurring secondary to a primary CNS tumor.

Hematogenous metastases to calvarium or dura are most commonly seen in patients with neuroblastoma, leukemia, and lymphoma. These lesions present as lytic calvarial lesions or as nonspecific, enhancing dural masses. CNS involvement by leukemia is also common, although this is more often diagnosed by lumbar puncture and pathologic evaluation of the CSF rather than imaging.

Hematogenously disseminated metastases to the brain parenchyma are rare in childhood. When they do occur, primary tumors are sarcomas, particularly rhabdomyosarcoma, Ewing sarcoma,[117] and osteosarcoma. A tumor with a particular propensity to metastasize to the brain is the rhabdoid tumor of the kidney, and imaging of the CNS is essential in children with this diagnosis. Parenchymal metastases are usually multiple and located at the interface between the gray and white matter. They are associated with marked vasogenic edema and demonstrate avid enhancement secondary to loss of the blood-brain barrier.

Although the imaging features may be variable depending on the primary malignancy, the presence of any focal area of signal abnormality or enhancement with a known primary tumor should raise suspicion for metastatic spread of disease to the CNS.

Spinal Cord Tumors

In comparison to pediatric brain tumors, spinal cord tumors are relatively rare in children. The majority of the intramedullary spinal tumors in children are low-grade astrocytomas, with the remainder being ependymomas.[118,119] The imaging features of these lesions mimic

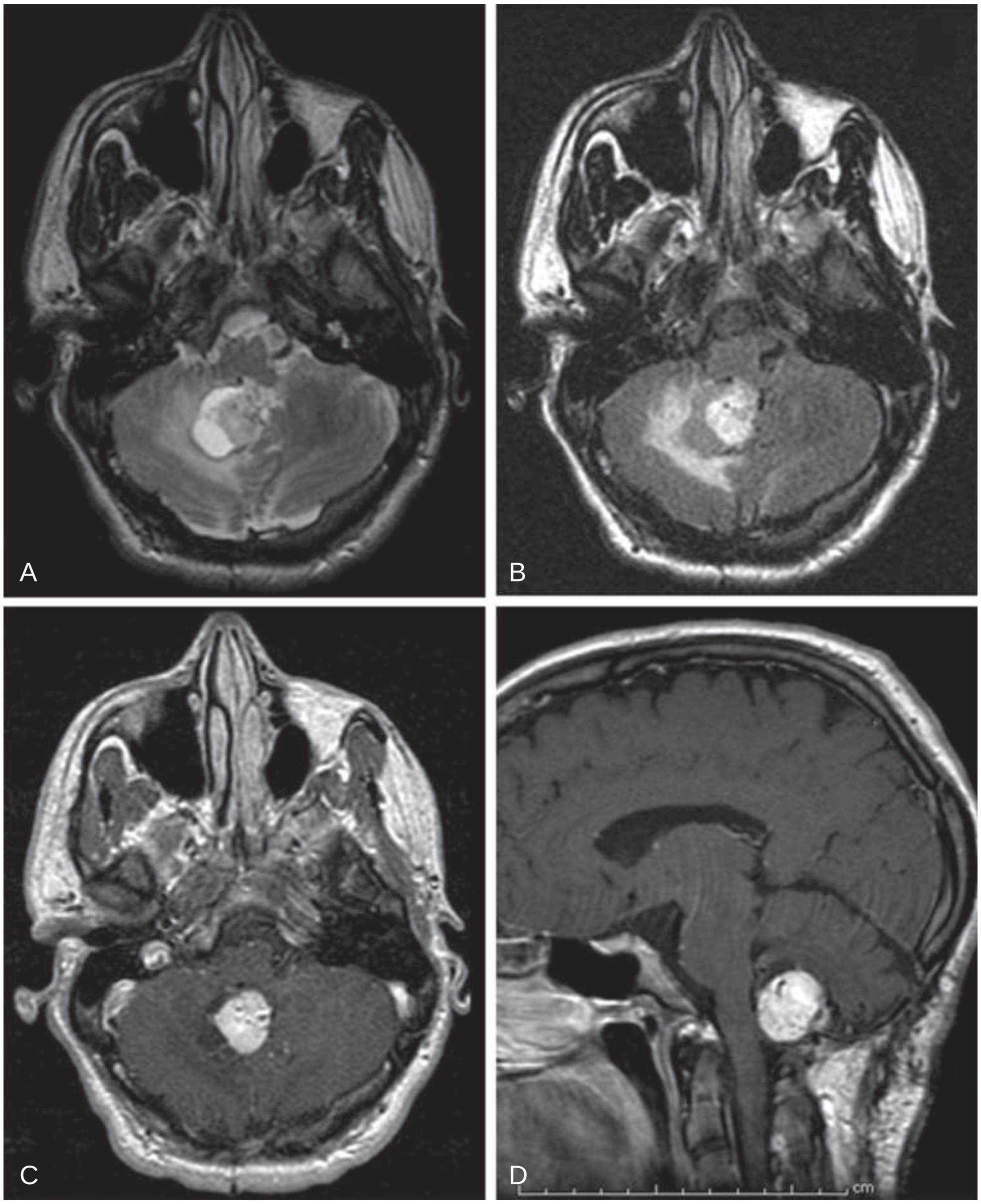

Figure 66-22 Hemangioblastoma. A 28-year-old patient with von Hippel-Lindau disease and cerebellar hemangioblastoma showing typical features on T2-weighted (**A**), fluid-attenuated FLAIR (**B**), and post-gadolinium enhanced images (**C** and **D**).

their intracranial counterparts. The imaging assessment is usually prompted by neurologic symptoms and should include multiplanar T1, T2, and post–gadolinium-enhanced imaging. Because it may be difficult to discriminate a primary intraspinal tumor from metastatic disease, imaging of the brain must also be performed.

As in the brain, the imaging examination should be directed at identifying the site of origin of the tumor. It should be possible to make a distinction between primary intramedullary tumors, extramedullary intradural tumors, and extradural/epidural lesions. This is important, because the majority of the spinal tumors in children are extramedullary. Intradural extramedullary lesions are, by definition, located within the subarachnoid space and are typically the result of drop metastases, which as discussed earlier, spread via the CSF from primary brain tumors (Fig. 66-23). Their appearance on imaging mimics their counterparts in the brain. Extradural extramedullary tumors may arise from local extension of other primary neoplasms such as neuroblastoma, lymphoma, or Ewing sarcoma (Fig. 66-24). In evaluating these other primary pediatric neoplasms, when their location is adjacent to the vertebral column, it is essential that a thorough imaging evaluation be undertaken to evaluate the presence and extent of intraspinal involvement. In particular, with neuroblastoma, this will influence staging and surgical resectability. The presence of intraspinal extension may be suggested by CT, but will be typically better delineated by MRI (Fig. 66-25).

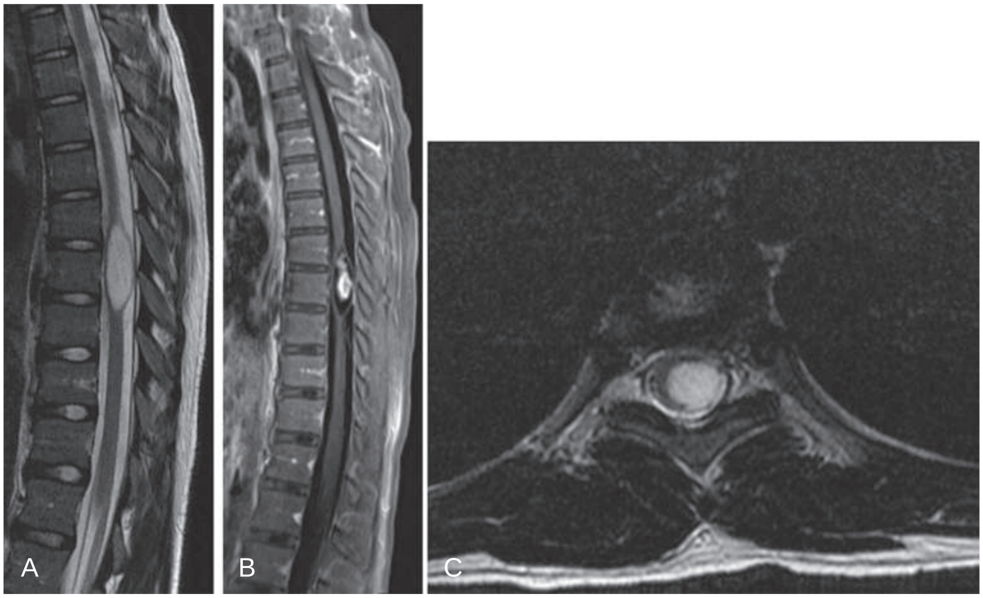

Figure 66-23 Spinal cord astrocytoma. Sagittal T2-weighted (**A**), sagittal post-Gd T1-weighted (**B**), and axial T2-weighted (**C**) images demonstrate a T2-hyperintense, ovoid lesion with enhancing central components consistent with a intramedullary thoracic spinal cord tumor. Pathology of resected specimen showed a low-grade astrocytoma.

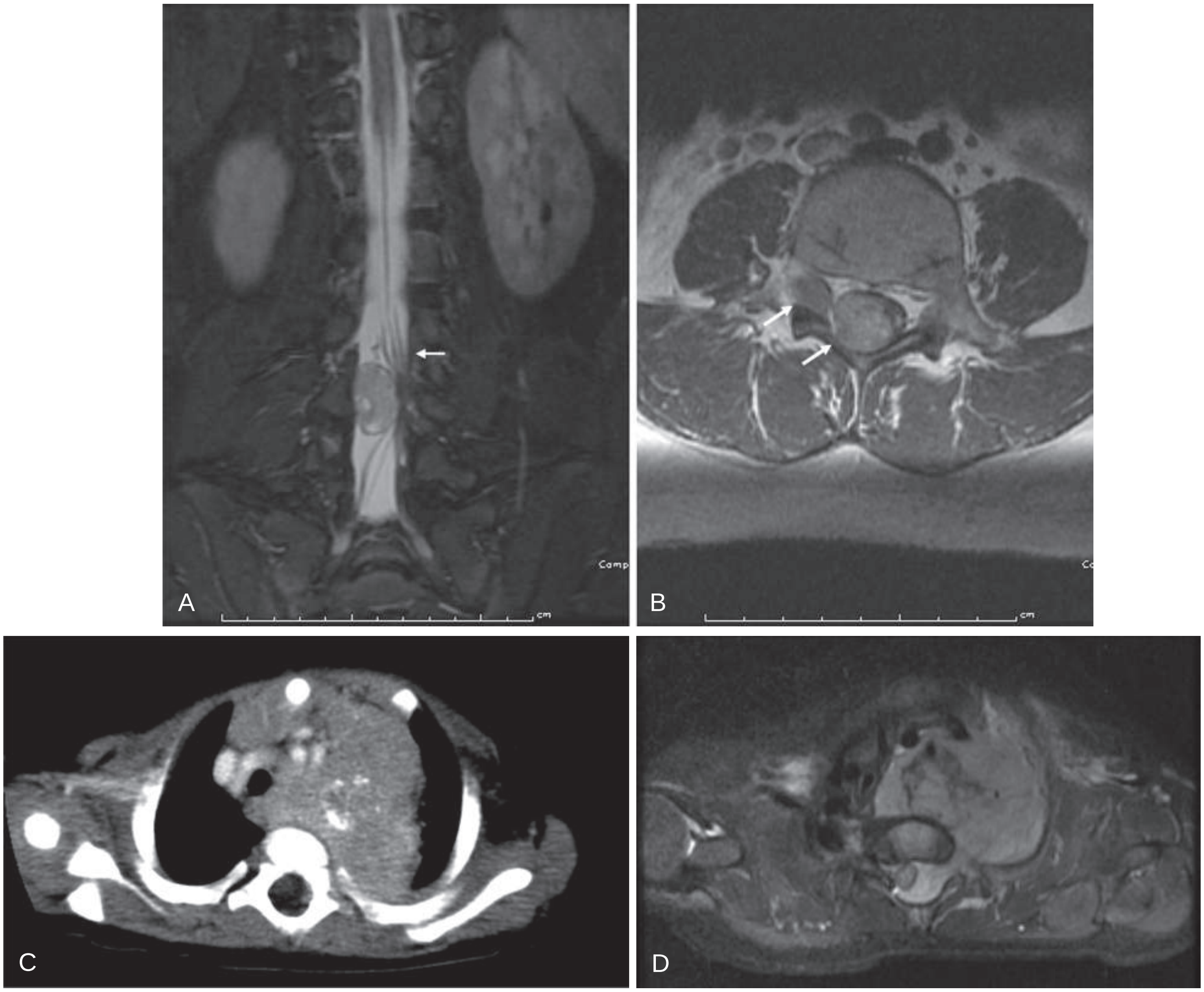

Figure 66-24 **A** and **B**, Coronal and axial MRI images show displacement of nerve roots and intension through the neural foramen in a patient with extramedullary intraspinal Ewing sarcoma. **C** and **D**, Patient with calcified left upper thoracic neuroblastoma initially detected on chest x-ray. MRI is superior in detecting the degree of intraspinal extension (**D**).

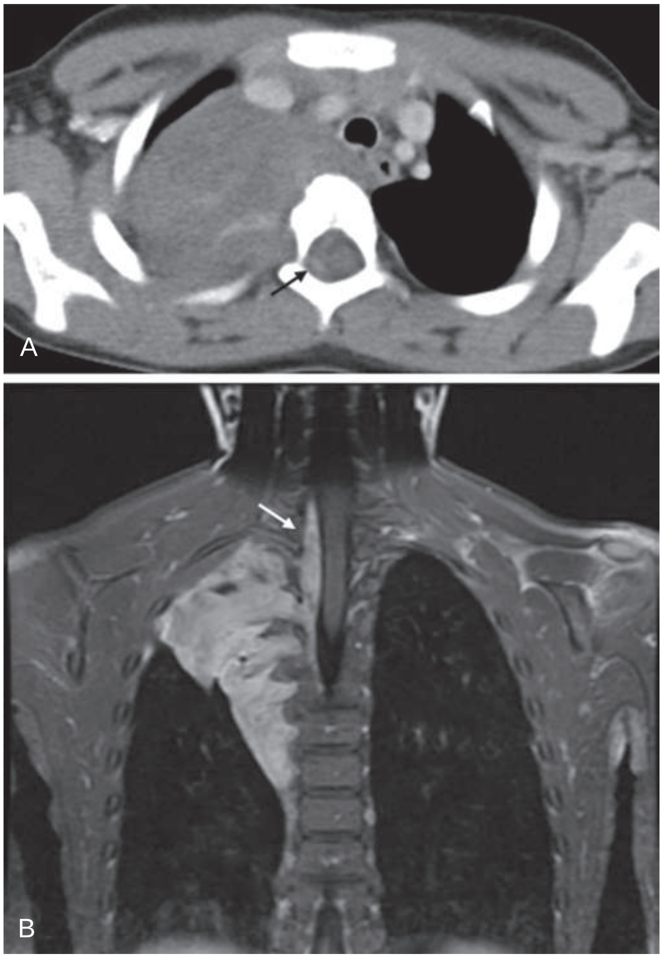

Figure 66-25 Upper thoracic paraspinal neuroblastoma. A 5-year-old patient with 2 weeks of upper-respiratory–infection symptoms. Chest x-ray revealed a right upper lobe opacity (not shown). This was found on CT to be a paraspinal mass. Intraspinal extension is evident on CT (**A**, *arrow*) but much better delineated by MRI (**B**, *arrow*).

PULMONARY AND INTRATHORACIC TUMORS

Thyroid and Parathyroid Tumors

Thyroid carcinoma in children is rare.[120] Three histologic subtypes are recognized in children, with the majority of patients (≈80%) having papillary carcinoma; follicular (≈15%) and medullary (≈5%) thyroid cancers occur less often. The risk is increased in patients with prior history or neck radiation or with predisposing familial syndromes (e.g., multiple endocrine neoplasia [MEN]).[120] Presentation is typically a painless mass in the thyroid gland. Ultrasound is the imaging modality of choice in evaluating the thyroid gland and is usually sufficient to characterize a palpable thyroid abnormality.[121] The goal of the ultrasound examination is to determine whether a cystic or solid mass is present (Fig. 66-26). The presence of increased flow and size of the mass is not reliable in distinguishing benign from malignant thyroid nodules. Malignant nodules are nearly always cold on ^{123}I-radioiodine scintigraphy relative to the surrounding thyroid gland.[121] Depending on the type of tumor, metastatic spread to adjacent lymph nodes and/or the lung may be present. In particular, papillary carcinoma of the thyroid often produces multiple small pulmonary nodules with a miliary distribution. These small pulmonary nodules may be challenging to detect by chest x-ray, and chest CT is indicated to provide increased sensitivity for lesion detection.[122] Bone metastases and thoracic lymph node involvement are common sites of extrapulmonary metastatic spread. ^{123}I or ^{131}I scintigraphy may also detect lesions not seen by conventional imaging (Fig. 66-27). The treatment of thyroid cancer in children includes total thyroidectomy and lymph-node dissection, followed by ^{131}I-radioiodine therapy for ablation of remnant thyroid tissue as well as to treat sites of micrometastatic disease not visualized by other imaging techniques or not amendable to surgical resection.[123] Diagnostic imaging is initially performed with ^{123}I-radioiodine to establish whether significant remnant tissue, iodine-avid lymph nodes, and metastatic disease are present in order to determine the appropriate therapeutic dose of ^{131}I-radioiodine. Higher doses are used for patients with extensive lymph-node involvement or

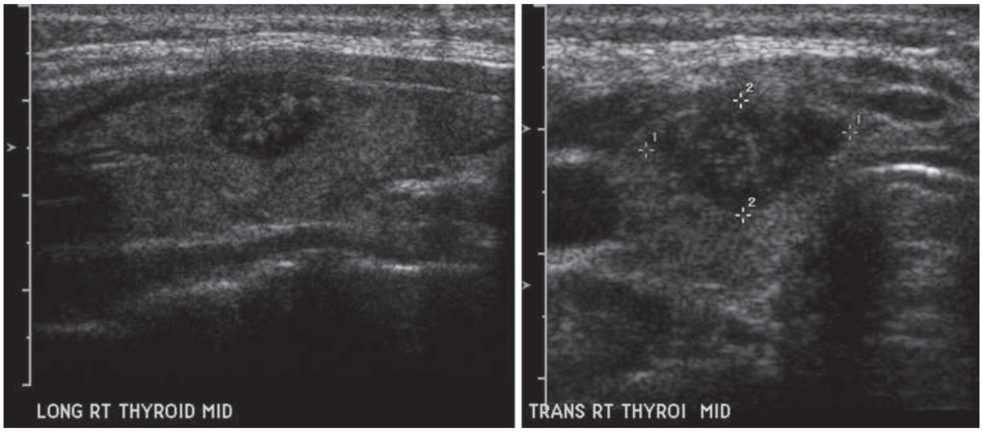

Figure 66-26 Follicular thyroid carcinoma. Palpable thyroid lesion with typical ultrasound imaging characteristics showing hypoechoic well-circumscribed mass. The mass is too small (≈1cm) to be accurately characterized by ^{123}I scintigraphy.

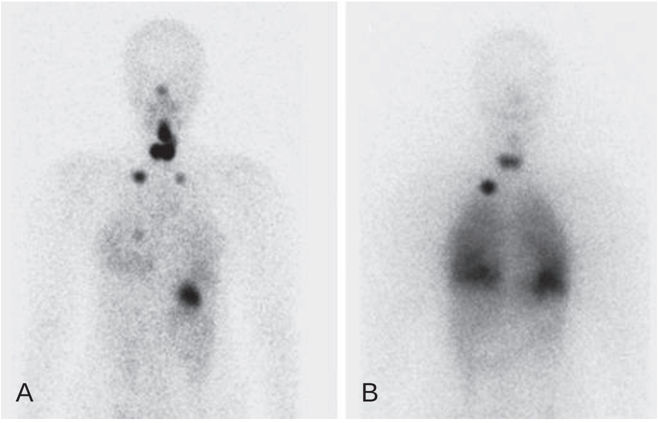

Figure 66-27 An 18-year-old patient with a history of stage-2 Hodgkin lymphoma treated with chemotherapy and radiation who developed a palpable thyroid nodule that was found to be papillary thyroid carcinoma. After thyroidectomy and lymph-node dissection, diagnostic imaging with ^{123}I radioiodine (**A**) shows intense uptake in the surgical bed, likely from remnant thyroid tissue, along with uptake in bilateral supraclavicular lymph nodes. Faint uptake is seen in the lungs. **B**, One week after therapy with ^{131}I radioiodine, remnant tissue and nodal uptake is reduced; however; intense pulmonary uptake is present, compatible with widespread pulmonary micrometastatic disease.

metastatic spread, as opposed to those with only remnant thyroid tissue in the surgical bed. As shown in Figure 66-27, the higher doses of ^{131}I used for therapy often allow sites of disease to be detected at the time of the post-therapy follow-up ^{131}I scintigraphy examination that were not apparent on the initial diagnostic ^{123}I scan.

Abnormalities of the parathyroid gland usually come to clinical attention during evaluation of hypercalcemic states. Functional parathyroid hyperplasia and parathyroid adenomas have a similar imaging appearance and can be very well characterized by ultrasound. There is usually no need for additional imaging by CT. Because of the variable anatomic locations of the parathyroid glands, it may be difficult to identify all potential sites of abnormality by conventional anatomic imaging techniques. ^{99m}Tc-Methoxyisobutylisonitrile (MIBI) scintigraphy is very sensitive and specific at showing focal accumulation of radiotracer in sites of functional parathyroid activity and can be used to identify sites of otherwise occult disease (Fig. 66-28).

Mediastinal Masses

The mediastinum is located in the center of the thorax, between the two thoracic cavities, the diaphragm, and thoracic inlet. It is conventional to divide the mediastinum into anterior, middle, and posterior mediastinal compartments based on their location as seen on the lateral chest radiograph. Although there are no distinct tissue planes that delineate these compartments, this system of classification is useful in characterizing diseases based on their tissue of origin.[124] Classifying mediastinal masses within a single mediastinal compartment helps narrow the differential diagnosis, calls attention to the potential effect of the mediastinal mass upon adjacent compartmental structures, and therefore commonly guides clinical decision making.[125]

Anterior Mediastinal Masses

The anterior mediastinum is defined as the prevascular space situated between the sternum and the heart, pericardium, and great vessels. The anterior mediastinum extends superiorly from the thoracic inlet to the level of the diaphragm. Organs located in the anterior mediastinum include thymus, thyroid, and parathyroid glands and prevascular-space lymphoid tissue. With respect to mediastinal mass formation, the thymus and anterior mediastinal lymph nodes are the two most important structures to be considered.

In infants, it is important to distinguish normal thymus from a mediastinal mass. The normal thymus typically has an undulating contour and on chest radiographs can be accurately identified based on subtle deformity by the adjacent anterior ribs' costal cartilages (Fig. 66-29). The normal thymus can be quite large and may extend posteriorly between the superior vena cava and aorta in the middle mediastinum or superiorly into the lower neck, making the distinction from neoplasm difficult.[125,126] In the appropriate clinical setting thymic hyperplasia may also be seen and is characterized by a homogeneously enlarged thymus. The thymus also may rapidly involute in response to physiologic stress, and this can also provide an indirect means of confirming its identity as normal thymus.

Lymphoma. Lymphoma is the most common tumor arising in the mediastinum and most commonly arises in either the anterior mediastinum or middle mediastinum. Both Hodgkin and non-Hodgkin lymphomas can present as mediastinal masses. The imaging features of Hodgkin lymphoma characteristically reveal a bulky anterior mediastinal mass with a nodular appearance. In addition, Hodgkin lymphoma is characterized by contiguous lymph-node spread, which may aid in the distinction

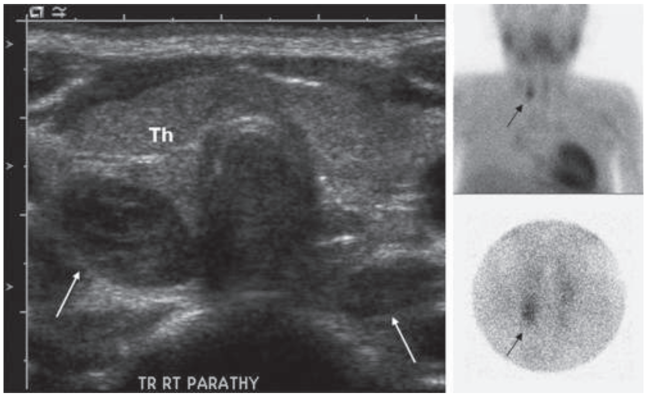

Figure 66-28 Parathyroid adenoma. Thyroid *(Th)* ultrasound showing a large hypoechoic mass on the right and a smaller lesion on the left *(arrows)* at the expected location of the parathyroid glands in an 11-year-old patient with end-stage renal disease and secondary hyperparathyroidism. The dominant mass on the *right* has corresponding increased uptake on ^{99m}Tc-MIBG scintigraphy, consistent with parathyroid adenoma. The lower levels of uptake in the smaller lesions are more in keeping with hyperplastic parathyroid tissue. ^{99m}Tc-MIBG is also effective at locating ectopically located parathyroid tissue (e.g., substernal) not visualized on thyroid ultrasound.

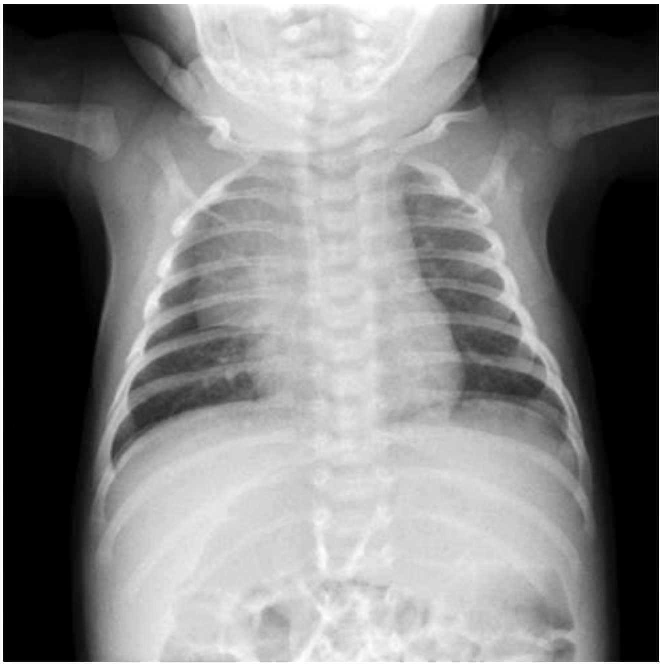

Figure 66-29 Anteroposterior chest radiograph of an infant shows prominent thymic shadow and undulating contour conforming to the adjacent ribs, findings all consistent with normal thymic tissue.

from other types of lymphoma. Approximately two thirds of pediatric patients with Hodgkin lymphoma show lymphadenopathy involving the mediastinum.[127] Non-Hodgkin lymphomas include lymphoblastic lymphoma and other subtypes.[127] Approximately one third of pediatric non-Hodgkin lymphomas have a mediastinal mass, and more than 50% of the lymphoblastic lymphomas have mediastinal involvement.[125] Although the distinction between some subtypes of non-Hodgkin and Hodgkin lymphoma may be difficult, acute T-lymphoblastic lymphomas often demonstrate a homogeneous infiltration and enlargement of the thymus gland with encasement/compression of the vessels (Fig. 66-30). All forms of lymphoma can result in significant tracheal compression/narrowing, vascular compression, and symptoms related to the superior mediastinal syndrome.[127,128] Pleural and pericardial effusions are seen in both non-Hodgkin and Hodgkin lymphomas of the mediastinum and are not a distinguishing feature. Bone-marrow involvement is seen in patients with either Hodgkin or non-Hodgkin lymphoma. Recent studies have shown that FDG-PET is a sensitive method for detecting bone-marrow involvement in children with Hodgkin lymphoma and can safely replace routine bone marrow biopsy for establishing the presence of marrow involvement.[129] Direct bone invasion by Hodgkin lymphoma is unusual; non-Hodgkin lymphomas often show more aggressive local involvement.

The imaging evaluation should always include a chest radiograph. In many instances this is the most important imaging study to guide the subsequent care of the patient. The presence of significant tracheal narrowing or tracheal displacement in the context of a large anterior mediastinal mass, particularly if accompanied by respiratory symptoms (see Figs. 66-2 and 66-30), should raise immediate concern for impending respiratory/cardiopulmonary compromise and prompt intensive care unit (ICU)–level monitoring.[128] Pneumomediastinum and pneumothorax may also be identified on these initial imaging studies (see Fig. 66-30). The placement of the patient in a recumbent position for CT scanning may further exacerbate the patient's already tenuous respiratory status, impair central venous return, and result in an acute cardiorespiratory event. In this setting it may be necessary to delay CT imaging, and the staging evaluation will have to be postponed until stabilization of the patient's clinical status.

In a stable patient, staging of anterior mediastinal lymphomas must include imaging of the neck to evaluate the Waldeyer ring of lymphoid tissue and should also include the abdomen and pelvis. Intravenous contrast should be used to provide an accurate assessment of the mediastinal vascular structures. Oral contrast should be provided to opacify the bowel and aid in the distinction from mesenteric lymphadenopathy.

MRI, particularly with faster scanning techniques, may be effective in evaluating mediastinal masses and staging lymphoma[130,131] but currently does not have an established role in the more acute stages of the evaluation. Gallium scintigraphy previously and currently FDG-PET imaging are recognized as standard of care in identifying sites of metabolically active lymphoma and in identifying sites of disease otherwise undetected by conventional imaging techniques (Fig. 66-31). FDG-PET scanning, in particular, has also been shown to play an important role in the posttreatment evaluation, helping to stratify patients into early responder and nonresponder groups to allow appropriate modulation of therapy based on treatment response (Fig. 66-32).[90,132,133-135]

After treatment, particularly of Hodgkin disease, it is common for the mediastinal mass to slowly regress and for residual inflammatory tissue to persist even months after therapy.[136,137] In contrast, lymphoblastotic lymphomas often respond quite rapidly to therapy with very little residual soft-tissue abnormality remaining in the anterior mediastinum, even after a relatively short duration of treatment (Fig. 66-33).[138] The use of FDG-PET for early response assessment in Hodgkin lymphoma is now considered standard of care, with images being simultaneously acquired and coregistered as fused PET-CT images to distinguish persistent metabolically active disease from residual posttreatment inflammatory tissue.[139-141] There is a large body of literature from both pediatric and adult patients showing that the presence of residual metabolically active disease after two cycles of chemotherapy is prognostically significant, presumably reflecting chemosensitive versus chemoresistant disease.[133,142,143,144-146] Patients with residual CT abnormalities that are FDG-avid have a worse overall outcome as compared with patients whose scans are FDG-negative, which has led to a paradigm shift in Hodgkin lymphoma therapy such that patients in whom early response assessment shows no metabolically active disease would be eligible for reduced chemotherapy and radiation in an effort to reduce treatment-related complications.[144,147] Whether this approach will maintain the good outcomes (>90%

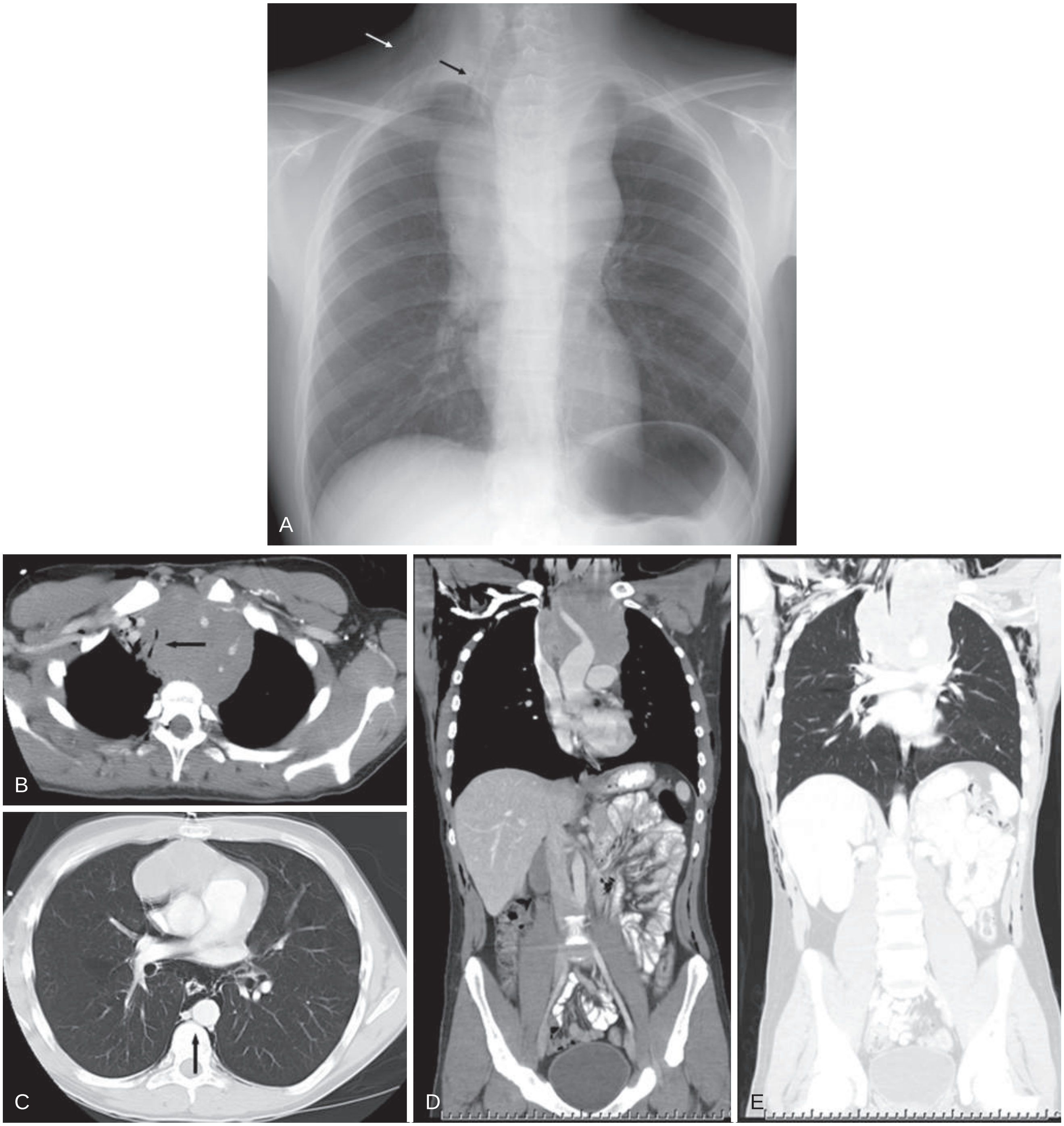

Figure 66-30 **A**, Chest x-ray showing marked tracheal deviation, pneumomediastinum, and subcutaneous emphysema along the right neck in a child with lymphoblastic lymphoma, emphasizing the severity of airway obstruction and potential for respiratory compromise *(arrows)*. **B-E**, CT shows large anterior mediastinal mass with mediastinal air around the heart, pulmonary vessels, aorta, and esophagus (**C**). The trachea is markedly narrowed *(arrow)*, likely the cause of the pneumomediastinum. Subcutaneous air is also evident in the anterior chest wall. Five days later the findings had progressed considerably (**C** and **D**).

overall survival) typically seen in Hodgkin lymphoma remains to be seen.[148,149] There is currently no convincing data showing the prognostic value of FDG-PET imaging in non-Hodgkin lymphoma.[150]

Germ-Cell Tumor. Mediastinal germ-cell tumors are primarily located in the anterior mediastinum near the thymus gland and make up about 10% to 20% of all childhood mediastinal tumors. Germ-cell tumors are second only to lymphoma as the cause of a thymic/anterior mediastinal mass. When they present as predominately solid masses, they may be difficult to distinguish from lymphoma. Histologically, mature teratomas are the most common germ-cell tumor arising in the

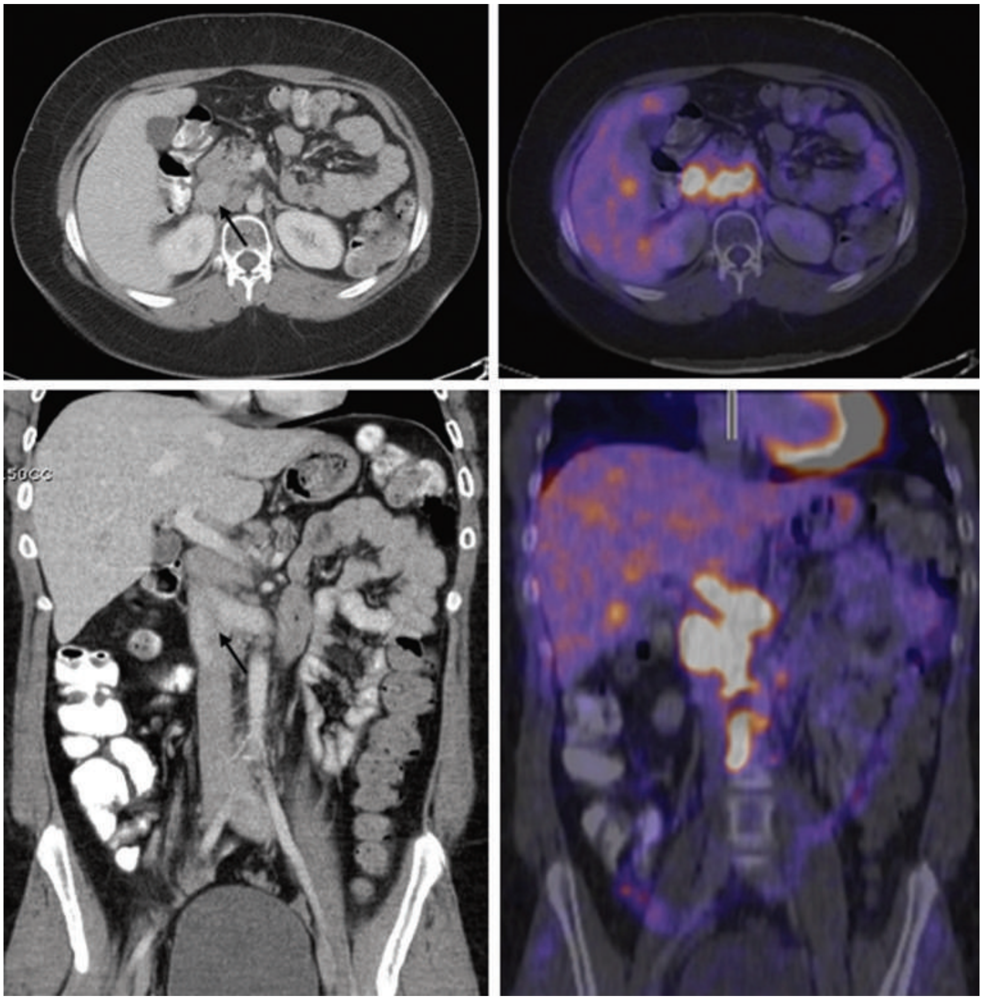

Figure 66-31 FDG-PET and image fusion delineate equivocal areas of abnormality on CT *(arrows)*, identifying foci of disease recurrence in a patient with Hodgkin lymphoma.

mediastinum.[151] At diagnosis, calcification of lymphoma is unusual, and the calcifications that are often present in teratoma may suggest the diagnosis. Germ-cell tumors are often asymptomatic and may be identified incidentally as mediastinal masses on chest radiographs obtained for other indications.[152] When symptoms are present, they are usually the result of compression of the tracheobronchial tree.

Either CT or MRI scanning are effective at further characterizing these masses. The presence of fluid attenuation, mixed attenuation fluids and gelatinous material, fat, and calcification all should suggest the diagnosis of mediastinal germ-cell tumor (Fig. 66-34). Ten percent to 20% of all mediastinal germ-cell tumors are malignant and present with signs of local invasion into the pleura or pericardium. Malignant tumors include seminomatous and nonseminomatous histologic subtypes. Seminomas tend to be noncalcified, bulky solid masses that either remain localized or spread to local lymph nodes, whereas other malignant germ-cell tumors display greater heterogeneity with cystic and solid elements and more aggressive, locally invasive features.[153] Germ-cell tumors may rupture into the pleural or pericardial spaces, the lung parenchyma, or the tracheobronchial tree. Imaging evaluation should be directed at determining the extent of rupture and sites of tissue involvement. Chemical pneumonitis can result from parenchymal rupture, and expectoration of blood, hair, and sebaceous material can result from rupture into the airways.[154]

Thymic Masses

Thymoma. Thymoma is rare in children, accounting for less than 4% of pediatric mediastinal tumors.[125] As with adults, thymoma may present with symptoms related to associated autoimmune disorders, including myasthenia gravis, diabetes, and Hashimoto thyroiditis. Thymomas are typically solid, lobulated masses arising in or around the thymus (Fig. 66-35). The imaging evaluation should be directed at assessing for aggressive features such as invasion into adjacent pericardial or pleural structures. MRI and contrast-enhanced CT reveal a heterogeneously enhanced low-density mass that is isointense on T1-weighted images and mildly T2 hyperintense relative to muscle.[125] Both CT and MRI are excellent in

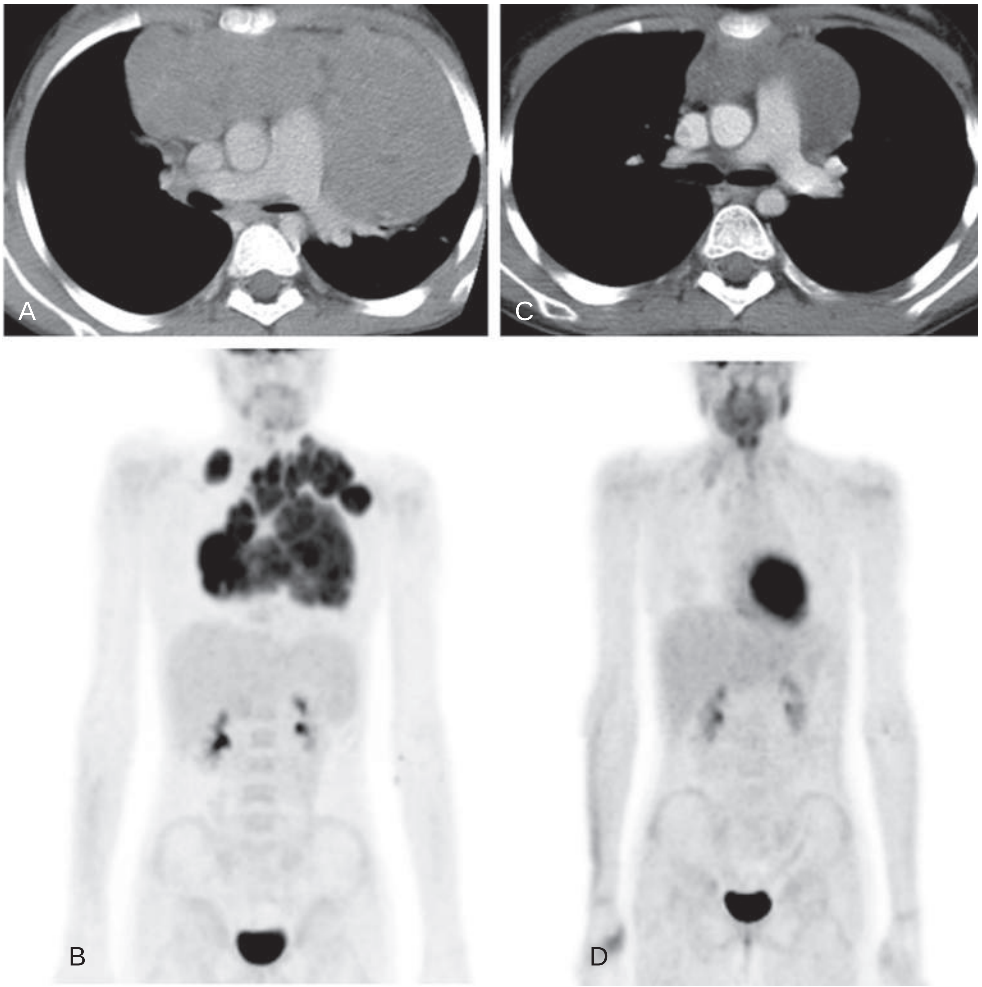

Figure 66-32 Hodgkin Lymphoma CT and FDG-PET obtained at diagnosis (**A** and **B**) and after 2 cycles of chemotherapy (**C** and **D**) show excellent functional and anatomic response to therapy. Residual mediastinal mass on CT with no residual FDG uptake emphasizes the potential importance of functional imaging in response assessment.

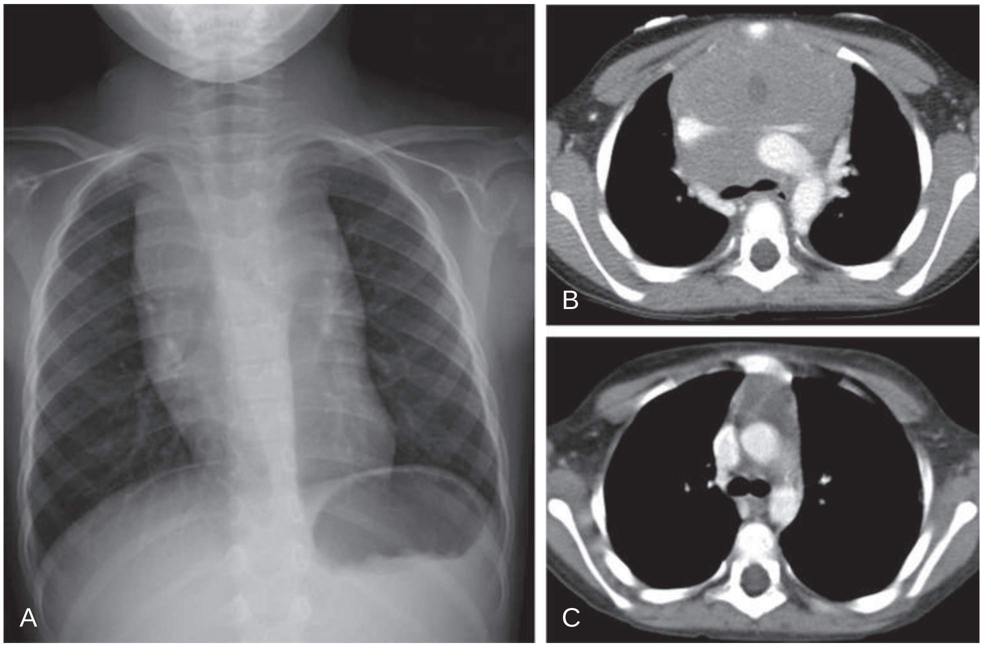

Figure 66-33 A 4-year-old child with T-cell ALL and a large mediastinal mass with airway and vascular compression seen by CXR (**A**) and CT (**B**), showing near complete resolution of the mass after just 2 weeks of therapy (**C**).

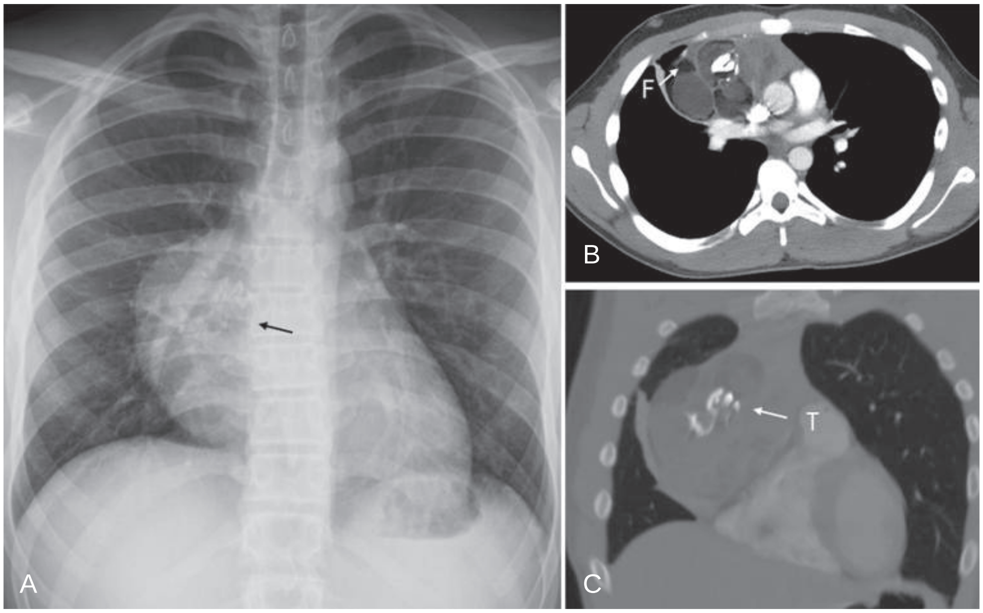

Figure 66-34 A 15-year-old boy with mediastinal mass seen incidentally on a chest x-ray obtained for pain after a football injury (**A**). Subsequent CT (**B** and **C**) shows fat *(F)* and adjacent solid elements in addition to calcification, in this case taking the form of tooth *(T)*, characteristic of germ cell tumor, in this case mature teratoma.

delineating the relationship of these masses to the adjacent vascular structures (see Fig. 66-35). Nodular pleural or pericardial thickening should raise concern for invasive thymoma. Because complete surgical resection is the most effective treatment for ensuring long-term survival for patients with thymoma, postsurgical radiation therapy may be considered when invasive features are present.[155]

Thymolipomas. Thymolipoma, in contrast to thymoma, is relatively more common, making up about 3% to 9% of pediatric thymic tumors.[156] These benign tumors are most often seen in older children, but they may present in infancy. These heterogeneous masses may contain calcification and cystic areas, making distinction from germ-cell tumors challenging. A predominantly fatty mass should, however, suggest the diagnosis of thymolipoma (Fig. 66-36). The fat characterization can be sensitively and specifically evaluated by MRI, showing a high signal-intensity mass on T1-weighted imaging with loss of signal on fat-suppressed images.[156] A low-attenuation mass with negative Hounsfield-unit density on either unenhanced or contrast-enhanced CT can also suggests the presence of a fat-containing mass (see Fig. 66-36).

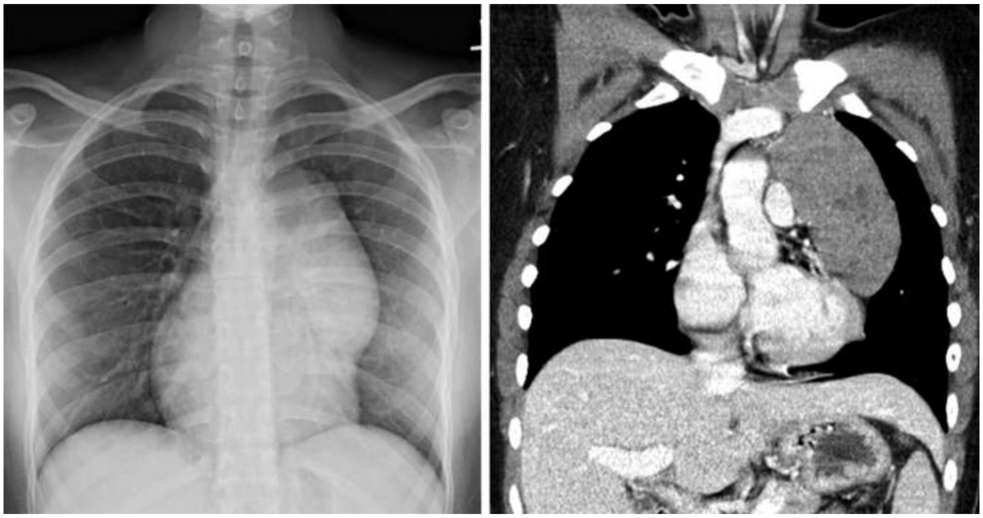

Figure 66-35 A 15-year-old girl with a 2-week history of chest pain. Chest x-ray and CT show a large, lobular, heterogeneous anterior mediastinal mass abutting the great vessels, with a poorly defined plane between the mass and normal mediastinal structures. Biopsy revealed thymoma, and the patient underwent complete resection.

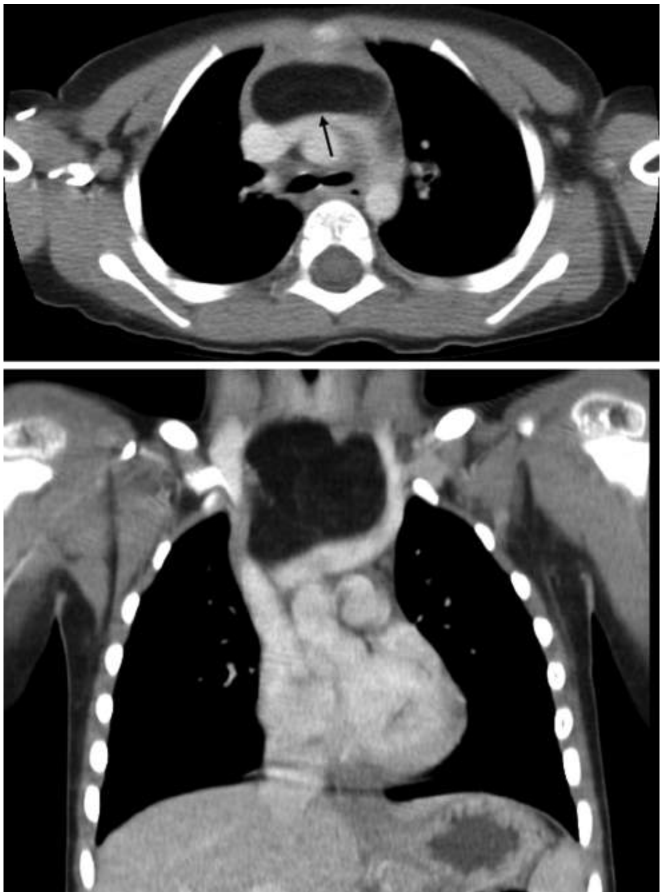

Figure 66-36 Thymolipoma in a child with an incidentally detected mediastinal mass on chest x-ray. Note the fat-density mass *(arrow)* located within the thymus.

Middle Mediastinum

Tracheobronchial Tree Masses. The middle mediastinum is defined as the vascular space including the pericardium/heart, the great vessels, and trachea/proximal bronchi and associated lymph nodes. Although the most common middle mediastinal mass is lymphoma, lymphoma is rarely isolated to the middle mediastinum and usually occurs in conjunction with a large anterior mediastinal mass. The most common masses isolated to the middle mediastinum are benign bronchopulmonary foregut malformations, including bronchopulmonary sequestration, congenital cystic adenomatoid malformation (C-CAM), and foregut/bronchogenic cysts.[124] These benign masses are not discussed further in this section except to emphasize that the distinction between cystic adenomatoid malformation and pleuropulmonary blastoma cannot be made on the basis of imaging alone (Fig. 66-37). Surgical resection and histopathologic evaluation is required to distinguish the benign C-CAM from its neoplastic counterpart.

Masses of the endobronchial tree are also exceedingly rare in children. The most common endobronchial neoplasm in children is the bronchial carcinoid tumor, making up about 50% of all bronchial neoplasms.[157] Although these tumors may secrete neuroendocrine peptides, the classic carcinoid syndrome is relatively unusual in children with bronchial carcinoids, and these patients are more likely to have presenting respiratory symptoms such as hemoptysis or lobar atelectasis/partial collapse caused by the obstructing endobronchial lesion (Fig. 66-38). These lesions are commonly hilar in location and may be very difficult to identify on chest radiographs. The presence of a persistent area of segmental or lobar collapse that does not resolve after appropriate therapy should prompt further investigation by CT scanning to directly assess the airways. Multidetector-row CT scans can provide exquisite 3D reformations of the airways and allow accurate detection of endobronchial abnormalities. Chest CT of patients with suspected endobronchial carcinoid tumor may be performed without intravenous contrast for the purpose of identifying the endobronchial lesion; however, contrast infusion typically shows prominent enhancement of these highly vascular lesions. There may be intraluminal, mural, and extraluminal components of these lesions, and the extraluminal extent of the lesion may exceed its intraluminal component.[158] Although the presence of calcification in an endobronchial mass should suggest the diagnosis of a carcinoid tumor, calcification is still relatively rare in childhood bronchial carcinoid tumors. Surgical resection is usually curative, and although the extent of local invasion around the primary lesion is variable, metastatic disease is relative rare (5% to 20%). Uptake of the neuroendocrine-specific radiotracer ^{111}I-octreotide provides highly specific scintigraphic imaging confirmation of the diagnosis (see Fig. 66-38, *C* and *D*) and may aid in identifying occult sites of metastatic disease. FDG-PET imaging of metabolically active carcinoid tumors has also been performed and, although not specific for neuroendocrine tumors, may prove to be a sensitive means of identifying occult metastases.

Mucoepidermoid carcinoma is the second most common primary bronchial neoplasm. These patients also have primarily respiratory presenting symptoms related to airway obstruction. Because primary airway neoplasia in children is rare and commonly characterized by nonspecific clinical symptoms, the diagnosis is often delayed and difficult to distinguish from infectious processes such as pneumonia. The most common finding on plain chest radiographs in patients with mucoepidermoid carcinoma is a central mass or nodule.[159,160] These tumors range from low to high grade, with high-grade neoplasms tending to invade the adjacent pulmonary parenchyma. As with carcinoid tumors, multidetector-row CT with multiplanar and 3D reformats can be used to accurately identify an airway abnormality.[160,161] However, in contrast to carcinoid tumors, mucoepidermoid carcinoma is relatively hypovascular and shows minimal enhancement. Recent studies have also suggested that FDG-PET imaging may be used to advantage in staging patients with mucoepidermoid carcinoma,[160] with intense FDG uptake visualized (Fig. 66-39).

Tumors of the heart and pericardium are very rare in children. The majority of these are rhabdomyomas arising from the neoplastic transformation of cardiac myocytes. There is a well-known association between cardiac rhabdomyoma and tuberous sclerosis (Fig. 66-40), with

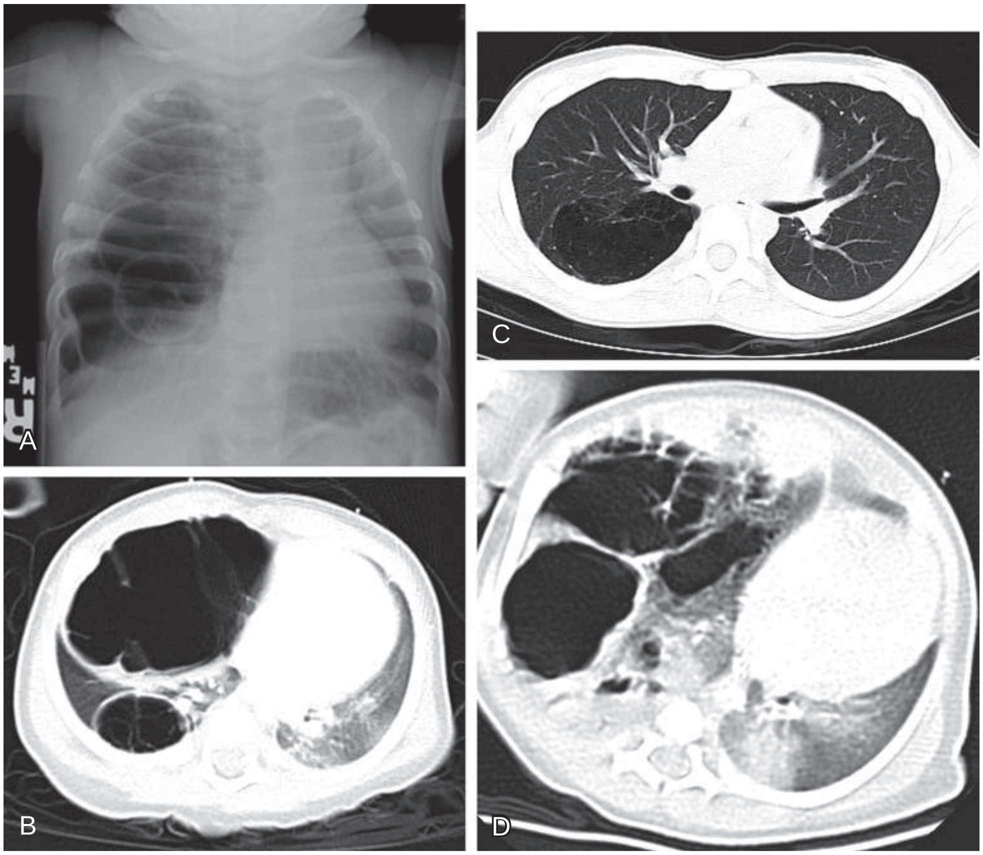

Figure 66-37 Pleuropulmonary blastoma (**A** and **B**). Note the difficulty in distinguishing this malignancy from C-CAM (**C** and **D**).

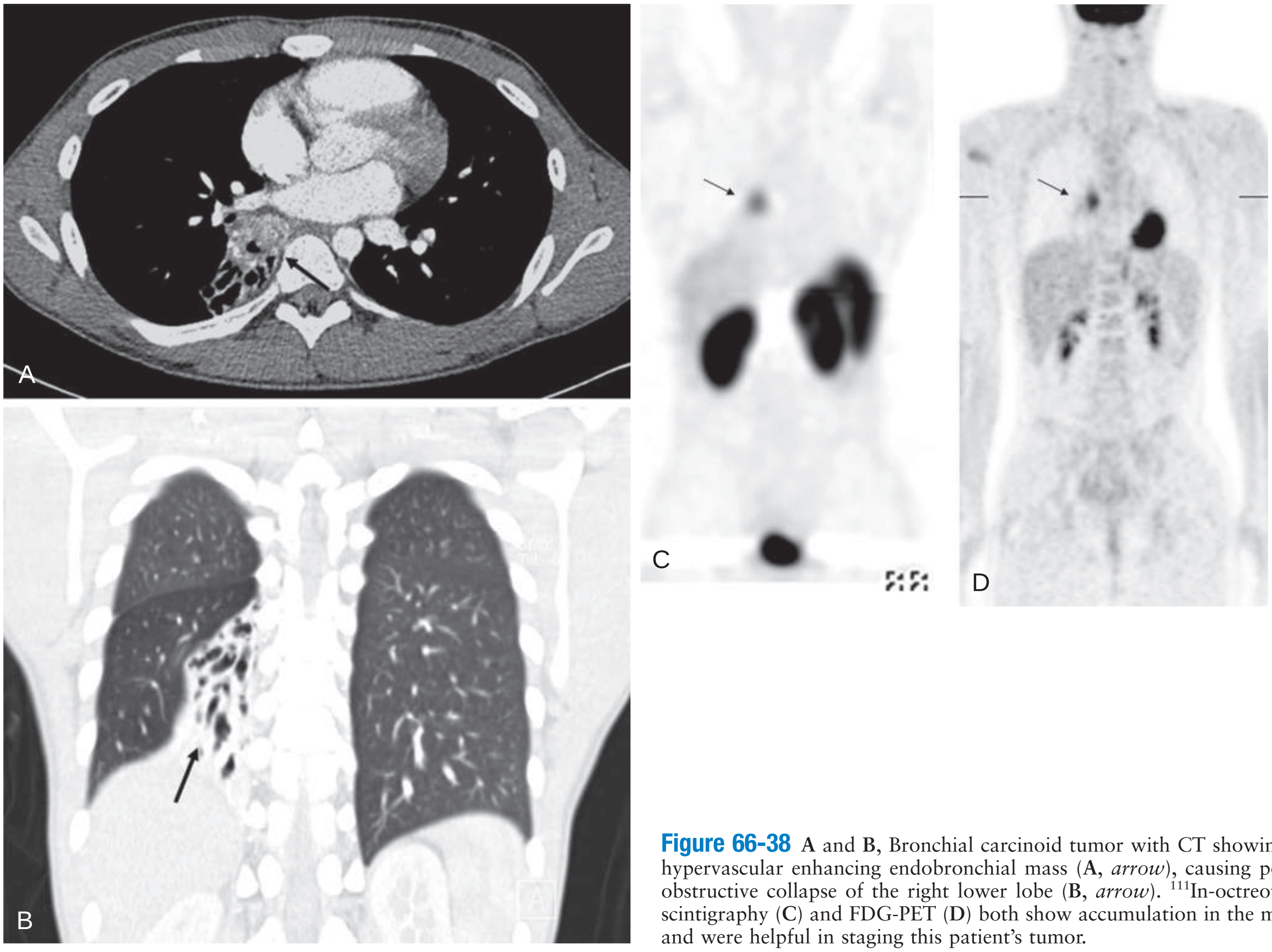

Figure 66-38 **A** and **B**, Bronchial carcinoid tumor with CT showing a hypervascular enhancing endobronchial mass (**A**, *arrow*), causing post-obstructive collapse of the right lower lobe (**B**, *arrow*). ^{111}In-octreotide scintigraphy (**C**) and FDG-PET (**D**) both show accumulation in the mass and were helpful in staging this patient's tumor.

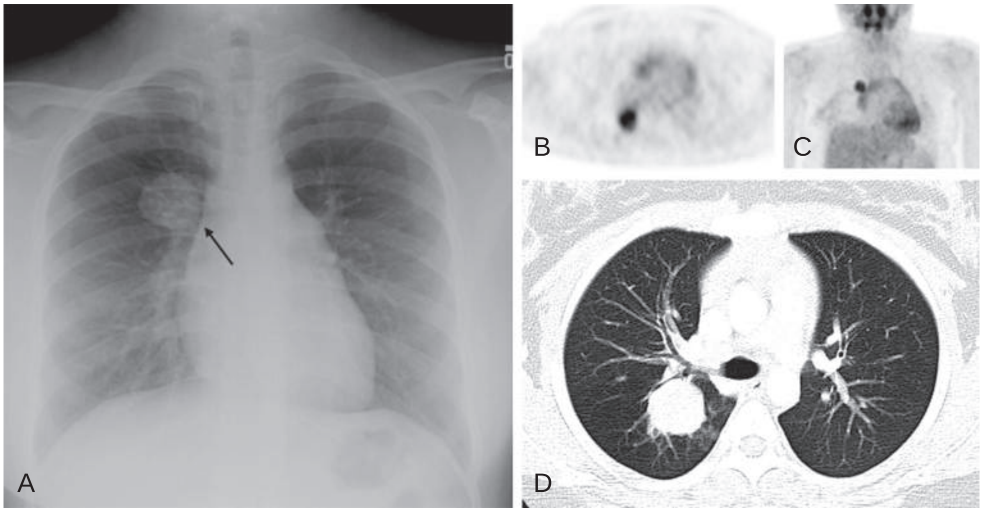

Figure 66-39 Mucoepidermoid carcinoma. Chest x-ray for symptoms of nonspecific upper respiratory infection showed right upper lobe mass (**A**). CT shows posterior segment upper lobe mass compressing the posterior segment bronchus with mild associated air-trapping (**D**). FDG-PET (**B** and **C**) showed intense uptake and was helpful for staging.

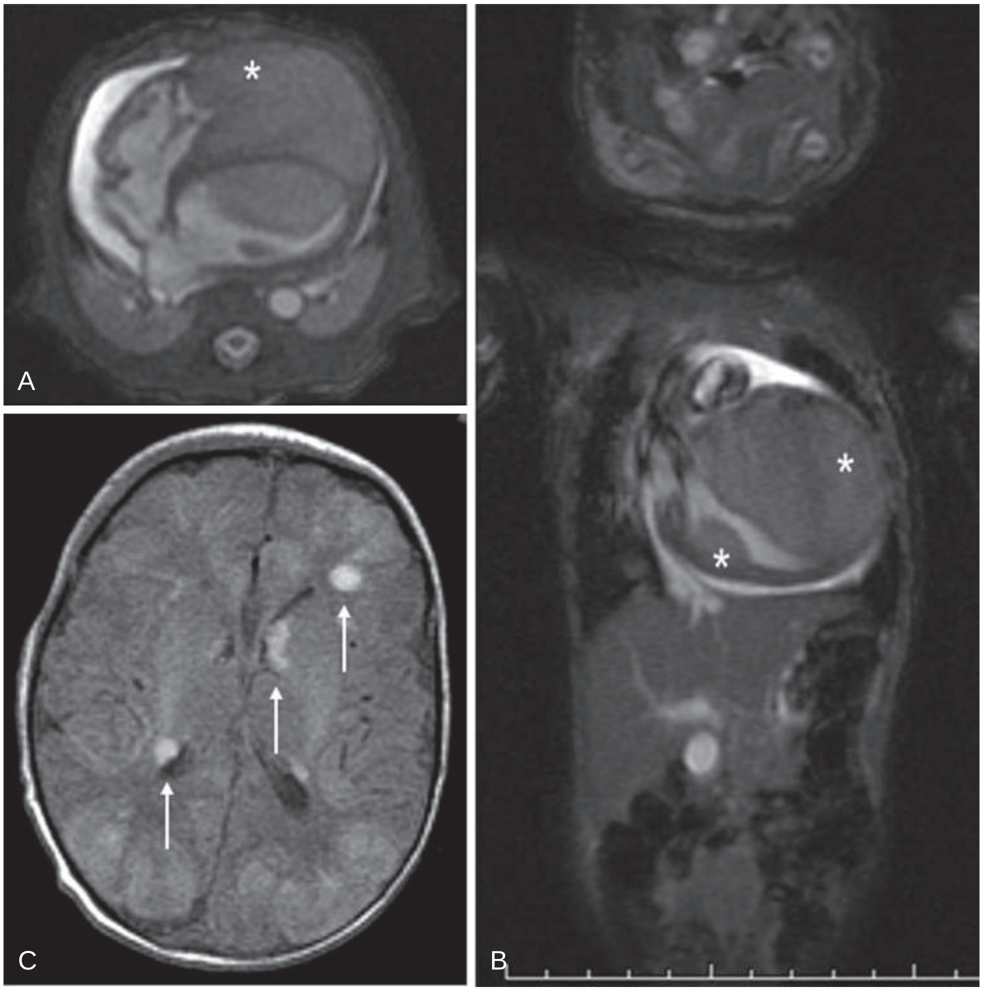

Figure 66-40 Cardiac rhabdomyoma. A 5-day-old infant with a large chest mass and tuberous sclerosis. **A**, Cardiac MRI showed multiple rhabdomyomas *(asterisks)*, the largest obliterating the right ventricle. **B**, Multiple smaller tumors are in the left ventricle, both adherent to the septum and along the free wall. **C**, Brain MRI shows multiple subependymal and cortical tubers *(arrows)*.

approximately half of the patients with this diagnosis having rhabdomyomas.[162] Indeed the presence of a rhabdomyoma should prompt further evaluation, because rhabdomyomas may be the initial presenting sign of tuberous sclerosis.

When symptoms are present, they are usually the result of tumor protruding into the cardiac lumen, resulting in outflow obstruction and congestive failure. Echocardiography is usually sufficient to suggest the diagnosis; however CT scanning and cardiac MRI are superior at defining the extent and attachments of these cardiac tumors and may be indicated for surgical planning. Cardiac fibromas and myxomas are much less common. Indeed it is more common to see secondary extension into the heart by other primary pediatric neoplasms such as neuroblastoma and Wilms tumor, both of which have a propensity to extend into the heart via the inferior vena cava (IVC); hepatoblastoma, which may show direct intracardiac extension (Fig. 66-41); and mediastinal lymphoma, which may result in chest wall/pericardial involvement. Contrast-enhanced CT scanning usually allows an accurate delineation of sites of local extension in the thorax. Either abdominal ultrasound, multiphase contrast-enhanced CT, or MRI can be used to demonstrate intravascular/intracardiac extension from primary abdominal malignancies.

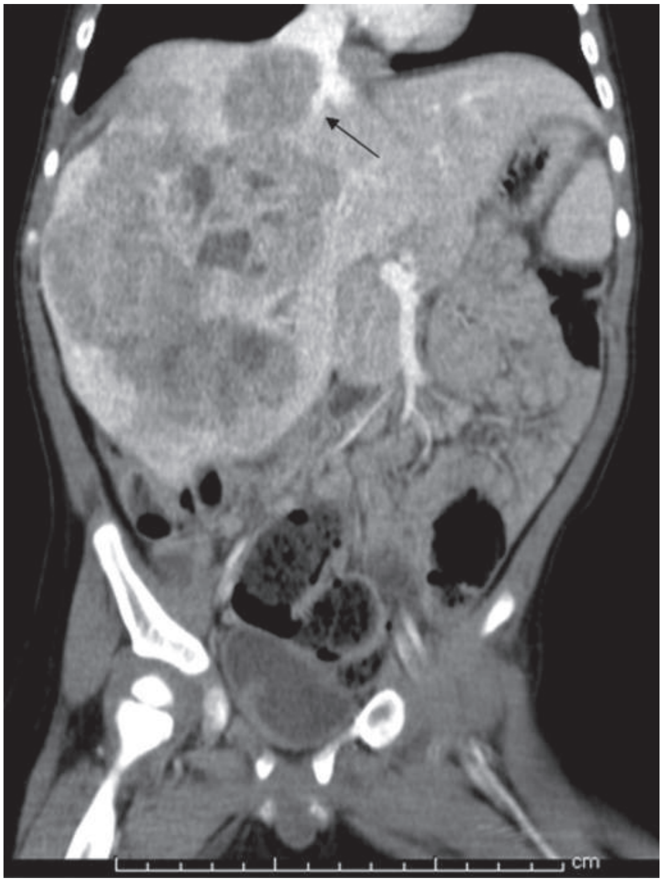

Figure 66-41 Coronal contrast-enhanced CT showing direct extension into the IVC in a 15-month-old patient with hepatoblastoma.

Posterior Mediastinal Masses

The posterior mediastinum is defined dorsally by the chest wall/vertebral column, ventrally by the pericardium and posterior wall of the great vessels, superiorly by the thoracic inlet, and inferiorly by the diaphragm. The primary components of the posterior mediastinum are the paravertebral sympathetic ganglia, azygos and hemiazygos veins, descending thoracic aorta, esophagus, and lymph nodes. The majority of the posterior mediastinal masses in children are neurogenic in origin[125,163] and include ganglion-cell tumors, nerve-sheath tumors, and other nervous-tissue neoplasms such as paragangliomas. Ganglion-cell tumors arise from sympathetic chain ganglia and range from well-differentiated and benign ganglioneuromas to malignant neuroblastomas. Ganglioneuroblastomas contain elements of both benign and malignant tissue types. The distinction between these different histologic subtypes cannot be made reliably based on imaging features, although age at presentation is important in narrowing the differential diagnosis. Neuroblastoma, the most common non-CNS solid tumor in children, typically occurs in young children with a median age at presentation of younger than 2 years and more than 95% of cases occurring by the age of 10 years. In contrast, the median age at presentation of ganglioneuroblastoma is approximately 5.5 years, whereas mature ganglioneuromas occur in later childhood/early adolescence, typically after the age of 10.[164] Whereas the majority of neuroblastomas occur in or around the adrenal gland, approximately 15% arise in the posterior mediastinum.[165]

It is important to emphasize that these lesions may be present in a child who is otherwise asymptomatic, and a thorough inspection of the chest radiograph using multiple windows and levels is necessary to identify small foci of disease. This is of particular importance, as shown in Figure 66-42, because a localized low-stage favorable histology neuroblastoma identified in a child under the age of 1 year may make the difference between being treated on low-risk protocols with higher likelihood of overall and disease-free survival versus more advanced stages of disease, when the patient's risk of relapse and treatment failure increases.[166] Primary intraabdominal neurogenic tumors may also have a significant posterior mediastinal extent and often have an associated posterior mediastinal/paravertebral component near the thoracic inlet (Virchow node). For this reason, it may be prudent to include imaging of the chest, in addition to the abdomen and pelvis, in the staging evaluation of patients with suspected neuroblastoma.

Radiographically, ganglion-cell tumors appear as paraspinal soft-tissue masses. Calcifications may be present. The presence of chest-wall invasion or destructive bony changes are more commonly seen with neuroblastoma, although benign ganglioneuromas can produce a considerable neural foraminal widening. As with primary adrenal neuroblastomas, staging should include ^{123}I-MIBG scintigraphy to assess for other sites of disease, including marrow involvement. ^{99m}Tc-MDP bone scintigraphy is also commonly performed to identify sites of

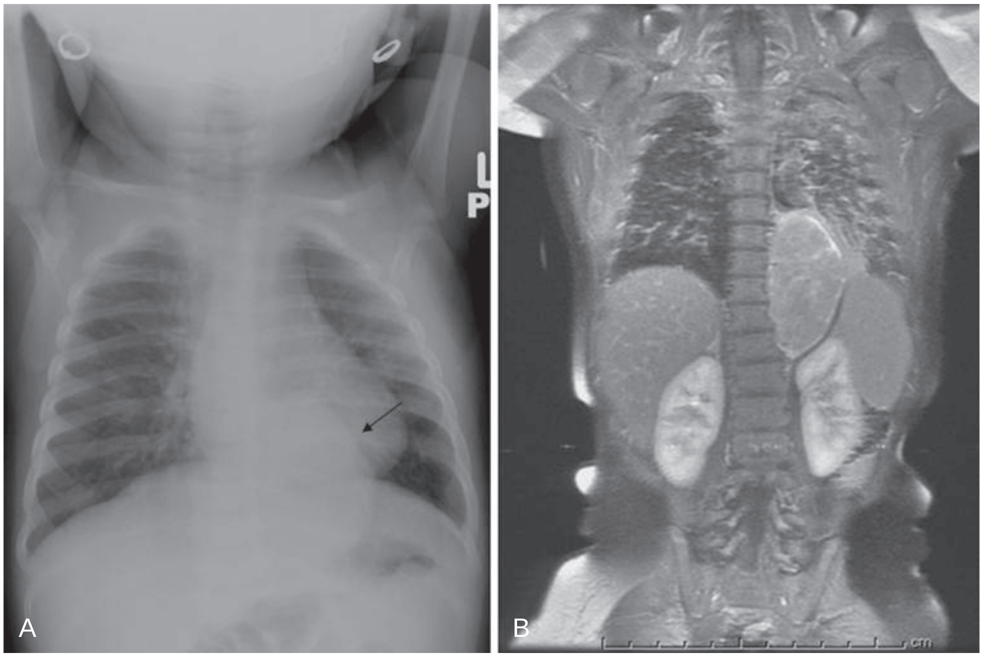

Figure 66-42 Posterior mediastinal neuroblastoma in a 5-month-old infant who showed symptoms of an upper respiratory infection. A posterior mediastinal mass was identified by chest x-ray (**A**) and MRI (**B**). Surgery revealed neuroblastoma. Subsequent staging confirmed low-stage disease.

cortical bone involvement,[91] although recent data suggests that bone scintigraphy rarely affects that clinical stage of the patient. The presence of a paraspinal mass can usually be adequately assessed by CT scanning, particularly with sagittal and coronal reconstructions. However, the extent of intraspinal extension is better depicted by MRI (Fig. 66-43).[125] MRI also provides a more accurate assessment of chest-wall involvement, nerve-root compression, and impingement upon the spinal cord. MRI of ganglion-cell tumors typically reveals a low signal-intensity lesion on T1-weighted images and intermediate to high signal intensity on T2-weighted and fast spin echo inversion recovery (FSEIR) images, with variable patterns of enhancement. Bone-marrow metastases are well demonstrated by MRI, with replacement of normal bone-marrow signal by areas of low signal intensity on T1-weighted images and high signal intensity on T2-weighted images. Indeed, several studies have suggested that MRI may be sufficient to identify sites of bone-marrow involvement in neuroblastoma, although is has not yet become common in clinical practice to rely on the MRI findings of bone-marrow involvement for staging of the patient.[167,168] The use of FDG-PET in neuroblastoma has received attention as an alternative to conventional imaging techniques.[91] However, a number of studies have shown variable patterns of FDG avidity in neuroblastoma, and FDG-PET has not yet been shown to offer a clear advantage over MIBG scintigraphy in either the staging or response assessment of patients with thoracic neuroblastoma.[169-171]

Nerve-sheath tumors, including schwannomas and neurofibromas may be similar in appearance to mature ganglion cell tumors, presenting as relatively sharply marginated, lobulated paraspinal masses. The presence of rib erosions and neural foraminal widening is commonly seen with nerve-sheath tumors, although intraspinal extension is less common. Paraspinal paragangliomas are catecholamine-secreting tumors arising from extra-adrenal chromaffin cells present in sympathetic chain ganglia and occur much less often than either primary ganglion-cell or nerve-sheath neoplasms. Distinguishing between these tissue types is difficult on the basis of imaging, and surgical resection is the mainstay of therapy. Local recurrence can occur, however, and close imaging follow-up is necessary in these patients.

Chest-Wall/Pleural-Based Neoplasms

Most of these tumors are of mesenchymal origin and are primary neoplasms arising from the chest wall. They are relatively rare in infants and children, with a reported incidence of approximately 2% of all pediatric tumors.[172] The most common chest-wall neoplasms are the Ewing sarcoma family of tumors and primitive neuroectodermal tumors (PNETs), collectively known as Askin tumors. These tumors have a peak incidence between 10 and 15 years of age and are more common in males. Because of their origin within the chest wall and propensity to invade the adjacent bone, symptoms are commonly of a palpable mass or pain secondary to local extension. Chest radiography should be the first imaging study in the evaluation of these patients and is usually accurate at depicting sites of bone destruction, pleural effusion, and pleural thickening. CT and/or MRI are almost always performed to better delineate the extent of local disease. CT scanning of the chest is essential to assess for the presence of pulmonary metastatic disease (Fig. 66-44). For paravertebral and intercostal masses, MRI may also be necessary to identify and accurately delineate the extent of intraspinal involvement. FDG uptake is usually seen with these

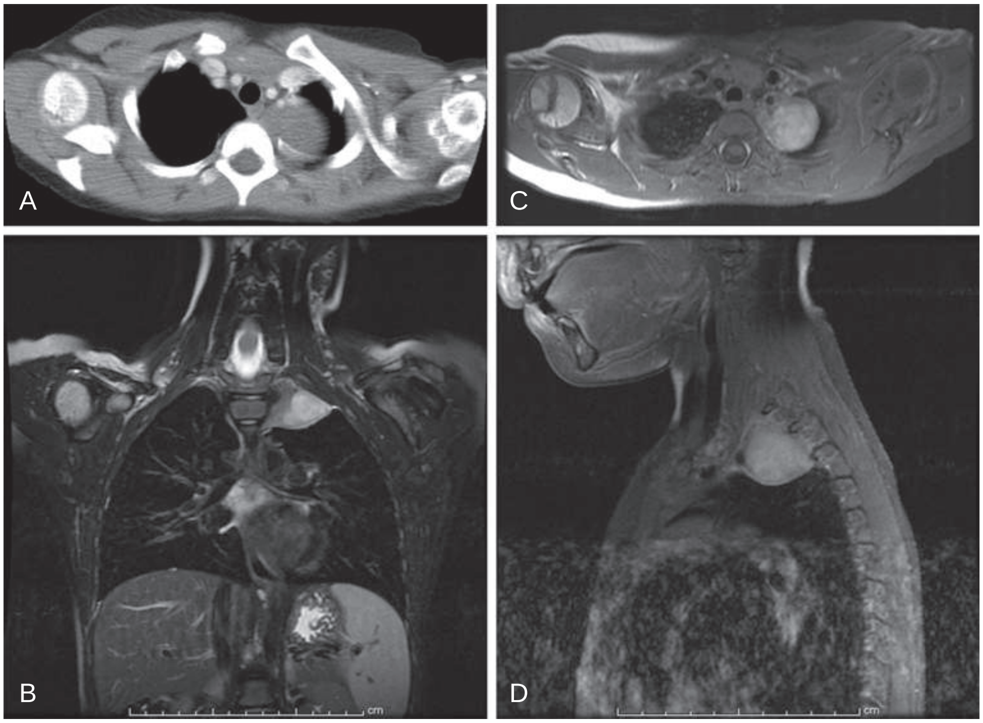

Figure 66-43 A 7-year-old child with a cough. Chest x-ray showed left upper lobe opacity. **A**, CT shows paraspinal mass. **B**-**D**, MRI shows the mass to much better advantage. Cor FSEIR and post–Gd-enhanced images (**C** and **D**) show the mass abutting the neural foramina and left subclavian vessels. MIBG scanning showed barely detectable uptake in the mature ganglioneuroma *(not shown)*.

tumors,[173] and as with Ewing sarcomas of the appendicular skeleton, FDG-PET imaging should be considered both for identifying sites of occult disease that would otherwise go undetected and for monitoring responses to therapy when complete surgical resection cannot be achieved.[174-176]

Wide excision of the primary tumor is critical for local disease control, with variable response to radiation therapy and chemotherapy.[177,178] These chest-wall neoplasms are extremely malignant and can be quite destructive, demonstrating extensive invasion into the airways, local vascular structures, and the lung parenchyma. There is a high rate of both metastatic spread and of local recurrence, and cure requires intensive therapy to control both distant and local disease. The presence of disseminated disease invariably results in a poor long-term outcome in patients with these aggressive poorly responsive tumors.[178]

Rhabdomyosarcoma is the most common soft-tissue sarcoma of childhood and is the second most common

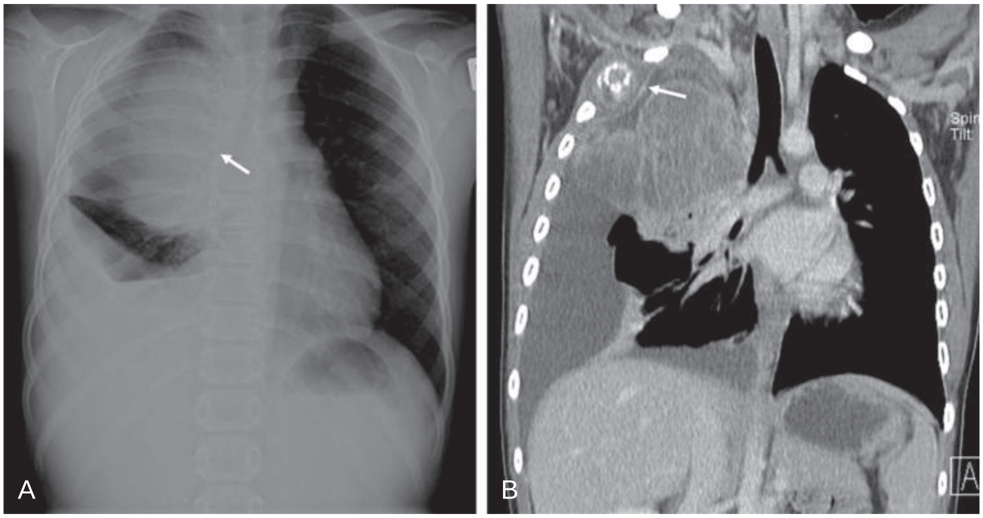

Figure 66-44 Ewing sarcoma of the chest wall in a 6-year-old patient with fever and a chest-wall mass detected by chest x-ray. **A**, Chest x-ray shows pleural effusion, central chest-wall mass *(arrow)*, and partial lung collapse. **B**, Contrast enhanced CT shows a large, locally invasive chest wall mass with pleural involvement and rib destruction *(arrow)*.

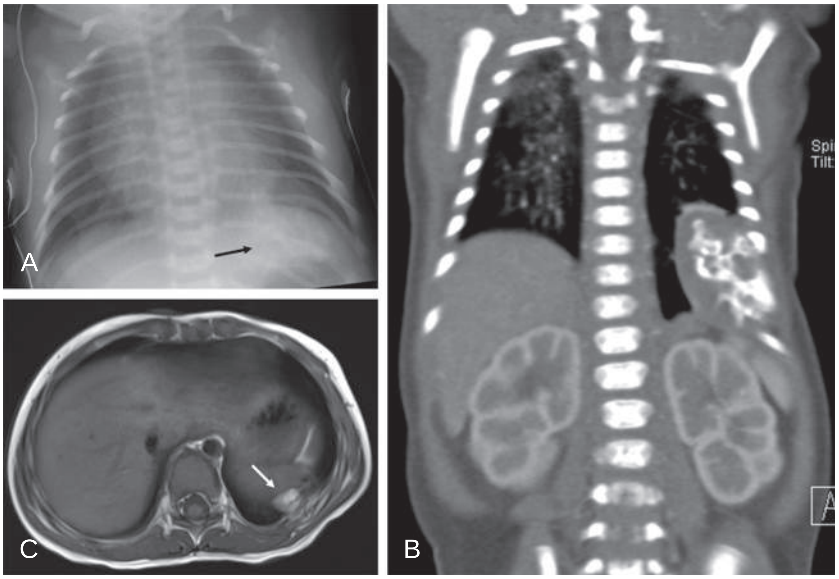

Figure 66-45 A 20-day-old infant with a prenatally diagnosed, calcified, left lower lung mass. Chest x-ray shows calcified mass (**A**, *arrow*) which on CT is seen to arise from posterolateral left ribs (**B**). T1-weighted MRI (**C**) shows presence of high signal intensity fat *(arrow)*, all consistent with the diagnosis of mesenchymal hamartoma.

malignancy of the chest wall in children.[177] Occurring in a younger population of children than thoracic Ewing sarcomas/PNETs, approximately 7% of pediatric rhabdomyosarcomas involve the chest wall and should be included in the differential diagnosis for a chest-wall mass occurring in children younger than the age of 10 years. Biopsy is necessary to make the diagnosis, and the imaging features are nonspecific, with considerable overlap between chest-wall Ewing sarcoma and rhabdomyosarcoma. Chest radiographs reveal a large unilateral chest mass, often with accompanying pleural thickening or effusion. As with Ewing sarcoma, there may be associated destructive rib changes. Cross-sectional imaging by either CT or MRI better delineates the extent of local disease spread. These tumors are heterogeneous in density on CT with variably increased signal on both T1- and T2-weighted MRI images. Contrast enhancement is heterogeneous, and these features likely reflect varying degrees of tumor necrosis. Tumor size, nodal status, and gross total tumor resection (upfront or delayed) have been shown to be significant predictors of event-free and overall survival. Tumors 5 cm or smaller were shown to be amenable to upfront surgical resection. Tumor size and the ability to achieve gross total resection were the strongest predictors of overall survival, with local recurrence after resection resulting in poor overall outcome.[179,180] As with other malignant sarcomas, FDG-PET has been used effectively in staging and response assessment in patients with rhabdomyosarcoma[181-184] and should be considered as an essential component of the imaging evaluation for these patients.

Mesenchymal Hamartomas

Mesenchymal hamartomas of the chest wall are rare, benign neoplasms occurring during infancy. The presence of a large chest-wall mass, often containing calcifications with associated rib destruction and chest-wall deformity should prompt this diagnosis. Because these lesions may contain a significant necrotic/cystic component, the distinction between Langerhans cell histiocytosis, lymphoma, and metastatic neuroblastoma may be difficult to make. These masses often become quite large, and the radiographic finding may be sufficient to suggest the diagnosis, showing a large calcified extrapleural mass with expansion/destruction of multiple ribs. CT or MRI allows more accurate measure of size, characterization of the soft-tissue component of the lesion, and mass effect on the lung and adjacent structures (Fig. 66-45).[185] In particular, the presence of hemorrhagic cystic components has been described as characteristic of these benign neoplasms.[185] There is some data to suggest that spontaneous resolution of these masses may also occur, although the extent of local invasion and tissue destruction may prompt surgical resection, which if complete, is usually curative. The importance of the imaging evaluation is to suggest this diagnosis in order to guide appropriate management of these benign lesions.

Pulmonary Metastases

Metastatic disease to the lung represents the most common pulmonary neoplasm encountered in childhood. The lung is a common site for metastatic disease spread but is an uncommon site in childhood for primary malignancy.

Extrathoracic pediatric tumors that are commonly associated with pulmonary metastases include Wilms tumor, rhabdomyosarcoma, hepatoblastoma, Ewing sarcoma, and osteosarcoma. Neuroblastoma does not typically result in hematogenously spread pulmonary metastases, although in its disseminated form, pulmonary metastases may be seen.

The imaging evaluation in a patient with a known primary malignancy with a propensity to metastasize to

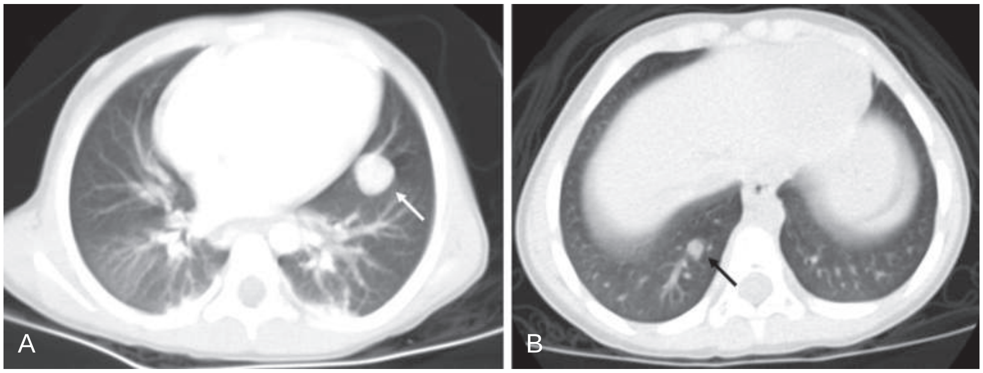

Figure 66-46 A 2½-year-old child with metastatic Wilms tumor. **A**, The nodule at the left lung base *(arrow)* was resected at diagnosis and found to be metastatic disease. **B**, The nodule at the right lung base discovered 1 year later was resected, and found to be a pulmonary hamartoma.

the lung should include chest CT. CT is more sensitive than conventional radiographs in detecting pulmonary metastatic disease, although the increased sensitivity of nodule detection has led to difficulty in distinguishing benign lesions from malignant pulmonary nodules (Fig. 66-46).[22,186] Once the staging evaluation has been completed and the absence of pulmonary metastatic disease and other sites of metastatic disease have been established, it may be reasonable to proceed with routine chest radiographs for subsequent follow-up care, reserving CT for patients with suspected relapse or at high risk for relapse. In particular, in osteosarcoma, which has a high frequency of developing pulmonary metastases and for whom resection of these metastases may be curative, routine follow up chest CT scanning is justifiable.

It is often difficult, particularly with small pulmonary nodules, to distinguish benign causes from malignancy.[22] Calcification within a pulmonary nodule is usually associated with benign causes (except in the case of osteosarcoma); however, there are no specific imaging findings that can be used to reliably make this distinction. Benign pulmonary nodules may result from postinflammatory change or represent small benign intrapulmonary lymph nodes. Short interval follow-up care and documentation of stability in areas of low suspicion is usually adequate to confirm the benign nature of these findings. For more suspicious or enlarging lesions, biopsy or surgical resection is necessary to arrive at a definitive diagnosis. The use of FDG-PET scanning has been advocated in an attempt to distinguish benign from malignant causes of pulmonary metastatic disease.[187] However, while CT can accurately identify lesions of only 1 to 2 mm in size, even the newest generation PET scanners have inherently lower limits of resolution, on the order of 5 mm, because of both the intrinsic spatial resolution of PET detectors and the distance traveled by positrons in the tissue before undergoing annihilation and emission of 511 keV coincident photons.

Controversy still exists as to the significance of pulmonary nodules detected by chest CT in patients with Wilms tumor.[188-190] There are conflicting data as to the importance of pulmonary nodules not visualized by chest radiography but detectable by chest CT in predicting relapse-free and overall survival in patients with Wilms tumor, with the most recent data from the National Wilms Tumor Study Group (NWTSG) 4 and 5 showing that patients with lung lesions visible only on CT have improved event-free, but not overall, survival and do not appear to benefit from pulmonary radiation.[191] Nonetheless it has been shown that the sensitivity of malignant nodule detection is enhanced by CT,[188,189] and current treatment protocols routinely advocate the use of chest CT in staging these patients. In an effort to develop a better understanding of CT features that might distinguish benign from malignant pulmonary nodules,[188] current Children's Oncology Group (COG) Wilms tumor protocols call for surgical resection or biopsy of persistent, suspicious lung nodules detected by CT in patients with intermediate and high-risk disease.

CT scanning of the chest for the purposes of identifying pulmonary metastatic disease need not include intravenous contrast, and the presence of hyperdense contrast material in small pulmonary vessels may confound the interpretation of a subtle parenchymal nodules. In addition, multidetector-row CT scanners allow the acquisition imaging data to be reviewed in 1-mm thick increments or less, as well as in sagittal and coronal planes. This allows the radiologist to make an accurate judgment as to the presence or absence of a suspicious pulmonary nodule and distinguish it from branching vessels. There is no data to suggest that additional high-resolution CT (HRCT) imaging, which is performed using a different image acquisition technique, provides any additional benefit or higher resolution over the 1-mm thick slices obtained during the multidetector helical acquisition.[192] MRI for the detection of pulmonary nodules has been shown to be feasible,[193-195] but there are no studies showing that MRI can replace CT for the routine identification of pulmonary metastatic disease.[196] Similarly low-dose attenuation-correction CT images of the lungs obtained as part of combined PET/CT examinations are not sufficient to exclude small pulmonary nodules, and a diagnostic chest CT obtained during suspended inspiration is still recommended for evaluation of patients at risk and in whom pulmonary metastases will impact clinical management.[197,198]

Although the imaging features of small pulmonary nodules may be nonspecific and the difficulty in distinguishing benign from nonneoplastic etiologies is challenging, the presence and persistence of lung nodules in a child with a solid tumor that has a propensity to metastasize to the lung is usually indicative of metastatic disease. The use of CT in detecting these sites of disease spread remains an essential component of the staging of most pediatric solid tumors.

GASTROINTESTINAL TUMORS

Tumors of the gastrointestinal tract, including the visceral abdominal organs, are relatively uncommon in childhood. In neonates, the majority of intraabdominal masses are not malignant. Children commonly have nonspecific complaints such as abdominal pain, weight loss, or failure to thrive. The imaging evaluation should begin with abdominal radiographs. These often provide valuable initial information, such as the presence of calcifications, mass effect on adjacent visceral organs, and the presence of bowel obstruction or perforation. Abdominal ultrasound is often used to gain further information about a suspicious mass. Doppler assessment may help distinguish cystic from solid masses and delineate patterns of blood flow in and around a tumor. These initial imaging studies are usually sufficient to narrow the initial differential diagnosis and to guide the subsequent imaging evaluation by CT, MRI, and/or PET.

Liver

Although rare in infants and young children, tumors of the hepatobiliary tract are the most common of the gastrointestinal tract tumors seen in childhood.[199-201] Usually a combination of diagnostic studies is obtained, including imaging findings, clinical findings, and serum markers, in order to arrive at a definitive diagnosis. Both benign and malignant tumors may be encountered. Hepatoblastomas are the most common primary malignant tumors in children under the age of 5.[201] Hepatoblastoma is usually encountered in infants and young children under the age of 3, with an average age at presentation of 16 months. At diagnosis the mass is typically quite large (greater than 10 cm).

Metastatic disease is encountered in approximately 20% of the patients, with pulmonary metastases most common. The imaging evaluation should be directed at determining surgical resectability, establishing sites of metastatic disease, and monitoring response to chemotherapy. More than 90% of the children have elevated α-fetoprotein (AFP) levels at diagnosis; the presence of metastatic disease, high AFP levels, and a high PRETEXT stage of liver involvement by tumor are all associated with poor outcome.[202-204] Surgical resection alone is insufficient to achieve cure in most cases, and the combination of surgery and chemotherapy has resulted in 5-year overall survival rates of over 70%.[204]

Abdominal radiographs typically show an abdominal mass. Calcifications may be present, and pulmonary masses may be visible at the time of diagnosis at the lung bases (Fig. 66-47). Ultrasound should be the initial imaging test in a child with an abdominal mass and in hepatoblastoma reveals a large echogenic, intrahepatic

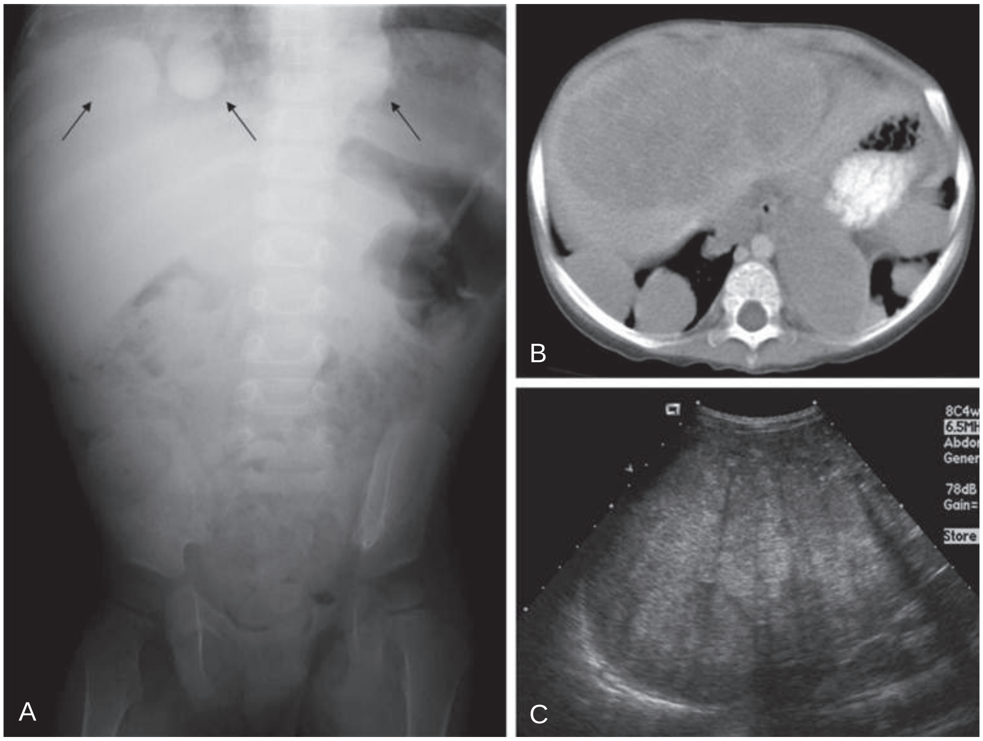

Figure 66-47 Hepatoblastoma. A 15-month-old patient with an abdominal mass found during the physical examination. **A**, Note lung metastases on the abdominal radiograph *(arrows);* **B**, extensive disease depicted on CT; and **C**, the difficulty discriminating the large echogenic mass from normal liver on ultrasound.

mass. It may be difficult with very large masses to define a margin between the hepatoblastoma and normal hepatic parenchyma (see Fig. 66-35). Hypoechoic areas may relate to necrosis or hemorrhage. Color Doppler imaging and pulsed-wave Doppler should be used routinely to assess for intravascular extension of tumor,[201,203] IVC invasion, and portal vein thrombosis, both of which may affect eligibility for liver transplantation.[205]

CT and or MRI are important to better define the relationship of the intra-hepatic mass to adjacent vascular structures, adjacent organs, and to determine the extent of hepatic involvement and surgical respectability.[203] This is best accomplished with biphasic CT or MR angiographic techniques and imaging should include scanning after early arterial and portal venous phases of contrast opacification, since small satellite lesions may only be visible on the early arterial phase imaging (see Fig. 66-2).[206] Typically, hepatoblastomas show a heterogeneous pattern of enhancement. Multiplanar reconstructions are also crucial, both in aiding surgical planning and in better delineating invasion into adjacent vascular structures (i.e., IVC invasion; portal venous thrombosis). MRI imaging typically shows heterogeneously T2-hyperintense lesions with variable patterns of enhancement after gadolinium contrast administration (Fig. 66-48). MRI may be of particular value in delineating subtle lesions that are inconspicuous on contrast enhanced CT scanning. The use of hepatobiliary contrast agents, which are taken up by normal functioning liver tissue but not by malignant liver lesions, as well as diffusion-weighted imaging, can further enhance lesion detection and characterization.[207] As shown in Fig. 66-49, the hepatobiliary agent gadoxetate (Eovist) identifies a poorly enhancing lesion on a background of normally enhancing hepatic parenchyma in a patent with unresectable hepatoblastoma.

The PRETEXT staging system was developed by the International Childhood Liver Tumors Strategy Group (SIOPEL) in an effort to develop a pretreatment imaging-based measurement of disease burden and surgical resectability as a basis for staging and risk stratification of children with hepatoblastoma.[203] In the PRETEXT system of staging the liver is divided into sections, based in part on Couinaud's system of segmental anatomy. PRETEXT staging is based on the number of adjacent liver sections involved with tumor (Table 66-1) and has been shown to be effective in predicting resectability and overall survival in patients who complete neoadjuvant chemotherapy.[208] Because the PRETEXT staging system relies almost entirely on the pretreatment imaging assessment, it is essential that MRI and/or CT scanning be performed using optimal scanning parameters (i.e., multiphasic technique, properly timed contrast bolus, sedation to minimize motion artifact) in order to provide and accurate assessment of the extent of liver involvement. The COG staging system, used widely throughout North America, historically had relied on intraoperative and postoperative assessment, with less dependence on preoperative imaging.[203] However, current protocols have adopted the SIOPEL recommendations for PRETEXT characterization to monitor response to neoadjuvant chemotherapy and determine surgical resectability.[209,210]

The presence of a rising AFP level in a patient previously free of detectable disease should prompt a thorough investigation to identify sites of recurrent disease. The detection of recurrent hepatoblastoma after chemotherapy and surgical resection may be challenging with conventional cross-sectional imaging techniques. Although there has been relatively little published experience using FDG-PET scanning to monitor early sites of disease recurrence, there are reports suggesting FDG-PET and/or CT can improve both the sensitivity and specificity of identifying early foci of disease recurrence.[72,211,212] Characterization by FDG-PET of unusual sites of disease relapse or equivocal abnormalities on CT and/or MRI

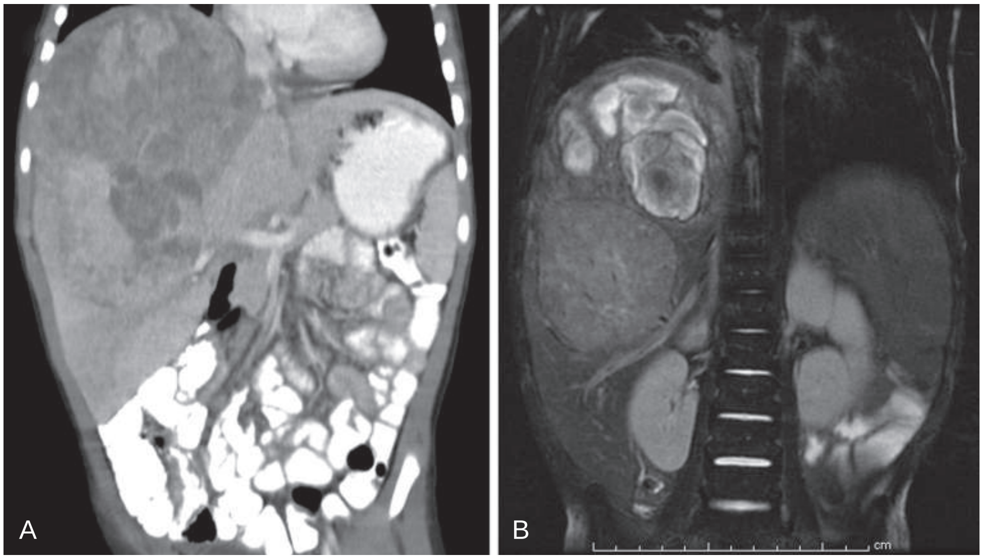

Figure 66-48 A 3-year-old patient with hepatoblastoma, showing superiority of MRI (**B**) in detecting the extent of liver involvement and the relationship to adjacent structures, as compared with CT (**A**).

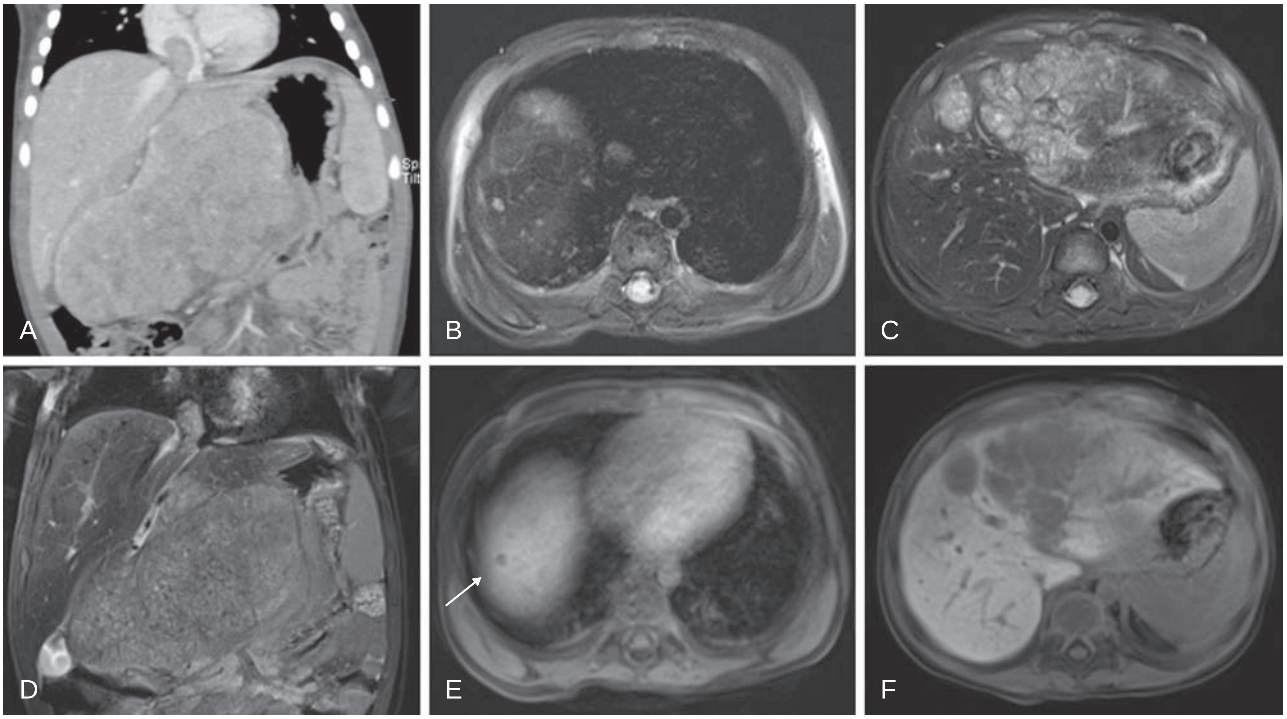

Figure 66-49 A 17-month-old infant with hepatoblastoma. CT angiogram (**A**), fat-suppressed T2-weighted (**B** and **C**) and contrast enhanced MRI (**D-F**) show a large exophytic liver mass with extension into the right atrium. Delayed postcontrast images after administration of the hepatocyte selective contrast agent gadoxetate (Eovist) identifies an additional tiny satellite lesion near the liver dome (**E,** *arrow*).

TABLE 66-1 Childhood Liver Cancer (Hepatoblastoma): PRETEXT Staging System[208]

Tumor Stage	Description
1	Tumor involves only one quadrant. Three adjoining liver quadrants are free of tumor.
2	Tumor involves two adjoining quadrants. Two adjoining quadrants are free of tumor.
3	Tumor involves three adjoining quadrants or two nonadjoining quadrants. One quadrant or two nonadjoining quadrants are free of tumor.
4	Tumor involves all four quadrants. There is no quadrant free of tumor.
Extrahepatic Considerations	
	V+: Involvement of the inferior vena cava and/or all three hepatic veins
	P+: Involvement of the main portal vein and/or both left and right branches
	E+: Extrahepatic disease in the abdomen
	M+: Distant metastases

PRETEXT, Pretreatment Extent of Disease.

may confirm the presence of metabolically active disease and guide subsequent treatment decisions (Fig. 66-50).

Hepatocellular Carcinomas

Hepatocellular carcinoma, although rare in childhood, is the second most common primary hepatic malignancy. It typically occurs in older children, with a mean age at diagnosis of approximately 12 years.[202] There is a slight male predominance and an association between development of hepatocellular carcinoma and preexisting liver disease, such as hepatitis B, biliary atresia, and Fanconi syndrome, as well as certain metabolic diseases such as hereditary tyrosinemia and glycogen storage disease.[202] The imaging features of hepatocellular carcinoma are not unique, and as for hepatoblastoma, ultrasound examination is often the first imaging study performed. Multiphase CT scanning with multiplanar reconstruction affords the greatest likelihood of detecting subtle lesions and in assessing relationships to adjacent structures and the vasculature,[200,206] although the use of diffusion-weighted MR imaging and hepatobiliary MR contrast agents has added to the appeal of MRI for evaluating these patients.[44,213] The CT appearance of hepatocellular carcinoma depends on tumor size and phase of contrast administration. Lesions are typically isointense or mildly hypointense relative to liver and show hyperattenuation on early arterial phase scanning, becoming isointense during later venous phases of imaging (Fig. 66-51). Early arterial phase, portal venous phase, and

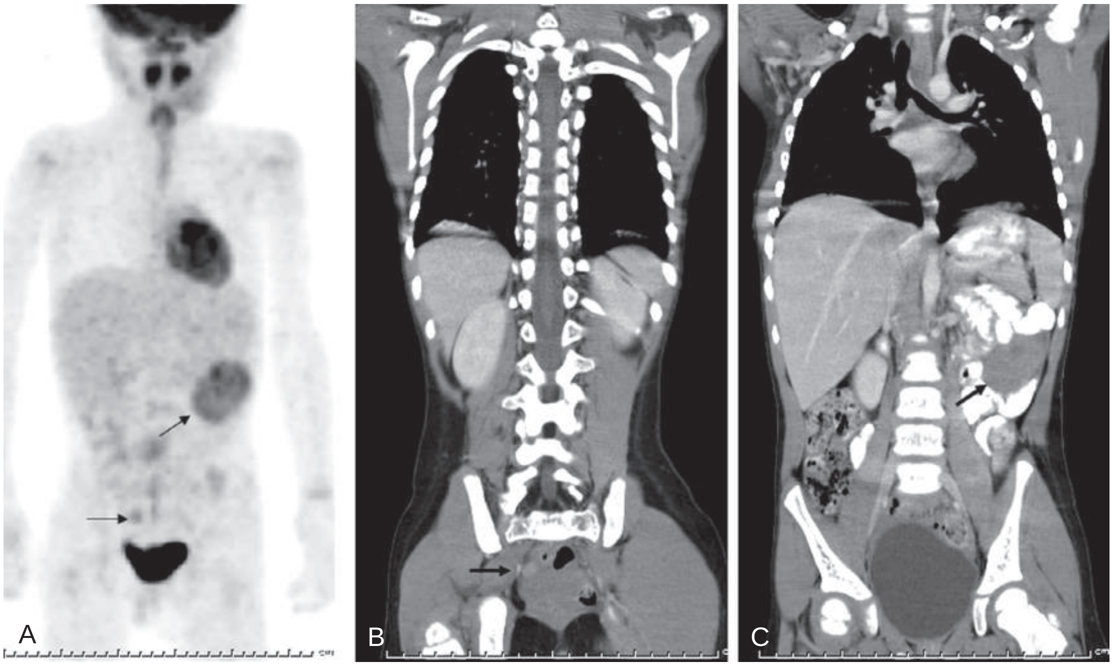

Figure 66-50 A 5-year-old girl with metastatic hepatoblastoma who had undergone resection of the primary mass. The patient was in remission with new rising AFP levels. FDG-PET (**A**) shows two foci of uptake *(arrows),* corresponding to soft-tissue masses on CT in the mesentery (**B**) and deep pelvis (**C**, *arrows*). Surgery confirmed metastatic disease.

delayed equilibration venous phase images have all been shown to contribute to enhancing lesion conspicuity, providing the greatest sensitivity and specificity in lesion detection.[214] MR imaging, with opposed-phase imaging to detect microscopic fat and hepatobiliary agents to identify lesions with hepatocyte function can help in distinguishing hepatocellular carcinoma from benign liver lesions such as hepatic adenoma and focal nodular hyperplasia (FNH)[201]; however, biopsy may still be necessary to confirm the diagnosis (Fig. 66-52). Gadolinium-enhanced MRI, like CT, shows hyperenhancing lesions during early arterial phase imaging, with larger tumors showing variable signal intensity and patterns of enhancement[215] and absent enhancement on delayed hepatocyte phase imaging when hepatobiliary agents are used. Superparamagnetic iron-oxide particles, which are taken up by Kupffer cells present in normal liver and in FNH, have been used to help differentiate hepatocellular carcinoma, which contains few if any Kupffer cells, from benign liver masses and to enhance lesion conspicuity relative to the surrounding liver tissue.[215]

The fibrolamellar form of hepatocellular carcinoma occurs in adolescents and has imaging features that are distinctive, showing a central scar and often associated with calcification (Fig. 66-53). Fibrolamellar hepatocellular carcinoma is not associated with elevated AFP, and overall survival is higher than in other forms of hepatocellular carcinoma, in part because of its presentation as a solitary localized mass amenable to complete surgical resection.[216] Ultrasound reveals a hypoechoic mass, often

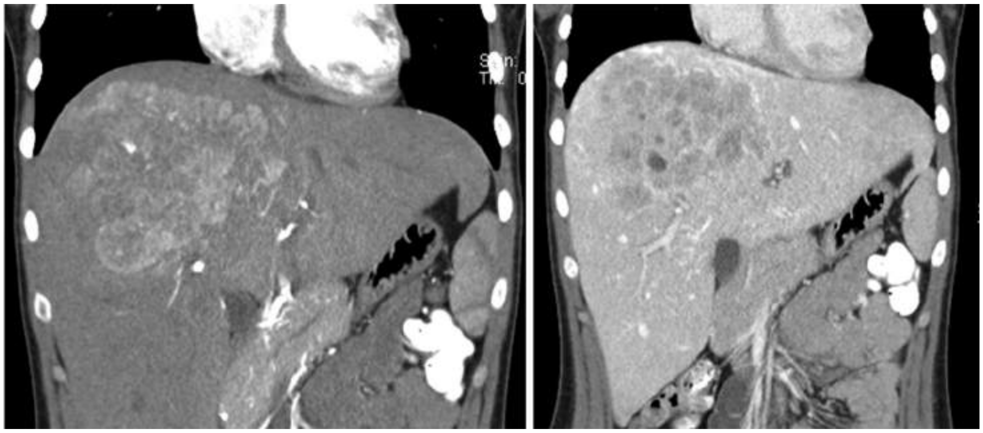

Figure 66-51 An 18-year-old female with fibrolamellar hepatocellular carcinoma. Biphasic contrast-enhanced CT demonstrates a large hyperenhancing lesion in the right lobe, which on delayed images becomes hypodense and less distinct from the background liver tissue.

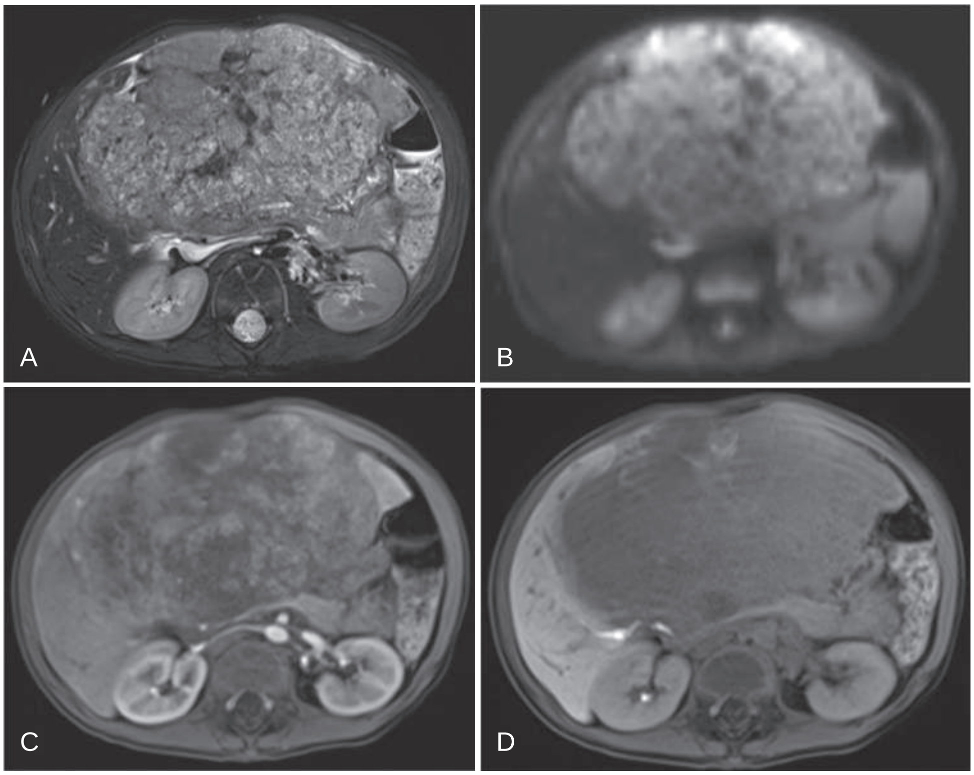

Figure 66-52 A 19-month-old child with hepatocellular carcinoma. T2-weighted (**A**) and DWI (**B**) images show a large heterogeneous mass in the left liver lobe with restricted diffusion. **C**, After Gd-contrast administration with gadoxetate (Eovist), early dynamic images shows heterogeneous enhancement. **D**, Delayed imaging shows absence of gadoxetate (Eovist) enhancement on the hepatocyte phase, in contrast to the enhancement of the adjacent normal liver.

with a focal central hyperechoic scar. After the initial characterization by ultrasound, CT or MRI is indicated for further staging. On CT, fibrolamellar hepatocellular carcinoma is usually relatively low attenuation, well-circumscribed mass with heterogeneous enhancement, initially becoming hyperintense relative to the surrounding liver parenchyma and ultimately becoming isointense to the liver during the equilibration phase of scanning. A nonenhancing central scar is seen in the majority of the cases (see Fig. 66-53).[217] On MRI, lesions are isointense to hypointense relative to the liver on T1-weighted images and isointense to hyperintense on T2-weighted sequences. The pattern on gadolinium enhancement is similar to that observed on CT, with lesions becoming hyperintense early after contrast infusion and ultimately isointense to liver on delayed scanning. The distinction between fibrolamellar hepatocellular carcinoma and FNH, both of which have similar imaging characteristics and patterns of early contrast enhancement, may be difficult to make (compare Figs. 66-53 and 66-54). However, as shown in Figure 66-54, the uptake and retention of a hepatobiliary contrast agent by the lesion on delayed hepatocyte phase imaging is nearly diagnostic of FNH.[213]

Embryonal Sarcomas (Hepatic Mesenchymomas)

Embryonal sarcomas of the liver are the fourth most common hepatic neoplasm in children after hepatoblastoma, hepatocellular carcinoma, and infantile hepatic hemangioma. These tumors are highly malignant, usually present in later childhood (between the age of 6 and 10), and are typically accompanied by symptoms of abdominal pain and an abdominal mass. Embryonal sarcomas may be difficult to distinguish from mesenchymal hamartomas, although the latter tumors are more commonly diagnosed before the age of 2 years. At presentation embryonal sarcomas are often large and contain both cystic and solid areas with areas of necrosis. Ultrasound reveals a heterogeneous echogenic mass with many features overlapping with mesenchymal hamartoma and infantile hemangioma. Calcifications are rare. CT reveals a large hypodense mass, often with septations and areas of internal high attenuation; MRI shows a high signal-intensity lesion on T2-weighted images (Fig. 66-55), with predominantly low signal on T1-weighted images. After contrast enhancement, imaging by either CT or MRI demonstrates peripheral enhancement and central areas of heterogeneous signal intensity that likely reflect solid tumor elements, cystic spaces, necrosis, and foci of hemorrhage.[218] Despite being highly malignant, complete surgical resection and adjuvant chemotherapy can be curative in the majority of these patients.[202,219]

Mesenchymal Hamartomas

Mesenchymal hamartoma, in contrast to hepatoblastoma, is a predominantly cystic mass most often occurring in infants under the age of 2 years. It commonly

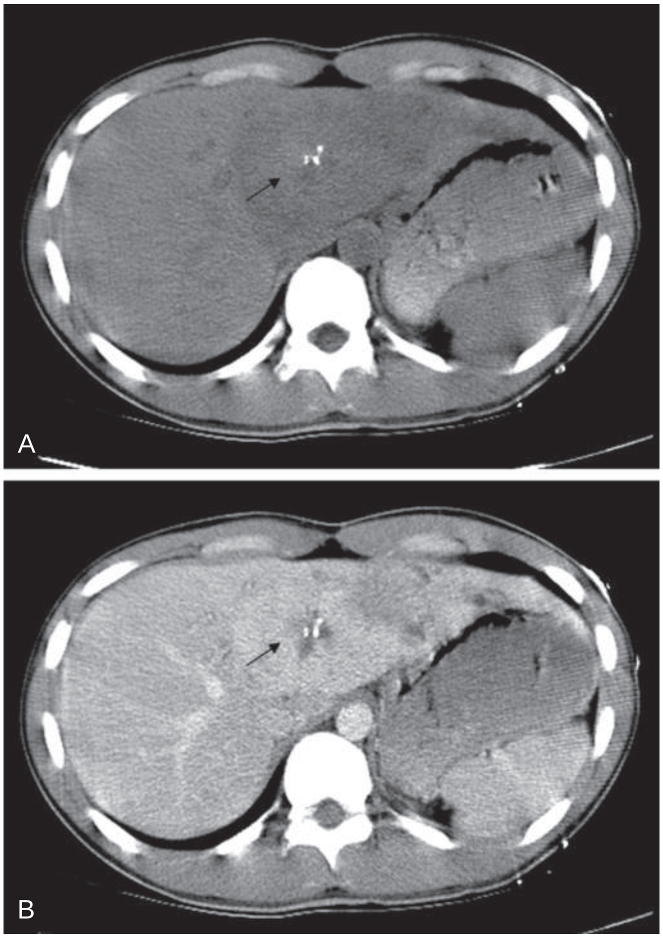

Figure 66-53 A 16-year-old patient with fibrolamellar hepatocellular carcinoma. Unenhanced (**A**) and contrast enhanced (**B**) CT show the calcifications and poorly enhancing central scar *(arrows)* typical of this form of hepatocellular carcinoma.

presents as a painless, palpable abdominal mass comprised of mesenchymal tissue, bile ducts, hepatocytes, and hematopoietic cells. In contrast to hepatoblastoma, which is the primary differential consideration in an infant, the AFP levels are normal. On imaging, radiographs reveal a large right upper quadrant mass. Calcifications, which are seen in hepatoblastoma, are unusual in mesenchymal hamartoma.[201] Ultrasound examination typically shows a complex, predominantly cystic mass. MRI and CT may be of value in further characterizing the lesion and typically show large multilocular cystic masses with septations. Only minimal enhancement is seen. Some of the masses may have a predominantly solid component yielding a so-called Swiss cheese appearance to the mass, and contributing to some difficulty in the differential diagnosis. MRI may also demonstrate the complex nature of the cystic fluid, with gelatinous, more complex fluids showing high signal on T1-weighted images instead of the more common T2 bright/ T1 dark serous fluid contained in the majority of the cysts (see Fig. 66-55). Treatment is surgical resection, and recurrence is uncommon.[202]

Infantile Hepatic Hemangiomas

Infantile hepatic hemangioma are proliferative endothelial cell neoplasms that may be solitary or may diffusely involve the liver. Infantile hepatic hemangiomas have characteristic phases of cellular proliferation followed by spontaneous involution. They are often referred to as *hepatic hemangioendothelioma*, creating some confusion in nomenclature and must be distinguished from epithelioid hemangioendothelioma.[220] The latter is a proliferative tumor with malignant potential that does not involute in contrast to the benign hepatic hemangioma. Infantile hepatic hemangiomas share the same growth characteristics as the more common cutaneous infantile hemangiomas. The majority present by 6 months of age, and about half are associated with cutaneous lesions. The majority of the infantile hepatic hemangiomas involute spontaneously, and adverse outcomes are only observed when there is massive hepatic involvement and arteriovenous (AV) shunting with accompanying high-output cardiac failure. The presence of extensive hepatic involvement and near complete replacement of the liver parenchyma occurs in the diffuse infantile hemangioma variant and is associated with profound hypothyroidism secondary to overproduction of type III iodothyronine deiodinase.[221]

Ultrasound is usually the first step in the evaluation and typically shows either focal or multiple hepatic masses with heterogeneous echotexture. It may be possible to identify a large draining varix or varices. Both arterial and venous waveforms are often detected, and calcifications may be present. More characteristic findings are seen on CT and MRI. MRI typically shows homogeneously hypointense lesions on T1-weighted imaging with heterogeneous hyperintense masses on T2-weighted images. After contrast enhancement, imaging by either CT or MRI reveals a typical pattern of peripheral contrast enhancement with gradual filling in centrally (centripetal enhancement) (Fig. 66-56, *A* and *B*).[222] Diffuse infantile hepatic hemangiomas may nearly completely occupy the entire liver parenchyma (see Fig. 66-56, *C* and *D*). The associated hypothyroidism is the result of the elaboration of deiodinase enzymes affecting thyroid hormone synthesis, which can in turn result in cardiac failure and neurologic impairment. These endocrinologic findings are important in securing the diagnosis, because the multiple hepatic masses may share overlapping imaging features with malignant hepatic lesions such as hepatoblastoma or metastatic neuroblastoma.

High-output cardiac failure associated with large varices and significant high-volume AV shunting can be effectively treated using interventional radiologic techniques, including coil and particle embolization. This may be essential in stabilizing the patient in order to initiate treatment to accelerate the involution of these benign proliferative lesions.[220]

Hepatic Adenoma

Other benign hepatic lesions include solitary hepatic adenomas. Hepatic adenomas are unusual in children in the absence of a predisposing condition, such as glycogen storage disease, galactosemia, or tyrosinemia. The

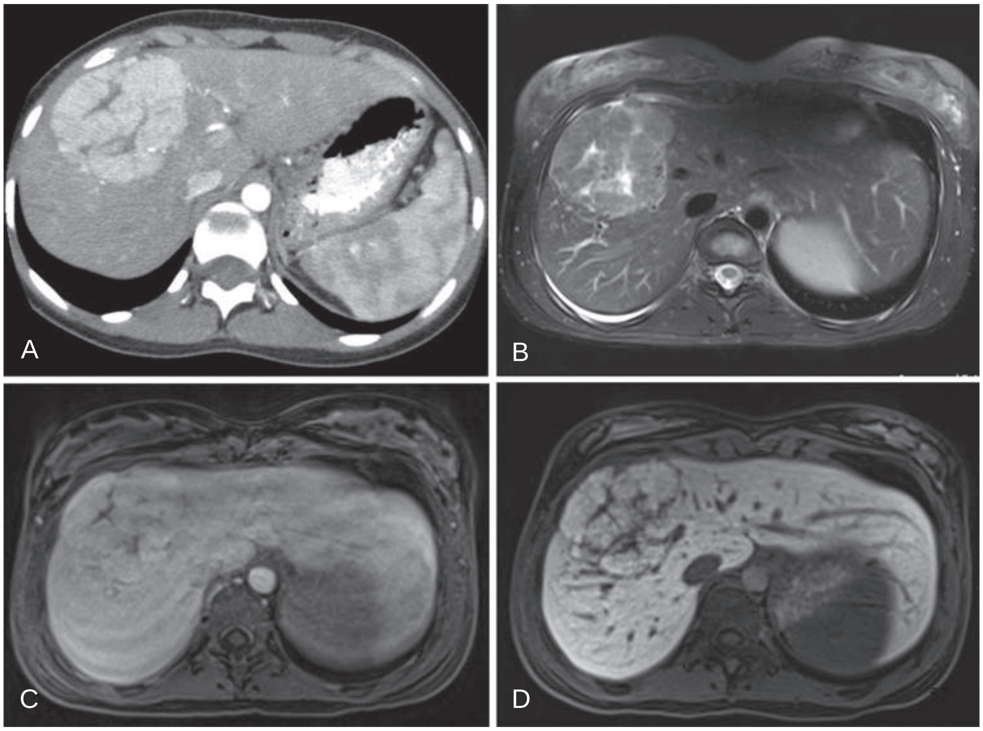

Figure 66-54 A 15-year-old patient with abdominal pain and a liver mass noted on outside CT (**A**). **B**, MRI performed with gadoxetate (Eovist) shows a mildly T2-hyperintense lesion that becomes isointense to liver shortly after contrast infusion (**C**), but that retains the contrast on delayed hepatocyte phase imaging (**D**). The features, together with the central nonenhancing scar, are characteristic of FNH.

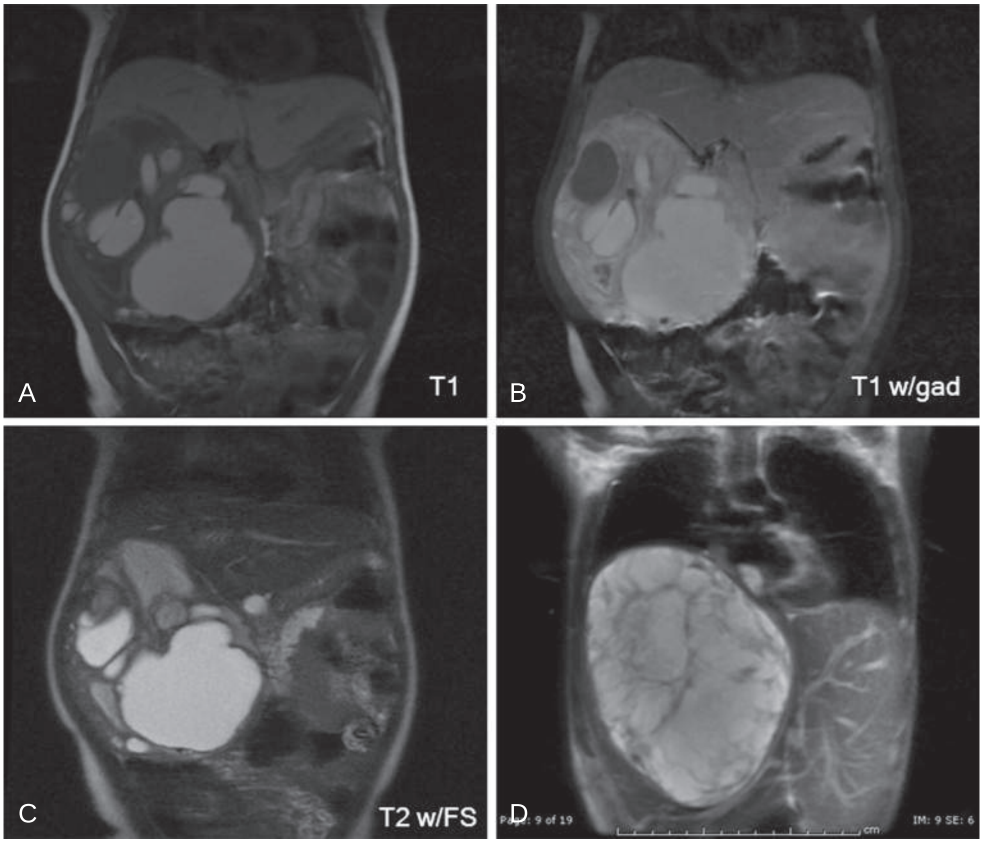

Figure 66-55 Mesenchymal hamartoma. MRI showing the complex nature of the multiple cysts in a 13-month-old infant with mesenchymal hamartoma. Note the importance of obtaining T1-weighted images before contrast administration (**A** and **B**), in addition to fluid-sensitive T2-weighted sequences (**C**), because areas of complex fluid may be T1 bright and should not be misinterpreted as enhancing solid components of the mass. Note the similarity to the large embryonal sarcoma shown in (**D**).

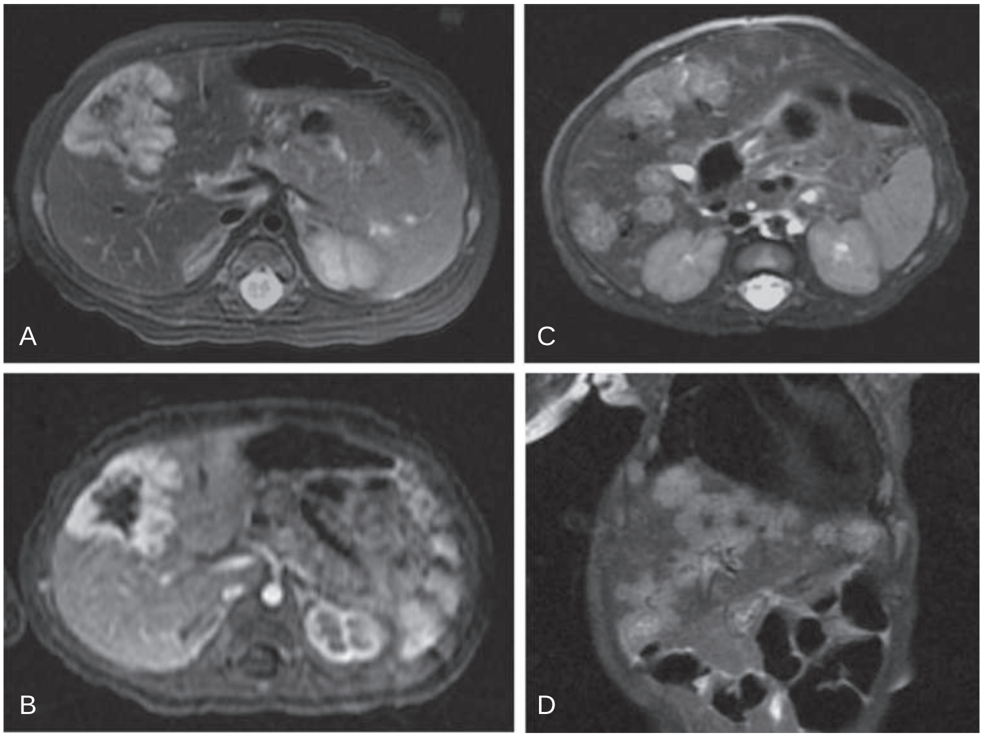

Figure 66-56 Involuting hepatic hemangioma and diffuse infantile hemangioma. Fat-suppressed T2-weighted (**A** and **C**) and post–gadolinium-enhanced (**B** and **D**) MRI showing large solitary peripherally enhancing lesion typical of involuting hepatic hemangioma (**A** and **B**) and the multifocal hepatic involvement in a patient with hypothyroidism and diffuse infantile hemangioma (**C** and **D**).

imaging features of hepatic adenomas are not specific, and imaging is usually directed at monitoring these lesions for increases in size and number. Rapid increase in size is generally considered to be an indication of degeneration of benign adenoma to hepatocellular carcinoma, particularly in patients with glycogen storage disease.[223] Regular surveillance, typically by ultrasound, is indicated in these patients. MRI should be considered to further aid in characterizing new or multiple lesions.

Focal Nodular Hyperplasia

FNH is rare in children, most commonly occurring in teenage and adolescent years.[224] It is usually found incidentally during imaging evaluation for other reasons. This benign lesion typically shows a central fibrous scar with surrounding hyperplastic hepatocytes and small bile ducts. As discussed, these imaging features are also seen in the fibrolamellar variant of hepatocellular carcinoma, and the distinction between FNH and fibrolamellar hepatocellular carcinoma may be difficult. CT and MRI scans usually show rapid homogeneous contrast enhancement during early arterial-phase imaging (see Figs. 66-53 and 66-54). Lesions then characteristically become isodense to the liver during later phases of enhancement, with the central scar remaining hypodense even during the delayed imaging period. With newer MRI imaging techniques performed either with iron-oxide particles that accumulate in reticuloendothelial cells (Kupffer cells) present in the hyperplastic FNH lesions or with hepatocyte selective gadolinium based agents such as gadoxetate (Eovist), the diagnosis of FNH[213,224] can generally be made with confidence. For equivocal cases, scintigraphy with ^{99m}Tc-sulfur colloid can also be performed, although in practice this is rarely necessary. These masses may be monitored by ultrasound to ensure stability and do not typically require surgical resection unless they become symptomatic or undergo progressive enlargement. FNH has also been observed in patients who have completed therapy for nonhepatic primary tumors.[225-227] Whereas larger masses may be by detected by ultrasound or CT, it has been found that dynamic contrast-enhanced MRI is a highly sensitive technique for identifying these lesions. Occasionally these lesions can be characterized by ultrasound using high-resolution, high-frequency transducers; however, they are unequivocally shown by contrast-enhanced MRI (Figs. 66-57 and 66-58). These FNH lesions are also isointense to the liver on conventional imaging sequences and display rapid early arterial enhancement, after which they become nearly isointense to the liver and indistinguishable from the surrounding parenchyma. Hepatobiliary contrast agents are now used routinely to further characterize these lesions as benign FNH (see Fig. 66-53) and distinguish them from hepatic metastatic deposits. It is important recognize these characteristic patterns of enhancement so as to not mistake this benign entity for recurrent disease or a new site of disease relapse.

Other lesions occurring in the liver include sarcomatous tumors such as malignant angiosarcomas and rhabdomyosarcomas of the biliary tree, malignant vascular tumors, and metastatic disease. The most common metastatic lesions occurring in the liver include neuroblastomas, locally invasive and hematogenously-disseminated

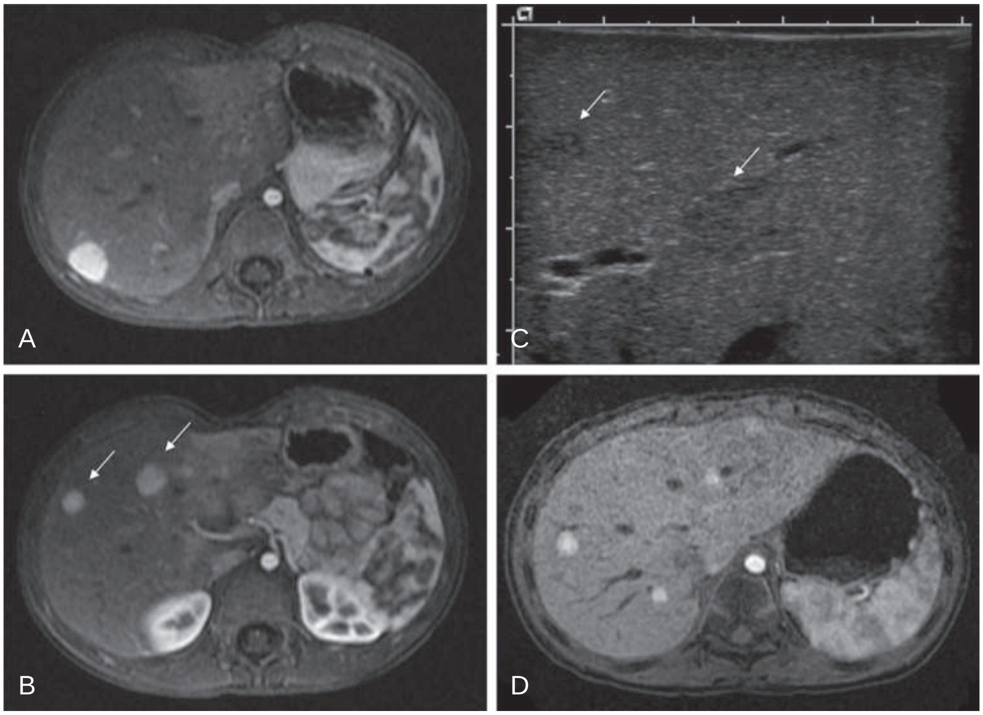

Figure 66-57 A 19-month-old boy with a history of treatment for stage 4 neuroblastoma. Follow-up MRI imaging using dynamic contrast enhancement shows multiple brightly enhancing lesions throughout the liver (**A** and **B**). These rapidly become isointense to liver during delayed scanning. They are not seen on any other sequences. The enhancement characteristics are typical of FNH. Intraoperative ultrasound localized lesions for biopsy (**C**) and confirmed them as FNH. Similar lesions in a 2-year-old patient treated in infancy for stage 4 neuroblastoma are shown in **D**.

Wilms tumors, rhabdomyosarcomas and Ewing sarcomas.[201] The imaging characteristics of these metastatic hepatic lesions are nonspecific with lesions characteristically hypoechoic on ultrasound relative to normal liver parenchyma, decreased attenuation on CT scan, low T1 and increased T2 signal intensity on T1- and T2-weighted MR images, respectively, and variable patterns of contrast enhancement with both CT and MRI.

Spleen

Primary tumors of the spleen are exceedingly rare.[228] Angiosarcomas have been reported but are not common in childhood. Involvement of the spleen by lymphoma and lymphoproliferative disease is the most common cause of malignant infiltration of the spleen.[229] Imaging evaluation by ultrasound often demonstrates multiple or

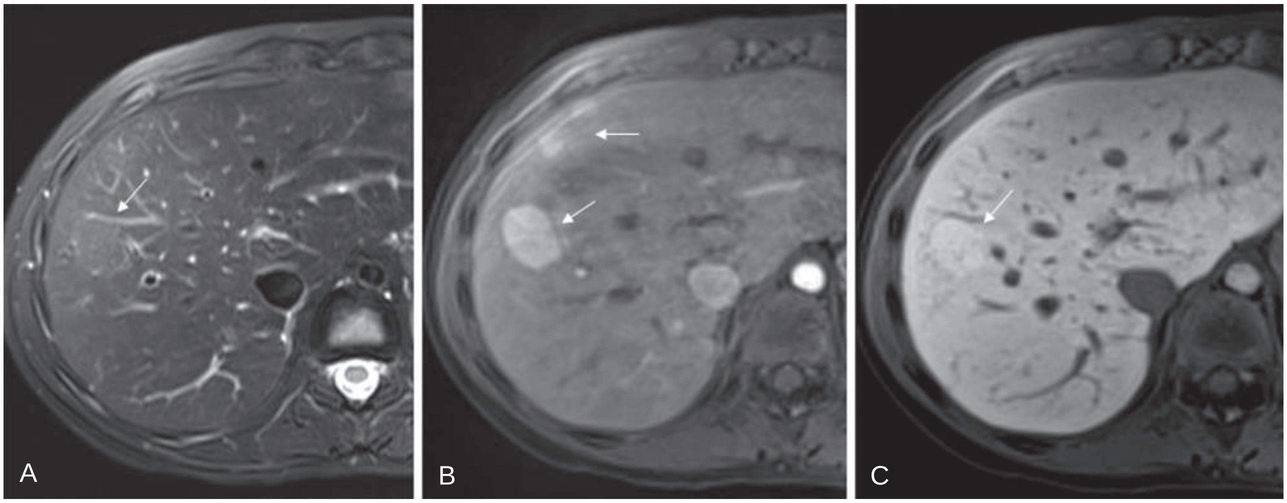

Figure 66-58 A 6-year-old patient treated for stage 4 neuroblastoma as an infant who developed multiple enhancing liver lesions proven to be FNH on biopsy. Subsequent MRI with gadoxetate (Eovist) shows representative lesions *(arrows)* to be isointense to liver on T2-weighted imaging (A), hyperenhancing on an early–arterial-phase image (**B**), and with persistent enhancement on the 20-minute delayed hepatocyte phase sequence (**C**). These features are characteristic of FNH.

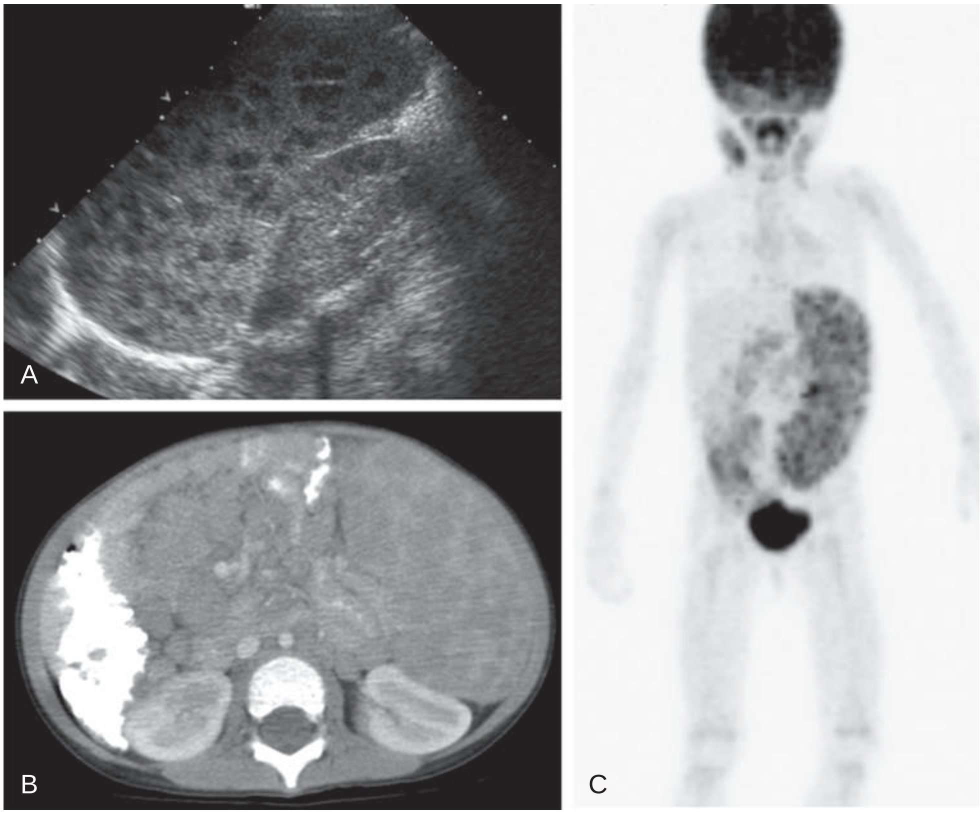

Figure 66-59 Lymphoproliferative disease. Ultrasound (**A**) and CT (**B**) demonstrate extensive splenic involvement and massive adenopathy in a patient with an unclassified lymphoproliferative disease. FDG-PET (**C**) shows multiple sites of disease and in particular confirms the extensive abnormalities in the spleen.

solitary hypoechoic masses (Fig. 66-59). CT scanning may be equivocal because of variable phases of splenic parenchymal enhancement. MRI, in contrast, can be very sensitive at detecting focal lesions that are occult by a CT and/or ultrasound. In the setting of lymphoma and lymphoproliferative disease, FDG-PET imaging has been shown to be an effective adjunctive imaging modality for detecting splenic involvement.[230,231] This is of importance both in determining the patient's stage of disease at diagnosis and in identifying sites of disease to be watched and assessed for response after treatment. In addition, the negative predictive value of PET/CT in discriminating between benign and malignant lesions has been emphasized both in patients with coexistent malignant disease and in patients without history of malignancy in whom a solid splenic mass is identified by other imaging techniques.[232]

Biliary Tract

Tumors of the gall bladder and bile ducts are rare in children, and include biliary rhabdomyosarcomas and cholangiocarcinomas, the latter of which occur in the setting of chronic ulcerative colitis. The initial imaging evaluation is usually by ultrasound, often for symptoms of biliary-tract obstruction such as pain, jaundice, and weight loss. Ultrasound may show evidence of bile-duct dilation and biliary-tract obstruction; however, identification of a focal mass usually requires additional evaluation by MRI/magnetic resonance cholangiopancreatography (MRCP), with diagnostic confirmation by endoscopic retrograde cholangiopancreatography (ERCP). Particularly in the setting of rhabdomyosarcoma, FDG-PET is of value in accurately determining the stage of disease.[184] Biliary rhabdomyosarcoma can arise from the intrahepatic or extrahepatic biliary bile ducts, gallbladder, cystic duct, or ampulla of Vater. At the time of presentation with obstructive jaundice and abdominal pain, these neoplasms are usually quite large, and as a result the distinction between biliary rhabdomyosarcoma, primary hepatic tumors arising in the hepatic hilum, pancreatic neoplasms, and renal/suprarenal masses may be challenging (Fig. 66-60).[233]

Pancreas

Primary tumors of the pancreas are rare in children, in contrast to adults. In addition, the types of tumors found in children differ from the adult tumors, in whom adenocarcinomas predominate. Pancreatic tumors can be classified into nonfunctional tumors of the exocrine pancreas and functional tumors of the endocrine pancreas, as well as secondary metastatic pancreatic neoplasia.[234]

Malignant epithelial neoplasms arising from the exocrine pancreas include pancreatoblastomas, and solid papillary epithelial neoplasms of the pancreas. Primary nonepithelial tumors of the pancreas are very rare and include lymphomas, rhabdomyosarcomas and primitive

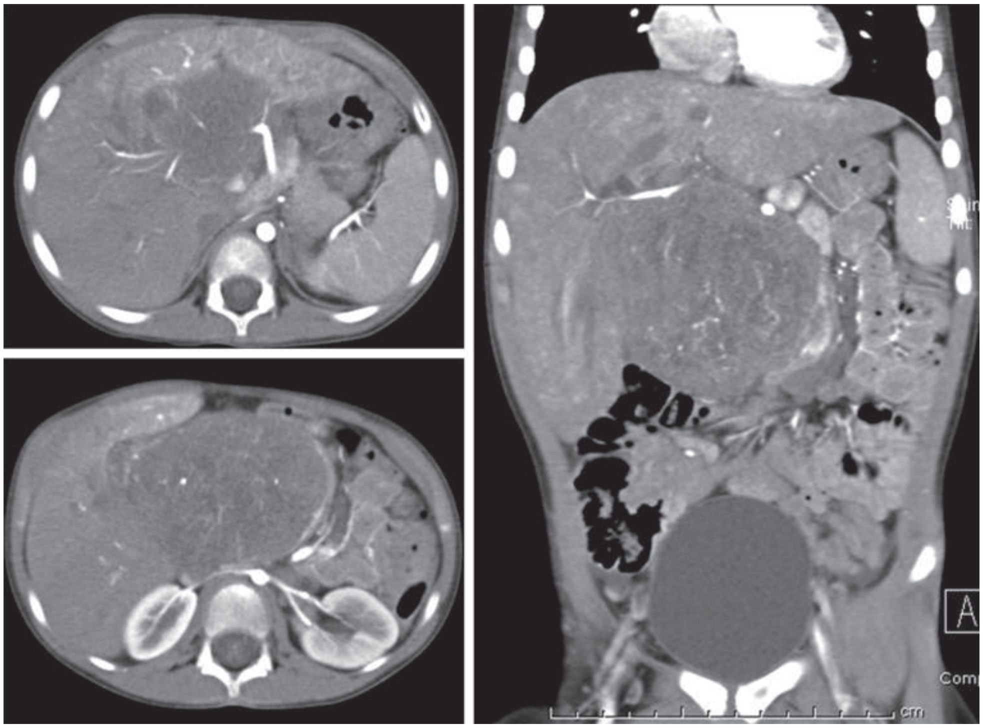

Figure 66-60 Biliary rhabdomyosarcoma. Dynamic contrast-enhanced CT reveals a large mass in the hepatic hilum, encasing the vessels and infiltrating adjacent structures. Note the lack of significant bile duct dilation, allowing the mass to become very large before producing symptoms of obstructive jaundice.

neuroectodermal tumors. Pancreatic endocrine neoplasms are rare in children, and they are usually associated with the multiple endocrine neoplasia syndrome. These include hormonally active tumors such as insulinomas, gastrinomas, VIPomas and glucagonomas.

Pancreatoblastomas

Pancreatoblastomas can occur at any age but are most common in children between the ages of 1 and 8 years. Pancreatoblastomas are reported as arising in or around the head of the pancreas in roughly half of the cases. Because they are nonfunctional, they are typically quite large at the time of diagnosis. Presenting symptoms include abdominal pain, nausea, and weight loss. Obstructive symptoms such as jaundice or pancreatitis are less common despite the large size of these masses, which has been attributed to the soft, gelatinous, and compliant consistency of these tumors.[235] These tumors are typically well encapsulated, have heterogeneous patterns of echogenicity on ultrasound, and show variable patterns of contrast enhancement on CT (Fig. 66-61). Calcifications are commonly seen and are probably the sequelae of hemorrhage and necrosis known to occur within these tumors. MRI shows a similar pattern of heterogeneous signal intensity and enhancement, with masses usually hypointense on T1-weighted images and variably hyperintense on T2-weighted and post–gadolinium-enhanced images. As with hepatoblastoma, pulmonary metastatic disease is often present, and the staging evaluation must include a CT of the chest. Because of their large size, the tissue of origin may be difficult to clearly delineate, and differential considerations includes exophytic hepatoblastoma, renal and suprarenal masses, and retroperitoneal neoplasms.[236]

Solid Pseudopapillary Neoplasms

Solid pseudopapillary neoplasms of the pancreas (also known as *solid* and *papillary epithelial neoplasms*)[237] are a rare primary pediatric pancreatic tumor. In contrast to other pancreatic tumors occurring in children, however, the prognosis after complete surgical resection is favorable.[238] Studies have reported up to a 90% survival rate after complete resection. In one small study, median age at presentation was 13 years, with abdominal pain, a palpable mass, dyspepsia, and pancreatitis the most common presenting symptoms.[238] On imaging, usually by ultrasound and/or CT, solid pseudopapillary tumors appear as solid, well-demarcated masses. On ultrasound there is heterogeneous echotexture, occasionally with fluid-filled cystic spaces (Fig. 66-62). CT shows a heterogeneous mass with peripheral contrast enhancement. Although these tumors are slow-growing tumors with low-grade malignant behavior, FDG uptake on PET imaging has been shown in solid papillary tumors of the pancreas.[239] FDG-PET scanning may therefore be of value in follow-up imaging to assess for recurrence. Despite

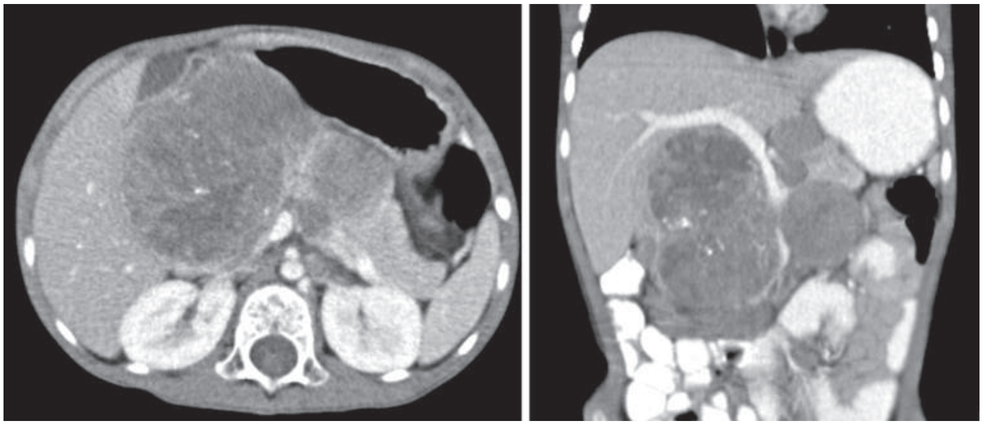

Figure 66-61 Pancreatoblastoma in a 4-year-old boy with a palpable abdominal mass. Contrast-enhanced CT shows a large, calcified retroperitoneal mass centered in the pancreas. Both adrenal glands were normal. Lung metastases were also present (not shown). The biopsy showed pancreatoblastoma.

high rates of overall 5-year survival, if metastases are present and not resectable, survival is poor with no clear, beneficial role reported for adjuvant chemotherapy or radiation therapy.[237]

Pancreatic Sarcomas

Mesenchymal tumors of the pancreas include rare synovial-cell sarcomas, myofibroblastic tumors, and metastatic rhabdomyosarcomas. Extremity rhabdomyosarcomas seem to have an unusual predilection for metastases to unusual cites such as the breast, ovary, testicle, and pancreas.[240] Two patients shown in Figure 66-63 had metastatic rhabdomyosarcoma to the pancreas. Local presentation may include pancreatitis and abdominal pain or may be incidental (see Fig. 66-48, *B*). A recent report from three major pediatric centers also noted an association of alveolar rhabdomyosarcoma with pancreatic metastasis,[241] emphasizing the importance of follow-up imaging in these patients and including an increasing role for routine FDG-PET/CT imaging to identify unusual sites of metastatic spread in patients with rhabdomyosarcoma.

Pancreatic Lymphomas

Burkitt lymphoma is well known to involve organs of the gastrointestinal tract, although isolated pancreatic involvement is unusual. Typically pancreatic involvement is accompanied by other sites of visceral and mesenteric disease. Diffuse pancreatic infiltration in the setting of Burkitt lymphoma is shown in Figure 66-64 in a child who had symptoms of pancreatitis. On ultrasound the pancreas was diffusely enlarged and heterogeneous in echogenicity. CT scan showed diffuse pancreatic enlargement, poor enhancement, and a homogeneous low attenuation. As is common for the rapidly dividing Burkitt lymphoma, a prompt response to chemotherapy was observed, with near complete resolution of pancreatic abnormalities 10 days after onset of chemotherapy.

Pancreatic Islet-Cell Tumors

Functioning islet-cell tumors of the pancreas are rare but important clinically because of symptomatic endocrine abnormalities that result from constitutive secretion of hormones normally tightly regulated by the endocrine

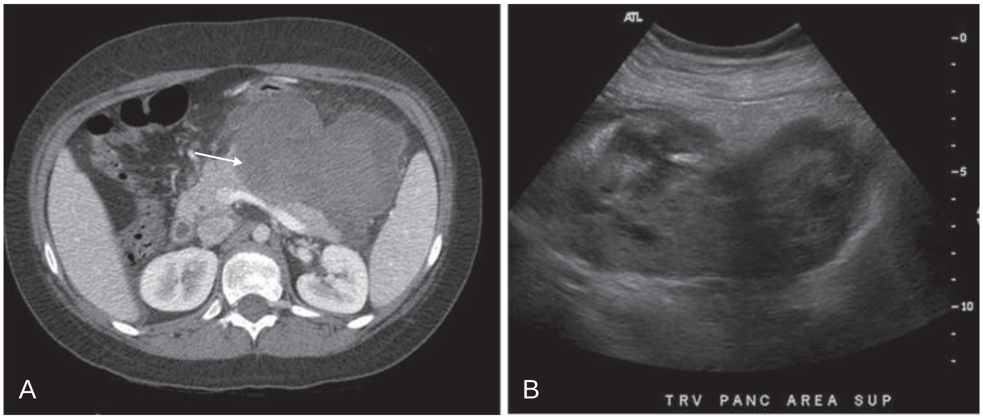

Figure 66-62 Pseudopapillary pancreatic tumor. This 9-year-old patient had hypertension and increased abdominal girth. **A**, CT shows a low attenuation, heterogeneously enhancing lobulated mass arising from the pancreatic body and tail *(arrow)*, which also abutted the greater curvature of the stomach (not shown). **B**, Ultrasound shows a lobulated solid mass that could not be distinguished from the pancreas.

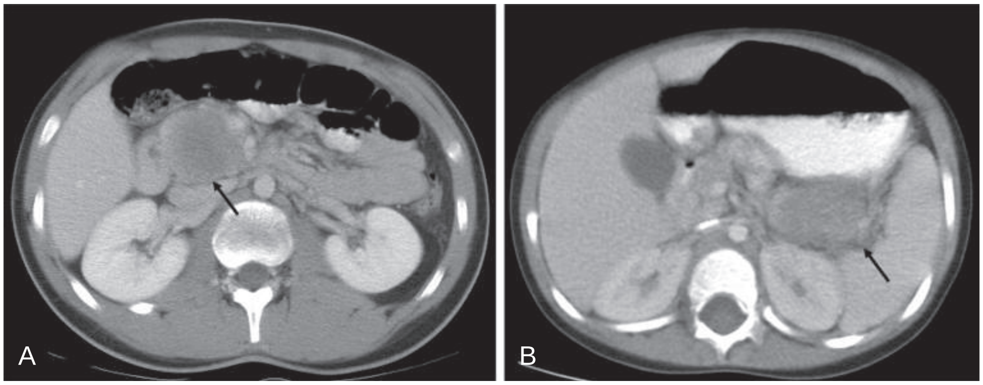

Figure 66-63 CT shows metastatic rhabdomyosarcoma to the pancreas in two patients with extremity rhabdomyosarcoma. **A**, The patient had symptoms of pain. **B**, The patient had no abdominal symptoms, and the lesion was detected by FDG-PET during routine surveillance.

pancreas. As shown in Figure 66-65, in which the patient had symptomatic hyperinsulinemic hypoglycemia, these tumors are often quite small and may be difficult to diagnose and characterize. As with other neuroendocrine neoplasms, optimal scanning techniques for detection of pancreatic islet-cell tumors include early arterial-phase scanning as part of a multiphase imaging technique. Thin sections (≈1 mm) are now routinely acquired by most multidetector-row CT scanners and the evaluation should include a careful review of sagittal and coronal reconstructed images. Although there are no controlled studies comparing CT and MRI in evaluating these tumors, a well-administered MRI examination with respiratory triggering/breath-holding, fast gradient-echo imaging, diffusion imaging, and dynamic-contrast administration is likely to yield comparable, if not superior results to CT. These small lesions are often difficult to see by conventional ultrasound; however, intraoperative ultrasound is invaluable for localizing small, nonpalpable lesions (see Fig. 66-65, *B*).

Alimentary Tract Tumors

Tumors of the gastrointestinal tract including the esophagus, stomach, and large and small bowel are rare in children. In the stomach, lymphomas, leiomyomas, lyomysarcomas, and adenocarcinomas are all rare as

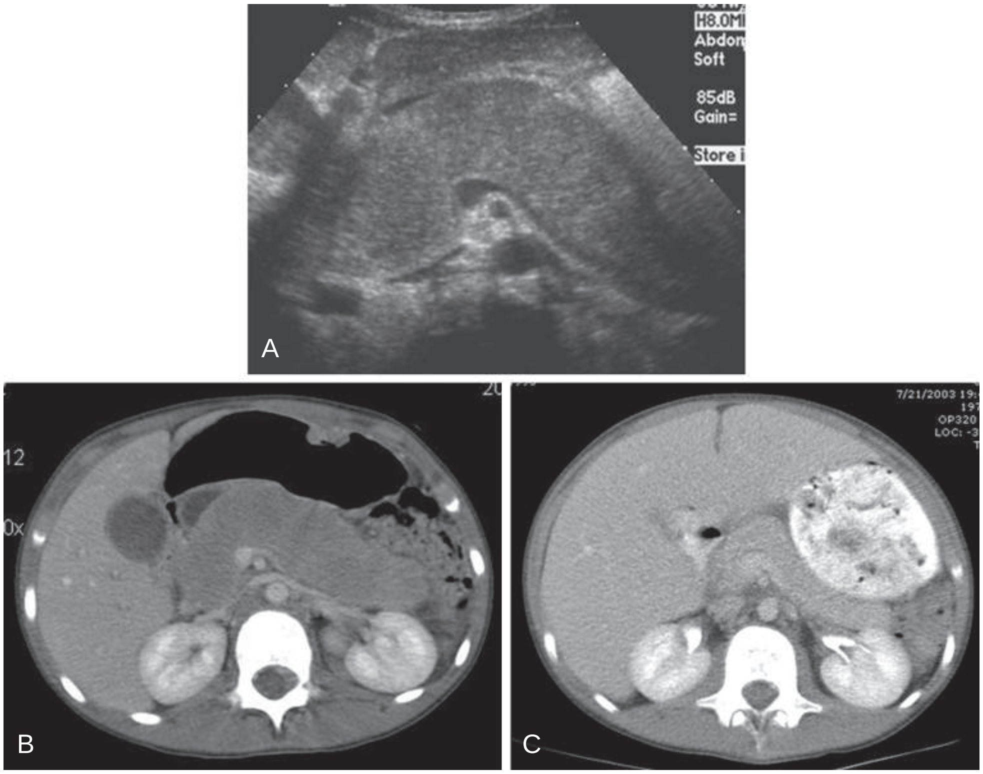

Figure 66-64 Burkitt lymphoma of the pancreas. An 8-year-old patient presented with pancreatitis. **A**, Ultrasound showed diffuse pancreatic infiltration, confirmed by CT (**B**), with disease limited to the pancreas. Ten days after initiating chemotherapy the pancreas appeared normal (**C**), typical of the rapid responses observed with Burkitt lymphoma.

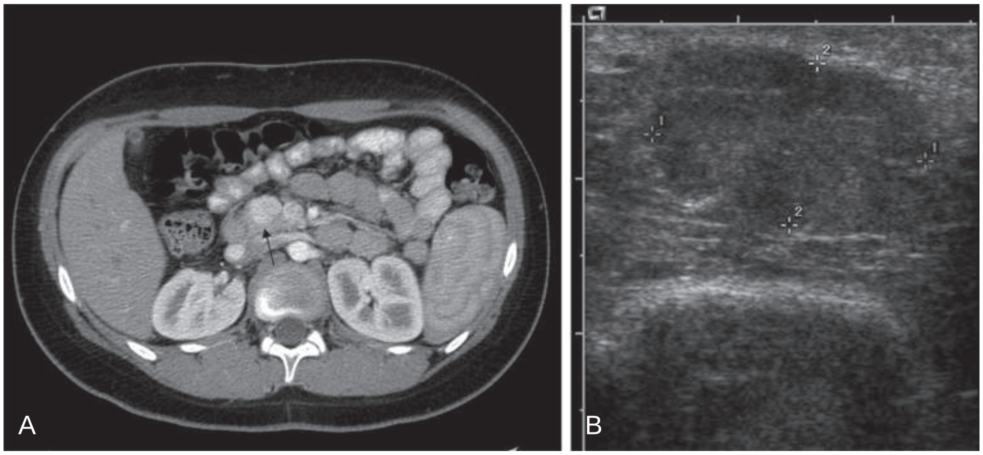

Figure 66-65 Pancreatic islet-cell tumor. This patient had profound hypoglycemia. **A**, CT shows a small enhancing lesion in the pancreatic head, adjacent to the superior mesenteric vein, detected only during the early arterial phase of scanning and consistent with insulinoma. **B**, Intraoperative ultrasound was essential to localize this small lesion before resection.

compared with adults. Gastrointestinal stromal tumors (GISTs) also have a low incidence in the pediatric population. Since early 2000, however, these tumors have attracted increased attention because of molecularly-targeted therapies (e.g., imatinib [Gleevec]) specifically inhibiting cell-surface receptors expressed on these cells. Although the majority of pediatric GISTs express a different cell-surface receptor mutation (*KIT* or *PDGFRA*)[242] and thus do not respond to imatinib like adult GISTs, the radiographic features are similar, with patients having a large mass closely apposed to the gastric wall. Depending on the size of the mass it may be difficult to distinguish between a pancreatic tumor invading the posterior gastric wall and a primary gastric tumor. On CT these tumors are often heterogeneous in attenuation with modest enhancement relative to the adjacent pancreatic tissue. For patients with localized disease, complete surgical resection is still the mainstay of treatment for this tumor, with targeted therapies being reserved for those patients with advanced or metastatic disease.[243] FDG-PET scanning has been shown to be important for staging and in follow-up evaluation, both as a measure of metabolic response to molecularly targeted therapy[243] and for early detection of small, local sites of relapse within the gastric wall that may be difficult to detect by conventional cross-sectional imaging techniques (Fig. 66-66).

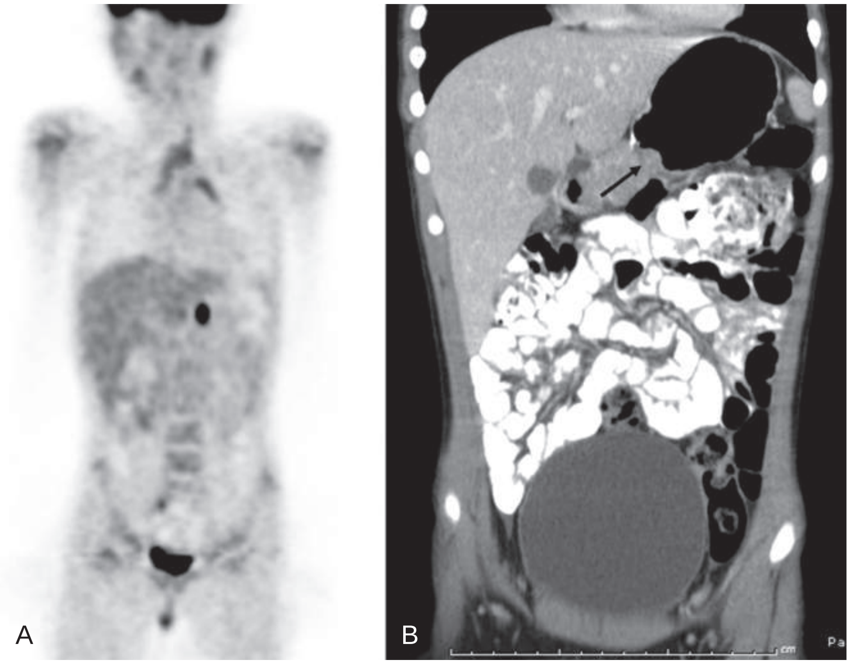

Figure 66-66 Images of a 9-year-old patient taken for routine follow-up examination after resection of a large GIST from the distal stomach. FDG-PET (**A**) shows intense focus of uptake in the upper abdomen, corresponding to a subtle nodular density seen by CT (**B**) along the greater curve of the stomach *(arrow)*. Endoscopic biopsy confirmed relapse at the resection margin.

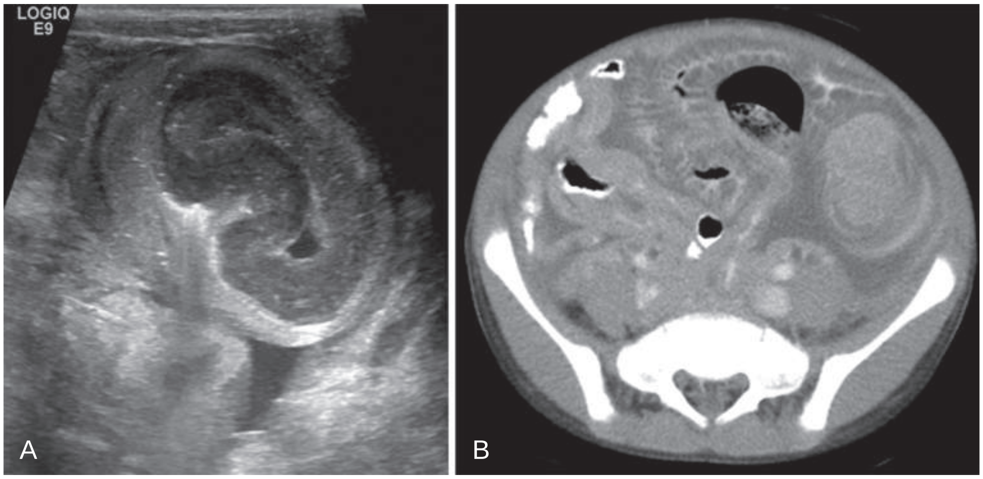

Figure 66-67 A 6-year-old patient with diffuse abdominal Burkitt lymphoma with symptoms of abdominal pain, diffuse bowel-wall thickening, adenopathy, ascites, and small-bowel intussusception, shown on both ultrasound (**A**) and CT (**B**).

The most common primary bowel malignancy in childhood is lymphoma. Small bowel is more often involved than large bowel, and this is most often non-Hodgkin lymphoma. Presentation is typically with abdominal pain, weight loss, anemia, gastrointestinal bleeding, or constipation. Intussusception or a palpable abdominal mass may bring the patient to attention sooner. In an older child (older than age 6 years) with intussusception, lymphoma should be the leading diagnostic consideration (Fig. 66-67). The majority of the small-bowel non-Hodgkin lymphomas are Burkitt lymphomas. These rapidly dividing tumors involve the bowel or small bowel/colonic mesentery and can become quite large and lead to intestinal obstruction. Burkitt lymphoma responds rapidly to chemotherapy and can be effectively treated with no residual imaging abnormalities seen.

The imaging evaluation may initially involve fluoroscopic/barium studies to assess nonspecific symptoms of abdominal pain. In the setting of suspected intussusception, conventional radiographs and ultrasound are usually sufficient to confirm the diagnosis. Attempts at hydrostatic or pneumatic enema reduction of intussusceptions occurring around pathologic lead points in the bowel because of lymphomatous infiltration of the bowel or mesentery should be undertaken with caution, because there is increased risk of perforation at the site of intussusception[244] and greater risk of failure at reducing the intussusception in these patients. Once the diagnosis of lymphoma has been made, a complete cross-sectional imaging (CT) staging examination, including the neck and chest, should be undertaken. Both intravenous and oral contrast are necessary for this evaluation. CT reveals bowel-wall thickening, mesenteric adenopathy, and often a large intraperitoneal/mesenteric mass. FDG-PET scanning has been shown to be effective in adult patients in staging and response assessment for non-Hodgkin lymphoma, and small case series have confirmed this finding in children, suggesting FDG-PET should probably be included in the diagnostic staging and posttreatment assessment of these patients.[245-248]

Adenocarcinomas of the colon and rectum are fortunately rare in children. As in adults, colonoscopy may provide the best early diagnosis for a suspected carcinomatous lesions. The staging evaluation for metastatic spread should involve imaging of the chest, abdomen, and pelvis, again with both intravenous and oral contrast. There is no clear role established for routine use of FDG-PET scanning in the staging of children with adenocarcinomas of the gastrointestinal tract.

Carcinoid tumors occurring in the gastrointestinal tract are rare and usually slow-growing but have malignant potential. The appendix is the most common location for carcinoid tumors. It is not uncommon for patients to show symptoms of acute appendicitis, with carcinoid tumors discovered at the time of appendectomy or incidentally during evaluation of the pathologic specimen.[249] When large, carcinoid tumors may also produce obstruction of the bowel or intussusception. Because of their propensity to metastasize to the liver, work-up should include imaging of the entire abdomen and pelvis with intravenous and oral contrast. Somatostatin receptor analogues (octreotide) are useful for occult sites of metastatic disease spread. These hepatic metastases are typically best demonstrated during the early arterial phase of contrast injection, which nicely delineates these hypervascular tumors relative to the surrounding hepatic parenchyma. Complete surgical resection remains the mainstay of therapy for disease localized to the appendix and is usually curative.[249]

GENITOURINARY TUMORS

Renal Masses

Wilms Tumors

Wilms tumors are the most common renal malignancy in children, representing 6% to 10% of all pediatric malignancies and making up greater than 80% of pediatric renal masses. The peak age of incidence is between 3 and 4 years, and bilateral disease is seen in between 5% and

TABLE 66-2 Childhood Wilms Tumor: National Wilms Tumor Study Group Staging[252]

Tumor Stage	Description
1	Tumor is limited to the kidney and is completely resected. The renal capsule is intact and tumor is not ruptured or biopsied before removal. No involvement of renal sinus vessels or evidence of the tumor at or beyond the margins of resection.
2	Tumor is completely resected, with no evidence of tumor at or beyond the margins of resection. Tumor extends beyond the kidney as evidenced by any one of the following: Regional extension of the tumor (i.e., penetration of the renal sinus capsule, or extensive invasion of the soft tissue of the renal sinus) Blood vessels within the nephrectomy specimen outside the renal parenchyma contain tumor.
3	Residual nonhematogenous intraabdominal tumor after surgery. Any one of the following may occur: Lymph nodes within the abdomen or pelvis are involved by tumor. Tumor implants are found on or penetrated through the peritoneal surface. Gross or microscopic tumor remains. Tumor is not completely resectable because of local infiltration. Tumor spillage occurs either before or during surgery, or tumor was biopsied before removal. Tumor is removed in more than one piece (e.g., tumor thrombus within the renal vein is removed separately from the nephrectomy specimen).
4	Hematogenous metastases (e.g., lung, liver, bone, brain) or lymph node metastases outside the abdominopelvic region are present.
5	Bilateral involvement by tumor is present at diagnosis.

10% of patients.[250,251] Wilms tumor is often associated with syndromes and nephroblastomatosis and typically presents as a painless, palpable abdominal mass. Occasionally, Wilms tumor is an incidental finding during a radiologic assessment for other indications such as trauma. Hypertension and hematuria only occur in about 25% of the cases.[250]

Tumor staging based on the NWTSG classification depends on surgical, pathological, and radiologic findings, and the imaging evaluation should be directed at determining spread to adjacent organs such as the pancreas, spleen, and liver and for intravascular extension into the renal vein, IVC, and right atrium (Table 66-2).[252] In addition, the imaging examination should assess for the presence of distant metastatic disease (lung and liver) and for the presence of contralateral kidney involvement.[251,253] Wilms tumor is a rapidly growing malignancy, and intraabdominal rupture at diagnosis, during chemotherapy, or at the time of surgical resection is a well-appreciated complication and a major risk factor for abdominal recurrence,[254] increasing the recurrence rate to as much as 20%.[255] Accurately identifying tumor rupture can be a challenge. In a recent review of 70 patients treated through the COG, preoperative CT imaging was found to have moderate specificity (88%) but relatively low sensitivity (62%) in the detection of Wilms tumor rupture, although ascites seen outside of the cul-de-sac, irrespective of fluid density, was most predictive of rupture.[256] When tumor rupture is identified, these patients are upstaged and treated as having stage 3 disease.[251,253]

The initial examination is often a conventional abdominal x-ray obtained for symptoms of abdominal pain or a palpable mass. The typical findings include displacement of bowel and a masslike opacity in the abdomen. An ultrasound should serve as the initial evaluation and usually confirms the renal origin of the suspected mass. Sonographically Wilms tumors are often quite large with relatively homogeneous echogenicity. There may be focal areas of hypoechogenicity or cystic change. Calcifications are uncommon. Ultrasound is also accurate and sensitive at detecting intravascular invasion, and both color and Doppler imaging are effective at establishing vascular patency (Fig. 66-68). Intravascular extension occurs in 4% to 10% of patients with Wilms tumor but is rare in neuroblastoma, helping to differentiate between these two tumors.[253] In a recent study evaluating the diagnostic performance of CT versus Doppler ultrasound in identifying intravascular extension of Wilms tumor, investigators from the COG found CT to be superior to ultrasound for detection of intravascular and cavoatrial thrombus.[257] Nonetheless because ultrasound is often the initial examination performed in these patients, early identification of intravascular extension into the right atrium is essential and may increase the sedation/anesthesia risk for a child for whom subsequent CT or MRI is planned.

Once the diagnosis has been suggested by ultrasound, further imaging of suspected Wilms tumor requires either CT or MRI to accurately stage the disease. Multidetector-row CT scanning allows for rapid scanning and in some instances may allow sedation to be avoided. High-resolution imaging techniques with multiplanar reconstructions are essential in determining the extent of tumor spillage, local invasion, invasion into vascular structures, and presence of pulmonary metastatic disease. Early studies suggested that pulmonary metastases detected only by CT should not result in upstaging of the patient, relying on the chest x-ray to identify pulmonary metastases.[253] Subsequent work by European investigators in the International Society of Paediatric Oncology (SIOP) and in North America with the NWTSG identified a small cohort of patients with pulmonary lesions visualized only by CT scanning in whom disease stage would be advanced based on the CT results.[253] One study showed an increased risk of relapse in patients with positive CT findings.[258] Others, however, reported either no difference in prognosis between patients with positive or negative chest CT scans[189,259] or improvement in event-free survival rates, but with no impact on the overall survival rate and with no benefit from whole-lung radiation for patients with CT-only pulmonary nodules.[191] These studies are limited by their retrospective nature, and it remains controversial whether patients would have benefited from increased therapy.[251,253] Furthermore other investigators

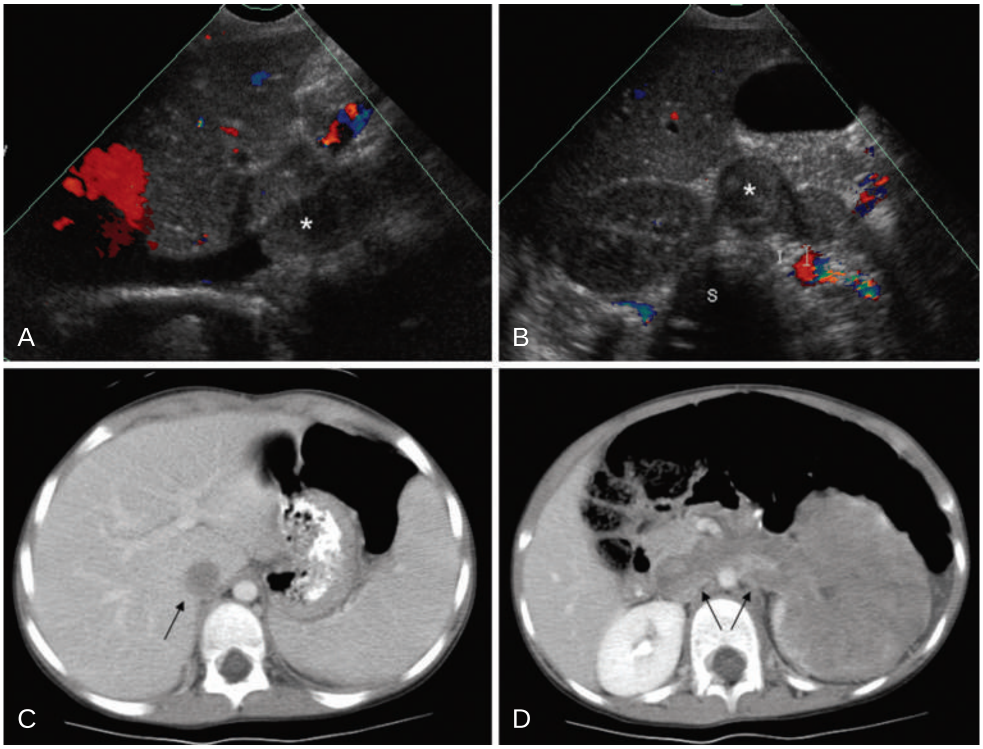

Figure 66-68 Wilms tumor. A 6-year-old child with a left renal Wilms tumor and left renal vein/IVC extension shown by ultrasound (**A** and **B**) and CT (**C** and **D**, *arrows*). Ultrasound confirms the complete absence of flow in the left renal vein and documents the level of intravascular invasion into the IVC *(asterisks)*. *s,* Spine.

have argued that CXR and CT data are concordant in the majority of cases,[190] and it as been shown that not all pulmonary lesions detected by CT represent metastases, even in the hands of experienced radiologists,[258] suggesting that surgical confirmation may be necessary to establish the malignant nature of suspicious lesions detected by CT in patients with Wilms tumor.[188] These considerations aside, it is current practice to obtain a chest CT for staging of all patients with Wilms tumor, with ongoing studies investigating the positive predictive value of this practice and its impact on overall clinical outcome.

Because of the need for chest CT in the staging evaluation, MRI is usually obtained for follow-up evaluation, either after resection or chemotherapy. In addition, MRI is the imaging modality of choice in monitoring nephrogenic rests that are seen in approximately 40% of patients with unilateral Wilms tumor and nearly all patients with bilateral Wilms tumors (Fig. 66-69).[260,261] Nephrogenic rests are remnants of primitive blastemal tissue that persist after birth and have the potential to transform into Wilms tumor. Nephroblastomatosis is the presence of multifocal or diffuse nephrogenic rests and is prevalent in patients with hemihypertrophy.[262] The distinction between nephrogenic rests and Wilms tumor may be difficult; however, there are certain distinguishing MRI features that may help discriminate between the two. On T1-weighted MRI images, Wilms tumors appear as well-defined heterogeneous masses with slightly lower signal intensity as compared with the adjacent renal cortex. On T2-weighted images Wilms tumors appear isointense to slightly hyperintense relative to the normal renal cortex. After intravenous gadolinium-contrast administration, Wilms tumors typically enhance heterogeneously but less than the surrounding renal parenchyma. Nephrogenic rests also exhibit decreased signal intensity on T1-weighted images, but in contrast to Wilms tumors maintain low signal on T2-weighted images.[263-265] Furthermore, nephrogenic rests show minimal enhancement after contrast infusion relative to the normal renal parenchyma, whereas foci of Wilms tumors show heterogeneous enhancement.[263,264] A role for FDG-PET imaging in the routine management of patients with Wilms tumor has not been established. However, studies documenting increased FDG accumulation in metabolically active Wilms tumors[266,267] suggest there may be role for FDG-PET imaging in Wilms tumor staging, posttherapy response evaluation, and possibly in helping discriminate benign nephrogenic rests from foci of transformed malignant tumor.

As discussed in the next section, the major differential considerations in patients with suspected unilateral Wilms tumor are clear-cell sarcoma of the kidney and rhabdoid tumor of the kidney, although there are no characteristic imaging features to make this distinction.

Clear-Cell Sarcomas

Clear-cell sarcomas of the kidney (also know as *bone-metastasizing renal tumor of childhood*) were previously thought to be a histologically aggressive variant of Wilms tumors. They occur less often than Wilms tumor (4% to 5% of pediatric renal neoplasms), with an estimated 20 new cases diagnosed annually in the United States.[268]

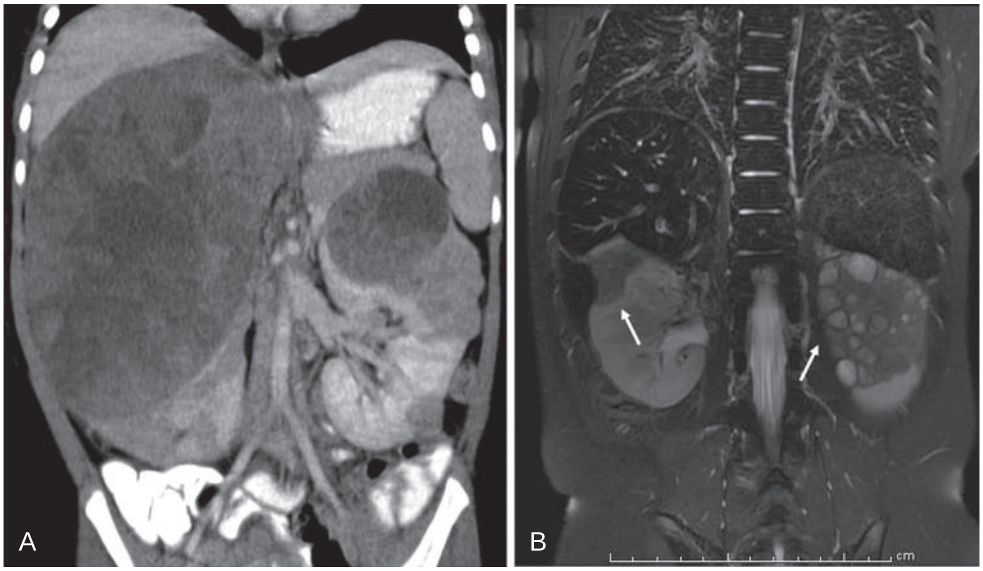

Figure 66-69 A 3-year-old patient with bilateral Wilms tumor, initially evaluated by CT (**A**). The patient underwent bilateral partial nephrectomies. **B**, MRI was used to monitor nephrogenic rests on both kidneys *(arrows)*, both for change in size and change in pattern of enhancement.

Although age at presentation ranges from infancy to the second decade, the peak incidence at about 2 to 3 years old closely parallels that of Wilms tumor, making differential diagnosis difficult. Bone metastases, which are unusual in Wilms tumor, occur commonly in patients with clear-cell sarcoma of the kidney and can have both osteolytic and osteoblastic activity.[250,262]

On imaging there are no specific features that allow distinction to be made from Wilms tumor.[269] Ultrasound confirms the renal origin of the mass, showing a heterogeneous mass with areas of hypoechogenicity and isoechogenicity reflecting cystic and solid elements within the mass. CT scanning is usually performed to more completely characterize the mass, assess for regional invasion, and check for metastatic disease. On CT, clear-cell sarcomas of the kidney are typically heterogeneous in attenuation with a variable pattern of contrast enhancement (Fig. 66-70). Vascular invasion is uncommon, and these tumors (in contrast to Wilms tumors) do not have a pseudocapsule and often infiltrate the adjacent renal parenchyma and perinephric spaces.[262] The most common site of metastatic spread is to locoregional lymph nodes, followed by metastases to the lung and bone.[270] Liver metastases also occur. As with Wilms tumor, the management of clear-cell carcinoma of the kidney involves a combination of nephrectomy and chemotherapy.[271] Clear-cell sarcomas are more aggressive than Wilms tumors with a higher relapse rate; the most common site of relapse is bone, and current recommendations include skeletal scintigraphy in follow-up screening examinations.

Rhabdoid Tumors of the Kidney

Rhabdoid tumors of the kidney are also extremely rare and are the most aggressive of the renal tumors in children. Previously thought to be a variant of Wilms tumor histology, they are now recognized as a discreet entity and account for approximately 2% to 3% of pediatric renal tumors. Rhabdoid tumors tend to occur in young infants, with more than 80% occurring in patients under the age of 2 years (Fig. 66-71).[272]

CT, ultrasound, and MRI all show a large heterogeneous intrarenal mass that often involves the central renal hilum. There may be associated vascular invasion, and calcifications may be seen. Although there are no distinctive imaging features to reliably distinguish rhabdoid tumor of the kidney from Wilms tumor (see Fig. 66-66), the findings of calcification, subcapsular hematoma, and a lobular, centrally located heterogeneous renal mass have been reported as characteristic imaging findings in patients with rhabdoid tumors of the kidney.[273] In addition, the association between rhabdoid tumors of the kidney and the presence of primary brain tumors (posterior fossa tumors, including medulloblastomas, PNETs, astrocytomas, and medulloblastomas) and synchronous or metachronous brain metastases are considered a unique and distinctive feature of this tumor.[272,274] Once the diagnosis of rhabdoid tumor of the kidney is established, therefore, the subsequent staging and follow-up evaluations should always include imaging of the brain to assess for intracranial disease.

Renal-Cell Carcinomas

As children enter their teenage years, the incidence of Wilms tumor decreases and the incidence of renal-cell carcinoma rises. Although there have been rare reports of renal-cell carcinoma occurring in infancy, the vast majority occur in children in late adolescence.[250,262] Syndromes associated with renal-cell carcinoma include von Hippel-Landau syndrome, tuberous sclerosis, and Beckwith-Wiedemann syndrome. In contrast to adults, in whom hematuria is a common presenting complaint, children with renal-cell carcinoma more often have vague abdominal pain and an abdominal mass.

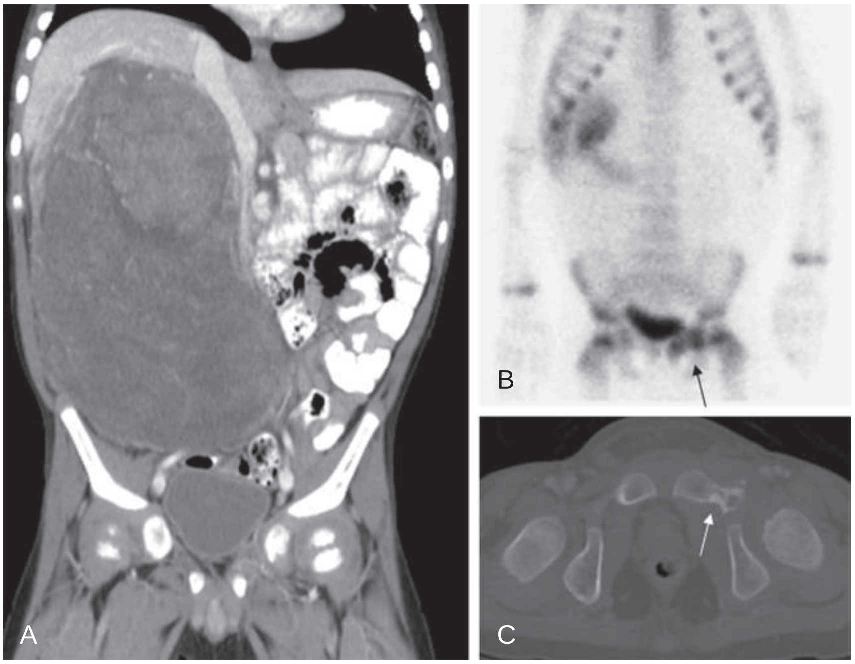

Figure 66-70 Clear-cell sarcoma of the kidney. A 2-year-old had symptoms of hip pain (x-rays were negative) and abdominal mass. Ultrasound (not shown) and CT (**A**) show heterogeneous masses with ill-defined margins, cystic and solid areas, and a variable pattern of contrast enhancement. Bone windows (**C**) revealed a destructive lesion in the left superior pubic ramus *(arrow)*, confirmed by bone scan (**B**), characteristic of the bone involvement seen with this renal neoplasm.

The imaging features of renal cell carcinoma and Wilms tumor overlap, although renal-cell carcinomas tend to show calcifications more often than Wilms tumors. Although renal cell carcinomas are more often bilateral and more commonly metastasize to bone than Wilms tumors, it is primarily the older age at presentation that favors renal-cell carcinoma over Wilms tumor. CT, MRI, and ultrasound all reveal a heterogeneous-appearing intrarenal mass. Multiple bilateral renal-cell carcinoma masses should suggest the diagnosis of von Hippel-Lindau syndrome.[272] Areas of cystic change may be seen, representing necrosis and hemorrhage, and calcifications are more common than in Wilms tumor (Fig. 66-72). After contrast infusion a heterogeneous pattern of contrast enhancement is seen on both CT and MRI. Local invasion into retroperitoneal lymph nodes and vascular invasion, particularly into the IVC, are common with renal-cell carcinoma and significantly influence the success of both primary surgical resection and overall prognosis. In some cases, when early-stage localized disease is established by CT and/or MRI, partial nephrectomy may be considered.[275,276] Intraoperative ultrasound can be valuable in guiding the surgeon and helping to define resection planes (Fig. 66-73). Because of increased incidence of bone metastases in renal-cell carcinoma, the staging evaluation should also include bone scintigraphy

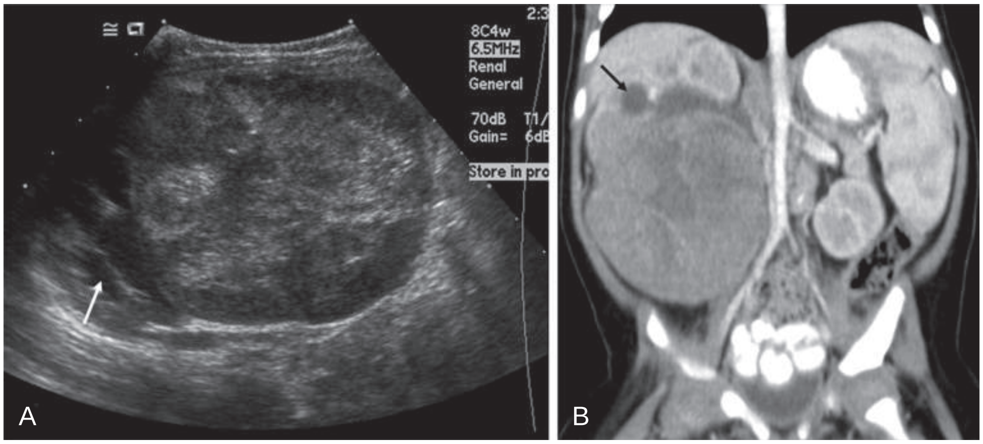

Figure 66-71 A 4-month-old patient with a palpable right renal mass. Ultrasound (**A**) and CT (**B**) show a centrally located lobulated mass causing obstruction of the right upper pole-collecting system *(arrows)*. On resection this was rhabdoid tumor. Note the difficulty in distinguishing this tumor from Wilms tumor (compare with Figs. 66-68 and 66-69).

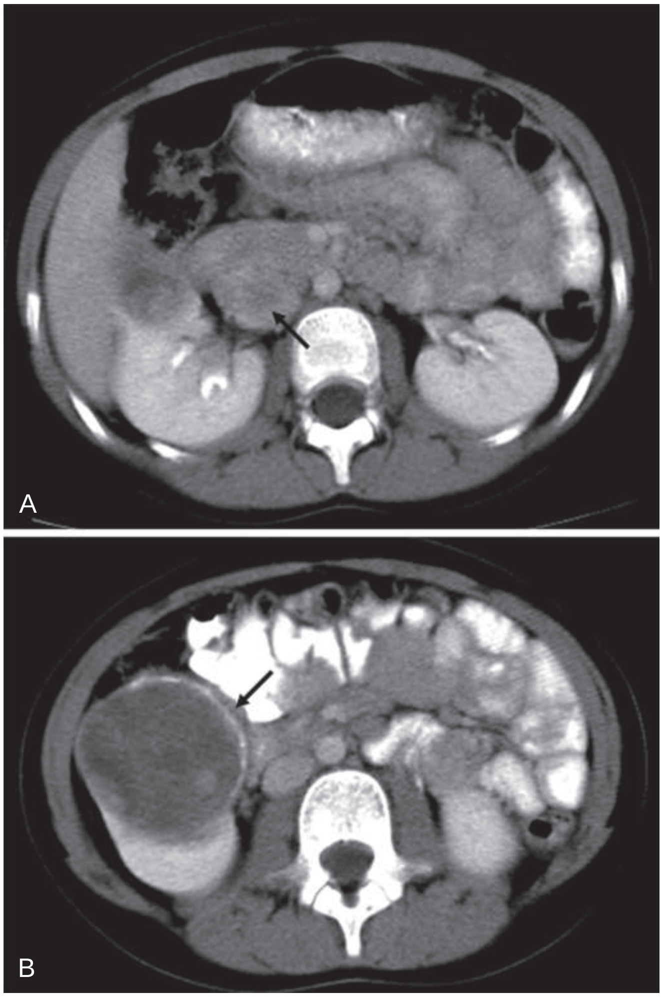

Figure 66-72 A 9-year-old child with a right renal mass that was found to be renal-cell carcinoma. Note the retroperitoneal lymph node spread (**A**, *arrow*), rim calcification (**B**, *arrow*), heterogeneous attenuation, and enhancement.

in addition to CT scanning of the chest, abdomen, and pelvis.

Congenital Mesoblastic Nephromas

Congenital mesoblastic nephromas, although not among the most common solid tumors in childhood, are the most common solid tumors of the neonatal period. They are benign tumor with almost three fourths of cases identified within the first year of life. With the advent of prenatal ultrasound screening, the incidence of early diagnosis is expected to increase. Infants typically have the presenting symptom of a large, palpable, abdominal mass. The imaging evaluation usually begins with ultrasound, showing a large, solid, renal mass that often involves the renal hilum (Fig. 66-74).[272] Although not a distinguishing feature, cystic elements are less commonly seen in mesoblastic nephroma than Wilms tumor. Congenital mesoblastic nephroma has been subdivided histologically into an aggressive high–cell-density variant and the classic variant, which has fewer active mitoses, less potential for local recurrence and metastasis, and overall better prognosis than the aggressive variant.[262,272] Hemorrhage, cysts, and necrosis, when present, are more often associated with the aggressive high–cell-density variant. Subsequent imaging by CT and/or MRI is usually performed to better characterize the intrarenal lesion and to assess for invasion into adjacent organs and structures (see Fig. 66-74). The imaging features alone do not allow distinction between mesoblastic nephroma and Wilms tumor,[270] and surgical resection is indicated. Because there may be poorly defined margins and the lack of a definable capsule, a surgical resection with wide margins is usually performed. The conventional benign histologic variant of this tumor is comprised primarily of benign connective tissue and mature mesenchymal cells, and prognosis is excellent if surgical resection is complete. Postoperative monitoring by ultrasound or MRI is still recommended, even with complete resection, for at least 1 year. With the aggressive cellular histologic variant, metastases and local spread of disease may require postoperative radiation and chemotherapy, and a more intensive follow-up imaging regimen should be directed at evaluating sites of local recurrence and typical metastatic spread (lungs, bone, and brain).[272]

Multilocular Cystic Nephromas

Multilocular cystic nephromas are benign renal tumors comprised almost entirely of coalescent cystic lesions with thin-walled septae.[277] Although the cystic appearance is distinctive, it is still not possible based on imaging features alone to discriminate between the cystic variant of Wilms tumor and the benign multilocular cystic nephroma. As with Wilms tumor, the typical age of presentation is between 2 and 5 years, with a smaller percentage occurring in young adults. Patients often have a large, painless, abdominal mass with no systemic symptoms.[272]

Radiographs may show a large abdominal mass displacing loops of bowel. The use of ultrasound is particularly important to demonstrate the cystic characteristics of these lesions, because CT scanning may simply show a large, low-attenuation mass with septations but not clearly demonstrate the hypoechoic cystic spaces revealed by ultrasound (Fig. 66-75). MRI shows characteristic fluid signal with low signal intensity on T1-weighted images and high signal intensity on T2-weighted images. After contrast enhancement, both by CT and MRI, enhancement of the thin-walled septae is seen, whereas the majority of the cystic mass remains unenhanced. Because the distinction between cystic Wilms tumor, clear-cell sarcoma, and multilocular cystic renal tumor cannot be made on the basis of imaging features, surgical resection is required for diagnosis.[270,271,277] Long-term prognosis is excellent; however; follow-up imaging is still necessary in the early stages postresection, because a low incidence of local recurrence is still possible.

Rare Renal Tumors

Two additional very rare tumors of infancy are the ossifying renal tumor of infancy and desmoplastic small round-cell tumors (Fig. 66-76). Both tumors characteristically calcify. Figure 66-76, *A* shows a renal hilar mass with

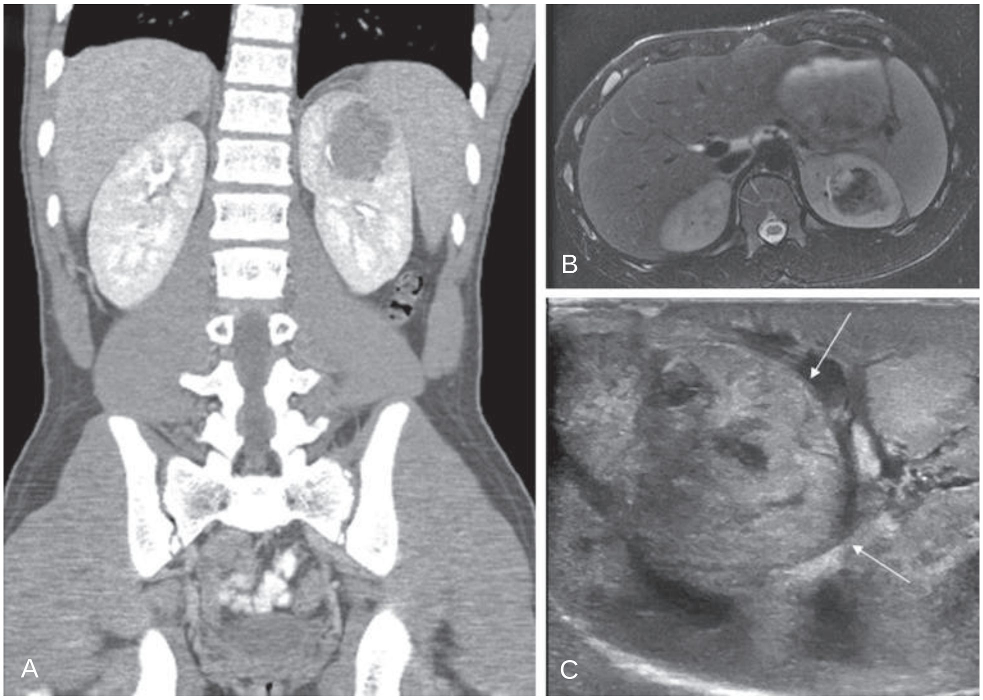

Figure 66-73 Renal-cell carcinoma in a 15-year-old patient with left flank pain and hematuria. Ultrasound showed left upper pole renal mass. CT (**A**) and MRI (**B**) show a low signal-intensity heterogeneously enhancing mass abutting the upper pole collecting system. Intraoperative ultrasound (**C**) defined a margin between the mass and renal parenchyma, vessels, and collecting system, allowing the patient to undergo partial nephrectomy of the upper pole.

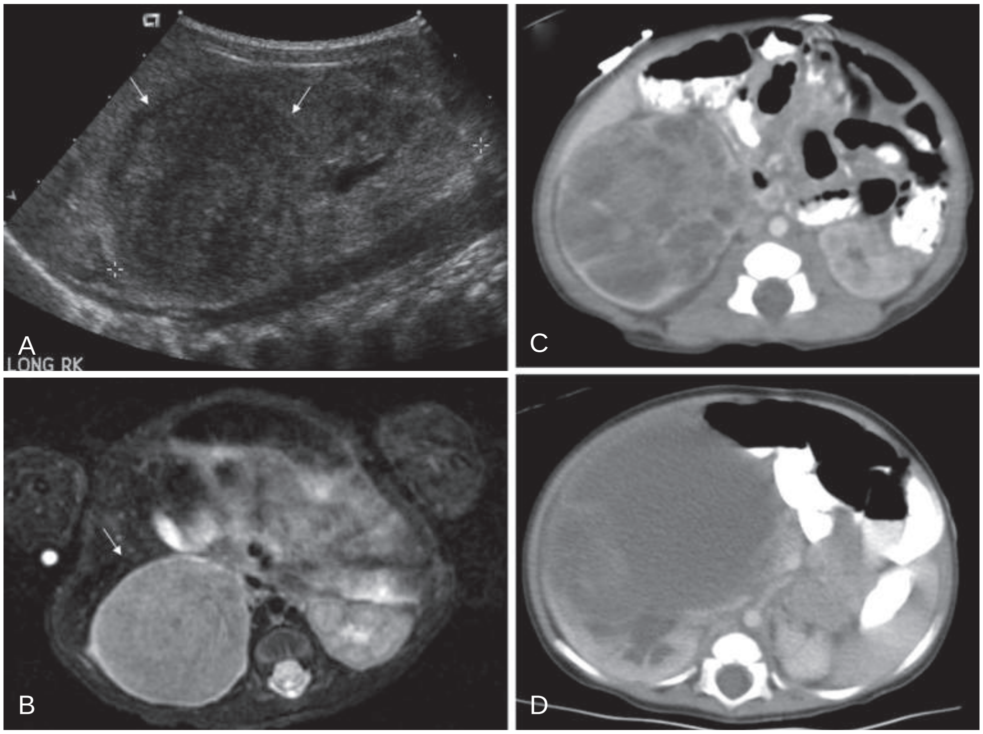

Figure 66-74 Congenital mesoblastic nephroma. **A** and **B** show a 7-day-old infant with a prenatally diagnosed solid right renal mass confirmed by ultrasound and MRI after birth. CTs from two other patients, newborn (**C**) and 5 months old (**D**), with large right renal masses showing both cystic and solid elements typical of congenital mesoblastic nephroma.

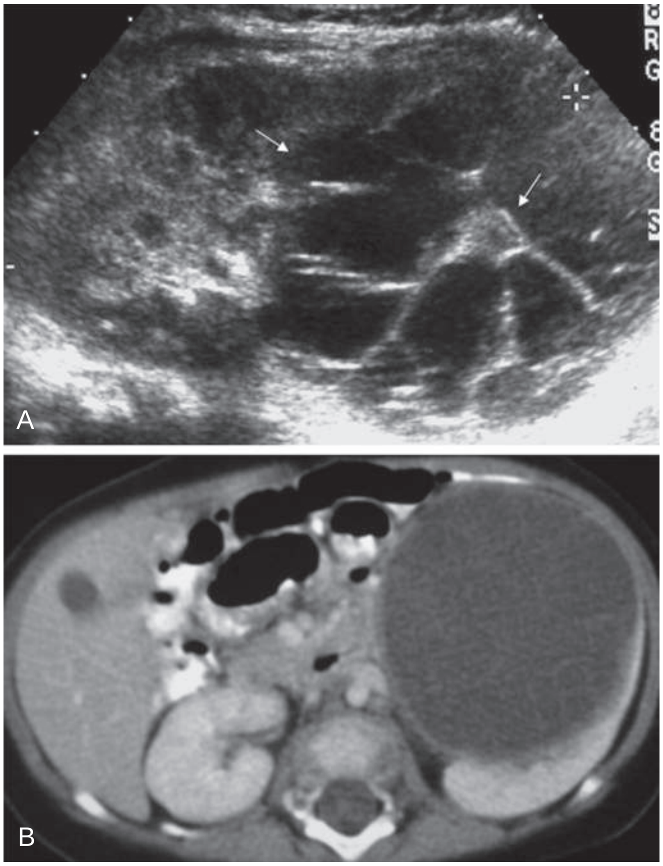

Figure 66-75 Multilocular cystic nephroma. Ultrasound shows a cystic left renal mass (**A**). Note how the cystic characteristics of these lesions and internal septations are not well demonstrated by CT (**B**), which simply shows a large low-attenuation mass, perhaps with thin internal septations.

extensive calcification that results from the ossifying renal tumors and is comprised of an osteoid core and osteoblastic tissue. Surgical resection is usually curative.[272] Desmoplastic small round-cell tumors are unusual as a primary renal neoplasm in children but have been reported.[278] As in other locations, these should be included in a differential for a calcified renal mass in a young child (see Fig. 66-76, *B*). Finally, metastatic disease to the kidney is unusual except in the settings of leukemia and lymphoma.[262] Leukemic involvement of the kidney typically manifests as diffuse nephromegaly. Lymphomatous involvement more often presents with discrete foci of lymphomatous infiltration and occurs in both non-Hodgkin and Hodgkin lymphoma subtypes. Burkitt lymphoma and T-lymphoblastic lymphoma are the non-Hodgkin lymphoma types most commonly presenting with renal involvement. This is probably the result of hematogenous metastasis, and in the setting of Burkitt lymphoma is usually associated with extensive mesenteric and retroperitoneal lymphadenopathy.[272] In the case of leukemia, diffuse nephromegaly may also be accompanied by hepatosplenomegaly; however, generalized adenopathy is less common. Ultrasound is usually sufficient to identify renal involvement by leukemia and lymphoma. Additional imaging by either CT or MRI confirms the diagnosis, demonstrating low-density lesions with little enhancement relative to the surrounding renal parenchyma (Fig. 66-77).

Bladder Neoplasms

In children, tumors involving the urinary bladder are uncommon, with rhabdomyosarcoma being the most common malignancy encountered. Adult-type tumors such as transitional-cell carcinoma, fibroepithelial polyps, and adenomatous lesions are unusual.

Rhabdomyosarcomas

Pelvic rhabdomyosarcomas arise from the bladder wall, prostate, and vagina. Tumors arising in the bladder and prostate often result in obstruction of urinary flow, symptoms of dysuria and hematuria, and urinary retention. Vaginal rhabdomyosarcomas present as pelvic masses with vaginal bleeding and occasionally prolapse of tumor from the vaginal orifice onto the perineum.[279]

Imaging evaluation is initially by ultrasound with rhabdomyosarcomas of the bladder presenting either as diffuse bladder-wall thickening or as polypoid or "botryoid soft-tissue masses" protruding from the bladder wall into the bladder lumen. Color and Doppler flow demonstrate increased vascularity of these lesions. Cystourethrography may be helpful to assess the degree of functional impairment and usually demonstrates filling

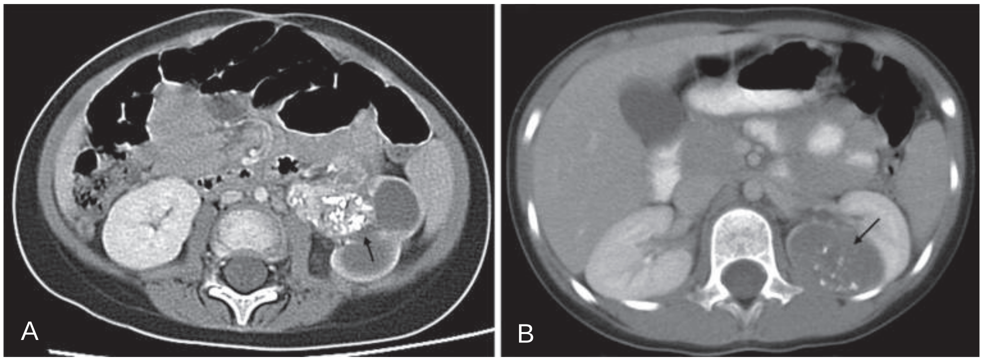

Figure 66-76 **A**, Ossifying renal tumor of infancy showing lobular renal hilar mass with extensive calcification *(arrow)* that results from the tumor being comprised of an osteoid core and osteoblastic tissue. **B**, Desmoplastic round-cell tumor of the kidney showing a well-circumscribed low-density mass in the left kidney with punctate calcifications *(arrow)* in a 6-year-old child who was being evaluated for hematuria.

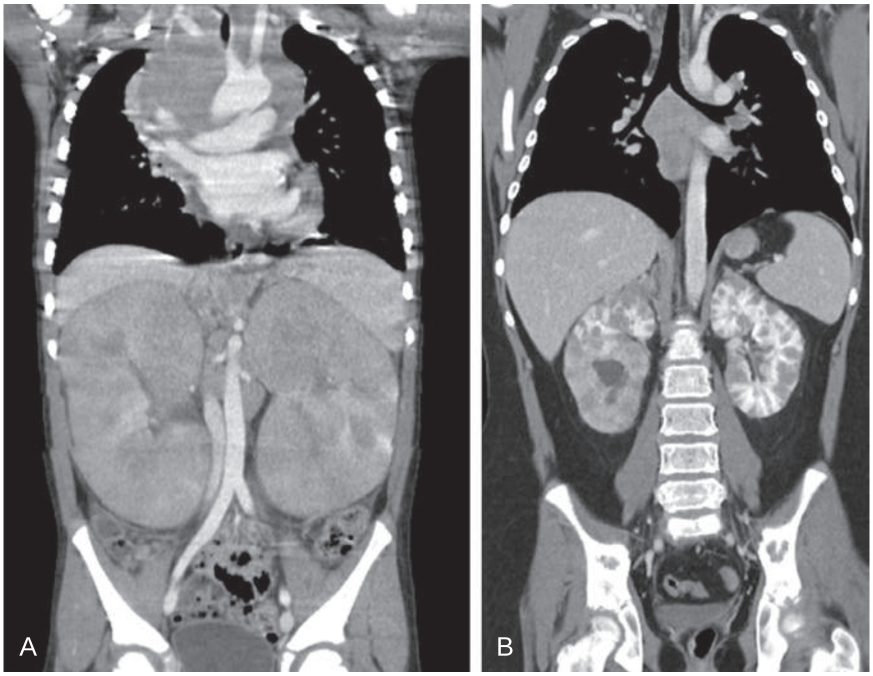

Figure 66-77 Renal involvement with leukemia and lymphoma. **A**, Diffuse renal enlargement and infiltration of the kidneys in a 6-year-old patient with T-cell lymphoblastic leukemia. This patient also had a large mediastinal mass at presentation. **B**, A 12-year-old patient with diffuse large B-cell lymphoma showing multifocal renal involvement as well as a mediastinal mass. Extensive bone involvement, seen under bone windows, is less evident here.

defects in the bladder, diminished bladder volume, and depending on the size and location of the lesion, obstruction to the flow of urine.

Contrast-enhanced CT scanning early after contrast infusion, after bladder filling, and after delayed bladder filling is helpful to assess the degree of local invasion. MRI, however, is preferable to characterize the lesion in multiple planes and to evaluate spread into adjacent pelvic organs and neurovascular structures.[280] Rhabdomyosarcomas typically show intermediate signal intensity on T1-weighted images with increased signal on T2-weighted images and heterogeneous or minimal enhancement on postcontrast images (Fig. 66-78). The imaging evaluation should be directed at assessing for local tumor extension into adjacent pelvic fat planes, adjacent lymph node enlargement, and involvement of the perivesical fat and adjacent musculature. Multiplanar fast-spin echo (FSE)–T2-weighted images with and without fat suppression are well-suited for detecting invasion of adjacent deep pelvic structures.

As with other rhabdomyosarcomas, metastases to the liver, bone, bone marrow, and lung occur, and the staging evaluation should include chest CT and bone scintigraphy. The role of FDG-PET in imaging rhabdomyosarcomas has not been the focus of any controlled prospective trials, but it has been observed[182,184,281] that rhabdomyosarcomas are usually FDG avid and that FDG-PET imaging can play in important role in identifying sites of occult disease, monitoring responses to therapy, and in evaluating equivocal foci of disease, either locally or at metastatic sites, that are identified by CT or MRI.[182]

ADRENAL/SUPRARENAL TUMORS

Neuroblastomas

Neuroblastomas are the most common non-CNS pediatric solid tumors and the third most common pediatric malignancy after leukemia and CNS tumors.[164,166] Greater than 90% of the cases occur in children younger than 5 years of age. Patients with neuroblastoma often have a painless abdominal mass. The most common site of origin is the retroperitoneum (adrenal gland), with other sites of origin in the posterior mediastinum, neck, and pelvis (at the level of the iliac bifurcation, the organ of Zuckerkandl).[164] Symptoms at presentation may also result from compression of adjacent organs, extension into the spinal canal, vascular compression/invasion, and bone involvement. Hypertension is seen in about 10% of patients and is caused by elevated catecholamine production. Paraneoplastic syndromes include the opsoclonus myoclonus syndrome and watery diarrhea. These symptoms commonly resolve after resection or chemotherapeutic treatment of the primary tumor.

The age and stage of disease remain the most important prognostic factors in neuroblastoma.[166] Infants (younger than 1 year of age) with localized disease have the highest rates of cure. Older patients with metastatic disease continue to be the most challenging population of patients to treat. The most widely-used staging system for neuroblastoma is the INSS (Table 66-3).[282,283] This involves a combination of radiographic, scintigraphic, surgical findings, and bone-marrow evaluation. Lower-stage disease is characterized by localized tumor involvement, whereas infiltration across the midline and/or

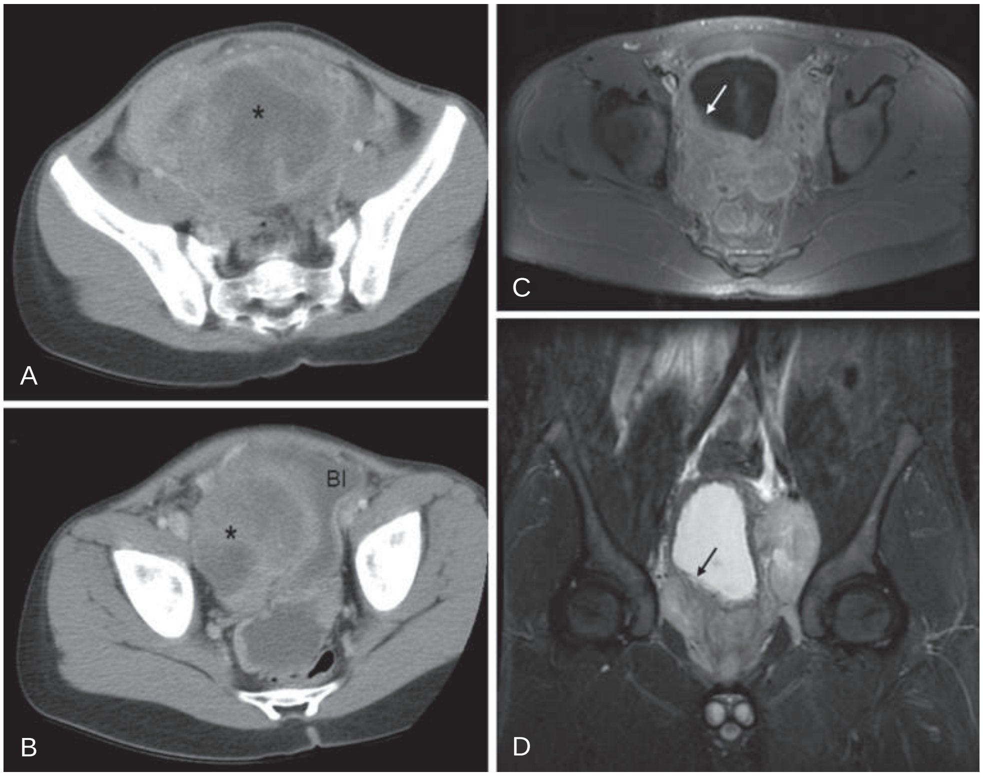

Figure 66-78 Rhabdomyosarcomas of the bladder and prostate. CT (**A** and **B**) shows large exophytic mass *(asterisk)* arising from the bladder wall and compressing the bladder lumen *(Bl)*. Contrast-enhanced (**C**) and FSEIR (**D**) MRI images show invasion of the bladder wall by prostatic rhabdomyosarcoma *(arrow)* showing the value of MRI over CT at demonstrating extent of invasion in the pelvis.

TABLE 66-3 Childhood Neuroblastoma: International Neuroblastoma Staging System[282,283]

Tumor Stage	Description
1	Localized tumor with complete gross excision with or without microscopic residual disease. Representative ipsilateral lymph nodes separate from the primary tumor are negative for tumor microscopically.
2A	Localized tumor with incomplete gross excision. Representative ipsilateral nonadherent lymph nodes are negative for tumor.
2B	Localized tumor with or without complete gross excision. Ipsilateral nonadherent lymph nodes are positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically.
3	Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement, or Localized unilateral tumor with contralateral regional lymph node involvement, or Midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement. The midline is defined as the vertebral column.
4	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, and/or other organs except as defined for stage 4S.
4S	Localized primary tumor as defined for stage 1, 2A, or 2B, and: Dissemination limited to skin, liver, and/or bone marrow. Limited to infants younger than 1 year. Minimal marrow involvement (i.e., <10% of total nucleated cells in aspirate). More extensive bone marrow involvement indicates stage 4 disease. MIBG scan should be negative for disease in the bone marrow.

MIBG, Metaiodobenzylguanidine.

encasement of major blood vessels that results in surgical unresectability define stage 3 disease. Widespread disease with cortical bone, bone marrow, or visceral organ involvement is defined as stage 4 disease. A unique feature to the staging of patients with neuroblastoma is the category stage 4S (special), which is defined as localized primary tumor with dissemination limited to liver, skin, and less than 10% bone-marrow involvement.[166] Because current approaches to risk classification and treatment stratification for children with neuroblastoma vary greatly throughout the world the International Neuroblastoma Risk Group (INRG) classification system has recently been proposed to establish a consensus approach for pretreatment risk stratification. Disease stage, age, histologic category, grade of tumor differentiation, the status of the *MYCN* oncogene, chromosome 11q status, and DNA ploidy were identified the most significant factors impacting clinical outcome.[284,285] New guidelines for staging and imaging of neuroblastoma have also been proposed as a result of the INRG recommendations, with recommendations for reporting two stages of localized disease based on whether image-defined risk factors (IDRFs) are present.[286] IDRFs are imaging findings based on preoperative imaging that are likely to impact surgical management, including success of gross total resection and risk of postoperative complications. A summary of the INRG imaging recommendations and IDRFs is shown in Table 66-4.

TABLE 66-4 International Neuroblastoma Risk Group Classification and Image-Defined Risk Factors

Descriptions of New International Neuroblastoma Risk Group Tumor Stages[286]	
Tumor Stage	**Description***
L1	Localized tumor not involving vital structures as defined by the list of IDRFs and confined to one body compartment.
L2	Local-regional tumor with presence of one or more IDRFs.
M	Distant metastatic disease (except stage MS tumor).
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow.
Descriptions of IDRFs[†286]	
Anatomic Region	**Description**
Multiple body compartments	Ipsilateral tumor extension within two body compartments (i.e., neck and chest, chest and abdomen, or abdomen and pelvis).
Neck	Tumor encasing carotid artery, vertebral artery, and/or internal jugular vein. Tumor extending to skull base. Tumor compressing trachea.
Cervicothoracic junction	Tumor encasing brachial plexus roots. Tumor encasing subclavian vessels, vertebral artery, and/or carotid artery. Tumor compressing trachea.
Thorax	Tumor encasing aorta and/or major branches. Tumor compressing trachea and/or principal bronchi. Lower mediastinal tumor infiltrating costovertebral junction between T9 and T12 vertebral levels.
Thoracoabdominal junction	Tumor encasing aorta and/or vena cava.
Abdomen and pelvis	Tumor infiltrating porta hepatis and/or hepatoduodenal ligament. Tumor encasing branches of superior mesenteric artery at mesenteric root. Tumor encasing origin of celiac axis and/or origin of superior mesenteric artery. Tumor invading one or both renal pedicles. Tumor encasing aorta and/or vena cava. Tumor encasing iliac vessels. Pelvic tumor crossing sciatic notch.
Intraspinal tumor extension	Intraspinal tumor extension (any location), provided that more than one third of spinal canal in axial plane is invaded, the perimedullary leptomeningeal spaces are not visible, or the spinal cord signal intensity is abnormal.
Infiltration of adjacent organs and structures	Pericardium, diaphragm, kidney, liver, duodenopancreatic block, and mesentery.

From References 285 and 286.
IDRF, Image-defined risk factors.
*Complete definitions of these stages are cited in the text.
†Conditions that should be recorded but are not considered IDRFs are multifocal primary tumors, pleural effusion with or without malignant cells, and ascites with or without malignant cells.

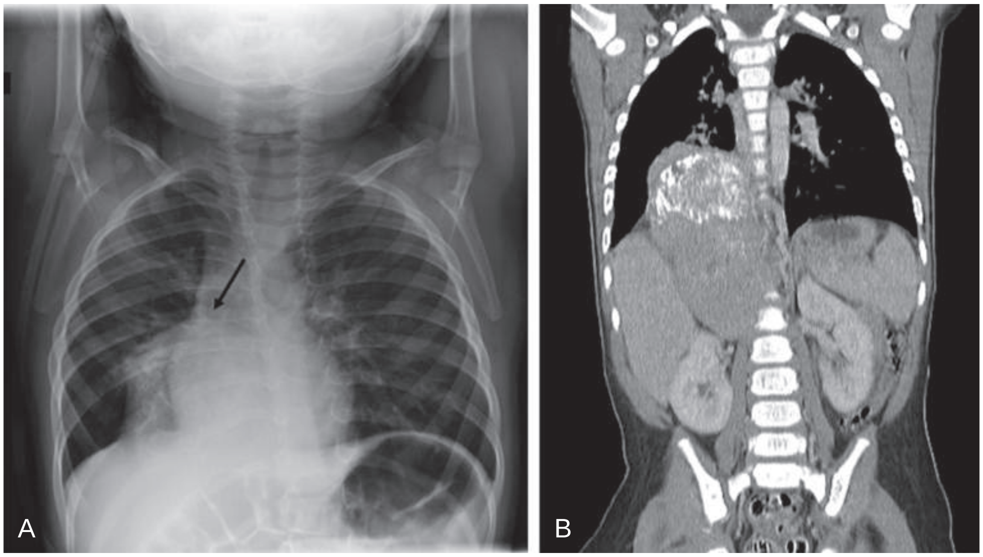

Figure 66-79 Neuroblastoma. **A**, Chest x-ray demonstrates calcified mass at the right lung base, seen on the lateral film to be posterior mediastinal (not shown). **B**, Coronal reconstructions from contrast-enhanced CT reveal a large, calcified right suprarenal mass with extension across the midline and into the thorax.

The initial imaging modalities used in evaluating patients with neuroblastoma are directed at the presenting symptoms. Abdominal films, chest radiographs, and skeletal films are usually obtained to assess abdominal distension, suspected pneumonia, or limp/extremity pain (see Figs 66-1 and 66-3; Fig. 66-79). Ultrasound is widely available and should be the first choice in evaluating a child with a suspected abdominal mass. Depending on the size of the mass, ultrasound can effectively demonstrate the presence of a retroperitoneal or suprarenal mass (Fig. 66-80) and is both sensitive and specific at evaluating for the presence of hepatic metastatic disease and localized vascular invasion. Calcifications are common in neuroblastoma and are seen sonographically as areas of acoustic shadowing. Depending on the age and size of the patient, intraspinal extension can also be detected.

CT scanning is widely used for defining the extent of primary tumor involvement and particularly with multiplanar reconstructions has become critical in the staging and surgical planning for patients with neuroblastoma (Fig. 66-81). The use of intravenous and oral contrast is essential in this evaluation, and scanning should include the chest, abdomen, and pelvis. The presence of intraspinal extension can often be detected by CT scanning; however, MRI is considered superior to CT in characterizing epidural extension or leptomeningeal spread of disease.[91] MRI, particularly with the advent of faster, whole-body scanning techniques together with diffusion weighted imaging, has also been advocated for use in determining extent of bone-marrow involvement.[167,287] In addition, MRI is effective in identifying unsuspected sites of bone and bone-marrow disease (Fig. 66-82), some of which may also be delineated by MIBG scintigraphy. MRI should also be considered for the monitoring of infants and children with low-risk disease, in whom treatment does not routinely involve radiotherapy and for whom reduction in radiation exposure is desirable. Guidelines for incorporating CT and MRI findings into the new INRG specifications for IDRFs have been published (see Table 66-4).[286]

The staging and monitoring of patients with neuroblastoma also depends on the use of scintigraphic studies, including bone scanning and MIBG scintigraphy. ^{99m}Tc-MDP scintigraphy has historically been used to detect the presence of cortical bone metastases and has been shown to be superior to conventional radiography,[91] although with current multimodality imaging and staging protocols, it is not clear that bone-scan findings impact the clinical stage of the majority of patient over the age of 1 year (see Fig. 66-77, C). MIBG is a catecholamine analogue taken up by neuroblastoma cells. ^{123}I-MIBG has been used to localize neuroblastoma at primary sites (Fig. 66-83), as well as in bone, bone marrow, and lymph nodes, and is effective in identifying sites of disease in more than 90% of the patients.[91,288] In particular, sites of previously unsuspected disease in the bone marrow are effectively detected by MIBG. Normal uptake by the liver, however, does limit the use of MIBG in detecting small foci of hepatic disease, and uptake by normal adrenal tissue after contralateral adrenalectomy may make postoperative follow-up observation challenging. Nonetheless, the relative sensitivity and specificity of MIBG for detecting disease not evident by other techniques has led most investigators to routinely include MIBG scintigraphy in the diagnostic and posttreatment evaluation of neuroblastoma patients.[289,290]

Although large controlled studies are lacking, a number of smaller consortium studies have shown that MIBG scanning at the time of staging has prognostic significance and that early response to therapy as detected by MIBG scintigraphy correlates with overall response and event-free survival.[291-295,296] As a result, MIBG scoring systems have been proposed to allow enumeration of the number

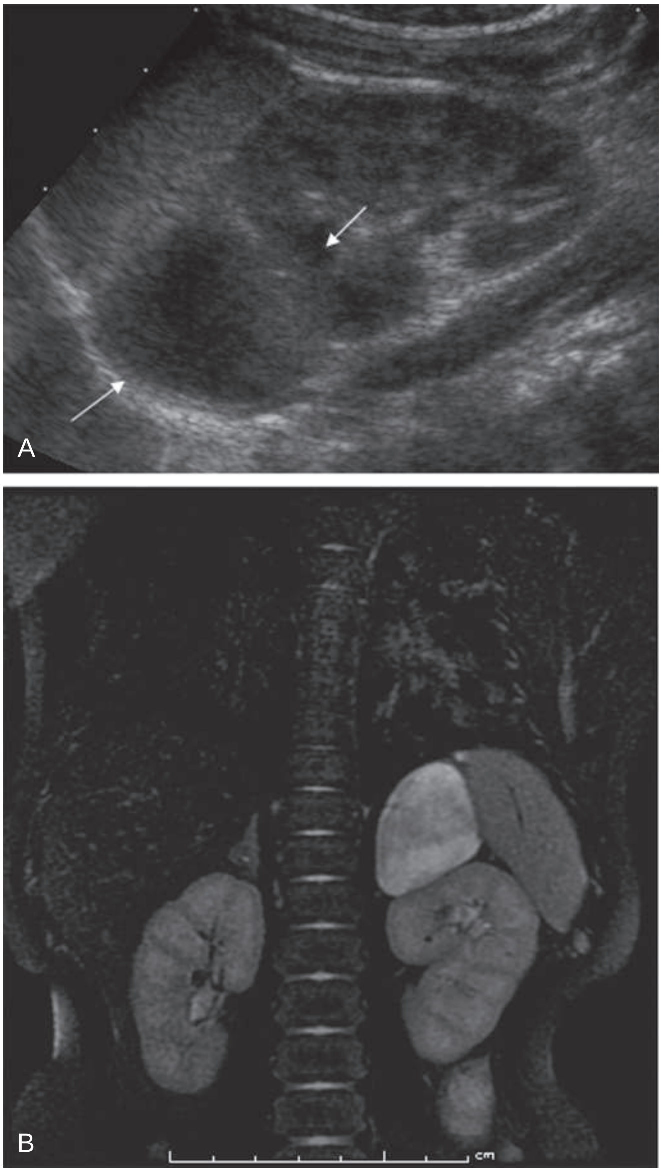

Figure 66-80 Neuroblastoma. Scans are of a 2-month-old infant being evaluated for vomiting. **A**, Ultrasound to assess for pyloric stenosis showed a well defined left suprarenal mass *(arrows)* confirmed by MRI (**B**).

of MIBG disease foci extent of MIBG avid disease, and have been used as a semi-quantitative means of monitoring responses to therapy, with good correlation with overall clinical response.[295,297,298]

FDG-PET imaging, although commonly used for many other tumor types, has not gained universal acceptance in the staging and follow-up evaluation of patients with neuroblastoma. PET scanning allows increased sensitivity and higher spatial resolution for disease detection.[70,299,300] However, discordant patterns of uptake,[169] combined with the general acceptance of MIBG scintigraphy and cross-sectional imaging for staging and response assessment, have resulted in the slow acceptance of FDG-PET in evaluation of patients with neuroblastoma.[91,169,301] Larger studies are needed to directly compare the sensitivity and specificity of FDG-PET with MIBG to determine the efficacy of FDG-PET imaging in the staging and post-treatment evaluation of patients with neuroblastoma.

Radiolabeled monoclonal antibodies, labeled with ^{131}I, ^{123}I, ^{99m}Tc, and ^{64}Cu, have all been used in both animal models and clinical models of neuroblastoma.[74,302,303] The antibodies upon which these imaging agents are based have mainly been directed against the disialoganglioside GD2, which has increased expression in neuroblastoma, and these same antibodies have been used clinically to treat patients with advanced stage disease.[304] However, the routine use of radioimmunodetection has still remained investigational in neuroblastoma patients.

Stage 4S neuroblastoma is unique among pediatric neoplasms. Identified in the early 1970s,[305] patients with this stage were identified as a "special" group with widespread neuroblastoma who had a favorable outcome. Criteria for defining these patients varied initially amongst cooperative groups. Currently, INSS stage 4S criteria include age younger than 1 year (increased to 18 months by the INRG) and localized primary tumor (not infiltrating across the midline and no contralateral lymph node involvement). Sites of metastatic involvement may include liver, skin, and bone marrow (less than 10% involvement). Patients with stage 4S disease comprise approximately 7% to 12% of all neuroblastoma cases, and despite extensive hepatic and primary site involvement, these tumors may completely resolve with observation alone and no additional chemotherapy (Fig. 66-84).

The importance of prenatal detection of neuroblastoma has also been emphasized recently in a study by Blackman, et al, in which a prenatal ultrasound and subsequent prenatal MRI delineated a large paraspinal mass.[306] In this case, the detection of spinal-cord compression by MRI combined with decreased fetal movement led to a rapid delivery, biopsy, further staging, and early initiation of treatment, which resulted in tumor response and preservation of lower-extremity neurologic function.

The opsoclonus myoclonus ataxia (OMA) syndrome is a paraneoplastic neurologic syndrome that has been associated with an occult neuroblastoma in up to 40% to 50% of patients.[307,308] Often the primary tumors associated with OMA syndrome are small, localized, and either paraspinal or extraabdominal. The identification of sites of disease is important, because symptoms may diminish or even resolve upon identification and treatment/resection of the neuroblastoma.[309,310] The imaging of patients with suspected OMA syndrome should include imaging of the chest, abdomen, and pelvis either by MRI or CT, as well as MIBG scintigraphy (Fig. 66-85).[91,311] MIBG scanning in particular has been shown to be both sensitive and specific for revealing occult neuroblastoma in patients with the OMA syndrome,[312] although with the availability of whole-body MRI, which affords a sensitive means of identifying sites of occult disease in a single examination, there is likely to be a shift in favor of whole-body MR screening for patients with OMA syndrome.

The distinction between neuroblastoma and its more differentiated counterparts ganglioneuroblastoma and ganglioneuroma cannot be made on the basis of imaging

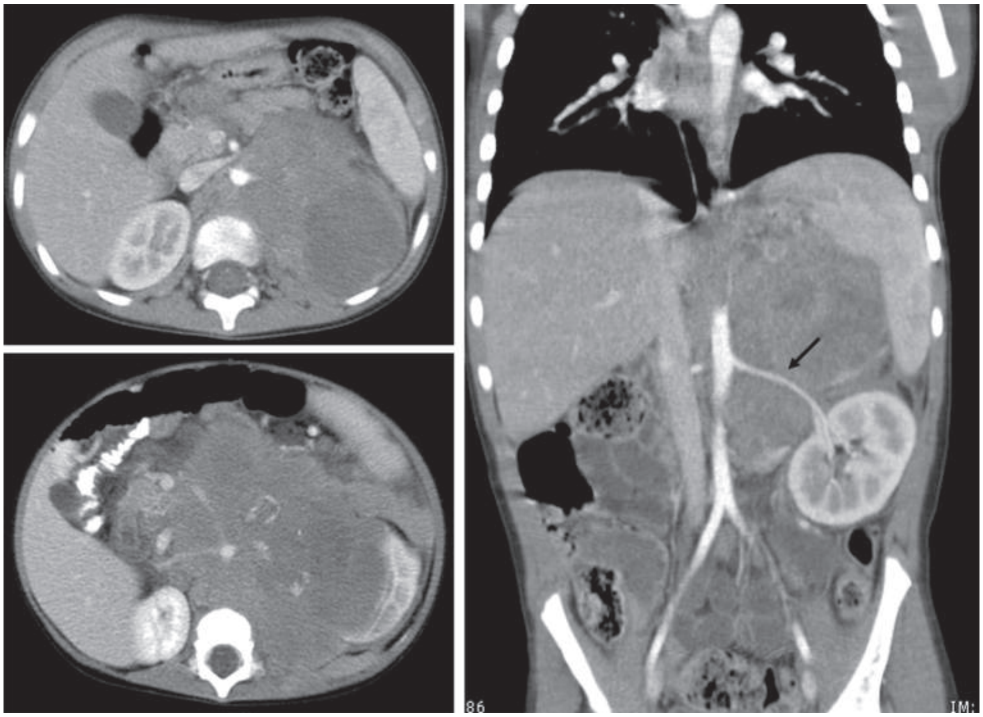

Figure 66-81 Neuroblastoma. Contrast-enhanced CT showing bulky retroperitoneal masses in two patients with encasement of vessels and extension across midline. Coronal reconstruction shows inferior displacement of the left kidney by the mass and demonstrate traction on the left renal artery *(arrow)*, which often leads to hypertension in such patients.

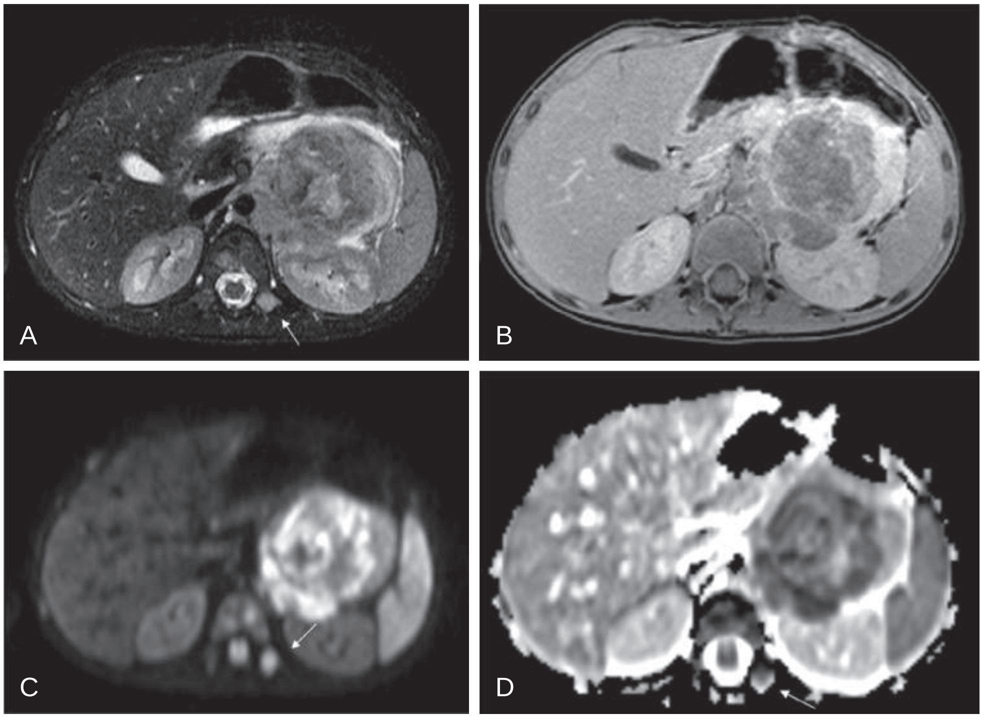

Figure 66-82 Stage 4 neuroblastoma. MRI shows left upper quadrant/retroperitoneal mass on T2-weighted (**A**) and post-Gd T1-weighted imaging (**B**). Diffusion-weighted imaging (**C**) and apparent diffusion coefficient (ADC) map (**D**) also reveal a focus of bone/bone-marrow involvement in the adjacent left vertebral pedicle *(arrow)* that is present but less conspicuous on the T2-weighted images and not seen on the post-Gd images.

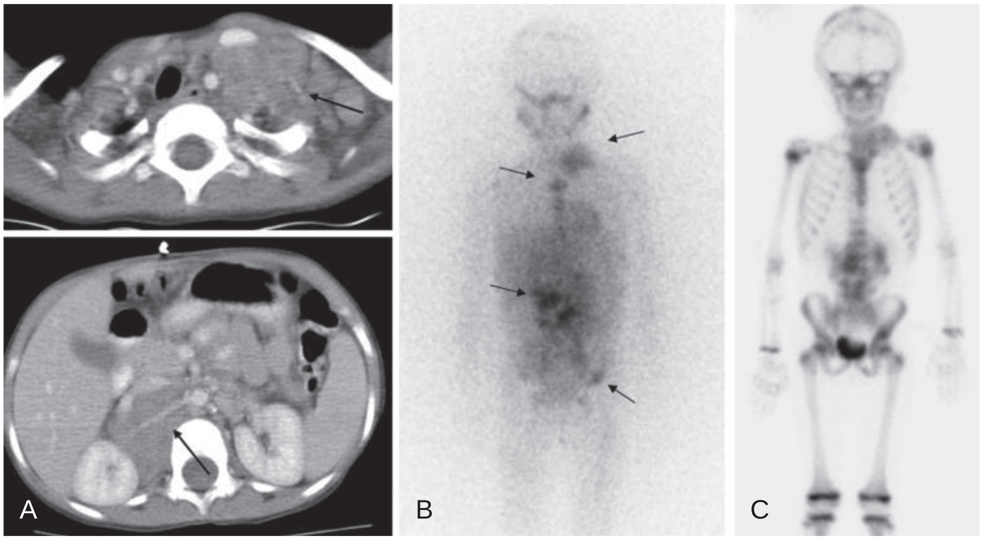

Figure 66-83 Neuroblastoma. Contrast enhanced CT (**A**) and MIBG (**B**) in a 5-year-old child showing intraabdominal, left supraclavicular, left acetabular, skull, and vertebral disease *(arrows)*. The thoracic vertebral disease is readily seen by MIBG but is not evident on CT. MDP bone scintigraphy (**C**) shows concordant uptake at most of these sites but does not reveal any additional sites of bone involvement; identifying additional sites would not have affected disease stage in this patient with stage 4 disease.

features alone.[164] Ganglioneuroma by definition shows completely differentiated stromal and cellular elements, with neuroblastoma containing less than 50% differentiated elements, and ganglioneuroblastoma intermediate between the two. Often patients who have responded to therapy will have residual lesions that remain MIBG avid and on biopsy are shown to be differentiated ganglioneuroma. In fact, novel treatment approaches are aimed at taking advantage of this potential for neuroblastoma to undergo differentiation, with cis-retinoic acid and its derivatives being used in an effort to accelerate the pathway toward differentiation.[313] Although biopsy/surgical resection is ultimately required to confirm the diagnosis, ganglioneuromas and ganglioneuroblastomas tend to be localized, with the most common sites of disease in the posterior mediastinum and retroperitoneum (Fig. 66-86). The age at presentation is also typically older than patients with neuroblastoma. Ganglioneuroma, like ganglioneuroblastoma and neuroblastoma, may accumulate MIBG, and MIBG uptake cannot be used to effectively discriminate between these distinct histopathologic entities.[314] Calcifications are often seen by CT and conventional radiography (see Fig. 66-86), and similar MRI appearances are seen with all three histopathologic types, with low signal intensity on T1-weighted images, intermediate to high signal intensity on T2-weighted images, and heterogeneous enhancement after gadolinium infusion. It is also important to recognize that sampling error can result after biopsy, and complete resection, if feasible, ensures a thorough sampling of the tumor specimen and a more confident diagnosis of ganglioneuroma.

Pheochromocytomas and Paragangliomas

Pheochromocytomas are rare pediatric tumors. They arise from chromaffin cells in the adrenal medulla or from cells of the extraadrenal sympathetic nervous system, where they are known as *paragangliomas*. In children these tumors are diagnosed from childhood to early adolescence, with a peak age range between 6 and 15 years. There is an increased incidence of pheochromocytoma in certain hereditary syndromes including the MEN syndromes IIA and IIB, von Hippel-Lindau disease, and neurofibromatosis type 1 (NF1). More recently pheochromocytoma/paraganglioma tumors have been associated with mutations in the succinate dehydrogenase gene family (*SDHB* and *SDHD*). The majority of these tumors (more than 80%) are located in the adrenal gland, with an increased likelihood of bilateral adrenal involvement in patients with MENII and von Hippel-Lindau disease.[315,316] Approximately 80% of patients have symptoms related to increased secretion of catecholamines, including sustained hypertension, headache, palpitations, anxiety, fatigue, and chest pain. Although these tumors are most often identified during the evaluation of clinical symptoms, they may also be discovered incidentally. The imaging features of pheochromocytoma are variable,[315] and while primary adrenal tumors are classically described on MRI as being hyperintense on T2-weighted images, low signal on T1 sequences, and showing a "salt and pepper" pattern of enhancement after contrast administration (Fig. 66-87),[315,317] these imaging characteristics are by no means specific for pheochromocytoma. ^{123}I-MIBG scintigraphy shows high specificity for pheochromocytoma and can be used to both identify lesions not seen by CT or MRI or to confirm the identity of abnormalities detected by cross-sectional imaging in patients with symptoms of catecholamine excess.[316,318] ^{18}F-dihydroxyphenylalanine (DOPA) PET and ^{18}F-fluorodopamine have both been shown to be superior to ^{123}I-MIBG, with greater sensitivity in detecting sites of metastatic disease,[319,320] although these agents are not in

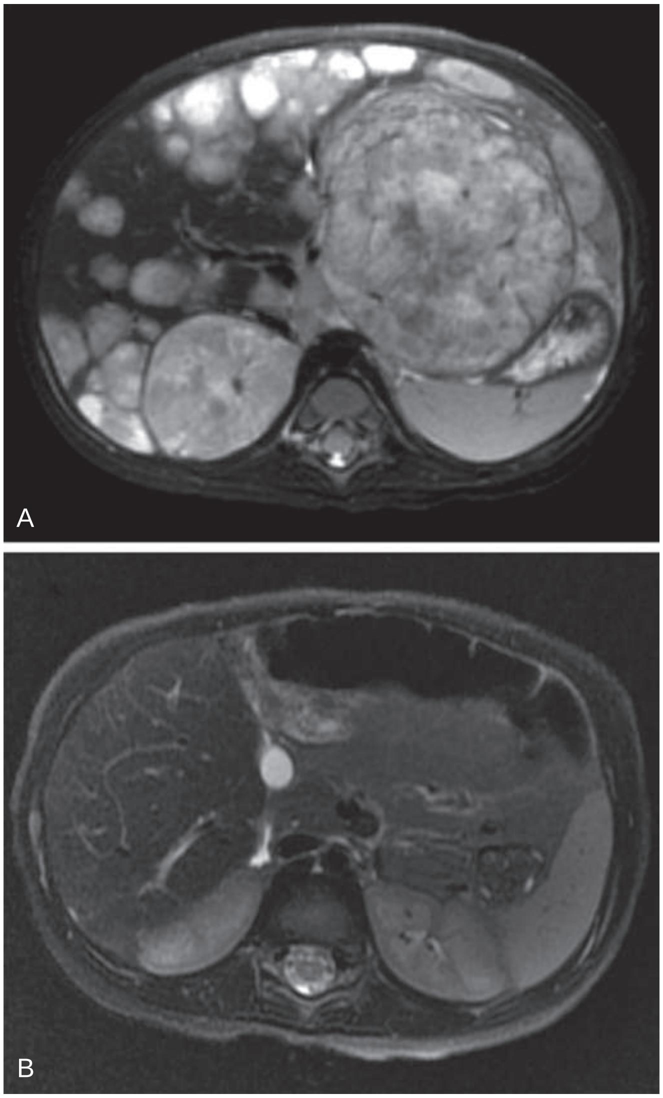

Figure 66-84 Stage 4S neuroblastoma. The patient is a 6-month-old baby with an abdominal mass. **A**, MRI shows extensive liver involvement. The infant was shown to have stage 4S disease. **B**, After 7 months of observation only and no chemotherapy, a repeat MRI shows near complete resolution of disease. This child continues with observation only.

routine use in the management of pediatric pheochromocytoma. Metastases are relatively uncommon in children (≈12%),[316] with the majority of patients having localized disease amenable to surgical resection. Higher rates of malignancy are seen in syndromic patients. Outcome is related to the presence and location of metastatic disease, with 5-year overall survival rates reported to be as high as 60% to 78% in patients with malignant metastatic tumors.[316]

GERM-CELL TUMORS

Gonadal and extragonadal germ-cell tumors are relatively rare in children, representing only about 1% of the tumors diagnosed in children under the age of 15 years. The most common extragonadal sites of disease in older children are the mediastinum and brain, and these tumors are discussed separately in those respective sections. Sacrococcygeal germ teratomas are the most common germ-cell tumors occurring in infancy and account for approximately 40% of all teratomas identified in children.

Ovarian Germ-Cell Tumors

Ovarian germ-cell tumors include both benign and malignant neoplasms.[279] The majority of the ovarian tumors are benign, with the most common tumor being the mature cystic teratoma/dermoid cyst. Malignant ovarian tumors include neoplasms of both stromal- and germ-cell origin, the former including Sertoli-Leydig cell tumors, granulosa thecal-cell tumors, and undifferentiated stromal tumors. Malignant ovarian tumors of germ-cell origin include dysgerminomas, immature teratomas, endodermal sinus tumors, embryonal carcinomas, and choriocarcinomas.

The imaging evaluation of malignant ovarian germ-cell tumors should be directed at characterizing the primary lesion and identifying possible sites of metastatic spread of disease. Ultrasound is usually the first imaging study performed to identify and characterize a suspected primary ovarian and/or pelvic mass.[321] Ultrasound reliably distinguishes between cystic and solid lesions and is helpful to assess blood flow to the lesion. The presence of associated ascites may also aid in the differential diagnosis. Additional evaluation by MRI and/or CT is typically performed as needed to further characterize the primary lesion and to assess for locoregional and metastatic spread of disease.

Sonographically the benign/mature cystic teratoma may be a homogeneous echogenic mass or alternatively a predominantly fluid-filled mass with an echogenic mural nodule. The presence of fat, calcification, or fat-fluid levels are all common findings in mature cystic teratoma (Fig. 66-88).[279] Large masses may be better characterized by CT and MRI, with CT scanning showing low-density lesions of fat density and heterogeneous enhancement of the solid components. Calcifications, particularly microcalcifications, are effectively identified by CT.

The MRI findings are variable depending on the characteristics and tissue composition of the teratomatous elements.[321] Typically mature teratomas have areas of both high and low signal on both T1- and T2-weighted images, with suppression of signal on fat-suppressed T1-weighted sequences. As shown in Figure 66-88, the presence of calcification, teeth, or hair may be useful in confirming the diagnosis. Even in the presence of mature elements, surgical resection is indicated, because the imaging features cannot clearly discriminate between malignant components of mixed germ-cell tumors and the entirely benign mature counterparts. The presence of lymph node involvement, extension into contiguous organs/structures, hematogenous spread, and evidence of peritoneal/omental seeding all should suggest the presence of malignant disease.[279]

It is important to correlate the imaging findings with the age of the patient. Functional ovarian neoplasms such

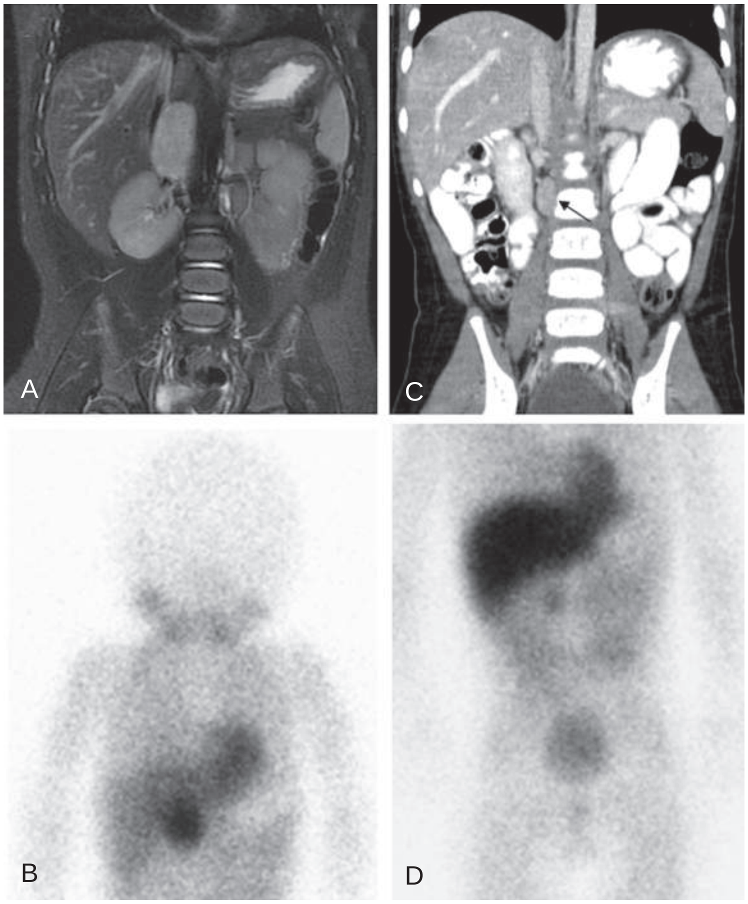

Figure 66-85 Two patients, ages 13 months (**A** and **B**) and 28 mo (**C** and **D**) with OMA syndrome secondary to occult neuroblastoma. MRI (**A**) and CT (**C**) show a right suprarenal mass (**A**) and subtle retroperitoneal lesion (**C**, *arrow*), both of which show focal MIBG uptake (**B** and **D**).

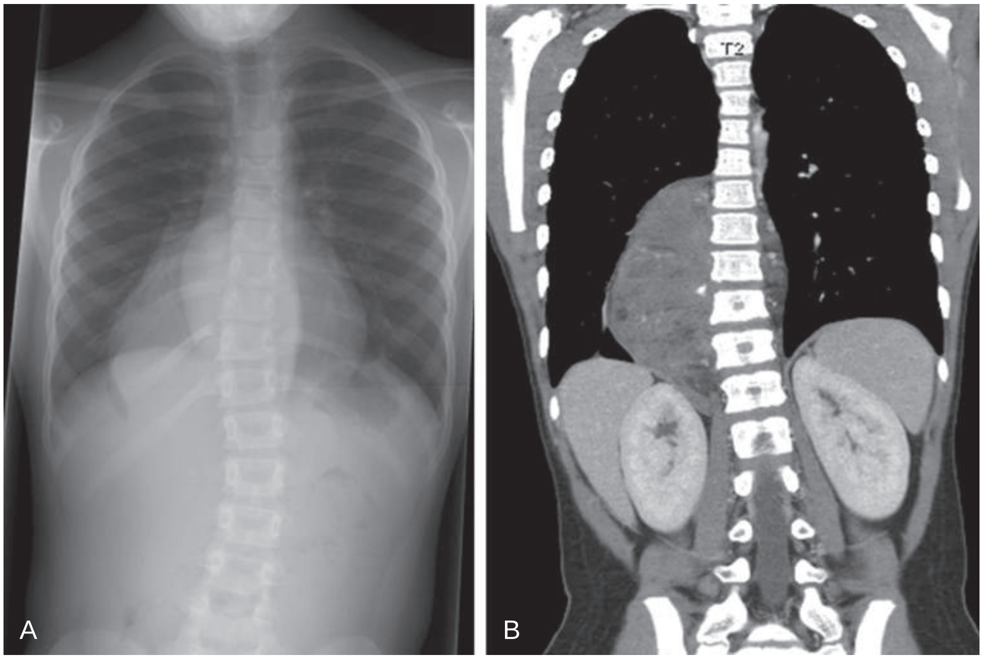

Figure 66-86 Ganglioneuroma in a 9-year-old child with back pain and scoliosis. Spine radiographs (**A**) and subsequent CT (**B**) showed a large paraspinal mass with calcifications also evident on CT. There was only faint MIBG uptake in the periphery of the mass (not shown), findings characteristic of mature ganglioneuroma.

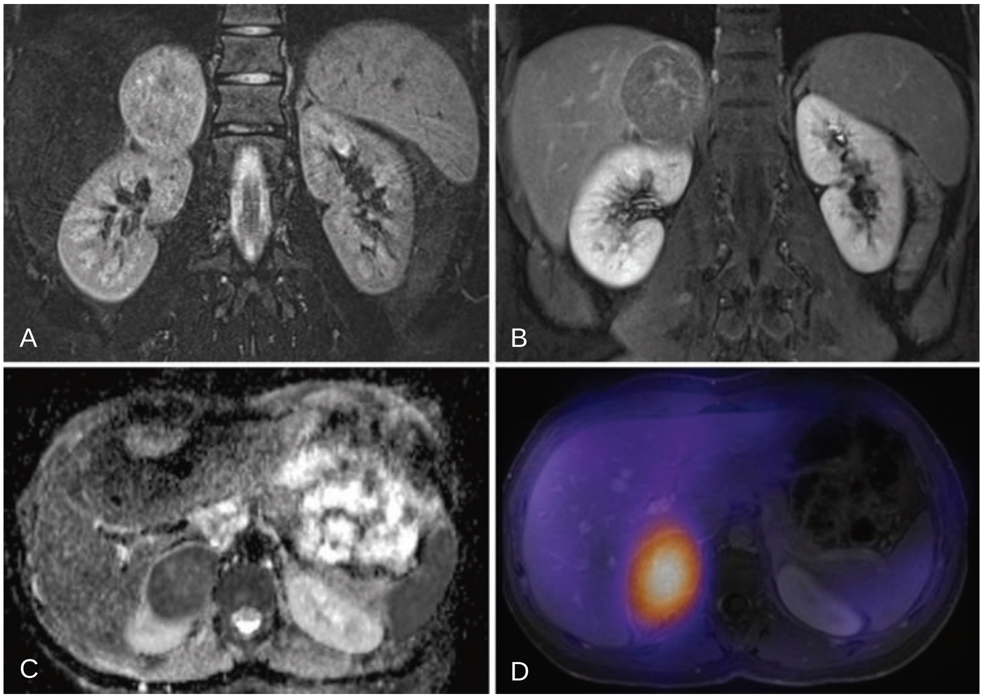

Figure 66-87 A 15-year-old patient with NF1 and an adrenal lesion detected on spine MRI. Coronal fat-suppressed T2-weighted (FSEIR) (**A**) and post-Gd fat-suppressed T1-weighted (**B**) images show a T2-bright lesion with a speckled ("salt & pepper") pattern of enhancement characteristic of pheochromocytoma. **C**, A DWI ADC map shows that the lesion has highly restricted diffusion. **D**, MIBG scintigraphy with SPECT images fused to the MRI show intense MIBG uptake in the lesion.

as granulosa thecal cell tumors may result in precocious puberty and sonographic findings that are discordant with the patient's age (Fig. 66-89). Correlation with serum hormone levels, AFP levels, and β human chorionic gonadotropin (HCG) levels is an important adjunct to the imaging evaluation.

The distinction between neoplastic masses of the ovary and nonneoplastic cysts, particularly hemorrhagic cysts, may be difficult. Nonneoplastic cysts are the most common etiologies for ovarian masses in infants and adolescent girls.[279,322] These hormonally responsive lesions arise as a result of follicular stimulation and corpus luteal maturation. Serial imaging by ultrasound is helpful in documenting stability and/or resolution of these benign processes and in making the distinction from their malignant neoplastic counterparts. Complex cysts and hemorrhagic cysts may be challenging to distinguish from solid neoplasms, and MRI with inclusion of fat suppression and magnetic susceptibility-weighted sequences can be helpful in discriminating fat-containing solid masses from complex hemorrhagic cysts.

Testicular Germ-Cell Tumors

Malignant testicular tumors are primarily of germ-cell origin, with the majority of these being malignant. The most common malignant testicular germ-cell tumor is the yolk sac, or endodermal sinus, tumor. Other malignant germ-cell tumors include embryonal carcinoma and teratocarcinoma. The benign teratomas seen more often in the ovary are less commonly encountered in the testes. Adult-type tumors such as seminomas and choriocarcinomas are less commonly encountered in infancy and begin to emerge around the time of puberty.[279]

Most testicular masses present with nontender scrotal swelling. Elevated serum AFP levels are common, and there is often spread to adjacent inguinal and retroperitoneal lymph nodes. The initial evaluation is usually by ultrasound to assess for the presence of a testicular mass and to distinguish between malignancy and nonneoplastic causes of scrotal swelling such as epididymorchitis, inguinal hernia or hydrocele, and testicular torsion. Sonographic features include an intratesticular mass with either increased or decreased echogenicity relative to the testicular parenchyma (Fig. 66-90). Color and Doppler assessments often reveal increased vascularity. The presence of an associated hydrocele, thickening of the spermatic cord, and extension of the mass through the tunica albuginea may indicate extratesticular spread of disease.[279] Once a mass has been confirmed, the staging evaluation should include imaging of the chest, abdomen, and pelvis to assess for regional lymph node spread and evidence of hematogenous disease spread, particularly to the liver and lungs.

FDG-PET imaging has been advocated in both the staging and follow-up evaluation of patients with malignant germ-cell tumors, with several studies showing the effectiveness of FDG-PET in identifying sites of disease that would otherwise have escaped detection on conventional cross-sectional imaging alone.[323-325] However, FDG-PET is unlikely to replace retroperitoneal lymph node sampling because of limitations in detecting small foci of disease.[326] In the only large prospective study

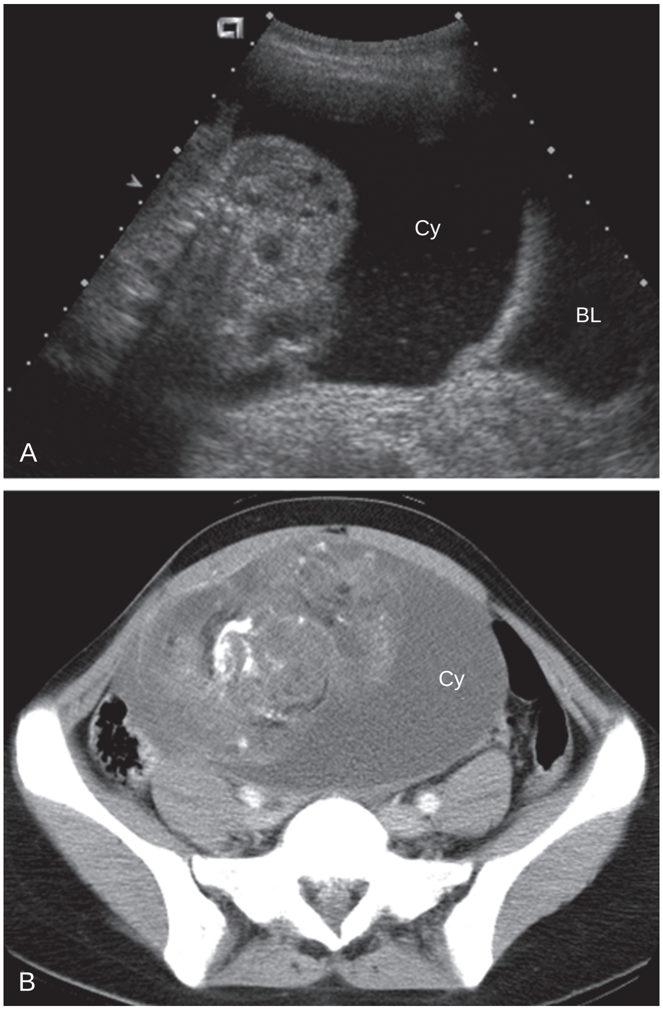

Figure 66-88 Mixed ovarian germ-cell tumor. Ultrasound shows a complex cystic and solid mass (**A**) that on CT showed areas of calcification within the solid mass (**B**), typical of germ-cell tumors.

available, posttherapy surveillance imaging with FDG-PET in patients with nonseminomatous germ-cell tumors did not confer a clinical benefit over CT and serum tumor markers for the prediction of tumor viability in residual masses.[327] Finally, there is a potential for false-negative findings on FDG-PET, particularly in the setting of mature, differentiated teratoma.[325]

Non–germ-cell tumors of the testes include Sertoli- and Leydig-cell tumors, both of stromal origin, as well as leukemia and lymphoma. Sertoli-cell tumors are more common in infancy, with Leydig-cell tumors occurring in early childhood and often accompanied by virilization or gynecomastia.

Sonographically, these testicular lesions are often heterogeneous and hypoechoic relative to the normal testicular parenchyma and usually demonstrate increased blood flow. MRI is not usually required to characterize the local site of disease; however, the MRI features of these testicular lesions include decreased signal intensity on T1-weighted images relative to the normal testicular parenchyma, increased T2 signal intensity, and heterogeneous enhancement. As with testicular germ-cell tumors, extension into the spermatic cord as well as inguinal and retroperitoneal lymph-node involvement, can be effectively demonstrated by MRI.

In children with cryptorchid testes, the presence of an undescended testicle is considered a predisposing factor leading to development of testicular tumors in childhood. Although the increased relative risk varies between published reports, there is little dispute that the risk of testicular cancer is increased in children and men with undescended testes.

Withdrawal of maternal estrogen at birth leads to a rise in testosterone and stimulation of testicular descent. Spontaneous descent of testes into the scrotum after 1 year of age is uncommon. The imaging evaluation of children with undescended testes should include a thorough ultrasound examination, with examination of the scrotum, inguinal canal regions, retroperitoneum, and kidneys. Often undescended testes can be located in the

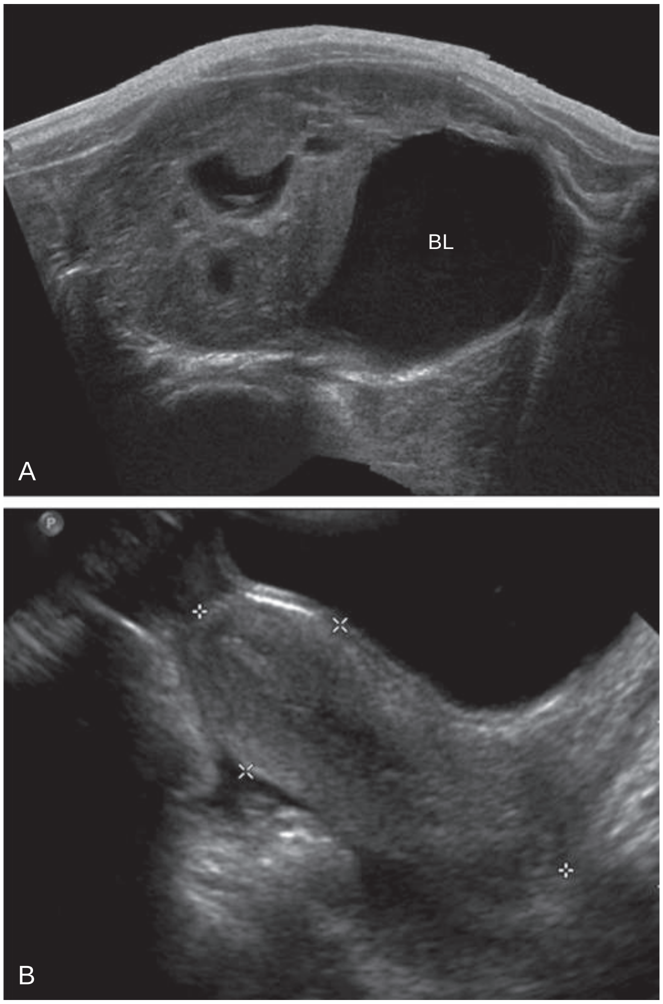

Figure 66-89 Granulosa-cell tumor. The images show a 5½-year-old child with the onset of menses. Luteinizing hormone/ follicle-stimulating hormone (LH/FSH): <0.1; estradiol: 144.5. Sagittal abdominal ultrasound image shows a mass adjacent to and above the bladder *(BL)* (**A**), likely arising from the left ovary. Note the post-pubertal appearance of the uterus (**B**), indicating a hormonally active mass.

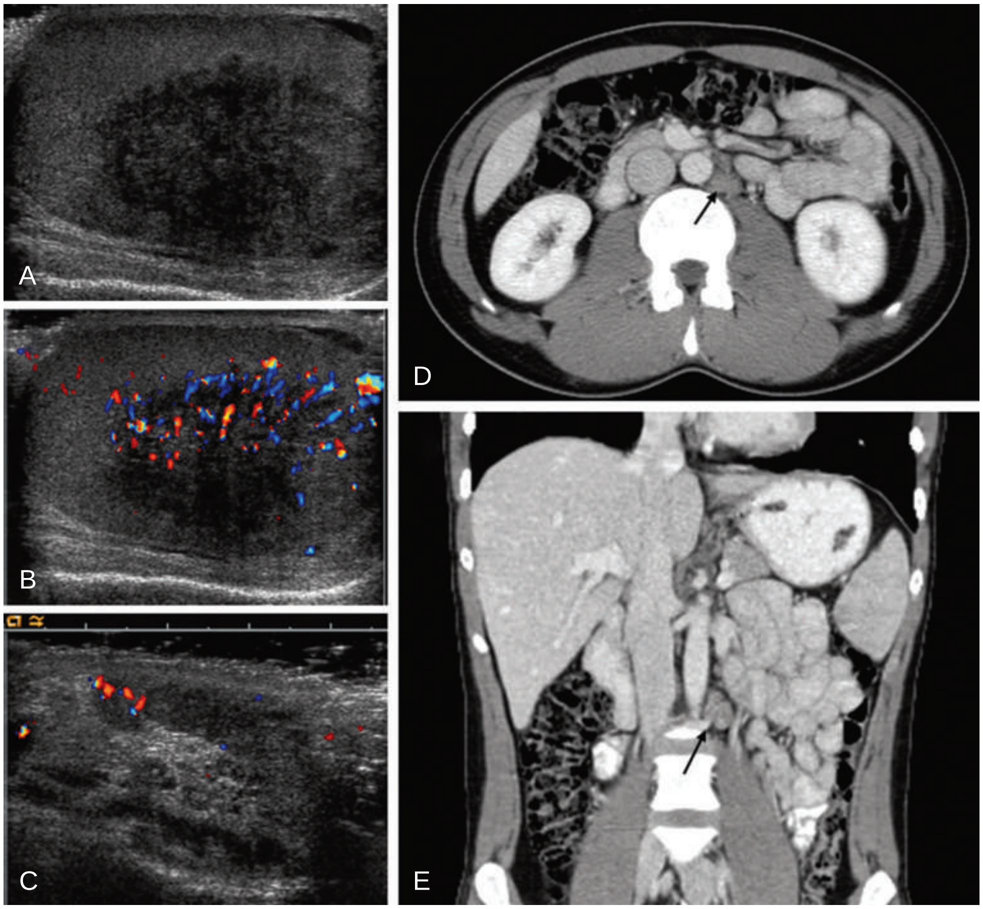

Figure 66-90 Testicular germ-cell tumor (embryonal carcinoma). Images show an 18-year-old patient with a palpable left-testicular mass. Ultrasound shows hypoechoic mass (**A**) with increased vascularity and thickening of the epididymis and spermatic cord (**B** and **C**). A small hydrocele was also present. CT shows an enlarged left retroperitoneal lymph node that is suspicious for extratesticular spread of disease (**D** and **E**, *arrows*).

inguinal canal and effectively relocated in the scrotum by orchidopexy. The retroperitoneal location of the undescended testes, although less common, is associated with a higher risk of malignancy as compared with those located in the inguinal canal. MRI may also be helpful in identifying undescended testes not detectable by ultrasound, particularly in older children where the homogeneously T2-bright testes can often be located along the expected pathway of descent of the developing testes.

Sacrococcygeal Germ-Cell Tumors

Sacrococcygeal germ-cell tumors are the most common germ-cell tumors in children. These germ-cell neoplasms include mature and immature teratomas and endodermal sinus tumors. Both mature and immature teratomas are considered benign, whereas mixed malignant germ-cell tumors indicate the presence of both benign teratomatous elements and malignant yolk sac tumor elements. Most sacrococcygeal teratomas are detected in the newborn period, whereas yolk sac tumors may be discovered later in childhood.[328] With the advent of prenatal sonography and prenatal MRI, an extensive prebirth evaluation of the tumor is often performed, facilitating a prompt surgical resection.

Sacrococcygeal teratomas are commonly classified according to the system of Altman[329] as type I, predominantly external with a very small presacral component; type II, predominantly external with a significant intrapelvic component; type III, predominantly internal with both pelvic and intraabdominal components; and type IV, entirely presacral without significant intraabdominal or external extension. The incidence of malignant elements is closely linked to the type of tumor, with almost 40% of type IV tumors having malignant components.[279]

In addition to the prenatal imaging evaluation, both CT and MRI can be used to effectively stage sacrococcygeal teratomas.[328] MRI is preferable in determining the extent of adjacent muscular, neurovascular, and intraabdominal invasion. MRI findings also reflect the heterogeneous nature of these tumors, with variable signal intensity on T1-weighted images, increased signal intensity on T2-weighted images, and heterogeneous enhancement after contrast administration (Fig. 66-91).[280] The presence of calcification, bone, and other more mature tissue elements may be better seen on CT. Metastatic disease to the lung, liver, and draining retroperitoneal lymph nodes can occur, and CT scanning of the chest and abdomen is an important component of the staging evaluation.

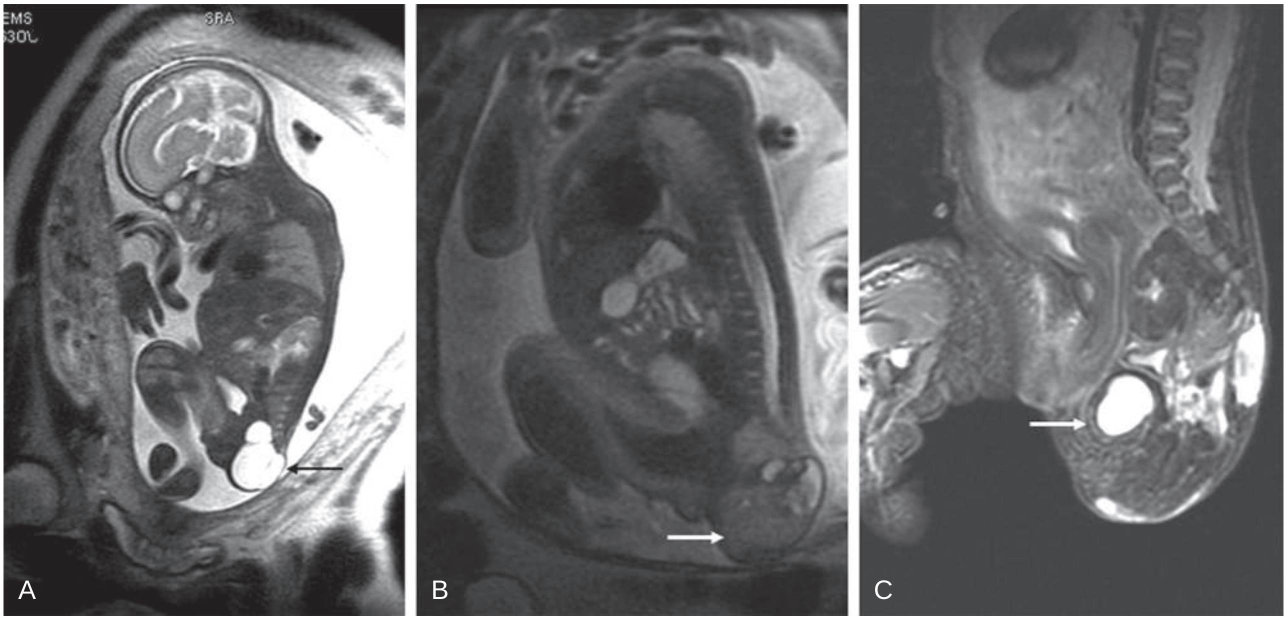

Figure 66-91 Sacrococcygeal germ-cell tumor. **A** and **B**, Prenatal MRI images show both cystic and solid elements within a large presacral mass. **C**, At birth the solid elements predominated, although large cystic spaces were still present.

Approximately 20% of these patients have pulmonary metastases at the time of diagnosis.[279]

These tumors arise from coccygeal tissue elements, and surgical resection must include resection of the coccyx. When complete resection can be accomplished, survival rates are greater than 95% with tumors that have benign mature teratomatous elements. The presence of malignant elements results in much lower rates of long-term survival.[279]

In addition to sacrococcygeal teratomas, other presacral solid masses must also be in the differential, including neuroblastoma and lymphoma. In particular, the distinction between presacral cystic neuroblastoma and a type IV germ-cell tumor may be difficult on the basis of imaging alone (Fig. 66-92). The presence of intraspinal extension, which is more commonly seen in neuroblastomas, is unusual in the setting of sacrococcygeal germ-cell tumors, aiding in the differential diagnosis. Predominantly cystic germ-cell tumors may also be difficult to distinguish from lumbosacral myelomeningoceles or the related fat-containing lipomyelomeningocele. Again the extent of intraspinal involvement, which is best demonstrated by MRI, may help in narrowing the differential diagnosis.

MUSCULOSKELETAL NEOPLASMS

Primary Bone Tumors

Pediatric bone tumors are relatively rare, comprising less than 5% of the new cancer diagnoses in children under the age of 9 years, and between 8% and 11% in children between the ages of 10 and 19 years. Osteosarcoma is the most common primary malignant bone tumor occurring in the first 2 decades of life. The remaining bone malignancies include the Ewing family of tumors (Ewing sarcoma) and primary lymphoma of the bone. Because symptoms are frequently nonspecific, there is often a delay in diagnosis. The goal of the imaging evaluation is to establish a preliminary diagnosis,[330] because about half of the bone lesions detected in children are benign. Based on these preliminary assessments, a more detailed imaging evaluation can then be undertaken. It is beyond the scope of this review to discuss the nonmalignant lesions that may mimic malignancy; however, chronic osteomyelitis, osteoid osteoma, bone cysts, and stress-related injury are all included among the differential considerations for primary bone lesions.[331]

Conventional radiography is essential as a first step in the evaluation.[332] Radiographs afford high spatial resolution, can be readily obtained, are relatively inexpensive, and do not require sedation. Most importantly the radiographs are critical in guiding the next step in the evaluation based on the pattern of bone reaction/involvement and surrounding soft-tissue involvement. MRI is usually the imaging modality of choice to further characterize primary bone lesions.[330,331] MRI sequences should include at a minimum, T1- and T2-weighted imaging in at least two orthogonal planes as well as post–gadolinium-enhanced imaging. Fat suppression sequences are nearly always of value in distinguishing focal areas of enhancement from intramedullary fatty marrow or surrounding soft-tissue fat, whereas non–fat-suppressed T1-weighted sequences are invaluable for delineating the margins of marrow-space involvement. Fluid-sensitive sequences are highly sensitive but lack specificity: tumor, edema, hemorrhage, and focal fluid collections all may show increased signal on fluid-sensitive sequences. More importantly the fluid-sensitive sequences allow the examiner to closely assess the relationship of signal abnormalities in the bone and soft tissues to adjacent neurovascular structures. It remains controversial whether post–gadolinium-enhanced images, simple T1-weighted images, or fat-suppressed

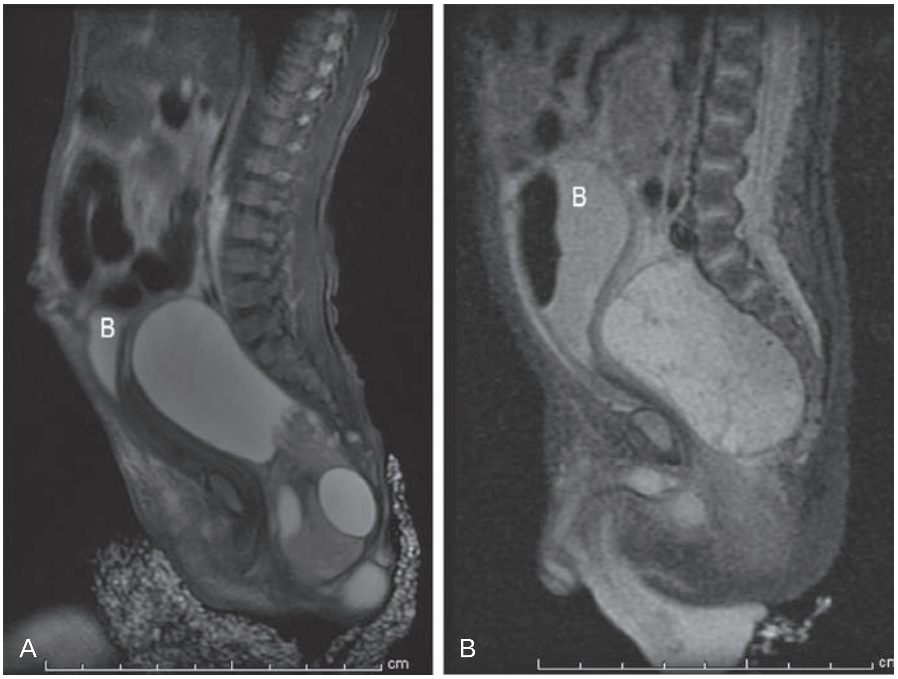

Figure 66-92 Sacrococcygeal germ-cell tumor (**A**) and cystic neuroblastoma (**B**). Two patients with prenatally diagnosed cystic presacral mass. Fluid-sensitive MRI showed similarly appearing complex cystic masses, both compressing the bladder *(B)*, with very similar imaging appearance, emphasizing the importance of including neuroblastoma in the differential for some sacrococcygeal teratomas.

T2-weighted images allow the best discrimination between the true intraosseous tumor margins and surrounding peritumoral edema and bone reaction. Dynamic contrast-enhanced MRI allows tumor vascularity and permeability to be measured directly, and in at one study of osteosarcoma patients, pretreatment patterns of intratumoral blood flow were identified as prognostic factors for event-free and overall survival, as well as being indicative of a histologic response to neoadjuvant therapy.[333] The technical challenges of performing these studies combined with complex postprocessing requirements has limited the more widespread acceptance of this technique.

CT has a relatively limited role in assessing the primary bone lesions, given the increased use of MRI. However, for focal bone lesions such as osteoid osteoma, CT is very helpful in characterizing the focal osseous abnormality. In addition, for primary bone tumors, particularly Ewing sarcoma involving the chest wall and ribs, CT may be preferable. The staging evaluation of primary bone tumors must also include a CT of the chest to assess for pulmonary metastatic disease. The use of bone scintigraphy, either using ^{99m}Tc or ^{18}F, is considered standard of care for assessing multifocal sites of disease.[90] FDG-PET imaging, although not in routine clinical practice, has been shown in several studies to be effective in detecting sites of lymph node spread and metastatic disease outside of the primary site and in helping determine the margin of metabolically active tumor involvement.[176,183,334-336] In addition FDG-PET has been effective at showing early responses to chemotherapy, and studies have shown that early responses to chemotherapy, manifest as decreased metabolic activity and decreased FDG uptake, correlate with improved outcome and overall survival.[174,183,337-339]

Osteosarcomas

Osteosarcomas are the most common primary bone tumor occurring in children older than the age of 10 years (Ewing sarcoma being more common in younger children).[340-342] This may relate to the period of rapid bone growth occurring in early to mid adolescence, which corresponds to the peak incidence of osteosarcoma. The majority of osteosarcomas are of the classic high-grade histologic type with telangiectatic and surface osteosarcomas occurring less often (the latter two entities being divided into parosteal and periosteal subtypes). Osteosarcomas most commonly occur in the metaphysis of the rapidly growing ends of the long bones including the distal femur, proximal tibia, proximal femur, and proximal humerus. Involvement of the axial skeleton and flat bones is much less common, except where osteosarcoma has developed secondary to radiation treatment or chemotherapy. At diagnosis, approximately 20% of patients have pulmonary metastatic disease detectable by chest CT. Other sites of metastatic disease spread are unusual.

The radiographic appearance of osteosarcoma varies widely; however, the typical lesion is an aggressive, destructive lesion centered in the metaphysis (Fig. 66-93). Smaller lesions may show periosteal reaction, most commonly a sunburst-type reaction. The Codman triangle formation indicates periosteal new-bone formation with the free edge of the periosteal new bone disrupted by growth of the tumor mass (Fig. 66-94). A lamellated or onion skin–type periosteal reaction may also be seen in

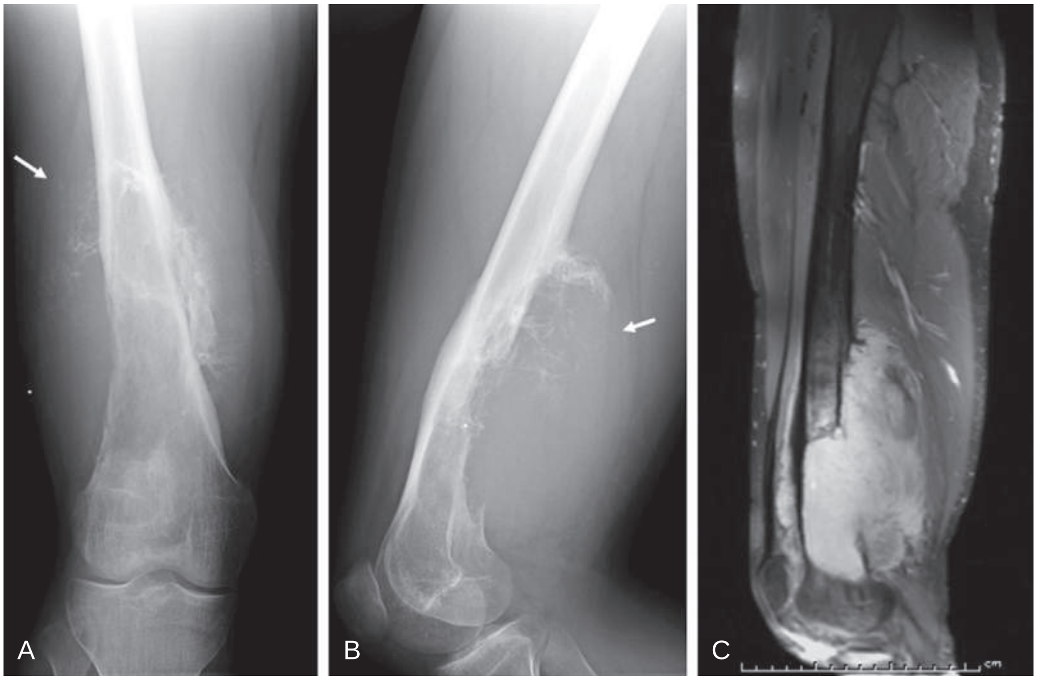

Figure 66-93 Osteosarcoma in a 19-year-old male with an enlarging right-leg mass. **A** and **B**, Radiographs show a large destructive lesion with extensive periosteal reaction. A large soft-tissue mass is also evident on the radiographs *(arrows)*. **C**, MRI shows better delineation of the associated soft-tissue mass and the extent of osseous involvement.

smaller lesions; however, this is more typically associated with Ewing sarcoma. Although the diagnosis of osteosarcomas is usually suggested by plain films, the films are relatively insensitive at determining the extent of disease. As noted, MRI is essential in determining the extent of long-bone involvement, the degree of soft-tissue involvement, and the extent of invasion into adjacent neurovascular structures (see Fig. 66-94 and Fig. 66-95).[341,343] Although epiphyseal involvement and crossing of the growth plate by osteosarcoma is seen as unusual, reports have suggested that this may occur in as many as 80% of patients.

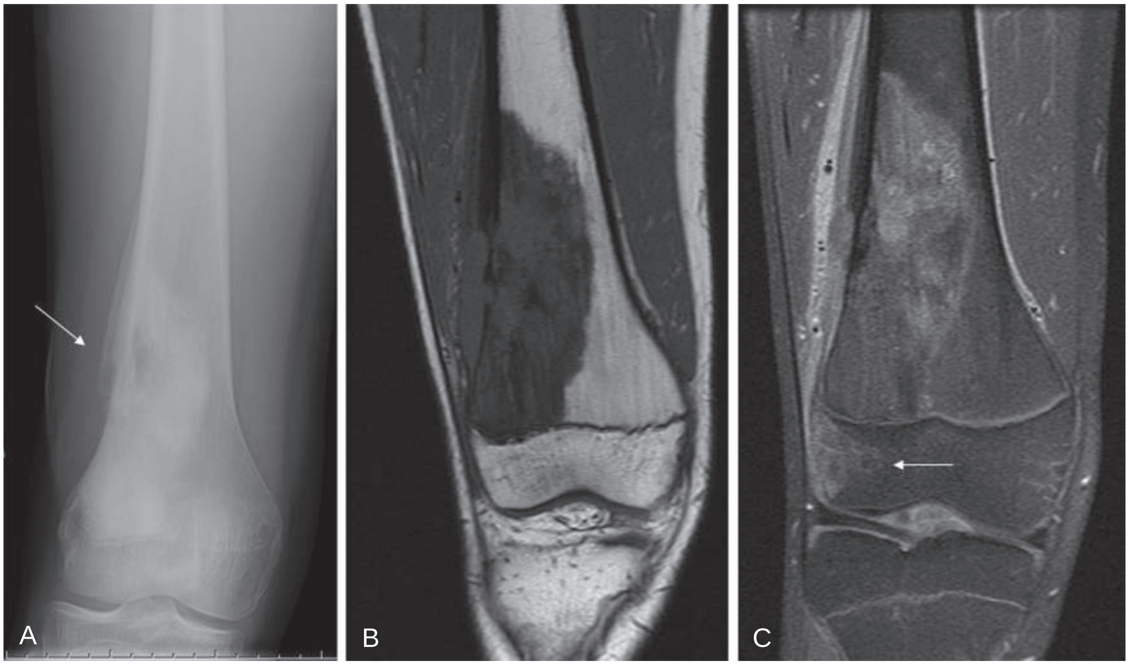

Figure 66-94 Osteosarcoma in a 16-year-old patient with leg swelling. **A**, Radiograph of the right femur shows a sclerotic lesion in the distal femoral metaphysis, with periosteal elevation and Codman's triangle *(arrow)*. **B** and **C**, T1-weighted MRI and post-Gd T1-weighted imaging show the extent of bone involvement and raise concern for epiphyseal extension of the tumor *(arrow)*.

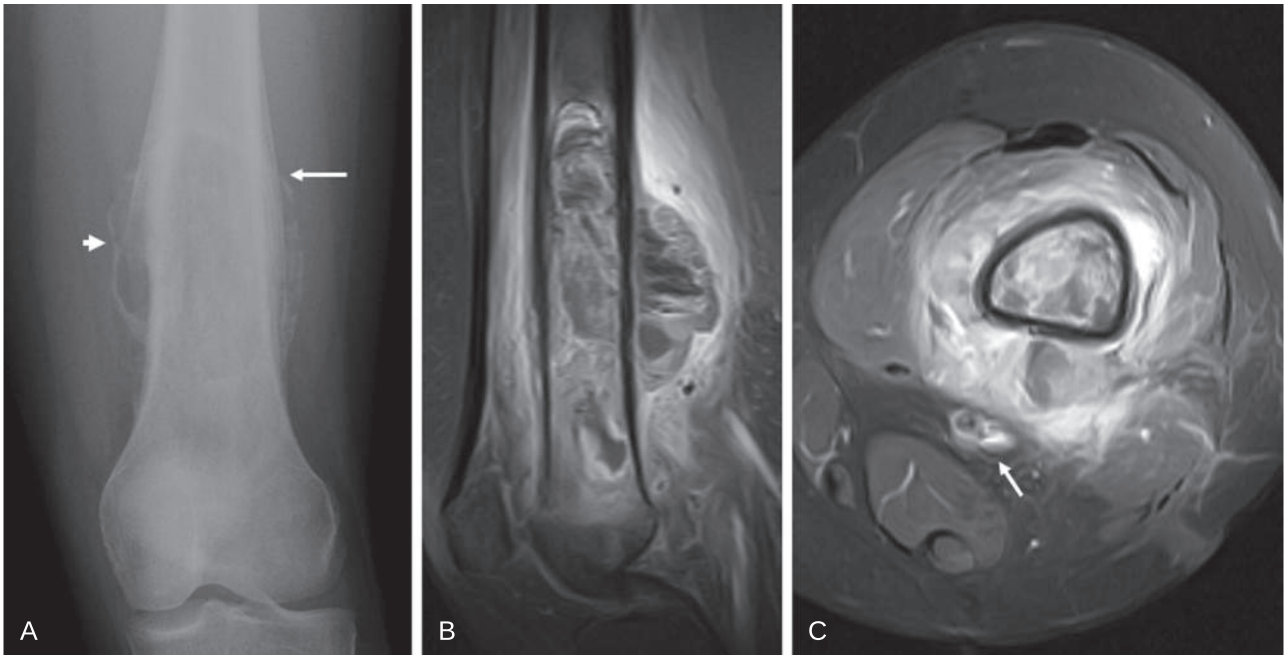

Figure 66-95 14-year-old girl with osteosarcoma. **A**, The radiograph shows a large, distal femoral destructive lesion with osteoblastic activity, lamellated periosteal reaction *(arrow)*, and a Codman triangle *(arrowhead)*. **B**, Contrast-enhanced MRI shows the associated soft-tissue mass to better advantage, as well as the extent of bone involvement. **C**, The axial MRI image shows the mass abutting the neurovascular bundle *(arrow)* but not encompassing it.

MRI is also helpful in characterizing the primary lesion. The presence of fluid-fluid levels, common to telangiectatic osteosarcoma, are best demonstrated by MRI (Fig. 66-96). The initial imaging evaluation should also assess for the presence of pathologic fracture and must include the joint proximal and distal to the primary lesion to aid in surgical planning and decision making. Skeletal scintigraphy and chest CT are necessary for the staging evaluation to assess for locoregional and distant metastatic disease.[341] Follow-up imaging is usually dictated by individual treatment protocols but typically includes imaging of the primary site of disease and chest

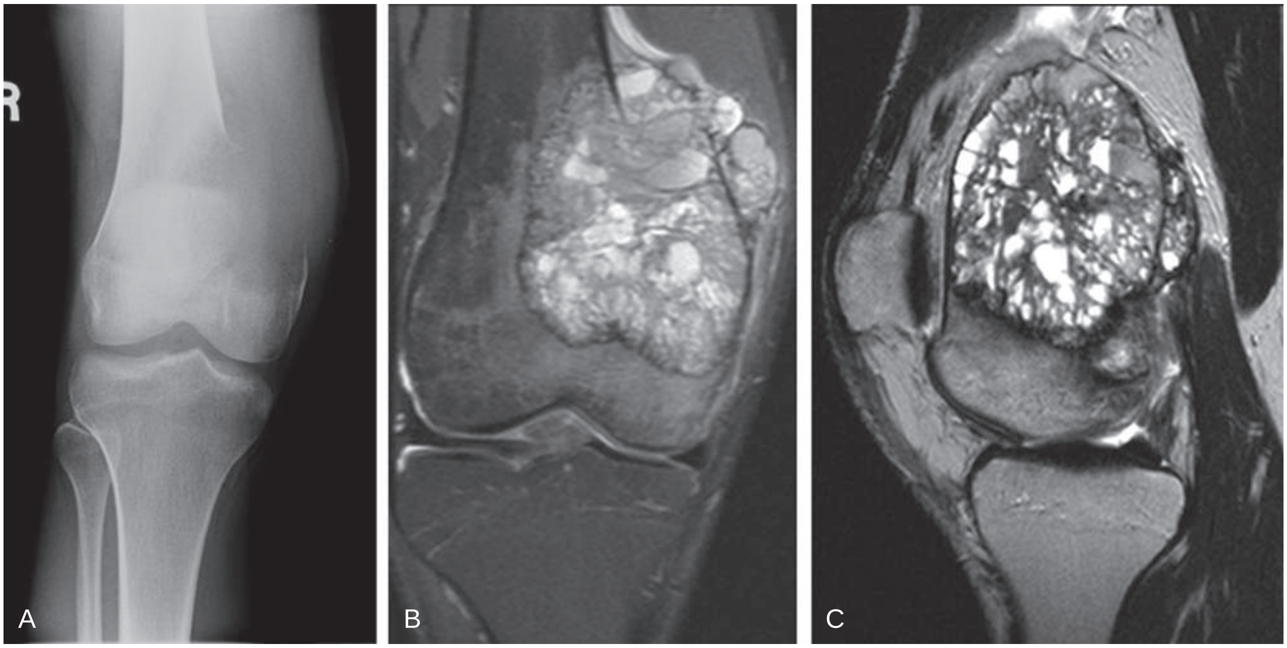

Figure 66-96 Osteosarcoma. This 18-year-old male experienced leg pain and swelling. **A**, Radiographs show a destructive lesion in the distal femur with an associated soft-tissue mass. Gadolinium contrast-enhanced (**B**) and fluid-sensitive FSEIR (**C**) MRI images demonstrate multiple fluids/fluid levels with heterogeneous contrast enhancement. The appearance is common in telangiectatic osteosarcoma, but in this patient with high-grade metastatic osteosarcoma the imaging features were the result of multiple solid and necrotic elements within the tumor.

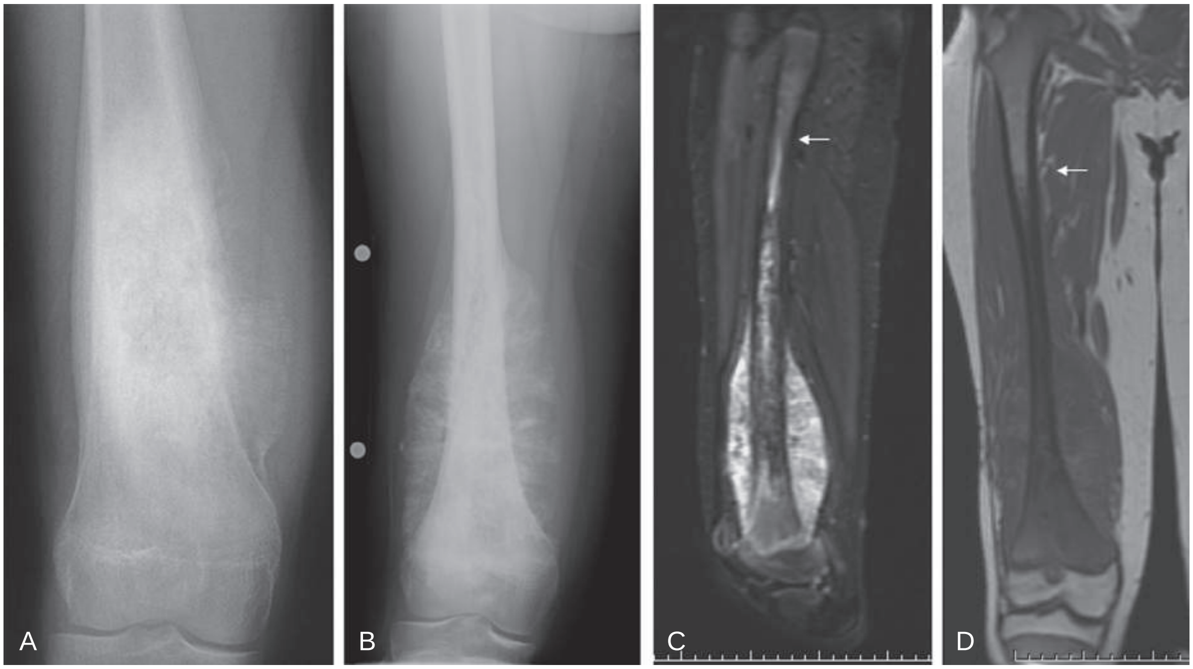

Figure 66-97 Ewing sarcoma. Radiographs from two patients with Ewing sarcoma show the "onion-skin" periosteal reaction characteristic of this tumor (**A**) and the sunburst periosteal reaction that is also commonly seen (**B**). FSEIR and T1-weighted MRI images from the patient demonstrate intramedullary high signal on the FSEIR images (**C**) and corresponding loss of normal intramedullary fat signal (**D**), indicating more widespread extension of tumor than was suggested by the radiographic findings and highlighting the importance of MRI in evaluating extent of disease in these patients.

CT. The presence of pulmonary nodules, both at diagnosis and as a site of relapse, is important prognostically,[344] and chest CT remains an essential part of the posttherapy evaluation in these patients. Unfortunately, there are no specific imaging features to distinguish benign from malignant pulmonary nodules,[22,186] and surgical resection remains essential for both definitive diagnosis and potential cure.[23]

Ewing Sarcomas

Ewing sarcomas are most often poorly differentiated tumors arising from the bone, although they may also arise from adjacent soft tissues. The primative neuroectodermal derivative of this tumor is known as the peripheral PNET (PPNET). Ewing sarcomas arising in a chest wall make up about 10% of the Ewing family of tumors. Clinically, pain and a palpable mass are the most common presenting complaints. As with osteosarcoma, the imaging evaluation should be initially directed at characterizing the primary site of abnormality. Anatomically, Ewing sarcomas occur with roughly equal distribution in the axial and appendicular skeleton, with about two thirds occurring in the pelvis and/or lower extremities. At the time of presentation, the size of the mass and extensive local invasion may make it difficult to determine whether the lesion has arisen primarily within the bone or within the surrounding soft tissue. Metastatic disease is also common at the time of diagnosis, with sites of metastatic disease reflecting hematogenous spread, including lung, bone, and bone marrow.

Radiographically, Ewing sarcoma of the bone is primarily a lytic lesion with a permeative destructive appearance.[345] The onion-skin or lamellated periosteal reaction is characteristically described with Ewing sarcoma, although it is not specific for this diagnosis. Other forms of periosteal reaction are also seen (Fig. 66-97), reflecting the variable degree of bone destruction and bone response to the tumor. There is usually a large accompanying soft-tissue mass. Particularly in the axial skeleton (pelvis and ribs), the soft-tissue component may predominate.

Plain radiographs should include imaging of the involved long bones, chest, and/or pelvis. MRI is indicated for further evaluating the appendicular skeleton and long bones, and as with osteosarcoma the entire length of the bone including the proximal and distal joints should be included. The choice of MRI pulse sequence is similar to that for osteosarcoma but should include at a minimum T1- and fat-suppressed T2-weighted images in orthogonal planes as well as post–gadolinium-enhanced images. The axial images, particularly T2-weighted, are useful for demonstrating the extent of subperiosteal and periosteal extension. Either sagittal or coronal images are effective at defining the margin of bone-marrow involvement and surrounding tissue edema.

Rib and chest wall lesions are best evaluated by CT. With multiplanar 3D image reconstruction capabilities on modern CT scanners, the extent of osseous involvement and adjacent lung/pleural involvement can usually be adequately demonstrated by CT (Fig. 66-98). For paraspinal lesions, MRI may be necessary to delineate intraspinal invasion. MRI is also useful in characterizing subtle abnormalities such as chest-wall muscle involvement and neurovascular involvement, particularly in the upper thorax and lung apices.

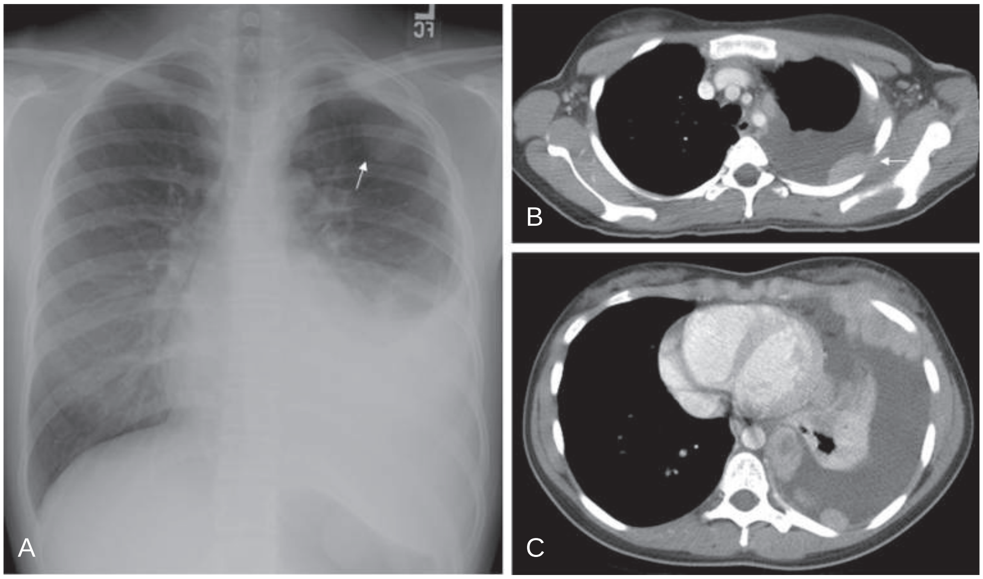

Figure 66-98 Ewing sarcoma of the chest wall. A 14-year-old girl with chest pain had a chest x-ray (**A**) that shows a large, left pleural effusion and multiple nodular densities *(arrow)* apparently arising from the chest wall/pleura. Contrast-enhanced CT (**B** and **C**) show extensive soft-tissue masses lining the chest cavity with large effusion and left lower lobe collapse. Biopsy showed Ewing sarcoma.

In contrast to osteogenic sarcoma, Ewing sarcoma does not typically show the presence of soft-tissue calcifications. Whole-body bone scintigraphy is effective at delineating sites of skeletal involvement; however, there is no clear evidence that skeletal scintigraphy is superior to MRI in characterizing local disease. FDG-PET imaging has been advocated in the staging evaluation of Ewing sarcoma, particularly with respect to identifying sites of occult bone-marrow metastatic disease. In addition, as with osteosarcoma, the use of FDG-PET has been advocated both for staging[336] and for monitoring response to therapy (Fig. 66-99).[80,174,335,337,339]

Other Bone Tumors

Primary lymphoma of the bone is a relatively unusual site of presentation in non-Hodgkin lymphoma but must be included in the differential diagnosis of a primary destructive lesion with intramedullary involvement. Radiographically the evaluation is similar to Ewing sarcoma, and biopsy is usually needed to confirm the diagnosis.

Although they are not primary bone neoplasms, leukemia and Langerhans-cell histiocytosis are both pediatric neoplastic conditions that may manifest with bone involvement (see Fig. 66-3). Leukemia, described below, often produces metaphyseal lucency and may result in an aggressive, permeative, moth-eaten appearance to the bone, occasionally accompanied by periosteal reaction. Pain is also common at this stage of bone reaction to the leukemic infiltration. Langerhans-cell histiocytosis can manifest either as localized disease or widespread osseous involvement. Typical osseous lesions are lytic on conventional radiographs. Because they are relatively slow growing and do not elicit a significant response from the surrounding bone, these lesions may go undetected by bone scintigraphy, and skeletal survey is still advocated to assess for the extent of osseous involvement in Langerhans-cell histiocytosis. Recent studies have also suggested that FDG-PET may be more sensitive both in determining sites of intraosseous involvement and in determining sites of soft-tissue involvement that would otherwise go undetected either by conventional scintigraphy or skeletal survey.[3]

Muscle/Soft-Tissue Tumors

The nonosseous musculoskeletal malignancies of childhood are typically sarcomas that arise from immature mesenchymal tissue. Rhabdomyosarcomas of skeletal-muscle origin comprise about half of the pediatric soft-tissue sarcomas.[342,346] The nonrhabdomyomatous soft-tissue sarcomas include fibrosarcomas, synovial sarcomas, alveolar soft-part sarcomas, malignant fibrous histiocytomas, and PPNETs. Ultimately the distinction between these different histiologic subtypes is made pathologically. The imaging approach is directed at determining the size and extent of primary tumor involvement, the relationship to critical adjacent structures such as nerves and blood vessels, and local involvement of bone or regional lymph nodes. All of the soft-tissue sarcomas have a propensity to metastasize to the lung, and a CT of the chest should be performed at the time of diagnosis. Bone scintigraphy is also indicated to assess for additional sites of skeletal involvement, and FDG-PET is increasingly be used for staging and response assessment in pediatric soft-tissue sarcoma.

Rhabdomyosarcomas are the most common soft-tissue sarcomas of childhood, making up more than half of all soft-tissue sarcomas in children.[342] Although the head and neck and genitourinary tract are common sites of rhabdomyosarcoma presentation, about one fourth of these tumors are also located in the extremities. These are typically of the alveolar or undifferentiated histologic type.[347] The imaging characteristics are not specific, but feature a

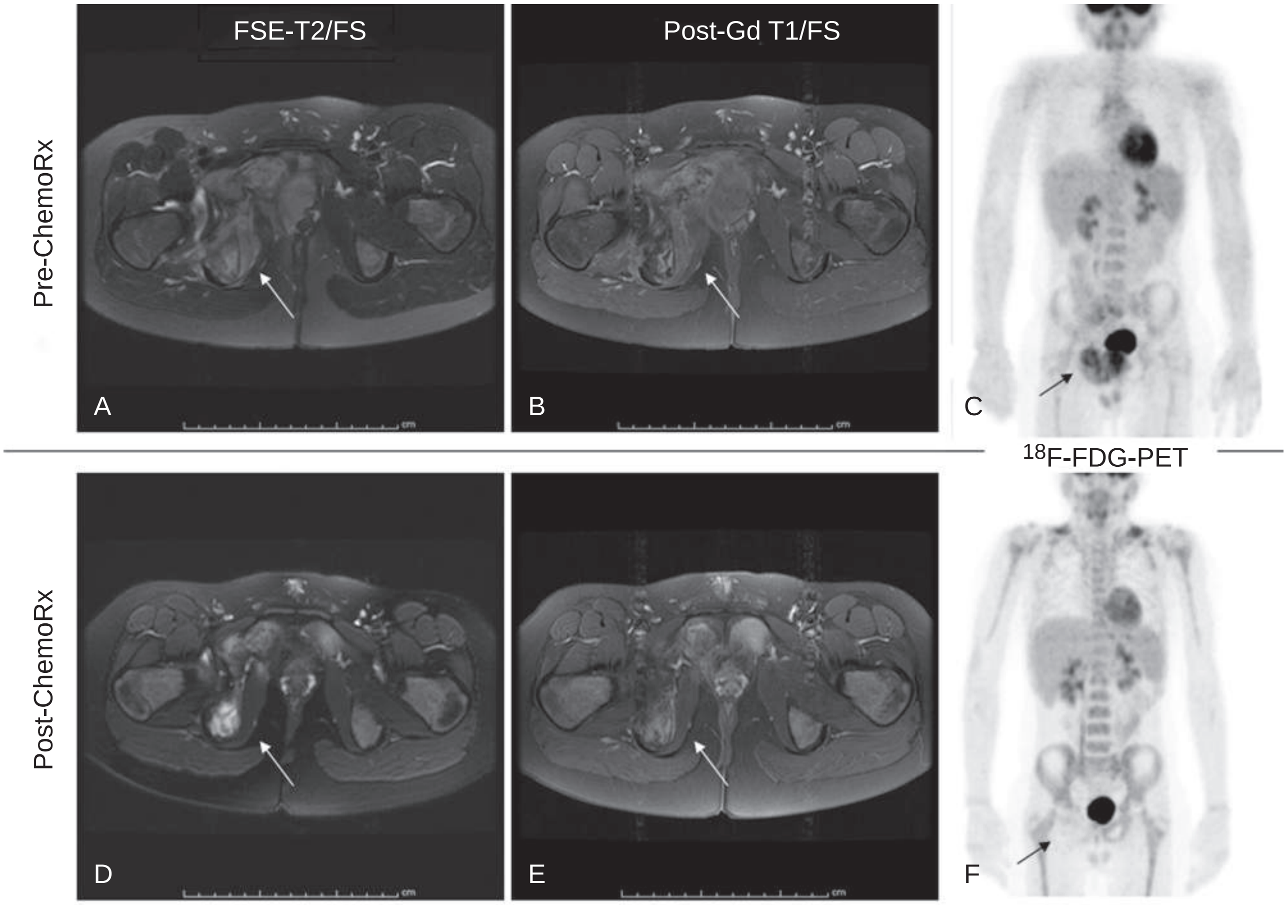

Figure 66-99 Ewing sarcoma response assessment. Images are of a 10-year-old child with nonmetastatic Ewing sarcoma of the right pelvis. T2-weighted (**A**) and contrast-enhanced T1-weighted (**B**) MRI images show an ill-defined enhancing mass centered in the right ischium with soft extension into the surrounding pelvis *(arrows)*. Pretreatment FDG-PET imaging (**C**) shows moderately intense uptake in the mass. After chemotherapy, MRI (**D** and **E**) shows persistent areas of increased T2 signal and enhancement *(arrows);* FDG-PET shows no residual metabolic activity in the mass (**F**), demonstrating an excellent response to therapy and suggesting that the MRI findings may relate to a necrotic tumor and enhancing scar/granulation tissue rather than active disease.

T2-bright lesion localized in the deep soft tissues with extension along fascial planes (Fig. 66-100). After contrast infusion, these tend to be hypervascular tumors with bright enhancement. FDG-PET has been shown in several studies to yield intense FDG uptake and is likely to be of value in identifying sites of occult disease at the time of staging and in assessing response to therapy.[183,184,281]

Synovial Sarcoma

Synovial sarcomas typically occur in patients during their teenage years. Synovial sarcomas are the most common nonrhabdomyosarcomatous soft tissues in children,[346] and despite their name, they arise from undifferentiated mesenchymal cells that are typically located in a paraarticular location. They are more common in the lower extremities than in the upper extremities or axial skeleton and are often mistaken for other benign or incidental conditions.[348] Conventional radiographs are indicated for extremity lesions and show calcifications in about 30% of cases. MRI shows a lobulated lesion that is usually isointense to muscle on T1-weighted images and isointense to slightly hyperintense on T2-weighted images with a relatively homogeneous appearance.[349] After contrast infusion, there is typically intense enhancement (Fig. 66-101). Although these findings are not specific for synovial sarcoma, the presence of a lobulated calcified lesion with the described MRI signal characteristics should suggest the diagnosis.

The clinical presentation is typically of a painless mass. Median duration of symptoms before presentation is approximately 14 months. The staging evaluation is important in predicting the overall survival of these patients. Patients with localized disease and/or with disease allowing gross total resection have an estimated 80% 5-year overall survival, as compared with patients who have metastatic disease at diagnosis and for whom the diagnosis is nearly always fatal.[350] Because these lesions most often occur in extremities, they may mimic benign disease such as synovial or ganglion cysts. The MRI examination must therefore include contrast enhancement in order to discriminate between a solid enhancing lesion and a predominantly fluid-containing cystic lesion (Fig. 66-102). Because of the importance of local control, imaging is also essential in determining resectability and in establishing the preoperative margins for surgical planning.

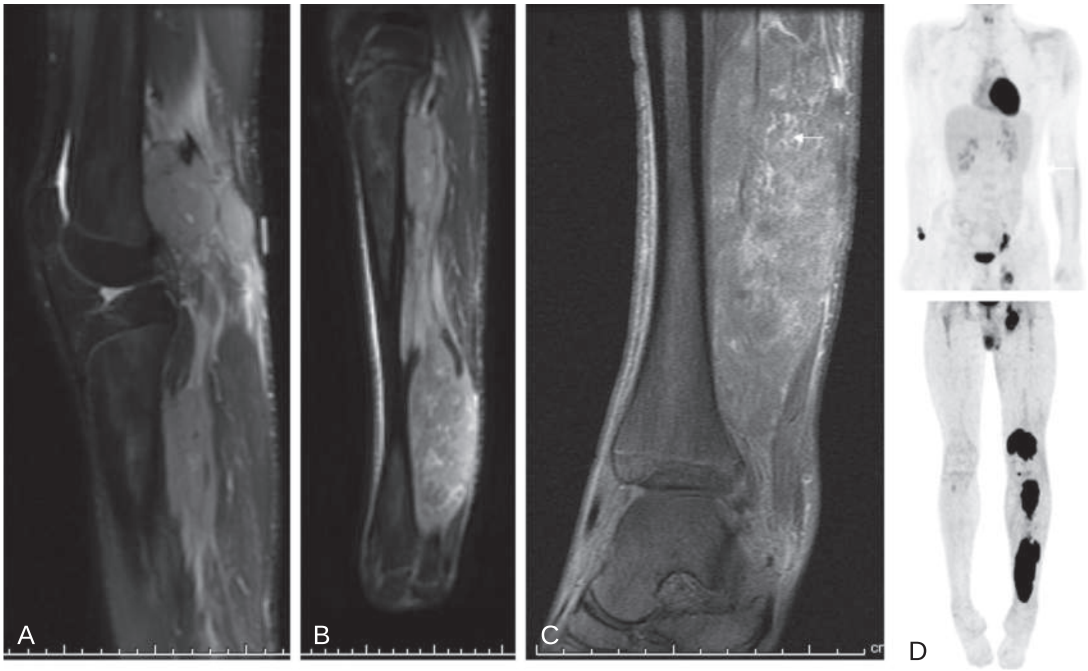

Figure 66-100 Rhabdomyosarcoma in a 16-year-old patient with a left leg mass. **A** and **B**, MRI findings show a T2 bright, enhancing (**C**) lesion localized in the deep soft tissues of the calf with extension proximally along fascial planes into the popliteal fossa. FDG-PET shows multiple sites of disease, including left inguinal lymph-node involvement that would not have been detected on the MRI. Biopsy showed alveolar rhabdomyosarcoma.

Tumor size has also been shown to be a key prognostic factor in synovial sarcoma, with lesions less than 5 cm resulting in a metastasis-free survival rate greater than 75%, in contrast to lesions greater than 5 cm in size. In patients with these lesions, the metastasis-free survival rate falls to less than 50%.[350] Synovial-cell sarcomas are typically FDG-avid tumors, and FDG-PET imaging should be considered at the time of staging to assess for locoregional lymph node spread and distant sites of metastatic disease that would adversely affect prognosis (see Fig. 66-102).

Malignant Peripheral Nerve-Sheath Tumor and Neurofibromatosis Type 1

Individuals with NF1 are at risk of developing benign and malignant tumors; the leading cause of mortality among these individuals is the development of malignant peripheral nerve-sheath tumors (MPNSTs). MPNSTs result from malignant degeneration of benign NF1 lesions, including plexiform neurofibromas (PNs).[351] In patients with extensive plexiform lesions, the diagnosis of malignant degeneration is challenging and classically relies on changes in lesion size and/or localized or unremitting pain, although these features are neither sensitive nor specific. Other than increase in size, no discriminating MRI features have been described to identify PNs potentially undergoing malignant degeneration into MPNSTs. A number of studies using FDG-PET in NF1 have demonstrated a significant difference in mean standardized uptake values (SUV) between benign and malignant lesions.[352-354] In a retrospective study of 19 children with NF1 who had cross-sectional imaging and correlative histopathologic data, Tsai et al. provided further data supporting the hypothesis that benign and malignant lesions are distinguishable by FDG-PET imaging in children and that there is a correlation between SUV_{max} and tumor grade.[355] It is the authors' current practice to perform FDG-PET imaging on all patients who have NF1 with new clinical symptoms, change in lesion size, or identification of new lesions by CT or MRI. Lesions with an SUV_{max} greater than 4 are considered worrisome, warranting either more intensive monitoring or surgical resection (Fig. 66-103).

Infantile (Desmoid-Type) Fibromatosis

Infantile fibromatosis is a benign fibrous proliferation, although it can be locally aggressive and deforming.[356] Infantile desmoid-type fibromatosis must be distinguished from the congenital generalized fibromatosis, also known as *infantile myofibromatosis*.[357] This latter disorder is characterized by lesions consisting of both smooth muscle and fibroblastic spindle cells in contrast to the more differentiated fibroblastic cells seen in desmoid fibromatosis. The presence of deep soft-tissue involvement as well as visceral involvement of organs such as lung and liver has a worse prognosis than localized disease. The extent of involvement is optimally determined by MRI (Fig. 66-104), with lesions typically characterized by low signal on both T1- and T2-weighted sequences because of the relatively high collagen content in these tumors.[357]

It may be difficult based on imaging findings alone to distinguish between aggressive multifocal fibromatosis and congenital fibrosarcoma. The desmoid-type lesions, in contrast, tend to be localized and have a less aggressive appearance.

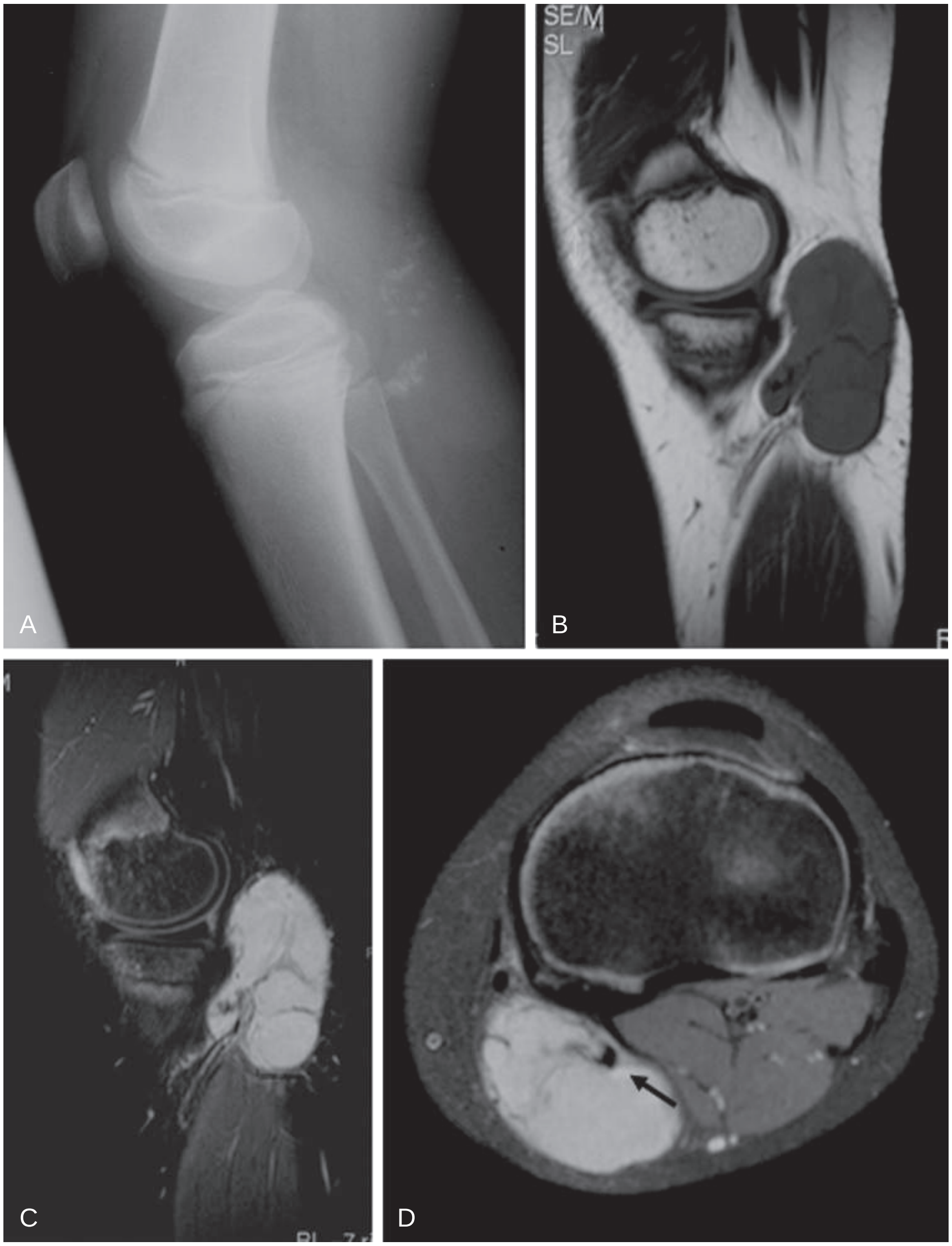

Figure 66-101 Synovial sarcoma. **A**, Radiograph of an 11-year-old child with a palpable leg mass showed a calcified mass behind the knee. MRI shows lobular mass in the medial compartment posterior to the knee, encasing the semi membranous tendon *(arrow)*, characterized by low signal on T1-weighted images (**B**), high signal on T2-weighted images (**C**) and intense contrast enhancement (**D**). These features are all characteristic findings in synovial sarcoma.

Other mesenchymal tumors, such as alveolar soft-part sarcomas, epithelioid sarcomas, and malignancy fibrous histiosarcomas are very rare in children and do not have distinctive imaging characteristics. As with the other mesenchymal tumors, lesions are typically isointense to slightly hyperintense on both T1- and T2-weighted images, with variable patterns of enhancement.

The distinction between liposarcoma and the benign lipoblastoma cannot be made on the basis of imaging alone, with both lesions showing high signal-intensity on T1-weighted images, decreased signal intensity on T2-weighted images, and minimal enhancement. As with the other malignant sarcomas, metastatic disease to the chest must be excluded, and the staging evaluation should include chest CT.

LYMPHOPROLIFERATIVE NEOPLASMS

Leukemias

Of the pediatric cancers approximately one third are leukemias, and together with brain tumors, leukemia accounts for over half of the childhood tumors.

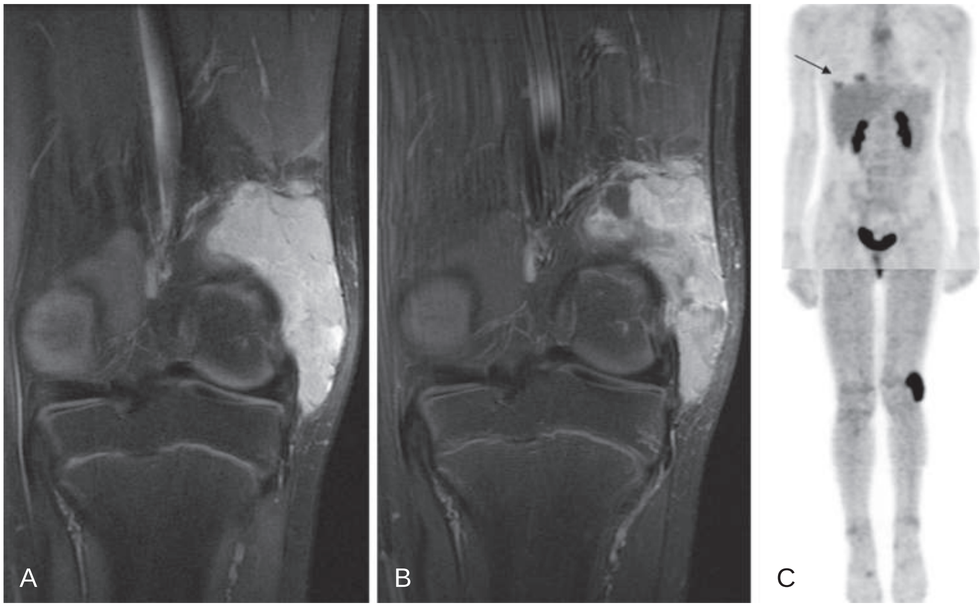

Figure 66-102 Synovial-cell sarcoma. Coronal fat-suppressed PD (**A**) and post-Gd T1-weighted MRI (**B**) shows a lobulated high signal-intensity enhancing lesion. The location and bright signal on fluid-sensitive sequences could be mistaken for a benign process; the pattern of contrast enhancement confirms a worrisome lesion. FDG-PET (**C**) shows intense uptake at the primary site and at least two focal pulmonary metastatic lesions *(arrow)*. This pattern of involvement and spread is common for synovial-cell sarcoma.

More than 75% of pediatric leukemia is acute lymphoblastic leukemia (ALL),[358] and the majority of the patients have B-lymphoblastic ALL, with 15% to 20% having T-cell ALL and 1% to 2% with mature B-cell ALL. Acute myelogenous leukemia (AML) makes up 15% to 20% of pediatric leukemia; the remainder are leukemic subtypes that are uncommon in children, such as chronic myelogenous leukemia and juvenile myelomonocytic leukemia.

The most common presenting symptoms and physical examination findings are nonspecific and include fever, bleeding, bone pain, lymphadenopathy, and splenomegaly. In addition to the fatigue, pallor, and anorexia, which

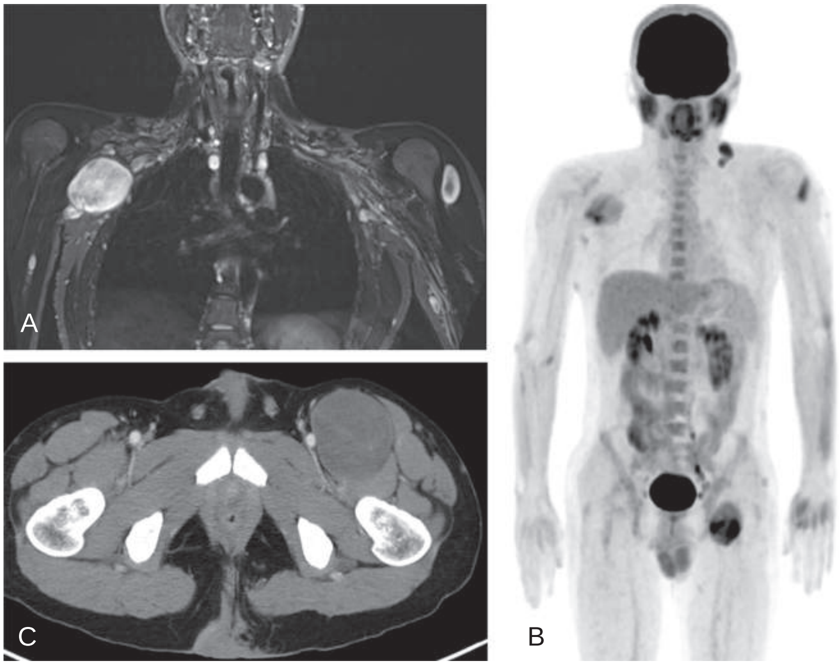

Figure 66-103 23-year-old patient with NF1 and multiple painful lesions. MRI shows T2-bright lesions in the right axilla and left deltoid (**A**), with corresponding FDG uptake (**B**). A CT scan of the pelvis shows low density left inguinal lesion (**C**) that has corresponding intense FDG uptake (**B**). SUV values and correlative histopathology of these lesions were: Left neck (not shown on MR) SUV: 4.97, atypical neurofibroma; right axilla SUV: 4.94, atypical neurofibroma; left deltoid SUV: 4.15:,atypical neurofibroma; left inguinal SUV: 9.33, low-grade malignant peripheral nerve sheath tumor.

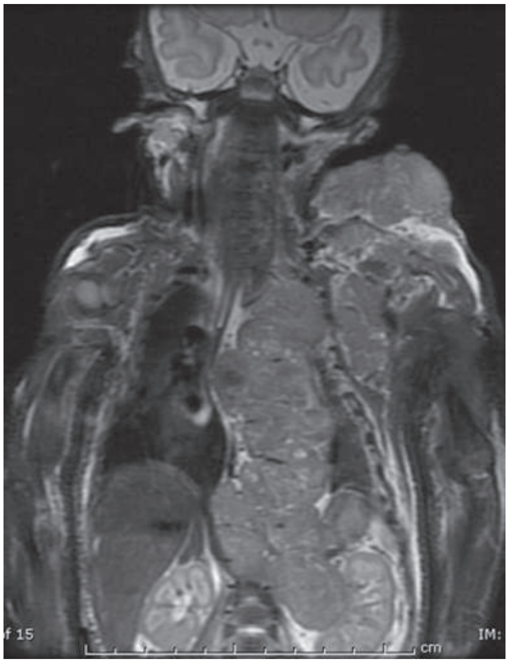

Figure 66-104 Infantile fibromatosis in a 4-week-old infant. The MRI shows extensive mediastinal, retroperitoneal, and intraabdominal disease. Biopsy showed infantile fibromatosis.

are seen in almost all patients with leukemia, hepatosplenomegaly and fever are seen in approximately two thirds of cases.[127]

Because of the nonspecific nature of presenting symptoms and physical examination findings, radiologic imaging may be useful in directing the next step in the evaluation. Common imaging findings include pulmonary edema secondary to high output cardiac failure in the setting of peripheral leukemic blast crisis (related to anemia); organomegaly including enlargement of the spleen, liver and heart; and pulmonary airspace opacities (related to leukostasis or infection).[359] Skeletal changes can range from subtle metaphyseal lucent bands to frank bone destruction resulting from leukemic infiltration. CNS hemorrhage may be encountered as a result of leukostasis and thrombocytopenia in patients with high peripheral leukemic-cell counts.[360] Although such imaging finding alone do not make the diagnosis of leukemia, the initial imaging evaluation can be critical in assessing the overall physiologic burden of the patient's disease.

Imaging Findings

Peripheral skeletal lesions may be detected on conventional radiographs in approximately 25% of patients newly diagnosed with ALL.[127] In the patient with fatigue and poorly localized bone pain, metaphyseal lucent bands (leukemic lines) may be seen (Fig. 66-105). This finding was initially thought to represent leukemic infiltration in the submetaphyseal bone, although the discovery of elevated parathyroid hormone levels in patients with leukemia suggests paraneoplastic effects on bone resorption likely contribute to the observed radiographic changes.[361] Metaphyseal lucent lines are not unique to leukemia and may also be seen in other marrow-infiltrative malignancies such as neuroblastoma (see Fig. 66-3) and lymphoma and in response to physiologic stress such as infection, systemic inflammatory reactions, and radiation.

Metaphyseal lucent lines may be difficult to distinguish from the diffuse metaphyseal lucency, which represents a combination of direct leukemic infiltration of the bone and accompanying bone resorption. This pattern of infiltration typically results in a broad irregularity and more poorly demarcated pattern of metaphyseal lucency. It is also associated with pain and localized tenderness, and depending on the degree of bone destruction it may also be accompanied by focal lytic lesions. Leukemic infiltration of the bone can also yield a moth-eaten appearance with a marked pattern of periosteal reaction.

More advanced imaging techniques such as MRI may be helpful in demonstrating the extent of leukemic infiltration by showing both focal and diffuse replacement of

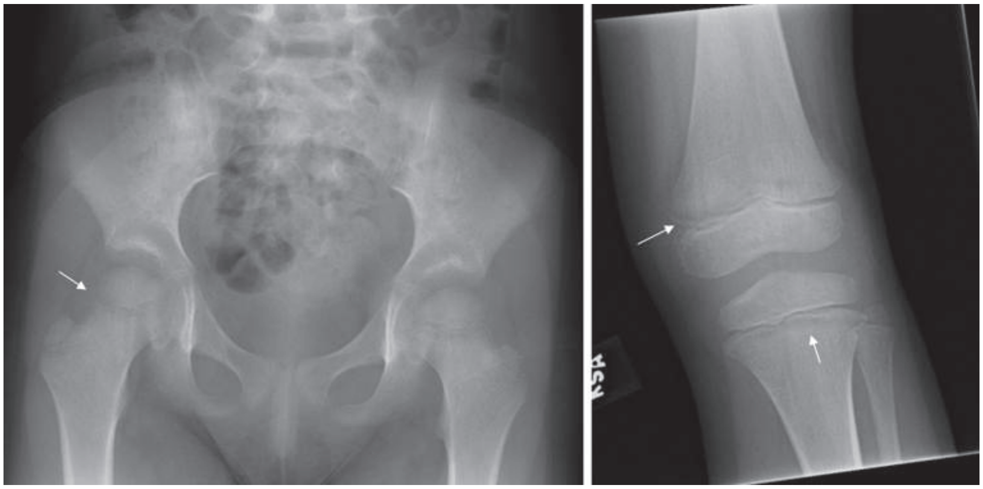

Figure 66-105 Leukemic lines. A 4½-year-old had child with limp and leg pain. Radiographs of the hips, pelvis, and knees show submetaphyseal lucency *(arrow)* and loss of the normal trabecular architecture, particularly in the femoral necks. Bone marrow aspiration confirmed leukemia.

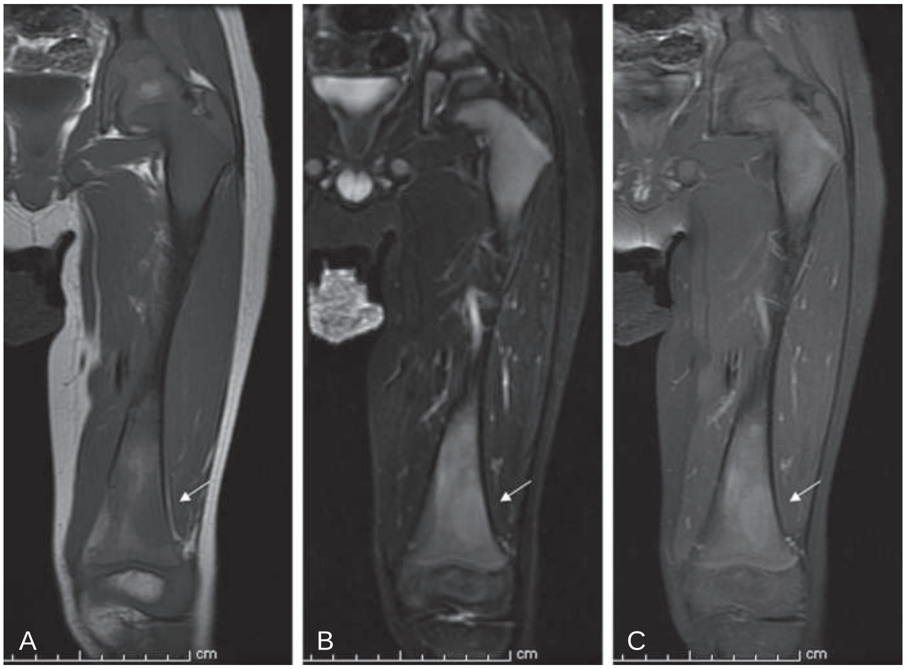

Figure 66-106 MRI of marrow infiltration in leukemia. A 3-year-old child experienced persistent limp and lower extremity pain. MRI showed patchy areas *(arrows)* of marrow replacement (A), heterogeneous increased signal (**B**), and enhancement (**C**) suggestive of diffuse marrow infiltration. Bone marrow aspiration confirmed B-cell ALL.

normal fatty marrow signal (Fig. 66-106).[127,362] However, heterogeneous patterns of marrow signal are a normal finding in children as hematopoietic marrow gradually converts to fatty marrow. Furthermore, therapeutic agents that stimulate bone-marrow recovery after ablative chemotherapy (e.g., granulocyte colony stimulating factor [G-CSF]) can mimic leukemic marrow replacement, and the extent of leukemic infiltration may be difficult to determine on the basis of MRI.[363,364]

In the setting of T-cell ALL, children and adolescents commonly experience wheezing as a result of accompanying large mediastinal masses (Fig. 66-107). The diffuse thymic infiltration seen in T-cell ALL (and T-cell lymphoblastic lymphoma) results in a bland, homogeneous-appearing mediastinal mass (Fig. 66-108). Pericardial and pleural effusions are common, as is leukemic infiltration of the kidneys. Despite the bulky disease that is often encountered at the time of presentation, T-ALL and lymphoblastic lymphomas respond rapidly to chemotherapy (see Fig. 66-33), and within a short time, radiographic studies typically return to normal, although the significance of incomplete response of the mediastinal mass to therapy with respect to overall outcome is uncertain.[365]

Childhood Acute Myelogenous Leukemia

AML represents a heterogeneous group of leukemias encompassing both the spectrum and extent of myeloid differentiation.[366] When patients with AML have high peripheral blast counts (leukocyte counts greater than 100,000/mm^3), clinical leukostasis may result and is characterized by global CNS changes or respiratory distress caused by pulmonary vascular involvement. Imaging findings in the brain may suggest diffuse microvascular thrombi (Fig. 66-109) without focal neurologic signs. CNS changes secondary to leukostasis may be difficult to detect by CT and standard MRI sequences, but they are readily demonstrated on paramagnetic susceptibility-sensitive gradient echo sequences because of the paramagnetic effects of hemoglobin breakdown products (i.e., hemosiderin) accumulating at sites of microvascular thrombus formation.

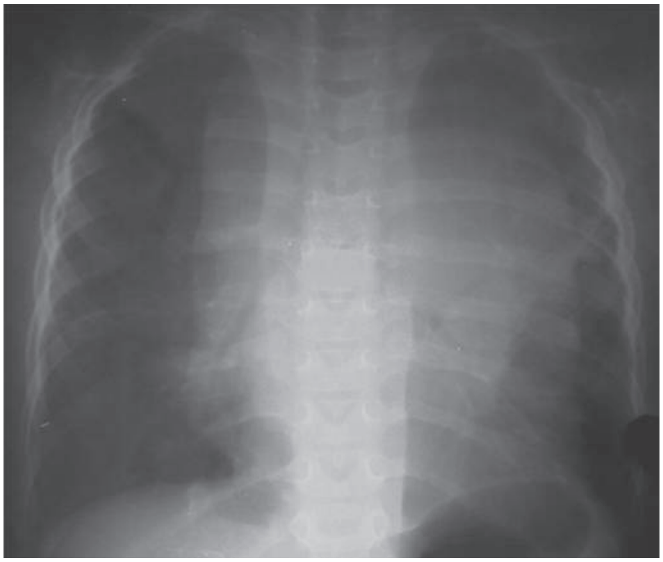

Figure 66-107 T-cell ALL in a 4-year-old child with first-time wheezing. Chest x-ray shows large mediastinal mass and narrowing of the proximal bronchi and air bronchograms, suggesting airway compression.

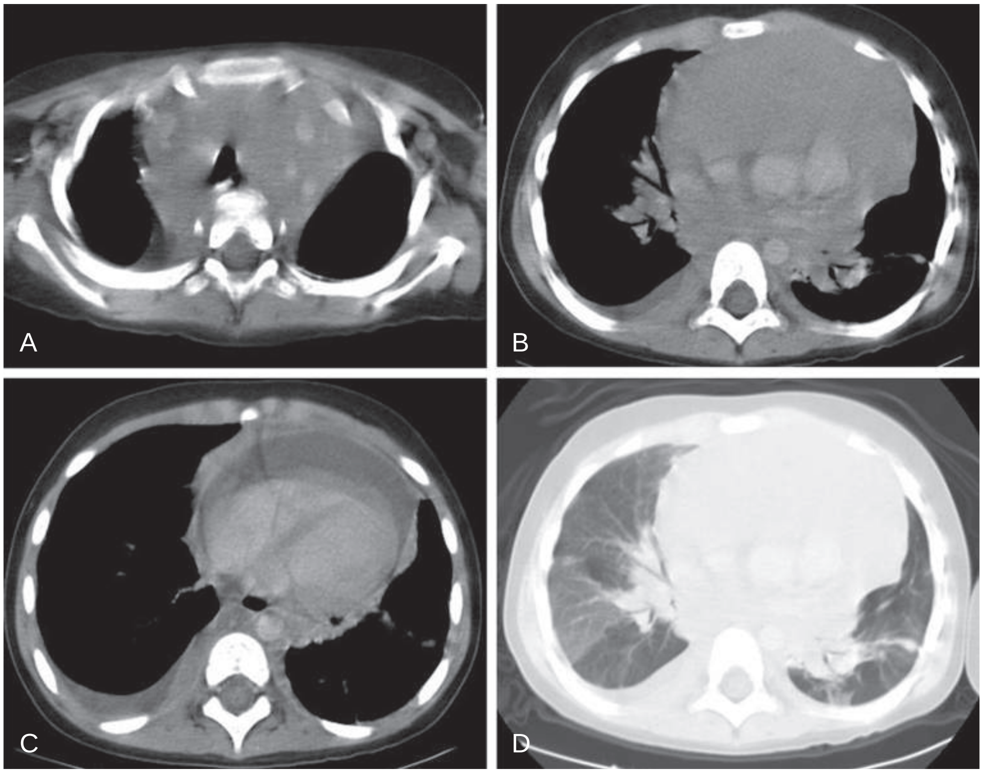

Figure 66-108 T-cell ALL. Shown are images of a 4-year-old child (see Fig. 66-107) with wheezing and mediastinal mass. Contrast-enhanced chest CT shows findings typical of T-cell lymphoblastic lymphoma, including mediastinal mass extending into the neck (**A**) with homogeneous density infiltrating the thymus (**A** and **B**). The mass causes marked airway compression and bronchial narrowing (**A** and **B**) and is associated with pericardial and pleural effusions (**C**) and mosaic lung attenuation (**D**) indicative of air-trapping and airway obstruction. Based on bone marrow involvement of greater than 50% marrow, the patient was diagnosed with T-cell lymphoblastic leukemia with a mediastinal mass.

Acute promyelocytic leukemia (APML) accounts for 5% to 10% of childhood AML and characteristically presents with a severe consumptive coagulopathy that is further exacerbated by cytotoxic chemotherapy because of cell lysis and release of procoagulant intracellular contents. The resulting coagulopathy can result in clinically significant bleeding, necessitating emergent imaging studies to assess for cerebral hemorrhage, which can be profound and rapidly progressive (see Fig. 66-109).

Extramedullary forms of AML, also called *chloromas* and *granulocytic sarcomas,* are seen in up to 10% of patients with AML and are also encountered in chronic myelocytic leukemia (CML) and myelodysplasia.[367] They are commonly asymptomatic; however, CNS involvement can result in mental status changes and lead to focal neurologic deficits. CNS chloromas are usually extraaxial and contiguous with the meninges. They may be virtually undetectable by CT, where they have similar to slightly increased attenuation relative to normal cortex. On MRI, these lesions are typically hypointense to isointense on T1-weighted imaging and heterogeneously isointense to hyperintense on T2-weighted imaging, with homogeneous contrast enhancement (Fig. 66-110).[367] Outside the CNS, chloromas may produce adjacent bone destruction and are often seen in the orbits, spine, paranasal sinuses, and the adjacent soft tissues,[367] as well as in the abdominal cavity (Fig. 66-111). Despite the uncommon presentation of this leukemic subset, extramedullary disease in any form—including that presenting without evidence of bone-marrow disease—is still considered evidence of systemic disease and is usually treated as such, with a combination of local control (surgery, radiation) and systemic chemotherapy.[368]

Lymphomas

Pediatric lymphomas are classified as either Hodgkin or non-Hodgkin lymphomas.[369,370] Because there is considerable overlap in the imaging features, age at presentation and sites of involvement are useful in helping to distinguishing the types of disease.[127]

Hodgkin Lymphoma

In Hodgkin lymphoma, the bulky mediastinal mass characteristically seen radiographically (Fig. 66-112) is made up primarily of inflammatory cells with the malignant Reed-Sternberg cell, from which the histologic diagnosis of Hodgkin lymphoma is made, comprising only 10% or less of the cells in the bulky lymph-node mass.[127] Hodgkin lymphoma is typified by involvement of contiguous lymph-node groups[371,372] and is staged according to the extent of lymph-node involvement in distinct nodal sites.

Staging. The most commonly used staging system is the Ann Arbor system (Table 66-5),[372,373] which involves determining the extent and pattern of lymph-node

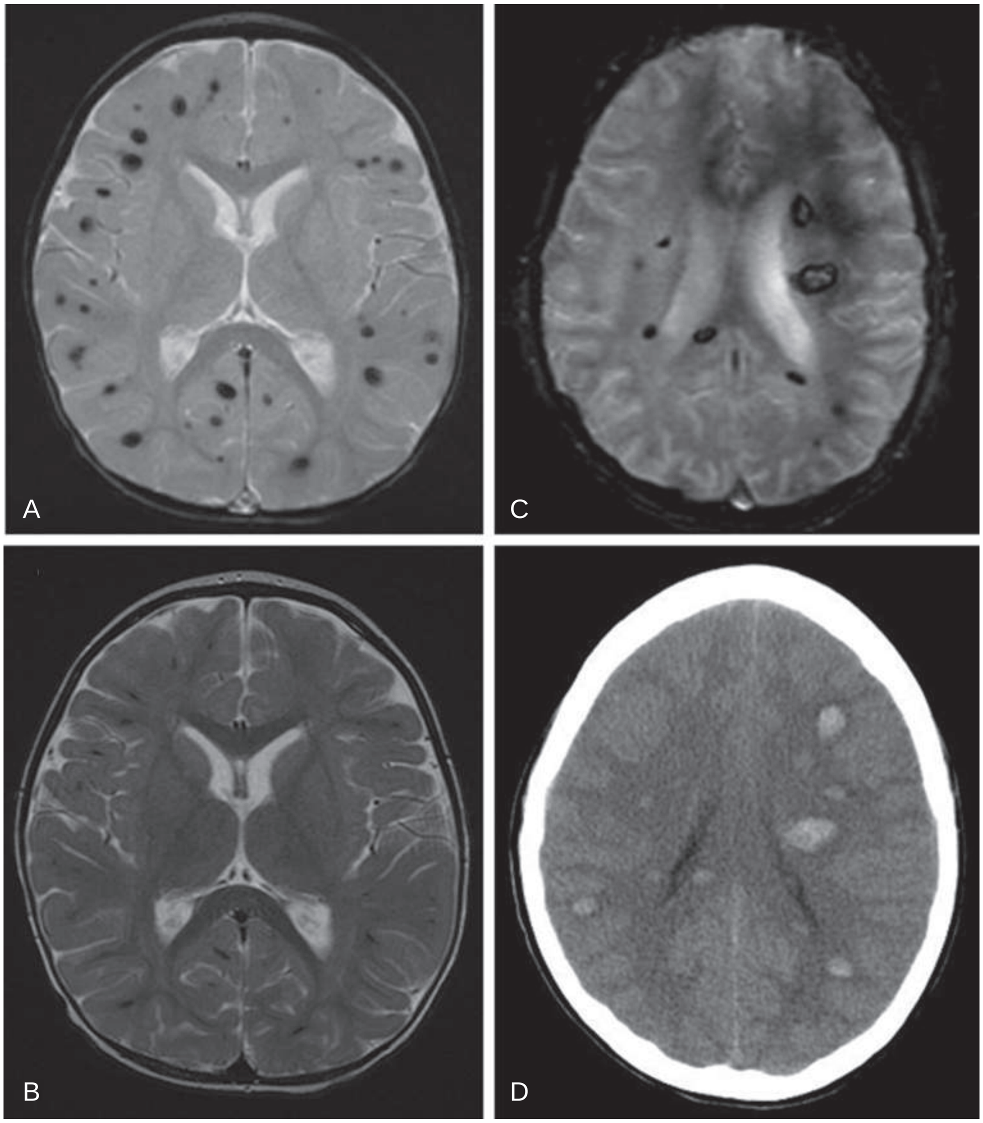

Figure 66-109 AML/APML. Gradient echo and FSE T2-weighted axial MRI images show the brain of a 17-month-old girl with AML who had a white blood cell count of greater than 300,000. Brain MRI obtained after the patient was stabilized showed multiple areas of paramagnetic susceptibility throughout the brain (**A**) with no corresponding T2 signal abnormality (**B**). Areas visualized were interpreted as foci of microthrombus formation secondary to leukostasis. A separate patient with APML had seizures, high white blood cell count, and a CT showing multiple foci of acute hemorrhage (**D**); the MRI showed multiple areas of paramagnetic susceptibility (**C**), likely caused by leukostasis with acute hemorrhage resulting from coagulopathy, reperfusion injury at sites of microthrombus formation, or both.

involvement. One unique feature of Hodgkin lymphoma relates to splenic involvement. For the purpose of disease staging, the spleen is consider localized lymphoid tissue, and lymphomatous infiltration of the spleen in Hodgkin lymphoma is not classified as stage 4 visceral metastatic disease, but rather as stage 3 disease.

The initial diagnosis is often prompted by chest x-ray evaluation for upper respiratory symptoms and vague constitutional symptoms such as fever and night sweats (see Fig. 66-112). At the time of diagnosis a mediastinal mass is encountered in more than two thirds of patients with Hodgkin lymphoma.[127,374] CT scanning is still the most commonly used cross-sectional modality for staging these patients. In addition to determining the extent of lymph-node involvement, pericardial and pleural effusions may be seen. Multiple sites of organ involvement may also be demonstrated, most commonly liver, spleen, and kidneys (Fig. 66-113). Children do not typically have symptoms referable to intraabdominal disease, so a thorough radiologic evaluation (intravenous and oral contrast-enhanced CT and FDG-PET) is essential to completely stage these patients. The staging evaluation for Hodgkin lymphoma should always include scanning of the neck to the level of the skull base in order to evaluate the nasopharyngeal and tonsillar lymphoid tissues (Waldeyer ring) for tumor involvement. Extensive involvement of the lymphoid tissue in Waldeyer ring may produce complete obliteration of the airway, requiring emergent intervention by a tracheostomy. As is true for all mediastinal lymphomas, the bulky anterior mediastinal mass

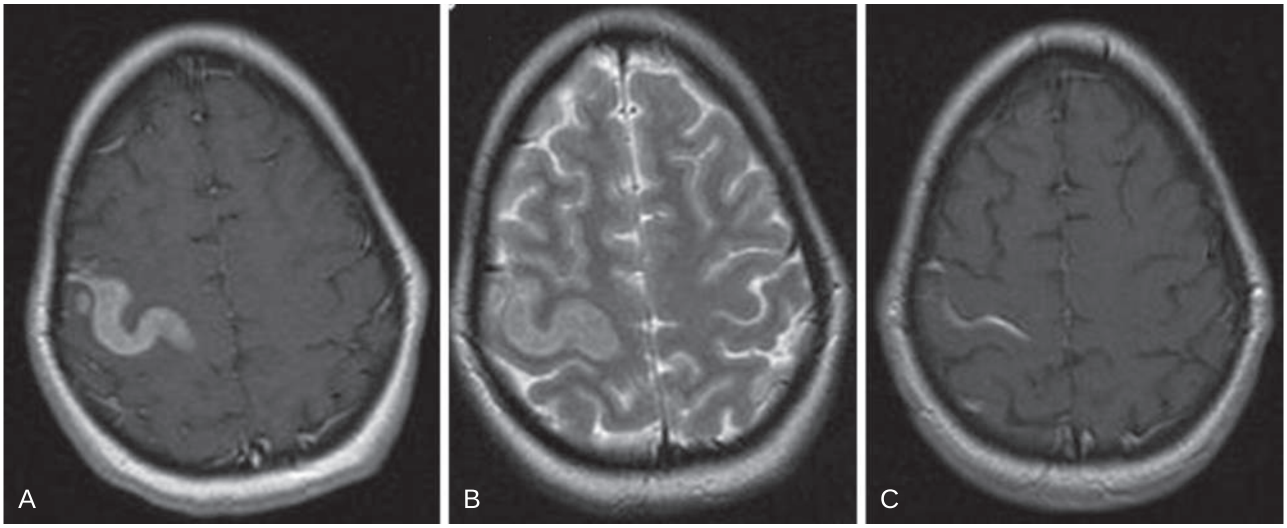

Figure 66-110 Chloroma in a 12-year-old patient with AML. Brain MRI shows typical findings of chloroma, including isointensity to cortex on T2-weighted images (**A**), homogeneous enhancement (**B**), and good response to radiation (**C**).

common in Hodgkin lymphoma may produce tracheal, bronchial, and central vascular compression (occasionally resulting in the superior vena cava [SVC] syndrome). Tracheal compression can result in acute airway compromise, particularly during procedures requiring general anesthesia and muscle relaxation. In addition, large anterior mediastinal masses can produce jugular vein thrombosis, venous sinus thrombosis, and CNS venous infarction (Fig. 66-114).

Pulmonary involvement in Hodgkin disease is unusual (seen in fewer than 5% of children under 10 years of age and fewer than 15% of adolescents),[374] but its identification often makes a difference in distinguishing stage 2 from stage 4 disease, resulting in dramatically different treatment regimens. Although pulmonary involvement can be quite extensive (Fig. 66-115), pulmonary nodules/masses that are associated with an ipsilateral hilar mass

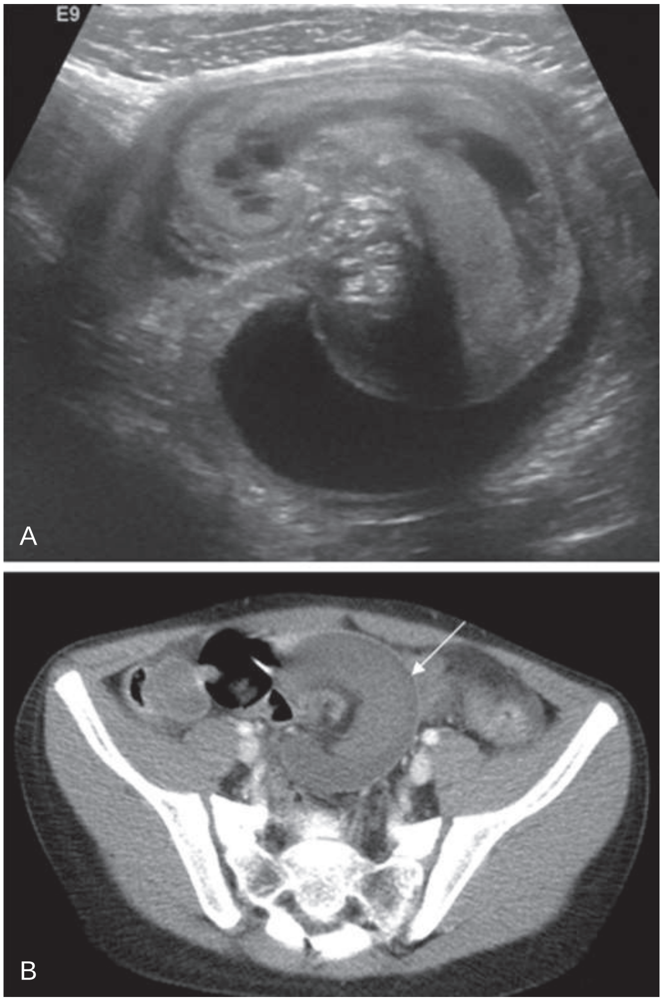

Figure 66-111 Extramedullary AML (chloroma). **A,** An 11-year-old boy with abdominal pain and vomiting had an ultrasound that revealed small-bowel intussusception. **B,** CT showed an abnormal mass involving the small bowel with apparent mural thickening *(arrow)*. At surgical resection this was found to be an isolated site of extramedullary AML (chloroma). The bone marrow biopsy was negative.

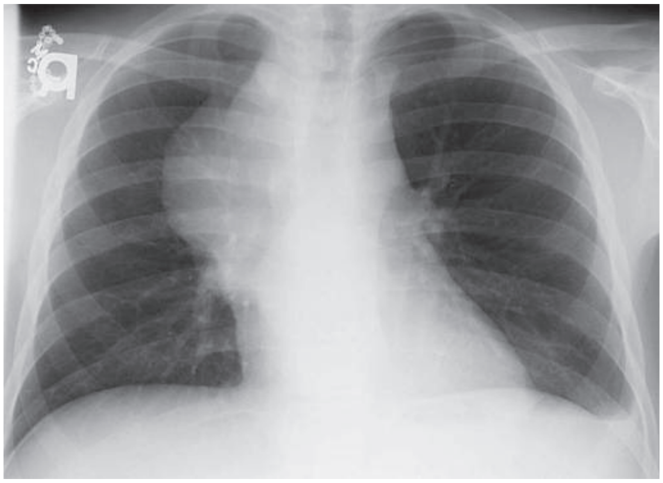

Figure 66-112 Hodgkin lymphoma. The chest x-ray of a patient with symptoms of a nonspecific upper respiratory infection shows an anterior mediastinal and paratracheal mass, as well as a small left effusion.

TABLE 66-5 Childhood Hodgkin Lymphoma: Ann Arbor Classification[372,373]

Tumor Stage	Description
1	Involvement of a single lymph node region, or Stage I(E): direct extension from that node to an adjacent extralymphatic region.
2	Involvement of two or more lymph node regions (number to be indicated) on the same side of the diaphragm, or Stage II(E): direct extension from any one of these lymph nodes to an adjacent extra-lymphatic organ.
3	Involvement of lymph node regions on both sides of the diaphragm. Stage III(E): may be accompanied by extension to an adjacent extralymphatic organ Stage III(S): may be accompanied by involvement of the spleen Stage III(E+S): involvement of both an adjacent extralymphatic organ and the spleen.
4	Noncontiguous involvement of one or more extralymphatic organs or tissues, with or without associated lymph-node involvement.

E, Extralymphatic spread of tumor into contiguous structures, to be distinguished from hematogenous spread; *S,* splenic involvement.

probably result in part from spread along peribronchial lymphatics,[371] although the distinction between local contiguous lymphatic spread and hematogenous dissemination can be challenging by CT.

As discussed in detail in the section on mediastinal masses, in addition to staging by CT, functional imaging with FDG PET is essential both for staging and monitoring response to therapy (see Figs. 66-31 and 66-32; Fig. 66-116). Historically, the time course to achieving gallium negativity was the initial functional imaging finding that correlated with event-free and overall survival.[375] However, with the ubiquitous use of FDG and modern day PET/CT scanners, gallium scintigraphy is rarely if ever performed for Hodgkin lymphoma.[135] FDG-PET imaging has been shown to impact staging and response assessment in a significant number of patients,* and its use in lymphoma has been incorporated into nearly all new clinical trials for Hodgkin lymphoma. As discussed earlier, there is growing evidence to suggest that early response to therapy, defined as rapid early resolution of previously FDG-avid sites of disease, even in the presence of residual disease by CT (see Fig. 66-32) or MRI, correlates with improved overall outcome.[144,149,378]

Using FDG-PET imaging to monitor changes in metabolic activity within the mediastinal mass provides an opportunity for making treatment decisions based on early response to therapy.[142,146] Based on studies in both adult and pediatric patients showing that patients with early metabolic responses to therapy have improved event-free and overall survival rates as compared with those who have residual PET abnormalities after the initial two cycles of therapy,[144,379] response-based treatment paradigms have been implemented clinically to directly test whether FDG-PET imaging is a reliable prognostic indicator for predicting chemosensitivity of the tumor and ultimately clinical outcome. Early results evaluating both change in size of the mediastinal mass and change in the metabolic activity have suggested that outcomes will likely be best predicted by a combination of factors, including resolution of FDG uptake and significant change in size of the patient's sites of disease involvement as measured by CT and/or MRI.[380,381] There have been no large multicenter prospective trials in either adults or children testing whether relying solely on FDG-PET to determine if early response to therapy can be used to reduce chemotherapy and radiation therapy in those patients achieving a rapid early response (reserving intensified treatment regimens for those patients not achieving an early response),[149,379] although one study reported a higher rate of early relapses in patients for whom involved-field radiation was omitted based on early resolution of FDG-avid disease.[382]

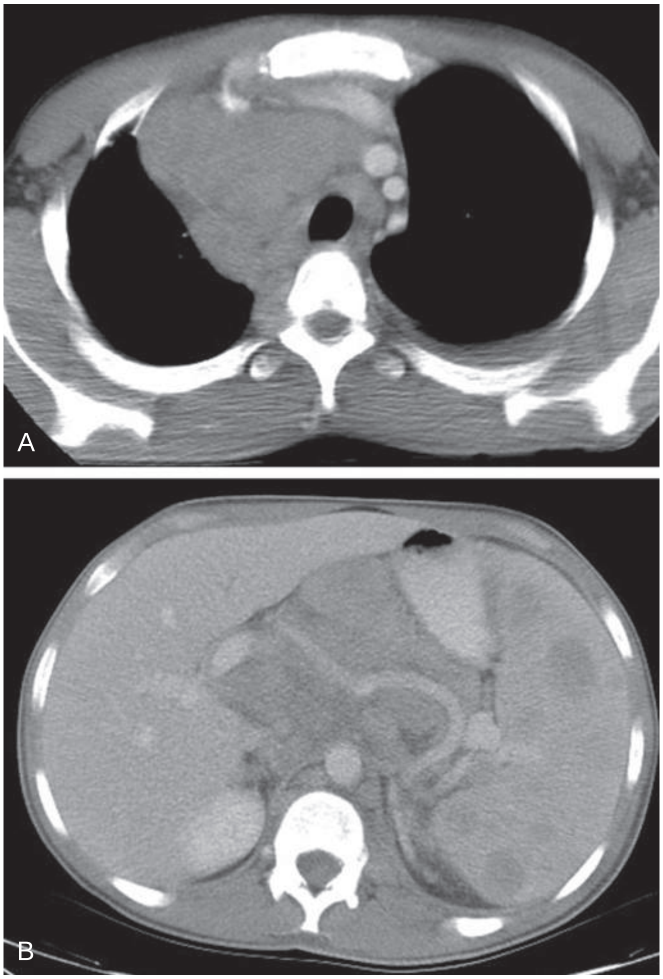

Figure 66-113 Hodgkin lymphoma. **A,** A contrast enhanced CT in patient whose chest x-ray is shown in Figure 66-112 shows the mediastinal mass and left pleural effusion. **B,** The images of the abdomen show extensive splenic, mesenteric, and retroperitoneal lymphadenopathy.

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*133, 134, 139, 142-146, 245, 376, 377

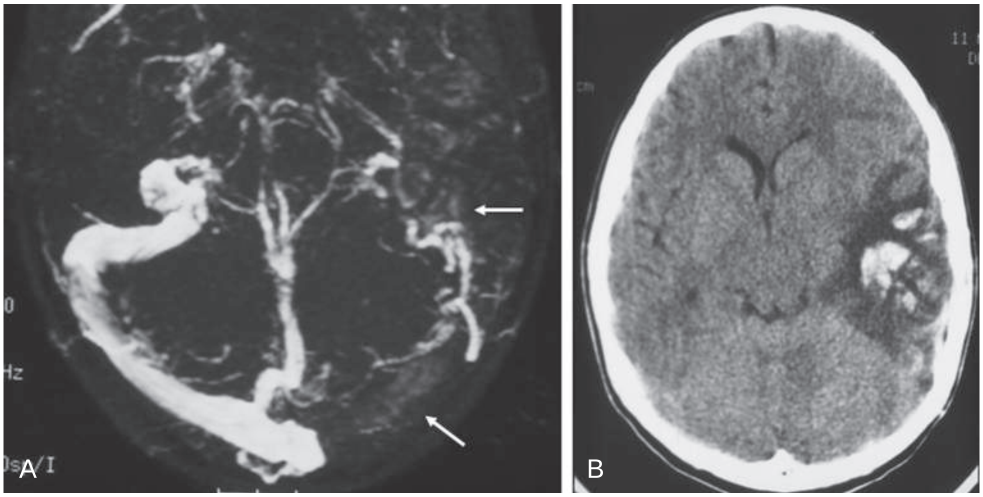

Figure 66-114 Hodgkin lymphoma. MR angiogram (**A**) and unenhanced head CT (**B**) in a patient with large mediastinal mass, brachiocephalic vein compression, and associated left jugular vein thrombosis show venous sinus thrombus involving the left transverse and sigmoid sinus *(arrows)* and associated hemorrhagic venous infarction. *(Images courtesy of Dr. Laureen Sena.)*

Given the importance of FDG-PET responses in dictating treatment decisions, an international team of experts reviewed the available data to arrive at the International Harmonization Project or International Working Group (IWG) consensus opinion regarding PET and CT criteria for determining disease and treatment response in lymphoma.[383,384] These are shown in Table 66-6, and are based on a revision of previous malignant lymphoma response criteria. Although the IWG criteria have not been validated in pediatric patients, the main challenge is developing objective standards for assessing FDG-PET response. A number of proposals have been suggested for distinguishing residual low-level neoplastic FDG activity from background uptake.[142] The use of SUV measurements is still considered experimental and has not been universally accepted. The Deauville Criteria—receiving the greatest acceptance—are based on a five-point scale, with uptake greater than mediastinal blood pool but less than liver, considered to be background, with uptake greater than the liver considered suspicious for residual

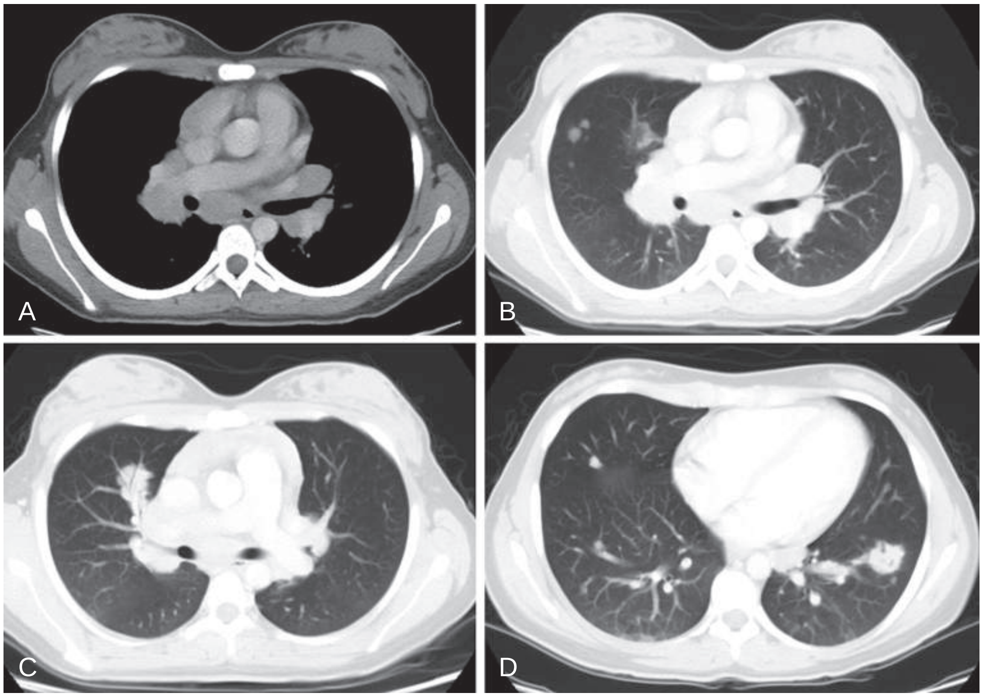

Figure 66-115 Hodgkin lymphoma and pulmonary involvement. This patient has extensive mediastinal and bilateral hilar nodal disease (**A**). The presence of discrete pulmonary nodules (**B** and **D**) may be the result of hematogenously spread disease, although contiguous spread along peribronchovascular lymphatics (**C** and **D**) is more common in Hodgkin lymphoma.

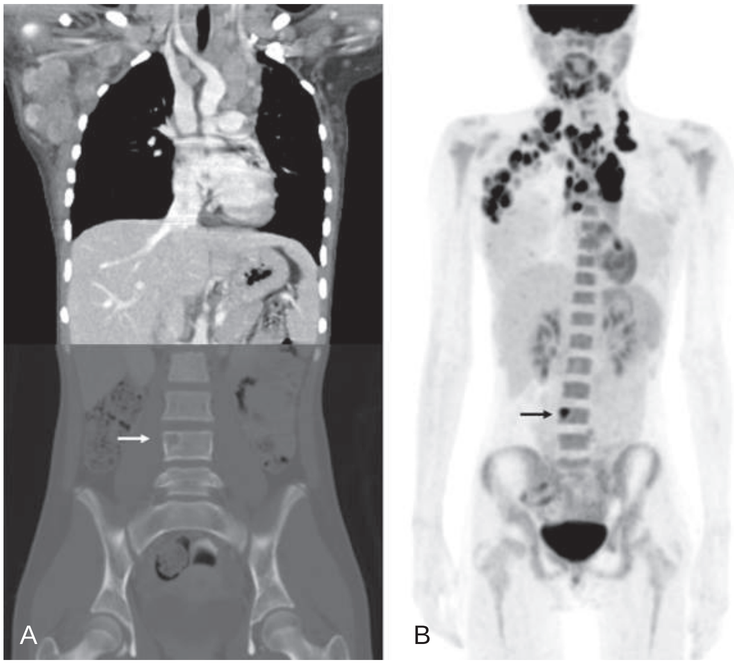

Figure 66-116 Hodgkin lymphoma and PET. Contrast-enhanced CT images of the chest, abdomen, and pelvis (**A**) show extensive mediastinal and axillary adenopathy, all of which is FDG avid. In addition, the PET scan (**B**) identifies a focus of FDG uptake at L4, corresponding to a subtle lytic lesion on CT, increasing the stage of this patient to stage 4. Note the background of low-level FDG uptake seen throughout the bone marrow, a common finding in Hodgkin lymphoma and reflective of a systemic inflammatory state associated with the disease rather an diffuse marrow infiltration.

metabolically active disease.[385] These criteria shown in Table 66-7 must be validated in larger treatment trials.

Non-Hodgkin Lymphomas

The non-Hodgkin lymphomas are a broad group of diseases.[127,370] Anaplastic large cell lymphoma often involves the soft tissues, skin, and bone, whereas Burkitt lymphoma most commonly presents with intraabdominal and mesenteric disease. Lymphoblastic lymphoma, particularly of the T-cell type, often presents with a mediastinal mass, although the appearance of the mass is characteristically distinct from the multilobulated bulky mass that is usually associated with Hodgkin disease. The distinction between T-cell lymphoblastic lymphoma and

TABLE 66-6 Summary of New International Working Group Criteria for PET and CT in Determining Response in Lymphoma[383]

Response	Criteria
CR*	FDG-PET completely negative. Residual lymph nodes/nodal masses allowed if FDG-negative. Bone marrow biopsy negative. Splenic/liver involvement must disappear. No new sites of disease.
PR	FDG positivity should be present in at least one previously involved site. Regression of measurable disease; no new sites of disease. ≥50% decrease in SPD of 6 dominant lymph nodes/nodal masses. ≥50% reduction in splenic/hepatic nodules, if present. Even if CR by other criteria, positive bone-marrow biopsy is considered PR.
SD	Failure to achieve PR, but not meeting PD criteria.
PD/Relapse	Any lesion increased in size by ≥50% from nadir. Any new lesion. PET should be positive in new/progressed lesions if ≥1.5 cm.

CR, Complete remission; *CT,* computed tomography; *FDG-PET,* 18-fluorodeoxyglucose positron emission tomography; *PD,* progressive disease; *PET,* positron emission tomography; *PR,* partial response; *SD,* stable disease; *SPD,* sum of the perpendicular diameters.

*New criteria include PET in definition of CR. PET considered positive if uptake is greater than mediastinal blood pool (lesions >2 cm) or above local background (lesions <2 cm).

TABLE 66-7 Five-Point Deauville Criteria for Determining FDG Response in Hodgkin Lymphoma[385]

Score	Semiquantitative Assessment of FDG Uptake
1	No uptake
2	Uptake less than mediastinal blood pool
3	Uptake greater than or equal to mediastinal blood pool but less than liver
4	Uptake greater than or equal to liver at any site
5	Markedly increased uptake at any site, including new sites of disease

FDG, Fluorodeoxyglucose.

TABLE 66-8 Childhood Non-Hodgkin Lymphoma: St. Jude (Murphy) Classification[386]

Tumor Stage	Description
1	A single tumor (extranodal) or nodal area is involved, excluding the abdomen and mediastinum.
2	Disease is limited to: A single tumor (extranodal) with regional node involvement, or Two or more tumors or nodal areas involved on one side of the diaphragm, or A primary gastrointestinal tract tumor (completely resected), with or without regional node involvement.
3	Tumors or involved lymph node areas occur on both sides of the diaphragm. Includes: Any primary intrathoracic (mediastinal, pleural, or thymic) disease Extensive primary intraabdominal disease Any paraspinal or epidural tumors
4	Tumors involve bone marrow (<25%) and/or central nervous system disease regardless of other sites of involvement.

its histologically-identical leukemic counterpart is made of the basis of bone-marrow aspirates; more than 25% marrow involvement results in a diagnosis of T-cell lymphoblastic leukemia.

Staging of non-Hodgkin lymphoma in most centers is according to the St. Jude Classification (Table 66-8).[386] Although there is considerable overlap in the imaging features of the lymphomas as shown in Figure 66-117, a localized anterior mediastinal mass that is not associated with other sites of significant contiguous lymph-node enlargement is more likely to be non-Hodgkin lymphoma. Non-Hodgkin lymphoma is also more likely to be disseminated at the time of diagnosis. Chest-wall and pericardial invasion is more common in the setting of non-Hodgkin lymphoma than in Hodgkin lymphoma (see Fig. 66-117).[370] The mediastinal mass in acute T-cell lymphoblastic lymphoma usually responds rapidly to chemotherapy, whereas other non-Hodgkin mediastinal lymphomas respond more slowly, with residual mediastinal abnormalities commonly seen even after multiple cycles of chemotherapy.

Non-Hodgkin lymphomas of the bone can present either primarily or in the setting of wide-spread metastatic disease. They often present with pathologic fracture and bone destruction[127] and may be difficult to distinguish from other processes.[387] MRI can be helpful in delineating the extent of marrow and adjacent soft-tissue involvement. Staging of non-Hodgkin lymphoma includes bone scintigraphy, which can be useful in distinguishing between localized osseous involvement and more widespread disease,[90] although it remains to be determined whether FDG-PET can replace bone scintigraphy in identifying sites of bone involvement in NHL.

Ileocecal involvement is common in non-Hodgkin lymphoma (Fig. 66-118). In the abdomen, intussusception in an older child should raise concern for a pathologic lead point caused by lymphoma. This is commonly seen in the setting of Burkitt lymphoma (see Figs. 66-67 and 66-118) but may also be seen in other forms of non-Hodgkin lymphoma.[127,370] The sporadic form of Burkitt lymphoma, which is more often encountered in Western and developed countries, is not etiologically linked to Epstein-Barr

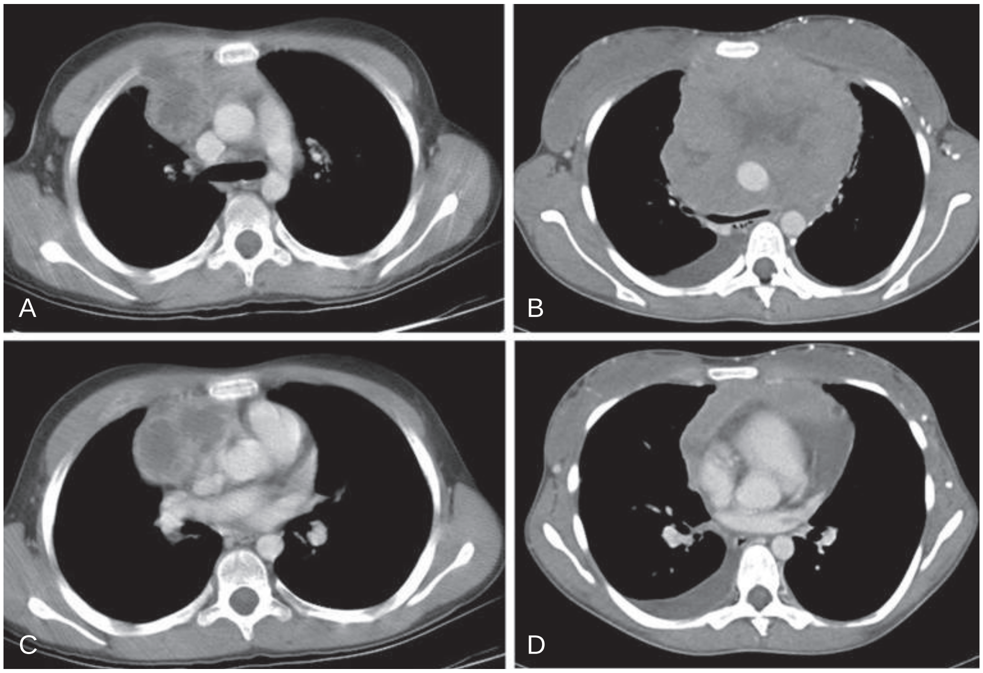

Figure 66-117 Non-Hodgkin lymphoma. Diffuse large B-cell (**A** and **B**) and B-cell–rich (**C** and **D**) non-Hodgkin lymphomas show chest wall and pericardial invasion that is more commonly seen in mediastinal non-Hodgkin lymphoma.

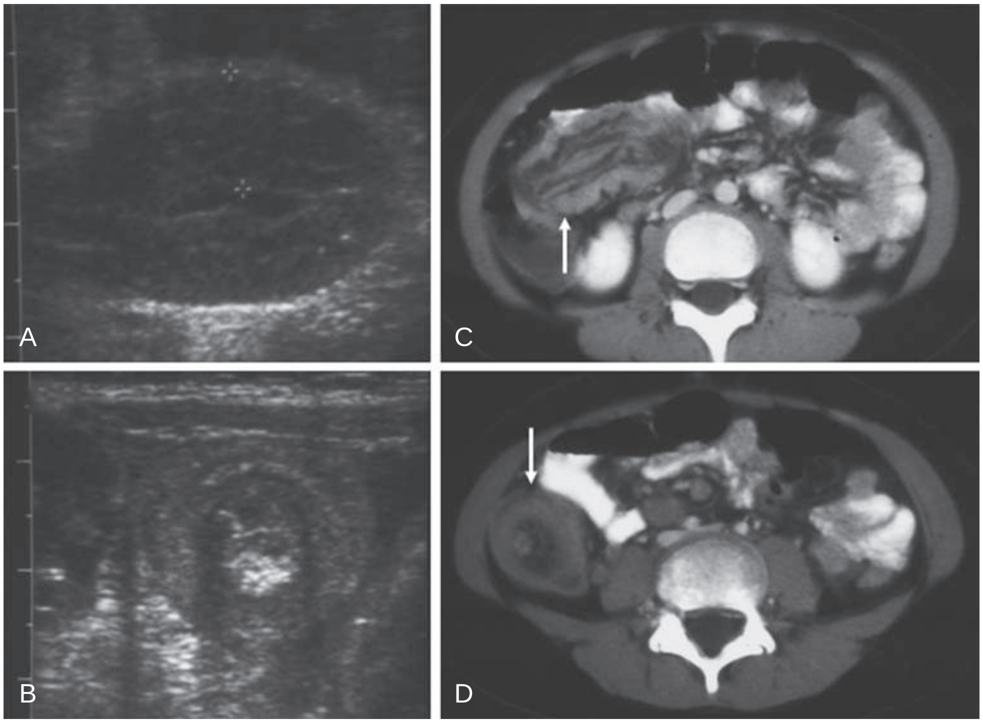

Figure 66-118 Non-Hodgkin lymphoma and intussusception. Ultrasound in a patient with Burkitt lymphoma (**A** and **B**) and CT in a patient with large-cell lymphoma (**C** and **D**) show typical features of intussusception, either small bowel/small bowel (**A** and **B**) or ileocolic (**C** and **D**).

virus (EBV) infection and has a pattern of bulky mesenteric and pelvic adenopathy, as well as involvement of multiple visceral organs including the liver, kidneys, and pancreas (see Fig. 66-64). Response of Burkitt lymphoma to chemotherapy is often quite rapid, with bulky disease responding within weeks. In contrast, the endemic form of Burkitt lymphoma, which is encountered in equatorial Africa, is usually associated with EBV infection (95%) and characteristically presents with jaw, orbit, paraspinal, and CNS involvement.[374]

Posttransplant lymphoproliferative disease (PTLD) is seen in the setting of both solid-organ and bone-marrow transplantation and results directly from the immunosuppression regimens used.[388] The lymphoproliferative disease, which is usually EBV-related, often responds to reduction in immunosuppression (Fig. 66-119). However, the lymphoproliferative disease may progress to an aggressive B-cell lymphoma, resulting in widespread malignant disease. PTLD can involve multiple organs and systems and responds variably to conventional lymphoma therapies. FDG-PET imaging is not routinely used in the management of PTLD, although some have advocated its use to improve early disease detection and staging.[389]

Just as with Hodgkin lymphoma, the role of FDG-PET imaging in non-Hodgkin lymphoma is currently being validated. Several studies of small cohorts of patients have indicated that FDG-PET is valuable both for staging and for predicting response to therapy in patients with

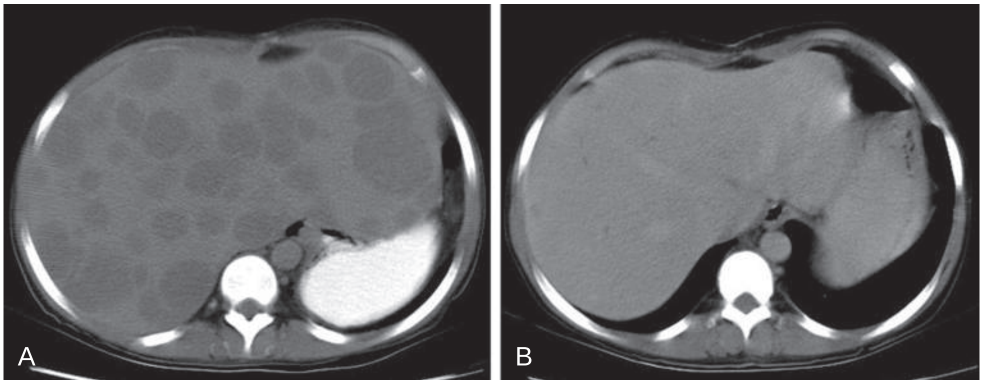

Figure 66-119 A 22-year-old patient with a renal transplant developed extensive PTLD involving the liver (**A**). **B**, Four months after reducing the immunosuppressive therapy, the PTLD had nearly completely resolved.

various forms of NHL.* As suggested by these studies, FDG-PET, particularly when combined with cross-sectional imaging either by CT or MRI, is extremely sensitive at detecting sites of disease that may not have otherwise been identified. It seems obvious that identifying the extent of disease should impact both staging and monitoring of treatment response; however; prospective trials are needed to determine whether FDG-PET response alone can by used to direct response-based modulation in therapy.

Sites of Relapse in Hematologic Malignancies

In the treatment of leukemia and lymphoma, radiologic evaluation plays an important role in monitoring for disease relapse. In addition to intramedullary (i.e., bone-marrow) relapse, common sites of extramedullary leukemic relapse include kidneys, spleen, CNS, and bone. Although less common, relapse to “sanctuary sites” such as the testicle can occur, both with diffuse organ infiltration and enlargement and with focal mass deposits (Fig. 66-120). Relapse to these sites may occur even in the setting of bone-marrow remission.

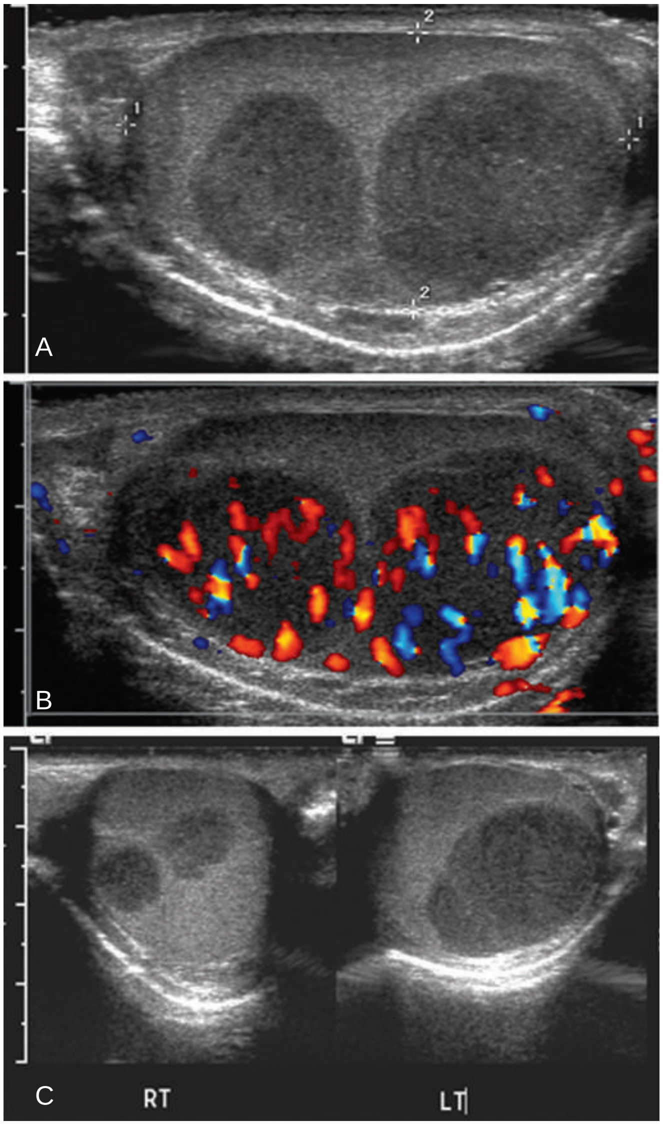

Figure 66-120 Scrotal relapse of leukemia. A 17-year-old patient with high risk ALL, s/p bone marrow transplant had painless scrotal swelling and palpable abnormalities on exam. Scrotal ultrasound shows multiple bilateral hypoechoic masses (**A** and **C**) (relative to testicular parenchyma) with increased blood flow to the masses (**B**), diagnostic of scrotal relapse.

IMAGING TREATMENT COMPLICATIONS

Complications resulting from the treatment of pediatric malignancies are myriad. It is important to note that the radiographic manifestations of fungal and other infectious diseases may not be manifest during periods of profound neutropenia (Fig. 66-121). Often, abnormalities on imaging studies emerge only after peripheral neutrophil counts recover and infection-fighting white blood cells become available to mount an inflammatory response. Infectious complications commonly include localized as well as disseminated fungal infection. The extent of hepatic and splenic involvement may be better detected by MRI (Fig. 66-122).[394]

Cardiomyopathy results from cardiotoxic chemotherapy agents such as doxorubicin. Echocardiography has become the imaging modality of choice in monitoring patients with this complication. Pulmonary toxicity can range from subtle ground-glass opacity, which can be an early manifestation of bronchiolitis obliterans with organizing pneumonia (BOOP, or cryptogenic pneumonia) to frank interstitial pulmonary fibrosis.[395]

Children with cancer also have poorly understood hypercoagulable state. Pulmonary emboli and other sites of venous thrombosis occur at an increased incidence in these patients and may be detected either incidentally (Fig. 66-123) or in response to specific clinical symptoms.[396] Clinically the probability of thromboembolic disease may be difficult to assess. The presence of multiple known risks factors, including indwelling catheters, concurrent infection, immobility, dehydration, and chemotherapy all increase the risk of thromboembolic events in these patients,[397] and the clinician and radiologist must be vigilant in their respective examinations to enable early detection and treatment of these potentially live-threatening complications.

Gastrointestinal complications occur during periods of neutropenia and can range from typhlitis (with localized inflammation of the cecum) to extensive pancolitis and bowel necrosis (Fig. 66-124).[398,399] Asparaginase, which is included in many chemotherapy regimens for ALL and lymphoblastic lymphoma, results in pancreatitis in approximately 5% to 10% of patients. The severity of pancreatitis can range from a mild chemical pancreatitis to frank pancreatic necrosis and subsequent pseudocyst formation (Fig. 66-125). This is best evaluated initially by ultrasound, with CT reserved for persistent or worsening clinical pancreatitis or to assess for complications such as pseudocyst.[400]

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*References 134, 136, 150, 246, 247, 390-393.

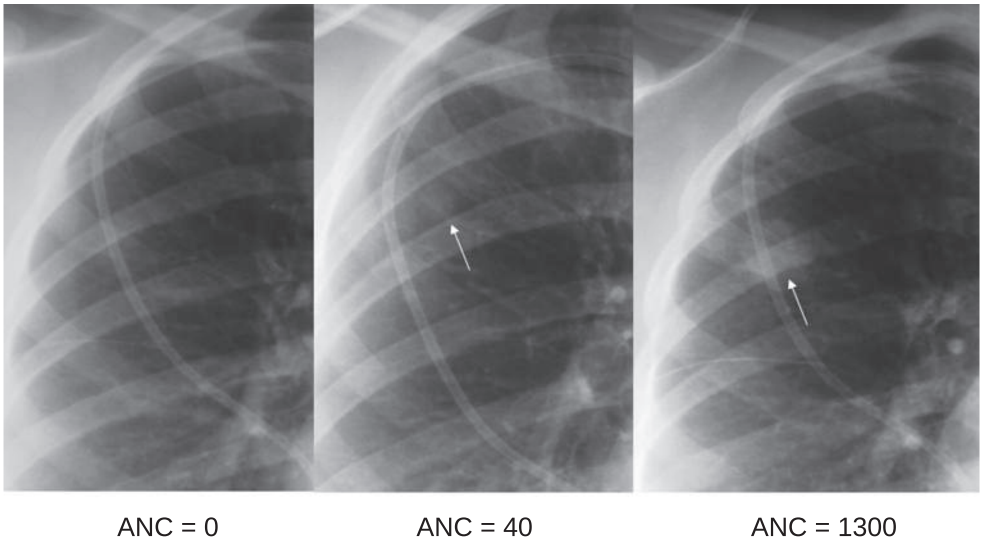

Figure 66-121 Imaging the patient with neutropenia. X-rays are of a 12-year-old patient with AML. Note the appearance of focal lung nodule *(Aspergillus)* as the patient's blood cell counts recover, suggesting that the appearance of infectious lesions in patients with neutropenia may be challenging to detect until the marrow has recovered after ablative chemotherapy.

Bony complications can arise after chronic treatment with corticosteroids and include osteopenia, fractures, bone-marrow infarcts, and osteonecrosis (Fig. 66-126), the latter of which is most typically seen in the hips and knees.[401] Second tumors, particularly musculoskeletal tumors, may be seen after radiation therapy. Osteochondromas involving the clavicles and sternoclavicular junction, for example, are common in patients with lymphoma who have received mantle radiation, particularly if radiation occurred at an early age. Malignant degeneration of these lesions is rare.

CNS changes can develop after radiation and chemotherapy. In one study, hemorrhagic events were seen in 4 of 120 children with ALL. All of these children had been disease-free for more than 2 years and had been treated with prior chemotherapy and cranial radiation.[402] These events are most commonly related to treatment-induced vasculopathy and may show diffuse or localized vasculopathy. Asparaginase may also produce venous sinus thrombosis, leading to profound neurologic deficits and areas of venous infarction (Fig. 66-127). Methotrexate, which is given both systemically as well as intrathecally for CNS prophylaxis in patients with high-risk ALL, may produce a leukoencephalopathy characterized by symmetric periventricular white-matter signal abnormalities. These changes may be transient or permanent. They are not specific to methotrexate and can be seen with other chemotherapeutic agents that penetrate the CNS.

Patients treated for cancer in childhood are also known to have an increased risk of developing second malignant neoplasms as a result of both cytotoxic chemotherapy and radiation therapy. In reports from the Childhood Cancer Survivor Study (CCSS), there was a 2.3-fold increase in second malignant neoplasms in children treated previously for cancer,[403] with patients who had Hodgkin lymphoma or Ewing sarcoma at greatest risk

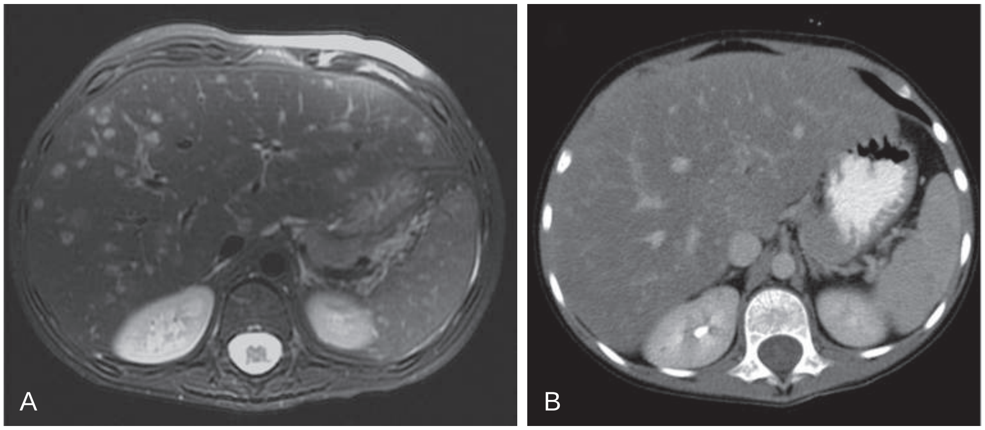

Figure 66-122 Imaging hepatosplenic fungal infection. MRI using fluid-sensitive FSEIR (or similar) sequences (**A**) is very sensitive for detecting lesions not seen with CT (**B**) or ultrasound.

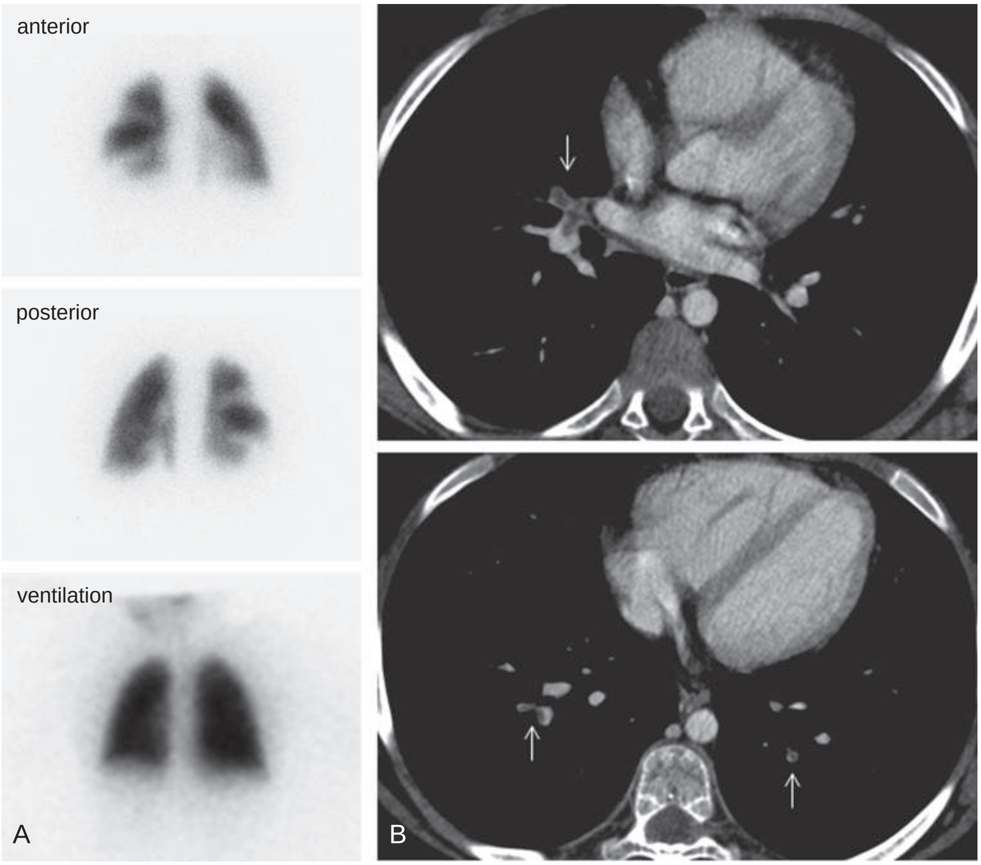

Figure 66-123 Pulmonary emboli. A 6-year-old boy with T-cell ALL had fever and neutropenia. CT scan of the chest identified multiple pulmonary emboli involving multiple lung segments (**B**). CT findings were confirmed by ventilation/perfusion scanning (**A**), which demonstrated multiple perfusion defects corresponding to involved segments identified by CT, but no corresponding ventilation defects.

(Fig. 66-128).[404] The most common second cancers include bone, breast, and thyroid cancer, as well as tumors of the CNS (meningioma and glioma) (Fig. 66-129).[405]

INTERVENTIONAL RADIOLOGY IN PEDIATRIC ONCOLOGY

Pediatric interventional oncology, also known as the "fourth pillar of cancer treatment" is an emerging specialty that offers an alternative resource when other options like surgery, chemotherapy, or radiation therapies have limited usefulness. This primarily involves use of minimally invasive techniques with image guidance for directly targeting tumors, both for diagnostic and for therapeutic reasons. When considering "tumors" broadly, to include nonneoplastic growths, pediatric interventional radiologists are involved in all aspects of management from diagnosis to treatment to offering palliative care. With regard to neoplastic disease, oncology-related pediatric interventional radiological procedures have traditionally been limited to diagnosis, management of complications, and procedures designed as auxiliaries to open surgery. Because of limitations such as the approval process for new devices, protocol-driven pediatric oncologic practice, and the inherent hesitation of trying new treatments in children, the development of interventional oncology for children has been challenging.[406] Newer landmarks in pediatric therapeutic oncological interventions are under active development, but standards remain to be established. Although many broad principles in adult and pediatric interventions are the same, some specific aspects related to procedures in children deserve discussion, and are enumerated in the following list.

1) Informed consent: Because most pediatric patients younger than 18 years of age have parents or legal guardians, a detailed preprocedural discussion is necessary to educate them regarding possible complications, some of which may remain present throughout life. Many adolescent patients are able to participate actively in the decision-making process. This usually requires a detailed conversation that is simple, clear, and addressed in a way to make the parents and the child comfortable without inducing unwarranted fear of the procedural risks. It is appropriate to create a "child-friendly environment" to provide a sense of safety, security, and dedicated pediatric care.
2) Sedation and anesthesia: Almost all the procedures in children need general anesthesia or higher levels of sedation to minimize motion, help children tolerate pain better, and allow positioning during the

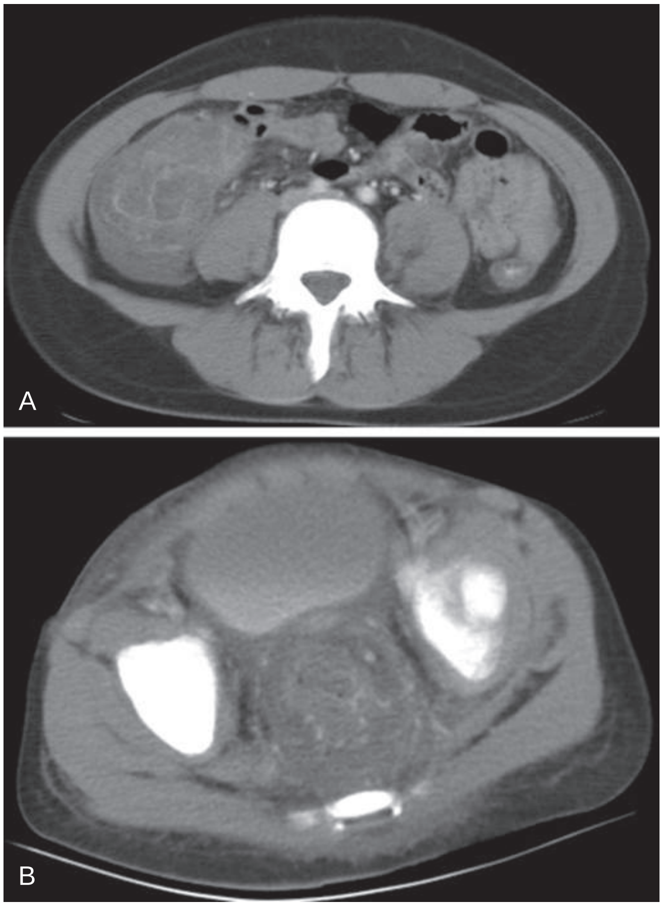

Figure 66-124 Contrast-enhanced CT in two patients with neutropenic colitis, showing disease primarily of the terminal ileum and cecum; i.e. typhlitis (**A**) and more extensive pancolonic disease with severe rectal necrosis that required diverting ileostomy (**B**).

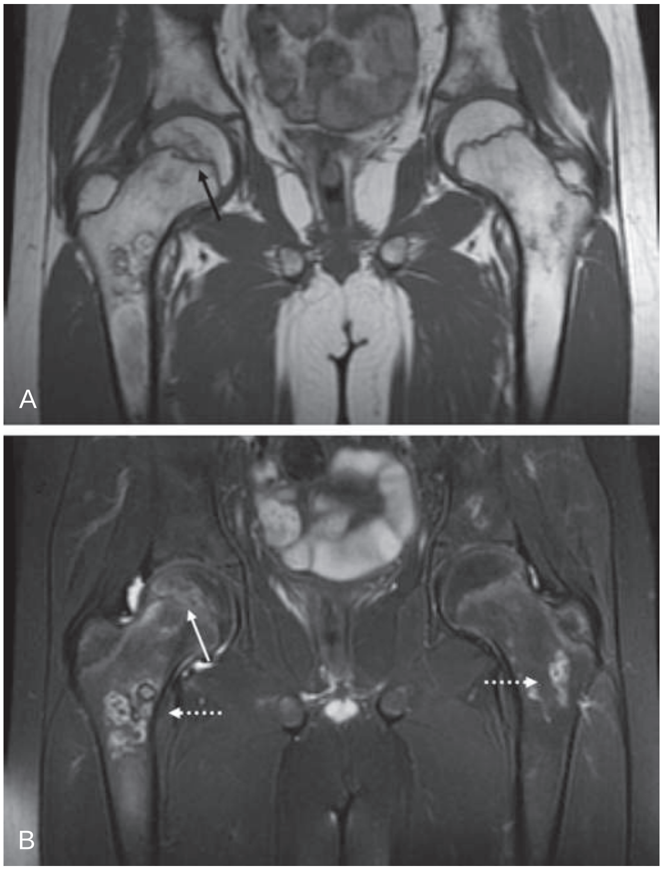

Figure 66-126 Osteonecrosis/avascular necrosis. A 13-year-old patient with a history of high-risk ALL, receiving high dose steroids, experienced right hip pain. Unenhanced T1-weighted (**A**) and Gd-enhanced (**B**) MRI of the hips and pelvis show osteonecrosis of the right femoral head *(arrows)* and evidence of bone infarcts in both femoral necks *(dashed arrows)*.

procedure. Deep sedation or general anesthesia can provide some postprocedural amnesia, unlike adults who can tolerate most procedures under mild sedation and local anesthesia.

3) Equipment: Most available devices are designed for use in adults. Hence bench modifications of devices developed for adults are often performed to match the smaller body habitus of children.
4) Contrast and radiation: It is imperative that contrast doses be diligently monitored in children to minimize renal toxicity. Likewise, maximal efforts are directed to limit the radiation dose, because the cumulative effects of radiation are of major concern in children. Most interventional radiologists practicing in adult populations concern themselves largely with the deterministic effects of radiation

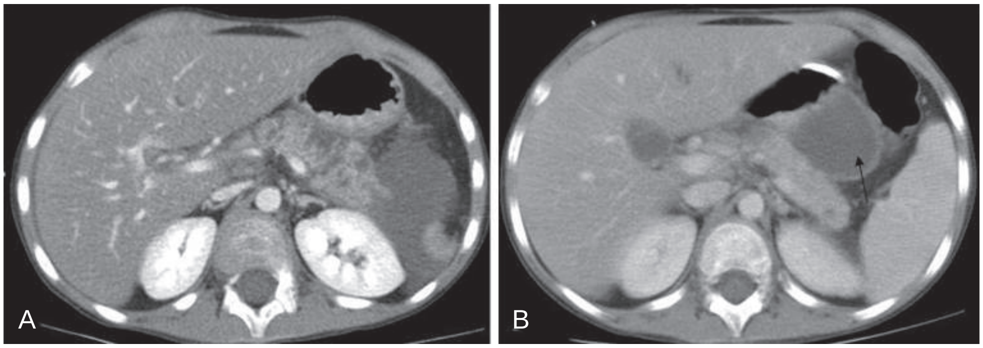

Figure 66-125 An 11-year-old with ALL receiving asparaginase developed severe pancreatitis (**A**) and subsequent pseudocyst formation *(arrow)*, with the pseudocyst closely adherent to the posterior wall of the stomach (**B**).

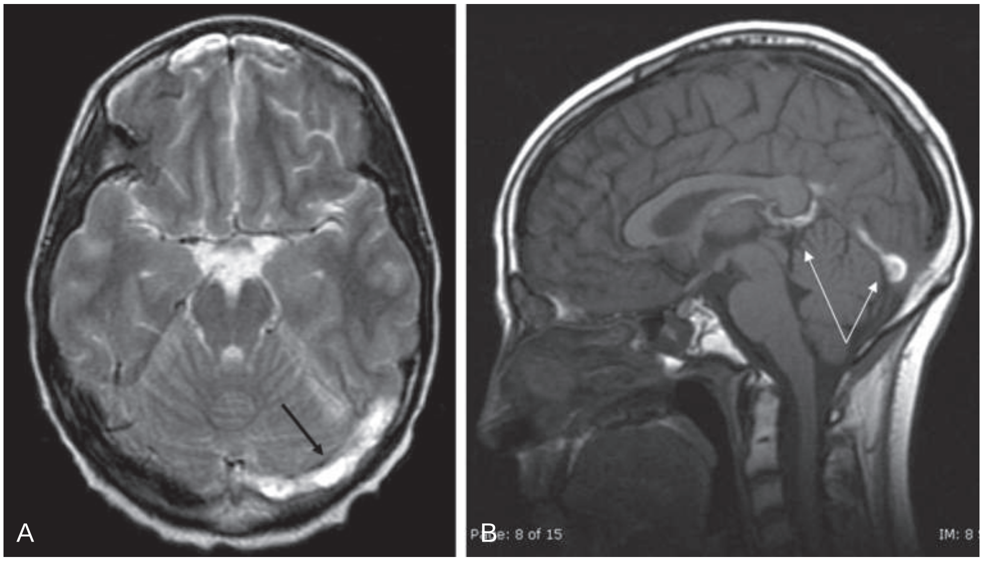

Figure 66-127 Cerebral sinus thrombosis. Axial T2-weighted (**A**) and sagittal T1-weighted (**B**) MRI in a 15-year-old patient with ALL, receiving asparaginase during induction therapy, shows high signal-intensity abnormality in the left transverse sinus, straight sinus, and deep cerebral veins *(arrows)*.

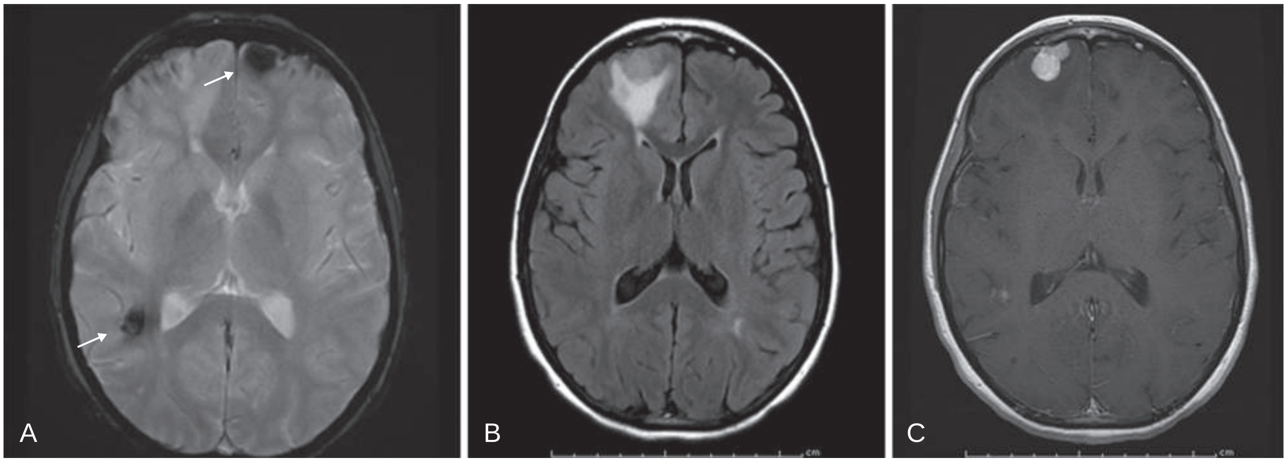

Figure 66-128 Radiation-related change and second malignancy. A 22-year-old patient was treated for ALL as a child with chemotherapy and craniospinal radiation therapy. Known cavernous angiomas (**A,** *arrows*) were being monitored routinely. She experienced seizures, and a new right frontal-lobe lesion seen on T2-FLAIR (**B**) and contrast-enhanced T1-weighted (**C**) MRI was found on resection to be high-grade sarcoma, presumably related to prior radiation.

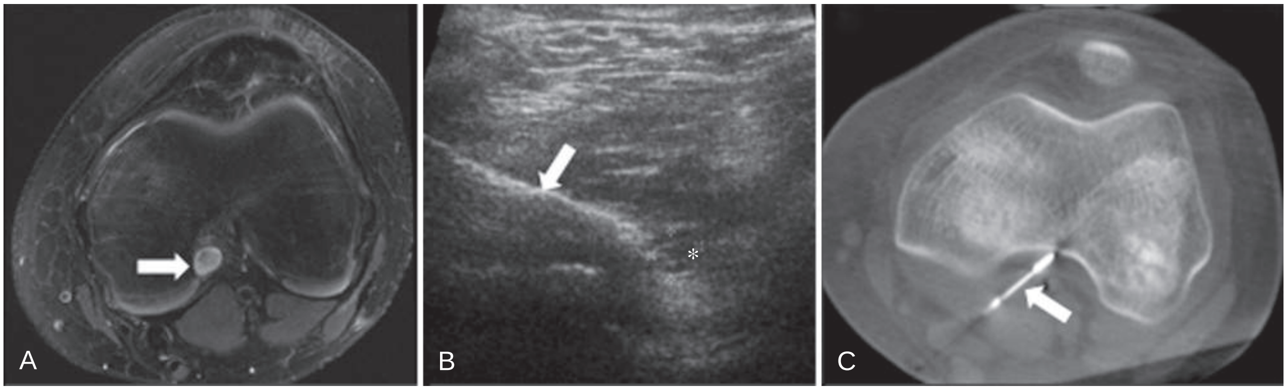

Figure 66-129 Percutaneous image-guided core needle biopsy. **A**, Axial T2-weighted fat inversion recovery MRI demonstrating a painful focal solid mass situated deep into the intercondylar notch of femur *(arrow)*. **B**, Ultrasound-guided access of the nodular lesion *(asterisk)* with a coaxial needle *(arrow)* **C**, Dyna CT images demonstrating the biopsy needle deployed into the lesion *(arrow)* to obtain samples.

related to dose and time of exposure at the time of the procedure. These effects are usually manifest as damage to bone marrow, gastrointestinal mucosa, or skin. It is, however, the stochastic effects, those cumulative effects that are related to any exposure to ionizing radiation regardless of time or dose, that are far more concerning in the pediatric population than the adult.[407]

There is no minimum threshold radiation dose for stochastic effects to occur, and stochastic exposure (primarily related to DNA damage) may not manifest itself for decades. It is specifically in young children with many years of life ahead that these issues are of greatest concern. Additionally, many oncology patients are exposed to high-dose external-beam radiation as part of their treatment protocol, adding to the risk. The ALARA concept of radiation exposure is ubiquitous in radiology but is held to much higher standard in pediatric diagnostic imaging and pediatric interventional radiology, with risk of radiation exposure always weighed against the benefits of a given image-guided procedure, and every attempt is made to use the lowest possible radiation dose. Limiting the fluoroscopy time, low-dose pulse fluoroscopy, aggressive coning and filtering applied to the smallest possible region of interest, and maximal utilization of imaging modalities that are nonionizing are used to reduce radiation exposure.

5) Anatomic considerations: Children have a smaller body mass index, which can limit accessibility. In compact regions like the neck, continuous image guidance using ultrasound imaging or CT fluoroscopy is preferred during biopsies to avoid major blood vessels and nerves. In children these procedures are done with the use of general anesthesia, which may obscure immediate signs of nerve injury. Because of the smaller volume of subcutaneous fat and muscle bulk, the skin can be also vulnerable to injuries during ablation of tumors. These aspects of the procedure must be carefully considered during preprocedure planning.
6) Physiologic responses: Physiologic responses to intervention can be different in a child versus an adult. For example, vasospasm is commonly seen in children, making simple access or further intravascular manipulation more challenging. Children react to fluid imbalances and medications more quickly. Therefore close monitoring of fluid balance and drug dosages based on weight or body-surface area is mandatory; vigilant care on the part of specialized pediatric nurses and a pediatric anesthesia team is invaluable.

Role of Interventional Oncology

Generally, oncological interventions depend on the kind of care that is provided. This can be divided into the following areas: 1) diagnostic evaluation; 2) therapeutic care; and 3) supportive and palliative care.

Diagnostic Evaluation

Percutaneous Needle Biopsy

Core biopsy can provide tissue both for accurate histopathology, immunohistochemistry, and molecular pathology necessary for diagnosis and differentiation of childhood tumors. In some tumors like neuroblastomas, biopsy provides tissue not only for histologic classification but also for analysis of prognostic factors, such as *MYCN* and ploidy status.[408] Almost any mass can initially be biopsied using image-guided percutaneous needle biopsy. Ultrasound guidance is usually the imaging modality of choice given its real-time capability. Most biopsy needles are sonographically visible, and precise real-time targeting of most masses is possible, even in locations adjacent to vital structures (see Fig. 66-129). Other guidance modalities can be used for targeting, including CT and MR; these typically require general anesthesia. These authors often use Dyna CT with the I-guide technology to target areas situated in critical or compact zones (Fig. 66-130). Correlation with MRI or FDG-PET can guide the targeting to viable segments of tumor.

Evaluating tissues located in prior operative sites or within radiation fields can be challenging diagnostically

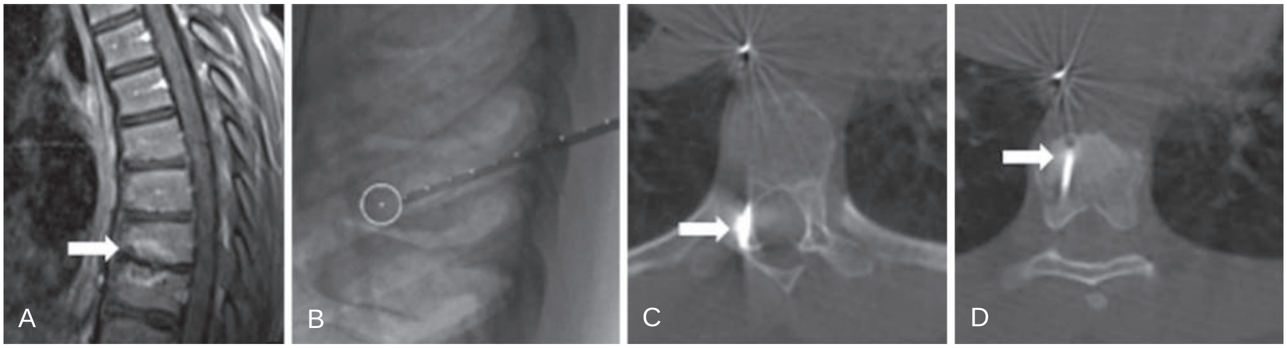

Figure 66-130 Percutaneous image-guided core needle biopsy. **A**, Sagittal T1-weighted contrast enhanced MRI of the spine demonstrating an enhancing destructive lesion of contiguous vertebral endplates *(arrow)*. **B**, The more destructive of the endplates was targeted using the I-guide technology *(circle)* on Dyna CT. **C**, Transpedicular course *(arrow)* of the biopsy needle is seen on the Dyna CT image. **D**, The biopsy needle tip is safely positioned at the proposed biopsy site *(arrow)*.

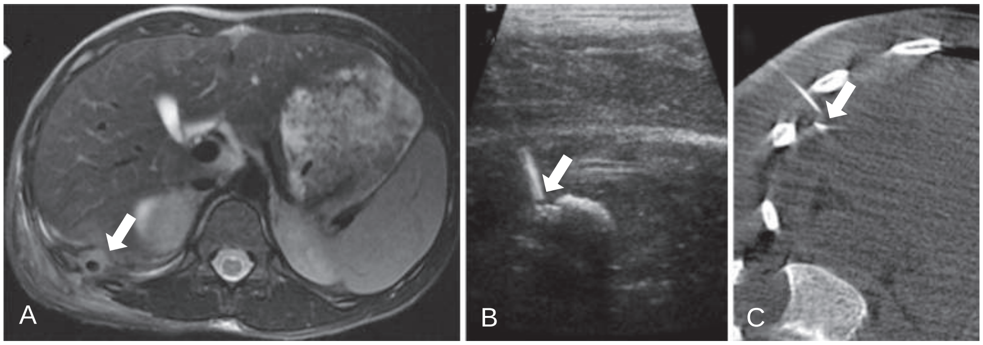

Figure 66-131 Needle localization. **A**, Axial T2 MRI of the abdomen demonstrating a hypointense, likely calcified, subhepatic nodule causing intense perilesional inflammation. An image-guided needle localization of this was requested before surgical exploration. **B**, Ultrasound-guided access toward the lesion *(arrow)* was performed. **C**, A hook was deployed *(arrow)* at the nodule to help localize the lesion at surgery as seen on Dyna CT image.

and can also be difficult to access, particularly when situated deep to vascular, neural, and bony structures. A multiple quadrant sampling or targeted sampling of FDG-avid areas should be planned, because intratumoral histologic heterogeneity could result in sampling artifacts that lead to incorrect prognostic histologic subgroup assignment,[408] which in turn may impact therapy. The most commonly biopsied structures include liver, renal and suprarenal tumors, mediastinal tumors, musculoskeletal tumors with associated soft tissue, and bony masses.

Obtaining a sample from the soft-tissue mass, bone mass and its interface is most helpful. Automated or semiautomated cutting needles in the range of sizes between 14-gauge and 20-gauge are available providing 1- to 2-cm throws. A coaxial core-needle biopsy technique is used, in which an initial puncture of the skin and the tumor capsule is made using a needle mounted on a coaxial outer cannula. A 16- or 18-gauge cutting needle (manual or spring-loaded) is then advanced via this outer cannula into the lesion, and several samples are obtained aiming at different tumor quadrants. This technique theoretically reduces the chance for tract seeding and also the possibility of bleeding and infection by limiting the number of punctures. Using the coaxial needle also allows the biopsy tract to be embolized if bleeding does not stop spontaneously or in patients with poor coagulation. For bone biopsies an 11- or 13-gauge needle permits the coaxial passage of a trephine Ackermann needle (Cook Medical, Bloomington, Ind.) to complete the bone biopsy. Smaller coaxial systems such as the Bonopty biopsy needle (Radi Medical Systems, Uppsala, Sweden) (12 and 14 gauge) are also available. Bony drills can be employed to obtain deeper access or access very hard bony lesions. Risks are smaller and rare and include bleeding, infection, and injury to the structures around the target.

Very tiny lesions or anatomically arduous areas can be subjected to fine-needle aspiration. Fine-needle aspiration can be obtained using a 20- to 22-gauge needle placed independently or coaxially via an 18- to 19-gauge needle. Whenever possible, the shortest and safest course to reach the lesion should be chosen. Accuracy, less invasiveness, low rate of complications, shorter recovery time, and ability to do targeted and multiquadrant sampling through minimal number of accesses are some of the major benefits of image-guided percutaneous biopsies.[409-412] Available pooled data suggests that 95% of image-guided needle biopsies provide adequate sample for tissue diagnosis in childhood malignancies.[413] CT or sonography can be used preoperatively, using similar technique as for biopsy, to place Kopans localization hook wires (Cook Medical, Bloomington, Ind.) (Fig. 66-131) or coils or to inject a dye to help the surgeon easily find the lesion during operation.[414] The interventional radiologist also has the ability to place central venous lines or chemotherapy ports as part of the same interventional radiological procedure, precluding the need for a separate operating room visit and additional anesthesia.

Therapeutic Care

Therapies providing local control of tumors are widely used in adult interventional oncology practice. Indications for regional cancer treatments in children are still evolving and are based largely on experience extrapolated from the adult literature. These can be categorized broadly based on their principle of use: 1) transarterial embolization; 2) tumor ablation; 3) high intensity focused ultrasound; and 4) irreversible electroporation.

Embolization Therapy

This technique involves performing catheter angiography to determine the arterial perfusion to the tumor. After this, feeding vessels are selectively catheterized to deliver tumoricidal drugs or to create tumor ischemia by stopping the blood flow in these vessels.

Chemoembolization. This is also known as *transarterial chemoembolization (TACE)* in which chemotherapy is delivered by injecting a mixture of the chemotherapeutic agent with Ethiodol or particles, or as chemotherapeutic agent-impregnated beads, also known as *drug-eluting beads (DEBs)*. This technique concentrates the chemotherapy drug in the target tissues, with the embolization effectively prolonging the dwell time of the agent in the target with less systemic effect than intravenous or oral chemotherapy. In adults TACE has been widely used for hepatocellular carcinoma and hepatic colonic metastases. It has significantly prolonged 2-year survival rates up to 63% as compared to 27% with supportive care.[415]

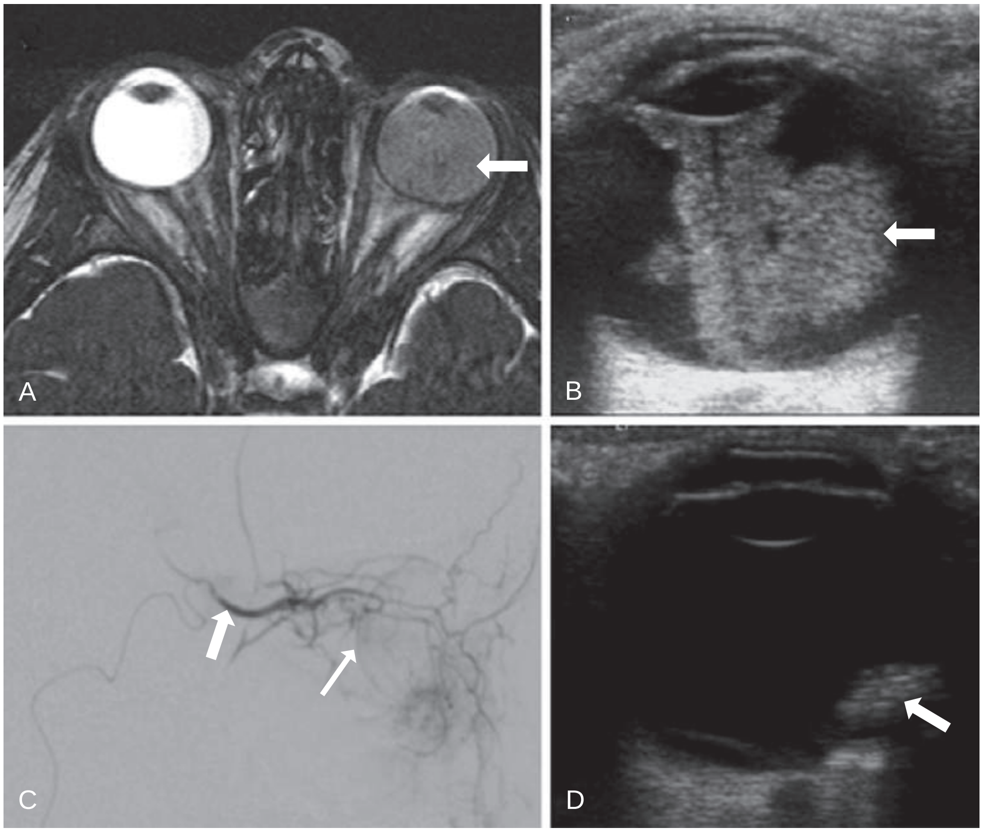

Figure 66-132 Intraarterial chemotherapy. **A**, Axial T2-weighted image of the orbits showing a hypointense retinoblastoma filling the entire left orbital cavity *(arrow)*. **B**, Ultrasound obtained during the first intraarterial chemotherapy demonstrating the hyperechoic intraorbital mass *(arrow)*. **C**, Selective left ophthalmic artery angiogram *(thick arrow)* before superselective chemotherapy demonstrating retinal blush *(thin arrow)*. **D**, Ultrasound obtained during the second intraarterial chemotherapy demonstrating the remarkable tumor shrinkage with a very small residual mass *(arrow)*.

Presurgical TACE for pediatric hepatoblastoma has been shown to decrease tumor volume without causing significant chemotoxicity, allowing for complete tumor removal.[416] In unresectable lung metastases having dual supply from pulmonary and bronchial arteries, TACE has been well tolerated as a palliative procedure.[417] TACE has also been used in osseous tumors[418] and in Wilms tumor, demonstrating prolonged time to tumor progression and increased survival compared with surgery alone.[419] Intraarterial chemotherapy for retinoblastoma has been widely accepted for pediatric tumors (Fig. 66-132).[420]

Radioembolization. In transarterial radioembolization (TARE) radioactive materials are used to embolize hepatocellular carcinomas with a technique similar to TACE. External radiation therapy in patients with diffuse hepatic malignancy does not improve overall survival,[421] in part because of the relatively low radiation tolerance of liver tissue when compared with the doses required for tumoricidal effect.[421,422] TARE is usually chosen for chemoinsensitive tumors or when chemotoxicity limit is reached. The technique also involves selective catheterization of the hepatic artery supplying the tumor and injecting yttrium-90 (^{90}Y) microspheres. This has been shown to have less toxicity, higher response rate with a longer time to disease progression, and a similar survival rate in patients with advanced hepatocellular carcinoma when compared with chemoembolization.[423] Potential complications include locoregional radiation effects, pulmonary irradiation, and gastric perforation. To reduce pulmonary or systemic irradiation a diagnostic study is initially performed by injecting ^{99m}Tc-labeled macroaggregated albumin (MAA) (the same tracer used for routine lung scans), allowing the hepatic-to-pulmonary artery shunt fraction to be estimated before embolization.[424] Preprocedure coil embolization of gastrointestinal collaterals prevents gastrointestinal irradiation.

Bland Embolization. This technique is mostly used palliatively in hypervascular tumors like hemangiomas[425] and angiosarcomas,[426] both to control symptoms and arrest tumor growth. It also used preoperatively to decrease the blood loss at surgical resection[427] or to reduce intractable blood loss in pediatric tumors.[428] It is occasionally used to arrest the growth of neuroblastoma metastases causing life-threatening hepatomegaly. The feeding arteries supplying the tumor are selectively catheterized and embolized with either polyvinyl alcohol (PVA) or Gelfoam particles, cutting off the blood supply to cause tumor shrinkage and necrosis (Fig. 66-133).

Ablation Therapy

Ablation results in local destruction of tumor using chemical or mechanical methods. Prior cross-sectional imaging

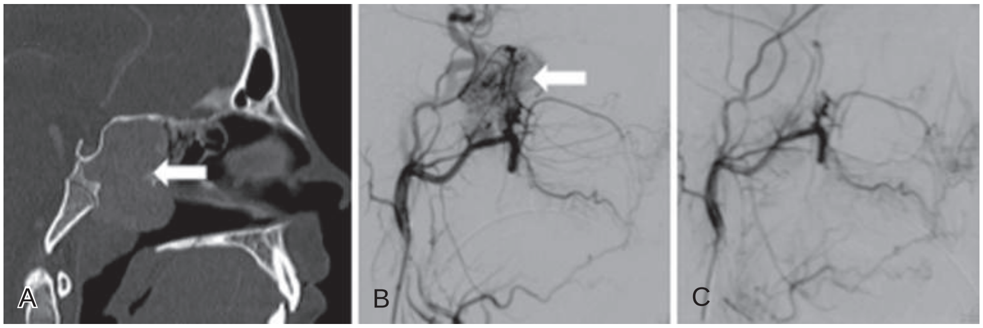

Figure 66-133 Juvenile nasopharyngeal angiofibroma. **A**, Sagittal-CT reconstructed image of the head demonstrates a large nasopharyngeal mass *(arrow)*. **B**, Selective internal maxillary artery injection demonstrating hypervascular nasopharyngeal mass *(arrow)*. **C**, After selective embolization with polyvinyl alcohol particles, there is near complete cessation of flow to the mass with preservation of the normal circulation.

is necessary in most patients to delineate the lesion and its relationship to critical structures like nerves, blood vessels, spinal cord, and hollow viscera, which are prone to injury during local ablation. This also helps plan the tract for probe placements and with assessment of the tumor volume to be treated. Local ablation techniques are most often used to treat hepatic tumors, desmoid tumors, and renal tumors and bony and pulmonary metastases.

Chemical Ablation. This is an established technique for treating hepatocellular carcinoma[429-433] and has been described for treating other solid tumors.[432] It is performed by percutaneously injecting and infiltrating the tumor with alcohol or acetic acid. The cytotoxic mechanism of action of ethanol is through cytoplasmic and membrane dehydration, denaturation of cellular proteins, and small-vessel thrombosis, resulting in coagulative necrosis. Acetic acid causes protein denaturation and dissolution of basement membrane and interstitial collagen, resulting in coagulative necrosis of tumor cells. Although these ablative techniques are technically simple, there is a small risk for nontarget tissue injury, including damage to adjacent blood vessels, nerves, organs, or overlying skin, especially if the tumor lacks a definite capsulation. Recently patients with desmoid tumors have been treated with acetic acid ablation.[434]

Thermal Ablation. These techniques provide cytolysis of the tumor cells either by thermal destruction or ionic agitation.

Radiofrequency Ablation. In this technique small probes are percutaneously directed into the tumor using image guidance like CT or Dyna CT. A radiofrequency generator generates high-frequency (375-500 kHz) alternating current applied directly via these probes, producing ionic agitation and frictional heat, thereby causing coagulation necrosis in tumor target tissue. Because this involves an electric circuit, grounding pads applied to the patient are necessary to allow the exit of the current. Local tissue temperatures reach 60 to 100° C, adequate to produce the cytotoxic effect. Temperatures greater than 100° C are generally not beneficial, because this produces tissue vaporization and carbonization, impeding conductance of the thermal energy and limiting the size of the ablation zone.[435] The burn time varies between 6 and 12 minutes. Proper placement of the grounding pads in full contact with the skin without any air pockets is necessary to avoid burns.[436]

Different tissues conduct electrical currents to different degrees. Blood vessels and bile ducts disproportionately absorb current, creating heat sinks that can confound planned heating of target ablation zones. Strategies to limit the temperature drop include internal cooling of the probe with chilled water, diffusion of water along the electrode tines, or preembolizing the feeding arteries adjacent to the tumor.[435] In children, radiofrequency ablation is most commonly used to treat osteoid osteoma (Fig. 66-134), osteoblastomas, and chondroblastomas.[437] Radiofrequency ablation has been used for large hepatic adenomas,[438] Wilms tumor,[439,440] and desmoplastic round-cell tumors in children.[441] A phase I study to determine the toxicity of radiofrequency ablation for solid tumors in children concluded that toxicity is limited and that radiofrequency ablation may offer a local control alternative in carefully selected pediatric patients.[442]

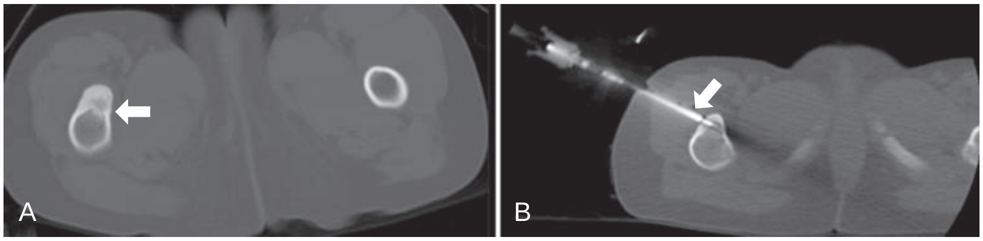

Figure 66-134 Radiofrequency ablation. **A**, Axial CT image of the hips demonstrates a painful sclerotic osteoid osteoma at the anterior cortex of the right femur *(arrow)*. **B**, The tumor was treated with radiofrequency ablation *(arrow)*, resulting in immediate resolution of pain and of the tumor at the follow-up examination.

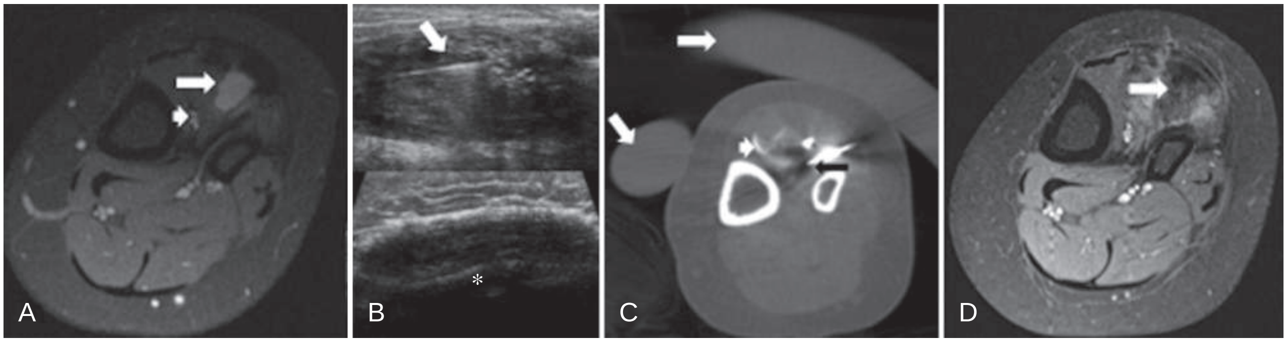

Figure 66-135 Cryoablation. **A**, Axial T1-weighted contrast-enhanced MRI of the left leg demonstrates a well-defined enhancing, recurrent desmoid tumor along the anterior aspect near the peroneus muscles *(arrow)*. The tibial neurovascular bundle lies in the vicinity *(arrowhead)*. **B**, The tumor was cryoablated using ultrasound guidance for probe placement *(arrow)*. Posterior acoustic shadow from the ice ball formation seen at intraprocedural ultrasound *(asterisk)*. **C**, Dyna CT monitoring during cryoablation. Contrast was used to create hydrodissection *(short arrow)* to protect the neurovascular bundle. A thermistor *(black arrow)* was positioned close to the neurovascular bundle to monitor temperature drop and prevent inadvertent nerve injury. Note the warm saline bags *(long arrows)* placed for skin protection during cryoablation. **D**, Follow-up post-contrast axial MRI of the leg demonstrates the loss of enhancement at the tumor ablation site *(arrow)*.

Radiofrequency ablation has shown to be effective in pain palliation of painful bony metastases.[443,444]

Cryoablation. This is one of the oldest ablation techniques used and produces tissue destruction by applying nitrogen to surgically exposed tumors.[445] This technique has been surgically used (open or laparoscopic) to treat tumors. With newer imaging techniques that detect small cancers in early stages, together with the availability of smaller cryoprobes, these procedures are now being performed percutaneously using image guidance. The system involves a set of 11- to 17-gauge cryoprobes through which gases from pressurized cylinders are circulated to produce the desired effect of freezing and thawing. Argon gas is used to produce freezing (cooling), and helium gas is used to produce thawing (warming). The Joule-Thompson effect of rapid gas expansion is the basis for rapid cooling of the metallic cryoprobes when there is sudden release of highly pressurized argon gas from the probe. As low as –100° C is reached within a few seconds. On a similar principle, there is production of heat caused by rapid expansion of helium gas that causes local warming. These effects are used alternatingly in a cycle with two 10-minute freezes separated by a 5-minute passive thaw to optimize tumor destruction. There is irreversible tissue destruction that occurs at temperatures below –20 to –30° C. Mechanisms causing cell death are by direct freezing, denaturation of the cellular proteins, cell-membrane rupture, cell dehydration, and ischemic hypoxia.[435] There is formation of an ice ball at the tip of the cryoprobe, the size of which can be either fixed or varied (V-probe), depending on the type of probe used. The size of the ice ball with respect to the tumor volume coverage can be carefully monitored using CT[446] intermittently every 2 to 3 minutes.

Once adequate freezing of the tumor volume is achieved, cryoablation is stopped, thus preventing injury to the adjacent normal structures. Unlike radiofrequency ablation, there is very little or almost no pain after cryoablation. Additionally, larger tumors adjacent to critical structures can be treated more safely. Nerve injury, skin blistering, and bowel injury have been reported with this modality. However, these effects can be minimized by image-based planning, use of temperature monitoring probes to measure adjacent tissue temperatures, careful monitoring of ice-ball formation, and use of tissue protection (Fig. 66-135) including hydro-dissection and warm saline bags over the skin. Cryoablation has been effectively used for treating desmoid tumors,[447] in palliation and local control of Ewing sarcoma,[448] and for painful bony metastases. Percutaneous cementoplasty has also been used as an adjuvant with radiofrequency ablation[449] and cryoablation in palliative pain management.

Microwave Ablation. This system consists of a microwave generator and a set of internally cooled antennas. Most microwave antennas are straight and needlelike with variations such as monopole, dipole, triaxial, choked, or slotted. These antenna shafts are as small as 17 gauge and are cooled by circulating chilled saline or by using compressed carbon-dioxide gas. This allows for delivery of higher powers for longer times to produce larger ablation zones. The generators emit 915 to 2450 MHz microwaves, creating high-speed microwaves that cause rapid vibration and rotation of the molecular dipoles within the ablation zone. This ionic agitation produces frictional heating and thermal coagulation of the tissue. Unlike radiofrequency ablation, the heating energy is deposited directly at the antenna rather than by creating a resistive heating circuit. This precludes the necessity of using grounding pads and chance of having skin burns. Additionally, there is less influence of tissue impedance factors like perfusion-mediated cooling. This system can create rapid heating of tumor tissue to greater than 100° C very quickly.[450] Multiple applicators can be safely placed in one session to optimize the ablation volume.[451,452] The use of microwave ablation has not been routine in children, but it is becoming more popular in adults for ablation of liver, lung,[453] and bone tumors.[454]

Laser Ablation. Although not widely used, laser ablation has also been used for treating tumors. An optical laser fiber transmits infrared light energy into the tumor to produce heat and coagulation necrosis.[435] The 17-gauge fibers are coaxially placed through 14-gauge guide needles, and the active tip is exposed. The size of induced zone of ablation depends on the laser wavelength, thermal and optical properties of the tissue, total duration of energy application, power used, diameter of the fiber, fiber tip, and number of fibers used sequentially or simultaneously for ablation.[455,456] An additional advantage of lasers is their MRI compatibility. Laser ablation has been used in the treatment of osteoid osteomas[457,458] and in lung metastases.[459]

High-Intensity Focused Ultrasound. Ultrasound waves generated by high frequency vibrations of up to 10 MHz are focused into a small discrete target region using a concave or parabolic arrangement.[460] When the local tissue absorbs this acoustic energy there is production of thermal energy, which creates coagulative tissue necrosis. Other changes, including tissue cavitation, microstreaming, and radiation forces generated during this process have been reported to contribute to the overall tumor lysis. Dose delivery to targeted tissues depends on the transducer array, the energy input, and the tissue attenuation that occurs between the transducer and the target.[461] This technique is entirely noninvasive and does not require incisions, general anesthesia, or blood transfusions, making it more appealing than the other modes of ablation. To minimize inadvertent injury to adjacent structures guidance and monitoring of acoustic therapy is critical. Sonography and MRI, especially in the obese,[462] can be used for image guidance and monitoring. Dynamic MRI temperature mapping to assess the ablative zone can also be performed.[463,464] Given the inherent behavior of ultrasound waves, this modality has limitations for lesions that are covered by bone or air-containing viscera, like bowel, which provide very high impedance to these waves. High-intensity focused ultrasound has been used in the treatment of desmoids,[465] bone,[466] and other solid tumors.[467]

Irreversible Electroporation. Irreversible electroporation (IRE) is novel technique in which high-voltage microsecond electrical pulses are applied through percutaneously placed probes into the tumor tissue. This technique causes tumor lysis by creating permanent pores in the cell membrane, which leads to apoptosis.[468] There is no generation of thermal energy; therefore critical structures like blood vessels, biliary ducts, and nerves are not adversely affected.[469,470] No grounding pads are necessary, thus avoiding possible skin burns. Most research on its application has been for pancreatic,[470] liver, pulmonary, and renal tumors.[471]

Supportive Care

Interventional radiologists play a major role in providing supportive care to cancer patients. These patients are usually referred after failed attempts at vascular accesses by other services or as an alternative to high-risk operations. By combining innovative approaches with image guidance and technical skill, these procedures provide a relatively safe and effective option in many difficult clinical situations for managing complex cancer patients.

Venous Access

Venous access in cancer patients is a major requirement for chemotherapy and nutritional support. It can also cause concern in small, underweight children with poor venous caliber. Maintenance of access can be problematic in patients with chronic venous stenosis resulting from long-standing line placement. Central line and peripherally-inserted central catheter (PICC) placements are commonly performed procedures in interventional radiology. These can be tunneled and partially buried underneath the skin at their exit points to provide a secure long-term intravascular access. Chemotherapy port placements can also be performed by interventional radiologists, a procedure ideally coordinated with the patient undergoing image-guided needle biopsy. This is shown to be safe and successful with a low rate of complications.[472,473] The initial vessel access is obtained using direct ultrasound guidance with a single anterior wall puncture, decreasing access time and complication rate and improving overall success of access.[474,475] Catheter position can be manipulated and confirmed simultaneously using fluoroscopy.

For central line placements or chemotherapy port placements, interventional radiologists prefer the internal jugular vein for access over the subclavian vein approach preferred by the surgeons (Fig. 66-136). This choice prevents the “pinch-off syndrome” caused by catheter kinking and is not associated with pneumothorax, hemothorax, and brachial plexus injury seen in the subclavian venous approach.[476] Chronic indwelling subclavian lines can also cause central venous stenosis. This has the disadvantage of precluding placement of dialysis fistulae in the upper extremity and making placement of peripherally inserted intravenous catheters more challenging.[477] Placement of these lines in interventional radiology is more accurate, less traumatic, and greatly reduces chances of inadvertent neurovascular injury. In patients with venous stenosis, venography can also be performed to assess for collateral patency and placement of venous lines using these pathways (Fig. 66-137). Paravertebral veins, internal thoracic veins, external jugular veins, although tenuous, can be safely accessed using sonographic guidance for placement of venous access in patients with inaccessible extremity veins.[478] Salvage procedures such as repositioning and removal of incorrectly positioned catheters (Fig. 66-138), fibrin stripping of occluded central venous catheters, and stenting of stenosed SVCs can also performed conveniently in interventional radiology.[476]

Ancillary Supportive Care

Interventional radiology also offers other supportive care in oncology patients, especially in palliative care. Enteric access for providing nutritional requirements becomes essential in cancer patients who feed poorly for a variety of reasons. Primary gastrostomy and transgastric jejunostomy tubes can be primarily placed with percutaneous nonendoscopic techniques using fluoroscopic guidance[479] and have the advantage of avoiding inadvertent bowel injury.[480] The transgastric tube may be inserted during the initial placement, avoiding the need for another tube

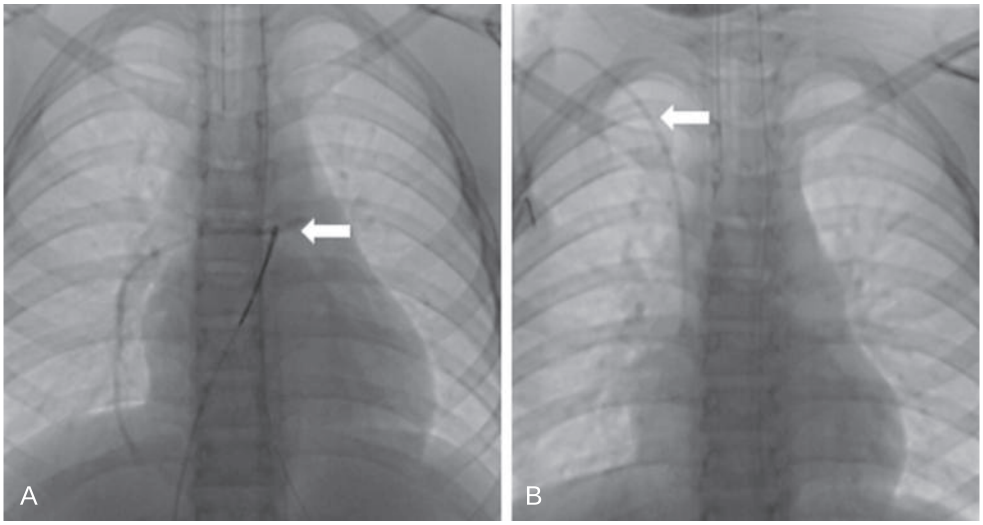

Figure 66-136 Port placement. **A,** Frontal chest radiograph demonstrates the migrated catheter of a chemotherapy port placed via the left subclavian vein. The proximal end of the catheter was floating in the main pulmonary artery with the distal end trapped in the lower branch of the right pulmonary artery. This was snared in the main pulmonary artery *(arrow)* and removed. **B,** A new chemotherapy port was then placed using a right internal jugular venous approach *(arrow)*.

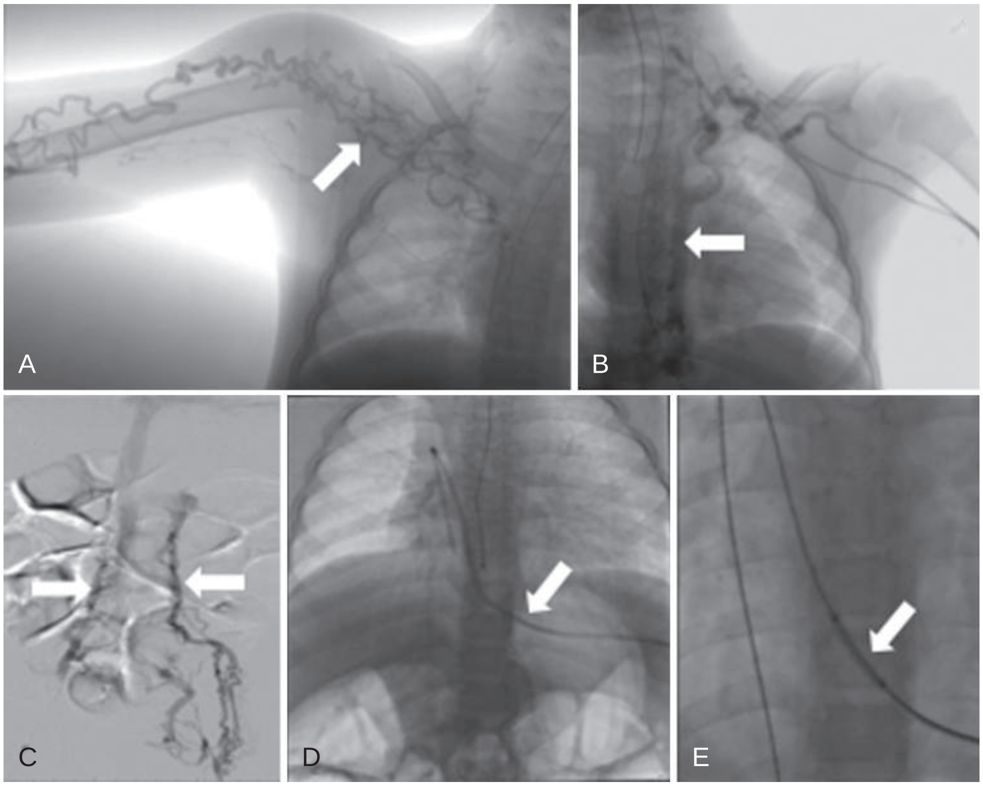

Figure 66-137 Difficult venous access. **A-C,** A patient with an imminent requirement for a central line had bilateral upper and lower extremity venograms demonstrating thrombosis of normal veins with extensive collateralization *(arrows)* diverting flow to the cava. **D,** Because of poor venous access, a posterior paravertebral vein was accessed, and a central line was inserted via the hemiazygos vein *(arrow)*. **E,** One year later there was line damage requiring an exchange. During the exchange there was extreme resistance at the access site with stenosis of the hemiazygos vein, which was angioplastied *(arrow)* to achieve successful exchange.

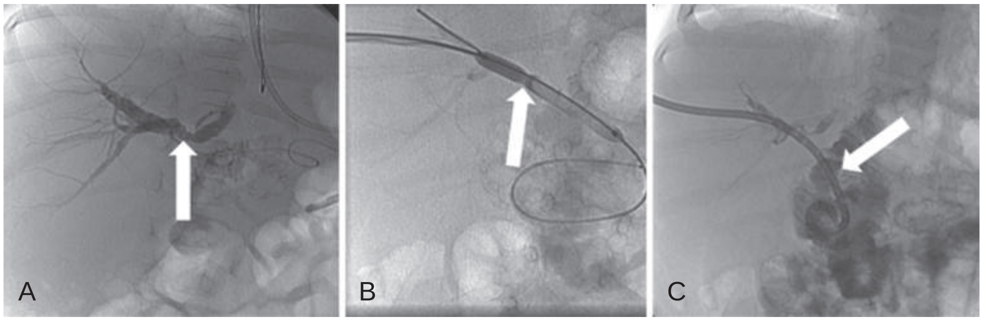

Figure 66-138 Hepatobiliary intervention. **A**, Percutaneous transhepatic cholangiogram in a liver transplant patient with elevated liver enzymes showing biliary strictures *(arrow)*. **B** and **C**, The stricture at the confluence was dilated (*arrow* in **B**) and a biliary drain to the jejunum (*arrow* in **C**) was placed, relieving the patient's symptoms.

conversion, as is required for endoscopically placed tubes. The routine exchange of these tubes is performed in interventional radiology, providing a dedicated and consistent team for support in the maintenance of these tubes.

Certain complex procedures, including biliary interventions such as percutaneous cholangiograms (PTCs), percutaneous transhepatic biliary drain (PTBD) placements (see Fig. 66-138), percutaneous transhepatic intrabiliary biopsies, and cholecystostomies are occasionally performed in pediatric oncology patients but are more commonly encountered in adults. Interventional radiology procedures like PTBD placement and hepatic vein dilation are most often used after liver transplant in cancer patients. These include percutaneous and transjugular liver biopsies performed with ultrasonographic and fluoroscopic guidance for the diagnosis of graft disease; angioplasty and stent placement for treatment of vascular and biliary occlusion, stenosis, and stricture; and catheter placement for drainage of fluid collections.[481,482] In patients with portal hypertension a transjugular intrahepatic portosystemic shunt can be placed to decompress the liver as a bridge to transplantation.[483] In the genitourinary system, percutaneous nephrostomy catheter placements, nephroureteral catheters, ureteral stents, and suprapubic catheter placements for extrinsic abdominal or pelvic tumors causing urinary obstruction are all performed in interventional radiology (Fig. 66-139).[484]

Occasionally catheter-directed thrombolysis of the deep vein thrombosis in cancer patients[485] and placement of IVC filters to prevent pulmonary embolism are other supportive procedures performed by interventional radiologists. Patients with multivisceral transplants, including bowel transplant, may suffer recurrent chylous fluid accumulation. Intranodal lymphangiogram performed by accessing a groin node is a novel procedure that helps diagnose this condition and allows percutaneous embolization (Fig. 66-140) to treat this challenging posttreatment complication.

NEW IMAGING TECHNIQUES

Molecular Imaging

Historically the standard tools of radiology—CT, MRI, and ultrasound—have provided primarily anatomic information, with relatively little direct functional information being evident. Nuclear medicine imaging has

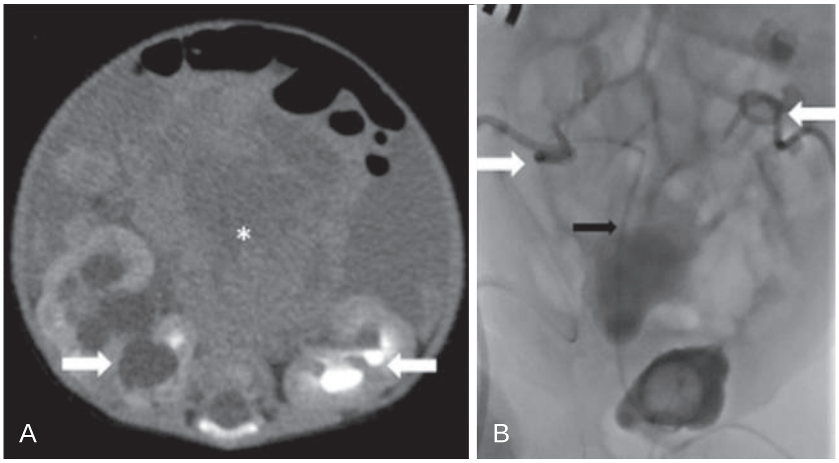

Figure 66-139 Genitourinary intervention. **A**, Axial CT image of the abdomen demonstrates a large rhabdoid tumor compressing the ureters and causing bilateral hydronephrosis. **B**, Bilateral percutaneous nephrostomy catheters *(white arrows)* and a right ureteric stent *(black arrow)* were placed to provide urinary diversion.

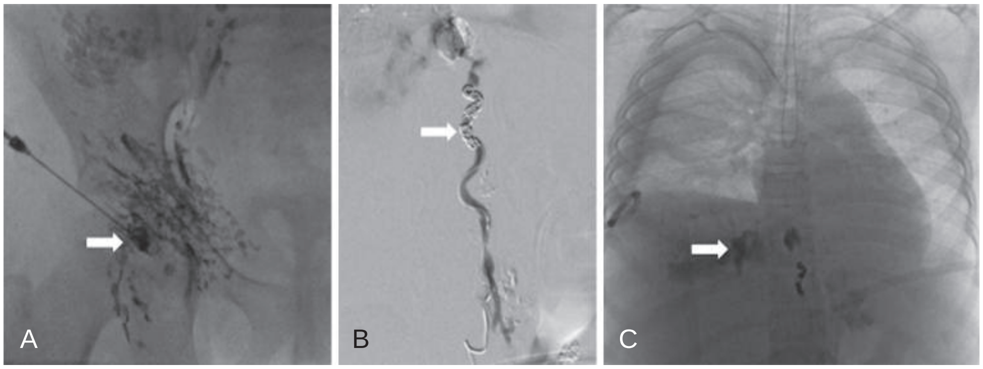

Figure 66-140 Intranodal lymphangiography and embolization. **A**, A child with recurrent chylous effusion after multivisceral transplant underwent intranodal lymphangiography *(arrow)*. **B**, The cisternae chyli was percutaneously embolized with coils and glue *(arrow)*. **C**, The persistent leak *(arrow)* that was causing recurrent pleural effusions stopped.

provided useful functional information but has been limited by the lack of accompanying anatomic detail available with conventional planar and SPECT techniques, although PET imaging has provided a considerable improvement in the anatomic resolution, particularly with the use of hybrid imaging approaches. Of the three major cross-sectional imaging technologies, MRI has come closest to providing direct information about cellular function, for example by employing diffusion-weighted imaging to assess regions of localized ischemia and using hydrogen (^{1}H) spectroscopy to derive metabolic information about specific regions being imaged. Functional MRI of the brain relies on relative changes in blood oxygen level in metabolically active tissues to yield exquisite information about cerebral activation in response to various stimuli. The relative success enjoyed by MRI in generating functional images derives mostly from the rich and varied information contained in the MR signal from different materials. Although most of the MR information being imaged routinely arises from the protons contained in hydrogen atoms from water molecules and lipids, a vast amount of metabolic information can also be derived from MR signals arising from molecules other than water.

The U.S. Food and Drug Administration (FDA), in its critical-path initiative, has emphasized the need to increase the speed, efficiency, and cost-effectiveness of drug development for cancer and other diseases.[486] Molecular imaging and functional imaging probes have great potential to identify and characterize disease-specific targets and to provide target- and treatment-specific imaging end points for therapy. In particular, as markers of biological response, imaging end points that characterize cellular vitality or apoptosis, receptor down-regulation, or functional inactivation, and treatment-specific changes to cellular metabolism will be critical in determining the success or failure of newer classes of molecular-based therapies. As has been learned with imatinib and gastrointestinal stromal tumors, noncytolytic therapies that slowly extinguish tumor metabolic activity do not immediately result in tumor shrinkage, and imaging techniques that directly measure tumor metabolism are necessary to directly reflect the response to therapy.[487] In addition, for many molecularly targeted agents such as antibodies or receptor-targeted agents, it is unclear whether the agents ever localize in tumor in vivo. For these compounds, it is critical to develop surrogate end points of response. The ability to directly image the distribution and targeting of such agents in vivo would significantly benefit this assessment, particularly when there is a poor overall clinical response to therapy. Phase 1 trials of therapeutic agents in particular offer an opportunity to use imaging as means of establishing drug activity at a time when therapeutic efficacy may not be the primary or anticipated goal of therapy. As shown in Figure 66-141, in a phase I/II study of the insulinlike growth factor 1 receptor (IGF1R)–targeted agent cixutumumab[488] after just one 4-week cycle of therapy a child with metastatic fibrosarcoma showed a nearly complete metabolic response to therapy despite minimal change in tumor size. Conventional measures of response would have concluded there was no activity in this patient, highlighting the importance of molecular imaging techniques in assessing novel noncytolytic approaches to therapy.

Newer functional imaging approaches are also expected to impact treatment strategy and drug design with an emphasis on response-based treatment regimens. This allows patients with a high likelihood of response to be treated less aggressively, whereas those not responding to therapy are identified early and redirected into more aggressive therapeutic regimens.

Molecular imaging has been defined by the Commission on Molecular Imaging of the American College of Radiology as the “spatially localized and temporarily resolved sensing of molecular and other processes in vivo”.[489] Although molecular imaging techniques, particularly in nuclear medicine, have been in use for many years, this terminology has recently been applied to the rapidly growing area of radiologic investigation aimed at developing tools to allow direct imaging of cellular and molecular events. Increasingly there has been recognition that the ability to perform multiparametric functional imaging is likely to provide the greatest insight into tumor or tissue biology and response to therapy by providing quantitative parameters (biomarkers) that derive from

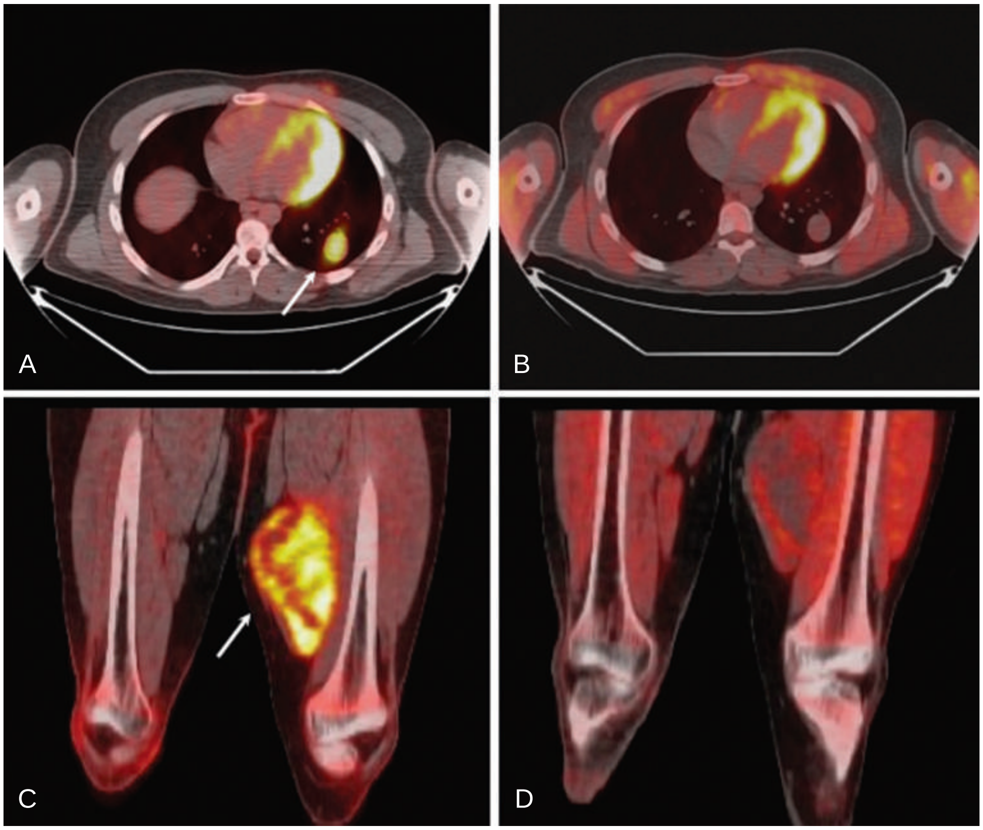

Figure 66-141 Reduction in FDG-PET activity with cixutumumab. Fused axial PET/CT of chest (**A** and **B**) and lower extremities (**C** and **D**) show increased uptake at baseline in metastatic left lower lobe lung nodule (**A**, *arrow*) and intense baseline uptake in a primary left midthigh mass at baseline (**C**, *arrow*) in a patient with metastatic fibrosarcoma. After 2 weeks of cixutumumab therapy, although the lung nodule had not changed in size, there was no significant residual FDG uptake remaining (**B**). Similarly, there was significant reduction in FDG uptake at the primary left thigh site with no apparent change in size of tumor (**D**) after 2 weeks of therapy. *(From Malempati, Weigel B, Ingle AM, et al: Phase I/II trial and pharmacokinetic study of cixutumumab in pediatric patients with refractory solid tumors and Ewing sarcoma: a report from the Children's Oncology Group.* J Clin Oncol *30:256–262, 2012.)*

merging the information obtained from different imaging techniques,[490] for example diffusion- and perfusion-weighted MRI results coupled with metabolic changes visible by PET imaging.[47]

Most of these investigations are still in the early stages of development[486,489,491,492] and include development of optical imaging probes, imaging gene expression, imaging marker genes, tracking cells in vivo, and developing targeted biomolecular conjugates to allow visualization of specific cellular and molecular events in vivo.[493,494] Perhaps the most fruitful area of research has been in the application of near-infrared fluorescent imaging to evaluation of malignant disease.[495-498] Fluorescence in the near-infrared range of the spectrum has been pursued, because these wavelengths penetrate biologic tissue further than visible or infrared light. Light in the visible spectrum is largely absorbed by abundant biomolecules such as hemoglobin that are present in most tissues, whereas light in the infrared range is almost completely absorbed by water.[499] The ability to image fluorescing tissues has long been in use in the laboratory setting but has not yet found clinical utility. The development of "smart" near-infrared fluorescent imaging probes has allowed tumor-specific enzymatic activity to be directly imaged by fluorescence. These probes are synthesized such that the near-infrared fluorophores are attached to a peptide scaffold that, by virtue of its amino acid sequence, can be selectively cleaved by tumor-specific proteases. Fluorescence of the near-infrared fluorophores is quenched because of their close proximity in the intact peptide scaffold. Upon enzymatic cleavage in the tumor, the fluorophores are released, and the resultant near-infrared fluorescent signal provides a specific signal, allowing tumor visualization. This technique has been used successfully to detect tumor-specific cathepsin activity in colon- and breast-cancer models[496] and more recently has been used for real-time hepatobiliary interventions.[493,500] Although the depth of penetration of near-infrared light still limits this technique to structures near the surface, near-infrared fluorescent imaging has been used with success intraoperatively for sentinel lymph node[501] identification and has great potential to provide guidance in determining tumor margins and sites of lymph node spread and in evaluating cavities and tissue linings such as the peritoneum, which are poorly imaged by other techniques.[502]

Magnetic Resonance Spectroscopy

Whole body MRI imaging uses the aggregate signal characteristics of a given tissue to generate high-resolution images that reveal information about both the anatomy and state of health of the tissue but give little direct information about the biochemical makeup of the tissue. In contrast, MR spectroscopy focuses on acquiring chemical information from small volumes of tissue, with the aim of characterizing the ongoing chemical processes that are unique to specific tissues. This is accomplished by separating the signal derived from specific tissue and cellular constituents into their different chemical forms. The protons contained within tissues experience a molecular environment that is unique to the tissue. These variations in molecular environment produce alterations or chemical shifts in the resonant frequency of the constituent protons. In theory, when the nuclear MR (NMR) signal is displayed as a function of resonant frequency, the different chemical forms in the tissue should yield a unique "spectroscopic fingerprint." In practice, large water and lipid peaks dominate the proton NMR spectra from biological tissues, with the less abundant elements giving lower signal. Because of the need to reduce heterogeneity of tissue within the voxel being interrogated, the small volumes of tissue evaluated by MR spectroscopy generally produce low signal, and prolonged imaging times are needed to acquire useful spectra. With prolonged imaging times, degradation of spectra from motion artifact occurs, and to date the majority of the routine spectroscopic examinations have focused on applications in the brain, where the tissues being examined remain relatively static.[52]

With higher field-strength magnets, faster scanning sequences, and advances in postprocessing techniques, ^{1}H-MR spectroscopy is increasingly being used for nonneurologic body applications. Some investigators have used ^{1}H-MR spectroscopy together with dynamic contrast-enhanced MRI to show that increases in vertebral marrow-fat content and accompanying decreases in marrow perfusion correlated with varying states of osteopenia and osteoporosis.[503] Others have evaluated the utility of ^{1}H-MR spectroscopy to characterize and monitor response to therapy in some pediatric solid tumors including neuroblastomas[504] and in adult prostate cancer.[505] Response of neuroblastoma to chemotherapy has been shown to correlate with decreases in cellular Cho peaks,[506] although this had not been validated against conventional imaging techniques in prospective clinical trials.

^{1}H-MR spectroscopy has also been used to characterize bone and soft-tissue tumors, with elevated Cho levels detected in lesions shown to have a malignant histology but not in the majority of benign lesions.[507] Quantitative spectroscopic measurement of trimethylamine concentration in MPNSTs has also been reported as having potential for the differentiation of benign and malignant lesions[508] and could be combined with FDG uptake for better characterization of these lesions. Several years ago, Preul et al. suggested that a pattern-recognition analysis of the biochemical information obtained from ^{1}H-MR spectroscopy of brain tumors could reliably distinguish benign, low grade, and high-grade tumors and suggested that specific tumor and nontumor "fingerprints" might ultimately provide an accurate noninvasive preoperative characterization of particular lesions.[509] Although considerable progress has been made, much work is still needed to develop a similar "library" of characteristic spectra for other pediatric solid tumors.

Other nuclei that can be evaluated with MR scanners, particularly at higher field strengths, include ^{31}P-phosphorus, ^{13}C-carbon, and ^{23}Na-sodium. These nuclei all have lower gyromagnetic ratios than protons and resonate at lower frequencies than protons for a given field strength. The molecules and ions containing these nuclei are also much less abundant than water protons, so that the signal-to-noise ratios from these nuclei are low compared with those associated with proton MRI. As such, large voxels must be sampled from a given tissue in order to obtain a sufficient signal-to-noise ratio with a reasonable scan time. ^{31}P-MR spectroscopy enables the observation of energy metabolism and intracellular compartmentation through the signals of phosphomonoesters (PMEs), phosphodiesters (PDEs), inorganic phosphate (P_is), and nucleotide triphosphates, mainly adenosine triphosphate (ATP). Depending on the tissue being studied, a peak from phosphocreatine may also be seen (such as brain and muscle). When compared to ^{1}H-MR spectroscopy in the evaluation of bone and soft-tissue tumors, ^{31}P-MR spectroscopy was superior in identifying lesions shown to have a malignant histology.[507] Whether ^{31}P spectroscopic measurement of intratumoral changes in a phosphate metabolite profile will be feasible and valuable in response assessment remains to be validated.

SURVEILLANCE IMAGING

Surveillance imaging in pediatric oncology presents a unique challenge. Since the mid 1960s new treatment developments have continued to result in dramatic improvements in event-free and long-term overall survival rates for patients with many pediatric cancers.[510] For example, in Hodgkin lymphoma, long-term survival rates of greater than 90% can now be expected, with even higher rates of survival in lower-stage disease.[137,511] Along with these improvements in overall survival there has been a shift in the treatment paradigm. With efforts to develop criteria for upfront risk stratification coupled with response-based reduction in treatment intensity, current approaches are aimed at reducing late effects and improving overall treatment-related complications while preserving excellent long-term treatment outcome and survival. At the same time, advances in diagnostic imaging have led to a corresponding increase in number of imaging evaluations performed on these same patients.[512] Increases in use of conventional radiography, CT, MRI, and PET imaging, although providing important information about disease stage and response to therapy, are also costly and contribute to cumulative exposure to ionizing radiation, particularly from CT and nuclear medicine examinations.[512,513] Even though the doses of radiation from diagnostic imaging examinations are relatively low (particularly when compared with therapeutic doses) they are not without potential risk. With the improvements in patient outcome, a number of studies have begun to question the need for repeated high-intensity surveillance imaging and additional exposure to potentially harmful ionizing radiation for a population of patients who do very well once they have successfully completed therapy.[514-516] Patients with Hodgkin lymphoma often receive a large number of off-therapy imaging studies with no effect on overall patient outcome.[514] Similarly CT protocols that routinely include the pelvis in off-treatment surveillance of patients with Wilms tumor had no effect on disease detection and exposed gonadal tissue to potentially harmful additional radiation.[517] A recent study of patients with low-stage gonadal germ-cell tumors showed that tumor markers provided a sensitive and specific means of detecting patients with disease relapse; no relapses were identified in patients who were marker-negative.[518]

Taken together these studies underscore the need to reassess the frequency and type of surveillance imaging needed for the routine off-therapy monitoring of patients who have had successful treatment of their disease. Practitioners must be cognizant of the need to provide reassurance that disease relapse, if present, will be detected as early as possible while at the same time balancing these assurances against performing unnecessary, costly, and potentially harmful studies that have no impact on outcome.[519]

CONCLUSION

The approach to imaging pediatric oncology patients is rapidly evolving. The era when the radiologist played a peripheral role in the care of the child with cancer has been left behind. As outlined in this chapter, the imaging of children with cancer is complex and the role of radiology has changed. The development of new diagnostic imaging techniques and image-guided therapeutic interventions has lead to a new paradigm in which treatment designs are increasingly relying on imaging endpoints, with exciting opportunities and challenges to better detect, stage, and monitor malignant disease. The next decade of imaging will require close collaboration between the pediatric oncologist and the pediatric imaging specialist in order to determine which of these imaging techniques are best suited to these patients.

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CHAPTER 67

Infectious Disease in the Pediatric Cancer Patient

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INTRODUCTION

Infections are important issues faced by children with cancer. Invasive bacterial and fungal infections are problematic mainly in those receiving intensive treatment. Viruses may also be life-threatening in specific circumstances. Furthermore, non–life-threatening infections such as upper respiratory tract infection may still affect treatment and quality of life, particularly if febrile illnesses occur during neutropenia. In an effort to provide a comprehensive discussion of infection in pediatric cancer patients, this chapter focuses on general issues such as risk factors for infection, infection classification, and then approaches to intervention. Next, the chapter reviews major opportunistic pathogens, the risk factors and epidemiology of fever and neutropenia (FN), the therapeutic options for prophylaxis, infectious issues in pediatric cancer that are specific to low- and middle-income countries (LMIC), and recent directions involving preclinical research.

GENERAL ISSUES RELEVANT TO INFECTIONS

Risk Factors for Infection

Children with cancer are predisposed to infection for several reasons. The most common and important risk factor is neutropenia caused by myelosuppressive chemotherapy and cancer itself. Greater depth and duration of neutropenia are directly related to the risk of invasive bacterial and fungal infection and infectious mortality.[1,2] Corticosteroids are commonly used as anticancer therapy and as supportive care. The negative consequences of corticosteroids on the host immune system predispose to infections such as *Pneumocystis jiroveci,* other fungi, and bacteria and may be an independent risk factor for sepsis and infectious mortality.[3] Intensive chemotherapy also predisposes to infection through disruption of important protective anatomic barriers. The development of mucositis after chemotherapy administration provides a portal of entry for organisms colonizing the oral cavity and is a particularly important risk factor for viridans group streptococcal bacteremia.[4,5] Likewise, compromise of the gastrointestinal mucosa may clinically manifest as enteritis, typhlitis, and colitis and can facilitate translocation of enteric pathogens to the bloodstream. Beyond chemotherapy and corticosteroids, certain procedures performed in this patient population may predispose to infection. In many countries central venous lines (CVLs) are routinely used in patients with cancer, resulting in a risk for catheter-related bloodstream and local CVL site infections. Additionally, many children require surgery for various reasons, including tumor resection, and such procedures predispose to surgical site infection. Finally, children who receive allogeneic hematopoietic stem cell transplantation (HSCT) have a unique predisposition to infections related to graft-versus-host disease (GVHD) and therapies used to prevent and treat this complication of transplantation.[6]

Infection Classification

Infections can be classified by etiology as bacterial, fungal, viral, or protozoal, and these pathogens may infect sterile or nonsterile sites. The overall risk of infection varies depending on many factors, with intensity of chemotherapy being very important. Children who receive the most intensive chemotherapy include those with acute myeloid leukemia (AML). One study demonstrated that more than 60% of children with AML have at least one microbiologically documented infection during each phase of therapy, and the cumulative risk of infection-related mortality was 11 ± 2%.[7] In a subsequent pediatric AML analysis, more than 80% of children experienced at least one sterile site bacterial infection and 14% experienced at least one sterile site fungal infection throughout chemotherapy. The risk of sterile site bacterial infection was 30% to 60% per chemotherapy course.[8] In contrast, some children who receive non-myelosuppressive therapy such as those receiving maintenance therapy for acute lymphoblastic leukemia (ALL), have a very low risk of sterile site bacterial or fungal infections.[9]

Infections may also be classified by type of infection: microbiologically documented, clinically documented, or fever of unknown origin (FUO). In an analysis of children with cancer or recipients of HSCT presenting with FN, 80% of episodes were ultimately classified as FUO, 13% had a microbiologically confirmed etiology, 6% were clinically documented infection, and 2% were invasive mycosis.[9] The distribution of the etiology of FN will change depending on the presence of the aforementioned risk factors, especially the intensity of chemotherapy administered.

Intervention Approaches

The major approaches to reduction of infectious morbidity and mortality are prophylactic, empiric, and definitive treatment approaches. With prophylaxis, antimicrobials are administered before onset of fever or other clinical signs of infection. Empiric therapy consists of the administration of antimicrobial agents with early signs of infection, such as fever. Definitive treatment consists of administering specific antimicrobials once an infection has been documented.

MAJOR PATHOGENS

Bacteria

Bacteria are the most common cause of invasive infection in pediatric cancer patients. Over the last three decades, there has been a shift from gram-negative organisms being responsible for most infections in patients with cancer to gram-positive infections predominating.[10] The shift toward gram-positive agents has been attributed to the wide-spread use of CVLs, chemotherapy regimens associated with mucositis, and routine use of antibiotics with gram-negative activity.[11] The most common gram-positive infections are coagulase-negative staphylococci, viridans group streptococci, enterococci, and *Staphylococcus aureus*. The major types of gram-negative infections are *Escherichia coli, Klebsiella* species, and *Pseudomonas aeruginosa*.[9] Bacterial sterile site pathogens are most commonly tested for and isolated from blood culture, followed by urine and, less frequently, cerebrospinal fluid.

Fungi

Pediatric patients requiring intensive chemotherapy treatment for cancers such as AML, relapsed ALL, and allogeneic HSCT have the highest risk for invasive fungal infection (IFI) because of the resultant depth and duration of neutropenia. Additional IFI risk factors include mucositis, corticosteroid use, and antibiotic exposure.[12-20] The most common fungal infections in pediatric cancer are *Candida* spp. and *Aspergillus* spp.[21] Sterile site candidal infections typically occur in the blood and urine. In contrast, invasive aspergillosis typically involves the lungs, sinuses, gastrointestinal tract, and brain. Emerging fungal pathogens that are increasingly being encountered in pediatric oncology are Mucorales (formerly referred to as zygomycetes) and *Scedosporium* spp. These are particularly concerning because they tend to be fatal and are challenging to diagnose antemortem.[22,23]

P. jirovecii is a yeastlike fungal species that classically causes pneumonia in children with acute leukemia who do not receive *P. jirovecii* pneumonia (PCP) prophylaxis. In addition to chemotherapy exposure, corticosteroid use and age younger than 2 years are also risk factors for PCP.[24,25] PCP prophylaxis is considered standard care for many pediatric malignancies, including acute leukemia and HSCT recipients. Trimethoprim-sulfamethoxazole (TMP-SMX) is the prophylactic drug of choice. Although the optimal dosing regimen is not clear, administration regimens of 2 or 3 days per week are most common and have been shown to be reasonably effective.[26,27] Alternative regimens for allergy or other toxicities include oral dapsone, oral atovaquone, and inhaled pentamidine. Intravenous pentamidine is often used in children younger than 5 years who cannot tolerate the other options. However, clinicians should be aware that the effectiveness of intravenous pentamidine is questionable.[25]

Viral Infections

There are many viruses that can result in infections in pediatric cancer patients.[28] The list of potential agents continues to evolve with improved diagnostic abilities for previously unrecognized pathogens such as human metapneumovirus (HMPV). In one pediatric cancer series, respiratory syncytial virus (RSV) (31%) and rhinovirus (23%) were the most frequently detected respiratory viruses, followed by parainfluenza (12%) and influenza A (11%).[29] RSV usually does not cause life-threatening infections in children with cancer. However, in patients with AML and HSCT recipients who do acquire RSV, infection may progress to lower respiratory tract involvement. In this setting RSV infection is associated with a 14% case fatality rate in patients with AML and a 50% case fatality rate in pediatric HSCT recipients.[7,30] Adenovirus may also cause serious infection, particularly in HSCT recipients,[31] and is a frequent cause of death in this setting.[32-35] Other viral infections, including varicella-zoster virus (VZV), influenza, and cytomegalovirus, are typically not life threatening in pediatric patients with cancer who are receiving less intense therapy, but they may cause severe infection and infectious mortality in the most intensively treated children. Conversely, some infections, such as severe acute respiratory syndrome (SARS), can cause fatal infection in immunocompetent patients but is rarely associated with severe illness in pediatric cancer.[36] It is hypothesized that an intact immune system is responsible for severity of illness and fatality in SARS, thus explaining this apparent paradox.

Protozoal Infections

In general, protozoal infections are not a common cause of fever in children with cancer in high-income countries, although these pathogens may be more prominent in some LMICs.[37]

FEVER AND NEUTROPENIA

FN is one of the most common complications of cancer therapy in pediatric patients. Episodes of FN are associated with considerable morbidity, reduction in quality of life (QoL), and high costs, and fatal outcomes still occur despite aggressive antimicrobial interventions.[2,38] Much research has been conducted in both adult and pediatric FN over the last several decades, which has allowed different therapeutic approaches to evolve while reducing mortality and improving other clinical endpoints.[39,40]

Initial Risk Stratification

Children with FN are not a homogenous group. Determining which children are at lower risk of complications can allow for a reduction in the intensity of therapy and monitoring. Conversely, identifying children at higher risk of complications can allow for escalation of therapy and closer observation.

In adults with FN, the risk stratification schema from the Multinational Association of Supportive Care in Cancer (MASCC) has been validated and is widely accepted as a standard approach. However, the MASCC score cannot be applied to children because age below 60 years and absence of chronic obstructive pulmonary disease are two items in the score and these are not applicable to pediatric patients.[41] At least 25 studies of risk prediction have been conducted in pediatric cancer.[42] These studies have been highly variable using different pediatric cancer populations and different endpoints (e.g., bacteremia, "serious infection," death, and intensive care unit admission), making the results more difficult to interpret.

Although six of these low-risk stratification schemas have been validated in children (Table 67-1), identification of a single schema to be applied across all clinical scenarios has not been feasible, likely because of the divergence in clinical settings. Therefore clinicians should review the six validated low-risk stratification schemas, choose which schema matches their clinical setting, and determine whether the application of that schema is feasible for their center. For example, some rules use biomarkers such as C-reactive protein and would require expeditious analysis and return of results to be useful. Others require clinical judgment and thus require trained health care professionals to be readily available. There are no validated risk stratification schemas to identify the pediatric high-risk patient with FN.

Initial Investigations

At initial presentation, an evaluation for the cause of fever should be conducted. The evaluation begins with a careful history and physical examination, with the assessment focusing on potential sites of infection. Sites that merit particular attention are the mouth to search for herpes simplex virus stomatitis and dental abscess, the exit site and tunnel of CVLs, perianal region for cellulitis and perirectal abscess, and fingers and toes for paronychia.

The standard evaluation should include blood cultures, with further diagnostic testing dictated by clinical signs and symptoms. Blood cultures should be obtained from each lumen of the CVL if present. The utility of a peripheral blood culture at the initial evaluation of FN is controversial. There are seven studies that evaluated the contribution of peripheral blood cultures in addition to CVL cultures in adults and children with cancer or undergoing HSCT.[43-49] When these results were combined, 13% (95% confidence interval [CI] 8% to 18%) of all true bacteremias were identified only by the peripheral blood culture. This result is likely explained by false-negative CVL blood cultures when obtained culture volumes are small.[50] However, it is not known how failure to identify these episodes of bacteremia affect patient outcomes or whether this problem can be overcome by optimizing CVL blood culture volumes. Consequently, the role of peripheral blood culture remains uncertain.

Urinalysis and urine culture testing for the detection of urinary tract infections (UTIs) is also considered controversial in the setting of FN. In immunocompetent children, a UTI is often diagnosed when there is both evidence of pyuria on urinalysis and a positive culture. The state of neutropenia limits the ability to use pyuria as a diagnostic criterion,[51] and reliance on nitrite testing is not ideal because the test may be negative in younger children with UTI.[52] Therefore urinalysis testing has limited utility in the setting of FN. It may be reasonable to obtain a sterile urine culture and define the presence of a UTI on the culture results alone. However, sterile urine

TABLE 67-1 Validated Pediatric Risk Stratification Strategies for Low-Risk Patients

	Rackoff (1996)[132]	Alexander (2002)[133]	Rondinelli (2006)[134]	Santolaya (2001)[135]	Ammann (2003)[136]	Ammann (2010)[137]
Patient and disease-related factors	None	AML, Burkitt lymphoma, induction ALL, progressive disease, relapsed with marrow involvement	2 points for central venous catheter, 1 point for age ≤5 years	Relapsed leukemia, chemotherapy within 7 days of episode	Bone marrow involvement, central venous catheter, pre–B-cell leukemia	4 points for chemotherapy more intensive than ALL maintenance
Episode-specific factors	Absolute monocyte count	Hypotension, tachypnea/hypoxia <94%, new CXR changes, altered mental status, severe mucositis, vomiting or abdominal pain, focal infection, other clinical reason for in-patient treatment	4.5 points for clinical site of infection, 2.5 points for no URTI, 1 point each for fever >38.5°C, hemoglobin ≤70g/L	CRP ≥90 mg/L, hypotension, platelets ≤50 g/L	Absence of clinical signs of viral infection, CRP >50 mg/L, white blood cell count ≤500/µL, hemoglobin >100 g/L	5 points for hemoglobin ≥90 g/L, 3 points each for white blood cell count <300/µL, platelet <50 g/L
Rule formulation	Absolute monocyte count ≥ 100/µL = low risk of bacteremia HSCT = high risk	Absence of any risk factor = low risk of serious medical complication HSCT = high risk	Total score <6 = low risk of serious infectious complication HSCT = high risk	Zero risk factors or only low platelets or only <7 days from chemotherapy = low risk of invasive bacterial infection	Three or fewer risk factors = low risk of significant infection HSCT = high risk	Total score <9 = low risk of adverse FN outcome HSCT = high risk
Demonstrated to be valid*	USA Madsen 2002[138]	United Kingdom Dommett 2009[139]	Brazil Rondinelli 2006[134]	South America Santolaya 2002[140]	Europe Ammann 2010[137], Macher 2010[141]	Europe Miedema 2011[142]

Adapted from Lehrnbecher et al.[79]

ALL, Acute lymphoblastic leukemia; *AML,* acute myeloid leukemia; *CRP,* C-reactive protein; *CXR,* chest radiograph; *FN,* fever and neutropenia; *URTI,* upper respiratory tract infection; *USA,* United States of America.

*"Valid" refers to clinically adequate discrimination of a group at low risk of complications.

collection can be difficult in young children and infants and often requires urinary catheterization, a procedure that is seldom recommended for a neutropenic patient. Therefore where a clean-catch or midstream urine can be collected, the results of urinalysis and urine culture should be included in the initial FN investigations. It is important to remember that antibiotic administration should not be delayed to obtain a urine sample.

There are have been four studies,[53-56] including 540 episodes of FN, that investigated the impact of chest radiography (CXR) as a component of the initial FN evaluation. These studies indicate that pneumonia is detected in fewer than 5% of children without respiratory symptoms and that the omission of routine CXR does not lead to adverse outcomes.[53,57] Thus routine CXR should not be performed in asymptomatic children with FN.

Initial Antibiotic Therapy

In general, empiric antimicrobial therapy choices should provide good coverage for gram-negative organisms with some gram-positive coverage. In particular, for high-risk patients with FN, antibiotic therapy should include coverage for viridans group streptococci and *P. aeruginosa*. Various factors must be considered when determining an empiric antibiotic regimen, route of administration, and location of therapy. These factors include patient and family social factors, clinical presentation, ability of the hospital to support an ambulatory approach, availability and cost of drugs, and local hospital resistance patterns.

The role of combination antibiotic therapy versus monotherapy for FN has been debated. Monotherapy was supported by two metaanalyses that compared monotherapy versus an aminoglycoside-containing regimen in FN[58] and in immunocompromised patients with sepsis.[59] These analyses demonstrated that monotherapy is not inferior and is less toxic than combination therapy. The meta-analysis in FN observed fewer treatment failures with monotherapy (odds ratio [OR] 0.88, 95% CI 0.78 to 0.99) but included only four trials that enrolled patients younger than 14 years of age.[58] In the pediatric setting monotherapy was supported by a meta-analysis that found similar clinical outcomes when antipseudomonal penicillin monotherapy was compared to antipseudomonal penicillin plus an aminoglycoside.[60] Therefore monotherapy is suggested for FN in pediatric patients who are clinically stable and who are treated at centers with a low rate of resistant pathogens.

Monotherapy regimens that have been evaluated in children include antipseudomonal penicillins such as piperacillin-tazobactam and ticarcillin-clavulanic acid, antipseudomonal cephalosporins such as cefepime, and carbapenems such as meropenem or imipenem. In two pediatric-specific evaluations, treatment failure, mortality rates, and adverse effects were similar when antipseudomonal penicillins were compared with antipseudomonal cephalosporins or carbapenems.[61,62] There are potential downsides of carbapenems and cefepime. Carbapenems were associated with more pseudomembranous colitis compared with other beta-lactam antibiotics in a large meta-analysis.[62] Cefepime was associated with increased all-cause mortality in another large meta-analysis when compared to other beta-lactam treated patients.[62] However, these findings were not replicated in other studies.[53,63] Consequently, cefepime remains an option for empiric therapy. Ceftazidime monotherapy lacks adequate coverage against viridans group streptococci and resistant gram-negative organisms and thus should not be used if these organisms are of concern.[64]

Empiric glycopeptides (e.g., vancomycin) are not routinely recommended. A largely adult meta-analysis of 14 randomized controlled trials (RCTs) illustrated that inclusion of a glycopeptide did not lead to a difference in success (if addition of glycopeptide in the control arm was not considered failure) but was associated with more adverse effects.[65] Empiric glycopeptides should be reserved for patients who are clinically unstable or who have signs or symptoms suggestive of a gram-positive infection.

Considerations for Low-Risk Patients with Fever and Neutropenia

Patients with FN who are at lower risk of adverse outcomes may be appropriate for a reduction in therapy intensity. Two strategies commonly considered are outpatient management and oral antibiotic administration. These two strategies are commonly used together, and in adults with low-risk FN, outpatient management with oral antibiotics is recommended for selected patients.[20,41] Some experts have expressed concern that these recommendations may not be generalizable to children.[66] However, over the last several years data have emerged related to efficacy and safety, costs, QoL and preference considerations for different management strategies in pediatric FN.

Efficacy and Safety of Outpatient Management and Oral Antibiotic Administration

The advantages of outpatient management include better QoL for children[67] and a reduction in costs,[68] nosocomial infection,[69] and acquisition of resistant microorganisms.[70] Outpatient management can be instituted at the onset of FN or after a brief period of hospitalization (step-down management). A meta-analysis synthesized the results of six RCTs, two of which were pediatric.[71] There was no difference in treatment failure with outpatient versus inpatient management (rate ratio [RR] 0.81, 95% CI 0.55 to 1.28) where the RR of less than 1 favored inpatient care. It is important to emphasize that failure was biased against outpatient care insofar as readmission was considered failure and this endpoint is applicable only to outpatients. No difference in mortality was demonstrated (RR 1.11, 95% CI 0.41 to 3.05). Results stratified by the two pediatric studies demonstrated similar findings as the overall analysis. The major concern with these data relates to the small number of children included; the two pediatric studies enrolled only 278 children. A subsequent systematic review combined all prospective randomized and nonrandomized pediatric trials that evaluated ambulatory or inpatient management within 24 hours of FN.[72] Among the 16 included studies, treatment failure was significantly less with outpatient management (15%) compared with inpatient management (27%, *P* = .04). There were no infection-related deaths among the 953 children treated as outpatients. Consequently, outpatient management appears to be a safe approach as long as appropriate patients can be selected and adequate follow-up monitoring is established. To institute outpatient management of FN, the institution must be able to identify low-risk patients and develop a program to monitor patients and expeditiously admit them in the case of deterioration. Social circumstances and travel considerations will dictate the feasibility of an ambulatory approach for a specific patient. The optimal frequency and nature of follow-up evaluations for children treated as outpatients for FN has not been determined, although daily clinic visits will rarely be feasible.

Oral antibiotic administration may be advantageous because it facilitates outpatient management, is usually less expensive, and does not require intravenous access. However, oral administration requires suspension formulation in younger children, and some children may refuse oral medication, particularly when they are unwell. There are two metaanalyses of RCTs that compared oral and parenteral antibiotic administration for FN; both did not restrict their review to low-risk patients. One included inpatient and outpatients (N = 2770),[73] whereas the other evaluated only outpatients (N = 1595).[71] Results were similar in both analyses, with no difference in treatment failure, mortality, or adverse effects of antibiotics by mode of administration. Results were similar when restricted to pediatric studies except for a trend toward lower risk of readmission for outpatient episodes treated with intravenous antibiotics compared with oral antibiotics (RR 0.52, 95% CI 0.24 to 1.09).[71] More data about the safety of oral administration were obtained from a meta-analysis of prospective pediatric trials, which instituted oral antibiotics within 24 hours of FN onset.[72] There was no difference in treatment failure among those who received oral versus intravenous antibiotics (20% versus 22%, *P* = .68). There was also no difference in the rate of antibiotic discontinuation resulting from adverse events (2% versus 1%, $P = .73$). No infection-related deaths were observed among the 676 children given oral antibiotics. To summarize, more readmissions were observed among children treated with oral antibiotics in the outpatient setting, although no difference in treatment failure or adverse events occurred and no child treated with oral antibiotics within 24 hours of FN onset died. Thus oral antibiotic administration may be appropriate if the health care team is confident that the child can tolerate this route reliably. Oral antibiotic regimens that have been used in pediatric FN include fluoroquinolone monotherapy, fluoroquinolone and amoxicillin-clavulanate, and cefixime.[72] One practical approach is to provide the first oral dose in the emergency or outpatient department to ensure that the child can tolerate oral administration of the planned empiric antibiotic. Discharge home would be contingent on successful administration. Even for children with low-risk FN who are managed as inpatients, oral administration may be advantageous because it is a more effective use of nursing resources and may facilitate early discharge depending on the reason for admission.

Costs

A pediatric cost-utility model demonstrated that outpatient management was the most cost-effective approach for children with low-risk FN.[68] Outpatient parenteral management was more cost-effective compared with outpatient oral management because of the higher rate of readmission among those who receive oral antibiotics. However, in sensitivity analyses, outpatient oral management may be more cost-effective depending on model assumptions. Inpatient management with intravenous antibiotic administration was the least cost-effective approach. These data suggest that outpatient management with intravenous or oral antibiotics is a better strategy for pediatric low-risk FN when probabilities, costs, and QoL are considered.

Preferences and Quality of Life

In implementing ambulatory and oral approaches for low-risk FN, consideration of patient and family preferences may facilitate program development. When asked which approach they preferred, approximately 50% of parents preferred inpatient intravenous management.[74,75] Both parents and children typically ranked inpatient intravenous management ahead of early discharge or ambulatory approaches.[75] A discrete choice experiment was used to assess preferences toward FN management.[76] Discrete choice experiment is an emerging method for the measurement of preferences in the face of multiple trade-offs in health care. Parents were willing to tolerate only 2.1 (95% CI 1.1 to 3.2) clinic visits weekly to accept outpatient oral management. If a program were developed with clinic visits three times weekly and a 7.5% chance of readmission, the probability of parental acceptance of such an ambulatory program was 43% (95% CI 39 to 48%).[76]

Estimation of QoL is also important to conduct cost-utility analyses, as previously described. In one study in which parents and health care professionals compared inpatient intravenous and outpatient oral management,[74] respondents rated child QoL as higher at home compared with hospital. Compared with parents, health care professionals overestimated QoL for children at home and underestimated QoL for parents in hospital. In another study in which parents rated their children's QoL with different FN management options, early discharge and outpatient intravenous therapy were associated with the highest anticipated QoL.[75]

These data suggest that parents may have reservations about an ambulatory oral antibiotic approach. In a qualitative study the major themes identified when parents make decisions regarding site of care and route of drug administration were convenience and disruptiveness for the family, the child's physical and emotional health, and modifiers of parental decision making.[77] Reasons for preferring an inpatient approach include the inconvenience of clinic visits, apprehension regarding whether they can adequately monitor their child, and concerns related to oral antibiotic administration. In summary, although the child's QoL is anticipated to be better with outpatient management, many parents prefer inpatient management.

Modification of Empiric Antibacterial Therapy

After therapy for FN has been initiated, the initial empiric regimen should be adjusted to provide appropriate coverage for any positive microbiology results or identified clinical focus of infection. In patients in whom empiric glycopeptides or dual gram-negative coverage was initiated, reassessment at 24 to 72 hours should be conducted. In the absence of a specific microbiological reason to continue these agents, they should be discontinued. In the event of persistent fever, careful evaluation for an undetected source of infection is important. In this setting modification of antibiotics, including addition of empiric vancomycin, is not warranted in children who remain clinically stable.[78] Children who clinically deteriorate warrant broadening of empiric antibacterial therapy to include coverage for resistant gram-positive, gram-negative, and anaerobic organisms.

Empiric antibiotics should be discontinued if cultures are negative, the child is clinically well, fever has resolved, and there is evidence of neutrophil recovery.[79] One randomized trial of pediatric low-risk patients found that cessation of antibiotics on day 3 irrespective of count recovery versus continuation of antibiotics was associated with similar outcomes.[80] However, *Enterobacter* bacteremia occurred in one child in the early cessation study arm. Consequently, it is reasonable to discontinue antibiotics on day 3 in low-risk children with FN who have become afebrile with negative cultures as long as careful monitoring is in place. In high-risk patients the optimal duration of antibiotic therapy is unknown in the setting of persistent profound neutropenia. A small study of 33 high-risk patients suggested that cessation of empiric antibiotics on day 7 is associated with bacteremia and poor infection outcomes compared with continuation for 14 days.[81] Thus continuation of empiric antibiotics for at least 14 days for high-risk FN in the absence of evidence of neutrophil recovery is a reasonable strategy.

Evaluation for Invasive Fungal Infection and Empiric Antifungal Therapy

Children with FN and persistent or recurrent fever that persists 96 hours or more after initiation of broad-spectrum antibiotics and who are at higher risk of IFI should undergo an evaluation for fungal infection, including careful physical examination, blood and urine cultures, and computerized tomography (CT) of the chest.[82-85] The role of routine CT sinuses and imaging of the abdomen have not been defined in the standard investigation of IFI, although routine CT sinuses should not be conducted in children 2 years of age or younger because of insufficient pneumatization of the sinus cavities.[79] In children with demonstrated pulmonary lesions, investigations may include bronchoalveolar lavage and lung biopsy, although fatal bleeding may occur with biopsy of any angioinvasive mold lesion. Thus the decision to biopsy requires careful consideration.

Empiric antifungal therapy should consist of either caspofungin or liposomal amphotericin B (L-AmB) because these two therapies are similarly effective and L-AmB is slightly better and less nephrotoxic than amphotericin B deoxycholate.[86-88] Empiric antifungal therapy may be discontinued at resolution of severe neutropenia if the patient is clinically well without evidence of an IFI.

PROPHYLACTIC STRATEGIES

Antibacterial Prophylaxis

The approach to routine antibacterial prophylaxis is uncertain because the overall balance of benefit and harm is not clear.[20] Most of the interest has been focused on fluoroquinolones because of broad spectrum of activity, relative preservation of gastrointestinal tract anaerobic flora, high concentration in the feces, systemic bactericidal activity, tolerability, and lack of myelosuppression. A large meta-analysis of RCTs composed mostly of adult subjects found that antibiotic prophylaxis decreased the risk of death (RR 0.67, 95% CI 0.55 to 0.81), infection-related mortality (RR 0.58, 95% CI 0.55 to 0.81), and bacteremia (RR 0.052, 95% CI 0.46 to 0.59).[89] Some uncertainty remains regarding whether these benefits are applicable to contemporary patients given the changing spectrum of gram-positive and gram-negative bacteria over time.

The decision to use routine antibacterial prophylaxis must include consideration of the negative consequences of prophylaxis. These consequences appear to be important to clinicians because most North American and German centers do not routinely use antibacterial prophylaxis for children with AML.[90] Potentially harmful effects include predisposition to IFI, *Clostridium difficile* colitis, and potential contribution to evolving antibiotic resistance. Additionally, the physician must consider the toxicities of the specific antibiotic chosen. The previously cited meta-analysis of antibacterial prophylaxis also showed that prophylaxis was associated with more adverse events (RR 1.57, 95% CI 1.33 to 1.86).[89] Parents also considered the frequency of administration, adverse effects, and costs in decision making related to antibacterial prophylaxis.[91]

Antifungal Prophylaxis

Because IFIs are relatively difficult to diagnose and effectively treat, there has been considerable interest in determining the effectiveness of a prophylactic approach, particularly in children undergoing allogeneic HSCT and children with AML, two populations at high risk for IFI.[92] Fluconazole prophylaxis was compared with placebo in two RCTs of allogeneic HSCT recipients.[93,94] Fluconazole decreased the occurrence of IFI because of a reduction in invasive candidiasis. Because children undergoing allogeneic HSCT are at risk for molds in addition to yeasts and because fluconazole does not provide any coverage against molds, there has been great interest in exploring the role of prophylactic antimold coverage. However, thus far agents with antimold activity, including micafungin, voriconazole, and amphotericin products, have not proved to have clinically significant advantages over fluconazole prophylaxis in the HSCT population.[95-97,98-100] Furthermore, some agents, such as itraconazole, showed a higher rate of toxicity leading to withdrawal[101] and agents such as amphotericin B led to infusion-related toxicities and renal toxicity.[102]

In pediatric AML a meta-analysis suggested that fluconazole significantly reduced the risk of IFI when the baseline rate was at least 15%.[103] The efficacy of itraconazole prophylaxis has also been explored. Although this agent has been shown to be effective compared with placebo or no therapy, no advantage over fluconazole or voriconazole has been demonstrated.[104-108] Posaconazole is an antifungal agent with broader antimold activity than voriconazole and thus represents a potentially better prophylactic option. In a randomized trial of patients with AML or myelodysplastic syndrome and prolonged neutropenia, posaconazole prophylaxis, when compared with fluconazole or itraconazole, reduced the rate of proven or probable IFI. Furthermore, overall survival rate was significantly better in posaconazole-treated patients.[109] However, only 16 adolescents between the ages of 13 and 18 years were included, and thus the generalizability of the results to children is limited. Furthermore, administration of posaconazole to children younger than 13 is challenging because of a lack of dosing information and the requirement to administer the drug orally with adequate food intake, which can be a challenge for children undergoing HSCT or those receiving chemotherapy for AML.

To better understand the contribution of mold-active prophylaxis, a systematic review of RCTs compared mold-active versus fluconazole prophylaxis in patients with cancer or those who underwent HSCT. Of 20 studies, children were included in four trials but only one trial was exclusively pediatric. Mold-active prophylaxis, when compared with fluconazole prophylaxis, significantly reduced the risk of IFI (RR 0.71, 95% CI 0.52 to 0.98), invasive aspergillosis (RR 0.53, 95% CI 0.37 to 0.75), and IFI-related mortality (RR 0.67, 95% CI 0.47 to 0.96). However, mold-active agents had significantly more associated adverse events leading to antifungal prophylaxis discontinuation or modification (RR 1.95, 95% CI 1.24 to 3.07). Mold-active prophylaxis did not influence overall mortality (RR 1, 95% CI 0.88 to 1.13).

Granulocyte and Granulocyte-Macrophage Colony Stimulating Factors

Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are hematopoietic colony-stimulating factors (CSFs) that reduce the duration and severity of neutropenia in chemotherapy recipients.[110,111] In a meta-analysis of 16 RCTs of prophylactic use of CSFs in pediatric patients with cancer, the mean rate of FN in the control arms was 57% (range 39 to 100%).[112] CSFs reduced the rate of FN (RR of 0.80, 95% CI 0.67 to 0.95), documented infection (RR 0.78, 95% CI 0.62 to 0.97), and amphotericin B use (RR 0.50, 95% CI 0.28 to 0.87). There was no difference infection-related mortality (RR 1.02, 95% CI 0.34 to 3.06).[112] A meta-analysis of prophylactic CSF administration in the HSCT setting found that CSFs reduced the risk of documented infections (RR 0.87, 95% CI 0.76 to 1) but did not significantly reduce infection-related mortality (RR 0.76, 95% CI 0.41 to 1.44). Finally, a meta-analysis of 148 trials that included any cancer or HSCT population found that CSFs did not reduce short-term all-cause mortality (RR 0.95, 95% CI 0.84 to 1.08). A reduction of infection-related mortality was shown, but this did not reach statistical significance (RR 0.82, 95% CI 0.66 to 1.02). CSFs reduced documented infections (RR 0.85, CI 0.79 to 0.92), microbiologically documented infections (RR 0.86, 95% CI 0.77 to 0.96), and FN (RR 0.71, 95% CI 0.63 to 0.80).[113] Consequently, CSFs reduce FN and infections but do not influence overall survival. Whether CSFs should be administered therefore should depend on preferences, QoL considerations, and costs.

Prevention of Central Line–Associated Bloodstream Infection

A central line–associated bloodstream infection (CLABSI) can be defined as a bloodstream infection occurring in the presence of a CVL (present during the 48-hour period before development of the infection) and not related to infection at another site.[114] The most common causes of CLABSI are coagulase-negative staphylococci, *S. aureus*, *Enterococcus* spp., and *Candida* spp.[115] An evidence-based guideline supported by multiple agencies, including the Centers for Disease Control and Prevention, has been developed.[116] Recommendations included providing education and training focused on insertion and maintenance of CVLs and in particular indications for their use and competencies around insertion technique. Other recommendations were to use maximum barrier precautions and greater than 0.5% chlorhexidine skin preparation before CVL insertion and to critically assess the need for a CVL on an ongoing basis. Finally, the guideline recommended consideration of chlorhexidine-impregnated sponges and antimicrobial- or antiseptic-impregnated short-term CVL if other strategies for CLABSI reduction are not successful.

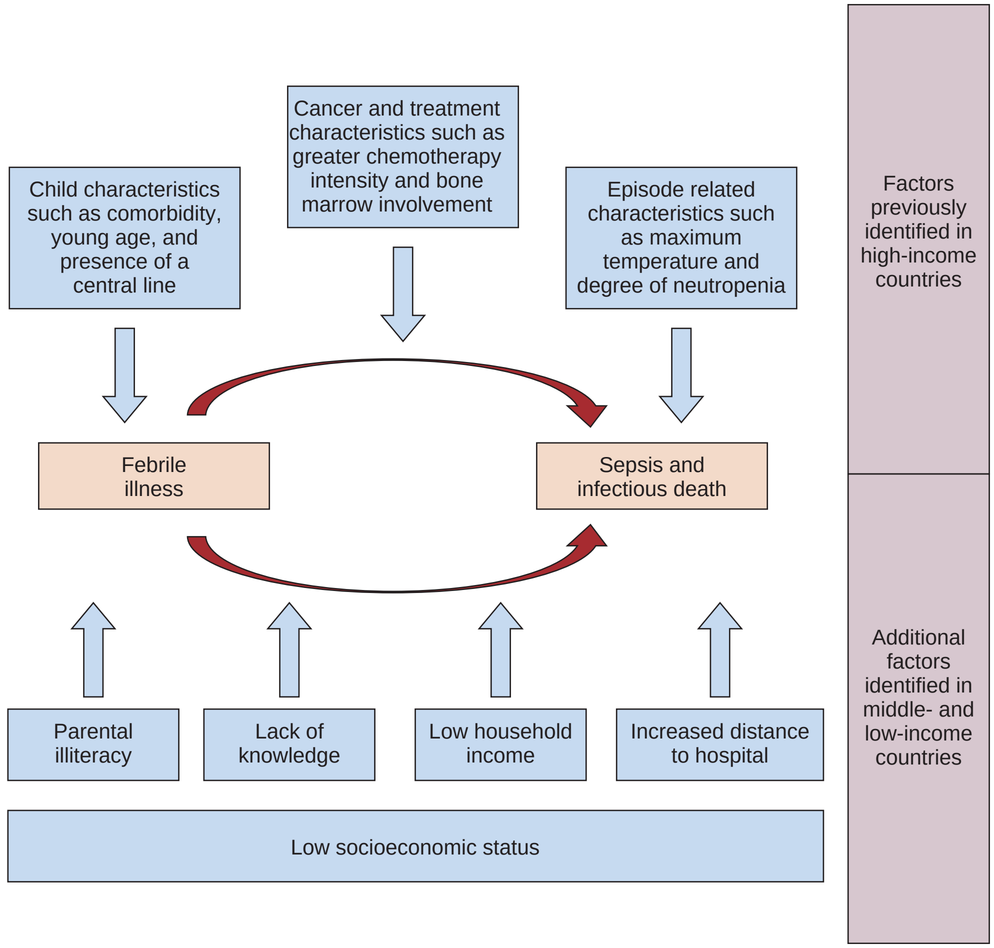
INFECTIOUS ISSUES IN PEDIATRIC CANCER PATIENTS IN LOW- AND MIDDLE-INCOME COUNTRIES

Children who live in low- and middle-income countries (LMICs) make up the majority of pediatric patients with cancer worldwide. As a group, they have not benefited from the same improvements in cancer outcomes compared with children who live in high-income countries.[117] Excessive infection-related mortality has contributed to this survival gap. In one study from El Salvador, treatment-related mortality (TRM) was responsible for 50% of deaths in children with ALL and AML;[118] the 2-year cumulative incidence of TRM was 12.5 ± 1.7% for ALL and 35.1 ± 6.4% for AML. The most important cause of TRM was infections. In a second study of FN among all cancer types in El Salvador, 12.3% of episodes were noted to result in death.[119]

Given the excessive risk of infectious toxicity, the identification of modifiable risk factors becomes paramount in this setting. Among children with ALL (but not AML), low monthly income and low parental education were associated with significantly higher TRM.[118] A subsequent prospective observational study in El Salvador sought to determine the association between socioeconomic status and time to assessment and treatment of fever and with rates of sepsis and infectious mortality.[120] Among 269 febrile outpatient episodes, the major source of delay was time from fever onset to decision to seek care, with 75% of parents waiting 5 hours or more. Once the decision to seek care had been made, the median time to reach the hospital was 1.8 hours. Parents who were illiterate had a 12- to 16-hour delay in deciding to seek medical care compared with parents who were literate. Once the decision to seek medical care had been made, parental literacy did not influence time to reach hospital. Maternal illiteracy was an independent risk factor for sepsis (OR 3.2, 95% CI 1.2 to 8.1), and lower annual household income was associated with infectious death (OR 13.9, 95% CI 1.6 to 119.1). These findings suggest that poor socioeconomic status translates into a delay in deciding to seek care and receiving medical attention in febrile children with leukemia. Figure 67-1 summarizes the different factors that affect the rates of infections and infection outcomes for children receiving cancer care in high-income countries and LMICs. The factors in high-income countries are predominantly treatment-related issues, whereas the factors in LMICs also include social and economic issues. Therefore any intervention employed to reduce TRM in LMICs must be tailored to these socioeconomic factors.

RECENT DIRECTIONS INVOLVING PRECLINICAL RESEARCH

Continued laboratory and translational investigation are critical to ensuring new advances in infectious diseases supportive care for future pediatric oncology patients. The fields of synthetic biology, immunotherapy, and gut mucosal immunology hold particular promise for


Cancer and treatment characteristics such as greater chemotherapy intensity and bone marrow involvement
Child characteristics such as comorbidity, young age, and presence of a central line
Episode related characteristics such as maximum temperature and degree of neutropenia
Factors previously identified in high-income countries
Febrile illness
Sepsis and infectious death
Parental illiteracy
Lack of knowledge
Low household income
Increased distance to hospital
Low socioeconomic status
Additional factors identified in middle- and low-income countries

* Based on a systematic review in pediatric oncology. Factors listed are illustrative and not meant to be exhaustive.

Figure 67-1 Factors associated with sepsis and infectious deaths in pediatric acute leukemia in low- and middle-income countries. *(From Gavidia R et al: Low socioeconomic status is associated with prolonged times to assessment and treatment, sepsis and infectious death in pediatric Fever in el salvador.* PLoS One *7:e43639, 2012.)*

novel methods of prevention and treatment of infectious diseases.

Synthetic biology is an emerging field focused on engineering biomolecular systems and cellular capabilities for a variety of applications, including the treatment of infections.[121] For example, bacteriophage (viruses that infect only specific bacteria) have been engineered to express a bacterial biofilm–degrading enzyme, dispersin B, that acts not only to rapidly degrade the biofilm matrix but also to kill 99.997% of the bacterial cells in the biofilm.[122] In another study synthetic adjuvants were designed by engineering bacteriophage to disrupt bacterial networks critical for antibiotic defense mechanisms, thereby resulting in significantly enhanced killing of bacterial strains by quinolones, beta-lactams, and aminoglycosides (in some cases a 5000-fold increase in killing).[123] Thus in the future bioengineered bacteriophages could help treat prosthetic device and CVL infections and also serve as adjuvants for treating antibiotic-resistant bacterial strains.

The use of immunotherapy in the prevention and treatment of infectious diseases is rapidly evolving. Adoptive transfer of antigen-specific T cells has proved to be an effective and powerful therapeutic tool in the prevention and treatment of viral infections (e.g., cytomegalovirus,[124] Epstein-Barr virus [EBV],[125] and adenovirus[126]) in immunocompromised hosts, but its use has been limited by the time needed to generate these cells. The technology has now advanced so that multivirus-specific (EBV, cytomegalovirus, adenovirus, BK, human herpes virus 6, RSV, and influenza) cytotoxic T lymphocytes (CTLs) can be generated within 10 days[127], and the possibility for using multivirus-specific CTLs for prophylaxis and treatment of viral infections, particularly in patients with leukemia and those undergoing HSCT, is encouraging.

The human microbiome, the aggregate of microorganisms that reside on and within the human host, has been shown to be critical for maintaining human immune function. Recent studies have shown that commensal microbes stimulate the gastrointestinal epithelium to produce antimicrobial proteins that act as a first line of defense against invading pathogenic bacteria.[128] When indigenous commensals are depleted after antibiotic administration, select pathogenic bacteria (e.g., vancomycin-resistant enterococci) can overgrow and cause invasive disease.[129] Interestingly, by stimulating intestinal Toll-like receptor (TLR) 4 by oral administration of lipopolysaccharide[129] or by stimulating TLR-5 with purified flagellin,[130] the antimicrobial protein RegIII gamma could be re-induced, and subsequently vancomycin-resistant enterococci levels in the murine gastrointestinal tract were significantly decreased and disseminated disease was prevented. As for human correlates, patients undergoing allogeneic HSCT uniformly developed reduced gut microbial diversity (as determined by deep 16S rRNA sequencing), with many patients exhibiting intestinal domination, defined as occupation of at least 30% of the microbiota by a single predominating bacterial taxon. As a predictor of outcomes, enterococcal domination increased the risk of vancomycin-resistant enterococcal bacteremia ninefold, and proteobacterial domination increased the risk of gram-negative bacilli bacteremia fivefold.[131] Thus methods for maintaining microbial homeostasis during chemotherapy and antibiotic therapies may provide a novel means for preventing colonization and dissemination of pathogenic microbes in cancer patients.

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CHAPTER 68

Oncologic Emergencies

Elizabeth A. Mullen and Eric Gratias

CHAPTER OUTLINE

ANTERIOR MEDIASTINAL MASSES
Definition
Clinical Presentation
Evaluation and Imaging
Treatment
Conclusions
SPINAL CORD COMPRESSION
Clinical Presentation
Evaluation and Imaging
Treatment
Late Effects
HYPERLEUKOCYTOSIS AND LEUKOSTASIS
Definition
Pathogenesis
Clinical Presentation
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TUMOR LYSIS SYNDROME
Overview
Pathophysiology
Incidence
Diagnosis
Clinical Manifestations and Treatment
Conclusions
COMPLICATIONS OF CHEMOTHERAPY
Vesicant Extravasation
Methotrexate-Induced Renal Dysfunction
Intrathecal Chemotherapy Overdose

Expert and timely management of emergent situations is crucial in the practice of pediatric oncology. Although many emergencies are common across the spectrum of pediatric patients, such as seizure, respiratory arrest, and electrolyte abnormalities, others stem from the underlying cancer or cancer-directed therapy and are unique to this population of patients. These types of emergencies require special consideration and specific management to minimize adverse outcomes. Emergencies related to space-occupying lesions, metabolic derangements caused by breakdown of tumor cells, and disruption of the hematopoietic system by invasion of cancer cells all in some way relate to disruption of the normal function of a cell or organ as a result of the abnormal presence of cancer cells. This disruption can be rapidly progressive and life-threatening, as when patients with T-cell lymphoblastic leukemia present with a mediastinal mass and respiratory compromise. The treatments directed toward cancer also carry significant toxicities and can result in uniquely emergent complications. Many chemotherapy agents result in a state of significant immune compromise and put patients at risk for unusual or opportunistic infections, as well as fulminant presentations of infection with more common organisms. All chemotherapy drugs carry extensive profiles of possible adverse effects, and errors of administration or dosage can magnify toxicity; any available therapeutic interventions must be administered immediately. Appropriate management of an emergent pediatric oncologic situation requires a clear understanding of the specific underlying disease process and of the effects and consequences of cancer-directed therapies.

Symptoms in children who present with new diagnoses of cancer or are receiving cancer treatment are myriad. Therefore recognition of an emergency is not always straightforward, particularly in the young pediatric population, for whom obtaining an accurate history can be challenging. It is therefore imperative that the clinician maintain a high level of vigilance for the presentation of potentially life-threatening situations. A child with cough and mild respiratory distress is most likely to have a viral infection, but these symptoms also might be consistent with a child presenting with a marked mediastinal mass, developing leukostasis syndrome, or the onset of Pneumocystis pneumonia. A complaint of lower back pain in a child may relate to new physical activity or could represent developing spinal cord compression from a tumor. Pediatric oncologists need to be familiar with all pediatric oncologic emergent situations to evaluate and manage

Box 68-1 **Commonly Encountered Pediatric Oncologic Emergencies**

DISEASE-RELATED

Anterior mediastinal mass
Increased intracranial pressure
Spinal cord compression
Leukocytosis and leukostasis
Tumor lysis syndrome
Disseminated intravascular coagulation

TREATMENT-RELATED

Fever and neutropenia, sepsis
Typhlitis
Pancreatitis
Vesicant infiltration
Methotrexate toxicity
All-*trans*-retinoic acid syndrome
Veno-occlusive disease
Intrathecal chemotherapy overdose
Central nervous system events such as seizure or CVA
Severe allergic reactions to chemotherapy agents or blood products

these high-risk patients appropriately. Rapid and appropriate triage of patient complaints is critical, because overlooked diagnoses, as well as inaction or inappropriate action, can have severe consequences.

Grouping of some emergent pediatric oncologic conditions into disease- and treatment-related events is presented in Box 68-1. Clear overlap exists among the conditions listed; for example, tumor lysis syndrome (TLS) can occur on presentation with a new diagnosis of leukemia or high-grade lymphoma, but it can be markedly worsened by initiation of chemotherapy. Similarly, superior vena cava syndrome (SVCS) can result from a new or recurrent presence of a mass lesion or as a complication of an indwelling catheter placed to deliver antineoplastic therapy. In this chapter our discussion will focus on several emergent situations unique to pediatric oncology patients, with a review of available data and overviews of current management strategies. The topics addressed in the chapter are anterior mediastinal mass, increased intracranial pressure, spinal cord compression, hyperleukocytosis and leukostasis, TLS, and selected chemotherapy toxicities. Several topics unique to the pediatric oncology patient are covered elsewhere in this text and will not be readdressed in this chapter (e.g., management of fever and neutropenia, typhlitis, veno-occlusive disease, all-*trans*-retinoic acid syndrome, transfusion medicine, and pain management). Situations that can occur in the pediatric oncology patient but that are not unique to this population, such as seizure, cerebral vascular injury, disseminated intravascular coagulation, and pancreatitis, will not be discussed here.

Although optimal management of many of these emergent situations has been difficult to study, given the relatively small number of patients presenting to each institution, a literature is evolving for many of these topics. Many institutions have established clinical practice guidelines to help manage the most common pediatric oncologic emergencies. Clinical practice guidelines provide uniform standards for management but must allow for adaptations for each specific clinical scenario. Uniform use of clinical guidelines with collection and analysis of outcomes has the potential to guide improvements in clinical practice.

ANTERIOR MEDIASTINAL MASSES

Definition

A commonly encountered oncologic emergency is the entity of anterior mediastinal mass. A clinician should always consider this entity in the differential diagnosis of a pediatric patient with new respiratory distress and must consider evaluation, even in asymptomatic patients presenting with any of the variety of cancers known to be associated with the occurrence of anterior mediastinal masses.

Space-occupying lesions occurring in the anterior mediastinum can impinge on vital structures, causing significant cardiac or airway compromise. The anterior mediastinum is defined as the anatomic compartment bounded by the sternum, thoracic inlet, and anterior border of the heart.[1] The presence of a mass in this region can cause SVCS, tracheobronchial compression, or compression of the heart or pulmonary vessels. The term *superior mediastinal syndrome* (SMS) has been used to denote the combination of great vessel and tracheobronchial tree compression. However, because these phenomena may be overlapping, particularly in the pediatric population, the terms SVCS and SMS are often used interchangeably.

Neoplasms that cause anterior mediastinal masses in the pediatric population and are associated with SVCS and/or tracheal compression include non-Hodgkin lymphoma (NHL), acute lymphoblastic leukemia (ALL), and Hodgkin lymphoma (HL),[2] as well as neuroblastoma, paraganglioma, germ cell tumors, sarcoma (including myeloid sarcoma), thymoma, desmoplastic round cell tumor, lymphangioma, inflammatory myofibroblastic tumor, and thyroid and parathyroid carcinaomas.[3-10] In a retrospective review of children with mediastinal masses treated at the Mayo Clinic, NHL and HL represented the majority of malignant causes of anterior mediastinal masses, although this review did not include patients with leukemia.[10] In a report on 3721 children with cancer treated at St. Jude Children's Research Hospital over a 15-year period, 24 children had superior vena cava (SVC) obstruction at initial presentation or with disease recurrence. Patients presenting with SVC obstruction at initial presentation included eight children with NHL, four with ALL, two with HL, one with neuroblastoma, and one with yolk sac tumor.[9] In contrast, in the adult population, small cell bronchogenic carcinoma is the most common underlying diagnosis associated with SVCS.[11]

SVCS was first described in a patient with a syphilitic aneurysm of the aorta in 1757.[12] The SVC is a thin-walled vessel with blood flowing under low pressure and is easily compressible by the enlargement of surrounding structures in the thorax. In persons with SVCS, compression and obstruction of the SVC cause restriction of blood return to the right atrium and impaired venous drainage from the upper extremities, head, and neck. Occlusion of

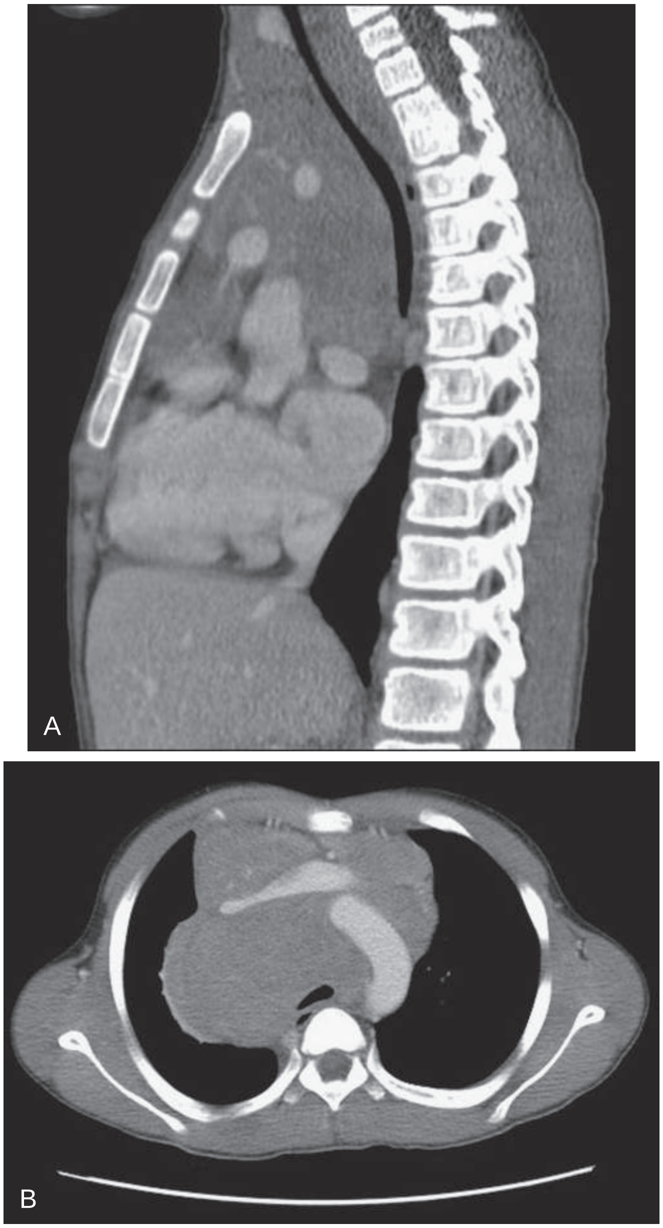

Figure 68-1 A chest computed tomography scan of a patient with Hodgkin disease. Sagittal (**A**) and axial (**B**) images show the significant effects of the mass on structures within the mediastinum, with marked narrowing of the trachea and superior vena cava. Remarkably, the patient was symptomatic only with exercise.

the SVC by thrombus—for example, as a complication of a central venous line—can also result in SVCS. Just as the SVC can be compressed by an anterior mediastinal mass, the trachea and main stem bronchi in children are relatively compressible and have smaller intraluminal diameters compared with these structures in adults, making the pediatric patient with an enlarging mass susceptible to respiratory compromise (Fig. 68-1).[10]

Clinical Presentation

Children with SMS can present with a constellation of signs and symptoms related to SVC and airway obstruction, including swelling, plethora, venous engorgement of the face, neck, and upper extremities, cough, hoarseness, orthopnea, cyanosis, anxiety, syncope, and altered mental status.[13,14] The child can present in extremis, with shock caused by cardiac compromise, or the child can present with respiratory failure, which more commonly occurs in malignancies with a higher growth fraction such as B- or T-lymphoblastic or mature B-cell lymphomas. Conversely, patients with more indolent tumors, such as HL, can present with impressively large masses and surprisingly little symptomatology (Fig. 68-2). Symptoms are often more profound with the patient in the supine position and may improve in the upright or prone position. In the supine position, thoracic volume is decreased because of decreased rib cage dimension and diaphragm position. In addition, blood flow to the mass itself can increase, causing further expansion of the mass.[1] Therefore pursuing testing such as computed tomography (CT) scanning in the supine position should be carefully considered, and patient tolerance of supine positioning should be evaluated before initiating cross-sectional imaging. Patients who are unable to tolerate supine positioning may require decubitus or prone positioning if CT evaluation is necessary during the initial workup, and some patients with extreme compression may not be able to tolerate any horizontal positioning prior to emergent cytoreductive therapy.

An anterior mediastinal mass may be suspected on the basis of medical history and physical examination and can be identified on a chest radiograph. Identifying patients at greatest risk for anesthetic complications is important in planning the diagnostic evaluation. Procedural sedation and anesthesia pose particular risks for

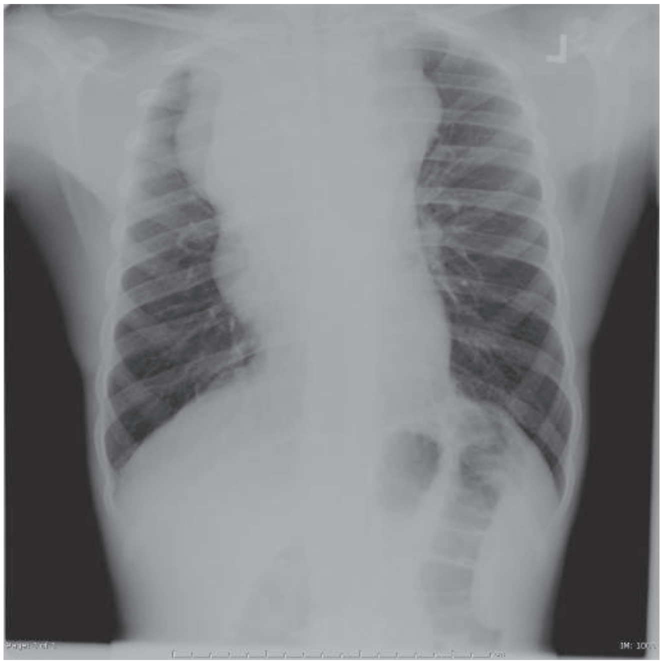

Figure 68-2 A chest radiograph of a patient with Hodgkin disease showing a markedly enlarged mediastinum. The patient had a several-month history of intermittent cough and had only mild respiratory symptoms at presentation.

patients with an anterior mediastinal mass, with the potential for cardiopulmonary collapse and death.[13-15] A patient whose respiratory status is stable while awake, with spontaneous respirations, may experience significant obstruction when anesthesia is induced. General anesthesia alters ventilatory mechanics, decreasing inspiratory muscle tone and functional residual capacity of the lung.[16] Chest wall tone is decreased by neuromuscular blockade. Airways are more compressible because of bronchial smooth muscle relaxation.[14] Positive pressure ventilation may further restrict an already narrowed airway.[17] In addition, general anesthetic agents can have negative inotropic effects and may contribute to decreasing cardiac output, precipitating hypoxemia, hypotension, or even cardiac arrest in patients with compression of the great vessels.[18]

Respiratory symptoms occur in 40% to 60% of children with anterior mediastinal masses, but correlation of symptoms with anesthetic risk can be challenging.[14,19] Orthopnea has been associated with increased anesthetic risk.[19,20] Posterior anterior and lateral chest radiographs can demonstrate tracheal compression but may not detect all cases.[19,21] CT can provide additional anatomic information, and patients with less than 50% of expected tracheal cross-sectional area on CT have been shown to be a high-risk group.[14,19] However, obstruction distal to the trachea may be more difficult to appreciate. Authors of a prospective study assessing tracheal area and pulmonary function testing have found that inducement of general anesthesia can be used safely in children who meet the minimum criteria of a tracheal area larger than 50% and predicted peak expiratory flow rate (PEFR) greater than 50%. Thirty-one children with mediastinal masses underwent pulmonary function testing in the sitting and supine positions along with CT prior to undergoing 34 surgical procedures. For children with less than 50% of predicted cross-sectional tracheal area (or with less than 50% of PEFR), only local rather than general anesthetic agents were used. Five patients had predicted tracheal areas larger than 50% but had a low PEFR and thus only local anesthetic agents were used. No intraoperative complications occurred in patients receiving anesthetic drugs when these criteria were used.[22]

Evaluation and Imaging

A definitive diagnosis is needed to initiate tumor-specific therapy, and the least invasive diagnostic methods possible should be pursued. If the peripheral blood smear is abnormal, a bone marrow aspiration or biopsy with use of a local anesthetic may prove sufficiently diagnostic. A biopsy of a peripheral lymph node, if present, may prove diagnostic. Serum α-fetoprotein or β-human chorionic gonadotropin may be positive in patients with mediastinal germ cell tumors, and these tests can be performed rapidly in many institutions. If a pleural effusion is present, pleural fluid aspiration may be possible with use of local anesthesia and may provide fluid for pathologic review and immunophenotypic and cytogenetic evaluation (Fig. 68-3).[23] Some patients whose only source of potential diagnostic tissue is the mediastinal mass will be able to tolerate percutaneous needle biopsy with use of a local anesthetic and anxiolytic-only sedation.

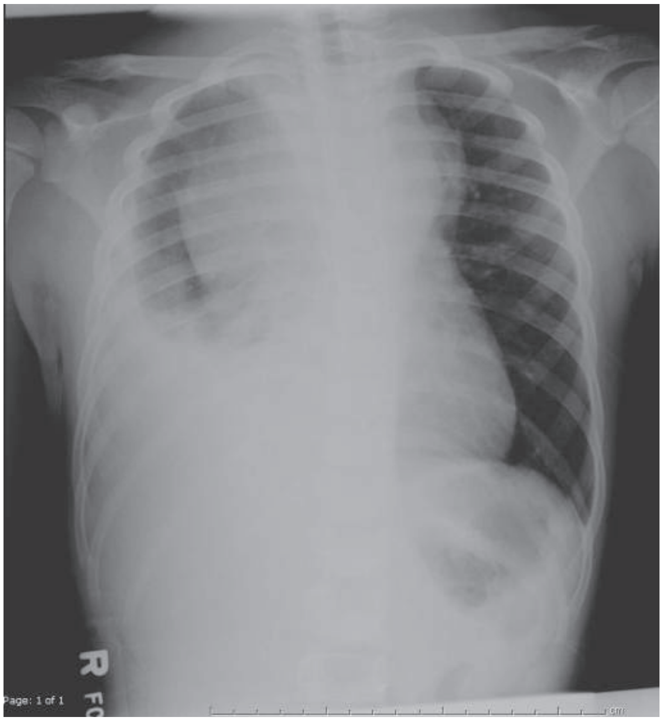

Figure 68-3 A chest radiograph of a patient who presented in near extremis, with facial swelling, plethora, and significant respiratory distress. The patient had a large anterior mediastinal mass and marked pleural effusion. T-cell lymphoblastic lymphoma was diagnosed from the pleural fluid.

An accurate tissue diagnosis allows for initiation of the optimal specific treatment regimen. For many patients with anterior mediastinal masses, a general state of anesthesia will be able to be induced safely.[24] However, the cases of patients in extremis or those with rapidly evolving respiratory compromise who require sedation or inducement of general anesthesia are particularly challenging to manage. When proceeding with general anesthesia, some persons have advocated the preservation of spontaneous respiration when at all possible, avoidance of the use of muscle relaxants, being prepared to change the patient's position, and having an expert bronchoscopist and emergency cardiopulmonary bypass available.[15] Use of bilevel positive airway pressure for safe anesthesia in these settings has been reported recently.[25] Successful suprasedative dosing of dexmedetomidine has also been reported.[26] Use of extracorporeal membrane oxygenation in persons with anterior mediastinal masses who are experiencing cardiopulmonary failure has been reported.[27] Use of inhaled heliox, a helium-oxygen mixture, has been advocated in case reports to decrease resistance to airflow in obstructed airways, although its use in the setting of anterior mediastinal masses has not been studied.[28]

In recent years the surgical literature has included an increased focus on novel operative techniques to allow safe biopsy and/or resection in patients with large anterior mediastinal masses. Percutaneous biopsy has long been a standard approach in patients too unstable for inducement of general anesthesia, but advances in imaging technique allow for additional applications of this methodology, such as real-time ultrasound fusion with CT for biopsy guidance.[29] Additional modalities recently described as successful in a majority of patients include awake video-assisted thoracoscopic biopsy, the hemiclamshell approach, and the Chamberlain procedure (also known as anterior mediastinotomy).[30-32]

Treatment

In some clinical situations the risk of proceeding with diagnostic procedures is considered too great, and empiric therapy aimed at reducing the mass must be initiated. Emergent treatment modalities include radiation, intravenous steroids, and combination chemotherapy. Radiation has traditionally been used in emergency management of anterior mediastinal masses, because most lymphomas are radiosensitive. Daily radiation doses are based on those used for the presumed diagnosis that best fits the clinical data. Depending on the radioresponsiveness of the mass, prebiopsy radiation treatment will frequently obscure the tissue diagnosis, even with low-dose radiation therapy. Loeffler and colleagues[33] reported that in 8 of 10 patients younger than 30 years who underwent emergent prebiopsy radiation for a mediastinal mass, a tissue diagnosis could not be established because of uninterpretable pathologic specimens. Also of concern in the pediatric population is a risk of postradiation respiratory deterioration caused by swelling of the trachea after radiation. Concurrent administration of a steroid (e.g., dexamethasone at a dosage for airway edema, 0.5 to 2 mg/kg/day in divided doses every 6 hours) can be considered with the goal of preventing this complication but may also contribute to obscurity of the diagnosis because most untreated lymphomas are sensitive to steroids.

The emergent use of empiric chemotherapy includes the use of intravenous (IV) steroids or combination chemotherapy. Steroid therapy should be considered in the emergent management of proven or presumed ALL or lymphoblastic lymphoma. Careful attention must be directed to fluid management given concerns for TLS in the patient with the potential for cardiovascular compromise. Consideration of the use of rasburicase may be warranted. Steroid dosing based on ALL treatment protocols can be used (e.g., methylprednisolone, 32 to 48 mg/m^2/day IV, divided every 8 to 12 hours). Steroid therapy may also be effective in the setting of other malignancies because of its potent antiinflammatory effect. Other chemotherapeutic agents for consideration in the emergent management of anterior mediastinal mass include cyclophosphamide, vincristine, and anthracyclines. Just as radiation may eliminate the ability to establish a definitive diagnosis, steroid therapy has also been reported to inhibit accurate diagnosis.[13] Without a definitive pathologic diagnosis, continued treatment of the patient for the presumed diagnosis that best fits the clinical data has been advocated. Masses that are not responsive to the previously described emergent therapies may require surgical intervention.

Conclusions

The evaluation of a child with a suspected anterior mediastinal mass or SVCS should be initiated rapidly once the diagnosis is considered to be a possibility. Great variability in the course of evaluation and treatment may be encountered, depending on the specifics of each clinical situation, including the clinical stability of the patient and the underlying diagnosis. A representative evaluation and treatment algorithm published in 2008 is outlined in Figure 68-4.[34] The clinician must adapt his or her care to the particular circumstances of the individual patient.

SPINAL CORD COMPRESSION

Spinal cord compression is overall a rare event in children, but several studies have shown an incidence of up to 5% in children with cancer.[35] The most frequent occurrence of spinal cord compression in children with cancer is during the initial presentation of the malignant process (67% of the time).[36] Although such a presentation often occurs with widely disseminated disease, spinal cord compression can also occur with a singular lesion as the initial presentation of a new diagnosis of cancer. In this scenario, along with preservation or restoration of lost neurologic functioning, proper diagnosis of the cancer through adequate tissue sampling is a management priority. Procurement of sufficient tissue for specialized testing and possible study enrollment is a lesser priority but still should be thoughtfully considered in the overall management plan. Spinal cord compression is also encountered in patients with relapsed and refractory disease, again as a single metastatic lesion or as widespread metastatic disease. In such situations, management strategies must take into account the likelihood of cure, the effect of interventions on the patient's quality of life, and the role of palliation.

The majority (71%) of tumors that cause spinal cord compression are extradural.[36] More than 50% of tumors causing spinal cord compression are found to be neuroblastoma, soft tissue sarcomas, Ewing sarcoma, or rhabdomyosarcoma. A wide variety of other tumors have been reported as causes of spinal cord compression and must be considered in the differential diagnosis. Tumors reported to date include osteosarcoma, Wilms tumor, metastatic central nervous system tumors, meningioma, pleuropulmonary blastoma, juvenile xanthogranuloma, adrenocortical carcinoma, acute myelogenous leukemia (AML), and lymphoma.[37-40]

Clinical Presentation

Back pain is the most common presenting sign of spinal cord compression and was present in 94% of 70 pediatric patients presenting with solid tumors and spinal cord compression.[36] Because recurrent back pain without known injury is a fairly uncommon complaint in children, spinal lesions and spinal cord compression should always be considered in the differential diagnosis of this complaint, even in the absence of neurologic findings. In pediatric patients known to have cancer, the index of suspicion must be particularly high. Any delay

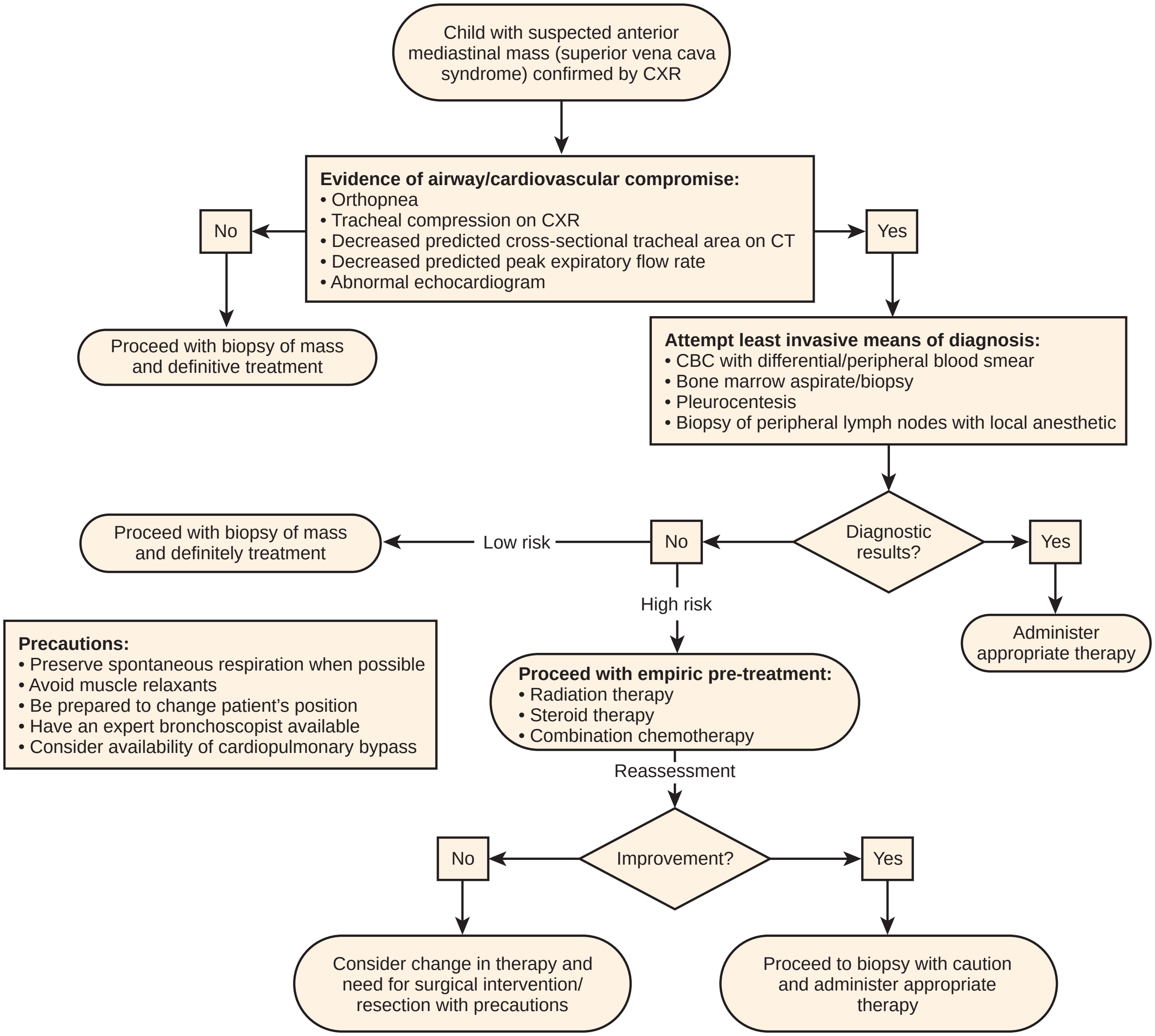
Child with suspected anterior mediastinal mass (superior vena cava syndrome) confirmed by CXR
Evidence of airway/cardiovascular compromise:
• Orthopnea
• Tracheal compression on CXR
• Decreased predicted cross-sectional tracheal area on CT
• Decreased predicted peak expiratory flow rate
• Abnormal echocardiogram
No
Yes
Proceed with biopsy of mass and definitive treatment
Attempt least invasive means of diagnosis:
• CBC with differential/peripheral blood smear
• Bone marrow aspirate/biopsy
• Pleurocentesis
• Biopsy of peripheral lymph nodes with local anesthetic
Diagnostic results?
No
Yes
Low risk
Proceed with biopsy of mass and definitely treatment
High risk
Administer appropriate therapy
Precautions:
• Preserve spontaneous respiration when possible
• Avoid muscle relaxants
• Be prepared to change patient's position
• Have an expert bronchoscopist available
• Consider availability of cardiopulmonary bypass
Proceed with empiric pre-treatment:
• Radiation therapy
• Steroid therapy
• Combination chemotherapy
Reassessment
Improvement?
No
Yes
Consider change in therapy and need for surgical intervention/ resection with precautions
Proceed to biopsy with caution and administer appropriate therapy

Figure 68-4 Algorithm for a patient with critical airways and an anterior mediastinal mass or superior vena cava syndrome.[34] *CBC,* Complete blood cell count; *CT,* computed tomography; *CXR,* chest x-ray.

in diagnosis of spinal cord compression may contribute significantly to morbidity. A thorough physical examination should accompany a complete history. The history should include characterization of pain, including onset and duration. Neurologic findings, if present, will vary, depending on the spinal level of the lesion and the degree of compression. In their series of children with systemic cancer and spinal cord disease, Lewis and coworkers[35] noted that localized spine tenderness is the most reliable clinical finding. A spectrum of ataxia, gait disturbance, and paraplegia can be observed. Sphincter dysfunction is most commonly seen as urinary retention or constipation. Localization of the level of epidural cord compression along the spine is suggested by the specific effects on strength, tendon reflexes, sensory level, Babinski reflex, sphincter abnormalities, and rate of progression (Table 68-1).[35,41]

Evaluation and Imaging

When the possibility of spinal cord compression is considered, immediate evaluation should be undertaken. Spinal radiography is often the first-choice modality because it is quickly and easily undertaken. Plain films may be able to provide valuable clinical information rapidly, but one must be aware that they are positive in only approximately 30% of patients with spinal cord compression.[35] The presence of a compressive spinal lesion may be seen on a radiograph as a paraspinal soft tissue mass. In cases of neuroblastoma, calcifications are often present within the paraspinal mass. Classically,

TABLE 68-1 Epidural Cord Compression: Clinical Localization

	LOCATION		
Sign	**Spinal Cord**	**Conus Medullaris**	**Cauda Equina**
Weakness	Symmetrical; profound	Symmetrical; variable	Asymmetrical; may be mild
Tendon reflexes	Increased or absent	Increased knee; decreased ankle	Decreased; asymmetrical
Babinski	Extensor	Extensor	Plantar
Sensory	Symmetrical; sensory level	Symmetrical; saddle	Asymmetrical; radicular
Sphincter abnormality	Spared until late	Early involvement	May be spared
Progression	Rapid	Variable; may be rapid	Variable; may be slow

Modified from Lewis DW, Packer RJ, Raney B, et al: Incidence, presentation, and outcome of spinal cord disease in children with systemic cancer. Pediatrics *78:438–443, 1986.*

neuroblastoma invading the spinal canal and intervertebral foramina may be visualized as a dumbbell-shaped lesion surrounding the spine. Other tumors can also have this appearance, emphasizing the importance of obtaining adequate tissue for diagnosis (Fig. 68-5). In many spinal lesions, lytic or sclerotic changes in adjacent bone are often observed on a radiograph. Diagnostic widening of interpeduncular distances and enlargement of the neural foramina can sometimes also be observed on radiographs.

Emergent magnetic resonance imaging (MRI) is essential when plain radiographs are negative and clinical suspicion still exists. MRI is also necessary for further delineation of lesions observed on plain radiographs. MRI has become widely accessible and thus it has replaced the previously used techniques of radionucleotide bone scanning, spinal CT with intrathecal contrast, and lumbar myelography. MRI offers the advantage of increased accuracy and a less invasive nature than previously used modalities. Careful comparison of axial, coronal, and sagittal images can help categorize spinal tumors as extradural, intradural-extramedullary, or intramedullary, thereby aiding in the differential diagnosis.[42] MRI sequences with and without use of gadolinium should be obtained for patients being evaluated for spinal cord compression. Contrast enhancement is less helpful for extradural tumors than for intradural-extramedullary or intramedullary lesions. Fat suppression techniques are most helpful for evaluating extradural lesions. It should also be remembered that it is important to image the entire spine to identify the possibility of involvement at several levels, even if the physical examination has localized the most likely area of compression.

MRI can also accurately detect compression and dislocation of the spinal cord. Coronal images can elucidate the degree of cranial and caudal extension on the intraspinal component of a mass lesion. Because of increased cellularity and high nuclear-to-cytoplasmic ratio, lesions are usually isodense to hypodense on T2-weighted images.[43] Cord edema can be seen as a hyperintense signal in T2-weighted images. Contrast enhancement is generally marked in the presence of intraspinal lesions. Diffusion tensor imaging has been recently described as a potentially beneficial additive sequence.[44]

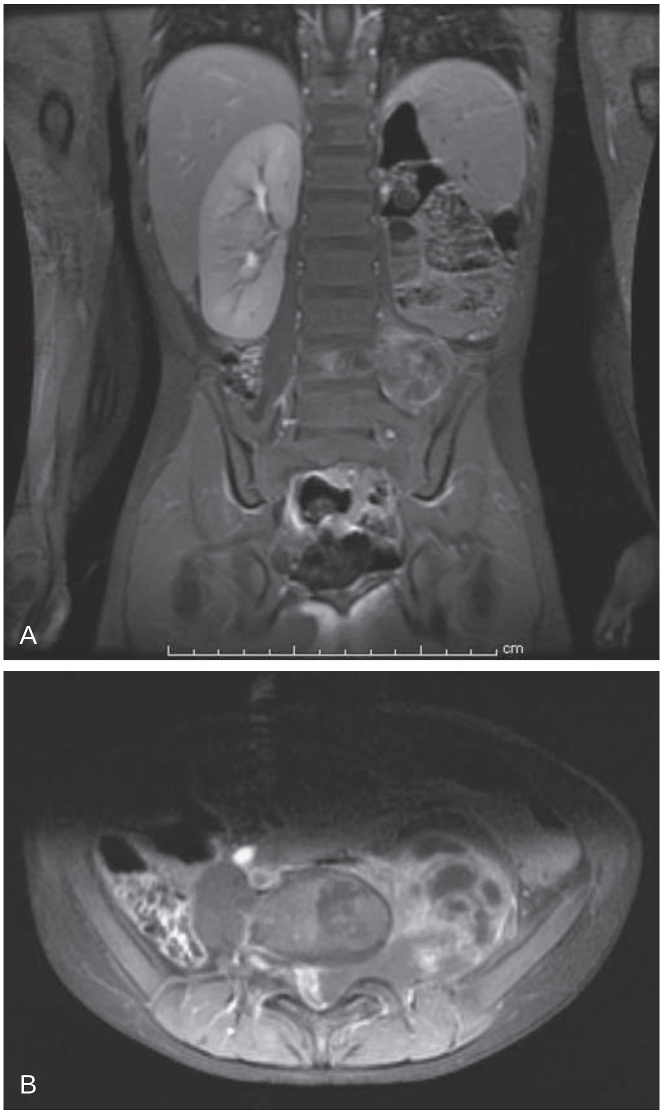

Figure 68-5 Magnetic resonance imaging after administration of gadolinium demonstrates a soft tissue retroperitoneal mass at the level of L4 with a dumbbell appearance on both coronal (**A**) and axial (**B**) views. The patient presented with a short history of leg pain and limp that progressed to the inability to ambulate, 2 years after the completion of therapy for Wilms tumor with a favorable histology.

If the clinical situation allows time for further radiologic evaluation, CT with bone windows can provide complementary information to MRI findings. Detailed information on bone architecture and cortical appearance is better obtained via CT.

Treatment

Treatment options for a patient presenting with spinal cord compression include surgery (laminectomy or laminotomy), chemotherapy, steroid therapy, and radiation therapy. The best choice of initial therapy will vary depending on the clinical situation. In many clinical situations use of more than one modality may be appropriate, and in some situations it is appropriate for more than one modality to be used simultaneously. The complexity of the choice of the best initial therapy stems largely from multiple competing therapeutic goals—the desire to act quickly to prevent further loss of neurologic function, the need to make an accurate diagnosis with sufficient biologic information for assignment of appropriate treatment, the hope of regaining any lost function existing at presentation, and the concern regarding the late effects of the available emergent treatment modalities. After providing a brief overview of the basic treatment options, we will present the conclusions of several clinical studies.

Surgery

Laminectomy is the most common surgical approach to relieve spinal cord compression caused by a solid tumor. Laminectomy involves removal of the bony arch, or lamina, of a vertebra. This procedure is often combined with biopsy and/or debulking of the tumor. The advantage of a laminectomy is the rapid decompression of the spinal cord. Laminectomy is generally accepted as the most likely modality to stabilize or improve lost neurologic function. Another significant advantage of laminectomy is the procurement of tumor tissue for proper histologic diagnosis and the option of obtaining tissue for relevant biology studies or possible protocol enrollment. The disadvantage of laminectomy is the high risk for late effects, with a significant percentage of patients experiencing kyphosis or scoliosis. Depending on the degree of spinal cord injury and how long symptoms have been present, laminectomy may not provide an advantage compared with treatment with chemotherapy only and may expose the child to risks of the surgical procedure and greater risks of long-term orthopedic sequelae.

The technique of osteoplastic laminotomy involves removal of part of the lamina above and below an affected nerve, with subsequent bracing for a period of 6 to 8 weeks. Although historically this technique is not commonly used, it may offer a decreased incidence of long-term sequelae and may be considered by the surgeon. Minimally invasive thoracoscopic approaches for decompression have been described.[45-48] Additionally, percutaneous approaches for decompression are increasing reported.[49,50]

Chemotherapy

Chemotherapy can be used successfully to reduce some neoplastic masses that cause spinal cord compression. Use of chemotherapy has been most studied in cases of neuroblastoma or Ewing sarcoma that present with spinal cord compression, and chemotherapy has been used successfully to treat germ cell tumors as well.[51] In the rare cases of leukemic or lymphomatous infiltrates, chemotherapy is the clear standard initial therapy once the diagnosis has been established.

Advantages of upfront chemotherapy for lesions causing spinal cord compression are that chemotherapy is anatomically noninvasive and long-term surgical consequences may be avoided. In addition, patients may proceed with tumor-specific therapy without the need for delay from postoperative healing. However, the use of chemotherapy depends on first establishing an accurate pathologic diagnosis. In cases of metastatic tumor, it may be possible to make a diagnosis by biopsy of other primary or metastatic lesions. Identifying isolated paraspinal masses can be more diagnostically difficult. If surgery is avoided completely and chemotherapy is started presumptively, the possibility of an incorrect diagnosis will exist. Also, if no tissue is obtained, even though an isolated paraspinal mass may eventually be diagnosed as neuroblastoma on the basis of elevated urine catecholamine levels or positive metaiodobenzylguanidine activity, the opportunity to obtain valuable biologic information, such as histology, v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog *(MYCN)* status, and other cytogenetic and molecular markers used to direct therapy could be lost.

In patients who experience spinal cord compression in the setting of relapsed disease, the choice of using chemotherapy rather than surgery or radiation therapy can become more complex, because the rate and overall likelihood of response can be substantially decreased in previously treated patients. Radiation therapy or surgeries may have been used previously and may limit these options in a relapse setting. Such clinical situations must be carefully weighed individually. The decision to initiate chemotherapy rather than to use surgery, radiation therapy, or comfort palliation must include consideration of the patient's overall chance of long-term cure, the history of treatment and response, and most importantly, the goals of treatment of the patient and family and the desired level of intensity of therapy.

In the patient with a new presentation of cancer, the choice of a chemotherapy regimen must be based on a clear identification of the malignant process. Once a diagnosis is known and a decision has been made to avoid surgery or radiation and commence with chemotherapy, standard frontline therapy for the malignancy that has been identified is accepted as the best initial chemotherapy in patients with spinal cord compression.

Dexamethasone

Dexamethasone is routinely recommended throughout the literature for the treatment of pediatric spinal cord compression. It is theorized that decreased swelling of the cord will help minimize neurologic damage through minimizing the effect of vasogenic edema and venous congestion. In a review of the adult literature compiled to create an evidence-based guideline for the emergency treatment of extradural spinal cord compression in adult patients, Loblaw and Laperriere[52] reviewed the available studies on the use of dexamethasone. They found strong evidence to support the use of high-dose dexamethasone with radiation therapy with improved outcome but significant associated toxicity.[53] They also reported evidence judged to be fair that dexamethasone does not need to be given to asymptomatic ambulatory patients with spinal cord compression receiving x-ray therapy.[54] Although no corresponding evidence is available to support the role of dexamethasone in improving outcome in spinal cord compression caused by solid tumors in pediatric patients, little substantial detrimental effect has been observed with the routine use of dexamethasone in the setting of acute spinal cord compression. Increased rates of infection and delayed wound healing are possible concerns. The use of gastric protectants is advised when administering dexamethasone in this setting and should include H2 blockers and/or proton pump inhibitors. Blood pressure and glucose levels should be monitored, and any increase above age norms should be treated appropriately.

In the case of a child presenting with a high index of suspicion of spinal cord compression caused by tumor and *any* neurologic compromise, rapid administration of dexamethasone at 1 to 2 mg/kg IV to be administered over 30 minutes is suggested. This dose should be administered prior to imaging. If no neurologic symptoms are present, lower oral dosing, 0.25 to 0.5 mg/kg every 6 hours, may be initiated while imaging is arranged. If the child is found to have a spinal lesion and radiation therapy is chosen as the modality for treatment of cord compression, use of moderate-dose dexamethasone is sometimes continued to decrease the effects of radiation-induced swelling and vasogenic edema.

Radiotherapy

Most tumors causing spinal cord compression in the pediatric population will respond to radiotherapy, and thus this modality can often be used emergently to relieve symptoms of spinal cord compression effectively. The benefits of its use include possible rapid response, reasonable tolerance, and avoidance of more invasive procedures. Potential drawbacks include interference with establishing a correct pathologic diagnosis, long-term effects on the spinal column (including stunted growth, cord damage, and scoliosis), thyroid dysfunction, and the risk of secondary malignancies. Additionally, young pediatric patients may require anesthesia for planning and delivery of radiotherapy fractions. For safety and accuracy, adequate imaging, usually through MRI, is required to define the length, depth, and width of the planned treatment field.

Several approaches can be used to deliver a therapeutic treatment dose.[55] Midline lesions, as well as lesions in the lumbar spine, may be treated through a parallel opposed anteroposterior-posteroanterior beam arrangement. A posterior field alone can be used for lesions in the thoracic spine. The cervical spine may be treated with opposed lateral fields in an effort to avoid the oral cavity. Conformal techniques can allow for more precise localization of the radiation therapy to the tumor volume. However, these methods require more elaborate planning and may delay the initiation of therapy. The potential benefit of a more conformal field must be weighed against the additional time and planning required.

Radiotherapy dose and schedule are determined by the pathologic diagnosis, extent of spinal involvement, possible coadministration of chemotherapy, and the patient's clinical situation (e.g., previously ambulatory or nonambulatory, overall life expectancy, likelihood of response to systemic therapy, and previous history of radiation therapy). A dosing range of 18 to 40 Gy can be used. Commonly used schedules include 30 Gy delivered in 10 fractions, 20 Gy given in 5 fractions, or up to 35 to 40 Gy spread over a more protracted course of 3 to 4 weeks. However, 8-Gy single fraction radiotherapy has been successfully used for palliation in patients with a grim prognosis and spinal cord compression, and a recent systematic review suggested that 30 Gy over 10 fractions was a preferred regimen for patients with a good prognosis.[56,57]

The concomitant use of dexamethasone with radiation therapy is often recommended. Dexamethasone is usually begun at a high dose prior to starting radiotherapy, continued at a lesser dose during the procedure, and tapered off after completion. The use of dexamethasone with a taper is thought to decrease some of the edema that can occur as a result of the radiotherapy. Although newer radiation modalities such as Gamma Knife radiosurgery and CyberKnife radiosurgery have been used in adult patients with spinal cord compression, pediatric use of these technologies for spinal cord compression has not been reported to date.[5,58]

Clinical Studies

Although much laudable investigation has been undertaken to address the best choice of initial therapy for children with spinal cord compression, no consensus has been reached to date. The available data are limited by relatively small sample sizes because of the overall rarity of spinal cord compression. Larger series tend to group multiple histologic diagnoses, which may respond differently to the interventions of chemotherapy, radiation, and surgery. Many of the studies also span long intervals and cannot necessarily take into account modernization of practice, improved imaging modalities, and improved therapeutic options.

Pollono and colleagues[36] have reported on a review of 70 pediatric patients with spinal cord compression treated at a single institution from 1984 to 2001; 71% were extradural tumors and 54% were soft tissue sarcomas or neuroblastoma. Figure 68-6 presents the algorithm by which these patients were diagnosed and treated. Patients with paraplegia of less than 96 hours' duration underwent laminectomy, as did patients with primary spinal cord tumor or isolated recurrence of main tumor, progression of symptoms after radiation or chemotherapy, or, if known, a radiation- and chemotherapy-resistant tumor. Surgery was avoided when the prognosis of the primary tumor was poor or risks were thought to outweigh benefits. Of patients with paraplegia, 57% became

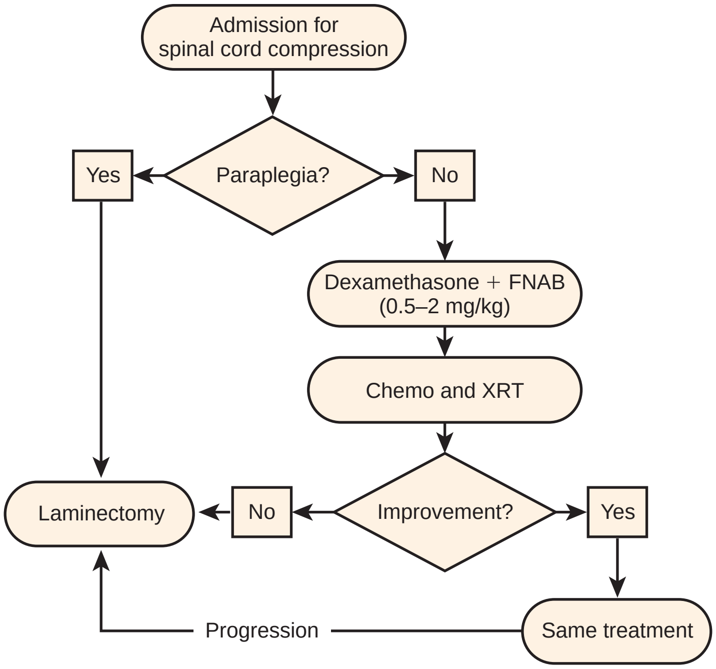

Figure 68-6 Algorithm for diagnosis and treatment of spinal cord compression. With tumor resection, paraplegia of less than 96-hour evolution in patients younger than 1 year and of less than 24 hours in older patients. *Chemo,* Chemotherapy; *FNAB,* fine-needle aspiration biopsy for diagnosis; *XRT,* x-ray therapy. *(Modified from Pollono D, Tomarchia S, Drut R, et al: Spinal cord compression: a review of 70 pediatric patients.* Pediatr Hematol Oncol *20:457–466, 2003.)*

ambulatory after undergoing a laminectomy, whereas only 14% improved after use of chemotherapy and radiation. In patients who presented without paraplegia and received medical treatment without surgery, 87% remained ambulatory. Overall survival was related to the original malignancy. These data suggest that patients with chemotherapy- and radiotherapy-sensitive tumors without evidence of progressive neurologic damage might be treated with chemotherapy and/or radiation therapy but that any indication of neurologic progression is a strong indication for laminectomy without delay.

The question of upfront chemotherapy versus surgery or radiation therapy for spinal cord compression has been most extensively studied in pediatric patients with neuroblastoma, because rapid improvement with initial chemotherapy has been demonstrated. The first reports on the use of chemotherapy as frontline therapy in children presenting with dumbbell neuroblastoma was published in 1984 by Hayes et al.[59] Eleven children with presenting neurologic symptoms were treated; 9 had complete recovery and 2 demonstrated sustained paraplegia. In 1989, Sanderson and coworkers[60] also reported encouraging results of two children with paraplegia and two with paraparesis who both demonstrated full neurologic recovery after treatment with vincristine, cisplatin, etoposide, and cyclophosphamide therapy. Since then, more investigations have been conducted on the use of frontline chemotherapy compared with neurosurgical intervention. Both prospective and retrospective studies have attempted to elucidate whether chemotherapy or surgery is the best initial choice of therapy, but the results yielded have conflicting conclusions. The variability in outcome likely reflects the difficulty of appropriately grouping patients based on initial presenting signs and extent of disease, as well as institutional practice variations and availability of resources.

A series of these studies has been well summarized by De Bernardi and colleagues.[43] Studies from several major international cooperative groups (Italy, Germany, Poland, United Kingdom, and United States) were reviewed. The Italian Cooperative Group for Neuroblastoma has reported a prospective study in which 26 patients with neuroblastoma and spinal cord compression were preferentially given chemotherapy rather than surgery or radiation therapy. Prior to this study, patients followed up between 1979 and 1998 by this group had been analyzed.[61] Retrospective analysis of the earlier patient group showed that radiotherapy, laminectomy, and chemotherapy have comparable ability to improve spinal cord compression. The prospective study also concluded that chemotherapy adequately relieves neurologic symptoms in most patients. Neurologic and orthopedic sequelae were seen in a large number of patients, including those who received chemotherapy only.

In another prospective study carried out by the French Society of Paediatric Oncology, 78 patients with intraspinal extension of neuroblastoma were treated, beginning in 1990.[62] Of these patients, 86% were initially treated with chemotherapy; 63% had a complete neurologic recovery and 21% had a partial neurologic recovery. Also, 13% failed to improve with chemotherapy, the disease of 1 patient progressed, 14% underwent primary surgery, 23% of initially symptomatic patients (10 of 43) had severe neurologic sequelae, 16% had mild sequelae, and 23% were reported to have severe orthopedic sequelae.

One of the largest series contained 83 patients registered for the Pediatric Oncology Group Neuroblastoma Biology Protocol 9047, in which 66 patients received upfront chemotherapy, 23 underwent primary laminectomy, 8 received initial radiotherapy, and 31 underwent initial surgical resection without laminectomy.[63] From this series of retrospectively collected data, it was concluded that patients with neurologic symptoms can be managed without laminectomy and that laminectomy should be reserved for patients who demonstrate progressive neurologic deterioration after initial chemotherapy. The likelihood of neurologic sequelae was found to be inversely related to the length of symptoms prior to initiation of treatment, and more severe sequelae were seen in patients presenting with more severe symptoms. Twenty-nine percent of patients had orthopedic sequelae, and the incidence was significantly lower in patients managed with chemotherapy compared with those who underwent laminectomy.

After a review of multiple studies, the primary conclusion of the neuroblastoma workshop was that a need exists for the creation of an international spinal cord compression registry.[43] The workshop group specified the need to standardize language and criteria to group patients with comparable degrees of neurologic compromise at presentation accurately and to characterize the late effects uniformly. A functional grading system proposed by Gilbert and colleagues[64] was used in some studies to aid in outcome analysis, but it has not been universally adopted (Table 68-2). This grading system notably does not take into account sphincter dysfunction, an important neurologic finding.

It was also concluded that chemotherapy is a reasonable initial approach, but laminectomy should be used for patients who demonstrate progressive neurologic symptoms after presentation or no restoration in the short term.[43] Patients with symptoms occurring within 96 hours of presentation may also be considered candidates for surgical intervention. It was also proposed that radiotherapy may be underused in spinal cord compression caused by neuroblastoma.

Because of the heterogeneity of pediatric malignancies, the findings of the neuroblastoma group cannot automatically be generalized for other types of cancer. Smaller series of outcomes of patients with Ewing sarcoma and intraspinal lesions have also described mixed use of upfront therapies, with variable outcomes. In a report of a series of seven patients with primary Ewing sarcoma of the spine, as well as a review of the literature, Sharafuddin and colleagues[65] noted the importance of the location (sacral or nonsacral) of the presenting lesion in the response to treatment. Differences in response in adult and pediatric patients were also noted. Figure 68-7 shows the algorithm that they proposed for the management of primary Ewing sarcoma of the spine. Initial chemotherapy or limited surgical decompression is recommended for all pediatric patients; surgical intervention is clearly indicated for patients who demonstrate progressive findings with medical treatment.

The rare cases of leukemia and lymphoma that present with spinal cord compression should be treated with initial chemotherapy. Radiotherapy can be considered for some special cases.

TABLE 68-2 Functional Grading Scale of Spinal Cord Compression

Grade	Patient Function
1	Ambulatory, with or without weakness of lower extremities or ataxia
2	Not ambulatory, but able to lift legs against gravity when supine
3	Paraplegic and unable to move legs against gravity

Modified from Lewis DW, Packer RJ, Raney B, et al: Incidence, presentation, and outcome of spinal cord disease in children with systemic cancer. Pediatrics *78:438–443, 1986.*

Late Effects

The long-term consequences of spinal cord compression and its management include neurologic compromise and orthopedic effects, and the choice of initial management heavily takes into account an attempt to minimize those late effects. A number of reports have supported the observation that neurologic sequelae are most related to the degree of presenting neurologic compromise; the more severe the neurologic compromise at presentation, the more likely long-term sequelae will be seen. Other studies have also shown that the duration of time between the development of neurologic symptoms and the initiation of therapy is inversely correlated with the degree of neurologic recovery.[49,63] Although laminectomy may be the most likely method to correct or prevent neurologic sequelae, it is clearly not necessary in all cases of spinal cord compression and carries the greatest risk of orthopedic sequelae. For patients with neuroblastoma in particular, the initial treatment for spinal cord compression did not demonstrate a clear impact on late effect frequency at a median follow-up of 8 years.[66] Orthopedic sequelae include scoliosis, growth impairment, and spinal instability. Orthopedic sequelae are related to the spinal level of the lesion and the age of the patient at treatment. The most severe sequelae are seen in patients with thoracic versus lumbar lesions and age younger than 12 months. Although a wide range of rates of sequelae has been reported, almost all studies have supported the observation of significantly greater orthopedic sequelae in patients who undergo surgery. In a study by Hoover and coworkers,[67] a scoliosis incidence of 67% was found in patients who underwent laminectomy compared with an incidence of 36% in patients who did not undergo laminectomy. Correlation between late effects, including differences in health-related quality of life and initial treatment, remain at the center of investigations into the optimal choice of initial therapy.

HYPERLEUKOCYTOSIS AND LEUKOSTASIS

Hyperleukocytosis is a clear, life-threatening pediatric oncologic emergency that requires immediate initiation of appropriate therapy. Marked metabolic abnormalities

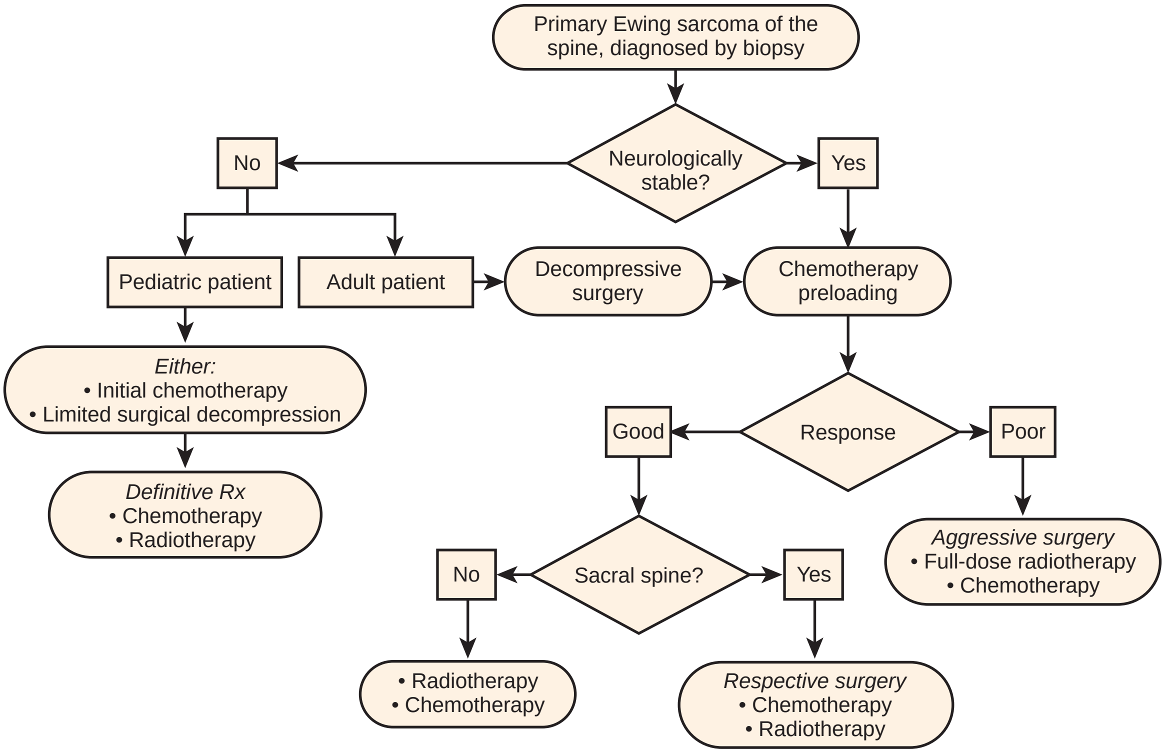
Primary Ewing sarcoma of the spine, diagnosed by biopsy
Neurologically stable?
No
Yes
Pediatric patient
Adult patient
Decompressive surgery
Chemotherapy preloading
Either:
• Initial chemotherapy
• Limited surgical decompression
Definitive Rx
• Chemotherapy
• Radiotherapy
Response
Good
Poor
Aggressive surgery
• Full-dose radiotherapy
• Chemotherapy
Sacral spine?
No
Yes
• Radiotherapy
• Chemotherapy
Respective surgery
• Chemotherapy
• Radiotherapy

Figure 68-7 Proposed algorithm for the management of primary Ewing sarcoma of the spine. *Rx,* Therapy. *(Modified from Sharafuddin MJ, Haddad FS, Hitchon PW, et al: Treatment options in primary Ewing's sarcoma of the spine: report of seven cases and review of the literature.* Neurosurgery *30:610–618, discussion 618–619, 1992.)*

and mild to severe pulmonary and neurologic symptoms can accompany the initial finding of hyperleukocytosis or can develop shortly after presentation. Stasis of leukemic cells within blood vessels and migration of blast cells into tissues can lead to the clinical entity of leukostasis, with mortality rates reported as high as 20% to 40%.[68-70] Despite the relative frequency of this true oncologic emergency and several varied therapeutic options, no data-driven management guidelines are available because of overall small numbers of patients, variability of clinical presentation, molecular differences in the underlying disease, and the difficulty of conducting randomized clinical trials with these constraints.

Definition

Hyperleukocytosis is generally accepted as a white blood cell (WBC) count higher than 100×10^9/L, although some studies have included patients with a WBC count higher than 50×10^9/L. It is estimated to occur in 5% to 20% of childhood leukemias and is seen most frequently in infant ALL and T-cell ALL. Hyperleukocytosis is more associated with certain subtypes of ALL and AML and is seen in almost all cases of childhood chronic myelogenous leukemia (Table 28-2). It has long been observed that the occurrence of leukostasis does not correlate directly with degree of hyperleukocytosis. Although hyperleukocytosis is seen more frequently in children with ALL, clinical leukostasis is seen more frequently in children with AML, particularly in M4 and M5 French-American-British subtypes. Clinical symptoms from hyperleukocytosis generally occur at WBC counts higher than 200 to 300×10^9/L in patients with AML and higher than 400 to 500×10^9/L in patients with ALL.[71] However, symptoms of leukostasis have also been observed in patients with WBC counts lower than 100×10^9/L.[69,72,73]

Clinical Presentation

Clinical symptoms of leukostasis are dependent on the system affected. Any small vessels, and therefore any organs, can be at risk. Although pulmonary and neurologic symptoms are most often observed, involvement of many other systems has also been reported. Hyperleukocytosis has been reported to result in leukostasis, causing renal failure, papilledema, dactylitis, priapism or clitorism, acute myocardial infarction, and cardiac failure.[74-76] Histopathologic studies have demonstrated aggregates of blast cells and thrombi leading to occlusion of small vessels in multiple organs of patients with hyperleukocytosis.[77] Corresponding to observed clinical symptoms, postmortem examinations have shown profound infiltration of multiple organs, including brain (including retinal vessels), liver, spleen, adrenals, and pulmonary vasculature, and in the alveoli and connective tissue of the lungs. A striking image of leukemic infiltration of a coronary artery was obtained by Thornton and Levis[75] (Fig. 68-8, Table 68-3).

The observance of the clinical expression of leukostasis and the accompanying related comorbidities has evolved to a description of a leukostasis syndrome, which has been defined as a critical condition with severe metabolic

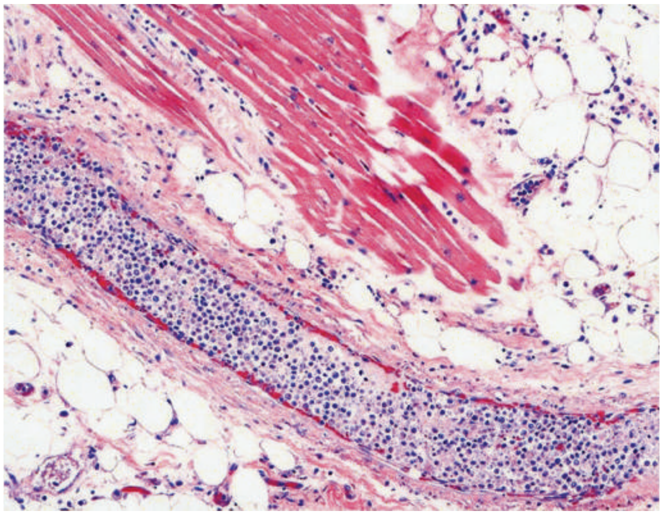

Figure 68-8 Small vessels throughout the body are at risk of occlusion in a patient with hyperleukocytosis. This image shows a coronary artery filled with fms-related tyrosine kinase 3–internal tandem duplication+ (FLT3-ITD+) acute myelogenous leukemia blasts. *(Modified from Thornton KA, Levis M: Images in clinical medicine: FLT3 mutation and acute myelogenous leukemia with leukostasis.* N Engl J Med *357:1639, 2007.)*

abnormalities, tumor lysis syndrome, severe coagulopathy, and multiorgan failure that occurs in patients presenting with hyperleukocytic leukemia.[78,79]

Pathogenesis

The pathogenesis of leukostasis is under continued investigation as researchers have realized the importance and potential clinical relevance of understanding the pathophysiology involved. Increased blood viscosity is often postulated to contribute to the development of leukostasis. At high flow rates (in larger vessels), bulk viscosity of the blood is a function of the deformability of the blood cells and the fraction of blood represented by these cells.[72] The overall viscosity of the blood generally has been found not to increase, even with the marked increase in leukocytic cells, in part because of the corresponding decrease in erythrocrit with increasing leukocrit. In patients with associated moderate to severe anemia, observed leukocrit levels greater than 20% to 25% are needed to demonstrate an objective increase in blood viscosity. Transfusion of red blood cells prior to reduction of the leukocyte count will increase the erythrocrit and can therefore result in hyperviscosity, which can be extremely deleterious to the patient, inducing clinical manifestations of hyperviscosity.

Blood viscosity in the microcirculation can increase because the viscosity is a function of the plasma viscosity and the deformability of the individual cells in the capillaries. Leukemic blasts are larger and less deformable than are erythrocytes or normal leukocytes. Flow in microvessels will slow if the diameter of the poorly deformable blast cell approaches that of the channel. Lymphoblasts range in size from 250 to 350 mm^3 and myeloblasts from 350 to 450 mm^3. It has been suggested that the larger size of myeloblasts compared with lymphoblasts helps account for the greater incidence of intracerebral hemorrhage that has been observed in patients who have AML compared with those who have ALL.[71] Histopathologic studies have revealed leukocyte thrombi and aggregates in the cerebral vasculature of patients with AML.

Appreciation of the role of adhesion molecules and the endothelial lining of the blood vessels is increasing. Laboratory observations have shown that expression of intracellular adhesion molecule–1, vascular cell adhesion molecule–1, and E-selectin can be induced in endothelial cells exposed to supernatants of blast cell cultures.[80] This effect was regulated by blast cell production of tumor necrosis factor-α and interleukin-1β. It is suggested that through this self-perpetuating interaction, leukemic blasts can promote their own adhesion to inactivated vascular endothelium. Alternate mechanisms of leukostasis have also been proposed and are being investigated, including molecular interactions directly between leukemic blast cells, leading to clumping and intravascular aggregates.

The molecular origin and subsequent cell phenotype is likely important to the role in developing leukostasis. The expression of CD56 neural cell adhesion molecule on leukemic blasts in a study of adult patients with AML was correlated with the development of severe clinical leukostatic syndrome.[78] In another study of adult patients with acute promyelocytic leukemia (APL, or level M3 AML), expression of CD13 (aminopeptidase N), a cell surface enzyme previously linked to tumor cell invasion, was highly associated with elevated leukocyte count and the development of all-*trans*-retinoic acid syndrome.[2] Further study in these intriguing areas of research is needed to confirm these findings and to test the applicability to pediatric patients. It is hoped that a better understanding of the underlying pathophysiology will lead to methods for early detection of patients at highest risk for severe leukostasis and eventually provide pharmacologic targets for disruption of the interaction of adhesion molecules, resulting in clinical improvements.

TABLE 68-3 Factors Associated With Occurrence of Hyperleukocytic Leukemias in Childhood

Acute Lymphoblastic Leukemia	Acute Myelogenous Leukemia
Age <1 year	Age <1 year
11q23	11q23
T cell with mediastinal mass	FAB M4 and M5
CNS involvement	FAB M3v
Hypodiploid	Inv 16
Philadelphia chromosome+	FLT3-ITD+*

CNS, Central nervous system; *FAB,* French-American-British; *FLT3-ITD,* fms-related tyrosine kinase 3–internal tandem duplication.

*FLT3 internal tandem duplication, shown in young adults.[74]

Clinical Presentation

Clinical manifestations of hyperleukocytosis and leukostasis can be myriad. Many patients with hyperleukocytosis are relatively asymptomatic. Clinical leukostasis can present subtly and evolve rapidly. Most commonly, pulmonary symptoms are observed. Tachypnea, oxygen desaturation, and dyspnea can occur and can progress rapidly to acute respiratory disease syndrome and respiratory failure. Neurologic symptoms can include headache, tinnitus, ataxia, behavioral changes, seizures, and stroke. Hemorrhagic stroke is a highly morbid complication of hyperleukocytosis and is seen most often in the microgranular variant of APL (level M3v AML). Often these patients have associated disseminated intravascular coagulation and thrombocytopenia, increasing their risk for hemorrhage.

Symptoms can rapidly worsen after initiation of therapy. It has been postulated that this phenomenon may be caused by release of intracellular components of blast cells after lysis, including enzymes that can lead to injury in surrounding tissue, such as alveolar damage and interstitial edema.[81] When using modern pediatric AML protocols for treatment and supportive care, early mortality in patients with AML who have hyperleukocytosis is very low, but hyperleukocytosis continues to be associated with inferior disease outcomes.[82,83] Additionally, Fong and coworkers[84] described unique anesthesia-related considerations for children with hyperleukocytosis and recommend close observation postoperatively for leukostasis-related pulmonary and neurologic events.

Authors of a number of studies have looked at the factors associated with more severe clinical events in patients with hyperleukocytic leukemias, with mixed conclusions. A clinical grading system has been proposed by Novotny and colleagues[79] (Table 68-4). The grading system groups patients into four categories: not present (0), possible (1), probable (2), and highly probable (3). The grading is based on the assumption that the severity of presenting symptoms may indicate the probability of leukostasis, provided another cause for the symptoms is not found. This assumption is supported by the knowledge that patients with respiratory distress or severe neurologic symptoms have a much increased mortality rate.[85] The investigators used this clinical scoring system in conjunction with initial flow cytometry data to show that the detection of CD56 expression at baseline in patients with AML level M4 and M5 disease may identify patients at highest risk for fatal leukostasis. This approach has clear clinical relevance but requires further validation prior to clinical application.

Treatment

Definitive treatment for hyperleukocytosis is the initiation of appropriate induction chemotherapy as soon as a diagnosis is established. Previously, for many years the initiation of IV hydration, urinary alkalinization, and allopurinol was the clinical standard for the immediate initial treatment of hyperleukocytic acute leukemias. In 1995, Basade et al.[86] reported an 81.5% reduction in WBC count within a median of 36 hours in a cohort of pediatric patients who had ALL with the use of IV hydration, urinary alkalinization, and allopurinol. No treatment-related complications occurred in their patient cohort. Although studies have not yet been published, the availability of urate oxalate is likely to modify this practice, because many of these patients clearly meet criteria for high risk of TLS. Early therapy with urate oxalate has the potential to decrease the risk for TLS markedly in patients who have acute hyperleukocytic leukemias, which could decrease the number of patients in whom leukostasis syndrome develops, thereby positively affecting overall morbidity and mortality.

TABLE 68-4 Grading of Symptoms in Hyperleukocytic Leukemia*

Group	Probability of Leukostasis Syndrome	Severity of Symptoms	Pulmonary Symptoms	Neurologic Symptoms	Other Organ Systems
0	Not present	No limitations	No symptoms and no limitations in ordinary activities	No neurologic symptoms	No symptoms
1	Possible	Slight limitations	Mild symptoms and slight limitation during ordinary activity, comfortable at rest	Mild tinnitus, headache, dizziness	Moderate fatigue
2	Probable	Marked limitations	Marked limitation in activity because of symptoms, even during less than ordinary activity, comfortable only at rest	Slight visual disturbances, severe tinnitus, headache, dizziness	Severe fatigue
3	Highly probable	Severe limitations	Dyspnea at rest, oxygen or respirator required	Severe visual disturbances (acute inability to read), confusion, delirium, somnolence, intracranial hemorrhage	Myocardial infarction, priapism, ischemic necrosis

Modified from Novotny JR, Muller-Beissenhirtz H, Herget-Rosenthal S, et al: Grading of symptoms in hyperleukocytic leukaemia: a clinical model for the role of different blast types and promyelocytes in the development of leukostasis syndrome. Eur J Haematol *74:501–510, 2005.*

*Probability of leukostasis deduced from the severity of symptoms attributable to leukostasis (no other obvious cause).

Additional measures to reduce the elevated WBC count and associated morbidities have been studied. Cranial radiation has been proposed to decrease the chance of intracerebral hemorrhage. Based on a study of adult patients with AML and a WBC count higher than 200 × 10^9/L, Wiernik and Serpick[87] have proposed the prophylactic use of 400 to 600 cGy of cranial radiotherapy to prevent intracranial hemorrhage. Several small series that used emergency cranial radiotherapy in pediatric patients with ALL and hyperleukocytosis were also reported, with a low incidence of intracranial hemorrhage.[88] However, subsequent experience has not consistently shown a beneficial effect of prophylactic cranial radiotherapy. Additionally, concerns exist about acute and chronic neuropsychiatric complications associated with cranial radiotherapy in pediatric patients, and thus it is rarely used for this indication in current practice.[89-91]

The use of low-dose prednisone as initial treatment for patients with ALL and hyperleukocytosis has been proposed, with the rationale of inducing a gradual blast cell lysis in an attempt to decrease the severity of metabolic derangements. Along with several other interventions, the use of low-dose prednisone was reviewed in a group of 124 patients with newly diagnosed ALL and a WBC count higher than 200 × 10^9/L who were followed up by the Children's Cancer Study Group from 1981 to 1983.[91] All patients received IV hydration, urinary alkalinization, and allopurinol. Some patients received additional measures (e.g., low-dose prednisone, cranial radiation, exchange transfusion, and leukapheresis) in an attempt to decrease complications. It was concluded that pretreatment with low-dose prednisone does not correlate with fewer electrolyte abnormalities. However, many of the patients in this study received more than one additional treatment, complicating the analysis in this relatively small group of patients.

Leukapheresis and exchange transfusion have both been shown to be effective in reducing the total WBC and blast count. Patients weighing less than 12 kg usually are unable to undergo leukapheresis but may safely undergo exchange transfusion. Studies in pediatric patients have shown a mean reduction of 50% to 60% in WBC with exchange transfusion and leukapheresis.[90,92] However, the rate of WBC clearance with leukapheresis is inconsistent, and the overall benefit of this cytoreduction is unclear.[93]

In patients with ALL, the use of leukapheresis may decrease the risk of severe TLS. Patients with ALL and hyperleukocytosis in the Children's Cancer Study Group cohort who received leukapheresis or exchange transfusion appeared to show some benefit of the procedures, with a lower incidence of severe electrolyte abnormalities and renal dysfunction.[91] The same conclusion was reached by Bunin and Pui[71] in a series of 161 patients with ALL and hyperleukocytosis.

In the three largest studies in patients with AML that examined the role of leukapheresis, differing conclusions were reached. In a group of 22 patients with newly diagnosed AML (ages 12 to 74 years), it was found that decreasing the total WBC count through leukapheresis by at least 30% correlates with improved outcome in those patients.[94] In contrast, in a study by Porcu and colleagues,[85] 48 patients ages 8 to 79 years who had AML or chronic myelogenous leukemia and were in blast crisis underwent immediate cytoreduction with leukapheresis, and no correlation was found between the degree of cytoreduction and early mortality rate. In the largest study, Giles and coworkers[95] reviewed outcomes of 146 patients (ages 16 to 86 years) with AML, 71 of whom underwent leukapheresis. An association with decreased early mortality rate was found, but no improvement occurred in the overall survival rate.

The use of leukapheresis in patients with APL is greatly discouraged because increased rates of mortality have been observed in these patients, which are largely caused by the increased incidence of hemorrhage. Vahdat and colleagues[2] reported fatal or near-fatal hemorrhage in 9 of 11 patients with APL who underwent leukapheresis.

Hydroxyurea is known to be effective in reducing overall WBC and blast counts.[96] At the American Society of Hematology 2006 Annual Meeting, preliminary results of a randomized study on the use of hydroxyurea versus leukapheresis in patients with AML were presented, with equivalent outcome noted at 8 and 28 days and no difference in death rate.[97]

The possible benefits from a rapid reduction in WBC and blast count that can be achieved with leukapheresis or exchange transfusion need to be weighed against the risks of these procedures, which include obtaining adequate venous access and anticoagulation. The decision to initiate leukapheresis must also take into account the time to arrange these procedures, including mobilization of specialized blood bank personnel and possible transfer to tertiary centers where such specialized procedures are available. Institution of these procedures can be particularly deleterious if they delay initiation of definitive chemotherapy treatments. With increased use of urate oxalate, the benefit of leukapheresis in patients with ALL may need to be reassessed.

Conclusions

Acute leukemias presenting with hyperleukocytosis pose immediate clinical challenges to the treating pediatric oncologist and must be addressed rapidly and appropriately. Although many components of treatment of hyperleukocytosis are generally accepted, much ambiguity still exists about the appropriateness of many possible interventions. Although further study is appropriate, carrying out such studies poses many challenges. Box 68-2 groups treatment guidelines into those that have been shown to be clearly beneficial, those that have been shown to be harmful, and those of less certain benefit. It is hoped that translational research into the underlying pathophysiology of the leukostasis syndrome will lead to novel therapeutic modalities and pharmacologic interventions.

TUMOR LYSIS SYNDROME

TLS is the most commonly encountered pediatric oncologic emergency. Diagnosis and treatment strategies have evolved during the past decade with the development of new drug therapy. Morbidity and mortality have improved as the entity is now recognized and treated earlier in

Box 68-2 **Clinical Guidelines for Patients With White Blood Cell Count Greater Than 50×10^9 and Diagnosis of Acute Leukemia**

RECOMMENDED FOR ALL PATIENTS WITH HYPERLEUKOCYTOSIS

- Institution of appropriate chemotherapy as soon as a diagnosis is made
- Careful clinical observation, neurologic examination, frequent laboratory evaluation per tumor lysis protocol, evaluation of renal function, chest radiograph, and oxygen saturation monitoring with supplemental oxygen as needed
- Treatment for TLS with IVF, alkalinization, and allopurinol OR rasburicase and no alkalinization; strong bias toward use of rasburicase if any parameters are met for high risk of TLS-increased uric acid, creatinine, LDH, potassium (see section on TLS)
- Correction of coagulopathy as appropriate with cryoprecipitate, FFP, vitamin K; special consideration of disseminated intravascular coagulation and risk of intracerebral hemorrhage in M4 or M5 level AML, and even greater risk in level M3v APL
- Platelet transfusion for platelet count <25 K or any clinical bleeding

RECOMMENDED IN SOME CLINICAL CIRCUMSTANCES

- Consider exchange transfusion or leukapheresis in patients with ALL and WBC >300-500 × 10^9, or AML and WBC >100-200 × 10^9, particularly if any pulmonary or neurologic symptoms exist
- Consider low-dose prednisone reduction phase prior to induction chemotherapy in patients with ALL and WBC >300-500 × 10^9 (may decrease chance of TLS)

CONTRAINDICATED

- Avoid leukapheresis in patients with APL (substantially increases the risk of intracerebral hemorrhage)
- Avoid use of diuretics or transfusion of PRBC in patients with ALL and WBC >300-500 × 10^9, or AML and WBC >100-200 × 10^9 (confers increased risk of hyperviscosity and clinical leukostasis)

ALL, Acute lymphoblastic leukemia; *AML,* acute myelogenous leukemia; *APL,* acute promyelocytic leukemia; *DIC,* disseminated intravascular coagulation; *FFP,* fresh-frozen plasma; *IVF,* intravenous fluid; *LDH,* lactate dehydrogenase; *PRBC,* packed red blood cells; *TLS,* tumor lysis syndrome; *WBC,* white blood cell count.

presentation. New therapeutic interventions have become available and are more widely used.

Overview

After the incorporation of cytotoxic chemotherapy into the treatment of hematologic malignancies and solid tumors, recurrent observations were made of severe metabolic abnormalities associated with the onset of therapy.[98,99] The consistent occurrence of these metabolic and electrolyte imbalances, particularly in the setting of lymphoma and leukemia, eventually became recognized as TLS. TLS describes the metabolic derangements that occur as a result of spontaneous or treatment-related breakdown of tumor cells. The release of intracellular contents from the tumor cells into the bloodstream leads to the characteristic triad of hyperuricemia, hyperkalemia, and hyperphosphatemia. TLS is most commonly associated with initiation of therapy for cancers with massive tumor burden or high proliferative rates, such as ALL and Burkitt lymphoma. Predictors of TLS include the presence of bulky disease, adenopathy, hepatosplenomegaly, and a high leukocyte count. Additional risk factors for the development of severe TLS include increased lactate dehydrogenase (LDH), uric acid, creatinine, and decreased urine output.[76,100,101] TLS can result in high morbidity and mortality, with possible rapid progression to multiorgan failure, and thus the prompt identification of patients at risk for this oncologic emergency and the institution of preventive measures are critical. Although the risk of developing TLS is highest at 12 to 72 hours after the initiation of chemotherapy, symptoms can also precede the initiation of therapy or occur as many as 7 days later. The main principles of TLS prevention and treatment include hydration and diuresis, careful monitoring of electrolyte abnormalities, and management of hyperuricemia. In this section, we will discuss the pathophysiology, incidence, and recent advances in the management of TLS.

Pathophysiology

Although TLS can occur spontaneously prior to the administration of chemotherapy, it is most commonly observed after the initiation of therapy. In tumors with a high proliferative rate, large tumor burden, and high chemosensitivity, the exposure of the tumor cells to cytotoxic chemotherapy leads to massive cell lysis, with the release of intracellular anions, cations, and breakdown products of nucleic acids and proteins into the bloodstream. This rapid efflux of intracellular contents can exceed the renal capacity for clearance and consequently can lead to the metabolic derangements and electrolyte imbalances observed in TLS. Hyperuricemia is the most common finding in TLS; it results from the release of intracellular nucleic acids and the subsequent catabolism of the purine nucleotides, adenosine and guanosine. Uric acid, the final product of both endogenous and dietary purine nucleotide catabolism, is normally generated in the liver by oxidation of xanthine to uric acid by xanthine oxidase (Fig. 68-9). Uric acid is a weak acid, with a pK_a of 5.4 to 5.7. At normal concentrations and at physiologic pH, 98% of uric acid is in the ionized form as urate, which is soluble in urine.[102] However, when uric acid is present in high concentrations in acidic urine, it can crystallize in the renal parenchyma, distal tubules, and collecting ducts, leading to intraluminal tubular obstruction and oliguria.

Hyperkalemia poses the greatest immediate threat of mortality because the rapid rise in serum potassium can result in severe arrhythmia and death. This life-threatening consequence of TLS is caused in part by the kidney's inability to excrete the massive quantities of intracellular potassium released from dying tumor cells. The kidney's inability to handle the excessive load of potassium can be amplified by renal dysfunction caused by precipitants such as uric acid. Hyperkalemia in the setting of TLS can be further exacerbated by concurrent metabolic acidosis or iatrogenic administration of potassium with IV fluids.

Hyperphosphatemia results from the rapid release of intracellular phosphorus from tumor cells, which can contain three to four times the amount of phosphorus in normal cells.[3,103] The development of hyperphosphatemia can also worsen in the setting of concurrent acute renal insufficiency as a result of uric acid precipitation. In TLS, the renal tubular capacity to reabsorb phosphorus can be

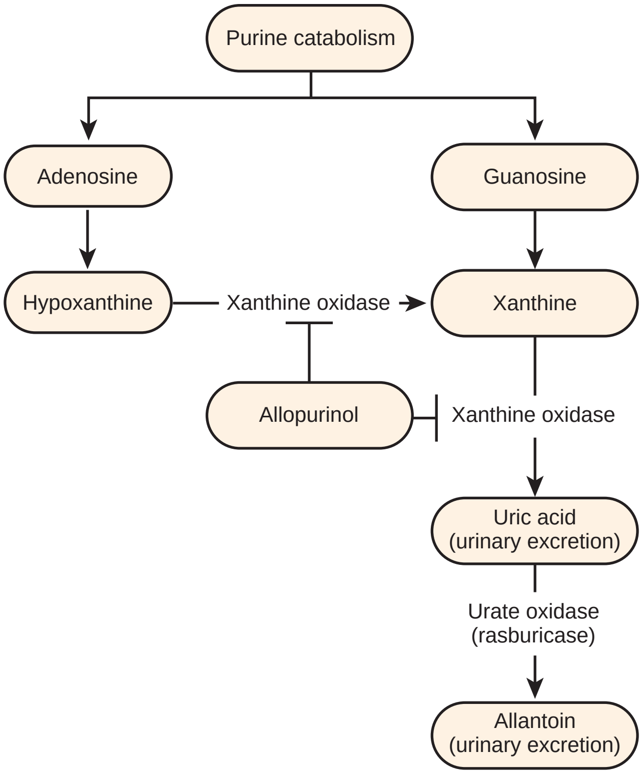

Figure 68-9 Pathophysiology of hyperuricemia in tumor lysis syndrome. Uric acid is a breakdown product of purine nucleotide catabolism. The current pharmacologic strategies for the management of hyperuricemia are allopurinol and rasburicase.

exceeded and lead to hyperphosphaturia. A cyclic process commences, in which the acute hyperphosphatemia and hyperphosphaturia can then lead to acute nephrocalcinosis and renal failure via precipitation of calcium phosphate crystals, further impairing the renal tubular reabsorption of phosphorus. The normal calcium × phosphorus product ranges between 30 and 55 mg^2/dL^2. When this product exceeds 70 mg^2/dL^2, the risk of calcium phosphate deposition in the kidney and other tissues is significant.[104] Metastatic calcification caused by calcium phosphate crystals in other soft tissues is rarely seen in TLS but has been described.[105] In addition, the precipitation of calcium phosphate can lead secondarily to asymptomatic or symptomatic hypocalcemia.

Incidence

The highest incidence of TLS occurs in ALL and high-grade NHL. A retrospective study of 102 patients with high-grade NHL found the incidence of TLS to be 42% based on laboratory evidence, although only 6% of the patients had clinically significant TLS.[106] Similarly, a pan-European retrospective chart review has reported TLS in 5.2% and 6.1% of patients with ALL and NHL, respectively.[107] This study also demonstrated a high incidence of mortality in patients with clinical TLS. Of the patients with TLS in this study, 17.5% died from TLS-related causes.

The incidence of TLS appears to be similar in the pediatric population. A retrospective study of 1192 pediatric patients with any form of NHL identified 63 patients (5.3%) with clinically significant TLS.[108] Among these 63 patients, two patients (3.2%) died within 48 hours of therapy because of electrolyte imbalances. However, another retrospective pediatric study has indicated that the incidence of TLS is significantly higher in patients with B-cell ALL or Burkitt lymphoma stage III or IV, with LDH greater than or equal to 500 units/L. Of the 218 patients who met these criteria initially (prior to the prophylactic use of urate oxidase), TLS developed in 35 (16.1%), and 20 (9.2%) became anuric.[109]

Other hematologic malignancies that have been less commonly associated with TLS include AML, chronic lymphocytic leukemia, and multiple myeloma. Only isolated case reports of TLS have been described in other hematologic malignancies, such as chronic myeloid leukemia in blast crisis, myeloproliferative disorders, and Hodgkin disease. TLS is also uncommon in solid tumors, which generally have a longer doubling time and a slow response to chemotherapy in comparison with lymphoproliferative malignancies. However, TLS can be seen in solid tumors that are highly chemosensitive or that have a high tumor burden, as in bulky, metastatic disease or in tumors that present with renal compromise. In the context of newly diagnosed solid tumors, renal compromise may occur from mass effect and compression. A literature review has identified 45 reported cases of TLS in solid tumors from 1977 to 2002.[110] Most of these cases occurred in tumors with a high response rate to cytotoxic therapy, such as small cell lung carcinoma, germ cell tumors, and breast carcinoma. Among the 45 reported cases, 16 patients (35.5%) died as a result of TLS. Risk factors for the development of TLS in persons with solid tumors were similar to those in persons with hematologic malignancies—increased LDH, hyperuricemia, and pretreatment renal impairment. However, the mortality rate caused by complications from TLS in these patients with solid tumors was higher than reported mortality rates in patients with hematologic malignancies. This finding may be the result of better implementation of prophylactic measures in patients with hematologic malignancies resulting from a greater awareness of TLS in this population.

Diagnosis

TLS should be anticipated in all new-onset oncology patients, with the highest concern for those presenting with a large tumor burden and concurrent laboratory abnormalities. Although many general criteria exist for TLS, a uniform, widely accepted definition for TLS is lacking. A standardized definition would enable a more exact determination of TLS incidence and would also improve the ability to evaluate and compare therapies for the prevention of TLS. Two classification systems for TLS have been developed and are variably followed, the Hande-Garrow system (Boxes 68-3 and 68-4) in 1993 and the Cairo-Bishop system in 2004. The Hande-Garrow classification system makes a distinction between laboratory and clinical TLS based on the observation that only a fraction of patients with TLS according to laboratory criteria experience clinically significant TLS.[106]

Box 68-3 **Hande-Garrow Definition of Laboratory Tumor Lysis Syndrome***

A 25% increase over pretreatment values in serum phosphate, potassium, uric acid, or urea nitrogen.
or
A 25% decline in serum calcium.

*Any two of the metabolic changes must occur within 4 days of treatment.

Box 68-4 **Hande-Garrow Definition of Clinical Tumor Lysis Syndrome***

Rise in serum creatinine >2.5 mg/dL.
Serum potassium level >6.0 mmol/L.
Decline in serum calcium to <6 mg/dL.
Development of life-threatening arrhythmia or death.

*Clinical tumor lysis syndrome is defined as the presence of laboratory tumor lysis syndrome and any one of the criteria in this box.

Laboratory TLS (LTLS) designates patients who have laboratory evidence of metabolic changes as a result of tumor cell breakdown but who do not require any therapeutic intervention. Patients with any of the following metabolic changes occurring within the first 4 days of treatment are defined as having LTLS: (1) a 25% increase compared with pretreatment values in serum phosphate, potassium, uric acid, or urea nitrogen levels or (2) a 25% decline in the serum calcium level. Clinical TLS (CTLS) is designated in any patient with LTLS who also experiences one of the following: (1) a rise in the serum creatinine level greater than 2.5 mg/dL; (2) a serum potassium level greater than 6.0 mmol/L; (3) a decline in the serum calcium level to less than 6 mg/dL; (4) the development of a life-threatening arrhythmia; or sudden death. In practice, this classification system has several limitations. First, in requiring a 25% increase in the baseline laboratory value, the definition of LTLS does not account for patients who have preexisting abnormal laboratory values at presentation. In addition, the definition requires that the laboratory changes occur within 4 days of starting therapy, which excludes patients with clinical evidence of TLS at presentation or patients in whom TLS develops beyond 4 days of therapy. Additionally, any symptomatic hypocalcemia should alone constitute CTLS.[111]

The Cairo-Bishop classification system is a modified version of the Hande-Garrow system that aims to be more clinically relevant (Table 68-5).[112] For example, the definition of LTLS has been extended to include a window of 3 days before and 7 days after the initiation of therapy. Similar to the Hande-Garrow system, the definition of CTLS requires the presence of LTLS, plus one or more of the three most significant clinical complications associated with TLS: renal insufficiency, cardiac arrhythmias or sudden death, and seizures. Cairo and Bishop also introduced a grading system for TLS that incorporates their definitions of no TLS, LTLS, and CTLS. This grading system was evaluated as part of a Children's Oncology Group study of chemoimmunotherapy and rasburicase in patients with newly diagnosed NHL (trial ANHL01P1). One of the specific aims of this study was to determine the incidences of TLS, consequent renal complications, and the requirement for assisted renal support (e.g., dialysis or hemofiltration) during the reduction phase and first induction phase of therapy in children with newly diagnosed advanced-stage leukemia or lymphoma. It was found that the rasburicase was safe and well tolerated. Increased initial LDH was correlated with increased uric acid level, increased incidence of TLS, and lower glomerular filtration rate. Patients treated with rasburicase have only a 9% and 5% incidence of LTLS and CTLS, respectively. Importantly, a significant improvement in estimated glomerular filtration rate from day 0 to day 7 was found in patients after administration of rasburicase, and only 3% of patients required new-onset renal-assisted support after administration of rasburicase.[113]

Building on the Cairo-Bishop and Hande-Garrow definitions of LTLS and CTLS, an algorithm for the assessment

TABLE 68-5 Cairo-Bishop Grading Classification of Tumor Lysis Syndrome

Grade	LTLS	Creatinine Level	Cardiac Arrhythmia	Seizure
0	–	≤1.5 × ULN	None	None
I	+	1.5 × ULN	Intervention not indicated	None
II	+	>1.5-3.0 × ULN	Nonurgent medical intervention indicated	One brief generalized seizure, seizure(s) well controlled by anticonvulsants, infrequent focal motor seizures not interfering with ADLs
III	+	>3.0-6.0 × ULN	Symptomatic and incompletely controlled medically or controlled with device (e.g., defibrillator)	Seizure in which consciousness is altered, poorly controlled seizure disorder, breakthrough generalized seizures despite medical intervention
IV	+	>6.0 × ULN	Life-threatening (e.g., arrhythmia associated with CHF, hypotension, syncope, shock)	Seizures of any type that are prolonged, repetitive, or difficult to control (e.g., status epilepticus and intractable epilepsy)
V	+	Death	Death	Death

Modified from Cairo MS, Bishop M: Tumour lysis syndrome: new therapeutic strategies and classification. Br J Haematol *127:3–11, 2004.*
ADLs, Activities of daily living; *CHF,* congestive heart failure; *LTLS,* laboratory tumor lysis syndrome; *ULN,* upper limit of normal.

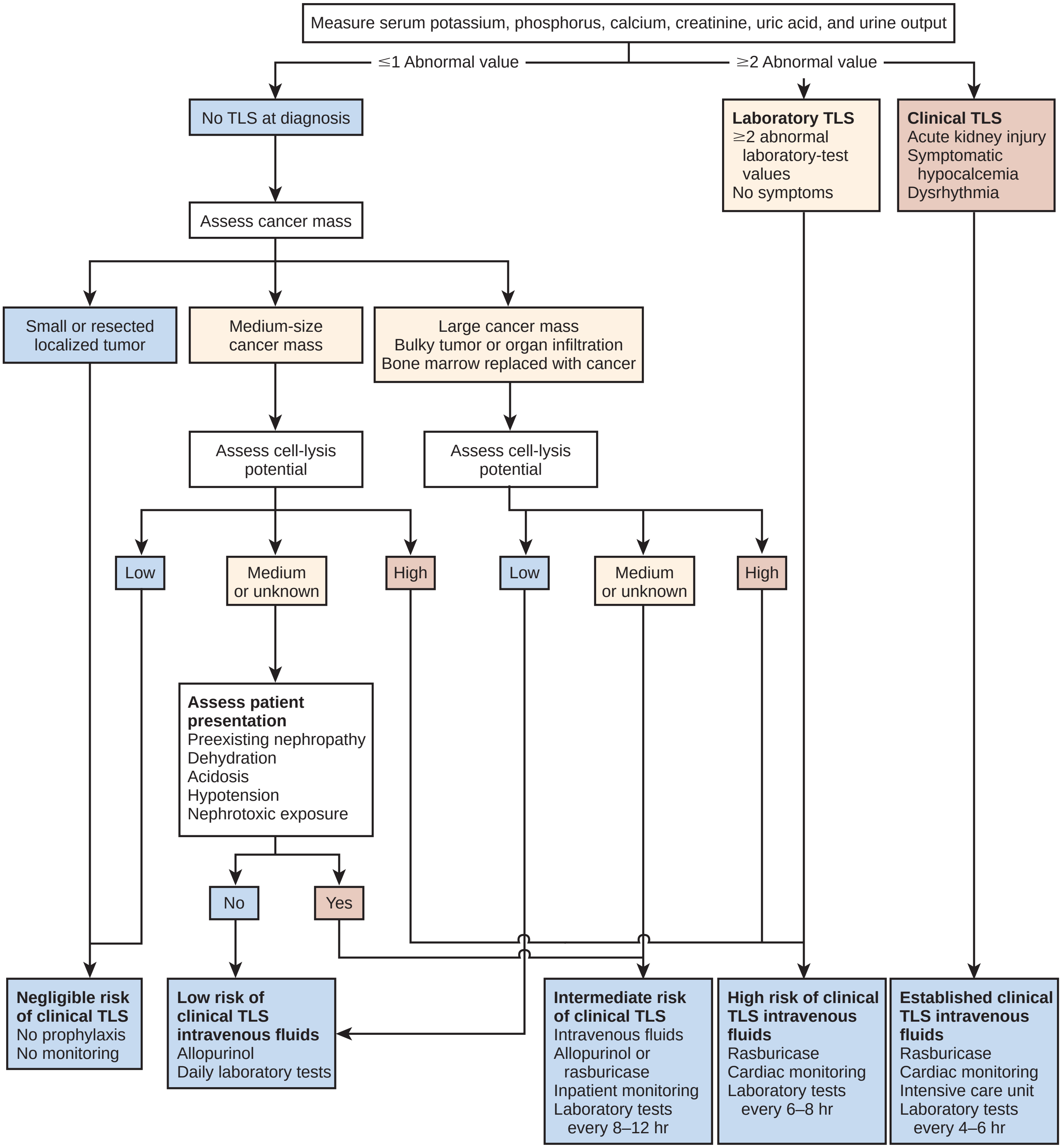
Measure serum potassium, phosphorus, calcium, creatinine, uric acid, and urine output
≤1 Abnormal value
≥2 Abnormal value
No TLS at diagnosis
Laboratory TLS ≥2 abnormal laboratory-test values No symptoms
Clinical TLS Acute kidney injury Symptomatic hypocalcemia Dysrhythmia
Assess cancer mass
Small or resected localized tumor
Medium-size cancer mass
Large cancer mass Bulky tumor or organ infiltration Bone marrow replaced with cancer
Assess cell-lysis potential
Assess cell-lysis potential
Low
Medium or unknown
High
Low
Medium or unknown
High
Assess patient presentation Preexisting nephropathy Dehydration Acidosis Hypotension Nephrotoxic exposure
No
Yes
Negligible risk of clinical TLS No prophylaxis No monitoring
Low risk of clinical TLS intravenous fluids Allopurinol Daily laboratory tests
Intermediate risk of clinical TLS Intravenous fluids Allopurinol or rasburicase Inpatient monitoring Laboratory tests every 8–12 hr
High risk of clinical TLS intravenous fluids Rasburicase Cardiac monitoring Laboratory tests every 6–8 hr
Established clinical TLS intravenous fluids Rasburicase Cardiac monitoring Intensive care unit Laboratory tests every 4–6 hr

Figure 68-10 Proposed algorithm for assessment of tumor lysis syndrome *(TLS). (Howard SC, Jones DP, Pui CH: The tumor lysis syndrome.* NEJM *364:1844–1854, 2011.)*

and initial management of TLS was proposed by Howard and colleagues (Fig. 68-10).[111] It is important to note that this guide is intended for a patient at the time of presentation, however; subsequent care and interventions should reflect the clinical course, expected or not, of the patient. When distinguishing the best classification group for a patient is difficult, it is always advisable to provide treatment as if the patient is in the higher risk group.

Clinical Manifestations and Treatment

Clinical manifestations of acute TLS include nausea, anorexia, cardiac arrhythmias, seizures, muscle cramps, tetany, oliguria or anuria, and alterations in consciousness. These symptoms primarily result from the electrolyte disturbances seen in TLS (described earlier). The most important objectives for the successful management of acute TLS are the prompt identification of patients at high risk for the development of TLS and aggressive institution of appropriate prophylactic measures to prevent and/or reduce the severity of the clinical manifestations of acute TLS (Box 68-5). Initial studies that can predict TLS and guide preventive management include serum electrolyte, calcium, phosphate, blood urea nitrogen, creatinine, and uric acid levels. This panel of laboratory assessments should be performed promptly for any patient presenting with new diagnosis of malignancy. The main preventive measures are hydration and optimization of urine output, along with prevention of uric acid synthesis by allopurinol or rasburicase. In addition to the specific treatment recommendations discussed later, patients at risk for TLS should be carefully observed with use of cardiovascular and respiratory monitors. Frequent vital signs and strict fluid assessments including intake and output should be recorded. Laboratory tests should be performed at a minimum every 6 to 8 hours initially, and the results should be evaluated immediately (see Box 68-5).

Box 68-5 **Management of Patients at Risk for Tumor Lysis Syndrome**

HYDRATION

Dextrose 5% in half-normal saline solution (without potassium)
Recommended rate: two times maintenance (125 mL/m^2/hour)

ALKALINIZATION

Routine alkalinization is not recommended; use with allopurinol when the primary concern is uric acid level and when use of rasburicase is contradicted or it is not available
Modify $NaHCO_3$ as needed to maintain urine pH at 7.0 to 8.0
Stop $NaHCO_3$ if the serum bicarbonate level is ≥30 mEq/L and/or urine pH is >8.0 or if hyperphosphatemia develops

DIURESIS

Maintain urine output at >100 mL/m^2/hour and urine specific gravity at <1.010
Consider furosemide (0.5 to 1.0 mg/kg) or mannitol (0.5 g/kg) for low urine output in the absence of hypovolemia

URIC ACID REDUCTION

Consider the role of allopurinol or urate oxidase (rasburicase), based on assessment of the potential of TLS, initial uric acid and phosphate level, drug availability, and G6PD status:
- Options: Allopurinol: by mouth, 100 mg/m^2 three times daily, or 10 mg/kg/day divided in three doses
- Rasburicase: IV, 0.2 mg/kg once daily for up to 5 days
  - Indicated when serum creatinine is >1.5 times the upper limit or normal, or when uric acid is >7.0 mg/dL; consider at lower levels if the patient is assessed to be at high risk of TLS or if hyperphosphatemia exists
  - Contraindicated in patients with G6PD deficiency
  - Discontinue allopurinol if using rasburicase
  - No bicarbonate necessary in IV fluid

METABOLIC ABNORMALITIES

Monitor serum electrolytes every 4 to 8 hours

HYPERKALEMIA

Moderate (≥6.0 mmol/L)
- Avoid IV and oral potassium
- Electrocardiogram and cardiorespiratory monitor
- Kayexalate (1 g/kg orally with 25% sorbitol every 6 hours)

Severe (>7.0 mmol/L and/or symptomatic)—same as for moderate hyperkalemia, plus:
- Albuterol nebulization treatment
- Insulin (0.1 units/kg) with concurrent 25% dextrose (2 mL/kg)
- Calcium gluconate (100 to 200 mg/kg)
- Dialysis

HYPERPHOSPHATEMIA

Moderate (≥5.0 mg/dL)—aluminum hydroxide 50-150 mg/kg/24 hours in divided doses every 4-6 hours
Severe (with symptomatic hypocalcemia)—dialysis, CVVH, and CVAH

HYPOCALCEMIA

Asymptomatic—no therapy
Symptomatic—calcium gluconate (50 to 100 mg/kg/dose)

CVAH, Continuous venoarterial hemofiltration; *CVVH,* continuous venovenous hemofiltration; *G6PD,* glucose-6-phosphate dehydrogenase; *IV,* intravenous; *$NaHCO_3$,* sodium bicarbonate; *TLS,* tumor lysis syndrome.

Fluids and Alkalinization

Unless the patient presents with signs of acute renal failure or oliguria, aggressive hydration is the most important intervention. IV fluids should be administered at a rate of 3000 mL/m^2/day, and brisk urine output should be maintained (more than 100 mL/m^2/hour), with a urine specific gravity goal of less than or equal to 1.010. This increased hydration and increase in urine flow promotes urinary excretion of uric acid and phosphate by enhancing renal blood flow and glomerular filtration. Potassium, calcium, and phosphate should not be added to initial hydration fluids to avoid iatrogenic aggravation of hyperkalemia, hyperphosphatemia, or calcium phosphate precipitation. If urine output remains low despite aggressive hydration (usually caused by third spacing), diuretics may be used as long as there is no evidence of acute obstructive uropathy and/or hypovolemia. Commonly used diuretics are mannitol (0.5 g/kg) or furosemide (0.5 to 1.0 mg/kg).

Urine alkalinization remains controversial, although historically it has been a general recommendation for the prevention and treatment of TLS. An alkaline urine (urine pH ≥6.5) promotes urinary excretion of urate, with maximal solubility of urate occurring at a pH of 7.5.[114] However, the solubility of its precursors, xanthine and hypoxanthine, significantly decreases at a pH greater than or equal to 6.5. Thus excessive systemic and urinary alkalinization may lead to metabolic alkalosis and obstructive uropathies from xanthine and hypoxanthine crystallization. In addition, alkalinization of urine favors precipitation of calcium phosphate crystals in the renal tubules, a risk that can be further exacerbated by concomitant hyperphosphatemia. Metabolic alkalosis can also worsen the neurologic manifestations of hypocalcemia. Thus sodium bicarbonate should be used judiciously, with checks of urine pH with every void. If rasburicase is used, urine should not be alkalinized. If allopurinol is being used, alkalinization can be considered, but its use should be discontinued once serum uric acid normalizes, the dose should be decreased for urine pH greater than 8, and it must be discontinued if hyperphosphatemia develops.

Hyperkalemia

In the setting of TLS, hyperkalemia results from the massive efflux of intracellular potassium from dying tumor cells. Clinical manifestations include neuromuscular signs and symptoms such as muscle weakness, cramps, and paresthesias. Cardiac manifestations may include peaked T waves on an electrocardiogram, malignant arrhythmias, and conduction disturbances. Hyperkalemia is usually defined by a serum potassium level greater than or equal to 6.0 mmol/L. However, in the clinical setting of ongoing tumor lysis, a serum potassium level greater than 5.0 mmol/L and/or a trend of increasing values also merit intervention. Treatment of hyperkalemia is based on three approaches: driving extracellular potassium into cells, removing excess potassium from the body, and antagonizing the membrane effects of potassium. In asymptomatic patients, it is reasonable to begin simply with increased hydration with alkalinized fluids and diuresis with furosemide. The treatment of choice after alkalinization and optimization of urine output often is a potassium-binding cation exchange resin, such as sodium polystyrene sulfonate (1 g/kg/dose orally every 6 hours). This resin can also be given as a retention enema, but oral administration is more efficacious. Each gram of resin may bind as much as 1 mEq of potassium and release 1 to 2 mEq of sodium. For symptomatic patients, more rapid-acting treatments are required, such as insulin and β_2-adrenergic agonists, which drive potassium into cells by enhancing the activity of the Na+,K+ adenosine triphosphatase pump in skeletal muscle. Insulin is usually given as a bolus (0.1 unit/kg) with concurrent glucose infusion (25% dextrose, 2 mL/kg). Commonly used β_2-adrenergic agonists in the treatment of hyperkalemia are albuterol nebulizer treatments and IV epinephrine. Interventions commonly used in the fluid management of TLS, such as alkalinization and diuretics, can also help in the reduction of serum potassium. The increase in systemic pH with sodium bicarbonate results in hydrogen ion release from the cells as part of the buffering reaction; this change results in the movement of potassium into the cells to maintain electroneutrality. Loop and thiazide diuretics can lead to a transient lowering of serum potassium. Calcium directly antagonizes the inactivation of sodium channels and decreased membrane excitability caused by hyperkalemia. Thus calcium gluconate (100 to 200 mg/kg) can be given to stabilize myocardial conduction in cases of severe hyperkalemia or the presence of electrocardiographic abnormalities. Because concomitant hyperphosphatemia and alkalinization in the setting of TLS can increase the risk of calcium phosphate or calcium carbonate precipitation, calcium infusions should be given only when absolutely necessary. Finally, dialysis can be used if the conservative measures listed earlier are ineffective or if the hyperkalemia is severe.

Hyperphosphatemia

Clinical manifestations of severe hyperphosphatemia include nausea, vomiting, diarrhea, lethargy, and seizures. Physiologically, hyperphosphatemia increases the risk of tissue precipitation of calcium phosphate, which can subsequently lead to intrarenal calcification, acute obstructive uropathy, or symptomatic hypocalcemia. Hyperphosphatemia is usually defined by a phosphorus level greater than or equal to 5.0 mg/dL in children. Initial treatment for significant hyperphosphatemia consists of administration of oral forms of phosphate binders, such as aluminum hydroxide at a dosage of 50 to 150 mg/kg/day divided into equal doses every 6 hours. Acute severe hyperphosphatemia with symptomatic hypocalcemia can be life-threatening. However, given the real risk of worsening precipitation of calcium phosphate complexes, patients with hyperphosphatemia should not receive calcium infusions unless they have clinical symptoms of hypocalcemia. Patients with impaired renal function may require more aggressive therapy, such as peritoneal dialysis, hemodialysis, or continuous venovenous hemofiltration. Of these modalities, hemodialysis appears to be the most efficacious at clearing phosphorus.[115]

Hypocalcemia

During acute TLS, hypocalcemia commonly occurs because of precipitation of calcium phosphate, which in turn is a consequence of hyperphosphatemia. Many patients experience symptoms when their serum ionized calcium concentration is less than or equal to 0.7 mmol/L or their serum total calcium concentration is less than or equal to 7 mg/dL. Muscular manifestations of hypocalcemia include muscle cramps and spasms, paresthesias, and tetany. Cardiac abnormalities include ventricular arrhythmias, heart block, and hypotension. Neurologic complications include confusion, delirium, and seizures. Treatment of asymptomatic hypocalcemia is generally not recommended, particularly in the setting of hyperphosphatemia. In patients with severe hypocalcemia, IV calcium gluconate (50 to 100 mg/kg/dose) may be administered to correct marked or life-threatening clinical symptoms. However, this treatment may increase the risk of calcium phosphate deposition.

Hyperuricemia

Hyperuricemia is defined as serum uric acid greater than or equal to 8.0 mg/dL or a 25% increase from baseline 3 days before or 7 days after the initiation of chemotherapy. The major route of urate clearance is through the proximal renal tubule, and thus hyperuricemia develops when the excretory capacity of the renal tubule is exceeded. In combination with an acidic pH, hyperuricemia can lead to the formation of uric acid crystals in the renal tubule, resulting in intraluminal renal tubular obstruction and the development of acute renal dysfunction. The current pharmacologic strategies for the management of hyperuricemia are allopurinol and rasburicase.

Allopurinol was first approved for clinical use by the U.S. Food and Drug Administration in 1966 to treat patients with gout.[116] An isomer of hypoxanthine, allopurinol is rapidly converted in vivo to oxypurinol, which functions as a competitive inhibitor of xanthine oxidase. This irreversible inhibition of xanthine oxidase prevents the metabolism of xanthine and hypoxanthine to uric acid (see Fig. 68-9). In 1965, one of the first studies to examine the use of allopurinol in persons with TLS reported that 15 patients with leukemia or lymphoma had lowering of serum and urine uric acid levels after daily administration of allopurinol during the initiation of therapy.[117] A similar study was published in 1966, in which 33 patients with chronic leukemia, acute leukemia, or lymphoma had a dose-related reduction in uric acid after treatment with allopurinol.[118] This agent has since formed the backbone of both TLS prevention and treatment.

Allopurinol is available in two preparations, oral and IV, and they have similar efficacies. However, the IV formulation is considerably more costly and should be reserved for patients who are unable to take oral drugs. Allopurinol is administered at a dosage of 100 mg/m^2 every 8 hours by mouth (maximum, 800 mg/day) or 200 to 400 mg/m^2/day in one to three divided doses IV (maximum, 600 mg/day). A significant limitation of allopurinol is that it cannot reduce uric acid produced prior to its initiation. Patients with hyperuricemia at presentation will not have a reduction in uric acid levels for 2 to 3 days after the initiation of allopurinol because the removal of existing uric acid is dependent on renal clearance.[119] Another concern is that treatment with allopurinol can lead to increased levels of the uric acid precursors hypoxanthine and xanthine via inhibition of xanthine oxidase. Because xanthine is less soluble than uric acid, it is possible that high levels of xanthine may lead to xanthine nephropathy or xanthine renal stones. The clinical significance of this hypothesis is not clear because xanthine nephropathy has been rarely reported. In a study of 19 children with ALL who received allopurinol for tumor lysis syndrome, precipitated xanthine was significantly more likely to be found in the urine sediment of patients with high urine xanthine levels (>350 mg/dL). However, no difference was found in the incidence of precipitated xanthine in patients who did and did not experience acute renal failure.[114]

Urate oxidase provides an alternate approach for treating hyperuricemia by promoting the catabolism of uric acid to allantoin. Compared with uric acid, allantoin is five to ten times more water soluble and is easily excreted in urine.[120] Urate oxidase is an endogenous enzyme found in many mammalian species but not in humans. A nonrecombinant form of urate oxidase purified from the mold *Aspergillus flavus* has been demonstrated to reduce uric acid levels in patients at risk for TLS and has been available in Europe for more than 20 years. In the United States, a pediatric trial of this nonrecombinant urate oxidase was conducted in 126 children with newly diagnosed non–B-cell ALL. When compared with historic control subjects who were treated with allopurinol, patients treated with urate oxidase had significantly greater decreases in their blood uric acid levels (median maximal level during treatment, 2.3 vs. 3.9 mg/dL; $P <$ .001).[121] However, the nonrecombinant urate oxidase was also associated with significant acute hypersensitivity reactions, including anaphylaxis, in 4.5% of patients receiving the drug.

Subsequently, a recombinant form of urate oxidase (rasburicase) produced by a genetically modified *Saccharomyces cerevisiae* strain has become available and appears to have a lower incidence of hypersensitivity reactions. Rasburicase offers a potential advantage over allopurinol by affecting the direct breakdown of uric acid and consequently leading to a more rapid lowering of serum uric acid levels. Faster normalization of uric acid levels also allows for earlier discontinuation of alkalinized fluids, which likely results in better excretion of other metabolites and avoids the increased risk of precipitation of calcium phosphate stones in the setting of overalkalinization. It should be noted that blood samples for uric acid measurement in patients receiving rasburicase therapy require special handling because of ex vivo degradation of uric acid by rasburicase at room temperature. To avoid falsely low uric acid levels, blood samples must be kept on ice and processed at 4°C within 4 hours of collection.

Pui and colleagues[122] initially administered rasburicase at a dose of 0.15 to 0.20 mg/kg IV for 5 to 7 days to 131 children with newly diagnosed leukemia or lymphoma. This study demonstrated a significant reduction of the median uric acid level by 4 hours after treatment, from 9.7 to 1 mg/dL in the 65 patients who presented with hyperuricemia and from 4.3 to 0.5 mg/dL in the remaining 66 patients. Rasburicase was very well tolerated in this study, with bronchospasm developing in 1 of 131 children. In addition, an open-label, multicenter, intent-to-treat, randomized, controlled trial was conducted to compare allopurinol with rasburicase in 52 pediatric patients with leukemia or lymphoma who were at high risk for TLS.[123] This study showed that patients randomized to rasburicase compared with allopurinol achieved an 86% versus 12% reduction of initial serum uric acid levels 4 hours after the first dose. In addition, no hypersensitivity reactions were observed in this study. Limitations of this study included its open-label design and the difficulty of generalizing these results to an adult population, although children up to age 17 years were included. Randomized controlled trials of allopurinol versus rasburicase have not been performed in the adult population.

Rasburicase is contraindicated in patients known to have glucose-6-phosphate dehydrogenase (G6PD) deficiency because the hydrogen peroxide generated in the breakdown of uric acid cannot be cleared by these patients.[120] The peroxide causes oxidative damage, resulting in hemolytic anemia and methemoglobinemia. The hemolytic anemia caused by rasburicase can be severe and can exacerbate renal damage in the setting of TLS. It is recommended that G6PD screening be performed before administration of rasburicase whenever possible.[124] Screening should be performed prior to transfusion with packed red blood cells to avoid false-negative results. If screening cannot be performed in a timely manner, the use of rasburicase should be avoided in patients who by ethnic background or family history have a significant risk of G6PD deficiency. Other adverse reactions associated with rasburicase include rare occurrences of hypersensitivity reactions, rash, increased liver enzyme levels, headache, vomiting, and nausea.[125] Because of the high incidence of hypersensitivity reactions associated with the nonrecombinant urate oxidase, several studies have measured antibody formation to rasburicase. In one study, no patients experienced hypersensitivity reactions and none of the 23 of 27 patient samples tested contained detectable levels of antibodies to rasburicase.[123] In another study, antibodies developed in 14% of patients, but none of these patients had hypersensitivity reactions.[123] It remains uncertain whether patients with detection of antibodies to rasburicase are at greater risk of hypersensitivity reactions with repeat courses. It has been suggested that patients with asthma and those with a high risk of hypersensitivity reactions based on allergy history should be monitored closely when receiving rasburicase.

Because of the high cost of rasburicase compared with allopurinol, it has been recommended that the use of rasburicase be reserved for patients with significant hyperuricemia at presentation or those at high risk of the development of severe TLS. The significant cost of rasburicase therapy has led several groups to evaluate the efficacy of single-dose therapy. Several published reports have demonstrated rapid reduction of uric acid levels with a single dose of rasburicase, with persistent effects for up to 7 days.[126,127] Thus single-dose therapy appears to be effective for both treatment and prophylaxis of hyperuricemia. However, when considering the use of rasburicase based on cost, it must be recognized that significant costs are also associated with complications of hyperuricemia and TLS, such as acute renal failure and dialysis. For example, a European study that examined the economic consequences of TLS management found that the main cost drivers in TLS are interventions requiring intensive care.[128] Thus studies aiming to analyze the cost difference between allopurinol and rasburicase should take into account the outcomes of acute renal failure and/or dialysis.

Uremia and Acute Renal Failure

Causes for renal dysfunction, azotemia, and rising creatinine levels can be multifactorial in persons with acute TLS. For example, any of the following conditions can result in renal injury: uric acid crystal obstructive uropathy, renal precipitation of calcium phosphate, renal tumor infiltration, xanthinuria, nephrotoxic drugs, or intravascular volume depletion. Despite appropriate medical management, some patients with increasing renal dysfunction and acute TLS require more aggressive measures, such as dialysis or hemofiltration. Indications for these therapies include metabolic derangements (e.g., hyperkalemia, hyperphosphatemia, hyperuricemia, hypocalcemia, and uremia) or volume overload that cannot be adequately controlled by medical management. Hemodialysis, peritoneal dialysis, and continuous hemofiltration (arteriovenous and venovenous) have been used in pediatric and adult populations for the treatment of TLS.[129-131] Peritoneal dialysis, although commonly used in many forms of acute renal failure, cannot be safely used in patients with intraabdominal tumors (e.g., Burkitt lymphoma) and is not recommended in the oncologic setting because of risk of infection in immunocompromised hosts.[115] The major advantage of hemodialysis compared with the other modalities is the rapidity with which electrolyte abnormalities can be corrected. However, hemodialysis can lead to large swings in plasma electrolyte levels and also can impose significant hemodynamic challenges. Continuous hemofiltration provides a more gradual method of correcting fluid and electrolyte disturbances and may serve as a safer alternative to hemodialysis in some cases. Unfortunately, little evidence relating to the comparison of the various dialysis modalities is available. Consultation with a nephrologist or an intensivist is recommended when weighing the options of hemofiltration and dialysis.

Conclusions

Tumor lysis syndrome is a constellation of metabolic derangements resulting from massive release of intracellular ions and nucleotides. Purine catabolism in the setting of TLS is particularly problematic for humans because of the lack of endogenous urate oxidase that can convert uric acid to the more soluble allantoin. The introduction of rasburicase has effectively reduced the incidence and severity of complications from hyperuricemia in patients at high risk for TLS. Although rasburicase appears to have great potential, many questions regarding its use remain unanswered.

COMPLICATIONS OF CHEMOTHERAPY

The use of chemotherapeutic agents carries risks for multiple acute and chronic toxicities and potential complications. Several oncologic emergencies related to the use of particular chemotherapeutic agents are summarized in this chapter. These emergencies must be recognized and managed effectively to prevent patient morbidity and mortality.

Vesicant Extravasation

Extravasation of vesicant chemotherapeutic agents can result in severe patient morbidity, with the development of extensive tissue damage. Vesicant agents include antitumor antibiotics (e.g., anthracyclines and dactinomycin), vinca alkaloids, epipodophyllotoxins, cisplatin, alkylating agents, and taxanes. Extravasation of anthracycline agents such as doxorubicin and daunorubicin can result in particularly severe and progressive tissue necrosis.[132] Importantly, the use of a central venous access device does not preclude the possibility of extravasation. Blood return or other evidence of patency, such as a dye study, is needed before a vesicant is administered in a central venous line. Once extravasation is suspected, usually because of swelling, redness, and pain at the injection site, administration of the agent should be stopped and assessment and treatment should be initiated.

Extravasation has traditionally been managed via the application of cold compresses, although this treatment is contraindicated with certain agents (such as vinca alkaloids and epipodophyllotoxins). Hyaluronidase may be used in certain situations to promote rapid dispersion of the infiltrate into surrounding tissue to lessen the severity of local tissue damage. Mechlorethamine extravasation can also result in tissue necrosis. The manufacturer recommends the local injection of sodium thiosulfate into the extravasation area, followed by topical application of ice. Vinca alkaloid extravasation management includes application of warm compresses and local injections of hyaluronidase.[133] Cooling the area has been recommended for taxane extravasation.[134]

Surgical debridement of affected areas may be required.[134] In 2007, the U.S. Food and Drug Administration approved dexrazoxane hydrochloride (Totect) for injection for the treatment of extravasation resulting from IV anthracycline chemotherapy.[135] In two studies, 57 evaluable patients underwent biopsy-proven extravasation of anthracycline from peripheral vein or central venous access sites. Totect was administered within 6 hours of extravasation for three doses, repeated 24 and 48 hours after the first dose. One patient required surgical intervention and the remaining patients had mild or no sequelae.[136] The use of Totect in the pediatric population has not been separately reported.

Methotrexate-Induced Renal Dysfunction

Because methotrexate is usually cleared exclusively by the kidneys, nephrotoxicity associated with high-dose methotrexate administration can be considered a true oncologic emergency. Although the strategies of hydration, alkalinization, and leucovorin rescue with close monitoring of methotrexate concentration and serum creatinine usually allow for safe administration of high-dose methotrexate, nephrotoxicity still occurs in some patients. Renal dysfunction was found to occur in approximately 1.8% of patients with osteosarcoma who were treated in clinical trials.[137] Renal dysfunction can result in persistently elevated methotrexate levels. Markedly delayed clearance of the drug amplifies methotrexate's other toxic effects, such as mucositis, hepatotoxicity, and myelosuppression.[138] In the patient with methotrexate-induced renal dysfunction, increasing leucovorin administration based on plasma methotrexate concentration remains a key intervention. Hemodialysis, hemoperfusion, therapeutic plasma exchange, and peritoneal dialysis have had limited success in this setting and may be useful in certain clinical situations.[137] Carboxypeptidase-G2 (known as CPDG2, glucarpidase, or Voraxaze), a recombinant bacterial enzyme, offers another emergency treatment option.[139,140] Glucarpidase provides hepatic elimination instead of renal clearance by metabolizing methotrexate to the inactive metabolite, 2,4-diamino-N10-methylpteroic acid.[141] Within minutes of administration, glucarpidase results in an average 97% reduction in plasma methotrexate concentration, allowing for continued management with leucovorin and supportive care. Glucarpidase is now commercially available and should be considered a standard component of treatment for patients with severe methotrexate-induced nephropathy.[142] Most cases of methotrexate-induced renal dysfunction resolve without long-term sequelae, and a single episode of nephrotoxicity does not automatically preclude future high-dose methotrexate use.[143]

Intrathecal Chemotherapy Overdose

Measures to prevent errors and overdose in the administration of intrathecal chemotherapeutic agents are key to avoiding this potentially devastating complication, and multistep safety systems have rendered these events rare in modern clinical practice. However, such errors continue to be reported, underscoring the importance of keeping plans for treatment and management in place in case such an adverse event occurs. Immediate response is crucial; case reports and small series highlight potential strategies.

Intrathecal overdose of methotrexate can result in seizures, coma, and death. Treatment options for intrathecal methotrexate overdose have traditionally included cerebrospinal fluid (CSF) drainage, ventriculolumbar CSF perfusion, systemic steroids, and systemic leucovorin.[137] Immediate removal of CSF has been advocated. The intrathecal use of leucovorin has not been advocated.[144] The intrathecal use of CPDG2 has also been reported.[145] In a 2004 report, Widemann et al.[137] presented results of a collaborative protocol assessing the intrathecal administration of CPDG2 in seven patients with cancer who had received accidental overdoses of intrathecal methotrexate, defined as a dose greater than 100 mg. These investigators found that methotrexate concentrations in the CSF declined by more than 98% in all patients and that all patients recovered completely from the overdose, except for two patients aged 61 and 77 years with memory impairment.[137] Four of the cases were pediatric cases, with ages ranging from 5 to 9 years. Three of these patients received a 600-mg dose of methotrexate, two underwent CSF exchange, and all were reported to have a complete recovery. It was concluded that CPDG2 should be considered for intrathecal methotrexate overdose.

One case report of an intrathecal overdose of cytarabine in a 4-year-old was managed with CSF exchange, and the patient survived.[146] At any dose, intrathecal vincristine is almost universally fatal and must be avoided at all costs.[147] As a result, the majority of active pediatric oncology practices have specific safety measures in place involving pharmacists, nurses, and physicians to prevent inadvertent administration of intrathecal vincristine. Clearly, preventive measures in prescribing, preparing, and delivering intrathecal chemotherapy remain essential.

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CHAPTER 69

Nursing Care of Patients with Childhood Cancer

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HISTORY OF PEDIATRIC ONCOLOGY NURSING

Nursing is the largest health care occupation in the United States, representing 2.7 million jobs—a number that is expected to grow by 26% or more before 2020, faster than the national average of 14%.[1] Because its roots are embedded in daily life and the caretaking role women traditionally assumed throughout history, nursing was not recognized as a profession until the mid-1800s, when Florence Nightingale identified the need for specialized education and training and developed the underpinnings of modern-day nursing practice.[2]

The first nurses were considered generalists and cared for patients of all ages. Pediatric nursing emerged as a specialty in the early 20th century, when free-standing children's hospitals became more common.[2] During this time, pediatric oncology patients were often cared for by pediatric nurses (sometimes called tumor therapy nurses) who learned to care for oncology patients through experience and self-directed, on-the-job training.[3-5]

The subspecialty of pediatric oncology nursing first appeared in the late 1940s and early 1950s, when courses specific to cancer nursing were introduced by the American Cancer Society, the University of Washington, and the University of Minnesota School of Public Health.[1,4,6] It was formally recognized as a subspecialty practice in the mid-1970s, when more nursing schools began offering education specific to pediatric oncology, and a small cohort of nurses formed the Association of Pediatric Oncology Nurses (APON).[4,7,8] APON changed its name in 2006 to the Association of Pediatric Hematology/Oncology Nurses (APHON) and is considered the leading professional organization for registered nurses caring for children and adolescents with cancer and blood disorders.[9,10]

Over the years, the role of pediatric oncology nurses has evolved to match advances in cancer treatment. During the 1880s and early 1900s, the predominant treatment modality for cancer was surgery, and nursing care was focused primarily on relieving pain and meeting other postsurgical needs of patients.[6] Until the latter half of the 1900s, cancer was a metaphor for death and nurses had few resources available to care for the patients it affected. Children diagnosed with cancer commonly died, diagnostic techniques and treatment were often not available, and care was typically palliative.[4,5] The general attitude among health care professionals was that there was little to offer the person with cancer except a cheerful manner and a few comfort measures.[11]

After World War II, single-agent chemotherapy was introduced. This new therapy was administered only by physicians until the 1960s, when it was proposed that nurses assume this responsibility.[6] Today, chemotherapy is almost always administered by nurses, who as a discipline have been instrumental in developing standards for safe practice and education related to chemotherapy administration and symptom management.[7,12-14] Pediatric oncology nurses are also leaders in patient and family education, pain management, and palliative care. Over the past few decades, a number of advanced-practice nursing roles have gained prominence. The term *advanced practice nurse* applies to nurses in specialized roles, including clinical nurse specialists, nurse practitioners, certified nurse midwives, and nurse anesthetists, who have completed additional formal education and training. In pediatric oncology settings, it is the clinical nurse specialist and the pediatric nurse practitioner who are most commonly employed. Although their responsibilities vary among institutions, the clinical nurse specialist generally focuses on the education of the staff and of patients and families, whereas the pediatric nurse practitioner usually provides direct patient care in consultation with the patient's oncologist. As a primary oncology provider, the pediatric nurse practitioner follows patients and families over time and helps to formulate, implement, and evaluate the patient's plan of care. Depending on institutional, state, and federal regulations, a pediatric nurse practitioner may also prescribe medications and perform procedures, such as bone marrow biopsies and lumbar punctures. Standards and guidelines regarding professional behaviors and scope of practice developed by APHON and the American Nurses Association also guide the practice of advanced practice nurses.[15]

Each year, 10,000 children will receive a diagnosis of childhood cancer and 1.16 million children younger than the age of 15 years will be hospitalized with a cancer-related diagnosis.[16,17]Although the incidence of pediatric cancer has increased 29% during the past 20 years, advances in treatment have resulted in a long-term childhood survival rate of 79% for all childhood cancers.[18,19] Some selected childhood cancers have realized long-term survival rates that now approach or exceed 95%.[16,18] Despite improving statistics where the 10-year survival rate is 75%, cancer is the second leading cause of death in children from birth to age 14 years.[20] Pediatric oncology nurses at all levels will continue to play critical roles in the care of children with cancer as new diagnostic and treatment techniques become available. Through persistence, vigilance, and dedication, nurses will shape the development and modification of practice standards that promote safe and effective pediatric cancer care.[7,21]

Nursing Education and Professional Certification

When nursing first emerged as a profession, nursing education was typically provided by hospital-based diploma programs.[22] Today, most diploma programs have closed and have been replaced by college- and university-based schools of nursing. Nurses specializing in pediatric oncology usually receive advanced training in all aspects of cancer and cancer treatment, including chemotherapy, management of complications related to therapy, pediatric oncology protocols, and the protection of human subjects.[23-25] In addition to this specialized training, APHON and most health care organizations recommend that pediatric oncology nurses obtain professional certification as another vehicle to confirm proficiency and demonstrate knowledge of pediatric oncology practice standards, although it is not a requirement for practice. Certification was first introduced in 1999, and since then more than 1700 registered nurses have become certified pediatric oncology nurses.[26,27-29]

OVERVIEW OF THE PEDIATRIC ONCOLOGY NURSE'S ROLE

The role of the nurse in the care of childhood cancer patients is well established.[30] Pediatric oncology nurses rely on their intellect, critical thinking abilities, expert communication skills, technical expertise, and up-to-date knowledge when caring for their patients.[25,31,32] These skills, when combined with intuition and caring, position pediatric oncology nurses to actively participate in and influence decisions concerning patient care and support, promote novel approaches to care, and optimize patient outcomes.[31-33]

When caring for patients, pediatric oncology nurses integrate information about the treatment modality with observations of how the patient and family are responding to treatment and whether additional education and support are needed. Much of a pediatric oncology nurse's practice is grounded in standards of care and professional performance defined by APHON. These standards apply to clinical settings across the continuum of care and serve as a guide for nurses practicing in all aspects of care, from prevention, early detection, ongoing physical and psychosocial care, and long-term survival. These standards of care also define activities associated with each step of the nursing process—a process that includes assessing the patient, identifying problems requiring nursing intervention, specifying expected outcomes, planning and implementing a nursing plan of care, and evaluating the child's progress (Table 69-1).[15] With the patient as the central

TABLE 69-1 The Nursing Process and Standards of Pediatric Oncology Nursing Care

Nursing Process	Standards of Care	Application: Case Study*
Assessment	The pediatric oncology nurse collects and documents data regarding the child and family.	• Obtained a patient history of severe chemotherapy-related nausea and vomiting during first treatment regimen.
Diagnosis	The nurse uses assessment data from nursing and other disciplines to identify problems and determine nursing diagnoses.	• Severe chemotherapy-related nausea despite antiemetics • New protocol regimen is highly emetogenic. • Assess the need for overnight hydration or next-day clinic infusion appointment for bolus to help with nausea control.
Outcomes Identification	The nurse identifies expected and desired outcomes specific to the patient and family and related to their physical and emotional health, education, growth and development, and effects of disease and treatment.	Expected outcomes: • Patient will experience little or no nausea during chemotherapy and postchemotherapy period. • Patient and family will communicate effectiveness of antiemetic and patient satisfaction with the regimen.
Planning	The nurse develops an individualized plan that prescribes interventions to attain expected outcomes.	• Resume previous antiemetics (ondansetron, corticosteroids, and scopolamine). Add new antiemetic, aprepitant. • Have patient maintain a diary describing nausea and individual drug effectiveness.
Implementation	The nurse implements the plan of care to achieve the expected outcomes for the child and family.	• Administer first antiemetic doses in clinic to monitor effectiveness, and review timing of doses.
Coordination of Care	The nurse coordinates the delivery of care to support transition across the continuum of care.	• College student describes past use of dronabinol and lorazepam to control nausea. Patient states that these agents made him feel "loopy" which impeded his ability to attend class and keep up with studies.
Health Teaching and Health Promotion	The nurse employs strategies to educate patient and family about maintaining health and providing a safe environment of care.	• Provided a detailed calendar outlining scheduled chemotherapy, predicted times of nausea, medication schedule, and escalation plan for unresolved nausea • Reviewed patient education materials associated with prescribed antiemetic agents. • Reviewed prn use of additional antiemetics
Evaluation	Working with other members of the care team, the nurse evaluates outcomes that have been achieved and follows the nursing process to adjust the plan of care as needed.	• Patient returned for cycle 2. Reviewed the drug diary with the patient to understand the duration of nausea and the patient's perspective on the effectiveness of the antiemetic regimen. • Patient reported no nausea, did not require supplemental antiemetics, and reported increased satisfaction with plan, noting that he had previously found the inability to control nausea extremely distressing.

From Association of Pediatric Oncology Nurses, American Nurses Association: Scope and standards of pediatric oncology nursing practice. *Silver Spring, Md., 2007, Nurses Books.*

*Applying the nursing process to the management of postchemotherapy nausea in a 19-year-old college student with relapsed Ewing sarcoma. Case study of a patient at Dana-Farber/Children's Hospital Cancer and Blood Disorders Center managed by primary nurse A. Carnes, BSN, RN, CPON.

focus, the ultimate goal is to reestablish a level of optimal wellness as defined by the patient and family.[34] The nursing process is dynamic and considers all dimensions of care; therefore, the plan of care is continually adjusted in response to changes in the patient's condition and treatment plan.[34]

As discussed in this chapter and highlighted in Box 69-1, pediatric oncology nurses are critical members of the oncology team, collaborating with colleagues and using an evidence-based approach to reduce the burden of cancer and meet the needs of patients and families.

The Nurse's Relationship with Patients, Families, and Other Providers

Meeting the varied needs of pediatric oncology patients and their families requires a broad range of professionals. For this reason, most pediatric cancer centers draw on an interdisciplinary team, replacing the physician-centered model that was more common in the early years of cancer care.[3] Today, members of the care team rely on one another's observations, knowledge, and skills, with physicians and nurses working as interdependent partners toward the care and cure of children with cancer.[5,35,36] Equally important is coordinated and ongoing interaction between the child, the family, and all members of the interdisciplinary team.

Patient- and Family-Centered Care

Partnering with patients and families is the cornerstone of patient- and family-centered care. A model of care delivery commonly practiced in pediatric oncology settings is based on the understanding that the family is the child's primary source of strength and support, that the perspectives and information provided by the family,

Box 69-1 **The Role of the Pediatric Oncology Nurse in Reducing the Burden of Cancer: Ensuring Families' Needs Are Met**

CARING PRACTICES

- Provides compassionate, developmentally appropriate care
- Considers cultural differences when developing and implementing a plan of care
- Minimizes pain and suffering
- Partners with patients and families to individualize approaches to care
- Develops and coordinates all aspects of patients' experiences across the continuum
- Collaborates with other disciplines to ensure optimal patient care
- Demonstrates caring practices toward all patients, families, and members of the health care system

COMMUNICATION AND COLLABORATION

- Serves as an advocate for the needs of the patients and families
- Exchanges ideas with other members of the clinical team to advance clinical practice and patient care
- Facilitates communication:
  - With the community
  - With the patients and families
  - Between the patients and physicians
  - Between the patients and other members of the health care team

EDUCATION AND LEARNING

- Teaches patients and families about the cancer diagnosis and its treatment
- Prepares patients and families to manage home care needs and transition back into the community
- Encourages techniques supporting compassionate clinical and emotional care
- Educates other members of the clinical team
- Creates educational materials for patients, families, and other health care providers
- Influences nursing practice at a local, national, and international level

CLINICAL INQUIRY AND SYSTEMS THINKING

- Uses clinical judgment and reasoning to care for patients effectively
- Guides pediatric oncology nursing practice by identifying key research areas
- Advances nursing science using the best available evidence as the basis for changes in practice
- Masters the skills and competencies necessary to maximize clinical outcomes
- Serves as a change agent
- Critically examines the environment, identifies opportunities for change, and introduces strategies to ensure safe care

the child, or the young adult are important to clinical decision making, and that the psychosocial and cultural needs of the family as well as the patient must be considered throughout the care process.[21,37,38,39] Guidelines developed by the Institute for Patient- and Family-Centered Care for clinicians interested in adopting a patient- and family-centered care model (Box 69-2) emphasize the importance of partnering with the patient and family throughout the course of treatment. Respecting the insights and perspectives of the parents regarding their child's needs, empowering the patient and family by providing them with information, and engaging the child in his or her own care are key aspects of the model. Organizations are also urged to involve patients and families in defining and improving institutional policies and care delivery systems.[37]

Although patient- and family-centered care is now widely embraced by pediatric health care professionals, there was a time when parental visitation was permitted only on a limited basis, information related to a child's diagnosis was often withheld from the patient and the family for fear of increasing anxiety, and decisions surrounding care were made within a paternalistic framework.[21,40] Nurses were often caught in the middle when a patient did not know the diagnosis or the family was not involved in shared decision making.[6,21] The cultural shift to a family-centered focus, along with improvements in survival rates, have helped members of the health care team develop a keener understanding of the long-term effects of diagnosis and treatment as well as an appreciation for how physiologic and psychological sequelae impact the patient and their family members.[21] The patient- and family-centered approach has also helped parents to gain confidence in their ability to care for their child throughout the course of illness and has helped children and young adults to manage aspects of their own health care.[37] Nurses routinely partner with patients and families throughout the cancer patient's care, from diagnosis to end of life, involving patients and families in establishing short- and long-term goals. This is particularly important for children because it gives them a feeling of control and enhances their sense of autonomy.[41] Although the ability of the child to participate in goal setting and decision making depends in part on their maturity and cognitive capacities, approaches and techniques usually can be tailored to match their capabilities.

Meeting the Emotional and Developmental Needs of Patients and Families

Pediatric oncology nurses often spend long stretches of time with patients and families, particularly when patients are hospitalized or receiving outpatient chemotherapy.

Box 69-2 **Patient- and Family-Centered Care: Guidelines for Providers**

- **Respect and dignity.** Health care practitioners listen to and honor patient and family perspectives and choices. Patient and family knowledge, values, beliefs, and cultural backgrounds are incorporated into the planning and delivery of care.
- **Information sharing.** Health care practitioners communicate and share complete and unbiased information with patients and families in ways that are affirming and useful. Patients and families receive timely, complete, and accurate information to effectively participate in care and decision making.
- **Participation.** Patients and families are encouraged and supported in participating in care and decision making at the level they choose.
- **Collaboration.** Patients and families are also included on an institution-wide basis. Health care leaders collaborate with patients and families in policy and program development, implementation, and evaluation; in health care facility design; and in professional education, as well as in the delivery of care.

From the Institute for Patient- and Family-Centered Care: www.ipfcc.org.

Because of their frequent interactions with patients and families, nurses are often the first to recognize practical issues faced by families. Over time, many families view nurses as advocates; however, the nurses' role is not to provide all of the support families need but rather to nurture support systems and identify available resources that might be of help.

Developing trusting relationships with patients and families is essential to effective oncology nursing practice.[41] Nurses often gain this trust simply through the care provided, the hours spent at the patients' bedsides, and the efforts undertaken to meet the needs of patients and families while facilitating communication with other providers. Among children and adolescents, trust is often more readily gained when the actions of the nurse reflect an appreciation for developmental needs and when nurses are forthright, honest, and maintain developmentally appropriate boundaries regarding the behavior of the child or adolescent.[42-44]

In addition to the emotional needs of patients and families, children undergoing treatment for cancer are at risk for significant delays in growth and development, which are often functions of the length and type of treatment and the side effects that children experience.[45] Therefore, nurses caring for children with cancer incorporate developmentally supportive care into all phases of treatment. Such care requires knowledge of normal growth and development as well as an appreciation for the variations and abnormalities that may be encountered among children who are ill. A variety of approaches are used to support a patient's growth and development to help them reach their maximum personal potential. Providing age-appropriate care individualized to each patient, encouraging parental involvement, and fostering normalcy whenever possible by maintaining routines and usual activities are just a few of the strategies nurses use. Child-life specialists are routinely consulted to establish interventions for hospitalized children that are appropriate to developmental milestones.[46,47]

Educating Patients and Families

A hallmark of comprehensive pediatric oncology care is educating patients and families about the diagnosis and treatment, empowering them to make informed decisions and become active partners in the treatment process.[48,49-51] Because the care process typically spans multiple settings, patients and families are likely to receive information and instruction from a variety of clinicians and care providers. As a matter of course, teaching is a part of most patient encounters to help patients and families build skills and gain the self-confidence necessary to actively participate in ongoing care processes. When teaching patients and families, nurses adapt teaching methods to meet the educational needs of individual patients and families. Nurses supplement didactic instructions with written materials that augment topics covered in teaching sessions. Frequent opportunities to observe and practice the skills needed to manage care at home are typically offered. The use of technology for simulation and teaching is increasing as new educational formats become available.

Nurses have been instrumental on local, national, and international levels in developing educational materials for parents and age-appropriate materials for patients. In many hospitals and other care-delivery settings, nurses coordinate efforts to develop and evaluate educational materials on a wide range of topics, have the materials translated into various languages, and make them accessible to patients and families. A number of nursing organizations have also developed education resources. For example, APHON has developed a wide range of disease-specific pamphlets and education programs for patients and families that outline treatment options, side effects, and how to care for children at home.[52] The Children's Oncology Group also offers an informative website, a handbook, and numerous networking opportunities for parents.[53]

CARING FOR PATIENTS AND FAMILIES DURING THE DIAGNOSTIC PHASE

Receiving a diagnosis of childhood cancer is devastating and life altering.[54] For parents, the diagnostic phase is marked by extreme emotional, physical, and spiritual distress, with many describing the period of waiting and not knowing as one of the most difficult times in the journey.[55-57] It is during this waiting period that parents are often acutely aware that cancer is the probable diagnosis, but the type of cancer, the expected treatment, and the likely outcome have yet to be determined.[57] As a result, this is a time of uncertainty, with many fears stemming from the unknown and questions the clinical team may not yet be ready to answer. Some of the questions known to be plaguing the parents during this phase include the following: Will our child survive? What will the future hold? Will our child suffer? Will we be able to comfort and protect our child from pain?[51,56]

The nurse caring for a child suspected of having cancer is in a unique position to assess needs, identify cultural preferences, offer support and guidance, as well as ensure access to information needed to provide informed consent.[55,58] Because this period typically involves interactions with a variety of health care providers, the nurse supports coordination of this process to optimize communication between the patients and families and members of the health care team.[59] To do this, the nurse must possess a thorough understanding of the prediagnostic and diagnostic phases of pediatric malignancies that include, but are not limited to, the various tests and procedures commonly used for diagnosis.[55]

The Diagnostic Workup

Confirming a cancer diagnosis can be a long process because many of the initial symptoms imitate normal childhood illnesses.[55] The diagnostic workup includes obtaining a complete history of the current illness, the incidence and duration of symptoms, and the existence of any predisposing factors.[60] It is during this phase that the nurse completes a comprehensive assessment that includes a physical examination, developmental assessment, and measurement of vital signs, height, and weight. The height and weight are used to calculate the body surface area, a key measure if cancer is confirmed because it is used to determine chemotherapy doses in patients 1 year of age and older (weight in kilograms, rather than body surface area, is typically used to calculate doses for children younger than 1 year). Accurate measurement of body surface area is critical; therefore, it is common practice to have two nurses independently measure the initial height and weight and verify the calculations of body surface area.

Because the diagnostic phase involves numerous tests to determine the extent of disease, the nurse's role is focused on collecting specimens, coordinating the scheduling of the diagnostic workup, and partnering with the patient and family.[60] The nurse not only is responsible for ensuring that preparative regimens for specific tests are observed but also must facilitate the sequencing of various tests to ensure that procedures do not conflict with one another, that sufficient time is allotted for traveling from one department to another, and that tests are scheduled in a manner that permits judicious use of sedation.[60] Before each test, the nurse speaks with the patient and family explaining the test and how long it will take, discussing whether a contrast medium or sedation will be used, and answering any questions they may have about the procedure.

The diagnostic phase is emotionally and physically difficult, with diagnostic tests provoking anxiety and causing pain. Studies regarding the patient perception of pain suggest that the pain associated with invasive procedures and ultimate treatment for cancer is greater than the pain associated with the disease itself.[61,62] Fortunately, there are a wide array of pharmacologic and nonpharmacologic interventions available to minimize distress associated with pain and anxiety.

Informing Patients and Families of the Diagnosis

Once the diagnostic workup is complete and the type and extent of the cancer is known, news of the diagnosis is shared with the parents or guardian during a meeting with the physician and nurse. In some centers, this meeting is called the "Day One Talk." The purpose of this meeting is to review the extent of the disease, discuss the proposed treatment, consider whether alternatives to that treatment are available, and introduce and obtain informed consent in the patient's preferred language. If the proposed treatment is a research protocol, all issues related to the protocol, including the intent of the research, must be clearly explained to ensure informed decision making.[57] Studies regarding informed consent have found that parents of pediatric cancer patients often do not understand some of the issues related to participating in clinical trials, particularly that the purpose of research may be to benefit future patients rather than their own child.[63]

The Day One Talk typically represents the first step toward developing the patient-provider relationship forming the basis for future care. Determining who should be present during the meeting is not always simple, and parents often ask the physician or nurse for advice about whether the patient should be in attendance. Consideration should be given to including adolescents because they may assume that information later received is not completely true and that the diagnosis and treatment are worse than those stated in the information they have been given.[57] Including adolescents from the beginning may also help in later efforts to engage them in their own care. In cases involving younger children, a separate discussion tailored to the developmental level of the child is suggested, with the goal of informing the child about the treatment and clinical trial to obtain assent.[60]

Receiving the news that a child has cancer and hearing the complex treatment plan can be devastating for parents who may be overwhelmed with feelings of anger, fear, guilt, sadness, and self-blame. Because of this, parents may have difficulty retaining, processing, and understanding the information. The signed consent form detailing the aspects of treatment and chemotherapy information sheets may serve as useful references for parents after the meeting.[57]

If the patient is an adolescent, consideration must be given to the potential impact the treatment may have on fertility, as well as possible fertility-preservation options. It is generally recommended that this conversation take place as early as possible before beginning treatment. Some physicians choose to discuss fertility-related issues during the Day One Talk with a follow-up referral to reproductive specialists.[64]

Supporting Patients and Families after Diagnosis

After the Day One Talk, nurses provide ongoing support as patients and parents express fears and concerns. Once the diagnosis is made, patients and families are suddenly thrust into a complex plan of treatment that involves hospitals, ambulatory clinics, and home care agencies. Nurses help to arrange and coordinate appointments and other aspects of treatment, as well as facilitate collaboration and communication among care providers in various locations. During the weeks and months after the diagnosis, nurses carefully monitor patients and families for signs that suggest they may be having difficulty coping. If any signs of distress are observed, nurses work with colleagues on the health care team to formulate a plan to help patients, parents, and other family members manage their stress to enhance the family's chances for the best possible outcomes.[51] Many patients, parents, and family members benefit from working with psychosocial clinicians, attending family support groups, or having regularly scheduled family meetings, whereas others may require more intensive interventions.

NURSING IMPLICATIONS OF PEDIATRIC CANCER TREATMENT

The treatment of childhood cancer has evolved into an increasingly complex set of treatment modalities and methods. Although advances in treatment are responsible for marked improvements in survival rates, they have resulted in a broad range of side effects that require diligent monitoring and supportive therapy. Knowledgeable and well-trained pediatric oncology nurses are crucial to the safe and effective implementation of surgical, radiologic, and pharmacologic approaches to cancer treatment as well as to the management and oversight of clinical trials.

Nurses interact with patients during each treatment phase and in every care setting, making critical assessments and observations that guide decisions regarding the patients' care. Administering care specified by the treatment protocol, nurses continually monitor patients for complications and unexpected developments, offer emotional support to the patients and their families, and teach them what to expect during each treatment phase. Throughout the weeks, months, and years of care, nurses often develop a strong rapport with patients and families, becoming trusted sources of support and information.

Nursing Care Associated with Various Modes of Treatment

In this section, the specialized nursing care associated with the primary forms of pediatric oncology treatment—surgery, chemotherapy, radiation therapy, biotherapy, and hematopoietic stem cell transplant—are discussed. Complementary and integrative therapies and the nurse's role in clinical trials are also examined.

Surgery

Surgery is a major treatment approach for many pediatric solid tumors. Preoperative nursing care involves preparing patients and their families for the expected surgery and recovery by providing individualized teaching that is tailored to meet the unique needs of each patient and their family. In an effort to demystify the surgical experience, many hospitals offer programs that allow patients to visit the inpatient surgical unit, meet operating room nurses and other members of the staff, and see much of the equipment that will be used as part of the intraoperative care.[65]

During the postoperative period, nurses support the recovery process and promote healing. Close monitoring of vital signs and the overall clinical status, coughing and deep breathing, frequent position changes, and early ambulation are just a few of the nursing interventions introduced after surgery to prevent complications. Comprehensive postoperative care includes ongoing assessment of airway clearance, level of pain, signs and symptoms of fluid shifts, and infection. Fever is a common postoperative complication warranting careful monitoring and prompt treatment in pediatric oncology.[66]

Controlling postsurgical pain is a primary focus. Pain can have a profound effect on physical and emotional well-being, as well as discourage children from engaging in activities that promote recovery. Medications for pain control are typically initiated intraoperatively and are commonly continued for at least 72 hours after surgery or until the child is able to take medication by mouth.[67-70] During the postoperative period, nurses regularly assess for the presence of pain and the effectiveness of prescribed analgesics by observing the patient for signs of discomfort using age-appropriate pain-rating scales. Analgesics may be administered prior to activities such as walking or dressing changes to promote comfort and reduce the anxiety associated with procedures or activities that may cause pain.

Preparing patients and their families for discharge from the hospital after surgery typically begins before or at the time of admission. Discharge teaching involves a review of the care regimen, as well as coaching caregivers on how to perform aspects of physical care at home. Emphasis is placed on ensuring caregiver understanding of the signs and symptoms of potential complications, when they should contact a provider, and how to reach someone in the case of an emergency. Follow-up appointments with the surgical team and pediatric oncology providers are usually scheduled before discharge.

Chemotherapy

Chemotherapeutic agents are considered high-risk medications because of their narrow therapeutic index and the complexity of the treatment regimens in which they are used. If administered inappropriately, there is the potential for serious harm and even death.[71] Unlike other medications, administering chemotherapeutic agents to children introduces challenges because of the physiology of children, broad dosing ranges and schedules, widely differing protocols, weight-based dosing, and a high risk for toxicities.[13,14,72,73] Statistics revealing the incidence of medication errors have triggered widespread changes and standardization with all aspects of the medication process, from prescribing, dispensing and administration. The majority of medication errors occur at the time of administration (41%), followed by dispensing errors (38%) and prescribing errors (21%)[74] Because administering chemotherapy is the primary responsibility of pediatric oncology nurses, they are commonly viewed as the patient's last line of defense against medication errors.[75] Therefore, the provider may be viewed as the first line of defense with prescribers initiating the medication process. Because prescribing errors related to chemotherapy agents can have serious and even lethal effects, nurses in most institutions follow a special process to verify chemotherapy orders before the agent is administered. Each step of the verification process is designed to ensure chemotherapy is administered safely, the treatment protocol is observed, required pretreatment test results are within acceptable ranges, and the correct chemotherapy agent and dose are administered.[76] To minimize the risk for error, nurses complete specialized training and follow standards of practice for chemotherapy administration developed by APHON. In addition, they receive ongoing education relative to new agents and protocols before any new agents are introduced.[71]

Before administration of chemotherapy to a patient, the nurse reviews the patient's medical record and interviews the patient and family to determine past experience with chemotherapy, level of tolerance, and antiemetics used. During the interview, the nurse explains the current treatment plan, discusses each agent, reviews potential side effects, and completes a medication reconciliation process. The medication reconciliation process includes a review of chemotherapeutic agents, antiemetics, over-the-counter preparations, as well as herbal and nutritional supplements taken since the last visit. Querying the patient and family about medications reinforces an understanding of the treatment plan and medication doses providing an opportunity to evaluate compliance with the prescribed plan. The nurse also conducts an assessment to determine drug-specific side effects (e.g., constipation) to determine whether the chemotherapy can be administered as prescribed and if the symptom management plan should be adjusted.[73,77]

Because many chemotherapeutic agents are highly toxic, the National Institute of Occupational Safety and Health has developed standards to protect health care workers when handling or administering chemotherapy and caring for patients who have received chemotherapy.[78] The standards identify personal protective equipment such as gowns, gloves, and eye shields that must be worn by those handling chemotherapeutic agents. In addition, standards recommend the use of closed-system drug transfer devices to prevent accidental disconnection and exposure. Research has shown that complying with these standards limits long-term exposure.[79] Although some patients and families may be frightened when they first see nurses and other clinicians using protective equipment and procedures, most are quickly accepting once they understand the rationale for such measures. The safety concerns and precautions related to chemotherapy administration also apply to the patient's family, who must learn how to handle chemotherapeutic agents and the patient's body fluids. Body fluids can be a source of cytotoxic drugs and may pose a threat to the environment. As a result, special precautions for their disposal must be observed for 48 hours after chemotherapy administration, and family members should be given appropriate personal protective equipment and written instructions on how to handle the fluids safely. Family members of child-bearing age or who are pregnant should not prepare or administer cytotoxic drugs in the home. If they must handle the agent, they should minimize their risk by wearing appropriate protective equipment.[78]

During the time a patient is receiving a dose of chemotherapy, the nurse monitors parameters specific to the particular agent. In addition to monitoring temperature, blood pressure, and heart rate, the nurse observes the patient for signs of an allergic reaction and watches the access site for signs of possible extravasation or leakage of intravenous fluid into the tissues. Special precautions must be taken to prevent extravasation when administering a vesicant because these agents can cause blistering, severe tissue injury, or tissue necrosis if they extravasate.[80] If extravasation does occur, institutional polices and guidelines typically determine the appropriate actions to take.

After the chemotherapy has been administered, the nurse evaluates the effectiveness of the antiemetic regimen and monitors the patient for signs of toxicity and adverse effects. Detecting adverse effects early and taking quick action can minimize their severity. The nurse records the patient's response to treatment carefully because these observations are used by the care team to evaluate tolerance to treatment and determine whether changes need to be made in the overall plan of care.

For many families, the chemotherapy treatment phase is particularly difficult because they must cope with their own fears and anxieties while supporting the child or adolescent through treatment. Families often turn to the nurse for support and reassurance and find it comforting to work with a familiar and trusted team of nurses and providers. In some cases, families are asked to administer chemotherapy at home. Nurses work closely with these families to establish administration schedules, learn how to prepare and administer each medication safely, and understand which side effects to watch for and report (Box 69-3). They also teach the family about special considerations related to administering some agents. Most protocols and manufacturers now include specific instructions for these situations.

Box 69-3 **General Guidelines for Administering Chemotherapy by Family Members in the Home: What Families Need to Know**

1. Treatment goals and plan, including the length of treatment, number of cycles and days of treatment
2. A schema or calendar that shows the general treatment plan
3. Names of all medications (brand and generic)
4. Indications for all medications
5. Doses of all medications, including the strengths
6. How to administer the medications
    a. Safe handling (e.g., disposal of waste products and management of chemotherapeutic exposure or spill)
    b. How to measure the medications, especially liquids
    c. What foods or drinks the medications can be mixed with or taken with
    d. What foods or drinks to avoid when taking the medications
7. How and when to take the medications (e.g., before or after meals, at bedtime)
8. The start and stop date or criteria for stopping
9. Expected and potential side effects and preventive measures for side effects
10. When to report side effects and whom to call

Data from references 71 and 77.

Radiation Therapy

Radiation therapy is a common form of treatment for pediatric malignancies. The advancement of new technologies such as proton beam therapy is promising. Evidence, however, is still emerging regarding the potential role these new technologies will have in maximizing disease control and achieving high-quality survivorship.[81] Often administered in an outpatient setting for a number of consecutive days and weeks, radiation therapy can have significant side effects in children. As part of the treatment planning process, most patients undergo radiation simulation to determine the radiation field. Simulation requires the use of markings, blocks, and immobilization devices to ensure that the radiation will be delivered consistently to the same location during each radiation session. Children younger than 4 years of age usually require sedation and central venous access for their simulation and radiation treatments.[82,83] Children between 4 and 6 years of age may be able to tolerate the sessions without sedation if distraction techniques and nursing support are made available. Children older than 7 years of age usually tolerate the treatments well and are able to cooperate during the sessions.[83]

Nursing care for a child undergoing radiation therapy includes discussing the treatment plan with the patient and family, describing what the simulation process involves, assessing the patient for side effects, and educating the patient and family how to prevent and manage side effects. Because the side effects of radiation therapy are related to the site that is irradiated, interventions to alleviate them vary accordingly. The mouth and skin are commonly affected, and all patients and families are taught proper mouth and skin care techniques before they begin treatment. Patients receiving radiation therapy in combination with chemotherapy or biotherapy are monitored closely because certain agents can produce an exacerbated effect known as radiation recall, a severe rash that requires special precautions and treatment.[82,83]

Biotherapy (Biologic Response Modifiers/Biologic Therapies/Targeted Therapy)

In recent years, biotherapy, also known as biologic response modifiers (BRMs), biologic therapies, and targeted therapy, has emerged as a treatment modality using immunologic mechanisms to eradicate tumor cells.[84] Biotherapy agents include a wide range of products such as vaccines, blood and blood components, allergenics, somatic cells, gene therapy, tissues, and recombinant therapeutic proteins.[76] For example, there are vaccines to help prevent cancers as well as those designed to treat cancer. Vaccines, such as the human papillomavirus vaccine, help protect against certain strains of viruses and ultimately may prevent some cancers. Alternatively, vaccines that treat cancers are intended to increase the patient's immune response with the goal of attacking cells with one or more specific antigens.[85]

Biologic therapies interact with the child's immune system to enhance the response to cancer and may cause toxicities influenced by the dose, route, and schedule of administration. The most common side effect of biotherapy is a flulike syndrome characterized by fever, chills, rigors, myalgias, headache, and fatigue.[86] The role of the nurse in caring for the patient receiving biotherapy includes obtaining baseline vital signs, administration of any prescribed medications, and close monitoring for side effects. During and after administration, the nurse observes the patient for tachycardia, hypotension, fever, chills, anaphylaxis, and signs of a local reaction. Nurses also teach the patient and family about potential side effects that might occur at home, when such side effects should be reported, and symptom management techniques.[86]

Hematopoietic Stem Cell Transplant

Hematopoietic stem cell transplant (HSCT) involves the intravenous infusion of autologous or allogeneic stem cells.[87] This modality is used to treat malignant and nonmalignant diseases, and more than 4000 HSCTs were performed in 2009-2010 in patients younger than the age of 20, revealing a 20% increase from 1993-1994.[87,88] Since its development in 1939 there have been advances in cell source; improved conditioning regimens; more exact human leukocyte antigen typing, thus improving donor and patient matches and increasing overall transplant survival; decreased incidence and severity of acute and chronic graft-versus-host disease; and improved rates of engraftment.[88,89]

In an effort to promote improvement in cellular therapy, regenerative medicine, and patient outcomes, more than 85% of all transplant facilities in the United States partner with the Foundation for the Accreditation of Cellular Therapy (FACT).[90] Founded in 1996, FACT has established standards for clinical and laboratory departments that apply to hematopoietic progenitor cells and other cells obtained from hematopoietic sources that include marrow, peripheral blood, and umbilical cord blood.[90] These standards are focused on processing the clinical use of cellular therapy products as well as ensuring that they are collected and processed using strict controls.[90] The primary role of the registered nurse is to participate in the coordination of the transplant and to ensure FACT standards related to nursing practice are understood and adhered to.

Preprocedure Phase. There are 75 pediatric FACT accredited HSCT centers in the United States.[90] As a result, many patients change providers and transfer care to one of these institutions for the duration of HSCT treatment. Nurses may help to facilitate patient transfer by coordinating aspects of the referral process, clinical evaluation, or preprocedure education in the patient and family's preferred language and counseling. The period before HSCT can be one of great anxiety for patients and families. Good communication between the referring institution and the transplant center helps to ensure a smooth transfer process and fosters trust between the families and the new provider. Preprocedure processes vary among institutions; however, most are focused on an exchange of information needed to appropriately plan for the transplant.

Inpatient Care. Although hospital mortality and complications after HSCT have decreased, hospital length of stay ranging between 2 weeks to several months requires an experienced, culturally competent multidisciplinary team.[91] Specially trained nurses familiar with the immune and hematopoietic systems, stem cell transplant concepts, and complex treatment protocols are key members of the team playing critical roles during each phase of the treatment process.[78] After admission, a core team of nurses is usually established to develop an individualized nursing plan of care and oversee the patient's nursing care throughout the hospitalization. By understanding the risk factors and potential complications associated with HSCT, the nursing team works closely with all members of the health care team to evaluate and revise the plan of care as needed. Throughout the patient's hospitalization nurses support the patient and family to ensure their understanding of the process, which includes careful monitoring, assessing the patient for side effects, and understanding adverse reactions associated with each type of transplant.[92]

Planning for discharge typically begins early in the hospitalization and becomes a higher priority as engraftment approaches. The nurse assists in the coordination of discharge, offering emotional support and providing education necessary to facilitate the transition of the patient and family back home. Discharge teaching should include instructions in the preferred language of the patient concerning medications that must be taken after discharge, the ambulatory treatment plan, the care that must be administered in the home, and arrangement of appropriate services. Careful discharge planning can reduce the anxiety many families experience as they transition home. In general, patients are required to remain in close proximity to the transplant center for the first 100 days after allogeneic transplant. Autologous transplant patients may be referred back to their primary physician once engraftment occurs and the risk for HSCT complications has decreased.[92]

Ambulatory Care and Acute Outpatient Follow-Up. After discharge from the hospital, outpatient care usually requires a combination of clinic and home care services. Some institutions provide housing close to the main hospital to allow for close supervision of the patient's status. Maintaining a consistent team of caregivers and good communication with the patient and family helps promote posttransplant recovery and minimizes toxicities and complications. HSCT patients continue to require treatment and close observation and may need to visit the oncology clinic as often as 5 days a week for the first few weeks. Outpatient nurses regularly assess the patient's physical status, administer prescribed treatment, and manage symptoms, particularly those associated with graft-versus-host disease and toxicities.

Clinical Trials

With approximately 60% of patients diagnosed with cancer younger than 29 years of age enrolling in clinical trials compared with less than 5% of adults with cancer, clinical trials are crucial to the treatment of children with cancer.[93] Most children receiving treatment today are either enrolled in a clinical trial or receiving therapy based on the findings of a previous clinical trial.

Permission to participate in a clinical trial must be obtained. If the patient is younger than the age of 18, permission must be granted by the patient's parent or guardian; however, many health care providers involved in treating young people believe that the child or adolescent should play a role in the decision to enter a research study, and they urge investigators to share information about the study with the child and obtain his or her assent or agreement to participate.[94,95] The National Commission for Protection of Human Subjects of Biomedical and Behavioral Research established age 7 as a reasonable minimum age for involving children in some kind of assent process.[94] After consenting to participate in a clinical trial, the child and family may have lingering questions about the study and turn to their nurse for clarification and more information. The nurse may arrange for them to meet again with the consenting physician to ensure that all questions have been addressed and that their decision to participate is truly informed.[95]

Once a patient has enrolled in a clinical trial, the research coordinator assigned to the trial, in partnership with the primary team, ensures that all required studies have been completed and that consent forms have been signed. Throughout the course of a trial, nurses help to oversee and maintain compliance with the study's protocol, provide ongoing staff education, and are primary collectors of the data used for dose adjustments and analyses. Nurses adhere to the principles of good clinical practices, including adequate human subject protection as a critical requirement to conducting research involving human subjects.[96] The role of the clinical research nurse includes, but is not limited to, administering investigational drugs; monitoring the patient's responses; assessing patient for adverse side effects in accordance with protocol criteria; collecting timed specimens according to the protocol schedule; and carefully documenting the start and stop times of drug administration.[97] Nurses also provide essential patient and family education and support, checking in with them frequently and bringing unresolved concerns to the attention of the team.[97,98]

Complementary and Alternative Medicine (Integrative Therapies)

It is not uncommon for a family whose child has been diagnosed with cancer to explore complementary and alternative medicine (CAM) at some point during the treatment process. CAM, also referred to as integrative therapies, includes practices and therapies that lie outside the realm of traditional medicine. They encompass a wide variety of approaches, including acupuncture, massage, imagery, energy healing, and prayer, as well as herbal, homeopathic, nutritional, and biologic therapies.[99] Although evidence that CAM improves immunity or promotes recovery is not well documented, the use of CAM among children and adolescents with cancer reportedly ranges between 46% and 85%.[100-102]

Even though CAM is not a replacement for standard pediatric oncology treatment, many patients and families view it as a form of adjunctive therapy that may be used to help relieve symptoms, cope with life-threatening illness, and improve well-being.[103] While working with a patient and family, pediatric oncology nurses and other providers should routinely assess whether the patient has used CAM. During all medication reviews, the patient and family should be asked to list any herbs and supplements the patient is taking. Although obtaining this information is an important part of the medication review process, many families report never having been asked about such practices.[103]

Patients often turn to nurses for information and insight about available therapies that might be useful to them. When trying to determine the best therapy for the patient, consideration should be given to the family's culture, spiritual beliefs, and practices and the patient's developmental level, education, and preferences.[99,104] Nurses' observations and documentation of a patient's response to CAM treatments are essential because they help providers gauge the impact of the treatments, as well as their potential interactions with individual therapies, and provide insight into the patient's coping strategies.

Symptom Management and Supportive Nursing Care

The advanced, complex treatment regimens designed to cure childhood cancer commonly produce multiple distressing side effects. Each treatment modality carries the potential for side effects that can occur during treatment or days, weeks, or even years after treatment. Side effects such as hair loss and low blood cell counts may be transient, whereas others, such as hearing loss and learning disabilities, can be permanent.[77,105] The most commonly reported side effects in children and adolescents with cancer include infection, bleeding, anemia, nutritional problems, nausea, vomiting, mucositis, fatigue, and pain.[61] Studies have found that children report nausea, fatigue, and pain as the most distressing.[106,107] Although many side effects can be effectively managed, some families believe suffering is to be expected among children with cancer. As a result, side effects may go unreported for a time and children may suffer unnecessary distress.[108,109]

Nurses help manage the side effects of cancer treatment by using strategies that have been tested and proven effective through research and evidence.[105] The nurse continually evaluates the effectiveness of symptom management strategies and, when necessary, collaborates with the interdisciplinary team to revise the plan so that it matches the needs and experiences of the individual patient. The following section summarizes specific nursing interventions associated with some of the most common and troubling side effects encountered in children with cancer.

Myelosuppression

Myelosuppression is a condition in which bone marrow activity is decreased, resulting in fewer red blood cells, white blood cells, and platelets.[110] It is the most common toxicity associated with radiation and many chemotherapeutic agents.[105] It can also occur with certain malignancies, such as leukemia, sarcoma, neuroblastoma, and lymphoma.[61] Patients receiving myelosuppressive agents commonly experience a nadir between 10 and 14 days after the end of treatment. With subsequent courses of myelosuppressive agents, the nadir and recovery from the nadir can be affected by the number and type of agents given and the timing of their administration.[105,111] Pediatric oncology nurses monitor a patient's response to treatment over time and anticipate complications of myelosuppression, such as infection, bleeding, and fatigue. Educating families to be aware of the signs and symptoms associated with complications related to myelosuppression, as well as when to report these symptoms, is an important part of the overall care of patients receiving cancer treatment.

Neutropenia

Neutropenia is characterized by an absolute neutrophil count of 1000 per mm^3 or less.[77] It is the most severe consequence of bone marrow suppression, increasing a patient's risk for potentially life-threatening infections, a risk that is higher when neutropenia is prolonged for more than 7 days. When neutropenia presents as fever, it must be treated as an emergency with broad-spectrum antibiotics administered as soon as possible.[112] The neutropenic patient often does not present with the routine signs of infection. In many cases, fever may be the only presenting symptom.[113,114]

Hand washing before and after contact with each patient minimizes the risk for microbial transmission and is the single most important method for preventing nosocomial infection.[115-117] BRMs can also help lower a patient's risk for infection by shortening the period of myelosuppression. These agents are usually given 24 hours after chemotherapy administration as a one-time subcutaneous injection or daily by the nurse, patient, or family member until the white blood cell count recovers.[118,119] Because many children associate subcutaneous injections with pain and fear, nurses use creative techniques and special devices to ameliorate the discomfort associated with these injections.

Major pathogens that cause infections in the neutropenic patients are bacteria, fungi, viruses, and protozoa, with gram-negative bacterial infections associated with higher mortality rates than all other causes of infection.[112] The administration of prophylactic antibiotics to prevent *Pneumocystis jiroveci* (formerly known as *Pneumocystis carinii*) pneumonia, a protozoal infection found in patients with prolonged neutropenia, is also a standard practice in many cancer treatment protocols.[120,121] These antibiotics are usually administered in an oral form and are commonly given throughout treatment. Getting children to take daily oral medications can be very distressing for parents of young children and adolescents. Teaching parents techniques that will help them to administer medications successfully and emphasizing the importance of the medications enhances the parents' ability to cope and has the potential to increase overall compliance with prescribed medication regimens.

Educating the patient and family about neutropenia is critically important. Elements of this education must include the meaning of blood cell counts, signs and symptoms of infection, an understanding that the time of greatest risk for infection occurs during nadir, and strategies to minimize the risk for infection (Box 69-4).[122,123] Because the family plays an important role in monitoring a patient for signs of infection, nurses also focus on teaching them when to contact the child's provider. Family members are taught how to take a temperature and that the presence of a fever may signal an emergency; they are instructed to call the provider whenever the child's temperature is higher than 38° C (100.4° F) on two occasions within 24 hours and to call immediately whenever it is higher than 38.5° C (101.3° F) (or per institutional guidelines). They are also taught to report any respiratory symptoms, shaking chills, and changes in the child's level of consciousness and are instructed not to administer acetaminophen unless directed to do so by the provider. In addition to reviewing these instructions regularly, nurses make sure family members have emergency contact information. Families must be taught the critical nature of fever and neutropenia and the importance of initiating antibiotics rapidly.

Early assessment and intervention are key to successfully managing a febrile neutropenic patient.[105] Whenever a fever is present, blood cultures are obtained from central venous access devices and peripheral sources. Broad-spectrum antibiotics are usually started within 60 minutes of the diagnosis of fever and neutropenia.[124] Treatment

Box 69-4 **Neutropenic Precautions: Guidelines for Patients and Families**

- Notify the physician if the child exhibits any of the following:
    - Oral or axillary temperature is ≥38.5°C (101.3°F) or is 38°(100.4°F)C two times within 24 hours
    - Oral lesions
    - Erythema at central venous access site
    - Open skin lesions
    - Perirectal laceration or irritation
    - Cough
    - Rhinorrhea
    - Tachypnea
    - Complaints of ear or throat pain
    - Diarrhea
    - Lethargy
- Perform meticulous hand washing.
- Do not take rectal temperatures or give suppositories.
- Avoid crowds and people who are sick.
- Do not share utensils with others.
- Do not let the child provide direct care to pets (e.g., the child should not change kitty litter).
- Do not keep reptiles or birds as pets.
- The child and siblings should not receive live-virus vaccines.
- Avoid exposure to mold (e.g., digging in soil).
- Practice good mouth care.

Data from references 122 and 123.

may be modified once the results of the culture and sensitivity are available and the specific organism has been identified. Antifungal therapy is often added if the patient's fever persists despite antibiotic treatment.

Most patients with fever and neutropenia are admitted to the hospital for treatment. During this time, nurses observe patients closely for signs and symptoms of septic shock by monitoring vital signs, peripheral perfusion, and intake and output. The patient's neurologic status is also monitored closely because lethargy, irritability, or a change in consciousness can indicate sepsis. Mouth care and perianal hygiene are performed on a routine basis. Rectal temperatures and suppositories are avoided in all neutropenic patients based on the risk for tearing the anal mucosa and introducing bacteria.

Anemia

Anemia can occur secondary to myelosuppression after chemotherapy or radiation therapy, most commonly appearing 7 to 10 days after treatment. It can also occur as a result of blood loss, metastasis to the bone marrow, or viral suppression.[105] Nurses routinely monitor all patients for symptoms of anemia, watching for pallor (particularly of the lips and conjunctiva), fatigue, tachycardia, gallop rhythm, headache, dizziness, dyspnea on exertion, and irritability.

Many children exhibit a high tolerance for low hemoglobin levels. Transfusions, however, are commonly necessary when the patient is symptomatic, hemoglobin is less than 7 g/dL, and the hematocrit is less than 21%. Recombinant human erythropoietin may be used to increase red cell recovery and decrease the need for transfusions, especially when the patient and family object to blood transfusions on the basis of religious beliefs. It takes erythropoietin 2 to 4 weeks to stimulate red cell precursors, whereas the effects of a transfusion are immediate.[118] Transfusion amounts are generally in the range of 10 to 20 mL/kg. Red blood cells should be leukoreduced to decrease alloimmunization and irradiated to inactivate T cells and reduce the risk for graft-versus-host reactions.[125]

Because the patient and family are often the first to note symptoms of anemia, the nurse focuses patient and family education on anemia, its symptoms, when it is most likely to occur, and how to manage symptoms at home, such as providing frequent rest periods. If a transfusion is ordered, nurses follow a standardized protocol to ensure that the blood product is administered safely and monitor the patient frequently for fever, chills, body aches, urticaria, pruritus, wheezing, respiratory distress, and other signs of reaction to transfusion.[105,126,127] Prior to the transfusion, a patient may require premedications to prevent transfusion reactions. Once the transfusion is complete, the nurse documents the patient's tolerance of the transfusion and whether premedications were administered. This information is important to guide future transfusions.

Thrombocytopenia

Thrombocytopenia, or a platelet count of less than 100,000/mm^3, can be caused by myelosuppressive therapy, disease, or coagulopathy.[127] Thrombocytopenic patients are at risk for internal bleeding when the platelet count falls below 15,000 to 20,000/mm^3.[127,128] Nurses instruct the patient and family in strategies for preventing injury, hence minimizing the chances that bleeding will occur (Box 69-5).[129] Nurses regularly assess a thrombocytopenic patient's risk for bleeding and communicate any

Box 69-5 **Bleeding Precautions (Platelet Count <20,000/mm^3): Guidelines for Patients and Families**

- Notify the physician if the child exhibits any of the following:
    - Increased bruising
    - Evidence of bleeding
    - If a nosebleed occurs, pinch the nostrils together for at least 10 minutes. (Use gauze to pinch the nostrils, holding it between your thumb and forefinger.) Go to the emergency department if the bleeding persists after this time.
    - Change in level of consciousness
- Whenever the platelet level drops below 50,000/mm^3, avoid skateboarding, trampolines, contact sports, and other activities that can cause bleeding.
- Always wear a helmet when riding a bicycle.
- Shave only with an electric razor.
- Clean teeth with a soft toothbrush or gauze. Do not use dental floss.
- Avoid sharp foods such as tortillas that can cause gum injury and bleeding.
- Avoid aspirin, aspirin products, and ibuprofen.
- To prevent straining, keep stools soft using prescribed laxatives and stool softeners.
- Avoid taking rectal temperature, enemas, and suppositories.
- Oral contraceptives may be prescribed to prevent excess bleeding during menses.
- Avoid sexual intercourse.

Modified from Elliott S: Thrombocytopenia. In Kline N, Brace-O'Neill J, Hooke M, et al, editors: Essentials of pediatric oncology nursing: a core curriculum, *2nd ed. Chicago, 2004, Association of Pediatric Oncology Nurses, p. 69–70.*

pertinent laboratory findings or symptoms to the clinical team. Platelets are commonly administered when patients are symptomatic, if below 50,000/mm^3 and scheduled for a lumbar puncture, or in accordance with institutional or protocol guidelines. Patients receiving platelets are monitored carefully during and after the transfusion for signs of transfusion reaction.[125,127,128]

Fatigue

Fatigue is a major side effect of cancer treatment in children and adolescents.[130-132] Although defining fatigue in children undergoing cancer treatment has been challenging, children and adolescents can reliably describe the physical and mental symptoms associated with fatigue. Nurses routinely assess for fatigue throughout the course of treatment to determine possible factors that may contribute to fatigue, such as nutritional problems, pain, dehydration, and patterns of activity.[130,131] While caring for patients, nurses incorporate interventions designed to minimize fatigue. For example, with hospitalized children, incorporating adequate sleep time into the plan for the day and minimizing noise during sleep hours can sometimes be effective. In outpatient settings, nurses work with other members of the care team to avoid long waiting times to conserve and maximize energy, ensure adequate time for rest, and work with patients and families to optimize the patient's nutritional status. Other interventions and treatments that may decrease fatigue include pharmacologic assistance, physical activity, and distraction techniques. Integrative therapies that promote well-being and relaxation, such as massage and Reiki therapy, are increasingly employed in symptom management and show promise for relieving fatigue and promoting well-being.[99,103,104]

Nausea and Vomiting

Nausea and vomiting are common side effects of cancer treatment and described by patients as the second most distressing symptom after fatigue.[106] Uncontrolled vomiting can produce its own complications because it may quickly lead to dehydration and electrolyte imbalance. In some cases, nausea and vomiting can become dose-limiting side effects of chemotherapy if resolution requires a delay in treatment or a reduction in the chemotherapy dose.[76,105]

For a patient just beginning chemotherapy, the first cycle offers predictors of the chemotherapy's emetogenic potential. Antiemetics are typically ordered on the basis of symptoms observed during and after the cycle and adjusted according to the patient's response to subsequent cycles. The antiemetic regimen can also be adjusted to accommodate escalating doses of chemotherapy, as well as to manage breakthrough nausea and vomiting. Other interventions that may benefit some patients include antispasmodic therapy, relaxation techniques, acupuncture, and guided imagery.

Nurses routinely assess patients for nausea and vomiting during each chemotherapy cycle, obtaining information that helps in establishing and evaluating the effectiveness of the antiemetic regimen. They often encourage patients and families to maintain a diary describing the response to the antiemetic treatment and to list management strategies that the patient finds effective. Such information can help providers fine-tune the antiemetic regimen to the individual patient. When teaching patients and families about the regimen, nurses stress the importance of continuing antiemetics for the prescribed length of time.

Mucositis

Mucositis, or damage to the gastrointestinal cells and ulcerations of the mucosa, can develop as a result of chemotherapy or radiation therapy.[133] Ulcers that occur in the mouth are a dose-limiting toxicity that affects quality of life. Painful mouth ulcers make it difficult to eat and drink and can compromise a patient's nutritional status.[134,135]

Before treatment begins, patients are instructed to obtain dental care to ensure good oral hygiene and integrity. If applicable, they are usually asked to have their braces removed to decrease the potential for infection and gum irritation. Once treatment starts, nurses work closely with patients and families to ensure adequate oral hygiene and decrease the risk for mucositis. Upon meeting with patients and their families, nurses assess the patient's oral cavity and their baseline oral hygiene habits. The nurse instructs them in the techniques of good oral care, including rinsing the mouth frequently with water using a saline solution and a soft toothbrush or gauze to clean the teeth and gums three to four times a day.[105,136,137] Nurses also assess patients for mouth pain and discomfort and work with the care team to alleviate pain and promote adequate nutrition.

A wide variety of agents are marketed for the treatment and prevention of mucositis. Recent studies have shown that cold substances administered to the oral mucosa during chemotherapy administration (e.g., having patients suck on flavored ice popsicles) can help prevent mucosal cell damage.[133]

Pain

Pain is a common and distressing side effect of pediatric cancer and cancer treatment. Although techniques for managing pain effectively in children and adolescents are widely available, patients with cancer still experience unnecessary suffering. Inadequate pain assessment and poor communication between patients and providers are just two of the barriers to successful pain management. Other obstacles include lack of knowledge by health care providers about the manifestations of pain in pediatric patients and about how it is best managed. Taking the time to assess patients regularly to determine the type, cause, location, and intensity of pain will assist in developing an individualized management plan to relieve pain and optimize quality of life.

The use of a procedure room should be considered for hospitalized patients undergoing painful procedures in an effort to preserve the bed as a safe place. In addition, limiting the number of attempts by a single health care provider is one technique used to minimize discomfort. Whereas institution-specific procedures may define the process for managing multiple unsuccessful attempts by

Box 69-6 **Common Myths and Barriers to Effective Pain Management in Children**

ASSESSMENT

A child reports pain to the nurse or doctor.
If a child does not complain of pain, the child is not suffering.
It is always possible to determine whether a child is faking pain or truly suffering.
Active or sleeping children cannot be in pain.
Pain must have an evident stimulus; without one, a child cannot be feeling pain.
If a child is in pain, the parent would know it.

DIAGNOSIS

Infants have immature nervous systems and therefore feel less pain.
Children do not remember painful events.
Children become accustomed to pain and no longer feel it.
Children cannot communicate the location and intensity of pain.
Neurologically impaired children do not feel pain.
Adolescents are drug seekers.

PAIN MANAGEMENT

It is unsafe to administer opioids to children because of respiratory depression and addiction.
The best way to give an analgesic is intramuscularly.
It is unsafe to give narcotics through a central line.
Administering morphine means the patient is dying.

Modified from Jodarski K, Wilson K: Pain. In Kline NE, Brace-O'Neill J, Hooke MC, et al, editors: Essentials of pediatric oncology nursing: a core curriculum, *2nd ed. Glenview, IL, 2004, Association of Pediatric Oncology Nurses, p. 155.*

one provider, if not otherwise specified it is recommended that a provider seek the assistance of another provider after three unsuccessful attempts.

Over the past decade, providers have become more aware of and concerned about pain in children, and efforts to understand and manage all aspects of pain have resulted in an overall improvement in pain management.[68,138] The creation of dedicated teams of clinicians who specialize in the treatment of pain has also contributed to the introduction of new drug combinations and techniques for the treatment and control of pain.[139]

Assessing Pain and Developing a Pain Management Plan. For pain to be effectively managed, dispelling the myths that exist regarding pain in children is important (Box 69-6) because believing any one of them can markedly compromise efforts to manage a patient's pain.[67,140] Although the most accurate and reliable indicator of pain remains the patient's report, many children cannot or do not consistently and independently report pain. Therefore health care providers must consider the physiologic and psychological components of pain and develop techniques for routine assessment.[67,68]

Finding the most effective pain management strategy begins with assessment. Nurses conduct routine pain assessments incorporating an understanding of the common types of pain and an appreciation of how pain is manifested by children at varying developmental stages. Because pain can affect patients differently, a comprehensive assessment typically includes the patient's pain threshold, fears related to particular drugs, cultural beliefs, anxiety level concerning pain control and, in adolescents and young adults, past use of alcohol or illicit drugs. A key component of the assessment also involves measuring the intensity of pain with an appropriate instrument. The most common scales use faces or numbers to gauge a patient's pain level. Scales that incorporate faces are particularly helpful in pediatrics because they can be understood by a variety of age groups and by patients who do not speak English.[141] Numerical scales, which generally ask the patient to rate pain on a scale of 1 to 10, are appropriate for children 5 years of age and older.[67,140,142]

Managing Pain and Stress during Invasive Procedures. Cancer treatment often involves numerous invasive procedures, such as bone marrow aspirations and lumbar punctures. Children with cancer perceive these procedures as extremely painful[143] and often report years later that the procedures were the most frightening and traumatic part of their cancer care.[134,144,145] Studies have demonstrated that children do not adapt to procedure-related pain; instead, the trauma and anxiety associated with procedures increases with repeated painful experiences.[61,143,146]

The pain and stress involved in procedures is commonly underestimated. Providers who believe that a procedure is brief and that sedation is time consuming may fail to manage procedural pain and anxiety with adequate attention. Offering support and preventing pain during procedures is the responsibility of nurses and physicians. A preprocedure assessment is usually conducted to determine the approach that is best suited to each particular patient.

Properly preparing a child to prevent trauma during the first procedure has been shown to have a direct impact on the ability of the child or adolescent to tolerate and cope with subsequent procedures.[134] A patient optimally managed through a combination of pain medication, careful timing, and parental support seems to be less anxious about and fearful of successive procedures.[142] A child who has had traumatic experiences during procedures in the past may demonstrate such symptoms as insomnia, anxiety, regression, and depression as the time for another procedure approaches.[61,140,145,146] If these symptoms do appear, providers should question whether past efforts to prevent or control pain have been adequate.

Researchers continue to build evidence for the best way to prepare a child for invasive procedures. Studies support general anesthesia as a safe and effective method of pain control and psychological support because procedural anesthesia does not require intubation and is accomplished quickly and effectively through the use of short-acting intravenous anesthetic agents.[143,147] If anesthesia services are not available, procedural sedation is recommended. Procedural sedation involves various pharmacologic combinations, most commonly an opioid analgesic such as fentanyl and a benzodiazepine such as midazolam, for anxiolysis and sedation.[142,144] Used in combination, these agents depress the level of consciousness while still allowing the child to maintain his or her airway independently and to respond to physical stimulation or verbal command. Carefully administering agents before and during the procedure can ensure a pain-free experience for children and adolescents.

A patient scheduled to receive general anesthesia or procedural sedation is ordered to have nothing to eat or drink for 6 to 8 hours before the scheduled procedure. An infant may have breast milk for up to 4 hours before and clear liquids until 2 hours before sedation. Ideally, the withholding of food and fluids begins at night and the procedure is performed early the following day to limit the child's distress. The nurse assisting with the procedure prepares the procedure room, ensuring that all necessary equipment such as oxygen saturation and cardiac monitor is available. For procedures performed with the patient under general anesthesia, the anesthesiologist ascertains that all the necessary anesthesia and emergency equipment is available, administers anesthetic agents, and monitors the patient. When procedural conscious sedation is used, the patient's physician or nurse practitioner usually determines the time of the procedure and prescribes the sedative agents to be used. The nurse assisting with the procedure initiates the time out, administers the prescribed sedative agents, and may also administer antiemetics if intrathecal chemotherapy is being given. During a procedure, the nurse carefully monitors vital signs and oxygenation. A pulse and oxygen saturation monitor is generally used throughout the procedure and for at least 30 minutes during the recovery period or until the patient's values return to baseline.[68,144]

For less invasive procedures, the application of a local anesthetic cream to the procedure site several hours before the procedure has been found to reduce pain. Applied in a thick layer (2 mm) and covered by an occlusive dressing that is left undisturbed for an hour, the anesthetic cream penetrates the skin and provides local anesthesia up to a depth of 5 mm for 2 to 3 hours. Anesthetic creams have been found to reduce the pain associated with a range of procedures, including venipuncture, lumbar puncture, injection, and accessing indwelling central venous lines.[148,149] In an ambulatory setting, a nurse may teach the parents to apply the cream before leaving home so it is at peak effectiveness on arrival at the hospital. The cream's effectiveness may be decreased by poor hydration and dark skin pigmentation and offers limited effectiveness in controlling the pain of bone marrow aspiration, which requires the addition of locally injected lidocaine and procedural sedation or anesthesia.

Stress Reduction Techniques. A child often benefits from having parents with them during a procedure. This is particularly true for toddlers and preschoolers for whom separation anxiety is a developmental issue. During the procedure, the parents should be positioned close to the child so they can offer reassurance and comfort and help the child use stress reduction and coping techniques. A parent who is uncomfortable staying with the child should be supported in that decision and given reassurance that the child will be well cared for and that a member of the team, such as a child life specialist or a member of the psychosocial staff, will be available to offer the child support.

A variety of behavioral techniques, such as music therapy, distraction, and relaxation methods, can be useful in reducing anxiety and managing the pain associated with cancer treatment and invasive procedures. Nurses routinely work with other members of the care team to identify techniques appropriate for the patient and to help the patient and family learn and use the techniques during treatment.[150] The appropriate stress-reduction techniques for a particular child are determined in part by the child's developmental age and individual preferences. An infant, for example, may benefit from sucking on a pacifier or sucrose nipple, whereas a toddler may be distracted by a book, a tactile toy, or music. Guided imagery often works well with a child of school age or older; an adolescent may prefer being distracted by a CD player with headphones or a video game. Medical play may also be used to help relieve a child's anxiety. Introduced before a procedure is performed, it allows the child to perform the procedure on a doll and to ask questions and examine equipment that will be used. Such play allows a child to gain insight into and understanding of the procedure and the sensations he or she will experience.[47,140]

Central Venous Catheters

Central venous catheters (CVCs) are one of the most important advances in the treatment and supportive care of children with cancer. The most commonly used CVCs are peripherally inserted central venous catheters, tunneled central venous lines, and implantable reservoirs inserted under the skin, also known as portacaths. Today, a CVC is considered integral to managing patient care; minimizing discomfort caused by placement of peripheral intravenous lines; and ensuring safe administration of blood products, parenteral nutrition, chemotherapy, antibiotics, and antifungal and other agents. CVCs can also be used to obtain blood samples and perform infusions in the patient's home.[149]

Complications associated with CVCs include local and systemic infections, central line–associated bloodstream infections (CLABSIs), septic thrombophlebitis, and endocarditis.[115] Other potential complications include bleeding, thrombus formation, and catheter damage or dislodgment. The complications can be significant, but the benefits of using CVCs generally outweigh the risks. To minimize and prevent complications, meticulous care of the CVC is required. Maintenance of a CVC requires special care to reduce the risk for infections. Extensive staff, patient, and family education must be conducted to ensure consistent and effective care of the CVC and the ability to identify the signs and symptoms of infection. To prevent the possibility of a nosocomial infection, caring for a patient with a CVC requires training in proper aseptic technique and good hand washing. In recent years, some institutions have found that a 15-second wipe of the cap prior to the entering line is the minimum necessary to remove bacteria. Guidelines developed by the manufacturer or institution-specific policies and procedures are designed to minimize the risk for infection and should be followed whenever a CVC is accessed.[117,121]

One of the most common complications in a CVC is a CLASBI. If a CLASBI is suspected, blood cultures are drawn immediately and antibiotic therapy is started. The administration of antibiotics for treatment of the infection should be alternated between the catheter's lumens, so that each lumen of a double- or triple-lumen catheter is used and the risk for colonizing an unused lumen is minimized. Depending on the institution's guidelines, the CVC may be removed, particularly if the patient has positive blood cultures 72 hours after antibiotics are initiated, evidence of tunnel infection, catheter-related septic shock, or occlusion of catheter that cannot be cleared by thrombolytic or chemical treatments.[124] If the CVC is not removed, parenteral antibiotic therapy usually continues for 7 to 10 days after a first negative blood culture is obtained in a patient who is not immunocompromised and for 10 to 14 days in an immunocompromised patient.[151,152]

Reduction of CLABSIs has become a national priority as well as an institutional one for many centers. CLABSIs are defined by the Centers for Disease Control and Prevention (CDC)/National Healthcare Safety Network (NHSN) and are a source of morbidity, mortality and increased medical costs.[153] The NHSN, established by the CDC, reported that in 2004 the mean among 54 pediatric intensive care units (PICUs) was 6.6 bloodstream infections per 1000 catheter-days, higher than many adult centers.[153] Recognizing the impact CLABSIs have on patients, families and institutions as well as in an effort to reduce infections, the National Association of Children's Hospitals and Related Institutions (NACHRI) partnered with clinical providers on a quality improvement initiative to examine bloodstream catheter infections in its (PICUs. Small tests of change were implemented and identified best practice, which included insertion and maintenance care bundles. Changes implemented resulted in a decrease of bloodstream infections from 5.40 per 1000 catheter-days to 3.10 per 1000 catheter-days. After 1 year the NACHRI PICU collaborative achieved a 43% reduction in catheter-associated bloodstream infection rates.[153] To date, pediatric oncology centers throughout the country are participating in a separate NACHRI collaborative specific to hematology, oncology, and stem cell transplant patients, with the goal of reducing bloodstream infections. Critical to prevention of CVC infections is focused education and training. Adherence to best practice and maintenance of the CVC, performance improvement plans, and nurse staffing are critical in prevention efforts to reduce bloodstream infections.[154]

Although a CVC is typically cared for by the nurse while a patient is in the hospital or clinic, the patient and family assume that responsibility at home. Patient and family education related to using and caring for the line at home is an important part of nursing care. Teaching begins before the line is inserted and covers a range of topics, such as proper technique for CVC dressing changes, how to flush the line with saline and heparin safely, and how to recognize the signs and symptoms of complications. Education of the patient and family consists of a series of demonstrations by the nurse of all procedures necessary to care for the CVC, followed by return-demonstrations by the parents. During the return-demonstration the nurse is able to evaluate technique and identify areas that need additional review. Other teaching materials, such as books and teaching dolls, may also be used. In addition to the management of the CVC, the patient and family are taught to identify the signs and symptoms of infection and to report untoward findings to the physician immediately. Family members are instructed to avoid giving antipyretics unless directed by the provider.

A patient with a CVC is also referred to a home care nursing service that provides nursing support and instruction in the child's home. The child's inpatient or clinic nurse works in collaboration with the home care agency to ensure that the supplies needed to care for the CVC and any prescribed medications are delivered to the patient's home.

Caring for Patients Across Multiple Treatment Locations

For many pediatric cancer patients, treatment spans months or years and takes place in multiple settings. Much of a patient's care is delivered through an ambulatory clinic, but many patients are hospitalized from time to time and also receive care from home care providers. Coordinating care across multiple sites and providers presents a complex challenge for the pediatric oncology care team as well as for the patient and family. Good communication, clear documentation, and careful planning are essential to safe and seamless care. Before a patient leaves a setting, the care team must develop a transition plan to ensure that the patient and family, as well as the team receiving the patient, are well informed about the patient's needs and treatment. In addition, nurses or social workers serving as case managers or care coordinators work with the primary care team to arrange for community services and obtain insurance approval for services and equipment the patient will need.[49,155]

Nursing agencies and other organizations in the community are often called on to help with a patient's care in the home. Some pediatric oncology patients may require home care nursing to assist with central line care, hyperalimentation, chemotherapy or antibiotic therapy, pain management, and psychological support. A home infusion company may also be involved to provide line care, infusion supplies, and pumps.

Although most patients receive the majority of their chemotherapy in an ambulatory clinic, some patients and families choose to administer chemotherapy at home, with the support of a home chemotherapy and infusion service. Research has shown that such home treatment programs are associated with lower total costs and yield no significant differences in patient outcomes.[156-158] Patients and families have noted that these programs not only help to decrease disruptions to their daily lives but also help reduce psychological distress.[159]

Whenever home care nurses or other community-based services are brought into a patient's home, the primary nurse from the inpatient or ambulatory setting should speak with the home care provider to provide a detailed patient history and outline specific home care needs. Written communication detailing specific flushing orders, cap changes, protocols, blood sampling, and when to call the doctor should also be provided. Once the patient is home, communication between the home care agency and the patient's providers must continue to ensure that the treatment plan is carried out appropriately and that the home care nurses know when and whom to call if they have questions or concerns.

PHARMACEUTICAL ADVANCES

Emerging Therapies

Advances in molecular biology and translational research will eventually allow selective targeting of tumor cells and radically change the way children with cancer are treated. Investigators who identify the underlying biology, potential obstacles to delivering the therapy, and the possible impact of new therapies on the practice of pediatric oncology will be preparing for the future of pediatric oncology. In recent years, treatment of genetically defined cancers—those cancers known to harbor specific genetic abnormalities—has moved away from general cytoxic chemotherapies toward agents that specifically target proteins and signaling pathways that cancer cells need for their growth. Called targeted therapies, these agents can induce dramatic clinical responses and offer significant therapeutic benefits in treating many previously hard-to-treat cancers, including certain melanomas, colorectal cancers, thyroid cancers, and advanced lung cancers.[160] As these therapies start to become available in clinical trials for children, pediatric oncology nurses will have to be educated regarding the agents, administration, adverse reactions, and side effects.

Patient Safety Considerations

Advances in the delivery of cancer therapies have reduced the risk for medication errors and improved patient safety. One of the most technologically advanced methods of reducing medication errors has been attributed to barcoding medication administration (BCMA). Until recently, general initiatives in place to reduce medication errors were limited to an institutional awareness of safety, examining the processes around medication administration to identify sources of error, and reviewing medication events. The advent of barcoding brought with it the ability of the nurse to verify the identification of the patient and prescribed medication via technology and thus not having to rely only on visually checking the medicine and patient to confirm that the "five rights" (right patient, drug, dose, route, and time) are correct. The literature has reported significant reductions in medication administration errors, from 50% to 90%, comparing retrospective data prior to BCMA with adverse event reporting after implementation and potential errors avoided.[75] One estimate is that BCMA may prevent more than 84,000 medication errors in the United States.[161]

SUPPORTING PATIENTS AND FAMILIES AT THE END OF TREATMENT

Usually, the patient and family eagerly await the completion of treatment. Its arrival, however, may be accompanied by uncertainty and fear. For some patients and families, ending treatment means a departure from the relative security of routine visits to the oncologist and signals a return to their pre-cancer world. This transition is typically accompanied by a whole host of worries about their child's future and the potential for recurrence. Some of the anxieties troubling a patient and family stem from the fear that once the cancer is no longer actively battled, the child will become more vulnerable to illness or disease. This anxiety is often heightened by the gradual loss of contact with the health care team as providers pay more attention to "sick families" and by the loss of the psychosocial support that the team has long provided.[21,162-164] Helping the patient and family transition from active treatment is an important part of comprehensive cancer care. The primary oncologist and nurse or nurse practitioner play key roles in this process because they provide much of the guidance and information that the patient and family need to transition from treatment successfully.[162,165]

The Emotional Impact of Transitioning from Treatment

For the patient and family, leaving the safety net provided by the health care team means they must adapt to living with the uncertainties of surviving cancer while reestablishing the life they had before cancer was diagnosed.[163-165] Often a patient finds it difficult to reassume the role of a well person and to integrate back into the contexts of family, school, and community.[163,164] Although the literature suggests that most childhood cancer survivors do relatively well from an emotional perspective, studies indicate that some survivors and their parents can exhibit trauma-related symptomatology and experience ongoing worry and alienation. For many survivors and families, the impact of the cancer experience stays with them long after treatment is over.[166-168]

The context of posttraumatic stress can help clinicians understand the long-term psychological reactions of the survivor and family.[168] The end of treatment may signal a return to normalcy from the providers' perspective, but it can hold a very different meaning for the patient and family. An individual's ability to reintegrate into the community and adjust to a "new normal" as a cancer survivor can be impacted by the psychological sequelae that result from prolonged uncertainty; the emotions, ranging from joy to unspeakable sadness; the restrictions the patient and family must still observe; and the extra work and overall loss associated with the cancer experience.[164,167]

Supporting the Transition

Patients' descriptions of their experiences after cancer treatment suggest that they and their families often need help in accepting and dealing with their new situations and circumstances.[162,163] Before providers can help patients and families, however, they must first understand the psychosocial impact of childhood cancer, as well as the heightened fears and the intense need for reassurance that families experience once treatment is over. These needs and fears may be especially evident when the child experiences common illnesses or during follow-up procedures and appointments.[21,165,166]

Nurses, physicians, and other providers can help to alleviate the fears of patients and families by validating the psychological distress that may surface at the end of treatment, by answering all questions, by providing honest feedback, and by helping them to begin to trust their ability to adjust to life after treatment.[41,164] Additional strategies include providing patients and families with ample opportunity to discuss concerns, acknowledging that fears of recurrence are normal, and preventing additional anxiety by ensuring that all posttreatment test results are reported to patients and families in as timely a manner as possible.[163]

The oncology team can also help to prepare patients and families for the challenges that lie ahead by teaching them about what to expect once treatment is over and by connecting them to programs that support the reintegration process. Support groups, programs that help with school reentry, camps that allow patients to socialize with other cancer survivors, and clinics that focus on cancer survival can all be instrumental in helping patients and families adjust to life after treatment.[163,169,170] Efforts to educate the patient and family and to support their transition from treatment can not only facilitate psychosocial adaptation but also improve the quality of life for cancer survivors.[165,171]

Because nurses are a primary source of support and information for patients and families, they play a critical role in ensuring a successful transition that is based on a unique understanding of the indicators of healthy transition: patient and family demonstration of an overall sense of well-being, observed mastery of skills required for home management, and well-being of the relationships among family members.[172] Nurses employ a variety of strategies to support transition after cancer treatment, which include, but are not limited to, an assessment of readiness, as well as the provision of materials and information about resources that patients can draw on once they leave the oncology setting. Information about programs and support groups for cancer survivors and written materials that outline follow-up care and potential side effects of treatment can be invaluable resources for patients and families as they leave the structure and security of the hospital and transition to other care settings.[162]

NURSING CARE OF CHILDHOOD CANCER SURVIVORS

The treatment of childhood cancer is one of the great medical success stories of the past 30 years. During this time, the cure rate for all childhood cancers has risen to 78%, resulting in a dramatic increase in the population of childhood cancer survivors.[173] Today, approximately 1 in 640 adults between the ages of 20 and 39 have a history of childhood cancer.[174] One unfortunate consequence of this success is that late effects of childhood cancer treatment are now more commonly seen, effects that can occur during childhood or many years afterward, sometimes with devastating consequences. As many as two thirds of survivors experience late effects, and as many as a fourth experience an effect that is life-threatening.[174] Among the late effects that pose risks to cancer survivors are learning disabilities, infertility, heart problems, secondary tumors, and genetic issues.[162,169]

Although systematic and consistent follow-up of childhood cancer survivors is recommended, there is no consensus about where this care should be provided, who should provide it, or the detailed components that such care should entail.[174] The appropriate time to transfer care is also controversial and is dependent on the provider, although many suggest transitioning to long-term follow-up once a child has been off therapy for 2 years.

The primary pediatric oncology team, which typically has an intense investment in the childhood cancer survivor, may be reluctant to transition care to the primary provider; however, planning for the transition is essential and should begin well before follow-up care is needed. Developing a plan that includes detailed written information about the patient's specific cancer and treatment protocol ensures that the primary provider will have the necessary information to provide appropriate follow-up care. Other necessary information includes the medications the patient received (including dosages); the radiation therapy administered (including dosages); the surgical procedures performed; and any complications of therapy that the patient experienced. The oncology team should also equip the patient with documentation describing the cancer, its treatment, and possible long-term effects. Such a document in the form of a medical passport, notebook, or other transferable records is an important reference for the patient and for follow-up providers alike.

The patient and family should begin learning about late effects and follow-up care long before the end of treatment to ensure that they have a good understanding of the information presented and have a chance to ask questions and discuss concerns. Research on survivors of childhood cancer has shown that most survivors are unfamiliar with specific details of their diseases and treatments and are unaware of their risks for late effects.[170] One study found that only 30% of childhood and adolescent cancer survivors could provide adequate histories of their diseases and treatments.[175,176]

Providing a patient and family with information not only empowers them to advocate for adequate follow-up care but also prepares them to navigate the health care system and play an active role in monitoring and managing late effects once the cancer treatment ends.[177] Pediatric oncology nurses play a critical role in preparing the patient and family for the transition to follow-up care by teaching them about the disease, its treatment, and possible physical or psychological late effects. Although being careful not to overwhelm the patient and family with too much information, nurses should also address risks specific to that patient's treatment and offer guidance about how to self-monitor for late effects.[174,176] Information about organizations that offer resources for childhood cancer survivors, such as the Candlelighters Childhood Cancer Foundation and the National Children's Cancer Society, is also helpful, as are books and other written materials that provide additional practical information.[175,178]

Follow-Up Care and Care Settings

Over the past 10 years, many oncology centers have developed childhood cancer survivor clinics that monitor patients for late effects and provide treatment to improve survival rates and quality of life.[174] Centers that belong to the Children's Oncology Group are required to offer such a service. Care in these clinics is often provided by advanced practice nurses in collaboration with one or more pediatric oncologists. The nurses are trained in oncology and have extensive knowledge about survivorship issues and follow-up guidelines. During visits, nurses evaluate the patient's growth and development and conduct comprehensive screenings for late effects associated with the patient's particular cancer or treatment.[68] They also monitor the patient for systemic toxicities resulting from the cancer drugs and therapies they received. Some follow-up clinics are multidisciplinary in structure and offer the support of specialists, such as cardiologists, endocrinologists, fertility and genetic counselors, and social workers.

Although the number of specialized follow-up clinics is growing, most pediatric cancer survivors are followed by adult primary care providers. Childhood cancer survivors can also be encountered in a variety of other practice settings, such as primary pediatric settings and adult specialty clinics. Nurses and other providers in nononcology settings often have little experience working with survivors of childhood cancer and may not know about the possible consequences of cancer treatment or what to watch for. In addition, they often have little incentive to learn about the complex care required by childhood cancer survivors because many practices see very few such patients.[171]

Goals for the follow-up care of pediatric cancer survivors include identifying, preventing, treating, and curing late effects and bridging primary and special care services through education and outreach. To help support nononcology clinicians who are caring for pediatric oncology survivors, the Children's Oncology Group has developed a comprehensive set of guidelines for detecting and managing the late effects of childhood, adolescent, and young-adult cancers.[179] These guidelines were developed in a collaborative effort between the Nursing Discipline Committee of the Children's Oncology Group and the Late Effects Committee and are designed to enhance providers' awareness of potential late effects and to standardize the follow-up care of childhood cancer survivors.[177] The guidelines are risk based and exposure related and provide specific clinical recommendations for screening and managing late effects, beginning 2 years after cancer therapy has been completed. These guidelines can be obtained at no cost and, because they are on the Internet, they are readily accessible to primary care physicians, nurses, and the public.[179] Education materials developed by nursing experts are also available and can be used along with the guidelines to enhance the process of educating patients and empower patients to become proactive participants in their long-term follow-up care.[177,179]

The care of patients followed in nononcology settings can also be augmented by regular communication with the patients' pediatric oncology providers. Documentation of the cancers and the treatments given to patients (e.g., the medical notebook and other transferable records described earlier) is also an important reference for follow-up providers. Such communication, along with continuing education for community primary care providers, helps to ensure that survivors of childhood cancer receive comprehensive care and ongoing monitoring and treatment of late effects.[162] Nurses in nononcology settings may not be aware of the guidelines for follow-up care and may not be knowledgeable about the late effects of childhood cancer. Although the guidelines of the Children's Oncology Group are helpful, additional methods of training nurses about specific interventions required by the survivor population are needed.[180,181]

Follow-Up Nursing Care

Much of the care required by pediatric cancer survivors is the same as that required by other patients. For example, studies have indicated that survivors are at risk for establishing unhealthy lifestyle behaviors.[181] Because of this, nurses should use appropriate health promotion strategies and encourage survivors to exercise and maintain a healthy weight and balanced diet, avoid smoking, protect themselves from excessive sun exposure, and keep their consumption of alcohol to acceptable limits. Nurses working with adolescents, in particular, need to clarify the risks of unhealthy behaviors and encourage them to participate actively in decisions related to their health and follow-up care. Preventive screening techniques recommended for the general population (e.g., for colon, breast, and cervical cancer) should also be observed as minimum recommended follow-up.[174]

In addition to employing standard prevention and screening measures, nurses caring for pediatric cancer survivors must also help to monitor patients for late effects of cancer treatment. One of the most disturbing late effects for children and families is neurocognitive deficits. Childhood cancer survivors who received intrathecal chemotherapy or cranial irradiation or who were treated for tumors in the central nervous system are at particular risk for this late effect and should be routinely assessed for its symptoms. Patients with neurocognitive deficits may experience psychosocial problems and may have difficulties with school performance and academic achievement.[159] Memory loss and impaired intellectual or motor function can also occur and are more common in children, particularly girls, who received treatment when they were younger than 6 to 8 years of age.[182]

Survivors who have neurocognitive deficits are often placed in special education programs or programs for the learning-disabled and tend to have lower self-esteem than their siblings.[174] Families often describe the difficulties they encounter when dealing with learning issues, cognitive deficits, attention disorders, behavior disorders, and other problems associated with late effects relating to neurocognition. It is common for these families to need preparation for and assistance in working with school systems and help in obtaining referrals to and resources for neurocognitive testing. Nurses can play key roles in helping patients and families to obtain the support they need and should educate the survivors and families about three federal laws that ensure equal access to education. The Individuals with Disabilities Education Act requires states to provide free and appropriate education to all children between the ages of 3 and 21 years. The Americans with Disabilities Act prohibits discrimination against people with actual and perceived disabilities or histories of disability. The Rehabilitation Act prohibits schools that receive federal funding from discriminating against qualified students who have a history of cancer.[174] Nurses caring for pediatric cancer survivors should also become familiar with school reentry programs and advocate for continued identification of appropriate cognitive programs to promote the success of these children.

Nurses also play roles in identifying late effects related to hormones and fertility. The potential fertility issues and options for their treatment, including sperm or egg banking, should be discussed with patients and families at the time of diagnosis. Once patients enter long-term follow-up, nurses should routinely assess and document patients' sexual development, including their histories of menses, sexual function, and Tanner scale staging, and should provide education and support to patients pursuing fertility options.

SUPPORTING PATIENTS AND FAMILIES DURING RELAPSE AND RECURRENCE

Although the cure rate for pediatric cancers has improved significantly over the past few decades, recurrence of the disease is still possible and is a source of great anxiety for patients and families. Identifying and diagnosing recurrence at an early stage is among the goals of posttreatment surveillance programs because early detection may enhance the chances of successful treatment by means of a second-line protocol.[183]

Some patients and families liken the possibility of relapse to having the sword of Damocles hanging over their heads. Many begin to express fears of relapse and uncertainty as the end of cancer therapy draws near or soon after treatment is over. For patients and families who experience actual relapse, the news can be more devastating than receiving the initial diagnosis of cancer. Nurses, physicians, and other providers working with cancer patients and their families must be aware of the fear surrounding relapse and be prepared to help patients and families manage their anxiety and cope with relapse should it occur. In addition, it is important for the nurse to understand mechanisms and the guiding framework of resilience to effectively assist the patients and families in their adjustment to relapse. Examples of resilience pathways include positive coping, hope-derived meaning, and a spiritual perspective.[184]

Helping Patients and Families Cope with Fear of Relapse

With infants and very young children, the onus of fear related to relapse is generally carried by the parents. Once children reach school age, however, they, too, can experience fear of relapse and require the support of the oncology team. Some patients and families begin to raise the possibility of relapse during conversations with providers as they approach the end of treatment, whereas others do not address it until treatment is over. The fear may persist even after children or adolescents experience 5 cancer-free years, a time limit that is generally recognized as indicating recovery. In many cases the fear of relapse is brought on or is heightened by trigger events, such as the occurrence of minor illnesses or an increase in bruising.

Because outpatient nurses encounter patients and families frequently and are commonly primary sources of support, they may be the first to notice signs suggesting that patients or families are worried about relapse and they may be the first of the oncology team to whom the patients and families speak about their concerns. In addition to sharing their observations with other members of the team, nurses may recommend that patients and families meet with the oncology team's psychologist or social worker, who can help them identify ways of coping with fears and anxieties.

While encouraging patients and family members to verbalize their fears, nurses also emphasize the importance of maintaining a positive attitude because this benefits patients and families alike. Nurses also try to assess patients' siblings because they, too, can harbor fears of relapse and must be encouraged to verbalize these fears. In many centers, nurses or other providers have developed support groups for siblings that help them to express their feelings while engaging them in various activities, such as outings, discussion groups, or arts and crafts projects that allow them to spend time with others who have shared the same experience.

Recurrence

More upsetting than the fear of relapse is the experience of an actual relapse. The fear of recurrence can have a profound effect on patients and families, but experiencing actual relapse can be devastating. How families respond to the news of relapse has marked effects on the patients' responses to the illness and on the psychosocial environments in which they must continue to grow and develop.[56] Once parents are informed of a relapse, nurses and other members of the care team must help them manage their immediate emotional reactions so they can take necessary and curative actions on their children's behalf.[185] The behaviors of the nurses and the rest of the care team are critical in helping the patients and families cope and adapt as they endure what may be the greatest challenge of their lives.

Immediately after patients and families are told of recurrence, they are thrust once again into a state of waiting and not knowing.[56] During these difficult times, nurses support the patient and family by listening and providing them with opportunities to express their feelings. They may be confused about some of the information they have received and may feel angry, helpless, and overwhelmed as they contemplate decisions about additional treatment and realize they must once again take on the immense challenge of making decisions about their children's care. Collaborating with the health care team and coordinating the day-to-day care of siblings and work responsibilities may also add to parental distress. At this early stage, parents are especially dependent on physicians, nurses, and other health care professionals to communicate clearly and to share information about the illness and treatment that will help them plan and carry out their lives over the next weeks and months.[56]

At the time of recurrence, patients and families may express dissatisfaction with the initial treatment choice and may blame themselves for the treatment's ineffectiveness, calling themselves failures.[186] Parents often vacillate between hope for another remission and anticipatory grief while they struggle to determine whether they need to fight for cure or prepare for loss. These conflicting feelings are a part of coming to terms with the relapse, a process that involves limiting immediate emotional responses so they can take appropriate action.[185]

As treatment begins, patients and parents may become extremely vigilant about many of the details of therapy, such as the time a medication should be administered and the results of laboratory tests. Nurses and other providers must understand that such selective and watchful awareness is normal at this time and that it reflects the patients' and parents' need to gain control of the situation. During this time, parents often benefit from having predictable care routines and periods of time, however brief, in which they are free of any immediate worries. Such periods, parents say, help them to rest and recover from the burden of recurrence.[185] It is critical that the patient's needs for support not be overlooked or minimized in the mistaken belief that children are too young to understand the implications of recurrence. Research has shown that nurses who realize that school-aged children may be aware of the uncertainty associated with relapse support patients more appropriately than do nurses who believe the patients are unaware of the status of their disease.[187]

Studies show that parents who have experienced the relapse of their child's illness note the value of being kept apprised of treatment options and of how their children are responding to treatment.[185] Nurses address this need by speaking with family members during each encounter, reviewing what the families have been told, verifying information, answering questions, and helping them put new information into perspective.[56] Parents who have gone through the experience of the relapse of their child's illness also note the comfort they felt whenever providers demonstrated personal knowledge of or fondness for their children. Such actions, they said, told them that regardless of the treatment's outcome, their children would never be forgotten.[185]

NURSING CARE OF CHILDREN AT THE END OF LIFE

When efforts to treat the disease are unsuccessful, end-of-life care becomes critical.[188] As noted in a landmark report by the Institute of Medicine, the goal for children and families who face life-threatening diseases should be access to a health care system that provides "competent, consistent, and compassionate care that families can count on for support and solace as they experience a loved one's grave illness or death."[189] Unfortunately, many children who die of terminal illness in the United States do not receive aggressive supportive care at the end of life.[190] Historically, palliative care research in pediatrics has been difficult to conduct, owing to the sensitive nature of the topic and the reluctance of providers and parents to accept the inevitability of the situation at hand. Although this has been improving over time, additional efforts are needed to deliver more effective end-of-life care, educate professionals, and design supportive public services.[189] In spite of these challenges, many nurses and other health care providers have found that participating in end-of-life and bereavement care is a richly rewarding experience.

Nurses play critical roles in delivering end-of-life care because they provide most of the physical care while the patients are in the hospital and are key sources of support for patients and families while they are at home. The nurse has a unique appreciation of the patient's clinical condition, emotional needs, and family dynamics. Their observations and assessments are crucial to the efforts to keep the child comfortable and enhance quality of life. The family, or legal guardians, rely on the nurse as a give or vital information and support because they often have many questions and concerns.

End-of-Life and Palliative Care

Many of the challenges encountered when caring for patients and families at the end of life begin before the child enters the terminal phase of illness. Managing pain and other symptoms and coping with anxiety are issues for many patients with cancer. Palliative care, a specialized area of practice that focuses on preventing or relieving suffering and ensuring the best possible quality of life, is particularly important for patients at the end of life.[190,191]

Palliative care is both a philosophy of care and an organized, highly structured system of delivering care. Palliative care seeks to enhance patients' quality of life in the face of terminal illness. Palliative, or supportive, care should begin as soon as the child receives a diagnosis of a life-threatening condition and should continue throughout treatment until death, should it occur. The term *hospice care* refers to palliative care services (including therapeutic interventions and medical equipment) that are generally provided by a multidisciplinary group of physicians, nurses, chaplains, health aides, and bereavement counselors.[192]

An increasing number of hospitals and other health care organizations have organized teams of trained palliative care providers that consult with the patient's primary care team and provide guidance regarding the physical, emotional, developmental and spiritual needs of the child and family. These teams include physicians, nurses, social workers, and chaplains.

One of the most difficult decisions a family can face is deciding when to shift the focus from finding a cure to managing the end of a child's life. Historically, the conclusion that no curative options remain was arrived at solely by the treating physician. More recently, however, the parents or guardians have been routinely included in making end-of-life decisions, and the dying child may be included as well. In some cases, the family's decision about whether to shift the focus to end-of-life care may be complicated by an offer to participate in a clinical trial or to receive some form of innovative care. For the patient and the family these opportunities are usually unexpected, and deciding whether to participate in a trial or try a new treatment can be very difficult. Deciding what is right can also be difficult for health care providers who are also clinical investigators. They may experience discordance between their desire to advance science and improve the care of other children and their desire to protect the dying child by doing all that is in the child's best interest. This discordance may be lessened by the certainty that the dying child (when possible) and the family have been given all of the available information about the study and that they are making a well-informed decision.[193]

When discussing further treatment options or end-of-life care with a patient and family, clinicians must take the time to communicate clearly. How they approach the discussion should reflect appreciation of the child's developmental stage, how the child understands death, and awareness of the family's culture. The nurses who are caring for the child should be involved in the discussions because the family often calls on the nurse to interpret the information they have been given. A recent integrative review on parent perspectives on the care of their child at end of life found that recurring themes in the literature included poor communication with the health care team, inadequate emotional support, the parent's need to maintain their relationship with the child during end-of-life, availability of health care services impacting the child and family's quality of life, and the difficult decision to terminate life support.[194]

The nurse's primary goal when caring for a child or adolescent dying of cancer is to maximize the patient's quality of life. Nursing interventions are based on the patient's needs and on the patient's and family's style, values, spirituality, culture, and ways of relating to and interacting with others. To identify and support the unique needs of each patient and family, nurses assess the family's structure, dynamics, coping mechanisms, cognitive and emotional functioning, previous history with loss, family support system, and spiritual and religious beliefs on a continual basis, and the plan of care is developed and revised accordingly.

Nursing Care in the Inpatient Setting

Nurses focus on preventing illness, alleviating suffering, and protecting, promoting, and restoring health. However, when the restoration of health is no longer possible, the care shifts to ensuring the highest possible quality of remaining life and a comfortable, dignified death. When a child is facing the death in the hospital, maintaining comfort and helping the patient and family cope are the major priorities. Often nurses provide all of the child's clinical and physical care so that parents and other family members are free to meet the emotional needs of the child.

During the hospital stay, a focus of care is to normalize the patient's daily activities. Depending on the child's age and developmental stage, this may mean encouraging parents to go for a walk with the child, read the child a story, or give the child a bath. Working with the other members of the care team, the nurse may introduce play therapy that helps the child to express and cope with fears and anxieties.[195] Throughout the child's hospitalization, the nurse should try to maintain consistent caregivers and routines, because this may decrease anxiety for the child and family.

Outpatient, Home, and Hospice Care

Many children with progressive cancer die at home. Helping a child or adolescent to die peacefully at home presents a multitude of challenges to the care team, but it is critically important because the support of providers can enhance the family's ability to cope with the child's death. In the United States, much of the palliative and hospice care outside the hospital is currently provided by specially trained community nursing agencies; however, palliative care is a rapidly growing specialty in medicine and nursing, and many new services are being developed by hospitals, skilled nursing facilities, and other groups and providers.

Nurses in hospital and clinic settings play central roles in helping to arrange for home-based services and to ensure good communication between home care providers and clinicians in the hospital and clinic. When a patient is hospitalized, an inpatient case manager (often a nurse or social worker) is usually charged with identifying and arranging the community resources. Once the patient is discharged, the outpatient pediatric oncology nurse usually coordinates communication between the clinic and the care providers in the community. If home care services are not available or are not covered by health insurance, outpatient nurses may play a more central role in coordinating care among the provider, patient, and family.[196]

Hospice is a philosophy of care that is rooted in the centuries-old idea of offering a place of shelter and rest, or "hospitality," to weary and sick travelers on long journeys. The term was first used in 1967 by Dame Cicely Saunders of St. Christopher's Hospice in London to describe specialized care for dying patients.[197] The hospice philosophy recognizes death as the final stage of life and seeks to enable patients to continue alert, pain-free lives and to manage other symptoms so that they may spend their last days with dignity and surrounded by their loved ones. Hospice affirms life and neither hastens nor postpones death. It treats the person rather than the disease and emphasizes the quality, rather than the length of, the person's life. Hospice care should be initiated when the patient can no longer benefit from curative treatment, and life expectancy is, at most, no longer than 6 months. If the patient's condition improves or the disease goes into remission, hospice discharge can be arranged and the patient can return to active treatment if they desire.

Hospice services provide family-centered care 24 hours a day, 7 days a week. Although it can be given in a hospital, nursing home, or private hospice facility, most hospice care in the United States is given in the patient's home, with a family member serving as the primary caregiver, and the hospice staff offering ongoing consultation, guidance, and support. Care is focused on relieving pain and other symptoms and providing social, emotional, and spiritual support. Many hospices also provide respite and bereavement care. Choosing a hospice service can be a difficult decision for patients and families, and nurses and other providers often help by assisting them in gathering information and helping them to choose a hospice service that will meet their needs. Because many providers are unfamiliar with the programs and hospice options that are available, The Joint Commission has created a list of questions that patients, families, and providers can use to guide their selection of a home care or hospice program.[198]

Unfortunately, pediatric hospice services are not as widely available as services for adults. Often the nursing agency that provides home care during treatment also provides palliative care at the end of life. Because round-the-clock, home-based pediatric nursing care is available only sporadically, most of the burden of care is placed on the parents. In some cases, enlisting the help of a surrogate caregiver to maintain daily routines and provide respite for the parents may be helpful.

Nurses are integral members of the hospice care team focusing on meeting the physical, emotional, and spiritual needs of patients and families. Hospice nurses work independently, providing care in settings that are removed from many of the resources available in hospitals. Although the limits, functions, and titles of nurses involved in hospice care may vary from state to state, all hospice nurses are guided by the same holistic hospice philosophy and by a position statement from the American Nurses Association regarding the care of the dying patient that reinforces the obligation to promote comfort and ensure aggressive efforts to relieve pain and suffering.

The primary role of hospice nurses is to coordinate the care of a patient at the end of life while maintaining a focus on comfort not cure. In the course of providing care within the patient's home, hospice nurses observe, assess, and record symptoms and work with the child's physician or nurse practitioner to develop and help the family implement a plan that ensures optimal end-of-life care. Family members caring for the patient rely on hospice nurses for guidance in identifying and treating symptoms and for instruction regarding who to call for assistance.

One of the major concerns at end of life is the fear of intractable pain that can lead to serious problems, such as sleeplessness, loss of morale, fatigue, irritability, restlessness, and withdrawal.[198] Because nurses often have the most frequent and continuous contact with patients, they play central roles in assessing and managing the patient's pain and other distressing symptoms at this critical time.

Bereavement Care

Grief and bereavement are highly individualized and are influenced by the family's culture, community, faith, and support system. For many grieving families, abruptly ending contact with health care providers can feel like abandonment.[188] There are organizations that developed bereavement care programs through which professionals, volunteers, other families, and community agencies work together to meet the needs of a family, should this be needed.

Nurses, physicians, and other care providers often experience grief when a patient dies. Some may identify with the family and feel sad about the death, whereas others may experience awkwardness and not know how to respond. Needs of health care providers working with dying children vary according to their personal and professional experiences. Contrary to popular belief, nurses and other clinicians do not become accustomed to working with dying children; however, caregivers who have developed coping skills over time are probably better equipped to deal with the care of a dying child.[186,196]

Nurses, and other providers, who cope effectively tend to recognize their own feelings, take advantage of support groups provided by the health care institution, have an established support network outside the hospital, understand their own limitations as health care providers, and feel their contributions to the patient's care have been positive. Those who detach themselves as the child's death approaches, or who become overly involved and focus on the dying child and the child's family to the exclusion of their own personal needs, tend to have a more difficult time coping with the child's death.[196]

Nursing Education about End-of-Life Care

The care of dying patients is enhanced when it is provided by nurses who are experienced in and knowledgeable about end-of-life care.[199] Among the barriers to providing optimal palliative care are lack of formal courses in palliative care, high reliance on trial-and-error learning, lack of strong role models, and limited access to services focusing on pain management and palliative care, although this has changed dramatically in the past 10 years.[200] Recognizing these barriers, many groups in the nursing community have made educating nurses regarding palliative and end-of-life care a priority.

In 1998, the American Association of Critical Care Nurses published a document specifying competencies that nursing students should acquire related to end-of-life care. Since then, APHON has developed a curriculum and standards related to end-of-life care for training nursing staff and the End-of-Life Nursing Education Consortium, which is composed of nationally recognized palliative care experts, has created a curriculum to train nurse educators in palliative and end-of-life care and prepare them for teaching nursing students and practicing nurses. To date, over 15,000 health care providers have been trained in the United States and 73 other countries.[199] Palliative care content has been included in licensing examinations for nurses and has been added to nursing textbooks.[200]

In addition to training staff nurses in end-of-life care, there is also a need for nurses who have advanced knowledge and skills in palliative care, and graduate nursing programs include palliative care tracks for nurse practitioner students.[200] Nurse practitioners and nurses at the bedside who have such expertise can play vital roles in caring for patients by leading organizations and programs that care for patients at the end-of-life.

ETHICAL NURSING PRACTICE

Complex patient care problems present themselves daily in pediatric oncology practices. Because of the interdisciplinary nature of pediatric oncology, many problems that involve ethical implications are foreseen, and anticipatory ethics is practiced.[201] Nurses dealing with ethical issues are guided by the Code of Ethics outlined by the American Nurses Association[202] (Box 69-7) and by the ethical standards for Pediatric Oncology Nursing Practice defined by APHON (Box 69-8).[15] These documents highlight fundamental principles of ethical practice, including respect for persons, beneficence, and justice that are considered basic to nursing and that underpin how nurses think when confronted by problems involving ethical tensions.[201,203]

Box 69-7 **The American Nurses Association Code of Ethics for Nurses**

1. The nurse, in all professional relationships, practices with compassion and respect for the inherent dignity, worth, and uniqueness of every individual, unrestricted by considerations of social or economic status, personal attributes, or the nature of health problems.
2. The nurse's primary commitment is to the patient, whether an individual, family group, or community.
3. The nurse promotes, advocates for, and strives to protect the health, safety, and rights of the patient.
4. The nurse is responsible and accountable for individual nursing practice and determines the appropriate delegation of tasks consistent with the nurse's obligation to provide optimum patient care.
5. The nurse owes the same duties to self as to others, including the responsibility to preserve integrity and safety, to maintain competence, and to continue personal and professional growth.
6. The nurse participates in establishing, maintaining, and improving health care environments and conditions of employment conducive to the provision of quality health care and consistent with the values of the profession through individual and collective action.
7. The nurse participates in the advancement of the profession through contributions to practice, education, administration, and knowledge development.
8. The nurse collaborates with other health professionals and the public in promoting community, national, and international efforts to meet health needs.
9. The profession of nursing, as represented by associations and their members, is responsible for articulating nursing values, for maintaining the integrity of the profession and its practice, and for shaping social policy.

From American Nurses Association: Code of ethics for nurses with interpretive statements, 2010. Accessed January 28, 2013 at http://www.nursingworld.org/MainMenuCategories/EthicsStandards/CodeofEthicsforNurses/Code-of-Ethics.pdf.

Box 69-8 **Association of Pediatric Hematology and Oncology Nurses: Ethical Standards of Pediatric Oncology Nursing Practice**

Standard: The pediatric oncology nurse respects the rights of all children and families and makes decisions and designs interventions that are in agreement with ethical principles.

Rationale: Advances in technology and genetics, along with scarce resources in health care, have created an environment in which ethical issues frequently arise. The pediatric oncology nurse should advocate for the rights of children with cancer as well as identify and help resolve ethical conflicts.

Measurement Criteria:

The pediatric oncology nurse

- Understands and applies the basic ethical principles of autonomy (right to self-determination), beneficence (do what is in the best interest of the patient), nonmaleficence (do minimal harm), justice, and veracity (truth telling).
- Examines own beliefs relating to autonomy, rights of a minor, quality of life, death, suffering, truth telling, equality, and access to care.
- Identifies available resources, including the *Code of Ethics for Nurses with Interpretive Statements* (ANA, 2001) when formulating ethical decisions.
- Maintains confidentiality.
- Provides quality care to all children, regardless of race, culture, educational background, religious beliefs, socioeconomic status, or the ability to pay.
- Delivers care in a manner that preserves and protects patient autonomy, dignity, and rights.
- Acts as a patient advocate and assists children and families in developing skills so they can advocate for themselves.
- Identifies ethical conflicts and seeks to resolve them through multidisciplinary team discussions, including the child and family as appropriate.
- Addresses advance directives with young adults 18 years of age and older.
- Seeks to include minors in decision making as appropriate.
- Ensures that all children and families receive truthful information regarding diagnosis and treatment.
- Participates in the informed consent process by witnessing the signing of consent documents, obtaining ongoing education about research trials, answering the child and family's questions regarding their participation in research, and ensuring the child and family's continued desire to participate in the research trial.
- Reports illegal, incompetent, impaired, or unethical practices.
- Maintains therapeutic professional nurse-patient relationship with appropriate boundaries.

From Association of Pediatric Hematology/Oncology Nurses: Scope and standards of pediatric oncology nursing practice. *Silver Spring, Md., 2007, Nurses Books.*

Several factors must be present for high-quality, ethical nursing care to occur. Perhaps the most important factor is that the nurses themselves must be moral individuals, possessing integrity, courage, honesty, and a sense of justice.[195] Further, they must be willing to use these virtues to protect patients' rights to self-determination and to ensure that an individual's wishes, goals, and viewpoints are considered when managing care.[195,204,205] Nurses who frequently confront ethical issues benefit from forums that facilitate moral and ethical discussions, communication, and inquiry and that promote the exploration of options for patients. Guided sessions that focus on sorting through choices and their implications help nurses to gain a deeper understanding of nursing ethics and encourage them to embrace their role as patient advocate. The education provided by such forums not only helps nurses to develop skills needed to work through options when difficult situations arise but also helps them to cultivate the field of nursing ethics.[201]

The ways in which nurses approach ethical practice are somewhat different from the approaches used by other disciplines.[206] The differences may have their origins in the very core of each profession. Nursing and medicine, for example, often play complementary and virtually inseparable roles but have functionally different foci: nursing, a health-oriented profession, is focused predominantly on the care of patients and families while preserving and restoring health; medicine traditionally has been oriented toward the treatment, prevention, and cure of illness.[207] Because of these differences, the relationship that develops between patients and nurses can be very different from that which develops between patients and physicians.[208] Although both nurses and physicians serve as patients' advocates, the nurses' perspectives are informed by their proximity to the bedside, a proximity that allows nurses to develop unique relationships with patients and to witness directly the converging influences that surround care versus cure.[204,207,209]

During the course of pediatric oncology care, the varied and often complementary perspectives of each discipline have legitimate places in supporting the needs of patients and families. Nurses and physicians share responsibility for ensuring high-quality patient care and for achieving the best possible outcomes.[203] Further, because the uncertain nature of cancer renders the children and their families vulnerable, the practices of both disciplines must possess the moral virtues of honesty, courage, and justice and must reflect commitment to ethical principles.[195,209]

Ethical dilemmas related to care are often unavoidable. Nurses and physicians alike have the responsibility to manage with care the trust shown them by their patients. And when the right or good thing to do is not clear, they must have the moral courage to analyze critically and, if necessary, to challenge practices and health policies so as to ensure the best possible outcomes.[203,195,210,211]

FUTURE TRENDS IN ONCOLOGY NURSING

Change is intrinsic to the nursing profession and to pediatric oncology nursing, which remains a dynamic subspecialty that is subject to constant advances in technology and science and the introduction of new treatment regimens.[3,4,7] Faced with the potential for a nursing shortage, direct and deliberate strategies to integrate these changes into nursing education, practice, and research are required to ensure that pediatric oncology nurses are positioned to continue providing high-quality care.[7] In the coming years, as new treatments are introduced and more aspects of care shift to the outpatient setting and the home, it is expected that the role of the pediatric oncology nurse will continue to expand and assume greater importance. Remaining current with new developments in the field will make it possible for nurses to influence pediatric nursing practice effectively and to develop innovative strategies for managing the care of patients and families.[4,7,22]

No single discipline can accomplish all that is necessary for a patient; therefore collaboration is critical.[13] Research has shown that interdisciplinary collaboration is associated with improved outcomes, suggesting that intensifying nurse-physician partnerships based on mutual trust, respect, teamwork, and open communication is a priority.[21,22,35,212] Even though both nurses and physicians recognize the importance of collaboration, developing such partnerships may be a challenge.[213]

The primary focus of pediatric oncology nurses is healing rather than curing, but nurses will continue to play strategic roles in supporting the pursuit of a cure whenever possible.[22,32,214] As the consistent provider at the bedside, nurses have a distinct advantage to support translational research efforts. Leveraging this position by combining theory, research, science, and practice allows nurses to bring knowledge from the bench to the bedside and from the bedside to the bench.[215,216] The wide-ranging interests of pediatric oncology nurses and concerns include problems with care delivery systems, worries about meeting the needs of individual patients and families, and worries about national and global issues that impede clinicians' efforts to provide the best possible care to children with cancer.[216]

Moral and Sociopolitical Influences

The United States spends more on health care than most developed countries yet does not compare favorably with quality indicators of other high-performing health care systems.[217] The signing of the Patient Protection and Affordable Care Act in 2010 has been a catalyst of insurance reform and the introduction of cost reduction strategies within health care institutions. Viewed by some with skepticism and concern, the intent is to improve desired outcomes such as access, quality, equity, efficiency, and overall public health.[217] Although the aftereffects of health care reform on the health and well-being of children with cancer are not yet fully understood, in a setting of scarce resources understanding the multifaceted aspects of social and moral justification for health policy and the relationship to the various dimensions of well-being for children with cancer may be an initial signal of moral urgency for nurses who have a professional obligation to promote good health.[208]

With cancer identified as 1 of the 10 most expensive medical conditions, having generated an annual cost of $124.6 billion in 2010 alone, the National Cancer Institute predicts that costs associated with cancer care will reach $160 billion within the next 10 years as new targeted therapies are introduced.[218] Although much of the published data surrounding the rising costs of cancer care is associated with adult cancer treatment, the incidence of pediatric cancer has increased by 29% during the past 20 years. Long-term childhood survival rate of 79% for all childhood cancers and empirical reports also suggest there is increased complexity associated with childhood cancer treatment when compared with adult cancer.[17,18] The emergence of improved cure rates in pediatric cancer is now highlighting not only the cost of pediatric cancer care but also the long-term costs associated with survivorship. Nurses will need to fully use their unique knowledge, skill, and insight into the human experience developed by the interrelationships that occur with patient and families to facilitate an informed dialogue to influence health policy and reform at a local and national level through involvement in professional organizations such as the American Nurses Association and APHON.

Cancer Treatment and the Roles of Nurses

Current trends toward shorter hospital stays and more outpatient therapy demand that pediatric oncology providers change their focus and pay more attention to evaluating the burden of care that is placed on families and to supporting patients and families through the treatment process.[3,8,159,219] Helping patients and families to strengthen their personal support networks, learn about the disease, understand treatment options, and become knowledgeable about managing symptoms and side effects must continue to be a major focus for nursing and the entire health care team.[50,109,220]

Helping patients adjust to the diagnosis of cancer has become an increasingly important part of pediatric oncology nurses' roles. Research has shown that hope has a powerful influence on a patient's psychological and physiologic defenses.[221] These findings have significant implications for nurses, who work with patients and families to capitalize on their determination, courage, and optimism, helping them to develop a vision of hope, regardless of the child's age or the extent of disease.[221]

In the coming years, nursing's paradigm of professional practice will continue to shift toward a more holistic approach that requires knowledge of integrative therapies.[21,222] Therapies and interventions that strengthen the ability of the body and mind to influence healing are increasingly popular among pediatric oncology patients and families. As noted earlier, studies have found that as much as 85% of children and adolescents have used some form of integrative therapy.[101,102] These figures highlight the idea that developing an understanding of the risks and benefits of combining integrative therapies with traditional medicine is an important consideration for pediatric oncology nurses, now and in the future.[104,223] A committee formed by the Children's Oncology Group has highlighted the role of pediatric oncology nurses in CAM research and has noted that nurses are critical to the success of research in this area.[99]

Patient Safety

In 1979, NASA's Aerospace Human Factors Research Division linked inadequate communication, leadership, coordination, and decision-making skills, rather than pilot error, to most aviation accidents.[224] Since then, many health care organizations have translated this work into training courses designed to maximize teamwork among health care workers.[224] Clinicians participating in these courses have found that learning to communicate effectively and developing an understanding of one another's roles, responsibilities, and skills yields valuable insights and can positively influence the quality of patient care.[36] Such training programs will become increasingly important as organizations search for ways to minimize medical errors and improve the safety of the care environment.

Systems issues and other factors make many of the actions of nurses and physicians especially vulnerable to error. Nurses and physicians routinely make high-consequence decisions based on their knowledge and experience and an understanding of the relationship between patient-specific variables and environmental factors, such as work-flow patterns and the activities and norms of the patient care unit.[225] At times, these decisions are made in the context of factors such as conflicting goals, obstacles, unpredictability, poorly designed technology, constant change, hazards, and missing data that contribute to medical errors or near misses.[225] Maintaining vigilance in analyzing systems of care, identifying factors that contribute to errors or omissions, and introducing sustainable practice changes to promote safe patient care will continue to be a critical part of patient safety efforts.[226-228]

In many organizations, the growing focus on patient safety has impacted the culture of the work environment, shifting it from one that condones silence and blame to one that encourages open disclosure and promoting a team approach to problem solving.[229] Nurses in direct care roles confront patient safety directly and are important members of these problem-solving teams. They will continue to play critical roles in preventing medical errors and promoting safety in the health care environment.[229]

Self-Care

Nurses and physicians alike must find ways to preserve their interest in and passion for the work of caring for others. Although working to save, improve, or prolong the lives of others is rewarding, health care professionals often find that such work entails significant stress. This is particularly true in subspecialty practices like pediatric oncology, where patients are incredibly challenged by their diseases and often face uncertain futures. In addition to the emotional stress that stems from the focus on healing and helping, pediatric oncology providers often encounter workload and system constraints that contribute to chronic secondary stress and may lead to burnout or compassion fatigue.[230]

Understanding the concept of self-care and self-management is a pragmatic consideration for pediatric oncology nurses. Knowing how to renew oneself is critical to maximizing professional effectiveness and satisfaction.[21,22]

Nursing Research

The research training, methods, and rigor of nurse scientists are consistent with those of any other scientific disciplines. Through the lens of professional nurses, research questions spanning the continuum of the cancer trajectory guide oncology nursing care. Based on a holistic model (Fig. 69-1), the needs of the patient and family are addressed using evidence-based strategies that help them cope with the physical and emotional sequelae of the cancer experience.

Nurse scientists practicing in academic medical centers at the juncture of clinical practice and clinical or

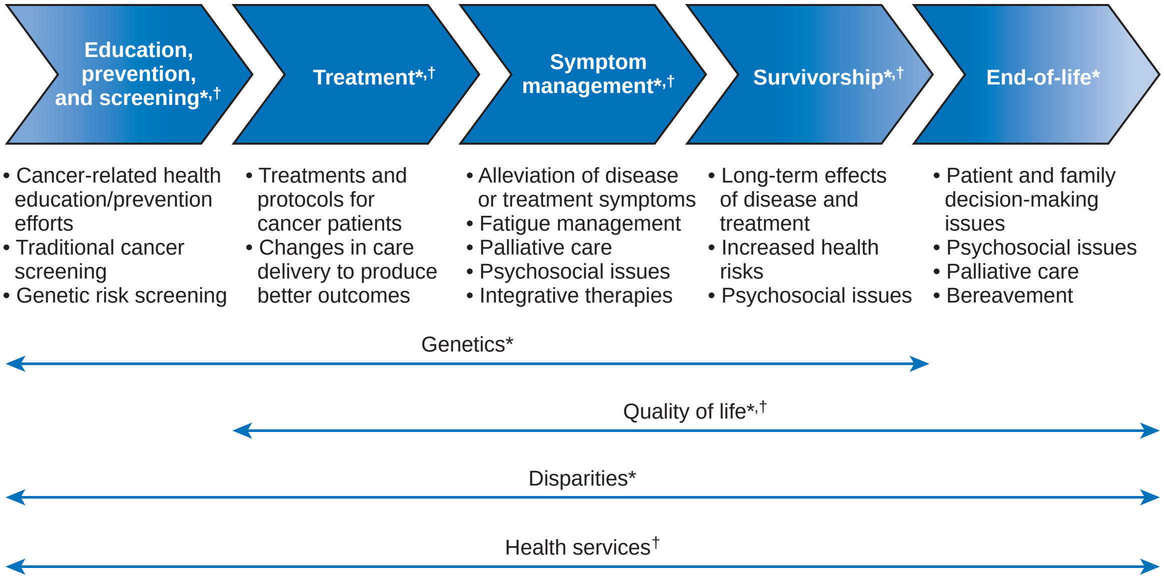
Education, prevention, and screening*,†
Treatment*,†
Symptom management*,†
Survivorship*,†
End-of-life*
• Cancer-related health education/prevention efforts
• Traditional cancer screening
• Genetic risk screening
• Treatments and protocols for cancer patients
• Changes in care delivery to produce better outcomes
• Alleviation of disease or treatment symptoms
• Fatigue management
• Palliative care
• Psychosocial issues
• Integrative therapies
• Long-term effects of disease and treatment
• Increased health risks
• Psychosocial issues
• Patient and family decision-making issues
• Psychosocial issues
• Palliative care
• Bereavement
Genetics*
Quality of life*,†
Disparities*
Health services†

Figure 69-1 A continuum of oncology nursing research. *Research emphasis area of the National Institute of Nursing Research; †Priority area of the Oncology Nursing Society. *(From Bauer-Wu S, Epshtein A: Dana-Farber Cancer Institute, 2007.)*

patient-oriented research have made noteworthy strides in the past decade as nurses have sought to change clinical practice on the basis of existing evidence. This trend toward evidence-based practice is widespread in the nursing community. The past decade has also witnessed a trend toward clinically based nurse-scientists who can bridge the knowledge gap between academia and clinical practice. More doctorally prepared nurses contributing to science will help to support a shift in the research paradigm to one that is multidisciplinary in nature, with nurse-scientists contributing to large multisite studies. This shift means more nurse scientists will have to be trained as members of larger multidisciplinary research teams.

A review of studies conducted by nurse researchers and of surveys among oncology nurses suggests that future nursing research will continue to focus on understanding the full scope of the patient experience and on identifying nursing interventions that ease the hardship of disease for patients and families alike.[231-233] Some of the high-priority areas for future nursing research include the following:

- Understanding issues related to long-term survivorship
- Enhancing patients' quality of life, with a focus on the management of pain and symptoms
- Evaluating and improving care at the end of life
- Evaluating and promoting safe and effective nursing practice in all care settings, including the home
- Understanding families' perspectives to find ways to support patients and families
- Developing new ways to incorporate nursing research into practice and to promote and support interdisciplinary research

These priorities are consistent with the strategic plan and overall mission of the National Institute of Nursing Research (NINR) to advance the science of health by enhancing health promotion and disease prevention; improving quality of life by managing symptoms of acute and chronic illness; improving palliative and end-of-life care; enhancing innovation in science and practice; and developing the next generation of nurse-scientists.[234] They also reflect pediatric oncology nurses' appreciation for the ways in which cancer impacts patients and families and the toll it takes on their physical, psychological, and social well-being. The priorities also exhibit nursing's continued commitment to preventing illness; to alleviating suffering; to protecting, promoting, and restoring health; and to ensuring a comfortable and dignified death and the highest possible quality of life when the restoration of health is no longer possible.

The advances in pediatric oncology that have occurred over the past half century are responsible for a remarkable increase in the number of patients who are cured; however, many patients still face uncertain futures and require ongoing care and support by health care professionals. Although the benefits to patients and families of an interdisciplinary approach to care cannot be underestimated, it is widely acknowledged that pediatric oncology nurses play a vital role in the care of patients and families. The knowledge, skills, and caring practices demonstrated by expert pediatric oncology nurses not only enhance the abilities of patients and families to cope with diagnoses of cancer but also help to reduce the overall burden of the disease.

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8. Forte K: Pediatric oncology nursing: providing care through decades of change. *J Pediatr Oncol Nurs* 18:154–163, 2001.

Pediatric oncology nursing practice has evolved over the years as new technology and trends in health care have necessitated change. Reflections of three pediatric oncology nursing leaders and a review of the literature demonstrate advances in treatment and supportive care.

15. Association of Pediatric Oncology Nurses, American Nurses Association: *Scope and standards of pediatric oncology nursing practice*, Silver Spring, MD, 2007, Nurses Books.

Standards of practice and standards of professional performance are outlined for the pediatric oncology nurse and the pediatric oncology advanced practice registered nurse (APRN). The standards are critical for guiding pediatric oncology nurses in delivering high-quality nursing care.

27. Heiney S, Wiley F: Historical beginnings of a professional nursing organization dedicated to the care of children and adolescents with cancer and their families: the Association of Pediatric Oncology Nursing from 1974–1993. *J Pediatr Oncol Nurs* 13:196–203, 1996.

In 1974, the Association of Pediatric Oncology Nurses (APON) was established based on the philosophy that pediatric oncology nursing is a specialty that requires specific knowledge and expertise in the care of children who have cancer. This distinction was formalized with the development of specialty certification in pediatric oncology nursing in 1993.

34. Curley M: *Synergy: The unique relationship between nurses and patients*, Indianapolis, IN, 2007, Sigma Theta Tau International.

This model describes what nurses do based on levels of expertise and building of optimal nurse-patient relationships. Nurses from all roles and subspecialties can apply this framework to their practice.

38. Ponte PR, Conlin G, Conway J, et al: Making patient-centered care come alive. *J Nurs Admin* 33:82–90, 2003.

The concept of patient-centered care is espoused in many health care organizations, yet implementing this framework has been challenging for many. This article describes how patient-centered care has been implemented within a major cancer center in the United States.

46. Hooke M: Psychosocial issues. In Kline N, Brace-O'Neill J, Hooke M, et al, editors: *Essentials of pediatric oncology nursing: a core curriculum*, ed 2, Chicago, 2004, Association of Pediatric Oncology Nurses, pp 196–227.

Psychosocial issues including care of families, culturally sensitive care, spirituality, school reentry, and professional nurse-patient relationships are discussed with emphasis on pediatric and adolescent and young adult cancer patients.

49. Holm K, Patterson J, Gurney J: Parental involvement and family-centered care in the diagnostic treatment phases of childhood cancer: results from a qualitative study. *J Pediatr Oncol Nurs* 20:301–313, 2003.

This qualitative study describes how parents of children with cancer participate in their child's medical care. Parents emphasized they advocated for their child during treatment, educated themselves about the disease and therapy, and worked with the medical team. Recommendations for practice included implementing a family-centered care approach and inclusion of parents regarding treatment decisions.

57. Mack J, Grier H: The day one talk. *J Clin Oncol* 22:563–566, 2004.

This article describes the salient features of the discussion with parents at the time of their child's cancer diagnosis. Three main features of the talk are emphasized: the cancer diagnosis, treatment and causation.

59. Vaartio-Rajalin H, Leino-Kilpi H: Nurses as patient advocates in oncology care: activities based on literature. *Clin J Oncol Nurs* 15:526–532, 2011.

This literature review found that oncology nurses assessed their patients both physically and psychologically and developed plans of care, starting with the diagnosis. The literature emphasizes the importance of patient education regarding cancer treatment and the informed consent process.

75. Cescon D, Etchells E: Barcoded medication administration: a last line of defense. *JAMA* 299:2200–2202, 2008.

The two processes from which preventable accidental disconnection and exposure most commonly arise are medication prescribing and administration. Barcoded medication administration (BCMA) systems are a last line of defense against medication errors and warrant greater advocacy and implementation.

78. Conley S: Safe handling of chemotherapy and biotherapy agents. In Kline N, Hesselgrave J, O'Hanlon-Curry J, editors: *The pediatric chemotherapy and biotherapy curriculum*, ed 3, Chicago, 2011, Association of Pediatric Oncology Nurses, pp 65–69.

Occupational exposure risks and exposure prevention guidelines including personal protective equipment, preparation and administration guidelines, disposal of chemotherapy, body fluid guidelines, exposure guidelines, spill management, and safe handling in the home are discussed.

108. Fochtman D: The concept of suffering in children and adolescents with cancer. *J Pediatr Oncol Nurs* 23:92–102, 2006.

Individual suffering needs to be defined and measured by self-report (as opposed to parent or staff report) to gain an accurate, complete holistic picture of the nature and scope of the child's and adolescent's suffering. Knowledge of how children and adolescents experience suffering would enable practitioners to design interventions to prevent or ameliorate this suffering.

111. Hockenberry M: Symptom management research in children with cancer. *J Pediatr Oncol Nurs* 21:132–136, 2004.

A review of the literature revealed that children with cancer continue to experience distressing physical symptoms caused by the disease and treatment. The purpose of this article is to provide a concise overview of the most common symptoms experienced by children with cancer including pain, nausea and vomiting, nutritional concerns, mucositis, and fatigue.

121. Kline N: Prevention and treatment of infection. In Baggott C, Kelly K, Fochtman D, et al, editors: *Nursing care of children and adolescents with cancer*, ed 4, Philadelphia, 2011, WB Saunders.

As immunity wanes during cancer treatment children and adolescents become susceptible to infections. A comprehensive review of bacterial, viral, and fungal infections is presented along with prevention and treatment guidelines.

153. Margolis P, Muething S, Brilli J, et al: Quality transformation efforts: decreasing PICU catheter-association bloodstream infections: NACHRI's. *Pediatrics* 125:206, 2010.

The purpose of this multiinstitutional study was to develop and evaluate effective catheter-care practices to reduce pediatric catheter-associated bloodstream infections (CA-BSI). Results suggested that the main reasons for additional reductions in pediatric CA-BSI rates are issues that surround daily maintenance care for central lines, and additional research is needed to define the optimal maintenance bundle to reduce CA-BSIs in children.

154. Secola R, Lewis M, Pike N, et al: "Targeting to zero" in pediatric oncology: a review of central venous catheter–related bloodstream infections. *J Pediatr Oncol Nurs* 29:14–27, 2012.

The purpose of this review of the literature is to summarize existing adult and pediatric data on central venous catheter–related bloodstream infections and explore nursing models of central venous catheter care that may improve pediatric oncology patient outcomes.

193. Hinds P, Kelly P: Helping parents make and survive end of life decisions for their seriously ill child. *Nurs Clin North Am* 3:465–474, 2010.

American parents of chronically ill children prefer to be involved in decision making about their ill child's end-of-life care. Parents report that having access to understandable information about their child's health status influences their ability to participate in end-of-life decisions.

196. Fochtman D: Palliative care. In Baggott C, Fochtman D, Foley G, et al, editors: *Nursing care of children and adolescents with cancer and blood disorders*, Chicago, 2011, Association of Pediatric Oncology Nurses, pp 468–501.

The focus of pediatric palliative care is to enhance the quality of life for the terminally ill child by preventing and alleviating suffering using the skills and knowledge of a specialized care team. Pediatric palliative care focuses on pain and symptom management, information sharing and advance care planning, practical, psychosocial and spiritual support, and coordination of care.

199. American Association of Colleges of Nursing: The End-of-Life Nursing Education Consortium (ELNEC), 2013. Accessed March 1, 2013 at <http://www.aacn.nche.edu/elnec/about/fact-sheet>.

The ELNEC program is a national initiative to provide nurses with training in palliative care. More than 17,500 nurses and other health care providers from all 50 states and 77 additional countries have received ELNEC training.

202. American Nurses Association: Code of Ethics for Nurses with Interpretive Statements, 2010. Accessed January 28, 2013 at <http://www.nursingworld.org/MainMenuCategories/EthicsStandards/CodeofEthicsforNurses/Code-of-Ethics.pdf>.

The code of ethics for nurses outlines the nonnegotiable ethical obligations and duties of individuals entering nursing practice and is an expression of nursing's understanding of its commitment to society.

224. Oriol M: Crew research management. *J Nurs Admin* 36:402–406, 2006.

Lessons from the aviation industry, which long ago acknowledged that most errors were the result of poor communication and coordination rather than individual mistakes, are applied to the health care setting. Examples are given of how health care organizations have successfully adopted aviation's curriculum called "Crew Resource Management," which promotes and reinforces the conscious, learned team behaviors of cooperation, coordination, and sharing.

234. National Institute of Nursing Research: Bringing Science to Life: NINR Strategic Plan, 2011. Accessed February 24, 2013 at <https://www.ninr.nih.gov/sites/www.ninr.nih.gov/files/ninr-strategic-plan-2011.pdf>.

Released in 2011, the NINR strategic plan identifies 25 years of accomplishments in nursing science, specifically those that have the greatest potential to improve the health of the nation. The plan is based on the idea that individuals would benefit from being actively involved in maintaining their own health from prevention of disease and self-management of illness.

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CHAPTER 70

Palliative Care in Pediatric Oncology

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CHAPTER OUTLINE

INTEGRATING PALLIATIVE CARE FROM THE TIME OF DIAGNOSIS
- Advantages of Early Integration of Palliative Care
- Enhancing Integration of Palliative Care When Cure is No Longer Possible

MAKING THE TRANSITION TO PALLIATIVE CARE
- Discussing a Change in Prognosis
- Discussing Goals of Care
- Eliciting Children's Goals
- Creating a Plan for Care Based on Goals
- Appointing a Health Care Proxy
- Resuscitation Status and Other Aspects of Advance Care Planning
- Making Recommendations

COMMUNICATING PLANS FOR CARE
- Talking with Children about Advanced Illness
- Techniques for Communication
- Listening

SPIRITUALITY
- Spirituality in Children
- The Spiritual Assessment

SCHOOL
- Advance Care Planning and the School System
- Grief and Bereavement in the School

INTERDISCIPLINARY CARE

COORDINATION OF CARE

LOCATION OF DEATH
- Preparation for Home Care
- Hospice Services

CANCER-DIRECTED THERAPY

SYMPTOM-DIRECTED THERAPY
- Pain
- Dyspnea
- Airway Obstruction
- Gastrointestinal Symptoms
- Nutrition and Hydration
- Fatigue
- Psychological Symptoms
- Sleep Disturbance
- Anemia and Bleeding
- Fever and Infection
- Central Nervous System Symptoms

WHEN DEATH IS IMMINENT
- Escalating Symptoms
- Palliative Sedation
- Talking with Parents About What to Expect
- Talking About Autopsy
- Care of the Child's Body after Death

AFTER DEATH
- Bereavement Care for Families
- Care of Siblings
- Care of Staff

ONGOING CHALLENGES

More than 2000 children die each year in the United States of cancer-related causes, and many more are living with advanced cancer.[1] In children with cancer, it is not always possible to determine whether the disease will be responsive to cancer-directed therapy, nor is it possible to determine which type of path the dying process will take. Some children may die suddenly and unexpectedly, such as a child undergoing bone marrow transplant who experiences a treatment-related complication. Others may experience a steady and fairly predictable decline, such as the child with a progressive brainstem glioma after radiation therapy. Most children with progressive cancer will experience varying periods of chronic illness punctuated by crises, one of which may prove fatal. An example of this type of path may involve a child with relapsed metastatic neuroblastoma who may be palliated long term before experiencing a life-ending event.

Although intensive, interdisciplinary supportive care, that is, palliative care, is essential for all children with cancer, it is especially critical for children living with

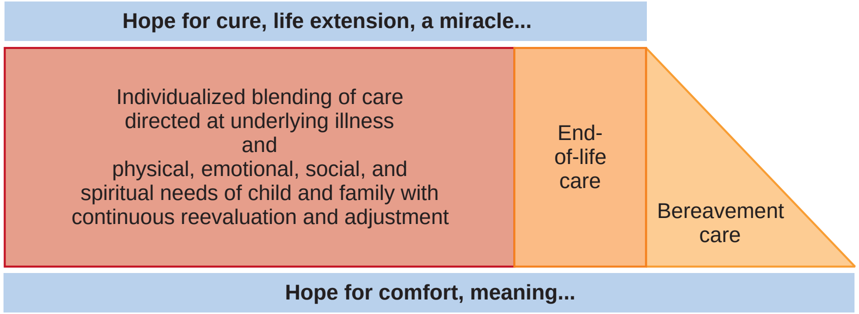
Hope for cure, life extension, a miracle...
Individualized blending of care directed at underlying illness and physical, emotional, social, and spiritual needs of child and family with continuous reevaluation and adjustment
End-of-life care
Bereavement care
Hope for comfort, meaning...

Figure 70-1 Pediatric palliative care includes individualized integration of palliative care principles to ensure combined hopes of varying degrees related to life extension and comfort, both of which can last throughout the child's life. End-of-life care is an important component of palliative care when the focus of care is almost entirely on comfort, although hope for a miracle can persist. Bereavement care needs can be intense and long lasting, gradually lessening over time.

more advanced stages of cancer. Involvement of palliative care services has been shown to improve symptom detection and identification of communication needs among children with advanced cancer,[2] but as of 2008, only 58% of Children's Oncology Group institutions had dedicated palliative care services available, suggesting that there is still room for improvement.[3] Nonetheless in recent years there has been greater awareness and integration of palliative care and, with this, care more consistent with its principles.[4]

Palliative care, as defined by the World Health Organization,[5] is an approach to care that improves the quality of life of patients with life-threatening illness and their families, through the prevention and relief of suffering by means of early identification, assessment, and treatment of pain and other problems, whether physical, psychosocial, or spiritual. The organization's definition of palliative care appropriate for children and their families is as follows:

- Palliative care for children is the active total care of the child's body, mind, and spirit and also involves giving support to the family.
- Palliative care begins when illness is diagnosed and continues regardless of whether a child receives disease-directed treatment.
- Health care providers must evaluate and alleviate a child's physical, psychological, and social distress.
- Effective palliative care requires a broad multidisciplinary approach that includes the family and makes use of available community resources; it can be successfully implemented even if resources are limited.
- Palliative care may be provided in tertiary care facilities, community health centers, and the home.

Hospice and Palliative Medicine (HPM) became a formal physician subspecialty in 2006, and, currently, to become board eligible, a physician needs to complete a 1-year accredited fellowship program. However not all children with cancer require care by a HPM subspecialist or pediatric palliative care (PPC) team. Basic palliative care knowledge, skills, and behaviors should be known to all clinicians who care for children with life-threatening illnesses and conditions. The role of the HPM subspecialist and team is to provide clinical consultation for more complex situations, to provide education and training, and to improve palliative care outcomes for all children and families through quality improvement and research.

In fact both data[6,7] and clinical experience favor a blended approach to care that includes disease-directed treatments together with palliative or comfort care. This approach is most often favored by parents, who commonly hope for life prolongation while also desiring maximal comfort and minimal pain and suffering for their child (Fig. 70-1). Pediatric palliative care should not be a choice between life-prolonging treatments (e.g., chemotherapy for cancer) and palliative care. Rather pediatric palliative care should be integrated into an overall care plan that is individualized and adaptable over changing circumstances.

INTEGRATING PALLIATIVE CARE FROM THE TIME OF DIAGNOSIS

Although palliative care and end-of-life care are often considered to be a single entity, it is important to note that palliative care can be initiated at any time, not just in the end-of-life period. Rather than being restricted to the last phase of life, palliation includes attention to symptoms from the time of diagnosis. Palliative care also allows for discussion of goals and preferences for care long before death occurs.

In addition rather than precluding intensive life-prolonging care, palliative care can be complementary to therapy with curative intent. With the inclusion of palliative care early in the disease course, children can benefit from attention to symptoms and quality of life concurrent with efforts to control disease. Parents often have many goals of care beyond cure or life prolongation. Parents of children who died of cancer, for example, recall holding simultaneous goals of extending life and minimizing suffering during their child's treatment course.[7,8] Concurrent disease-directed care and attention to the physical, psychosocial, and spiritual needs of the child and family may be the optimal approach to meeting these seemingly conflicting goals.

Among some pediatric cancer patients, such as children with pontine gliomas, the pattern of progression is relatively predictable, making planning for the end of life a logical part of care from diagnosis. Patients with other cancers, however, may have long periods of disease control and relative clinical stability. Although ultimately the child may be expected to die of the disease, which relapse or acute decompensation will result in death can be very difficult to predict. Early relapses may be responsive to intensive treatment, and the timing of death may be difficult to predict.

For a child with relapsed neuroblastoma, for instance, many years may elapse between the first recurrence and death. At any moment in time, the use of intensive chemotherapy may seem reasonable and may offer significant hope of life prolongation and remediation of symptoms. However repeated intensive therapies including chemotherapy-related toxicity may not coincide with the family's wishes for the end of the child's life and may be associated with unnecessary suffering for the child. Even when families choose life-extending measures, they should have the opportunity to do so in the context of an understanding of the expected path of illness.

Advantages of Early Integration of Palliative Care

Before the patient is clearly in the last phase of life, physicians may find it difficult to initiate conversations about a poor prognosis.[9] But the majority of parents of children with cancer say they want prognostic information, even when the news is upsetting.[10] Patients and families who are poorly prepared for death tend to choose more intensive disease-directed care at the end of life.[8,11] Without realistic information, they may not have the opportunity to make choices for care that are consistent with their values.

Just as physicians sometimes prefer not to discuss prognosis, pediatricians sometimes consider palliative care to be equivalent to hospice care. As a result they may integrate palliative care concepts such as advanced care planning and anticipatory guidance only once curative options have been exhausted.[12] Yet families may find earlier integration of palliative care to be helpful. A study of bereaved family members of adult cancer patients, for example, found that half believed that palliative care was provided too late in the disease course, whereas less than 5% believed palliative care referrals occurred too early.[13] Physician communication was found to be an important contributing factor; families who believed that the physician had communicated effectively about the patient's prognosis and end-of-life care were less likely to believe that palliative care was introduced too late.

Early initiation of palliative care facilitates attention to symptoms and quality of life. Involvement of palliative care services improves symptom detection and identification of communication needs among children with advanced cancer.[2] Similarly adults with advanced cancer tend to experience improvement in symptoms, including pain, anorexia, nausea, vomiting, sleeplessness, and constipation, after referral to palliative care services. In addition patients who receive palliative care frequently experience improvements in overall quality of life and well-being.[14]

It is also possible that early discussions about palliative care can help parents to cope with the death. Mothers who experience the sudden death of a child tend to have more intense grief than mothers whose children die after a chronic condition.[15] These findings raise the possibility that emotional and psychological adjustment before a child's death can help parents to cope with the loss of the child over time.

Perhaps most importantly parents value preparation for the end-of-life period. In a study of bereaved parents of children with cancer, physician communication about what to expect in the end-of-life care period was a component of high-quality care.[16] Parents who felt prepared for the circumstances surrounding the child's death were also more likely to consider care to be of high quality overall. Communication with parents in advance of the child's death may allow them to process and be prepared for the death before it happens and to make choices about end-of-life care that are right for them and their child.

Box 70-1 gives some general guidance for discussion about end-of-life preferences.[17,18] Even if the patient's outcome is uncertain, death can be discussed as a possibility so that the patient has opportunities to express goals for care.

Enhancing Integration of Palliative Care When Cure is No Longer Possible

Although integration of palliative care from the time of diagnosis is often ideal, sometimes a clinical event such as relapse or a severe complication makes it necessary to discuss a poor prognosis for the first time. A transition from care intended to cure the child can be experienced as a terrible loss for families. However as with discussions about care at the time of diagnosis, honest communication about the child's prognosis can help the family make reasonable decisions about care, based on their values.[19]

Delays in parental understanding of a poor prognosis can affect the care they choose for their children. In a study of parents of children who died of cancer, parents recalled understanding that the child was likely to die about 3 and one half months after the physician realized this. Earlier parental realization of the low likelihood of cure was associated with increased hospice use and decreased cancer-directed therapy in the end-of-life care period.[8] Although parents sometimes have difficulty accepting a poor prognosis, the difficulty physicians have in communicating a poor prognosis also contributes to parents' overly optimistic expectations about cure.[20]

MAKING THE TRANSITION TO PALLIATIVE CARE

Discussing a Change in Prognosis

A statement that physicians can use when introducing this discussion with parents is, "Although I had hoped that your child's disease would be cured and your child could live a long life, I am now worried that this is no longer possible."[18] Acknowledging the sadness of the situation, responding to the parent's emotions, and allowing for silence can also be helpful. Parents may ask about treatment options, but such discussion should not obscure the fact that the child's disease is unlikely to be cured.

Box 70-1 **Communicating with Children and Families About Integrating Palliative Care**

BEGINNING THE CONVERSATION

"What is your understanding of what is ahead for your child?"

"Would it be helpful to talk about how his or her disease may affect him or her in the months and years ahead?"

"As you think about what is ahead for your child, what would you like to talk about with me? What information can I give you that would be helpful to you?"

INTRODUCING THE POSSIBILITY OF DEATH

"I am hoping that we will be able to control the disease, but I am worried that this time we may not be successful."

"Although we do not know for certain what will happen for your child, I do not expect that your child will live a long and healthy life; most children with this disease eventually die because of the disease."

"I have been noticing that your child seems to be sick more and more often. I have been hoping that we would be able to make him or her better, but I am worried that his or her illness has become more difficult to control and that soon we will not be able to help him or her to get over these illnesses. If that is the case, he or she could die of his or her disease."

ELICITING GOALS OF CARE

"As you think about your child's illness, what are your hopes?"

"As you think about your child's illness, what are your worries?"

"As you think about your child's illness, what is most important to you right now?"

"You mentioned that what is most important to you is that your child be cured of his or her disease. I am hoping for that, too. But I would also like to know more about your hopes and goals for your child's care if the time comes when a cure isn't possible."

INTRODUCING PALLIATION

"Although I hope that we can control your child's disease as long as possible, at the same time I am hoping that he or she feels as good as possible each day."

"Although it is unlikely that this treatment will cure your child's disease, it may help him or her to feel better and possibly to live longer."

TALKING ABOUT WHAT TO EXPECT

"Would it be helpful to talk about what to expect as your child's illness gets worse?"

"Although we cannot predict exactly what will happen to your child, most children with this disease eventually have [difficulty breathing]. If that happens to your child, our goal will be to help him or her feel as comfortable as possible. We can use medications to help control his or her discomfort."

TALKING TO CHILDREN

"What are you looking forward to most of all?"

"Is there anything that is worrying you or making you feel afraid?"

"Is there anything about how you are feeling that is making you feel worried or afraid?"

From Mack JW, Wolfe J: Early integration of pediatric palliative care: for some children, palliative care starts at diagnosis. Curr Opin Pediatr *18:10–14, 2006.*

The physician might respond to such requests by saying, for instance, "It is possible that treatment might help your child to feel better and possibly live longer; the hope would be for the treatment to control the cancer for as long as possible." Although these conversations are difficult for families and for medical providers, such honesty can help parents to make the best possible decisions for their child. In addition although honest provision of information is important, physicians may be able to clarify the goals of care without needing to have parents necessarily openly acknowledge that cure is not likely; this approach may be preferable to some parents.

Discussing Goals of Care

An important aspect of providing care for children with cancer is an understanding of the goals of care. Often these goals are identified by the parents, but adolescents and even younger children are often able to define priorities for their care as well.[21] Understanding goals of care allows the medical team to make recommendations for care that fit with patient and family values. In addition goals of care, and conversations about these goals, provide another window into parents' perceptions of what is ahead.

A discussion about goals of care can be started by asking parents what is most important to them as they think about what is ahead for the child.[18] Specific questions about hopes for the future and worries about what is ahead can also be useful. Often parents will verbalize general goals, such as maintaining a good quality of life for as long as possible, minimizing suffering, prolonging life, or curing the child's cancer. Parents often hold more than one goal and may have separate goals for cancer-directed and symptom-directed therapy; for example, goals of cancer-directed therapy may be to cure or to prolong life, whereas goals of symptom-directed therapy may be to lessen suffering and maximize quality of life.[8,7] Although such goals may appear to be conflicting, holding more than one goal of care is normal, and physicians can make plans for care with both sets of goals in mind.

Acceptance of the parents' goals is an important aspect of acknowledging the parents' values, even when the goals are not in concert with the physicians' goals. At times, however, parents will continue to articulate a goal of cure, when the medical team believes that such an outcome is not possible. Understanding what the parent would want if the child cannot be cured can help the medical team to make plans for care. Back and colleagues[22] have recommended that the physician ask what the family would want if cure is not possible, a strategy described as hoping for the best but preparing for the worst. This strategy allows the medical team to have a conversation about what the family would want in such a situation.

Even when parents hold goals of cure, physicians need to have clear discussions about the goals of treatment, especially cancer-directed treatment. Depending on the circumstances, the oncologist may want to express goals of controlling the disease for as long as possible, for example. When the purpose of treatment is to help cancer research, with little hope of direct benefit to the child, that goal also should be stated.

In addition to the specific nature of goals of care, parents also consider a number of other values while they make these difficult decisions. For example parents sometimes think about what it means to be a good parent to their child as one compass for decision making.[23] Parents may believe, for instance, that a good parent would focus on preserving the child's life, would make unselfish decisions in the child's best interest, or show the child that he or she is loved. In addition to helping parents clarify their own wishes, having conversations about the meaning of the good parent role can help clinicians to reinforce that role as the child's illness progresses.

Similarly parents may use phrases to sum up the things that are most important to them: "I want the best for my child;" "I have to fight;" "I want my child to have quality of life."[24] By listening for and probing these phrases, clinicians partner with parents in clarifying their goals and making decisions. Ultimately these conversations about parents' values allow clinicians and the parents themselves to recognize their values, enact them in the child's care, and work together during these very difficult times.

Eliciting Children's Goals

Children and adolescents may express goals in general terms, like their parents, or they may have very specific wishes, such as staying at home, spending time with siblings, or minimizing invasive procedures.[21,25,26,27] Knowing about these goals, which can be elicited by asking children for their hopes and worries, and what is most important to them when they think about what is ahead, can allow the medical team to work toward meeting any specific hopes or needs.

Creating a Plan for Care Based on Goals

Once goals of care have been elicited, then a plan of care can be made. It is important to acknowledge that this plan is flexible and may change frequently. Importantly each family is unique, and even with similar goals there may be very different plans of care. As previously mentioned, pediatric palliative care should be integrated while a family is seeking a life-prolonging or potentially curative therapy. Therefore a plan of care may include preventative therapies, looking for life-prolonging or curative therapies, rehabilitation, and intensive quality of life and symptom management.[28] Having parents consider these questions may help them with decision making.

- How will this treatment affect my child's quality of life?
- What is likely to happen without this treatment?
- How will the treatment change my child's prognosis?
- What will it be like for my child (and family) to go through this treatment?
- Are there other options we should consider that might have the same outcome but with a different approach?

Particularly as cure becomes less likely, helping parents to think deeply about what brings a child joy or which experiences provide quality of life may help their decision making. Ideally the child will be included in these decisions and plan of care. In one study, children aged 8 to 17 years wanted to be told if they were dying and adolescents in particular wanted to participate in palliative care decisions.[29]

Appointing a Health Care Proxy

For patients older than 18 years, the decision of whom to appoint as a health care proxy may provide an opportunity for discussion of care preferences. This document, often completed on admission to the hospital, allows the patient to designate someone to make medical decisions if the patient is unable to do so. The proxy ideally makes decisions based on knowledge of what the patient would want in various situations and not on what the proxy would want or believe to be appropriate. Most hospitals have standard forms that are completed by the patient and an admissions staff person. This could be an opportunity to explore the true wishes of the young adult patient and lead to rich discussion and meaningful decision making.

Resuscitation Status and Other Aspects of Advance Care Planning

Advance care planning may include (1) designation of a decision maker or, for those older than 18 years old, choosing a health care proxy; (2) discussion of the illness and prognosis; (3) establishment of goals of care; (4) discussion of preferences for resuscitation, artificial nutrition and hydration, and palliative sedation; (5) primary location of care and death; and (6) discussion about organ or tissue donation, including autopsy, and preferences for funeral arrangements.[30] Advanced care planning is a process that requires time and often occurs through multiple conversations and at different phases of the child's illness.

Of note many clinicians believe that these conversations happen later than they should.[31] Clinicians cite multiple barriers to discussions about advance care planning, especially unrealistic parent expectations about the child's illness, differences between clinicians' and parents' understanding of prognosis, and a lack of parent readiness to have these conversations. Clinician perceptions about parent understanding or readiness, however, should not preclude having these conversations. Physicians also report that they often do not know what to say.[31]

Fortunately several resources are available to assist in these discussions. *Five Wishes* (for those >18 years) (available at www.agingwithdignity.org) is a legal document in 37 states. This document presents information and questions in a way that enhances communication about these difficult subjects, and it addresses what kinds of nonmedical interventions would be wanted, such as music, prayer, presence of family, and so on. *My Wishes* (for those <18 years) (also available at www.agingwithdignity.org) is not a legal document, but through simple questions and the opportunity to write and draw it affords the child or adolescent the opportunity to communicate his or her wishes. In addition *Voicing My Choices* has been recently developed for adolescents and young adults aged 16 to 28, using materials from *Five Wishes* and modified after interviews with adolescent and young adult patients with cancer or who are infected with human immunodeficiency virus.[26] This work involves four priority areas, which can serve as a model for younger patients as well: the ability to express and document preferences for (1) the medical treatment they want and the treatment they do not want; (2) how they would like to be cared for; (3) information they want family and friends to know; and (4) how they want to be remembered.

Other techniques may be helpful, such as asking an adolescent to name his or her wishes, realistic or not. The provider may also ask a question such as, “What is your understanding of your illness now?” Another technique is to suggest that the adolescent write, with a trusted person, such as a child life specialist, psychosocial clinician, or parent, what questions he or she would like to ask of the health care team. Ideally advance care planning among adolescents is family centered, with the family and adolescent both taking part in shaping wishes for care, in an effort to develop a plan for care that all can support.[32] Doing so can allow families to understand their adolescent’s wishes and ultimately carry out these wishes.

Making Recommendations

Even when curative therapies are no longer sought, families report wanting recommendations regarding available interventions. Most do not want the burden of making decisions completely on their own. Providers may believe that, especially when there are treatments with uncertain or marginal benefit, plans for care should be the parents’ choice. However parents can often be best supported by providers who clearly discuss the positives and negatives of each treatment or therapy and then recommend a treatment in the context of the family’s goals and values. This can apply to a wide spectrum of situations such as participating in a phase I or II clinical treatment trial, putting in a chest tube for pleural effusions, using intravenous antibiotics to treat an infection, or instituting a do not resuscitate (DNR) order. Clinicians who make recommendations can alleviate this burden from the parents and help to minimize the parents’ sense of regret and responsibility for choices that ultimately allow death to occur.

COMMUNICATING PLANS FOR CARE

A plan of care must then be communicated to all involved in the child’s care. Consistency and honesty are among what bereaved parents tell us is most important.[33] Written documentation of team meetings, family meetings, and the care plan is essential. Documentation can also help to communicate plans of care with other providers, such as the home care team or those providing emergency services. Along with a home DNR or comfort care form, for instance, a letter that documents the child’s situation and plans for care can be carried by the parents to help ensure that any care providers they encounter know their goals for care. By partnering with the child and family, the health care team in the hospital or home has the opportunity to enhance quality of life while the child is living and affect the grieving and bereavement that will follow for the family.

Talking with Children about Advanced Illness

To tell or not to tell? This is the question that typically arises when parents and health care providers of children with life-threatening illnesses face the issue of discussing prognosis with children. Health care providers have reported a wide range (10% to 80%, with a median 45% prevalence) of open discussions among parents and children regarding the child’s impending death.[34] Of a sample of 429 parents in Sweden, 34% discussed death with their child, and none of those parents regretted having this discussion. When children were given the information that their illness was terminal, most (63%) received it from the parent and physician together.[35] In addition, several studies have shown that children as young as 6 to 10 years old are willing to participate in end-of-life decision making.[21,36] Parents’ and health care providers’ distress in navigating these communication processes (especially regarding death) may prevent children and families from accessing appropriate and timely pediatric palliative care.[37]

Parents may be reluctant to discuss death with their child because of the understandable worry that such a conversation may be very upsetting to the child. However children may have some understanding that death is likely but, because they sense the parents’ distress, may feel unable to discuss this topic with a parent. This choice often stems from concern for the parent’s well-being. Providing the child with an opportunity to talk, however, can be therapeutic for the child. We therefore often encourage parents to allow for such an opportunity. For parents to initiate discussion, they need to be supported for their efforts in broaching and carrying out these difficult conversations with their child.[38] When parents were asked how they best could be supported during end-of-life care of their child, they responded by saying that respect for the family’s role, comfort, spiritual care, access to care and resources, communication, support for parental decision making, and caring/humanism were central.[39]

By describing to parents a nonthreatening mode of communication when carrying out discussions with their child, centered on exploring what the child knows and what the child may be worrying about and hoping for in the time ahead, we can often reassure parents that this conversation will be sensitive and guided by the needs of the child.

The needs and wishes of the individual child should guide how communication takes place and its content. A child’s age and level of cognitive development as well as the changing comprehension of death by age can help shape the appropriate conversations regarding the prognosis, treatment choices, and end-of-life decisions; however, all this information cannot necessarily predict what a child understands about death.[40] Furthermore depending on the disease (e.g., brain tumor), the act of communication may be functionally difficult on the part of the child.[41] Thus careful assessment of a child’s physical abilities, maturity, level of comprehension, and coping abilities rests on the family and health care team. Himelstein[30] explains appropriate communication interventions (i.e., language, details, expression, participation) based on age (Table 70-1). Chesler and colleagues found that the age at which the child was diagnosed with cancer was strongly and significantly related to the amount of information the parents provided about the illness: the older the child, the more information was given.[42]

TABLE 70-1 Development of Death Concepts and Spirituality in Children

Age Range	Characteristics	Predominant Concepts of Death	Spiritual Development	Interventions
0-2 yr	Has sensory and motor relationship with environment. Has limited language skills. Achieves object permanence. May sense that something is wrong.	None	Faith reflects trust and hope in others. Need for sense of self-worth and love.	Provide maximal physical comfort, familiar persons, transitional objects (favorite toys), and consistency. Use simple physical communication.
>2-6 yr	Uses magical and animistic thinking. Is egocentric. Has thinking that is irreversible. Engages in symbolic play. Is developing language skills.	Believes death is temporary and reversible, like sleep. Does not personalize death. Believes death can be caused by thoughts.	Faith is magical and imaginative. Participation in ritual becomes important. The need for courage is important.	Minimize separation from parents. Correct perceptions of illness as punishment. Evaluate for sense of guilt and assuage if present. Use precise language (dying, dead).
>6-12 yr	Has concrete thoughts.	Develops adult concepts of death. Understands that death can be personal. Is interested in physiology and details of death.	Faith concerns right and wrong. Eternal interpretations may be accepted as the truth. Ritual is connected with personal identity.	Evaluate child's fears of abandonment. Be truthful. Provide concrete details if requested. Support child's efforts to achieve control and mastery. Maintain access to peers. Allow child to participate in decision making.
>12-18 yr	Has generality of thinking. Sees reality as objective. Is capable of self-reflection. Sees body image and self-esteem as paramount.	Explores nonphysical explanations of death.	Internal interpretations start to be accepted as the truth. Relationship with God or higher power evolves. Meaning, purpose, hope, and the value of life are sought.	Reinforce child's self-esteem. Allow child privacy. Promote child's independence. Promote access to peers. Be truthful. Allow child to participate in decision making.

From Himmelstein BP, Hilden JM, Boldt AM, et al: Pediatric palliative care. N Engl J Med *350:1752–1762, 2004.*

However, in another study that retrospectively collected information regarding psychosocial variables on 45 pediatric oncology patients who died, it was found that children between the ages of 3 to 6 years had more information about their own death from their parents than older children between the ages of 7 to 11 years. The investigators concluded that perhaps children in the younger age group had a "magical concept" of death, thus making it easier for caregivers to discuss end of life with these younger children.[43] Hurwitz and colleagues provide questions and statements children raise about dying at various ages, thoughts that guide the behavior, their underlying developmental understanding of death, and strategies and responses that can help children in these situations (Table 70-2).

Assessing the child's level of autonomy, perception of threat and safety, and individual coping style can help the clinician determine how to communicate with the child regarding palliative care. Independence and individuation will vary. For example, some adolescents, especially those who have close relationships with their parents, may not make a lot of the day-to-day decisions on their own and depend on their parents' authority and life experience for critical decision making. Other adolescents may be accustomed to "heading" the household and making many of the daily decisions for a family.[44]

Understanding the child's family history with regard to illness and experience with death are important components to consider when broaching sensitive issues within palliative care. A child who has previously witnessed a relative die or suffer from a serious illness and observed his or her family's grief may have unique needs related to this experience. Children who are accustomed to making decisions along with their siblings may request to have siblings present during important decision-making events.

Understanding whether the child has a current and/or premorbid history of anxiety and/or depression is also critical in how information is delivered during advanced care planning discussions.[45]

TABLE 70-2 Statements About Dying at Various Stages of Development

Examples of Questions and Statements About Dying	Things That Guide Behavior	Developmental Understanding of Dying	Strategies and Responses to Questions and Statements About Dying
1-3 Years			
“Mommy, after I die, how long will it be before I’m alive again?” “Daddy, will you still tickle me while I’m dead?”	Understanding of accidental events, of future and past time, and of the difference between living and not living is limited.	Death is often viewed as continuous with life. Life and death are often considered alternate states, like being awake and being asleep, or coming and going.	Maximize physical comfort, familiar persons, and favorite toys. Be consistent. Use simple physical contact and communication to satisfy the child’s need for sense of self-worth and love. “I will always love you.” “You are my wonderful child, and I will always find a way to tickle you.”
3-5 Years			
“I have been a bad boy, so I have to die.” “I hope the food is good in heaven.”	Concepts are crude and irreversible. The child may not distinguish between reality and fantasy. Perceptions dominate judgment.	The child sees death as temporary and reversible and not necessarily universal (only old people die). Because of their egocentricity, the child often believes that he or she has somehow caused the death or views it as a punishment. Death is like an external force that can get you and may be personified (e.g., the bogeyman).	Correct the child’s perception of illness as punishment. Maximize the child’s time with his or her parents. A child of this age may be concerned about how the family will function without him or her. Help parents accept and appreciate the openness of these discussions. Reassure the child and help parents lessen guilt that the child may feel about leaving by using honest and precise language. “When you die, we will always miss you, but we will know that you are with us and that you are in a safe, wonderful place” (perhaps with another loved one who has died).
5-10 Years			
“How will I die? Will it hurt? Is dying scary?”	The child begins to demonstrate organized, logical thought. Thinking becomes less egocentric. The child begins to problem-solve concretely, reason logically, and organize thoughts coherently. However he or she has limited abstract reasoning.	The child begins to understand death as real and permanent. Death means that your heart stops, your blood does not circulate, and you do not breathe. It may be viewed as a violent event. The child may not accept that death could happen to himself or herself or to anyone he or she knows but starts to realize that people he or she knows will die.	Be honest and provide specific details if they are requested. Help and support the child’s need for control. Permit and encourage the child’s participation in decision making: “We will work together to help you feel comfortable. It is very important that you let us know how you are feeling and what you need. We will always be with you, so you do not need to feel afraid.”
10-13 Years			
“I’m afraid if I die my mom will just break down. I’m worried that when I die, I’ll miss my family or forget them or something.”	Thinking becomes more abstract, incorporating the principles of formal logic. The ability to generate abstract propositions, multiple hypotheses, and their possible outcomes becomes apparent.	The child begins to understand death as real, final, and universal. It could happen to him or her or family members. The biologic aspects of illness and death and details of the funeral may begin to interest the child. The child may see death as a punishment for poor behavior. The child may worry about who will care for him or her if a parent or caregiver dies. He or she needs reassurance that he or she will continue to be cared for and loved.	Help reinforce the adolescent’s self-esteem, sense of worth, and self-respect. Allow and respect the adolescent’s need for privacy, but maintain his or her access to friends and peers. Tolerate the teenager’s need to express strong emotions and feelings. Support the need for independence, and permit and encourage participation in decision making. Although I will miss you, you will always be with me and I will rely on your presence in me to give me strength.”

TABLE 70-2 **Statements About Dying at Various Stages of Development** (Continued)

Examples of Questions and Statements About Dying	Things That Guide Behavior	Developmental Understanding of Dying	Strategies and Responses to Questions and Statements About Dying
14-18 Years			
"This is so unfair!" "I cannot believe how awful this cancer has made me look." "I just need to be alone!" "I can't believe I'm dying. … What did I do wrong?"	Thinking becomes more abstract. Adolescence is marked by risk-taking behavior that seems to deny the teenager's own mortality. At this stage, the teenager needs someone to use as a sounding board for his or her emotions.	A more mature and adult understanding of death develops. Death may be viewed as an enemy that can be fought against. Thus dying may be viewed by the teenager as a failure, as giving up.	"I can't imagine how you must be feeling. You need to know that despite it all, you are doing an incredible job of handling all of this. I'd like to hear more about what you are hoping for and what you are worrying about."

From Hurwitz CA, Duncan J, Wolfe J: Caring for the child with cancer at the close of life: "there are people who make it, and I'm hoping I'm one of them." JAMA *292:2141–2149, 2004.*

Within the framework of the American culture is the diversity of the various ethnic and religious cultural subgroups that utilize a range of guiding principles with regard to illness and truth-telling. It is critical to assess the family's identification with their particular culture and inquire what their beliefs are with regard to communication with children about issues pertaining to advanced care,[46] because this may vary from the protective approach (shielding young patients and siblings from full knowledge of the disease) to a more open approach.[42,47] Although every family's style of disclosing sensitive information may be different for a variety of reasons, health care providers should consider how parents may feel if they do not prepare their child for impending death, because this has been shown to have long-term implications for parental grief.[48]

Techniques for Communication

In many instances communicating with children regarding palliative care issues is best received when healthcare professionals are aware of the child's level of desired information and concurrently reinforce the primary role of parents.[49] Children between the ages of 8 and 17 years who were interviewed about disclosure regarding their diagnosis and prognosis reported a range of views about the form of disclosure they preferred: a few believed it was better to hear the news at the same time as their parents, some thought it was more appropriate for their parents to be told first, and others reported no strong feelings either way.[50] Patients reported the roles their parents performed in facilitating communication with their health care team: asking questions for them, being the source of information, and reframing the information from the health care team so they could understand it better. Some children reported feeling "protected" by the parent's executive role in screening the communication with doctors and "marginalized" by the nonparticipant status.[49] Parents of cancer survivors indicated that the shielding of information that took place (i.e., preference regarding whether to provide information to their child about prognosis and treatment options) depended on their child's age and emotionality.[51]

The range of views reported by pediatric patients exemplifies the necessity for tailoring one's approach of communication to the individual child. The most beneficial method for determining the most appropriate way to communicate with a particular child is by spending time with him or her. A provider who knows the child well may be best suited for this conversation whenever possible. Prior to the delivery of information, it is helpful to uncover what the child already knows. This can be done through direct conversation or through nonverbal communication (e.g., drawing and symbolic play). Storytelling, such as using the experience of another family with a child or a loved one facing death, may be another modality that can be used by parents and health care providers to broach sensitive topics related to death.[52] Ensuring privacy, confidentiality, and support can facilitate the trust that is needed for the child to disclose what it is that he or she already knows. The next step is finding out what the child wants to know. Then the clinician can give information in small amounts, responding to the child's reactions and feelings; and can remind the child that there will be additional opportunities to discuss the issues of concern.[40]

Listening

Listening to a child cannot be underrated nor underutilized. Because a child confronting a life-threatening illness is at his or her most vulnerable, listening to the child can be a very powerful tool in placing some of the control back in the hands of the child.[53] Allowing moments for silence in the discussion only reinforces this acknowledgement that the clinician is listening.[53] Providing a child with a tape recorder can be another method for empowering the child. This allows the child to record his or her questions for the upcoming appointment with the clinician, and also allows them to record the clinician's answers so they can be reviewed if necessary.[54] Asking about and listening to the child's priorities and providing choices, when possible, may allow the child to participate in "life-enhancing experiences even at the close of life."[55(p 746)] Listening to the voice of the parent(s) goes hand-in-hand with listening to the child to provide comprehensive family-centered care; it can provide helpful information about the child and make the health care provider a better listener for the child. Listening to the parent without the presence of the child can also be a valuable tool in certain instances when the child's presence may restrict communication.[56]

SPIRITUALITY

Spirituality can be regarded as a multidimensional (mind, body, spirit), unique expression that brings individuals hope, meaning, and purpose.[57] Spirituality is related to, but different from, religion, such that "spirituality is a dynamic state of being in which the individual seeks connectedness, whereas, religion represents the beliefs, values, practices, and rituals that are an observable aspect of a person's spirituality."[58(p 56)] Spirituality is often "inexpressible" whereas religion is marked by greater structure.[57] A child can have a "highly developed awareness of spiritual concepts without ever having been part of a formal religious group."[59(p 271)]

Spirituality in Children

Similar to the development of cognitive abilities, children display a developing awareness of spiritual needs, meaning, or purpose within their lives at various ages. During infancy, children find meaning in their daily life through unconditional love and trust. Toddlers desire self-assertion, worthiness, and success when performing new skills. Preschoolers and school-aged children tend to derive their satisfaction from learning concepts of right/wrong, impulse control, and the benefits of peer socialization. Adolescents find purpose in their developing independence, individuality, and a deeper relationship with a higher being.[59] For a child with a life-threatening illness, spiritual concerns often include a need for unconditional love, forgiveness, hope, safety and security, and development of a legacy. Children also often experience loneliness and loss of wholeness.[60] It can be helpful for children of all ages to be assured that they bring meaning to their families lives and that when they are no longer alive they will be remembered by their family.[57,61]

Children, in general, more than adults, tend to attribute an illness to "internal" (personal) factors, as opposed to "external" (environmental) factors. Children younger than the age of 7 years, who have yet to develop the ability to think abstractly, often see illness as a form of punishment for something they have done wrong (immanent justice belief).[58] Although some older children may attribute the illness to internal causes to maintain control, others are able to attribute the illness to external causes but also seek out the meaning and purpose of their illness—a task that is very often spiritual.[58]

The Spiritual Assessment

Because children tend to reflect the religious and spiritual values of their parents and family, a family-centered assessment of spirituality is often helpful. Providing spiritual care for parents can prepare them for the questions children ask, such as, "Why is God doing this?" or "What is heaven like"? Parents need assurance that there is no specific "right answer."[62]

One method for conducting a spiritual assessment is through the use of the B-E-L-I-E-F questions:[63]

*B*elief system (e.g., Does your family take part in religious events/rituals? Have you discussed the idea of afterlife with your child? Do you believe in a higher power that influences your life?)

*E*thics or values (e.g., What are the values you believe are most central to your family?)

*L*ifestyle (e.g., Are there rituals or dietary practices that you/your family follow?)

*I*nvolvement in a spiritual community (e.g., Are you involved with a spiritual community?)

*E*ducation (e.g., Do you/your family receive or have received religious education?)

*F*uture events (In the near future, are there any religious rites of passage set to occur for you or your family members? What role would you like the members of your faith community to play in the spiritual care of your child?).

However most spiritual counselors and chaplains discourage the use of a "checklist format" in assessing spirituality. Instead questions could be posed to the family and/or to the child in a conversation format depending on the situation.[59] Clinically effective pediatric spiritual assessments are conducted in narrative form and may use words, pictures, music, and play, thus inviting a child to explain his or her sources of strength and areas of fear. Discussions with children and their families regarding spirituality early on in the disease course can help bring meaning to what the family is enduring and can provide hope and comfort at end of life.[60] Assessing children's developmental understanding of faith using Fowler's stages of faith[64] can be helpful in knowing how to lead these spiritual conversations with children directly and how to guide parents in doing so:

Stage 1: Intuitive Projective Faith (2-7 years) is when children's thinking is filled with fantasy and notions of faith are based on stories usually told by parents and other caretakers.

Stage 2: Mythic-Literal Faith (7-12 years) incorporates children's knowledge of cause-and-effect relationships into separating fact from fantasy when constructing an understanding of faith and their religious expression.

Stage 3: Synthetic-Conventional Faith (12-21 years) is when adolescents are able to use abstract reasoning skills to develop their own spiritual identity by separating out what has been told to them and creating new connections between faith-based stories and their own beliefs.

Facing obstacles, challenges, and adversity in life often engenders spiritual thoughts and prompts adults and children alike to ask questions of a higher power, such as, "why me?" "why my child?" It is natural that spirituality is a significant issue for families of children with cancer.[57,65] Pastoral care providers at children's hospitals estimate that more than half of child patients can benefit from spiritual care for fear, anxiety, coping with pain, and familial difficulties.[66] Providers in this study identified three major barriers to providing spiritual care: "inadequate training of health care providers to detect patients' spiritual needs, inadequate staffing of the pastoral care office, and being called to visit with patients and families too late to provide all the spiritual care which could have been provided."[67(p e70)] When the focus of clinical care is on "the cure" for children, treatment discussions focus on medical facts and issues centered on resolving the "problem" with medications or therapies; spirituality, in palliative care, by contrast, is more about a journey of meaning, where there may be no clear solution.[57] Providing spiritual care to dying children and families requires the professional to face his or her own issues with spirituality, something not all individuals have done or are ready to do.[57] Children's spirituality tends to be overlooked and, as a result, children may be more likely to feel unheard, invalidated, and alone in the end-of-life period.[68] A focus on spiritual beliefs during the end-of-life period is not only critical to the dying patient but also can have implications for bereavement outcomes, such that people who profess stronger spiritual beliefs may experience a more rapid and complete sense of closure after the death of a loved one.[69]

SCHOOL

Going to school provides multiple benefits for the school-aged child with cancer. It is the child's peer group; it is the child's chance for discovery, mastery, and socialization.[70] Most children want to continue with their class unless they have not been well supported or they feel unable to participate. With the family and child's permission, creative solutions for participation can allow the child to remain in school often until just days before death. Preparation of school staff and classmates is important, as is a willingness on the family's part to allow for candid information about the child's illness and treatment course.

Ideally this information is shared soon after a child is diagnosed. Often hospital or clinic staff will travel to the child's school and provide information through a "back to school" program. The question frequently arises during these sessions as to whether the child/friend/peer will survive. Most school-aged children have known an adult with cancer, some who have survived and some who have not. Addressing this question in a hopeful way while acknowledging the uncertainty is essential. In all instances if the child dies, it is important for schools and communities to have a plan about ways to communicate this information and support classmates' questions, fears, and feelings.

One possible disturbing aspect of school attendance by the ill child may be the change in the child's physical condition. Reassuring classmates of the child's unchanging personhood may be necessary. Explaining absences, altered appearance, and altered ability to participate in regular class activities may also be helpful.

For the ill child to attend school, school staff and medical providers may need to be creative. Perhaps the child is allowed to attend for half days or just for lunch or on special activity days. Even limited time can be meaningful for the child and his or her peers. An individualized education plan (IEP) tailored to the needs of the child can also allow resources to be mobilized. An ill child should be able to receive home or hospital schooling, and some may be able to complete computer courses or stay in touch with the classroom via teleconferencing.

Advance Care Planning and the School System

As a child's disease progresses, the family and medical team may decide together that a DNR order should be in effect. This can present challenges as to how to honor this within a child's community. Many states are in the process of adopting medical orders for life-sustaining treatment[71] to provide consistent communication regarding advanced care decisions for loved ones no matter what the location of care. However most school districts do not have guidelines or policies to deal with this kind of situation, and those that do have policies typically prohibit school staff from honoring an order that would limit attempts on resuscitation.[72] Nonetheless ethical principles of beneficence, nonmaleficence, and autonomy have been offered as justification for honoring limits on attempts at resuscitation for the student.[72,73] At this point each child, family, and school will need to come to some clear agreement of whether abiding by medical orders for life-sustaining treatment would be observed if the child was at school during a life-ending event.[74-76]

Grief and Bereavement in the School

Finally anticipatory grief and bereavement needs of classmates and school staff must be considered. Resources are available (e.g., www.dougy.org); however affording the training and opportunities for staff to integrate this into their knowledge and experiential repertoire may be challenging. It may be helpful for the oncology clinicians who helped treat the child as well as palliative care staff to do an in-service or advise school personnel.[77] Because children with life-threatening illness are living longer, however, acceptance of their goals to enhance their quality of life must be honored whenever possible.

INTERDISCIPLINARY CARE

The interdisciplinary team is an essential aspect of palliative care for children.[28] How else could we possibly address the physical, social, emotional, and spiritual needs of the child with a life-threatening illness and the family as well?[78] Ideally the team is providing care from the time of diagnosis through death and bereavement.[18,28,79,80] Because care may occur in the hospital, home, or community, most often the child has a primary team (pediatrician or oncologist) whose members take the lead in day-to-day management. Most often the team consists of a physician, nurse practitioner or nurse, and social worker. Other core team members include a psychologist, chaplain, child life specialist, and case manager. The needs of the child and family require an entire team to provide the holistic care that is synonymous with palliative care.

Interdisciplinary care occurs with the child and family as partners. Priorities of the family are elicited, with their values, beliefs, and culture taken into account as recommendations are made that inform the necessary decision making. Family meetings, especially those used to address goals of care, include the parents, the child if appropriate, and interdisciplinary team members, so that information, emotional cues, and nonverbal language may be appreciated from different perspectives. This format also allows for a response that may address the issues holistically. In addition to those already mentioned, at times an interpreter, physical therapist, teacher, respiratory therapist, nutritionist, or pharmacist is present for the family meeting. Importantly, research has shown that when the physician is working with a psychosocial clinician there is closer agreement between the physician and parents in the understanding of the child's prognosis.[8]

Interdisciplinary care is essential so that not only the child's and parents' needs can be addressed but also those of the extended family, community groups, and the hospital, clinic, and home care staff. Particularly when doing a home visit, it is helpful to have several team members present so that physical and emotional needs of the patient, parents, and siblings may receive attention. Each team member carries the responsibility to inform, teach, and reach out to colleagues. Depending on one's clinical setting, there inevitably will be times that different team members are called on to take the lead.

Pediatric palliative care is particularly challenging. As providers, we have "an obligation to nurture relationships that can hold within their embrace both vulnerability and suffering: that which is experienced by our child patients and their families, and that which we experience within ourselves."[81] The depth and meaning of this care touches us at our core. We need the expertise, support, and companionship that interdisciplinary care provides.

COORDINATION OF CARE

The coordination of care for a child with a life-threatening illness who moves between hospital, home, clinic, and perhaps a rehabilitation facility is daunting. Despite the efforts of many providers, parents often report feeling overwhelmed with the responsibility of care and coordination of services, particularly in the home. There are many barriers to coordination of pediatric palliative care services, including lack of available staff to provide home-based services; limited training of staff in pediatric palliative and hospice care; few resources for services; limited agencies with comprehensive services; and insufficient systems to support communication and coordination between sites of care.[30] Ideally each hospital, clinic, community, or home care agency has designated personnel to perform coordination tasks for the child and family and has protocols specifying a standard of practice.[28]

Thankfully, in the United States a growing number of home care agencies have developed palliative care programs that serve as a bridge to hospice services for children not yet meeting hospice eligibility criteria (<6 months to live) or for those seeking intensive disease-directed therapies. Previously the reimbursement structure and use of medical technology (e.g., intravenous nutrition, assistive respiratory support), experimental therapies, and/or need for block hours of nursing care had precluded enrollment of children on the hospice benefit.

Importantly section 2302 of the recent Patient Protection and Affordable Care Act, termed the "Concurrent Care for Children Requirement" (CCCR) has eliminated the requirement that Medicaid patients younger than 21 years forgo curative or life-prolonging therapies to be eligible for hospice. Therefore Medicaid programs in every state are now required to provide concurrent curative/life-prolonging treatment and hospice services for hospice-eligible children. The development of systems to make such concurrent care a reality has been slow and, in the meantime, a number of state-based pediatric palliative care coalitions have formed to jumpstart access to home-based pediatric hospice/palliative care services, using strategies such as Medicaid waivers or state plan amendments to increase coverage for hospice services. For example Massachusetts implemented a statewide pediatric palliative care program in 2006.[82]

LOCATION OF DEATH

At the end of life, children and families often benefit from intensive support. It is generally assumed that children would prefer to be at home to die, although approximately half of children with progressive cancer die in the hospital,[83] as do the majority of infants and children who die in the United States.[84] In a study from the United Kingdom, research showed that of children dying in the hospital, 85.7% died in the intensive care unit, with the trend having increased over the years from 1997 to 2004.[85] Families and children need to feel safe and well cared for and given permission, if possible, to choose location of care, be that at home, in the hospital, or a hospice house. Asking children where they would most like to be even if they are very sick and what is most important to them is often very instructive.

Although seemingly contradictory, the philosophy of palliative care can be successfully integrated into a hospital setting, including the intensive care unit, when the focus of care also includes the prevention or amelioration of suffering and improving comfort and quality of life. All interventions that affect the child and family need to be assessed in relationship to these goals. This proactive approach focuses on what "we can do" not what we cannot do. We might ask what can we offer that will improve the symptoms and quality of this child's life (e.g., radiation therapy, a trip to the garden, a special visit from a pet) and provide the most meaning and control for their family instead of asking what interventions are not going to be offered. Staff needs education, support, and guidance because pediatric palliative care, like other types of intensive care, is an area of specialty.

Preparation for Home Care

Preparing parents for events that may occur at home is essential. Even if they may not want to talk about this, written materials can be offered. Anticipating likely symptoms, ways to manage the symptoms, what resources will best match needs, and who will be available on call 24/7 to the family is key to making the transition to home successful. The child's primary inpatient or clinic providers must work closely with the case managers and social workers who know the intricacies of insurance benefits, home care nurses, and durable medical equipment vendors and who can determine what each can and will provide. It is also important to reach out to the primary care provider, who may have important insights to the home environment and resources in the community.

Even the most experienced families still need maximum physical and emotional supports in the home to care for a child who is facing the end of life. The goal is to have enough support in place so that parents can simply be "parents" and so that family interactions and events may become meaningful experiences.

Hospice Services

For those families and children who know they want to be at home, hospice services can be invaluable. Many hospice agencies now have palliative care programs, which offer greater flexibility and broaden the scope of care. There are many barriers, however, to provision of hospice services to children even with the new legislation section 2302 of the Patient Protection and Affordable Care Act.[78] Many fewer children than adults die, and so maintaining a staff that is willing and able to provide care to children can be challenging. Families and practitioners often see hospice as "giving up," and it remains difficult to change this assumption. There is a prognostic requirement of life expectancy of less than 6 months, and this can be a significant emotional barrier for families and clinicians. Financially provision of hospice may also be a struggle, because hospice is mandated to provide all the personnel, medications, equipment, and supplies that the child needs and this is capped at a per diem rate.[86] The financial concerns may be mitigated with successful implementation of the Affordable Care Act, which should be reassessed over time.

The first free-standing pediatric palliative care center in the United States, the George Mark House, opened in 2004, and other pediatric hospice houses have followed. Some adult hospice houses are now willing and equipped to accept a child, with the hospice staff often appreciating the support of a previously involved pediatric palliative care team. In addition to hospice care there is a monumental unmet need for many families who are in desperate need of respite care for their child, who may have a prolonged palliative care path ahead of them.

Despite these barriers, palliative care, respite care, and hospice care for all children with life-threatening conditions is the recommendation of the Committee on Bioethics and Committee on Hospital Care.[80] Importantly when experienced in caring for children, hospice teams are ideally suited to provide comprehensive home-based services to the child, siblings, parents, and extended family. Hospice programs often have physicians, nurses, social workers, chaplains, and bereavement staff, art therapists, music therapists, pet therapists, and volunteers who can provide integrative therapies such as massage and Reiki.[87]

CANCER-DIRECTED THERAPY

Parents often wish to continue some form of cancer-directed therapy in the end-of-life care period and are more likely to favor using cancer treatment at the end of life than clinicians.[88] The choice of cancer-directed therapy should be considered in the context of goals of care. For example if a parent's primary goal is to minimize the child's suffering, then an option that causes few distressing side effects and that necessitates few interventions may be a reasonable choice, if parents wish to continue to provide cancer-directed therapy.

Some regimens with limited toxicity have established efficacy in relapsed or refractory tumors. For example, daily oral etoposide has been used in children with refractory solid tumors, with limited resulting toxicity and with the possibility of tumor response.[89-93] Oral temozolomide has had similar results in children with neuroblastoma.[94] In refractory acute lymphoblastic leukemia, maintenance-type regimens of 6-mercaptopurine, low dose methotrexate, vincristine, and prednisone may be effective without causing significant side effects. Antibody therapies, such as rituximab for children with B-cell malignancies, and molecular agents, such as imatinib in potentially responsive cancers, may also offer palliation with limited toxicity. Other examples of therapies with limited toxicity can be found in the literature.

Other parents may wish to continue more intensive regimens of chemotherapy. Recent work has found that, when prescribing chemotherapy for children with no realistic chance for cure, oncologists consider the potential toxicity of the chemotherapy, family preferences, and the potential that therapy will decrease cancer-related symptoms.[95] However, nearly half of oncologists studied had prescribed chemotherapy to meet parental wishes, and many oncologists' recent experiences with end-of-life chemotherapy did not meet their goals of prescribing. Thus physicians should ensure that such regimens offer reasonable hope of meeting the parents' and child's goals of care.

Parents may also wish to pursue experimental options such as those provided on phase I clinical trials. In general the likelihood of significant benefit from agents offered on phase I trials is limited, typically under 8%, even in children.[96] Misconceptions about the purpose of phase I trials are common; as with other clinical trials, many subjects believe that the purpose of such a trial is to benefit them personally rather than to benefit future patients.[97] When clinical trials are offered, clarity about the purpose of the trial is important so that parents and children can make informed decisions about whether such therapy will help to meet their goals. In addition such therapies do not preclude concurrent attention to symptoms and quality of life. In fact attention to life prolongation and symptoms simultaneously may best meet the needs of children with advanced cancer and their families.[98,99]

Other cancer-directed options to be considered include local irradiation or surgery. If a single lesion is causing significant pain or other symptoms, then localized radiation therapy may offer palliation with few systemic side effects. Radiation therapy directed at an isolated painful bony metastasis is one such example. Surgery can be similarly beneficial, but a frank team discussion with the surgeon about the possibility of recovery and the likelihood of recurrence can help in consideration of the likelihood that the child will benefit from the surgery. Debulking of a rapidly growing abdominal tumor, for example, may provide little benefit, in contrast to control of a single painful metastasis by radiation or surgery. A consistent recommendation between the oncologist and surgeon or radiation oncologist should be provided to the family.

Physicians sometimes feel an obligation to describe cancer-directed therapy as either “curative” or “palliative.” Such a distinction may be difficult, however, because at times cancer-directed therapy may offer some chance of cure, even if this chance is low, as well as some possibility of symptom palliation and life extension. In addition this distinction may be difficult for families, who may hold more than one goal for care. We recommend rather than using a label of curative or palliative, that cancer-directed therapy be discussed in terms of the goals for care. For example we may be able to tell parents that the goals of a particular therapy are to control the disease for as long as possible, with a very small possibility of cure, and with a higher likelihood of decreasing symptoms for a period of time. If cure is clearly not a goal of therapy, then that also should be stated.

Families who have been accustomed to using cancer-directed therapy at every recurrence may continue to pursue such options even when the medical team believes that the likelihood of benefit is extremely low. Unfortunately previous work suggests that parents may regret decisions made about end-of-life cancer treatment in retrospect,[100,101] especially if treatment involved suffering.[100] Thus clinicians should do everything possible to ensure thoughtful decision-making prospectively. In addition when such situations arise, the team should reach consistent recommendations about cancer-directed therapy and should not offer therapies with no chance of benefit and a high risk for harm. However patients and families will have different preferences for end-of-life care; and for some families, continuing to “battle cancer” until the time of death may hold personal meaning. The team should therefore follow the same standards for communication that we have described earlier: assess the family’s understanding of prognosis, allow for further communication about the prognosis if needed, elicit goals of care, come to a team consensus about plans for care that fit with goals, and make recommendations for care based on family and patient goals and the team’s consensus. Intensive interventions may be chosen by families who have overly optimistic perceptions of prognosis[8,11]; ongoing communication about the child’s prognosis may allow families to readjust goals and to choose interventions that offer realistic chances of benefit. The team should never be obligated to provide care that is considered to be inappropriately harmful and in such cases should work with hospital ethics teams to define appropriate responses to family requests. But beyond the reaches of harm, families who make thoughtful decisions about care for their children should be allowed to do so, even when plans for care do not match the ideals of staff members.

SYMPTOM-DIRECTED THERAPY

Managing symptoms for the child with a life-threatening condition is of utmost importance. Parents and children are partners in care and the experts regarding the symptoms and treatments that make the most sense based on their goals and values. Managing symptoms can be complicated and difficult. In research by Wolfe and colleagues, parents reported that 89% of children who died of cancer suffered a lot or a great deal from at least one symptom in the last month of life.[6] In a nationwide study in Sweden, parents of children who died of malignancies reported that pain in particular had a profound impact on their children.[102] Not surprisingly parents are quite distressed by these symptoms, especially changes in behavior and appearance, pain, weakness, fatigue, and dyspnea.[103]

Importantly there is also evidence of progress. A more recent study by Wolfe and associates documented less suffering from pain and dyspnea near death for children who died in the years after their initial study.[4] Thus a culture of awareness and attention to symptoms as well as intensive efforts at management may be able to mitigate the suffering children experience at the end of life.

Many of the symptoms we describe are addressed in detail in Chapter 71, “Symptom Management in the Child with Cancer.” We have therefore limited our discussion to management of common symptoms in the setting of more advanced illness. Medications used in managing common symptoms are listed in Table 70-3.

Pain

Pain should be considered a medical emergency; over 80% of children with advanced cancer experience pain.[6,104] Pain affects not only the physical being but also the emotional and spiritual being. Often pain is accompanied by anxiety and emotional or spiritual distress. It is important to address each coexisting or contributing factor to pain. Parents often express extreme helplessness and distress when the child is suffering in pain.

The child’s report of pain is an essential aspect of assessment.[105] Reliable scales that are consistent in the hospital and home are available, but assessment can be challenging, particularly with the nonverbal child. A knowledge of the child’s usual behaviors and previous experience with pain management and with children with similar diagnoses can be helpful.[106] Tailored assessment tools can allow parents to equate behaviors with comfort or pain on an individualized rating scale.[107,108]

Treatment of pain should be followed by assessment for effective relief. The current WHO pain guidelines

TABLE 70-3 Medication Guidelines for Symptom Management

Symptom	Medication	Dosage	Comment
Anorexia	Megestrol acetate	100 mg PO bid; if no effect in 2 wk, double dose to 200 mg bid	Use only in children >10 yr
	Dronabinol	2.5-5 mg/m^2/dose q4h	Use only in children >6 yr; contraindicated in depression
	Dexamethasone	0.3 mg/kg/day PO/IV	Use only in children >2 yr
	Cyproheptadine	0.25 mg/kg/day divided twice daily; age-dependent maximum daily dose: ≤6 yr: 12 mg/day; 7-14 yr: 16 mg/day; ≥15 yr: 32 mg/day	
Agitation	Haloperidol	0.01 mg/kg PO tid prn; for acute onset: 0.025-.05 mg/kg PO; may repeat 0.025 mg/kg in 1 hour prn	
Anxiety	Fluvoxamine	25 mg/day; may increase by 25 mg every 4-7 days up to 200 mg/day	Rarely can worsen depression or cause suicidality
Constipation	Glycerin suppository	One suppository PR qd	
	Lactulose	5-10 mL q2h until stools	
	Polyethylene glycol (MiraLax)	0.8 g/kg/day PO; maximum dose 17 g/day PO	Always dissolve dose in 4-8 oz of liquid
	Children's Senokot liquid	2-6 yr: 2.5-3.75 mL qd 6-12 yr: 5-7.5 mL qd	
	Pediatric Fleets enema	One PR qd prn	
	Methylnaltrexone	0.15 mg/kg SQ every other day prn	
Depression	Fluvoxamine	25 mg/day; may increase by 25 mg every 4-7 days up to 200 mg/day	Rarely can worsen depression or cause suicidality
	Methylphenidate	5 mg orally in the morning and at noon	Not recommended for children <6 yr
Dyspnea	Morphine sulfate immediate release	0.1 mg/kg PO q1-4h	Starting dose, no ceiling
	Lorazepam	Starting dose 0.025-0.1 mg/kg PO/IV q4h Maximum dose: 0.5 mg/kg	
Fatigue	Methylphenidate	5 mg orally in the morning and at noon	Not recommended for children <6 yr
Fever	Acetaminophen	15 mg/kg PO q4h	
	Ibuprofen	10 mg/kg PO q6-8h	
Insomnia	Melatonin	3 to 5 mg given 3 to 4 hours before an imposed sleep period	
	Trazodone	0.75-1 mg/kg PO at bedtime	Not recommended in children <10 years of age
	Nortriptyline	0.1 mg/kg PO at bedtime	
Nausea and vomiting			
Mild-moderate	Diphenhydramine	1 mg/kg PO q6-8h prn	
	Metoclopramide	0.25 mg/kg PO q8h prn	
Severe	Ondansetron	0.15 mg/kg PO q8h prn	
	Dexamethasone	0.3 mg/kg/day PO/IV	Maximum dose 10 mg/kg/day PO/IV
	Lorazepam	0.025-0.1 mg/kg q8h prn	Maximum 3 mg/day
Pruritus	Diphenhydramine	1 mg/kg PO/IV q4h prn	
	Hydroxyzine	0.5 mg/kg PO q8h prn	
	Hydrocortisone 1% cream	q6-8h	

Continued on following page

TABLE 70-3 Medication Guidelines for Symptom Management (Continued)

Symptom	Medication	Dosage	Comment
Pain			
Mild pain	Acetaminophen	15 mg/kg PO q4h prn or around the clock	Available in 160 mg/5 mL suspension or 80 mg/mL
Moderate to severe pain	Morphine sulfate immediate release	*<6 mo*: 0.1 mg/kg PO q3-4h *>6 mo to 50 kg*: 0.3 mg/kg PO q3-4h for opioid-naïve patient Titrate by 50%-100%/dose Prescribe breakthrough dose of 0.15 mg/kg PO q15-30 min.	
	Time-released morphine (MS Contin, Oramorph, Kadian)	Total daily dose divided by 2 determines q12h dose (approximately 1 mg/kg PO q8 or 12h). Breakthrough dose approximately 10% of the 24 hour total given q1-2h	
	Oxycodone	0.1 mg/kg q3-4h PO	
	Hydromorphone	0.03-0.08 mg/kg q4h PO	
	Fentanyl patch	*2-12 yr:* Do not exceed 15 µg/kg every 72 hours.	
	Methadone	0.1 mg/kg PO/IV bid or tid	No dose adjustments for 72 hours due to long half-life of drug; available in liquid
Adjuvant analgesics	Ibuprofen	10 mg/kg PO q6h	
Bony metastases	Choline magnesium trisalicylate	25 mg/kg PO tid	
Neuropathic pain	Nortriptyline	0.5mg/kg at bedtime	Maximum dose: 150 mg/day
	Gabapentin	*Initial dose:* 10-15 mg/kg/day divided q8h. Titrate if needed to 50 mg/kg/day; not to exceed 2400 mg/day	
Severe visceral distention or bony disease	Prednisone	*Oral solution:* 1 mg/kg/day or bid with food	
Secretions	Scopolamine	1.5-mg patch, apply topically, every 72 hours	
	Glycopyrrolate	0.04-0.1 mg/kg PO q4-8h	
	Hyoscyamine sulfate	*<2 yr:* 4 gtts PO q4h prn (0.125 mg/mL solution) *2-12 yr:* 8 gtts PO q4h prn (0.125 mg/mL solution)	Maximum: 24 gtts/day
Seizures	Lorazepam	0.1 mg/kg PO/SL/PR, may repeat q 15 min × 3 doses 4 mg single dose	Parents should be advised to contact physician if used
	Diazepam rectal gel (Diastat)	*2–5 yr:* 0.2 mg/kg q 15 min × 3 doses *6-11 yr:* 0.3 mg/kg q 15 min × 3 doses *>11 yr:* 0.2 mg/kg q 15 min × 3 doses	Parents should be advised to contact physician if used

recommend the first step for mild pain and the second step for moderate to severe pain.[109,110] This approach suggests moving from weak analgesics, such as acetaminophen, to stronger analgesics such as opioids, although, depending on the presenting pain, strong analgesics may be initiated at the outset. Codeine should no longer be used in children. Although it was previously recommended, prescribing codeine should generally be avoided because of its side effect profile and lack of superiority over nonopioid analgesics. Furthermore, relatively common genetic polymorphisms in the *CYP2D6* gene lead to wide variation in codeine metabolism. Specifically, 10% to 40% of individuals carry polymorphisms causing them to be "poor metabolizers" who cannot convert codeine to its active form, morphine, and therefore are at risk for inadequate pain control; others are "ultrametabolizers" who may even experience respiratory depression from rapid generation of morphine from codeine. It is therefore preferable to use a known amount of the active agent, morphine.[111]

Plans for treatment should be based on the child's report of pain, the type of pain, the history of pain medicine use, and the parent's understanding of the pain and possible treatment options.

The child and parents should be educated about the basic principles of self-report, around-the-clock dosing, and the need for frequent reevaluation. Particularly when a family is facing the end of the child's life, the meaning of a recommendation for a medicine such as morphine sulfate cannot be underestimated.[112-115] Often myths of opioid use need to be explored and education, reassurance, and rationale provided. Using opioids also necessitates the prophylactic treatment of common side effects.

In addition to analgesic agents, there are a variety of adjuvant therapies that may be used to alleviate pain (see Chapter 71, "Symptom Management in Children with Cancer"). Increasingly families are also being encouraged to use nonpharmacologic strategies such as distraction, heat, cold, positioning, and touch. Children may also benefit from acupuncture, hypnosis, Reiki, and guided imagery, particularly if it has been used during the earlier days of treatment.[116-121]

Occasionally a child suffers from severe pain due to malignancy and needs massive opioid infusions. Collins and colleagues found that 6% of children with terminal malignancy were in this category of needing more than 3 mg/kg of morphine dose equivalent per hour. Eight of 12 required epidural or subarachnoid infusion and/or sedation.[122] Children may also experience rapidly escalating symptoms of pain, dyspnea, and or agitation. For these situations it has been beneficial to have templated orders, created by an interdisciplinary group of clinicians, so that sound principles and standard management can be executed at the bedside.[123]

Transitioning a child from the hospital to home with adequate symptom management may pose challenges to the home care system. Strategies such as using concentrated elixir that can be administered buccally, by mouth, or by gastrostomy tube or using transdermal medications such as a fentanyl patch[124,125] may be of benefit. Long-acting agents such as methadone and transdermal fentanyl may be given in conjunction with shorter-acting agents administered intravenously, subcutaneously, or orally. Compounding pharmacies can assist with multiple drug combinations for ease of administration in a variety of preparations. Many home care agencies, including hospice, are able to provide intravenous patient-controlled analgesia in the home setting as well. Finally exploring the potential use of midazolam or propofol infusions with the home care or hospice agency[126] may help determine what can realistically be provided for the child in extreme circumstances at home.

Dyspnea

Similar to pain dyspnea is subjective and can be caused by a multitude of factors. Having the child report the degree of distress associated with dyspnea, using a scale similar to a visual analogue scale for pain, may be helpful.[127] Recommended interventions should be congruent with the child's and family's goals, whether for extending life, relieving suffering, being in the hospital, or remaining at home. For instance if pneumonia is diagnosed, some families may choose parenteral antibiotics, others enteral antibiotics, or others strictly symptom relieving measures, such as treating fever and dyspnea. Diuretics may be given for situations involving fluid overload, and thoracentesis can be considered for pleural effusions, although such interventions may not provide effective palliation in patients with extensive parenchymal disease.[128] Transfusion can be considered if the level of red blood cells is low with the hope of increasing oxygenation potential.

Oxygen is often considered for dyspnea.[129] In a study in adult patients with advanced cancer, Philip and colleagues found that patients reported relief with either room air or oxygen by nasal prongs.[130] Interestingly they also found poor correlation between dyspnea complaints and measured oxygen saturation levels. In the hospital it may be easy to provide oxygen, whereas at home opening a window or providing a fan, to provide facial cooling in the areas subserved by the second and third branches of the trigeminal nerve, in addition to positioning and relaxation techniques, may be useful.

Finally opioid medications intravenously, subcutaneously, or orally can provide relief[131,132] and can be used without hastening death or suppressing respirations. Dyspnea can often be relieved with a lower opioid dose than that used for pain; a starting dose that is 25% to 30% of the opioid dose used for pain is often effective, although the dose should then be titrated to symptom relief. Nebulized opioids have been used, although studies have not confirmed efficacy of this route.[133] Emerging data among adults also suggest that low-dose long-acting opioids may improve symptom relief in chronic dyspnea.[134] In addition anxiety often precedes or follows episodes of dyspnea and may be relieved by benzodiazepines,[135,136] hypnosis, or other relaxation techniques.

Airway Obstruction

Airway obstruction and the resulting "noisy breathing" from excessive secretions and relaxation of oropharyngeal muscles can be very distressing to families. Usually deep suctioning is not helpful and may cause discomfort. Rather explaining that this is a natural result of the physiologic decline and that it is not distressing to the child may be reassuring. Anticholinergics may be used to decrease secretions[137,138] as well as decreasing parenteral or enteral fluids, if they are being given. However overly thickened secretions can lead to airway obstruction and greater distress; thus careful titration of interventions is warranted.

A somewhat different situation occurs when tumor progression causes airway obstruction. In our experience, the respiratory distress that occurs in this situation can be quite difficult to control, even with massive opioid doses. If the child appears to be gasping and uncomfortable despite opioid therapy, then palliative sedation may be warranted. In this case extensive discussions with the family and providers must occur so consensus is reached and it is clear that the goal is to sedate the child and relieve his or her symptoms and not to hasten death.

Gastrointestinal Symptoms

Nausea and Vomiting

Just over half of parents of children who have died of cancer report that nausea and vomiting were present at the end of life, often with significant associated suffering.[6,139,140] Such symptoms sometimes go unrecognized and untreated by physicians,[6,140,141] and even when treatment is offered, efficacy may be limited. Physicians should therefore assess for nausea and vomiting carefully, attend to symptoms promptly, and pay close attention to the efficacy of treatment.

Vomiting is stimulated by the gastrointestinal tract, after local receptor stimulation by obstruction, stasis, or toxins; by the pharynx, due to mucous or mucosal breakdown; by the medullary vomiting center, in response to cortical, gastrointestinal, or chemoreceptor trigger zone stimulation; or by the cortex, in response to elevated intracranial pressure or emotional and sensory stimuli. Chapter 71, "Symptom Management in Children with Cancer," provides further detail on causes of nausea and vomiting in cancer patients. Because the cause may define the most effective therapy, the most likely cause should be considered in evaluation and treatment of the patient.[142]

At end of life chemoreceptor trigger zone stimulation by toxins such as medications (chemotherapeutic agents, antibiotics, and opioids) and metabolic byproducts of uremia or hepatic failure in the blood and cerebrospinal fluid is a particularly common cause of nausea. In addition to effects on the chemoreceptor trigger zone, opioids induce nausea by decreasing gut motility and thereby causing gastroparesis and constipation. Patients may particularly experience nausea and vomiting after dose escalation, but this typically resolves after 3 to 4 days at a stable dose.[136] Opioid-induced nausea and vomiting are usually responsive to antiemetic therapy, especially with 5-hydroxytryptamine antagonists.[136,143,144]

Another important cause of nausea and vomiting in the end-of-life period is bowel obstruction, which deserves particular consideration in patients with abdominal tumors. Management of obstruction is discussed later in this chapter.

Refractory nausea and vomiting in the end-of-life setting have been effectively treated with antipsychotics such as olanzapine[145] and haloperidol. Also of note dexamethasone may have limited efficacy against nausea and vomiting in the end-of-life setting,[146] in contrast to its efficacy with chemotherapy-induced nausea, unless specific corticosteroid-responsive causes such as increased intracranial pressure or bowel obstruction are present. Finally because sights, smells, and the child's emotional state can contribute to nausea, management of distress and any environmental triggers should be considered. Nonpharmacologic therapies such as hypnosis and acupuncture may also be effective.

Constipation

Constipation is a common cause of pain and distress at the end of life,[6,139,140] particularly owing to opioid use for pain. Opioids cause constipation by decreasing colonic motility and secretions and by increasing fluid absorption in the colon. Preventive measures should always be considered at the time of initiation of opioids, with an osmotic and motility agent typically prescribed at initiation. Although many opioid-related side effects tend to decrease over time, constipation tends to remain a problem throughout the period of opioid use.[136]

Other important causes of constipation in the end-of-life care period are decreased patient mobility and decreased oral fluid and nutritional intake.[147] In addition cord compression and bowel obstruction due to intraabdominal tumor should be considered as possible causes.

Unless evaluation reveals an underlying cause such as bowel obstruction[148] or cord compression, therapy should be similar to that used for any child with cancer, regardless of phase of life. Because fluid intake is often limited at the end of life, however, fiber supplementation is not generally useful and can sometimes increase constipation. Similarly stool softeners alone are often inadequate and should instead be replaced by or combined with osmotic agents or stimulants.

For opioid-induced constipation, subcutaneous methylnaltrexone can also be used to relieve constipation. About half of patients will experience laxation after the first dose,[149] and subsequent doses can be given every other day as needed. Despite properties as a mu opioid receptor antagonist, methylnaltrexone has not been found to precipitate pain or withdrawal.[149]

Bowel Obstruction

Intestinal obstruction, such as from an abdominal tumor, is an important cause of refractory nausea and vomiting in the end-of-life care period.[142] Goals of care and the patient's quality of life may guide the choice of treatment. Therapeutic surgical options include bypass of the intestinal obstruction or placement of a venting gastrostomy tube. However nonsurgical alternatives exist for patients who wish to avoid surgery or who have limited ability to tolerate invasive measures.[150] Options for medical management include corticosteroids, which can be useful in relieving obstruction,[151] and octreotide, which can markedly decrease nausea and vomiting by reducing gastrointestinal secretions.[152] Octreotide can be administered subcutaneously and can be used in the outpatient setting if desired. The combination of morphine, scopolamine, and haloperidol has also been used to relieve cramping and nausea and vomiting.

Anorexia and Cachexia

Cachexia is a common finding in patients with advanced cancer. Poor nutritional intake and increased metabolic demands related to cancer appear to be its primary underlying causes. Mechanisms for increased catabolism, including lipolysis and proteolysis, are complex and likely mediated in part by cytokines.[153-155]

Anorexia compounds the problem. This too is mediated by a number of factors, including gastrointestinal (nausea, constipation, mucositis, dysphagia), other physical (pain), medication-related (impaired taste, decreased hunger), and psychological (depression) factors. Often multiple factors contribute to anorexia in any patient, and treatment should be directed at the individual causes when such causes can be discerned.[154,155]

Plans for care should include assessment of the meaning of this syndrome to individual patients and families. Some children and families will not be significantly bothered by anorexia and cachexia, but other children and families will find wasting to be extremely distressing, conjuring up the concern that the child is starving to death. Although severe cancer-related wasting is associated with poor outcomes in adult cancer patients,[156] increased nutrition is not enough to reverse this process.[157,158] Physicians may be able to address concerns about starvation by explaining that the cancer itself may be responsible for this condition and that increased nutritional intake often cannot halt this process. Goals for management should instead focus on symptoms, such as nausea or constipation, associated with this process. Another important goal is the maintenance of the child's level of function.[159]

Treatment of any underlying causes including associated psychological and spiritual issues should be a first step in management. Additional options such as enteral or parenteral[160] nutrition can be considered in light of overall goals of care and the efficacy of treatment for underlying causes. If the family wishes to institute artificial nutrition, it can be helpful to discuss the fact that, as the child's cancer progresses, there may be some point at which supplemental nutrition is no longer providing significant benefits relative to its actual or potential harms. The family and physician may wish over time to reassess the degree to which supplemental nutrition is meeting the goals of care, with the thought that, at some point, discontinuation of supplemental nutrition may make the most sense for the child. Discussion of this issue with families at the time of institution of supplemental nutrition may make reconsideration of this intervention less distressing for families as the child's illness progresses.

Pharmacologic therapeutic options include corticosteroids, which can increase appetite and nutritional intake. Their effect tends to wane after 4 weeks. Cyproheptadine is a safe and effective way to promote weight gain in children with cancer.[161] Cannabinoids such as dronabinol can also increase appetite, as can megestrol acetate.[162] Because megestrol acetate is associated with severe adrenal suppression in children with cancer,[163] it should be used with caution; some have recommended routine corticosteroid replacement for children on megestrol.[163]

Nutrition and Hydration

Near the end of life, fluid and nutritional needs diminish. Because parents often identify provision of nutrition as one of their important roles, physicians should prepare parents for the expected decrease in fluid and nutritional needs as the end of life approaches. Preparation for decreased urine output, as a manifestation of waning perfusion rather than of dehydration, can also be helpful.

Most studies suggest that nutrition and hydration can be withheld or limited in the end of life period without causing suffering.[164-166] In fact continued feeding via gastrostomy or nasogastric tube may be associated with abdominal discomfort or nausea when the child is in the last stages of life. In a study of bereaved parents who had made decisions to forgo artificial nutrition and hydration for their children, parents perceived deaths to be peaceful and comfortable.[167] Many of these parents chose to forgo artificial nutrition and hydration when the child developed feeding intolerance, suggesting that continued artificial nutrition may have increased discomfort.

One study of terminally ill adult cancer patients, however, suggests that some degree of parenteral hydration may improve symptoms associated with dehydration.[168] Even in this study, limited volumes of parenteral fluid were sufficient to improve symptoms. Parenteral hydration also has the potential to affect other choices about care, such as the location in which families feel most comfortable receiving care, the need for medical services at the end of life, and the need for intravenous or subcutaneous access.

When the choice is made not to provide supplemental hydration or nutrition at the end of life, it is helpful to pay close attention to symptoms of dehydration, hunger, or thirst, with the goal of treating any symptoms that arise. Often noninvasive measures such as moistening the lips or providing small tastes of food or fluid are enough to provide comfort.

Fatigue

Fatigue shapes the types of activities that children with life-limiting illnesses choose to participate in and has the potential to significantly diminish a child's quality of life.[169] Parents consider fatigue to be the most common symptom in the last month of life and the course of greatest distress for dying children.[6,170] Unfortunately this symptom is also the least likely to be treated.[6]

In general fatigue is a multidimensional symptom with both physical elements (muscle weakness, decreased energy) and cognitive or affective elements (difficulty concentrating, maintaining attention, lack of motivation and interest).[171] In a retrospective study of children who died of cancer, parents reported that 96% of children experienced fatigue in the last month of life. Nausea and vomiting, anorexia, and fear were other symptoms independently associated with fatigue.[172]

Younger children and adolescents with cancer define fatigue differently; younger children emphasize the physical aspects of the symptom, whereas adolescents describe multiple dimensions and distinguish between mental and physical fatigue.[173] Parents of children with cancer note that physical and emotional dimensions of fatigue create significant disruptions for the entire family.[174] Parents describe mood changes in the child and also report delays around the household and family activities because of the need to accommodate the fatigued child.[174] Fatigue also tends to limit children who wish to attend school until the very end of the illness, rather than receive private tutoring from a teacher in the hospital.[169] For children who remain in the hospital, fatigue also impacts cognitive function and mood due to interrupted sleep patterns that are mostly a result of frequent awakenings throughout the night and daytime hours.[175]

Opioid analgesics contribute to diminished levels of energy as well as a decreased ability to concentrate in children with advanced disease.[169] Coexisting or contributing factors include anemia, infection, pain, depression, and anxiety.[171,176] Assessment instruments commonly utilized to assess fatigue in cancer patients measure severity, onset, course, duration, and distress and concurrently assess other correlated symptoms, yet these assessment tools are not widely used. Furthermore the development of more sensitive instrumentation could assist practitioners in conducting a more comprehensive assessment of fatigue and, thus, provide more effective treatment of this symptom.[177]

Fatigue can seriously affect pediatric quality of life.[169] Multimodal approaches to treatment may be most effective; treatment of anxiety, depression, and sleep disturbance can also mitigate fatigue. Psychostimulants such as methylphenidate can be used to increase wakefulness, particularly when patients have opioid-related somnolence,[178] although they have not been formally evaluated in children. Because methylphenidate has a short duration of action, patients can control the timing of doses to coincide with important events during the day, such as time with family and friends. Blood transfusions, exercise programs (including physiotherapy),[179] psychosocial interventions, acupuncture, rest and relaxation (including playing/socializing), and nutrition and hydration counseling can also help alleviate fatigue.[171,174,180] A qualitative study conducted by Hinds and colleagues[173] reported differing views regarding successful fatigue interventions among patients, parents, and staff: whereas parents and staff considered themselves the primary responders to the child's fatigue and source of alleviation, patients stated rest and distraction as their first choice for alleviating symptoms of fatigue.

Psychological Symptoms

Psychological symptoms (including worrying, sadness, nervousness, and irritability) are among the most common symptoms experienced by children with cancer.[181,182] Some authors have reported that children with cancer as young as 7 years "can report clinically relevant and consistent information about their symptom experience" including psychological symptoms.[181(p 14)] Through interviews with children with life-threatening illness, Woodgate and colleagues[183] have identified a symptom experience described by children as: "I am hurting … My heart is sad." Children may have difficulty distinguishing between such symptoms, instead experiencing them as more of an "overall state," such as "experiences that cause … mental distress or suffering … from feeling sick to being scared."[183(p 811)] In adult studies of cancer patients, the notion of neuropsychological symptom clusters (including depressive symptoms, cognitive disturbance, fatigue, sleep disturbance, and pain) may help shed light on the mechanisms behind these symptoms. It is hypothesized that proinflammatory cytokines may underlie the first stage of development of these symptoms; however, more research is needed to fully understand the role of these cytokines and how this information can be used to effectively treat this cluster of symptoms.[184] This phenomenon of a "cluster" of symptoms is also noted in quantitative studies in which children reported high levels of symptom distress, suggesting an "interplay between psychological and physical symptoms," especially between psychological symptoms and the physical symptom of pain.[182] Additionally when disease advances, it may be challenging for children to discuss with their loved ones and with the health care team some of the emotional issues that arise.

Depression

Children with life-threatening illness have been reported to experience symptoms of depression and may also meet diagnostic criteria for clinical depression. Gothelf found that 16 of 63 adolescents with cancer (25.4%) met the *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM-IV) criteria for major depressive disorder.[185] Children older than 12 years, with a diagnosis of acute lymphocytic leukemia, receiving opiate analgesics, or undergoing radiation therapy were all significantly more likely to receive antidepressant medication.[186] Adolescents with higher levels of self-reported depression and lower levels of self-esteem had higher rates of nonadherence to medication; these same teens had lower rates of survival at a 6-year follow up.[187] Collins and colleagues found that roughly 36% of 159 children with cancer between the ages of 10 and 18 reported "feeling sad"; of this group, 17.5% rated its frequency as "a lot to almost always," 59.6% rated its intensity as "moderate to very severe," and 39.5% rated the distress it caused as "quite a bit to very much."[182]

In a younger cohort of children (ages 7 to 12) with cancer, Collins and colleagues[181] determined that 10.1% of the children surveyed reported feeling sad. Of this group, 53% reported feeling sad "a medium amount to almost all the time" on a scale of frequency, 60% reported feeling sad "a medium amount to a lot" on a scale of intensity, and 50% reported their sadness causing "a medium amount to very much" distress.[181] A slightly different story unfolds when Dejong and colleagues conducted a review of studies on depression in pediatric cancer published between 1980 and 2004. The majority of studies reviewed was cross-sectional and conducted, at the most, 5 years after diagnosis. In almost all the studies either no significant differences between depression rates in children with cancer and normal control subjects or significantly lower depression scores among the cancer group were found.[188]

There is mixed evidence that rates of sadness in children with cancer increase as disease advances. Parents have reported that a significant portion (up to 65%) of their children experience sadness during the terminal phase[139,189] and that sadness was the psychological symptom their child experienced most during the child's physical decline.[189] The well-being of children who died between the ages of 9 and 15 years was moderately or severely affected by depression, although similar results were not found among children who died before the age of 4 years.[139]

Risk factors for depression in children with cancer include parental depression and perceived stress and social support; age and illness severity are not consistently associated with depression.[188] However variation in findings across studies may be attributed to factors such as having multiple informants about the child's symptoms. Another confound in conducting accurate assessments of depression relate to the presence of other cancer symptoms or to the medications being taken to alleviate these symptoms, which may exacerbate or mask the symptoms of depression.[190]

Anxiety

Anxiety appears to be quite common in children with life-threatening illnesses.[181,182] In a sample of 149 children between the ages of 7 and 12, 20.1% reported feeling "worry."[181] Of those children, 43% experienced it a "medium amount to a lot" (intensity), 43% experienced it "a medium amount to almost all the time" (frequency), and 30% reported that it caused them "a medium amount to very much" distress. Among 160 older children with cancer,[182] 35.4% reported feeling "worry." Of these children, 66.1% reported that worry was "moderate to very severe," 28.6% reported a frequency between "a lot and almost always," and 27.2% reported the worry caused "quite a bit to very much" distress.

In children with advanced disease, levels of anxiety have been described by parents and health care providers. Researchers studied the medical records of 28 children who died of cancer to understand their end-of-life experiences; 53.6% of the children were documented as experiencing anxiety.[140] In a separate study of 164 symptom assessments collected in the last month of life, 50.0% of children reported "no problem" with anxiety/depression, 27.4% reported a "minor problem," and 17.7% reported a "major problem."[190] Anxiety was found to significantly influence the prevalence of pain.[191] Additionally exposure to physical pain early in life and/or a history of frequent painful medical procedures is associated with fear and anxiety during successive medical procedures, and, in turn, may have implications for future medical nonadherence and other related problems.[192] In the documentation of the symptom experiences of 30 children who died of cancer, worrying and nervousness were two of three symptoms "for which the proportion of children suffering a high level of distress ('quite a bit' to 'very much') was more than 50%."[170(pp 597–598)]

Parents of children who died of cancer were surveyed about their child's symptom experiences during the last month of life and reported, that among other symptoms, their child's sense of well-being was moderately or severely affected by anxiety.[139] Children older than 9 years were reported to be more troubled by anxiety than younger children.[139]

Qualitatively, children have described anxiety and fear together: "I am scared … I don't know what is going to happen to me."[193(p 811)]; often children and families experienced a fear so strong that they could "not focus their thoughts on anything but the cancer and [it paralyzed] children and families from participating in life."[193(p 811)] In children undergoing cancer treatment, anxiety has been commonly linked to the experience of nausea, vomiting, insomnia, nightmares, and rashes. Younger children may act out behaviorally (kicking, fighting).[194] In children with advanced disease, children who identified themselves as feeling "scared" explained that the feeling was often the result of "painful treatments or procedures, experiencing unexpected and new symptoms, and experiencing feelings that there was a greater chance for relapse or death … sometimes the fear experienced by children would be so great this led them to not to talk about the symptom(s) or event(s) that caused them to experience such intense fear and anxiety."[193(p 811)] Parents have described how they handled the fear that their children with cancer experience and note that they dealt with their child's fear by providing a sense of reassurance and security for their child up until the point when they needed to reprioritize their energies on their child's physical health rather than assuage their fears.[195]

Treatment of Depression and Anxiety

Pain, anxiety, distress, and suffering in children with advanced disease are interrelated; thus, providing relief for each of these symptoms is important. Given this reality, depression and anxiety, especially as they relate to other symptoms such as pain, should be treated simultaneously. Depression and anxiety screening programs[196,197] have been documented as operating successfully in oncology outpatient settings and should be adopted as standard practice to the continuum of pediatric care in all ages.

When treating depression and/or depressive symptoms of children in palliative care, a combination of medication and psychosocial therapies is optimal. Fluvoxamine and other types of serotonin reuptake inhibitors may be effective for those children with cancer who also exhibit symptoms of depression and anxiety. In one study of adolescents receiving chemotherapy for advanced cancer, 25.4% of those given fluvoxamine experienced a significant decrease in the somatic and nonsomatic symptoms of major depressive disorder with few side effects.[185] Methylphenidate also has antidepressant properties and may have a more rapid onset of antidepressant effects than other agents.[198] Other antidepressants may also be effective, as may counseling or psychotherapy or, ideally, a combination of medications and psychotherapy when indicated.

In contrast benzodiazepines in isolation are believed to be overused for chronic anxiety. Although medications can be effective in palliative care, therapeutic interventions that address a child's anxiety must be tailored to his or her condition, age, and temperament.[199] In children undergoing cancer treatment as well as at the terminal phases of the disease, supportive therapies such as hypnosis appear to be beneficial.[200,201] Pretend play has been suggested for younger children undergoing cancer treatment,[121] whereas telehealth[202] and virtual reality[203] technologies are on the cutting edge of providing effective forums to provide cost-efficient and convenient methods of support for diverse age groups of pediatric patients and their families.

Sleep Disturbance

Sleep disturbance in children who are critically ill typically takes the form of insomnia-type symptoms (involving difficulties in falling asleep, trouble staying asleep, early-morning awakening, complaints of nonrestorative sleep) and/or periods of too much sleep. When Goldman and colleagues[191] surveyed the records of children with terminal cancer, the prevalence of children who reported that too much sleep was a problem almost doubled between the point of entry into the study ("palliative phase") to the last month of life.

Data on the quality of sleep of children with critical illness found that children infected with human immunodeficiency virus slept approximately 1 hour less than healthy children, had more nightmares, and had more general sleep problems.[204] Children with serious illness [205] also slept significantly less on nights when they were experiencing pain, compared with nights that were pain free, and "reported poor sleep on an average of 43% percent of days with pain and 3% of days without pain."[206]

Predictors of sleep disturbance among cancer patients include pain, breathing difficulty, headaches, hot flashes, limb movements, frequent urination, nausea, and vomiting.[207] Patients who received greater amounts of radiation had poorer sleep.[208] In a study of adolescents with chronic pain, the investigators found a correlation between more frequent pain and daytime sleepiness and longer-lasting pain and later bedtimes; furthermore, sleep/wake problems (irregular sleep habits, hard time falling asleep or waking up) were associated with reduced social/emotional well-being.[211]

Psychological consequences of impaired sleep include feelings of fatigue, impaired daytime functioning, mood disturbances,[210] as well as an negative impact on quality of life, ability to cope, and perception of illness severity.[207] Cancer patients with sleep disturbances also reported high correlations between difficulty falling asleep and fatigue, early awakening and fatigue, and difficulty falling asleep and anxiety. Study results also support the idea that depression, anxiety, and sleep disturbances are related as are cancer-related pain, fatigue, and sleep disturbances.[211,212]

Treatments for problems with sleep include pharmacologic and nonpharmacologic modalities. Pharmacologic treatments include melatonin,[213] the tetracyclic antidepressant trazodone and the tricyclic antidepressants,[210] and antipsychotics (especially for cancer patients with delirium).[207] Psychological and behavioral interventions for sleep disturbance include stimulus control, sleep restriction, sleep education, relaxation training, and combinations of the aforementioned therapies.[210] In cases in which sleep problems are linked to symptoms or correlated with a disorder, treatment of the underlying condition may improve the sleep problems.

Anemia and Bleeding

Decisions about treatment of anemia should center on the child's quality of life and the expectations of the family and providers. For a child whose major complaint is fatigue, significant dizziness, tachycardia or dyspnea, a red blood cell transfusion may add to quality of life.[214] For some children and families, however, the need to travel to a center and spend half a day for associated medical care may make transfusions less desirable.

Fear of profuse bleeding in the setting of thrombocytopenia at the end of life is worrisome for providers and family members. As with red cells, platelet transfusions are not possible in the home setting; therefore careful planning and consideration are necessary so as not to disrupt the child's last days. Families may tolerate only treating symptomatic bleeding or may choose prophylactic scheduling of transfusions at a clinic or nearby hospital.[215] When children are at high risk for bleeding at home, parents and providers may want to develop a plan that minimizes distress to the child if this should occur. For example, ways to apply pressure for a nosebleed or having dark-colored linens on hand may be recommended in this situation.

Mucosal bleeding can sometimes be controlled with less invasive measures.[216] For example, aminocaproic acid can be used orally or intravenously to inhibit fibrinolysis.[217] Nausea is a common side effect, and aminocaproic acid should be avoided in patients who have hematuria. Topical options include fibrin sealants.[217] The tannins present in black teas can also help to stop bleeding. At home, patients can wet a tea bag with cool water and press it onto bleeding gums.

Fever and Infection

Parents of children with cancer are well aware of the consequences of fever and neutropenia during the child's treatment course. At the end of life, it may be appropriate to think about the role of antibiotics in a different way. Fevers may be due to infection; however, they may be due to the underlying disease. If a fever and associated symptoms can be ameliorated with acetaminophen and other simple measures, this may be most beneficial to the child. Other families may feel more comfortable with a course of oral or parenteral antibiotics, whether the child is an inpatient or outpatient. If this is the case, it may be helpful to plan for a time-limited course, after which antibiotics may be discontinued if no infection has been detected. Over time, parents who prefer antibiotic therapy may become more comfortable with only antipyretic treatment if they see that their child can tolerate this management strategy. Meeting with parents and the child to determine what investigative measures and treatment fit with their family goals will help to determine provider recommendations.[218]

Central Nervous System Symptoms

Seizures

Although seizures tend to be associated with limited suffering for the patient, parents often find seizures to be highly distressing at the end of life. Seizures may occur near the end of life as a result of central nervous system disease, electrolyte abnormalities, fever, or hypoxia. For any patient at home and deemed to be at risk for seizures, rectal diazepam should be available. At times seizure prophylaxis with an antiepileptic agent or benzodiazepine may also be desirable, especially if a patient is at high risk.

Increased Intracranial Pressure

Children with intracranial tumors are at risk for experiencing symptoms of increased intracranial pressure as the disease progresses. Common signs and symptoms include headache, vomiting, and somnolence. Although invasive therapies, such as surgical debulking or ventricular shunting, can be considered, the efficacy of such treatments may not be enduring in the end-of-life setting; tumor growth can quickly block a shunt or lead to recurrence of elevated pressure. Administration of dexamethasone can be effective in alleviating symptoms, and the dosing can be titrated as new symptoms arise or if initial symptoms wane. Dexamethasone-associated side effects, including mood changes, cushingoid appearance, and hunger with subsequent weight gain can be a source of distress to patients, and such side effects should therefore be part of ongoing discussion about dose titration. Radiation therapy may also be considered to decrease tumor size and thereby decrease symptoms at least temporarily.

Spinal Cord Compression

Spinal cord compression is relatively uncommon in the end-of-life setting for children with cancer, but it has the potential to severely impact quality of life at the end of life. Early diagnosis and management maximizes the chances of preservation of function.[219] Presenting symptoms include sensory and motor changes as well as bowel and bladder function abnormalities. Prompt imaging, typically with magnetic resonance imaging, can confirm the diagnosis. Dexamethasone can be used to decrease edema at the site of compression, thereby reducing symptoms and preserving neurologic function.[220] A local intervention such as neurosurgical intervention or radiation therapy is the mainstay of treatment, because decompression surgery plus radiation therapy increases the chances of recovery to ambulation.[221] However the specific intervention recommended should take the patient's overall functional status and goals of care into consideration.

Swallowing Impairment

Patients with brainstem involvement of a central nervous system tumor may develop swallowing impairment as one manifestation of disease progression. Such impairment has important implications for nutrition and hydration and may place patients at risk for aspiration pneumonia. Symptoms of coughing or choking when eating or drinking should be regarded as signs of aspiration and evaluated carefully. A swallowing study can establish which consistencies of liquids and solids the patient can tolerate without aspirating. If thin liquids cannot be tolerated, then additives can be used to thicken liquids until they are of tolerable consistency.

Some patients are unable to tolerate adequate nutrition and hydration without a significant risk for aspiration. A trial of a nasogastric tube may be a short-term option, or gastrostomy tube placement can allow long-term sustenance while minimizing food-related aspiration risks. This choice may be most appropriate in patients who are expected to live and to have a reasonable quality of life for some time. Even with a gastrostomy tube, patients remain at risk for aspiration of oropharyngeal secretions. Pharmacologic drying of secretions can offer some help, but most patients remain at risk for aspiration pneumonia. Chemical pneumonitis is also a risk in patients with reflux, and so acid control may also be beneficial.

For some families, placement and use of a gastrostomy tube may not be consistent with a goal of minimizing procedures or risks as opposed to allowing a more natural end of life to unfold. Such families may choose to either limit nutrition and hydration or to continue to provide oral nutrition and hydration despite the inherent risks. When eating provides significant pleasure to the patient, choosing to maintain oral feeding or even continuing to offer "tastes" may positively impact quality of life. For ease some families may choose to use a gastrostomy tube for nutrition and medication administration. Such decisions should be made with sensitivity to the role of parents in providing sustenance for their children and the social interaction inherent in meal time.

WHEN DEATH IS IMMINENT

Escalating Symptoms

For children with chronic conditions and progressive disease, escalating symptoms may occur over days, hours, or minutes. Ideally family goals are known and interventions can be recommended that are consistent.[79] A palliative care team often can assist the primary providers by anticipating what interventions are likely to be the most helpful and to cause the least suffering and in strategizing about how to carry out plans for care. It may be helpful to have an emergency symptom management plan (including pharmacologic and nonpharmacologic interventions) in place to initiate during an inpatient admission or at home. This process facilitates efforts of all involved to anticipate and proactively manage physical and emotional symptoms.

Families need to be reassured that there is no "right" place to be as the child nears end of life. Instead, families should choose what best meets the needs of the child and whole family. Homecare services vary widely between regions and countries.[87,222,223] Hospitals may have the advantage of multiple providers, quicker access to therapies, and even a special room such as a "comfort corner," but it is never "home."

For a patient experiencing extreme distress in the hospital, one strategy is to have algorithms or template orders in place for intravenous administration of medications with starting doses and resources to be accessed. Such an escalating situation for a child at our institution prompted nurses, physicians and pharmacists to develop a set of orders for management of escalating pain, dyspnea or agitation, using morphine, fentanyl, or hydromorphone, to improve care in these relatively rare but highly demanding cases.[123] Some general recommendations for rapid escalation include the following:[123]

- Bedside titration when symptoms arise, with intravenous bolus dosing every 10 to 15 minutes until pain is relieved
- Initial bolus dose of 10% of the 24-hour intravenous morphine dose for patients already on opioids
- Increase of intravenous bolus dose by 30% to 50% every third dose for unrelieved pain

- Initiation of continuous infusion once pain is relieved, with additional dosing, approximately equivalent to hourly rate, for breakthrough pain
- Increase of hourly intravenous rate and bolus by 30% to 50% when pain is unrelieved by existing regimen

At some institutions, a 24-hour on-call palliative care service or pain treatment service is available for assistance. Often allowing children who are facing the end of life to be directly admitted to their usual inpatient unit, with staff members who know them, will lessen the anxiety of the family and child and allow for faster, individualized care.

Palliative Sedation

Palliative sedation may be considered in rare circumstances. Sedation for refractory symptoms requires pain and palliative care expertise and a trusting relationship with the child and parents of the dying child. It must be clear that it is not the same as euthanasia and is not intended to hasten death, although parents who are desperate to see their child at peace may ask for that. When a child's refractory or unendurable symptoms cannot be managed by the "best standard" with opioids, benzodiazepines, and all other adjuncts, then palliative sedation may be warranted.[224,225]

Palliative sedation has the expressed outcome of relieving patient suffering and is considered only when physical and/or psychological symptoms are refractory to all other reasonable medical and complementary therapies.[226,227] Medications are most commonly started by a continuous infusion and titrated to the effect that the patient and/or parents and medical team have agreed upon. The most common agents used are anxiolytic sedatives, sedating antipsychotics, barbiturates, or general anesthetics.[126,226,228,229] Suggested dosing may be found in *Fast Facts*[230] available at www.eperc.mcw.edu or the *National Hospice and Palliative Care Compendium.*[231]

Before beginning palliative sedation, a frank discussion needs to be held with the family as well as the health care team so that any questions, concerns, or misgivings may be addressed.[232] Staff and/or family members may express ethical concerns that may be addressed by the pain or palliative care team, chaplaincy, or ethics consultation service. Ethical principles, including the "principle of double effect" and "principle of proportionality," justify the use of such measures when the benefits outweigh possible unintended but foreseeable consequences. Allowing for a full discussion of any ethical concerns, as well as the consistent intent to relieve suffering,[232] may be helpful. The patient and/or family and staff need to agree on the symptoms, amount of distress, and reasonable outcomes of interventions. Being certain that the patient and family have had the opportunity to express any final thoughts or feelings while the loved one is awake and conversant may be important to reduce the subsequent potential for regret.

Talking with Parents About What to Expect

Parents usually want to know and often ask what to expect as their child nears death.[16] In particular parents often want to know how to achieve comfort for their child, both with medications and with nonpharmacologic interventions. Once it is clear that there is very little likelihood of the child's clinical situation being stable or improving, families want to know when their child will die. Using parameters of prognosis such as "hours to days," "days to weeks," or "weeks to months" can be helpful because so often more specific prognostication is wrong.

Families want to know what death will look like and what to look for, so that they may be ready and be present at the moment of death. Some may wish to keep cardiac and respiratory monitors turned on to help them be prepared and know when it might be "safe" to leave the bedside, if only momentarily. It may be helpful to describe likely physical changes such as decreased mobility, weakness, loss of interest in eating or drinking, and decreased interest in interacting.[83] Being frank about decreased urine output, cool and mottled extremities, vital sign and breathing changes, respiratory congestion, and decreased level of consciousness in the final days can allow the parents to recognize the situation when it occurs.

At this time, it is important to help families focus on being with their child in whatever way is helpful to the child and family. Highlighting what family members can do for their loved one, for example, a simple hand or foot massage, retelling favorite family stories, playing music, praying, reading poems, and simply being present are examples.

Despite our best efforts, not every death is peaceful. Although assuring families that we will do everything possible to alleviate suffering and allow for a peaceful death, we cannot make promises. Some families interpret this as their child "fighting to the end." Making sure staff is present, listening, and responding to anyone's distress. During such times, it is important to reaffirm the positive role of the parents, siblings, and grandparents throughout the child's life.

Talking about Autopsy

The discussion about autopsy is often dreaded by staff, and sometimes it is avoided altogether, although ideally it is discussed by a trusted caregiver before the death. Some families ask about organ/tissue donation, and autopsy may be discussed similarly. Some families may believe that the child has "been through enough"; however some believe that this is a way to advance science and help other children who may suffer from the same condition. Autopsy may answer some of the questions about disease, cause of death, or side effects of treatments. Tissue or organ donation, including donation to science through autopsy, may be a part of a child's legacy. Studies have shown adults and children are altruistic even when facing death,[233] and so autopsy may be offered as a kind of "gift" to future affected children, to medical science, and to the training of physicians who are seeking cures to diseases. In addition to inviting the family to meet for a bereavement visit, inviting them to review the autopsy results with trusted providers and a pathologist may allow lingering questions or concerns that may be causing parental distress to be explored.[234]

Care of the Child’s Body after Death

After death, whether at home or in the hospital, families often value the opportunity to have time for memory-making activities, family rituals, sharing of stories, and having staff come to pay respect. They may also want to participate in washing or dressing the body. In the hospital, one of the most difficult moments comes when the family leaves the hospital without their child. Having one staff member stay with the body while another accompanies the family to the lobby may be comforting. At home sometimes a parent chooses to carry the body to the hearse rather than have staff remove the body. Again having home care/hospice staff be present through this time may assist the family by bearing witness and holding the sacred space in an unspoken way. Finally, reassuring the family of bereavement follow-up allows them to know that continued contact will occur and is available.

AFTER DEATH

Bereavement Care for Families

Bereavement is a term used to describe the overall experience of family members and friends in the periods before, during, and after the death of a loved one.[235] It includes the unique grief (the emotional/internal process experienced as a result of the loss) that family members and loved ones feel as well as the mourning process (the more external process related to changing one’s environment as a result of the loss) for the loss of a child.[236]

Bereavement begins upon the diagnosis of any pediatric life-limiting condition, because the diagnosis, in itself, signifies a loss of normal life and the beginning of significant emotional and physical adaptations on the part of the family. Health care providers can begin to support the family before the death of the child by helping the family to stay connected with the child, communicate effectively with the child, process the concept of the “appropriate death,” and develop memories. In addition, health care providers may help the family to negotiate the medical system, obtain respite care, and assist with the “prethinking” of the funeral.[237] Having this type of system in place before the death of the child can help the family begin to adapt to life without their child.[238]

The grieving process for families who have just lost a child is typically fraught with the ache of coming to the realization that they will no longer see or be with their child. This process is often layered with a sense of guilt as well—guilt that stems from questioning their treatment decisions, thoughts that they caused the illness, as well as guilt from perhaps giving more attention to the ill child than other siblings.[237] The two-track model of bereavement, described by Rubin,[239,240] can guide interventions in dealing with the grief of family member by first (track one) assessing the individual’s functioning (for example his or her feelings of anxiety, depression, guilt, return to work) and then (track two) assessing the relationship of the individual to the deceased person, even as it continues to evolve and change in meaning over the course of the surviving relative’s life span. Similarly via review of recent literature on parent grief, Davies rejects certain notions that bonds with the deceased individual must be broken for grief to be resolved or to obtain closure. Instead she concludes that “exploring the significance of their children’s lives, as well as the continuing influence they have through continuing bonds, may be regarded as a positive means [of] dealing with bereavement.”[241(p 510)] Others emphasize attempting to understand patterns of individual differences in grieving families and how continuing the bond with the deceased while relinquishing certain ties can be adaptive to the family functioning on a long-term basis.[242]

Spinetta and colleagues measured the after-death (up to 3 years after the death) adaptation of parents whose child had died from cancer. Of 23 sets of parents interviewed, most had “adequately mastered” a return to normal activities, become active and zestful again, become able to confront physical reminders of their child, and had no regret of the past. Other tasks, such as resolving remaining questions and healing relationships with siblings, were found to be more difficult for the bereaved parents.

Four to 6 years after the death of a child due to cancer, bereaved parents were more likely to report depression, low to moderate psychological well-being, and anxiety compared with nonbereaved parents. Overall self-reports of continued grief were associated with more anxiety and depression. The risk for depression and anxiety for bereaved parents resembled that of nonbereaved parents 7 to 9 years after the death of the child; however parents were at an increased risk if their child was 9 years or older at the time of death.[243,244]

Immediate supportive bereavement interventions can take place during end-of-life care. In-depth qualitative interviews with 10 mothers of children who died of a variety of life-limiting illnesses revealed four main needs of the mothers during the end of their children’s’ lives: time with their dying child, space and privacy to be with their dying child, time to be with their child’s body after death, and space and privacy to be with their child’s body after death. Mothers who did not have time, quiet space, and dignity expressed distress and anguish over the unavailability of these things.[245] In a study of 156 bereaved parents, Lichtenthal and colleagues[246] explored how parents brought meaning to the loss of their child. Through qualitative analysis of these parent interviews, *meaning making* was broken down into two themes: (1) sense-making (the use of spirituality and religious beliefs in finding meaning) and (2) benefit-finding (the increased need to offer help and compassion for others’ suffering). The support of *meaning making* for bereaved parents can be a valuable tool for balancing the act of maintaining ties with their deceased child while also facilitating the relinquishing of some ties that may complicate grief.

Shortly after their child’s death, many parents cited that the attendance of members of health care team at their child’s memorial or funeral service tended to be helpful in coping with their loss. Parents also found that formal support groups where they could speak with other parents who had similar experiences, follow-up contact with the health care team to discuss results of the autopsy, contact with the health care team on significant days (e.g., anniversaries of the death, Mother's or Father's Day), and guidance in how to help siblings cope with the loss of a brother or sister proved to be beneficial while they mourned their loss.[247] Other, more long-term, types of supportive care for bereaved families included individual grief counseling for one or both parents, couples counseling, family counseling, and social support groups,[237] including Internet support groups.[248]

Care of Siblings

Siblings of children with cancer are deeply affected by their experiences of the sibling's illness and its effects within the family.[249] Siblings experience profound life changes as a result of the illness and experience intense positive and negative feelings.[250] Bereaved siblings are at increased risk for low self-esteem, have lower levels of personal maturity, and experience more difficulties falling asleep than nonbereaved siblings.[251] In addition the family dynamic and their role within the family are often altered by the illness.[250]

The experiences of bereaved siblings are particularly intense. Their bereavement is often marked by feelings of anger, guilt, jealousy, and sadness.[252] A particular area of distress reported by siblings is a lack of involvement in the dying process.[252] Parents in particular may choose not to involve the sibling closely in the end-of-life period so as to protect them.[253] However it is possible that limited involvement is even more problematic for some siblings than being closely involved in the end-of-life period. Care providers should encourage open communication and allow involvement in care to the extent that siblings wish. Helping parents to understand that such involvement can be healing may be useful.

Care of Staff

Providing care to children with life-limiting illnesses and their families can be one of the most worthwhile acts a health care professional can offer. At the same time providing pediatric palliative care services can place both uniquely physical and emotional demands on the health care team. Pediatric oncologists need to be aware of the impact of these stressors on themselves and colleagues and take the appropriate steps in maintaining both a healthy physical and emotional well-being. Zambrano and associates[254] investigated how general practitioners processed the death of their adult patients. The general practitioners who participated in the study used the metaphor of a "death journey" that involved five different moments that depicted their experience with dying patients: private acknowledgment, communication of prognosis, continuity of care, the moment of death, and looking after the family. A model of self-awareness for grief in caregiving as described here is a positive step in acknowledging the very normal emotions that practitioners experience when caring for sick patients and thus opens the door for self-care and prevention of burnout at each stage of the caregiving process.

Stressors identified by hospice nurses and physicians have included care of patients with intractable symptoms, difficult communication issues, administrative concerns and paperwork, too many patients dying at the same time, the expectation (among family members, other staff, or self-imposed) that all the patient's problems can be fixed, as well as becoming overinvolved in the care of their patients.[255-257] When pediatric residents were asked to rate their personal experiences regarding death, similar emotional stressors such as guilt and feelings of responsibility over the child's death were prevalent.[258]

Self-care can take the form of staff support sessions, educational seminars, as well as mechanisms created within the medical system to relieve professionals of patient service time to restore physical and emotional well-being. Methods for staff support can include regular, confidential debriefing sessions consisting of time for reflection with opportunities for staff to share challenges as well as effective ways for coping with these challenges.[259] These debriefing sessions can either have a specific focus (e.g., processing the death of an individual child) or can have a more general focus that might take into account the day-to-day issues that arise from providing pediatric oncology and palliative care services.[260] Other types of staff support sessions can take the form of annual or semi-annual memorial services to give health care teams a designated day to remember those children they cared for throughout the year.[255,261]

Often a combination approach of providing educational seminars regarding palliative care topics as well as time set aside to discuss and process emotions can be quite effective in gathering a larger crowd of professionals who may not necessarily attend one type or another. For example a combination approach could include a 1-day[262] or several-day death and bereavement retreat for residents to help them achieve the following goals: gain experience in talking about child death with parent; practice being in emotional situations; provide knowledge about autopsy, organ donation, and ministry; provide resources for supporting the family; gain insight into the impact of death on themselves and prevent burnout from stress; and provide resources to better understand the parents' perspective.[263-265]

Sumner suggests opportunities for professional growth for the seasoned professional as well as new providers of pediatric oncology and palliative care services through mentorship programs. This type of program would encourage, for example, a new physician or pediatric oncology provider be partnered with an "expert colleague."[255] The mentor, in turn, may develop the sense of satisfaction of self-efficacy in training a new member of the team in providing pediatric palliative care.

ONGOING CHALLENGES

High-quality palliative care is now an expected standard,[28,80] and Hospice and Palliative Medicine has been recognized as a formal medical subspecialty.[266] The American Academy of Pediatrics set the following as a minimum standard for pediatric palliative care: "Excellence in pediatric palliative care is essential for hospitals and other facilities caring for children. Program development in pediatric palliative care, along with community outreach and public education, must be a priority of tertiary care centers serving children.”[80]

Although the principles of pediatric palliative care have been defined and refined over recent decades, notable challenges remain in all domains of care, including communication, symptom management, coordination of care, and bereavement. The field of pediatric palliative care is still in need of rigorous research efforts, and these efforts should be integrated into curricula across disciplines and at all levels. Further advocacy aimed at creating greater opportunity for children to receive palliative care across care settings is essential.[28]

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CHAPTER 71

Symptom Management in Children with Cancer

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CHAPTER OUTLINE

PAIN
Epidemiology
Pathophysiology
Assessment
Approach to the Child with Pain
Analgesic Medications and Interventions
Adjuvant Treatments
Invasive Approaches to Pain Management
Psychological and Nonpharmacologic Approaches to Pain
Pediatric Cancer Pain Syndromes
Postoperative Pain
Procedure-Related Pain
Reassessing Interventions for Pain
Barriers to Effective Pain Management
NAUSEA AND VOMITING
Pathophysiology
Chemotherapy-Induced Nausea and Vomiting
Radiation-Induced Nausea and Vomiting
Postoperative Vomiting
Vomiting from Other Causes
Nonpharmacologic Interventions
Role of the Patient and Family
Future Directions
OTHER GASTROINTESTINAL SYMPTOMS
Constipation
Bowel Obstruction
Diarrhea
DYSPNEA
Pathophysiology
Diagnosis and Assessment
Management
Future Directions
COUGH
FATIGUE
Impact
Approach
Measurement and Assessment of Fatigue in Children
Associated Factors
Interventions
Future Directions
PRURITUS
Interventions
MOOD DISORDERS AND MENTAL STATUS CHANGES
Depression
Anxiety
Delirium
Corticosteroid-Induced Mood and Behavior Disturbance

Progress in understanding the causes of pediatric cancer, as well as advances in cancer-directed therapies, hold great promise for curing and extending the lives of many children diagnosed with cancer. However, as advances in medicine and technology improve the survival of children with life-threatening illnesses, attention to health-related quality of life and progress in symptom management have not kept pace with advances in disease-directed therapies. As a result a population of children exists who are living with cancer and have suboptimally controlled symptoms.

Amelioration of symptoms experienced by a child with cancer does far more than reduce the suffering caused by the troublesome symptoms. Control of physical symptoms may allow attention to be focused on other issues faced by family members who are living with a child's cancer, such as psychosocial concerns or existential distress, and it may allow them to participate in activities and interactions that are important for maximizing quality of life. In fact, symptom burden is highly associated with quality of life in children with cancer[1] and is one of the most significant determinants of quality of life in adolescents and young adults with cancer.[2] In some instances, improved control of symptoms may enhance the delivery of optimal cancer-directed therapy as well. Finally, optimal symptom control throughout the illness trajectory may also shape the child's and family's long-lasting impressions of their experience with cancer.

In many instances, relief from distressing symptoms is possible with a myriad of modalities available today. Pediatric oncologists play a key role in managing symptoms in their patients. Symptom management requires actively partnering with families to assess not only the presence of symptoms but also the impact of uncontrolled symptoms on their daily lives, as well as to implement appropriate symptom-directed interventions. Ideally such attention to symptoms occurs in the context of attention to the child's overall condition so that the impact of symptoms on the child's overall quality of life is appreciated.

The vast majority of children who have cancer experience multiple symptoms that could be ameliorated but are not addressed.[3,4,5,6,7] For example, Collins and colleagues[3] asked 10- to 18-year-old children about the symptoms they experienced during the past week. Symptoms included lack of energy (49%), pain (49%), nausea (45%), lack of appetite (40%), and itching (33%).[3] The prevalence of uncontrolled symptoms in children with cancer is likely to stem from a variety of systemwide causes, including inadequate formal training dedicated to symptom management, a focus on cancer-directed treatment, and time constraints.

In addition, a lack of systematic research in the pediatric population, particularly with regard to non–pain-related symptoms, leads to a lack of evidence or standards on which to base interventions. Many symptom-directed interventions for children at this time are based on extrapolation from adult studies or even individual or anecdotal experiences. Some guidelines exist that are strongly evidence based, such as the National Comprehensive Care Network (NCCN) Guidelines for Pediatric Cancer Pain, which is encouraging.[8] As advances in pediatric oncology extend into the realm of symptom management, additional guidelines for non–pain-related symptoms will become available to help pediatric oncologists attend to their patients' symptoms.

The promising survival rates that have resulted from discoveries in pediatric oncology have led to increasing attention to health-related quality of life and the human costs of cancer care.[9] With such a shift, efforts to understand and ameliorate the myriad of symptoms experienced by children with cancer are likely to be increasingly supported in the coming years.

PAIN

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage."[10] This symptom is the most studied and best understood of all cancer symptoms in adults and children alike. However, despite such attention, pain is often suboptimally controlled.

Families and children confronting cancer often worry about potential pain due to the disease and its treatment. In fact, pain is the most feared problem for children with cancer.[11] To some extent, their concern is justified. For example, Collins and colleagues[3] demonstrated that children with cancer experience multiple symptoms, including pain. Among 160 children ages 10 to 18 years with cancer, pain was the second most common symptom, with a prevalence of 49.1%. In addition, 61.6% reported pain that was moderate to very severe in the 48 hours prior to completing the questionnaire, and 40.9% reported pain that occurred "a lot" to "almost always." Moreover, the pain experienced by children was distressing—"quite a bit" to "very much" in 39.1% of respondents.

Pain control provides a variety of benefits beyond the amelioration of suffering. Prompt pain relief is needed to prevent central sensitization, a centrally mediated hyperexcitability response that may result in escalating pain. Uncontrolled pain also leads to a physiologic stress response with a variety of effects such as altered metabolism and immune function. Control of pain with appropriate analgesia in the perioperative period can reduce some of these effects and prevent complications.[12]

Epidemiology

In general, pain experienced by children with cancer may be caused by a variety of entities, including the disease itself (e.g., tumor invasion of bone, viscera, or the peripheral or central nervous system [CNS] or compression of the spinal cord), treatment (e.g., mucositis, radiation-induced dermatitis, and drug-induced neuropathy) or procedures (e.g., venipuncture, lumbar puncture, and bone marrow aspiration or biopsy, as well as postoperative pain). Authors who performed a cross-sectional analysis of pain in inpatient children with cancer found that the most frequent cause of pain was adverse effects of antitumor therapy.[13] Evidence also indicates that children who have solid tumors outside of the CNS have more pain and higher opioid requirements.[14]

Because the majority of pediatric cancers respond at least initially to treatment, most of the pain that children experience early in the disease trajectory is related to procedures and treatment. Later, if the cancer progresses, pain is more likely to be due to tumor extension. In a series of structured interview surveys conducted by Ljungman and colleagues,[15] 49% of children with cancer experienced pain at diagnosis. Procedure- and treatment-related pain were the most significant types of pain at the start, and although procedure-related pain improved, treatment-related pain did not improve. In addition, measurement of pain intensity was rarely performed.[15]

A significant barrier to pain management in children stems from the fact that research and development of evidence-based practice guidelines in pediatrics lag behind that in adults. The NCCN and World Health Organization (WHO) have developed comprehensive guidelines for managing pain in children with cancer,[16] but in many instances, pain management in children relies on extrapolation from adult data, anecdotal reports, and personal experience.

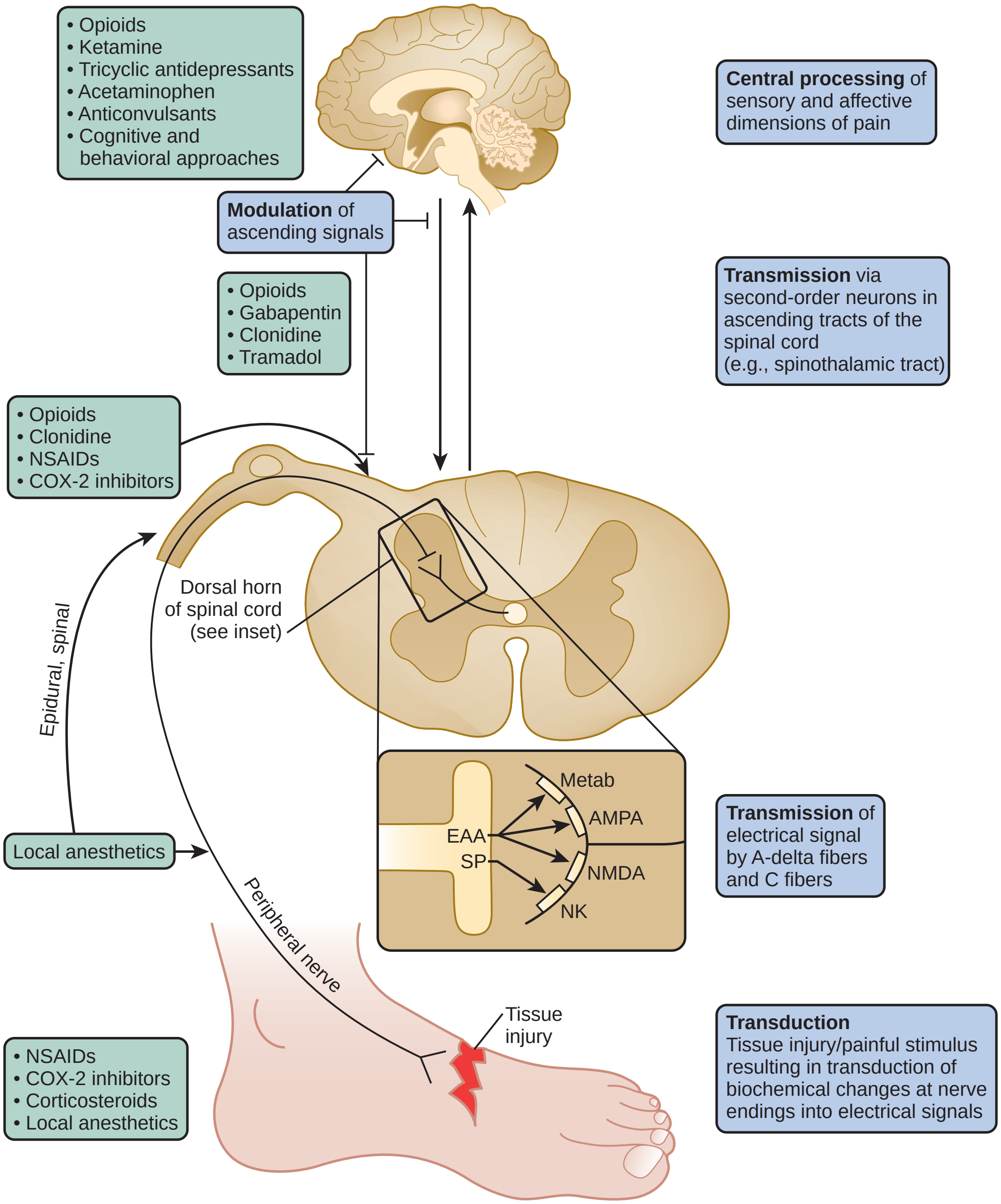

• Opioids
• Ketamine
• Tricyclic antidepressants
• Acetaminophen
• Anticonvulsants
• Cognitive and behavioral approaches
Central processing of sensory and affective dimensions of pain
Modulation of ascending signals
• Opioids
• Gabapentin
• Clonidine
• Tramadol
Transmission via second-order neurons in ascending tracts of the spinal cord (e.g., spinothalamic tract)
• Opioids
• Clonidine
• NSAIDs
• COX-2 inhibitors
Epidural, spinal
Dorsal horn of spinal cord (see inset)
Metab
AMPA
EAA
SP
NMDA
NK
Transmission of electrical signal by A-delta fibers and C fibers
Local anesthetics
Peripheral nerve
Tissue injury
Transduction Tissue injury/painful stimulus resulting in transduction of biochemical changes at nerve endings into electrical signals
• NSAIDs
• COX-2 inhibitors
• Corticosteroids
• Local anesthetics

Figure 71-1 Anatomic illustration of a nociceptive pathway and corresponding analgesic action sites. Major elements of nociception are indicated in *blue boxes*. Analgesics are indicated in *green boxes*. *Inset,* Neurotransmission and neuromodulation in the dorsal horn of the spinal cord. Analgesics: *COX-2,* Cyclooxygenase-2; *NSAIDs,* nonsteroidal antiinflammatory drugs. Neurotransmitters: *EAA,* Excitatory amino acids; *SP,* substance P. Receptors: *AMPA,* Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic; *Metab,* metabotropic; *NK,* neurokinin; *NMDA,* N-methyl-d-aspartate.

Children may also experience incidental, non–cancer-related pain. Other pain-inducing disorders such as migraines, recurrent abdominal pain, or injuries seen in the general pediatric population can also occur in children with cancer. These causes of pain unrelated to the cancer diagnosis should always be included in the differential diagnosis of pain in children who have cancer.

Pathophysiology

Nociception is a complex process whereby actual (or potential) tissue damage is perceived as pain by an individual. In many instances pain is a protective mechanism that alerts a person to tissue injury. Nociception may be thought to occur on three levels: peripheral, spinal, and supraspinal (Fig. 71-1). Through transduction, the primary afferent nociceptors, which are thinly myelinated A-delta and unmyelinated C fibers, transmit biochemical changes at the sensory nerve endings that are generated by painful (chemical, thermal, or mechanical) stimuli into electrical signals. Stimulation of such nerve endings in the periphery may be increased or decreased by molecules such as prostaglandins, leukotrienes, bradykinin, and histamine. For example, the bradykinin and histamine that accompany tissue inflammation can directly activate nociceptors, in addition to reducing their threshold and increasing their response to suprathreshold stimulation.[17] The analgesic effect of nonsteroidal antiinflammatory drugs (NSAIDs) lies in their ability to inhibit prostaglandin synthesis and thus desensitize nociceptors.[18]

The electrical signals generated are propagated, or transmitted, to second-order neurons that synapse with sensory nociceptors in the dorsal horn of the spinal cord. Second-order neurons convey signals via ascending tracts in the spinal cord, including the anterolateral spinothalamic tracts, to supraspinal sites that include the brainstem, thalamus, and cortical areas involved in the sensory and affective dimensions of pain.

For example, descending inhibitory pathways from the thalamus and brainstem modulate excitatory transmission through such inhibitory neurotransmitters as serotonin, norepinephrine, and endogenous opioids. Several pharmacologic interventions such as opioids and tricyclic antidepressants (TCAs) exert their analgesic effects through such inhibitory processes at the spinal and supraspinal levels.

Pain is a complex sensory and emotional experience that involves nociception but is modified by a range of contextual and psychological factors. Because the experience of pain is subjective, the degree of tissue injury and therefore nociceptive input does not necessarily correlate with the intensity of pain experienced. Interventions that may reduce the perception of pain include hypnosis and relaxation techniques.

Pain that results from stimulation of intact neurons by impulses reflecting tissue injury or inflammation is called *nociceptive pain*. On the other hand, pain resulting from abnormal excitability of neurons (for example, due to neuronal damage) is called *neuropathic pain*. Even if tissue damage initially accompanied neuropathic pain, neuropathic pain may persist long after the damage has resolved. Nociceptive pain often may be distinguished from neuropathic pain by its characteristics. Neuropathic pain frequently is described as burning or shooting and is often associated with paresthesias or allodynia (i.e., elicitation of pain by normally nonpainful stimuli such as light touch).

Factors Influencing a Child's Experience of Pain

As a subjective experience, many factors have an impact on a child's experience of pain. Recognizing such factors can facilitate an understanding of the pain as it is experienced by the child and facilitate development of an effective pain treatment plan. The experience of pain shapes learning in infancy and throughout life. The neurobiologic mechanisms underlying nociception develop during the third trimester. Specific cortical responses to noxious stimuli can be demonstrated in 32-week preterm infants using evoked potentials or near-infrared spectroscopy. The nature of pain as a conscious experience in young infants remains a subject of speculation and controversy.[19]

Factors influencing the perception and meaning of pain that occurs after infancy are both individual and contextual and include developmental and cognitive factors (e.g., understanding, control, expectation, and relevance) and behavioral and emotional factors (e.g., anxiety, fear, frustration, anger, guilt, and isolation), as well as familiar and cultural factors.[20-22] A variety of other factors including age, gender, pain acceptance, and pain tolerance have been hypothesized to influence pain perception in the pediatric population and have been summarized elsewhere.[23]

Importantly, previous encounters with pain may heighten the experience of subsequent encounters with pain. For example, children newly diagnosed with cancer who had inadequate procedural analgesia when undergoing a first bone marrow aspirate or lumbar puncture had more severe distress during subsequent procedures, even when efficacious pain relief was subsequently provided.[24] This finding highlights the need to provide effective analgesia to prevent both present and future painful experiences.

Assessment

Pain assessment and measurement provide the foundation for addressing pain effectively. Regular assessment of pain may improve pain management[25,26] and should be conducted in a developmentally appropriate manner. When permitted by the child's developmental status, self-report is considered the gold standard in pain assessment. Because self-report can include bias and error, behavioral observations, physiologic changes, and clinician/parental report may be incorporated into the pain assessment. However, these methods also have inherent limitations. For example, tachycardia may reflect fever or intravascular volume depletion rather than pain.

Physiologic and behavioral signs and symptoms can indicate pain, but lack of these signs does not indicate absence of pain, particularly in chronically or very ill children, in whom these indicators are unreliable. In one study comparing a behavioral pain measure with two self-report measures, many children who reported severe pain showed few behavioral pain indicators.[27] In addition, these signs are not necessarily specific for pain itself and may reflect distress that may or may not be related to pain. Parental and clinician estimates of the child's pain also have limitations, because clinicians and parents frequently underestimate pain.[28,29]

Examples of symptom assessment tools for children of various ages and developmental capacities are demonstrated in Figure 71-2. These tools may be helpful in assessing symptoms and measuring severity before and after interventions. However, they are not all validated specifically for the population of children with cancer.[30] A given tool should be used consistently with a given child. When the assessment reveals the presence of pain, further inquiry regarding the nature of the pain (e.g., the character of the pain and aggravating and alleviating factors) and the meaning that the pain holds for the child and the family is in order.

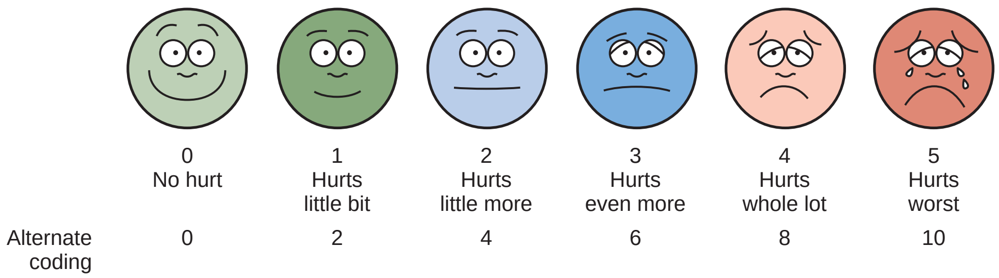
Self-Report Instruments

Many children 3 years of age or older are able self-report their symptoms. Because some young children, FLACC Behavioral Scale: Recommended for Children <3 Years of Age

Categories*	Scoring* 0	1	2
Face	No particular expression or smile	Occasional grimace or frown, withdrawn, disinterested	Frequent to constant quivering chin, clenched jaw
Legs	Normal position or relaxed	Uneasy, restless, tense	Kicking, or legs drawn up
Activity	Lying quietly, normal position, moves easily	Squirming, shifting back and forth, tense	Arched, rigid, or jerking
Cry	No cry (awake or asleep)	Moans or whimpers; occasional complaint	Crying steadily, screams or sobs, frequent complaints
Consolability	Content, relaxed	Reassured by occasional touching, hugging, or being talked to, distractable	Difficulty to console or comfort

A

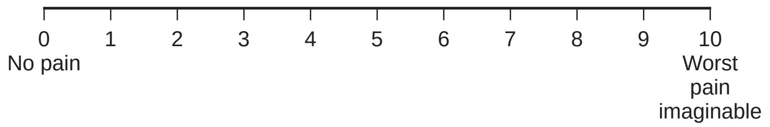
Wong-Baker FACES Pain Rating Scale: Recommended for Children ≥3 Years of Age



Brief word instructions: Point to each face using the words to describe the pain intensity. Ask the child to choose face that best describes own pain and record the appropriate number.

B

Numerical Rating Scale: Recommended for Children ≥8 Years of Age



C

Figure 71-2 Pain assessment instruments. **A,** The Face, Legs, Activity, Cry, Consolability *(FLACC)* Scale, recommended for use in children younger than 3 years. Each of the five categories is scored from 0 to 2, which results in a total score between 0 and 10. **B,** The Wong-Baker FACES Pain Rating Scale, recommended for children 3 years of age and older. Brief word instructions: Point to each face, using the words to describe the pain intensity. Ask the child to choose the face that best describes his or her own pain and record the appropriate number. **C,** The Numerical Pain Rating Scale, recommended for children 8 years and older. *(**A,** From Merkel SI, Voepel-Lewis T, Shayevitz JR, et al: The FLACC: A behavioral scale for scoring postoperative pain in young children.* Pediatr Nurs *23:293–297, 1997;* **B** *and* **C,** *Modified from Hockenberry MJ, Wilson D, Winkelstein ML:* Wong's essentials of pediatric nursing, *ed 7, St. Louis, 2005, Mosby, p. 1259.)*

particularly those who are chronically ill, may be more mature than their chronologic age, the responses even of young children should be heeded. Self-report scales for children include faces scales, color analogue scales, visual analogue scales,[31] the Poker Chip Tool,[32] and numeric rating scales. With the faces scales, such as the Wong-Baker Faces Scale[33] and the Bieri Faces Pain Scale,[34] the child is asked to match how he or she feels with one of the faces. Color analogue scales and faces scales can be used by most children ages 4 years and older. Faces scales differ psychometrically; an example is the use of a smiling face for the "no pain" anchor in the Wong-Baker Faces Scale versus a neutral face for the "no pain anchor" in the Bieri Faces Pain Scale. Although children often report that they like using the Wong-Baker Scale, some researchers regard the use of a neutral face for the "no pain anchor," as in the Bieri Faces Pain Scale, to be psychometrically more specific. With the visual analogue scale, a child selects a point on a line that represents the intensity of his or her pain. These scales have been extensively studied and are appropriate for children 8 years and older.

Numeric rating scales do not require any equipment, are simple to use, and are already used frequently with adults. These scales require numeracy and the ability to think and express oneself in quantitative terms and thus are most appropriate for use in children who are at least 8 years of age.[35] Children younger than 8 years may provide an unreliable numeric response because, although they can count, they have do not have an understanding of the quantitative meaning of numbers.[35] All quantitative scales are based on the concept of counting, which is a universal concept for children who have this developmental capacity. It is therefore possible to develop quantitative tools for pain measurement that are appropriate for children of virtually all cultures.[36]

Behavioral Observation Scales

Behavioral and physiologic cues are particularly useful in preverbal children and in children who are not able to verbalize their symptoms because of cognitive impairment, developmental capacity, or sedation. Such scales may rely on facial expressions,[37] motor or verbal responses, or combinations of behavioral and autonomic responses.[38] Behavioral scales may actually rate distress, including fear and anxiety, rather than specifically assessing pain per se.

When using behavior to assess pain, it is important to partner with parents or other caretakers who are particularly familiar with the child, because knowing the child, having familiarity with children who have the same or similar conditions, and having a thorough grasp of the science of symptom management are all important components of effective pain relief.[39] For example, the Paediatric Pain Profile was created to be a usable document for parents to assess and record their child's pain behavior. It is a well-validated instrument that uses behavioral cues, including changes in facial expression, vocal sounds, posture and movements, sleeping, eating, and mood, to assess pain.[40] The Individualized Numeric Rating Scale is an individualized scale for nonverbal children.[41] This scale is an adaptation of the numeric rating scale; it allows parents or other clinicians who know the child well to identify the child's typical pain behavior and rank that behavior on a standard scale from 0 to 10.

The Face, Legs, Activity, Cry, Consolability (FLACC) tool was originally designed to score postoperative pain in children ages 2 to 7 years[42] but has also been validated for postoperative use in children with cognitive impairment.[43] Use of this instrument may be advantageous because it employs a variety of types of measures such as activity and facial expression. Further work in the area of pain assessment in children with developmental or cognitive impairment is needed because children in these vulnerable populations are less likely to be assessed for pain, in addition to receiving less analgesic medication.[44]

The previously discussed assessment scales largely reduce pain assessment to the measurement of pain intensity. Although such pain assessment is an oversimplification, the scales permit rapid evaluation of pain and rapid institution of interventions aimed at ameliorating pain. Such scales are also key outcome measures in evaluations of pain-relieving interventions. Findings from a recent study, however, highlight the fact that such a simple screening for pain intensity fails to identify many persons who have significant functional interference from pain or pain significant enough to trigger a physician visit.[45] Regardless of the scale used, if clinical pain indicators are unclear, a trial of measures to ameliorate pain may clarify the cause of distress or pain.

Beyond Assessment Instruments

Beyond use of formal assessment tools, key elements in the history include alleviating/exacerbating factors; the quality, location, onset, and severity of pain; and the degree of impact on the child's function and well-being. Understanding the child's previous experiences with pain and strategies that have been used successfully in the past to address pain are also key components of the history and may inform the treatment plan. To this end a multidimensional indicator of pain can help the clinician understand the existence, intensity, and location of pain that a child is experiencing. Use of techniques that employ more than one measure (e.g., self-report, behavioral, and physiologic) permit a more accurate pain assessment.

In addition, it is necessary to understand the holistic nature of pain, rather than pain as merely a physiologic phenomenon. For example, the meaning of the pain, the degree of distress it causes, and the impact of pain and pain treatment on the child's functional capacity and quality of life are crucial elements of the history. Exploration of these issues may elicit exacerbating and potentially modifiable factors related to the pain experience. Finally, it is important to gain a clear understanding of the beliefs that the child and family have about pain, as well as their treatment goals in terms of pain control.

Approach to the Child with Pain

Anticipation and prevention of pain is the most effective treatment approach. Just as children and parents need information regarding their cancer treatment so they know what to expect, informing them in a sensitive manner about the symptoms they may expect and strategies available for reducing their symptoms can reduce anxiety about the unknown. In many cases, without open conversations in which expectations can be addressed, the imagined reality that is substituted is far worse than the true reality. By helping children understand what will happen and how the treatment or procedure will work, their fear and anxiety may be allayed, thereby reducing the symptoms they may actually experience.

When a child reports pain, careful assessment with use of history and physical examination is imperative to generate a complete differential diagnosis. Determination of the underlying cause of pain may inform consideration of which modalities are likely to be most effective in alleviating it. Both pharmacologic and nonpharmacologic (e.g., cognitive, behavioral, physical, and supportive) approaches should be considered, including addressing factors that are likely to affect the child's experience of pain. Once a pain treatment plan is implemented, pain should be regularly reassessed. The goal of pain management should be to achieve the degree of comfort that the child finds satisfactory.

To provide optimal pain control and prevent breakthrough pain, analgesics should in most cases be administered "By the ladder, by the clock, by the appropriate route, by the child" (WHO), and behavioral, physical, and cognitive supports should be provided throughout treatment. Pain of a moderate to severe rating should be treated with analgesics that are administered around the clock, with rescue doses of the same or an alternative analgesic available. Families often find it helpful to understand that preemptive analgesia is far more effective than catch-up analgesia administered for established pain.

Finally, the dosing interval as determined by the pharmacokinetics of the agents considered and the feasibility of their administration by caregivers should also be considered.

WHO Guidelines

The WHO guidelines previously provided a three-step approach to cancer-related pain in children and adults. For mild pain in a child who is not taking any analgesics, an NSAID or acetaminophen was suggested. For moderate pain, a short-acting weak opioid (e.g., codeine) was recommended, and for severe pain, a strong opioid was recommended (e.g., morphine).

The three-step WHO guidelines, which were widely publicized for cancer-related pain, provided an effective approach to pain control for many adults, with published success rates of 69% to 100%.[46-48] However, the three-step ladder consisting of nonopioids, weak opioids, and strong opioids may not be appropriate for some patients with cancer, particularly those with advanced disease. In fact, studies have shown that a two-step ladder in which treatment passes directly from step I (nonopioids) to step III (strong opioids) for mild to moderate pain provides superior pain relief.[49,50] Some medications historically used in step II (e.g., codeine and tramadol) are unlikely to provide better control of moderate pain compared with continuation of acetaminophen and NSAIDs (unless contraindicated) along with titrated doses of a strong opioid. Furthermore, codeine presents safety and efficacy concerns (discussed later), and few data exist to guide the administration of tramadol or other intermediate-potency analgesic agents in children. For these reasons, the most recent WHO guidelines for the management of pain in children with cancer (or other medical illness) are now based on a two-step approach.[51]

The WHO guidelines, which provide an effective, systematic approach to pain that includes strong opioids, have facilitated other WHO initiatives, such as increasing worldwide access to essential medicines for children with painful conditions. The WHO approach, however, may not adequately emphasize a multidisciplinary approach from the start, and thus opportunities to use adjuvant medications (e.g., steroids and local anesthetics) and cognitive or behavioral interventions to reduce pain and pathologic responses to pain may be missed.

Analgesic Medications and Interventions

Nonopioid Analgesics

Nonopioid analgesics are appropriate for mild pain in a child who is not already receiving analgesics. Nonopioid analgesics include acetaminophen, NSAIDs, salicylates, and selective cyclooxygenase (COX-2) inhibitors.

Nonopioid analgesics are frequently used as monotherapy for mild pain and are used together with opioids for more severe pain. Unlike opioids, they do not cause sedation, tolerance, and respiratory depression. When given as an adjuvant to opioids, they enhance analgesia.[52,53] To this end they may be opioid-sparing (i.e., decreasing the amount of opioid required for adequate analgesia),[54,55] thereby also limiting opioid-associated adverse effects. A recent randomized double-blind study of infants who underwent noncardiac surgery demonstrated that children randomly assigned to receive intermittent intravenous (IV) paracetamol with morphine as needed for rescue required only about one third as much rescue morphine as children randomly assigned to receive an IV placebo.[56] Combinations of nonopioids and opioids are available, although dosing of such a combination is often limited by the maximum dose of the nonopioid.

Salicylates and NSAIDs

Salicylates and NSAIDs nonspecifically inhibit cyclooxygenase enzymes, thereby blocking production of a variety of prostaglandins that mediate pain, inflammation, fever, and platelet function, protect gastric mucosa, and maintain a physiologic distribution of blood flow in the liver and kidneys. The permanent effects of aspirin on platelet function via permanent acetylation of cyclooxygenase are of particular concern in patients who have thrombocytopenia because the antiplatelet effect lasts even after the drug has been metabolized. Choline magnesium salicylate (Trilisate) is a salicylate that provides many of the same benefits as those provided by other salicylates or NSAIDs without known antiplatelet activity. Salicylates, however, have been associated with Reye syndrome in children younger than 2 years.

No particular NSAID or route of administration has been found to be superior over another. Even ketorolac, the only parenteral NSAID widely used in the United States, is no more effective than orally administered NSAIDs, especially if doses are compared in an equitoxic range. A single NSAID dose is roughly equivalent to 5 to 10 mg of intramuscular morphine in adults and has fewer adverse effects.[57] NSAIDs are commonly regarded as having a ceiling effect with no added benefit at supramaximal doses, although the doses commonly recommended (based on safety concerns) remain well below the ceiling in most cases. NSAIDs can cause nephropathy, gastritis, and bleeding from reversible platelet dysfunction, a characteristic that may limit their use in patients with cancer who are thrombocytopenic. COX-2 inhibitors (e.g., celecoxib) selectively block production of prostaglandins that mediate inflammation and pain without impairing platelet function and with fewer effects on gastric mucosal integrity compared with traditional NSAIDs, particularly with short-term use.[58,59] Concerns have been raised regarding effects of COX-2 inhibitors on the risk of cardiovascular events in adults. Evidence does not currently indicate that these concerns extend to children with neoplasms, including the small subgroup of children with tumors or vascular anomalies who have an increased risk of thrombotic events.

Acetaminophen

Acetaminophen is a nonopioid analgesic that reduces pain and fever. It may act in part by inhibiting prostaglandin synthesis in the CNS. Unlike salicylates and NSAIDs, which inhibit peripheral cyclooxygenases, it does not have peripheral antiinflammatory properties. Acetaminophen is available in a variety of oral formulations, as an IV formulation (approved in the United States for children ≥2 years), and as a rectal suppository, although rectal administration should be avoided in neutropenic patients. When not contraindicated, rectal administration is helpful for children unwilling or unable to take oral medications, although its absorption is slow and variable, peaking at 70 minutes.[60] Because rectal absorption is less efficient, single doses of 30 to 40 mg/kg can be administered.[60] Subsequent doses should be smaller (20 mg/kg), and the interval between doses should be increased to 6 to 8 hours.[60,61] Dosing of oral or IV acetaminophen is limited to 75 mg/kg/day or a maximum of 4 g/day (whichever is smaller) because of the risk of hepatic toxicity.

Opioid Analgesics

General Considerations. Guidelines for initial opioid dosages are presented in Table 71-1 and are discussed in

TABLE 71-1 Guidelines for Initial Dosages for Opioid Analgesics[*]

	EQUIANALGESIC DOSES		USUAL STARTING IV OR SUBCUTANEOUS DOSES AND INTERVALS			USUAL STARTING ORAL DOSES AND INTERVALS	
Drug	**Parenteral**	**Oral**	**Child <50 kg**	**Child ≥50 kg**	**Ratio of Parenteral to Oral Dose**	**Child <50 kg**	**Child ≥50 kg**
Morphine	10 mg	30 mg (long term) 60 mg (single dose)	Bolus: 0.1 mg/kg every 2-4 h Infusion: 0.03 mg/kg/h	Bolus: 5-8 mg every 2-4 h Infusion: 1.5 mg/h	1:3 (long term) 1:6 (single dose)	Immediate release: 0.3 mg/kg every 3-4 h Sustained release: 20-35 kg: 10-15 mg every 8-12 h 35-50 kg: 15-30 mg every 8-12 h	Immediate release: 5-20 mg every 3-4 h Sustained release: 30-45 mg every 8-12 h
Oxycodone	NA	15-20 mg	NA	NA	NA	0.1-0.2 mg/kg every 3-4 h	5-10 mg every 3-4 h
Methadone[†]	10 mg	10-20 mg	0.1 mg/kg every 4-8 h	5-8 mg every 4-8 h	1:2	0.1-0.2 mg/kg every 4-8 h	5-10 mg every 4-8 h
Fentanyl	100 µg (0.1 mg)	NA	Bolus: 0.5-1.0 µg/kg every 1-2 h Infusion: 0.5-2.0 mg/kg/h	Bolus: 25-50 µg every 1-2 h Infusion: 25-100 µg/h	NA	NA	NA
Hydromorphone	1.5-2 mg	6-8 mg	Bolus: 0.02 mg/kg every 2-4 h Infusion: 0.006 mg/kg/h	Bolus: 1 mg every 2-4 h Infusion: 0.03 mg/h	1:4	0.04-0.08 mg/kg every 3-4 h	2-4 mg every 3-4 h
Meperidine (pethidine)[‡]	75-100 mg	300 mg	Bolus: 0.8-1.0 mg/kg every 2-3 h	Bolus: 50-75 mg every 2-3 h	1:4	2-3 mg/kg every 3-4 h	100-150 mg every 3-4 h

Modified from Berde CB, Sethna N: Analgesics for the treatment of pain in children. N Engl J Med *347:1094–1103, 2002. All doses are approximate and should be adjusted according to clinical circumstances. Recommendations are modified from previous summary tables, including those of a consensus statement from the World Health Organization and the International Association for the Study of Pain.*

IV, Intravenous; *NA,* not applicable.

[*] Doses are for patients older than 6 months of age. In infants younger than 6 months, initial per-kilogram doses should begin at roughly 25% of the per-kilogram doses recommended here. Higher doses are often required for patients receiving mechanical ventilation.

[†] Use of methadone requires additional vigilance because it can accumulate and produce delayed sedation. If sedation occurs, doses should be withheld until sedation resolves. Thereafter, doses should be substantially reduced; the interval between doses should be extended to 8 to 12 hours, or both.

[‡] Use of meperidine, especially long-term use, should generally be avoided if other opioids are available, because its metabolites can cause seizures.

greater detail in this section. Ultimately, the correct dose of opioid is the dose that provides the desired analgesia with acceptable adverse effects. In general, intolerable adverse effects are usually the dose-limiting factor, as opposed to opioids having a ceiling effect. This situation should be explained to parents, as well the fact that, with rare exceptions, *children with cancer who receive opioids for pain control do not become addicted to opioids*. Addiction is an aberrant psychiatric condition in which the person exhibits maladapted, drug-seeking behavior. True addiction is comparatively rare in patients with cancer-related pain. Children who exhibit exaggerated pain behaviors (e.g., demanding pain medication and engaging in manipulative behaviors) are far more likely to be demonstrating pseudoaddiction, in which case their behavior is a reflection of poorly controlled pain.

Addiction should be distinguished from dependence, that is, a physiologic response to opioids in which abrupt removal leads the patient to experience symptoms of withdrawal. It may be helpful to draw a parallel to blood pressure medication, the abrupt cessation of which causes an undesired rebound hypertensive effect because the body has adjusted to the presence of the antihypertensive drug. Addiction should also be distinguished from tolerance, which is another physiologic response of the body to the presence of opioids that requires increasing doses to achieve the same analgesic effect. *The need to adjust dosing to account for tolerance is not an indication of addiction*. Other processes that can resemble tolerance, including opioid-induced hyperalgesia, are discussed later in this chapter.

Opioids do not need to be saved for cases of extreme pain, because increasing pain can often be managed by increasing the opioid dose. In addition, when escalating doses provide marginal benefit, rotating to an alternative opioid may provide better analgesia. It may be helpful to explain to parents that good pain control from the start may improve pain control overall and actually minimize the amount of opioid that is ultimately needed because when good pain control is achieved, the need to use large doses required to catch up to uncontrolled pain can be avoided.

Some children who have cancer, particularly advanced cancer, require extremely high doses of opioids. For example, in a sample of children with advanced cancer, Collins and colleagues[62] found that in some patients the opioid infusions ranged more than 100-fold from 3.8 to 518 mg per hour of morphine equivalent.

Some studies conducted in vitro and in animal models have demonstrated that opioids may promote cancer cell growth by affecting processes such as tumor cell proliferation and migration. For example, opioids at physiologically relevant concentrations promote tumor angiogenesis.[63,64] In addition, the opioid receptor antagonist naloxone and the COX-2 inhibitor celecoxib inhibit angiogenesis, tumor growth, and metastasis in rodents.[64,65] However, other studies demonstrate growth-inhibiting effects of opioids and have been reviewed elsewhere.[66] No evidence to date demonstrates opioid-associated promotion of tumor growth in humans. These preliminary findings should not, by themselves, lead to the conclusion that use of opioids to relieve cancer-related pain should be avoided. The data accumulated to date regarding the potential risks of opioids and the benefits of adequate analgesia are insufficient to recommend limiting opioid use for analgesia.

Developmental Pharmacology. Elements of renal clearance such as glomerular filtration and tubular secretion increase in the first few weeks of life, such that renal clearance commensurate with adult clearance is achieved by 8 months. When compared with children and adults, neonates and infants have reduced hepatic clearance as a result of hepatic enzyme immaturity. In addition, children 2 to 6 years of age have higher clearance than do adults because of a larger liver mass to body weight ratio.[67] As a result, drugs may need to be administered more frequently in children than they are in adults. Other age-related differences, such as changes in body composition and plasma concentrations of drug-binding proteins, can also influence pharmacokinetics.

Choice of Opioid. The usual starting opioid is morphine because of its low cost, wide availability, multiple routes of administration, and familiarity to clinicians. Because of its long history of use in children, it should be considered the first-line opioid in this population unless specific reasons exist to consider alternatives. Alternative opioids including oxycodone, hydromorphone, and semisynthetic and synthetic compounds such as fentanyl and methadone can be used in children. Use of alternative opioids may be predicated on availability, route of administration, presence of organ impairment, and the patient's prior experience with particular opioids.

Renal Failure. Metabolites of morphine, oxycodone, and hydromorphone (discussed later) may accumulate during renal failure. Some metabolites have analgesic activity, which may lead to delayed opioid toxicity (opioid neurotoxicity will be discussed later). For this reason, dosing intervals may need to be increased in patients with renal dysfunction. The pharmacokinetics of alternative opioids such as fentanyl and methadone are not changed with renal impairment, making these opioids better choices in this setting.

Hepatic Failure. Because glucuronidation is relatively well preserved in persons with liver failure, opioids metabolized by glucuronidation (i.e., morphine, hydromorphone, and buprenorphine) are generally better choices than those metabolized by oxidation via liver cytochromes (e.g., oxycodone, fentanyl, and methadone) in this setting.[68] However, because of shunting in persons with liver cirrhosis, the bioavailability of glucuronidated opioids may be increased. For this reason, initial opioid doses should be lower in persons with hepatic impairment.

Weak Opioids. Tramadol is an atypical analgesic with some direct noradrenergic and serotonergic agonist action and with an active metabolite that is a weak opioid. Tramadol has numerous drug-to-drug interactions that should be considered. For example, it can produce seizures by itself, but this risk is greatly increased when tramadol is administered in combination with several classes of antidepressants. It is available in oral immediate and extended-release preparations and in combination formulations with acetaminophen.

Although codeine has historically been recommended as a weak opioid for mild to moderate pain, it has several limitations. The variable expression of the CYP2D6 enzyme that biotransforms codeine leads to unpredictable levels of the active metabolite, morphine. In the fetus, CYP2D6 activity is absent, and in children younger than 5 years, CYP2D6 activity is less than 25% that of adult activity.[51] Furthermore, certain genotypes are associated with reduced enzyme activity, regardless of age. In a study of 96 children randomly assigned to receive either morphine and diclofenac or codeine and diclofenac after an adenotonsillectomy, it was found that 47% of children had genotypes associated with reduced enzyme activity and that neither morphine nor its metabolites were detected in 36% of children with reduced CYP2D6 activity genotypes who received codeine, although the study did not account for other factors influencing catalytic rate, such as gene copy number.[69] Such "poor metabolizers" of codeine were more likely to have uncontrolled pain and to require rescue medication.

On the other hand, persons who are "ultrarapid metabolizers" may possess multiple copies of the *CYP2D6* gene responsible for codeine metabolism or have genotypes associated with rapid metabolism and may therefore be at risk for adverse effects such as respiratory depression from rapid generation of morphine from codeine. Several pediatric deaths from codeine administered after a tonsillectomy and/or an adenoidectomy have resulted from such ultrarapid metabolism. It is for this reason that the U.S. Food and Drug Administration (FDA) has issued a "boxed warning" and deemed codeine contraindicated in this setting.

In a variety of painful conditions in children, provision of codeine provides no benefit greater than that of NSAIDs. When codeine, ibuprofen, and acetaminophen monotherapy were compared in children with musculoskeletal trauma who presented to an emergency department, ibuprofen provided superior analgesia.[70] In the posttonsillectomy setting, codeine in combination with acetaminophen caused more nausea with no difference in pain or postoperative bleeding.[71] A meta-analysis also found that weak opioids (i.e., codeine) in combination with NSAIDs fail to provide superior analgesia to that provided by NSAIDs alone[57] but do have significantly more adverse effects. Based on these findings, in our view, very few instances exist in which codeine is a preferred choice among opioids.

Some opioids exhibit partial mu receptor agonist activity (e.g., buprenorphine), kappa agonist activity (e.g., nalbuphine), or mixed agonist activity (e.g., butorphanol and pentazocine). Although these opioids have predominantly agonist activity, some have significant antagonist activity as well, and thus they may reduce the effect of pure mu agonists given concurrently. In general they do not provide superior analgesia, although they may have fewer gastrointestinal (GI) or respiratory adverse effects and may be considered for individual patients who have limiting adverse effects with other opioids. Currently the greatest use of buprenorphine in the United States is for substance abuse treatment. It has been used by multiple routes for treatment of cancer-related pain in children, particularly in countries with greater impediments to the prescribing of morphine, methadone, or other opioids. Authors of a recent small prospective case series reported reasonable effectiveness and tolerability of transdermal buprenorphine for children with cancer.[72]

Strong Opioids. Meperidine is a strong opioid that should be avoided in most cases because of its adverse effect profile and lack of superiority to the strong opioids described in the next sections. For example, repeated doses of meperidine lead to accumulation of its metabolite, normeperidine, which in turn causes neuroexcitatory symptoms including agitation, tremors, myoclonus, and seizures.[73] In a double-blind trial comparing morphine with meperidine administered via patient-controlled analgesia (PCA), it was found that morphine resulted in better analgesia and had no more adverse effects than did meperidine.[74] In low doses, meperidine reduces postoperative shivering or rigors associated with amphotericin infusion.

Morphine. Morphine is the most frequently prescribed opioid in children and is the best studied opioid in this population. It offers flexibility in terms of routes of administration. It is also available in a controlled-release formulation, and multiple randomized controlled trials have shown that this formulation can effectively control cancer-related pain when administered every 12 hours.[75] Morphine is extensively metabolized by glucuronidation in the liver to morphine 3-glucuronide (M3G) and morphine 6-glucuronide (M6G). M3G does not bind mu receptors and has no analgesic activity but may contribute to some of the neuroexcitatory adverse effects of morphine.[76] On the other hand,M6G does bind mu receptors and is a potent analgesic.[77,78]

Oxycodone. Oxycodone is a semisynthetic derivative of morphine. Although it is frequently categorized as a weak opioid appropriate for mild to moderate pain (and is frequently administered in combination with acetaminophen), this categorization is a reflection of its use at low doses. In fact, a meta-analysis showed that oxycodone is as efficacious an analgesic as morphine or hydromorphone.[79] This meta-analysis also showed no difference between oxycodone and morphine in terms of adverse effects such as dry mouth, sedation, or nausea.[79] Oxycodone itself has no ceiling effect or dose limit, although dosing may be limited when it is administered in combination with nonopioid agents such as acetaminophen. Although it is available in parenteral formulations in other countries, in the United States it is only available in an oral formulation. The oral formulation is available in an extended-release preparation.

Oxycodone is predominantly metabolized by CYP3A4 to inactive products. A secondary pathway involving CYP2D6 may lead to generation of the active opioid oxymorphone in patients with ultrarapid metabolizing variants. Although cases of apnea and death associated with excessive conversion from oxycodone to oxymorphone have been published, the prevailing impression is that pharmacogenomic variation overall leads to less variance in effect for oxycodone compared with codeine. Oxycodone pharmacokinetics have been studied in children,[80] but there is a need for additional pediatric pharmacokinetic/pharmacodynamic studies that include oxymorphone assay, pharmacodynamic end points, and contemporary genotyping.

Hydromorphone. Hydromorphone may be administered orally, intravenously, or subcutaneously. Although it was previously thought that hydromorphone has less neurotoxicity, hydromorphone metabolites have recently been shown to convey neuroexcitatory adverse effects. One advantage of hydromorphone compared with morphine is that its higher potency allows for smaller subcutaneous volumes to be delivered when this route of administration is utilized.

Fentanyl. Fentanyl may be given intravenously with rapid onset of action and short duration of action (20 to 30 minutes). For this reason fentanyl is frequently used as an analgesic for brief, painful procedures. Rapid administration of fentanyl may cause chest wall rigidity that requires reversal with naloxone or even neuromuscular blockade and positive pressure ventilation.[81] In occasional patients, fentanyl may be better tolerated than other opioids, in part because it is associated with less histamine release[82] and creates no metabolites that may produce neurotoxicity.

Fentanyl transdermal patches last 72 hours and are a convenient parenteral mode of drug delivery that is preferred by many adults.[83,84] These patches have also been used successfully in children who have chronic pain.[85] Although wide within-individual variability in fentanyl absorption exists, intraindividual absorption is reported to be stable.[86,87] In addition, hyperhidrosis, hypertrichosis, and the localization of patches on the skin do not appear to affect fentanyl absorption.[86] Because the onset of action is at least 12 hours, and because some fentanyl remains in the system for 72 hours after patch removal, transdermal fentanyl lacks flexibility for close titration for rapidly changing pain severity. The smallest patch size (12 μg/h) may be too large a dose for some children. For children as young as 2 years who had previously taken opioids and had developed some degree of tolerance, transdermal fentanyl was found to be a safe and well-tolerated alternative to oral opioid treatment.[85] The reservoir design of the patch prevents the patch from being cut to adjust the dose delivered. Transdermal fentanyl should be avoided in patients who have not previously taken opioids because it may result in respiratory depression.

In addition to rapid onset of action and transdermal application, fentanyl provides several other benefits. When renal function is impaired, fentanyl does not accumulate to the same extent as morphine.[88] Some studies have demonstrated that transdermal fentanyl appears to cause less constipation than does oral morphine, but it is unknown whether this observation is related to the route or the drug.[89-91]

In adults or children already receiving 60 mg/day of morphine, oral transmucosal fentanyl citrate (OTFC, or Actiq) provides extremely rapid control of incident pain, with an onset of action of 5 to 10 minutes. This rapid onset of action is due to its lipophilic nature, as well as because this route bypasses first-pass hepatic metabolism. Because of the rapidity of its onset of action, OTFC has been used to provide analgesia for brief, painful procedures without the requirement for IV access.[92] OTFC is also quite useful for patients who have breakthrough pain. In a double-blind, double-dummy, randomized, crossover study of adult patients with cancer who had incident (breakthrough) pain, it was found that OTFC reduced pain intensity more effectively than did immediate-release oral morphine, and OTFC was favored over immediate-release morphine sulfate by more patients after the study.[93] No conversion ratio is available for OTFC, and careful titration is needed to determine the correct dose. No correlation exists between the effective OTFC dose and the around-the-clock dose of an opioid.[94] For this reason the lowest strength (200 μg) should be tried first. If inadequate pain relief is achieved in 20 minutes, this dose may be repeated.

The fentanyl buccal tablet is another preparation of fentanyl that is rapidly absorbed through effervescent action through the oral mucosa. Patients should be taking at least 60 mg/day of oral morphine so they have opioid tolerance to safely receive a fixed dose of fentanyl with such rapid onset of action through the oral mucosa. The need for this degree of tolerance is highlighted by recent experiences with buccal/sublingual fentanyl tablets, which, when (inappropriately) administered to opioid-naive patients, may result in life-threatening respiratory depression.

Methadone. Methadone may be given orally (it is available as a tablet or liquid) or intravenously. One advantage to methadone in the pediatric population is that it is the only long-acting opioid widely available in liquid formulation. In addition, it is relatively inexpensive to manufacture, costing 90% less than extended-release morphine.[95] Methadone also exhibits unique receptor-binding properties in that the l-isomer binds mu opioid receptors and the d-isomer binds the N-methyl-d-aspartate (NMDA) receptor. Because the NMDA receptor is involved in opioid tolerance, opioid hyperalgesia, and neuropathic pain, it may be a useful opioid in these clinical situations, which are discussed later. For these reasons, administration of methadone for analgesic purposes has become more popular in recent years.

The variable pharmacologic half-life of methadone, which ranges from 12 to 150 hours, may result in delayed toxicity (e.g., sedation and hypoventilation) that can occur many days after initiation of the drug.[96] The analgesic half-life of methadone is commonly cited as 4 to 6 hours,[97] although some patients can require minimal rescue analgesia with dosing at 8- or 12-hour intervals. In addition, equianalgesic dose conversion from other opioids is variable and depends in part on the dose of the previous opioid. Its potency relative to morphine is highly dependent on the previous morphine dose.[98,99] A very convenient website, www.globalrph.com/narcoticconv.htm, can be used to assist in dose conversions. It should be emphasized that even with these calculations, enormous individual variability exists, and close follow-up is required to avoid delayed oversedation.

Methadone is metabolized through several cytochromes and therefore interacts with a variety of other medications. Individual variation in cytochrome expression may account in part for significant blood concentration variability in patients.[100,101] Methadone, in conjunction with other medications, may prolong the QTc interval.[102] It is unclear whether this phenomenon explains the otherwise unexplained increased incidence of sudden cardiac arrest in adults receiving methadone therapy.[103] Until the potential for methadone to pose cardiotoxicity is better understood, it should be used cautiously in children who have underlying cardiac conditions or those at risk for prolonged QTc. Although methadone has several advantages, it also has some unique features that require familiarity with this agent for safe and effective use. The majority of pediatric oncologists rarely, if ever, prescribe methadone.[104] Lack of familiarity with methadone pharmacodynamics, effectiveness, and dosing equivalence are the most common reasons cited for prescribing other opioids rather than methadone.

Oxymorphone. Oxymorphone is the active metabolite of oxycodone and is available as a rectal suppository. An extended-release oral formulation has also recently been approved.

Opioid Starting Doses. For the opioid-naive patient, recommended starting doses are listed in Table 71-1. For infants younger than 6 months, the starting dose should be roughly one fourth the weight-scaled dose suggested in the table and titrated to effect. Opioids should be administered with caution in patients who have disordered control of respiration, altered mental status, or altered drug metabolism. This is not to say, however, that opioids should be withheld from these patients. In fact, opioids can be safely delivered and adequate analgesia achieved with careful titration to effect.

Routes and Methods of Administration. Medication should be given by the simplest, most effective, and least distressing route. Other considerations that should guide the choice of route include the severity and type of pain, the ability of the child to tolerate a given route due to developmental or personal factors, and the ability of the caregivers to administer medication via certain routes.

Oral. When possible, the oral route of administration should be attempted first. In general, the time for opioids to reach peak effect is about 60 minutes with the oral route. Most extended-release preparations are available in tablets or capsules. For children who cannot swallow tablets or capsules but who would benefit from an extended-action preparation or agent, liquid methadone can be used. If the child has a gastrostomy tube, ultra–extended-release morphine (given every 24 hours) may be suspended *(but not crushed)* and administered via a gastrostomy tube. Ultra–extended-release morphine allows once-daily oral dosing, but unintended chewing or crushing may lead to overdose from immediate release of the morphine.[105,106] Opening the capsules and sprinkling the drug onto applesauce may be an appropriate administration technique for adults[105] but should be avoided in young children. Ultra–extended-release morphine may be considered for adolescents, but in younger children who may be at risk for accidentally chewing the capsules, these preparations should generally be avoided.

For intermittent dosing, oral opioids prepared as concentrated drops may provide analgesia without the requirement of swallowing larger volumes of liquid. Concentrated morphine may be helpful for children who are unable to reliably take oral opioids because of their neurodevelopmental capacity or nausea and vomiting.

Rectal. Suppositories containing hydromorphone and morphine may be administered rectally. In addition, controlled-release morphine tablets may be given rectally.[107] Although the published potency of rectal opioids approximates that of oral opioids,[108] the pharmacokinetic properties of morphine, that is, first-pass metabolism to the active metabolite M6G by the portal circulation, should be considered when considering rectal administration. For example, Wilkinson and colleagues[107] determined that the area under concentration-time curves for morphine metabolites were approximately twice those achieved after rectal administration. The maximal concentration of morphine and its metabolites was lower and the time to achieve peak levels was longer for rectal administration. In this study, the variation in morphine kinetics did not correspond with altered pain ratings.[107] Although it is reasonable to start with 1 : 1 (oral to rectal) equianalgesic dosing of morphine, adjustments in the dose or dosing interval should be anticipated.

Transdermal. Fentanyl and buprenorphine are the only opioids manufactured in transdermal formulations. The transdermal route for delivery of fentanyl or buprenorphine provides some advantages. Other opioids such as morphine may be compounded as transdermal formulations, but absorption of these other opioids via this route is unreliable, and alternative means of opioid administration are almost invariably available.

Intravenous. Use of the IV route, when appropriate, may provide rapid and reliable administration. In general, the time for morphine to reach its peak effect is about 15 minutes via this route. The IV route is frequently used for patients who have severe pain or for children who are unable to take oral medications, such as children who are in the final stages of life.[14]

Subcutaneous. All IV opioids may be administered subcutaneously, although methadone may cause local irritation if infused continuously.[109] Delivery of opioids by this route dose adds approximately 30 minutes to the time of peak effect obtained by IV administration. This route is simple to use and requires only a portable syringe driver to administer the medication through a butterfly needle. In addition, it confers consistent delivery and easy titration without the requirement for IV access. Needles are changed every week or more often if skin irritation occurs, and this task can often be performed by a family member. In our experience, children can absorb 2 mL per hour, whereas adults can absorb 3 to 5 mL per hour. Higher doses of opioids may exceed these volume limits. In such cases, switching to a more potent opioid, such as hydromorphone, may be necessary.

Patient-Controlled Analgesia. PCA can provide a continuous IV or subcutaneous infusion of opioid to provide basal control of pain, as well as a bolus, which provides relief from breakthrough pain. The PCA delivery system allows patients to manage their pain themselves, and no lag time exists between the request and delivery of a bolus dose for uncontrolled pain, increasing their sense of control over their pain. In one study comparing continuous infusion morphine with PCA, it was found that patients who used PCA required less total opioid while receiving equivalent control of the pain of mucositis associated with bone marrow transplantation.[110]

Because opioid-induced sedation generally occurs before respiratory depression, it is rare for a patient to administer boluses to himself or herself to the point of respiratory depression. PCA delivery has been used in the pediatric population with safety and efficacy. PCA does not increase the incidence of opioid-related complications, including sedation.[111] Clinical protocols for calculating the PCA commencement opioid dose and subsequent opioid-dose escalation can facilitate the safe and efficacious implementation of PCA.[112] Thorough assessment of PCA use includes the total daily dose, ratio between continuous and bolus opioid, amount of baseline and breakthrough pain, and response to the bolus dose. A common recommendation is to adjust the continuous (basal) rate to supply roughly two thirds of the patient's daily opioid requirement. Although this starting point is reasonable for most oncology patients with persistent disease-related pain, individual circumstances exist in which the parameters should be modified. For example, in the setting of brief, severe, episodic pain, it may be reasonable to use a lower basal rate and give more generous boluses. In postoperative care, considerable variation exists in the use of basal infusions. Our practice is to use them for operations expected to result in more severe pain and/or in patients who are likely to underdose themselves. Conversely, we tend to avoid basal infusions or use very low basal rates for patients who have less painful surgery, for patients who have received other nonopioid methods of analgesia (e.g., peripheral nerve blocks or plexus blocks), or for patients who have factors that increase respiratory risks.

PCA has been used successfully in children as young as 4 years of age.[113] PCA may also be administered by surrogates, commonly as nurse-controlled analgesia or parent-controlled analgesia, or collectively as PCA-by-proxy. In theory the safety of PCA is maximized when the patient self-administers the medication, because when the patient falls asleep, he or she stops pushing the button. Because the proxy assesses the child's pain and provides the bolus dose, the safety feature inherent in a PCA to prevent respiratory depression is overridden, but when it is used appropriately it is associated with only rare respiratory or neurologic complications.[114] Overall, the safety of nurse-controlled analgesia is well established and is widely used for both opioid-tolerant and opioid-naive infants and children. Greater controversy persists with parent-controlled PCA, although experience with this arrangement is mounting.[115] Our general practice is to greatly limit its use for routine care of opioid-naive postoperative infants and children but to use it widely for opioid-tolerant infants and children in palliative care, especially at home. Guidelines to increase the safety of PCA by proxy have been proposed.

Intrathecal. Opioids may be administered intrathecally and are considered later in the "Invasive Approaches to Pain Management" section.

Regardless of the route utilized, if the patient has continuous pain, a regimen providing continuous pain control should be implemented. To establish a patient's true analgesic needs, short-acting opioids, which can be easily titrated, may be administered for the first 24 to 48 hours. Based on the amount of opioid required during the initial interval, a longer acting opioid may then be substituted, with provision of an as-needed short-acting opioid available for incident or breakthrough pain.

Whatever the regimen, ensuring proper follow-up assessment is critical to ensure appropriate analgesia and to evaluate for potential opioid-associated adverse effects. Because constipation is a predicted and preventable adverse effect of opioids, a bowel regimen to prevent this adverse effect should always be instituted and adjusted as necessary whenever opioids are started or escalated.

Breakthrough Pain. Breakthrough pain is a transitory exacerbation of pain that occurs when pain is otherwise relatively controlled or stable at baseline. Patients with chronic cancer-related pain and superimposed breakthrough pain have worse overall pain, more impaired functioning, and higher psychological distress than do patients with chronic cancer-related pain alone.[116] Breakthrough pain may occur as a result of incident pain (i.e., pain due to a stimulus, such as movement or coughing) or end-of-dose failure, or it may be spontaneous in nature (such as lancinating pain attacks in persons with postherpetic neuralgia).

In a prospective study of pediatric inpatients with cancer it was found that 57% experienced one or more episodes of breakthrough pain during the preceding 24 hours, with each episode lasting seconds to minutes and most commonly characterized by the children as "sharp" and "shooting."[117]

Breakthrough pain may be challenging in that its onset may be unpredictable and rapid, and it may be more severe than pain typically experienced at baseline. For these reasons, use of a pain diary may be particularly important in detecting patterns and factors associated with breakthrough pain. If end-of-dose breakthrough pain occurs, the total daily around-the-clock dose may be increased by 25%, although this increase may lead to intolerable adverse effects. Alternatively, the dosing interval may be shortened.

Breakthrough pain should be approached as other types of pain are approached, with (1) consideration of the underlying triggers and interventions aimed at the underlying problem, (2) optimization of the scheduled analgesic regimen, and (3) use of adequate analgesics for episodic pain, that is, rescue medication. The rescue dose to treat such breakthrough pain should be the equivalent to the dose used every 4 hours, or 5% to 10% of the total daily opioid dose. For predictable incident pain, a short-acting opioid given just prior to the activity may be helpful. When the breakthrough pain has neuropathic features, consideration should be given to adjunctive use of analgesics with specificity for these types of pain, that is, anticonvulsants or antidepressants, as will be discussed later.

OTFC may safely provide very rapid pain relief in patients receiving the equivalent of 60 mg/day of oral morphine, as was previously discussed. It may be especially useful when breakthrough pain is unpredictable and short-lived. Other hydrophilic agents such as morphine are often administered sublingually for breakthrough pain but have a longer onset of action. A recent small study indicates that the rapid onset of action of methadone may make it a useful agent for treatment of breakthrough pain.[118]

Dose Escalation. When a patient who is already receiving oral or IV opioids has severe pain that persists despite a dose of breakthrough opioid medication, the dose should be increased by 50% to 100% and repeated. Once adequate analgesia is reached, the total amount given over 4 hours to determine the “effective dose” for every 4 hours should be calculated.[8] If more than two rescue doses are used in a 24-hour period, the total standing dose should be increased.

For patients receiving continuous IV opioids, a rescue dose for breakthrough pain should be provided that consists of 50% to 200% of the hourly infusion every 15 minutes as needed. For the sake of simplicity, use of multiple different short-acting opioids is discouraged.

Opioid Tolerance. The response to acquired tolerance to an opioid is usually to increase the opioid dose. When this approach is not feasible because of opioid adverse effects such as neuroexcitability, rotating to a different opioid may be an option. Because the NMDA receptor plays a role in opioid tolerance, methadone, with its attendant NMDA antagonist properties, may control pain that is otherwise refractory to opioids.[119] The addition of adjuvant NMDA receptor antagonists such as ketamine may also reverse opioid tolerance. In animals, as well as in some patients, chronic administration of opioids can generate a condition of generalized hyperalgesia, meaning that new painful stimuli produce a greater intensity of pain than would have occurred with the same stimulus for that subject in an opioid-naive state. Opioid-induced hyperalgesia is discussed in greater detail later.

Opioid Rotation. Opioid rotation, or switching, may also be indicated as a result of intolerable adverse effects or route of administration. Equianalgesic tables are useful for conversion from one opioid to another and are widely available. Although no evidence exists to demonstrate the superiority of one opioid over another in such a switch, the strategy of changing opioids may be successful in alleviating dose-limiting adverse effects or opioid tolerance in children. For example, Drake and colleagues[120] found that opioid rotation resolved adverse effects in 90% of children without loss of analgesia or the need to increase morphine equivalents. In fact, because of the phenomenon of incomplete tolerance, the equianalgesic dose of the second opioid should be decreased by 20% when a patient is transitioned from one opioid to another. The phenomenon of incomplete tolerance is particularly pronounced when converting to methadone,[98,99] likely because of the ability of the d-isomer of methadone to act as an NMDA receptor antagonist.[121]

Opioid Withdrawal and Opioid Tapering. Opioid withdrawal is a physiologic response. Sudden discontinuation of opioids in a patient who has been taking long-standing opioids may prompt a withdrawal syndrome characterized by irritability, restlessness, dysphoria, anxiety, muscle aches, sweating, piloerection, diarrhea, nausea, vomiting, yawning, and sneezing. Withdrawal may be prevented by tapering opioids. Opioid doses may be safely cut in half without precipitating withdrawal. Continued tapering may be achieved by halving the dose every 3 days. An alternative strategy is to reduce the total daily dose by 10% per day. Maintaining the rescue dose at its original dose during the tapering process allows for effective treatment of breakthrough pain that may occur as the dose is tapered.

Opioid Adverse Effects. Nonrespiratory adverse effects from opioids include constipation, nausea, pruritus, somnolence/sedation, and, particularly at high doses, neurotoxicity. A prospective study of pediatric oncology patients found that in children receiving morphine, postoperatively, 38% had vomiting, 32% had nausea, and 24% had constipation.[122] The high incidence of nausea and vomiting in this population is likely confounded by postoperative nausea and vomiting.

Opioid-associated sedation typically self-resolves within a few days of the institution of opioids. In some cases the sedation observed when instituting opioids is not an adverse effect of opioids per se but rather a result of an exhausted patient finally able to sleep once pain is controlled. Nausea experienced by some patients upon starting opioids similarly self-resolves within a few days and is often relieved with use of an antiemetic. Switching to a different opioid due to nausea within the first 3 days of opioid therapy may be premature. Opioid-associated urinary retention is an uncomfortable symptom that may respond to a switch to an alternative opioid. Anecdotally, urinary retention in some patients may respond to selective alpha-1A antagonists such as tamsulosin, which are commonly prescribed for adults with lower urinary tract outflow obstruction. Urinary retention, frequency, or urgency should prompt a focused consideration of a range of oncologic and neurologic causes, as well as effects of other medications in addition to opioids, such as anticholinergics and antihistamines. Management of opioid-associated sedation/fatigue, constipation, pruritus, and nausea are further discussed in the sections of this chapter dedicated to these specific symptoms.

If adverse effects persist despite appropriate interventions, rotation to a different opioid may be helpful. Overall, at this point no clear evidence exists that one particular opioid has a different adverse effect profile than another opioid. Patients may exhibit different sensitivities to different opioids because of individual variability in opioid receptors, pharmacokinetics, and metabolism. Inroads have recently been made in understanding which genetic variants may influence a particular person's response to opioids. For example, certain variants of the multidrug resistance 1 gene (formerly *MDR1,* now called *ABCB1*) or the gene encoding catechol-O-methyltransferase *(COMT)* are associated with CNS adverse effects, such as drowsiness or confusion.[123] A very promising recent approach to reducing opioid adverse effects involves identifying opioid agonists with "ligand biasing" of actions on opioid receptors that preferentially act via G proteins rather than via beta-arrestin signaling.[124]

Opioid-associated adverse effects may be also improved with a reduction in opioid dose, which may be achieved without loss of analgesic effect if a coanalgesic medication is added. For example, when gabapentin and morphine are combined they provide better analgesia from neuropathic pain at lower doses of each drug than either does as a single agent.[125]

Opioid-Induced Neurotoxicity. Opioid-induced neurotoxicity is typically encountered when opioid doses are rapidly increased, when high doses of an opioid are used, or in the setting of renal failure. The patient may describe increased sensitivity to pain (hyperalgesia), pain in response to nonpainful stimuli (allodynia), worsening pain despite increasing doses of opioids, or pain that appears to spread. Other findings of neurologic hyperexcitability such as myoclonus, delirium, or seizures may also be present. Opioid-induced hyperalgesia is likely due to accumulation of neurotoxic opioid metabolites such as M3G or hydromorphone-3-glucuronide. Such metabolites activate presynaptic calcium channels that release glutamate, which in turn activates NMDA receptors and depolarized postsynaptic neurons in the CNS.[126]

If signs or symptoms of opioid neurotoxicity develop, the opioid should be decreased or changed to one with potentially less neurotoxic effects, such as fentanyl or methadone. The addition of a nonopioid analgesic may lessen the amount of opioid required. An alternative mode of analgesia such as intrathecal, regional, or local analgesia may be used in place of systemic opioids. If needed, parenteral ketamine, an NMDA receptor antagonist, may also be initiated to reduce NMDA receptor-mediated neurotoxicity.[127]

Myoclonus is frequently relieved by a benzodiazepine such as diazepam. Opioids may also play a role in the development of delirium; the reader is referred to the section in this chapter dedicated to mental status changes.

Management of Opioid Overdose. Respiratory depression occurs as a result of opioid receptor blockade of CO_2 chemoreceptors in the medulla. The risk of respiratory depression from opioids is indeed small when opioids are dosed and titrated judiciously. Scenarios in which opioids may cause respiratory depression are the development of renal failure or a sudden decrease in pain, such as after a neurolytic block if opioid doses are not adjusted.

Respiratory depression characterized by hypopnea alone is not in and of itself an indication for opioid reversal with naloxone. In fact, administration of oxygen and reduction of the subsequent dose may be the only actions needed. For a true respiratory emergency, naloxone should be administered by (1) diluting the 0.4 mg/mL ampule in 10 mL saline solution and (2) administering 1 mL IV or subcutaneously every 3 minutes until respiratory depression improves. If the patient is taking long-acting opioids, repeated doses of naloxone or a naloxone infusion may be required, with the hourly dose being the dose initially required to overcome respiratory depression. Overadministration of naloxone will block opioid receptors, resulting in withdrawal that may be characterized by severe pain and sympathetic instability.

Adjuvant Treatments

Radiotherapy

Radiotherapy is commonly used to relieve pain such as localized bone metastases. In a review of 13 randomized trials in adults with painful bone metastases who received either radiotherapy or radioisotope injection for pain, it was found that 27% achieved total pain relief and 42% attained a 50% level of pain relief. The largest trial found that the median duration of complete pain relief was 12 weeks. A variety of fractionation schedules were used, and no clear difference between schedules was found.[128]

Antidepressants

TCAs and serotonin and norepinephrine reuptake inhibitors (e.g., duloxetine) may relieve neuropathic pain and are discussed in more detail later.

Steroids

Pain attributable to swelling, such as headache from increased intracranial pressure, bone pain, nerve pain from compression by a tumor, and hepatic capsular pain, may be responsive to steroids. A common starting dose is 10 mg followed by 4 mg four times a day in larger patients. Dosages of 10 mg/m^2 followed by 4 mg/m^2 four times a day can be used in smaller patients. If the response is favorable, the steroid dose should gradually be reduced to the lowest effective dose. A medication to provide concurrent gastric protection should also be prescribed.

Anticonvulsant Agents

Anticonvulsant agents control neuropathic pain by preventing peripheral nerve excitation. Carbamazepine has been widely studied for neuropathic pain such as trigeminal neuralgia. However, carbamazepine interacts with many other drugs and may suppress bone marrow function, limiting its usefulness in patients with cancer. Oxcarbazepine is a carbamazepine derivative that acts via use-dependent blockade of sodium channels. Overall, compared with carbamazepine, oxcarbazepine appears to result in fewer risks for hematologic, dermatologic, and hepatic complications. Hyponatremia can occur, and electrolytes should be monitored in the first weeks of therapy or with dose escalation. Gabapentin and pregabalin are newer anticonvulsants that lack such interactions and bone marrow toxicity. These agents target both excitatory and inhibitory neurotransmitters through inhibition of sodium and voltage-gated calcium channels.

Gabapentin may be sedating, requiring gradual initiation (i.e., a 5 to 7 mg/kg/dose by mouth three times a day, with a gradual increase every 3 days) and titration to effect. Reports of pediatric uses of gabapentin and pregabalin for treatment of chronic neuropathic pain are limited to case series. For any of the anticonvulsants, effects on mood, sedation, and mental clarity are quite variable.

Clonidine

Clonidine is an alpha-agonist drug that historically has been used to treat hypertension or opioid withdrawal. It may also be used for neuropathic pain or to enhance analgesia from opioids. Clonidine may be administered orally or transdermally, which is an additional benefit in the pediatric population. Clonidine has also been used in children for hyperactivity or steroid-associated psychiatric symptoms. Sedation is common.

Ketamine

Ketamine is an effective adjuvant analgesic that is useful for its opioid-sparing and opioid-sensitizing capabilities, particularly in persons experiencing difficult pain syndromes such as neuropathic pain.[129] Although the evidence is scant, reports exist of its usefulness in relieving refractory pain in children with cancer.[130] Ketamine may on occasion cause hallucinations and depersonalization/derealization. Such effects are more common in adults and may be prevented or treated with a benzodiazepine.

Invasive Approaches to Pain Management

In a small subgroup of patients, aggressive titration of analgesics and adjuvants still results in a circumstance of inadequate analgesia and/or intolerable adverse effects, including intolerable sedation. In some situations consideration of regional anesthetic approaches may be appropriate; the most common approaches are infusions of analgesics and local anesthetics via indwelling intrathecal, epidural, or plexus catheters. A number of considerations influence the choice of these methods, including the wishes of the patient and his or her surrogate in terms of balancing analgesia, alertness, and the risks and inconveniences of a technologic approach; availability of expert personnel for both catheter placement and ongoing management; and consideration of the nature of the pain, the expected course of the disease, and disease-related symptoms. Because a number of technical and management issues cannot be readily extrapolated from similar infusions in adults or from perioperative infusions in children, consultation with clinicians who have experience in these techniques is encouraged. In adults, a track record exists of using implantable programmable pumps that can be refilled at a frequency ranging from weekly to every several months. One study indicated benefits of these systems with regard to pain, quality of life scores, and even longevity.[131]

In children with cancer, it is generally necessary to combine small amounts of a local anesthetic along with opioids in the spinal infusion. Because of the relatively low potency and low solubility of existing local anesthetics, this requirement makes it impractical to use the programmable pumps commonly used in adults. Therefore in a majority of these cases, our preference has been to use implanted intrathecal ports rather than implanted pumps. The procedure to place these ports is performed in the operating room with use of general anesthesia while the patient is in the lateral position. The intrathecal catheters are advanced cephalad from spinal entry near L3-L4 under fluoroscopic guidance to position the tips near the spinal dorsal horn levels (rather than the exiting nerve root levels) relevant to the sites of greatest pain. The reader is referred to Figure 564 in the atlas by Clemente[132] for illustration of the spinal dorsal horn levels.

As an example, for refractory pain due to pelvic or lower extremity tumors predominantly involving lumbosacral dermatomes, we advance the catheter tips to a level around T11. Catheters are tunneled subcutaneously and connected to a port that is positioned over the lower ribs near the anterior axillary line.

In the setting of refractory pain due to advanced cancer, our view is that coagulopathy in most cases should not be regarded as a major contraindication to this procedure. If necessary, platelets and/or fresh-frozen plasma are infused immediately before and during the procedure as guided by coagulation parameters.

Use of indwelling ports is preferable for the sake of sterility and skin care. Ports are accessed with Huber needles similar to those used for IV ports. Dilute local anesthetics are generally required for adequate analgesia (particularly with movement), and they can generally be titrated in a manner that preserves reasonable lower body motor functioning. Depending on the adverse-effect profile seen with opioids and local anesthetics, other spinal analgesics may be included in the mixture, including clonidine and ketamine.

Although the epidural route can be used, our experience is that the intrathecal route is more reliable over a longer time frame because of several factors, including the development of adhesions or fibrosis in the epidural space over periods of months with use of high-concentration infusions and because of the phenomenon of tolerance to local anesthetics, which can be overcome very readily with intrathecal dosing but with much greater difficulty with prolonged epidural dosing. Clinicians' estimates of prognosis and longevity are often imprecise. Our general recommendation is that, if invasive approaches are being considered in the first place, the most versatile system available should be chosen to be implanted—that is, one most likely to work successfully for the short term (i.e., a few weeks) as well as longer term (i.e., months to a year) and least likely to require a repeat procedure. One potential early complication of this procedure is cerebrospinal fluid leak, leading to headache in the days after placement. In two cases we have returned to the operating room several days later to resolve this problem with a fluoroscopically guided epidural blood patch. The introduction of spinal analgesic medication and the corresponding adjustment of systemic analgesics require careful attention because of the potential for oversedation and even respiratory depression. Because the intensity of afferent nociceptive transmission is diminished by the spinal medications, the stimulating effect of this nociceptive activity on alertness and respiratory drive is also diminished.

Occasionally in patients who have a tumor that involves a single peripheral nerve or nerve plexus, indwelling tunneled catheters can be placed percutaneously along the nerve or in the plexus sheath. Our preference is to use a combination of ultrasound and nerve stimulation guidance. These peripheral and plexus catheters can be extremely helpful if the pain remains highly localized. In a majority of cases, however, the tumor spreads beyond these more limited distributions, and we have therefore generally favored intraspinal infusions because of greater versatility in covering pain arising from a broader area. For similar reasons, our general preference is to choose intraspinal drug infusions rather than neurolytic nerve blocks except in very restricted circumstances, such as the use of a neurolytic celiac plexus blockade for a tumor that is almost entirely restricted to the upper abdominal solid viscera. Celiac plexus blockade has a good track record for treatment of refractory pain due to pancreatic cancer in adults. It affects visceral innervation only and produces no somatic sensory or motor deficits. Adverse effects such as diarrhea or orthostatic hypotension tend to be relatively short-lived and tolerable. For children and adolescents, our preference is to perform this procedure with use of general anesthesia with the patient in the prone position, using alcohol as the neurolytic agent. We use a posterior approach with computed tomography (CT) guidance, in collaboration with an interventional radiologist. Interventional approaches for pain in children were recently reviewed.[133]

Psychological and Nonpharmacologic Approaches to Pain

Because of the multifactorial nature of the experience of pain, a multidisciplinary approach is often helpful in optimizing pain management. With use of psychosocial assessment it may be possible to identify and address factors that contribute to pain that analgesic interventions alone cannot address.

Depending on the developmental capacity of the child, interventions such as preparatory play, distraction, imagery, relaxation, deep breathing, hypnosis, and behavioral management can reduce anxiety and fear associated with pain. A child life specialist, psychologist, complementary medicine practitioner, or other specialist may be able to provide some of these interventions.

A variety of cognitive and behavioral approaches to pain and invasive procedures may be helpful to reduce pain and distress experienced by the child. In a study of 56 children aged 3 to 13 years, delivery of a cognitive-behavioral intervention was compared with preprocedure administration of diazepam or cartoon watching prior to bone marrow aspiration.[134] The cognitive-behavioral intervention consisted of breathing exercises, positive reinforcement, and imagery or distraction to provide the child with imaginative and cognitive tools to cope with procedural stress. Overall, children who received the cognitive-behavioral intervention had lower pulse rates and distress and pain scores when compared with children who either received diazepam or watched cartoons. However, during the actual aspiration procedure, no difference was found between groups, suggesting that it did not ameliorate distress associated with the intense experience of the actual procedure.[135] Cognitive behavioral therapy (CBT) does provide children with mechanisms for coping with distressing procedures and may explain why children who receive CBT display less distress after the procedure compared with children who receive an anesthetic via a mask but do not receive CBT.[136]

Hypnotherapy can be performed in children as young as 4 years.[137] Hypnosis can reduce procedural pain (e.g., from a bone marrow aspirate) and postoperative pain in children, as well as anticipatory nausea and dyspnea. In a randomized controlled trial of 30 children (ages 5 to 15 years) with cancer who were undergoing bone marrow aspiration, children who received either hypnosis or CBT reported less pain and anxiety compared with their baseline symptoms or control subjects who did not receive either intervention. Children who underwent hypnosis also demonstrated less procedure-related distress than did those in the other two groups.[138]

Music, art, and play therapy may also be helpful in reducing anxiety associated with procedures. For example, soft lullaby music reduces heart rates in children undergoing the application or removal of a cast.[139] Even when specific nonpharmacologic techniques are not employed, some behavioral strategies should always be used, including appropriately preparing the child, giving the child choices when possible, providing developmentally appropriate and honest explanations, and providing positive reinforcement. Patient education is also an important component of addressing pain.

Many patients find integrative therapies (i.e., therapies complementary to traditional medical approaches) to be of benefit in coping with stress, reducing the effects of treatment and illness, providing a sense of control, and enhancing quality of life.[140] In a survey of adults participating in cancer clinical trials, 63% used at least one type of integrative therapy, with an average use of two therapies per patient.[140] Few clinicians are aware of these practices and thus should routinely inquire about them.

Although many practices such as acupuncture, massage, and healing touch seem promising in reducing cancer-related pain, the evidence base supporting these therapies is weak. Barriers to the publication of such trials include issues of study quality and design such as small study size, high attrition rates, and lack of a comparison arm, particularly in fields where the placebo effect may be high.[141] Studies with adequate power and duration and sham controls are needed to evaluate the efficacy of these interventions for cancer-related pain.

Pediatric Cancer Pain Syndromes

Although pain should be approached with a systematic approach and the use of analgesics, including opioids and adjuvants, certain cancer-related pain syndromes may respond to particular treatments.

Tumor-Mediated Pain

GI obstruction may cause pain. Management of GI obstruction is discussed in the section entitled "Other Gastrointestinal Symptoms." Visceromegaly or invasion of organs, which often presents as a poorly localized, dull pain, is best treated with opioids, radiation, nerve blockade, or epidural/intrathecal techniques. Involvement of nerve or bone may result in neuropathic or bone pain, which is discussed later. Liver capsular pain may be alleviated by steroids.[142] Elevated intracranial pressure may present as headache and vomiting and may respond to steroids or surgical decompression. In situations in which inflammation is thought to contribute to pain, the pain may respond to steroids or NSAIDs.

Pain Related to Administration of Antineoplastic Therapy

Antineoplastic therapy may produce a variety of pain syndromes. Injection of chemotherapy into a peripheral vein may cause local transient pain or phlebitis. Extravasation of a vesicant such as vincristine or anthracycline may cause local pain, burning, swelling, and/or redness, as well as tissue necrosis. These symptoms are most likely to occur when the agent is injected peripherally. Preclinical data showed that dexrazoxane, a reversible topoisomerase II catalytic inhibitor, reduced the number, severity, and duration of wounds that developed in a dose-dependent manner after extravasation.[143] In a single-arm, open-label study of 53 humans with anthracycline extravasation, dexrazoxane was well tolerated. In this uncontrolled trial, one patient required surgical resection (1.8%).[144]

Intrathecal chemotherapy may cause arachnoiditis, which is associated with fever, headache, nuchal rigidity, nausea, and vomiting. A new liposomal formulation of cytarabine appears to have an increased risk of arachnoiditis in children, which can be mitigated with systemic dexamethasone.[145]

Prolonged steroid administration may result in avascular necrosis, which occurs most often in the hip.[146] NSAIDs may help provide relief for this painful condition, although if the pain is severe, opioid therapy may be indicated. Pamidronate is a bisphosphonate that holds promise in reducing symptoms and increasing function in children who have painful osteonecrosis due to steroid therapy for acute lymphoblastic leukemia (ALL).[147,148]

Bone Pain

Infiltration of bone is often experienced as a constant aching, which may be relieved by steroids, NSAIDs, or opioid analgesics. Isolated bone metastases may be treated with local treatments such as external beam radiation or surgical techniques such as vertebral body or long bone stabilization or other more invasive treatments for pain (see "Invasive Approaches to Pain Management"). Bisphosphonates, which reduce osteoclastic bone resorption, may be helpful when analgesics or radiotherapy are inadequate for the management of painful bone metastases.[149] Diffuse bone metastases may also be treated with radioisotopes, but radioisotopes may cause myelosuppression.

Bone pain may also be caused by filgrastim or pegfilgrastim.[150] Although the mechanism underlying such pain has not been clearly established, it is frequently attributed to filgrastim-stimulated marrow expansion. In a study of 100 adults receiving care in a community oncology practice, 79% experienced either moderate or severe bone pain due to use of pegfilgrastim. Pain was either diffuse or located in the lower extremities/hips or back. For persons with the most severe pain, the mean duration of pain was 2.8 days after the injection. NSAIDs provided relief for 74% of persons with moderate pain. Some persons with severe pain received opioids, but opioids provided relief in only 45% of patients. Interestingly, patients with lymphoma who were receiving concurrent steroids as lymphoma-directed therapy did not have significantly less bone pain.

Filgrastim-induced bone pain has not been well studied in children, although anecdotally it appears to occur less frequently in children than in adults. In a relatively small study of 28 children with cancer who received pegfilgrastim (100 μg/kg with a maximum dose of 6 mg for a total of 126 doses) after myelosuppressive chemotherapy, four children reported bone pain.

Although use of antihistamines to treat filgrastim-associated bone pain has been reported,[151] no clinical trials have been performed to evaluate this practice. Until evidence indicates that antihistamines reduce this type of bone pain, antihistamines should not be used at the exclusion of established analgesic practices.

Mucositis

Mucositis is a common problem for children after chemotherapy, radiation, or bone marrow transplantation. It is particularly problematic in children undergoing high-dose chemotherapy or head and neck radiotherapy. Mucositis is associated with increased risk of infection and may impair intake of adequate nutrition. It usually self-resolves within 14 days or upon recovery of the bone marrow. The WHO has developed a mucositis rating scale that ranges from 0 (no visible evidence of mucositis, no pain, able to eat solids, and able to drink) to 4 (erythema, ulceration present, significant pain, and inability to eat or drink). Prophylactic antiviral or antifungal therapy does not decrease the incidence of mucositis. However, superinfection should be considered and cultures should be obtained when mucositis appears to be more severe or prolonged than anticipated.

Historically the mainstay of pain control for mucositis has been parenteral opioid therapy, usually via PCA. This mechanism of analgesia delivery is both safe and effective for this purpose.[112] An adjuvant mouthwash containing diphenhydramine, 2% viscous lidocaine, and liquid antacid (e.g., Maalox) in a 1:1:1 formulation may be used in children older than 2 years who can spit out the medication rather than swallowing it. Topical application of straight viscous lidocaine carries the risk of systemic absorption if the mucositis is widespread. Although the true risk of aspiration due to local anesthesia of glottic structures is unclear, some centers avoid using this strategy because of this concern. A supersaturated calcium phosphate mouth rinse moistens and lubricates the oral cavity. Although results of studies of this agent have been mixed, the majority of studies support its use to reduce the severity/duration of mucositis and the pain associated with it. The Children's Oncology Group is currently conducting a study of this rinse in preventing mucositis in children who are undergoing hematopoietic stem cell transplantation.

Other measures to alleviate mucositis include regular administration of oral sucralfate during chemotherapy, low-dose laser therapy, and gum chewing.[152-154] Results of studies evaluating the efficacy of the free radical scavenger amifostine in reducing mucositis have been inconsistent.[155] Severe mucositis can also cause abdominal pain, which is best treated with opioids. Perineal pain due to mucositis may also be addressed with a topical barrier cream to soothe and protect the area.

Recent advances in the understanding of the pathogenesis of mucositis have led to the development of targeted therapies. For example, palifermin is a keratinocyte growth factor that mediates epithelial cell growth and reduces epithelial cell apoptosis, factors that both appear to be important processes in the evolution of mucositis. Palifermin reduces mucosal injury as manifested by decreased mucositis duration and severity and a reduced need for analgesics and parenteral nutrition in adults undergoing high-dose chemotherapy and radiotherapy requiring peripheral blood stem cell rescue. In one study of adults undergoing high-dose therapy with autologous stem cell rescue, the incidence of WHO grade 4 mucositis was reduced from 62% to 20% ($P < .001$).[156]

The role of L-glutamine in mucositis has also been recently investigated. Depletion of L-glutamine is associated with epithelial damage, and increased levels are associated with healing. The role of uptake-enhanced L-glutamine suspension (AES-14) in reducing mucositis has recently been studied. In a placebo-controlled crossover study of 326 women undergoing chemotherapy for breast cancer, AES-14 reduced mucositis incidence and severity.[157] In addition, AES-14 demonstrated a carryover effect, with patients receiving AES-14 before placebo having a lower risk of mucositis.

Neuropathic Pain

Neuropathic pain may occur from infiltration or compression of nerves, nerve damage from surgery, herpes zoster, or adverse effects from antineoplastic therapies such as vinca alkaloids, cisplatin, oxaliplatin, or bortezomib. Children often describe it as burning or shooting pain, and it may be accompanied by dysesthesia or hyperalgesia.

Neuropathic pain may be related to chronic pain states whereby chronic afferent input into the dorsal horn of the spinal cord leads to a hyperactive state. Mediators of this condition are excitatory amino acids, such as glutamate, which bind to the NMDA receptor. The NMDA/glutamate receptor is a calcium channel involved in opioid-resistant pain, neuropathic pain, allodynia, and hyperalgesia. NMDA receptor binding may lead to lasting increases in neuronal excitability, leading to persistence of neuronal hyperactivity. The end result of such a phenomenon is hyperalgesia, allodynia, spontaneous pain, and phantom limb pain, even after the painful stimulus has resolved. Methadone, with its NMDA receptor antagonist properties, may be the most appropriate opioid for neuropathic pain. However, no clear evidence from studies in humans shows that methadone is superior to other opioids in treating neuropathic pain.[158] In addition, other approaches such as nerve blocks or adjuvant NMDA receptor antagonists may disrupt ongoing nociceptive input and neuronal hyperexcitability.

Most therapies for neuropathic pain have been evaluated in non–cancer-related neuropathic pain syndromes such as diabetic neuropathy. Medications for neuropathic pain include TCAs and the newer atypical antidepressants venlafaxine and duloxetine. TCAs often relieve neuropathic pain more quickly and at lower doses than are required to treat depression. TCAs reduce neuropathic pain by inhibiting serotonin and norepinephrine uptake, which increases transmission of inhibitory signals in the spinal cord. For children experiencing disturbed sleep, amitriptyline, a TCA that leads to greater sedation, may be a good choice. However, amitriptyline also has more anticholinergic effects such as xerostomia and constipation, in which case nortriptyline is an alternative TCA.

Duloxetine is the first antidepressant to be approved by the U.S. FDA for treatment of neuropathic pain, and in a recent multicenter randomized placebo-controlled crossover trial, it was demonstrated to reduce chemotherapy-induced painful peripheral neuropathy.[159] Anticonvulsants such as topiramate, pregabalin, or gabapentin may also be effective.[125,160,161] In the cancer population, gabapentin effectively reduced tumor-related neuropathic pain in a randomized controlled study of adults already receiving opioids, with lower average pain intensity scores and less dysesthesia in the gabapentin group.[162] In some instances when tumors incite pain because of spinal cord compression, dexamethasone may reduce pain.[163] Other interventions such as topical lidocaine or capsaicin, transcutaneous electrical nerve stimulation, surgical decompression, or nerve blocks may also relieve neuropathic pain.

Phantom Limb Pain

Phantom limb pain is a common phenomenon in children who undergo amputation of an extremity.[164] It may be experienced as either stump pain or the type of pain experienced preoperatively, and it appears to improve over time.[164,165] Phantom limb pain appears to be associated with preoperative limb pain.[164] For this reason it is best approached with preemptive regional anesthesia. In our practice, regional anesthesia is used widely for anesthetic and analgesic management of amputations in children. The widespread use of ultrasound guidance has increased the reliability of continuous peripheral nerve blockade via indwelling catheters.

Additional measures to treat phantom limb pain include anticonvulsants, TCAs, and topical agents such as Lidoderm. Some small series have reported success with gabapentin[166] and topiramate for phantom limb pain.[167] A preliminary observational study found that use of a prosthesis to support limb use was associated with reduced phantom limb pain when compared with cosmetic prosthesis use.[168] A few studies suggest that therapy with mirrors may relieve phantom limb pain, although the limitations of these studies, including small sample size, preclude definitive conclusions.[169,170]

Pain Related to Graft-Versus-Host Disease

Graft-versus-host disease (GVHD) may cause a variety of pain syndromes. It is a common cause of abdominal pain in children undergoing bone marrow transplantation.[171] Along with diarrhea, nausea/vomiting, weight loss, dysphagia, and early satiety, abdominal pain/cramping may occur in gut GVHD.[172] Antimuscarinic/anticholinergic agents such as scopolamine may reduce colic from smooth muscle spasms. However, gut GVHD may be so severe that opioids are required for analgesia.[113]

Oral GVHD usually does not cause pain. Development of oral pain is associated with ulceration or involvement of the soft palate.[173,174] When evaluating pain in the setting of oral GVHD, one should also consider a concomitant infection such as herpes simplex virus or candidiasis. Oral GVHD pain is usually reduced with GVHD-directed treatment such as topical steroids or topical FK506 rinses. Pain associated with vulvovaginal GVHD may also respond to topical steroids or topical cyclosporine.[175,176] Other types of GVHD, such as myositis or fasciitis, may require systemic analgesia.

Postoperative Pain

A broader discussion of treatment of postoperative pain in children and adolescents than that presented here can be found elsewhere.[177,178]

Cancer surgery is often distressing and anxiety provoking for children and their parents. Unless specifically contraindicated, anxiolytic premedication with benzodiazepines should be considered routine, using oral or IV routes as appropriate. For patients who were treated with opioids on a regular basis before surgery, it is necessary to increase postoperative analgesic dosing accordingly. It is a common mistake to select routine parameters for opioid infusions or PCA for these patients, which invariably results in underdosing and inadequate analgesia.

For major tumor resections in the lower extremities, pelvis, abdomen, and thorax, we make extensive use of epidural infusions for postoperative analgesia. Epidural analgesia in children requires specific training regarding the technical aspects, as well as pediatric-specific management protocols and a level of acute pain service coverage that permits optimal dose adjustment. For patients receiving epidural infusions who were opioid tolerant before surgery, it may be necessary to provide a systemic opioid along with an epidural opioid postoperatively (despite a widespread "taboo" against this combination); alternately, larger than usual dosing of epidural opioids, the addition of epidural clonidine, or other approaches may be necessary to account for the patient's preexisting opioid tolerance, which may be accompanied by hyperalgesia. Peripheral nerve and plexus catheters placed via ultrasound guidance are increasingly being used for surgery on the limbs. For thoracotomies, ultrasound-guided continuous paravertebral catheters are a promising alternative to thoracic epidural catheters.

Procedure-Related Pain

Brief procedures involving the use of needles, including venipunctures, IV cannulation, lumbar punctures, and bone marrow biopsies and aspirates, are a major component of cancer care and a major source of distress for patients. Approaches to management of these interventions should be individualized, based on consideration of the child's age, temperament, coping style, and past pain experiences, as well as the intensity of the procedure (e.g., bone marrow biopsy is more painful than a simple venipuncture).

Explanations provided prior to procedures should be tailored according to the child's age, developmental status, and coping style. The presence of the parents can be helpful, especially when the parents have been coached to help facilitate positive coping. Parents should be present to provide support, not to hold the child or assist the clinicians.

A variety of products are available to provide needle-free skin analgesia. These products include local anesthetic creams such as eutectic mixture of local anesthetics (EMLA), ELA-Max, tetracaine gel (Ametop), patches such as Synera, needleless injection systems such as Zingo, and lidocaine iontophoresis. These products differ in their onset time, propensity for vasodilation or vasoconstriction, cost, and availability in different countries. In a busy clinic, use of these topical anesthetic techniques is facilitated by standing order policies so they can be applied at an appropriate time before the planned procedure.

Several cognitive-behavioral approaches have been shown to be effective in reducing a child's procedure-related distress, including hypnosis, guided imagery, and related approaches to achieving a relaxed or dissociated state. These approaches have the virtue of safety and generalizability. A patient who learns these techniques for one setting can apply them to other situations.

Conscious sedation, deep sedation, or brief general anesthesia can be very effective in alleviating the pain and distress of lumbar punctures and bone marrow biopsies and aspirates. The approach chosen will depend in part on the individual patient, the intensity of pain involved in the procedure, the degree of immobility required (e.g., for radiation therapy or magnetic resonance imaging), and available resources.

Considerable variation exists in the choices of drugs, the depth of sedation, and the personnel involved in different aspects of sedation. In some hospitals a two-tiered approach is taken, with lower risk patients receiving sedation administered by oncologists and nurses and higher risk patients cared for by pediatric anesthesiologists.

Other hospitals use a different two-tiered approach, with sedation for low- to intermediate-risk patients being administered by nurses and pediatric subspecialists from a sedation service who have specific expertise (e.g., pediatric intensivists or pediatric emergency physicians) and utilization of pediatric anesthesiologists to care for higher risk patients, those having more extensive procedures, or those requiring higher degrees of immobility. For example, for a cooperative, relatively healthy 12-year-old undergoing a lumbar puncture, a regimen that involves conscious sedation with midazolam and fentanyl and topical local anesthesia followed by local anesthetic infiltration is likely to be safe and effective. Conversely, a 2-year-old with a history of a very difficult to manage airway and cardiomyopathy who is undergoing bone marrow aspiration, biopsy, and a lumbar puncture would be at higher risk and therefore might be referred for management by a pediatric anesthesiologist.

In our view, regardless of local decisions about which specific specialists are involved in which procedures, pediatric oncologists need to advocate for sufficient institutional resources to ensure that effective and safe approaches to conscious sedation and general anesthesia are available for all children with cancer. Some general recommendations for sedation programs are listed in Box 71-1.

More detailed discussions of individual drugs and outcomes of pediatric sedation may be found elsewhere.[179,180]

Reassessing Interventions for Pain

Careful reassessment to determine response to therapy is a key component in effectively addressing pain. In addition to reducing pain, interventions seek to reduce distress and functional impairment and enhance quality of life. Pain relief measures do not necessarily achieve all of these goals. For example, in one survey of adults with non–cancer-related pain, even when the degree of pain was controlled for, persons receiving opioid therapy did not have improved quality of life or improved function.[181] Therefore in addition to evaluation of whether pain relief is attained, determination of whether increased functional capacity and increased quality of life is achieved is crucial in symptom management.

Box 71-1 **Recommendations for Pediatric Oncology Sedation Programs**

1. A standardized preprocedure history and physical should be performed to identify factors that might modify risk (e.g., airway anomalies, respiratory difficulties, and cardiac compromise).
2. Use of procedure checklists should be encouraged.
3. Nothing by mouth guidelines should be consistent with those recommended for children undergoing general anesthesia.
4. Clinicians who are performing the procedure should have undivided attention during the procedure (e.g., their beeper and phone should be carried by someone else), and they should ensure that all necessary supplies (including kits, sterile supplies, chemotherapy, and tubes) are already in the room before bringing the child into the room or initiating sedation.
5. Children receiving sedation should receive continuous monitoring, including a dedicated observer, continuous use of pulse oximetry, and regular recording of vital signs.
6. All procedure locations should have at least the following supplies, equipment, or systems at hand:
   a. Wall or tank oxygen supplies
   b. Wall or portable suction devices
   c. Monitoring equipment
   d. Airway equipment, including bags and suitably sized masks, laryngoscopes, endotracheal tubes, oral airways, and laryngeal mask airways
   e. An emergency cart, such as a code cart
   f. Communication protocols (often assisted by a code button or phone system)
7. After the procedure, children require observation until they meet standardized recovery criteria, and often for a period of time thereafter. During recovery, and especially when they no longer appear sedated, children remain at significant risk for injuries caused by falling, hitting their head on bed rails, and so forth.
8. Observation and administration of medications should be performed by a clinician with no additional responsibilities—that is, not by the clinician performing the procedure. These clinicians should have training and competency in pediatric acute care and pediatric airway management.
9. Clinics and hospitals performing pediatric sedation should track outcomes related to efficacy (from a child-centered viewpoint) and safety to facilitate system-based improvements in quality of care.

Barriers to Effective Pain Management

Increasing interest in pain and pain treatment in recent years has resulted in a better understanding of pathophysiologic mechanisms of pain, better treatment options, and greater availability of treatment for pain. However, these advances have not necessarily resulted in a reduced prevalence of pain in patients with cancer.[182]

This paradox is due in part to the multitude of barriers to effective pain management. Clinician-related barriers include a focus on cure at the exclusion of attention to symptoms, lack of familiarity with pain management, fear of either regulatory repercussions for prescribing opioids or diversion of the medications to persons for whom the opioid is not prescribed, and underappreciation of the multifactorial nature of the experience of pain. Patient-related factors may include fear of addiction; fear of adverse effects, including respiratory depression; fear that treating pain with opioids is equivalent to "giving up"; and the misperception that opioids should be "saved" for severe pain.

Educational strategies for clinicians and families alike are likely to surmount many of these barriers to pain management. In addition, partnership with the child and family may enhance attention to this important issue and provide added opportunities for addressing barriers to treating pain adequately. For example, a randomized clinical trial tested the effectiveness of the PRO-SELF Pain Control Program, which actively involves adult patients and families in the pain management plan; findings included decreased pain intensity scores, an increased number of appropriate analgesic prescriptions, and increased analgesic intake in oncology outpatients who had pain from bone metastasis when compared with standard care.[183] Such an approach is endorsed by the American Pain Society and is likely to contribute greatly to overcoming barriers to successful pain management.

NAUSEA AND VOMITING

Pathophysiology

Vomiting may be caused by a wide variety of triggers, including motion, insult to the GI tract, hormonal changes, medications, and emotions. Vomiting is a complex process that involves both the central and peripheral nervous systems, with both biologic and psychological mechanisms playing a role.

During the past three decades strides have been made in understanding the pathophysiologic components of

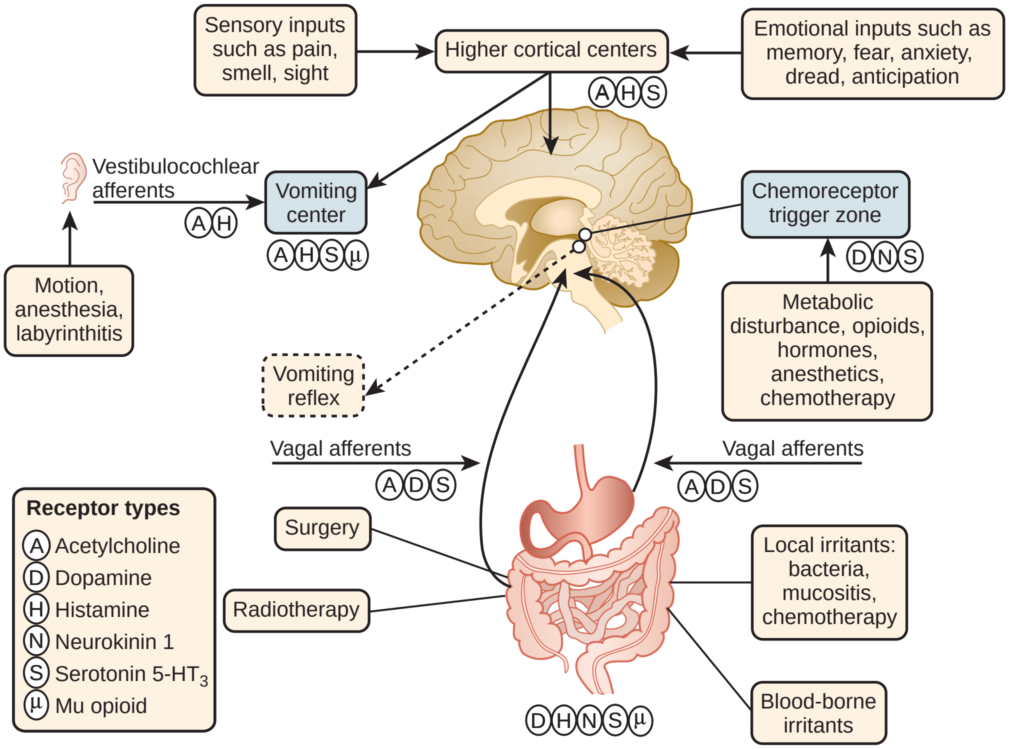

Sensory inputs such as pain, smell, sight
Higher cortical centers
Emotional inputs such as memory, fear, anxiety, dread, anticipation
A H S
Vestibulocochlear afferents
A H
Vomiting center
A H S μ
Chemoreceptor trigger zone
D N S
Motion, anesthesia, labyrinthitis
Metabolic disturbance, opioids, hormones, anesthetics, chemotherapy
Vomiting reflex
Vagal afferents
A D S
Vagal afferents
A D S
Receptor types
A Acetylcholine
D Dopamine
H Histamine
N Neurokinin 1
S Serotonin 5-HT_3
μ Mu opioid
Surgery
Radiotherapy
Local irritants: bacteria, mucositis, chemotherapy
Blood-borne irritants
D H N S μ

Figure 71-3 The pathogenesis of chemotherapy-induced nausea and vomiting.

nausea and vomiting and in the development of agents aimed at them. Investigations have focused primarily on chemotherapy-induced nausea and vomiting (CINV). Prior to the 1990s, dopamine was the major neurotransmitter recognized as being important in mediating CINV, and dopamine blockade was the mainstay of therapy for CINV.

Vomiting is mediated by the vomiting center in the medullary lateral reticular formation. This center is not a discrete entity but instead is a group of loosely organized neurons in the medulla that are activated in a sequential manner during emetogenesis.[184,185] Sources of afferent input to the nucleus tractus solitarius in the vomiting center include the chemoreceptor trigger zone (CTZ), the viscera (via vagal and sympathetic afferents), the midbrain, the vestibular system, and the cerebral cortex (Fig. 71-3). Vomiting occurs when the vomiting center emits coordinated efferent impulses to several targets including the diaphragm, abdominal wall musculature, esophagus, and stomach.

Chemotherapy-Induced Nausea and Vomiting

Chemotherapy can lead to nausea and vomiting through several mechanisms. One mechanism is by stimulation of the CTZ, which lies in the area postrema, in the floor of the fourth ventricle. Although the CTZ is part of the CNS, it contains chemoreceptors outside the blood-brain barrier that can detect emetogenic agents present in the circulation, as well as in the spinal fluid. However, evidence suggests that chemotherapy stimulates the CTZ indirectly because (1) it is unlikely that the CTZ has specific receptors that are activated by each type of chemotherapy agent; (2) the latency time to the onset of emesis is not compatible with direct action of the drugs on the CTZ; and (3) vagotomy and sympathectomy prevent cisplatin-induced emesis in the ferret, indicating that these inputs are needed for chemotherapy-induced vomiting to occur.[186,187]

Chemotherapy can also stimulate the vomiting center directly.[184] In addition, chemotherapeutic agents can stimulate the gut, leading to release of serotonin. This release in turn stimulates the vagus nerve, which activates the vomiting center, either directly or via impulses to the CTZ.[188] The central cortex may also be involved in CINV by mediating emotions such as anxiety that play a role in the genesis of nausea.

Several neurotransmitter pathways are known to play a role in nausea and vomiting. Whereas histamine and muscarinic receptors are prominent in vomiting associated with motion sickness,[189] serotonin, dopamine, and substance P appear to be particularly important in CINV.[190] Central blockade of the D2 subtype of dopamine receptors in the area postrema and vomiting center is the mechanism of action of antidopaminergic antiemetics such as metoclopramide. It was later found that in addition to dopamine receptor antagonism, metoclopramide was an antagonist of the 5-hydroxytryptamine 3 (5-HT3) receptor,[191,192] prompting interest in the role of this receptor in nausea and vomiting.[184]

Serotonin is produced by enterochromaffin cells in the gut and is also found in multiple locations in the CNS. Several observations in both animals and humans confirmed that serotonin plays a major role in acute CINV. Cisplatin and cyclophosphamide-induced vomiting in the ferret was drastically reduced with the administration of intravenous, intraperitoneal, or intracerebral injection of serotonin S3 receptor antagonists.[193] Serotonin receptor blockers and serotonin receptor blockade inhibited chemotherapy-induced vomiting in ferrets[186,194-197] and in other animal systems.[198,199] Depletion of serotonin stores was shown to also prevent cisplatin-induced vomiting.[200]

In humans, studies by Cubeddu et al.[201] demonstrated that strongly emetogenic regimens containing cisplatin or dacarbazine resulted in a marked increase in serotonin metabolites in plasma and urine with a time course similar to CINV symptoms in patients. In addition, treatment with a serotonin synthesis inhibitor inhibited both cisplatin-induced vomiting and increases in serotonin metabolites.[201] The relationship between other chemotherapeutic agents and markers of serotonin metabolism has been further explored, with more emetogenic regimens resulting in higher serotonin metabolite levels.[201] Chemotherapeutic agents appear to be toxic to the enterochromaffin cells lining the upper small intestine, resulting in generation of free radicals and release of serotonin.[187]

5-HT3 receptors are present in the GI tract as well as vagal afferents. They are also found throughout the human brainstem, including a high concentration in the CTZ.[202] Serotonin antagonists injected into the area postrema inhibit cisplatin-induced emesis in the ferret,[193] but it appears that 5-HT3 receptor antagonists also exert their antiemetic effect at the level of the peripheral nervous system via serotonin blockade in the vagal nerve.[203,204]

Substance P, which binds to the tachykinin neurokinin NK1 receptor, has more recently been implicated in mediating vomiting. Substance P is a neuropeptide found in both the CNS and in the GI tract. Because of its localization pattern and its ability to induce vomiting when administered intravenously, substance P was suspected to play a role in the genesis of vomiting.[205] NK1 receptor antagonists appeared promising in that they inhibited vomiting induced by a range of agents known to produce vomiting by both central and peripheral mechanisms in the ferret.[206,207] The site of antiemetic action of NK1 receptor antagonists has not yet been clearly defined. It may occur peripherally, at the level of vagal motor neurons that control relaxation of the gastric fundus.[185] Investigation of the time course of the antiemetic effects of a 5-HT3 antagonist and the NK1 receptor antagonist aprepitant indicates that serotonin may mediate the early vomiting process (within the first 8 to 12 hours of cisplatin administration), whereas later vomiting may be mediated by the binding of substance P to NK1 receptors.[203]

Classification of CINV

Acute and Delayed CINV. Acute CINV, also known as posttreatment CINV, is traditionally defined as nausea and vomiting that occurs within the first 24 hours after administration of chemotherapy. Delayed CINV occurs 24 hours after chemotherapy and may last up to 5 days. Delayed CINV is typically associated with certain chemotherapeutic agents such as cisplatin and peaks 48 to 72 hours after administration of chemotherapy.[208] The incidence of delayed CINV is as high as 89% in patients who receive cisplatin-based chemotherapy without antiemetic prophylaxis.[209] The incidence of delayed CINV in patients who do receive antiemetic prophylaxis is as high as 73%.[210]

Because not all acute CINV antiemetics are effective for delayed CINV and specific treatment for delayed symptoms may not be prescribed, delayed CINV may be more severe than acute CINV. The recent introduction of the NK1 receptor antagonist aprepitant has provided new options for controlling delayed CINV. However, it remains clear that the single best predictor of delayed CINV is acute CINV and that *control of acute CINV is a key factor in preventing delayed CINV*.[208,211,212] For example, in a cohort of 705 patients receiving chemotherapy for the first time, when acute CINV was completely controlled with ondansetron plus dexamethasone, 92% had complete control of delayed CINV. However, of the patients who did have acute CINV, only 41% had complete control of delayed CINV.[212]

Although some pharmacologic agents to control delayed CINV have been developed, lack of recognition of this problem remains a major barrier to its effective management. Grunberg et al.[213] demonstrated that in a cohort of patients receiving moderately emetogenic chemotherapy for the first time, clinicians overestimated control of CINV, and this discrepancy was particularly notable for delayed CINV, in which case control of symptoms was overestimated by 21% to 28%. More than 75% of clinicians underestimated the incidence of delayed CINV.[213] This misperception may in part explain the observation that fewer than half of persons who experience delayed CINV receive adequate prophylaxis that conforms to established guidelines.[214] Given the extent to which delayed CINV negatively affects the lives of persons undergoing chemotherapy,[215] improved practice, in conjunction with pharmacologic advances, is needed to reduce suffering from delayed CINV.

Relatively little information has been published regarding delayed vomiting in children. Pinkerton and colleagues[216] found that of 27 children who received cisplatin, cyclophosphamide, or carboplatin and ondansetron, 9 (33%) experienced delayed emesis. In a larger prospective study of pediatric patients who did not receive antiemetics during the delayed phase after chemotherapy, two of six children (33%) who received highly emetogenic antineoplastics (cyclophosphamide, cisplatin, or carboplatin) and 12 of 110 children (11%) who received other chemotherapeutic agents experienced delayed vomiting, although the accuracy of the incidence of delayed vomiting in the “high risk” group is limited by the small number of children in that group.[217] As has been appreciated in adult patients, children who vomited in the acute phase were significantly more likely to have delayed vomiting (45%) compared with children who did not experience significant acute vomiting (13%). In addition, chemotherapy lasting 2 or more consecutive days was associated with a significantly higher rate of delayed vomiting (39%) compared with chemotherapy administered on a single day (13%). In our experience, many children experience delayed CINV.

Anticipatory Nausea

Anticipatory nausea and vomiting is a conditioned response that occurs in anticipation of planned chemotherapy when vomiting has been poorly controlled in previous cycles. In adults, anticipatory nausea and vomiting occurs in one quarter of adults receiving chemotherapy[218] and is associated with higher anxiety, a history of severe acute CINV, and a history of delayed CINV.[218-221]

A similar pattern is seen in the pediatric population, with the severity of distress from nausea/vomiting, greater expectation for severe nausea/vomiting, and the severity of the nausea/vomiting that is actually sustained all corresponding with a higher likelihood of anticipatory CINV.[222-224] Anticipatory nausea/vomiting is most likely to develop within the first 4 months of therapy, usually occurs hours before treatment, and is most severe at the actual time that chemotherapy is administered.[222] Anticipatory nausea and vomiting in children appears to have features consistent with a conditioned response. These features suggest that this type of nausea and vomiting is a learned response, which explains why it is refractory to typical antiemetics.

Measurement and Assessment

Measurement. Although nausea and vomiting are often lumped together, as in the term "CINV," they may be regarded as two distinct phenomena. Nausea may be accompanied by physiologic changes such as pallor, sweating, or feeling hot or cold, but it is essentially a subjective experience that is measured by patient report. For this reason nausea cannot be studied in models other than humans. On the other hand, vomiting can be objectively measured. For this reason it is invariably used as the outcome of choice when evaluating interventions for nausea and vomiting. Complete control of CINV is a frequently used outcome and is defined as the absence of any vomiting for a defined period after administration of chemotherapy. That being said, vomiting does not always accompany nausea, and changes in vomiting do not necessarily reflect a concomitant change in nausea.

Although they can occur together, nausea occurs more frequently than does vomiting.[210,213,225] Much attention has been focused on the prevention of vomiting, which is an important goal because previous uncontrolled vomiting may stimulate anticipatory vomiting in subsequent cycles of chemotherapy. However, nausea is also an important symptom to assess and control because it too impairs functioning and quality of life, even in the absence of vomiting.[215,226-229] Finally, although advances such as the 5-HT3 receptor antagonists may have improved chemotherapy-associated vomiting, related posttreatment nausea has not improved.[230] For all these reasons, attention to both nausea and vomiting is needed. Research that reports only vomiting as an outcome is a limited endeavor, and interpretations of assessments that rely solely on vomiting should be made with care.

Instruments have been developed to measure the subjective phenomenon of nausea in adults.[231,232] Instruments to assess nausea in children, including children who are preverbal, have been described but are not in widespread use.[233,234] One strategy for assessing nausea in children is to have them use a simple analogue or numeric scale to rate the severity of their nausea. It is known that children 5 years and older can reliably use a color analogue scale to report the severity of their pain[235] and that children 7 years and older can report pain with a visual analogue scale[31] or a numeric scale, and thus it is likely that they can report their nausea with similar reliability. A nausea scale of 0 to 10 with six faces (the Baxter Retching Faces BARF scale) for use in children ages 7 to 18 years who have nausea for a number of reasons has recently been demonstrated to have convergent and discriminant validity, as well as responsiveness.[236]

Hinds et al.[237] have shown that a symptom distress instrument that includes nausea assessment, which was designed for adults, is reliable and valid in adolescents with cancer. Other symptom distress scales that include nausea assessment, most notably the Memorial Symptom Assessment Scale, are also reliable and valid in children.[3,4]

Within the pediatric population, parental report is also an important source of information when assessing nausea and vomiting. In one small study of CINV, parent-child dyads were found to have a moderate to strong association when reporting symptoms of nausea and vomiting.[238] Studies by Zeltzer et al.[239,240] have also showed a high degree of correlation between parental and child reports of nausea, although Tyc et al.[241] have found that parents tend to underestimate their child's nausea.

Assessment. When nausea and vomiting in patients with cancer are considered, attention is often focused on CINV. When evaluating a patient with nausea/vomiting, however, it is imperative that other causes be considered in the differential diagnosis.

A full assessment of a patient with nausea and vomiting that extends beyond quantification of the severity of the symptom permits the generation of a complete differential diagnosis. Only in this way can all possible mechanisms and therefore all potentially efficacious interventions be entertained.

Features such as whether the nausea and vomiting is acute or chronic and intermittent or constant, along with whether it is associated with any particular factors, are important to consider. Other elements of the history, such as whether vomiting is projectile, bowel patterns, current medications, and prior history of nausea and vomiting, can also be helpful in delineating the cause and therefore finding potentially effective interventions. For example, obtaining a history that suggests constipation as the cause of nausea and vomiting will lead to a workup and treatment plan that is entirely different than if the cause is suspected to be chemotherapy or labyrinthitis.

Another important component of the history is an assessment of the impact of the symptom on the patient's

Box 71-2 Patient-Related Risk Factors for Developing Chemotherapy-Induced Nausea and Vomiting

- History of previous chemotherapy and prior chemotherapy-induced nausea and vomiting
- Prechemotherapy nausea
- Prechemotherapy anxiety
- Female sex
- History of motion sickness
- Low performance status
- Low social functioning

daily functioning and quality of life. Specific instruments to address these aspects have been developed, such as the Morrow Assessment of Nausea and Emesis, which is a self-report form that allows adults to assess their experience with acute, delayed, and anticipatory CINV.[242]

Predictors of Nausea and Vomiting

The likelihood of CINV development and CINV severity is determined by both treatment-related and patient-related factors (Box 71-2). The particular agent used is the primary treatment-related risk factor for CINV, but higher dosages of the agent, shorter infusion rates, combinations with other agents, and repeated cycles of chemotherapy can also increase the risk of CINV.[204,243,244] When combination chemotherapy is given, antiemetics should be prescribed based on the most emetogenic chemotherapy drug and with the consideration that some combinations may potentially act synergistically in creating CINV.

Patient-related factors, as determined in the adult population, also appear to affect the risk for developing CINV (Box 71-2).[226,245,246] In one multivariate analysis, low social functioning, prechemotherapy nausea, and female sex were found to be predictive of more severe CINV.[226] Patients with a history of vomiting in a previous cycle are at greater risk of vomiting with the subsequent cycle[247] in part, perhaps, because of anticipatory vomiting. Use of patient-reported outcomes from previous chemotherapy cycles is critical when prescribing antiemetics. An assessment of the intended treatment, as well as the patient's individual risk factors, may help determine which patients are more at risk for the development of CINV and therefore in need of more intensive prophylaxis or rescue therapy.

Principles of Pharmacologic Therapy

Prevention of acute CINV is a key element in reducing delayed CINV. Because (1) the development of anticipatory CINV is based on prior experience, (2) severity of acute CINV predicts delayed CINV severity, and (3) rescue therapy is inadequate once nausea and vomiting have developed, the best approach to CINV and other situations in which nausea and vomiting may develop (e.g., in the postoperative period) is to prevent these symptoms from occurring in the first place. Although it is best to take a targeted approach based on the inciting cause and the suspected underlying pathophysiology and/or evidence demonstrating superiority of certain agents or classes in different situations, it is not always possible to know which agent to choose first. Guidelines for the initial choice of antiemetics, as well as dosing, are provided in Tables 71-2 and 71-3. In this situation, use of one (or two) agents with titration of the dose and the addition of other agents if necessary might be the best strategy. For CINV, which is thought to be mediated through several pathways, a combination of antiemetics is usually warranted. This approach is particularly necessary with use of highly emetogenic chemotherapy, in which case monotherapy is almost always insufficient.

Drugs used to manage CINV include true antiemetics and adjuvants. Adjuvant medications may be used to allay anxiety, induce amnesia, or induce sleep. Although many of these agents have been investigated in adults, evidence regarding their use in children, including optimal dosing and potential adverse effects, is frequently much more limited. When evidence regarding dosing in the pediatric population is available, it is presented in this chapter. However, in many circumstances we must rely on evidence from adult studies and extrapolate appropriate dosing. Furthermore, guidelines and standard practices in

TABLE 71-2 Schematic Approach to Chemotherapy-Induced Nausea and Vomiting*

Type of CINV	Prophylaxis	Breakthrough[†]
Acute CINV		
High risk	5-HT3 receptor antagonist Dexamethasone Aprepitant[‡] Lorazepam ± scopolamine patch, metoclopramide, or cannabinoid if needed	Same as prophylaxis, initially as needed, then scheduled
Moderate or low risk	5-HT3 receptor antagonist Dexamethasone ± lorazepam, scopolamine patch, metoclopramide, or cannabinoid if needed	
Delayed CINV	Ondansetron Aprepitant Dexamethasone ± metoclopramide, cannabinoid, as needed	Same as prophylaxis, initially as needed, then scheduled
Anticipatory CINV	Prevention of CINV Lorazepam Nonpharmacologic approaches	Lorazepam

CINV, Chemotherapy-induced nausea and vomiting; *5-HT3,* 5-hydroxytryptamine 3.

*Points to consider:

- Choose appropriate prophylactic antiemetics based on emesis risk, determined by patient-related risk factors and the emetogenicity of the chemotherapeutic agent(s) being used.
- Assess the effect of the antiemetic regimen regularly.

[†] If breakthrough CINV occurs, strategies may be to (1) add a new agent, (2) change the dose or schedule of antiemetics in use but not already maximized, or (3) change administration of existing "as-needed" antiemetics to scheduled administration. In addition, the prophylaxis regimen for subsequent chemotherapy cycles should be augmented.

[‡] If ≥12 years of age.

TABLE 71-3 Classes of Antiemetics, Dosing, and Routes of Administration

Class	Dose and Route	Indication	Notes
5-HT3 Receptor Antagonists			
Ondansetron	0.15 mg/kg dose PO every 8 h or 0.45 mg/kg/dose PO every 24 h, not to exceed 24 mg 0.15 mg/kg (not to exceed 8 mg/dose) IV every 8 h; no single dose should exceed 16 mg due to risk of QT prolongation	First-line therapy for prophylaxis of high-, moderate-, or low-risk CINV or breakthrough CINV	May cause constipation and/or headache; available as an oral disintegrating tablet (that contains phenylalanine) or may be extemporaneously prepared For patients with electrolyte abnormalities, congestive heart failure, bradyarrhythmias, or patients taking other medications with the potential to cause QT prolongation, consider ECG monitoring
Granisetron	<50 kg: 20 μg/kg IV/PO daily; maximum dose 1 mg ≥50 kg: 1 mg IV/PO daily	First-line prophylaxis for high-, moderate-, or low-risk CINV or breakthrough CINV	Available as an oral solution For patients with electrolyte abnormalities, congestive heart failure, bradyarrhythmias, or patients taking other medications with the potential to cause QT prolongation, consider ECG monitoring
Dolasetron	≥2 yr: 1.8 mg/kg IV or PO as a single dose; maximum single dose 100 mg		May be extemporaneously prepared For patients with electrolyte abnormalities, congestive heart failure, bradyarrhythmias, or patients taking other medications with the potential to cause QT prolongation, consider ECG monitoring
Dopamine Antagonist			
Metoclopramide	0.5 mg/kg IV or PO daily (for prophylaxis) 0.5 mg/kg IV or PO every 6 h (for breakthrough CINV) 1 mg/kg IV or PO every 6 h (for severe breakthrough CINV or if lower dose is ineffective)	High-risk prophylaxis; breakthrough CINV	High-dose metoclopramide: Prescribe with diphenhydramine and continue diphenhydramine for 24 h after the last dose to prevent EPS; if the metoclopramide dose is ≤1 mg/kg and the patient weighs >40 kg, scopolamine may be considered Treat EPS with diphenhydramine or benztropine
Corticosteroid			
Dexamethasone	≤BSA 1 m^2: 10 mg/m^2 IV or PO daily BSA >1 m^2: 10-12 mg IV or PO daily; may use 20 mg as a single dose on day 1	High-risk prophylaxis for acute or delayed CINV; breakthrough CINV	Contraindicated in patients receiving pulmonary radiation therapy Consider lower dose if given with an NK-1 receptor antagonist
NK1 Antagonist			
Aprepitant	≥45kg: 125 mg PO ×1 on day 1, then 80 mg PO daily on days 2 and 3	Prophylaxis of acute or delayed CINV	Limited data in pediatrics CYP3A4 substrate and inhibitor of metabolism of drugs that are CYP3A4 substrates including chemotherapeutic drugs (such as vincristine, irinotecan, imatinib, among others) and nonchemotherapeutic drugs (e.g., corticosteroids)
Anticholinergic			
Scopolamine	1.5-mg patch applied transdermally; change every 72 h	High-risk prophylaxis; breakthrough CINV	Use only if >40 kg (the patch cannot be cut); may cause dry mouth and/or blurred vision
Cannabinoid			
Dronabinol	2.5-5 mg/m^2/dose PO every 6 h (10 mg/dose maximum)	High-risk prophylaxis; breakthrough CINV	Do not use in children <6 yr; use with caution if 6-12 yr; may cause confusion, ataxia, hallucinations; no IV form
Anxiolytic			
Lorazepam	<20 kg: 0.025 mg/kg IV or PO every 6 h 20-40 kg: 0.0125 mg/kg IV or PO every 6 h >40 kg: 0.5-2 mg IV or PO every 6 h	High-risk prophylaxis; breakthrough; anticipatory CINV	May cause confusion, sedation, hallucinations, or memory impairment; avoid use of dose greater than 0.5 mg unless proven tolerance of 0.5 mg

BSA, Body surface area; *CINV*, chemotherapy-induced nausea and vomiting; *ECG*, electrocardiogram; *EPS*, extrapyramidal side effects; *IV*, intravenous; *NK1*, neurokinin 1; *PO*, by mouth.

adults may involve formulations of medications that cannot be adjusted for pediatric patients (e.g., capsules or patches). Use of medications in children that are only available in tablet or capsule form also may not be feasible for developmental or psychological reasons.

Dopamine Receptor Antagonists

Dopamine receptor antagonists include the butyrophenones (e.g., droperidol), phenothiazines (e.g., prochlorperazine), and substituted benzamides (e.g., metoclopramide; refer to the "Metoclopramide" section later in this chapter). Until the advent of 5-HT3 receptor antagonists and other antiemetics in the 1990s, dopamine-blocking agents were widely used. Use and efficacy of these agents are limited by their adverse effects, which include sedation and extrapyramidal symptoms (EPS). Children may be at higher risk for EPS, although EPS can often be prevented by concomitant administration of an anticholinergic agent such as diphenhydramine. Scopolamine, an antiemetic in its own right, is sometimes also used for this purpose. We have experience using scopolamine for EPS prophylaxis when the metoclopramide dose is less than or equal to 1 mg/kg/day and the patient weighs 40 kg or more.

Metopimazine, a phenothiazine derivative, is used as an adjunct to 5-HT3 antagonists to treat CINV. Although it is not marketed in North America, metopimazine is used for children in Europe, and a recent small study demonstrated improved emetic control when it was combined with ondansetron compared with ondansetron monotherapy. Olanzapine, an atypical antipsychotic agent, has fewer extrapyramidal effects than do older agents. In small studies in which it is combined with other antiemetics for CINV prophylaxis, olanzapine showed promise in preventing or treating CINV.[248] Chlorpromazine and droperidol both may cause prolongation of the QT interval, and droperidol has been associated with serious cardiac adverse events. Extreme caution, including electrocardiogram monitoring, should be exercised if these agents are used, particularly in patients at risk for arrhythmias. Today use of droperidol is usually limited to the operative period in patients who do not respond to other agents.

5-HT3 Receptor Antagonists

5-HT3 receptor antagonists became the mainstay of therapy in the prevention and treatment of acute CINV since their introduction in the 1990s, when they were shown to be superior to the majority of preexisting antiemetics such as prochlorperazine[249] and metoclopramide,[250-255] even when highly emetogenic chemotherapy such as cisplatin is administered.[251-253]

Compared with other agents, the superiority of 5-HT3 receptor antagonists in preventing acute CINV in children has also been confirmed.[256-260] The efficacy of these agents appears to be further enhanced by the addition of a corticosteroid.[261-263] In one study of 33 children, ondansetron plus dexamethasone provided a complete response in 61% of patients, whereas ondansetron alone provided a complete response in only 21% of patients.[262] Ondansetron has also been shown to reduce acute CINV compared with placebo when chemotherapy is delivered intrathecally.[264]

First-generation agents in this class include ondansetron, dolasetron, and granisetron. Taken together, the data on these agents indicate that these agents are equally effective when given at equivalent doses.[265-271,272] These agents also appear to be equivalent in children, including in situations involving high-dose therapy such as preparative regimens for hematopoietic stem cell transplant.[273-275] In one small study it was found that, compared with tropisetron, ondansetron better controlled acute CINV resulting from use of moderately emetogenic agents. However, no difference was found between the two agents with regard to controlling acute CINV resulting from use of highly emetogenic agents.

5-HT3 antagonists are quite well tolerated by children,[263] with the most common adverse effects reported being mild headache and constipation.[264] 5-HT3 inhibitors, in conjunction with a corticosteroid, are now recommended as first-line therapy for children who receive moderately to highly emetogenic chemotherapy.[246,276]

Efficacy and tolerability of daily administration of high-dose ondansetron (24 mg or 32 mg by mouth daily) has been compared with a smaller dose (8 mg by mouth) given twice daily in adults. A large dose given daily is well tolerated in adults.[277] In a large multicenter, randomized trial, the 24-mg daily dose provided the highest complete control of nausea and vomiting.[278] The superiority of more intensive dosing has been confirmed by other investigators.[279-282] The efficacy of 32 mg compared with 24 mg has also been confirmed by Tsavaris.[283] However, because of the risk for QT interval prolongation in the IV formulation, the 32-mg IV dose is no longer available, and single (daily) doses exceeding 24 mg are not recommended.

Oral and IV routes of administration have been shown to be equally effective in both adults and children.[284-287] Ondansetron, dolasetron, and granisetron are all available in IV formulations and as an oral tablet. Ondansetron is available as an oral dissolving tablet and as an oral liquid; these two preparations are especially attractive in the pediatric population. Granisetron may be extemporaneously compounded by an apothecary. Successful administration of 16 mg of ondansetron daily as a suppository has been described in adults[288] but not in children.

A second-generation 5-HT3 receptor antagonist, palonosetron, differs from its predecessors in its stronger affinity for the 5-HT3 receptor and its prolonged plasma half-life (four times that of the other 5-HT3 antagonists), qualities that may enhance its duration of action.[289,290] In randomized trials in adults, single doses of palonosetron appear to be at least as good in the prevention of acute CINV as single doses of first-generation antagonists delivered 30 minutes before administration of moderately emetogenic chemotherapy. Furthermore, the single dose of long-lasting palonosetron provides better protection from delayed CINV.[291-293] Because of these findings, palonosetron is recommended rather than first-generation 5-HT3 receptor antagonists for adults receiving highly or moderately emetogenic chemotherapy.[294] In pediatric patients palonosetron may prove to be a useful agent in the prevention of acute CINV during multiple-day therapy (in which acute CINV is repeatedly induced) and for the prevention of delayed CINV. Although administration of palonosetron for the prevention of CINV in children has been described,[295,296] further study of dosing, safety, and efficacy is needed before palonosetron can be recommended for children.

Corticosteroids

Corticosteroids are frequently used as antiemetics, although their mechanism of action is unclear. Dexamethasone is the best-studied corticosteroid and the primary one in use today, although success with methylprednisolone has been described. The typical dexamethasone dose used for adults is 10 to 20 mg per day. Our starting dose for children is 10 mg/m^2, to a maximum of 10 mg/day. For persistent vomiting, this dose may be doubled to a maximum of 10 mg given twice a day. If administered with an NK1 receptor antagonist, the corticosteroid dose should be halved.

Dexamethasone provides moderate protection from CINV, including delayed CINV, when used alone.[297-299] Dexamethasone has been shown to be as effective as metoclopramide for acute CINV in adults receiving moderately and highly emetogenic chemotherapy without the extrapyramidal effects of metoclopramide.[300,301]

Dexamethasone is especially useful in preventing delayed CINV from cisplatin, cyclophosphamide, and doxorubicin[302-307] and may be even more effective for this purpose when combined with metoclopramide.[209] Dexamethasone is also very effective in potentiating the action of other antiemetics, including 5-HT3 inhibitors[308] and metoclopramide.[304,306,307,309-312]

In the pediatric population, dexamethasone combined with ondansetron provided significantly more protection from acute CINV that occurred as a result of highly emetogenic agents than did ondansetron monotherapy; 77% of children receiving ondansetron monotherapy had at least one episode of vomiting, compared with 39% who received ondansetron with dexamethasone.[262]

The benefit of improved protection from CINV must be weighed against the risks of corticosteroids. Adverse effects of corticosteroids include metabolic effects, gastritis, insomnia, hypertension, immune dysregulation, impaired wound healing, and adverse psychiatric effects such as emotional lability and, more rarely, psychosis. In general, short courses of corticosteroids do not usually produce significant adverse effects and are often very well accepted and tolerated by patients.[297] Potential beneficial effects of corticosteroids are preservation of appetite and promotion of energy.

Other potential risks include a decrease in action of a biologic response modifier or induction of radiation pneumonitis when a steroid is withdrawn from patients who have undergone lung radiation.[313,314] Caution is advised if corticosteroids are used as antiemetics in conjunction with a treatment regimen that is associated with a high risk of infection or GI toxicity such as induction regimens for acute myelogenous leukemia. Concomitantly administered corticosteroids may further increase these risks. Concerns about the impact of steroids on the blood-brain barrier have led to a desire to avoid the use of steroids as an antiemetic in patients with brain tumors, although chemotherapy-specific data are lacking.[315] The use of steroids may be prohibited when they are already part of a patient's chemotherapeutic regimen.

Metoclopramide

Metoclopramide, a procainamide derivative, exerts antiemetic effects via both a central mechanism (dopamine receptor blockade in the CTZ) and a peripheral mechanism (promotion of gastric emptying).[316] It has been recognized as an effective antiemetic for adults for the past two decades[317] as monotherapy or in conjunction with dexamethasone,[318] lorazepam,[319] or both.[320] Low-dose metoclopramide (i.e., 0.1 mg/kg/dose) is effective in treating postoperative nausea and in promoting gastric emptying.

At high doses, metoclopramide also serves as a serotonin receptor antagonist[191,192] and provides better protection from CINV.[247] For these reasons, metoclopramide is usually given at high doses for CINV. At such higher doses, however, patients—and, in particular, children—are at a greater risk for extrapyramidal adverse effects such as dystonia and akathisia. In a dose-related toxicity study in children, significant extrapyramidal toxicity was observed at doses of 2 mg/kg or higher,[321] and for this reason, high-dose metoclopramide is given with diphenhydramine to decrease the risk of extrapyramidal adverse effects.[247] Extrapyramidal adverse effects can occur up to 24 hours later in patients who receive multiple daily doses of metoclopramide, and thus diphenhydramine administration should continue until 24 hours after the last dose of metoclopramide. We have also had personal experience using transdermal scopolamine as an anticholinergic agent in patients receiving no more than 1 mg/kg/day of metoclopramide. Because of the association of tardive dyskinesia with high-dose or long-term administration of metoclopramide, the FDA issued a boxed warning about this risk in 2009.

Cannabinoids

Delta-9-tetrahydrocannabinol (THC) is the active ingredient in cannabis, or marijuana. THC was approved by the FDA in 1985 for the treatment of emesis. Synthetic THC, called dronabinol (Marinol), has since become available. It is formulated in sesame oil and is available as 2.5- or 5-mg gelatin capsules. A homologue of THC, nabilone, is also now available in the United States.

THC binds to the CB1 receptor found in the central and peripheral nervous systems, as well as the CB2 receptor, which is found in nonneural tissues. THC exerts its antiemetic effect as a receptor agonist, in contrast to other antiemetics, which typically serve as receptor antagonists. THC also interacts with dopaminergic, serotonin, monoaminergic, noradrenergic, and opioid systems, which are pathways that mediate both emesis and pain.

Sallan and colleagues[321a] demonstrated that in patients who have CINV that is not controlled by standard antiemetic therapy, THC provided better complete protection from CINV (in 36 of 79 subjects) than did prochlorperazine (in 16 of 78 subjects). Interestingly, patients younger than 20 years had a higher proportion of complete responses than did older patients. Persons in the THC group also had significantly higher food intake. Adverse effects from cannabinoid administration include sedation, mood alterations (such as euphoria and dysphoria), dizziness, hallucinations, and arterial hypotension. Because of these potential adverse effects, cannabinoids are not typically considered first-line agents for CINV prophylaxis but are added to a regimen if first-line agents are not adequate for prophylaxis or breakthrough CINV.

An advantage of cannabinoids is their utility in treating pain. They are known to bind to kappa and delta receptors and act synergistically with opioids, a feature that may be useful when treating children with both nausea and pain.[322] In addition, they are frequently used to stimulate appetite in patients who are not experiencing nausea or vomiting per se.

NK1 Receptor Antagonists

Aprepitant and its prodrug, fosaprepitant, are the only approved NK1 receptor antagonists. NK1 receptor antagonists are particularly effective in preventing delayed CINV, a historically difficult symptom to prevent. A study was performed to compae cisplatin-naive patients who received granisetron plus dexamethasone on day 1 and either (1) aprepitant (then called L-754,030) on day 1 or (2) aprepitant on days 1 through 5 or (3) placebo on days 1 through 5. In the aprepitant arms, 93% and 94% of subjects had no acute vomiting, compared with 67% in the placebo arm who had no vomiting. Subjects in the aprepitant arms also experienced significantly better complete protection from delayed vomiting (82% and 78%) compared with subjects who received a placebo (33%). In addition, minimal or no nausea was noted in 49% and 48% of subjects in the aprepitant arms and in 25% of subjects who received a placebo.[323]

Other studies have replicated these impressive findings in terms of control of delayed CINV, but they have not demonstrated the improved protection from acute CINV seen in the aforementioned study.[324,325] For example, in a double-blind multicenter trial of 351 patients, aprepitant with dexamethasone provided no benefit in preventing acute emesis when compared with the combination of granisetron and dexamethasone; however, the addition of aprepitant to granisetron and dexamethasone more than doubled the efficacy of this regimen in preventing delayed vomiting.[324]

Aprepitant is generally very well tolerated by adults and adolescents and is easily administered by mouth daily. In addition, it appears to decrease delayed cisplatin-induced CINV that has been established to be refractory to the combination of 5-HT3 antagonists and dexamethasone.[326] The efficacy of aprepitant appears to be sustained over multiple cycles of chemotherapy.[326,327]

Aprepitant is usually administered for 3 consecutive days, because administration on additional days has not been shown to provide better protection from CINV. A pilot study comparing 1-day versus 3-day administration of aprepitant in the prevention of acute and delayed CINV in patients who were receiving highly emetogenic chemotherapy demonstrated no significant difference between the regimens.[328] Additional studies are needed to confirm the noninferiority of the single-day aprepitant regimen.

Studies of the efficacy and safety of aprepitant in children are rare and quite limited methodologically. Use of aprepitant in children as young as 11 years using adult dosing (125 mg on day 1 and 80 mg daily on days 2 and 4) has been reported.[329-331] In the only published prospective trial of aprepitant in children, 46 children aged 11 to 19 years who received emetogenic chemotherapy along with ondansetron and dexamethasone for 4 days were randomly assigned to also receive aprepitant or placebo for 3 days. In this study, which was designed to evaluate pharmacokinetics and tolerability, it was found that aprepitant was well tolerated but that febrile neutropenia occurred more often in children who received aprepitant, although the overall infection rate was higher in the placebo group. The limited sample size may have contributed to these findings and prevented statistical significance in the assessment of efficacy. The study demonstrated that pharmacokinetics in adolescents is similar to that of adults, and it is therefore reasonable to administer the adult dose to this age group.[331] Administration of a lower dose (80 mg/day for 3 days) to smaller children (<20 kg) has been described, but the efficacy of this regimen in preventing CINV was not reported.[330]

Fosaprepitant is an IV prodrug of aprepitant that is approved for administration in adults as a one-time dose prior to the administration of chemotherapy. Administration of this one-time dose given with ondansetron and dexamethasone provides protection from acute and delayed CINV that is equivalent to a regimen of aprepitant, ondansetron, and dexamethasone.[332] Fosaprepitant dosing in children is currently being studied.

Aprepitant is a CYP3A4 enzyme pathway substrate and inhibitor and therefore may alter levels of drugs metabolized via this pathway. Concomitant administration with aprepitant may raise the level of drugs such as dexamethasone, methylprednisolone, fentanyl, cyclophosphamide, thiotepa, vincristine, or etoposide.[333,334] For this reason aprepitant should be prescribed with care to avoid potential interactions, and the patient should be monitored closely for such interactions. It has been suggested that when aprepitant is coadministered with dexamethasone for CINV prophylaxis, the dexamethasone dose should be decreased by 50%.[335] Aprepitant does not appear to alter the pharmacokinetics of ondansetron or granisetron.[336] Aprepitant should be used with caution in patients who take warfarin.[337]

Other Agents

Lorazepam is often used as an adjuvant to true antiemetics because of its anxiolytic and amnestic properties. Early studies evaluating the addition of lorazepam found that patients reported less anxiety and preferred regimens that contained this additional agent, although it did cause more sedation.[338-340] The acute antiemetic effect of several agents is enhanced when lorazepam is given concomitantly.[341-343] Interestingly, the addition of lorazepam to regimens that contained dexamethasone as prophylaxis against delayed CINV from cisplatin appears to decrease delayed CINV as well.[303,341]

Scopolamine is a muscarinic antagonist that is known to reduce motion sickness. It is inadequate as monotherapy for CINV,[344] but in conjunction with other antiemetics such as metoclopramide and dexamethasone, it reduces cisplatin-induced CINV.[345] Scopolamine is administered as a 1.5-mg transdermal patch applied behind the ear that releases 0.5 mg/day and must be changed every 3 days. Because the patch is only available in the 1.5-mg size and cannot be cut, we normally reserve it for children who weigh 40 kg or more. Adverse effects from scopolamine include dry mouth, blurry vision, and mydriasis from systemic effects. Mydriasis on the side ipsilateral to the patch from unintentional touching of the patch followed by rubbing of the eyes can also occur.[346]

Antihistamines such as dimenhydramine, hydroxyzine, and diphenhydramine are effective in reducing the nausea associated with vertigo and motion sickness. For CINV management, diphenhydramine is often given in regimens containing high-dose metoclopramide to prevent extrapyramidal effects. Diphenhydramine does not, however, enhance the antiemetic effect of metoclopramide,[347,348] and apart from preventing extrapyramidal effects, it should not be used for CINV management.

Olanzapine is an atypical antipsychotic agent that binds to several receptors, including dopamine, serotonin, and to a lesser extent histamine and muscarinic receptors. Because of its action at multiple receptor sites implicated in CINV, it may hold promise as a therapy for CINV. In a small study of 10 adult patients receiving moderately to highly emetogenic chemotherapy with olanzapine, palonosetron, and dexamethasone as antiemetic prophylaxis, 100% had complete protection from nausea and vomiting in the first 24 hours after chemotherapy, with 50% and 75% of patients protected from nausea and vomiting, respectively, on days 2 to 5.[349] Olanzapine is consequently included in some guidelines for adults with CINV. No studies evaluating olanzapine for children with CINV have been published.

Gabapentin has also been evaluated for CINV in adults.[350] One study showed a decrease in peak nausea scores for both acute and delayed CINV, indicating that this agent may also hold promise as a therapy for CINV.

Special Cases

Delayed Vomiting. Until very recent years, steroids and metoclopramide were the primary agents used to control delayed vomiting,[209,305,320] with modest protection from delayed CINV in 48% to 57% of adult patients. Some studies have demonstrated efficacy of 5-HT3 antagonists in preventing delayed CINV[351,352] and providing better protection when compared with existing drugs such as prochlorperazine.[353] However, the majority of studies have demonstrated that the addition of a 5-HT3 receptor antagonist to the standard therapy, dexamethasone, does not confer greater protection from delayed CINV.[211,212,354-357] In a meta-analysis of the efficacy of 5-HT3 antagonists, these antagonists did not confer protection from delayed CINV significantly beyond that provided by dexamethasone monotherapy. When ondansetron plus dexamethasone was compared with metoclopramide plus dexamethasone in randomized controlled trials, 5-HT3 was equivocal at best.[358-360]

In one pediatric study evaluating ondansetron for acute and delayed CINV after treatment with carboplatin, cisplatin, Adriamycin with cyclophosphamide, or ifosfamide, ondansetron prevented acute CINV in 87% of children. Its efficacy in preventing delayed CINV was not nearly as high; it prevented only 20% of children from experiencing nausea and 50% of children from vomiting after administration of cisplatin or ifosfamide. A reasonable approach would be to start with dexamethasone and metoclopramide. In children who do not tolerate or who do not respond to metoclopramide, a 5-HT3 antagonist such as ondansetron may then be tried as an alternative.

In adults, some of the best protection from delayed CINV is conferred by the combination of a single dose of palonosetron with three doses of aprepitant and concurrent dexamethasone. In one study, this regimen provided a complete response (no emesis and no rescue medication) for 88% of patients in the acute period and 78% during the delayed period.[361] Evidence for the efficacy of palonosetron in preventing delayed CINV is limited to single-day administration regimens. Interest in consecutive daily dosing of palonosetron to cover multiday chemotherapy has been expressed. Multiple dosing of palonosetron has recently been approved for adults.

Numerous studies in adults have demonstrated the effectiveness of aprepitant for delayed CINV, as previously described. Because most studies of aprepitant have been in the setting of single-day emetogenic treatment, it can be difficult to know how best to utilize this drug in the setting of the multiday regimens common in pediatrics. It is our practice to give the drug for up to 5 days, which may or may not continue beyond the end of the emetogenic treatment.

Anticipatory Nausea. Because anticipatory CINV it is a learned response, typical antiemetics are generally ineffective. The best strategy for preventing CINV is the prevention of CINV in previous courses of chemotherapy.[213] Because anxiety plays a key role in anticipatory nausea, use of an anxiolytic such as lorazepam may be effective.

Breakthrough Emesis. Breakthrough emesis is defined as vomiting that occurs in spite of optimal preventive therapy. Although no studies of breakthrough emesis have been performed and no widely accepted standards are available, a reasonable approach is (1) ensuring that the patient is receiving maximal doses of current antiemetics and (2) adding a rescue agent from a category different from those the patient is already receiving. Although breakthrough agents may be started on an as-needed basis, scheduled administration may be needed during the current or future cycles.

Radiation-Induced Nausea and Vomiting

Radiation-induced nausea and vomiting occurs acutely in more than 90% of adult patients who receive total body

TABLE 71-4 Emetogenicity of Chemotherapeutic Agents and Radiotherapy Fields Commonly Used in Pediatrics

	RISK OF EMESIS			
Modality	**High (>90%)**	**Moderate (30%-90%)**	**Low (10%-30%)**	**Minimal (<10%)**
Chemotherapy	Carmustine (BCNU) Cisplatin Cyclophosphamide (>1500 mg/m^2) Dacarbazine (DTIC) Dactinomycin Ifosfamide (≥500 mg/m^2) Lomustine (CCNU) Mechlorethamine (nitrogen mustard) Procarbazine Thiotepa (≥300 mg/m^2)	Arsenic trioxide Carboplatin Clofarabine Cyclophosphamide (750-1500 mg/m^2) Cytarabine (>1000 mg/m^2) Cytarabine (intrathecal) Daunorubicin Doxorubicin Epirubicin Idarubicin Ifosfamide (<500 mg/m^2) Irinotecan Melphalan (>50 mg/m^2) Methotrexate (>1000 mg/m^2)	Cyclophosphamide (≤750 mg/m^2) Cytarabine (<1000 mg/m^2) Etoposide (VP-16) 5-Fluorouracil Gemcitabine Imatinib Methotrexate (50-1000 mg/m^2) Mitomycin Mitoxantrone Topotecan Vinblastine Vorinostat	2-Chloroxydeadenosine (cladribine) Asparaginase Bortezomib Bleomycin Busulfan Decitabine Fludarabine Hydroxyurea Mercaptopurine Methotrexate (<50 mg/m^2) Monoclonal antibodies (e.g., rituximab) Thioguanine Vincristine Vinorelbine
Radiotherapy	Total-body irradiation Upper abdomen (moderate to high risk)	Abdominal-pelvic Craniospinal Hemibody irradiation	Cranium Lower thorax Mantle	Other (e.g., extremity)

irradiation for bone marrow transplantation and within 30 to 60 minutes in 80% of adults who receive single high-dose/large-field hemibody irradiation. Radiation-induced nausea and vomiting may also occur within 2 to 3 weeks in about 50% of adults who receive fractionated radiotherapy to the abdomen.[362] The incidence and severity of radiotherapy-induced nausea and vomiting is largely related to the location of the radiation field, as indicated in Table 71-4. In a prospective study of adults undergoing radiotherapy, the two radiotherapy-related risk factors for nausea and vomiting were the site of irradiation and the field size, with significantly more vomiting in persons who received radiation to the upper abdomen and in those with a radiation field size greater than 400 cm^2.[363] The only patient-related factor was previous experience with cancer chemotherapy. Because radiation to the upper/mid hemibody results in an increase in circulating serotonin metabolites and because of the efficacy of 5HT-3 receptor antagonists in this setting, it has been proposed that serotonin mediates radiation-induced emesis.[364]

In patients receiving single-dose radiation to the upper abdomen, ondansetron has been demonstrated to be superior to metoclopramide in reducing vomiting and nausea.[365] In a randomized controlled trial comparing ondansetron with prochlorperazine in adults receiving fractionated radiotherapy to the abdomen, 43 (61%) of patients in the ondansetron arm and 23 (35%) of patients in the prochlorperazine arm had a complete response, that is, complete protection from emesis throughout their entire treatment course (P = .002). However, no difference was found in the incidence or severity of nausea in the two groups.[366] Although no controlled trials have been performed in the pediatric population, studies have demonstrated its efficacy and tolerability during radiotherapy.[367] Dolasetron has also been used successfully to prevent nausea after a single high-dose fraction of radiotherapy to the upper abdomen.[368]

The addition of dexamethasone to ondansetron appears to confer a slight benefit to ondansetron, even for multiply fractionated radiation to the upper abdomen.[369] Current adult guidelines recommend prophylaxis with a 5-HT3 receptor antagonist before each fraction and for 24 hours after the last fraction for patients at high or moderate risk of radiation-induced vomiting (i.e., total body irradiation and radiation directed at the upper abdomen); prophylaxis or rescue with a 5-HT3 receptor antagonist in the low-risk group (i.e., those with radiation directed at the lower thorax, pelvis, or cranium); and rescue with a dopamine or 5-HT3 antagonist in the minimal risk group (i.e., patients with radiation directed at the head and neck, extremities, and breast).[370] One study evaluating ondansetron in children undergoing radiotherapy has been performed.[367] In this study it was found that ondansetron provided 60% protection in children undergoing radiation therapy for a brain tumor, although no control subjects were used for comparison.

Postoperative Vomiting

Postoperative nausea and vomiting (PONV) is a common problem in all pediatric patients. Recent studies have demonstrated that dexamethasone is effective in preventing acute and late PONV.[371-373] Droperidol is also an effective agent, but given the high level of sedation, risk of EPS, and the recent concern of cardiotoxicity, this agent is no longer considered first-line prophylaxis.[374] A significant number of studies have evaluated the efficacy of ondansetron in preventing PONV in children, and this agent is considered the drug of choice for this situation.[374,375] Pooled analyses, however, show that the efficacy lies primarily in an antiemetic effect rather than an antinausea effect.[375]

A recent controlled study of adults undergoing surgery also showed that scopolamine may be a promising agent in preventing PONV in adults, although it caused the symptom of dry mouth to a considerable degree.[376] Two large adult studies have recently shown that one dose of aprepitant is superior to ondansetron in preventing postoperative vomiting; however, only one study found a significant reduction in postoperative nausea.[377,378]

Vomiting from Other Causes

Opioid-Induced Nausea and Vomiting

Although reactions to opioids are frequently, and incorrectly, labeled as an allergy, they can cause nausea and vomiting. Such effects are mediated through (1) direct effects of opioids on the CTZ, (2) effects on the vestibular apparatus, and (3) signals from the gut as a result of constipation. Except when it is due to constipation, opioid-related nausea and vomiting tends to improve with repeated dosing of the opioid. Therefore a reasonable strategy is to provide an antiemetic with the first few doses of the opioid, particularly if the patient is at risk for nausea and vomiting. This strategy prevents the problem of the patient being labeled as "allergic" to the opioid.

Although no evidence is available to indicate that one opioid is more emetogenic than another, it is commonly believed that morphine and codeine are the most likely opioids to cause nausea and vomiting. If nausea and vomiting in response to an opioid persist despite use of antiemetics, it may be reasonable to consider changing the opioid or route of administration. Because tolerance to the emetogenic effect of the initial opioid usually develops in a few days, however, it may be difficult to know whether it was tolerance or the change in opioid that improved symptoms.

Data comparing antiemetics for opioid-induced nausea and vomiting are limited. One study compared 24 mg of daily ondansetron with either metoclopramide, 10 mg by mouth three times a day, or placebo. No differences were detected in the three arms, although the study was terminated prematurely as a result of accrual difficulties.[379] In another study of adults receiving opioids for postsurgical pain, single doses of either 8 or 16 mg of ondansetron provided better emetic control than did a single 10-mg dose of metoclopramide. One report also indicates that ondansetron may be effective in preventing nausea and vomiting from morphine administered via the spine.[380] Recent publications have also demonstrated that the addition of a low-dose naloxone infusion may reduce opioid-related adverse effects, including nausea, without affecting analgesia in both adults and children.[381-383]

Disease-Related Vomiting

Outside of direct treatment-related nausea and vomiting, disease processes themselves may cause nausea and vomiting through a variety of mechanisms, including increased intracranial pressure, GI obstruction, altered gut motility, organ capsule distension, or GVHD. For example, altered gut motility appears to contribute to nausea and vomiting in patients recovering from bone marrow transplantation.[384-386] Interestingly, recent reports have indicated that mirtazapine, a new serotonin-norepinephrine reuptake inhibitor, improves nausea and vomiting in patients with gastroparesis from a variety of causes, but mirtazapine has not been studied for this purpose in patients undergoing bone marrow transplantation or in children.[387-390]

In addition, other processes that may or may not be related to the disease process or its treatment, such as GI infection, may be present. The mnemonic V.O.M.I.T. may be used to review the possible underlying causes of nausea and vomiting so that a targeted approach to the situation may be undertaken.[391] This mnemonic indicates that nausea and vomiting may be due to **v**estibular problems, **o**bstruction of the bowel (including constipation), gut dys**m**otility, **i**nfection and inflammation, and **t**oxins. Remarkably little is known about the effectiveness of antiemetics for these causes, and treatment of the underlying problem may be the best strategy. Ondansetron has a modest effect in controlling vomiting that occurs as a result of acute gastroenteritis.[392]

Nonpharmacologic Interventions

Acupuncture and behavioral therapies are some of the best-studied nonpharmacologic interventions for CINV. Electroacupuncture reduced vomiting but not nausea in women undergoing myeloablative chemotherapy when compared with control subjects.[393] In children, P6 acupoint injections are as effective as droperidol in preventing postoperative nausea and vomiting.[394]

Acupressure and acustimulation have also been studied as interventions to mitigate CINV. In one relatively large study of 739 patients who were randomly assigned to use an acupressure band, an acustimulation band, or no band, persons who received acupressure bands had less nausea on the day of treatment, but no differences in delayed nausea were found.[395] In pooled analyses of trials evaluating acupuncture-point stimulation, these interventions reduced the proportion of acute vomiting but not the severity of nausea. When broken down by modality, stimulation with needles and electroacupuncture reduced vomiting but not nausea. Acupressure reduced nausea but not vomiting, but these studies were not controlled.[396]

In children receiving chemotherapy, behavioral interventions such as hypnosis and cognitive distraction reduce nausea, vomiting, and the extent to which these symptoms bothered the children, and these effects were maintained even after the interventions were discontinued and chemotherapy was continued.[239] Behavioral interventions such as hypnosis and systemic desensitization also appear to be useful in treating anticipatory nausea, a phenomenon with a strong learned component.[397,398] Progressive muscle relaxation has been shown to reduce the duration of CINV in women with breast cancer but not the intensity or frequency of nausea or vomiting episodes.[399]

Role of the Patient and Family

Because patients who anticipate CINV may be more likely to actually experience it, discussing the role of interventions to prevent or mitigate CINV may enhance the efficacy of these interventions. Patients and families should also be encouraged to record their symptoms in a diary and to notify their medical team of uncontrolled symptoms when they occur, particularly because uncontrolled acute nausea is associated with increased incidence of both delayed nausea and anticipatory nausea with future chemotherapy. The phenomenon of delayed nausea should specifically be addressed, because children and their parents are usually at home and unable to be observed at the time the child may experience these symptoms.

Strategies to enhance patients' sense of control over their treatment may also improve their experience. In one small study of adults with CINV that was controlled with IV antiemetics via a pump, the patients who were able to control the pump themselves used less antiemetic.[400] More recent studies in both adults and children indicate that a continuous infusion, patient-controlled pump is well tolerated, safe, and effective in controlling CINV.[401,402] Patients should be encouraged to explore strategies for nonpharmacologic management if these options are of interest, because they appear to have little downside and can also add to the patient's sense of control over his or her experience.

Future Directions

Significant advances have been made in the past few decades with regard to managing nausea and vomiting, particularly CINV; however, more remains to be done to improve the management of these symptoms in children. More knowledge is needed regarding assessment of the symptom of nausea, looking beyond the quantification of vomiting, along with the determination of a child's risk for the development of nausea to begin with.

Pharmacogenomics may play a role in finding candidate genes that may predict emetic sensitivity and responsiveness to antiemetic therapy, which may help clinicians identify patients who are unlikely to respond to conventional therapies. Optimal regimens for multiple-day and high-dose chemotherapy or bone marrow transplantation, as well as new strategies for control of delayed and refractory symptoms, are also needed. Exploration of drugs, such as the new atypical antipsychotic medications or new small molecules, may contribute to this area.

For children in particular, more evidence of efficacy and adverse effects are needed, as are evidence-based guidelines for CINV management to address the wide variations in practice that exist. Finally, knowledge of nausea and vomiting in children historically has been based on CINV or postoperative situations. Much remains to be learned regarding non–chemotherapy-related nausea and vomiting.

OTHER GASTROINTESTINAL SYMPTOMS

Constipation

Constipation is the passage of hard feces that typically occurs with difficulty and decreased frequency. Most children who experience constipation have no history of bowel dysfunction prior to their cancer diagnosis. Children who have cancer are predisposed to the development of constipation when compared with healthy children because of their decreased fluid intake, variable diet, and decreased mobility, in addition to specific cancer- and treatment-related causes. Constipation may be caused by direct effects of the cancer such as a tumor that obstructs the intestinal lumen, infiltrates enteric nerves and muscles, or causes spinal cord compression or cauda equina syndrome. Cancer-directed therapy, particularly neurotoxic agents such as vinca alkaloids, may also cause constipation. In addition, use of opioids may be one of the most important causes of constipation in this population.

Constipation may lead to painful bowel movements, which some children respond to by withholding stool, further exacerbating the problem. Constipation may lead to significant distress, discomfort (e.g., pain, nausea, and bloating), and embarrassment for some children. Medical complications may include GI obstruction, urinary obstruction, or infection.

Constipation is usually due to altered GI motility and/or altered fluid handling. Intestinal motility is controlled by neuronal, endocrine, and luminal factors. Acetylcholine is the chief neurotransmitter that mediates peristalsis. Serotonin also plays an important role in mediating the gut's response to luminal contents. Opioids lead to constipation by reducing intestinal motility and secretions and increasing fluid absorption and blood flow,[403] as well as by decreasing sensitivity to luminal contents. The effects of opioids on the gut do not appear to be strongly related to the dose.

Evaluation of the patient with constipation includes a thorough history and physical examination. If not contraindicated by a condition such as neutropenia or mucositis, a rectal examination may facilitate the distinction of lower (rectosigmoid) constipation from "colonic inertia" or high obstruction and may also allow assessment of anal sphincter tone and examination for fissures or hemorrhoids. When the diagnosis of constipation is unclear, a radiograph of the kidneys, ureters, and bladder may facilitate the diagnosis.

In addition to medical interventions, the treatment of constipation includes strategies such as provision of a comfortable, easily accessible toilet or commode and addressing issues such as lack of privacy and the need for care providers to help the patient with toileting. Although physical activity is associated with colonic peristalsis, increasing exercise does not necessarily reduce constipation.[404] In addition, the child's medications should be reviewed and dosing of constipating medications such as opioids, TCAs, antihistamines, and 5-HT3 receptor antagonists such as ondansetron and neuroleptics should be altered, if possible. For example, transdermal fentanyl may be less constipating than oral morphine.[405] Patients taking methadone have less of a laxative requirement than do patients taking morphine or hydromorphone, perhaps because methadone provides analgesia by binding to both opioid and NMDA receptors.[406]

Medical management of constipation relies on stool softeners, osmotic agents, and irritant stimulants that increase bowel motility. Softeners, by acting as detergents, soften the stool. Osmotic agents include lactulose, polyethylene glycol, and magnesium salts and increase secretion of water into the lumen. Osmotic agents that increase stool volume actually also facilitate contraction of the bowel wall musculature, thereby increasing bowel motility. Polyethylene glycol is a tasteless agent that may be mixed with a beverage of the child's choice, making it an attractive option for children. In addition, because it is a large polymer that is not degraded by bacteria, it causes less bloating and flatulence than other osmotic agents.[407]

Irritant stimulants such as senna and bisacodyl stimulate contraction and increase secretion. Both of these agents act quickly and are most appropriately used in single doses for temporary constipation. When a hard, leading edge of stool is detected in the rectal vault on examination, it may need to be addressed with use of rectal laxatives (i.e., stimulant suppositories or enemas) if these are acceptable to the child and are not contraindicated because of neutropenia. In the neutropenic child requiring rectal laxatives, administration of antibiotics may prevent bacterial translocation during these maneuvers. As a last resort, manual disimpaction may be required for impacted, hard stool in the rectal vault. Manual disimpaction is a very uncomfortable and distressing procedure for many children and should be avoided if possible.

Although increased fiber intake may be a useful strategy for reducing constipation in healthy persons, patients with cancer may be unable to consume enough fiber to prevent constipation. Inadequate fluid intake directly contributes to constipation but also reduces the efficacy of fiber and increases the risk of obstruction due to a gelatinous mass from inadequately hydrated fiber.[403]

The most effective approach to predictable constipation due to opioids or vinca alkaloids is prevention. Because constipation is one adverse effect of opioids to which patients do not develop tolerance, opioids should always be prescribed with a bowel regimen including a stool softener and stimulant. Despite wide recognition of the constipating effects of opioids, as many as 26% of health care providers who prescribe opioids fail to provide bowel prophylaxis.[408] Other barriers to effective prevention of constipation include (1) difficulty in convincing patients to take a medication to *prevent* another symptom, (2) multiple daily dosing for softeners and laxatives, and (3) adverse effects from these agents such as abdominal pain, cramping, and loose stools.[403]

Methylnaltrexone, a novel agent that may be given orally, intravenously, or subcutaneously to treat opioid-induced constipation, has recently been evaluated in adults.[409,410] This opioid receptor antagonist binds to peripheral mu opioid receptors and does not cross the blood-brain barrier. As such it does not reverse analgesia or induce withdrawal but reliably results in a prompt bowel movement, often within minutes of parenteral administration. It may provide added benefits of treating other peripheral opioid adverse effects such as nausea, pruritus, and urinary retention. It provides the added benefit of relieving opioid-induced constipation without the addition of an enteral medication. Methylnaltrexone is contraindicated in the setting of mechanical GI obstruction or an acute surgical abdomen. Although a pediatric study is required to determine the optimal pediatric methylnaltrexone dose, in one retrospective study it was found that after administration of methylnaltrexone at a mean dose of 0.15 mg/kg/dose, 10 of 19 patients (53%) had a bowel movement within 30 minutes and an additional 4 patients (for a total of 14/19, or 74%) did so within 4 hours of administration.[383]

Bowel Obstruction

Bowel obstruction may result from tumor (malignant bowel obstruction) or other entities such as strictures or postradiation fibrosis obstructing the intestinal lumen. Factors that reduce bowel propulsion such as electrolyte imbalance, opioids, and vinca alkaloids may also contribute to bowel obstruction. Bowel obstruction may occur anywhere from the gastroduodenal junction distally and typically causes abdominal pain, colic, distension, and nausea and vomiting.

Management options for obstruction depend on the site and the underlying cause of the obstruction, the patient's functional status and prognosis, and the goals of care of the patient and family. Treatment modalities for obstruction include surgery, endoscopic procedures, radiation, and medical interventions. For these reasons, decisions regarding management of obstruction may likely involve an interdisciplinary discussion with the family.

Surgery, either by laparoscopy or laparotomy, is often the primary treatment for a mechanical bowel obstruction. Such techniques may allow for resection or bypass of the obstruction and perhaps placement of a venting gastrostomy or a stoma distally. Obstructions may also sometimes be stented endoscopically. Consultation with a surgeon to determine the likelihood of success of the procedure and the potential for obstruction to recur in light of the patient's medical condition may be helpful. Even if obstruction is likely to recur, for some patients it may be reasonable to consider surgery if it is likely to restore bowel function or reduce symptoms for a period. For example, a relatively minor surgery such as gastrostomy placement may allow for venting of an upper obstruction, reducing nausea and vomiting and allowing the patient to eat for comfort.

For some patients, such as those with advanced disease and poor functional status, medical management may be more appropriate than surgery. A variety of medical interventions may break the cycle of obstruction, distension, and secretion. These interventions are aimed at relieving symptoms and potentially reducing the obstruction to allow some degree of enteral nutrition.

Antimuscarinic/anticholinergic agents such as scopolamine reduce colic from smooth muscle spasm and reduce GI secretions. Scopolamine may be administered intravenously, subcutaneously, or transdermally. Because it crosses the blood-brain barrier, it may cause CNS effects such as delirium; glycopyrrolate, which does not cross the blood-brain barrier, is sometimes used as an alternative.

Octreotide is a somatostatin analogue that is a potent antisecretogogue. By inhibiting several hormones, as well as neurotransmission, in the GI tract, it reduces splanchnic blood flow and peristalsis, reduces gastric and intestinal secretions, and increases the absorption of water and salts from the intestine. Octreotide may be given subcutaneously or intravenously, and a long-acting injection formulation is available for once-monthly dosing for patients taking a stable dose.

In a randomized trial of 68 adults with an inoperable malignant bowel obstruction who were receiving an anticholinergic agent, antiemetics, and an opioid, those who also received octreotide had significantly reduced nausea, vomiting, anorexia, and fatigue, although no difference in pain was appreciated.[411] In an uncontrolled study of 27 adults who had an inoperable malignant bowel obstruction, a combination of octreotide, dexamethasone, metoclopramide, opioids, and other agents produced a 90% recovery rate.[412] This regimen reduced vomiting, restored intestinal motility, and allowed for oral feedings.

Other medications that may be helpful include prokinetic drugs such as metoclopramide, which reverses peristalsis from obstruction and decreases nausea and need not be reserved only for partial obstruction. Other pharmacologic interventions include corticosteroids, which decrease inflammation and edema and reduce nausea. For some patients, a venting gastrostomy tube may reduce symptoms. IV hydration may reduce nausea and sedation.[413] Both surgical approaches and medical hydration/nutrition, however, are supportive measures that should be considered in light of the patient's prognosis and the goals of the patient and family.

Diarrhea

Diarrhea is defined as the frequent occurrence of loose stools, often with urgency. Patients' definitions of diarrhea may vary, and when it is a presenting complaint, it must be accurately characterized, particularly with regard to how the patient's bowel habits have changed compared with his or her baseline. In addition to potentially causing significant medical complications, diarrhea may be debilitating for patients. It may cause life-threatening dehydration and electrolyte imbalance in addition to malnutrition and immune impairment. Attendant mucosal injury may also predispose patients to sepsis. Diarrhea may cause physical discomfort with cramping and painful perianal skin breakdown and emotional distress for children and caregivers.

Diarrhea may occur as a direct effect of a malignancy (e.g., a carcinoid, a GI stromal tumor, or medullary cancer of the thyroid), although this occurrence is relatively rare in pediatric cancers. Secretory diarrhea can occur in rare cases of neuroblastoma with associated vasoactive intestinal polypeptide secretion. Diarrhea may also result from chemotherapy, as will be discussed later. Radiation to the abdomen or pelvis may cause mucosal damage, prostaglandin release, and bile salt malabsorption, which in turn increase peristalsis and cause diarrhea. Radiation-induced enteritis frequently occurs during the third week of fractionated therapy.[414] Another form of treatment-related diarrhea is gut GVHD after bone marrow transplant. In gut GVHD, inflammatory infiltrates of activated lymphocytes, plasma cells, and eosinophils cause intestinal mucosal damage, in turn leading to diarrhea and sometimes bleeding.[415] Mucosal damage from cancer treatment may increase the risk of superinfection, which can further exacerbate diarrhea and other GI symptoms.

Other causes of diarrhea in patients with cancer include bacterial overgrowth, which may occur with changes in anatomy after surgery, as well as bona fide infection. In particular, patients with cancer are prone to *Clostridium difficile* infections largely as a result of the frequent administration of antibiotics. Infection leads to diarrhea through mucosal injury and impaired absorption, although malabsorption may occur for other reasons, such as a surgically shortened gut or fistula that reduces absorptive capacity.

Diarrhea may also be caused by medications, including antibiotics and magnesium (in electrolyte supplements or antacids). Furthermore, diarrhea may be seen in association with constipation, either as a result of impaction constipation with overflow or leakage or laxatives used to treat constipation.

In general, treatment of diarrhea depends on the underlying cause. If needed, rehydration should be undertaken with correction of electrolyte imbalance. Patients should be advised that food intake is secondary to maintenance of hydration during an episode of diarrhea. Dairy products should be avoided because of secondary lactase deficiency. In addition, a topical skin barrier such as Desitin may be applied to protect the skin from irritation. Symptomatic management with opioids, and in the case of severe diarrhea, octreotide, is discussed later in the context of chemotherapy-induced diarrhea (CID), but it is applicable to diarrhea from a wide variety of causes.

Chemotherapy-Induced Diarrhea

CID is seen with increasing frequency as an adverse effect of new chemotherapeutic agents. It is a common adverse effect of agents such as 5-fluorouracil, irinotecan, capecitabine, and docetaxel. Irinotecan may cause acute diarrhea immediately after administration of the drug through cholinergic mechanisms; the diarrhea may be treated with an anticholinergic agent such as atropine. Irinotecan commonly causes delayed diarrhea, usually at least 24 hours after administration. Irinotecan is conjugated by the liver to form SN-38, which is then deconjugated back to the active agent in the intestinal lumen by bacteria. The irinotecan is then able to directly exert toxic effects on colonic epithelium, resulting in diarrhea.[416,417] The late onset secretory diarrhea is best prevented by antibiotics, and if necessary, it is treated with loperamide.

Loperamide is the opioid of choice for diarrhea because it acts locally in the gut and is only very minimally absorbed. By slowing peristalsis and promoting water reabsorption, it reduces stool frequency and volume, as well as urgency. If systemic absorption is less of a concern, other opioids such as morphine may be used as well. Loperamide may be discontinued after the patient has been free of diarrhea for 12 hours, but in the case of radiation-induced diarrhea, loperamide should be continued for the duration of radiation treatment.[414] In cases in which CID is severe or complicated (i.e., with moderate to severe cramping, decreased performance status, fever, neutropenia, and bleeding or dehydration), administration of octreotide should be considered.[414] Octreotide is also effective in cases of diarrhea refractory to loperamide,[418] and it has been shown to be effective in reducing diarrhea associated with GVHD.[419] In a small study of pediatric patients receiving irinotecan, it was found that activated charcoal taken three times a day during irinotecan administration reduced the severity and frequency of diarrhea and improved chemotherapy tolerability and continuation rates.[420]

DYSPNEA

Dyspnea is defined as the uncomfortable or abnormal awareness of breathing. Patients may describe dyspnea in a variety of ways, including chest tightness, air hunger, choking, or heavy breathing. Dyspnea is clearly an important source of suffering in children and adults alike, triggering feelings of fear, anxiety, and loss of control and impairing quality of life.[421] In addition, family members are distressed by witnessing a loved one with this symptom.[422]

Dyspnea may occur in patients with malignant involvement of the respiratory tract. Direct effects of malignancy such as airway obstruction by tumor or a malignant pleural effusion are possible. Treatment-related complications such as pulmonary fibrosis, pneumonitis, or GVHD after hematopoietic stem cell transplantation may cause dyspnea. Other respiratory processes that result in dyspnea include pulmonary edema, pulmonary embolus, pneumonitis, or a restrictive physiology due to tumor, ascites, or organomegaly. Patients with cardiac pathologic conditions such as anthracycline-induced congestive heart failure or pericardial effusion may also experience dyspnea. Despite the multitude of potential causes of dyspnea, it may also occur in patients who have no respiratory or cardiac involvement at all. For example, in the National Hospice Study it was found that 24% of terminally ill adults with cancer who reported dyspnea had no known cardiac or pulmonary disease.[423] In cases in which no known underlying respiratory or cardiac disease exists, dyspnea may stem from multiple causes, including muscle weakness from deconditioning or poor nutritional status, anemia, metabolic derangements, fluid shifts, or anxiety.

Dyspnea may occur at any point during the clinical course but is most notably observed at the end of life. In the National Hospice Study it was found that 70% of 1754 terminally ill adults experienced dyspnea in the last 6 weeks of life.[423] Dyspnea, particularly dyspnea at the severe end of the spectrum, is associated with shorter survival. For example, as a symptom by itself or in association with other parameters, it is a prognostic indicator of more rapid mortality in adults with advanced cancer.[424]

Pathophysiology

The respiratory center that processes input from the respiratory system and coordinates respiratory activity is located in the medulla and pons. Afferent input arises from peripheral mechanoreceptors in respiratory muscles, the chest wall, the lungs, and the upper airway, as well as central and peripheral chemoreceptors and pulmonary vagal afferents (Fig. 71-4). Chemoreceptors detect low P_{O_2} and high P_{CO_2} in the blood and send this input to

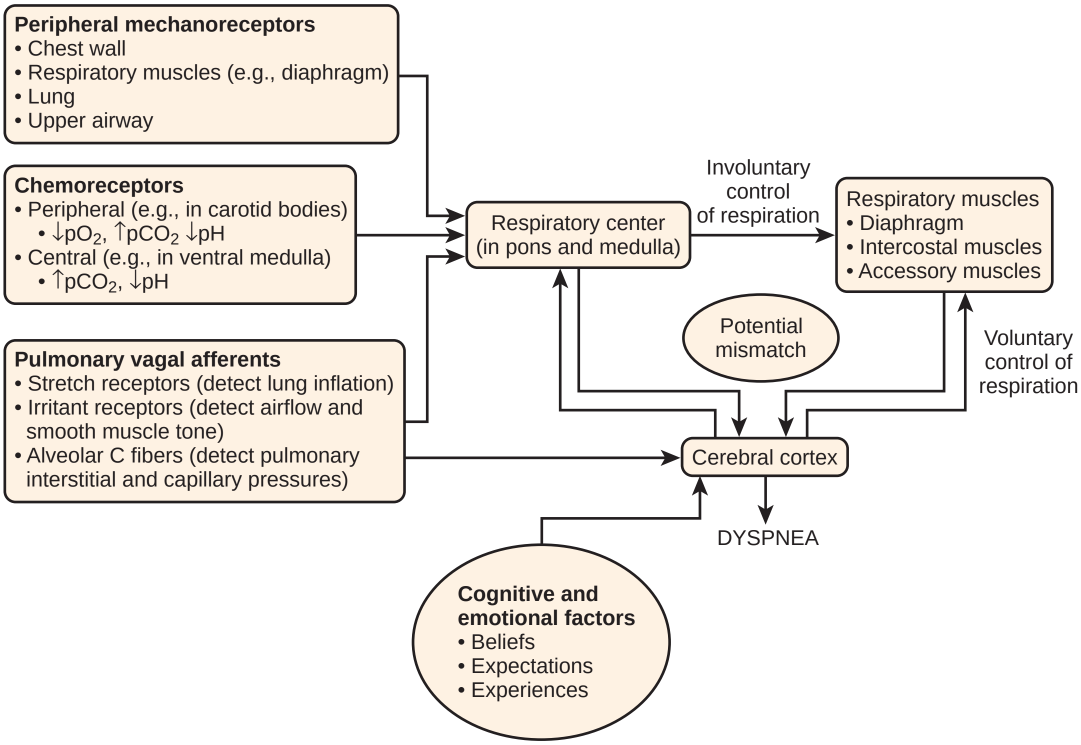

Peripheral mechanoreceptors
• Chest wall
• Respiratory muscles (e.g., diaphragm)
• Lung
• Upper airway
Chemoreceptors
• Peripheral (e.g., in carotid bodies)
• ↓pO_2, ↑pCO_2 ↓pH
• Central (e.g., in ventral medulla)
• ↑pCO_2, ↓pH
Pulmonary vagal afferents
• Stretch receptors (detect lung inflation)
• Irritant receptors (detect airflow and smooth muscle tone)
• Alveolar C fibers (detect pulmonary interstitial and capillary pressures)
Respiratory center (in pons and medulla)
Involuntary control of respiration
Respiratory muscles
• Diaphragm
• Intercostal muscles
• Accessory muscles
Potential mismatch
Voluntary control of respiration
Cerebral cortex
DYSPNEA
Cognitive and emotional factors
• Beliefs
• Expectations
• Experiences

Figure 71-4 The pathophysiology of dyspnea.

the respiratory center. Pulmonary vagal afferents are activated by input from stretch receptors and irritant receptors, and they also trigger activity in tracts that project to the cerebral cortex. Based on this sensory input, the respiratory center coordinates the respiratory apparatus consisting of the diaphragm, intercostal muscles, and accessory muscles. The cerebral cortex integrates sensory input, motor output, and cognitive and emotional input to create the sensation of breathing.[425]

The phenomenon of dyspnea occurs when the afferent sensory input to the brain—for example, input from mechanoreceptors in the chest wall—fails to match the outgoing motor signal emanating from the brain.[426] Situations in which such mismatch may occur include (1) increased work of breathing such as when breathing against increased resistance or with weakened muscles; (2) chemical changes such as hypercapnia and hypoxemia; and (3) neuromechanical dissociation such as when sensory input from a given inspiratory effort does not match the input anticipated by the brain.[425] The interpretation of this mismatch, or perceived dyspnea, by the cerebral cortex is affected by the individual's expectations, experiences, and beliefs, making the sensation of dyspnea highly subjective and individual.

Diagnosis and Assessment

Neither clinical signs such as respiratory rate or oxygen saturation nor laboratory data such as arterial blood gases and hemoglobin concentration are reliable predictors of dyspnea. Because objective data correlates poorly with dyspnea, the gold standard for measuring and assessing dyspnea is the patient's self-report. The visual analogue scale[427] and Borg scale[428] are among the most widely used in adults. The Borg scale is a 10-point scale with descriptive anchors at the ends. A numeric rating scale has also been evaluated in adult oncology patients, with a 0/1 out of 10 score having a 98% sensitivity and a 54% specificity. A score greater than 1 is predictive of dyspnea that may impair daily function and is recommended as a threshold score requiring further evaluation.[429] The Dalhousie dyspnea scale was developed to meet the need for a pediatric dyspnea assessment instrument.[430] This pictorial instrument actually contains three scales depicting three subconstructs of dyspnea including throat closing, chest tightness, and effort. This instrument was tested in children with asthma or cystic fibrosis and in healthy children. Children 8 years or older used the instrument reliably. Our review of the literature did not yield any instruments that have been specifically developed for the measurement of dyspnea in the younger pediatric population.

Further assessment of dyspnea necessitates a history that should include triggers or exacerbating factors, alleviating factors, severity, and description of degree of functional impairment. Indications of complications such as infection, GVHD, and cardiac compromise should be sought, as should potential treatment-related causes such as chemotherapy and radiotherapy. Potential causes of dyspnea unrelated to malignancy such as asthma should also be considered. Finally, contributing psychosocial factors such as anxiety should be explored because emotional and cognitive factors may influence how the individual interprets dyspnea as a symptom.

Evaluation beyond a thorough physical examination depends upon the suspected cause of dyspnea. Studies that may provide useful information include a complete blood cell count, noninvasive measures including pulse oximetry and capnometry, selective assessment of venous or arterial blood gases, and a chest radiograph. Additional tests that may also be considered include pulmonary function testing, an axial chest CT scan, an echocardiogram, an electrocardiogram, and a spiral CT or ventilation-perfusion scan to assess for a pulmonary embolus.

Management

Targeted approaches to treat dyspnea depend upon the underlying cause and may include medical therapies such as chemotherapy, antibiotics, or diuretics. Procedural interventions may include pleurocentesis and stenting of the airways to relieve the obstruction or surgical removal of the obstruction. Consideration of such interventions should weigh the likely benefits and burdens for the child in light of his or her medical condition.

Rapid and effective strategies for symptomatic management of dyspnea are available and applicable both for patients who are candidates for targeted approaches and for those in whom such interventions are not appropriate. The mainstays of symptomatic management include opioids and oxygen. Other agents such as anxiolytics, steroids, and bronchodilators may also be of benefit, particularly if an inflammatory or bronchospastic component is suspected.

Opioids

Several studies in adult patients with cancer support the use of opioids as an effective intervention for dyspnea.[431-434] Bruera et al.[431] and Mazzocato et al.[433] conducted randomized placebo-controlled crossover studies demonstrating that opioids relieved dyspnea and furthermore did not cause clinically important respiratory depression. No studies demonstrating the efficacy of opioids for dyspnea in children have been published, although based on clinical experience, they appear to be as effective and safe in children as in adults.

The mechanism(s) by which opioids relieve dyspnea has/have not yet been clearly elucidated. Opioid receptors present in both the peripheral nervous system and the CNS may play a role relieving dyspnea when they are bound. Opioids may alleviate dyspnea by blunting the effect of hypercapnia or hypoxia on ventilation, relieving anxiety, or modifying the sensation of dyspnea, akin to their ability to modify the sensation of pain.

The dose required to relieve dyspnea is often relatively small, even when a patient is not opioid-naive. For example, Allard and colleagues[434] showed that a 25% increase in the baseline dose controlled breakthrough dyspnea for up to 4 hours. Our standard practice for opioid-naive patients is to start by administering 0.025 mg/kg of morphine intravenously (i.e., 25% of 0.1 mg/kg, the typical starting dose for pain) and titrating as indicated. In addition to short-acting preparations, long-acting preparations such as sustained-relief morphine are effective treatments for dyspnea.[435]

Because opioid receptors are present in the lower respiratory tract, it was postulated that nebulized opioids might alleviate dyspnea. Case reports of inhaled morphine and fentanyl in adolescents with cystic fibrosis[436,437] and a small uncontrolled study in patients with cancer who used inhaled morphine fentanyl to reduce dyspnea held promise in this regard.[438] A crossover study conducted by Bruera and colleagues[439] comparing subcutaneous and nebulized morphine suggested that both routes of administration may reduce dyspnea, but perhaps because of a small sample size or placebo effect, a difference between the two routes could not be excluded. In addition, several other studies have not demonstrated efficacy of nebulized opioids. Until larger studies are conducted that use standardized doses of opioid and consistent delivery methods, evidence is insufficient to support this method of delivery for dyspnea.[440]

Oxygen

When hypoxemia is suspected to be the cause of dyspnea, administration of oxygen to reverse hypoxemia may relieve this symptom. However, it is likely that most patients who have dyspnea either are not hypoxemic or do not have dyspnea as a result of hypoxemia alone. For example, in a study of adults with advanced cancer and dyspnea, only 40% were hypoxic.[441]

Studies evaluating oxygen compared with air for relief of dyspnea in both hypoxemic and nonhypoxemic adults show no clear superiority of oxygen compared with air.[442-445] An explanation for the comparability of flowing oxygen and air in relieving dyspnea is based on the ability of both to stimulate mechanoreceptors in the trigeminal nerve. Receptors in the distribution of the trigeminal nerve appear to influence induced dyspnea in healthy subjects.[446] In addition, administration of cold air appears to improve dyspnea in patients with chronic obstructive pulmonary disease.[447] Therefore provision of flowing air or oxygen could potentially reduce dyspnea through this mechanism. Trigeminal nerve stimulation may explain why experiencing cool, moving air provided by a fan or open window also seems to reduce dyspnea.

Anxiolytics

Although anxiety may worsen perceived dyspnea and dyspnea may induce anxiety, evidence to date does not support the routine use of anxiolytics to treat dyspnea, particularly not as a first-line agent. Dyspnea is a common symptom in persons who have panic attacks or anxiety disorder. However, anxiety may contribute less to coexisting dyspnea than has been traditionally thought. Recent work reveals that in adults with advanced cancer, anxiety explained only 10% of the variance in dyspnea. Use of an anxiolytic agent to alleviate anxiety may be appropriate when anxious symptoms are particularly prominent and are thought to have a significant influence on a patient's interpretation of the sensation of dyspnea. Anxiolytics may also be indicated for sedation to manage escalating dyspnea at the end of life, as discussed in Chapter 69, which covers palliative care.

Nonpharmacologic Interventions

To the extent that the cerebral cortex mediates the sensation of dyspnea, interventions that modify cognitive and emotional factors such as breathing/relaxation training that influence the interpretation of dyspnea may be helpful. In healthy adults undergoing induced dyspnea, distraction of subjects did not change dyspnea severity ratings but did decrease the perceived unpleasantness of the dyspnea.[448] Increased support, such as is provided in the nurse-run dyspnea clinic studied by Bredin and the one described by Booth, may reduce dyspnea by providing education, cognitive and behavioral training, and psychosocial support.[449]

Escalating Dyspnea

The symptom of dyspnea may escalate at the end of life. Respiratory changes and the development of secretions are discussed in Chapter 69, which covers palliative care. Refractory, distressing dyspnea such as may occur at the end of life is an indication for consideration of palliative sedation, as described in Chapter 70.

Future Directions

Much remains to be learned regarding the physiologic features and mechanisms of dyspnea. Once these features and mechanisms are better understood, targeted interventions may be developed to ameliorate this challenging symptom for which we currently have relatively few treatment options. Within the pediatric realm, a better understanding of how children sense and interpret this highly subjective symptom is needed. This understanding in turn may enable development of methods to measure and assess this symptom, allowing the care team to better understand how it affects children and evaluate dyspnea-directed interventions.

COUGH

Cough can be a distressing symptom and may lead to dyspnea, vomiting, sleep disruption, and chest or throat pain. Topical antitussives act on receptors in the respiratory tree to reduce cough. Nebulized lidocaine has been described to ease an intractable cough at the end of life.[450] Symptomatic treatment for a significant cough usually entails administration of an opioid to suppress the cough reflex in the medulla. Dextromethorphan and codeine are most commonly used, although all opioids have antitussive activity.

FATIGUE

Cancer-related fatigue has been defined by the NCCN as a "distressing persistent, subjective sense of tiredness or exhaustion related to cancer or cancer treatment that is not proportional to recent activity and interferes with usual functioning."[451] Fatigue is a complex symptom that may have physical, cognitive, and emotional components. In addition, fatigue is subjective and highly individual, and thus patients may experience and interpret fatigue differently. Patients may describe fatigue in a wide variety of ways (Box 73-3). Nevertheless, adults with cancer-related fatigue almost universally report that (1) it is unresponsive to rest, (2) it is not proportional to activity, and (3) it pervades multiple areas of their life. Clearly, this type of fatigue is not the fatigue of everyday life. Recent work in adult cancer survivors reveals that fatigue may be long-lived, lasting for months to years after cancer-directed treatment has been completed.[452,453] In pediatric cancer survivors, fatigue is the only factor associated with both poor physical and psychosocial health-related quality of life.[454]

Impact

It is well established that fatigue is the most common symptom in patients with cancer.[455,456] One survey of published studies on cancer-related fatigue in adults found that fatigue was present in 50% to 75% of patients at diagnosis, with the prevalence increasing to 80% to 96% in patients undergoing chemotherapy and to 60% to 93% in patients receiving radiotherapy.[457] In children, fatigue is less well studied but has been shown to be the most common symptom.[5] For example, Wolfe and colleagues[5] found that fatigue was the most common symptom in children with advanced cancer, with 96% of children experiencing fatigue. Collins also found that in a cross-sectional study of children aged 10 to 18 years who had current or previous cancer, lack of energy was the most common symptom, affecting 49.7%.

According to adult patients with cancer, fatigue is the most distressing of all the symptoms they experience, creating profound physical, psychological, and financial burdens that can impair quality of life and diminish hope.[458] It not only leads to a decrement in physical ability but also to a sense of loss of control, loneliness, and isolation. In the Fatigue II study of 379 adults undergoing treatment for cancer, 60% ranked fatigue as the symptom affecting their life the most.[458] According to the parents in the study by Wolfe et al.,[5] fatigue caused "a great deal" or "a lot" of suffering in 57% of children approaching the end of life. In another study, Jalmsell et al.[6] reported that of 449 parents of children who had died from cancer, 86% reported that fatigue significantly affected their child's well-being. Adolescents who have cancer also report that fatigue significantly affects their physical, social, and psychological well-being.[459] Higher self-reported fatigue in adolescents is also negatively associated with quality of life.[460]

In addition to causing suffering, concerns regarding fatigue as an adverse effect of disease-directed or symptom-directed treatment may limit therapy. Fatigue is documented to be the most common adverse effect of chemotherapy and radiotherapy in adults.[457,461] In adults, treatment-related fatigue can be severe enough to prevent maximal treatment and disease control,[451,462] although in children, fatigue is rarely cited as a reason to reduce therapy. In terms of symptom-related treatment, pain may be inadequately relieved when concerns regarding opioid-related fatigue or sedation inhibit optimal opioid administration.[463,464] When fatigue is not controlled, patients are forced to choose between adequate analgesia with somnolence and mental clouding and less sedation at the price of increased pain.

Approach

Despite its prevalence and impact, fatigue frequently is not addressed by clinicians and patients alike. In a large multicenter study of 1317 adults with cancer, only 52% of those with fatigue reported it to their physician.[465] One significant barrier to addressing fatigue is the perception on the part of the care team that fatigue, although undesirable, does not have a significant adverse affect on patients. In the Fatigue I study it was found that although oncologists believe that pain adversely affects their patients to a greater degree than fatigue (61% vs. 37%), patients with cancer believed that fatigue adversely affected their daily lives more than pain (61% vs. 19%).[456] This disparity may be due in part to clinicians' personal understanding of day-to-day fatigue that leads them to believe they understand the reality of cancer-related fatigue. Other barriers to addressing fatigue may include (1) a belief that fatigue is "normal" or "to be expected" in a patient with cancer; (2) a paucity of descriptors for patients to adequately convey the various manifestations of fatigue; (3) the clinician's lack of familiarity with options to ameliorate fatigue; (4) a disease-directed focus to the exclusion of attention to symptoms; and (5) system-related problems such as time pressures or reimbursement difficulties.

Measurement and Assessment of Fatigue in Children

The NCCN provides guidelines for fatigue management in adults and children that calls for screening for fatigue at every visit using a 1 to 10 scale.[451] Although use of such a scale has not been validated in children, this type of scale may generally be used reliably in children 7 years and older.

Once screening identifies fatigue, it should be further assessed, although universally accepted means for assessing fatigue in either adults or children do not exist. Several unidimensional and multidimensional scales for assessment in adults and a few pediatric instruments are available. One, developed by Hockenberry and colleagues[466] is for children with cancer. Although it is part of a series of instruments that evaluate fatigue in pediatric oncology patients from the perspective of the child, parent, and staff,[466] it was derived from interviews with only 13 children and has not yet been prospectively evaluated. Another instrument, the PedsQL Multidimensional Fatigue Scale,[467] measures child and parent perceptions of fatigue in pediatric patients. This instrument has been studied in healthy children and in children with cancer.[467]

Even when a formal assessment instrument is not used to assess fatigue, clinicians should explore a variety of possible manifestations of fatigue that the child/adolescent and the adult caregiver may identify (Box 71-3). Exacerbating and alleviating factors, the pattern of fatigue, and the degree to which the symptom is affecting the child's life can then be explored. In assessing fatigue it may be helpful to bear in mind that children tend to conceptualize fatigue as a physical sensation, whereas adolescents alternate or merge the physical concept with mental

Box 71-3 **Manifestations of Fatigue**

PHYSICAL

- Weakness
- Physical tiring
- Heaviness

COGNITIVE

- Mental clouding
- Poor concentration
- Impaired memory

EMOTIONAL

- Depression
- Apathy
- Irritability
- Decreased motivation

ENERGY

- Tiredness
- Lethargy
- Low energy
- Decreased endurance

SLEEP

- Insomnia
- Hypersomnia
- Somnolence
- Nonrestorative sleep

From Ullrich C, Mayer O: Assessment and management of fatigue and dyspnea in pediatric palliative care. Pediatr Clin North Am *54:735–756, 2007.*

tiredness.[468] On the other hand, parents and staff conceptualize fatigue as a symptom that interferes with the child's ability to participate in a variety of activities and may be manifested by physical, emotional, and mental changes. Investing the time to fully assess a report of fatigue may frequently yield important clues regarding the cause of the fatigue; these clues are valuable because few diagnostics tests exist to determine the cause of fatigue.

Associated Factors

Fatigue may stem from concomitant, interrelated causes including physical factors, psychological factors, and disrupted sleep (Fig. 71-5). Investigations aiming to uncover the cause of fatigue exist only in adults and consist of retrospective and prospective analyses of factors associated with fatigue. No studies have been published that evaluate objective factors associated with fatigue in children with cancer.

For simplicity, factors considered to be associated with fatigue may be categorized into physical and psychosocial factors, with the realization that many patients experience fatigue as a result of a combination of factors. For example, in adults undergoing chemotherapy or radiotherapy, fatigue correlates with both physical factors (symptom distress) and psychological factors (e.g., depression, anxiety, anger, or confusion).[469] Another study of adult patients with cancer similarly determined that both physical factors (severity of pain and dyspnea) and psychological symptoms (anxiety and depression) were associated with the experience of fatigue.[455]

In adults, certain sociodemographic factors are also associated with fatigue. For example, the demographic factors of being employed or living alone were significantly associated with the symptom of fatigue.[470] Although children are unlikely to experience fatigue related to these exact sociodemographic considerations, the stress induced by these financial or caregiver concerns can affect children and the fatigue that they experience.

Physical Factors

Physical factors contributing to fatigue include direct effects of the underlying disease and adverse effects of treatment of the underlying illness. Unrelieved symptoms are also likely to contribute to a patient's fatigue. In adults, overall symptom distress is associated with the report of fatigue.[471] Conversely, treatment for symptoms such as pain or dyspnea may also contribute to fatigue.

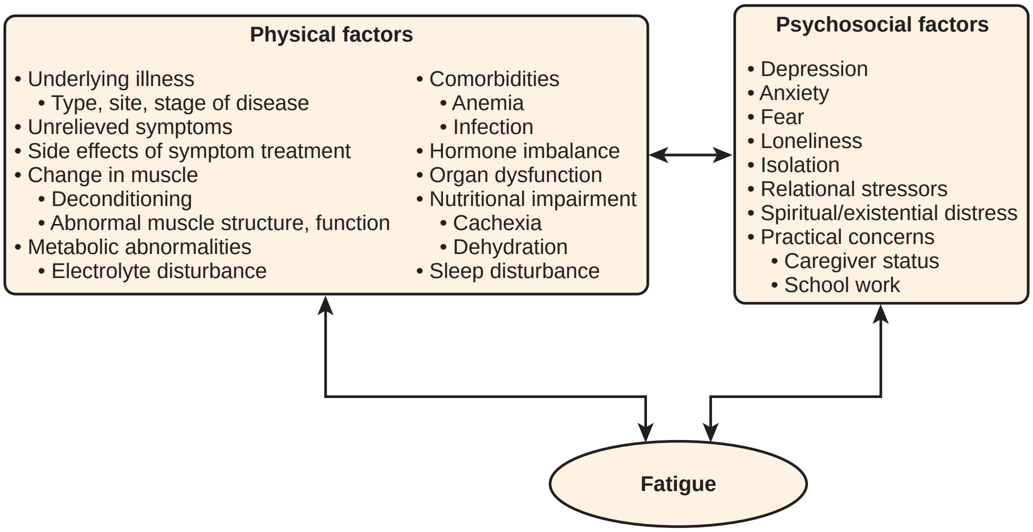

Physical factors
• Underlying illness
• Type, site, stage of disease
• Unrelieved symptoms
• Side effects of symptom treatment
• Change in muscle
• Deconditioning
• Abnormal muscle structure, function
• Metabolic abnormalities
• Electrolyte disturbance
• Comorbidities
• Anemia
• Infection
• Hormone imbalance
• Organ dysfunction
• Nutritional impairment
• Cachexia
• Dehydration
• Sleep disturbance
Psychosocial factors
• Depression
• Anxiety
• Fear
• Loneliness
• Isolation
• Relational stressors
• Spiritual/existential distress
• Practical concerns
• Caregiver status
• School work
Fatigue

Figure 71-5 A conceptual model of fatigue.

Because a variety of medications including benzodiazepines, opioids, and antihistamines may cause fatigue, thoroughly reviewing the patient's medications may reveal medications that cause fatigue. Finally, a variety of comorbidities such as organ dysfunction (e.g., renal, hepatic, cardiac, pulmonary, or endocrine dysfunction), electrolyte imbalance, poor nutritional status, and infection may contribute to fatigue.

Anemia is a well-established cause of fatigue in a variety of patient populations. In adults with cancer, it has been demonstrated to be associated with fatigue and impaired quality of life. In some adults with advanced cancer, anemia is not significantly related to fatigue.[472,473] Thus anemia may not fully account for the fatigue experienced by some patients, suggesting that correction of anemia may or may not decrease fatigue.

Like anemia, hypothyroidism is associated with fatigue in patients with and without cancer. Other endocrine abnormalities that are associated with the experience of fatigue include hypothalamic-pituitary axis alterations[474] and hypogonadism, which is also associated with negative mood and cachexia.[475-477]

Deconditioning from decreased activity may lead to fatigue, which in turn lessens activity and may further exacerbate fatigue. A variety of muscle abnormalities that have been described in patients with cancer may be associated with fatigue.[478] Finally, muscle wasting (cachexia) is associated with fatigue, perhaps because of the cytokines proposed to mediate cachexia such as tumor necrosis factor, which are also thought to contribute to the sensation of fatigue.[474]

Psychosocial Factors

Psychosocial factors such as depression and anxiety, existential or spiritual suffering, and stress from practical concerns may all be related to fatigue. When psychological factors such as fatigue and depression coexist, depressed mood may be a contributor or a consequence of fatigue. For example, when characterized by withdrawal and decreased participation in activities in an adolescent, fatigue may be a manifestation of depression. On the other hand, the experience of fatigue may lead to isolation, decreased engagement in activities, and impaired quality of life, all of which may contribute to depression.

Sleep Disturbance

Sleep disturbance, manifested as decreased quantity or quality of sleep, results from factors including unrelieved physical or psychological symptoms or environmental factors, such as distortion of sleep architecture by medications or sleep disruptions that occur in the hospital.[479,480] Impaired sleep may in turn worsen the physical or psychological distress experienced by patients. Although a decrement in the quantity or quality of sleep may contribute to fatigue, cancer-related fatigue may occur independent of changes in sleep.[481]

Interventions

An algorithm for approaching the symptom of fatigue is presented in Figure 71-6. Although fatigue is frequently multifactorial, targeting underlying factors that are suspected to contribute to fatigue can improve this symptom. Even when no particular contributing factor is identified, nonspecific interventions may ameliorate fatigue. For example, exercise and stimulants are effective interventions that reduce fatigue. Educating the patient and his or her family about fatigue is important in helping them understand fatigue and its impact, as well as the available options for addressing it. Strategies to optimize function and facilitate adaptation to fatigue such as realistic goal setting, modifying activities, and conserving energy should also be reviewed.

Exercise

Curtailing activity is a natural reaction to fatigue, and oncologists report recommending activity in response to the complaint of fatigue.[456] Several randomized controlled trials in adults have demonstrated that exercise actually reduces fatigue, and in some instances it also ameliorates psychological symptoms such as depression and anxiety. For example, a 6-week walking program for women undergoing radiotherapy for breast cancer improved fatigue as well as anxiety, depression, and sleep disturbance.[482] Finally, exercise can also provide a variety of physical benefits, including improved strength and function.

Psychosocial Interventions

Strategies to address psychological factors include both pharmacologic and nonpharmacologic approaches. Psychological interventions such as psychotherapy and support group participation may reduce fatigue,[483] as can increased support from clinicians such as intensive nursing support.[484] If depression or anxiety are suspected to contribute to fatigue, a trial of a pharmacologic agent such as an antidepressant or anxiolytic may be of benefit, recognizing that some drugs in each of these classes may exacerbate sedation and fatigue. If other stressors are uncovered, such as spiritual suffering or concerns about practical matters, professionals such as a chaplain, resource specialist, or staff at school should be approached for assistance.

Sleep

If sleep disturbance is suspected, reducing factors that impair sleep and strategizing with patients to improve sleep hygiene are a necessary first step. If indicated by persistent or severe sleep impairment, pharmacologic interventions such as benzodiazepines, benzodiazepine receptor agonists, and antidepressants with sedating qualities (e.g., TCAs, trazodone, and mirtazapine) may be tried. It is important to keep in mind, however, that benzodiazepines and antihistamines in particular may have paradoxic effects in children and that the benzodiazepine receptor agonists have not yet been well studied in young children.

Pharmacologic Interventions

Correction of Anemia. Correcting anemia associated with cancer or chemotherapy with erythropoietin is a strategy often used in adult oncology. Some studies support this practice in adults, demonstrating that

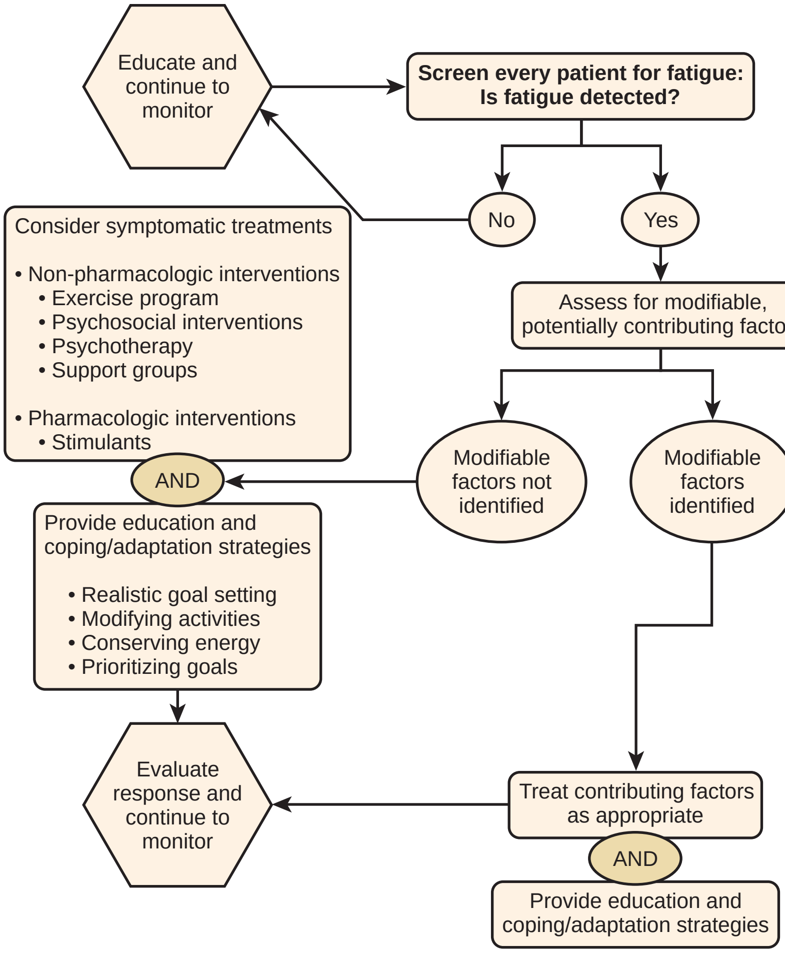

Educate and continue to monitor
Screen every patient for fatigue: Is fatigue detected?
No
Yes
Consider symptomatic treatments
• Non-pharmacologic interventions
• Exercise program
• Psychosocial interventions
• Psychotherapy
• Support groups
• Pharmacologic interventions
• Stimulants
AND
Provide education and coping/adaptation strategies
• Realistic goal setting
• Modifying activities
• Conserving energy
• Prioritizing goals
Assess for modifiable, potentially contributing factors
Modifiable factors not identified
Modifiable factors identified
Evaluate response and continue to monitor
Treat contributing factors as appropriate
AND
Provide education and coping/adaptation strategies

Figure 71-6 An algorithm for fatigue management.

improving anemia may also improve fatigue-related quality of life.[485] However, correction of anemia with erythropoietin has not been as widely pursued in pediatric oncology practice, in part because no evidence exists that treatment of anemia with erythropoietin improves quality of life. The one published study examining this question in children failed to show that health-related quality of life improved with correction of anemia.[486] The authors provide several possible explanations for this finding, but the lack of association between correction of anemia and quality of life and fatigue suggests that focusing on anemia at the exclusion of other suspected causes of fatigue and decreased quality of life may lead to inadequate treatment of fatigue. If erythropoietin is used, recent concerns that it is associated with more rapid tumor progression, thrombosis, and increased mortality in adults[487-489] should be kept in mind. If anemia is suspected as a cause of fatigue, transfusion support is an alternative to erythropoietin therapy.

Stimulants. The stimulant with the best-established efficacy in ameliorating fatigue in adults who have serious illness is methylphenidate. This agent reduces fatigue in adults with cancer and human immunodeficiency virus, regardless of the underlying cause.[490,491] In one study of adults with cancer-related fatigue, patients had significantly less fatigue after they started taking methylphenidate.[492] However, studies have not consistently demonstrated the efficacy of methylphenidate in reducing fatigue. These discrepancies are likely due to the variation in dosing and titration procedures, as well as heterogeneity in fatigue assessment instruments and the study populations evaluated. Methylphenidate is advantageous in that it improves cognition and exerts direct analgesic and antidepressant actions,[490,492,493] and one study reports success in improving cognition in children who have survived cancer.[494] A case series documenting successful treatment with methylphenidate of opioid-related sedation in children who have cancer has been published,[495] but currently no randomized controlled trials have been published that evaluate its efficacy or tolerability in children with cancer-related fatigue.

Methylphenidate has a history of extensive use in children with attention deficit hyperactivity disorder[496] and has a well-established track record in terms of being safe and well tolerated by children. The most prevalent adverse effect of methylphenidate is decreased appetite, which we attempt to mitigate by giving the first dose after breakfast. Despite concerns that methylphenidate should be used with caution in children at risk for seizure, open-label and controlled trials in adults and children with attention deficit hyperactivity disorder and epilepsy do not support this concern (reviewed by Baptista-Neto et al.[497] in 2008). For example, a recent study in children with epilepsy found no increase in seizure frequency and in fact observed beneficial electroencephalogram changes with methylphenidate.[498] Modafinil may be considered as an alternative to methylphenidate, although significantly less pediatric experience exists with this agent. Modafinil has been used with success to treat children with narcolepsy[499] and excessive daytime sleepiness associated with Prader-Willi syndrome[500] and to treat adults with fatigue associated with multiple sclerosis or sedation due to opioids.[501,502] However, no studies evaluating its use in children to treat cancer-related fatigue have been published.

Integrative Therapies

Adult patients with fatigue are more likely to use dietary and herbal supplements,[503] which may be related to patients' perceptions that conventional medicine offers no effective interventions for fatigue. Patients reporting fatigue should be questioned regarding potential use of dietary and herbal supplements. A randomized controlled study evaluating ginseng in adults found that subjects who received 1000 mg per day for 8 weeks had significantly improved fatigue scores when compared with subjects who received a placebo.[504]

Future Directions

Despite its prevalence and impact, fatigue is frequently underrecognized and underappreciated, particularly in comparison with other symptoms. Particular areas of need in the management of fatigue include a better developed assessment method, increased understanding of causes of fatigue, and interventions targeted at specific causes of fatigue.

PRURITUS

Pruritus, also referred to as itching, may occur in children who have cancer for a variety of reasons. As a paraneoplastic symptom it may be associated with malignancy, such as Hodgkin lymphoma or other types of lymphoma. Other disorders such as mastocytosis, iron deficiency, and polycythemia vera may cause pruritus. Pruritus may also occur with organ dysfunction such as hypothyroidism, hyperparathyroidism, liver dysfunction (i.e., cholestasis) or renal dysfunction (uremia),[505] as well as infection of the skin. Pruritus may also be iatrogenic and occur as a result of cancer-directed therapy, as in the pruritic rash caused by growth factor receptor inhibitors[506]—for example, in skin GVHD after hematopoietic stem cell transplantation. Pruritus may also result from a variety of other medications, as well as blood products. Rarely, pruritus may be a manifestation of an underlying psychiatric or neurologic disorder. In general, pruritus associated with a systemic disease is generalized, more gradual in onset, and accompanied by normal appearing skin.[505]

Although pain and pruritus are distinct sensations, they are mediated by the same neural pathways. Stimulation of unmyelinated C fibers that are situated in the dermal-epidermal junction (which are more superficial than those responsible for detecting pain) creates impulses that travel to the dorsal root ganglia and cross to the contralateral spinothalamic tract and ultimately to the somatosensory cortex. Neurotransmitters thought to mediate the sensation of pruritus include the amines, histamine and serotonin, opioids, eicosanoids, neuropeptides, and cytokines. Histamine, which is released primarily in the skin from mast cells, mediates pruritus via H1 and H2 receptors. The superficial C fibers are especially sensitive to histamine release. By binding to 5-HT3 receptors on C fibers, serotonin also plays a role in inducing pruritus. Finally, endogenous opioids also appear to affect itching, potentially through both central and peripheral mechanisms.

Pruritus triggers the urge to scratch through a spinal reflex, and scratching transiently relieves pruritus. However, persistent scratching can worsen pruritus and lead to more serious consequences, such as skin breakdown and infection. Pruritus is likely worsened by a variety of factors including dry skin, pain, boredom, or anxiety. Conversely, distraction and competing stimulation of the skin such as by painful stimuli may reduce this symptom.

Opioid-induced pruritus is a common problem in patients treated with systemic opioids. For example, Maxwell and colleagues[382] found that as many as 77% of children who received IV morphine by PCA experienced pruritus. The incidence of pruritus with use of opioids administered via the spine is also high, occurring in 20% to 90% of patients, particularly in the postoperative setting. The incidence of itching with spinal opioids appears lower in opioid-tolerant patients who have advanced cancer.[507] Whereas the itching associated with systemic opioids is usually widespread, the itching observed with spinal opioids tends to be more segmental.[505]

Although opioids do cause histamine release from mast cells, this phenomenon does not seem to account for most of the pruritus attributed to opioids, for several reasons. First, fentanyl does not cause histamine release, but it may still cause pruritus.[507] Second, opioids administered via the spine (e.g., morphine in doses up to fiftyfold lower than those used systemically) can produce intense and prolonged itching despite evoking negligible peripheral histamine release. Third, as will be detailed later, antihistamines are much less effective for opioid-induced itching compared with interventions that influence opioid-receptor coupling to G-protein–mediated intracellular actions. Possible explanations for opioid-induced pruritus include central activation of itch centers, activation of serotonergic pathways, and opioid-receptor–mediated activation of G-stimulatory proteins.

Interventions

Effective control of pruritus is important to provide comfort, improve quality of life, and prevent potential worsening of any underlying dermatologic condition or complications, such as infection. Treatment should be targeted to the suspected underlying cause, if possible. For example, the most effective treatment for pruritus resulting from Hodgkin lymphoma is cancer-directed therapy. Similarly, stenting of the bile duct, if clinically appropriate, is the most effective treatment for cholestatic pruritus. Depending on the degree and extent of involvement, pruritic GVHD may be treated with systemic immunosuppression, topical steroids, or topical tacrolimus.[508] Pruritus from histamine-mediated reactions (e.g., allergic reactions) often responds to antihistamines such as diphenhydramine. These agents should not be used indiscriminately or without regard for the underlying cause because they may contribute to mental status changes such as sedation, confusion, and delirium.

Antihistamines are typically ineffective in persons with cholestatic or uremic pruritus. Cholestyramine is effective in reducing pruritus associated with cholestasis, presumably by binding bile salts or other accumulated pruritogens. Randomized placebo-controlled trials have shown that cholestatic pruritus is relieved by naloxone or its oral equivalent, naltrexone, perhaps through opioid receptors thought be activated in this clinical condition.[509-511] Ondansetron may also reduce itch from cholestasis.[512-514] Cholestatic pruritus has also been reported to respond to midazolam in the absence of sedation, with the effect purportedly achieved through increased γ-aminobutyric activity.[515] For uremic pruritus, studies have yielded conflicting results for both the efficacy of ondansetron[516,517] and naltrexone.[518,519] Uremic and postburn pruritus, both considered forms of neuropathic itch, are relieved with use of either gabapentin or pregabalin.[520,521]

Biologic cancer treatments, such as epidermal growth factor receptor inhibitors, commonly cause pruritus. A single published study supports the use of aprepitant (125 mg on day 1 and 80 mg daily on days 2 and 3) for pruritus associated with erlotinib.[2] It is hypothesized that neuropeptide substance P activates mast cells in the lesional skin of persons who are treated with erlotinib, and because it is an NK-1 receptor antagonist, aprepitant may ameliorate itch.[522]

Interventions for Opioid-Associated Pruritus

Despite the widespread use of antihistamines for treatment of opioid-induced pruritus, data on their effectiveness in this setting are remarkably sparse, and some randomized controlled trials indicate lack of effectiveness.[523] One study found that hydroxyzine was superior to placebo in a postoperative setting, but most other trials have shown no differences between antihistamines and placebo.[523] Similarly, we have been unable to identify any trials showing effectiveness of the less-sedating antihistamines, such as cetirizine, fexofenadine, or loratadine, for opioid-induced itching.

Several studies, including a prospective, randomized controlled trial in children and adolescents receiving postoperative analgesia with a morphine PCA, have shown that low-dose naloxone infusions (0.25 μg/kg/h) reduce opioid-induced pruritus, and to a lesser extent nausea, without increasing opioid requirements or pain scores.[381,382,524] More recently, a prospective dose-finding study found a higher naloxone dose (1 μg/kg/h) to be more consistently effective in preventing opioid-induced adverse effects.[383] When low-dose naloxone infusions and nalbuphine are used to treat itching in patients who are quite opioid-tolerant, some uncertainty exists regarding whether to begin with lower or higher infusion rates or doses, but the 1 μg/kg/h rate appears to be appropriate for this population as well.

Systemic opioid-induced pruritus may be addressed by opioid rotation.[525] In one study of 90 children receiving morphine, hydromorphone, or fentanyl for postoperative pain, it was found that pruritus was more severe in the morphine group.[526] To our knowledge, however, no other studies have demonstrated superiority of one opioid over another in terms of pruritus.

Low-dose systemic infusions of opioid antagonists are widely used for patients receiving opioids administered via epidural and spinal routes. Nalbuphine is a mixed-agonist antagonist that is widely used to treat itching (as well as nausea and other adverse effects) from systemic and neuraxial opioids. A common dosing schedule is 0.01 to 0.02 mg/kg IV every 6 hours, to a maximum total dose of 1.5 mg. Note that these doses are fivefold to tenfold lower than the starting doses recommended when nalbuphine is administered as an analgesic.

Ondansetron has been shown to reduce systemic opioid-associated scratching, although the reduction in opioid-induced itching was not statistically significant.[527] Ondansetron reduces pruritus from intrathecal morphine,[528-530] although the mixed opioid agonist nalbuphine may be superior to ondansetron for this purpose.[531]

Our clinical impression, unconfirmed by data, is that pruritus is a more significant problem in the initial weeks of opioid use and that for many patients with advanced cancer who are receiving escalating doses of opioids, pruritus becomes less problematic as opioid tolerance progresses.

Topical Approaches

Any potential skin irritants such as perfumed skin products and harsh detergents should be avoided. When dry skin is thought to compound itching, application of a moisturizer may be helpful. Medicated baths containing oatmeal or baking soda, as well as the application of ice or cold compresses, may quell the itching sensation. Topical agents such as capsaicin (0.025% cream) or Sarna lotion containing camphor and menthol may also soothe skin. Successful use of topical naltrexone to relieve pruritus in persons with atopic dermatitis has recently been reported, suggesting that opioid receptors are involved in a variety of pruritic conditions, although this strategy remains experimental at this point.[532]

Other Potential Therapies for Pruritus

Several antidepressants have been used to treat pruritus. In adult dermatology practice, TCAs, especially doxepin, are widely used. TCAs appear to reduce itch and pain due to postherpetic neuralgia. Paroxetine has been implicated in providing relief from pruritus in a variety of situations, including paraneoplastic itch, opioid-induced itch, and pruritus associated with polycythemia vera. In a small randomized controlled trial of 26 patients with pruritus from a variety of nondermatologic causes, Zylicz[532a] found that one third of patients experienced a modest improvement in pruritus with paroxetine, although it was not reported which patients benefited. In addition, some patients received concurrent cisapride, a 5-HT3 receptor antagonist, which could have accounted for the improvement in pruritus through serotonergic mechanisms.[533] The antidepressant mirtazapine, which has 5-HT2, 5-HT3, H1 antagonist, and noradrenergic activity, was reported to ameliorate pruritus in a case series of four patients with either cholestatic, uremic, or paraneoplastic pruritus. It is unclear whether it is serotonergic blockage at the peripheral or central level that reduces pruritus or whether sedation per se contributed to its efficacy. Other medications used for neuropathic pain, such as anticonvulsants and systemic local anesthetics, have also occasionally been used for patients with refractory pruritus.

MOOD DISORDERS AND MENTAL STATUS CHANGES

Depression

Children with cancer may experience depression that predates their cancer diagnosis or develops after their cancer diagnosis. The reported prevalence of depression in children with cancer varies, likely because of differences in assessment techniques and the populations studied. In general, it does not appear that children with cancer have more psychological symptoms, including depression, than do other children.[534-536] For example, Noll and colleagues[535] found no significant differences in measures of depression, anxiety, or loneliness between children with cancer and their classroom peers. Psychologists have proposed that the similarity in depression diagnosis rates is due to use of avoidant or repressive styles of coping with the cancer experience.[537,538]

Physical symptoms stemming from cancer or its treatment can also occur in people with depression and in fact may be diagnostic criteria for depression. Adults diagnosed with major depression have been shown to have higher rates of such nonspecific symptoms, including fatigue, decreased appetite, and sleep disturbance.[539] Standardized screening measures for healthy children have not been validated in the pediatric oncology population. Clinicians may find it helpful to rely on psychological symptoms such as guilt, hopelessness, worthlessness, or anhedonia to make the diagnosis of depression. However, clinicians have been demonstrated to underestimate psychosocial symptoms such as depression and anxiety in children with cancer.[540]

Risk factors for the development of depression in children with cancer include perceived stress, lack of social support, not receiving open information about their diagnosis and prognosis at the initial stage of the disease, and parental anxiety.[537,541,542] Cancer survivors are also at risk for depression.[543,544] For example, adolescents who had cancer as children are 1.5 times more likely to have depression or anxiety[544] and are at higher risk for suicidal ideation than are their siblings.[545] Depression in this case may be due to impaired social functioning as a result of cancer or its treatment.[543]

Oncologists play a crucial role in screening patients for psychological disorders and for implementing first-line treatments for depression and anxiety (described later). Involvement of a psychosocial team is crucial. Most oncologists prescribe selective serotonin reuptake inhibitors (SSRIs), although some only do so in conjunction with a mental health consultation for the child.[546] Options for medical interventions for depression include TCAs, SSRIs, and newer selective serotonin norepinephrine reuptake inhibitors. TCAs are rarely used for depression because of their anticholinergic adverse effects and the lack of efficacy data in children. SSRIs appear to be the most commonly prescribed class of antidepressants in children with cancer.[547,548]

No particular antidepressant has been demonstrated to be superior to another in the pediatric cancer population. Instead, the choice of antidepressant should be determined by the characteristics, including adverse effect profiles, of different agents. For example, mirtazapine may be an optimal agent for a child with anorexia or difficulty sleeping. Likewise, a TCA may be the treatment of choice for a child with concomitant neuropathic pain. A modest increase in suicidal ideation has been noted in studies evaluating children treated with SSRIs compared with placebo.[549] The FDA now requires labeling of all antidepressants to warn clinicians of such a risk.[550] Although the meaning of the increased risk is unclear, initiation of an antidepressant warrants discussion of the risks and benefits with families and close monitoring of children for worsening depression or signs of suicidality. Some medications such as tramadol, triptans, and linezolid interact with SSRIs, increasing the risk of serotonin syndrome. Children with depression who do not respond to these measures, and children with suicidal ideation or complex psychological symptoms, should be referred for formal psychiatric evaluation.

Evaluation of screening for depression, longitudinal studies of mood disorders and their predictors, and prospective evaluation of interventions for children with cancer and depressive symptoms are clearly needed to better understand and ameliorate depression in children with cancer.

Anxiety

Children with cancer may experience anxiety that is situation-dependent (e.g., related to treatment). Such anxiety may respond to anxiolytic medications administered prior to treatment, as well as cognitive or behavioral interventions. Such nonpharmacologic interventions are described in more detail in the section on pain. Other types of anxiety in children should be approached with an exploration of potentially contributing factors (e.g., stress related to family or school) and referral for increased psychosocial support. A child with anxiety who meets the criteria for a psychiatric anxiety disorder (e.g., generalized anxiety disorder and panic disorder) or a child whose anxiety significantly impairs his or her function should be referred for psychiatric evaluation.

Delirium

Delirium is a state of disturbed consciousness or level of arousal characterized by an inability to focus or to maintain or divert attention. Delirium has a rapid onset and results in significant confusion for the patient. Delirious patients may have mumbling speech, perceptual disturbances with delusion or hallucinations, or alterations in their sleep/wake cycle. Patients may exhibit hyperalert-hyperactive delirium with anxiety, agitation, or even aggression. Alternatively, patients may have hypoalert-hypoactive delirium, characterized by slowed reactions and difficulty focusing. Delirium is a disturbing symptom for patients and may be extremely distressing for caregivers.[551]

Delirium may be caused by metabolic disturbances (e.g., hypoglycemia or electrolyte abnormalities), CNS pathology, liver or kidney dysfunction, infection, constipation, or urinary obstruction. The most common cause of delirium is a drug effect. The most common offending medications are anticholinergics, corticosteroids, benzodiazepines, and opioids. For this reason, delirium should never be treated with escalation of opioid therapy unless uncontrolled pain is suspected. Instead, the opioid dose should be decreased if possible or converted to an alternative opioid. In addition, psychoactive medications should be discontinued, if possible, and a neuroleptic such as haloperidol should be inititated. In fact, a randomized controlled trial of haloperidol versus chlorpromazine or lorazepam for adults with human immunodeficiency virus and delirium was halted early because of adverse effects in the lorazepam arm. Haloperidol should be initiated by dose escalation similar to the process used for treating pain.

Atypical antipsychotic medications have also been used successfully in adults with delirium, although no trials comparing them with haloperidol have been performed.[552] For delirium with significant agitation, benzodiazepines may be considered, but they may cause paradoxic exacerbation of symptoms. Regardless of the pharmacologic agent used, concomitant nonpharmacologic interventions should always be used as well. Measures to reduce stimulation in the environment or to orient the patient, such as a familiar person remaining with the patient and frequent orientation to time and place, may be helpful.

Corticosteroid-Induced Mood and Behavior Disturbance

Psychiatric abnormalities including depression, mania, hypomania, and psychosis, as well as cognitive changes due to corticosteroid therapy, are well described. Among children with ALL, both dexamethasone and prednisone are associated with neurobehavioral adverse effects, and preschool-aged children appear to be at greater risk for these effects.[553] Such alterations are reversible with dose reduction or discontinuation of treatment. However, dose reduction or discontinuation of corticosteroids administered for antineoplastic purposes, as in therapy for ALL, may not be possible. Reports of successful use of olanzapine or promethazine for steroid-associated psychosis or mood disturbance have been published. Risperidone also appears to be an effective short-term pharmacologic agent for corticosteroid-related mood disturbance or psychosis related to corticosteroid treatment for ALL.[554]

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CHAPTER 72

Childhood Cancer Survivorship

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CHAPTER OUTLINE

HISTORY AND OVERVIEW
Recognition of Late Effects
Early Mortality
Identifying Risk Factors for Late Effects
Interpreting Late Effects Research
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Secondary Solid Tumors
Hematologic Malignancies
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Cardiovascular System
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Central Nervous System
Reproductive System
Renal System
Genitourinary System
Gastrointestinal System
OTHER LATE EFFECTS
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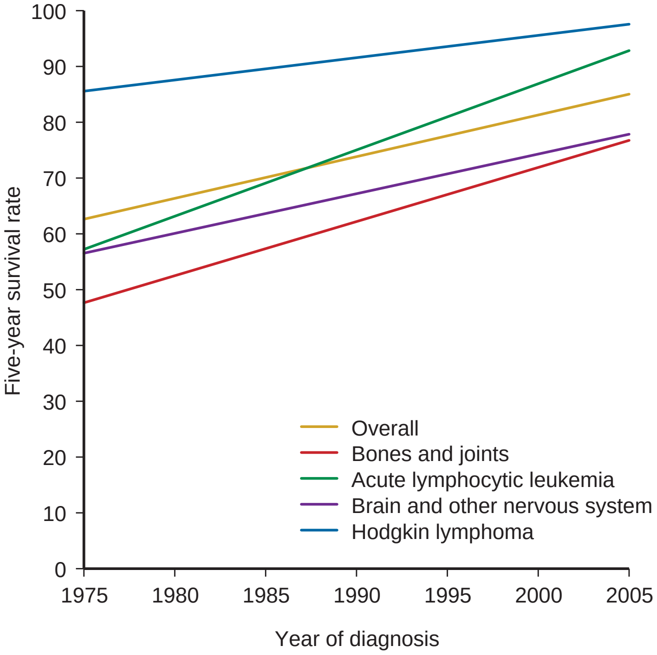
Dental Problems
Psychosocial Function
SPECIAL CONSIDERATIONS FOR THE HEMATOPOIETIC STEM CELL TRANSPLANT SURVIVOR
FOLLOW-UP CARE OF CHILDHOOD CANCER SURVIVORS
Models of Survivorship Care
Comprehensive Clinical Evaluation
FUTURE DIRECTIONS

With ongoing advances in the diagnosis and treatment of childhood cancers, increasing numbers of children and adolescents diagnosed with cancer are becoming long-term survivors. However, even as higher cure rates are achieved, there is increasing recognition that the diagnosis and treatment of cancer during childhood and adolescence can have lifelong implications for health and well-being. Cancer therapy administered during critical periods of growth and development render childhood cancer survivors vulnerable to a variety of physical and emotional disorders often referred to as “late effects.” Some late effects can be diagnosed soon after completion of cancer-directed therapy, and others may not manifest until decades later. The severity of late effects ranges from minor disabilities to chronic conditions and even life-threatening morbidities, including second cancers, cardiac disease, and pulmonary dysfunction. Severe late effects can reduce a survivor’s life expectancy.

Although remarkable progress has been made in advancing our understanding of the adverse health outcomes associated with treatment of childhood cancer, survivors continue to experience significant long-term morbidity related to late effects. Increasingly it is recognized that the care of the child or adolescent diagnosed with cancer does not end with the completion of therapy but continues on the path into adulthood. Clinical focus during survivorship care shifts from treatment of the primary cancer and screening for its recurrence to the prevention, detection, and management of adverse consequences of prior therapy. Ultimately, ongoing research may result in the reduction or even elimination of late effects and in improved quality of life for childhood cancer survivors.

HISTORY AND OVERVIEW

Prior to 1950, children and adolescents who developed cancer rarely survived. The first generation of survivors primarily consisted of children with Hodgkin lymphoma, sarcoma, and Wilms tumor who were successfully treated with surgery and high-dose, large-field radiation therapy.[1-4] The widespread use of multiagent chemotherapy, beginning in the 1970s, resulted in dramatic increases in survival for the most common childhood cancer, acute



Note: Data represent linear trendline.
Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov)

Figure 72-1 Trends in 5-year cancer survival by selected diagnosis, age younger than 20 years, 1975 to 2005. *(Data from Howlader N, Noone AM, Krapcho M, et al, editors: SEER cancer statistics review, 1975-2010. Bethesda, Maryland, National Cancer Institute. Accessed at* http://seer.cancer.gov/csr/1975_2010/. *Modified from November 2012 SEER data submission, posted to the SEER website, April 2013.)*

leukemia, and continued improvement in survival for other pediatric cancers as well.[5] By the mid-1970s, many forms of childhood cancer became treatable, and potentially curable. Continuing advances in treatment options for childhood cancer have continued to improved survival. Children with cancer have also benefited from improvements in diagnostic testing, resulting in earlier and more precise diagnosis and more timely delivery of necessary treatment. Enhanced supportive care including advances in intensive care, blood banking, and management of infectious disease and acute toxicities of therapy also contributed to successful outcomes. Currently, the overall 5-year survival for childhood cancer exceeds 80%, and for some cancer diagnoses 5-year survival is above 95% (Fig. 72-1).[6] There are estimated to be more than 300,000 adult survivors of childhood cancer in the U.S. population, and this number continues to grow by approximately 10,000 survivors each year.[7]

Recognition of Late Effects

Along with these remarkable advances in survival there is a growing recognition that treatment for childhood cancer is associated with long-term complications or “late effects.” Late effects can be defined as adverse health outcomes experienced by childhood cancer survivors associated with previous cancer–directed therapy; these outcomes reduce survival or affect the “quality” of survival. Some late effects can be life threatening and contribute to the increased risk for early mortality observed in adults who are long-term survivors of childhood cancer. Other late effects, such as neurocognitive impairment, physical deformity, and infertility, can limit survivors’ social or economic opportunities and impair their overall quality of life. In contrast, some late effects may be “positive” effects, such as increase in psychological resiliency and decrease in smoking rates and the rates of other high-risk behaviors compared with those in the rest of the population.[8,9]

Second cancers, cardiopulmonary disease, and infertility were among the first treatment-associated late effects recognized in childhood cancer survivors. These first descriptions were based on clinical observations in small numbers of the earliest survivors of childhood cancer treated primarily with surgery and irradiation in the 1960s.[10-12] Early clinical observations created awareness about late effects and identified the need for further research defining treatment-related health outcomes in the growing numbers of survivors of childhood cancer.

Early Mortality

Follow-up studies of the earliest cohorts led to the observation that childhood cancer survivors are at risk for early mortality. These studies showed that late recurrence of the primary cancer and treatment-associated complications resulted in decreased long-term survival, 80% at 20 years after diagnosis compared with 97% expected survival in the age-matched general population.[13,14] The majority of premature deaths observed in survivors in these early studies were attributed to late relapse, whereas treatment-associated second cancers and major organ toxicity accounted for 30% of deaths.[13-16] It was also recognized that the risk for dying of a treatment-related cause increased with increasing intensity of therapy.[15,16] Although late recurrence accounts for the majority of premature deaths observed in childhood cancer survivors, the risk for relapse diminishes over time while the risk for therapy-related mortality persists beyond 25 years of follow-up.[17-21] Figure 72-2 depicts all-cause mortality in 5-year survivors of childhood cancer, and Figure 72-3 demonstrates all-cause mortality by underlying cancer diagnosis.[21] It remains to be determined if current treatment protocols that are specifically designed to reduce late effects will continue to improve very long-term survival by reducing the risk for therapy-related mortality.

Identifying Risk Factors for Late Effects

After the early descriptive studies, registry-based investigations of larger groups of childhood cancer survivors were undertaken with the aim of identifying specific risk factors for late effects. These included the National Cancer Institute’s “Five Center Study,” a registry-based cohort that enrolled 2490 adult 5-year survivors diagnosed with cancer between 1945 and 1975 at age younger than 20 years who were followed prospectively for reproductive outcomes, second cancers, and mortality[22]; the “Late Effects Study Group,” a multiinstitutional cohort that analyzed the occurrence of second cancers in 9170

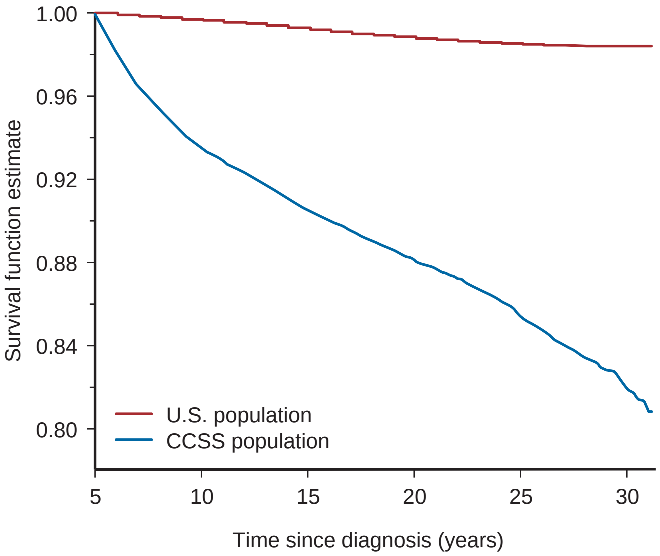


Figure 72-2 All-cause mortality in 5-year survivors of childhood cancer diagnosed from 1970 to 1986, age younger than 21 years. *(From Armstrong GT, Liu Q, Yasui Y, et al: Late mortality among 5-year survivors of childhood cancer: a summary from the Childhood Cancer Survivor Study.* J Clin Oncol *27:2328–2338, 2009).*

two-year survivors of childhood cancer diagnosed between 1936 and 1979[23]; and cancer registry–based studies in the United Kingdom,[24] the Netherlands,[25] and the Nordic countries (Denmark, Finland, Iceland, Norway, Sweden)[26] from the same early treatment era and beyond. These epidemiologic studies analyzed the association between specific treatments and adverse health outcomes of interest. Relative risks were calculated to compare rates of outcomes under study in cancer survivors with rates in a control population. Collectively these investigations made significant contributions to the understanding of late effects. Limitations of such studies of late-occurring outcomes included that recruitment was often not complete, resulting in a possible overrepresentation of childhood cancer survivors who were experiencing health conditions related to their prior therapy. In addition, some of the late effects observed in these cohorts were associated with historic treatments that have been replaced by more modern modalities. However, despite such limitations, these studies identified the relationships, now well accepted, between cancer therapy and early mortality, radiation therapy and secondary cancers, and alkylating agents and gonadal dysfunction.[16,22,27,28]

To address the limitations of prior studies, the development of "survivor" cohorts to be followed prospectively was undertaken by investigators in North America and Europe. In North America, a multiinstitutional follow-up study of a cohort of over 14,000 childhood cancer survivors (the Childhood Cancer Survivor Study [CCSS]) was created, evaluating late effects in survivors diagnosed from 1970 to1986, at age younger than 21 years, and surviving at least 5 years from diagnosis.[29] A recent expansion of the CCSS to include survivors treated between 1986 and 1999 will yield important analyses of the effects of more "modern" therapies, as well as reflect a more ethnically and racially diverse population in North America.[30] The CCSS uses self-reported questionnaires to obtain health outcomes and analyzes the association between outcome and treatment variables documented in abstracted medical records. The primary end points investigated by the CCSS included second cancers, mortality, and heart disease. Large registry-based cohort studies of childhood cancer survivors treated in Great Britain, France, the Netherlands, and the Nordic countries are ongoing and continue to contribute to our understanding of survivorship.[20,31-33] Within these cohort studies, the prevalence of and the risk factors for the development of late effects after childhood cancer have been better elucidated.

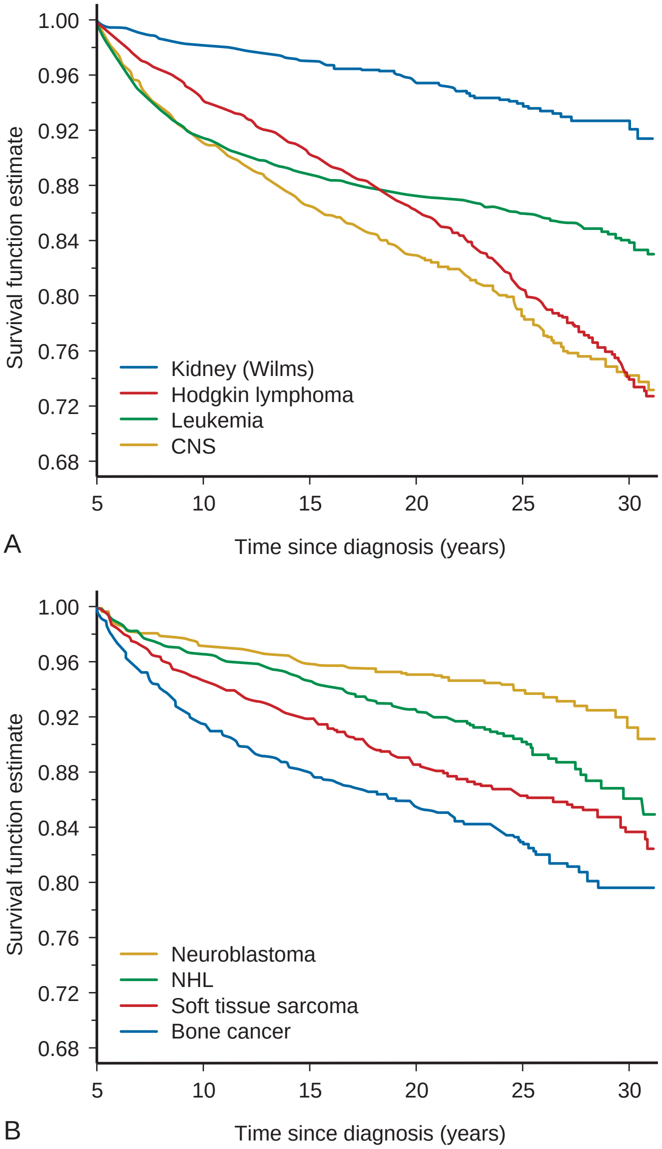


Figure 72-3 **A** and **B**, All causes of mortality, 5-year survivors of childhood cancer diagnosed 1970 to 1986, age younger than 21 years, by original cancer diagnosis. *CNS*, Central nervous system; *NHL*, non-Hodgkin lymphoma. *(From Armstrong GT, Liu Q, Yasui Y, et al: Late mortality among 5-year survivors of childhood cancer: a summary from the Childhood Cancer Survivor Study.* J Clin Oncol *27:2328–2338, 2009.)*

Interpreting Late Effects Research

Advances continue to be made in recognizing and categorizing adverse health outcomes in childhood cancer survivors. However, the study of late effects in cancer survivors has inherent challenges that must be considered when interpreting research findings. First, there is not universal implementation of a definition for "cancer survivor." The definition chosen for a particular investigation determines eligibility for studies and dictates how results can be interpreted and generalized to specific populations of survivors. The Office of Cancer Survivorship at the U.S. National Cancer Institute considers an individual a cancer survivor from the moment of cancer diagnosis through the balance of his or her life regardless of degree of cancer control.[34] Although this definition is meaningful, particularly from an advocacy perspective, it is not effective for the purposes of understanding late outcomes in a cured population. Survival 5 years beyond cancer diagnosis is the definition of cancer survivor that is often, but not universally, used for the purpose of research on late effects.

Another consideration is how investigators measure the late effect or outcome of interest. Self-report is frequently used to measure outcomes in epidemiologic studies of late effects. Although it is cost-effective, self-report can lead to reporting bias that results in inaccurate assessments of the incidence or severity of outcomes. Studies of smaller groups of survivors utilizing direct observation to delineate outcomes help to confirm self-reported outcomes, refine our understanding of these outcomes, and allow direct measurement of asymptomatic or early morbidity not evident by self-report. The disadvantages of this type of clinical studies are cost, complexity, and small sample size, which can limit interpretation of results.

The timing of assessments can also influence the results and interpretation of late effects research. Although it can be efficient to recruit survivors for follow-up studies during or soon after treatment, some late effects are not observed until decades after initial therapy. Studies with inadequate periods of follow-up might yield results that underestimate or overestimate late effects. Retaining survivors in observational cohorts over long periods of follow-up is challenging, and results may be biased toward retention of patients experiencing the outcomes under investigation. Consideration of the duration of follow-up is particularly important when applying results to populations of pediatric survivors, in whom late effects may emerge in the course of developmental change.

Another challenge when interpreting the results of late effect studies is that most results are reported as relative risks, the rate of an outcome observed in a cohort of survivors relative to the rate of the outcome observed in a control population. Elevated relative risks must be interpreted and communicated with caution, because even the smallest increase in an otherwise very rare outcome results in a very large relative risk; however, even a small or statistically insignificant increase in relative risk might have important clinical implications.

Box 72-1 **Risk Factors for Late Effects in Childhood Cancer Survivors**

TREATMENT FACTORS

Time since treatment
Dose and intensity of treatment
Concurrent treatments
Prior treatment toxicities

INDIVIDUAL FACTORS

Age
Developmental stage
Gender
Genetic predisposition
Health behaviors
Comorbidities

Finally, an inherent difficulty with studying late effects is that treatments for cancer continue to evolve; therefore the health outcomes of interest in follow-up studies are often reflective of historic or outdated treatments. Methodology does not exist to address this issue, so treatment era must be taken into consideration when interpreting the results of these investigations. Duration of follow-up, treatment era, cohort eligibility, and outcome measures are all aspects of study design that merit careful consideration when interpreting results of late effects research studies.

General Principles of Late Effects

Epidemiologic studies investigating the association between treatment and risk for adverse health outcomes have led to general principles that contribute to our thinking about an individual survivor's potential for developing late effects (Box 72-1). A childhood cancer survivor's risk for developing late effects depends on the nature of prior treatment as well as individual characteristics, such as age at exposure, gender, or time since exposure.

First, the dose and intensity of the treatment is frequently correlated with risk for late effects. In general, survivors exposed to larger doses of therapy have worse outcomes. For example, risk for male infertility after treatment with gonadotoxic chemotherapy is directly related to dose; males exposed to the highest cumulative doses have the greatest risk for future infertility.[35] In addition, a survivor's likelihood for late effects is increased if there is exposure to more than one modality with related toxicities. For example, survivors treated concurrently with mediastinal radiation and cardiotoxic chemotherapy are at greater risk for cardiac late effects than those treated with either of those modalities alone.[36]

Organ toxicity associated with a single treatment exposure can manifest clinically as different late effects depending on the timing of the clinical assessment. For example, consider a postmenarchal female adolescent treated with gonadotoxic chemotherapy. During treatment she might experience acute reversible amenorrhea. Years later, after resolution of acute menorrhea, the woman might experience infertility secondary to premature menopause.

Over time, the same woman might have osteoporosis as a secondary late effect of chronic estrogen deficiency. Neurocognitive impairment after central nervous system (CNS) therapy provides another example of how the timing of assessment influences detection of late effects. Children treated with CNS therapy might not manifest treatment-related neurocognitive deficits until they reach a time when school work, employment responsibilities, or social interactions require more complex cognitive function. The clinical manifestations of a single treatment exposure can change over time and are influenced by the physical, emotional, and social needs of the individual survivor.

Duration of follow-up also influences the potential for late effects. Some irradiation-associated late effects, especially secondary malignancies, have long latency periods, with increases in cumulative incidence over time. In contrast, other treatment-related toxicities can be acute and resolve (bone marrow suppression) or occur late and be progressive (pulmonary fibrosis). The leukemogenic potentials of specific forms of chemotherapy appear to be associated with fairly discrete time frames after exposures: topoisomerase inhibiters are associated with occurrence of secondary leukemias within months of exposure and rarely beyond 5 years, whereas alkylating agents are associated with a later onset and a later, but still predictable plateau in cumulative incidence at 10 years after exposure.[37] In addition, acute chemotherapy organ toxicity can predict late toxicity. For example, chemotherapy-associated renal toxicity diagnosed during therapy can persist as a chronic condition or resolve and be diagnosed again as chronic renal disease decades after treatment. Although treatment exposures are the largest determinants of future late effects, a survivor's age or developmental stage during cancer treatment and his or her sex, family history, existing comorbidities, and health behaviors can all modulate risk for the development of late effects. In general, younger age at treatment is associated with a higher risk and/or greater severity for late effects. Children treated with cranial irradiation during the critical period of rapid brain development (<2 years old) are at greater risk for neurocognitive late effects than children treated when they are older.[38] In addition, female survivors are at greater risk than males for some late effects, including chemotherapy-associated cardiomyopathy, neurocognitive impairment, and second cancers, although the explanation for this gender difference is not clear.[39-42]

Underlying genetic predisposition for cancer (contributing etiologically to the primary cancer occurrence) will influence the risk for secondary cancers, as has been observed in hereditary retinoblastoma and nevoid basal cell carcinoma syndrome. In some patients, the only marker of this predisposition will be family history or a feature of the primary tumor itself (young age at occurrence, occurrence in paired organs). Predisposition to late effects based on genetic background is an area of great interest in survivorship research. Those survivors with a genetic predisposition to chronic medical conditions, such as heart disease, may be at greater risk for treatment-associated chronic conditions, such as radiation-associated coronary artery disease.[43] Recent genome-wide association studies have identified risk-specific polymorphisms that may become clinically relevant for predicting the risk for late effects and allow for potential tailoring of primary cancer therapy or follow-up. Most associations that have been described—for example, for ototoxicity after cisplatin exposure[44] or second cancer after irradiation[45]—await further validation. In addition, common medical comorbidities associated with aging, such as hypertension or hypercholesterolemia, may contribute to a survivor's risk for a variety of medical late effects, including chronic renal disease, heart disease, and cerebrovascular disease. Modifiable health behaviors such as diet, exercise, smoking, and sun exposure may also modulate a survivor's risk for late effects.

High rates of chronic health conditions have been described in adult survivors of childhood cancer.[46,47] In the CCSS cohort, the relative risk for a severe or life-threatening chronic condition in survivors when compared with siblings was 8.2 (95% confidence interval [CI], 6.9 to 9.7).[46] In the St. Jude Lifetime Cohort Study, the cumulative prevalence of a chronic condition was 95.5% (9%% CI, 94.8% to 98.6%) by age 45 years and 80.5% (95% CI, 73.0% to 86.6) for a severe or life-threatening or disabling condition.[47] The following section provides an overview of late effects experienced by survivors of childhood cancer. A summary of selected treatment-associated late effects is presented in Table 72-1.

SECOND CANCERS

Second cancers are the leading cause of treatment-related mortality in 5-year survivors. In the CCSS cohort, second cancers accounted for 13% of deaths overall and for 60% of deaths defined as treatment related.[17] Most often, a new cancer diagnosed in a survivor can be associated with a prior treatment exposure, such as radiation therapy or alkylating agent chemotherapy. Genetic cancer predisposition may also contribute to a survivor's risk for subsequent cancers. In addition, survivors, like all aging individuals, develop cancers that are not related to prior treatment or known genetic predisposition and these cancers will become more prevalent as the childhood cancer survivor population ages. To date, there are no data that address the comparative morbidity and mortality from these adult-onset cancers and whether survivors will be compromised in their ability to receive or respond to known effective therapies.

Overall, the likelihood of a childhood cancer survivor being diagnosed with a second primary cancer is three to six times greater than the risk in the aged-matched general population.[33,48-51] At 20 years after diagnosis, the cumulative incidence of second cancers in childhood cancer survivors is estimated at 3.2% to 4.2%.[48-50] In the British Survivorship Cohort, 5% of survivors had developed a secondary cancer by 38 years of age; this cumulative incidence of cancer would be expected at age 54 in a comparable, nonsurvivor population.[33] Clinical and demographic factors that increase the risk for second cancers include primary diagnosis, exposure to radiation, attained age, and chemotherapy with alkylating

TABLE 72-1 Selected Late Effects by Treatment Exposure

Treatment	Late Effect	Comments	Reference
Alkylating agents (busulfan, carmustine, lomustine, mechlorethamine, procarbazine, cyclophosphamide, ifosfamide, dacarbazine, melphalan)	Secondary leukemia/ myelodysplasia	Melphalan and mechlorethamine more leukemogenic than cyclophosphamide Median latency 4-6 years Dose-response relationship	23-25, 143-145
	Premature menopause	Risk factors: increasing cumulative total dose, concurrent pelvic radiation, increasing age, older age at treatment Myeloablative doses are associated with ovarian failure. Cryopreservation of oocytes or embryos are interventions to preserve fertility.	341, 343, 344
	Male infertility	Risk of azoospermia increases with increasing cumulative dose; spermatogenesis may recover over time. Highest dose is associated with impaired testosterone production. Pretreatment semen cryopreservation and testicular sperm extraction are interventions to preserve fertility.	35, 349, 351, 352
Anthracyclines (daunorubicin, doxorubicin, mitoxantrone, idarubicin)	Clinical heart failure	Risk factors: female gender, age <5 yr, cumulative doxorubicin equivalent dose >250-300 mg/m^2, concurrent radiation	152, 155, 156, 158, 175-177
Bleomycin, carmustine, busulfan (pulmonary toxic chemotherapies)	Pulmonary fibrosis	No plateau in incidence observed Radiation exposure an additional risk factor	215, 219
Platinum compounds (cisplatin, carboplatin)	High-frequency hearing loss	Cumulative dose cisplatin >450 mg/m^2 Risk factors: dose, age <5 yr Carboplatin ototoxic at myeloablative dose	326-329
	Speech frequency hearing loss	Cumulative dose cisplatin >720 mg/m^2 Rarely observed cumulative dose cisplatin <360 mg/m^2 Risk factors: dose, age <5 yr Carboplatin ototoxic at myeloablative dose	326-329
Topoisomerase II inhibitors (epipodophyllotoxins and anthracyclines)	Secondary leukemia	Median latency 1-3 yr Frequent administration increases risk (weekly/twice weekly).	24, 146-148, 149, 150
Any irradiation	Secondary sarcoma	Risk factors: increasing radiation dose, concurrent alkylating agent or anthracycline chemotherapy, young age at diagnosis, primary diagnosis of sarcoma or retinoblastoma, family history of sarcoma	66, 110-115
	Nonmelanomatous skin cancer	Fifty percent of survivors will have multiple lesions. Basal cell carcinoma—linear dose-response	118-120
	Melanoma	Risk factors: radiation dose >15 Gy, prior history melanoma, retinoblastoma, familial melanoma	115, 116
Cranial and head/neck irradiation (including TBI)	Growth hormone deficiency	High prevalence (85%-100%) with median dose 45-55 Gy Observed at 18-24 Gy, with lower prevalence and longer latency	223, 229-231
	Obesity	Risk factors: ≥20 Gy cranial radiation, female gender	282, 283, 285
	Hypothyroidism	Primary—direct neck radiation ≥10 Gy Central hypothyroidism—cranial dose >35 Gy Dose-response relationship with no plateau in incidence observed after neck radiation	93, 223, 226
	Thyroid cancer	Risk factors: radiation dose 15-30 Gy, young age at treatment, female gender	93-103
	Brain tumors	The most common pathologic processes are meningioma and glioma. Risk increases with increasing dose.	41, 122, 126
	Head and neck carcinoma	Parotid gland carcinoma most common; secondary head and neck carcinoma Risk of salivary gland carcinoma increases linearly with dose.	51,67,68, 138
	Stroke	Mean cranial radiation dose ≥30 Gy increased risk for both leukemia and brain tumor survivors. Highest risk after cranial dose ≥50 Gy	210, 212
	Dental abnormalities	Risk factors: age at diagnosis <5 yr, higher-dose radiation	416-419

TABLE 72-1 Selected Late Effects by Treatment Exposure (Continued)

Treatment	Late Effect	Comments	Reference
Chest irradiation (chest, mantle, lung, mediastinal, TBI)	Coronary artery disease	Increased risk with high-dose exposure	32, 36, 188, 192
	Cardiac valve disease	Increased risk with high-dose exposure	36, 43, 198
	Breast cancer	Risk factors: increasing radiation dose, age 10-30 years at diagnosis Latency: 10-15 years Ovarian failure reduces risk.	61, 82-85
	Lung fibrosis	Pulmonary—toxic chemotherapy an additional risk factor	216-219
	Lung cancer	Greatest risk in Hodgkin lymphoma survivors Increased risk in smokers	51, 65, 67, 85
	Thyroid cancer	Risk factors: radiation dose 15-30 Gy, young age at treatment, female gender	93-103
	Hypothyroidism	Risk factors: increasing radiation dose, older age at diagnosis, female gender No plateau in incidence observed over time	93, 223
Abdominal irradiation (abdominal, periaortic, flank)	Gastrointestinal cancer	Risk factors: dose >30 Gy; concurrent treatment with alkylating agents; increasing radiation dose and increasing radiation volume	72, 73,130
Pelvic irradiation (pelvic, inverted "Y," TBI)	Genitourinary cancer	Modestly increased risk overall; greatest risk for cancers of the female genital tract, bladder cancer, and renal cell carcinoma Risk factors: renal cell carcinoma renal irradiation, platinum-based chemotherapy, primary diagnosis of neuroblastoma	51, 67, 131
	Premature menopause	Risk factors: dose (≥1 to ≤20 Gy) and concurrent alkylating agent chemotherapy Pelvic radiation >10 Gy associated with ovarian failure Cryopreservation of oocytes or embryos is intervention to preserve fertility.	341-344
	Male hypogonadism	Risk of irreversible azoospermia >2 Gy Higher dose associated with impaired testosterone production, >20 Gy Pretreatment semen cryopreservation and testicular sperm extraction are interventions to preserve fertility.	264, 339, 349

TBI, Total-body irradiation.

agents or epipodophyllotoxins.[33] Diagnoses that confer the greatest risk for subsequent cancers include Hodgkin lymphoma (standardized incidence ratio [SIR], 8.7),[51] Ewing sarcoma (SIR, 8.5),[51] and retinoblastoma (relative rate [RR], 15).[49]

Genetic predisposition for second cancers results from an inherited or spontaneous germline mutation in a known or presumed cancer susceptibility gene. Characteristic findings in family cancer predisposition syndromes are younger age at cancer diagnosis, cancer in two or more generations, two or more cancers in an individual, and rare forms of cancer in combination.[52,53] An example of a rare cancer predisposition syndrome that is associated with pediatric malignancies is Li-Fraumeni syndrome, which is associated with germline mutations in the tumor suppressor gene *TP53*. It is inherited as an autosomal dominant trait and has a high penetrance with cancer risk of 50% by age 40 years.[52] The cancers most commonly observed in Li-Fraumeni syndrome are soft tissue sarcomas, breast cancer, leukemia, osteosarcoma, adrenocortical cancer, and brain tumors.

Survivors with an underlying genetic predisposition for cancer are more susceptible to treatment-associated secondary malignancies. This is demonstrated in survivors of retinoblastoma who carry a mutation in the *RB1* gene. Those with hereditary retinoblastoma treated with irradiation for their primary cancer have a cumulative risk for second cancers of 58% at 50 years, compared with 26% if not treated with irradiation.[54] There are other rare genetic syndromes that are associated with an increased susceptibility to pediatric cancers and subsequent adult-onset secondary cancers, and increasing evidence suggests that a substantial percentage of children with cancer may have an underlying cancer predisposition syndrome that might inform risk for subsequent cancers.[55] Genome-wide association studies of patients with and without secondary cancers may reveal polymorphisms associated with increased risk; for example, a study of Hodgkin lymphoma survivors who received irradiation identified two variants at genetic locus 6q21 that are associated with increased risk for a subsequent cancer.[45] Further work in this field may determine which survivors should undergo more intensive surveillance or prophylaxis for secondary cancer. All survivors of childhood cancer should be assessed for risk for secondary cancer based on a possible cancer predisposition, with attention to the family history as it evolves, development of benign disorders (e.g., polyps or hamartomatous lesions), as well as assessment of the pediatric history, including the primary diagnosis and age at diagnosis. Referral for genetic counseling for survivors of childhood cancer is increasingly important, because recent studies suggest that early genetic diagnosis and subsequent tumor surveillance may result in early diagnosis of secondary cancers in patients with known cancer predisposition syndromes (Box 72-2).[56,57]

Secondary Solid Tumors

Cancer that develops in a prior radiation field is the most common type of second cancer observed in survivors.[33,51] Risk estimates for second cancers have been reported from populations of survivors treated with irradiation during historic treatment eras when the modes of radiation therapy routinely used in clinical practice were different than current practices. In current oncology practice, orthovoltage radiation has been eliminated, thus reducing radiation doses to nontumor tissues such as skin, bones, and soft tissue. Large-dose extended-field radiation therapy is increasingly being replaced by involved-field lower-dose radiation therapy or conformal radiation therapy, often in combination with chemotherapy. It is assumed that this change in clinical practice aimed at reducing exposure to radiation will result in a lower incidence of radiogenic cancer; however, no “safe” dose of therapeutic radiation has been established and irradiation-associated cancers have been observed at what would be considered the lowest treatment doses.[58,59] In general, risk factors for irradiation-associated second cancers include treatment with radiation doses greater than 30 Gy, radiation in combination with alkylating agent chemotherapy, young age at exposure, more than 10 years beyond treatment, and the presence of a cancer predisposition syndrome.[25,27,60] Of particular concern is the observation that the cumulative incidence of irradiation-associated cancers does not plateau in groups of survivors followed for up to 50 years after their therapy for childhood cancer.[27,61-64] Second cancer risk is modulated by lifestyle choices; for example, adult survivors of Hodgkin lymphoma who smoke after exposure to chest irradiation have a 20-fold greater risk for secondary lung cancer compared with survivors who are nonsmokers.[65]

Box 72-2 **Indications for Cancer Genetic Counseling in Childhood Cancer Survivors**

FAMILY HISTORY

- Cancer in first-degree relatives
- Relatives with cancer at early age
- Cancer in two or more generations
- Individual with two or more cancers
- Rare cancers in combination
- Cancer in paired organs

PRIMARY CHILDHOOD CANCER DIAGNOSIS

- Adrenal cortical carcinoma
- Choroid plexus carcinoma
- Melanoma
- Pleuropulmonary blastoma
- Renal cell carcinoma
- Retinoblastoma
- Rhabdoid tumor
- Sarcoma

MEDICAL HISTORY

IMMUNODEFICIENCY SYNDROME

- X-linked lymphoproliferative syndrome
- Wiskott-Aldrich syndrome

GENETIC SYNDROME

- Beckwith-Weidemann syndrome
- WAGR/Denys-Drash syndrome
- Hereditary leiomyomatosis and renal cell carcinoma
- Familial adenomatous polyposis
- Juvenile polyposis syndrome
- Peutz-Jeghers syndrome
- Cowden syndrome
- Bannayan-Riley-Ruvalcaba syndrome
- Von Hippel-Lindau disease
- Hereditary paraganglioma–pheochromocytoma
- Nevoid basal cell carcinoma
- Noonan syndrome
- Blooms syndrome
- Diamond-Blackfan anemia
- Fanconi anemia
- Ataxia-telangiectasia
- Neurofibromatosis
- Tuberous sclerosis
- Carney complex
- Xeroderma pigmentosum
- Turcot syndrome
- Rubenstein-Taybi syndrome
- Simpson-Golabi-Behmel syndrome
- Costello syndrome

KNOWN CARRIER OF CANCER PREDISPOSITION GENE MUTATION

- *ATM*
- *TP53*
- *APC*
- *RB1*
- *WT1*
- *PTCH*
- *MLH1, MLH2*
- *BRCA1, BRCA2*
- *NF1, NF2*
- *MEN1, MEN2*
- *DICER-1*
- *PTEN*
- *VHL*

PHYSICAL FINDINGS

- Dysmorphic features
- Skin lesions
- Hemihypertrophy

Data from Genetics Home Reference: Your guide to understanding genetic conditions. Accessed at http://ghr.nlm.nih.gov; and Schneider K: Counseling about cancer: strategies for genetic counseling. *New York, 2011, John Wiley & Sons.*

All tissues and organs are vulnerable to cancer induction by exposure to radiation. However, some tissues, such as thyroid and breast tissue, have greater sensitivity to radiation. Excluding low-grade nonmelanomatous skin cancer and (benign) meningiomas, the most frequently reported irradiation-associated second cancers in 5-year survivors of childhood cancer followed in the CCSS are, in order, breast cancer, thyroid cancer, CNS cancers, soft tissue sarcomas, and bone sarcomas.[48,66] Additional irradiation-associated second cancers reported in survivors, especially with longer follow-up intervals, include carcinomas of the head and neck (primarily parotid gland), colon cancer and other gastrointestinal cancers, lung cancer, and carcinomas of the genitourinary tract.[33,51,67,68-72,73] The most common second cancers observed in childhood cancer survivors are described next.

Breast Cancer

Breast cancer is one of the most frequently diagnosed irradiation-associated second cancers in childhood cancer survivors. Women treated with mantle, mediastinal, chest, spinal, whole-lung, and total-body irradiation during childhood or adolescents are at risk for secondary breast cancer.[27,61,62,68,74,75,76-81] Large cohort studies of childhood Hodgkin lymphoma survivors report a fifteen- to thirtyfold increased risk for irradiation-associated secondary breast cancer compared with the age-matched general population. Risk estimates vary by characteristics of the cohort, including radiation dose, age at therapy, and duration of follow-up. The cumulative incidence of secondary breast cancer is as high as 12% to 35% at 20 years after therapy for Hodgkin lymphoma, and the incidence does not plateau with longer duration of follow-up.[27,61,62,68,74-79]

Female survivors at greatest risk for secondary breast cancer are those treated between the age of 10 and 30 years with chest radiation doses greater than 20 Gy.[76,82] Studies have demonstrated a linear dose-response relationship between radiation dose and risk for secondary breast cancer.[76,83,84] The typical latency of irradiation-associated breast cancer is 10 to 15 years after therapy; however, secondary breast cancer has been reported in women as young as 20 years of age and as soon as 4 years after primary diagnosis.[61,85] Secondary breast cancer is most often associated with chest irradiation for Hodgkin lymphoma; however, it is important to recognize that women treated with chest irradiation for any childhood cancer diagnosis have increased risk for subsequent breast cancer, as illustrated in Figure 72-4.[61] Survivors of primary sarcomas not treated with chest irradiation also have an increased risk for secondary breast cancer (RR, 7).[61] Interestingly, survivors with therapy-induced ovarian dysfunction have a relative decreased risk for irradiation-associated breast cancers compared with survivors with normal ovarian function.[61,76,83] Although very rare, sporadic cases of male breast cancer have been reported in men treated with whole-lung irradiation as children.[10]

The most common type of breast cancer diagnosed in survivors is invasive ductal carcinoma; adverse biologic features (e.g., hormone-receptor negativity) may be more common in these childhood cancer survivors than in the general population.[61,74,86-88] Similar to findings in the general population, secondary breast cancer is detectable by radiographic imaging and has an excellent prognosis when treated in the earliest stages.[86] Breast cancer treatment options for survivors, however, can be limited by the therapy received for their childhood cancer. Specifically, prior anthracycline exposure and concern for potential cardiotoxicity may limit the use of adjuvant systemic chemotherapy with doxorubicin and the concern for tissue necrosis with repeated exposure to chest irradiation limits the option of breast-conserving surgery for some women.[86]

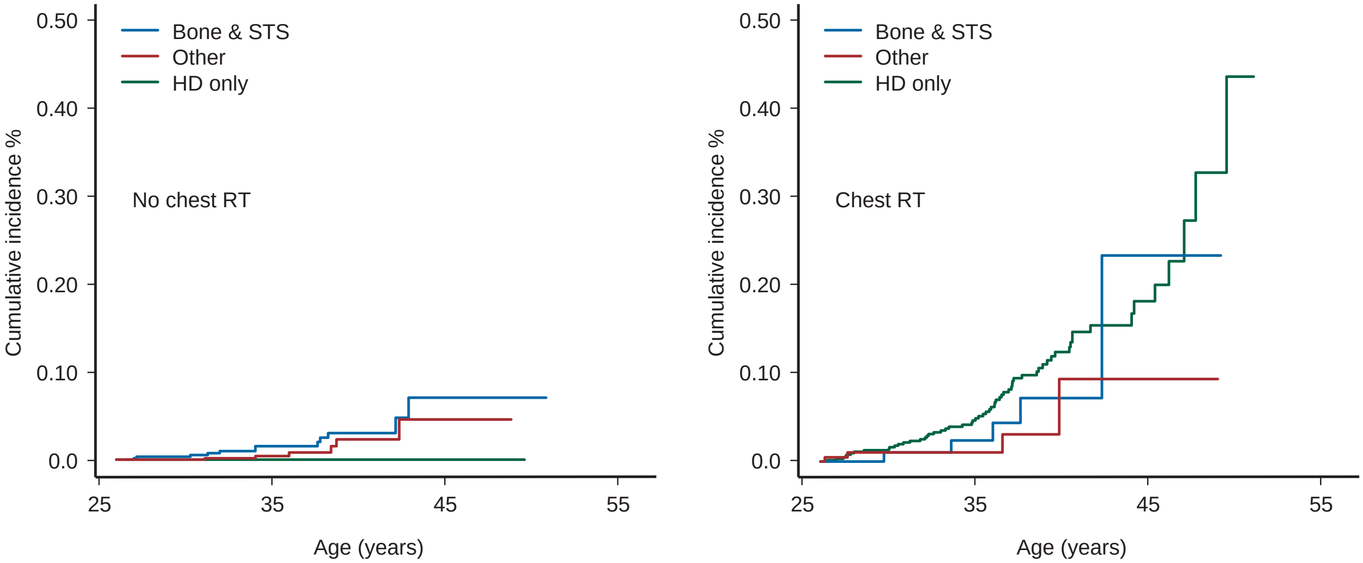


Figure 72-4 Cumulative incidence of breast cancer in survivors of Hodgkin lymphoma, sarcomas, and other diagnosis exposed and not exposed to chest radiation therapy *(RT)*, as a function of attained age. *STS,* Soft tissue sarcoma. *(From Kenney LB, Yasui Y, Inskip PD, et al: Breast cancer after childhood cancer: a report from the Childhood Cancer Survivor Study.* Ann Intern Med *141:590–597, 2004.)*

TABLE 72-2 Evaluation of Childhood Cancer Survivors for Irradiation-Associated Second Cancers

	Treatment Risk Factors	Clinical Evaluation	Special Considerations
Breast Cancer	Any chest irradiation Spinal irradiation TBI	Monthly breast self-examination Biannual clinical breast examination Breast MRI Mammogram	Current recommendations are to begin annual radiologic screening 8 years after irradiation or age 25 years, whichever occurs last.
CNS Tumor	Cranial irradiation Head /neck irradiation TBI	Annual neurologic examination Head MRI	Imaging studies are indicated for new or persistent neurologic symptoms.
Colorectal Cancer	Any abdominal irradiation Pelvic irradiation Spinal irradiation TBI	Colonoscopy Computed tomographic colonoscopy (CTC)	Current recommendations are to begin colonoscopy 10 years after radiation or age 35, whichever occurs last. CTC is not universally recommended as an alternative to colonoscopy in high-risk populations.
Thyroid Cancer	Neck irradiation Chest irradiation TBI MIBG	Annual clinical thyroid examination Thyroid ultrasound evaluation	Routine ultrasound screening is not currently recommended but should be considered for survivors with difficult clinical examination.
Skin Cancer	Any irradiation	Annual skin cancer screening Monthly skin self-examination	Practicing skin cancer prevention has potential to reduce risk.

Data from www.cancer.org/healthy/findcancerearly/cancerscreeningguidelines*;* www.uspreventiveservicestaskforce.org/recommendations.htm*; and* www.survivorshipguidelines.org.

TBI, Total-body irradiation.

Current screening recommendations for childhood cancer survivors treated with chest radiation published by the Children's Oncology Group include monthly self-breast examination, annual clinical breast examination, and annual breast magnetic resonance imaging (MRI) and mammography starting 8 years after treatment or age 25 whichever comes last.[89] Guidelines published by the American Cancer Society also advocate mammography and annual breast MRI, but suggest MRI only for women treated with chest irradiation between the ages of 10 and 30 years.[90] The addition of MRI to mammography has been reported to increase the sensitivity of breast cancer screening, although an impact on breast cancer outcomes has not been described (Table 72-2).[91]

Thyroid Cancer

Irradiation of the head and neck and upper thorax, total-body irradiation, and treatment with radioactive iodine (e.g., with ^{131}I-labeled-metaiodobenzylguanidine [MIBG])[92] place childhood cancer survivors at risk for subsequent thyroid cancers. Large cohort studies of childhood cancer survivors estimate relative risks for secondary thyroid cancer of between 4 and 36,[51,77,85,93-98] with a cumulative incidence of 1.3% at 30 years reported for female survivors and 4.4% in Hodgkin disease survivors.[85,99] Well-differentiated papillary carcinoma is the most common pathologic process (63% to 80%); however, follicular carcinomas are also observed.[68,85,96,97,100] The minimum observed latency period for secondary thyroid carcinoma is 5 years,[85,96,100] although cases have been reported closer to the time of original diagnosis.[85] At 20 years of follow-up, the relative risk for thyroid carcinoma remains increased[85,101] and a plateau in incidence has not been observed beyond 40 years of follow-up.[102] The risk for thyroid cancer is related to radiation dose; but unlike most other radiation-induced second cancers, the risk for thyroid cancer is greatest at lower doses of 15 to 30 Gy and beyond 30 Gy there is a decline in risk.[95,96,103] This finding demonstrates that thyroid tissue has enhanced sensitivity to radiation toxicity and supports the "cell kill" hypothesis that suggests higher doses of radiation cause cell death, which reduces the initiation of carcinogenesis observed at lower doses.[95] Although there is conflicting evidence, chemotherapy does appear to modify risk for irradiation-associated secondary thyroid cancer when the radiation dose is less than 20 Gy.[95,97,99,103] Female gender and young age at treatment are independent risk factors for secondary thyroid cancer.[85] Survivors of neuroblastoma treated with ^{131}I-MIBG are also at risk for subsequent thyroid cancer. In a single-institution case series of 16 neuroblastoma survivors treated with ^{131}I-MIBG and no external-beam irradiation, followed for a median of 15 years, 9 of 16 had nonpalpable thyroid nodules detected on thyroid ultrasonography, 6 of the 9 went on to biopsy, and 2 of those 6 were diagnosed with papillary thyroid carcinoma.[92]

Radiation-induced thyroid cancer in young survivors has an excellent prognosis; therefore many experts have recommended clinical screening. Appropriate screening schedules for secondary thyroid cancers and recommended modality for screening are controversial.[104-107] At a minimum, annual palpation of the thyroid for nodules by an experienced examiner is recommended.[89] Routine screening to detect subclinical thyroid nodules with high-resolution ultrasonography has been recommended by some investigators but remains controversial.[105,106] Ultrasound screening has the benefit of detecting smaller nonpalpable nodules but has the consequence of false-positive findings and potential for unnecessary biopsies.[106,107] Thyroid nodules do have ultrasound characteristics that are of concern for malignancy (hypoechogenicity, irregular margins, microcalcification). However, these findings are not diagnostic[108] and fine-needle aspiration of nodules larger than 1.0 cm or with suspicious features is usually necessary to evaluate for malignancy. Serial ultrasonography is then recommended to monitor thyroid nodules that are small or benign on biopsy.[108] Although clinical practice is moving toward using ultrasound to screen asymptomatic high-risk survivors for thyroid cancer, screening should be considered only after a discussion of the potential risks and benefits (see Table 72-2).

In general, irradiation-associated thyroid cancer is treated the same as other thyroid cancers and has an excellent prognosis. Thyroid surgery is indicated for nodules that are enlarging, are of indeterminate pathology on fine-needle aspiration, or are positive on fine-needle aspiration for malignancy.[108] Total thyroidectomy is often recommended to survivors because irradiation-associated thyroid cancers can be multicentric and because benign nodules can accompany clinically undetectable malignant lesions.[108] Finally, there is indirect evidence to support that exogenous thyroid hormone use reduces the risk for secondary thyroid cancer by suppressing thyroid stimulating hormone (TSH)-induced proliferation of thyroid nodules. Although not proven to reduce risk for thyroid cancer, the use of exogenous thyroid hormone to suppress thyroid proliferation is supported by data that show a decrease in incidence of new thyroid nodules in patients treated with exogenous thyroid hormone after partial thyroidectomy (34% vs. 14%).[109]

Sarcomas

Survivors treated with radiation are at increased risk for secondary cancer of the bone and soft tissue in the radiation field. Although secondary sarcomas are most often associated with irradiation, survivors of primary sarcoma and hereditary retinoblastoma who have not had prior radiation therapy also have an increased risk, suggesting a genetic predisposition.[54,110,111] Risk estimates for secondary sarcoma vary by type of sarcoma and composition of the study cohort.[60,63,110,112,113] The CCSS cohort, which did not include survivors of retinoblastoma, reports an overall ninefold increased risk for secondary sarcomas and an absolute excess risk of 32.5 per 100,000 person-years.[110] Secondary sarcomas occurring after childhood cancer include multiple histologic subtypes such as osteosarcoma, nonrhabdomyosarcoma soft tissue sarcomas, primitive neuroectodermal tumors (Ewing sarcoma), and malignant peripheral nerve sheath tumors, as well as rarer sarcoma subtypes.[110] The median latency for secondary sarcomas is 11 years, with increased risk persisting beyond 20 years.[63,110] Reported risk factors for subsequent sarcomas include higher doses of radiation, primary diagnosis of sarcoma or hereditary retinoblastoma, younger age at diagnosis, alkylating agent exposure, anthracycline exposure, and family history of sarcoma.[63,110,112-114] A nested case-control study of secondary sarcomas in the CCSS cohort identified a dose-response relationship between radiation dose and risk for secondary sarcomas, with elevations in risk ratios with higher radiation doses: 10 to 29.9 Gy (odds ratio [OR], 15.6; 95% CI, 4.5 to 53.9), 30 to 49.9 Gy (OR, 16.0; 95% CI, 3.8 to 67.8), and more than 50 Gy (OR, 114.1; 95% CI, 13.5 to 964.8).[66] Risk factors other than irradiation associated with increases in risk for secondary sarcoma in this study included primary cancer diagnosis of sarcoma or Hodgkin lymphoma and anthracycline exposure.[66]

For survivors of hereditary retinoblastoma treated with irradiation, the risk for subsequent bone sarcomas is as high as 400-fold with a cumulative incidence at 20 years of 14.1% and the relative risk for soft tissue sarcomas is 140 with 50-year cumulative incidence of 13%.[60,63,111] Radiation exposure and underlying genetic predisposition both contribute to risk for secondary sarcomas in retinoblastoma survivors.[60,63,111]

Routine radiographic screening for secondary sarcomas is currently not recommended. Survivors at risk for secondary sarcomas should be counseled to report new pain, lesions, or masses in the radiation field and should be promptly evaluated for any signs or symptoms suggestive of a secondary sarcoma. As survivors enter follow-up care, providers should consider obtaining baseline imaging of any previously irradiated site, so these studies are then available for comparison should symptoms suggestive of a new malignancy develop. There is no established role for secondary sarcoma surveillance for survivors of hereditary sarcoma predisposition syndromes (Li-Fraumeni syndrome, hereditary retinoblastoma), although the role of whole-body MRI is currently under investigation.

Skin Cancer

Melanoma and nonmelanomatous skin cancers are reported with increased incidence in survivors. The relative risk for melanoma is 2.5 to 9.0 in childhood cancer survivors, with a median latency of 15 to 18 years.[48,51,115,116] Survivors of hereditary retinoblastoma, familial melanoma syndromes, and those with a prior history of melanoma all have an increased risk for subsequent melanoma independent of irradiation.[49,55,111,117] Although the association between therapeutic radiation and melanoma is not firmly established,[116] a case-control study of 16 survivors with secondary melanoma in a European cohort of 4000 childhood cancer survivors found an association between secondary melanoma and exposure to more than 15 Gy of radiation.[115] In contrast, basal cell carcinomas and squamous cell carcinomas are known to be more common in radiation-exposed survivors (RR, 6.3), with 90% of all nonmelanomatous skin cancers diagnosed in previously irradiated fields.[118,119] A linear dose-response relationship is described for basal cell carcinomas, with an excess odds ratio of 1.09 per Gy of radiation.[120] Also of note, these low-grade skin cancers can be recurrent, and almost half of survivors diagnosed will have multiple lesions.[119] The prognosis for secondary skin cancers is excellent with early diagnosis; therefore routine screening

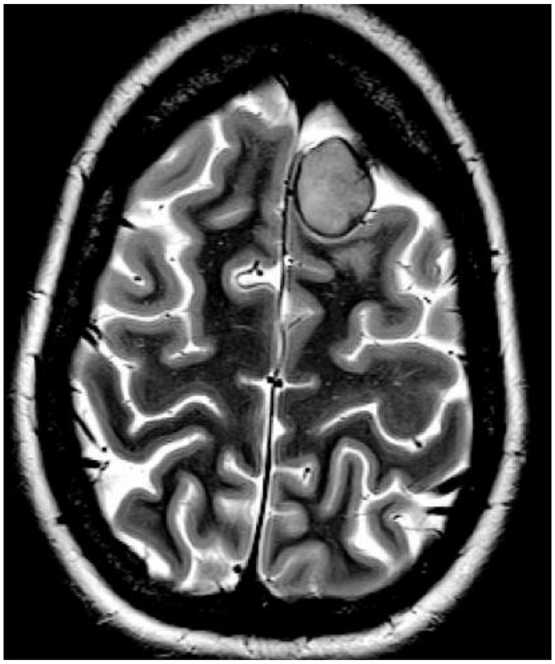


Figure 72-5 MR image of meningioma in a 20-year-old with a history of relapsed acute lymphoblastic leukemia, with therapy including total-body irradiation, presenting with headache. Note frontal mass. Pathology was consistent with meningioma.

with skin examinations is recommended.[121] Because ultraviolet radiation from the sun is known to contribute to skin cancer risk; patients should be routinely educated about preventive strategies such as aggressive sun exposure protection (see Table 72-2).

Central Nervous System Tumors

Past exposure to cranial irradiation is considered the most important risk factor for the development of subsequent CNS tumors in survivors of childhood cancer.[41,122] The most common pathologic processes include meningioma (Fig. 72-5) and glioma; however, other tumors such as primitive neuroectodermal tumor or medulloblastoma have been reported.[41,49,123-125] In a report from the British Childhood Cancer Survivor Study, the standardized incidence ratio for development of a subsequent glioma was 10.8 (95% CI, 8.5 to 13.6),[126] and in the CCSS cohort it was 8.7 (95% CI, 6.2 to 11.6).[41] Higher standardized incidence ratios have been reported for meningioma,[122] and meningioma is the most common subsequent CNS tumor 10 years or more after a childhood cancer diagnosis.[41,124] There is an established dose-response relationship between cranial radiation dose and incidence of secondary CNS tumors. In the CCSS cohort, Neglia and colleagues reported an excess relative risk of 1.06 per Gy of cranial irradiation for meningiomas and 0.3 per Gy for gliomas.[41] In the British Childhood Cancer Survivor Study cohort, Taylor and colleagues reported that the risk for glioma and for meningioma each increased linearly with increasing radiation dose.[126] Several studies have suggested that the increased risk for development of meningioma does not appear to plateau.[118,123,127] In a report from the CCSS, among survivors with no previous diagnosis of meningioma at 25 years after a primary CNS tumor, the cumulative incidence of meningioma at 30 years was 3.5% (95% CI, 0.9% to 6.1%).[123] The contribution of chemotherapy to the risk for CNS malignancies is controversial. Laboratory investigations have suggested an association between radiation-induced brain tumors and thiopurine-based chemotherapy related to a deficiency in the enzyme thiopurine methyltransferase (TPMT).[128] In the British Childhood Cancer Survivor Study cohort, a higher cumulative dose of intrathecal methotrexate was associated with the development of meningioma.[126] In the CCSS cohort, after adjusting for radiation dose, chemotherapy exposure did not alter the survivor's secondary brain tumor risk.[41]

The role of routine surveillance for secondary CNS tumors with neuroimaging in asymptomatic childhood cancer survivors requires further investigation. Single-institution series of neuroimaging screening have reported high rates of meningioma.[127,129] For example, in a prospective study of 76 survivors of acute lymphoblastic leukemia that was treated with cranial irradiation and who were screened with MRI every 3 to 6 years for 20 years, Goshen and colleagues detected 16 meningiomas (15 asymptomatic) and one glioma.[127] Cumulative incidence in this screened population was 14.8% at 20 years.[127] Surgical resection of these secondary CNS tumors was recommended for 12 of the 16 survivors, with one surgical complication reported.[127] Whether early detection of meningiomas in asymptomatic survivors might reduce morbidity and minimize neurologic complications by facilitating surgical intervention remains to be determined. Survivors with a prior history of cranial irradiation should be educated about the potential for late-occurring CNS tumors and instructed to report new or persistent neurologic signs or symptoms (see Table 72-2).

Other Cancers

Secondary carcinomas of the gastrointestinal tract, genitourinary organs, lung, and head and neck region are observed in survivors of childhood cancer and are associated with radiation exposure and longer follow-up times. In general, irradiation-associated carcinomas (like colon cancers) occur at an earlier age than is seen in the general population, thereby supporting recommendations for early initiation of cancer screening and aggressive evaluation of symptoms suggestive of possible secondary malignancy.

Large cohorts of childhood cancer survivors report relative risks for gastrointestinal cancers between 4.6 and 7.0.[33,49,67,68,72,73] A European cooperative cohort study found the risk for secondary gastrointestinal malignancies greatest after abdominal radiation dose greater than 30 Gy (SIR, 9.7); however, lower-dose radiation was also associated with increased risk (10 to 29 Gy, SIR 5.2).[72] Alkylating agent chemotherapy is consistently found to be independently associated with an increased risk for gastrointestinal cancer.[72,73,130] The most common irradiation-associated secondary gastrointestinal malignancy is colorectal cancer, with a standardized incidence ratio of 3.6 to 13 and as high as 63.4 (95%

CI, 15.7 to 71.8) reported in a cohort of Hodgkin lymphoma survivors.[68,72,73,130] In a British cohort of 18,000 childhood cancer survivors, the 50-year cumulative incidence of colorectal cancer for those treated with direct abdominal radiation was 1.4% (95% CI, 0.7 to 2.6).[33] The St. Jude Children's Research Hospital cohort of over 13,000 childhood cancer survivors found a 40-year cumulative incidence of secondary colorectal cancer of 1.4% ± 0.53%).[130] In this study, the risk for colorectal cancer increased by 70% with each 10-Gy increase in radiation dose and increasing radiation volume also significantly increased risk. Current recommendations for screening of survivors treated with abdominal radiation therapy are to begin colon cancer screening earlier than what is recommended for the general population (10 years after completing radiation therapy or age 35 years, whichever comes last), although the benefit of early surveillance has not be established in this high-risk population (see Table 72-2).[89]

Of note, gastric cancers and esophageal cancers are also observed in greater numbers than expected for age in survivors of pediatric Hodgkin lymphoma.[68,85] Common symptoms such as dysphagia and dyspepsia should be evaluated aggressively, with a low threshold for utilizing endoscopy or imaging to rule out malignant or premalignant conditions in exposed survivors.

Overall, the relative risk for genitourinary cancer is modestly elevated (SIR, 1.9; 95% CI, 1.6 to 2.4),[33] with elevated relative risks for secondary malignancies of the female genital tract (SIR, 2.1; 95% CI, 1.4 to 3.2),[51] secondary renal cell carcinoma (SIR, 8.0; 95% CI, 5.2 to 11.7),[131] and secondary bladder cancer (SIR, 5.1; 95% CI, 2.1 to 12.4).[67] Data from the CCSS show the risks for secondary renal carcinoma are primary diagnosis of neuroblastoma (SIR, 85.8; 95% CI, 38.4% to 175.2%), renal-directed radiation therapy of 5 Gy or greater (RR, 3.8; 95% CI, 1.6 to 9.3), and platinum-based chemotherapy (RR, 3.5; 95% CI, 1.0 to 11.2).[131] Secondary urinary bladder cancer is associated with both radiation therapy and cyclophosphamide chemotherapy, with the risk dependent on dose and duration of therapy.[67,132-135] An adult series of more than 6000 non-Hodgkin lymphoma survivors treated with cyclophosphamide reported 31 cases of bladder cancer (RR, 4), and no significantly increased risk was observed at doses less than 20 g/m^2.[133] In a British cohort of 18,000 childhood cancer survivors, 17 were diagnosed with bladder cancer (RR, 4); risk factors were diagnosis of hereditary retinoblastoma, treatment with chemotherapy, male gender, and longer follow-up.[135] In another series of adults treated with prolonged cyclophosphamide for nonmalignant disease, 5% developed secondary bladder cancer at a median of 8.5 years and all diagnoses were preceded by symptoms of microscopic hematuria.[136] Although the absolute excess risk for bladder cancer in childhood cancer survivors is low, routine monitoring with urinalysis of survivors exposed to pelvic radiation, large cumulative dose of cyclophosphamide, and those with hereditary retinoblastoma should be considered.

Cancer of lung and bronchus is diagnosed more commonly in childhood cancer survivors than the general population (SIR, 3.4; 95% CI, 1.9 to 6.1),[51] with greater risk observed in cohorts of pediatric Hodgkin lymphoma survivors (SIR, 5.1 to 27.2).[67,85] In a meta-analysis of secondary lung cancer in Hodgkin lymphoma survivors, the largest risk for secondary lung cancer was observed among patients diagnosed age 15 to 24 years (RR, 8.76; 95% CI, 4.55 to 16.89).[137] Treatment with alkylating agents and irradiation are both associated with increased lung cancer risk.[67] In addition, studies of adult survivors of Hodgkin lymphoma have shown that smoking increased lung cancer risk more than twentyfold. Aggressive smoking cessation interventions and routine surveillance with radiographic imaging have been suggested for this high-risk population of survivors, although the use of spiral computed tomography has not been studied in the survivors who had chest irradiation.

Survivors are also at increased risk for secondary head and neck carcinomas (SIR, 10.8 to 14.1), with mucoepidermoid carcinoma of the parotid gland the most frequently observed diagnosis.[51,67,68] In a CCSS analysis of secondary salivary gland carcinomas, the incidence was thirty-ninefold higher in survivors compared with the general population (95% CI, 25.4 to 57.8).[138] The analysis also demonstrated that risk increased linearly with radiation dose (excess RR, 0.36/Gy; 95% CI, 0.06 to 2.5).[138] Interestingly, modifiable health behaviors including cigarette smoking and alcohol intake were not associated with increased risk for head and neck cancers in childhood cancer survivors.[138]

Hematologic Malignancies

Childhood cancer survivors are at an increased risk for developing subsequent hematologic malignancies, particularly secondary acute myelogenous leukemia. Overall, the relative risk for secondary acute leukemia in childhood cancer survivors is reported from 7 to 20.[23,24,48,49,68,77,139,140] An increased risk for secondary leukemia is described after treatment of a primary solid tumor[139] and after treatment of acute lymphoblastic leukemia, particularly with exposure to a topoisomerase II inhibitor.[141] In an analysis of survivors of cancer diagnosed when younger than 20 years of age, utilizing the Surveillance, Epidemiology, and End Results (SEER)-9 database, the cumulative incidence of secondary hematologic malignancy at 5 years was 0.2% (± 0.03%) and at 10 years it was 0.4% (± 0.04).[142] In contrast to irradiation-associated malignancies, the latency for leukemogenesis associated with chemotherapy is within the first 10 years after treatment, with the greatest risk observed in the first 5 years off therapy. In a study of 4202 survivors of childhood solid tumors, the relative risk of leukemia was 20 at 3 to 5 years and then decreased to 2.2 by 10 years off therapy.[139] Of interest, in this same study a second peak in secondary leukemia was observed at 20 years or more of follow-up (RR, 14.8); this finding has not been observed in studies with shorter follow-up periods.[24,48,62]

Acute leukemias and myelodysplasia are associated with exposure to high cumulative doses of alkylating agents and topoisomerase II inhibitors, including epipodophyllotoxins and anthracyclines. The alkylating agents presenting the greatest concern for secondary leukemia risk are melphalan and mechlorethamine, when compared with cyclophosphamide and ifosfamide.[23,25,143] Secondary leukemias induced by exposure to high-dose alkylating agents are classically considered to be preceded by myelodysplasia, have a median latency of 4 to 6 years, are more often diagnosed in older children, and are often associated with losses or deletions of chromosome 5 or 7.[37] Higher cumulative dose of alkylating agents is associated with a greater risk for the development of secondary acute myelogenous leukemia.[144] Interestingly, more recent pediatric studies demonstrated lower overall rates of leukemia after alkylating agent exposure[139,145] than earlier studies,[23,24] likely reflecting the trend of treatment with lower cumulative doses of less leukemogenic alkylating agents in more modern cohorts.

Topoisomerase II–induced leukemias, on the other hand, typically do not have an indolent course, have a relatively short latency (median, 1 to 3 years), are diagnosed in younger children, and are associated with a balanced chromosomal translocation 11q23 and, less frequently, 21q22.[24,37,146,147] Some have reported an increased risk for secondary acute myelogenous leukemia with higher cumulative epipodophyllotoxin dose. For example, in a case-control study of 61 cases of secondary leukemia in childhood survivors, Le Deley and colleagues reported a greater than twentyfold increased risk for leukemia after exposure to greater than 6 g/m^2 of epipodophyllotoxins and a sevenfold increased risk after exposure to either 1.2 to 6 g/m^2 epipodophyllotoxins or greater than 170 mg/m^2 of anthracyclines compared with lower doses of these agents.[145] However, other investigators have not demonstrated a relationship between higher cumulative epipodophyllotoxin dose and the development of secondary acute myelogenous leukemia.[148,149] Schedule of administration of epipodophyllotoxins may contribute to the risk for secondary leukemia, because prolonged infusions (twice weekly, weekly) have been found to be an independent risk factor for development of leukemia.[148,150]

The contributory role of therapeutic doses of radiation in the development of secondary leukemia is debated. Although some studies find no association between leukemia and exposure to radiation therapy,[49,139,148] others studies have documented subsequent leukemia in survivors after exposure to radiation in the absence of chemotherapy.[24,37,139] In addition, specific host factors, such as polymorphisms of DNA repair genes, may contribute to susceptibility to secondary acute myelogenous leukemia.[144]

The prognosis of secondary acute myelogenous leukemia is generally considered to be poor.[37,146] In the SEER-9 analysis cited earlier, the 5-year overall survival for patients with secondary hematologic malignancies was 31% ± 4.7%.[142] In a report of 24 children with secondary myelodysplastic syndrome or acute myelogenous leukemia, those with secondary myelodysplastic syndrome or acute myelogenous leukemia had lower rates of remission with induction therapy and poorer overall and event-free survival when compared with patients with de novo acute myelogenous leukemia.[151] Further investigation is needed to determine the optimum strategies for preventing and treating secondary leukemia.

ORGAN SYSTEM LATE EFFECTS

Cardiovascular System

Cardiac disease is the leading cause of treatment-related morbidity and noncancer mortality observed in childhood cancer survivors.[152] In an analysis of late mortality among 5-year survivors of childhood cancer from the CCSS cohort, childhood cancer survivors were seven times more likely to die of cardiac-related events compared with expected population-based rates of cardiac-related deaths.[21] Indeed, childhood cancer survivors have been reported to be at significantly increased risk, compared with a sibling cohort, for the development of congestive heart failure (RR, 15.1; 95% CI, 4.8 to 47.9,), coronary artery disease (RR, 10.4; 95% CI, 4.1 to 25.9), and cerebrovascular accidents (RR, 9.3; 95% CI, 4.1 to 21.2).[46] The treatments most often associated with cardiac morbidity and mortality are anthracycline chemotherapy and cardiac irradiation.[21] A recent cohort study assessing the long-term risk for clinically validated, symptomatic cardiac events in 5-year survivors of childhood cancer demonstrated that higher cumulative anthracycline dose or higher cumulative radiation dose was associated with higher risk ratio for cardiac events. Survivors with both anthracycline and cardiac irradiation exposures had the highest cumulative incidence of cardiac events, with nearly one in eight survivors treated with both exposures developing a serious cardiac event.[36] Risk factors for late onset anthracycline cardiotoxicity (occurring 1 year beyond completion of therapy) have been assessed in multiple studies and include higher cumulative anthracycline dose, history of acute cardiac toxicity, combined exposure with other cardiotoxic therapy, female sex, younger age at exposure (<5 years old), and longer duration of follow-up.[42,153-158] Irradiation of the heart as a consequence of irradiation to the chest for treatment of childhood cancer can cause both functional and anatomic cardiac disease. Mantle field, whole-lung fields, left- or whole-abdominal irradiation, spinal irradiation, and total-body irradiation all put a survivor at risk for cardiac late effects.[155,159-162] Irradiation-associated cardiac late effects include constrictive pericarditis,[163] myocardial fibrosis,[164] cardiomyopathy, coronary artery disease, valvular abnormalities, conduction disturbances, and vascular disease.[43,155,159] Similar to other irradiation-associated late effects, cardiac toxicity is observed more frequently in those survivors treated at the youngest age, receiving higher radiation dose volumes to the heart, and followed the longest.[159,160]

Nonanthracycline chemotherapeutic agents, including cyclophosphamide, cytarabine, cisplatin, and ifosfamide, have also been associated with cardiotoxicity.[152] In addition, children and adolescents are increasingly being treated with newer agents with unclear cardiac risks. For example, tyrosine kinase inhibitors (imatinib mesylate, sorafenib, and sunitinib) have been associated with cardiac toxicity with exposure in adulthood.[165,166] Cardiac risks with prolonged exposures to newer agents in large cohorts of pediatric patients are yet to be defined.

The spectrum and nature of cardiac late effects experienced by childhood cancer survivors suggest a need for regular screening with one or more cardiac imaging modalities. Risk-based screening recommendations for childhood cancer survivors exposed to cardiotoxic therapies have been published by the Children's Oncology Group as part of the "Late Effect Guidelines" as well as by other groups.[89,167,168] However, these guidelines have not been tested for efficacy in terms of reduction of cardiac mortality or morbidity. Survivors exposed to cardiotoxic therapy should be informed about their risk for cardiac disease and should also be made aware of activities that present potential stressors to already impaired cardiac function, including smoking, pregnancy, and anesthesia.

Cardiomyopathy

Cardiomyopathies diagnosed in childhood cancer survivors after completion of therapy are associated with both anthracycline chemotherapy and cardiac irradiation. Survivors are at risk for both systolic and diastolic left ventricular dysfunction, which can lead to clinical heart failure.[169-171] Anthracycline-associated cardiotoxicity can be classified as acute, early onset (<1 year after anthracycline exposure), or late onset (>1 year after anthracycline exposure),[158] although it is not clear to what extent these categories reflect differences in underlying pathophysiology.[172] Anthracyclines are considered to be directly toxic to cardiac myocytes, with exposure resulting in reduced myocyte mass and impaired function of cardiac muscle. Subclinical anthracycline cardiotoxicity is measured as diminished left ventricular contractility and a thinning of the left ventricular wall. Thinning of ventricular walls causes elevated wall stress, which increases afterload, and ultimately can progress to symptomatic left ventricular failure. In children, loss of cardiac myocytes at a young age can result in impaired cardiac growth and function relative to somatic growth, which contributes to the observed decrease in left ventricular systolic function.[42,173] Anthracycline-associated late cardiac toxicity can manifest as asymptomatic cardiac dysfunction detected by echocardiogram or other modalities or symptomatic dysfunction and the classic signs of heart failure.[155,156] Longitudinal and long-term follow-up data support that cardiac abnormalities diagnosed in childhood cancer survivors treated with anthracyclines are persistent and progressive.[174,175] In a Dutch cohort of more than 800 anthracycline-exposed childhood survivors, the cumulative incidence of anthracycline-induced clinical heart failure increased with the duration of follow-up; there was a cumulative incidence of 2.6% at 10 years and 5.5% at 20 years, with no evidence of plateau in incidence with longer duration of follow-up.[157] Of further concern, the incidence of asymptomatic left ventricular dysfunction detected by cardiac screening is reported to be as high as 50% to 60%.[153,176] However, the long-term clinical implications of subtle echocardiographic abnormalities in asymptomatic individuals are not fully determined.

Many risk factors for anthracycline-associated cardiomyopathy have been identified. The major risk factor for anthracycline cardiomyopathy is cumulative dose ("doxorubicin equivalent"). Equivalent dose takes into consideration exposure to different anthracyclines and can be calculated using conversion factors (Table 72-3). Survivors exposed to cumulative doses of doxorubicin equivalents greater than 250 to 300 mg/m^2 appear to be at greatest risk for cardiomyopathy.[19,42,157,175,177,178] The dose-dependent relationship between anthracyclines and subsequent congestive heart failure was demonstrated in the Dutch cohort, where the greatest risk for congestive heart failure was at a dose greater than 300 mg/m^2 (RR, 8), and no congestive heart failure was observed at doses less than 150 mg/m^2.[157] However, others have shown that survivors treated with low cumulative dose (45 mg/m^2) also have asymptomatic cardiac abnormalities detectable on an echocardiogram,[174] suggesting that exposure to anthracycline at any dose poses potential risk for late cardiotoxicity.

TABLE 72-3 Calculation of Anthracycline (Doxorubicin) Isotoxic Dose Equivalents*

Anthracycline	Conversion Factor
Doxorubicin	Total dose (mg/m^2) × 1
Daunorubicin	Total dose (mg/m^2) × 1
Mitoxantrone	Total dose (mg/m^2) × 4
Idarubicin	Total dose (mg/m^2) × 5
Epirubicin	Total dose (mg/m^2) × 0.67

From the Children's Oncology Group long-term follow-up guidelines for survivors of childhood, adolescent, and young adult cancers, *Version 4.0. Accessed at www.survivorshipguidelines.org, October 2013.*

*Estimation of anthracycline isotoxic dose equivalents for use in calculation of total cumulative anthracycline dose.

Additional reported risk factors for the development of anthracycline-associated cardiomyopathy include history of acute cardiac toxicity during treatment, combined exposure with other cardiotoxic therapy, female sex, younger age at exposure (<5 years of age), and longer duration of follow-up.[42,153-157] Cardiotoxicity diagnosed during or within 1 year of completion of therapy is considered a risk factor for late cardiotoxicity.[152] Furthermore, survivors exposed to anthracycline therapy and concurrent mediastinal radiation are considered to be at risk for cardiomyopathy at lower total anthracycline dose.[160] Increasingly, genetic variants that may predispose to anthracycline cardiotoxicity are being explored.[177,179] A recent evaluation from the Children's Oncology Group demonstrated that patients with *CBR3* V244M homozygous G genotype with low- to moderate-dose anthracycline exposure were at an increased risk for cardiomyopathy.[177]

Interventions aimed at preventing or reducing long-term anthracycline-associated cardiotoxicity in childhood cancer survivors include limiting anthracycline doses in most therapeutic regimens to less than 300 mg/m^2, adding cardioprotectants such as dexrazoxane to anthracycline-based regimens, and prescribing afterload-reducing agents to prevent progression of asymptomatic dysfunction to overt heart failure. Data from clinical trials in childhood ALL show that the addition of cardioprotectants did not adversely affect survival.[180] Although an early analysis of a Pediatric Oncology Group clinical trial for Hodgkin lymphoma, which randomized the use of dexrazoxane as a cardioprotectant, demonstrated a possible increase in second cancers with dexrazoxane exposure,[181] a larger cohort of survivors of childhood acute lymphoblastic leukemia treated with dexrazoxane did not demonstrate an increased risk for second cancers.[182] Childhood cancer survivors diagnosed with anthracycline-associated left ventricular dysfunction have been treated with angiotension-converting enzyme inhibitors with the aim of reducing afterload and preventing the progression of left ventricular dysfunction to heart failure. In a longitudinal study of 18 survivors with left ventricular dysfunction, treated with enalapril and followed for a median of 10 years, all initially showed improvement in left ventricular function. The effects, however, were transient, lasting 6 to 10 years, followed by deterioration of left ventricular function over time.[183] However, given results from large studies of medical interventions for patients with asymptomatic left ventricular dysfunction (who have not received anthracyclines), as well as data from adult cancer patients,[184,185] many cardiologists who evaluate and treat adults with anthracycline-induced cardiomyopathy will intervene with angiotensin-converting enzyme inhibitors. Future investigations should be directed at testing interventions to prevent cardiac injury during therapy and to prevent progression of asymptomatic left ventricular dysfunction to overt cardiac disease in survivors.

Irradiation-associated cardiomyopathy is most often restrictive, with impaired diastolic function, although systolic dysfunction may also be observed.[163,164] Radiation is believed to induce injury to the myocardial microvasculature, which in turn causes myocardial ischemia, and ultimately myocardial fibrosis, resulting in diastolic dysfunction.[37] Studies have demonstrated a significant incidence of diastolic dysfunction in asymptomatic patients who undergo radiation therapy.[159,186,187] In a small cohort of Hodgkin lymphoma survivors treated with a median mediastinal dose of 35 Gy, evaluated at a mean of 9 years after therapy, 20% had evidence of diastolic dysfunction on cardiac imaging.[186] The extent to which these changes are progressive, and whether subclinical dysfunction will result in clinical heart failure over time, is unknown. As noted earlier, there is a significantly increased risk for cardiotoxicity with combined exposure to both anthracyclines and irradiation.[36,159,188]

Screening for cardiomyopathy with serial echocardiograms is currently recommended for survivors treated with anthracyclines and/or thoracic radiation therapy, with the frequency of echocardiogram screening depending on the individual survivor's risk for cardiomyopathy as determined by age at exposure, cumulative treatment dose, and concurrent treatment with thoracic irradiation.[89] The use of newer modalities for screening, such as cardiac MRI, is an area of active investigation.[189] The extent to which screening will result in a reduction in cardiac morbidity and mortality in survivors remains unknown.

Coronary Artery Disease

Survivors treated with thoracic irradiation are at increased risk for the development of radiation-associated coronary artery disease (CAD), which is an established cause of early mortality.[109,190] Radiation injury to the coronary arteries is considered to be caused by acute endothelial damage followed by subsequent initiation or acceleration of atherosclerosis.[152,160] Irradiation-associated coronary artery stenosis is most common in the proximal coronary arteries, which are not necessarily shielded during cardiac blocking.[191] In a historic cohort, childhood Hodgkin lymphoma survivors treated with more than 40 Gy of mediastinal radiation had a fortyfold excess risk for fatal myocardial infarction compared with an age-matched general population.[192] A report on 314 Hodgkin lymphoma survivors treated at age younger than 20 years found a fivefold increased risk for myocardial infarction at a median of 19 years after treatment,[188] and a British cohort that included 2158 Hodgkin lymphoma survivors treated at age younger than 25 years found a nineteenfold increased risk for death from myocardial infarction.[32] A recent study assessing the long-term risk for clinically validated, symptomatic cardiac events in 5-year survivors of childhood cancer demonstrated a significantly increased 30-year cause-specific cumulative incidence of cardiac ischemia for survivors treated with cardiac irradiation compared with survivors treated without cardiac irradiation (2.2% [95% CI, 0% to 4.7%] vs. 0.3% [95% CI, 0% to 0.8%], $P = .01$)].[36] Modern irradiation techniques are designed to reduce radiation to the coronary arteries, although reduction in irradiation-associated CAD has not yet been proven.

The risk for asymptomatic CAD in survivors is described in many studies; however, optimal screening and interventions to detect and treat irradiation-associated cardiovascular disease have not been determined. Survivors at risk for irradiation-associated CAD are advised to adopt preventive measures commonly used to reduce the risk for atherosclerotic CAD in the general population (aggressive treatment of hypertension, diabetes, obesity, dyslipidemias). Although the efficacy of such measures is not proven in this population, at least one cardiac risk factor was present in all survivors who developed CAD in a single-institution study of 415 Hodgkin lymphoma survivors[43]; these data suggest that standard cardiovascular risk–reducing strategies might be beneficial to survivors.

Stress echocardiograms can be used as a noninvasive method to detect asymptomatic ischemic heart disease by observing stress-induced wall motion abnormalities. Noninvasive nuclear scintigraphy has also been considered to screen survivors for asymptomatic CAD. A report of screening 294 asymptomatic Hodgkin lymphoma survivors treated at a median age of 25 years with more than 35 Gy found 20% had electrocardiographic findings consistent with prior ischemia and 14% had abnormal stress echocardiograms or stress-induced perfusion defects by nuclear scintography.[193] The survivors with abnormal nuclear perfusion studies who went on to cardiac catheterization were diagnosed with irradiation-induced microvascular damage in the myocardium that was not amendable to surgical intervention.[193] This study showed that the increased sensitivity of adding perfusion scanning in addition to stress echocardiography was at the expense of decreased specificity, with false-positive detection of disease not amendable to intervention. This calls into question the benefit of adding nuclear studies to routine screening of asymptomatic survivors for CAD. The roles of modalities such as CT angiography, MR angiography, and coronary artery calcium scores in the childhood cancer survivor population are yet to be established.[152,194]

Surgical treatment of CAD in survivors with prior radiation exposure can be complicated by mediastinal fibrosis, myocardial fibrosis, and the coexistence of other irradiation-associated morbidities, including valvular disease, conduction abnormalities, and pulmonary dysfunction.[195] In a recent report from Wu and colleagues, adults with irradiation-associated heart disease undergoing cardiothoracic surgery had significantly higher mortality compared with a matched control population undergoing similar procedures.[196] The high surgical risk in patients with irradiation-associated heart disease should be carefully considered when planning surgical interventions.

Arrhythmias

A full spectrum of cardiac arrhythmias and conduction disturbances have been reported in childhood cancer survivors treated with both irradiation and anthracyclines. These include atrioventricular nodal bradycardia, sick-sinus syndrome, complete and incomplete heart block, prolonged QT interval, and serious ectopy (ventricular tachycardia).[197] In a series of 48 survivors of pediatric Hodgkin lymphoma treated with mediastinal irradiation (median dose, 40 Gy), 75% were found to have conduction abnormalities.[198] The most frequent conduction disturbance was right anterior bundle branch block, the most anterior structure in the conduction system.[198] These arrhythmias are rarely symptomatic; however, the clinical concern is that conduction abnormalities may be progressive.[197,198] Because conduction abnormalities typically are asymptomatic, they usually are detected in association with other irradiation-induced cardiac abnormalities.[197,198]

Valvular Disease

The mitral, aortic, and tricuspid valves can all be affected by irradiation-associated fibrous endocardial thickening.[199] Valvular insufficiency is more common than valvular stenosis, but stenosis tends to be more severe, requiring intervention.[43,199,200] The frequency of left-sided valvular regurgitation is reported to be 16% to 24% in chest-irradiated survivors of Hodgkin lymphoma screened by echocardiography at a median of 10 years, and valvular dysfunction was not observed at a radiation dose less than 30 Gy.[200,201] In a cohort of 48 long-term survivors of pediatric Hodgkin lymphoma (median age at diagnosis, 16 years; median dose, 40 Gy), left-sided regurgitation was noted in 36% and valvular defects were detected in 42%.[198] In a recent report, the 30-year cause specific cumulative incidence of symptomatic valvular disease in survivors treated with cardiac irradiation was 2.5% (95% CI, 0% to 5.0%), compared with 0.1% (95% CI, 0% to 0.3%) for those treated without cardiac irradiation.[36] Valvular defects can be progressive, which is of particular concern for young survivors.[199] In a single-institution series of 415 survivors of Hodgkin lymphoma who received mediastinal irradiation, clinically significant valvular dysfunction developed in 6.2% at a median of 22 years.[43] In this cohort, aortic stenosis was the most common clinically significant valve lesion and when results were compared with those of an age-matched general population the relative risk for needing valve surgery was eightfold higher in survivors. The only treatment risk identified for significant valvular disease in this cohort was irradiation technique that resulted in a higher total dose to the heart.[43] Symptoms of valvular heart disease are the same in survivors as the general population, and valve function can be monitored by routine echocardiographic screening.

Pericarditis

The incidence of late-occurring irradiation-associated pericarditis has been significantly reduced by modern irradiation techniques.[202,203] In historic cohorts of children treated with orthovoltage mediastinal irradiation, 7% to 15% developed symptomatic constrictive pericarditis,[203,204] compared with 0% to 2.5% of children treated with megavoltage therapy.[203,205,206] Although the incidence of pericarditis has been significantly reduced in recent cohorts, older childhood cancer survivors continue to be at risk for pericarditis. Presenting signs and symptoms include pleuritic chest pain, shortness of breath, a friction rub, and electrocardiographic changes. A survivor presenting with symptomatic pericarditis is at risk for progressing to cardiac tamponade.[198] Management includes pericardiocentesis and pericardiectomy,[203] which are high-risk procedures in this patient population with known risks for other irradiation-associated cardiac morbidities.

Cerebrovascular Disease

Survivors treated with irradiation to the head and neck are at risk for the development of clinically significant premature cerebrovascular disease. Irradiation-induced cerebrovascular disease can encompass numerous abnormalities, such as moyamoya disease, vascular malformations including venous-based cavernous malformations (cavernomas) or intracranial aneurysms, and extracranial internal carotid stenosis.[207] Abnormalities such as cavernous malformations may be asymptomatic and noted incidentally on follow-up imaging or may present as neurologic symptoms such seizures, headache, or with hemorrhage, potentially necessitating surgical interventions.[208] Intracranial aneurysms can be life threatening.[209] There are limited long-term data on the risk for late-occurring cerebrovascular disease in survivors of childhood cancer. Data from the CCSS, based on self-report, demonstrated an increased risk for stroke in survivors of brain tumors (RR, 29) and of leukemia (RR, 6.4) when compared with sibling controls, and risk for stroke was associated with a mean cranial irradiation dose greater than or equal to 30 Gy. In addition, among leukemia and brain tumor survivors who did not receive cranial radiation therapy, the risk for stroke remained significantly increased when compared with siblings.[210] CCSS data also demonstrate an increased risk for stroke among survivors of Hodgkin lymphoma (RR, 4.32), with mantle irradiation strongly associated with subsequent stroke.[211] Importantly, in the CCSS cohort, smoking was associated with a threefold excess risk for stroke in survivors of Hodgkin lymphoma. In a study investigating cerebrovascular mortality in a French/United Kingdom cohort of 4227 five-year survivors of childhood cancer, at a median follow-up 29 years after radiation to the brain, the risk for death from cerebrovascular disease increased linearly with local radiation dose to the prepontine cistern. Survivors who received greater than 50 Gy to the prepontine cistern had a notably increased risk for death from cerebrovascular disease (RR, 17.8).[212]

Risk-based surveillance for cerebrovascular disease currently includes careful assessment by history and physical examination for neurologic and neurocognitive signs and symptoms. Neuroimaging is indicated for diagnostic assessment in symptomatic patients. The Children's Oncology Group Guidelines recommend consideration of baseline Doppler ultrasonography of carotid vessels 10 years after radiation therapy to the neck in those with radiation exposure of more than 40 Gy.[89] In a report on 30 childhood cancer survivors who received neck irradiation, Meeske and colleagues noted an increase in carotid disease on carotid ultrasound assessment compared with healthy control subjects, including in those treated with lower doses of radiation (18 to 30 Gy).[213] Further investigations are needed to better define risks and to inform screening recommendations. Abstinence from smoking should be emphasized. Aggressive medical management of other risk factors associated with cerebrovascular disease in the general population should also be advised.

Modifiable Cardiovascular Risk Factors

As noted earlier, attention to modifiable cardiovascular risk factors, such as obesity, hypertension, diabetes mellitus, dyslipidemia, and smoking, may be particularly important in the care of survivors of childhood cancer. A recent assessment from the CCSS found that two or more cardiovascular risk factors were reported by 10.3% of survivors. In this report, acquisition of modifiable cardiovascular risk factors, particularly hypertension, increased the risk for severe, life-threatening, and fatal cardiac events.[214] These results reinforce the importance of early diagnosis and careful management of modifiable cardiovascular risk factors in survivors.

Pulmonary System

Pulmonary toxicities as a consequence of cancer-directed therapy can range in severity from asymptomatic abnormalities on pulmonary function testing to life-threatening disease. Childhood cancer survivors are at increased risk for death from pulmonary complications. In an analysis of late mortality among 5-year survivors from the CCSS cohort, childhood cancer survivors were 8.8 times more likely to die of a pulmonary event compared with the U.S. population (age-, calendar year-, and sex-matched).[21] Therapeutic exposures, including radiation therapy, certain chemotherapeutic agents (bleomycin, carmustine, lomustine, busulfan, and cyclophosphamide), thoracic surgery, and hematopoietic stem cell transplantation, are associated with late pulmonary complications.[215] Recognition of these toxicities informed changes in upfront cancer-directed therapies, such as the reduction or elimination of radiation therapy and bleomycin in the treatment of Hodgkin lymphoma.

The late effects of radiation therapy on the respiratory system include reduced lung volumes, pulmonary fibrosis, and decreased chest wall growth. Risk factors for radiation-induced pulmonary late effects include concurrent pulmonary toxic chemotherapy, lung radiation dose greater than 15 Gy, spinal irradiation, and total-body irradiation.[216-218] Data from the CCSS cohort showed an increased risk for pulmonary fibrosis (RR, 4.3), emphysema (RR, 2.0), abnormal chest wall development (RR, 5), and chronic pneumonia (RR, 2.2) in those survivors who had chest irradiation. At 20 years after diagnosis, chest irradiation was associated with a cumulative incidence of pulmonary fibrosis of 3.5%.[219]

Treatment with chemotherapy including bleomycin, busulfan, cyclophosphamide, and nitrosoureas is also associated with the development of pulmonary fibrosis.[215,220,221] In the CCSS cohort, an increased risk for pulmonary fibrosis was seen in those exposed to bleomycin (RR, 1.4), carmustine (RR, 1.6), and busulfan (RR, 2.1).[219] A plateau in cumulative incidence of pulmonary fibrosis was not observed up to 15 years after diagnosis in survivors treated with chemotherapy alone or in combination with radiation therapy.[219]

Current long-term follow-up guidelines include obtaining a baseline chest radiograph and pulmonary function testing, including spirometry and diffusing capacity, in survivors with significant risk factors for late pulmonary toxicity.[89] Attention to smoking abstinence and cessation is also recommended. Reports of worsening bleomycin-associated pulmonary fibrosis after exposure to 100% oxygen suggest that survivors receive a pulmonary evaluation before undergoing anesthesia and avoid situations with increased oxygen concentrations such as scuba diving.[222]

Endocrine System

Hypothalamic-Pituitary Dysfunction

Hypothalamic pituitary dysfunction is a potential late effect in childhood cancer survivors who undergo radiation therapy for CNS, orbital, facial, or nasopharyngeal cancers. CNS tumors of the optic pathway, hypothalamus, or suprasellar region can also cause long-term hypothalamic-pituitary dysfunction as a result of hypothalamic injury from tumor growth or surgical resection. The incidence of pituitary hormone deficiencies after cranial irradiation varies by radiation dose and other treatment factors, such as age at therapy and duration of follow-up.[223] The reported range in prevalence of anterior pituitary deficiencies after a median dose of 45 to 55 Gy cranial radiation is as follows: growth hormone (GH), 85% to 100%; TSH, 3% to 60%; luteinizing hormone/follicle-stimulating hormone (LH/FSH), 5% to 40%, and adrenocorticotropic hormone, 4% to 30%.[223] This variability is a result of differences in cohort composition as well as different measures used to define hormone deficiencies. The GH pathway is the most sensitive to radiation and, therefore, GH deficiency is the most likely manifestation of hypothalamic hypopituitarism in survivors treated with cranial irradiation. The pituitary-thyroid, gonadal, and adrenal axes are less sensitive to late onset radiation toxicity, and, as such, centrally mediated deficiencies of these hormones are usually not observed in survivors treated with cranial irradiation below the range of 35 Gy.[186,224-228]

Growth Hormone Deficiency. The severity and latency of GH deficiency diagnosed in survivors is related to cranial irradiation dose. GH deficiency can be observed after doses as low as 18 to 24 Gy with late onset typically more than 10 years after therapy, whereas a cranial dose of more than 30 Gy can result in GH deficiency within 5 years after treatment.[229] This suggests that after a dose threshold of 18 to 25 Gy the incidence of GH deficiency increases with increased period of follow-up.[230,231] Clinical features of GH deficiency in survivors who have cranial irradiation can include poor growth, increased fat mass, reduction in lean body mass, decreased strength and exercise tolerance, adverse lipid profile, and decreased bone mineral density (BMD). These clinical features can be improved by GH replacement.[232] However, the use of GH replacement in GH-deficient survivors after they obtain final height is controversial, with more to be determined regarding the long-term benefits, safety, and risks for GH replacement in GH-deficient survivors in adulthood.[230,233]

The safety of GH replacement in childhood cancer survivors with regard to the risk for cancer recurrence or the development of a second primary cancer is controversial. Several studies report a lack of association between history of treatment with GH and primary cancer recurrence.[234-237] In a report from the CCSS, there was no documented increased risk for primary cancer recurrence in GH-treated survivors. However, exposure to GH was associated with an increased risk for second neoplasms (RR, 3.21; 95% CI, 1.88 to 5.46). An updated report from the CCSS continued to demonstrate this association (RR, 2.15; 95% CI, 1.33 to 3.47).[235,238] The association between development of second cancers and GH treatment is based on relatively few events and could be confounded by ascertainment bias (GH-treated patients could have had increased surveillance) and suggests the need for further study. The frequency and duration of screening for GH deficiency in childhood cancer survivors, the long-term effects and safety of GH replacement therapy, and the utility of long-term replacement therapy beyond attainment of final height require further investigation.

Skeletal Toxicities

Skeletal toxicities, including low BMD and osteonecrosis are increasingly recognized potential complications of therapy for childhood cancer. In children and adolescents undergoing treatment for cancer, acquisition of peak bone mass may be compromised. Cancer-directed treatment exposures, nutritional deficiencies, or physical inactivity can all contribute to decreased BMD in the setting of treatment of childhood cancer.[239] Risk factors for low BMD identified in cohorts of childhood cancer survivors include prior treatment with corticosteroids, methotrexate, cranial irradiation, and total-body irradiation. Survivors with hypogonadism, nutritional deficiencies, and prolonged immobilization or physical inactivity are also at risk for low BMD.[240-243] In the general population, osteoporosis-associated fractures result in significant morbidity and mortality; however, the association between low BMD and fracture risk has not been established for childhood cancer survivors. A report from the CCSS did not note an increased prevalence of fracture in adult survivors of childhood cancer compared with siblings[244]; however, the risks for long-term skeletal morbidity with advancing age remain to be defined.

Baseline bone density evaluation has been advised upon entry into survivorship care for those with treatment exposures associated with low BMD.[89] Dual-energy x-ray absorptiometry (DXA) is a commonly used modality that provides quantitative measurement of bone mineral content and indirect calculation of BMD. DXA has well-established normative pediatric values. However, as a two-dimensional technique, DXA has limitations and cannot provide direct volumetric measurements of BMD and is confounded by bone size.[245] Use of other modalities such as quantitative computed tomography (QCT) has been largely investigational.[246] Nutritional counseling to ensure sufficient intake of calcium and vitamin D in the diet, and supplementation in the setting of inadequate intake, has been recommended.[239] Management of conditions associated with decreased BMD, such as GH deficiency and hypogonadism, could potentially enhance bone health; however, the overall potential risks and benefits of such interventions with limited available data on long-term bone health outcomes must be considered.

Childhood cancer survivors are at increased risk for the development of osteonecrosis (also referred to as avascular necrosis). Osteonecrosis typically effects weight-bearing joints and can result in pain, loss of function, and joint collapse requiring surgical intervention including joint replacement. Cancer treatment exposures associated with the development of osteonecrosis include glucocorticoid therapy and irradiation,[247-249] with several studies indicating that exposure to dexamethasone is associated with a greater risk for osteonecrosis than exposure to prednisone.[249,250] Treatment as an adolescent has consistently been identified as a risk factor for the development of osteonecrosis.[249,250] Osteonecrosis has been identified as an acute toxicity of cancer therapy, with potential long-term impact on the quality of life of survivors. In the CCSS cohort, the 20-year cumulative incidence of osteonecrosis was 0.43%, with survivors 5.6 times more likely to have reported osteonecrosis compared with siblings. The risk for osteonecrosis was greatest among survivors of allogeneic stem cell transplant for underlying diagnoses of leukemia and in survivors of acute lymphoblastic leukemia, acute myelogenous leukemia, and bone sarcoma.[249] In this study, the cumulative incidence of osteonecrosis increased with time after treatment. Whether this reflects the worsening of previously asymptomatic bony lesions or a continued risk for the development of new lesions over time is unclear. Indeed, data are limited on the longitudinal outcomes of those diagnosed with osteonecrosis and more remains to be determined about the risk for late-occurring osteonecrosis.

In addition, bone and soft tissue in treatment fields are vulnerable to irradiation-induced hypoplasia and fibrosis, resulting in reduced or uneven skeletal growth. Survivors treated with irradiation that included the spine (>12 Gy) and long bones are at risk for scoliosis, shortened trunk height, and growth asymmetry.[251,252]

Disorders of Glucose Metabolism

Survivors of childhood cancer appear to be at increased risk for the development of insulin resistance and diabetes mellitus (DM). In a cross-sectional evaluation of survivors of childhood acute lymphoblastic leukemia, Oeffinger and colleagues noted that survivors of this type of leukemia treated with and without cranial irradiation had an increased prevalence of insulin resistance when compared with a cohort composed of older individuals from the same community.[253] In a report from the CCSS, the prevalence of DM was almost twice as high in childhood cancer survivors compared with siblings, with an increased risk for DM associated with total-body irradiation (OR, 7.2; 95% CI, 3.4 to 15; P <.001), abdominal irradiation (OR, 2.7; 95% CI, 1.9 to 3.8; P <.001), and younger age at diagnosis.[254] This increased risk for DM with abdominal radiation exposure in this cohort appeared to be independent of body mass index (BMI). Survivors of acute lymphoblastic leukemia who had cranial irradiation were 1.6 times as likely as siblings to report DM (after adjustment for BMI). [254] Survivors treated with total-body irradiation or with abdominal irradiation have been reported to have an increased risk for DM in several recent reports.[254-257] A report of 2520 survivors from France and the United Kingdom demonstrated a dose-response relationship between irradiation to the tail of the pancreas, where beta cells are concentrated, and risk for DM (RR of DM, 11.5% [95% CI 3.9 to 34] with 10 Gy or more to the tail of the pancreas).[257] Irradiation to other part of the pancreas was not associated with an increased risk for DM in this study. Further investigation of the mechanisms of development of DM and the interplay between treatment-related and modifiable risk factors in survivors is needed. Screening for DM should be considered in survivors, with implementation of aggressive risk-reduction strategies as clinically indicated.

Thyroid Disorders

The thyroid gland is extremely sensitive to radiation toxicity; subsequently, thyroid abnormalities are among the most common irradiation-induced morbidities after childhood cancer therapy. Childhood cancer survivors exposed to thyroid radiation as a consequence of direct neck irradiation for Hodgkin lymphoma, sarcomas, nasopharyngeal or oropharyngeal carcinomas, and other tumors are at risk for thyroid abnormalities. The clinical manifestations of thyroid irradiation injury include hypothyroidism, hyperthyroidism, thyroid nodules, and thyroid cancer. Because even low doses and scatter radiation can damage thyroid tissue, survivors who received seemingly distant irradiation such as whole-lung irradiation, craniospinal irradiation, and total-body irradiation for bone marrow transplant are also at risk for thyroid abnormalities. In addition, neuroblastoma survivors treated with ^{131}I-MIBG are at risk for hypothyroidism.[92,258] Although coadministration of potassium iodide is considered protective, a single-institution follow-up study of 16 survivors, a median 15 years after treatment, found 8 of 16 with clinical hypothyroidism. Primary hypothyroidism (low thyroxine [T_4] level with elevated TSH or compensated normal T_4 with elevated TSH) is the most common thyroid abnormality observed after thyroid irradiation.[93] Survivors in the CCSS cohort had a seventeenfold increased risk for hypothyroidism, compared with siblings.[93] The incidence of hypothyroidism increases with increasing radiation dose to the thyroid; at 20 years after treatment with 35 to 44.99 Gy the cumulative incidence is 35%, and with 45 Gy or more the cumulative incidence is 50%.[93] Risk factors identified for hypothyroidism are larger dose of radiation, older age at diagnosis, and female gender.[93] Although the greatest risk for hypothyroidism occurs the first 5 years after treatment, no plateau for thyroid disease has been observed and cumulative incidence continues to rise beyond 20 years of follow-up.[93] Central hypothyroidism (low TSH, low T_4) can be observed as a late toxicity of cranial irradiation to the hypothalamic pituitary axis, although centrally mediated deficiencies are usually not observed in survivors treated with cranial irradiation below 35 Gy.[224,226,229] Hyperthyroidism is an uncommon late effect and is sometimes observed after high-dose thyroid irradiation greater than 35 Gy.[93] Irradiation-induced thyroid nodules are commonly detected in survivors treated with head and neck irradiation. A recent cohort study of over 3000 survivors reported the cumulative incidence of irradiation-associated thyroid adenomas as 5.5% (95% CI, 4.2 to 7.2) at 40 years of follow-up.[259] A small cohort study of Hodgkin lymphoma survivors reported the median latency of thyroid nodules as 10 years (range, 1 to 25 years) after therapy, and in this study only 10% of nodules detected by physical examination or screening ultrasonography were malignant.[107]

Recommended screening for survivors at risk for irradiation-associated thyroid disease include annual palpation of the thyroid by a skilled examiner and annual measurements of serum thyroid hormones beginning 1 year after completion of therapy.[89,93] Thyroid replacement therapy is successful in treating irradiation-associated hypothyroidism. Treatment with thyroxine to suppress elevated TSH may reduce the frequency of benign nodules.[260] Routine ultrasound screening of asymptomatic survivors is controversial, because the majority of thyroid nodules detected in survivors have an indolent course and a minority of the nodules are malignant.[107] Survivors with palpable thyroid nodules are advised to undergo high-resolution thyroid ultrasonography with fine-needle aspiration of any large or suspicious nodules. Nodules with benign pathology can be followed by serial ultrasonography and resampled if there is interval change, and patients whose nodules that have indeterminate or malignant pathology are referred for thyroidectomy. Total thyroidectomy is often recommended to survivors because irradiation-associated thyroid cancers can be multicentric, the remaining thyroid is at risk for malignancy, and occult malignancy has been detected in nodules believed preoperatively to be benign.[108]

Parathyroid Disorders

Neck irradiation is associated with an increased incidence of hyperparathyroidism. Studies of adults treated with head and neck irradiation for nonmalignant conditions during childhood show a 2.5-fold increased risk for hyperparathyroidism, with a prolonged latency of 25 to 50 years.[223,261-263] Hyperparathyroidism was diagnosed in 10% (5/53) of childhood cancer survivors treated with head and neck irradiation who underwent surgery for thyroid disease at a single institution, with a latency of 15 to 34 years.[264] Survivors treated with neck irradiation as children should have monitoring of their serum calcium concentration as they approach older adulthood, especially if they have thyroid disease.

Short Stature

Many factors can contribute to growth disturbances in childhood cancer survivors, and it may be challenging to isolate a specific cause for diminished height in an individual child. Final height in survivors can be directly affected by cancer therapy and indirectly affected by treatment-related hormonal deficiencies and poor nutrition. Identified independent risk factors for diminished final height in childhood cancer survivors include treatment with cranial irradiation (related to GH deficiency), treatment with total-body irradiation, treatment with spinal irradiation, and young age at diagnosis (<age 13 years, with children <age 4 years at greatest risk).[265-269] Survivors of pediatric brain tumors and of leukemia who undergo cranial or craniospinal irradiation are at particular risk for short stature. In a report from the CCSS, among survivors of acute lymphoblastic leukemia significant risk factors for short stature included diagnosis before puberty, female sex, higher-dose cranial radiation therapy (≥20 Gy), and irradiation of the spine.[270] In a CCSS report assessing 921 brain tumor survivors, nearly 40% of survivors were below the 10th percentile for height. Risk factors included earlier age at diagnosis and higher doses of radiation therapy to the hypothalamic-pituitary axis.[267]

Irradiation-associated bone hypoplasia and fibrosis can directly impede skeletal growth, such that radiation fields that include the spine or lower extremities can result in diminished adult final height.[267,271,272] Irradiation-associated spinal deformities (kyphosis or scoliosis) can also impact final adult height.[251,252,271,272] Prolonged use of corticosteroids can impair longitudinal growth and reduce final adult height. Proposed mechanisms of corticosteroid-induced growth suppression include suppression of pituitary growth hormone secretion, reduction of insulin-like growth factor type 1 production by the liver, and possibly impaired action of insulin-like growth factor type 1 on bone, all of which contribute to diminished growth.[241,271,273] Antimetabolite chemotherapy is associated with decreased bone growth and diminished final height in some clinical studies.[241] This finding is supported by animal and in vitro models, which demonstrate that antimetabolite chemotherapy damages growth plate chondrocytes and impairs growth.[274]

Loss of height velocity during a prolonged course of treatment without subsequent catch-up growth can also result in diminished final attained height. Interruption of growth during the active treatment period can be related to the malignant process itself, poor nutrition, or growth suppression by therapy.[266,275] In a single-institution study of final height in survivors of childhood acute lymphoblastic leukemia, decrease in height growth scores in children undergoing active treatment was noted as soon as 6 months after beginning therapy.[266]

Treatment-induced deficiencies of GH, thyroid hormone, testosterone, and estrogen can all contribute to reduced final height in survivors. The effect of hormone deficiencies on final height can be minimized by early recognition and use of hormone replacement therapy.[276,277] Precocious puberty, a late effect observed after cranial irradiation, can also indirectly result in diminished final height because early onset of puberty reduces the time interval of prepubertal growth.[278] Cranial irradiation for CNS leukemia prophylaxis (18 to 24 Gy) is associated with relatively early and precocious puberty in girls, and higher doses of cranial irradiation often used to treat brain tumors can precipitate early puberty in both males and females.[278]

All childhood cancer survivors, and especially survivors exposed to therapies particularly associated with growth impairment such as total-body irradiation and cranial irradiation, should be monitored for appropriate growth during and after therapy.

Obesity

Obesity has been reported as a potential late complication in survivors of childhood cancer. Obesity is frequently defined as a BMI greater than the 95th percentile for age, or greater than or equal to 30 after age 21 years.[279] Irradiation-induced hypothalamic damage has been implicated in the pathogenesis of obesity in childhood cancer survivors, resulting in impaired growth, low metabolic rate, altered body composition, and dysregulation of eating behaviors.[265,280,281] Indeed, cranial irradiation has been identified as a risk factor for obesity in adult survivors of acute lymphoblastic leukemia.[282,283] A study of 1765 young adult acute lymphoblastic leukemia survivors enrolled in the CCSS identified female gender (RR, females 2.6, males 1.9), younger age at diagnosis in females (<4 years of age), and treatment with a radiation dose greater than 20 Gy during cranial irradiation as risk factors for obesity.[282] Treatment with lower-dose radiation therapy or chemotherapy only was not associated with an increased risk for obesity in this study. In a follow-up longitudinal analysis of changes in obesity and BMI among adult survivors of acute lymphoblastic leukemia from the CCSS, survivors treated with cranial radiation therapy had a significantly greater increase in BMI when compared with the sibling cohort. The rate of increase was not significantly increased for survivors treated with chemotherapy alone.[283] In contrast, a prospective study measuring BMI of 422 children with acute lymphoblastic leukemia and non-Hodgkin lymphoma before, during, and after completion of therapy found no difference in the rate of BMI increase between irradiated and nonirradiated children and concluded that high BMI at diagnosis was the best predictor of adult obesity in survivors of these malignancies.[284]

Physical inactivity as a consequence of treatment-associated morbidities, including cardiopulmonary disease that limits exercise tolerance, neurocognitive disabilities that limit coordination and participation, chronic pain, genetic factors, and social factors, all likely contribute to the obesity observed in survivors. Green and colleagues assessed risk factors for obesity among 9284 adult CCSS participants. In a multivariable analysis, impaired physical function, hypothalamic/pituitary irradiation, use of paroxetine, and younger age at cancer diagnosis were each independent predictors of obesity, whereas meeting the Centers for Disease Control and Prevention guidelines for physical activity and a reported moderate amount of anxiety were associated with a reduced risk for obesity. This study, including the findings of an association of a specific antidepressant and of poor physical functioning with obesity, highlights the importance of further investigation of the interplay between obesity, pharmacologic exposures, physical activity, and other modifiable risk factors.[285] Survivor care should include education regarding the long-term health consequences of obesity and the reduction of modifiable risk factors for this late effect.

Metabolic Syndrome

An increased risk for metabolic syndrome and/or its component parts has been observed in survivors of childhood cancer.[286-288] Metabolic syndrome is a grouping of component physiologic disturbances that is associated with an increased risk for developing cardiovascular disease and diabetes mellitus. Although there are various definitions of metabolic syndrome in the literature, definitions generally include measures of obesity, insulin resistance, dyslipidemia, and hypertension.[289] Identified treatment-related risk factors associated with metabolic syndrome in childhood cancer survivors include cranial irradiation and total-body irradiation.[287,288] Older attained age and a sedentary lifestyle have also been implicated.[290] Because metabolic syndrome is an established risk factor for cardiovascular disease and diabetes mellitus in the general population, aggressive management of modifiable cardiovascular risk factors in survivors is warranted.

Central Nervous System

Therapy for childhood cancer can result in long-term adverse sequelae on function of the CNS and sensory organs. Surgery, radiation therapy, and chemotherapy used to treat brain tumors and solid tumors in the head and neck region and cranial irradiation and chemotherapy used as CNS prophylaxis for leukemia and lymphoma can all contribute to neurologic, cognitive, and sensory deficits observed in survivors.

Neurocognitive Impairment

There is a broad spectrum of neurocognitive impairments observed in childhood cancer survivors. These include deficits in executive functioning (planning and organization), sustained attention, memory (visual sequencing and temporal memory), processing speed, visuomotor integration, and diminished intelligence. As a result of these deficits, survivors may experience learning difficulties, academic failure, and behavioral changes.[38,291-297] Neurocognitive impairment is observed in survivors treated with cranial radiation. Methotrexate, administered intrathecally as CNS prophylaxis or at high doses systemically (single dose >1000 mg/m^2), is also associated with neurocognitive late effects.[291,298] Indeed, neurocognitive impairment has been noted in adult survivors of acute lymphoblastic leukemia treated with chemotherapy alone (without cranial irradiation).[299] Brain tumor survivors treated with surgery alone are at risk for neurocognitive impairment attributable to direct brain injury from tumor growth, surgical resection, hydrocephalus, and seizures.[300]

Risk factors for neurocognitive impairment in childhood cancer survivors treated with radiation include higher dose, young age at exposure (<3 years), female gender, and personal or family history of learning disabilities.[38,293-295,301,302] Additional risk factors include total-body irradiation exposure and concurrent treatment with dexamethasone, methotrexate, or cytarabine.[293] Lower-dose cranial irradiation as CNS prophylaxis for leukemia/lymphoma (18 to 24 Gy) is more commonly associated with information processing or learning disabilities,[38] whereas higher-dose cranial irradiation necessary to treat brain tumors is more often associated with global cognitive impairments and diminished intelligence.[294] Neurocognitive deficits are persistent,[299] and new deficits may emerge over time or become evident during periods of increasing cognitive demands.[293] Armstrong and colleagues reported that adult survivors of childhood acute lymphoblastic leukemia who received 24 Gy of cranial irradiation had increased immediate and delayed memory impairment. In addition, survivors who received 24 Gy of cranial irradiation were found to function at a level one to two decades older than their chronologic age on delayed memory assessments, raising concern for early onset of cognitive impairment.[303]

Formal neurocognitive testing is used to diagnose cognitive impairments. A full battery of tests administered by specially trained psychologists is indicated to evaluate the full spectrum of potential deficits. Survivors diagnosed with neurocognitive impairments should be referred to specialized services at the treating center or in the community to facilitate access to educational, rehabilitation, social, and vocational resources. Cognitive remediation, educational interventions with individualized learning programs, and family support that address survivor specific needs are all necessary for survivors to meet full educational potential.[293,304]

Neurologic Deficits

Surgery for brain tumors and head and neck cancers can result in permanent neurologic deficits that are specific to the location of the primary cancer. For example, postoperative cerebellar mutism may persist years beyond the surgical resection of a posterior fossa tumor.[305] In addition, postoperative hydrocephalus, infections, and seizures can evolve into chronic neurologic conditions that result in neurologic deficits that can be stable or progressive over time.[40]

Peripheral sensory and motor neuropathy presenting as weakness, paresthesias, or pain and episodic vasospasm of the digits (Raynaud phenomenon) are potentially late effects associated with vincristine and vinblastine therapy.[306-308] Treatment-induced neuropathy might restrict survivors from physical activity; however, impaired motor performance, measured in a cohort of 128 childhood cancer survivors, was not associated with cumulative vincristine dose,[309] nor was prolonged vincristine exposure associated with physical inactivity observed in acute lymphoblastic leukemia survivors in the CCSS cohort.[310] Management of these late effects is primarily symptomatic, and a trial of agent used to treat neuropathic pain or prevent peripheral vascular spasm can be considered.[89,306]

Irradiation-induced cerebrovascular disease, such moyamoya disease, vasculitis, and vascular malformations (venous-based cavernous malformations or intracranial aneurysms) have been reported.[207,311-313] Late onset of seizures was reported in 6.5% of brain tumor survivors in the CCSS cohort and cortical irradiation of greater than 30 Gy was associated with a twofold risk for this complication.[40] Also in the CCSS cohort, an increased risk for late-occurring stroke was reported among survivors of brain tumor (RR, 29), leukemia (RR, 6.4), and Hodgkin lymphoma (RR, 4.32).[210,211] Rare neurologic late effects of radiation therapy associated with higher doses of radiation (>50 Gy) include focal brain necrosis and spinal cord myelitis.

Leukoencephalopathy, presenting as spasticity, ataxia, dysarthria, dysphagia, hemiparesis, or seizures, is a rare complication of treatment of childhood cancer and has been associated with cranial irradiation with doses of radiation greater than 18 Gy as well as chemotherapy with methotrexate and dexamethasone. Signs and symptoms of leukoencephalopathy may present without imaging abnormalities (brain MRI, MR angiography, CT); conversely, findings on neuroimaging (white matter changes, cerebral atrophy, dystrophic calcifications, mineralizing microangiopathy) do not necessarily correlate with clinical severity.[314-316] Leukoencephalopathy has also been described as a late manifestation of childhood histiocytic syndromes with no known treatment association.[317]

Late-onset neurologic complications can present as a variety of nonspecific neurologic symptoms, including headache, seizure, hemiparesis, or other focal findings. Neuroimaging is indicated for diagnostic assessment. Neurovascular abnormalities or irradiation-associated abnormalities of the brain parenchyma can also be incidental findings on neuroimaging obtained for other indications. The role of screening with neuroimaging in asymptomatic survivors is not yet defined, with no current data to support that screening asymptomatic survivors reduces morbidity or mortality from neurologic complications. However, cancer survivors who have received cranial irradiation and have nonspecific neurologic symptoms might benefit from early evaluation by a neurologist and/or early neuroimaging.

Vision

Childhood cancer survivors' vision may be impaired by surgery, localized irradiation, total-body irradiation, or chemotherapy with corticosteroids. Visual late effects are both anatomic and functional, including cataracts, orbital hypoplasia, lacrimal gland atrophy, conjunctival corneal damage, retinopathy, glaucoma, and optic nerve damage.[40,318-320] Cataracts are a visual late effect frequently diagnosed in bone marrow transplant survivors and survivors treated with cranial irradiation. Bone marrow transplant conditioning regimens that include total-body irradiation are associated with significant risk for cataract development that can require surgical intervention.[321,322] Chemotherapy-based conditioning regimens are not as likely to induce cataracts, and those that develop are usually less severe and do not requiring intervention.[321,322] Survivors with visual symptoms, those with a history of an ocular tumor, those who received total-body irradiation, or those treated with cranial, orbital, or ocular irradiation are at highest risk for late-onset ocular complications and should receive ongoing follow-up by an ophthalmologist.[40,89,320,321]

Hearing

Hearing loss is a late effect experienced by childhood cancer survivors exposed to head and neck radiation and/or platinum-based chemotherapy. Radiation fields that include the temporal bone and adjacent soft tissues can cause impaired auditory function.[40] Low-dose cranial irradiation alone (as was administered to leukemia patients) is unlikely to cause hearing loss; however, sensorineural hearing loss can occur at cranial doses greater than 50 Gy and at lower doses (40 to 50 Gy) when combined with ototoxic chemotherapy, especially when the treatment sequence is chemotherapy administered after irradiation.[40,323,324] In a series of 157 children with brain tumors treated by radiation therapy only with a median dose of 54 Gy, the cumulative incidence of hearing loss in the speech frequency range was 27% at 5 years after therapy.[325] The cumulative incidence of hearing loss steadily increased over the 9-year follow-up period, and no plateau was observed.[325]

The effect of platinum-based chemotherapy exposure on hearing is dose and age dependent. Children treated at a younger age (<5 years) are more vulnerable to cisplatin ototoxicity at any dose level.[326,327] The incidence of high-frequency hearing loss (4000 to 8000 Hz) is up to 50% in children treated with more than 450 mg/m^2 cumulative dose of cisplatin.[326] Hearing loss in the speech range (500 to 3000 Hz) is rarely reported at cumulative cisplatin doses less than 360 mg/m^2; however, it has been reported at cumulative doses as low as 360 mg/m^2, and the incidence is as high as 22% to 25% at cumulative dosages greater than 720 mg/m^2.[323,326] Cisplatin ototoxicity is typically bilateral and irreversible, and children with normal hearing at the completion of platinum therapy, who have not undergone cranial irradiation, are unlikely to have subsequent loss of hearing.[326] Survivors treated with carboplatin in nonmyeloablative doses do not appear to be at risk for hearing loss, but carboplatin-containing conditioning regimens used in stem cell transplant can contribute to hearing impairment.[328,329] Genetic polymorphisms associated with an increased risk for cisplatin-induced hearing loss in children have been identified.[44] Such findings await further validation but reflect a potential for tailoring treatment and/or follow-up based on the genetic risk for chemotherapy-associated ototoxicity.

The role of otoprotectants, such as amifostine, administered before or during cancer therapy, has not been established, and studies are ongoing in this area.[330] To minimize risk, clinicians should minimize exposure to other ototoxins, including aminoglycoside antibiotics and furosemide. In assessing a survivor, prior and current exposure to all ototoxic agents should be considered in the assessment of auditory function. The contribution of environmental factors (e.g., use of headphones, occupational exposures) to hearing loss in the young survivor population has not been documented.[329] Survivors with treatment exposures that put them at risk for hearing loss or those with symptoms of hearing impairment should have serial auditory evaluations, and those diagnosed with hearing impairments should be prescribed hearing aids and classroom or workplace accommodations as indicated. Early intervention for auditory problems is particular important in the survivor population because hearing loss has the potential to compound already existing social, developmental, and cognitive impairments.

Reproductive System

Reproductive function depends on the complex interaction of hormonal, anatomic, and psychosocial factors. Treatment of childhood cancer directed at the brain, spinal cord, pelvis, genitourinary organs, and gonads can all impair future reproductive potential. Therapy that is directly toxic to the ovaries and testes predisposes survivors to primary hypogonadism resulting in both diminished sex hormone production (testosterone, estrogen) and damage to oocytes and spermatocytes. Secondary hypogonadism, impaired secretion of gonadotropins (LH, FSH), can be the result of radiation and surgery to the hypothalamic-pituitary axis. Alternation of the neurovascular or structural anatomy of the reproductive organs or reduction in the volume of gonadal tissue by radiation and surgery can also impair reproductive function. As with most late effects, chemotherapy-associated gonadotoxicity is directly related to the individual agent and cumulative dose. Specifically, the alkylating agents cyclophosphamide and procarbazine pose a greater risk for gonadal dysfunction than cisplatin and dacarbazine.[35,331-333] In general, the ovary is less vulnerable to chemotherapy-induced damage than the testis. As with other late effects, irradiation-associated toxicity to the ovaries and testes is dependent on the radiation dose and field. The availability of established fertility preservation strategies for patients newly diagnosed with cancer (semen cryopreservation, oophoropexy, oocyte cryopreservation) may improve long-term fertility outcomes for survivors treated with gonadotoxic therapy.[334-337]

Females

Ovarian dysfunction can be a result of acute ovarian toxicity that persists or premature ovarian failure. The majority of prepubertal and adolescent girls receiving standard-dose chemotherapy will retain or recover ovarian function; however, those receiving high-dose alkylating agents, conditioning for bone marrow transplant, or those exposed to pelvic irradiation are at risk for irreversible ovarian toxicity, which manifests as both estrogen deficiency and infertility.[338-341] Of note, irradiation to the pelvis, abdomen, or spine may include the ovaries in the field. Ovarian doses greater than 10 Gy can result in complete ovarian failure, requiring hormone replacement therapy.[342,343] Survivors treated with both radiation therapy and gonadal-toxic chemotherapy have a worse prognosis for ovarian function.[344,345] Cranial irradiation that has a radiation dose greater than 22 Gy to the hypothalamic-pituitary access is associated with reduced fertility, and use of more than 30 Gy is associated with ovarian failure as a result of impaired secretion of gonadotropins (LH, FSH).[346] Survivors diagnosed with ovarian failure should be offered treatment with hormone replacement therapy in consultation with an endocrinologist.

Survivors who retain or recover ovarian function after completing therapy are at risk for premature menopause (defined as cessation of menses prior to age 45 years). Risk factors for premature menopause include increasing age, older age at treatment, increasing radiation dose to the ovaries, and increasing cumulative dose of alkylating agents.[344,345] The pathophysiology of therapy-induced premature menopause is reduction in number of ovarian follicles, which supports the finding that prepubertal girls with the greatest reserve of follicles are less vulnerable to premature ovarian failure than older survivors. In the historic Five-Center Study, which included 1067 female survivors, the risk for reaching early menopause (age <25 years) for those treated between the ages of 13 and 19 years was four times greater than sibling controls.[347] Increased risk for premature menopause was observed in survivors treated with irradiation below the diaphragm (RR, 3.7), alkylating agents (RR, 9.2), or both (RR, 27). In this historical cohort, 42% of women treated with irradiation below the diaphragm and alkylating agents had reached menopause by age 31 years.[347] The overall relative risk for premature menopause in survivors in the CCSS cohort was thirteenfold higher than sibling controls, with a cumulative incidence of 8% by age 40.[345] In this study, the cumulative incidence of menopause for survivors exposed to alkylating agents only was 5% at age 35 years and increased to 15% by age 40 years, and survivors exposed to both radiation therapy and alkylating agents had a cumulative incidence of menopause of 15% at age 30 years that increased to 30% by age 40 years.[345] Of note, the threshold dose of cumulative alkylating agent exposure below which ovarian function is preserved has not been determined, and in pelvic irradiation even the lowest radiation doses (1 to 99 cGy) are associated with premature menopause.[345]

Survivors who retain ovarian function but are at risk for premature menopause should be educated about their potentially reduced window of fertility and offered referral to fertility specialists for counseling and intervention. Clinical tests to predict the precise timing of menopause are not available; however, the serum antimüllerian hormone level is promising as a predictor of ovarian reserve and is likely to be recommended for routine monitoring of cancer survivors at risk for premature menopause in the near future.[341] Hormonal interventions to potentially prolong ovarian function have not been clinically effective.[341] Cryopreservation of embryos (which require a partner or sperm donor) is an option for a survivor who anticipates early menopause. Recent successes in the techniques of cryopreservation of eggs during the period of ovarian function create new options for survivors at risk for premature menopause.[341,348]

Males

Males treated with alkylating agents as children and adolescents are at risk for infertility secondary to impaired testicular germ cell function and low testosterone resulting from Leydig cell insufficiency.[349] Alkylating agent gonadal toxicity is dose dependent, and pubertal status or age at exposure is not protective.[35,225,350] Of note, the majority of male survivors with alkylating agent–induced infertility will have normal testosterone production, with normal sexual function and secondary sexual characteristics. Abnormal semen analyses have been observed with cumulative cyclophosphamide doses greater than 5.0 to 7.5 g/m^2. However, Leydig cell insufficiency (elevated serum LH, normal testosterone) or Leydig cell failure (elevated LH, low testosterone) is associated with higher total cyclophosphamide doses (>20 g/m^2).[35,351,352] Survivors treated with unilateral orchiectomy and not exposed to additional gonadal toxic therapy usually maintain adequate testicular function.[353]

A survivor's risk for irradiation-induced testicular dysfunction depends on radiation dose and age at exposure. Testicular germ cells are very sensitive to radiation damage. Changes in spermatogenesis can be observed after doses as low as 0.1 Gy, and irreversible azoospermia usually occurs after doses greater than 2 Gy.[339] In contrast, testosterone production is usually preserved with testicular doses up to 20 Gy. Irreversible damage to testicular germ cell and endocrine function usually occurs after exposure to more than 20 Gy of radiation, requiring androgen replacement therapy. However, survivors who are beyond puberty at the time of diagnosis can usually tolerate higher doses of radiation before loss of testicular endocrine function.[265]

Although reduced testicular volume on physical examination, elevated serum gonadotropins (e.g., FSH), and low serum inhibin B suggest infertility, the only definitive measures of a survivor's potential fertility is by semen analysis or paternity. Of note, male survivors with ejaculatory azoospermia diagnosed by semen analysis soon after completion of therapy have the potential to recover spermatogenesis many years later.[352,354] Thus a sexually active survivor who is not contemplating paternity must be counseled about birth control, even if he has had an abnormal semen analysis in the past. Furthermore, survivors with ejaculatory azoospermia who desire biologic paternity may be candidates for the surgical procedure of testicular biopsy with sperm extraction.[355] These men should be referred to a urologist with this expertise for consultation.

Survivors with delayed onset of puberty or symptoms of hypoandrogenism should be screened with morning serum testosterone, LH, and FSH evaluation. If results are abnormal, testosterone replacement therapy should be guided by the appropriate subspecialist. Of note, serum testosterone levels normally decline with advancing age, so survivors at risk for treatment-associated hypoandrogenism should have serum testosterone levels reevaluated as they reach older adulthood.

Sexual Function

Surgery or irradiation of the pelvis or lumbar spine, treatment-associated hormonal insufficiencies, medical comorbidities, and the social and emotional changes associated with a cancer experience may predispose childhood cancer survivors to sexual dysfunction. An emerging literature on the prevalence and risk factors for sexual dysfunction in young adult survivors of childhood cancer shows a higher than expected prevalence of both males (20% to 32%) and females (37% to 52%) reporting a problem in one or more areas of sexual function.[356-359] Normal sexual function is dependent on the complex interplay of both physical and social and emotional factors. Physical factors that can cause sexual dysfunction include disruption of normal pelvic anatomy from pelvic or spinal surgery or irradiation, hormonal deficiency, increasing age, and emotional distress.[349] Survivors at the greatest risk for psychosexual dysfunction include those diagnosed during adolescence, those with a brain tumor, and those with poor health status; these factors may be related to social isolation and delayed psychosexual development.[356,358,359] Because sexual function is an important aspect of quality of life and all survivors are at risk for psychosexual dysfunction related to their cancer experience, it is recommended that all adolescent and young adult survivors have an assessment of their sexual function as part of follow-up care.[360] The assessment for sexual dysfunction in the childhood cancer survivor includes a thorough psychosexual history, sexual history, medical history, and physical examination, including Tanner staging. Sexual history should include detailed questions about social relationships; body image; sexual experiences; libido; for women, lubrication genital sensation, orgasm, dyspareunia, and postcoital bleeding; and for men, nocturnal emissions, spontaneous erections, orgasm, and quality of ejaculate. If sexual dysfunction is a concern, consultation with gynecologist, urologist, or psychotherapist is indicated.

Pregnancy

Women who undergo abdominal or pelvic irradiation who are able to conceive children are at risk for complications of their pregnancy and delivery. Irradiation-induced damage to uterine muscle and blood flow adversely affects survivors' prognosis for a healthy full-term pregnancy.[361] Pelvic and abdominal radiation fields that include the uterus are associated with pregnancy complications, including spontaneous abortions, preterm labor, low-birth-weight infants, infants who are small for gestational age (SGA), neonatal death, and postpartum hemorrhage.[343,362,363,364-369] A report on more than 4000 pregnancies from the CCSS showed that the only risk factor for adverse pregnancy outcomes was pelvic irradiation, which was associated with low-birth-weight infants (RR, 1.84). No other treatment factor was a risk for any adverse pregnancy outcome in this large cohort.[362,363] When the analysis was restricted to 2201 liveborn children of 1264 survivors in the CCSS cohort, maternal uterine irradiation (>5 Gy) was associated with prematurity (OR, 3.5), low birth weight (OR, 6.8), and SGA infants (OR, 4.0).[364] Estrogen therapy does increase uterine size and stimulate blood flow but is not shown to improve uterine muscular function.[370]

Survivors with therapy-associated cardiac toxicity (either subclinical or overt) are at risk for pregnancy-associated congestive heart failure and should be monitored before pregnancy, during pregnancy, and after delivery by obstetricians aware of potential complications.[157,371] Peripartum acute congestive heart failure has been described in survivors treated with anthracyclines and chest irradiation; however, the incidence has not been determined.[157,371] One study of 37 anthracycline-exposed pregnant women showed that prepregnancy left ventricular dysfunction was a risk factor for acute congestive heart failure during pregnancy, supporting the need to closely monitor at-risk survivors.[371] Survivors with risks for other organ toxicities such as endocrinopathies, pulmonary fibrosis, and renal insufficiency should also be meticulously evaluated and monitored before pregnancy, during pregnancy, and after delivery.

Offspring

Many studies have investigated the possible association between exposure to cancer therapy during childhood and a mutagenic or teratogenic effect on future offspring. Fortunately, no study to date has found in increased risk for either congenital anomalies or cancer in the offspring of adult survivors of nonhereditary childhood cancer treated before conception,[35,372-381] This is true for both male and female survivors of childhood cancer and survivors treated with both chemotherapy and radiation therapy. Of note, survivors of childhood cancers that are associated with genetic syndromes and hereditary cancer predisposition (e.g., Cowden syndrome, Li-Fraumeni syndrome, retinoblastoma) are at increased risk for their offspring being diagnosed with cancer. These survivors should be referred for genetic counseling and genetic testing as desired and appropriate (see Box 72-2). Preimplantation genetic testing may be available to survivors with a known mutation in a cancer predisposition gene who are contemplating pregnancy.[382,383]

Renal System

Chronic renal disease is a late effect associated with irradiation to the kidneys as part of an abdominal field or total-body irradiation; chemotherapy with ifosfamide and high-dose cisplatin; nephrectomy, especially in combination with other nephrotoxic therapy; and treatment with other nephrotoxic agents, including antibiotics and immunosuppressants. The pathophysiology of irradiation injury to the kidney is progressive arteriolonephrosclerosis, resulting from irradiation injury of the renal microvasculature with subsequent secondary damage to the glomeruli and tubules. Irradiation-associated renal injury is dose related, and the threshold dose is approximately 15 Gy; however, nephrectomy and exposure to other nephrotoxic agents lower that threshold.[384,385] In a cohort of bone marrow transplant survivors treated with fractionated total-body irradiation, 12 to 14 Gy over 3 to 4 days, late renal dysfunction was reported in 35% of acute lymphoblastic leukemia survivors and 71% of neuroblastoma survivors.[385] Modern conditioning regimens with altered fractionation should reduce the incidence of renal insufficiency in transplant survivors.

Chemotherapy with cisplatin at doses greater than 200 mg/m^2 can cause nephropathy secondary to glomerular or tubular injury. This injury is usually acute and reversible; however, it can be stable or progressive.[386] Carboplatin does not appear to be associated with long-term nephrotoxicity.[387] Ifosfamide nephrotoxicity is also a result of glomerular or tubular injury and presents most often as renal tubular acidosis.[388] The greatest risk for ifosfamide nephrotoxicity is at total dose greater than 45 g/m^2, and age younger than 3 years.[388] Combination nephrotoxic chemotherapy, nephrectomy, and therapy with nephrotoxic antibiotics all contribute to survivors' risk for long-term renal damage.[389-392]

The risk for chronic renal disease in survivors treated with unilateral nephrectomy alone appears to be minimal.[393] However, data suggest that patients treated with unilateral nephrectomy do manifest subclinical evidence of early reversible renal injury detectable by elevated urine levels of microalbumin.[394,395] A study linking Wilms tumor survivors in the National Wilms Tumor Study group to the U.S. End-Stage Renal Disease registry followed for a median of 11 years showed a low risk for end-stage chronic renal failure in nonsyndromic Wilms tumor survivors (cumulative incidence, 0.6% at 20 years).[393] These data suggest that Wilms tumor survivors with clinically detectable renal dysfunction will not routinely progress to end-stage renal disease.[393] Of note, this cohort has not reached an age when survivors might be at the greatest risk for chronic renal failure.[393] Survivors of Wilms tumor with the highest cumulative incidence of chronic renal failure at 20 years are survivors with predisposition syndromes such as Denys-Drash syndrome (74%), WAGR syndrome (36%), genitourinary abnormalities (7%), and survivors with bilateral disease

(12%).[396-398] Survivors of Wilms tumor with progressive tumor in the contralateral kidney or nephrogenic rests and survivors who have undergone irradiation have also been identified as having an increased risk for renal dysfunction.[396-398]

Survivors treated with nephrotoxic therapy should be routinely monitored for early clinical signs of renal dysfunction, including hypertension, proteinuria, and elevated serum creatinine concentration. Survivors with the earliest signs of renal injury might benefit from therapeutic interventions with angiotensin-converting enzyme inhibitors to reverse or halt progression of renal injury. Survivors with a single kidney should be counseled to adopt health behaviors to maintain kidney health, including hydration, avoidance of prolonged use of nonsteroidal antiinflammatory drugs, prompt treatment of urinary tract infections, and meticulous control of coexisting diabetes and hypertension.

Genitourinary System

In addition to the renal late effects discussed previously, survivors of childhood cancer are also at risk for toxicity to the genitourinary system from surgery, treatment with pelvic and whole-abdominal irradiation, and alkylating agent chemotherapy. Genitourinary surgery can result in long-term functional impairments, including urinary incontinence and sexual dysfunction. The primary late effects associated with irradiation include fibrosis or hypoplasia of the anatomic components of the male and female genitourinary systems, including urinary bladder, ureter, urethra, prostate, vagina, and uterus, resulting in chronic pain or dysfunction. Survivors experiencing anatomic or functional genitourinary late effects, including sexual dysfunction, should be referred to a gynecologist or urologist as indicated for evaluation and management of their symptoms.

Chemotherapy with cyclophosphamide and ifosfamide has been associated with protracted hemorrhagic cystitis and bladder fibrosis, although this late effect has been reduced by concurrent therapy with mesna.[399,400] Hemorrhagic cystitis that develops beyond the time of therapy more often has a viral etiology or is associated with chronic graft-versus-host disease (GVHD) in transplant survivors.[401] Although the risk for bladder pathology in childhood cancer survivors is low, routine monitoring with urinalysis of survivors exposed to cyclophosphamide and ifosfamide is clinically indicated.

Gastrointestinal System

Potential late effects of cancer therapy on the gastrointestinal system are late onset surgical complications such as adhesions, small bowel obstruction, and fecal incontinence[402]; irradiation-induced functional impairment; and secondary neoplasms of the gastrointestinal tract and hepatobiliary system.[67] Hepatic fibrosis is a rare late complication of antimetabolite chemotherapy (methotrexate, mercaptopurine, thioguanine), and the fibrosis typically follows acute hepatic toxicity such as venoocclusive disease.[403-405] As with other organ systems, irradiation can result in functional impairment and second cancers of the gastrointestinal organs consequently exposed to radiation as a result of location in a treatment field. The clinical spectrum of irradiation-induced gastrointestinal toxicity that has been reported includes salivary gland dysfunction, esophageal stricture, Barrett esophagus, hepatic fibrosis and cirrhosis, focal nodular hyperplasia, cholelithiasis, and chronic enterocolitis.[405,406] In the CCSS, survivors had nearly twice the risk for gastrointestinal disease than that observed in the sibling cohort. Survivors were more likely to require colostomy/ileostomy (RR, 5.6 [95% CI, 2.4 to 13.1]), undergo liver biopsy (RR, 23.1 [95% CI, 7.5 to 77.8]), or self-report cirrhosis (RR, 8.9 [95% CI 2.0 to 40.0]).[407] Risk factors for gastrointestinal toxicity in long-term survivors included older age at diagnosis (>10 years), high-dose alkylating agent and/or anthracycline exposure, abdominal irradiation, and abdominal surgery.[407] Of note, survivors were more likely to have more than one gastrointestinal condition than their siblings and more frequently reported common conditions such as diarrhea, ulcers, polyps, and heartburn.[407]

Common gastrointestinal symptoms reported by a childhood cancer survivor, such as abdominal pain and heartburn, should be evaluated in the context of his or her treatment history. A survivor's risk for a serious gastrointestinal pathologic process should be considered when planning a diagnostic workup and therapy for common symptoms. Children requiring abdominal surgery as part of their cancer therapy are at risk for late onset small bowel obstruction and should be educated about his late effect.[408] Because survivors exposed to abdominal irradiation are considered to be at higher risk for colon cancer, and self-report polyps at greater frequency,[407] they are advised to begin colon cancer screening earlier than what is recommended for the general population (10 years after completing radiation therapy or age 35 years, whichever comes last).[67,89]

OTHER LATE EFFECTS

Infections

Late-occurring infectious complications can result from surgery, transfusions, immunosuppression, and radiation therapy. Childhood cancer survivors who had a surgical splenectomy as part of their staging for Hodgkin lymphoma are at lifelong risk for potentially fatal bacterial sepsis with encapsulated organisms. Survivors who received more than 40 Gy of abdominal or left flank irradiation are considered functionally asplenic and have a similar risk for sepsis.[409] These survivors must be continually reminded of their risk and educated to receive immediate medical evaluation and prophylactic antibiotic therapy for fever or other signs of sepsis. Immunizations against *Streptococcus pneumoniae, Haemophilus influenzae,* and *Neisseria meningitidis* are also recommended for this high-risk group.[410] Hematopoietic stem cell transplant survivors receiving immunosuppression for chronic GVHD are also at risk for ongoing infectious complication and should be considered for prophylactic antibiotics.[411] Older survivors treated in the United States are at risk for transfusion-acquired chronic hepatitis B if they received blood products prior to 1972; for hepatitis C if they received blood products prior to 1993; and for human immunodeficiency virus if they received blood products between 1977 and 1985.[412] Irradiation to the sinuses for a primary sarcoma or CNS tumor at doses greater than 30 Gy is associated with chronic bacterial sinusitis.[413] Survivors who received therapy that is associated with a risk for pulmonary disease such as lung irradiation or total-body irradiation and those with cardiac disease should receive annual influenza vaccine. Survivors with known irradiation-associated valve disease should receive appropriate antibiotic prophylaxis for subacute bacterial endocarditis.[414] Survivors at risk for infectious complications should be educated about their risks and informed of the necessity for preventive strategies.

Dental Problems

Childhood cancer survivors are at risk for dental late effects. Survivors treated with head and neck irradiation are at risk for root shunting, microdontia, hypodontia, and craniofacial abnormalities resulting in severe cosmetic or functional impairment necessitating surgical or orthodontic intervention.[415-417] The risk for dental facial abnormalities is greatest in survivors treated at the youngest age and with higher doses of radiation. Survivors treated with chemotherapy prior to the complete eruption of their permanent teeth are at risk for tooth agenesis, root thinning or shortening, and enamel dysplasia.[417,418] Neuroblastoma survivors and leukemia survivors treated with cranial irradiation have a particularly high incidence of dental abnormalities (71% and 94%, respectively) most likely associated with young age at treatment.[416,417] In a report from the CCSS, when compared with the sibling cohort, survivors were more likely to report microdontia, hypodontia, root abnormalities, abnormal enamel, teeth loss (six or more), severe gingivitis, and xerostomia. Radiation exposure of 20 Gy or more to dentition was associated with an increased risk for one or more dental abnormalities. Children diagnosed at age younger than 5 years treated with alkylating agents also had increased risk for one or more dental abnormalities.[419] All childhood cancer survivors should be advised to have regular dental care, including cleanings, fluoride applications, and appropriate imaging to evaluate tooth development.[89]

Psychosocial Function

The experience of having childhood cancer and its associated treatment can have long-term effects on a survivor's emotional and social functioning. Common disorders of emotional function, such as depression, anxiety, and posttraumatic stress disorders are reported more frequently in populations of childhood cancer survivors than in both their siblings and the general population.[420,421] Serious emotional distress, such as suicidal symptoms, is also reported more commonly in survivors. In a report from the CCSS, adult survivors of childhood cancer were at increased risk for suicidal ideation compared with the sibling control cohort (7.8% of survivors reported suicidal ideation compared with 4.6% of controls [OR, 1.79; 95% CI, 1.4 to 2.4]). In survivors, suicidal ideation was associated with a primary diagnosis of CNS malignancy, depression, and poor current physical health.[422] There is no known increased incidence of psychiatric disorders such as schizophrenia or other psychoses in childhood cancer survivors, with the exception of survivors of brain tumors. A Dutch study of over 3700 childhood cancer survivors found that brain tumor survivors had an excess risk for psychiatric hospitalizations for diagnoses that included psychoses of somatic and cerebral causes, psychiatric disorders in somatic disease, and schizophrenia.[423]

Challenges to normal social functioning such as educational remediation, employment discrimination, insurance discrimination, marital dissatisfaction, social isolation, and poor health-related quality of life have all been reported more frequently in survivors of childhood cancer.[424-433] The majority of childhood cancer survivors will not experience significant psychosocial dysfunction as a result of their cancer experience or cancer therapy; however, survivors of childhood CNS malignancies and those treated with cranial irradiation are more vulnerable to psychosocial morbidity. These survivors, in particular, should be routinely monitored for emotional distress and social disability.

SPECIAL CONSIDERATIONS FOR THE HEMATOPOIETIC STEM CELL TRANSPLANT SURVIVOR

The comprehensive clinical evaluation of the hematopoietic stem cell transplant (HSCT) survivor requires that the clinician consider all aspects of the survivor's pretransplant history, transplant course, posttransplant immune function, and disease status. In assessing the risk for late effects in a survivor of HSCT, both the treatments associated with the condition that led to the transplant and the therapies associated with the transplant must be considered. The modality, dose, and intensity of the conditioning regimens for HSCT are major factors that determine the survivor's risk for late effects. Total-body irradiation used as conditioning for HSCT may contribute to the organ-specific toxicity of prior chemotherapy, such as cardiotoxicity from anthracyclines,[188] ototoxicity from platinum agents,[324] and gonadotoxicity of alkylating agents.[344] The transplant history should be reviewed for complications that occurred during the course of transplant, such as acute pneumonitis or renal failure, which might add to the survivor's risk for late onset organ toxicity.[221,385] Survivors who received an allogeneic HSCT are at risk for chronic GVHD that can be associated with severe chronic illness. Long-term consequences of chronic GVHD can include joint contractures, polymyositis, vaginal stenosis, alopecia, vitiligo, scleroderma, lichenoid skin lesions, xerostomia, lichenoid or atrophic oral lesions, xerophthalmia, keratoconjunctivitis, esophageal strictures, malabsorption, cholestasis, bronchiolitis obliterans, and obstructive airway disease. Survivors with chronic GVHD and those on chronic immunosuppressive regimens have an increased risk for infection.[411] Comprehensive assessment of the HSCT must also include posttransplant immunization history to ensure revaccination was accomplished at appropriate intervals after transplant.

FOLLOW-UP CARE OF CHILDHOOD CANCER SURVIVORS

The need for specialized follow-up care for childhood cancer survivors is evident, given the numerous morbidities associated with childhood cancer treatment and the potential for survivors to develop complications decades after treatment. The prevalence of chronic health conditions observed in young adult survivors of childhood cancer is 62% in the CCSS cohort and 58% in a single-institution cohort of survivors from the United Kingdom.[46,434] In the CCSS, almost a third of patients had a severe or life-threatening health condition, and the cumulative incidence of a severe or disabling health condition approached 50% at 30 years after the diagnosis of childhood cancer.[46] A survivor's self-reported health status, including self-assessment of functional health, general health, mental health, and pain, has been described in several cohorts of young adult survivors of childhood cancer. Impaired health status is reported in more than 75% of adult survivors surveyed in Canada, 58% of adult survivors in a British cohort, and 48% of adult survivors in the CCSS.[434-436]

Although childhood cancer survivors are at risk for a variety of treatment-related health conditions as they age, they are not necessarily knowledgeable about the specifics of their cancer diagnosis and treatment, nor are they proactive about their future health or adherent to follow-up recommendations. Of the young adult survivors surveyed in the CCSS cohort, only 72% could accurately report their diagnosis; and although the majority were knowledgeable about the general type of therapy they received (e.g., chemotherapy, surgery, irradiation), only 70% of those receiving radiation therapy could report the site.[437] Furthermore, an analysis of cancer screening practices of the young adults in the CCSS cohort showed that adherence was not optimal[438] and in a separate analysis it was shown that the proportion of survivors who remain in long-term follow-up decreases with increasing years since completion of therapy.[439] The high prevalence of chronic health conditions, poor health status, knowledge deficits, and suboptimal adherence to follow-up recommendations all support the need for comprehensive specialized follow-up care of childhood cancer survivors, including both provision of health care services and risk education.

Models of Survivorship Care

Comprehensive survivor programs have been developed at pediatric cancer centers to provide long-term, specialized, risk-based, follow-up care to childhood cancer survivors. A survey of Children's Oncology Group institutions found 87% provide survivorship care and 59% have specialized late effects programs.[440] The ideal components of follow-up care systems include review of disease treatment and history, screening for and diagnosing late effects, a plan for long-term surveillance, coordination of subspecialists, provision of psychosocial support, counseling on education and occupation, coordination of transition to adult care, education and outreach to survivors in the community, education of professionals (primary care providers, educators), and research. The optimal patterns and models of care for childhood cancer survivors have not been well studied. A model endorsed by leaders of existing pediatric long-term follow-up clinics contains the following elements: clinic services under the direction by a pediatric oncologist with expertise in survivorship care; staffing by advanced degree nurses to provide and coordinate patient care; and access to social workers, psychologists, a referral network of medical and surgical specialists with survivorship expertise, and ongoing care provided in the community with periodic center-based follow-up.[441] This model assumes frequent bidirectional communication between survivors, primary care providers, and oncologists with clear delineation of responsibilities for ongoing care. In general, these comprehensive, center-based programs have the proven advantage of providing multidisciplinary expertise and survivor education and have the facility for research in cancer survivorship. Common challenges these programs face are limited financial and personnel resources; inadequate capacity to meet the needs of growing numbers of survivors; and limited access because of geography, insurance, or lack of awareness on the part of the primary care providers or the individual survivor.[441,442] These and other alternative models for extending and improving care delivered to childhood cancer survivors have been proposed but have not been formally evaluated in terms of feasibility and efficacy.

Current efforts to retain survivors in long-term follow-up have focused on introducing and reinforcing the concept of life-long follow-up at critical transition points on the cancer care continuum (Fig. 72-6). Two specific points that have become the focus of educational interventions are the time periods around completion of planned cancer treatment and again when a survivor is an emerging adult. Transition programs for those who recently completed therapy are designed to educate survivors regarding treatment-related risks and to introduce issues related to survivorship early after cancer treatment. The goal of these programs is to bridge the period between active therapy and survivorship follow-up care and subsequently reduce the knowledge deficit and loss to follow-up seen in prior cohorts of long-term survivors.[443] Similarly, transition programs for survivors who have reached the age of adolescence or young adulthood are designed to support adolescent or young adult survivors as they transfer care to adult-oriented health care services. Ideally, these transition programs address the transfer of knowledge about disease, treatment, and future health risks from parents to adolescent or young adult survivor; enhance the survivor's cognitive competence/self-efficacy; and help with access to and confidence in adult providers.[444] Research is needed to test the efficacy of these transition specific programs to improve the participation of childhood survivors in lifelong term follow-up care.

Comprehensive Clinical Evaluation

Independent of the setting in which it occurs, the clinical evaluation of the childhood cancer survivor should include a comprehensive treatment summary, thorough

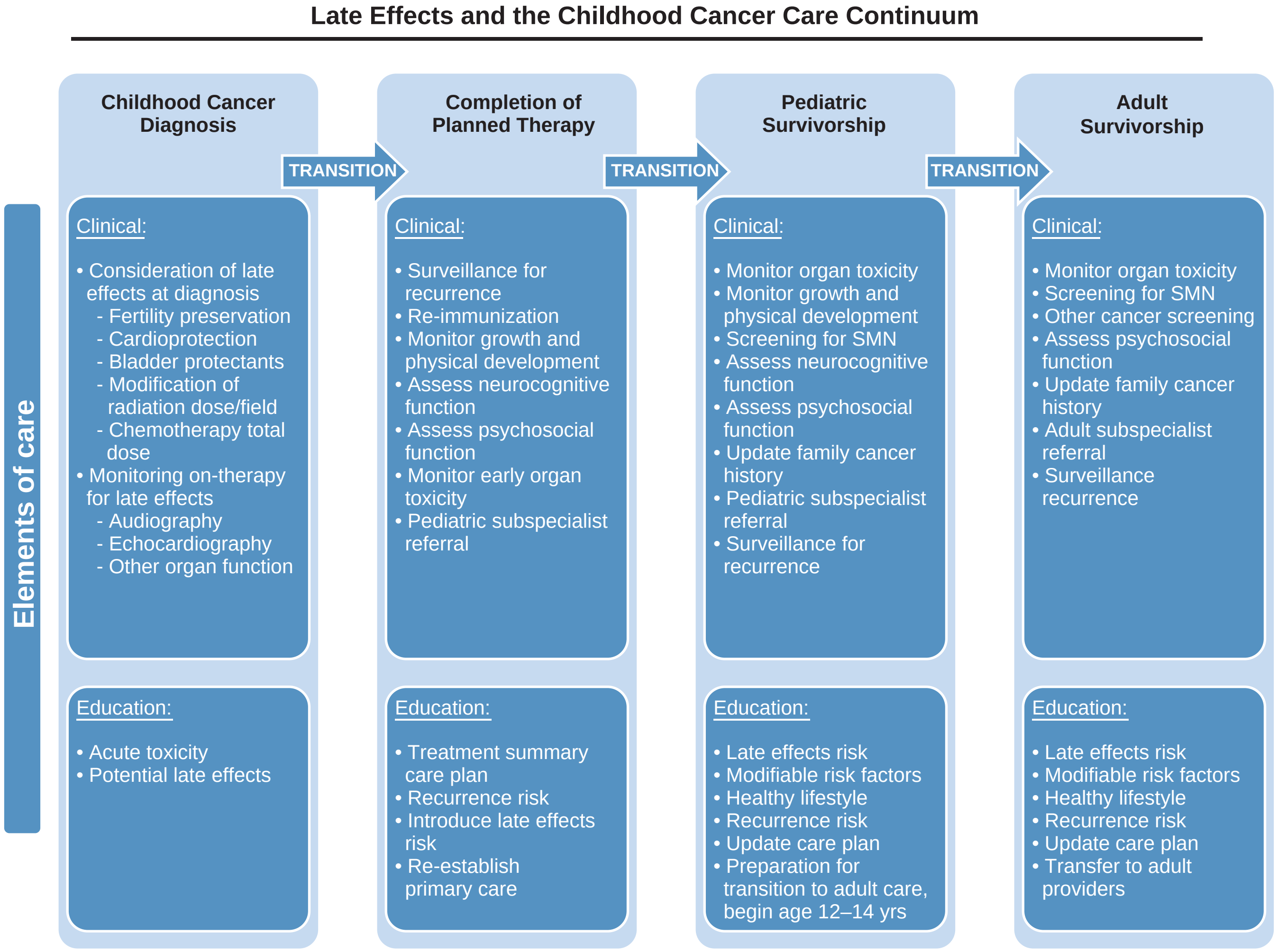

Late Effects and the Childhood Cancer Care Continuum
Elements of care
Childhood Cancer Diagnosis
TRANSITION
Completion of Planned Therapy
TRANSITION
Pediatric Survivorship
TRANSITION
Adult Survivorship
Clinical:
• Consideration of late effects at diagnosis
- Fertility preservation
- Cardioprotection
- Bladder protectants
- Modification of radiation dose/field
- Chemotherapy total dose
• Monitoring on-therapy for late effects
- Audiography
- Echocardiography
- Other organ function
Clinical:
• Surveillance for recurrence
• Re-immunization
• Monitor growth and physical development
• Assess neurocognitive function
• Assess psychosocial function
• Monitor early organ toxicity
• Pediatric subspecialist referral
Clinical:
• Monitor organ toxicity
• Monitor growth and physical development
• Screening for SMN
• Assess neurocognitive function
• Assess psychosocial function
• Update family cancer history
• Pediatric subspecialist referral
• Surveillance for recurrence
Clinical:
• Monitor organ toxicity
• Screening for SMN
• Other cancer screening
• Assess psychosocial function
• Update family cancer history
• Adult subspecialist referral
• Surveillance recurrence
Education:
• Acute toxicity
• Potential late effects
Education:
• Treatment summary care plan
• Recurrence risk
• Introduce late effects risk
• Re-establish primary care
Education:
• Late effects risk
• Modifiable risk factors
• Healthy lifestyle
• Recurrence risk
• Update care plan
• Preparation for transition to adult care, begin age 12–14 yrs
Education:
• Late effects risk
• Modifiable risk factors
• Healthy lifestyle
• Recurrence risk
• Update care plan
• Transfer to adult providers

Figure 72-6 Late effects and the childhood cancer care continuum.

physical examination, and a care plan that includes recommendations for diagnostic or screening tests as indicated. The results of the evaluation should be summarized, documented, and communicated to the survivor and all health care providers participating in the survivor's care.

Treatment Summary

Information about prior diagnosis and treatment exposures is necessary for a health care provider to begin a comprehensive childhood cancer survivor's evaluation. This critical information is likely contained in a pediatric oncology "treatment summary." Details about diagnosis that are necessary for the survivor follow-up evaluation include age at diagnosis, presenting symptoms, results of diagnostic and staging evaluation, and pathology. Specifics of radiation therapy (field and dose); chemotherapy (specific agents and cumulative doses); surgeries (splenectomy, organ resections, and biopsies); and blood transfusions are all important component of a survivor's treatment history that contribute to the assessment of future health risks. Information about any required modifications in treatment, major acute complications of therapy, and ongoing treatment-related issues, such as chronic GVHD, should be available at the time of evaluation. For a provider not familiar with a survivor's prior therapy, a treatment summary can usually be obtained from the treating institution. A sample treatment summary template is provided in Figure 72-7. A summary of late effects associated with curative treatments for the most common childhood cancer diagnoses is presented in Table 72-4.

Medical History and Physical Examination

As in all medical assessments, obtaining and documenting a history of current and past medical problems is important to the childhood cancer survivor evaluation. Past medical history, including birth history, surgeries, and medical conditions that preceded the cancer diagnosis, may be pertinent. For older survivors, menstrual history, reproductive history, and obstetric history all contribute important information to the follow-up evaluation. Information about current medications and chronic medical conditions should also be obtained. Survivors should specifically be asked about any late effects diagnosed or treated to date, including subsequent cancers.

Treatment Summary

Current Name:	Medical Record Number:
Name at Diagnosis:	Date of Birth:

Diagnosis:	Site(s):
Presenting Symptoms:	
Date of Diagnosis:	Protocol:
Date of Recurrence:	Site(s):
Relapse Protocol:	Date of Treatment Completion:
Complications During Treatment	

Chemotherapy

Agent:	Total Dose:
Agent:	Total Dose:
Agent:	Total Dose:
Agent:	Total Dose:
Agent:	Total Dose:

Radiotherapy

Date:	Site:	Dose:

Bone Marrow Transplant

Date:	Allo/Auto	Allo Donor / HLA Matching	
Chemotherapy Conditioning (include doses):			
TBI / Other Radiotherapy	Site:	Dose:	Fractions:

Acute GvHD (Grade, site)	Chronic GvHD (Grade, site)	Treatment

Surgery

Site:	Date:

Medical History

Complications After Treatment Completion:

Family History:

Recent Studies

Test:	Date:	Results:

Figure 72-7 Example of childhood cancer survivor treatment summary template. *(Modified from Skinner R, Wallace WHB, Levitt GA, editors:* Therapy-based long-term follow-up practice statement, *2nd ed. United Kingdom, 2005, Children's Cancer Study Group. Accessed at http://www.ukccsg.org.uk/public/followup/PracticeStatement/APPENDICES-g.pdf.)*

TABLE 72-4 Potential Late Effects Associated With Curative Therapies for Common Childhood Cancers

	Categories of Treatment-Associated Late Effects					
Primary Cancer	Secondary Malignancy *(Associated Therapy)*	Cardiovascular/Pulmonary *(Associated Therapy)*	Musculoskeletal *(Associated Therapy)*	Neurologic/Developmental *(Associated Therapy)*	Endocrine/Reproductive *(Associated Therapy)*	Other *(Associated Therapy)*
Leukemia	MDS/leukemia *(Alkylating agents, topoisomerase inhibitors)* Skin cancer Meningiomas CNS tumors *(Cranial irradiation)*	Cardiomyopathy Arrhythmias *(Anthracyclines)*	Decreased bone mineral density *(Corticosteroids, MTX)* Osteonecrosis *(Corticosteroids)* Short stature *(Corticosteroids, Cranial irradiation)* Dental abnormalities *(Cranial irradiation)*	Learning disabilities *(Cranial irradiation, intrathecal therapy)* Vasculopathy *(Cranial irradiation)* Cataracts *(Corticosteroids, cranial irradiation)* Peripheral neuropathy Raynaud phenomenon *(Vincristine)*	GH deficiency Obesity *(Cranial irradiation)* Hypogonadism Azoospermia *(Testicular irradiation)* Infertility Hypogonadism *(Alkylating agents)*	Alopecia *(Cranial irradiation)*
Brain tumor	CNS tumors Meningiomas Skin cancer *(Cranial/spinal irradiation)* MDS/leukemia *(Alkylating agents, topoisomerase inhibitors)*	Cardiac dysfunction *(Spinal irradiation)*	Scoliosis Short stature *(Cranial/spinal irradiation)* Dental abnormalities *(Cranial irradiation)*	Learning disabilities *(Cranial irradiation/ neurosurgery)* Cataracts Neurosensory deficits Vasculopathy *(Cranial irradiation)* Seizures Hydrocephalus *(Neurosurgery)* Hearing loss *(Platinum compounds, cranial irradiation)* Peripheral neuropathy Raynaud phenomenon *(Vincristine)*	Hypothalamic-hypopituitarism GH deficiency Obesity *(Cranial irradiation)* Infertility Hypogonadism *(Alkylating agents)*	Renal insufficiency *(Platinum compounds, ifosfamide)* Alopecia *(Cranial irradiation)*
Hodgkin lymphoma	Thyroid cancer Breast cancer Lung cancer Upper GI cancer Sarcomas Skin cancer *(Mantle irradiation)* Colon cancer GU cancers Skin cancer *(Abdominal, pelvic irradiation)* MDS/leukemia *(Alkylating agents, topoisomerase inhibitors)*	Carotid artery stenosis Coronary artery disease Valvular heart disease Cardiomyopathy Pericardial fibrosis Arrhythmia *(Mantle irradiation)* Cardiomyopathy Arrhythmias *(Anthracycline)*	Scoliosis Short stature *(Thoracic irradiation)* Osteopenia Osteoporosis *(Corticosteroids)*		Thyroid disease *(Mantle irradiation)* Infertility Hypogonadism *(Pelvic irradiation/ alkylating agents)*	High-risk pregnancy *(Irradiation, anthracyclines)* Life-threatening bacterial sepsis *(Splenectomy)* GI strictures *(Mantle irradiation)* Pulmonary fibrosis *(Mantle irradiation, bleomycin)*

Neuroblastoma	Skin cancer Sarcomas *(Any irradiation)* Breast cancer Thyroid cancer *(Thoracic irradiation)* MDS/leukemia *(Alkylating agents, topoisomerase inhibitors)*	Cardiomyopathy Arrhythmias *(Anthracycline)*	Scoliosis Short stature *(Thoracic irradiation, TBI)*	Hearing loss *(Platinum compounds)*	Thyroid disease *(Thoracic irradiation)* Infertility Hypogonadism *(Alkylating agents)*	Renal insufficiency *(Platinum compounds, ifosfamide, abdominal irradiation)* Pulmonary fibrosis *(Thoracic irradiation)* Small bowel obstruction *(Abdominal surgery)* Horner syndrome Scoliosis *(Thoracic surgery)*
Wilms tumor	Colon cancer Skin cancer Sarcoma *(Abdominal irradiation)* Skin cancer Breast cancer Lung cancer *(Lung irradiation)*	Cardiomyopathy Arrhythmias *(Anthracycline)*	Scoliosis Short stature *(Abdominal irradiation)*	Peripheral neuropathy Raynaud phenomenon *(Vincristine)*	Infertility Hypogonadism *(Alkylating agents)*	Small bowel obstruction *(Abdominal surgery)* Renal insufficiency *(Nephrectomy, abdominal irradiation)* Pulmonary fibrosis *(Lung irradiation)* High-risk pregnancy *(Irradiation, anthracyclines)*
Sarcoma	MDS/leukemia *(Alkylating agents, topoisomerase inhibitors)* Cancer of the skin/bone/ soft tissue/organs in the treatment field *(Irradiation)*	Cardiomyopathy Arrhythmias *(Anthracycline)*	Hypoplasia of the bone/soft tissue in irradiation field *(Irradiation)* Deformity of bone/soft tissue *(Surgery)* Dental abnormalities *(Irradiation)*	Hearing loss *(Platinum compounds)* Peripheral neuropathy Raynaud phenomenon *(Vincristine)*	Infertility Hypogonadism *(Alkylating agents)*	Renal insufficiency *(Platinum compounds, ifosfamide)* High-risk pregnancy *(Irradiation, anthracyclines)* Dysfunction of the organs in the treatment field *(Irradiation)*

CNS, Central nervous system; *GH*, Growth hormone; *GI*, gastrointestinal; *GU*, genitourinary; *MDS*, myelodysplastic syndrome; *MTX*, methotrexate; *TBI*, total-body irradiation.

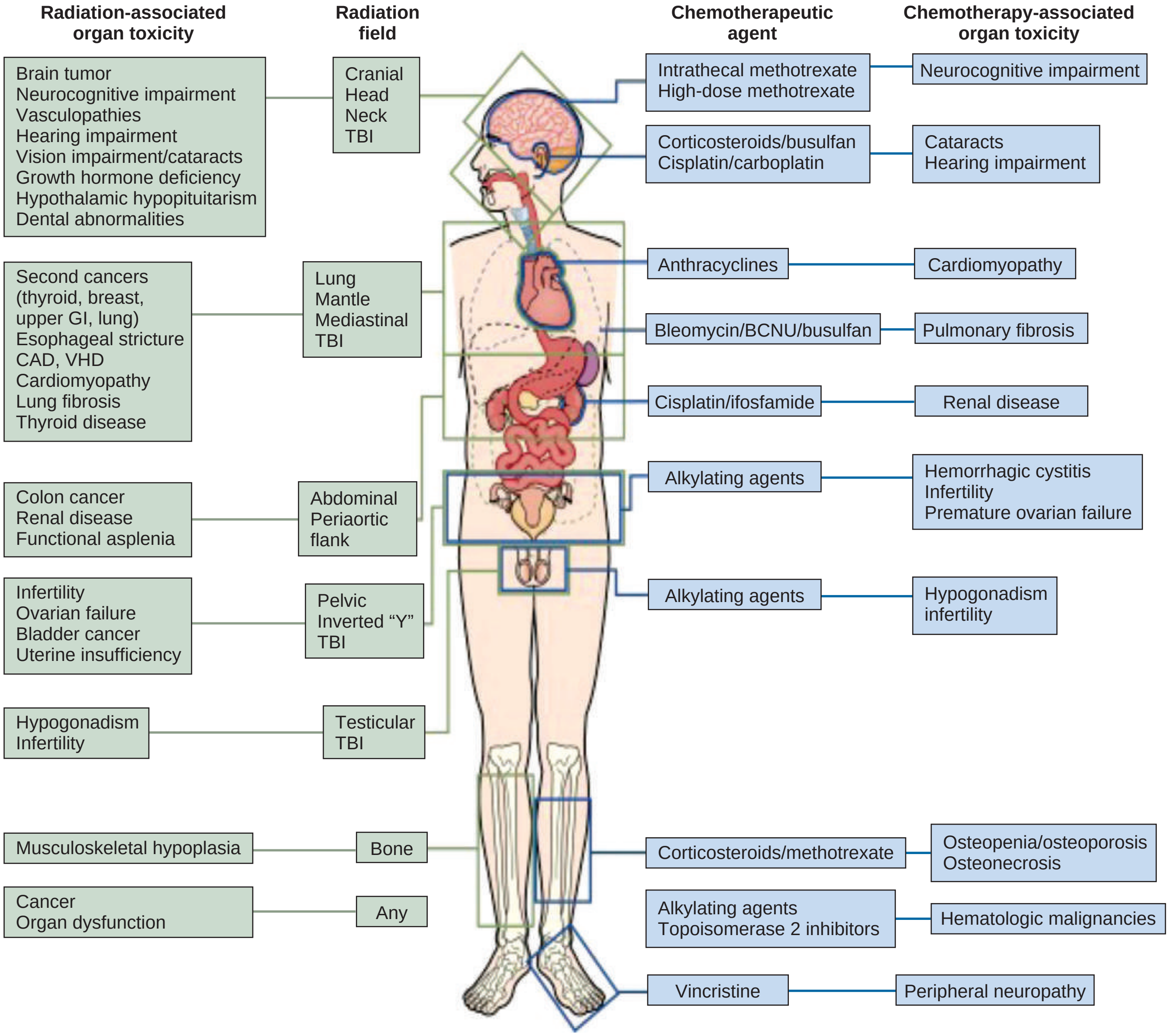

Radiation-associated organ toxicity
Radiation field
Chemotherapeutic agent
Chemotherapy-associated organ toxicity
Brain tumor
Neurocognitive impairment
Vasculopathies
Hearing impairment
Vision impairment/cataracts
Growth hormone deficiency
Hypothalamic hypopituitarism
Dental abnormalities
Cranial
Head
Neck
TBI
Intrathecal methotrexate
High-dose methotrexate
Neurocognitive impairment
Corticosteroids/busulfan
Cisplatin/carboplatin
Cataracts
Hearing impairment
Second cancers
(thyroid, breast,
upper GI, lung)
Esophageal stricture
CAD, VHD
Cardiomyopathy
Lung fibrosis
Thyroid disease
Lung
Mantle
Mediastinal
TBI
Anthracyclines
Cardiomyopathy
Bleomycin/BCNU/busulfan
Pulmonary fibrosis
Cisplatin/ifosfamide
Renal disease
Colon cancer
Renal disease
Functional asplenia
Abdominal
Periaortic
flank
Alkylating agents
Hemorrhagic cystitis
Infertility
Premature ovarian failure
Infertility
Ovarian failure
Bladder cancer
Uterine insufficiency
Pelvic
Inverted “Y”
TBI
Alkylating agents
Hypogonadism
infertility
Hypogonadism
Infertility
Testicular
TBI
Musculoskeletal hypoplasia
Bone
Corticosteroids/methotrexate
Osteopenia/osteoporosis
Osteonecrosis
Cancer
Organ dysfunction
Any
Alkylating agents
Topoisomerase 2 inhibitors
Hematologic malignancies
Vincristine
Peripheral neuropathy

Figure 72-8 Late effects associated with common treatments for childhood cancer by organ systems. *CAD,* Coronary artery disease; *GI,* gastrointestinal; *TBI,* total-body irradiation; *VHD,* valvular heart disease.

Family history of chronic diseases, mental illness, and family cancer history are also an important part of the comprehensive survivor evaluation. Cancer diagnosed in first-degree relatives (parents, offspring, siblings) in a pattern suggestive of a hereditary cancer predisposition syndrome should be noted and appropriate referrals to cancer geneticists considered (see Box 72-2). A family history that includes other children with cancer raises particular concerns about hereditary cancer syndromes, as does a family history of genetic syndromes associated with cancer predisposition. A survivor's family history of common chronic diseases should be noted, with special attention to conditions for which the survivor may have treatment-related risk factors, such as heart disease, osteoporosis, or depression. Family history should be updated and reassessed at each subsequent evaluation.

All survivors should be asked about educational attainment and special education services or needs. For older survivors, employment history and insurance status are also critical to determining health care needs. Health behaviors including exercise, diet, sleep, tobacco use, alcohol use, and nonprescription drug use are also important aspects of the follow-up assessment. To further evaluate a survivor's emotional and physical needs not addressed in a medical history, survivors should be asked about their relationships with family and peers, sexual activity, and current living arrangements.

A comprehensive childhood cancer survivor's evaluation also includes a review of current and recent symptoms. This review of symptoms should include special consideration of symptoms known to be associated with specific late effects. Symptoms should be evaluated in light of the increased prior probability of significant morbidity compared with similar-aged patients. For example, a complaint of chronic headaches or sinus problems in a patient with a history of head and neck irradiation should be evaluated in light of the risk for radiogenic tumors; a young patient with dyspnea or chest pain might undergo a cardiac evaluation with a lower threshold than a patient who was not exposed to cardiotoxic therapies as a child.

A thorough physical examination including a skin examination and neurologic examination are also part of a childhood cancer survivor's routine evaluation. Radiation fields in particular should be examined carefully for any skin changes, masses, or lesions. A careful thyroid examination is indicated, in particular for patients who received radiation to the head, neck, chest or upper spine. Head, trunk, and limbs should be examined for growth disturbances related to surgery or irradiation, such as limb-length discrepancies, spinal deformity, and facial bone deformity. Major organ toxicity associated with specific chemotherapy agents and radiation fields commonly used to treat pediatric cancers is illustrated in Figure 72-8. Transplant survivors should be examined for signs of chronic GVHD.

Diagnostic and Screening Tests

The health care provider evaluating the childhood cancer survivor should arrange for diagnostic or screening tests and subspecialty consultations based on the survivor's treatment risks, symptoms, or other findings. The provider should recommend surveillance studies assessing primary disease recurrence if indicated; studies to assess organ function in any organs potentially affected by cancer therapy; and specialist consultation to evaluate findings or potential health risks. Depending on exposures, age, and gender, screening studies to assess for second cancers such as mammography or colonoscopy should be considered. Clinical guidelines developed by the Children's Oncology Group are available to help direct clinicians in the evaluation of survivors of childhood, adolescent, and young adult cancer.[89] These risk-based, treatment-related guidelines are intended to provide screening recommendations for late effects commonly observed in survivors.

Assessment and Follow-Up Plan

A childhood cancer survivor's risk for each late effect should be interpreted in light of his or her treatment history, past medical history, family history, symptoms, and physical examination. All findings and test results from the comprehensive survivor evaluation should be summarized in a survivorship care plan, and recommendations and plans for follow-up should be documented and communicated to the patient and all providers involved in the patient's care. Acknowledging the potential complexity of the survivorship care plan, the provider should prioritize the follow-up recommendations for the survivor and family and emphasize that this advice is limited by our current knowledge of late effects and based on future probabilities.

In summary, the successful follow-up care for the childhood cancer survivor depends on thorough evaluation with attention to possible late effects and effective communication with patient, family, and other providers involved in ongoing care.

FUTURE DIRECTIONS

Advances in the treatment of childhood cancer have resulted in growing numbers of survivors. Epidemiologic studies show that survivors of childhood cancer are likely to experience adverse health-related consequences of their treatment, and the potential to develop late effects appears to be lifelong. Although remarkable progress has been made in describing the late effects experienced by childhood cancer survivors and their associated risk factors, future research is needed to continue to decrease incidence and morbidity and to improve the quality of life for current and future survivors. In response, survivorship research is focused on three main areas: changes in primary cancer therapy that are focused on decreasing incidence of late toxicity; improvements in care to decrease the incidence or severity of particular late effects; and changes in the health care systems to ensure that all survivors have access to best practices to detect and manage late effects.

Current treatment protocols for childhood cancer are designed to minimize late effects without compromising survival. The strategies that have been investigated or are under investigation include eliminating or reducing exposures to specific chemotherapies with known late toxicities, such as anthracyclines and alkylating agents, developing chemoprotectant agents, reducing radiation dose and field, and delaying radiation therapy for very young children.[445-451] Studies to quantify the efficacy of these strategies to obviate late effects while maintaining survival are forthcoming. Current strategies for primary treatment of standard-risk acute lymphoblastic leukemia, localized Hodgkin lymphoma, Wilms tumor, and neuroblastoma designed to reduce late effects have resulted in excellent outcomes, with low incidence of both recurrence and treatment-related late effects, to date.[180,448,451,452]

Another strategy to reduce late morbidity in childhood cancer survivors is the identification of individuals with heightened susceptibility for specific treatment-associated late effects. One potential method of identification is genetic polymorphism testing, which identifies individuals with a genotype that is known to increase susceptibility to treatment-associated toxicity, such as second cancers, obesity, and cardiac disease.[128,453-455] Testing could be done before cancer treatment and the results used to modify treatment plans to account for an individual's genetic susceptibility for late effects. For example, lower-dose radiation therapy might be indicated for an individual identified by genetic polymorphism testing as having a genotype associated with irradiation-induced second cancers. Similarly, genetic polymorphism testing could be done decades after therapy to identify survivors at risk for late effects, which would inform screening recommendations and eligibility for preventive interventions.

Health services research focused on understanding cancer survivor care and studying improvements in care is ongoing. As the current population of childhood cancer survivors ages, health care delivery to this group will become more complex and will include integration of treatment-related risks and adult-onset morbidity. Optimal and innovative methods of patient (and health care provider) education that achieve this integration will need to be elucidated and disseminated to ensure the best possible quality of life for survivors of childhood cancer.

The care of the child diagnosed with cancer does not end with the completion of therapy. Consequently, pediatric oncology providers need to have a working knowledge of the potential late effects of curative therapy for childhood cancer and are responsible to communicate information about late effects to survivors, their families, and other health care providers involved in the care of childhood cancer survivor. Pediatric survivorship research has made significant contributions to advance our understanding of long-term treatment–associated morbidities, and current therapeutic protocols are designed with the intention of minimizing known late effects. However, despite progress, childhood cancer survivors remain vulnerable to many long-term treatment related morbidities. Ongoing efforts in research and clinical care for childhood cancer survivors must be directed toward improving and extending follow-up care, at developing interventions for individuals identified as being at risk for specific late effects, and at optimizing management of late effects with the ultimate aim of improving quality of life for all current and future childhood cancer survivors.

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This study is one of the first descriptions of the association between treatment of childhood cancer and the development of second primary malignancies decades after treatment. In this single-institution study researchers reported the 20-year cumulative probability of a second cancer (12% SE 4%). They describe the association between therapeutic radiation and second cancers as well as suggest that host susceptibility may be an etiologic factor.

21. Armstrong GT, Liu Q, Yasui Y, et al: Late mortality among 5-year survivors of childhood cancer: a summary from the Childhood Cancer Survivor Study. *J Clin Oncol* 27:2328–2338, 2009.

This is a comprehensive summary of the CCSS mortality data based on a cohort of 20,483 childhood cancer survivors, representing 337,334 person-years of observation, linked to the National Death Index and death certificates. Overall cumulative mortality, patterns of late death over time, cause-specific mortality, and specific treatment-related risk factors for late mortality are described.

29. Robison LL, Mertens AC, Boice JD, et al: Study design and cohort characteristics of the Childhood Cancer Survivor Study: a multi-institutional collaborative project. *Med Pediatr Oncol* 38:229–239, 2002.

The CCSS is a consortium of 25 North American institutions representing one of the largest and most extensively characterized cohorts of childhood and adolescent cancer survivors (diagnosed prior to age 21 years with selected cancer diagnoses, diagnoses between 1970-1986, and survival for at least 5 years). This reference is a description of the methodology used to develop the CCSS, which serves as a resource for addressing risk for second malignancies, endocrine and reproductive outcomes, cardiopulmonary complications, and psychosocial implications among childhood cancer survivors.

33. Reulen RC, Frobisher C, Winter DL, et al: Long-term risks of subsequent primary neoplasms among survivors of childhood cancer. *JAMA* 305:2311–2319, 2011.

The British Childhood Cancer Survivor Study is a population-based cohort of 17,981 5-year survivors of childhood cancer diagnosed with cancer when younger than 15 years between 1940 and 1991 in Great Britain, followed up through December 2006. In this study the cohort is used to investigate the long-term risks of subsequent primary neoplasms in survivors of childhood cancer, to identify the cancer types that contribute most to long-term excess risk, and to identify subgroups of survivors at substantially increased risk for particular subsequent primary neoplasms.

36. van der Pal HJ, van Dalen EC, van Delden E, et al: High risk of symptomatic cardiac events in childhood cancer survivors. *J Clin Oncol* 30:1429–1437, 2012.

This cohort study assessing the long-term risk for clinically validated, symptomatic cardiac events in 5-year survivors of childhood cancer demonstrated that higher cumulative anthracycline dose or higher cumulative radiation dose was associated with higher risk ratio for cardiac events. Survivors with both anthracycline and cardiac irradiation exposures had the highest cumulative incidence of cardiac events, with nearly one in eight survivors treated with both exposures developing a serious cardiac event.

46. Oeffinger KC, Mertens AC, Sklar CA, et al: Chronic health conditions in adult survivors of childhood cancer. *N Engl J Med* 355:1572–1582, 2006.

This report from the Childhood Cancer Survivor Study details chronic health conditions in adult survivors of childhood cancer. The relative risk for a severe or life-threatening chronic condition in survivors when compared with siblings was 8.2 (95% CI, 6.9-9.7).

47. Hudson MM, Ness KK, Gurney JG, et al: Clinical ascertainment of health outcomes among adults treated for childhood cancer. *JAMA* 309:2371–2381, 2013.

This report of clinically ascertained health outcomes among adult survivors of childhood cancer from the St. Jude Lifetime Cohort Study presents the prevalence and adverse health outcomes of 1713 adult survivors of childhood cancer. Survivors underwent comprehensive, risk-based clinical assessment. The cumulative prevalence of a chronic condition was estimated as 95.5% (95% CI, 94.8%-98.6%) and the cumulative prevalence of a severe or life-threatening/disabling condition was estimated as 80.5% (95% CI, 73.0%-86.6%) by age 45 years.

51. Friedman DL, Whitton J, Leisenring W, et al: Subsequent neoplasms in 5-year survivors of childhood cancer: the Childhood Cancer Survivor Study. *J Natl Cancer Inst* 102:1083–1095, 2010.

In this study, the CCSS cohort was used to update the incidence of and risk for subsequent neoplasms occurring 5 years or more after the childhood cancer diagnosis from 14,359 5-year survivors in the CCSS who were at a median age of 30 years (range, 5 to 56 years) at follow-up. Cumulative incidence of subsequent neoplasm at 30 years, standardized incidence ratios, excess absolute risks for invasive second malignant neoplasms, and relative risks for subsequent neoplasms are presented. The study confirms that as childhood cancer survivors progress through adulthood, risk for subsequent neoplasms increases and there is no evidence of risk reduction with increasing duration of follow-up.

55. Garber JE, Offit K: Hereditary cancer predisposition syndromes. *J Clin Oncol* 23:276–292, 2005.

This review highlights the most common hereditary cancer syndromes and a number of rare syndromes in which particular progress has been made. The prevalence, penetrance, tumor spectrum, and underlying genetic defects are discussed and then summarized in a table in which a comprehensive listing of known hereditary cancer syndromes is provided.

61. Kenney LB, Yasui Y, Inskip PD, et al: Breast cancer after childhood cancer: a report from the Childhood Cancer Survivor Study. *Ann Intern Med* 141:590–597, 2004.

This study identifies risk factors for secondary breast cancer beyond chest radiation therapy among female survivors of childhood cancer. In the CCSS cohort, breast cancer risk for female survivors was independently associated with prior chest radiation therapy, primary diagnosis of bone and soft tissue sarcoma, family history of breast cancer, and history of thyroid disease. In addition, prior pelvic radiation was found to reduce breast cancer risk.

67. Bassal M, Mertens AC, Taylor L, et al: Risk of selected subsequent carcinomas in survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *J Clin Oncol* 24:476–483, 2006.

This study describes childhood cancer survivors' risk for subsequent carcinomas other than the three most common (breast, thyroid, and skin) using standardized incidence ratios calculated from the CCSS cohort and Surveillance, Epidemiology, and End Results program data. Young survivors of childhood cancers were found to be at increased risk for developing subsequent carcinomas typically associated with older adulthood, including those of the genitourinary system, head and neck area, and gastrointestinal tract.

73. Henderson TO, Oeffinger KC, Whitton J, et al: Secondary gastrointestinal cancer in childhood cancer survivors: a cohort study. *Ann Intern Med* 156:757–766, 2012.

This analysis from the CCSS characterizes risk factors for childhood cancer survivors to develop secondary gastrointestinal cancer. Results show the greatest risk for subsequent gastrointestinal cancer is prior abdominal radiation; however, high-dose procarbazine and platinum chemotherapy were also found to independently increase the risk.

75. Travis LB, Hill DA, Dores GM, et al: Breast cancer following radiotherapy and chemotherapy among young women with Hodgkin disease. *JAMA* 290:465–475, 2003.

This matched case-control study of 3817 female Hodgkin lymphoma survivors provides a detailed analysis of the relative risk for secondary breast cancer associated with both radiation dose delivered to site of breast cancer or to ovaries and with the cumulative dose of alkylating agent chemotherapy. Results of this study support the importance of hormonal stimulation for the development of radiation-induced breast cancer, as evidenced by the reduced risk associated with ovarian damage from alkylating agents or ovarian radiation.

89. Children's Oncology Group: *Long-Term follow-up guidelines for survivors of childhood, adolescent and young adult cancers, version 3.0*, Arcadia, CA, 2008, Children's Oncology Group. Accessed at: http://www.survivorshipguidelines.org/.

These Children's Oncology Group Long-Term Follow-Up Guidelines for Survivors of Childhood, Adolescent, and Young Adult Cancer (COG-LTFU Guidelines) are evidence-based recommendations for screening and management of late effects of therapeutic exposures. They are updated by a multidisciplinary panel based on current literature review and expert consensus.

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Treatment of childhood cancer is associated with a variety of thyroid abnormalities. This early analysis from the CCSS comparing Hodgkin lymphoma survivors to sibling controls describes relative risks for hypothyroidism, hyperthyroidism, thyroid nodules, and thyroid cancer. It also characterizes the interaction between various patient and treatment variables. Female sex, radiation dose, and younger age at diagnosis were found to be independent risk factors for thyroid disease.

110. Henderson TO, Whitton J, Stovall M, et al: Secondary sarcomas in childhood cancer survivors: a report from the Childhood Cancer Survivor Study. *J Natl Cancer Inst* 99:300–308, 2007.

Childhood cancer survivors are at increased risk for secondary sarcomas. This analysis from the CCSS determined the incidence of secondary sarcomas and examined factors associated with the risk for developing secondary sarcomas. In a multivariable model, increased risk for secondary sarcoma was associated with radiation therapy, with a primary diagnosis of sarcoma, and with treatment with anthracyclines.

126. Taylor AJ, Little MP, Winter DL, et al: Population-based risks of CNS tumors in survivors of childhood cancer: the British Childhood Cancer Survivor Study. *J Clin Oncol* 28:5287–5293, 2010.

A large cohort study of CNS tumors in survivors of childhood cancer from the British Cancer Survivor Study linked to national population–based cancer registries investigated treatment-related risk factors. Investigators found that the risk of secondary meningioma increases linearly and independently with increased dose of radiation to meningeal tissue and also with increased dose of intrathecal methotrexate. The risk for secondary glioma/primitive neuroectodermal tumors also increased linearly with dose of therapeutic radiation.

130. Nottage K, McFarlane J, Krasin MJ, et al: Secondary colorectal carcinoma after childhood cancer. *J Clin Oncol* 30:2552–2558, 2012.

In this nested case-control study of 13,048 childhood cancer survivors from St Jude Children's Research Hospital, the long-term risk for secondary adenocarcinoma of the colon or rectum is reported to be 10-fold greater in survivors compared with the age-matched general population. Matched-pairs analyses found that radiation and alkylator exposure are associated with increased risk for secondary colorectal carcinoma and that the risk is proportional to dose and volume of radiation received. These data support the recommendation for early colon cancer screening for survivors receiving high-risk exposures.

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This report assesses the incidence of and risk factors for late-occurring stroke among survivors of childhood leukemia and brain tumors. The relative risk for stroke for leukemia survivors was 6.4 (95% CI, 3.0-13.8) and for brain tumor survivors it was 29.0 (95% CI, 13.8-60.6) compared with a sibling comparison group.

214. Armstrong GT, Oeffinger KC, Chen Y, et al: Modifiable risk factors and major cardiac events among adult survivors of childhood cancer. *J Clin Oncol* 31:3673–3680, 2013.

In this assessment of adult survivors of childhood cancer from the CCSS, two or more cardiovascular risk factors were reported by 10.3% of survivors. In this report, acquisition of modifiable cardiovascular risk factors, particularly hypertension, increased the risk for a severe, life-threatening, and fatal cardiac event, reinforcing the importance of early diagnosis and careful management of modifiable cardiovascular risk factors in survivors.

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In this report from the CCSS, survivors of childhood cancer were found to have a significantly increased risk for pulmonary complications compared with siblings, including an increased risk of lung fibrosis, recurrent pneumonia, and use of supplemental oxygen.

223. Cohen LE: Endocrine late effects of cancer treatment. *Endocrinol Metab Clin North Am* 34:769–789, xi, 2005.

This review article describes the endocrinopathies that are associated with the treatment of childhood cancers, specifically hypothalamic-pituitary, thyroid, and gonadal dysfunction, as well as osteopenia-osteoporosis and obesity. The article also addresses the risk factors for endocrine complications, including the underlying disease, treatment with cytotoxic agents, and radiation therapy.

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In the CCSS cohort, the 20-year cumulative incidence of osteonecrosis was 0.43%, with survivors 5.6 times more likely to have reported osteonecrosis compared with siblings. The risk for osteonecrosis was greatest among survivors of allogeneic stem cell transplant for underlying diagnoses of leukemia in survivors of acute lymphoblastic leukemia, acute myelogenous leukemia, and bone sarcoma.

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    This is a comprehensive review of the literature that informed the COG-LTFU guidelines task force recommendations for the assessment and management of female reproductive complications after treatment of childhood, adolescent, and young adult cancers. Topics include all aspects of female reproductive and sexual health, including hypogonadism, precocious puberty, and reduced fertility. Also reviewed is current knowledge in other areas relevant to the reproductive health of female survivors of childhood cancer, including investigational fertility preservation, interventions for infertility, and sexual function.

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    This study from the Childhood Cancer Survivor Study cohort evaluates the incidence of long-term gastrointestinal disease for childhood cancer survivors and identifies treatment-related risk factors. Compared with siblings, survivors had increased risk for late onset complications of the upper gastrointestinal tract, liver, and lower gastrointestinal tract. Older age at diagnosis, intensified therapy, abdominal radiation, and abdominal surgery increased the risk for certain gastrointestinal complications.

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CHAPTER 73

Psychosocial Care of the Child and Family

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CHAPTER OUTLINE

CONCEPTUAL MODELS GUIDING THE PSYCHOSOCIAL CARE OF CHILDREN AND FAMILIES
PROFESSIONAL ROLES AND A TEAM APPROACH TO PSYCHOSOCIAL CARE
CRISIS OF A CANCER DIAGNOSIS
Presenting the Diagnosis and Plan for Treatment: Day One Talk
Parents' Reactions to a Child's Diagnosis
Children's Reactions to Diagnosis
Education and Information about Treatment
ADJUSTING TO TREATMENT
Psychosocial Assessment of the Patient and Family
Support for Parents and Families during Treatment
EXPECTABLE CHALLENGES DURING TREATMENT
Coping with Treatment and Adverse Effects
Maintaining Child and Family Routines
Transitions and Changes in Care
Home Care
SPECIAL ISSUES THAT MAY ARISE DURING TREATMENT
Marital Adjustment
Nonadherence
Depression and Anxiety
Relapse and Recurrence
SPECIAL POPULATIONS
Adolescents
Neurooncology
Hematopoietic Stem Cell Transplant

The diagnosis and treatment of cancer represents a major challenge for the family and the individual child. For most families, coping with a child's cancer will be the most stressful and difficult experience they will face. Although the majority of families will make an adequate adjustment,[1-4] coping with the diagnosis and the subsequent challenges of treatment is a difficult and demanding process.[5-9] In this chapter we will address the emotional and psychosocial needs of children and their families while the child receives a diagnosis of cancer and undergoes therapy, and describe some resources and interventions that may be useful. This chapter is intended primarily for oncologists, nurse practitioners, and other medical professionals who are seeking to understand and attend to the psychosocial needs of children with cancer. In each section we highlight the issues facing the child and family and suggest supportive strategies or interventions that may be helpful. A description of the psychosocial clinician's role is included in certain sections so that oncology providers can better understand and collaborate with psychosocial clinicians, but readers looking for a detailed description of psychosocial assessment and treatment techniques will need to look beyond this chapter to more comprehensive treatments of the subject.[4,10,11,12,13] Given the great variability in oncology diagnoses and treatments, as well as individual differences in children and families, we will focus on the issues that are most common in this population. Beginning with the crisis of diagnosis and proceeding chronologically through the treatment period to the end of therapy, we will address the issues that are expected to arise for most families.

CONCEPTUAL MODELS GUIDING THE PSYCHOSOCIAL CARE OF CHILDREN AND FAMILIES

In approaching the topic of psychosocial care for the child and family, we have adopted a conceptual model developed by Anne Kazak[14] that illustrates the different levels of need seen in families of children with cancer (Fig. 73-1). In this model, adapted from the preventative mental health model,[14] Kazak represents the distribution of families along the continuum of needs in the form of a pyramid, with the least acute needs at the bottom and

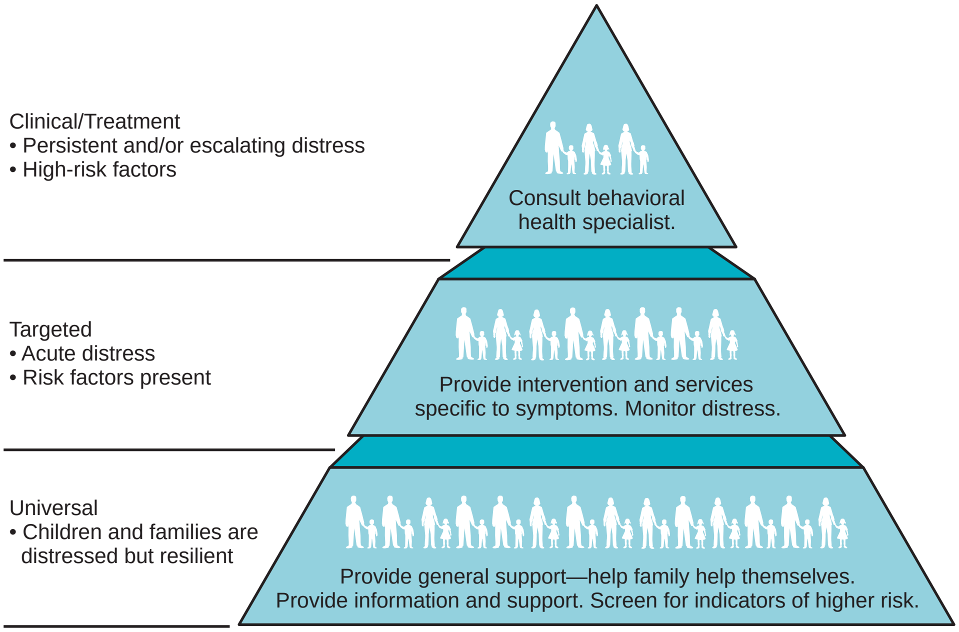
Clinical/Treatment
• Persistent and/or escalating distress
• High-risk factors
Consult behavioral health specialist.
Targeted
• Acute distress
• Risk factors present
Provide intervention and services specific to symptoms. Monitor distress.
Universal
• Children and families are distressed but resilient
Provide general support—help family help themselves.
Provide information and support. Screen for indicators of higher risk.

Figure 73-1 Pediatric psychosocial preventative health model. *(From Kazak AE: Pediatric psychosocial preventative health model [PPPHM]: research, practice, and collaboration in pediatric family systems medicine.* Fam Syst Health *24:381–395, 2006.)*

the most acute needs at the top. The largest section of the pyramid at the bottom represents the majority of patients and families who have expected levels of distress but generally have adequate resources and may benefit from information and psychosocial support. Moving up the pyramid, the second largest group of families represented have more significant needs according to Kazak, either because of existing risk factors or the presence of more acute distress. Finally, at the top of the pyramid is the smallest group of families, whose level of distress is in the clinical range and for whom some significant behavioral or psychiatric intervention is required. This chapter focuses on describing the needs of the largest group of families with “universal” or expected needs and discussing the support strategies and interventions that can be helpful for them. In addition we address some of the special issues that are likely to be seen in the “targeted” group of families such as noncompliance or marital conflicts that are not typical but that the treatment team should be aware of and be prepared to address. In the less common situations in which patients or parents require significant mental health interventions, referrals to specialists are necessary; these situations will be discussed briefly at the end of this chapter.

The ecological and developmental perspectives are also essential for understanding the experience of the child and family going through cancer therapy. Urie Bronfenbrenner (1917-2005) created the modern concept of the ecology of human development.[15] In his model no one factor operates in isolation, and the biopsychosocial model is considered within a developmental context. At the core of the model, the child has genetic and environmental influences on emotions, cognition, and behavior. In addition the child’s own responses to the environment not only influence his or her emotions, thoughts, and behaviors but in turn influence the microenvironment.[16,17] For example a child who responds to new situations with aggression or fear will elicit different responses from medical providers than will a child who responds with curiosity, and in that way the child shapes the environment to which he or she was initially reacting. At the next level of the model, the family system is a powerful factor that directly affects the child and shapes many of the child’s interactions with the environment. Family factors including family composition, parental emotional states, socioeconomic status, and other family stressors will influence the child’s experience of illness.[18-22] For example parental depression might reduce the extent of nurturing and support provided to a child with cancer. Recommendations for family-centered care as the primary model of care delivery for children are supported by this aspect of the model (Fig. 73-2).[20]

Finally, at the broadest level, racial, ethnic, community, and cultural factors can have an impact on the entire family/child system (Fig. 73-3).[23]

From a developmental perspective, children go through five recognizable stages of development from the newborn period until adulthood. These stages typically are thought of as infant, toddler, preschool, school age, and adolescence. Different developmental tasks are associated with each stage, and the needs for information, support, and care will vary in relation to the developmental stage of the child. A review of how to communicate with sick children during different developmental stages is provided by Rushforth.[24] Pediatric health care providers are accustomed to tailoring their general approach to patients and families according to the child’s developmental stage and will draw on these skills and experience

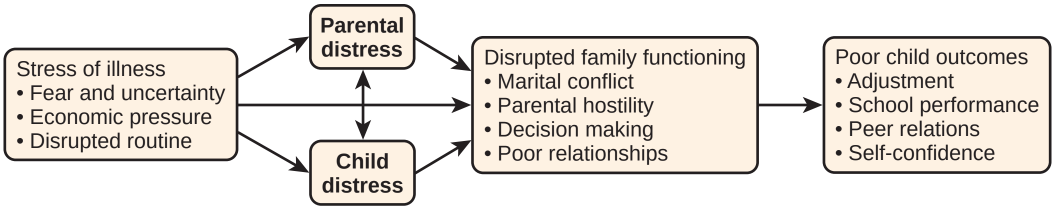

Figure 73-2 Family stress model. *(Modified from Conger KJ, Rueter MA, Conger RD: The role of economic pressure in the lives of parents and their adolescents: the family stress model. In Crockett LJ, Silbereisen RJ, editors:* Negotiating adolescence in times of social change. *Cambridge, England, 2000, Cambridge University Press, p. 201–233.)*

when working with children who have cancer. For example infants generally need a significant degree of parental soothing and physical contact, as do toddlers, yet toddlers need room for explanation and consistency in the environment. Preschool-aged children can understand simple information that can help reduce fears of the unknown. School-aged children need to have opportunities for peer interaction, as well as clear communication. Adolescents have a need for the development of autonomy and identity formation. When little room for individual choice is perceived by the adolescent, the drive for independence can lead to maladaptive coping, such as nonadherence to medications or refusals to come to the clinic or undergo medical procedures. Adolescents with cancer by necessity become more dependent upon their parents for physical and emotional needs and may have self-image issues related to weight loss or baldness, because during this developmental period perceptions of peer acceptance are important and sexuality develops as well.[25,26] The specific needs of the adolescent patient are discussed in a separate section.

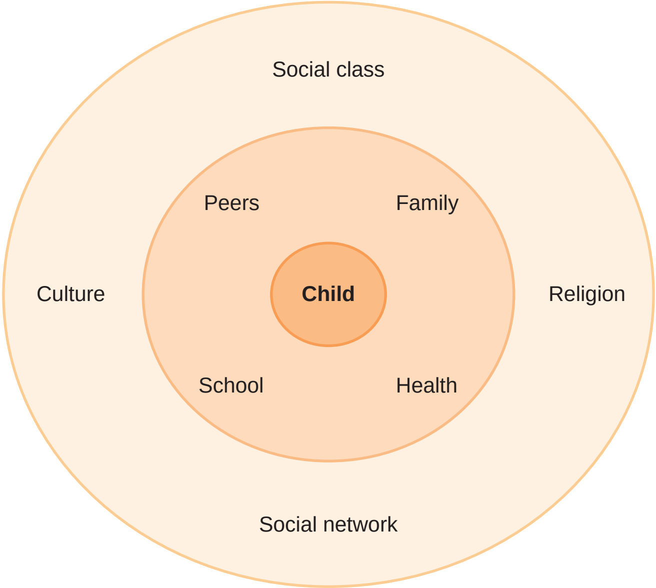

Figure 73-3 Child health from an ecological perspective. *(Modified from Kazak AE: Pediatric psychosocial preventative health model [PPPHM]: research, practice, and collaboration in pediatric family systems medicine.* Fam Syst Health *24:381–395, 2006.)*

PROFESSIONAL ROLES AND A TEAM APPROACH TO PSYCHOSOCIAL CARE

No single individual is able to provide for all the psychosocial needs of the child and family undergoing cancer therapy. In many pediatric oncology settings a team approach[27-30] is used, with the team composed of both medical and psychosocial care providers. The composition of the medical members of the team vary from setting to setting, but the team generally includes an attending oncologist, a surgeon, and a radiation oncologist (if these treatment modalities are indicated), as well as other oncology care providers such as an oncology fellow, a nurse practitioner or physician's assistant, and an oncology nurse. Psychosocial members of the team may include a resource specialist, social worker, psychologist, child life specialist, or psychiatrist. Variation in the professions included in the psychosocial support team in a particular treatment setting generally has more to do with local culture and tradition than with the specific training or skills of a particular profession. Although each psychosocial discipline has its particular focus or specialized skill set[31-38] (e.g., prescriptive authority or psychological testing) in working with children who have cancer and their families, considerable overlap between their roles is likely.

Psychosocial professionals have several important roles on the care team. The primary role may be as a consultant to other members of the team, including the patient and the family.[39-42] Because all team members will be interacting and providing some level of information and support, they will need to attend to psychosocial concerns, and developing a consistent approach to support of the family is essential.[43] As a consultant, the psychosocial team members should conduct a preliminary assessment of the child and family to understand their history, preferred ways of coping, important supports, and potential sources of vulnerability. Using this knowledge of the family, the psychosocial care provider may be able to help the other team members understand the family's individual needs and consider best approaches for supporting them. Similarly when the patient or family has questions and concerns that are not resolved in the course of routine care, the psychosocial care provider may help them work on solving the issue and think about how to help them raise their concerns with the medical team. The emphasis of these consultations is on improving communication between the family and the medical team, and in most instances these consultations focus on clarifying and correcting misunderstanding rather than resolving conflicts. In cases in which the family is very reluctant to address a concern with the medical team, or when a more significant conflict exists with the medical team, the psychosocial team member may be asked to play a more formal consultant role. Psychosocial team members also fulfill the role of direct service providers to patients and families, providing individual or family meetings to offer support to the parents, patient, or family, as well as education and preparation for surgery and other medical procedures. Support groups run by psychosocial and/or nursing staff in the inpatient or outpatient setting may be very helpful for meeting families' educational and emotional needs.[44-47] Finally, the psychosocial provider's role often includes consultation and liaison with outside agencies and community resources.[48-51] This aspect may include assisting families in accessing financial and insurance resources, contacting schools, tutors, and other child care agencies, and helping identify additional support services in the community, including referrals for psychotherapy when appropriate.

CRISIS OF A CANCER DIAGNOSIS

The cancer diagnosis and the events leading up to it are typically frightening, complex, and unfamiliar.[7,8,52] The diagnosis period, which can be thought of as beginning when the child first comes to medical attention until a clear diagnosis and treatment plan are made, is foremost a period of uncertainty. At some point in this period, parents are told cancer is possible or even likely, but it may take time before a definitive diagnosis is made. During this period their child's symptoms may persist and the child often must face painful diagnostic tests and procedures.

Until their child is diagnosed, most families have never thought about childhood cancer and don't know anyone who has had to face it. Whereas families facing common stressful life events can be guided by their prior experiences and the experience of their friends and family, the cancer diagnosis pushes them beyond the realm of ordinary experience. Not surprisingly parents are typically overwhelmed emotionally and cognitively while they try to understand their child's illness. In addition parents may describe feelings of unreality, "like this is not really real." Children's immediate reactions to illness depend largely on their age, their symptoms, and the medical procedures they have had to undergo. Except in cases of older adolescents, it is most often the parents who are most anxious to learn the diagnosis and are the first ones to be informed of the diagnosis of cancer.

Presenting the Diagnosis and Plan for Treatment: Day One Talk

The diagnostic period comes to a close when the treatment team has made a clear diagnosis of the child's cancer and communicates this diagnosis and related treatment plan to the patient and family. Often referred to as the "day one talk," this conversation with the family usually takes place in the form of a treatment conference between the family and several members of the care team, including physicians, nurses, and psychosocial care providers. The day one talk is a comprehensive discussion and usually the first to address the child's prognosis and specific treatment recommendations. For many families this meeting is a pivotal event in their lives, marking the real beginning of their child's cancer. Throughout the diagnostic phase most families maintain hope that the child does not in fact have cancer, so the day one talk is similar to other situations in which a physician must communicate bad news to the family. The literature on communicating bad news about a child's health[53-57] may be relevant, and recommendations from these noncancer settings may be instructive. Box 73-1 provides a summary of essential points for communicating the diagnosis.

Although the medical details discussed will vary from one family to the next, challenges in communicating diagnostic, prognostic, and treatment information are consistent across families and can be considered and planned for.[58-61] Planning is essential for a successful meeting with the family, and although there may be pressure from family or medical staff to have the conversation as soon as possible, some minimal amount of scheduling and organization will be required. A written protocol for communicating the diagnosis has been recommended,[60] with the expectation that this protocol will

Box 73-1 **Summary of Essential Points: Principles for Communicating the Diagnosis**

STRUCTURE—ORGANIZATION

- Establish an initial plan for communication.
- Communicate in a private and comfortable space where interruptions will be minimized.
- Communicate with the parents, child, and other family members, if desired. Nursing staff and psychosocial workers should also be included whenever possible.
- Hold a separate session with the child if he or she is not present for the initial communication.

CONTENT—INFORMATION TO DISCUSS

- Solicit questions from the parents and child, especially regarding their understanding of the present illness.
- Share information about the diagnosis, treatment, and the plan for cure and/or goals of treatment.
- Share information on lifestyle and psychosocial issues, particularly surrounding causation (e.g., the cancer is neither the child's nor family's fault).

PROCESS—PROCESS OF COMMUNICATION

- Communicate immediately at diagnosis and follow-up later.
- Communicate at a pace the family can follow, allowing the child's or family's emotional reaction to guide the flow of the conversation.
- Communicate in ways that are sensitive to cultural differences (e.g., for families who are not native English speakers, arrangements should be made for an interpreter).
- Encourage the entire family to talk together.

Modified from Krahn GL, Hallum A, Kime C: Are there good ways to give bad news? Pediatrics *91:578–582, 1993; Mack JW, Grier HE: The day one talk.* J Clin Oncol *22:563–566, 2004; and Masera G, Chesler MA, Jankovic M, et al: SIOP Working Committee on Psychosocial Issues in Pediatric Oncology: guidelines for communication of the diagnosis.* Med Pediatr Oncol *28:382–385, 1997.*

be tailored to meet the needs of the patient and family. Conducting the meeting in a private space while all participants are free from interruptions is very important, as is scheduling the meeting so that the important members of the family and care team can attend. Because families typically find the day one talk overwhelming[58,62-64] and may not remember many of the details discussed, some practitioners recommend providing written material or making a tape recording of the meeting for the parents to review subsequently.[63,65-68] Reassuring families that they are not expected to remember all of the details discussed can reduce their sense of information overload, as will making explicit the critical pieces of information they are being asked to focus on.

In addition to the oncologists directly caring for the patient, the day one talk should include nurses and psychosocial caregivers whenever possible.[69,70] The organization and roles of the medical center staff are often confusing to families, and taking time to introduce the different members of the team and describe their roles is essential. Both parents should attend, and they should be offered the opportunity to include other family members involved in the care of the child. Whether to include the child in all or part of this session or to arrange for a separate session for the child will depend largely on the age of the child and the parents' preference. Although a subsequent meeting with a smaller group consisting of parents and one or two medical professionals may be more appropriate for younger children,[71-73] including adolescents in the day one talk can help them develop trust in the medical team and invest in their own care.[58,74]

The principal goal of the meeting is to provide the family with an overview of the child's diagnosis, the treatment recommendations, and the prognosis. It is important to directly elicit questions the patient or family may have and to provide sufficient time to discuss their concerns. Mack and Grier[58] emphasize the opportunity to address the family's questions about what may have caused the child's cancer and particularly to correct misconceptions that may lead to a sense of blame or responsibility for the cancer. Communicating prognostic information to the family is critical because it is likely to have an impact on their understanding of the treatment recommendations,[75,76] but delivering this information can be difficult, especially when the prognosis is guarded or poor. How much to rely on a numeric presentation of this information is a question that frequently arises, and some evidence indicates that patients may misunderstand the information[77-79] if it is not carefully explained. Numeric information about risk, such as 5-year survival rates, should not be avoided altogether because many families want this information,[80] but care should be taken to ensure that parents receive the information in the way they find most useful and that they understand the information that is provided. Recommendations for treatment should be presented, beginning with a general overview of the modalities used and the timing and setting for treatments. The introduction of consent forms and discussion of known and likely adverse effects (short term and long term) should follow after the family understands the general outline of the treatment.

If a separate session for the child is held, the same general information about diagnosis, prognosis, and treatment plan should be covered. The information will need to be tailored to the developmental level and the particular concerns of the patient, such as the ability to return home and to school, to see friends, and to participate in sports or other activities.[74] Children should be told about specific expectable adverse effects such as hair loss, with an emphasis on concrete ways in which their appearance or activities may be affected. Older children and adolescents may be able to engage directly with the medical providers, but younger children may be more comfortable talking with their parents, who can elicit their questions and reinforce what medical providers communicate to the child directly. Most professionals will emphasize the importance of the children knowing the name of their disease and that it is cancer. Parents may initially shy away from this direct approach and particular words like "cancer," and they may need some preparation for how to talk with their child and what words to use. Understandably many parents feel protective toward their children and may wish to avoid using the words "cancer" or "leukemia." It is important to help parents understand that honesty, even about potentially frightening information, can decrease anxiety and be supportive for children in the long term because they can grow to trust that adults are not withholding information and that they need not fear unpleasant surprises. Similarly it is important to explain that the word "cancer" is commonly used with pediatric patients and that it is important for children not to feel that their condition is so frightening it cannot be named or spoken about. It can be helpful to share with parents that the climate of open communication with young patients comes from lessons learned in past treatment eras when communication was not open, which contributed to children's anxiety and fear about their disease. With this sort of preparation, most parents can begin to feel comfortable with open communication with the child. Parents from different cultural backgrounds,[73,81,82] or those who are unused to open discussions of illness, may not easily accept this openness and naming of the disease, and some careful negotiation and compromise may be required to reach an agreement of what words to use and topics to discuss with the patient. Over time families may become more comfortable being open with the patient themselves, while they meet other families and children whom they see are comfortable with this type of open discussion. In an elegant description of the day one talk in pediatric oncology, Mack and Grier[58] highlight the importance of forming an alliance between the family and the medical provider and the ways in which this alliance can affect adjustment to the diagnosis. In particular they emphasize the importance of listening closely, making an emotional connection with the family, and acknowledging the overwhelming impact of the diagnosis on every aspect of the family's life. Although they provide some very specific guidelines for the information that should be discussed, they conclude, "…listening and the openness of silence may be more important than the words themselves."[58]

Parents' Reactions to a Child's Diagnosis

Many factors can contribute to the overwhelming nature of the cancer diagnosis. The word "cancer" continues to have a significant social stigma,[83-88] as well as personal connotations. For many parents, their personal experiences with cancer may be limited to relatives who were diagnosed later in life and may not have survived long after diagnosis. Although it can be helpful to learn about these personal associations with cancer, it is very important to clearly correct parents' explicit or implicit assumptions as they apply to their child's illness. Families who have lost family members to cancer may need very explicit explanations that a parent's or grandparent's experience with prostate or lung cancer may have little in common with a child's diagnosis of cancer.

In the context of a new diagnosis, most parents will experience many intense emotional reactions and may even report psychiatric symptoms of acute stress.[5,89] They are typically frightened and anxious about their child's prognosis and distraught at the thought of their child's physical suffering. If many days or weeks elapse between the child's initial symptoms and the diagnosis, parents may feel angry at medical providers, whom they believe "missed the diagnosis" or did not listen to them or to their child. Parents' belief that there was a delay in diagnosis may also contribute to feeling angry at themselves. Parents are ordinarily active agents in shaping every aspect of their child's care and well-being and may feel quite dismayed at how helpless they feel to protect their ill child. This sense of responsibility may lead many parents to feel responsible for their child's situation, perhaps blaming themselves for not bringing the child to medical attention earlier or for not understanding the significance of a child's initial symptoms.[2,7,8,90,91] In some cases this sense of responsibility and the need to understand the cause of the child's illness may make parents (or other family members) wonder if they themselves are responsible for the cancer. Examples of these thoughts, many of which are not entirely rational, might be wondering if allowing their child to use certain electrical devices, or bringing some kind of contamination into the home from a work site, may have contributed to the cancer.[2,7,8,91] For families coping with neurofibromatosis, Li-Fraumeni syndrome, or other genetic predispositions, a child's diagnosis of cancer may weigh more heavily on parents and leave them more vulnerable to psychosocial distress. The psychosocial care provider can play a crucial role in helping families reframe these difficulties, which will allow them to cope more effectively. In addition encouraging families to pursue a consultation with a genetics counselor can be an important intervention in helping families gain increased knowledge about the genetic predisposition to cancer and develop strategies to decrease stigma and fear and increase appropriate management of risk. Listening to the family in crisis and providing reassurance can be the most useful responses for all members of the care team. Normalizing parents' reactions by letting them know that parents almost universally feel overwhelmed and that intense emotional reactions are normal responses to their child's illness can help reassure them that they are not "going crazy" and they won't be judged for their reactions by the care team.[2,3,7,44,92,93] One reason parents feel overwhelmed is their awareness that they are facing a more complicated, frightening, and demanding challenge than they may have ever faced before. Although it is helpful to acknowledge the feeling of facing this seemingly overwhelming challenge, it is also useful to let parents know that families do cope well, and with some assistance and support, there is every reason to believe their family will cope successfully, even though that may be difficult to imagine at the beginning of treatment.[52,94-97]

During the crisis of diagnosis, many families will benefit from concrete structuring from the care team. Parents often need to be encouraged to focus on their own self-care and may need encouragement to mobilize their social supports to meet their acute needs, such as arranging extended time off from work or asking friends and extended family to help with other tasks. Many parents can benefit from some active problem solving,[52,92,98-100] with staff helping them to identify needs, generate possible solutions, and then select and implement a potential solution. Social workers and resource specialists on the care team may be helpful in identifying sources of practical support (e.g., transportation, meals, and lodging), sorting through insurance issues, and helping solve problems with regard to meeting the needs of the patient and family.

Occasionally some parents experience such a strong need to act in the face of their child's illness that they make decisions that can be premature or even impulsive. For example some parents have quit their jobs, mortgaged their homes, or moved their residence even before knowing the details of their child's treatment and taking time to consider these major life decisions. Although parents need to decide for themselves how their family will adjust and cope with the demands of the child's illness and treatment, it is appropriate for members of the care team to caution against making impulsive decisions and support them in making short-term plans for managing the health crisis that can be revised as circumstances change or new information becomes available.

During the crisis period parents can experience a wide range of intense emotional reactions. In most cases parents' reactions can be managed with education and support from the care team. Specialized services from a psychosocial care provider, usually a psychologist or social worker, can be particularly useful in helping parents identify and begin to cope with their emotional reactions.[92,101-103] Many of these reactions will be of short duration because parents often report feeling less overwhelmed after they have heard a definitive diagnosis and can focus on the demands of a treatment plan. Research has demonstrated that the initial psychological distress experienced by mothers at the time of diagnosis tends to steadily improve as long as 6 months after the diagnosis.[104] In rare cases parents may have emotional reactions that are more enduring or problematic,[2,12,105,106] which may require more targeted intervention during the crisis period. Situations involving confusion about the child's diagnosis or requiring a prolonged diagnostic evaluation

TABLE 73-1 Factors Associated with Increased Psychosocial Distress

Family Factors	Disease Factors	Environment/ Resource Factors
Single-parent families	Unclear diagnosis(es)	Financial problems (e.g., job loss or debts)
Preexisting chronic or mental health	Poor prognosis	Isolation (e.g., lack of family and/or problems with peer support)
Concurrent illness or injury in family (e.g., parent or sibling illness)	Prolonged or intense treatment(s) (e.g., bone marrow transplant)	Language differences (e.g., foreign national or immigrant)
Marital problems (e.g., separation and/or divorce)	Disfiguring disease or treatment(s) (e.g., amputation)	Transportation (e.g., no car to get to the hospital)
Family problems (e.g., emotional or learning difficulties)	Disease status (e.g., relapse or recurrence)	Minimal or no health insurance
Recent stressful life events (e.g., job and/or school problems, a move, or relocation)		

Modified from Hersh SP, Wiener LS: Psychosocial support for the family of the child with cancer. In Pizzo PA, Poplack DG, editors: Principles and practice of pediatric oncology. *Philadelphia, 1989, J.B. Lippincott Company, p. 897–891; Kazak AE, Cant MC, Jensen MM, et al: Identifying psychosocial risk indicative of subsequent resource use in families of newly diagnosed pediatric oncology patients.* J Clin Oncol *21:3220–3225, 2003; and Lansky SB, List MA, Ritter-Sterr C: Psychiatric and psychological support of the child and adolescent with cancer. In Pizzo PA, Poplack DG, editors:* Principles and practice of pediatric oncology. *Philadelphia, 1989, J.B. Lippincott Company, p. 885–896.*

period are more stressful for parents and may be more likely to cause significant distress. Similarly parents who are managing other significant stressors such as having a parent or other family member with medical needs and parents with preexisting psychological problems will be more likely to have symptoms that interfere with their ability to function (Table 73-1).[2,12] Examples of these problematic reactions might include manifesting signs of clinical depression or anxiety, not being able to care for themselves adequately (i.e., not sleeping or eating), or being so frustrated and angry that they cannot consistently collaborate with medical providers. In these situations in which parents' reactions will have a negative impact on the child and have the potential to interfere with the treatment process, it is important to address the situation directly with the parents and make appropriate referrals for intervention.

Interventions for parents experiencing these problematic reactions may require more intensive services from some members of the care team, as well as consultation with and referral to other individuals. The underlying problems may predate the child's cancer, but the focus of intervention during the crisis of diagnosis may appropriately be alleviation of symptoms. Parents showing signs of severe distress should be evaluated by an appropriate psychosocial team member or be referred to a mental health provider who can assess their psychological status and refer them for any urgent intervention they may need. In addition to supportive therapy and the problem-solving approaches previously described, psychopharmacologic intervention may be particularly helpful for parents who have severe anxiety or depressive reactions, severe sleep disturbance,[107,108] or a preexisting psychopathologic diagnosis. Pastoral counseling may be useful for parents whose faith is an important source of support and for those experiencing a "crisis of faith" as they struggle to make meaning of their child's illness.[109-111]

Children's Reactions to Diagnosis

Children will react to their diagnosis and initial treatment on the basis of their developmental level, their own individual temperament and past experiences, and to a large degree on how their parents cope with the new diagnosis and treatment.[112,113] Typically the infant, toddler, and especially the preschooler will be more concerned about changes in routine and the normal home environment, separation from family, especially the primary caregiver, and bodily intrusion through physical examinations and medical procedures for diagnostic or treatment purposes. Strange environments will disrupt normal awake/sleep patterns, and sleep deprivation can reduce the child's normal self-regulation effectiveness. For example infants who had been able to sleep through the night at home might have constant awakenings. Separation from the primary caregiver(s) then becomes an even larger issue if the parent is not present during the night or during bodily intrusion episodes to help comfort the child. Clearly this need for the parents suggests that, when possible, it is better for parents to create "shifts" to be with the child as much as possible, day and night. Such parental support also means that the issue of support for parents, both emotionally and in practical ways, needs to be addressed.[114] Stressed parents who cope poorly will not be able to help their child self-soothe well.[15,115]

One issue that the medical team can address is the manner in which examinations and procedures are carried out. For example Chen and colleagues[116,117] found that children had memories of stressful and painful procedures that affected their experience and behaviors during subsequent procedures. Asking the parent to leave the room while a team of doctors and nurses in white coats enter the child's room to carry out examinations is more likely to help create an uncooperative child who will become conditioned to be anxious around anyone with a white coat. Having one member of the team develop a relationship with the child, from toddlerhood through adolescence, will be helpful in establishing the child's trust of the nonfamily "team." Establishing a system for families in the hospital that sets clear guidelines for providers as they enter a child's room can prevent significant distress. For example psychosocial providers or child life specialists can create a sign for the door that reminds providers to introduce themselves when they enter the room, let the child know up front what is going to be required of them, and hold medical discussion outside the room when appropriate. Respecting such requests fosters rapport, reiterates the message that the child and family are part of the team, and promotes the sense of being "heard" by the medical team.

For the very young child, procedures that can be performed competently and quickly while distracters are present (e.g., iPad apps, bubbles, party blowers, cartoons, and stories) can reduce fear of future procedures and help the child develop a sense of control and cooperation. The use of positive reinforcement is helpful in an effort to pair "scary" novel and intrusive experiences with positive, pleasurable experiences. The use of behavioral strategies (e.g., breathing, relaxation, imagery, and behavioral charts) can provide parents with ways to support their child in these novel and intrusive situations and help the child develop a sense of security and mastery, and they give parents the opportunity to feel successful in the context of challenging medical demands. Additionally it may be useful to encourage the family to bring in a "transitional object" for their child (e.g., a favorite blanket or stuffed animal) to bring the familiarity of "home" into the hospital.

School-aged children will be interested in concrete answers to their questions about hospitalization, expectations with regard to medical examinations, and specifics about what to expect in unfamiliar situations. Encouraging parents to answer questions directly, even when the topics may be uncomfortable, will foster open communication and minimize the chance that the child will create "answers" based on faulty assumptions that can be anxiety provoking.[118-120] Parents should be reminded that this is not a one-time discussion and that providing their child with information, even upsetting information, can decrease uncertainty about the illness and potentially improve psychological functioning.[121]

While children develop an increased understanding of their illness, they may ask, "Am I going to die?" Psychosocial providers can help guide parents in providing answers to this question based on a child's age and diagnosis. It is important for parents to acknowledge the severity of the disease while highlighting the fact that the medical team has a treatment for the illness. Answers to this question will vary based on a child's medical condition, particularly in the case of relapse. Exploring a child's thoughts and fears around illness and death can be helpful; however, providing reassurance that he or she will be well taken care of and not left alone and that his or her symptoms will be treated is critical.[119]

Adolescents are likely to want to know more information about their illness and treatment. Simple responses to questions often will not satisfy them, and parents will need to understand that adolescents may potentially turn to alternative sources beyond their parents for important information. A child or adolescent who has formed a relationship with a member of the treating team can ask questions of that team member that might seem difficult to ask parents.[122,123] Some adolescents feel a need to "protect" their parents from more stress by not acknowledging to their parents their own fears, anger, and worries. Encouraging adolescents to talk about what they have learned from their medical providers, the Internet, and their peers will help eliminate misinformation and confusion while empowering adolescents to seek information in their own way. Developing a sense of mastery over the medical information can lead to a feeling of control and confidence that can be helpful for teenagers.

Finally, children of all ages often regress when they are scared, and parents need to know that changes in the child's behavior are not unusual.[124] Psychosocial providers can normalize behavioral reactions for parents and subsequently minimize the distress associated with particular behaviors. For instance a preschooler who has just been toilet trained may begin having accidents, and it is imperative that parents recognize that this behavior is a normal response to a stressor and that they should not focus too heavily on that behavior during a time of crisis. The following guidelines are related to children's reactions to diagnosis and treatment:[125]

- Many parents need help talking with their child, who is often scared and needs some explanation and education along with his or her parents.
- A variety of disciplines, such as child life, nursing, social work, and psychology, can all be helpful to parents, siblings, and pediatric patients. Interventions relating to preparation for medical procedures, supportive work with parents and other family members, and psychoeducation are important. Connections with other children and families who are having similar experiences can be helpful.
- Keys to getting through diagnostic procedures are good preparation and good pharmacologic and psychological pain and anxiety management.

Education and Information about Treatment

Most treatment regimens put an enormous information burden on the family. Children are often treated with several modalities (such as surgery, radiation, and/or chemotherapy) and may receive a large number of medications, as well as a variety of blood tests and other diagnostic procedures. Although adolescents may be active in their own care to some extent, parents are responsible for understanding and managing their child's treatment, especially during outpatient phases of treatment when parents will need to work with home care agencies, administer treatments themselves, and monitor their child's health. To meet these demands, parents require detailed information about their child's treatment regimen and reactions that can be expected.[101,102,126,127] Most of this detailed information will be provided by nursing and medical care providers. Patients and families are generally quite resilient and successfully accommodate even the most demanding treatment regimens, but initial apprehension about being able to understand and implement these regimens is common. Staff may need to present information several times and reinforce verbal exchanges with written materials. While remaining optimistic and encouraging families to develop competence, it is also important for providers to recognize the complexity of what they are asking of families and the burden it imposes. Normalizing the common feelings of anxiety and "information overload" while focusing on problem solving in the face of new demands is an important role for the psychosocial clinician. Talking with other families who are undergoing similar treatment can be particularly helpful,[102,103,126,128,129] and many centers offer education and support groups for new patients and families. In addition a wide variety of books useful to parents[130-135] and helpful for patients and siblings[136-146] may be available from hospitals, libraries, and booksellers.

Among older children and adolescents, interest in or openness to learning more about their illness and treatment can be highly variable. Because they have more developed cognitive and language abilities than younger children, older children can ask more questions and understand more about their treatment, and like parents, they may receive educational information from nursing staff as previously described. To the extent of their capabilities, adolescents should be encouraged to participate in their own treatment by learning about their medications and schedule, helping with simple self-care activities such as routine care for a central venous line (CVL), and learning the best ways for them to cope with adverse effects or medical procedures. In some cases adolescents may not be interested in some of this information or may feel disinclined to assume even basic self-care responsibilities. It is not uncommon for adolescents to feel overwhelmed by information about their disease, particularly given the typical developmental milestones they are attempting to navigate.[147] Feelings of distress associated with a loss of autonomy may lead adolescent patients to become more withdrawn. It can be helpful to empathize with adolescents' plight of feeling "pushed around" while actively working with them to develop adaptive coping strategies that may lessen their feelings of helplessness. It is often important to point out that although many aspects of the situation cannot be changed (i.e., you have cancer, you need treatment, and the treatment will cause you to miss school), some aspects can be changed (e.g., you can maintain ties with friends through texting, social media, or visits, you can keep up with school work, and you can plan a party or an outing to celebrate an upcoming treatment milestone). It can be overwhelming for parents to include adolescents in difficult conversations about their illness and treatment, but adopting a model of shared decision making with adolescent patients in the early stages of treatment can empower patients, improve comprehension and understanding of the treatment plan and toxicities, and enhance communication among parents, patients, and providers.[148,149]

The Internet

In addition to obtaining medical information from medical teams, outside consultations, and family and friends, parents also gather information from the Internet. The ability to access information quickly and conveniently is appealing to parents who can feel overwhelmed during medical discussions. Given that it is common for patients and parents to use the Internet to gain knowledge about their disease and treatment, it is crucial that medical providers help families identify appropriate websites where they can access accurate information. In particular providers should consider providing parents with a list of recommended websites. These websites may include local resources affiliated with the treating institution, disease-specific resources, and general pediatric cancer sites facilitated by reputable organizations such as the Children's Oncology Group, Pediatric Cancer Research Foundation, National Cancer Institute, and the American Childhood Cancer Organization (e.g., www.curesearch.org, www.pcrf-kids.org, www.cancer.gov, and www.acco.org). The National Cancer Institute website offers educational materials in both English and Spanish. Ensuring accuracy of information is important not only for the parents' understanding but also for that of adolescent patients who frequently access the Internet.[150,151] Using a search engine to research a cancer diagnosis and treatment does not always lead to relevant content.[152] In addition the readability of websites is highly variable,[152] and the information provided on some websites may be difficult for some parents and patients to understand, potentially leading to additional confusion at an already challenging time. Potential exposure to difficult material (e.g., prognosis, individual narratives, and grief) without the immediate support of the medical team can be a particular concern. Having a discussion with families about the dangers of exploring too broadly on the Internet early in the diagnosis and treatment period is important so they can avoid exposure to potentially upsetting information.

Despite the potential challenges associated with gathering information via the Internet, this avenue provides some clear advantages for parents and patients, and research is burgeoning in this area to best meet the needs of the families.[153] Open patient-physician communication with regard to gathering information, asking questions, and researching treatment options and plans can help minimize confusion or distress based on misinformation obtained via the Internet. This approach represents a movement away from the more paternalistic approach, and medical providers can encourage families to conduct their own research and come back with questions or concerns.

ADJUSTING TO TREATMENT

While the child and family come to understand the diagnosis and treatment plan, their focus begins to shift from managing uncertainty to coping with the demands of treatment. Although parents in particular will continue to worry about their child's prognosis for years to come, the day-to-day demands of treatment along with the other demands of work and family life become increasingly salient. Families often experience a subjective sense of relief associated with a reduction in uncertainty, but they may also have an increased sense of burden. During the crisis of a new diagnosis, parents usually take time off from work and temporarily set aside many practical concerns. With the initiation of treatment, parents and children typically need to return to their regular roles and responsibilities while also taking on the new responsibilities associated with treatment. During this period of adjusting to treatment, families often benefit from continued psychosocial assessment, education, and support. In actual practice, encounters with the patient and family will blend these three objectives, especially at the initiation of treatment, but they are presented separately here for clarity of presentation.

Psychosocial Assessment of the Patient and Family

Experienced psychosocial care providers generally have a way of conducting an assessment that is comfortable for them and generates the relevant information. For illustrative purposes, a general approach to the assessment interview will be outlined here and in Box 73-2. The process of the interview should include time for the family members to speak about their concerns, but the provider will need to actively direct the conversation to ensure that the necessary topics are covered. In an interview with both parents if possible, the provider may begin by asking about the general family history and then focus on the patient's premorbid functioning, a developmental history, and the relevant history of any other children. Details of the patient's functioning at school and relationships with peers and siblings should be solicited, as well as prior experience with stressful situations and any behavioral, developmental, or emotional concerns prior to the cancer diagnosis. In learning more about the parents, it is useful to know the basic outline of their families of origin, their current work or family roles, and any religious and cultural traditions that may affect their ability to cope with the illness. It is important to know if the family has had to cope with significant stressors in the past and if any other significant problems exist in the family at present. If the parents are divorced or not married, understanding this history, as well as the practical and legal aspects of any custody arrangements, is critical. Understanding the family's practical and social resources is also an important component of the assessment.

The assessment of the pediatric patient will vary significantly depending on the child's age and medical status. With an infant or toddler, the child will be seen along with the parent, and the goals may be to observe the parent-child interaction, appreciate the child's temperament and reaction to the medical setting, and establish the psychosocial provider as a friendly and nonthreatening presence. With older children the goals will also include directly assessing their reaction to their illness as they are able to express it verbally or in play. Because children typically have met many health care providers in the hospital or clinic, it is important for the provider to begin by introducing himself or herself, describing the purpose of the meeting, and reassuring the child that there will be no medical examination or use of needles during the meeting. With most children, even into adolescence, the assessment can be greatly facilitated by including action, such as unstructured play, a walk in the hospital, a craft, or a game.

With preschool and school-aged children, familiar activities such as drawing or symbolic play with dolls or puppets can serve the dual purpose of engaging them and providing an expressive medium through which they can relate their concerns. When children are engaged in this type of play, the provider can assess them by observing or participating in the play, as well as by asking them questions about their illness, their medical care, and how they are coping (e.g., "What has been the hardest part about being a patient?"). For older children, questions

Box 73-2 **Psychosocial Assessment Interview of the Patient and Family**

STRUCTURE OF INTERVIEW

- One to three intake meetings with patients and parents, conducted by a mental health professional.
- Meetings held in a relaxed, nonthreatening setting.
- Written summary of assessment to be shared with the treatment team.

ASSESSMENT CONTENT

ILLNESS CONTEXT

- Initial presentation of the illness.
- Diagnosis, prognosis, and anticipated treatment.
- Previous child and family experiences with physical health problems and behavioral problems.
- Family's coping, especially cohesiveness, communication style, and ways of resolving conflicts.

CHILD PATIENT

- History
  - Developmental—milestones, temperament, delays.
  - Medical—previous diagnoses and responses to medical care.
  - Behavioral—energy level, moods, and sleeping/eating habits before and after the illness.
  - School and Social—relationships with parents, siblings, and peers, educational history, and functioning in school.
- Response to diagnosis and coping style.

PARENT AND FAMILY

- Background and History.
  - Basic structure and involvement of extended family.
  - Sources of social support.
  - Relationships, sources of conflicts, and cohesion.
  - Physical and mental health.
  - Sibling relationships.
  - Parent education and employment.
  - Religious beliefs and cultural traditions.
  - Parenting styles.
- Initial Response to the Diagnosis
  - Knowledge and understanding of diagnosis.
  - Level of distress—emotional and physical symptoms.
  - Strength and vulnerabilities during the crisis.
  - Coping styles and experienced need for support.
  - Expectations and sources of stress and anxiety.

RESOURCES

- Housing.
- Names and ages of family members living at home (e.g., siblings and grandparents).
- Available support for work, neighborhood, and school.
- Financial status.
- Health insurance.
- Involvement within the community and social supports.
- Travel time and transportation to treatment center.
- Resources available during the child's ongoing medical treatment.

Modified from Hersh SP, Weiner LS, Figueroa V, et al: Psychiatric and psychosocial support for the child and family. In Pizzo PA, Poplack DG, editors: Principles and practice of pediatric oncology*, ed 3, Philadelphia, 1997, Lippincott-Raven, p. 1241–1266.*

about life before cancer including friends, hobbies, and school activities can be a good starting place before asking about their illness and how they are coping with their medical care. It is important to ask older children directly about any worries, anxiety, and symptoms of depression or hopelessness, as well as how the adults involved in their care can best help and support them.

Adolescents may have very personal concerns about how their illness will affect them, such as by causing them to miss an upcoming social event or interfering with a particular friendship or a dating or romantic interest; such concerns may not be immediately apparent to the parents or the medical team. Asking explicitly about what they anticipate will be difficult about their treatment plan is an important part of the assessment, as is inquiring about ways they believe they will deal with the challenges of treatment. Family systems often shift in response to a child's illness, and it can be important to understand the changes adolescents may have experienced and how those changes may be affecting them, especially because adolescents in treatment may become more dependent on their parents and spend more time with them than they normally would. Because adolescents often believe that the patient role limits their privacy, agreeing ahead of time with parents and adolescents about what information from the adolescent interview will need to be discussed with parents and what information everyone agrees can be kept confidential will be important.

After concluding the interview with the patient, it is important to give feedback to the parents about what was discussed, the child's main concerns, and generally how the patient is coping with the illness and the demands of treatment. If the psychosocial care provider has specific recommendations for further assessment or intervention, they should be explained to the parent and patient. For illustrative purposes this description has emphasized the information that should be gathered in the assessment, but the process of the interview should accommodate both the goal of assessment and the goals of education and support.

Support for Parents and Families during Treatment

Families may require very different levels of psychosocial support during treatment, and even within a single family, needs will vary over time. Ideally psychosocial support can be offered to patients and families in a flexible way that can be tailored to their individual needs. This flexible approach requires some ongoing assessment and communication with families about their coping and adjustment, as well as the ability to quickly adjust the level of services offered to each family. Families typically require more intensive involvement from the psychosocial care provider during the diagnostic period and the beginning of treatment,[104,154] as previously described. Some families may continue to have high levels of emotional distress or ongoing problems adjusting to treatment (Table 73-1) and will require continued regular meetings with a psychosocial provider, but many families will not have this need. For families who are adjusting adequately to the routine of treatment, their need for psychosocial care often decreases. For these families the psychosocial care provider may briefly check in with patients and parents in the context of their regular medical care. These brief visits may be useful for assessing their adjustment and reinforcing previously offered suggestions and interventions. The psychosocial team may also offer information about educational and support groups, which can be particularly helpful for parents adjusting to treatment.

With any decrease in intensity of direct psychosocial involvement, care should be taken to ensure that patients and families do not feel abandoned by the psychosocial care provider. In most clinical settings a team model is used in which medical providers and psychosocial clinicians are in frequent communication so parents can be assured that when important medical changes occur that have potential psychological implications, psychosocial care providers will be informed and will intervene as appropriate. It is important to review with parents the expected challenges that may arise during the course of treatment (described in the next section) and encourage them to contact the psychosocial care provider if they need assistance with these or other issues that arise.

If some adjustment problems arise during the course of treatment, such as problems at school or difficulties with discipline, the psychosocial care provider should offer symptom-focused interventions such as educational support, behavioral intervention, or other counseling. Typically this intervention may involve a small number of sessions delivered as part of the patient's regular medical visits or a referral for short-term therapy within the treating institution or in the community. While the presenting issue is resolved, children and families will typically return to their previous level of adjustment, and the intervention can be tapered off and discontinued.

Some families will experience brief periods of intense emotional distress related to the stress of cancer treatment. These episodes may occur when they are facing painful or unpleasant therapies or when treatment complications arise. Intervention for moderate to severe distress could include an increase in the intensity of routine psychosocial support services, consultation with additional mental health providers to provide crisis intervention, psychiatric assessment, and use of psychopharmacology for patients or other family members. In the outpatient setting, referral to outpatient providers in the community will also be important.

Some children and families will require a more intensive level of psychosocial care consistently throughout treatment. In most cases these families have high emotional distress, multiple stressors, or limited resources, which can often be recognized during the psychosocial assessment (see the previous section). For most of these children and families, regular ongoing psychosocial services will need to be provided for much or all of the treatment period. In these cases the psychosocial care provider should consider several types of intervention, such as play therapy, cognitive-behavioral treatment, and parent guidance. Referral to other specialists, either within the treating medical center or in the community, is useful when specialized services are needed (e.g., psychopharmacology) or the child or family needs more intensive treatment than can be accommodated within the oncology clinic. Referral for outside services such as psychopharmacology or individual or family therapy is particularly useful in cases of some preexisting mental illness, family conflicts, or multiple major stressors.

Few families will present with psychological distress or behavioral problems that cannot be successfully managed with routine psychosocial care along with some provision for brief intensive interventions.[14] Usually families that cannot be managed in this way are those with many other stressors occurring at the same time or families that include an individual with a preexisting psychiatric disorder, such as major mental illness, substance abuse, or severe personality disorder. When families have multiple stressful events occurring simultaneously, such as a second illness in the family, a major loss, or economic instability, they may require more psychosocial support at the cancer center, as well as referral to additional outside agencies. Preexisting psychiatric disorders should be evaluated as part of the psychosocial assessment. Vulnerable persons at high risk for worsening of symptoms or relapse should be referred for mental health treatment or counseled about the need to access treatment quickly if symptoms recur. Communication between the psychosocial providers on the oncology team and outside mental health providers can be helpful to share a common understanding of the child's cancer treatment status and updates about the mental health status of the family member in treatment, but this communication needs to be undertaken in a limited manner that preserves privacy.

Families with consistently active psychosocial needs can present a challenge to the care team because of their emotional distress and the demands they may make on staff time. By providing consultation to the nursing and oncology staff, the psychosocial care provider can help the treatment team formulate an understanding of the family's needs and coordinate an appropriate and consistent response.

School

Shortly after diagnosis, questions often arise regarding the child's academic needs and how these needs will be met. Will the child be able to attend school, or will a tutor be provided until the child is able to return to the classroom? How is the treatment course likely to interfere with the child's ability to succeed academically? Will the child meet the academic requirements to be promoted to the next grade, or is it likely that the child will need to repeat a grade? These types of questions will need to be addressed by the family, school, and medical team.

School represents an important locus for the child's cognitive, social, and emotional development, and maintaining participation in or reconnecting with school should be a priority when planning the child's treatment and care.[155,156] For the child resuming school responsibilities can feel overwhelming given the demands of treatment. Adverse effects of treatment can have a negative impact on motivation, concentration, and processing speed, making the transition back to school challenging. Despite the issues resuming academic work promptly will minimize the disruption to academic progress and maximize the child's ability to reengage in school-related tasks. Because many children will be unable to attend school during at least part of their treatment, home- or hospital-based tutoring along with a structured schedule that provides the child with the exact time and duration of school-related work often promotes compliance.

Parents and caregivers must commit themselves to providing the necessary structure and support to promote the child's academic progress, even when juggling numerous other responsibilities. Parents should be encouraged to let the tutors be responsible for assisting children with their academic work and avoid the urge to "help" children with their homework. Extended absences from school can lead to increased anxiety at the time of reentry and potential avoidance behavior. Psychosocial providers can work closely with patients, parents, and school personnel to help ease a child back into school while addressing anxiety issues.

The medical team is often called upon to provide the school with relevant information about the child's ability to attend to academic responsibilities or any vulnerability the child may have as a result of treatment (e.g., risk of infection, susceptibility to illness, and potential cognitive and/or physical deficits). School personnel may be especially anxious when the child initially returns to school, resulting in multiple calls to the treating physician. Parents will play a critical role in educating the school about their child's situation and advocating for the child's needs. Many parents benefit from consultation with psychosocial providers about how best to negotiate the return to school and often find written guides for parents[131,135] to be helpful. Some clinics have established "school reentry" programs in which staff members (e.g., child life, nursing, and mental health professionals) visit schools to present information to teachers and classmates about the child's diagnosis and treatment.[157-159] This proactive approach can be effective in providing medical information to the school nurse and other staff, as well as helping to answer questions that teachers and classmates may have about the returning child's condition and needs. In most cases, these efforts will result in a plan for school reentry that is appropriate and comfortable for the child and parents.

If a child will need specialized instruction because of his or her illness or consequences of treatment, an individualized education plan (IEP) will be required. Federal laws, as well as the laws in several states, require that children with disabilities be accommodated in their schools,[160-162] and parents who suspect their child will need some accommodation should speak with the school's special education director and refer to their state's Department of Education website for information about the special education procedures in their location. In most cases, parents will need to begin the process by making a written request for an evaluation. This evaluation is conducted by the school, often with significant input from medical and psychosocial providers involved in the child's cancer treatment. If as a result of the evaluation the child is found to be eligible for special education services, an IEP will be developed and implemented by the school. If the child does not qualify for specialized education services, he or she may still qualify for accommodations under the Federal Rehabilitation Act, often referred to as a "504 plan."[163] A 504 plan may ensure appropriate accommodation for problems in physical mobility, fatigue, attendance, or other needs of the child that affect school performance but do not require specialized instruction. The IEP determination process can be time consuming and frustrating for parents who have already had to negotiate complex health and insurance systems for their child. Parents should educate themselves about their state's education laws and the federal laws that govern accommodation of children with medical conditions and disabilities[161,162,164] and consult with psychosocial providers who understand the system and academic needs. If significant issues are encountered in securing appropriate accommodations, parents may want to consider enlisting the services of educational advocates[165] to assist them in negotiating with their school.

Peer Relationships

Relationships with peers at any developmental level provide a child or adolescent with crucial components for successful adaptation, including a sense of connection, friendship, and support. This role of peer relationships is as true for children diagnosed with cancer as it is for their healthy peers. Several research studies have focused on the role of peer relationships in the coping and adjustment of children and adolescents with cancer.[166-170] Although parents have been identified as a primary source of nurturance, support, and information for children with cancer,[168] research findings suggest that relationships either with healthy peers or with peers diagnosed with cancer are also extremely important. For example adolescents diagnosed with cancer who report close friendships have been noted to experience increased social support,[148,167] increased feelings of hope,[171] and an ease in communicating about their cancer experience.[168] Additional research suggests that adolescents with cancer view peer relationships as vital to the continued development of their independence and identity, which are areas that can easily be derailed by illness.[169,172]

Studies examining other aspects of peer interactions reveal somewhat mixed results. Some findings have suggested that both children and adolescents who are diagnosed with cancer have fewer friends than their same-aged peers[168,173] and that this trend may continue after treatment has been completed.[174,175] Other findings have indicated that children diagnosed with cancer may be socially isolated and withdrawn,[176] despite the fact that their popularity and number of friendships are comparable with those of their peers.[177] Whether these results reflect a change in the nature or quality of peer interactions because of illness-related factors (e.g., prolonged hospitalizations, significant school absences, or the inability to engage in recreational activities) is difficult to determine.

Given the importance of peer relationships in the course of normal development and the potential for disruption to these relationships during diagnosis and treatment, members of the medical team must support connections with peers whenever possible.[178] This support may include encouraging parents to facilitate peer interactions at home and at the hospital and to work with educators to establish opportunities for the child to visit school for lunch or a single class so that peer connections can continue when the child is not well enough to attend school regularly. When appropriate, medical staff can also support an adolescent patient who wishes to share the clinic experience with a friend and invite the friend to attend an outpatient appointment. These opportunities, although often limited by the impact of treatment, help children with cancer and their peers maintain crucial relationships that will improve posttreatment adjustment. Encouraging parents to allow children and adolescents to have contact with peers who are also diagnosed with cancer can be extremely important. The Internet and social media sites can be very helpful when treatment prevents school attendance. Although parents will want to ensure that the sites that children are using are appropriate, technology that provides ways for children to share pictures, play video games with friends online, video chat, or text can be important ways for them to maintain contact with peers. Participation in summer camps or activities offered through the clinic or hospital provide opportunities for children and adolescents to enjoy activities with peers who have a shared medical experience and can improve self-concept.[179]

EXPECTABLE CHALLENGES DURING TREATMENT

Although psychosocial needs vary considerably between families, certain challenges commonly arise while children progress through their cancer treatment. Some of these challenges are directly related to coping with the demands of treatment, and others have to do with managing the ongoing demands of work, school, and family life while also managing the added stressors of cancer and its treatment. Understanding these challenges can help families anticipate when and how they may need to mobilize their resources. Psychosocial care providers can provide anticipatory guidance with regard to these concerns and facilitate access to appropriate intervention as needed.

Coping with Treatment and Adverse Effects

About 25 years ago, adolescents with cancer reported that their treatment and its adverse effects were the worst things about having cancer.[180] The nausea and vomiting accompanying chemotherapy and the pain associated with procedures such as lumbar punctures and bone marrow aspirations were particularly distressing.[181-184] Treatment of these adverse effects has significantly improved since then. Fairly effective antiemetic drugs are available, and for most patients, procedures are carried out after induction of light or deep sedation or general anesthesia. Despite these advances, children still find chemotherapy, radiation therapy, and various procedures to be very unpleasant and a source of significant apprehension and distress.[185-190] A variety of studies have documented the effects of hypnotherapy, memory evaluation, and other behavioral therapies in reducing adverse effects in this population.[116,191-198] Reducing procedure-related pain, anxiety, and nausea and vomiting associated with chemotherapy and radiation therapy is critically important for reduction of risk factors for posttraumatic stress disorder, long-term pain, and psychological distress in survivors.[1,105,199-210] The topics of pain and symptom management are treated more extensively in Chapter 71.

Maintaining Child and Family Routines

Basic Needs

In the aftermath of a pediatric cancer diagnosis, family members must find ways to attend to basic needs and responsibilities. Although this process can be difficult, family members must eventually shift their focus from the diagnosed child and the isolation of the hospital room to life outside of the hospital. For many families, this process may include working to understand their insurance coverage and medical costs or negotiating with employers regarding the use of vacation or sick leave to care for their child. Grocery shopping, cleaning the house, paying bills, and keeping up with the laundry are additional activities that constitute a typical week for the average family—activities that were likely ignored or attended to by others during the early stages of diagnosis and treatment.

Transitioning from managing a medical crisis to managing the more mundane tasks of everyday life can be challenging but can also reintroduce a sense of normalcy. The reality of financial pressures can necessitate the transition back to work and create additional stress on the family system. Even with excellent insurance coverage, the costs associated with treatment can be significant, particularly for parents or caregivers who have been away from work since the child's diagnosis.

After the initial phase of treatment, parents and caregivers typically attempt to piece together a plan that will allow the family to function effectively and attend to basic needs. This plan will likely take into account the child's medical appointments (both scheduled and unscheduled), the child's school reentry and ongoing attendance, parent or caregiver work responsibilities, attention to the academic and social needs of the child and the child's siblings, and other family commitments and responsibilities. The ability of the family to successfully develop and implement this plan is dependent upon a number of factors, including characteristics of individual family members (e.g., coping style, organizational skills, and cognitive abilities), the functioning of the family as a whole (e.g., cohesion, adaptability, and flexibility), and the response of community members (e.g., a supportive network of friends, the flexibility of employers, and the commitment of school personal).

Understandably attending to basic needs and securing resources is crucial to the successful treatment of the child and the overall functioning of the family, and thus it is essential that members of the medical team support the family in their efforts to meet basic needs whenever possible. This support might include the timely completion of medical forms that will facilitate the implementation of educational services, helping eligible families secure appropriate financial resources, helping families access specialized nursing services in the home, allowing for flexibility in the scheduling of appointments to accommodate a long commute to the clinic, or simply being willing to listen empathically when parents discuss the challenges they face.

Discipline

The diagnosis and treatment of cancer in a child can disrupt every facet of family functioning, including expectations that parents have about their child's behavior and their ability to effectively set limits and exercise appropriate discipline. Understandably many parents and caregivers are unable or unwilling to discipline their children given the difficult medical circumstances in which the family finds itself. A child's behavioral outbursts that may include hitting, kicking, and screaming are seen as a natural consequence of a very difficult and unnatural situation. Disruptions to daily routines, invasive medical procedures, medication adverse effects, and the child's "just feeling lousy" can provide more than sufficient rationale to let the child's behavior remain unchecked. In addition parents and caregivers may be wrestling with their own feelings of guilt, sadness, anxiety, or exhaustion that can make it increasingly difficult to impose behavioral expectations, even for parents who previously demonstrated consistent and appropriate behavior management techniques with their children.

For children whose parents or caretakers are unable to provide effective discipline during treatment, the short- and long-term consequences can be disastrous. For example a child who does not experience appropriate limit setting may feel out of control and unsafe, leading to further behavioral disruption. Behavioral outbursts and noncompliance can interfere with medical care and have an impact on effective working relationships with staff. Inappropriate behavior can spread quickly to siblings who learn through observation that parental authority has been eroded, increasing the overall chaos within the family system. By the time the child's treatment ends months or years later, it can be extremely difficult, if not impossible, to reestablish expectations for appropriate behavior.

To avoid these short- and long-term consequences, parents and caregivers need to provide clear and consistent expectations for their child's behavior as quickly as possible after diagnosis. It is essential to acknowledge to the child that the pain and disruption associated with diagnosis and treatment often causes frustration, sadness, anger and despair and that this is expected. However it is equally essential to convey to the child that expressing emotions through appropriate means is imperative.

Members of the medical team should work with parents to reestablish appropriate discipline practices. During the initial phase of treatment, the team's assessment of the family should include questions about the parents' approach to discipline and strategies that are both effective and ineffective in managing their child's behavior. Understanding parenting styles[211] and the child's prior experiences with discipline can be extremely helpful in predicting possible reactions in both the child and parent. Encouraging parents to utilize an authoritative approach—that is, clear, firm limit setting coupled with appropriate encouragement of independence—will help children be more successful at negotiating the difficulties associated with diagnosis and treatment. A more punitive and authoritarian style will likely increase the anxiety and fear the child is already experiencing, whereas an indulgent or permissive style can foster noncompliance and increased disrespect of others.

A number of behavioral techniques can be used to increase a child's compliance and to minimize disruptive behavior. First, taking the parent aside, acknowledging the challenges of disciplining a sick child, and offering suggestions will be more effective than openly criticizing the parent's poor behavior management in front of the child. When the child's behavior interferes with medical care, staff should encourage and support authoritative parenting. Such encouragement might include staff modeling of behavior management techniques such as giving the child clear instructions ("You need to sit on the table, and you can hold dad's hand"), praise ("I really appreciate how you answered my questions; you've done a great job"), and attention ("Because you were so cooperative and we finished your dressing change so quickly, I have a few minutes to look at your baseball cards, if you'd like to show them to me"). Praise and attention are examples of positive reinforcement, approaches that increase the likelihood that the child will behave the same way in the future.

Behavioral techniques such as "sticker charts" can be implemented with the help of mental health practitioners or child life specialists. Developing a sticker chart includes identifying a behavior the team needs the child to perform (e.g., taking medication), as well as a reward that increases the child's willingness to engage in the behavior. Each time the child behaves appropriately, a sticker is applied to the chart, and after a specified number of stickers have been achieved, a predetermined reward is given. The reward must be given frequently enough that the child experiences the benefits of his or her efforts. When available these techniques should be used in conjunction with psychosocial services from mental health practitioners familiar with the treatment of medically compromised children. Parent education and guidance, as well as counseling or psychotherapy, can be helpful in supporting parents and caregivers in maintaining discipline while providing children with appropriate outlets for their feelings. Many parents will benefit from referral to parenting support groups and books about effective discipline.[212,213]

Siblings

The siblings of children diagnosed with cancer have a unique and often difficult road from the moment a brother or sister is diagnosed with cancer. The uncertainty experienced in the context of a cancer diagnosis and the impact it has on a family system has significant implications for siblings. Siblings spend a significant amount of time with caregivers other than parents, and despite the best parental intentions, they can get shuffled around from place to place while the ill sibling is in the hospital. Although parents may work diligently to attend to the needs of all their children, the parents are often overwhelmed and overburdened, and as a result the siblings of the diagnosed child often feel ignored and excluded, contributing to feelings of jealousy, guilt, anxiety, and sadness.[214,215] The type of challenges that are faced and how a sibling responds are greatly dependent on developmental level. For example preschoolers may experience significant anxiety related to prolonged separation from caregivers who remain at the hospital with the patient, whereas adolescents may be more concerned about details of their sibling's treatment and prognosis.

Siblings of children with cancer have been the subjects of numerous studies designed to investigate their adjustment and adaptation after the diagnosis of their brother or sister.[214,216-223] Findings suggest that most siblings experience some level of stress associated with the treatment of the ill child, especially in the first few months,[154,224] and that this stress may lead to deterioration in normal functioning. Siblings may demonstrate a range of externalizing and internalizing problems, including withdrawal from friends and activities, disturbances in eating and sleeping, temper tantrums, depressed mood, and anxiety.[219,220,225-229] A few studies have documented positive effects, particularly in older siblings, who may exhibit higher levels of empathy and maturity.[216,222,230-232]

Despite years of research highlighting the challenges encountered by siblings of children with cancer, relatively few targeted interventions have been developed for this group. A systematic review of the literature identified studies using individual intervention, camp, and group support to evaluate the impact on sibling psychological adjustment. Findings indicated that participation in interventions improved siblings' depressive symptoms, medical knowledge, and health-related quality of life.[233] Ideally interventions should address sibling adjustment throughout the treatment process rather than at specific phases (e.g., diagnosis or at the time of a bone marrow transplant).[234] General guidelines for improving sibling adjustment include involving the sibling in developmentally appropriate discussions as early as possible, educating the sibling about the illness and treatment course, encouraging the sibling to visit the hospital, and providing opportunities for supportive interactions (e.g., sibling support groups, individual counseling, and summer camp experiences).[215,235] Programs such as SuperSibs, a nonprofit organization for siblings of pediatric cancer patients,[236] provide opportunities for siblings to feel valued and heard through activities and services. Feelings of frustration, anger, jealousy, or guilt need to be acknowledged and normalized, and younger siblings may need to be reassured that the illness of their brother or sister was in no way attributable to anything they may have said or done. Having siblings maintain a routine by attending school or day care, socializing with friends, or engaging in various extracurricular activities provides structure and a sense of normalcy during a very challenging time for the family and increase the opportunities for social support when parents and caregivers are busy attending to the ill child. For siblings who demonstrate ongoing emotional distress or significant difficulties in returning to their regular school, family, and social routines, a consultation with a psychosocial provider should be recommended. Depending on the sibling's needs and available resources, they may be able to provide some short-term supportive therapy or make a referral to community or school-based resources.

Transitions and Changes in Care

While families transition from one phase of cancer care to another, they often experience the change as a loss. In each phase of treatment, children and families adapt, even unwittingly, to difficult circumstances of treatment, and when these circumstances change, they experience disruption in their new routines and habits. Parents may be particularly susceptible and often appreciate the irony of having spent days or week feeling "trapped" in the hospital, only to find themselves later terrified to leave it. Children may miss the special care they received in certain settings, the reassurance and attention they received from nursing staff, relationships they developed with other children, and the relaxed expectations that may apply when they are undergoing intensive treatment. Many kinds of transitions in care can be challenging to families, but discharges from the hospital and the transition from active treatment to going "off therapy" may be among the most stressful,[237-241] especially for parents. During these transitions it is especially important for providers to be sensitive to the likelihood that families will feel anxious and unsure, even while the treatment is "progressing." Providers may naturally tend to emphasize only the positive aspects of a transition, but this approach is likely to increase feelings of anxiety and isolation. Providers should normalize families' feelings by asking about their uncertainty or ambivalence with regard to moving to a new phase of treatment and should reassure them that these reactions are common. Asking, "How does it feel now that you are getting ready to go home?" is much more useful to the family than a well-intentioned statement of "Oh, you must be so happy to be going home." Acknowledging that a transition may require the patient and family to adapt to new routines and providers or manage aspects of the medical care more independently is important, as is reassuring the family that there will be continued support for them during the transition period.

For families beginning a new phase of treatment, such as outpatient radiation or chemotherapy, a tour of the new treatment area, introductions to new providers, and opportunities to meet other children and families can be very helpful. Child life specialists can be particularly helpful to children acclimating to new treatment settings and modalities. Families transitioning from active therapy to off-treatment care can experience significant uncertainty and emotional distress, especially when treatment has lasted for a year or longer. Although some families find the end of therapy a great relief, many families are surprised to find themselves experiencing anxiety, uncertainty, and feelings of helplessness reminiscent of feelings they had during the crisis period of diagnosis.[238] For these families it is helpful to normalize their reactions, helping them to understand that during active therapy their anxiety was kept at bay by the demands of treatment, the frequent contact with providers, and the rituals of medical care. While the demands of treatment decrease, they may find themselves looking back and reflecting on all they have been through since diagnosis. In addition the cessation of treatment raises concerns about the possibility of relapse, and less frequent visits to the oncologist may make parents in particular worry about how their child's health will be monitored. Because the off-therapy transitions can be a time of increased stress and uncertainty for many families, it is helpful to anticipate these needs by offering additional psychosocial support either in individual meetings with families or through parent support groups. In addition to providing increased emotional support, programs providing tailored medical information for families ending treatment, such as a summary of the child's treatment and their schedule for off-treatment and follow-up care, can be effective for reducing anxiety and uncertainty.[238]

Home Care

Children's cancer treatment will often require that some specialized care be provided in the home. Depending on the specifics of the child's treatment, specialized care may include taking oral medications or nutritional supplements, caring for a CVL, or receiving injections or intravenous medications. Care in the home setting is critical to allowing children and families to leave the medical setting and return home to normal routines, but it also represents a significant new responsibility for parents and children.[242,243] Over time, providing care in the home can give both parents and patients a way to feel active and competent in managing their own care, but at least initially it can be a source of burden and anxiety. Parents may feel overwhelmed both by the technical aspects of home care they must learn to provide, such as flushing a CVL or giving an injection, as well as by the sense of responsibility for monitoring their child's health. Children can feel confused about why their home setting is being "invaded" by the medical world they had hoped to leave behind in the hospital, and some resistance to treatment may emerge. Receiving care from the parent rather than a medical provider can be a relief for some children but may raise problems for other children. Parents' discomfort or unfamiliarity can be unsettling for children, and the parent as medical provider can be an awkward change of roles in the family. Young children may experience difficulty in having parents shift from an emotionally supportive role to being the hands-on person providing their care, and thus they may become resistant to certain aspects of their care, such as taking oral medications. Older children and adolescents may want to assert their independence and be directly involved in their care but may not know how to do so. They may also be ambivalent, with some adolescents alternately striving for independence and exhibiting dependence and passivity.

Practical education from nursing staff is vital for parents and children assuming responsibility for home care.[242,244] The availability of home care nurses to provide some of this care, monitoring, and education is also critical. Nursing and psychosocial professionals can also offer important emotional support by normalizing feelings of uncertainty and responsibility and by promoting the development of a sense of competence by patients and families. For children of all ages it is useful for parents and providers to consider how the child can be involved in his or her care in a way that will help the child feel some mastery and sense of control. If problems with a child's compliance are anticipated or develop at home, the psychosocial care provider should work with the family to develop a behavioral plan to address the problems. Supporting the parents in their role, helping them to set clear and consistent expectations, implementing a reward system with tokens, and helping the child find a way to be appropriately active in his or her care can all be useful interventions. With older children and adolescents, a token reward system such as a sticker chart is less likely to be effective, and some negotiation of roles and responsibilities will often be more helpful. Family members may need to be encouraged to be more flexible and try involving different family members or different schedules to manage their home care. In these cases the psychosocial care provider may want to involve other members of the treatment team such as nurses, nutritionists, or pharmacists in considering how the home care plan can be adapted for the needs of a particular family. Peer support from other families and patients may also be helpful.

SPECIAL ISSUES THAT MAY ARISE DURING TREATMENT

Marital Adjustment

A pediatric cancer diagnosis has immediate and significant consequences for those connected to the ill child, most notably the parents. Although the level of care a child will require through the treatment process will depend on several factors, including the severity of illness and the child's developmental level, most children undergoing cancer treatment will require significant support from at least one parent or caregiver. Many families find that managing the child's hospitalizations, clinic visits, daily medications, adverse effects of treatment, and other illness-related factors is a full-time job, often leaving the responsible adult feeling overwhelmed. With one parent now dedicated to caring for the ill child, the other parent is often left to support the family financially, attend to other children, and ensure that the household continues to function effectively. Not surprisingly these circumstances can present significant challenges to all aspects of family functioning and can test the marital relationship. Understanding how these circumstances affect the child and family is complex and necessitates the consideration of several factors, such as parental coping, family cohesion and organization, emotional expression, and marital satisfaction.[245]

Given the challenges that parents must face after their child has been diagnosed, one might predict that marital relationships would suffer as a result. Research has suggested that although parents of children with cancer were no more likely to divorce than other parents and that marriages generally remain stable,[246-248] marital dissatisfaction and distress do increase.[249-251] Specifically Hoekstra-Weebers and colleagues[249] noted that marital dissatisfaction was associated with higher levels of psychological distress 6 and 12 months after diagnosis, but not at the time of diagnosis. Schuler and colleagues[250] found that parents, especially mothers, experienced distress with regard to adjusting to new demands. Similarly Dahlquist and colleagues[94,251] reported that marital distress was predicted by general emotional distress and a discrepancy between the couple's perceived anxiety levels. Although pediatric cancer may strain marital relationships and accentuate differences in parental coping styles, these results suggest that couples are committed to remaining in their marriages, perhaps to minimize any additional disruption to the family and to the diagnosed child. Given that marital distress appears to increase over the course of the child's treatment and may not be evident at diagnosis when clinical assessments are initiated, it may be helpful for medical staff to monitor parental functioning over time and to seek consultation from mental health colleagues when marital tension is evident and could potentially affect the adjustment of the child and family.

Nonadherence

The ability to adhere to treatment regimens is an important clinical issue that has ramifications for overall disease outcome and clinical trial data.[252] In pediatric oncology, nonadherence can be used to refer to failure to attend medical appointments, undergo chemotherapy or take other medications as directed, and comply with clinical research requirements.[253] Nonadherence can have significant implications such as inaccurate conclusions regarding medication efficacy, skewed research findings for experimental protocols, and even a decreased chance for survival.[254] In one study nonadherence with oral cancer treatment was found in nearly 60% of adolescents and 33% of children younger than 13 years.[255]

The adherence literature has consistently demonstrated that adolescents are at greater risk for nonadherence compared with younger or older patients.[254,256-258] The nonadherence rates for adolescents receiving cancer treatment are consistent with findings of nonadherence in other chronic diseases such as cystic fibrosis[259] and diabetes.[260] The nonadherence demonstrated in the literature is likely related to the developmental tasks associated with adolescence. On a practical level, because adolescents spend more time away from their parents, the parents (especially those who are working or caring for other children) may naturally expect the adolescent to remember when to take medications and to take them more independently. Psychologically adolescents naturally develop an increased sense of autonomy and move away from being dependent on parents, which may make it more complicated to negotiate how their medication use will be monitored.[147] Although most parents and adolescents may fully intend to adhere to medication schedules, the combination of the adolescent's need to exert some level of independent functioning, the need to fully integrate adolescents into school and social settings where parents may have less opportunity for oversight, and multiple demands on parents can increase noncompliance and create opportunities for miscommunication and conflict with regard to who is responsible for ensuring adherence to treatment.[252]

Despite the belief of adolescents that they can take care of themselves, they face a number of challenges in adhering to a complicated oral treatment regimen. Their neurocognitive abilities may not be appropriately developed, and executive functioning challenges may interfere with their capacity to organize medication schedules. In addition many adolescents may not adhere to treatment recommendations if the treatment schedule or medication adverse effects interfere with pleasurable activities. Psychosocial care providers can be helpful in terms of potentially preventing adherence issues by encouraging parents and providers to include adolescents, when developmentally appropriate, more fully in their medical care. Simply raising the issue can be valuable, because letting families know that trouble with ongoing adherence to medication is not unusual can help many parents and patients acknowledge the issue and talk more openly about it. Research has demonstrated that including adolescents in the treatment decision-making process increases their sense of autonomy and improves treatment adherence.[257] In addition, working with the medical providers to factor in flexibility, when appropriate, to accommodate the social needs of adolescent patients may help increase adherence.

A number of individual, family, and treatment characteristics have been linked to treatment adherence. Die-Trill and Stuber[255] found that characteristics of the child such as behavioral problems, poor self-image, poor insight regarding prognosis, and poor coping mechanisms were positively correlated with nonadherence. Family predictors of nonadherence included parental depression and an increase in the number of siblings. Treatment intensity and the number of medications needed for effective treatment also increase nonadherence. Die-Trill and Stuber[255] identified additional factors that promote treatment adherence, including increased anxiety in female patients and increased obsessive-compulsive behaviors in parents of male patients.

Recent research on adherence has begun to utilize advances in technology to move beyond self-report and pill counting to incorporate electronic monitoring devices on pill bottles and blood work. In one such study Bhatia and colleagues[254] identified nonadherence in 44% of children with acute lymphoblastic leukemia and found that 59% of the relapses were related to nonadherence to medication regimens. In addition ethnic differences in adherence were reported, with Hispanic patients having significantly higher rates of nonadherence when compared with other ethnic groups. Patients from single-parent homes were also at greater risk for nonadherence. It is likely that in such households, adolescents are more likely to be responsible for medications, and given the issues previously raised, they have difficulty adhering to the recommended medical treatment.

Although a significant amount of literature has been devoted to nonadherence in adolescent patients, issues associated with adherence can also be a result of parent error. Protocols that involve oral regimens have increased, subsequently requiring parents to be responsible for appropriate dosing and administration. A multisite study of medication errors in the home revealed that one in two patients were exposed to at least one, if not more than one, medication error when medications were administered in the home. These errors mostly entailed inaccurate dosing or missed doses.[261] It is important that the medical team consistently monitor patients and parents regarding the use of oral medications, and if nonadherence or errors are suspected, education and training should be provided to support families in adhering to what can often be complicated treatment regimens.

Given that adherence is essential for the safe and successful treatment of pediatric cancer, a number of interventions can be incorporated to increase adherence, such as educating parents and patients about the prescribed medical treatment and the medical ramifications of not taking medications as recommended. It is also important to understand the neurocognitive capabilities of patients and families and factor that knowledge into helpful strategies that can be used to aid organizational and memory issues, thus helping parents to develop supportive relationships with their children that will allow the use of appropriate behavioral techniques.

Depression and Anxiety

Given the many challenges that children and adolescents face during the course of cancer treatment, many investigators have hypothesized that children with cancer are at risk for higher levels of depression and anxiety. Research findings to date are mixed, with some studies suggesting emotional and behavioral maladjustment, including depression and anxiety, in children with cancer[262-264] and other studies showing less behavioral or emotional disruption in these children.[265,266] Social support and effective parenting throughout treatment appear to provide protection from the adversity encountered by the child with cancer[266] and may be important buffers against serious psychological distress in patients.

Given the stress associated with the diagnosis and treatment of pediatric cancer, some level of heightened anxiety and depression is to be expected, although these symptoms may not be of sufficient intensity to reach clinical significance and may not be consistent with a psychiatric diagnosis. The inability to attend school, participate in extracurricular activities, and socialize with friends, coupled with invasive procedures, nausea, and hair loss, would leave even the most well-adjusted child or adolescent struggling to maintain appropriate functioning. Given the likelihood that most patients will experience at least some transient symptoms of anxiety and depression, the tasks for providers and parents is to identify these symptoms, assess their level of intensity and functional significance, and plan for appropriate interventions to address them.

The medical team, with the assistance of parents or caregivers, should consistently assess the child's mood and behavior for signs of emotional difficulty, comparing current functioning with functioning prior to diagnosis. The team may ask why a child is no longer playing his or her favorite video games, being disrespectful toward nursing staff, refusing physical therapy, or ignoring visitors. Anhedonia, sleep disturbance, irritability, and social isolation are some of the signs and symptoms of depression that are commonly seen in children and adolescents coping with cancer treatment. For most patients these symptoms will be mild to moderate in severity and can be thought of as reflecting expectable reactions to the illness and demands of treatment. Supportive care provided by nurses, parents, and child life specialists to minimize physical discomfort, empathize with the frustrations and disappointments of being a patient, and promote opportunities to find some pleasurable activities can be useful for most of these individuals. Similarly nightmares, heightened reactivity, and worry about future health problems or medical procedures are indicators of anxiety commonly encountered in children with cancer. Parents and providers can often help patients with these symptoms by providing reassurance, offering information about the medical course, providing the opportunity to express their worry openly, and teaching some relaxation and distraction techniques.

When symptoms of depression or anxiety do not respond to routine supportive care, cause significant distress, or otherwise interfere with the child's ability to engage in essential daily activities or comply with medical care, a specialized mental health evaluation should be sought. This evaluation drawing on information from the patient, family, and medical team, should be focused on determining the extent and severity of behavioral and psychological symptoms, the relevant psychiatric disorders, if any, as well as deficits in knowledge or coping skills that may need to be addressed. Based on this information, the mental health specialist should be able to recommend which of a variety of types of interventions such as cognitive-behavioral therapy, expressive therapy, play therapy, psychopharmacology, or family therapy would be most appropriate. Psychological assessment can be complicated by questions of whether a pediatric patient's physical symptoms are secondary to the physical cancer or are evidence of psychological disturbance.[267-269] The physical effects of treatment and adverse effects of medications may mimic symptoms of depression or anxiety and interfere with an accurate diagnosis. While working to understand how medical factors can contribute to the presentation of psychological symptoms, it is important not to dismiss these symptoms too quickly as manifestations of physical illness. Hedstrom and colleagues[270] found that medical and nursing staff underestimate the level of depression and anxiety in adolescents with cancer, underscoring the need to carefully consider depression and anxiety as problems in this group.

Relapse and Recurrence

Patients and families who experience a relapse or recurrence of cancer can be expected to enter a second crisis period. Similar to the initial crisis of diagnosis, families experience a cognitive and emotional overload; they often are overwhelmed with fear and anxiety at the same time they are trying to understand the medical implications of the return of the cancer. Families typically feel shocked by news of a relapse. For many families, faith in their medical care, trust in the world, and hope for future treatment are severely challenged.

The initial approach to this new crisis should draw on the elements of psychosocial support described in the section on the crisis of diagnosis, including provision of psychosocial support and participation in a new treatment conference or "day one talk." In the context of a relapse, emotional distress and disbelief are to be expected, and parents, older patients, and siblings may be distraught. Presenting information about prognosis after relapse can be even more difficult, because the prognosis is typically less favorable, and patients and families who previously may have been reassured may now need to be told that cure is less likely or even not to be expected. Families need to have accurate medical information presented to them in a way they can understand, but perhaps just as important, they need to feel that the physicians and other providers are committed to continuing to care for them. For some patients and families a recurrence may be experienced as a personal failure, and the team should be careful to readdress this issue of blame and causality in the context of relapse or recurrence. In cases in which a new treatment plan means transferring care to a new clinical service (e.g., as in the case of a bone marrow transplant) or referral to a new care team or hospital, great care should be taken to ensure that patients and families do not feel abandoned.

The psychosocial challenges facing the patient and family after relapse depend to a large extent on the subsequent treatment that is planned and the extent to which cure remains the goal of treatment. When potentially curative treatment is planned, patients face the challenge of reengaging in a new and potentially more intensive therapy regimen. Patients and families already burdened by an initial treatment may have more trouble adjusting to new demands along with increased anxiety and the psychological experience of "starting over" with a new treatment. Psychosocial care providers should be prepared to reassess the patient and family in this new context and offer emotional support and crisis intervention. Psychosocial providers should also anticipate new issues that could emerge such as financial stress, parent depression, patient behavioral problems, or emotional distress and be prepared to help the family address these issues.

For some patients the likelihood of cure after relapse or recurrence is remote. For these patients, treatment options may include palliative care or experimental therapies. Learning that a cure for the cancer is unlikely and that the patient may be expected to die of his or her disease is devastating for the family. Parents and patients are likely to be terrified and overwhelmed, and care providers may feel unsure about how to help these families. Communication between the physicians and other members of the team is especially important at this juncture,[80] and care should be taken to communicate directly to patients as well as parents and to provide honest information in a sensitive manner. Psychosocial providers should participate in team meetings with the family, assess the needs of the patient, parents, and other family members, and offer crisis intervention and emotional support. They should anticipate that symptoms of depression, hopelessness, and anxiety may become significant in one or more family members and be prepared to offer clinical care and/or referrals to other specialists or community agencies. A more comprehensive discussion of care for the dying child is included in Chapter 70.

SPECIAL POPULATIONS

Adolescents

Adolescence is traditionally seen as a time of transition and growing independence. The diagnosis of cancer during this developmental stage has the potential to disrupt normal functioning and limit the attainment of specific goals that move the adolescent from childhood into adulthood. Research has considered several important areas related to the psychosocial adjustment of adolescents with cancer, including family factors, psychological factors, and socialization factors.[26,209]

Mental health professionals have noted that children and adolescents often function as well or as poorly as their parents during stressful life events. The psychological adjustment of adolescents with cancer was noted to be better when maternal distress was low[271] and maternal adjustment was high.[272] In addition adolescents benefited from the ability of family members to introduce a sense of normalcy and felt more supported during treatment when family members demonstrated flexibility.[148] Some adolescents noted that stressful family relationships impeded successful adjustment to the illness.[273] These data taken together suggest that the adolescent's family is crucial to the adolescent's successful coping and illness management. The medical team should encourage open communication between family members that will promote positive interactions and allow for the ongoing assessment of individual functioning. Psychosocial support should extend beyond the adolescent patient to all family members to ensure the healthy functioning of the entire family system. When problems in an adolescent's behavior or coping are identified, providers should be mindful that some family-focused assessment and intervention may be indicated.

Psychologically adolescents typically possess cognitive abilities that allow for a more sophisticated understanding of their illness and treatment than would be found with younger children. Adolescents often seek information[102,126] and feel empowered by their understanding, which may in turn facilitate their active participation in treatment decisions, thereby increasing their use of effective coping strategies and their overall sense of control.[25] However the ability to understand illness-related information does not guarantee increased psychological adjustment. In fact some adolescents may feel overwhelmed by extensive medical information and corresponding treatment decisions, increasing the level of stress and potential psychological maladjustment. To ensure that the adolescent is appropriately informed, a careful assessment must be conducted to evaluate how information is processed and understood by the adolescent and what amount and type of information will be most helpful. Because adolescents may feel that opportunities to achieve increased independence (e.g., attaining a driver's license) are limited as a result of their illness, including the adolescent in treatment discussions, facilitating effective coping strategies, and encouraging participation in the decision-making process may promote an increased sense of autonomy.[274]

Socially the adolescent's ability to engage with peers, participate in extracurricular activities, or attend school while undergoing treatment is often very limited. Immediately after diagnosis, extensive support is often available to the adolescent and the adolescent's family. However, as treatment progresses and the initial shock associated with the diagnosis decreases, support from friends, teachers, and others in the community may be less consistent. With time friends may return their focus to school and other activities, leaving the diagnosed adolescent feeling isolated and left out.[126,175,209,275] Evan and Zeltzer[25] suggest that developmental level plays a significant role in the type of support that an adolescent both provides and seeks. Encouraging flexibility and support with regard to an adolescent's participation in his or her usual activities, such as attending school, attending social events, and spending time with friends, can help adolescents feel "normal" and may increase adherence to treatment.[253] Encouraging the adolescent patient to bring a friend to a clinic appointment can also be helpful to bridge the divide between peers and the medical setting for some adolescents. Research findings suggest that adolescents with cancer seek social support from same-aged peers encountering similar developmental issues[276] and from parents and other adults who have a wider range of life experiences that may be relevant to overcoming their illness.[275]

Studies have considered several factors related to the development of successful adolescent social relationships, including the importance of self-esteem and sexual identity.[26,277-282] For example, findings suggest that adolescents who are absent from school may begin to experience academic difficulties that have a negative impact on their self-confidence and self-esteem. This decrease in self-confidence and self-esteem undermines the adolescent's attempt to form and sustain social relationships. Other findings highlight sexual identity and the significant role that sexual identity plays in adolescent relationships and developing self-esteem.[26] The adolescent diagnosed with cancer may have a negative body image as a result of treatment-related adverse effects (e.g., hair loss, procedural/surgical scars, and weight gain or loss)[283-286] and more limited opportunities for both formal and informal sex education, impeding healthy sexual development and positive self-esteem.[287] Evan and colleagues[26] suggest assessing the adolescent's cognitive, social, developmental, and family functioning, both to determine the most appropriate ways to enhance sexual health and self-esteem and to recommend opportunities to assume personal control and interact with other adolescents who may be encountering similar experiences.

Social support and social relationships are essential to successful adjustment during the treatment course and beyond. Although parents and other family members typically provide the primary, day-to-day support needed by the adolescent patient to successfully complete treatment, peer relationships are equally valuable, because they allow adolescents to remain connected to other key aspects of their life that exist outside of their treatment. Members of the medical staff can also provide important interpersonal connections that can greatly improve the adolescent's social functioning. The medical team should work to understand how cancer is adversely affecting the adolescent's peer relationships and encourage peer interactions by supporting the adolescent's return to school and participation in peer-related activities when feasible. For adolescents who have been dating, cancer often interferes with their ability to develop these relationships or to maintain contact with a boyfriend or girlfriend. For the sexually active adolescent, the illness may also raise questions about whether they need to limit their sexual activities because of treatments or risks for infection. Counseling and information on contraception and prevention of sexually transmitted diseases should be provided to sexually active adolescents in keeping with recommended practices for this age group.[288,289] Because parents may not be privy to all of their adolescent child's romantic or sexual activities, it is important that providers discuss these issues with adolescents in private and provide appropriate support and information.

Neurooncology

The literature has indicated that, in general, children and adolescents treated for cancer will have a positive adjustment to treatment and that once the acute crisis of diagnosis has passed, they tend to adapt positively.[290,291] The diagnosis of a pediatric brain tumor is often more complicated given the location of the tumor, the toxicities of treatment, the possibility of long-term medical late effects, and the poor prognosis associated with many pediatric brain tumors.[292,293] Research has often indicated that patients diagnosed with central nervous system (CNS) tumors are at greater risk for psychological difficulties, neurocognitive deficits, poor functional outcomes, and decreased quality of life when compared with children treated for other cancers.[174,290,294-298] In addition disease-related fear, uncertainty, loss of control, and depression have been found to be heightened among parents of children treated for a CNS tumor when compared with parents of children who have acute lymphoblastic leukemia or acute myeloid leukemia.[294] Issues associated with diagnosis, the impact of treatment on immediate and long-term functioning, and the morbidity associated with CNS tumors are factors contributing to the increased risk for psychological distress among parents.

The terms "benign" and "malignant" are often the nomenclature by which we understand the gravity of a particular diagnosis. However in pediatric neurooncology, very little is benign. Children and adolescents die of both benign and malignant tumors. Regardless of the label, persons with both benign and malignant tumors require treatment (e.g., surgery, chemotherapy, radiation, or autologous stem-cell transplant) that comes with significant demands that can be challenging for patients and families. Parents are often in the position of consenting to a treatment that will cause significant late effects and will have a negative impact on quality of life for their child, all in the hope for a cure. The stress associated with making this decision in the context of trying to integrate new diagnostic information, the input of family and friends, and the abundance of material on the Internet can be extremely overwhelming for parents. Many families opt to seek second opinions at other institutions, which can be helpful but also can make treatment decisions more complex. Psychosocial care providers can help parents process information, encourage additional conversations with medical providers, and facilitate coping styles that most effectively support decision making.

Given that the cancer is located in the brain, all treatment interventions come with a number of risks. An insult to the brain can have an impact on motor skills, cognition, vision, speech/language, and emotional regulation. Although some of the postoperative complications can improve, it is also possible that a patient may not return to his or her presurgical level of functioning,[299] which has significant implications for self-esteem and self-identity. Psychosocial providers working with children with brain tumors need to recognize the impact of loss, grieving related to permanent changes in functioning, despair pertaining to the need for extensive rehabilitation, and uncertainty about the future. These issues are prevalent not just for the patient but for the family as a whole. Providing the space in which a patient and family can grieve, while dealing with ongoing medical stressors, is important for future psychological adjustment. Patients may believe that they are unable to engage with peers because of their limitations; however, guidance about how to encourage participation in activities is crucial. For patients who may not be able to engage in athletics, ongoing participation on the sports team through a team manager position or scorekeeper can be instrumental in keeping patients connected to peers, bolstering self-confidence, and fostering the attainment of developmental milestones.

Although significant improvements have occurred in the survival rate for some pediatric brain tumors, some diagnoses are still associated with a very poor prognosis. The medical team is often faced with the difficult task of telling a family that their child is not likely to survive, while preserving some hope through the form of treatment options. Psychosocial care providers can serve as an important support during the meeting, provide guidance with regard to having additional family members at the meeting, and validate the challenges of making treatment decisions. Because of advancements in treatment, parents are often faced with the challenge of deciding between clinical trials, none of which has data regarding efficacy. Parents are often overwhelmed at this time while they process the news of the diagnosis, the prognosis, and the treatment options. It is crucial that psychosocial clinicians assess the parents' psychological history and current functioning to identify interventions that are required immediately to help facilitate decision making and treatments that will provide symptom relief on a long-term basis.

Hematopoietic Stem Cell Transplant

Similar to patients with CNS tumors, patients and families for whom stem-cell transplant is the recommended treatment are at increased risk for psychosocial complications during and after treatment in terms of their overall psychological adjustment.[300,301] Increased vulnerability to psychological distress and the posttraumatic symptoms described in the literature that are associated with the stem-cell transplant experience can be attributed to factors associated with treatment risks, morbidity, the stress associated with simply trying to get to the point of transplantation, restrictions and isolation, and the late effects associated with treatment.

The process of getting disease in remission or eliminating any residual disease can be an anxiety-provoking start to the challenges of transplantation. Patients and families often experience anticipatory anxiety as they await news from screening tests that will clear the patient to move forward with the transplant process. At the same time, significant stress is associated with finding an appropriate donor. Many patients have siblings who are eligible to be the donor, and although they are certainly fortunate in terms of having a good match, this process becomes complicated and plays into multiple layers of family dynamics. Sibling donors have been shown to be at risk for posttraumatic stress disorder, anxiety, and low self-esteem.[302-304] Psychosocial care providers play an important role by conducting donor evaluations that provide the medical information donors need to ensure that they understand the process. Psychosocial care providers also provide the psychological support and care required to ensure that the donor is not being coerced into the procedure and understands the possibility of both a positive and negative outcome and that neither outcome is associated with the donor process. Parents now have to worry about the emotional and medical functioning of both the sibling and the patient, leading to increased vulnerability for distress.[305]

Parents with a child who requires a bone marrow transplant are in the position of choosing a treatment that will irrevocably change the course of their child's life because of the toxicities of treatment. The adverse effects of transplantation can include hearing loss, infertility, renal failure, cardiac dysfunction, growth and gonadal failure, secondary malignancies, neurocognitive delay, and possible death. Discussion of the potential risk of death is part of the consenting process and indicates the intensity of the treatment and the potential psychological trauma that can result for both patients and parents. Indeed retrospective studies have indicated that parental distress tends to be at its highest before the actual transplant procedure.[306,307] This information is crucial in terms of determining points of intervention, given that the literature has shown that fewer maternal depression symptoms are associated with improved patient outcomes after the transplant procedure.[308] Ensuring that patients and families have been provided with appropriate medical information up front and ample opportunity to ask questions of the medical team is one way that psychosocial care providers can help empower families before the transplant procedure.[309] A thorough assessment that includes identification of significant parental psychological distress well before the start of transplant process is important to ensure that potential psychotropic interventions have ample opportunity to take effect before the patient enters the hospital.

Given the intensity of the treatment, the extended period of hospitalization, and the potential for significant medical complications, families should be followed closely by their psychosocial care provider throughout the transplant process. Use of cognitive-behavioral interventions such as relaxation strategies and guided imagery can be helpful for both patients and caregivers. Implementation of behavioral plans that provide positive reinforcement to the patient with regard to the significant demands of treatment can be very helpful. Given the significant stress associated with transplant procedure, as well as the isolation that can occur for caregivers, parent support groups can be extremely helpful in connecting parents with others going through similar experiences, allowing for parental self-care, and providing a forum for discussion of worries and challenges. Many medical institutions also incorporate family-centered rounds or regularly scheduled family meetings to keep lines of communication open, provide an opportunity for parents to ask questions of the team, and address any logistical or systemic issues that may be negatively affecting the family's adjustment to the transplant. Keeping in close contact with medical, nursing, and child life care providers is critical to providing the best care for these families who are at significant psychosocial risk.

Once patients have completed the transplant and are discharged from the hospital, they must participate in an intensive outpatient plan that can include multiple trips to the hospital or clinic per week, use of multiple medications, and restriction in terms of social contact. Given the immunocompromised status of patients after transplantation, they are not allowed to attend school or be in an enclosed space with a lot of people. This restriction can be extremely difficult for children and adolescents who can be essentially cut off from their peers. This period is also anxiety provoking because parents are concerned about the possibility of complications, infections, and the potential for disease relapse. Parents are often on high alert with increased vigilance regarding oral intake, participation in physical/occupational/speech therapies, and/or hygiene. Adolescent patients in particular may have significant difficulty in managing a hypervigilant parent in the context of their limited control. Power struggles between patients and parents are not uncommon, and anticipatory guidance with regard to these potential challenges early in the process with a focus on prevention can be an important role for the psychosocial clinician.

With the significant limitations placed on daily functioning as a result of the intensive treatment and the very real possibility of disease relapse despite the transplant, it is understandable that patients who have undergone bone marrow transplant are at increased risk for psychological distress, both acutely and long term.[301] Clinicians should be aware that subsets of patients are more likely to experience psychological difficulties, specifically patients who are dealing with debilitating adverse effects such as graft-versus-host disease (GVHD) and its treatment. Results from the Bone Marrow Transplant Survivor Study indicated that active chronic GVHD was associated with somatic and global psychological distress.[310] In addition, the use of corticosteroids in the management of GVHD is another factor associated with depression and diminished quality of life.[311]

Despite the risks, some reports in the literature demonstrate that many survivors of bone marrow transplant have a good health-related quality of life within 12 months after the transplant procedure, particularly if they are not affected by severe adverse effects of treatment.[300,312] Parents and children may have very different perspectives on the child's progress and adaptation after transplant, making it important to include both patient and parent perspectives whenever possible.[301,313,314] Parents may experience elevations in distress during the acute phase of the transplant process, but the distress typically resolves approximately 4 to 6 months after the transplant for most parents. Nonetheless subgroups of parents exhibit distress after the transplant that is significant enough to meet the criteria for posttraumatic stress disorder or to be classified as having posttraumatic stress symptoms; families with lower socioeconomic backgrounds have been noted to be at particular risk.[315] With this in mind, clinicians should incorporate assessment of social and psychological functioning of the patient and the family into the routine follow-up of patients undergoing a transplant. Informing patients and families that ongoing psychological or social problems are not uncommon after transplant may help families be forthcoming about their functioning and help those who have significant difficulties to accept a referral for more specialized assessment and treatment.

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    This article reviews the evidence-based literature available with regard to caring for adolescents and young adults with cancer. Factors pertinent to the psychological adjustment of adolescent and young adult patients and the clinical implications of late effects are discussed. Practical examples of empirical interventions to address psychosocial challenges for adolescent and young adult patients are presented.

43. Masera G, Spinetta JJ, Jankovic M, et al: Guidelines for a therapeutic alliance between families and staff: a report of the SIOP Working Committee on Psychosocial Issues in Pediatric Oncology. *Med Pediatr Oncol* 30:183–186, 1998.

    This document was developed by the International Society of Paediatric Oncology Working Committee on Psychosocial Issues in Pediatric Oncology. It addresses the therapeutic alliance between families and staff and the overarching goal of curing cancer while minimizing medical and psychological toxicities.

58. Mack JW, Grier HE: The day one talk. *J Clin Oncol* 22:563–566, 2004.

    This article outlines a thoughtful and sensitive approach for physicians who need to inform the family of a child's cancer diagnosis. The article provides concrete suggestions for the content and structure of the "day one" talk, emphasizing an approach that is empathic and supportive to patients and families.

64. Levi RB, Marsick R, Drotar D, et al: Diagnosis, disclosure, and informed consent: learning from parents of children with cancer. *J Pediatr Hematol Oncol* 22:3–12, 2000.

    The authors report on a retrospective study of parental perception surrounding their child's cancer diagnosis and the process of informed consent. Focus groups were conducted and themes associated with receiving the diagnosis, how to improve the informed consent process, and lack of control were discussed. Limitations were identified in parental understanding of the distinction between the treatment, research participation, and other aspects of the cancer experience.

80. Mack JW, Wolfe J, Grier HE, et al: Communication about prognosis between parents and physicians of children with cancer: parent preferences and the impact of prognostic information. *J Clin Oncol* 24:5265–5270, 2006.

    The authors of this study examined parents' preferences for prognostic information in regard to their child's cancer diagnosis. Parents were surveyed and findings indicated that the majority of parents wanted as much prognostic information as possible and wanted that information to be in numbers. Although some parents found the information distressing, they were no less likely to want the information.

104. Dolgin MJ, Phipps S, Fairclough DL, et al: Trajectories of adjustment in mothers of children with newly diagnosed cancer: a natural history investigation. *J Pediatr Psychol* 32:771–782, 2007.

     This study assessed posttraumatic stress symptoms (PTSS) in mothers of children who were diagnosed with cancer, the relationships between sociodemographic and psychosocial distress over time, and whether particular subgroups of mothers could be identified based on trajectory of adjustment. Mothers completed standardized measures at the time of diagnosis, 3 months, and 6 months. Findings indicated mildly elevated negative affectivity and PTSS at the time of diagnosis, but improvement over time. The study was able to identify subgroups of mothers who were likely to have particularly trajectories of adjustment.

105. Kazak AE, Boeving CA, Alderfer MA, et al: Posttraumatic stress symptoms during treatment in parents of children with cancer. *J Clin Oncol* 23:7405–7410, 2005.

     This study examined posttraumatic stress symptoms (PTSS) in mothers and fathers of children receiving treatment for cancer and in relation to treatment variables. Parents completed questionnaires and data were obtained from oncologists regarding treatment intensity. With the exception of one parent, all participants reported PTSS. In families in which both parents participated in the study, 80% had at least one parent report moderate to severe

PTSS. Treatment-related variables were minimally associated with PTSS.

117. Chen E, Craske MG, Katz ER, et al: Pain-sensitive temperament: does it predict procedural distress and response to psychological treatment among children with cancer? *J Pediatr Psychol* 25:269–278, 2000.

This intervention study examined pain sensitivity and distress in children undergoing lumbar punctures (LPs). Baseline data were gathered on pain sensitivity, and then children were randomized into the psychological intervention arm or control group. Findings demonstrated that children who reported high pain sensitivity had higher levels of anxiety and pain prior to and during the LP. Children who had higher pain sensitivity and who received the intervention had significant decreases in distress with time, whereas children with high pain sensitivity who did not receive the intervention reported elevated LP distress with time.

130. Sourkes BM: *Armfuls of time: the psychological experience of the child with a life-threatening illness*, Pittsburgh, 1995, University of Pittsburgh Press.

This seminal work on the emotional experience of the ill child provides clinical material that highlights the value and importance of a multidisciplinary approach. Using play therapy vignettes and stories from patients and their families, a rich description is provided of the challenges faced when experiencing a life-threatening disease and the interventions that can provide support around the issues of diagnosis, treatment, and loss.

154. Houtzager BA, Oort FJ, Hoekstra-Weebers JE, et al: Coping and family functioning predict longitudinal psychological adaptation of siblings of childhood cancer patients. *J Pediatr Psychol* 29:591–605, 2004.

This study examined the relationships between coping and family functioning in siblings of children with cancer. Assessments were conducted at four time points across 2 years since the cancer diagnosis. Findings indicated that at initial assessment (1 month after the diagnosis), psychological adjustment was impaired, but that it normalized over time. Optimism and having the sibling involved were noted as protective factors. Sibling age, gender, and coping style were noted to help identify at-risk siblings.

172. Zebrack B, Bleyer A, Albritton K, et al: Assessing the health care needs of adolescent and young adult cancer patients and survivors. *Cancer* 107:2915–2923, 2006.

In response to the literature demonstrating that adolescent and young adult (AYA) patients have not had improvement in cancer outcomes compared with other populations, this study sought to identify the health and supportive care needs of AYA patients and survivors. Findings from surveys of medical providers and young adults survivors indicated agreement in a number of areas of importance across health care professionals and AYA survivors. However the importance of meeting same-aged peers with cancer was rated significantly higher for AYA survivors. This study identified areas in which resources could be useful in potentially changing the gap in cancer outcomes between the population as a whole and AYA patients.

174. Vannatta K, Gartstein MA, Short A, et al: A controlled study of peer relationships of children surviving brain tumors: teacher, peer, and self ratings. *J Pediatr Psychol* 23:279–287, 1998.

This original research evaluated the behavioral reputation and acceptance of pediatric brain tumor survivors. Children (ages 8 to 18 years) were compared with nonchronically ill peers and data were gathered from peers, teachers, and patients. In comparison with same-aged classmates, children diagnosed with brain tumors were reported to be more socially isolated and received fewer friendship nominations. Issues associated with fatigue and school absence were reported. This article represents the initial work that highlighted the significant social struggles experienced by pediatric brain tumor survivors.

198. Zeltzer LK, Dolgin MJ, LeBaron S, et al: A randomized, controlled study of behavioral intervention for chemotherapy distress in children with cancer. *Pediatrics* 88:34–42, 1991.

This article reports on a prospective trial comparing hypnotherapy for reduction of chemotherapy-related distress with distraction/relaxation therapy and an attention control condition. Hypnotherapy was found to be superior to other conditions, with the results discussed in the context of complex interactions between biologic and psychological factors contributing to nausea and related adverse effects of chemotherapy.

210. Zebrack BJ, Gurney JG, Oeffinger K, et al: Psychological outcomes in long-term survivors of childhood brain cancer: a report from the childhood cancer survivor study. *J Clin Oncol* 22:999–1006, 2004.

This study evaluated the psychological outcomes of the pediatric neurooncology participants in the Childhood Cancer Survivor Study. Data for survivors and siblings were utilized and levels of psychological distress were consistent with reports in the general population. Survivors reported high levels of distress and depressive symptoms compared with siblings. Higher distress was associated with female gender, low household income, lower educational attainment, unemployment, poor health, and not being married. This study indicates that although treatment for pediatric brain tumors may not be associated with higher distress than the general population, the social difficulties experienced by survivors may be what puts them at greater risk for psychological challenges.

215. Spinetta JJ, Jankovic M, Eden T, et al: Guidelines for assistance to siblings of children with cancer: report of the SIOP Working Committee on Psychosocial Issues in Pediatric Oncology. *Med Pediatr Oncol* 33:395–398, 1999.

This document establishes a framework for the psychosocial care of siblings in pediatric oncology as developed by the International Society of Paediatric Oncology Working Committee on Psychosocial Issues in Pediatric Oncology, instituted in 1991. It outlines the general principles for helping siblings throughout various phases of treatment, including relapse, palliation, and death.

216. Sloper P: Predictors of distress in parents of children with cancer: a prospective study. *J Pediatr Psychol* 25:79–91, 2000.

This study investigated parental psychological distress in both mothers and fathers of children with cancer at 6 and 18 months after diagnosis. Relationships between illness, illness appraisal, resources, and coping were examined. Findings demonstrated elevated levels of distress for both mothers and fathers that were consistent with time. Internal factors such as appraisal and coping were associated with maternal distress, whereas external factors such as employment issues and the number of hospital admissions were associated with paternal distress. This article highlights the importance of identifying at-risk parents so appropriate supports can be put into place early during the treatment process.

253. Butow P, Palmer S, Pai A, et al: Review of adherence-related issues in adolescents and young adults with cancer. *J Clin Oncol* 28:4800–4809, 2010.

This review article examines studies that investigated medication adherence in the adolescent and young adult (AYA) oncology population between the years 1980–2008. Lack of adherence has been associated with higher risk of relapse, more adverse effects, and poor outcomes. The review highlighted the methodologic issues found in the AYA adherence literature, including a lack of clarity around defining the AYA age range and a lack of representation of oncologic diagnoses beyond leukemia and lymphoma. Reported rates of nonadherence range are broad, from 27% to 60%. Consistent predictors of adherence include openness of family relationships and support.

254. Bhatia S, Landier W, Shangguan M, et al: Nonadherence to oral mercaptopurine and risk of relapse in Hispanic and non-Hispanic white children with acute lymphoblastic leukemia: a report from the Children's Oncology Group. *J Clin Oncol* 30:2094–2101, 2012.

This original research report describes a prospective study of the prevalence and impact of nonadherence with oral mercaptopurine in a cohort of 327 patients treated for acute lymphoblastic leukemia. Using a medication event-monitoring system, findings show that nonadherence is associated with relapse of disease, ethnicity, and patient age.

283. Pendley JS, Dahlquist LM, Dreyer Z: Body image and psychosocial adjustment in adolescent cancer survivors. *J Pediatr Psychol* 22:29–43, 1997.

This study evaluated body image and social adjustment in adolescent cancer survivors and healthy peers. Participants completed questionnaires and a videotaped interview. Raters who were blind to medical history rated the attractiveness of the participants.

Survivors reported being less involved in social activities compared with the comparison group. Survivors who had been off treatment for a longer period reported lower self-worth, higher social anxiety, and more negative body image. Despite the self-report around these difficulties, these long-term survivors were not rated as less attractive by observers. This article provides evidence that psychological adjustment issues and body image difficulties may not fully emerge until well after treatment completion.

290. Zeltzer LK, Recklitis C, Buchbinder D, et al: Psychological status in childhood cancer survivors: a report from the Childhood Cancer Survivor Study. *J Clin Oncol* 27:2396–2404, 2009.

This article reviews findings from the Childhood Cancer Survivor Study regarding psychosocial adjustment, health-related quality of life (HRQOL), and life satisfaction. Comparisons are made between siblings and survivors and to normative data when available. Overall most survivors are psychologically healthy and report satisfaction with their lives; a significant proportion of survivors report more symptoms of psychological distress and poorer physical HRQOL. Risk factors for psychological distress and poor HRQOL are brain tumor diagnosis, female sex, lower educational attainment, unmarried status, presence of a major medical condition, and treatment with cranial radiation and/or surgery. Psychological distress also predicted poor health behaviors, including smoking, alcohol use, fatigue, and altered sleep.

310. Sun CL, Francisco L, Baker KS, et al: Adverse psychological outcomes in long-term survivors of hematopoietic cell transplantation: a report from the Bone Marrow Transplant Survivor Study (BMTSS). *Blood* 118:4723–4731, 2011.

The authors report on a large cohort study of survivors of hematopoietic cell transplantation. Psychological outcomes were assessed in bone marrow transplant (BMT) survivors and sibling control subjects. Survivors of BMT reported significantly higher levels of psychological distress than did their siblings, with those who had been exposed to prednisone reporting global distress across somatization, anxiety, and depression scales. High levels of somatization were reported, particularly for patients with graft-versus-host disease.

312. Parsons SK, Phipps S, Sung L, et al: NCI, NHLBI/PBMTC First International Conference on Late Effects after Pediatric Hematopoietic Cell Transplantation: health-related quality of life, functional, and neurocognitive outcomes. *Biol Blood Marrow Transplant* 18:162–171, 2012.

The authors of this article review the late effects in survivors of pediatric hematopoietic stem cell transplantation in the domains of health-related quality of life, physical function, and neurocognitive function.

314. Jobe-Shields L, Alderfer MA, Barrera M, et al: Parental depression and family environment predict distress in children before stem cell transplantation. *J Dev Behav Pediatr* 30:140–146, 2009.

This study examined the factors of parental and family environment variables in relation to the severity of distress in children slated to receive a stem cell/bone marrow transplant. Parental depression, family cohesion, and expressiveness were found to predict child distress, and parental depression, when high, was associated with elevated child distress regardless of family environment. This study provides evidence that parental psychological functioning prior to transplant is a predictor of child psychological adjustment.

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CHAPTER 74

Ethical Considerations in Pediatric Oncology Clinical Trials

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CHAPTER OUTLINE

HISTORIC PERSPECTIVE
WHAT IS "CONSENT" IN PEDIATRICS?
ASSENT IN CHILDHOOD CANCER TRIALS
PEDIATRIC CLINICAL TRIALS
Minimal Risk
Phase I and II Trials in Pediatric Oncology
Phase III Trials and Clinical Equipoise
SPECIAL CONSIDERATIONS FOR CANCER TRIALS USING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION
MODELS AND TOOLS FOR ETHICAL DECISION MAKING IN PEDIATRIC CLINICAL TRIALS
FURTHER CHALLENGES IN PEDIATRIC CLINICAL ONCOLOGY TRIALS
Bioinformatics and Genomics
Globalization
CONCLUSION

Ethical decision making is a fundamental goal common to both clinical medicine and clinical research trials of all types. Broad attempts have long been made to thoroughly explicate the elements of ethical decision making that are common to both settings. However this goal has been pursued with increased vigor since the exposure of human moral abuses in the United States Public Health Service–sponsored Tuskegee Syphilis Study and by the Nuremberg trials and President Clinton's Advisory Committee on Human Radiation Experiments. The practical yield has been the production of highly influential documents such as the Belmont Report, the Declaration of Helsinki and its several versions, guidelines produced for the International Arena by the Council for International Organizations of Medical Sciences, and most recently, the adoption of the Universal Declaration of Bioethics and Human Rights in October 2005 by the United Nations Educational, Scientific, and Cultural Organization. Common to these efforts are principles such as respect for persons, beneficence, and justice. Table 74-1 lists selected guidelines for research ethics. But how do general principles translate into practical action that is ethically sound for the practitioner faced with a particular clinical situation, for the clinical research physician considering whether to offer a phase I trial to a patient, and for the investigator whose nontherapeutic trials pose some risks?

The issues become considerably more complex when the patient or research participant is a child and the decision maker is not the person facing the possibility of risk or benefit. This issue poses special challenges in both pediatric clinical medicine and pediatric clinical research. The challenges are made more acute by the fact that they are not static. The considerations change as a child grows in age and experience and as we move from standard-of-care therapy to clinical trials, which, as recently argued, involve the standard of care for future children, the results of which have been a remarkable success story.[1,2,3] Indeed it may be that survival percentages for adolescents and young adults with "pediatric" cancers have lagged behind those of younger patients in part because of their lower rates of participation in clinical trials.[4] Further the issues do not remain static across all types of clinical trials.[5] A child enrolled in a phase III clinical trial for newly diagnosed, standard-risk leukemia faces a situation very different from that of a child who has had multiple relapses of leukemia and whose family must choose between a phase I trial and a Make-A-Wish trip to the Grand Canyon.

This chapter begins with a brief historic review of the unique issues in pediatric medicine and pediatric clinical trials. It then moves to issues of parental permission and assent—issues that draw on, but are not fully informed by, the many studies of informed consent in adults. The differences in language and approach required as patients progress from phase III trials to phase I trials and palliative care are examined. After these more general issues, we will consider more specific questions, such as those raised by autopsy, stem cell transplantation, advances in

TABLE 74-1 Selected Guidelines on Ethics of Biomedical Research with Human Subjects

Guideline	Source	Year and Revisions
Nuremberg Code	Fundamental; Nuremberg Military Tribunal decision in *United States v Brandt*	1947
Declaration of Helsinki	World Medical Association	1964, 1975, 1983, 1989, 1996
Belmont Report	National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research	1979
International Ethical Guidelines for Biomedical Research Involving Human Subjects	Council for International Organizations of Medical Sciences in collaboration with World Health Organization	Proposed in 1982; revised, 1993
45 CFR 46, Common Rule	Other: U.S. DHHS and other federal agencies	DHHS guidelines in 1981; Common Rule, 1991
Guidelines for Good Clinical Practice Trials on Pharmaceutical Products	World Health Organization	1995
Good Clinical Practice: Consolidated Guidance	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use	1996
Convention of Human Rights and Biomedicine	Council of Europe	1997
Guidelines and Recommendations for European Ethics Committees	European Forum for Good Clinical Practice	1997
Medical Research Council Guidelines for Good Clinical Practice in Clinical Trials	Medical Research Council, United Kingdom	1998
Guidelines for the Conduct of Health Research Involving Human Subjects in Uganda	Uganda National Council for Science and Technology	1998
Ethical Conduct for Research Involving Humans	Tri-Council Working Group, Canada	1998
National Statement on Ethical Conduct in Research Involving Humans	National Health and Medical Research Council, Australia	1999

From Emanuel EJ, Wendler D, Grady C: What makes clinical research ethical? JAMA *283:2701–2711, 2000.*
CFR, Code of Federal Regulations; *DHHS,* Department of Health and Human Services.

technology, conflict of interest, insurance, ethics consultation, and international bioethics.

HISTORIC PERSPECTIVE

For hundreds of years, medical research has involved children as subjects.[6] Edward Jenner first used an experimental smallpox vaccine on his own 1-year-old son at the end of the eighteenth century. At the end of the nineteenth century, a 9-year-old child was the first human recipient of Louis Pasteur's rabies vaccine.[7] However it was not until the first half of the twentieth century that regulations governing such experimental therapy began to be established and the importance of consent and parental permission became clearer.

The informed consent of human research subjects was first officially advocated in the Nuremberg Code, a code of ethics that grew out of the trial of German physicians who conducted egregious human experiments in Nazi concentration camps during World War II. However because this important code emphasized the absolute requirement of informed consent, it implicitly excluded children—who cannot give informed consent—from participating in research on human subjects.[8]

The issue was more explicitly addressed in 1964, when the World Medical Association adopted a set of research ethics principles now known as the Declaration of Helsinki.[9,10] This set of principles established the priority of the human subjects' interests over those of science and society, and it sanctioned the participation of children in research if permission was given by the child's responsible guardian.

Interestingly experiments were performed on children after the publication of the Nuremberg Code, which implicitly excluded children from research, and before the Declaration of Helsinki, which explicitly allowed such research under specific conditions. One of the most important of these experiments was a series of radiation exposure experiments. Among participating institutions was the Fernald School in Framingham, Massachusetts and the Massachusetts Institute of Technology with funding from the Quaker Oats Company, in which children deemed mentally retarded were fed radioactive iron and calcium in their cereal.[11] President Clinton's Advisory

Committee on Human Radiation Experiments revealed the ethical consequences of inadequate parental permission in these experiments.[12]

Another important set of experiments on children at that time occurred at the Willowbrook institution (located on New York's Staten Island) and was designed to follow the natural history of hepatitis in children deemed mentally retarded.[13,14] Newly arrived children were infected with hepatitis virus by housing them with others already known to be infected. Participating children were kept in a special unit with better conditions and nutrition, and children whose parents agreed to participation were admitted more rapidly. The lead investigator did require a thorough consent process that included a 2-week waiting period for full deliberation. However because Willowbrook was so crowded, critics subsequently argued that the expedited admission and special treatment amounted to coercion. These two cases underscored the need for ethical review and oversight of human subjects research involving children, even when parental permission is obtained.

The United States Congress became increasingly concerned about research ethics during the early 1970s, in part because of the syphilis study involving poor black men conducted by the U.S. Public Health Service at the Tuskegee Institute. As a result the National Commission for the Protection of Research Subjects of Biomedical and Behavioral Research was formed, and it published the Belmont Report in 1979.[15] This report embraced three principles that are now familiar and accepted as crucial for research involving human subjects: respect for persons, beneficence, and justice. The principle of beneficence acknowledges the Hippocratic maxim "do no harm" and extends it to include maximizing the possible benefits and minimizing the possible harms to research subjects (minimizing harms is often formulated separately as a fourth principle, nonmaleficence).[16] The principle of beneficence is operative in any discussion weighing the risks and benefits of research on human subjects, especially when the subject is a minor. The principle of justice concerns the right and fair distribution of the benefits of research, as well as the burdens, an issue that has a particular urgency when clinical treatment and research are considered globally. The concept of respect for persons includes two principles: that individuals be treated as autonomous agents and that those with less autonomy are entitled to protection. The latter principle is especially relevant in pediatrics and is somewhat fluid in its definition because of the growing autonomy of pediatric patients as they approach adulthood.

Given the past abuse of research subjects who were harmed in studies that they did not understand and for which they had not given meaningful permission, it is understandable that "informed consent" has become the dominant concept in discussion about ethical research on human subjects. However recent arguments have been put forth that informed consent is not always necessary for ethical clinical research, nor is it sufficient to qualify research as ethical. A recent review of major codes and declarations relating to research with human subjects led to the proposal that there be seven requirements for ethical clinical research.[17-20] The research must lead to enhancement of health or knowledge. It must be methodologically rigorous. Selection of study sites and of individual subjects should be determined by scientific objectives and the potential for and distribution of risks and benefits. Given standard clinical practice and the research protocol, risks should be minimized, the potential for benefits should be enhanced, and risks must be outweighed by potential benefits to individuals and knowledge for society. Individuals should be informed about the research and provide voluntary consent. Research subjects should have their privacy protected, have the opportunity to withdraw, and have their well-being monitored. These requirements are thorough and persuasive, but they still must be adapted to the conditions under which the research is conducted. Factors of health, economy, culture, religion, and technology will affect how these requirements are translated into concrete, ethical action. For this reason, the specific context in which the research is conducted will affect ethical deliberation.

Ethical decision making in therapeutic and research efforts for children comprises all of the previously discussed considerations, plus the profoundly salient fact that the recipient of the risk or benefit is not the person who makes the decisions. The minor may not be capable of assimilating sufficient information to meaningfully participate in an "informed choice." One need not enter the arena of international bioethics to find challenges to the role of "autonomy" in ethical decision making: such challenges are the daily fare in pediatric medicine and research, because children's "autonomy" is an issue that changes from day to day.

WHAT IS "CONSENT" IN PEDIATRICS?

The Nuremberg Code provides one starting point for understanding the meaning and importance of informed consent. The statement in this code that "the voluntary consent of human subjects is absolutely essential" has been interpreted as legal capacity, a power of free choice based on knowledge and comprehension.[21] Consent, which was once seen as a single event, has come to be understood to be more of a process.[22] It is not surprising, with the development of guidelines and goals for consent, that the process has become more intentional and more highly scrutinized.

The dominant theoretical framework for morally valid informed consent requires that four criteria be met: disclosure, understanding, voluntariness, and competence. Briefly, the core set of information that must be *disclosed* includes (1) facts (such as risks, benefits and alternatives) that patients (subjects) and providers believe are relevant to the decision, (2) the recommendation of the professional, and (3) the purpose, nature, and limitations of consent. *Understanding* goes beyond disclosure because, although the elements disclosed can be objectively stated, true understanding involves many variables and is more difficult to assess. Establishing and documenting understanding remains a great challenge, because information that has been disclosed but not understood contributes little to the ideal paradigm of informed consent.

Voluntariness—another complex notion susceptible to interpretation—means at least that a decision is made without constraints of coercion or manipulation. Finally, *competence*, which is conventionally understood as the ability to perform a task, has also become a complex concept the definitions of which derive from law, psychiatry, and philosophy. At base a person's competence (or perhaps more accurately expressed as cognitive capacity) to make a particular decision relates to the person's ability to understand and think rationally about available choices, to weigh the benefits and burdens and risks within the context of his or her life and values, and then to use that understanding and logic to make a rational decision.

In the context of complex pediatric medical therapy or research, the process of consent is influenced by the fact that parents are making high-stakes decisions on behalf of their own child. The Children's Cancer Group (CCG) conducted studies that showed that a sense of pressure is perceived by parents when making the consent decision for intervention in the context of childhood cancer.[23] However a more recent study indicated that despite significant dissatisfaction with the consent process among CCG clinician-investigators, most parents were satisfied with it.[24] The same study indicated that the parents' satisfaction did not constitute adequate informed consent.

Even among clinicians assessment of the process of informed consent varies. The experience of the clinician is relevant in this assessment. Simon et al.[25] found that clinicians with 10 or fewer years of experience were more likely to say that the most important goal of informed consent is to explain the disease and treatment and were more likely to suggest to parents that other children might benefit from the research. The same study found that in the end, when reports from clinicians and parents are compared, clinicians are dissatisfied with aspects of consent that parents seem far more satisfied with. For example, clinicians expressed concern about information overload and about the fact that the consent discussion often occurs while the parents are still in a state of shock about the diagnosis.

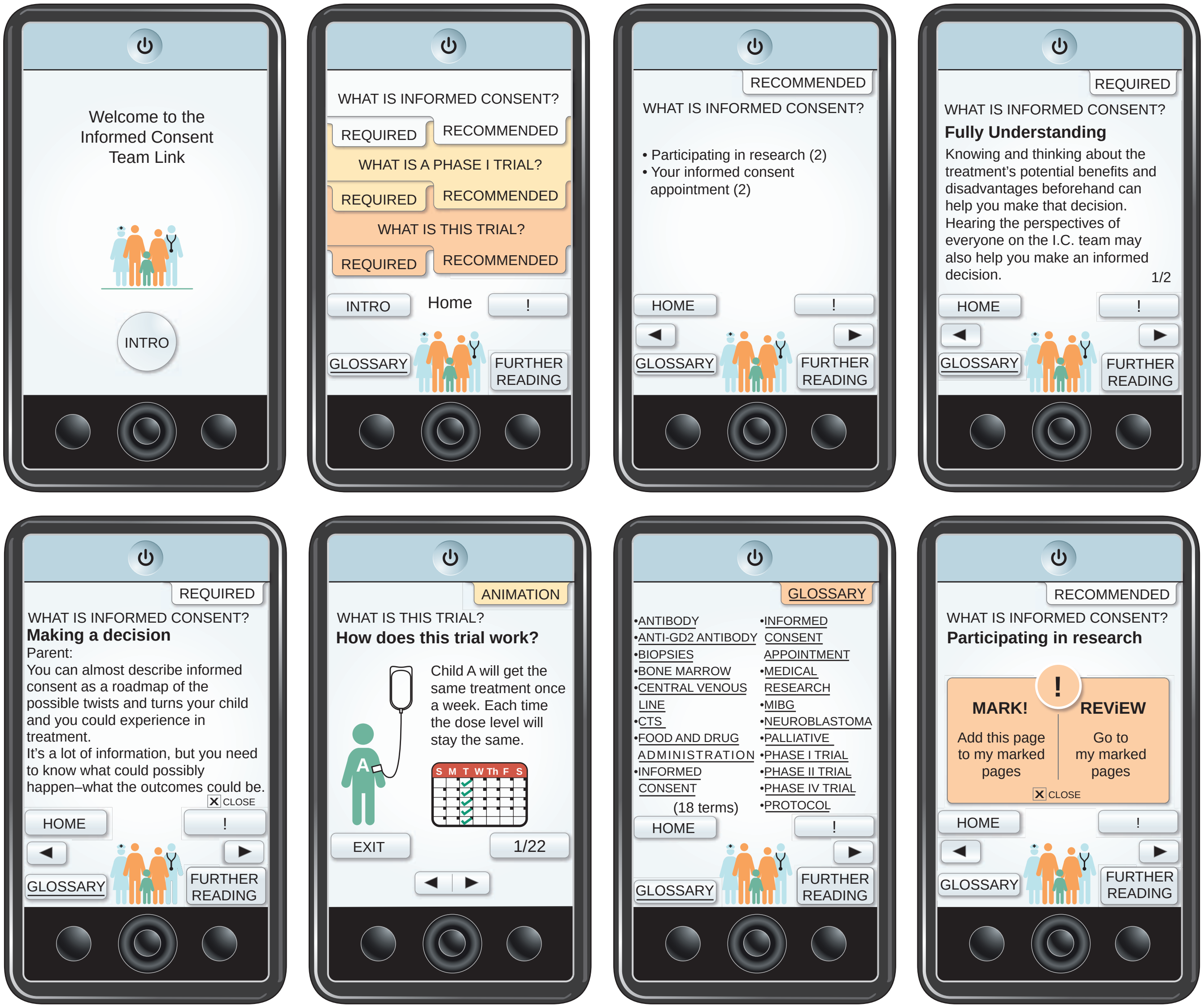
Further insights into this difference have been gained from a number of studies addressing the perspectives of parents of children with cancer. One such study used three focus groups of parents of children with cancer to retrospectively examine their perceptions of the informed consent process.[26] High levels of stress were consistently reported, which were attributed to efforts to cope with multiple demands, including assimilating their child's diagnosis, nurturing and supporting the child, understanding the information given to them about the diagnosis and treatment, getting to know an entirely new group of people involved in the child's care, and participating in the child's treatment. Another important point was that the parents did not consistently distinguish research from their child's medical treatment. This finding underscores the importance of clearly explaining that research is optional during the process of "informing." If no distinction is made between research and treatment, the goal of informing has not been met. Physician-investigators often experience tension between their role as the patient's physician and their role as a researcher offering participation in a clinical trial designed to contribute to generalizable knowledge. This tension suggests that someone besides the physician-investigator should conduct the consent process. The study also suggested that the nature of clinical research, as well as the difference between research and proven current therapy, might be difficult for parents to grasp.

These conclusions were supported by a second study in which parents of children with newly diagnosed cancer were interviewed.[27] All of the participants recalled the diagnosis, and most (80%) recalled survival statistics. However only slightly more than half knew that the treatment protocol involved research and understood the concept of randomization. This finding is striking when coupled with the fact that three fourths of the parents thought that alternatives to enrollment in a randomized protocol had been insufficiently discussed. Because randomization is such an important tool for answering certain clinical questions, this finding suggests that greater emphasis be placed on explaining unfamiliar concepts during the process of "informing." Again, in this study, the majority of participants were satisfied with the consent process.

A study by Kodish and colleagues[28] confirmed the difficulty of effectively conveying the meaning of randomization during the consent process in pediatric leukemia trials. In this multisite study, informed consent conferences were observed and audiotaped and were then compared with information acquired in interviews with parents shortly after the conference. The investigators found that although randomization was explained in 83% of cases, 50% of the parents did not understand it. Further, parents who did not understand randomization were more likely to consent to the randomized study than did those who did understand it, although this difference was not statistically significant ($P = .07$). These findings led to several recommendations for improving understanding, including a clear explanation of the differences between the randomized trial and off-study therapy, assessment of parental understanding of randomization, and further explanation when the idea was not yet grasped.

Other efforts have been made to improve the process of informed consent in pediatrics, especially in the case of complex clinical trials. For example, a study by the Children's Oncology Group (COG) assessed the possibility of staged consent; investigators had the option of obtaining consent over a 28-day period using a staged approach.[29] This option allowed parents and patients more time to discuss and absorb facts about the disease, the purpose of the trial, the design of the study, and the potential risks. Several measures in this study suggested benefit from the staged approach. There was greater understanding of treatment choices and of the distinction between a randomized controlled trial and standard therapy with the staged approach (80% understanding) than in the other studies (62.5%, $P = .05$).

Contemporary medicine will require the introduction of new methods of informing patients and parents to meet the ethical imperative of informed consent in pediatric trials. Informed consent documents for cancer



Welcome to the
Informed Consent
Team Link

RECOMMENDED
WHAT IS INFORMED CONSENT?
- Participating in research (2)
- Your informed consent appointment (2)

REQUIRED
WHAT IS INFORMED CONSENT?
Fully Understanding
Knowing and thinking about the treatment's potential benefits and disadvantages beforehand can help you make that decision. Hearing the perspectives of everyone on the I.C. team may also help you make an informed decision.
1/2

REQUIRED
WHAT IS INFORMED CONSENT?
Making a decision
Parent:
You can almost describe informed consent as a roadmap of the possible twists and turns your child and you could experience in treatment.
It's a lot of information, but you need to know what could possibly happen–what the outcomes could be.
☒ CLOSE

WHAT IS THIS TRIAL?
How does this trial work?
Child A will get the same treatment once a week. Each time the dose level will stay the same.

- ANTIBODY
- ANTI-GD2 ANTIBODY
- BIOPSIES
- BONE MARROW
- CENTRAL VENOUS LINE
- CTS
- FOOD AND DRUG ADMINISTRATION
- INFORMED CONSENT
- INFORMED CONSENT APPOINTMENT
- MEDICAL RESEARCH
- MIBG
- NEUROBLASTOMA
- PALLIATIVE
- PHASE I TRIAL
- PHASE II TRIAL
- PHASE IV TRIAL
- PROTOCOL

(18 terms)

RECOMMENDED
WHAT IS INFORMED CONSENT?
Participating in research
Add this page to my marked pages
Go to my marked pages

Figure 74-1 The Informed Consent Team Link, a prototype device to augment the process of informed consent in phase I trials. This device was developed through multidisciplinary user-centered research and design. The aim of such efforts is to use technological and educational advances to address the widening gap between the average capacity to understand concepts and the advances in medical technology and research.

clinical trials are sometimes long and difficult to understand, with language that serves less to protect human subjects than to decrease institutional liability.[30] Therefore some advances may take the form of technologic and educational methods to explain ever more complex medicine and research to people not formally trained in a medical discipline. One such example is a device known as the Informed Consent Team Link (Fig. 74-1). This device was designed to help families understand phase I trials, which present daunting challenges to fully informed consent. The design was based on information from focus groups of parents, physicians, nonphysician health care workers, and teenaged patients. Among the elements considered important were (1) the need for a "big picture" overview that would create a context for details, (2) delivery of information in small chunks, (3) use of multiple modes of information delivery, and (4) prioritization of information. Such basic observations provided a basis for the design of a device that uses interactive technology, voice, animation, and health education principles to deliver information about phase I trials. Because of children's apparently natural affinity for interactive electronic devices, this approach may be helpful in addressing assent as well.

Concepts about the nature of consent in pediatrics are evolving with changes in contemporary medicine. For example some persons argue that informed consent may be too restrictive a concept and that "valid consent" should be substituted.[31] The three aspects of valid consent are personal competence (Does the patient have the capacity to make the decision?), procedural competence (Is the consent given correctly?), and material competence (Is the procedure consented to appropriate for valid consent?). The concept of valid consent has been explored in the pediatric context by the Society for Industrial and Organizational Psychology Working Committee on

Psychosocial Issues in Pediatric Oncology.[32] The value of this notion, they suggest, is that it emphasizes the patients' or parents' understanding of what is being consented to and recognizes that there are both rational and nonrational aspects to the decision-making process that must be understood. The underlying concern is that informed consent has come to mean legally signed documentation rather than real understanding, insofar as that is possible. Because parents come from different backgrounds and because children of different ages differ in their ability to grasp complex concepts, the level of understanding that is attainable may vary from situation to situation. The notion of valid consent acknowledges this fact and counters the idea that a signed document is equivalent to informed consent.

The four elements of informed consent previously mentioned—disclosure, understanding, voluntariness, and competence—are useful for informed consent in adults, but informed consent in pediatrics is complicated by the fact that three parties are involved (parent, child, and clinician/investigator) and that the research subject is the child. In pediatrics, it is not autonomy (the basis of the four components of consent) that takes precedence but rather the best interest of the child.[33] The best-interests notion is clearer in a purely clinical setting than in a research setting, in which interventions are designed to contribute to general knowledge. Despite the difficulty of approximating truly informed consent in the setting of pediatrics, and especially pediatric research, the obligation to advance pediatric medicine lends urgency to continued efforts to offer the closest possible approximation to informed consent.

One important aspect of the difference between informed consent in adult and pediatric medicine and research is the fact that in pediatrics, informed consent is better thought of as a combination of parental permission and (as appropriate) the more complex concept of the assent of the child.

ASSENT IN CHILDHOOD CANCER TRIALS

The ethics of assent is one of the most difficult issues faced in pediatric clinical trials. Many adolescents, and even some younger children, possess the elements of competence or health care decision-making capacity (setting aside for the moment the legal definitions, which hinge on age rather than capacity). This is especially true, perhaps, of children who are exposed to long-term clinical trials and to the environment of a children's hospital for long periods. Certainly younger children are not developmentally capable of comprehending complex protocols, but they do have some level of understanding that increases with age and experience.[34] How should assent be understood and when should it be required? But first, what do we actually mean by "assent" and how can it be distinguished from consent?

Consent refers to an active affirmative agreement to something by an autonomous agent (i.e., a person). It is capable of being given by anyone who meets the minimal criteria of being in the state of mind and body to be an autonomous agent; generally, most adults would qualify as long as they were not placed in a situation in which their decisions might be suspected to be the result of some form of coercion. The ability to give consent is also context and subject dependent in the sense that most people are generally not competent in all spheres of their lives: for instance, many persons who are perfectly capable to make health care decisions or even agree to participate in research may not be competent to make (informed) decisions about automobile repair. For the purposes of this discussion, the relevant area in which cognitive capacity is important is in the domain of health care (and associated human subjects research). In the United States the legal age of consent is 18 years for most realms (nearly all jurisdictions, however, permit older adolescents who have not yet reached the age of 18 years to make some types of health care decisions [i.e., give consent], usually in regard to reproductive medical issues).[35] Assent is a more complex concept; in general it describes a less active form of agreement to a proposed procedure or action, rather than the more active and involved consent. It is used in an attempt to engage children and adolescents in the process of consent that must be given by parents or other authorized surrogate decision makers.[36,37] However what may qualify as acceptable assent (a grudging or sullen nod of the head or a more energetic and participatory form of positive interaction) remains an area of reasonable concern.

Far less is known about assent than about consent. Much remains to be learned about the practices of institutional review boards (IRBs), the perceptions of parents, clinicians, and children about consent, and the methods that might be most effective in the process of assent. Several studies have looked into these issues and documented the need for further work to address variability in IRB practices and the implementation of assent. One study found that half of IRBs have a method they require investigators to follow when determining which children are capable of assent, whereas half have no such method, and the majority of IRBs rely on investigators' judgment.[38] IRBs need guidance on the implementation of requirements regarding assent. Authors of a second study compared standards for assent, as well as consent forms approved by 55 local IRBs, when reviewing three standardized multicenter research protocols.[39] Standards varied widely; 35 had separate forms and simplified language for assent, and 31 specified lower age ranges for obtaining assent in three studies. For a hypertension study, the age at which assent was required ranged from 6 to 15 years;, for a pain study, the age range was 6 to 12 years; and for a respiratory failure study, the range was 7 to 12 years. It is not clear why some IRBs consider a child of 6 years to be capable of assent while others do not require assent until a child is 15 years old.

Do the regulations help us understand assent? Most research on children is governed by subpart D of 45 Code of Federal Regulations (CFR) 46, which provides additional protections for children (as vulnerable subjects) beyond those specified in subpart A of 45 CFR 46. Subpart D, added in 1983, outlines three categories of research that a local IRB can approve: research not

TABLE 74-2 Categories of Pediatric Research and Requirements for Institutional Review Board Approval

	PROSPECT OF DIRECT BENEFIT	
Level of Risk	**No**	**Yes**
Minimal risk	Approval by IRB permitted (46.404), conditioned on: • Permission from one parent or guardian • Assent of child, unless waived on capacity grounds	
Greater than minimal risk	Approval by IRB permitted (46.406), conditioned on: • Minor increase over minimal risk • Research involves experiences commensurate with those inherent in children's actual or expected medical, dental, psychological, social, or educational circumstances • Intervention or procedure is likely to yield generalizable knowledge about the subject's disorder or condition that is of vital importance for understanding or ameliorating that condition or disorder • Permission from both parents, if reasonably available • Assent of child, unless waived on capacity grounds	Approval by IRB permitted (46.405), conditioned on: • Risks are justified by anticipated benefit to subjects • Relation of anticipated benefit to risk is at least as favorable as that presented by available alternatives • Permission from one parent or guardian • Assent of child, unless waived on capacity grounds or grounds of prospect of direct benefit that "is available only in the context of the research"
Not otherwise approvable, but presents the opportunity to understand, prevent, or alleviate a serious problem affecting the health or welfare of children	Approval requires U.S. Department of Health and Human Services approval after consultation with an expert panel and opportunity for public review and comments (46.407), conditioned on: • Research presents reasonable opportunity to further understanding, prevention, or alleviation of a serious problem affecting health or welfare of children • Permission from both parents, if reasonably available • Assent of child, unless waived on capacity grounds	

From Joffe S, Fernandez CV, Pentz RD, et al: Involving children with cancer in decision-making about research participation. J Pediatr *149:862–868, 2006.*

involving greater than minimal risk to participants (45 CFR 46.404), research involving greater than minimal risk but with the prospect of direct benefit to individual participants (45 CFR 46.405), and research involving no greater than a minor increase over minimal risk (with no prospect of direct benefit) but likely to provide generalizable knowledge of the subject's condition or disorder that is vital to understanding or ameliorating it (45 CFR 46.406; Table 74-2).[40] Local IRBs cannot approve research that does not fall into one of these categories, and performance of the research is possible only if two conditions are met: (1) the local IRB finds that the research provides a reasonable opportunity to further the understanding, prevention, or alleviation of a serious problem affecting the health and welfare of children, and (2) the protocol is approved by the Secretary of Health and Human Services, after soliciting the opinions of an expert panel and providing for a period of public comment (45 CFR 46.407). According to these regulations governing research on children, assent means "a child's voluntary affirmative agreement to participate in research. Mere failure to object should not, absent affirmative agreement, be construed as assent" (45 CFR 46.402). If the IRB determines that the participants in certain categories of age and maturity are capable of providing assent, investigators must obtain it to proceed. When assent is required, a child's refusal is generally binding.

Can an IRB waive the requirement for assent? Yes, but only if (1) the intervention or procedure offers the possibility of direct benefit that is important to the health or well-being of the child and this intervention or procedure is only available in the research context (45 CFR 46.408[a]) or (2) the IRB determines that children below a certain age in a certain situation or with a certain condition have such a limited capacity to participate in the decision that they cannot reasonably be consulted.

The requirements are difficult to apply, especially in the context of clinical trials for childhood cancer. Most of the children are enrolled in research studies in which the therapy is prolonged and carried out in the context of profound physical, spiritual, and psychological stress. The Bioethics Committee of the COG convened an international multidisciplinary task force to address assent in 2003. This group identified a number of problems with the regulatory framework.[41] In brief the regulations do not take adequate account of the dynamic moral and cognitive development of children and thus allow a child either to have no formal role in decision making or to have the power of veto. Nor do the guidelines make clear what constitutes meaningful assent, and so they leave IRBs and investigators with uncertainty about when they have or have not hit the mark (although other groups have provided guidance in this area).[42,43] The regulations do not take into account the fact that some clinical research is more complex than other research, and thus some research may be more accessible to the understanding of children than other research. The regulations say that permission from the guardians and assent from the child are distinct decisions, and they do not take into account the manner in which parents and children make decisions together. Finally, cultures in which autonomy is less emphasized are not accommodated by a regulation

Box 74-1 **Principles Governing Children's Participation in Research Decisions**

1. Investigators should always respect children as persons. In particular, investigators together with parents should honor children's developing autonomy in decisions about research.
2. Investigators should respect parents' roles in guiding their children's moral development and assessing their best interests. For example, parents should have discretion to determine the degree to which children should be encouraged to participate in activities that benefit others.
3. Policies regarding assent, as well as decisions of Institutional Review Boards with respect to particular protocols, should be sufficiently flexible to accommodate the wide range of medical, psychological, and contextual circumstances seen in pediatric oncology.

From Joffe S, Fernandez CV, Pentz RD, et al: Involving children with cancer in decision making about research participation. J Pediatr *149:862–868, 2006.*

that potentially sets parental prerogatives against a child's veto power.[44]

Taking into account these factors, the COG Task Force offered three principles governing children's participation in research decisions (Box 74-1). A number of specific recommendations followed from these principles. The Task Force acknowledged that in some settings the principles offered will sometimes conflict. Therefore the recommendations emphasize the importance of establishing procedures for the resolution of conflict rather than attempting to define universal rules. In that way the absence of universal rules governing decisions in families with, perhaps, different cultural backgrounds and assumptions does not end conversation and negotiation. This approach is especially valuable in an increasingly pluralistic society and a global culture of information sharing. Much work remains to be done to understand the processes of communication and learning and the interactions that comprise the permission-assent process.

The flexibility of the approach using conflict resolution acknowledges that the obligation to obtain assent may change, depending on the time and situation, even for the same child. For example assent considerations may differ between a 9-year-old child with standard-risk leukemia who may obtain considerable benefit from enrollment in a phase III leukemia trial and a 9-year-old child who has leukemia that has relapsed multiple times and for whom a phase I trial is being considered. Similarly, a child at the beginning of a 2 and one half-year course of therapy for leukemia will be different in age, maturity, vocabulary, experience, and capacity to understand by the end of that course of therapy (Fig. 74-2). Developmentally appropriate approaches to help the child understand the experience do not have the restrictions and limitations that accompany the binding aspects of assent.

For older adolescents assent should be approached in the same way as adult informed consent, even though parental permission may be required.[45,46] Some adolescents may have the capacity for consent but may choose to have their parents make decisions about their participation in research.[47] Several studies have recently assessed assent in adolescents.[48,49] Our understanding of the differences between parents' and adolescents' perceptions of research is growing as we learn more about the assessment of risk (i.e., concern for physical safety) versus aversion (psychological discomfort).[50] More than 3000 adolescents in the United States alone die of chronic illness and cancer each year. In such situations when adolescents meet the criteria for functional competence, the broad ethical consensus is that decisional authority should be granted to them regardless of their legal decisional status.[51] However this is an area in which a wide divergence exists between what ethics may demand but the law allows. Most states and jurisdictions do not permit minors to be sole participants in medical decision making, including consenting to participate in research, unless they have been emancipated according to the laws of the state in which they reside. This restriction does not imply that adolescents should not fully participate in the decision-making process, and there certainly is no legal proscription against their doing so; it simply underlines the difference between good ethical clinical (and research) practice and the legal framework. It might perhaps be better stated that in these situations, if the adolescent is going to be a research subject, assent is necessary but not sufficient.

PEDIATRIC CLINICAL TRIALS

The majority of children with cancer are enrolled in clinical trials.[52] This systematic evaluation of interventions is largely responsible for the dramatic improvement in survival during the past 40 years. A number of important ethical concepts influence the design and execution of these trials. Permission and assent have already been discussed. Other important issues include the distinction between therapeutic and nontherapeutic research, the various concepts of minimal risk and clinical equipoise, and the considerations that distinguish phase I and II trials from phase III trials.

As previously described and in Table 74-2, reports published by the National Commission for Subjects of Biomedical and Behavioral Research formed the basis of current regulations governing research with human subjects, including children. One of the most important

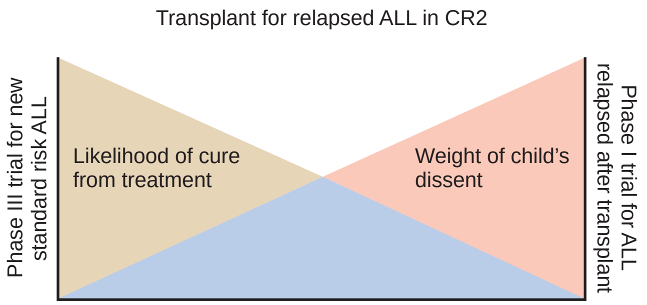

Transplant for relapsed ALL in CR2
Phase III trial for new standard risk ALL
Likelihood of cure from treatment
Weight of child's dissent
Phase I trial for ALL relapsed after transplant

Figure 74-2 The weight of childhood dissent changes as the likelihood for direct benefit (in the form of cure or extension of life) diminishes, as with a patient who goes through a phase III trial for standard risk leukemia, relapses, undergoes transplantation in second remission, relapses, and goes on to consider phase I trials. *ALL,* Acute lymphoblastic leukemia; *CR2,* second complete remission.

distinctions made was between research that does, versus does not, offer the prospect of direct benefit to the child. Some elements of a clinical trial may offer the prospect of direct benefit (for example, chemotherapy for acute lymphoblastic leukemia), whereas other elements may not (e.g., biologic and pharmacokinetic studies designed to improve future trials). What are the general criteria used to determine whether the latter sorts of research are permissible?

Minimal Risk

The central ethical concept that guides discussion of pediatric research that has no prospect of direct benefit is "minimal risk." The Belmont Report defines minimal risk as "the probability and magnitude of physical or psychological harm that is normally encountered in the daily lives, or in the routine medical or psychological examination, of healthy children." The question that immediately arises is which children's lives should be the basis for deciding what is "normally encountered in daily life." These risks can vary widely, not only from country to country but within subgroups in a single country. Even if the notion of "harms or discomforts that average, healthy, normal children may encounter" (as clarified by the National Bioethics Advisory Commission) can be roughly agreed upon as a standard, the question remains whether it is ever acceptable for a parent to give permission for any risk greater than minimal risk in the absence of direct benefit to the child.

Because of concern that the "minimal risk" standard would exclude some pediatric research that is important and appropriate, the National Commission allowed parental discretion in giving permission for research that exposes children to a "minor increase" over minimal risk, given the fact that parents routinely permit their children to participate in activities, such as contact sports, that present more than a minimal risk, and given the potential benefits of such research to others. Therefore a "minor increase" over minimal risk is permissible if the proposed research involves only procedures that such children might normally experience because of their medical condition and if the proposed research has the potential to yield vitally important knowledge for understanding or ameliorating the child's particular condition.

The permissibility of a "minor increase" over minimal risk provides a conceptual way to avoid barriers to important pediatric research. However a difficult question remains: exactly what constitutes a "minor increase"? The term is used in the federal regulations, but it is not defined. Wendler and Emanuel[53] proposed five possible standards for defining a "minor" increase consistently among IRBs. These standards include (1) a fixed percentage above daily risks, (2) the confidence intervals around the risks of daily life, (3) the risks an ill child might encounter in an examination, (4) the "scrupulous parent" standard, in which minor risks are sufficiently similar to the child's past experiences in daily life, and (5) the "socially acceptable" risk standard.

Wendler and Emanuel[53] argue that the fifth standard is the most reasonable. The "socially acceptable" risk standard takes as its starting point the fact that some children encounter greater risks in daily life than others. The socially acceptable risk standard allows risks that are greater than those faced every day by healthy children, but it limits the risks to those that are socially acceptable. The socially acceptable risks greater than those experienced by the average healthy child may be more closely defined through research. In any case this standard tethers the concept of "minor increase over minimal risk" to an evaluation that is nonarbitrary, that does not impede important beneficial research, and that does not justify greater risks for ill children simply because their daily lives are filled with more risk (due to their disease and its treatment).[54] An alternative to the approach taken by Wendler and Emanuel has been suggested by Nelson and Ross.[55] They argue that the "scrupulous parent" standard is ethically justifiable and that such an approach incorporates both minimal risk and a minor increase over minimal risk within a uniform standard that should be applicable to all children.[55]

Phase I and II Trials in Pediatric Oncology

Phase I studies have the unique primary objective of assessing the maximum tolerated dose and estimating toxic effects in human subjects, in contrast to phase II and phase III studies, which explicitly assess the efficacy and relative safety of interventions. Phase I studies in children are important because the pharmacokinetics and toxic effects of drugs differ between children and adults. Therefore if drugs were used in the general pediatric population without phase I testing in children, the incidence of adverse effects would be unpredictable. Additionally some diseases are unique, or nearly unique, to the pediatric population, including a number of pediatric cancers.[56] Prohibition of phase I studies in children would limit the ability of the research community to develop new drugs that target uniquely pediatric diseases.

Phase I studies are typically dose-escalation trials in which some children receive a dose unlikely to be of any benefit while others receive a dose that is toxic. Therefore phase I studies do not fit the category of minimal risk nor, many would argue, of a "minor increase" over minimal risk. Much of the controversy about phase I studies in children, therefore, centers on whether they offer sufficient direct benefit to justify the risks, both from a purely ethical point of view and from the regulatory point of view.

What is the likelihood of harms and benefits in phase I oncology trials? A recent review of abstracts and reports of the results of phase I cancer trials submitted to the American Society of Clinical Oncology (ASCO) from 1991 to 2002 found that the death rate from toxicity was 0.54%, with an overall objective response rate of 3.8%, and that risks decreased over time.[57] A National Cancer Institute–sponsored study of adult phase I oncology trials run between 1991 and 2002 found an overall response rate of 10.6%. The response rates in "classic" single-agent phase I trials were lower (4.4%), whereas those that included at least one Food and Drug Administration (FDA)–approved anticancer agent had a response rate of 17.8%.[58] In pediatric oncology phase I trials, the response rate (both partial and complete responses) has been reported to be between 4% and 9.6%.[59,60] However the (causal) relationship or correlation between dose and response is rarely reported, and the small number of subjects at any dose level would preclude any sort of definitive conclusion from being drawn. The overall rate of death due to toxicity was 0.49%. That said, in any single phase I trial centered around a single drug, considerable variability in outcomes can exist among the research subjects recruited. Hence it is reasonable to conclude that few children entered in phase I trials die from study-drug–related toxicity and a very small percentage may derive some therapeutic benefit. Unfortunately no feasible mechanism exists to abstract what might be called the "pain and suffering" effects of trial participation or whether—as has been reported for adults enrolled in similar studies—there may be an avoidance of engaging in appropriate end-of-life discussions and planning.[61] Finally, at least one study has suggested that parents may feel compelled by their child's physician to enroll in phase I trials.[62] These observations suggest the idea of "collusion" between doctors and patients to temper the discussion of bad news with the hope that may be imparted by treatment.[63]

Can phase I trials be viewed as offering a prospect of direct benefit in the context of pediatric oncology? The pediatric medical and bioethics communities are divided on this question. The fact is that most people who enroll in phase I trials hope for personal benefit, although they know that the trial is designed to test toxicity.[64-66] The concern exists that such a "therapeutic misconception" may encourage false hope for cure and lead parents to make decisions that do not take into account all of the elements that make up "the child's best interest."[67,68] This phrase refers to the mistaken belief by clinical trial subjects (especially those participating in early-phase studies, such as phase I) that the principal purpose or goal of the trial is therapeutic, rather than data generation.[69,70,71,72,73] Clearly the primary scientific goal is not therapeutic, and the fundamental purpose of these trials is to gather data on toxicity, physiologic tolerability, and dosage. However it is very difficult to communicate such bare facts to families. Much of the confusion is derived from the emotional tension of the situation, the hopes and fears of parents (and patients), the fact that treating physicians often are involved in discussing the clinical options available for terminally ill patients, and the mixed messages that are both transmitted and received by families during these conversations.[74-76] The simple fact of the matter is that parents may not wish to hear "the truth" and their physicians may not wish to transmit it. Although many persons consider this problem to be almost insuperable, some attempts have been made to combat it with mixed success.[77] Nevertheless it remains a significant complicating factor that contributes to the ethical complexity of performing pediatric phase I trials.

On the other hand because only patients for whom known therapies have failed are usually eligible for phase I trials, these trials may be viewed as offering a relative prospect of direct benefit, although the chance of controlling the disease is small.[78] Such a view would bring phase I research under CFR 46.405 of the federal regulations, but it has been criticized because such a classification (in which risk is justified by potential benefit) fails to take into account the researcher's focus on safety rather than efficacy. From the latter point of view, phase I research would be approvable only under CFR 46.407, in which the research is reviewed and approved by a panel appointed by the Secretary of the Department of Health and Human Services—a time-consuming effort that requires both national and local IRB review.

One critic of the "direct benefit" argument also questions the approval of phase I trials under CFR 46.407 because the required review process delays providing benefit to children and adds little to the meaningful protection of children. This author has suggested that a new category of research offering the potential for "secondary direct benefit" be added to the regulations.[79] Such a category would require (1) that the risks be justified by the likelihood that the research will yield generalizable knowledge, (2) that the research be commensurate with the lived experience of those with the disease or condition, (3) that the research offer a potential secondary benefit that is otherwise not available (a potential therapeutic benefit even though the trial lacks therapeutic intent), and (4) that consent requirements be met. Work that addresses gaps in the understanding of phase I trials and the federal regulations governing them should take high priority in pediatric medical ethics research. A recent essay supports phase I trials in children using an interesting and somewhat novel argument.[80] Morris[80] suggests that most children who would be eligible for the types of phase I trials that may involve more than minimal risk (independent of how it may be defined) would of course be afflicted with the disease or condition for which the agent under study is under investigation (for future possible therapeutic use). Furthermore it is likely that the experience of this disease would have acquainted both the child and his or her parents with many of the tests and procedures used as part of the trial (such as phlebotomy). Because they would also have an inherent interest in the outcome of such studies (both for the present and the future), they (and local, institutional IRBs) should be extended some moral flexibility in interpreting what should be interpreted as "minimal risk."

Phase II trials play an important role in all areas of cancer therapy. They provide the initial assessment of treatment efficacy and identify agents for further investigation in phase III studies.[81] These studies, although experimental, are clearly therapeutic in intent and thus the therapeutic misconception is not as germane. Phase II studies are likely to increase in number as high-throughput screening of compounds identifies more candidate anticancer drugs and more molecularly targeted agents and biologic agents become available. This fact will raise many important ethical questions in pediatric clinical trials, because phase II studies specifically evaluate drug efficacy before benefit is clearly demonstrated. Therefore it is vital that families and patients be fully informed about alternatives.

We should mention the attraction for incorporating nonrandomized phase II trials into therapeutic phase III studies, the so-called "phase II window." This approach was quite popular for some time because of the belief that previously untreated, "chemotherapy-naive" patients would constitute a population that would better represent the efficacy of a given agent compared with heavily pretreated patients whose response rates might be diminished both because of cross-resistance to other drugs and decreased performance status. This technique has come under significant ethical and statistical criticism, and in 2010, a consensus statement from the National Institute Clinical Trial Design Task Force was issued that called for the elimination of single-arm studies of this type and their replacement with better designed trials (although the Task Force did express the caveat that unique situations may exist in which nonrandomized, window designs might be appropriate).[82,83] Many stand-alone phase II COG studies have had this design, and it is rarely used in phase III studies (only one as of this writing). Therefore although phase II windows and single-arm studies still exist, the future of this form of design is questionable.

Finally, a word should be said about the use of drugs that are still under investigation in an "off-label" capacity. FDA-approved agents have been demonstrated to be safe and effective by FDA standards and thus receive approval for one or more indications. Many of these approved agents that have not yet been shown to have similar efficacy or a justifiable safety profile in children are nonetheless used in a phase II–like practice, but without the protections of an IRB-approved clinical trial (including rigorous and supervised informed consent and an adverse event reporting framework). Data about the frequency of this practice in children have not yet been published, but Peppercorn and colleagues[84] have reported this practice among adult oncologists, even when a clinical study incorporating the same drug(s) and for which the patient is eligible is available. Although it may be imagined that many studies use drugs that are only available via the trial, this situation is not actually the fact in adult oncology. In pediatrics this observation would be even more germane because it is rare for an agent to be available for children (especially for phase II and III trials) without first being tested and approved in adults.[85] The widespread use of this practice is worrisome for several reasons: First, it tends to undermine the clinical trial enterprise if physicians are willing to prescribe (off-label) investigational drugs in the same clinical setting in which they are being investigated. Such use also can lead to the failure to notice uncommon toxicities that may be more easily detected within the stricture of a formal clinical trial. Second, it is argued that such use results in the conduct of research without the significant human subjects oversight. Third, this practice exposes children to more than minimal risk, but the (relative) safety of patients cannot be ensured as it would be in a clinical trial with formal tracking of adverse events.[86] Finally, this practice represents a missed opportunity for more phase II trials to be conducted with sufficient statistical power to enable substantive conclusions about their significance to be drawn.[87,88] Thus this practice exposes patients (subjects) to potential risk without even the compensatory benefit of contributing valuable scientific knowledge to the general community.

Phase III Trials and Clinical Equipoise

If the concept of minimal risk demarcates the upper limit of nontherapeutic research, the notion of clinical equipoise is central (although not unproblematic) to ethical thought about randomization in clinical research. Clinical equipoise requires that if patients are to be randomly assigned to one of two interventions in a clinical trial, real doubt must exist—in the medical community as a whole, if not in the mind of the investigator—about which of the two interventions is superior. This requirement is said to provide one answer to the possible conflict of interest that clinical investigators can experience in the dual role of researcher and physician.[89]

At least two sources of confusion are presented by all forms of clinical trials, especially in pediatrics. First, sick children are first and foremost patients, which creates a special and unique fiduciary relationship between the patient and his or her doctor. This relationship also establishes a duty on the part of the doctor toward the patient that confers certain responsibilities to act in the patient's best interest.[90,91] This relationship should be distinguished from that of an investigator to a research subject, which is defined within the parameters of federal regulations (previously described).[92] In pediatric hematology-oncology, physicians often occupy both roles simultaneously, which can present significant conflicts (although not necessarily straightforward conflicts of interest). For example if the treating physician is also in some way involved in the design or implementation of the clinical trial under discussion with a patient and his family, she (the doctor) may have a conflict. Enrolling in the trial may not necessarily be to the best advantage or well-being of the patient as defined by him (and his surrogates) and his physician, but participating in the trial as a subject could be beneficial to the investigator (the doctor). In these situations it may be advisable for the treating physician to avoid caring for patients with diagnoses that may make them eligible for the trial for which he or she is responsible. For physicians whose relationship to the study is peripheral, in a manner that could perhaps be described as administrative (as is often the case for many, if not most, treating physicians with COG clinical trials), the conflict is minimal. However in a number of institutions, participation in "therapeutic" (i.e., phase II and III) trials may be remunerative, if not to the doctor, to the local group that employs the physician, by way of financial reimbursements to (partially) cover the cost of performing clinical trials. Although these rewards are hardly profitable, they do raise the specter of a conflict and may need to be addressed. A commitment to transparency may be sufficient in these cases. Second—and we admit that this may be more of a theoretical concern—years of experience have demonstrated the enormous benefit bestowed by the cooperative group clinical trial enterprise in pediatric oncology. Of course this progress would only have been possible with the recruitment (by physicians) and participation (by patients/subjects) in many clinical trials during the past four decades. The ongoing dedication of families, patients, and pediatric oncologists to this endeavor has been crucial to its success. Hence it could be perceived that there is a not-so-subtle pressure to enroll as many (eligible) patients as possible in group clinical studies (both therapeutic and nontherapeutic); indeed, failure to enroll sufficient numbers may result in institutional sanctions, up to and including suspension from the Group and thus an inability to access study protocols and documents (see the COG website for details). However, the recognition that this cooperative endeavor has been both productive and protective of patient (and subject) welfare is well understood and accepted. Nevertheless, the conflicts and the potential for their intensification and complexity remains and actually could increase as more pharmaceutical industry clinical trials are incorporated into the cooperative clinical trial group apparatus.

Does the physician's "therapeutic obligation" require the provision of the best possible care or simply of competent care?[93] The concept of clinical equipoise has been challenged from a number of perspectives. Some critics argue that therapeutic medicine and clinical research are distinct types of activity that require distinct ethical frameworks and that the concept of "clinical equipoise" cannot provide a unifying ethical framework.[94,95] Clinical equipoise has also been criticized as especially problematic in developing countries, where the lack of resources may not support trials that test new treatments against established therapies or placebo. The requirement for clinical equipoise could make it difficult to justify the search for interventions that, although they provide significant benefit for less money, are known not to be the most effective interventions.[96,97]

Several other considerations are important in evaluating the concept of clinical equipoise. First, the conditions for establishing true equipoise are not objective, based as they are on unverified judgments that are open to bias.[98,99] Second, if clinical equipoise is not always a reliable foundation for randomized studies, how do we resolve the conflicts that can be posed by clinical investigators' dual roles? Finally, although adults may be able to negotiate the ambiguities of clinical equipoise in a clinical trial, children cannot do so. In light of the previous discussion regarding the best interest of the child (or the attempt to avoid harm to the child), no phase III randomized pediatric trial should be conducted unless genuine doubt exists about which of two or more alternatives is better.

Recently a new approach to bringing together research and clinical care has begun to emerge. The Institute of Medicine has called for the transformation of health systems into "learning health care systems" that are able to study and continuously improve their practices. Learning health care systems pursue a wide range of investigations, including those aimed at quality improvement and better clinical effectiveness. An active question is whether the data collection and monitoring central to learning health care systems should be construed as research, and if so, what type of ethical oversight it should have.[100]

Finally, a great deal of discussion has ensued about the responsibility of investigators to communicate the results of clinical trials to patients and their families, irrespective of the outcome of the studies. Surveys have suggested that patients who have enrolled in trials (or who have been enrolled by their parents) retain an interest in the outcome.[102-105] Thus there is good reason to believe that a continuation of the respect we pay to patients, and the diligence and responsibility owed to study participants/subjects, would warrant the formal reporting of the results of trials to them, even taking into consideration the time lag that is intrinsic to the completion of a large study, the maturing of the data, and the challenges associated with communicating complex information of this type. Furthermore, the variety of data that can be collected from the trial—including intricate genetic and other biologic marker data—will require careful consideration to decide what information would be most important to communicate. The issue of conveying genetic or genomic information has proven to be particularly problematic, and to date the challenge has resisted consensus solutions.[106-108]

SPECIAL CONSIDERATIONS FOR CANCER TRIALS USING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Many patients with high-risk cancers (especially leukemias) that are resistant to standard chemotherapy may be cured with hematopoietic stem cell transplantation (HSCT). As in other areas of pediatric oncology, most such patients are treated according to research protocols or treatment plans recently derived from research protocols. Because of the continuing, rapid changes in the field of HSCT, ethical considerations, such as the specifics of the risk/benefit analysis, are rapidly changing as well.

The issues that apply to pediatric oncology trials in general are also relevant to those that include HSCT. However HSCT raises additional questions. Unlike most phase III trials, transplantation for cancer is usually a second-line medical procedure aimed at cure after a patient has been treated with other therapies. Common indications for transplantation in patients with malignant disease include lack of response to conventional chemotherapy and a known high risk of relapse despite conventional therapy. In both cases patients have been treated prior to transplantation, and the education received in this experience will be relevant to the consent process (the same, of course, can be said for phase I trials). Patients' and parents' familiarity with the language of pediatric oncology makes the discussion about transplantation easier in some ways than the discussion about a phase III trial in the context of a newly diagnosed childhood malignancy. However these considerations are primarily cognitive. What about other issues?

No studies have examined noncognitive considerations in consent for pediatric HSCT, but one study addressed the relative value of receiving full information in decision making among adult patients.[109] The findings provide interesting information that might in part guide future assessments of the unique characteristics of consent and assent in pediatric HSCT. The study had an unusual hypothesis: in the context of a potentially life-saving procedure, when there are no treatment alternatives that have a reasonable chance of cure, factors other than the effort to fully inform have the greatest influence on the patient's decision-making process. The notion of "voluntary" action is complex in such cases, because the patients' life-threatening illness has "forced" them to consider HSCT as an option. Four factors were considered by patients, according to this study: (1) a full understanding of the treatment, (2) trust in the physician, (3) trust in the treatment team, and (4) the best chance for a good outcome. The most important factor was "best chance for a good outcome," and the least important factor was "a full understanding of the treatment."

This finding is relevant to any high-risk, complex intervention (such as transplantation) in which the patient's life is at stake. The lack of alternative therapies that offer the real possibility of cure limits the voluntariness of the decision, and trust in the physician and the health care team may become more significant than understanding of the treatment. Given the vulnerability of these patients, this fact underscores the importance of careful attention to the therapeutic rationale of treatment, as well as the relationship of the patient and family to the health care team, especially when the intervention has not been established as the standard of care.

Another significant difference between cancer chemotherapy trials and those using allogeneic HSCT is the fact that HSCT involves two patients—the recipient and the donor. Different issues arise depending on whether the donor is a matched unrelated, matched related, cord blood, or haploidentical donor. These differences are, in part, based on the fact that the matched unrelated donor is a stranger, the matched related donor is a brother or sister, and the haploidentical donor is usually a parent. The patient's course and outcome affect the donor in various ways, depending on the relationship of the donor to the patient, and this effect is relevant to the ethical issues involved in HSCT cancer clinical trials.

More than 7 million people are registered as potential bone marrow or peripheral blood stem cell donors for the 70% of candidates for HSCT who do not have a matched sibling donor.[110] The risks of bone marrow donation are small; life-threatening complications occur in approximately 0.1% of healthy donors.[111] Ninety-five percent of peripheral blood stem cell (PBSC) donors do well with only minor complaints.[112] In matched unrelated–donor transplantation, one must consider the donor's rights as well as the patient's rights. These issues were outlined in a review by the World Marrow Donor Association.[113] First, donor autonomy must always be honored. At a minimum, this means that the consequences of each act in the process (as they bear on both the donor and the patient) must be explained to the donor. The informed consent process and document must stipulate the donor's right to withdraw at any time (even during the pretransplant conditioning) and must state the possible consequences of withdrawal. The donor registry cannot pressure or coerce donors, although the center/registry has the right to exclude a donor.

No one is obligated to join a donor registry, but a donor who does join a registry with clear knowledge of the expectations voluntarily incurs some moral obligation. This obligation grows as the process continues: a donor at the point of donation for a patient who is being conditioned has a greater obligation than does a person who is only at the stage of registering, because the patient has undergone a treatment that creates considerable vulnerability and has done so because of the expectation that the donor will follow through. Therefore donor centers are obligated to communicate clearly with donors to avoid disrupting the recipient's treatment plan if possible.

Limits exist to a donor's obligation. Sometimes a clinical situation is such that optimal care of a patient would require additional cell products from the donor, such as T cells or additional stem cells. The donor does not have a legal obligation to continue donating cells or bone marrow after the original donation.

Donor unavailability remains a problem that bears upon decision making because the treatment plan is dependent upon such variables. When donor unavailability rates over 1 year were examined by the National Marrow Donor Program, the worldwide registry of volunteer unrelated stem cell donors, some interesting facts were discovered.[114] Initially when a donor search is started, a request is made for confirmatory typing. Approximately 20% of all donors were permanently deferred at the time of confirmatory testing, and another 12% were temporarily unavailable (because of such things as pregnancy, high-risk exposure, job change, and location change). Donation centers differed considerably. The self-identified racial or ethnic group of the donor affected the likelihood that the donor would be available when requested. These findings suggest important areas in which practical improvement might be achieved: better medical screening, better collection of contact information, better education about the details of the commitment, and movement toward a pressure-free environment. Other important issues that bear upon ethical decision making, such as cultural sensitivity and route of contact, must be studied further. The overall goal is to structure the donor registry in such a way that donor availability is maximized through better recruitment, retention, and contact.

A second important source of hematopoietic stem cells in pediatric oncology transplant trials are sibling donors, many of whom are minors. Although the process of donation has been designed to minimize the coercion of unrelated donors, sibling donors may experience a variety of pressures. The ethical question centers on the coercion of a younger child who is dependent on the parents to provide permission for the donation. One central related ethical question concerns what, if anything, constitutes a benefit to the donor. The sibling donor can experience psychological, spiritual, and physical stress like that experienced by the HSCT recipient. The pain and anxiety among adult sibling donors of bone marrow or PBSC has been described.[115-118] Further, refusal to donate can be perceived as resulting in the loss of a sibling, even though the loss is actually due to the underlying disease. Because the variable of developmental age is added in pediatrics, the issue is more complex. Whether or not the transplant actually occurs, if the sibling dies, the complexity is compounded.[119] In many areas of family life, parents are allowed to give more weight to the interest of one child than to those of another or of the family in general; therefore some persons advocate leaving the decision of whether a minor child should donate bone marrow to a sibling in the hands of the parents.[120] Regardless of how such issues are ultimately resolved, the sibling donors clearly deserve all developmentally appropriate information at the beginning of the process and full psychological support throughout and after the procedure.

Sibling donor interviews provide helpful information. Irrespective of transplant outcome, pediatric sibling donors report a perception of having "no choice" about becoming a donor, whether the sense of constraint is derived from outside pressure (indicated by participants' use of the words "guilt," "propaganda," "privileged," and "conned") or from the donor's own beliefs.[121] Hesitancy may arise not from a lack of concern for the sibling's health but rather from fear of the procedure and of pain. Donors whose recipient sibling died offered a unique perspective. Anger, guilt, and blame were common emotions expressed and seemed to be most difficult to deal with in cases in which the death was directly related to graft failure or graft-versus-host disease. These issues have not been studied enough to draw substantial conclusions, but enough is known to raise questions about competence to consent, the weight accorded to a child's refusal to donate, the limits of the parents' decision-making power, and the worrisome specter of what may constitute battery in the face of a child's refusal. One might override the refusal of a 5-year-old, but what about that of a 10-year-old, a 13-year-old, or a 17-year-old?

Another important issue in the case of sibling donors for pediatric HSCT is the fact that siblings who are not chosen as donors are susceptible to ambivalent feelings of relief, disappointment, and guilt.[122] If siblings are involved in the screening process, issues of psychological well-being and respect for persons meet. In one study that compared sibling donors with sibling nondonors, nondonors had more school-related problems than did donors, and a third of siblings in both groups reported moderate to severe posttraumatic stress.[123-125] Medical encounters with a nondonor sibling may well show suffering that would not have occurred apart from the process of being screened for stem cell donation. Caregivers owe this person as much respect as they owe any patient.

Both case and common law have reasonably established that potential donors who have achieved legal majority (age 18 years or emancipation by a court) may not necessarily or generally be compelled to donate bone marrow (or other organs) irrespective of who the prospective beneficiary may be and the relationship between the two (although the legal history of this situation is complex).[126] The state of affairs for minors who are possible donors is considerably more involved. In 2010 the Committee on Bioethics of the American Academy of Pediatrics (AAP) endorsed the view that minors could serve as donors provided that five conditions were met: (1) "there [should] be no medically equivalent histocompatible adult relative who is willing and able to donate; (2) there [must] be a strong personal and positive relationship between the donor and recipient … this is important, in part, to increase the likelihood that the donor will experience some psychological benefit; (3) there [should] be some likelihood that the recipient will benefit from transplantation; (4) the clinical, emotional, and psychosocial risks to the donor be minimized and be reasonable in relation to the benefits expected to accrue to the donor and the recipient; and (5) parental permission and donor assent be obtained."[127] The AAP also suggested that a "donor advocate" be appointed to help the donor "… (and parents) understand the process and procedures and to protect and promote the interests and well-being of the donor. As such the donor advocate … should not be involved in direct patient care of the potential transplant recipient." The statement did not describe any further the identity of this advocate. Although presumably he/she could be a guardian ad litem appointed by a court, we suspect most institutions would wish to avoid the potential cumbersome process of applying to a court unless necessary. Not surprisingly this position has generated some controversy, including the opposition of the parent (whose child had severe aplastic anemia and who received a sibling marrow transplant) and other objections to the donor advocate requirement.[128,129] The AAP (and others) defended this requirement, and at present their position stands.[130,131]

However a recent case from Alabama in which a 6-year-old girl was required to "donate" split-thickness skin for grafting onto her severely burned identical twin sister suggests that the legal landscape for these cases may still be in a state of some flux.[132] Does this case (and others like it) suggest that under certain conditions involving desperately ill children who may possibly benefit from an allogeneic stem cell transplant, a minor sibling can be compelled to be a donor? Although the image of a child being forced to undergo a painful procedure, protesting all the way, is disconcerting, one can certainly imagine forms of parental pressure that are brought to bear to persuade a reluctant child to proceed with donation. Indeed the opposition to the possibly interdictive role of the "advocate" is the source of at least some of the objections to the AAP position. However most of the cases under discussion (or dispute) concern potential donors who are quite young. For those who may be in the mid to late teenage years, the answer is quite a bit more complicated. As children mature, they become more able to participate in health care decision making, as previously noted. However this increasing cognitive capacity does not accord with the laws in most states that do not extend legal decision-making authority to so-called "mature minors." Nevertheless the legal facts may conflict not only with our moral intuitions but with more mundane biology: it may be physically very difficult (if not completely improper) to force a 15- or 16-year-old to submit to bone marrow or PBSC harvesting if he or she does not agree to be a donor. Indeed the role of the advocate under these circumstances would be superfluous. Unfortunately this fact implies that the objections of a (small) 6-year-old count for far less than those of an adult-sized teenager, even though legally they are similarly inconsequential in the overwhelming majority of states.

A third, increasingly prominent source of stem cells is cord blood. Initial concerns about early clamping of the umbilical cord to obtain the maximum number of cells have been allayed, but other ethical questions remain.[133] Should the cord blood be linked to the identity of the donor so that unsafe units can be identified if the donor is later disqualified (e.g., by genetic testing)? The controversy centers on concerns about loss of privacy for donors. Because the cord blood deoxyribonucleic acid holds genetic information (the social implications of which are not clear at this time), the donor is potentially vulnerable to inappropriate disclosure.[134] A related issue concerns what the limits for cord blood testing ought to be and what one should do with the results. Clearly, some testing is needed to avoid unnecessary risk to the recipient. However in 1994, the Institute of Medicine recommended that children should not be tested for abnormal genes unless the disease has an effective curative or preventative treatment that must be initiated early in life.[135] This view was also endorsed more recently in a comprehensive report from the President's Commission on Bioethics.[136] Thus it may be that samples should be tested broadly to protect the recipient but that the testing should be performed in a blinded way to protect the donor. The details of this issue will require further thought and debate as more is learned about the human genome and prediction of disease. However this approach does not address the use of these samples for general genetic research, which is a related but altogether different question (discussed later).

The fourth source of stem cells used in pediatric clinical oncology trials is the haploidentical donor, usually a parent. The advantage of this sort of transplant is the ready availability of a partially matched donor. No research has been performed on the impact of this sort of transplant on parents and families, but it would not be surprising if the issues seen with sibling donation were compounded when the parent is the donor, including feelings of guilt when the child has severe graft-versus-host disease or graft failure. Another important question is whether there is a limit to acceptable parental sacrifice, and if so, what it is. For example one would not allow a parent to donate his or her heart, but intermediate scenarios include those in which a parent donates a portion of a lung, kidney, or liver in the face of posttransplant complications.

Finally, autologous stem cell reconstitution after myeloablation, mostly for solid tumors (frequently inaccurately referred to as "transplants"), is worth mentioning. Although these procedures do not raise the types of ethical challenges associated with allogeneic transplants of various sorts, they do often take the form of phase II single-arm trials for which little evidentiary support may exist and that are statistically underpowered, as explained earlier in this chapter. The classic example of this situation was the story of stem cell treatment for advanced breast cancer, but similar cases have occurred with childhood tumors as well, including Ewing sarcoma and rhabdomyosarcoma in which apparently excellent outcomes are compared with historic control subjects.[137-140] This observation does not imply that there are not clinical situations in which this therapy may provide significant benefits (as in stage IV, poor-risk neuroblastoma or relapsed Hodgkin disease).[141-143] Although this issue would appear to be straightforward and its resolution similarly so, it has proven to be surprisingly resilient over the years. The significant point is that this intervention can, and should, be used in a manner that is designed to address an important clinical question in a manner that could provide a reliable (and presumably reproducible) answer.

MODELS AND TOOLS FOR ETHICAL DECISION MAKING IN PEDIATRIC CLINICAL TRIALS

As important as the individual issues (such as consent, assent, and clinical equipoise) are, they make the most sense in both the clinical and research settings when they are considered within a larger framework for ethical decision making. Such a framework takes into account fundamental moral principles such as respect for persons, individual and cultural variation, the importance of full information, and the relevance of an individual's experience of illness to the concrete process of decision making. Although it is essential to have a sound foundational understanding of ethical principles as they apply to both the clinical and research settings, it is equally important to emphasize the vital role that facts play. "Good ethics begins with good facts" is an epigram often cited but not trivialized by its pithiness. Comprehensive knowledge about the facts of a case and possession of much information as possible about the patient and his or her family situation can be critical to the pediatric oncologist's role as caregiver and advisor. Incorporated in this approach is also an understanding of the prognosis, along with an appreciation (which should be communicated to the patient and family) of the limits and inherent uncertainty of the prognosis and what this signifies.

The Individualized Care Planning and Coordination (ICPC) model for ethical decision making developed by Baker and colleagues[144] combines the various elements of decision making in such a way that they are intentionally and systematically addressed (to ensure nothing is missed) and revisited using the care model (so that changes over time are acknowledged and responded to). The model begins by establishing a foundation based on the principles of competence, empathy, compassion, communication, and quality (Fig. 74-3). The aims of this model are to attend to quality of life, healing, and suffering from the start of a life-threatening illness. The ICPC model has three steps, each of which has three components (Fig. 74-4, Table 74-3).

The first step, relationship, is made up of three interrelated components: understanding the illness experience, sharing relevant information, and needs assessment. Without comprehending the illness experience of the child and the family, the clinician will be unable to proceed to seeking the best interest of the child and the decision-making process. This may be especially true at the extreme of consideration of phase I clinical trials toward the end of the child's life. Similarly, no decision

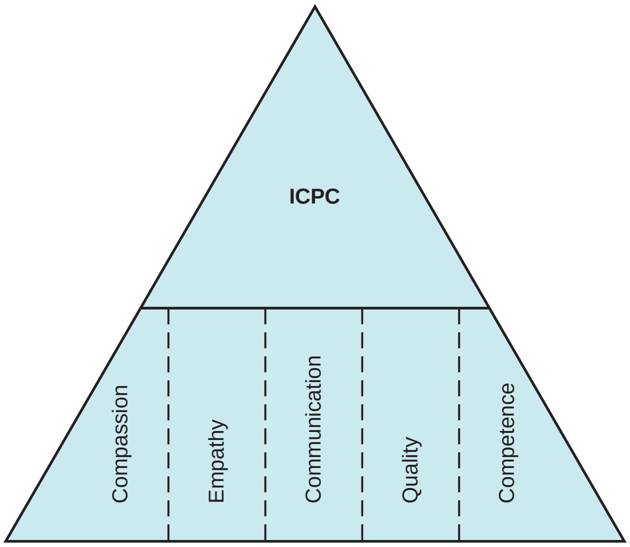


Figure 74-3 Foundation of the Individualized Care Planning and Coordination *(ICPC)* model. *(Modified from Baker JN, Barfield RC, Hinds PS, et al: A model to facilitate decision making in pediatric stem cell transplantation: the individualized care planning and coordination model.* Biol Blood Marrow Transplant *13:245–254, 2007.)*

can realistically address the best interest of the child unless all persons involved in the decision-making process have the needed information. This issue can be difficult when the relevant information is bad news; several studies have shown that the bearer of bad news can elicit strong negative reactions from family members that, in turn, make the clinician reluctant to deliver bad news (an effect known as the "MUM effect").[145-147] Communication of sufficient information for ethical decision making can also be truncated in approximately 10% of cases by lack of training and communication of the pediatric oncologist.[148] A comprehensive needs assessment—not just an assessment of medical needs—is also vital to the process of ethical decision making in both clinical medicine and clinical oncology trials (see Table 74-3). Such comprehensive assessments can inform the decision-making processes by incorporating relevant changes in a patient's needs.

The second step in the ICPC model is negotiation in which goals, prognoses, and treatment options are considered. Although the prognosis for cure can be dynamic because of advances in the science of pediatric oncology, prognostic information is helpful to patients and families as they make decisions.[149,150] Another relevant consideration is that more than one relevant prognosis exists—more, that is, than the prognosis for cure—such as the prognosis for life prolongation and the prognosis for relief of suffering. These two prognoses are relevant, relate intimately to the goals of care, and are profoundly important aspects of ethical decision making in pediatric oncology.

After the range of prognostic elements has been considered, it is possible to move on to a discussion of what the goals are to be—goals that may change as the prognosis changes (Fig. 74-5).[151] In a phase III clinical trial, cure may be the dominant goal. However for a child who has had multiple relapses and whose family is considering a phase I trial, ethical decision making will involve a reassessment of goals in light of the best interest of the child, including a reassessment of the child's expressed values. Assessment and prioritization of the values of the child—especially, perhaps, in the context of a low probability of cure—is an area in which much research remains to be done. Among novel projects being assessed is the use of interactive technologies (tapping into the "video-game" culture) to augment the expressive ability of younger children (Fig. 74-6).

Each potential goal with its associated prognosis is related in turn to a treatment option aimed at achieving the goal to the extent possible (i.e., as limited by scientific progress). If families see that supportive care is available to help them achieve their goals, they are less likely to feel abandoned and to fear discontinuation of therapies with curative intent.[152] Ethical decision making takes into account prognoses, goals, and treatment options and

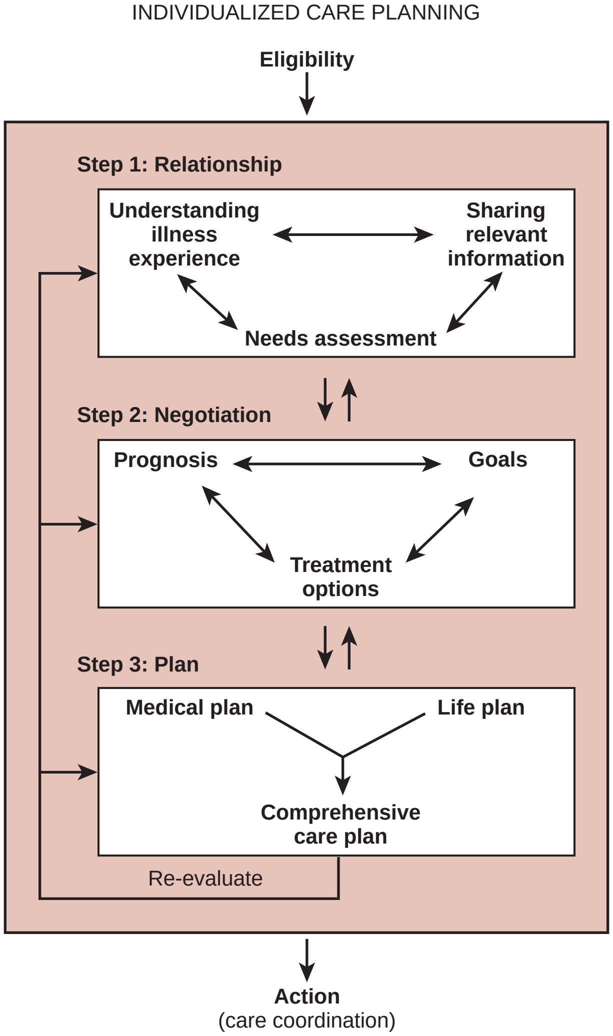


Figure 74-4 The three-step individualized care planning and coordination model. *(Modified from Baker JN, Barfield RC, Hinds PS, et al: A model to facilitate decision making in pediatric stem cell transplantation: the individualized care planning and coordination model.* Biol Blood Marrow Transplant *13:245–254, 2007.)*

TABLE 74-3 Domains of Interdisciplinary Needs Assessment

Assessment Domain	Examples
Structure and process of care	Specialty-level trained symptom management experts are available. The setting of care should meet the preferences, needs, and circumstances of the patient and family. The care plan is based on a comprehensive interdisciplinary assessment.
Physical aspects of care	Symptoms and adverse effects are assessed and managed in a timely, safe, and effective manner. Symptoms and side effects are assessed and managed in a manner that is patient and family centered.
Psychological and psychiatric aspects of care	Psychological and psychiatric needs are assessed and managed in a timely, safe, and effective manner. Grief and bereavement program is available to assess and manage patient, family, and staff grief.
Social aspects of care	Comprehensive interdisciplinary assessment identifies the social needs of patients and families.
Spiritual, religious, and existential aspects of care	Spiritual and existential dimensions are assessed and responded to based on the best available evidence. Spiritual and existential dimensions are approached in a manner that is acceptable to the patient and family as they pertain to the patient's illness.
Cultural aspects of care	Needs are assessed in a culturally sensitive manner. Specific patient and family cultural needs are assessed.
Care of the imminently dying	During this stage of the illness trajectory, the comprehensive needs assessment continues. Signs and symptoms of impending death are assessed, recognized, communicated, and treated as needed.
Ethical and legal aspects of care	Health care professionals assess and attempt to incorporate the values, goals, and preferences of each patient and family. The need for ethics consultation should be assessed based on the comprehensive needs assessment and discordant steps in the process (i.e., cure as a primary goal in end-stage cancer).
Informational aspects of care	Comprehensive interdisciplinary assessment identifies the informational and educational needs of patients and families. Specific patient and family information is provided in a timely manner.
Relational aspects of care	As a part of the generation of a life plan, the relational needs of the patient and family are assessed. Relationships are assessed and augmented throughout the illness trajectory.

From Baker JN, Barfield RC, Hinds PS, et al: A model to facilitate decision making in pediatric stem cell transplantation: the individualized care planning and coordination model. Biol Blood Marrow Transplant *13:245–254, 2007.*

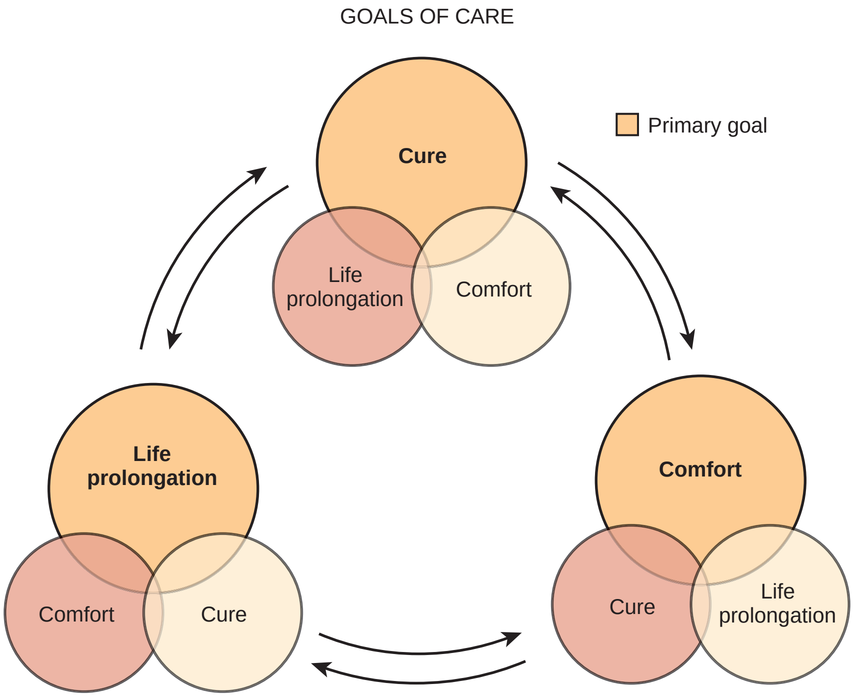


Figure 74-5 Goals of care. *(Modified from Baker JN, Barfield RC, Hinds PS, et al: A model to facilitate decision making in pediatric stem cell transplantation: the individualized care planning and coordination model.* Biol Blood Marrow Transplant *13:245–254, 2007.)*

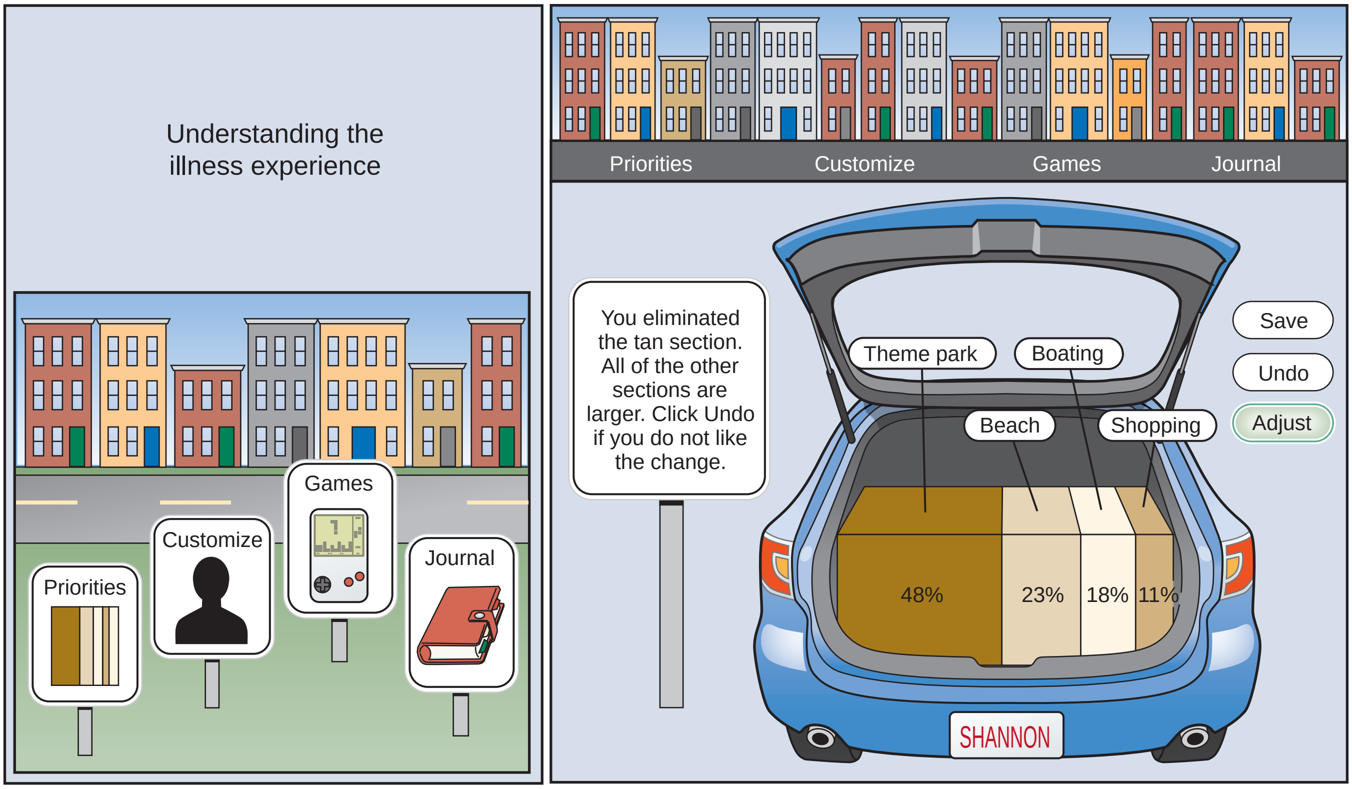


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Figure 74-6 "The Treasure Box" prototype uses children's attraction to electronic games to help them express their priorities and values in the context of ethical decision making regarding phase I trials.

involves shared decision making by the family, the patient, and the clinicians. This process leads to the third step of the ICPC, the comprehensive care plan.

The ethical decision-making process that yields a comprehensive plan takes into account consent, assent, full disclosure of information, and attention to the variety of ways that families and individuals prioritize values. The comprehensive care plan includes both a medical plan (decisions about medical treatment and symptom management) and a life plan (addressing personal goals and family needs). The former might carry more weight when the goals emphasize cure, and the latter might carry more weight for a patient with incurable disease.[153]

The value of such an approach is that it can clarify complex ethical decision making in settings such as the intensive care unit (with the extraordinary advances in sustaining biologic life) and can clarify the goals of care. A useful tool that complements the ICPC is the "four box" method developed by Jonsen and colleagues,[154] which offers specific questions that can add content to the information categories relevant to comprehensive ethical decision making (Fig. 74-7). This more detailed set of questions in the categories of medical information, family preferences, contextual details, and quality of life expands those put forward by the President's Commission in 1983 and by the publication *Deciding to Forgo Life-Extending Treatment*.[155] The five considerations offered by the Commission for determining the best interest of the child in such situations are the amount of suffering and potential for relief, the severity of dysfunction or the potential for restoration of function, expected duration of life, potential for personal satisfaction and enjoyment of life, and the potential to develop a capacity for self-determination.

These issues are very complex, and it is beyond the scope of this chapter to discuss them in depth. However both the moral and spiritual outlook of families (and the emerging and solidifying views of patients) and physicians can become entangled in an elaborate mixture of feelings and facts that may have a profound influence on the types of decisions that emerge during discussions at or near the end of life.[156,157,158,159,160,161] The often-heard directive to "do everything" may be interpreted in a variety of ways, including offering further therapies both in and out of the context of sanctioned clinical trials. Complicating these clinically challenging situations is the influence of both radical uncertainty about prognosis and the underrecognized influence of anecdotal experience.[162-164] When to transition to comfort care only in the dying pediatric cancer patient—or, for that matter, when to concede that a patient is actually dying—remains a dilemma, but one that should be managed in an individualized manner for each patient and his or her family.

FURTHER CHALLENGES IN PEDIATRIC CLINICAL ONCOLOGY TRIALS

Bioinformatics and Genomics

In the past two decades we have seen an unprecedented increase in the international sharing of information and

Demographic Information

Name:
Date:
Length of visit:
Physician of record:
Prepared by (name of scribe):
Present (all participants with titles for future reference):
Care coordinator:
____ First decision--making meeting
____ Update

History of Present Illness

Summary of history of present illness.

Medical Indications	**Patient and Family Preferences**
Comment on the principles of beneficence and nonmaleficence: • What is the patient's medical problem? History? Diagnosis? Prognosis? • Is the problem acute? Chronic? Critical? Emergent or reversible? • What are the goals of treatment? • What are the probabilities of success? • What are the plans in case of therapeutic failure? • In sum, how can this patient and family be benefited by medical and nursing care, and how can harm be avoided?	Comment on the principle of respect for autonomy: • Is the child-patient capable of participating in the decision--making process? Is there evidence of incapacity (e.g., age, cognitive or developmental status)? • If able to participate, what is the extent of that participation? Is the child--patient making decisions about care? If so, what is the patient stating about preferences for treatment? • If able to participate, has the child--patient been informed of benefits and risks, understood this information, and given consent? • If incapacitated or unable to participate, who is making decisions on his or her behalf? Is the parent or surrogate using appropriate standards for decision-making? • Has the patient, parent, or surrogate expressed prior preferences (e.g., Advance Directives)? • Is the patient, parent, or surrogate unwilling or unable to cooperate with medical treatment? If so, why? • In sum, are the patient and family's rights to choose being respected to the extent possible in ethics and law? Do they know they have a choice?

Quality of Life	**Contextual Features**
Comment on the principles of beneficence, nonmaleficence, and respect for autonomy: • What are the prospects, with or without treatment, for a return to normal life? • What physical, mental, and social deficits is the patient likely to experience if treatment succeeds? What is the impact on the family? • Are there biases that might prejudice the provider's evaluation of the patient's quality of life? • Is the patient's present or future condition such that his or her continued life might be judged undesirable? • Is there any plan and rationale to forego treatment? • Are there plans for comfort and palliative care?	Comment on the principles of loyalty and fairness: • What are the rights of the child relative to the rights of the family? • Are there family issues that might influence treatment decisions? • Are there provider issues that might influence treatment decisions? • Are there financial and economic factors? • Are there religious or cultural factors? • Are there limits on confidentiality? • Are there problems of allocation of resources? • How does the law affect treatment decisions? • Is clinical research or teaching involved? • Is there any conflict of interest on the part of the providers of the institution?

Discussion

Summary of key discussion points.

Plan

Summary of action plan.

Figure 74-7 Decision-making communication tool. *(From Jonsen AR, Siegler M, Winslade WJ: Clinical ethics: a practical approach to ethical decisions in clinical medicine, ed 6, New York, 2006, McGraw-Hill.)*

ideas. This fact has allowed more rapid dissemination of useful information and has also increasingly uncovered disparities in access to technology, availability of resources, and ethical values, all of which bear on the ethics of pediatric clinical oncology trials when considered from a global perspective.

Technological advances that are revolutionizing the diagnosis and treatment of childhood cancer (and other childhood disorders) bring with them new ethical issues.[165] These complex ethical questions challenge us to develop a coherent approach that accommodates the diversity of political, religious, cultural, philosophical, and vocational backgrounds that bear upon the discussion.

Bioinformatics is one area demonstrating rapid advances. Bioinformatics attempts large-scale organization of information, including genomic data, protein expression data, and molecular interactions.[166] Genomics is revolutionizing the diagnosis and treatment of cancer.[167] High-throughput microarrays can profile the expression patterns of tens of thousands of genes simultaneously.[168] This technology offers much promise. Similarly, it raises a number of important ethical questions. If a disease gene or disease susceptibility gene profile is discovered, should a subject be notified? Should the subject's family also be notified, or is this a breech of confidentiality?[169]

ASCO has recommended that genetic testing for cancer predisposition be offered when (1) the individual has a personal history, family history, or features suggestive of genetic cancer susceptibility, (2) the test can be adequately interpreted, and (3) the result will aid in a diagnosis or in the medical or surgical management of a patient or family member who has a hereditary risk for cancer.[170] ASCO has also recommended that the regulatory oversight of laboratories providing such tests be strengthened and that federal laws be established to prohibit discrimination by health care providers, employers, and insurance agencies on the basis of such information. Although some persons maintain that genetic discrimination will not affect insurance coverage, several surveys have shown that the public fears this possibility.[171,172,173] One important legislative step that has been taken to address these concerns is the Genetic Nondiscrimination Act of 2008 (Box 74-2).[174]

Box 74-2 Genetic Information as Defined by the Genetic Information Nondiscrimination Act of 2008

- An individual's genetic tests (including genetic tests done as part of a research study).
- Genetic tests of the individual's family members (defined as dependents and up to and including fourth-degree relatives).
- Genetic tests of any fetus of an individual or family member who is a pregnant woman, and genetic tests of any embryo legally held by an individual or family member utilizing assisted reproductive technology.
- The manifestation of a disease or disorder in family members (family history).
- Any request for, or receipt of, genetic services or participation in clinical research that includes genetic services (genetic testing, counseling, or education) by an individual or family member.
- Genetic information does not include information about the sex or age of any individual.

From Office for Human Research Protections, Department of Health and Human Resources: Genetic Information Nondiscrimination Act of 2008. Accessed June 10, 2014 at www.hhs.gov/ohrp/policy/gina.pdf.

In pediatric oncology, the ethical issue is even more complex because surrogate decision makers make decisions that may affect the research subjects' future employability, insurability, and privacy. Further, as technology grows more complex, the gap between technology and meaningful understanding on the part of the family/patient widens (a growing challenge to "informed consent"). Can the surrogate decision makers understand fully the implications of their decision? How much can be expected of a parent without special training?

Pharmacogenomics is the subcategory of genomics research that has perhaps the most immediate potential impact in pediatric clinical oncology trials. This area, too, has enormous potential to benefit children but also creates the potential for social and economic discrimination and abuse of information in genomic databases. Pharmacogenomics combines human genomics with pharmacogenetics to study the role of the individual's genetic makeup in the individual's response to a drug.[175] The promise of this approach is that patients can receive individually tailored medical therapy and dosing. This technology advances the important concept of risk stratification, which historically has led to better protection of children with lower risk cancers from excess toxicity while ensuring that children with higher risk disease are not undertreated.

Pharmacogenomics raises several ethical questions. For example an economic question exists: Might pharmacogenomic criteria eventually become regulatory requirements for the development and testing of medicines?[176] Because the pharmaceutical industry functions on a business model, it is unlikely to develop medicines that will benefit only a small, narrowly defined group of patients. This dilemma is familiar to investigators in pediatric oncology. Orphan drugs might therefore become more common. How should economic risk be weighed against the potential clinical benefit for a small group of patients? Second, if pharmacogenomics information suggests only that a child is more or less likely to benefit from a drug, what likelihood threshold would exclude the child from the use of a drug? Ten percent? Thirty percent? If a child is less likely to respond to a drug, is an insurance company obligated to pay for the drug?[177] If genetic variance peculiar to an ethnic group is found, what are the political, social, and economic implications? Might these be used to decide whether to develop certain medicines for certain populations?[178]

Globalization

Such questions about the impact of technology present a contrast to the ethical issues involved in pediatric oncology trials in low-income countries.[179] The cure rates of many diseases in children differ substantially between high-income and low-income countries.[180] The interventions that have the greatest impact in such countries are often not therapies per se but are rather aimed at reducing the substantial relapse rate caused by, for example, abandonment of treatment—the main cause of treatment failure in low-income countries. Remedial interventions

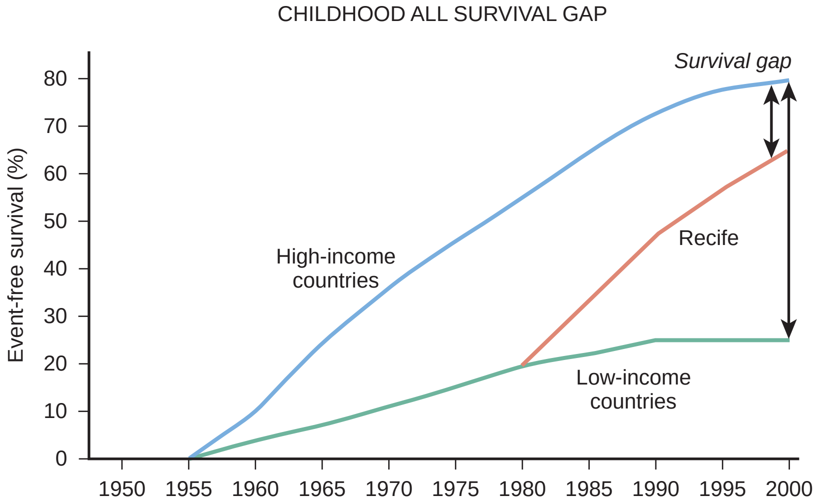

Figure 74-8 Five-year event-free survival of children with acute lymphoblastic leukemia in high-income countries versus low-income countries. Interventions to reduce the loss of patients to follow-up (e.g., making work available near the hospital so the parents are able to make sufficient income to feed other children) increased the survival rate in Recife, Brazil. *(Modified from Howard SC: Delivering global oncology care. Accessed June 10, 2014 at* http://www.cure4kids.org/public/873.*)*

can have a significant impact.[181] St. Jude Children's Research Hospital collaborated with a pediatric cancer center in Recife, Brazil, to reduce abandonment of therapy by providing family support such as housing, work, and food, and the 5-year event-free survival rate of children with acute lymphoblastic leukemia rose from 32% in the 1980s to 63% in 2000 (Fig. 74-8). Similarly, in El Salvador, the 4-year survival rate for children with acute lymphoblastic leukemia increased from 5% to 46% for high-risk cases and 69% for standard-risk cases.[182] Such success has underscored the promise of international collaborative clinical care and research for children with cancer. Many challenges remain, however.

One important challenge is that of ethical oversight of international research in pediatric oncology. Researchers in low-income countries often have little or no access to a functional research ethics committee. However institutions in high-income countries require (in part under the guidance of federal regulations) that adequate ethical oversight be in place as a prerequisite for cooperative clinical research. Collaborative research that uses the strategy of "twinning" institutions in high-income countries with those in low-income countries can produce important advances, as previously described, but the lack of ethical oversight can preclude these collaborations. Researchers cannot report such work in high-profile journals. The International Committee of Medical Journal Editors requires that reports of research involving human subjects document established mechanisms of human subject protection.

The Council for International Organizations of Medical Sciences guidelines state that researchers and institutions in high-income countries sponsoring research in low-income countries have an ethical obligation to improve the capacity for human participant protection in these countries. However in a survey of 203 clinical researchers in low-income countries, 44% reported that their studies were not reviewed by the country's ministry or department of health, and 25% reported that their studies underwent no ethical review by a research ethics committee, IRB, or ministry or department of health.[183]

Federal regulations require that clinical trials conducted in low-income countries in collaboration with U.S. institutions must meet the ethical standards for trials based in the United States. However within the framework of these ethical standards, the research procedures can be adapted to local needs, conditions, and culture.[184] Advancing the development of research ethics in developing countries will open future possibilities for international collaboration that can increase the survival of children with cancer worldwide. Such trials do not necessarily require advanced technology to address the root causes of the disparate survival rates of children in low-income versus high-income countries. Addressing this disparity is itself an ethical imperative.

Although performing clinical trials in impoverished countries in an ethically justifiable manner is laudable, it fails to address the issue of whether the results of such studies will be able to be used to practically benefit future patients. For example within the confines of a trial in a developing country, the sponsor may provide the drugs free of charge to the subjects. However once the trial was complete and, say, the experimental arm was proven to be a significant improvement over the control arm of a phase III study for leukemia, would the expensive agents required to bestow the benefits of the therapy be available for future patients to receive and hence gain the advantages of such treatment? It is unlikely that multiple pharmaceutical companies would continue to make their products available without cost in perpetuity. Another approach that has been suggested is to perform trials with truncated versions of the "first world" model in an attempt to discern if less expensive and perhaps simpler options exist to obtain similar therapeutic outcomes. Of course this would be adjusting treatment to acknowledge the reality of the clinical situations in poor countries and hoping to transmit some (rather than no) benefit, but it also preserves an altered standard of care for people (patients) who already endure the excessive burdens associated with poverty.[185,186] In addition the enactment of the (U.S.) Pediatric Exclusivity Provision, which provides a financial incentive to drug companies to study agents in children that have heretofore only been approved in adults, has generated a large amount of international research, but it is unclear whether the subjects and future children for whom the drugs are appropriate but who live outside the home countries of these pharmaceutical companies will continue to benefit.[187,188] How this situation will ultimately settle out and what the landscape will look like remain to be resolved.[189,190]

CONCLUSION

Pediatric clinical trials present some of the most challenging ethical issues in modern medicine. Children are inherently vulnerable, and yet they are in need of the best, most effective, and least toxic therapies. Much is at stake in childhood cancer. New therapies are developed through research, and yet in pediatric trials, the person who is experiencing the risk is not the same person who is deciding whether the child participates. Many questions will require further thought and discussion and empiric research for the questions amenable to such approaches. Children do not have legal status as decision makers, and yet persons who care for children in pediatric clinical trials know how mature a child's perception can be, even that of a young child. What weight should be given to their opinions? More globally, how should we respond to the disparity of wealth in prioritizing pediatric research goals, even within the boundaries of a developed nation? How should we navigate cultural and political differences that influence the ethical considerations in international clinical trials?

Advancing excellent care of children around the world who have catastrophic diseases such as cancer depends upon clinical trials. Good clinical trials require ethical decision making at every level. Two questions regarding advances in therapy for childhood cancer must always be asked at the same time: "What *can* be done next?" and "What *should* be done next?" The means for answering the first question is scientific. The means for answering the second question is often more philosophical, drawing on many sources of wisdom and insight that are not intrinsically scientific in their approach. History has taught us that scientific advances without ethical considerations can become horrific. Similarly, wisdom without scientific advances cannot further the effort to cure sick children. Medical science committed to ethical research offers the best possibility for continuing to advance the care of children with cancer.

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   Incidence, survival, and mortality data for childhood cancers were analyzed in this article to provide an overview of current statistics to examine the impact of past research discoveries as a means to direct future research.

25. Simon C, Eder M, Raiz P, et al: Informed consent for pediatric leukemia research: clinician perspectives. *Cancer* 92:691–700, 2001.

   Clinicians from five major medical centers were interviewed to learn their perspectives on the informed consent process for a pediatric leukemia trial. Clinicians' race, gender, and experience practicing were correlated with several items. Clinicians provided their view of the informed consent process and provided suggestions for improvement.

27. Kupst MJ, Patenaude AF, Walco GA, et al: Clinical trials in pediatric cancer: parental perspectives on informed consent. *J Pediatr Hematol Oncol* 25:787–790, 2003.

   Parents of children newly diagnosed with cancer were interviewed to learn their perspective on the informed consent process for a clinical trial. Recollection for items such as diagnosis, treatment, and general statistics were high, but understanding of certain aspects of clinical trials was low.

28. Kodish E, Eder M, Noll RB, et al: Communication of randomization in childhood leukemia trials. *JAMA* 291:470–475, 2004.

   This report analyzed physicians' explanations of and parents' understanding of randomizations in randomized clinical trials for childhood leukemia. Physicians explained randomization in most cases, but half of the parents did not understand the concept. The presence of a nurse and discussing specific aspects of the clinical trial were shown to increase the understanding of randomization, and parents who did not understand randomization were likely to be of the racial minority and lower socioeconomic status.

39. Kimberly MB, Hoehn KS, Feudtner C, et al: Variation in standards of research compensation and child assent practices: a comparison of 69 institutional review board-approved informed permission and assent forms for 3 multicenter pediatric clinical trials. *Pediatrics* 117(5):1706–1711, 2006.

   This study was carried out to review child participant assent and compensation by looking at informed permission, assent, and consent forms. These forms were found to offer compensation in three instances: reimbursement for travel and food expenses, for inconveniencing the participant, and for the participant's time. Compensation varied between $0 and $1400. Most of the forms included assent documentation, and a little over half included a separate form written for children and specified at which age to provide assent.

50. Brody JL, Scherer DG, Annett RD, et al: Voluntary assent in biomedical research with adolescents: a comparison of parent and adolescent views. *Ethics Behav* 13:79–95, 2003.

   Parent and adolescent perspectives on an asthma research vignette, including a final decision of whether or not to participate, were compared; 74% of the time parents and their children agreed on research participation, with both claiming to be the responsible party for the decision.

53. Wendler D, Emanuel EJ: What is a "minor" increase over minimal risk? *J Pediatr* 147(5):575–578, 2005.

   The additional protections for children involved in research require the interpretation and application of three different levels of risk: minimal risk, minor increase over minimal risk, and more than a minor increase over minimal risk. Wide variability exists in the categorization of various research procedures according to these levels of risk. Noting that the federal regulations do not define a minor increase over minimal risk, the authors propose that this level of risk be interpreted as socially acceptable risks that are nevertheless greater than the risks ordinarily encountered by healthy children (i.e., minimal risk).

55. Nelson RM, Ross LF: In defense of a single standard of research risk for all children. *J Pediatr* 147:565–566, 2005.

   In response to the article on minor risk by Wendler and Emmanuel[53] (2005), this article argues that the ethical justification for this standard appears tautological, endorsing those risks that are "acceptable" to society and rejecting those that are "unacceptable" to society without explaining why certain risks would or would not be considered socially acceptable. As an alternative, the authors believe that the "scrupulous parent" standard is ethically justifiable and incorporates both minimal risk and a minor *increase over minimal risk within a uniform standard that should be applicable to all children.*

59. Kim A, Fox E, Warren K, et al: Characteristics and outcome of pediatric patients enrolled in phase I oncology trials. *Oncologist* 13:679–689, 2008.

    Analyses presented in this article aimed to define characteristics, toxicity risk, and outcomes of pediatric phase I trial participants. A higher risk for a dose-limiting toxicity was associated with drug dose and prior radiation, and the median survival rate was 5 months from the time of subject enrollment.

62. Maurer SH, Hinds PS, Spunt SL, et al: Decision making by parents of children with incurable cancer who opt for enrollment on a phase I trial compared with choosing a do not resuscitate/terminal care option. *J Clin Oncol* 20:3292–3298, 2010.

    Decision making for parents who just enrolled their child on a phase I trial and those who had chosen a do not resuscitate order or terminal care were compared. They were asked for the rationale of their decision, how they define a good parent, and what clinician behaviors supported this definition. Both groups desired clinician support and believed a good parent was supportive, present, and sacrificed for the child. Participants who chose a clinical trial indicated that they felt compelled to continue with care, whereas the other group expressed quality of life concerns.

66. Tomlinson D, Bartels U, Hendershot E, et al: Factors affecting treatment choices in paediatric palliative care: comparing parents and health professionals. *Eur J Cancer* 47:2182–2187, 2011.

    Factors influencing parents' and health care professionals' decision making for chemotherapy or supportive care in pediatric palliative care were compared. Parents rated hope, increased survival time, and the child's quality of life as most important to their decision, which were significantly higher than the physicians' ratings. Physicians placed a higher importance on the family's financial considerations.

71. Miller FG, Brody H: A critique of clinical equipoise. Therapeutic misconception in the ethics of clinical trials. *Hastings Cent Rep* 33:19–28, 2003.

    A predominant ethical view holds that physician-investigators should conduct their research with therapeutic intent. Because a physician offering a therapy would not prescribe second-rate treatments, the experimental intervention and the best proven therapy should appear equally effective. "Clinical equipoise" is necessary. The authors argue that this perspective is flawed, that the ethics of research and of therapy are fundamentally different, and that clinical equipoise should be abandoned.

76. Cousino MK, Zyzanski SJ, Yamokoski AD, et al: Communicating and understanding the purpose of pediatric phase I cancer trials. *J Clin Oncol* 30:4367–4372, 2012.

    Parents of children enrolled in a phase I clinical trial were interviewed and their responses were analyzed for understanding of three core components of a phase I trial: dose escalation, dose limiting toxicity, and safety. Physician disclosure was also measured. The child was present in 83 of the 85 conferences analyzed. Only a third of parents exhibited understanding of the scientific goals, and another third exhibited no understanding. Parents of a higher socioeconomic status and racial majority status were more likely to understand. Physician explanation of goals were associated with parent understanding but were not explained in all cases.

77. Marshall PA, Magtanong RV, Leek AC, et al: Negotiating decisions during informed consent for pediatric phase I oncology trials. *J Empir Res Hum Res Ethics* 7:51–59, 2012.

    Sixteen informed consent conferences for a pediatric phase I trial were examined to identify communication steps and what influences the negotiation. It was found that families and patients attempt to gain control and autonomous decision making by negotiating microdecisions in drug logic and logistics and administration and/or scheduling, which unfold in a four-step process.

108. Kollek R, Petersen I: Disclosure of individual research results in clinico-genomic trials: challenges, classification and criteria for decision-making. *J Med Ethics* 37:271–275, 2011.

    Although an ethical obligation to report findings of clinical research to trial participants is increasingly recognized, it is unclear how such a process should be organized. The authors present a classification of different actors, processes, and data involved in the feedback of research results pertaining to an individual. They go on to discuss the types of difficulties that must be faced when returning individual research results to trial participants and present a stepwise model to trigger the individual feedback process.

121. MacLeod KD, Whitsett SF, Mash EJ, et al: Pediatric sibling donors of successful and unsuccessful hematopoietic stem cell transplants (HSCT): a qualitative study of their psychosocial experience. *J Pediatr Psychol* 28:223–230, 2003.

    This study was a qualitative review of the psychosocial experience of pediatric sibling donors' successful and unsuccessful hematopoietic stem cell transplants. Siblings who were in the unsuccessful transplant group had a higher negative impact, reported feelings of guilt, and were less likely to experience positive effects. According to both groups, psychological aspects were of greater importance than physical aspects.

126. Coleman DL, Rosoff PM: The authority of "mature minors" to consent to general medical treatment. *Pediatrics* 131:786–793, 2013.

    The law has developed a broad "mature minor exception" to the general requirement of parental consent in abortion cases and has additionally carved out numerous specific status-based and condition-based exceptions to that requirement. Because of this, it is not always easy for medical professionals who treat adolescents to ascertain the applicable law. In this article, the authors discuss the underlying differences between medical ethics and law that have caused some of the confusion in this area, and they set out the most current legal rules governing adolescent decision-making authority in general medical settings.

134. Sugarman J, Kaalund V, Kodish E, et al: Ethical issues in umbilical cord blood banking. Working Group on Ethical Issues in Umbilical Cord Blood Banking. *JAMA* 278:938–943, 1997.

    Umbilical cord blood (UCB) technology is increasingly important. In this article the authors argue for several important conclusions regarding UCB. UCB banking for autologous use is associated with even greater uncertainty than is banking for allogeneic use. Marketing practices for UCB banking in the private sector require close attention. There must be continued work to ensure that recruitment for banking and use of UCB are equitable. The process of obtaining informed consent for collection of UCB should begin before labor and delivery.

144. Baker JN, Barfield RC, Hinds PS, et al: A model to facilitate decision making in pediatric stem cell transplantation: the individualized care planning and coordination model. *Biol Blood Marrow Transplant* 13:245–254, 2007.

    The authors introduce the individualized care planning and coordination (ICPC) model as a practical approach to facilitate ethical and effective decision making in pediatric stem cell transplantation settings. The ICPC is a three-step model comprising (1) relationship—understanding the illness experience from the perspective of the patient and family, sharing relevant information, and assessing ongoing needs; (2) negotiation—prognosticating, establishing goals of care, and discussing treatment options; and (3) plan—generating a comprehensive plan of care that includes life and medical plans. Based on a foundation of a care of competence, empathy, compassion, communication, and quality, the ICPC model aims to diminish contentious family-staff interactions that can lead to mistrust and help guide treatment decision making.

148. Hilden JM, Emanuel EJ, Fairclough DL, et al: Attitudes and practices among pediatric oncologists regarding end-of-life care: results of the 1998 American Society of Clinical Oncology survey. *J Clin Oncol* 19:205–212, 2001.

    Members of the American Society of Clinical Oncology were surveyed to gain an understanding of the attitudes, practices, and challenges its members experience when caring for end-of-life care patients. Clinicians reported a lack of formal education in pediatric palliative care and a need for strong mentors. The absence of a palliative care team was reported as impeding quality care for patients and reducing the effectiveness of communication between clinicians and patients.

153. Whitney SN, Ethier AM, Fruge E, et al: Decision making in pediatric oncology: who should take the lead? The decisional priority in pediatric oncology model. *J Clin Oncol* 24:160–165, 2006.

    The authors propose a new model of decision making in pediatric oncology to reconcile the discrepancies that exist between *how ethicists and clinicians view the decision-making process for patients. Emphasis is placed on distinguishing decisional priority from decisional authority and on having clinicians encourage parents to assume decisional priority for their child.*

156. Curlin FA, Chin MH, Sellergren SA, et al: The association of physicians' religious characteristics with their attitudes and self-reported behaviors regarding religion and spirituality in the clinical encounter. *Med Care* 44:446–453, 2006.

The relationship between physicians' religious beliefs and their self-reported behaviors on religion and spirituality in the clinical setting was examined. Clinicians indicated that it was appropriate to discuss these topics when the patient initiates the discussion and that they often are encouraging of the patients' own beliefs and practices. Clinicians who indicate they are more religious are more likely to address religion and spirituality in the clinical setting.

158. Ecklund EH, Cadge W, Gage EA, et al: The religious and spiritual beliefs and practices of academic pediatric oncologists in the United States. *J Pediatr Hematol Oncol* 29:736–742, 2007.

The religious and spiritual beliefs of pediatric oncologists were compared with those of the general public, because it is recognized that religion and spirituality are important to the care of seriously ill patients. Many oncologists described themselves as spiritual, while also reporting that they did not have a religious identity.

160. Seale C: The role of doctors' religious faith and ethnicity in taking ethically controversial decisions during end-of-life care. *J Med Ethics* 36:677–682, 2010.

In this study the author compares ethnicity and religious faith in the medical and general United Kingdom populations and reports on their associations with ethically controversial decisions taken when providing care to dying patients. Based on the study's findings, the author advocates for greater acknowledgement of the relationship of doctors' values with clinical decision making.

164. Fagerlin A, Wang C, Ubel PA: Reducing the influence of anecdotal reasoning on people's health care decisions: is a picture worth a thousand statistics? *Med Decis Making* 25:398–405, 2005.

This study was designed to assess participants' decision making when given a quiz or pictograph versus anecdotal information and how heavily each was relied upon. The use and influence of anecdotal information decreased when participants were presented with statistical information in a pictograph.

165. Feigin RD: Prospects for the future of child health through research. *JAMA* 294:1373–1379, 2005.

Advances made possible through genomics, proteomics, and the application of nanosystem technology, coupled with a greater understanding of the influence of the environment on human genes, will enhance our ability to prevent, modify, or cure numerous childhood disorders. This article reviews some of the more pressing and important causes of morbidity and mortality in children, discusses the manner in which some of the newer technologies may be applied to investigations of these disorders, and offers predictions concerning the effect that new discoveries may have in ameliorating the morbid consequences of childhood diseases. The author highlights the need to design and implement prospective long-term studies to determine the most effective ways to reduce the burden of preventable problems, which are rooted in societal issues.

172. Nowlan W: Human genetics. A rational view of insurance and genetic discrimination. *Science* 297:195–196, 2002.

The Human Genome Project has committed a portion of its annual budget to the study of the ethical, legal, and social implications (ELSI) of genetics research. The assertion that discrimination in health insurance is a real problem pervades the ELSI literature, despite compelling evidence that at worst the threat is theoretical. The author reviews this evidence and also argues that genetic discrimination in the area of life insurance is unlikely to become a significant social problem.

182. Howard SC, Pedrosa M, Lins M, et al: Establishment of a pediatric oncology program and outcomes of childhood acute lymphoblastic leukemia in a resource-poor area. *JAMA* 291:2471–2475, 2004.

The cure rate for childhood acute lymphoblastic leukemia (ALL) differs markedly between developed and developing countries. The authors assess the effect of a multidisciplinary team approach and protocol-based therapy on the event-free survival of children with ALL in an area with limited resources over 22 years. The 5-year event-free survival improved steadily with implementation of a range of changes over time. The authors conclude that the treatment of childhood ALL in a dedicated pediatric oncology unit using a comprehensive multidisciplinary team approach, protocol-based therapy, and local support and funding is associated with improved outcomes in a resource-poor area.

185. Glickman SW, McHutchison JG, Peterson ED, et al: Ethical and scientific implications of the globalization of clinical research. *N Engl J Med* 360:816–823, 2009.

Economic globalization is an important development of the past half century. The authors address several important questions related to this development. Who benefits from the globalization of clinical trials? What is the potential for exploitation of research subjects? Are trial results accurate and valid, and can they be extrapolated to other settings? In this article, they discuss recent trends in and underlying reasons for the globalization of clinical research, highlight important scientific and ethical concerns, and propose steps for the harmonization of international clinical research.

188. Pasquali SK, Burstein DS, Benjamin DK, et al: Globalization of pediatric research: analysis of clinical trials completed for pediatric exclusivity. *Pediatrics* 126:e687–e692, 2010.

This study was conducted to assess the setting in which pediatric research is conducted and its influence in the globalization of pediatric research. Studies under the U.S. Pediatric Exclusivity Provision were included that reported trial data between 1998 and 2007. Fifty-four countries were represented in conducting pediatric research, and the majority of published research was conducted at locations outside the United States.

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APPENDICES

Reference Values in Infancy and Childhood

Carlo Brugnara

APPENDIX 1 Hematologic Values* in Normal Fetuses at Different Gestational Ages

Week of Gestation	Hemoglobin (g/dL)	RBCs ($\times 10^6$/mL)	Hematocrit (%)	Mean Corpuscular Volume (fL)	Total WBCs ($\times 10^6$/μL	Corrected WBCs ($\times 10^6$/μL)	Platelets ($\times 10^6$/μL)
18-21 (N = 760)	11.69 ± 1.27	2.85 ± 0.36	37.3 ± 4.32	131.1 ± 11.0	4.68 ± 2.96	2.57 ± 0.42	234 ± 57
22-25 (N = 1200)	12.2 ± 1.6	3.09 ± 0.34	38.59 ± 3.94	125.1 ± 7.8	4.72 ± 2.82	3.73 ± 2.17	247 ± 59
26-29 (N = 460)	12.91 ± 1.38	3.46 ± 0.41	40.88 ± 4.4	118.5 ± 8.0	5.16 ± 2.53	4.08 ± 0.84	242 ± 69
>30 (N = 440)	13.64 ± 2.21	3.82 ± 0.64	43.55 ± 7.2	114.4 ± 9.3	7.71 ± 4.99	6.4 ± 2.99	232 ± 87

Modified from Forestier F, Daffos F, Catherine N, et al: Developmental hematopoiesis in normal human fetal blood. Blood *77:2360, 1991.*
RBCs, Red blood cells; *WBCs,* white blood cells.
*Hematologic data obtained with a Coulter S plus II instrument. Total WBC count included nucleated red blood cells. Corrected WBC count included only WBCs, after subtracting the nucleated red cell component, based on a 100-cell manual differential.

APPENDIX 2 WBC Manual Differential Counts in Normal Fetuses at Different Gestational Ages

Week of Gestation	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)	Basophils (%)	Monocytes (%)	Nucleated RBCs (% of WBCs)
18-21 (N = 186)	88 ± 7	6 ± 4	2 ± 3	0.5 ± 1	3.5 ± 2	45 ± 86
22-25 (N = 230)	87 ± 6	6.5 ± 3.5	3 ± 3	0.5 ± 1	3.5 ± 2.5	21 ± 23
26-29 (N = 144)	85 ± 6	8.5 ± 4	4 ± 3	0.5 ± 1	3.5 ± 2.5	21 ± 67
>30 (N = 172)	68.5 ± 15	23 ± 15	5 ± 3	0.5 ± 1	3.5 ± 2	17 ± 40

From Forestier F, Daffos F, Catherine N, et al: Developmental hematopoiesis in normal human fetal blood. Blood *77:2360, 1991.*
RBCs, Red blood cells; *WBCs,* white blood cells.

APPENDIX 3 Hematologic Values for Cord Blood (Vaginal Delivery and Cesarean Section*)

	Study Sample (N = 167)		**Vaginal Delivery (N = 63)**		**Cesarean Section (N = 104)**		
Characteristic	Median	Range	Median	Range	Median	Range	**P-value[2]**
WBC ($\times10^9$ per l)	15.1	5.54-39.7	18.4	12.0-34.1	13.6	8.54-39.7	<0.0001
RBC ($\times10^{12}$ per l)	4.7	3.46-6.62	4.78	3.89-6.30	4.62	3.46-6.62	NS
Hb (g l^{-1})	174	130-234	176	140-230	171	130-234	NS
Hct (%)	53.6	40.1-73.1	54.7	41.9-73.1	52.6	40.1-72.2	NS
MCV (fl)	112	97.7-127	114	105-127	112	97.7-125	NS
MCH (pg)	36.5	31.4-41	36.5	31.4-41	36.6	32-39.9	NS
MCHC (g l^{-1})	324	303-359	323	308-359	324	303-344	NS
RDW (%)	17.4	14.2-23.6	17.4	14.9-23.6	17.4	14.2-23.3	NS
PLT ($\times10^9$ per l)	270	161-607	297	169-607	254	161-424	0.0053
MPV (fl)	8.7	7.5-11.5	8.7	7.7-11.4	8.8	7.5-11.5	NS
Plateletcrit (%)	0.24	0.15-0.48	0.26	0.15-0.48	0.23	0.15-0.36	0.0056
CD34+ cells ($\times10^6$ per l)	43.9	7.14-253	47.7	15.9-253	39.9	7.14-120	0.007

From Eskola M, Juutistenaho S, Aranko K, et al: J Perinatol *2011; 258–262. Data obtained with Sysmex K-1000 analyzer (Sysmex, Kobe Japan).*

Hb, Hemoglobin; *Hct,* hematocrit; *MCH,* mean corpuscular hemoglobin; *MCHC,* mean corpuscular hemoglobin concentration; *MCV,* mean corpuscular volume; *MPV,* mean platelet volume; *NS,* not significant; *plateletcrit,* MPV × PLT; *PLT,* platelet; *RBC,* red blood cell; *RDW,* red blood cell distribution width; *WBC,* white blood cell.

*aP-values of the differences between vaginal delivery and cesarean section. The concentrations were standardized to exclude the varying effect of the anticoagulant.

APPENDIX 4 Hematologic Values* for Normal Cord Blood

	Mean ± SD		**Mean ± SD**
Red Blood Cells		% HYPER (MCHC >41 g/dL)	0.6 ± 0.3
Hb (g/dL)	15.3 ± 1.3	% MICRO (MCV <61 fL)	0.8 ± 0.3
Hct (%)	49 ± 5	% MACRO (MCV >120 fL)	31.8 ± 9.7
RBC ($\times10^6/\mu$L)	4.3 ± 0.4	**Reticulocytes**	
MCV (fL)	112 ± 6	%	3.63 ± 1.11
MCH (pg)	36.2 ± 2.2	Absolute reticulocytes ($\times10^9$/L)	156.1 ± 47.7
MCHC (g/dL)	30.9 ± 1.3	MCVr (fL)	125.8 ± 7.3
CHCM (g/dL)	30.4 ± 1.2	CHCMr (g/dL)	25.6 ± 1.2
% HYPO (MCHC <28 g/dL)	17.3 ± 11.9	CHr (pg)	31.3 ± 1.4

Modified from Diagne I, Archambeaud MP, Diallo D, et al: Parametres erythrocytaires et reservés en fer dans le sang du cordon. Arch Fr Pediatr *2:208, 1995; and from G Tchernia, personal communication. Data obtained in 142 specimens with H*2 Technicon analyzer.*

CHCM, Cell hemoglobin concentration mean; *CHCMr,* reticulocyte cell hemoglobin concentration mean; *CHr,* reticulocyte hemoglobin content; *Hb,* hemoglobin; *Hct,* hematocrit; *% HYPER,* % hyperchromic red cells; *% HYPO,* % hypochromic red cells; *% MACRO,* % macrocytic red cells; *MCH,* mean corpuscular hemoglobin; *MCHC,* mean corpuscular hemoglobin concentration; *MCV,* mean corpuscular volume; *MCVr,* reticulocyte mean corpuscular volume; *% MICRO,* microcytic red cells; *RBC,* red blood cell; *SD,* standard deviation.

*Values obtained with Technicon H*2 and H*3 Hematology analyzers (Bayer Diagnostics) in neonates delivered at term with weight ≥2500 g.

APPENDIX 5 Red Cell and Reticulocyte Indices, Serum Iron Status Markers in Cord Blood

Parameter	Mean	SD	Reference Range*
Blood Count and Cellular Indices			
Hb, g/L	159	15	146-189
HCT, L/L	0.49	0.05	0.44-0.58
MCV, fL	109	4	102-118
MCVr, fL	124	6	115-136
MCH, pg	35	1	33-38
MCHC, g/L	325	10	306-342
%Retic, %	4.0	0.8	2.6-5.4
IRF-H, %	24.1	7.8	10.2-40.0
CHm, pg	34.9	1.3	32.5-37.2
CHr, pg	35.6	1.3	33.1-38.6
%HYPOm, %	3.0	3.0	0.4-9.9
%HYPOr, %	42.0	15.6	18.3-76.8
Serum Measurements			
TfR, mg/L	2.0	0.7	1.2-4.0
Ferritin, µg/L	198	137	45-636
TfR-F index	0.95	0.43	0.49-2.1
Iron, µmol/L	27.4	7.7	12.2-42.1
Transferrin, g/L	2.0	0.4	1.2-2.9
TfSat, %	55	19	21-111

From Ervasti M, Kotisaari S, Sankilampi U, et al: The relationship between red blood cell and reticulocyte indices and serum markers of iron status in the cord blood of newborns. Clin Chem Lab Med *45:1000–1003, 2007. Hematologic data obtained in 199 full-term newborn infants with a ADVIA 120 analyzer (Siemens Diagnostic Solutions, Terrytown, NY).*

CHm, Cellular hemoglobin in red blood cells; *CHr,* cellular hemoglobin in reticulocytes; *Hb,* hemoglobin; *HCT,* hematocrit; *%HYPOm,* percentage of hypochromic red blood cells; *%HYPOr,* percentage of hypochromic reticulocytes; *IRF-H,* high immature reticulocyte fraction; *MCH,* mean cell hemoglobin; *MCHC,* mean cell hemoglobin concentration; *MCV,* mean cell volume of red blood cells; *MCVr,* mean cell volume of reticulocytes; *%Retic,* proportion of reticulocytes; *TfR,* transferrin receptor; *TfR-F* index, transferrin receptor/log (ferritin); *TfSat,* transferrin saturation.

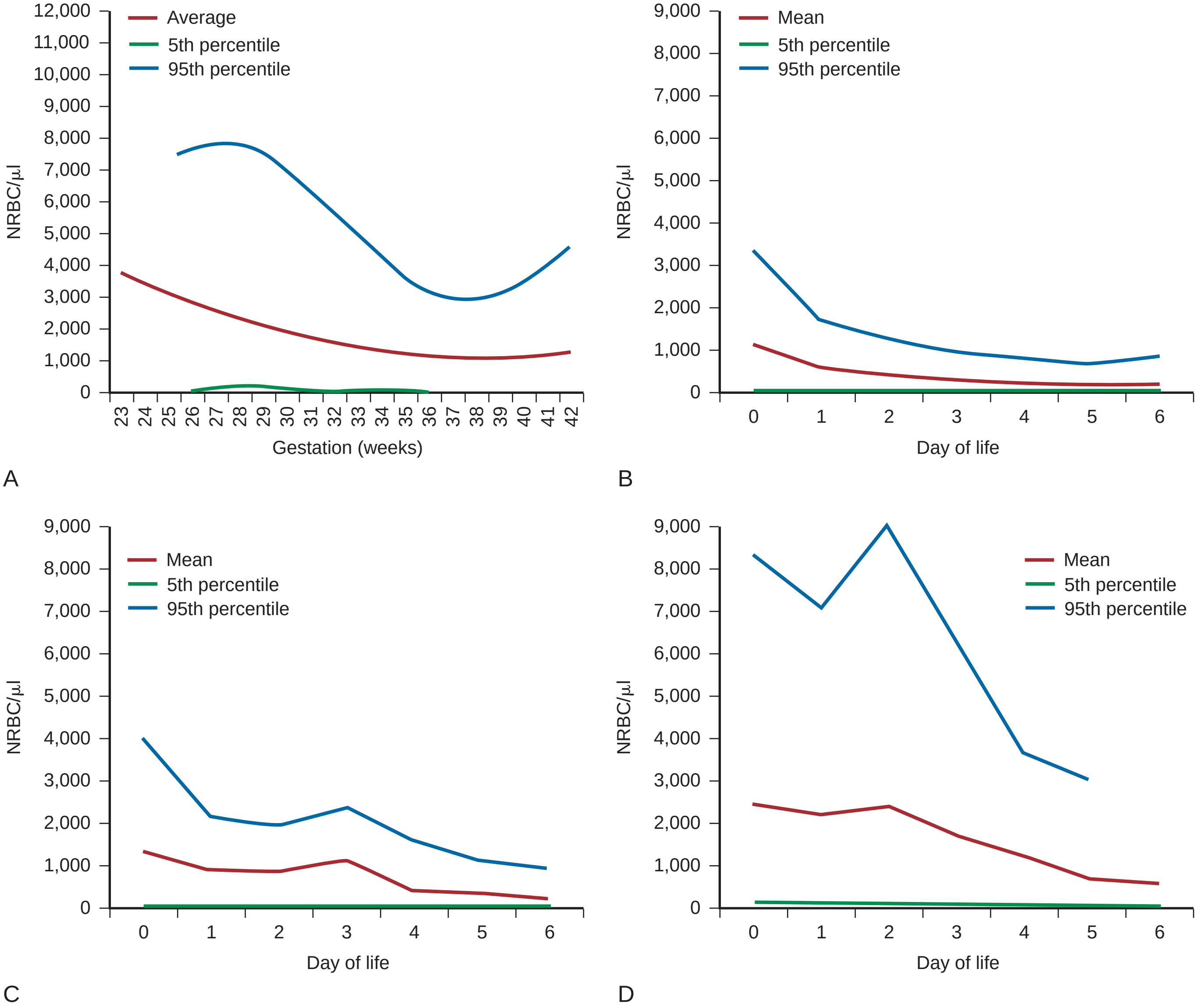
*For reference range calculations, only samples in which Hb was greater than 146 g/L were included.

APPENDIX 6 Erythroblast and Leukocyte Counts in Umbilical Cord Blood*

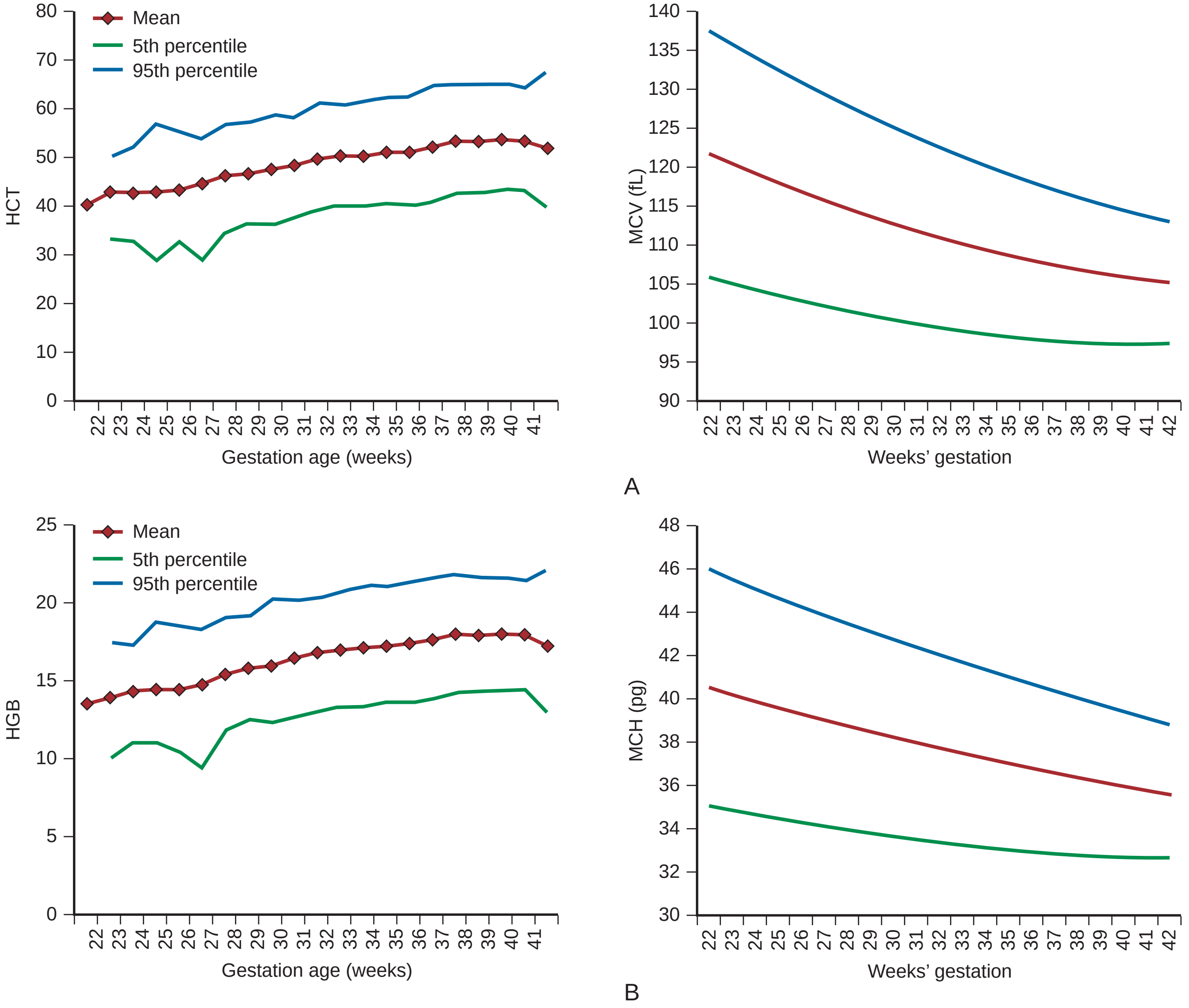
Type of Delivery	Erythroblast Count (×10⁹/L)	Leukocyte Count (×10⁹/L)
Spontaneous, vaginal (N = 55)	0.75 (0.0-5.3)	13.8 (7.25-48.0)
Elective cesarean section (N = 39)	0.30 (0.0-0.49)	10.6 (6.2-17.7)
Emergency cesarean section (N = 55)	1.10 (0.0-15.9)	13.5 (4.2-40.3)

From Thilaganthan B, Athansasiou S, Ozmen S, et al: Umbilical cord blood erythroblast count as an index of intrauterine hypoxia. Arch Dis Child *70:F192, 1994.*

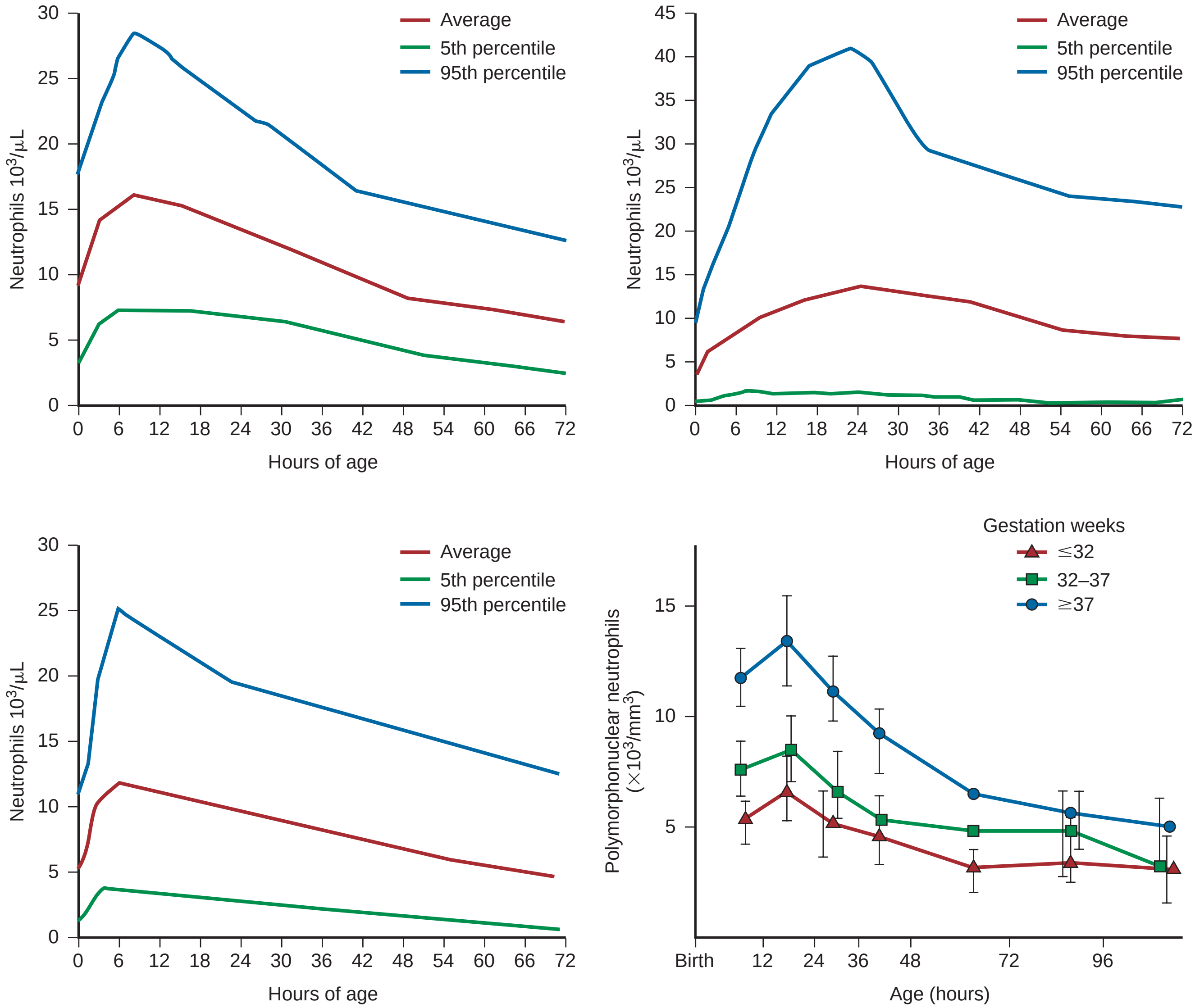
*Values are expressed as mean (range). Erythroblast counts were significantly higher in the spontaneous vaginal and emergency cesarean section groups compared with the elective cesarean group.



Appendix 7 **A**, Reference ranges for blood concentrations of NRBC on the day of birth are displayed according to gestational age. The lower and upper lines represent the 5% and 95% limits, and the middle line represents the mean value. **B-D**, Reference ranges for blood concentrations of NRBC during the first 7 days following birth are shown. The lower and upper lines represent the 5% and 95% limits, and the middle line represents the mean value. **B**, Reference range for neonates 1 to 36 weeks' gestation at birth. **C**, Range for neonates 30 to 35 weeks' gestation at birth. **D**, Range for neonates 1 to 30 weeks' gestation at birth. *(From Christensen RD, Henry E, Andres RL et al. Reference ranges for blood concentrations of nucleated red blood cells in neonates.* Neonatol *99:289–294, 2011.)*



Appendix 8 *Left Column*, Reference ranges (5th percentile, mean, and 95th percentile) for hematocrits *(top)*, Hemoglobin *(bottom)*, both obtained during the first 6 hours after birth among patients 22 to 42 weeks gestation. *Right Column (top and bottom)*, Expected range of values for mean corpuscular volume (MCV, *a*) and mean corpuscular hemoglobin (MCH, *b*) for neonates on the first day after birth. The upper and lower boundaries incorporate 95% of the measured values. *(Left column, From Christensen RD, Henry E, Jopling J, et al: The CBC: reference ranges for neonates.* Semin Perinatol *33:3–11, 2009; Right column, From Christensen RD, Jopling J Henry E et al. The erythrocyte indices of neonates, defined using data from over 12000 patients in a multihospital health care system.* J Perinatol *28:24–28, 2008.)*



Appendix 9 *Top Left,* Reference ranges for blood neutrophil concentrations during the first 72 hours after the birth of term and near-term (36 weeks' gestation) neonates. A total of 12149 values were obtained for the analysis. The 5th percentile, the mean, and the 95th percentile values are shown. *Bottom Left,* Reference ranges for blood neutrophil concentration during the first 72 hours after the birth of 28 to 36 weeks' gestation preterm neonates. A total of 8896 values were obtained for the analysis. The 5th percentile, the mean, and the 95th percentile values are shown. *Top Right,* Reference ranges for blood neutrophil concentration during the first 72 hours after the birth of 28 weeks' gestation preterm neonates. A total of 852 values were obtained for the analysis. The 5th percentile, the mean, and the 95th percentile values are shown. *Bottom right,* Changes in polymorphonuclear neutrophils after birth in three groups with different gestational ages. Total white blood cell (WBC) counts were obtained with a Coulter S analyzer; manual differential counts were performed on 200 nucleated cells. *(Top and Bottom Left and Top Right, From Christensen RD, Henry E, Jopling J, et al: The CBC: reference ranges for neonates.* Semin Perinatol *33:3–11, 2009; Bottom Right, Modified from Coulombel L, Dehan M, Tchernia G, et al: The number of polymorphonuclear leukocytes in relation to gestational age in the newborn.* Acta Paediatr Scand *68:709, 1979.)*

APPENDIX 10 Reference Ranges for Hb, HCt, Reticulocyte Parameters, and S-TfR During the First 15 Weeks of Life

	Hb			**Hct**			**Reticulocyte**			**CHr**			**IRF**			**S-TfR**		
		Reference Interval			Reference Interval			Reference Interval			Reference Interval			Reference Interval			Reference Interval	
Age, Week	MEAN	LOWER	UPPER	MEAN	LOWER	UPPER	MEAN	LOWER	UPPER	MEAN	LOWER	UPPER	MEAN	LOWER	UPPER	MEAN	LOWER	UPPER
0.5	150.5	101.8	222.4	0.45	0.30	0.65				35.7	31.5	39.9	36.6	13.5	59.7			
1.5	138.7	97.9	196.3	0.41	0.29	0.58	59.5	20.2	175.6	35.0	31.1	38.9	35.5	13.7	57.2	1.4	0.9	2.4
2.5	128.8	94.0	176.5	0.38	0.28	0.52	66.8	24.7	181.1	34.3	30.6	38.1	34.3	13.6	55.1	1.3	0.8	2.3
3.5	120.6	90.0	161.5	0.36	0.27	0.47	74.1	29.2	187.9	33.7	30.1	37.4	33.3	13.1	53.4	1.3	0.8	2.1
4.5	113.8	86.2	150.3	0.34	0.26	0.44	81.2	33.7	195.8	33.1	29.5	36.7	32.2	12.4	52.1	1.2	0.7	2.0
5.5	108.3	82.5	142.1	0.32	0.25	0.42	87.9	37.8	204.3	32.6	29.0	36.2	31.3	11.6	50.9	1.2	0.7	1.9
7.0	101.9	77.8	133.7	0.30	0.23	0.39	96.7	43.1	216.7	31.9	28.3	35.5	29.9	10.3	49.6	1.1	0.7	1.8
9.0	96.9	73.5	127.7	0.29	0.22	0.38	105.2	48.4	228.8	31.2	27.5	34.8	28.4	8.6	48.1	1.1	0.7	1.8
11.0	94.9	71.7	125.7	0.28	0.21	0.37	109.0	51.3	231.9	30.6	26.9	34.3	27.0	7.4	46.7	1.1	0.7	1.9
13.0	96.1	72.6	127.3	0.28	0.21	0.37	107.6	51.4	225.2	30.2	26.5	33.9	25.9	6.7	45.0	1.2	0.7	2.0
15.0	100.5	75.9	133.1	0.30	0.22	0.39	101.2	48.1	212.9	30.0	26.4	33.6	24.9	6.7	43.3	1.3	0.8	2.2

From Mäkela E, Takal TI, Suomine P, et al: Hematological parameters in preterm infants from birth to 16 weeks of age with reference to iron balance. Clin Chem Lab Med *46:551–557, 2008. Hematologic data obtained with ADVIA 120, Siemens Medical Solutions, Tarrytown, NY. S-TfR measured with an automated immunoturbidimetric methos (IDeA sTfR-IT, Orion Diagnostica), Espoo, Finland. Ferritin assayed with Elecsys ferritin electrochemiluminescence immunoassay on a Modular E analyzer (Roche Diagnostics).*

CHr, Reticulocyte hemoglobin content; *Hb,* hemoglobin; *Hct,* hematocrit; *IRF,* immature reticulocyte fraction; *S-Tfr,* soluble transferrin receptor.

APPENDIX 11 Reference Ranges for Ferritin During the First 15 Weeks of Life

	Ferritin*			Ferritin, No Transfusions*		
		Reference Interval			Reference Interval	
Age, Week	MEAN	LOWER	UPPER	MEAN	LOWER	UPPER
1.5	221.4	77.0	636.8	215.5	73.5	631.6
2.5	199.9	65.6	609.2	178.5	56.9	559.8
3.5	180.4	52.8	616.5	149.2	43.9	507.2
4.5	162.8	41.4	640.2	125.9	34.1	465.4
5.5	147.0	32.3	668.4	107.2	26.8	429.7
7.0	126.0	22.6	702.3	85.7	19.2	382.1
9.0	102.6	14.9	708.7	65.7	13.3	324.6
11.0	83.5	10.6	659.1	52.3	10.1	271.7
13.0	68.0	8.2	563.0	43.2	8.3	224.0
15.0	55.3	6.9	444.8	37.0	7.5	182.9

From Mäkela E, Takal TI, Suomine P, et al: Hematologic parameters in preterm infants from birth to 16 weeks of age with reference to iron balance. Clin Chem Lab Med *46:551–557, 2008. Ferritin assayed with Elecsys ferritin electrochemiluminescence immunoassay on a Modular E analyzer (Roche Diagnostics).*

*The information under the column "Ferritin" shows values calculated for the neonates who had not been transfused during the preceding 2 weeks of measurements. The information under the column "Ferritin, no transfusions" shows the values calculated for those neonates who had not been transfused at all (indicating ferritin level development in the most stable preterm neonates).

APPENDIX 12 Reference Ranges for CBC, Reticulocytes, Ferritin, and S-TfR During the First Year of Life in Preterm and Full-Term Infants

Age, Weeks	Hb, g/L		Hct		RBC count, × 10^{12}/L		MCV, fL		MCH, pg		Retic, × 10^9/L	
	Mean	95% RI	Mean	95% RI	Mean	95% RI	Mean	95% RI	Mean	95% RI	Mean	95% RI
Preterm												
20	112.2	92.0-136.8	0.327	0.271-0.395	4.03	3.15-4.92	81.6	73.2-90.0	28.1	25.2-31.0	77.4	39.0-153.5
25	116.8	98.7-138.1	0.340	0.287-0.401	4.27	3.48-5.07	80.1	72.3-88.0	27.7	24.8-30.5	69.7	36.7-132.5
30	120.8	103.6-140.9	0.351	0.300-0.411	4.47	3.70-5.25	79.0	71.2-86.7	27.3	24.4-30.3	64.1	34.3-119.6
35	124.3	106.7-144.7	0.360	0.309-0.421	4.64	3.83-5.44	78.1	70.1-86.0	27.1	24.0-30.2	60.1	32.4-111.8
40	127.0	108.5-148.6	0.368	0.315-0.431	4.76	3.92-5.60	77.5	69.3-85.7	26.9	23.7-30.1	57.6	30.9-107.2
45	129.0	109.6-151.8	0.374	0.319-0.438	4.85	3.98-5.71	77.3	68.9-85.6	26.8	23.5-30.0	56.3	30.1-105.1
50	130.2	110.3-153.6	0.378	0.323-0.442	4.89	4.03-5.75	77.3	69.0-85.6	26.7	23.6-29.9	56.1	30.0-105.2
55	130.6	111.1-153.6	0.380	0.326-0.442	4.90	4.07-5.73	77.6	69.6-85.7	26.8	23.7-29.8	57.2	30.3-107.7
60	130.2	112.0-151.3	0.379	0.329-0.437	4.87	4.11-5.62	78.2	70.7-85.8	26.9	24.0-29.7	59.4	31.1-113.5
Full-Term												
20	120.0	102.2-140.9	0.345	0.293-0.407	4.32	3.76-4.87	80.3	74.2-86.4	27.9	25.6-30.2	45.5	25.3-82.2
25	118.9	101.9-138.8	0.345	0.296-0.402	4.38	3.84-4.92	79.0	73.1-84.9	27.3	24.9-29.6	45.2	25.1-81.5
30	118.3	101.5-137.7	0.345	0.298-0.400	4.44	3.90-4.98	78.1	72.3-83.9	26.8	24.3-29.2	45.4	26.1-78.9
35	118.0	101.5-137.3	0.346	0.300-0.400	4.48	3.93-5.03	77.6	71.8-83.4	26.5	23.9-29.0	46.0	27.6-76.6
40	118.2	102.0-137.1	0.348	0.303-0.400	4.51	3.96-5.06	77.4	71.6-83.2	26.3	23.7-28.9	47.1	28.8-76.8
45	118.9	102.9-137.3	0.351	0.307-0.401	4.54	3.99-5.08	77.6	71.7-83.5	26.3	23.6-29.0	48.7	28.7-82.5
50	120.0	104.2-138.1	0.354	0.313-0.402	4.55	4.01-5.08	78.2	72.2-84.1	26.5	23.8-29.2	50.9	26.9-96.3
55	121.5	105.4-140.0	0.359	0.319-0.403	4.55	4.04-5.06	79.1	73.1-85.1	26.9	24.2-29.6	53.7	23.9-121.0

Age, Weeks	Ferritin, μg/L		S-TfR, mg/L		CHr, pg		WBC count, × 10^9/L		Platelet count, × 10^9/L	
	Mean	95% RI	Mean	95% RI	Mean	95% RI	Mean	95% RI	Mean	95% RI
Preterm										
20	37.8	7.37-193.9	1.52	1.07-2.16	29.8	26.6-33.1	9.48	5.63-16.0	478	294-777
25	30.1	7.48-120.8	1.54	1.10-2.15	29.6	26.4-32.8	9.60	5.73-16.1	465	290-744
30	24.7	7.06-86.6	1.56	1.12-2.16	29.4	26.2-32.7	9.71	5.82-16.2	452	286-714
35	21.1	6.41-69.5	1.57	1.14-2.17	29.3	26.0-32.6	9.83	5.92-16.3	439	280-687
40	18.6	5.80-59.9	1.59	1.16-2.19	29.2	25.9-32.6	9.94	6.01-16.5	427	274-663
45	17.1	5.41-53.8	1.61	1.17-2.21	29.2	25.8-32.6	10.1	6.09-16.6	415	268-642
50	16.2	5.25-49.8	1.63	1.18-2.25	29.3	25.8-32.7	10.2	6.17-16.8	403	260-623
55	15.9	5.31-47.5	1.65	1.18-2.30	29.4	25.8-32.9	10.3	6.24-17.0	391	252-607
60	16.2	5.44-47.9	1.67	1.18-2.36	29.5	25.9-33.2	10.4	6.31-17.2	380	244-593
Full-Term										
20	71.7	21.5-239.7	1.49	1.06-2.08	29.8	27.4-32.1	9.36	5.84-15.0	426	282-646
25	51.1	16.1-162.2	1.52	1.08-2.12	29.2	25.7-32.6	9.19	5.73-14.7	415	269-640
30	38.5	11.5-128.7	1.54	1.09-2.19	28.8	24.7-32.9	9.02	5.57-14.6	404	258-634
35	30.6	8.76-107.0	1.57	1.09-2.27	28.7	24.5-32.9	8.85	5.37-14.6	394	247-628
40	25.8	7.46-89.1	1.60	1.08-2.37	28.9	25.1-32.6	8.68	5.14-14.7	383	237-621
45	22.9	7.01-75.0	1.63	1.07-2.48	29.2	26.4-32.1	8.52	4.88-14.9	373	227-614
50	21.6	6.78-68.6	1.66	1.05-2.62	29.9	27.6-32.2	8.36	4.61-15.2	363	218-607
55	21.5	5.90-78.0	1.69	1.04-2.76	30.8	27.0-34.5	8.21	4.33-15.5	354	209-599

From Takale TI, Mäkela E, Suomine P, et al: Blood Cell and iron status analytes of preterm and full-term infants from 20 weeks onward during the first year of life. Clin Chem Lab Med *48:1295–1301, 2010. Hematologic data obtained with ADVIA 120, Siemens Medical Solutions, Tarrytown, NY. S-TfR measured with an automated immunoturbidimetric methos (IDeA sTfR-IT, Orion Diagnostica), Espoo, Finland. Ferritin assayed with Elecsys ferritin electrochemiluminescence immunoassay on a Modular E analyzer (Roche Diagnostics).*

CBC, Complete blood count; *CHr*, reticulocyte hemoglobin content; *Hb*, hemoglobin; *Hct*, hematocrit; *MCH*, mean corpuscular hemoglobin; *MCV*, mean corpuscular volume; *RBC*, red blood cell; *Retic*, reticulocyte; *RI*, reference interval; *S-Tfr*, soluble transferrin receptor; *WBC*, white blood cell.

Hematocrit (%)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	31.0		36.0		39.0		39.5		32.0		37.0		40.0		41.0	
2-3	32.0		36.0		39.0		39.5		33.0		37.0		40.5		41.0	
4-6	33.0		37.0		41.0		42.0		34.0		38.0		42.0		42.5	
7-10	34.0		38.0		42.0		42.5		35.0		39.5		43.5		45.0	
11-14	35.0	33.0	40.0	38.0	45.0	43.0	46.0	43.5	36.5		42.0	40.0	47.5	44.0	48.0	45.0
15-18	38.0	32.0	44.0	38.0	49.0	42.0	51.0	43.5	40.0	34.0	46.0	40.0	50.0	43.5	51.5	44.5

Hemoglobin (g/dL)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	10.4		12.0		13.1		13.2		10.5		12.5		13.7		13.9	
2-3	10.8		12.0		13.4		13.5		11.0		12.6		13.9		13.9	
4-6	11.0		12.5		14.0		14.1		11.7		12.9		14.2		14.2	
7-10	11.2		12.7		14.1		14.4		12.0		13.5		14.7		15.0	
11-14	11.8	10.6	13.6	12.9	15.2	14.4	15.4	14.7	12.6	12.3	14.3	13.7	16.0	14.9	16.1	15.0
15-18	12.9	10.7	14.9	12.8	16.5	14.2	16.7	14.6	13.7	11.5	15.4	13.7	17.0	14.9	17.2	15.0

Red blood cell count ($10^6/mm^3$)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	4.10		4.65		5.25		5.40		4.10		4.60		5.20		5.50	
2-3	4.00		4.50		5.30		5.50		4.00		4.50		5.05		5.10	
4-6	3.90		4.50		5.30		5.40		4.00		4.50		5.05		5.20	
7-10	4.00		4.50		5.25		5.45		4.05		4.60		5.20		5.30	
11-14	4.10	3.70	4.70	4.45	5.50	5.00	5.80	5.20	4.30	4.00	4.85	4.60	5.50	5.10	5.60	5.25
15-18	4.10	3.60	5.00	4.40	5.80	5.00	5.90	5.30	4.45	3.80	5.15	4.50	5.65	4.95	5.75	5.05

Mean cell volume (fL)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	63.0		77.0		87.0		88.0		71.0		79.5		84.0		85.5	
2-3	64.0		80.0		88.0		88.5		74.0		81.5		88.5		89.0	
4-6	67.0		82.5		89.0		90.5		77.0		84.0		90.0		90.5	
7-10	72.0		84.0		91.5		92.5		78.0		85.0		90.0		90.5	
11-14	73.0	71.0	85.5		93.5		95.0		80.0		87.0		92.5		93.5	
15-18	75.0	71.0	87.0		94.5		95.5		81.0		89.0		95.0		95.5	

Mean cell hemoglobin concentration (g/dL)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	32.3		33.4		34.8		35.0		32.4		34.0		35.0		35.1	
2-3	31.7		33.6		34.9		35.0		32.6		34.1		35.4		35.7	
4-6	31.8		33.8		35.2		35.4		32.9		34.2		35.5		35.7	
7-10	31.4		33.8		35.0		35.2		32.6		34.1		35.4		35.7	
11-14	31.7		33.7		34.8		34.8		32.6		34.0		35.1		35.1	
15-18	32.0		33.4		34.6		34.8		32.5		33.9		35.1		35.2	

Mean cell hemoglobin (pg)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	20.2		25.8		29.4		29.5		23.5		27.0		29.0		29.3	
2-3	21.0		26.7		29.9		30.8		24.5		28.0		30.0		30.3	
4-6	22.0		27.7		30.7		30.8		26.0		28.8		30.0		31.0	
7-10	23.3		28.3		31.2		31.6		26.3		29.0		31.0		31.2	
11-14	24.0	22.8	29.0		31.0	32.0	32.2		26.6		29.5		31.5		31.9	
15-18	24.2	22.9	29.3		32.0		32.2		27.0		30.2		32.5		32.8	

Red cell distribution width ($10^9/L$)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	11.5		13.4		15.6		15.9		11.8		13.1		15.5		17.0	
2-3	11.6		12.7		14.8		15.2		11.4		12.4		14.1		14.6	
4-6	11.4		12.4		13.7		15.1		11.4		12.2		13.2		13.5	
7-10	11.3		12.5		13.7		14.1		11.4		12.2		13.2		13.4	
11-14	11.4		12.4		14.0		14.5	16.0	11.3		12.2		13.4		13.6	
15-18	11.2		12.5		13.5	15.9	13.6	18.3	11.4		12.2		13.2	13.8	13.6	14.4

White blood cell count ($10^3/mm^3$)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	5.0		8.0		11.5		13.2		6.0		9.5		16.5		17.5	
2-3	4.0		6.8		10.0		11.0		5.5		8.0		12.0		13.0	
4-6	4.0		6.5		9.2		10.0		5.0		7.5		11.5		12.5	
7-10	3.3		6.0		9.3		11.4		4.5		7.3		11.0		11.5	
11-14	3.3		6.0		9.0		10.0		4.5		7.0		11.0		11.5	
15-18	3.0	4.0	5.5	6.3	9.0		9.7		5.1		7.0		11.0		12.0	

Neutrophil count ($10^3/mm^3$)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	1.0		2.6		4.6		4.6		1.5		3.1		6.2		7.4	
2-3	1.0		2.4		4.8		7.5		1.5		3.3		6.3		7.5	
4-6	1.0		2.8		5.2		5.8		1.7		3.5		6.7		7.9	
7-10	1.1		2.5		5.7		6.8		1.8		3.6		6.7		7.2	
11-14	1.2		2.8		5.7		5.6	7.0	1.9		3.4	4.1	6.7	7.5	7.4	8.2
15-18	1.1	1.6	2.7	3.2	5.7		6.8	5.9	2.3		3.5	4.6	7.0	7.9	7.9	8.3

Lymphocyte count ($10^3/mm^3$)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	1.8		4.1		6.0		6.7		2.5		5.0		8.5		9.5	
2-3	1.1		3.4		5.0		5.4		5.0		3.6		6.0		6.3	
4-6	1.1		2.7		4.6		5.1		1.5		3.0		4.8		4.7	
7-10	1.3		2.3		3.7		4.0		1.5		2.7		4.0		4.5	
11-14	1.3		2.3		3.4		3.7		1.3		2.4		3.6		3.8	
15-18	1.4		2.1		3.2		3.3		1.2		2.0		3.6		3.5	

Monocyte count ($10^3/mm^3$)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	0.40		0.60		1.38		1.66		0.45		0.80		1.40		1.50	
2-3	0.30		0.60		0.92		1.03		0.35		0.60		0.95		0.95	
4-6	0.30		0.50		0.90		0.90		0.35		0.60		0.95		1.10	
7-10	0.20		0.50		0.90		1.00		0.30		0.60		0.90		0.90	
11-14	0.25		0.50		0.80		0.85		0.30		0.55		0.90		0.97	
15-18	0.30		0.50		0.88		0.95		0.35		0.55		0.87		0.90	

Eosinophil ($10^3/mm^3$)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	0.10		0.30		1.00		1.10		0.10		0.20		0.70		0.80	
2-3	0.00		0.20		0.70		0.90		0.10		0.20		0.75		0.90	
4-6	0.05		0.30		0.80		0.95		0.05		0.25		0.75		0.80	
7-10	0.10		0.20		0.90		1.00		0.10		0.20		0.75		1.00	
11-14	0.00		0.20		0.55		0.60		0.05		0.15		0.70		0.80	0.70
15-18	0.00		0.15		0.45		0.55		0.05		0.15		0.55	0.40	0.50	

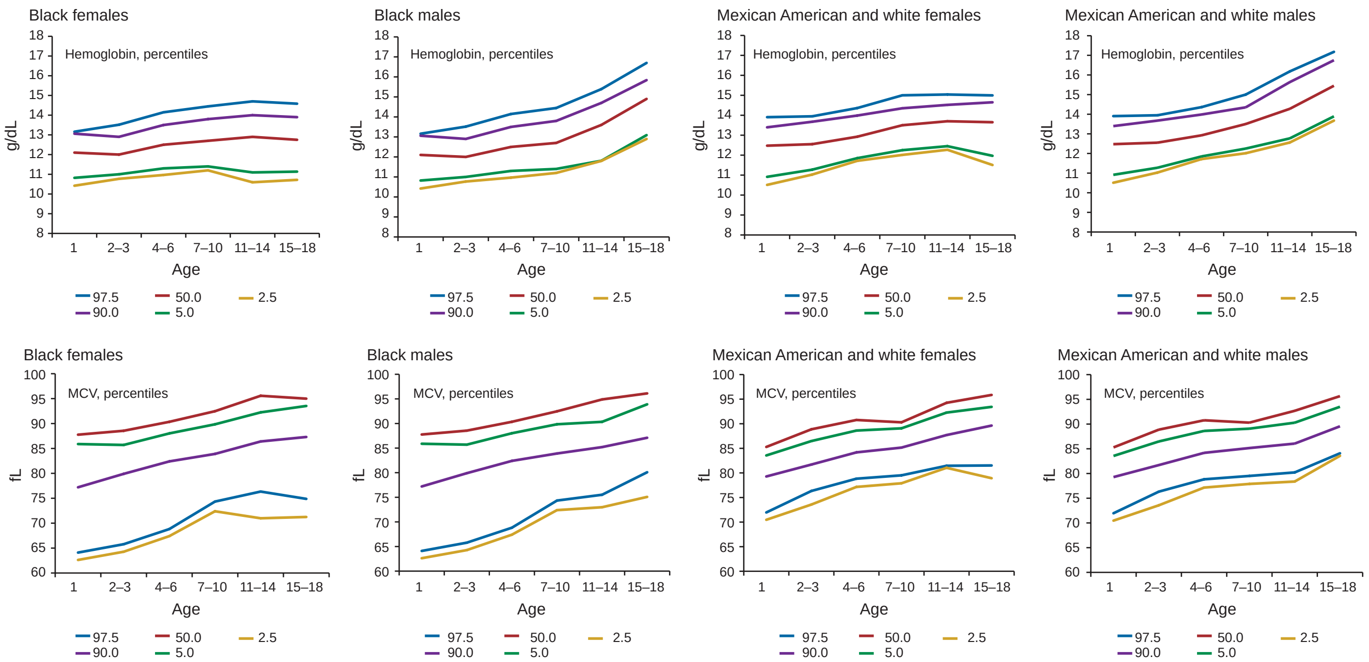
Platelet count ($10^3/mm^3$)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	225		330		475		480		235		375		510		555	
2-3	220		335		480		490		225		335		470		530	
4-6	220		315		440		470		225		325		440		460	
7-10	210		310		420		435		200		295		410		435	
11-14	180		275		380	410	410	430	190		280		495		420	
15-18	165	185	245	265	340	375	360	400	180		270		345	400	360	420

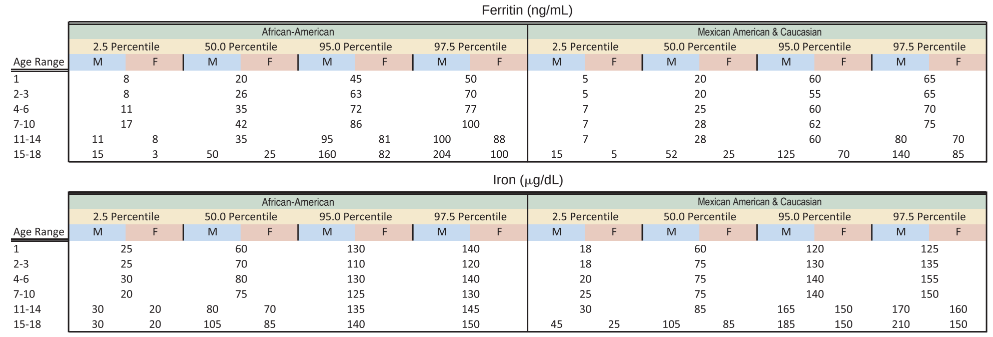
Mean platelet volume (fL)

	African-American								Mexican American & White							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	6.3		7.6		8.8		9.0		6.3		7.2		8.0		8.4	
2-3	6.5		7.5		8.7		8.8		6.2		7.3		8.6		8.8	
4-6	6.4		7.7		9.2		9.6		6.5		7.5		8.6		8.9	
7-10	6.7		8.1		9.1		9.4		6.6		7.8		9.2		9.6	
11-14	6.8		8.2		9.7		10.3		6.7		8.1		9.5		9.7	
15-18	7.0		8.2		10.1		10.8		6.9		8.2		9.9		10.2	

Appendix 13 *From Cembrowski GS, Chan J, Cheng C, Bamforth FJ. NHANES 1999-2000 Data used to create comprehensive health-associated race-, sex-, and age-stratified pediatric reference intervals for the Coulter MAXM.* Lab Hematol *10:245–246, 2004. Presented at the annual meeting of the International Society for Laboratory Hematology, Barcelona, Spain, May 2004. Numerical summary kindly provided by GS Cembrowski, MS Cembrowski, KA Versluys, 2013. Original Graphs at* http://www.mylaboratoryquality.com/hematologyri1x.htm.



Appendix 14 Hb and MCV percentile curves for African American and Mexican American/Caucasian girls and boys. *Top row,* Hb percentile curves for African American girls and boys and for Mexican American and Caucasian girls and boys. *Bottom row,* MCV percentile curves for African American girls and boys and for Mexican American and Caucasian girls and boys. *(From Cembrowski GS, Chan J, Cheng C, Bamforth FJ: NHANES 1999-2000 Data used to create comprehensive health-associated race-, sex-, and age-stratified pediatric reference intervals for the Coulter MAXM.* Lab Hematol *10:245–246, 2004. Presented at the annual meeting of the International Society for Laboratory Hematology, Barcelona, Spain, May 2004. Numerical summary kindly provided by GS Cembrowski, MS Cembrowski, KA Versluys, 2013. Original Graphs at* http://www.mylaboratoryquality.com/hematologyri1x.htm.*)*



Ferritin (ng/mL)

	African-American								Mexican American & Caucasian							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	8		20		45		50		5		20		60		65	
2-3	8		26		63		70		5		20		55		65	
4-6	11		35		72		77		7		25		60		70	
7-10	17		42		86		100		7		28		62		75	
11-14	11	8	35		95	81	100	88	7		28		60		80	70
15-18	15	3	50	25	160	82	204	100	15	5	52	25	125	70	140	85

Iron (µg/dL)

	African-American								Mexican American & Caucasian							
	2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile		2.5 Percentile		50.0 Percentile		95.0 Percentile		97.5 Percentile	
Age Range	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	25		60		130		140		18		60		120		125	
2-3	25		70		110		120		18		75		130		135	
4-6	30		80		130		140		20		75		140		155	
7-10	20		75		125		130		25		75		140		150	
11-14	30	20	80	70	135		145		30		85		165	150	170	160
15-18	30	20	105	85	140		150		45	25	105	85	185	150	210	150

Appendix 15 *From Cembrowski GS, Chan J, Cheng C, Bamforth FJ. NHANES 1999-2000 Data used to create comprehensive health-associated race-, sex-, and age-stratified pediatric reference intervals for the Coulter MAXM.* Lab Hematol *10:245–246, 2004. Presented at the annual meeting of the International Society for Laboratory Hematology, Barcelona, Spain, May 2004. Numerical summary kindly provided by GS Cembrowski, MS Cembrowski, KA Versluys, 2013. Original Graphs at* http://www.mylaboratoryquality.com/hematologyri1x.htm.

APPENDIX 16 Reference Ranges for WBC, Hb, Hct, and MCV in African-American Children and Adolescents[*]

		African-American [Hb (g/dL)]		African-American [Hct (%)]		African-American [MCV (fl)]	
Age (years)	African American (mean WBC)	Mean	–2 STD	Mean	–2 STD	Mean	–2 STD
2-5	7.33	11.94	10.37	35.98	31.54	79.81	74.65
6-10	6.74	12.44	10.3	37.27	32.71	81.44	76.45
11-15 male	6.32	13.2	10.97	39.29	32.91	83.20	77.99
11-15 female	6.58	12.67	10.1	37.95	33.41	83.60	78.01
16-18 male	5.91	14.35	11.30	43.50	37.62	86.02	80.85
16-18 female	6.92	12.55	10.27	37.39	31.87	85.26	79.43

From Robins EB, Blum S: Hematologic reference values for African American children and adolescents. Am J Hematol *82:611–614, 2007.*
Hb, Hemoglobin; *Hct,* hematocrit; *MCV,* mean corpuscular volume; *STD,* standard deviation; *WBC,* white blood cell.
[*] Sample size is 5039. Mean values obtained from NHANES (National Health and Nutrition Examination Survey) and Robins studies.

APPENDIX 17 WBC and ANC Mean Values and Reference Intervals[*]

	12-18 Years: WBC		>18 Years: WBC		12-18 Years: ANC		>18 Years: ANC	
	N		*N*		*N*		*N*	
NHBM	401	3.2-9.3 (5.6)	547	3.1-9.9 (6.1)	401	1.0-6.2 (2.9)	547	1.3-6.6 (3.1)
MAM	493	4.1-11.4 (6.9)	776	4.1-11.4 (7.1)	492	1.8-8.2 (4.1)	776	2.1-7.9 (4.2)
NHWM	342	4.1-11.4 (6.8)	1473	4.1-11.8 (7.0)	342	1.8-7.2 (3.8)	1471	2.1-8.0 (4.3)
MAM, NHWM	835	4.1-11.4 (6.8)	2249	4.1-11.6 (7.1)	834	[†]	2247	2.1-8.0 (4.3)
NHBF	329	3.7-10.1 (6.2)	344	3.4-11 (6.3)	329	1.2-6.6 (3.1)	344	1.4-7.5 (3.4)
MAF	401	4.1-12.0 (7.6)	552	4.5-11.8 (7.5)	401	1.9-8.4 (5.3)	550	2.2-8.0 (4.9)
NHWF	322	4.1-11.3 (7.2)	1222	3.9-11.6 (7.0)	322	1.9-7.7 (4.0)	1221	2.0-7.9 (4.7)
MAF, NHWF	723	[†]	1774	[†]	723	[†]	1771	2.0-7.9 (4.8)

From Lim EM, Cembrowski G, Cembrowski M, et al: Race-specific WBC and neutrophil count reference intervals. J Lab Clin Hematol *32:590–597, 2010.*
ANC, Absolute neutrophil count; *MAF,* female Mexican American; *MAM,* male Mexican American; *NHBF,* female non-Hispanic black; *NHBM,* male non-Hispanic black; *NHWF,* female non-Hispanic white; *NHWM,* male non-Hispanic white; *WBC,* white blood cell.
[*] Data are presented as reference intervals (2.5-97.5 percentile limits) and mean values (in parentheses).
[†] Could not be pooled because of variation within and between populations.

APPENDIX 18 Activities of RBC Enzymes

Enzyme	**Activity at 37° C (mean ± SD)**
Acetylcholinesterase	36.93 ± 3.83
Adenosine deaminase	1.11 ± 0.23
Adenylate kinase	258 ± 29.3
Aldolase	3.19 ± 0.86
Bisphosphoglyceromutase (2,3- diphosphoglyceromutase)	4.78 ± 0.65
Catalase	$15.3 \pm 2.4 \times 10^4$
Enolase	5.39 ± 0.83
Epimerase	0.23 ± 0.06
Galactokinase	0.029 ± 0.004
Galactose-1-phosphate uridyltransferase	28.4 ± 6.94
Glucose phosphate isomerase	60.8 ± 11.0
Glucose-6-phosphate dehydrogenase	8.34 ± 1.59
WHO method	12.1 ± 2.09
Glutamic oxaloacetic transaminase without PLP	3.02 ± 0.67
Glutamic oxaloacetic transaminase with PLP	5.04 ± 0.90
γ-Glutamylcysteine synthetase	0.43 ± 0.04
Glutathione peroxidase	30.82 ± 4.65
Glutathione reductase without FAD	7.18 ± 1.09
Glutathione reductase with FAD	10.4 ± 1.50
Glutathione S-transferase	6.66 ± 1.81
Glyceraldehyde phosphate dehydrogenase	226 ± 41.9
Hexokinase	1.78 ± 0.38
Hypoxanthine phosphoribosyltransferase	1.72 ± 0.30
Lactate dehydrogenase	200 ± 26.5
Methemoglobin reductase	2.60 ± 0.71
Monophosphogylceromutase	37.71 ± 5.56
NADH-methemoglobin reductase	19.2 ± 3.85 (30°)
NADPH diaphorase	2.26 ± 0.16
Phosphofructokinase	11.01 ± 2.33
Phosphoglucomutase	5.50 ± 0.62
Phosphoglycerate kinase	320 ± 36.1
Phosphoglycolate phosphatase	1.23 ± 0.10
Pyrimidine 5' nucleotidase	0.11 ± 0.03
Pyruvate kinase	15.0 ± 1.99
6-Phosphogluconate dehydrogenase	8.78 ± 0.78
Triose phosphate isomerase	2111 ± 397

From Beutler E, Blum KG: In Altman PL, Dittmer DS, editors: Human health and disease. *Bethesda, Md., Federation of American Societies for Experimental Biology, 1977, p. 156; and Beutler E:* Red cell metabolism: a manual of biochemical methods, *3rd ed, Orlando, Fla., Grune & Stratton, 1984.*

FAD, Flavin adenine dinucleotide; *NADH,* reduced nicotinamide adenine dinucleotide; *NADPH,* reduced nicotinamide adenine dinucleotide phosphate; *PLP,* pyridoxal 5-phosphate; *SD,* standard deviation; *WHO,* World Health Organization.

APPENDIX 19 Levels of Intermediate Metabolites in Normal Adult Erythrocytes

		CONCENTRATION (mean ± 1 SD)		
Metabolite	**Abbreviation**	**nmol/g Hb**	**nmol/mL Red Cells**	**µmol/L in Whole Blood**
Adenosine-5′-diphosphate	ADP	6635 ± 105	216 ± 36	—
Adenosine-5-monophosphate	AMP	62 ± 10	21.1 ± 3.4	—
Adenosine-5-triphosphate	ATP	4230 ± 290 (whites) 3530 ± 301 (blacks)	1438 ± 99 1200 ± 102	—
2,3-Diphosphoglycerate	2,3-DPG	1227 ± 1870	4171 ± 636	—
Glutathione	GSH	6570 ± 1040	2234 ± 354	—
Glutathione (oxidized)	GSSG	12.3 ± 4.5	4.2 ± 1.53	—
Glucose-6-phosphate	G6P	82 ± 22	27.8 ± 7.5	—
Fructose-6-phosphate	F6P	27 ± 5.8	9.3 ± 2.0	—
Fructose-6-diphosphate	FDP	5.6 ± 1.8	1.9 ± 0.6	—
Dihydroxyacetone phosphate	DHAP	27.6 ± 8.2	9.4 ± 2.8	—
3-Phosphoglyceric acid	3-PGA	132 ± 15.0	44.9 ± 5.1	—
2-Phosphoglyceric acid	2-PGA	21.5 ± 7.35	7.3 ± 2.5	—
Phosphoenolpyruvate	PEP	35.9 ± 6.47	12.2 ± 2.2	—
Creatine		1310 ± 310 (male) 1500 ± 250 (female)	445 ± 105 510 ± 85	—
Lactate		—	—	932 ± 211
Pyruvate		—	—	53.3 ± 21.5

From Beutler E: Red cell metabolism: a manual of biochemical methods, *3rd ed. Orlando, Fla., 1984, Grune & Stratton.*
SD, Standard deviation.

APPENDIX 20 Red Cell Enzyme Activity in Premature and Term Neonates, Adults, and Adult Reticulocytes

	Premature Infants (N = 11)	Term Infants (N = 10)	Normal Adults (N = 50)	95% Adult* Reticulocytes
Hexokinase (HK)	3.6 ± 0.6[a]	2.7 ± 0.3[a]	1.15 ± 0.35	9.9[a]
Glucosephosphate isomerase (PGI)	94.5 ± 15.2[a]	88 ± 16[a]	67.7 ± 12.0	95.7[c]
Phosphofructokinase (PFK)	8.0 ± 1.3[d]	7.1 ± 1.5[c]	8.4 ± 3.4	14.1[d]
Aldolase (ALD)	4.3 ± 0.8[c]	5.3 ± 1.7[b]	3.55 ± 1.07	9.5[a]
Triosephosphate isomerase (TIM)	2961 ± 1378[d]	1787 ± 380[d]	2700 ± 700	3220[d]
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	131 ± 76[d]	164 ± 45[d]	134 ± 93	240[d]
Phosphoglycerate mutase (PGK)	591 ± 114[b]	436 ± 119[b]	312 ± 86	438[d]
Diphosphoglycerate mutase (DPGM)	8.3 ± 3.6[c]	6.7 ± 1.9[c]	5.3 ± 2.5	7.3[d]
Enolase (ENO)	15.1 ± 3.4[a]	14.6 ± 2.1[a]	7.8 ± 2.2	8.1[d]
Pyruvate kinase (PK)	19.4 ± 3.5[a]	17.3 ± 2.5[a]	11.4 ± 2.8	54.2[a]
Glucose-6-phosphate dehydrogenase (G6PD)	21.3 ± 3.8[a]	19.1 ± 4.3[a]	10.9 ± 3.8	30.6[a]
6-Phosphogluconate dehydrogenase (6PGD)	9.1 ± 2.1[d]	9.9 ± 1.6[c]	8.4 ± 1.0	14.4[a]
Glutathione reductase (GR)	9.5 ± 1.7[a]	11.0 ± 2.6[a]	7.2 ± 2.2	11.3[c]
Acetyl choline esterase (ACE)	17.5 ± 3.5[a]	18.5 ± 4.1[a]	36.9 ± 3.8	44.4[c]

From Jansen G, Koenderman L, Rijksen G, et al: Characteristics of hexokinase, pyruvate kinase, and glucose-6-phosphate dehydrogenase during adult and neonatal reticulocyte maturation. Am J Hematol *20:203–215, 1985.*

*Obtained after Percoll density gradient centrifugation of red cells from a patient with a hemolytic syndrome. Statistical interpretation versus normal adult group: [a]*P* <.001; [b]*P* <.01; [c]*P* <.1; [d]not significant.

APPENDIX 21 Red Cell Glycolytic Intermediate Metabolites in Normal Adults, Term Infants, and Premature Infants*

Metabolite	**Normal Adults (10)**	**Term Infants (10)**	**Premature Infants (11)**	**Normals (5)**
Glucose-6-phosphate	24.8 ± 9.8	45.2 ± 8.7	66.8 ± 34.8	27 ± 2.4
Fructose-6-phosphate	5.4 ± 1.0	9.9 ± 2.3	20.5 ± 8.9	11 ± 2.5
Fructose, 1,6-diphosphate	4.6 ± 1.0	3.8 ± 0.7	3.6 ± 0.8	5 ± 0.9
Dihydroxyacetone phosphate	4.9 ± 3.5	11.9 ± 5.0	18.6 ± 10.7	12 ± 3.7
Glyceraldehyde-3-phosphate	2.6 ± 0.7	1.9 ±1.6	6.5 ± 3.2	4 ± 1.5
3-Phosphoglycerate	61.6 ± 12.4	58.2 ± 14.4	47.5 ± 14.2	48 ± 16.1
2-Phosphoglycerate	4.3 ± 1.8	4.9 ± 1.6	4.4 ± 2.5	7 ± 1.7
Phosphoenolpyruvate	8.8 ± 2.6	7.6 ± 2.9	7.4 ± 3.0	12 ± 0.9
Pyruvate	73.5 ± 33.1	70.4 ± 32.3	78.4 ± 4.15	71 ± 17.7
2,3-Diphosphoglycerate	4423 ± 1907	3609 ± 800	3152 ± 2133	4000

From Oski FA: Red cell metabolism in the newborn infant: V. Glycolytic intermediates and glycolytic enzymes. Pediatrics *44:87, 1969.*

*Infant samples were obtained from newborns weighing more than 2800 g whose gestational age was 39 weeks or greater. Blood was drawn within 24 hours of birth. All the newborns were clinically healthy. Adult samples were obtained from healthy normal volunteers.

APPENDIX 22 RBC Enzyme Activity* in ELBWI Infants and Premature and Normal Adults (Whole Blood and Reticulocyte Rich)

	Normal Adult (N = 28) Mean ± SD	**ELBWI (N = 28) Mean ± SD**	**Reticulocyte Rich (N = 20) Mean ± SD**	**ELBWI Versus Reticulocyte Rich**	**ELBWI Versus Normal Adults**
Hexokinase (HK)	1.05 ± 0.11	3.28 ± 0.47	3.45 ± 1.78	NS	High (*P* <.0001)
Glucosephosphate isomerase (GPI)	58.4 ± 5.0	83.4 ± 6.2	65.2 ± 8.5	High (*P* <.0001)	High (*P* <.0001)
Phosphofructokinase (PFK)	13.3 ± 1.7	10.1 ± 1.6	15.8 ± 2.4	Low (*P* <.0001)	Low (*P* <.0001)
Aldolase (ALD)	3.09 ± 0.53	4.73 ± 5.13	5.12 ± 0.94	NS	High (*P* <.0001)
Triosephosphate isomerase (TPI)	1760 ± 253	2215 ± 339	2077 ± 375	NS	High (*P* <.0001)
Glyceraldehyde-3-phosphate dehydrogenase (GA3PD)	191 ± 34	252 ± 39	232 ± 51	NS	High (*P* <.0001)
Phosphoglycerate kinase (PGK)	288 ± 31	469 ± 67	355 ± 43	High (*P* <.0001)	High (*P* <.0001)
Monophosphoglycerate mutase (MPGM)	23.5 ± 2.8	33.8 ± 6.9	29.9 ± 5.3	High (*P* = .0412)	High (*P* <.0001)
Enolase (ENL)	6.9 ± 1.0	23.9 ± 3.0	9.3 ± 1.7	High (*P* <.0001)	High (*P* <.0001)
Pyruvate kinase (PK)	16.3 ± 2.1	29.7 ± 4.0	27.6 ± 8.1	NS	High (*P* <.0001)
Lactate dehydrogenase (LDH)	195 ± 17	248 ± 28	264 ± 34	NS	High (*P* <.0001)
Glucose-6-phosphate dehydrogenase (G6PD)	7.6 ± 0.8	18.1 ± 2.2	12.8 ± 2.0	High (*P* <.0001)	High (*P* <.0001)
6-Phosphogluconate dehydrogenase (6PGD)	8.1 ± 1.0	8.2 ± 1.5	13.2 ± 1.8	Low (*P* <.0001)	NS
Glutathione reductase (GR)	7.1 ± 1.2	9.2 ± 1.8	7.8 ± 1.8	High (*P* = .0090)	High (*P* <.0001)
GR + flavin adenine dinucleotide (FAD)	9.9 ± 1.6[b]	10.9 ± 1.7[b]	10.4 ± 1.7	NS	NS
Glutathione peroxidase (GSH-Px)	31.8 ± 5.7	22.5 ± 4.0	41.3 ± 7.4	Low (*P* <.0001)	Low (*P* <.0001)
Adenylate kinase (AK)	252 ± 35	132 ± 18	253 ± 26	Low (*P* <.0001)	Low (*P* <.0001)
Adenosine deaminase (ADA)	1.25 ± 0.33	0.96 ± 0.30	1.60 ± 0.60	Low (*P* <.0001)	Low (*P* = .0010)
Acetylcholinesterase (AchE)	31.7 ± 4.7	16.7 ± 3.3	37.6 ± 10.1	Low (*P* <.0001)	Low (*P* <.0001)
Pyrimidine 5'-nucleotidase	9.1 ± 1.4	17.0 ± 4.0	19.1 ± 4.5	ns	High (*P* <.0001)
NADH methemoglobin reductase (MetHbR)	14.9 ± 1.8[b]	11.5 ± 2.1[a]	14.1 ± 2.3[a]	Low (*P* = .0087)	Low (*P* <.0001)
Catalase (CAT)	13.6 ± 2.1[b]	11.8 ± 2.7[a]	15.6 ± 3.2[a]	Low (*P* = .0048)	Low (*P* = .0459)
Reduced glutathione (GSH) (mg/dL RBC)	67.8 ± 9.3	76.9 ± 13.8	69.4 ± 12.0	NS	High (*P* = .0059)

From Miyazono Y, Hirono A, Miyamoto Y, et al: Erythrocyte enzyme activities in cord blood of extremely low-birth-weight infants. Am J Hematol *62:88–92, 1999.*

ELBWI, Extremely low-birth-weight infants (birth weight <1000 g); *Hb*, hemoglobin; *NS*, not significant; *RBC*, red blood cell; *SD*, standard deviation.

*All enzyme activities are represented as units/g Hb, except for CAT (10^4 units/g Hb) and P5N (μmol Pi liberated/hr/g Hb). [a]N = 12; [b]N = 20.

APPENDIX 23 G6PD Reference Values for G6PD-Normal and –Deficient Males and Females with Probable Genotypes

Category (U/g Hb)	No.	Median Value	IQR	Min-Max	G6PD Phenotype	Probable Genotype*†
Male (N = 1502)						
<7.0	243 (16.2%)	0.28	0.21-0.39	0.01-6.20	Deficient	Deficient hemizygote
≥9.0	1256 (83.8%)	18.76	17.27-21.68	9.00-34.66	Normal	Normal hemizygote
Female (N = 1298)						
<7.0	81 (6.2%)	4.84	0.45-6.16	0.06-6.96	Deficient	Deficient homozygote
7.0-9.4	64 (4.9%)	8.61	7.67-9.00	7.04-9.46	Intermediate	Heterozygote
≥9.5	1153 (88.8%)	18.36	16.21-20.63	9.54-35.50	Normal	Normal homozygote

*In females, because of nonrandom X chromosome inactivation among heterozygotes, accurate genotype categories may be difficult to determine based on phenotype. The term “probable” genotype is therefore used.

†Male genotype based on phenotype may be assumed with reasonable accuracy.

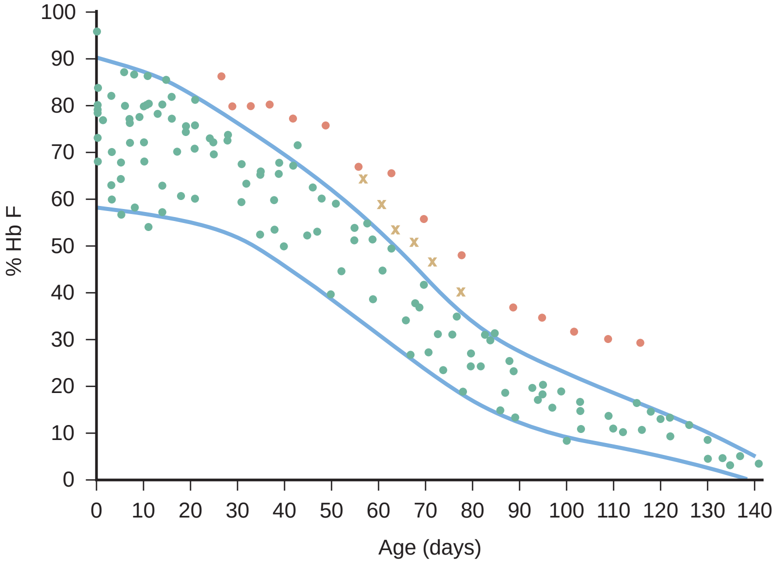
APPENDIX 24 Hemoglobin A Content in Male and Female Newborns According to Birth Weight

Birth Weight (g)	Males Hb A (%)	Females Hb A (%)
<1501	7.1 ± 1.3 (9)	13.2 ± 11.3 (14)
1501-2000	12.2 ± 8.2 (36)	13.1 ± 10.4 (44)
2001-2500	12.6 ± 5.2 (139)	14.9 ± 6.5 (206)*
2501-3000	15.8 ± 6.4 (635)	17.8 ± 6.2 (776)*
3001-3500	18.2 ± 6.5 (1289)	19.9 ± 6.8 (1204)*
3501-4000	20.0 ± 6.6 (803)	20.9 ± 7.2 (590)*
4001-4500	19.7 ± 6.3 (200)	22.6 ± 8.6 (94)*
>4500	21.7 ± 7.0 (45)	20.9 ± 7.2 (20)

From Galacteros F, Guilloud-Bataille M, Feingold J: Sex, gestational age, and weight dependency of adult hemoglobin concentration in normal newborns. Blood *78:1121, 1991.*

HbA, Hemoglobin A.

**P* <.05 between male and female newborns.



Appendix 25 Relative concentration of hemoglobin F in infants and its variation with age. The region between the curved lines contains 120 observations in 17 normal children. *(From Garby L, Sjolin S. Development of erythropoiesis.* Acta Paediatr *51:245, 1962.)*

APPENDIX 26 Percentage of Hemoglobins F and A2 in the Newborn and Adult*

	% Hb F (Gα:Aα ratio)	% Hb A2
Newborn	60-90 (3:1)	<1.0
Adult	<1.0 (2:3)	1.6-3.5

From Altman PL, Dittmer DS, editors: Human health and disease. *Bethesda, MD, 1977, Federation of American Societies for Experimental Biology, p. 159.*

Hb, Hemoglobin.

*The α chains of fetal hemoglobin contain either a glycyl residue or an alanyl residue at position 136. The Gα:Aα ratio in the newborn undergoes a considerable change between the third and fourth months of life, at which time it approximates that of the Hb F of adults.

APPENDIX 27 Methemoglobin Levels in Normal Children*

	METHEMOGLOBIN (g/dL)					METHEMOGLOBIN AS PERCENTAGE OF TOTAL HEMOGLOBIN				
	No. Cases	**No. Det.**	**Mean**	**Range**	**Standard Dev.**	**No. Cases**	**No. Det.**	**Mean**	**Range**	**Standard Dev.**
Prematures (birth-7 days)	29	34	0.43	(0.02-0.83)	±0.07	24	28	2.3	(0.08-4.4)	±1.26
Prematures (7-72 days)	21	29	0.31	(0.02-0.78)	±0.19	18	23	2.2	(0.02-4.7)	±1.07
Prematures (total)	50	63	0.38	(0.02-0.83)	±0.10	42	51	2.2	(0.08-4.7)	±1.10
Cook County Hospital, prematures (1-14 days)	8	8	0.52	(0.18-0.83)	±0.08	—	—	—	—	—
Newborns (1-10 days)	39	39	0.22	(0.00-0.58)	±0.17	25	30	1.5	(0.00-2.8)	±0.81
Infants (1 month-1 year)	8	8	0.14	(0.02-0.29)	±0.09	8	8	1.2	(0.17-2.4)	±0.78
Children (1-14 years)	35	35	0.11	(0.00-0.33)	±0.09	35	35	0.79	(0.00-2.4)	±0.62
Adults (14-78 years)	30	30	0.11	(0.00-0.28)	±0.09	27	27	0.82	(0.00-1.9)	±0.63

From Kravitz H, Elegant LD, Kaiser E, et al: Methemoglobin values in premature and mature infants and children. Am J Dis Child *91:2, 1956.*

*The premature and full-term infants were free of known disease. None had respiratory distress or cyanosis. Analysis of milk and water ingested by these infants revealed a nitrate level less than 0.027 ppm. The premature infants routinely received vitamin C orally each day from the seventh day of life.

APPENDIX 28 Hemoglobin, Serum Transferrin Saturation, Serum Ferritin*, and Red Cell Indices in Adolescents (15-16 Years of Age)†

		PERCENTILE				
	Mean ± SD	**10th**	**25th**	**50th**	**75th**	**90th**
Boys						
Hb (g/dL)	14.7 ± 0.831	135	14.2	14.7	15.2	15.7
Transferrin saturation (%)	32.7 ± 10.25	21.2	25.7	31.7	38.6	46.3
Serum ferritin (μg/L)	26.4 ± 17.71	13.2	22	29	40.8	52
MCV (fL)	88.4 ± 3.87	83.5	85.5	88.1	90.9	93.2
RDW (%)	13.0 ± 0.95	12.3	12.5	12.9	13.2	13.5
MCH (pg)	29.4 ± 1.43	27.7	28.6	29.2	30.2	31.0
Girls						
Hb (g/dL)	13.4 ± 0.763	12.3	12.9	13.4	13.9	14.3
Transferrin saturation (%)	29.9 ± 10. 7	17.2	22.8	29	35.2	43.4
Serum ferritin (μg/L)	18.2 ± 1.98	7.5	12	18.2	28.5	41.5
MCV (fL)	90.1 ± 3.86	84.8	87.6	90.3	92.8	94.8
RDW (%)	12.8 ± 0.71	12.1	12.4	12.7	13.2	13.6
MCH (pg)	29.6 ± 1.43	27.8	28.8	29.5	30.6	31.3

From Hallberg L, Hulten L, Lindstedt G, et al: Prevalence of iron deficiency in Swedish adolescents. Pediatr Res *34:680–687, 1993.*

Hb, Hemoglobin; *MCH,* mean corpuscular hemoglobin; *MCV,* mean corpuscular volume; *RDW,* red blood cell volume distribution width; *SD,* standard deviation.

*Data for serum ferritin are presented as geometric means ± antilog of logarithmic values.

†Data collected in 197 to 207 boys and 215 to 220 girls, from ages 15 to 16.

APPENDIX 29 Hemoglobin and Iron Status Indicators According to Age

	2 m			6 m			9 m		
	Mean ± SD	**5-95 Percentiles**	**N**	**Mean ± SD**	**5-95 Percentiles**	**N**	**Mean ± SD**	**5-95 Percentiles**	**N**
Hemoglobin									
(g/L)	115 ± 8	101-130	68	116 ± 9	105-130	57	117 ± 7	103-129	94
(mmol/L)	7.1 ± 0.5	6.3-8.1		7.2 ± 0.6	6.5-8.1		7.3 ± 0.5	6.4-8	
Serum ferritin* (µg/L)	301	134-675	63	59	23-140	54	37	17-78	84
Erythrocyte protoporphyrin (nmol/l erythrocytes)	679 ± 185†	479-1148	65	475 ± 109	304-659	48	556 ± 133†	369-763	83
Serum transferrin (µmol/L)	26 ± 4†	20-33	65	31 ± 4	24-38	57	35 ± 7†	26-47	90
Serum iron (µmol/L)	13.1 ± 3.5†	7.5-19.1	59	9.9 ± 3.1	6.3-17.3	46	8.8 ± 3.4	3.9-14.9	80
Transferrin saturation (%)	26 ± 8†	15-40	59	16 ± 6	10-29	46	13 ± 6	5-25	77
Mean red cell volume (fl)	86 ± 5†	78-94	64	76 ± 4	69-82	49	76 ± 6	68-85	89

From Michaelsen KF, Milman N, Samuelson G: A longitudinal-study of iron status in healthy Danish infants—effects of early iron status, growth velocity and dietary factors. Acta Paediatr *84:1035–44, 1995.*

m, Months; *SD*, standard deviation.

*Geometric mean values.

†Significantly different (*P* <.001) from values at 6 months (Student's paired *t*-test).

APPENDIX 30 Serum Ferritin, Iron, Total Iron-Binding Capacity, and Transferrin Saturation in Children and Adolescents

Age	**Male Subjects**		**Female Subjects**	
Ferritin	**ng/mL**	**µg/L**	**ng/mL**	**µg/L**
1-30 d*	6-400	6-400	6-515	6-515
1-6 m*	6-410	6-410	6-340	6-340
7-12 m*	6-80	6-80	6-45	6-45
1-5 y†‡	6-24	6-24	6-24	6-24
6-9 y†‡	10-55	10-55	10-55	10-55
10-14 y‡	23-70	23-70	6-40	6-40
14-19 y‡	23-70	23-70	6-40	6-40
Iron	**µg/dL**	**µmol/L**	**µg/dL**	**µmol/L**
1-5 y†‡	22-136	4-25	22-136	4-25
6-9 y†‡	39-136	7-25	39-136	7-25
10-14 y‡	28-134	5-24	45-145	8-26
14-19 y‡	34-162	6-29	28-184	5-33
Iron-Binding Capacity				
1-5 y†‡	268-441	48-79	268-441	48-79
6-9 y†‡	240-508	43-91	240-508	43-91
10-14 y‡	302-508	54-91	318-575	57-103
14-19 y‡	290-570	52-102	302-564	52-101

APPENDIX 30 Serum Ferritin, Iron, Total Iron-Binding Capacity, and Transferrin Saturation in Children and Adolescents (Continued)

Age	Male Subjects		Female Subjects
Transferrin Saturation			
1-5 y[†‡]	0.07-0.44		0.07-0.44
6-9 y[†‡]	0.17-0.42		0.17-0.42
10-14 y[‡]	0.11-0.36		0.02-0.40
14-19 y[‡]	0.06-0.33		0.06-0.33
Transferrin		**U/L (Males and Females)**	
0-5 d[§]		1.43-4.46	
1-3 y[§]		2.18-3.47	
4-6 y[§]		2.08-3.78	
7-9 y[§]		2.25-3.61	
10-13 y[§]		2.24-4.42	
14-19 y[§]		2.33-4.44	

d, Days; *m*, months; *y*, years.

[*] Soldin SJ, Morales A, Albalos F, et al: Pediatric reference ranges on the Abbott IMx for FSH, LH, prolactin, TSH, T_4, T_3, free T3, T-uptake, IgE, and ferritin. *Clin Biochem* 28:603, 1995. Study based on hospitalized patients; values represent 2.5th and 97.5th percentiles.

[†] No significant differences between males and females; range derived from combined data.

[‡] Lockitch, G; Halstead, AC; Wadsworth, et al: Age- and sex-specific pediatric reference intervals and correlations for zinc, copper, selenium, iron, vitamins A and E, and related proteins. *Clin Chem* 34:1625, 1988. Study based on healthy children; values represent the 0.025 and 0.975 fractiles. Transferrin saturation calculated from iron (µmol/L)/total iron binding capacity. Note that the lower reference limits for serum iron and transferrin saturation in this study are below the limits used to define acceptable levels for these two analytes (O'Neal RM, Johnson OC, Schaefer AE: Guidelines for classification and interpretation of group blood and urine data collected as part of the National Nutrition Survey. *Pediatr Res* 4:103, 1970.)

[§] Lockitch, G; Halstead, AC; Quigley G, et al: Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. *Clin Chem* 34:1618, 1988. Results are 2.5th and 97.5 percentiles.

APPENDIX 31 Reference Ranges for Erythrocyte Zinc Protoporphyrin in Children and Adolescents

	Blood ZPP Reference Ranges 2.5th–97.5th Percentiles (µg/dL)[*]		**Blood ZPP Reference Ranges 2.5th–97.5th Percentiles (µmol/mol heme)**		**N**	
Age Groups	Males	Females	Males	Females	Males	Females
0-12 months	8.5-34.5	9-40	15.6-63.5	16.6-73.6	145	203
13-24 months	10-34	11-32	18.4-62.6	20.2-58.9	605	725
2-5 years	5-35	10-31	9.2-64.4	18.4-57.0	1926	1822
6-9 years	6-31	9-30	11.0-57.0	16.6-55.2	522	408
10-17 years	5.5-31.5	5-33.5	10.1-58.0	9.2-61.6	61	61
Total					3259	3219

From Soldin OP, Miller M, Soldin SJ: Pediatric reference ranges for zinc protoporphyrin. Clin Biochem *36:21–25, 2003.*

ZPP, Zinc protoporphyrin.

[*] Multiply µg/dL by 1.83 to convert to µmol/mol heme.

APPENDIX 32 Reference Ranges for Serum Transferrin Receptor in Children and Adolescents*

Age Groups	2.5% Reference Limit mg/L	97.5% Reference Limit mg/L
6 months to 4 years	1.5 (1.4-1.5)	3.3 (3.1-3.4)
4 to 10 years	1.3 (1.3-1.4)	3.0 (2.9-3.2)
10 to 16 years	1.2 (1.1-1.2)	2.7 (2.7-2.8)
>16 years	0.9 (0.9-1.0)	2.3 (2.2-2.4)

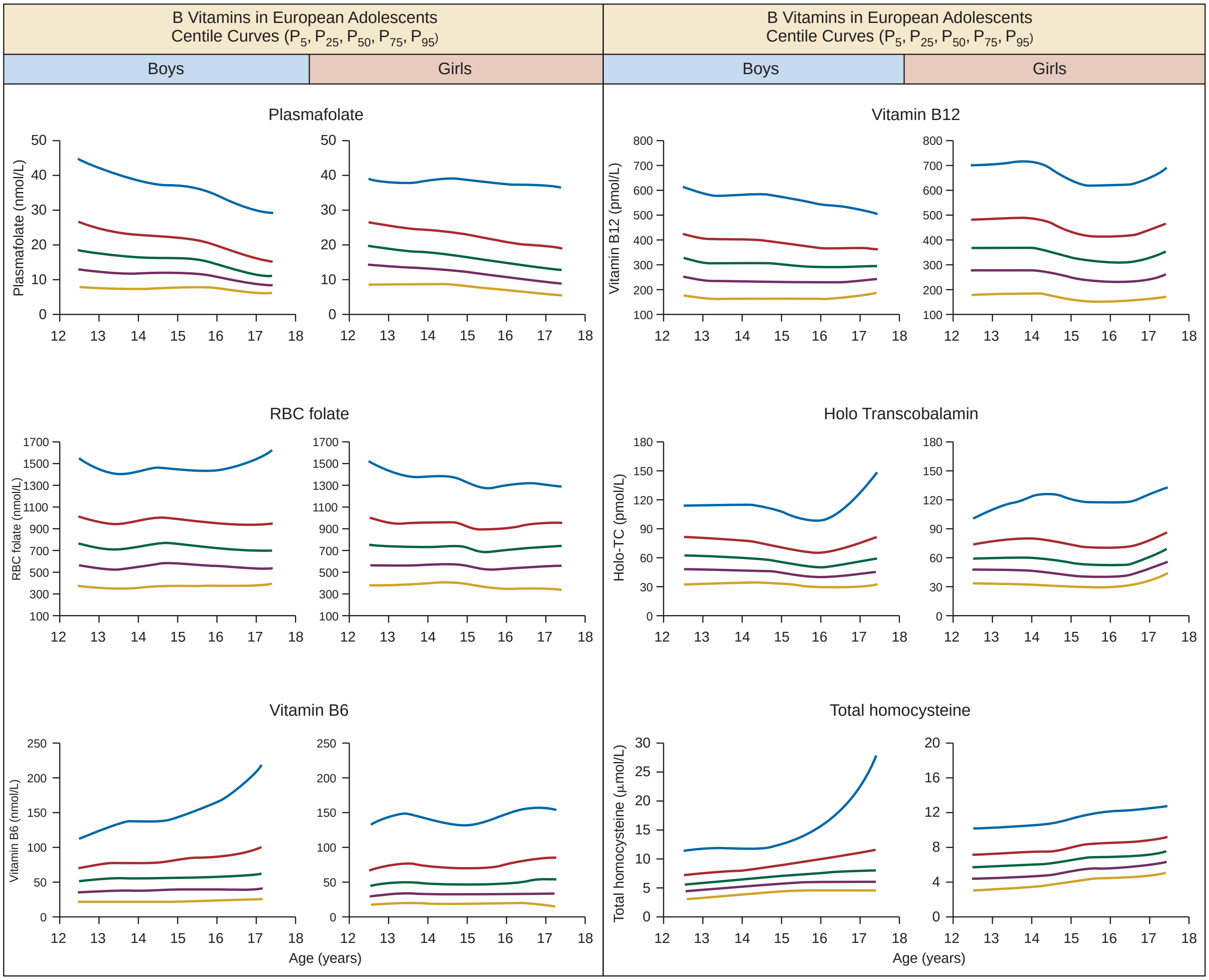
From Suominen, P, Virtanen A, Lehtonen-Veronmaa M, et al: Regression-based reference limits for serum transferrin receptor in children 6 months to 16 years of age. Clin Chem *47:935–937, 2001.*

*The 95% confidence interval for each limit is given in parentheses.

APPENDIX 33 Percentile Distributions for Plasma Folate, RBC folate, Vitamins B_6 and B_{12}, Holotranscobalamin, and Total Homocysteine of Healthy European Adolescents 12.5 to 17.49 Years of Age

		PF(nmol/L)	RBC Folate nmol/L	PLP (nmol/L)	Cbl (pmol/L)	Holo-TC (pmol/L)	tHcy (μmol/L)
	N	1049	1040	974	1051	1017	1050
	Mean	18.7	784.2	63.2	349.4	63.4	7.4
	SD	10.5	340.9	51.9	143.5	33.7	3.8
Percentile	2.5	6.4	315.5	16.6	154.4	27.4	3.7
	5	7.6	378.8	20.0	170.8	30.9	3.9
	10	9.1	428.9	25.0	193.0	35.2	4.4
	25	11.8	553.0	34.7	240.0	44.4	5.4
	50	16.0	721.9	49.1	319.0	57.8	6.7
	75	22.1	942.3	75.7	434.9	73.0	8.4
	90	31.7	1214.0	113.9	557.0	91.7	10.5
	95	39.0	1389.3	149.7	638.0	107.1	12.3
	97.5	46.4	1582.6	187.3	696.0	127.1	15.7

Cbl, Cobalamin; *Holo-TC,* holotranscobalamin; *PF,* plasma folate; *PLP,* pyridoxal 5-phosphate; *RBC,* red blood cell; *SD,* standard deviation.



Smoothed (LMS method) centile curves (*from the bottom to the top*: P5, P25, P50, P75, P95) of plasma folate (nmol/L), red blood cell folate (nmol/L), vitamin B_6 (pmol/L), vitamin B_{12} (pmol/L), holo-transcobalamin (pmol/L), and total homocysteine (µmol/L) concentrations in males and females. *Cbl,* Cobalamin; *Holo-TC,* holo-transcobalamin; *PF,* plasma folate; *PLP,* pyridoxal phosphate or Vitamin B_6; *RBC folate,* red blood cell folate; *tHcy,* total homocysteine. *(From Gonzalez-Gross M, Benser J, Breidenassel C, et al: Gender and age influence blood folate, vitamin B12, vitamin B6, and homocysteine levels in European adolescents: the Helena Study.* Nutr Res *32:817–826, 2012.)*

APPENDIX 34 Plasma Levels of Vitamin E (α-Tocopherol) in Children and Adolescents

	MALES AND FEMALES	
Age	**μmol/L**	**μg/mL**
Premature[*]	1-8	0.5-3.5
Full Term	2-8	0.5-3.5
2-5 m[*]	5-14	2.0-6.0
6-24 m[*]	8-19	3.5-80
2-12 y[*]	13-21	5.5-9.0
1-6 y[†]	7-21	3.0-9.0
7-12 y[†]	10-21	4.0-9.0
13-19 y[†]	13-24	6.0-10.1

[*] Meites S, editor: *Pediatric clinical chamistry,* 3rd ed. Washington, DC, 1989, AACC Press, p. 295–296.

[†] Lockitch G, Halstead AC, Wadsworth L, et al: Age- and sex-specific pediatric reference intervals for zinc, copper, selenium, iron, vitamins A and E and related proteins. *Clin Chem* 34:1625, 1988. Study based on healthy children; values represent the 0.025th and 0.975th fractiles.

APPENDIX 35 Serum Erythropoietin Levels During the First Year of Life[*]

Days After Birth	**Erythropoietin (mU/mL)**	**Hemoglobin (g/dL)**	**RBC ($\times 10^6/\mu L$)**
0-6	33.0 ± 31.4 (11)	15.6 ± 2.2 (11)	4.51 ± 0.74 (11)
7-50	11.7 ± 3.6 (7)	12.8 ± 1.1 (5)	3.92 ± 0.35 (5)
51-100	21.1 ± 5.5 (13)	11.4 ± 1.0 (10)	4.09 ± 0.51 (10)
101-150	15.1 ± 3.9 (5)	11.2 ± 1.1 (3)	4.21 ± 0.31 (3)
151-200	17.8 ± 6.3 (6)	—	—
>201	23.1 ± 9.7 (10)	11.8 ± 0.8 (9)	4.57 ± 0.25 (9)

From Yamashita H, Kukita J, Ohga S: Serum erythropoietin levels in term and preterm infants during the first year of life. Am J Pediatr Hematol Oncol *16:213, 1994.*

RBC, Red blood cell.

[*] Values are expressed as mean ± standard deviation; number of specimens is within parentheses.

APPENDIX 36 Plasma Erythropoietin Reference Ranges in Children and Adolescents[*]

	MALE SUBJECTS		FEMALE SUBJECTS	
Age (y)	**2.5%**	**97.5%**	**2.5%**	**97.5%)**
1-3	1.7	17.9	2.1	15.9
4-6	3.5	21.9	2.9	8.5
7-9	1.0	13.5	2.1	8.2
10-12	1.0	14.0	1.1	9.1
13-15	2.2	14.4	3.8	20.5
16-18	1.5	15.2	2.0	14.2

From Krafte-Jacobs B, Williams J,. Soldin SJ, Plasma erythropoietin reference ranges in children. J Pediatr *126:601, 1995.*

y, Years.

[*] Data obtained from a total of 1122 hospitalized and outpatient children ages 1 to 18 years. Levels found in anemic patients cannot be compared with normal values. In fact, as long as the erythropoietin-generating apparatus in the kidney is efficient, serum levels increase exponentially as the hematocrit concentration decreases. Serum erythropoietin must therefore be evaluated in relation to the degree of anemia, and every single laboratory should determine the exponential regression of serum erythropoietin versus hematocrit (or hemoglobin) in a home-made reference population of anemic subjects and define the 95% confidence limits. The patients gathered to calculate a reference regression equation should have an anemia with a single simple mechanism and no evidence of either renal failure or excessive cytokine production (i.e., normal values for C-reactive protein and α_2-globulins). Patients with chronic iron-deficiency anemia caused by nonneoplastic and noninflammatory chronic blood loss have the advantages of being easily found, unequivocably defined, and homogeneous. They could become the universal reference population, although patients with hemolytic anemia or thalassemia intermedia also may be studied as reference subjects. Serum erythropoietin levels are also much higher for hemoglobin concentration in hypoplastic than in hyperplastic states. Thus for the same hemoglobin concentration, serum erythropoietin levels are lower in thalassemia intermedia than in Diamond-Blackfan anemia.

APPENDIX 37 Membrane Lipid Composition of Fetal Erythrocytes

	G1 20-25 wk(N = 8)	G2 28-35 wk (N = 7)	G3 38-41 wk (N = 7)	G4 Adults (N = 10)
Cholesterol (µg/µg prot.)	0.22 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.23 ± 0.01
Phospholipids(µg/µg prot.)	0.70 ± 0.14	0.81 ± 0.07	0.89 ± 0.16	0.93 ± 0.11
CH/PL	0.36 ± 0.05	0.30 ± 0.03	0.26 ± 0.02	0.27 ± 0.02
Phospholipids				
Sphingomyelin (%)	30.11 ± 0.91	30.64 ± 0.65	31.97 ± 2.62	30.83 ± 0.3
Phosphatidylcholine (%)	30.30 ± 0.48	30.17 ± 1.0	26.78 ± 0.30*	26.88 ± 0.4*
Phosphatidylinositol (%)	3.76 ± 0.29	3.70 ± 0.24	3.39 ± 0.2	3.38 ± 0.21
Phosphatidylserine (%)	11.75 ± 1.24	11.87 ± 0.8	12.73 ± 0.31	12.02 ± 0.81
Phosphatidic acid (%)	2.98 ± 0.11	2.99 ± 0.22	2.79 ± 0.12	2.81 ± 0.18
Phosphatidylethanolamine (%)	20.93 ± 0.6	20.68 ± 1.33	22.25 ± 0.63	21.82 ± 0.43
Fatty Acids				
16:0 (%)	22.51 ± 0.92	25.31 ± 0.78*	22.95 ± 0.73	23.02 ± 0.79
18:0 (%)	17.74 ± 0.92	17.52 ± 0.51	18.18 ± 0.33	17.92 ± 0.46
18:lw9 (%)	13.03 ± 0.21	11.74 ± 0.38*	10.70 ± 0.30	10.57 ± 0.33
18:l bw7 (%)	1.75 ± 0.12	1.57 ± 0.07	1.73 ± 0.08	1.59 ± 0.07
18:2w6 (%)	4.81 ± 0.36	4.85 ± 0.42	4.38 ± 0.31	4.18 ± 0.32
20:3w6 (%)	2.40 ± 0.14	2.79 ± 0.1 1	3.42 ± 0.11*†	3.59 ± 0.11*
20:4w6 (%)	21.95 ± 0.5	23.13 ± 0.4	22.36 ± 0.46	22.47 ± 0.48
22:4w6 (%)	3.76 ± 0.34	4.28 ± 0.19	4.98 ± 0.23*	5.31 ± 0.40*
22.5w3 (%)	1.29 ± 0.19	1.45 ± 0.04	1.76 ± 0.17	1.63 ± 0.15
22:6w3 (%)	9.41 ± 0.71	8.79 ± 0.26	9.49 ± 0.46	9.48 ± 0.52
Fatty Acids of Phosphatidycholine				
16:0 (%)	40.40 ± 2.10	40.78 ± 1.74	35.75 ± 2.76	32.12 ± 2.58
18:0 (%)	9.36 ± 0.48	9.32 ± 0.53	12.27 ± 0.57*	13.07 ± 0.51*
18:lw9 (%)	20.10 ± 0.82	15.96 ± 0.65*	14.63 ± 0.56*	12.23 ± 0.88*
18:l bw7 (%)	3.51 ± 0.15	3.05 ± 0.15	3.62 ± 0.21	3.45 ± 0.18
18:2w6 (%)	6.37 ± 0.47	6.94 ± 0.5	8.71 ± 0.78	10.42 ± 0.9*
20:3w6 (%)	1.97 ± 0.15	2.92 ± 0.20*	4.89 ± 0.28	6.05 ± 0.21*
20:4w6 (%)	16.23 ± 0.98	17.18 ± 14.2	14.21 ± 1.17	14.12 ± 1.74
22:4w6 (%)	0.54 ± 0.06	0.57 ± 0.07	0.73 ± 0.09	0.73 ± 0.08
22.5w3 (%)	0	0.35 ± 0.07	0.42 ± 0.03	0.22 ± 0.03
22:6w3 (%)	1.93 ± 0.16	2.6 ± 0.47	3.73 ± 0.43*	4.37 ± 0.49*
Fatty Acids of Phosphatidylethanolamine				
16:0 (%)	29.83 ± 0.83	30.19 ± 1.71	30.20 ± 1.44	28.55 ± 1.23
18:0 (%)	11.62 ± 0.68	10.36 ± 0.64	9.16 ± 0.57*	7.63 ± 0.58*
18:lw9 (%)	17.25 ± 1.20	16.44 ± 0.22	17.36 ± 0.33	16.17 ± 0.62
18:l bw7 (%)	1.63 ± 0.08	1.22 ± 0.05	1.59 ± 0.15	1.28 ± 0.13
18:2w6 (%)	3.01 ± 0.2	2.71 ± 0.15	3.11 ± 0.20	4.52 ± 0.26*
20:3w6 (%)	1.61 ± 0.11	1.82 ± 0.16	2.16 ± 0.14*	2.4 ± 0.12*
20:4w6 (%)	22.85 ± 0.68	24.22 ± 1.4	21.60 ± 0.98	23.6 ± 1.5
22:4w6 (%)	3.95 ± 0.29	4.92 ± 0.52	5.14 ± 0.27*	5.5 ± 0.43*
22.5w3 (%)	1.52 ± 0.16	1.37 ± 0.09	1.65 ± 0.17	1.72 ± 0.12
22:6w3 (%)	7.81 ± 0.30	7.27 ± 0.45	8.01 ± 0.42	9.00 ± 0.88

From Colin FC, Gallois Y, Rapin D, et al: Impaired fetal erythrocytes' filterability: relationship with cell size, membrane fluidity, and membrane lipid composition. Blood *79:2148, 1992.*

CH, Cholesterol; *PL,* phospholipids; *SEM,* standard error of the mean; *wk,* weeks.

Results are expressed at mean ± SEM, *P* <.05 versus G1(*) or G2(†).

APPENDIX 38 Geometric Data[*] for Unfractionated, Top, and Bottom Neonatal and Adult Erythrocytes

	Neonatal	Adult
Volume (fL)		
Unfractionated	107.3 ± 5.6[†‡]	90.5 ± 4.4[‡]
Top	130.8 ± 13.1[†‡]	99.2 ± 6.3[‡]
Bottom	89.4 ± 5.9[†]	80.3 ± 4.0
Surface Area (μm^2)		
Unfractionated	153.5 ± 7.0[†‡]	137.1 ± 6.7[‡]
Top	186.1 ± 10.6[†‡]	150.6 ± 8.4[‡]
Bottom	108.5 ± 8.2[†]	118.6 ± 6.2
Surface Area/Volume		
Unfractionated	1.43 ± 0.04[†‡]	1.51 ± 0.04
Top	1.43 ± 0.05[†]	1.51 ± 0.03
Bottom	1.21 ± 0.05[†]	1.51 ± 0.04
Diameter (μm)		
Unfractionated	8.8 ± 0.4[‡]	7.9 ± 0.4
Top	9.8 ± 0.4[†‡]	8.6 ± 0.5[‡]
Bottom	6.9 ± 0.5	7.5 ± 0.4
Mean Thickness (μm)		
Unfractionated	1.76 ± 0.10[‡]	1.84 ± 0.13
Top	1.70 ± 0.11	1.71 ± 0.11[‡]
Bottom	2.39 ± 0.13[†]	1.83 ± 0.14
Surface Area Index		
Unfractionated	1.40 ± 0.05[‡]	1.41 ± 0.04[‡]
Top	1.49 ± 0.07[‡]	1.46 ± 0.05[‡]
Bottom	1.12 ± 0.04[†]	1.33 ± 0.04

From Linderkamp O, Friederichs E, Meiselman HJ: Mechanical and geometrical properties of density-separated neonatal and adult erythrocytes. Pediatr Res *34:688–693, 1993.*

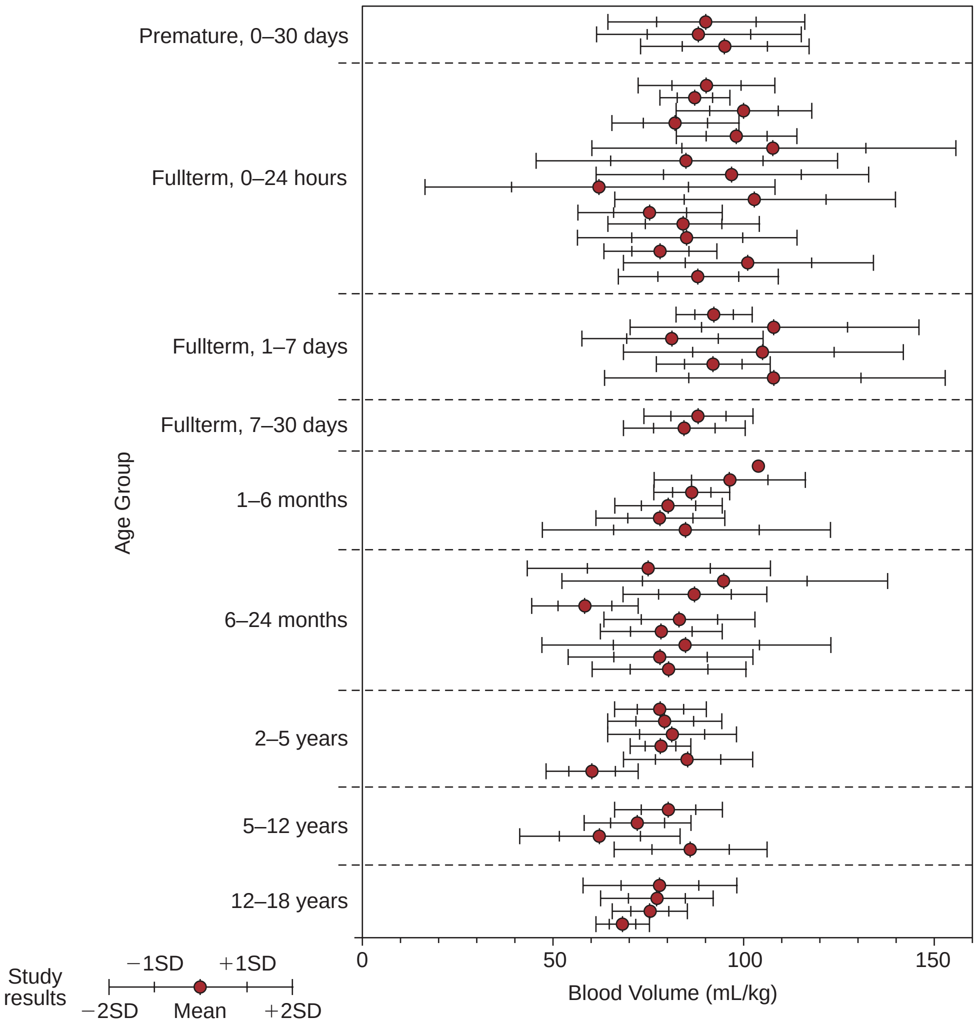
[*] Data are presented as mean ±SD for 10 nenonatal and 10 adult samples.

[†] Significant difference ($P < .05$ unpaired † test) between adult and neonatal erythrocytes.

[‡] Significant difference ($P < .05$ paired † test) between top or bottom fraction and unfractionated erythrocytes.

Age Group	No of Studies	Estimated Mean Circulating Blood Volume, mL/kg (95% CI)	Test of Homogeneity*	
			Q Statistic	*p*
All subjects	21	80.0 (77.4–82.5)	46.53	0.16
Premature newborns 0–30 d	3	91.5 (77.3–105.7)	0.18	0.91
Full-term newborns 0–24 h	16	87.7 (83.2–92.3)	11.69	0.70
Full-term newborns 1–7 d	6	91.7 (84.0–99.4)	2.40	0.79
Full-term newborns 7–30 d	2	86.0 (75.6–96.4)	0.09	0.76
Children 1–6 mo	6	84.0 (77.2–90.7)	2.68	0.75
Children 6–24 mo	9	75.4 (68.5–82.3)	8.74	0.36
Children 2–5 yr	6	75.7 (70.7–80.7)	7.26	0.20
Children 5 –12 yr	4	74.9 (67.3–82.6)	2.98	0.39
Children 12–18 yr	4	71.4 (66.2–76.5)	1.90	0.59

This compilation was obtained using study-level data from all studies.
*$p > .05$ indicates that results of the included studies are homogeneous and can be combined.



Appendix 39 Circulating blood volume by age group. Bars on the table indicate 1 and 2 standard deviations with the center point indicating study mean blood volume. *(From Riley AA, Arakawa Y, Worley S, et al. Circulating blood volumes: a review of measurement techniques and a meta-analysis in children.* ASAIO *56:260–264, 2010.)*

APPENDIX 40 Percentages and Absolute Counts for Total B cells and B-Cell Subsets*

	Number	Cord Blood	0-6 Months	6-12 Months	1-2 Years	2-3 Years	3-4 Years	4-6 Years	6-9 Years	9-12 Years	12-15 Years	15-18 Years
Total B cells	*90*	11.5 (7.6-15.5)	31.3 (20.5-40.9)	26.1 (11.1-45.4)	21.1 (16.3-26.8)	25.7 (17.3-30.3)	20.0 (14.4-25.1)	17.9 (7.9-22.5)	16.5 (8.5-20.2)	13.1 (4.3-18.2)	12.3 (7.8-23.7)	11.5 (7.8-15.1)
RBE B cells	*83*	15.5 (5.6-30.7)	27.6 (17.1-32.2)	15.4 (9.3-26.7)	14.8 (9.7-17.9)	10.2 (3.6-21.4)	9.1 (6.4-13.9)	9.3 (6.8-11.5)	5.8 (3.4-9.0)	6.4 (2.9-23.8)	4.8 (1.5-7.3)	5.2 (1.5-19.1)
Naive B cells	*83*	75.9 (55.6-81.3)	60.2 (55.2-73.9)	73.7 (57.5-84.7)	65.7 (61.5-68.7)	68.7 (50.4-78.5)	66.5 (49.7-77.1)	59.1 (52.3-72.1)	52.6 (47.8-69.8)	57.2 (51.5-73.3)	70.6 (64.6-80.1)	64.7 (59.0-81.1)
CD27^{+}IgM^{+}IgD^{+}memory B cells	*145*	4.6 (1.3-11.5)	4.5 (1.6-10.1)	5.9 (3.4-14.0)	10.2 (4.6-15.0)	7.6 (5.1-12.3)	8.3 (4.8-16.1)	11.4 (6.7-18.1)	10.4 (6.3-22.0)	10.3 (6.5-22.2)	9.0 (4.7-15.5)	7.6 (4.6-16.8)
CD27^{+}IgM^{+}IgD^{-}memory B cells	*145*	0.1 (0.0-0.3)	0.4 (0.1-0.7)	1.1 (0.4-2.6)	1.9 (1.6-2.6)	2.3 (1.6-6.6)	4.5 (0.9-9.3)	4.3 (2.0-9.5)	6.9 (2.0-11.8)	2.9 (1.8-13.3)	2.8 (1.6-11.3)	3.8 (0.9-9.1)
CD27^{+}IgG^{+}memory B cells	*100*	0.2 (0.1-0.5)	0.3 (0.1-0.4)	1.0 (0.4-1.7)	2.3 (1.5-4.2)	3.3 (0.8-6.3)	4.3 (1.8-6.2)	5.6 (0.8-10.3)	6.1 (2.7-14.0)	4.7 (1.5-8.8)	5.4 (2.1-9.4)	4.7 (0.8-11.7)
CD27^{+}IgA^{+}memory B cells	*100*	0.2 (0.1-0.6)	0.4 (0.2-0.5)	0.7 (0.3-1.0)	1.3 (0.8-1.7)	1.4 (0.7-2.9)	1.8 (1.0-3.5)	2.6 (1.0-3.9)	3.5 (1.1-6.1)	2.4 (1.3-6.1)	2.6 (1.2-3.8)	2.6 (0.2-4.6)
CD27^{-}IgG^{+}memory B cells	*100*	1.4 (1.1-2.3)	0.7 (0.5-0.8)	1.2 (0.4-2.9)	2.1 (0.7-2.6)	1.7 (0.5-3.4)	1.5 (1.1-3.9)	2.4 (0.7-6.8)	4.1 (1.8-6.5)	2.6 (0.7-4.4)	3.1 (1.1-8.0)	2.6 (0.6-28.4)
CD27^{-}IgA^{+}memory B cells	*100*	2.5 (2.2-4.7)	1.8 (1.4-2.4)	2.4 (1.4-4.4)	2.5 (1.0-4.3)	2.1 (1.3-5.8)	2.5 (1.3-4.9)	3.3 (1.6-4.9)	4.1 (1.1-5.7)	2.6 (1.2-10.3)	3.1 (1.1-5.3)	4.0 (0.8-5.6)
Total B cells	*90*	858 (559-1054)	1623 (961-3679)	1717 (571-3680)	1115 (871-1553)	1157 (686-1732)	709 (359-1552)	593 (278-1022)	418 (296-784)	338 (116-555)	284 (119-578)	210 (114-436)
RBE B cells	*73*	89 (72-164)	459 (164-1184)	214 (70-821)	143 (109-278)	143 (41-248)	72 (23-95)	41 (24-98)	33 (13-63)	24 (12-35)	14 (2-41)	10 (6-41)
Naive B cells	*73*	698 (439-800)	988 (627-2136)	1378 (420-2181)	738 (586-955)	757 (346-1356)	470 (244-724)	344 (149-618)	263 (154-413)	233 (60-300)	193 (83-398)	153 (72-257)
CD27^{+}IgM^{+}IgD^{+}memory B cells	*90*	25 (24-34)	61 (49-179)	71 (30-396)	116 (40-177)	87 (50-148)	62 (27-210)	62 (32-164)	48 (24-135)	35 (9-109)	21 (10-74)	20 (10-39)
CD27^{+}IgM^{+}IgD^{-}memory B cells	*90*	0 (0-2)	10 (6-19)	16 (2-42)	21 (16-34)	33 (19-53)	27 (6-113)	24 (7-98)	32 (7-65)	9 (3-34)	7 (3-39)	10 (2-40)
CD27^{+}IgG^{+}memory B cells	*90*	2 (1-3)	4 (1-15)	13 (9-44)	28 (13-55)	38 (6-57)	29 (13-95)	32 (4-73)	26 (13-74)	16 (4-49)	11 (7-32)	8 (2-51)
CD27^{+}IgA^{+}memory B cells	*90*	1 (1-1)	5 (3-10)	69 (21-106)	16 (8-20)	17 (8-26)	10 (6-54)	13 (4-26)	13 (5-35)	9 (3-26)	7 (4-13)	4 (1-20)
CD27^{-}IgG^{+}memory B cells	*90*	12 12-16)	12 (5-20)	22 (8-48)	22 (8-35)	20 (4-51)	18 (4-27)	15 (4-28)	17 (8-35)	7 (4-20)	8 (3-22)	5 (1-49)
CD27^{-}IgA^{+}memory B cells	*90*	21 (14-24)	30 (17-50)	51 (15-85)	26 (11-48)	31 (10-70)	18 (5-75)	17 (6-42)	18 (5-34)	10 (7-35)	10 (1-17)	10 (2-17)

IG, Immunoglobulin; *RBE*, recent bone marrow emigrants.

*Total B cells (defined as CD19+ lymphocytes) as percentage of total lymphocytes. B-cell subsets as percentage of total B cells. Values are presented as medians (value range). Recent bone marrow emigrants were defined as CD19+IgD+CD10+ B cells, which brightly express CD38 and are CD27– and IgM+.

APPENDIX 41 Lymphocyte Subsets in Term and Premature Neonates in the First Week of Life*

		Term (N = 21)	**Premature (N = 104)**	**Healthy Premature (N = 36)**	**Sick Premature (N = 68)**
Total leukocytes	—	15.44 ± 1.42	13.63 ± 0.94	13.81 ± 1.12	13.54 ± 1.32
Lymphocytes	—	3.51 ± 0.38	5.47 ± 0.23	6.04 ± 0.36	5.18 ± 0.28
CD2	%	67 ± 4	72 ± 1.5	77 ± 3	70 ± 2
	A	2.65 ± 0.34	3.97 ± 0.20	4.61 ± 0.33	3.63 ± 0.24
CD4	%	45 ± 2	47 ± 1.5	52 ± 2	45 ± 2
	A	1.56 ± 0.20	2.58 ± 0.14	3.16 ± 0.14	2.29 ± 0.17
CD8	%	13 ± 1	12 ± 0.5	12.6 ± 0.7	12 ± 0.6
	A	0.57 ± 0.13	0.67 ± 0.04	0.73 ± 0.07	0.64 ± 0.05
CD20	%	5.3 ± 0.4	6.4 ± 0.4	5.6 ± 0.4	6.8 ± 0.6
	A	0.18 ± 0.03	0.34 ± 0.03	0.34 ± 0.04	0.34 ± 0.03
CD21	%	9.1 ± 0.6	8.6 ± 0.5	6.8 ± 0.6	9.5 ± 0.7
	A	0.33 ± 0.06	0.44 ± 0.03	0.39 ± 0.04	0.47 ± 0.05

From Series IM, Pichette J, Carrie C, et al: Quantitative analysis of T and B cell subsets in healthy and sick premature infants. Early Hum Dev *26:143, 1991.*

A, Absolute cell count; *SEM*, standard error of the mean.

*Values are expressed as mean ± SEM; %, relative %; A, Absolute cell count × 10^9/L.

APPENDIX 42 Absolute Counts for Total T Cells, T-Cell Subsets, sjTRECs, and T-Cell Ratios in Children and Adolescents

	No.	Cord Blood	0-6 Months	6-12 Months	1-2 Years	2-3 Years	3-4 Years	4-6 Years	6-9 Years	9-12 Years	12-15 Years	15-18 Years
Leukocytes (×10^9/l)	98	nd	10.8 (4.7-14.8)	12.1 (6.6-13.6)	8.7 (7.4-14.3)	9.5 (6.6-12.9)	8.5 (5.0-11.2)	8.4 (6.7-11.9)	7.0 (4.0-12.5)	6.0 (5.3-11.2)	5.9 (4.1-8.3)	5.9 (4.8-7.4)
Lymphocytes (×10^9/l)	98	nd	5.2 (3.2-9.8)	6.4 (3.8-9.3)	5.6 (4.6-6.0)	4.4 (2.8-6.4)	3.7 (2.2-5.9)	3.4 (2.8-4.8)	3.0 (1.8-5.0)	2.7 (2.1-4.0)	2.4 (1.5-2.8)	2.1 (1.5-2.7)
Total CD4$^+$ T cells	98	nd	1985 (1294-4012)	2721 (1327-4455)	2073 (1902-2977)	1469 (925-2477)	1044 (646-2331)	1186 (1000-1931)	992 (641-1453)	970 (695-1473)	904 (608-1217)	809 (560-1067)
CD25$^+$CD127$^-$CD4$^+$ T cells (regulatory)	73	nd	208 (64-282)	136 (74-280)	107 (79-162)	63 (42-69)	78 (30-126)	58 (41-86)	47 (18-86)	36 (23-89)	41 (25-64)	26 (19-41)
CD27$^+$ CD45RO$^-$ (naive)	73	nd	175 (55-251)	123 (60-237)	100 (57-147)	41 (27-49)	58 (16-93)	33 (21-58)	28 (10-61)	25 (9-52)	20 (10-40)	16 (7-23)
CD45RO$^+$ (memory or effector)	73	nd	21 (9-36)	25 (11-43)	18 (9-26)	20 (13-27)	20 (10-38)	26 (11-31)	20 (8-30)	20 (10-36)	19 (12-32)	11 (8-23)
CD27$^+$CD45RO$^-$CD4$^+$ T cells (naive)	98	nd	1856 (1164-3712)	2512 (1134-4204)	1797 (1565-2794)	1153 (685-2055)	882 (430-1871)	894 (630-1414)	661 (375-1096)	596 (441-1109)	523 (311-781)	469 (335-725)
CD31$^+$	73	nd	1684 (781-2695)	1920 (788-2564)	1188 (961-1597)	824 (438-1037)	908 (296-1446)	644 (493-1054)	460 (244-894)	442 (313-785)	304 (239-477)	325 (207-585)
CD27$^+$CD45RO$^+$CD4$^+$ T cells (memory)	98	nd	130 (110-299)	230 (119-355)	241 (112-314)	293 (210-442)	236 (155-476)	357 (222-502)	266 (195- 27)	262 (215-489)	328 (232-373)	242 (201-419)
CD27$^-$CD45RO$^+$CD4$^+$ T cells (memory effector)	98	nd	6 (1-17)	9 (5-67)	12 (4-18)	16 (10-34)	16 (5-51)	39 (11-50)	28 (14-97)	45 (26-75)	29 (19-59)	25 (15-78)
CD27$^-$CD45RO$^-$CD4$^+$ T cells (effector)	98	nd	7 (1-14)	4 (3-37)	8 (3-11)	3 (1-12)	4 (2-10)	3 (1-7)	3 (1-10)	10 (0-28)	2 (1-101)	2 (1-21)
CD45RO$^+$CD4$^+$ T cells	98	nd	139 (111-315)	240 (126-378)	245 (121-329)	311 (224-456)	251 (167-499)	399 (234-534)	304 (216-497)	322 (246-564)	357 (252-417)	273 (216-490)
Total CD8$^+$ T cells	98	nd	747 (394-1865)	850 (593-1517)	892 (667-1473)	733 (394-1197)	868 (365-1255)	852 (602-1203)	628 (249-1440)	652 (426-991)	447 (228-577)	399 (216-499)
CD27$^+$CD45RO$^-$CD8$^+$ T cells (naive)	98	nd	669 (345-1635)	789 (499-1423)	834 (637-1113)	526 (336-927)	673 (248-999)	573 (442-733)	426 (203-961)	448 (267-683)	325 (165-424)	257 (118-312)
CD27$^+$CD45RO$^+$CD8$^+$ T cells (memory)	98	nd	28 (14-260)	40 (26-127)	34 (8-127)	66 (38-239)	105 (32-228)	156 (62-402)	103 (35-256)	100 (72-262)	82 (32-153)	96 (30-166)
CD27$^-$CD45RO$^+$CD8$^+$ T cells (memory effector)	98	nd	1 (0- 18)	1 (0-67)	1 (0- 47)	5 (2- 77)	18 (2-106)	36 (3-67)	16 (2-109)	18 (3-51)	5 (3-26)	10 (1-26)
CD27$^-$CD45RO$^-$CD8$^+$ T cells (effector)	98	nd	5 (0-50)	3 (1-123)	5 (1-193)	15 (5-174)	25 (7-218)	40 (5-310)	20 (3-198)	50 (8-155)	5 (3-59)	20 (3-65)
CD45RO$^+$CD8$^+$ T cells	98	nd	29 (14-268)	41 (26-177)	35 (8-174)	74 (41-314)	151 (36-246)	196 (79-425)	134 (38-296)	123 (77-303)	89 (35-163)	111 (33-189)

CD4$^+$ T-cell sjTREC content (per cell)	*78*	0.098 (0.080-0.173)	0.180 (0.083-0.343)	0.087 (0.035-0.317)	0.225 (0.047-0.251)	0.130 (0.018-0.377)	0.093 (0.016-0.299)	0.057 (0.023-0.342)	0.032 (0.014-0.190)	0.030 (0.013-0.102)	0.045 (0.007-0.108)	0.037 (0.017-0.058)
CD8$^+$ T-cell sjTREC content (per cell)	*77*	0.112 (0.096-0.172)	0.145 (0.060-0.222)	0.084 (0.028-0.341)	0.151 (0.043-0.206)	0.078 (0.022-0.285)	0.085 (0.021-0.239)	0.036 (0.015-0.289)	0.032 (0.011-0.152)	0.034 (0.016-0.104)	0.047 (0.009-0.120)	0.031 (0.012-0.055)
CD4$^+$ T-cell sjTREC number	*71*	nd	484 (188-1206)	384 (50-953)	436 (105-626)	142 (25-585)	102 (36-625)	71 (24-433)	33 (13-289)	22 (19-118)	36 (5-94)	24 (14-56)
CD8$^+$ T-cell sjTREC number	*70*	nd	131 (41-345)	69 (17-551)	113 (39-145)	65 (12-267)	54 (19-293)	32 (13-181)	17 (5-219)	18 (8-85)	10 (3-68)	13 (4-26)
Total CD4$^+$/CD8$^+$ T-cell ratio	*109*	2.8 (2.2-4.3)	2.6 (1.9-5.5)	2.7 (1.9-5.4)	2.7 (1.4-3.7)	2.4 (1.3-3.0)	1.6 (0.9-2.7)	1.6 (0.9-2.3)	1.6 (1.0-3.0)	1.6 (0.9-2.5)	2.2 (1.5-3.7)	2.2 (1.4-4.0)
Naive CD4$^+$/CD8$^+$ T-cell ratio	*109*	2.9 (2.2-4.3)	2.8 (1.9-5.5)	2.9 (1.8-5.4)	2.7 (1.5-3.5)	2.2 (1.4-2.8)	1.7 (0.9-2.5)	1.7 (1.0-2.3)	1.5 (0.9-2.6)	1.6 (0.8-2.1)	1.7 (1.1-3.1)	1.7 (1.3-4.6)
Naive/memory CD4$^+$ T-cell ratio	*109*	18.4 (11.8-61.9)	13.2 (9.0-18.6)	8.7 (5.5-18.6)	7.1 (4.8-22.8)	3.8 (2.6-5.4)	3.1 (1.9-5.4)	2.8 (1.4-4.3)	1.9 (1.3-3.2)	1.9 (1.2-3.1)	1.5 (1.0-2.7)	1.7 (1.0-2.7)
Naive/memory CD8$^+$ T-cell ratio	*109*	22.2 (11.5-71.8)	25.0 (5.1-36.3)	17.8 (3.7-31.3)	23.4 (8.4-116.1)	7.6 (2.3-14.4)	4.6 (2.0-15.9)	3.1 (1.4- 9.2)	3.8 (2.0-6.5)	3.7 (1.5- 6.4)	3.6 (2.3-7.8)	2.4 (1.1-8.6)
Naive/(memory or effector) Treg ratio	*80*	5.7 (3.4-18.1)	6.9 (4.9-8.8)	5.3 (4.2-9.2)	4.2 (2.5-13.2)	1.9 (1.5-3.0)	2.4 (1.1-5.0)	1.5 (0.8-3.1)	1.5 (1.0-2.5)	1.2 (0.6-2.4)	1.1 (0.6-2.0)	1.2 (0.6-2.4)

From van Gent R, van Tilburg CM, Nibbelke EE, et al: Refined characterization and reference values of the pediatric T- and B-cell compartments. Clin Immunol *133:95–107, 2009.*

Absolute counts × 10^6 per liter blood, unless stated differently. Values are presented as medians (value range). Counts of T-cell subsets in boldface, whereas counts of specific cell populations within these T-cell subsets in italics. *No.*, Number of individuals; *nd*, not determined.

sjTREC numbers × 10^6 per liter blood. Values are presented as medians (value range). *No.*, Number of individuals; *nd*, not determined. Naive = CD27$^+$CD45RO$^-$; memory = CD27$^+$CD45RO$^+$; effector = CD27$^-$CD45RO$^+$; *Treg* indicates regulatory T cells, which are defined as CD25$^+$CD127$^-$CD4$^+$ T cells.

APPENDIX 43 Absolute Numbers of T Lymphocytes

Population	**N**	**Cord Blood**	**N**	**1 w-2 m**	**N**	**2-5 m**	**N**	**5-9 m**	**N**	**0-15 m**	**N**	**15-24 m**	**N**	**2-5 y**	**N**	**5-10 y**	**N**	**10-16 y**	**N**	**>16 y***	**N**
Lymphocytes (total)	136	5.4 (3.1-9.4)	18	5.7 (2.9-11.4)	11	6.5 (3.4-12.2)	12	5.8 (1.8-18.7)	13	6.3 (3.2-12.3)	10	4.1 (1.4-12.1)	10	2.7 (1.4-5.5)	11	2.4 (1.2-4.7)	15	2.4 (1.4-4.2)	15	2.3 (1.2-4.1)	21
B lymphocytes	136	0.54 (0.14-2.0)	18	0.81 (0.18-3.5)	11	1.1 (0.52-2.3)	12	0.90 (0.13-6.3)	13	0.94 (0.11-7.7)	10	0.76 (0.16-3.7)	10	0.49 (0.18-1.3)	11	0.29 (0.10-0.80)	15	0.30 (0.12-0.74)	15	0.23 (0.064-0.82)	21
NK cells	136	1.2 (0.5-3.1)	18	0.51 (0.14-1.9)	11	0.44 (0.097-1.99)	12	0.52 (0.068-3.9)	13	0.50 (0.071-3.5)	10	0.47 (0.055-4.0)	10	0.18 (0.061-0.51)	11	0.20 (0.070-0.59)	15	0.33 (0.092-1.2)	15	0.34 (0.10-1.2)	21
T lymphocytes	136	3.1 (1.4-6.8)	18	4.0 (1.9-8.4)	11	4.5 (2.2-9.2)	12	4.0 (1.4-11.5)	13	4.4 (2.4-8.3)	10	2.5 (0.7-8.8)	10	1.9 (0.85-4.3)	11	1.8 (0.77-4.0)	15	1.6 (0.85-3.2)	15	1.5 (0.78-3.0)	21
Helper T lymphocytes	136	2.2 (1.0-4.8)	18	3.0 (1.5-6.0)	11	3.3 (1.6-6.5)	12	2.7 (1.0-7.2)	13	3.0 (1.3-7.1)	10	1.6 (0.4-7.2)	10	1.1 (0.5-2.7)	11	1.0 (0.4-2.5)	15	0.9 (0.4-2.1)	15	1.0 (0.5-2.0)	21
Cytotoxic T lymphocytes	136	0.8 (0.2-2.7)	18	0.9 (0.3-2.7)	11	1.0 (0.3-3.4)	12	1.1 (0.2-5.4)	13	1.2 (0.4-4.1)	10	0.7 (0.2-2.8)	10	0.6 (0.2-1.8)	11	0.6 (0.2-1.7)	15	0.6 (0.3-1.3)	15	0.5 (0.2-1.2)	21
Naïve helper T cells	125	1.8 (0.9-3.9)	18	2.7 (1.3-5.7)	10	3.1 (1.6-6.0)	11	2.5 (0.8-7.6)	11	2.7 (1.1-6.4)	10	1.3 (0.2-7.5)	10	0.8 (0.3-2.3)	11	0.7 (0.2-2.5)	13	0.6 (0.2-1.7)	15	0.5 (0.1-2.3)	16
Terminally differentiated helper T cells	125	0.00079 (0.00021-0.003)	18	0.0005 (0.00017-0.0015)	10	0.0011 (0.00024-0.0053)	11	0.0027 (0.0000047-0.4)b	11	0.0021 (0.0000081-0.077)b	10	0.0015 (0.0001-0.033)b	10	0.0017 (0.000061-0.016)b	11	0.0011 (0.000025-0.025)b	13	0.0016 (0.00004-0.051)b	15	0.0037 (0.000098-0.068)b	16
Effector memory helper T cells	125	0.00067 (0.00017-0.0026)	18	0.0017 (0.00011-0.026)	10	0.0064 (0.002-0.021)	11	0.0093 (0.0012-0.072)	11	0.013 (0.00043-0.12)b	10	0.011 (0.00085-0.15)	10	0.018 (0.0035-0.089)	11	0.023 (0.003-0.17)	13	0.033 (0.0051-0.21)	15	0.053 (0.013-0.22)	16
Central memory helper T cells	125	0.39 (0.075-2.0)	18	0.39 (0.09-1.7)	10	0.35 (0.053-2.2)	11	0.32 (0.083-1.3)	11	0.39 (0.19-0.8)	10	0.17 (0.001-0.65)b	10	0.33 (0.16-0.66)	11	0.18 (0.0037-0.51)b	13	0.3 (0.12-0.74)	15	0.43 (0.18-1.1)	16
Naïve cytotoxic T cells	125	0.36 (0.023-1.3) b	18	0.55 (0.14-2.2)	10	0.69 (0.29-1.65)	11	0.7 (0.15-3.2)	11	0.58 (0.14-2.46)	10	0.31 (0.03-3.1)	10	0.24 (0.053-1.1)	11	0.24 (0.042-1.3)	13	0.22 (0.078-0.64)	15	0.13 (0.016-1.0)	16
Terminally differentiated cytotoxic T- cells	125	0.095 (0.012-0.75)	18	0.086 (0.015-0.48)	10	0.1 (0.013-0.82)	11	0.22 (0.017-2.8)	11	0.22 (0.029-1.7)	10	0.16 (0.055-0.46)	10	0.12 (0.025-0.53)	11	0.14 (0.057-0.34)	13	0.12 (0.035-0.42)	15	0.084 (0.025-0.28)	16
Effector memory cytotoxic T cells	125	0.044 (0.0074-0.26)	18	0.042 (0.0094-0.19)	10	0.056 (0.0024-0.4)b	11	0.1 (0.0076-1.4)	11	0.13 (0.0076-2.1)	10	0.1 (0.016-0.63)	10	0.12 (0.024-0.59)	11	0.14 (0.045-0.41)	13	0.11 (0.016-0.81)	15	0.16 (0.04-0.64)	16
Central memory cytotoxic T cells	125	0.04 (0.0011-0.16)b	18	0.071 (0.013-0.38)	10	0.059 (0.0006-0.19)b	11	0.037 (0.0018-0.15)b	11	0.034 (0.012-0.092)	10	0.022 (0.0048-0.11)	10	0.017 (0.0043-0.064)	11	0.016 (0.0061-0.043)	13	0.014 (0.0023-0.086)	15	0.024 (0.0047-0.12)	16
CD197+/– cytotoxic T cells	125	0.56 (0.13-2.4)	18	0.65 (0.18-2.3)	10	0.81 (0.31-2.1)	11	0.96 (0.18-5.0)	11	0.86 (0.25-3.0)	10	0.5 (0.1-2.4)	10	0.37 (0.12-1.2)	11	0.38 (0.1-1.5)	13	0.36 (0.16-0.8)	15	0.23 (0.06-0.83)	16
Recent thymic emigrants	126	1.7 (0.71-4.2)	18	2.2 (1.0-4.9)	10	2.7 (1.4-5.2)	11	2.2 (0.8-6.2)	11	2.3 (0.9-5.8)	10	1.1 (0.17-7.4)	10	0.71 (0.19-2.6)	11	0.59 (0.2-1.7)	14	0.48 (0.15-1.5)	15	0.34 (0.05-2.4)	16
NK-T cells	136	0.034 (0.0056-0.21)	18	0.025 (0.0069-0.091)	11	0.035 (0.013-0.09)	12	0.047 (0.0044-0.51)	13	0.049 (0.0075-0.33)	10	0.038 (0.0065-0.23)	10	0.06 (0.015-0.25)	11	0.064 (0.012-0.34)	15	0.075 (0.016-0.35)	15	0.096 (0.023-0.41)	21

CXCR5+ memory helper T cells	102	0.0019 (0.00052-0.0066)	14	0.0063 (0.00067-0.06)	10	0.024 (0.0062-0.089)	11	0.041 (0.015-0.11)	10	0.048 (0.011-0.2)	10	0.045 (0.015-0.14)	10	0.048 (0.013-0.17)	9	0.047 (0.013-0.16)	8	0.047 (0.014-0.16)	10	0.067 (0.024-0.19)	10
Regulatory T cells	124	0.144 (0.065-0.319)	18	0.23 (0.083-0.64)	10	0.25 (0.12-0.53)	11	0.21 (0.06-0.74)	11	0.21 (0.063-0.69)	10	0.12 (0.02-0.77)	10	0.076 (0.039-0.15)	11	0.073 (0.02-0.27)	15	0.078 (0.033-0.19)	12	0.067 (0.025-0.18)	16
TCR-α/β	127	2.8 (1.1-6.9)	18	3.7 (1.6-8.6)	10	4.3 (2.2-8.3)	11	3.8 (1.2-12.0)	11	4.0 (2.0-8.0)	10	2.2 (0.6-8.5)	10	1.6 (0.6-4.3)	11	1.5 (0.6-3.7)	15	1.4 (0.7-2.8)	15	1.4 (0.6-3.3)	16
Double negative T cells	123	0.028 (0.01-0.079)	18	0.022 (0.0055-0.086)	10	0.034 (0.0082-0.14)	11	0.046 (0.016-0.14)	11	0.04 (0.012-0.14)	10	0.033 (0.011-0.1)	10	0.047 (0.016-0.14)	11	0.032 (0.01-0.1)	15	0.027 (0.009-0.078)	14	0.023 (0.0069-0.074)	13
Helper T cells	123	1.9 (0.8-4.7)	18	2.8 (1.2-6.2)	10	3.1 (1.6-6.0)	11	2.5 (0.83-7.8)	11	2.7 (1.0-7.0)	10	1.4 (0.3-7.0)	10	1.0 (0.4-2.8)	11	0.91 (0.36-2.8)	15	0.85 (0.34-2.1)	14	0.85 (0.3-2.4)	13
Cytotoxic T cells	123	0.73 (0.23-2.4)	18	0.85 (0.23-3.1)	10	1.1 (0.4-2.8)	11	1.2 (0.27-5.2)	11	1.2 (0.36-3.8)	10	0.65 (0.17-2.5)	10	0.54 (0.17-1.7)	11	0.57 (0.21-1.5)	15	0.54 (0.21-1.5)	14	0.45 (0.12-1.7)	13
Double positive T cells	123	0.039 (0.0073-0.21)	18	0.049 (0.015-0.17)	10	0.059 (0.014-0.24)	11	0.035 (0.0063-0.19)	11	0.027 (0.0066-0.11)	10	0.014 (0.0023-0.088)	10	0.012 (0.0033-0.043)	11	0.0081 (0.0036-0.018)	15	0.0091 (0.0032-0.026)	14	0.012 (0.0023-0.06)	13
TCR-γ/δ	127	0.087 (0.03-0.25)	18	0.12 (0.013-1.0)	10	0.17 (0.056-0.51)	11	0.18 (0.038-0.89)	11	0.21 (0.07-0.63)	10	0.16 (0.041-0.64)	10	0.16 (0.027-0.96)	11	0.16 (0.027-0.96)	15	0.15 (0.039-0.54)	15	0.071 (0.025-0.2)	16
Double negative T cells	123	0.059 (0.02-0.17)	18	0.075 (0.0077-0.73)	10	0.094 (0.026-0.34)	11	0.12 (0.032-0.43)	11	0.14 (0.047-0.43)	10	0.12 (0.027-0.5)	10	0.13 (0.021-0.85)	11	0.13 (0.033-0.54)	15	0.13 (0.039-0.44)	14	0.056 (0.019-0.17)	13
Helper T cells	123	0.014 (0.0034-0.061)	18	0.02 (0.0039-0.1)	10	0.03 (0.01-0.086)	11	0.017 (0.0056-0.051)	11	0.013 (0.00092-0.17)	10	0.008 (0.00087-0.073)	10	0.0047 (0.001-0.021)	11	0.0028 (0.00051-0.015)	15	0.0022 (0.00041-0.012)	14	0.001 (0.00012-0.0076)	13
Cytotoxic T cells	123	0.011 (0.0027-0.046)	18	0.012 (0.00025-0.14)b	10	0.04 (0.012-0.13)	11	0.043 (0.004-0.45)	11	0.046 (0.008-0.27)	10	0.03 (0.005-0.19)	10	0.019 (0.0019-0.2)	11	0.019 (0.0051-0.069)	15	0.02 (0.0021-0.19)	14	0.013 (0.0018-0.094)	13
Double positive T cells	123	0.0005 (0.000098-0.0026)	18	0.00082 (0.00012-0.0057)	10	0.0021 (0.00029-0.015)	11	0.0011 (0.000097-0.012)	11	0.0058 (0.000067-0.005)	10	0.00041 (0.000038-0.0045)	10	0.0003 (0.000038-0.0045)	11	0.0002 (0.000034-0.0011)	15	0.0002 (0.00002-0.0021)	14	0.0002 (0.000035-0.0011)	13

From Schatorjé EJH, Gemen EFA, Driessen GJA, et al: Paediatric reference values for the peripheral T cell compartment. Scand J Immunol *75:436–444, 2012.*

m, Months; *w*, weeks; *TCR*, T-cell receptor; *y*, years.

*The right limit of this tolerance interval was based on the original values (see Methods).

APPENDIX 44 Reference Ranges for Dendritic Cell Subpopulations in Peripheral Blood for Different Age Groups[*]

Dendritic Cell Subpopulations	AGE GROUPS			
	0-<2 (N = 16)	2-<5 (N = 19)	5-<10 (N = 27)	10-<18 (N = 38)
mDC/µl PB	16 (7-30)	15 (6-28)	13 (6-24)	10 (5-19)
mDC (%)	0.16% (0.08-0.24)	0.19% (0.09-0.30)	0.19% (0.09-0.34)	0.16% (0.08-0.33)
pDC/ µl PB	17 (8-38)	16 (7-34)	15 (6-29)	13 (5-23)
pDC (%)	0.19% (0.09-0.38)	0.19% (0.09-0.38)	0.20% (0.08-0.39)	0.20% (0.08-0.40)

From Heinze A, Elze MC, Kloess S, et al. Age-matched Dendritic cell subpopulations reference values in Childhood. Scand J Immunol *77:213–220, 2013*

%, Relative count per peripheral leukocytes; *mDC*, myeloid dendritic cell; *PB*, peripheral blood; *pDc*, plasmacytoid dendritic cell.

[*] Absolute counts (cells/µL), percentage of dentritic cells within the leucocytes population in PB: median and percentiles (5th to 95th percentile). Age expressed in years.

APPENDIX 45 Tibial Bone Marrow Cell Populations of Normal Infants from Birth to 18 Months of Age[*]

Cell Type	MONTH										
	0 (N = 57)[†]	1 (N = 71)	2 (N = 48)	3 (N = 24)	4 (N = 19)	5 (N = 22)	6 (N = 22)	9 (N = 16)	12 (N = 18)	15 (N = 12)	18 (N = 19)
Small lymphocytes	14.42 ± 5.54	47.05 ± 9.24	42.68 ± 7.90	43.63 ± 11.83	47.06 ± 8.77	47.19 ± 9.93	47.55 ± 7.88	48.76 ± 8.11	47.11 ± 11.32	42.77 ± 8.94	43.55 ± 8.56
Transitional cells	1.18 ± 1.13	1.95 ± 0.94	2.38 ± 1.35	2.17 ± 1.64	1.64 ± 1.01	1.83 ± 0.89	2.31 ± 1.16	1.92 ± 1.39	2.32 ± 1.90	1.70 ± 0.82	1.99 ± 1.00
Proerythroblasts	0.02 ± 0.06	0.10 ± 0.14	0.13 ± 0.19	0.10 ± 0.13	0.05 ± 0.10	0.07 ± 0.10	0.09 ± 0.12	0.07 ± 0.09	0.02 ± 0.04	0.07 ± 0.12	0.08 ± 0.13
Basophilic erythroblasts	0.24 ± 0.25	0.34 ± 0.33	0.57 ± 0.41	0.40 ± 0.33	0.24 ± 0.24	0.47 ± 0.33	0.32 ± 0.24	0.31 ± 0.24	0.30 ± 0.25	0.38 ± 0.37	0.50 ± 0.34
Early erythroblasts	0.27 ± 0.26	0.44 ± 0.42	0.71 ± 0.51	0.50 ± 0.38	0.28 ± 0.30	0.55 ± 0.36	0.41 ± 0.30	0.39 ± 0.28	0.39 ± 0.27	0.46 ± 0.36	0.59 ± 0.34
Polychromatic erythroblasts	13.06 ± 6.78	6.90 ± 4.45	13.06 ± 3.48	10.51 ± 3.39	6.84 ± 2.58	7.55 ± 2.35	7.30 ± 3.60	7.73 ± 3.39	6.83 ± 3.75	6.04 ± 1.56	6.97 ± 3.56
Orthochromatic erythroblasts	0.69 ± 0.73	0.54 ± 1.88	0.66 ± 0.82	0.70 ± 0.87	0.34 ± 0.30	0.46 ± 0.51	0.38 ± 0.56	0.39 ± 0.48	0.37 ± 0.51	0.50 ± 0.65	0.44 ± 0.49
Extruded nuclei	0.47 ± 0.46	0.16 ± 0.17	0.26 ± 0.22	0.19 ± 0.12	0.16 ± 0.17	0.14 ± 0.11	0.16 ± 0.22	0.22 ± 0.25	0.23 ± 0.25	0.17 ± 0.12	0.21 ± 0.19
Late erythroblasts	14.22 ± 7.14	7.60 ± 4.84	13.99 ± 3.82	11.40 ± 3.43	7.34 ± 2.54	8.16 ± 2.58	7.85 ± 4.11	8.34 ± 3.31	7.42 ± 4.11	6.72 ± 1.80	7.62 ± 3.63
Early/late erythroblasts ratio[‡]	1:50	1:15	1:18	1:22	1:23	1:15	1:17	1:19	1:17	1:15	1:10
Fetal erythroblasts	14.48 ± 7.24	8.04 ± 5.00	14.70 ± 3.86	11.90 ± 3.52	7.62 ± 2.56	8.70 ± 2.69	8.25 ± 4.31	8.72 ± 3.34	7.81 ± 4.26	7.18 ± 1.95	8.21 ± 0.19
Blood reticulocytes	4.18 ± 1.46	1.06 ± 1.13	3.39 ± 1.22	2.90 ± 0.91	1.65 ± 0.73	1.38 ± 0.65	1.74 ± 0.80	1.67 ± 0.52	1.79 ± 0.79	2.10 ± 0.91	1.84 ± 0.46

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APPENDIX 45 Tibial Bone Marrow Cell Populations of Normal Infants from Birth to 18 Months of Age (Continued)

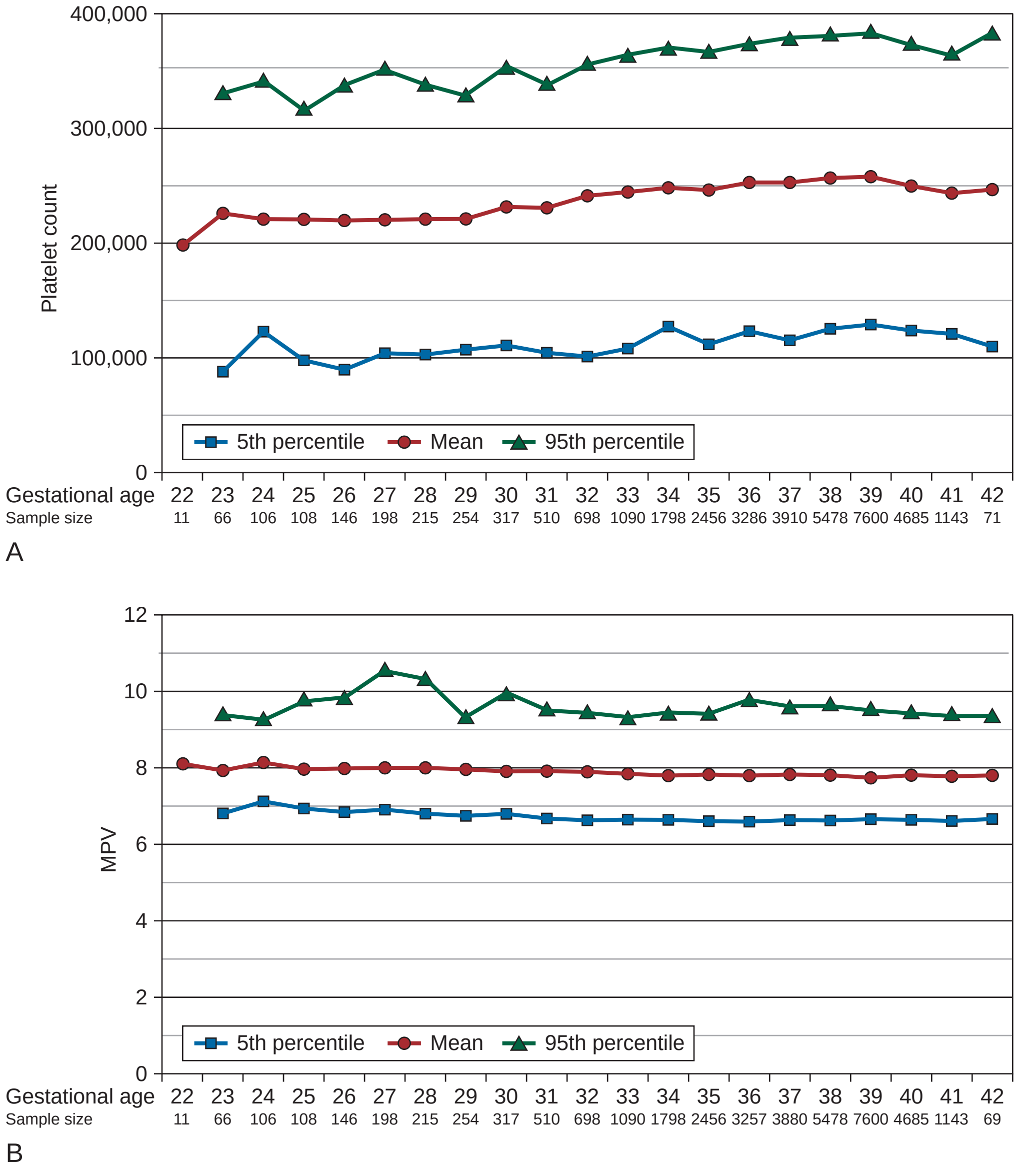
	MONTH										
Cell Type	**0 (N = 57)[†]**	**1 (N = 71)**	**2 (N = 48)**	**3 (N = 24)**	**4 (N = 19)**	**5 (N = 22)**	**6 (N = 22)**	**9 (N = 16)**	**12 (N = 18)**	**15 (N = 12)**	**18 (N = 19)**
Neutrophils											
Promyelocytes	0.79 ± 0.91	0.76 ± 0.65	0.78 ± 0.68	0.76 ± 0.80	0.59 ± 0.51	0.87 ± 0.80	0.67 ± 0.66	0.41 ± 0.34	0.69 ± 0.71	0.67 ± 0.58	0.64 ± 0.59
Myelocytes	3.95 ± 2.93	2.50 ± 1.48	2.03 ± 1.14	2.24 ± 1.70	2.32 ± 1.59	2.73 ± 1.82	2.22 ± 1.25	2.07 ± 1.20	2.32 ± 1.14	2.48 ± 0.94	2.49 ± 1.39
Early neutrophils	4.74 ± 3.43	3.27 ± 1.94	2.81 ± 1.62	3.00 ± 2.18	2.91 ± 2.01	3.60 ± 2.50	2.89 ± 1.71	2.48 ± 1.46	3.02 ± 1.52	3.16 ± 1.19	3.14 ± 1.75
Metamyelocytes	19.37 ± 4.84	11.34 ± 3.59	11.27 ± 3.38	11.93 ± 13.09	6.04 ± 3.63	11.89 ± 3.24	11.02 ± 3.12	11.80 ± 3.90	11.10 ± 3.82	12.48 ± 7.45	12.42 ± 4.15
Bands	28.89 ± 7.56	14.10 ± 4.63	13.15 ± 4.71	14.60 ± 7.54	13.93 ± 6.13	14.07 ± 5.48	14.00 ± 4.58	14.08 ± 4.53	14.02 ± 4.88	15.17 ± 4.20	14.20 ± 5.23
Mature neutrophils	7.37 ± 4.64	3.64 ± 2.97	3.07 ± 2.45	3.48 ± 1.62	4.27 ± 2.69	3.77 ± 2.44	4.85 ± 2.69	3.97 ± 2.29	5.65 ± 3.92	6.94 ± 3.88	6.31 ± 3.91
Late neutrophils	55.63 ± 7.98	29.08 ± 6.79	27.50 ± 6.88	31.00 ± 11.17	31.30 ± 7.80	29.73 ± 7.19	29.84 ± 6.74	29.86 ± 7.36	30.77 ± 8.69	34.60 ± 7.35	32.93 ± 7.01
Early/late neutrophil ratio	1:12	1:9	1:9	1:9	1:11	1:8	1:10	1:12	1:10	1:10	1:10
Total neutrophils	60.37 ± 8.66	32.35 ± 7.68	30.31 ± 7.27	34.01 ± 11.95	34.21 ± 8.61	33.12 ± 8.34	32.75 ± 7.03	32.33 ± 7.75	33.79 ± 8.76	37.76 ± 7.32	36.06 ± 7.40
Total eosinophils	2.70 ± 1.27	2.61 ± 1.40	2.50 ± 1.22	2.54 ± 1.46	2.37 ± 4.13	1.98 ± 0.86	2.08 ± 1.16	1.74 ± 1.08	1.92 ± 1.09	3.39 ± 1.93	2.70 ± 2.16
Total basophils	0.12 ± 0.20	0.07 ± 0.16	0.08 ± 0.10	0.09 ± 0.09	0.11 ± 0.14	0.09 ± 0.13	0.10 ± 0.13	0.11 ± 0.13	0.13 ± 0.15	0.27 ± 0.37	0.10 ± 0.12
Total myeloid cells	63.19 ± 9.10	35.03 ± 8.09	32.90 ± 7.85	36.64 ± 2.26	36.69 ± 8.91	35.40 ± 8.54	34.93 ± 7.52	34.18 ± 8.13	35.83 ± 8.84	41.42 ± 7.43	38.86 ± 7.92
Monocytes	0.88 ± 0.85	1.01 ± 0.89	0.91 ± 0.83	0.68 ± 0.56	0.75 ± 0.75	1.29 ± 1.06	1.21 ± 1.01	1.17 ± 0.97	1.46 ± 1.52	1.68 ± 1.09	2.12 ± 1.59
Miscellaneous											
Megakaryocytes	0.06 ± 0.15	0.05 ± 0.09	0.10 ± 0.13	0.06 ± 0.09	0.06 ± 0.06	0.08 ± 0.09	0.04 ± 0.07	0.09 ± 0.12	0.05 ± 0.08	0.00 ± 0.00	0.07 ± 0.12
Plasma cells	0.00 ± 0.02	0.02 ± 0.06	0.02 ± 0.05	0.00 ± 0.02	0.01 ± 0.03	0.05 ± 0.11	0.03 ± 0.07	0.01 ± 0.03	0.03 ± 0.07	0.07 ± 0.12	0.06 ± 0.08
Unknown blasts	0.31 ± 0.31	0.62 ± 0.50	0.58 ± 0.50	0.63 ± 0.60	0.56 ± 0.53	0.50 ± 0.37	0.56 ± 0.48	0.42 ± 0.50	0.37 ± 0.33	0.46 ± 0.32	0.43 ± 0.45
Unknown cells	0.22 ± 0.34	0.21 ± 0.25	0.16 ± 0.24	0.19 ± 0.21	0.23 ± 0.25	0.17 ± 0.22	0.10 ± 0.15	0.14 ± 0.17	0.11 ± 0.14	0.13 ± 0.18	0.20 ± 0.23
Damaged cells	5.79 ± 2.78	5.50 ± 2.46	5.09 ± 1.78	4.75 ± 2.30	4.80 ± 2.29	4.86 ± 1.25	5.04 ± 1.08	4.89 ± 1.60	5.34 ± 2.19	4.99 ± 1.96	5.05 ± 2.15
Total	6.38 ± 2.84	6.39 ± 2.63	5.94 ± 1.94	5.63 ± 2.36	5.66 ± 2.30	5.66 ± 1.41	5.78 ± 1.16	5.55 ± 1.74	5.90 ± 2.03	5.65 ± 2.02	5.81 ± 2.16

From Rosse C, Kraemer MJ, Dillon TL, et al: Bone marrow cell populations of normal infants; the predominance of lymphocytes. J Lab Clin Med *89:1228, 1997.*

*Percentages of cell types (mean ± standard deviation) is tibial bone marrow of infants from birth to 18 months of age. Data were obtained from normal American infants of black, white, and Asian racial origin. The changes in the marrow during the first 18 months of postnatal life are based on different counts of 1000 cells classified on stained smears on each of 10 serial marrow samples aspirated from the same population of infants. Criteria for including bone marrow data in this study consisted of absence of any clinical evidence of disease, normal rate of growth, and normal serum protein and transerffin saturations.

[†] N = number of infants studied at each stage.

[‡] Expressed in round figures for facilitating comparison. Means ± standard deviation were calculated from values obtained in individual infants, and statistical comparisons were performed.



Appendix 46 The first recorded platelet counts (**A**) and MPV determinations (**B**) obtained in the first three days after birth, are shown for neonates of 22 to 42 weeks gestation. Mean values are given by the red line and the 5th and 95th percentiles are given by the green and blue lines. **A,** Initial platelet counts. (b) Initial MPV measurement. Platelet values were measured using automated Beckman Coulter Hematology Analyzers (Beckman Coulter Inc., Fla., USA). *(From Wiedmeier SE, Henry E, Sola-Visner MC, et al: Platelet reference ranges for neonates, defined using data from over 47 000 patients in a multihospital healthcare system.* J Perinatol *29:130–136, 2009.)*

APPENDIX 47 Circulating Platelet Counts and Serum TPO Levels at Different Ages[*]

Age Categories	**Platelet Count (10^9/L)**	**Serum TPO (fmol/L)**
Core blood	288 ± 53	3.73 ± 1.48 (1.44-6.74)
2 days	303 ± 48	5.92 ± 1.4 (4.33-9.18)
5 days	338 ± 59	4.32 ± 0.94 (2.38-5.76)
1 month	343 ± 72	3.77 ± 1.45 (2.25-7.60)
2-11 months	365 ± 49	2.10 ± 0.69 (1.04-4.24)
1-2 years	314 ± 78	2.23 ± 0.89 (0.43-4.54)
3-4 years	304 ± 66	1.97 ± 0.67 (0.98-3.73)
5-6 years	303 ± 65	1.67 ± 0.66 (0.58-3.14)
7-10 years	295 ± 58	1.39 ± 0.63 (0.53-2.94)
11-15 years	251 ± 40	1.24 ± 0.40 (0.49-1.99)
Adult	234 ± 48	0.83 ± 0.36 (0.25-1.72)

From Ishiguro A, Nakahata T, Matsubara K, et al: Age-related changes in thrombopoietin in children: reference interval for serum thrombopoietin levels. Br J Haematol *106(4):884–888, 1999.*

TPO, Thrombopoietin.

[*] All values for children are significantly different (*P* <.001, Mann-Whitney U test) from values for adults. Data are expressed as mean ± standard deviation. The range of values for TPO is reported in parentheses.

APPENDIX 48 Comparison of Selected Coagulation Factor Values in Newborns[*]

Age	**Fibrinogen (mg/dL)**	**F II (U/mL)**	**F VIII (U/mL)**	**F IX (U/mL)**	**F XII (U/mL)**	**Antithrombin (U/mL)**	**Protein C (U/mL)**
Term							
Hathaway and Bonnar (1987) and Manco-Johnson et al. (1988)[†]	240 (150)	0.52 (0.25)	1.5 (0.55)	0.35 (0.15)	0.44 (0.16)	0.56 (0.32)	0.32 (0.16)
Andrew et al. (1987, 1988)[‡]	283 (177)	0.48 (0.26)	1.0 (0.50)	0.53 (0.25)	0.53 (0.20)	0.63 (0.25)	0.35 (0.17)
Corrigan (1992)[§]	246 (150)	0.45 (0.22)	1.68 (0.50)	0.40 (0.20)	0.44 (0.16)	0.52 (0.20)	0.31 (0.17)
Preterm							
Hathaway and Bonnar (1987) and Manco-Johnson et al. (1988)[†]	300 (120)	0.45 (0.26)	0.93 (0.54)	0.41 (0.20)	0.33 (0.23)	0.40 (0.25)	0.24 (0.18)
Andrew et al. (1987, 1988)[‡]	243 (150)	0.45 (0.20)	1.1 (0.50)	0.35 (0.19)	0.38 (0.10)	0.38 (0.14)	0.28 (0.12)
Corrigan (1992)[§]	240 (150)	0.35 (0.21)	1.36 (0.21)	0.35 (0.10)	0.22 (0.09)	0.35 (0.10)	0.28 (0.12)

From Hathaway W, Corrigan J: Report of scientific and standardization subcommittee on neonatal hemostasis. Thromb Haemost *65:323, 1991.*

[*] Data are expressed as mean and lower limits of normal. Preterm = 30- to 36-weeks' gestational age.

[†] Hathaway W, Bonnar J: *Hemostatic disorders of the pregnant woman and newborn infant*. New York, 1987, Elsevier; Manco-Johnson M, Marlar R, et al: Severe protein C deficiency in newborn infants. *J Pediatr* 113:359, 1988.

[‡] Andrew M, Paes B, Milner R, et al: Development of the human coagulation system in the full-term infant. *Blood* 70:165, 1987; Andrew M, Paes B, Milner R, et al: Development of the human coagulation system in the healthy premature infant. *Blood* 72:1651, 1988.

[§] Corrigan JJ Jr: Normal hemostasis in fetus and newborn. coagulation. In Polin RA, Fox WW, editors: *Fetal and neonatal physiology.* Philadelphia, 1992, Saunders, p. 1368–1371.

APPENDIX 49 Reference Ranges for Coagulation Parameters*, Inhibitors, and Fibrinolysis in Preterm Newborns

	Premature SGA Newborns (N = 68) (Mean ± SD and 2.5th to 97.5th Percentile)	Premature SGA Newborns (N = 71) (Mean ± SD and 2.5th to 97.5th Percentile)	S
[1]INR	1.35 ± 0.22 (1.02-2.09)	1.32 ± 0.20 (1.02-1.85)	NS
[1]PT(sec)	16.6 ± 2.1 (13.2-23.1)	16.4 ± 1.98 (13.3-21.4)	NS
[2]APTT(sec)	51 ± 11 (35.4-97.6)	51 ± 12 (34.2-102.9	NS
[1]Fibrinogen (mg/dL)	158 ± 46 (65-243)	183 ± 80 (64-478)	**0.029**
[1]IIc (%)	37.6 ± 6.5 (23.4-53)	37.2 ± 9 (20.5-58)	NS
[1]Vc (%)	61 ± 22 (23-128)	62 ± 20.4 (22.6-120)	NS
[1]VIIc (%)	61.2 ± 20.5 (17.9-116.5)	68.8 ± 23.7 (27.8-124.2)	NS
[1]VIIIc (%)	142 ± 80 (26-389)	116 ± 57 (28-276)	**0.041**
[2]IXc (%)	32 ± 22 (11.8-107.5)	28 ± 11 (11.6-60.5)	NS
[1]Xc (%)	41.2 ± 9.7 (18.2-65.5)	41.5 ± 10 (27.0-68.7)	NS
[1]XIc (%)	33.5 ± 14.2 (11.3-76.9)	30.5 ± 11.0 (13.2-60.4)	NS
[1]XIIc (%)	50 ± 22 (16-110.7)	47 ± 24 (12.7-119.6)	NS
[1]AT Act (%)	37.2 ± 11.0 (19.5-67.8)	39.0 ± 13.7 (16.7-80.3)	NS
[2]PC Act (%)	24 ± 8 (11.0-50.3)	24 ± 9 (10.4-52)	NS
[1]Free PS Act (%)	31.5 ± 9.0 (17-57)	31.1 ± 5.9 (20-47.6)	NS
[2]APCR	2.3 ± 0.30 (1.32-3.13)	2.2 ± 0.34 (1.43-3.16)	**0.017**
[2]tPA (ng/ml)	13.8 ± 8.2 (4.5-44.1)	11.4 ± 7.0 (3.2-34.6)	**0.021**
[2]PAI-1 (ng/ml)	55.3 ± 24.4 (14.9-102.6)	46 ± 24 (16.5-122)	**0.019**
[1]VWF Ag (%)	202 ± 64 (105-355)	193 ± 59 (95-339)	NS

From Mitsiako G, Giougi E, Chatziioannidis I, et al: Haemostatic profile of healthy premature small for gestational age neonates. Thromb Res *126:103–106, 2010.*

1, T test; *2,* Mann-Whitney U test; *Act,* activity; *Ag,* antigenic value; *c,* coagulant activity; *D,* difference; *NS,* not significant; *S,* significance.

*All parameters were determined utilizing a STA Compact Analyzer and reagents from Diagnostica Stago (Asnières sur Seine, France).

APPENDIX 50 Reference Ranges for Coagulation Tests in Healthy, Full-Term Newborns Compared with Normal Adults*

Test	Newborns	Adults	*P*<
PT (sec)	13.1 ± 0.9	11.9 ± 0.6	.0001
aPTT (sec)	35 ± 4.5	28.8 ± 2.7	.0001
Platelets (×10^9/L)	214 ± 55	258 ± 66	.0001
Fibrinogen (mg/dL)	251 ± 51	262 ± 44	NS
Factor II (%)	73 ± 7	100 ± 15	.0001
Factor V (%)	93 ± 13	98 ± 19	NS
Factor VII (%)	88 ± 12	95 ± 18	.005
Factor VIII (%)	113 ± 38	92 ± 21	.0001
Factor IX (%)	86 ± 18	94 ± 16	.003
Factor X (%)	72 ± 10	97 ± 15	.0001
Hematocrit (%)	59 ± 3.0	44 ± 2.5	.0001

From Cerneca F, de Vonderweid U, Simeone R, et al: The importance of hematocrit in the interpretation of coagulation tests in the full-term newborn infant. Hematologica *79:25, 1994.*

*Data were obtained in 71 newborns and 100 adults and expressed as mean ± standard deviation. Samples were collected with a constant anticoagulant-to-blood ration, based on a previous determination of hematocrit level.

APPENDIX 51 Template Bleeding Time in Neonate According to Gestational Age and in Children and Adolescents*

	≤28 Weeks			29-32 Weeks			33-37 Weeks			≥ 38 Weeks		
DOL	**BT**	**PL**	**MPV**	**BT**	**PL**	**MPV**	**BT**	**PL**	**MPV**	**BT**	**PL**	**MPV**
1	204 ± 80	255.5 ± 69.4	8.9 ± 1.2	207 ± 105	286.6 ± 81.1	10.8 ± 1.0	157 ± 68	307.0 ± 106.1	10.6 ± 0.8	107 ± 38	315.9 ± 80.9	10.5 ± 1.4
10	152 ± 59	286.0 ± 81.1	9.7 ± 1.3	146 ± 79	300.9 ± 124.3	10.4 ± 1.4	163 ± 92	347.6 ± 93.9	10.7 ± 1.1	88 ± 31	344.2 ± 99.4	10.5 ± 1.6
30	104 ± 45	277.5 ± 99.8	9.9 ± 1.4	173 ± 86	306.1 ± 125.0	10.5 ± 1.4	146 ± 92	314.1 ± 120.9	10.3 ± 1.3	82 ± 39	352.3 ± 139.3	11.3 ± 1.2

From Del Vecchio A, Latini G, Henry E, et al: Template bleeding times of 240 neonates born at 24 to 41 weeks gestation. J Perinatol *28:427–431, 2008.*

BT, Bleeding time; *DOL,* day of life; *MPV,* mean platelet volume; *PL,* platelets.

*Patients were tested on either day of life 1 (N 1/420 in each gestational age group), day of life 10 (N 1/420 per group), or day of life 30 (N 1/420 per group).

Bleeding Time in Children*

Age (y)	Number of Subjects	Bleeding Time (s)
0-2	33	180 ± 30
2-4	33	240 ± 60
4-9	75	300 ± 60
9-18	66	300 ± 70
0-18	201	270 ± 60
Adults	90	320 ± 90

From Aversa LA, Vázquez A, Peñalver JA, et al: Bleeding time in normal children. J Pediatr Hematol Oncol *17:25, 1995.*

s, Seconds; *y,* years.

*Values are expressed as mean ± standard deviation, using a disposable Simplate method.

APPENDIX 52 Reference Ranges for Global Coagulation Assays in Children and Adolescents

Assay	Method	1-6 Months N = 29† (14M/15F)	7-12 Months N = 25‡ (19M/6F)	1-5 Years N = 57 (35M/22F)	6-10 Years N = 56 (29M/27F)	11-18 Years N = 50§ (24M/26F)	> 19 Years N = 52 (27F/25M)
PT (sec)	Thromborel S	12.5/12.8*‖	12.2/12.4*‖	12.1/12.2*‖	12.6/12.6*‖	12.8/12.6*‖	11.7/11.8*
	BCS	11.2-15.5	11.4-13.5	11.2-13.4	11.5-14.0	11.4-13.8	10.7-12.9
	Innovin	10.7/10.7 *	10.6/10.6*	10.6/10.6 *	10.9/10.9*‖	10.8/10.9*‖	10.5/10.6*
	CA-1500	**10.0-12.7**	**9.5-12.8**	**10.0-11.4**	**10.2-11.6**	**10.1-11.9**	**9.7-11.4**
PT (%)	Thromborel S	92/89*‖	95/93*‖	97/96*‖	91/91*‖	89/91*‖	101/101*
	BCS	**64-108**	**81-105**	**81-108**	**76-104**	**78-105**	**88-116**
	Innovin	103/104*	106/106*	106/106*	100/100*‖	101/100*	108/108*
	CA-1500	**72-122**	**71-128**	**89-121**	**86-116**	**81-118**	**89-129**
APTT (sec)	Pathromtin SL	41/42*‖	39/39*‖	36/37*‖	37/37*‖	35/36*‖	34/34*
	BCS	**33-56**	**32-49**	**31-44**	**31-44**	**30-43**	**27-40**
	Actin FS	29/29*‖	28/28*‖	27/27*‖	28/28*‖	27/27*‖	25/25*
	CA-1500	**21-33**	**24-33**	**24-30**	**25-32**	**25-30**	**22-28**
TT (sec)	Thromboclotin	19.2/20.0‖	18.0/18.0	17.0/17.2	17.5/17.4	17.4/17.8	17.4/17.5
	CA-1500	**16.2-24.9**	**15.4-21.1**	**15.3-19.7**	**14.5-19.9**	**15.2-24.0**	**15.5-20.5**
BT (sec)	Batroxobin	21.0/21.4‖	20.2/20.5	20.2/20.3	20.2/20.2	19.8/19.9	20.1/20.1
	Reagent CA-1500	19.7-25.0	19.1-24.0	18.8-22.7	19.1-21.5	18.8-21.5	18.7-22.4

Grimminck B, Geerts J, et al: Age dependency of coagulation parameters during childhood and puberty. J Thromb Haemost *10:2254–2263, 2012.*

APTT, Activated partial thromboplastin time; *BCS,* Behring Coagulation System; *BT,* batroxobin time; *CA-1500,* Sysmex CA-1500 Analyzer; *F,* female; *M,* male; *PT,* prothrombin time; *sec,* seconds; *TT,* thrombin time.

*Indicates statistically significant difference between devices for the student's t-test.

†n = 28 for APTT with the BCS.

‡n = 24 for PT, TT, and BT with the CA-1500.

§n = 49 for batroxobin time (one sample was excluded because of an extremely outlying result of 13.3 seconds). Data are presented as median/mean with t-test results of between methods and age comparisons in the first row, whereas the second row shows the boundaries including 90% of the central population.

‖Indicates statistically significant difference between child groups and adults for the student's t-test.

APPENDIX 53 Reference Ranges for Single Coagulation Factors in Children and Adolescents

Assay	Method	1-6 Months N = 29[†] (14M/15F)	7-12 Months N = 25[‡] 19M/6F	1-5 Years N = 57[§] 35M/22F	6-10 Years N = 56[¶] 29M/27F	11-18 Years N = 50[‖] 24M/26F	> 19 Years N = 52[†] 27F/25M
Fbg (g/L)	Multifibren U	2.2/2.3[#]	2.3/2.6[#]	2.5/2.7[*]	2.3/2.6[#]	2.3/2.5[#]	2.9/3.0
	BCS	**1.5-3.8**	**1.8-4.8**	**1.9-3.9**	**2.0-3.9**	**1.9-3.7**	**2.1-4.2**
	Dade Thrombin	1.9/2.0[#]	2.2/2.3[#]	2.4/2.5[#]	2.3/2.4[#]	2.3/2.4[#]	2.7/2.8
	CA-1500	**1.3-3.3**	**1.6-4.0**	**1.7-3.5**	**1.8-3.6**	**1.8-3.3**	**2.0-3.9**
FII (%)	Thromborel S	93/91[#]	98/99[#]	104/105[#]	99/99[#]	96/99[#]	117/119
	BCS	**66-112**	**83-132**	**85-126**	**78-121**	**78-132**	**96-147**
	Innovin	86/86[#]	95/97[#]	102/103[#]	97/98[#]	92/94[#]	114/116
	CA-1500	**60-109**	**77-134**	**81-126**	**77-116**	**70-120**	**93-151**
FV (%)	Thromborel S	114/114	114/117[*]	108/111	97/99[#][*]	95/99[#][*]	112/113[*]
	BCS	**82-145**	**97-148**	**85-153**	**80-123**	**76-132**	**84-149**
	Innovin	118/110[#]	102/102[#][*]	102/104[#]	93/92[*]	87/87[*]	89/91[*]
	CA-1500	**56-148**	**66-141**	**68-143**	**62-127**	**55-119**	**57-128**
FVII (%)	Thromborel S	98/97[#]	96/98[#]	99/99[#][*]	96/98[#][*]	100/101[#][*]	105/108
	BCS	**54-126**	**74-131**	**81-117**	**79-119**	**75-130**	**86-142**
	Innovin	93/91[#]	89/88	84/85[#][*]	86/87[#][*]	86/86[#][*]	101/101
	CA-1500	**38-129**	**41-148**	**61-111**	**61-127**	**55-115**	**67-146**
FVIII (%)	Pathromtin SL	90/96[#]	95/100[#]	109/109[#][*]	100/101[#][*]	109/108[#][*]	123/123[*]
	BCS	**58-144**	**59- >152**	**76-143**	**68-137**	**70-148**	**87- >152**
	Actin FS	108/107[#]	116/119[#]	124/125[#][*]	118/119[#][*]	118/122[#][*]	133/140[*]
	CA-1500	**67-141**	**70-213**	**83-170**	**75-163**	**80-166**	**96-216**
FIX (%)	Pathromtin SL	53/57[#]	64/68[#]	77/78[#]	78/80[#]	84/85[#]	102/104[*]
	BCS	**41-87**	**42-109**	**58-99**	**57-106**	**60-117**	**78-139**
	Actin FS	57/57[#]	71/72[#]	78/78[#]	77/80[#]	87/89[#]	110/116[*]
	CA-1500	**44-78**	**46-114**	**63-97**	**60-108**	**72-116**	**87-174**
FX (%)	Thromborel S	90/90[#]	100/99[#]	104/104[#]	95/95[#]	88/94[#][*]	112/115
	BCS	**66-132**	**74-124**	**84-129**	**74-120**	**73-128**	**90-149**
	Innovin	88/87[#]	97/99[#]	101/100[#]	92/92[#]	84/86[#][*]	110/114
	CA-1500	**55-120**	**67-146**	**75-124**	**69-118**	**66-117**	**78-159**
FXI (%)	Pathromtin SL	83/80[#]	86/88[#]	100/100	95/96[#]	88/91[#]	104/104[*]
	BCS	**54-101**	**65-125**	**72-134**	**75-127**	**72-122**	**77-130**
	Actin FS	85/82[#]	88/91[#]	104/104[#]	99/100[#]	93/95[#]	115/113[*]
	CA-1500	**57-105**	**64-129**	**74-134**	**78-131**	**78-122**	**83-158**
FXII (%)	Pathromtin SL	75/72[#]	88/81[#]	95/92[#]	96/90[#]	96/89[#]	102/101
	BCS	**29-112**	**35-113**	**44-127**	**41-122**	**44-116**	**52-140**
	Actin FS	76/74[#]	82/82[#]	88/87[#]	92/88[#]	92/88[#]	106/108
	CA-1500	**28-116**	**31-126**	**36-122**	**37-123**	**43-122**	**53-165**
FXIII (%)	Berichrom FXIII	96/99[#]	97/97[#]	99/100[#]	104/103[#]	99/97[#]	116/115
	BCS	63-152	42-128	71-139	76-133	64-133	68- >156

Appel IM, Grimminck B, Geerts J, et al: Age dependency of coagulation parameters during childhood and puberty. J Thromb Haemost *10:2254–2263, 2012.*

BCS, Behring Coagulation System; *CA-1500,* Sysmex CA-1500 Analyzer; *F,* female; *Fbg,* fibrinogen; *M,* male; *PT,* prothrombin time; *sec,* seconds.

[*] Indicates statistically significant difference between devices for the student's t-test.

[†] *n* = 28 for FVII, FVIII, FIX, FX with the BCS and *n* = 27 for FXIII.

[‡] *n* = 24 for Fbg, FII, FV, FVII, FX, and FIX with the CA-1500, *n* = 23 for FXI with the BCS and *n* = 18 for FXIII.

[§] *n* = 53 for FXI with the BCS and *n* = 50 for FXIII.

[‖] *n* = 48 for FXI with BCS and FXIII; *n* = 51 for FXI with BCS, and *n* = 49 for FXIII. Data are presented as median/mean with t-test results of between methods and age comparisons in the first row, whereas the second row shows the boundaries including 90% of the central population.

[¶] *n* = 55 for FII with the CA-1500, *n* = 53 for FXI with BCS, and *n* = 51 for FXIII.

[#] Indicates statistically significant difference between child groups and adults for the student's t-test.

APPENDIX 54 Reference Values for the Inhibitors of Coagulation in Healthy Children Aged 1 to 16 Years Compared with Adults[*]

	AGE			
	1 to 5 y	**6 to 10 y**	**11 to 16 y**	**Adult**
Coagulation Inhibitors	Mean (Boundary)	Mean (Boundary)	Mean (Boundary)	Mean (Boundary)
ATIII (U/mL)	1.11 (0.82-1.39)	1.11 (0.90-1.31)	1.05 (0.77-1.32)	1.0 (0.74-1.26)
α_2M (U/mL)	1.69 (1.14-2.23)[†]	1.69 (1.28-2.09)[†]	1.56 (0.98-2.12)[†]	0.86 (0.52-1.20)
C$_1$-Inh (U/mL)	1.35 (0.85-1.83)[†]	1.14 (0.88-1.54)	1.03 (0.68-1.50)	1.0 (0.71-1.31)
α_1AT (U/mL)	0.93 (0.39-1.47)	1.00 (0.69-1.30)	1.01 (0.65-1.37)	0.93 (0.55-1.30)
HCII (U/mL)	0.88 (0.48-1.28)[†]	0.86 (0.40-1.32)[†]	0.91 (0.53-1.29)[†]	1.08 (0.66-1.26)
Protein C (U/mL)	0.66 (0.40-0.92)[†]	0.69 (0.45-0.93)[†]	0.83 (0.55-1.11)[†]	0.96 (0.64-1.28)
Protein S				
Total (U/mL)	0.86 (0.54-1.18)	0.78 (0.41-1.14)	0.72 (0.52-0.92)	0.81 (0.60-1.13)
Free (U/mL)	0.45 (0.21-0.69)	0.42 (0.22-0.62)	0.38 (0.26-0.55)	0.45 (0.27-0.61)

From Andrew M, Vegh P, Johnston M, et al: Maturation of the hemostatic system during childhood. Blood *80:1998, 1992.*

y, Years.

[*] All values are expressed in units per milliliter, where for all factors pooled plasma contains 1.0 U/mL, with the exception of free protein S, which contains a mean of 0.4 U/mL. All values are given as a mean, followed by the lower and upper boundary encompassing 95% of the population. Between 20 and 30 samples were assayed for each value for each age group. Some measurements were skewed because of a disproportionate number of high values. The lower limits, which exclude the lower 2.5% of the population, are given.

[†] Values that are significantly different from those of adults.

APPENDIX 55 Reference Ranges for Coagulation Inhibitors in Children and Adolescents[‖]

Assay	**Method**	**1-6 Months N = 29[†] 14M/15F**	**7-12 Months N = 25[‡] 19M/6F**	**1-5 Years N = 57 35M/22F**	**6-10 Years N = 56 29M/27F**	**11-18 Years N = 50 24M/26F**	**>19 Years N = 52 27F/25M**
AT (%)	INNOVANCE	105/104[§]	110/109[§]	110/109[§]	108/107[§]	104/104[§]	116/115
	AT, **BCS**	**81-126**	**90-132**	**93-128**	**92-122**	**90-119**	**97-133**
	Berichrom AT	106/103[§]	110/108[§]	113/113[*]	110/109[§]	105/106[§]	113/114
	CA-1500	**78-129**	**88-132**	**97-129**	**97-122**	**93-122**	**98-131**
PS (%)	Protein S Ac	78/19[§]	81/80[§]	85/83[§]	84/84[*][§]	82/86[§]	101/105[*]
	BCS	**60-103**	**61-95**	**65-99**	**63-97**	**69-119**	**83- >130**
	Protein S Ac	84/83[§]	85/82[§]	85/87[§]	87/89[*][§]	90/90[§]	116/114[*]
	CA-1500	**59-99**	**59-110**	**60-115**	**63-116**	**62-126**	**86- >130**
PC (%)	Protein C	70/71[§]	83/85[§]	97/97[§]	98/97[§]	100/103[§]	120/118
	Reagent, **BCS**						
	Berichrom	**41-115**	**60-117**	**63-133**	**62-134**	**71-144**	**78-148**
	Protein C	66/67[§]	76/78[§]	88/92[§]	90/92[§]	93/96[§]	114/115
	CA-1500	**43-102**	**59-103**	**71-125**	**75-120**	**70-131**	**83-153**

Appel IM, Grimminck B, Geerts J, et al: Age dependency of coagulation parameters during childhood and puberty. J Thromb Haemost *10:2254–2263, 2012.*

AT, Antithrombin; *BCS,* Behring Coagulation System; *CA-1500,* Sysmex CA-1500 Analyzer; *F,* female; *M,* male; *PT,* prothrombin time; *sec,* seconds.

[*] Indicates statistically significant difference between devices for the student's *t*-test.

[†] N = 28 for PS with CA-1500 and N = 27 for PC with BCS.

[‡] N = 24 for AT with CA-1500.

[§] Indicates statistically significant difference between child groups and adults for the student's *t*-test.

[‖] Data are presented as median/mean with t-test results of between methods and age comparisons in the first row, whereas the second row shows the boundaries including 90% of the central population.

APPENDIX 56 Reference Ranges for D-dimer, α_2-Antiplasmin, and Plasminogen in Children and Adolescents

Assay	Method	1-6 Months N = 29[†] 14M/15F	7-12 Months N = 25[†] 19M/6F	1-5 Years N = 57 35M/22F	6-10 Years N = 56 29M-27F	11-18 Years N = 50 24M/26F	>19 Years N = 52 27F/25M
D-dimer (mg/L FEU)	INNOVANCE D-dimer, BCS	0.28/0.50[*]	0.25/0.46[*]	0.19/0.25	0.19-0.24	<0.17/0.28	<0.17/0.21
		<0.17-2.81	<0.17-3.32	<0.17-0.64	<0.17-0.49	<0.17-0.99	<0.17-0.44
	INNOVANCE D-dimer, CA-1500	0.33/0.39[*]	0.27/0.34[*]	0.20/0.27	0.20/0.26	<0.19/0.27	<0.19/0.23
		<0.19-3.49	<0.19-10.9	<0.19-0.65	<0.19-0.52	<0.19-0.75	<0.19-0.48
α_2-antiplasmin (%)	Berichrom α_2- antiplasmin, CA-1500	122/121	123/125[*]	128/128[*]	119/121	114/113[*]	118/119
		103-139	100-151	107-145	103-140	97-126	103-133
Plasminogen (%)	Berichrom Plasminogen, CA-1500	81/79[*]	93/94[*]	104/106[*]	99/99[*]	95/99[*]	112/117
		56-102	66-115	84-130	75-126	83-128	92-150

Appel IM, Grimmick B, Geerts J, et al: Age dependency of coagulation parameters during childhood and puberty. J Thromb Haemost *2254–2263, 2012.*

BCS, Behring Coagulation System; *CA-1500,* Sysmex CA-1500 Analyzer; *F,* female; *FEU,* fibrinogen equivalent units; *M,* male; *PT,* prothrombin time; *sec,* seconds.

Data are presented as median/mean with t-test results of between methods and age comparisons in the first row, whereas the second row shows the boundaries including 90% of the central population.

[*] Indicates statistically significant difference between child groups and adults for the student's t-test. Differences between devices for D-dimer were statistically not significant for student's t-test.

[†] N = 28 for D-dimer with CA-1500; N = 24 for α_2-antiplasmin and plasminogen.

APPENDIX 57 Reference Intervals for FVIII: C, VWF: Ag, and VWF: RCo and Relation to ABO (H) Blood Group[*]

	AGE GROUP					
Coagulation Factors (%)	**1-3 Months**	**4-6 Months**	**7-12 Months**	**13 Months-4 Years**	**5-9 Years**	**10-18 Years**
FVIII: C						
All	96	93	117	113	110	119
	(68-179)	(71-147)	(67-178)	(78-177)	(78-182)	(79-185)
	N = 40	N = 47	N = 45	N = 242	N = 127	N = 71
	26M/14F	20M/27F	30M/15F	188M/54F	89M/38F	39M/32F
Blood group A/B/AB	95	97	117	122[†]	120[†]	134[†]
	(67-176)	(78-144)	(78-178)	(85-182)	(81-189)	(84-202)
	N = 22	N = 29	N = 26	N = 127	N = 62	N = 32
Blood group O	93	88	119	104	99	102
	(70-181)	(65-181)	(59-187)	(70-153)	(78-166)	(68-161)
	N = 16	N = 18	N = 19	N = 108	N = 64	N = 39
VWF: Ag						
All	143	110	105	89	90	99
	(81-191)	(66-190)	(60-154)	(54-149)	(59-151)	(55-170)
	N = 38	N = 47	N = 45	N = 240	N = 126	N = 71
	25M/13F	20M/27F	30M/15F	187M/53F	89M/37F	39M/32F
Blood group A/B/AB	122	113	108	99[†]	106[†]	124[†]
	(88-186)	(77-166)	(66-146)	(65-150)	(60-174)	(85-193)
	N = 20	N = 29	N = 26	N = 126	N = 62	N = 32
Blood group O	163	100	95	80	76	80
	(78-191)	(60-245)	(51-183)	(53-125)	(58-126)	(51-150)
	N = 16	N = 18	N = 19	N = 107	N = 63	N = 39
VWF: RCo						
All	136	105	96	87	87	89
	(72-196)	(54-206)	(54-151)	(51-145)	(47-159)	(45-163)
	N = 40	N = 47	N = 45	N = 242	N = 127	N = 70
	26M/14F	20M/27F	30M/15F	188M/54F	89M/38F	38M/32F
Blood group A/B/AB	135	120	111	93[†]	97[†]	113[†]
	(83-186)	(57-206)	(60-153)	(55-153)	(51-181)	(50-181)
	N = 22	N = 29	N = 26	N = 127	N = 62	N = 32
Blood group O	139	91	85	78	72	73
	(67-205)	(52-288)	(35-151)	(40-126)	(40-133)	(43-135)
	N = 16	N = 18	N = 19	N = 108	N = 64	N = 38

Klarmann D, Eggert C, Geisen C, et al: Association of ABO(H) and I blood group system development with von Willebrand factor and Factor VIII plasma levels in children and adolescents. Transfusion *50:1571–1580, 2010.*

[*] For each assay the first two rows show the median and the central 90% reference intervals. The second two rows show the number *(N)* of individual samples and the ratio of males *(M)* to females *(F)* for each group.

[†] Blood group A/b/AB values are significantly different from blood group O values (*P* <.01 Mann-Whitney test).

APPENDIX 58 Reference Values for the Fibrinolytic System in Healthy Children Aged 1 to 16 Years Compared with Adults

	AGE			
	1 to 5 y	**6 to 10 y**	**11 to 16 y**	**Adult**
	Mean (Boundary)	***Mean (Boundary)***	***Mean (Boundary)***	***Mean (Boundary)***
Plasminogen (U/mL)	0.98 (0.78-1.18)	0.92 (0.75-1.08)	0.86 (0.68-1.03)	0.99 (0.77-1.22)
Tissue plasminogen activator (TPA) (ng/mL)	2.15 (1-4.5)*	2.42 (1.0-5)*	2.16 (1-4)*	4.90 (1.4-8.4)
α_2-antiplasm (α-AP) (U/mL)	1.05 (0.93-1.17)	0.99 (0.89-1.10)	0.98 (0.78-1.18)	1.02 (0.68-1.36)
Plasminogen activator inhibitor (PAI) (U/mL)	5.42 (1-10)	6.79 (2-12)*	6.07 (2-10)	3.60 (0-11)

From Andrew M, Vegh P, Johnston M, et al: Maturation of the hemostatic system during childhood. Blood *89:1998, 1992.*

For α2-AP, values are expressed as units per millimeter, where pooled plasma contains 1 U/mL. Values for TPA are given as nanograms per millimeter. Values for PAI are given as U/mL, where 1 U of PAI activity is defined as the amount of PAI that inhibits 1 IU of human-single chain TPA. All values are given as the mean, followed by the lower and upper boundary encompassing 95% of the population (boundary).

*Values that are significantly different from those of adults.

APPENDIX 59 Endogenous Plasma Concentrations of Thrombin-Antithrombin Complexes (TATs) and Prothrombin Fragment 1.2 (F1.2) in Children and Adults*

	1-5 Years	**6-10 Years**	**11-16 Years**	**20-45 Years**
TAT (μg/L)	2.3 ± 0.08	2.38 ± 0.13	2.8 ± 0.18	2.15 ± 0.09
F1.2 (nm/L)	1.04 ± 0.06	0.87 ± 0.07	0.82 ± 0.06	0.83 ± 0.06

From Andrew M, Mitchell L, Vegh P, et al: Thrombin regulation in children differs from adults in the absence and presence of heparin. Thromb Haemost *72:836, 1994.*

*Data are expressed as mean ± standard error of the mean.

APPENDIX 60 Coagulation Screening Tests and Factor Levels in Fetuses and Full-Term Newborns

	FETUSES (WEEKS' GESTATION)				
Parameter	**19-23 (N = 20)**	**24-29 (N = 22)**	**30-38 (N = 22)**	**Newborns (N = 60)**	**Adults (N = 40)**
PT (s)	32.5 (19-45)	32.3 (19-44)†	22.6 (16-30)*	16.7 (12.0-23.5)*	13.5 (11.4-14.0)
PT (INR)	6.4 (1.7-11.1)	6.2 (2.1-10.6)†	3.0 (1.5-5.0)*	1.7 (0.9-2.7)*	1.1 (0.8-1.2)
APTT (s)	168.6 (83-250)	154.0 (87-210)†	104.8 (76-128)†	44.3 (35-52)*	33.0 (25-39)
TCT (s)	34.2 (24-44)*	26.2 (24-28)*	21.4 (17.0-23.3)	20.4 (15.2-25.0)†	14.0 (12-16)
Factor					
I (g/L Von Clauss)	0.85 (0.57-1.50)	1.12 (0.65-1.65)	1.35 (1.25-1.65)	1.68 (0.95-2.45)†	3.0 (12-16)
I Ag (g/L)	1.08 (0.75-1.50)	1.93 (1.56-2.40)	1.94 (1.30-2.40)	2.65 (1.68-3.60)†	3.5 (2.50-5.20)
IIc(%)	16.9 (10-24)	19.9 (11-30)*	27.9 (15-50)†	43.5 (27-64)†	98.7 (70-125)
VIIc (%)	27.4 (17-37)	33.8 (18-48)*	45.9 (31-62)	52.5 (28-78)†	101.3 (68-130)
IXc (%)	10.1 (6-14)	9.9 (5-15)	12.3 (5-24)†	31.8 (15-50)†	104.8 (70-142)
Xc (%)	20.5 (14-29)	24.9 (16-35)	28.0 (16-36)†	39.6 (21-65)†	99.2 (75-125)
Vc (%)	32.1 (21-44)	36.8 (25-50)	48.9 (23-70)†	89.9 (50-140)	99.8 (65-140)
VIIIc (%)	34.5 (18-50)	35.5 (20-52)	50.1 (27-78)†	94.3 (38-150)	101.8 (55-170)
XIc (%)	13.2 (8-19)	12.1 (6-22)	14.8 (6-26)†	37.2 (13-62)*	100.2 (70-135)
XIIc (%)	14.9 (6-25)	22.7 (6-40)	25.8 (11-50)†	69.8 (25-105)†	101.4 (65-144)
PK (%)	12.8 (8-19)	15.4 (8-26)	18.1 (8-28)†	35.4 (21-53)†	99.8 (65-135)
HMWK (%)	15.4 (10-22)	19.3 (10-26)	23.6 (12-34)†	38.9 (28-53)†	98.8 (68-135)

From Reverdiau Moalic P, Delahouse B, Body G, et al: Evaluation of blood coagulation activators and inhibitors in the healthy human fetus. Blood *88:900, 1996.*

Ag, Antigenic value; *c,* coagulant activity.

Values are the mean, followed in parentheses by the lower and upper boundaries including 95% of the population.

**P* <.05

†*P* <.01

APPENDIX 61 Coagulation Inhibitors in Fetuses and Full-Term Newborns

	FETUSES (WEEKS' GESTATION)				
Parameter	**19-23 (N = 20)**	**24-29 (N= 22)**	**30-38 (N = 22)**	**Newborns (N = 60)**	**Adults (N = 40)**
AT (%)	20.0 (12-31)*	30.0 (20-39)	37.1 (24-55)[†]	59.4 (42-80)[†]	99.8 (65-130)
HCII (%)	10.3 (6-16)	12.9 (5.5-20)	21.2 (11-33)[†]	52.1 (19-99)[†]	101.4 (70-128)
TFPI (ng/ML)	21.0 (16.0-29.2)	20.6 (13.4-33.2)	20.7 (10.4-31.5)[†]	38.1 (22.7-55.8)[†]	73.0 (50.9-90.1)
PC Ag (%)	9.5 (6-14)	12.1 (8-16)	15.9 (8-30)[†]	32.5 (21-47)[†]	100.8 (68-125)
PC Act (%)	9.6 (7-13	10.4 (8-13)	14.1 (8-18)*	28.2 (14-42)[†]	98.8 (68-129)
Total PS (%)	15.1 (11-21)	17.4 (14-25)	21.0 (15-30)[†]	38.5 (22-55)[†]	99.6 (72-118)
Free PS (%)	21.7 (13-32)	27.9 (19-40)	27.1 (18-40)[†]	49.3 (33-67)[†]	98.7 (72-128)
Ratio of free PS to total PS	0.82 (0.75-0.92)	0.83 (0.76-0.95)	0.79 (0.70-0.89)[†]	0.64 (0.59-0.98)[†]	0.41 (0.38-0.43)
C4b-BP (%)	1.8 (0.6)	6.1 (1-12.5)	9.3 (5-14)	18.6 (3-40)[†]	100.3 (70-124)

From Reverdiau Moalic P, Delahouse B, Body G, et al: Evaluation of blood coagulation activators and inhibitors in the healthy human fetus. Blood *1996;88:900, 1996.*

Values are the mean, followed in parentheses by the lower and upper boundaries including 95% of the population.

Ag, Antigenic value; *AT,* antithrombin; *c,* coagulant activity; *HCII,* heparin cofactor II; *TFPI,* tissue factor pathway inhibitor; *PC,* protein C; *PS,* Protein S; *C4b-BP,* C4b binding protein.

**P* <.05

[†] *P* <.01

APPENDIX 62 Age-Specific and Sex-Specific Pediatric Reference Intervals for C3, C4, and high sensitivity C-reactive Protein

		CHEMISTRY									
		FEMALE REFERENCE INTERVAL					MALE REFERENCE INTERVAL				
Analyte	**Age**	**Lower Limit**	**Upper Limit**	**No. of Samples**	**Lower Limit Confidence Interval**	**Upper Limit Confidence Interval**	**Lower Limit**	**Upper Limit**	**No. of Samples**	**Lower Limit Confidence Interval**	**Upper Limit Confidence Interval**
C3, mg/dL	0-14 days	50	121	155	42-57	118-130	50	121	155	42-57	118-130
	15 days to < 1 year	51	160	151	31-55	150-165	51	160	151	31-55	150-165
	1 to <19 years	83	152	877	82-87	151-157	83	152	877	82-87	151-157
C4, mg/dL	0 to <1 year	7	30	353	5-8	28-32	7	30	353	5-8	28-32
	1 to <19 years	13	37	864	12-13	36-38	13	37	864	12-13	36-38
Hs-CRP, mg/L	0 to 14 days	0.3	6.1	139	0.2-0.4	5.4-6.3	0.3	6.1	139	0.2-0.4	5.4-6.3
	15 days to <15 years	0.1	1.0	653	0.1-0.1	0.9-1.1	0.1	1.0	653	0.1-0.1	0.9-1.1
	15 to <19 years	0.1	1.7	196	0.1-0.1	1.6-1.9	0.1	1.7	196	0.1-0.1	1.6-1.9

From Colantino DA, Kyriakopoulou, Chan MK, et al: Closing the gaps in pediatric laboratory reference intervals: a CALIPER Database of 40 biochemical markers in a healthy and multiethnic population of children. Clin Chem *58:854–868, 2012.*

APPENDIX 63 Serum Acid Glycoprotein (AGP), α_1-Antitrypsin (α_1-AT), and Haptoglobin Levels Reference Ranges at Different Ages

Age (Years)	Acid Glycoprotein (g/L)			α_1-Antitrypsin (g/L)			Haptoglobin (g/L)		
	2.5th	50th	97.5th	2.5th	50th	97.5th	2.5th	50th	97.5th
Males									
1	0.52	0.88	1.47	0.87	1.40	1.91	0.07	1.11	3.1
4	0.50	0.84	1.42	0.89	1.44	1.96	0.05	0.83	2.31
7	0.49	0.83	1.39	0.90	1.45	1.98	0.04	0.74	2.05
10	0.47	0.80	1.34	0.91	1.46	1.99	0.04	0.68	1.90
14	0.44	0.74	1.25	0.86	1.38	1.88	0.27	0.81	1.63
18	0.46	0.78	1.31	0.83	1.34	1.83	0.31	0.93	1.87
20	0.47	0.80	1.34	0.83	1.33	1.82	0.32	0.98	1.97
30	0.51	0.87	1.46	0.82	1.31	1.79	0.38	1.15	2.31
Females									
1	0.63	1.07	1.80	0.92	1.48	2.02	0.08	1.38	3.84
4	0.54	0.92	1.55	0.90	1.44	1.97	0.06	0.95	2.63
7	0.51	0.86	1.45	0.89	1.43	1.95	0.05	0.81	2.26
10	0.49	0.83	1.40	0.88	1.42	1.94	0.04	0.74	2.05
14	0.48	0.80	1.35	0.88	1.41	1.93	0.31	0.95	1.90
18	0.46	0.78	1.31	0.92	1.48	2.02	0.35	1.07	2.14
20	0.46	0.77	1.31	0.92	1.49	2.03	0.37	1.11	2.23
30	0.50	0.83	1.40	0.90	1.45	1.98	0.40	1.22	2.45

From Ritchie, RF, Palomaki GE, Neveux LM, et al:Reference distributions for the positive acute phase proteins, alpha1-acid glycoprotein (orosomucoid), alpha-antitrypsin, and haptaglobin: a comparison of a large cohort to the world's literature. J Clin Lab Anal *14(6):265–270, 2000; see also Ritchie, RF, Palomaki GE, Neveux LM, et al: Reference distributions for the positive acute phase serum proteins, alpha1-acid glycoprotein (orosomucoid), alpha1-antitrypsin, and haptoglobin: a practical, simple and clinically relevant approach in a large cohort.* J Clin Lab Anal *14(6):284–292, 2000.*

APPENDIX 64 Plasma Levels of Immunoglobulin A in Males and Females

Age	g/L	mg/dL
Lockitch et al (1988)*		
0-12 m	0.00-1.00	0-100
1-3 y	0.24-1.21	24-121
4-6 y	0.33-2.35	33-235
7-9 y	0.41-3.68	41-368
10-11 y	0.64-2.46	64-246
12-13 y	0.70-4.32	70-432
14-15 y	0.57-3.00	57-300
16-19 y	0.74-4.19	74-419
Children's Hospital†		
Newborn	0.00-0.11	0-11
1-3 m	0.06-0.05	6-50
4-6 m	0.08-0.90	8-90
7-12 m	0.16-1.00	16-100
1-3 y	0.20-2.30	20-230
3-6 y	0.50-1.50	50-150
Adult	0.50-2.00	50-200

m, Month; *y*, year.

*Lockitch G, Halstead AC, Quigley G, et al: Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. *Clin Chem* 34:1618, 1988. Results are expressed as 2.5th and 97.5th percentiles.

†Children's Hospital, Boston, Massachusetts, by laser nephelometry (Dade-Behring BN2).

APPENDIX 65 Serum Levels of Immunoglobulin D in Males and Females

Age	mg/L
Cord blood*	0.04-1.02
Adult†	2.0-173

*Ownby DR, Johnson CC, Peterson EL: Maternal smoking does not influence cord serum IgE or IgD concentrations. *J Allergy Clin Immunol* 88:555, 1991. Values constitute 95% of the measured samples.

†Tozawa T, Nakata N, Adachi K: Serum IgD concentrations in normal individuals 20-39 years of age. *Rinsho Byori Jpn J Clin Pathol* 42:656, 1994.

APPENDIX 66 Serum Levels of Immunoglobulin E in Males and Females

Age		IU/mL
Cord blood[*]		0.02-2.08
<1 y[†]		0-6.6
1-2 y[†]		0-20.0
2-3 y[†]		0.1-15.8
3-4 y[†]		0-29.2
4-5 y[†]		0.3-25.0
5-6 y[†]		0.2-17.6
6-7 y[†]		0.2-13.1
7-8 y[†]		0.3-46.1
8-9 y[†]		1.8-60.1
9-10 y[†]		3.6-81.0
10-11 y[†]		8.0-95.0
11-12 y[†]		1.5-99.7
12-13 y[†]		3.9-83.5
13-16 y[†]		3.3-188.0
<3 y[‡]		<30
<10 y[‡]		<200
10-14 y[‡]		<500
Adult[‡]		<120
	Females (KIU/L)	**Males (KIU/L)**
0-12 m[§]	0-20	2-24
1-3 y[§]	2-55	2-149
4-10 y[§]	8-279	4-249
11-15 y[§]	5-295	7-280
16-18 y[§]	7-698	5-268

KIU/L, Kilo international units/L; *m*, months; *y*, years.

[*] Ownby DR, Johnson CC, Peterson EL: Maternal smoking does not influence cord serum IgE or IgD concentration. *J Allergy Clin Immunol* 88:555, 1991. Values constitute 95% of the measured samples.

[†] Lindenberg RE, Arroyave C: Levels of IgE in serum from normal children and allergic children as measured by an enzyme immunoassay. *J Allergy Clin Immunol* 78:614, 1986.

[‡] Children's Hospital, Boston, Massachusetts.

[§] Soldin SJ, Morales A, Albalos F, et al: Pediatric reference ranges on the Abbott IMx for FSH, LH, prolactin, TSH, T4, T3, free T3, T-uptake, IgE, and ferritin. *Clin Biochem* 28:603, 1995. Study based on hospitalized patience; values represent 2.5th and 95.7th percentiles.

APPENDIX 67 Plasma Levels of Immunoglobulin M in Males and Females

Age	g/L	mg/dL
Lockitch et al. (1988)[*]		
0-12 m	0.0-2.16	0-216
1-3 y	0.28-2.18	28-218
4-6 y	0.36-3.14	36-314
7-9 y	0.47-3.11	47-311
10-11 y	0.46-2.68	46-268
12-13 y	0.52-3.57	52-357
14-15 y	0.23-2.81	23-281
16-19 y	0.35-3.87	35-387
Children's Hospital[†]		
Newborn	0.05-0.30	5-30
1-3 m	0.15-0.70	15-70
4-6 m	0.10-0.90	10-90
7-12 m	0.25-1.15	25-115
1-3 y	0.30-1.20	30-120
3-6 y	0.22-1.0	22-100
Adult	0.50-2.0	50-200

m, Months; *y*, years.

[*] Lockitch G, Halstead AC, Quigley G, et al: Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. *Clin Chem* 34:1618, 1988. Results are expressed as 2.5th and 97.5th percentiles.

[†] Childen's Hospital, Boston, Massachusetts, by laser nephelometry (Dade-Behring BN2).

APPENDIX 68 Plasma Levels of Immunoglobulin G in Males and Females

Age	g/L	mg/dL
Lockitch et al. (1988)[*]		
0-12 m	2.73-16.60	273-1660
1-3 y	5.33-10.78	533-1078
4-6 y	5.93-17.23	593-1723
7-9 y	6.73-17.34	673-1734
10-11 y	8.21-18.35	821-1835
12-13 y	8.93-18.23	893-1823
14-15 y	8.42-20.13	842-2013
16-19 y	6.46-18.64	646-1864
Children's Hospital[†]		
Newborn	7.0-1.30	700-1300
1-3 m	2.80-7.50	280-750
4-6 m	2.0-12.0	200-1200
7-12 m	3.0-15.0	300-1500
1-3 y	4.0-13.0	400-1300
3-6 y	6.0-15.0	600-1500
Adult	6.0-13.4	600-1344

m, Months; *y*, years.

[*] Lockich G, Halstead AC, Quigley G, et al: Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. *Clin Chem* 34:1618, 1988. Results are expressed as 2.5th and 97.5th percentiles.

[†] Childen's Hospital, Boston, Massachusetts, by laser nephelometry (Dade-Behring BN2).

APPENDIX 69 Reference Intervals for IgG Subclasses on Dade Behring Nephelometer by Age Groups

Age Group (months)	Patients (N)	Median (g/L)	2.5th Percentile (g/L)	97.5th Percentile (g/L)
1 to 6 m				
IgG	12	4.3	2.3	9.5
IgG1	12	3.0	1.5	7.9
IgG2	12	0.84	0.36	1.40
IgG3	12	0.22	0.09	0.86
IgG4	12	0.048	0.006	0.460
6 to 12 m				
IgG	17	4.1	2.3	7.3
IgG1	15	3.2	1.7	5.8
IgG2	15	0.51	0.26	1.30
IgG3	17	0.25	0.10	0.92
IgG4	15	0.022	0.004	0.370
12 to 18 m				
IgG	16	6.8	4.1	12.1
IgG1	16	5.3	3.2	9.2
IgG2	16	0.76	0.26	1.50
IgG3	16	0.31	0.12	0.88
IgG4	14	0.060	0.007	0.370
18 m to 2 y				
IgG	21	6.2	2.8	9.9
IgG1	21	5.0	2.6	7.8
IgG2	21	0.77	0.42	2.20
IgG3	21	0.22	0.11	0.97
IgG4	20	0.063	0.017	0.750
2 to 3 y				
IgG	47	7.0	3.7	11.7
IgG1	47	5.6	2.7	9.4
IgG2	46	0.99	0.44	1.90
IgG3	47	0.22	0.09	0.63
IgG4	46	0.101	0.023	0.590
3 to 4 y				
IgG	37	8.6	3.8	17.9
IgG1	37	6.3	2.8	13.7
IgG2	37	1.15	0.44	3.00
IgG3	37	0.29	0.13	0.84
IgG4	37	0.129	0.005	1.140
4 to 6 y				
IgG	76	9.4	5.6	15.4
IgG1	76	6.8	3.8	11.7
IgG2	76	1.43	0.73	2.90
IgG3	76	0.37	0.13	0.75
IgG4	73	0.282	0.013	1.570
6 to 9 y				
IgG	62	9.4	6.0	14.6
IgG1	62	6.3	4.2	9.9

Continued on following page

APPENDIX 69 Reference Intervals for IgG Subclasses on Dade Behring Nephelometer by Age Groups (Continued)

Age Group (months)	Patients (N)	Median (g/L)	2.5th Percentile (g/L)	97.5th Percentile (g/L)
IgG2	62	1.72	0.63	3.50
IgG3	62	0.38	0.17	0.88
IgG4	61	0.288	0.010	1.210
9 to 12 y				
IgG	43	9.4	5.5	15.2
IgG1	43	6.7	3.6	11.2
IgG2	43	1.92	0.89	3.60
IgG3	43	0.43	0.23	0.83
IgG4	43	0.381	0.052	1.560
12 to 18 y				
IgG	86	10.7	6.5	14.6
IgG1	86	6.9	3.9	10.0
IgG2	86	2.47	1.02	4.50
IgG3	86	0.42	0.14	1.02
IgG4	84	0.412	0.061	1.860

From Lepage N, Huang SH, Nieuwenhuys, et al: Pediatric reference intervals for immunoglobulin G and its subclasses with Siemens immunonephelometric assays. Clin Biochem *43:694–696, 2010.*

APPENDIX 70 Serum Levels of Immunoglobulin Light Chains in Males and Females

Age	Kappa g/L	Lamba g/L
Newborn	7.7-8.7	1.7-1.9
Premature	3.1-4.9	1.6-1.9
1 m	3.6-4.8	1.6-2.0
2 m	2.4-2.7	1.6-1.7
3 m	1.7-2.5	1.0-1.3
4 m	1.9-2.4	0.8-1.0
5 m	2.0-3.2	1.0-1.1
1 y	3.0-5.3	0.9-1.2
2 y	3.6-6.4	1.3-1.5
3 y	4.1-5.4	1.1-1.4
4 y	4.8-8.3	1.2-1.7
5 y	5.4-7.1	1.3-1.7
6 y	6.0-8.5	1.6-1.7
7 y	4.6-7.5	1.4-1.8
8 y	7.3-9.1	1.4-1.7
9 y	7.1-9.6	1.5-1.8
10 y	8.2-9.6	1.3-1.9
11 y	6.0-8.3	1.4-1.8
12 y	8.2-9.6	1.7-2.0
13 y	8.2-10.5	1.4-1.7
14 y	8.0-10.8	1.9-2.2
15 y	7.2-11.0	1.7-2.3
16 y	7.0-11.2	1.5-2.2

From Herkner KR, Salzer H, Bock A, et al: Pediatric and perinatal reference intervals for immunoglobulin light chains kappa and lambda. Clin Chem *38:548, 1992.*

m, Months; *y*, years. Results expressed as 10th and 90th percentiles.

APPENDIX 71 Selected Biochemical Parameters in Cord Blood

	Cord Blood (Term)	Cord Blood (Preterm)	Adult or Maternal Blood
Erythrocytes			
Magnesium (mmol/L)[*]	1.76 ± 0.15 (44)	—	—
Copper (μmol/L)[*]	12.9 ± 3.0 (39)	—	—
Zinc (μmol/L)[*]	40.4 ± 13.6 (44)	—	—
Ferritin (ag/cell)[†]	265 (47)	92.7 (47)	
Serum/Plasma			
Ferritin (μg/L)[†]	56.8 (47)	17.7 (47)	—
Free riboflavin (nM)[†]	20.2 (31)[‖]	25.0[‖]	4.7 (31)
Flavocoenzymes (nM)[‡]	55.8 (31)[‖]	71.8[‖]	90.7 (31)
Vitamin B2 (nM) [‡]	77.2 (31)[‖]	92.6[‖]	94.9 (31)
Flavocoenzymes uptake(nmol/min per kg)[‡]	0.4	1.5[¶]	—
Free riboflavin release (nmol/min per kg)[‡]	0.2	0.4[¶]	—
Selenium (μmolL)[§]	1.04 ± 0.21[‖]	0.89 ± 0.27[‖]	1.56 ± 0.27
Retinol (μmol/L)[§]	0.66 ± 0.22[‖]	0.52 ± 0.12[‖]	1.26 ± 0.45
Alpha-Tocopherol (μmol/L)[§]	7.10 ± 2.1[‖]	8.81 ± 2.8[‖]	32.4 ± 9.2
Glutathione peroxidase (U/L)[§]	456 ± 108[‖]	305 ± 89[‖]	873 ± 176

[*] Speich M, Micat A, Acget JC, et al: Magnesium total calcium, phosphorus, copper and zinc in plasma and erythrocytes of venous cord blood from infants of diabetic mothers: comparison with a reference group by logist discriminant analysis. *Clin Chem* 38:2002, 1992. Values expressed as mean ± SD; number of samples in parentheses.

[†] Carpani G, Marini F, Ghisoni C, et al: Red cell and plasma ferritin in a group of normal fetuses at different ages of gestation. *Eur J Haematol* 49:260, 1992.

[‡] Zempleni J, Link G, Bitsch I: Intrauterine vitamin B2 uptake of preterm and full-term infants. *Pediatr Res* 38:585, 1995. Concentrations measured in the umbilical vein are reported in this table; values for umbilical artery are reported in the chapter text as well.

[§] Dison PJ, Lockitch G, Halstead AC, et al: Influence of maternal factors on cord and neonatal plasma micronutrient levels. *Am J Perinatol* 10:30, 1993. Data obtained in 107 term, 23 preterm (<32 weeks) and 58 maternal samples.

[‖] *P*<.05 compared with full term.

[¶] *P*<.05 compared with adult/maternal.

APPENDIX 72 Reference Ranges for Serum Ferritin, Folate, and Cobalamin in Children and Adolescents*

		MALE RIs						FEMALE RIs					
Analyte	**Age**	**No. of Samples**	**Geometric Mean**	**Lower Limit**	**Upper Limit**	**Lower Limit CI**	**Upper Limit CI**	**No. of Samples**	**Geometric Mean**	**Lower Limit**	**Upper Limit**	**Lower Limit CI**	**Upper Limit CI**
Ferritin, ng/mL	4 to <15 days	127	288.4	99.6	717.0	63.1 to 122.9	648.7 to 741.2	127	288.4	99.6	717.0	63.1 to 122.9	648.7 to 741.2
	15 days to <6 months	172	109.2	14.0	647.2	9.8 to 22.1	521.5 to 734.2	172	109.2	14.0	647.2	9.8 to 22.1	521.5 to 734.2
	6 months to <1 year	140	41.7	8.4	181.9	4.6 to 8.9	167.1 to 250.4	140	41.7	8.4	181.9	4.6 to 8.9	167.1 to 250.4
	1 to <5 years	126	25.1	5.3	99.9	4.0 to 9.6	80.9 to 167.0	126	25.1	5.3	99.9	4.0 to 9.6	80.9 to 167.0
	5 to <14 years	497	34.3	13.7	78.8	12.9 to 15.4	73.0 to 86.6	497	34.3	13.7	78.8	12.9 to 15.4	73.0 to 86.6
	14 to <16 years	**65**	**39.3**	**12.7**	**82.8**	**10.0 to 15.7**	**74.5 to 87.9**						
	16 to <19 years	**88**	**55.6**	**11.1**	**171.9**	**7.6 to 17.3**	**149.7 to 188.4**						
	14 to <19 years							**151**	**25.7**	**5.5**	**67.4**	**4.4 to 5.9**	**61.4 to 78.8**
Folate (serum), ng/mL	5 days to <1 year	235	NA	10.6	NA	8 to 11.7	NA	235	NA	10.6	NA	8 to 11.7	NA
	1 to <3 years	50	NA	3.9	NA	0 to 6.4	NA	50	NA	3.9	NA	0 to 6.4	NA
	3 to <6 years	90	NA	11.9	NA	11.1 to 12.8	NA	90	NA	11.9	NA	11.1 to 12.8	NA
	6 to <8 years	70	NA	13.1	NA	10.2 to 14.4	NA	70	NA	13.1	NA	10.2 to 14.4	NA
	8 to <12 years	284	NA	11.4	NA	11.2 to 12.6	NA	284	NA	11.4	NA	11.2 to 12.6	NA
	12 to <14 years	144	NA	11.9	NA	10.8 to 12.3	NA	144	NA	11.9	NA	10.8 to 12.3	NA
	14 to <18 years	342	NA	7.9	NA	6.6 to 8.7	NA	342	NA	7.9	NA	6.6 to 8.7	NA
Cobalamin, pg/mL	5 days to <1 year	257	652	259	1576	181 to 274	1509 to 1620	257	652	259	1576	181 to 274	1509 to 1620
	1 to <9 years	267	744	283	1613	229 to 340	1420 to 1769	267	744	283	1613	229 to 340	1420 to 1769
	9 to <14 years	374	596	252	1125	234 to 291	1078 to 1207	374	596	252	1125	234 to 291	1079 to 1208
	14 to <17 years	217	493	244	887	214 to 261	848 to 925	217	493	244	888	214 to 262	848 to 926
	17 to <19 years	127	428	203	811	192 to 224	783 to 885	127	428	203	812	192 to 225	783 to 885

From Bailey D, Colantonio D, Kyriakopoulou L, et al: Marked biological variance in endocrine and biochemical markers in childhood: establishment of pediatric reference intervals using healthy community children from the Canadian Laboratory Initiative for Pediatric Reference Intervals Cohort Clinical Chemistry Clin Chem *59:1393–405, 2013.*

CI, Confidence interval; *NA*, not applicable.

*Bold and underlined values indicate sex-specific differences within age partitions.

APPENDIX 73 Reference Ranges for Selected Biochemical Markers in Children and Adolescents*

		FEMALE REFERENCE INTERVAL					MALE REFERENCE INTERVAL				
Analyte	Age	Lower Limit	Upper Limit	No. of Samples	Lower Limit Confidence Interval	Upper Limit Confidence Interval	Lower Limit	Upper Limit	No. of Samples	Lower Limit Confidence Interval	Upper Limit Confidence Interval
Bilirubin (direct), mg/dL	0 to 14 days	0.33	0.71	171	0.29-0.36	0.67-0.73	0.33	0.71	171	0.29-0.36	0.67-0.73
	15 days to <1 year	0.05	0.30	108	0.04-0.06	0.27-0.33	0.05	0.30	108	0.04-0.06	0.27-0.33
	1 to <9 years	0.05	0.20	281	0.05-0.05	0.18-0.20	0.05	0.20	281	0.05-0.05	0.18-0.20
	9 to <13 years	0.05	0.29	181	0.05-0.10	0.28-0.33	0.05	0.29	181	0.05-0.10	0.28-0.33
	13 to <19 years	**0.10**	**0.39**	**177**	**0.05-0.11**	**0.34-0.46**	**0.11**	**0.42**	**170**	**0.10-0.12**	**0.40-0.43**
Bilirubin (total), mg/dL	0 to 14 days	0.19	16.60	166	0.10-0.29	15.04-17.88	0.19	16.60	166	0.10-0.29	15.04-17.88
	15 days to <1 year	0.05	0.68	245	0.05-0.10	0.63-0.73	0.05	0.68	245	0.05-0.10	0.63-0.73
	1 to <9 years	0.05	0.40	270	0.05-0.05	0.36-0.42	0.05	0.40	270	0.05-0.05	0.36-0.42
	9 to <12 years	0.05	0.55	135	0.05-0.10	0.50-0.61	0.05	0.55	135	0.05-0.10	0.50-0.61
	12 to <15 years	0.10	0.70	161	0.05-0.10	0.64-0.81	0.10	0.70	161	0.05-0.10	0.64-0.81
	15 to <19 years	0.10	0.84	219	0.05-0.11	0.81-0.89	0.10	0.84	219	0.05-0.11	0.81-0.89
Iron, μg/dL	0 to <14 years	16	128	588	15-21	123-138	16	128	588	15-21	123-138
	14 to <19 years	**20**	**162**	**143**	**13-31**	**138-185**	**31**	**168**	**138**	**11-42**	**153-184**
Magnesium, mg/dL	0 to 14 days	1.99	3.94	183	1.80-2.19	3.77-4.11	1.99	3.94	183	1.80-2.19	3.77-4.11
	15 days to <1 year	1.97	3.09	145	1.85-2.11	3.01-3.21	1.97	3.09	145	1.85-2.11	3.01-3.21
	1 to <19 years	2.09	2.84	897	2.09-2.11	2.82-2.87	2.09	2.84	897	2.09-2.11	2.82-2.87
	15 to <19 years	130	250	227	124-142	239-257	130	250	227	124-142	239-257
LDH, U/L	0 to 14 days	309	1222	197	267-360	1116-1257	309	1222	197	267-360	1116-1257
	15 days to <1 year	163	452	145	94-173	428-483	163	452	145	94-173	428-483
	1 to <10 years	192	321	370	189-199	314-333	192	321	370	189-199	314-333
	10 to <15 years	**157**	**272**	**141**	**130-162**	**258-308**	**170**	**283**	**125**	**138-175**	**277-286**
	15 to <19 years	130	250	227	124-142	239-257	130	250	227	124-142	239-257

From Colantonio DA, Kyriakopoulou, Chan MK, et al: Closing the gaps in pediatric laboratory reference intervals: a CALIPER database of 40 biochemical markers in a healthy and multiethnic population of children. Clin Chem *58:854–868, 2012.*

LDH, Lactate dehydrogenase.

*Bold and underlined values indicate sex-specific differences within age partitions.

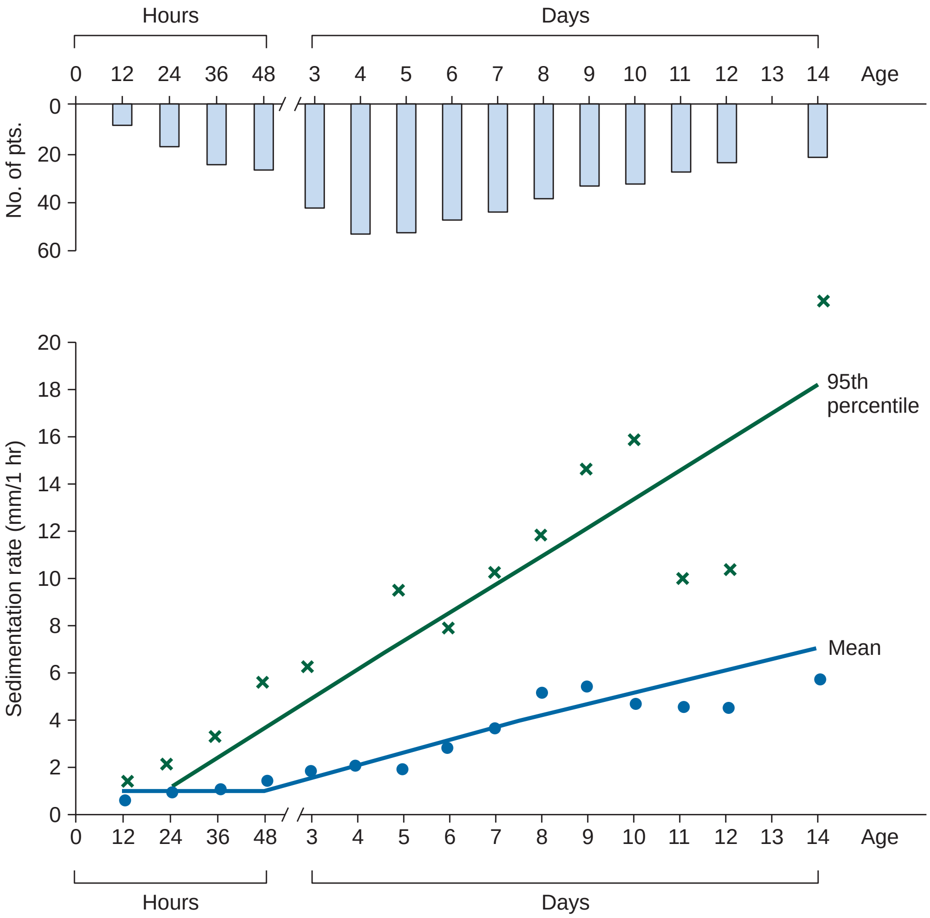
APPENDIX 74 Reference Ranges for Selected Biochemical Markers in Children and Adolescents*

Analyte	Age	Lower Limit	Upper Limit	No. of Samples	Lower Limit Confidence Interval	Upper Limit Confidence Interval	Lower Limit	Upper Limit	No. of Samples	Lower Limit Confidence Interval	Upper Limit Confidence Interval
Albumin G, g/dL	0 to 14 days	3.3	4.5	191	3.2-3.3	4.4-4.6	3.3	4.5	191	3.2-3.3	4.4-4.6
	15 days to <1 year	2.8	4.7	156	2.6-3.0	4.5-5.3	2.8	4.7	156	2.6-3.0	4.5-5.3
	1 to <8 years	3.8	4.7	298	3.8-3.9	4.6-4.7	3.8	4.7	298	3.8-3.9	4.6-4.7
	8 to <15 years	4.1	4.8	388	4.1-4.1	4.8-4.9	4.1	4.8	388	4.1-4.1	4.8-4.9
	15 to <19 years	**4.0**	**4.9**	**119**	**3.9-4.1**	**4.8-4.9**	**4.1**	**5.1**	**123**	**4.1-4.2**	**5.0-5.2**
Albumin P, g/dL	0 to 14 days	2.8	4.1	182	2.6-2.9	4.0-4.2	2.8	4.1	182	2.6-2.9	4.0-4.2
	15 days to <1 year	2.5	4.6	153	2.1-2.7	4.4-4.7	2.5	4.6	153	2.1-2.7	4.4-4.7
	1 to <8 years	3.5	4.5	298	3.5-3.6	4.5-4.6	3.5	4.5	298	3.5-3.6	4.5-4.6
	8 to <15 years	3.7	4.7	390	3.7-3.8	4.6-4.7	3.7	4.7	390	3.7-3.8	4.6-4.7
	15 to <19 years	**3.5**	**4.9**	**119**	**3.5-3.6**	**4.8-4.9**	**3.8**	**5.0**	**123**	**3.7-3.9**	**4.8-5.0**
Prealbumin, mg/dL	0 to 14 days	2	12	127	2-2	10-12	2	12	127	2-2	10-12
	15 days to <1 year	5	24	137	4-7	22-25	5	24	137	4-7	22-25
	1 to <5 years	12	23	148	12-13	22-23	12	23	148	12-13	22-23
	5 to <13 years	14	26	359	13-14	25-27	14	26	359	13-14	25-27
	13 to <16 years	18	31	187	15-18	30-33	18	31	187	15-18	30-33
	16 to <19 years	**17**	**33**	**96**	**0-17**	**32-34**	**20**	**35**	**95**	**0-20**	**34-36**
Total protein, g/dL	0 to 14 days	5.3	8.3	158	5.0-5.4	8.0-8.5	5.3	8.3	158	5.0-5.4	8.0-8.5
	15 days to <1 year	4.4	7.1	152	4.2-4.6	6.9-7.4	4.4	7.1	152	4.2-4.6	6.9-7.4
	1 to <6 years	6.1	7.5	209	5.8-6.2	7.5-7.6	6.1	7.5	209	5.8-6.2	7.5-7.6
	6 to <9 years	6.4	7.7	118	6.3-6.5	7.6-7.8	6.4	7.7	118	6.3-6.5	7.6-7.8
	9 to <19 years	6.5	8.1	588	6.5-6.6	8.0-8.2	6.5	8.1	588	6.5-6.6	8.0-8.2
Haptoglobin, mg/dL	0 to 14 days	0	10	64	0-0	9-10	0	10	64	0-0	9-10
	15 days to <1 year	7	221	129	7-7	203-234	7	221	129	7-7	203-234
	1 to <12 years	7	163	444	7-7	150-168	7	163	444	7-7	150-168
	12 to <19 years	7	179	418	7-12	172-191	7	179	418	7-12	172-191
Transferrin, mg/dL	0 to <9 weeks	104	224	188	99-111	217-233	104	224	188	99-111	217-233
	9 weeks to <1 year	107	324	104	93-118	310-337	107	324	104	93-118	310-337
	1 to <19 years	220	337	878	216-223	333-342	220	337	878	216-223	333-342

From Colantonio DA, Kyriakopoulou, Chan MK, et al: Closing the Gaps in Pediatric Laboratory Reference Intervals: A CALIPER Database of 40 Biochemical Markers in a Healthy and Multiethnic Population of Children. Clin Chem *58:854–868, 2012.*

Albumin G, Albumin assay with bromcresol green; *Albumin P*, albumin assay with bromcresol purple.

*Bold and underlined values indicate sex-specific differences within age partitions.



Appendix 75 Sedimentation rate in the newborn period. The erythrocyte sedimentation rate was measured in capillary blood in healthy full-term and low-birth-weight newborns. Normal values ranged from 1 mm/h at 12 hours of age to 17mm/h at 14 days of age. All values of neonates with hematocrit values less than 40% were corrected to 40%. *(From Adler SM, Denton RL: The erythrocyte sedimentation rate in the newborn period.* J Pediatr *86:942, 1975.)*

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